Acta Universitatis Sapientiae

Alimentaria

Volume 2, No. 1, 2009

Sapientia Hungarian University of Transylvania
Scientia Publishing House

Contents

Acta Univ. Sapientiae, Alimentaria, **2**, 1 (2009) 5–30

The D-amino acid content of foodstuffs (A Review)

J. Csapó[1,2]
email: csapo.janos@ke.hu

Cs. Albert[2]
email: albertcsilla@sapientia.siculorum.ro

Zs. Csapó-Kiss
email: csapo.janosne@ke.hu

[1]University of Kaposvár,
Faculty of Animal Science,
Guba S. u. 40, 7400 Kaposvár, Hungary

[2]Sapientia–Hungarian University of Transylvania,
Csíkszereda Campus, RO-530104, Libertatii 1., Miercurea-Ciuc

Abstract. D-amino acids occurring in dietary proteins originate as a consequence of technological intervention while basic materials are being prepared for consumption. Foodstuffs are the most significant sources of D-amino acids, as in the process of cooking or during the various processing procedures used in the food industry dietary proteins undergo racemisation to a greater or lesser degree. Food stores are now selling increasing quantities of foods (such as breakfast cereals, baked potatoes, liquid and powdered infant foods, meat substitutes and other supplements) which in some cases contain substantial quantities of D-amino acids, which in turn possess characteristics harmful with respect to digestion and health. Alkali treatment catalyses the racemisation of optically active amino acids. The degree of racemisation undergone varies from protein to protein, but the relative order of the degree of racemisation of the individual amino acids within proteins shows a high level

Key words and phrases: protein, D-amino acids, racemisation, digestibility of protein

of similarity. The principal factors influencing racemisation are the pH of the medium, heat treatment, the duration of the application of alkaline treatment and the structure of the respective amino acids. D-amino acids formed in the course of treatment with alkalis or heat give rise to a deterioration in quality and reduce the extent to which food thus treated can be used safely. The presence of D-amino acids in proteins leads to a decrease in digestibility and the availability of the other amino acids. This results in a reduction in the quantities of the L-enantiomers of the essential amino acids, as the peptide bonds cannot split in the normal way. Some D-amino acids can exert an isomer-toxic effect and have the capacity to give rise to changes in the biological effect of lysinoalanine. On the other hand, certain D-amino acids may also be of benefit (e.g. in pain relief), and proteins containing D-amino acids of lower digestibility can be used in, for example, diets designed for weight loss.

1 Introduction

Foods contain large quantities of non-natural substances of external origin which influence their digestibility to a considerable degree [44]. An example is the D-stereoisomer amino acids, which are formed from common L-stereoisomer amino acids, either in the course of the production process or as a consequence of changes in the microbiological quality of the foodstuff. The presence of these D-stereoisomer amino acids results in a substantial reduction in the digestibility of dietary protein and the availability of the transformed amino acid. However, despite the fact that D-amino acids in foods are considered undesirable, some hold the opinion that in certain cases D-amino acids can nevertheless be beneficial to the human organism.

As in many other aspects, Pasteur [79] also accomplished pioneering work in this field. He demonstrated that aspartic acid derived from vetch is optically active (chiral), whereas that produced by the heating of ammonium fumarate does not exhibit optical activity. It was subsequently realised that the proteins occurring in the living organism are constructed exclusively from L-amino acids, despite the fact that D- and L-stereoisomers (enantiomers) possess the same chemical and physical properties, with the exception of the rotation of the plane of polarised light. The two stereoisomers rotate this plane in different directions. The stereospecific synthesis of proteins in the living organism [92] could not initially be explained, as a result of which this sphere of issues occupied scientists for almost a century [4].

As methods developed for the separation and determination of amino acid enantiomers have been perfected it has been found that, contrary to previous

belief, D-amino acids occur in a great variety of organisms. For example, bacterial cell wall peptidoglycans contain D-aspartic acid, D-glutamic acid and D-alanine [3, 21, 83]; in some marine worms and invertebrates the cellular fluid contains D-amino acids as a main component [20, 36, 40, 74]; in certain marine shellfish quantities of D-amino acids can exceed 1% [41, 82]; and higher plants also contain D-amino acids [84]. Metabolically stable proteins in mammals of longer life span contain major quantities of D-aspartic acid derived from racemisation [1]: the D-aspartic acid concentration of the white matter of the human brain amounts to 3%, the clarified basic protein of the spinal cord to 10% [45, 72]. Clarke [19] verified that aspartic acid racemises in vivo in human tissues, but due to rapid metabolism does not accumulate in measurable quantities.

The chiral amino acids can be transformed into racemic mixtures, the reaction mechanism of this transformation process necessitating the splitting off of the hydrogen of the α-position carbon atom and the formation of the structure of the planar carbanion. The degree of racemisation occurring depends on whether the amino acid occurs free or in bound form in the peptide chain, and is naturally chiefly dependent on temperature and pH, and also on the nature of the R group occurring in the amino acid [2]. On examination of the racemisation of free amino acids Bada [2] and Steinberg et al. [89] established that at 100 °C and at pH between 7 and 8 the half-life of racemisation (i.e., the time taken for the D/L ratio to reach 0.33) for serine is 3 days, for aspartic acid 30 days, for alanine 120 days, and for isoleucine 300 days. Liardon and Lederman [68] reported that at pH 9 and at 83 °C for casein the half-life of racemisation for the above four amino acids, respectively, is as follows: 16 hours, 19 hours, 11 days and 57 days; Friedman and Liardon [49] gave these respective values for soya protein at 75 °C in 0.1 M sodium hydroxide as 9 minutes, 20 minutes, 5 hours and 25 hours. As can be seen from these collected data, in different conditions the respective amino acids show racemisation times of different duration, but the order of the degree of racemisation among the amino acids remains to a certain extent unchanged. The racemisation of serine, cystine and threonine results not only in the corresponding D-enantiomer, but also in an amino acid not constituting one of the components of proteins. For example, in the inter-carbanion state serine can readily lose its OH group in the formation of dehydroalanine. Reaction of dehydroalanine with the ϵ-amino group of lysine results in lysinoalanine [47, 70, 73], an amino acid of which the alanine part is racemic while the lysine part is optically active. In dietary proteins this reaction can result in cross-linking, leading to a reduction in protein digestibility [18, 50]; the lysinoalanine content of the resultant foodstuff also

bears toxic effect [62].

From the aspect of nutrition the racemisation of essential amino acids is of the greatest significance. The digestibility and metabolism of the D-enantiomers of the essential amino acids have been studied for some considerable time. It is evident from the work of Neuberger [76] and Berg [7], both of whom summarised earlier studies, that in mammals the D-enantiomers of essential amino acids are utilised to very low degrees, in some cases act as growth inhibitors, and are for the most part excreted in the urine. The following authors have corroborated the results obtained in earlier research [48, 51, 64, 88].

The half-life of racemisation for the essential amino acids has only recently been subjected to investigation. At pH between 7 and 8 Bada [2] measured the half-life of racemisation at 100 °C for isoleucine, leucine and valine at 300 days, and for phenylalanine and tyrosine at 50 days. Working under the same conditions Engel and Hare [39] determined the half-life of racemisation for lysine at 40 days, while Liardon and Lederman [68] measured the half-life of racemisation at pH 9 and 83 °C at 40 days for tryptophan, 20 days for threonine and 2 days for cysteine. Boehm and Bada [10] obtained a value of 30 days for the half-life of racemisation for methionine at 100 °C at pH between 7 and 8. It appears from the empirical data that cysteine is particularly susceptible to racemisation, while the amino acids with aliphatic side-chains are the most stable in this respect. For most of the essential amino acids the half-life of racemisation is longer than that for aspartic acid.

Food proteins exposed to alkali treatment processes or to lengthy heat treatment contain considerable concentrations of amino acids derived from racemisation. Dakin [34] was the first to demonstrate that the digestibility of proteins decreases on exposure to heat or strong alkalis. It is now evident that this reduction in digestibility is related to the formation of lysinoalanine and the racemisation arising [13, 18, 50, 52, 60, 70].

2 D-amino acids of dietary origin

Despite the fact that some insects, worms and marine invertebrates contain substantial quantities of D-amino acids, since such organisms do not constitute main components of the human diet these quantities are insignificant, and their importance can therefore be disregarded. However, in communities in which marine shellfish represent an important source of food account should be taken of D-amino acids consumed in large quantities, not only with respect to nutrition, but also from the toxicological aspect [41], as, for example, quantities

of D-amino acids occurring in marine shellfish can exceed 1%. According to Preston [82] in marine molluscs quantities of D-amino acids can vary between 0.11 and 1.6 mM related to body tissue of 70% water content.

The majority of food treatment procedures, performed for the purposes of improving flavour, consistency or non-perishability, and including cooking and baking, involve heat treatment, and in some cases alkaline conditions are also applied. Racemisation induced by such intervention gives rise to D-amino acids in proteins. Fuse et al. [52], Jenkins et al. [63], Liardon and Hurrel [67] and Masters and Friedman [73] demonstrated that considerable quantities of D-amino acids are to be found in some commercially available foodstuffs which have been subjected to the effect of technological processes. Lysinoalanine is present almost universally in food substances [70]. In addition, synthetically manufactured products such as aspartame dipeptide are particularly susceptible to racemisation [9]. Investigations performed by the authors indicate that 10 to 40% of the amino acid content of feather meal produced by means of alkaline hydrolysis undergoes racemisation, the degree of this being dependent on the production parameters [33].

3 Natural basic materials

Milk, meat and the various types of grain, which do not contain substantial quantities of D-amino acids, are often exposed, in the course of preparation for consumption, to conditions which may give rise to racemisation. Milk and dairy products serve as examples of how the composition of natural substances can change [72]. Although untreated (i.e., raw) milk is available in some food stores, most dairy products are first pasteurised (involving heating for 30 minutes at 68–72 °C) or ultrapasteurised (involving heating for 15 seconds at 135–145 °C). They are subsequently subjected to homogenisation and condensation, until a particular product such as milk for commercial consumption, yoghurt or cheese derived from the various milk protein fractions is finally obtained. The latter two dairy products are fermented by means of bacteria, this process also constituting a source of D-amino acids. (The concentration of D-amino acids is hereafter given in accordance with the following: % D-amino acid = (D/D+L)×100).

Payan et al. [80] studied changes occurring by the effect of milk treatment by measuring D-aspartic acid concentration. They found that untreated raw milk contains the lowest levels of D-aspartic acid (1.48%), but that quantities rise with increasing extent of treatment involved in processing (acidophilus

milk: 2.05%, fat-free milk powder: 2.15%, kefir: 2.44%, evaporated milk: 2.49%, yoghurt: 3.12%, milk-based infant formula: 4.95%). Thus, products whose production requires heating may have a D-aspartic acid content as high as 5%. The highest ratio of D-aspartic acid is found in infant formulae, which are subjected to technological intervention procedures such as powder drying or heat sterilisation.

On studying the effect of heat treatment and bacteria on the free and protein-bound D-amino acid content of milk Gandolfi et al. [53] established that the free D-amino acid content of raw milk does not increase by the effect of pasteurisation, ultrapasteurisation or sterilisation. The above authors determined free D-alanine content of between 3 and 8%, D-aspartic acid content of between 2 and 5%, and D-glutamic acid content of between 2 and 4% for the milk samples examined. They also ascertained that the free D-amino acid content of raw milk samples increases substantially during storage at 4 °C; therefore, they recommend that D-alanine content be used for the purposes of monitoring bacterial contamination of milk. The D-amino acid content detected in milk protein is attributed to racemisation occurring during protein hydrolysis.

The past few years have seen the development of a number of methods for the determination of the proportion ascribable to microbial origin of the nitrogen-containing matter. Some of these methods were published by Csapó and Henics [21] and Csapó et al. [23, 26]. Recently, in examining the D-amino acid content of foodstuffs, particularly that of milk and dairy products, the attention of the authors has been turned to whether D-glutamic acid (D-Glu) and D-aspartic acid (D-Asp) can be detected in quantities similar to those determined for D-Ala, principally in products associated with bacterial activity. Based on their investigation the authors recommend that the above two D-amino acids be included alongside DAPA as markers for bacterial protein.

Palla et al. [77] determined the free D-aspartic acid content of milk powder at 4–5 and its D-alanine content at 8–12%. With respect to yoghurt, free D-alanine content was measured by the above authors at 64–68%, free D-aspartic acid content at 20–32%, and free D-glutamic acid content at 53–56%. For mature cheese content values for the same D-amino acids of 20–45%, 8–35% and 5–22% respectively were obtained. The free D-phenylalanine content of mature cheese was found to be between 2 and 13%, D-leucine also being detected in minimal quantities in mature cheese. The D-aspartic acid content of roast coffee proved to be 23–38%, its D-glutamic acid content 32–41%, and its D-phenylalanine content 9–12%. On the basis of the measurements recorded the above authors draw attention to the fact that it is not foodstuffs

subjected to lengthy heat treatment which contain substantial quantities of D-amino acids, but rather those which have undergone a process of bacterial fermentation.

On examination of free D-amino acids in milk, fermented milk, fresh cheese and curd cheese Bruckner and Hausch [11] established that considerable quantities of D-amino acids occur both in raw milk and in fermented dairy products manufactured from it. The empirical data obtained by the above authors are presented in *Table 1.*

Table 1: Free amino acid content of milk and fermented milk products[1] (mg/100 g)

Amino acid	Raw/Pasteurised milk	Kefir	Yoghurt	Curdled milk	Fresh cheese	Harz cheese
D-Ala	0.003 – 0.012	0.31	1.35	0.46	1.07	2.48
D-Asx[3]	0.017 – 0.038	0.35	0.31	0.25	0.38	0.37
D-Glx[3]	0.070 – 0.190	0.50	1.09	0.58	0.75	2.13
D-Val	-	0.03	-	0.04	0.09	-
D-Leu	-	0.11	-	0.15	0.16	-
D-Lys	-	0.09	-	0.13	0.44	1.49
D-allo-Ile[2]	-	0.07	-	0.02	-	0.27
D-Ser	-	0.02	-	-	-	-
D-Pro	-	-	-	-	-	2.18
Free amino acids (mg/100 g)	3.29 - 10.3	26.2	28.4	36.8	39.2	159
Free D-amino acids (mg/100 g)	0.09 - 0.24	1.48	2.75	1.63	2.89	8.92

[1]% D=(D/D+L)×100
[2]% D-allo-Ile=D-allo-Ile/(D-allo-Ile+L-allo-Ile+D-Ile+L-Ile)
[3]Asx=Asp+Asn, calculated as aspartic acid; Glx=Glu+Gln, calculated as glutamic acid

It may be ascertained from the data given in the above table that yoghurt and cheese contain substantial quantities of D-alanine (1.35–2.48 mg/100 g), D-aspartic acid (0.31–0.37 mg/100 g) and D-glutamic acid (1.09–2.13 mg/100 g), while the quantities of D-lysine (1.49 mg/100 g) and D-proline (2.18 mg/100 g) present may also be considerable. In addition, trace quantities of D-valine, D-leucine, D-allo-isoleucine and D-serine were also detected in fermented dairy products by the above authors. On analysis of the origin of D-amino acids they established that the occurrence of these can, for the most part, be traced back to microbiological intervention, or to microbial contamination in the case of raw or pasteurised samples, or possibly to the unintentional addition to the

composite milk of milk derived from cows with subclinical mastitis.

Csapó et al. [31] used ion exchange column chromatography to determine the total free amino acid content of mature Ardrahan Irish and Camembert cheese (in both cases samples being taken from the half centimetre thick external crust and from the inner part), and that of Danish blue, Emmental, Gouda, Mozzarella, Parmesan and Cheddar cheeses produced by various procedures; the free D-aspartic acid (D-Asp), D-glutamic acid (D-Glu) and D-alanine (D-Ala) content of the same cheeses was determined by means of high performance liquid chromatography. Of the free D-amino acids *(Table 2)* average concentrations of 58 μmol/100 g (30.3%) for D-Asp, 117 μmol/100 g (15.8%) for D-Glu and 276 μmol/100 g (37.2%) for D-Ala were determined in the various cheeses. The figures in brackets give the proportions of these D-amino acids in terms of percentage of total free amino acids. The quantities of the respective D-amino acids showed substantial differences between the individual cheeses: the percentage composition of D-amino acids represented by D-Asp varied between 13.9 and 46.3%, by D-Glu, between 12.9 and 26.6%, and by D-Ala, between 16.1 and 48.1%. Apart from the above three D-amino acids only trace quantities of the other D-amino acids were detected in the cheeses, with values on the limit of detectability. Higher D-amino acid content was determined in those Cheddar cheeses for which lactobacilli are also used in the manufacturing process.

In seeking to ascertain what gives rise to the D-amino acid content of commercially available milk Csapó et al. [27, 28, 29] determined the free D-amino acid content of the first jets of milk produced by healthy cows, that of the composite milk excluding the first milk jets, and that of milk samples corresponding to the various grades applied in the mastitis test *(Tables 3 and 4)*. It was ascertained that both the first jets of milk produced and milk from diseased udders contain substantial quantities of D-Asp, D-Glu, D-Ala and D-allo-Ile. In addition to the above amino acids, D-Ser, D-Pro, D-Val, D-Leu and D-Lys were also detected in the milk derived from mastitis-affected udders. The quantities and ratios of D-amino acids determined in milk derived from diseased udders were observed to increase in accordance with the grades designated in the mastitis test. The study verifies that the D-amino acid content of commercially available milk might be attributable to the first jets of milk produced or to milk originating from cows with subclinical mastitis.

Table 2: Principal[1] D-amino acid content of the various cheeses (μmol/100 g)

Cheese	D-amino acid					
	D-Asp	**D-Asp %**	**D-Glu**	**D-Glu %**	**D-Ala**	**D-Ala %**
Mature Ardrahan Irish: external crust	74	27.2	173	13.1	433	27.1
Mature Ardrahan Irish: inner part	70	23.2	235	14.4	393	28.2
Camembert: external crust	42	13.9	122	12.9	334	18.0
Camembert: inner part	36	14.0	176	14.8	259	16.1
Danish blue	89	31.1	149	20.2	212	42.4
Emmental	42	26.8	195	26.6	405	45.6
Gouda	61	28.5	244	22.7	462	38.4
Mozzarella	5.2	28.9	9.6	24.0	52	33.3
Parmesan	57	20.8	72	10.6	752	37.3
Commercial Cheddar	74	46.3	45	14.1	96	45.3
Cheddar: expt. 1	74	43.5	62	12.5	153	46.3
Cheddar: expt. 2	89	41.4	65	12.4	165	48.1
Cheddar: expt. 3	59	45.4	53	12.5	161	47.9
Cheddar: expt. 4	41	33.4	42	10.9	125	46.1

[1]% D=(D/D+L)×100 [2]All the D-amino acids were analysed, but apart from a few exceptions, the other D-amino acids proved to be present in very low concentrations, determination of these being unreliable.

Table 3: Free D-amino acid content of milk from healthy and mastitis-affected cows[1]

D-amino acid	Groups examined, on the basis of the mastitis test				
(mg/100 cm^3)	neg. (5)	+(5)	++(5)	+++(5)	++++(5)
D-Asp	0.021	0.17	0.23	0.32	0.32
D-Ser	-	-	0.02	0.04	0.04
D-Glu	0.053	0.74	0.99	1.48	1.53
D-Pro	-	-	0.04	0.09	0.10
D-Ala	0.043	-	1.13	2.32	2.41
D-Val	-	0.48	0.09	0.09	0.12
D-allo-Ile	-	0.08	0.10	0.12	0.15
D-Leu	-	0.08	0.12	0.17	0.17
D-Lys	-	0.06	0.27	0.36	0.37
Total free		0.11			
D-amino acids	0.117	1.72	2.99	4.99	5.21

[1]Mean for the milk of 5 cows.

Table 4: Free D-amino acid content of milk from healthy and mastitis-affected cows in terms of percentage of total free amino acids[1]

D-amino acid	Groups examined, on the basis of the mastitis test				
%	neg. (5)	+(5)	++(5)	+++(5)	++++(5)
D-Asp	17.5	19.0	20.1	23.0	22.0
D-Ser	-	-	6.5	8.2	7.7
D-Glu	6.2	29.2	31.0	32.0	32.5
D-Pro	-	-	9.5	11.5	11.2
D-Ala	12.6	22.0	33.0	47.5	48.9
D-Val	-	11.3	12.2	11.1	13.0
D-allo-Ile	-	27.6	28.6	29.6	34.1
D-Leu	-	28.6	31.6	24.6	25.8
D-Lys	-	22.4	32.5	32.0	32.5

[1]Mean for the milk of 5 cows.

4 Foodstuffs subjected to various technological procedures

Modern food industry technology applies a diverse range of procedures for the purpose of modifying the characteristics of proteins in order to improve

flavour, consistency and non-perishability. Treatment with heat or alkalis is used preferentially for the manufacture of products possessing particular characteristics, form and function. For example, soya protein is treated with alkalis and heat for the purposes of obtaining, through extrusion, a product of fibrous structure suitable for consumption as a meat substitute. Alkali treatment is also applied in order to obtain flaked maize and tortillas from maize protein. *Table 5* gives the D-amino acid content of various alkali-treated foodstuffs in comparison with untreated controls.

Table 5: D-amino acid content of various foodstuffs (%)[1]

Treated product	**Amino acids**					
(Untreated control; ref.)	**Asp**	**Ala**	**Phe**	**Leu**	**Val**	**Met**
Toast[2]	10.5	2.8	2.4	2.7	1.1	1.7
Bread, [13]	5.6	2.4	2.3	3.2	0.9	2.3
Extruded soya bean meal	7.6	2.2	2.4	2.7	0.8	-
Soya bean meal, [13]	4.4	2.5	2.8	1.4	1.0	-
Soya protein[3]	27.7	9.9	19.7	3.1	1.0	18.2
Untreated, [49]	0.5	0.2	0.5	0.2	0.03	0.3
Zein[4]	40.2	17.6	31.3	5.0	2.9	19.5
Not heat treated, [63]	3.4	0.7	2.2	0.7	0.4	0.9
Hamburger[5]	5.5	2.8	2.7	3.2	1.5	2.9
Raw meat, [13]	6.2	3.2	2.8	3.1	1.6	2.4
Chicken muscle[6]	22.4	0.5	0.4	0.1	-	-
Raw chicken, [67]	2.9	-	-	-	-	-
Bacon, 180 °C[7]	10.7	2.4	3.1	3.1	1.6	-
Not heat treated, [52]	2.4	-	1.8	3.3	0.7	-
Casein, 230 °C[7]	31.0	12.0	-	7.0	4.4	-
Not heat treated, [58, 59]	3.1	1.5	-	-	-	-

[1]D-amino acids % = (D/D+L)×100
[2]The white bread was heated for 1 minute 45 seconds, only its surface having been analysed.
[3]3 hours, 65 °C, 0.1 N NaOH.
[4]4 hours, 85 °C, 0.2 N NaOH.
[5]The hamburger was fried on both sides for 4 minutes. The temperature of the pan was 250 °C. Only the surface was analysed.
[6]Heating at 121 °C for 4 hours.
[7]Heated for 20 minutes.

Heat treatment or combined heat and alkali treatment in every case gave rise to D-amino acids in measurable quantities. The highest D-aspartic acid content (31%) was determined in the casein heated to 230 °C for 20 minutes. Comparison of the racemised amino acids reveals that the highest degree of racemisation occurred in aspartic acid. Certain amino acids not included in the table, such as serine and cysteine, probably racemise more rapidly than aspartic acid. It may be stated in general that the essential amino acids do not racemise rapidly unless exposed to high temperature. However, it may also be the case with the essential amino acids that a combination of high temperature and alkali treatment is accompanied by a substantial degree of racemisation.

Authors of other studies have also reported on the high D-amino acid content of treated foods. On examination of the D-Asp content of a number of commercially available foods Masters and Friedman [73] established very high ratios of this D-amino acid in textured soya protein (9%), bacon (13%) and non-milk fat (17%). Finley [43] determined substantial quantities of D-Asp in savoury crackers made from wheat flour (9.5%), wheat cake (11.9%), Mexican pancake (11.6%) and corn cake (15.4%). The data for the fried hamburger indicate that racemisation occurs to only an insignificant degree in that particular food in the course of the frying process. The high ratios of D-amino acids detected in the toasted white bread, the cooked bacon and the chicken meat demonstrate that in some foods substantial degrees of racemisation can arise in the process of cooking, baking or frying.

On examining the effect on food proteins of microwave treatment Lubec et al. [69] fairly recently ascertained that by the effect of microwave treatment of 10 minutes duration the cis-3 and cis-4 hydroxyproline content of all three infant foods examined increased, and only microwave-treated formulae contained D-proline in detectable quantities. The concentration of the cis isomer was found to be 1–2 mg per litre. The above authors point out that if the cis isomer is incorporated into a protein instead of the trans isomer, structural, functional and immunological changes can result.

5 Manufactured foods and artificially produced peptides

This category includes every type of food subjected to substantial levels of technological treatment, or synthetically produced (e.g. aspartame). In some liquid foods the protein is combined with carbohydrate, in the process of which

the protein may undergo considerable change. Antibiotic peptides may contain substantial quantities of D-amino acids [8, 87], as may some drugs used in chemotherapy [15]; the residues of these may subsequently result in significant D-amino acid content of foodstuffs produced. On evaluation of data in the literature it may be ascertained that synthetic products contain considerably higher levels of amino acids than natural basic materials, the former being the main sources of the D-amino acid content of foods. Liquid food formulae based on soya protein, actually purchased from health food stores, has been found to contain 13% D-aspartic acid, this being a substantially higher level than that determined in soya-based infant formulae. Finley [43] reported that food products formulated to induce weight loss which had been subjected to alkali treatment proved to contain 50% D-serine, 37% D-aspartic acid and 26% D-phenylalanine; these high quantities of D-amino acids might pose a risk if consumed as the sole source of dietary protein. Such extreme cases are relatively rare, but it should nevertheless be noted that in foodstuffs subjected to lengthy alkali or heat treatment processes a high proportion of the amino acids present may undergo racemisation.

On studying racemisation in aspartame sweetener Boehm and Bada [9] reported that both aspartic acid and glutamic acid racemised rapidly at neutral pH at 100 °C. Racemisation occurs when the sweetener is transformed into a cyclical dipeptide, these being highly susceptible to racemisation. The importance of awareness of this lies in the fact that if sweetener is added to food before, for example, cooking, a high degree of racemisation may result.

6 D-amino acid metabolism

The points outlined above provide clear evidence that D-amino acids can occur in substantial quantities in foods. What is the fate of these unnatural stereoisomers? Since the publication of the pioneering work of Krebs [65] it has been generally known that mammals possess specific enzymes for the purposes of a D-amino acid metabolism. D-amino acids metabolise primarily in the series of reactions of D-amino acid oxidase, in the formation of α-ketoacids [6, 7, 14, 66, 76]. These α-ketoacids may subsequently undergo stereospecific transamination, which results in the L-enantiomer of the original amino acid, which in turn enters the usual metabolic process, or alternatively is broken down directly in another reaction, e.g. by means of oxidative decarboxylation. The transformation of D-amino acids into α-ketoacids takes place principally in the kidneys; thus, dietary D-amino acids first have to diffuse across the mem-

branes to enable them to metabolise via this route. However, the transport mechanisms are stereo-selective and discriminative with respect to D-amino acids [54, 86].

The respective amino acids are oxidised to different degrees by D-amino acid oxidase. The D-enantiomer of aspartic acid, the amino acid which according to investigations is the most susceptible to racemisation, is a highly unsuitable substrate for D-amino acid oxidase. Despite this, Dixon and Kenworthy [38] reported that there occurs in mammals D-amino acid oxidase specific to D-aspartic acid; however, there is none for the other amino acids. Essential amino acids such as lysine and threonine racemise more rapidly than alanine, and are also very unsuitable substrates for D-amino acid oxidase. On the other hand, proline, which does not racemise to a significant degree in the preparation of food products, is the best possible substrate for this enzyme [67]. Thus, it appears that there is no relation between susceptibility to racemisation and readiness to react with D-amino acid oxidase. It can therefore be asserted that the D-amino acid oxidase system in mammals is not sufficiently developed to be capable of meeting the challenge posed by racemised amino acids of food origin. Krebs [65, 66] was still in doubt as to the biological function of D-amino acid oxidase; however, the view now generally held is that D-amino acid oxidase detoxifies D-amino acids which happen to be present or which arrive via bacterial protein [5]. This is corroborated by the fact that rats reared in a germ-free environment show far lower levels of D-amino acid oxidase activity than those reared in a normal environment. Despite this, D-glutamic acid constituting a component part of peptidoglycan occurring in bacterial cell walls is the least suitable substrate for D-amino acid oxidase, undergoing only very slow oxidation with D-aspartic acid oxidase [38]. Although D-amino acid oxidase enzymes enable mammals to metabolise D-amino acids, this route is inefficient and obviously overburdened, since when racemic amino acids enter the organism a high proportion of D-amino acids is excreted in the urine [7, 76]. Free D-amino acids can also be transformed by means of racemases into racemic mixtures or the corresponding L-amino acids. However, since racemases occur primarily in bacteria, this is not a route for the metabolism of D-amino acids in mammals. It is now known that amino acid transaminases also occur only in bacteria.

The main sources of D-amino acids in human foodstuffs are industrially produced proteins. Before D-amino acids present in these proteins are able to metabolise in the series of reactions of D-amino acid oxidase they must be liberated by means of the metabolic enzymes. The first stage of the digestion of dietary proteins results in free amino acids and peptides consisting of

small numbers of amino acids [5, 55]; the peptides are subsequently further hydrolysed by peptidases [81, 85]. It is quite evident that peptides containing D-amino acids resist enzyme hydrolysis in the digestive process. Studies involving synthetic peptides indicate that D-aspartic acid [75] and D-methionine [78] are not liberated from the peptide bonds in the course of enzyme hydrolysis, even if all the other adjacent amino acids are L-enantiomers. A number of published works have reported that amino acids racemised to a large extent by the effect of heat and alkali treatment resist proteolytic hydrolysis. On studying the relation between phenylalanine racemisation and protein digestibility Chung et al. [18] established that as the degree of racemisation increases digestibility decreases rapidly. As phenylalanine racemises more slowly than aspartic acid, serine or cysteine, it is evident that proteins containing significant quantities of racemised amino acids are only partially broken down in the course of proteolysis.

The products of the proteolytic hydrolysis of proteins contain racemised amino acids and peptides of low molecular weight containing D-amino acids. Di- and tripeptides diffuse across the membranes, while peptides consisting of larger numbers of amino acids are simply excreted in the faeces. Di- and tripeptides containing D-amino acids are not particularly suitable substrates for D-amino acid oxidase [14, 66]. Under in vitro conditions at pH 7 dipeptides rapidly undergo cyclic transformation into cyclic peptides (diketopiperazine) [89]. Tripeptides are hydrolysed rapidly by non-enzyme processes in vitro in the course of internal ammonolysis, which results in cyclic dipeptides and a free C-terminal amino acid [90]. Cyclic dipeptides are highly susceptible to in vivo racemisation [57, 89]. Thus, if the hydrolytic process were also to occur in vivo, this would lead to the formation of other D-amino acids. However, studies of the metabolism of D-amino acids have not, as yet, devoted attention to the presence of diketopiperazine.

7 D-amino acid digestion

There is not yet sufficient knowledge of the effect on the human organism of long-term consumption of proteins containing racemised amino acids. Masters and Friedman [73] pointed out that no authors have performed specific investigations on the effect exerted by racemised amino acids on the human organism, i.e., how racemisation affects digestibility and the availability of amino acids.

Harmful effects of D-amino acids: The utilisation of D-amino acids bound

in proteins depends on whether such D-amino acids are liberated from the L–D, D–L and D–D bonds, and whether the liberated D-amino acids can be transformed efficiently into L-amino acids. At the beginning of the century Dakin and Dudley [35] were the first to observe that in dogs a large proportion of alkali-treated casein was excreted, undigested, in the faeces. Other researchers subsequently determined the digestibility of alkali-treated and non-treated protein. In each case the treated samples exhibited reduced digestibility, attributed primarily to racemisation and/or to the formation of lysinoalanine. On studying racemisation of the amino acids in alkali-treated proteins Hayashi and Kameda [61] reported that even a minor degree of racemisation provokes a major decrease in the digestion of such proteins. The above authors ascribed this reduced digestibility to the fact that racemised amino acids are not substrates for proteases, and also exert an effect on the capacity for liberation of adjacent non-racemised amino acids. Thus, the racemisation of some amino acids may also give rise to substantial loss with respect to the neighbouring essential amino acids, thus reducing the proteolytic digestibility of the protein.

Friedman et al. [50] examined the effect of treatment temperature, duration and pH on the digestibility of alkali-treated casein, trypsin and chymotrypsin. The above authors observed that a decrease in the digestibility of aspartic acid and phenylalanine was accompanied by an increase in lysinoalanine cross-linking and racemisation. Bunjapamai et al. [13] were the first to succeed in distinguishing the effects on in vitro digestibility of racemisation and cross-linking. The main conclusion of this research was that reduced digestibility was caused primarily by racemisation. According to Schwass et al. [86] one D-amino acid is sufficient to render a peptide unfit for transport. These authors stated that racemisation is the one process which alone leads to a reduction in in vitro digestibility and in the in vivo assimilation of enzyme-digested protein.

One highly important issue is whether D-amino acids present in foodstuffs are toxic. It may be established from the outset that the various D- and L-amino acids are equally toxic, as verified by their LD_{50} values [56]. D-proline may be an exception to this, higher lethality in chickens having been ascertained for this enantiomer than for L-proline [17]. It has already been stated that D-proline is the best possible substrate for D-amino acid oxidase. Masters and Friedman [73] reported that some D-amino acids exert their toxic effect over a protracted period. The investigations performed by the above authors indicated that D-serine, lysinoalanine and the various alkali-treated proteins present in foodstuffs provoke pathological changes in the kidneys of rats. Free lysinoalanine is much more highly nephrotoxic than that bound in peptide

bonds; it follows from this that the nephrotoxic effect of bound lysinoalanine within alkali-treated proteins will be substantially weaker [47]. According to Degroot et al. [37] rats are particularly sensitive to the nephrotoxic effect of alkali-treated proteins and lysinoalanine, and it is evident from the studies performed by these authors that different species of animal show different degrees of sensitivity in this respect.

Lysinoalanine is an in vitro inhibitor to carboxypeptidases and aminopeptidases, as is D-alanine occurring in alkali-treated proteins [51, 62]. The inhibitory effect of lysinoalanine is manifest in its forming of a complex with the enzyme metal ion involved in the enzyme reaction [62]. The question of whether lysinoalanine and D-amino acids of dietary origin are inhibitors to metabolic enzymes has not yet been investigated; nor are there available at present data on the effect on inhibition of treatment of longer duration.

Beneficial effects of D-amino acids: Reduced digestibility of dietary proteins due to D-amino acids may in certain cases prove advantageous with respect to nutrition, on condition that the substances remaining after proteolytic digestion are not toxic. Racemised proteins may be consumed for a period of a few days in diets designed for weight loss, and in consequence of the very low digestibility of these proteins considerable loss of weight may be anticipated within a short period. It has been demonstrated [16] that D-phenylalanine and D-leucine exert an analgesic effect, due to which they are applied in cases of persistent pain [12]. This analgesic effect is based on the inhibition of carboxypeptidase A and similar enzymes playing a role in the breakdown of opioid pentapeptide in the brain and spinal cord [12]. Friedman et al. [51] reported that the lysinoalanine and D-amino acid content of alkali-treated food proteins also inhibits carboxypeptidase A. The above research findings allow the conclusion to be drawn that the presence of racemic amino acids in food proteins may be beneficial in the relief of pain.

