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Structural and quantitative analysis of exopolisaccharides and oligosaccharides produced by lactobacillus. I. Basic information, isolation, quantitative determination, molecular mass and monosaccharide composition

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Abstract. There are a number of lactic acid bacteria capable to produce exopolysaccharides (EPS), mucoid bacteria cultures, which, when utilized in yogurt production, can improve the structure of the product, leading to more favourable rheological properties and increased viscosity. The current focus of interest in the field of oligosaccharides are those compounds, which contain 2-10 molecules of glucose and/or fructose and galactose; these are called galacto-oligosaccharides (GalOS) and are considered to have a positive effect on the intestinal function, promote beneficial bifidobacteria proliferation, reduce the pH and the amount of unwanted products generated during the putrefaction process. In order to use the EPS and the GalOS in the food industry and to examine their physiological effects, there is a need to develop analytical methods, which allow the determination of the quantity and the composition of these carbohydrates.

In order to examine the EPS, they need to be extracted from their medium. For this process, in addition to the traditional methods, preparative chromatographic purification or enzymatic breakdown of unwanted materials has also been used. The amount of EPS was usually determined directly from the matrix or from the partially purified fermented liquid and rarely from the aqueous solution of the prepared extract. The quality characteristics examination of these products include the determination of the molecular weight, the determination of the monosaccharide composition and the determination of the interconnection region, the isomerism, the chain structure and the phosphor content of the monosaccharides with various methods.

For the quantitative measurement of EPS, the following methods were used: mass measurements, measurement of absorbance after derivatization, preparative ion chromatography, and most of the authors determined the molecular weight by using gel chromatography, also known as size-exclusion chromatography. To determine the monosaccharide composition of the EPS, gas chromatography or high-performance liquid chromatography was used with or without derivatization.

1 Introduction

Several bacteria produce such kind of polysaccharides, which they do not incorporate into their cell structure but excrete them outside the cell. These kinds of substances form mucus in the intercellular space or become fixed to the cell wall, surrounding it as a shell (*Sutherland*, 1972). These kinds of polysaccharides are called exopolysaccharides (hereinafter referred to as: EPS) because the polysaccharides can be found outside the cell wall.

A number of lactic acid bacteria are also capable of producing EPS (*Sutherland*, 1972; *Cerning*, 1990; *Cerning et al.*, 1988; *Doco et al.*, 1990; *Ariga et al.*, 1992). The use of these kinds of mucoid cultures in the yogurt production can improve the structure of the product (*Cerning*, 1990; *Andres*, 1982; *Schellhaass* & *Morris*, 1985; *Teggatz* & *Morris*, 1990) because of the effect of EPS results in a more favourable rheological property and an increased viscosity (*Macura* & *Townsley*, 1984; *Manca De Nadra* et al., 1985), while the breakup of the gel structure and the syneresis will be avoidable (*Cerning et al.*, 1988). By using some kind of EPS-producing lactic acid bacteria species, the utilization of stabilizers of vegetal origin is avoidable, producing this way a "natural," "without additives" yogurt (*Marshall* & *Rawson*, 1999; *De Vuyst et al.*, 1998; *Uemura et al.*, 1993) or ice cream (*Christiansen et al.*, 1999). The presence of EPS is not desirable in any kind of food product; the consistence of wines will be mucous and oily from it (*Dueñas-Chasco et al.*, 1998).

Some EPS could have positive effects on human health because they are non-digestible food ingredients (*Gibson* & *Roberfroid*, 1995), on the one hand, and they have been attributed to have anti-carcinogenic (*Oda et al.*, 1983; *Kitazawa et al.*, 1991) and cholesterol-lowering (*Nakajima et al.*, 1992) properties, on the other hand. In recent years, extensive studies have been carried out on the mapping of the structure of EPS (*Dueñas-Chasco et al.*, 1998; *Casteren et al.*, 1998; *Urashima et al.*, 1999; *De Vuyst et al.*, 1998; *Uemura et al.*, 1998) and the determination of the amount of EPS (*Dueñas-Chasco et al.*, 1998; *Casteren et al.*, 1998; *Sebastiani* and *Zelger*, 1998; *Urashima et al.*, 1999; *Marshall* and *Rawson*, 1999; *De Vuyst et al.*, 1998; *Uemura et al.*, 1998; *Casteren et al.*, 1998) produced by the various types of lactic acid bacteria species. According to some authors, depending on the fermentation conditions, the resulting structure of the EPS may differ even in the case of the same bacteria (*Petit et al.*, 1991; *Kojic et al.*, 1992; *Cerning et al.*, 1994; *Grobet et al.*, 1996). The amount of EPS produced, varies widely depending on the fermentation conditions and the species used. To exploit the benefits of the EPS-producing species in food industry, we have to know the quantity, structure as well as the production conditions of the EPS produced by any particular bacteria species used, on the one hand; on the other hand, we have to know if a particular amount of EPS with a particular structure significantly improves the physical properties of the product. In the case of undesirable EPS (e.g. glutinous wine), the goal is to design an enzymatic treatment which can reduce the viscosity, and for this the knowledge of the structure of the particular EPS is required as well (*Dueñas-Chasco et al.*, 1998).

On the basis of their chemical structure, EPS produced by lactic acid bacteria can be divided into three groups (*Cerning*, 1995):

- α-glucans, which are mainly formed from glucose units linked by $\alpha-1,6$ and $\alpha-1,3$ bonds, for example, dextrans;
- fructans, which are mainly formed from fructose molecules linked by $\beta-2,6$ bonds, for example, levans;
- heteropolysaccharides, which are composed of several monosaccharides.

The oligosaccharide structures containing 2-10 molecules of glucose and/or fructose and galactose, which are called galacto-oligosaccharides (hereinafter referred to as GalOS) (*Hyun-Jae Shin et al.*, 1998), have been currently in the focus of interest (*Hyun-Jae Shin et al.*, 1998; *Rustom et al.*, 1998; *Yanahira et al.*, 1998). GalOS have a positive effect on the intestinal function: they promote the growth of beneficial bifidobacteria, reduce the pH and the amount of products formed during the putrefaction process (*Yanahira et al.*, 1998). Since they are non-digestible, they have a physiological effect similar to the fibres, i.e. they lower the blood pressure and the level of cholesterol in the blood serum (*Tomomatsu*, 1994), and serve as a low-energy-containing sweetening agent applicable as a food or cosmetic additive (*Hyun-Jae Shin et al.*, 1998). Due to the above reasons, the industrial production of GalOS is also studied, which – according to our current knowledge – is possible in three different ways (*Hyun-Jae Shin et al.*, 1998):

- chemical or enzymatic hydrolysis of galactan and lactose (*Prenosil et al.*, 1987; *Iwasaki et al.*, 1996; *Bonnin* and *Thibault*, 1996);
- chemical or enzymatic synthesis (*Cote* & *Tao*, 1990; *Thien*, 1995);
- fermentation with eukaryotic cell cultures (*Gorin et al.*, 1964; *Ohtsuka et al.*, 1990; *Shin et al.*, 1995).

Due to its high lactose content, whey is a suitable raw material for the enzymatic production of GalOS (*Rustom et al.*, 1998). The productivity of enzymatic methods has been studied (*Hyun-Jae Shin et al.*, 1998), and in the case of a particular method the fermentation parameters have been optimized (*Rustom et al.*, 1998). These studies also require the quantitative (*Hyun-Jae Shin et al.*, 1998; *Rustom et al.*, 1998) and structural (*Yanahira et al.*, 1998) determination of GalOS.

As the lactose digestion of individuals with lactose intolerance is not as effective as those of healthy individuals, there is a need to reduce the amount of

lactose intake, which can only be done if the lactose content of dairy products is known. *Richmond et al.* (1987) investigated the changes of lactose, glucose and galactose during the production of yogurt. It was found that the lactose content is reduced during the initial heat treatment and the subsequent fermentation.

In summary, it can be stated that in order to use EPS and GalOS in food production and to examine its possible physiological effects there is a need for the existence of analytical methods which enable the quantification and the determination of the composition of these kinds of carbohydrates.

2 Determination of the structure and the quantity of the exopolisaccharides (EPS)

Before the examination, EPS have to be extracted from the medium, which is mostly a fermented liquid, a complex biological system containing living and dead cells, macromolecules "released" into the solution and small molecules as well. This complex task involves the use of traditional methods (separation based on different sedimentation or solubility rates), on the one hand, while in the final step, purification with preparative chromatographic methods or by the application of enzymatic methods for the breakdown of the unwanted materials. The amount of EPS is usually determined directly from the matrix or from the partially purified ferment, and rarely from the aqueous solution of the obtained mixture. The quality characteristics examination of these products includes the determination of the molecular weight, of the monosaccharide composition and that of the interconnection region, the isomerism, the chain structure and the phosphor content of the monosaccharides with various methods.

2.1 Isolation of the exopolysaccharides

The optional steps of the extraction process of exopolysaccharides are the following:

- removal of microorganisms by centrifugation,
- precipitation of proteins from the supernatant solution,
- precipitation of the EPS using an organic solvent,
- cleaning of the EPS with enzymes,

- purification and breakdown into fractions of the EPS by ion-exchange chromatography.

The medium from which the EPS has to be extracted is usually a dairy-based ferment. *Dueñas-Chasco et al.* (1998) first removed the cells with direct centrifugation (20,000 g, 30 min.) and then with centrifugation (11,000 g, 20 min.) applied after cooling (6 °C, 1 hour) (*Urashima et al.*, 1999). In both cases, the following step was the alcoholic precipitation of the EPS from the supernatant. The step of removing the proteins was omitted in the first case (*Dueñas-Chasco et al.*, 1998), while in the second case the authors attempted to remove the proteins by ion-exchange chromatography (*Urashima et al.*, 1999). To precipitate the proteins, *Casteren et al.* (1998) stirred the "raw EPS," which was obtained after centrifugation, ultrafiltration and then lyophilization of the ferment in a solution containing 4% trichloroacetic acid for 2 hours at a temperature of 4 °C followed by centrifugation (28,100 g, 30 min.).

Other authors (*Sebastiani* and *Zelger*, 1998; *Marshall* and *Rawson*, 1999; *De Vuyst et al.*, 1998; *Uemura et al.*, 1998) omitted the first step and began immediately with the precipitation of the proteins followed by the removal of the precipitate. Most of the authors used the trichloroacetic acid method (*Sebastiani* and *Zelger*, 1998; *Marshall* and *Rawson*, 1999; *De Vuyst et al.*, 1998); *Uemura et al.* (1998) reached their goal by acidifying the solution and then boiling it. *Sebastiani & Zelger* (1998) added 70 cm^3 of 80% trichloroacetic acid to 400 cm^3 of the solution, leaving it to stand for one night and then centrifuged at a temperature of 4 °C (13,000 g, 30 min.). *Marshall & Rawson* (1998) added 85% trichloroacetic acid to 1 cm^3 of the yogurt during the precipitation of the protein. *De Vuyst et al.* (1998) added an equal volume of 20% trichloroacetic acid to the ferment and removed the proteins and the cells by centrifugation (25,000 g) for 20 minutes. After diluting the ferment to the two-fold of its initial volume, *Uemura et al.* (1998) precipitated first the casein fraction by adjusting the pH of the ferment to the value of 4.6. The casein and the microbes were removed from the whey applying centrifugation (10,000 g, 4 °C) for 20 minutes. After the neutralization process, the whey was boiled in a water bath for 30 minutes and the insoluble proteins were sedimented by centrifugation (10,000 g, 20 min).

In the third step, most of the authors (*Dueñas-Chasco et al.*, 1998; *Sebastiani & Zelger*, 1998; *Urashima et al.*, 1999; *Marshall & Rawson*, 1999; *Uemura et al.*, 1998; *Casteren et al.*, 1998) precipitated the EPSs with a concentrated alcoholic solution. *De Vuyst et al.* (1998) examined the functionality

of methanol, isopropanol and acetone for this purpose, and decided to use acetone. They added an equal volume of acetone to the deproteinized solution, left it to stand overnight and then centrifuged it (25,000 g, 30 min, 4 °C). After the aqueous dissolution of the pellet, another trichloroacetic acid clarification and a centrifugation followed, closing the procedure with a second precipitation with acetone.

With the exception of *Casteren et al.* (1998), the authors cited that they repeated the clarification with alcohol several times: they dissolved the precipitate formed as a result of the addition of ethanol in water and removed the insoluble parts, then added ethanol to the supernatant again. After the removal of the cells, *Dueñas-Chasco et al.* (1998) added three units of cold ethanol to one unit of supernatant, left the solution stand overnight at 4 °C and then centrifuged it at 4,500 g for 20 min. The resulting precipitate was solved in distilled water and then repeated the treatment with ethanol three times, after which they dissolved the final precipitate in distilled water and dialysed it. *Sebastian & Zelger* (1998) added also three units of alcohol to one unit of the deproteinized solution, kept the mixture at 20 °C for 30 minutes, and centrifuged it at 4 °C for 30 minutes at 13,000 g. The precipitate was dried up under vacuum and then, depending on the amount and solubility of pellets, dissolved it in 5-25 cm^3 of sterile distilled water. The insoluble fraction was separated from the soluble fraction by centrifugation. The insoluble fraction was suspended in distilled water again and three times as much ethanol was added again to the soluble fraction. After centrifugation, the pellet was drained again and dissolved in water. *Urashima et al.* (1999) added three units per volume of cold ethanol to the one unit per volume supernatant. They left it to stand overnight at 4 °C and then centrifuged it at 5,000 g for 10 minutes. The pellet was dissolved in hot water and centrifuged again in the solution for 10 minutes. This operation was repeated three times, after which the resulting polysaccharide was dialysed and lyophilized. *Marshall & Rawson* (1999) added an equal unit per volume of ethanol to the supernatant resulted after the treatment with tricloroacetic acid, suspended the precipitate and carried out a second alcohol precipitation treatment. *Uemura et al.* (1998) also used the same unit per volume of 99.5% ethanol as the volume of the aliquot part of the deproteinized solution to precipitate the EPS. The solution was stirred overnight at 4 °C; then the precipitate was obtained by centrifugation, after which it was dissolved in water and the ethanol precipitation was repeated twice more. After neutralizing the tricloroacetic acid supernatant with 2 M NaOH, *Casteren et al.* (1998) realized the extraction of EPS by adding two units per volume of 96% ethanol at 4 °C. After the centrifugation (28,100 g,

30 min, 4 °C), the precipitate was dissolved in distilled water, was dialysed and lyophilized, and then the sugar content of the fractions was subsequently determined. The "raw" EPS contained 51% by weight of sugar, the EPS precipitated with tricloroacetic acid, consisting mainly of protein substances, contained 17% by weight of sugar, the substances precipitated with ethanol contained 63% by weight of sugar and the supernatant of the alcohol precipitation contained 9% by weight of sugar. As expected, the precipitate of the ethanol solution contained the decisive part of the sugar. The sugar content increased from 51% to 63% after the removal of proteins. The difference in the polysaccharide-monosaccharide composition of the "raw" EPS and the alcohol precipitation was very low.

After purification with tricloroacetic acid and alcohol, *Uemura et al.* (1998) removed the remaining proteins and DNAs with enzymes. The polysaccharides were dissolved in a 0.05 M Tris-HCl buffer (pH=8.0), which also contained 1 mM $MgCl_2$, and were treated at 37 °C for 6 hours with 2 $\mu g/cm^3$ Dnase (type IV, EC.3.1.21.1, Sigma, St. Louis, USA) and RNase (type I-AS, EC.3.1.27.5, Sigma). The protein contamination in the sample was degraded by adding 0.2 mg/cm^3 proteinase K (EC.3.4.21.14, Sigma) at 37 °C for overnight. The reaction was stopped with a heat treatment at 90 °C for 10 minutes. The EPS fraction was precipitated with ethanol, dialysed with distilled water, and then lyophilized.

Urashima et al. (1998), *Uemura et al.* (1998) and Casteren et al. (1998) realized the removal of the remaining proteins as well as the fractionation of EPS by preparative ion-exchange chromatography (IE-HPLC). *Dueñas-Chasco et al.* (1998); *Sebastiani & Zelger* (1998); *Marshall & Rawson* (1999) and *De Vuyst et al.* (1998) omitted this purification and at the same time the purity control step, too. *Sebastiani & Zelger* (1998) did not perform chromatographic purity test, and thus, according to their statement, they "could not detect either peptides or proteins in their EPS mixture".

Urashima et al. (1999) introduced the aliquot part of the polysaccharide solution through a DEAE- Sephadex A-50 (Pharmacia Fine Chemicals) 1.5×20 cm ion-exchange column. The column was equilibrated with a 50 mM Tris- aminomethane – HCl buffer (pH 8.7) and the same buffer was also used for eluting with 250 cm^3 of eluent; after this, a linear NaCl gradient was supplied with further 250 cm^3 of liquid until reaching a concentration of 0-1 M. The presence of proteins was determined using a spectrophotometric detector by measuring the absorbance at 280 nm, while the presence of sugars was determined using the phenol – sulphuric acid method (*Hodge & Hofreiter*, 1962), measuring the wavelength at 490 nm. Using this method, it was possible

to separate well the neutral polysaccharides (which are found at the beginning of the chromatogram) from a fraction containing mainly proteins. However, in addition to the proteins, this fraction contained carbohydrates too, which are probably acidic carbohydrates with acidic properties on the pH of the eluent, whereas they were bound to the column better and could be eluted from the column only due to the "ion exclusion" effect of the NaCl gradient from the column.

Casteren et al. (1998) dissolved the previously purified EPS in 5 mM NaOAc (pH=6) and equilibrated the analytical column with the same buffer (DEAE Sepharose fast flow (52×5 cm) (Pharmacia), Biopilot system). They increased the flow rate during the measurement from 10 cm^3/min to 50 cm^3/min, while the concentration of NaOAc increased from the initial 5 mM to 2 M. The substances which remained on the column were cleaned off the column by applying a flow rate of 20 cm^3/min and a concentration of 0.5 M NaOH. The absorbance of the eluent was measured at 280 nm, while the concentration of the proteins was measured based on the β-casein extinction factor (*Swaigsgood*, 1992). The sugar content of the fractions (approx. 100 mL) was measured with the orcinol method (*Tollier & Robin*, 1979), using a standard glucose curve. In this case, the chromatogram contained two large carbohydrate fraction peaks, of which the second peak, similar to the chromatogram provided by the *Urashima et al.* (1999), coincided with the protein fractions peak.

In such circumstances, *Urashima et al.* (1999) and *Casteren et al.* (1998) were able to purify only the neutral EPS from the proteins in the ion-exchange column. If there is a need to purify the acidic EPS fraction too, a new chromatographic method has to be developed, which allows the separation of the acidic EPS and the proteins, or the sample preparation methods have to be changed. During the pretreatment, *Casteren et al.* (1998) applied a trichloroacetic acid treatment, while *Urashima et al.* (1999) applied no trichloroacetic acid treatment. Despite this fact, *Casteren et al.* (1998) detected a significant amount of proteins; so, the trichloroacetic acid treatment (4% trichloroacetic acid solution, stirring at 4 °C for 2 hours, followed by centrifugation) alone was not a sufficient method for the deproteinization. Based on the reported information, the comparison of the carbohydrate/protein ration of the two methods in the co-eluting fraction (acidic EPS and protein) is not possible, as *Urashima et al.* (1999) communicate only the absorbance values and do not supply information about the concentration of the carbohydrate and protein fractions. The initial composition of the mixture was also different. Therefore, it is possible to suggest the application of tricholoroacetic acid treatment during the sample preparation, because of its property to re-

duce the amount of the protein fraction interfering with the acidic EPS, only on the basis of theoretical considerations.

Uemura et al. (1998) loaded the EPS dissolved in 0.05 M Tris - HCl buffer (pH 8.6) into a DEAE 650M Toyopearl 650 M anion-exchange column (Tosoh, Tokyo, Japan, 2.6×20 cm), equilibrating it previously with the same buffer. After loading, the column was washed with 350 cm^3 buffer having the same composition as the equilibrating solution, and then a NaCl linear gradient ranging from 0 M to 0.5 M was applied. For the detection of neutral sugars in the eluent, the method based on the phenol-sulphuric acid reaction, designed by *Dubois et al.* (1956), was applied and the absorbance of the proteins was measured at the wavelength of 280 nm. The carbohydrate containing fraction was lyophilized after dialysing it at 4 °C by treating it first with 2 M NaOH and then with distilled water. The EPS was separated into two main fractions, non-binding neutral polysaccharide (NPS) and binding acidic polysaccharides (APS). Based on the chromatogram reported, none of the resulting EPS fractions contained a significant amount of eluted proteins. The deproteinization processes associated with the acidification, boiling, and subsequent enzymatic breakdown processes probably removed the bulk amount of the proteins efficiently, or larger amounts of proteins eluted from the column only during its regeneration. However, during the studies based on gel chromatography applications, a protein fraction appeared on the chromatogram, which was separated from the APS.

In summary, if there is a need to have protein-free APS fractions after the isolation of EPS, the steps described by *Uemura et al.* (1998) have to be used, which are the following:

- precipitation of the protein by acidifying and then boiling the solution,
- precipitation of the EPS with ethanol,
- purification of the EPS with enzymes,
- purification and breakdown into fractions of the EPS by ion-exchange or gel chromatography.

If the goal is to extract only NPS fraction, it is sufficient to apply the pretreatment methods described by *Urashima et al.* (1999), which are the following:

- removal of the microorganisms by centrifugation,
- precipitation of the EPS with ethanol,

- purification and breakdown into fractions of the EPS by ion-exchange chromatography.

2.2 Determination of the quantity of exopolysaccharides

The quantitative measurement of the EPS was carried out using the following procedures. *De Vuyst et al.* (1998) and *Uemura et al.* (1998) measured the weight of the EPS extracted from a mixture with a particular volume. *Marshall & Rawson* (1999) and *Sebastiani & Zelger* (1998) generated a colour reaction by adding the adequate reagents to the aqueous solution of the purified EPS, then determined the extinction and from this the concentration. *Urashima et al.* (1999), *Uemura et al.* (1998) and *Casteren et al.* (1998) reacted the fractions, which were separated by preparative ion-exchange chromatography, with reagents leading to colour reaction in the presence of carbohydrates, then determined the concentration after measuring the extinction.

