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Editorial office:

Central Food Research Institute
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Phone: (36-1) 355-8244 ext. 149
Fax: (36-1) 355-8991

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MANUFACTURE OF LOW FAT ZABADY USING DIFFERENT FAT SUBSTITUTES

K. M. K. KEBARY and S. A. HUSSEIN

Department of Dairy Science and Technology, Faculty of Agriculture, Menofiya University, Shibin El-Kom. Egypt

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Fifteen batches of zabady were made to study the effect of different fat replacers on zabady quality. Milk fat was standarized to 4% fat (control), 2% and 0.0%. Simplesse (a protein-based fat replacer) and Cerestar (a carbohydrate-based fat replacer) were added separately or their mixture at the rate of 100 and 50% of reduced fat. Total solids and calorific value decreased ($P \leq 0.05$) by decreasing the fat content, increased ($P \leq 0.05$) by increasing concentration of fat replacer, while they were not affected ($P > 0.05$) by the type of fat replacers. Protein, carbohydrates and ash content were affected ($P \leq 0.05$) by the type and concentration of fat replacers. Protein and ash increased by using Simplesse, while carbohydrates increased by adding Cerestar. Acidity of zabady increased ($P \leq 0.05$) during storage, while total solids, carbohydrates and pH decreased. Syneresis decreased ($P \leq 0.05$), while viscosity and organoleptic scores increased ($P \leq 0.05$) by adding fat replacers. Simplesse was more effective to reduce syneresis, increase viscosity and organoleptic scores of the resultant zabady. Syneresis of whey decreased ($P \leq 0.05$) in all zabady treatments gradually up to 6 days then increased up to the end of storage period. Organoleptic scores of zabady were not affected ($P > 0.05$) by storage up to 6 days, then they gradually decreased ($P \leq 0.05$) as the storage period proceeded. Zabady made from 2% fat milk and added 2% Simplesse received the highest scores followed by control zabady (made from 4% fat milk) and zabady made from skim milk and added 4% Simplesse, while zabady made from skim milk scored the lowest. So it is possible to reduce the energy value by 25% without detrimental effects on zabady quality.

Keywords: fat substitutes, zabady, Simplesse, maltodextrin

Zabady is a set-type yoghurt. It is one of the oldest fermented dairy products and the most popular of these products in Egypt and worldwide. Consumption of zabady in Egypt has nearly doubled in the past three years.

The value of yoghurt in human diet is determined by the nutritive value of milk from which it is made, increased digestibility, prophylactic and healing effects (RASIC & KURMANN, 1978; MARSHALL, 1984 and BUTTRISS, 1997). Digestibility of protein and fat and bio-availability of minerals improved compared with ordinary milk (RASIC & KURMANN, 1978; MARSHALL, 1984; GURR, 1987 and BUTTRISS, 1997). Moreover

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many health benefits have been attributed to yoghurt such as improved lactose tolerance, protection against gastrointestinal infections, effective treatment for specific types of diarrhoea, relief of constipation, improved immunity, cholesterol reduction and protection against cancer (RASIC & KURMANN, 1978; AGERBAEK et al., 1995; SCHAAFSMA, 1996; TVEDE, 1996; YAESHIMA, 1996; BADAWY & EL-SONBATY, 1997 and BUTTRISS, 1997).

Lipids play vital functional and sensory roles in food products. They carry, enhance and release the flavours of other ingredients. Lipids also interact with other ingredients to develop and mould texture, flavour perception, flavour stability, flavour generation and the overall sensation of foods (GIESE, 1996 and DE ROOS, 1997). Over the past decade, there has been substantial interest in the development of a new range of dairy products which are similar to the existing products but in which the fat content is substantially reduced to avoid the health problems associated with fat such as diabetes, hypertension, atherosclerosis, gallbladder disease and heart disease (WILLIAMS, 1985 and GIESE, 1996). Low fat zabady can be achieved by lowering the fat content and/or using fat substitutes to replace the fat (TAMIME et al., 1994 and GIESE, 1996). KHADER (1994) had to increase the total solids of buffalo skim milk to 12% to improve the quality of fat free zabady. Using fat substitutes to replace fat in food while keeping the same functional and organoleptic properties as fat has attracted great attention in past few years. Available fat substitutes can be classified as carbohydrate-based, protein-based and fat-based fat replacers (GIESE, 1996). Reduced and low fat foods have relied on a combination of fat replacers because for a single fat replacer it has not been possible to replace all the functions of fat (GIESE, 1996).

The objectives of this study were to evaluate the possibility of manufacturing low and fat-free zabady using different fat substitutes and study the effects of these fat replacers on the chemical, physical, rheological and organoleptic properties of the resultant zabady.

1. Materials and methods

1.1. Bacterial strains

Streptococcus salivarius subsp. *thermophilus* EMCC 1043 and *Lactobacillus delbrueckii* subsp. *bulgaricus* EMCC 1102 were obtained from Cairo Mircen (Ain Shams University, Egypt). These strains were activated by three successive transfers in sterile 10% reconstituted non-fat dry milk.

1.2. Manufacture of zabady

Fresh bulk buffalo milk (produce of the herd of Faculty of Agriculture, Menofiya University, Shibin El-Kom, Egypt) was separated and then the cream was remixed to standardize the fat content as illustrated in Fig. 1. Fourteen different types of low fat zabady in addition to the control (4% fat) were produced using either Simplesse 100 (protein-based fat replacer) (The Nutra Sweet Kelco Co., Deerfield, II. USA), Cerestar C*De Light 01970 (carbohydrate-based fat replacer) (Cerestar is a company of Eridania Béghin-Say, Vilvoorde, Belgium) or a mixture from them. The chemical composition of Simplesse 100 was moisture <4%, protein 53.5±2.0 on dry basis, fat <4%, ash <7% and carbohydrate 35.9%, while the composition of Cerestar was moisture 4.7%, protein <0.3%, traces of fat, carbohydrates ≃ 95% and ash <0.1%. Fat replacers were added at the rate of 50% or 100% of reduced fat (Fig. 1). Zabady was produced from all mixes as illustrated in Fig. 1. Zabady was stored for 12 days (6–8 °C).

All zabady batches were sampled for analysis at days 1, 3, 6, 9 and 12. This experimental design was triplicated. Samples designations are shown in Fig. 1.

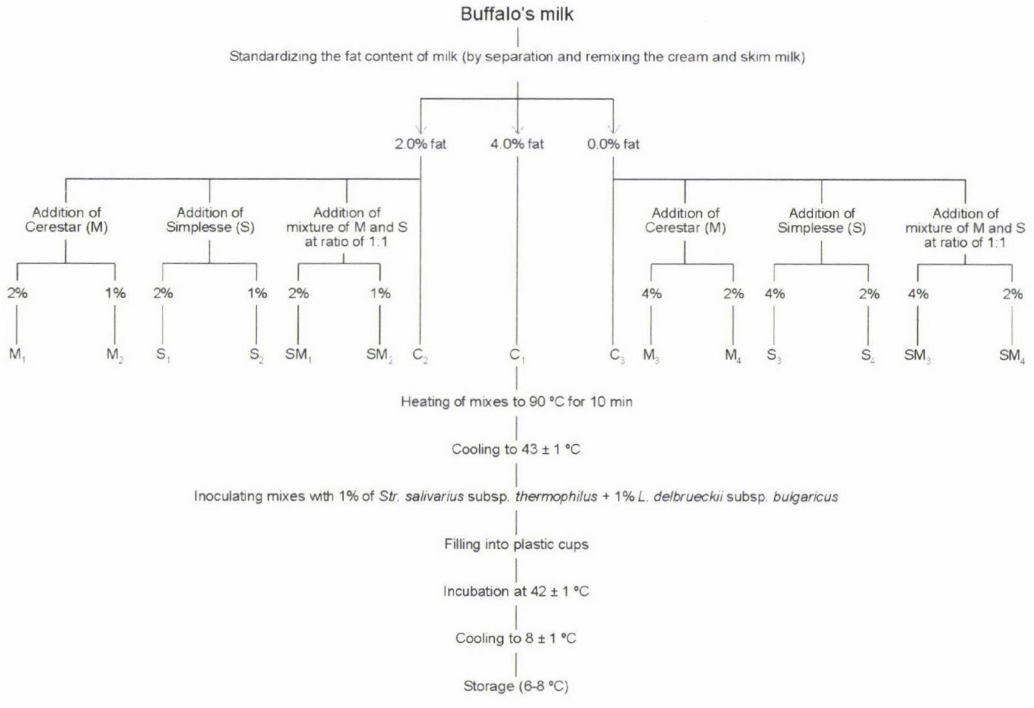

Fig. 1. Processing steps of zabady made with fat substitutes

1.3. Rheological analysis

Viscosity was determined after 1 day storage using Bohlin Viscometer (Bohlin V88, Sweden) attached to a work station loaded with soft ware V88 viscometry program. Measurement of viscosity was carried out at room temperature 22±1 °C. Viscosity was measured at 2 min intervals up to 10 min.

Syneresis was determined according to the method of DANNENBERG and KESSLER (1988) with slight modification. Hundred g zabady in plastic cup was cut into four sections and transferred into a funnel fitted with 120 mesh metal screen. The whey was drained into a graduated cylinder. The amount of whey drained off was measured after 15, 30, 45, 60, 90 and 120 min at room temperature (20±1 °C) on zabady stored for one day and after 2 h on zabady stored for 3, 6, 9 and 12 days.

1.4. Chemical analysis

Zabady samples were analyzed for total solids, total nitrogen, fat, ash, titratable acidity, pH values according to LING (1963). Carbohydrate was calculated by difference. Total energy of zabady was calculated based on conversion factors as follows; protein 4, carbohydrate 4 and fat 9 and expressed as kCal/100 g zabady.

1.5. Sensory evaluation

Zabady samples were evaluated for flavour, appearance, acidity and body and texture by 15 panelists of the staff members of Department of Dairy Science and Technology according to NELSON and TROUT (1981). Samples were presented to judges in plastic cups in random order. Judges were provided with room temperature rinse water, plastic spoons and score sheets.

1.6. Statistical analysis

Randomized block design and 2×3 factorial design were used to analyze the data. Duncan's test was used to make the multiple comparisons (STEEL & TORRIE, 1960). Significant differences were determined at $P \leq 0.05$.

2. Results and discussion

2.1. Chemical composition

Changes in chemical composition during storage of zabady made by substituting fat with either Simplesse (S) (a protein-based fat substitute) or Cerestar (M) (carbohydrate-based fat substitute) and combinations of them (SM) are shown in Tables 1 and 4. Batches made from 2% fat milk and addition of 2% of either fat replacers or

those made from skim milk and added 4% of either fat replacers (M_1, S_1, M_3, S_3, SM_1 and SM_3) as well as control made from 4% fat milk (C_1) had the highest total solids contents followed by batches made from 2% fat milk and addition of 1% fat replacers (M_2, S_2, SM_2), then batches made from 2% fat milk (C_2) or those made from skim milk and addition of 2% of either fat substitutes (M_4, S_4 and SM_4) and finally zabady made from skim milk (C_3). Batches made with addition of Simplesse were not significantly ($P>0.05$) different from corresponding batches made with addition of Cerestar which might be due to the almost similar moisture contents (3.9 and 4.7%). Total solids contents of all batches decreased slightly throughout the storage period (EL-SHIBINY et al., 1979; KHADER, 1994; SALAMA & HASSAN, 1994; ABD EL-SALAM et al., 1996 and KEBARY et al., 1996).

Replacement of fat with Simplesse (a protein-based fat replacer) increased significantly ($P\leq0.05$) the protein content of zabady. Protein contents of zabady increased ($P\leq0.05$) as the amount of added Simplesse increased (Tables 1 and 4). However, protein content of zabady from different treatments did not change significantly ($P>0.05$) during storage (KHADER, 1994).

Fat content of zabady from different batches decreased significantly ($P\leq0.05$) by reducing the fat content of milk used in the manufacture of zabady (Tables 1 and 4). Neither the type nor the concentration of fat replacers affected significantly ($P>0.05$) the fat content of zabady, except zabady made from skim milk with addition of 4% Simplesse, which might be due to the fat content of Simplesse (4.3%). Fat content of all zabady treatments did not change significantly ($P>0.05$) during storage (Tables 1 and 4).

Replacement of fat with Simplesse increased significantly ($P\leq0.05$) the ash content when added at 4 and 2% (S_3, S_1, SM_3) which might be attributed to the ash content of Simplesse (Tables 1 and 4). Neither addition of Cerestar nor storage of zabady affected significantly ($P>0.05$) the ash content of all zabady treatments (Tables 1 and 4).

Carbohydrates content of zabady increased significantly ($P\leq0.05$) by substituting the fat with Cerestar (a carbohydrate-based fat substitute) and it increased as the concentration of added Cerestar increased (Tables 1 and 4). Carbohydrates decreased slightly ($P\leq0.05$) during storage period which may be due to the fermentation of carbohydrates during storage (KHADER, 1994; SALAMA & HASSAN, 1994; KEBARY et al., 1996 and ABD EL-SALAM et al., 1996).

Calorific values of zabady decreased significantly ($P\leq0.05$) as fat content decreased (Tables 1 and 4). Neither the type of fat replacer nor storage period affected significantly ($P>0.05$) the calorific values of the corresponding zabady treatments. However, increasing the concentration of fat replacers increased significantly ($P\leq0.05$) the calorific values of zabady treatments made from milk containing the same fat content (Tables 1 and 4).

Table 1

Composition of zabady made with different fat substitutes stored for 12 days

Zabady samples	Total solids (%)		Total protein (%)		Fat (%)		Ash (%)		Carbohydrates (%)		Energy value (KCal/100 g)	
	1 day	12 days	1 day	12 days	1 day	12 days	1 day	12 days	1 day	12 days	1 day	12 days
C_1[a]	15.29	14.70	5.39	5.41	4.0	4.1	1.09	1.12	4.81	4.07	76.80	74.82
C_2	13.09	12.77	5.40	5.36	2.1	2.0	1.07	1.12	4.52	4.29	58.58	56.60
M_1	15.25	14.57	5.37	5.40	2.0	2.1	1.08	1.12	6.80	5.95	66.68	64.30
M_2	14.02	13.68	5.31	5.29	2.0	2.0	1.07	1.12	5.64	5.27	61.80	60.24
S_1	15.28	14.75	6.54	6.58	2.1	2.2	1.19	1.24	5.45	4.73	66.86	65.04
S_2	14.15	13.69	5.75	5.67	2.0	2.0	1.09	1.11	5.31	4.91	62.24	60.32
SM_1	15.28	14.65	5.83	5.76	2.0	1.9	1.09	1.13	6.36	5.86	66.76	63.58
SM_2	14.15	13.62	5.60	5.61	2.0	2.0	1.08	1.13	5.47	4.88	62.28	59.96
C_3	11.15	11.01	5.37	5.40	0.1	0.1	1.08	1.11	4.60	4.40	40.78	40.10
M_3	15.11	14.49	5.34	5.30	0.1	0.1	1.08	1.13	8.59	7.96	56.62	53.94
M_4	12.89	12.35	5.35	5.26	0.1	0.1	1.07	1.13	6.37	5.86	47.78	45.38
S_3	15.22	14.58	7.31	7.27	0.3	0.2	1.29	1.31	6.32	5.80	57.22	54.08
S_4	13.05	12.66	6.56	6.46	0.2	0.2	1.22	1.23	5.07	4.77	48.32	46.72
SM_3	15.22	14.61	6.42	6.37	0.2	0.1	1.18	1.21	7.42	6.93	57.16	54.10
SM_4	12.99	12.68	5.97	5.91	0.1	0.1	1.10	1.14	5.82	5.53	48.06	46.66

Each value in the table is the mean of three replicates

[a] See Fig. 1

Table 2

Changes in titratable acidity and pH values during storage of zabady made with different fat substitutes

Zabady samples	Titratable acidity (%)					pH value				
	Storage period (days)					Storage period (days)				
	1	3	6	9	12	1	3	6	9	12
C_1[a]	0.90	0.97	1.00	1.16	1.21	4.66	4.52	4.37	4.18	4.01
C_2	0.91	0.99	1.03	1.17	1.23	4.67	4.50	4.40	4.16	4.00
M_1	0.92	0.95	1.01	1.18	1.25	4.67	4.57	4.38	4.12	4.01
M_2	0.85	0.95	1.01	1.18	1.24	4.70	4.58	4.40	4.12	3.99
S_1	0.93	0.97	1.02	1.10	1.23	4.72	4.61	4.43	4.18	4.02
S_2	0.83	0.96	1.00	1.17	1.23	4.73	4.62	4.41	4.16	4.00
SM_1	0.92	0.96	1.02	1.18	1.23	4.67	4.58	4.42	4.16	4.00
SM_2	0.82	0.94	1.00	1.15	1.24	4.69	4.63	4.40	4.14	4.01
C_3	0.92	0.99	1.06	1.21	1.26	4.61	4.48	4.29	4.11	3.98
M_3	0.94	0.96	1.04	1.20	1.25	4.65	4.58	4.37	4.12	3.98
M_4	0.92	0.98	1.01	1.18	1.23	4.65	4.59	4.38	4.12	3.98
S_3	0.78	0.93	1.00	1.13	1.23	4.75	4.64	4.41	4.16	4.00
S_4	0.77	0.96	1.01	1.17	1.24	4.76	4.61	4.40	4.16	3.99
SM_3	0.92	0.97	1.01	1.18	1.23	4.66	4.59	4.39	4.12	4.00
SM_4	0.90	0.96	1.00	1.12	1.25	4.67	4.60	4.41	4.16	3.98

Each value in the table is the mean of three replicates

[a] See Fig. 1

Table 3

Scores of organoleptic properties during storage of zabady made with different fat substitues

Zabady samples	Flavour (out of 45)					Body and texture (30)					Appearance (15)					Acidity (10)					Total score (out of 100)				
	Storage period (days)					Storage period (days)					Storage period (days)					Storage period (days)					Storage period (days)				
	1	3	6	9	12	1	3	6	9	12	1	3	6	9	12	1	3	6	9	12	1	3	6	9	12
C_1[a]	42	42	41	40	37	27	27	25	25	24	13	12	12	11	11	9	9	8	7	6	91	90	85	83	78
C_2	39	38	37	37	35	26	25	23	22	21	12	11	10	9	8	8	7	6	6	5	85	81	76	74	69
M_1	40	40	39	39	36	26	25	24	23	22	12	12	10	10	9	7	7	7	6	6	85	84	80	78	73
M_2	40	39	38	37	35	25	24	23	22	21	11	11	10	10	9	7	8	7	6	6	83	82	78	75	71
S_1	43	42	41	40	39	27	27	27	26	24	13	13	12	11	10	9	9	8	8	7	92	92	89	86	81
S_2	40	40	39	39	37	26	26	24	23	22	12	12	11	10	9	8	8	7	6	5	86	86	81	77	72
SM_1	43	42	42	38	36	26	25	25	24	22	12	12	11	10	9	7	7	7	6	5	88	86	85	77	72
SM_2	41	40	40	39	37	25	25	25	24	22	12	12	11	10	8	9	8	7	7	6	86	84	81	78	71
C_3	38	35	34	33	31	23	21	22	21	20	9	9	8	7	6	8	7	6	6	5	78	72	70	66	61
M_3	39	38	37	36	34	23	23	23	21	20	10	10	9	8	8	7	7	7	6	5	79	78	76	71	67
M_4	38	38	37	36	33	22	21	21	20	19	10	10	9	8	7	7	7	7	6	5	77	76	74	70	64
S_3	42	41	40	40	38	27	27	26	24	23	12	12	12	11	10	9	8	8	7	6	90	88	85	81	76
S_4	39	38	37	36	35	23	23	22	21	20	10	10	10	9	8	8	8	7	7	5	80	79	76	73	68
SM_3	41	40	40	38	36	25	24	23	22	20	10	10	9	9	8	8	7	6	6	6	84	81	78	75	70
SM_4	40	40	39	39	37	23	23	23	21	21	10	10	9	9	8	8	8	7	6	5	81	81	78	75	71

Each value in the table is the mean of three replicates

[a] See Fig. 1

Table 4

Statistical analysis of zabady properties

Properties of zabady	Effect of treatments															Effect of storage (days)					
	Mean squares	Multiple comparisons[a]														Mean squares	Multiple comparisons[a]				
		C_1[b]	C_2	M_1	M_2	S_1	S_2	SM_1	SM_2	C_3	M_3	M_4	S_3	S_4	SM_3	SM_4		1	3	6	9	12
T.S. (%)	21.697*	A	C	A	B	A	B	A	B	D	A	C	A	C	A	C	16.574*	A	AB	AB	AB	B
Protein (%)	5.322*	E	E	E	E	B	C	C	D	E	E	E	A	B	B	C	0.492	A	A	A	A	A
Fat (%)	6.644*	A	B	B	B	B	B	B	B	D	D	D	C	D	D	D	0.312	A	A	A	A	A
Ash (%)	0.004*	C	C	C	C	B	C	C	C	C	C	C	A	B	B	C	0.045	A	A	A	A	A
Carbohydrate (%)	211.18*	DE	DE	B	C	D	D	BC	CD	DE	A	B	BC	D	B	C	6.187*	A	AB	AB	AB	B
Calorific value	7.125*	A	D	B	C	B	C	B	C	F	D	E	D	E	D	E	0.367	A	A	A	A	A
pH value	0.010*	CDE	E	CDE	BCD	A	AB	ABCD	ABC	F	DE	DE	A	AB	CDE	ABCD	3.555*	A	AB	B	CD	D
Titratable acidity (%)	0.005*	BCD	ABC	ABC	CD	DE	DE	ABC	DE	A	AB	ABC	F	EF	ABC	CD	0.665*	D	CD	C	B	A
Synersis after 2 h (%)	73.526*	H	C	G	D	I	F	H	E	A	C	A	HI	B	G	E	14422.5*	A	B	E	DE	C
Organoleptic properties:																						
Flavour	50.644*	AB	DE	D	DE	A	C	B	C	G	EF	F	B	EF	C	C	137.211*	A	AB	ABC	D	E
Body and texture	29.957*	AB	D	C	DE	A	C	C	C	G	F	G	B	F	E	F	104.349*	A	AB	ABC	CD	E
Appearance	13.790*	A	D	C	DE	A	C	C	CD	G	G	G	AB	FG	F	F	59.778*	A	AB	ABC	D	E
Acidity	2.833*	AB	DEFG	CDEFG	CDE	A	CD	EFG	B	G	FG	FG	AB	C	CDEF	CD	48.293*	A	AB	BC	D	E
Total score	283.531*	AB	D	C	D	A	C	C	C	G	E	EF	B	DE	CD	D	1346.449*	A	AB	ABC	D	E

[a] Each different letter (in the same row) means that the multiple comparisons are different from each other, letter A is the highest mean followed by B, C,...

* Significant at 0.05

[b] See Fig. 1

Addition of fat substitutes affected significantly ($P \leq 0.05$) the development of acidity in zabady from different treatments (Tables 2 and 4). Zabady treatments made with addition of Cerestar (M_1, M_3 and M_4) had higher acidity than those made with addition of Simplesse (S_1, S_3 and S_4). This may be due to the availability of fermentable carbohydrates which enhance the acid production in treatments made with addition of Cerestar and on other hand increasing the buffering capacity of treatments made with Simplesse as a result of increasing protein content. Acidity increased slightly during the first 6 days of storage then increased gradually ($P \leq 0.05$) up to the end of storage period (Tables 2 and 4). These results are in agreement with those reported by BADRAN (1986), FAROOQ and HAQUE (1992), KHADER (1994), SALAMA and HASSAN (1994), ABD EL-SALAM and co-workers (1996), KEBARY and co-workers (1996) and BADAWI and EL-SONBATY (1997). Changes in pH value of zabady treatments as affected by the type of fat substitute or storage period followed almost contrary trends to acidity as shown in Tables 2 and 4 (BADRAN, 1986; KHADER, 1994; SALAMA & HASSAN, 1994; ABD EL-SALAM et al., 1996 and BADAWI & EL-SONBATY, 1997).

2.2. Syneresis

Syneresis of zabady from different batches was measured at different times after one day storage (Fig. 2). Increased separation of whey from zabady was observed as the time was increased up to 2 h (Fig. 2), so it was decided to measure syneresis throughout the storage period for 2 h. Similar trends were obtained by HARWALKAR and KALAB (1983). Addition of fat replacers or their mixtures resulted in a reduction ($P \leq 0.05$) of syneresis compared to control containing the same fat content (Fig. 3 and Table 4). Similar results were obtained by FAROOQ and HAQUE (1992), who used sugar esters, HARWALKAR and KALAB (1983) and DANNENBERG and KESSLER (1988) who found that heating of milk decreased separation of whey from yoghurt made from this milk. Addition of fat replacers might lead to form a complex with casein micelles and prevent them from excessive fusion during storage and form a fine-meshed gel network which is less susceptible to whey separation (DANNENBERG & KESSLER, 1988). Addition of Simplesse was more effective to reduce syneresis of whey from zabady than Cerestar. This might be due to its higher ability to bind water (water holding capacity) and/or higher ability to form a complex with casein micelles which led to reduce whey separation. Moreover syneresis decreased significantly ($P \leq 0.05$) as the concentration of fat replacers was increased in the corresponding treatments (Fig. 3 and Table 4). Syneresis of zabady treatments made without fat replacers increased as the fat content was decreased (Fig. 3 and Table 4). Similar results were reported by KEBARY and MORRIS (1990) for rennet-coagulated curd, DIMOV and MINEVA (1962) and STORRY

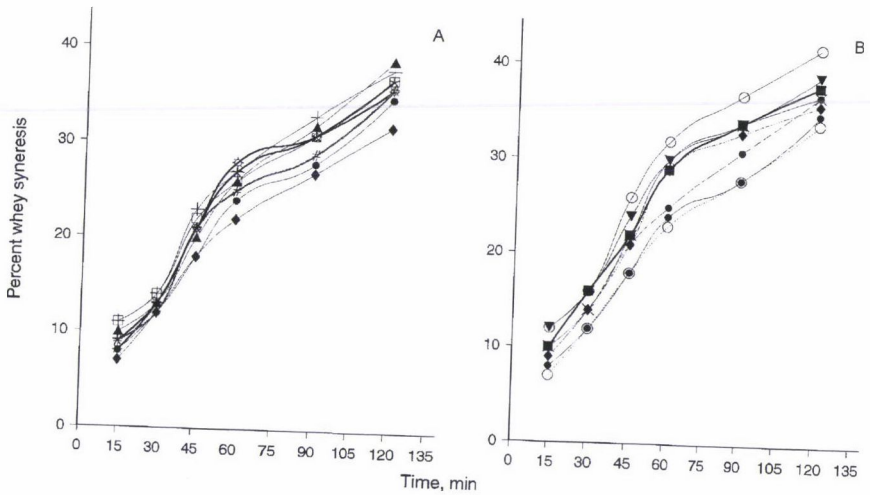

Fig. 2. Syneresis of whey (%) of zabady made by different fat substitutes.

A ● C_1[a] +C_2 ▲ M_1 □ M_2
◆ S_1 * S_2 # SM_1 ⊞ SM_2

B ● C_1[a] ○ C_3 X M_3 ▼ M_4
--o-- S_3 ■ S_4 --•-- SM_3 ◆ SM_4

[a] See Fig 1

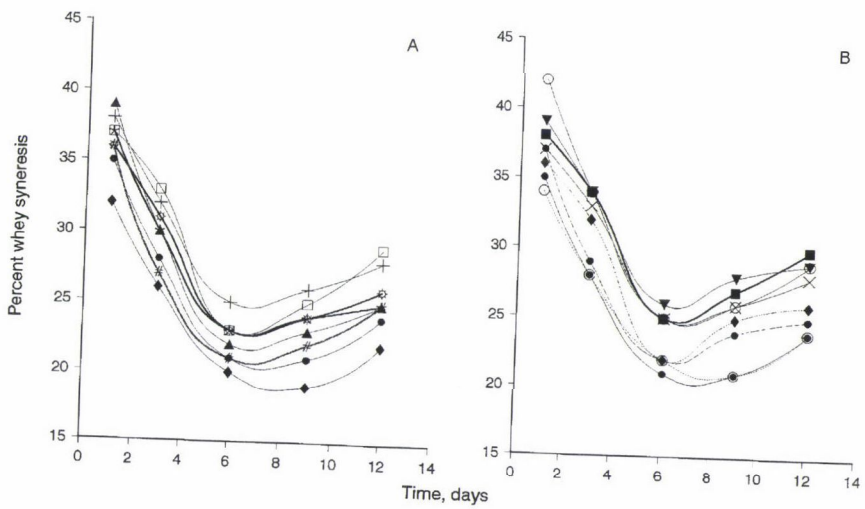

Fig. 3. Changes in syneresis of zabady made with different fat replacers. (Test was carried out for 2 h).

A ● C_1[a] +C_2 ▲ M_1 □ M_2
◆ S_1 * S_2 # SM_1 ⊞ SM_2

B ● C_1[a] ○ C_3 X M_3 ▼ M_4
--o-- S_3 ■ S_4 --•-- SM_3 ◆ SM_4

[a] See Fig 1

and co-workers (1983). Syneresis from zabady treatments decreased gradually ($P \leq 0.05$) as storage period proceeded up to 6 days then increased ($P \leq 0.05$) up to the end of storage period (Fig. 3 and Table 4). These results are in agreement with those reported by BADRAN (1986), FAROOQ and HAQUE (1992) and ABD EL-SALAM and co-workers (1996). This increase in syneresis can be attributed to the developed acidity during storage and consequently contraction of curd which help to expel the whey from curd.

2.3. Viscosity

Successive measurement caused a reduction in the apparent viscosity of zabady (Fig. 4). These results were in agreement with those reported by ABD EL-SALAM and co-workers (1997) who reported that such behaviour might be due to the continuous distruction of zabady structure by successive sheering. Viscosity of zabady increased significantly ($P \leq 0.05$) by adding fat replacers and their mixtures. Addition of Simplesse was more effective to increase viscosity than treatments made with Cerestar (Fig. 4). Viscosity increased as the concentration of fat replacers was increased in the corresponding zabady treatments. Moreover viscosity decreased as fat content was decreased in zabady treatments made without fat replacers (Fig. 4).

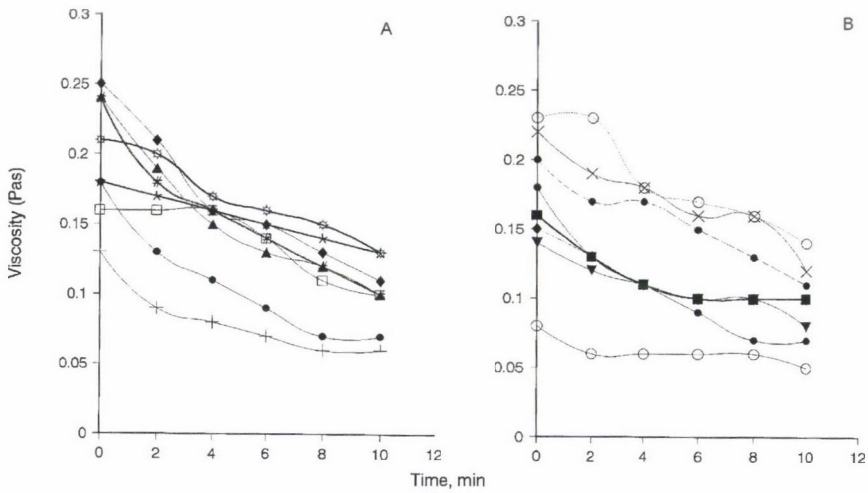

Fig. 4. Effect of fat substitutes on viscosity of zabady.

A ● C_1[a] +C_2 ▲ M_1 □ M_2 ◆ S_1 * S_2 # SM_1 ⊕ SM_2

B ● C_1[a] ○ C_3 X M_3 ▼ M_4 --o-- S_3 ■ S_4 --•-- SM_3 ◆ SM_4

[a] See Fig 1

2.4. Sensory evaluation

Scores of organoleptic properties (flavour, body and texture, appearance and total scores) as affected by type and concentration of fat replacers and storage period followed approximately similar trends (Tables 3 and 4). Adding fat replacers increased ($P \leq 0.05$) scores for flavour, body and texture and appearance of the corresponding zabady treatments. Simplesse was more effective to improve flavour, body and texture, appearance, acidity and increase the total scores of organoleptic properties than zabady made with Cerestar (Tables 3 and 4). Storage of zabady did not affect significantly ($P > 0.05$) the scores of organoleptic properties up to the 6th day then decreased significantly ($P \leq 0.05$) up to the end of storage period (Tables 3 and 4) (BADRAN, 1986; FAROOQ & HAQUE, 1992; KHADER, 1994 and KEBARY et al., 1996). Treatment made from 2% milk fat with 2% Simplesse received the highest scores for flavour, body and texture, appearance and acidity, followed by treatments C_1 (4% fat) and S_3 (made from skim milk with added 4% Simplesse). Also the rates of deterioration of S_1 and S_3 were lower than those of C_1, C_2 and C_3, respectively (Tables 3 and 4).

3. Conclusions

It could be concluded that addition of fat replacers to low or fat free milk to make zabady caused a significant reduction in syneresis and increased the viscosity and organoleptic scores of the resultant zabady. Simplesse was more effective to improve the rheological and sensoric properties of zabady. It is possible to reduce the energy by 13% and 26% without detrimental effects on quality of zabady by making zabady from milk containing 2% fat and adding 2% Simplesse or from skim milk and adding 4% Simplesse, respectively, which also has better keeping quality.

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SUPERCRITICAL FLUID EXTRACTION OF ROSEMARY AND SAGE ANTIOXIDANTS

D. BAUMAN[a], M. HADOLIN[b], A. RIŽNER-HRAŠ[b] and Ž. KNEZ[b,c]

[a] PINUS, Chemical Works, SLO-2327 Rače, Slovenia
[b] University of Maribor, Faculty of chemistry and chemical engineering, Smetanova 17, SLO-2000 Maribor, Slovenia

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A newly developed process for isolation of antioxidative components from rosemary (*Rosemarinus officinalis*) and sage (*Salvia officinalis*) with supercritical fluid as a solvent is presented. In the first stage of the process the essential oils are separated. Later at higher pressure the antioxidative components are extracted.

The efficiency of antioxidative extracts, isolated from rosemary and sage, is compared with synthetic antioxidants. Higher efficiency of natural antioxidants is presented.

Keywords: dense carbon dioxide, extraction, rosemary, process parameters

High pressure technology offers the industry an enormous opportunity to develop novel products of high value. High isostatic pressure is able to inactivate microorganisms and enzymes and thus can be applied for preservation of some foodstuffs which is usually performed by high temperature treatment.

On the other hand, sub- and supercritical fluids have been used as a solvent for a wide variety of extractive applications. Recently supercritical fluids have also been applied as a solvent for non extractive applications in high pressure micronisation, in chromatography and as chemical and biochemical reaction media. The advantages of using sub- and supercritical fluids to perform and achieve separations are well documented in several reviews (KNEZ, 1987; KNEZ, 1997; ŠKERGET et al., 1998). Extraction solvents, generally regarded as safe (GRAS) in compliance with good manufacturing processes (GMP) are: propane, butane, butyl acetate, ethyl acetate, ethanol, carbon dioxide, acetone, nitrous oxide (EEC, 1988). Four of the eight permitted solvents are gases. Selectivity of gases under high pressure (sub- or supercritical) is largely attributed to their density, which may be varied over a wide

[c] To whom the correspondence should be addressed. Phone: +386/62/22 94 461, fax: +386/62/22 50 13, + 386/62/22 77 74, E-mail:ZELJKO.KNEZ@UNI-MB.SI

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range by small changes of temperature and pressure near the critical point as can be seen from P–T diagram (Fig. 1).

The solvation power of carbon dioxide may be altered by the presence of a cosolvent. Other very important characteristics of supercritical gases are: very low dynamic viscosity and very high diffusion coefficients. Therefore supercritical fluids are especially suitable for diffusion-controlled separation processes.

The order of magnitude of physico-chemical properties of a typical gas, liquid and supercritical fluid is summarised in Table 1.

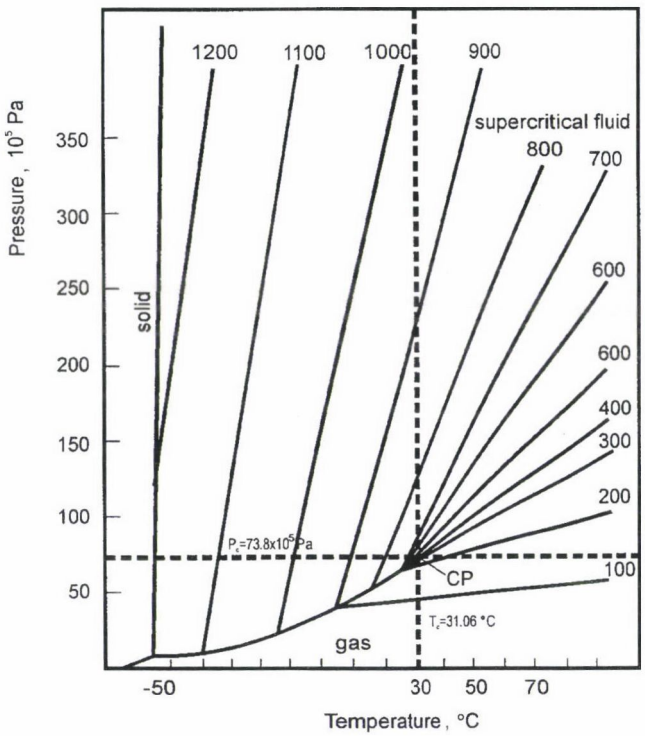


Fig. 1. P-T diagram for CO_2 with lines of constant density (KNEZ, 1987). CP: critical point

Table 1

Order of magnitude of physico-chemical properties of typical gas, liquid and supercritical fluid

Property	Unit	Gas	Supercritical fluid		Liquid
		1 bar 25 °C	P_c, T_c	$4P_c$, T_c	1 bar 15 °C
Density	kg m^{-3}	1	200–500	400–900	1000
Viscosity	kg m^{-1} s^{-1}	10^{-5}	$(1–3)\cdot10^{-5}$	$(3–9)\cdot10^{-5}$	10^{-3}
Diffusivity	m^2 s^{-1}	10^{-5}	$0.7\cdot10^{-7}$	$0.2\cdot10^{-7}$	10^{-9}

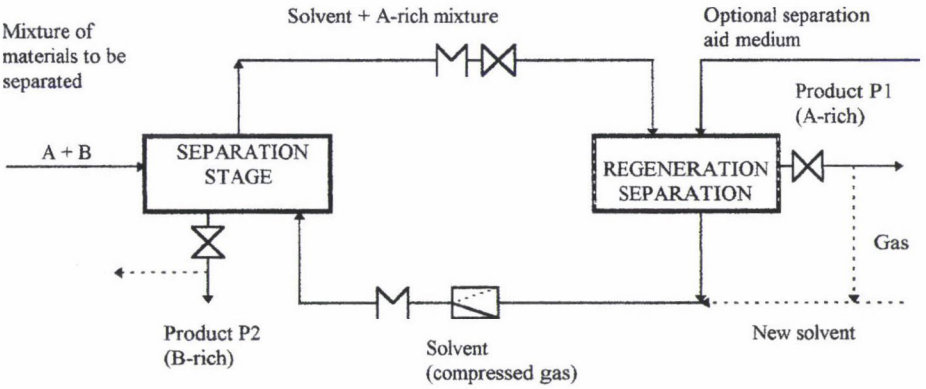


Fig. 2. Flow diagram of process for gas extraction (KNEZ, 1997)

A typical high-pressure extraction process comprises basically an extraction stage for the feedstock and a separation stage for the extract (Fig. 2). The description of the extraction process can be found in the literature (BRUNNER, 1994; KNEZ, 1987; KNEZ, 1997; ŠKERGET et al., 1998).

Near the critical point the latent heat of evaporation is low, so a simple separation of extract and solvent recovery with low energy consumption is feasible. The product contains no organic solvent and fractionation of the product is possible.

It is well known that in foods antioxidants retard oxidative rancidity caused by atmospheric oxidation and thus protect oils, fats and fat soluble components such as vitamins, carotenoids and other nutritive ingredients. In addition, they delay undesirable change brought about by oxidation in foods, for example discoloration in meat

and meat products, browning or "scald" on fruit and vegetables. Antioxidants act by interfering with the oxidation process, they cannot eliminate oxidative products that have already been produced. Consequently, antioxidants should be added to the fresh product as early as possible because they can not reverse any oxidation that has already occurred. Antioxidants do not render a rancid fat or spoilt food palatable, nor do they suppress hydrolytic rancidity, which is enzymatically catalysed hydrolysis of fats. Antioxidants do not have any effect on hydrolytic rancidity resulting from hydrolysis of fats and oils to yield free fatty acids. This type of rancidity usually takes place in the presence of moisture and/or lipolytic enzymes and can be controlled by heat to destroy natural lipases and by removing all moisture from fat/oil (HUDSON, 1990).

Antioxidants may be added directly to the food system or as a solution in the food's oil phase, in a food grade solvent or in an emulsified form which can be sprayed onto the food product. The type of food to which antioxidants may be added is variable, ranging from dry (cereal-based products), convenience and snack foods, biscuits, nuts, mayonnaise, fruit drinks, chewing gum and meat products, to oils and fats (ARUOMA et al., 1992; BELITZ & GROSCH, 1987; BRACCO et al., 1981; CHRASTIL, 1982; COENEN & KRIEGEL, 1983; CUVELIER et al., 1994; DJARMATI et al., 1991; EISERICH & SHIBAMOTO, 1994; FRANKEL, 1993; FRANKEL et al., 1994; FRANKEL et al., 1996; HOPPE, 1975; HUANG et al., 1994; HUDSON, 1990; INATANI et al., 1983; JACOB, 1994; KNEZ et al., 1991; LŐLIGER, 1991a; LŐLIGER, 1991b; POTER et al., 1989; ROSE & BODE, 1995, SCHWARZ & TERNES, 1992a; SCHWARZ & TERNES, 1992b; SCHWARZ & TERNES, 1992c; STROH, 1994).

It should be pointed out that antioxidants must not be added above a certain level, not only because of legal restraints, but also because a pro-oxidant effect would occur (ALLEN & HAMILTON, 1983).

Lipids and lipid-soluble substances which may be susceptible to oxidation are present in almost all foods. They include edible fats and oils, triglycerides, as well as mono- and diglycerides (emulsifiers), sterols, fat soluble vitamins, phospholipids, flavours and aromas, carotenoids and others. The most common cause of deterioration of oils and fats is autoxidation of polyunsaturated fatty acids, which can lead to:

- unpleasant taste and flavour,
- formation of toxic polymers,
- lost in nutritional and physiological value of fatty acids and consequently deterioration of fat soluble vitamins and essential fatty acids,
- discoloration of pigments,
- structural changes and
- other chemical changes.

Autoxidation is a free radical chain reaction, catalysed by oxygen or by some metal ions. The primary oxidation products are hydroperoxides with no taste and

flavour, but the secondary degradation products are very potent taste and flavour modifiers. As colour, odour and aroma are the main subjective criteria used in assessing product quality, the choice of packing and storage conditions and the inclusion of antioxidants are factors, extending the shelf-life of food and cosmetic products.

Antioxidants, traditionally used in industries, consist of two major groups.

- Synthetic (chemical) antioxidants and
- Natural antioxidants.

Synthetic antioxidants is the largest group in use today. They mostly consist of phenolic derivatives:

- Butylated hydroxyanisole (BHA),
- Butylated hydroxytoluene (BHT),
- Tertiary butylhydroquinone (THBQ) and
- Propyl galate (PG).

These antioxidants are manufactured by chemical processes. Their use in food industry is severely restricted to both application and level of use.

A further group of chemical antioxidants used in foods comprise the sequestering agents nitric acid, ascorbic acid, sodium erythroborate etc., none of which probably have direct antioxidant activity. They are often used in conjunction with antioxidants. Natural food and cosmetic antioxidants are extracted from plant materials or by-products. There are currently three major groups:

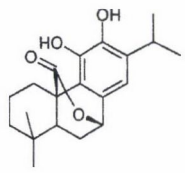
Carotenoids: Carotenoids have long been known for their antioxidant effect and their possible effect on a number of degenerative diseases. Many fruits and vegetables, carrots, pumpkins, spinach, tomatoes, oranges, hot chili peppers, contain at least one member, if not more, of the carotene family (known as carotenoids).

Tocopherols: These are a mixture of alpha, beta, gamma and delta tocopherols, obtained as a by-product from vegetable oil refining industry. They include vitamin E (alpha-tocopherol) and they represent the largest group of natural antioxidants currently in use.

Spice extracts: Certain natural spices have long been known to exhibit antioxidant properties. The strongest in this regard are rosemary and sage. As the demand for clean labeling and chemical-free all-natural food products has increased, a number of natural antioxidants derived from rosemary and sage are now being marketed. These include Rosemary Deodorised (Cal-Pfizer), Spice Extract AR (Nestle), Herbalox (Kalsec), Flavor Guard (OM Ingredients), Labex (NORAC) and ROS. (Pinus-Slovenia).

Despite the lower cost-effectiveness of the spice antioxidants, their use is increasing due to market pressures.

Most research work on this area is concentrated on herbs from Labiatae family that includes also rosemary and sage.



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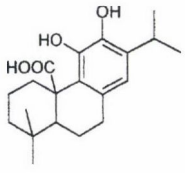

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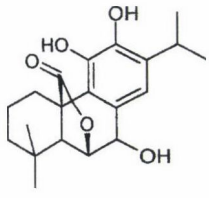

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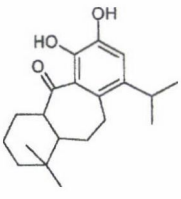

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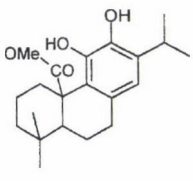

e)

Fig. 3. Antioxidative components from rosemary: a) carnosol, b) carnosic acid, c) rosmanol, d) rosmaridiphenol and e) methyl carnosate

The most active antioxidative components in rosemary are: carnosic acid, carnosol, rosmanol, rosmaridiphenol and methyl carnosate. Chemical structures of these components are shown in Fig. 3 (HUDSON, 1990).

1. Materials and methods

1.1. Materials

The rosemary plant material originated from KRKA (Novo mesto, Slovenija). All chemicals for analytics were obtained from Merck (Darmstadt, Germany). Carnosic acid as a standard for HPLC method was made by purification of rosemary extracts. The purified carnosic acid was better than 90% pure (RICHHEIMER et al., 1996).

Oils and fats for peroxide value measurements were supplied by GEA (Slovenska Bistrica, Slovenia).

CO_2 (purity 99.97%) was obtained from Linde plin (Celje, Slovenia).

1.2. Methods

1.2.1. Extraction process

Supercritical extractions with CO_2 were performed in the UHDE GmbH (Hagen, Germany) pilot plant.

The extraction of antioxidative components was performed in two stages. In the first stage essential oils were removed. In the second stage antioxidative components were extracted at higher pressure and temperature. These components were separated in a separator. In both stages, volume flow was the same. All extraction experiments were performed at different pressure, volume flow and temperature to obtain optimal extraction conditions.

1.2.2. Analytics

1.2.2.1. HPLC analysis of rosemary and sage extracts – The method was similar to that used by RICHHEIMER and co-workers (1996). The HPLC system consists of a constaMetric 3000 (Milton Roy) pump, spectroMonitor 3100 (Milton Roy) UV-VIS detector, HP 3396 integrator (Hewlett Packard) and Rheodyne injector (Cotati, California). The column was LiChrosorb RP-18, 7 µm (Merck, Germany). The column was run at 2 ml min^{-1} with a 65:35 mixture of acetonitrile and water contained 0.5% phosphoric acid and 1 mM EDTA. The sample injected to the column was 10 µl. The detection wavelength was 230 nm. Carnosic acid dissolved in alcohol with 0.5% H_3PO_4 and 1 mM EDTA was used as an external standard to estimate the concentrations of carnosic acid and carnosol.

1.2.2.2. Estimation of peroxide value – Peroxide values (PVs) were measured to determine the antioxidative activity of the extracts. Therefore antioxidants were dissolved in oil and the solution was stored at 98 °C. Peroxide values were determined periodically, using A.O.A.C. official method (A.O.A.C., 1997).

About 1 g of sample was weighted into 250 ml glass Erlenmeyer flask each hour. Thirty ml of acetic acid was added and swirled to dissolve. 0.5 ml of saturated KJ solution was added and shaked for 1 min. Finally, 30 ml of water was added.

The liberated iodine was titrated with the 0.01 *N* sodium thiosulphate solution with vigorous shaking until yellow was almost gone. One ml of 1% starch solution was added and the solution was titrated until blue disappeared.

The peroxide value (PV), expressed in millimoles of active oxygen per kg of sample, is given by the formula:

$$PV = \frac{V \cdot T}{m} \cdot 1000$$

where V is the ml of the standarised sodium thiosuphate solution, used for the test, corrected to take into account the blank test, T is the exact normality of the sodium thiosuphate solution used, m is the mass, in g, of the test portion.

PV for each sample was analysed three times and SD value was calculated.

1.2.2.3. Costs evaluation – Costs determination was made for the use of synthetic and natural antioxidants. Price data for synthetic ones were taken from CHEMICAL MARKET REPORTER (1997).

2. Results

The results of supercritical extraction of rosemary are presented in Table 2. The yield of extraction was also measured versus time and temperature, as can be seen in Fig. 4. The mass of the extract was measured at constant pressure at two different temperatures, 40 °C and 100 °C, and was higher at 100 °C. The yield is dependent on pressure, temperature and time of extraction. With different extraction pressures the optimal conditions of extraction were determined. At pressures under 25 MPa, the extracts contain no antioxidative components and only essential oils were extracted. At a pressure of 35 MPa, the content of antioxidative components was still low, at a pressure of 47.5 MPa, the content was maximal, but the product had a strong smell of rosemary essentials oils.

Therefore extraction experiments were performed in two steps. At a pressure of 25 MPa and 100 °C essential oils were extracted and at 47.5 MPa the antioxidative components were isolated. The yield of essential oil at the above mentioned conditions was 7–8% and the yield of antioxidant extracts, obtained at 47.5 MPa and 100 °C, was between 5 and 6% (see Table 2).

Table 2

Extraction of rosemary with supercritical CO_2. $T=100$ °C, $Q_v=35\ l\ h^{-1}$

P(MPa)	Yield%
10	2.10
20	4.05
25	7.56
35	11.13
47.5	15.44

The antioxidative efficiency of extracts was determined by measuring of peroxide value. The fatty acid composition of different oils used is shown in Table 3.

With the peroxide value measurements a quantitative content of peroxide bounded oxygen was determined, expressed in milimol of active oxygen per kilogram oil or fat. The comparison between peroxide values by adding synthetic and natural antioxidant to beef tallow, groundnut oil and sunflower oil is presented in Table 4. Natural antioxidants are more efficient than synthetic (BHA).

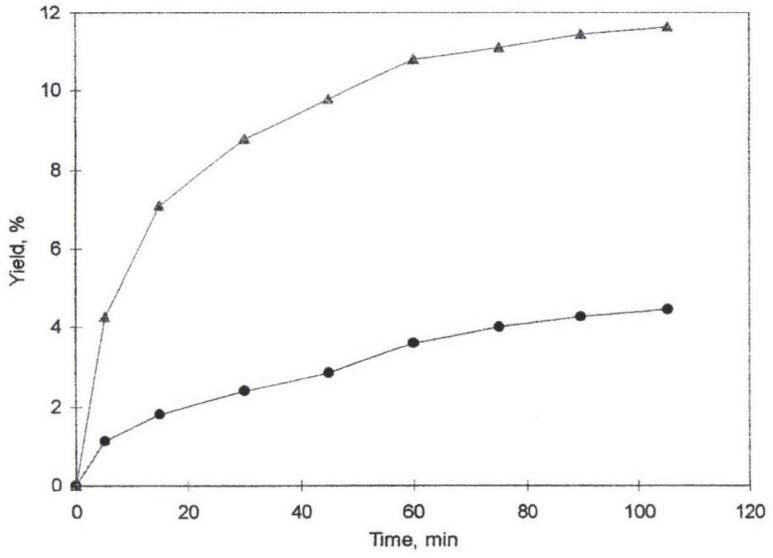

Fig. 4. Yield depending on time and temperature of extraction (pressure 35 MPa)
●: Yield, 40 °C; ▲: Yield, 100 °C

Table 3

Fatty acid composition of oils used in experiments

Oil	Saturated fatty acids (%w/w)	Mono-unsaturated fatty acids (%w/w)	Poly-unsaturated fatty acids (%w/w)
Sunflower oil	10	20	70
Groundnut oil	20	55	25

Table 4

Comparison between peroxide value by adding synthetic and natural antioxidants to beef tallow, groundnut oil and sunflower oil (conc. BHA and rosemary=0.02% w/w). Measurements were made at 98 °C and SD value is 0.01

t(h)	Peroxide value (Milliequivalent peroxide/kg sample)								
	Beef tallow			Groundnut oil			Sunflower oil		
	No AO contr.	BHA	Rose-mary	No AO contr.	BHA	Rose-mary	No AO contr.	BHA	Rose-mary
1	0.88	1.46	0.40	1.77	0.67	0.33	3.50	2.38	1.42
2	1.28	1.26	0.46	2.54	1.57	0.64	6.55	3.37	1.58
3	1.85	1.31	0.55	3.33	1.70	0.61	8.34	3.59	1.65
4	2.77	1.38	0.65	3.75	2.30	0.56	9.82	4.43	1.91
5	3.78	1.00	0.92	4.00	2.52	0.52	11.60	5.49	2.02
6	4.66	0.90	1.33	4.32	2.97	0.79	13.10	6.57	2.02
7	3.55	0.89	2.00	4.55	3.14	0.81	14.20	6.95	2.15
8	3.44	2.56	2.07	5.09	3.30	0.92	15.50	7.50	2.22
9	3.30	2.89	2.67	5.30	3.52	0.95	16.20	8.03	2.35
10	3.80	2.72	2.87	5.63	3.72	0.70	–	8.85	2.54

AO: antioxidant
contr.: control

Various synthetic antioxidants, such as BHA, BHT, PG, and blends of rosemary antioxidants were tested. For analysis, a sunflower oil was used, and also the following quantities of antioxidants: 100 mg/kg of fat for PG and 200 mg/kg of fat for all other antioxidants (BHA, BHT, blends of rosemary antioxidant) were used.

The results of peroxide value measurements are shown in Fig. 5.

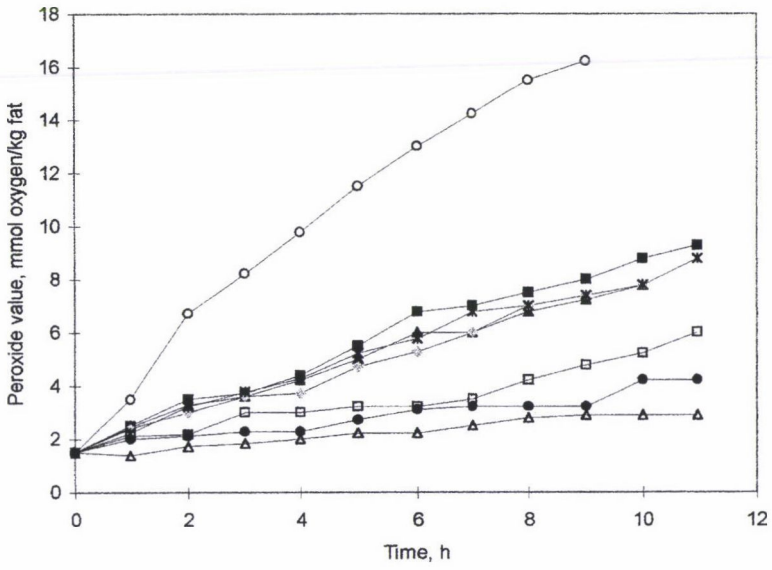

Fig. 5. Peroxide value of sunflower oil at 98 °C. (ROS.AP: mixture of rosemary extract and ascorbyl palmitate, ROS. AP.C.: mixture of rosemary extract, ascorbyl pamitate and ascorbic acid, ROS. AP. ALC: mixture of rosemary extract, ascorbyl palmitate and ethyl alcohol, ROS. OLEO: mixture of rosemary extract and vegetable oil). O: control; ■: BHA; ▲: BHT; ◊: PG; Δ: ROS.AP; ●: ROS. AP. C; □: ROS.AP.ALC; *:ROS.OLEO

The peroxide value of control sample, containing no antioxidant, rises rapidly. It can be seen that blends of natural antioxidant, obtained from rosemary, have better or at least equal antioxidative activity than synthetic antioxidants.

Peroxide values of extracts of rosemary and sage are presented in Fig. 6. The antioxidants isolated from sage are more efficient than those isolated from rosemary. The content of antioxidative components of the extract is higher than in rosemary extracts but the total yield in extraction process is lower.

From costs evaluation (Fig. 7) it can be seen that natural antioxidants are more expensive than synthetic, but the costs of antioxidants added to the fat containing food are very low and are practically negligible to the total selling prices of the food products. Other advantages, which are also very important, are: GRAS for all uses (as a spice extract), no restrictions on applications of levels of use, "ALL NATURAL" label claims, oil and water dispersible forms, no label declaration of function (as spice extract).

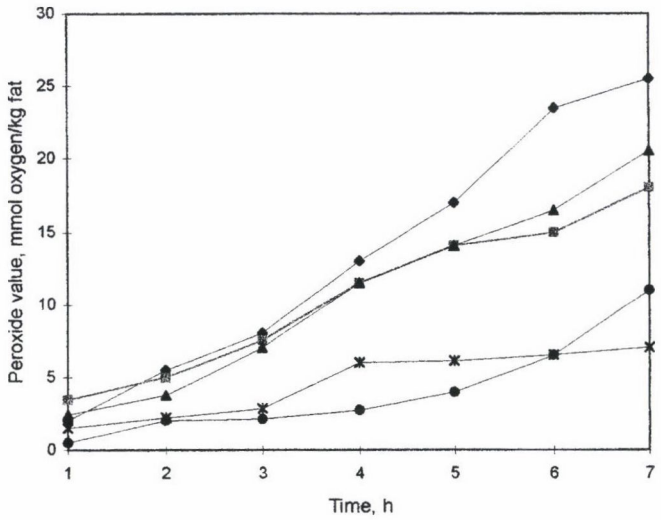

Fig. 6. Comparison between antioxidative efficiency of rosemary and sage antioxidants (ROS: rosemary extract, SAL: sage extract). ◆: control; ■: PG(0.01%); ▲: BHT(0.02%); ●: ROS(0.02%); *: SAL(0.02%)

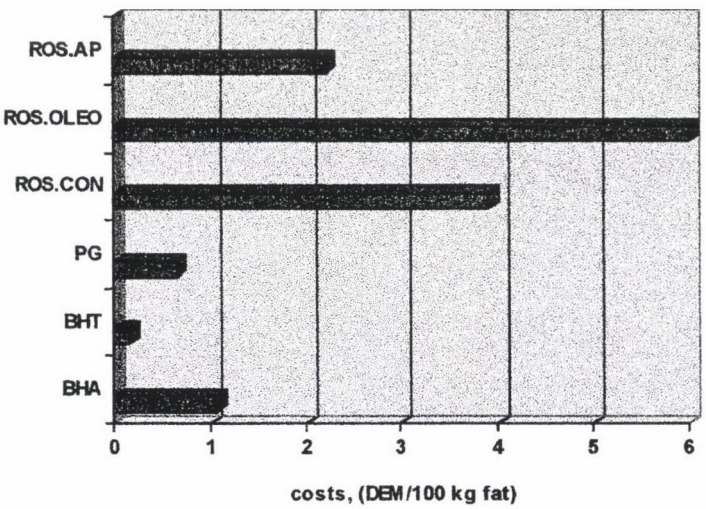

Fig. 7. Costs comparison between synthetic and natural antioxidants

3. Conclusions

In this contribution a newly developed process for isolation of antioxidative components from rosemary and sage with supercritical carbon dioxide as a solvent is presented. In food containing fats, the unsaturated fatty acids undergo autoxidation when exposed to air. This process can be inhibited by adding the antioxidants, which prolong the lifetime of the product.

Because of their convenient physico-chemical properties, supercritical fluids are being used as a solvent in extraction processes. Supercritical fluid extraction is very appropriate for isolation of natural thermolabile substances. The product do not contain residual organic solvents as in conventional extraction processes, which makes these products suitable for use in food, cosmetic and pharmaceutical industry. The efficiency of antioxidative extracts of rosemary and sage is higher compared to synthetic antioxidants such as BHA, BHT and PG. Various blends of rosemary antioxidants were tested and higher efficiency was found. The optimal process parameters for isolation of antioxidative components from rosemary and sage were: P_{ext}=47.5 MPa, T=100 °C, but in the first stage at P_{ext}=25 MPa and T=100 °C, the essential oils must be separated. The process can be performed also as a single step process where separation has to be done in two stages.

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EFFECT OF STORAGE CONDITIONS ON THE STABILITY OF PIGMENTS OF PAPRIKA (*CAPSICUM ANNUUM*) STUDIED BY HPLC AND MULTIVARIATE METHODS

P. RODRIGUES[a], H. MORAIS[a], T. MOTA[a], S. OLIVERA[a], E. FORGÁCS[b] and T. CSERHÁTI[b]

[a] Instituto Nacional de Investigacao Agraria, Oeiras, Portugal
[b] Institute of Chemistry, Chemical Research Center, Hungarian Academy of Sciences, P.O. Box 17, 1525 Budapest, Hungary

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The effect of storage conditions and the addition of ascorbic acid on the stability of the colour pigments of paprika (*Capsicum annuum*) was studied. Pigments were separated by reversed-phase high performance liquid chromatography and the influence of the storage conditions on the rate and selectivity of the decomposition was assessed by spectral mapping technique followed with two-dimensional nonlinear mapping and stepwise regression analysis. It was established that the length of storage exerts the highest impact on the decomposition rate whereas the selectivity of decomposition depends on the concentration of the added ascorbic acid, on the exposition to light and on the length of storage.

Keywords: spectral mapping technique, high-performance liquid chromatography, pigments, *Capsicum annuum*

The development and commercialization of automated chromatographic instruments enormously increased the flow of retention data/time of various substances. The evaluation of large data sets containing a high number of data is practically impossible with the traditional methods of calculation. The efficiency of many multivariate methods for the evaluation of retention data matrices has been tested. However, the overwhelming majority of methods such as principal component analysis (MARDIA et al., 1979), factor analysis (MALINOWSKI & HOWERY, 1980), and cluster analysis (WILLETT, 1987) do not separate the solvent strength and solvent selectivity, only classify the retention data taking into consideration simultaneously both solvent strength and selectivity. When the chromatographer is interested in the classification of chromatographic systems or solutes separately according to the strength and selectivity of retention the methods mentioned above cannot be used. Spectral mapping (SPM) technique developed for quantitative structure – activity relationship calculations in the design of new drugs overcomes this difficulty (LEWI, 1989). This method separates the

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strength of the effect from its selectivity. As the evaluation of the multidimensional selectivity map is difficult with traditional methods, the dimensionality of the map can be reduced by a two-dimensional nonlinear mapping (NLM) technique (SAMMON, 1969).

Multivariate methods have been frequently used for the evaluation of chromatographic retention data in food science and technology, too. Thus, principal component analysis has been successfully employed for the differentiation between various Spanish wines according to the composition of volatile aroma compounds determined by gas chromatography (GARCIA-JARES et al., 1995a; GARCIA-JARES et al., 1995b), for the assessment of the authenticity of edible oils according to the composition of fatty acids determined by GC (SCHWAIGER & VOJIR, 1994), and for the classification of raspberry cultivars according to the enantiomeric and isotopic ratio of flavour compounds (CASABIANCE & GRAF, 1994). According to our knowledge, SPM has never been applied for the evaluation of retention data of foods and food products.

The carotenoids form one of the most important groups of natural pigments found in all families of the vegetable and animal kingdoms. The natural pigments are more acceptable to consumers as they have always been present in natural foods and are readily metabolized. The carotenoids are the compounds which are responsible for the desirable colour of paprika. The carotenoid pigments are fairly stable in their natural environment but when the foodstuff is harvested they became more labile. Colour impairment during storage of paprika powder (*Capsicum annuum* L.) is a serious problem in many factories and enterprises (BIACS et al., 1992) and this degradation is attributable to many factors, namely variety of paprika (LEASE & LEASE, 1956), moisture content (MALCHEV et al., 1982), ripeness stage at harvest, and healthy state of dry fruits before grinding. In an attempt to control colour degradation of paprika powders during storage, ground seeds have been added to the paprika powder. Paprika seeds contain an effective antioxidant (τ-tocopherol) which can inhibit the oxidation of the carotenoids. Ascorbic acid exerts a considerable influence on the stability of colour pigments of paprika powders (MARTINEZ-SANCHEZ et al., 1993; CARNEVAL et al., 1995). This effect is probably due to the antioxidant effect of ascorbic acid (KANNER et al., 1977).

The objectives of our work were the separation and quantitative determination of colour pigments of paprika powders, and elucidation of the influence of various environmental conditions (time of storage, concentration of ascorbic acid added to the powder, presence or absence of light) on the stability of pigments using multivariate mathematical-statistical methods such as SPM, NLM and stepwise regression analysis.

1. Materials and methods

Fresh red pepper (*Capsicum annuum*) was purchased at the local market (Oeiras, Portugal). All solvents and reagents used were of analytical or HPLC grade (Merck, Darmstadt, Germany). Capsanthin and β-carotene standards were purchased from Extrasynthese (Genay, France). The fruits were washed, drained, and after removal of the stems and seed vessels, were chopped (approximately 8 cm^2). The pieces of the pulp were dried at a temperature of 55 °C for 2 days. After drying the pericarp was ground and passed through a sieve of 35 mesh. Ascorbic acid was mixed with the ground pulp at different weight ratios. The samples were stored in plastic bags (oxygen permeability 60 $ml/m^2/24$ h at 1 atm and 23 °C) sealed with metal clips and shrunk by heat at 90 °C. Half of the samples were kept in a dry dark place and the other half were kept in a metal box with a 40 W cool white fluorescent tube inside. Every day the tube was placed in a different position and every 18 days samples were taken for HPLC analysis. A sample with a dry weight of about 2 g was extracted with 3×15 ml of acetone in an Ultra Turrax homogenizer (IKA ULTRATURRAX T25, IKA Labortechnik, Janke & Kunkel GMBH & Co. KG Labortechnik, Staufen, Germany) and was dried in a rotary evaporator (Rotavapor Büche, R114/v, Büchi Labortechnik AG, Flawil, Switzerland) in vacuum at 35 °C. The residue was dissolved in 10 ml of acetone, filtered and stored at –20 °C until subsequent analysis by HPLC. The separation of carotenoid pigments was carried out in an HPLC system consisting of a L-4200 UV-VIS detector, a L-6200 Intelligent pump and D-2000 Chromato-Integrator (Hitachi Ltd, Tokyo, Japan), a 20 µl loop using a Lichrocart Merck end-capped, reversed-phase C_{18} column (250×4 mm i.d., particle size 10 µm), and a precolumn (Lichrocart 4 mm×4 mm, Lichrosphere 100 RP-18, particle size 5 µm, Merck, Darmstadt, Germany). The eluents were: A acetone-water 75:25 (v/v):B acetone-methanol 75:35 (v/v). The binary gradient was: from 0% B to 65% B in 10 min, to 100% B in 60 min at a flow-rate of 1 ml/min^{-1} (FISHER & KOCIS, 1987). Detection wavelength was set to 510 nm and the injection volume was 20 µl. To avoid carotenoid degradation the analyses were carried out under subdued light. Quantification of identified pigment fractions was accomplished using external standards since it has been previously proved that the method of standard addition does not offer any advantage in the HPLC analysis of carotenoids.

As we were interested in the strength and selectivity of the overall effect of treatments on the stability of colour pigments and the strength and selectivity of the overall response of pigments towards treatments we used a spectral mapping (SPM) technique to extract these pieces of information from the data matrix. Spectral mapping technique divides the information into two matrices using the logarithm of the concentration values. The first one is a vector containing so-called potency values

proportional to the overall effect of treatments on the stability of pigments, i.e. it is a quantitative measure of the effect. As the potency values are related to concentration dependent responses, a higher potency value indicates a higher efficacy of the treatments or higher tolerance of the pigments. The second matrix (selectivity map) contains the information related to the spectrum of activity, i.e., the qualitative characteristics of the effect of treatments in the various tests. SPM calculations were carried out twice:

a. The treatments were the variables and the average of the peak area of 16 pigment fractions separated by HPLC were the observations (SPM calculated the strength and selectivity of the response of pigments towards treatments).

b. The average of the peak areas of 16 pigment fractions separated by HPLC were the variables and the treatments were the observations. (SPM calculated the strength and selectivity of the effect of treatments on the stability of pigments).

As the evaluation of the multi-dimensional spectral map is fairly difficult, the dimensionality of the map was reduced to two by the two-dimensional nonlinear mapping technique. The iteration was carried out to the point where the difference between the last two iterations was less than 10^{-8}. To find the relationship between the stability of pigments and the conditions of treatments, stepwise regression analysis was employed (MAGER, 1982). In the traditional multilinear regression analysis the presence of independent variables (conditions of treatments) that exert no significant influence on the dependent variable (strength and selectivity of the effect) lessens the significance level of the independent variables that significantly influence the dependent variable. To overcome this difficulty, stepwise regression analysis automatically eliminates from the selected equation the insignificant independent variables having no significant impact on the strength and selectivity, thus increasing the information power of the calculation. The independent variables were the sampling time (days), concentration of ascorbic acid added to the paprika powder (w/w%) and the presence or absence of light. Calculation was carried out three times, the potency values and the first and second coordinates of the spectral map being separately the dependent variables. The number of accepted independent variables was not limited and the acceptance limit was set to 95% significance limit.

Similar calculations were used to elucidate the relationship between the stability of the independent pigment fractions and their lipophilicity as calculated from the retention time.

Calculations were carried out on an IBM AT computer, both spectral mapping and nonlinear mapping software were prepared by Dr. Barna Bordás, Plant Protection Institute of Hungarian Academy of Sciences, Budapest, Hungary. The software for stepwise regression analysis was the product of COMPUDRUG Ltd, Budapest, Hungary.

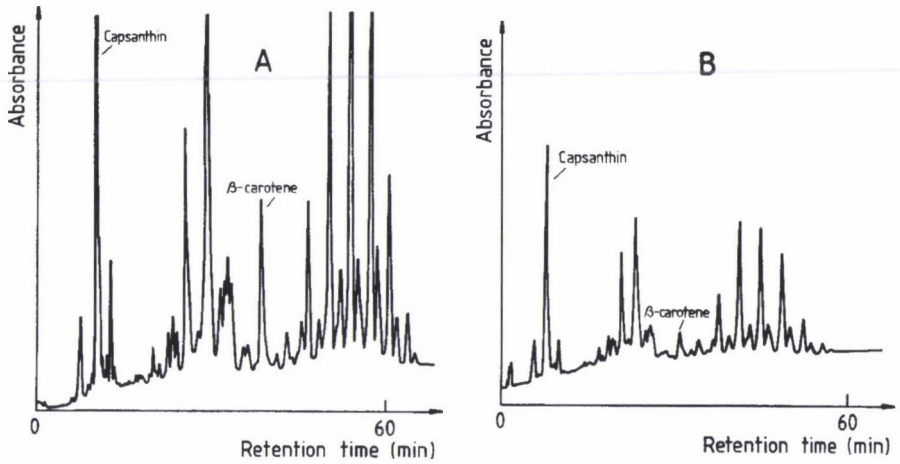

Fig. 1. Separation of the pigments of paprika powder on an octadecylsilica HPLC column. Detection wavelength 510 nm. For chromatographic conditions see Experimental. A = control; B = paprika powder containing 0.23% w/w of ascorbic acid, after 54 days of storage in light

2. Results and discussion

The pigments of paprika powder were well separated in many fractions under the reversed-phase HPLC conditions as demonstrated in Fig. 1. The chromatograms indicate that the overall pigment content of paprika powder decreases considerably during storage. Some of the quantitative data (the effect of various treatments on the pigment composition of paprika powder represented by the peak areas of various pigment fractions separated by HPLC) are listed in Table 1. The ratio of fractions is different in both the fresh and stored samples suggesting that the sensitivity of individual pigment fractions to storage conditions show considerable variations.

The potency values calculated with the spectral mapping technique showed high differences as demonstrated in Table 2. The data prove that the decomposition rate of pigments depend considerably on the environmental conditions. The response of individual pigment fractions towards treatments also show considerable diversity. This finding indicates that the stability of pigments is markedly different and the overall decomposition rate observed by traditional spectroscopic methods is the mean of the highly different decomposition rates of the individual pigment fractions. It can be also established that the selection and use of cultivars with higher ratio of stable pigments may facilitate the commercialization and storage of paprika powders.

Table 1

Effect of various storage conditions on the pigment composition of paprika powder (peak areas × 10^{-3} of pigment fractions separated by reversed-phase high-performance liquid chromatography). Concentration of ascorbic acid 0.02% w/w. A: control; B: stored in dark for 18 days; C: stored in dark for 36 days

No. of fraction	A	B	C
1	7.50	6.25	4.25
2	59.08	48.94	28.84
3	7.68	5.22	2.46
4	3.36	2.47	1.06
5	4.56	4.03	1.98
6	30.54	35.51	10.49
7	7.16	7.34	3.32
8	12.50	12.49	3.98
9	27.11	23.61	12.60
10	66.93	62.30	30.42
11	10.48	10.34	4.64
12	80.69	67.90	31.78
13	16.08	13.87	5.31
14	63.70	56.25	25.25
15	6.57	10.29	4.18
16	20.04	17.06	6.37

Table 2

Overall effect of storage conditions on the pigment content of paprika powder. Potency values calculated by the spectral mapping technique (I: control; II: after 18 days of storage in dark; III: after 18 days of storage in light; IV: after 36 days of storage in dark; V: after 36 days of storage in light)

Number of treatments	Potency values (arbitrary units)
I	98.66
II	95.96
III	78.94
IV	50.00
V	44.23

Table 3

Influence of various environmental conditions on the strength (potency) and selectivity ($spmap_1$, and $spmap_2$[a]) of the decomposition rate of pigments in paprika powder.

(Results of stepwise regression analysis; n = 24)

I Potency $= a + b_1 \cdot x_1 + b_2 \cdot x_2$

II $Spmap_1 = a + b_1 \cdot x_1 + b_2 \cdot x_2 + b_3 \cdot x_3$

Parameters	No. of equation	
	I	II
a	109.92	130.01
b_1	−1.05	0.97
s_{b1}	0.33	0.32
b_2	−21.14	21.84
s_{b2}	9.08	8.79
b_3	0	2.15
s_{b3}	0	0.53
b_1'%	68.41	31.76
b_2'%	31.59	26.01
b_3'%	–	42.23
$F_{calc.}$	8.73	11.26
$F_{99\%}$	5.85	5.85
r^2% a	45.40	62.81

x_1: time of storage (days); x_2: light; x_3: concentration of ascorbic acid added to the paprika powder (w/w%); a: intercept value of eqs I and II; b_1, b_2 and b_3: partial regression coefficients indicating the effect of the time of storage, light and the concentration of added ascorbic acid on the strength and selectivity of the decomposition of pigments, respectively; s_{b1}, s_{b2} and s_{b3}; standard deviations of b_1, b_2 and b_3, respectively; b_1'%, b_2'% and b_3'%: standard partial regression coefficients of b_1, b_2 and b_3, which are normalized to unity; $F_{calc.}$: calculated F value indicating the fitness of the equation to the experimental data; $F_{99\%}$: tabulated F value indicating the significance level of the fitness of the equation to the experimental data; r^2%: coefficient of determination expressed in %.

[a] No significant relationship was found between $spmap_2$ and the environmental conditions

The results of stepwise regression analysis are compiled in Table 3. Both the strength and selectivity of the effect of the treatments depended significantly on the environmental conditions. The length of storage and the exposure of samples to light influenced significantly (significance level over 95%) both the overall velocity of decomposition rate and its selectivity proving again the different decomposition rates of the individual pigment fractions. The concentration of ascorbic acid in the samples has

also a significant impact on the selectivity of the decomposition of pigments. This finding can be tentatively explained by the supposition that ascorbic acid selectively inhibits or slows down the oxidative deterioration of the individual pigment fractions resulting in modified selectivity of decomposition. The path coefficients (b'% values, indicating the effect of various parameters on the stability of pigments independently of their original dimensions) suggest that the effect of the environmental conditions on the stability of pigments are similar that is the length of storage, the exposure to light and the addition of ascorbic acid influence to a similar extent the decomposition rate of pigment fractions.

Although stepwise regression analysis found significant correlations between the stability parameters of paprika pigments and the environmental conditions, the ratio of variance is fairly low (see r^2 values). This result suggests that other parameters not included in the calculations may also have a considerable impact on the stability of pigments.

Treatments do not form clusters on the two-dimensional non-linear selectivity map (Fig. 2). This finding entirely supports our previous conclusions based on the results of stepwise regression analysis that each environmental condition exerts a similar impact on the decomposition of colour pigments of paprika powders.

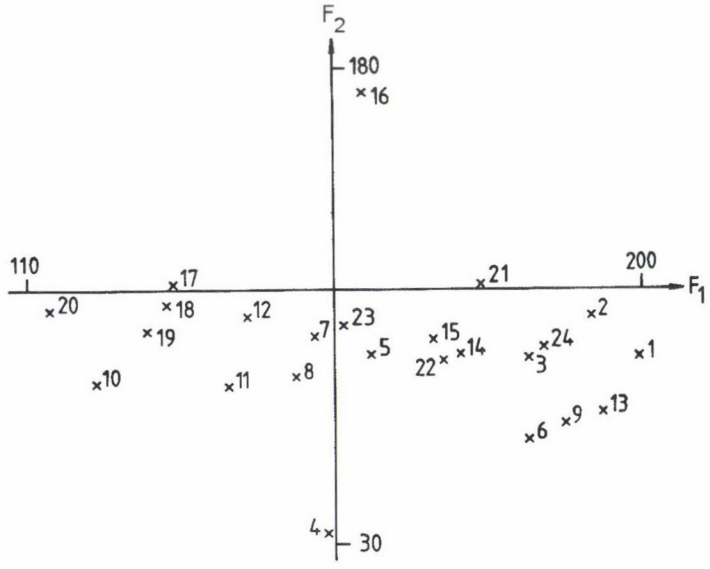

Fig. 2. Similarities and dissimilarities between the selectivities of treatments towards colour pigments in paprika (*Capsicum annuum*) powders. Two-dimensional nonlinear selectivity map. No. of iterations: 117; maximal error: $1.82.10^{-2}$

Neither the potency values of the individual pigment fractions, nor the coordinates of their two-dimensional selectivity map showed significant relationship with the molecular lipophilicity (data not shown), indicating that the hydrophobicity parameters of pigments do not influence the decomposition rate.

3. Conclusions

The data indicated that the pigments of paprika powder can be successfully separated and quantitatively determined by reversed-phase high-performance liquid chromatography using gradient elution. Multivariate mathematical-statistical methods such as spectral mapping technique combined with stepwise regression analysis can be employed for the evaluation of the retention data matrices and for the study of the impact of storage conditions on both the overall decomposition rate and the selectivity of decomposition. The calculations proved that the length of storage and the exposure to light exert a similar influence on the stability of pigments whereas ascorbic acid modifies only the selectivity of the decomposition of the individual pigment fractions.

*

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VANADIUM CONTENT OF SOME COMMON EDIBLE, WILD MUSHROOM SPECIES

J. VETTER

Department of Botany, University of Veterinary Sciences, H-1400 Budapest, P.O. Box 2. Hungary

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The vanadium content of samples of 21 common, edible wild mushroom species was measured. The mushroom samples were gathered from different locations (habitats) of Hungarian forests and the analysis was performed with ICP. The V contents were found between 0 (i.e. under the detections limit) and 1 mg/kg d.m. Bioaccumulation of V was not found, the species *Xerocomus porosporus* and *Clitocybe odora* (practically: 1 mg/kg d.m.) had the relative highest level, those of *Hypholoma capnoides*, *Laccaria amethysthina*, *Tricholoma imbricatum*, *Xerocomus subtomentosus*, are practically near to detection limit. The actual V content of these mushrooms is a result of taxonomic and ecological factors.

The V content of these common mushrooms is not problematical from toxicological point of view; some of these can be a natural source of V supplement for the human organism.

Keywords: vanadium, wild edible mushrooms

Vanadium belongs to the micro elements of living organisms which has different roles and functions in plants, animals, humans and in fungi, respectively. This element has today an environmental role, too. The effect of vanadium on plants is different and widely investigated on wheat (*Triticum aestivum*, ZADE et al., 1995) barley (*Hordeum vulgare*: NOWAKOWSKI & SADKOWSKI, 1994), bean (*Phaseolus vulgaris*: MARTIN & SACO, 1995), lettuce (*Lactuca sativa*: GILL et al., 1995), broad bean (*Vicia faba*: PETERKOVA, 1989), and on *Cicer arietinum* (ZADE et al., 1992). The problem of vanadium accumulation have been investigated in bean (*Phaseolus vulgaris*: KAPLAN et al., 1990), in pea (*Pisum sativum*: NOWAKOWSKI, 1993).

Vanadium as a micro nutrient of fungi has a relative specific situation. According to the earlier investigations, the accumulator function was established only in a non edible, poisonous species (*Amanita muscaria* = fly agaric). This fungus contains an extreme level of V in cap and stem (VETTER, 1989). The reason of this fact is the biosynthesis of the amavadine, a vanadium-binding protein molecule. This vanadium containing complex has the formule $C_{12}H_{20}N_2O_{11}$. All other fungus species, including the other *Amanita* species, contain very low level of this element. According to BYRNE

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and co-workers (1976) the average level was 0.39 mg/kg d.m., others found an average 0.22 mg/kg d.m. for 309 fungus samples. Our earlier results (VETTER, 1989) indicated V concentrations between 0 and 0.1 mg/kg d.m. in wild (edible and poisonous) fungi of Hungary. The V concentrations in the most important cultivated mushrooms are 0–0.11 mg/kg for *Agaricus bisporus* and 0.11 mg/kg d.m. for *Pleurotus ostreatus*, respectively (VETTER, 1994).

Analysis of edible wild mushroom samples was performed to compare and evaluate the V content of samples of 22 species in the last three years.

1. Materials and methods

The fruit bodies of fungi were gathered from different locates of Hungary in years 1993–1995 (see Fig. 1). The edible character of fungi was judged according to the worldwide known book of MOSER (1978). We tried to visit the same sites and to gather the same species for evaluation of the variability of V content from year to year.

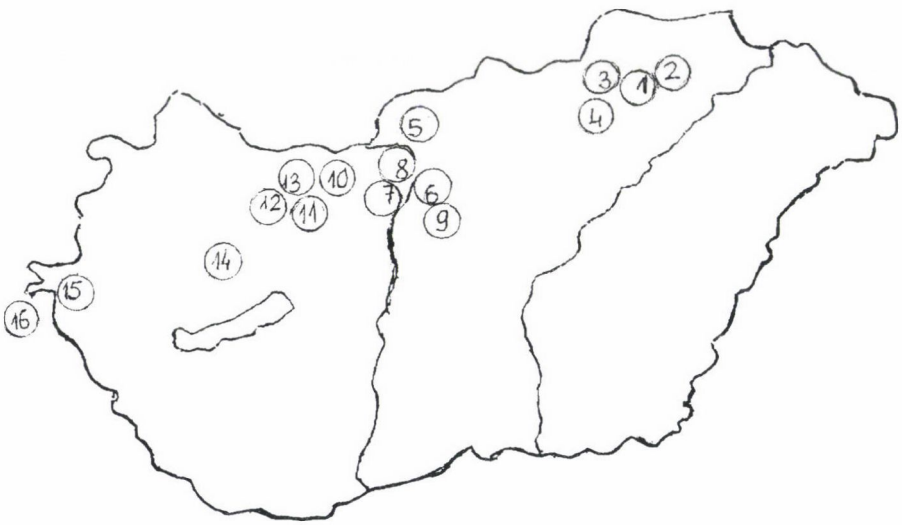

Fig. 1. The sites of gathering of the samples. (Miskolc/1 – 1; Miskolc/2 – 2; Miskolc/3 – 3; Mt. Bükk – 4; Mt. Börzsöny – 5; Halmi Wood – 6; Kamara Wood – 7; Mt. Budai – 8; Botanical Garden of Soroksár – 9; Mt. Pilis – 10; Tatabánya/1 – 11; Tatabánya/2 – 12; Tatabánya/3 – 13; Mt. Bakony – 14; Őrség – 15; Loipersdorf – 16)

The V content of fruit bodies was determined by inductively coupled plasma emission spectrometry (ICP) in trials. The V content of samples is characterized by arithmetical mean and standard deviation (SD). If the concentration was below the detection limit (it is about 0.15 mg/kg d:m.) then it is indicated by the abbreviation n.d. (not detectable). The different species are characterised by the average values of all analysed samples of the same species.

2. Results and discussion

The data of V content of analysed mushrooms are listed in Table 1 in alphabetical order of the Latin names of species. The Table contains the V content of all analysed samples and the average of the samples of the same species.

The highest average V content was measured in *Clitocybe odora* (Fig. 2) and *Xerocomus porosporus* (0.98 mg/kg d.m.), high concentrations were found in *Lactarius deliciosus*, *Lepista nuda*, *Macrolepiota rhacodes* (in all three species: 0.48 mg/kg d.m.). The second group of species has a vanadium content between 0.2–0.4 mg/kg d.m.: *Clitocybe nebularis*, *Lepista luscina*, *Macrolepiota procera*, *Stropharia aeruginosa*, *Tricholoma scalpturatum*, *T. terreum*. The third group of species contains V below 0.2 mg/kg d.m. (*Armillaria mellea*, *Laccaria laccata*, *Lepista inversa*, *Xerocomus chrysentheron*, *Lycoperdon perlatum*, *Hericium chlathorides*. The V level of the last group of species is under the detection limit (*Hypholoma capnoides*, *Laccaria amethystina*, *Lepista gilva*, *Tricholoma imbricatum*, *Xerocomus subtomentosus* (Fig. 3).

According to investigations here presented, the V content of the most common wild edible fungi is between 0 and 1 mg/kg d.m. Our earlier results (VETTER, 1989; 1994) indicated a lower average V concentration. Secondly, bioaccumulation was not found because the only accumulator species is not edible (*Amanita muscaria*).

The first logical question of discussion of our data: does a connection exist between the taxonomic position and the V content of the fungi? The answer: this is not characteristic. The analysed *Clitocybe*, *Tricholoma* or *Xerocomus* species show remarkable differences (for example: *Xerocomus chrysentheron*: 0.17, but *X. porosporus*: 0.98 mg/kg d.m.; or *Clitocybe nebularis*: 0.39; but *C. odora*: 1.01 mg/kg d.m.). The second problem is the effect of location, of samples of the same species. It is undoubtable that the different locations have different soil and other environmental parameters. Therefore, differences might occur between the samples of the same species. In the case of *Clitocybe odora* the samples of the Central Mountain of Hungary (Tatabánya) have significantly higher V contents than those from the North-Middle Mountain of Hungary (Miskolc).

It seems that the location (different soil, substrate, geography) has a real, undeniable effect on V content of mushrooms.

Table 1

Vanadium content of some wild, edible mushroom species

Species and year	Site of gathering	V content (mg/kg d.m.) (standard deviation)
Armillaria mellea (Vahl. in Fl. Dan.: Fr.)		
1993	Mt. Budai	0.26 (0.06)
1993	Miskolc/3	(n.d.)
1993	Tatabánya/3	0.27 (0.01)
1993	Tatabánya/4	0.11 (0.02)
1994	Tatabánya/2	0.90 (0.12)
1994	Miskolc/1	(n.d.)
1994	Miskolc/1	0.16 (0.07)
1994	Miskolc/2	0.12 (0.05)
1995	Mt. Budai	(n.d.)
1995	Miskolc/3	(n.d.)
1995	Tatabánya/1	0.25 (0.04)
1995	Tatabánya/2	(n.d.)
1995	Tatabánya/4	(n.d.)
1995	Kamara Wood	0.27 (0.21)
	Average:	0.16
Clitocybe nebularis (Fr.) Harmaja		
1993	Mt. Börzsöny	0.45 (0.04)
1993	Mt. Bakony	0.21 (0.03)
1994	Mt. Bükk	0.39 (0.13)
1995	Miskolc/1	0.52 (0.26)
	Average:	0.39
Clitocybe odora (Bull.:Fr) Kummer		
1993	Tatabánya/1	0.78 (0.05)
1993	Miskolc/l	0.31 (0.11)
1993	Tatabánya/2	1.89 (1.33)
1994	Miskolc/1	0.38 (0.02)
1994	Tatabánya/2	1.72 (0.10)
	Average:	1.01

Table 1 (cont.)

Species and year	Site of gathering	V content (mg/kg d.m.) (standard deviation)
Hypholoma capnoides (Fr.):Fr. Kummer		
1993	Mt. Pilis	(n.d.)
1993	Miskolc/3	(n.d.)
1994	Mt. Pilis	0.09 (0.01)
	Average:	0.03
Lactarius deliciosus Fr.		
1993	Miskolc/3	0.97 (0.28)
1995	Miskolc/3	(n.d.)
	Average:	0.48
Laccaria laccata (Scop.: Fr.) BK et Br.		
1995	Miskolc/3	(n.d.)
1995	Őrség/County Zala/	0.34 (0.08)
	Average:	0.17
Laccaria amethystina		
1995	Őrség/County Zala/	(n.d.)
1995	Loipersdorf	(n.d.)
	Average:	(n.d.)
Lepista gilva (Pers.: Fr.) Roze		
1994	Miskolc/1	(n.d.)
1994	Miskolc/1	(n.d.)
1995	Miskolc/2	(n.d.)
1995	Loipersdorf	(n.d.)
	Average:	(n.d.)
Lepista nuda (Bull.:Fr.) Cke.		
1993	Tatabánya/2	0.74 (0.09)
1993	Mt. Börzsöny	1.51 (0.04)
1993	Mt. Bakony	0.52 (0.13)
1993	Mt. Bakony	0.37 (0.26)
1995	Őrség/County Zala/	0.27 (0.04)
	Average:	0.48

Table 1 (cont.)