It has been known for some considerable time that D-amino acid sequences are present in most antibiotic peptides. It is therefore conceivable that in the proteolytic breakdown of racemised dietary proteins peptides with antibiotic properties may be formed.

8 Acknowledgement

This work has been accomplished with the financial support of OTKA (National Foundation for the Subsidy of Research, Hungary). The number of the contract is T061587. The Sapientia - Hungarian University of Transylvania,

Institute of Research Programs, also granted the research. Their financial assistance is gratefully acknowledged.

References

[1] J.L. Bada, In vivo racemization in mammalian proteins, *Methods Enzymol.,* 106 (1984) 98–115.

[2] J.L. Bada, *Racemization of amino acids,* In: Chemistry and biochemistry of amino acids, London-New York, Chapman & Hall. 1985. 399–411.

[3] J.L., Bada, J.R. Cronin, M.S. Ho, K.A. Kvenvolden, J.G. Lawless, On the reported optical activity of amino acids in the Murchison meteorite, *Nature,* 310 (1983) 494–497.

[4] J.L. Bada, S.L. Miller, Racemization and the origin of optical active organic compounds in living organisms, *In: Dietary D-amino acids. Ann. Rev. Nutr.,* 7 1987. 209–225.

[5] D.A. Bender, *Amino acid metabolism,* Wiley Chichester/New York, 2^{nd} ed. 1985. 1–153.

[6] A.E. Bender, H.A. Krebs, The oxidation of various synthetic α-amino acids by mammalian D-amino acid oxidase, L-amino acid oxidase of cobra venom and the L- and D-amino acid oxidases of Neuospora crassa, *Biochem. J.,* 46 (1950) 210–219.

[7] C.P. Berg, Utilization of D-amino acids, *In: Protein and amino acid nutrition.* A.A. Albanese, New York Academic Press, 1959. 57–96.

[8] M. Bodansky, D. Perlman, Antibiotic peptides, *Science,* 163 (1969) 352–358.

[9] M.F. Boehm, J.L. Bada, Racemization of aspartic acid and phenylalanine in the sweetener aspartame at 100 °C, *Proc. Natl. Acad. Sci.,* 81 (1984) 5263–5266.

[10] M.F. Boehm, J.L. Bada, Investigations of in vivo methionine racemization in mammalian tissues, *Biochem. Int.,* 8 (1984) 603–608.

[11] H. Brückner, M. Hausch, D-amino acids in dairy products: Detection, origin and nutritional aspects. I. Milk, fermented milk, fresh cheese and acid curd cheese, *Milchwissenschaft,* 45 (1990) 357–360.

[12] K. Budd, Use of D-phenylalanine, and enkephalinase inhibitor, in the treatment of intractable pain, *Adv. Pain Res. Ther.,* 5 (1983) 305–308.

[13] S. Bunjapamai, R.R. Mahoney, I.S. Fagerson, Determination of D-amino acids in some processed foods and effect of racemization on in vitro digestibility of casein, *J. Fd. Sci.,* 47 (1982) 1229–1234.

[14] K. Burton, D-amino acid oxidase from kidney, *Methods Enzymol.,* 2 (1945) 199–204.

[15] P.K. Chakravarty, P.L. Carl, M.J. Weber, J.A. Katzenelknbogen, Plasmin-activated prodrugs for cancer chemotherapy. 2. Synthesis and biological activity of peptidyl derivatives of dexorubicin, *J. Med. Chem.,* 26 (1983) 638–644.

[16] R.S.S. Cheng, B. Omeranz, Correlation of genetic difference in endorphin systems with analgesic effects of D-amino acid in mice, *Brain Res.,* 177 (1979) 583–587.

[17] A. Cherkin, J.L. Davis, M.W. Garman, D-proline stereospecifity and sodium chloride dependence of lethal convulsant activity in the chick, *Pharmacol. Biochem. Behav.,* 8 (1978) 623–625.

[18] S.Y. Chung, H.E. Swaisgood, G.L. Catignani, Effect of alkali treatment in the presence of fructose on digestibility of food proteins as determined by an immobilized digestive enzyme assay (IDEA), *J. Agric. Fd. Chem.,* 34 (1986) 579–584.

[19] S. Clarke, *The role of Asp and Asn residues in the aging of erythrocyte proteins: Cellular metabolism of racemized and isomerized forms by methylation reactions.,* In: Cellular and molecular aspects of aging: The red cells as a model, New York, 1985. 91–103.

[20] J.J. Corrigan, D-amino acids in animals, *Science,* 164 (1969) 142–149.

[21] J. Csapó, Z. Henics, Quantitative determination of bacterial protein from the diaminopimelic acid and D-alanine content of rumen liquor and intestines, *Acta Agronomica Hungarica,* 1-2 (1991) 159–173.

[22] J. Csapó, I. Tóth-Pósfai, Zs. Csapó-Kiss, Separation of D- and L-amino acids by ion exchange column chromatography in the form of alanyl dipeptides, *Amino Acids,* (1991) 1 331–337.

[23] J. Csapó, S. Gombos, Zs. Csapó, J. Tossenberger, A bakteriális eredetű fehérje mennyiségi meghatározása a bendőfolyadék és a béltartalom diaminopimelinsav és D-alanin tartalma alapján, *Állattenyésztés és Takarmányozás,* 5 (1991) 431–441.

[24] J. Csapó, S. Einarsson, Élelmiszerek és takarmányok D-aminosav tartalma. 1. Az aminosav enantiomerek szétválasztása és meghatározása az 1-(9-fluorenil)etil-kloroformáttal történő származékképzés után forditott fázisú folyadékkromatográfiával, *Élelmiszervizsg. Közl.,* 39 (1993) 290–302.

[25] J. Csapó, S. Folestad, A. Tivesten, Élelmiszerek és takarmányok D-aminosav tartalma. III. Jelentőségük, meghatározásuk és fiziológiai hatásuk a szakirodalom alapján, *Élelmiszervizsg. Közl.,* 4 (1994) 299–316.

[26] J. Csapó, Zs. Csapó-Kiss, E. Csordás, S. Folestad, A. Tivesten, T.G. Martin, S. Némethy, Rapid method for the determination of diaminopimelic acid using ion exchange column chromatography, *Analytical Letters,* 28 (1995) 2049–2061.

[27] J. Csapó, T.G. Martin, Zs. Csapó-Kiss, J. Stefler, S. Némethy, Influence of udder inflammation on the D-amino acid content of milk, *J. Dairy Sci.,* 78 (1995) 2375–2381.

[28] J. Csapó, Zs. Csapó-Kiss, J. Stefler, E. Csordás, T.G. Martin, S. Némethy, L. Wágner, T. Tálos, A tőgygyulladás hatása a tej D-aminosav tartalmára, *Szaktanácsok,* 1-4 (1996-97) 38–52.

[29] J. Csapó, Zs. Csapó-Kiss, L. Wágner, T. Tálos, T.G. Martin, S. Némethy, S. Folestad, A. Tivesten, Hydrolysis of proteins performed at high temperatures and for short times with reduced racemization, in order to determine the enantiomers of D- and L-amino acids, *Anal. Chim. Acta,* 339 (1997) 99–107.

[30] J. Csapó, Zs. Csapó-Kiss, J. Stefler, Influence of mastitis on D-amino acid content of milk, *Agriculturae Conspectus Scientificus,* 62 (1997) 162–167.

[31] J. Csapó, Zs. Csapó-Kiss, E. Csordás, P.F. Fox, L. Wágner, T. Tálos, Különböző technológiával készült sajtok összes szabad- és szabad D-aminosav tartalma, *Tejipar,* 57 (1997) 25–30.

[32] J. Csapó, Zs. Csapó-Kiss, É. Vargáné Visi, G. Andrásyné Baka, É. Balla Terlakyné, Élelmiszerek D-aminosav tartalma, *Acta Agraria Kaposváriensis,* 1 (1997) 3–20.

[33] G. Pohn, É. Varga-Visi, É Terlakyné Balla, L. Kametler, J. Csapó, A különböző technológiával készült toll-lisztek D-cisztein tartalma, *Műszaki Kémiai Napok'99,* Veszprém, 1999. 48-49.

[34] H.D. Dakin, Note on the relative rate of absorption of optically isomeric substances from the intestine, *J. Biol. Chem.,* 4 (1908) 437–439.

[35] H.D. Dakin, H.W. Dudley, The action of enzymes on racemized proteins and their fate in the animal body, *J. Biol. Chem.,* 15 (1913) 271–277.

[36] A. Danielo, A. Giuditta, Presence of D-aspartate in squid axoplasm and in other regions of the cephalopod nervous system, *J. Neurochem.,* 31 (1978) 1107–1108.

[37] A.P. Degroot, P. Slump, V.J. Feron, L. Van Beek, Effects of alkali treated proteins: feeding studies with free and protein-bound lysinoalanine in rats and other animals, *J. Nutr.,* 106 (1976) 1527–1538.

[38] M. Dixon, P. Kenworthy, D-aspartate oxidase of kidney, *Biochem. Biophys. Acta,* 146 (1967) 54–76.

[39] M.H. Engel, P.E. Hare, Racemization rates of the basic amino acids, *Carnegie Inst. Washington Yearb.,* 81 (1982) 422–425.

[40] H. Felbeck, Occurrence and metabolism of D-aspartate in the gutless bivalve Solemya reidi, *J. Exp. Zool.,* 234 (1985) 145–149.

[41] H. Felbeck, S. Wiley, Free D-amino acids in the tissues of marine bivalves, *Biol. Bull.,* 173 (1987) 252–259.

[42] L.R. Finch, F.J.R. Hird, The uptake of amino acids by isolated segments of rat intestine. II. A survey of affinity for uptake from rates of uptake and competition for uptake, *Biochim. Biophys. Acta,* 43 (1960) 278–287.

[43] J.W. Finley, *Environmental effects of protein quality,* In: Chemical changes in food during processing. Inst. Food Technologists Basic Symp. Ser., AVI Publ. Westport Conn. 1985. 443–482.

[44] J.W. Finley, D.E. Schwass, (eds), *Xenobiotics in foods and feeds,* ACS Symp. Ser. No. 234. Washington, DC. Ann. Chem. Soc., 1983. 421.

[45] G.H. Fisher, N.M. Garcia, I.L. Payan, R. Cadilla-Perezrios, W.A. Sheramata, E.H. Man, D-aspartic acid in purified myelin and myelin basic protein, *Biochem. Biophys. Res. Commun.,* 135 (1986) 683–687.

[46] S. Folestad, A. Tivesten, J. Csapó, Élelmiszerek és takarmányok D-aminosav tartalma. 2. Az aminosav enantiomerek szétválasztása és meghatározása származékképzés után, *Élelmiszervizsg. Közl.,* 40 (1994) 17–26.

[47] M. Friedman, Crosslinking amino acids – Stereochemistry and nomenclature, *Adv. Exp. Med. Biol.,* 86B (1977) 1–27.

[48] M. Friedman, M.R. Gumbman, The utilization and safety of isomeric sulfur-containing amino acids in mice, *J. Nutr.,* 114 (1984) 2301–2310.

[49] M. Friedman, R. Liardon, Racemization kinetics of amino acid residues in alkali-treated soybean proteins, *J. Agric. Food Chem.,* 33 (1985) 666–672.

[50] M. Friedman, J.C. Zahnley, P.M. Masters, Relationship between in vitro digestibility of casein and its content of lysinoalanine and D-amino acids, *J. Food Sci.,* 46 (1981) 127–134.

[51] M. Friedman, D.K. Grosjean, J.C. Zahnley, Carboxypeptidase inhibition by alkali-treated food proteins, *J. Agric. Food Chem.,* 33 (1985) 208–213.

[52] M. Fuse, F. Hayase, H. Kato, Digestibility of proteins and racemization of amino acid residues in roasted foods, *J. Jpn. Soc. Nutr. Food Sci.,* 37 (1984) 348–354.

[53] I. Gandolfi, G. Palla, L. Delprato, F. Denisco, R. Marchelli, C. Salvadori, D-amino acids in milk as related to heat treatments and bacterial activity, *J. Food Sci.,* 57 (1992) 377–379.

[54] Q.H. Gibson, G. Wiseman, Selective absorption of stereoisomers of amino acids from loops of the small intestine of the rat, *Biochem. J.,* 48 (1951) 426–429.

[55] G.M. Gray, H.L. Cooper, Protein digestion and absorption, *Gastroenterology,* 61 (1971) 535–544.

[56] P. Gullino, M. Winitz, S.M. Birnbaum, J. Cornfield, M.C. Otey, J.P. Greenstein, Studies on the metabolism of amino acids and related compounds in vivo. I. Toxicity of essential amino acids, individually and in

mixtures, and the protective effect of L-arginine, *Arch. Biochem. Biophys.,* 64 (1956) 319–332.

[57] P. Gund, P. Veber, On the base-catalysed epimerization of N-methylated peptides and diketopiperazines, *J. Am. Chem. Soc.,* 101 (1979) 1885–1887.

[58] F. Hayase, H. Kato, M. Fujimaki, Racemization of amino acid residues in protein during roasting, *Agric. Biol. Chem.,* 37 (1973) 191–192.

[59] F. Hayase, H. Kato, M. Fujimaki, Racemization of amino acid residues in proteins and poly (L-amino) acids during roasting, *J. Agric. Food. Chem.,* 23 (1975) 491–494.

[60] R. Hayashi, I. Kameda, Racemization of amino acid residues during alkali treatment of proteins and its adverse effect on pepsin digestibility, *Agric. Biol. Chem.,* 44 (1980) 891–895.

[61] R. Hayashi, I. Kameda, Decreased proteolysis of alkali treated proteins: consequences of racemization in food processing, *J. Food Sci.,* 45 (1980) 1430–1431.

[62] R. Hayashi, Lysinoalanine as a metal chelator: an implication for toxicity, *J. Biol. Chem.,* 257 (1982) 13896–13898.

[63] W.L. Jenkins, L.R. Tovar, D.E. Schwass, R. Liardon, K.L. Carpenter, Nutritional characteristics of alkali-treated zein, *J. Agric. Food Chem.,* 32 (1984) 1035–1041.

[64] C. Kies, H. Fox, S. Aprahamian, Comparative values of L, D/L and D-methionine supplementation of an oat-based diet for humans, *J. Nutr.,* 105 (1975) 809–814.

[65] H.A. Krebs, Metabolism of amino acids. III. Deamination of amino acids, *Biochem. J.,* 29 (1935) 1620–1644.

[66] H.A. Krebs, The D- and L-amino acid oxidases, *Biochem. Soc. Symp.,* 1 (1948) 2–19.

[67] R. Liardon, R.F. Hurrel, Amino acid racemization in heated and alkali-treated proteins, *J. Agric. Food. Chem.,* 31 (1983) 432–437.

[68] R. Liardon, S. Lederman, Racemization kinetics of free and protein-bound amino acids under moderate alkaline treatment, *J. Agric. Food. Chem.,* 34 (1986) 557–565.

[69] G. Lubec, C.H.R. Wolf, B. Bartosch, Amino acid isomerisation and microwave exposure, *The Lancet,* March 31 (1990) 792.

[70] J.A. Maga, Lysinoalanine in foods, *J. Agric. Food. Chem.,* 32 (1984) 955–964.

[71] E.H. Man, G.H. Fisher, I.L. Payan, R. Cadilla-Perezrios, N.M. Garcia, D-aspartate in human brains, *J. Neurochem.,* 48 (1987) 510–515.

[72] H. Man, J.L. Bada, Dietary D-amino acids, *Ann. Rev. Nutr.,* 7 (1987) 209–225.

[73] P.E. Masters, M. Friedman, *Amino acid racemization in alkali treated food proteins–chemistry, toxicology, and nutritional consequences.,* In: Chemical Deterioration of Proteins, ACS Symp. Ser., Washington, 123, 165-194. Am. Chem. Soc., 1980. 268.

[74] O. Matsushima, H. Katayama, K. Yamada, Y. Kado, Occurrence of free D-alanine and alanine racemase activity in bivalve molluscs with special reference to intracellular osmoregulation, *Mar. Biol. Lett.,* 5 (1984) 217–225.

[75] E.D. Murray, S. Clarke, Synthetic peptide substrates for erythrocyte protein carboxyl methyltransferase, *J. Biol. Chem.,* 259 (1984) 10722–10732.

[76] A. Neuberger, The metabolism of D-amino acids in mammals, *Biochem. Soc. Symp.,* 1 (1948) 20–32.

[77] G. Palla, R. Marchelli, A. Dossena, G. Casnati, Occurrence of D-amino acids in food. Detection by capillary gas chromatography and by reversed-phase high-performance liquid chromatography with L-phenylalanine amides as chiral selectors, *J. Chromatography,* 475 (1989) 45–53.

[78] A. Paquet, W.C. Thresher, H.E. Swaisgood, G.L. Catignani, Syntheses and digestibility determination of some epimeric tripeptides occurring in dietary proteins, *Nutr. Res.,* 5 (1985) 891–901.

[79] L. Pasteur, Untersuchungen über Asparaginsäuren und Apfelsäure, *Ann. Chem.,* 82 (1852) 324–335.

[80] I.L. Payan, R. Cadilla-Perezrios, G.H. Fisher, E.H. Man, Analysis of problems encountered in the determination of amino acid enantiomeric ratios by gas chromatography, *Anal. Biochem.,* 149 (1985) 484–491.

[81] T.J. Peters, Intestinal peptides, *Gut.,* 11 (1970) 720–725.

[82] R.L. Preston, Occurrence of D-amino acids in higher organisms: A survey of the distribution of D-amino acids in marine invertebrates, *Comp. Biochem. Physiol.,* 87B (1987) 55–62.

[83] D.A. Reaveley, R.E. Burge, Walls and membranes in bacteria, *Adv. Microb. Physiol.,* 7 (1972) 1–81.

[84] T. Robinson, D-amino acids in higher plants, *Life Sci.,* 19 (1976) 1097–1102.

[85] E.M. Rosen-Levin, K.W. Smithson, G.M. Gray, Complementary role of surface hydrolysis and intact transport in the intestinal assimilation of di- and tripeptides, *Biochim. Biophys. Acta,* 629 (1980) 126–134.

[86] D.E. Schwass, L.R. Tovar, J.W. Finely, *Absorption of altered amino acids from the intestine,* In: Xenobiotics in Foods and Feeds. ACS Symp. Ser., Washington, 234, 187–201. Am. Chem. Soc., 2 1983. 01.

[87] J.I. Shoji, Recent chemical studies on peptide antibiotics from the genus Bacillus, *Adv. Appl. Microbiol.,* 24 (1978) 187–214.

[88] L.D. Stegnick, E.F. Bell, L.J. Filer, E.E. Ziegler, D.W. Anderson, Effect of equimolar doses of L-methionine, D-methionine and L-methionine-dl-sulfoxide on plasma and urinary amino acid levels in normal adult humans, *J. Nutr.,* 116 (1986) 1185–1192.

[89] S. Steinberg, J.L. Bada, Diketopiperazine formation during investigations of amino acid racemization in dipeptides, *Science,* 213 (1981) 544–545.

[90] S. Steinberg, J.L. Bada, Peptide decomposition in the neutral pH range via the formation of diketopiperazines, *J. Org. Chem.,* 48 (1983) 2295–2298.

[91] S. Steinberg, P.M. Masters, J.L. Bada, The racemization of free and peptide-bound serine and aspartic acid at 100 °C as a function of pH: implications for in vivo racemization, *Bioorg. Chem.,* 12 (1981) 349–355.

[92] T. Yamane, D.L. Miller, J.J. Hopfield, Discrimination between D- and L-tyrosyl transfer ribonucleic acid in peptide chain elongation, *Biochemistry,* 20 (1981) 7059–7065.

Received: August, 2008

Acta Univ. Sapientiae, Alimentaria, **2**, 1 (2009) 31–43

Influence of mastitis on D-amino acid content of milk

G. Pohn[1]
email: pohn.gabriella@ke.hu

Cs. Albert[2]
email: albertcsilla@sapientia.siculorum.ro

Zs. Csapó-Kiss[1]
email: csapo.janosne@ke.hu

J. Csapó[1,2]
email: csapo.janos@ke.hu

[1]University of Kaposvár,
Faculty of Animal Science,
Guba S. u. 40, 7400 Kaposvár, Hungary

[2]Sapientia–Hungarian University of Transylvania,
Csíkszereda Campus, RO-530104, Libertatii 1., Miercurea-Ciuc

Abstract. The California Mastitis Test was used as an indicator of mastitis. Five cows were chosen for each of the five scores from 0 to 4. Milk samples were analysed for free amino acids and free D-amino acids. The contents of free amino acids, free D-amino acids and the ratio of free D-amino acids to free amino acids increased significantly as score increased. The free D-amino acids content of foremilk (first milk jets) from nonmastitic cows (score = 0) was approximately five times that of samples drawn later from the same udders. Contents of free amino acids and free D-amino acids were highly associated with score and udder inflammation. Very low concentrations of free D-amino acids are normal for raw milk. Higher concentrations of free D-amino acids could be attributed to inclusion of foremilk and milk from cows having subclinical mastitis in the bulk tank milk.

Key words and phrases: free amino acids, D-amino acids, mastitis, foremilk, milk

1 Introduction

Foods have recently been shown to contain considerable quantities of D-AA [1, 4, 9, 10, 13, 15, 16, 18, 19] attributed to both processing technology and microbiological status of the foods. Milk and milk products have been reported [2, 3, 11, 20] to contain significant amounts of D-AA, which were generally attributed to biological status rather than to processing technology. The pertinent literature does not reveal an explanation for the presence of D-AA in raw milk from healthy cows.

The presence of D-AA in food products has been shown to cause a decrease in digestibility of protein [4, 9, 13, 18] and bioavailability of essential AA. It was suggested [9, 18] that some D-AA may provide the basis for formation of toxic products. Absorption rates of L-AA in the intestine were reported to be greater than those of the respective D-enantiomers [7]. Milk products produced by bacterial fermentation contained levels of D-AA [18], and the bacteria were implicated as a biological source of D-AA.

Because D-AA are often products of bacterial metabolism, and mastitis is an inflamation of the udder of bacterial origin, we investigated the influence of mastitis as a possible explanation of D-AA content of raw milk. The cow produces leukocytes to counteract the infection, and, in early studies, direct microscopic count of leukocytes was used to measure the severity of mastitis. Shalm and Noorlander [21] utilized a chemical reaction test that could be read in one of five classes (0 to 4) and was highly correlated with leukocyte count [17, 22]. The California Mastitis Test (CMT) has been used in many studies. Electronic procedures have been used to count cells, and the SCC has also served as the measure of mastitis.

Harmon [12] stated that the primary factor influencing SCC or CMT is intramammary bacterial infection. He also reported that cows with mastitis, as indicated by high SCC, produced milk that differed in composition from milk produced by cows with healthy udders. High SCC was associated with reduced casein and increased concentrations of whey protein, albumin, and immunoglobulin [12]. Another study [5] reported changes in mineral contents. Mastitic milk contained more sodium, chloride, calcium, iron, and manganese, and normal milk was higher in potassium, phosphorus, zinc, and copper. The protein and mineral contents of mastitic milk were similar to those of colostrum. In related studies [8, 17], the foremilk from nonmastitic cows was reported to have higher SCC or CMT than milk drawn later in the milking process.

The objectives of this research were 1) to determine the concentrations of

free AA and free D-AA in milk from cows having different CMT scores and 2) to compare the D-AA contents of foremilk and milk drawn later from cows having a negative CMT.

2 Material and methods

2.1 Assignment of cows and sampling milk

The CMT, as describes for use in Hungary [14] was used in a herd of 1020 Holstein-sired cows. For this experiments, 25 individual cows were identified with each having the same CMT score for all four quarters of the udder. There were five cows in each of the five groups based on CMT score: 0, 1, 2, 3, and 4. The CMT reaction [21] is disintegration of leukocytes when milk is mixed with the reagent (NaOH and an anionic surface active agent). In a negative sample (0 score), the mixture of milk and reagent remains liquid and produces no precipitate. As score increases, the degree of precipitation increases and, when score = 4, a distinct gel with central peak is formed. CMT score is based on number of leukocytes in milk, and mean counts for scores 0, 1, 2, 3, and 4, respectively, were 67, 118, 401, 1737, and 6964×10^3 per ml [17].

At the time of sampling, the amount of milk needed to conduct CMT on each quarter was drawn from each cow. For negative (0 score) cows, the sample was drawn from well-mixed complete yield of the udder with the remainder going to the bulk tank. For positive (CMT of 1, 2, 3, or 4) cows, a volume of 1 l was manually milked, mixed and sampled with the remainder being milked and discarded.

The comparison of D-AA contents of foremilk and later milk required the selection of 5 cows, each of which had negative CMT for all quarters. The foremilk sample consisted of two hand-milked jets from each of the four quarters of the udder. The udder was then milked out completely, and a sample was drawn from the well-mixed remainder.

All milk samples for both experiments were cooled immediately in ice-water and, within 2 h, were placed in a deep freeze at −25 °C. The samples were stored at −25 °C until preparations for AA analysis were initiated.

2.2 Preparation of milk samples for analysis

After defrosting and heating to 30 °C, the samples were centrifuged at 5000 g at room temperature for 20 min to skim the milk and deposit particulate matter at the bottom of the centrifuge tube. To 50 ml of sample, 50 ml of a 25%

trichloroacetic acid solution were added and the mixture was allowed to stand for 20 min. The resulting precipitate was centrifuged at room temperature for 30 min at 10 000 g. To determine total AA, pH of the supernatant was adjusted to pH 2.2 with 4 M sodium hydroxide. To conduct D-AA determinations, the pH was adjusted to pH 7. Using a 10 °C hot plate, the solutions were lyophilized. For total AA determinations, the resulting solid material was dissolved in 10 ml of citrate buffer at pH 2.2, and, for determinations of D-AA, dissolution was in 1 ml of twice-distilled water. Samples prepared were stored at −25 °C until analysis.

2.3 HPLC and ion-exchange column chromatography for the determination of D-AA and total free AA

Instruments. The chromatographic system was assembled from ISCO 100 DM syringe pumps (Isco Inc. Lincoln, Nebraska, USA) and a Rheodyne (Berkeley, California, USA) injector equipped with a 20 μl loop. The separation process was monitored and chromatograms stored on an ISCO Chem Research (Isco Inc. Lincoln, Nebraska, USA) system. The derivative formation and sample injection were performed manually. The excitation and observation wavelengths were 325 and 420 nm, respectively.

Reagents. Acetonitrile and methanol were purchased from Rathburn Ltd (Walkeburn, England). The AA standards, the *o*-phthalaldehyde and the TATG (2,3,4,6,-tetra-O-acetyl-1-thio-β-D-glucopyranozide) were obtained from Sigma Chemical Co., Inc. (St. Louis, MO). The buffers used for elution were prepared from mono- and disodium phosphate. The pH was adjusted with 4 M sodium hydroxide.

Synthesis of derivatives. The reaction was carried out in a 120 μl microvial which was placed in another vial (volume, 1.8 ml) that had Teflon^{R} coating, internal cover plate, and a screw cap. The sample (free AA or protein hydrolysate evaporated in a nitrogen atmosphere), dissolved in 90 μl borate buffer (0.4 M; pH 9.5), was mixed with 15 μl of reagent (8 mg of *o*-phthalaldehyde and 44 mg of TATG dissolved in 1 ml of methanol). The mixture was then homogenized by bubbling through approximately 100 μl of nitrogen and left standing for 6 min. Then, 25 μl of the reaction mixture were injected into the analytical column. After injection, the system was rinsed three times with approximately 100 μl of a 70:30 acetone-water (v/v) solution. Synthesis of derivatives was performed manually and mixing of reagent solution was made

with the aid of an IKA Vibro Fix instrument (Janke and Kunkel, IKA-WERK, Breisgau, Germany).

Separation and quantitation of the enantiomers. Separation of the enantiomers was made according to the method of Einarson et al. [6], using a reversed-phase analytical column packed with Kromasil octyl C-8 (250×5.6 mm internal diameter; 5 μm particle size, EKA Nobel AB, Bohus, Sweden). To increase the lifetime of the column, a safety column was fitted between the sample injector and the analytical column (RP-8, Newguard, 25×3.2 mm internal diameter, 7 μm particle size, EKA Nobel AB, Bohus, Sweden), and a cleaning column (C-18, 36×4.5 mm internal diameter, 20 μm particle size, Rsil, EKA Nobel AB, Bohus, Sweden) was installed between the pump and the sample injector. In order to separate the enantiomers, the two component gradient system had the following composition: A = 40% methanol in phosphate buffer (9.5 mM, pH = 7.05) and B=acetonitrile. The flow rate was 1 ml/min, and the elution of the gradient as a function of time is shown in *Table 1.*

Table 1: The elution gradient, as a function of time, for the two components used to separate and quantitate the enantiomers

Time	**A**[1]	**B**[2]
(min)	**%**	
0	95	5
10	95	5
35	83	17
55	72	28
56	67	33
74	67	33
75	62	38

[1]40% methanol in phosphate buffer (9.5 mM, pH = 7.05).
[2]Acetonitrile

Determination of total free AA was performed with an LKB Model 4101 automatic AA analyzer, following postcolumn derivative synthesis with ninhydrin.

3 Results

3.1 Free AA

Concentrations of total (D- and L-) free AA are shown in *Table 2* for the five CMT scores.

Table 2: Mean free AA (D- plus L-) contents of milk from cows with various California Mastitis Test scores

Amino acid (mg/100 ml)	CMT score[1]					SEM
	0	1	2	3	4	
Asp	0.12^{d}	0.89^{c}	1.13^{b}	1.38^{a}	1.45^{a}	0.021
Thr	0.12^{b}	0.16^{b}	0.18^{ab}	0.20^{ab}	0.24^{a}	0.010
Ser	0.23^{b}	0.27^{b}	0.31^{b}	0.49^{a}	0.52^{a}	0.014
Glu	0.96^{d}	2.53^{c}	3.19^{b}	4.63^{a}	4.71^{a}	0.043
Pro	0.05^{d}	0.09^{d}	0.42^{c}	0.78^{b}	0.89^{a}	0.016
Gly	0.04^{c}	0.06^{bc}	0.09^{b}	0.19^{a}	0.22^{a}	0.006
Ala	0.34^{d}	2.18^{c}	3.42^{b}	4.88^{a}	4.92^{a}	0.113
Cys	0.11^{c}	0.17^{b}	0.19^{b}	0.26^{a}	0.27^{a}	0.009
Val	0.67^{b}	0.17^{b}	0.74^{b}	0.81^{ab}	0.92^{a}	0.025
Met	0.01^{b}	0.01^{b}	0.02^{ab}	0.03^{a}	0.03^{a}	0.002
Ile	0.03^{d}	0.29^{c}	0.35^{b}	0.42^{a}	0.44^{a}	0.010
Leu	0.04^{d}	0.21^{c}	0.38^{b}	0.69^{a}	0.66^{a}	0.014
Tyr	0.09^{c}	0.11^{bc}	0.14^{ab}	0.17^{a}	0.18^{a}	0.007
Phe	0.10^{d}	0.13^{cd}	0.17^{bc}	0.21^{ab}	0.23^{a}	0.008
Lys	0.28^{d}	0.49^{c}	0.83^{b}	1.14^{a}	1.14^{a}	0.017
His	0.12^{b}	0.25^{b}	0.68^{a}	0.71^{a}	0.89^{a}	0.061
Arg	0.09^{d}	0.19^{c}	0.38^{b}	0.57^{a}	0.62^{a}	0.013
Total (sum)	3.40^{e}	8.73^{d}	12.62^{c}	17.56^{b}	18.32^{a}	0.097

[1]Five cows were in each group. 0 = no precipitate, 1 = slight precipitate, which disappears, 2 = distinct precipitate, but no gel formation, 3 = mixture thickens with some suggestion of gel formation and 4 = distinct gel with central peak.
[a,b,c,d,e]Means in the same row and having no superscripts in common differ ($P \leq 0.01$).

Based on analysis of variance and comparison of paired means, differences among the five CMT score groups were significant ($P \leq 0.05$) for each of the 17 AA. Variation among cows within CMT class was very small, as indicated by the small standard errors of means shown in *Table 2.* The milk samples with

CMT of 2, 3, or 4 had free AA composition similar to that of colostrum. This tendency has also been reported [5, 12] for protein components and minerals. Compared with concentrations of normal milk, the most spectacular increases were seen for Ile, Ala, Asp, Pro, and Leu which were more than 10 times the concentrations observed in milk samples scored 0 by the CMT.

The nature of differences among CMT classes was investigated by regression analysis. *Table 3* shows the significant ($P \leq 0.01$) linear contrast for each of the 17 free AA and for the sum. The concentration of free AA increased linearly for CMT scores 0 to 3. The differences between CMT scores of 3 and 4 were significant only for Pro and for the sum of all AA. This result tended to cause the quadratic contrast to account for more variation than the cubic contrast. Based on free AA contents, the CMT classifications could be reduced to four: 0 to 3.

Table 3: Regression of free amino acid content on CMT score: significance of linear (L), quadratic(Q), and cubic (C) orthogonal contrasts and proportion of variance (R^2) associated with regression.

Amino acid	F-Values[1]			R^2		
	L	Q	C	L	L, Q	L, Q, C
Asp	2149**	306**	27**	0.85	0.97	0.99
Thr	81**	0	1	0.80	0.80	0.81
Ser	318**	7*	14**	0.87	0.88	0.92
Glu	4995**	186**	11**	0.94	0.98	0.98
Pro	2171**	7*	113**	0.94	0.94	0.99
Gly	787**	18**	22**	0.90	0.92	0.96
Ala	1101**	63*	5*	0.92	0.97	0.98
Cys	221**	2	1	0.87	0.88	0.89
Val	55**	4	1	0.70	0.74	0.75
Met	80**	0	0	0.84	0.84	0.84
Ile	903**	171**	26**	0.80	0.95	0.98
Leu	1493**	23**	63**	0.92	0.93	0.97
Tyr	274**	1	2	0.84	0.85	0.86
Phe	171**	2	2	0.87	0.88	0.89
Lys	1972**	51**	66**	0.92	0.94	0.97
His	107**	4	1	0.91	0.94	0.95
Arg	1321**	5*	32**	0.96	0.96	0.99
Total (sum)	15921**	503**	76**	0.96	0.99	0.99

*$P \leq 0.05$, **$P \leq 0.01$
[1]F-Values (1, 20 d. f.)

A quadratic function of CMT score explained 99% of the variation in the sum of free AA and 97% of the variation in contents of Asp, Glu, and Ala. Conversely, the free AA content could be used as an indicator of severity of mastitis.

3.2 Free D-AA

Concentrations of free D-AA are shown in *Table 4.* Milk samples rated as negative score, CMT of 0, contained free D-Asp, D-Glu, and D-Ala. However, the quantities present (0.02, 0.05, and 0.04 mg/100 ml, respectively) were almost negligible compared with the quantities occurring in the milk samples that had CMT of 2, 3, or 4. For samples scored 1, D-Val, D-allo-Ile, D-Leu, and D-Lys were also present. D-Ser and D-Pro were identified in samples scored 2, 3 or 4. Not even traces could be identified of the D-enantiomers of other AA, which are the building blocks of proteins.