According to *De Vuyst et al.* (1998), "PS determinations are based mainly on indirect methods. The colorimetric techniques result in that the amount of EPS is given in glucose or dextran equivalent values, and there is not necessarily a relationship between the results of the viscosity measurements and the amount of EPS." The amount of EPS was determined by applying the already described EPS isolation procedure on 0.5 L sample, and then washed the polysaccharides, which have been considered to be pure with acetone, dried it for 48 hours at 42 °C, and then measured its weight. The quantity of EPS was expressed in polymer solids/L (mg PDM/L).

Marshall & Rawson (1999) dissolved the purified EPS in 1 cm^3 distilled water, and added 1 cm^3 5% phenol solution and 5 cm^3 concentrated sulphuric acid. The extinction values of the solution were measured at 490 nm, and then determined the concentration of the EPS using standard glucose curves (*Dubois et al.* 1956).

Sebastiani & Zelger (1998) worked with two methods. In the case of the phenol-sulphuric acid method, they dissolved 0.2 cm^3 of EPS preparation in 1.8 cm^3 of bi-distilled water, and then added 50 µl distilled phenol and 5 cm^3 97% sulphuric acid. The mixture was held at room temperature for 10 minutes, stirred with rotation, and then incubated for another 15 minutes at 27 °C. The samples were put into quartz cuvettes and their absorbance was measured at 490 nm against water as a blank. The concentration of glucose was determined using standard glucose calibration curve.

During the other study, 4 cm^3 of ferment was stirred with 0.7 cm^3 80% tricloroacidic acid, and then centrifuged at 2,000 g for 10 minutes. 900 µl

has been examined from the supernatant applying the dextran test method designed by *Keniry et al.* (1969), which was modified according to *Garcia-Garibay & Marshall* (1991). The amount of EPS was determined using calibration curves made of diluted dextran solutions.

During the study of the ferment containing multiple different lactic acid bacteria species or bacteria variety, the two methods gave contradictory results: the dextran test showed detectable levels of EPS only in two cases of five, while the sulphuric acid method produced measurable results in all five cases. The explanation cannot be the greater sensitivity of the latter method since, according to the sulphuric acid test, the difference between the content of the five EPS fractions may be at most a three-fold one. Moreover, the sulphuric acid method resulted in a lower EPS content in the case of a ferment which was detected by the dextran method compared to the ferments where the dextran method did not show the presence of any EPS.

To find the cause of the discrepancy, the EPS mixture was examined for its content of glucose, galactose or lactose by the *Boehringer-Mannheim* enzyme test. The result was negative, after which no proteins or peptides could be detected from the mixture. Performing a quantitative determination of the amount of monosaccharides by HPLC, after the hydrolysis of monosaccharides, the concentration of monosaccharides showed a good match with the EPS results of phenol sulphuric acid method.

From those performing the ion-exchange examination, *Urashima et al.* (1999) and *Uemura et al.* (1998) determined the EPS content of the eluent with the phenol-sulphuric acid method already mentioned (*Dubois et al.*, 1956). *Uemura et al.* (1999) measured the EPS content of 1 L ferment to be 58.4 mg. *Casteren et al.* (1998) measured the sugar content of the fractions using the orcinol method calibrating with glucose solutions.

The applied indirect photometric methods (phenol-sulphuric acid test and dextran method) gave conflicting results. Based on a method comparison reported by *Sebastiano & Zelger* (1998), it seems that the phenol-sulphuric acid method resulted in a better match with the HPLC measurement results than the use of the dextran method; so, the former method is more preferred. Almost all the authors applied the phenol-sulphuric acid method (*Sebastiani & Zelger*, 1998; *Urashima et al.*, 1999; *Marshall & Rawson*, 1999; *Uemura et al.*, 1998), only *De Vuyst et al.* (1998) and *Casteren et al.* (1998) deviated from this method. The exclusive weight determination applied by De Vuyst et al. (1998) can only be used to determine the amount of EPS if the mixture does not contain significant quantities of contaminants (e.g. proteins) or if there is no significant EPS loss during the purification procedures.

2.3 Determination of the molecular weight of the exopolysaccharides

Most authors carry out the molecular weight determination by gel chromatography methods, also known as size exclusion chromatography methods. *Urashima et al.* (1999) determined the molecular weight of the EPS by HPLC, equipped with a Tohsoh SD 8022 pump, TSK gel G6000 PWXL (7.8 mm ID×30 cm) column and a refractometric detector (Tohsoh RI-8020), using distilled water as eluent during the measurement. The molecular weight was determined with the help of a pullulan standard kit (P-800, Shodex). According to their evaluation, the neutral EPS fraction appeared in a single peak on the chromatogram, its molecular weight was around 4.5×106 Da. The acidic fractions and the protein contamination have not been studied.

Uemura et al. (1998) worked also with gel chromatography, using an Asahipak GS-710 type column (7.6×500 mm, Asahi Chemical Industry Co., Kawasaki, Japan). The eluent was a 5 mM acetic acid-triethylamine buffer (pH=5.0). The calibration of the retention volumes was carried out with the elements of the pullulan kit, P-800, P-400, P-200, P-100, P-50, P-20, P-10 and P-5 (Showa Denko KK, Tokyo, Japan). The molecular weight of the NPS eluted as a single peak; it was estimated to be 1.2×10^6 Da. A lower molecular weight protein containing component also appeared in the chromatogram (5.8×10^3 Da), which was completely separated from the APS with a molecular weight of 1.1×10^6 Da.

Casteren et al. (1998) determined the molecular weight using a high-performance size-exclusion chromatography (HPSEC). The measurements were carried out by a SP8700 (Spectra-Physics) HPLC apparatus, equipped with a 300×7.8 mm Bio-Gel TSK column series (60XL, 40XL and 30XL), each of them including a TSK XL column (40×6 mm). The eluent was 0.4 M NaOAc (pH=3.0), the temperature of the column space was 30 °C, the flow rate was 0.8 cm^3/min. The eluent flowed through a Viscotek refractometer or online; for the calibrations, there were used dextrans, where the molecular weight varied from 4,000 to 500,000. Static "light-scattering" (SLS) detector was also used during the analyses. The molar mass of the purified EPS was measured to be 6.8×10^5 g/mol.

2.4 Monosaccharide composition of exopolysaccharides

The determination of the monosaccharide composition consists of the following possible steps:

- breakdown of the polysaccharide to monomer units by acid hydrolysis or by methanolysis,
- derivatization (pyridyl amine monomers, alditol acetates, trimethylsilylated derivatives),
- qualitative and quantitative determination by high-performance liquid chromatography (HPLC) or gas chromatography (GC).

Sebastiani & Zelger (1998) incubated 2 cm^3 of EPS with 1 M sulphuric acid solution in a 5 cm^3 hermetically sealed tube for 3 hours at 100 °C. The determination of the monosaccharide composition was carried out by HPLC, by separating 1 cm^3 mixture (REZEX organic acid column, 50 °C). Water was used as mobile phase at a flow rate of 0.6 mL/min. As EPS components glucose, galactose and rhamnose were detected.

De Vuyst et al. (1998) purified the already purified EPS once again by precipitating it with acetone, and then lyophilized the precipitate. 15 mg/L of the lyophilized solution was hydrolysed with 1-1 cm^3 at 100 °C for 3 hours with 6 M of trifluoroaceti acid or at 100 °C for 4 hours with 0.6 M of hydrochloric acid. The monosaccharide composition of the hydrolysates was calculated as the average value of the four HPLC measurements, where the relative ratio of the peak areas was used to estimate the monomer composition. For the measurements, a Waters HPLC was used (Waters Corp., Milford, MA, USA) equipped with the following modules: Waters 410 differential refractometer, a Waters column thermostat, "Waters 717 plus" sampler and Milennium software version 2.10. 30 µl of the sample was loaded at 35 °C to a Polispher OA KC column (Merck, Darmstadt, Germany). The mobile phase was 0.005 M sulphuric acid solution, the flow rate was 0.4 cm^3/min. In addition to the concentration of glucose and galactose, it was also possible to determine the concentration of lactose and lactic acid; thus, the HPLC assay was suitable to determine the fermentation profile in addition to the EPS composition.

Uemura et al. (1998) determined the sugar composition according to *Hase et al.* (1978) by HPLC method. The samples were hydrolysed in a 2 M trifluoroacetic acid solution at 100 °C for 2 hours, after which a pyridyl amination (PA) and a reduction occurred. The PA-monosaccharides were identified using a HPLC apparatus equipped with the following elements: Hitachi L-6200 "Intelligent Pump" (Hitachi Ltd., Tokyo, Japan), Palpak "A-type" column (4.6 mm × 150 mm, Takara, Kyoto, Japan) and an F-1080 fluorescence detector (Hitachi Ltd.). The elution took place under isocratic conditions using a ratio of 9:1 (v/v) of 0.7 M potassium borate buffer (pH=9.0)/acetonitrile, at a flow rate of 0.3 cm^3/min and at temperature of 65 °C. The detection of PA sugars

was carried out based on the values of their fluorescence (Ex: 310 nm, Em: 380 nm). The area under the peaks was determined using D-5500 Chromate software (Hitachi Ltd.). The assay showed that the EPS contained glucose and galactose.

Casteren et al. (1998) incubated the preparations treated with acid and ethanol, by first using a 12 M sulphuric acid solution at 30 °C for an hour, and then hydrolysing it according to the method of *Sebastiani & Zelger* (1998), with the use of an internal inositol standard by using a 1 M sulphuric acid solution at 100 °C for 3 hours. From the liberated sugars, they formed alditol acetate derivatives according to the method described by *Englyst & Cummings* (1984). These were separated from each other with a Carlo Erba 4200 gas chromatograph, on a 15 m × 0.53 mm size J W DB-225 column. The temperature programme was as follows: 1 min at a temperature of 180 C, heating from 180 °C to 220 °C at a speed of 2.5 °C/min, and then isothermal at 220 °C for 3 minutes. The temperature of the flame ionization detector (FID) was 275 °C and helium was used as a carrier gas. This method could be used to detect the following monomers: glucose, galactose, rhamnose, mannose and xylose.

Using the chemical modifications performed on the EPS, the authors concluded that if the carbohydrate chains contain galactose phosphate esters, there is a risk that the hydrolysis does not take place perfectly in the case of galactose phosphate bonds, thus obtaining a lower amount of alditol-acetate derivatives, and the detected amount of galactose will be lower than the real amount.

Dueñas-Chasco et al. (1998) have analysed the monosaccharides in the form of trimethylsilylated methyl glycosides based on the method of *Chaplin* (1982). The polysaccharides were treated at 80 °C with 0.625 M hydrochloric acid methanol solution for 16 hours, then silylated at 80 °C for 16 hours with the mixture of 1:1 ratio of pyridine-BSTFA. Isobutanol was added to the mixture and then dried in a nitrogen gas stream. The TMS derivatives were analysed by GLC-MS (gas-liquid chromatography–mass spectrometry), whereby a Kratos MS80RFA device was connected to a 25 m × 0.32 mm id. sized CP-Sil5-CB (WCOT ("Wall Coated Open Tubular") column. The temperature of the column space was isotherm for 2 minutes at 140 °C, and then increased to 250 °C at a speed of 8 °C/min.

Urashima et al. (1999) methanizated the polysaccharides in a 2% hydrochloric acid methanol solution at 80 °C for 20 hours, followed by the N-acetylation with acetic anhydride in methanol solution (1:3, v/v). The 2% hydrochloric acid methanol solution was prepared from a 5% HCl methanol solution so

that the solution was diluted with anhydrous alcohol. The methanol isolate was trimethyl silylated with a TMS-HT kit (Tokyo Kasei Co., Tokyo, Japan) and the derivatives were analysed using a Shimadzu 13B GC. The detector was a FID, the column was a 30-m-sized quartz capillary (123-1732 DB-17, Shimadzu, Kyoto, Japan). During the measurement, a temperature gradient from 150 °C to 250 °C was used at a speed of 3 °C/minute. D-galactose and L-rhamnose could be detected in the EPS.

The steps of composition determination were carried out in different ways. In the case of some HPLC methodologies, there is no need for derivatization, while in the case of GC analysis this step cannot be omitted since the reactive groups of alcohol must be sealed with the adding of a less active group. There is no report about the qualitative identification process of the monomers. Only one author used MS (*Dueñas-Chasco et al.*, 1998) to detect monosaccharide derivatives; in the case of the rest of the authors, the identification was perhaps based on the retention time of the standard materials. The principle of quantification is not detailed except by *De Vuyst et al.* (1998), who used the relative ratio of the peak area to determine the ratio of the monomers.

References

The references are located at the end of the second part of the article.

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Structural and quantitative analysis of exopolisaccharides and oligosaccharides produced by lactobacillus. II. The connection status-, configuration-, phosphorous content-, modification-, structure of monosaccharides, exopolysaccharides in yogurt, galacto-oligosaccharides

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Abstract. After derivatization and hydrolysis, it was possible to determine the amount and proportion of the various forms of chemical bonds. The configuration and phosphor content was also examined, and in order to determine the structure of the EPS as well as its physical and chemical properties, there have been carried out chemical modifications, too. By using nuclear magnetic resonance spectroscopy, it was also possible to study the fine structure of the constituent atomic groups of the EPS, elucidating this way the structure of the repeating units and the monomers of these units, the isomerism of the anomeric carbon atoms and the frequency of occurrence of some atom groups. In the second part of the presentation, the glucose, galactose and lactose content of yogurt were determined by the following steps: extraction of the saccharides from yogurt, determination of the quality and quantity of the saccharides by HPLC, and the evaluation of the methods. At the end of the article, different methods are reviewed for the determination of the composition and quantity of the galacto-oligosaccharides.

1.1 The connection status between monosaccharides

All the authors who dealt with the analysis of the binding sites marked the carbon atoms involved in the glycosidic bond, with an acetate group (*Dueñas-Chasco et al.*, 1998; *Urashima et al.*, 1999; *Uemura et al.*, 1998; *Casteren et al.*, 1998). Polysaccharides were first methylated in the free hydroxyl group, and then hydrolysed. After the hydrolysis of the partially methylated sugars, the liberated glycosidic and non-glycosidic hydroxyl groups were converted into acetate, whereby partially methylated alditol acetates were obtained, for which the location of the acetate groups was identical with the binding sites of the monomers before the hydrolysis. Knowing the quantity of derivatives, it is also possible to determine the ratio of the various bonding forms. The main steps were as follows:

- methylation
- purification
- hydrolysis
- acetylation

Dueñas-Chasco et al. (1998) methylated the polysaccharides two times according to the method of *Ciucanu & Kerek* (1984). The product was purified by reverse-phase chromatography using Sep-pak C18 column (*Waeghe et al.*, 1983), and then hydrolysed with 2 M trifluoroacetic acid. The products were reduced according to the method of *Blankeney et al.* (1983), and then acetylated. The partially methylated alditol acetates were analysed by GLC-MS, whereby the temperature programme began with a one-minute heat isotherm phase at 100 °C, then increasing the temperature to 250 °C with a speed of 5 °C/min.

Urashima et al. (1999), *Uemura et al.* (1998) and *Casteren et al.* (1998) used the *Hakomori* method for the methylation of the purified EPS. *Urashima et al.* (1999) purified the methylated polysaccharide further on a silica gel column (Wakogel S-1). The partially methylated alditol acetates were produced from the per-methylated polysaccharides according to the method of *Stellner et al.* (1973). The analysis of the derivatives was performed on a Hitachi 163 GC, equipped with a FID detector and a 02 × 200-cm-sized glass column filled with chromosolv W and moistened with 2% OV- 17. During the analysis, a temperature gradient of 3 °C/min ranging from 150 °C to 250 °C was applied. The GC-MS analysis was performed on a Jeol HX -105 mass spectrometer, in which the ionization power was 100 μA, the voltage was of 70 eV, the column linked to the MS was of type MXT-5, with an internal diameter of 0.25 mm and a length of 60 m. The temperature of the column space increased during the measurements from 150 °C to 250 °C with a speed of 3 °C/min. The determination of the amount of alditol acetates was on the basis of the area under the GC peak.

Uemura et al. (1998) purified the per-methylated carbohydrates on a silica gel column chromatography (Wakogel S-1: 0.3 × 3 cm, Wako Pure Chemicals, Osaka, Japan). The sample was hydrolysed with 90% formic acid at 100 °C for one hour (formolysis), and then hydrolysed with 2 M trifluoroacetic acid at 100°C for 5 hours. The partially methylated monosaccharides were converted into alditol acetates derivatives. The derivatives were separated on a Hitachi 163 GC 0.8 mm × 30 m UA -1 capillary column (Hitachi Co. Ltd., Japan). During the analysis, the temperature was raised from 150 °C to 240 °C with a speed of 1 °C/min.. The identification was carried out by a GC HP 5890A/Jeol JMS HX-105 GC-MS system, the electron acceleration energy was of 70 eV. During the separation, a 0.28 mm × 60-m-sized MXT-5 capillary column (Restek, Bellfoute, USA) was used, the determination of the molar ratios was carried out by measuring the areas under the peak. The identification of the mass spectra was based on the comparison of the obtained mass spectra

with an already known, partially methylated alditol acetate spectrum.

Casteren et al. (1998) dialysed the methylated EPS with water, and then lyophilized them. The hydrolysis was carried out with a 2 M trifluoroacetic acid for 1 hour at 121 °C. After the solution was concentrated under a stream of air at a temperature lower than 20 °C, the partially methylated sugars were converted into alditol acetates based on the method of *Englyst & Cummings* (1984). The analysis was carried out according to the method described by *Vincken et al.* (1994) on a Carlo Erba HRGC 5160 Series GC with flame ionization detector. The amount of partially methylated alditol acetates were determined based on the effective carbon number (Sweet et al., 1975). The identification of the components was performed on HP 5973 mass selective detector to which an HP-6890 GC was attached, equipped with a 25 m × 0.25 mm, 0.2 μm thick, CP Sil 19 CB quartz capillary (Chrompack). During the temperature programme, the first heating range was carried out from 160 °C to 185 °C with a speed of 0.5 °C/min, while the second one from 185 °C to 230 °C with a speed of 10 °C/min, and then held the column at 230 °C for 5.5 minutes. For the data analysis, a Hewlett-Packard ChemStation software was used.

Casteren et al. (1998) have shown that, when determining the binding sites, in the case of phosphorus-containing polysaccharides, the incomplete hydrolysis of the phosphate ester bonds could pose a problem: it may result in the underestimation of the monomers involved in the phosphate binding.

1.2 Configuration of the monosaccharides

Dueñas-Chasco et al. (1998) determined the optical isomers by creating enantiomeric pairs and then analysing them with gas chromatography. After the methanolysis with 0.625 M HCl methanol of the polysaccharides, these were treated with 0.625 M (+)-2-butanol HCl solution under the same conditions, such as in the case of sugar composition analysis, and then trimethylsilylated. The trimethylsilylated 2-butyl glycosides were analysed by GLC-MS, under conditions identical to the monosaccharide composition determination assay, being the only difference in the temperature gradient, which was isothermal at the beginning of the measurement at 130 °C, and then raised to 250 °C with a speed of 2 °C/min.

1.3 Examination of the phosphorous content of the exopolysaccharides

The phosphorus content of NPS and APS was determined by *Casteren et al.* (1998) based on the methods defined by *Dittmer & Wells* (1969). After removing the phosphorus from the APS and NPS by perchloric acid hydrolysis, the resulting derivatives were treated with ammonium molybdate to result in a colour reaction. Measuring the resulting colourful derivative with photometric methods, it was found that the NPS did not contain phosphorus, while the APS contained 0.1% of phosphorus. This was in accordance with the acidic character of the latter one, which came to light during the ion-exchange column chromatography.

Casteren et al. (1998) determined the total phosphorus content of the samples with a colour reaction by treating them with 72% perchloric acid at 180 °C for 20 minutes and the free phosphorus content with colour reaction without any treatment. After the dilution of the treated and the untreated samples, these were analysed based on the methods of *Chen et al.* (1956). To find out whether the hydrolysis carried out during the sugar composition analysis broke all the sugar – phosphate bonds down, the free phosphorus content of the hydrolysed samples was also determined.

1.4 Chemical modification of the exopolysaccharides

Chemical modifications of the EPS were carried out by *Casteren et al.* (1998) in order to examine the structure of the EPS and the physical and chemical properties of the modified EPS. During the treatment with sulphuric acid, 25 mg of EPS was dissolved in 6.25 cm^3 of distilled water, and then 6.25 cm^3 of 0.6 M sulphuric acid was added to the solution. The solution was incubated for 2 hours at 37 °C, and then cooled on ice and neutralized with 2 M NaOH. During the treatment with hydrogen fluoride, to 50 mg EPS 2.5 cm^3 of 28 M (approximately 48%) HF was added at 0 °C, stirred for 48 hours, followed by neutralization with 6 M NaOH under ice-water cooling. During the treatment with NaOH, 25 mg EPS was dissolved in 12.5 cm^3 of distilled water, and then 12.5 cm^3 of 4 M NaOH solution containing 1 mg/cm^3 $NaBH_4$ was added; it was left at room temperature for 3 minutes, then heated to 80 °C for 4 hours, then cooled with ice water and neutralized with 2 M acetic acid.

After the chemical modification, the samples were analysed by HPAEC. The remaining samples were dialysed and divided into two parts: one part was concentrated and then analysed by HPSEC and with "light scattering";

the other part was lyophilized and used for the monosaccharide composition analysis and the phosphorus analysis.

The measurements of the monosaccharides released as a result of the chemical treatments were performed with high-performance anion exchange chromatography (HPAEC) as follows. The Dionex system device consisted of the following modules: a gradient pump, eluent degassing unit (He), 4×250 mm CarboPac PA1 column with CarboPac PA100 column, a pulsed electrochemical detector (PED-2) in pulsed amperometric detection (PAD) mode and a Spectra Physics AS3000 sampler. The chromatograms were recorded using PC1000 software. The eluent was measured with gold electrode containing PED-2 detector; the reference electrode was Ag/AgCl. For the T1 0.4 s, T2 0.2 s and T3 0.4 s retention times, the following potentials were applied: E1 0.1; E2 0.7; E3 0.1. The gradient elution was created by mixing Millipore distilled water and 0.1 M NaOH, 1 M NaOAc and 0.1 M NaOH. After equilibration with 16 mM NaOH, 20 ĺµL of sample were injected onto the column. The eluent programme was as follows: $0 \rightarrow 20$ minutes 16 mM NaOH; $20 \rightarrow 25$ minutes, $0 \rightarrow 1$ M NaOAc; $25 \rightarrow 30$ minutes, 1 M NaOAc; $30 \rightarrow 35$ minutes, 0.1 M NaOH; $35 \rightarrow 40$ minutes, 0.1 M$\rightarrow$ 16m M NaOH; $40 \rightarrow 55$ minutes, re-equilibration with 16 mM NaOH.