Species and year	Site of gathering	V content (mg/kg d.m.) (standard deviation)
Lepista luscina		
(Fr.) Sing.		
1993	Kamara Wood	0.55 (0.04)
1993	Mt. Börzsöny	0.09 (0.05)
1993	Mt. Börzsöny	0.17 (0.03)
	Average:	0.27
Lepista inversa		
(Scop.:Fr.) Pat		
1993	Halmi Wood	0.50 (0.02)
1993	Mt. Bakony	0.16 (0.11)
1993	Mt. Pilis	0.09 (0.05)
1995	Tatabánya/1	(n.d.)
	Average:	0.18
Macrolepiota rhacodes		
(Vitt.) Sing		
1993	Mt. Pilis	0.37 (0.11)
1993	Mt. Pilis	0.22 (0.09)
1993	Miskolc/3	1.49 (0.20)
1994	Miskolc/2	0.34 (0.05)
1995	Miskolc/1	0.23 (0.06)
1995	Miskolc/2	0.24 (0.15)
	Average:	0.48
Macrolepiota procera		
(Scop.: Fr.) Sing		
1993	Miskolc/2	(n.d.)
1994	Kamara Wood	0.67 (0.07)
1994	Miskolc/1	0.19 (0.09)
1995	Miskolc/1	0.23 (0.03)
	Average:	0.27
Stropharia aeruginosa		
(Curt.: Fr) Quél.		
1993	Miskolc/3	(n.d.)
1993	Miskolc/1	0.47 (0.06)
1994	Mt.Bükk	0.22 (0.06)
	Average:	0.23

Table 1 (cont.)

Species and year	Site of gathering	V content (mg/kg d.m.) (standard deviation)
Tricholoma imbricatum (Fr.:Fr.) Kummer		
1993	Miskolc/3	(n.d.)
1994	Miskolc/3	(n.d.)
1995	Miskolc/3	(n.d.)
	Average:	(n.d.)
Tricholoma scalpturatum (Fr.) Quél.		
1993	SBK	0.12 (0.04)
1993	Tatabánya/1	0.40 (0.02)
1993	Tatabánya/4	0.35 (0.06)
	Average:	0.29
Tricholoma terreum (Schff.:Fr.) Kummer		
1993	SBK	0.56 (0.10)
1995	SBK	0.17 (0.06)
	Average:	0.36
Xerocomus subtomentosus (L:Fr.) Quél		
1993	Halmi Wood	(n.d.)
Xerocomus chrysentheron (Bull.: St. Amans) Quél.		
1993	Halmi Wood	0.14 (0.04)
1993	Mt. Börzsöny	0.45 (0.11)
1994	Miskolc/2	0.12 (0.07)
1995	Miskolc/1	(n.d.)
	Average:	0.17
Xerocomus porosporus Imler		
1993	Mt. Börzsöny	0.79 (0.05)
1993	Mt. Börzsöny	1.18 (0.22)
	Average:	0.98

Table 1 (cont.)

Species and year	Site of gathering	V content (mg/kg d.m.) (standard deviation)
Lycoperdon perlatum (Pers.: Pers.)		
1993	Mt. Pilis	0.17 (0.04)
1993	Miskolc/3	0.14 (0.03)
1993	Tatabánya/1	0.48 (0.11)
1994	Miskolc/1	0.34 (0.03)
1994	Miskolc/1	0.14 (0.05)
1994	Miskolc/1	0.11 (0.06)
1994	Miskolc/2	(n.d.)
1995	Miskolc/1	(n.d.)
	Average:	0.17
Hericium chlatrhoides		
1993	Mt. Bükk	(n.d.)
1994	Tatabánya/2	(n.d.)
1994	Mt. Budai	0.21 (0.03)
	Average:	0.07

(Abbreviations: SBK:Botanical Garden of Soroksár; n.d.: not detectable)

Fig. 2. *Clitocybe odora*, a mushroom species of relatively high V content

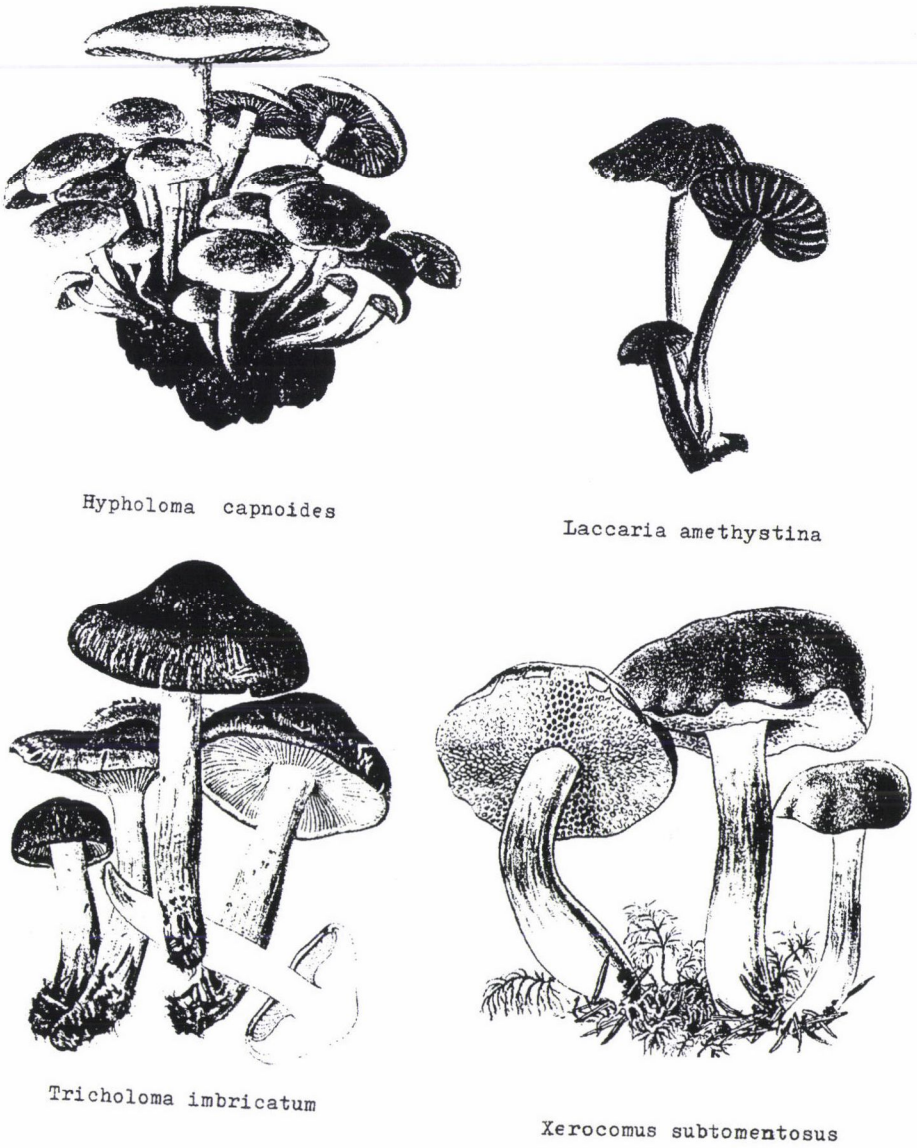

Fig. 3. Mushroom species containing V under the detection limit (after MICHAEL et al., 1958–1970)

The last question: the effect of nutrition and life types of mushrooms (i.e. mycorrhizal, saprotroph or wood rotting species) on the vanadium content. Most of the species belong to saprotroph type, some are mycorrhizal (*Xerocomus*) and some belong to wood rotting species (*Hypholoma capnoides*, *Armillaria mellea*). From that point of view we can not see such differences or connections.

The analysed, common edible mushroom species are valuable food sources generally and have a supplemental role in the modern food practice (protein content and quality, low lipid and energy contents, positive aromatic substances, different minerals /P, K/ and vitamins). Our results do not indicate bioaccumulation of V in the analysed wild, edible species, on the other hand these species can have a role in the normal mineral supply for humans, too.

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EXTRACTIVE FERMENTATION OF ETHANOL USING ALGINATE GEL CO-ENTRAPPED YEAST CELLS (*SACCHAROMYCES BAYANUS*) AND LIPASE ENZYME

M. A. KISS[a], T. VENYIGE[b], É. STEFANOVITS-BÁNYAI[a], CS. SISAK[b] and L. BOROSS[a]

[a] University of Horticulture and Food Industry, Dept. of Chemistry and Biochemistry, H–1518 Budapest, P.O. Box 53. Hungary

[b] Institute of Chemical Engineering of the Hungarian Academy of Sciences, Dept. of Bioengineering, H–8200 Veszprém, P.O. Box 125. Hungary

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Our earlier experiments were further continued with systems containing gel particles in oleic acid. In these cases the fermentation culture media were inside the gel beads only. To build such systems we prepared complex sterile solution containing sodium-alginate, yeast cells, their additives and various amount of glucose (100–400 g l^{-1}). These solutions were dropped in Ca-chloride precipitating solutions. The gel beads were suspended directly in oleic acid.

The glucose was uptaken by yeast cells quantitatively during 24–72 h. The uptake rate of glucose is dependent on the concentration of glucose. It was similar to the free cell fermentation: in cases of higher concentrations of glucose the glucose uptake in the second period was stopped.

In accordance with changes of the concentration of glucose the concentration of ethanol increased rapidly in the first day of fermentation and thereafter slowed down.

We supposed that by co-immobilization of lipase in the same gel particles the inhibitory effect of produced ethanol would be solved. Using of lipase we can transform ethanol to ethyl-oleate, which is soluble in oleic acid. According to our data, at least 4 U ml^{-1} lipase is needed to increase ethanol production significantly.

Summarising we can conclude that in our system the maximum ethanol production can be achieved using 100 g l^{-1} glucose and 4 U ml^{-1} lipase for 48 h. In this system 50 g l^{-1} ethanol can be produced by 48 h fermentation and practically a steady state concentration of ethanol is maintained after it.

Keywords: extractive fermentation, ethanol, oleic acid, co-entrapped yeast and lipase

In our previous experiments for ester synthesis using immobilised biocatalysts a problem was arisen. In aqueous phase lipase catalyses hydrolysis instead of synthesis. We wanted to minimise the rate of aqueous phase by constructing a fermentation system, in which the glucose and other nutrients of fermentation were dissolved inside the gel beads. The gel particles were surrounded only by oleic acid.

In the extractive fermentation of ethanol, the ethanol producing (for this reason ethanol tolerant) yeast strain and the lipase enzyme are used simultaneously (AIRES-BARROS et al., 1987; MARQUES et al., 1989).

The biological function of lipase enzymes (EC 3.1.1.3.) is to catalyse the hydrolysis and/or synthesis of triglycerides. The molecules of lipase are acting at the interface of the aqueous and the organic phase, moreover this interface is the main necessity to establish the activity of enzyme (KNEZ et al., 1990).

The base of the integrated extractive fermentation process is that the generated ethanol, which is one of the substrates of lipase is removed from the fermentation medium via extractive enzyme reaction (OLIVIERA & CABRAL, 1991).

Entrapment of viable cells into a Ca-alginate gel is a gentle and simple kind of immobilisation. It efficiently protects cells against mechanical harm and inhibition effect of high substrate concentration. Protecting effect of Ca-alginate gel results in higher number of viable cells by one or two orders of magnitude within the gel than that of in a free cell fermentation.

Saccharomyces bayanus cells and lipase enzyme originated from *Mucor miehei* were co-immobilized in Ca-alginate gel beads. The glucose and other nutrients (N-sources, mineral salts) were dissolved inside the gel beads. The generated ethanol – diffusing to the interface of the aqueous and organic phase, surrounding the gel beads – is capable to take part in lipase catalysed ester synthesis reaction with oleic acid.

That process is suitable for biofermentation of other ethyl esters, too.

1. Materials and methods

1.1. Chemicals

Sodium-alginate (medium viscosity); β-nicotinamide-adenine-dinucleotide (NAD^+); 3,5-dinitro-salicylic acid are from SIGMA (St. Louis, USA).

Oleic acid and the other chemicals are from REANAL Ltd. (Budapest, Hungary).

1.2. Enzyme

Lipase (EC 3.1.1.3.) originated from *Mucor miehei*, is a gift from NOVO Industry A/S (Copenhagen, Denmark).

1.3. Yeast strain

Saccharomyces bayanus, is a gift from Instituto Superior Tecnico (Lisbon, Portugal). This ethanol producing yeast strain was selected in L'Institute d'Oenologie de Paris (Paris, France).

1.4. The fermentation

The aqueous phase was only inside the gel beads, thus all of the substrates of fermentation were inside the gel. The content of the gel solution ("fermentation broth"): 30 g l^{-1} sodium-alginate, different amount of glucose (100–400 g l^{-1}), 5.0 g l^{-1} peptone, 5.0 g l^{-1} yeast extract, 5.0 g l^{-1} ammonium-sulphate, 1.0 g l^{-1} potassium-dihydrogen-phosphate, 0.5 g l^{-1} magnesium-sulphate. The gel beads were being shaken in Erlenmeyer flasks on water heated shaker at 28 °C temperature. The volume ratio of gel beads and oleic acid was 1:3.

1.5. Immobilisation of cells and enzyme

The cell suspension (at the start 10^6 per ml gel solution), the lipase (1 or 4 unit per ml of gel solution) and the 30 g l^{-1} Na-alginate gel solution, containing all substrates, were dropped into 20 g l^{-1} Ca-chloride solution, which was stirred (the precipitating solution containing glucose at the same concentration of gel solution), to avoid the dissolving of glucose out of the gel beads. After hardening of the gel the Ca-chloride solution was removed and the gel beads were replaced onto oleic acid.

1.6. Dissolution of gel beads

To measure the ethanol and glucose concentration and to count the cell number inside the beads the gels have to be dissolved. The gel-dissolvent (pH 9.2) contains 20 g l^{-1} NaCl and 2.7 g l^{-1} Selecton B_2. By increasing the pH and concentration of EDTA the dissolution of gel beads can be accelerated. The method above is suitable to dissolve the gel beads from layer to layer (BOROSS et al., 1990).

1.7. Analytical processes

1.7.1. Determination of glucose concentration. The glucose concentration inside the gel particles was measured by the dinitro-salicilic-acid method.

1.7.2. Determination of ethanol concentration. The ethanol concentration inside the gel particles was measured by the enzymatic analytical method based on the Boehringer method. The root of this method is that the ethanol, in the presence of NAD^+ coenzyme, and in a reaction catalysed by alcohol-dehydrogenase (EC 1.1.1.1) turned into acetaldehyde. The conversion of $NAD^+ \Rightarrow NADH+H^+$ was detected on the spectrophotometer at 340 nm wavelength, therefore we measured the concentration of NADH, which is proportional to the concentration of ethanol of the sample.

1.7.3. Counting of cell number. After dissolving the gel particles the cell number was determined by measuring the optical density at 530 nm wavelength.

2. Results

2.1. Kinetics of cell number during the fermentations

The cell number inside the gel particles decreases with increasing glucose concentration. In case of a gel containing 100 g l^{-1} glucose the cell number increases by two orders of magnitude (about 100-fold), while in the presence of 400 g l^{-1} glucose the increase is only 10-fold.

Because of the outside organic phase (oleic acid) the cells grow only inside the gel particles.

Figure 1 shows the time dependence of cell number in the presence of different glucose concentration. In case of gels containing 100 or 200 g l^{-1} glucose cell numbers reach the maximum value (10^8 cells per ml gel) in a 24 h period and after that time it stays at the same level. However, in case of higher glucose concentration (300–400 g l^{-1}) the maximum cell number appears later, after 36–48 h. The concentration of lipase has no influence on the kinetics of cell number, the glucose concentration is the only limiting factor.

2.2. Kinetics of glucose concentration during the fermentation

The glucose consumptions are shown in Fig. 2 in case of different initial loading glucose concentrations.

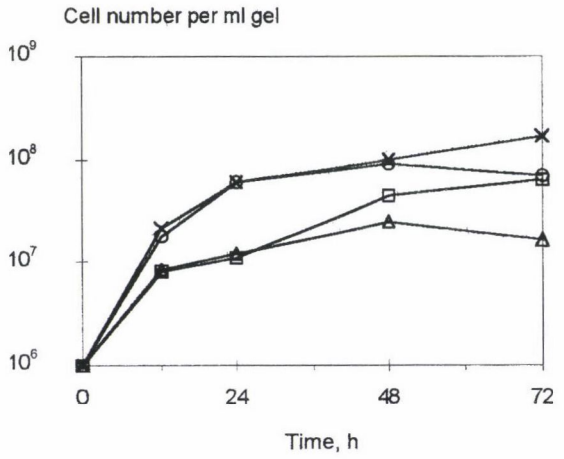

Fig. 1. Increasing of the cell number during 72 h fermentation in the presence of different glucose concentration. Initial glucose concentration of gel beads, g l^{-1}: ⊠ : 100; ⊖ : 200; ⊟ : 300; ⍋ : 400

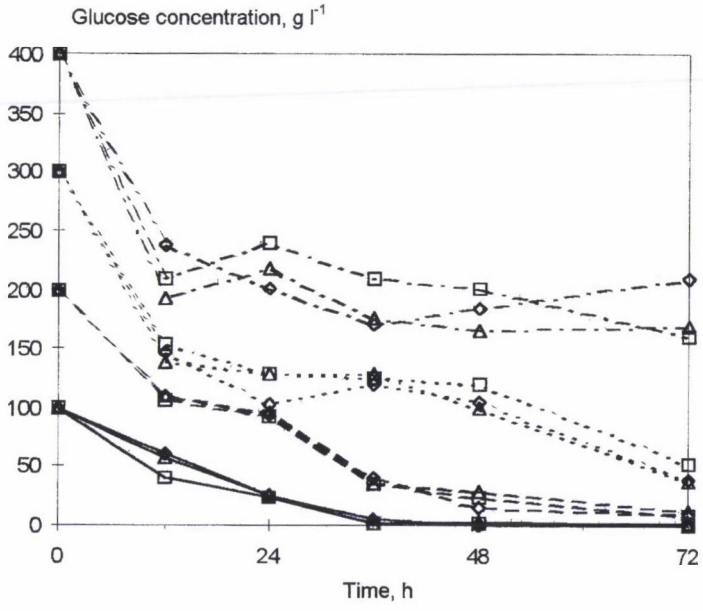

Fig. 2. Consumption of glucose in the presence of 100–400 g l^{-1} initial glucose concentration of gel beads. Initial glucose: —: 100 g l^{-1}; - - - -: 200 g l^{-1}; · · · ·: 300 g l^{-1}; – · – · –: 400 g l^{-1}; ◇: control; □: 1 U ml^{-1} lipase; △: 4 U ml^{-1} lipase

In the gel beads containing 100 g l^{-1} glucose the total amount of glucose is consumed by yeast cells in 36 h.

It can be seen that no more than 200 g l^{-1} of initial glucose concentration can be applied to avoid the inhibitory effect of extra glucose in cell growth. Since there is no substantial difference in cell growth using either 100 or 200 g l^{-1} of glucose (cf. Fig. 1) we suggest that in our system 100 g l^{-1} of glucose seems to be optimal as far as the cell number is concerned.

Apparently the lipase has not any effect on the glucose uptake of yeast cells.

The consumption of glucose inside the gel particles showed the kinetic of free cell fermentations (Fig. 3). In gels containing higher concentration of glucose (300–400 g l^{-1}) the cells do not consume the total amount of glucose.

2.3. Kinetics of ethanol concentration during the fermentation

Ethanol production of cells in the presence of 100–300 g l^{-1} of glucose are shown in Figs 4–6. It can be seen that with increasing glucose concentration the efficiency of ethanol production is decreased. The highest quantity of ethanol was produced in the presence of 100 g l^{-1} glucose.

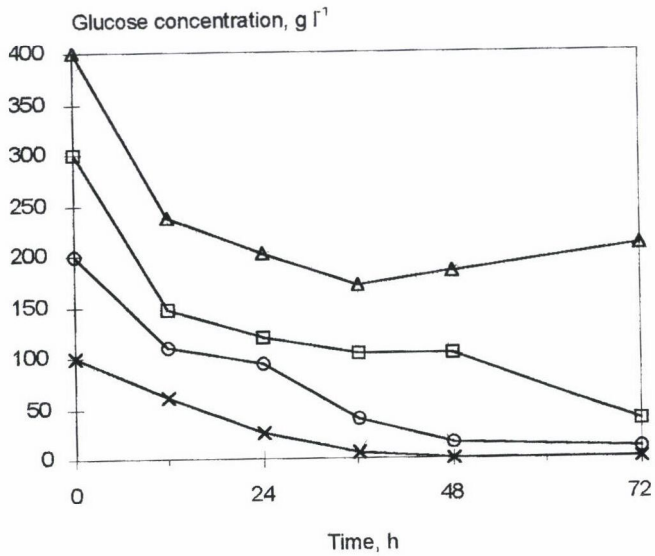

Fig. 3. Consumption of glucose inside the gel particles (in fermentations without lipase). Initial glucose concentration of gel beads, g l^{-1}: ⊠: 100; ⊖ : 200; □ : 300; △ : 400

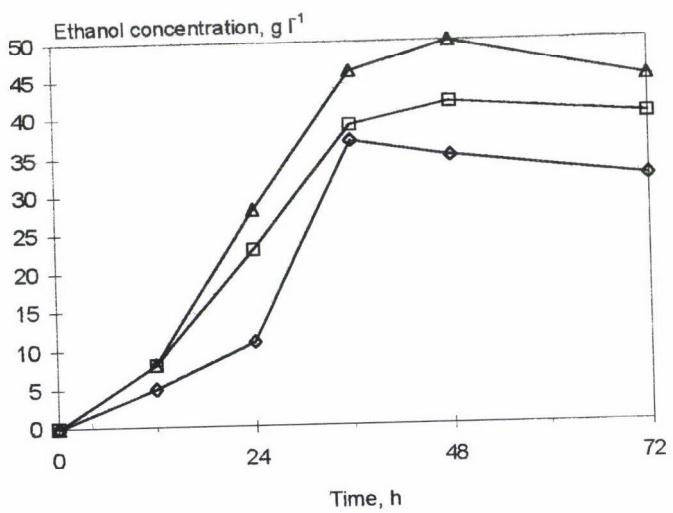

Fig. 4. Production of ethanol in the presence of 100 g l^{-1} initial glucose concentration of gel beads. ◇ : Control; □: 1 U ml^{-1} lipase; △: 4 U ml^{-1} lipase

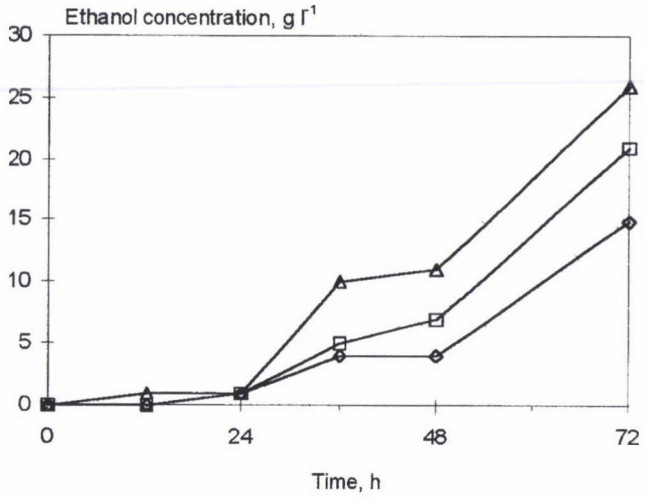

Fig. 5. Production of ethanol in the presence of 200 g l^{-1} initial glucose concentration of gel beads. ◇: Control; □: 1 U ml^{-1} lipase; △: 4 U ml^{-1} lipase

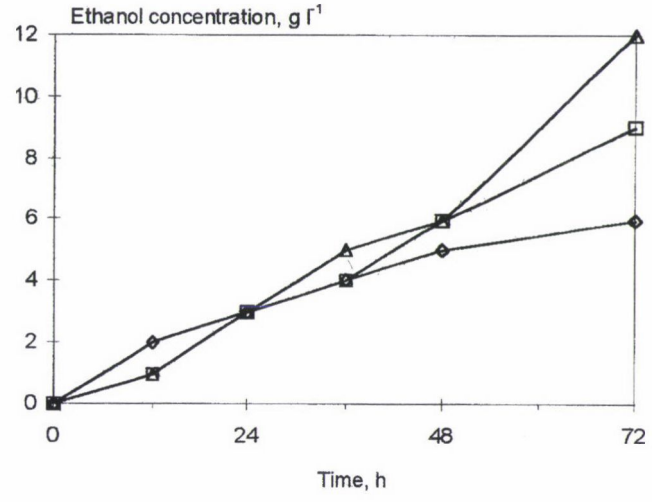

Fig. 6. Production of ethanol in the presence of 300 g l^{-1} initial glucose concentration of gel beads. ◇: Control; □: 1 U ml^{-1} lipase; △: 4 U ml^{-1} lipase

According to our data at least 4 U ml^{-1} lipase is needed to increase ethanol production significantly (Figs 4–6).

Summarising we can conclude that in our system the maximum ethanol production can be achieved using 100 g l^{-1} glucose and 4 U ml^{-1} lipase for 48 h. In this system 50 g l^{-1} ethanol can be produced (Fig. 4) by 48 h fermentation and practically a steady state concentration of ethanol is maintained after it.

3. Discussion

In the literature there was not any reference found similar to this process.

The experiments under these conditions – the aqueous phase inside the gel particles – were similar to a free cell fermentation. This method of gel-entrapment has not got a real preventing function on the viable cells against the harmful effect of high glucose concentration. On the other hand using this immobilisation process, the cells can be protected against the effects of ethanol and oleic acid. Our results show that the cells are capable to produce ethanol under these circumstances.

At this stage of our experiments we determined only the consumption of glucose and the amount of ethanol produced and not the formation of other by-products and formation of ethyl-oleate. Under stress conditions (i.e. immobilisation, high glucose concentration) the amount of by-products is increased (SROKA & RZEDOWSKI, 1993; TAIPA et al., 1993). Maybe that is the reason of the different amounts of potentially and effectively produced ethanol. We supposed that using co-entrapment of lipase in the same gel particles, where the yeast cells were entrapped, can solve the problem of ethanol inhibition by esterification of produced ethanol with oleic acid.

The experiments to characterise the diffusion rate of ethanol and the esterification reactions are in progress, because our main purpose is to produce ethyl-oleat (and other flavour esters) by extractive fermentation process.

*

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PUMPKIN AND CANOLA SEED PROTEINS AND BREAD QUALITY

E. H. MANSOUR[a,d] E. DWORSCHÁK[b], ZS. POLLHAMER[c], Á. GERGELY[b] AND J. HÓVÁRI[b]

[a] Department of Food Science and Technology, Faculty of Agriculture, Menofiya University, Shibin El-Kom. Egypt
[b] National Institute of Food Hygiene and Nutrition, H–1097 Budapest, Gyáli út 3/a. Hungary
[c] Company of Baking Industry, 8000 Székesfehérvár, Palotai út 117. Hungary

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Incorporation of pumpkin seed meal, pumpkin protein concentrate and isolate and canola protein concentrate and isolate in the production of breads was evaluated. Pumpkin seed meal and protein concentrate and canola protein concentrate and isolate can be added to wheat flour up to 18% protein concentration, while pumpkin protein isolate can be added to wheat flour up to 22% protein concentration without a detrimental effect on dough or loaf quality. Further incorporation larger than 18 and 22% protein concentration yielded dense, compact, unacceptable breads. The addition of pumpkin and canola proteins increased the protein, lysine and mineral (Ca, P, Cu, Fe and Mg) contents of the breads by 11–38%, 90–200% and 70–135%, respectively. Lysine and tyrosine were the first limiting amino acids for control breads and bread enriched with canola protein concentrate, respectively. Valine was the first limiting amino acid in breads enriched with canola protein isolate, pumpkin seed meal, pumpkin protein concentrate and isolate. The chemical scores and essential amino acid indices of breads enriched with pumpkin and canola proteins were increased by 55–80% and 14–27%, respectively, compared to chemical scores and essential amino acid indices of control breads.

Keywords: Pumpkin seed, canola seed, wheat flour, bread quality, lysine

The nutritional quality of wheat protein is low in certain amino acids, especially lysine. The nutritional value of wheat flour can be improved by replacing part of the wheat flour with legumes and oil seeds. Soybean, cottonseed, sesame, sunflower, peanut flours and selected other protein sources are used to produce bread with a high protein content and quality (ROONEY et al., 1972; KHAN et al., 1975; GONZALEZ-GALAN et al., 1991; YUE et al., 1991).

MANSOUR and co-workers (1992, 1993a, 1993b, 1993c) investigated the preparation, functional properties and nutritional quality of pumpkin seed meal,

[d] To whom correspondence should be addressed. Phone & Fax No. 20 48 223942

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pumpkin protein concentrate and isolate and canola protein concentrate and isolate. Pumpkin and canola seed proteins exhibit unique functional properties, and high lysine content, which suggest their incorporation in bakery products.

The objective of this study was to determine the effect of partial replacement of wheat flour by pumpkin seed meal, pumpkin protein concentrate and isolate, canola protein concentrate and isolate and protein concentrations of pumpkin and canola protein blends on the physical properties of dough breads and loaf volume, sensory properties, chemical composition and amino acid profile of breads.

1. Materials and methods

1.1. Wheat flour

Wheat flour (60% extraction) of Alföld cultivar (*Triticum aestivum*) was obtained from the Baking Industry Company (Székesfehérvár, Hungary). The flour contained 15.8% crude protein.

1.2. Pumpkin and canola seed proteins

Defatted double zero canola (*Brassica napus*) meal and peeled pumpkin (*Cucurbita pepo* cv. kakai 35) seeds were obtained from the Research Institute of the Vegetable Oil and Detergent Industry (Budapest, Hungary). Defatted pumpkin seed meal (PM) was prepared by extraction of the ground seeds with hexane in a Soxhlet apparatus. Hexane was removed from the extracted ground seeds by heating at 50 °C for 2 h. The resultant defatted meal was ground and passed through a 400 µm sieve.

Pumpkin protein concentrate (PPC) and pumpkin protein isolate (PPI) were prepared according to the method of MANSOUR and co-workers (1993a). The percentage of crude protein in PM, PPC and PPI, was 72.1, 76.7 and 96.3, respectively. Canola protein concentrate (CPC) and canola protein isolate (CPI) were prepared as described earlier (MANSOUR et al., 1992). The percentage of crude protein in CPC and CPI was 89.4 and 92.2% respectively.

1.3. Preparation of pumpkin and canola seed proteins–wheat flour blends

Each of pumpkin and canola seed proteins partially replaced wheat flour to produce blends with final protein concentrations 18%, 20%, 22%, 24% and 25%. Quantities of pumpkin and canola seed proteins blended with wheat flour are presented in Table 1.

Table 1

Pumpkin and canola seed proteins–wheat flour blends

Protein concentration percentage in blends	Pumpkin and canola seed proteins (g/100 g blend)				
	PM	PPC	PPI	CPC	CPI
18	3.91	3.61	2.73	2.99	2.88
20	7.46	6.90	5.21	5.71	5.50
22	11.01	10.08	7.70	8.42	8.12
24	14.56	13.46	10.19	11.14	10.73
25	16.34	15.11	11.43	12.50	12.04

1.4. Baking and loaf volume

The procedure of POLLHAMER (1981) was used for baking and loaf volume determination. For each test, 50 g flour, 0.3 g dry yeast, 0.5 g salt and water (as determined by a farinograph) were incorporated. The dough was kneaded for 3 min in the 100 g pan of the farinograph. The dough was fermented at 30 °C for 70 min in a raising cabinet.

The fermented dough was placed in a slightly oiled gradual glass cylinder and levelled with a cylindrical piece of wood. The dough was left at 30 °C for 60 min in a raising cabinet. The bread was baked in an oven at 260 °C for 15 min. After cooling at room temperature (~20 °C) the mean volume of the bread was evaluated from the side of the gradual glass cylinder.

Breads were divided into two parts, one for the sensory properties evaluation and others were dried at 40–45 °C for 12 h. in an electric air draught oven. The dried breads were ground (National Matsushita Elec. grinder Ind. Co., LTD., Japan) and passed through a 400 μm sieve. The ground breads were packed in screw cap glass jars and left at room temperature for chemical analysis.

1.5. Physical properties of dough

Water absorption, dough stability and dough softening of the selected blends were determined with a farinograph with a 100 g mixing bowl according to A.A.C.C. (1983).

1.6. Analytical methods

Moisture content (method No. 14.004), crude protein (method No. 2.057), crude oil (method No. 7.056) and total ash (method No. 14.006) were determined according to A.O.A.C. (1980).

Proteins were hydrolyzed in 6M HCl at 110 °C for 24 h in a nitrogen atmosphere. Amino acids were determined using a Mikrotechna AAA 881 (Prague, Czech) automatic amino acid analyzer according to the method of MOORE and STEIN (1963). Sulfur containing amino acids were determined after performic acid oxidation (MOORE, 1963). Tryptophan was chemically determined by the method of MILLER (1967).

Chemical scores of amino acids were calculated using the FAO/WHO (1973) reference pattern. Essential amino acid index (EAAI) was calculated according to OSER (1959).

Phosphorus was assayed photometrically according to A.O.A.C. (1980). Na, K and Ca were determined according to the method of LINDNER and DWORSCHÁK (1966) using a Flamom flame photometer (Budapest, Hungary). Cu, Zn, Fe, Mn, and Mg were measured by Perkin Elmer Model 403 atomic absorption spectrophotometer (Überlingen, Germany).

1.7. Sensory properties

The freshly sliced bread was cut into 5×5 cm pieces and served to trained sensory panelists at room temperature. Five trained members of the Baking Industry Company (Székesfehérvár, Hungary) evaluated the breads for taste, odor, crust color and crumb color using a 5-point scale, where 5 = excellent, 4 = good, 3 = satisfactory, 2 = fair and 1 = poor.

1.8. Statistical analysis

Loaf volume, sensory properties and chemical composition data were analyzed using Statistical Analysis System (SAS, 1985). Significant differences between treatments were determined at the 5% level.

2. Results and discussion

Addition of pumpkin and canola proteins to wheat flour resulted in an increase in water absorption (Fig. 1). Blends of wheat flour with PM, PPC and CPI absorbed more water than blends of wheat flour with PPI and CPC, except the blends of wheat flour with 18% PM. Blends of wheat flour with PPC at 18, 20, 22, 24, 25% protein concentration absorbed more water than other blends, except the blend of wheat flour

with 25% CPI. RASCO and co-workers (1990), GONZALEZ-GALAN and co-workers (1991) and YUE and co-workers (1991) reported that water absorption of wheat flour increased substantially with the addition of 5–15% native sunflower protein concentrate and isolate.

Blends of pumpkin and canola proteins decreased dough stability, except the blends of wheat flour with 18 and 20% PPC (Fig. 2). The blend of wheat flour with 18% PPC exhibits slight improvement in the dough stability compared to wheat flour. ANJUM and co-workers (1991) reported that the dough stability was decreased by increasing the concentration of sunflower protein concentrate and isolate.

Dough softening was 110 BU for wheat flour (Fig. 3). Blends of wheat flour with pumpkin and canola proteins were higher than 110 BU, except for blends of wheat flour with PM up to 20% or PPC up to 24%. Dough softening of the blends of wheat flour with PM up to 20% or PPC up to 24% was improved (less than 110 BU) compared to wheat flour. RANGA RAO and co-workers (1980) reported that incorporation of wheat flour with 5–20% wheat germ on weight basis decreased water absorption, stability and softening of bread dough.

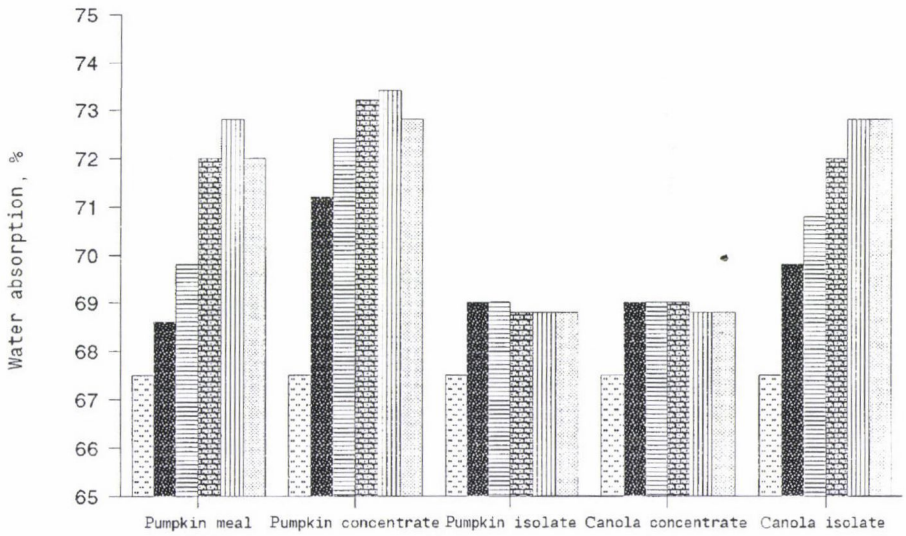

Fig. 1. Water absorption of protein–wheat flour blends. : Control; : 18% protein level; : 20% protein level; : 22% protein level; : 24% protein level; : 25% protein level

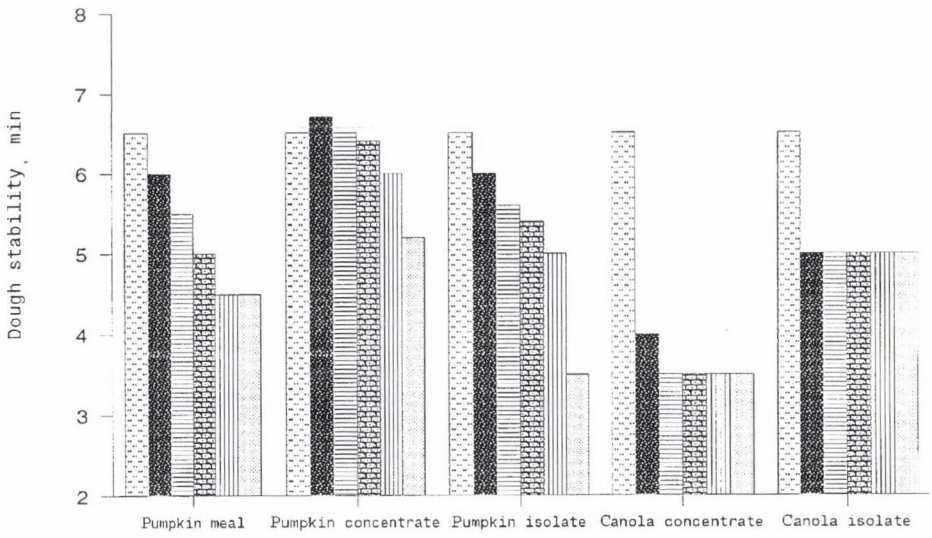

Fig. 2. Dough stability of protein–wheat flour blends. : Control; : 18% protein level; : 20% protein level; : 22% protein level; : 24% protein level; : 25% protein level

Increasing the concentrations of pumpkin and canola proteins in bread flour decreased the loaf volume of breads (Table 2). There was no significant difference ($P>0.05$) in loaf volume between control breads and breads containing PPI at selected protein concentrations. However, the loaf volume of the control bread was significantly larger ($P<0.05$) than that of breads containing PM, PPC, CPC or CPI. Breads containing PPI were significantly ($P<0.05$) larger in loaf volume than breads containing PM, PPC, CPC or CPI.

There were no significant differences ($P>0.05$) in taste and odor among breads containing up to 18% PPC or CPI or up to 20% PM, PPI or CPC. Also, breads containing 22% PM, PPI or CPC were not significantly different in odor from each other and control breads. However, breads containing high protein concentrations received lower taste and odor scores ($P<0.05$) than control breads.

There was no significant difference ($P<0.05$) in crust color between control breads and breads containing PPC, PPI or CPC up to 25%, PM up to 22% or CPI up to 20% protein concentration.

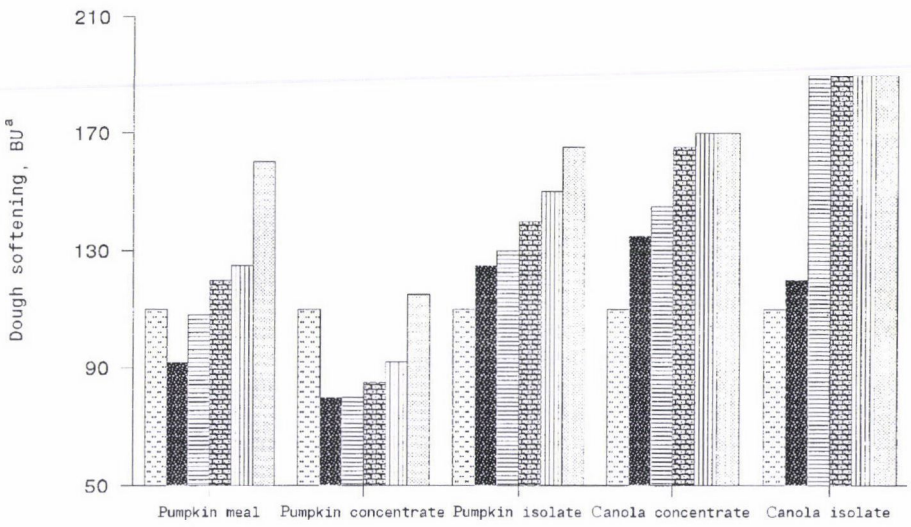

Fig. 3. Dough softening of protein–wheat flour blends. [a]Brabender units; : Control; : 18% protein level; : 20% protein level; : 22% protein level; : 24% protein level; : 25% protein level

There were no significant differences ($P>0.05$) in crumb color among breads containing up to 18% PM or PPC, 20% CPC or CPI, or 22% PPI. However, breads baked with higher protein concentrations were darker in crumb color than control breads. Bread quality decreased as the concentration of pumpkin or canola proteins increased. HANSMEYER and co-workers (1976) and YUE and co-workers (1991) reported a detrimental effect on loaf quality with the addition of succinylated sunflower protein concentrate and isolate to breads. Flour blends containing 5% native sunflower protein concentrate and isolate produced acceptable breads, but the quality of the breads deteriorated at 10–15% of the same fractions.

Bread baked with PPI was similar in moisture content to control bread (Table 3). However, the breads baked with PM, PPC, CPC and CPI were significantly ($P<0.05$) higher in moisture content than control breads. Addition of PPI to bread increased ($P<0.05$) the protein content by 38%, however, the other protein additives increased ($P>0.05$) by 11–12%. There were no significant differences ($P>0.05$) in fat or ash content between control breads and other breads, except breads containing PPI which have a lower ash content than other breads. RASCO and co-workers (1989) reported that partial incorporation of wheat flour with distillers dried grains from soft white winter wheat flour increased crude protein content in breads.

Table 2

Pumpkin and canola proteins–wheat flour blends and bread quality

Bread	Loaf volume cm^3	Taste	Odor	Crust color	Crumb color
Control	240	4.0	4.0	3.0	4.6
Pumpkin seed meal					
18%	205*	4.0	4.0	4.0	4.0
20%	180**	4.0	3.3	3.0	3.7*
22%	170**	3.0*	3.3	3.0	3.0**
24%	165**	2.7**	2.7**	2.0**	2.0**
25%	160**	2.7**	2.3**	2.0**	2.0**
Pumpkin protein concentrate					
18%	205*	3.7	4.0	3.0	4.0
20%	180**	3.0*	3.0*	3.0	3.7*
22%	170**	2.0**	3.0*	3.0	3.0**
24%	160**	2.0**	2.0**	3.0	2.0**
25%	160**	2.0**	2.0**	3.0	2.0**
Pumpkin protein isolate					
18%	225	4.0	4.0	3.0	4.3
20%	225	3.7	3.7	3.0	4.3
22%	230	3.7	3.7	3.0	4.0
24%	225	2.3**	3.0*	3.0	3.0**
25%	225	2.0**	2.3**	3.0	3.0**
Canola protein concentrate					
18%	175**	3.7	4.0	3.0	4.3
20%	170**	3.3	4.0	3.0	4.0
22%	160**	3.0*	4.0	3.0	3.7*
24%	155**	2.3**	3.0*	3.0	3.0**
25%	155**	2.0**	3.0*	3.0	3.0**
Canola protein isolate					
18%	195*	3.7	3.7	2.7	4.0
20%	175**	3.0*	3.0*	2.3	4.0
22%	165**	2.7**	2.7**	2.0**	3.3**
24%	160**	2.3**	2.3**	1.6**	2.0**
25%	160**	1.7**	1.7**	1.3**	2.0**

* = $P<0.05$

** = $P<0.01$

Addition of pumpkin and canola proteins to wheat flour increased the concentration of lysine, sulfur containing amino acids and tryptophan in the resultant breads (Table 4). The concentration of lysine of bread baked with PPI was three times

greater than that of the control bread. Lysine content was increased by 90–162% in breads supplemented with PM, PPC, CPC or CPI compared to control breads. However, sulfur containing amino acids and tryptophan contents increased by 7–12% and 17–150%, respectively. The chemical scores and EAAI of breads made with pumpkin and canola proteins increased by 55–80% and 14–27%, respectively. HANSMEYER and co-workers (1976) reported that the lysine content in breads increased twice by the addition of wheat bran protein concentrate to wheat flour. CARLSON and co-workers (1981) reported that supplementation of the wheat flour with 10–20% tomato seed flour increased lysine content by 40–69% in breads. MACIEJEWICZ-RYS and HANCZAKOWSKI (1990) reported that supplementation of wheat flour with 33% leaf protein concentrate from the green matter of barley improved the chemical scores from 42 to 50% and the essential amino acid indices increased from 66 to 74% of the resultant blends.

Mineral content of breads were increased by the addition of pumpkin and canola proteins (Table 5). Breads made with PM or PPC were higher in Cu, Zn, Fe, Mn, Mg and K than breads made with CPC or CPI. Na, Ca and P contents were higher in breads made with CPC or CPI than breads made with PM or PPC. Bread made with PPI was lower in P, Zn, Fe, Mn and Mg than breads made with PM, PPC, CPC or CPI.

Table 3

Proximate composition of breads[a]

Bread	Moisture	Crude protein g/100 g	Crude fat	Ash
Control	9.4^{A}	16.2^{A}	2.4^{A}	1.6^{A}
Pumpkin seed meal[b]	10.6^{B}	18.1^{A}	2.9^{A}	1.6^{A}
Pumpkin protein concentrate[b]	10.0^{C}	18.2^{A}	2.6^{A}	1.7^{A}
Pumpkin protein isolate[c]	9.4^{A}	22.3^{A}	2.6^{A}	1.4^{B}
Canola protein concentrate[b]	10.3^{BC}	18.1^{A}	2.4^{A}	1.6^{A}
Canola protein isolate[b]	10.2^{BC}	18.0^{A}	2.3^{A}	1.5^{AB}

[a] Dry weight basis

[b] 18% protein concentration

[c] 22% protein concentration

[A,B,C] Means in the same column with different superscripts are significantly different ($P<0.05$)

Table 4

Amino acid composition (g/16 g N), chemical scores and essential amino acid indices of breads

Amino acid	Bread					
	Control	PM[a]	PPC[a]	PPI[b]	CPC[a]	CPI[a]
Isoleucine	3.4	4.6	3.3	3.8	3.8	3.6
Leucine	7.9	7.8	8.0	7.7	7.4	7.6
Lysine	2.1	4.4	4.0	6.3	5.5	4.1
Cystine	1.9	2.0	1.9	1.9	1.5	1.8
Methionine	0.9	1.4	1.1	1.2	1.6	1.5
Total sulfur amino acids	2.8	3.4	3.0	3.1	3.1	3.3
Tyrosine	5.0	5.2	4.8	5.0	3.0	4.1
Phenylalanine	5.5	5.9	5.2	5.3	4.9	4.9
Total aromatic amino acids	10.5	11.1	10.0	10.3	7.9	10.0
Threonine	4.4	4.2	3.8	3.7	3.9	3.8
Tryptophan	0.6	0.9	1.1	1.2	0.7	1.5
Valine	3.2	4.0	3.2	3.8	3.9	3.8
Total essential amino acids	34.9	40.4	36.4	39.9	36.2	37.7
Histidine	3.2	3.7	3.1	3.5	3.0	3.3
Arginine	3.9	6.9	5.9	7.0	4.3	5.1
Aspartic acid	5.2	5.5	6.6	6.8	5.6	5.6
Glutamic acid	24.5	20.5	20.1	18.0	24.4	22.0
Serine	6.1	4.9	6.2	6.0	5.4	5.6
Proline	14.2	10.7	12.5	10.0	12.9	12.2
Glycine	4.1	3.9	4.9	4.5	4.3	4.5
Alanine	3.9	3.5	4.3	4.3	3.9	4.0
Total non-essential amino acids	65.1	59.6	63.6	60.1	63.8	62.3
Chemical score %	40	71	62	68	64	72
First limiting amino acid	Lys	Val	Val	Val	Try	Val
Second limiting amino acid	Try	Lys	Lys	Met+Cys	Val	Lys
EAAI%	60.4	76.4	70.2	76.7	68.9	75.0

[a] 18% protein concentration
[b] 22% protein concentration

Table 5

Mineral content of breads[a]

Bread	Na	Ca	K	P	Cu	Zn	Fe	Mn	Mg
					mg/100g				
Control	485.7	149.2	99.9	88.4	0.3	3.0	1.5	0.9	49.7
Pumpkin seed meal[b]	485.0	214.1	117.1	148.9	0.6	3.3	4.5	2.0	123.0
Pumpkin protein concentrate[b]	485.2	213.4	115.9	146.2	0.5	4.0	4.6	2.2	105.6
Pumpkin protein isolate[c]	911.8	255.8	103.5	116.8	0.6	1.9	2.3	0.6	44.2
Canola protein concentrate[b]	936.9	344.7	101.6	195.5	0.4	2.0	3.3	0.9	103.1
Canola protein isolate[b]	583.1	239.8	104.6	157.3	0.5	3.1	3.1	1.8	100.2

[a] Dry weight basis
[b] 18% protein concentration
[c] 22% protein concentration

3. Conclusion

Supplementation of wheat flour with PM, PPC, PPI, CPC and CPI increased the water absorption and dough softening as the protein level increased; however, dough stability and loaf volume decreased. Pumpkin protein isolate and other pumpkin and canola products could be added to wheat flour up to 22% and 18%, respectively, without a detrimental effect on sensory properties. The addition of pumpkin and canola proteins increased the content of protein, lysine, sulfur containing amino acids, tryptophan and minerals. Therefore, pumpkin and canola proteins can be added to bread formulations not only as functional agents, but also as nutrients supplement.

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APPLE JUICE AROMA CONCENTRATION FROM EVAPORATOR CONDENSATE BY REVERSE OSMOSIS

I. INFLUENCE OF PROCESS PARAMETERS ON RETENTION AND FINAL AROMA CONCENTRATION

A. POZDEROVIĆ and T. MOSLAVAC

University J. J. Strossmayer Osijek, Faculty of Food Technology, 31000 Osijek, F.Kuhača 18. Croatia

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Application of reverse osmosis (RO) for apple juice aroma concentration from an evaporator condensate was investigated. Feed evaporator condensate was prepared by diluting the apple aroma concentrate with water. Laboratory apparatus for RO with plate-and-frame membrane configurations was used. Volatile aroma components of apple juice in the feed condensate, retentate and permeate were determined by gas chromatography using a head space method. Total aroma changes were monitored, whereby aroma was determined by summation of the peak areas excepting ethanol. The influence of processing pressure, temperature and aroma concentration in the retentate on recovery and permeation was studied. The results proved that concentration of apple aroma from evaporator condensate with RO was possible. Recovery and aroma concentration in the retentate was higher at a lower temperature and higher pressure. Pressure increase had a bigger influence on retention and aroma recovery in the retentate than temperature decrease. Aroma loss in the permeate was bigger when aroma concentration in the feed condensate and retentate was bigger.

Keywords: apple juice, aroma, aroma compounds, aroma rentention, aroma recovery, concentration, volatile compounds, reverse osmosis

There is a 90% loss of volatile aroma compounds in fruit juice concentration by evaporation. This loss is partially amended by applying an aroma recovery unit with rectification column (POZDEROVIĆ et al., 1988; KONJA et al., 1987, 1988; SANCHO & RAO, 1993).

KOLLMANNSBERGER and BERGER (1994a, 1994b) examined thermal recovery of aroma compound by fractional distillation and plate condensation during the processing of fruit juice concentrate and aroma concentrate. Both processes showed similar performances for the transfer of aroma compounds into aroma concentrate. Heat applied in this process may cause chemical changes of aroma compounds. Various studies examined application of other process solutions of aroma recovery in fruit juice

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concentration by evaporation, e.g. extraction techniques (GOMEZ et al., 1992; SIMON et al., 1992; ARTOZOUL, 1994; SCHOFIELD, 1994, SCHOFIELD & RILEY, 1998), adsorption recovery (DI CESARE et al., 1988), pervaporation and reverse osmosis. Fruit juice aroma concentration from evaporator condensate by pervaporation was examined: BENGTSSON and co-workers (1989; 1992; 1993), BEANMELLE and co-workers (1992), KARLSSON (1994); KARLSSON and TRAEGARD (1993), KARLSSON and co-workers (1995). BRADDOCK and co-workers (1991), KANE and co-workers (1995) have found a high percentage of aroma compounds recovery in reverse osmosis concentration of aqueous-phase citrus juice essence.

Many authors have found that a loss of volatile aroma compounds is significantly smaller in fruit juice concentration by reverse osmosis compared to the concentration by evaporation. (SHEU & WILEY, 1983; CLAUSS et al., 1988; CHOU et al., 1991; SANCHO & RAO, 1993). KONJA and CLAUSS (1991) reported that aroma retention in the retentate was 88% in fruit juice concentration by RO with HR 98 membrane. This was the basis of our research on reverse osmosis application for the concentration of aroma aqueous condensate and apple juice evaporator condensate, respectively, obtained by the separation of aroma compounds from an apple juice in concentration evaporation.

1. Materials and methods

1. 1. Preparation of feed evaporator condensate

Feed evaporator condensate was prepared from an apple aroma concentrate obtained by concentrating an evaporator condensate on an apple juice concentration plant with aroma recovery unit. One liter of one hundred-fold concentrated evaporator condensate was diluted with distilled water to 10 or 20 l, respectively. Solution diluted to ten liters was considered as the basic solution and its dilution was labelled as 1/10. Solutions diluted to 20 l were labelled 1/20. Both solutions were used as feed evaporation condensate for RO concentration.

1. 2. RO Equipment

Research was conducted using the laboratory equipment for RO Lab Unit 20 with plates and frames (Dow Danmark Separation Systems De Sukkerfabrikker, Copenhagen, Denmark).

The pumping action was furnished by a 16.5 high pressure Rannie pump. The pumping rate was 7.5 l min^{-1} at 40 bar pressure and 25 °C. The composite membranes type HR-98 were used for the concentration of the feed condensate obtained from the same firm as the equipment. Twenty membranes were used cut from a membrane roll stock containing 0.58 m^2 area.

1. 3. Concentration procedure

Twenty liters of the feed condensate with 1/10 and 1/20 dilution were concentrated in each experiment, excluding one, by RO on a batch basis to the retentate final volume of 3 l. The concentrate stream was conveyed to the feed tank for reconcentration while the permeate was collected in a separate container. Experiments were conducted at the operating pressure of 38 bar and the operating temperature of 25 and 40 °C with 10 l of feed condensate diluted to 1/10. Other experiments were also carried out at the operating pressure of 10, 20, 30 and 38 bar and the operating temperature of 25 °C and at 30 bar and 10 °C with 10 l of feed condensate diluted to 1/20. There was a special experiment of concentration conducted with 20 l of the feed condensate diluted to 1/20 up to 1.5 l of the concentrate (retentate) at the operating pressure of 30 bar and the operating temperature of 18 °C. Operating temperature was controlled either by heating or refrigerating the feed condensate to the appropriate temperature, using a heat exchange for controlling the temperature bath. During the experiment the samples were taken from the feed condensate, retentate, total permeate and from each liter of the permeate which was separated during the process.

1. 4. Aroma analysis

Aroma components were determined in the feed condensate, permeate and retentate (concentrate) by gas chromatography using a head space method (direct vapor) (POZDEROVIĆ & BABIĆ, 1991; POZDEROVIĆ & LOURIĆ, 1986; POZDEROVIĆ et al., 1988). A Perkin-Elmer gas chromatograph model Sigma 3 with a flame ionization detector and HS6 head space injector were used for the analysis. The column used for the analysis had a diameter of 1.75 mm, a length of 2 m and was packed with 3% DEGS on a Chromosorb P80-100 mesh.

Operating conditions were the following: nitrogen was used as a carrier gas at 38 ml min^{-1} through the column, detector and injector at 150 °C, an oven at 40 °C (4 min) to 60 °C (5 °C min^{-1}), at 60 °C (26 min), HS block 90 °C, gas pressurization time into the HS sample (4 min), volume of head space sample (2 ml), area of signal weakening (4×1) (POZDEROVIĆ & BABIĆ, 1991).

Total apple aroma was determined by summation of the peak areas from the chromatograms. The analysis produced 13 peaks of the volatile components of apple aroma. N-pentanol as an internal standard was added to all samples for quantitative results. Prior to analysis the samples were diluted with water to be analyzed at the same area of signal weakening.

1. 5. Data calculation

To study the processes of concentration and recovery of apple aroma volatile components, the following parameters were calculated using the formulas (CHOU et al., 1991):

% concentration index of total aroma in retentate

$$(I_{Rt}) = \frac{A_{Rt} \times D_{Rt}}{A_{Fc} \times D_{Fc}} \times 100$$

% concentration index of total aroma in permeate

$$(I_{Pt}) = \frac{A_{Pt} \times D_{Pt}}{A_{Fc} \times D_{Fc}} \times 100$$

% recovery of total aroma in retentate

$$(R_{Rt}) = I_{Rt} \times \frac{V_{Rt}}{V_{Fc}}$$

% recovery of total aroma in permeate

$$(R_{Pt}) = I_{Pt} \times \frac{V_{Pt}}{V_{Fc}}$$

% permeation of total aroma

$$(P) = \frac{A_{Pt} \times D_{Pt}}{A_{Rt} \times D_{Rt}} \times 100$$

Where A_{Rt}, A_{Pt}, A_{Fc} = summations of the peak areas; D_{Rt}, D_{Pt}, D_{Fc} = dilutions before HSGC analysis; V_{Rt}, V_{Pt}, V_{Fc} = volume, Rt - retentate; Pt - permeate; Fc - feed evaporate condensate.

Experiments were repeated twice and the mean values were taken to present experiment results.

1. 6. Statistical analysis

Statistical data analysis was done by correlation coefficient (r) and standard deviation (± s). PC and the Excel 7.0 were used to analyze data. The number of replicates was two.

2. Results

Experimental research data of the influence of processing temperature on the concentration and recovery of total aroma in the permeate and retentate are shown in Tables 1 and 2. Table 1 shows the concentration results of the feed condensate with 1/10 dilution concentrated from 10 l to 3 l at 38 bar and at temeperatures 25 °C and 40 °C. Data showed that temperature increase from 25 to 40 °C reduced aroma

Table 1

Concentration index and percent recovery of total aroma in retentate and permeate at 38 bar at 25 ° C and 40 ° C (dilution of feed aroma condensate 1/10, initial volume 10 l, final volume 3 l)

Pressure (bar)	Temperature (°C)	Concentration index of total aroma (%)				Recovery of total aroma (%)			
		retentate		permeate		retentate		permeate	
		$\bar{x}$	±s	$\bar{x}$	±s	$\bar{x}$	±s	$\bar{x}$	±s
38	25	260.9	2.35	28.6	0.39	78.2	0.70	20.0	0.31
	40	230.9	2.54	43.0	0.79	67.4	0.91	30.1	0.56

Table 2

Concentration index and percent recovery of total aroma in retentate and permeate at 30 bar at 10 ° C and 25 ° C (dilution of feed aroma condensate 1/20, initial volume 10 l, final volume 3 l)

Pressure (bar)	Temperature (°C)	Concentration index of total aroma (%)				Recovery of total aroma (%)			
		retentate		permeate		retentate		permeate	
		$\bar{x}$	±s	$\bar{x}$	±s	$\bar{x}$	±s	$\bar{x}$	±s
30	10	253.2	3.11	33.2	0.42	75.9	1.01	23.2	0.40
	25	249.0	2.31	37.1	0.66	74.7	1.17	26.0	0.51

concentration index in the retentate from 260.9% to 230.9%. Concentration index in the permeate increased from 28.6% to 43.0%. The obtained aroma recovery in the retentate was 78.2% and 67.4%, in the permeate 20.0% and 30.1% at 25 °C and 40 °C, respectively.

Concentration results of the feed condensate with 1/20 dilution concentrated from 10 l to 3.0 l at 30 bar pressure and temperatures of 10 °C and 25 °C are shown in Table 2.

The results also showed that a temperature increase from 10 °C to 25 °C decreased concentration index and aroma recovery in the retentate but it increased them in the permeate. Thus, an increase of processing temperature tended to increase the permeation rate of aroma compounds and to decrease aroma compounds retention.

Research data on the influence of processing pressure on concentration, recovery and permeation of aroma components are shown in Figs. 1, 2 and 3. Figure 1A and B illustrates that the concentration index of total aroma (1A) and recovery of total aroma (1B) in final retentate are increased by the increase of pressure. Pressure increase from 10 to 38 bar resulted in the final retentate with the concentration index of 204.9% and 261.0%, respectively. Hereby, the recovery of total aroma in the retentate increased from 61.4% to 78.3%. An adequate increase of the concentration index and total aroma

recovery also appeared with a pressure increase from 10 to 20 and 30 bar. Data demonstrated that a high percentage of recovery (61%) and concentration index (204.9%) were achieved by aroma concentration from evaporate condensate at a relatively low pressure for RO (10 bar). Figure 1A data showed that the 10 bar pressure increase tended to increase concentration index by approximately 20%.

The influence of pressure on concentration index and aroma compounds recovery in the permeate is shown in Fig. 2A and B. Concentration index (2A) and total aroma recovery (2B) in the permeate were decreased by aroma compounds concentration in evaporate condensate at a higher processing pressure. Concentration aroma index in the permeate was ten times lower in the permeate than in the retentate at the highest applied pressure of 38 bar. At the same time, aroma recovery in the permeate was about 20% and in the retentate about 80%, concentration index was 30% and 260%, respectively. Higher retention and aroma recovery in the retentate and lower aroma recovery in the permeate were due to lower permeation of total aroma through the membrane at higher processing pressure (Fig. 3). The pressure increase from 10 to 38 bar caused a decrease of total aroma permeation from 22 to 13%. CHOU and co-workers (1991) reported that increasing pressure caused increasing flux which reduced the processing time of fruit juice concentration by RO. Thus, decreased processing time resulted in less loss of aroma compounds from the retentate due to volatilization, adsorption onto the membrane and possible losses due to shear.

Figures 4 and 5 showed the results of the feed concentrate concentration with 1/20 dilution from 20 l to 1.5 l at 18 °C and 30 bar. This experiment was aimed at monitoring aroma retention and recovery in the retentate and permeate during the concentration process. Figure 4 data clearly showed that the decreasing volume of the retentate during the concentration time significantly increased aroma concentration index in the retentate. If the retentate volume was 10 l, aroma concentration in the retentate would be 1.8 times higher than in the feed condensate. If the volume was 5 l it would be 3.2 times higher and if the final volume was 1.5 l it would be 6.8 times higher. These results, if compared to the previous ones, demonstrated that if the feed condensate was concentrated to a smaller final volume, higher concentration index would be achieved, namely higher aroma concentration in the retentate. Thus, a significant aroma concentration by RO from feed evaporate condensate is possible.

Data in Fig. 5A and B showed that the increasing aroma concentration in the retentate during concentration caused greater permeation of aroma compounds through a membrane. This illustrated the dependence of the concentration index of aroma (5A) and aroma recovery (5B) in the permeate on the aroma concentration index in the retentate. Therefore, permeation of aroma compounds through the membrane, namely aroma retention on the membrane, depended on the processing pressure and temperature as well as on the aroma concentration in the retentate.

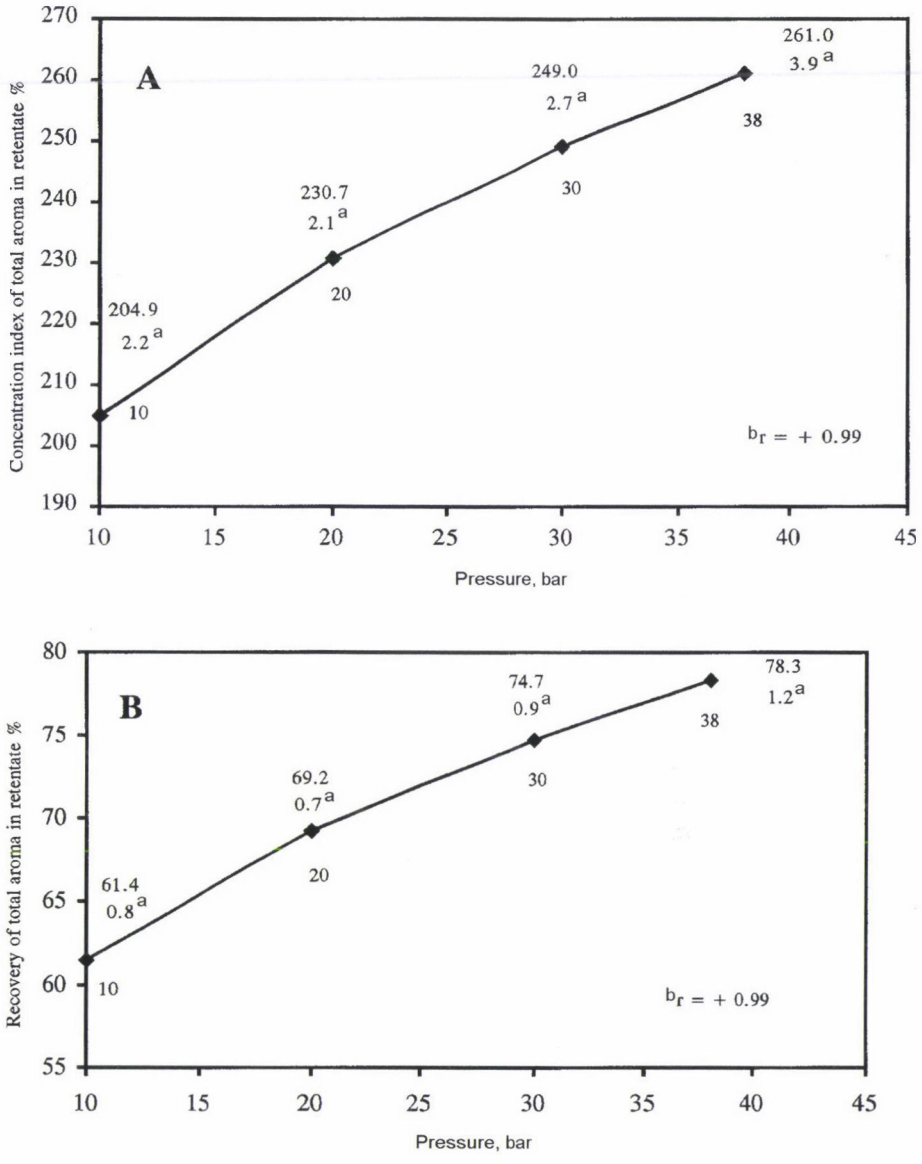

Fig. 1. The effect of pressure on concentration index (A) and aroma compounds recovery (B) in the retentate at 25 °C, dilution of feed aroma condensate 1/20, initial volume 10 l, final volume 3 l.
[a]Standard deviation (±s); [b]correlation coefficient (r)

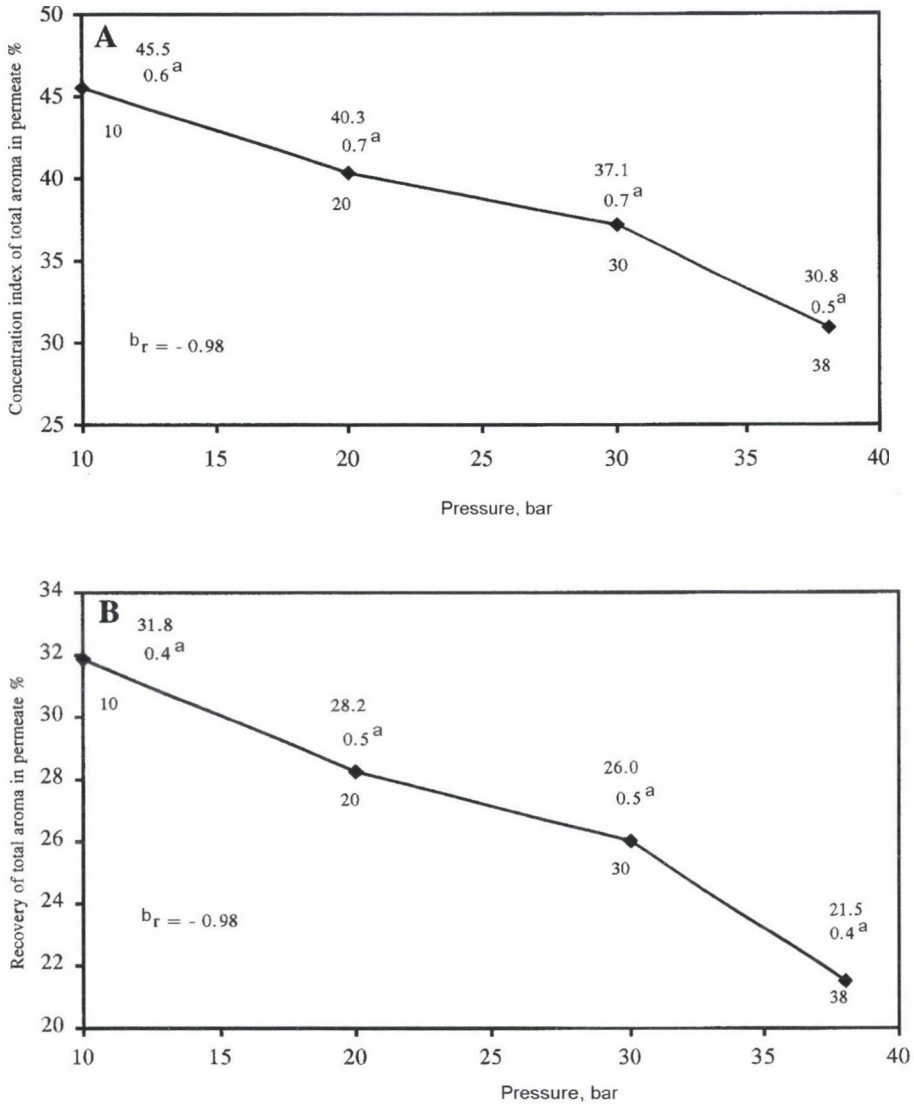

Fig. 2. The effect of pressure on concentration index (A) and aroma compounds recovery(B) in the permeate at 25 °C, dilution of feed aroma condensate 1/20, initial volume 10 l, final volume 3 l. [a]Standard deviation (±s); [b]correlation coefficient (r)

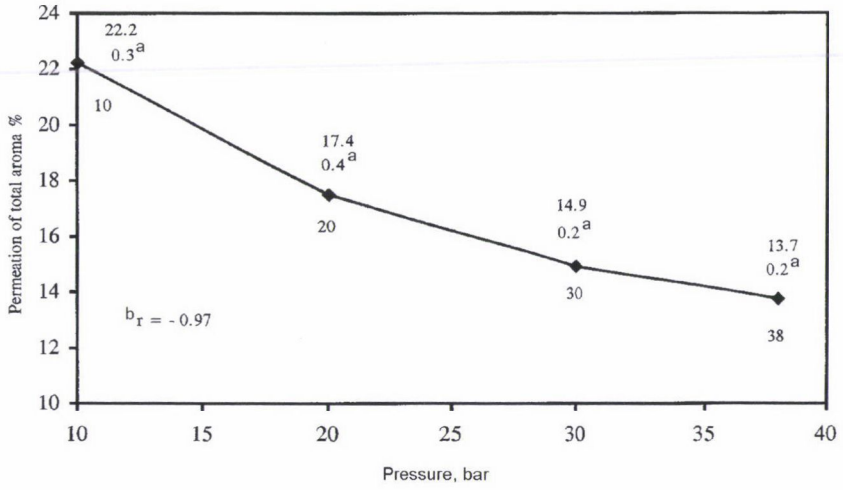

Fig. 3. The effect of pressure on permeation of total aroma through the membrane at 25 °C, dilution of feed aroma condensate 1/20, initial volume 10 l, final volume 3 l.
[a]Standard deviation (±s); [b]correlation coefficient (r)

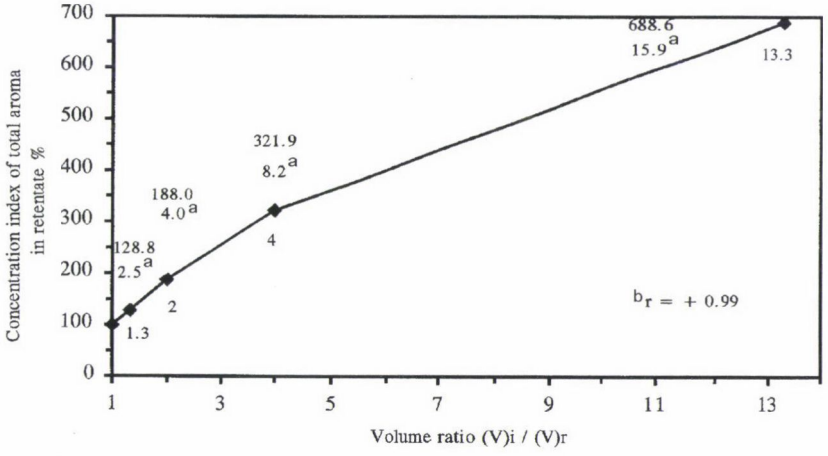

Fig. 4. Change of concentration index of total aroma in retentate by decreasing of retentate volume at 30 bar at 18 °C, dilution of feed aroma condensate 1/20, initial volume 20 l, final volume 1.5 l. Volume ratio (V)i/(V)r represent initial volume/retentate volume
[a]Standard deviation (±s); [b]correlation coefficient (r)

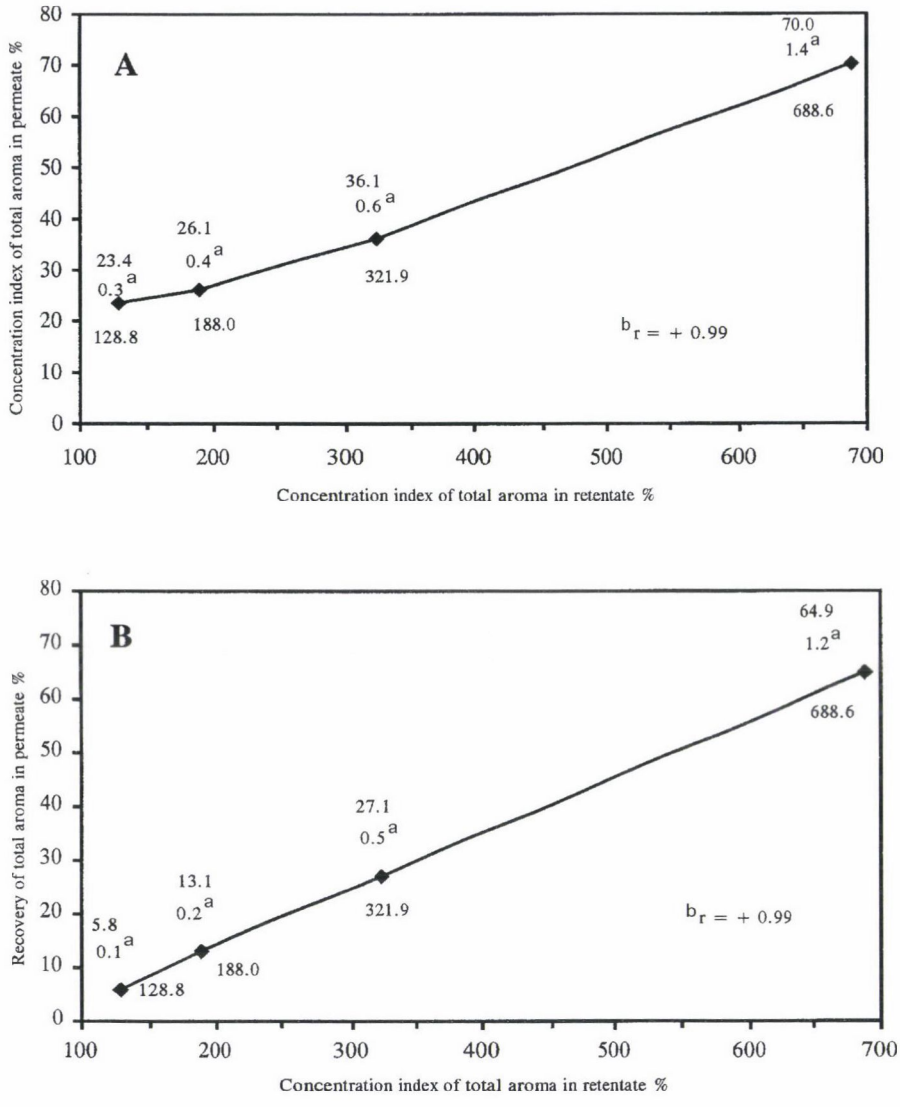

Fig. 5. The influence of change of concentration of aroma compound in retentate on concentration index (A) and recovery of aroma (B) in permeate at 30 bar at 18 °C, dilution of feed aroma condensate 1/20, initial volume 20 l, final volume 1.5 l.

[a]Standard deviation (±s); [b]correlation coefficient (r)

When fruit juice was concentrated by RO using the membrane HR98, KONJA and CLAUSS (1991), SHEU and WILEY (1983) achieved high juice aroma retention (88%) and high aroma recovery (86%).

Aroma retention in the concentrate, namely retentate, achieved in this study was 688% in relation to the feed condensate (100%).

Since one part of aroma may permeate through the membrane, multiple-stage concentration was required to obtain successful and complete aroma recovery from evaporate condensate, i.e. concentration of obtained permeate had to be repeated.

3. Conclusion

Retention and recovery of aroma compounds in the retentate during RO concentration from evaporate condensate depended on the processing pressure, temperature and aroma concentration in the retentate. Higher retention and aroma recovery in the retentate were achieved at higher processing pressure and lower processing temperature. Increasing processing pressure had significantly higher influence on retention, namely aroma concentration, than decreasing processing temperature. Increasing processing pressure and decreasing temperature caused lower aroma recovery in the permeate due to decreased aroma permeation through the membrane.

Retentate volume decrease during concentration time increased the concentration of the total aroma in the retentate. An increase of the total aroma concentration in the retentate increased aroma permeation and recovery of the total aroma in the permeate. Retentate concentration from 10 l to 3 l and from 20 to 1.5 l resulted in the concentration of the total aroma in the retentate 2.6 and 7 times, respectively. The results indicated a substantial recuperation of aroma compounds in the permeate, approximately 20 and 60%. Concentration of aroma compounds by RO from aqueous solutions and evaporator condensate, respectively, was possible.

High recovery of aroma compounds in the final concentrate demanded multiple-stage concentration, or repeated permeate concentration, respectively.

In commercial RO concentration of apple aroma from evaporator condensate, lowering condensate temperature prior to RO processing at maximum pressure permitted with concentration of the permeate repeated would provide a finished concentrate with a relatively high aroma component content.

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DETERMINATION AND CONTROL OF BEE-ACARICIDE FLUMETHRIN IN HONEY AND BEEWAX

M. SZERLETICS-TÚRI

"Fodor József" National Center of Public Health, National Institute of Food Hygiene and Nutrition, H–1097 Budapest, Gyáli út 3/a. Hungary

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In the last decades considerable economic damages were caused by *Varroa* mite in European and also in Hungarian apiaries. For the control fumigant strips, solutions and aerosols containing acaricide active ingredients were introduced. Plastic strips impregnated with synthetic pyrethroids show a high efficacy against these mites. The long-term (2–6 weeks) treatment, however, increases the problem of residues in honey and honeycomb.

An analytical method was introduced in Hungary for the determination of pyrethroid type active ingredient (flumethrin) residues. The residue content of honey and beewax samples were determined after field experiments. The results served as a basis for registration for marketing and application of Bayvarol strips (Bayer) in Hungary.

Keywords: bee-acaricide, flumethrin, Bayvarol, pyrethroid residues

In the last decades several products with different active ingredients (e.g. amitraz, bromopropylate, fluvalinate) were registered and tested, respectively, for control of Varroatosis in honey-bees in Hungary.

In the course of the registration and application of the products the protection of the environment and the food chain is essential. Already, in the phase of the registration, for the food-hygienic evaluation of the products – beside several basic data and results of animal experiments – the knowledge, determination of residue levels, getting into the human organism via food are required.

The honey-bee infecting ectoparasite, formerly only in Asia present, *Varroa jacobsoni* mite appeared 20–25 years ago in Europe (KOENIGER & FUCHS, 1988a). Hungary has been infected since 1978 (KOLTAI, 1985) and in the early eighties the mite caused enormous economical damages in the beekeeping farms, having endangered the honey export, too. For the control, acaricide active ingredient containing fumigant strips, solutions and aerosols were introduced (KOENIGER & FUCHS, 1988a). From veterinary point of view however, some problems arised as the products were effective

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only for a short time and did not kill protected forms of mites in different developmental phases.

Products with active ingredients such as impregnated plastic strips meant some advance as they are present for some weeks continuosly in the beehive (KOENIGER & FUCHS, 1988b, RUTTNER, 1988) and thus eliminate the above mentioned problems. The active ingredients of these veterinary products belong to the group of synthetic pyrethroides, e.g. Bayvarol strips (Bayer AG) contains flumethrin in a quantity of 3.6 mg/strip (RUIJTER & EIJNDE, 1991; MOOSBECKHOFER, 1990; KOENIGER, 1991). Figure 1 shows the structure of the active ingredient.

The continuous (2–6 weeks) treatment, however increases the problem of residues in honey and honeycomb, respectively. For this reason an analytical method was introduced for the determination of the active ingredient (flumethrin) residues in apicultural products. After experimental treatments, the amount of active ingredient residue in honey and beewax samples – taken from beehives treated with Bayvarol – were determined.

The first treatment was performed in Gödöllő, in the experimental apiary of the Agricultural University by members of the Department of Zoology and Ecology, according to the technology prepared upon detailed examinations of the effectiveness (RUIJTER & EIJNDE, 1991; KOENIGER, 1990; MOOSBECKHOFER, 1991). Five bee-families (not treated against *Varroa jacobsoni* in the last 7 months) were treated by hanging 4 Bayvarol strips into every beehive for 4 weeks.

Further treatments were performed in a private apiary in another part of the country. Strips were suspended into the spaces between the combs in the central brood-rearing area in such a way that could be occupied by bees on both sides, as it can be seen in Fig. 2.

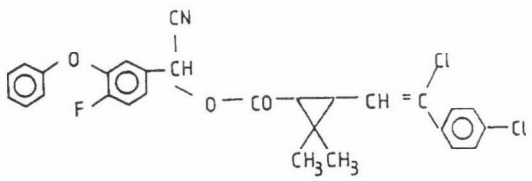

Fig. 1. Structure of flumethrin. Active ingredient: the mixture of trans-Z_1 and trans-Z_2 configuration diastereoisomers of 3[2-chloro-2(4-chlorphenyl)-ethenyl]-2,2-dimethyl-cyclopropane carboxylic acid cyano-(4-fluoro-3-phenoxyphenyl)-methyl-ester

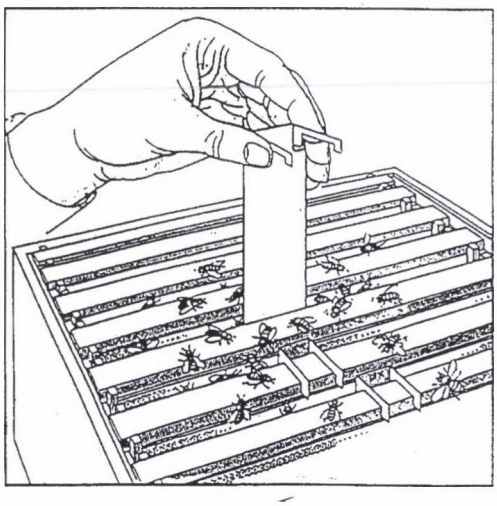

Fig. 2. How to place Bayvarol strips into the beehive

1. Methods

One week after removing the strips from the treated beehives samples were taken in Gödöllő. In the course of the second treatment the time of sampling was at the end of the 4 weeks' treatment.

Comb-honey pieces, having arrived in our laboratory, were moderately warmed (60 °C) and honey and wax were separated by pressing. The appearance of the lipophil active ingredient was expected first of all in the beewax, therefore the residue content was determined separately from honey and beewax, according to the method presented in Figs 3 and 4.

From honey, active ingredient was extracted with the mixture of toluene, dichloromethane and methanol. The concentrated extract was purified on silicagel column (RIEDGER, 1986). The residue was determined by gas chromatography with electron capture detection.

The beewax sample was dissolved in heated isopropanol, then precipitated out again by adding methanol and water. After filtration and repeating the extraction procedure, the extract was evaporated. After two solvent-solvent partitions (water and ethylacetate/cyclohexane 1/1, then acetonitrile/hexane) and purification on silicagel column (RIEDGER, 1986), the flumethrin residue was determined by gas chromatography with electron capture detection.

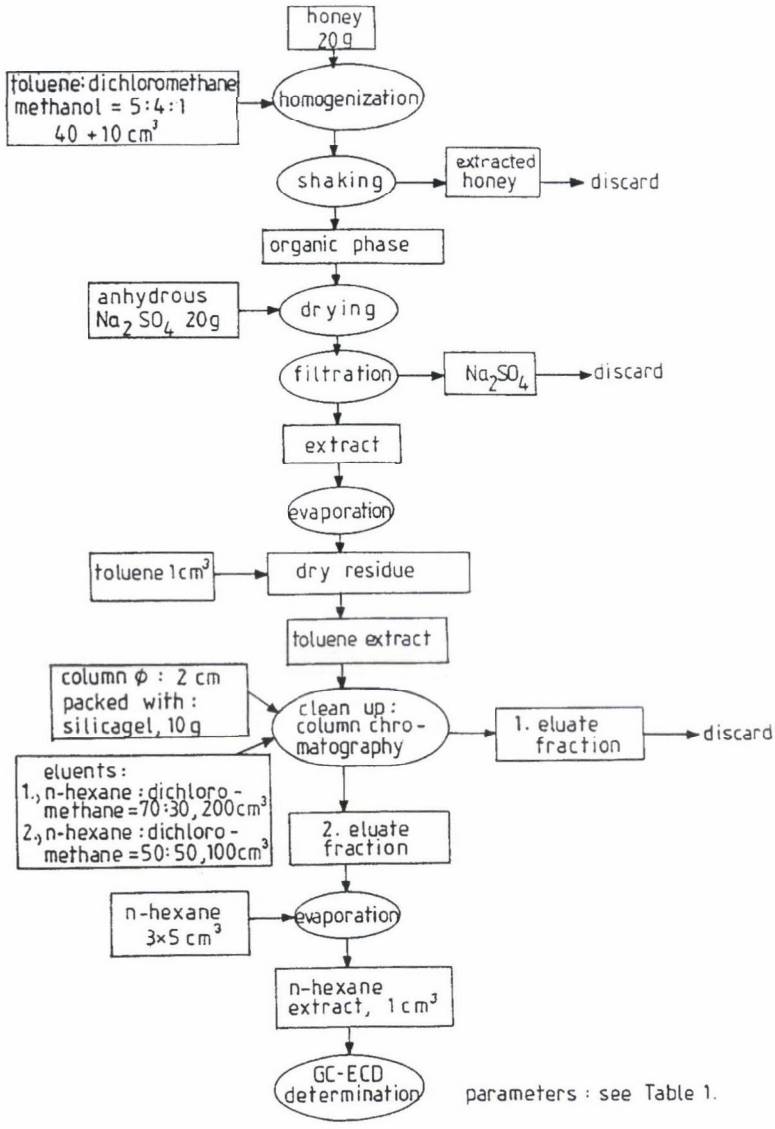

Fig. 3. Analytical method for the determination of flumethrin residues in honey

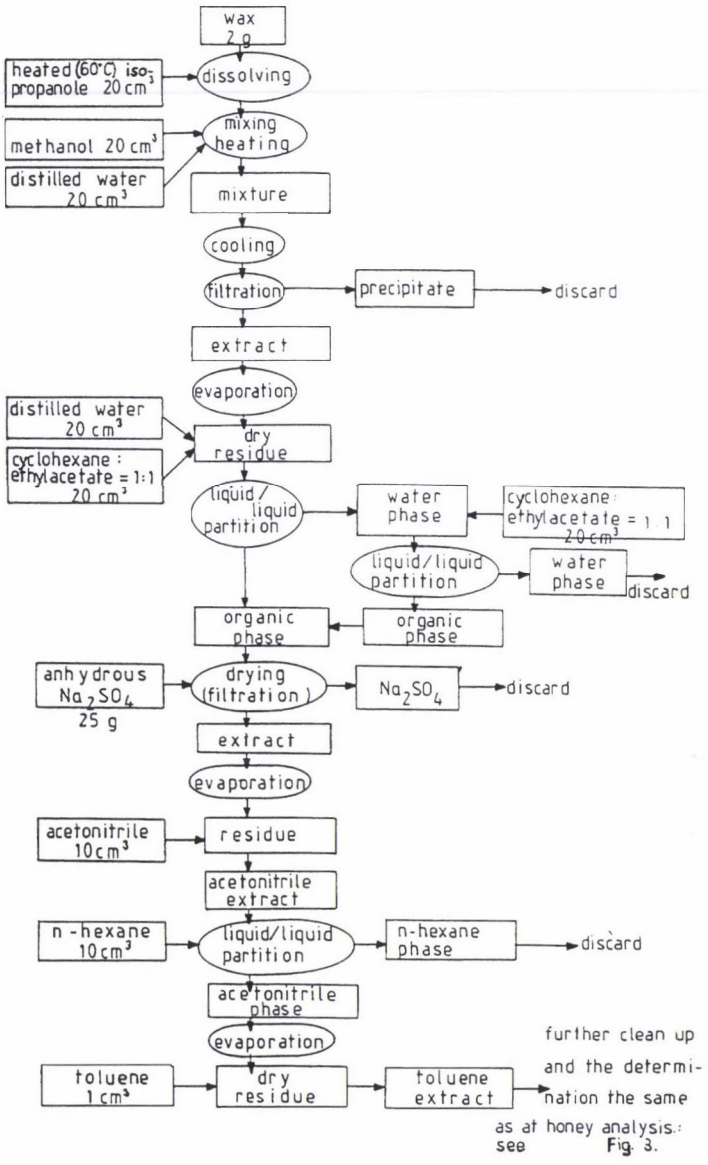

Fig. 4. Analytical method for the determination of flumethrin residues in beewax

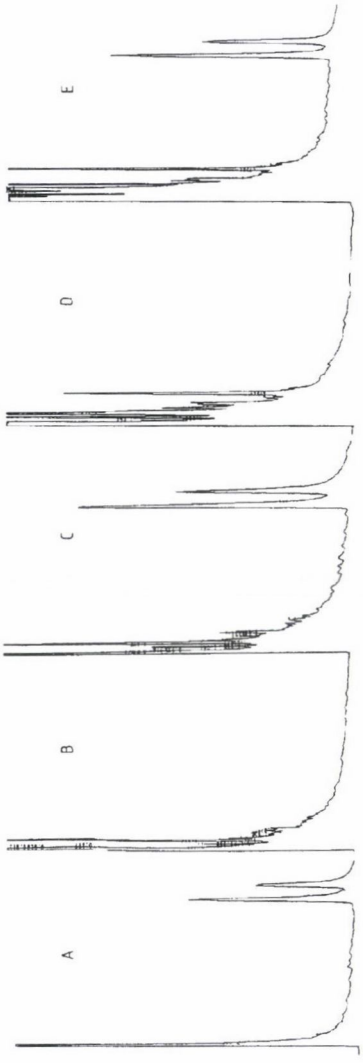

Fig. 5. Chromatograms of flumethrin standard (A), honey from beehive treated with Bayvarol strips (B), spiked honey (C), beewax from hive treated with Bayvarol strips (D), spiked wax sample (E)

The parameters of gas chromatography are shown in Table 1.