Table 4: Mean free D-AA contents of milk from cows with various California Mastitis Test scores.

D-AA	**CMT score**[1]					**SEM**
(mg/100 ml)	**0**	**1**	**2**	**3**	**4**	
D-Asp	0.02^{d}	0.17^{c}	0.23^{b}	0.32^{a}	0.32^{a}	0.008
D-Ser	0^{c}	0^{c}	0.02^{b}	0.04^{a}	0.04^{a}	0.003
D-Glu	0.05^{c}	0.08^{c}	0.99^{b}	1.48^{a}	1.53^{a}	0.027
D-Pro	0^{c}	0^{c}	0.04^{b}	0.09^{a}	0.10^{a}	0.005
D-Ala	0.04^{c}	0.05^{b}	1.13^{b}	2.32^{a}	2.41^{a}	0.020
D-Val	0^{b}	0.08^{ab}	0.09^{ab}	0.09^{ab}	0.12^{a}	0.017
D-allo-Ile	0^{d}	0.08^{c}	0.10^{bc}	0.12^{ab}	0.15^{a}	0.006
D-Leu	0^{d}	0.06	0.12^{b}	0.17^{a}	0.17^{a}	0.008
D-Lys	0^{b}	0.11^{c}	0.27^{b}	0.36^{a}	0.37^{a}	0.012
Total (sum)	0.11^{e}	0.63^{d}	2.99^{c}	4.99^{b}	5.21^{a}	0.018

Data expressed as in *Table 2.*
^{a,b,c,d,e}Means in the same row and having no superscripts in common differ ($P \leq 0.01$).

Free D-AA increased as CMT score increased for all nine free D-AA and for the sum of free D-AA. The nature of the regression pattern *(Table 5)* was slightly different; the cubic contrast was relatively more important than was the quadratic contrast. California Mastitis Test classes 3 and 4 had similar free D-AA concentrations, as was observed previously. However, differences in free D-AA contents of samples scored 0 and 1 also tended to be small. Therefore, the graphic expression of free D-AA content relative to CMT score tended to be sigmoid in shape. Thus CMT score can be used as an accurate predictor of concentration of free D-AA, but the prediction equation would be a cubic function: $y = a + bx + b_2x_2 + b_3x_3$.

Table 5: Regression of free D-amino acid content on CMT score: significance of linear (L), quadratic (Q), and cubic (C) orthogonal contrasts and proportion of variance (R^2) associated with regression.

D-AA	F-Values[1]			R^2		
	L	Q	C	L	L, Q	L, Q, C
D-Asp	692**	72**	0	0.87	0.96	0.96
D-Ser	148**	0	20**	0.80	0.80	0.99
D-Glu	5261**	13**	243**	0.89	0.90	0.98
D-Pro	638**	3	28**	0.86	0.87	0.94
D-Ala	25775**	14**	1246**	0.91	0.91	0.99
D-Val	216**	33**	34**	0.71	0.82	0.94
D-allo-Ile	333**	17**	13**	0.87	0.91	0.95
D-Leu	295**	17**	3	0.88	0.93	0.94
D-Lys	5195**	33**	12**	0.91	0.95	0.97
Total (sum)	9047**	27**	572**	0.94	0.94	0.99

Data expressed as in *Table 3.*

The increase in free D-AA could be easily attributed to the availability of larger amounts of free AA for conversion to D-AA. However, the concentration of free D-AA relative to free (D- and L-) AA increases as CMT increases *(Table 6).* Free D-Ala represented almost 50% of the total free Ala when CMT score was 3 or 4. Conversely, the increase in percentage D-Asp (17 to 23%) was small. For the sums of all AA, the percentage D-AA was only 3% for samples scored 0 compared with 28% for these scored 3 or 4 by CMT. Both absolute quantity and percentage (relative to total free) of D-AA increased as CMT increased.

Table 6: Free D-amino acid concentration as a percentage of free (D and L) amino acid content of milk from cows with various California Mastitis Test scores.

D-AA	CMT score[1]					SEM
(mg/100 ml)	0	1	2	3	4	
D-Asp	17.0^{c}	19.0^{bc}	20.7^{ab}	23.3^{a}	21.9^{ab}	0.92
D-Ser	0^{b}	0^{b}	6.6^{a}	8.6^{a}	7.7^{a}	0.74
D-Glu	5.6^{a}	3.0^{b}	31.1^{a}	31.9^{a}	32.5^{a}	0.79
D-Pro	0^{c}	0^{c}	8.4^{b}	11.8^{a}	11.1^{a}	0.89
D-Ala	12.9^{c}	2.2^{d}	33.1^{b}	47.6^{a}	49.1^{a}	1.03
D-Val	0^{b}	11.0^{a}	12.5^{a}	11.0^{a}	13.0^{a}	0.79
D-allo-Ile	0^{c}	27.6^{b}	27.6^{b}	29.3^{b}	34.7^{a}	1.61
D-Leu	0^{b}	29.1^{a}	31.7^{a}	24.4^{a}	25.8^{a}	1.91
D-Lys	0^{c}	22.0^{b}	32.6^{a}	32.0^{a}	32.8^{a}	1.81
Total (sum)	3.4^{d}	7.0^{c}	23.7^{b}	28.4^{a}	28.4^{a}	0.40

Data expressed as in *Table 2.*
^{a,b,c,d}Means in the same row and having no superscripts in common differ (P≤0.05).

3.3 Free D-AA content of foremilk

Foremilk has been shown to have higher CMT score and higher SCC [8, 17, 22] than that of milk drawn later in the milking process. The D-AA concentrations of foremilk and later milk from cows having CMT score of 0 are shown in *Table 7.*

Table 7: Free D-amino acid content of foremilk and mixed total milk from five cows having negative (zero) California Mastitis Test scores

D-AA	Milk sample			Conf. Limits[2]	
(mg/100 ml)	Foremilk	Milk	Difference[1]	LL	UL
D-Asp	0.132	0.021	0.111^{**}	0.086	0.136
D-Glu	0.214	0.053	0.161^{**}	0.124	0.198
D-Ala	0.203	0.043	0.160^{**}	0.111	0.209
D-allo-Ile	0.061	0	0.061^{**}	0.045	0.077
Total (Sum)	0.610	0.117	0.493^{**}	0.443	0.543

[1]Difference (foremilk-milk) calculated for each cow and averaged (D).
[2]Lower (LL) and upper (UL) 99% confidence limits for average difference (D) = D±t (S.E.D) with t5 d.f. and α= 0.01 and S.E.D = $\sqrt{s_{\mathrm{D}}^2/5}$
** P≤0.01

The foremilk was two jets of milk drawn from each quarter at the beginning of milking. Free D-AA contents of foremilk were approximately five times those of later drawn milk ($P \leq 0.01$). Negative (CMT score of 0) samples had extremely low concentrations of free D-AA in later milk. The free D-AA contents of foremilk from cows whose milk scored 0 was typical of the concentrations reported for milk from cows whose milk scored 1 *(Table 4).*

4 Implications

Based on the results, we concluded that very low concentrations of D-AA are normal for raw milk. Higher D-AA can be traced to inclusion of the bacteria-rich foremilk and milk from cows with subclinical mastitis in the bulk tank. Data presented here would support the hypothesis that D-AA content of raw milk is associated with mastitis and, consequently, with bacterial activity in the udder.

5 Acknowledgments

The authors acknowledge the support of the Hungarian National Research Fund through Grant OTKA T 12769, and OTKA T 14916.

References

[1] M.F. Boehm, J.L. Bada, Racemization of aspartic acid and phenylalanine in the sweetener aspartame at 100 °C, *Proc. Natl. Acad. Sci.,* 81 (1984) 5263–5266.

[2] H. Brückner, M. Hausch, D-Amino acids in dairy products: detection, origin and nutritional aspects. I. Milk, fermented milk, fresh cheese and acid crude cheese, *Milchwissenschaft,* 45 (1990) 357–360.

[3] H. Brückner, M. Hausch, D-Amino acids in dairy products: detection, origin and nutritional aspects. II. Ripened cheeses, *Milchwissenschaft,* 45 (1990) 421–425.

[4] S. Bunjapamai, R.R. Mahoney, I.S. Fagerson, Determination of D-amino acids in some processed foods and effect of racemization on in vitro digestibility of casein, *J. Food Sci.,* 47 (1982) 1229–1234.

[5] J. Csapó, Zs. Csapó-Kiss, J. Máté, I. Juricskay, Kísérletek a masztitiszes tej részarányának meghatározására elegytejekből, *Állattenyésztés és Takarmányozás,* 35 (1986) 337–343.

[6] S. Einarsson, S. Folestad, B. Josefsson, Separation of amino acid enantiomers using precolumn derivatization with o-phthalaldehyde and 2,3,4,6,-tetra-O-acetyl-1-thio-β-glucopyranoside, *J. Liquid Chromatogr.,* 10 (1987) 1589–1598.

[7] L.R. Finch, F.S.R. Hird, The uptake of amino acids by isolated segments of rat intestine. 2. A survey of affinity for uptake and competition for uptake, *Biochim. Biophys Acta,* 43 (1960) 278–287.

[8] T.L. Forster, U.S. Ashworth, L.O. Luedecke, Relationship between California Mastitis Test reaction and production and composition of milk from opposite quarters, *J. Dairy Sci.,* 50 (1967) 675–682.

[9] M. Friedman, J.C. Zahnley, P.M. Masters, Relationship between in vitro digestibility of casein and its content of lysinoalanine and D-amino acids, *J. Food Sci.,* 46 (1981) 127–134.

[10] M. Fuse, F. Hayase, H. Kato, Digestibility of proteins and racemization of amino acid residues in roasted foods, *J. Jpn. Soc. Food. Nutr.,* 37 (1987) 348–354.

[11] I. Gandolfi, G. Palla, L. Delprato, F. DeNisco, R. Marchelli, C. Salvadori, D-amino acids in milk as related to heat treatments and bacterial activity, *J. Food Sci.,* 57 (1992) 377–379.

[12] R.J. Harmon, Physiology of mastitis and factors affecting somatic cell counts, *J. Dairy Sci.,* 77 (1994) 2103–2112.

[13] R. Hayashi, I. Kameda. Decreased proteolysis of alkali treated proteins: consequences of racemization in food processing, *J. Food Sci.,* 45 (1980) 1430–1431.

[14] Hungarian Standard No. 12320-85. Nyers tehén és juhtej vizsgálat masztiteszt próbával. (Testing of cow's and ewe's milk with mastitis test.) Hungarian Standardisation Bureau. Budapest. Hungary.

[15] R. Liardon, S. Lederman, Racemization kinetics of free and protein-bound amino acids under moderate alkaline treatment, J. *Agric. Food. Chem.,* 34 (1986) 557–565.

[16] G. Lubec, C.H.R. Wolf, B. Bartosch, Amino acid isomerisation and microwave exposure, *Lancet,* 334 (1989) 1392–1393.

[17] L.O. Luedecke, T.L. Forster, U.S. Ashworth, Relationship between California Mastitis Test reaction and leucocyte count, catalase activity and A-esterase activity of milk from opposite quarters, *J. Dairy Sci.,* 50 (1967) 1592–1596.

[18] H. Man, J.L. Bada, Dietary D-amino acids, *Ann. Rev. Nutr.,* 7 (1987) 209–225.

[19] P.E. Masters, M. Friedman, *Amino acid racemization in alkali treated food proteins chemistry, toxicology, and nutritional consequences,* In: Chemical Deterioration of Protein, Ser. 123 165. 165–194.

[20] G. Palla, R. Marchelli, A. Dossena, G. Casnati, Occurrence of D-amino acids in food. Detection by capillary gas chromatography and by reversed-phase high-performance liquid chromatography with L-phenylalaninamides as chiral selectors, *J. Chromatogr.,* 475 (1989) 45–53.

[21] O.W. Schalm, D.O. Noorlander, Experiments and observations leading to the development of the California Mastitis Test, *J. Am. Vet. Med. Assoc.,* 130 (1957) 199.

[22] J.W. Smith, W.D. Schultze, Preliminary evaluation of the California Mastitis Test as a method of estimating the inflammatory condition of individual quarters, *J. Dairy Sci.,* 48 (1965) 820.

Received: August, 2008

Acta Univ. Sapientiae, Alimentaria, **2**, 1 (2009) 45-53

Effect of microorganisms on free amino acid and free D-amino acid contents of various dairy products

Cs. Albert[1]
email:
albertcsilla@sapientia.siculorum.ro

G. Pohn[2]
email: pohn.gabriella@ke.hu

K. Lóki[2]
email: loki.katalin@ke.hu

J. Csapó[1,2]
email: csapo.janos@ke.hu

[1]Sapientia–Hungarian University of Transylvania,
Csíkszereda Campus, RO-530104, Libertatii 1., Miercurea-Ciuc

[2]University of Kaposvár,
Faculty of Animal Science,
Guba S. u. 40, 7400 Kaposvár, Hungary

Abstract. Free amino acid and free D-amino acid contents of milk samples with different microorganism numbers and composition of dairy products produced from them were examined. Total microorganism number of milk samples examined varied from 1.25×10^6 to 2.95×10^6. It was established that with an increase in microorganism number concentration of both free D-amino acids and free L-amino acids increased, however, increase in D-amino acid contents was bigger considering its proportion. There was a particularly significant growth in the microorganism number range of 1.5×10^6 to 2.9×10^6. Based on analysis of curds and cheese samples produced using different technologies we have come to the conclusion that for fresh dairy products and for those matured over a short

Key words and phrases: D-amino acids, free amino acids, dairy products, microorganism number, milk, cheese

time there was a close relation between total microorganism number and free D-amino acid and free L-amino acid contents. At the same time it was found that the ratio of the enantiomers was not affected by the total microorganism number. For dairy products, however, where amino acid production capability of the microbial cultures considerably exceeds production of microorganisms originally present in the milk raw material, free amino acid contents of the milk product (both D- and L-enantiomers) seem to be independent of the composition of milk raw material.

1 Introduction

From our earlier examinations [4, 5, 9] it is obvious that free amino acid and free D-amino acid contents of milk are significantly influenced by the technology, in the first place, however, by the microbiological condition of milk raw material. It is known that D-stereoisomer amino acids are not or not easily utilized by the human organism, their harmful effects were reported in several publications [2, 3, 6, 7]. It is also known that presence of D-amino acids in the proteins reduces digestibility and in bigger volumes they can act as growth inhibitors [8]. In nutritional scientific respect an important fact is that D-amino acids and peptides containing D-amino acids have a different taste than the corresponding L-stereoisomers [1].

In case of countries recently joined the European Union milk manufacturers are reduced to occassionally produce various dairy products complying with the standards out of milk with microorganism number of several millions considered to be unsuitable for human consumption in EU countries. Because of the above we aimed at examinating of total free and free D-amino acid contents of milk with various total microorganism numbers in order to establish a relationship between microorganism number and total free and free D-amino acid contents of milk. Subsequently we were trying to answer the question how free amino acid contents of milk raw material influenced free amino acid composition of dairy products manufactured from it.

2 Material and methods

2.1 Milk samples examined

Milks with different total microorganism numbers and dairy products were obtained from a dairy company in Székelyland out of those mixed milk samples from which the company produced consumption milk and various dairy

products. Total microorganism number of obtained milk samples varied from 1.23×10^6 to 2.95×10^6. As a control sample milk with total microorganism number less than 100.000 was used, obtained from the cattle farm of the University of Kaposvár, Faculty of Animal Science, and which was taken from a mixed milk of around 100 Holstein-Friesian cows having lactation milk production of around 10.000 liters. Subsequent to the sampling and determination of the total microorganism number milk samples were cooled down to $-25\,^\circ$C and kept at this temperature until the preparation for chemical analysis.

2.2 Determination of total microorganism number

For determination of the microbe number direct counting of the bacteria was applied. The milk sample taken into a sterile test tube was thoroughly mixed through a rapid rotation movement. A 1/10 dilution was prepared (for the dilution 0.85% sodium chloride solution was used that was sterilized in autoclave beforehand). One cm^3 of the pasteurized milk sample was added to $9\,\mathrm{cm}^3$ of sterile dilutant water, then $1\,\mathrm{cm}^3$ of the thoroughly mixed diluted sample was pipetted onto a sterile Petrifilm plate with a culture medium. The Petrifilm plate was incubed at $37\,^\circ$C for 24 h, and the developed colonies were directly counted with the use of a culture counter.

2.3 Dairy products examined

The examined dairy products included yoghurt, Sana, curds and some types of cheeses (Telemea, Dalia and Rucăr), all obtained from a Transylvanian dairy company for analysis. The company documentation showed which dairy product from milk of what average total microorganism number was produced, so the examined products could be sorted one by one as per microorganism number.

Sana is a soured dairy product, manufactured by lactic acidic coagulation of milk using a lyophilized culture mixture (consisting of *Lactococcus lactis lactis, Lactococcus lactis cremoris, Lactococcus lactis diacetilactis).* Telemea is a feta-type cheese, produced by mixed coagulation i.e. using both a lyophilized culture mixture (consisting of *Streptococcus thermophilus, Lactococcus lactis lactis, Lactococcus lactis cremoris, Lactobacillus bulgaricus),* and rennet (chymosin). During its production Telemea is matured over 2 days in a brine of 20–21%, at 12–14 °C. Dalia is a semi-hard cheese, produced by mixed coagulation, using a lyophilized culture mixture (consisting of *Streptococcus thermophilus, Lactococcus lactis lactis, Lactococcus lactis cremoris, Lactococcus acidophilus)*

and rennet (chymosin). Dalia is matured over 2 weeks at 14 °C at a relative humidity of 75–80%. Cow's curds were produced by mixed coagulation using a lyophilized culture mixture (consisting of *Streptococcus thermophilus, Lactococcus lactis lactis, Lactococcus lactis cremoris, Lactococcus diacetilactis)* and chymosin.

Out of dairy products examined, curds, yoghurt, Sana and Telemea are considered as products matured over a short time, while cheeses Dalia and Rucăr as products matured over a longer time. The examined milk products were manufactured by keeping the Romanian standards and specifications as well as hygienic regulations.

2.4 Sample preparation

Preparation of milk and dairy products for analysis. Preparation of the samples was carried out at the University of Kaposvár, Faculty of Animal Science, Department of Chemistry and Biochemistry. In case of cheese sample analysis, as much cheese was homogenized with distilled water so that the dry matter contents of the mixture obtained similarly to milk be between 12–15%. Subsequently, the completely milk-like homogenized samples were treated as they had been milk samples. The milk samples stored deep-frozen were after defrosting and warming up to 30 °C centrifuged at 8.000 g for 10 min in order to remove the cellular elements and milk fat. Subsequently, to 25 cm^3 of sample 25 cm^3 of 25% trichloroacetic acid were added, left standing for 20 min, and centrifuged at 10.000 g for 10 min. The supernatant was poured down and its pH was adjusted to be 7 with 4.0 M NaOH. The obtained solution was lyophilized at −10 °C, and the residue (pH = 7) was solved in sodium acetate buffer for determination of total free amino acid contents. Prepared samples were stored at −25 °C until analysis.

Determination of total free amino acids and free D-amino acids. Determination of free amino acid and free D-amino acid contents were carried out using a Merck-Hitachi HPLC instrument, for collecting and evaluating the measured data D-7000 HPLC System Manager software was used.

For determination of total free amino acids cyclic derivatives were formed from the amino acids with *o*-phthaldialdehyde and 2-mercaptoethanol, the formed derivatives were separated on a Licrospher (C-18) analytical column (dimensions: 125×4 mm; particle size: 4m μm) using a gradient system consisting of methanol and sodium acetate buffer. Derivatives were detected at an excitation wavelength of 325 nm and emission wavelength of 420 nm. For de-

termination of free D-amino acids diastereomer derivatives were formed from the amino acid enantiomers with *o*-phthaldialdehyde and 1-thio-β-D-glucose tetraacetate, the enantiomers were separated in the above described system on a Superspher (C-8) analytical column using a gradient system consisting of methanol, acetonitrile and phosphate buffer, the derivatives were detected at an excitation wavelength of 325 nm and at emission wavelength of 420 nm.

3 Results

Free L-amino acid and free D-amino acid contents of milks with various total microorganism numbers by 50.000 CFU, are presented in *Table 1.*

Table 1: Free L-amino acid and free D-amino acid contents of milks with different total microorganism numbers (mg/100 g sample) and proportion of D-amino acids ((D/D+L)×100)

Total CFU	**Amino acid**								
	Aspartic acid			**Glutamic acid**			**Alanine**		
$\times 10^6$	**L**	**D**	**ratio**	**L**	**D**	**ratio**	**L**	**D**	**ratio**
0.1	0.12	0.015	11.11	0.96	0.053	5.23	0.32	0.043	11.85
1.23	0.34	0.042	10.99	1.22	0.084	6.44	0.67	0.102	13.21
1.53	0.54	0.087	13.88	1.47	0.124	7.78	0.91	0.235	20.52
2.00	0.84	0.145	14.72	2.79	0.455	14.02	1.69	0.454	21.17
2.20	0.88	0.257	22.60	2.80	0.715	20.32	1.85	0.942	33.73
2.95	1.48	0.321	21.97	4.53	1.534	25.30	4.83	2.419	33.37

CFU: Colony Forming Unit

It was established that in the control milk sample proportion of D-aspartic acid to total free aspartic acid was 11.11%, proportion of D-glutamic acid was 5.23%, and that of D-alanine was 11.85%. In case of samples with total microorganism numbers between 1.25×10^6 and 1.53×10^6 there was no substantial change in the quantity of either free L-amino acids or free D-amino acids, although both concentration of free L-amino acids and proportion of D-amino acids grew continuously with increasing total microorganism number. This minimal change continued up to total microorganism number of 2.20×10^6 where there was an explosion in both total free amino acid quantity and free D-amino acid quantity, and this sudden increase also applied to the propor-

tion of D-amino acids to the total free amino acids. It appears that up to a microorganism number of 1.5×10^6–1.6×10^6 there are no significant changes in free amino acid and free D-amino acid contents of milk. Afterwards, subsequent to a short period there is an explosion. In summary, in case of each examined free amino acids concentration of both free D-amino acids and free L-amino acids increases, however, increase of D-amino acids is bigger in its proportion considered since for aspartic acid compared to the control milk up to the microorganism number of 2.95×10^6 this proportion increased from 11.11% to 21.97%, for glutamic acid from 5.23% to 25.30% and for alanine from 11.85% to 33.37%.

After having determined the development of milk raw material composition as a function of microorganism number, we examined what effect the increased quantity of free D- and L-amino acids had on the composition of dairy products produced from this raw milk. Again, it was focused on aspartic acid, glutamic acid and alanine since these three amino acids are contained in peptidoglycan that compose cell wall of bacteria, and when released they give a major part of D-amino acid contents of milk products. After bacteria die, subsequent to the lysis these amino acids contribute to the formation of taste, aroma and nutritional value of dairy products. Knowing the relationship between total microorganism number of milk raw material and D-amino acid concentration it can be assumed that the milk raw material can affect composition of dairy product manufactured from it. In order to prove this hypothesis composition of 4 Sana, 4 Dalia, 3 Telemea, 2 curds, 1 Rucăr and 1 yoghurt, manufactured from 4 milks with different total microorganism numbers was examined. We do not want to draw any definitive conclusions from our examinations because of the low sample number in case of curds, Rucăr and yoghurt, results are published here only for orientation. Results are presented in *Table 2.*

The four Sana were manufactured from milks with total microorganism numbers of 1.23, 1.35, 1.53 and 2.95×10^6. Based on the obtained results the conclusion can be drawn that with increasing total microorganism number of milk raw material the quantity of both D- and L- enantiomers increases for all the three amino acids. This increase becomes substantial after a microorganism number of 1.5×10^6 as Sana produced from milk with total microorganism number of nearly 3×10^6 contains the most of both L- and D-amino acids. No significant changes could be experienced regarding D- and L-ratios of the individual amino acids. Proportion of D-glutamic acid is the least with 22.4–26.4%, followed by that of D-aspartic acid with 31.3–32.4%, and by that of D-alanine with 37.6–41.9%.

For the cheese Dalia, free amino acid contents of cheeses produced from

Table 2: Free L-amino acid and free D-amino acid contents (mg/100 g sample) of dairy products manufactured from milk with various total microorganism numbers and proportion of D-amino acids ((D/D+L)×100)

Total CFU $\times 10^6$	Dairy products	Amino acid								
		Aspartic acid			Glutamic acid			Alanine		
		L	D	ratio	L	D	ratio	L	D	ratio
1.228	Sana	0.552	0.251	31.34	1.624	0.583	26.41	0.698	0.462	39.81
1.351	”	0.567	0.259	31.42	2.144	0.619	22.39	0.861	0.519	37.63
1.530	”	0.725	0.320	30.64	2.548	0.834	24.65	1.265	0.790	38.42
2.945	”	1.132	0.543	32.43	4.556	1.542	25.09	1.735	1.251	41.90
1.250	Dalia	13.419	5.593	29.42	42.535	12.791	23.12	21.706	15.621	41.85
2.000	”	15.309	6.142	28.63	43.049	12.852	22.99	26.379	17.601	40.02
2.800	”	16.754	6.231	27.11	48.247	13.439	21.85	27.347	17.803	39.43
2.912	”	15.170	6.324	29.42	41.381	13.516	24.62	24.816	17.004	40.66
1.320	Telemea	0.861	0.389	31.14	3.057	0.752	19.73	1.688	1.071	38.81
1.664	”	1.027	0.428	29.42	3.493	0.841	19.41	1.904	1.223	39.12
2.200	”	1.504	0.610	28.99	3.212	0.935	22.54	1.973	1.349	40.60
1.560	Curds	0.081	0.038	32.14	0.458	0.109	19.23	0.187	0.124	41.62
1.684	”	0.101	0.051	33.51	0.492	0.112	18.54	0.213	0.133	38.43

CFU: Colony Forming Unit

milk with total microorganism number of 1.25; 2.00; 2.80 and 2.91×10^6 were analyzed. Proportion of D-aspartic acid varied from 27.11 to 29.42%, that of D-glutamic acid from 21.85 and 24.62%, and appeared to be, similarly to aspartic acid, independent of microorganism number of milk raw material. Percentage of D-alanine exceeded with the exception of one sample 40%, ranging between 39.43 and 41.85%.

In case of Telemea, products manufactured from milks with total microorganism numbers of 1.32; 1.66 and 2.20×10^6 were analyzed. In this total microorganism number range with the exception of L-glutamic acid there was an increase for all amino acids and enantiomers, but since the total microorganism number range was not wide enough in this case, definitive conclusions similar to those in case of the two previous dairy products could not be drawn from our investigations. Similarly to the previous two cheeses, percentage of D-glutamic acid was found to be the lowest with 19.73–22.54%, whereas quantity of D-aspartic acid ranged between 28.99–31.14%, and proportion of D-alanine between 38.81–40.60%. It appears that in case of Telemea there is no relation between total microorganism number of milk raw material and the examined products manufactured from the milk raw material.

In case of the two curds, one Rucăr, and one yoghurt of course no conclusions can be drawn on the effect of microorganism number. Compared the amino acid composition of the curds to that of all of the other dairy products it

can be established that the quantity of both D- and L-amino acids is less by almost one order of magnitude than that of the other products examined, while proportion of the D-amino acids shows only a slight difference compared to the others.

Summarized the results of our investigations, we can say that in case of the milk raw material with increasing total microorganism number concentration of both free D-amino acids and L-amino acids increases, however, the increase for the D-amino acids is bigger its proportion regarded since compared to the control sample the ratio of D-amino acids increases to a multiplied value.

Examined the relationship between the quality of dairy products manufactured from milk raw material of different total microorganism numbers it was established that the percentage of D-amino acids to the total free amino acid contents was not affected by either the total microorganism number of the milk raw material or the fact what kind of dairy it was about. Proportion of D-aspartic acid was found to be around 30% for most of the examined dairy products, although in case of Sana and the curds this was a little more, while for Dalia somewhat less. Percentage of D-glutamic acid varies between 18–27%, this ratio is higher for Sana than for Dalia and the lowest for Telemea. Proportion of D-alanine is around 40% for each dairy products independently of total microorganism number of the milk. Out of the examined three amino acids proportion of D-glutamic acid is the smallest, that of D-alanine is the biggest, while D-aspartic acid has a value between these two, nearer to that of D-glutamic acid.

For fresh dairy products and for those are matured for a short time (Sana, yoghurt, curds, Telemea) a relationship can be established between total microorganism number and D-amino acid contents and this relation applies in most cases also to the L-enantiomers. Despite the fact that total microorganism number has a substantial effect on concentration of both enantiomers, ratio of the enantiomers is not affected by the total microorganism number. For those dairy products, however, which are matured over a longer time and for those where amino acid production capability of microbial cultures significantly exceeds production of microorganisms originally present in the milk raw material no effect of the milk raw material can be expected, thus, free amino acid contents of the milk products seem to be independent of the composition of milk raw material.

4 Acknowledgements

Authors are grateful to the Sapientia Foundation, Institute of Research Programs for the financial support.

References

[1] M.F. Boehm, J.L. Bada, Racemization of aspartic acid and phenylalanine in the sweetener aspartame at 100 °C, *Proc. Natl. Acad. Sci.*, 81 (1984) 5263–5266.

[2] H. Brückner, M. Hausch, D-amino acids in dairy products: Detection, origin and nutritional aspects. I. Milk, fermented milk, fresh cheese and acid curde cheese, *Milchwissenschaft*, 45 (1990) 357–360.

[3] H. Brückner, M. Hausch, D-amino acids in dairy products: Detection, origin and nutritional aspects. I. Ripened Cheeses, *Milchwissenschaft*, 45 (1990) 421–429.

[4] J. Csapó, T.G. Martin, Zs. Csapó-Kiss, J. Stefler, S. Némethy, Influence of udder inflammation on the D-amino acid content of milk, *Journal of Dairy Science*, 78 (1995) 2375–2381.

[5] J. Csapó, Zs. Csapó-Kiss, J. Máté, I. Juricskay, Kísérletek a masztitiszes tej részarányának meghatározására elegytejekből. *Állattenyésztés és Takarmányozás*, 35 (1986) 337–343.

[6] M. Fuse, F. Hayase, H. Kato, Digestibility of proteins and racemization of amino acid residues in roasted foods, *J. Jpn. Soc. Nutr. Food Sci.*, 37 (1984) 348.

[7] I. Gandolfi, G. Palla, L. Delprato, F. DeNisco, R. Marchelli, C. Salvadori, D-amino acids in milk as related to heat treatments and bacterial activity, *J. Food Sci.*, 57 (1992) 377–379.

[8] H. Man, J.L. Bada, Dietary D-amino acids, *Ann. Rev. Nutr.*, 7 (1987) 209–225.

[9] G. Pohn, J. Csapó, Free D-amino acid content of milk from mastitic udder, *Acta Agraria Kaposváriensis*, 6 (2002) 149–157.

Received: August, 2008

Acta Univ. Sapientiae, Alimentaria, **2**, 1 (2009) 55–64

The influence of manufacture on the free D-amino acid content of Cheddar cheese

É. Varga-Visi[1]
email: visi@ke.hu

K. Lóki[1]
email: loki.katalin@ke.hu

Cs. Albert[2]
email:
albertcsilla@sapientia.siculorum.ro

J. Csapó[1,2]
email: csapo.janos@ke.hu

[1]University of Kaposvár,
Faculty of Animal Science,
Guba S. u. 40, 7400 Kaposvár, Hungary

[2]Sapientia–Hungarian University of Transylvania,
Csíkszereda Campus, RO-530104, Libertatii 1., Miercurea-Ciuc

Abstract. The changes in the concentration and that of composition of alanine, aspartic acid and glutamic acid enantiomers were investigated during manufacture of Cheddar cheese. The amount of D-alanine increased continuously during ripening following the liberation of L-alanine originated from the proteolysis of milk proteins. There was slightly more D-aspartic and D-glutamic acid in the dry matter of curd after pressing than before pressurization. The D-amino acid content and the ratio of the D-enantiomers related to the total amount of free amino acids differed significantly among cheeses produced with different single-strain starters. The D-amino acid composition changed during manufacture, but the influence of the strain selection was not significant on the D-amino acid pattern.

Key words and phrases: D-alanine, D-aspartic acid, D-glutamic acid, Cheddar cheese manufacture, autolysis

1 Introduction

The D-amino acid content of raw milk with low cell count was reported to be not significant. Neither pasteurization nor UHT heating resulted in significant increase in the amount of D-amino acids [2, 8]. In contrast to heating, the concentration of D-amino acids in fermented milk products was significantly higher than that of the raw material. Among fermented products ripened cheeses are the richest source of D-amino acids. Mostly D-alanine (D-Ala) was present in the highest quantities and with a few exceptions D-glutamic acid (D-Glu) and D-aspartic acid (D-Asp) were also present [2, 4].

The origin of free D-amino acids related with bacterial activity is supposed to be connected with the autolysis of cells and the presence of bacterial racemases [1, 4, 9]. The Gram positive bacteria applied in starters during cheesemaking have cell walls with D-amino acid containing peptidoglycan and substituted teichonic acid giving appr. 50% of the dry material of the cell [9, 11]. The retardation and the death phase of the bacterial cell life-span is concerned with cheese ripening [12]. During this period the number of non-viable cells increases. These cells in cheese may be more-or-less intact with complete cell walls or being in sphaeroplast form with membranes with varying degree of disintegration depending on the state of lysis [14]. In the latter stage the D-amino acids may be liberated from the constituents of the cell wall and intracellular izomerases could also release from the cells. Among D-amino acids the amount of D-Ala in fruit juices and milk was reported to be associated with the stage of the bacterial growth. D-Ala content began to increase when the bacteria population was in the retardation phase [8, 9]. Because the stage of bacterial growth determines the die-off rates, the increment of D-Ala could also be associated with the number of dead cells. In cheeses this part of the life-span associated with ripening, therefore similar tendencies could be possible during the ripening of cheeses in the D-Ala content. On the other hand, besides the stage of manufacture the intensity of lysis can depend on the manufacturing conditions applied to a given type of cheese varieties. Actually in the case of the same variety of cheese, e. g., starter culture strains with different susceptibility to lysis can be applied [14]. In our experiment the manufacture of one of the most popular cheeses was investigated and the influence of the processing stages and the selection of starter culture on the free D-amino acid content were investigated.

2 Material and methods

2.1 Cheese manufacture

The conventional Cheddar cheesemaking procedure was applied [12]. The raw material of each trial was 100 l of bulkmilk from cows. The casein/fat ratio was adjusted between 0.7 and 1.0 after pasteurization (72 °C, 15 s). The starter culture used was a 2% (v/v) inoculum of a single-strain *Lc. lactis* ssp. *cremoris* 303 or *Lc. lactis* ssp. *cremoris* AM2. The rennet was added when acidity reached 0.20–0.22%. After cutting the curd/whey the mixture was stirred and cooked (0.2 °C/min to 40 °C). Curds were pitched and after the whey was removed the curds were cheddared until the acidity of whey draining reached 0.68–0.85%. After milling the curd was salted at a rate of 2% (w/w), pressed (75 kPa, 16 h), vacuum wrapped and stored at 8 °C. The first sampling was carried out on the day of the beginning of the processing (0. day) after salting and before pressing. The next sample was taken after pressing (1. day), and the following ones were taken during ripening on the 7^{th}, 28^{th} and 63^{rd} days. Samples were grated and stored at −24 °C after lyophilization. Cheeses were made at the Faculty of Food Science and Technology, strains were obtained from the culture bank of the Department of Microbiology, University College Cork (Ireland).