The amount of the released galactose-1-phosphate was determined with HPAEC, with the already presented method. The eluent composition (flow rate 1 mL/min) has been changed as the following: 0.1 M NaOH solution and 1 M NaOAc containing 0.1 M NaOH solution was mixed. The eluent programme was as follows: $0 \rightarrow 5$ minutes, 0.1 M NaOH isocratic; $5 \rightarrow 72$ minutes, linear gradient $0 \rightarrow 0.6$ M NaOAc; $72 \rightarrow 77$ minutes, 0.6 M NaOAc isocratic; followed by the washing step, $77 \rightarrow 82$ minutes $0.6 \rightarrow 1$ M NaOAc; $82 \rightarrow 87$ minutes, 1 M NaOAc; $87 \rightarrow 102$ minutes; re-equilibration with 0.1 M NaOH. α-D-galakctose-1-phosphate (Sigma) was used as a standard.

After the chemical treatments, the sugar composition and the phosphorus contents of the resulting polymers were determined. After sulphuric acid treatment, the monosaccharide content of the samples was analysed by HPAEC, and there have been found only galactose monomers. As the sugar-1-phosphate bonds are sensitive to diluted acids, the galactose has probably bounded terminal to the phosphate, although the amount of the released galactose was less than it could have been expected on the basis of the assumed structure of the EPS. This may be either owing to the fact that only a part of the terminal galactose molecules have been released due to the sulphuric acid treatment, or owing to the fact that not every repeating unit contained terminally bounded galactose in this structure. The later NMR studies revealed that

the sulphuric-acid-treated EPS contained only phosphomonoester bonds in a significant amount; thus, almost all of the terminally linked galactose could be removed with the treatment. Thus, the amount of the measured galactose was less than expected because the EPS chain structure was different from the presumed one.

The treatment with HF removed the total amount of phosphorus contained in the EPS; thus, the treated polysaccharide contained less galactose and rhamnose, while the glucose content remained unchanged. During the treatment with HF, the sugar phosphate bonds cleaved while the sugar-sugar bonds were unchanged (*Hancock & Poxton*, 1980; *Nakajima et al.*, 1992). In addition, several authors have found that the rhamnose bonds could also cleave as a result of HF treatment (*Lugowski & Jennings*, 1984; *Janssen et al.*, 1998; *Robijn et al.*, 1995). The hydrodynamic volume of the polymer was measured by HPSEC and with static "light scattering" detector, according to the method described by *Tuinier et al.* (1998). As the hydrodynamic volume of the molecule did not change greatly after the removal of phosphate and rhamnose, these have to be present in the side groups of the EPS molecule and not in the main chain. The appearance of the galactose after treatment indicated that it was linked to the main chain via the phosphate group.

The treatment with HF removed all the phosphorus as well as all the terminally linked galactose while the sulphuric acid treatment released most of the terminally linked galactose, but left the phosphorus almost unchanged. The polymer galactose content subjected to the sulphuric treatment was found to be relatively less than in the case of the HF treatment, from which it was concluded that in the presence of phosphorus only a small part of the galactose contained in the main chain could be analysed, i.e. only a part of the galactose phosphate bonds have been degraded during the hydrolysis and thus the free galactose, which could convert to alditol-acetate, was less. The correctness of the assumption was examined by measuring the total and free phosphorus content after each step of the monosaccharide composition determination assay. The free phosphorus content was almost negligible in the raw EPS; however, after the hydrolysis, 30-40% of the total phosphorus amount was free phosphorus. This meant that only a part of the galactose-phosphate bonds of the main chain were hydrolysed, which resulted in the fact that only 30-40% of the galactose contained in the main chain could be detected; however, in the case of terminal galactose, the total quantity of galactose could be detected, as it was fully released during the hydrolysis.

The NaOH treatment resulted in polymers with lower phosphorus and galactose content as compared to the HF treatment; the NaOH treatment did not

remove all of the phosphorus from the EPS and the rhamnose bonds remained unbroken.

From the effects of chemical modifications, it can be concluded that the amount of galactose in the main chain of the purified EPS was underestimated due to incomplete hydrolysis of galactose-phosphate esters in the main chain.

In the case of the analysis (methylation, hydrolysis, acetylation) dedicated to the exploration of the monosaccharides binding sites, the phosphorus content was measured as well. The samples were divided into three parts: one of them was subjected to treatment with HF before methylation; another part of the samples was treated prior to alditol acetate derivatization after methylation; in the case of the third part, no HF treatment was applied. The untreated EPS contained, according to the results, 1,4-linked glucose and rhamnose (1,4-di-O-acetyl derivatives), and traces of 1,2,4-linked and 1,2,3,4-linked galactose.

Several authors mentioned that in the case of phosphorylated polysaccharides, during the methylation, a partial dephosphorylation occurs and the hydrolysis of phosphate esters is also not complete (*Janssen et al.*, 1998; *Nakajima et al.*, 1992; *Robijn et al.*, 1995; *Robijn et al.*, 1996). As an effect of the treatment with HF after the methylation, the recovery of the 1,2,3,4-linked galactose increased as compared to untreated EPS, which was explained with the following: as all the sugar-phosphate bonds broke up during treatment with HF, the phosphorus did not alter the hydrolysis anymore and therefore all the galactose contained in the main chain could be analysed. Due to the effects of the treatment with HF before methylation, instead of 1,2,3,4-linked galactose, there has been found 1,2,4-linked galactose in nearly identical quantity. As the binding site of the removed phosphate group was constituted from the point of view of methylation to be a free group, this site was methylated; therefore, no acetylation could occur. Thus, the 1,2,3,4-linked galactose in the EPS chain is substituted on the third carbon atom with phosphate.

During the methylation analysis, "under-methylation" occurred, probably because of the poor dissolution of the EPS in the methylating agent. However, the composition of the non-methylated substances was similar to that of the EPS sugar composition. The results were well reproducible, and in the case of the terminally linked rhamnose and 1,4-linked glucose no di-O-methyl and mono-O-methyl sugars have been observed; therefore, it was assumed that the methylated part of the EPS represents the total EPS.

Regarding the structure of the EPS, the results obtained by *Casteren et al.* (1998), except for one, were identical with the results obtained by other authors. *Casteren et al.* (1998) did not detect terminally linked galactose, but this was expected in the case of HF treated samples as the terminally linked

galactose linked to the main chain by phosphate was cleaved because of the treatment, and the subsequent dialysis removed it. In contrast, in the case of untreated EPS, the absence of the terminally linked galactose was unexpected. As the pH was strongly alkaline during the structure exploratory analysis, it seemed likely that the galactose-3-phosphate had cleaved, after which the resulted galactose-1-phosphate was removed during the dialysis. This contradicts the fact that the HF treatment occurred after the methylation had resulted in the formation of 1,2,3,4-linked galactose derivatives, which means that the phosphate group was still present during the methylation. *Nakajima et al.* (1992) found some amount of terminally linked galactose after the purification of the methylated, HF treated and re-methylated polysaccharide – but less than expected. As the surplus HF was removed with vacuum using a KOH desiccator, the terminally linked galactose was retained which was detached during the HF treatment. If the terminally linked galactose would have been released during the methylation, this would have been also retained. Thus, the differences appearing regarding the recovery amount of the terminally linked galactose may be due to the differences in the purification process.

In summary, it can be concluded that after the chemical treatments the released sugars and the composition of the residual polymer were also analysed. During the sulphuric acid treatment, units linked to the phosphate through 1-glicosidic bonds were released; thus, their structure and quantity could also be determined. The HF treatment removed all the phosphate and a part of the rhamnose, and with the determination of the hydrodynamic volume of the molecule it was possible to realize whether these components are situated mainly in the main chain or in the side chain. The analysis of the components of the residual polymer gave rise to the suspicion that in the presence of phosphate, the hydrolysis of the polymer is not perfect, which was confirmed during the determination of the total and free phosphate content after hydrolysis. As an effect of the HF treatment, the recovery amount of the phosphate-linked monosaccharide increased as the hydrolysis inhibitor phosphate-ester bond was cleaved prior to hydrolysis. The combination of the methylation analysis with the HF treatment allowed the identification of the binding site of the phosphorus-containing substituent (HF treatment prior to methylation, then after methylation).

With the help of chemical treatments, the following results have been achieved: the determination of the structure and quantity of the units bond through 1-glicosidic linkage to the phosphate, the determination of the location of phosphate and rhamnose in the molecule (main or side chain) with the measurement of the hydrodynamic volume, the determination of the binding

sites of the phosphorus-containing substituents (combination of methylation with HF treatment), and finally it was determined that the recovery amount of the monosaccharides linked to phosphate increased due to the HF treatment.

1.5 Examination of the structure of monosaccharides by nuclear magnetic resonance spectroscopy

These kinds of examinations allow an extensive exploration of the structure, suitable not only for the determination of atomic groups, but also for the fine-structure studies. With the study of the signals generated by the chemical shifts (the electron cloud alters the local magnetic field acting on the nucleus, and thereby the frequency of the nucleus resonance) and the spin-spin interactions (the spin orientation of the protons acts upon the neighbouring groups; the effect is mediated by the binding electron pairs), even low environmental differences can be detected. The applied analyses in the case of EPS were as follows: the detection of the repeating units in the structure, identification of the monomers forming the repeating units, the analysis of the isomerism of the anomeric carbon atoms (α or β), determination of the linkage mode of monomers and the occurrence and frequency of certain atomic groups.

Dueñas-Chasco et al. (1998) treated the samples with deuterium for several times; then removed the deuterium with freeze-drying and analysed the solution (3 mg/cm^3), solving it in 99.98% D_2O. The spectrums were collected at 303 or 333 K with a Bruker AMX 500 spectrometer, where the frequency of the electromagnetic radiation was 500.13 MHz (^{1}H) and 125.75 MHz (^{13}C), respectively. The chemical shift was given in ppm, the signal of HDO (4.75 ppm, 303 K or 4.33 ppm, 333 K) (^{1}H), or using the signal of an external standard dimethyl-sulfoxide (39.5 ppm) (^{13}C) as a reference.

In the 1D^{1}H-NMR spectrum, the following was determined: the number of different resonant frequency shift indicating anomeric protons (i.e. the hydrogen atoms linked to anomeric carbon atoms) in different environments. By determining the configuration of the anomers (α or β) and their ratio, it was revealed how many different α-glucopyranose and different β-glucopyranose units are there in the EPS and what their ratio is. Based on the initial NMR examination and on the studies regarding the linkage position of the monomers, it was likely that the EPS is a mixture of two kinds of polysaccharides: one part consists of α configuration unit (α PS), the other part of β-configuration unit (PS β). This assumption was confirmed by ^{1}H-NMR analysis of the fractions of EPS precipitated with alcohol, where the α/β polysaccharide ratio was measured in the anomeric region of the spectrum of the fractions.

The PSa unit was analysed in detail with NMR spectroscopy. The NMR signals of the ^{1}H and ^{13}C fractions were analysed for their chemical shift, whereby the signals of three different resonant frequency transmitter anomeric protons were observed on the one-dimensional ^{1}H-NMR spectrum: PSα was constituted of three type of α-D glucopyranoses (A, B, C). The resonant signals of the protons belonging to carbon atoms $(1, 2, \ldots 6)$ of individual subunits (A, B, C) were also observed and the spin-spin interactions were also analysed, specifically the interaction between the three different spin systems (A, B, C). The C-2 (the second carbon atom of the monomer) and the associated H-2 resonant signals showed chemical shifting towards the lower magnetic field strength in the A region compared to the identical nucleus of B and C units. It followed that the second carbon atom of unit A, unlike the other units, was linked with some type of substituent; thus, the unit containing 1,2,6 bonds is the side ramification point of the main chain. The C-6 resonant signal of the B unit showed chemical shifting towards the higher magnetic field strength as compared to units A and C, which means that the 6 carbon atom is unsubstituted in unit B, i.e. the B terminal unit is nothing else than the α-D-glucopyranose linked with its anomer carbon atom to the chain. The C unit is unsubstituted and forms the α-D-glucopyranose main chain with its 1,6 bonds.

Urashima et al. (1999) dissolved the polysaccharide in 1 cm^3 heavy water (99.96 D atomic%, Aldrich, USA) and then placed it into the NMR sample holder. The chemical shift value was given relatively compared to the 2,2-dimethyl-2-silapentan-5-sulphonic acid sodium salt (DSS), but during the analysis the chemical shift was measured compared to the acetone. The ^{1}H-NMR (proton NMR) spectrum was determined in heavy water (99.96 D atomic%, Aldrich, USA), at a frequency of 270 MHz, using a Jeol JNM-GSX-270 spectrometer, in which the sample temperature was 300K. Based on the different chemical shift values showing anomeric resonant signals of the ^{1}H-NMR spectrum, the structure of the polysaccharide was formed by repeating rings consisting of seven units. The rings were formed by five α-glycosides and two β-glycosides. The rhamnose (H-6) resonant signal was also observed on the spectrum.

Uemura et al. (1998) determined the ^{1}H-NMR spectrum also in D_2O (Merck, 99.75%, Darmstadt, Germany), at the temperature of 300 of 354 K, using acetone as an internal standard. The NMR apparatus was a Varian Unity INOVA 600 model, which operated in Pulse Fourier Transform mode. There were found five, H-1 (the hydrogen atom belonging to the first carbon atom of the monomer) resonant signals with nearly identical intensity on the ^{1}H-

NMR spectrum determined in a NPS at 50 °C and D_2O solution at 600 MHz. Based on the chemical shift, the five signals formed two different groups; the first group was formed by three nearly identical signals, while the second group was formed by two nearly identical signals. From this, it was concluded that a pentasaccharide unit repeated in the chain (five H-1 with different environment), in which three members were of α spatial orientation and two members of β spatial orientation. The NMR spectrum of the APS included also five H-1 signals with similar chemical shift values as in the case of NPS. This indicated that the same pentasaccharide repeating unit was present in the APS as well as in the NPS. None of the following was found on the spectrum: methyl group, 6-deoxi-saccharide, amino group of the hexosamine and resonant signal of sialic acid (H-3). Based on the knowledge of the NMR spectrum and the chemical composition, the carbohydrate structure was identical in the case of the APS and NPS; there were differences only in the phosphorus content.

Casteren et al. (1998) analysed the resonance of the nucleus different than protons. During the ^{31}P NMR analysis, the spectrum of the sample was determined in 5-10% D_2O containing water at 121.500 MHz using a Bruker AMX-300 type spectrometer. The sample holder was of 10 mm, the temperature was 27 °C. The chemical shift was determined compared to the internal standard L-α-glycero-phosphoryl choline (GPC, Sigma). Chemical shifts caused by changes in pH were measured to determine whether there are phosphomonoesters or/and phosphodiesters in the sample. The pH of the sample was adjusted with 25 mM HCl or 25 mM NaOH solution.

The 1D, 400, 13 MHz ^{1}H-NMR spectrum of the HF treated EPS and the spin decoupled 100.63 MHz ^{13}C NMR spectrum of the proton was determined in 8 mg/cm^3 D_2O at 60 °C with a Bruker DPX-400 spectrometer, where the sample holder was of 5 mm. The ^{31}P NMR spectrum of the purified EPS included one resonance signal at neutral pH, which confirmed the existence of a single type of phosphorus-containing group in a repeating unit (*Moreau et al.*, 1998). During the titration experiments, the resonance frequency was not shifted substantially at the range of pH 6.1 to 10.0, which indicates the presence of phosphodiesters (*Ilg et al.*, 1996). After the purification, there were no phosphate monoesters found, which indicates that the galactose-1-phosphate bond – sensitive to acids – did not become cleaved during the tricloroacetic acid purification and that all the phosphate groups were present in the form of diester. However, in the case of EPS exposed to the effect of tricloroacetic acid for a longer time, the breakdown of phosphodiesters could also appear. In such a case, in addition to the phosphodiester resonance signal, the phosphomonoester resonance signal is also observable in the

^{31}P NMR spectrum.

At pH 4.0, the sulphuric-acid-treated samples gave a resonance line, which was shifted during the titration experiments. This phenomenon indicates the presence of titratable phosphomonoester. The signal of the phosphodiester was very low, from which it was concluded that almost all the galactose (linked terminally to the chain with phosphate-ester bond) was removed with the sulphuric acid treatment. In the case of EPS treated with HF, there were no resonance signals when the ^{31}P NMR was measured, whereas the treatment resulted in the complete dephosphorylation of the EPS. The spectrums taken with ^{1}H-NMR and ^{13}C NMR 1H NMR techniques have been compared to EPS spectrums of other origins, analysed by other authors, and it was used for the study of repeating structural units.

1.6 Glucose, galactose and lactose content of yogurt

1.6.1 Extraction of the saccharides from yogurt

Richmond et al. (1987) measured 10 g of homogenized yogurt at room temperature into a centrifuge tube, and layered onto it an amount of absolute ethanol until the final ethanol concentration became 80% (v/v%). The slurry was mixed and left it to stand for 20 minutes at room temperature until the proteins had sedimented. Subsequently, 80% by volume of ethanol was added to the mixture so that the total volume of the solution became 50.0 cm^3, and the precipitate was centrifuged. The supernatant was discarded and the precipitate was washed with 25 cm^3 of 80% v/v of ethanol. The extracts and the washing liquid were pooled, and then the alcohol was removed with a rotary rapid evaporator at 25-27 °C. The volume of the extracts was filled until 25 cm^3 with water and filtered over a Whatman No. 42 filter paper. The samples and the standard solutions were also filtered over a 0.45 μm Metricel membrane and were stored in vials frozen at -10 °C until the start of the analysis.

1.6.2 Determination of the quality and quantity of the saccharides by HPLC

The HPLC system consisted of the following elements: Waters Assoc. (Milford, MA) M-45 solvent delivery unit, Waters U6K septum free sampler, Waters Model RI-401 differential refractometer, and a Linear Instruments Model 232 recorder. The column was a Bio-Rad Aminex HPX-87 carbohydrate column of the size of 300 mm × 7.8 mm. The column was held at the temperature of 80 °C by an Alltech Assoc. water jacket (catalogue Nr.: 9502) equipped with

a Precision Scientific 66600 water bath and a 62538 thermometer. The unwanted anions were removed with a Bio-Rad Aminex type A-25, 40 mm × 4.6 mm sized Microguard Anion/OH cartridge patron system column. The eluent was ammonia-free water deionized by reverse osmosis, which was degassed by vacuum and held at 50 °C until the start of the HPLC analysis to lower the re-dissolution of the gases. The samples and the standards were injected into the device with a 10 μL Hamilton syringe. Qualitative identification was made by retention time, quantitative identification was made by external standard calibration, based on the equation of the straight fitted onto six measurement points (lactose concentration range: 0.20-2.00 g/100 cm^3; glucose and galactose concentration range, one by one: 0.1-1.00 g/100 cm^3).

1.6.3 Examination of the methods

The recovery was analysed in the case of lactose and galactose. The extraction steps were applied first to standard solution, then to yogurt-sugar preparation. 10 g of standard solution (2.00 g/100 cm^3 lactose and 1.00 g/100 cm^3 galactose) and 10 g yogurt were mixed, the mixture was extracted, and then the sugar content of the extract was measured. 1 g of the standard sugar solution was mixed with 10.0 g of yogurt and was also extracted. The carbohydrate content of the following samples was determined repeatedly: untreated standard solution, extracted standard solution, yogurt, yogurt and added carbohydrates. The recovery of lactose was of 103.6% and that of the galactose was 103.4%. During the analysis of the heat-treated samples, a new peak appeared on the chromatogram, which was identified as lactulose based on the retention time of the standards.

2 Determination of the composition and quantity of the galacto-oligosacharides

Hyun-Jae Shin et al. (1998) determined the quantity of GalOS, while *Yanahira et al.* (1998), in addition to the quantity, determined their structure, too. *Hyun-Jae Shin et al.* (1998) analysed the total carbohydrate content (mono- and oligosaccharides) of the ferment, using high-performance liquid chromatography (HPLC). The Waters (USA) device was equipped with reflective index (RI) detector and Sugar Pak I column (Waters, USA). The mobile phase was 10 mM Ca-EDTA solution, the flow rate was 0.5 cm^3/min and the column space temperature was 90 °C. The authors did not report the chromatogram,

and they observed di- and a trisaccharides as oligosaccharides; as their identification was not carried out, the authors described their structure based on data from literature.

Rustom et al. (1998) determined the lactose, glucose+galactose and oligosaccharide content of the permeates with HPLC based on the method described by *Jeon & Mantha* (1985). The device was constituted of the followings: 20 μL manual sampler, pump (Waters, M-6000A), a pre-column (Nucleosil 120-7NH_2, 30 × 4 mm ID) and a 250 × 4-mm-sized column (Nucleosil 120-7NH_2), reflective index detector (Varian R1-4, 16, temperature at 35 °C) and a Crome Jet SP 4400 integrator (AT=8, C5=0.5 cm/min). The mobile phase was acetonitrile/water mixture (75%:25% v/v), which was filtered over a microfilter with the pore size of 0.22 μm and degassed in an ultrasonic bath for 30 minutes. The samples were diluted to the twenty-fold with bi-distilled water, filtered over a filter with the pore size of 0.22 μm, injected at room temperature, then eluted with a 2.0 cm^3/min flow rate. Each measurement was repeated three times. The retention time of glucose and galactose was so close to each other that their quantity could be determined only together. Only the amount of the oligosaccharide concentration was reported, structural identification was not carried out.

Yanahira et al. (1998) studied the structure of oligosaccharides with a HPLC "LC module 1" device, "model 490" UV (208 nm) and "model 410" refractive index detector (Waters, Tokyo, Japan) under the following circumstances: "A" version: Bior-Rad HPX-87H 6×250-mm-sized ion exclusion column, 0.005 M sulphuric acid solution as eluent, flow rate of 1.0 cm^3/min. "B" version: Shodex SH-1821 (Showa Denko Co.) column; 0.005 M sulphuric acid solution as eluent, flow rate of 0.75 cm^3/min. The N1 signed acidic oligosaccharide could be separated into two substances (N1-1; N1-2) by carbon column chromatography with a 5.0 × 50-cm-sized column. During the measurement, applying a linear gradient, the quantity of ethanol varied between 0% → 15%. The FAB-MS spectrum was determined in negative ion operation mode using a Jeol JMS-700 mass spectrometer with 6 kV equivalent kinetic energy Xe atoms, using glycerin as matrix.