Table 1

Parameters of gas chromatographic determination

Instrument	Chrompack 9000
Detector	Ni 63 ECD
Column	25 m × 0.32 mm i.d. CP Sil PAH CB df=0.12 μm
Carrier gas	nitrogen of high purity, 3.2 cm^3/min
Make up gas	nitrogen of high purity, 30 cm^3/min
Split proportion	5 : 1
Temperatures	
injector	250 °C
column	245 °C
detector	300 °C
Flumethrin retention times	11.5 min 12.5 min
Detection limit	
from honey	0.005 mg/kg
from beewax	0.025 mg/kg

Table 2

Recoveries in the course of the determination of flumethrin residue

Examined sample	Fortification level (mg/kg)	Recovery(%)	mean±s
Control honey	0.25	95	87±6.7
	0.10	81	
	0.05	85	
Control beewax	2.50	68	68±6.5
	1.00	74	
	0.20	61	

Some of the chromatograms are presented in Fig. 5.

Parallel to the treated samples we examined control honey and beewax samples from an untreated beehive and samples spiked with active ingredient. Flumethrin was added to honey and beewax, respectively on 0.05–2.50 mg/kg levels and after the procedure the recovered quantities were determined. Mean recoveries were, in the case of honey samples, 87%, in the case of beewax samples 68% (Table 2).

2. Results

Results are summarized in Table 3. Data represent the means of the results of 2–2 parallel measurements.

Blinds of the chemicals did not show any disturbing contamination. Flumethrin residues could not be detected in the extracts of control honey and beewax samples, respectively (in honey <0.005 mg/kg; in beewax <0.025 mg/kg).

3. Conclusions

Flumethrin residues were not detectable in any of the processed honey samples. Small amounts of active ingredient were measured in 4 wax samples, but flumethrin could not be detected in the further 12 samples.

From food-hygienic point of view, it is favourable that during the treatments honey does no get "contaminated" with flumethrin.

Based on Hungarian and foreign residue examinations, in the course of the registration of Bayvarol strips – applied according to the recommendations of the manufacturer – the following prescriptions were recommended: 0.01 mg/kg maximum residue level (MRL) in honey; 0.2 mg/kg MRL in beewax and no withdrawal period (0 day).

By the use of the given method, we are going to continue the work in our Institute by more comprehensive examinations of samples taken in different places and times. For health protection and to maintain the exportability of Hungarian honey, further examinations will be performed with flumethrin and other active ingredients of bee-acaricides (fluvalinate, amitraz, acrinathrin).

Table 3

Residue content of honey and beewax samples taken from beehives treated with Bayvarol strips against Varroa *mites*

First experimental treatment (one sample taken from each beehive)

Sample		Flumethrin residue content (mg/kg)
Honey	1st family (beehive)	<0.005
	2nd family (beehive)	<0.005
	3rd family (beehive)	<0.005
	4th family (beehive)	<0.005
	5th family (beehive)	<0.005
Beewax	1st family (beehive)	<0.025
	2nd family (beehive)	<0.025
	3rd family (beehive)	<0.025
	4th family (beehive)	<0.025
	5th family (beehive)	<0.025

Second treatment (samples taken from different parts of the single beehives)

Sample			Flumethrin residue content (mg/kg)
Honey	1st family	1st sample	<0.005
		2nd sample	<0.005
		3rd sample	<0.005
		4th sample	<0.005
Honey	2nd family	1st sample	<0.005
		2nd sample	<0.005
		3rd sample	<0.005
		4th sample	<0.005
		5th sample	<0.005
Beewax	1st family	1st sample	<0.025
		2nd sample	0.044
		3rd sample	<0.025
		4th sample	<0.025
Beewax	2nd family	1st sample	<0.025
		2nd sample	<0.025
		3rd sample	<0.025
		4th sample	0.030
		5th sample	<0.025
		6th sample	0.025
		7th sample	0.025

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Short communications

DETERMINATION OF VOLATILES FROM RED WINES MADE BY CARBONIC MACERATION USING SOLID PHASE MICROEXTRACTION (SPME) TECHNIQUE

GY. VAS[a] and GY. LŐRINCZ[b]

[a] Research Institute of Viticulture and Enology, H–3300 Eger, P.O. Box 83. Hungary
[b] EGERVIN Vineyard Co., H–3300 Eger, Széchenyi út 3. Hungary

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A winemaking technology termed "maceration carbonique" (MC), affecting the volatile components, was examined. Solid Phase Microextraction sample preparation and GC technique were used to examine the volatile components of the wines. The wines made by carbonic maceration had higher quantity of diethyl succinate and ethyl lactate, but lower methanol and hexanol contents than the skin-fermented wine.

Keywords: carbonic maceration, anaerobic metabolism, SPME, volatiles

During technology whole, mature grape brunches are treated in anaerobic conditions under CO_2 before usual winemaking steps (crushing, destemming, fermentation). This process uses the natural enzymatic transformation, which occurs in the intact berries. The carbonic maceration produces early-maturing wines with soft taste and special aroma. The method is useful for making white and also red wines.

Several authors studied the aromatic composition of carbonic macerated wines. Some of them suppose that typical compounds are produced during the process like ethyl cinnamate (VERSINI & TOMASI, 1983), diethyl succinate (TESNIÉRE et al., 1989), benzaldehyde (SALINAS et al., 1996), ethyl-9-decenoate (DUCRUET, 1984; LŐRINCZ et al., 1996), volatile phenols (ETIÉVANT, 1989). The differences in flavour between wines made by carbonic maceration and traditional skin-fermentation are due to different proportion of volatile components, and mainly to major compounds such as ethyl esters and fusel alcohols (DUCRUET, 1984).

In this study we examined some volatile components in red wines made by carbonic maceration (MC) and skin-fermentation.

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1. Materials and methods

We made wines by MC with different technological parameters from Kékfrankos grape variety. We used stainless steel tanks (75 l) for carbonic maceration. The tanks were put in a warm chamber equipped with heat insulation. The chamber was heated. The sound and mature grapes were held in sealed tanks for 19 days at 26 °C in CO_2 atmosphere. After the treatment time the free run must was separated from the berries which was pressed. The free run must (MCf) and the press must (MCp) were fermented respectively after yeast inoculation. The third treatment was the following. After the carbonic maceration the berries were crushed and fermented on skin for two days before pressing (MC+S).

The control wines were made by traditional skin maceration (19 days). At the end of the fermentation all wines were racked, treated with sulfur dioxide and were stored in glass carboys (25 l) for 10 months.

The analysis of aromatic compounds was made at the Research Station of Viticulture and Enology.

1.1. Sample preparation and capillary – GC (CGC) analysis

Wine samples were studied with the SPME technique according to the following protocol. The sample (125 ml) was placed into a 130 ml sampling bottle. A 100 µm polydimethylsiloxane (PDMS) coated SPME fiber (Supelco Inc., Bellefonte, PA USA) was inserted into the head space and held in place for 10 min at ambient temperature. During this time the liquid phase (wine) was stirred with magnetic stirrer. The fiber was then inserted into the GC injector (held at 250 °C) for 5 min to desorb the aroma compounds, which were then analysed by GC.

In the experiments discussed, quantitation (peak area measurement) was performed by GC using FID detector. A Hewlett Packard (USA) 5890 series II gas chromatograph equipped with two-channel Electronic Pressure Control and FID detector was used with a Supelco 30 m×0.25 mm fused silica capillary column coated with a poly-alkylene-glycol (PAG). The PAG phase has a lower polarity, but similar characteristic to the PEG phase, so retention indices are somewhat different.

This can be advantageous, particulary if some peaks of interest are not resolved on a PEG column. The injector and the FID detector temperatures were 250 °C, the splitless purge valve was closed for 5 min, the carrier gas was hydrogen (UCAR, purity 5.5), the gas flow was 1.8 ml min^{-1}. The temperature program of the GC was the following: initial temperature, 35 °C (5 min hold), 1st ramp: 5 °C min^{-1} to 100 °C (0 min hold), second ramp: 3 °C min^{-1} to 200 °C (1 min hold) and 3rd ramp: 10 °C min^{-1} to 220 °C (0 min hold).

The compounds were identified by mass spectrometric analysis (GC-MS) and retention indices. In these analyses the same GC with a Hewlett Packard 5972 MSD mass selective detector in electron impact ionization mode (70 eV) was used. GC run parameters were the same as described above, but the carrier gas was He. Retention indices were calculated from retention times using external calibration, twice a day, utilising a software written by J. Harangi (Hewlett Packard Hungary). The calibration mixture contained 20 aliphatic hydrocarbons (C_8–C_{27}). Day to day reproducibility of retention index determination was ±2 units.

The evaluation is based on the statistical average of three measurements. The results are expressed in percentage compared to the control.

2. Results and discussion

The main products of anaerobic metabolism are the ethanol and CO_2. Ethanol content in the juice comes from yeasts' fermentation, while the same in the berries is the result of intracellular metabolism and also that of the ethanol-diffusion from the environmental space of berries.

The anaerobic metabolism makes the ester amount increase. In our experiments it was the MC-wines which proved higher ethyl dodecanoate quantity. An earlier experiment (LŐRINCZ, unpublished data) resulted in higher quantity of diethyl succinate in MC-wines; this was validated in our experiment presented here. The amount of this compound proved higher in every MC-wine than in control sample.

By modification of metabolic pathways, ethanol enrichment in anaerobiosis could lead to lactate formation (TESNIÉRE et al., 1989). The MC-made wines showed significantly higher amount of ethyl lactate.

By being the first step in MC-winemaking, the anaerobic maceration makes the process of hexanol production limited (DUCRUET, 1984). This phenomenon is evidenced by observing the data (Table 1.). In the table, the amounts (average of three measurements) of volatile compounds are presented in percentage ratios related to control values.

The wines produced by maceration carbonique showed significantly less hexanol content than the skin-fermented ones. The C_6 components and, also the hexanol among them, are compounds with unplesant, grassy smell. Hexanol has derived from linolenic and linoleic acids; it was produced in the course of the winemaking process by oxidation (BRANDER et al., 1980). In the MC-wines made in the classical way (without plus skin-fermentation), 3-hexene-1-ol quantity also proved less, similarly to hexanol amount. According to DUCRUET (1984), hexyl acetate content in MC-wines was less, as well. For lack of standard solution, the determination of this compound could not be performed.

Table 1

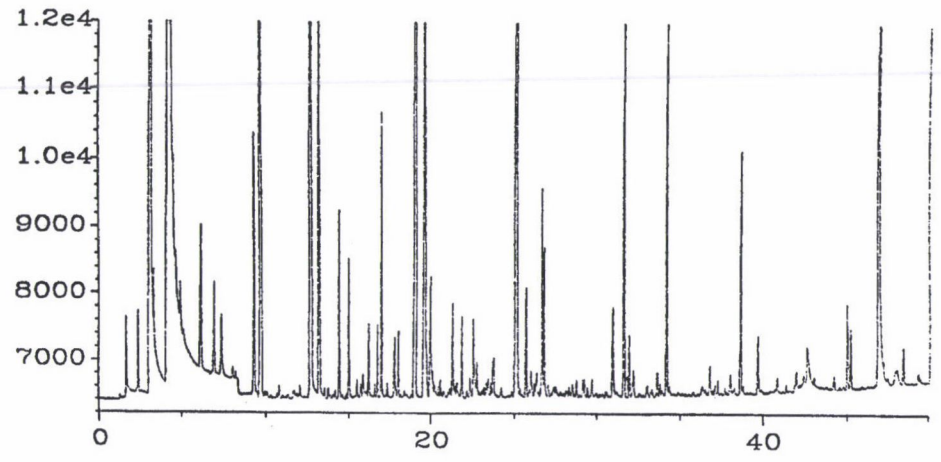
Values of some examined volatile compounds, expressed in percentages in relation to control values

Compounds	Ret.index	Control	MCf	MCp	MC+S
Acetaldehyde	541.4	100	116.0	140.0	117.0
Methanol	746.0	100	8.8	19.5	10.2
Ethyl lactate	1358.3	100	215.9	424.5	158.4
Hexanol	1366.9	100	19.8	13.6	21.4
3-Hexene-1-ol	1396.2	100	73.7	53.9	103.7
Linalool	1561.7	100	97.7	88.0	134.0
Linalyl acetate	1563.7	100	112.2	312.3	183.9
Diethyl succinate	1698.3	100	245.5	200.0	223.7
Ethyl dodecanoate	1850.0	100	129.4	119.7	124.3
Number of compounds		122	110	132	128

Control = skin fermented; MCf = maceration carbonique (19 days, 26 °C) free-run wine; MCp maceration carbonique (19 days, 26 °C) press wine; MC+S = maceration carbonique (19 days, 26 °C) + skin fermentation (two days)

Carbonic macerated press wines are taken for "potentially" richer in aromatic and flavour compounds than the free run wines produced in carbonic maceration way (FLANZY et al., 1987). This was evidenced also by results of our experiments (Fig. 1). 132 volatile components were examined in the press wine of classical MC, whilst in the freerun wine, the components detected were 110.

According to BITTEUR and co-workers (1992), in the course of carbonic maceration winemaking, the anaerobiosis enhances both the directly discernible aroma (free terpenols) and the potential aroma (bound terpenols) of the grape. We extended the experiments by studying the terpenols and the derivatives of them. On the basis of the method applied, linalool and linalyl acetate could be identified. The linalool amounts proved nearly equal in the wines of the experiment, though, MC+S sample contained somewhat higher quantity. As the analysis was performed 10 months after winemaking, a turning of the linalool content into other aromatic component, in the mean time, could be supposed. MC-wines proved significantly larger linalyl acetate content than the control wine. It could be also observed that this component showed larger enrichment in press wines than in the free-run wine.



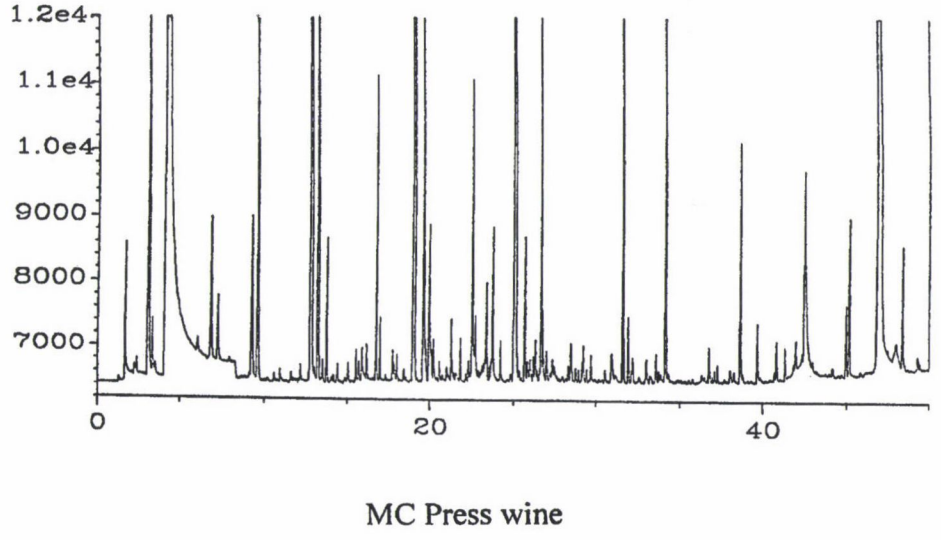


Fig. 1. Chromatograms of the control (skin-fermented) wine and the same wine made by carbonic maceration (MC press wine)

Previously, it had been observed that the MC-wines contained less methanol than the skin-fermented, control wines (NAVARRO et al., 1988). At the experiments disclosed here, we also observed significant methanol enrichment in the control wine made in skin-fermentation way. A fermentation together with long-treated skin structure made the methanol content increase.

This is an outcome of the ezymatic decomposition of pectines, principally. Methanol content in press wine surpassed the level taken in the free-run wine.

3. Conclusions

It may be emphasized that odour and flavour of the MC-produced wines are predominated by a special aroma. This flavour is mainly attributed to the different proportion of components. There are less from some of the flavour components in MC-wines than in the control wine, while the quantity of some other flavour components such as ethyl lactate, diethyl succinate has increased in the carbonic macerated wines etc.

The control, skin-fermented wine contained more methanol, hexanol. Furthermore, detailed examination is necessary to determine the flavour components giving special flavour to MC wines.

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We express our thanks to Mr. T. ERDŐSS for his help.

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IDENTIFICATION OF IRRADIATION IN CODED BLACK PEPPER SAMPLES BY DIFFERENT PHYSICAL METHODS (VISCOSIMETRY AND ESR)

Z. FORMANEK[a], S. BARABÁSSY[a], J. RAFFI[b], S. CHABANE[b], C. MOLINA[c], P. STOCKER[c] and V. DEYRIS[d]

[a] Department of Refrigeration and Livestock Products Technology, Faculty of Food Science, University of Horticulture and Food Science, H–1118 Budapest, Ménesi u. 45. Hungary
[b] Laboratory of Food Quality Research (LARQUA), Faculty of Saint Jérôme, Marseille. France
[c] Laboratory of Chemistry of Natural Products, Faculty of Saint Jérôme, Marseille. France
[d] Laboratory of Applied Biochemistry, Faculty of Saint Jérôme, Marseille. France

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The experiment had been carried out on four black pepper samples coded A, B, C, D. Two physical methods (viscosimetry with two different sample preparation and electron spin resonance) were used to detect irradiated samples. Both viscosimetry preparation methods (10% for heat gelatinization, 8% for non heat gelatinization) showed significant differences in apparent viscosity of coded samples and gave similar results at the examined concentrations. However, heat gelatinization gives greater differences in viscosity between the different samples. All the examined methods showed that sample D was not irradiated, but samples A and C were irradiated. Regarding the results of viscosity measurements sample A and C had to be irradiated at about 8 kGy (according to the calculations of identification number from the literature and our experience). Sample B could have been irradiated at a very low level or long ago according to ESR results, but it was not irradiated according to the results of viscosimetry. Identification of irradiation >8 kGy in black pepper samples is possible using these methods after one month storage at ambient temperature. In well organised conditions all the measurements can be carried out in 3 h.

The applied dose for coded black pepper samples were the following:
A = 8 kGy, B = 0 kGy, C = 8 kGy, D = 0 kGy

Keywords: detection, ESR, EPR, identification, irradiation, pepper, viscosity

For international trade of different irradiated foods there is a need for simple and reliable methods to identify irradiated foodstuffs. In case of spices, the most important methods are viscosity measurement, electron spin resonance (ESR) and thermoluminescence (TL).

Viscosity measurement was reported to be a promising method for identification of irradiated spices by MOHR and WICHMANN (1985) and FARKAS and co-workers

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(1990). They described a method with heat gelatinization of starch of different spices. FORMANEK and co-workers (1994) and BARABÁSSY and co-workers (1995, 1996) suggested an alternative method which was less time-consuming because of neglection of heat gelatinization; in case of cinnamon and allspice (15% suspensions, particle size <0.16 mm) the apparent viscosities seemed to be as sensitive to irradiation dose as those of heat gelatinised spices.

ESR is known to be a very sensitive method but in case of spices it did not lead to favourable conclusions because the main radio-induced signal decreased too fast with storage time and disappeared before the maximal usual commercial storage time (RAFFI & STOCKER, 1996), however, other authors suggest longer existence of cellulose peaks (POLONIA et al., 1995) and paprika was included in the European protocol (ANON, 1995).

Our aim was to compare the sensitivity of two methods (viscosimetry and electron spin resonance) and to identify irradiated black pepper from coded samples after 1 month storage at ambient temperature.

1. Materials and methods

1.1. Black pepper

About 1 kg black pepper of Vietnamese origin was ground and sieved. One hundred g from particles below 0.5 mm were packed into four plastic bags and sealed. The four bags were marked as A, B, C, D.

1.2. Irradiation

Two of the packed black pepper samples were irradiated in a Co-60 gamma irradiation facility by AGROSTER Ltd (Hungary). We received the four bags with codes A, B, C and D.

1.3. Viscosity

For measurement of apparent viscosity of samples a Rheomat, Mettler RM 180 rotational viscosimeter was used. Two types of sample preparation were carried out simultaneously in order to find a shorter preparation for a screening method. The first one was heat gelatinization described by FARKAS and co-workers (1990). The other one was without heat gelatinization (FORMANEK et al., 1994, BARABÁSSY et al., 1995, 1996). The main separation steps are put in Table 1.

Table 1

Preparation of black pepper samples for viscosity measurements

Preparation steps	With heat gelatinization	Without heat gelatinization
Preparation of 100 ml X% suspension	10%	8%
Homogenisation	yes, 15.000–20.000 r.p.m. for 30 s	not necessary, mixing with glass rod
pH setting	pH=12.5 by adding NaOH	pH=12.5 by adding 10 ml 33% NaOH
Heat gelatinization at 100 °C for 30 min	yes	no
Cooling samples at room temperature for 1 h	yes	no

Measurements of black pepper samples were carried out at 25±1 °C in the velocity gradient range of 190–1291 s^{-1} in three replicates. To calculate the value of normalised viscosimeter, Shell Helix 15W-40 commercial mineral oil was used as standard material. It was measured in different measuring systems (MS 11, MS 22) covering together the viscosity range of 0.0030–30.0000 Pas. MS 22 for heat gelatinised samples and MS 11 for non heat gelatinised samples were used. Viscosity measurements for heat gelatinised samples were carried out immediately after cooling. For the samples which were not heat gelatinised, the apparent viscosity measurement was done after 1 and 4 h of pH setting.

Normalised viscosity was calculated by dividing the measured viscosity of the sample at a given velocity gradient by the viscosity of the mentioned mineral oil. Normalised viscosity could be used to compare the measured viscosities of spices on different instruments. Identification parameter was calculated by dividing the highest viscosity (supposed to be the control) at a given velocity gradient by the viscosity of the sample.

1.4. Electron Spin Resonance (ESR)

A Bruker EMS 104 spectrometer was used. Samples were filled into measuring tubes (i.d. 3 mm) and measured with different parameters. The final measuring parameters can be seen in Table 2.

Table 2

ESR measuring parameters for detecting irradiation in coded black pepper samples

Part of the instrument	Parameter (abbreviation)	Value
Receiver	Receiver gain (RRG)	3.556559e+05
	Phase (RPH)	0.000000e+00 deg
	Harmonic (SH)	1.000000e+01
	Modulation – requency (RMF)	1.00000e+02 kHz
	Modulation amplitude (RMA)	1.009532e+01 G
Signal channel	Sweep time (RST) =	1.677722e+02 ms
	Conversion (RCT)	
	Time constant (RTC)	2.048000e+01 ms
Field	Centre field (HCF)	3.480000e+03 G
	Sweep width (HSW)	3.000000e+02 G
	Resolution (ANZ)	1024 points
Microwave	Frequency (MF)	9.792196 Ghz
	Power (MP)	7.905694e-01 mV

Number of scans 7

2. Results and discussion

2.1. Apparent viscosity

The viscosity of mineral oil could only be measured with both measuring systems in the velocity gradient range of 190–348 s^{-1}. After determining the viscosity of oil the same value was found in both measuring systems in the examined velocity gradient region. The apparent viscosity values of coded samples can be seen in Table 3.

There were no significant differences between the apparent viscosities of black pepper samples which were prepared without heat gelatinization and were kept at room temperature for 1 and 4 h (with one exemption, sample B). The summary of normalised parameters can be seen in Table 4. Normalized parameters of sample B and D were higher than those of A and C.

Table 5 shows the identification values which were calculated from the viscosity values of black pepper samples. Identification values of sample A and C were higher than those of samples B and D.

Sample D was regarded to be the control sample as having the highest viscosity. According to both preparation methods, samples B and D seemed to be non irradiated or only at a very low level. Sample A seemed to be irradiated at somewhat higher dose than sample C. According to the method without heat gelatinization (in 8% suspensions) samples A and C were irradiated in the same conditions.

Table 3

Calculated apparent viscosities of black pepper samples (accuracy of 10–15%)

Method	Measuring time [h]	Measured apparent viscosity values [Pas]							
		at 190 s^{-1}				at 348 s^{-1}			
		A	B	C	D	A	B	C	D
With heat gelatinization	Immadiately after cooling	0.0425	0.4090	0.0574	0.4473	0.0364	0.2724	0.0462	0.3039
Without heat gelatinization	1	0.0397	0.2706	0.0350	0.2242	0.0279	0.1901	0.0297	0.1678
Without heat gelatinization	4	0.0391	0.2168	0.0389	0.2168	0.0280	0.1554	0.0270	0.1554

Table 4

The normalised parameters of heat gelatinised and non heat gelatinised black pepper samples

Method	at 190 s^{-1}				at 348 s^{-1}			
	A	B	C	D	A	B	C	D
With heat gelatinization	0.24	2.32	0.32	2.53	0.18	1.38	0.23	1.54
Without heat gelatinization	0.22	1.37	0.21	1.25	0.13	0.77	0.13	0.72

Viscosity of oil at 190 s^{-1} was 0.1765+0.0057 (at 25.3+0.2 °C) and at 348 s^{-1} it was 0.197+0.022 (24.85+0.15 °C)

Table 5

Identification values of coded black pepper samples calculated from the apparent viscosities of black pepper samples

Method	at 190 s^{-1}				at 348 s^{-1}			
	A	B	C	D	A	B	C	D
With heat gelatinization (10%)	10.52	1.09	7.79	1.00	8.34	1.11	6.58	1.00
Without heat gelatinization (8%)	5.60	0.91	5.97	1.00	5.78	0.94	5.70	1.00

Identification parameter for sample A and B with this preparation procedure was between 5–6. An identification parameter of ~5.2 with heat gelatinization procedure could be calculated from data of POLONIA and co-workers (1995) for black pepper samples which were irradiated at 10 kGy.

2.2. Electron Spin Resonance

The ESR spectra of samples consisted of a strong central signal, which was present in all samples, and one small signal from cellulose radicals on the left of the main signal, which was only observed in samples A and C. POLONIA and co-workers (1995) found the same cellulose radical signal after 8 months of storage at room temperature of irradiated (10 kGy) black pepper. The small signal on the right could not be seen because it was overlapped by the signal of Mn^{2+} (RAFFI & STOCKER, 1996). Our measurements can be seen in Fig. 1. Sample A presents a peak at 3458.7 G, sample B presents a shoulder, sample C a peak at 3458.4 G and sample D contains no peak nor shoulder left from the central peak.

According to the ESR results sample A and C were irradiated. Sample B is different from D and different from A and C. Sample B could have been irradiated at a very low level or very long time ago. Sample D was not irradiated.

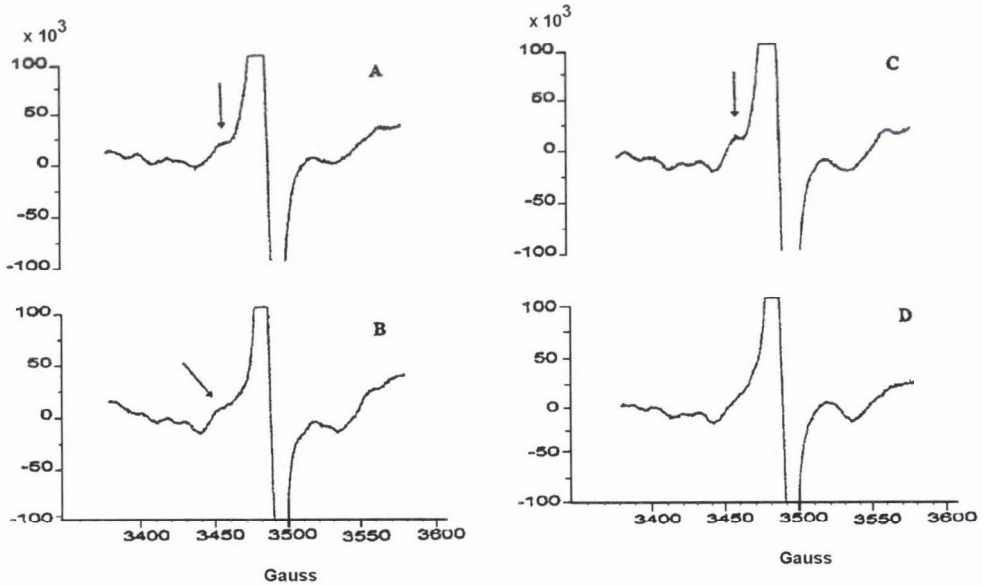

Fig. 1. First derivative ESR spectra of coded black pepper samples (A, B, C, D)

3. Conclusions

Both viscosimetry preparation methods resulted in significant differences in apparent viscosity of coded samples and gave similar results, however at these concentrations heat gelatinization seemed to be more sensitive to differences in viscosity between the samples. Both of the examined methods (ESR and viscosimetry) showed that sample D was not irradiated. Sample A and C was irradiated according to all the methods. According to viscosity measurement these samples might have been irradiated with more than 8 kGy (according to the calculations of identification number from the literature and our experience). Sample B could be irradiated at very low level or long ago according to ESR results but it was not irradiated according to the results of viscosimetry. The information we got from the radiation processor after the analysis revealed that samples B and D were untreated whereas A and C were irradiated at 8 kGy. Therefore, identification of an irradiation treatment of 8 kGy is possible in black pepper samples using these two methods at least one month of post-irradiation storage. In well established conditions all the measurements can be carried out in 3 h.

*

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Acta Alimentaria, Vol. 28 (1), pp. 111–112 (1999)

Book review

Edible coatings and films to improve food quality

J. M. KROCHTA, E. A. BALDWIN, M. O. NISPEROS-CARRIEDO (Eds)

Technomic Publishing Co., Inc., USA, 1994, ISBN No. 1-56676-113-1, 379 pages

Edible coatings of foods seem to be a new invention, however, they are used already for centuries as a method of preserving food products. As chapter 1 cites, dipping of oranges and lemons in wax to retard water loss was practised in China already in the twelfth and thirteenth centuries. About 400 hundred years ago in England food products were coated with fat (larding) to prevent loss of moisture. In our days the application of edible coatings and films is quite wide, i.e. casings for sausages, wax coatings of fruits, chocolate coatings for different products, etc. The trend is to widen the application of this technology further in order to fulfill the consumer needs for fresh, healthy and safe food. It also may reduce the environmental consequences of packaging and in most cases materials used for the production of edible coatings and films are present as renewable sources in the agriculture.

The book collects and digests the information and knowledge in the field of materials used for coatings and films production, their properties (supported with mathematics), their application as coatings, encapsulation materials, and as carriers of food additives, preservatives. Different food commodities, with special interest to fruits and vegetables – fresh and minimally processed – are also discussed in details.

The chapters are as follows: Chapter 1.: Edible films and coatings: characteristics, formation, definitions, and testing methods; Chapter 2: Edible coatings for fresh fruits and vegetables: past, present and future; Chapter 3: Development of edible coatings for minimally processed fruits and vegetables (including the problems connected with the minimal processing techniques and the osmotic membrane (OSMEMB) process); Chapter 4: Edible coatings and films for processed foods (next to the fruits and vegetables the meat, poultry, seafood, nuts, cereals and cereal-based products, confectioneries, and heterogeneous products are also discussed); Chapter 5: Flavour encapsulation; Chapter 6: Edible coatings as carriers of food additives, fungicides and natural antagonists; Chapter 7: Permeability properties of edible films (permeability to water vapor, permanent gases, lipids, their mechanism and mathematics, solubility and diffusivity analysis, and values for water vapor and gas permeability are included); Chapter 8: Application of coatings (the commodities, methods of application and some problem-solving advice are enlisted); Chapter 9: Edible coating and films based on proteins (the largest chapter dealing with the development, properties and application of protein-based films and coatings also outlining the trends for future research necessary in this field); Chapter 10: Edible coatings from lipids and resins; Chapter 11: Edible coatings and films based on polysaccharides (cellulose, starches and their derivatives, pectins, seaweed extracts, exudate, seed, microbial fermentation and gellan gums, chitosan are discussed); Chapter 12: Mathematical modeling of moisture transfer in food systems with edible coatings.

0139-3006/99/$ 5.00

Each chapter is written by outstanding experts of the subjects. List of references closes the chapters. A well-compiled index helps the orientation.

This comprehensive study is very useful for those who would like to get informed on the development and present situation as well as the problems and future trends of edible coatings and films, that is for graduate and post-graduate students, technologists, microbiologists in the research and production.

J. BECZNER

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LUGASI, A., ALMEIDA, D. P. F. & DWORSCHÁK, E.

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IMPROVING VIABILITY OF BIFIDOBACTERIA BY MICROENTRAPMENT AND THEIR EFFECT ON SOME PATHOGENIC BACTERIA IN STIRRED YOGHURT

S. A. HUSSEIN and K. M. K. KEBARY

Department of Dairy Science and Technol., Faculty of Agriculture,
Menofiya University, Shibin El-Kom. Egypt

(Received: 14 April 1998; accepted: 15 December 1998)

Fifteen batches of stirred yoghurt were made to study the effect of microentrapment on the viability of bifidobacteria and their ability to inhibit the growth of *E. coli* and *Staph. aureus*. Entrapped cells of *Bifidobacterium bifidum* and *Bifidobacterium infantis* were able to produce antimicrobial agents which inhibited *E. coli* and *Staph. aureus* used as test organisms. Viable counts of unentrapped bifidobacteria decreased sharply, while entrapped cells of bifidobacteria were quite stable during refrigerated storage of stirred yoghurt. *Bif. infantis* was more tolerant to storage conditions than *Bif. bifidum*. Microentrapment of bifidobacteria improved their survival during storage of stirred yoghurt, especially *Bif. bifidum*, whose viability was not significantly ($P>0.05$) different from entrapped *Bif. infantis*. Viable counts of *E. coli* decreased during storage of stirred yoghurt. Addition of bifidobacteria caused a sharp decrease in the viability of *E. coli*. *E. coli* growth was not dectected at the 5th day, when entrapped cells of bifidobacteria were added to stirred yoghurt, while *E. coli* growth was not detected at the 7th day of storage in yoghurt containing unentrapped bifidobacteria. Addition of *Bif. bifidum* inhibited the growth of *E. coli* more effectively than *Bif. infantis*. *Staph. aureus* showed similar patterns to *E. coli*, except that *Staph. aureus* was more tolerant to storage conditions. The counts of total bacteria, lactobacilli and *Streptococcus salivarius* subsp. *thermophilus* increased up to the third day then declined till the end of storage. Titratable acidity increased gradually during the first 3 days of storage then increased slightly up to the end of storage, while pH values dropped during storage. Adding of bifidobacteria, *E. coli* and *Staph. aureus* did not affect significantly ($P>0.05$) the counts of lactobacilli and *Str. salivarius* subsp. *thermophilus*, acidity and pH value.

Keywords: viability, bifidobacteria, microentrapment, stirred yoghurt, pathogenic bacteria

Yoghurt is the most popular fermented milk produced in Egypt and worldwide. Its consumption in Egypt has almost doubled in the past three years. The value of yoghurt in human nutrition is based, not only on the nutritive effect of the milk from which it is made and increased digestibility, but also on the beneficial effect of intestinal microflora, prophylactic and healing effects (RASIC & KURMANN, 1978;

MARSHALL, 1984; GURR, 1987; AGERBAEK et al., 1995; SCHAAFSMA, 1996; TVEDE, 1996; YAESHIMA, 1996; BADAWI & EL-SONBATY, 1997 and BUTTRISS, 1997).

The majority of yoghurt brands are made in modern dairy plants but still some brands are made in dairy stores under poor hygienic conditions. Yoghurt is very sensitive to changes in temperature and could be exposed to spoilage. Also yoghurt could be contaminated during processing and handling. Coliform bacteria, *Escherichia coli* and staphylococci have been found in yoghurt collected from retail markets (ABOU-DONIA et al., 1975; SAAD et al., 1987; IBRAHIM et al., 1989 & YOUSEF, 1996).

Bifidobacteria are the predominant gut flora in breast-fed infant (RASIC & KURMANN, 1983). These bacteria are becoming recognized worldwide because of their health and nutritional benefits such as potential beneficial roles in the human intestinal tract (KURMANN & RASIC, 1991; ROBINSON & SAMONA, 1992; VIJAYENDRA & GUPTA, 1992; HOOVER, 1993). Antitumorigenic activity, improvement of lactose-tolerance, reduction of serum cholesterol levels, reduction of ammonia and free serum phenol in patients with liver disease, synthesis of vitamins, increased immunocompetence and antagonistic effects towards enteropathogenic bacteria have all been deviled (POUPARD et al., 1973; ANAND et al., 1984; KAGEYAMA et al., 1984; OKAMURA et al., 1986; UEDA, 1986; YAMAZAKI et al., 1985; HUGHES & HOOVER, 1991; HOOVER, 1993; ISHIBASHI & SHIMAMURA, 1993; DAVIDE, 1995; KEBARY, 1995; SUZUKI, 1995; JIANG et al., 1996; ZOMMARA et al., 1996; BADAWI & EL-SONBATY, 1997; KAILASAPATHY & RYBKA, 1997; ATHRAYILKKALATHIIL & PRASAD, 1997). It is estimated that over 70 products containing bifidobacteria are produced worldwide (HUGHES & HOOVER, 1991; SHAH, 1997). They include fermented milk, butter milk, sour cream, frozen dessert, cheese, baby foods, pharmaceutical preparations and livestock feed supplements (KURMANN & RASIC, 1991; HEKMAT & MCMAHON, 1992; HUNGER & PEITERSEN, 1992; DINAKAR & MISTRY, 1994; GOMES et al., 1995; TAMIME et al., 1995; BLANCHETTE et al., 1996; FORBES et al., 1996; HAYES et al., 1996; KEBARY, 1996; MARTIN, 1996; SEZGING et al., 1996; KEBARY et al., 1998).

It has been speculated that the minimal number of viable cells of bifidobacteria in a product should be more than 10^5 g^{-1} to achieve the therapeutic effects (SAMONA & ROBINSON, 1991; HUNGER & PEITERSEN, 1992; LEE et al., 1996). Survival of bifidobacteria in fermented dairy products depends on the strain, the species, fermentation conditions and storage temperature (MARTIN & CHOU, 1992; LANKAPUTHRA et al., 1996; BLANCHETTE et al., 1996; MARTIN, 1996; SHIN et al., 1996; BADAWI & EL-SONBATY, 1997; MICANEL et al., 1997; DAVE & SHAH, 1997). Even the most resistant strains of bifidobacteria are likely to decrease during storage of fermented milks. Although *Bifidobacterium bifidum* is commonly used by manufacturers, it appears to be less tolerant to pH than *Bifidobacterium infantis*

(MARTIN, 1996). Microencapsulation has been used to protect bifidobacteria in cheese and frozen ice milk (DINAKAR & MISTRY, 1994; KEBARY et al., 1998).

The objectives of this study were to investigate the effect of microentrapment of bifidobacteria on the production of antimicrobial substances, to study the behaviour of different strains of bifidobacteria during storage of stirred yoghurt, to study the effect of microentrapment on the survival of bifidobacteria and to study the effect of adding bifidobacteria to stirred yoghurt on the growth of some pathogenic bacteria.

1. Materials and methods

1.1. Bacterial strains

Streptococcus salivarius subsp. *thermophilus* EMCC1043 and *Lactobacillus delbrueckii* subsp. *bulgaricus* EMCC1102 were obtained from Cairo Mircen (Ain Shams University, Cairo, Egypt). *Bifidobacterium bifidum* DI was provided by Diversitech Inc (Gainesville, FL), while *Bifidobacterium infantis* 4038 was provided by Prof. Morsi El-Soda (Dairy Sci. Department, Faculty of Agriculture, Alexandria University, Egypt). *Escherichia coli* K12 and *Staphylococcus aureus* K12C600 were obtained from the Department of Microbiology, College of Pharmacy, Tanta University, Egypt. *Lactobacillus bulgaricus* and *Streptococcus thermophilus* were activated by three successive transfers in sterile 10% reconstituted non-fat dry milk. Bifidobacteria strains were activated by three successive transfers in modified lactobacilli MRS broth and incubated under anaerobic conditions (VENTLING & MISTRY, 1993). *E. coli* and *Staph. aureus* were activated by two successive transfers in MacConkey and Staphylococcus Medium 110 broth, respectively.

1.2. Preparation of entrapped bifidobacteria

Five milliliters from each active bifidobacterial strain was inoculated separately into flasks containing 95 ml of modified MRS and incubated for about 18 h at 37 °C under anaerobic conditions. Cells were harvested by centrifugation at 1500×g for 15 min and washed twice with sterile saline solution. Bifidobacterial cells from each strain were suspended individually in sterile saline to about 1.0×10^{10} CFU ml^{-1}. Cells from each strain were microentrapped individually in calcium alginate gels according to the method described by SHEU and MARSHALL (1993). One part of cell suspension was mixed with four parts of 3.0% sodium alginate solution (BDH Chemicals Ltd Poole, England). One part of the alginate-cell mixture was then added dropwise by a syringe to five parts of corn oil containing 0.2% Tween 80 (in a 1000 ml beaker), which was magnetically stirred at 200 r.p.m. A uniform turbid emulsion was obtained within 10 min. Calcium chloride (0.05 mol) was added quickly at the side of the beaker, while it

was magnetically stirred until the water-oil emulsion was broken. Calcium alginate beads were formed within 10 min. The beads were collected by centrifugation at 350×g for 10 min and washed with sterile distilled water. Beads were kept at 5–8 °C in a small volume of sterile distilled water until usage to prevent their collapse and adherence.

1.3. Production of antimicrobial substances

This test was carried out to study the effect of microentrapment on the production of antimicrobial substances. Tubes containing 10.0 ml of sterile modified MRS broth were inoculated with about 1.0×10^6 viable cells of *Bifidobacterium bifidum* or *Bifidobacterium infantis* either entrapped or unentrapped separately. Tubes were incubated anaerobically at 37 °C for 48 h. The fermented cultures were centrifuged at 8000 r.p.m. for 30 min to obtain the cell free broth, which was used to measure the inhibitory activity by the disc assay procedure (PULUSANI et al., 1979). *Staph. aureus* K12C600 and *E. coli* K12 were seeded on Staphylococcus Medium 110 and MacConkey agar C and used as test organisms.

1.4. Manufacture of yoghurt

Fresh buffaloe's milk was obtained from the herd of the Faculty of Agriculture, Shibin El-Kom, Egypt. Yoghurt was made as illustrated in Fig. 1. The amount of entrapped and unentrapped bifidobacteria were calculated to get approximately 1.5×10^7 CFU g^{-1} of stirred yoghurt. Also *E. coli* and *Staph. aureus* were added to levels of about 1.0×10^7 CFU g^{-1} stirred yoghurt. Stirred yoghurt was distributed into 120 ml plastic containers and stored in the refrigerator for 7 days. The experiment was performed in triplicate. All treatments were sampled at zero time and 1st, 3rd, 5th and 7th day of storage for bacteriological, pH and acidity analysis. Sample designations are shown in Fig. 1.

1.5. Bacteriological analysis

Total viable bacterial counts were enumerated on standard plate count agar (MESSER et al., 1985). MRS medium was used to enumerate *L. delbrueckii* subsp. *bulgaricus* (DEMAN et al., 1960). *Str. salivarius* subsp. *thermophilus* was enumerated on yeast-lactose agar (SKINNER & QUESNEL, 1978). *E. coli* and *Staph. aureus* were enumerated on violet red bile agar and Staphylococcus Medium 110 agar, respectively (DIFCO, 1984). Bifidobacterium counts were enumerated on modified MRS agar (VENTLING & MISTRY, 1993) with NPNL solution (neomycin sulfate 0.2%, paromomycin sulfate 0.2%, nalidixic acid 0.03% and lithium chloride 6.0%) (SAMONA & ROBINSON, 1991). Samples containing beads were suspended in 9.0 ml of sterile phosphate buffer (1 mol, pH 7.5) followed by gentle shaking at room temperature for 10 min to release bifidobacteria from beads (SHEU et al., 1993).

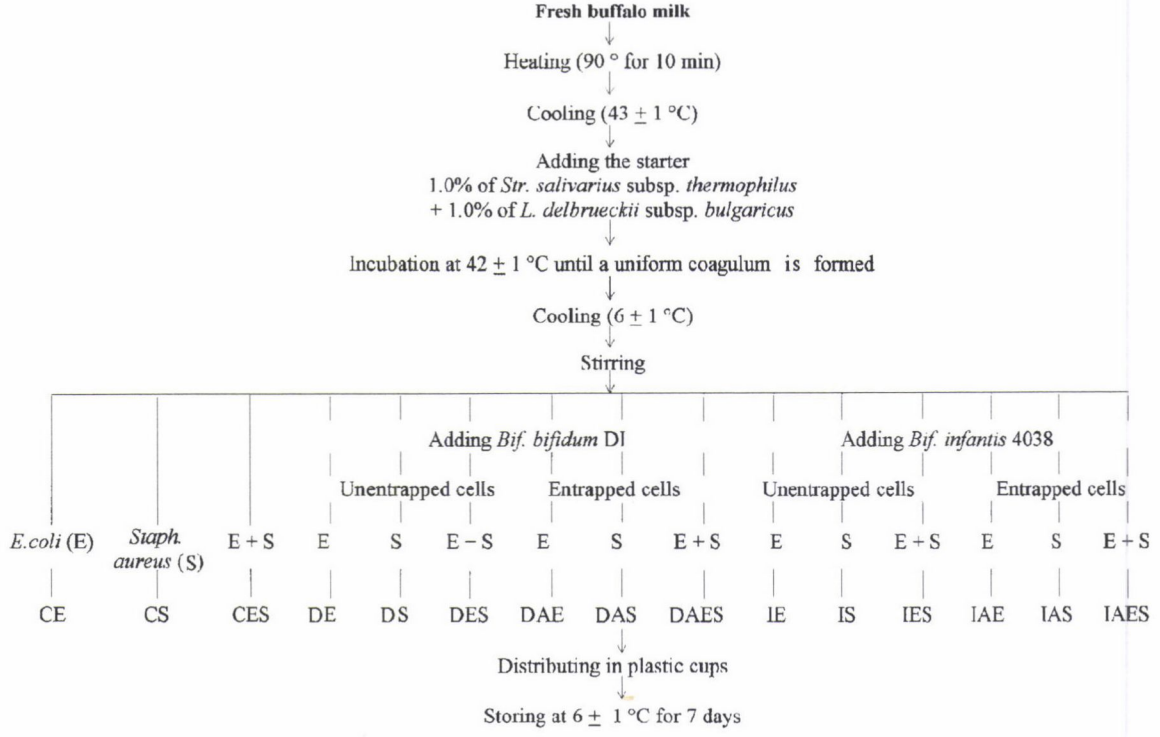

Fig. 1. Manufacturing steps of stirred yoghurt. E: *E. coli*, S: *Staphylococcus aureus*

1.6. Chemical analysis

Titratable acidity and pH value were determined according to LING (1963).

1.7. Statistical analysis

A two factors ×3 replicates factorial design was used to analyze the data for each effect and Duncan's test was used to make the multiple comparisons (STEEL & TORRIE, 1960). Significant differences were determined at $P \leq 0.05$ level.

2. Results and discussion

2.1. Production of antimicrobial agent by bifidobacteria

Inhibitory activity of bifidobacteria against gram-positive and gram-negative-bacteria has been reported (HOOVER, 1993; ISHIBASHI & SHIMAMURA, 1993; GIBSON & WANG, 1994; DAVIDE, 1995; KEBARY, 1995; BADAWI & EL-SONBATI, 1997). Antagonistic effects of *Bif. bifidum* DI and *Bif. infantis* 4038 against *Escherichia coli* K12 and *Staphylococcus aureus* K12C600 are shown in Figs 2 and 3. It is obvious that entrapped cells of *Bif. bifidum* or *Bif. infantis* were able to produce antimicrobial substances and inhibit the growth of *E. coli* or *Staph. aureus* (Figs 2 and 3). Supernatant of *Bif. bifidum* culture was more effective at inhibiting the growth of *E. coli* or *Staph. aureus* than that of *Bif. infantis*, which might be due to the differences in the amount and/or the kind of antimicrobial substances produced by each strain (GIBSON & WANG, 1994; BADAWI & EL-SONBATY, 1997).

2.2. Survival of bifidobacteria

Product should contain levels of bifidobacteria higher than 10^5 CFU ml^{-1} to produce therapeutic benefits. Therefore this work is concerned with improving viability of bifidobacteria during storage of yoghurt. The viability of both *Bif. bifidum* and *Bif. infantis* increased up to the first day of storage then declined during the storage of stirred yoghurt (Fig. 4 and Table 5) (MARTIN & CHOU, 1992; LANKAPUTHRA et al., 1996; KEBARY et al., 1996; MARTIN, 1996; BADAWI & EL-SONBATY, 1997; DAVE & SHAH, 1997; MICANEL et al., 1997). SHAH (1997) reported that the growth of most strains of *Bifidobacterium* is significantly retarded below pH 5.0. Unentrapped *Bif. infantis* showed better survival than unentrapped *Bif. bifidum* during storage of stirred yoghurt (Fig. 4 and Table 5). *Bif. bifidum* appeared to be less tolerant to storage conditions (LANKAPUTHRA et al., 1996; MARTIN, 1996; SHAH, 1997).

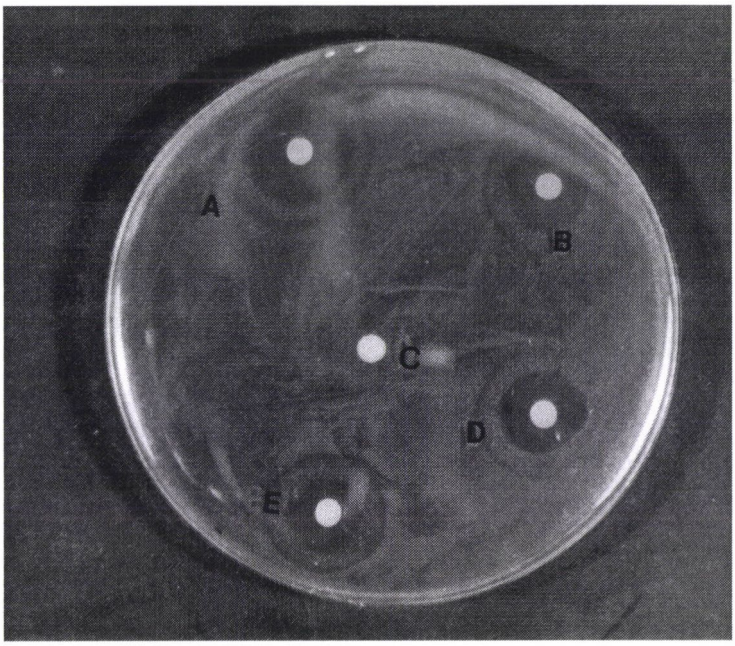

Fig. 2. Photography showing the inhibitory effect of supernatant fluid of bifidobacteria on the growth of *Escherichia coli* K12. A: Supernatant of unentrapped *Bif. bifidum*; E: Supernatant of entrapped *Bif. bifidum*; C: Free disk; B: Supernatant of unentrapped *Bif. infantis*, D: Supernatant of entrapped *Bif. infantis*

These results revealed that tolerance of bifidobacteria to storage conditions of stirred yoghurt is species dependent. The counts of unentrapped cells of *Bif. bifidum* and *Bif. infantis* decreased almost one log cycle, while counts of entrapped cells of both strains were almost stable during refrigerated storage of stirred yoghurt (Fig. 4 and Table 5). These results indicate that microentrapment of *Bif. bifidum* cells increased their viability about one log cycle during storage for only 7 days (Fig. 4 and Table 5). Entrapped cells of *Bif. bifidum* survived well and became more tolerant to storage conditions which might be due to the protection provided by microencapsulation of bifidobacterial cells. Survival of entrapped *Bif. bifidum* and *Bif. infantis* was not significantly different ($P>0.05$) from each other (Fig. 4 and Table 5). The counts of bifidobacteria, even unentrapped cells, after 7 days of refrigerated storage remained higher than the recommended level ($>10^5$ CFU ml^{-1}) that should be present in yoghurt to achieve the therapeutic benefits (Fig. 4).

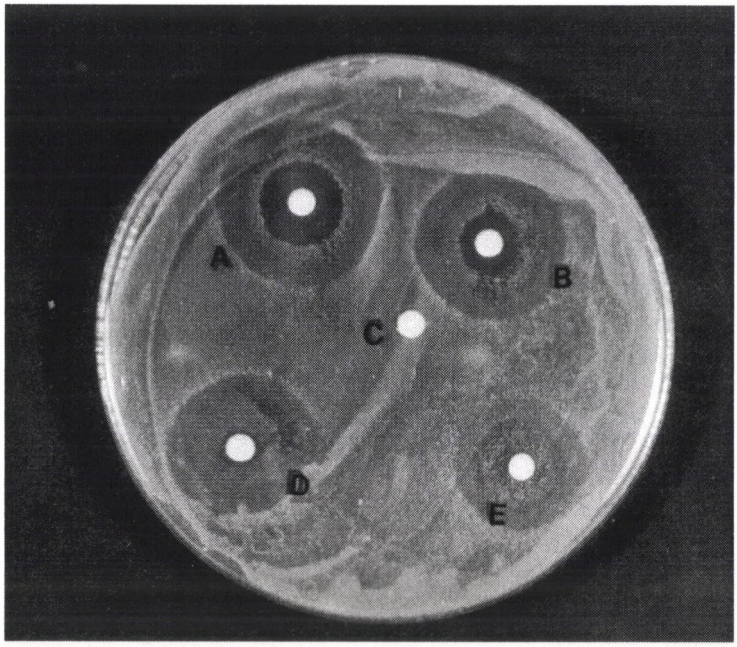

Fig. 3. Photography showing the inhibitory effect of supernatant fluid of bifidobacteria on the growth of *Staphylococcus aureus* K12 C 600. A: Supernatant of unentrapped *Bif. bifidum*; B: Supernatant of entrapped *Bif. bifidum*; D: Supernatant of unentrapped *Bif. infantis*; E: Supernatant of entrapped *Bif. infantis*; C: Free disk

2.3. Fate of pathogenic bacteria

Changes in the counts of *E. coli* during refrigerated storage of stirred yoghurt are presented in Table 1. The cold storage and acidity had an adverse effect on the viability of *E. coli*. Although, the counts of *E. coli* decreased markedly about 5 log cycles during storage in yoghurt without added bifidobacteria, *E. coli* showed faster decay in the presence of bifidobacteria (Tables 1 and 5). This sharp decrease might be due to the production of antimicrobial agents by bifidobacteria (GIBSON & WANG, 1994; KEBARY, 1995; BADAWI & EL-SONBATY, 1997). The viable count of *E. coli* dropped sharply in yoghurt samples containing bifidobacteria and no *E. coli* growth was detected at the 7th day of storage (Tables 1 and 5). *E. coli* growth was not observed in yoghurt samples containing entrapped bifidobacteria at the 5th day of storage (Table 1), which could be attributed to the higher populations of bifidobacteria and subsequently higher production of antimicrobial agents. Samples containing *Bif. bifidum* showed more effective inhibition of the growth of *E. coli* than those containing *Bif. infantis*, which

might be due to the differences in the amount and kind of antimicrobial agents produced. The counts of *Staph. aureus* showed similar patterns to those of *E. coli* except that staphylococci were more tolerant to storage conditions than *E. coli* (Tables 2 and 5). *Staph. aureus* survived until the 7th day of storage in yoghurt samples without bifidobacteria and with *Bif. infantis,* while no *Staph. aureus* growth was detected at the 7th day in yoghurt samples containing *Bif. bifidum* and entrapped cells of *Bif. infantis* (Tables 2 and 5). This means that adding bifidobacteria to yoghurt could inhibit the growth of some pathogenic and undesirable bacteria, which could reach yoghurts by post contamination.

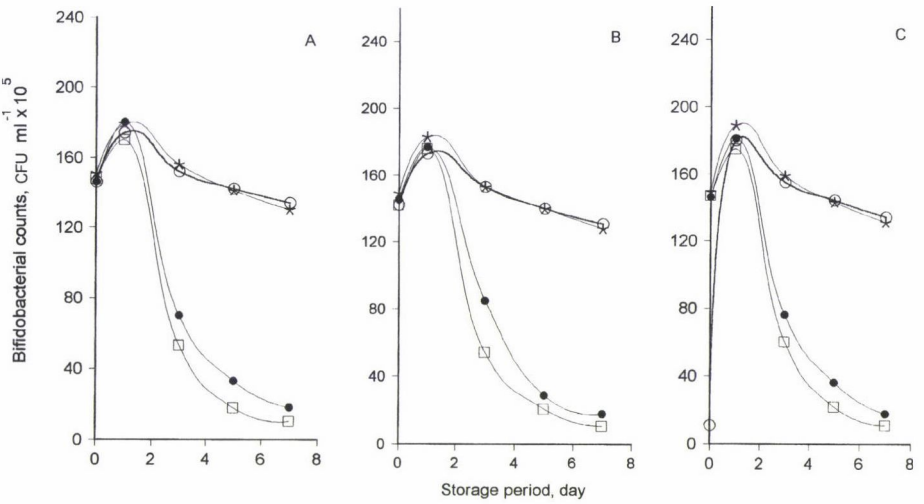

Fig. 4. Survival of bifidobacteria during storage of stirred yoghurt to which bifidobacteria, *E. coli* and *Staph. aureus* were added after manufacturing (each value in the Figure is the mean of three replicates). A: ● IE* ✶IAE ⊟ DE ⊖ DAE; B: ● IS* ✶IAS ⊟ DS ⊖ DAS; C: ● IES* ✶IAES ⊟ DES ⊖ DAES; *See Fig. 1

Table 1

Changes in viable counts (CFU ml^{-1}) of Escherichia coli *during storage of stirred yoghurt to which bifidobacteria,* E. coli *and* Staph. aureus *were added after manufacturing*

Samples	Storage period (days)				
	0	1	3	5	7
CE[a]	96×10^5	31×10^5	73×10^3	28×10^2	17×10
CS	NG	NG	NG	NG	NG
CES	94×10^5	46×10^5	64×10^3	21×10^2	13×10
IE	97×10^5	98×10^3	70×10^2	13×10	NG
IS	NG	NG	NG	NG	NG
IES	96×10^5	86×10^3	63×10^2	9×10	NG
IAE	94×10^5	93×10^3	58×10^2	NG	NG
IAS	NG	NG	NG	NG	NG
IAES	97×10^5	86×10^3	52×10^2	NG	NG
DE	97×10^5	77×10^3	47×10^2	6.3×10	NG
DS	NG	NG	NG	NG	NG
DES	98×10^5	72×10^3	45×10^2	4.5×10	NG
DAE	94×10^5	83×10^3	35×10^2	NG	NG
DAS	NG	NG	NG	NG	NG
DAES	96×10^5	72×10^3	32×10^2	NG	NG

Each value in the table is the mean of 3 replicates.
[a] See Fig. 1.
NG: no growth.

2.4. Behaviour of total bacterial, lactobacilli and streptococci counts

Changes in total bacterial counts are illustrated in Fig. 5. Counts of total bacteria increased up to the third day of storage of all yoghurt samples, which could be due to the residual activity during this period (KEBARY et al., 1996; BADAWI & EL-SONBATY, 1997). Total bacterial counts decreased after the third day to the 7th day by about 40–50% of the maximum survival at the third day of storage (Fig. 5 and Table 5). The decrease in total bacterial counts was more obvious in yoghurt samples containing bifidobacteria (Fig. 5 and Table 5), which might be due to the inhibitory effects of antimicrobial agents produced by bifidobacteria (GIBSON & WANG, 1994; KEBARY, 1995; KEBARY et al., 1996).

Table 2

Changes in viable counts (CFU ml^{-1}) of Staphylococcus aureus *during storage of stirred yoghurt to which bifidobacteria,* E. coli *and* Staphylococcus aureus *were added after manufacturing*

Samples	Storage period (days)				
	0	1	3	5	7
CE[a]	NG	NG	NG	NG	NG
CS	95×10^5	80×10^5	41×10^4	53×10^3	81×10
CES	97×10^5	73×10^5	31×10^4	38×10^3	58×10
IE	NG	NG	NG	NG	NG
IS	93×10^5	90×10^4	28×10^3	37×10^2	8.7×10
IES	94×10^5	75×10^4	16×10^3	22×10^2	4.8×10
IAE	NG	NG	NG	NG	NG
IAS	97×10^5	86×10^4	20×10^3	10.1×10^2	NG
IAES	98×10^5	74×10^4	11×10^3	9.5×10^2	NG
DE	NG	NG	NG	NG	NG
DS	95×10^5	79×10^4	10.8×10^3	9.2×10^2	NG
DES	94×10^5	69×10^4	10.4×10^3	8.5×10^2	NG
DAE	NG	NG	NG	NG	NG
DAS	96×10^5	79×10^4	9.3×10^3	6.0×10^2	NG
DAES	93×10^5	68×10^4	9×10^3	5.7×10^2	NG

Each value in the table is the mean of 3 replicates.

[a] See Fig. 1.

NG: no growth.

Viable counts of lactobacilli and *Streptococcus salivarius* subsp. *thermophilus* (yoghurt bacteria) showed similar patterns of increase or decrease during storage of stirred yoghurt (Figs 6, 7 and Table 5). Counts of yoghurt bacteria increased and reached their maximum survival at the third day of storage then declined up to the end of storage (Figs 6, 7 and Table 5) (KEBARY et al., 1996). Adding bifidobacteria and/or *E. coli* and *Staph. aureus* did not affect significantly ($P>0.05$) the count of lactobacilli and *Str. salivarius* subsp. *thermophilus* (Figs 6, 7 and Table 5).

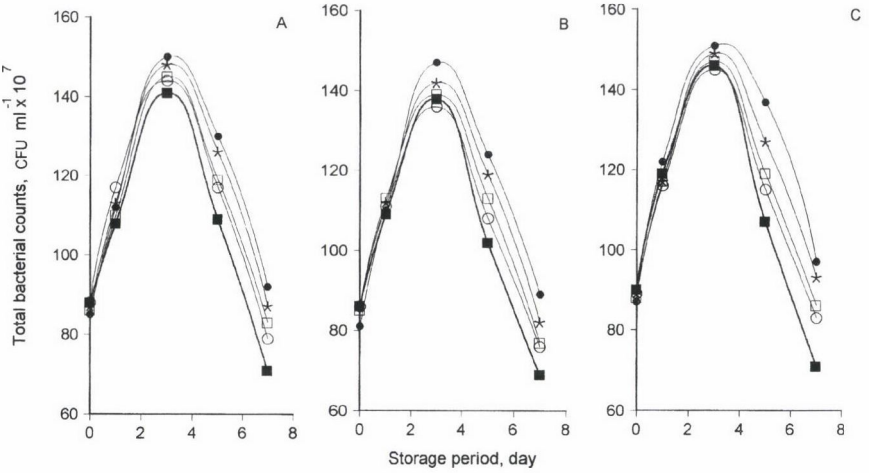

Fig. 5. Total bacterial counts during storage of stirred yoghurt to which bifidobacteria, *E. coli* and *Staph. aureus* were added after manufacturing (each value in the figure is the mean of three replicates). A: ● CE* ✶IE □ IAE ○ DE ■ DAE; B: ● CS* ✶IS □ IAS ○ DS ■ DAS; C: ● CES* ✶IES □ IAES ○ DES ■ DAES; *See Fig. 1

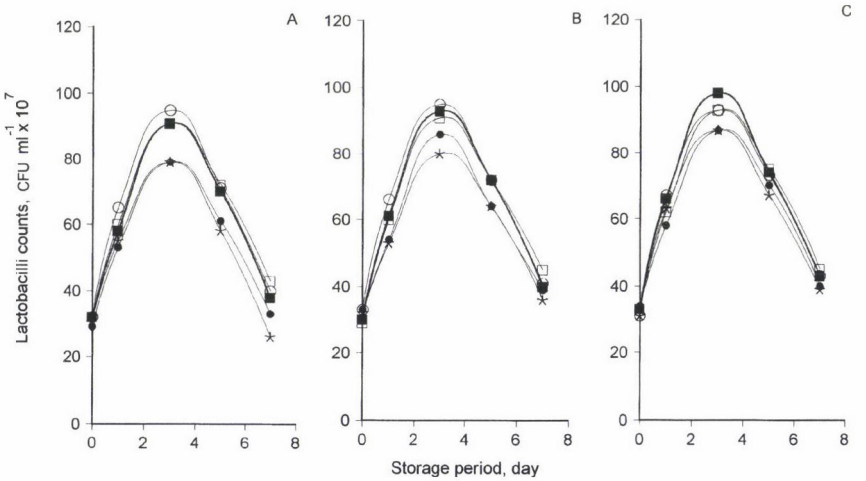

Fig. 6. Lactobacilli counts during storage of stirred yoghurt to which bifidobacteria, *E. coli* and *Staph. aureus* were added after manufacturing (each value in the figure is the mean of three replicates). A: ● CE* ✶IE □ IAE ○ DE ■ DAE; B: ● CS* ✶IS □ IAS ○ DS ■ DAS; C: ● CES* ✶IES □ IAES ○ DES ■ DAES; * See Fig. 1

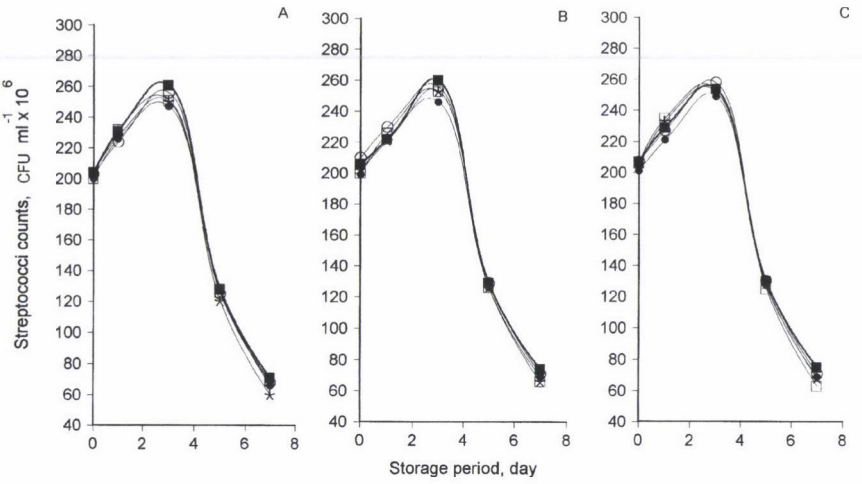

Fig. 7. *Streptococcus salivarius* subsp. *thermophilus* counts during storage of stirred yoghurt to which bifidobacteria, *E. coli* and *Staph. aureus* were added after manufacturing (each value in the figure is the mean of three replicates). A: ● CE* ✶ IE □ IAE ○ DE ■ DAE; B: ● CS* ✶ IS □ IAS ○ DS ■ DAS; C: ● CES* ✶ IES □ IAES ○ DES ■ DAES; * See Fig. 1

Table 3

Changes in titratable acidity (%) during storage of stirred yoghurt to which bifidobacteria, E. coli *and* Staph. aureus *were added after manufacturing*

Samples	Storage period (days)				
	0	1	3	5	7
CE[a]	0.93	1.09	1.14	1.18	1.20
CS	1.00	1.16	1.16	1.19	1.21
CES	1.03	1.12	1.17	1.20	1.24
IE	1.04	1.15	1.17	1.22	1.26
IS	1.02	1.17	1.18	1.21	1.23
IES	1.04	1.15	1.18	1.23	1.26
IAE	1.01	1.13	1.15	1.17	1.23
IAS	0.95	1.12	1.15	1.19	1.24
IAES	1.01	1.12	1.16	1.20	1.24
DE	0.98	1.17	1.19	1.24	1.27
DS	1.05	1.18	1.22	1.26	1.29
DES	0.98	1.17	1.18	1.27	1.31
DAE	0.95	1.16	1.17	1.19	1.21
DAS	0.95	1.16	1.16	1.18	1.20
DAES	0.99	1.18	1.19	1.23	1.26

Each value in the table is the mean of 3 replicates.
[a] See Fig. 1.

2.5. Changes in acidity

Titratable acidity increased gradually during the first 3 days of storage, then increased slightly up to the end of the storage period (Tables 3 and 5). The gradual increase coincides with the increase in yoghurt bacterial counts. Addition of bifidobacteria and pathogenic bacteria did not affect significantly ($P>0.05$) the titratable acidity. Conversely, pH values dropped during the storage period of all yoghurt samples (Tables 4 and 5) (KEBARY et al., 1996, DAVE & SHAH, 1997). Neither bifidobacteria nor pathogenic bacteria affected significantly ($P>0.05$) the pH values of yoghurt (Tables 4 and 5).

Table 4

Changes in pH value during storage of stirred yoghurt to which bifidobacteria, E. coli *and* Staph. aureus *were added after manufacturing*

Samples	Storage period (days)				
	0	1	3	5	7
CE[a]	4.86	4.43	4.13	4.03	3.88
CS	4.83	4.53	4.23	4.00	3.80
CES	4.73	4.33	4.16	3.93	3.73
IE	4.80	4.33	4.06	3.88	3.63
IS	4.80	4.36	4.13	3.96	3.73
IES	4.76	4.33	4.10	3.88	3.60
IAE	4.86	4.46	4.16	4.10	3.88
IAS	4.90	4.43	4.13	4.00	3.73
IAES	4.83	4.43	4.13	3.96	3.73
DE	4.83	4.30	4.03	3.83	3.60
DS	4.76	4.23	4.00	3.80	3.53
DES	4.86	4.30	4.06	3.73	3.43
DAE	4.93	4.43	4.16	4.03	3.76
DAS	4.90	4.40	4.10	4.10	3.83
DAES	4.83	4.35	4.10	4.00	3.73

Each value in the table is the mean of 3 replicates.
[a] See Fig. 1.

Table 5

Statistical analysis of yoghurt properties

Properties of yoghurt	Effect of treatments																Effect of storage period (days)					
	Mean squares	Multiple comparisons[a]															Mean squares	Multiple comparison[a]				
		CE[b]	CS	CES	IE	IS	IES	IAE	IAS	IAES	DE	DS	DES	DAE	DAS	DAES		0	1	3	5	7
Bifidobacterial counts	12305*	NG	NG	NG	C	C	C	A	AB	A	D	D	D	A	A	A	9176*	B	A	C	D	E
E. coli counts	2.299*	A	NG	A	B	NG	B	D	NG	D	C	NG	C	E	NG	E	0.632*	A	B	C	D	E
Staphylococci counts	3.988*	NG	A	A	NG	B	B	NG	D	D	NG	C	C	NG	E	E	0.6719*	A	B	C	D	E
Total bacterial counts	3.388*	A	A	A	B	B	B	B	B	B	B	B	B	B	BC	B	2957*	D	BC	A	B	D
Lactobacilli counts	1076	AB	A	A	ABC	AB	A	A	A	A	A	A	A	A	A	A	18561*	DE	BC	A	B	D
Str. salivarius subsp. *thermophilus*	4.054	A	A	A	AB	A	A	A	A	AB	A	A	A	A	A	A	13687*	C	B	A	D	E
Titratable acidity	0.0103	ABCD	ABCD	ABCD	AB	ABCD	ABC	ABC	ABCD	ABCD	ABC	AB	AB	ABCD	ABCD	ABC	0.430*	E	D	C	AB	A
pH value	0.0936	ABC	AB	ABC	ABC	ABC	ABC	A	ABC	ABC	ABC	ABC	ABC	ABC	ABC	ABC	8.321*	A	B	C	D	E

[a] For each effect, different letter (in the same row) means the multiple comparisons are different from each other, letter A is highest means followed by B, C,... E.

[b] See Fig. 1.

* Significant at 0.05.

NG: No growth.

3. Conclusion

It could be concluded that unentrapped cells of bifidobacteria decreased one log cycle during storage for 7 days, but entrapped cells were stable during storage. *Bif. infantis* was more tolerant to storage conditions than *Bif. bifidum*. Entrapment of bifidobacteria increased their viability by one log cycle. Viable counts of *E. coli* and *Staph. aureus* declined during storage, while in the presence of bifidobacteria the decline was greater. *Bif. bifidum* was more effective for inhibiting the growth of *E. coli* and *Staph. aureus*. Adding bifidobacteria and pathogenic bacteria did not affect the acidity, pH value, the counts of lactobacilli and *Str. salivarius* subsp. *thermophilus*. These results revealed that microentrapment of *Bif. bifidum* and *Bif. infantis* is a promising method to improve the viability of bifidobacteria especially that of *Bif. bifidum*, which became more stable and tolerant to storage conditions of yoghurt. Therefore *Bif. bifidum* will be the most likely choice to use as dietary adjuncts in yoghurt because it is preferred by the manufacturers and, in addition, it has a higher inhibitory activity on some pathogenic bacteria.

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EXAMINATION OF AROMA PRODUCTION KINETICS OF DIFFERENT COMMERCIAL WINE YEASTS IN FERMENTING MUSCAT OTTONEL WINES WITH THE HELP OF SPME HEAD-SPACE SAMPLING AND FAST GC ANALYSIS

GY. VAS[a], I. BLECHSCHMIDT[b], T. KOVÁCS[c] and K. VÉKEY[d]

[a] Research Institute for Viticulture & Enology of Agricultural Ministry, H-3301 Eger, Kőlyuktető P.O. Box 83. Hungary; e-mail address: h12232vas@ella.hu

[b] Frederich Schiller University, Faculty of Chemistry, Institute for Analytical and Inorganic Chemistry, D–07745 Jena, Lessingstrasse 8. Germany; e-mail address: cbi@dagobert.rz.uni-jena.de

[c] University of Horticulture and Food Industry, Department of Food Chemistry and Nutrition, H–1118 Budapest, Somlói út 14–16. Hungary

[d] Research Institute for Chemistry of the Hungarian Academy of Sciences, Laboratory of Mass Spectrometry, H–1025 Budapest, Pusztaszeri u. 59–67. Hungary; e-mail address: vekey@cric.chemres.hu

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Solid phase microextraction (SPME) coupled to fast capillary gas chromatography was used for monitoring the wine fermentation process. This combination offers a simple, quick and sensitive approach suitable for characterization of head-space components of wines during the fermentation process without a complicated sample preparation procedure. In this work this method was used to observe the differences in aroma production between three different commercial yeasts and the indigenous yeast flora.

Keywords: fermentation, head-space sampling, fast GC analysis, SPME, wine yeast

Yeasts have a great effect on wine quality. Different yeasts liberate the glucosidally linked primary aroma compounds to different extent and synthesize the secondary aroma substances in various quantities. Thus, certain yeast strains are more suitable for the fermentation of the different grape musts than the others, because they make the aroma character of the cultivated grape varieties dominant in several different ways. The effect of yeasts is very important in winemaking. DITTRICH (1992) in a Wachau lecture stated that the primary aim of the present enologists is to produce unique wines bearing the identical aroma characteristics of the particular type in question, and therefore one of the most important future tasks of yeast strain selection is to find and isolate the best *Saccharomyces cerevisiae* variants for the various varieties.

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The goal of our work was to find some yeast strains suitable for developing the characteristic fermentation products of certain Eger Region grape musts. In solving this problem a solution simpler than the yeast strain selection has been chosen. In 1996 and 1997 microvinification pilot fermentation experiments were performed by the lyophilized high quality yeast starter cultures of the firm Uvaferm in the Research Institute for Viticulture and Enology in Eger.

The firm wanted to explore which strains are the most suitable for the reductive fermentation of wines under the climatic and provincial conditions in question. Investigations have been carried out with the following grape varieties: Muscat Ottonel, Leányka and Chardonnay. The present paper reports on the Muscat Ottonel fermentation experiments in 1997.

For the fermentation of Muscat Ottonel must a strain producing elegant Muscat flavour and freshness had to be found. Secondary aroma substances synthesized by the yeast were not allowed to be too heavy and depress the identity of the wine.

The analysis used in the research is a special new sample preparation method, SPME, and the samples were measured with microbore column and EPC equipped GC-FID instrument. Solid phase micro extraction (SPME) is a revolutionary new sample preparation procedure (YANG & PEPPARD, 1994). The first wine aroma analytical application dealing with the SPME measurement of monoterpene-alcohol content can be found in the publication of DE LA CALLE GARCIA and co-workers (1996). Since that time head-space SPME has become a frequently applied preparatory method in wine analysis (VAS et al., 1998). It is very sensitive, fast, reproducible, non destructive and environmentally friendly procedure.

By using microbore (100 μm i.d.) capillaries, the time of analysis can be reduced by half or one third (appr. 15 min) compared to the time required for conventional columns.

Fast sample preparation and analysis enabled us to measure the changes in aroma content of the microvinification experiments with satisfying frequency. Thus, the investigation has become suitable to follow the kinetics of the maturation process, too. Analysis has been aimed at examining the changes in aroma compounds of wines and allowing comparison of the final aroma content between different batches.

The change of individual primary aroma compounds (e.g. linalool, linalyl-acetate), that of the alcohols (fusel oils, iso-amylalcohol) ethyl- and acetate-esters synthesized during the fermentation have been determined.

Sensory evaluation of the wines has also been performed after the fermentation.

1. Materials and methods

1.1. Wines

Wines (and previously grape musts) from the Muscat Ottonel grape variety were analysed in this study. Muscat Ottonel is a popular grape in the Eger wine region, Hungary. It exhibits a distinctive, strong muscat character. The grapes were picked on 16th September in 1997. Sugar content of grape was 190 g l^{-1}. The quality of grape was much better than in 1996, and this year we could start to control spontaneous fermentation also in our experiment. After the crushing of grape berries the juice was soaked for 4 h with the addition of 50 mg l^{-1} SO_2. The must was clarified overnight at cellar temperature. After the clarification process we prepared the following batches (we made two of each, and so the analytical results are the averages of two parallels):

- Spontaneous: Grape must fermented by natural yeast flora.
- Batch CM: Grape must fermented by Uvaferm CM yeast.
- Batch 228: Grape must fermented by Uvaferm 228 yeast.
- Batch ALB: Grape must fermented by Uvaferm ALB yeast.

The Uvaferm SC and CEG yeasts have not been used this year, because they yielded poor results with variety Muscat Ottonel in the previous year.

The yeast concentrations by inoculation of batches with starter cultures were regulated at 40 g l^{-1} to eliminate the effect of natural flora.

The wines were fermented at 12 °C (cellar temperature).

1.2. Sample preparation using SPME extraction

Wine aroma analytes were concentrated with the SPME method according to the following protocol: each of the above mentioned sample categories were analyzed at nearly the same time. (This was possible due a very short chromatographic and sample preparation time). A volume of 2 ml of each sample was placed in a 7 ml sampling bottle. The SPME fiber was inserted into the head space and remained exposed there for 10 min, while the liquid sample was subjected to low-speed agitation. The average temperature of the sample during this procedure was 20 °C.

Thermal desorption of analytes (when using SPME extraction) was performed in the chromatograph injector where the fiber remained for 5 min at 250 °C.

The fiber used was 100 µm PDMS (polydimethylsixolane) phase coating (Supelco Inc., Bellefonte, PA, USA). It must be noted that PDMS coating is regularly used for the extraction of low molecular weight or volatile apolar compounds.

1.3. Instrumental analysis

The gas-chromatograph was a Hewlett Packard (USA) 5890 series II Gas Chromatograph equipped with two channels EPC (Electronic Pressure Control). A Chrompack (Middleburg, The Netherlands) CP-WAX 52CB 10 m×0.1 mm fused capillary column coated with 0.2 µm bonded polyethylene-glycol was used. The temperature of the injector was held constant at 250 °C during analysis. Splitless purge valve was closed for 5 min. SPME splitless inlet liner was used with 0.75 mm internal diameter. The detector was a FID whose temperature was held constant at 280 °C.

H_2 (purity 5.5) was used as the carrier gas: Gas linear velocity was 45 cm sec^{-1} at 26.6 psig head-pressure. EPC had a controlled constant flow of 0.5 ml min^{-1}.

The GC oven temperature was programmed as follows: initial: 35 °C (0 min hold), 1st ramp: 10 °C min^{-1} to 220 °C (1 min hold).

1.4. Retention indices

A test mixture containing 20 aliphatic hydrocarbons (C_8–C_{27}) was used for external calibration, which was performed once a day. Retention indices were calculated with a software. Day to day reproducibility of retention indices determined in this way was ±2 unit (HARANGI & VAS, 1998).

2. Results and discussion

The selected dried wine yeasts began the fermentation fast. The yeasts 228 and CM fermented with almost the same intensity. The slowest fermenting Uvaferm yeast was the ALB. The strain 228 showed the most reliable fermentation features. The natural yeast flora fermented in the most uncertain way (late start of fermentation – possible oxidation problems; the fermentation was protracting).

The dominating monoterpene alcohols in the aroma profile of Muscat varieties are linalool, geraniol, nerol, citronellol and alpha-terpineol (RAPP, 1988). The changing in quantity of primary flavour materials and derivatives of them (linalool, nerol, geraniol, alfa-terpineol, citronellol) has been measured in the Muscat Ottonel samples under examination.

In Fig. 1 we can observe the changing of total quantity of terpene alcohols (linalool, terpineol, citronellol, geraniol). It is clearly observable that the quantity of terpenols in microvinification of wines is practically unchanged during the first 48 h. After this time the concentration of terpene alcohols decreased greatly in the headspace of batches inoculated with three selected dried wine yeasts. The explanation of this is that the fermentation became faster and took place with higher intensity in the wines fermented by selected wine yeasts, and so the concentration of ethanol increased more

quickly than in the spontaneous samples. Ethanol retains the terpene alcohols better in solution, according to the measuring results of DE LA CALLE GARCIA and co-workers, (1996) and so the concentration of the terpenols decreased only apparently in the steam space of wines fermented with Uvaferm yeasts. At the end of fermentation of all the samples, the wine with the highest total terpene content was produced by CM. The quantity of terpene alcohols was less in the wines produced by ALB, and even smaller concentrations were produced by 228 compared to CM.

In spite of this, the alcohol content of the wine fermented by natural flora was the lowest (in this case the terpene alcohol concentration in the head space is greater at the same terpenol content), the quantity of measured terpenols and terpene derivatives was here found to be the lowest.

The analytical results supported the results of sensory evaluation of wines, because after the end of fermentation the most varietal character was shown by CM.

Ethyl-esters of straight-chain fatty acids and acetates of higher alcohols are the dominating esters of wines, and they are formed during the alcoholic fermentation (RAPP, 1988).

The fatty acid esters with the large number of combinations form the biggest group of aroma components between the secondary flavour materials. Examination of the changing quantities of ethyl-acetate, ethyl-butanoate, ethyl-hexanoate, -octanoate, -decanoate and diethyl-succinate was conducted in our microvinification experiments.

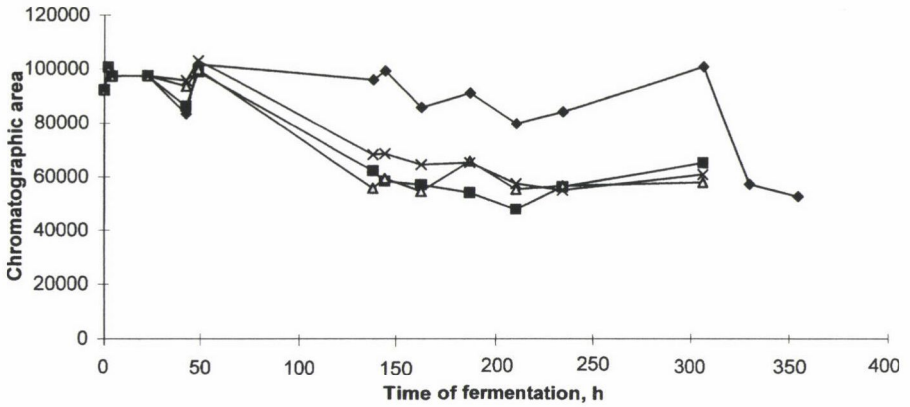

Fig. 1. Changing of terpene alcohols during the fermentation. ♦: Control; ■: CM; Δ: 228; X: ALB.

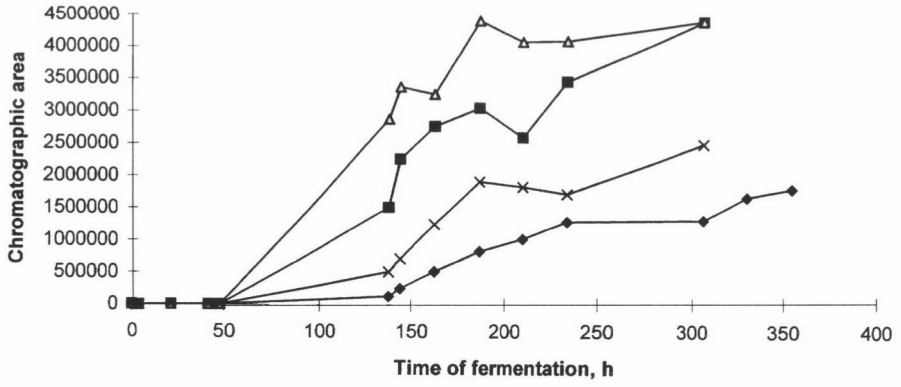

Fig. 2. Changing of ethyl-esters during the fermentation. ♦: Control; ■: CM; Δ: 228; X: ALB.

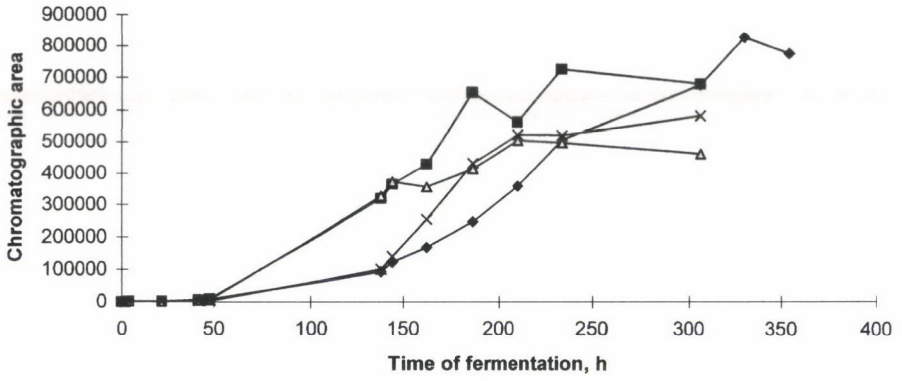

Fig. 3. Changing of acetate-esters during the fermentation. ♦: Control; ■: CM; Δ: 228; X: ALB.

Figure 2 displays changes in the total quantity of ethyl-esters during fermentation. These esters do not originate from the prefermentory process. The quantification of esters was measurable from the 48th hour. The strain 228 produced these compounds with the largest intensity. Not until the end of the fermentation did the CM reach the ethyl-ester concentration of the strain 228. The ALB produced less from these aroma components. The production of these aroma compounds of the control was the smallest.

The quantity of the following acetate-esters was determined in our measurements: linalil-acetate, hexyl-acetate, isoamyl-acetate, buthyl-acetate. Figure 3 shows the quantitative changes of acetate-esters with time. Though the total intensity of

these compounds is only 1/5th of the intensity of ethyl-esters, they are important flavour materials. The acetate-esters give the fruity character to the wines.

The concentration of acetate-esters increased most dramatically with yeasts 228 and CM during the first 100 h of the fermentation. After this the intensity of acetate-ester production of strain 228 went back, and at the end of the fermentation the concentration of these materials was the lowest for this type of yeast. Not until the end of fermentation did the ALB produce more fruity esters than the 228. The CM and the sample with spontaneous fermentation was the richest in acetates.

Figure 4 summarizes the quantity formation of the aroma materials.

Besides the above mentioned aroma compounds the hexanol, octanol (primary aroma materials), fusel alcohols, phenylethyl-alcohol etc. were measured and there were numerous aroma compounds which we could not identify, because we did not have standards for these combinations.

The ethyl- and acetate-esters gave 65–70% of the total aroma quantity in our measurements. In compliance with it, the total aroma content in the samples changed with almost the same dynamics, like the whole ester content.

The aroma content of the batches were practically unchangeable until the 48th hour. After this the two fastest fermenting strains were the CM and 228. The intensity of aroma production of CM and 228 yeasts were the largest. At the end of fermentation, the wines produced by CM and 228 contained the most aroma. The ALB, compared with the most aromatic sample, produced 20–25% less flavour materials. The spontaneous fermentation gave 30–35% less aroma than the CM and 228.

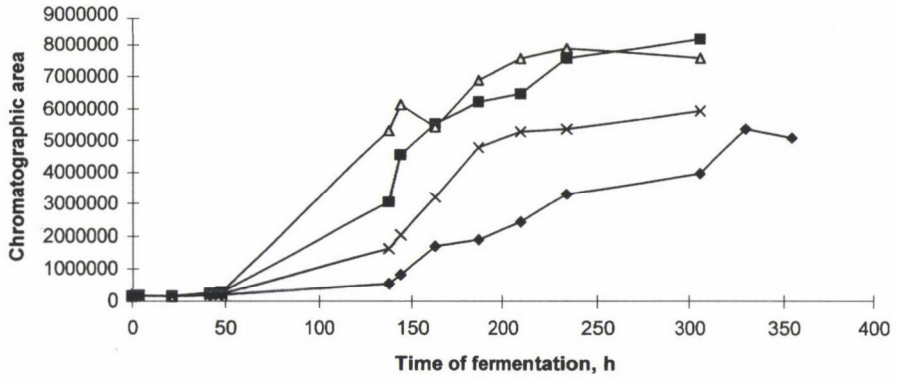

Fig. 4. Changing of total aroma during the fermentation. ♦: control; ■: CM; Δ: 228; X: ALB.

The result of sensory evaluation of wines was similar to our findings. The most aromatic wines were the batches fermented by CM. The only problem with this yeast was that the wines of it were very poor in extract, and it can be a problem for the qualification of wine, because, according to the Hungarian regulations the wines fermented by CM can only be table wines, not quality wines. The second most aromatical wines were produced by the yeast 228. This strain brought enough extract to the wines, and so the 228 was the most suitable yeast for the fermentation of Muscat Ottonel from the 5 tested strains during the two vintages. The spontaneous flora produced organoleptically more aromatical wines than the ALB, against the analytically measured results.