2.2 Chemical analysis

Lyophilized cheese samples were pulverized with a Microculatti grinder. 5 g of sample was weighed into a 100 ml Erlenmeyer flask and 20 ml of 0.1 M HCl was added. The suspensoid was stirred for 3 hours with a magnetic stirrer then it was left to steep overnight at 5 °C. The following day the two-phase system was shaken up again, and then centrifuged at 500 g for 10 minutes. Protein was precipitated from the supernatant with equal volume of 25% (w/v) trichloroacetic acid solution with the final concentration of trichlorocetic acid of 12.5%. The suspensoid was centrifuged (500 g, 10 min) after 30-minute standing. Then 4 ml from the supernatant were placed in a 10 ml volumetric flask and the solution was neutralized with 4 M NaOH solution following dilution with distilled water. The extract was filtered through a 0.45 μm membrane filter before analysis.

During precolumn derivatization with OPA (*o*-phthaldialdehyde) and TATG (1-thio-β-D-glucose tetraacetate) (Sigma, St. Louis, MO, USA) diastereoisomer pairs of the amino acids were produced [5, 6]. Derivatization and analysis were carried out with a MERCK-Hitachi HPLC comprised of a L-7250 pro-

grammable autosampler, L-7100 pump, L-7350 column thermostat, L-7480 fluorescence detector, and AIA data conversion utility for the D-7000 HPLC system manager. The compounds were separated on a 125 mm×4 mm i.d. column packed with Superspher 60 RP-8e (MERCK, Darmstadt, Germany). The initial mobile phase composition was 28% (v/v) methanol and 72% phosphate buffer (50 mM, pH=7). After ten-minute isocratic elution the ratio of acetonitril was increased from 0 to 17% and the volume of phosphate buffer decreased from 72% to 55%. From the 40th minute the volume of acetonitril was increased from 17 to 40% while the ratio of the phosphate buffer changed from 55 to 36% and that of methanol from 28 to 24%. The flow rate was 1 ml/min, and the oven temperature was 40 °C. Solvents (acetonitrile and methanol) were HPLC gradient grade (MERCK, Darmstadt, Germany). The derivatives were detected with a fluorescence detector (λ_{ex} 325 nm, λ_{em} 420 nm).

2.3 Statistical analysis

Cheese manufacture was repeated three times with both strains (*Lc. lactis* ssp. *cremoris* 303 and *Lc. lactis* ssp. *cremoris* AM2). The influence of processing steps and the starter culture selection on the D-amino acid content of the products was evaluated with multiple analysis of variance. Besides the concentration of free D-amino acids, the D-amino acid composition $\left(\frac{\text{D-aminoacid}}{\sum \text{D-aminoacid}} \times 100\right)$ and the percentage of the D-enantiomer $\left(\frac{\text{D}}{\text{D+L}} \times 100\right)$ were calculated and regarded as variables.

The equation of the used linear model was the following:

$$\boldsymbol{Y_{ijk} = \mu + B_i + C_j + BC_{ij} + e_{ijk}}$$

Where:

Y_{ijk}	=	the k^{th} observation in the ij^{th} treatment combination,
μ	=	the least squares mean,
B_i	=	the effect of the i^{th} class of factor B (manufacturing step) expressed as a deviation from μ,
C_j	=	the effect of the j^{th} class of factor C (strain) expressed as a deviation from μ,
BC_{ij}	=	the interaction effect of the i^{th} class of factor B and the j^{th} class of factor C expressed as a deviation from $\mu + B_i + C_j$ and
e_{ijk}	=	the random error associated with the k^{th} observation in the ij^{th} treatment combination.

In order to compare the influence of the cheesemaking steps on the of D-amino acid content within one processing protocol the manufactures with two different strains were separately evaluated with one way analysis of variance. In these particular cases the equation of the linear model was:

$$\boldsymbol{Y_{ij} = \mu + B_i + e_{ij}}$$

Where:

Y_{ij}	=	the j^{th} observation in the i^{th} treatment,
μ	=	the least squares mean,
B_i	=	the effect of the i^{th} class of factor B (manufacturing step) expressed as a deviation from μ,
e_{ij}	=	the random error associated with the j^{th} observation in the i^{th} treatment.

In case of significant difference among treatment means ($P<0.05$) the comparison of that was accomplished with the Student-Newman-Keuls test. Data analysis was carried out with the use of SPSS for Windows 10.0 (1999) statistical program.

3 Results

Firstly the D-amino acid content of the semi-finished Cheddar cheeses was evaluated separately depending on the type of the starter (*Table 1,* part A and B). The D-Ala values at the beginning of the manufacture (day 0 and 1) were very close to the concentration that was determined in yoghurt and fresh cheese [2]. The amount of D-Ala showed a continuous increase during processing, especially in the ripening period. Though, the extent of this increment was not outstanding. By the time of the last sampling (9^{th} week of ripening) it did not reach the values that were detected in ripened cheeses [3]. Probably the length of the ripening period was not enough for the accelerated release of this D-amino acid.

The D-Asp and the D-Glu content of curd slightly increased during pressing. As values were counted on the basis of dry material this change cannot be attributed to the decrease of the water content. During pressing there is an acceleration of growth of the cell count [12] and the number of dead starter cells is theoretically low. The value of the pressure applied to form the curd into a shape was four orders of magnitude lower than pressure values used in order to reduce the cell count [10]. However, it cannot be excluded that more

traits together such as pressing and the increase of salt content and that of osmotic pressure may induce the destruction of certain cells, or exert some stress on microorganisms which resulted in the production of more D-Asp and D-Glu.

Table 1: The D-alanine, D-aspartic acid and D-glutamic acid content of semi-finished Cheddar cheese in the function of the stage of manufacture and the used starter strain (mg/100 g dry matter) (n=3)

A. *Lactococcus lactis* subsp. *cremoris* 303

Examined amino acid	Elapsed time from the beginning of the manufact. (days)				
	0	1	7	28	63
	Stage of manufacture				
	Before pressing	After pressing	Ripening	Ripening	Ripening
D-Ala	$1.8^{a}\pm0.8$	$2.4^{ab}\pm0.5$	$3.1^{abc}\pm0.9$	$3.8^{bc}\pm0.8$	$4.5^{c}\pm0.6$
D-Asp	$0.98^{a}\pm0.33$	$1.9^{b}\pm0.4$	$2.2^{b}\pm0.6$	$2.2^{b}\pm0.5$	$2.4^{b}\pm0.4$
D-Glu	$4.1^{a}\pm0.5$	$6.0^{b}\pm0.4$	$6.6^{b}\pm1.1$	$6.4^{b}\pm0.8$	$6.4^{b}\pm0.5$

B. *Lactococcus lactis* subsp. *cremoris* AM2

Examined amino acid	Elapsed time from the beginning of the manufact. (days)				
	0	1	7	28	63
	Stage of manufacture				
	Before pressing	After pressing	Ripening	Ripening	Ripening
D-Ala	$1.0^{a}\pm0.7$	$1.2^{a}\pm0.6$	$1.7^{b}\pm0.7$	$2.0^{b}\pm1.0$	$2.3^{c}\pm0.9$
D-Asp	$0.59^{a}\pm0.31$	$1.1^{b}\pm0.7$	$1.4^{b}\pm0.7$	$1.5^{b}\pm0.9$	$1.5^{b}\pm0.8$
D-Glu	$2.7^{a}\pm1.4$	$3.6^{a}\pm2.4$	$4.3^{a}\pm2.3$	$4.2^{a}\pm3.1$	$4.1^{a}\pm2.3$

^{abc} Averages in one row with common superscript do not differ ($P\geq0.05$).

Though these two amino acids are present in the cell wall [11, 13], their concentration did not change significantly during the maturation of Cheddar until the 9^{th} week. It is in agreement with the findings of others [8, 9], who stated that D-Ala is the first D-amino acid whose amount began to increase due to bacterial activity while the amount of D-Asp and D-Glu remained unchanged.

The six manufacturing processes with the two different strains were also

evaluated together with multiple analyses of variance. In this case, with the exception of D-Ala, the influence of the processing steps has less effect on the D-amino acid content than the selection of the starter culture. The choice of strains exerted a significant effect on the D-Ala, D-Asp and D-Glu content ($P<0.01$) of the semi-finished products *(Table 2)*. Cheeses inoculated with *Lc. lactis* ssp. *cremoris* 303 contained more free D-amino acids than the ones acidified with *Lc. lactis* ssp. *cremoris* AM2. This result can be connected with the difference between strains in the D-amino acid formation capacity. Supposedly their susceptibility to autolysis is different, but this hypothesis could be accepted or rejected with the knowledge of the activity of intracellular enzymes.

Table 2: The influence of the stage of manufacture and the selection of starter culture strain on the D-alanine, D-aspartic acid and D-glutamic acid content and the D-amino acid composition of semi-finished Cheddar cheese

Factors	D-AA concentration (mg/100 g dry matter)				D-AA composition (D-AA/∑D-AA) (%)		
	D-Ala	D-Asp	D-Glu	∑D-AA	D-Ala	D-Asp	D-Glu
Manufacturing steps (B)	**	*	NS	NS	**	* * *	* * *
Strain (C)	* * *	**	* * *	**	NS	NS	NS
Interaction B x C)	NS	NS	NS	NS	NS	NS	NS

*P<0.05, **P<0.01, ***P<0.001

The D-amino acid composition continuously changed during cheese manufacture. The ratio of D-Asp was slightly higher after pressing than prior to pressurization. During ripening the ratio of D-Ala increased, and that of D-Glu decreased and the proportion of D-Asp remained unchanged *(Table 3)*. Despite of the processing steps, the choice of the starter strain did not exert an effect on the D-amino acid pattern of the products *(Table 2)*.

The percentage ratio of the D-enantiomer within a given free amino acid content was also calculated *(Table 3)*. The ratio of D-Ala practically did not change during ripening because the concentration of both enantiomers increased at a similar rate and the ratio of the enantiomers was approximately 50–50 per cent. While the release of D-Ala can be associated with the lysis of bacteria the appearance of free L-Ala is connected with the enzymatic proteolysis of para-casein owning to starter proteinases and peptidases hydrolyzing

peptides to lower molecular weight peptides and free amino acids. The increase of the concentration of D-Ala followed the liberation of L-Ala originated from milk proteins. Due to this tendency the question of the origin of free D-Ala may arize. Some part of it could be originated from the cell wall, but besides this it may be formed from free L-Ala if the bacterial alanine racemase could operate outside the bacteria. If it were possible the L-D conversion could accelerate during ripening. With the greater extent of bacterial lysis the activity of the released racemase may increase, on the other hand the amount of its substrate (free L-Ala) also increases due to hydrolysis of milk proteins.

Table 3: The free D-amino acid composition and the percentage ratio of the D-enantiomer of free amino acids in Cheddar cheese during manufacture (mg/100 g dry matter) (n=6)

D-AA composition (%)	**Elapsed time from the beginning of the manufact. (days)**				
	0	**1**	**7**	**28**	**63**
	Stage of manufacture				
	Before pressing	**After pressing**	**Ripening**	**Ripening**	**Ripening**
D-Ala/$\sum$D-AA	$24^{ab}\pm5$	$23^{a}\pm3$	$25^{ab}\pm3$	$29^{bc}\pm3$	$32^{c}\pm3$
D-Asp/$\sum$D-AA	$14^{a}\pm1$	$18^{b}\pm1$	$18^{b}\pm1$	$19^{b}\pm1$	$18^{b}\pm1$
D-Glu/$\sum$D-AA	$62^{c}\pm5$	$59^{c}\pm3$	$57^{bc}\pm3$	$53^{ab}\pm3$	$50^{a}\pm3$
D-enantiomer ratio (D/D+L)×100 (%)	**Elapsed time from the beginning of the manufact. (days)**				
	0	**1**	**7**	**28**	**63**
	Stage of manufacture				
	Before pressing	**After pressing**	**Ripening**	**Ripening**	**Ripening**
Ala	$54^{b}\pm15$	$47^{a}\pm13$	$48^{a}\pm13$	$45^{a}\pm13$	$44^{a}\pm13$
Asp	$28^{a}\pm8$	$36^{b}\pm7$	$39^{b}\pm7$	$38^{b}\pm6$	$36^{b}\pm7$
Glu	$25^{a}\pm14$	$26^{a}\pm7$	$52^{b}\pm15$	$82^{c}\pm4$	$81^{c}\pm2$

^{abc} Averages in one row with common superscript do not differ ($P \geq 0.05$).

The ratio of the D-enantiomers in the free amino acid pool was higher ($P<0.01$) in Cheddar cheeses inoculated with *Lc. lactis* ssp. *cremoris* 303 than in cheeses acidified with *Lc. lactis* ssp. *cremoris* AM2 because the D-amino acid content was higher in the first case, but the amount of the released L-enantiomer did not differ significantly among strains.

4 Acknowledgements

The cheese samples were produced at the Faculty of Food Science and Technology, University College Cork. The professional help of Prof. Dr. Patrick F. Fox, Dr. Paul McSweeney and Baukje Folkertsma is gratefully acknowledged. Authors are grateful to the Sapientia Foundation, Institute of Research Programs for the financial support.

References

[1] E. Adams, *Racemases and epimerases,* In: The enzymes, P.D. Boyer (ed) Academic Press, New York, 1972. 479.

[2] H. Brückner, M. Hausch, D-amino acids in dairy products: detection, origin and nutritional aspects. I. Milk, fermented milk, fresh cheese and acid curd cheese, *Milchwissenschaft,* 6 (1990) 357–360.

[3] H. Brückner, M. Hausch, D-amino acids in dairy products: detection, origin and nutritional aspects. II. Ripened cheeses, *Milchwissenschaft,* 7 (1990) 421–425.

[4] H. Brückner, P. Jaek, M. Langer, H. Godel, Liquid chromatographic determination of D-amino acids in cheese and cow milk. Implication of starter cultures, amino acid racemases, and rumen microorganisms on formation, and nutrirional considerations, *Amino Acids,* 2 (1992) 271–287.

[5] J. Csapó, Zs. Csapó-Kiss, J. Stefler, T.G. Martin, S. Némethy, Influence of mastitis on D-amino acid content of milk, *J. Dairy Sci.,* 78 (1995) 2375–2381.

[6] S. Einarsson, S. Folestad, B. Josefsson, Separation of amino acid enantiomers using precolumn derivatization with o-phtaldialdehyde and 2,3,4,6,-tetra-O-acetil-1-thio-β-glucopyranoside, *J. Liquid Chrom.,* 10 (1987) 1589–1598.

[7] M. Friedman, Chemistry, nutrition, and microbiology of D-amino acids, *J. Agric. Food Chem.,* 47 (1999) 3457–3479.

[8] I. Gandolfi, G. Palla, L. Delprato, F. De Nisco, R. Marchelli, C. Salvadori, D-amino acids in milk as related to heat treatments and bacterial activity, *J. Food Sci.,* 2 (1992) 377–379.

[9] I. Gandolfi, G. Palla, R. Marchelli, A. Dossena, S. Puelli, C. Salvadori, D-alanine in fruit juices: a molecular marker of bacterial activity, heat treatments and shelf-life, *J. Food Sci.,* 1 (1994) 152–154.

[10] K. Koncz, L. Mészáros, J. Farkas, K. Pásztorné Huszár, R. Helt, N. Lechner, A nyers tej kezelése nagy hidrosztatikus nyomással, *Tejgazdaság,* 1 (2003) 10–14.

[11] K.H. Schleifer, O. Kandler, Peptidoglycan types of bacterial cell walls and their taxonomic implications, *Bact. Rev.,* 4 (1972) 407–477.

[12] R. Scott, *Bacteriology in relation to cheesemaking,* In: Cheesemaking practice, R. Scott (ed) Aspen Publishers Inc., Gaithersburg, Maryland, 1998. 66–68.

[13] D.J. Tipper, A. Wright, *The structure and biosynthesis of bacterial cell walls,* In: The bacteria, J.R. Sokatch, L.N. Ornston (eds) Academic Press, New York, 1979. 291–426.

[14] M.G. Wilkinson, T.P. Guinee, D.M. O'Callaghan, P.F. Fox, Effect of cooking temperature on the autolysis of starter, Lactococcus lactis subsp. cremoris AM2, and the maturation of Cheddar cheese, *Milchwissenschaft,* 7 (1995) 376–380.

Received: August, 2008

Acta Univ. Sapientiae, Alimentaria, **2**, 1 (2009) 65–79

The influence of extrusion on loss of and racemization of amino acids

É. Varga-Visi[1]
email: visi@ke.hu

K. Lóki[1]
email: loki.katalin@ke.hu

Cs. Albert[2]
email:
albertcsilla@sapientia.siculorum.ro

J. Csapó[1,2]
email: csapo.janos@ke.hu

[1]University of Kaposvár,
Faculty of Animal Science,
Guba S. u. 40, 7400 Kaposvár, Hungary

[2]Sapientia–Hungarian University of Transylvania,
Csíkszereda Campus, RO-530104, Libertatii 1., Miercurea-Ciuc

Abstract. The influence of the operation conditions (temperature and residence time) of a thermic treatment on the total amount (free and protein-bound) of amino acid enantiomers of dry fullfat soya was investigated. Total amino acid content was determined using conventional ion-exchange amino acid analysis of total hydrolyzates and chiral amino acid analysis was performed with HPLC after precolumn derivatization with *o*-phthaldialdehyde and 1-thio-β-D-glucose tetraacetate. Contrary to corn that was investigated previously notable racemization was detected even at lower temperatures. At 140 °C the ratio of the D-enantiomer was 0.87% for glutamic acid, 2.81% for serine and 1.92% for phenylalanine, at 220 °C the ratio of the D-enantiomer in the case of the above amino acids was 1.43%, 4.61% and 4.68%, respectively. The concentration of several L-amino acids decreased. At 220 °C there was 10% less L-glutamic acid,

Key words and phrases: racemization, D-amino acid, fullfat soybeans, extrusion temperature, residence time

17% less L-serine, 5% less L-phenylalanine, 6.6% less L-aspartic acid and 21% less L-lysine than in the control and their loss can be assigned to the L – D conversion in a different degree. While transformation of L-phenylalanine nearly completely can be attributed to the racemization, the main cause of the loss of L-lysine is not racemization. The treatments in the same order of magnitude resulted in the formation of more D-amino acids and greater extent of racemization of amino acids in fullfat soya than that of maize.

1 Introduction

Epimerization (partial racemization) of protein-bound and free amino acids may occur during the processing of food if the operation conditions involve application of heating and/or alkaline conditions [11, 12, 32]. Heating in alkaline medium has been shown to yield significant amount of D-amino acids through the mechanism of base-catalyzed racemization [10, 18, 19, 21]. Albeit the medium is usually neutral or weakly acidic during food processing, reducing sugars can induce partial racemization of free L-amino acids in the course of the Maillard reaction [3, 8], and D-amino acids can also be formed via the mechanism of acid-catalyzed racemization [9, 18]. Epimerization of L-aspartic acid can also occur through transpeptidation reactions [14].

The digestibility of the proteins decreases when significant ratio of the protein-bound amino acids is in the D-configuration due to the stereospecificity of the proteinases and peptidases [11, 12, 31]. The rate of absorption can be discriminative to D-amino acids [25, 29] and the bioavailability due to the restricted efficiency of D-amino acid oxidase system can be diminished [22, 24]. The activity of D-amino acid oxidases depends on several factors (species, age, organ, tissue, substrate) and there is a big variation in the efficiency of utilization of the D-amino acids among species [12]. In the case of mammals only small ratio of the D-amino acids were utilized following oral consumption and the D-stereoisomers of the essential amino acids in some cases caused growth inhibition and were mainly excreted in the urine [22]. The value of relative oral bioavailability (RBV) for D-Met is only 30% for humans [1]. From a nutritional standpoint, racemization could result in the loss of protein that is one of the most valuable components of the food.

On the other hand the oral consumption of D-serine, lysinoalanine [4, 15, 16] and D-proline [17] have been claimed to induce histological changes in the rat kidney while others found no sign of organic disorders in the case of D-proline and D-aspartic acid [28]. Recently it has been shown that D-

amino acids that are present in the different organs and tissues of animals and humans have specific biological functions. In the central nervous system, D-serine and D-aspartate occur in considerable concentrations [13]. D-serine is synthesized and metabolized endogenously by human serine racemase [23] and the same might account for D-aspartate. Some part of D-serine in brain can be originated from exogenous source through the blood-to-brain transfer [2].

The food industry is nowadays aware of the potential risk of the treatment of proteinaceous food, and the aim is to define conditions when the aim of the treatment is completed without significant change in the structure of the biological valuable components.

During thermic processing the integrity of food components is disrupted due to the effect of heat and pressure and a spongoid structure is formed. Heat sensitive antinutritive factors are totally or partially inactivated, and the number of microorganisms is also diminished. The task is to determine the conditions of good manufacturing practice in which the above-mentioned aims are accomplished without significant loss of amino acids. The decrease of the amino acid content in corn grain due to extrusion has been investigated, but in these studies the ratio of the enantiomers was not determined [26, 27]. In our previous experiment the influence of extrusion conditions on the D-amino acid content of corn was investigated [30]. In the present work the thermic treatment of an important vegetal protein source is investigated and the results are compared.

2 Materials and methods

2.1 Extrusion

The raw material of the extrusion was fullfat soya (Glycine max (L.) Merr., 'Borostyán' variety). The basic chemical composition was as follows: the dry matter content was 98.1% and the ash content 4.5% (g/100 g sample). Fullfat soya consisted of 33.7% crude protein, 22.9% crude fat; 3.4% crude fibre and 33.6% nitrogen-free extractable material. The starch content was 5.4% and the total sugar content 8.9%. The amino acid content of fullfat soya and the amino acid composition of its proteins can be seen in *Table 1.*

Table 1: The amino acid content and the amino acid composition of untreated fullfat soybean grain

Amino acid	AA content (g AA/ 100 g sample)	AA composition (g AA/ 100 g protein)
Asp	3.83	11.6
Thr	1.25	3.8
Ser	1.77	5.3
Glu	6.39	19.3
Pro	1.73	5.2
Gly	1.57	4.7
Ala	1.51	4.6
Cys	0.38	1.1
Val	1.43	4.3
Met	0.50	1.5
Ile	0.96	2.9
Leu	2.57	7.8
Tyr	1.36	4.1
Phe	1.64	4.9
Lys	2.26	6.8
His	1.10	3.3
Arg	2.47	7.5
NH_3	0.42	1.3

The raw material was ground with a hammer grinder and the particle size distribution was determined. Due to the high oil content conditioning was not necessary prior to extrusion. Ten kg of material was used for each trial. Extrusion was carried out using a Do-Corder DC 2001 type Brabender machine equipped with a 19 mm i.d. barrel (21:1 length to diameter ratio); a screw with the length of 400 mm with increasing screw diameter from 12 to 17 mm, and a cylindrical die which consists of two parts: a 55 mm long by 8 mm i. d. following a 22 mm long by 5 mm. The barrel and the die were heated by electrically controlled split ring resistance heaters, and the screw speed was also kept under control. The barrel and the die temperatures were monitored by thermocouples mounted in shallow wells. Extrusion trials with the full

cross-classification of the applied nominal temperature and screw speed levels *(Table 2)* were repeated three times on three different days. From the two reported zone temperatures (T_1, T_2), one value was calculated (T) to characterize the effect of temperature. Minimum residence time was determined by introducing a small amount of dye into the feeding port and measuring the time required for the first colored extrudate to exit the die. Prior to sampling, the machine was allowed to equilibrate to the desired temperature, then appr. 200 g sample was collected and allowed to cool down before being homogenized, and sealed in polyethylene bags and stored at −20 °C. Control samples were taken from each batch and treated in the same way as extruded samples.

Table 2: Nominal and measured properties of extrusion of fullfat soya

Levels	$\mathbf{T_{nom}}$ **(°C)**	$\mathbf{T_{meas}}$ **average±s.d. (°C)(n=12)**	**Screw speed (s^{-1})**	**Residence time (s) average±s.d. (n=12)**	**Throughput (kg/h) average±s.d. (n=12)**
1	100	101±4	50	29±0.2	1.6±0.4
2	140	140±3	90	17±0.2	2.8±0.8
3	180	180±3	130	12±0.8	4.1±1.1
4	220	220±3	170	10±1.4	4.8±1.4

T_{nom} = Nominal temperature
T_{meas} = Measured temperature

2.2 Chemical analysis

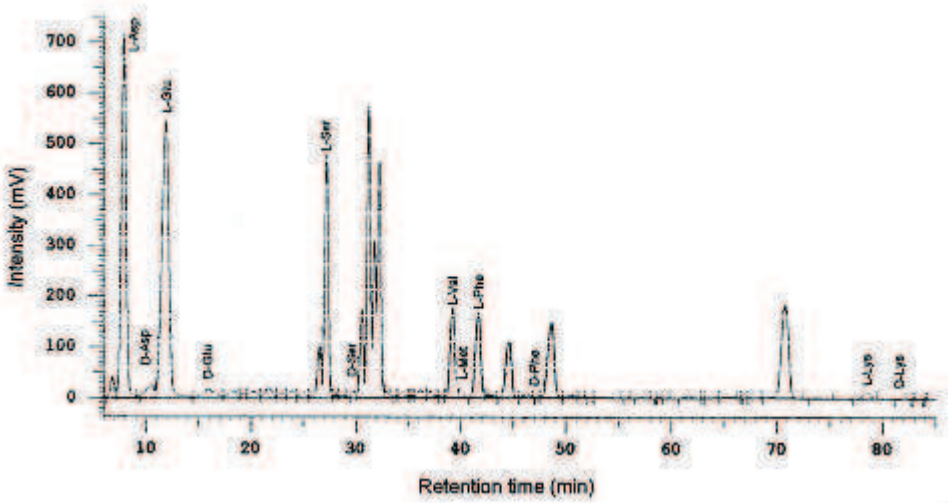
The moisture content was determined with the standard procedure of MSZ ISO 1442, the crude protein content measurement based on the basic method of Kjeldahl (MSZ EN ISO 5983-1:2005). Crude fat (MSZ 6369-15:1982), total ash (MSZ ISO 749:1992), crude fibre (MSZ ISO 6865:2000), starch (MSZ 6830-18:1988) and total sugar content examinations (MSZ 6830-26:1987) were carried out with the use of standard procedures approved by the Hungarian Standards Institution.

Prior to amino acid analysis the samples were dissolved in hydrochloric acid (6 M; 5 cm^3) and proteins were hydrolyzed at 105±1 °C for 24 h. The amino acid content and composition was determined with an INGOS AAA 400 amino acid analyzer (INGOS, Praha, Czech Republic) equipped with a 35×0.37 cm

column packed with OSTION Lg ANB.

The concentration of the amino acid enantiomers was also determined from the total hydrolizate of the samples. After cooling, the pH was set to pH = 7 with sodium hydroxide solution, then diastereomers were produced with OPA (*o*-phthaldialdehyde) and TATG (1-thio-β-D-glucose tetraacetate) (Sigma, St. Louis, MO, USA) during precolumn derivatization and separated with HPLC following detection with a fluorescence detector as described elsewhere [5, 7, 30]. A typical chromatogram of the derivatives of the examined L- and D-amino acids of soybean can be seen in *Figure 1.* Before the analysis of soybean samples standard solutions of D- and L-amino acids were derivatized and analyzed, and calibration curves were established and response factors were calculated for each analyzed component. The amount of the amino acid enantiomers of the samples was calculated based on these calibration curves. Moreover, the concentration of the enantiomers was determined after the hydrolysis of the total amount of samples thus the sum of free and protein-bound amino acids was determined.

Figure 1: The chromatogram of the OPA/TATG derivatives of the amino acid enantiomers obtained from hydrolyzed fullfat soya proteins



See conditions in the text.

2.3 Statistical analysis

Data analysis was carried out with the use of SPSS for Windows 10.0 (1999) statistical program (Statistical Package for Social Sciences). There were four levels of temperature factor and four levels of screw speed factor. The number of replication was three; sampling was repeated on three different days with the full cross-classification of the applied levels of factors. The extent of racemization was defined as the percentage of the D-enantiomer within the total (D+L) amino acid content (D/(D+L)×100) [12]. In the above formula 'D' and 'L' means the concentration of the D- and L-enantiomer of the given amino acid calculated with the use of the relevant response factors. The influence of temperature and residence time on the D-amino acid content and the degree of racemization were evaluated with multiple analysis of variance. The equation of the used linear model was the following:

$$\boldsymbol{Y_{ijk} = \mu + T_i + F_j + TF_{ij} + e_{ijk}}$$

Where:

Y_{ijk}	=	the k^{th} observation in the ij^{th} treatment combination,
μ	=	the least squares mean,
T_i	=	the effect of the i^{th} class of factor T (temperature) expressed as a deviation from μ,
F_j	=	the effect of the j^{th} class of factor F (screw speed) expressed as a deviation from μ,
TF_{ij}	=	the interaction effect of the i^{th} class of factor T and the j^{th} class of factor F expressed as a deviation from $\mu + T_i + F_j$ and
e_{ijk}	=	the random error associated with the k^{th} observation in the ij^{th} treatment combination.

If the treatment means differed significantly ($P<0.05$), the comparison of that was accomplished with the Student-Newman-Keuls test.

3 Results

3.1 The influence of extrusion on the D-amino acid content of fullfat soya

Similarly to extrusion of corn residence time did not change when the same screw speed was used at different temperatures [30]. Thus residence time and screw speed could be regarded as traits substituting each other without con-

founding with temperature when their influence on the D-amino acid formation was analyzed *(Table 2).*

In order to determine the D-amino acid producing capacity of extrusion, the measured D-amino acid values of treated samples have to be corrected with the D-amino acid content of control samples [31] because some formation of D-amino acids during acidic hydrolysis of proteins occurs [6, 21]. There were significant differences in the D-glutamic acid, D-serine and D-phenylalanine content of soybean treated at different temperatures ($P<0.05$). The amount of these amino acids showed a notable increase when the extrusion temperature was increased from 101 to 140 °C *(Table 3),* and their concentration was higher in the products extruded at 140 °C than in control samples without extrusion. The variance of D-aspartic acid content was higher than that of the other amino acids and thus significant differences cannot be detected. In the case of corn samples the extrusion on the same instrument below 144°C of 28–72 s did not induce significant ($P<0.05$) racemization [30].

Table 3: Influence of the extrusion temperature on the total (free and protein-bound) D-amino acid content of fullfat soya (mg/100 g dry matter)

Average ± s.d.[1,2] (n=12)	**Temperature (T)**			
	101 °C	**140 °C**	**180 °C**	**220 °C**
D-Glu	$40^{a}\pm9$	$57^{b}\pm13$	$65^{b}\pm24$	$89^{c}\pm20$
D-Ser	$29^{a}\pm27$	$49^{b}\pm20$	$67^{c}\pm12$	$74^{c}\pm12$
D-Phe	$5^{a}\pm23$	$27^{b}\pm16$	$42^{b}\pm18$	$66^{c}\pm10$
D-Asp	$29^{a}\pm17$	$20^{a}\pm56$	$47^{a}\pm92$	$83^{a}\pm106$

[abc] Averages in one row with common superscript do not differ ($P\geq0.05$).
[1] Corrected with control values obtained from untreated fullfat soya.
[2] Averages and standard deviations of samples extruded at the same temperature with different residence times.

The influence of the screw speed (residence time) on the D-amino acid content was not significant at the investigated range.

High-temperature treatments related to the low-temperature treatments resulted in more significant increase of the extent of racemization ($D/(D+L)\times100$, *Table 4)* than that of the amount of D-amino acids *(Table 3)* because the L-amino acid concentration decrease *(Table 5)* exceeded the D-amino acid concentration increase. Namely besides izomerization the intensity of other pro-

cesses that alter the structure of the amino acids was also significant. In the case of corn the loss of aspartic acid and lysine was reported during high temperature extrusion [26, 27, 30].

Table 4: Influence of the extrusion temperature on the degree of partial racemization of the examined amino acids in corn (D/(D+L)×100)

Average ± s.d.[1,2] (n=12)	Temperature (T)			
	101 °C	140 °C	180 °C	220 °C
Glu	0.57^{a}±0.13	0.87^{b}±0.18	0.99^{b}±0.38	1.43^{c}±0.36
Ser	1.51^{a}±1.41	2.81^{b}±1.04	3.90^{c}±0.70	4.61^{c}±0.69
Phe	0.23^{a}±1.56	1.92^{b}±1.18	2.88^{b}±1.17	4.68^{c}±0.72
Asp	0.69^{a}±0.40	0.44^{a}±1.35	1.06^{a}±2.15	1.93^{a}±2.46

[abc] Averages in one row with common superscript do not differ (P≥0.05).
[1] Corrected with control values obtained from untreated fullfat soya.
[2] Averages and standard deviations of samples extruded at the same temperature with different residence times.

Table 5: The total (free and protein-bound) L-amino acid content of soybean treated at different temperatures (g/100 g dry matter)

Average ± s.d.[1] (n=12)	Control	Temperature (T)			
		101 °C	140 °C	180 °C	220 °C
L-Asp	3.70^{b}±0.17	3.77^{b}±0.39	3.59^{ab}±0.21	3.56^{bc}±0.15	3.38^{a}±0.28
L-Glu	6.74^{b}±0.26	6.73^{b}±0.52	6.41^{bc}±0.42	6.43^{bc}±0.35	6.05^{a}±0.53
L-Ser	1.83^{c}±0.06	1.82^{c}±0.15	1.69^{b}±0.10	1.66^{b}±0.06	1.52^{a}±0.10
L-Val	1.53^{bc}±0.06	1.57^{c}±0.08	1.50^{bc}±0.09	1.45^{bc}±0.07	1.38^{a}±0.13
L-Met	0.54^{a}±0.01	0.55^{a}±0.07	0.55^{a}±0.05	0.56^{a}±0.03	0.55^{a}±0.04
L-Phe	1.40^{bc}±0.04	1.46^{c}±0.10	1.37^{bc}±0.16	1.41^{bc}±0.06	1.34^{bc}±0.08
L-Lys	2.39^{b}±0.39	2.10^{b}±0.30	1.96^{a}±0.19	1.99^{a}±0.22	1.87^{a}±0.23

[abc] Averages in one row with common superscript do not differ (P≥0.05).
[1] Averages and standard deviations of samples extruded at the same temperature with different residence times.

The concentration of most of the L-amino acids under the scope of the study decreased when the temperature of the heat treatment increased *(Table 5)*. The sample extruded at the highest temperature contained 6.6% less L-aspartic acid than the control. The amount of the formed D-enantiomer

(0.08 g/100 g) was account for 25% of the difference (0.32 g/100 g). In the case of L-glutamic acid and L-serine there was a concentration decrease of 10% and 17%, respectively, and 13% (L-serine: 24%) of the loss can be attributed to formation of the D-enantiomers. The degree of the decomposition of L-phenylalanine (5%) was almost the same as the extent of the formation of the D-enantiomer. The highest concentration decrease was detected in the case of L-lysine (21%). Since the degree of racemization of lysine was less than 2%, it can be account for not more than 8% of the concentration decrease of L-lysine (0.52 mg/100 g). Similarly like in the case of corn the main cause of the loss of L-lysine is not racemization.