The ^{1}H-and ^{13}C-NMR spectrums were determined in 2-3% D_2O containing solution at a temperature of 25 °C with a Jeol Alpha-500 NMR spectrometer. The chemical shift was given in ppm with a magnetic field strength decrease orientation compared to the signal of 4,4-dimethyl-4-silapentanoate, related to 1,4-dioxane. The analysis of acidic oligosaccharides was based on NMR measurements. Acidic oligosaccharides were purified by anion exchange chromatography (5.0 × 50 cm, Dowex 1, acetate form). The column was washed

amply with water, removing the neutral sugars, and then the acidic oligosaccharides were eluted with the linear gradient ($0 \rightarrow 0.2$ M) of sodium-acetate solution. Each fraction was desalted with a Micro Acilyzer S1 (Asahi Chemical Ind. Co Ltd.) device, and then concentrated in vacuum, and finally they were lyophilized. Four different acidic GalOS (N1, N2, N3, N4) could be separated with HPLC. Under the "A" analytical circumstances, N1 and N2 oligosaccharides were separated. Under the "B" analytical circumstances, G1 and G2 oligosaccharides were separated. The N1 signed oligosaccharide could be further separated into two different substances (N1-1; N1-2), using carbon column chromatography. For the means of oligosaccharide yield measurement, GalOS were extracted from the reaction mixture by anion-exchange chromatography, and the same preparations were used for the structure identification studies.

The detailed structure of acidic GalOS was determined by NMR spectroscopy. The H1-H6 resonance signals of the N1 acidic GalOS galactosyl residue were identified based on the spectrums, whereby the anomeric proton was identified first (β-galactose H1). Based on the chemical shifts, there was no α-galactose (H1) signal visible on the N1-1 spectrum, from which it was concluded that the galactose unit contained in N1-1 was situated on the non-reducing chain end; thus, the glycosidic OH group of galactose was in bond. The signal of N-acetyl muramic acid (NeuAc) axially situated H3 proton and equatorially situated H3 proton were also observed in the spectrum, and with the help of these signals the sequence of protons H4, H5 and H6 could also be determined. In order to select the NeuAc H7, H8 and H9 signals, first, the signal of H9, then that of H7 and H8 were identified using the spectrum. Subsequently, it was controlled with the spectrum if the signals of N1-1 constituting carbon atoms were identified correctly. The C8 resonance signal of the NeuAc unit showed chemical shift towards lower field strength because of its involvement in the glycosidic bond. From these results, the GalOS structure was determinable: one β-galactose molecule was linked with $1 \rightarrow 8$ bonding to the NeuAc molecule.

Similarly to the previous ones, the ^{1}H- and ^{13}C-NMR data of the N1-2 revealed that the non-reducing galactose group has similar chemical shifts as the N1-1 galactose group. The C9 signal of the NeuAc unit showed chemical shift towards lower field strength on the N1-2 ^{13}C-NMR spectrum, which was also confirmed by the distortion-free increase of the polarization transfer. Based on these results, the N1-2 structure was the following: one β-galactose molecule linked with a $1 \rightarrow 9$ bonding to the NeuAc molecule.

Among the N2 GalOS ^{1}H- and ^{13}C-NMR data, chemical shift signals of the non-reducing galactosyl group and the NeuAc were similar to that of N1-1,

and the C'3 signal of the internal galactose unit showed chemical shift towards the lower field strength because of the glycosylation. The N2 structure was as follows: galactose β-(1 → 3)-galactose β-(1 → 8)-NeuAc. The structure identification of G1 and G2 GalOS was performed also with the study of ^{1}H- and ^{13}C-NMR spectrum according to the method described earlier.

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Statistical analysis of the basic chemical composition of whole grain flour of different cereal grains

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Abstract. Samples of whole grain flour of five cereals (wheat, rye, barley, oats and buckwheat) were analysed for ash, starch, fat, cellulose and protein content. Coefficient of variation shows that within the same sample of whole grain flour variation of starch, protein, fat and ash content is relatively small, rarely exceeding 3%. The variability of the cellulose content is relatively high. The significance of the difference between chemical compositions of two independent samples of the same whole grain flour has been tested by Student's t-test. With the exception of protein content, the difference between two samples of buckwheat whole grain flour

Keywords and phrases: cereals, whole grain flour, basic chemical composition, statistical analysis.

was significant. With the exception of wheat whole grain flour, for other cereals, the difference in ash content between two samples was significant. In all the other cases, there was no significant difference between two independent samples of the same whole grain flour. The significance of the difference in chemical compositions between the whole grain flour from different cereals has been tested by analysis of variance. Barley whole grain flour shows significant difference in ash content, while wheat whole grain flour significantly differs in starch content compared to other flour samples. All investigated samples significantly differ in fat content. Oat (higher content) and rye (lower content) whole grain flour significantly differ to other samples considering the protein content.

1 Introduction

Cereals are the fruits of cultivated grasses, members of the Gramineae family (*Kent*, 1975). Although a great number of cereal grains exist, eight are considered to be a principal cereal crops (cereals of commerce): wheat, corn, barley, rye, oat, rice, sorghum and millet (*Hoseney & Faubion*, 1992). Buckwheat is not a true cereal and belongs to the Polygonaceae family (*Marshall & Pomeranz*, 1982), but it is typically associated with the grain family due to similar composition. Although different types of grain differ markedly in their proximate physical composition, all share the same basic anatomical structures: an outer bran layer, a germ fraction and a starchy endosperm (*Hoseny*, 1998). Each of the main parts of the grain has different structural characteristics and chemical composition, and it is further subdivided into various layers (*Kent*, 1975). Depending on the type of grain, bran constitutes approximately 3-30% of its dry weight. The outer bran layer includes the seed coat (pericarp and testa), while the inner layers are composed of aleurone cells, positioned next to the starchy endosperm. Some cereal grains (oat, rice and barley) also possess outer hulls, which are tightly bound to the bran layer. Both the amount and composition of bran are highly variable across the different types of grain (*Hoseny*, 1998). The bran layer is associated with a wealth of macro- and micronutrients, including fibre, protein, B vitamins, minerals and flavonoids. The germ makes up 4-17% of the dry weight of a whole grain. The germ is a rich source of proteins, lipids, B vitamins and vitamin E. The starchy endosperm makes up approximately 65-75% of the dry weight of a cereal grain, and it is composed of starch, non-starch polysaccharides and small amounts of protein and lipids (*Marquart et al.*, 2002).

Traditional flour milling process produces refined flour. During this process, the bran and germ layers of grain are removed in order to stabilize the raw material and to increase the keeping quality. Unfortunately, the type of milling used for refined flour leads to the loss of certain nutrients (Nestle, 2006). If the bran, germ and endosperm components are retained during the milling process, the resulting flour is classified as whole grain (*Franz & Sampson*, 2006). Whole grains contain all the essential parts and the same balance of nutrients that are found in the original grain seed. Compared to refined flour, whole grains are nutritionally superior; they are richer in dietary fibre, protein, antioxidants, dietary minerals and vitamins. Diet rich in whole grain foods has been associated with decreased risk of cardiovascular disease, diabetes, obesity and certain cancers (*Jacobs et al.*, 1998; *McKeown et al.*, 2002; www.healthgrain.com). These are the reasons why the consumption of whole grain flour attracts more and more attention.

Although cereal grains have many structural similarities, they do differ in the relative proportion of their principal components and subsequently in the chemical composition of whole grain flours. The aim of this study was to test the significance of the difference in basic chemical compositions between the whole grain flour from different cereals by analysis of variance (ANOVA).

2 Materials and method

Materials

Commercially available whole grain flours of five different cereals, namely wheat, rye, barley, oat and buckwheat were used for the present study. Two independent samples (2×1 kg) of each of the whole grain flour type were analysed.

Methods

For each sample chemical characteristics, namely moisture, starch, cellulose, fat, protein and ash, analyses were carried out as per standard methods. Moisture content has been determined based on the weight loss suffered by the sample when dried at a temperature of 130 to 133 °C (*ICC standard method No.110/1*). Starch content has been determined according to Ewers polarimetric method (*ISO 10520*), using the Elmer-Perkin polarimeter. Crude cellulose content has been determined according to Kirschner-Ganakova's procedure (*Ćirić et al.*, 1975). Crude lipids were extracted from the samples in a Soxhlet extractor with ether. The crude fat content was determined gravimetrically

after oven-drying. The crude protein content was calculated by multiplying the corresponding total nitrogen content, which was determined according to the Kjeldahl method (*ICC standard method No.105/2*), by a factor of 6.25. To determine the ash content, the samples were placed in a muffle furnace at 900 °C for 2 hours, and weighed before and after (*ICC standard method No.104/1*). The results are expressed as percentage by weight of sample, and presented on a dry matter basis. Each analysis was carried out in triplicate and mean values ± standard deviation reported.

Statistical analysis

The data were analysed by the following statistical procedures: coefficient of variation, t-test and ANOVA (performed by STATISTICA 12). The means were compared using the Tukey test at the 95% significance level.

3 Results and discussion

Representative values for the proximate composition of the investigated whole grain flour samples are shown in *Table 1*. However, for any cereal, a wide range of values for each chemical constituent can be encountered when a series of samples is analysed. This is the reason why the figures given in *Table 1* are limited only to reveal the major differences between the investigated whole grain flours.

The highest protein and fat (lipid) contents were registered in whole grain oat flour. Compared with other cereals, oats are known to contain high amount of protein and especially lipids. The protein content (16.91-17.46%) was within the range (15-20%) as reported by Robbins et al. (1971) and *McMullen* (1991). The lipid content (6.41-6.49%) also was within the range (5-9%) reported previously (*Youngs*, 1986; *Saastamoinen et al.*, 1989). The highest ash and cellulose contents were registered in whole grain barley flour. The mineral and cellulose (fibre) contents are higher in barley compared to other cereals as a consequence of the presence of hull. The hull (10% of the grain dry weight) is undesirable for human consumption and it needs to be removed before the covered grain can be used as a raw material for flour production. After removing the hull, the differences in mineral and cellulose contents are much reduced. However, since the parts of the outer layers of the kernel are removed, some may not regard this flour as a whole grain (*Andersson & Aman*, 2008). As expected, the highest starch content was registered in whole grain wheat flour.

Table 1: Ash, starch, cellulose, fat and protein content in the samples of whole grain flour

Sample	Ash $(\%)_{dm}$	CV (%)	Starch $(\%)_{dm}$	CV (%)	Cellulose $(\%)_{dm}$	CV (%)	Fat $(\%)_{dm}$	CV (%)	Protein $(\%)_{dm}$	CV (%)
Barley 1	2.52 ± 0.007	0.27	60.98 ± 1.404	2.30	3.25 ± 0.382	11.74	1.87 ± 0.038	2.02	15.25 ± 0.328	2.15
Barley 2	2.42 ± 0.017	0.68	59.50 ± 0.241	0.41	2.85 ± 0.126	4.44	1.82 ± 0.044	2.42	14.53 ± 0.067	0.46
Oat 1	1.69 ± 0.017	0.56	62.69 ± 1.436	2.29	1.33 ± 0.196	14.69	6.41 ± 0.306	4.77	17.46 ± 0.769	4.40
Oat 2	2.01 ± 0.009	0.97	63.57 ± 0.931	1.46	1.40 ± 0.078	5.56	6.49 ± 0.162	2.50	16.91 ± 0.010	0.06
Wheat 1	1.57 ± 0.004	0.26	69.31 ± 2.100	3.03	2.24 ± 0.096	4.28	1.48 ± 0.032	2.14	14.78 ± 0.092	0.62
Wheat	21.57 ± 0.027	1.72	69.82 ± 2.010	2.87	2.01 ± 0.437	21.67	1.53 ± 0.012	0.77	14.98 ± 0.448	3.00
Buckwheat 1	2.00 ± 0.024	1.21	60.35 ± 0.457	0.76	2.87 ± 0.027	0.93	2.51 ± 0.060	2.40	15.70 ± 0.329	2.10
Buckwheat 2	1.60 ± 0.146	9.14	62.97 ± 0.893	1.42	2.59 ± 0.121	4.66	2.38 ± 0.053	2.25	15.15 ± 0.194	1.28
Rye 1	1.52 ± 0.007	0.46	62.23 ± 0.849	1.36	1.71 ± 0.097	5.66	1.17 ± 0.124	10.60	13.74 ± 0.355	2.58
Rye 2	1.66 ± 0.036	2.19	62.44 ± 0.627	1.00	1.85 ± 0.045	2.42	1.23 ± 0.087	7.03	13.98 ± 0.146	1.04

Values are mean ± standard deviation of three independent determinations; dm – dry matter basis CV – coefficient of variation

Generally speaking, the values presented in *Table 1* are within the range of values previously reported for the chemical composition of wheat (*Kent*, 1975), buckwheat (*Marshall & Pomeranz*, 1982; *Steadman et al.*, 2001), rye (*Vinkx & Delcour*, 1996), oat (*Kaukovirta-Norja* & Lehtinen, 2008) and barley (*Andersson & Aman*, 2008).

The statistical analysis was performed in three steps. In the first step, the coefficient of variation showed that within the same sample of each of the investigated whole grain flour the variation of starch, protein, fat and ash content is relatively small, rarely exceeding 3%, and in lot of the cases even below 1% (*Table 1*). Ash content in the Buckwheat 2 sample and the fat content in both of the rye samples can be considered an exception from this general conclusion. On the contrary, the variability of the cellulose content was relatively high (up to 21.67%).

In the second step, the significance of the difference between the chemical compositions of two independent samples of the same whole grain flour was tested using the Student's t-test. With the exception of protein content, the difference was significant between the two samples of buckwheat whole grain flour. With the exception of wheat whole grain flour, for other cereals, the difference in ash content between the two samples was significant. In all the other cases, there was no significant difference between two independent samples of the same whole grain flour. In the third step, the significance of the difference between the chemical compositions of the whole grain flour from different cereal grains was tested by the ANOVA (*Table 2*).

Table 2: The significance of the difference between the chemical composition of whole grain flour samples tested by ANOVA

Sample	Cellulose $(\%)_{dm}$	Ash $(\%)_{dm}$	Protein $(\%)_{dm}$	Starch $(\%)_{dm}$	Fat $(\%)_{dm}$
Barley	3.03 a	2.47 a	14.89 b	60.24 c	1.85 a
Oat	1.37 c	1.85 b	17.19 a	63.13 b	6.45 b
Buckwheat	2.73 a	1.80 bc	15.43 b	61.66 bc	2.44 c
Rye	1.78 b	1.59 c	13.87 c	62.34 bc	1.20 d
Wheat	2.13 b	1.57 c	14.88 b	69.57 a	1.50 e

Values are the means of six independent determinations; dm – dry matter basis. Means with the same letter do not differ significantly.

ANOVA showed that, in terms of cellulose content, there was no statistically significant difference between barley and buckwheat flour or between wheat and rye flour, while the oat flour is significantly different from all other types of whole grain flours. By ash content, barley flour is allocated in a separate group. A relative closeness exists between oat and buckwheat flour, while at the same time the buckwheat flour is in the same group with wheat and rye flour. Considering the protein content, oat flour (higher content) and rye flour (lower content) significantly differ to other flour samples. Considering the starch content, the investigated flours were classed into three groups. Wheat flour is in a separate group. Barley and oat flour are different from each other; however, they are not significantly different from buckwheat and rye flour. All investigated whole grain flour samples significantly differ in fat content.

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Effect of carbon sources on the production of the biofungicide by *Streptomyces hygroscopicus*

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Abstract. Fungi from the genera *Alternaria*, *Colletotrichum* and *Fusarium* are listed among the most important storage pathogens of apple fruits. Isolate of *Alternaria sp.* was obtained from apple fruit samples expressing rot symptoms. Biological control of plant pathogens by means of microorganisms is considered as an attractive alternative to chemical-based treatments, with minimal impact on the environment. Actinomycetes are known to have a great potential for control of plant fungal diseases and their antifungal activity greatly depends on the medium

Keywords and phrases: *Streptomyces hygroscopicus*, carbon sources, biofungicides, *Alternaria sp.*

used for their cultivation. The aim of this study was to determine the influence of ten different carbon sources on the production of the biofungicide produced by *Streptomyces hygroscopicus* against *Alternaria sp.* as a test microorganism. *In vitro* antifungal activity of the cultivation liquids on *Alternaria sp.* grown on potato dextrose agar were examined using wells technique. The maximum inhibition zone was reached after 72 hrs of incubation at 28 °C in mediums with fructose and starch (diameter >20 mm). However, the efficacy of other eight carbon sources was significantly higher compared to the control. The obtained results indicate that tested isolate of *Streptomyces hygroscopicus* shows great potential as a tool for the biological control of *Alternaria sp.*

1 Introduction

Post-harvest losses are caused by fungal pathogens due to high amount of nutrients and water in fruits, low pH and loss of intrinsic resistance that protects them while they are attached to the plant (*Nunes*, 2012). Well-known mycotoxin producers are *Aspergillus*, *Fusarium*, *Alternaria* and *Penicillium* species, and they are listed among the most important storage pathogens of apple fruits (*Andersen et al.*, 2006). In apples, some Alternaria spp. causes various types of post-harvest fruit rot. Also, *Alternaria sp.* can grow at low temperatures, which means that the contamination of refrigerated foodstuffs during transport and storage is possible.

Post-harvest pathogens may be the most suitable target organisms for biological control due to two reasons: first of all, they are managed in controlled environment, such as storage, and, secondly, the use of synthetic fungicides after harvest is already prohibited in many European countries (*Adaskaveg* & *Forster*, 2010). Biological control using microbial antagonists has emerged as one of the most promising alternatives, either alone or as part of integrated pest management to reduce pesticide use. During the past 30 years, several biocontrol agents have been exploited and widely investigated against different post-harvest fungal pathogens (*Saravanakumar et al.*, 2008).

According to *Kunoh* (2002), endophytic *Streptomyces* may play an important role in the development and health of plants because it affects plant growth due to its assimilation of nutrients and production of secondary metabolites (*Dhanasekaran et al.*, 2012). Streptomycetes are known to have a great potential for the control of plant fungal diseases (*Doumbou et al.*, 2001) and their antifungal activity greatly depends on the medium used for their cultivation. With regard to carbon sources, species-specific variation occurs within

Streptomyces for cell growth and production of secondary metabolites. Microorganisms usually break down high molecular weight carbon sources into small molecules, convert these to amino acids, nucleotides, vitamins, carbohydrates and fatty acids, and finally build these basic materials into proteins, coenzymes, nucleic acids, mucopeptides, polysaccharides and lipids used for growth. Glucose, which commonly is an excellent carbon source for cell growth, has been shown to influence the formation of several antimycotics. Other carbohydrates, such as glycerol, maltose, mannose, sucrose and xylose, have also been reported to interfere with the production of secondary metabolites. However, the optimal carbon source and the morphology varies between many antimycotic producing *Streptomyces* (*Jonsbu et al.*, 2002).

In the present study, the ability of *Streptomyces hygroscopicus* to assimilate different carbon sources and produce high-value metabolic compounds with antifungal activity against isolate of *Alternaria spp.* was investigated.

2 Materials and methods

Fungal pathogen

Isolates of *Alternaria sp.* were obtained from apple fruit samples expressing rot symptoms. Apple samples were collected during 2012 from Ultra Low Oxygen storages in Vojvodina Province, Serbia. The pathogen was identified according to pathogenic, morphological and ecological characteristics. The isolates were initially grown on PDA (Potato Dextrose Agar) plates for seven days. After seven days, a small amount of mycelium of each isolate was added to flasks containing 50 ml of potato dextrose broth. The flasks were incubated for 48 hrs on a rotary shaker (150 rpm) at 25 °C. Before use, culture liquid was filtered through the double layer of sterile cheesecloth.

Antifungal component production

Production microorganism *Streptomyces hygroscopicus* was isolated from the natural environment and stored in the Microbial Culture Collection of the Faculty of Technology in Novi Sad. The medium used for the growth of production microorganism had the following composition (g/L): glucose (15.0), soybean flour (10.0), $CaCO_3$, (3.0), NaCl, (3.0), $MgSO_4$, (0.5), $(NH_4)_2HPO_4$, (0.5), K_2HPO_4, (1.0). The pH of the medium was adjusted to 7.2 ± 0.1 prior to autoclaving.

For the preparation of the fermentation mediums, we used the following ten carbon sources: glucose, starch, lactose, mannitol, arabinose, galactose, fructose, maltose, sucrose and glycerol. Other components were the same as in the medium used for growth. The pH of mediums was adjusted to 7.2 ± 0.1 prior to autoclaving. The isolate was grown in a 100 cm^3 shake flask containing 30 cm^3 of the culture medium. The fermentation medium was inoculated with 10% (v/v) of a preculture after 48 hrs of growth and incubated at 26 ± 1 °C for 7 days under conditions of spontaneous aeration. Rotary shaker at 150 rpm was used for the mixing of fluids during the cultivation.

After cultivation, the sample of the cultivation medium was centrifuged at 10,000 g for 10 min and the supernatant of the cultivation medium was used for *in vitro* antagonistic activity assay.

In vitro antagonistic activity assay

In vitro antagonistic activity assay was performed in 85-mm Petri plates using wells technique (*Segy*, 1983). In short, two layers of PDA medium were spread in plates. The first layer consisted of 2% PDA medium. After solidification, a new layer composed of 1.2% PDA and filtered fungal culture liquid (35%) was added. Three wells per plate with a diameter of 10 mm were made and two plates represented one treatment. For each treatment, 100 µl of test liquid was added in each well. The treatments included: supernatant of *Streptomyces hygroscopycus* cultivation medium and sterile distilled water served as negative control treatment. After 72 hrs of incubation at 28 °C, the radius (mm) of mycelial growth inhibition zone around wells was measured.

The values were subjected to further analysis by factorial analysis of variance (factorial ANOVA) and the Duncan's multiple range test, using software Statistica 12 (Statistica, 2012).