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SYNTHESIS OF CROWN ETHER COMPOUNDS AS POTENTIAL INHIBITORS OF ENZYMATIC DISCOLOURATION OF FOODS

I. SYNTHESIS OF POSSIBLE INHIBITORS

R. VUKOVIĆ,[a] A. ERCEG,[a] V. PILIŽOTA,[b] and D. ŠUBARIĆ[b]

[a] INA, Research and Development Institute, HR-10000 Zagreb. Croatia
[b] Faculty of Food Technology, University of Osijek, F. Kuhača 18, HR-31000 Osijek. Croatia

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The synthesis of the following crown ether containing compounds as potential polyphenol oxidase inhibitors is described: 4'-N(benzo-crown-5)succinamic acid (I), 4'-N(benzo-15-crown-5)malemic acid (II), ester of 4'-N(benzo-15-crown-5)bis[4'-N(benzo-15-crown-5)]phthalamic acid (III) and complex of benzo-18-crown-6 with 2-propenyl-acrylamide (IV). The complexes of I, II and IV with potassium 2-propenyl-acrylamide are also described.

Keywords: complexes of crown ethers containing 2-propenyl-acrylates, inhibitors of polyphenol oxidase, synthesis of crown ether amides

The discovery of crown compounds by Pedersen during the late 1960s was followed by intensive synthesis, characterization and application of these compounds. The most important crown compounds are macrocyclic ethers, which – due to their unique properties to form complexes with metal ions – have found numerous applications in various fields, such as organic synthesis, especially polymer synthesis, analytical chemistry, capture and separation of metal ions, resolution of optical isomers and in biochemistry and biophysics (PEDERSEN, 1967a,b; 1970a,b; 1972). In a recently published review article from our Laboratory in Osijek, part of the work by Cram and co-workers concerning the application of atropisomeric crown compounds as enantioselective supports in chromatography is described (PILIŽOTA, 1998). Over one thousand scientific papers and patents related to crown compounds were published during the last few years. Recently a naturally occurring crown compound, β-cyclodextrin, has found practical use in food technology as inhibitor of enzymatic browning of fruits and vegetables (HICKS et al., 1996).

The mechanism of enzymatic discolouration of foods is described in a large number of papers and monographs (VÁMOS-VIGYÁZÓ, 1981; SAPERS, 1993; PILIŽOTA

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& ŠUBARIĆ, 1998). Based on extensive studies it is considered that the enzymatic browning is connected with the hydroxylation of mono-phenols to diphenols, under the subsequent oxydation of diphenols to the coloured o-quinones.

These reactions are catalysed by polyphenol oxidase which is an oligomeric metalloporphirine containing copper as prosthetic group. Based on a large number of studies it has been established that inhibitors of enzymatic discolouration, which react with copper ions, are efficient stabilizers of food products. One of these stabilizers is ascorbic acid oxidase, which also contains copper ion bound to protein (BURNS & GREENBERG, 1963).

In view of the unusual properties of the complexing of crown compounds with metal ions, including copper ions, we have prepared several compounds which contain macrocyclic crown ethers as potential inhibitors of food discolouration caused by polyphenol oxidase. The macrocyclic ethers, which are part of the compounds described in the experimental section of this work, are benzo-15-crown-5 and benzo-18-crown-6, respectively. In a recently published paper we have described the complexing ability of benzo-15-crown-5 and poly(benzo-15-crown-5-alt-α-methyl-styrene) with sodium and potassium ions, and it was found that these complexes are very stable. The sodium ion forms a 1:1 complex with benzo-15-crown-5, while potassium ions form 1:2 complexes, respectively. Both complexes are soluble in methylene dichloride (POONIA, 1974; FLEŠ et al., 1994).

As shown by PEDERSEN and FRENDSDORFF (1972), the stability of crown ether/metal ion complexes depends on the relative sizes of the cation and the cavity of the crown ether: the more closely they match, the more stable is the complex formed. For the crown compounds used in this work, Pedersen calculated the cavity diameters on the basis of atomic models and obtained the following values: for benzo-15-crown-5 the cavity diameter was 1.7–2.2 Å (lower values are estimated from Corey-Pauling-Koltum atomic models, while higher values are calculated from Fisher-Hilschfelder-Taylor models). According to the data tabulated in the monography "Crown compounds, their characterization and application" by HIRAOKA (1982) the ionic diameter of Cu^{2+} has a value of 1.44 Å and is therefore able to form stable complexes with benzo-15-crown-5 and benzo-18-crown-6, respectively.

1. Materials and methods

1.1. 4-(Aminobenzo-15-crown-5)

The compound was prepared by reduction of 4-(nitrobenzo-15-crown-5) following the procedure published by FLEŠ and co-workers (1992).

1.2. 4'-N(Benzo-15-crown-5)succinamic acid(I)

A mixture of 4-(aminobenzo-15-crown-5) (0.566 g; 0.002 mol) and 0.2 g (0.002 mol) of succinic anhydride was dissolved under heating in 4 ml of chloroform and left overnight at room temperature. The crystalline product was removed by filtration yielding 0.41 g (53%) of product melting at 150–154 °C. A sample for analysis was recrystallized from ethanol, m.p. 153–154 °C. Recrystallization from water did not change the melting point.

Analysis. Calculated for $C_{18}H_{25}NO_8$ (383,39): C, 56.39; H, 6.57; N, 3.65; Found: C, 55.89; H, 5.23; N, 3.71.

1.3. Complex of potassium salt of 4'-N(benzo-crown-5)succinamic acid with potassium 2-propenyl acrylamide (Ia)

4'-N(Benzo-15-crown-5)succinamic acid (0.793 g: 0.002 mol) is dissolved in 20 ml of n-KOH under the addition of 0.150 g (0.001 mol) of potassium 2-propenyl-acrylate. The clear filtered solution is evaporated in vacuum to dryness, yielding 1.02 g of white powder soluble in water.

Analysis. Calculated for $C_{42}H_{53}N_2O_{18}K_3$ (991.2); N, 2.82; Found: N, 2.49.

1.4. 4'-N(Benzo-15-crown-5)maleamic acid II

Compound II was prepared according to the procedure described by FLEŠ and co-workers (1992). 4-Aminobenzo-15-crown-5 (0.566 g; 0.002 mol) was dissolved in 4 ml of chloroform and mixed under cooling with 0.200 g (0.002 mol) of maleic anhydride dissolved in 4 ml of chloroform. After standing overnight at room temperature the solvent was evaporated in vacuum, and the dark coloured crystalline product was recrystallized from water, yielding analytically pure II: Yield 95%, m.p. 152–154 °C.

Analysis. Calculated for $C_{18}H_{23}NO_8$ (381.4); C, 56.68; H, 6.08; N, 3.67; Found: C, 56.40; H, 6.10; N, 3.50.

1.5. Complex of potassium salt of N(benzo-15-crown-5)maleamic acid with potassium 2-propenyl acrylate (IIa)

Complex IIa was prepared in the same manner as previously described for Ia: 0.381 g, 0.001 mol of N-substituted maleic acid I was dissolved in 1 ml of n-KOH followed by the addition of 0.075 g (0.0005 mol) of potassium 2-propenyl-acrylate. After standing overnight at room temperature, the dark brown solution was evaporated to dryness yielding 0.41 g of semisolid residue which was triturated with ether; the resulting white powder was filtered off and dried in vacuum.

Analysis. Calculated for $C_{42}H_{51}N_2O_{18}K_3$ (989.18); C, 50.99; H, 5.20; N, 2.85; Found: C, 49.46; H, 4.27; N, 2.71.

1.6. Ester of 4'-N(benzo-15-crown-5)-bis[4'-N(benzo-15-crown-5)]phthalamic acid (III)

A mixture of 0.566 g (0.002 mol) of 4-amino-benzo-15-crown-5 and 0.296 g (0.002 mol) of phthalic anhydride is heated in an oil bath at 140 °C. Water and ammonia are formed under foaming and after 30 min a solid crystalline mass is formed. The crystalline product is dissolved in 10 ml of chloroform, and crystallized by the addition of 10 ml of petroleum ether (b.p. 40–70 °C; yield 0.48 g, 74%; m.p. 188–189 °C. A sample (150 mg) was recrystallized from 20 ml of isopropanol, m.p. 188–189 °C.

Analysis. Calculated for $C_{50}H_{61}NO_{18}$ (963); 62.27; H, 6.38; N, 1.45; Found: C, 62.69; H, 5.46; N, 1.48.

1.7. Complex of benzo-18-crown-6 with 2-propenyl-acrylic acid (IVa)

Benzo-18-crown-6 (0.312 g, 0.001 mol) was mixed with 0.113 g (0.001 mol) of 2-propenyl-acrylic acid and dissolved in 2 ml of water. The residue obtained upon evaporation of water in vacuum was triturated with ether yielding 0.42 g (100%) of the title complex in the form of white powder.

Analysis. Calculated for $C_{22}H_{32}O_8$ (424.5); C, 62.25; H, 7.60; Found: C, 61.93; H, 6.55.

1.8. Complex of benzo-18-crown-6 with potassium 2-propenyl-acrylate (IVb)

Complex IVb was prepared in the same manner as described for IVa.

Analysis. Calculated for $C_{22}H_{31}O_8K$ (462.58); C, 57.12; H, 6.76; Found: C, 56.41; H, 7.06.

Results and discussion

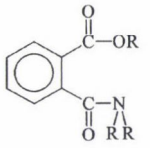
In the experimental part of this paper the preparation of the following crown ether containing compounds is described: 4'-N(benzo-15-crown-5)succinamic acid (I), 4'-N(benzo-15-crown-5)maleamic acid (II), and ester of 4'-N(benzo-15-crown-5)bis [4'-N(benzo-15-crown-5)]phthalamic acid (III).

$HOOC\text{-}CH_2\text{-}CH_2\text{-}CONHR$

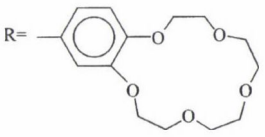
I

$HOOC\text{-}CH{=}CH\text{-}CONHR$

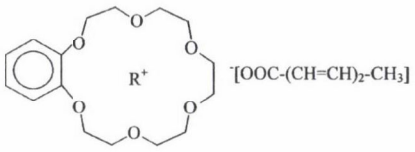
II



III



Compounds I and II are prepared by condensation of 4-amino-benzo-15-crown-5 with succinic anhydride or maleic anhydride, respectively. In order to increase the solubility of the amides I and II in water and to improve their efficiency as food stabilizers, the complexes with potassium 2-propenyl-acrylate (Ia and IIa) were prepared. Potassium 2-propenyl-acrylate is a well known fungistatic agent widely used as food stabilizer under the name of sorbic acid (THE MERCK INDEX, 1976). An attempt to prepare benzo-15-crown-5-phthalic acid was unsuccessful since melting of the equimolar mixture of amino-benzo-15-crown-5 with phthalic anhydride leads to the loss of water and ammonia under the formation of ester-diamide III. Complexes IVa and IVb are under investigation in our Laboratory as potential browning inhibitors of cut fruits and fruit juices (ŠUBARIĆ & PILIŽOTA, 1998).



IVa R=H
IVb R=K

3. Conclusions

Addition of 4'-N(aminobenzo-15-crown-5) to succinic anhydride and maleic anhydride gave the corresponding amides: 4'N(benzo-15-crown-5) succinamic acid (I) and 4'-N(benzo-15-crown-5)maleamic acid (II). The reaction of amino benzo-15-crown-5 with phthalic anhydride gave the ester of 4'-(benzo-15-crown-5)-bis[4'-N(benzo-15-crown-5)]phthalamic acid (III). Crown compounds I and II gave complexes with potassium 2-propenyl-acrylates (Ia and IIa). One to one complexes were formed by mixing benzo-18-crown-6 with 2-propenyl acrylic acid and potassium 2-propenyl-acrylate. The compounds I to III and complexes Ia, IIa and IVa and b were prepared as potential inhibitors of enzymatic discolouration of food.

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EVALUATION OF THE MICROBIAL HAZARDS DURING COOLING OF THE BECHAMEL USED TO ELABORATE COMMERCIAL CROQUETTES

M. G. CÓRDOBA[a], J. J. CÓRDOBA[b] and R. JORDANO[a,c]

[a] Departamento de Bromatología y Tecnología de los Alimentos. Facultad de Veterinaria. Universidad de Córdoba. E-14071 Córdoba. Spain

[b] Higiene y Tecnología de los Alimentos. Facultad de Veterinaria de Cáceres. Universidad de Extremadura. E-10071 Cáceres. Spain

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In the commercial processing of the croquettes a 80 °C heat treatment for 30 min is applied to the raw materials to make a semi-solid paste called "bechamel". Since the heat treatment is not that of sterilization, some micro-organisms surviving it may proliferate in the next steps, which could introduce two microbial hazards; the presence of pathogenic bacteria and croquettes spoilage. To evaluate microbiological hazards, microbiological analysis of the bechamel cooled and stored under refrigeration following commercial processing were developed at different times of cooling. In addition a fast cooling method was assayed. Immediately after heat treatment used to obtain the bechamel, microbial counts were always under 1 log CFU g^{-1}, except for mesophilic aerobes which were at levels of 1.2 log CFU g^{-1}. During cooling used in commercial process of croquettes, levels of most of micro-organisms investigated showed high increases as compared with bechamel after cooking. In this step mesophilic organisms, psychrotrophic organisms and yeast reached levels of around 6 log CFU g^{-1}. Enterococci counts increased during cool storage, although always were at levels below 3.2 log CFU g^{-1}. *Enterobacteriaceae* and *Clostridium* sp. only slightly increased at the end of cold storage reaching levels of around 3 log CFU g^{-1}. Although rates which could be considered as being inadmissible were reached at day 5, the use of over 1-day old bechamel could be associated with microbiological sanitary hazards or with the spoilage of the croquettes made afterwards. In contrast, the bechamel cooled at –20 °C air temperature for 12 h, only showed total aerobic counts of under 1 log CFU g^{-1}. Cooling of bechamel is a decisive critical control point and a fast cooling should be considered in the implementation of an HACCP program in the prepared and frozen croquettes process.

Keywords: microbial, hazards, cooling, bechamel

[c] Corresponding author
Departamento de Bromatología y Tecnología de los Alimentos, Campus Universitario Rabanales. Edificio C-1. Anexo. Ctra. NIV Km. 396.A, E-14071 Córdoba (Spain), Tel.+34 957 212006, Fax +34 957 212000
E-mail:bt1josar@lucano.uco.es

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Croquettes are prepared and frozen foods whose main raw materials are meat or fish in addition to other ingredients such as onion, garlic, flour, condiments, water, powdered milk, starch and whey. Croquettes and similar prepared and frozen foods have been highly demanded in the last years given the actual preference for fast methods of food preparation and minimally processed foods (ANON., 1994; KNABEL, 1995).

This kind of prepared and frozen foods may have a highly varied microbial population due to the great diversity of raw materials used in their manufacture (CÓRDOBA et al., 1998a). Among these micro-organisms have been reported pathogens such as *Staphylococcus aureus* and *Clostridium perfringens* which may pose a health hazard for the consumers (CÓRDOBA et al., 1998a). In the manufacturing process of these foods there is a heating step which can modify the microbial load present in the raw materials. In the commercial processing of the croquettes an 80 °C heat treatment for 30 min of the raw materials is applied to make a semi-solid paste called "bechamel" (CÓRDOBA et al., 1998b). Then, this paste is cooled and extruded to obtain the croquettes.

During the heat treatment of the bechamel inactivation of most of the micro-organisms present in raw materials is achieved. However, the temperature used is not that of sterilization, and thus some micro-organisms surviving this heat treatment may proliferate in the next steps, which could introduce two microbial hazards; the presence of pathogenic bacteria and croquettes spoilage. Thus, cooling of the bechamel could be a critical step to obtain a final product of appropriate microbial quality. Due to technological problems, in the commercial process the semi-solid paste should be cooled in containers in refrigerated rooms for at least 24 h. In some industrial process, the bechamel is stored under refrigeration for up to 5 days. During this time, there may be a proliferation of micro-organisms surviving heat treatment, with the corresponding sanitary and technological repercussions in the finished product (ROBERTS, 1982; DAVEY, 1985; BRYAN et al., 1992a, 1992b, 1992c).

The objective of this work was to evaluate the microbiological hazards of the bechamel used to elaborate chicken croquettes, cooled and stored under refrigeration, following their commercial processing. It was aimed to establish a possible maximum storage time at which no sanitary and/or spoilage problems appear.

1. Material and methods

A firm manufacturing chicken croquettes on a large-scale was selected where the raw meat material was received at temperature below 3 °C. After mixing and cooking the raw materials at 80 °C for 30 min, the bechamel obtained was placed in 0.50 m^3 metal containers, covered with plastic foils. Containers were separated in two different batches. One batch was cooled in a cold-storage room (air temperature and speed of

2 °C and 2 m sec^{-1} respectively) following the commercial process. Temperature of geometric center of the bechamel reached 5 °C in 24 h. Refrigerated storage of the bechamel was prolonged for 5 days, as happens in some industrial processes.

The second batch of bechamel was cooled in a cold room (air temperature and speed of –20 °C and 2 m sec^{-1} respectively) for 12 h, to achieve a faster cooling. Then semisolid paste was stored for a further 12 h in the same conditions of the bechamel of batch 1.

1.1. Sampling

In batch 1, samples for analysis were taken each 24 h. From this batch a total of 110 samples were analyzed during 5 days of refrigerated storage, 60 of which corresponded to the microbiological analysis made of the geometric centre of the container and the remaining 50 on the bechamel adhering to its sides, in order to assess the possible influence of contamination from the utensils used. In batch 2, sampling was done at the 24 h and 10 samples were taken.

At each sampling point, five sample units were taken and placed in sterile bags, sealed and considered as a single sample. Then, samples were immediately transported in a refrigerated containers to the laboratory. At the same time as the samples were taken, the temperature of the mixture was measured with a Crison probe coupled with a model Pt 100 sensor.

For microbiological control to determine microbial population at the inner surfaces of the containers before adding the bechamel, the swab method was used (SVEUM et al., 1992). Previously sterilized templates with a 15 ml opening were used. The surface selected was sampled with the swab which was then rapidly replaced in its tube with 10 ml of 0.1% peptone-water. The swab samples were taken to the laboratory in refrigerated containers to carry out the microbiological determinations.

1.2. Microbiological analysis

Samples of food were aseptically removed from plastic bags, and 10 g were homogenized in 90 ml sterile diluent (0.1% peptone-water) by use of Stomacher (Lab Blender, Model 4001, Seward Medical, London, England) for 30 sec. From those and from swab samples serial dilutions were made in peptone-water, and 1 ml amounts were plated in each of the media shown in Table 1 to enumerate total aerobic organisms, *Enterobacteriaceae*, total and fecal coliforms, *Escherichia coli*, *Enterococcus* spp., psychrotrophic organisms, *Staphylococcus* spp., *Clostridium* spp., *Pseudonomas* spp., moulds and yeasts. Temperature and incubation time followed in each method is indicated in Table 1.

Table 1

Media and incubation conditions used for sample analysis

Microbal count type	Incubation			
	Time (h)	Temperature (°C)	Atmosphere	Growth medium
Total aerobic count	48–72	31±1	Aerobic	Plate Count Agar (PCA, Oxoid™)
Enterobacteriaceae	24	35–37	Aerobic	Violet Red Bile Glucose Agar (VRBG, Oxoid™)
Total coliforms	24	37	Aerobic	Violet Red Bile Agar (VRBA, Oxoid™)
Fecal coliforms	24	45	Aerobic	Violet Red Bile Agar (VRBA, Oxoid™)
Enterococcus spp.	48	37	Aerobic	Slanetz & Bartley Medium (Oxoid™)
Psychrotrophs	175–240	5	Aerobic	Plate Count Agar (PCA, Oxoid™)
Yeast & moulds	96	22–25	Aerobic	Potato Dextrose Agar (PDA, Oxoid™) pH=3.5;Lactic Acid 10% Solution
Clostridium spp.	24–48	45–46	Anaerobic	Sulphite Polymixin Sulfadiazine (SPS) Agar (Merck™)
E.coli	24–48	44.5±0.5	Aerobic	Eosin Methylene Blue Agar (Levine, Oxoid™)
Pseudomonas spp.	24–48	42	Aerobic	Pseudomonas Agar (Cultimed™)
Staphylococcus spp.	30–48	35–37	Aerobic	Baird-Parker Medium (Oxoid™) Egg Yolk-Tellurite Emulsion (Oxoid™)

In food samples *Salmonella* spp. and *Listeria monocytogenes* were investigated. For *Salmonella* spp. 25 g samples were enriched in 225 ml of lactose broth (Oxoid™ Unipath Ltd., Basingstoke, England) at 35 °C for 24 h. For selective enrichment, 1 ml of lactose broth was transferred to 9 ml each of tetrathionate and selenite cystine broth (Oxoid™) and incubated in water bath at 43 °C for 24 h. Brilliant green agar (Oxoid™), xylose lysine desoxycholate agar (XLD, Oxoid™) and bismuthe sulphite agar (Oxoid™) were used as plating media. Representative colonies from these three cultures were further screened using TSI (Triple Sugar Agar, Oxoid™) and LIA (Lysine Iron Agar, Oxoid™). Typical colonies were tested using the PASCO identification system (Difco Laboratories, Detroit, M.I., USA).

Table 2

Saturated saline solutions and respective relative humidities (ERH) used to create atmospheres of constant relative humidity for the determination of water activity

Salts	Concentration ($g \times 100\ ml^{-1}$)	ERH at 20 °C %
Potassium sulfate	12.0	97
Potassium nitrate	20.0	94
Potassium chloride	35.0	84
Barium chloride	38.0	91
Ammonium sulfate	90.0	79

For *Listeria* spp. detection, an enrichment was achieved with 25 g samples in Listeria enrichment broth and Listeria plating medium at 30 °C for 48 h. One ml of enrichment broth was plated in Palcam agar (Merck™, Darmstadt, Germany). Colonies with a black point in the centre were observed using translumination at 45 °C. Suspected colonies were also tested for catalase activity, tumbling motility at 25 °C and with the API-10 Listeria Kit (bioMérieux™ Vitek, Inc., Hazelwood, MO) (BILLE et al., 1992).

1.3. Physicochemical analyses

1.3.1. Determination of pH. The pH meter Crison probe coupled with a model Pt 100 sensor and the method used were described by BANDEIRA and co-workers (1990). For this, 5 g of the previously homogenized samples were taken and homogenized with 45 ml of distilled water. The pH measurement was done at 20 °C temperature.

1.3.2. Determination of the water activity (a_w). To determine water activity 2 g samples were taken and placed in microenvironmental chambers with constant relative humidity (ERH) atmospheres (Table 2) maintaining them there at 20 °C for 24 h. The calculation of the different losses or gains in weight for each ERH was done following the equation a_w=(P1–P2)/P1 (P1: weight of the sample before placing it in the constant ERH atmosphere; P2: Weight of the sample after staying in the constant ERH atmosphere) (LANDROCK & PROCTOR, 1951).

1.4. Statistical analysis

Data were analyzed using the general linear models procedures of the Statistical Analysis System (SAS Institute, Cary, NC). A significance level of $P<0.05$ was employed. Tukey's multiple range test was use to determine differences among means.

2. Results and discussion

Figures 1 and 2 show the results of the microbiological analysis of the bechamel from batch 1 in both the centre and the walls of the containers, respectively. It can be seen that immediately after heat treatment all the microbiological parameters analyzed have counts of under 1 log CFU g^{-1} except for mesophilic aerobes whose values were slightly lower than the foregoing level. During cold storage, it was observed how the number of those bacteria increased, even reaching levels of 7 log CFU g^{-1} after 5 days of refrigerated storage. Psychrotrophic micro-organisms and yeasts also reached significant levels ($P<0.05$) after 5 days of storage. Indeed, all these microbial groups showed high counts (>4 log CFU g^{-1}) from the second or third day of cold storage although the increases were not statistically significant ($P<0.05$) until the fifth day. An important increase was also noted of enterobacteria, enterococci and even clostridia (Fig. 1). All these increases can be explained by the proliferation of micro-organisms surviving the heat treatment since it was only of 80 °C. Similar or lower temperatures to 80 °C in the centre of the product only destroys vegetative forms but the spores of the different micro-organisms survive, and even when boiling the foods at temperatures of close to 95 °C, spores resistant to high temperature heat treatment remain (MICHAINE et al., 1988; WORSFOLD & GRIFFITH, 1995; GRAHAM et al., 1996). In addition the slow cooling allows that bechamel, mainly that located at the centre of containers, can be for several hours at a temperature over 10 °C, which have been reported as the main factor contributing to the growth of pathogenic micro-organisms in cooked foods during cooling (ROBERTS, 1982; DAVEY, 1985; BRYAN & BARTLESON, 1985; BRYAN et al., 1988a, 1988b; BRYAN et al. 1992a, 1992b). BRYAN and co-workers (1992c) found strains of *C. perfringens* and *B. cereus*, as well as high counts of mesophilic aerobes in most foods which, after being cooked, were left to cool at room temperature. Increases in the levels of the coliforms, *Staphylococcus* spp., *C. perfringens* and *B. cereus* of cooked foods that were cooled slowly from one day to the next have also been reported (BRYAN et al., 1988a, 1988b; BRYAN et al., 1992c). In the present work, although increases in most of the microbial parameters analyzed were observed, no *E. coli*, *Staphylococcus* spp., *Pseudomonas* spp., *Salmonella* or *L. monocytogenes* were detected.

Although the a_w did not change during cold storage at 2 °C and was maintained at high values of around 0.99, the pH fell from 6.27 to 4.95 (Tables 3 and 4). At this water activity value growth of any micro-organisms is allowed, that could be responsible of the pH decrease.

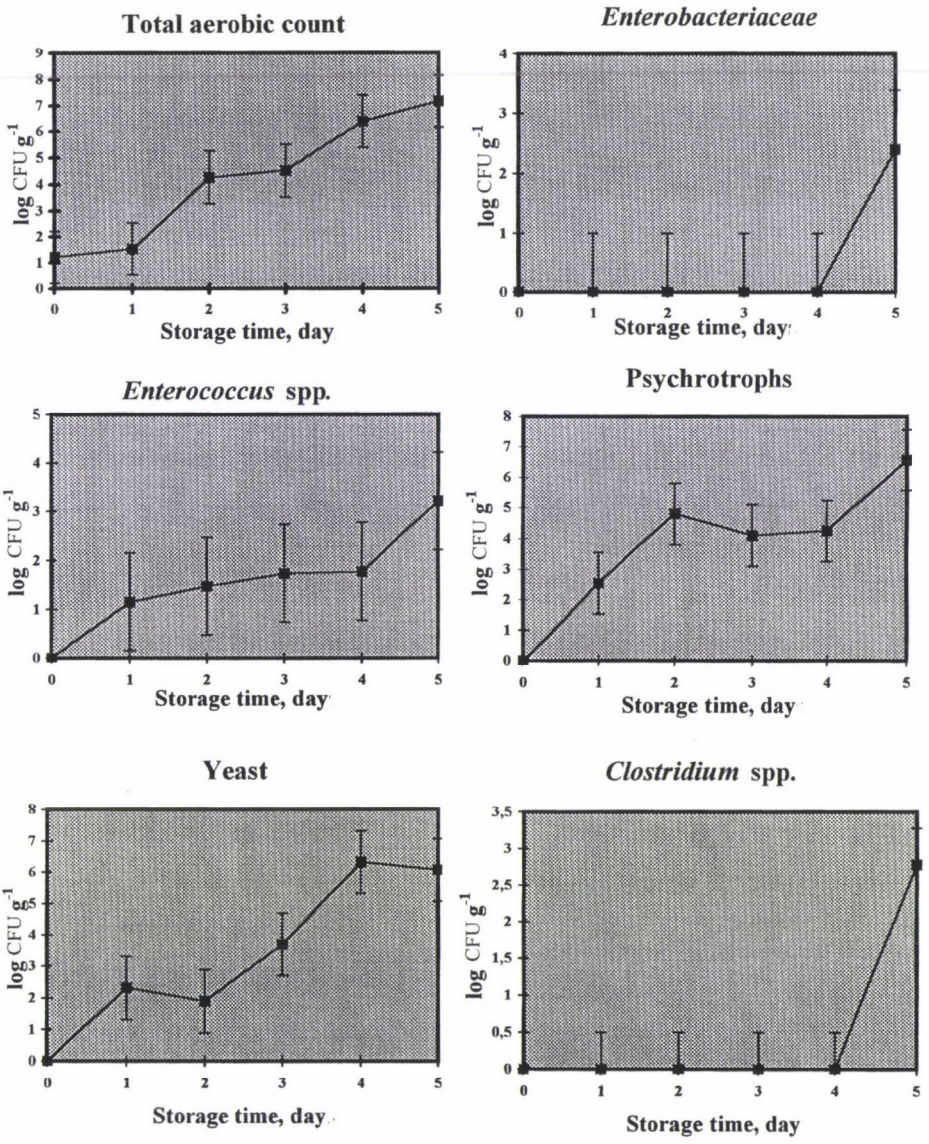

Fig. 1. Microbiological analysis of the bechamel from batch 1, located at the central part of the container, during cooling and subsequent refrigerated storage at 2 °C

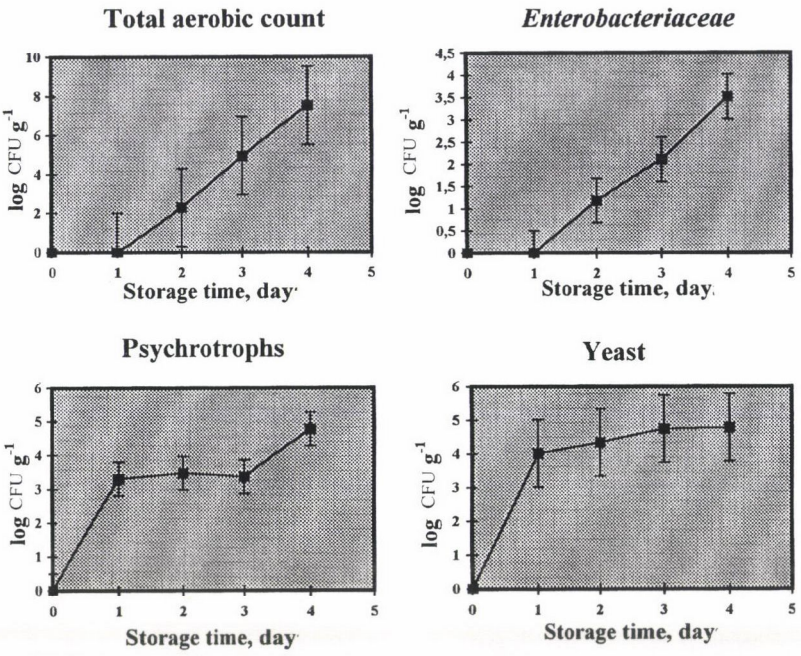

Fig. 2. Microbiological analysis of the bechamel from batch 1, adhering to the sides of the container, during cooling and subsequent refrigerated storage at 2 °C

Table 3

Mean values of pH, water activity and temperature of central part of bechamel from batch 1 during cooling and subsequent refrigerated storage at 2 °C (n=10)

	Cooling and refrigerate storage (days)					
	0	1	2	3	4	5
pH	6.27	5.98	5.58	5.40	5.05	4.95
a_w	0.98	0.96	0.99	0.99	0.98	0.98
Temperature (°C)	81.00	5.85	4.20	3.50	3.00	2.40

Table 4

Mean values of pH, water activity and temperature of bechamel from batch 1, adhering to the sides of the container during cooling and subsequent refrigerated storage at 2 °C (n=10)

	Cooling and refrigerated storage (days)				
	0	1	2	3	4
pH	6.25	5.78	5.70	4.89	4.05
a_w	0.97	0.98	0.99	0.98	0.98
Temperature (°C)	42.00	5.70	4.80	3.80	3.80

Table 5

Microbiological contamination of surface of the containers and plastic foil used during cooling and subsequent refrigerated storage of bechamel at 2 °C

	Aerobic count log CFU g^{-1}	*Enterobacteriaceae* log CFU g^{-1}	*Enterococcus* log CFU g^{-1}	Psychrotrophs log CFU g^{-1}	Yeast log CFU g^{-1}
Central part of the container	1.45	<1	<1	<1	<1
Sides of the containers	3.62	1.20	1.00	0.95	2.83
Lateral walls of the containers	2.62	<1	<1	2.45	2.46
Plastic foil	1.46	<1	<1	<1	<1

Regarding the bechamel adhering to the sides of the container, it was seen that already from the first day of storage, this showed high levels (3–4 log CFU g^{-1}) of psychrotrophs and yeasts and a statistically significant ($P<0.05$) increase in mesophilic aerobes was observed, reaching values of over 7 log CFU g^{-1} on the fourth day of storage (Fig. 2). The high increases of micro-organisms during the first day of storage may be related to the microbial load found in the inside surfaces of the containers before adding the bechamel. In Table 5 it can be observed that the levels of the different micro-organisms studied are higher at the corners of the container because of the possible difficulties in its cleaning and sanitation. Likewise, a marked decrease in the pH values of the bechamel from this part of the containers was noticed (Table 4). The decrease of temperature is higher in the centre (Table 3), the pH decrease is smaller.

During cold storage, the growth of different micro-organisms is slower but their proliferation does not halt. It has been seen that pathogenic micro-organisms such as *Salmonella*, *S. aureus* and some strains of *E. coli* are able to grow at temperatures as low as 8–8.5 °C and can even produce toxins. Also of special interest is the group of

spoiling pathogenic micro-organisms which can grow at temperatures close to 0 °C such as *L. monocytogenes*, *Yersinia enterocolitica* (CID et al., 1995). This is related to the storage time, so that a temperature of 0 °C means that the preservation time is twice as long as at 5 °C (ROSSET et al., 1977). In our experiment, a 4 and 5 days storage time at 2 °C has been necessary to reach high levels of micro-organisms which caused the acidification of the bechamel. Although it was at day 5 that levels which could be considered as being inadmissible were reached, the use of over 1-day old bechamel could be associated with microbiological sanitary hazards or with the spoilage of the croquettes made afterwards.

Bechamel from batch 2 sampled after 24 h cooling, showed total aerobic counts of under 1 log CFU g^{-1} (data not shown). Thus, microbiological levels were considerably lower than those found after 24 h cooling in bechamel from batch 1. It should be noted that in bechamel from batch 2, 5 °C was reached after 12 h cooling while in batch 1, 24 h was necessary to reach 5 °C. Thus, it is demonstrated that temperature of cooling is the main factor responsible for the high microbial numbers observed in batch 1. Cooling bechamel at –20 °C air temperature for 12 h could be proposed in commercial processing of croquettes to obtain final product of appropriate microbial quality.

In conclusion, in commercial croquette production there is a hazard of microbial proliferation during cooling of the bechamel after its cooking. Thus, cooling of bechamel should be considered as important critical control point in the implementation of an HACCP program in the prepared and frozen croquettes process.

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STABILITY OF THE ANTHOCYANINS FROM *ACALIPHA HISPIDA* AND COPIGMENTATION EFFECT

M. A. BAILONI, P. A. BOBBIO and F. O. BOBBIO

Deparmento de Ciência de Alimentos, FEA-UNICAMP – C.P 6121.
CEP: 13081-970 – Campinas – SP. Brasil

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The stability and stabilization of the anthocyanins to light and air in the crude and partially purified extracts of the leaves of *Acalipha hispida* were studied at pH 3.0. Addition of non anthocyanic flavonoid fractions from *A. hispida* to the partially purified extracts did not significantly improve the time of half life of the extracts, whereas addition of tannic acid resulted in an increase of $t_{1/2}$ of 60–67%.

Keywords: anthocyanins, copigmentation, *A. hispida*, stability

Consumer's concern (MARKAKIS, 1982) about the use of artifical colours in food and pharmaceutical products has been an important factor in promoting the search for natural non toxic substitutes of some of the artifical colours, mainly red ones.

Anthocyanins are among the natural colours with red to bluish shades that are non toxic, water soluble and could eventually become the most important substitutes for the artificial red colours. Two main problems make this substitution difficult presently: the lack of reliable and cheap sources and the poor stability of anthocyanins to light and high pH. The search for reliable sources of stable anthocyanins has been the subject of many publications i.e.: MOK and HETTIARHCACHY (1991), SHI and co-workers (1992), DOUGALL and co-workers (1997), DUHARD and co-workers (1997). A potential source of anthocyanins for use in food and pharmaceutical products are the leaves of *Acalipha hispida* for which long term toxicity studies are yet to be made.

According to INAMI and co-workers (1996), acylation improves both heat and light stability, whereas glycosidation only stabilized anthocyanins in the presence of light. The use of tannic acid to stabilize the colour of orange juice from Italian red oranges was proposed in a paper by MACCARONE and co-workers (1987).

In a previous paper (BAILONI et al., 1998) the preparation of a methanolic extract from the leaves of *A. hispida* as well as its concentration and partial purification by reverse-osmosis were reported. The main anthocyanin in the extract was cyanidin-3-arabinosyl-glucoside.

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The present paper reports on the stability of the extract and of purified fractions from it as well as on the stabilizing effect of copigmentation.

1. Materials and methods

1.1. Stability of the extract E_1 to light at pH 3.0 under N_2 or air

Five hundred mg of the crude extract, free of methanol, prepared as described in a previous paper (BAILONI et al., 1998), was dissolved in sufficient amount of citrate-phosphate buffer at pH 3.0. The pH of the solution was adjusted to 3.0 by the addition of a few drops of 0.1 N HCl and the volume of the solution made up to 100.00 ml with the buffer solution. The slightly turbid solution was filtered and the clear liquid distributed into 10 ml screw-cap tubes. The head space was thoroughly flushed with N_2 when needed. The screw-cap tubes were divided in 2 lots. One was kept in the dark at 21±1.0 °C, while the other was irradiated at the same temperature between two 40 W lamps, day-light type, with nominal intensity of 2500 lm. Absorptions at 530 nm were measured from time to time until the loss of absorption reached aproximately 50%.

1.2. Fractionation of extract E_1

Extract E_1 was chromatographed on Whatman No. 3 and the chromatogram was developed with 1% HCl (FRANCIS, 1982).

A strong red zone, E_2, near the top was eluted and dried at 36 °C under vacuum.

A strip of paper from the chromatogram, when exposed to ammonia vapor turned yellow indicating the presence of a large zone of non anthocyanic flavonoids (NAF) preceding the E_2 zone. The NAF fraction was eluted and evaporated under vacuum at 36 °C to a pale yellow syrup which turned brown on exposure to air and light. Both E_2 and NAF were stored under N_2 at –18 °C.

Part of fraction E_2 was rechromatographed and the chromatogram was developed with BAW (n-BuOH-HAc-H_2O 6:1:2) (FRANCIS, 1982). A strong red zone, E_3, near the middle part of paper was eluted, dried at 36 °C and stored at –18 °C under N_2.

Part of the E_3 fraction was rechromatographed using Bu-HCl (n-BuOH-2NHCl 1:1 upper phase) as a developer (FRANCIS, 1982). A narrow red zone E_4, in the lower portion of the chromatogram was eluted and dried at 36 °C under vacuum. Fraction E_4 was stored under N_2 at –18 °C.

1.3. Stability of the purified fractions (E_2, E_3, E_4) to light and air

Solutions of each fraction were prepared as in 1.1 and their stability to light and air was estimated as described for fraction E_1.

1.4. Effect of copigmentation on the stability of the anthocyanic fractions

Solutions of each fraction (E_{1-4}) were prepared as described in 1.1. To each fraction sufficient tannic acid was added in order to attain a 3:1 proportion of tannic acid:anthocyanin (w:w) (BOBBIO et al., 1990; 1992).

Approximately the same weight of the fractions E_2 and NAF were used to estimate the copigmentation effects of NAF on the anthocyanins.

2. Results and discussion

Results presented in Table 1, $t_{1/2}$, were obtained from data of Figs 1–9 which represent the average of the absorbance readings from 2 samples. All values of $t_{1/2}$ were calculated from absorbance readings up to a maximum of 50% loss of the initial absorbance.

It has been reported as early as 1931 by ROBINSON and ROBINSON, that NAF can act as a stabilizing agent for anthocyanins by copigmentation, and the NAF-anthocyanin association has been considered responsible for the stability of the anthocyanins in plants tissues (ASEM et al., 1972).

Purification of E_1 with separation of colourless flavonoids did not significantly affect the $t_{1/2}$ values of fraction (E_2) (Table 1), but a considerable decrease of the $t_{1/2}$ occurred when purifying E_1, which besides removing flavonoids (NAF) also removed other minor anthocyanins as in fractions E_3–E_4.

Table 1

Time of half life (h) for E_1 and purified extracts E_2, E_3, E_4 with copigment at pH 3.0, 20 °C

Reaction conditions		$t_{1/2}$ (h)
E_1+N_2+D	(Fig. 1)	2772
E_1+N_2+L	(Fig. 2)	721
E_1+A+L	(Fig. 3)	445
$E_1+A+L+TA(1:3)$	(Fig. 4)	1103
E_2+A+L	(Fig. 5)	459
$E_2+A+L+TA$ (1:2)	(Fig. 6)	1388
E_3+A+L	(Fig. 7)	202
E_4+A+L	(Fig. 8)	103
$E_2+A+L+NAF$	(Fig. 9)	476

A: air; D: dark; E_1: crude extract; E_2: ext. purified by p.c. 1% HCl; E_3: purified ext. by p.c. BAW; E_4: purified ext. by p.c. Bu-HCl ; L: light; NAF: non anthocyanins flavonoids; TA: tannic acid

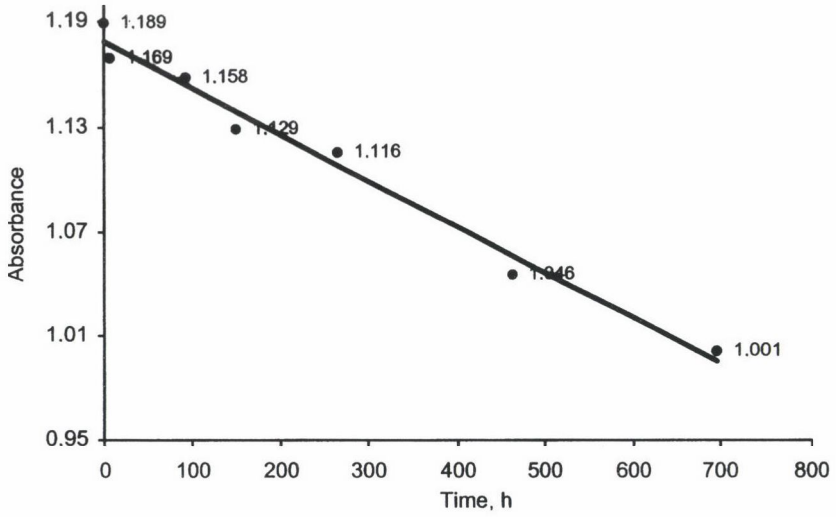

Fig. 1. Loss of absorbance for solutions of *A. hispida* E_1 under N_2 in the dark. Values are the average of two readings from two samples. $y = -0.0003\ x + 1.1784$; $R^2 = 0.9827$

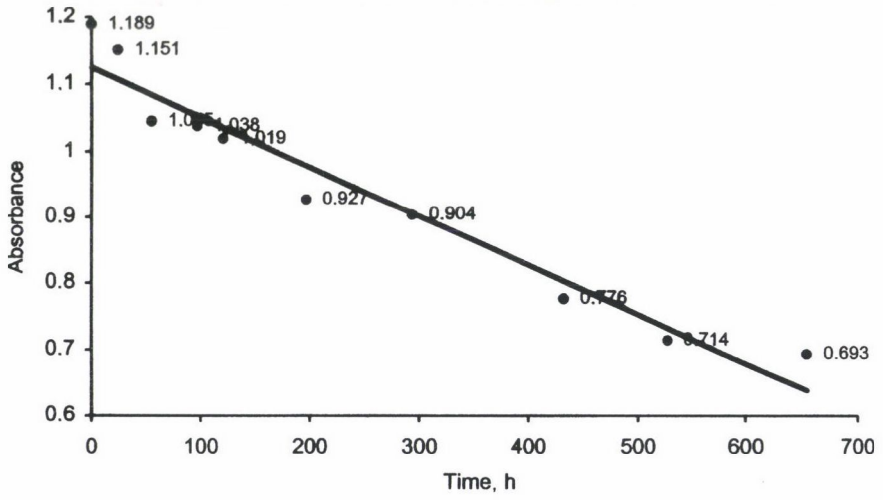

Fig. 2. Loss of absorbance for solutions of *A. hispida* E_1 under N_2 and light. Values are the average of two readings from four samples. $y = -0.0007\ x + 1.1241$; $R^2 = 0.9466$

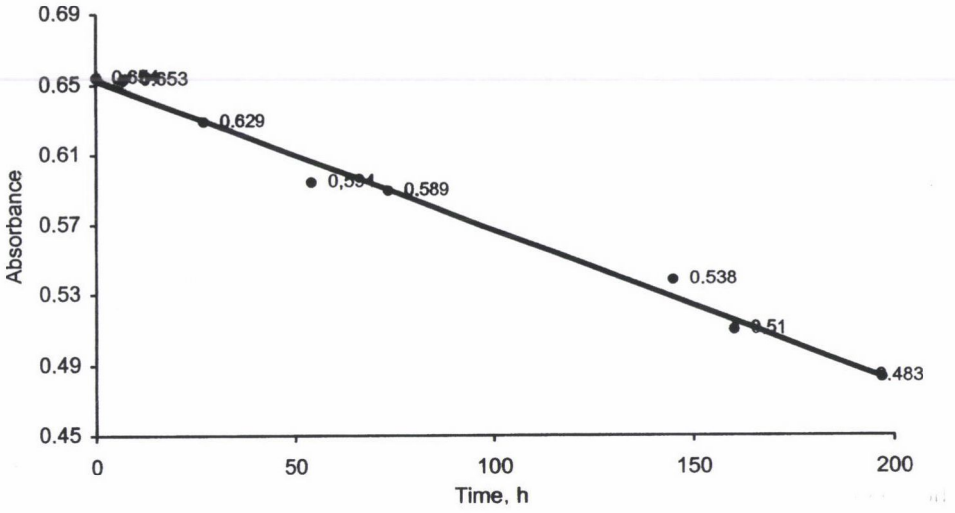

Fig. 3. Loss of absorbance for solutions of *A. hispida* E_1 under air and light. Values are the average of two readings from two samples. $y = -0.0009\ x + 0.6524$; $R^2 = 0.9892$

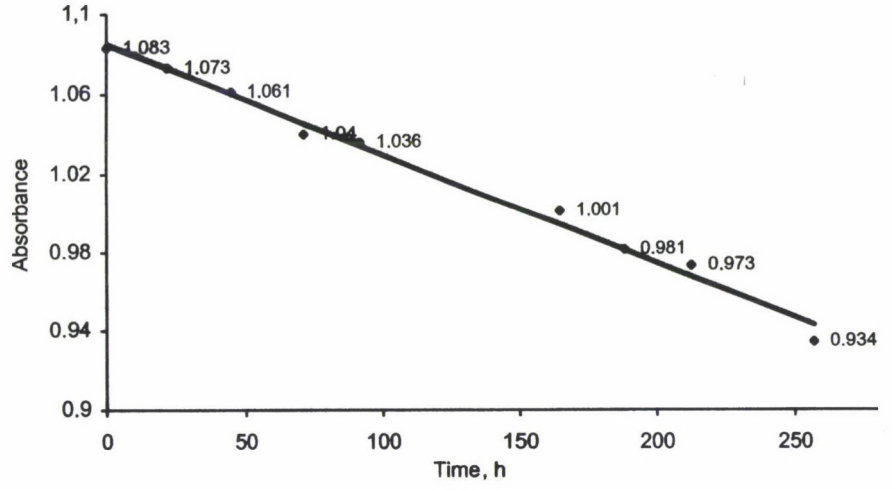

Fig. 4. Loss of absorbance for solutions of *A. hispida* E_1 under light and air with addition of tannic acid (1:3). $y = -0.0006\ x + 1.0848$; $R^2 = 0.9908$

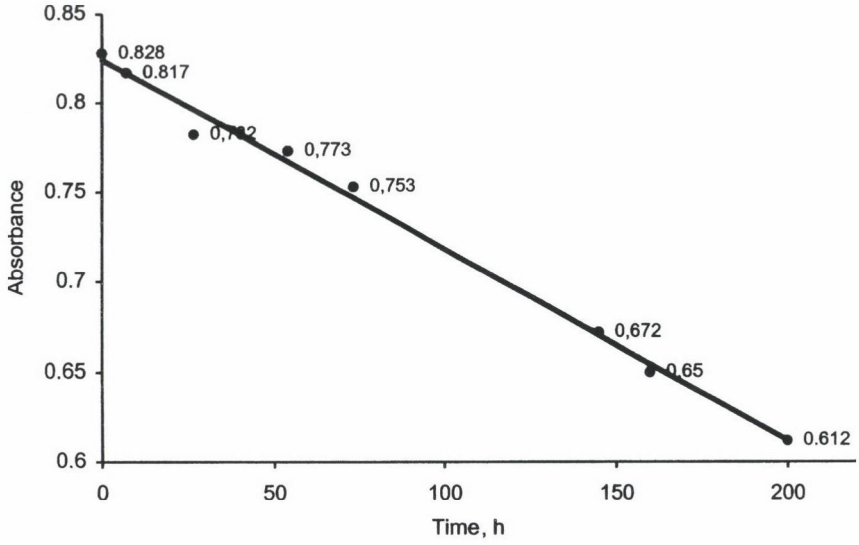

Fig. 5. Loss of absorbance for solutions of *A. hispida* E_2 under air and light. Values are the average of two readings from two samples. $y = -0.0011\ x + 0.8242$; $R^2 = 0.9932$

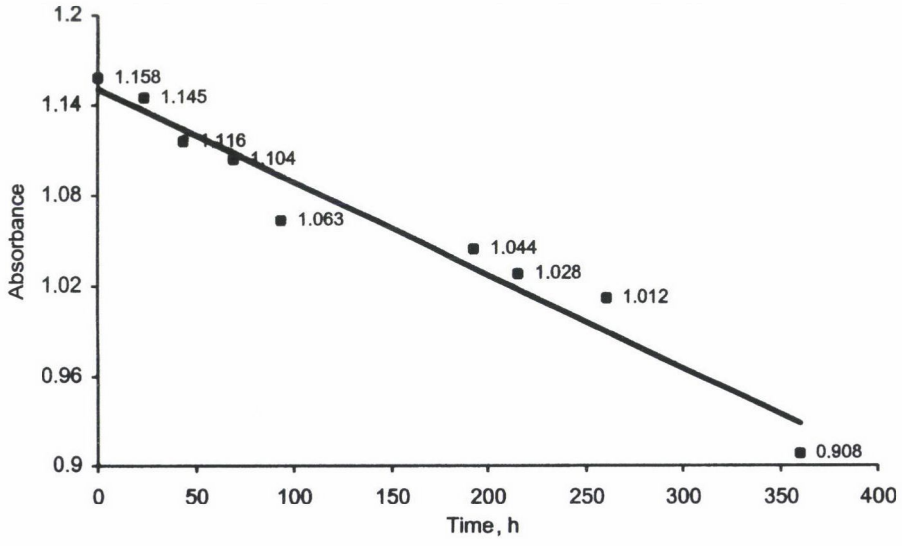

Fig. 6. Loss of absorbance for solutions of *A. hispida* E_2 under light and air with addition of tannic acid (1:2). $y = -0.0006\ x + 1.1505$; $R^2 = 0.953$

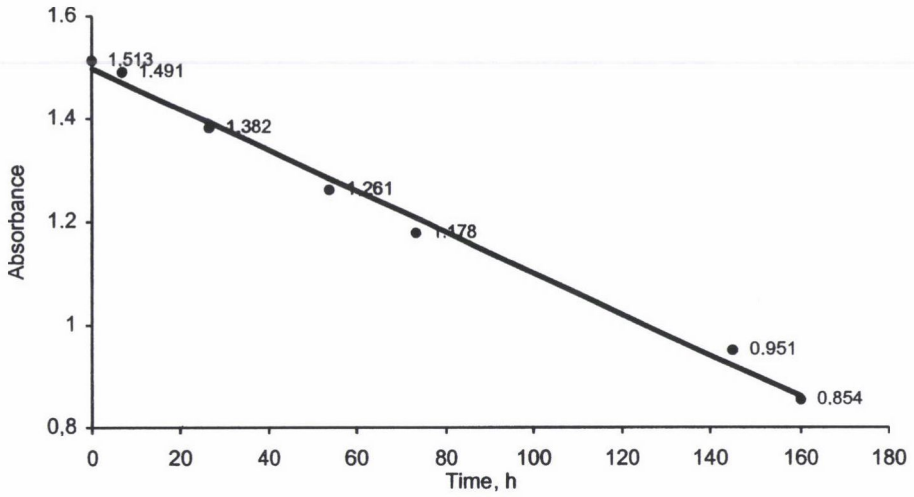

Fig. 7. Loss of absorbance for solutions of *A. hispida* E_3 under air and light. Values are the average of two readings from two samples. $y = -0.004\ x + 1.4976$; $R^2 = 0.9922$

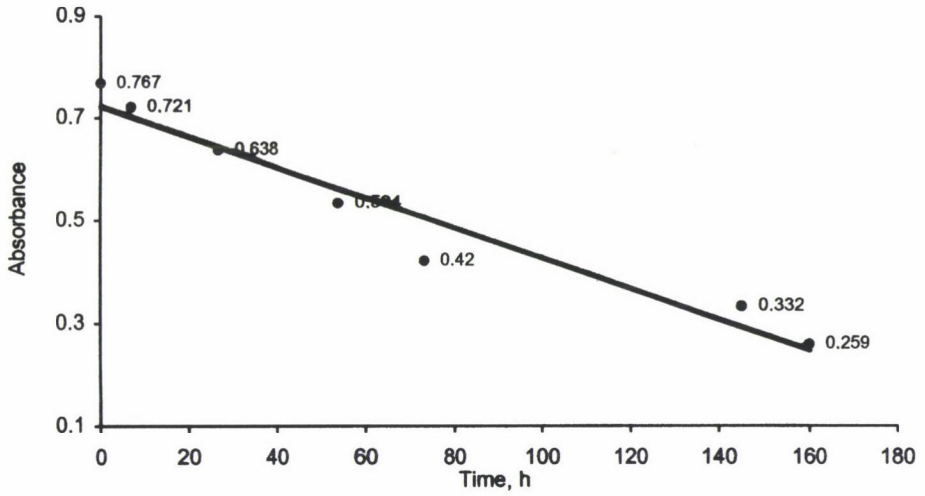

Fig. 8. Loss of absorbance for solutions of *A. hispida* E_4 under air and light. Values are the average of two readings from two samples. $y = -0.003\ x + 0.7215$; $R^2 = 0.9464$

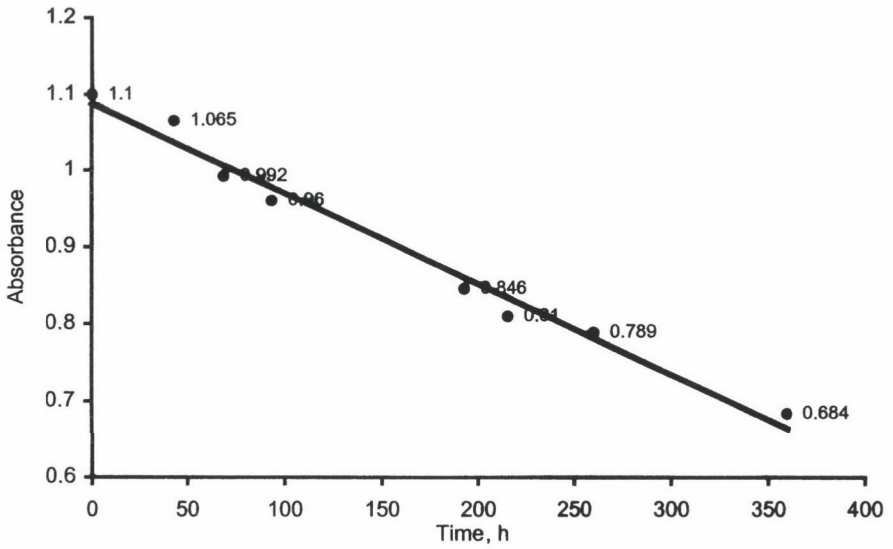

Fig. 9. Loss of absorbance for solutions of *A. hispida* E_2 under air and light, with addition of NAF. $y = -0.0012\ x + 1.0872$; $R^2 = 0.9816$

The addition of the recovered NAF to fraction E_2 did not significantly increase its stability (Table 1). The rapid darkening of the NAF fraction by air and light could be in part responsible for its lack of protective effects. Addition of ascorbic acid to the NAF fraction effectively prevented its darkening but the possibility of using ascorbic acid was precluded by the reported destructive effect of ascorbic acid on anthocyanins (MARKAKIS, 1982).

Intermolecular association of anthocyanins has been considered responsible for the stability of the anthocyanins in plants (SCHEFFELDT & HRAZDINA, 1978). This association as well as copigmentation is easily destroyed by methanol or ethanol (MINIATI et al., 1992). The significant decrease of $t_{1/2}$ from fraction E_1–E_4 parallels the increase in purity of the main anthocyanin by the progressive elimination of minor anthocyanins, therefore decreasing the possibility of hetero intermolecular associations and stabilization.

Tannic acid, on the other hand, was quite effective in stabilizing the anthocyanin, particularly fraction E_2 (Table 1). The presence of tannic acid apparently retarded the hydration reaction, which becomes important at pH 3.0 and retarded the formation of the hemiacetalic structure, but did not increase the concentration of the flavilium ion since no significant increase in $\lambda_{max.}$ value was observed.

By far the most destructive effect on the anthocyanins (Table 1) resulted from the combination of purification, have the light and oxygen. Tannic acid was an effective stabilizing agent, and for fraction E_2 under light and oxygen, increased the $t_{1/2}$ from 459 h to 1380 h (Table 1).

*

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HIGH RESOLUTION COMPUTED TOMOGRAPHY DETECTION OF ALIMENTARY FACTORS RELATED TO ARTHROPATHIES IN INFLAMMATORY BOWEL DISEASES

Á. MESTER[a], E. MAKÓ[a], S. NAGYLUCSKAY[b], K. KARLINGER[a], E. MÁRTON[a] and T. GYÖRKE

Semmelweis University of Medicine,
[a] Department of Radiology and Oncotherapy, H–1082 Budapest, Üllői út 78/A. Hungary
[b] Institute of Public Health, H–1089, Budapest, Nagyvárad tér 4. Hungary

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Alimentary factors of enterogen arthropathies were studied, in particular the early morphological detection, to prevent the disease progression by nutritional prescriptions. Authors compared the method of high resolution computed tomography (HRCT) with conventional X-ray and nuclear medicine investigations. The results showed the superiority of HRCT to detect cartilage erosions and ligamental calcifications in the sacroiliac joint and lumbar facet joint. The importance of nutritional factors is briefly discussed. The need for high quality and high quantity food of patients suffering from inflammatory bowel diseases (IBD) is well known. Elemental diet is effective for remission of IBD. Folic acid intake is extremely important. Total parenteral nutrition in acute active disease (Crohn's fistulas) has not been proved to effect IBD. Elimination of whole protein as a possible luminal factor for long term application can help, if elementary amino acids are in the diet. Eicosapentaenoic acid and docosahexaenoic acid (major components of fish oils) have beneficial effects. Polyunsaturated fatty acids in the diet can decrease the inflammation. Antioxidants, glutamine are essential in the diet. Removal of fat is effective to get remission. Alimentary complication in IBD patients can be the osteoporosis, so there is a need for regular bone densitometry. Conclusion of the study suggests that HRCT offers the most sensitive detection of enterogen arthropathy related changes. The predictive value of this diagnostic method is accurate enough to advise restrictive and/or supplemental diets for IBD patients. Dietary therapy allows circumvention of the adverse side-effects of repeated courses of steroids.

Keywords: arthritis, Crohn, inflammatory bowel diseases, sacroileitis

Alimentary factors associated with inflammatory bowel diseases (IBD) associated in arthropathies have got a rising importance in the recent period of scientific and clinical research.

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Alimentary factors can explain, why the different localisation (small bowel/large bowel) of the inflammation, – for example Crohn's disease (CD) versus ulcerative colitis (UC) – involves more or less frequently the joints. Small bowel Crohn's manifestation versus colonic CD and/or ulcerative colitis causes a higher prevalence of the arthritis.

Vegetarian diet can have a beneficial influence on arthritis, such as rheumatoid arthritis (RA) (KJELDSEN–KRAGH et al., 1991). This can be connected to the immune globulin production (MIELANTS et al., 1995). FELTELIUS and co-workers (1994) found increased jejunal IgM in untreated ankylotic spondylitis (AS) patients.

Bowel inflammation and joint inflammation can occur by a common pathologic origin, on one hand, and on the other hand the arthropathy can be a secondary consequence of changes in the intestinal absorption. The third possibility is a combined manifestation of both action mechanisms (BRANDTZAEG et al., 1997).

Arthritis types, on one hand, are transient monarthritis episodes, which are characteristic. Destructive (erosive) arthritis and granulomatous synovitis are less common on the other hand (GRAVALLESE et al., 1988; NORTON et al., 1993; HERMANS, 1984). Sacroileitis is the most important axial articular involvement with a possible progression up to ankylosis (see ankylosing spondylitis). Sacroileitis is a more common central arthritis in IBD than in ankylosing spondylarthritis (AS).

Enterogen artropathies are multifactorial diseases. Genetic factors, such as human leukocyte antigen (HLA)–B27 are associated with ankylosing spondylitis (AS) with later occurrence of enteritis, and sometimes with Crohn's disease or ulcerative colitis which develop many years after the initial arthritis (PURRMANN et al., 1988; DE VOS et al., 1989).

Bacterial enteral infections can possibly be the first disease (*Salmonella*, *Shigella*, *Campylobacter*, *Yersinia*) and consecutive reactive arthritis (ReA) can be a later manifestation (these bacteria express lipopolysaccharides on their outer membrane, and can exist intracellularly). In case of the Reiter's syndrome urethritis and conjunctivitis combine with arthritis.

The influence of the intestinal mucosal permeability – increased by inflammation – has an important, but not single pathogenic role. In case of CD patients, their healthy first degree relatives had increased intestinal permeability, too (KATZ et al., 1989; MIELANTS et al., 1991).

The antigen presentation (of bacteria) by HLA class I molecules to (memory)T–lymphocytes can explain why the strong correlation in–between bacterial exposure and genetic factors exists. In this context class II alleles, like DR103 may have importance, too. These molecules can bind and present fragments of class I molecules to the lymphocytes (molecular mimicry), which stimulate/prolongate inflammation and/or autoimmune mechanisms (ORCHARD et al., 1997).

The inflammation causes changes in the adhesion properties of the mucosal microvascular endothelium. By this process the "gatekeeper" function of the B and T cells (excreted by the Peyer's patches in the normal mucosa) decreases. Gut derived lymphoblasts have dual endothelial ("homing") affinity: both intestinal mucosa and synovial membrane of the joints (JALKANEN et al., 1986). In ReA mucosal macrophages transport bacterial antigens to the synovial endothelial cells (SALMI et al., 1997).

Nutritional factors of inflammatory bowel diseases

Nutrition and intestinal functions are strongly interrelated. The 1/2–2/3 of patients with Crohn's disease were weight-depleted and 3/4 of adolescents growth-retarded. Chronic malnutrition impairs digestive and absorptive function because food and nutrients are not only the major trophic factors to the gut but also provide the building blocks for digestive enzymes and absorptive cells. Weight loss greater than 30%, accompanying a variety of diseases is associated with a reduction in pancreatic enzyme secretion of over 80%, villus atrophy and impaired carbohydrate and fat absorption (O'KEEFE et al., 1996). Specific nutrients can induce disease, for example, gluten-sensitive enteropathy, whilst dietary factors such as fibres, among them resistant starch, short-chain fatty acids, glutamine and fish-oils are joined in prevention of gastrointestinal diseases such as diverticulitis, diversion colitis, ulcerative colitis, colonic adenomatosis and colonic carcinoma. The role of dietary antigens in the aetiology of Crohn's disease is controversial, but controlled studies have suggested that elemental diets may be as effective as corticosteroids in inducing a remission in patients with acute Crohn's disease.

A general need for high quality and high quantity food of IBD patients predominantly in the adolescent and young adult age groups, is evident. The IBD diseases often cause weight loss, by decreasing the appetite, and/or by diarrhoea. Chronic blood loss is common in the UC patients, with a consecutive need of iron intake. Lactose intolerance requires lactose free milk, or yoghurt to supply calcium. Unexpected osteoporosis can occur even in young adults, if calcium absorption decreases because of chronic IBD. In coeliac disease the prescription of wheat protein gluten is essential to prevent enteral inflammation (THOMPSON et al., 1993).

Elemental diet is effective for both inducing and maintaining remission of IBD. MUNAKATA and co-workers (1997) reported equal effectiveness between elemental diet, oligopeptide diet and intact nutrients treating active Crohn's disease. Although corticosteroid is also useful for treating active Crohn's disease, low dose of it is not effective for maintaining remission. Steroids have important role in combined therapy with elemental diet.

The importance of folic acid intake is extremely important, if 6–mercaptopurine (6MP) or sulfasalazine therapy is being used. It is also required to measure the B_{12} vitamin absorption ("Schilling test"). In malabsorption or bile salt deficiencies A, D and K vitamin support is recommended. Restrictive (so called "natural") diets are dangerous in severe cases (sepsis, obstructing lesions or extensive fistulas).

In case of IBD there has been no major alimentary factor like gluten in coeliac disease. Total parenteral nutrition in actual active disease (fistulas) has not proved to effect in IBD. Elimination of whole protein as a possible luminal factor for long term application can help, if elementary amino acids are in the diet. (O'MORAIN and co–workers /1984/ found this to be similarly effective to prevent exacerbation of Crohn's disease like prednisolone.) In ulcerative colitis fish–oil seems to have beneficial effects, because of its effect on the mucosal production of prostaglandins and leukotriens.

Dietary intake can be the initiator of intestinal inflammation (PRIKAZSKA et al., 1997). In this connection they reported observations about a group of patients suffering from Crohn's disease and ulcerative colitis. In patients with Crohn's disease, a preferred consumption of flour products, decreased intake of vegetables and fruit, lowered tolerance to milk and milk products, increased sugar consumption, increased proportion of smokers, no differences in diet regimen between sick and healthy subjects were noticed.

Antigens in food can

- induce/influence immune response: aggravation of inflammation,
- pass through an abnormally permeable mucosa.

Malnutrition is a common consequence of advanced IBD, predominantly in adolescence, and if small bowel shortening exist (fistulas and/or surgical resections).

Polyunsaturated fatty acids in the diet can decrease the inflammation by altering the metabolic pathway with formation of less active leukotriene B5 rather than leukotriene B4. FRENCH and co–workers (1997) compared Crohn's patients, normal subjects and subjects with inactive Crohn's disease consuming a high polyunsaturated to saturated fat ratio diet. He concluded that

- absorption of labelled [1–13C] 10:0 and [1–13C] 16:0 ingested with a test meal is reduced in Crohn's patients, and
- consumption of a high polyunsaturated to saturated fatty acid ratio diet improves the utilization of dietary C16:0 by Crohn's patients.

Eicosapentaenoic acid and docosahexaenoic acid are major components of fish oils, and strong competitive inhibitors of the synthesis from arachidonic acid of (inflammatory) prostaglandins.

Antioxidants (ascorbic acid, alpha tocopherol, beta-carotene, selenium, methionine) can decrease the inflammation caused by damaging oxygen species of leukocytes and other inflammatory cell activities.

Glutamine is an essential metabolic substrate for enterocytes. It can diminish the intestinal permeability (LENNARD-JONES et al., 1996).

Removal of large molecules from the food has a beneficial effect on gut inflammation:

- complete bowel rest (intravenous parenteral nutrition) doesn't influence significantly the IBD activity, because the essential substrates for epithelial cell metabolism are removed,
- elemental diet (amino acids, glucose, little fat with minerals and vitamins) and
- partially hydrolysed (polymeric) "peptide" diet (peptides, oligosaccharides, medium-chain length triglycerides) equally can influence bowel metabolism, reducing inflammation similarly to steroid therapy (OKADA et al., 1990; SEIDMAN et al., 1986; GORARD et al., 1993; GONZALEZ-HUIX, 1993; PARK, 1991).

Liquidized normal food has an advantage in case of intestinal strictures, by which stasis and its consequence: altered, pathogenic bacterial flora and their products, like formyl-methionyl-leucyl-phenylalanine (FMLP) can increase the inflammation.

Removal of fat can increase the beneficial effect of liquid diets. Monounsaturated fatty acids have less disadvantages. Linoleic acid (precursor of arachidonic acid) and long chain triglycerides can aggravate the inflammation (MIDDLETON, 1994). Short chain fatty acids (derived from bacterial fermentation of unabsorbed carbohydrate) are essential for colonocytes (HEATON et al., 1979; RITCHIE et al, 1987).

In cases of less severe patients, the alimentary advises are more simple: they can take a bland diet, poultry, white fish, lamb, white bread, cooked fruit and potato.

Alimentary complication in IBD patients can be the osteoporosis, even in young people late diagnosed with a high grade (GENANNT et al., 1976). The high grade osteoporosis (with compression of the vertebral body) causes asymmetric partial overload of the apophyseal facet joints, resulting in spinal pain syndrome (RYAN et al., 1992).

Enteral feeding has been shown to be as effective as primary therapy for Crohn's disease. However, in adolescents with growth failure and when corticosteroid therapy is contra-indicated or has failed, it may become the treatment of choice. Furthermore, diet therapy allows circumvention of the adverse side-effects of repeated courses of steroids. Comparison of feeds with differing composition suggests that a low fat content increases efficacy and various explanations have been offered. The reduction of colonic bacterial load may also be important. Because symptoms of Crohn's disease may be provoked by eating, there is a risk of falsely attributing symptoms to specific foodstuffs. However, in many individuals foods can be identified which affect disease activity, and their exclusion leads to prolongation of disease remission. Dietetic supervision during food testing is important to avoid detrimental effects on nutrient and micronutrient intake (KING et al., 1997).

The aim of our study was to find a highly sensitive and precise diagnostic tool, which can help the selection of patients, who need an additional alimentary therapy to stop the articular disease progression, increase the sensitivity of the detection of sacroiliac joint erosions, and to help by this the precise diagnosis of extraintestinal IBD manifestations.

The early morphological detection of erosions in sacroileitis is insufficient with conventional radiological techniques: X-ray detects erosions in 15% only, but bone scan is positive in 70%, suggesting transient sacroileitis (GRAVALLESE et al., 1988). In case of the apophyseal facet joint's erosions the conventional radiography is not useful at all. Bone scan can not differentiate in between degenerative and inflammatory apophyseal facet joint's involvement.

1. Materials and methods

Patients suffering of inflammatory bowel diseases (IBD), as Crohn's disease (CD), ulcerative colitis (UC) and malabsorbtion syndrome/coeliacia were investigated.

The diagnoses of IBD was established with abdominal CT (1500 ml of per oral iodine-benzol-alkyl contrast material, 10 g% iodine and/or 1500 ml diluted barium sulphuric suspension, 10 g%). The CT investigation was followed by fluoroscopic enteroclysis: naso-jejunal catheter (with 50 g% diluted barium sulphuric suspension). The inflammatory activity of the bowel was measured by 99m-Tc labelled monoclonal anti-granulocyte antibody radioisotope scan. The actual inflammatory activity of the joints was measured by 99m–Tc labelled pyrophosphate scans.

The sacroiliac joint high resolution computed tomography (HRCT) and lumbar facet joint HRCT were done in 25 cases with proved IBD. The HRCT was carried out with Siemens (DRG2 and SomatomPlus4) equipment, using thin section (1 or 2 mm) scanning with 10 mm interslice gap, and high resolution image reconstruction algorithm. The X-ray tube-detector system ("gantry") angulation was 20° in the caudo-cranial direction.

To evaluate the HRCT results, 99m-Tc radiolabelled pyrophosphate bone scintigraphy (whole body planar scanner) and X-ray plain films were compared.

2. Results and discussion

Sacroiliac joint erosions were detected in 8 of 25 cases, with plain films, but the HRCT visualised 17 of 25, i.e. only 8 of 17 was seen on the plain films. Unilateral was the sacroileitis in 4 of 8 plain film positive cases, but only in 5 of 17 had unilateral involvement with HRCT. (The HRCT changed unilateral category into bilateral.)

Calcifications in the ligamental parts of the sacroiliac joint/lumbar facet joint's capsular region were with erosions in 3, without erosions in 7 of 25 cases.

The nuclear medicine investigation was positive in 7 cases, but in the majority others, than the HRCT. There was no strong correlation in–between the HRCT and nuclear medicine matching.

Lumbar facet joint's erosions were not seen on any plain films. The HRCT detected erosive lesions in 5 of 14 enterogen arthropathy cases. Nuclear medicine bone scan positivity was found in 2 of 5 erosive cases, but in 2 non erosive lesions, too (suggesting degenerative changes).

The enterogen apophyseal facet joint erosions – detected first by our research – using HRCT, can occure as primary inflammatory manifestations. Enterogen arthropathy has two components in this case. First the destructive monarticular apophyseal lesions of the CD patients could be connected to their specific granulomatous inflammatory changes (LINDSTROM et al., 1972; NUGENT et al., 1976; HERMANS et al., 1984; AL–HADITI et al., 1984; TOUBERT et al., 1985). The other component is a secondary antibacterial immune–reaction, i.e. "post infect" arthritis (related to the pathologically increased bowel absorbtion). The third component of the morphology can be a degenerative joint space narrowing (with or without osteoporosis related overloading).

The lymphoma patient's erosive joint inflammation was most probably an enterogen arthropathy, too (by a prae-lymphomatic sprue/malabsorbtipon). The enterogen and other SNSA sacroileitis/apophyseal facet joint inflammation can not be visualised much more accurately with HRCT, versus plain film or radionuclide scan. Even the SPECT can not increase highly the detection (RYAN et al., 1992), because it is only positive for a short period of actual (florid) sacroileitis. The HRCT visualises all residual morphological changes of the cartilage. Rarely hyperuricaemia/gout can cause apophyseal facet joint erosions.

The HRCT sensitivity gives the possibility to detect sacroiliac/lumbar facet joint involvement in IBD patients earlier. The articular involvement detection can influence the drug therapy and alimentary prescriptions as well.

The correction from pseudo-unilateral into bilateral involvement can help the differentiation of enterogen and other seronegative/infect arthritis. By the accurate diagnosis the indication of correct causal therapy – included food restrictions – can be supported.

Erosions and ligamental calcifications are both connected with arthritis. The mechanism is most probably different, because they appear only partially together,

and often separated. Our hypothetical suggestion about the differences of the two versions could be:

- erosive lesions primary intraarticular manifestation of granulomatous inflammation, parallel with IBD versus
- secondary metabolic changes (by the increased bowel permeability) resulting inflammation/calcification in the ligamental parts of the joints.

The theory is based on the higher prevalence of erosive lesions in case of Crohn's disease versus malabsorbtion and ulcerative colitis.

The articular erosions in IBD patients are similar to the erosions of other (seronegative spondylarthritis) SNSA, but some pathological differences are present: granulomatous inflammation, similar with the intestinal granulomatous manifestation was present (NUGENT et al., 1976; HERMANS et al., 1984; AL-HADITI et al., 1984; TOUBERT et al., 1985). The possible mechanism seems to be the repeated infectious agents provoke joint inflammation. The reasons could be:

- changes of the bowel anatomy, for example following bypass surgery,
- autoimmune mechanisms by the molecular mimicry, for example after diarrhea, or similar to ankylosing spondylitis (AS, Bechterew) pathogenesis,
- the changed intestinal permeability after milk allergy or coeliacia,
- toxin mediated synovitis, for example pseudomembranosus enterocolitis.

The changes of the intestinal permeability can cause direct in situ antigen deposition (PHILLIPS et al., 1989). In cases of reactive arthritis colonoscopy proved relationship in-between the length of the inflamed intestinal mucosa and the arthritis (MIELANTS et al., 1987).

Other mechanism of intestinal seronegative sterile arthritis is the post-infect (*Salmonella*, *Shigella*, *Yersinia*, *Campylobacter*) reactive arthritis. Involvement of lymph nodes and bacteriemia (except *Shigella*) was detected (MIELANTS et al., 1987). The typical manifestation is, that after a period of 1–3 month acute "self-limiting" inflammation and specific (bacterial) immune–complexes can be detected in the [illegible] fluid of larger joints. Rarely true septic arthritis can occur (predominantly as [illegible]*neila* complication in children) with direct cartilage destruction.

The HRCT offers the differentiation:

- in-between degenerative and inflammatory changes (joint space/subchondral lesions),
- intraarticular cartilaginous erosions / intraarticular ligamental calcifications / periartikular capsular / periarticular ligamental calcifications.

3. Conclusion

Elemental, hydrolysed and polymeric liquid diets appear to give equivalent results in reducing inflammation, and can be used as a first line of treatment instead of (or prior to) a corticosteroid drug therapy and/or in combination with steroids.

HRCT of axial joint involvement can be a predictor for the need of restrictive and/or supplemental diets.

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CHLOROGENIC ACID CONTENT AND ANTIOXIDANT PROPERTIES OF POTATO TUBERS AS RELATED TO NITROGEN FERTILISATION

A. LUGASI[a], D. P. F. ALMEIDA[b], and E. DWORSCHÁK[a]

[a] National Institute of Food Hygiene and Nutrition, Gyáli út 3/a, Budapest, Hungary. H–1097
[b] University of Trás-os-Montes e Alto Douro, Crop Science Department, Apartado 202, 5001 Vila Real Codex. Portugal

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The chlorogenic acid and the total polyphenol content were analysed in two different potato varieties (Kennebec, Agria) grown under five different nitrogen fertiliser rates (0, 75, 150, 225, 300 kg ha^{-1}). Chlorogenic acid content ranged between 6.0–22.3 mg kg^{-1} fresh weight and was not influenced by fertiliser levels. The chlorogenic acid in potato tubers accounted for almost 90% of the total polyphenols. Free radical scavenging and antioxidant acitivities of the tubers were also analysed. Ethanolic extracts of the tubers showed marked hydrogen-donating activity in the experiment using 1,1-diphenyl-2-picrylhydrazyl (DPPH), they had reducing power as measured by the Fe(III)→Fe(II) reaction, but did not exhibit H_2O_2 scavenging activity assessed with a chemiluminescence method. Potato extracts showed significant, although weak Cu(II)-chelating activity and inhibited the autoxidation of linoleic acid as measured by the thiocyanate method. Chlorogenic acid containing extract of potato, can act as primary and secondary antioxidant in prevention of oxidative stress. The strong correlation between the antioxidant activity and the level of total pholyphenols suggests that the phenolic compounds are important antioxidant components of whole potato tubers. Variety had minimal, while nitrogen fertiliser rate had no effects on the levels of the studied characteristics.

Keywords: antioxidant, free radical scavenger, chlorogenic acid, chemiluminescence, potato, nitrogen fertiliser

Foods of plant origin, including potato, contain several secondary metabolites: phenolic compounds (chlorogenic acid, caffeic acid), glycoalkaloids (α-chaconine, α-solanine), protease inhibitors, and phytoalexins which protect the plant tissues against pathogens and pests (DAO & FRIEDMAN, 1992). Potato is considered a good source of antioxidants such as ascorbic acid, α-tocopherol and polyphenolic compounds, which act synergistically to inhibit the free radical-induced tissue damage (BYERS & PERRY, 1992).

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Chemistry, biochemistry, and the dietary role of potato polyphenols were recently reviewed by FRIEDMAN (1997). Chlorogenic acid (5-O-caffeoyl-quinic acid) constitutes about 90% of the total polyphenol content of potatoes (DAO & FRIEDMAN, 1992). This compound may be responsible for the "after-cooking blackening or darkening" of boiled and steamed potatoes. The blackening appears to be caused by the formation of a ferrous ion – chlorogenic acid complex. Following exposure to oxygen in the air, the colourless ferrous complex is oxidised to a dark ferric complex (HUNGHES et al., 1962). Chlorogenic acid also participates in enzymatic browning which can lead to nutritional damage (MOLNAR-PERL & FRIEDMAN, 1990). Chlorogenic acid is oxidised by polyphenol oxidase to a highly reactive o-quinone intermediate which could interact with different amino acids in nucleophil addition and in polymerisation reactions. These reactions destroy essential amino acids, decrease the nutritional quality and may also result in the formation of toxic compounds (FRIEDMAN, 1992, 1996).

Recently, several studies have been focused on the antioxidant activity of phenolic compounds in potato. ONYENEHO and HETTIARACHCHY (1993) evaluated the effectiveness of freeze-dried extracts from the peel of potato varieties for their ability to prevent soybean-oil autoxidation. Chlorogenic and protocatechuic acids were the main antioxidants in the extracts. RODRIGUEZ DE SOTILLO and co-workers (1994a, 1994b) confirmed the strong antioxidant activity of freeze-dried extract of potato peel waste in sunflower oil. These results have suggested that potato peel has possible preventive effects against oxidative rancidity of food products. AL-SAIKHAN and co-workers (1995) found that phenolic content and antioxidant activity of four potato cultivars were genotype-dependent. VINSON and co-workers (1995) showed that chlorogenic acid and other polyphenols exhibit strong in vitro antioxidant activity in the oxidation process of lipoproteins. In vivo oxidation of low-density lipoproteins appears to be a major cause of coronary heart disease (CDH). It is thus possible that foods containing chlorogenic acid and other polyphenols may decrease the incidence of CDH.

FELICE and co-workers (1976) observed that caffeic acid and chlorogenic acid were effective scavengers of peroxyl radicals and more reactive than the corresponding quinones which are formed by oxidation of the phenolic acids mentioned above. On the other hand, quinones are better metal-chelating antioxidative agents than the phenolic acids. Both effects are involved in the antioxidant behaviour of polyphenolic compounds. Chlorogenic acid and other polyphenols are reported as inhibitors of cancer development due to their ability to scavenge and trap potentially DNA-damaging electrophiles and free radicals, to inhibit enzymes that activate pre-carcinogens, and to induce carcinogen-detoxifying enzymes (STEVENS et al., 1995). Chlorogenic acid suppresses the elevation of serum cholesterol level by alcoholism, stimulates bile acid secretion, inhibits lipid peroxidation in liver mitochondria and

microsomes, and shows protective effects on paraquat-induced oxidative stress in rats (LAZAROV & WERMAN, 1996; TSUCHIYA et al., 1996).

DAO and FRIEDMAN (1992) called attention to the fact that food processing technologies such as heating induce a decrease in chlorogenic acid content of potatoes. This compound can be partially or even completely destroyed by the usual cooking conditions. This decrease appeared to depend on the nature of the heat used, microwaves seemed to be the least destructive, boiling and oven-baking intermediate and the most destructive, respectively. Commercial potato chips and mashed potato flakes did not contain chlorogenic acid (DAO & FRIEDMAN, 1992), thus querying the potential health benefits of the potato phenolics.

The objectives of our work were to investigate [1] the chlorogenic acid and the total polyphenol content of two potato cultivars grown under five different fertiliser rates, [2] the free radical scavenging and antioxidant activity of the potato and [3] to establish the relationships among the characteristics mentioned above.

1. Materials and methods

1.1. Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), tetramethyl murexide (TMM), chlorogenic acid, luminol, microperoxidase, and linoleic acid were obtained from Sigma Chemical (Co(MO, USA). Absolute ethanol and hexamine were purchased from Merck (Germany). All other chemicals and reagents were of analytical grade from Reanal (Hungary).

1.2. Plant material

Potato (*Solanum tuberosum* L. cvs. Agria and Kennebec) were grown in the spring of 1995 in Vila Real, Portugal. Five nitrogen levels – 0, 75, 150, 225 and 300 kg N ha^{-1} – were combined with both cultivars in completely randomised block design with four replications. All the nitrogen fertilisers were applied at planting in the form of NH_4NO_3. At harvest, samples of 5 tubers including peel with diameter between 35–55 mm, showing no signs of pests or diseases, were collected, freeze-dried, and kept in a sealed plastic bag until analysed.

1.3. Preparation of the potato extract

The freeze-dried potato powders were first defatted with *n*-hexane. A portion of defatted powder (500 mg) was then refluxed for 6 h with 20 ml of ethanol in Soxhlet extractor (FRIEDMAN et al., 1989). The refluxed sample was then filtered

through Whatman 4 filter paper and the filtrate adjusted to a volume of 20 ml with ethanol. The extracts were stored in a refrigerator at 4 °C until analysed.

1.4. Chlorogenic acid determination

For the chlorogenic acid determination (and for the other spectrophotometric measurements used in this study) a Perkin-Elmer Lambda3 UV-VIS spectrophotometer was used. The UV spectrum of the potato extracts was determined at 250–400 nm according to DAO and FRIEDMAN (1992). The concentration was calculated from the absorption maximum at 325 nm for a standard of chlorogenic acid. The molar extinction coefficient of chlorogenic acid was determined as 18130 M^{-1} cm^{-1}.

1.5. Total polyphenol determination

The total polyphenol content present in the ethanolic extract of freeze-dried potato powder was determined spectrophotometrically using Folin-Denis reagent (A.O.A.C. 1990). The ethanolic extract of potatoes (1 ml) was diluted with distilled water to 7 ml. Folin-Denis reagent (0.5 ml) was added, and the content of the tube was mixed thoroughly. After 3 min, 1 ml of saturated Na_2CO_3 was added and the final volume adjusted to 10 ml with distilled water. The mixture was allowed to stand for 30 min at room temperature. The absorbance was determined at 760 nm using tannic acid as standard.

1.6. Hydrogen-donating ability

Hydrogen-donating ability of potato extracts was determined in the presence of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical as described by BLOIS (1958) and modified by HATANO and co-workers (1988). Ethanolic extract of potato (1 ml) and a portion ethanol (3 ml) were added to a methanolic solution of DPPH (1 mM, 1 ml). The mixture was shaken and left to stand at room temperature for 30 min. The absorbance of the resulting solution was measured spectrophotometrically at 517 nm. Hydrogen-donating ability of the extracts was given by the inhibition of the colour development expressed as percentage of the control, which contained ethanol instead of the potato extract.

1.7. Reducing power

The reducing power of the potato extracts was determined according to the method of OYAIZU (1986). Ethanolic extract (1 ml) was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and 1% potassium ferricyanide /$K_3Fe(CN)_6$/ (2.5 ml); the mixture was incubated at 50 °C for 20 min. Trichloracetic acid (2.5 ml, 10%) was

added to the mixture, which was then centrifuged at 1.500 g for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and freshly prepared $FeCl_3$ solution (0.5 ml, 0.1%). The absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicates elevated reducing power.

1.8. Copper(II)-chelating activity

The chelating activity of potato extracts on copper(II)-ions was measured according to SHIMADA and co-workers (1992), with a minor modification. An aliquot of potato extract (1 ml) was added to hexamine buffer (1.0 ml, 10 mM, pH=5.0) containing KCl (10 mM) and $CuSO_4 \cdot 5H_2O$ (3 mM). The reaction mixture was shaken well and after 2 min tetramethylmurexide (TMM) (0.1 ml, 1 mM) was added. Absorbances at 485 and 530 nm were measured. Copper(II)-ions in hexamine buffer show an absorption maximum at 485 nm in the presence of TMM. Strong chelating agents such as EDTA and polyphenols form new complex molecules with copper ions that give absorbance maximum at 530 nm. The position of the absorption maximum depends on the concentration and the copper-binding ability of the compounds. Chelating activity was determined as the absorbance ratio at 485 vs 530 nm. Lower absorbance ratios indicate higher chelating activity compared to the control.

1.9. Scavenging activity in H_2O_2/·OH-luminol system

The scavenging activity of the potato extracts in H_2O_2/·OH-luminol system was measured with the use of a Berthold Lumat LB 9501 luminometer according to HEIDE and BÖGL (1986), and modified by BLÁZOVICS and co-workers (1992). Unstable free radicals formed from H_2O_2 in the presence of transitional metal ions via Fenton reaction decompose the luminol to inactive amino-phthalic acid while monochromatic light (λ=425 nm) is emitted. The reactions can be catalysed by hemin, or microperoxidase. We used microperoxidase in our experiments. The intensity of the chemiluminescence expressed as relative light unit (RLU) is reduced by free radical scavenged substances. The highest chemiluminescence intensity – the standard light – is given by the H_2O_2/·OH-luminol system. The reaction mixture contained 0.3 ml of H_2O_2 (10^{-4}M), 0.05 ml of luminol ($7 \cdot 10^{-7}$ M), 0.3 ml of microperoxidase (10^{-7} M) and 0.2 ml of sample. Measurements were made over a 30 sec period.

1.9. Antioxidant activity

The antioxidant activity of potato extracts was determined according to the thiocyanate method (MITSUDA et al., 1966) with a slight modification. An aliquot of potato extract (0.5 ml) was added to 9.5 ml linoleic acid emulsion (500 mg linoleic acid was dissolved in 40 ml of absolute ethanol, mixed well and then 40 ml pH=7.0

phosphate buffer was added and diluted to 100 ml with distilled water.) The reaction mixture was stored for 7 days at 40 °C. After the storage period 0.1 ml portion of the reaction mixture was added to 9.7 ml ethanol (750 ml l^{-1}) and 0.1 ml of thiocyanate solution (30%). Three min later, the mixture was completed by the addition of a $FeSO_4$ solution (0.1 ml, 0.02 M in 3.5% HCl solution). The absorbance was read at 500 nm, and the antioxidant activity was expressed in percentage of the inhibition of the linoleic acid autooxidation compared to the control mixture.

1.10. Statistical analysis

The field experiment was designed as a randomised complete block with two factors (cultivar and fertiliser rate). The data were analysed by two-factor ANOVA and the means separated using the Fisher's least significant difference test.

2. Results and discussion

The chlorogenic acid concentrations of the tubers ranged from 12.9 to 14.5 mg 100 g^{-1} fresh weight in the cultivar Agria and from 13.3 to 17.9 mg 100 g^{-1} in Kennebec. The N fertiliser rate had no effect on chlorogenic acid content (Table 1). Chlorogenic acid affects the taste and flavour of potatoes (MARTENS & BAARDSETH, 1987), and is involved in the defence against insects and phytopathogens (SINDEN et al., 1988) and has strong antioxidant activity (PRATT, 1993). Chlorogenic acid has superoxide radical scavenging activity in a concentration-dependent manner (TSUCHIYA et al., 1996). This radical scavenging ability is stronger than that of β-carotene and synthetic butylhydroxyanisol but weaker than that of vitamin E. The concentration of total polyphenols in the potato tubers varied between 14.0–16.6 mg 100 g^{-1} f.w. in the cultivar Agria and 14.0–18.5 mg 100 g^{-1} f.w. in Kennebec and was not affected by the N rate (Table 1). The levels of chlorogenic acid made up 88.9±10.3% of the total polyphenols in potato tubers, which are consistent with the values reported by DAO and FRIEDMAN (1992) and MONDY and GOSSELIN (1988).