3.2 Comparison of the effect of extrusion on soya and corn with respect to the formation of D-amino acids

Thermic treatment of fullfat soya resulted in higher amount of D-amino acids related to dry matter than in the case of corn. It can be attributed to the fact that the protein content of fullfat soya is about four-fold higher than that of corn. Contrary to D-amino acid content, the D/(D+L)×100 ratio does not depend on the absolute amount of protein. As the ratio of the amino acids that are most susceptible to racemization (that is serine, glutamic and aspartic acid) is similar in soya and in corn proteins, their common transformation can be investigated. The degree of partial racemization of these amino acids in soya extruded at 180 °C was slightly higher than that of corn at 200 °C. Thus similar heat treatment seems to cause a higher extent of L–D amino acid conversion in the soya proteins than in corn proteins.

The influence of the screw speed (residence time) on the D-amino acid content and racemization was not significant in the case of either of the raw material. It can be attributed to the fact that in the function of screw speed there was only three-fold change of the residence time. 10°C temperature increase resulted in 2.2–5.5-fold increase in the first order reaction rate constant (k) of amino acid racemization [12]. Due to the relationship of reaction time (t) and 'k' in the first order reaction kinetic equitation of racemization, three-fold residence time increase exert about the same effect on the D-amino acid content than 10 °C temperature increase. Therefore within the examined temperature and time intervals, the change in the treatment temperature has only visible effect on the racemization stage of the proteins and the D-amino acid content of products.

3.3 Comparison of the effect of extrusion on soya and corn with respect to the loss of L-amino acids

The rate of contribution of racemization and the other processes to the loss of L-amino acids seems to depend both on the sort of the amino acid and the type of the protein source. In the case of soya the 'non-racemization loss' of L-aspartic acid, L-glutamic acid, L-serine and L-lysine related to the whole concentration decrease was 75, 87, 76 and 92%, respectively. In the case of corn 22% of the loss of L-Asp and 98% of the concentration decrease of L-Lys was not related with racemization only with other processes. Due to heat treatment alteration of side chains of the amino acids and crosslink formation can occur, e.g. serine (after β-elimination as dehydroalanine) and lysine can form lysinoalanine, the side chain of asparagine and glutamine can form imide-type crosslink with lysine, the carboxyl group of acidic amino acids esterify the hydroxyl group of serine. Furthermore, the loss of lysine can also be attributed to the reaction of the ϵ-amino group with reducing sugars in the Maillard reaction. In contrast with the above four amino acids the degree of the concentration decrease of L-phenylalanine was practically the same as the amount of D-enantiomer formed, namely there was no significant concentration decrease due to other processes than racemization.

Both in soya and in corn the decomposition of L-lysine was the highest among amino acids. The loss of L-amino acids was more significant in case of soya than that of corn. High temperature (200 °C) extrusion of corn reduced the amount of L-lysine and L-aspartic acid, while in soya significant decrease of the following other amino acids were also detected such as L-serine, L-glutamic acid and L-phenylalanine. The ratio of lysine within the soya protein (6.8%) was almost three-fold higher than that of corn protein (2.5%) and the main cause of the loss of L-lysine was not the racemization but other processes. Supposedly the greater amount of lysine in the soya protein can form more crosslinks with serine and the acidic amino acids than in corn protein and thus the L-amino acid loss could be higher in the case of soya. This hypothesis is supported by the fact that contrary to corn when racemization is the main cause of the loss of L-aspartic acid (78%), the concentration decrease detected in soya can be attributed to a lesser extent to racemization (25%) than other reactions (75%).

In sum dry extrusion of fullfat soybeans can result in significant loss of the amino acids. Within the decomposition the ratio of racemization and that of the other processes was evaluated. Among essential amino acids the concentration decrease of lysine was the most significant (21%). In nutritional point

of view one can avoid drawing considerable consequences because analytical results only gave gross values and utilization of amino acids depend on several factors. This study pointed out the need of conducting biological tests in order to estimate the possible loss of the bioavailability of amino acids of fullfat soybean due to dry extrusion at different species.

References

[1] D.H. Baker, Comparative species utilization and toxicity of sulfur amino acids, *J. Nutr.,* 136 (2006) 1670–1675.

[2] D. Bauer, K. Hamacher, S. Broer, D. Pauleit, C. Palm, K. Zilles, H.H. Coenen, K.J. Langen, Preferred stereoselective brain uptake of D-serine – a modulator of glutamatergic neurotransmission, *Nucl. Med. Biol.,* 32 (2005) 793–797.

[3] H. Brückner, J. Justus, J. Kirschbaum, Saccharide induced racemization of amino acids in the course of the Maillard reaction, *Amino Acids,* 21 (2001) 429–433.

[4] F.F. Carone, S. Nakamura, B. Goldman, Urinary loss of glucose, phosphate and protein by diffusion into proximal straight tubules injured by D-serine and maleic acid, *Lab. Invest.,* 52 (1985) 605–610.

[5] J. Csapó, Zs. Csapó-Kiss Zs, S. Einarsson, S. Folestad, A. Tivesten, Methods for determination of D-amino acid content of foods and feeds, *Acta Alimentaria,* 24 (1995) 125–126.

[6] J. Csapó, Zs. Csapó-Kiss, L. Wágner, T. Tálos, T.G. Martin, S. Némethy, S. Folestad, A. Tivesten, Hydrolysis of proteins performed at high temperatures and for short times with reduced racemization in order to determine the enantiomers of D- and L-amino acids, *Anal. Chim. Acta,* 339 (1997) 99–107.

[7] S. Einarsson, S. Folestad, B. Josefsson, Separation of amino acid enantiomers using precolumn derivatization with o-phtalaldehyde and 2,3,4,6,-tetra-O-acetil-1-thio-ß-glucopyranoside, *J. Liquid. Chrom.,* 10 (1987) 1589–1598.

[8] T. Erbe, H. Brückner, Studies on the optical isomerization of dietary amino acids in vinegar and aqueous acetic acid, *Eur. Food Res. Technol.,* 211 (2000) 6–12.

[9] H. Frank, W. Woiwode, G.J. Nicholson, E.Bayer, Determination of the rate of acidic catalyzed racemization of protein amino acids, *Liebigs. Ann. Chem.,* (1981) 354–365.

[10] M. Friedman, J.C. Zahnley, P.M. Masters, Relationship between in vitro digestibility of casein and its content of lysinoalanine and D-amino acids, *J. Food Sci.,* 46 (1981) 127–131.

[11] M. Friedman, *Formation, nutritional value, and safety of D-amino acids,* In: M. Friedman (ed) Nutritional and toxicological consequences of food processing. Plenum Press, New York 1991. 447–481.

[12] M. Friedman, Chemistry, nutrition, and microbiology of D-amino acids, *J. Agric. Food. Chem.,* 47 (1999) 3457–3479.

[13] S.A. Fuchs, R. Berger, L.W.J. Klomp, T.J. de Koning, D-amino acids in the central nervous system in health and disease, *Molecular Genetics and Metabolism.,* 85 (2005) 168–180.

[14] T. Geiger, S. Clarke, Deamidation, isomerization, and racemization at asparaginyl and aspartyl residues in peptides. Succinimide-linked reactions that contribute to protein degradation, *J. Biol. Chem.,* 262 (1987) 785–794.

[15] K. Imai, T. Fukushima, T. Santa, H. Homma, Y. Huang, M. Shirao, K. Miura, Whole body autoradiographic study on the distribution of ^{14}C-D-serine administered intravenously to rats, *Amino Acids,* 15 (1998) 351–361.

[16] J.P. Kaltenbach, C.E. Ganote, F. Carone, Renal tubular necrosis induced by compounds structurally related to D-serine, *Exp. Mol. Pathol.,* 30 (1979) 209–214.

[17] D. Kampel, R. Kupferschmidt, G. Lubec, *Toxicity of D-proline,* In: G. Lubec, G.A. Rosenthal (eds) Amino acid: chemistry, biology and medicine. Escom, Leiden, The Netherlands 1990. 1164–1171.

[18] R. Liardon, M. Friedman, G. Philippossian, Racemization kinetics of free and protein bound lysinoalanine in strong acid media, *J. Agric. Food Chem.,* 39 (1991) 531–537.

[19] R. Liardon, S. Ledermann, Racemization kinetics of free and protein bound amino acids under moderate alkaline treatment, *J. Agric. Food Chem.,* 34 (1986) 557–565.

[20] R. Liardon, M. Friedman, Effect of peptide bond cleavage on the racemization of amino acid residues in proteins, *J. Agric. Food Chem.,* 35 (1987) 661–667.

[21] P.M. Masters, M. Friedman, *Amino acid racemization in alkali treated food proteins - chemistry, toxicology and nutritional consequences,* In: J.R Whittaker, M. Fujimaki (eds) Chemical deterioration of proteins. Am Chem Soc, Washington DC 1980. 165–194.

[22] H. Man, J.L. Bada, Dietary D-amino acids, *Ann. Rev. Nutr.,* 7 (1987) 209–225.

[23] J. De Miranda, A. Santoro, S. Engelender, H. Wolosker, Human Serine Racemase: molecular cloning, genomic organization and functional analysis, *GENE,* 256 (2000) 183–188.

[24] Y. Nagata, R. Yamada, H. Nagysaki, R. Konno, Y. Yasumura, Administration of D-alanine did not cause increase of D-amino acid oxidase activity in mice, *Experientia Birkhäuser Verlag,* 47 (1991) 835–838.

[25] D.L. Oxender, Stereospecificity of amino acid transport for Ehrlich tumor cells, *J. Biol. Chem.,* 240 (1965) 2976–2982.

[26] Zs. Ormainé Cserhalmi, E. Horváth, J. Petres, B. Czukor, Extrudálás az élelmiszeriparban II., *Élelmezési Ipar,* 10 (1988) 366–370.

[27] Zs. Ormainé Cserhalmi, B. Czukor, Az extrudálás hatása a kukorica és a rizs fehérjeemészthetőségére és aminosavtartalmára, *Élelmezési Ipar,* 5 (1991) 168–172.

[28] A. Schieber, H. Brückner, M. Rupp-Classen, W. Specht, S. Novitzki-Grimm, H.G. Classen, Evaluation of D-amino acid levels in rat by gas chromatography-selected ion monitoring mass spectrometry: no evidence for subacute toxicity of orally fed D-proline and D-aspartic acid, *J. Chrom. B,* 691 (1997) 1–12.

[29] D.E. Schwass, L.R. Tovar, J.W. Finley, Absorption of altered amino acids from the intestine, *ACS Symp.,* 234 (1983) 187–201.

[30] É. Vargáné Visi, P. Merész, É. Terlakyné Balla, J. Csapó, The effect of the extrusion temperature and the residence time on the D-amino acid content of corn extrudates, *Acta Agraria Kaposváriensis,* 1 (2004) 59–68.

[31] M. De Vrese, R. Frik, N. Roos, H. Hagemeister, Protein-bound D-amino acids, and to a lesser extent lysinoalanine, decrease true ileal protein digestibility in minipigs as determined with ^{15}N-labeling, *J. Nutr.,* 8 (2000) 2026–2031.

[32] J. Zagon, L.I. Dehne, K.W. Bögl, D-amino acids in organisms and food, *Nutrition Research,* 3 (1994) 445–463.

Received: August, 2008

Acta Univ. Sapientiae, Alimentaria, **2**, 1 (2009) 81–88

Influence of the microwave heating on the water soluble vitamin and D-amino acid content of meat

J. Csapó[1,2]
email: csapo.janos@ke.hu

Sz. Salamon[2]
email: salamonszidonia@sapientia.siculorum.ro

É. Varga-Visi[1]
email: visi@ke.hu

Zs. Csapó-Kiss[1]
email: csapo.janosne@ke.hu

[1]University of Kaposvár,
Faculty of Animal Science,
Guba S. u. 40, 7400 Kaposvár, Hungary

[2]Sapientia–Hungarian University of Transylvania,
Csíkszereda Campus, RO-530104, Libertatii 1., Miercurea-Ciuc

Abstract. The authors examined the vitamins B_1, B_2 and C content of meatballs and also the D-aspartic acid and D-glutamic acid content plotted against the cooking time. The vitamin B_1 mixed in 50 mg/100 g concentration to the normal-sized meatball changed minimal in 10 minutes, but during 20 minutes baking, 70% decomposed. The vitamin B_2 seems to resist more against microwave treatment since its concentration in 10 minutes reduced from 50 mg/100 g to 43 mg/100 g, and after 20 minutes of microwave treatment it reduced to 35 mg/100 g. The vitamin C content of the meatballs (50 mg/100 g initial concentration), applying the two methods of our own was 20–22 mg/100 g after

Key words and phrases: meat, microwave treatment, vitamins, vitamin B_1, vitamin B_2, vitamin C, D-aspartic acid, D-glutamic acid

10 minutes and 13–14 mg/100 g after 20 minutes. No significant differences were found in the vitamin content of inside and outside of the meatball. The D/L aspartic acid ratio was 0.048 in the non-treated sample, the D/L glutamic acid ratio was 0.027. This ratio concerning the aspartic acid increased to 0.069 in 10 minutes, 0.151 in 20 minutes, and concerning the glutamic acid increased to 0.042 in 10 minutes, and 0.047 in 20 minutes.

1 Introduction

Several articles were published in the scientific press on the harmful effects of microwave and heat treatment concerning the vitamin content of foods [1, 2, 3, 4, 5, 7, 12, 10, 16]. Several articles are also known according to which microwave and heat treatment can promote the amino acid racemisation [14, 15]. In the case of baby-foods such an account was given that due to microwave treatment D-allo-hidroxiproline was formed in a great contentration [13]. Japanese researchers also presented that the solutions of L-amino acid due to microwave treatment racemised completely which was used to produce D-amino acid from L-amino acid [8, 9]. Since, as far as we know, no such investigations were done in Hungary, we have set ourselves the task to analyse the changes of water-soluble vitamins B_1, B_2 and C under the influence of microwave treatment. From the same samples the greatest proportion of protein, the aspartic acid's and the glutamic acid's D-enantiomers were also determined examining the effects of microwave treatment on the racemisation of amino acids.

2 Material and methods

Preparation of meatballs 5 kg non-fatty pork chop was minced on a laboratory grinding mill, it was made homegeneous, and then 600 g of the homogenised material was used for our experiments. For the minced meat as much vitamins B_1, B_2 and C was given in 1% concentration that 100 g of the meatball made of the homogenised material consisted of 50 mg vitamin. The meatball enriched with vitamins were treated in microwave oven for 2, 5, 10, 15 and 20 minutes on 750 W energy, then after cooling down the samples were equalized with laboratory homogeniser, and the vitamin content and also the D-amino acid content was analysed from these homogenised samples. The experiments were completed, parallel with the homogenised sample, in the inner and outer layer of the meatball.

Vitamin determination Before the determination of vitamins B_1, B_2 and C 50 ml extraction mixture was added to 5 g homogenised sample, which was assembled from the water solution of 800 ml 1% KH_2PO_4 and 200 ml metanol. After making the mixture the pH was adjusted to 3. The samples were treated in ultrasonic bath for 5 minutes for 3 times with 2 minutes intervals, then were filtered with the help of Büchner funnel and water jet pump. The material remained on the filter was washed with 2×5 ml extraction mixture, then the filted material was vacuum dried, centrifuged for 10 minutes in 5000 g, and 20 μl from the supernatant was injected to the 250×4 mm RP-18 column purospher of the MERCK Hitachi LaChrom HPLC. The separation of the vitamins was made with pH=2.8; 0.04 M and pH=2.8; 0.02 M phosphate puffer and acetonitril according to the following gradient *(Table 1).*

Table 1: Gradient used for the separation of vitamins B_1, B_2 and C

Time	**0.04 M phosphate buffer**	**0.02 M phosphate buffer**	**Acetonitril**
0	100	0	0
2	100	0	0
3	0	98	2
8	0	88	12
12	0	83	17
16	0	83	17
16.1	0	50	50
26.0	0	50	50
26.1	100	0	0
30.0	100	0	0

The detection was made on 254 nm with LaChrom UV-detector. The flow rate was 1 ml/min. Parallel with the HPLC determination the vitamin C content was determined with the traditional 2,6-dichlorophenol-indophenol reaction.

D-amino acid determination The determination of the D-amino acid content of the meatballs was made with HPLC after the 6 M HCl hydrolysis, and after OPA/TATG precolumn derivatization with fluorescent detection besides 325 nm extinction and 420 nm emission wavelength [6, 11].

Evaluation of results The results were evaluated with the help of Microcall origin programpackage.

3 Results

Evaluating the results of our experiments for determining vitamins B_1, B_2 and C and for separating vitamins B_1, B_2 and C we can ascertain that the separation of the mentioned vitamins is good, no overlaying emerged disturbing the determination. *Table 2* shows the formation of dry material, vitamins B_1, B_2 and C content plotted against microwave treatment.

Table 2: Formation of dry material, vitamins B_1, B_2 and C content plotted against microwave treatment

Baking time (minutes)	vitamin B_1 (mg/100 g)	vitamin B_2 (mg/100 g)	vitamin C (HPLC) (mg/100 g)	vitamin C (titration) (mg/100 g)
0	49.02	49.14	49.07	48.76
2	48.89	47.82	41.24	39.66
5	48.73	45.97	34.34	29.60
10	48.79	43.22	22.70	19.44
15	32.26	35.55	16.72	14.96
20	14.57	34.90	12.84	13.79

Evaluating the data of *Table 2,* which shows the formation of vitamin content plotted against baking time, we can state that the vitamins B_1, B_2 and C content of the untreated sample is around 49 mg/100 g, so the recovery of the added vitamins is almost 100% in the raw, untreated sample. No significant changes were experienced until the 10^{th} minute, after 10 minutes of treatment in the 15^{th} minute, the vitamin content decreased to 32, in the 20^{th} minute to 15 mg/100 g. In the case of vitamin B_2 the concentration decreased to 43.2 in 10 minutes, and in 20 minutes to 34.9 mg/100 g. It seems that the vitamin B_2 bears the long lasting microwave treatment better than the vitamin B_1, in 5–10 minutes no considerable decomposition is to be expected concerning the vitamins. The situation is completely different concerning vitamin C, which decreases to 41.2 in 2 minutes, 34.3 in 5 minutes, 22.7 in 10 minutes, 16.7 in 15 minutes and 12.8 mg/100 g in 20 minutes. It seems that vitamin C is much

more sensitive to microwave treatment than vitamins B_1 and B_2. It is clearly demonstrated in the table that there is no significant difference between the vitamin content determined by HPLC and the classic method determined by titration, so in the case of meatballs for analysing the added vitamin C content both the HPLC and the titration method is convenient.

The effect of the microwave treatment on the D-Asp and D-Glu content of the meatballs is shown in *Table 3.*

Table 3: The effect of microwave treatment on the D-Asp and D-Glu content of meatballs

Baking time (minutes)	**D/L-Asp**	**D/L-Glu**
0	0.0480	0.0270
2	0.0699	0.0376
5	0.0664	0.0380
10	0.0689	0.0417
15	0.1439	0.0442
20	0.1506	0.0466

The D/L aspartic acid ratio of the untreated meatballs was found 0.048, and the D/L glutamic acid ratio 0.027. This initial hydrolysis resulted very likely due to the protein hydrolysis, derivatization and during the determination of D-amino acids. Under the influence of 2 minutes of treatment in the case of aspartic acid it decreases to 0.07, concerning glutamic acid to 0.038 and this ratio is not practically likely to change until 10 minutes of treatment. Major racemisation results at the aspartic acid between the 10 and 15 minutes - treatment, and the maximum of the D/L aspartic acid ratio is reached at 20 minutes treatment with 0.151. The D/L ratio of the glutamic acid after 15 minutes of treatment is 0.044 and after 20 minutes is 0.047. It seems so that racemisation of the aspartic acid is much more considerable than of the glutamic acid, under our applied circumstances. In the case of 20 minutes of microwave treatment we did not get any considerable amount of D-amino acids neither concerning aspartic acid nor glutamic acid, but in the case of exceeding the ten minutes treatment 15% of the aspartic acid, 4–5% of the glutamic acid changed into D-enantiomers.

Parallel to our previous experiments we analysed the vitamin content of

the meatballs' outer 1 mm thick layer and its inside, and also its D-amino acid contents. We ascertained that the water soluble vitamin content of the skin is a little higher than that of the inner parts, which is probably due to the movement of the water taking the vitamins and then evaporating on the surface. Considering D-amino acids in the case of the aspartic acid on the influence of 10 minutes of treatment no significant difference was experienced between the skin and the inner parts, neither concerning aspartic acid, nor glutamic acid, but in the 20^{th} minute it seems that the surface of the meatball contains substantially more D-aspartic acid and slightly more D-glutamic acid than its inner parts.

From our researches we can come to the conclusion that due to the impact of microwave treatment used in households, out of the 3 water soluble vitamin analysed by us, vitamins B_1 and B_2 hardly changes, but vitamin C can decrease about 20% even at two minutes of microwave treatment. 10 minutes of microwave treatment destroys more than half of vitamin C. Concerning D-amino acids, 10 minutes of microwave treatment just slightly increases the amount of D-amino acids, while due to a longer microwave treatment the amount of D-amino acids can be considerable. Further on we would like to measure the change in the concentration of vitamin B_6 and B_{12} during microwave treatment, and we would also like to complete the microwave treatment with other sorts of food-products and analyse its effects on the vitamin and D-amino acid content.

4 Acknowledgements

Authors are grateful to the Sapientia Foundation, Institute of Research Programs for the financial support.

References

[1] I.A. Abd El-Gawad, M.M. El-Abd, F.H. Ragab, M.A. El-Aasar, Study on vitamin B_2 in milk and some milk products, *Egypt. J. Food Sci.,* 16 (1988) 175–192.

[2] I. Andersson, R. Öste, Loss of ascorbic acid, folacin and vitamin B_{12}, and changes in oxygen content of UHT milk, *Milchwissenschaft,* 47 (1992) 233–234.

[3] I. Andersson, R. Öste, Nutritional quality of pasteurized milk. Content of vitamin B_{12}, folacin and ascorbic acid during storage, *Int. Dairy J.,* 4 (1994) 161–172.

[4] S. Areekul, K. Quarom, J. Doungbarn, Determination of vitamin B_{12} and vitamin B_{12} binding proteins in human and cow's milk, *Mod. Med. Asia,* 13 (1977) 17–23.

[5] J.O. Bosset, P. Eberhard, U. Butikofer, Evaluation of criteria of milk deterioration after various heat and mechanical treatments as well as light exposure of diferent durations. Part I. Vitamin C, *Mitteilungen aus dem Gebeite der Lebensmitteluntersuchung und Hygiene,* 82 (1991) 433–456.

[6] J. Csapó, S. Einarsson, D-amino acid content of foods and feeds. 1. Separation and determination of amino acid enantiomers by HPLC after precolumn derivatization with 1-/9-fluorenyl/ethil-chlorophormate, *Élelmiszervizsgálati Közlemények,* 39 (1993) 290–302.

[7] H.R. Chapman, J.E. Ford, S.K. Kon, Further studies of the effect of processing on some vitamins of the B complex in milk, *J. Dairy Res.,* 34 (1967) 197–197.

[8] S.T. Chen, S.H. Wu, K.T. Wang, Rapid racemization of optically active amino acids by microwave oven-based heating treatment, *Int. J. Peptid Protein. Res.,* 33 (1989) 73–75.

[9] S.T. Chen, S.H. Chiou, Y.H. Chu, K.T. Wang, Rapid hydrolysis of proteins by means of microwave technology and its application to amino acid analysis, *Int. J. Peptide Protein Res.,* 30 (1987) 572–582.

[10] S. Demel, I. Steiner, J. Washuettl, G. Kroyer, Chemical and microbiological studies on microwave-treated milk, *Z. Ernaehrungswiss.,* 29 (1990) 299–303.

[11] S. Folestad, A. Tivesten, J. Csapó, D-amino acid content of foods and feeds. 1. Separation and determination of amino acid enantiomers by HPLC after precolumn derivatization with OPA/TATG, *Élelmiszervizsgálati Közlemények,* 40 (1994) 17–26.

[12] G.S. Haddad, M. Loewenstein, Effect of several heat treatments and frozen storage on thiamine, riboflavin and ascorbic acid content of milk, *J. Dairy Sci.,* 66 (1983) 1601–1606.

[13] G. Lubec, C.H.R. Wolf, B. Bartosch, Amino acid isomerisation and microwave exposure, *The Lancet,* March 31 (1990) 792.

[14] H. Man, J.L. Bada, Dietary D-amino acids, *Ann. Rev. Nutr.,* 7 (1987) 209–225.

[15] R. Liardon, R.F. Hurrel, Amino acid racemization in heated and alkali-treated proteins, *J. Agric. Food. Chem.,* 31 (1983) 432–445.

[16] K. Wagner, H. Graf, G. Schaarmann, G. Flackowsky, *Influence of cooking and microwave treatment on the vitamin A content of milk,*. Vitamine und weitere Zusatstoffe bei Mensch und Tier 4. Symposium, Jena (Thuringen) (G. Flachowsky and R. Schubert, eds.) 1993. 49–52.

Received: August, 2008

Acta Univ. Sapientiae, Alimentaria, **2**, 1 (2009) 89–100

The influence of the season on the fatty acid composition and conjugated linoleic acid content of the milk

R.V. Salamon[1]
email:
salamonrozalia@sapientia.siculorum.ro

É. Varga-Visi[2]
email: visi@ke.hu

Zs. Csapó-Kiss[2]
email: csapo.janosne@ke.hu

A. Győri[3]
email: gyoria@agr.unideb.hu

Z. Győri[3]
email: gyori@agr.unideb.hu

J. Csapó[1,2]
email: csapo.janos@ke.hu

[1]Sapientia–Hungarian University of Transylvania,
Csíkszereda Campus, RO-530104, Libertatii 1., Miercurea-Ciuc

[2]University of Kaposvár,
Faculty of Animal Science,
Guba S. u. 40, 7400 Kaposvár, Hungary

[3]University of Debrecen
Centre of Agricultural Scieneces,
Debrecen, H-4032 Böszörményi út 138.

Abstract. The purpose of the research was to determine the fatty acid composition of milk of general varieties in Hungary that is Hungarian Simmenthal, Red Holstein Friesian and Black Holstein Friesian and the

Key words and phrases: conjugated linoleic acid, fatty acids, milk fat, season, Hungarian Simmenthal, Red Holstein Friesian, Black Holstein Friesian

changes in the fatty acid composition of their milk fat throughout the year with special respect to the conjugated linoleic acid content. The amount of unsaturated fatty acids (oleic acid, linoleic acid and linolenic acid) including conjugated linoleic acid was higher in summer than in winter. In the case of the saturated fatty acids (butyric acid, caproic acid, caprylic acid, capric acid, myristic acid, palmitic acid and stearic acid) an opposite tendency has been shown. The amount of conjugated linoleic acid ranged from 0.8 to 1.4%, with an average value of 1.1%.

1 Introduction

Milk fat as a fat source for the human nutrition used to be regarded as harmful to health because it is a rich source of the saturated fatty acids. New scientific results have demonstrated that milk fat contains components with advantageous impact on health and these materials proved to be effective against cancer or atherosclerosis [4, 5, 6, 9, 10, 11]. Certain long chain unsaturated monocarboxylic acids that is conjugated linoleic acids (CLA) also belong to this group. CLA means several positional and geometrical isomers of octadecadienoic acid with the two double bond in the conjugated position. When the positive effect of CLA on health was proven by many researcher, the question arose how to increase the CLA intake of human. The CLA content of different sort of foods has been surveyed and now the factors that exert an effect on the CLA concentration of food is under studying. On the one hand CLA can be originated from the processes of biological hydrogenization occurs in the rumen of cow and other ruminants, on the other it can be formed during the heat processing of food.

In the case of milk fat $c9$,$t11$-C18:2 ($c9$,$t11$-CLA) is the main component giving more than 80% of the total CLA content [1, 3, 12]. The CLA content of the milk fat has been shown to vary from 0.2 to 2.0 g CLA/100 g milk fat. In Sweden the CLA content of the milk fat was found between 0.25 and 1.77 g CLA/100 g milk fat [8], in the EU the average amount of $c9$,$t11$-CLA was 0.76 g CLA/100 g milk fat and the extreme values were 0.13 and 1.89 g CLA/100 g milk fat [13]. Among the factors that affects the CLA content of milk the influence of season and housing can be attributed to the different feeding techniques. The most important factor connected with feeding is the unsaturated fatty acid content of the fodder, that means mostly linoleic acid and linolenic acid content, because these fatty acids are supposed to be the precursors of CLA.

The first author was *Riel* [14] who pointed out that the CLA content of

milk in summer had been twice as high (1.46%) than in winter (0.78%) based on the results of spectrofotometric determinations. This finding was supported by others who mesaured significantly higher CLA content in milk from cows drown up to the pasture than cows feeding with hay and/or silage using chromatographic methods [2]. An experiment on the milk of cows from the EU countries has shown that the poliunsaturated fatty acid (PUFA) intake of animals grazing on the pasture is higher than that of animals kept in the cow-sheed and fed with preserved fodders [13]. The type of the farming (conventional or ecological) has also an effect on the CLA content of milk [7]. The amount of CLA ranged from 0.34 g CLA/100 g milk fat (animals kept in cow-sheed) to 0.8 g CLA/100 g milk fat (animals kept in ecological farms).

The CLA content of milk of cow and the changes in the CLA content of milk fat throughout the year has not been determined yet in Hungary. The purpose of the present research was to determine the fatty acid composition of milk of general varieties in Hungary and the changes in the fatty acid composition of milk fat with special respect to CLA content. The present work is supposed to be a preliminary research with limited number of repetition in order to establish a higher volume research involving higher number of individuals and repetitions.

2 Materials and methods

2.1 Animals and feeding

The milk samples were collected from the selected individuals of 210 cows throughout a year at the dairy plant called "Új Élet" at Hencida. Half of the cows were Black Holstein Friesian 15% of them Red Holstein Friesian and 30% was the ratio of Hungarian Simmenthal. During the summer period (from 10^{th} May until 15^{th} October) the animals were kept on the pasture and the grass was supplemented with 3.5 kg of fodder that contained 20% dairy concentrate 60% corn and 20% wheat. The diet was supplemented with phosphorus and calcium and 10–15 kg corn silage. During winter alfalfa and grass hay were supplied ad libitum and the diet consists of 3.5 kg of dairy forage, mineral supplementation, 15 kg of slice of sugar-beet and 15 kg of corn silage.

The bulk milk of the individual cows was sampled and in the case of each variety three milk samples were drown that means 100 ml of milk. The samples were immediately cooled down and stored frozen at $-25\,^{\circ}\text{C}$ until the chemical analysis.

2.2 Chemical analysis

Determination of fatty acid composition: The laboratory analysis of samples was carried out at the Department of Chemistry and Biochemistry, Faculty of Animal Science, University of Kaposvár. The homogenized sample was weighed into a flask, 8 ml concentrated hydrochloric acid was added and it was boiled for 60 minutes. After cooling down 7 ml ethanol was added then 15 ml diethylether following one-minute-shaking. The next extraction was with 15 ml petrolether (b.p.<60 °C). After phase separation organic phase that contains about 150–200 mg fat was separated and evaporated under vacuum on a rotadest. Then 4 ml 0.5 M sodium-hydroxide in methanol was added, and boiled on a water bath for 5 minutes. Then 4 ml 14% boron-trifluoride in methanol was added and boiled for 3 minutes following the addition of 4 ml n-hexane. It was boiled for one minute then the level of the organic phase was brought to the neck of the flask with saturated sodium-chloride solution. When phases were separated samples were taken for the analysis from the organic phase, and it was dry on sodium sulfate.

The fatty acid methyl esters (FAMEs) were separated on a 100 m×0.25 mm wall coated open tubular (WCOT) column equipped with CP-SIL 88 (FAME) stationary phase. The quantitation of FAMEs was obtained with a flame ionization detector (FID) at 270 °C. The temperature of the splitter injector was 270 °C, the carrier gas was helium with the head pressure of 235 kPa. The oven was temperature programmed from 140 °C (10 min.) with 10 °C/min increase up to 235 °C (26 min). The injected volume varied between 0.5 and 2 μl. The instrument was a Chrompack CP 9000 gas chromatograph.

Determination of conjugated linoleic acid content: 10 ml milk sample was weighed and 80 ml organic solution mixture (a mixture of hexan:i-propanol in the ratio of 3:2, HIP) was added. The sample was dispersed with an IKA Ultra-turrax dispersion instrument and filtered. The filtration apparatus was rinsed three times with 10 ml HIP. The water was eliminated from the organic phase with the addition of 5 g anhydrous sodium sulphate. The HIP mixture was evaporated to dryness from the lipids and the residue was washed with hexane into a volumetric flask. Transesterification (methylation) of lipids was accomplished with 4 M sodium methylate solution in methanol. The reaction was completed at 50 °C for 30 minutes. The resulting FAMEs were extracted with hexane and injected into the same column as was used in the case of the determination of the other FAMEs. The oven was kept at 140 °C for 10 min and the temperature was raised with 5 °C/min until 235 °C then held

for 30 minutes.

3 Results

The changes in the fatty acid composition and CLA content in the function of the months can be seen in *Table 1.* The results are given as the averages of the three varieties. Among the isomers of CLA the $c9,t11$-C18:2 isomer is present at the highest amount in the milk fat and has been reported to has health protecting role, therefore the amount of this isomer was determined. *Figures from 1 to 5* show the changes in the ratio of different fatty acids of milk fat. The milk of different genotypes is assigned with various lines. Due to the limited financial opportunities there were only three analyses per variety and per samples. The mean of the three analyses can be seen in *Table 1.* The absence of standard deviation values and statistical analysis is due to the limited number of repetition.