3 Results and discussion

It is well known that designing an appropriate fermentation medium is of crucial importance in the production of secondary metabolites. To design effective medium, it is necessary to evaluate the effects of different carbon sources on the production of bioactive metabolites. Carbon source is required for the synthesis of microorganism cells and as an energy source. In the case of secondary metabolites, special attention is paid to the choice of carbon source because of their inhibitory effect on the biosynthesis of secondary metabolites. Also, biomass yield and cell morphology are strongly influenced by carbon

source. Media which contains starch, lactose, glycerol or fructose as a carbon source may result in reduced growth with low biomass yield (*Brzonkalik et al.*, 2011). Carbon sources which are rapidly metabolized, such as glucose, often lead to the maximum growth rate of the biomass, but a reduced production of many secondary metabolites (*Gallo* & *Katz*, 1972). However, some carbon sources have better influence on the biomass growth, while others significantly affect the synthesis of secondary metabolites. There is a diversity in carbon sources that can be metabolized by different *Streptomyces* species.

In order to examine the growth of *Streptomyces hygroscopicus* on different carbon sources, shake flask cultures were carried out. A selection of different carbon sources was tested, which included representatives of monosaccharides, disaccharides, polysaccharides and alcohols. As expected, the evaluated treatments had significant ($p \leq 0.01$) influence on the mycelial growth inhibition radius (mm).

Table 1: Mean values of the inhibition zone diameter [mm] after 72 hours of incubation at 28 °C and significance of differences at 5% level probability

Carbon source	**Inhibition zone (mm)±Sd**
Sucrose	20.6 ± 1.2^{c}
Maltose	23.0 ± 3.6^{c}
Galactose	26.3 ± 1.5^{b}
Glycerol	27.6 ± 2.1^{ab}
Glucose	27.6 ± 0.6^{ab}
Arabinose	28.0 ± 1.7^{ab}
Lactose	28.6 ± 1.2^{ab}
Mannitol	30.0 ± 0.0^{a}
Starch	30.3 ± 2.5^{a}
Fructose	31.0 ± 1.7^{a}

*The mean values with the same lowercase letters in the column "Inhibition zone radius [mm]" are not significantly different at 5% level of probability. (Duncan's multiple range test).

The results shown in *Table 1* indicate that the tested isolate of *Streptomyces hygroscopicus* shows great potential as a tool for the biological control of Alternaria rot on apple, and that the medium containing different carbon sources ensures its high activity (diameter > 20 mm).

The results indicate that there was no statistically significant difference between inhibition zone diameters when seven different carbon sources were applied for medium preparation. The efficacy of the other three carbon sources was also on a significantly higher level compared to the control. In *Figure 1*, the inhibition zones formed around wells with 100 µl of *Streptomyces hygroscopicus* are shown for isolates with fructose (1), starch (2) and control plates (3) after 72 hrs of incubation at 28 °C. The least efficiency to test microorganism showed mediums with sucrose and maltose.

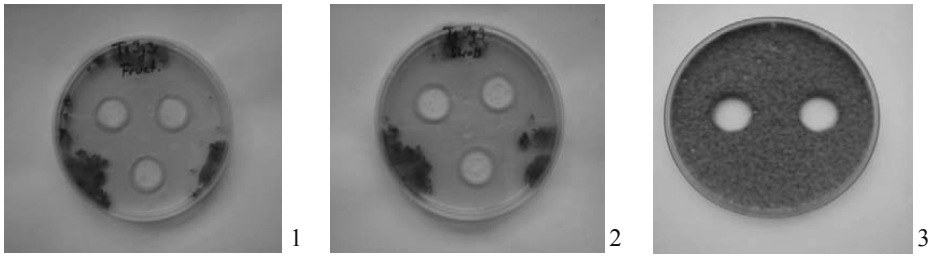

Figure 1: Inhibition zones formed around wells with 100 µl of *Streptomyces hygroscopicus* for isolates with fructose (1), starch (2) and control plates (3) after 72 hrs of incubation at 28 °C.

However, between glycerol, glucose, arabinose, lactose, mannitol, starch and fructose, as the seven best carbon sources in this experiment, there were no statistically significant differences. On the other hand, sucrose, maltose and galactose showed the least efficiency to test microorganism. *Singh et al.* (2009) also showed that the addition of carbon sources, such as maltose, sucrose and galactose, to the medium favoured the growth of *Streptomyces tanashiensis*, but the antibiotic production was less when compared with glucose, for example.

The choice of the carbon source, which will be used for potential industrial production, greatly depends on its availability and price. For example, an increase in biodiesel production results in an increase in the amount of waste glycerol. Waste glycerol constitutes a versatile carbon source with many possible applications in industrial fermentations, so it can be used in industrial microbiology for the production of valuable products, such as biofungicides (*Reungsang et al.*, 2013). That means that the use of glycerol as waste material significantly affects the price of biofungicide production process. *Jonsbu et al.* (2002) showed that glycerol was the carbon source that supported high specific growth rate at the same time as high nystatin production by *Streptomyces noursei*.

However, lactose and fructose showed a great potential as a carbon source for the production of antifungal compounds in this experiment. *Vinogradova et al.* (1985) detected a high level of heliomycin on lactose and *Sanchez & Demain* (2002) have reported positive effects of lactose on the biosynthesis of penicillin and erythromycin (*Gesheva et al.*, 2005). On the other hand, *Jonsbu et al.* (2002) concluded that fructose showed, in comparison to glucose, a trend of a more efficient utilization for the production of nystatin by *Streptomyces noursei*.

Demain & Fang (1995) have investigated that polysaccharides (e.g. starch) and oligosaccharides (e.g. lactose, maltose, sucrose) are often preferable for fermentations yielding secondary metabolites. This was in accordance with our work, where starch and disaccharides have proved to be very good carbon sources for the production of components for biological control of *Alternaria* rot on apple.

The development of efficient fermentation processes for the production of secondary metabolites by *Streptomyces* requires the examination of a diversity of species-specific features, including carbon-source nutrition and morphology (*Jonsbu et al.*, 2002). In conclusion, the findings of the present study showed that naturally occurring actinomycetes have a great potential to assimilate different carbon sources and produce high-value metabolic compounds with antifungal activity against the isolate of *Alternaria spp.* This fact allows that for the production of targeted antifungal components we can use different carbon sources depending on their availability on the market and prices.

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Evaluation of mass transfer kinetics and efficiency of osmotic dehydration of pork meat

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Abstract. In order to analyse mass transfer kinetics during osmotic dehydration, pork meat (*M. triceps brachii*) was dehydrated in three different osmotic solutions (sugar beet molasses, ternary solution and the combination of these solutions in a 1:1 ratio) under atmospheric pressure, at room temperature (20 °C), with and without manual stirring in every 15 minutes. The aim was to examine the influence of different osmotic solutions, immersion time and mixing on the mass transfer kinetics of water and solids, and the efficiency of osmotic treatment. The most significant kinetic parameters of the process, water loss-WL, solid gain-SG, weight reduction-WR, rate of water loss-RWL, rate of solid gain-RSG, rate of weight reduction-RWR and dehydration efficiency index-EI, were determined after 1, 3 and 5 hours of dehydration. Better results

Keywords and phrases: osmotic dehydration, pork meat, mass transfer kinetic, sugar beet molasses, ternary osmotic solution.

were obtained by performing the process with stirring. According to the results, all three solutions are satisfying osmotic agents and the diffusion occurred most rapidly during the first 3 hours of the process.

1 Introduction

The osmotic dehydration (OD) process is an important method for preserving solid food, which involves partial water removal from food stuff immersed in hypertonic aqueous solutions. Due to low energy consumption and mild temperatures, which is considered minimal processing, OD is suitable as a pretreatment for many processes to improve nutritional, sensorial and functional properties of food without changing its integrity (*Vieira et al.*, 2012; *Manivann et al.*, 2011; *Bellary et al.*, 2011; *Mavroudis et al.*, 1998; *Moreno et al.*, 2011). The difference in the chemical potential of water between the raw material and the osmotic medium is the driving force for dehydration. During this process, three types of mass transfer in counter-current flux take place: water loss from the sample to the solution, solute transference from solution to the sample and natural solute flux from sample to osmotic solution, which is quantitatively negligible compared to the other two, but it is significant for the final product quality. The existence of these simultaneous and opposite fluxes is one of the major difficulties in modelling osmotic dehydration kinetics (*Moreira et al.*, 2008; *Koprivca et al.*, 2010; *Mercali et al.*, 2010). Working temperature, concentration of osmotic solution and immersing time are the most important variables in the osmotic process. Increasing the osmotic solution concentration induces an increase in the mass transfer (*Ferrari et al.*, 2011; *Corrêa et al.*, 2010; *Silva et al.*, 2012). Great influence on the kinetics of water removal and solid gain has the type of osmotic agent. Ternary aqueous solutions containing salt and sugar are usually used as osmotic agents for meat dehydration (*Damez et al.*, 2008; *El-Aouar et al.*, 2006). Research has shown that sugar beet molasses represents an excellent osmotic medium for dehydration process, primarily due to the high content of dry matter (80%), which provides high osmotic pressure in the solution as well as the specific chemical composition characterized by high contents of vitamins, minerals, antioxidants and betaine (*Šušić et al.*, 1989; *Kowalska et al.*, 1998; *Mišljenović et al.*, 2009). Meat treatments by soaking in concentrated solutions allow the elimination of sequenced operations of salting and dehydration that are commonly practised in traditional meat processing by dehydrating the product and impregnating it with solutes, commonly with salt, in only one operation step (*Santchurn et al.*, 2007). Water content in meat has a ma-

jor impact on its physicochemical, sensorial and technological properties. In meat, water is held in myofibrils, functional organelles, but also it may exist in the intracellular space between myofibrils and sarcoplasm. The water content in meat depends on many factors, including the tissue itself and how the product is handled (time, temperature, treatments) (*Barat et al.*, 2009). The knowledge of the kinetics of water and salt transfers during the processing is of great technological importance because it allows estimating the immersion time of meat cuts in an osmotic solution to obtain products with determined salt and moisture contents (*Schmidt et al.*, 2009). The aim of this work was to examine the influence of different types of osmotic solutions, immersion time and agitation on the efficiency of the osmotic dehydration process of pork meat. The most important kinetics parameters and the rate of mass transfer were defined.

2 Materials and methods

Experimental circumstances

Pork meat (*M. triceps brachii*) was purchased at the local butcher shop in Novi Sad, shortly before use. The initial moisture content of the fresh meat was $74.64 \pm 0.48\%$. Prior to the osmotic treatment, fresh meat was cut into cubes of nearly $1 \times 1 \times 1$ cm dimension. Three different solutions were used as hypertonic mediums. The first one, the ternary osmotic solution, was made from sucrose in the quantity of 1,200 g/kg water, NaCl in the quantity of 350 g/kg water and distilled water (in further text indicated as solution 1). The second osmotic solution was the combination of the first and third in a 1:1 ratio (in further text indicated as solution 2). The third, sugar beet molasses, with an initial dry matter content of 80.00%, was obtained from the sugar factory Pećinci, Serbia (in further text indicated as solution 3). The material to solution ratio was 1:5 (w/w). Dehydration was performed at room temperature (20 °C) with and without stirring in every 15 minutes under atmospheric pressure. Samples from all three solutions after 1, 3 and 5 were taken out to be lightly washed and gently blotted to remove excess water. The dry matter content of the fresh and treated samples was determined by drying at 105 °C for 24 hrs in a heat chamber (Instrumentaria Sutjeska, Serbia) until reaching a constant weight. All analytical measurements were carried out in accordance with *AOAC* (2000). In order to follow the mass transfer kinetics of the OD, three key process variables were measured: moisture content, change in weight and change in the soluble solids. Considering water loss (WL),

weight reduction (WR) and solid gain (SG), the rate of water loss (RWL), rate of solid gain (RSG), rate of weight reduction (RWR) and the dehydration efficiency index (EI) were calculated as described by *Koprivica et al.*, 2010. The analysis of variance (ANOVA) was performed using StatSoft Statistica, for Windows, ver. 10 programme.

3 Results and discussion

Table 1. shows average values and standard deviations of dry matter content in the samples of pork meat during OD with and without agitation in every 15 minutes, as a function of different type of osmotic solution and dehydration time. Along with changes in dry matter content, changes in kinetic parameters occurred and they are shown as well in Table 1. The increase of immersion time during the process resulted in increased dry matter content in pork meat samples and the higher values were obtained when the process was performed with agitation. The highest value was obtained in solution 2 after five hours of immersion ($60.08 \pm 0.09\%$) with agitation, while the highest value when the process was performed without agitation was achieved in solution 3 after five hours ($58.01 \pm 0.53\%$). ANOVA showed that for DMC, WL and WR values there was no significant statistical difference between the values of the meat dehydrated in solutions 1, 2 and 3. There was a significant statistical difference between the values of the meat samples dehydrated for 1, 3 and 5 hours and between the values of the meat samples when the process was performed with and without agitation. This indicates that time and agitation have a significant influence, while the nature of the osmotic solution does not have a significant influence on the DMC, WL and WR of the meat samples. The agitation of the osmotic medium recovers the thick diffusion layer of water that diffused from the meat cube into the osmotic medium (*Filipović et al.*, 2012). As a consequence of the process, the weight of meat samples was reduced. The samples' weight was more reduced when the process was performed with agitation. The highest values of WR parameter were obtained after 5 hrs in solution 3, when the process was performed with (0.3922 ± 0.0366 g/g initial sample weight (in future text: i.s.w.)) and without agitation (0.3271 ± 0.0425 g/g i.s.w.). SG value shows the degree of penetration of solids from hypertonic solution into the meat samples (Koprivica et al., 2009). SG, during the osmotic dehydration of pork meat, showed a tendency to increase with increasing the immersion time. The lowest value of SG parameter after five hours of process was obtained in samples dehydrated in solution 2 (0.2641 ± 0.0011 g/g i.s.w.) when the process was performed with agitation.

Table 1: Average values and standard deviations of dry matter content and the kinetic parameters during the osmotic dehydration of pork meat without and with agitation

Type of osmotic solution	Time (h)	DMC, % dry matter content	WR, g/g initial sample weight	SG, g/g initial sample weight	WL, g/g initial sample weight	EI-dehydration efficiency index
Osmotic dehydration without agitation						
Solution 1	1	42.36 ± 0.72^{a}	0.1518 ± 0.0180^{a}	0.2187 ± 0.0122^{a}	0.2585 ± 0.0061^{a}	1.1822 ± 0.0381^{a}
	3	51.89 ± 0.44^{b}	0.2692 ± 0.0233^{b}	0.2660 ± 0.0064^{b}	0.3978 ± 0.0032^{b}	1.4952 ± 0.0238^{b}
	5	52.88 ± 0.11^{c}	0.2753 ± 0.0339^{c}	0.2713 ± 0.0017^{c}	0.4074 ± 0.0008^{b}	1.5020 ± 0.0061^{b}
Solution 2	1	44.08 ± 0.28^{a}	0.1789 ± 0.0035^{a}	0.2063 ± 0.0045^{a}	0.2858 ± 0.0023^{a}	1.3854 ± 0.0195^{c}
	3	52.43 ± 1.99^{b}	0.2677 ± 0.0083^{b}	0.2767 ± 0.0293^{c}	0.4013 ± 0.0148^{b}	1.449 ± 0.1006^{d}
	5	58.62 ± 0.64^{c}	0.3006 ± 0.0085^{c}	0.3322 ± 0.0089^{d}	0.4615 ± 0.0045^{c}	1.3892 ± 0.0239^{c}
Solution 3	1	43.06 ± 1.24^{a}	0.1908 ± 0.0145^{a}	0.2006 ± 0.02003^{a}	0.2872 ± 0.0101^{a}	1.4318 ± 0.0932^{d}
	3	52.89 ± 0.96^{b}	0.2758 ± 0.0316^{b}	0.2791 ± 0.0139^{d}	0.4094 ± 0.0071^{b}	1.4667 ± 0.0481^{b}
	5	58.01 ± 0.53^{c}	0.3271 ± 0.0425^{c}	0.2788 ± 0.0071^{b}	0.4651 ± 0.0035^{c}	1.6686 ± 0.0297^{e}
Osmotic dehydration with agitation						
Solution 1	1	42.53 ± 0.09^{d}	0.1802 ± 0.0191^{d}	0.2158 ± 0.0015^{a}	0.2864 ± 0.0007^{a}	1.3268 ± 0.0058^{f}
	3	50.90 ± 2.63^{e}	0.2995 ± 0.0012^{e}	0.2152 ± 0.0369^{a}	0.4105 ± 0.0183^{b}	1.9081 ± 0.2458^{g}
	5	57.03 ± 0.95^{f}	0.3321 ± 0.0179^{f}	0.2894 ± 0.0127^{e}	0.4731 ± 0.0064^{c}	1.6349 ± 0.0498^{e}
Solution 2	1	43.27 ± 0.11^{d}	0.2057 ± 0.0084^{d}	0.2120 ± 0.0017^{a}	0.3082 ± 0.0008^{a}	1.4413 ± 0.0074^{d}
	3	52.73 ± 0.57^{e}	0.3325 ± 0.0057^{e}	0.2138 ± 0.0076^{a}	0.4401 ± 0.0038^{b}	2.0757 ± 0.0566^{h}
	5	60.08 ± 0.09^{f}	0.3801 ± 0.0187^{f}	0.2641 ± 0.0011^{b}	0.5108 ± 0.0005^{d}	1.9339 ± 0.0059^{g}
Solution 3	1	41.79 ± 0.60^{d}	0.2007 ± 0.0275^{d}	0.1749 ± 0.0097^{f}	0.2905 ± 0.0048^{a}	1.6614 ± 0.0644^{e}
	3	51.83 ± 0.6236^{e}	0.3443 ± 0.0046^{e}	0.1813 ± 0.0082^{f}	0.4383 ± 0.0041^{b}	2.4175 ± 0.0867^{j}
	5	59.93 ± 2.3930^{f}	0.3922 ± 0.0366^{f}	0.2675 ± 0.0291^{c}	0.5199 ± 0.0147^{d}	1.9432 ± 0.1571^{g}

^{abcdefghj} Different letters in the superscript in the same column indicate significant statistical difference between the values at a level of significance $p < 0.05$.

The values of the SG parameter were slightly higher when the process was performed without agitation (the lowest value after five hours was 0.2713 ± 0.0017 g/g i.s.w. for samples dehydrated in solution 1).

ANOVA showed that there was a significant statistical difference between the values of SG of the meat dehydrated in solutions 1, 2 and 3. Also, there was a significant statistical difference between the values of SG of the meat samples dehydrated for 1, 3 and 5 hours. However, there was no significant statistical difference between the values of SG in meat when the process was performed with and without agitation, except for meat samples dehydrated in solution 3 for 1 and 3 hours with agitation. This indicates that time and the nature of the osmotic solution have a significant influence on the SG of the meat samples while agitation does not have a significant influence. The amount of the solute penetration from the osmotic solution into the sample can be reduced by applying starch edible coatings (*Mišljenović et al.*, 2009). Increasing the dehydration time causes a greater water loss of the meat samples. The highest WL values were noticed in samples dehydrated for 5 hrs in solution 3 (with agitation 0.5199 ± 0.0147 g/g i.s.w. and without agitation 0.4651 ± 0.0035 g/g i.s.w.). The value of EI (WL/SG ratio) is the most important indicator of the effectiveness of the OD process (*Lević et al.*, 2007). In general, the increased concentration of the osmotic medium favours the diffusion of solids into the sample, which leads to decline in the value of EI. This ratio is considered to best predict the efficiency of the osmotic treatment. High EI ratios point to intensive water removal from the samples accompanied with minimal solid gain. In contrast, low EI are associated with an increased diffusion of solute to the sample with minimal water removal, which is unacceptable considering the purpose of the dehydration process. By changing the process variables (temperature, concentration and time), one tends towards finding the optimal conditions under which the process is most efficient (maximum EI). According to results in tables 1 and 2, OD treatment is more effective when the process is performed with agitation. The highest value of EI (2.4175 ± 0.0867) was achieved by using sugar beet molasses as osmotic solution after 3 hrs of treatment performed with agitation.

ANOVA showed that process time, the type of osmotic solution and agitation have a significant influence on the EI of the meat samples.

Tables 2 and 3 show the mass transfer rate during the osmotic dehydration with and without agitation, respectively, as a function of the immersion time and type of osmotic solution. According to the obtained results, osmotic dehydration was the most intensive at the beginning of the process.

Table 2: Mass transfer rate during the osmotic dehydration of pork meat without agitation

Type of osmotic solution	Time (h)	RWL g/(gi.s.w.·s)·10^5	RSG (gi.s.w.·s)·10^5	RWR (gi.s.w.·s)·10^5
Solution 1	1	7.1817	6.0748	4.2181
	3	3.6835	2.4636	2.4927
	5	2.2639	1.5070	1.5297
Solution 2	1	7.9395	5.7310	4.9703
	3	3.7155	2.5625	2.4783
	5	2.5642	1.8458	1.6703
Solution 3	1	7.9793	5.5729	5.2992
	3	3.7906	2.5845	2.5536
	5	2.5842	1.5487	1.8175

Table 3: Mass transfer rate during the osmotic dehydration of pork meat with agitation

Type of osmotic solution	Time (h)	RWL g/(gi.s.w.·s)·10^5	RSG (gi.s.w.·s)·10^5	RWR (gi.s.w.·s)·10^5
Solution 1	1	7.9550	5.9957	5.0047
	3	3.8014	1.9923	2.7735
	5	2.6283	1.6076	1.8449
Solution 2	1	8.5612	5.9399	5.7134
	3	4.0752	1.9632	3.0799
	5	2.8380	1.4675	2.1119
Solution 3	1	8.0705	4.8576	5.5745
	3	4.0584	1.6788	3.1876
	5	2.8882	1.4863	2.1788

Higher values of mass transfer rate were obtained when the process was performed with agitation, due to the already mentioned recovering of the thick diffusion layer of water. In this way, the forming of the concentration gradient in solution is avoided. The rate of mass reduction, the rate of water loss and

the rate of solid gain were the highest during the first hour of the process. Mass transfer rate decreased continuously from the first to the third hour and, after the third hour, it showed a tendency of slowing down. The mass transfer rate was slightly more intensive when meat samples were immersed in solution 3, due to greater difference between the osmotic pressures of the hypertonic medium and the animal tissue.