The levels of chlorogenic acid and total phenolics reported here are similar to those previously published by AL-SAIKHAN and co-workers (1995), by DAO and FRIEDMAN (1992, 1996) and by KARIM and co-workers (1997), but lower than the results presented by THOMAS and JOSHI (1977). Differences may be attributed to genotypes and environmental conditions in which the potatoes were grown. AL-SAIKHAN and co-workers (1995) showed that the accumulation of phenolics is genotype-dependent. The levels of chlorogenic acid in seven potato varieties ranged from 9.7 to 18.7 mg 100 g^{-1} fresh weight (DAO & FRIEDMAN, 1992).

Table 1

Concentration of chlorogenic acid and total phenols in tubers of potatoes grown with several nitrogen application rates

N rate (kg ha^{-1})	Chlorogenic acid (mg 100 g^{-1} f.w.)		Total polyphenols (mg 100 g^{-1} f.w.)	
	Agria	Kennebec	Agria	Kennebec
0	14.0±1.6a	13.5±1.3a	16.6±2.3a	17.0±2.2a
75	13.3±1.0a	14.6±1.0a	15.8±1.1a	16.5±2.1a
150	13.6±2.0a	13.3±1.2a	14.1±1.8a	14.9±1.6a
225	14.2±1.9a	17.5±1.7a	16.0±2.0a	19.5±2.2a
300	12.9±1.1a	17.9±0.6a	15.3±0.8a	18.5±0.3a
Average	13.6±0.6a	15.4±0.7a	15.5±0.7a	17.3±0.8a

Values are means ±SE of 4 replications. Mean separation by Fisher LSD test, 5% level. Mean separation by rows for the N levels and by columns for the averages over N levels

Different parts of the potato plant had much wider range of chlorogenic acid content than the tubers. The highest concentration was found in sprouts, followed by leaves, roots and finally the tubers, indicating that chlorogenic acid concentrations are also organ-dependent.

Ethanolic extracts of potato tubers had strong hydrogen donating ability in the presence of DPPH radical (Table 2). Hydrogen-donating ability is an index of the primary antioxidant activity of the tubers. Primary or chain-breaking antioxidants give hydrogen to free radicals, particularly the lipid hydroperoxide radicals which are the major propagator of the chain autoxidation of fats. This conversion leads to non-radical species, therefore inhibiting the propagation phase of lipid peroxidation. We found no correlation between hydrogen-donating activity and the concentration of chlorogenic acid and/or total polyphenols. H-donating activity was higher in the cultivar Kennebec.

All the samples exhibited strong reducing power (Table 2). Reducing power can be interpreted as an index of secondary antioxidant activity. Secondary or preventive antioxidants can reduce the rate of chain initiation in the lipid peroxidation process or can react with the products of lipid peroxidation. This conversion leads to more stable non-radical, non-deleterious products. A significant linear correlation was found between the reducing power and both the chlorogenic acid ($r=0.817$; $n=40$) and the polyphenol content ($r=0.859$; $n=40$) as shown in Figs 1/a and 1/b.

Table 2

Hydrogen-donating ability and reducing power of ethanolic extracts from tubers of potatoes grown with several nitrogen application rates

N rate (kg ha^{-1})	H-donating ability (% inhibition)		Reducing power (Abs 700 nm)	
	Agria	Kennebec	Agria	Kennebec
0	28.5±4.1a	36.5±4.5a	0.16±0.02a	0.25±0.06a
75	33.0±0.9a	39.0±8.2a	0.16±0.01a	0.23±0.03a
150	37.0±6.6a	35.8±2.4a	0.20±0.03a	0.20±0.02a
225	38.0±4.1a	45.5±4.0a	0.21±0.02a	0.26±0.04a
300	36.3±3.9a	53.3±3.1a	0.21±0.03a	0.24±0.01a
Average	34.6±1.9b	42.0±2.5a	0.19±0.01a	0.24±0.02a

Values are means ±SE of 4 replications. Mean separation by Fischer LSD test, 5% level. Mean separation by rows for the N levels and by columns for the averages over N levels

Although chelating agents are not antioxidants, they play a valuable role in stabilising fatty foods against rancidity. During the propagation phase of lipid peroxidation, decomposition of the lipid hydroperoxides catalysed by transition metal ions (Fe^{2+}, Cu^{2+}) results in an explosive increase of free radicals and consecutive chain reactions. The presence of chelating agents inhibits the metal catalysis. Controls, which contained no chelating agents, had an absorbance ratio at 485 vs 530 nm of 3.55±0.05.

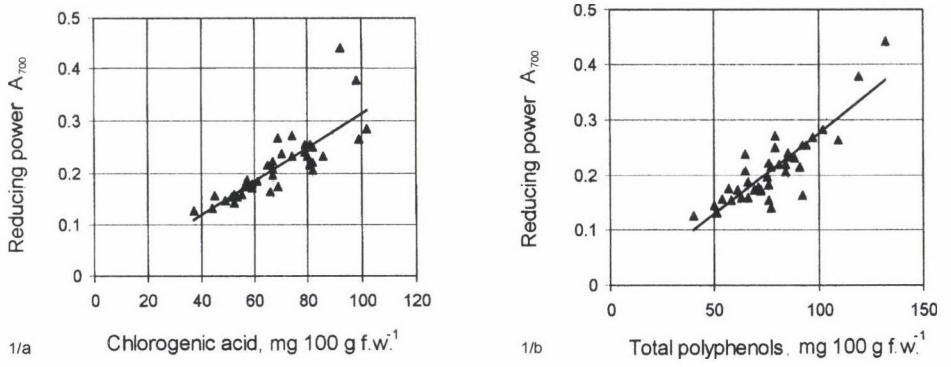

Fig. 1. Linear correlations between the chlorogenic acid content and reducing power (1/a) and total polyphenol content and reducing power (1/b) of potato tubers. 1/a: y = 0.0033x–0.0124, R = 0.817; 1/b: y = 0.0029x–0.0157, R = 0.859

Table 3

Copper(II)-chelating ability of the potato extracts

Sample	Chelating ability (Abs_{485}/Abs_{530})
Control (Cu^{2+})	3.55±0.05a
Agria	2.99±0.04b
Kennebec	3.00±0.03b

Values are the mean ±SE of 20 observation. Values followed by the same letter are identical of the 5% level as determined by the Fisher LSD test

This ratio decreased significantly with the addition of potato samples. The copper chelating ability of potato tubers from both cultivars was around 3.00 compared with 3.55 of the controls (Table 3), and was not affected by N fertiliser rates.

Hydrogen peroxide has a weak effect on the initiation of lipid peroxidation. However, its activity as active oxygen species comes from its potential to produce the highly reactive hydroxsyl radical through the Fenton reaction ($Fe^{2+}+H_2O_2 \rightarrow Fe^{3+}+OH^-+ \cdot OH$). The hydroxyl radical is an extremely reactive species formed in biological systems and reacts rapidly with most biomolecules such as sugars, amino acids, phospholipids, DNA, and organic acids (HALLIWELL & GUTTERIDGE, 1984). Hydroxyl radicals are capable of abstracting hydrogen atoms from membrane lipids thus bringing about lipid peroxidation (KITADA et al., 1979). Similar to the chelating ability, the scavenging activity of the potato extracts was not affected by N application. The ethanolic extracts from potato tubers did not reduce the chemiluminescence intensity of the $H_2O_2/ \cdot OH$-luminol-microperoxidase system under the experimental conditions, although pure chlorogenic acid (0.2 w/v % in methanol, 0.01 ml) induced a significant decrease on chemiluminescence (Table 4). Toxic free radicals can be generated from flavonoids and other polyphenols depending on their concentration under special circumstances (GYÖRGY et al., 1992, SINDEN et al., 1988). It is possible that such mechanism could modify the total scavenging characteristic of the potato extracts in our experimental system.

Ethanolic extracts of potato tubers inhibited the autoxidation of the linoleic acid measured by thiocyanate method (Table 5). Total antioxidant activity was significantly correlated with the level of chlorogenic acid ($r=0.782$; $n=40$) and the total polyphenols ($r=0.686$; $n=40$) as it can be seen in Figs 2/a and 2/b. The water-soluble glycoprotein patatin has been considered a major source of antioxidant activity. AL-SAIKHAN and co-workers (1995) found a 68.7% inhibition of the autoxidation of β-carotene/linoleic acid in potato extracts, a much higher activity than that of the other vegetables like onion,

carrot and bell pepper. In contrast with our results AL-SAIKHAN and co-workers found a poor correlation between antioxidant activity and total polyphenols. Other plant proteins have also been reported to have antioxidant activity (PRATT, 1972). Glutathione, quercetin and ascorbic acid are biologically active compounds found in potato tubers that have shown marked antioxidant activity in different experimental systems (JONES et al., 1992, PRATT & WATTS, 1964). Epidemiological data also confirm the beneficial health effect of quercetin and other polyphenols (KANDASWAMI & MIDDLETON, 1997).

Table 4

H_2O_2/·OH scavenging capacity of the extracts of potato measured by the luminol-dependent chemiluminescence method

Sample	Chemiluminescence intensity (RLU)
Standard light (H_2O_2/·OH-luminol)	13,496,605 ± 403,216a
Agria	11,309,700 ± 604,936a
Kennebec	9,059,518 ± 1,496,157a
Chlorogenic acid (0.01 ml, 0.2 w/v%)	91,681 ± 1,645b

Values are means ±SE of 20 observations per cultivar

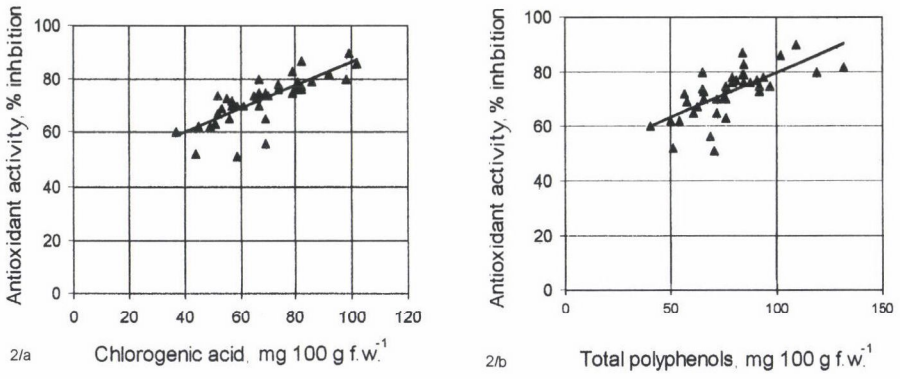

Fig. 2. Linear correlations between the chlorogenic acid content and the antioxidant activity (2/a) and the total polyphenol content and the antioxidant activity (2/b) of potato tubers. 2/a: $y = 0.4391x+42.356$, $R = 0.781$; 2/b: $y = 0.3292x+46.89$, $R = 0.688$

Table 5

Antioxidant activity (% inhibition) *of potato tubers grown with several N fertiliser rates*

N rate (kg ha^{-1})	Agria	Kennebec
0	71.8±5.7a	70.5±6.8a
75	65.5±4.0a	74.0±1.9a
150	70.8±7.7a	70.8±3.2a
225	76.5±4.6a	76.5±2.3a
300	72.3±4.9a	76.8±0.9a
Average	71.4±2.3a	73.7±1.6a

Values are means ±SE of 4 replications. Mean separation by Fisher LSD test, 5% level. Mean separation by rows for the N levels and by columns for the averages over N levels

3. Conclusion

Potato tubers have high concentration of phenolic compounds with chlorogenic acid accounting for up to 90% of the total. Nitrogen fertiliser rates had minimal or no effect on the concentration of chlorogenic acid, polyphenols, and also on hydrogen donating ability, reducing power, chelating property and antioxidant activity of potato tubers. Ethanolic extracts of the potato tubers proved to be good hydrogen donors and reducing agents. These properties make potatoes good primary and secondary antioxidants, possibly conferring a protective role against the damage caused by free radicals and active oxygen species. Phenolic compounds, mainly chlorogenic acid, are likely to be the major contributors for the reducing power and the antioxidant activity of the tubers. Potato extracts contain copper-binding substances and significantly inhibited the autoxidation of linoleic acid. These properties are probably relevant to the antioxidant activity of potatoes. The overall antioxidant effect of phenolics on lipid peroxidation might be attributed to its properties of scavenging free radicals and active oxygen species. Given the importance of potatoes in the diet they represent a major source of antioxidants. Development of in vitro system to characterise the antioxidant effect of natural plant products merits further investigations.

*

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S-L-G (SOLID-LIQUID-GAS) PHASE TRANSITION OF COCOA BUTTER IN SUPERCRITICAL CO_2

K. KOKOT[a], Ž. KNEZ[a,c] and D. BAUMAN[b]

[a] University of Maribor, Faculty of Chemistry and Chemical Engineering, Smetanova 17, SI–2000 Maribor. Slovenia

[b] PINUS, Chemical Works, Grajski trg 21, SI–2327 Rače. Slovenia

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Phase equilibrium data (solid-liquid-gas) for cocoa butter and carbon dioxide were determined in view of their importance in design of PGSS (particles from gas saturated solutions) micronization process. The mutual solubility in the system cocoa butter/CO_2 was measured by the static-analytic method in the temperature range of 30–80 °C and pressure range of 1–30 MPa. The experiments on solid-liquid (S–L) transition for cocoa butter in the presence of carbon dioxide were performed by the modified capillary method in a high pressure optical cell. For the production of finely dispersed cocoa butter by expanding CO_2-saturated solutions, the initial guess indicates that the starting conditions should be near the liquefaction curve in order to allow the solid-liquid region to be reached after expansion.

Keywords: Solid-liquid-gas phase transition; cocoa butter; supercritical carbon dioxide.

For the design of processes with supercritical fluids developed on industrial or research scale, solubility or phase equilibria data are very important. Recently, supercritical fluids have been applied as solvents for non extractive applications in high pressure micronisation processes. Many substances are extremely unstable under conventional conditions and the product is contaminated with residual solvent. At the same time waste solvent streams are also produced. Supercritical fluids can therefore be used as an excellent agent for micronisation process. The process for the production and fractionation of fine particles from gas saturated solutions (PGSS) consists of dissolving the substance which has to be produced and fractionated in a medium under pressure followed by the expansion of the obtained gas saturated solution and the separation of the obtained particles from gas (WEIDNER et al., 1997c). Through the choice of the appropriate combination of solvent and operating conditions suitable for a particular compound, PGSS can eliminate some of the disadvantages of traditional

[c] To whom the correspondence should be sent. Phone: +386/62/22 94 461; fax: +386/62/22 77 74, E-mail: ZELJKO.KNEZ@UNI-MB.SI

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methods of particle size redistribution in material processing. Solid formation by PGSS, therefore shows great potential for the production of crystalline and amorphous powders with narrow and controllable size distribution.

Cocoa butter (CB) is a vegetable fat, present in chocolate at levels up to 40%. Cocoa butter is a complex compound, containing different types of triglycerides. The characteristic physical properties of cocoa butter are related to the arragement of the fatty acids in the triglycerides: there is a high content of symmetrical monounsaturated triglycerides, which have the unsaturated fatty acid in position 2 and saturated fatty acids in the position 1- and 3, respectively. These triglycerides consist of 2-oleoyl-1-palmitoyl-3-stearoylglycerol (POS), 2-oleoyl-1,3-distearoylglycerol (SOS), and 2-oleoyl-1,3-dipalmitoylglycerol (POP), with POS being present in the largest amount. The melting behaviour of cocoa butter can be related to the melting points of these major triglycerides (POS, SOS, POP). Each of these triacylglycerols is polymorphic, they can solidify in various crystallographic forms. The process of solidification of cocoa butter is more complex than in the case of other fats. It can crystallize in five different polymorfic forms, each with its own melting point. The melting points of the major polymorfic forms (in order of increasing stability) are: γ (16–18 °C), α (21–24 °C), β_1 (27–29 °C), β (34–35 °C) and β_2 (36–37 °C). Phase β is always stable below its melting temperature, but its kinetics of nucleation and growth is very slow, so that under direct cooling a less stable phase is generally formed (ROUSSET et al., 1996). The hardness of cocoa butter and crystallinity has a major effect on organoleptic characteristics of chocolate.

Several authors (BHASKAR et al., 1996; LI et al., 1992; MAJEWSKI et al., 1994; SEBALD et al., 1996) have studied the solubility of cocoa butter in supercritical fluids (SCF's) because of its relevance to fractionation as well as extraction of cocoa butter from cocoa beans.

The possibility of cocoa butter micronization with supercritical carbon dioxide (SC–CO_2) has not been explored yet. No value on phase equilibrium solid-liquid for the binary system cocoa butter/CO_2 and for the equilibrium solubility data of CO_2 in the liquid phase of cocoa butter can be found in the literature.

Solid-liquid-gas phase transition: Solid-liquid-gas (S-L-G) equilibria are of great technical interest, because they form the basis for the design of several particle formation processes using dense fluids (MATSON et al., 1987; DEBENEDETTI, 1990; DIXON et al., 1993). At the moment four processes are known:

- Particles from gas saturated solutions (PGSS);
- Rapid expansion of supercritical solutions (RESS);
- Gas antisolvent recrystallization (GASR), and
- Crystallization from supercritical solutions (WEIDNER et al., 1997).

In all processes it is very important to understand the influence of pressure on the melting point of the substance in the presence of gas and phase equilibrium data of the system. The first type with a negative slope dP/dT is found for the system where the supercritical gas has a relatively high solubility in the molten heavy component (ARONS et al., 1963). In the second type, the three-phase curve shows a temperature minimum (TUMINELLO et al., 1995). The third type of solid-liquid-gas (S-L-G) lines is for systems, where the supercritical fluids are only slightly soluble in the molten heavy component, and therefore the increase in hydrostatic pressure raises the melting temperature (WEIDNER et al., 1997a). The mutual solubility in binary system of cocoa butter/CO_2 is influenced by pressure and temperature (KUMAR et al., 1988; CHRASTIL, 1982; KNEZ et al., 1992).

Solid-liquid-gas (S-L-G) line for the cocoa butter-CO_2 system in P-T projection belongs to the category of binary solid-SCF fluid systems with a temperature minimum (CHEONG et al., 1986).

1. Materials and methods

1.1. Materials

Pure prime pressed cocoa butter was obtained from LEX – Portorož, Slovenia (CAS number 8008-31-1). The melting point was 32.1 °C (confirmed by DSC method). Carbon dioxide (purity 99.97%) was kindly donated by Linde Plin Celje, Slovenia.

1.2. Methods

1.2.1. Determination of melting point under the pressure of CO_2. The values of melting points were determined by the modified capillary method. The method used to determine the melting point of a substance in the presence of gas under pressure is similar to one used at atmospheric pressure. A glass capillary tube is filled with the substance in question and inserted into a high pressure view cell along with the thermocouple. Fig. 1 gives a schematic drawing of the equipment for the determination of melting points under the pressure of supercritical fluids.

An optical cell with a volume of 14 ml was used (NWA GmbH, Lörrach, Germany); this cell can operate at pressures up to 50 MPa and temperatures up to 250 °C. The cell has two saphire windows which are fixed to the cell with screws. There are also three openings, one for entering the gas, one for emptying the gas, and one for inserting the thermocouple. The thermocouple was calibrated with some pure substances (o-nitrophenol, T_m=45.0 °C; azobenzol, T_m=68.0 °C; benzil, T_m=98.0 °C; acetanilid, T_m=115.0 °C) with known melting points. A standard curve was constructed and used to adjust the recorded experimental data. Pressurized gas was inserted via a

high-pressure pump. The pressure was measured by electronic pressure manometer (Digibar PE 200 Hottinger-Baldwin to ±0.1%), and the cell was electrically heated with a heating jacket to ±0.5 °C. The temperature was measured with an accuracy of ±0.1 °C with a thermocouple.

The gas was inserted into the system and the temperature was raised using a heating coil. The melting of the substance was observed by a video camera. The recordings of the temperature and pressure were made at the beginning and end of melting.

In screening experiments it was observed whether the melting-temperature range depended on the mode of operation (pressure/temperature increase: "upward strategy", or pressure/temperature decrease: "downward strategy"). No hysteresis was found within the experimental accuracy (±0.5 °K, ±0.05 MPa).

1.2.2. Determination of liquid-vapour phase transition under the pressure of carbon dioxide. For the determination of mutual solubility in the system cocoa butter/CO_2 in the temperature range 30–80 °C and pressure range 1–30 MPa, a static-analytic method was described in our previous work (KNEZ et al., 1995).

The 500 ml autoclave was loaded with 150–200 g of cocoa butter. The CO_2 from the tank was cooled to a liquid state and compressed into the equilibrium cell by a high-pressure pump. The temperature in the autoclave was regulated with a heating jacket (accurate to ±1.0 °C), and the pressure was measured by a Digibar manometer (Hotinger Baldwin Messtechnik, accuracy ±0.1%).

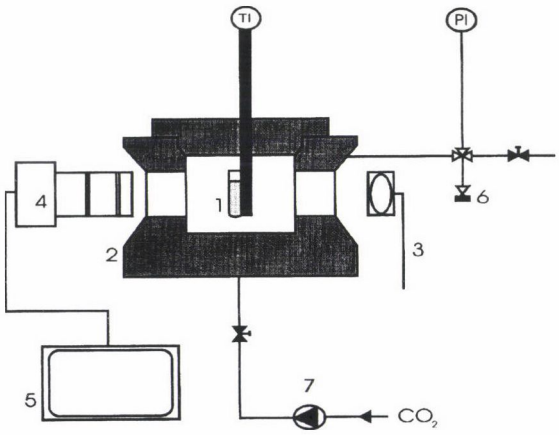

Fig. 1. Basic scheme of experimental equipment. 1: glass capillary with sample, 2: high pressure cell, 3: lamp, 4: video camera, 5: computer, 6: safety valve, 7: high pressure pump

Because of the spinodal and binodal effects (WEIDNER et al., 1997b), different values were obtained when the pressure in the system was increased or decreased. In all cases where "upward strategy" was applied, CO_2 had to diffuse into the unsaturated liquid. The binary mixture was mixed and equilibrium was established. After 1 h of phase separation, samples of the lower phase (rich in cocoa butter) and the upper phase (rich in CO_2) were taken. The amount of CO_2 released was measured by a flow meter (Elser-type, ϕ=±2%), and the mass of cocoa butter was measured gravimetrically (± 0.1 mg). The cocoa butter/CO_2 mass ratio was calculated for both samples.

2. Results

2.1. Solid-liquid phase transition under the pressure of CO_2

The P-T projection of the solid-liquid-gas (S-L-G) equilibrium lines has been measured for binary mixture of cocoa butter/CO_2. The three-phase curve for the binary system shows a temperature minimum. The experimental data for this binary system are presented in Table 1 and Fig. 2.

Table 1

Melting points of cocoa butter under the pressure of carbon dioxide

Mean pressure [MPa]	Mean temperature [°C]
31.23	24.7
24.08	24.6
20.50	24.6
17.88	24.8
16.00	24.6
14.85	24.7
13.70	24.2
11.15	23.4
9.28	22.4
7.28	22.3
6.05	22.1
5.98	22.4
3.95	23.1
3.30	27.1
2.55	30.2
1.65	30.9
0.80	32.2
0.70	32.4
0.10	32.1

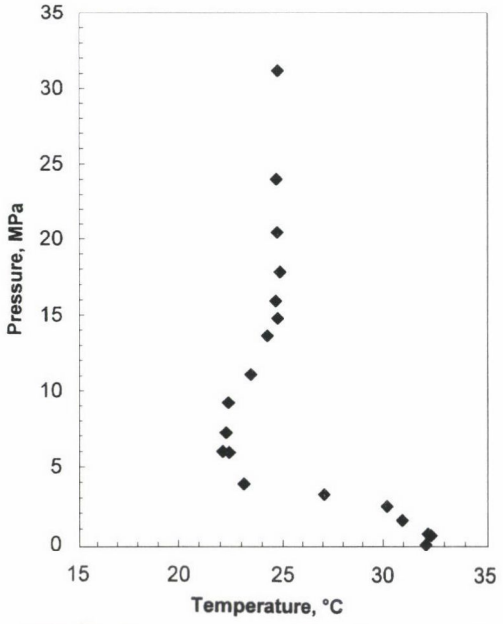

Fig. 2. P-T diagram for cocoa butter-carbon dioxide system

In Fig. 2 the mean value between the upper and lower melting temperatures is presented.

Each mean value was measured at least three times and the experimental accuracy was ±0.5 °K and ±0.05 MPa. The accuracy of the method was ±0.5%. Because the quantity of the sample was sufficiently small compared to the volume of the equilibrium cell, further experiments could have been done after obtaining equilibrium. The reliability and efficiency of the solubility measuring technique (especially in the lower range) have been established previously by measuring the solubility of β-carotene and oleic acid in CO_2 and by comparing obtained data with the literature data (ŠKERGET et al., 1995).

For cocoa butter the liquefaction temperature increased very little when the pressure rose to about 1 MPa as compared to the melting point at 0.1 MPa. In this pressure range (0.1–1 MPa) the solubility of CO_2 in CB was low, and therefore no increase in the liquefaction temperature, due to the solubilized gas in CB, was observed. At pressure higher than 1 MPa, the transition temperature of the CB investigated decreased. Based on the high solubility of CO_2 in the molten mixture, at pressures higher than 1 MPa, the transition temperature of the cocoa butter decreased. For cocoa butter, the decrease went up to 6.05 MPa (from 32.1 °C at 0.1 MPa CO_2, to 22.1 °C at 6.05 MPa).

Table 2

Liquid-vapour phase equilibrium data for the binary system cocoa butter/carbon dioxide
(w = mass fraction of carbon dioxide, ^{l} = liquid phase, ^{g} = gas phase)

T (°C)	P (MPa)	w^l (%)	w^g (%)	T (°C)	P (MPa)	w^l (%)	w^g (%)
30	1.50	2.92	98.89	40	0.85	2.89	99.23
	2.20	5.95	98.92		2.45	8.90	99.53
	3.30	11.86	95.53		2.80	10.51	98.18
	5.50	16.03	97.17		4.00	12.07	98.30
	6.65	28.02	97.21		5.00	14.45	99.39
	7.20	32.05	96.54		6.45	20.84	99.48
	8.20	36.95	95.44		7.35	25.48	99.61
	9.35	46.04	95.80		8.15	22.89	99.89
	10.95	41.32	99.12		10.00	33.27	99.20
	12.15	42.65	95.63		12.40	33.27	96.30
	12.70	45.84	97.51		12.95	36.07	99.5
	13.65	46.32	96.04		15.55	39.20	99.70
	14.70	44.47	98.71		16.45	44.30	99.20
	15.95	42.84	99.00		18.05	41.10	98.99
	17.10	42.52	98.22		19.00	44.61	99.50
	19.30	45.18	96.61		19.40	43.42	98.89
	19.95	44.09	98.35		22.75	45.41	99.60
	22.35	44.20	98.95		24.60	46.22	99.00
	23.14	45.11	99.48		26.50	47.76	99.75
	23.45	42.98	98.57				
	23.80	43.30	99.16				
	25.80	50.14	99.91				
	26.90	50.54	99.63				
60	0.90	0.98	95.84	80	1.00	1.60	94.00
	2.35	4.03	97.65		2.50	5.28	97.70
	4.00	10.50	98.04		3.90	8.44	96.00
	5.90	13.27	94.38		6.55	14.12	97.00
	8.10	19.76	99.74		8.30	21.33	99.26
	9.90	26.75	99.87		10.30	23.81	97.85
	11.65	34.48	99.95		12.55	33.48	97.96
	14.00	42.23	99.88		14.55	35.48	97.27
	14.30	45.31	99.83		15.90	37.61	98.82
	15.75	43.26	99.97		18.00	37.40	99.59
	16.50	41.38	99.89		20.00	37.26	99.79
	18.55	40.22	99.65		21.10	35.15	99.68
	21.40	39.45	99.26		22.30	36.39	96.72
	22.60	40.00	99.70		23.90	36.96	99.82
	24.40	40.5	99.00		26.15	38.02	99.02
	26.70	44.49	98.20		27.25	38.36	99.00
	28.80	43.60	97.90				

The melting point data allows us to determine solubility due to the colligative properties of solutions (ARONS et al., 1963). This explanation is based on the fact that when a supercritical gas is pressed on a melting heavy component, there are two opposite temperature effects: because of the increase of hydrostatic pressure the melting temperature will rise (in most cases), and because the gas will dissolve in the molten heavy component there will be a melting point depression. The deviations are greater in case of higher solubility of gas in the equilibrium liquid. When SCF diffuses into substances, the melting point changes. The molecules of supercritical fluid interfere with the crystal structure of the substance and reduce the energy needed to break the intramolecular bonds.

It was concluded that the pressure effect on the solid-liquid transition outweighs the solubility effect in the pressure range from 0.1 to 1 MPa. The depression of the liquefaction temperature with the observed minimum can also be explained by the competing effects of the shrinking molar volume of CO_2 and the increase in the melting point of cocoa butter due to higher pressure (Clausius-Clapeyron relationship). At lower pressure, the molar volume of CO_2 decreases more rapidly with increasing pressure, and therefore in the pressure range 1–6.05 MPa this effect is dominant. In the pressure range 6.05–14.85 MPa the liquefaction temperature slowly increases. At pressures greater than 14.85 MPa, the transition temperature is practically constant. In this pressure range, a further increase in pressure does not significantly enchance the solubility of CO_2 in cocoa butter (see Fig. 3).

2.2. Liquid-vapour phase transition under the pressure of carbon dioxide

The experimental liquid-vapour equilibrium line for cocoa butter/CO_2 has important implications in interpreting the solubility behaviour for this system. The results of the determination of the solubility of CO_2 in cocoa butter and cocoa butter in supercritical phase are presented in Table 2 and Fig. 3.

Each data point in Fig. 3 represents average of the least three measurements. The maximum deviation among the three measurements was 2.5%. This deviation of 2.5% gives a good indication of the expected accuracy of the results because the individual experimental errors that contribute to the overall error (i.e. in pressure, temperature, concentration and volume) are all significantly below this level. The 2.5% variation is due to random experimental errors associated with the difficulties of working with high-pressure supercritical fluids.

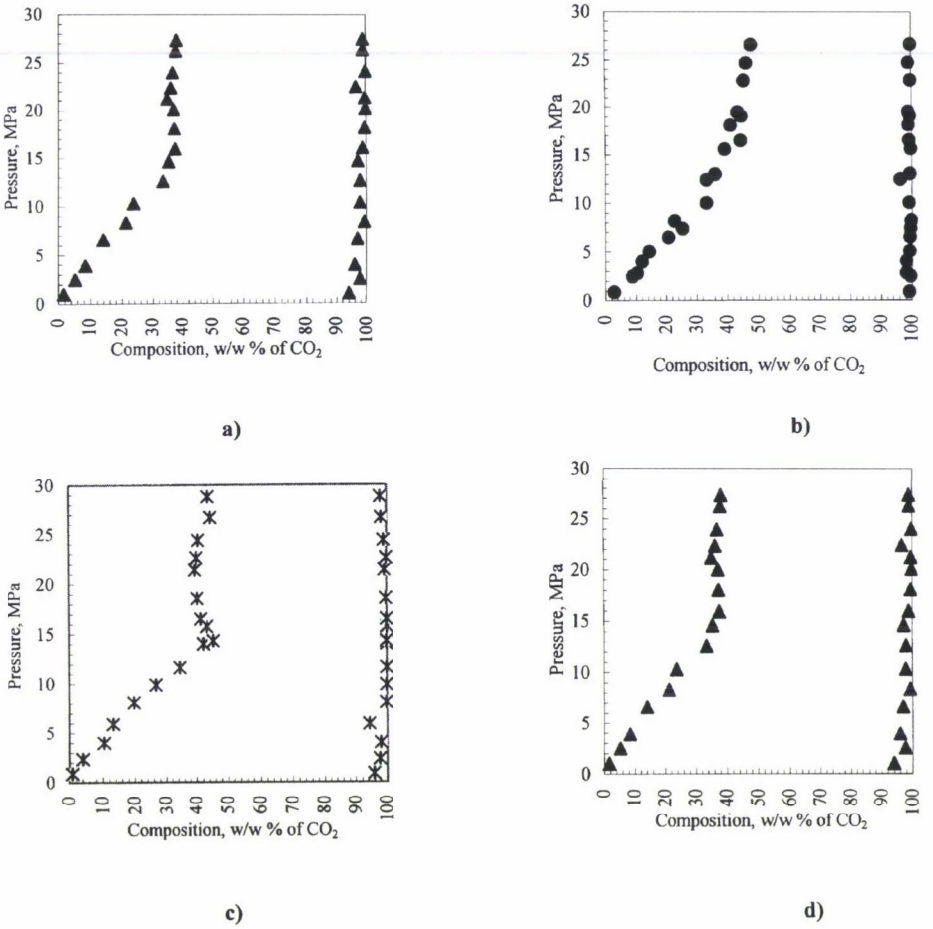

Fig. 3. Equilibrium solubility data for binary system Cocoa Butter/CO_2 at: a) T=30 ° (303.15 K); b) T=40 °C (313.15 K); c) T=60 °C (333.15 K) and d) T=80 °C (353.15 K)

It is shown that the solubility of fats and oils in SC-CO_2 is strongly correlated to pressure and temperature (YU et al., 1992). The experimental equilibrium solubility data were determined at the temperatures of 30, 40, 60 and 80 °C and in the pressure range from 1 to 30 MPa. Temperature and pressure effect on the solubility show the usual trends. The solubility of CO_2 in cocoa butter is high and the maximal equilibrium solubility is 50.5 w/w% of CO_2, at pressure 26.9 MPa and 30 °C. At constant pressure and with increasing temperature, the solubility of CO_2 decreases.

The equlilibrium solubility data are presented as a pressure-weight composition plot (Fig. 3). The solubility of CO_2 in cocoa butter increases with decreasing temperature, and increases with increasing pressure. The isotherms at 40, 60 and 80 °C in Fig. 3/b,c,d have practically the same equilibrium solubility values, especially at pressures lower than 15 MPa. Typical values for the concentration of cocoa butter in CO_2 are lower than 5%. This observation is also confirmed by the data for cocoa butter of LI and co-workers (1992) and BHASKAR and co-workers (1996).

2.3. Pressure-temperature diagram with isopleths

The phase equilibrium data were plotted in a P-T diagram. From the phase diagram, lines of constant liquid/vapour composition were determined and are shown in Fig. 4.

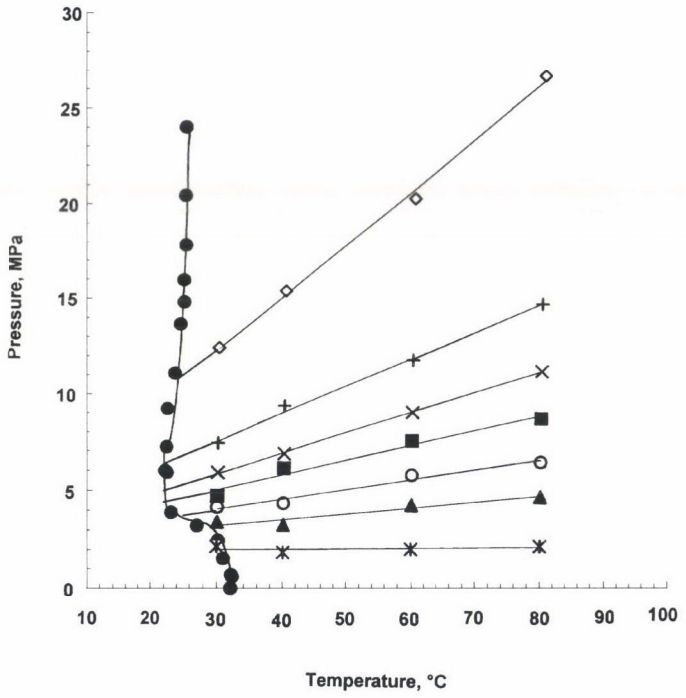

Fig. 4. P-T diagram with melting point and constant composition lines for cocoa butter. ●: Melting curve of cocoa butter under pressure of CO_2, ✳: w=5% (w/w), ▲: w=10% (w/w), ○: w=15% (w/w), ■: w=20% (w/w), ×: w=25% (w/w), +: w=30% (w/w), ◊: w=45% (w/w)

The lines of constant composition are linear functions of pressure and temperature. The slopes of constant composition lines depend on vapour pressure of a component in gas saturated solution. In a P-T projection, the vapour-liquid equilibrium is presented by the lines of constant compositions (isopleths), which are plotted as a function of the temperature.

Extrapolation of constant composition lines to the S-L line (liquefaction line, Fig. 4) shows the pressure and temperature at which a solution of cocoa butter-CO_2 with a certain composition can be obtained.

3. Conclusions

The experimental liquid-vapour equilibrium solubilities in the binary system cocoa butter/carbon dioxide were determined at temperatures 30, 40, 60 and 80 °C and at pressures ranging from 1 to 30 MPa. The effect of pressure on the mutual solubility follows the expected trends, with the solubility increasing with increasing pressure for all four temperatures studied.

Presented data are important for design of PGSS-micronisation process. From the obtained S-L-G phase transition data it can be concluded that the gas saturated solution is established at pressures and temperatures which are on the right of the liquefaction curve (S-L-G). On the left, the liquefaction curve of solid cocoa butter coexists with gaseous CO_2. The starting conditions have to be chosen so that the maximum loading of CB with CO_2 be obtained and that the final conditions after the expansion are in the S-V region of the binary system.

*

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Acta Alimentaria, Vol. 28 (2), pp. 209–212 (1999)

Book reviews

Lawrie's meat science

R. A. LAWRIE

Woodhead Publishing Ltd, Cambridge, Sixth edition, 1998, ISBN 1 85573 395 1, 336 pages

Ralston Lawrie, Emeritus Professor of Food Science at the University of Nottingham, is one of the world's prominent authorities in meat science. The first edition of this famous book, which remains a standard work for both students and professionals in the meat industry, was published in 1966, followed by Spanish (1967), German (1969), Japanese (1971) and Russian (1973) editions, confirming the success and popularity of this work. Its basic theme remains the outstanding importance of biochemistry in understanding the production, storage, processing and sensory attributes of meat, providing a clear guide from the growth and development of meat animals, through the conversion of muscle to meat, to the point of consumption.

The new, sixth edition also includes the recent advances in meat science of the past decade: e.g. the more thorough understanding of the structure of muscular tissue, the prediction of microbial spoilage patterns, the biochemical changes during postmortem ageing as well as the aberrations in DNA which lead to the development of the pale, soft and exudative (PSE) syndrome in meat, etc.

As in previous editions, the book does not include a systematic discussion of the many types of manufactured meat products; the text concentrates only on the scientific backgrounds which are common to all meat and meat products.

"Currently there is no reason to alter the long-established view that meat is an excellent source of nutrients and organoleptically enjoyable commodity for the vast majority of consumers; and one which cannot be faulted because meat is potentially hazardous for the few" – emphasizes Professor Lawrie in the Preface of his well systematized, perspicuous book.

A brief overview of contents is given as follows:

1. Introduction: Meat and muscle. The origin of meat animals: sheep, cattle and pigs.

2. Factors influencing the growth and development of meat animals: Genetic aspects. Nutritional aspects. Exogenous manipulation. Growth control.

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3. The structure and growth of muscle: The growth of normal muscle. Abnormal growth and development in muscle.

4. Chemical and biochemical constitution of muscle.

5. The conversion of muscle to meat: Pre-slaughter handling. Death of the animal. Conditioning (ageing).

6. The spoilage of meat by infecting organisms: Symptoms of spoilage. Factors affecting the growth of meat-spoilage microorganisms. Prophylaxis.

7. The storage and preservation of meat I.: Temperature control (Refrigeration. Storage below of freezing point. Thermal processing).

8. The storage and preservation of meat II.: Moisture control (Dehydration. Freeze dehydration. Curing).

9. The storage and preservation of meat III.: Direct microbial inhibition (Ionizing radiation. Antibiotics. Chemical preservatives).

10. The eating quality of meat: Colour. Water holding capacity and juiciness. Texture and tenderness. Odour and taste.

11. Meat and human nutrition: Essential nutrients. Toxins and residues. Meat eating and health.

12. Prefabricated meat: Manipulation of conventional meat. Nonmeat sources. Upgrading abattoir waste.

Some 2230 literary sources are listed in the Bibliography.

L. KÖRMENDY

Practical dehydration

M. GREENSMITH

Woodhead Publishing Ltd., Cambridge, Second edition, 1998 ISBN 1 85573 394 3, 274 pages

The volume treats the pieces of information on food drying in a richly illustrated form, according to practical aspects. This book has been one of the basic professional manuals of food industrials. Its special strength is the summary of international practice in food drying procedures. As there were radical changes occurring since the first edition on the domain of the manufacture and marketing trends for dried products, the book had to be completed with the most recent procedures of fruit- and vegetable drying, with the pieces of information on the economy of the various methods and on

quality requirements and test methods. A new chapter has been elaborated in connection with the new developments of spray drying.

Maurice Greensmith has been working for fifteen years in the domain of drying in Great Britain as well as in the whole world. Among others, he worked as managing director in Ireland at the Company Batchelor and was active as consulting expert at several multinational companies producing food machinery such as Guinness Peat Group of Companies and APV. He worked in Western- and Eastern-Europe, in South-America, in the Caribeans, in Africa, in Asia and in the Middle-East. His scope of duties comprised feasibility studies, monitoring of actual manufacturing technics and assistance to the management of a factory in the improvement of production.

In the last two decades, radical changes happened in the marketing possibilities of dried products. The domains of application of the goods were reduced. In consequence, the number of factories manufacturing dried products has been reduced greatly in the United Kingdom. As a contribution to this, operating costs increased, due to the oil-crisis in the Middle-East.

Naturally, market demands have changed, too. Dried vegetables lost their popularity in the households as well as in the catering. Actually, their importance is growing, as they are used in the production of value-added food products. Here we can mention snack foods, instant soups, gravies and sauces, ethnic specialities, and at last but not at least the foods required by health-conscious consumers. As an example, dried fruits are very popular used in breakfast cereals, and as components of müzli food products.

Thus, there is a hope that with the help of this book modern drying practice can gain a lot. The growing markets are controlled mainly by international companies and these multinationals are protecting the reputation of their brands. Those factories, who want to take part in supplying these clients, have to satisfy all requirements, quality prescriptions being tested in their own laboratories.

Thirteen chapters summarize the pieces of information on practical drying. At the end of the volume, an index helps to find the part searched for. First we are informed on the history, growth and potential of drying. We can see the goods imported to the United Kingdom of the year 1986, presented in tables according to goods and countries. Hungary can be found in the table on dried onions at a very noble place.

After the detailed presentations on factory organization (factory site, raw material-, working power demand, water- and power supply, heat production, fuels, effluents, etc.), the phases of raw material processing are reviewed. After the machines and procedures of raw material preparation, drying equipment is detailed: ovens and cabinet driers, tunnel driers, batch driers with through-flow, conveyor driers with single and multiple pass, fluidized driers, rotary driers, vacuum driers, freeze-driers, drum driers, foam-mat driers, spray driers, all these working in the dairy and food industries.

Chapters are dealing with potato products, various vegetables and fruits. A special chapter deals with spray drying, meat drying and the formulation of dried soups. Finally, the selecting and separating machines and instruments are detailed (filters, electronic colour sorting, packaging, bulk packaging). Quality control (laboratory tests, specifications, analytical methods) and the factors of economic operation are dealt with.

In general, food processing industry is highly competitive on the world market and here, drying is playing a very significant role. The successful operation is influenced by numerous factors, thus by the climate, the continuous work, the length of the seasons, the domain of processed goods, the distribution of manual and mechanical work, the quantity of wastes and by-products and their use, and the size of costs (raw materials, additives, fuels, wages, operating costs, maintenance, means for the operation, water, electricity, packaging-, commercial-, and transport costs).

J. BARTA

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RECENTLY ACCEPTED PAPERS

Chemical composition and evaluation of protein quality by amino acid score method of edible brown marine algae Arame (*Eisenia bicyclis*) and Hijiki (*Hijikia fusiforme*)
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Combined microwave – convective drying of *Saccharomyces cerevisiae* based yeast
BERECZ, L., NEMÉNYI, M. & PUIGGALI, J. R.

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CHEMICAL COMPOSITION AND EVALUATION OF PROTEIN QUALITY BY AMINO ACID SCORE METHOD OF EDIBLE BROWN MARINE ALGAE ARAME (*EISENIA BICYCLIS*) AND HIJIKI (*HIJIKIA FUSIFORME*)

N. KOLB[a,c], L. VALLORANI[b] and V. STOCCHI[b]

[a] Faculty of Food Technology and Biotechnology, University of Zagreb, Pierottijeva 6, 10 000 Zagreb. Croatia

[b] Instituto di Chimica Biologica "Giorgio Fornaini" Università degli studi di Urbino, Via Saffi, 2, 61 029 Urbino. Italia

(Received: 6 April 1998; accepted: 18 January 1999)

Crude proteins and their amino acid composition, fats, carbohydrates, cellulose, ashes, nucleic acids and minerals were determined in two edible and commercially available brown marine algae (*Phaeophyceae*), Arame (*Eisenia bicyclis*) and Hijiki (*Hijikia fusiforme*). The essential amino acid ratios for five key essential amino acids as well as the amino acid score based on the first limiting amino acid, Lys-Met-Cys score, Lys-Met-Cys-Trp score and Lys-Met-Cys-Trp-Thr score were calculated. The results have shown:

- rather high contents of proteins, containing all essential amino acids
- high amino acid ratios which are nearly as high as the value suggested by FAO/WHO/UNU pattern or higher
- the first limiting amino acid in both analysed algae is tryptophane
- very low contents of fats and nucleic acids
- high contents of cellulose and other carbohydrates
- large quantities of minerals and very low amounts of heavy metals.

Keywoods: Arame, Hijiki, chemical composition, protein quality, amino acid scoring

Eisenia bicyclis and *Hijikia fusiforme*, commercially named Arame and Hijiki, respectively, are marine macroalgae classified as brown algae or *Phaeophyceae*. In Japan and the Far East these marine algae are regulary utilised in human alimentation since ancient times, while in European countries their use is only occasional (LAHAYE, 1991). With the spreading of the so called alternative diet like macrobiotic way of nutrition, the edible marine algae although in small quantities are consumed daily.

[c] Author to whom correspondence should be addressed.

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Generally the marine algae are a good source of carbohydrates (FLEURY & LAHAYE, 1991), proteins, vitamines and minerals (FUJIWARA-ARASAKI et al., 1984). The algal carbohydrates: carrageenan, agar, alginate, cellulose, laminarans, fucans are undigestible by man and therefore considered dietary fibres (FLEURY & LAHAYE, 1991). Furtheremore, due to their polysaccharidic structure, brown algae are particularly suitable for binding metallic ions, so lead and cadmium can be easily removed from dilute solutions by algal biomass (VOLESKY & HOLAN, 1995).

The algal proteins contain all essential amino acids, and the vitamine B-complex, β-carotene and vitamin C are determined.

The contents of iodine, calcium and iron are claimed to be greater than in vegetables and fruits used in traditional western diet (FUJIWARA-ARASAKI, 1984).

Marine algae are the source of safe and effective natural antioxidants (CAHYANA et al., 1992). As for some medical applications, the marine algae can develop antibiotic activity against multi-antibiotic resistant bacteria (MAHASNEH et al., 1995), and play a protective role against carcinogens (OKAI et. al., 1997).

The objective of this work was to evaluate Arame and Hijiki as natural and useful food supplements in our western staple diet.

1. Materials and methods

Arame (*Eisenia bicyclis*) and Hijiki (*Hijikia fusiformis*) imported from Japan, were bought dry in a local health store. The samples were pulverised and the chemical composition (crude proteins (N × 6.5), fat, cellulose, ash and iodine) was determined according to A.O.A.C. method (1990). Minerals were determined by atomic absorption spectrophotometer Perkin Elmer 3030 B according to A.O.A.C. (1990). Phosphorous was determined by spectrophotometric method according to A.O.A.C. method (1990). Nucleic acids were determined by spectrophotometric method (RUT, 1973).

1.1 Amino acids analyses

1.1.1. Chemicals. DABS–Cl was purchased from Fluka (Buchs, Switzerland). A standard calibration mixture of amino acids of the highest grade and 6 mol l^{-1} hydrochloric acid (constant boiling, sequanal grade) were obtained from Pierce (Pierce Chemical Co., Rockford, IL). Acetonitrile, methanol, acetone and ethanol of HPLC grade and NaOH were obtained from Carlo Erba (Farmitalia, Italy). Analytical reagent grade potassium dihydrogen phosphate was purchased from Merck (Darmstadt, Germany). Triple-distilled water was prepared in the laboratory and used for the preparation of buffers. The buffer A used for the HPLC analyses was filtered through a 0.22 μm Millipore filter (Millipore Corp., Milford, MA).

1.1.2. Preparation of DABS-derivatives. DABS–Cl solution (4 nmol μl^{-1} acetonitrile) was prepared according to the method of CHANG and co-workers (1983) with slight modifications (STOCCHI et al., 1985; STOCCHI et al., 1989). Dried samples were resuspended in 50 µl of 0.2 mol l^{-1} sodium hydrogen cabonate, pH 9.0 and treated with 100 µl of DABS–Cl solution (4 nmol μl^{-1} acetonitrile). The mixture was left at 70 °C for 10 min and then dried under vacuum. The residue was redissolved in a proper volume of 70% (v/v) ethanol, and 20 µl of this volume was used for amino acids determination.

1.1.3. Liquid phase hydrolysis with HCl (c=6 mol l^{-1}). The pyrex hydrolysis tubes (10 cm×7 mm i.d.) for high sensitivity amino acids analysis were freed from amino acid contaminations by heating at 600 °C overnight. Hydrolysis was carried out as follows: 10 mg of each dried and powdered sample was placed into a pyrex tube, treated with 1 ml of 6 mol l^{-1} hydrochloric acid (constant boiling, sequanal grade), sealed under vacuum and left at 110 °C for 22 h.

Twenty µl of the hydrolysed sample were pipetted into a test tube, dried under vacuum and derivatized (STOCCHI et al., 1989).

1.1.4. Alkaline hydrolysis with NaOH. Alkaline hydrolysis, using 4.2 mol l^{-1} NaOH, was performed to check the recovery of tryptophane in protein samples with high sugar concentration. The hydrolysis was performed according to the method of HUGLI and MOORE (1972).

1.1.5. HPLC analysis. A Gold liquid chromatographic system from Beckman (Beckman, Berkeley, CA) was used for amino acid analyses. The HPLC apparatus consisted of two Model 126 pumps, a PC-8300 solvent programmer, a Model 210 sample injection valve, a 20 µl injection loop, and a Model 166 variable wavelength UV-visible range detector, equipped with a 12 µl flow cell. Integration of peak areas was obtained by means of the Gold software. Separation of DABS-amino acids was performed using a 3 µm Supelcosil LC-18 T column (15 cm×4.6 mm i.d.) protected with a 5 µm Supelcosil LC-18 T guard column (2 cm×4.6 mm i.d.) obtained from Supelco (Supelco, Bellefonte, PA). Solvent A was 25 mM potassium dihydrogen phosphate buffer, pH 7.05 and solvent B was acetonitrile and methanol (70:30). The gradient was 1 min at 20% of solvent B, 5 min up to 25% of solvent B, 14 min at 25% of solvent B, 1 min up to 32% of solvent B, 6 min at 32% of solvent B, 7 min up to 40% of solvent B, 9 min up to 60% of solvent B, 1 min up to 75% of solvent B, 5 min at 75% of solvent B.

The gradient was then returned to 20% of solvent B. The flow rate was 1.5 ml min^{-1} and detection was performed at 436 nm.

2. Results and discussion

Results are shown in Tables 1–5.

The protein contents in analysed algae are 11.1 g/100 g d.w. for Arame and 7.6 g/100 g d.w. for Hijiki (Table 1). The amino acid analysis revealed the presence of all amino acids including all essential amino acids (Table 2). The amount ofamino acids in Arame and Hijiki was found to be 9.2% of dry weight and 6.7% of dry weight, respectively. It is interesting that in both analysed algae valine is present in the highest amount i.e. 14,0% of total amino acid content, followed by glutamic acid with 13.0% in Arame and 11.0% in Hijiki (Table 2). The presence of essential amino acids is 49.3% in Arame and 45.2% in Hijiki of the total amino acid content. Tryptophane, methionine, cystine, lysine and threonine are five key essential amino acids which are likely to be deficient in mixed human diet (SARWAR & MC DONOUGH, 1990). In both analysed algae tryptophane was found in the lowest amount (Tables 2, 3) and represents the first limiting amino acid with amino acid scores of 27 for Arame and 18 for Hijiki (Table 4). In Hijiki cystine was not determined, but the presence of a fairly high content of methionine with an amino acid ratio of 21 is about as high as the contents of sulfur containing amino acids (methionine + cystine) in FAO/WHO/UNU reference protein (FAO/WHO/UNU, 1985) (Table 3). In Arame and Hijiki the amino acid ratio of lysine is about 20% lower compared with the reference protein, while threonine in both analysed algae exceeds the recommended value (Table 3). In macrobiotic diet Arame and Hijiki are consumed with rice (LEDVINKA, 1986). As the first limiting amino acid in rice is lysine followed by threonine (HOSENEY, 1994), the amino acid content of Arame and Hijiki could be considered supplementary. Rather high values of amino acid scores Lys-Met-Cys, Lys-Met-Cys-Trp and Lys-Met-Cys-Trp-Thr (Table 4) would indicate a good protein quality. However, these data should be corrected for the protein digestibility in vivo (SARWAR et al., 1989).

The fat content (Table 1) in analysed algae are very low (0.90 g/100 g d.w. in Arame and 0.70 g/100 g d.w. in Hijiki). According to the literature the algae are richer in unsaturated fatty acids than the land plants. In *Phaeophyceae* the unsaturated fatty acids are mainly oleic and arachidonic acids (HARWOOD et al., 1988).

Table 1

Chemical composition of marine algae Arame (Eisenia bicyclis) *and Hijiki* (Hijikia fusiforme) *in g/100 g d.w.*

Chemical composition	Arame		Hijiki	
	$\bar{x}$	±s	$\bar{x}$	±s
Proteins	11.10	0.75	7.50	0.20
Fats	0.90	0.05	0.70	0.02
Cellulose	21.50	1.20	25.50	1.40
Ash	12.00	0.80	17.10	0.90
Carbohydrates	54.50	3.60	49.20	2.50
Nucleic acids	0.61	0.08	0.54	0.07

$\bar{x}$: means of 3 determinations

±s: standard deviation of 3 determinations

Table 2

Amino-acid composition of marine algae Arame (Eisenia bicyclis) *and Hijiki* (Hijikia fusiforme) *in mg/g d. w.*

Amino acid	Arame		Hijiki	
	$\bar{x}$	±s	$\bar{x}$	±s
Aspartic acid	7.18	0.36	6.86	0.34
Glutamic acid	11.95	0.52	7.48	0.30
Serine	3.51	0.25	2.63	0.26
•Threonine	6.24	0.59	3.34	0.31
Glycine	5.26	0.25	4.20	0.20
Alanine	8.88	0.42	4.96	0.23
Arginine	2.23	0.15	1.75	0.08
Proline	3.37	0.34	2.90	0.28
•Valine	12.86	1.15	9.47	0.85
•Methionine	1.44	0.13	1.54	0.14
•Isoleucine	4.35	0.37	4.48	0.40
•Leucine	6.99	0.34	5.99	0.26
•Phenylalanine	4.40	0.20	3.95	0.19
•Lysine	5.02	0.25	3.61	0.18
•Histidine	3.57	0.18	1.92	0.09
Tyrosine	2.33	0.12	1.99	0.09
Cystine	1.84	0.07	N.D.	N.D.
•Tryptophane	0.35	0.028	0.17	0.015
Total	91.76	5.718	67.24	4.215

•: Essential amino-acids

$\bar{x}$: means of 3 determinations

±s: standard deviation of 3 determinations

Table 3

The essential amino-acid ratios of Arame (Eisenia bicyclis) *and Hijiki* (Hijikia fusiforme) *compared with FAO/WHO/UNU suggested pattern of amino acid requirements in mg/g of protein*

Amino acid	Arame	Hijiki	FAO/WHO/UNU (1985) reference protein
HIS	32	25	19
ILE	39	60	28
LEU	63	80	66
LYS	45	48	58
MET+CYS	29	21	25
PHE+TYR	61	80	63
THR	56	45	34
TRP	3	2	11
VAL	116	126	35
Total	444	487	339
MET	13	21	
CYS	16	N.D.	
TYR	21	27	
PHE	40	53	

The amount of carbohydrates is high: 54.5 g/100 g d.w. for Arame and 49.8 g/100 g d.w. for Hijiki (Table 1). In brown algae carbohydrates represent the dietary fibres and consist of laminarans, alginates, fucans (LAHAYE, 1991). As the consumption of low-fibre diets is considered a common etiological factor in "civilisation" diseases like digestive problems, metabolic and cardiovascular diseases (TROWELL, 1976), the consumption of algae with other food rich in dietary fibres is obviously an important factor in self-protection of health and improvement of life. Furthermore, in *Phaeophyceae* the major polysaccharidic component is alginate which is known to have a particular affinity for binding bivalent heavy metals, and form slightly soluble salts, which are excreted by faeces (CONCON, 1988).

The contents of nucleic acids (Table 1) are low. If consumed by man the nucleic acids are metabolised to uric acid, which although mainly excreted by kidney can enter blood circulation. Too high amounts of uric acid in blood circulation could increase the risk to some diseases like gout, arthritis and kidney disorders (LEHNINGER, 1982), so the daily intake from unconventional sources like marine algae should not exceed 2.0 g of nucleic acids in addition to the usual diet (EDOZIEN, et al., 1970). It means that the intake of about 300 g d.w. of Arame and 440 g d.w. of Hijiki should be permitted.

Table 4

Amino acid scores (%) for Arame (Eisenia bicyclis) *and Hijiki* (Hijikia fusiforme)

Algae	Amino acid score	Lys-Met-Cys score	Lys-Met-Cys Trp score	Lys-Met-Cys Trp-Thr score
Arame	27	96	73	96
Hijijki	18	83	62	79

According to the results in (Table 5) it can be seen that Arame and Hijiki have high contents of calcium, magnesium and phosphorus. The recommended daily dose for adults up to 25 years old of calcium is 800 mg, of magnesium 350 mg and of phosphorous 800 mg (WHITNEY & ROLFES, 1996). The presence of high calcium contents can represent a very good supplement in a diet which is often deficient in calcium for insufficient consumption of milk.

Table 5

Mineral composition of marine algae Arame (Eisenia bicyclis) *and Hijiki* (Hijikia fusiforme) *in mg/100 g d.w.*

Minerals	Arame		Hijiki	
	$\bar{x}$	±s	$\bar{x}$	±s
Fe	2.69	0.06	4.35	0.09
Zn	1.32	0.04	0.891	0.021
Ca	946	20	1156	29
P	490	15	625	10
Mg	410	12	634	19
I	245	7	30	3
Cu	0.989	0.025	0.946	0.020
Mn	0.242	0.015	0.628	0.040
Cd	0.027	0.006	0.018	0.005
Ni	0.058	0.01	0.035	0.007
Cr	<0.005		<0.005	
Pb	0.046	0.012	0.095	0.038
Hg	0.025	0.008	0.027	0.005

$\bar{x}$: Means of 3 determinations
±s: Standard deviation of 3 determinations

Arame and Hijiki contain 245 and 30 mg/100 g d.w. of iodine, respectively (Table 5). As the essential trace element iodine is an integral part of two hormones released by the thyroid gland, and its recommended daily dose for adults is 150 μg (0.150 mg) (WHITNEY & ROLFES, 1996). The daily requirement of iodine would therefore be satisfied with 61 mg d.w. of Arame and 500 mg d.w. of Hijiki. The deficiency of iodine as well as its excessive accumulation in the tissue can cause serious health impairments. The toxic dose of iodine for adults is over 2000 μg (2 mg) per day (WHITNEY & ROLFES, 1996), meaning that the intake of 816 mg d.w. of Arame and 6.67 g of Hijiki per day would be allowed.

The same daily intake of Arame and Hijiki would supply 8 and 77 mg of calcium, 4 and 42 mg of phosphorous, respectively while the contribution of magnesium would be 3 and 42 mg, respectively. The allowed daily amount of Hijiki would therefore supply as much as 10% of daily dose in calcium, 5% in phosphorous and 12% in magnesium.

Three heavy metals mercury, lead and cadmium are the most toxic for the man (WHITNEY & ROLFES, 1996). The determined quantities of mercury (Table 5) is much inferior to the tolerable weekly intake, of 0.3 mg total mercury per person (CONCON, 1988) i.e. cca 0.04 mg daily, established by FAO/WHO Expert Committee in 1972. Allowed daily cadmium intake in humans established by FAO/WHO Expert Committee is 50–150 μg (0.05–0.15 mg), meaning that daily intake of 200 g d.w. of analysed algae hardly reaches the critical quantity of cadmium. In 1971 WHO suggested a limit for lead of 100 μg l^{-1} of drinking water i.e. 250 μg daily, if it is assumed that 2.5 l of water is consumed daily (CONCON, 1988). In Hijiki where the quantity of lead is found to be twice as high as in Arame, the daily intake of cca 300 g d.w. would be allowed.

3. Conclusions

– The proteins of analysed algae contain all essential amino acids with high values of amino acid ratios except for tryptophane, which represents the first limiting amino acid.

– The high contents of undigestible carbohydrates contribute to the total amount of dietary fibres consumed daily, representing an important factor of self-protection.

– The analysed algae, especially Arame, are the source of iodine, the quantity of which should limit algal daily intake.

– In combination with other food the analysed algae contribute to satisfy the recommended doses in calcium, magnesium and phosphorous.

– Considering the low amount of nucleic acids and heavy metals, the analysed algae consumed as food supplement can improve the nutritive value of our staple diet.

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COMBINED MICROWAVE–CONVECTIVE DRYING OF *SACCHAROMYCES CEREVISIAE* BASED YEAST

L. BERECZ[a], M. NEMÉNYI[b] and J. R. PUIGGALI[c]

[a] Process Engineering of Agricultural Products, Research Group of the Hungarian Academy of Sciences, Mosonmagyaróvár, H–9200, Vár 2. Hungary

[b] Pannon Agricultural University, Institute of Agricultural Engineering, Mosonmagyaróvár, H–9200, Vár 2. Hungary

[c] ENSGTI – Université de Pau et des Pays de l'Adour, Rue Jules Ferry F-6400 Pau. France

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Attention is paid to drying as a downstream processing of foodstuff as it is used finally for human consumption. In some cases the conventional (convective, contact or infrared) drying processes can damage the quality of food due to crusting phenomena, overheating or protein denaturation. Better results can be obtained using combined microwave–convective dehydration.

The paper mainly focuses on the investigation of drying *Saccharomyces cerevisiae* pulp using the method of dielectric dehydration. A dried product with a residual fermentative activity of over 80% was produced with the application of a microwave–convective drying system. As a result the moisture content was lower than 10% calculated on dry basis. We permanently regulated the incident microwave power manually in order to eliminate the mentioned disadvantageous effects. This type of regulation was applied when the surface temperature reached 45 °C.

Keywords: microwave–convective drying, *Saccharomyces cerevisiae*

The rise of temperature of a dielectric material being exposed to an electromagnetic field can be explained by the conversion of electromagnetic energy into heat. This new heating method was discovered about 40 years ago, although the magnetron, which is used to make it, was already used in radar equipment during World War II.

The microwave radiation ranges from 300 MHz frequency up to 300 GHz, which corresponds to wavelength 1 mm–1 m. From this range 2.45 GHz frequency was selected for the household microwave ovens.

Microwave heating and drying differ from the conventional heating methods essentially. The latter methods are based on the internal friction between weakly bound electrons and molecules. Microwave energy transmission in wet solids can be explained by polarisation of randomly oriented water dipoles. In the 2.45 GHz alternating field the dipoles are forced to change their direction 4.9×10^9 times per second. A part of the

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input energy is converted into heat and results in a very rapid heating. ZAGROUBA and co-workers (1993) found that electromagnetic energy at a frequency of 2.45 GHz can penetrate an object with a thickness of 100 mm uniformly.

The power dissipated and converted into heat in a unit volume of the material can be expressed by:

$$P_v = 2\pi f E^2 \varepsilon_0 \varepsilon'' \quad (1)$$

were f: frequency, E: electric field strength, ε_0: permittivity of free space, ε'': dielectric loss factor. The higher the frequency and the electric field strength the higher the amount of energy converted into heat in the material. The dielectric loss factor is a material parameter, which indicates the amount of input microwave loss throughout the volume of the material. The values of dielectric loss factor of water was compiled by ZAGROUBA and co-workers (1993) at frequency 2.45 GHz in function of the temperature (Table 1). It obviously depends on the temperature.

One of the most important applications of the microwave technology is drying. In convective drying the heat gradient is of the opposite direction compared to the mass gradient. Consequently, if the material is dried too rapidly, an impermeable outer crust can form and will not permit moisture diffusion. Moreover, this crust can result in overheating of the surface. Since the direction of the heat gradient is the same as the mass gradient during microwave drying, the mentioned undesirable phenomena can be avoided, therefore the microwave technology is ideal for drying thermosensitive materials. Thermosensitive materials can be favourably dried by combining convective and microwave methods. In this case the moisture migration inside the material can be influenced by the microwave power mainly, and the evaporated water is carried out by the air ventilation at a moderate temperature.

ZAGROUBA and co-workers (1993) investigated the behaviour of a gelatine film and a polyacrylamid sphere during microwave-convective drying. Analysing the obtained drying characteristics, they found the presence of an over pressure drying period owing to the microwave energy, which can seriously damage the product by bursting it.

Table 1

Dielectric loss factor of water at different temperatures

Temperature (°C)	0	20	40	60	80	100
Dielectric loss factor	20	12	7	4.5	2.7	2

AYAPPA and co-workers (1991) determined the conditions of the approximate applicability of Lambert's law which was conventionally used to model the microwave heating of finite slabs. The correct power dissipation was computed from Maxwell's equations. They determined the critical slab thickness, above which the Lambert's law is valid. They compiled a wide range of dielectric properties of foods.

BODOR and co-workers (1993) carried out experiments applying microwave treatment to concentrate L-ascorbic acid solutions. They stimulated the crystallisation process using infrared and microwave energy. With special regard to the thermodegradable material, they did not heat it over 50 °C.

TURNER and co-workers (1998) built up a mathematical model for convective and microwave enhanced convective drying of pine wood. Drying periods, rate, moisture profiles and temperature profiles, effective diffusivity were modelled and analysed. They found that the use of combined microwave and convective drying can reduce time and increase drying rate as compared to the convective equivalent. The reduction of drying time depends on the level of incident microwave power.

Combined microwave–convective investigations were carried out on baker's yeast suspension by SZALAY and co-workers (1993). They found that the critical time and critical moisture content were reduced compared to the convective method. They emphasized the importance of the accurate temperature control of the material during the process.

BERECZ and NEMÉNYI (1996) investigated the internal temperature profile of baker's yeast during infrared drying. They determined the critical moisture content of the material, at which crust formation started, by means of temperature profiles and weight changes.

1. Materials and methods

Commercial compressed *Saccharomyces cerevisiae* based yeast was used in the experiments as model material. Its moisture content varied from 200% to 300% on dry basis. The purpose of the work was to produce dried yeast with

- maximum 10% moisture content according to GINZBURG (1979)
- at least 80% of its original fermentative activity retained (BÉNDEK, 1987).

In the case of yeast drying it is essential to preserve the fermentative activity, which refers to its biological value and characterizes the drying method used. To determine a value for the fermentative activity, the dried yeast was dissolved in a 10% (w/w) glucose solution and poured into a test tube. After closing the tube with a plug pierced with a capillary tube, the test tube was vertically turned by 180°. Then it was put into a water bath of 20 °C to assure the necessary temperature for fermentation. Then the fermentation, namely the evolution of carbon dioxide started. The solution was

forced by carbon dioxide to drop out of the tube and its weight was registered and plotted at intervals by means of a PC. The slope of this line represented the rate of evolution of CO_2 (Fig. 1). There were three typical periods: first the "lag" period of microbes, second the exponential growing period and third the falling-rate period of growing when the decrease of substrate molecules began to limit fermentation. The fermentation capacity was characterized by the maximal rate of evolution of CO_2 (v_1). It is obvious that the rate of gas evolution was in direct proportion to the rate of measured weight.

After t_1 time passed a peak of rate of gas evolution could be observed, then the falling-rate period began. Each fermentation experiment was carried out twice, first using a dehydrated material, then a fresh material without drying with the same dry matter. Maximal rates of gas evolution of dried material (v_{1d}) and fresh material (v_{1f}) were then determined. The residual fermentative activity of dried material was given by

$$A = v_{1d}/v_{1f} \tag{2}$$

The drying apparatus (ENSGTI, France) was a convective drying tunnel (Fig. 2) equipped with a magnetron (2.45 GHz) with a modulable power between 0 and 800 W.

The convective drying parameters could be varied in the following ranges:

- the air velocity between 0.5 m s^{-1} and 2.5 m s^{-1},
- the temperature between the ambient one and 155 °C,
- the relative air humidity between 12 and 99%.

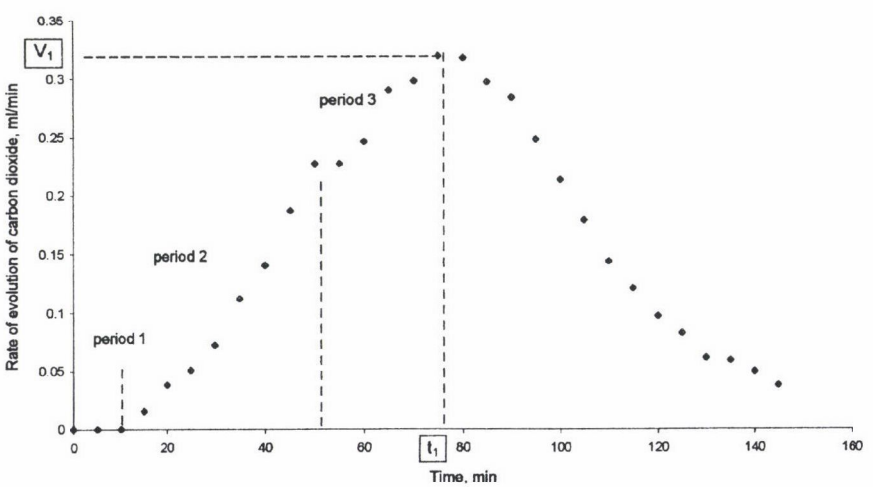

Fig. 1. Evolution rate of carbon dioxide during fermentation

An example drying rate curve is presented in Fig. 3 in order to get a better insight into the process of drying achieved by means of the mentioned drying tunnel. It describes convective drying. Moisture content is indicated on the abscissa. The process is started at point No. 1 and finished at point No. 4, which is the equilibrium state. The following generalities can be noted: curve of mass flux (right ordinate) can be divided into three principal sections: the constant-rate drying section (A) and the first and second section of falling-rate drying (B, C). The first section of falling-rate drying extends from point No. 2 (as it is the first critical moisture content) to point No. 3 (as it is the second critical moisture content) and the second section of falling-rate drying extends from point No. 3 to point No. 4. The drying rate is controlled by the surrounding drying air during constant-rate period. The surface temperature (left ordinate) of the material is the same as that of the wet bulb temperature of the drying air.

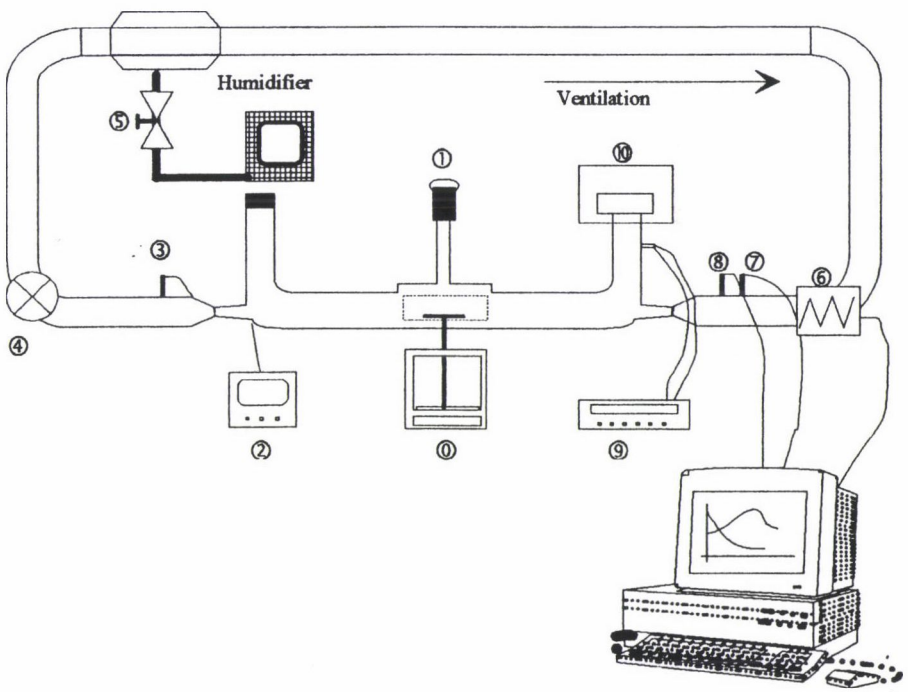

Fig. 2. Experimental drying tunnel. 0: Balance, 1: optic pyrometer, 2: powermeter, 3, 8: thermohygrometer, 4: fan, 5: valve, 6: heater, 7: anemometer, 9: optic thermometer, 10: generator

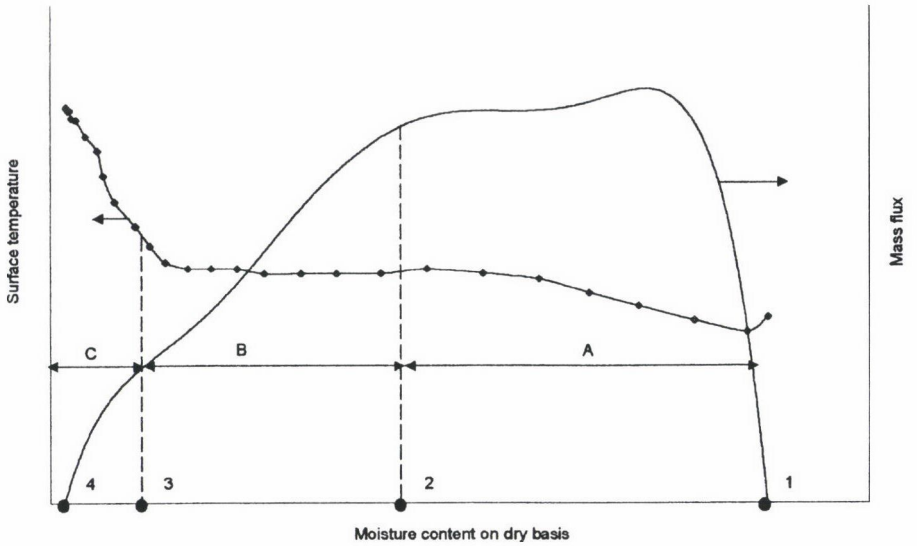

Fig. 3. Drying rate curve with the surface temperature curve. A: constant-rate period, B: first falling-rate period, C: second falling-rate period

The process is controlled by the resistance of the product to heat and mass flows in the falling-rate stages. Most of the undesirable quality deficiencies (e.g. shrinkage, protein denaturation) can occur during these periods. Surface temperature exceeds the wet-bulb temperature and reaches the dry-bulb temperature.

During the completed drying experiments the layer thickness, air velocity and relative humidity of inlet air remained constant (1 mm, 1.2 m s^{-1} 20%, respectively). The effect of drying temperature on the fermentative activity was investigated at four inlet air temperatures: 39 °C, 45 °C, 48 °C and 54 °C.

In combined experiments incident microwave power amounted to 1 W and 3 W. The samples were disc shaped with 0.1 cm thickness and 2.5 cm radius, so the volumetric power was calculated as 0.509 W cm^{-3} and 2.036 W cm^{-3}, respectively.

2. Results

2.1. Convective method

The results of fermentation after convective drying process are represented in Fig. 4. The activity after drying at 45 °C was close to the required value (74% and 80%, respectively). Drying at 48 °C and 54 °C resulted in a product, which retained 37% of

the original fermentative activity. Degradation, denaturation of enzymatically active proteins started at a temperature of 45–48 °C. The yeast cells preserved their fermentative nature at 39 °C well. The lower drying temperature provided good circumstances for the viability of microbes, but at atmospheric pressure the drying time could be longer.

2.2. Combined convective–microwave method

A possible way to shorten drying time is to combine convective method with microwave power. The drying curves and surface temperature values are shown in Fig. 5. Chapter 2.1. indicates that the ideal inlet air temperature should be 39 °C. Incident microwave power of 0.509 W cm^{-3} and 2.032 W cm^{-3} was added to the convective process. Figure 6 represents the calculated fermentative activity levels which did not reach the required minimum activity value. According to Fig. 5, surface temperatures did not exceed 46.7 °C, therefore this considerable activity loss (more than 30%) demands an explanation. The possible reason is that oscillated microwave power took effect on the material within the sampled time and resulted in a brief and undetected overheating.

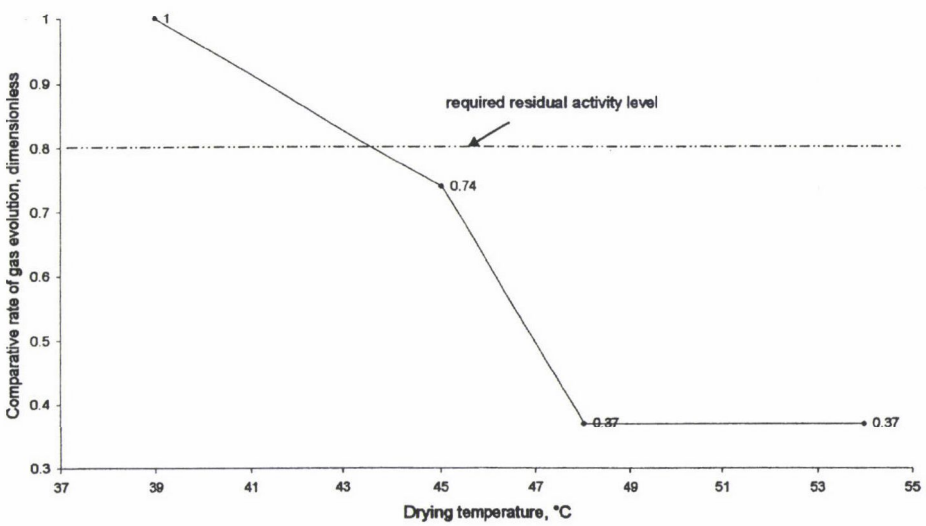

Fig. 4. Fermentation activity of yeast after convective drying at different temperatures

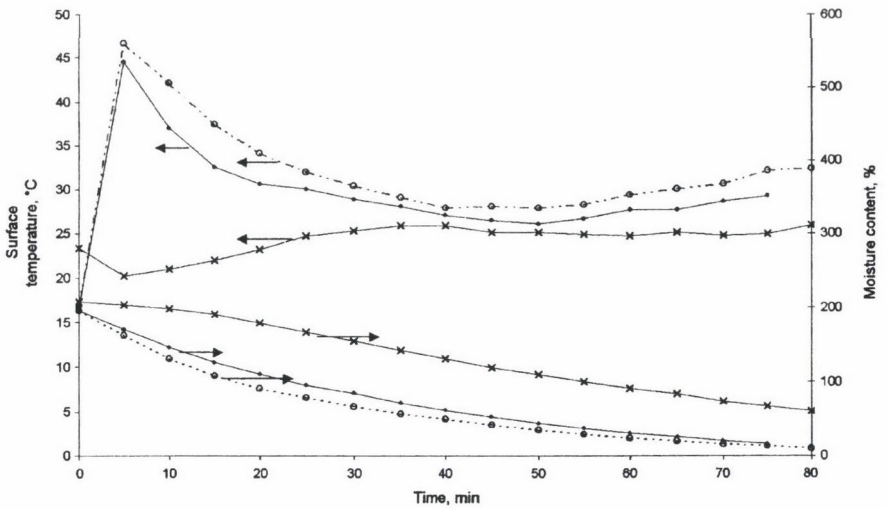

Fig. 5. Convective and microwave enhanced convective drying curves and surface temperatures curves of yeast. X: pure convection at 39 °C ●: convection at 39 °C with 0.509 W cm^{-3} microwave power
O: convection at 39 °C with 2.032 W cm^{-3} microwave power

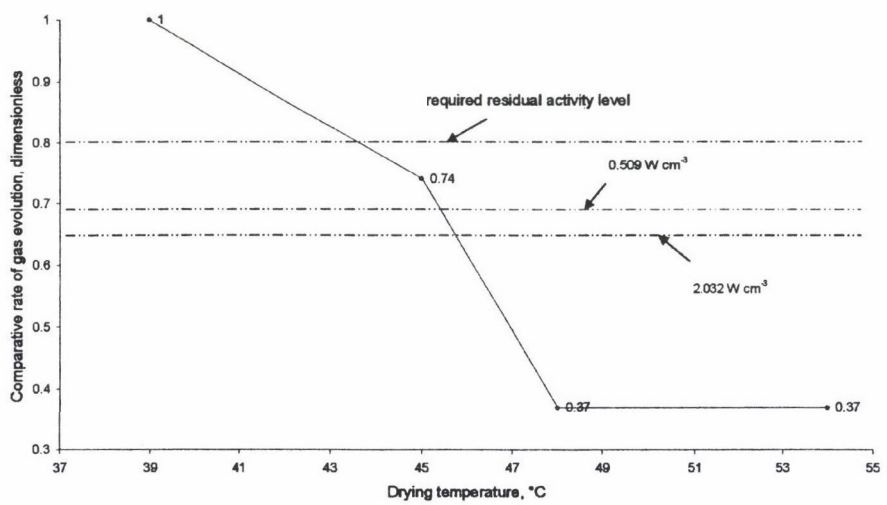

Fig. 6. Fermentation activity of yeast after convective drying at different temperatures (solid line) and combined drying at 39 °C (dashed line)

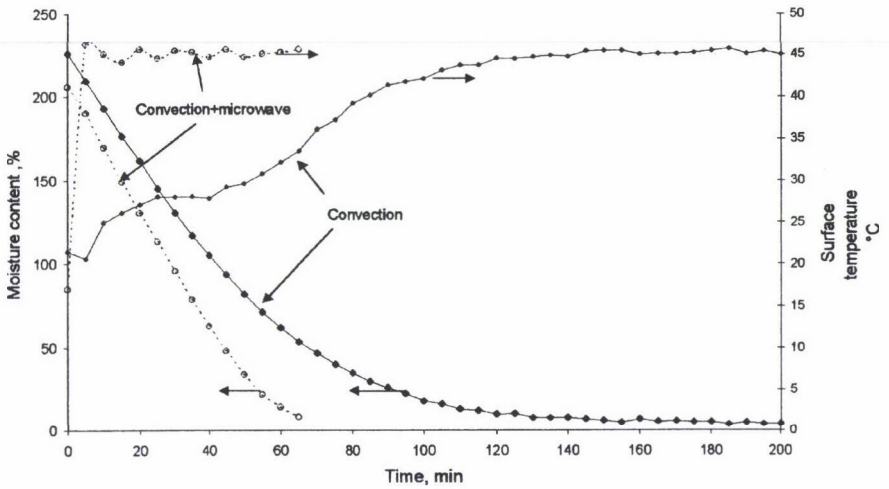

Fig. 7. Convective and enhanced convective drying curves with manual regulation of the incident microwave power

2.3. Combined convective-microwave method with manual regulation of incident microwave power

In order to avoid overheating phenomena permanent manual regulation of incident microwave power was performed taking the surface temperature as a basis. The regulation was set into action when the surface temperature attained 45 °C, and the inlet air temperature was 39 °C.

In Fig. 7 a comparison is shown between pure convection (temperature level was 45 °C) and the convection method enhanced by microwave. During pure convective process 10% moisture content was reached after 130 min whereas manual regulation of the indicent microwave power resulted in the same moisture level after 65 min.

The results of the gas evolution experiments for determining the biological value are shown in Table 2. The maximal rate of carbon dioxide formation (0.205 g min^{-1}) in the dried material was reached after 130 min. Using fresh material we got values of 0.235 g min^{-1} after 75 min. In order to compare the results, the specific rate of carbon dioxide evolution was introduced, which means the maximal rate of gas evolution divided by the dry matter. In this way the rate of gas evolution can be calculated per unit of dry matter. The final fermentative activity referring to the biological activity of the material can be obtained by dividing the specific gas evolution rate of the dried material

by the specific gas evolution rate of the fresh material. For getting a percentage, the results are to be multipled by 100:

$$0.447/0.550 \times 100 = 81.27\%$$

Table 2

Results of yeast fermentation after manually regulated convective-microwave drying

	Dried material	Fresh material
t_1 (min)	130	75
v_1 (g min^{-1})	0.205	0.235
Dry matter (g)	0.429	0.427
Specific rate evolution of carbon dioxide (min^{-1})	0.447	0.550

This value met 80% of the residual activity level, as required.

3. Conclusions

Our study indicated the effect of microwave enhanced convective drying on the activity of *Saccharomyces cerevisiae* based yeast. The drying time shortened and the drying intensity increased.

The temperature profiles of the material are of primary importance because of the presence of thermosensitive enzymes. The use of constant incident microwave power could be the cause of either overheating of the material or inefficient dehydration during the process. Consequently, the incident microwave power had to be regulated on the basis of the material temperature. A dried product with a moisture content of 8% was produced, which retained 81% of its original fermentative activity.

Further investigations are needed to be able to determine the yeast's dielectric properties being essential to calculate some process parameters.

*

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N-3 FATTY ACID ENRICHMENT AND OXIDATIVE STABILITY OF BROILER CHICKEN

(A REVIEW)

H. A. MANILLA and F. HUSVÉTH

Department of Animal Physiology, Pannon University, Georgikon Faculty of Agricultural Sciences, H–8361 Keszthely, Deák F. u. 16. Hungary

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Increasing awareness of the health benefits of n-3 fatty acids has led to studies related to the manipulation of the fatty acid composition of animal products. These fatty acids, especially eicosapentaenoic acid (EPA; C20:5n-3) and docosahexaenoic acid (DHA; C22:6n-3), are abundant in foods of marine origin. Fish consumption is, however, limited by seasonal availability, affordability and consumers' preference. Recent studies on the provision of n-3 fatty acid rich foods have therefore centred on the enrichment of products such as poultry meat through feeding fish oil diets. However, decreased quality (storage and flavour) has been associated with products from poultry fed such diets. Other dietary sources of n-3 fatty acids such as fish meal and plant seed oils result in minor improvement of the quality and low levels of EPA and DHA in the enriched product. Supplementation of high levels of vitamin E or other synthetic antibiotics in diets may increase oxidative stability and hence the storage quality of n-3 fatty acid enriched broiler meat. However, their reported influence on off-flavour is conflicting. Other methods of reducing off-flavour in enriched meat involving the use of processed n-3 PUFA sources although may reduce off-flavour, result in reduced deposition of EPA and DPA. Marine algae (MA) is an attractive source of n-3 fatty acids because it is a primary rich source of DHA and contains naturally occurring carotinoids, which are useful for their antioxidant activity. Investigations into the use of MA and identification of cheaper sources of n-3 PUFA for the enrichment of broiler chicken are needed. In addition, the search for viable methods of reducing off-flavour in n-3 enriched broiler meat should continue. The production of high quality and affordable broiler meat is essential for realising the full benefits associated with the consumption of n-3 fatty acid enriched products.

Keywords: n-3 fatty acids; broiler chicken; oxidative stability; marine algae; vitamin E

1. Introduction

In recent years there has been an increase in morbidity and mortality from cancer, atherosclerosis, coronary heart diseases (CHD) and other related diseases the world

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over. Research has established a positive relationship between dietary fat intake and fatty acid composition of foods, and incidence of these diseases in humans (BURR et al., 1989; HRBOTCKY & WEBER, 1993). Current studies, involving the production of high quality animal products, have therefore been directed towards enriching animal products with health beneficial fatty acids of the n-3 family. A number of these studies have, however, focused on the enrichment of broiler chickens with these fatty acids through dietary sources. The tendency of broiler chicken meat so enriched to high rate of lipid peroxidation, has also prompted investigations into the influence of dietary n-3 fatty acid sources on the oxidative stability of the resultant product and on ways of reducing the rate of lipid peroxidation.

This review presents the physiological and health benefits of essential fatty acids in human diet. Current studies on dietary manipulation of the fatty acid composition of broiler chickens with emphasis on the n-3 polyunsaturated fatty acids (PUFA) are reviewed. The various methods of improving the oxidative stability of n-3 fatty acid enriched products are also discussed.

2. Essential fatty acids (n-3 and n-6 PUFA) in human health

Investigations on the diet–health relationship among various populations, North Americans (SINCLAIR, 1953), Greenland Eskimos (BANG & DYERBERG, 1972) and Nigerians (HOLMAN et al., 1996), have established that consumption of fish reduces the risks of CHD in humans.

The effect of fish consumption on the incidence of CHD is suggested to be related primarily to the long chain n-3 PUFA (eicosapentaenoic acid, EPA; and docosahexaenoic acid, DHA) found in fish oils.

Apart from their effects on CHD, n-3 PUFA (especially EPA and DHA) have been shown to posses anti-promotional effects on some types of cancer, rheumatoid arthritis, and multiple sclerosis (BRITISH NUTRITION FOUNDATION, 1992) as well as inflammatory bowel diseases (IBD; MESTER et al., 1999). Evidence from animal studies suggests that retinal function and learning ability are affected by nutritional deficiency of DHA during development (NEURINGER et al., 1988; BOURRE et al., 1989).

3. Relationship between n-3 and n-6 fatty acids

HOLMAN and co-workers (1996) reported an imbalance between n-3 and n-6 fatty acid in the food and plasma of many populations and a negative correlation between the plasma content of these two families of fatty acids in humans. Earlier, HOLMAN and MOHRHAUER (1963) observed that an increase in linolenic acid (C18:3n-3) intake

suppresses the metabolic products of linoleic (C18:2n-6) acid and enhances those of linolenic acid. Also it has been suggested that PUFA of the n-3 family exert an inhibiting effect on the metabolism of arachidonic acid (C20:4n-6; DYERBERG & BANG, 1978; HEROLD & KINSELLA, 1986), and the conversion of dietary linoleic acids to arachidonic acid (LANDS et al., 1973). Reduction in the concentration of arachidonic acid and its metabolites is suggested as one of the reasons why n-3 fatty acids reduce the risk of CHD (LEAF & WEBER, 1988). Also, a high intake of n-6 PUFA has been shown to increase the risk of gallstone in humans (STURDEVANT et al., 1973), reduce high density lipoprotein concentration (MATTSON & GRUNDY, 1985) and suppress the immune system (SANDERS, 1988; RASMUSSEN et al., 1994).