Figure 1: The butyric acid (C4:0) and caproic acid (C6:0) content of milk fat in the function of months expressed in the relative weight-percentage of the fatty acid methyl esters

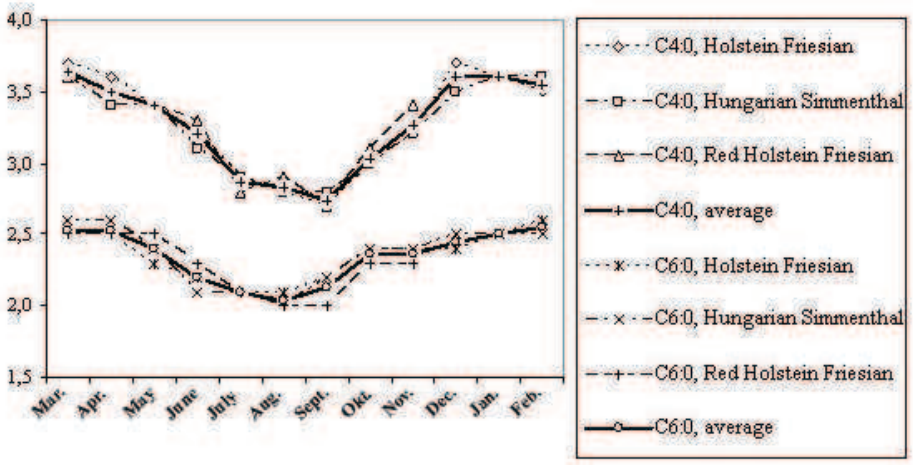


Figure 2: The caprylic acid (C8:0) and capric acid (C10:0) content of milk fat in the function of months expressed in the relative weight-percentage of the fatty acid methyl esters

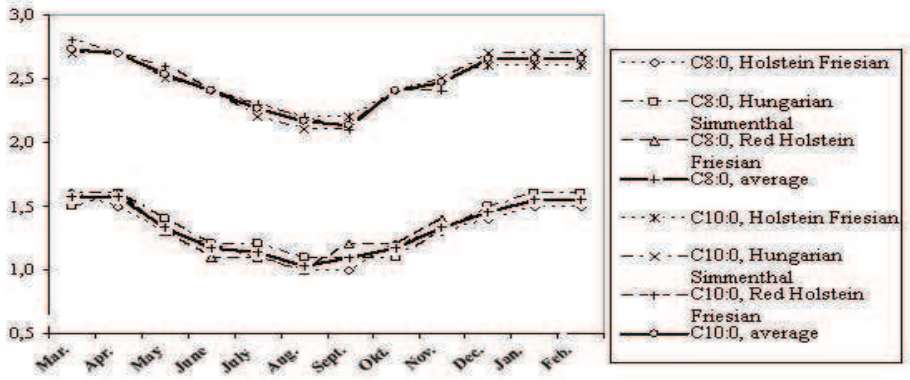


Figure 3: The palmitic acid (C16:0) and oleic acid (18:1) content of milk fat in the function of months expressed in the relative weight-percentage of the fatty acid methyl esters

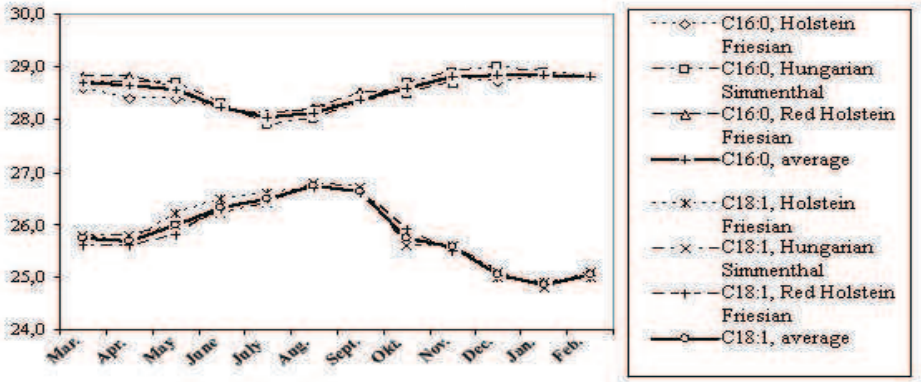


Figure 4: The linoleic acid (C18:2) and linolenic acid (C18:3) content of milk fat in the function of months expressed in the relative weight-percentage of the fatty acid methyl esters

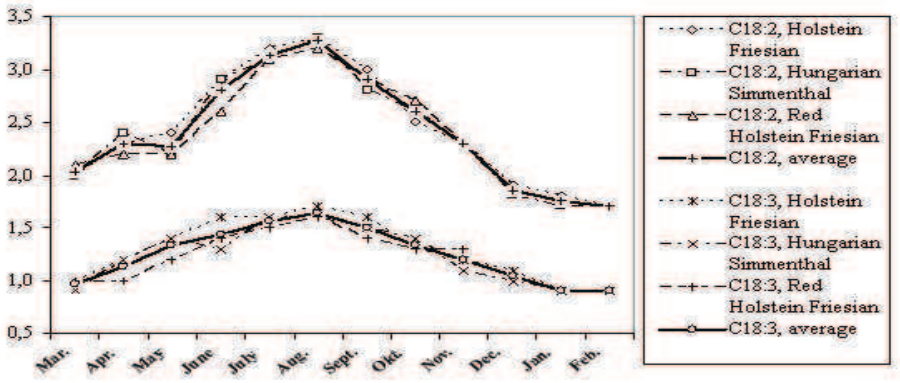


Figure 5: The conjugated linoleic acid content of milk fat in the function of months expressed in the relative weight-percentage of the fatty acid methyl esters

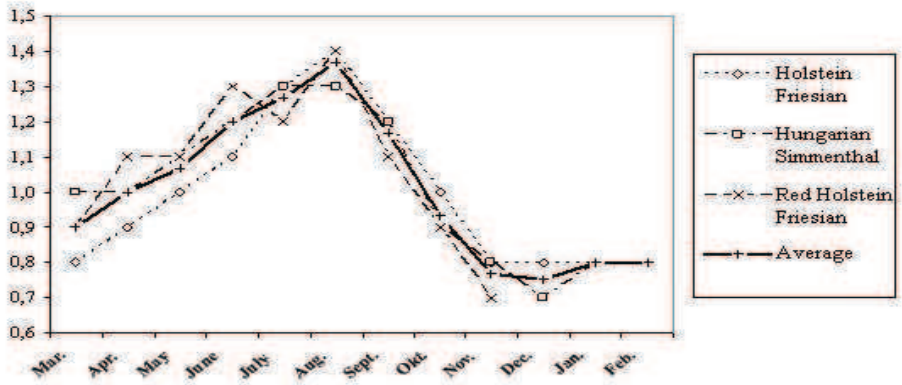


Table 1: The average fatty acid composition of the milk fat of three genotypes expressed in the relative weight-percentage of the fatty acid methyl esters

	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.
Butyric acid C4:0	3.6	3.5	3.4	3.2	2.9	2.8	2.8	3.0	3.2	3.6	3.6	3.5
Caproic acid C6:0	2.5	2.5	2.3	2.2	2.0	2.1	2.2	2.4	2.4	2.4	2.5	2.6
Caprylic acid C8:0	1.6	1.5	1.4	1.2	1.2	1.1	1.0	1.2	1.3	1.4	1.5	1.5
Capric acid C10:0	2.7	2.7	2.5	2.4	2.3	2.1	2.1	2.4	2.5	2.6	2.7	2.7
Lauric acid C12:0	3.4	3.2	3.3	3.3	3.3	3.2	3.4	3.4	3.4	3.5	3.5	3.4
Myristic acid C14:0	11.1	11.0	11.1	10.9	11.0	11.0	11.3	11.4	11.4	11.5	11.7	11.6
Myristoleic acid C14:1	1.4	1.3	1.2	1.3	1.5	1.4	1.5	1.6	1.5	1.6	1.6	1.6
Pentadecanoic acid C:15:0	1.2	1.1	1.1	1.0	1.1	1.2	1.1	1.2	1.1	1.2	1.2	1.2
Palmitic acid C16:0	28.6	28.6	28.5	28.5	28.0	28.1	28.3	28.7	28.8	28.7	28.9	28.8
Palmitoleic acid C16:1	2.6	2.6	2.5	2.5	2.4	2.3	2.4	2.5	2.6	2.6	2.6	2.6
Heptadecanoic acid C17:0	1.0	1.1	1.1	1.0	1.1	1.0	1.1	1.2	1.3	1.3	1.3	1.3
Stearic acid C18:0	10.6	10.6	10.6	10.6	10.4	10.4	10.5	10.5	10.6	10.7	10.7	10.8
Oleic acid C18:1	25.8	25.9	26.2	26.3	26.7	26.9	26.5	25.7	25.6	25.1	24.7	25.0
Linoleic acid C18:2	2.0	2.3	2.4	2.9	3.2	3.3	3.0	2.5	2.3	1.9	1.8	1.7
Linolenic acid C18:3	1.0	1.2	1.4	1.6	1.6	1.7	1.6	1.3	1.2	1.1	0.9	0.9
CLA c9,t11-C18:2	0.9	0.9	1.0	1.1	1.3	1.4	1.2	1.0	0.8	0.8	0.8	0.8

The figures show that the fatty acid composition of the milk fat of the three varieties is almost the same and their changes in the function of the season did not depend on the sort of the variety. Based on the results of this preliminary research the difference between varieties is negligible. It seems to be not probable that with the increase of the number of individuals significant

differences among varieties could be shown. Higher variation was observed in the case of CLA among fatty acids, but this is not connected with the difference of varieties. It can be connected with the uncertainty of the analytical method and the seasonal changes in the composition of forage. The absolute value of the standard deviation is not higher that in case of the other fatty acids, but due to the minor amount of CLA in milk fat, the relative standard deviation is higher. However, the means of the different varieties are almost the same.

When the different fatty acids are evaluated individually, it can be concluded, that the butyric acid has a minimum level of 2.6–2.8% between June and September, and a maximum value of 3.5–3.7% between December and April. The changes in the ratio of caproic acid, caprylic acid and capric acid have a similar tendency in the function of the months like butyric acid. They dropped to their minimum levels between July and September, and reached their maximum values during the winter and early spring months. The minimum level of caproic acid is 2.1–2.2% in August and in September, the maximum value is 2.6–2.7% between December and April. Among the short carbon chain fatty acids the concentration of caprylic acid is the lowest in the milk fat of cows under study. The lowest concentration was between 1.1 and 1.2% measured between July and September and the highest amount of it was 1.6% between January and April. Among the fatty acids of the milk fat palmitic acid and oleic acid are present in the highest quantities. The changes in the percentage ratio of palmitic acid throughout the year is very similar to the tendency that the short chain fatty acids show, its minimum value is 28.1–28.3% between July and August, the maximum level is 28.7–29.0% during the winter and the early spring months.

The changes in the ratio of the unsaturated fatty acids in the milk fat show an opposite tendency throughout the year related to that of saturated fatty acids. The oleic acid, that is present in the milk fat of the second highest concentration, has the highest concentration of 26.5–26.7% between July and September, and its amount dropped to 25.0% during winter months. Regarding the effect of the season, linoleic acid and linolenic acid show similar tendency than oleic acid, namely both polyunsaturated fatty acids reach their maximum between July and September. The milk fat during summer contains 3.2–3.3% of linoleic acid and its amount in winter is 1.7–1.8%. In August the linolenic acid has a maximum with 1.6% that is dropped to 0.8–0.9% during the winter and the early spring month. The CLA content of milk fat is the highest in August that is 1.35% calculated as the average value of the varieties. Between July and September the CLA content of milk fat of each variety exceeds 1.2% and this value rapidly decreases during the autumn month and

drops to 0.75–0.80% during winter months. These results are in agreement with the statements of Riel [14] that the CLA-content of raw milk is twice as high in summer than in winter, but in the present work the difference between the two extreme values was smaller (from 0.8 to 1.4%). Our findings are similar to that of Dhiman et al. [2] that is the CLA content of the milk increases when the cows are driven out to the pasture. The CLA content of milk fat in the case of cows were kept in the cow-sheed during winter was measured 2–2.5 times higher than Jahreis and coworkers observed [7] and the CLA-values measured during the summer were also higher than that of measured on an ecological farm. The range between the extreme values of CLA (0.8–1.4%) was smaller than in the other authors' experiments (0.2–2.0 CLA/100 g milk fat, Chin et al. [1], Parodi [12], Fritsche and Steinhart, [3]). It can be explained with the different formulation of the diet, the variance in the genotypes of the animals, and perhaps the alteration among the applied analytical methods. The average of the CLA content of milk fat was 1.1% that is slightly higher than Precht and Molkentin [13] determined, and the extreme values are also closer to each other. In the case of the other fatty acids present, the results are similar to the results of the other authors that are not cited in this article.

In sum it can be concluded that the majority of the saturated fatty acids dropped to a minimum value during summer and reached their maximum during winter and the early spring months. In contrast to this, the amount of unsaturated fatty acids, including CLA has the highest concentration during the summer, and the lowest concentration was measured in winter and the early spring months. The findings of the present work are in good agreement with the results of the other authors regarding to the season variation tendency. In the case of the absolute values there is a slight difference. Based on the results it can be concluded that the milk fat in summer consists of more linoleic acid, linolenic acid, oleic acid and CLA than in winter – independently of the variety of cow – and therefore the milk in summer is more suitable for the purpose of health preservation. Since the animals were kept under identical feeding conditions – mainly they consumed grass in summer and hay and silage in winter – the higher CLA content of the summer milk can be associated with the higher unsaturated fatty acid content of pasture grass, maybe the higher CLA content or the influence of the higher ultraviolet radiation.

In the future this experiment needs to be repeated with higher number of individuals and repetitions in order to achieve statistical analysis for studying the factors that could affect the fatty acid composition of the milk fat, that is feeding, variety of cows and others. In the next experiment the fatty acid composition of the forage will also be determined, because it is assumed that

the pasture grass contains more CLA-precursor than the preserved fodder, and the connection between the fatty acid composition of feed and CLA content of milk fat will be evaluated.

4 Acknowledgements

This work has been accomplished with the financial support of OTKA (National Foundation for the Subsidy of Research, Hungary). The number of the contract is T049405. The Sapientia - Hungarian University of Transylvania, Institute of Research Programs, also granted the research. Their financial assistance is gratefully acknowledged.

References

[1] S.F. Chin, W. Liu, K. Albright, M.W. Pariza, Tissue levels of cis-9,trans-11 conjugated dienoic isomer of linoleic acid (CLA) in rats fed linoleic acid (LA), *Faseb J.*, 6 (1992) A1396.

[2] T.R. Dhiman, G.R. Anand, L.D. Satter, M.W. Pariza, Dietary effects on conjugated linoleic acid content of cow's milk, *87th AOCS Annul Meeting and Expo*, USA, (1996).

[3] J. Fritsche, H. Steinhart, Amounts of conjugated linoleic acid (CLA) in German foods and evaluation of daily intake, *Z. Lebensm Unters Forsch A.*, 206 (1998) 77-82.

[4] Y.L. Ha, N.K. Grimm, M.W. Pariza, Anticarcinogens from fried ground beef: heat-altered derivatives of linoleic acid, *Carcinogenesis*, 8 (1987) 1881-1887.

[5] Y.L. Ha, J. Storrkson, M.W. Pariza, Inhibition of benzo(a)pirene-inducted mouse forestomach neoplasis by conjugated dienoic derivatives of linoleic acid, *Cancer Res.*, 50 (1990) 1097-1101.

[6] C. Ip, S.F. Chin, J.A. Scimeca, M.W. Pariza, Mammary cancer prevention by conjugated dienoic derivative of linoleic acid, *Cancer Res.*, 51 (1991) 6118-6124.

[7] G. Jahreis, J. Fritsche, H. Steinhart, Conjugated linoleic acid in milk fat: high variation depending on production system, *Nutr. Res.*, 17 (1997) 1479-1484.

[8] J. Jiang, L. Björck, R. Fondén, M. Emanuelson, Occurrence of conjugated cis-9,trans-11-octadecadienoic acid in bovine milk: effects of feed and dietary regimen, *J. Dairy Sci.*, 79 (1996) 438-445.

[9] K.N. Lee, D. Kritchevsky, M.W. Pariza, Conjugated linoleic acid and atherosclerosis in rabbits, *Atherosclerosis*, 108 (1994) 19-25.

[10] R.J. Nicolosi, L. Laitinen, Dietary conjugated linoleic acid reduces aortic fatty streak formation greater than linoleic acid in hypercholesterolemic hamsters, *Faseb J.*, 10 (1996) 2751.

[11] M.W. Pariza, W.A. Hargraves, A beef-derived mutagenesis modulator inhibits initiation of mouse epidermal tumours by 7,12 dimethylbenz(a)anthracene, *Carcinogenesis*, 6 (1985) 591-593.

[12] P.W. Parodi, Conjugated linoleic acid: An anticarcinogenetic fatty acid present in milk fat, *Journal of Dairy Techology*, 49 (1994) 93-97.

[13] D. Precht, J. Molkentin, Frequency distributions of conjugated linoleic acid and trans fatty acid contents in European bovine milk fats, *Milchwissenschaft*, 55 12 (2000) 687-691.

[14] R.R. Riel, Physico-chemical characteristics of Canadian milk fat. Unsaturated fatty acids, *J. Dairy Sci.*, 46 (1963) 102-106.

Received: August, 2008

Acta Univ. Sapientiae, Alimentaria, **2**, 1 (2009) 101–109

Changes in fatty acid composition of different milk products caused by different technology

R.V. Salamon[1]
email:
salamonrozalia@sapientia.siculorum.ro

Zs. Mándoki[2]
email: mandoki.zsolt@ke.hu

Zs. Csapó-Kiss[2]
email: csapo.janosne@ke.hu

A. Győri[3]
email: gyoria@agr.unideb.hu

Z. Győri[3]
email: gyori@agr.unideb.hu

J. Csapó[1,2]
email: csapo.janos@ke.hu

[1]Sapientia–Hungarian University of Transylvania,
Csíkszereda Campus, RO-530104, Libertatii 1., Miercurea-Ciuc

[2]University of Kaposvár,
Faculty of Animal Science,
Guba S. u. 40, 7400 Kaposvár, Hungary

[2]University of Debrecen,
Centre of Agricultural Scieneces
Debrecen, H-4032 Böszörményi út 138.

Abstract. The fatty acid composition of cow's milk with fat contents of 3.6%, Dalia cheese with fat contents of 44%, butter with fat contents of 80% and margarine with fat contents of 24% was determined after a

Key words and phrases: milk, cheese, butter, margarine, fatty acid composition, cis-trans isomers, microwave

heat treatment performed on cooking plate and microwave treatment, respectively of different durations. The biggest difference was obtained for oleic acid and elaidic acid since with the exception of the margarine in each case proportion of the cis-configurated oleic acid decreased while that of the trans-configurated elaidic acid increased. For all of the other fatty acids in the foodstuffs examined no such differences were obtained regarding change in fatty acid composition, which differences could influence healthy nutrition to considerable extent, therefore we can take it as a fact that neither heat treatment performed on a traditional cooking plate nor microwave treatment affects considerably the composition of food fats.

1 Introduction

Role of fats and that of fatty acids, they are composed of, in the human nutrition is well-known. Fats contained in foodstuffs provide substantial amount of energy for the human organism, and the essential and semi-essential fatty acids are – as the human organism cannot produce them – indispensible to the human organism. Recently, there have been many discussions about trans fatty acids, some experts considered their harmful effects to be proven, while others could not report on such negative effects. Some were of the opinion that trans fatty acids increased fragility of red blood cells, changed the aggregation of thrombocytes [1, 2, 3] and evidenced their negative effects on the metabolism of linolenic acid and arachidonic acid [8]. It was established that they caused lack of essential fatty acids [6], inhibited synthesis of prostaglandin [7] and increased the risk of certain cancers. Lately, it has been reported that incorporation of trans fatty acids into the phospholipids of the membranes affected its properties and mainly the activity of enzymes attached to the membrane, in fact, in recent times a positive relation has been established between allergic diseases and trans fatty acid consumption [5, 10].

Cis–trans transformations can take place due to several technological interventions, as well. The most important such operation is partial hydrogenation as a result of which a part of cis-configurated bonds transforms into trans configuration. By appropriate choosing of technological parameters it can be achieved that this transformation possibly be the slightest and that the products contain trans isomers in minimal quantity. Heating of fats can also cause isomerization, cooking in oils can result in appearance of multiple unsaturated trans fatty acids, and heat treatment of fats can produce trans fatty acids and even also a cyclic fatty acid derivatives [4].

It was reported that fatty acids can convert as a result of microwave treatment, as well. For soya bean, after being microwave-treated for 12 min transformation and decomposition of great volume of fatty acids was experienced. Compared changes in fatty acid composition during food making procedures with the effect of microwave treatment some experts have come to the conclusion that considerable changes could be expected during such treatment and they suggested choosing other kind of procedure instead of microwave treatment for warming up foods [9].

Because of the above, during present research we aimed at analysing changes of fatty acid composition of milk and foodstuffs with high fat contents (cheese, butter, margarine) as an effect of traditional heat treatment and microwave treatment. We paid special attention to the cis-configurated oleic acid and trans-configurated elaidic acid which forms from the former by isomerization.

2 Material and methods

2.1 Samples examined

Milk sample with fat contents of 3.6% was obtained from a seven years old Simenthal cow, feeded mainly with hay and minimal feed supplementation, and it was producing in the second month of lactation. Sample was taken from the mixed milk of the completely milked-out udder. Other components of the milk corresponded to the values characteristic for normal cow's milk in every respect.

In case of the cheese examined was a commercially obtainable cheese with the trade name of Dalia, which was semi-hard, coagulated with mixed coagulated rennet, pressed, formed in brine of 10%, then matured over 2 weeks at 13–14 °C. Its dry matter contents were 55% and fat contents referred to dry matter contents were 44%. The other cheese used in our experiment was commercially obtainable under the trade name of Telemea, a type of feta cheese, coagulated with Lactobacillus acidofilus pure culture, with enzymatic rennet, pressed, sliced and matured over 2 days. Dry matter contents of this cheese were 55% and its fat contents referred to dry matter contents were 44%.

The butter we used was a commercially obtainable butter of trade name Alpenbutter. The margarine examined was of a Holland-24 type, commercially obtainable margarine.

2.2 Heat treatment and microwave treatment

Heat treatment was carried out on a laboratory hot plate, for 2 and 8 min, respectively, after the boiling point was reached. For microwave treatment we applied a treatment over 1, 2, 4, and 8 min using a normal Electrolux EMN 2015 microwave oven at electrical power of 450 W. Subsequent to the heat treatment the samples were cooled down immediately and stored at −25 °C until the preparation for analysis.

2.3 Determination of fatty acid composition

Sample preparation A sample quantity containing approx. 0.5–1.0 g fat was destructed with 8–20 ml of hydrochloric acid (37%) for 1 hour on hot water bath. After having cooled down, 7 ml of ethanol was added. Lipids were extracted with 15 ml diethylether and 15 ml benzine (b.p.<60 °C), and the organic layers were combined. From a portion of this solution, containing approx. 150–200 mg fat, the solvents were removed under reduced pressure (a complete evaporation not necessary). To the residue 4 ml of 0.5 M sodium hydroxide methanol solution was added and boiled until all the fat drops disappeared (approx. 5 min), then 4 ml of 14% boron trifluoride methanol solution was added, boiled for 3 min, finally 4 ml of hexane, dried on water-free sodium sulphate, was added and boiled for 1 min, and the mixture was allowed to cool down. Saturated aqueous sodium chloride solution was added and after having separated the organic layer was collected into a 4 ml vial containing water-free sodium sulphate and was directly examined by gas chromatography.

Conditions of the gas chromatographic analysis Instrument: Varian 3380 CP gas chromatograph. Column: 100 m×0.25 mm id, CP-Sil 88 (FAME) phase. Detector: FID 270 °C. Injector: splitter, 270 °C. Carrier gas: H_2, 235 kPa. Temperature program: 140 °C for 10 min; at 10 °C/min up to 235 °C; isotherm for 26 min. Injected volume: 1 μl.

3 Results

3.1 Change of fatty acid composition of milk as an effect of traditional heat treatment and microwave heat treatment

Change in fatty acid composition of raw milk for the control sample and for the samples heat treated for 2 and 8 min, on cooking plate and in microwave oven, respectively, is shown in *Table 1.*

Table 1: Change in fatty acid composition* as an effect of heat treatment performed on cooking plate and of microwave treatment, respectively

Fatty acid	Control	Microwave treatment		Cooking plate	
		2 min	8 min	2 min	8 min
Miristic acid, C14:0	11.45	11.62	11.35	11.05	10.94
Palmitic acid, C16:0	42.23	42.17	43.21	42.95	42.27
Stearic acid, C18:0	10.07	11.58	11.80	11.27	11.42
Linoleic acid, C18:2	1.38	1.32	1.31	1.36	1.30
Linolenic acid, C18:3	1.17	1.19	1.15	1.09	1.12

*In relative weight % of fatty acid methyl esters.

Only fatty acids with concentrations higher than 9–10%, as well as linoleic acid and linolenic acid are shown. Oleic acid and elaidic acid are shown in *Table 3.*

As it can be seen from the data of *Table 1,* the raw milk contains somewhat more fatty acid than the microwave-treated milk, no significant differences, however, could be found between the microwave treatments. Similarly, no significant differences could be found in palmitic acid and stearic acid contents, which fatty acids are mounting major part of the total fatty acid contents. In the raw milk the milk fat contained 16.26% oleic acid and 1.53% elaidic acid; the cis configuration was 91.36% and the trans configuration was 8.64% of the total C18:1 fatty acids. After a cooking for 2 min the proportion of the cis-configuration reduced by 2%, and after cooking for 8 min it reduced by 4%; the proportion of the trans configuration increased as an effect of cooking (for 2–8 min) by around 15–20%. Similar changes could be observed due to microwave treatment: after a treatment of 2 min the proportion of the cis configuration reduced by 2%, and after 8 min by nearly 10%, whereas the proportion of the trans configuration increased by 10–15% after a treatment of 2 min, and by 40–50% after a treatment of 8 min. Hence, the conclusion can be drawn that heat treatment carried out for 2 and 8 min at 100 °C, and microwave treatment performed for 2 and 8 min at 450 W reduces proportion of the cis-configurated oleic acid and increases proportion of the trans-configurated elaidic acid to a significant extent.

In case of the cheeses Dalia and Telemea changes occured due to microwave treatment are summarized in *Table 2,* changes of oleic acid and elaidic acid

contents of examined dairy products as an effect of traditional and microwave heating are summarized in *Table 3.*

Table 2: Changes of fatty acid composition* of cheeses Dalia and Telemea due to microwave treatment

Fatty acid	Dalia			Telemea		
	Control	2 min	8 min	Control	2 min	8 min
Miristic acid, C14:0	9.83	9.39	9.27	10.23	10.87	10.11
Palmitic acid, C16:0	29.24	28.99	30.33	30.79	31.77	32.14
Stearic acid, C18:0	14.41	14.66	15.45	13.13	13.42	13.85
Linoleic acid, C18:2	1.80	1.79	1.72	1.89	1.85	1.83
Linolenic acid, C18:3	1.50	1.42	1.40	1.08	1.06	1.04

*In relative weight % of the fatty acid methyl esters.

For the cheese of Dalia-type with 44% fat contents in the control sample not microwave-treated proportion of oleic acid was 83.84%, that of elaidic acid was 16.16% to the whole C18:1 fatty acids. As an effect of microwave treatment of 2 min proportion of cis configuration decreased by 1.5%, after treatment of 8 min it decreased by 2%. During 2 min proportion of the trans configuration increased by 8% and after 8 min by 9–10%. For all of the other fatty acids no significant changes were experienced owing to the microwave treatment, and composition of treated samples were practically identical with that of the control sample. In case of the examined two cheese samples fatty acids behaved completely the same way towards microwave treatment.

Oleic acid contents of the butter with fat contents of 80% was measured to be 23.37%, elaidic acid contents were measured to be 3.62%, in percentage of total fatty acids. Within C18:1 fatty acids in untreated butter oleic acid was 86.58%, elaidic acid 13.42%. These proportions changed slightly for samples treated both on cooking plate at 215 °C and in microwave oven at 450 W. Increasing from 2 min to 8 min the duration of the treatment with a cooking plate of 215 °C proportion of oleic acid decreases by 1%, and similar results are obtained when time of microwave treatment is increased from 2 to 8 min. Looking at the proportions for both treatment durations and both heat treatment methods decrease of oleic acid corresponds within the limits of error to the increase of elaidic acid. From our experiments we can draw the conclusion that with increasing duration of the heating in case of both experiment performed with cooking plate and that performed with microwave oven the quantity of cis-configurated oleic acid reduces whereas that of trans-configurated elaidic acid grows.

Table 3: Changes of oleic acid and elaidic acid contents* of various dairy products due to conventional and microwave heat treatment, respectively

Sample examined		C18:1 fatty acid ratio	
		Oleic acid	**Elaidic acid**
Milk control		91.36	8.64
heat treatment	2 min	89.64	10.36
	8 min	87.18	12.82
microwave treatment	2 min	90.08	9.92
	8 min	84.11	15.89
Dalia control		83.84	16.16
microwave treatment	2 min	82.46	17.54
	8 min	82.21	17.79
Telemea control		84.73	15.27
microwave treatment	2 min	82.19	17.81
	8 min	81.47	18.53
Butter control		93.63	6.37
heat treatment	2 min	90.86	9.14
	8 min	90.49	9.51
microwave treatment	2 min	91.23	8.77
	8 min	90.63	9.37
Margarine control		14.36	85.64
heat treatment	2 min	14.23	85.77
	8 min	14.40	85.60
microwave treatment	2 min	14.29	85.71
	8 min	14.73	85.27

*Percentages of C18:1 fatty acids (total C18:1 fatty acid contents=100%).

In case of margarine with 25% fat contents it appears so that the heat treatment and microwave treatment, respectively, do not affect concentration of cis and trans fatty acids. This contradicts our previous examinations, our experiences until now, and the literature as well, since the cis configuration is substantially less stable than the trans, so after a heat treatment in principle quantity of fatty acids with cis configuration should be decreased and that of fatty acids with trans configuration should be increased. By the way, as an effect of heat treatment concentration of unsaturated fatty acids should be reduced due to various oxidative reactions and chain-crackings at the double bonds.

Summarized the effect of heat treatment and microwave treatment it can be said that with the exception of oleic acid and elaidic acid, in case of various samples examined by us the differences in fatty acid composition are so slight that they do not indicate any harmful effect of either heat treatment or microwave treatment. For these fatty acids there is no difference in the effect of the duration of heat treatment and microwave treatment, either, since no difference could be evidenced between the treatments with duration of 2 min and 8 min, respectively. We can take it as a fact therefore, that for the examined foodstuffs time and energy combination used does not result in considerable deviation in the fatty acid composition, thus, there is no need to be afraid that during microwave treatment any artificial product harmful to human beings would form or that biological value and utilization in the human organism of fat of foodstuff treated this way would reduce to significant extent.

In case of oleic acid and elaidic acid it was established that by both heat treatment performed on a cooking plate and microwave treatment the proportion of the cis-configurated oleic acid decreased and that of the trans-configurated elaidic acid increased. This decrease and increase, respectively, do not reach, however, such an extent, which could affect the healthy nutrition.

4 Acknowledgements

This work has been accomplished with the financial support of OTKA (National Foundation for the Subsidy of Research, Hungary). The number of the contract is T049405. The Sapientia–Hungarian University of Transylvania, Institute of Research Programs, also granted the research. Their financial assistance is gratefully acknowledged.

References

[1] A. Ascherio, M.B. Katan, M. Stampfer, Trans fatty acids and coronary heart disease, *N. Engl. J. Med.,* 340 (1999) 1994-1998.

[2] A. Ascherio, Epidemiologic studies on dietary fats and coronary heart disease, *Am. J. Med.,* 113 (2002) 9S-12S.

[3] A. Ascherio, C. Hennekens, J. Buring, C. Master, M. Stamper, W. Willett, Trans fatty acids intake and risk of myocardial infraction, *Circulation,* 89 (1994) 94-101.

[4] K. Hansen, T. Leth, *Surveillance of margarines,* Ministeriet for Fødevarer, Landbrug og Fiskeri, Fødevaredirektoratet, København, 2000.

[5] D. Kritchevsky, Trans fatty acids and cardiovascular risk, *Prostaglandins, Leukotrienes and Essential Fatty Acids,* 57 4-5 (1997) 399-402.

[6] F.A. Kummerow, Q. Zhou, M.M. Mahfouz, M.R. Smiricky, C.M. Grieshop, D.J. Schaeffer, Trans fatty acids in hydrogenated fat inhibited the synthesis of the polyunsaturated fatty acids in the phospholipid of arterial cells, *Life Sci.,* 74 (2004) 2707-2723.

[7] L. Kushi, E. Giovannucci, Dietary fat and cancer, *Am. J. Med.,* 113 (9B) (2002) 63S-70S.

[8] E. Larque, F. Perez-Llamas, V. Puerta, M.D. Giron, M.D. Suarez, S. Zamora, A. Gil, Dietary trans fatty acids affect docosahexaenoic acid concentration in plasma and liver but not brain of pregnant and fetal rats, *Pediatr. Res.,* 47 (2000) 278-283.

[9] T. Sachiko, Y. Hiromi, Microwave heating influences on fatty acid distributions of triacylglycerols and phospholipids in hypocotyls of soybeans, *Food Chemistry,* (2002) 66.

[10] S. Stender, J. Dyerberg, Influence of trans fatty acids on health, *Ann. Nutr. Metab.,* 48 (2004) 61-66.

Received: August, 2008

Acta Univ. Sapientiae, Alimentaria, **2**, 1 (2009) 111–117

Evaluation of the inactivation of heat sensitive antinutritive factors in fullfat soybean

É. Varga-Visi[1]
email: visi@ke.hu

Cs. Albert[2]
email:
albertcsilla@sapientia.siculorum.ro

K. Lóki[1]
email: loki.katalin@ke.hu

J. Csapó[1,2]
email: csapo.janos@ke.hu

[1]University of Kaposvár,
Faculty of Animal Science,
Guba S. u. 40, 7400 Kaposvár, Hungary

[2]Sapientia–Hungarian University of Transylvania,
Csíkszereda Campus, RO-530104, Libertatii 1., Miercurea-Ciuc

Abstract. The regular quality control on the adequacy of heat treatment of fullfat soybeans requires the application of rapid chemical methods. In the present work the trypsin inhibitor activity test and the urease test were applied on fullfat soya samples that were cooked in a pressured steam (toasted) or extruded at different temperatures and speed rates. In the case of toasting both of the results of the laboratory examinations proved that the heating was adequate, while in the case of the extruded samples the two tests gave different results. In the case of certain temperature and time combinations the more rapid and less accurate urease test claimed that the heat treatment reached the aim, while the results of the trypsin inhibitor activity test showed that the level of the inhibitors is still high and the fullfat soya is underheated.

Key words and phrases: fullfat soybeans, dry extrusion, toasting, trypsin inhibitor activity, urease test

1 Introduction

Soybeans are the primary vegetable protein source in animal feed. Nowadays the use of soya without oil extraction, that is fullfat soybeans, has a great importance. Apart from its high protein content with unique biological value, its fat content contribute to the energy required for protein synthesis. It is suitable to formulate high-energy diets, thereby part of the cereals can be replaced. Fullfat soya contains antinutritive factors that reduce the digestibility and utilization of amino acids in nonruminants and inmature ruminants. The effect of proteinaceous antinutritive compounds can be eliminated by heat treatments [3]. The objectives of heating processes for fullfat soybeans are to maintain an optimum balance between degradation of antinutritive factors on the one hand and maintenance of bioavailability of essential amino acids on the other [2, 4]. The best way to evaluate the adequacy of processing and the quality of the product is conducting biological tests. However, the cost, time requirement and complexity of biological tests mean that reliable laboratory procedures, of which trypsin inhibitor activity (TIA) determination perhaps the most appropriate still have a valuable role to play in quality control procedures [3]. The urease test is an indirect method, which based on the inactivation of urease by heat. Due to its rapidness, low skill and minimum amount of laboratory equipment requirements it is suitable for quality control of heating in the plant.

The current study was undertaken to investigate the influence of two sorts of heat treatments on the TIA and urease activity of fullfat soybeans and comparison of the results of the two tests is discussed.