Based upon the presented results, it can be concluded that all three solutions are satisfying osmotic mediums. The process was more efficient when it was performed with agitation due to the better homogenization of the osmotic medium. The best results regarding dry matter content were achieved using solutions 2 and 3. At the end of the treatment, the solid gain values were the lowest in samples immersed in solutions 1 and 2. However, the best results considering water loss, weight reduction and dehydration efficiency index were achieved using sugar beet molasses as osmotic agent, which is economical, considering that molasses is a by-product of sugar industry. During the osmotic dehydration of pork meat, in all three osmotic solutions, the water removing process was the most intensive at the beginning and, after 3 hours, it had a tendency of slowing down; therefore, processing time can be limited to 3 hours.

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Microbiological profile of fish dehydrated in two different osmotic solutions

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Abstract. Fish is a substantial source of animal protein in human nutrition, but high water activity ($\mathfrak{a}_w$) value and moisture content of fish tissue are favourable to the growth of microorganisms. To extend its shelf life, fish needs to be processed. Dehydration of foods by osmosis involves the contact of the material with a concentrated aqueous solution. The aim of this research is to examine the influence of two different hypertonic mediums (sugar beet molasses and ternary solution) on the

Keywords and phrases: osmotic dehydration, fish, sugar beet molasses, ternary solution.

microbiological profile of fish (*Carassius gibelio*) after the process of osmotic dehydration. The process was carried out in laboratory jars under atmospheric pressure at a constant solution temperature of 20 °C for 5 hours. The sample to solution ratio was 1:5 (w/w) to neglect the changes of solution concentration during the process. In every 15 minutes, the fish samples were manually stirred. Both osmotic solutions have proved to be efficient in reducing a_w and the moisture content of samples providing quality and safe fish semi-product.

1 Introduction

Preservation of fish meat by drying involves a decrease in water content in order to reduce or inhibit microbiological growth (*Tsironiand & Taoukis*, 2014). Using osmotic dehydration to remove water from fish tissue reduces a_w, while the nutritional, sensorial and functional properties of food are improved (*Byrne et al.*, 2001; *Lee & Lim*, 2011. The osmotic dehydration process has many advantages in comparison to other drying methods such as water removal in liquid form, usage of mild temperatures, reduction of drying time, osmotic solution reusing, improvement of texture, flavour and colour, no chemical pretreatment and energy efficiency (*Ćurčić et al.*, 2013). The most important part of the osmotic treatment is the immersion into concentrated solutions of different salts and sugars and their combinations (*Agustinelli et al.*, 2013). For the osmotic dehydration of fish, usually binary (sodium chloride, sucrose) or ternary (salt-sucrose, salt-corn syrup) aqueous solutions are used as hypertonic mediums (*Oladele et al.*, 2008). According to a recent research, sugar beet molasses as a hypertonic solution improves the dehydration process primarily because of high dry matter content and specific nutrient composition (*Koprivica et al.*, 2013), and it can be successfully used for the osmotic dehydration of fruits, vegetables (*Mišljenović et al.*, 2011) and meat (*Filipović et al.*, 2012). Sugar beet molasses has a complex chemical composition (approximately 51% sucrose, 1% raffinose, 0.25% glucose and fructose, 5% proteins, 6% betaine, 1.5% nucleosides, purine and pyramidine bases, organic acids and bases) and the high content of solids (around 80%) provide high osmotic pressure in the solution; therefore, molasses appears to be an excellent osmotic medium (*Pezo et al.*, 2013).

The goal of this research was to examine the efficiency of the osmotic dehydration process, comparing the influence of two different osmotic mediums on water removal and on the microbiological profile of fish meat.

2 Materials and method

Experimental circumstances

The osmotic dehydration was carried out in laboratory jars under atmospheric pressure at a constant solution temperature of 20 °C. Fish (*Carassius gibelio*) was purchased on a local market in Novi Sad, Serbia, shortly prior to the experiment. The initial moisture content of untreated samples was 75.34%. Fish samples were filleted and cut into shapes (1 × 1 cm) using kitchen slicer and scissors. Hypertonic solution 1, sugar beet molasses, was obtained from the sugar factory Pećinci, Serbia with an initial dry matter content of 85.04% w/w; hypertonic solution 2, ternary aqueous solution (TAS) of sodium chloride and sucrose, was made from commercial sucrose and NaCl in the quantity of 1,200 and 350 g/kg of distilled water, respectively. After preparation, samples were measured and immersed in hypertonic solutions for 5 hours. The sample to solution ratio was 1:5 (w/w), which can be considered high enough to neglect the changes of solution concentration during the process. In every 15 minutes, the fish samples in the osmotic solutions were stirred to provide a better homogenization of the osmotic solution, considering the amount of diffused water from the samples. After 5 hours, the fish samples were taken out from the solutions, lightly washed with distilled water, gently blotted with paper to remove excess water from the surface and then weighed.

Methods

The dry matter content of the fresh and treated samples was determined by drying the material at 105 °C for 24 hours in a heat chamber (Instrumentaria Sutjeska, Croatia). a_w of the osmotically dehydrated samples was measured using a water activity measurement device (TESTO 650, Germany) with an accuracy of ± 0.001 at 25 °C. The soluble solids content of the molasses solution was measured using Abbe refractometer, Carl Zeis, Jenna, at 20 °C. All analytical measurements were carried out in accordance with AOAC (2000). In order to describe the mass transfer of the osmotic dehydration process, the experimental data for three key process variables are usually used, and these are: the moisture content, the change in the weight and the change in the soluble solids. Using these, the water loss and solid gain values were calculated as described by *Mišljenović et al.* (2012).

The determination of the total number of bacteria, *Escherichia coli*, Sulphite-reducing Clostridia and coagulase-positive Staphylococci was done by the SRPS EN ISO 4833, SRPS ISO 16649-2, ISO 15213 and SRPS EN ISO 6888-1, respectively.

3 Results and discussion

The osmotic dehydration process was studied in terms of common kinetic parameters such as dry matter content (DM), water loss (WL), solid gain (SG) and a_w. In *Table 1*, the changes in DM content in the samples of fish meat after the osmotic dehydration as a function of different type of osmotic solution are shown. The process resulted in higher dry matter content in fish meat samples dehydrated in both osmotic solutions, but a slightly higher value was achieved in samples dehydrated in sugar beet molasses ($58.339 \pm 4.471\%$).

Along with changes in dry matter content, as a consequence of the osmotic dehydration process, changes in water content occurred, causing a great water loss from the fish tissue. Both hypertonic solutions appear to be efficient in the water removal process; however, the higher WL value (0.530 ± 0.003 g/g i.s.w.) was noticed in samples dehydrated in sugar beet molasses. SG value shows the degree of penetration of solids from the hypertonic solution into the fish meat samples. SG, after the osmotic dehydration of fish meat, increased and the lower value of SG parameter was obtained in samples dehydrated in AOS (aqueous osmotic solution) (0.099 ± 0.008 g/g i.s.w.).

Table 1: Average values and standard deviations of kinetic parameters of the dehydrated fish

Kinetic parameter	Fresh fish meat	Fish meat dehydrated in molasses	Fish meat dehydrated in AOS
Dry matter content, %	23.975 ± 1.965	58.339 ± 4.471	52.680 ± 2.256
Water loss*, g/g i.s.	0.000 ± 0.000	0.530 ± 0.003	0.474 ± 0.004
Solid gain*, g/g i.s.	0.000 ± 0.000	0.111 ± 0.003	0.099 ± 0.008
a_w	0.944 ± 0.007	0.845 ± 0.023	0.848 ± 0.036

*mass in grams of WL or SG per mass in grams of initial sample

Table 1 shows the average a_w values and the standard deviation of the fresh and dehydrated fish in sugar beet molasses and in the AOS solution. Fresh

samples of fish before treatment had an average a_w of 0.944 ± 0.007, which is close to the optimum growth level of most microorganisms (*Nićetin et al.*, 2012). After the process of osmotic dehydration, lower a_w values of fish meat samples dehydrated in both osmotic solutions were observed. The obtained a_w values of samples dehydrated in sugar beet molasses and AOS solution were 0.845 ± 0.023 and 0.848 ± 0.036, respectively. Sugar beet molasses was slightly more effective in lowering the a_w of fish samples. It may be concluded that the process of osmotic dehydration ensures a_w values which are within a specified range for fish meat quality and safety, considering that most meat spoilage bacteria do not grow below a_w value of 0.91 (*Vereš*, 1991).

The results of the microbiological analysis of the fresh and dehydrated fish meat are presented in *Table 2*. The total number of bacteria in fresh fish was $6.67 \cdot 10^5 \pm 3.4 \cdot 10^4$ CFU/g. After the osmotic dehydration process, the total number of bacteria in dehydrated samples in sugar beet molasses and AOS were $4.23 \cdot 10^4 \pm 2.6 \cdot 10^3$ and $7.33 \cdot 10^4 \pm 7.6 \cdot 10^3$ CFU/g, respectively. The reductions of the total number of bacteria in dehydrated samples in comparison to the initial total number of bacteria in the fresh fish meat was 93.66% for samples dehydrated in sugar beet molasses and 89.01% for samples dehydrated in AOS. These results prove that the process of osmotic dehydration has an important influence on the reduction of the total number of bacteria in the osmotically treated fish.

Table 2: Microbiological analysis of the fresh and dehydrated fish meat in two osmotic solutions

Hygiene and food safety criteria	Fresh fish meat	Fish meat dehydrated in molasses	Fish meat dehydrated in AOS
Total number of bacteria, CFU/g	$6.6 \cdot 10^5 \pm 3.4 \cdot 10^5$	$4.23 \cdot 10^4 \pm 2.6 \cdot 10^3$	$7.33 \cdot 10^4 \pm 7.6 \cdot 10^3$
Escherichia coli (CFU/g)	0	0	0
Sulphite-reducing Clostridia (CFU/g)	< 10	< 10	< 10
Coagulase-positive Staphylococci (CFU/g)	< 100	< 100	< 100

The number of *Escherichia coli*, coagulase-positive Staphylococci and sulphite-reducing Clostridia in fresh fish meat samples was in accordance with the hygiene production criteria of the Serbian National Regulation (72/2010). There

was no observed increase in the number of these bacteria in the dehydrated fish post-osmotic treatment.

The microbiological profile of dehydrated fish meat samples indicates that the osmotic dehydration is a hygienically safe process. A better reduction of the present microorganisms in fish meat was obtained in the samples dehydrated in sugar beet molasses. Both osmotic solutions have proved to be efficient in reducing the water content and the a_w of samples, providing quality and safe fish semi-product. Sugar beet molasses was proved to be more than a good alternative to the conventional hypertonic solution.

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Comparison of N and S contents of different winter wheat flour samples

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Abstract. Sulphur is the fourth macro-element, which mostly occurs in crops in organic forms, as components of sulphur-containing amino acids (methionine, cystine, cysteine). In a number of cases, these amino acids together with lysine act as limiting amino acids. Furthermore, in winter wheat, the S-S cross binding of gluten components (cystine) plays a vital role in forming a suitably structured soft part of bread as sulphur deficit may cause serious quality disorders, the decline of the viscoelasticity of dough. In Hungary, the amount of the atmospheric deposition of sulphur and superphosphate application containing 10-11% sulphur has decreased significantly over the past twenty years. Since winter wheat flours can be found in one of the important raw materials for the foods, we considered important to examine the contents of their nitrogen and sulphur. We analysed different wheat varieties for long-term mineral fertilization experiments. The nitrogen and sulphur contents of flours were determined by using the combustion (Dumas) method. We found that both the genotype and the mineral fertilizer treatments had significant effects on the N and S contents of winter wheat flours, but the interaction between these factors was not proved. The N contents varied between 1.90 and 2.85% on dry matter base. The S content of samples was between 0.09 and 0.14% on dry matter base and the pace of changing was similar to the changing of N content. As the overall conclusion, the characteristics of the N/S ratio of four species were established (GK Ati 17.8 to 20; Ukrainka 19 to 20.5, 19 to 23.3 Lupus; Mv Suba 21.8-23.3), the rates of which were higher than the values previously reported for the grain of wheat.

Keywords and phrases: winter wheat, flour, N and S content, mineral fertilization.

1 Introduction

Sulphur is the fourth macro-element, which mostly occurs in crops in organic forms as a component of sulphuric amino acids (metionyne, cystine, cysteine). In a number of cases, these amino acids together with lysine act as limiting amino acids. Furthermore, in winter wheat, the S-S cross binding of the gluten components (cystine) plays a vital role in forming a suitably structured soft part of bread as sulphur deficit may cause serious quality disorders. A close correlation has been established between the sulphur contents of wheat grains and the volumes of bread loaves (*Zhao et al.*, 1999). The reduction in the percentage of polymeric protein in flour as a result of sulphur deficiency was due to a decrease in LMW glutenin subunits, which are normally present in larger amounts than the HMW subunits (*MacRitchie & Gupta*, 1993).

Scherer (2001) described that wheat has a relatively low S requirement (to about 20 kg/ha S necessary to 8 t/ha grain yield) (*McGrath et al.* 1996), but several findings reveal that – as regards the quality of wheat – a good sulphur supply is essential since low sulphur supplies will decrease cystine contents and, as a result, the disulphide bonds on the gluten will not be adequate to ensure a satisfactory resilience. The S deficiency symptoms are not easily identifiable in wheat because they can be confused with those of the N deficiency (*Zhao et al.*, 1996). The marks of sulphur deficiency from barley are the accumulation of amides in roots and the accumulations of asparagine and glutamine in leaves (*Karmoker et al.*, 1991).

When analysing the sulphur contents of wheat varieties in England, *McGrath et al.* (1993) found that sulphur contents had significantly decreased over the ten previous years. The reason for the decrease in Hungary is caused by the decreasing atmospheric deposition and the decreasing mineral fertilization (especially superphosphate). In Hungary, *Győri* (2005) published data from the sulphur contents of different winter wheat varieties in different cropping sites. His results show that the average sulphur content of winter wheat grain is 1500±120 mg/kg in Hungary. Mars et al. (2006) investigated the effect of different sulphur fertilizers on wheat yield and their baking quality. They found that the foliar sulphur (5 kg/ha) fertilization increased both the gluten content and the farinograph (BU) value number. Because the bakery products are among staple foods, it was important to examine the nitrogen and sulphur contents of winter wheat grains and flours.

2 Materials and methods

The samples examined were taken to the Experimental Station of the University of Debrecen at Látókép in 2004. The type of soil was a calcareous chernozem soil. In the experiment, we applied the following treatments: control, 30 kg ha^{-1} nitrogen (as ammonium nitrate), 22.5 kg ha^{-1} P_2O_5 (as superphosphate) and 26.5 kg ha^{-1} K_2O (as potassium chloride) and the double, triple, four- and fivefold amounts of these doses in four repetitions.

For the milling process, we used a FQC-106 laboratory mill (MSZ 6367/9-1989) with a 250 μm sieve (InterLab Kft., Budapest). We examined the quality parameters of four winter wheat varieties (GK Ati, Ukrainka, Mv Suba and Lupus). The determination of the N and S contents of the flours was done with Elementar VarioMax equipment (Hanau, Germany), based on the combustion method by Dumas (*AACC 046– 0, 2000*) in the Central Laboratory of the University of Debrecen, Centre for Agricultural Sciences. The certified sample was BCR CRM 189 wheat. Statistical analyses were performed using Microsoft Excel 2007 and SPSS 22.0 for Windows.

3 Results and discussion

The adequate sulphur supply of wheat is an important task even after the atmospheric deposition of sulphur pollution has decreased significantly. By using superphosphate, which contains sulphur during fertilization, the essential question is how to develop the composition of wheat flour. Gluten properties and their sulphur-protein compounds play an important role in the bread making process and its quality. The quantity and quality of these compounds, different for each variety and fertilizer treatment, may bring about different responses. We found that both the genotype and the mineral fertilizer treatments had significant effects on the N and S contents of winter wheat flours, but the interaction between these factors was not proved (*Table 1*).

There were significant differences between the nitrogen contents of the varieties even as regards the control treatments, as they showed the following readings: Ukrainka 1.9%, Gk Ati 2.18%, Lupus 2.3% and Mv Suba 2.5%. The N contents varied between 1.9 and 2.85% on dry matter base (*Figure 1*). The responses of these varieties to the increasing fertilizer ratios were diverse; the N content of GK Ati variety increased continuously with the treatments, while the other genotypes showed maximum values at lower nutrient levels (Ukrainka at 120 kg ha^{-1} N+PK, Mv Suba at 60 kg ha^{-1} N+PK, Lupus at 30 kg ha^{-1} N+PK), which were followed by slow decreases.

Table 1: Results of the two-way ANOVA of the grain N and S content showing the F values and P levels of the main effects and the interaction

	N content		S content		N/S ratio	
	F value	P level	F value	P level	F value	P level
Genotype	50.741	0.0000	7.585	0.0010	11.296	0.0001
Mineral fertilizer treatment	27.504	0,0000	4.449	0.0052	1.637	0.1886
Genotype x mineral fertilizer treatment	1.768	0.1034	0.580	0.8618	0.500	0.9167

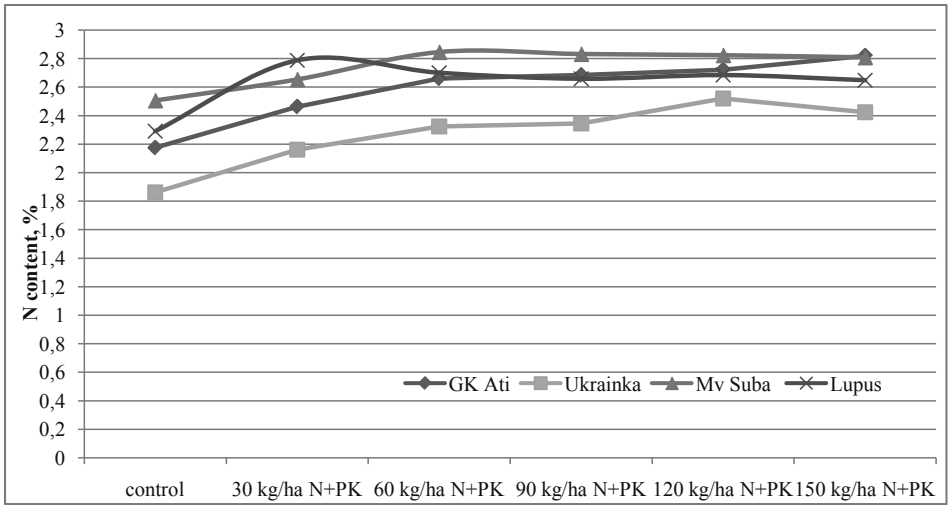

Figure 1: Effect of mineral fertilization on the N content of wheat flours

The S contents of the samples were between 0.09 and 0.14% on dry matter base (*Figure 2*) and the rate of change was similar to the changes in the N contents; so, winter wheat variety Ukrainka showed the strongest fertilizer response and Mv Suba showed the most stable quality in the response to the different levels of mineral fertilization. In the case of variety Lupus, the S content showed a peak with the highest N content at 30 kg ha^{-1} N+PK and the higher NPK doses resulted in a decline, but at the N150+PK dose the sulphur content of 1,400 mg/kg was the highest.

An important parameter indicating the nutritional status of winter wheat is the N:S ratio in the grains.

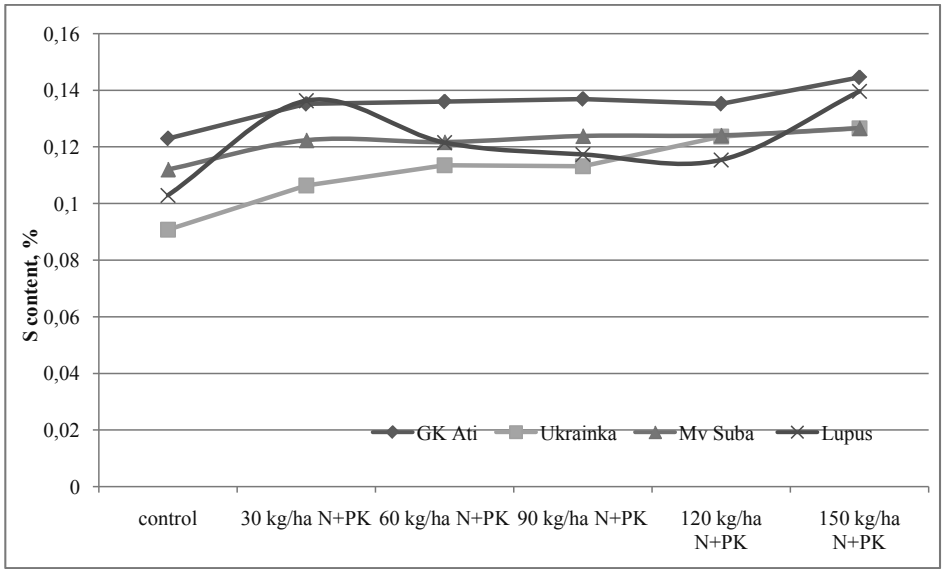

Figure 2: Effect of mineral fertilization on the S content of wheat flours

According to the data reported in my previous article (*Győri*, 2005), the values of this ratio were around 15, in accordance with the values in the literature for the case of good plant nutrition. The N/S ratios of flours, as the statistical analysis suggested, were stable values by genotypes (Figure 3).