These findings have led to the suggestion that it may be desirable to give attention not only to the n-3 PUFA level but also to the n-6 to n-3 fatty acid ratio in human foods (LANDS, 1989; BRITISH NUTRITION FOUNDATION, 1992). YEHUDA and CARASSO (1993) in studies with rats found the optimum functional ratio between n-6 and n-3 fatty acids to be 4:1. BRITISH NUTRITION FOUNDATION (1992) recommended the consumption of n-6 and n-3 fatty acids to be in the ratio of 6:1.

4. Broiler chicken as a source of n-3 fatty acids in human diet

Broiler chicken can provide an excellent alternative source of PUFA of the n-3 family for humans (CHANMUGAM et al., 1992; HARGIS & VAN ELSWYK, 1993). Poultry meat is naturally low in fat content and rich in PUFA (IGENE & PEARSON, 1979). HULAN and co-workers (1988) calculated the amount of n-3 fatty acids that can be made available to a consumer of fat modified broiler chicken meat and reported that 100 g of such chicken meat would contain approximately 142 mg of EPA+DPA+DHA. This provides slightly higher amount of these fatty acids than the same amount of cod flesh (138 mg). In a follow-up study, HULAN and co-workers (1989) reported that feeding a 12% red fish meal diet to broiler chickens would provide approximately 197 mg of EPA+DPA+DHA per 100 g meat.

5. Dietary manipulation of lipid composition of poultry meat

Early studies involving the production of high quality poultry products were focused on the manipulation of total fat content for leaner meat and low cholesterol content of eggs (CARTWRIGHT, 1991). Relatively few studies were directed towards the manipulation of the fatty acid composition of poultry meat. Table 1 shows the n-3 fatty acid composition of breast muscles from broilers fed a standard no added fat diet and 4% cod liver or linseed oil diets. These data clearly demonstrate the remarkable

changes that occur in the n-3 fatty acid composition of broiler muscle as a result of the influence of diet.

OLOMU and BARACOS (1991) fed flax oil (highly polyunsaturated) to broilers with the aim of evaluating its effects on the incorporation of n-3 fatty acids into the skeletal muscle tissue. They reported an increased accumulation of n-3 fatty acids (linolenic acid and its long chain products, EPA, DPA and DHA) in skeletal muscle lipids with significant decrease in n-6 fatty acids levels. Their observation is in agreement with that of an earlier study by PHETTEPLACE and WATKINS (1989) who also reported a general increase in the tissue contents of linolenic acid and its long chain derivatives with the feeding of flax oil to chickens. CHANMUGAM and co-workers (1992) fed diets supplemented with corn, linseed, or menhaden oil to broilers. Birds supplemented with linseed oil (rich in linolenic acid) had significantly higher levels of n-3 fatty acids and higher n-3 to n-6 fatty acid ratio than those supplemented with the same level of menhaden oil primarily due to an accumulation of linolenic acid. Levels of EPA were increased in all the groups fed menhaden oil and higher levels of linseed oil when compared with the controls fed the same levels of corn oil. In a similar study, YAU and co-workers (1991) fed broiler diets containing safflower (highly polyunsaturated), olive (highly monounsaturated) and coconut (highly saturated) oils and reported that the ratios of specific fatty acids and the fatty acid profile of the breast meat were similar to those of the dietary oils. In an earlier study, MARION and WOODROOF (1963) investigating the effects of dietary corn oil (highly polyunsaturated) and tallow (highly saturated), on the fatty acid composition of breast, thigh, and skin tissues of broiler chickens, reported that each of the tissues analysed exhibited a fatty acid composition similar to those of the diet.

Table 1

N-3 fatty acid composition of the breast muscle of chickens fed a standard no added fat diet, cod liver and linseed oil diets (MANILLA, 1998)

	Standard no added fat diet	4% cod liver oil diet	4% linseed oil diet
	% total fatty acids		
C18 : 3n-3	0.6	1.7	10.0
C20 : 5n-3	1.0	4.2	2.7
C22 : 5n-3	1.8	4.0	3.1
C22 : 6n-3	3.4	9.9	2.6
Total n-3	6.8	19.8	18.3

The results of these studies indicated that feeding diets having the fatty acid composition desired of the resulting tissue might customise fatty acid profile of broiler tissues. In general, high n-3 PUFA diets increase tissue n-3 fatty acid concentration and depress those of n-6 and monounsaturated fatty acids. Dietary fish oils result in high deposition of EPA and DHA.

The influence of dietary fatty acid composition on various tissues of broiler chicken has also been widely studied (MARION & WOODROOF, 1963; MILLER and ROBISCH, 1969; LIN et al., 1989a; HUANG et al., 1990; YAU et al., 1991; PINCHASOV & NIR, 1992; HRDINKA et al., 1996) with reports of significant differences in fatty acid profile between muscle and adipose tissues of chicks fed diets of similar fatty acid composition.

Most of the above studies, reported higher deposition of EPA and DHA in the breast when compared with the adipose tissues. Similar results of increased levels of EPA and DHA deposition in the broiler muscle tissue, when compared with adipose (EDWARDS & MAY, 1965; MILLER et al., 1967a,b), with differences in deposition level between the breast and the thigh muscles (MARION & WOODROOF, 1963), with the feeding of various dietary oils have been made in earlier reports.

The observation of preferential deposition of n-3 fatty acids within muscle fat depots may be of interest due to the fact that these are the consumers' choice carcass portions.

6. Sources of n-3 fatty acids for broiler diets

6.1. Fish oil and fish meal

Most studies on the n-3 fatty acid enrichment of poultry meat through dietary sources have focused on the use of marine sources of n-3 fatty acids especially fish oil and fish meal (CARRICK & HAUGE, 1926; MARION & WOODROOF, 1963; EDWARDS & MAY, 1965; MILLER et al., 1967a,b; MILLER & ROBISCH, 1969; HULAN et al., 1988; ACKMAN et al., 1988; HULAN et al., 1989). Studies cited above reported substantial enhancement of EPA, DHA, and other n-3 fatty acids in the tissues of chickens with the supplementation of various fish oil or fish meal in the diets. HULAN and co-workers (1988) fed chickens with a diet containing 5% fish meal and reported a substantial increase in EPA, DHA and other n-3 PUFA in the total carcass and edible meat lipids. Earlier, MILLER and ROBISCH (1969), in a broiler feeding trial with menhaden, herring and safflower oils, reported the highest level of deposition of EPA and DHA for diet supplemented with menhaden oil followed by herring oil (8% less than menhaden in total 20-carbon n-3 fatty acids).

DYERBERG and BANG (1978), and HEROLD and KINSELLA (1986) suggested that the beneficial effect of seafood consumption in reducing the risk of CHD is due to their high content of n-3 PUFA (especially EPA and DHA). Fish oil is rich in EPA and DHA which are considered the group of fatty acids contributing to the anti ischeamic disease effect of PUFA enriched products (NETTLETON, 1991). These findings may have led to the idea that it may be desirable to increase the n-3 fatty acids of chickens with marine sources.

The feeding of fish oil have been reported to strongly influence flavour and storage quality of carcass. In an early study, CARRICK & HAUGE (1926) reported significant off-flavour in meat samples from chickens fed 4% dietary cod liver oil. EDWARDS and MAY (1965) using untrained panellists, reported that meat samples from broilers fed menhaden oil alone were poorer in flavour (using mean flavour scores) than those fed menhaden in combination with plant seed oils. Unacceptable odours have also been observed by other authors in carcasses of chickens fed fish oil at levels of 4% (DANSKY, 1962), 2.5% (HOLDAS & MAY, 1966), 2% (CARRICK & HAUGE, 1926; EDWARDS & MAY, 1965) and 1.8% (HARDIN et al., 1964). In studies with turkey, feeding of fish oil at levels of 2- or 5% (ASMUNDSON et al., 1938; KLOSE et al., 1953) also produced off-flavour.

Decreased product quality associated with feeding fish oil prompted investigations to examine the effects of dietary fish meal as an alternative to fish oil on the flavour of broiler chicken meat. The feeding of various levels of fish meal of over 14% has been associated with unacceptable flavours (DEAN et al., 1971; HULAN et al., 1989; RATNAYAKE et al., 1989). In an earlier study, ASMUNDSON and co-workers (1938) replaced 2–10% (commonly used levels) fish oil with fish meal in broiler diet and reported decreased off-flavour in meat when compared with fish oil. RATNAYAKE and co-workers (1989) found no off flavour with meat samples from broiler chickens fed 4% or 8% dietary red fish meal. However, they noted that meat samples from chicks fed 12% red fish meal were less preferred in flavour than those birds fed lower dietary fish meal levels.

Although a large number of the studies reviewed tended to suggest that fish meal decreases off-flavour in n-3 fatty acid modified poultry meat, the resultant flavours were identified as "less preferred" by panellists using flavour scores.

6.2. Oils of plant origin

Because of the association of off-flavour of poultry meat products with dietary fish oils, several investigations were conducted to investigate the effects of plant sources of n-3 fatty acids in diet on the fatty acid composition and quality of broiler chicken meat. PHETTEPLACE and WATKINS (1989) compared the effects of 5% dietary linseed oil (rich in linolenic acid) with 5% menhaden oil when fed to broilers and reported that

while both oils resulted in significant increases in total tissue n-3 fatty acids, muscle deposition of the sum total of EPA and DHA was significantly less (2.9 and 2.2 weight % in breast and thigh muscles, respectively) in linseed oil fed chicks than in those fed menhaden oil (20 and 17 weight % in breast and thigh muscles, respectively). Linseed oil resulted predominantly in linolenic acid deposition in muscle tissue. Recent studies on the effect of dietary linseed oil on off-flavour of broiler meat appear to be lacking. Early work by KLOSE and co-workers (1951, 1953) studied the effects of dietary linseed on off-flavour of turkey meat and reported moderate fish flavour with birds fed 2% or 5% dietary linseed when compared with corn oil or beef tallow. OLOMU and BARACOS (1991) using various combinations of animal tallow and flaxseed oil (FSO) reported that linolenic acid was the primary constituent of muscle lipids of broilers fed 4.5% dietary flax seed oil, increasing from less than 1% (in muscle tissues of birds fed 6% animal tallow) to 8.9% of muscle lipids in response to dietary flax seed oil. Deposition of EPA and DHA in muscle tissue was significantly low. Flaxseed oil contains about 50% linolenic acid, making it the richest plant source of n-3 fatty acids.

The evaluation of the effect of other n-3 PUFA rich plant oils such as rapeseed oil (HAWRYSH et al., 1980), canola oil (SALMON et al., 1981), safflower oil (MILLER & ROBISCH, 1969) have also been conducted with report of lower tissue deposition of EPA and DHA and improved flavour when compared with fish oil. Plant sources of n-3 fatty acids (oils) are rich in linolenic acid (LANDS, 1986) and contain low concentrations of EPA and DHA (NETTLETON, 1991). Therefore, the reduction in occurrence of off-flavour reported in the above studies with dietary plant oils may be related to the high linolenic acid, and low EPA and DHA content of the products. Results from these studies also reveal that the ability of broiler chickens to synthesise long chain n-3 PUFA from linolenic acid (C18:3n-3) is apparently limited. Despite the improvement in flavour of meat enriched with n-3 fatty acids through the feeding oils of plant origin, the resulting n-3 fatty acid of the meat product must be considered.

6.3. Marine microalgae (MA)

A recent development in the search for optimum source of n-3 fatty acids for poultry feed has been the use of fermented natural marine microalgae (MA) with a high DHA content (BARCLAY et al., 1994). MA is a rich source of DHA which has been shown to inhibit diseases which inhibition is associated with EPA consumption (GAUDETTE & HOLUB, 1991). Studies have demonstrated that DHA can be converted in the animal tissue to EPA (FISCHER et al., 1987; GRONN et al., 1991; ROSENTHAL et al., 1991). REITAN and co-workers (1993) examined the effects of dietary MA on the performance of fish and reported an increased tissue deposition of DHA. In a recent study HERBER and VAN ELSWYK (1996) investigated the influence of MA as a poultry feed supplement in the enrichment of shell egg with n-3 fatty acids. They observed that

levels of total n-3 fatty acids in yolk were significantly increased with a reduction in yolk n-6 fatty acids. Yolk DHA deposition was efficient with the highest yolk DHA level attained in eggs from hens fed 4.8% MA as compared to a typical corn-soybean control, 1.5% menhaden oil and 2.4% of MA.

MA is an attractive source of n-3 fatty acids. It is a primary source in the natural fish diet. It is also a source of natural carotinoids (canthaxanthin and beta-carotene) which are useful for their antioxidant activity (BENDICH, 1989; BURTON, 1989). The presence of these will enhance oxidative stability of broiler tissue while providing humans with the fatty acids associated with health benefits. Apart from these benefits, microalgae can be effectively preserved (GRIMA et al., 1994) and cultured to give a high biomass and PUFA content (COCCHI et al., 1994).

These studies suggest that there are numerous viable n-3 fatty acid sources for poultry ration. However, it must be realised that the fatty acid composition of the final product varies depending on the dietary source. The optimum dietary source of n-3 fatty acids will be one that supplies both similar fatty acids as fish oil and offers oxidatively stable fat.

7. Oxidative stability and n-3 PUFA enriched broiler meat quality

To fully realise the benefits associated with consumption of n-3 fatty acid enriched poultry meat and products, they must meet consumer's requirement in taste and storage quality. The decrease in product quality (flavour and storage) of n-3 PUFA enriched poultry meat product has been associated with the oxidation of highly unsaturated fatty acids in the tissues (SALMON & O'NEIL, 1973). KLOSE and co-workers (1953) investigated the oxidative stability of meat from turkey fed linseed and fish oil (highly unsaturated) and reported positive correlation between oxidation activity (peroxide and aldehyde production) in carcass and dietary PUFA levels. Unsaturated fats easily undergo oxidation as a result of their double bonds, to produce oxidation products (peroxides and aldehydes) which are responsible for the off-flavour in oxidised tissues (ALLEN & FOEGEDING, 1981; MACDONALD et al., 1982; PERSON et al., 1983) and reduction in storage quality (GRAY & CRACKEL, 1991).

Studies have also shown that the triacylglycerol fraction of the n-3 PUFA modified meat containing significant levels of long chain fatty acids such as EPA and DHA are positively correlated with tissue off-flavour (WESSELS et al., 1973).

8. Improving the oxidative stability of n-3 fatty acid enriched broiler chicken meat

8.1. Use of antioxidants

Attempts have been made at reducing lipid peroxidation of meat through the use of antioxidants. LIN and co-workers (1989b) reported that an antioxidant stabilisation of dietary oil improved the shelf life of n-3 fatty acid enriched meat products. Similarly, EDWARDS and MAY (1965) reported improved flavour scores for meat samples from birds fed menhaden oil together with antioxidant in the diet. Antioxidants such as santoquin (EDWARDS & MAY, 1965) and ethoxyquin (ATKINSON et al., 1972; LIN et al., 1989b; MONHAN et al., 1990) have been studied with varying results on their effects on meat flavour and n-3 PUFA deposition in the muscle of broiler chickens.

8.2. Dietary vitamin E supplementation

Recent investigations on the improvement of the oxidative status of poultry meat have been directed towards the supplementation of diets with high level of vitamin E. Studies have shown that dietary supplementation of both free α-tocopherol or its acetate ester in the diets of broilers and turkey increases tocopherol content of tissues, delays lipid oxidation and extends the shelf life of meat (MECCHI et al., 1956; MARUSICH et al., 1975; UEBERSAX et al., 1978; COMBS & RENGENSTEIN, 1980; BARTOV & BORNSTEIN, 1981; BARTOV et al., 1983). LIN and co-workers (1989a,b), investigating the effects of dietary supplementation of α-tocopherol on oxidative stability of broiler meat reported that meat from broilers given 100 IU kg^{-1} α-tocopherol in feed was more stable than meat from those fed unsupplemented diets. In addition, the oxidative stability of the thigh and the breast muscle were significantly improved during storage. When oxidised oils were used in the diet 200 IU kg^{-1} α-tocopherol supplementation in feed improved the oxidative stability of broiler meat when compared with unsupplemented diet (LIN et al., 1989b). SHELDON (1984) fed turkeys 275 IU α-tocopherol acetate/kg feed for three weeks before slaughter and reported 3 times higher tissue concentration of α-tocopherol than those from the control chicks fed 1.63 IU kg^{-1}. Furthermore, tissues from the supplemented diets were more stable in cold store. AJUYAH and co-workers (1993), evaluating the stability of n-3 enriched broiler meat after supplementing full-flax seed based broiler diet with vitamin E, reported a significant reduction in meat susceptibility to oxidation.

Several other recent studies have also reported improvement in the oxidative stability of chicken meat (SHEEHY et al., 1993; KLAUS et al., 1995) and pork meat (LESKANICH et al., 1997; FLACHOWSKY et al., 1997) with dietary supplementation of α-tocopherol. CHERIAN and co-workers (1996) studied the effect of dietary oils with added tocopherols on the fatty acid composition of liver, adipose tissue, thigh and breast meat

in broiler chickens and reported a significant increase ($P<0.05$) in the content of EPA and DHA in adipose tissue and breast meat from birds fed menhaden oil with added tocopherol diet when compared with that without tocopherol. AJUYAH and co-workers (1993) reported an elevation of the n-3 PUFA in the broiler muscle with dietary antioxidant supplementation.

8.3. Other methods

The off-flavour observed with n-3 fatty acid modified meat has also been attributed to the quality of the dietary sources, especially of fish meal and oils. STANSBY (1990) indicated that the off-flavour associated with meat samples from dietary fish oil and fish meal is due to volatile compounds and a range of other substances from peroxidation during processing and storage. It does appear that extensive processing with the resulting increased surface area of fish and oils make these sources susceptible to oxidative deterioration, which enhances the formation of volatile compounds. FRY and co-workers (1965) recommended the use of high grade fish oil and fish meal as dietary sources of n-3 fatty acids for poultry enrichment. In an early study, CRUIKSHANK (1939) reported no off-flavour and fishy-flavour with feeding high and low-grade fish meal, respectively.

To reduce the high levels of oxidation compounds (by-products) in dietary n-3 sources they were subjected to water, acid and ethanol extraction (WESSELS et al., 1973) before feeding. Improved flavour scores were observed for the three-extraction methods. However, a significant reduction (85%) of the n-3 fatty acid deposition was observed for ethanol extraction, which gave the highest flavour scores. The results suggest that the extraction methods modify the fatty acid composition of these sources, which may result in reduced deposition of n-3 fatty acids especially EPA and DHA in the product.

Heat stabilisation of dietary oil (ATKINSON et al., 1972) has also been studied with report of no significant improvement of off-flavour.

The effects of starving chicks (DEAN et al., 1971) and withdrawal of fish oil form diet (CARRICK & HAUGE, 1926; ASMUNDSON et al., 1938; MILLER & ROBISCH, 1969) before slaughter on the off-flavour of meat have also been studied with reports of reduced off-flavour. However, these methods may reduce weight gain and levels of deposited n-3 fatty acids, respectively.

9. Conclusion

Broiler chicken may effectively be enriched with n-3 PUFA through dietary means. However, the high rate of lipid peroxidation of such enriched product, which affects flavour and storage quality, may still be a limiting factor to its full acceptance by consumers. High (supernutritional) levels of vitamin E and other antioxidants in diet may improve the oxidative stability and hence storage quality of enriched products. Investigations on ways of reducing off-flavour have not produced a viable method. Investigations into the use of marine algae (MA) and identification of other cheap sources of n-3 PUFA for broiler ration are needed. In addition the search for viable methods of reducing off-flavour should continue. Quality and affordability will play an increasing role in the acceptability and marketing of n-3 enriched broiler meat products.

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RELATIONSHIP OF INCREASE IN BODY WEIGHT, FATTENED LIVER WEIGHT AND LIVER QUALITY IN GEESE OF DIFFERENT BREEDS, DETERMINED ON THE BASIS OF FORCE FEEDING METHODS

J. SZIGETI[a], ZS. TURCSÁN[b], E. BIRKÁS[b], F. BONYHÁDI[b], and A. VARGA[b]

[a] Institute of Food Science, Faculty of Agricultural Sciences, Pannon University of Agricultural Sciences, H–9200 Mosonmagyaróvár, Lucsony u. 15–17. Hungary
[b] MERIÁN, Orosháza Co. H–5901 Orosháza, Október 6. u. 8. Hungary

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From the age of 9 weeks, 90-90 Kolos, Gourmaud and Babati goose breeds were force fed with 2 different types of technology and 3 types of feedstuff. Force feeding technologies were the traditional Hungarian and Israeli soft groats quick methods. In the case of the latter, feedstuff was fed both in a pre-fermented (*Lactobacillus plantarum* lactic acid bacteria) form and without fermentation. Frequency of daily force feeding was gradually increased from 2 to 6 until the 21st day of force feeding. Live weight before and after fattening and liver weight were measured in the case of each breed and treatment. Liver quality was also determined. On the basis of our results, differences in liver weight average were significantly influenced only by the genetic property of the breed. Liver quality was also influenced by the method of force feeding of one breed (Babati). Feedstuff tested did not result in significant differences in liver weight or in liver quality. Independently of the breed very close correlation was found between "fattening weight" and liver weight ($r=0.98$) and between "fattening weight" and liver quality grade ($r=-0.97$).

Keywords: goose fatty liver weight, body weight, liver quality grade, force-feeding

Many publications aimed to find correlation between parameters that might influence the fattened liver weight of ducks and geese. It was found by BABILE and co-workers (1987) that after force feeding of 3 groups of Muscovy ducks there was a significant relation between liver weight (LW) and total feed (DM) intake. Experimental results suggest connection between LW and increase in body weight (BW) during force feeding, independently of sex and breed of ducks. In another paper, BABILE and co-workers (1988) established that the type of diet did not, while genotype differences of ducks did affect LW significantly. However, CHEN and co-workers (1985) found a significant difference of about 30 g between LW of geese fed with diet of cooked or

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uncooked maize plus 0.8% NaCl. LW was slightly, yet significantly heavier in geese given alfalfa meal as major dietary fibre sources than those given barley bran (CHEN et al., 1993).

MAMAEV (1986) measured LW of above 400 g in geese that weighed 4.2 kg to 4.3 kg at the start of fattening. These geese have gained 78–85% of their initial body weight during the 3 weeks of feeding. SHALEV and co-workers (1986) revealed by regression analysis that LW increased by 69 g for 1 kg of increase in BW after fattening and that it increased by 100 g for a 1 kg increase in BW before fattening.

POUJARDIEU and co-workers (1994) found that, in Italian and Polish goslings, there was no relationship between LW and liver quality grade (LQG). Studies by BÓDI (1992) showed significant correlation between 14 weeks' BW and carcass weight of males and females of Hungarian geese.

Several authors, including JOUGLAR and co-workers (1992), found relationship between LW and carcass and body weight. Carcass weight increased from 3.183 g to 5.611 g after 34 force-feeding sessions, while liver weight increased tenfold in 9 groups of 20 caged Mulard ducks. It means that the increase in carcass weight was caused about 20% by liver weight. After a short force period of 12 days AUVERGNE and co-workers (1995) noticed that 37% of the total weight gain resulted from increase in LW. After force feeding of 13 days, ROUVIER and co-workers (1994) measured BW, paletot weight and LW in some duck breeds and found a good correlation between BW and LW. Besides examining the effect of genotype on LW in geese PENKOVA and BÓDI (1995) found correlation between LW and BW except for the breed of Hungarian white breeds.

Two different breeds of Hungarian white and a crossbreed were force-fed on a diet of maize, fresh meal-and-bone, sunflower oil meal and vegetable oil by VEREMEENKO and KUTNYUK (1995) and among others body weight, body fat and liver weight were measured before and after finishing. Calculated data are given with respect to liver weight and growth weight. Correlation of about $r=0.55$ was found between LW and BW after finishing. It can be figured out from these results that the foie gras weight gain (LW) exceeded 12% of live weight gain after finishing of both breeds.

This paper reports the results of investigations carried out to determine the goose breed which produces the heaviest foie gras with the best quality and whether there is a correlation between finished live weight BW and LW independently of the force-fed technology and breed of goose.

1. Materials and methods

1.1. Goose breeds examined

It was a common property of the geese tested that they were bred from Toulouse goose for different selection purposes. All three liver hybrids tested belonged to the Landes group of breed.

1.1.1. Kolos liver hybrid. It was bred in Denmark by phenotype selection of Landes goose. The purpose of its breeding was large body growth and extremely large liver growth. Selection has proved to be successful, this hybrid can grow large liver of 800 g to 1000 g. However, one direction selection had a negative influence on prolification. An average goose has approximately 20–22 offsprings.

1.1.2. Gourmaud liver hybrid. This is a Landes genotype liver hybrid goose bred for liver production. This liver hybrid has good liver growth ability (600 g to 800 g) and prolification is also satisfactory (30–35 offsprings per goose).

1.1.3. Babati liver hybrid. This hybrid is a crossbred of Hungarian white gander and Landes geese. Its liver production is 600 g to 700 g/goose, prolification is 28–30 offsprings per goose.

Flocks consisting of 500–500 geese belonging to the same breed were formed and then 90–90 groups with a gander ratio of 50% were randomly selected and further divided into 3 sets, according to fattening type.

1.2. Fattening method

According to "traditional" technology known in Hungary, geese were fed for 21–23 days with cooked maize of 13.5 kg to 14.0 kg, soy groats of 0.5 kg containing 42% crude protein, feedstuff pea of 0.3 kg with 28% crude protein, 1.0–2.0% NaCl calculated feedstuff weight, goose fat of 1% to 1.5% and zeolite of 0.3% to 0.4% per bird.

Another fattening technology applied was the Israeli soft-groat technology, where 15–17 kg wet ground maize, 0.8 kg soy groats containing 42% crude protein content, 0.3–1.0% NaCl, 0.2–0.7% goose fat and 1% granulated bentonite as binding agent were used for each bird.

In the case of the third fattening method, Israeli soft groats were pre-fermented at a temperature of 20 °C for 24 h with *Lactobacillus plantarum* (NCAIM B1415) homofermentative lactic acid bacteria. Rate of inoculation of liquid culture was adjusted in such a way, that pH of feedstuff would reach 4.5–5.0 value a day later. Birds were fed with feedstuff mixture 2 and 3 with Israeli quick force feeding equipment. Force-feeding was performed by the same tender in the case of all of three goose breeds.

The fattening time was 21 days in all three fattening methods. Number of force feedings was defined as 2 in the first 3 days then 3 in the following 4 days etc.

1.3. Evaluation of results

Cutting and disassembling were made separately. A staff of 5 experts did weight measurements (body and liver weight) and liver classification. Quality classification system of goose livers is illustrated in Table 1. Although these principles of liver classification are not fixed in standards they are generally accepted in the Hungarian poultry industry. Evaluation of results was figured out by two factor (breed and fattening method) variance analysis and linear regression analysis (increase in weight during force feeding – liver weight and liver quality grade).

If blood was found in vessels or tissue of liver, classification was altered according to the following way: blood in arteria hepatica, vena portae or vena hepatica, value of quality decrease is 0.2; blood in liver capillaries or interstitial blood decreases class with 0.1–0.5 value.

Table 1

Directives of liver classification

		First class (1.0)	Second class (2.0)	Third class (3.0)
1	Liver weight	500 g to 850 g	450 g to 850 g	400 g
2	Substance of liver	– well-matured lobes – putty-like to touch – elastic substance -slightly soft	– less putty-like to touch – one-third part of lobule is fleshy to touch	– both loose or contact substance allowed
3	Colour of liver	– uniformly light – occasional haemorrhage disappears in ice	– discoloration on the one-tenth part of liver allowed (in spots)	– deeper discoloration on the one-fourth part of liver surface allowed
4	Intact character of liver	– intact liver, smooth surface – intact Glysson membrane	– surfacial injuries 1-5 mm deep	– injured, deep haemorrhagic regions

2. Results and discussion

2.1. Liver weight and liver quality grade as a function of various goose breeds and methods of force feeding

Liver weight averages in the case of the three hybrids and the three force feeding technologies are shown in Table 2 and Fig. 1.

LSD 95% value depending on breed or force feeding methods was 34.7 g in liver weight whereas that depending on breed and force feeding method proved to be 65.4 g. On the basis of these findings, the following conclusions can be drawn.

– Liver production of Kolos is better than that of Gourmaud, while Gourmaud geese liver production reached greater value than that of Babati.

– The examined force feeding methods did not indicate significant difference in liver weight.

Liver quality grade in the case of various hybrids and experimental force feeding methods are shown in Table 3.

Table 2

Liver weight of different liver hybrid geese, using various force feeding methods

Force feeding method	Liver weight average (g) and standard deviation (n = 30) Breed		
	Kolos	Gourmaud	Babati
Traditional	667.5 ± 74.2*	590.4 ± 133.3	482.5 ± 115.9
Israeli	662.1 ± 70.2	597.9 ± 139.7	484.3 ± 111.1
Israeli (with pre-fermented feedstuff)	665.4 ± 120.5	543.9 ± 125.3	500.4 ± 101.9

* LSD 95% between lines or columns is 34.7 g and between treatments is 65.4 g

Table 3

Liver quality grade in the case of various hybrids and force feeding methods

Force feeding method	Liver quality grade and standard deviation (n = 30) Breed		
	Kolos	Gourmaud	Babati
Traditional	1.11 ± 0.31*	1.53 ± 0.88	2.07 ± 1.38
Israeli	1.07 ± 0.26	1.32 ± 0.67	1.61 ± 0.83
Israeli-modified	1.18 ± 0.47	1.61 ± 0.91	1.93 ± 1.12

* LSD 95% between lines or columns is 0.26 and between treatments is 0.45

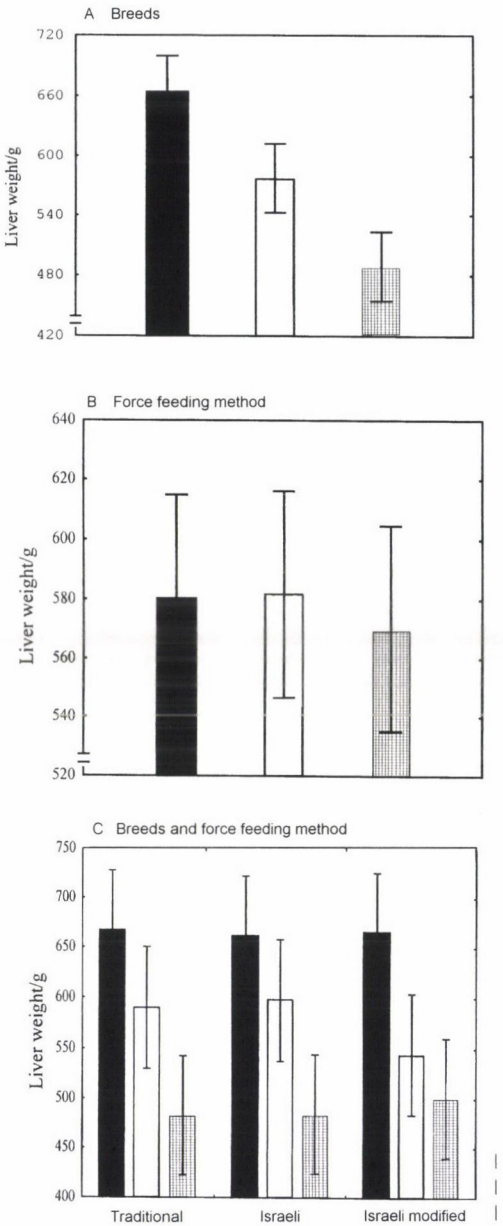

Fig. 1. Liver weight average of different geese hybrids and of various force feeding methods. A,C: ■ Kolos, □ Gourmaud, ▦ Babati; B: ■ Traditional, □ Israeli, ▦ Israeli modified

As for liver quality grades, LSD 95% value depending on breed or force feeding method is 0.26 whereas that depending on breed and force feeding method is 0.45. Evaluating these results we can conclude that:

– Liver quality – where bleeding, colour, etc. are also important besides weight – further emphasises superiority of Kolos and Gourmaud over Babati, which is also reflected in the results of liver weight measurements.

– Israeli force feeding method might be successful without pre-fermentation of feedstuff and it results in better liver quality in the case of breeds having originally poor liver quality (Fig. 2).

2.2. Relationship of increase in body weight during fattening with liver weight and liver quality grade

The terms "increase in body weight" or "fattening weight" mean the difference in live weight between starting force fed and before slaughtering.

Investigations have shown that increase in body weight does not depend on the method of force-feeding and it slightly rests with breed. The larger the "fattening weight" is, the larger the liver weight will be (Fig. 3).

A similar relationship was found between fattening weight and liver quality. Independently of breed, a larger fattening weight resulted in better quality (Fig. 4).

Our investigations have revealed that liver production is better in the case of hybrids showing a larger increase in weight during fattening. Moreover, within a given population, the liver weight of geese, which have a larger increase in weight, is also larger. We have to emphasise that confidence interval of the regression line showing fattening weight dependent change of liver quality classification and liver weight is occasionally greater under industrial circumstances. Considering that experiments like ours could not be found in the literature, comparison of our results with those of other authors was not possible.

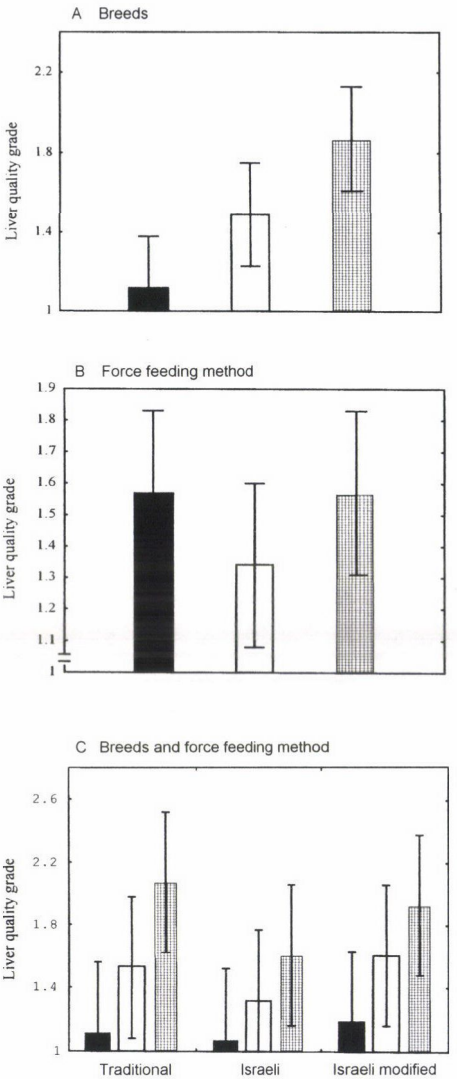

Fig. 2. Liver quality grade on the effect of different geese hybrids and of force feeding methods. A,C: ■ Kolos, □ Gourmaud, ▦ Babati; B: ■ Traditional, □ Israeli, ▦ Israeli modified

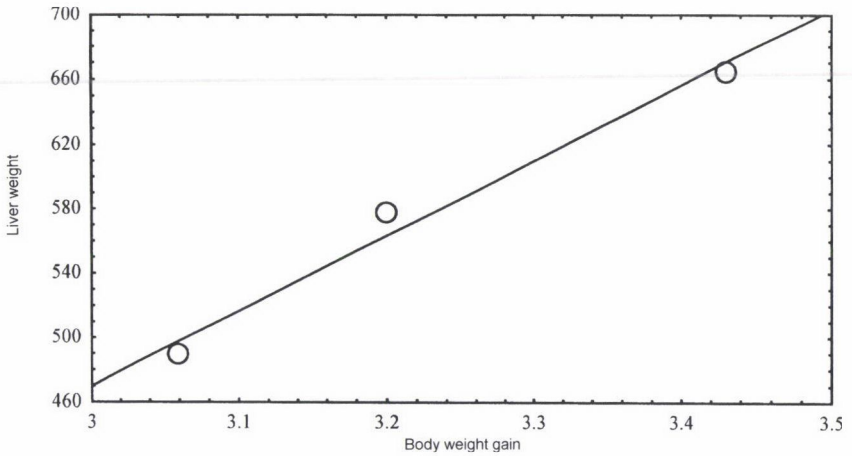

Fig. 3. Correlation between liver weight and body weight gain during force feeding (LSD 95% around straight is ±22 g), r=0.98

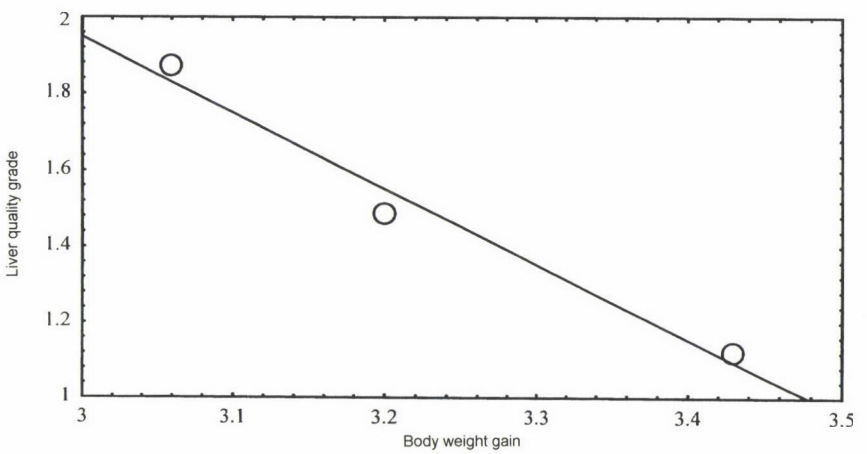

Fig. 4. Relation between liver quality grade and body weight gain during force feeding (LSD 95% around regression line is ±0.11), r=–0.97

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IMMUNOCHEMICAL ANALYSIS OF BUCKWHEAT PROTEINS, PROLAMINS AND THEIR ALLERGENIC CHARACTER

E. AUBRECHT and P. Á. BIACS

Central Food Research Institute H–1022 Budapest, Herman Ottó út 15. Hungary

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Prolamin content of buckwheat flour and processed foods was 24.2–42.1 mg/kg dry material measured by ELISA. According to in vitro results buckwheat is suitable for use in coeliac diet, although it contains some antinutritive materials, protease inhibitors and tannin. The allergenic properties of buckwheat are poorly understood. In our investigation intensity of the 24 kD protein band of buckwheat, of which allergenic activity is known has decreased, and 30–35 kD protein associations have been formed after heat treatment. Immunochemical reaction of buckwheat proteins were studied with blood specimens of coeliac and healthy persons.

Keywords: buckwheat prolamin, gluten-free, coeliac, allergen

Currently, the only help for coeliac patients, who do not tolerate wheat gliadins in their food, is a gluten-free diet. The main problem connected with the gluten-free food produced from seeds of maize and rice is the quick staling. This problem could be eliminated by the use of buckwheat seed. The products prepared from it can be kept fresh for a long time.

The buckwheat (*Fagopyrum esculentum* Moench) belongs botanically to *Polygonaceae*, it is far from *Graminaceae* family. Buckwheat seed can be applied for human nutrition only after dehulling, as there are toxic substances included in the hull of the seeds. Dehulled seed, flour milled from this seed and grits can be used for the preparation of meals. Protein and carbohydrate contents of buckwheat seed are nearly similar to those of corn and rice. The predominant storage protein of buckwheat seed is 13 S globulin. This protein has a hexametric structure with disulfide-bonded subunits composed of acidic and basic polypeptides. This structure is common for all legume-like storage proteins (MAKSIMOVIC et al., 1996). Buckwheat flours are rich in albumin and globulin, but they have a lower content of prolamin and glutenin than wheat flour (WEI et al., 1995). Buckwheat is rich in minerals, micro-elements and vitamins (especially vitamin B_1 and B_2), and it is an important source of vitamin E.

The aim of our immunochemical studies was to study in coeliac and non-coeliac patients whether buckwheat flours, grits and their products can be applied for the

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nutrition of patients with wheat flour sensitivity or not. This work is important as gluten-free diets exclude wheat gliadin and other proteins with homologous structure. Gluten content should not exceed the 100 mg gliadin/kg dry material amount in the gluten-free diet. This limit has been proposed by the Codex Alimentarius, in the recommendation of FAO/WHO (1998), it was published as ALINORM 97/26, Appendix V.

1. Materials and methods

1.1. Materials

1.1.1. Samples. Buckwheat seeds, grits, flours, bread, cake (muffin) were gifts from Food Technology Department of Central Food Research Institute (KÉKI). Positive sample GK Öthalom wheat flour was a product of the Cereal Research Institute, Szeged. Rice and glucono-delta-lakton (GDL) were purchased at store.

1.1.2. Anti-gliadin rabbit serum was developed in our laboratory against 30 kD gliadin, antigen was bought from Serva Co. Human sera specimen of coeliac (C) and healthy (H) people were derived from the Department of Dermatology of the Semmelweis University Medical School, Budapest.

1.1.3. Chemicals, reagents for ELISA, and SDS-PAGE. All chemicals were of analytical grade. Antigliadin-rabbit IgG was developed at KÉKI. Antigliadin-rabbit-horse-radish-peroxidase (HRP) conjugate were prepared at the Biology Department of KÉKI according to NAKANE and KAWASI (1974).

Goat anti-human IgG-HRP-conjugat and goat anti-rabbit IgG were purchased from Sigma Co. 3,3', 5,5'-tetramethyl benzidine (TMB) substrate (Sigma Co.) was used. Microtiter plates were bought from the Company of Instrument and Plastic Material, Sülysáp, Hungary.

1.2. Methods

1.2.1. Prolamin/gliadin extraction. Samples (flours and grits, 100 mg) and food were mixed with 1 cm^3 of 70% (v/v) aqueous ethanol in Eppendorf tube and extracted with vigorous shaking on a flash shaker (IKA-Schüttler MTS 4, 700 r.p.m. for 30 min at 4 °C). Following extraction, the samples were centrifuged (T 24 D, 5000 r.p.m. for 20 min at room temperature), the supernatant was separated and buckwheat samples were concentrated by evaporation of alcohol solution and then used for ELISA.

1.2.2. Sandwich ELISA for prolamin content (AUBRECHT & TÓTH, 1995). Wells of microplate were incubated overnight at 4 °C with anti-gliadin rabbit IgG antibody diluted 1:200 from stock solution in 0.05 mol Na-carbonate-bicarbonate buffer (pH 9.6) (as a coating buffer). Following washing, the wells were incubated with 0.5% gelatine for 1 h at 37 °C. After washing, samples diluted in 0.1 mol PBST pH 7.4 (1:2, 1:50,

1:200) and gliadin standard in 0.1 mol PBST (3 000 ng, 1 000 ng, 300 ng, 100 ng, 50 ng, 25 ng, 12.5 ng, 6.25 ng, pH 7.4) were incubated in wells for 1 h at 37 °C. After 3 cycles of washing anti-gliadin IgG-HRP-conjugate was added, and incubated for 1 h at 37 °C. Then the plate was washed and dried. Solution of 42 mmol TMB, containing 0.01% (v/v) H_2O_2, was added to each well. After 15 min the reaction was stopped by adding 0.07 cm^3 of 2 mol sulfuric acid and optical densities in the wells were measured at 450 nm.

1.2.3. Indirect ELISA for immunochemical reaction of human sera. Microtiter plates were incubated overnight at 4 °C with antigens: gliadin 30 kD (3 000 ng, 1 000 ng, 300 ng, 100 ng, 50 ng, 25 ng, 12.5 ng, 6.25 ng) wheat flour GK Öthalom as a positive sample (1:200) and buckwheat samples (1:5) in coating buffer (pH 9.6). After 3-cycle washing 0.5% gelatine was added and incubated for 1 h at 37 °C. After washing, coeliac and healthy human sera were added in 1:20 dilution. After incubation at 37 °C for 1 h the wells were washed, then 0.1 cm^3 of horse-radish peroxidase-conjugated goat anti-human IgG diluted in 0.1 mol PBS (pH 7.4, 1:500) was added to each well. Reagents were then incubated at 37 °C for 1 h and the excess of reagents was removed by washing with PBST (3×0.3 cm^3/well). TMB substrate was added according to the sandwich ELISA.

1.2.4. SDS-PAGE analysis of buckwheat prolamin and protein. For the analysis of extracted samples (total proteins and prolamins) the modified discontinuous buffer system (LAEMMLI, 1970), stacking gel 5% PAA (polyacrylamide) (5%, C 2.70%) separating gel 10% PAA (T 10%, C 2.7) were used. The vertical SDS-PAGE was carried out with the LKB 2050 Midget Electrophoresis Unit. The slabs were stained at room temperature in a solution of 0.2% brillant Blue R-250 dissolved in 25% (v/v) methanol 1–10% (v/v) acetic acid. The slabs were evaluated by BIOTEC-Fischer laser video densitometer.

1.2.5. A-PAGE (Acid-PAGE). Prolamin and proteins of buckwheat and wheat gliadin were separated by PAGE at pH 3.1 (A-PAGE) according to LAFIANDRA and KASARDA (1985). The lanes on the slab were evaluated by densitometer.

1.2.6. Baking. Bread was made with a special bread-making technology with glucono-delta-lacton (GDL) from 100% buckwheat flour. (The recipe is under patenting process.) Muffin was made of 100% buckwheat flour and pie was made of 100% wheat flour with ingredients: margarine, egg, salt, and $NaHCO_3$ in a usual way.

2. Results

Prolamin content of buckwheat flour and processed food (as presented in Table 1) is under the permitted limit (FAO/WHO, 1998). The prolamin concentrations were 24.2–42.1 mg/kg dry material measured by ELISA with polyclonal rabbit sera. The

wheat gliadin and buckwheat prolamin had reaction with human sera. The results of immunochemical reactions can be seen in Table 2.

It is well-known that human sera (normal sera) contain circulating antibody against gliadin in a lower level. Healthy people have a lower immunochemical reaction with wheat gliadin than coeliac patients. Higher values of patients 3 (C) and 5 (C) are caused by diet abuse according to KÁRPÁTI (1998). Both investigated sera (coeliac and healthy) gave reaction with prolamins of buckwheat, but the absorbance values were low.

Table 1

Determination of prolamin content of buckwheat flour and processed food by ELISA method with anti-gliadin polyclonal rabbit serum

Samples	Prolamin content (mg/kg dry material)
Buckwheat flour "Bio"	24.2 ± 2.81
Buckwheat bread with GDL	38.0 ± 2.19
Buckwheat bread mix with GDL	42.1 ± 3.12
GK Öthalom wheat flour as a positive sample	5094 ± 18.49
Rice flour as a negative sample	31.0 ± 4.18

The baked products were made of 100% buckwheat

Table 2

Immunochemical reaction of immune serum of coeliac and healthy people with 70% ethanol soluble wheat and buckwheat flour

Healthy (H) and coeliac (C) sera	Absorbance of immunochemical reaction with	
	buckwheat flour	wheat flour
1 (C)	0.064	0.463
2 (C)	0.080	0.363
3 (C)	0.078	0.553
4 (C)	0.141	0.433
5 (C)	0.153	0.591
1 (H)	0.099	0.291
2 (H)	0.032	0.296
3 (H)	0.096	0.191
4 (H)	nd	0.119
5 (H)	nd	0.201

nd: no detection

In our SDS-PAGE investigation ethanol soluble buckwheat prolamin fraction was a 12 kD protein, which consisted of three protein subfractions (AUBRECHT & KÁRPÁTI, 1995; AUBRECHT et al., 1998). They were studied by acid-PAGE. The separated subfractions of 12 kD protein, compared with wheat gliadin can be seen in Fig. 1. The three subfractions are marked in the electrophoretogram (1,2,3↓↓↓). This investigation has proved that buckwheat was gluten-free because the molecular weight distribution of the three subfractions differed from that of wheat. The acid-PAGE is special for wheat gliadin and it did not give the same result with buckwheat prolamins.

The other question in our investigation was that whether the 24 kD protein caused allergenic reaction in human diet (BIACS & AUBRECHT, 1998). The pattern of whole buckwheat protein from different origin, the wheat and oat samples can be seen in Fig. 2. The 24 kD protein band is predominant, intensive protein on the slab. The 24 kD buckwheat protein was shown by immunoblotting analysis to be the most frequently recognised allergen, binding to IgE antibodies from the patients' sera (KONDO et al., 1996) with RAST (Radio Allergosorbent Test). After baking muffin from buckwheat, intensity the predominant protein band has decreased and 30 kD and 35 kD bands have risen on slab in Fig. 2. The allergenic activity of the latest bands have not yet been known until now.

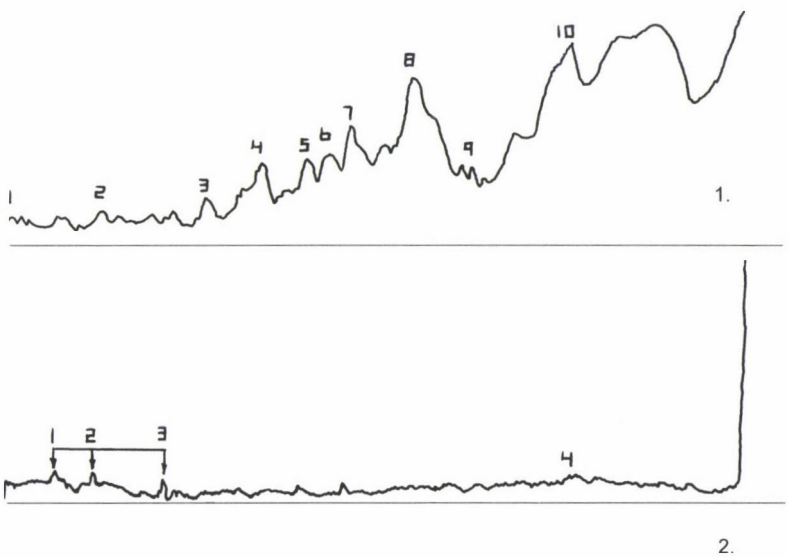

Fig. 1. Electrophoretogram of wheat gliadin (1) and buckwheat prolamin bands (2)

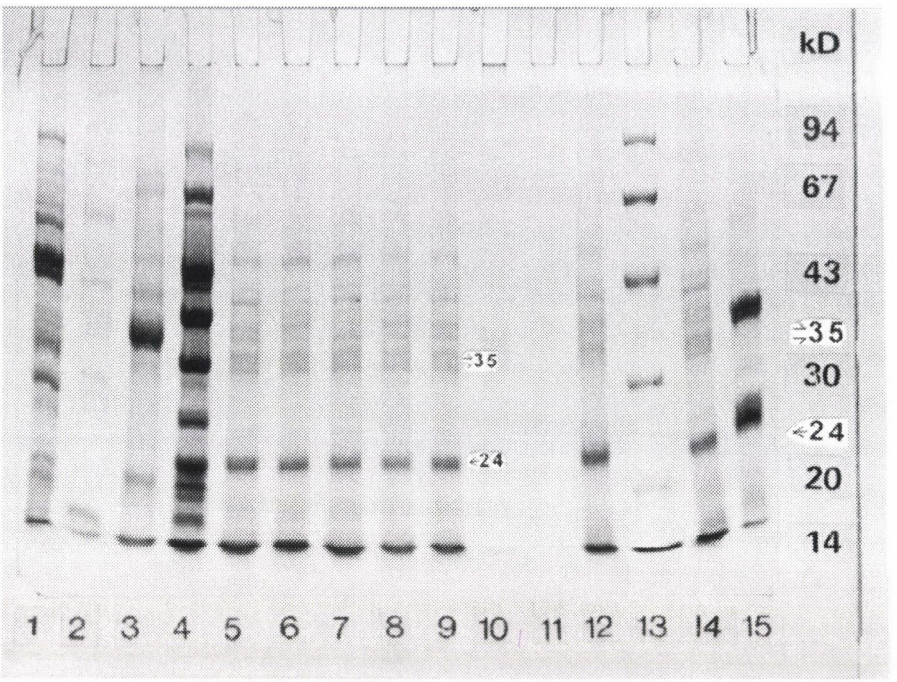

Fig. 2. SDS PAGE of whole protein of buckwheat and different cereals. Lane 1: Kamut organic culture of wheat; lane 2: GK Öthalom wheat; lane 3: Tiszadur durum wheat; 4: Tönköly (Spelta) wheat; lane 5: buckwheat flour; lane 6: buckwheat flour France; lane 7: buckwheat flour Slovene: lane 8: pancake powder 1; lane 9: pancake powder 2; lane 10: cake from 100% buckwheat flour (muffin); lane 11: cake from 100% wheat flour (pie); lane 12: noodles made of 100% buckwheat; lane 13: molecular weight standard; lane 14: buckwheat flour; lane 15: oat bran

3. Discussion

It has been proved that buckwheat and wheat prolamins differ. The in vitro results mentioned above suggested that buckwheat can be used in gluten-free diet. Although buckwheat seed is a source of well-balanced protein (POMERANZ & ROBINS, 1972), seeds have a high level of endogenous antinutrients, protease inhibitor and tannin persisting after cooking (IKEDA et al., 1991).

According to the findings of FRANCISCHI and co-workers (1994) the effect of buckwheat flour ingested by coeliac patients was evaluated by indirect immunofluorescence technique. Serum samples were collected 30 days after the flour ingestion. The assay has revealed that buckwheat flour is not present in the coeliac

patients. It was observed that anti-prolamin buckwheat antibodies have not been formed during a 30-day assay period. In spite of promising immunological results the allergenic properties have to be studied. KONDO and co-workers (1996) investigated the allergen in buckwheat antigen using the immunoblott method, radioallergosorbent test (RAST) and RAST inhibition assay. Results indicated that the 24 kD buckwheat protein was the major allergen among buckwheat antigens. Buckwheat and rice are frequent foods in Japan and they cause food allergy in some cases (YANO et al., 1989). Our present result suggested that the quantity of allergen could be decreased under baking conditions, however, it has not yet been proved whether muffin (cake made of 100% buckwheat flour, under the given technology) after digestion will contain reactive groups of 24 kD allergen protein or not. As a result of degradation of 30 kD protein in gut, heat resistant allergen peptides can form and might cause allergenic reaction, too. We are planning to continue our research work to know more about allergenic activity after digestion of the major fractions (24 kD, 30 kD) in bakery products.

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STUDIES OF THE POTATO MICROSTRUCTURE DURING BLANCHING

P. BONTOVICS[a] and A. SEBŐK

Campden & Chorleywood Food Industry Development Institute Hungary,
H–1094 Budapest, Márton u. 3/b. Hungary

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The texture changes of potatoes (variety Desire) at different blanching times (0–240 s) and temperatures (85 °C, 95 °C, 100 °C) were examined within this study.

After blanching the breaking forces (N) were determined with an INSTRON texture analyser (type 1140). The logarithm of the breaking force as a function of blanching time show that 3 different stages (0–40 s; 40–160 s; 160–240 s) were found at all temperatures (85 °C, 95 °C, 100 °C).

Photographs of potato cells were taken from scanning electron microscopy studies. Results show that the swelling and gelatinization of the potato starch has only little influence on the texture during the first (0–40 s) of the 3 different observed stages. In this initial stage the breaking forces increased with time.

The water uptake of the potato cells was determined gravimetrically. The results show that the increase of the breaking forces during the first 40 s results from the water uptake.

The examinations with the scanning electron microscopy and the texture analyser proved that the main reason for the changes in texture are due to softening and destruction of the cell walls. In the second (40–160 s) and third stage (160–240 s) of the blanching process a linear relationship was found between the logarithm of the breaking force and the blanching time.

Keywords: potato, blanching, texture, microstructure

The aim of blanching. Due to different activity losses of the enzymes with decreasing temperature, changes in colour, scent taste and aroma composition occur even at freezing temperatures. A minimum temperature for enzymatic reactions cannot be given. Activities for catalase and peroxidase were found at a temperature of –15 °C, for lipase and lipoxidase at –30 °C, and for invertase at –40 °C.

Most vegetables must be blanched before freezing to inactivate enzymes. Beside the inactivation at low energy consumption the blanching prevents the leaching of nutritive and effective substances. Air which is trapped within the cells is removed.

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[a] Present address: SOLVENT Kereskedőház Rt, 1097 Budapest, Gubacsi út 25-29. Hungary

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During blanching not only enzyme catalysed reactions are prevented but also the texture of vegetables changes. These textural changes can be measured by different test procedures. Among these are also analytical methods (HARADA & PAULUS, 1986). The most common methods are scanning electron microscopy and mechanical tests.

Many papers can be found that describe the textural changes. But by reviewing them it is not possible to associate the softening of the cell wall with only a few effecting factors. We separated the factors as product and process related.

Product related factors. Following WARREN and co-workers (1975) it is difficult to evaluate the influence of a single factor without looking at others. One of the most important factors that influences the texture of raw and blanched potatoes is the chemical composition (BOURTON, 1989; LINEHAN & HUGHES, 1969; WARREN & WOODMAN, 1973).

Starch: Much attention is paid to the relationship between starch content and texture. Still it is not possible to identify the role of the starch exactly.

MICA (1985) concludes that potato starch has not got a dominant influence upon the texture although potatoes with a high starch content have a firmer structure than those with a low starch content. It is shown that the texture is influenced rather by the physical properties of the starch than by its content.

REEVE (1977) describes cell separation by cell expansion as an important factor for the softening of cell tissues by computing microscopic photographs. During the gelatinization of the starch the volume of each starch granule increases. The volume increase builds up a pressure directed to the cells walls. This pressure is named swelling power (HAYDAR et al., 1980).

Whereas VOISEY (1976) infers from microscopic photographs of blanched potato cells that the influence of starch on the cell damage found is neglectable.

Process related factors. Following REEVE (1977) the softening of cell tissues is influenced by the processing of different potato species. NONAKA (1980) associate the changes with different blanching processes.

Structural changes during blanching: The softening of the cell tissues and therefore the textural changes can be quantified by determining the breaking or shearing force as a function of blanching time.

PAULUS and SAGUY (1980) found a logarithmic relationship between objective textural parameters and the blanching time at constant temperature. In a series of studies the entire range of blanching (0–15 min) was examined with larger time intervals between the single data points.

HARADA and co-workers (1985) used shearing force measurement to determine the cooking kinetics of different potato species (Bintje, Mentor, Desire) with different blanching temperatures (90 °C, 100 °C, 110 °C). The measurements helped to predict the cooking behaviour of different species.

VERLINDEN and co-workers (1995) developed a model which describes the textural changes of potatoes with first order reaction kinetics.

Results presented by BOURNE (1987) indicate that the softening of vegetable tissues (carrots, green beans) can also be described with first order reaction kinetics, but with different slopes for two different phases.

The structural changes during the first 4 minutes have so far not been studied, although this time interval plays the most important role for the blanching of vegetables in the frozen food industry. It was the aim of the study presented here to examine this initial period of the blanching process.

1. Material and methods

For the study fresh potatoes (variety Desire; storage 10 °C) from the regular supply of the Hungarian frozen food industry as test samples were used with a starch content of 14 to 17%, the dry substance 22 to 24 g/100 g. Cylindrical specimens (diameter: 25 mm; height: 14 mm) were cut from potatoes.

Samples (15 parallel) were blanched in a temperature regulated laboratory water bath (5 l) at different bath temperatures (85 °C, 95 °C, 100 °C) and different time intervals between 5 to 240 s and cooled in the air flow at 20 °C.

After blanching the breaking forces of 15 samples were determined at 25 °C using INSTRON texture analyser (Type 1140) with a cylindrical probe (diameter: 57 mm) and a crosshead speed of 200 mm min^{-1}. For the breaking force the first peak on the force-deformation curve was used. For the better understanding of the results samples of the raw potatoes were also measured.

Changes in the microstructure were additionally examined with a scanning electron microscope (TESLA BS 300). For the sample preparation potato pieces (3×5× 5 mm) were taken from the same position at different potatoes and fixed in a glutaraldehyd solution (5%) for 2 h. After that the fixing liquid was removed by washing the samples three times with a phosphate buffer solution (pH 7.2).

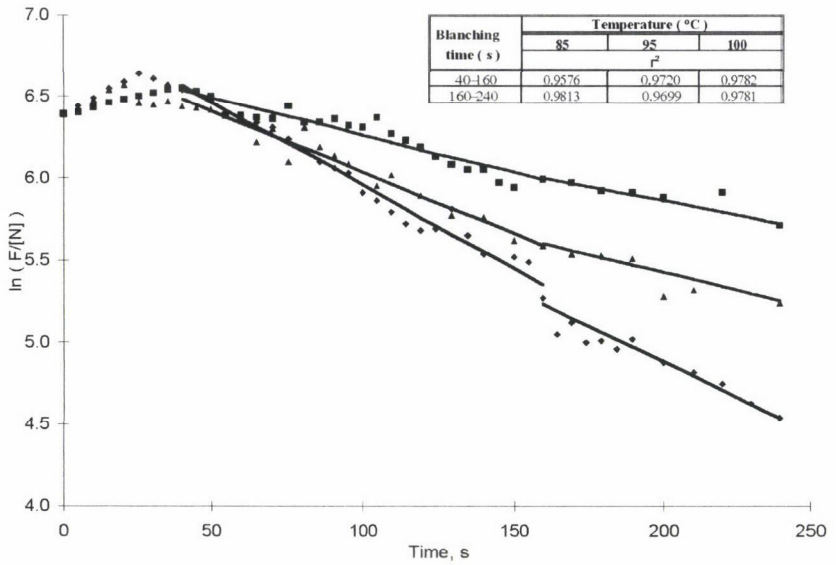
In a second fixing step an osmium-tetraoxid solution (1%) was used during a 1.5 h period followed by washing with the buffer solution as described before.

In a last step the potato samples were dehydrated with acetone and dried to the critical point with liquid carbon dioxide in BALZERS CPD 020.

A gold layer of 30 nm was sputtered on the dry potato samples using a cathodic sputter (BALZER SCD 040).

The starch molecules were removed during the samples preparation. The surface of the samples was cut away later in order to show the starch particles.

The water uptake (all measured data contain 15 samples analyses) of the potato cells was determined gravimetrically.



Blanching time (s)	Temperature (°C)		
	85	95	100
	r^2		
40-160	0.9576	0.9720	0.9782
160-240	0.9813	0.9699	0.9781

Fig. 1. Changes of the breaking force (ln F) of potatoes during blanching. ■: 85 °C; ▲: 95 °C; ◆: 100 °C, 0–240 s

2. Results

The changes in breaking forces for blanched potatoes were examined with the texture analyzer. A transient stage, which lasted for the first 40 seconds, was found at all temperatures (85 °C, 95 °C, 100 °C). During this transient stage the breaking forces increase as a function of blanching time (Fig. 1). After this initial phase two phases with different slopes were found for the examined blanching temperatures. The relationship between the logarithm of breaking forces and blanching times follows first order kinetics. The coefficients were determined using non-linear regression analysis (Jandel Scientific, Table Curve 2D for Windows). Correlation coefficient r^2 are plotted in Fig. 1 for the different phases.

A slight vaulting of the otherwise straight cell wall level was also found by REEVE (1977). He relates the vaulting to gelatinization of the starch.

The following results of the study presented here show that the increase of the breaking forces is not due to the gelatinization but to the water uptake of the potato cells.

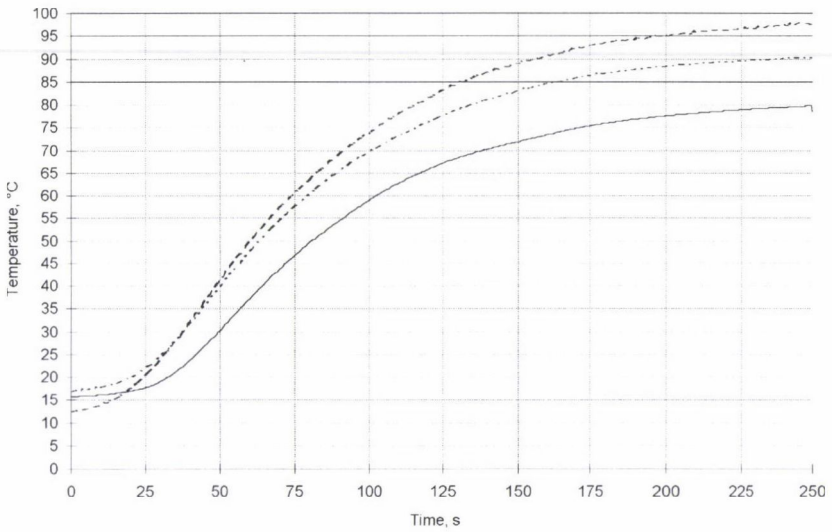


Fig. 2. Core temperature of a potato sample during blanching at —— 85 °C; - - - 95 °C; and — — 100 °C, 0–250 s

The core temperature of the potato samples was measured during blanching at 85 °C, 95 °C and 100 °C . The results are presented in Fig. 2. As Fig. 2 shows the maximum core temperature at 100 °C blanching temperature at 40 s reaching not more than 35 °C. Following PRAVISANI and co-workers (1985) even a temperature of 67.5 °C is not sufficient for the gelatinization of starch.

The examinations with the scanning electron microscopy support the statement that the increase of the breaking forces during the first 40 s is resulting from the water uptake.

Figure 3 shows that the potato cells take up water. As a results the intracellular pressure increases and the cells vault. Figure 4 shows the difference between raw potato cells (Fig. 4a) and vaulted potato cells (Fig. 4d). Only a small number of starch particles can be seen on the photographs (Fig. 4). Some of the starch molecules were removed during the sample preparation.

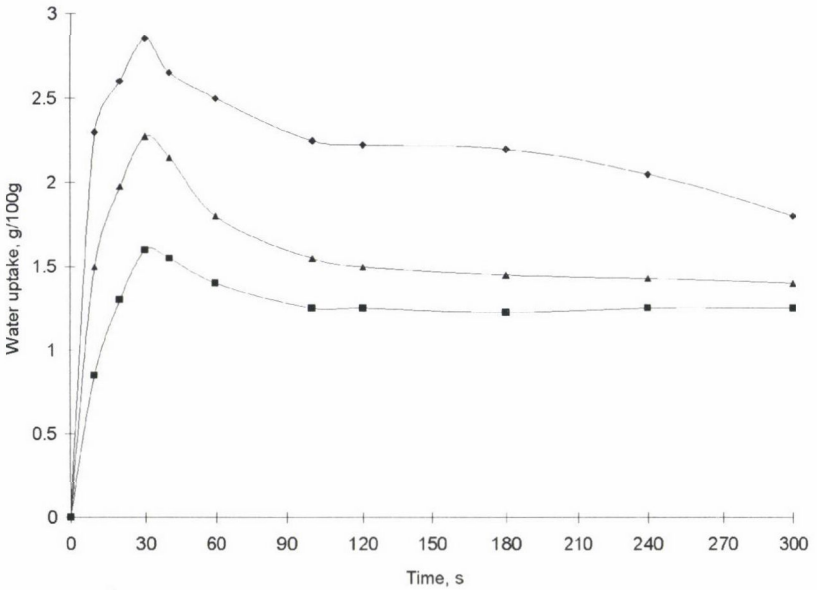


Fig. 3. Water uptake of potatoes during blanching. ■: 85 °C; ▲: 95 °C; ◆: 100 °C, 0–300 s

The surface of the samples was cut away later in order to show the starch particles (Figs 5 and 6.). This lead to the destruction of the cell walls. With the latter samples, examples are presented in Fig. 5, it is possible to prove that the starch remained intact within the cells even after 30 s of blanching. Therefore it can be stated that starch does not play an important role during this initial phase of blanching.

During the first linear phase between 40–160 s the starch has only limited influence upon the textural changes of potatoes.

When samples are compared after 120 s which were blanched at 95 °C and 100 °C (Fig. 6) it can be shown that at 95 °C not more than 50% of the starch gelatinized while at 100 °C nearly all of the starch gelatinized. As no statistically significant differences in the breaking force could be measured it becomes clear that the differences are not related to the starch. The decrease of the breaking forces in this phase can be explained with the softening of the potato cell tissues.

Changes in the texture which are related to the shrinking of the cell walls and to the softening of cell tissues are presented in Fig. 4f, g.

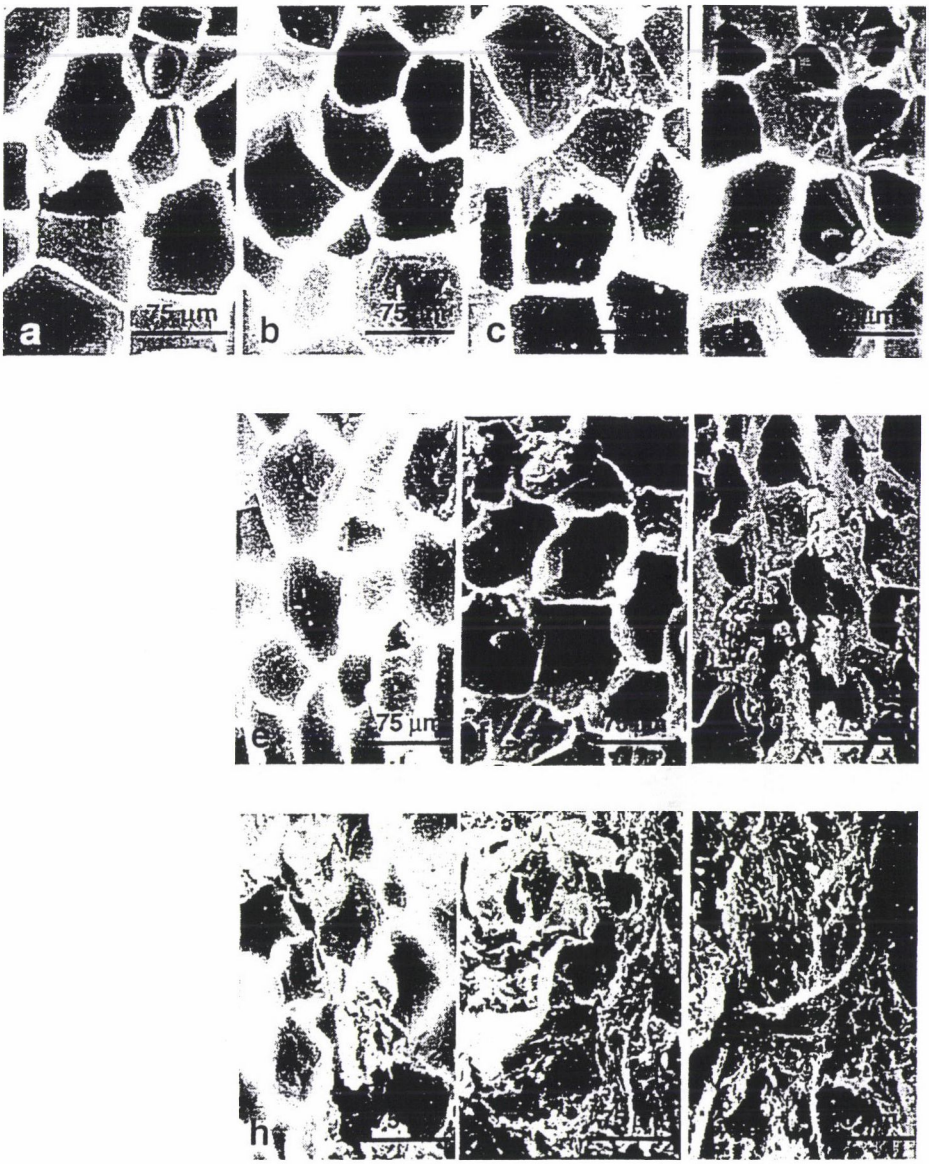


Fig. 4. Ultrastructure (SEM) of the heat-treated (in water) potatoes tissue. (Some of the starch molecules were removed during the sample preparation.)
a: raw; b: 85 °C, 30 s; c: 95 °C, 30 s; d: 100 °C, 30 s; e: 85 °C, 120 s; f: 95 °C, 120 s; g: 100 °C, 120 s; h: 85 °C, 240 s; i: 95 °C, 240 s; j: 100 °C, 180 s

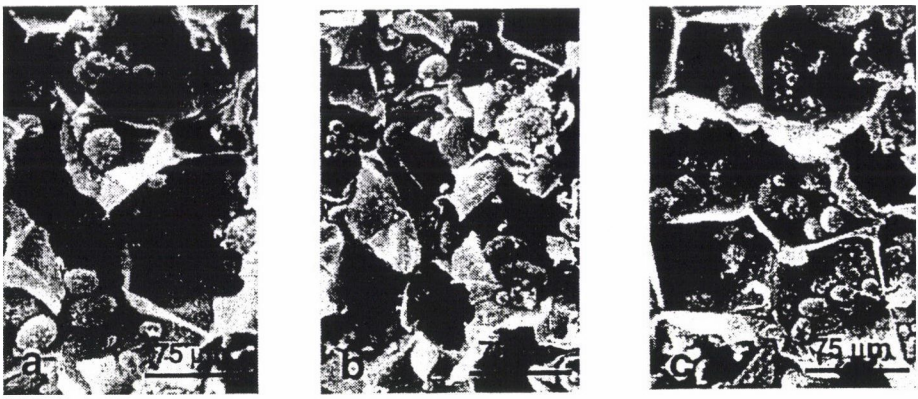


Fig. 5. Ultrastructure of starch particles in potato cells during blanching at different temperatures. (The surface of the samples was cut.)
a: 85 °C, 30 s; b: 95 °C, 30 s; c: 100 °C, 30 s

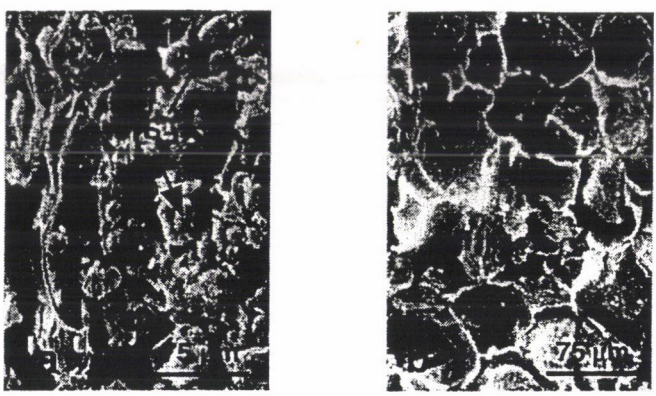


Fig. 6. Amount of gelatinization of starch particles in potato cells at different temperatures. (The surface of the samples was cut.)
a: 95 °C, 120 s; b: 100 °C, 120 s; S: starch particles

From the results presented so far it can be concluded that potato starch does not play a dominant role during the first two phases of blanching (0–160 s) at the examined temperatures. The entire cell wall structure is destroyed at 100 °C between a blanching time of 120 and 180 s. An amorphous matrix substance is shown in Fig. 4j. A very similar cell structure is obtained after blanching at 95 °C for 240 s (Fig. 4i). Also the

breaking forces were similar (Fig. 1) for those blanching conditions. Opposite to this the texture change is much slower at 85 °C (Fig. 4h). This slower change can be explained with the activity of the enzyme pectinmethylesterase and the protein denaturation (LIU et al., 1993).

On Fig. 4b, e thickenings in the cell walls can be seen which are due to the denaturation of proteins.

During the last phase (160 to 240 s) it cannot be excluded that the gelatinization of the starch plays an important role which leads to the amorphous structure at 95 °C and 100 °C.

Finally, it can be concluded that under none of the examined blanching temperatures the potato starch is the dominant factor for changes of the texture between 0 and 240 s.

3. Discussion

The objectives of this research work were to study structural changes in potatoes during blanching of different time intervals (0–240 s) at different temperatures (85 °C, 95 °C, 100 °C) and to explain the causes of changes found.

The influence of starch upon the structural changes as one of the possible causes was discussed. It could be shown that starch neither plays a dominant role during the transient initial phase (0–40 s) nor during the following phase where a linear correlation exists between the logarithm of the breaking force and the blanching time (40–160 s). During the last observed phase (160–240 s) the influence cannot be entirely excluded. Again, a linear relationship between the logarithm of breaking force and time is found but with a different slope. An increase in the breaking force at the beginning of the blanching can be explained with the water uptake. In the linear phases a decrease of the breaking forces can be observed which is related to the shrinking of the cell walls and the softening of the cell tissues.

It could be shown in this study that the observed changes are temperature dependent and are slower at lower temperatures. If the temperature dependent change of breaking forces is known, blanching processes can be optimised with respect to product quality.

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A KINETICAL APPROACH OF TEXTURE CHANGES OF VEGETABLES DURING BLANCHING

A. SEBŐK, P. BONTOVICS and M. BLESZKÁN

Campden & Chorleywood Food Industry Development Institute Hungary,
H–1094 Budapest, Márton u. 3/b. Hungary

(Received: 27 November 1998; accepted: 22 March 1999)

The texture changes during the initial phase of blanching of potatoes, carrots and green peas at different blanching times (0–240 s) and temperatures (85 °C, 95 °C, 100 °C) were investigated. The breaking force (N) was determined by compression or Back extrusion tests with an Instron texture tester. Electronmicroscopic studies (SEM) were made to support the interpretation of the results.

For each vegetable several sections of changes of the breaking force (lnF) were identified. For potatoes a three phase change of the breaking force by all temperatures (85 °C, 95 °C, 100 °C) was observed. In the first of the 3 different observed stages of potato blanching the breaking forces increased with time (0–40 s). In the second and third stage of the blanching process of potato a linear relationship was found between the logarithm of the breaking force and the blanching time. The second phase observed was faster (40–160 s) than the consecutive slower third phase (160–240 s).

For green peas the change of the breaking force (lnF) can be described by two consecutive first rate reactions. A faster decreasing period between 0–25 s was followed by a slower decreasing one between 70–140 s. Between the first and second stage of the blanching process there was an initial lag period (25–70 s), which will not be described here. In the period after 140 s, there was not any change, this period is constant.

For carrots the fastest change can be observed at 100 °C compared to the results at 85 and 95 °C. A three-phase curve was observed as well. An initial lag period (0–90 s) was followed by a faster (90–190 s) and a slower (190–240 s) decreasing one. In the second and third stage of the blanching process of carrots a linear relationship was found. For the changes of the breaking force (lnF) a kinetical approach was applied, reaction rate constants and apparent activation energies were calculated. The kinetical approach helps to compare and forecast changes at different process conditions.

Keywords: blanching, microstructure, reaction rate, texture, vegetables

The softening of texture of vegetables during blanching was described in details, but less information is available about the process and kinetics of the changes. Most of the authors described the kinetics of the softening by a single first order reaction (BOURNE, 1976; HARADA et al., 1985a, b). HARADA and PAULUS (1987) used first order

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kinetics to describe the changes of shear force of potatoes at different cooking parameters. KOZEMPEL (1988) found lag periods before the first order equation was applied for the changes of Back extrusion peak forces of potato samples during cooking. The Arrhenius plot was used to calculate kinetic constants. VERLINDEN and co-workers (1995) developed a compartmental kinetic texture model for potatoes during cooking, where all texture changes related to the degradation of the cell wall were described by an apparent first order reaction and the effect of starch gelatinization was described with an other first order reaction.

In plant tissues softening of the texture by cooking is caused by several factors: the loss of turgor pressure and occluded air, the degradation of middle lamella pectin and other cell wall polysaccharides and starch gelatinization (AGUILERA & STANLEY, 1990).

STANLEY and co-workers (1995) reported that in beans and carrots the main reason of softening was the thermal degradation of the middle lamella, which resulted in separation of the cells.

BOURNE (1987) found that during blanching of carrots and green beans between 0–40 min cooking time a rapid softening was observed in the first period, followed by a nearly linear softening. He proved that the softening curves are similar at higher and lower temperatures, however the actual firmness values differed significantly.

The objective of this study was to describe the first transitional period of textural changes during blanching, and to use these results for the interpretation of the changes.

1. Materials and methods

Potatoes (variety Desire; storage 10 °C), carrots (variety Chatenay; storage 10 °C) and green peas (variety Polar; storage 10 °C) from the regular supply of the Hungarian frozen food industry were used as test samples.

From potatoes cylindrical specimens (height 14 mm, diameter 25 mm) were cut out with a starch content of 14–17% and dry substance of 22–24 g/100g.

Green peas were used after vining and sieving. The starch content varied between 17.5–17.75%, the dry substance between 22.9–23.4 g/100g and the size between 8–9 mm.

From carrots cylindrical specimens (height 14 mm, diameter 25 mm) from xylem were cut. The dry substance of carrots was determined between 13–13.5 g/100g.

Samples (15 parallel) were blanched in hot water at 85 °C, 95 °C, 100 °C within a temperature regulated laboratory waterbath (5 l). Individual pieces (5 samples) of potatoes and carrots or a bulk of peas were taken out of the waterbath at different time intervals (5–240 s) and cooled in the air flow at 20 °C before the measurements were made.

The breaking force (15 samples) was determined at 25 °C by compression of individual cylinders of carrots and potatoes by an Instron 1140 Texture Tester (crosshead speed 200 mm min^{-1}) with a cylindrical probe (diameter: 57 mm) as the first peak on the force-deformation curve. For green peas the average height of the plateau of a Back Extrusion test was determined (crosshead speed 200 mm min^{-1}, cell gap: 4 mm).

The breaking force values measured by Instron were plotted against blanching time (Fig. 1, A). Results were plotted as ln F-t (Fig. 1, B), and kinetical rate constants were calculated from the linear part at different temperatures. The dependence of logarithmed breaking force on blanching time as described by the following equation is:

$$\ln F_t = \ln F_0 - k \times t$$

where F_t is the breaking force at any time t (N), F_0 is the breaking force of unblanched vegetables (N), k is the kinetical rate constant (min^{-1}), t is the blanching time (min).

The apparent activation energies were calculated from the slope of the ln k–1/T plot as well (Fig. 1C). The dependence of kinetical rate constant on blanching temperature as described by the following equation is:

$$\ln k_t = \ln k_0 - E_a/R \times 1/T$$

where k_t is the kinetical rate constant at any time (min^{-1}), k_0 is the kinetical rate constant of unblanched vegetables (min^{-1}), E_a is the apparent activation energy (J mol^{-1}), R is the gas constant (1.987 J mol^{-1}), T is the absolute temperature (K).

For the electron microscope studies the method of DAVIS and GORDON (1980) was followed: The samples were fixed with glutaraldelyd, washed with phosphate buffer, fixed with osmiumtetraoxide, washed with phoshate buffer and dehydrated with acetone, followed by dehydration with liquid CO_2 in BALZERS CPD 020. The dried samples were gold covered by BALZERS SCD 040 as in BONTOVICS & SEBŐK, 1999). For the electron microscopy studies a TESLA BS 300 scanning electron microscope was used (20 kV).

2. Results

The results showed that the process of texture changes during blanching of vegetables can be described by several phases and different patterns can be observed for different vegetables.

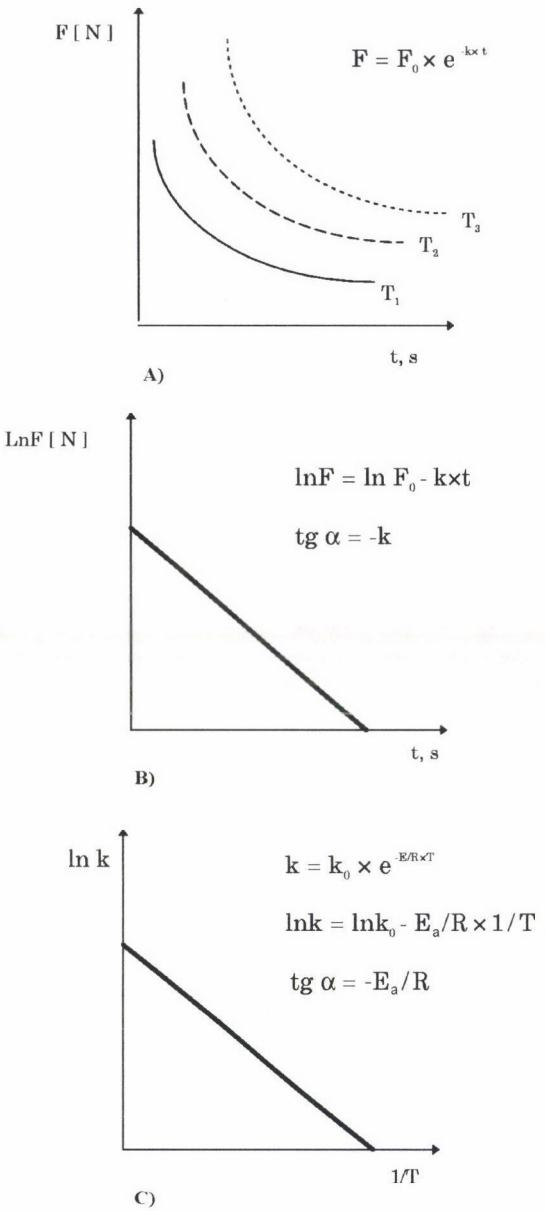


Fig. 1. Kinetical approach. F_t: breaking force at any time t; F_0: breaking force of unblanched vegetables; k: kinetical rate constant; k_t: kinetical rate constant at any time; k_0: kinetical rate constant of unblanched vegetables; t: blanching time; E_a: apparent activation enegry; R: gas constant; T: absolute temperature

For potatoes (Fig. 2) a slight increase of the breaking force was observed at the initial phase (0–40 s). At this phase higher blanching temperature results in higher maximum value. This first period (I. phase between 0–40 s) is followed by two linear phases on the semilogarithmic plot, the second phase (II. phase between 40–160 s) shows faster, the third phase (III. phase between 160–240 s) slower softening at 85 °C, where the difference of softening rate of the second and third phases wasn't significant, but the correlation coefficient shows that the three phases practically can be used, for a kinetical approach (BONTOVICS & SEBŐK, 1999).

Higher blanching temperature results in softer texture and faster softening rate. The apparent activation energies for these two linear phases (II. and III. phases) are of similar magnitude E_{II} (40–160 s) = 56.94 kJ mol^{-1} and E_{III} (160–240 s) = 58.24 kJ mol^{-1} (Table 1).

For green peas four phases were observed as well (Fig. 3.). A rapid softening in the first phase (I. phase) 25 s E_I (0–25 s) = 89.27 kJ mol^{-1} was followed by a lag period in the second (25–70 s) and a slower decrease in the third (III. phase) E_{III} (70–140 s) = 28.36 kJ mol^{-1} phases (Table 1), and then it ended (IV. phase between 140–240 s) in a very slow softening in the last period at all temperatures.

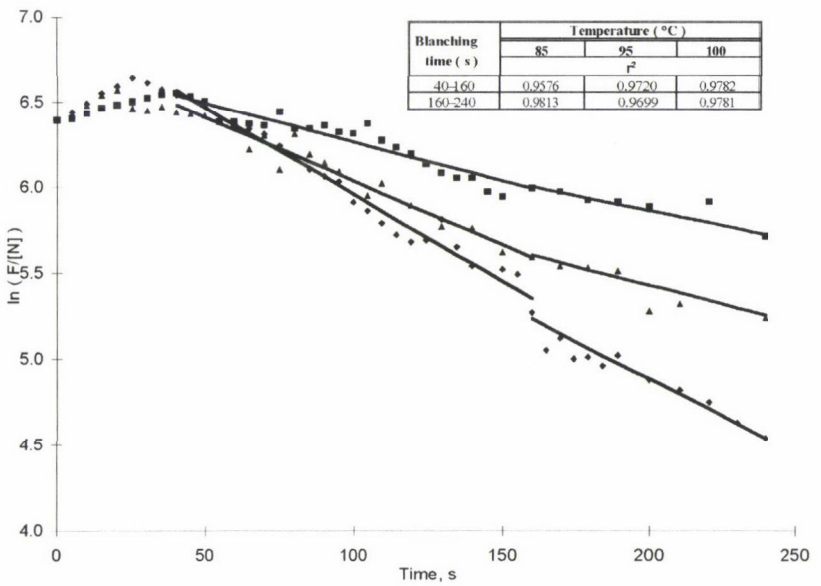


Fig. 2. Changes of the breaking force during blanching of potatoes. ■: 85 °C, ▲: 95 °C, ◆: 100 °C, 0–240 s

For carrots a three phase softening was observed as well (Fig. 4). In case of carrots a lag period was observed in the first 90 s, the texture is not constant even during the lag period, but an initial increase of firmness is followed by a decrease and an increase again before the faster softening period starts. Apparent activation energies were E_{II} (90–190 s) = 159.56 kJ mol^{-1} and E_{III} (190–240 s) = 66.99 kJ mol^{-1} (Table 1) respectively.

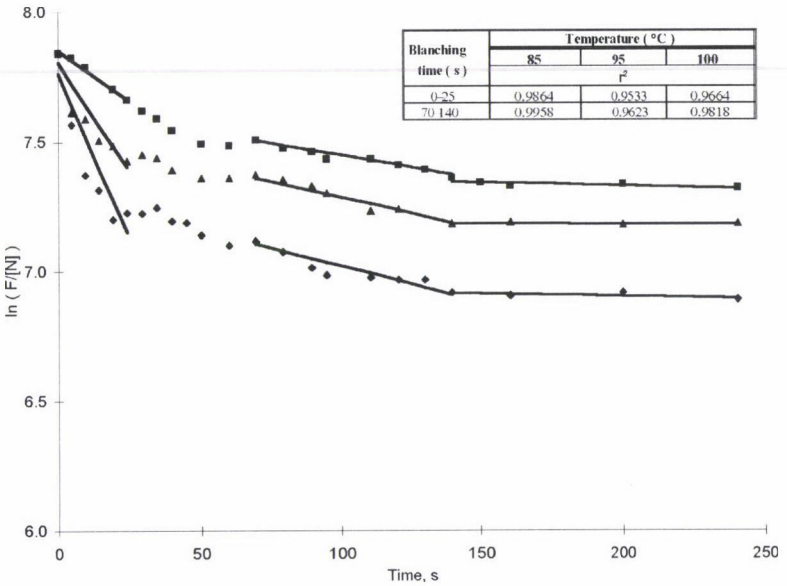
Electron microscopic photographs help to interpret the texture changes. During the cooking of potatoes at 100 °C (Fig. 5) at 30 s (Fig. 5b) the parenchyma cells are swollen as a result of the diffusion of water into the tissues but the cell walls are intact. These pictures explain that the first increase of the breaking force can be caused by the increased internal pressure of the tissues as a result of water uptake.

At 60 s (Fig. 5c) starch granules start to swell, and the thermal destruction of the cell walls has been started. Between 90–120 s (Fig. 5d–e) the rapid progress of thermal destruction of the middle lamella is well observable, while the swelling and gelatinization of the starch continue.