2 Materials and methods

Pressurized steam cooking (toasting) Fullfat soybeans were processed at the Bóly Stock Company (Bóly-Állomáspuszta, Hungary). Soybeans were cracked into 9–12 pieces then boiled in a KAHL HR-1600 hydrothermic reactor (toaster). In this stirrer autoclave soya was heated with pressurized steam at 120 °C for 30 minutes. After steam processing the product called "hydrothermic soya" were air-dried and cooled. In the manufacture of the other type of product called "hydrothermic soya grain" an additional step followed that is grinding in a hammer mill and both products were stored at −20 °C prior to laboratory analyses.

Extrusion The extrusion experiment was carried out at the Budapest University of Technology and Economics, Department of Biochemistry and Food Technology. Fullfat soybeans (Borostyán sp.) were ground with a hammer grinder and the particle size distribution was determined. Extrusion was carried out using a Do-Corder DC 2001 type laboratory-scale Brabender machine which has been described in detail elsewhere [5]. Extrusion trials with the full cross-classification of the applied nominal temperature and screw speed levels *(Table 1)* were repeated three times. From the two reported zone temperatures (T_1, T_2), one value was calculated (T) to characterize the effect of temperature. Minimum residence time was determined by introducing a small amount of dye into the feeding port and measuring the time required for the first colored extrudate to exit the die. Prior to sampling, the machine was allowed to equilibrate to the desired temperature, then the sample was collected and after cooling it was homogenized and sealed in polyethylene bags and stored at $-20\,^{\circ}\mathrm{C}$ until chemical analyses began. Control samples were taken from each batch and treated in the same way as extruded samples.

Table 1: Extrusion of fullfat soybean. Nominal temperature and screw speed levels

Temperature levels	**T_1 (°C) 1. zone (barrel)**	**T_2 (°C) 2. zone (barrel)**	**Screw speed levels**	**Screw speed (rpm)**
1	100	100	1	50
2	140	140	2	90
3	180	180	3	130
4	220	220	4	170

Chemical analyses The trypsin inhibitor activity (TIA) of samples was determined according to the EN ISO 14902 standard [1]. The method based on the measurement of activity decrease of trypsin in a model solution due to the inhibitors that were dissolved from the sample. An artificial substrate benzoyl-L-arginine-p-nitroanilide (L-BAPA) was added to the solution containing trypsin and the sample extract, and the quantity of the released p-nitroaniline was measured spectrometrically. The trypsin inhibitor content was expressed as mg trypsin inhibited per g of the sample. The acceptable

level of TIA depends on the protein content of the material. The European Federation of Feed Manufacturers recommended the next upper TIA limits for fullfat soybeans [3]:

% of protein in the feed	TIA content (mg/g)
50	5
40	4
30	3

The urease test was conducted as following: 50 cm^3 phosphate buffer (0.07 M, pH = 7.5) was added to 1.000 g soybean grain (first solution), and 50 cm^3 buffered urea solution was added to 1.000 g of the same sample (second solution). The buffered urea solution consisted of 30 g urea in 1000 cm^3 phosphate buffer (0.07 M, pH = 7.5). The two solutions were incubated at 35 °C for 30 minutes after stirring. In the presence of significant urease activity the pH of the second solution increases due to the release of ammonia from urea. After incubation the pH of the solutions should be determined rapidly and the degree of heating was estimated basing on the pH difference between the first and the second solution.

Soybean product	pH difference
Raw or not heated	1.7–2.5
Under cooked	0.2–1.7
Well cooked	0–0.2

3 Results

The influence of pressurized steam cooking on the trypsin inhibitor activity and urease activity of fullfat soybean. The results of the heat treatment evaluating analyses can be seen in *Table 2.* The data clearly show that the activity of the trypsin inhibitors was reduced successfully below the required level for both of the products and the adequacy of the heat treatments were also verified with the results of the urease test. However, the pH difference was slightly higher in the case of hydrothermic soya product than that of hydrothermic soya grain. In any case, the differences in the size of the particles of the products and thus higher surface area of grained material cannot be important in the point of view of toasting because grinding was carried out after steam cooking.

Table 2: The trypsin inhibitor activity (TIA) and urease activity of toasted fullfat soybean products (n=3)

Chemical examination	Fullfat soya samples		
	Control	Hydrothermic soya grain	Hydrothermic soya
TIA (mg/g)	17.2 ± 0.5	1.1 ± 0.2	1.2 ± 0.3
Urease test (ΔpH)	1.5 ± 0.1	0.05 ± 0.02	0.14 ± 0.02

The influence of dry extrusion on the trypsin inhibitor activity and urease activity of fullfat soybean. The theoretical and the measured properties of extrusion can be seen on *Table 3.* The adjusted screw speed levels and residence time values can be substituted each other because the temperature did not exert a significant effect on the residence time due to minor changes in the viscosity of the material.

Table 3: Nominal and measured properties of extrusion of fullfat soya

Levels	T_{nom} (°C)	T_{meas} (°C) average ± s.d. (n=12)	Screw speed (s^{-1})	Residence time (s) average ± s.d. (n=12)	Throughput (kg/h) average ± s.d. (n=12)
1	100	101 ± 4	50	29 ± 0.2	1.6 ± 0.4
2	140	140 ± 3	90	17 ± 0.2	2.8 ± 0.8
3	180	180 ± 3	130	12 ± 0.8	4.1 ± 1.1
4	220	220 ± 3	170	10 ± 1.4	4.8 ± 1.4

T_{nom} = Nominal Temperature
T_{meas} = Measured Temperature

At samples extruded at low temperatures (100 °C and 140 °C) the level of TIA remained almost as high as was in control and the effect of the lengthening of residence time was also negligible *(Table 4).*

Based on the result of the urease test of fullfat soybeans extruded at 180 °C for 29 s ($50\,s^{-1}$) it can be claimed that the extent of the heat treatment is adequate, while the result of the TIA measurement clearly shows, that the

Table 4: The trypsin inhibitor activity (TIA) and urease activity of fullfat soybean products extruded at different temperatures with different screw speed (residence time, n=3)

Temperature (°C)	Screw speed (s^{-1})	TIA (mg/g)	Urease test (ΔpH)
100	50	17.0±1.0	1.47±0.10
100	90	16.6±0.3	1.47±0.06
100	130	16.8±0.3	1.49±0.08
100	170	16.6±0.3	1.50±0.09
140	50	16.4±0.7	1.49±0.08
140	90	16.7±0.5	1.43±0.05
140	130	16.4±0.3	1.47±0.07
140	170	16.1±0.9	1.47±0.09
180	50	11.4±0.6	0.10±0.08
180	90	13.4±3.3	0.95±0.08
180	130	15.8±0.8	1.31±0.09
180	170	15.5±0.7	1.44±0.06
220	50	5.0±0.4	0.03±0.01
220	90	9.2±0.5	0.08±0.05
220	130	12.4±1.0	0.53±0.32
220	170	14.0±0.6	1.18±0.16
Control		17,2±0.5	1.53±0.10

activity of trypsin inhibitors barely decreased. Similar tendency can be seen in the case of samples extruded at 220 °C for 17 and 29 s (90 and 50 s^{-1}, respectively). In the last case the TIA value almost dropped to the required level that is 4 mg inhibited trypsin/g sample in fullfat soybean samples with protein content of 37%.

The authors are aware of the fact that the exact chemical characterization of a protein source in the point of view of the adequacy of heat treatment could require more additional laboratory examinations. The aim of this work was solely to draw the attention to that urease test that is often used in plants as a quality control test may not in all the cases gives reliable results compared to the more accurate TIA determination.

References

[1] EN ISO 14902 Animal Feeding stuffs–Determination of trypsin inhibitor activity of soya products (ISO 14902:2001)

[2] F.G. Kaankuka, T.F. Balogun, T.S.B. Tegbe, Effects of duration of cooking of full-fat soya beans on proximate analysis, levels of antinutritional factors, and digestibility by weanling pigs, *Anim. Feed Sci. Technol.,* 62 (1996) 229–237.

[3] S. Monary, *Fullfat soya handbook,* American Soybean Association, Brussel, Belgium 1996. 1–46.

[4] G. Qin, E.R. ter Elst, M.W. Bosch, A.F.B. van der Poel, Thermal processing of whole soya beans: Studies on the inactivation of antinutritional factors and effects on ileal digestibility in piglets, *Anim. Feed Sci. Technol.,* 57 (1996) 313–324.

[5] É. Vargáné Visi, P. Merész, É. Terlakyné Balla, J. Csapó, The effect of the extrusion temperature and the residence time on the D-amino acid content of corn extrudates, *Acta Agraria Kaposváriensis,* 1 (2004) 59–68.

Received: August, 2008

Acta Univ. Sapientiae, Alimentaria, **2**, 1 (2009) 119–131

Composition of mare's colostrum and milk I. Fat content, fatty acid composition and vitamin contents

R.V. Salamon[1]
email:
salamonrozalia@sapientia.siculorum.ro

Sz. Salamon[1]
email:
salamonszidonia@sapientia.siculorum.ro

Zs. Csapó-Kiss[2]
email: csapo.janosne@ke.hu

J. Csapó[1,2]
email: csapo.janos@ke.hu

[1]Sapientia–Hungarian University of Transylvania,
Csíkszereda Campus, RO-530104, Libertatii 1., Miercurea-Ciuc

[2]University of Kaposvár,
Faculty of Animal Science,
Guba S. u. 40, 7400 Kaposvár, Hungary

Abstract. Changes in the fat content, fatty acid composition and vitamin contents of mare's colostrum and milk during the first 45 days of lactation were studied. Milk samples (300–800 ml) from 29 lactating mares, were collected daily at the beginning of the lactation and weekly from 5 to 45 days postpartum. Colostrum and early milk samples were obtained by hand, without oxytocin administration, while the foal nursed. Later milk samples were from mixed milk of the totally-milked udder. Each sample was analysed for total solids, fat content, fatty acid

Key words and phrases: Mare's colostrum and milk, fat content, fatty acid composition of butterfat, determination of cow milk in mare's milk, vitamin contents

composition and vitamin contents by conventional methods and Packard gas chromatograph.

The total solids and the fat contents, respectively, of the colostrum and milk were 24.25 to 26.28% and 2.85 to 2.93% on the first day of lactation, 12.15 to 12.78% and 2.05 to 2.17% on the 2^{nd} to 5^{th} days and 10.37 to 10.61% and 1.04 to 1.32% on the 8^{th} to 45^{th} days of lactation. The concentrations of octanoic, decanoic, dodecanoic, miristic and palmitoleic acids increased over time while stearic, oleic, linolic and linolenic acids decreased. The fatty acid composition of mare's milk fat was very different from that of cow's milk fat. Mare's milk fat contained octanoic, decanoic, dodecanoic, linolic, linolenic, stearic, miristic and palmitic acids, respectively, in ratios of approximately 9.6, 3.1, 2.1, 4.4, 224, 0.2, 0.6 and 0.5 times those of concentrations in cow's milk. On the basis of the differences in fatty acid composition, a new method was developed to determine the amount of cow's milk mixed with mare's milk.

Contents of vitamins A, D_3, K_3 and C of colostrum (0.88, 0.0054, 0.043, 23.8 mg/kg) were found to be 1.4 to 2.6 times the levels in normal milk (0.34, 0.0032, 0.029, 17.2 mg/kg). There was no significant difference found between vitamin E contents of colostrum and milk (1.342 and 1.128 mg/kg). Vitamin contents of mare's milk were very similar to those of cow's milk.

1 Introduction

The relative importance of the horse industry in Hungary has changed greatly in recent years due to mechanisation in agriculture. In 2000, there were 70 000 horses in Hungary, with most of the industry being associated with sport horses and slaughter horses. Currently, there is considerable interest in the use of mare's milk for human consumption in Western Europe. It has been suggested that mare's milk may be curative agent for metabolic and allergic diseases and, consequently, the price paid for mare's milk has increased greatly. This suggests a new possibility for producing income from the horse industry. It also suggests that research is needed to evaluate the value of mare's milk as a human food. Most of the earlier research on composition of mare's milk was directed toward evaluating the value of milk as related to nutrition of the foal.

Duration of lactation has been reported to be 5 to 8 months [13, 34] and estimated milk production of mares was 2000–3000 kg [30]. During a single milking, composition of the milk changes, so the mixed milk of the totally-milked udder must be sampled [5, 24]. Butterfat content undergoes the largest

change [9, 30], and can be 10 to 20 times more at the end than that at the beginning of milking [8, 9, 12]. Sampling method and milking interval [15, 26] also influence the composition of milk. Most authors suggest that foals should be present at sampling [1, 3, 4, 15, 20, 25, 28, 29, 31, 36]. Some others advise an injection of oxytocin [25, 31].

The composition of mares' colostrum was analysed by several authors [20, 27, 33]. Colostral period of mares was found to be much shorter than that of cows, and the colostrum showed significant differences from normal milk only on the first day after foaling [20, 29, 35]. The dry matter content of mare's milk decreased drastically from colostrum to normal milk, due primarily to a decrease in protein content [17, 29]; the fat content and fatty acid composition of milk fat showed much smaller changes over time [2, 17].

The fat content of mare's milk is very low [10, 19]. However, it can be influenced by environment and ranged form 0 to 7.9% [29, 30]. Analysis of the fatty acid composition of butterfat of mare's milk showed [2, 17, 18, 22] that it contains very small quantities of stearic and palmitoleic acids, and high quantities of linolenic and linolic acids. This could be explained by the fact that unsaturated fatty acids are not hydrogenated in the digestive system and horses consume a very large amount of forage, which is rich in unsaturated fatty acids.

Among the factors influencing milk composition, stage of lactation is the most important, but the stage of lactation may [17] or may not [18] influence the fatty acid composition of milk fat. Most authors [21, 29] have not reported breed to affect milk composition, but Boulot [3] reported a significant effect of breed. Increasing the fat content of feeds did not increase the quantity of milk and caused no change in milk composition [36], which differed from effects on cow's milk [10]. Others found higher milk fat content to be associated with higher fat input [7].

Holmes et al. [16] and Kulisa [22] published data on water soluble vitamin contents of mare's milk. No estimates of fat soluble vitamin contents of mare's milk were found in the literature.

Based on the fact that data on the fatty acid composition of mare's milk are limited and that there are virtually no data comparing the fatty acid composition of mare's milk with cow's milk in the same trial, a study was initiated at the University of Kaposvár to evaluate the composition of mare's milk and cow's milk simultaneously. Additional objectives of the experiment were to evaluate time changes in milk composition from foaling to 45 days after foaling and to obtain more detailed information on the quantities of polyunsaturated essential fatty acids present in mare's milk.

2 Material and methods

In the spring of 2001, the experiments were initiated and milk samples of 29 mares (16 Hungarian Draughts, 4 Haflingers, 6 Bretons and 3 Boulonnais) were collected and analysed. Mares were on pasture of relatively good quality (1 ha per mare-foal/pair), from spring to autumn; supplemental feeding, when needed was 3 kg oats per day. Winter feeding was 3 kg hay, 2 kg concentrate and ad lib. straw daily.

Mares were milked on the following schedule: they were driven in the stable at 06:00 hours. Each mare and foal were tied. First milking was started at 09:00 and finished at 11:00 hours. During milking time, foals were released, but retied following milking. Second milking time was between 11:15 and 12:30 hours. The foals were not retied after the second milking because mares and foals were returned to the pasture until 06:00 hours the following day. Milking of mares was accomplished with a Westfalia milking machine (model RPSZ 400).

Colostrum and milk samples (80–100 ml) were taken directly after foaling and on the second and third days of lactation by hand milking. On the 5^{th}, 10^{th}, 30^{th} and 45^{th} days of lactation, the mixed milk of the totally-milked udder was sampled. Colostrum and milk samples were frozen and stored at $-25\,^{\circ}\text{C}$. At the time of analysis, the frozen material was thawed and mixed. Dry matter of colostrum and milk samples was determined by Hungarian Standard No. 3744–67 by drying to constant weight at $105\,^{\circ}\text{C}$. Fat content was determined by the Gerber method according to Hungarian Standard No. 3703–78.

The fatty acid contents of the milk fat were determined in the form of fatty acid methyl esters by a Packard 419 type gas chromatograph, a flame ionisation detector and a Hewlett-Packard 33900 type electronic integrator. In the quantitative evaluation, the weight percentage proportions of the methyl esters were regarded as equal to the proportions of the corresponding peaks in the chromatogram [6].

To determine vitamins A-, D_3- and E-contents of milk, samples (5 ml) were saponified by alcoholic pirogallol solution and 2.5 ml 80% potassium hydroxide. The resulting material was extracted in an alcohol – n-hexane system. The extract was distilled and diluted in 200 μl methanol; 20 μl of the solution was injected on a 250×5 mm column packed with 10 μm granulation Partisil ODS, and the vitamin concentrations were determined on a Pye UNICAM LC-XP HPLC. Elution was carried out with a 85 : 15 solution of methanol : water at 1.4 ml/minute drift speed. The basis for quantitative evaluation was vitamin standards made by MERCK. Vitamin K_3 was determined on a solution ob-

tained by a chloroform extraction of an alkalescent substrate. The extracted vitamin K was detected on 251 nm. Vitamin C content of milk samples was determined by the method of Radeff [32].

3 Results

Dry matter content and changes associated with stage of lactation (colostrum to 45 days) for mares of the four breeds are shown in *Table 1.* Changes in the fat content of colostra and milks for the same period are in *Table 2. Table 3* shows fatty acid contents of milk fat and time changes, expressed as relative percentages of fatty acid methyl esters. Investigations related to comparison of fatty acid composition of mare's milk, cow's milk and blends are summarised in *Table 4.*

Table 1: Dry matter contents of the colostrum and milk of mares (g/100 g milk)

Breed	**No. of mares**	**Days post-partum**		
		0–0.5	2–5	8–45
Haflinger	4	24.25	12.87	10.61
S.D.		4.34	1.49	2.12
Breton	6	24.65	11.93	10.39
S.D.		6.38	2.05	1.24
Boulonnais	3	25.42	12.15	10.37
S.D.		4.12	2.22	1.73
Hungarian Draught	16	26.28	12.78	10.40
S.D.		3.16	1.64	1.57
Mean	29	25.57	12.55	10.42
S.D.		4.10	1.32	1.54

The dry matter content of colostrum immediately after foaling ranged from 14.65 to 29.35%. The mean and standard deviation were 25.57% and 4.10. Due to the large variation, breed differences were not significant ($P \times 0.25$). Dry matter content decreased quickly following foaling, and values found on day 2 differed only slightly from those obtained 5 days after foaling. The dry matter content of transition milk, on days 2 to 5 of lactation, averaged 12.55%. The dry matter content of normal milk obtained on days 8 to 45 averaged

Table 2: Fat contents of the colostrum and milk of mares (g/100 g milk)

Breed	No. of mares	Days post-partum		
		0–0.5	2–5	8–45
Haflinger	4	2.87	2.05	1.04
S.D.		0.462	0.183	0.610
Breton	6	2.91	2.10	1.32
S.D.		0.381	0.214	0.483
Boulonnais	3	2.85	2.17	1.29
S.D.		0.294	0.331	0.390
Hungarian Draught	16	2.93	2.16	1.26
S.D.		0.455	0.163	0.540
Mean	29	2.91	2.13	1.25
S.D.		0.431	0.189	0.499

10.42% with S.D. = 1.54. There were no significant differences among breeds ($P \times 0.25$) in the dry matter content of their colostrum or milk samples.

The fat content of colostrum immediately after foaling averaged 2.91% while that of transition milk and normal milk, respectively, averaged 2.13 and 1.25%. There were no significant differences ($P \times 0.25$) among breeds in the fat content of their colostrum, transition milk or normal milk. These changes in the fat content of colostrum and milk over time after foaling confirm results of other authors [20, 23]. Those authors who reported an increase in the fat content of colostrum to 48 h [35] or to day 7 of lactation [14] probably made a sampling mistake due to the fact that the udder cannot be easily milked totally immediately after foaling and the fat content increases dramatically during milking. No relationship was found between milk quantity and fat content.

Comparison of the fatty acid composition of butterfat of colostrum and milk showed that the fat of colostrum contained less octanoic, decanoic, dodecanoic, miristic, palmitic and palmitoleic acids than that of normal milk. On the other hand, the fat of normal milk contained less stearic, linoleic and linolenic acids than that of colostrum. There were no significant differences among breeds regarding fatty acid content, and data shown in *Table 3* represent the arithmetic average of 29 mares, ignoring breed.

Table 3: Mean and standard deviations for the fatty acid composition of the lipids in colostrum and milk of mares (Relative percentages of the fatty acid methyl esters)

Fatty acid	Days post-partum							
	Mare						Cow	
	0–0.5		2–5		8–45		5–270	
	x	S.D.	x	S.D.	x	S.D.	x	S.D.
Caprylic acid C 8:0	1.39	0.18	2.56	0.94	2.79	0.91	0.29	0.022
Capric acid C 10:0	5.41	0.47	8.59	2.89	8.05	2.25	2.61	0.219
Lauric acid C 12:0	7.90	1.57	9.89	3.19	8.97	2.10	4.35	0.362
Miristic acid C 14:0	6.30	0.26	9.67	1.89	8.72	1.97	14.00	0.998
Palmitic acid C 16:0	21.32	1.58	25.63	2.99	23.28	3.58	44.06	2.10
Palmitoleic acid C 16:1	2.80	1.97	5.07	1.14	3.96	1.52	2.08	1.009
Stearic acid C 18:0	2.36	0.53	1.63	0.51	1.55	0.79	7.94	1.001
Oleic acid C 18:1n9	17.12	0.21	13.77	5.38	13.72	2.58	17.25	1.533
Oleic acid C 18:1n6	0.78	0.29	0.74	0.21	0.69	0.24	*	
Linoleic acid C 18:2n6	9.78	0.83	6.40	0.90	7.53	1.47	1.72	0.198
γ-linolenic acid C 18:3n6	0.75	0.13	0.51	0.03	0.61	0.19	*	
Linolenic acid C 18:3n3	24.11	2.57	15.53	1.99	20.12	4.12	0.09	0.02

* not determined

Results of these analyses were in agreement with the data in the literature. Exceptions were linoleic acid which was significantly less and linolenic acid, which was significantly higher than data in the literature. These differences can be explained by the composition of the diet of mares. The fatty acid composition of feedstuffs have a greater influence on the fatty acid composition of milk fat in case of horses than of ruminants. Microbial action in the rumen

results in more modification of dietary fats than would occur in the horse.

When comparing the fatty acid composition of milk fat of mares at day 45 of the lactation and cows, it can be stated that the fat of mare's milk contains 2.1 times as much dodecanoic acid, 3.1 times as much decanoic acid, 4.9 times as much linoleic acid, 9.6 times as much octanoic acid and 224 times as much linolenic acid as cow's milk fat. On the other hand, the fat of mare's milk contains only 0.62 as much miristic acid, 0.53 as much palmitic acid and 0.2 as much stearic acid as cow's milk fat. The fatty acids which are higher in mare's milk are unsaturated or short chain fatty acids which suggests that mare's milk fat is a more desirable dietary constituents than cow's milk fat. This huge difference between the fatty acid contents of the milk fat of the two species suggested that we could use a function of fatty acid composition to detect the presence of cow's milk in a blend of milks from the two species.

The fatty acid content of cow's milk and mare's milk are shown in the first and last columns of *Table 4.*

A ratio of fatty acid contents, designated as the f-factor, was calculated as the product of fatty acids higher in mare's milk divided by the product of fatty acids higher in cow's milk.

$$f = \frac{\text{caprylic} \times \text{capric} \times \text{lauric} \times \text{linoleic} \times \text{linolenic}}{\text{miristic} \times \text{stearic}}$$

Based on the averages, the f-value for mare's milk was 2257 and that of cow's milk was 0.005. Using day 45 milk of 10 mares and milk of 10 individual cows, f-values were calculated for each individual and the standard deviations were calculated as 112 and 0.0007, respectively, for mares and cows.

Assuming the fat contents of mare's and cow's milks to be 1.5 and 4.0%, respectively, the fatty acid contents of various blends were calculated and entered in *Table 4.* The f-values for 1, 5, 10, 25 and 50% cow's milk were, respectively, 1840, 816, 390, 57, and 5.3. Mare-cow pairs were formed randomly at each blend and 10 f-values were calculated for each blend. These values were used to calculate the standard deviations for each blend, which are shown in *Table 4.* The t-value for 9 degrees of freedom and probability of 0.01 ($t = 3.25$) was used to calculate the 99% confidence band for each blend. Confidence limits = Mean $\times$ 3.25 (S.D.). The confidence limits are shown at the bottom of *Table 4* and it can be seen that we could not be 99% certain of detecting adulteration with cow's milk at a level of 1%, but we could be 99% certain of detecting cow's milk at the level of 5%.

All of the above results were based on calculations. The method was tested by creating five blended samples of individual mares and cows at the level of

Table 4: Fatty acid composition of mare's milk blended with different proportions of cow's milk (calculated values)

Fatty acid	Percentage of cow's milk in the blend						
	Mare's milk	**1**	**5**	**10**	**25**	**50**	**Cow's milk**
Caprylic acid C 8:0	2.79	2.73	2.45	2.23	1.62	0.98	0.29
Capric acid C 10:0	8.05	7.92	7.28	6.82	5.42	4.07	2.61
Lauric acid C 12:0	8.97	8.88	8.29	7.93	6.39	5.62	4.35
Miristic acid C 14:0	8.72	8.87	9.24	9.94	11.21	12.56	14.00
Stearic acid C 18:0	1.55	1.70	2.29	3.00	4.55	6.20	7.94
Linoleic acid C 18:2n6	7.53	7.38	6.71	6.21	4.80	3.31	1.72
Linolenic acid C 18:3n3	20.12	19.61	17.40	15.55	10.71	5.56	0.09
f-values							
mean	2257	1840	816	390	57	5.3	0.005
S.D.	112	62.4	16.2	12.8	4.45	0.44	0.0007
99% confidence limits for f [Mean ± 3.25 (S.D.)]:							
Upper limit	2621	2043	869	432	72	6.7	0.007
Lower limit	1893	1778	763	348	43	3.9	0.003

$f = \frac{\text{caprylic} \times \text{capric} \times \text{lauric} \times \text{linoleic} \times \text{linolenic}}{\text{miristic} \times \text{stearic}}$

5% cow's milk. The f-values ranged from 763 to 824 and, when compared with the calibration curve developed from the calculated values, predicted 4.95% cow's milk. Based on the results, the f-value can be used as a means of detecting small quantities of cow's milk blended with mare's milk and the rate of dilution can be quite accurately predicted.

The analysed vitamin contents of colostrum and milk *(Table 5)* showed that colostrum contained 2.6, 1.7, 1.4 and 1.5 times as much vitamins A, D_3, C and K_3, respectively, as mare's milk between 8^{th} and 45^{th} days of lactation. Vitamin E contents of colostrum and milk were similar. Mare's milk contained practically the same amounts of vitamins A, D_3 and K_3 as cow's milk, but

Table 5: Vitamin contents of mare's colostrum and milk

Vitamin (mg/kg)	Days postpartum Mare		Cow
	0–0,5	8–45	5–270
A	0.88	0.34	0.352
D_3	0.0054	0.0032	0.0029
E	1.342	1.128	1.135
K_3	0.043	0.029	0.032
C	23.8	17.2	15.32

vitamin C content was slightly higher.

Since the fat content of cow's milk is 2.5 to 3.0 times as high as that of mare's milk, the concentrations of liposoluble vitamins in milk fat is much higher in mare's milk fat than in cow's milk fat. The values reported in *Table 5* could appear to be the first reported for contents of vitamins A, D_3, E and K_3. Vitamin C was reported to be 14.7 mg/kg [16] which is slightly lower than the 17.2 mg/kg in *Table 5.*

4 Acknowledgement

The financial support of Hungarian Research Fund (OTKA T 12769) is gratefully acknowledged.

References

[1] A. Aschraft, W.J. Tyznik, Effect of diet on volume and composition in mare's milk, *J. Anim. Sci.,* 43 (1976) 248.

[2] J. Base, K. Zadrazil, *Fatty acid of milk fat in mare's milk,* Proc. XXI. Intern. Dairy Congr., Moscow, 1982. 621–622.

[3] S. Boulot, *L' ingestion chez la jument. Etude de quelques facteurs de variation au cours du cycle gestation-lactation. Implications, nutritionelles et métaboliques,* Thése Doct. Ing., ENSA Rennes, Univ. Rennes I. 1987.

[4] H. Bouwman, W. Van der Schee, Composition and production of milk from Dutch warm-blooded saddle horse mares, *Z. Tierphysiol. Tierern. u. Futtermittelk.,* 40 (1978) 39–53.

[5] J. Csapó, *Colostrum and Milk Composition of Several Genotypes of Cattle,* Ph. D. Thesis. PATE, Kaposvár, 1984.

[6] J. Csapó, L. Sugár, A. Horn, Zs. Csapó-Kiss, Chemical composition of milk from red deer, roe and fallow deer kept in captivity, *Acta Agronomica Hungarica,* 3–4 (1986) 359–372.

[7] K.E. Davison, G.D. Potter, L.W. Greene, J.W. Evans, W.C. McMullan, *Lactation and reproductive performance of mares fed added dietary fat during late gestation and early lactation,* Proc. 10^{th} Eq. Nutr. Physiol. Symp., Colorado State Univ., (1987) 87–92.

[8] H. Dittrich, A study of the composition of mare's milk, *Milchwirtschaftliche Forschungen,* 19. (1938) 406–412.

[9] M. Doreau, S. Boulot, W. Martin-Rosset, H. Dubroeucq, Milking lactating mares using oxytocin, *Reprod. Nutr. Dév.* 26 (1986) 1–11.

[10] M. Doreau, J.P. Bruhat, W. Martin-Rosset, Effects du niveau des apports azotés chez la jument en début de lactation, *Ann. Zootechn.,* 37 (1988) 21–30.

[11] M. Doreau, S. Boulot, Methods of measurement of milk yield and composition in nursing mares, *Le Lait,* 69 (1989) 159–161.

[12] K. Dyusembin, Uneven distribution of major milk components in consecutive portions of a milking in farm animals, *Trudy Inst. Fiziol. Alma-Ata,* 17 (1972) 42–45.

[13] J.D. Feist, D. McCullough, Behaviour patterns and communication in feral horses, *Z. Tierpsyhol.,* 41 (1976) 337–371.

[14] I.A. Forsyth, P.D. Rossdale, C.R. Thomas, Studies on milk composition and lactogenic hormones in the mare, *J. Reprod. Fert.,* 23 (1975) 631–635.

[15] P.D. Gibbs, G.D. Potter, R.W. Blake, W.C. McMullan, Milk production of quarter horse mares during 150 days of lactation, *J. Anim. Sci.,* 54 (1982) 496–499.

[16] A.D. Holmes, B.V. McKey, A.W. Wertz, H.G. Lindquist, L.R. Parkinson, The vitamin content of mare's milk, *J. Dairy Sci.,* 29 (1946) 163–171.

[17] F. Intrieri, L. Minieri, Composition of milk of Hafling mares, *Dairy Sci. Abstr.,* 32 (1970) 665.

[18] J. Jaworski, H. Jaworska, R. Tomczynski S. Smoczynski, Sklad kwasow tlusz czowyh tluszczu mleka klaczy w okresie laktacii, *Zesz. Nauk. Akad. Roln. Tech. Olsztynie,* 17 1982) 85–94.

[19] R. Jenness, *The composition of milk,* In: Lactation of mare, Comprehensive Treatise. Vol III. Nutrition and Biochemistry of Milk (Larson B.L. & Smith V.R. eds.) Academic Press, London, 1974. 3–107.

[20] R.H, Johnston, L.D. Kamstra, P.H. Kohler, Mare's milk composition as related to foal heat scours, *J. Anim. Sci.,* 31 (1970) 549–553.

[21] M. Kulisa, The composition of mare's milk in three horse breeds with reference to N-acetylneuraminic acid, *Acta Agrar. Silv. Ser. Zootech.,* 27 (1977) 25–37.

[22] M. Kulisa, *Selected amino acids, fatty acids and N-acetylneuraminic acid in mare milk,* Proc. 37th Annu. Meet. EAAP, Budapest, 1986. 442.

[23] R.G. Linton, The composition of mare's milk, *J. Agric. Sci.,* 21 (1931) 669–688.

[24] R.G. Linton, The composition of mare's milk, *J. Dairy Sci.,* 8 (1937) 143–172.

[25] J.L. Linzell, Milk yield, energy loss in milk and mammary gland weight in different species, *Dairy Sci. Abstr.,* 34 (1972) 351–360.

[26] W. Martin-Rosset, M. Doreau, J. Cloix, Etude des activités d' un troupeau de pouliniéres de trait et de leurs poulains au paturage, *Ann. Zootech.,* 27 (1978) 33–45.

[27] T.C. McGuire, T.B. Crawford, J.B. Henson, *The isolation, characterization and functional properties of equine immunoglobulin classes and subclasses,* 3rd Internat. Conf. Equine Infectious Diseases, 1972. 364–381.

[28] N. Miraglia, F Quintavalla, P. Mariani, A.L. Catalano, *Plasma biochemistry changes in mares and foals in relation to nutritional aspects,* Proc. 37th Annu. Meet. EAAP, Budapest, 1986. 11.

[29] R. Neseni, E. Flade, G. Heidler, H. Steger, The yield and composition of mare's milk throughout lactation, *Archiv für Tierzucht,* 1 (1958) 91–129.

[30] U. Neuhaus, Milch und Milchgewinnung von Pferdestuten, *Z. Tierzucht.,* 73 (1959) 370–392.

[31] O.T. Oftedal, H.F. Hintz, H.F. Schryver, Lactation in the horse: Milk composition and intake by foals, *J. Nutr.,* 113 (1983) 2196–2206.

[32] T. Radeff, Eine Methode zur Bestimmung des Vitamin-C in der Milch, *Milchw. Forsch.,* 19 (1938) 187–192.

[33] B.T. Rouse, D.G. Ingram, The total protein and immunoglobulin profile of equine colostrum and milk, *Immunology,* 19 (1970) 901–907.

[34] S. Tyler, The behaviour and social organisation of New-Forest ponies, *Anim. Behav. Monogr.,* 5 (1972) 85–196.

[35] D.E. Ullrey, R.D. Sruther, D.G. Hendricks, B.E. Brent, Composition of mare's milk, *J. Anim. Sci.,* 25 (1966) 217–222.

[36] R.A. Zimmerman, *Effect of ration on composition of mare's milk,* Proc. 9th Eq. Nutr. Physiol. Symp., Michigan State Univ., 1985. 96–102.