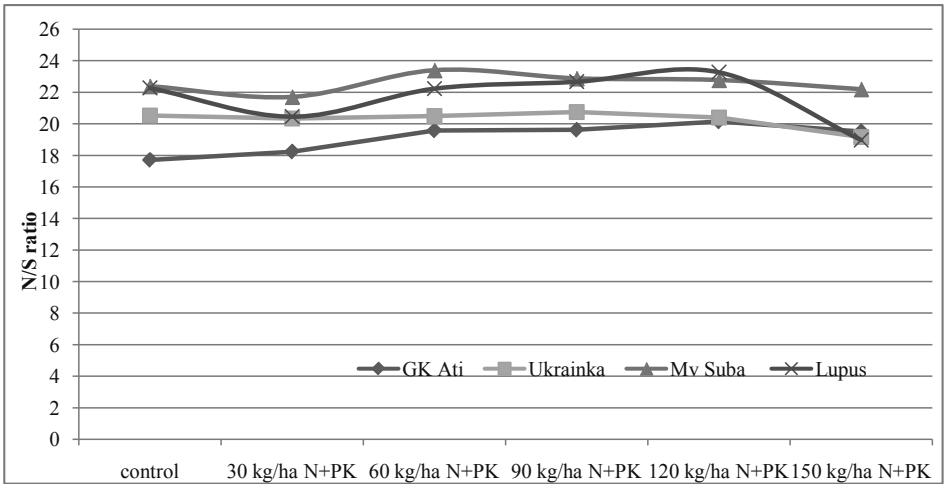

Figure 3: Effect of mineral fertilization on the N/S ratio of wheat flours

In all of the four varieties analysed, the S:N ratios decreased in the case of the highest ratios of fertilizer application to either a smaller (GK Ati) or a larger extent (Lupus) in relation to the other treatments. The effect of increasing fertilizer doses resulted in no changes in the N/S ratio of Ukrainka to a N120 + PK kg/ha treatment, but in contrast, in the case of GK Ati, the rate increased. In the case of Lupus and Mv Suba varieties, the rate was higher than in the previous two varieties, but the effects of the fertilizers were also more varied.

As the overall conclusion, the characteristics of the N/S ratios in four species were established (GK Ati 17.8 to 20; Ukrainka 19 to 20.5, 19 to 23.3 Lupus; Mv Suba 21.8-23.3), the rates of which were higher than the values previously reported for the grain of wheat (*Győri*, 2005; *Byers* & *Bolton*, 2006).

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Effect of salt forms and concentrations on the valorigraphic parameters of winter wheat flour

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Abstract. Salt (NaCl) is a basic component of our foodstuffs. Its taste is required by customers and it has effects on the technological properties, too. Nutrition science considers it as one of the hazardous food additives due to the negative health aspects of sodium. It is especially a current topic in Hungary; the average national intake is about threefold-fourfold of the recommended value for men and twofold-threefold for women. This question is especially interesting in the case of bakery products; it is found that a significant amount of sodium intake is due to the baked product consumption. The aim of this study was to evaluate that whether the gluten network influencing the effect of the salt concentrations and forms can be measured by Valorigraph. Results show that the sodium chloride addition significantly influences the Valorigraph parameters. An increase was found in the case of dough development time and stability, while the value of softening decreased and therefore the baking value of flour improved. The different salt forms also change the readings; so, the gluten network modification effects of salt forms and concentrations can be characterized by Valorigraph test.

Keywords and phrases: winter wheat flour, Valorigraph, sodium, salt forms.

1 Introduction

Sodium chloride (NaCl) is one of our most ancient food raw materials and additives. Its role is giving taste to the food (it is one of the four general basic tastes); it is one of the first preservatives (due to its water-activity decreasing function) and in several cases it has effects on the behaviour of raw materials and therefore on their technological quality. These functions can be experienced in bakery use: the sodium chloride stabilizes the fermentation processes also during rising and baking. Saltless dough is gassy; a sour one and the bread made from it has poor texture (*Matz*, 1992). The increase in osmotic potential resulted by the salt addition significantly increases the fermentation time or makes the increase of the amount of added yeast necessary. The leaven or the indirect dough-making technology is this microbe-hindering role of salt; the fifth-sixth part of the original yeast addition is necessary for leaven bread making, but the process time increases to 6-8 hours.

Beside its effect on fermentation, salt has an important influence on gluten and dough structure. It was found that increase in sodium chloride addition decreases the water absorption of flour and increases the development time of dough while increasing its strength too (*Hlynka*, 1962; *Preston*, 1989; *Tanaka et al.*, 1967). Its main reasons are the changes in the pH and the ionic conditions of dough. The liquid phase of saltless dough has a pH around 6 and the gluten network has stable positive charges due to the weakly acidic medium, while the side-chains repulse each others, resulting in a weaker gluten network. Sodium chloride addition results in a stronger gluten structure due to the increase of pH and the decrease in positive charges. These changes can be experienced to 1.5 and 2% salt addition; a further increase in concentration hinders the dough development (*Preston*, 1981; *Danno & Hoseney*, 1982). On the other hand, by the appearance of Na^+ and Cl^- ions in the dough, their protein-stabilizing (non-chaotropic) character makes the proteins less hydrated, resulting less water absorption (*Cacace et al.*, 1997; *Miller & Hoseney*, 2008). About 35% of gluten proteins are hydrophobic and the ionic concentration and composition of the liquid phase of dough modifies their solubility as well as the hydrophobic interactions within the gluten structure (*Danno & Hoseney*, 1982).

The salt intake of today's people is found to be high by the nutritionists. The recommended daily intake is 5 g/day sodium chloride, but the people consume a higher (and, in several cases, much more) amount almost worldwide. Based on an international survey, the Hungarian consumers are leading in salt intake: 17 g/day is the average consumption for men and 12 g/day for

women, and the reading for children is also high (*Martos*, 2010). Salt intake reduction programmes started worldwide to decrease people's sodium increase, as significant connection was found between the intake and the occurrence of high blood pressure and cardiovascular diseases (*He & MacGregor*, 2007; *Jones*, 2008; *Satin*, 2008), and the decrease in intake results in the immediate decrease in blood pressure (*Kurtzman*, 2001). About one-third of the sodium is consumed via the bakery products (*Cauvain*, 2007) and – as this product group and other wheat-based products have significant role in the groups of staple foods (*Véha*, 2007; *Véha et al.*, 2012) – the Hungarian National Salt Reduction Programme started in 2010 prescribes decrease in the salt content of bakery products.

There are only a few products available for the substitution of salt. Generally, potassium chloride is used for this purpose, but its metallic taste in bread and its potential health risk hinder its use – it is used only to a maximum of 50% substitution for sodium chloride (*Matz*, 1992). While the effects of sodium chloride on dough and the gluten properties are widely evaluated, the effects of other salt forms are investigated much less. The aim of this study is the evaluation of the effect of salt forms and concentrations on the rheologic properties of winter wheat dough and the detailed exploration of their effects on the Valorigraph parameters.

2 Materials and method

Materials

The evaluated flour samples were BL55 ones and bought from a local supermarket. The evaluated salt forms were sodium chloride, potassium chloride, sodium acetate, potassium acetate and calcium acetate (VWR, Belgium). The ion-exchanged water used for tests was performed by a MILLIPORE water purifier (Millipore, France).

Valorigraph tests were performed by FQA-205 valorigraph (METEFÉM, Hungary). All the analyses were done in the laboratory of the University of Debrecen, Faculty of Agricultural and Food Sciences and Environmental Management, Institute of Food Science.

Methods

Valorigraph tests were performed by the MSZ ISO 5530-3:1995 Hungarian Standard. Salt solutions were prepared in 0.5; 1.0; 1.5 and 2.0% w/v%. All

the measurements were performed in two repetitions. The results were analysed by one-way analysis of variance using SPSS 15.0 for Windows statistical programme package (SPSS Inc.), while Tukey's post-hoc test was used to reveal significant differences. The tables present means and standard deviations.

3 Results and discussion

The rheologic properties measured by Valorigraph were influenced by the sodium chloride addition. The dough development time increased by the increasing salt concentration; 2% sodium chloride addition resulted in a 34%-increase regarding this value, but only the salt addition had proved to be effective statistically; this parameter was not influenced significantly by the concentration of sodium chloride, although an increasing tendency can be seen in the further results (*Table 1*). The stability also increased significantly by the salt addition and the effect of higher concentrations (1.5 and 2.0%) resulted in a statistically proved increase. In the readings of baking value, a significant increase was found again, but the water absorption capacity was not influenced by the increase of salt concentration in contrast to the references.

Table 1: Valorigraph readings of dough made from BL55 flour and sodium chloride solution

Salt concentration, %	Water absorption capacity,%	Dough development time, min	Stability, min	Baking value
0.0	64.7 ± 0.4 a	3.5 ± 0.1 a	7.3 ± 0.1 a	57.9 ± 1.5 ab
0.5	64.9 ± 0.4 a	4.1 ± 0.1 b	7.6 ± 0.1 a	55.4 ± 1.3 a
1.0	65.6 ± 0.6 a	4.1 ± 0.1 b	8.0 ± 0.3 a	61.9 ± 0.7 b
1.5	64.7 ± 0.1 a	4.6 ± 0.1 b	10.6 ± 0.2 b	68.1 ± 0.6 c
2.0	64.8 ± 0.3 a	4.7 ± 0.2 b	10.4 ± 0.4 b	65.1 ± 1.6 bc

Means marked with the same letter in the same column were not significantly different at the 5% confidence level on the basis of Tukey's test.

Similar tendencies were found in the case of potassium chloride addition (*Table 2*). The KCl addition did not influence water absorption capacity, but the increasing concentration improved the valorigraph readings significantly. The increases in dough development time, stability and baking value were remarkable and higher than the ones experienced in the case of NaCl use.

Table 2: Valorigraph readings of dough made from BL55 flour and potassium chloride solution

Salt concentration, %	Water absorption capacity,%	Dough development time, min	Stability, min	Baking value
0.0	64.7 ± 0.4 a	3.5 ± 0.1 ab	7.3 ± 0.1 a	57.9 ± 1.5 ab
0.5	64.4 ± 0.3 a	4.2 ± 0.3 abc	7.9 ± 0.4 ab	54.3 ± 1.0 a
1.0	64.3 ± 0.4 a	3.4 ± 0.2 a	9.1 ± 0.2 b	62.0 ± 0.8 b
1.5	64.0 ± 0.1 a	4.8 ± 0.4 bc	12.2 ± 0.4 c	71.5 ± 1.5 c
2.0	64.1 ± 0.1 a	4.9 ± 0.5 c	12.3 ± 0.5 c	72.7 ± 1.1 c

Means marked with the same letter in the same column were not significantly different at the 5% confidence level on the basis of Tukey's test.

The effects of sodium, potassium and calcium acetate were also negligible on the water absorption capacity readings; this parameter varied from 63.0 to 64.7% in all cases. Sodium acetate did not have a statistically proved influence on the dough development time in contrast to the sodium chloride addition, but the increasing tendency can be seen in the results (*Table 3*).

Table 3: Valorigraph readings of dough made from BL55 flour and sodium acetate solution

Salt concentration, %	Water absorption capacity,%	Dough development time, min	Stability, min	Baking value
0.0	64.7 ± 0.4 a	3.5 ± 0.1 a	7.3 ± 0.1 a	57.9 ± 1.5 a
0.5	64.3 ± 0.1 a	4.0 ± 0.7 a	10.8 ± 0.1 b	60.9 ± 0.8 ab
1.0	63.5 ± 0.7 a	3.8 ± 0.4 a	10.3 ± 1.1 ab	61.8 ± 3.7 ab
1.5	64.3 ± 0.4 a	4.3 ± 0.4 a	11.8 ± 0.3 b	66.3 ± 0.1 bc
2.0	64.0 ± 1.4 a	4.5 ± 0.7 a	12.4 ± 1.5 b	69.9 ± 1.9 c

Means marked with the same letter in the same column were not significantly different at the 5% confidence level on the basis of Tukey's test.

However, the increasing concentrations of the other two tested acetates significantly increased this parameter to a similar extent than the chlorides and the sodium acetate. The stability was influenced by the increasing acetate concentrations in statistically proved ways and the highest increase was resulted by the highest concentration of sodium acetate. All the increases are higher than the one that was experienced in the case of NaCl addition. The

baking value readings were also improved by the increasing salt concentrations and the potassium salt resulted in the highest increase (*Table 4* and *Table 5*).

Table 4: Valorigraph readings of dough made from BL55 flour and potassium acetate solution

Salt concentration, %	Water absorption capacity,%	Dough development time, min	Stability, min	Baking value
0.0	64.7 ± 0.4 a	3.5 ± 0.1 a	7.3 ± 0.1 a	57.9 ± 1.5 a
0.5	64.4 ± 2.3 a	4.0 ± 0.1 ab	9.2 ± 0.5 b	56.9 ± 3.3 a
1.0	64.7 ± 1.0 a	4.3 ± 0.4 ab	9.3 ± 0.2 b	58.7 ± 2.6 a
1.5	64.1 ± 0.4 a	4.3 ± 0.4 ab	10.5 ± 0.1 b	64.4 ± 2.2 ab
2.0	65.1 ± 0.1 a	4.5 ± 0.1 b	11.1 ± 1.3 c	71.2 ± 0.6 b

Means marked with the same letter in the same column were not significantly different at the 5% confidence level on the basis of Tukey's test.

Table 5: Valorigraph readings of dough made from BL55 flour and calcium acetate solution

Salt concentration, %	Water absorption capacity,%	Dough development time, min	Stability, min	Baking value
0.0	64.7 ± 0.4 a	3.5 ± 0.1 a	7.3 ± 0.1 a	57.9 ± 1.5 a
0.5	64.1 ± 0.7 a	3.3 ± 0.4 a	9.6 ± 0.1 ab	58.1 ± 3.5 a
1.0	64.2 ± 0.3 a	4.0 ± 0.1 ab	8.9 ± 0.6 ab	58.4 ± 0.3 a
1.5	64.2 ± 0.3 a	4.3 ± 0.4 ab	8.6 ± 1.3 ab	60.6 ± 0.9 ab
2.0	63.0 ± 0.6 a	4.8 ± 0.4 b	11.1 ±1.3 b	69.0 ± 3.3 b

Means marked with the same letter in the same column were not significantly different at the 5% confidence level on the basis of Tukey's test.

Based on our experimental results, it was concluded that all the examined salt forms had influence on the valorigraph parameters of dough: not only the sodium chloride addition improving the quality of dough but the other evaluated salt forms, too. The increasing concentrations resulted increase in the parameters (with the exception of the water absorption capacity), but the potassium salts (chloride and acetate) had the highest effects: its use in 1.5 and 2.0% concentrations resulted advances in the quality group, from B1 to A2, based on the Hungarian qualification system. Surprisingly, calcium acetate

addition also resulted in similar findings; therefore, it is not unequivocal that the added ions or the changes in pH caused by the saline are the reasons for the effects.

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Analysis of age differences in the risk perception of food additives: Results of focus group interviews

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Abstract. Hungarian consumers have a high level of worry about food additives, which shows differences according to various socio-demographic factors like age. Focus group interviews were conducted in order to analyse different age groups' risk perception as well as to recognize their knowledge, opinions and their information sources about food additives. Results revealed that young adults (18–24 yrs.) and elderly people (over 45 yrs.) did not care about foodstuff label information, including additives and "E-numbers". Adults' (25–44 yrs.) shopping decisions were influenced by the ingredients (e.g. carbohydrates, gluten and additives); however, their exact knowledge about the avoided components was limited. Group members were able to list a number of negative information and food-related scandals, but just a few were acquainted with food additives (aspartame, guar gum). The main source of information about foodstuffs and additives is the Internet. Youths show negligent attitude

Keywords and phrases: additives, E-numbers, focus group interview, shopping decision.

regarding food additives, adults try to pay attention to the composition of their foodstuffs, while elderly people try to return to home-made production and preservation methods. Mistrust against foodstuffs and producers as well as the demand for accurate information is common. This can be decreased with the understandable and truthful information and furthermore with the indication of the proper sources.

1 Introduction

Only 64.4% of the Hungarian consumers believe that foodstuffs present on the market are safe (*Eurobarometer*, 2011), and most of them (82%) think that foods and drinks might contain chemicals (*Eurobarometer*, 2013). Hungarian consumers have a high worry about food additives (*Eurobarometer* 2006a, 2010; *Szűcs et al.*, 2012), in which the negative (e.g. polluted guar gum, colourings can cause hyperactivity (*McCann et al.*, 2007), carcinogenic sweeteners) and often enhancing effect of misleading media news may have a dominant role (*Kasza*, 2009). Due to this, consumers tend to change their consumption behaviour (e.g. change a shop or a product) (*Eurobarometer*, 2011). Furthermore, Hungarian consumers have ambiguous knowledge about food additives (*Tarnavölgyi*, 2003, 2004; *Szűcs & Bánáti*, 2010a; *Szűcs et al.*, 2012). For example, only 61.8% of them know correctly that every food additive can be linked to an "E-number" (*Szűcs et al.*, 2012). This may be due to the fact that they use inadequate information sources. As a result, these Hungarian consumers conceive that avoidance of food additives is part of the healthy diet (*Eurobarometer*, 2006b), and their shopping decisions are significantly influenced by the presence of food additives (*GFK*, 2007; *Marián et al.*, 2011; Marketing Info, 2013). Consumers' uncertainty is verified by the fact that almost more than half of them trust in independent consumer organizations (66.5%), public authorities (68.5%) and that sellers/providers (61.4%) protect and respect their rights (*Eurobarometer*, 2011).

Risk perception of food additives shows differences according to the sociodemographic factors. A number of studies have stated that males (*Dosman et al.*, 2001; *Eurobarometer*, 2006a; *Dickson-Spillmann et al.*, 2011), youthful (*Eurobarometer*, 2006a; *Food Standard Agency*, 2010; *Szűcs et al.*, 2012), highly educated (*Kajane & Pirtillä-Backman*, 1999) and well-off people (*Dosman et al.*, 2001) perceive a lower level of risk in the case of food additives.

The aim of the present study was to analyse different age groups' risk perceptions regarding food additives as well as to recognize their knowledge, opinions and information sources about this topic.

2 Materials and methods

In order to analyse our targets, focus group interviews were conducted with the help of three age-homogenous groups: young adults (18–24 years), adults (25–44 years) and elderly people (over 45 years). The separation of these age groups can be justified by the fact that these three groups use and accept different information sources as authentic concerning healthy nutrition (*Szűcs et al.*, 2010b; *Szakály*, 2011); furthermore, it was assumed that members of the same age range can communicate easier with each other (*Vicsek*, 2006). There were six members in each focus group, and they were mixed regarding genders and place of residence (capital and countryside). The interviews were semi-structured and the guidelines were planned ahead. Besides the raising of questions, word associations were also applied. This helps to form the feeling of active participation, gives energy, entertains the respondents and contributes to their "warming up" (*Gordon & Langmaid*, 1997). In order to explore the knowledge and to recognize differences between the associations about "E-numbers" and food additives, different questions were asked. Data analysis was conducted with a rapid method on the basis of the notes taken during the interviews (made by a note maker). The voice recordings were checked again to refine the citations. After the conversation, the note maker and the moderator briefly evaluated the interview ("debrief") (*Vicsek*, 2006) in order to be taken into consideration during the data analysis. The word associations were analysed quantitatively, while other data were evaluated by qualitative content analysis.

3 Results

Factors effecting the shopping decisions

Young adults pay attention to the composition of foodstuffs. Some of them avoid certain substances (e.g. trans fatty acids), while others avoid well-specified food additives (e.g. aromas, colourings). The importance of the price–value ratio was mentioned only in one case. Enriching of the foodstuffs (e.g. with vitamin E) is not important for them.

> "I avoid colourings if I can, but it is difficult." (young adult)
>
> "Do not contain smoke aroma!" (young adult)

Amongst the influencing factors, adults have also alluded certain substances like carbohydrates, gluten and 'E-numbers' as well as the price–value ratio.

The importance of packaging was mentioned in many cases. If the packaging is attractive for them, they are ready to pay more for that foodstuff. The expiration date and the origin (importance of Hungarian product) were mentioned in one case.

> "It is hard to avoid 'E-numbers,' they are present in everything." (adult)
>
> "I check what kind of 'E'-s are there in it." (adult)

The foodstuff shopping decisions of the elderly people are strongly influenced by the price. Ingredients were also mentioned many times; however, they noted that they are not always able to read the small letters on the labels. The origin of the foodstuffs is also a significant characteristic. The Hungarian products are more attractive for them, mainly in the case of fruits and vegetables.

> "My glasses are not strong enough to see the ingredients." (elderly).
>
> "If I can choose, first of all, let it be Hungarian." (elderly).

Associations and feelings regarding the "E-numbers"

The associations were grouped into six topics in order to facilitate their analysis. Young adults and elderly people linked "E-numbers" to food additives. The preservation effect of food additives was mentioned in all three interviews; furthermore, the colourings in the case of young adults and elderly people. A number of allusions referring to the harmful health effects and their uncertainty occurred many times amongst the young adults and adults, while less often amongst the elderly people. Additionally, a common observation was the query of their utilization's reasonableness (*Table 1*).

> "Not all of them are bad or harmful." (young adult)
>
> "In order to decide, it has to be known which one is which and what kind of effect they have. But this is impossible because there are a lot." (young adult)
>
> "There are changing and diverse information about them." (adult)
>
> "There are too much negative beliefs about them." (adult)
>
> "Negative feelings because the media enhance these." (elderly)
>
> "Eventually harmful substances should not be put in it." (elderly)

Table 1: Associations and feelings of the age groups regarding "E-numbers"

Young adults	Adults	Elderly	Topics
"food additives" "abbreviation of substances"	"grouping" "product improver"	"food additives"	**Meaning**
"preservative" "colouring"	"preservation" "flavour enhancer"	"colouring" "preservative" "aroma"	**Technological function**
"can be harmful or not harmful" "carcinogenic" "some of them are bad for the body"	"unhealthy" "there are some harmless ones" "harmful" "can cause hyperactivity" "risk"	"not all of them are harmful"	**Health effect**
"unknown effect" "negative"	"not understandable" "incomprehensive" "claptrap" "influencing" "mixed information" "uncertainty"	"mystic"	**Uncertainty**
"too much does not mean good" "must not buy products with too much 'E'-s" "some of them is necessary" "bad but necessary"	"unnecessary" "artificial"	"good or bad, but necessary" "the less the better" "can be natural or artificial"	**Judgement of their utilization**
"I do not care about it"	–	"not important to me" "I do not care about it"	**Neutrality**

Associations and feelings regarding "food additives"

The connections of food additives and "E-numbers" were mentioned several times by the elderly participants. There was one person from the young adults who felt that food additives were more positive than "E-numbers," while among the adults just the opposite was mentioned. Most of the associations were oriented towards the judgement of utilization and the technological functions of food additives. Utilization of food additives is not reasonable according to the participants. They feel that there are marketing aims, economic pressure and commercial needs behind their application.

Young adults feel that they are cheated because foodstuffs are modified by the additives like fruit yogurt without fruit, frankfurter which contains more soya than meat, ham which is coloured by artificial pickle. Adults consider food additives as artificial substances. During the interview with elderly people, the question came up that it was not necessary to buy foodstuffs containing additives; at home, you can prepare them without these (*Table 2*).