Between 160–180 s (Fig. 5f–g) the cell structure disappears and the process of starch gelatinization is nearly complete, which explains the third phase of the texture changes. At this phase the volume and the weight loss of the potatoes are nearly constant. This third phase is the initial part of that long softening period, which is described as nearly linear in a semilogarithmic plot in the majority of the studies, where larger time span of potato cooking (0–40 min) was investigated (HARADA et. al., 1985a; KOZEMPEL, 1988).

These results support the assumption that the softening of potato is caused mainly by the thermal destruction and decomposition of the pectin material and other cell wall polysaccharides, and by cell separation, while starch gelatinization has a secondary effect only.

For green peas where turgor pressure and starch structure have much more significance the first (Fig. 3, 0–25 s 85 °C, 95 °C, 100 °C) rapid decrease of the breaking force can be explained by the loss of turgor, and the start of the thermal destruction of the cell wall. This is well supported by the electron microscopic photographs (Fig. 6). At 25 s (Fig. 6b,c,d) starch granules start to swell and the process of starch gelatinization at 100 °C (Fig. 6d) is nearly complete. The second phase (Fig. 3 70–140 s) of the reduction of the breaking force may be caused by the gelatinization of the starch (the gelatinization of the starch continues Fig. 6e,f,g), while the third phase can be characterised by the progress of the thermal decomposition of the cell walls (Fig. 6h,i,j).



Blanching time (s)	Temperature (°C)		
	85	95	100
	r^2		
0-25	0.9864	0.9533	0.9664
70-140	0.9958	0.9623	0.9818

Fig. 3. Changes of the breaking force during blanching of green peas. ■: 85 °C, ▲: 95 °C, ◆: 100 °C, 0–240 s

For carrots the two processes i.e. the swelling of cells (Fig. 7b,c) caused by diffusion of water into the cells and the loss of turgor take place nearly parallelly (Fig. 7d,e), which result in the first apparent lag period. The phase of permanent softening may be connected to the heat destruction of the cell structure of the tissue, while the third phase may represent a slower decomposition of the cell wall polysaccharides (Fig. 7f,g).

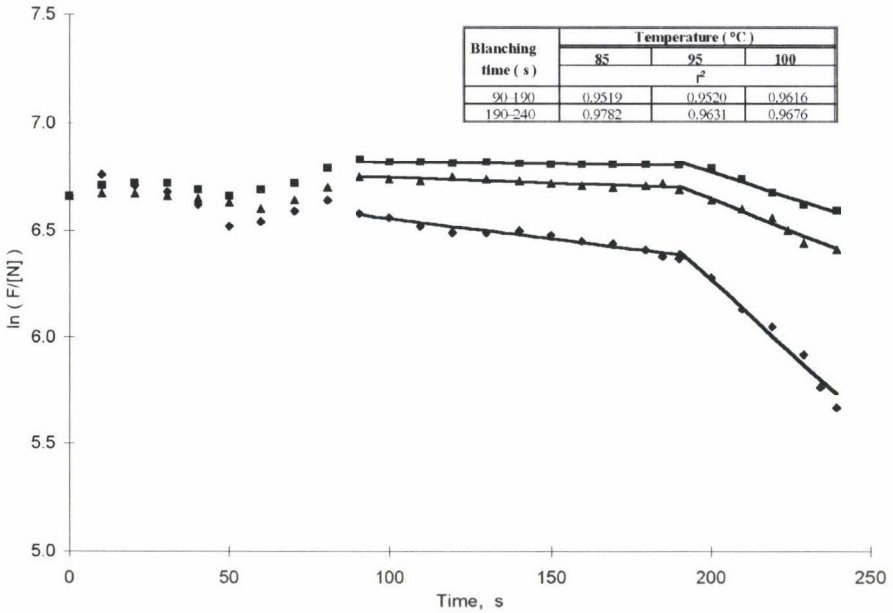


Fig. 4. Changes of the breaking force during blanching of carrots. ■: 85 °C, ▲: 95 °C, ◆: 100 °C, 0–240 s

Table 1

Apparent activation energies

Blanching time (s)	Activation energies (kJ mol^{-1})
	Potatoes
40–160	56.94
160–240	58.24
	Green peas
0–25	89.27
70–140	28.36
	Carrots
90–190	159.56
190–240	66.99

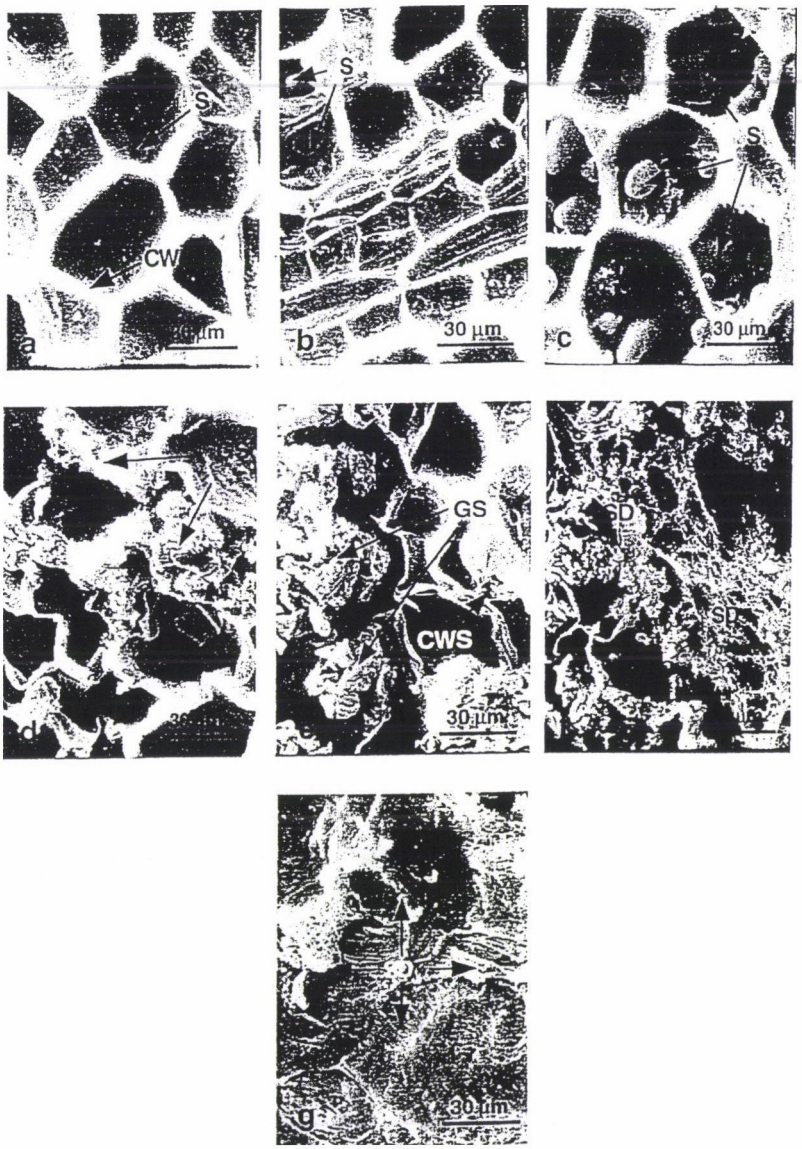


Fig. 5. Ultrastructure (SEM) of the blanched potato tissue (in water 100 °C). a: raw; b: 100 °C, 30 s; c: 100 °C, 60 s; d: 100 °C, 90 s; e: 100 °C, 120 s; f: 100 °C, 160 s; g: 100 °C, 180 s; S: starch granule; GS: gelatinized starch; SD: structural damage; CW: cell wall; CWS: cell wall separation

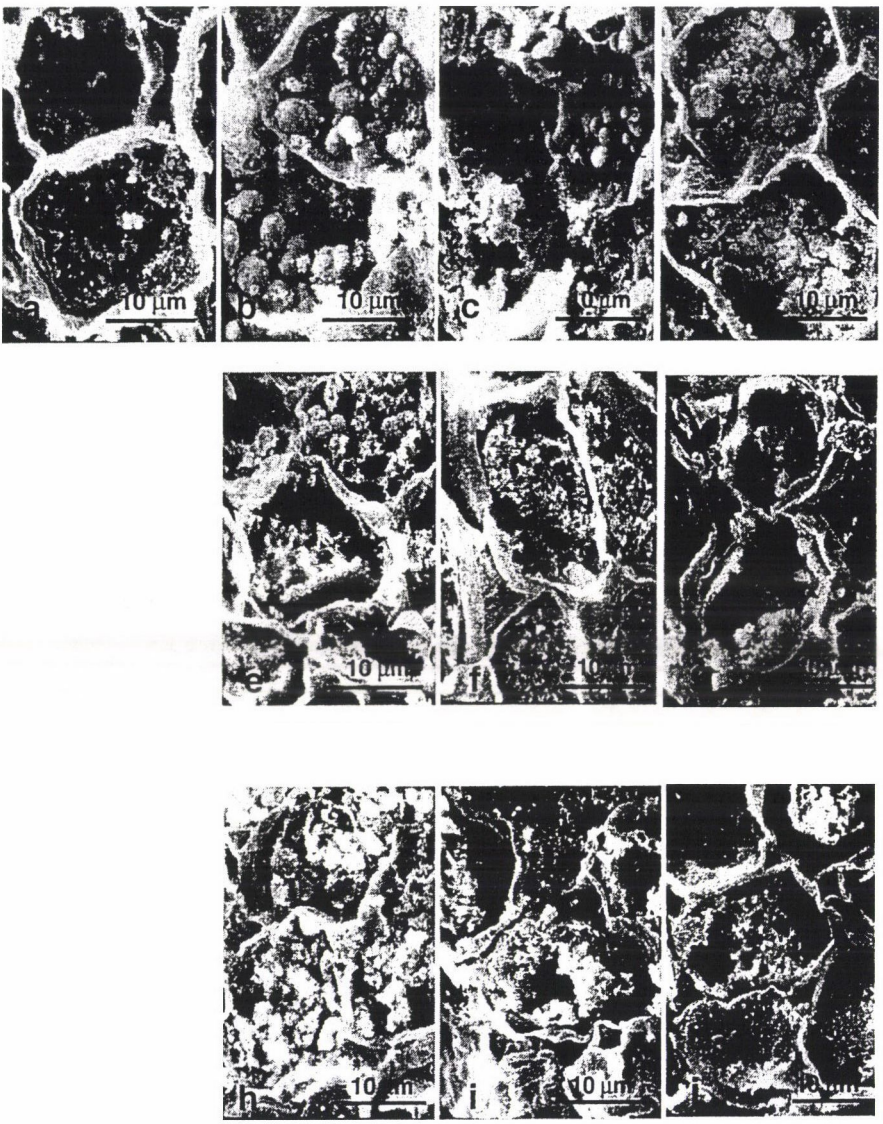


Fig. 6. Ultrastructure (SEM) of the blanched green peas tissue (in water 85 °C, 95 °C, 100 °C). a: raw; b: 85 °C, 25 s; c: 95 °C, 25 s; d: 100 °C, 25 s; e: 85 °C, 150 s, f: 95 °C, 150 s; g: 100 °C, 150 s; h: 85 °C, 240 s; i: 95 °C, 240 s; j: 100 °C, 240 s

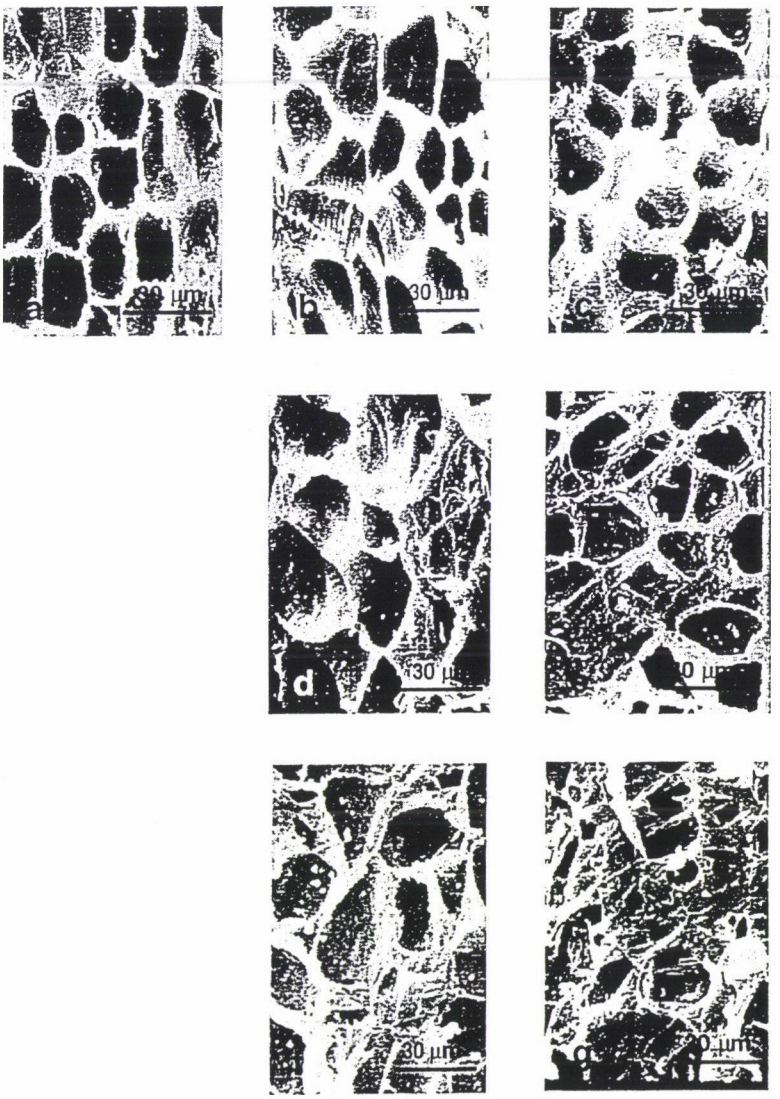


Fig. 7. Ultrastructure (SEM) of the blanched carrot tissue (in water 95 °C, 100 °C). a: raw; b: 95 °C, 60 s; c: 100 °C, 60 s; d: 95 °C, 120 s; e: 100 °C, 120 s; f: 95 °C, 240 s; g: 100 °C, 200 s

3. Conclusions

Results proved that the investigation of the initial transient phases of blanching of different vegetables provides useful information for the interpretation of the processes causing changes in the microstructure.

For each vegetable the texture change in the initial part of the blanching processes can be described as combinations of different sections. Most of these individual sections can be approximated by first rate kinetics.

These results confirmed that the softening of texture during blanching is mainly influenced by the thermal destruction of cell wall polysaccharides – potatoes, carrots – but the loss of turgor (peas, carrots, potatoes) and starch gelatinization may have an influence, which is significant in case of peas and less pronounced for potatoes.

The kinetical approach of the processes – the calculation of reaction rates and apparent activation energies – provides a valuable tool for comparison of the experimental curves and for better interpretation of the results.

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Short communication

GC-MS INVESTIGATION AND ANTIMICROBIAL ACTIVITY OF THE ESSENTIAL OIL OF *CARUM COPTICUM* BENTH & HOOK

M. SRIVASTAVA, A. SAXENA and P. BABY

Department of Chemistry, Lajpatrai (P. G.) College, C. C. S. University, Sahibabad - 201 005 (Ghaziabad). India

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The fruit oil of *Carum copticum* showed the presence of eleven components with carvacrol (45.20%) and δ–cymene (41.98%) as the major constituents by GC-MS analysis. The essential oil exhibited considerable in vitro antimicrobial activity with most of the gram-positive and gram-negative bacteria tested and the results are comparable with the standard bactericide. The pure oil inhibited the growth of *Phomopsis destructum*, *Aspergillus niger* and *A. flavus*.

Keywords: *Carum copticum*, agar overlay technique, zone of inhibition, microbicidal activity, essential oils

Carum copticum Benth & Hook (Syn. *Trachyspermum ammi* Linn.) is the Bishop's weed widely cultivated all over India (ASOLKAR et al., 1992). The seeds and its extractions are extensively administered in the Indian system of medicine as antiseptic, carminative and stimulant. MHASKAR and CAIUS (1931) suggested it as a remedy for snake bite. GEORGE and MARIAM (1957) investigated the plant for isolation of antibodies. QADRY and ATAL (1967) gave the pharamacognocy of its fruits. HANDA and co-workers (1986) formulated a liver protecting drug from the plant. SAXENA (1987) and BUCH and co-workers (1988) studied the effect of its volatile oil on ejaculated human spermatozoa.

RAMASASTRY (1983) reported Ca, Fe and its oxalate constituents. KARTA and KHAN (1969) isolated Δ^{5-6} and Δ^{7-8} octadecanoic acids from its seed fats. The effect of γ-rays on the thymol content of the oil was studied by THENGANA and DHYANSAGAR (1985) and GUPTA (1958) extracted demethylated azowan oil. MALLAIAH (1969) carried out the epoxidation studies of the oil and flavonoid pattern in its fruits was revealed by HARBORNE and WILLIAMS (1972).

Hot water infusion of the fruit is a household remedy for a number of intestinal disorders. On account of the folklore medicinal use of the azowan, its essential oil was analysed by the capillary GC-MS method, and in order to rationalise its curative properties the oil was screened for its antimicrobial activity in the present studies.

1. Materials and methods

The fruits for the present study were collected from the plants specially grown in our botanical garden. A voucher specimen was preserved in the herbarium of the botany department. The essential oil was extracted using the volatile oil extraction apparatus (Paul Scientific, Calcutta). It consists of one round bottom flask, where weighed quantity of fruits were placed and distilled. The vapours were condensed and the volatile oil isolated was separated that gave a yield of 4.52% w/w. The characteristically smelling, colourless etherial oil was stored at 4 °C after drying over anhydrous sodium sulfate.

The isolated volatile oil was fed to a gas chromatograph (GC) where it was partitioned between the stationary liquid and the mobile gaseous phases. A peak was registered when its components emerged. Based on the number and areas of peaks recorded, its components were qualitatively and quantitatively estimated. The eluants were passed to the mass spectrograph (MS) which registered its mass fragmentation pattern indicating its molecular structure.

GC-MS analysis was carried out with the combined GC-MS system consisting of a Hitachi 163 GC and Hitachi M-80A mass spectrometer. A DB-1 fused silica column (60 m×0.28 mm i.d.) was used with helium as carrier gas. The temperature was held at 70 °C for 5 min then programmed at 240 °C at 3 °C min^{-1} and ionization voltage was 20 eV with scan rate of 0.0445 scans s^{-1}. Peak identification was confirmed by comparing GC retention index and mass spectrum with that of an authentic sample. Mass spectral data searching was carried out on Hitachi 0101 data processor.

The in vitro antimicrobial screening of the oil was carried out by the agar overlay technique (JASSEN et al., 1986) adopting the modified Bondi's paper disc method (LOUIS & ANDERSON, 1989). Bacteria were maintained and grown in Potato Dextrose Agar (PDA) and the fungi in chloramphenicol agar media. All the tests were conducted in triplicate and the average zones of inhibition were recorded. Blank and control tests were also performed. Activity of standard fungicides and bactericides was also simultaneously assessed for comparison.

2. Results and discussion

The percentage of the components present in the seed are shown in Table 1 and the zones of inhibition with different microorganisms at various dilutions are given in Tables 2–4. It showed good bactericidal activity with *Staphylococcus pyogenes* which causes rheumatic fever and infections in respiratory tract and *Corynebacterium diphtheriae* that causes diphtheria, though the activity dimmed with dilution. The oil showed considerable activity with gram-negative pathogenic bacteria, *Escherichia coli* causing diarrhoea and gastroenteritis and aerobic sporeformers (ASF) causing general infections. The activity of the pure oil was comparable with those of standard bactericides.

Table 1

Volatiles of Carum copticum

Compound	%
α-Pinene	0.38
β-Pinene	1.38
Myrcene	0.15
δ-Cymene	41.98
1,8-Cineole	0.23
Limonene	0.26
γ-Terpinene	1.79
Terpinene-4-ol	0.22
α-Terpineol	0.11
Thymol	0.48
Carvacrol	45.20
Unidentified	3.90

Table 2

Antimicrobial efficacy of the essential oil of Carum copticum *against gram-positive bacteria at different dilutions*

S. No.	Name of the bacterium	Average diameter of zone of inhibition in mm					
		Bactericide[a]	Pure oil	Dilutions			
				1:100	1:250	1:500	1:1000
1	*Staph. pyogenes*	25	22	20	10	10	10
2	*Strep. pyogenes*	26	16	R	R	R	R
3	*Strep. pneumoniae*	28	18	10	10	10	10
4	*Mycobacterium sp*	24	14	R	R	R	R
5	*Corynebacterium sp*	22	22	16	14	14	10
6	*Bacillus sp*	24	20	16	9	9	9
7	*A.S.F.*	18	16	14	10	10	10

[a] Bactericide is Streptomycin sulfate 10 mg ml^{-1}

R indicates resistance

Table 3

Antimicrobial efficacy of the essential oil of Carum capticum *against gram-negative bacteria at different dilutions*

S. No.	Name of the bacterium	Average diameter of zone of inhibition in mm					
		Bactericide[a]	Pure oil	Dilutions			
				1:100	1:250	1:500	1:1000
1	*Escherichia coli*	22	22	14	8	8	8
2	*Bordetella sp*	20	R	R	R	R	R
3	*Shigella sp*	18	R	R	R	R	R

[a] Bactericide is Streptomycin sulfate 10 mg ml^{-1}

R indicates resistance

Table 4

Antimicrobial efficacy of the essential oil of Carum capticum *against fungi at different dilutions*

S. No.	Name of the fungi	Average diameter of zone of inhibition in mm[a]					
		Fungicide[b]	Pure oil	Dilutions			
				1:100	1:250	1:500	1:1000
1	*Rhizopus sp*	17	10	R	R	R	R
2	*Sordaria sp*	19	11	9	9	9	9
3	*Phomopsis sp*	22	12	9	9	9	9
4	*Aspergillus niger*	16	14	R	R	R	R
5	*Aspergillus flavus*	18	14	R	R	R	R

[a] Includes diameter of the paper disc (6 mm)

R indicates resistance

[b] Fungicide is Nystatin 50 mg ml^{-1}

Terpinene 4-ol was reported to be the active component of juniper oil, which is administered in urinary bladder infections. Similarly the use of thymol as medicine is mainly because of its activity against dermatophytes. Likewise the therapeutic value of the present oil may be attributed to δ-cymene and carvacrol, the major constituents. The therapeutic use of Bishop's weed is further confirmed by the present study.

*

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2nd International Symposium on Food Packaging Ensuring The Safety And Quality Of Foods, 8-10 November 2000, Vienna, Austria

Objectives of the meeting

Following the success of the Symposium on Food Packaging: Ensuring The Quality And Safety Of Foods held in Budapest in September 1996, this follow-up symposium will have the same objectives as the first, to look at the advances being made in the underlying science relating to the safety and quality of packaged foods, to disseminate results of on-going research and to stimulate debate, while examining the implications for the future. It will bring together those involved in basic studies, those responsible for bringing developments to the market place, and those charged with ensuring the safety and quality of the end-product.

Programme

The programme will consist of a number of invited keynote lectures together with contributed oral papers and a poster session. It will cover a wide range of subjects related to food packaging.

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EVALUATING OF SOME QUALITY PARAMETERS OF DIFFERENT APRICOT CULTIVARS USING HPLC METHOD

K. DOLENC-ŠTURM, F. ŠTAMPAR and V. USENIK

Institute for Fruit Growing, Viticulture and Vegetable Growing, Agronomy Department, Biotechnical Faculty, Jamnikarjeva 101, SLO-1111 Ljubljana, Slovenia

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High performance liquid chromatography (HPLC) was used for separation, identification and quantification of sugars, sugar alcohol sorbitol and non-volatile acid content in puree of 15 apricot cultivars (*Prunus armeniaca* L.). Sugar analyses included sucrose, glucose, fructose, xylose and sorbitol. The main organic acids characterizing apricot puree are malic, citric and fumaric acid. Variation in the content of the analyzed compounds mentioned and also in the content of soluble solids (SS), titratable acids and pH value was found among the cultivars.

Sensory evaluation showed that the content of individual organic acids and sugars as well as their relations could be crucial in forming the taste.

Knowledge of the qualitative and quantitative composition of acids and sugars in apricot fruits may prove to be a powerful tool in evaluating fruit maturity and quality.

Keywords: apricot (*Prunus armeniaca* L.), organic acids, sorbitol, sugars

Fruits represent an important and rich compound of healthy nutrition and are vital for human's health, well being and active life style. Fruits are generally high in dietary fiber and low in fats and oils, and are therefore important in diets that are designed to reduce the risk of coronary heart disease.

Stone fruits have long been appreciated for their gustatory and aesthetic qualities. In spite of these characteristics, they have not been highly favored as specimens in biochemical research. Nowadays, new chemical analyses allow a specific and objective determination of the content of individual substances in fruit of different varieties. Subjective estimations, which have been used to evaluate the quality of fruits from the visual aspect to sensory characteristics, are thus supplemented. Taste of an edible product, which is perceived by specialized taste buds on the tongue, represents one of the quality attributes consumers try to correlate with visual parameters of the products. Before the introduction of new apricot varieties in extensive production, growing and pomological characteristics have to be evaluated, because some important characteristics of varieties might depend on the environment. Of the many factors that

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can affect the taste quality of a product, cultivar, ripeness, maturity, irrigation and fertilization are especially important.

The content of soluble solids, free sugars, organic acids, minerals, vitamins, etc., fruit colour, taste and firmness of fruit depends on the activity of photosynthesis and the use of its products (FAUST, 1989). These compounds are common indicators of metabolic activity (HUDINA & ŠTAMPAR, 1998) or may indicate changes in quality, since sensory changes often accompany changes in organic acids, sugars and alcohol (DOYON et al., 1991). Chromatographic investigations of sugars and organic acids in different fruits were made by RICHMOND and co-workers (1981), of apricot cultivars by KOVÁCS and DJEDRO (1994) and by LO VOI and co-workers (1995), of raspberry cultivars by RIAZ and BUSHWAY (1994), of peach genotypes by BROOKS and co-workers (1993), of cherry cultivars by DOLENC and STAMPAR (1998) etc. High performance liquid chromatography (HPLC) is often the method of choice in quality control laboratories when analyzing for sugars (FÖLDHÁZI, 1994), sugar alcohols and organic acids. Recently, due to such advantages as rapidity, reliability, sensitivity and cost-effectiveness the method has been of increasing interest in the analysis of food constituents (MACRAE, 1981).

The modern industry relies on a rather narrow array of cultivars that are characterized by the size, firmness and eye appeal, and standards necessary for successful marketing. Consumers concern about poor taste is also increasing in certain European markets. Descriptions of end results, e.g. changes in soluble solids, colour and firmness of the fruit are of interest and value to the management of orchards (ROMANI & JENNINGS, 1971). BASSI and SELLI (1990) suggest two possible ways to improve fruit quality in apricots: developing new genotypes that express the flavour and taste before the fruit becomes too soft, and enhancing flavour in cultivars with firm fruits that never reach a satisfactory quality even fully ripe. The search for maturity indices results from the recurrent need to decide when fruits should be harvested. Knowledge of the qualitative and quantitative composition of acids and sugars in fruits may prove a powerful tool in evaluating fruit maturity and quality (MOLNÁR-PERL & MORVAI, 1992).

This paper presents the amount of total and individual sugars, titratable acids and individual acids in apricot fruits of different cultivars. Those results could be used together with optical measurements (i.e. fruit size, firmness, skin and mesocarp colour) to determine inner fruit quality, which is especially important for the popularity of individual cultivars by consumers being the aim of every fruit grower.

1. Materials and methods

Pomological characteristics were determined for 15 apricot cultivars: 'Laycot', 'Palummella', 'Pellecchiella', 'Tomcot', 'Hargrand', 'Icapi', 'Aurora', 'Bella d'Imola', 'Giada' 'Dulcinea', 'Harcot', 'Goldrich', 'Perla', 'Orange Red' and 'Sabbatani'. Apricots sampled on the location Stara Gora in the Fruit Growing Center Bilje were analysed at the Institute for Fruit Growing, Viticulture and Vegetable Growing of Biotechnical Faculty in 1998.

Apricots have been picked in identical conditions, at stage called "commercial maturity", i.e., when they are still having 10% of remaining green colour localized at stitch point.

Five fruits per cultivar were separately evaluated for aroma, sweet/sour ratio and eating quality by five panelists. Aroma and eating quality were assessed according to IBPGR (1984) apricot descriptors, where eating quality is a combined assessment of flavour, acidity, sweetness, aroma and astringency. From the results obtained a modus was calculated for each parameter.

1.1. Chemical and physical analyses

High performance liquid chromatography method was used for separation, identification and quantification of individual compounds in apricot puree. The HPLC system consisted of Thermo Separation Products (TSP) equipment with a model P1000 pump, autosampler model AS1000, column heater and OS/2 Warp IBM Operating system (1994)-work station. Solute elution was monitored using a variable wavelength UV detector set at 210 nm and differential refractive index RI (model Shodex-71RI).

Samples for sugar determination (glucose, fructose, sucrose, xylose and sorbitol) and organic acids (malic, citric and fumaric) determination were prepared from 1.5 kg of fresh apricots and then divided into three subsamples. They were converted into pulp individually by a mixer and homogenized with Ultra -Turrax T-25 (Ika-Labortechnik). The fruit puree (10 g) was diluted to 50 ml of bidistilled water and clarified by centrifugation at 6000 *g* for 15 min. The extract was filtered through 0.45 μm Millipore filters and a 20 μl sample was used for HPLC analysis of sugars and organic acids.

Sugars and sugar alcohol sorbitol analyses were performed isocratically on Aminex HPX-87C cartridge at a flow rate of 0.6 ml min^{-1} at a temperature of 85 °C with bidistilled and on-line degassed water used as eluent. Attenuation of the refractive index detector was set at 16x. Sugars present in each sample were identified by comparison of the retention time of each peak with those of standard sugars. The concentration of each sample was calculated by comparison of peak areas to the area of calibrated sugar solutions of known concentrations. The reproducibility of the chromatographic separation of the components was determined by making six injections

of the standard solutions and apricot sample. The results expressed as relative standard deviation (RSD%) are as follows: 0.29 for sucrose, 0.27 for glucose, 0.28 for fructose and 0.26 for sorbitol.

Organic acids were determined by HPLC analysis using an Aminex HPX-87H column, containing a 9 μm particle size, thermostated at 65 °C, which separates organic acids using primarily ion exclusion and reversed phase mechanisms. Using diluted 4 mmol sulfuric acid as eluent; organic acids elute from the column in order of increasing pK_a. Organic acids were identified and quantified by using a UV detector with wavelength set at 210 nm and by comparison of retention times and peak areas with standard solutions of known organic acids. Results of reproducibility study of chromatographic separation for organic acids expressed as relative standard deviation (RSD%) are as follows: 0.30 for malic acid, 0.31 for citric acid and 0.13 for fumaric acid.

A value for total acidity, expressed as malic acid monohydrate, was obtained by titrating a 10 g fresh sub-sample blended with distilled water (100 ml) to end-point pH 8.1 with 0.1 *N* NaOH. The soluble solids, expressed as Brix degrees, were determined in the juice of each sample using a refractometer at 20 °C.

1.2. Standard materials

Standards for sucrose, glucose, fructose, xylose and sorbitol as well as citric and fumaric acids were obtained from Fluka Chemical (New York, NY, U.S.A.). Malic acid was obtained from Merck Chemicals (Darmstadt, Germany).

Linearity of the response to UV and RI detection was tested for each compound with five different concentrations prepared in bidistilled water and all correlation coefficients were in the required range.

2. Results

The results (modus) of testing panel of tested apricot cultivars are presented in Table 1.

Fruits rich in aroma were those of cvs. 'Palummella', 'Pellecchiella', 'Laycot', 'Harcot' and 'Tomcot'. Fruits of cvs. 'Orange Red' and 'Perla' had little aroma, whereas the fruits of other cultivars demonstrated aroma of intermediate quality.

Fruits of 'Orange red' cultivar were very sweet but without the desired acid, whereas cultivar 'Sabbatani' demonstrated distinctly sour fruits. The best eating quality resulting from a combined assessment of flavour, acidity, sweetness, aroma and astringency was demonstrated by cvs. 'Laycot', 'Palummella' and 'Pellecchiella'.

Table 1

Results of testing panel of aroma, sweetness/sourness and eating quality of tested apricot cultivars

	Aroma	Sweetness/sourness	Eating quality
Laycot	rich	sweet-sour	excellent
Palummella	rich	sweet-sour	excellent
Pellecchiella	rich	sweet-sour	excellent
Tomcot	rich	sweet-sour	good
Hargrand	intermediate	sweet-sour	good
Icapi	intermediate	sweet-sour	good
Aurora	intermediate	sweet-sour	good
Bella D'Imola	intermediate	sweet-sour	good
Giada	intermediate	sweet-sour	good
Dulcinea	intermediate	sour-sweet	good
Harcot	rich	sour-sweet	fair
Goldrich	intermediate	sour-sweet	fair
Perla	little	sour-sweet	fair
Orange Red	little	sweet	fair
Sabbatani	intermediate	sour	fair

Table 2

The content of sugars and sorbitol in apricot puree

	Sucrose (g/kg)	Glucose (g/kg)	Fructose (g/kg)	Xylose (g/kg)	Sorbitol (g/kg)
Laycot	66.0	29.0	13.4	5.0	6.1
Palummella	55.2	37.3	12.3	3.6	0.8
Pellecchiella	74.1	28.1	14.0	4.8	6.4
Tomcot	61.4	30.5	14.8	4.7	5.2
Hargrand	79.8	31.9	11.0	5.5	5.2
Icapi	65.8	32.4	20.5	1.5	2.0
Aurora	43.4	23.9	6.4	0.4	0.2
Bella D'Imola	34.5	34.2	13.4	7.4	2.9
Giada	58.6	29.1	12.7	1.4	0.4
Dulcinea	65.1	35.1	15.4	5.0	10.2
Harcot	79.5	22.7	10.1	5.6	4.8
Goldrich	63.5	31.1	12.9	4.7	2.4
Perla	45.3	36.5	14.6	4.3	0.3
Orange Red	54.9	35.4	17.5	4.3	2.8
Sabbatani	45.3	35.2	11.7	5.8	2.6
min.	34.5	22.7	6.4	0.4	0.2
average	59.5	31.5	13.4	4.3	3.5
max.	79.8	37.3	20.5	7.4	10.2
STD	13.3	4.4	3.2	1.9	2.8

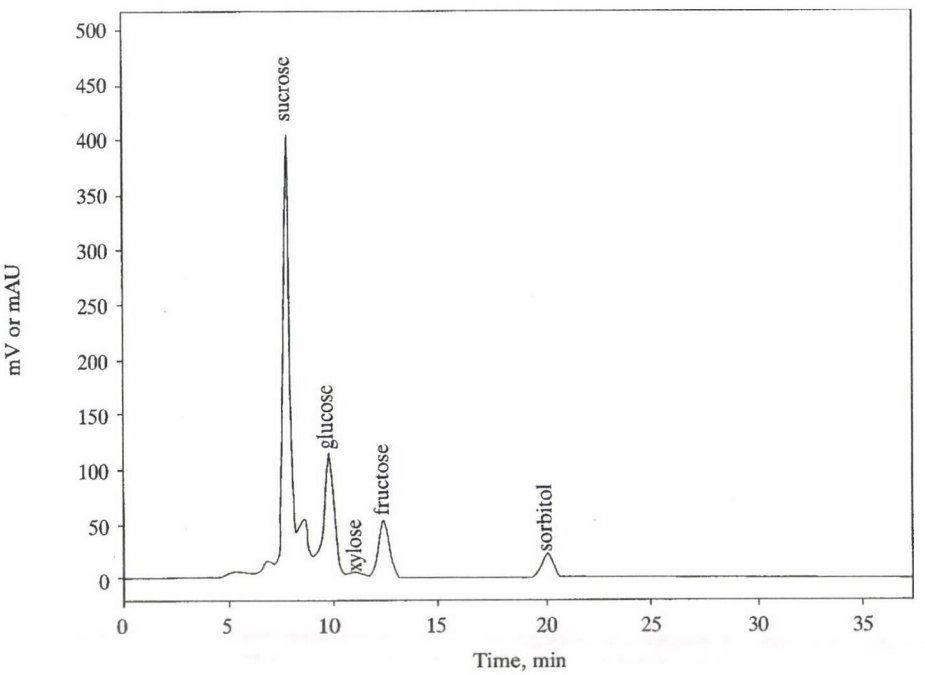

Fig. 1. Chromatographic separation of sugars in apricot puree. Column: Aminex HPX-87C (300×7.8 mm); column temp. 85 °C; mobile phase: BD water; flow rate 0.6 ml min^{-1}, refractive index detector

The analytical data on the carbohydrate fraction of 15 apricot cultivars are presented in Table 2. Each value is a mean of three measurements (one measurement per subsample). The content of sucrose in analyzed samples varied from 34.5 g/kg with cv. 'Bella d'Imola' to 79.5 and 79.8 g/kg in cvs. 'Harcot' and 'Hargrand', respectivelly. The variability in the content of glucose ranges from 22.7 g/kg with cv. 'Harcot' to 37.3 g/kg in cv. 'Palummella' with a mean of 31.5 g/kg for the different cultivars tested. The amount of fructose in the apricot puree varied from 6.4 g/kg in cv. 'Aurora' to 20.48 g/kg in cv. 'Icapi' with a mean value of 13.4 g/kg. Xylose content is also very variable; its range is from 0.4 g/kg to 7.4 g/kg, with an average value of 4.3 g/kg.

The content of sugar alcohol sorbitol was extremely low in cultivars 'Aurora', 'Perla' and 'Giada', whereas the content of sorbitol in cv. 'Dulcinea' was 10.2 g/kg. Normally, with larger amount of soluble solids in apricot cultivars, the content of individual sugars and especially sorbitol, increased too.

Figure 1 shows the chromatogram of sugars in apricot puree.

The analytical data on individual organic acids, total acidity (g/kg) and pH value of apricot puree are presented in Table 3. Each value is a mean of three measurements (one measurement per subsample). Malic acid content varied from 10.9 g/kg in cultivar 'Pellecchiella' to 32.6 g/kg in cultivar 'Aurora'. The mean value was 18.9 g/kg. The citric acid content was from 0.5 in cv. 'Sabbatani' and 0.6 in cv. 'Aurora' to 20.3 g/kg in 'Laycot' with an average value of 11.6 g/kg.

The average value of fumaric acid was 16.8 mg/kg, with the lowest content in cultivar 'Laycot' (9.9 mg/kg) and the highest content in cv. 'Perla' (30.0 mg/kg). Mean value for total acidity in apricot puree was 37.6 g/kg. The highest content of free acids was obtained in cvs. 'Aurora' and 'Bella d'Imola' and the lowest content of free acids were in cvs. 'Icapi' and 'Orange Red'. The mean pH value for analyzed apricot puree was 3.6, with a lower value 3.2 in 'Aurora' and upper value 3.9 in cultivar 'Pellecchiella'.

Table 3

The content of organic acids, pH value and total acidity in apricot puree

	Malic acid (g/kg)	Citric acid (g/kg)	Fumaric acid (mg/kg)	pH value	Total acidity (g/kg)
Laycot	14.3	20.3	9.9	3.5	44.3
Palummella	18.9	14.7	21.3	3.3	43.2
Pellecchiella	10.9	16.3	20.6	3.9	30.4
Tomcot	15.8	17.8	12.8	3.6	40.9
Hargrand	18.1	9.9	17.7	3.8	30.3
Icapi	11.0	11.0	16.2	3.5	24.6
Aurora	32.6	0.6	13.3	3.2	54.3
Bella D'Imola	30.8	6.5	18.6	3.3	50.0
Giada	15.4	11.0	19.9	3.7	31.1
Dulcinea	18.4	16.7	12.8	3.5	41.3
Harcot	21.4	14.6	12.7	3.5	39.3
Goldrich	16.4	15.4	17.0	3.5	42.5
Perla	16.6	7.2	30.0	3.7	27.6
Orange Red	15.5	12.2	16.3	3.8	26.9
Sabbatani	27.7	0.5	12.2	3.4	36.6
min.	10.9	0.5	9.9	3.2	24.6
average	18.9	11.6	16.8	3.6	37.6
max.	32.6	20.3	30.0	3.9	54.3
STD	6.8	11.6	5.0	0.2	8.8

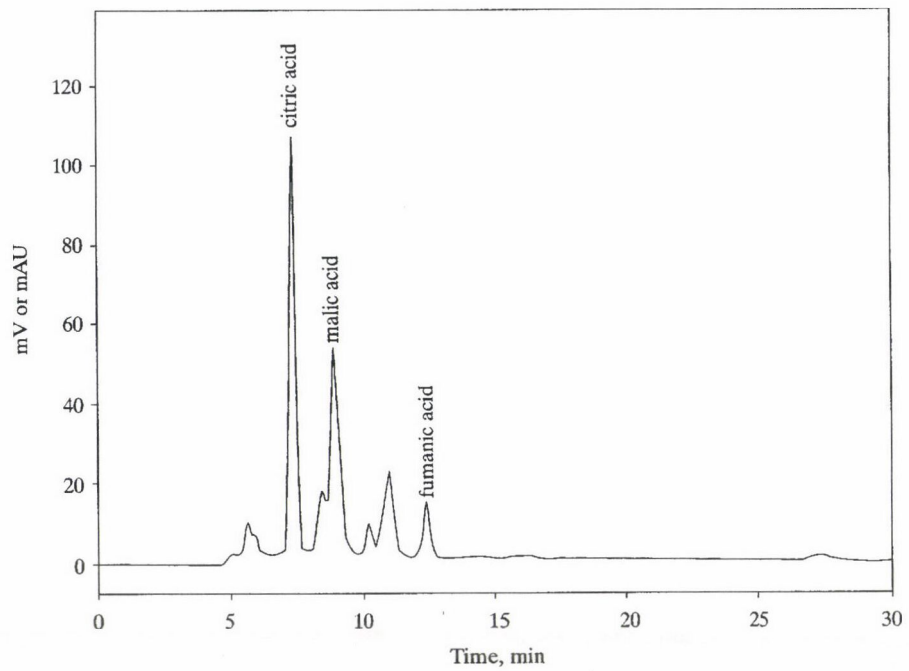

Fig. 2. Chromatographic separation of organic acids in apricot puree. Column: Aminex HPX-87H (300×7.8 mm); temp. 65 °C; mobile phase: 4 mmol sulphuric acid; flow rate 0.6 ml min^{-1}; UV detector at 210 nm

Figure 2 shows the chromatogram of organic acids in the apricot puree.

Analytical data on soluble solids (SS), total sugars (g/kg), glucose/fructose, Brix°/acidity and malic/citric ratio in apricot puree are reported in Table 4. The content of soluble solids (SS) of different apricot cultivars was from 10.2 to 18.2 degrees of Brix. Cultivars 'Aurora', 'Giada' and 'Perla', ripening early in June had the lowest content of SS, whereas the cv. 'Dulcinea' had the highest content (18.2 Brix°) of soluble solids. The average content of soluble solids was 13.8 Brix°.

The average glucose to fructose ratio of tested cultivars was 2.5, with the lowest ratio in cv. 'Icapi' and the highest ratio in cv. 'Aurora' (3.7). Those ratios are usually significant for varieties and lower values of that ratio could easily be linked to abnormalities in the storage process. Higher ratios in products can indicate that glucose was added to hide high acidity. The average value of sweet/sour ratio is calculated by dividing the sugar content of fruit, expressed as degrees Brix, by its percentage of acids.

Table 4

The content of soluble solids (SS), total sugars (g/kg), glucose/fructose, Brix°/acidity and malic/citric ratio in apricot puree

	SS Brix°	Total sugars (g/kg)	Glu./fru. ratio	Brix°/acidity ratio	Malic/citric acid ratio	Total acidity/ malic acid
Laycot	15.2	119.6	2.2	3.5	0.7	3.1
Palummella	12.2	109.1	3.0	2.8	0.8	2.3
Pellecchiella	14.2	127.4	2.0	4.7	0.7	2.8
Tomcot	12.6	116.6	2.1	3.1	0.9	2.6
Hargrand	17.2	133.2	3.0	5.7	1.8	1.7
Icapi	14.6	122.2	1.6	5.8	1.0	2.2
Aurora	10.2	74.3	3.7	1.9	55.2	1.7
Bella D'Imola	13.6	92.4	2.6	2.7	4.7	1.6
Giada	10.2	102.2	2.3	3.3	1.4	2.0
Dulcinea	18.2	130.7	2.3	4.4	1.1	2.2
Harcot	17.0	122.2	2.2	4.4	1.5	1.8
Goldrich	14.2	114.6	2.4	4.6	1.1	2.6
Perla	10.2	101.1	2.5	3.6	2.3	1.7
Orange Red	12.8	144.9	2.0	4.7	1.3	1.7
Sabbatani	14.2	100.6	3.0	3.8	53.0	1.3
min.	10.2	74.3	1.6	1.9	0.7	1.3
average	13.8	107.6	2.5	3.9	8.5	2.1
max.	18.2	144.9	3.7	5.8	55.2	3.1
STD	2.5	32.2	0.5	1.1	18.5	0.5

The Brix°/acid ratio for apricot fruit ranged from 1.9 for cv. 'Aurora' to 5.7 and 5.8 in cvs. 'Hargrand' and 'Harcot', respectively. So low ratio in cv. 'Aurora' is mostly due to low soluble solids content and high total acidity content.

The ratio between the content of malic and citric acid varied from 0.7 in cvs. 'Laycot' and 'Pellecchiella' to 53.0 in cv. 'Sabbatani' and 55.2 in cv. 'Aurora'. Such variability is almost due to high malic acid content in those cultivars. It is interesting that cultivars with low ratio ('Laycot', 'Pellecchiella' and 'Palummella') were very tasteful, whereas the cv. 'Sabbatani' was found to be acidic. The average value for the ratio between total acidity and the content of malic acid was 2.1, but cultivars with higher values were more appreciated.

3. Discussion

Taste of a fruit is largely determined by a balance between the sugar and acid contents. Low acid and high sugar contents result in a bland taste, and high acid and low sugar contents give a sour taste. The content of soluble solids is the function of several factors of which total sugars and organic acid constitute the major part (SHARMA & SHARMA, 1990). The average content of soluble solids (13.8 Brix°) of investigated cultivars was higher in comparison with results of LO VOI and co-workers (1995), who found an average value of 10.6. Sucrose was the major sugar in apricots with glucose and fructose present in higher amounts too, whereas xylose was present as a minor component. Comparison of results with the data of WILLS and co-workers (1983) and LO VOI and co-workers (1995) shows, that sugars (glucose, fructose and sucrose) in analyzed cultivars are present in higher amounts. In addition to the variety influence on the composition of the fruit, weather and nutritional conditions may also affect the concentration of the individual compounds and may cause an increase in sugar and a decrease in acid content, especially in citric acid (RIAZ & BUSHWAY, 1994).

High accumulation of sugars in the fruit is a result of the direct translocation of sorbitol from nearby leaves (WILLIAMS et al., 1967). Sorbitol is a specific and major translocation carbohydrate in mature apricot leaves (BIELESKI & REDGWELL, 1985). In fruits it is not accumulated but continuously converted into fructose, sucrose and glucose (ACKERMANN et al., 1992). During the early stages of growth, reducing sugars like glucose and fructose predominate, while sucrose appears at the start of second phase of growth and thereafter increases along with reducing sugars till picking maturity (NIGAM & SHARMA, 1987). The variability of sorbitol in the investigated apricot cultivars ranges from 0.2 to 10.2 g/kg with a mean value of 3.5. These findings are in agreement with the literature, although the variations are higher than those reported by LO VOI and co-workers (1995). It is quite interesting that cultivars with the highest sucrose content ('Harcot', 'Hargrand' and 'Pellecchiella') demonstrate lower content of glucose and fructose. Fruits of cv. 'Sabbatani' inspite of subsequent chemical determination of high content of glucose and xylose were evaluated as very sour during the tastings. Such an estimate is explained by the fact that fructose was found to be sweeter than sucrose and sucrose sweeter than glucose (BASSI et al., 1996). Sorbitol probably has no special impact on the sweetness, and has the relative sweetness index of 50–60% if compared to sucrose (RAPAILLE et al., 1993). Initially, polyols like sorbitol and mannitol were used to sweeten food for diabetics. Alcohol sugar sorbitol has an advantage over sucrose in dietetic nutrition (RAPAILLE et al., 1993), because the different metabolism of sorbitol in comparison to other carbohydrates results in lower energetic input to the body.

Organic acids are important constituents of fruit. Apart from being important organic metabolites (KAYS, 1991), they play a decisive role not only in the determining the quality of fruit and form but also important part in human diet (SHARMA & NIGAM, 1992). The most important acids in apricot fruit are malic and citric acids, whereas fumaric acid is only a minor acid, contributing to the taste. The average content of citric acid (11.6 g/kg) is in agreement with results reported by LO VOI and co-workers (1995), who found citric acid levels of 12.0 g/kg, whereas the malic acid levels (18.9 g/kg) were higher than those reported by the same author (11.8 g/kg). In appreciation of sensory qualities of apricots, acidity is a determining element of choice, especially as its level and alterability after picking will greatly modify the consumers' reactions. To characterize it, we have just used two criteria: pH and titratable acidity content, which correspond to the content of free organic acids in the pulp of fruit. Varieties having high free acidity and low level of total sugars would hardly be accepted by consumers, because cultivars with higher content of citric acid (cvs. 'Palummella, 'Pellecchiella', 'Tomcot' and 'Laycot') were more appreciated in testing panel than cv. 'Sabbatani', the latter with higher content of malic acid. Compared with citric acid, malic acid has a much stronger apparent acidic taste. From this point of view we could conclude that the optimal ratio between malic and citric acid is near the value of 0.8.

4. Conclusions

Quality of a fruit is a subjective and complex parameter, therefore it is difficult to define, formulate and determine solid parameters, both in terms of production/marketing context and research. It is to be mentioned that only recently quality has been given a high priority as a price determinant by producers and consumers due to the combination of increased production, increased availability, and increasing consumer acquaintance.

Values from chemical analyses are useful for screening new cultivars and breeding selections for their potential acceptability, or for growers as targets to ensure that cultivation practice favourably affects fruit quality. In addition to the aforementioned objective parameters, in some countries other objective factors, which contribute to consumer preference, are considered important. These can include the combinations of the shape or form, the colour, the consistency of the pulp, the taste of the fruit that is often particular and closely linked to local habits and customs. Also, more attention should be given to correlating sensory evaluations and chemical composition of the fruit in order to identify the components that have major influences on consumer acceptance.

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AN IMPROVED METHOD FOR THE DETERMINATION OF SULPHACHLOROPYRAZINE IN MEAT AND LIVER OF BROILERS DURING AND AFTER THEIR TREATMENT FOR COCCIDIOSIS

LJ. KOSTADINOVIĆ[a], S. PAVKOV[a] and F. GAÁL[b]

[a] Scientific Veterinary Institute, 21000 Novi Sad, Rumenački put 6. Yugoslavia
[b] Institute of Chemistry, Faculty of Sciences, 21000 Novi Sad, Trg D. Obradoviča 3. Yugoslavia

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The paper presents results of the HPLC determination of sulphachloropyrazine residues (active component of the drug "Esb_3 30%") in muscle tissue and liver of broiler chickens inoculated with laboratory-grown coccidium in the course and after treatment with this sulphonamide.

Extraction of sulphachloropyrazine from samples of broiler muscle tissue and liver was carried out with a mixture of solvents dichloromethane-methanol-acetic acid (90:5:5, v/v/v), followed by extract purification by chromatographic separation on a XAD-2 column and elution of sulphachloropyrazine residues with dichloromethane. The HPLC determination of sulphachloropyrazine residues was accomplished on a Bio Sil C-8 HL 5 µm column with a mobile phase consisting of 60% aqueous solution of acetonitrile and NH_3 (pH=9.5), using a UV detector at 254 nm.

The method developed allows quantitative determination of the residues of the anticoccidial agent in broiler tissue samples with a detection limit of 0.02 µg g^{-1}. Recovery of the method for this type of samples with a complex matrix was satisfactory, the results ranging from 79.2±0.6 to 86.7±0.2% for muscle tissue and from 81.7±0.8 to 87.3±0.7% for liver.

Keywords: broiler, HPLC, sulphachloropyrazine, tissues

Coccidiosis is an infective disease of the digestive tract which is most frequent with poultry, causing a decrease in daily increment, prolonged fattening, poorer skin pigmentation, slower feed conversion and increased mortality. The disease is caused by Protozoas from the genera of *Eimeria*, *Isospora* and *Cryptospora*, and it is manifested by damaging the intestine epithelial cells, less frequently the bile duct and renal tubuli (RADOSTITS et al., 1994). If coccidiosis is manifested in its clinical form, the treatment is most often accomplished by sulphonamide-based derivatives, i.e. the derivatives of p-aminobenzolsulphonic acid (KATZUNG, 1995).

0139-3006/99/$ 5.00

Sulphachloropyrazine, 4-amino-N(chloropyrazinyl)monosodium, monohydrate (active substance of the drug "Esb_3 30%" – Novartis) is a sulphonamide of a wide spectrum of action, capable of stopping the development cycle of coccidia and bacteria and thus their propagation, blocking primarily the synthesis of folic acid (BEVILL, 1988).

As chicken meat plays an important role in human nutrition, it is of essential importance from the health point of view to study the presence of sulphachloropyrazine in muscle tissue and liver of broilers during their treatment and in the post-treatment period. By determining the sulphachloropyrazine residues and establishing the withdrawal time, the risk of introducing this sulphonamide into the organism is diminished.

In the methods for determining sulphonamides in meat and meat products and other biological materials the most important, and at the same time the most difficult, step is the process of extraction and extract clean-up to remove matrix components that could interfere in the sulphonamide determination.

In the last decade or so, a number of instrumental techniques have been introduced for the determination of sulphonamide residues, such as thin-layer chromatography (THOMAS & SOROKA, 1982; HAAGSMA, 1985), immuno-enzymatic method (NOUWS et al., 1985), supercritical fluid chromatography (GUGGISBERG et al., 1992), and gas chromatography coupled with mass spectrometry (STOUT et al., 1984; SIMPSON et al., 1985). However, the most prominent place among them is occupied by high performance liquid chromatography (HPLC). By this method, sulphonamide residues are determined either directly after the separation on a chromatographic column using a UV detector (VAN'T KLOOSTER et al., 1991; KOSTADINOVIĆ, 1998), or indirectly by applying derivatization procedures followed by measurements on a fluorescent detector (TAKEDA & AKIYAMA, 1991; AERTS et al., 1986).

Sulphachloropyrazine is a new-generation sulphonamide which has been introduced in our country relatively recently. As far as we know, there are no reports concerning the determination of this sulphonamide in the muscle tissue of treated broilers. We can only mention the method recommended by the producer of this coccidiocide (ANDERSON et al., 1990). It consists of the extraction of sulphachloropyrazine from tissue samples with a mixture of solvents dichlormethane--methanol-acetic acid (90:5:5, v/v/v), extract cleaning on a cationic exchanger and HPLC determination using the TSK-ODS column with a mixture of 0.25 mol dm^{-3} ammonium acetate (pH=5.2) and acetonitrile (82.5:17.5 v/v) as mobile phase.

In this work we employed the same extraction procedure, whereas in the part of the clean-up and determination we introduced a number of modifications.

1. Materials and methods

1.1. Inoculation of broilers with laboratory-grown coccidia species

Heavy-line broiler chickens Arbor acres of both sexes (70 chicken), 28 days old, with average body weight of 1030 g, were inoculated with laboratory-grown coccidia species by p.o. administration of 1.00 cm^3 of coccidia suspension so that each chick received 2×10^4 oocista *Eimeria* (*E. necatrix*, *E. tenella*, and *E. mitis*). When the first clinical signs of coccidiosis appeared the broilers were treated with the preparation "Esb_3 30%", according to the producer's instructions. The drug was administered through drinking water: 2.00 g of the preparation were dissolved in 1.00 dm^3 of water, and the treatment was carried out following the regime of three-day treatment, two-day break, three-day treatment.

1.2. Taking samples of muscle tissue and liver

Samples of the muscle tissue and liver were taken after broilers sacrifice by randomly chosen 10 chickens, carried out on the third day of treatment, on the first day of the break following the three-day treatment (4th day), on the last day of treatment (8th day), and then on each of the subsequent three days after completing the treatment (9–11th day).

1.3. Preparation of samples

The extraction efficiency and recovery of the HPLC method for the determination of sulphachloropyrazine residues were studied by the standard addition method. To 10 g samples of broiler muscle tissue and liver the amounts of 1.0, 5.0, and 10.0 μg of sulphachloropyrazine standard were added. Three replicates were carried out for each sample with standard addition. Blank probes (samples of muscle tissue and liver of broilers not treated with sulphachloropyrazine) were run in parallel, and all the results were corrected for sulphachloropyrazine content in the blank.

1.3.1. Extraction procedure. Samples of 10 g of minced meat or liver were homogenized on a vibration shaker with 60 cm^3 of the solvent mixture dichlormethane--methanol-acetic acid (90:5:5 v/v/v) for 30 minutes. The extract was decanted and the extraction was repeated two more times, using the same volume of the extractant. The precipitated proteins were removed by filtration through the Whatmann No. 1 filtering paper. The filtrate was heated in a vacuum evaporator to the acetic acid fraction. By adding dropwise a solution of 0.1 mol dm^{-3} NaOH, the pH was adjusted to 7.0.

1.3.2. Clean-up procedure. In our procedure the extract cleaning was accomplished by passing it through a glass column (20×1 cm) filled with the XAD-2 adsorbent (grain size 0.3–1.0 mm; Serva; USA). The effluent was discarded and the sulphachloropyrazine residues were eluted with dichlorometane in 5 cm^3 fractions. It was found that the whole amount of sulphachloropyrazine absorbed is removed with 15 cm^3 of the eluent.

1.4. Calibration diagram

To construct the calibration diagram for the HPLC determination of sulphachloropyrazine 50 µl of its standard solution with the contents of 2, 4, 8, 12, and 16 µg cm^{-3} were taken whereby the obtained signals correspond to the amounts of 0.1, 0.2, 0.4, 0.6, and 0.8 µg of sulphachloropyrazine per probe. On the basis of the obtained chromatographic peaks we constructed the calibration graph presented in Fig. 1 (inset).

1.5. HPLC determination of sulphachloropyrazine residues

Contents of sulphachloropyrazine in the investigated samples were determined by HPLC on a Bio Rad system (Model 2800) with a Bio Rad UV spectrophotometric detector 1801. The column used was a C-8 HL (4.6×200 mm) 5 µm with the mobile phase: 60% acetonitrile in water with pH=9.5 adjusted with NH_3. Before use, the mobile phase was degassed on an ultrasonic bath. The other parameters of the HPLC determination were as follows: injected volume 50 µl; total flow-rate 1 cm^3 min^{-1}; wavelength 254 nm; column temperature 22 °C.

Under the given determination conditions the retention time for sulphachloropyrazine was 11.41 min.

2. Results and discussion

2.1. Determination of the relationship between chromatographic peak area and sulphachloropyrazine concentration

On the basis of the calibration diagram for the HPLC determination of sulphachloropyrazine (Fig. 1 (inset)), it is evident that a successful quantification of this coccidiocide is possible in the concentration range from 2 to 16 µg cm^{-3}, as the correlation coefficient for the linear relationship between the peak area and concentration is very good (r=0.999). In case of the determination of higher concentrations it would be necessary to change the measurement conditions. Such new conditions would allow the determination of higher concentration but the detection limit would then be higher.

2.2. Recovery

The extraction efficiency and the recovery achieved by the HPLC procedure for the determination of sulphachloropyrazine residues in muscle tissue and liver samples were tested by standard addition method, using the sulphachloropyrazine spikes of 0.1, 0.5, and 1.0 μg/g.

In Fig. 1 the chromatograms obtained for the extract of broiler muscle tissue with 0.1 μg/g of sulphachloropyrazine standard addition (a), for the muscle tissue of broilers receiving no sulphachloropyrazine (b), and for the muscle tissue of after the three-day treatment and one-day break (c) are presented.

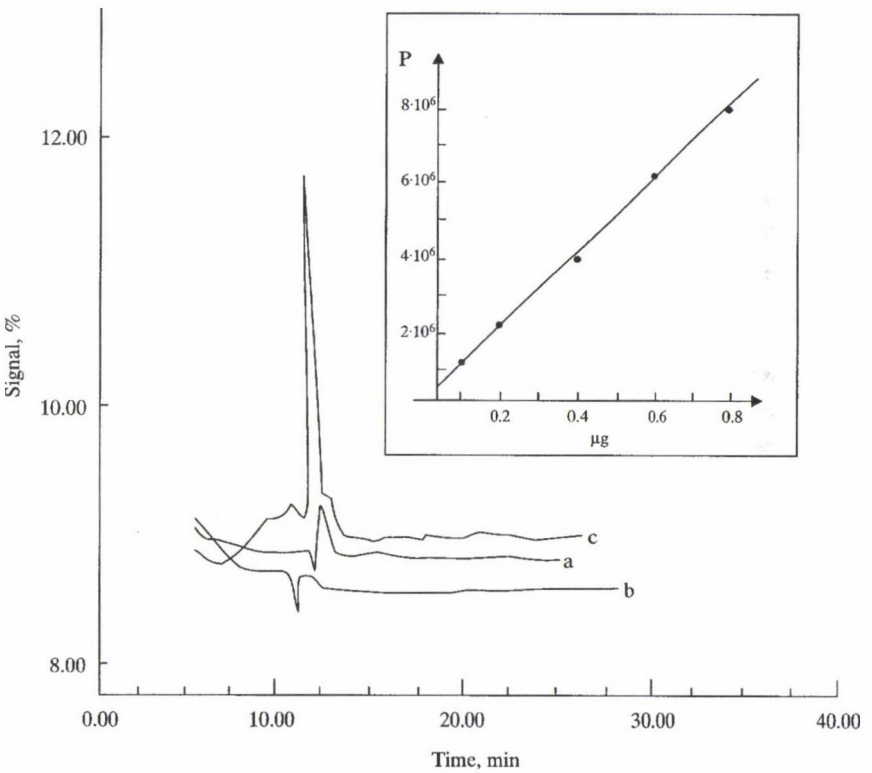

Fig. 1. Chromatograms of the broiler muscle tissue with standard addition 0.1 μg/g of sulphachloropyrazine (a), muscle tissue of broilers receiving no sulphachloropyrazine (b), and muscle tissue of broilers on the first day of the break following the three-day treatment (c). Inset: calibration graph

Using standard addition method it was found that the detection limit for sulphachloropyrazine was 0.02 μg/g.

The results of studying the efficiency and recovery of the applied HPLC procedure for the determination of sulphachloropyrazine in the investigated biological samples with addition of sulphachloropyrazine are presented in Table 1.

The results demonstrate that the developed procedures for extraction and HPLC determination can be successfully used to determine sulphachloropyrazine residues in the samples of muscle tissue and liver of broilers. The recovery of the method was in the range from 79.3±0.6 to 86.7±0.2% for muscle tissue and from 81.7±0.8 to 87.3±0.7% for liver samples. Having in mind the complexity of the matrix, these recovery values should be considered as satisfactory.

2.3. Results of the determination of sulphachloropyrazine residues

Using the above procedure we determined the contents of sulphachloropyrazine in samples of the muscle tissue and liver of coccidia-inoculated broilers and in those treated with a therapeutic dose of sulphachloropyrazine. The treatment with sulphachloropyrazine was carried out according to the regime: three-day treatment, two-day break, three-day treatment, samples for the analysis were taken on the third day of treatment, on the first day of the break after the three-day treatment (4th day), on the last day of treatment (8th day), and on the subsequent three days after completing the treatment (9–11th day). The results are presented in Table 2.

Table 1

Results of assessing the efficiency of the determination of sulphachloropyrazine in samples of broiler muscle tissue and liver

Sample	Added (μg/g)	Found ± SD (μg/g)	Recovery ± SD (%)
Muscle tissue	0.1	0.087 ± 0.002	86.7 ± 0.2
Muscle tissue	0.5	0.39 ± 0.06	79.3 ± 0.6
Muscle tissue	1.0	0.83 ± 0.05	83.3 ± 0.5
Liver	0.1	0.087 ± 0.065	87.3 ± 0.7
Liver	0.5	0.409 ± 0.078	81.7 ± 0.8
Liver	1.0	0.825 ± 0.005	82.5 ± 0.5

SD: standard deviation

Table 2

Results of the determination of contents of sulphachloropyrazine residues in muscle tissue and liver of treated broilers

Sample	Sampling day	Found[a] µg/g±SD
Muscle tissue	3rd	22.3 ± 5.4
	4th	0.79 ± 0.24
	8th	2.57 ± 0.02
	9th	0.29 ± 0.03
	10th	0.07 ± 0.01
	11th	0.02 ± 0.02
Liver	3rd	32.4 ± 6.2
	4th	2.00 ± 0.09
	8th	2.33 ± 0.01
	9th	0.06 ± 0.01
	10th	ND
	11th	ND

ND: not detected

[a] results were not corrected for the % of recovery

The presented results indicate that there is a minimal risk of the appearance of sulphachloropyrazine residues in tissues of the broilers treated with this coccidiocide. On the first day of the break following the complete treatment, the content of sulphachloropyrazine residues in the muscle tissue was three times higher than the maximal allowed content of 0.1 µg/g given in the pertinent REGULATIONS (1992), and on the subsequent day it was already below this value.

On the first day of the break after the completed treatment (9th day from the beginning of treatment) the content of sulphachloropyrazine residues was already below the tolerated level, and on the third day of the break (11th day) it was below the detection limit of the HPLC method employed in the liver samples of the sulphachloropyrazine treated broilers.

The obtained results confirmed that sulphachloropyrazine has a short withdrawal time, which for broiler meat is one day. In this respect it is advantageous over other sulphonamide agents used to treat coccidiosis, for which this period is 5 to 6 days. Our method of the extract cleaning-up and detection of sulphachloropyrazine enables a simple, selective and reproducible determination of this coccidiocide in samples with a complex matrix. Compared to the original method (ANDERSON et al., 1990), the determination is simplified, the analysis time is shortened and the potential losses due to transferring the extract to alkaline pH range are eliminated.

3. Conclusions

The developed HPLC method enables successful quantitative determination of sulphachloropyrazine residues in samples of muscle tissue and liver of broilers. The detection limit of 0.02 μg/g and the recovery ranging from 79.2±0.6 to 86.7±0.2% for the muscle tissue and 81.7±0.8 to 87.3±0.7% for the liver samples confirm the applicability of the method.

By determining content of sulphachloropyrazine in the coccidia-inoculated broilers treated with therapeutic doses of this anticoccidial agent it was established that there is a minimal risk of the appearance of its residues in their meat and liver. On the first day of the break following the complete treatment, the content of sulphachloropyrazine residues in broiler muscle tissue was three times higher than the tolerable level of 0.1 μg/g given by the pertaining regulations, which confirms the necessity of establishing the withdrawal period for sulphachloropyrazine, which in case of broiler meat is one day.

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EFFECT OF ULTRAFILTRATION OF BAKERS' AND BREWERS' YEAST EXTRACTS ON THEIR NITROGEN CONTENT AND TURBIDITY

C. P. CHAMPAGNE[a,d], H. GAUDREAU[b] and J. CONWAY[c]

[a] Food Research and Development Center, Agriculture and Agri-Food Canada, 3600 Casavant Blvd. West, St-Hyacinthe Qc, Canada J2S 8E3
[b] Fondation des Gouverneurs, 3600 Casavant Blvd. West, St-Hyacinthe Qc, Canada J2S 8E3
[c] Lallemand Inc., 1620 Préfontaine, Montréal Qc, Canada H1W 2N8

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Five commercial yeast extracts (YE) were fractionated by ultrafiltration (UF) with 10 000, 3000 and 1000 Da molecular weight cutoff membranes in the aim of evaluating the effect of UF on the turbidity and total nitrogen content of YE. Membrane pore size had much more influence on UF permeation rates than the source of the YE. UF filtration rates were on the average 4 times lower when the YE were treated with the 3000 Da membrane as compared to the 10 000 Da filter, and the 1000 Da unit gave rates approximately 40% lower than those observed with the 3000 Da pore size membrane. Pre-filtration with a 8 µm membrane reduced between 47 and 96% the original YE turbidities, while UF with a 1000 to10 000 Da membranes gave filtrates having between 80 and 99.9% less turbidity than the original YE. On the average, UF with the 10 000 Da unit removed 12% of total solids, while UF with 3000 and 1000 Da cutoff membranes generated the retention of 20 and 23% of solids, respectively. Brewers' YE had lower total nitrogen content than bakers' YE, and UF increased the total nitrogen content of the dried yeast extract filtrates (YEF) obtained. The powders obtained after freeze-drying of brewers' YEF tended to have higher moisture contents than bakers', and this was quite significant with the YEF powders obtained with the filtrates generated with the 1000 Da membrane.

Keywords: amino acids, peptides, membrane pore size, bakers', brewers', yeast

Yeast extracts (YE) are used as flavouring ingredients in sauces, soups and various other food products (DZIEZAK, 1987; NAGODAWITHANA, 1992). They are also used as fermentation nutrients in many growth media destined for the production of food-related cultures, such as lactic acid bacteria used in cheese, yoghurt, sauerkraut or dry sausage manufacture.

[d] Corresponding author: C. P. Champagne; E-mail: ChampagneC@em.agr.ca

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In analytical studies of YE, ultrafiltration (UF) has been used to remove salts from the products (HALÁSZ & SZAKÁCS-DOBOZI, 1993). Industrially, the technological steps that lead to the production of YE may include filtration (PEPPLER, 1982; SCHOENBERG, 1993), especially in instances where clear YE solutions are required. Although filtration is carried out commercially in some YE manufacturing processes, the technological data regarding the effect of this processing step on characteristics of YE seems to be proprietary. There is information on the filtration of brewers' yeasts (YOSHIKAWA et al., 1994), but no comparative data are found on the UF of bakers' and brewers' YE.

The aim of this study is to examine the effects of UF with three pore size membranes (1000, 3000 and 10 000 Da) on the physical (turbidity) and chemical (solids, nitrogen) characteristics of bakers' and brewers' YE.

1. Materials and methods

1.1. Yeast extracts

Five commercial YE were obtained from the following suppliers : Bio Springer (Maisons-Alfort, France), Difco (Detroit, MI, USA), Lallemand (Montréal, QC, Canada), and Red Star (Juneau, WI, USA). Since variability between lots has been reported (POTVIN et al., 1997) two lots of each source were used. The products were coded A to E so as to prevent any prejudice to the companies. Three YE were from bakers' yeasts (A, B, E) while two were from brewers' yeasts (C, D).

1.2. Ultrafiltration of yeast extracts

YE were suspended in deionized water to obtain a solution containing 10% (w/v) solids. This solution was pre-filtered on a 8 µm Whatman (No. 2 filter paper) membrane. The filtrate was further processed by UF, using a tangential filtration system (Minitan Filter plates, Millipore, Bedford, USA) with membranes having molecular cutoffs of 10 000, 3000 or 1000 Da. The system enabled the use of four 30 cm^2 membranes for a total filtration surface of 120 cm^2. A 500 ml solution of 10% YE was used, and the UF process was stopped after the recovery of 300 ml of filtrate. The yeast extracts filtrates (YEF) were lyophilized in a LyoTech (Lyo San Inc, Lachute, Canada) at 24 °C for 72 h and stored at –20 °C until used. Total nitrogen and water contents of the powders as well as total solids and turbidity of YE and YEF solutions were evaluated. All chemical analyses were done in duplicate for each lot tested and the results presented are the means of the two values obtained.

1.3. Chemical analyses of YE and YEF

The total nitrogen determination was done using a FP-428 LECO apparatus (LECO Corporation, Saint Joseph, MI), operated under the following conditions: sample size, 150 mg; oxidation Furnace temperature, 900 °C; oxidation standby temperature, 650 °C; purge cycles, 3; minimum timeout, 30 s; comparator level, 1.00; loop select low range, flow constants at high; gases, oxygen 99.99% and helium 99.99%. The calibration standard was composed of 150 mg EDTA (No 502–092, 9.56± 0.03% Nitrogen, LECO Corporation, Saint Joseph, MI).

Turbidity of YE and YEF solutions were determined with a Orbico-Hellige turbidimeter (Model 965; Farmingdale USA). Hydrazine sulfate standards (VWR; West Chester PA, USA) were used to calibrate the turbidimeter.

Water content of the YE, filtrates and YEF powders were obtained by dry weights after an incubation at 105 °C for 16 h.

1.4. Statistical analyses

Statistical analyses (variance and *t*-tests) were carried out on InStat (GraphPad, San Diego, CA, USA) software.

2. Results

2.1. Filtration rates

In the time required to obtain 300 ml of filtrate from the original 500 ml solution, filtration rates with 10 000 Da membranes showed only small decreases (Fig. 1). This is related to the high filtration surface (120 cm^2), which enabled completion of the process in less than 10 min. The YE source had an effect, as brewers' YE (products C and D) showed lower filtration rates. The membrane used had the greatest effect on filtration rates (Fig. 2). Filtration rates were on the average 4 times lower when the YE were treated with the 3000 Da membrane as compared to the 10 000 Da filter. However, the difference between filtration rates obtained with the 3000 and 1000 Da membranes were much lower, as the 1000 Da unit gaves rates approximately 40% lower than those observed with the 3000 Da pore size membrane. Therefore, with respect to ultrafiltration rates, the membrane pore size had much more influence than the source of the YE.

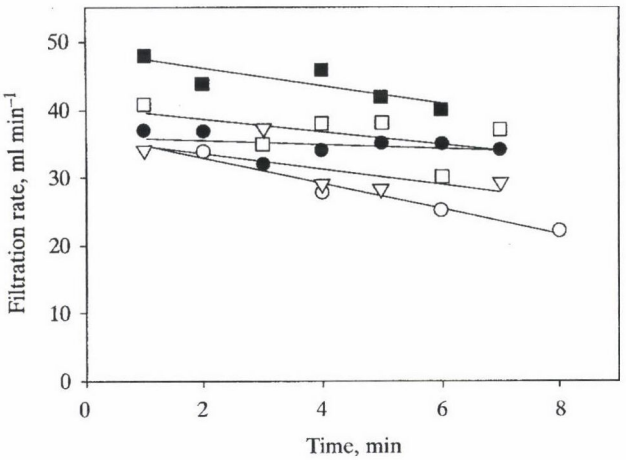

Fig. 1. The effect of yeast extract source on permeation rates during ultrafiltration of yeast extract solutions (10%) with membranes of 10 000 Da cutoff. ■: A-bakers', ●: B-bakers', ❍: C-brewers', ▽: D-brewers', □: E-bakers'

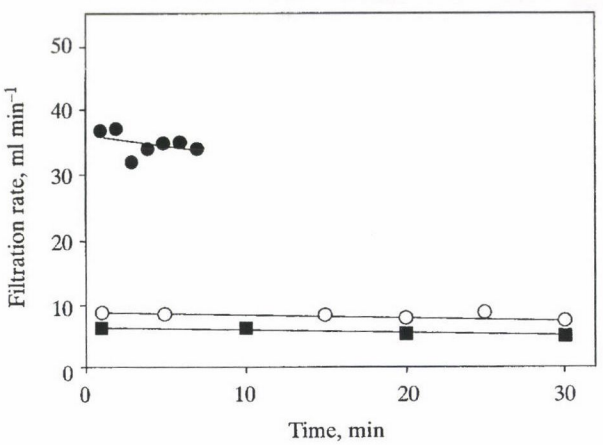

Fig. 2. The effect of membrane pore size on the permeation rates during ultrafiltration of yeast extract B. ●: 10 000 Da, ❍: 3000 Da, ■: 1000 Da

There was no correlation (r of –0.28) between the turbidity of the YE and the initial filtration rates.

Table 1

Chemical composition and turbidity of yeast extracts and yeast extracts fractions

Yeast	Filtration	Turbidity (NTU)	Solid retention by membrane (%)	Total nitrogen (g/100g)
A	None	6.1 c	0 f	10.9 cdef
(Bakers')	8 µm	1.6 c	ND	ND
	10 000 Da	1.2 c	10 de	11.6 bc
	3000 Da	0.9 c	21 bcd	11.0 cde
	1000 Da	0.8 c	22 bcd	10.9 cdef
B	None	87.2 b	0 f	11.9 bc
(Bakers')	8 µm	45.9 be	ND	ND
	10000	0.6 c	5 e	13.4 a
	3000	0.6 c	20 bcd	12.9 ab
	1000	0.6 c	22 bcd	12.7 ab
C	None	60.6 be	0 f	7.7 g
(Brewers')	8 µm	30.2 de	ND	ND
	10 000 Da	0.6 c	24 abc	9.5 f
	3000 Da	0.5 c	32 ab	9.7 ef
	1000 Da	0.7 c	35 a	9.8 ef
D	None	361.3 a	0 f	9.8 def
(Brewers')	8 µm	12.7 d	ND	ND
	10 000 Da	0.4 c	12 de	11.0 cde
	3000 Da	0.5 c	14 cde	10.9 cdef
	1000 Da	0.6 c	19 cd	10.9 cdef
E	None	6.5 cd	0 f	10.8 cdef
(Bakers')	8 µm	1.4 c	ND	ND
	10 000 Da	0.7 c	10 de	11.6 bc
	3000 Da	0.7 c	14 cde	11.3 cd
	1000 Da	0.6 c	20 bcd	11.1 cde

For a given column, means that are followed by the same letter are not significantly different ($P>0.05$)
ND: not determined

2.2. Effect of filtration on the turbidity of YE

The YE solutions before ultrafiltration had large variations (between 6 to 361 NTU) in turbidity (Table 1). Native YE B, C and D gave visibly turbid solutions. Pre-filtration (8 µm) reduced between 47 and 96% the original YE turbidities, with an average of 74%. UF with a 10 000 Da membrane gave filtrates having between 80 and

99.9% less turbidity than the original YE, with an average of 95%. There were no further gains by using membranes of 3000 of 1000 Da. Thus, filtration of YE solutions with a 10 000 Da membrane cutoff was enough to produce a clear YE solution, and filtration with membranes having lower MW cutoffs were not required for this purpose.

2.3 Effect of filtration on total solids

Ultrafiltration of the YE resulted in significant retention of solids (Table 1). The ultrafiltration of brewers' YE was very difficult and solutions had to be pre-filtered with the 8 μm membranes. On the average, UF with the 10 000 Da membrane removed 12% of total solids, while UF with 3000 and 1000 Da cutoff membranes generated the retention of 20 and 23% of solids, respectively. Although, a variance analysis did not consider the differences in solids retention between 1000 and 3000 Da filtrations as being significant (Table 1), the increased retention of solids with the 1000 Da membrane is systematic, and paired *t* tests of the 1000/3000 data show that the difference is real.

2.4. Effect of filtration on total nitrogen

In all instances, UF with the 10 000 Da membrane resulted in products that had higher total N contents (Table 1). This suggests that the compounds removed by UF at 10 000 Da had little protein content, and were presumably glycan cell wall fractions. However, further filtration with the 3000 and 1000 Da membranes tended to reduce the total N content. Since YE contain amino acids and various peptides (OHLY, 1998) these data suggest that peptides constitute a significant fraction of the YE compounds found in the 1000–10 000 Da range. Consequently, the UF process that enables the production of the YEF powders having the highest total N content is with the 10 000 Da membrane.

The source of YE had an effect, as brewers' YE had lower total N content than bakers' YE (Table 1), confirming results of COHAS and COHAS (1991). UF improved considerably the total N content, and one brewer' YEF became similar to bakers' YEF A and E with respect to total N content.

2.5. Residual humidity in the YEF powders

The water content of the commercial YE powders were not significantly different (Table 2) and averaged 7.6%. The brewers' YEF powders obtained after freeze-drying tended to have higher moisture contents, and this was quite significant with the YEF powders obtained with the filtrates generated with the 1000 Da membrane.

Table 2

Humidity contents (g water/100g powder) of the original yeast extracts and the products obtained after ultrafiltration and freeze-drying

Yeast extract source	Yeast extract fraction				
	Original	0–10 000 Da retentate	0–10 000 Da filtrate	0–3000 Da filtrate	0–1000 Da filtrate
A	6.8 a	6.7 ab	6.6 a	7.9 a	8.0 a
B	7.9 a	7.8 b	9.7 b	8.0 a	8.3 a
C	8.8 a	6.6 ab	12.1 c	11.9 b	11.7 b
D	7.9 a	7.9 b	9.1 b	10.2 c	11.3 c
E	6.8 a	5.1 a	6.6 a	7.2 b	7.1 b

For a given column, means that are followed by the same letter are not significantly different ($P>0.05$)

Filtration influenced the residual moisture of the products. YEF powders, which contained the low molecular weight compounds of YE, generally had higher water contents than the powders obtained by their retentates. These results are thus in line with those of COHAS and COHAS (1991), who had found that non-fractionated brewers' YE has higher water contents than the equivalent bakers' YE.

The commercial drying of the YEF would probably be carried out by spray drying rather than freeze-drying, and it remains to be determined if these results can extend to this technology. Nevertheless, the data does show that UF changes the properties of YE to drying.

3. Conclusions

UF may be a method of improving the nitrogen content of YEF, particularly in low molecular weight nitrogen compounds such as peptides and amino acids. This can have applications in food technology as well as in fermentation technology. In food technology amino acids such as glutamate enhance the flavour of foods (NAGODAWITHANA, 1992; WARMKE & BELITZ, 1993), and peptides contribute as well to their flavour (KURAMITSU et al., 1996; RAKSAKULTHAI & HAARD, 1992); YE are indeed recognized as useful ingredients for the enhancement of food flavour (DZIEZAK, 1987; LEE et al., 1981). With respect to fermentation technology, amino acids and peptides are also growth factors of many microbial cultures (HOLT et al., 1994). Thus, UF could be used to modify the flavour profiles and biological value of YE. Studies are currently under way in our laboratory to determine the growth-promoting value of the YEF on various lactic acid bacteria destined for food fermentations.

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INTERFACIAL ENZYME PARTITIONING AS A TOOL FOR CONSTRUCTING BIOSENSORS

N. ADÁNYI, J. SZAMOS, E. E. SZABÓ and M. VÁRADI

Central Food Research Institute, H-1022 Budapest, Herman Ottó út 15. Hungary

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To explore new possibilities of enzyme immobilization, we investigated bioactive layers prepared by a new procedure based on three-phase partitioning (TPP) of proteins. By this method a third phase or midlayer as a protein layer can be developed at the interface of a protein system containing two phases (organic solvent/aqueous salt solution). Proteins of meat origin partitioned together with bioselective material (e.g. an enzyme) after centrifugation resulted in excellent bioactive layers.

In the newly developed sensor, glucose oxidase was immobilized in a layer, which was fixed on the surface of a platinum ring electrode. The biosensor was built in a flow injection analyzer (FIA) system, where the hydrogen peroxide generated during the enzymatic reactions was determined by an amperometric cell. The parameters for biochemical and electrochemical reactions (ion concentration and pH of buffer, flow rate) were optimized. The linear range of analysis by the newly developed sensor was from 0.5 to 10 mmol l^{-1} glucose. The biosensor could be used for more than 300 analysis.

Keywords: bioactive protein gel layer, three phase partitioning, amperometric cell, FIA system

When planning a new method for constructing biosensors, the most important question is how to immobilize the enzyme/enzymes or cells so that the biochemical-electrochemical reactions be complete without the disturbing agents reaching the surface of the electrode. One of the most important requirements in a new method used in on-line processes is the easy and reproducible preparation of enzyme layer. Entrapment in polymeric gels prevents diffusion of biomolecules to the reaction mixture. On the other hand, small substrates can easily permeate. The gel entrapment is a mild procedure, the biomolecules are not covalently bound to the matrix. Contrary to gel entrapment, covalent binding is often accompained by loss of activity.

Slices of animal tissues or plant origin are the most complex biosystems applied so far in biosensors. Tissues containing large amounts of the enzymes of interest have been widely used. This sensor benefits from the high stability in its native environment. Fitting into the trend towards improvement of availability and simplification of the

preparation of biocatalytic layers for biosensors, the use of crude materials has been explored (SCHELLER & SCHUBERT, 1992).

Three-phase partitioning (TPP) used in this work to prepare protein layers is originally a protein enrichment method, in which proteins are collected in form of a middle layer between aqueous (ammonium sulfate solution) and organic (tert-butanol) phases at room temperature (ODEGAARD et al., 1984; LOVRIEN et al., 1987; SZAMOS & HOSCHKE, 1992). PIKE and DENNISON (1989) investigated the partitioning process for several proteins, however details of the mechanism are unknown.

An analytical application of TPP for drips (multicomponent meat protein solutions, SZAMOS et al., 1998) was used for developing a method to prepare protein layers of definite size. To prepare drips the centrifugal method of PENNY (1975) was used with modifications. Solutions having an initial interfacial tension of 1 mN m^{-1} were used in each case for three phase partitioning. Consistency of these layers proved to be suitable for biosensors, so an enzyme in a solution preserving its activity during the partitioning process could be loaded into the system recovering in the layer by centrifugation. We used glucose oxidase (GOD) to investigate whether this method is acceptable as a new immobilization process for biosensor technology.

1. Instrumentation and materials

1.1. Materials and reagents

Glucose oxidase (EC. 1.1.3.4): 25 U mg^{-1}, lyophilized from *Aspergillus niger* and tert-butanol (HPLC grade) were obtained from Sigma (St. Louis, MA, USA).

All other reagents were commercially available and of analytical grade.

1.2. Instrumentation

A flow injection analyzer (FIA) consisting of a buffer reservoir, a peristaltic pump (Minipuls 3, Gilson Co., Villiers-le-Bel, France), an injection valve (20 µl sample loop, Rheodyne Inc., Cotati, CA, USA) and an amperometric cell connected to polarograph (Radelkis, Budapest, Hungary) was the basic instrumentation of our research. A platinum-ring electrode was used as measuring electrode while the reference electrode was a silver/silver chloride (Ag/AgCl) electrode. The polarization potential was ensured and fixed (+600 mV).

The protein layers were washed in a buffer solution and fixed on the electrode surface with a nylon net and connected to the flow-through amperometric cell (Fig. 1). The layers were easy to change after cleaning the Pt-ring electrode.

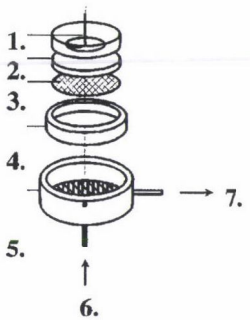


Fig. 1. Construction of biosensors, 1: Platinum-ring electrode, 2: protein layer with GOD, 3: nylon net, 4: seal, 5: plexi block, 6: inlet, 7: outlet

1.3. Preparation of protein layers

Pieces of meat (leg of pork, chicken and turkey, rump of beef) of 2 cm in diameter were cut out by a stainless steel cork-borer from 1–2 cm thick slices. Instead of Teflon discs perforated with channels (PENNY, 1975), glass beads (4 mm diameter) were used as holder in 50 ml Falcon tubes filled up to 7.5 ml. Meat discs were placed on beads and centrifuged (Beckman J2-21, JS-7.5 swinging bucket rotor) at 450 *g* for 20 min at 15 °C. The spun drip from the meat was collected by Finnpipette, freeze-dried, and then kept at room temperature.

Lyophilized drips were suspended in distilled water. Aliquots of 50 µl were added to 950 µl ammonium sulfate solution in screw capped 2 ml polypropylene tubes at 25 °C (thermostat). After 20 min 310 µl tert-butanol was added to each tube, shaken thoroughly by hand, then let to stand for 15 min. The tubes were centrifuged at 4500 *g* for 6 min at 20 °C. The thickness of the protein layers was measured and the concentration of freeze-dried drip was calculated to obtain layers about 1.5 mm thick. This approach resulted in layers about 1.5 mm thick with the exception of turkey (Table 1).

1.4. Preparation of layers containing GOD

Calculated amounts of GOD were added to freeze-dried drips of meat (e.g. for one layer of chicken drip containing 20 U GOD, 0.48 mg enzyme and 1.4 mg drip were weighed) and suspended in 50 µl distilled water. This solution was added to 950 µl of ammonium sulfate solution, then processed by the above procedure. The layers containing GOD can be stored in refrigerator for two months.

Table 1

Change in the thickness of the layer with changing the initial drip concentration

	Beef	Pork	Chicken	Turkey
mg drip ml^{-1}	5.0	5.0	5.0	5.0
Thickness, mm	3.0	5.0	5.0	4.0
mg drip ml^{-1}	1.7	1.3	1.4	1.3
Thickness, mm	1.0	1.5	1.5	0.5

2. Results and discussion

2.1. Characteristics of the protein layers

Equidimensional protein layers of 1.0–1.2 mm thickness and 54 mm^2 cross section were prepared from drip of beef, pork, turkey and chicken by TPP, tested for their physical properties, and checked whether they are permeable to hydrogen peroxide.

Protein layers containing GOD were produced using different relative saturation of ammonium sulfate (30, 40, 50, 60%, respectively) to study the effect of salt concentration and the changes in physical properties. The results are summarized in Table 2. The layers were classified by thickness, permeability for hydrogen peroxide and the signal produced by injected glucose standards (pH 6.0).

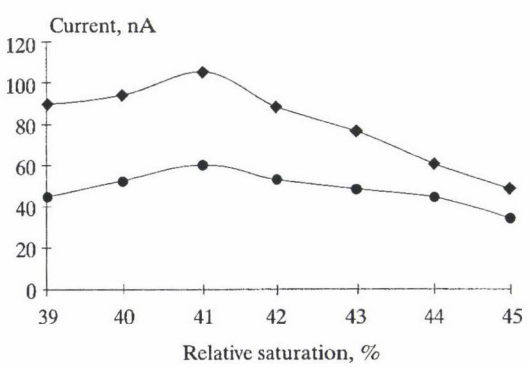


Fig. 2. Effect of relative saturation of ammonium sulfate at TPP (pH 6). ●: 5 mmol l^{-1} glucose ◆: 10 mmol l^{-1} glucose

Table 2

Properties of protein layers containing GOD using different relative saturations of ammonium sulfate

	Ammonium sulfate,%	Thickness mm	H_2O_2 1 mmol l^{-1} nA	Glucose signal, nA 5 mmol l^{-1}	10 mmol l^{-1}
Beef	30	0.5	26	16	28
	40	0.5	18	20	36
	50	1.0	16	18	30
	60	2.0	–	–	–
Turkey	30	1.5	12	12	28
	40	1.0	12	16	30
	50	1.5	12	18	32
	60	1.0	18	22	40
Chicken	30	1.5	18	14	30
	40	1.5	18	30	54
	50	2.0	16	20	38
	60	5.0	–	–	–
Pork	30	0.5	2	9	18
	40	0.5	28	16	36
	50	2.5	36	38	60
	60	5.0	–	–	–

The layers from chicken drip prepared at about 40% saturation of ammonium sulfate showed acceptable behaviour in consistency, permeability, signal for glucose and ease in handling. To determine the optimal relative saturation, a range of 38 to 45 relative % was examined (Fig. 2). The maximal peak was obtained using layers with 41% relative saturation.