Received: August, 2008

Acta Univ. Sapientiae, Alimentaria, **2**, 1 (2009) 133–148

Composition of mare's colostrum and milk II. Protein content, amino acid composition and contents of macro- and micro-elements

J. Csapó[1,2]
email: csapo.janos@ke.hu

Sz. Salamon[2]
email: salamonszidonia@sapientia.siculorum.ro

K. Lóki[1]
email: loki.katalin@ke.hu

Zs. Csapó-Kiss[1]
email: csapo.janosne@ke.hu

[1]University of Kaposvár,
Faculty of Animal Science,
Guba S. u. 40, 7400 Kaposvár, Hungary

[2]Sapientia–Hungarian University of Transylvania,
Csíkszereda Campus, RO-530104, Libertatii 1., Miercurea-Ciuc

Abstract. Changes in the protein content, protein fractions and amino acid composition of mare's colostrum and milk, and biological value of milk protein during the first 45 days of lactation were studied. Milk samples (averaging 300–800 cm^3) from 29 lactating mares were collected daily at the beginning of the lactation and weekly from the 5^{th} to 45^{th} days post-partum. Colostrum samples were obtained by hand milking without oxytocin administration, while the foals nursed and milk samples

Key words and phrases: Mare's colostrum and milk, protein content, protein fractions, amino acid composition, biological value, ash content, macro- and micro-elements

were obtained from the mixed total daily production. Each sample was analysed for total protein, true protein, whey protein, true whey protein, casein and non-protein nitrogen (NPN) content using a Kjel-Foss nitrogen analyser and for amino acid composition by LKB amino acid analyser. The biological value of the milk protein was calculated on the basis of amino acid composition by the method of Morup and Olesen [27]. Ash and macro and micro-elements contents of colostrum and milk were also determined.

The total protein, whey protein, casein and NPN contents, respectively, were 16.41, 13.46, 2.95 and 0.052% for colostrum immediately after parturition; 4.13, 2.11, 2.02 and 0.043% for milk between the 2^{nd} and 5^{th} days and 2.31, 1.11, 1.20 and 0.031% for milk in the 8^{th} to 45^{th} days of lactation. The ratios of true protein and whey protein to total protein decreased, while the comparable ratios of casein and NPN increased from foaling to 45 days. The amino acid contents of colostrum and milk decreased during the first 45 days of lactation. Most of the essential amino acids (threonine, valine, cystine, tyrosine, lysine) decreased, while glutamic acid and proline increased in the milk protein after parturition. Therefore, the biological value of the milk protein is highest (132.3) immediately after parturition due to very high levels of threonine and lysine. This value decreases in the course of 5 days to 119.7 and to 107.9 on the 45^{th} day of lactation. The essential amino acid composition and biological value of mare's milk protein was much higher than that of bovine milk proteins.

Ash content of colostrum (0.592%) was significantly higher than that of normal milk (0.405%). Calcium content was lowest immediately after foaling (747.7 mg/kg) and reached a maximum at day 5 (953.7 mg/kg). Zinc and copper contents decreased after reaching a maximum on day 5, while manganese content increased to day 5 and maintained that level. The macro- and micro-element contents (mg/kg) of colostrum and milk, respectively, were: potassium, 928.6 and 517.2; sodium, 320.0 and 166.6; calcium, 747.7 and 822.9; phosphorus, 741.7 and 498.8; magnesium, 139.7 and 65.87; zinc, 2.95 and 1.99; iron, 0.996 and 1.209; copper, 0.606 and 0.249 and manganese, 0.0447 and 0.0544.

1 Introduction

The first report in this series [6] reviewed the reasons for initiating studies on the composition of mare's milk and reported dry matter and fat contents of mare's colostrum and milk and the fatty acid composition of milk fat. This report concerns contents of protein and protein fractions of colostrum and milk, amino acid composition of milk and milk protein, biological value of

mare's milk protein and contents of macro- and micro-elements of colostrum and milk.

Mare's colostrum was reported to contain more than 10% protein and almost 80% of the protein content was immunoglobulins [9, 16, 28]. Those concentrations decreased rapidly after foaling. Most foals are born with agammaglobulinaemia or hypogammaglobulinaemia, and colostrum with a high immunoglobulin content is needed as soon as possible to endow passive immunity [4, 23, 24].

Following the colostral period, the total protein content of mare's milk (N%×6.38 [28]) was 1.7 to 3% [3, 11, 14, 18, 25, 26, 28, 36, 39]. Casein, as a proportion of total protein, was less than 50% in most cases, while whey protein was more than 50%. In mare's milk, the percentage of non-protein nitrogen (NPN) was surprisingly great compared with other farm animal species, and it may represent 10% of total nitrogen content. The NPN fraction contains much free amino acids [10]. The proteose-peptone fraction of mare's milk was reported to be 0.16–0.19% [37, 42]. After the colostral period, the whey protein fraction of mare's milk contains 11 to 21% immunoglobulin, 2–15% serum albumin, 26–50% α-lactalbumin and 28–60% β-lactoglobulin [25, 35, 42, 43].

There are few reliable data on the amino acid composition of colostrum and milk of mares. The amino acid composition of protein showed little change during the colostral period, and was similar to that of ruminants with the exception of arginine and threonine, and was very similar to that of sow's milk [33]. Others [13, 19, 30] reported that the amino acid composition of mare's milk differed significantly from that of other farm animals due to higher cystine and glycine contents. Mare's milk contains much free serine and glutamic acid but is low in methionine.

Among factors which influence protein composition of mare's milk, stage of lactation is the most important. The protein content of mare's milk decreases rapidly to the second week of lactation and continues to decrease slowly to the end of second month [3, 14, 22]. NPN content did not change during the first two months of lactation [29]. After the colostral period, stage of lactation had no significant effect on either the ratio of milk protein fractions [9, 18, 25, 26], or the total amino acid composition [11].

Most authors [9, 13, 18, 28] reported no breed effect on milk composition, but Boulot [2] reported a significant effect. Though there were considerable individual variations [1, 2, 17, 29], there was a low correlation between milk yield and milk composition.

The protein content of mare's milk decreased when the energy content of feed increased [11], which differs from results experienced with cattle [31]. Several

authors [14, 30] have not found a relationship between the nitrogen content of feed and the total protein content of mare's milk, while others reported a dramatic decrease in the levels of protein and NPN in milk if nitrogen content of feed was reduced [10].

Most authors determined only the main components of the protein of mare's milk and very few of them determined the total and free amino acid composition of milk. There is very little information on how protein content and amino acid composition of milk protein change after parturition and during the lactation. There are very few data concerning the free amino acid content of mare's milk and the amino acid composition and biological value of mare's milk protein in comparison with cows' milk and bovine milk protein determined at the same time and by the same method.

Table 1: Macro-element contents of mare's colostrum and milk

Author	Time after foaling (day)	Ash %	K mg/kg	Na mg/kg	Ca mg/kg	P mg/kg	Mg mg/kg
Doreau	7	-	-	-	1350	460	-
et al. [11]	28	-	-	-	1180	420	-
	56	-	-	-	970	360	-
Holmes	28	-	790	-	1060	710	112
et al. [15]	112	-	640	-	1020	630	90
Kulisa [19]	-	0.295	-	-	-	394	29
Linton [20]	28	0.35	-	-	1265	1205	-
	112	0.26	-	-	945	865	-
Oftedal	-	0.42	-	-	-	-	-
et al. [29]							
Neseni	28	0.45	624	112	847	580	-
et al. [28]	112	0.30	303	75	485	467	-
Schryver	7	0.61	664	237	1345	943	118
et al. [34]	28	0.45	469	161	1070	659	86
	105–119	0.32	341	115	700	540	43
Sutton	1	-	-	-	1000	900	-
et al. [38]	30	-	-	-	1000–1200	500–600	-
Ullrey	28	0.46	580	186	1186	358	65
et al. [39]	112	0.27	370	161	614	216	43

Mineral content of mare's milk has been reported to be lower than that of milk of other farm animals. Ash content *(Table 1)* was generally reported to be 0.3 to 0.5% with extremes of 0.2 and 0.7%. Macro-element contents expressed

as mg/kg also varied greatly among the various reports in the literature *(Table 1)* with ranges of 485 to 1350 for calcium, 216 to 1205 for phosphorus, 29 to 118 for magnesium, 75 to 237 for sodium and 303 to 990 for potassium. Mare's milk was found to have 61% of calcium, 31% of phosphorus and 16% of magnesium in colloidal form [8]. Reports of trace elements contents of mare's milk *(Table 2)* are limited in number and the reported concentrations are extremely variable.

Table 2: Micro-element contents of mare's colostrum and milk

Author	**Time after foaling (day)**	**Zn mg/kg**	**Fe mg/kg**	**Cu mg/kg**	**Mn mg/kg**
Kulisa [19]	-	0.89	1.46	0.25	-
Lonnerdal et al. [21]	-	1–2	0.3–1.0	0.2–0.4	-
Schryver et al. [34]	7	3.1	-	0.85	-
	28	2.2	-	0.55	-
Ullrey et al. [40]	0	6.4	1.31	0.99	-
	0.5	2.8	0.95	0.83	-
	1	3.6	1.05	0.73	-
	8	3.3	0.88	0.44	-
	35	2.2	0.71	0.25	-
	120	2.4	0.49	0.20	-
Underwood [41]	-	-	-	0.20–0.36	0.05

Bouwman and Van der Schee [3] reported that ash, calcium and phosphorus contents of milk increased from day two to day three of lactation and then decreased to the 28^{th} day of lactation. Calcium content was reported to decline between the 7^{th} and 56^{th} days of lactation [11]. Ash content, most macro-element contents and some micro-element contents were reported to decrease during lactation [20, 34].

2 Material and methods

Protein and protein fraction contents, amino acid composition and biological value of milk and milk protein of colostrum and milk produced up to the 45^{th} day of lactation by 16 Hungarian Draught, 4 Haflinger, 6 Breton and 3 Boulonnais mares were determined. The feeding, milking and sampling

techniques were described previously [6].

Milk samples, frozen to −25 °C, were thawed in water of 35 °C and blended. Total protein content and protein fractions of colostrum and milk were measured by the Kjel-Foss nitrogen analyser (protein content = N%×6.38). Separation of protein fractions was done as described by [5]. The amino acid composition of milk protein was measured by automatic amino acid analyser (type: LKB 4101). Protein was hydrolysed by 6 M HCl, the sulphur-containing cystine was determined in the form of cysteic acid and the tryptophan content of milk protein was determined by the barium-hydroxide hydrolysis method [7]. The biological value of milk protein was calculated by the method of [27] on the basis of amino acid composition.

Ash content was determined by the Hungarian Standard (MSZ-3926/2-76). The macro- and micro-elements which were present in the ash as metallic oxides, were converted to chlorides by hydrochloric acid and taken into solution. The metallic contents were determined by UNICAM Solaar M6 type atomic absorption spectrofotometer. Phosphorus content was determined by Spekol photometer by measuring the blue colour created by ammonium molibdenate.

The composition of cow's milk was determined on samples from 32 cows by the same methods as were used for mare's milk. The cows were under summer feeding conditions, based principally on grass. The sample consisted of 17 Holstein-Friesian sired crossbred cows (62.5% Holstein-Friesian, 25% Jersey and 12.5% Hungarian red spotted) which were in second or third lactation and 15 Hungaro-Friesian cows in first lactation.

3 Results

The concentration of protein and protein fractions, their changes in mare's colostrum and milk to the 45^{th} day of lactation and distribution of protein fractions are shown in *Table 3*. *Table 4* shows the free amino acid content of mare's colostrum and milk while the total amino acid concentrations in mare's colostrum and milk, expressed as g/100 g fluid and g/100 g protein, are shown in *Table 5*. Ash content and concentrations of macro- and micro-elements are shown in *Table 6*.

There were no significant differences among the four breeds with regard to the protein content of colostrum, transition milk or milk. Breed was found not to influence distribution of protein fractions, amino acid composition, biological value of colostral or milk protein or contents of ash, macro-elements and micro-elements. The results reported are for 29 individual draught mares.

The total protein content of colostrum immediately after foaling ranged from 13.2 and 22.0% and averaged 16.41%. This value decreased to 4.13% in transitional milk (2^{nd} to 5^{th} days) and to 2.13% in milk (8 to 45 days after foaling). Due to the fact that the true protein content was calculated by subtraction of NPN from total protein, the changes in true protein coincided with those of total protein. There were similar large changes in whey protein and true whey protein contents over time. These two components, respectively, decreased from 13.5 to 13.1%, measured immediately after foaling, to 2.1 and 1.8% days 2 to 5 and to 1.1 and 0.9% days 8 to 45.

Corresponding changes in casein and NPN contents were much smaller. The casein content of colostrum was 2.95% immediately after foaling, 2.02% days 2 to 5 and to 1.20% days 8 to 45. The NPN content of colostrum was about 20% higher than that of transition milk and 40% higher than that of normal milk.

The distribution of protein fractions, expressed as percentages of total protein *(Table 3),* changed over time. The true protein content decreased from 97–98% for colostrum to 91% for milk. Contents of whey protein and true whey protein, respectively, decreased from 80 and 82% to 39 and 48%, while casein and NPN contents, respectively, increased from 18 to 52% and from 2.0 to 8.6% during the 45 days after foaling. Most of the observed changes occurred during the first 24 h after foaling, and the remainder of the changes from colostrum to milk occurred gradually over the first five days.

The composition of milk secreted after the 5^{th} day of lactation was almost identical to that of milk on the 45^{th} day of lactation. The authors cited earlier published very few data on colostrum, and these data had much greater standard deviations than we observed. The range of 4.8–25.0% for the protein content of colostrum reported by Linton [20] and the range of 10.6–25.0% reported by Rouse & Ingram [32] indicated great differences among individual mares. The smaller range in present study (13.2 to 22.0%) was probably due to use of consistent sampling methods. We considered first milked colostrum to be only that sample which was taken immediately after foaling before the foal could suckle. If the foal suckles prior to sampling, the sample will be diluted due to initiated milk secretion and the composition is altered significantly. Precise timing of the first sample is much more important for mares than for cows because the quantity of colostrum is much less than that in the mammary gland of cows; therefore, the dilution after sucking is much greater. The value of 16.41% for the total protein content of first milked colostrum measured by us was 2.5% lower than that reported by Ullrey et al. [39] and 5–8% higher than that measured by Sutton et al. [38].

Table 3: Means and standard deviations of protein contents and protein fractions of mare's colostrum and milk (g/100 g milk), and distribution of mare's milk protein fractions as percentages of total protein

Protein fractions	Days postpartum					
	0–0.5		2–5		8–45	
	x	S.D.	x	S.D.	x	S.D.
Total protein	16.41	3.21	4.13	0.77	2.31	0.50
True protein	16.08	2.98	3.86	0.72	2.11	0.48
Whey protein	13.46	2.63	2.11	0.61	1.11	0.32
True whey protein	13.13	2.41	1.84	0.50	0.91	0.25
Casein	2.95	0.34	2.02	0.26	1.20	0.14
NPN × 6.38	0.34	0.041	0.27	0.015	0.20	0.052

Protein fractions	Days postpartum		
	0–0.5	2–5	8–45
Total protein	100	100	100
True protein	97.96	93.44	91.38
Whey protein	82.02	51.09	48.05
True whey protein	79.98	44.53	39.43
Casein	17.98	48.91	51.95
NPN × 6.38	2.04	6.56	8.62

There are no previous reports concerning the protein fractions of colostrum. The results reported here for the protein fractions of milk are difficult to compare with data of authors cited earlier because they reported casein contents ranging from 41 to 65% of the total protein while we obtained a value of 49 to 52%. There are no reports on changes in protein fractions from colostral period to 45th day of lactation. Values observed for the NPN content of mare's milk were slightly higher than those discussed earlier.

The free amino acid content *(Table 4)* of colostrum, with the exception of threonine, serine and glutamic acid, were about twice as high as those of normal milk. When the composition of free amino acids is expressed in percentages, it is clear that colostrum contained approximately five times as much basic (histidine, lysine, arginine) amino acids, and only about 1/3 to 1/2 as much acidic amino acids as normal milk. The concentration of free amino acids in colostrum was 63.68 mg/100 g, which is 19.01% of NPN. These values in case of milk were 31.19 mg/100 g and 15.67%, respectively. Therefore, ap-

proximately 16–20% of NPN of colostrum and milk of mares is in the form of free amino acids. It has been reported [19] that mare's milk contains more free serine and glutamic acid than free methionine. The values in *Table 3* would indicate that this statement could be expanded to say that the proportion of all other investigated free amino acids were greater than that for methionine. The sulphur-containing amino acids (methionine and cystine) represented much lower proportions of the free amino acids than any of the other amino acids investigated.

Table 4: Free amino acid contents of mare's colostrum and milk

Amino	**Free amino acid content**			
acid	**mg free AA/100 g milk**		**g free AA/100 g free AA**	
	colostrum	milk	colostrum	milk
Asp	2.90	0.60	4.6	1.9
Thr	2.90	3.57	4.6	11.5
Ser	5.59	8.97	8.8	28.8
Glu	9.21	9.92	14.5	31.8
Pro	2.50	1.61	3.9	5.2
Gly	4.01	1.01	6.3	3.2
Ala	3.68	0.66	5.8	2.1
Cys	0.53	0.06	0.8	0.2
Val	9.21	1.67	14.5	5.4
Met	0.39	0.03	0.6	0.1
Ile	1.84	0.16	2.9	0.5
Leu	3.75	0.35	5.9	1.1
Tyr	1.38	0.28	2.2	0.9
Phe	1.51	0.57	2.4	1.8
Lys	6.32	0.88	9.9	2.8
His	6.38	0.66	10.0	2.1
Arg	1.58	0.19	2.5	0.6
Totals	63.68	31.19	100.2	100.0

The amino acid compositions of colostrum and milk *(Table 5)* show that changes in amino acid content paralleled those of total protein content relative to time after foaling. It means that each amino acids decrease, without exception, from colostrum to milk (8 to 45 days). For example, threonine content declined from 1.13 g/100 g colostrum to 0.10 g/100 g milk and the corresponding change for glutamic acid was 2.28 to 0.47. When the amino acid composition was expressed as g AA/100 g protein, the changes were much less

apparent. Threonine decreased from 6.9 to 4.3 g/100 g protein while glutamic acid increased from 13.8 to 20.1 g/100 g protein. The sum of five essential amino acids (threonine, valine, cystine, tyrosine and lysine) decreased from 26.4 to 21.3 g/100 g protein, while the total of two nonessential amino acids (glutamic acid and proline) increased from 21.9 to 28.5 g/100 g protein.

Table 5: Amino acid composition of mare's colostrum and milk, amino acid composition of colostrum and milk proteins and amino acid composition of cow's milk and cow's milk proteins

Amino acid	Free amino acid content						Cow's milk	Cow's milk protein
	Days postpartum			Days postpartum				
	0–0.5	2–5	8-45	0–0.5	2–5	8-45		
	g AA/100 g sample			g AA/ 100 g protein			g AA/100 g milk	g AA/100 g protein
Asp	1.543	0.404	0.246	9.3	9.7	10.4	0.26	7.8
Thr	1.132	0.235	0.101	6.9	5.7	4.3	0.15	4.5
Ser	1.444	0.306	0.147	8.7	7.4	6.2	0.16	4.8
Glu	2.281	0.702	0.474	13.8	16.9	20.1	0.77	23.2
Pro	1.346	0.339	0.197	8.1	8.2	8.4	0.32	9.6
Gly	0.558	0.124	0.045	3.4	3.0	1.9	0.06	1.8
Ala	0.673	0.157	0.076	4.1	3.8	3.2	0.10	3.0
Cys	0.164	0.033	0.014	1.0	0.8	0.6	0.02	0.6
Val	0.853	0.198	0.097	5.2	4.8	4.1	0.16	4.8
Met	0.213	0.054	0.035	1.3	1.3	1.5	0.06	1.8
Ile	0.492	0.132	0.090	3.0	3.2	3.8	0.14	4.2
Leu	1.444	0.388	0.229	8.7	9.3	9.7	0.29	8.7
Tyr	0.771	0.182	0.101	4.7	4.4	4.3	0.15	4.5
Phe	0.738	0.200	0.111	4.5	4.8	4.7	0.16	4.8
Lys	1.444	0.351	0.189	8.7	8.4	8.0	0.27	8.1
His	0.492	0.116	0.056	3.0	2.8	2.4	0.10	3.0
Arg	0.706	0.186	0.123	4.3	4.5	5.2	0.11	3.3
Trp	0.229	0.054	0.028	1.4	1.3	1.2	0.05	1.5
Totals	16.523	4.161	2.359	100.1	100.3	100.0	3.33	100.0

These results seem to contradict data reported by Doreau et al. [11] who reported no change in the amino acid composition of mare's milk protein between the 7^{th} and 56^{th} day of lactation. However, they did not investigate the critical period of the first five days after foaling when the largest changes occurred in our investigation. We did not find a publication which reported data on the amino acid composition of colostrum milked immediately after foaling.

Data for the amino acid composition of milk protein, with the exception of the two sulphur-containing amino acids, agree with results of Doreau et al. [11] and Peltonen et al. [30]. In the case of some amino acids, our results differed from results reported by Kulisa [19] and Sarkar et al. [33].

The biological value of milk protein was calculated by the method of Morup & Olesen [27] based on amino acid composition. The biological value of colostrum milked immediately after foaling (132.3) almost reached the maximum of the method (140), which was due to the very high threonine and lysine contents. During days 2 to 5, this value decreased to 119.7 due to the reduced quantities of essential amino acids. From the 8^{th} to the 45^{th} day, the biological value of milk protein was 107.9. This is a very high biological value compared to that of cow's milk which was 80.2 based on data in *Table 3.* These differences can be explained by the higher proportion of whey protein and higher quantities of essential amino acids, especially threonine, in mare's milk. There are no comparable data in the literature.

Ash content of mare's colostrum averaged 0.592% during the first 48 hours of lactation with a range of 0.515 to 0.804 *(Table 6).* There were no literature values reported prior to day 7 *(Table 1).* Ash content decreased to 0.513% (range 0.499 to 0.542%) on days 3 to 5 and to 0.405% (range 0.301 to 0.479%) in the period 8 to 45 days. The latter value is comparable of the mean of literature values in *Table 1.* The values of 0.61% [34] would seem to be high while values of 0.30% or lower [19, 28, 39] would seem to be low.

All macro-elements except calcium decreased in the colostral period and at the beginning of lactation. Decrease was most evident for magnesium, but significant decrease was also experienced for potassium and sodium. Phosphorus content showed little change prior to 5^{th} day of lactation. Calcium content of mare's colostrum was lowest right after foaling (747.7 mg/kg), reached a maximum of 953.7 mg/kg on the 5^{th} day of lactation and then declined. Ullrey et al. [39] observed maximum calcium content on the 8^{th} day after foaling. Macro-element contents of mare's milk were in good agreement with literature values in *Table 1.*

Time trends for micro-element contents of mare's colostrum and milk showed that zinc and copper decreased continuously, iron decreased after reaching a maximum on the 5^{th} day, manganese increased to the 5^{th} day and then stayed constant. Ullrey et al. [40] published data on micro-element contents of mare's colostrum and reported higher zinc, iron and copper contents of colostrum than we observed in this study. Differences were negligible for later periods of the lactation. Comparison of micro-element contents of mare's milk *(Table 6)* with literature data shows that 1.99 mg/kg for zinc content agrees well with about

Table 6: Ash, macro-element and micro-element contents of mare's milk and cow's

Analysis	Days postpartum							
	Mare						Cow	
	0–2		3–5		8–45		5-270	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Ash, %	0.592	0.091	0.526	0.019	0.405	0.063	0.753	0.031
Macro-elements (mg/kg)								
Potassium	928	75	709	138	517	65	1204	68
Sodium	320	86	177	44	167	72	504	34
Calcium	748	190	953	86	823	125	1287	143
Phosphorus	742	109	638	121	499	83	996	111
Magnesium	140	81	86	15	66	16	139	12
Micro-elements (mg/kg)								
Zinc	2.95	1.36	2.08	0.50	1.99	0.28	5.63	0.19
Iron	1.00	0.54	1.58	0.90	1.21	0.63	1.07	0.32
Copper	0.61	0.30	0.25	0.12	0.23	0.09	0.30	0.06
Manganese	0.045	0.025	0.053	0.022	0.054	0.029	0.093	0.013

half of the literature data, and is slightly lower then the others. Iron, copper and manganese contents were also in general agreement with values shown in *Table 2.*

Cow's milk contained almost twice as much ash, potassium, phosphorus, magnesium and manganese, 50% more calcium, iron and copper and almost three times as much sodium and zinc as mare's milk. The low sodium content of mare's milk is a particularly desirable attribute for a dietary component for cardiovascular and hypertension patients.

4 Acknowledgement

The financial support of Hungarian Research Fund (OTKA T 12769) is gratefully acknowledged.

References

[1] H. Balbierz, M. Nikolajczuk, A. Poliwoda, M. Ruda, Study of whey proteins of mares' colostrum and milk during nursing, *Polskie Archiwum*

Weterynaryjne, 18 (1975) 455–465.

[2] S. Boulot, *L' ingestion chez la jument. Etude de quelques facteurs de variation au cours du cycle gestation-lactation. Implications, nutritionelles et métaboliques,* Thése Doct. Ing., ENSA Rennes, Univ. Rennes I. 1987.

[3] H. Bouwman, W. Van der Schee, Composition and production of milk from Dutch warm-blooded saddle horse mares, *Z. Tierphysiol. Tierern. u. Futtermittelk.,* 40 (1978) 39–53.

[4] A. Conti, J. Godovac-Zimmerman, J. Liberatori, G. Braunitzer, The primary structure of monomeric beta-lactoglobulin from horse colostrum, *Hoppe-Seyler's Z. Phisiol. Chem.,* 365 (1984) 1393–1401.

[5] J. Csapó, *Colostrum and Milk Composition of Several Genotypes of Cattle,* Ph. D. Thesis. PATE, Kaposvár, 1984.

[6] J. Csapó, J. Stefler, T.G. Martin, S. Makray, Zs. Csapó-Kiss, Composition of mares' colostrum and milk. I. Fat content, fatty acid composition, and vitamin contents, *International Dairy Journal,* 5 (1994) 393–402.

[7] J. Csapó, I. Tóth-Pósfai, Zs. Csapó-Kiss, Optimization of hydrolysis at determination of amino acid content in food and feed products, *Acta Alimentaria,* 15 (1986) 3–21.

[8] D.T. Davies, C. Holt, W.W. Christie, *The composition of milk,* In: T.B. Mepham, Biochemistry of lactation. Elsevier, Amsterdam, 1983. 71–117.

[9] S. Deskur, I. Leonhard-Kluz, K. Grochowalski, M. Rychwalsa-Nahlik, Wystepowanie biegunek u zrebiat w czasie rui pozrebietnej klaczymatki a sklad bialek serwatkowych mleka. *Rocz. Nauk. Roln.,* 5 (1978) 115–127.

[10] M. Doreau, J.P. Bruhat, W. Martin-Rosset, Effects du niveau des apports azotés chez la jument en début de lactation, *Ann. Zootechn.,* 37 (1988) 21–30.

[11] M. Doreau, S. Boulot, J.P. Barlet, P. Patureau-Mirand, Yield and composition of milk from lactating mares: effect of lactation stage and individual differences. *J. Dairy Res.,* 57 (1990) 449–454.

[12] K.I. Duisembaev, Mare milk proteins, *Trudy Alama-Atinskogo Zooveterinarnogo Instituta,* 23 (1973) 76–80.

[13] K.I. Duisembaev, B.R. Akimbekov, Variation of milk yield and its relationship with milk composition of mares at a koumiss farm, *Dairy Sci. Abstr.,* 46 (1982) 652.

[14] P.D. Gibbs, G.D. Potter, R.W. Blake,W.C. McMullan, Milk production of quarter horse mares during 150 days of lactation, *J. Anim. Sci.,* 54 (1982) 496–499.

[15] A.D. Holmes, A.F. Spelman, C.T. Smith, J.W. Kozmesk, Composition of mare's milk as compared with other species, *J. Dairy Sci.,* 30 (1947) 385–395.

[16] F. Intrieri, L. Minieri, Sul contenuto in acidi grassi della quota lipidica del colostro e del latte di cavalla. Indagini su soggetti di razza avelignese, *Acta Med. Vet. Napoli,* 16 (1970) 89–98.

[17] R.H. Johnston, L.D. Kamstra, P.H. Kohler, Mare's milk composition as related to foal heat scours, *J. Anim. Sci.,* 31 (1970) 549–553.

[18] M. Kulisa, The composition of mare's milk in three horse breeds with reference to N-acetylneuraminic acid, *Acta Agrar. Silv. Ser. Zootech.,* 27 (1977) 25–37.

[19] M. Kulisa, *Selected amino acids, fatty acids and N-acetylneuraminic acid in mare milk,* Proc. 37th Annu. Meet. EAAP, Budapest, 1986. 442.

[20] R.G. Linton, The composition of mare's milk, *J. Dairy Sci.,* 8 (1937) 143–172.

[21] B. Lonnerdal, C.L. Keen, L.S. Hurley, Iron, copper, zinc and manganese in milk, *Rev. Nutr.,* 1 (1981) 149–174.

[22] V.K. Lukas, W.W. Albert, F.N. Owens, A. Peters, Lactation of Shetland mares, *J. Anim. Sci.,* 34 (1972) 350.

[23] T.C. McGuire, T.B. Crawford, J.B. Henson, *The isolation, characterization and functional properties of equine immunoglobulin classes and subclasses,* 3rd Internat. Conf. Equine Infectious Diseases, 1972. 364–381.

[24] T.C. McGuire, M.J. Poppie, K.L. Banks, Hypogammaglobulinemia predisposing to infection in foals, *JAVMA,* 166 (1975) 71–75.

[25] L. Minieri, F. Intrieri, Ricerche ellettroforetiche sulle frayioni proteiche del colostro e del latte di cavalle di rayya avelignese, in ropporto alla distanya dal parto, *Acta Med. Vet. Napoli,* 16 (1970) 73–88.

[26] N. Miraglia, F. Quintavalla, P. Mariani, A.L. Catalano, *Plasmabiochemistry changes in mares and foals in relation to nutritional aspects,* Preliminary trials,. Proc. 37$^{\text{th}}$ Annu. Meet. EAAP, Budapest, 1986. 11.

[27] K. Morup, E.S. Olesen, New method for prediction of protein value from essential amino acid pattern, *Nutrition Reports International,* 13 (1976) 355–365.

[28] R. Neseni, E. Flade, G. Heidler, H. Steger, The yield and composition of mare's milk throughout lactation, *Archiv für Tierzucht,* 1 (1958) 91–129.

[29] O.T. Oftedal, H.F. Hintz, H.F. Schryver, Lactation in the horse: Milk composition and feed intake by foals, *J. Nutr.,* 113 (1983) 2196–2206.

[30] T. Peltonen, V. Kossila, L. Huida, *Effect of protein supplementation on milk composition of the mare and growth rate of their foals,* Proc. 31st Annu. Meet. EAAP, Munchen, 1980. 6.

[31] B. Rémond, Influence de l'alimentation sur la composition du lait de vache. 2. Taux protéique: facteurs généraux, *Bull. Tech. C.R.Z.V. Theix, INRA,* 62 (1985) 53–67.

[32] B.T. Rouse, D.G. Ingram, The total protein and immunoglobulin profile of equine colostrum and milk, *Immunology,* 19 (1970) 901–907.

[33] B.C.R. Sarkar, A.J. Rykala, C.W. Duncan, The essential amino acid content of the proteins isolated from milk of the cow, ewe, sow, and mare, *J. Dairy Sci.,* 36 (1953) 859–864.

[34] H.F. Schryver, O.T. Oftedal, J. Williams, L.V. Soderholm, H.F. Hintz, H.F. Lactation in the horse: the mineral composition of mare milk, *J. Nutr.,* 116 (1986) 2142–2147.

[35] B. Senft, F. Meyer, *Changes in the concentration of protein fraction in the blood and milk of mares and in the blood of their foals,* Proc. 31$^{\text{st}}$ Annu. Meet. EAAP, München, 1980. 6.

[36] S. Smoczynsky, R. Tomczynski, A study of chemical composition of mare milk. I. Chemical composition of mare milk in first ten days of lactation, *Zesz. Nauk. Akad. Rol. Tech. Olszt.,* 17 (1982) 77–83.

[37] G. Storch, Untersuchungen über einige Inhaltsstoffe und Eigenshaften von Stutenmilch und Kumyss unter besonderer Berücksichtigung diätetischer Fragestellungen, *Thesis Univ. Giessen, in Dairy Sci. Abstr.,* 48 (1985) 873.

[38] E.I. Sutton, J.P. Bowland, W.D. Ratcliff, Influence of level of energy and nutrient intake by mares on reproductive performances and blood serum composition of the mares and foals, *Can. J. Anim. Sci.,* 57 (1977) 551–558.

[39] D.E. Ullrey, R.D. Sruther, D.G. Hendricks, B.E. Brent, Composition of mare's milk, *J. Anim. Sci.,* 25, (1966) 217–222.

[40] D.E. Ullrey, W.T Ely, R.L. Covert, Iron, zinc and copper in mare's milk, *J. Anim. Sci.,* 38 (1974) 1276-1277.

[41] E.J.Underwood, *The mineral nutrition of livestock,* CAB, Slough, 1981. 180.

[42] Z.K. Urbinisov, G.K. Servetnik-Chalaya, E.A. Izatullaev, Protein content of mare's milk, *Molochnaya Promyschlennost,* 2 (1981) 45.

[43] V. Ustinova, A. Kazantseva, R. Baturina, T. Dementeva, *Siberian koumiss. Konevodstvo Konnyi Sport,* 4 (1983) 17.

Received: August, 2008

Acta Universitatis Sapientiae

The scientific journal of the Sapientia University publishes original papers and deep surveys in several areas of sciences written in English.
Information about the appropriate series can be found at the Internet address http://www.acta.sapientia.ro.

Acta Universitatis Sapientiae, Alimentaria

Contact address and subscription:
Acta Universitatis Sapientiae, Alimentaria
RO 400112 Cluj-Napoca
Str. Matei Corvin nr. 4.
Email: acta-alim@acta.sapientia.ro

This volume contains two issues.

Sapientia University

Scientia Publishing House

ISSN 1844-7449

http://www.acta.sapientia.ro

Information for authors

Acta Universitatis Sapientiae, Alimentaria publishes original papers and deep surveys in all field of Food Science. All papers will be peer reviewed.

Papers published in current and previous volumes can be found in Portable Document Format (pdf) form at the address: http://www.acta.sapientia.ro.

The submitted papers should not be considered for publication by other journals. The corresponding author is responsible for obtaining the permission of coauthors and of the authorities of institutes, if needed, for publication, the Editorial Board disclaiming any responsibility.

Submission must be made by email (acta-alim@acta.sapientia.ro) only, using etheir the LaTeX style and sample file at the address: http://www.acta.sapientia.ro or a Word format. Beside the LaTeX or Word source file a pdf format of the paper is needed too.

References should be listed alphabetically using the following examples:

For papers in journals:

D. Precht, J. Molkentin, Frequency distributions of conjugated linoleic acid and trans fatty acid contents in European bovine milk fats, *Milchwissenschaft*, 55, 12 (2000) 687-691.

For books:

J. M. Walker, *The protein protocols Handbook*, Humana Press 2000. pp. 1146.

For papers in contributed volumes:

P.M. Masters, M. Friedman, *Amino acid racemization in alkali treated food proteins - chemistry, toxicology and nutritional consequences,* In: J.R Whittaker, M. Fujimaki (eds) Chemical deterioration of proteins. Am Chem Soc, Washington DC 1980. 165–194.

For internet sources:

E. Ferrand, An Analogue of the Thue-Morse Sequence, *Electron. J. Comb.* 14 (2007) #R30, http://www.combinatorics.org/

Illustrations should be given in Encapsulated Postcript (eps) format.

Authors are encouraged to submit papers not exceeding 15 pages, but no more than 10 pages are preferable.

Each author is entitled to an issue containing his paper free of charge. No reprints will be available.

Publication supported by