2.2. Effect of flow rate

The flow rates in FIA systems have significant effect on both the sample throughput and the detection limit. The disadvantages of lower flow rates are lower sample throughput and increased dispersion. On the other hand, using the higher flow rate more samples can be analyzed, the peaks become more narrow, but the detection limit gets higher at the same time. To determine the optimal flow rate, measurements were carried out using various flow rates in the range of 0.2 to 1.6 ml min^{-1}. In fact, the diffusion rate through the layers turned out to be more determinative than the flow rate. In a fairly wide range of flow rate (0.4–0.9 ml min^{-1}) peaks were just the same. When the flow rate was slower, a larger proportion of the samples entered the layer, the enzyme-substrate conversion was more complete, but the peaks of hydrogen peroxide

produced became wider and the measurement was very slow. At higher flow rates only a limited amount of substrate entered the layer, the peaks were much smaller. After analyzing the results, the optimal flow rate was found to be 0.7 ml min^{-1}. In this case 20–30 samples were measured in an hour. (Data not shown.)

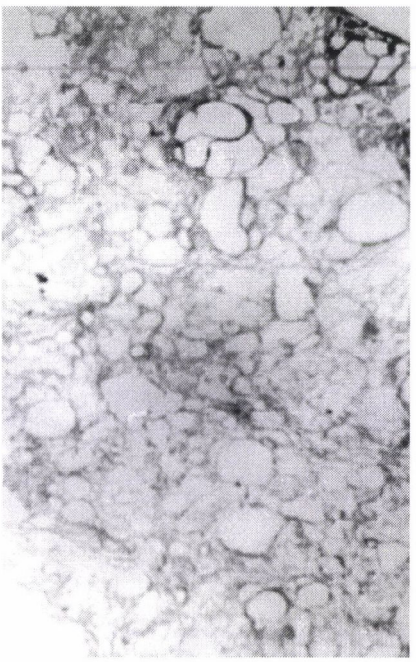
2.3. Effect of enzyme quantity

Layers were prepared at different enzyme concentrations. The enzyme added to partitioning systems was 12, 16, 20, 24 and 28 U respectively (from 0.47 mg GOD/1.38 mg to 1.1 mg GOD/1.38 mg lyophilized drip). The interrelationship can be followed on Fig. 3, where the microscopic structures (40 × magnification) of the gels containing increasing amount of GOD (0, 12, 20, 28 U respectively) are shown. On the photos it can be observed, that the size of vesicles became smaller and more indistinct with increasing enzyme amount. The layer prepared by 12 U GOD was very thin, permitting only a few measurements. The layers with 16, 20 and 24 U GOD gave acceptable signals, while the last one (28 U) was thick, and not permeable enough.

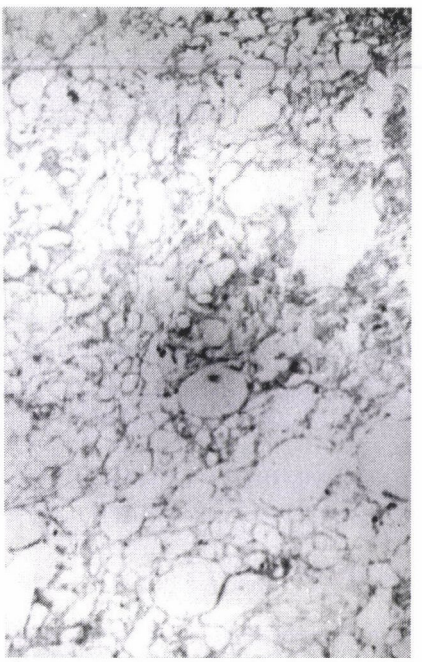
2.4. Effect of buffer pH

To study the effect of buffer pH on the behavior of the enzymes entrapped in the membranes, the protein layers were always washed out with the proper buffer which was used for the measurements. The investigated range of pH was from 4.5–7.2. The results are shown in Fig. 4. According to results the peaks increased in the range of 4.8–5.4 pH. The optimal pH value for measuring glucose was found to be 5.1, the same as for dissolved enzyme. It is surprising because during the immobilization process the pH optimum of the enzymes usually changes (VÁRADI et al., 1995). Considering this result, it can be assumed that the enzyme molecules are in dissolved state in this chicken protein matrix and are only captured by the gel.

Fig. 3. Structure of protein gel layers containing GOD (a-d, 0, 12, 20, 28 U, GOD, respectively) → (Microscopic photo, 40x magnification.)



a



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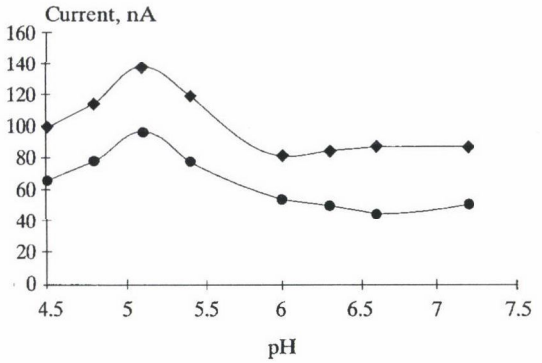

Fig. 4. Effect of pH. ●: 5 mmol l^{-1} glucose ◆: 10 mmol l^{-1} glucose

2.5. Effect of buffer concentration

Since enzyme inhibition and the electrochemical reaction are influenced by the total ion concentration of buffer solutions, this effect had to be studied. Applying Michaelis buffers of 0.067–0.3 mol l^{-1} concentration and of pH 5.1, always the same buffers were used for washing the layers as for the measurements. The optimum of the buffer concentration was 0.2 mol l^{-1}. Using this buffer, the peaks became sharper, and the analysis of glucose could be performed in a relatively wide linear range from 0.5 mmol l^{-1} to 10 mmol l^{-1}. (Data not shown.)

2.6. Lifetime of the protein membranes and statistical parameters

The biosensors were used intensively for ten days to determine the stability of the layer and the number of detectable samples (Fig. 5). After completing the cell it took only a few minutes until the amperometric current signal became permanent. In the next period of time the samples were measured with proper punctuality, the activity of the cell was stable, the rate of the reaction was determined by the diffusion. In the last part of the observed time the amperometric signal decreased quickly. By this time the rate of the reaction was already determined by the enzyme reaction. The enzyme cell ran down after measuring about 300 samples.

When injecting standard solutions the average of the measurements and the residual standard error within 15 samples were 80.6 nA and 2.5 nA, respectively.

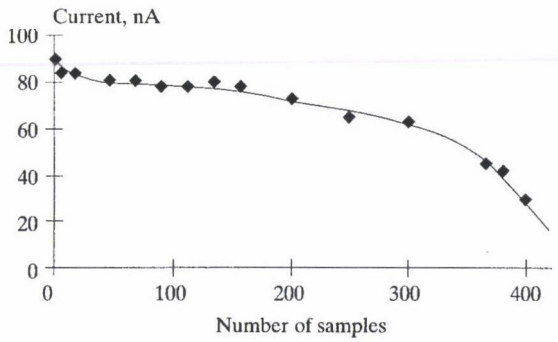

Fig. 5. Lifetime of the protein layer containing GOD

2.7. Determination of glucose content in juice samples

Glucose concentration of 10 juice samples was measured and compared with results obtained by the reference UV photometric method (D-Glucose UV-Test No. 716251 Boehringer, Mannheim). The average concentrations measured by both methods are shown in Fig. 6. The correlation between the two methods was very good, the correlation coefficient (r) was 0.992.

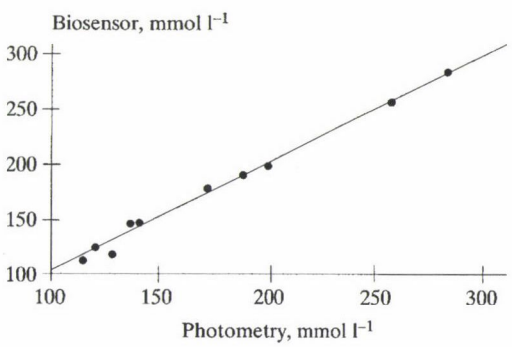

Fig. 6. Correlation between glucose measurement with reference method and with biosensor. (Correlation coefficient = 0.993; $r^2 = 0.986$)

3. Conclusion

Protein layers, containing glucose oxidase, were prepared by combining TPP with middle speed centrifugation (4500 g). Beyond entrapping the enzyme in its active state, the layer proved to be permeable to electroactive components such as hydrogen peroxide. The most effective layer could be prepared from chicken drip. The sensors constructed by protein layers prepared from chicken drip and glucose oxidase with their partitioning between water and organic phase, met the overall requirements for biosensors. An outstanding uniformity of layers containing GOD was achieved by strict control of the partitioning process and centrifugation. During the measurements we never found any microbial contamination in the protein layers used for entrapping the enzymes. The novel procedure elaborated is simple and fast. In the present work the enzymes bind to proteins of meat origin by only physical way giving the immobilized layer of the biosensor. On the basis of these results, it can be assumed, that enzymes which are immobilized together with proteins of meat origin by TPP preserve their original activity whereas layers usually prepared for biosensors containing enzymes which meet solubility criteria loose some of their activity during the covalent immobilization.

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MODULATION OF PHYSICO-CHEMICAL PROPERTIES OF BOVINE β-CASEIN BY NONENZYMATIC GLYCATION ASSOCIATED WITH ENZYMATIC DEPHOSPHORYLATION

M. DAREWICZ, J. DZIUBA[a], H. MIODUSZEWSKA and P. MINKIEWICZ

Department of Food Biochemistry, Olsztyn University of Agriculture and Technology, Plac Cieszyński 1, 10-726 Olsztyn-Kortowo, Poland

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A major bovine casein fraction, β-casein was chemically glycated and/or enzymatically dephosphorylated. Ten glucose and nine lactose moieties were attached while all phosphate groups were removed. Glycation shifted the pI to acidic pH range and decreased the solubility at acidic pHs while dephosphorylation shifted the pI to neutral pH range and increased the solubility at acidic pHs. Dephosphorylation led to longer retention time measured using the reversed-phase high-performance liquid chromatography and affected UV-spectra of β-casein which suggested structural changes. Glycation did not affect these properties. Both modifications decreased the calcium sensitivity of β-casein, making it to keep α_{S1}-casein in solution in the presence of Ca^{2+}.

Keywords: β-casein, dephosphorylation, glycation, solubility, stabilization ability

The casein fraction of milk comprises four major proteins: α_{S1}-; α_{S2}-; β- and κ-casein. In comparison with typical globular proteins, such as the whey proteins the structures of the caseins are quite unique. Perhaps the most unusual feature is the amphiphilicity of their primary structure (SWAISGOOD, 1992).

β-Casein (β-cn) consists of a polypeptide chain composed of 209 amino acids and has a molecular weight of ~24 kDa (SWAISGOOD, 1992). This protein is a flexible molecule with a polar head and unpolar tail. So the behaviour of β-cn is expected to be soap-like. A number of attempts have been made to alter physicochemical properties of proteins by chemical and enzymatic treatment (IMAFIDON et al., 1997). Non-enzymatic glycation (glycosylation) is the covalent binding of single sugars to α- or ε- amino groups on proteins. In glycosylation catalysed by glycosyl transferases, short oligosaccharide chains become attached to asparagine, threonine or serine side chains through glycosidic bonds. In completely different, e.g. chemically-catalysed, glycation,

[a] To whom correspondence should be sent. Fax: (00 48 89) 527 39 08, Phone: (00 48 89) 523 37 15

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sugars attack N-terminal amino acids or side chain lysine groups (FURTH, 1988). The preparation of neoglycoproteins by the covalent attachment of carbohydrates has been done to immobilize enzymes (KAMATA et al., 1990), to increase the heat stability of enzymes and inhibitors (MER et al., 1996) and to improve functional properties of proteins (WANISKA & KINSELLA, 1984; KITABATAKE et al., 1985; COURTHAUDON et al., 1989; SAEKI, 1997). In all cases, better solubility was found for these carbohydrate-derived proteins.

Among the non-proteolytic enzymes acid and alkaline phosphatases have been used for casein modification. Dephosphorylation of casein micelles has been shown to lead to their disintegration and to reduce the formation of phosphopeptides (MEISEL & SCHLIMME, 1995). Changes in solubility and decrease in calcium sensitivity were reported for whole casein by VAN HEKKEN and STRANGE (1993). The release of phosphate groups resulted in lower calcium sensitivity and softer curd during cheese making. LORENZEN and REIMERDES (1992) reported that the modification with alkaline and acid phosphatases increased the emulsion stability. In contrast, HUSBAND and co-workers (1997) showed a decrease in emulsion stability and an increase in foamability for dephosphorylated β-cn (dp-β-cn). Mild enzymatic and chemical treatments may have a beneficial effect on the physicochemical properties of β-casein.

In our earlier studies (DZIUBA et al., 1998; DAREWICZ et al., 1998) we reported about the changes of bovine β-cn upon 15-day of glycation with glucose. HENLE and KLOSTERMEYER (1993) reported that the reactivity of individual lysine residues for saccharide attack was extremely different, with Lys-28/29, Lys-32, Lys-99 and Lys-107 being preferably glycated. One of our intentions in this study was to increase the stabilizing properties of β-cn for α_{S1}-cn in the presence of Ca^{2+}. By terminating the reaction of coupling the carbohydrates after 32 h, we wanted the β-cn molecular status to be assimilated to κ-cn as the stabilizer for α_{S1}-cn fraction. The release of five phosphates from β-cn also may increase its stabilizing ability when dp-β-cn is interacted with α_{S1}-cn. The objective of our studies was to investigate the effects of these modifications (i.e. covalent attachment of saccharides and enzymatic release of phosphate groups) on solubility of modified proteins in media of different ionic strength and pH. The chromatographic (reversed-phase high-performance liquid chromatography) and spectral (UV spectra) properties of modified proteins were also studied.

1. Materials and methods

1.1. Materials

Potato acid phosphatase [EC 3. 1. 3. 2.] was obtained from Boehringer Mannheim (Germany, cat. No. 108219). α-D-Glucose and α-D-lactose anhydrates were purchased

from Merck (Darmstadt, Germany). Unless otherwise stated, all other chemicals were of analytical reagent grade obtained from Sigma (England) and Serva Feinbiochemica (Germany).

1.2. Preparation of α_s- and β-casein

The details of the casein fraction preparation were described earlier (DZIUBA et al., 1998; DAREWICZ et al., 1998). Briefly, the bovine casein was prepared from fresh skim milk from selected, homozygous at the four casein loci cow (genotype α_{S1}-cn BB; β-cn BB and κ-cn AB) by isoelectric precipitation at pH 4.6 (ANDREWS & ALICHANIDIS, 1983). α_{S1}- and β-Casein were prepared and purified by ion-exchange chromatography (THOMPSON, 1966). The purity of fractions was checked by isoelectric focusing (IEF) (see Section 1.6). The fractions were freeze dried. The protein content of the samples was estimated by Lowry's method using bovine serum albumin as a standard (LOWRY et al., 1965).

1.3. Chemical modification of β-casein

Samples were prepared by incubation of intact β-cn in 0.5 mol l^{-1} glucose, galactose or lactose for 32 h, at 37 °C, in 0.05 mol l^{-1} phosphate buffer (pH 7.4) containing 3 mmol l^{-1} sodium azide. After dialysis against demineralized water, the fractions were freeze dried. The amount of sugars bound to proteins was quantified according to the procedure described by NESSAR and FURTH (1991).

1.4. Enzymatic modification

Intact and glycated β-cn were dephosphorylated with acid phosphatase. Samples (10.0 mg l^{-1}) were incubated with enzyme at 20 °C in 0.01 mol l^{-1} imidazole buffer (pH 7.0). Maximally dephosphorylated samples were obtained by adding 0.05 U of enzyme per 1 mg of protein samples and incubating for 1 h. The reaction was terminated by heating at 100 °C for 5 min, after that the samples were dialyzed (24 h) against demineralized water and freeze dried. The degree of dephosphorylation was estimated by microscale colorimetric method according to IDF standard (FIL-IDF 1987) and Fiske-Subbarow method modified by BARTLETT (1959).

1.5. Polyacrylamide gel electrophoresis

SDS-PAGE was performed according to LAEMMLI (1970) in 12.5% (30.8% T, 2.7% C) polyacrylamide gel slabs of 1 mm thickness. Protein bands were stained with Coomassie Brilliant Blue R-250. Additionally, we checked the effect of galactose binding on electrophoretic behaviour of β-cn.

1.6. Isoelectrofocusing analysis

IEF was performed in 0.3 mm thick 8% polyacrylamide gel (40% T, 3.9% C). An aliquot (5 µl) of sample solutions containing 1.5 mg ml^{-1} casein, 8 mol l^{-1} urea, 11% (v/v) glycerin and 3% 2-mercaptoethanol were loaded into the gel. Whole casein, intact and completely dephosphorylated β-cn (A^1A^2) were used as standards. The values of pI of each sample before and after modification were determined according to SEIBERT and co-workers (1985).

1.7. Reversed-phase high-performance liquid chromatography (RP-HPLC) and UV spectroscopy

RP-HPLC analyses were performed using the Hewlett-Packard HP 1050 equipment with photodiode-array detector, linked to Hi-Pore RP 318 4.6×250 mm (Bio Rad Laboratories, USA). Chemstation A.03.03 program was used for data acquisition and processing. The solvent composition was as follows: acetonitrile (ACN):water:trifluoroacetic acid (TFA) 100:900:1 and 900:100:0.8 v/v/v in solvents A and B, respectively. ACN and TFA (J. T. Baker, The Netherlands) were of HPLC grade. The gradient of solvent B was from 27% to 42% during 20 min. The protein concentration was c.a. 1.5 mg ml^{-1} in solvent A containing 6 mol l^{-1} urea with pH=2.2 adjusted using TFA. Injection volume was 100 µl. Other details of separations were described elsewhere (MINKIEWICZ et al., 1996). Apparent ratios of aromatic amino acids were calculated from the second-derivative UV spectra using the procedure described by PERRIN and co-workers (1995).

1.8. Assessment of solubility

β-Casein (intact and modified) samples were dissolved in demineralized water at 25 °C. The pH was adjusted with 0.1 mol l^{-1} HCl or 0.1 mol l^{-1} NaOH. Final ionic strength was 0.1, 0.5 or 1 mol l^{-1} (adjusted using NaCl). An aliquot (5 ml) of each sample was centrifuged for 15 min/5000×g. Protein concentration in the resulting supernatant was determined by measuring the absorbance at 280 nm and calculating the concentration from standard curves made from diluted protein stock solution. Solubility was expressed as the percentage of protein in solution.

1.9. Ca^{2+} sensitivity

Stock solutions of 0.5% β-cn (intact and modified) and 0.5% α_{S1}-cn in 10 mmol l^{-1} imidazole buffer (pH 7.0) were prepared (VAN HEKKEN & STRANGE, 1993). An aliquot (5 ml) of stock solutions was adjusted with 1 mol l^{-1} $CaCl_2$ to a Ca^{2+}

concentration range from 0.0 to 30.0 mmol l^{-1}. After centrifugation, the protein concentration in the supernatant was determined as described above.

1.10. Ability of β-casein to stabilize α_{S1}-casein

Stock solutions of α_{S1}- and β-cn were prepared as described in Section 1.9. The casein solutions were then mixed at 1:1 (v/v) ratio. After 1 h of continuous stirring, the samples were incubated at 26 °C for 15 min. Then the supernatant suspension was clarified with 0.1 mol l^{-1} sodium citrate, and the samples were centrifuged at 3000×*g* for 30 min. The protein content of the supernatant was measured as described in section 1.8.

1.11. Statistical analysis

Statistical significance of differences was evaluated using the *t*-test.

2. Results and discussion

2.1. Characterization of glycated and dephosphorylated β-casein

It was found that 10 molecules of glucose and 9 molecules of lactose were bound to one molecule of β-cn. Under the conditions described in section Materials and methods, the percentage of glycosylated amino groups was 83% with glucose and 75% with lactose. These values are in good agreement with those found by COURTHAUDON and co-workers (1989) for covalent binding of glucosyl residues to whole casein. In all cases the samples were completely dephosphorylated (100% dp). It could mean that even potential structural changes in β-cn after glycation did not hinder the approach of acid phosphatase to the susceptible bonds, which in turn led to the same result of dephosphorylation of intact and glycated β-cn.

2.2 Effect of glycosylation and dephosphorylation on electrophoretic behaviour of β-casein

The effects of glycation and dephosphorylation studied by polyacrylamide gel electrophoresis under denaturing conditions are presented in Fig. 1. The electrophoretic mobility was rather unmodified by the glycosylation and dephosphorylation. The glycated β-cn fraction bands were less stained probably because of their increased solubility (as discussed below) and decreased binding of Coomassie Brilliant Blue R-250 by the lysyl residues, which are responsible for dye-protein interactions (DZIUBA & MIODUSZEWSKA, 1997). Moreover, the lanes with dephosphorylated β-cn had bands additional to the main casein band. This may indicate a proteolytic activity of the acid phosphatase, resulting in peptide formation. LORENZEN and REIMERDES (1992) also reported on proteolytic activity of acid phosphatase resulting in the formation of

hydrophobic peptides. These additional peptide bands were visible on the IEF electoropherograms. Binding of single sugar molecules suggests evident differences between the isoelectric points determined for intact, glycated and/or dephosphorylated β-cn. Table 1 shows the influence of glycation and dephosphorylation on pI values. The pI value determined for intact β-cn is in good agreement with the value found by SEIBERT and co-workers (1985). Glycation for 32 h slightly decreased the pI values for β-cn modified with glucose and lactose. This was due to a slight increase in the net negative charge of the β-cn molecule, since the attachement of saccharides replaced the positively charged $-NH_3^+$ groups of lysine with uncharged sugar residues. Dephosphorylation of β-cn results in a decrease of the net negative charge of the N-terminal 50 amino acids at pH 7.0 from –11 to –3. Consequently the increase of pI of dephosphorylated β-cn was estimated. When both modifications were applied to β-cn, pI values were affected similarly as for dephosphorylated β-cn. The differences were significant at $P<0.01$.

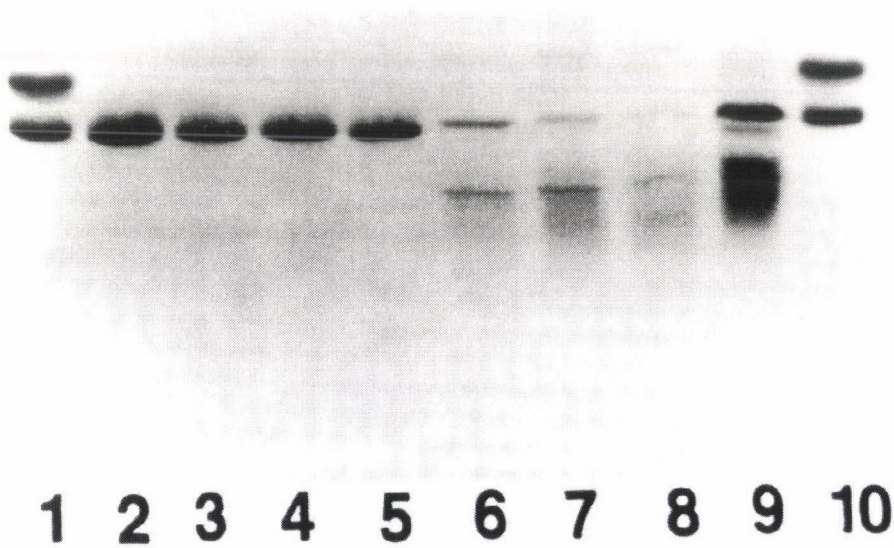

Fig. 1. Polyacrylamide gel electrophoresis of intact, glycated and dephosphorylated β-casein. For conditions see Materials and methods. 1: control (whole) casein; 2: intact β-casein (-cn); 3: glucosylated β-cn; 4: galactosylated β-cn; 5: lactosylated β-cn; 6: glucosylated and dephosphorylated β-cn; 7: galactosylated and dephosphorylated β-cn; 8: lactosylated and dephosphorylated β-cn; 9: dephosphorylated β-cn; 10: control (whole) casein

Table 1

The influence of glycation and dephosphorylation on pI values and solubility at the pI of intact and modified β-casein[a]

Sample[b]	Mean value of pH n=6	Standard deviation (±sd)	Corrected pI[c]	Solubility (%)[d]
Intact β-cn	5.72	0.08	4.98	22 ± 0.8
Glu-β-cn	5.63	0.05	4.89	44 ± 1.2
Lac-β-cn	5.62	0.03	4.88	40 ± 1.4
Dp-β-cn	6.48	0.03	5.74	20 ± 0.2
Dp-glu-β-cn	5.36	0.04	5.62	42 ± 0.9
Dp-lac-β-cn	5.34	0.04	5.60	40 ± 1.2

[a] For conditions see Materials and methods

[b] Key: β-casein (β-cn); glucosylated β-casein (glu-β-cn); lactosylated β-casein (lac-β-cn); dephosphorylated β-casein (dp-β-cn); dephosphorylated and glucosylated β-casein (dp-glu-β-cn); dephosphorylated and lactosylated β-casein (dp-lac-β-cn)

[c] The error of urea pH value (0.74)

[d] Differences are significant at $P<0.01$

2.3 RP-HPLC

Chromatograms of intact and dephosphorylated and glucosylated β-cn (dp-glu-β-cn) are presented in Fig. 2a and b. Dephosphorylated and lactosylated β-cn (dp-lac-β-cn) showed the same HPLC pattern as dp-glu-β-cn (data not shown). Intact β-cn, glucosylated β-cn (glu-β-cn) and lactosylated β-cn (lac-β-cn) had the same retention times (RT), (data not shown). BURR and co-workers (1996) have presented similar results for intact and lactosylated β-lactoglobulin. Dephosphorylation caused an increase of RT of sugar-free, glu- and lac-β-cn. This result is consistent with earlier results of MOLLÉ and LÉONIL (1995) as well as MINKIEWICZ and co-workers (1996), who found that RT of bovine caseinomacropeptide decreases with an increase of the number of phosphate groups. The peak with the RT value corresponding to this intact (phosphorylated) β-cn, indicated in Fig. 2b, can not be attributed to this protein, considering the fact that all phosphate groups were removed. We can rather suppose that it is a product of proteolysis. This may be supported by the SDS-PAGE and IEF results and the fact that RT of peptides with similar hydrophobicity decreases with the molecular weight according to the literature data (MANT et al., 1988).

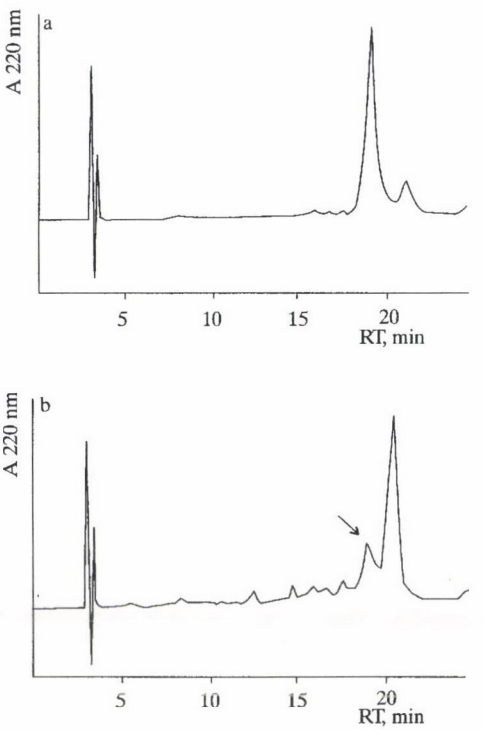

Fig. 2. The RP-HPLC chromatogram of intact (a) and lactosylated and dephosphorylated β-casein (b). For conditions see Materials and methods. Arrow indicates the peak with the retention time (RT) corresponding to intact β-casein

2.4 Second-derivative UV spectra

The second-derivative UV spectrum of dp-glu-β-cn is presented in Fig. 3. It was possible to measure accurately the apparent tryptophan/phenylalanine and tyrosine/phenylalanine molar ratios only for peaks of β-cn and dp-β-cn (both sugar-free and glycated). Other peaks have too low ratio of absorbances at the top of peak and at the saddle. The minima corresponding to aromatic amino acid residues, present within the range given by GREGO and co-workers (1986), were taken into consideration. The observed Trp/Phe ratios were 0.106 (sd=±0.006; n=4) and 0.115 (sd=±0.013; n=4) for β-cn (including glycated forms) and dp-β-cn (including glycated forms), respectively. There were statistically significant differences in the apparent Trp/Phe ratio between phosphorylated and dephosphorylated β-cn as well as between apparent ratios and true ratio for β-cn (0.111) calculated from the amino acid composition (SWAISGOOD, 1992).

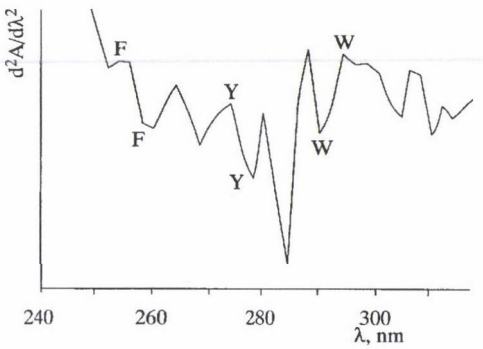

Fig. 3. The second-derivative UV spectrum of intact β-casein. For conditions see Materials and methods. F, Y, W: minima and maxima corresponding to phenylalanine, tyrosine and tryptophan, respectively

The observed Tyr/Phe ratios were: 0.35 (sd=±0.07; n=4) and 0.54 (sd=±0.06; n=4) for β-cn (including glycated forms) and dp-β-cn (including glycated forms), respectively. There were differences at $P<0.01$ between β-cn and dp-β-cn, respectively, as well as between both forms and the true ratio (0.44) at $P<0.05$. Glycation did not cause changes in the UV spectra. Second-derivative UV spectra reflect the amino acid composition of oligopeptides (PERRIN et al., 1995). In the case of proteins, the differences between second derivative of absorbance at the maxima and minima may also depend on the structure of proteins and solvent accessibility of individual aromatic amino acid residues (MACH & MIDDAUGH, 1994). In our case, the observed changes in UV spectra may be explained by the sensitivity of spectral properties of the tyrosine residues to the structural changes in β-cn particle caused by dephosphorylation. Our results differ from those reported by MACH and MIDDAUGH (1994). These authors found that the magnitude of peaks corresponding to Trp residues was more sensitive to the structural changes and environment than that corresponding to Tyr residues, but their experiments were performed in water solutions and in mixtures containing ethanol or polyethylene glycol in neutral pH. Our spectra were collected in highly acidic ACN: water mixtures used for RP-HPLC separations. Moreover, UV spectra of β-cn forms could be affected by the irreversible structural changes caused by the interactions of protein with urea during sample preparation. The influence of urea on protein spectral properties was observed by BOBE and co-workers (1998).

2.5 Effect of glycosylation and dephosphorylation on the solubility of β-casein

The covalent attachment of glucosyl or lactosyl residues to β-casein improved the solubility in the pH range of isoelectric points (Table 1). Changes in the solubility of

glycated fractions can be caused by the production of the steric hindrance of the β-cn molecule. It leads to higher electrostatic repulsion between the modified molecules. Removing five phosphate groups from β-casein causes a decrease in the net negative charge of the molecule and consequently decreases the solubility in the pH range of isoelectric point, as compared to the intact molecule. It is due to a decrease in the hydrophilicity, electrostatic repulsive forces and steric repulsion between the dephosphorylated molecules. Dephosphorylation of β-cn samples modified with glucose and lactose did not alter their solubility, as compared to glu- and lac-β-cn.

The effect of pH range from 2.0 to 8.0 and ionic strength 0.0, 0.1, 0.5 and 1.0 on the β-cn samples solubility is shown in Tables 2–5. Generally, in all cases, when the ionic strength increased, solubility also increased at the pH range of the isoelectric points. This phenomenon is due to the well known salting-in effect which decreases the electrostatic attractions between molecules and competition of Na^+ with protons. For intact β-cn, the solubility was slightly decreased at the acidic pH range when ionic strength increased. For glu- and lac-β-cn (results not shown, tendency the same as for glu-β-cn) the solubility increased at neutral and alkaline pH ranges, while it decreased at acidic pH range. Dephosphorylation significantly increased the solubility of β-cn molecules at acidic pH range and ionic strengths used. It caused however, slight decrease in solubility of dp-β-cn at neutral and alkaline pH ranges. Similar trends in behaviour of modified casein molecule in solutions at different pH and ionic strength was observed by COURTHAUDON and co-workers (1989) as well as VAN HEKKEN and STRANGE (1993). Dephosphorylation associated with glycation increased the solubility of the samples in the whole pH range, as compared to both intact and dp-β-cn samples. Generally, the improvement in the solubility can be attributed to the introduction of saccharide residues onto the β-cn molecule. The solubility of a protein has been reported to be a manifestation of the equilibrium between protein-solvent and protein-protein interactions (KINSELLA et al., 1985). Therefore, the attachment of hydrophilic carbohydrate moieties shifts the equilibrium away from protein-protein interactions and enhances solubility of the β-cn molecule. On the other hand the removal of the hydrophilic phosphate groups creates circumstances unfavoured for protein-solvent interactions (KINSELLA et al., 1985). In this case it seems that protein-protein interactions are increased more than protein-solvent interactions. It could lead to aggregation or even precipitation of modified β-cn molecule.

Table 2

Effect of pH and ionic strength on the solubility, % (± SD) of intact β-casein[a]

pH	Ionic strength			
	0.0	0.1	0.5	1.0
2.0	65.1 ± 0.3	76.0 ± 0.8	61.0 ± 0.4	60.0 ± 4.2
3.0	76.4 ± 0.4	77.0 ± 1.8	66.0 ± 0.5	66.0 ± 0.4
4.0	58.0 ± 1.2	63.0 ± 3.2	56.0 ± 0.5	57.0 ± 0.4
5.0	20.0 ± 1.7	31.0 ± 0.4	40.0 ± 1.2	40.0 ± 4.6
6.0	81.0 ± 1.2	88.0 ± 1.0	80.0 ± 0.4	77.0 ± 1.9
7.0	76.0 ± 0.7	91.0 ± 1.5	82.0 ± 1.2	75.0 ± 0.8
8.0	77.0 ± 1.5	90.0 ± 1.0	81.0 ± 1.9	75.0 ± 0.2

[a] For conditions see Materials and methods

Table 3

Effect of pH and ionic strength on the solubility, % (± SD) of glucosylated β-casein[a]

pH	Ionic strength			
	0.0	0.1	0.5	1.0
2.0	80.0 ± 1.6	78.0 ± 0.3	69.0 ± 0.6	70.0 ± 1.1
3.0	73.0 ± 0.1	55.0 ± 1.3	58.0 ± 0.7	58.0 ± 0.5
4.0	49.0 ± 0.6	50.0 ± 0.3	56.0 ± 0.4	54.0 ± 0.5
5.0	44.0 ± 1.5	49.0 ± 0.7	50.0 ± 0.4	52.0 ± 0.7
6.0	78.0 ± 0.8	79.0 ± 0.6	85.0 ± 1.1	85.0 ± 0.3
7.0	90.5 ± 0.4	100.0 ± 2.3	90.0 ± 1.1	99.0 ± 0.4
8.0	90.0 ± 1.0	90.0 ± 0.8	99.0 ± 0.2	100.0 ± 0.7

[a] For conditions see Materials and methods

2.6. Effect of β-casein glycation and dephosphorylation on its and α_{S1}-casein Ca^{2+} sensitivity

The Ca^{2+} solubility curves for intact, glycated and dephosphorylated β-cn and β-cn associated with α_{S1}-cn in solution are presented in Figs 4 and 5, respectively. The solubility of intact β-cn decreased significantly between 0 and 7 mmol l^{-1} Ca^{2+}.

Table 4

Effect of pH and ionic strength on the solubility, % (± SD) of dephosphorylated β-casein[a]

	Ionic strength			
pH	0.0	0.1	0.5	1.0
2.0	78.0 ± 0.1	89.0 ± 0.3	90.0 ± 0.8	88.0 ± 0.4
3.0	77.0 ± 0.8	85.0 ± .03	79.0 ± 0.4	85.0 ± 0.3
4.0	78.0 ± 0.1	78.0 ± 0.2	72.0 ± 0.1	77.0 ± 0.8
5.0	22.0 ± 0.3	32.0 ± 0.5	48.0 ± 1.7	42.0 ± 0.2
6.0	64.0 ± 0.2	59.0 ± 1.3	60.0 ± 0.4	58.0 ± 0.6
7.0	72.0 ± 0.4	64.0 ± 0.7	68.0 ± 0.5	76.0 ± 0.1
8.0	80.0 ± 1.3	76.0 ± 0.3	78.0 ± 0.6	70.0 ± 0.9

[a] For conditions see Materials and methods

Table 5

Effect of pH and ionic strength on the solubility, % (± SD) of dephosphorylated and glucosylated β-casein[a]

	Ionic strength			
pH	0.0	0.1	0.5	1.0
2.0	89.0 ± 1.2	97.0 ± 0.6	98.0 ± 0.2	99.0 ± 0.3
3.0	90.0 ± 1.5	98.0 ± 0.2	99.0 ± 0.9	97.0 ± 1.1
4.0	79.0 ± 0.1	93.0 ± 1.1	88.0 ± 0.4	92.0 ± 0.2
5.0	40.0 ± 1.7	53.0 ± 1.2	54.0 ± 1.4	55.0 ± 0.4
6.0	69.0 ± 0.2	90.0 ± 2.5	95.0 ± 0.4	97.0 ± 0.5
7.0	82.0 ± 0.5	91.0 ± 0.9	93.0 ± 1.2	96.0 ± 2.2
8.0	83.0 ± 0.6	90.0 ± 1.8	92.0 ± 1.7	97.0 ± 1.3

[a] For conditions see Materials and methods

Then reversal of solubility was detected up to 30 mmol l^{-1} Ca^{2+}. This behaviour can be due to the presence of carboxyl groups. Binding the Ca^{2+} can reverse their charge or displace a proton to produce a charged group. The appearance of electrostatic repulsion can prevent the β-cn molecule from polymerization and precipitation. The phenomenon of solubility reversal was observed for all our samples. Dephosphorylation caused the

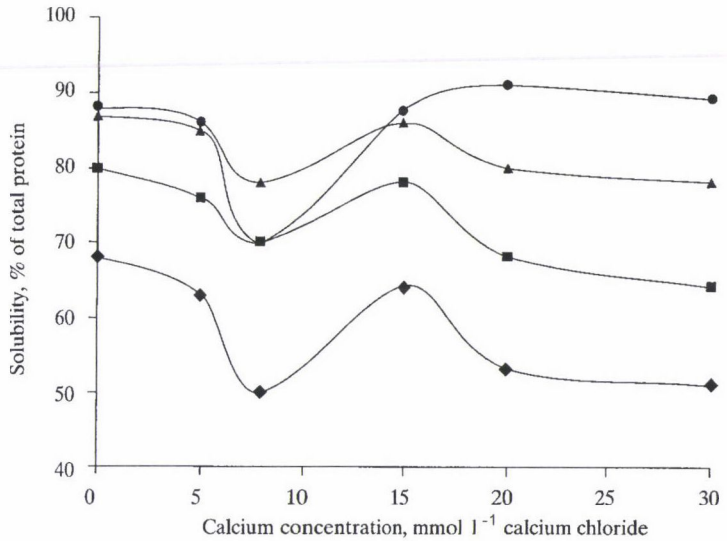

Fig. 4. Effect of Ca^{2+} concentration on the solubility of intact, glucosylated and/or dephosphorylated β-casein. ◆: intact β-casein; ●: glucosylated β-casein; ■: dephosphorylated β-casein; ▲: glucosylated and dephosphorylated β-casein. For conditions see Materials and methods. Maximum standard deviation (SD) was ±1.85

calcium sensitivity of β-cn to be decreased. Similar result was observed for glu- and lac-β-cn (result similar to the first one, not shown), but at a higher percentage of protein in solution. This may be due to the higher solubility of glycated β-cn, as compared to dephosphorylated one. This is the only way we can explain the lowest calcium sensitivity of glycated samples which still contain phosphate groups. Such explanation can be supported by the results obtained for glycated and dephosphorylated samples. Removal of negative phosphate groups, which are the binding sites for Ca^{2+}, decreases the association of β-cn molecule in solution. So the dp-β-cn is less likely to form a micelle and is better soluble in the presence of Ca^{2+}. In the presence of the α_{S1}-cn in solution the Ca^{2+}-independent dp-β-cn can protect the first one against the calcium precipitation. Because β-cn still possesses the amphiphilic structure, it can associate with α_{S1}-cn and, in this way, is free in solution. Glycation can deepen the behaviour of the molecule presented above by increasing the solubility of β-cn.

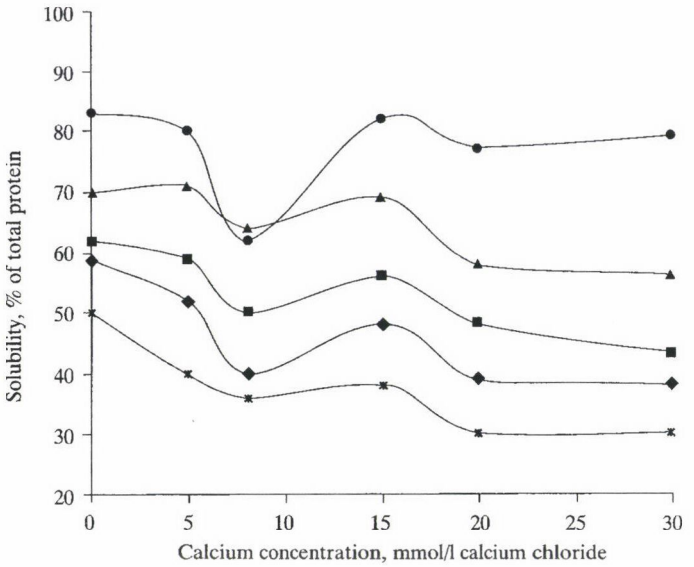

Fig. 5. Effect of Ca^{2+} concentration on the solubility of intact, glucosylated and/or dephosphorylated β-casein associated with α_{s1}-casein. ◆: intact β-casein with α_{s1}-casein; ●: glucosylated β-casein with α_{s1}-casein; ■: dephosphorylated β-casein with α_{s1}-casein; ▲: glucosylated and dephosphorylated β-casein with α_{s1}-casein; ✶: α_{s1}-casein; For conditions see Materials and methods. Maximum standard deviation (SD) was ±1.66

3. Conclusions

Binding of carbohydrate moieties with β-casein through glycation increased the interactions between the protein and polar environment, shifting the pI value to the acid pH range and leading to increased solubility. Dephosphorylation increased the retention time measured using RP-HPLC and changed the spectral properties of β-cn. Glycation did not affect the RP-HPLC patterns as well as the second-derivative UV spectra of protein. It suggests that dephosphorylation caused changes in the structure of peptide backbone of β-casein. The removal of charged phosphate groups shifted the pI value to the neutral pH range and decreased the solubility of β-cn. On the other hand both treatments significantly altered the solubility of β-cn. Moreover, both modifications (alone or combined) decreased the calcium sensitivity of β-cn, making it to keep α_{S1}-cn in solution in the presence of Ca^{2+}. All these modifications should also appreciably

modify other functional properties, but the data concerning the foaming and emulsifying properties of modified β-cn are now being prepared. To investigate the modified β-cn structure-function relationship in more detail we will focuse in our future studies on the secondary structure of β-cn in solution and adsorbed state.

*

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Short communications

ANTIFUNGAL EFFECTS OF *MICROMERIA MYRTIFOLIA* BOISS. & HOHEN. IN BOISS. AND *PRANGOS UECHTRITZII* BOISS. HAWSSKN DECOCTIONS

M. ÖZCAN

Department of Food Engineering, Faculty of Agriculture, Selçuk University, 42031 Konya, Turkey

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Antifungal effect of *Micromeria myrtifolia* Boiss. & Hohen. in Boiss. and *Prangos uechtritzii* Boiss. Hawsskn decoctions was tested against *Alternaria alternata, Aspergillus niger, Aspergillus parasiticus, Botrytis cinerea, Fusarium oxysporum* f.sp. melonis and *Penicillium digitatum*. Of the 2 substances tested *Pr. uechtritzii*, being present at 75 to 80% concentration in potato dextrose agar, partly inhibited growth of *A. alternata, B. cinerea* and *P. digitatum*. *Pr. uechtritzii* had higher antifungal effect than *M. myrtifolia* on mycelial growth during incubation. *M. myrtifolia* partly affected mycelial growth of *A. alternata* and *A. niger* at the beginning of incubation. But the mycelial growth of *F. oxysporum* was not inhibited by *M. myrtifolia* concentrations during incubation. Also, *Pr. uechtritzii* did not have any affect on mycelial growth of *A. niger* during incubation *P. digitatum*, the most sensitive microorganism to both decoctions. Higher decoction concentrations of plants used in study will be probably inhibit mycelial growth of microorganisms.

Keywords: antifungal effect, decoction, inhibition, moulds

Micromeria myrtifolia Boiss. & Hohen. in Boiss. and *Pr. uechtritzii* Boiss. Hawsskn grow as wild, and are known as taş çayı and çaşır in Turkey, respectively. *M. myrtifolia* is drunk as tea. But *Pr. uechtritzii* is used as vegetable and pickling product. Although spices are used primarily for their desirable flavour and odour, they may play other important roles in food systems. They are highly valued for their use as antimicrobial agents. Antimicrobial properties of spices and of their essential oils have been documented. The preservative action of herbs and spices has only recently received attention in the literature where studies have been reported that mycotoxin-producing moulds may be inhibited by some herbs and spices. Past investigations have indicated that the antimicrobial factor of spices resides in the essential oil/or oleoresin fraction. Aromatic plants/spices, herbs and derivatives widely used in foods were used

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as antimicrobial agents against several microorganisms under in vitro conditions. While antimicrobial activities of several spices in culture media have been reported over the years, few tests have been conducted in food systems (BEUCHAT, 1976; SHELEF et al., 1980, HITOKOTO et al., 1980; AZZOUZ & BULLERMAN, 1982; ZAIKA et al., 1983; FARAG et al., 1989; BENJILALI et al., 1984; GRAHAM & GRAHAM, 1987; BOYRAZ & ÖZCAN, 1997).

There are several chemicals that can be used as antimicrobial agents. For instance, acetic acid and sulfur dioxide are widely used as food preservatives. However, these chemicals require caution in handling since they are corrosive and their vapours can irritate the eyes and respiratory tract. On the contrary, herbs and their derivatives possessing antimicrobial activity, might have beneficial effect, but cause no health problems to the handler and consumer. In this respect, various essential oils of spices were tested for their inhibitory activity towards the growth of some microorganisms.

The objective of this work was to evaluate the inhibitory potency of *M. myrtifolia* and *P. uechtritzii* decoctions on *A. alternata*, *A. niger*, *A. parasiticus*, *B. cinerea*, *F. oxysporum* and *P. digitatum* in vitro.

1. Materials and methods

1.1. Materials

Micromeria myrtifolia Boiss. & Hohen. in Boiss. (*Labiatae*) and *Prangos uechtritzii* Boiss. Hawsskn (*Apiaceae*) used in the experiments were collected from Mersin (Gülnar) and Kayseri, respectively, in 1997. Plants were identified at the Department of Biology, Faculty of Science and Education, Selçuk University.

1.2. Organisms

Moulds used in this study were: *A. parasiticus* NRRL 2999, obtained from USDA, Agricultural Res. Service, National Center for Agricultural Utilization Res. Service, Illinous, USA; *A. alternata*, *A. niger*, *B. cinerea*, *F. oxysporum* and *P. digitatum* obtained from Department of Food Engineering, Faculty of Agriculture, Selçuk University.

1.3. Medium

Potato dextrose agar (E. Merck, Darmstadt) was used as main medium in the experiment. Plants used in this study were boiled in water (1:2, w/v) for one hour. After the decoctions were filtered, they were cooled and stored in refrigerator until use. Decoctions to be used in the experiment were prepared as 50, 66, 75 and 80% concentrations. Then, each medium of about 120 ml quantity, prepared from different concentrations, separately was put into 250 ml Erlenmeyer flasks and sterilized by autoclaving at 121 °C for 15 min.

1.4. Analysis

The effect of decoctions at different concentrations (50, 66, 75 and 80%) was determined against *A. niger*, *A. parasiticus*, *A. alternata*, *B. cinerea*, *F. oxysporum* and *P. digitatum* grown on Czapek Dox agar. Potato dextrose medium containing the decoctions in different concentrations was dispensed into petri dishes (20 ml/dish). Five mm discs of the test fungi, cut from periphery of 7 day old cultures, were inoculated upside down separately to each assay plate and incubated at 28 °C. The colony diameter was measured and percent mycelial inhibition was calculated (DEANS & SVOBODA, 1990). Four replicates of each treatment were similarly maintained and averages calculated. Control sets were simultaneously run without using decoctions of plants.

$$I = [(C–T)/C] \times 100$$

I: Inhibition (%)
C: Colony diameter of mycelium from control petri plate (mm)
T: Colony diameter of mycelium from test petri plate (mm)

2. Results and discussion

The inhibitory effects of different concentrations of plant decoctions were tested. The results are shown in Tables 1 and 2.

While decoctions of *Pr. uechtritzii* were effective against *A. alternata*, *A. parasiticus*, *B. cinerea* and *P. digitatum* during incubation, *M. myrtifolia* was effective also against *P. digitatum* and *B. cinerea*. The decoction at concentration of 80% of *P. uechtritzii* had the largest effect on mycelial growth of *A. alternata*, *A. parasiticus* and *P. digitatum*. However, none of the concentrations of *Pr. uechtritzii* showed inhibitory effect against *A. niger*. Also, *F. oxysporum* showed resistance against all concentrations after five days. High concentrations usually showed high inhibitory effect.

Table 1

Inhibitory effect of M. myrtifolia *decoction at different concentrations (% inhibition)*

Incubation days	Concentrations %	*A. alternata*	*A. niger*	*A. parasiticus*	*B. cinerea*	*F. oxysporum*	*P. digitatum*
3	50	2	–[a]	–	4	–	43
	66	3	29	–	5	–	48
	75	13	29	–	6	–	57
	80	13	33	–	28	–	65
4	50	5	–	–	5	–	66
	66	11	31	–	7	–	70
	75	20	31	–	8	–	71
	80	29	37	–	25	–	72
5	50	–	–	–	6	–	54
	66	11	43	–	24	–	68
	75	19	49	–	24	–	68
	80	30	51	–	27	–	69
6	50	–	–	30	10	–	41
	66	13	–	33	24	–	58
	75	21	–	35	37	–	60
	80	36	–	54	38	–	61
7	50	–	–	32	32	–	37
	66	18	–	35	35	–	47
	75	28	–	38	42	–	49
	80	44	–	53	51	–	50
8	50	–	–	19	36	–	32
	66	–	–	24	36	–	41
	75	–	–	31	47	–	44
	80	–	–	47	52	–	48
9	50	–	–	9	42	–	31
	66	–	–	14	42	–	37
	75	–	–	22	50	–	41
	80	–	–	41	52	–	43
10	50	–	–	–	42	–	30
	66	–	–	–	44	–	33
	75	–	–	–	49	–	37
	80	–	–	–	52	–	39

[a] No inhibition

None of the concentrations of *M. myrtifolia* showed inhibitory effect against *F. oxysporum* through incubation. Also, not all concentrations showed inhibitory effect against *A. parasiticus*, *A. niger* and *A. alternaria* during incubation. The most sensitive mould tested against all concentrations of *M. myrtifolia* were *P. digitatum* and partly *B. cinerea*. However, the concentration of 80% in accordance with other concentrations of *M. myrtifolia* showed higher inhibitory effect against some mould used in the experiment. *Pr. uechtritzii* did not affect the mycelial growth of *A. niger* during incubation at all.

Consequently, these two decoctions had partly inhibited the growth of some microorganisms used in the experiment. Inhibitory effect of both decoctions was lower than that of the spices themselves and derivatives such as essential oils or oleoresin (AZZOUZ & BULLERMAN, 1982; SHELEF, 1983; ÖZCAN, 1998). This decrease can be probably due to the evaporation of their essential oils during boiling, because their components have got antimicrobial effect (BEUCHAT, 1976, SHELEF et al., 1980).

Table 2

Inhibitory effect of Pr. uechritzii *decoction at different concentrations (% inhibition)*

Incubation days	Concentrations (%)	*A. alternata*	*A. niger*	*A. parasiticus*	*B. cinerea*	*F. oxysporum*	*P. digitatum*
3	50	36	–[a]	4	4	32	24
	66	45	–	8	6	39	47
	75	48	–	10	15	55	49
	80	48	–	12	19	59	57
4	50	38	–	6	5	17	50
	66	47	–	8	7	34	64
	75	49	–	11	13	41	71
	80	51	–	14	27	54	71
5	50	37	–	10	7	15	52
	66	44	–	10	12	27	57
	75	46	–	18	28	36	69
	80	57	–	29	29	50	71
6	50	31	–	23	10	–	54
	66	34	–	32	23	–	59
	75	44	–	39	28	–	68
	80	57	–	61	39	–	79
7	50	29	–	18	26	–	53
	66	34	–	32	54	–	54
	75	41	–	40	57	–	70
	80	59	–	59	60	–	77
8	50	31	–	12	31	–	52
	66	39	–	18	58	–	56
	75	44	–	29	60	–	65
	80	57	–	48	62	–	75
9	50	36	–	11	32	–	50
	66	42	–	14	59	–	51
	75	45	–	25	60	–	58
	80	47	–	39	61	–	62
10	50	24	–	4	39	–	49
	66	32	–	8	63	–	50
	75	39	–	12	63	–	61
	80	43	–	14	67	–	69

[a] No inhibition

As a result, the effect of decoctions was not 100% on mycelial growth, *Pr. uechtritzii* showed the highest inhibitory effect against all the moulds tested. While all the concentrations of *Pr. uechtritzii* showed inhibitory effect against *A. alternata*, *A. parasiticus*, *B. cinerea* and *P. digitatum* during incubation, *M. myrtifolia* showed inhibitory effect against only *P. digitatum* and *B. cinerea*. The most sensitive microorganism to both water decoctions was *P. digitatum*. So, the higher decoction concentrations of both plants will probably inhibit the mycelial growth of *A. alternaria*, *B. cinerea* and *P. digitatum*.

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β-GLUCOSIDASE PRODUCTION OF TWO DIFFERENT *ASPERGILLUS* STRAINS

A. BRUMBAUER[a] and K. RÉCZEY

Department of Agricultural Chemical Technology, Technical University, Budapest, H-1521 Budapest, P.O. Box 91. Hungary

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β-Glucosidase has an important role in cellulose degradation by cleaving the cellobiose to glucose units. Supplementation of *Trichoderma* cellulase with exogenous β-glucosidase is needed to prevent the inhibition effect of cellobiose on exoglucanases and endoglucanases.

Production of β-glucosidase by *Aspergillus niger* and *Aspergillus phoenicis* has been investigated under different fermentations. Both strains were appropriate for enzyme production at high level under the applied conditions.

Cultivation of *A. phoenicis* on Mandels' medium containing a complex nitrogen source has resulted in a higher β-glucosidase activity than on Vogel's medium. In an air-lift fermenter *A. phoenicis* grew in the shape of beads with 1.41 IU g^{-1} cell-associated enzyme activity. The fungal pellets can be used as in situ immobilized enzyme preparation.

A. niger produced extracellular β-glucosidase at the level of 2.1 IU ml^{-1} in stirred-tank fermenter with a yield of 210 IU g^{-1} glucose and productivity of 21.8 IU l^{-1} h^{-1}.

Keywords: β-glucosidase, fermentation, *A. niger*, *A. phoenicis*

Enzymatic hydrolysis of lignocellulosic materials appears to be one of the most promising ways to produce renewable energy. In the hydrolysis of cellulose – the major component of plants – three main components of the cellulase enzyme system are acting simultaneously, i.e. 1,4-β-D-glucan-4-glucanohydrolases (endoglucanase, EC 3.2.1.4), 1,4-β-D-glucan-cellobiohydrolases (exoglucanase, EC 3.2.1.91) and β-1,4-glucosidase (β-glucosidase, EC 3.2.1.21). The hydrolysis of insoluble native cellulose carried out by the synergistic action of exoglucanases and endoglucanases results in soluble cellodextrins and cellobiose; then β-glucosidase cleaves the cellobiose units to glucose (ENARI, 1987).

However *Trichoderma reesei*, which is one of the best cellulase producing microorganisms (MANDELS, 1975) secretes β-glucosidase at a low level. The cellobiose accumulating during the hydrolysis inhibits the activity of endoglucanase and

[a] Tel: 36 1 463 1279, Fax: 36 1 463 2598; E-mail: brumbauer@chem.bme.hu

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exoglucanase, decreasing the overall rate of hydrolysis (HOWELL & STUCK, 1975, HSU et al., 1980, MAGUIRE, 1977). One the other hand cellobiose is not fermentable by *Saccharomyces cerevisiae* (WOODWARD & WISEMAN, 1982). Therefor β-glucosidase activity should be kept at an optimal level related to cellulase in order to increase the saccharification rate of cellulose (STERNBERG et al., 1977, KHAN et al., 1985). One way to diminish the effect of cellobiose inhibition is the addition of exogenous β-glucosidase. Fungi belonging to the genus *Aspergillus* produce extracellular and cell-associated ß-glucosidase in high yield, which is compatible with *T. reesei* cellulases (ALLEN & STERNBERG, 1980, RÉCZEY et al., 1989, FLACHNER et al., 1999).

In this study *Aspergillus phoenicis* QM 329 and *Aspergillus niger* BKM F-1305 were examined, grown in shake flask and different kind of fermenter (air-lift, stirred-tank fermenter). The aim of the work was to compare the extracellular and intracellular β-glucosidase production of the above *Aspergilli* under different fermentation conditions.

1. Materials and methods

1.1. Microorganisms

Aspergillus niger BKM F-1305 and *Aspergillus phoenicis* QM 329 were maintained on agar slant containing 5% malt extract, 18 g l^{-1} bacto agar, at 30 °C. After 14 days the spores were used for inoculation. The strains were obtained from the Culture Collection of the Dept. of Agricultural Chemical Technology, Technical University of Budapest.

1.2. Media

Inoculum was prepared the following way. One percent of spore suspension, containing 5×10^5 conidia ml^{-1}, was used to initiate growth on 5% malt extract at 5.4–5.6 pH. The inoculum preparation was complete after 2 days at 31 °C and 350 r.p.m.

Two different nutrient media were used. The composition of Vogel's medium was (g l^{-1}): glucose 10, proteose peptone 1.0 and citric acid 0.5. After sterilization 2% Vogel's stock solution was added to the medium (VOGEL, 1964).

The composition of Mandels medium was (g l^{-1}): urea 0.3, $(NH_4)_2SO_4$ 1.4, KH_2PO_4 2, $CaCl_2$ 0.3, $MgSO_4$ 7 H_2O, 0.3, proteose peptone 0.75, yeast extract 0.25, and trace elements were added to the medium in the following concentrations from 1% solutions: 3.3 ml l^{-1} $FeSO_4$. 7 H_2O, 1.1 ml l^{-1} $MnSO_4$, 0.9 ml l^{-1} $ZnSO_4$ and 1.3 ml l^{-1} $CoCl_2$ together with 10 g l^{-1} glucose as carbon source (MANDELS & WEBER, 1969).

1.3. β-Glucosidase production

Shake flask cultures were made in 750 ml Erlenmeyer flasks containing 150 ml medium at 30 °C and 350 r.p.m. Inoculation was made with 15 ml inoculum, described above. Samples were taken once a day to observe consumption of reducing sugar and production of β-glucosidase; pH was adjusted daily to 5.8–6.0. Three parallel flasks were run with both fungi.

The stirred-tank reactor was a 24 l BIOSTAT U (Braun, Germany) fermenter with working volume of 18 l, 0.5–1.0 v/v min^{-1} aeration and 250–300 r.p.m. stirring. The inoculum constituted 10% of the total volume. The initial pH was 5.8–6.0 and it was adjusted to 6.0 after the first day, when it was let to go down to pH 3.0, by addition of 10% NaOH and 10% H_2SO_4. Enzyme production was continuing on for 4 days, samples were regularly withdrawn and analysed for reducing sugar content and β-glucosidase activity. The calculated yield and productivity were based on extra- and intracellular β-glucosidase activity.

The air-lift fermenter was a Pyrex glass cylinder with a diameter of 70 mm and a height of 500 mm with 1200 ml working volume. The bottom of reactor was a porous glass filter with a pore size of 40–90 μm. Agitation and aeration were performed by letting sterile air through the glass filter at a rate of 450–500 l h^{-1}. Temperature was maintained at 30 °C, the initial pH was 5.2, not adjusted. The air-lift fermenter was directly inoculated with conidia resulting in 10^5–10^6 conidia ml^{-1} broth, determined with Bürker counting chamber.

One experiment was run with each *Aspergillus* strain in stirred-tank fermenter and in air-lift fermenter as well.

1.4. Assays

The substrate consumption was determined colorimetrically using dinitrosalicylic acid (DNS) reagent described by MILLER (1959). Samples of 0.1–0.5 ml were made up to 1.5 ml with distilled water and 3 ml of DNS reagent was added. The colour obtained after boiling the mixture for 5 min and diluting with 16 ml of distilled water was measured at 550 nm. The relative standard deviation of this method was ≤2.8%.

Extracellular β-glucosidase activity was measured with 5 mmol l^{-1} pNPG in 50 mmol l^{-1} citrate-buffer, pH 4.8. One ml of substrate and 0.1 ml of correspondingly diluted culture filtrate were incubated for 10 min at 50 °C (NORKRANS, 1957). The reaction was stopped by addition of 2 ml 1 mol l^{-1} Na_2CO_3. After cooling, 10 ml of distilled water was added and the absorbance was read at 400 nm. The activity was calculated as IU ml^{-1}. One international unit is defined as one μmol p-nitrophenol produced by 1.0 ml of enzyme per minute under the assay conditions. The assays were

done in duplicate on each sample and the mean of the obtained values was used. Relative standard deviation was ≤2.0%.

For cell-associated, intracellular β-glucosidase activity measurement the pellets were separated from the fermentation broth by filtration through a Büchner funnel. Wet pellets were directly used instead of 0.1 ml of culture filtrate and treated as above. Intracellular activity was calculated as IU g^{-1} wet pellet. The determinations were done in triplicate on each sample and the mean was used. Relative standard deviation was ≤4.5%.

2. Results and discussion

2.1. Extracellular β-glucosidase production on two different nutrient media

The comparison of β-glucosidase production of *Aspergillus phoenicis* on two different nutrient media was carried out in shake flasks with 10 g l^{-1} glucose as carbon source, inoculated by spore suspension. The results on Fig. 1A and 1B show that the reducing sugar concentration decreased in two days to zero on Mandels' medium while on Vogel's medium it lasted for three days. The starting pH 5.5–6.0 decreased to about 3 on both media but the reduction was much quicker on Mandels' medium. In both cultivations pH were adjusted to 6.0, but only on Vogel's medium occurred a drastic pH drop again. The greatest difference between the two media was in extracellular β-glucosidase production. On Vogel's medium enzyme activity appeared in the fermentation broth after a rather long lag phase of 3 days, while on Mandels' medium it reached 0.9 IU ml^{-1} already on the first day of cultivation. Maximum activity, yield and productivity were five times higher on Mandels' medium than on Vogel's after 7 days of fermentation (Table 1).

Table 1

Comparison of extracellular β-glucosidase activity, yield and productivity on two different culture media

Culture medium[a]	Vogel's medium	Mandels' medium
Activity (IU ml^{-1})	0.46	2.38
Yield (IU g^{-1} glucose)	46	238
Productivity (IU l^{-1} h^{-1}	2.7	14.2

[a] Cultivation was performed in shake flasks with *A. phoenicis*.

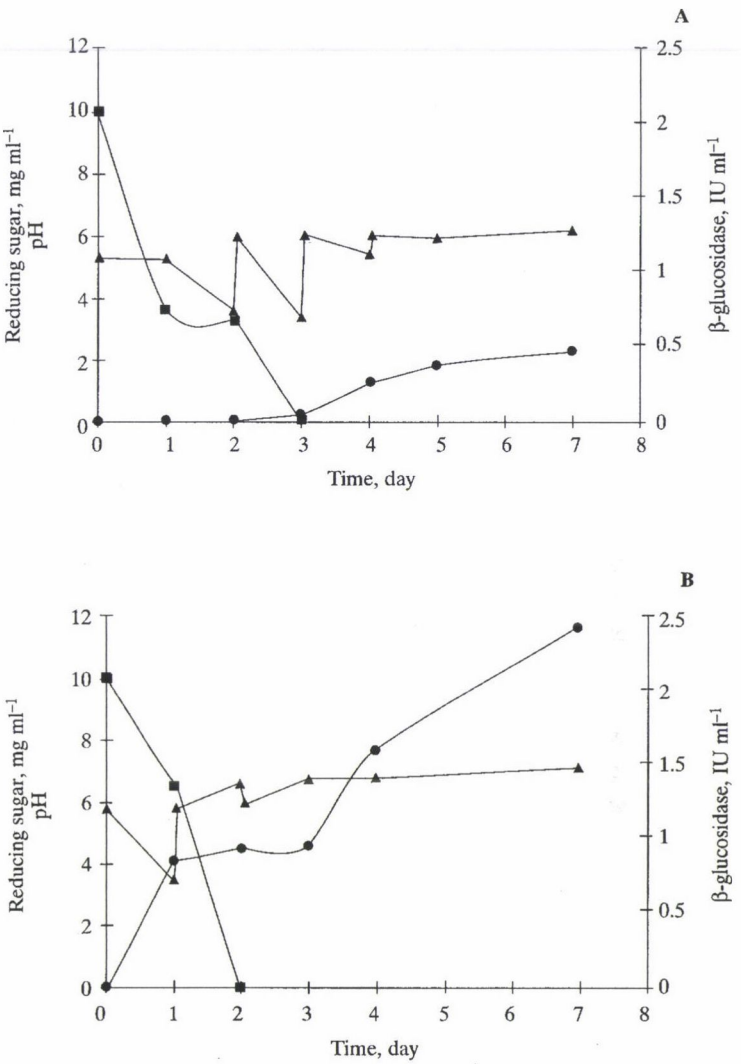

Fig. 1. Comparison of extracellular β-glucosidase production of *A. phoenicis* on different nutrient media. The cultivations were carried out in shake flasks with 10 g l^{-1} carbon source. A: Cultivation on Vogel's medium. B: Cultivation on Mandels' medium. —■—: reducing sugar (mg ml^{-1}); —▲—: pH; —●—: extracellular β-glucosidase activity (IU ml^{-1})

2.2. β-Glucosidase production in shake flask and stirred-tank fermenter

Strains, *Aspergillus niger* and *Aspergillus phoenicis* were cultivated in shake flasks and stirred-tank fermenter in order to compare the extracellular and intracellular ß-glucosidase production under the same circumstances. Mandels' medium was used with 10 g l^{-1} glucose as carbon source. Both strains showed the same behaviour in shake flasks resulting in 2.80 IU ml^{-1} extracellular enzyme activity. The yield of both cultivation was 280 IU g^{-1} based on the added amount of glucose and 19.5 IU l^{-1} h^{-1} productivity was reached after a 6-day cultivation.

Whereas experiments performed in stirred-tank fermenter have shown different pH profile and carbon source utilization (Fig. 2A and 2B). *A. phoenicis* has consumed the carbon source somewhat slower but on the second day the reducing sugar concentration was zero in both experiments. In *A. niger* fermentation the pH dropped more rapidly and reached a minimum value of pH 1.6 at 22 h of fermentation. Change in pH was not so drastic in *A. phoenicis* cultivation, where minimum was pH 2.4 at 41 h of fermentation. Appearance of the β-glucosidase happened at about the same time, when pH has dropped under 3.0 and the carbon source was consumed. Final extracellular and intracellular β-glucosidase activity were 2.4-fold and 2.7-fold higher in *A. niger* cultivation than in *A. phoenicis* culture. The yield and productivity of *A. phoenicis* based on extra- and intracellular activity were only 38 and 39%, respectively, related to *A. niger* (Table 2).

Table 2

β-Glucosidase production on Mandels' medium in stirred-tank fermenter

Strains	*A. niger*	*A. phoenicis*
Extracellular enzyme activity (IU ml^{-1})	2.1	0.86
Inracellular enzyme activity (IU g^{-1} wet pellet)	12.5	4.68
Yield[a] (IU g^{-1} glucose)	185.8	70
Productivity[a] (IU l^{-1} h^{-1})	29.9	11.7

[a] Yield and productivity were based on extra- and intracellular enzyme activities

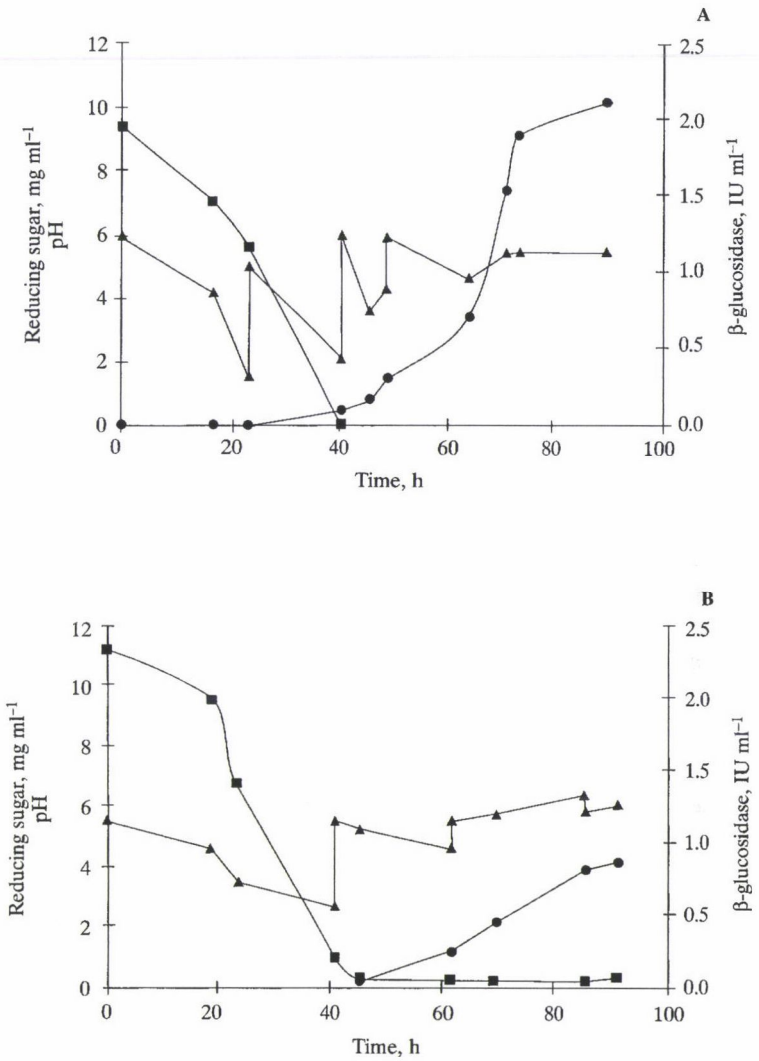

Fig. 2. Comparison of cultivation of *A. niger* and *A. phoenicis* in a 24-l stirred-tank fermenter on Mandels' medium. The working volume was 18 l with 10% of inoculation. The carbon source was glucose at 10 g l^{-1}. A: Cultivation of *A. niger* BKM F-1305. B: Cultivation of *A. phoenicis* QM 329. —■—: reducing sugar (mg ml^{-1}); —▲—: pH; —●—: extracellular β-glucosidase activity (IU ml^{-1})

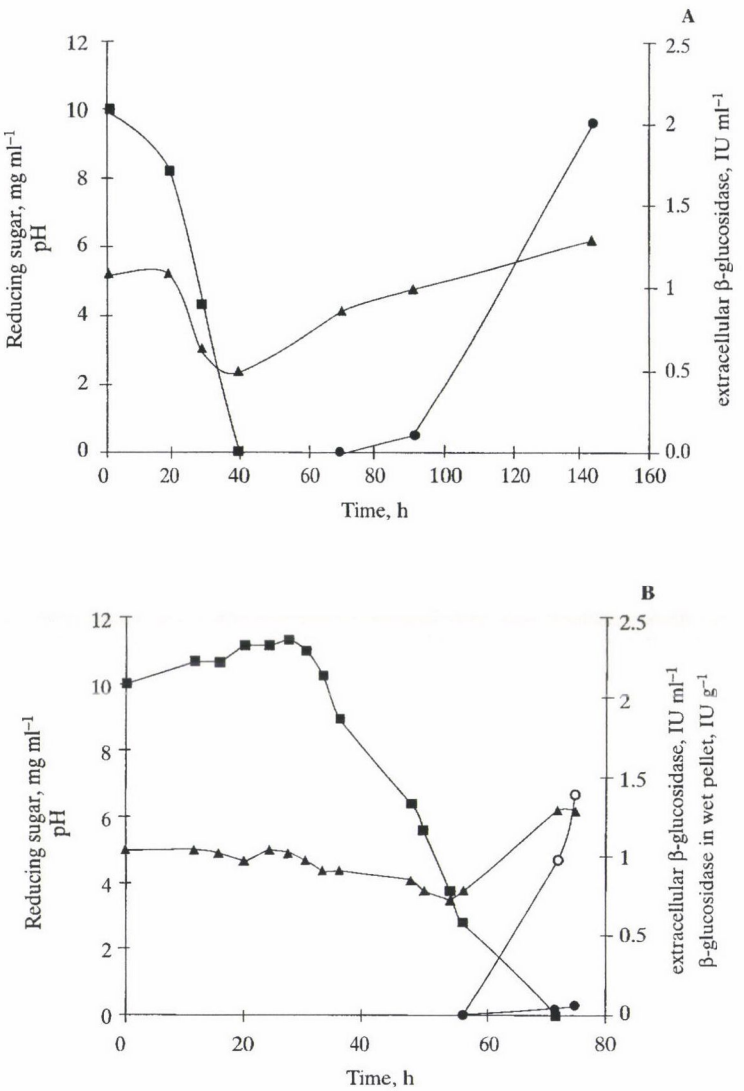

Fig. 3. Comparison of cultivation of *A. niger* and *A. phoenicis* in air-lift fermenter. The media were Mandels' and Vogel's medium, respectively. The working volume was 1200 ml with 10 g l^{-1} glucose as carbon source. A: Cultivation of *A. niger* BKM F-1305. B: Cultivation of *A. phoenicis* QM 329. —■—: reducing sugar (mg ml^{-1}); —▲—: pH; —●—: extracellular β-glucosidase activity (IU ml^{-1}); —○—: wet pellet activity (IU g^{-1})

2.3. Enzyme production in air-lift fermenter

Both *A. niger* and *A. phoenicis* were cultivated in air-lift fermenter using Mandels' and Vogel's media, respectively (Fig. 3.). *A. niger* consumed glucose in 39 h while there was a rapid decrease in pH to 2.4. In the *A. phoenicis* cultivation glucose was exhausted after 72 h. During this cultivation pH dropped quite slowly and reached a minimum, pH 3.5 in 54 h. *A niger* has grown in filamentous and micropellet shapes producing 2.01 IU ml^{-1} extracellular β-glucosidase activity after 6 days of cultivation. It was not possible to detect intracellular enzyme activity owing to these growing forms. Under the same circumstances *A. phoenicis* has grown in the shape of beads with 1.41 IU g^{-1} wet pellet activity and only 0.08 IU ml^{-1} enzyme was detectable in the supernatant (Fig. 3A, Fig. 3B).

3. Conclusions

Both *Aspergillus* strains can produce β-glucosidase at high level to supplement cellulase of *Trichoderma*, corresponding to earlier studies (ALLEN & STERNBERG, 1980, KERNS et al., 1987). However our results show that while *A. phoenicis* is recommended for immobilized enzyme production using air-lift fermenter, *A. niger* is recommended for soluble enzyme production in stirred-tank fermenter.

Cultivating *A. niger* in stirred-tank fermenter 12% and 56% higher extracellular enzyme productivity can be achieved than in shake flasks and in air-lift fermenter, respectively (Table 3.).

Comparing the inoculation method of *Aspergillus phoenicis*, using the 2-day-inoculum on malt extract 37% higher productivity was achieved than by using a direct inoculation with conidia (Table 1 and 2.2. Chapter).

Table 3

Productivity of extracellular β-glucosidase activity with Aspergilli *in different type of fermenters (IU l^{-1} h^{-1})*

Strains	*A. phoenicis*	*A. niger*
Shake flasks	19.5	19.5
Stirred-tank fermenter	9.3	21.8
Air-lift fermenter	0.56	13.9

*

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LIPID INTAKE AND SERUM CHOLESTEROL LEVEL IN CYSTIC FIBROSIS PATIENTS

A. MADARASI,[a] E. GREINER,[b] R. ÚJHELYI,[c] E. SÓLYOM[d], L. BÍRÓ,[b] E. MOZSÁRY,[b] A. REGÖLYI-MÉREI[b] and M. ANTAL[b]

[a] Buda Children's Hospital, H-1023 Budapest, Bolyai u. 5–7. Hungary
[b] NCPH-National Institute of Food Hygiene and Nutrition, H-1097 Budapest, Gyáli út 3/a. Hungary
c Heim Pál Children's Hospital, H-1089 Budapest, Üllői út 86. Hungary
[d] Borsod County Teaching Hospital, Child Health Center, H-3501 Miskolc, Szentpéteri kapu 72–76. Hungary

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The authors evaluated the data of 30 patients with cystic fibrosis (18 male, 12 female), between ages 2–18 (average age: 9.57 years). In 60% of the patients serum cholesterol was below the 5th percentile value according to the data of the NATIONAL CHOLESTEROL EDUCATION PROGRAM (1991). They examined the connection between the serum cholesterol levels and the total fat, fatty acids and cholesterol intake. No significant differences were found between total fat, fatty acids and cholesterol intake of patients with serum cholesterol levels below the 5th percentile value and patients with levels above the 5th percentile value. The analysis of the nutritional data showed that lipid intake was in accordance with the proportion prescribed for patients with CF (35.56% of energy). The intakes of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) were 11%, 11% and 8.3% of total energy, respectively, the PUFA/SFA ratio was 0.78. The ratio of linoleic to alfa-linolenic acid was especially high: 29. The clinical condition of the patients in the low cholesterol level group, as shown by the Shwachman-Kulczycki score was significantly lower ($P<0.05$) than that of the group with serum cholesterol levels above the 5th percentile value. The authors therefore suggest the importance of the regular monitoring of serum cholesterol levels in patients with cystic fibrosis. Observing the changes in serum cholesterol levels is a fast, informative and inexpensive method of evaluating the clinical course of the disease.

Keywords: cystic fibrosis, serum cholesterol, nutrient intake

Cystic fibrosis (CF) is one of the most common life-threatening, metabolic diseases with an autosomal recessive pattern of inheritance. There is virtually no other chronic disease, where the nutritional state plays such a crucial role in the progression (ROULET, 1994). The fate of patients with CF is determined mainly by the state of their lungs (BEDDOES et al., 1981; DODGE, 1992). It has been conclusively proven that adequate nutritional state slows the progression of the pulmonary process and results in

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increased life expectancy and better life conditions for the patients (ANTHONY et al., 1998.; MIETTIEN, 1972.; ROULET, 1994.). This is why physicians treating CF patients place special emphasis on the evaluation of the nutritional state and related issues (CABRERA et al., 1994; CANNELA et al.,1993, MARCUS et al., 1991; PLATA-SALAMÁN, 1996; SÓLYOM, 1996).

The aim of our study was to examine the cause and clinical significance of the decreased levels of serum cholesterol seen in patients with CF. We compared the nutrients intake – focusing on total fat, fatty acids and cholesterol – of patients with low levels of serum cholesterol and patients with serum cholesterol level above the 5th percentile value.

1. Methods

The study was carried out on 30 patients (18 male, 12 female) between 1 and 18 years of age (average age: 9.57 years) with cystic fibrosis, recruited from the outpatient department for a scheduled follow up visit. All patients and their parents volunteered for the study, which had been accepted by the ethical committee of Haynal Imre University of Health Sciences. The disease was diagnosed based on the clinical signs and the elevated levels of sweat chloride (>60 mmol l^{-1}); all of the patients underwent mutation studies (Table 1).

The clinical condition of the patients was evaluated with the Shwachman-Kulczycki score (SHWACHMAN & KULCZYCKI, 1958). As the analysis of stool lipid excretion was not feasible for all of the children involved in the study, the dosage of the enzyme substitution was set ensure one or maximum two stools of normal consistency daily. The average lipase intake of our patients was ~5000 IU kg^{-1}/day.

Table 1

Mutations of cystic fibrosis patients (n=30)

Number of patients	Mutation
15	ΔF508/ΔF508
4	ΔF508/N
2	ΔF508/R553X
2	ΔF508/G542X
1	R553X/N
1	N/W1282X
5	N/N

Participants had previously received three questionnaires prepared for recording meals consumed on 2 weekdays and on the last Sunday before the examination. The method of 3×24 h dietary record has been applied together with 1×24 h recall made by a trained dietitian. The energy and nutrient intakes were calculated on the basis of the nutrient data bank and computer program, both developed by the National Institute of Food Hygiene and Nutrition (BÍRÓ et al., 1996., GANJI et al., 1998).

Laboratory measurements were performed as follows:

– Hepatic enzymes, alkaline phospatase, albumin were measured on a Hitachi 704 clinical analyzer with commercially available kits.

– Total cholesterol, triglyceride, HDL-cholesterol, LDL-cholesterol, Apo A, Apo B concentrations were measured with Roche analytical kits with a Cobas Mira Plus analyzer.

Statistical analysis was performed with the SPSS software program: means were calculated for all values, comparison was performed with the ANOVA method (significance level $P<0.05$) and linear regression was calculated (significance level $P<0.05$).

2. Results

Table 2 summarizes the most important clinical data and nutrient intake of the children involved in the study. The children were assigned to one of two groups based on their serum cholesterol level (Table 3). Group 1 consisted of patients whose serum cholesterol level was above the 5th percentile value for their age group and sex. Group 2 consisted of patients, whose serum cholesterol level did not reach the 5th percentile value (NATIONAL CHOLESTEROL EDUCATION PROGRAM, 1991).

The analysis of the two groups reveals the following:

– there was no significant difference between values of the body mass index

– the Shwachman-Kulczycki score, indicative of the clinical condition of the patient was significantly higher in children, whose level of serum cholesterol was above the 5th percentile norm ($P<0.05$)

– there was no significant difference between the two groups in total energy, fat-, cholesterol-, carbohydrate and protein energy intake,

– PUFA, MUFA- and SFA-energy percentages of the two groups did not show significant differences either,

– PUFA/SFA ratio was 0.78,

– ratio of linoleic acid to alfa-linolenic acid was exceptionally high: 29,

– the intake of fibers was 23 g.

Table 2

Some clinical data and nutrient intake of cystic fibrosis patients with different serum cholesterol levels

Clinical data and nutrient intake	Group 1 n=12		Group 2 n=18	
	Mean	±SD	Mean	±SD
Age (year)	9.6	3.15	9.6	3.67
Body mass index (BW kg m^{-2})	15	1.91	16	3.13
Shwachman-Kulczycki score	89.2	12.03a	81.1	8.50a
Energy (kcal BW kg^{-1})	105.7	32.22	104.6	32.70
Cholesterol (mg)	408	206.2	376	95.5
Cholesterol (mg BW kg^{-1})	14.7	4.29	15.1	5.54
Fat % of energy	35.6	3.26	35.5	3.68
Carbohydrate % of energy	49	3.5	49	4.0
Protein % of energy	15.1	1.85	15.5	2.08
PUFA % of energy	7.8	1.77	8.8	2.52
MUFA % of energy	11.1	1.91	10.5	1.61
SFA % of energy	11.7	1.90	11.1	2.15
P/S	0.7	0.23	0.8	0.40
Fibers (g)	25.3	5.81	22.4	7.06
Linoleic acid (g)	22.4	7.23	24.2	7.43
α-Linolenic acid (g)	0.9	0.34	0.8	0.27
Linoleic/α-Linolenic acid	26	8.2	33	13.2

Means followed by the same letters are significantly different at $P<0.05$ level between the columns
Group 1: serum cholesterol level >5th percentile
Group 2: serum cholesterol level <5th percentile

Table 3

Serum total cholesterol 5th percentil levels according to the National cholesterol Education Program, (1991), USA

Age (year)	Serum total cholesterol level (mmol l^{-1})	
	Males	Females
0–4	3.03	2.97
5–9	3.20	3.36
10–14	3.18	3.28
15–19	2.98	3.18

Table 4

Biochemical indices of cystic fibrosis patients with different serum cholesterol levels

Biochemical indices	Group 1 n=12		Group 2 n=18	
	Mean	±SD	Mean	±SD
Serum cholesterol (mmol l^{-1})	3.9	0.53b	2.7	0.38b
Serum HDL-cholesterol (mmol l^{-1})	1.2	0.29b	0.9	0.20b
Serum LDL-cholesterol(mmol l^{-1})	2.2	0.58b	1.3	0.37b
Serum ApoA (g l^{-1})	1.4	0.22a	1.2	0.19a
Serum ApoB (g l^{-1})	0.7	0.12b	0.6	0.09b
Serum triglycerides (mmol l^{-1})	1.0	0.38	0.8	0.36
Serum uric acid (mmol l^{-1})	259.4	78.97	294.9	69.26
Serum albumin (g l^{-1})	45.2	1.75a	43.6	2.23a
SGOT (U l^{-1})	27.3	12.82	23.4	6.67
SGPT (U l^{-1})	22.9	14.66	16.5	7.31
GGT (U l^{-1})	16.5	8.67	16.7	8.92
Serum alkaline phosphatase (U l^{-1})	661.7	204.30	684.9	196.65

Means followed by the same letters are significantly different at a: $P<0.05$, b: $P<0.01$ between the column, respectively

Group 1: serum cholesterol level >5th percentile

Group 2: serum cholesterol level <5th percentile

Table 4 shows some clinical data and laboratory findings of the two groups as follows:

– from the biomarkers relating to nutritional status, serum albumin was significantly lower in patients with serum cholesterol below the 5th percentile value ($P<0.05$),

– there was no significant difference between the values of the hepatic enzymes and alkaline phosphatase,

– serum cholesterol, HDL-cholesterol, LDL-cholesterol, Apo A and Apo B concentrations were significantly higher in group 1.

3. Discussion

The basis of cystic fibrosis is a mutation of the cystic fibrosis transmembrane regulator (CFTR) gene located on the long arm of the 7th chromosome, resulting in the inability of the CFTR protein in the plasma membrane of the epithelial cells to regulate the cAMP stimulated chloride channels of the cell membrane. This results in the

production of mucus with abnormal composition, which causes irreversible damage primarily in the airways and the digestive system. Faultless functioning of the plasma membrane necessitates adequate quantities of cholesterol (BÍRÓ, 1990.; IRIBARREN et al., 1995.). This phenomenon call attention to the importance of our observation that a considerable proportion of our patients treated for cystic fibrosis (60% in this study) the serum levels of cholesterol were below the 5th percentile value, in spite of the fact that the intake of fat, fatty acids and cholesterol - both in absolute value and per kg of body weight – in these children was not less than that of the patients with cystic fibrosis with serum cholesterol levels above the 5th percentile value.

We did not find any difference between the amount – which was in accordance with the guidelines set for children with cystic fibrosis 35.6% of energy) (DODGE, 1992., MACDONALD, 1996) – or composition of the lipid intake of the two groups: SFAs, MUFAs and PUFAs were approximately 11%, 11% and 8% of total energy, respectively. Therefore we propose that the low levels of serum cholesterol were due to a disorder in the endogenous cholesterol synthesis. Due to the parthomechanism of the disease, this disorder can manifest in all patients with cystic fibrosis, as the lack of the CFTR protein affects the epithelial cells of the liver and the biliary tract. Long-term course of the disease lead to biliary cirrhosis, which can result in decreased levels of cholesterol in multiple ways: decreased synthesis of cholesterol, and decrease in the quantities of cholesterol salvaged via the entero-hepatic circulation (GYLLING et al., 1995; MIETTIEN, 1972). Apo A and Apo B are the main components of HDL- and LDL-cholesterol, respectively. The major sites of the synthesis of these proteins are the liver and the intestine. It is a clear trend towards the low levels of total cholesterol and Apo A reflecting the severity of liver injury. Moreover, the low Apo B concentration may be connected to the impaired hepatic synthesis of this apolipoprotein (GRUNDY, 1999, RIESEN & KLOER, 1989). Our data are in good accordance with these statements. It should also be noted that treatment with modern enzyme products do not ensure perfect absorption of fats, cholesterol and other nutrients (BERNABDESLAM et al., 1998, KALIVIANAKIS et al., 1999).

We studied the levels of hepatic enzymes, serum alkaline phosphatase and found no significant difference between the two groups. In other words we need a different kind of parameter which is suitable for the evaluation of hepatic damage. For this reason we suggest that the monitoring of serum cholesterol levels is an important and sensitive method for all patients with cystic fibrosis.

We also investigated the importance of the high linoleic acid intake (EGMOND et al., 1996) and the high ratio of linoleic to alfa-linolenic acid intake of our patients. An increase in linoleic acid (n-6 PUFA) intake results in a lowering of plasma LDL-cholesterol concentration. This effect may not be linear and most of the benefits appear to be gained by moving from lower to moderate (~4–5% of energy) intake. Whereas a

shift between 15 and 20% of energy as linoleic acid, is often without effect. Moreover, it is worthy to note that the effects of dietary n-6 PUFAs are less than half of that of lowering dietary saturated fatty acids. Therefore, if total fat intake is maintained, the LDL-cholesterol lowering effects of increasing linoleic acid intake are greatly enhanced if saturated fatty acid intake is decreased (HODGSON & WAHLQVIST, 1999). To maintain the alveolar membrane integrity a sufficient docosahexaenoic acid (n-3, DHA C22:6) content is absolutely necessary. When the ratio of linoleic to alfa-linolenic acid intake is so high, the synthesis of DHA from alfa-linolenic acid is inhibited. So it would be absolutely important to improve ratio of linoleic and alfa-linolenic acid with increasing alfa-linolenic acid content of the diet of patients with cystic fibrosis (KATZ et al., 1996).

Summarizing our results it has been found that low serum cholesterol level with adequate lipid, cholesterol intake and enzyme substitution implies an impaired cholesterol absorption and/or hepatic lesion with connection to the severity of cystic fibrosis. We would like to stress the importance of our finding, which shows a significant correlation between low cholesterol levels and the Shwachman-Kulczycki (SCHWACHMAN & KULCZYCKI, 1958) score, used since 1958 for the evolution of the clinical status of patients with cystic fibrosis: children with decreased levels of cholesterol have lower scores, their clinical condition is worse.

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