> "Today, everything is too convenient and most of the food additives are in the ready-to-eat foods." (young adult)
>
> "We do not think that natural substances are applied nowadays." (adult)
>
> "If you do not like something, do it on your own." (elderly)

Opinions about food additives

Food additives are harmful to health and risky substances according to the young adults. This is due to the lack of information on them. A member of the group mentioned that there are some food additives which have favourable effects; however, in the case of unknown components, they are ambiguous. They get their information from talking with their friends and from the Internet; however, they are not always sure in the truthfulness of the sites. Young adults are distrustful of producers. The opinion of the authorities is more acceptable for them. Independence is the most important in authenticity.

> "More information is needed to decide about their harmfulness." (young adult)
>
> "The opinion of a professional is not always believable because you can never know what her/his position is linked to." (young adult)

The application of food additives is not reasonable according to the adults.

Table 2: Associations and feelings of the age groups regarding "food additives"

Young adults	Adults	Elderly	Topics
"E-number" "only small amount of food components" "less negative than 'E-numbers'"	"broad phrasing" 'worst than the 'E-numbers'" "product improver"	"E-numbers"	**Meaning**
"make products more appealing to the eye" "revise foodstuffs" "influence product characteristics" "stabilizers" "flavour enhancer"	"flavour enhancer" "preservative"	"improve/modify product characteristics"	**Technological function**
–	"unknown/not evidenced effect"	–	**Health effect**
–	"unknown"	"not sure that necessary"	**Uncertainty**
"ready-to-eat products" "unnecessary marketing aims"	"artificial" "not natural" "obligatory standard" "false/appearance/synthetic" "economic pressure/lobby"	"advance" "trade necessity" "not just in foodstuffs" "unnecessary" "unavoidable" "understandable"	**Judgement of their utilization**
–	–	"not important to me"	**Neutrality**

It is directed by economic reasons, and the aim is the rapid and cheap food production. Unhealthy effects of food additives were mentioned. The advantage of their utilization is that the storage life of the foodstuffs becomes longer. They try to avoid food additives mainly if they buy a foodstuff for their children. In the media, they continuously meet negative and contradictory news. Their main information source is the Internet, but the authenticity of the sites can be queried. Truth and reliable (e.g. trade, producers) information is missed.

> "We continuously meet the negative information from the media." (adult)
>
> "Probably they say today that it is healthy and after five years that they are not." (adult)

The health risk of food additives according to elderly participants is mixed; however, they agree that children have to avoid these substances. Utilization of food additives is associated with mass production; thus, it is thought to be reasonable. As a disadvantage, it came up that consumers are damaged by their application as well as it can aggregate in the case of composite foodstuffs. However, they do not feel that they are cheated because everything is on the label. If they are interested in a concrete additive, they search it on the Internet, but they are not sure that the obtained information is always reliable.

> "Foodstuffs cannot be produced without food additives in the mass production." (elderly)
>
> "Theoretically, the composites are written on it." (elderly)

Perception of food scandals

In the course of the interviews, participants could recall only two scandals regarding food additives: the guar gum and the aspartame (however, much more in connection with foodstuffs). Food sophistications like meat (e.g. horse, beef), Hungarian red paprika and infant nutrition (melamin) were mentioned in all groups as well as the negative news about fast food restaurants (McDonald's) as among the young adults and adults. The main source of information in these topics is the TV, while in the case of the elderly people the newspaper was also mentioned.

The shopping decisions of the young adults and adults are strongly influenced by these media news; they avoid the named foodstuffs. The main reason of this is that they do not believe in the recall of the problematic products and they feel that the references are not independent and creditable enough (*Table 3*).

Table 3: Allusions regarding food scandals

Young adults	Adults	Elderly	Topics
"guar gum"	"guar gum" "aspartame"	"aspartame"	**Food additives**
"infant nutrition/melamine"	"McDonald's"	"red paprika"	**Other**
"meat colouring (beef from pork)"	"chocolate santa"	"Chinese milk powder"	
"hygienic problems"	"beef sophistication"	"meat sophistication"	
"horse meet"	"horse meet scandal"	"beef sophistication"	
"trans fatty acids"	"cucumber scandal"	"cinnamon"	
"red paprika"	"organic product re-labelling"	"re-labelling"	
"McDonald's"	"red paprika"	"horse meet scandal"	
"soya in infant nutrition"	"egg content of pastas"	"dioxin scandal"	
	"colouring of eggs"	"honey sophistication"	
	"animal traces in bread"	"wine sophistication"	
	"infant nutrition"	"syrup sophistication"	
	"mad cow disease"		

"It remains as a bad feeling." (young adult)

"Other name is given to the foodstuffs, e.g. milk – morning drink, coffee – chicory coffee." (adult)

Elderly people believe in the recall of the objected products; thus, their shopping habits are not influenced by these scandalous items of news. However, the accurate information is prominently important for them, while they also miss the appropriate punishment of the producers.

"Punishment does not have enough retentiveness." (elderly)

4 Conclusions

The conducted focus group interviews pointed out many differences and identities among the age groups. The composition of the foodstuffs appeared as a remarkable factor in the shopping decision in all groups. However, the importance of food additives and "E-numbers" – contrary to other studies (*GFK*, 2007; *Marián et al.*, 2011; *Marketing Info*, 2013) – were mentioned only by the young adults and the adults. Elderly people showed a price-sensitive attitude.

The conception of "E-number" evoked health damaging and ambiguous feelings in the case of young adults and adults, while in the case of elderly people this was not so typical. Verifying the results of previous studies (*Tarnavölgyi*, 2003, 2004; *Szűcs & Bánáti*, 2010a; *Szűcs et al.*, 2012), participants had uncertain knowledge about food additives; there were queries about their application necessity, and it was a common view. Adults felt that food additives are artificial substances.

Respondents could not separate negative news about food additives from food scandals. According to this, it can be noted that – in spite of the uncertainty and negative feelings about food additives – they do not pay too much attention to negative news about food additives; they simply treat them as negative information about foodstuffs.

Furthermore, as a difference between the age groups, it can be stated that while young adults and adults try to avoid foodstuffs involved in negative news – as it was found in the survey of the *Eurobarometer* (2011) –, elderly people prefer home-made food production and think of it this as a solution. Mistrust against consumer organizations, authorities and producers was noticeable among the young adults and the adults (*Eurobarometer*, 2011).

Elderly respondents showed a lower level of worry about food additives, which is not in line with the results of the literature (*Eurobarometer*, 2006a;

Food Standard Agency, 2010; *Szűcs et al.*, 2012).

Common mistrust found in the course of the interviews can be explained by the questionable reliability of the media news as well as by the utilization of improper information sources. In order to increase consumers' trust – which will lead to the decrease of consumers' risk perception –, the accurate information as well as the appropriate and truthful references have to be treated with a high priority. Further analysis of the topic is reasonable to identify additional differences and features of the age groups.

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Consumer acceptance of combined hot-air and microwave vacuum-dried apple pieces

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Abstract. The microwave vacuum-dried apple has a puffy and crispy structure; so, it can be a new promising product for the replacement of traditional snack products, which contains a high level of fat and salt. In order to analyse the consumer acceptance regarding the microwave (MW) vacuum-dried apple pieces, a rating-based conjoint analysis with sensory tasting (MW vacuum-dried piece and hot-air dried slice) was done. This study was performed with the help of three attributes: appearance (piece/slice), applied technology (MW vacuum-/HA drying) and price (average/+20%). Data were analysed with SPSS Conjoint. On the basis of the results of 420 respondents, it was found that without tasting respondents could not decide which product they would have liked to buy: MW vacuum-dried apple piece on average +20% price or HA-dried apple slices on average price. However, having tasted, the "piece" appearance resulted a significantly positive utility, while the "slice"a negative one. Significant difference was not observed between the utility values of the applied technologies. Average and higher price resulted negative utility. Although the utility values of the technology did not show a significant

Keywords and phrases: microwave, vacuum drying, conjoint analysis, consumer acceptance.

difference, these had the highest impact on the shopping decision, closely followed by the appearance. It can be concluded that a general rejection against the MW vacuum drying technology was not observed. The sensorial characteristics of the microwave dried apple pieces had a favourable impact on the shopping decision.

1 Introduction

Apple is harvested in the largest amount (650,595 tons/year) (*KSH*, 2013a) and it is the most frequently consumed (9.6 kg/capita/year) fruit in Hungary (*KSH*, 2013b). The popularity of this fruit can be partly due to the fact that it is freshly available all year round in a great variety. Its remarkable nutritional values make dried apple products become an excellent option for the replacement of traditional snack products, which contain a high level of fat and salt.

Dehydration – which is one of the oldest preservation methods – helps to preserve foods and extend the shelf-life of the products without the addition of chemical substances. Conventional drying at high temperature and for a long time causes significant nutritional and sensorial (e.g. flavour, colour, aroma) damages in fruits and vegetables (*Drouzas et al.*, 1999; *Drouzas & Schoubert*, 1996; *Lin et al.*, 1998; *Mousa & Farid*, 2002). To avoid these losses, microwave (MW) drying is a promising option. The single MW drying has some drawbacks, which include uneven heating, possible texture damage and limited product penetration of the microwave into the product (*Zhang et al.*, 2006); this can be solved by its combination with other methods like vacuum drying.

Consumers' product choices are often influenced not only by the attributes of the product, but also by the method based on which the product was produced, including factors such as origin, working conditions and production technology (*Grunert et al.*, 2004; *Nielsen et al.*, 2009). Studies about the level of worry regarding MW treatments are limited; however, it can be stated that – even though it has been used in the households for a long time – it evokes a moderate concern (*Cardello*, 2003).

Consumer acceptance of dehydrated products depends on characteristics such as structural, textural, sensorial, microbiological and rehydration properties (*Giri & Prasad*, 2013). Several studies show that – compared to conventional dried products – MW vacuum-dried products result in better sensorial characteristics, such as colour or flavour (*Ferenczi et al.*, 2012; *Giri & Prasad*, 2013; *Maskan*, 2002), as well as crisp and porous texture (*Ferenczi et al.*, 2010;

Krulis et al., 2005), contrary to the undesirable hard-crust surface formed by the traditional hot-air (HA) drying (*Li et al.*, 2011).

Therefore, the aim of this study was to analyse the consumers' acceptance regarding the MW vacuum-dried apple pieces compared to an HA-dried slice product.

2 Materials and methods

Conjoint analysis design

In order to get detailed information on the consumers' willingness to buy and their preference of MW vacuum-dried apple piece products – compared to the HA-dried apple slice products currently found on the market –, a conjoint analysis combined with sensorial analysis was done among Hungarian consumers over 18 years in the autumn of 2013. The conjoint analysis is a technique which helps to determine the relative importance of the product characteristics and the utilities of the different levels of the characteristics according to the consumers (*Hoffmann et al.*, 2000). For the conjoint analysis, three product characteristics were used: the "appearance" (emotional characteristic), the "applied technology" as well as the "price". All characteristics had two levels (*Table 1*). The selection of characteristics was explained by the fact that the HA-dried apple slice product had already been on the market, while the MW vacuum-dried apple piece was a new product developed in the pilot plant of the NARIC FSRI (National Agricultural Research and Innovation Centre – Food Science Research Institute). As for levels of the "price," an average (based on market data) (200 HUF, approx. 0.6 EUR) and a +20% price was taken into account, and this can be justified by the extra cost of MW technology compared to the price of dried-apple slice products present on the market. All of the created model products (cards) appeared in 50-g packages.

Table 1: Levels of the analysed product characteristics

Product characteristic	**Level 1.**	**Level 2.**
Appearance	Piece	Slice
Applied technology	Microwave vacuum drying	Hot-air drying
Price	Average	Average+20%

In the questionnaire, first, the applied technologies were demonstrated in form of a short definition (*Figure 1*); then the participants were asked – in the possession of the technological information, without tasting – to decide which dried-apple product they would have liked to buy. Since the appearance and the sensorial attributes in this case were not relevant, two cards were shown for the respondents: MW vacuum-dried apple slices in a 50-g package for an average+20% price (product developed in the pilot plan), and HA-dried apple slices in a 50-g package for average price (product already present on the market). If the participants were not able to decide between the two products, they could choose "both".

Hot-air drying: is a traditional method which removes the whole humidity content of the fruit with the circulating of hot and dry air.

Microwave vacuum drying: is a two-step method in which, at first, the product is partly dehydrated, then the remaining humidity content is removed with low pressure (vacuum) microwave heating. This is a considerate method because of lower heat loading.

Figure 1: Definitions of the applied technologies

As the next step, respondents were asked to taste the two samples (microwave vacuum-dried piece and hot-air dried slice) and to decide on a 1 to 7 Likert scale – in the case of each card respectively – how likely they would have bought the model product (1: not likely at all; 7: very likely). Both of the tasted samples were produced from the same raw material (Idared, *Malus domestica*); however, the technological methods were not presented for the participants; thus, their benefits could not be consciously linked to the samples. For the conjoint study, eight cards were formed with the help of the orthogonal design of the SPSS statistical software and the created cards were set out in a fixed order.

Furthermore, in the questionnaire – in order to get acquainted with the marketing opportunities –, respondents were invited to evaluate the consumption possibilities of potato chips and dried apple products as well as some socio-demographic questions were asked. The final conjoint questionnaire was developed after two group interviews (six participants respectively). After the random asking of people, 420 evaluable questionnaires were collected. For the data analysis, univariate (frequency) and multivariate (cluster analysis) methods and conjoint analysis were done with the help of the SPSS statistical software.

Participants

Regarding the respondents' socio-demographic distribution, it can be said that more females (61.9%) participated than males (38.1%). More than half of the respondents (54.1%) were between the age of 25 and 44 years and most of them were inhabitants of the capital (40%), while fewer of them were from small cities (14.8%). More than two-thirds of the samples were highly educated people (66.7%). Less than half of the participants had children over 18 years of age and almost three quarter of the sample (72.5%) answered that they live under average circumstances. 26% of the respondents consume potato chips only 1–3 times or less often, while 34.1% of them consume dried fruit or vegetable products.

3 Results

Willingness to buy dried apple products without tasting them

Having understood the technological definitions without tasting the samples, the participants were not able to decide unambiguously which product they would have liked to buy. 37.6% answered that they would have bought the HA-dried apple slices in a 50-g package for average price, 32.9% the MW vacuum-dried apple slices in a 50-g package for average+20% price and 29.5% both foodstuffs.

Willingness to buy dried apple products after tasting – conjoint analysis

Following the "piece" tasting (MW vacuum-dried), appearance had a significantly more positive influence on the respondents' shopping decisions than the "slice" format (HA-dried). Significant difference was not observed between the utilities of the "applied technology" levels, while the higher price resulted negative utility values.

Regarding the relative importance data, the "applied technology" was the most important factor influencing the participants' shopping decisions (*Table 2*).

After the tasting and the judgment of the cards, respondents were asked to decide which product they preferred (piece/slice/both/none of them). More than half (60.3%) of the participants preferred the "piece" product and 21.5% the "slice" one. 16.5% of the tasters were not able to make any difference and only 1.7% did not like the tasted samples.

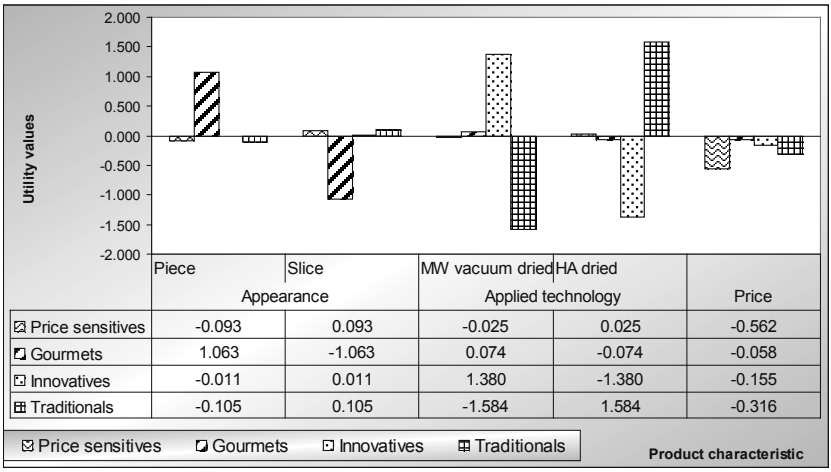
Table 2: Relative importance of each product characteristic and utility values for each level

Product characteristic	Level	Utility	SE	Relative importance (%)
Appearance	Piece	0.167[a]	0.043	32.924
	Slice	-0.167[a]	0.043	
Applied technology	MW vacuum drying	0.007	0.043	41.815
	HA drying	-0.007	0.043	
Price	Average	-0.349	0.085	24.546
	Average+20%	-0.699	0.170	

[a] Significant differences between the levels of the characteristic according to paired t-test ($p < 0.05$)

Cluster analysis

For the exploration of the differences among the shopping preferences and for the formation of homogenous consumer groups with the help of the utility values, cluster analysis (K-mean) was done. This resulted four significantly (p=0.000) different and well-explainable groups. The "applied technology" (F= 482.362) had the strongest effect on the cluster creation, while "price" (F= 12.439) the weakest one. The respondents' price-sensitive attitude appeared – more or less – in the clusters, too. Utility vales and relative importance data are shown in figures 2 and 3.



	Piece	Slice	MW vacuum dried	HA dried	Price
	Appearance		Applied technology		
Price sensitives	-0.093	0.093	-0.025	0.025	-0.562
Gourmets	1.063	-1.063	0.074	-0.074	-0.058
Innovatives	-0.011	0.011	1.380	-1.380	-0.155
Traditionals	-0.105	0.105	-1.584	1.584	-0.316

Figure 2: Cluster utilities of each level

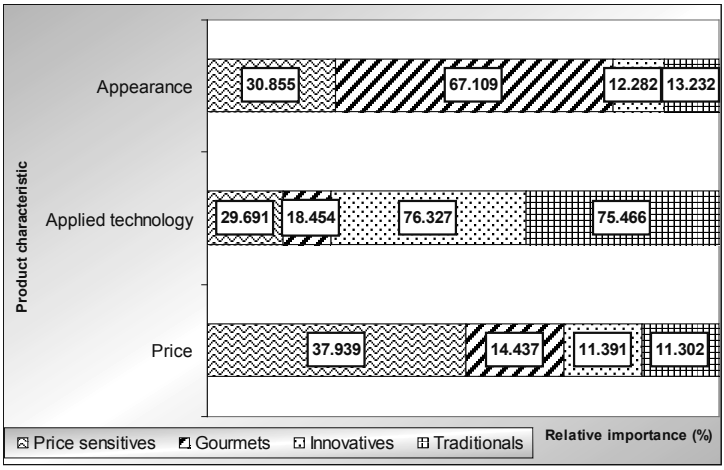


Figure 3: Relative importance of each product characteristic based on clusters

Price sensitives (N= 198)

Most of the participants belong to this cluster. For these consumers, "price" is the most important product characteristic influencing their dried-apple-product-shopping decisions, while "appearance" and "applied technology" have almost the same importance. Utility values show the price-sensitive attitude of these cluster members.

Gourmets (N= 90)

During the shopping decisions of the second biggest cluster members, "appearance" has an outstanding role. "Piece" appearance has a strong positive effect on their intention. "Applied technology" is not a dominant characteristic for them, and the participants of this cluster showed the lowest price sensitivity.

Innovatives (N= 71)

"Applied technology" has a prominently high relative importance, while "appearance" and "price" have more or less the same role in their shopping decisions. Members of this cluster do not feel aversion towards the "MW vacuum drying"; on the contrary, it has a positive impact on their intention. "Appearance" was less important; however, "price" is a relevant factor for them.

Traditionals (N= 61)

"Applied technology" – as in the case of the "innovatives" – is a highly important characteristic for the members of this cluster. However, as the smallest cluster, its participants reject the "MW drying" technology and prefer the "HA-dried" apple products.

"Traditionals" do not only show aversion towards the technological innovations, but their willingness to pay is also low.

Consumption possibilities of dried apple products

The consumption possibilities of the dried apple products were found to be more varied than that of the potato chips. Respondents can imagine dried apple consumption during a journey or travelling, watching TV, as snacking, crunching for children, for slimming diet, for guests as well as for adding to the muesli. Participants could not imagine the consumption of any of these products in the cinema, neither as a small meal nor as a composition of dishes (*Table 3*).

Among the other opportunities, some allusions were made at snack in kindergarten and primary schools, during working schedules, and it can also be taken into account as decoration for cakes.

Table 3: Consumption possibilities of dried apple and chips products

	Dried apple	**Potato chips**	**Both**	**None of them**
During a journey or travelling	50.5%	10.2%	13.1%	26.2%
During watching TV	35.2%	17.4%	21.0%	26.4%
Snacking during the day	50.7%	6.9%	8.1%	34.3%
For children to crunch	69.5%	4.3%	5.2%	21.0%
As a small meal	30.5%	4.8%	3.6%	61.2%
For amusement	32.9%	14.3%	28.6%	24.3%
For dishes as a component	27.9%	2.4%	1.4%	68.3%
In cinema	14.3%	19.8%	7.1%	58.8%
For slimming diet	55.5%	1.7%	1.4%	41.4%
For morning muesli	62.1%	2.9%	1.9%	33.1%

4 Conclusions

Results of the study showed that the "applied technology" has an outstanding importance during respondents' dried-apple-shopping decisions. At the same time, the utility values did not show significant differences between the two treatments, what is due to the diversification of the opinions. Based on the creation of homogenous consumer groups, it can be highlighted that the "applied technology" was the most important product characteristic in the dried-apple-shopping decisions of the "traditional" and the "innovatives" clusters. The "innovatives" strongly accepted the utilization of "MW vacuum drying"; however, the "traditionals" rejected it.

"Appearance" – and, in this way, the sensorial attributes of the samples produced from the same raw material – influenced notably the willingness to buy. The puffy and crispy texture of the MW vacuum-dried apple pieces were favourable characteristics – mainly for the members of the "gourmet" cluster – compared to the slightly dry texture of the HA apple slices. On the basis of these results, an outstanding attention is needed for the familiarization and tasting of these products during the launching of the MW vacuum-dried piece version. The presentation of the product in the ambience preferred by the consumers (e.g. trip, travelling, watching TV, snacking, for slimming diet, children and guests) can result further advantages. It is important to note that even though the "price" did not result high importance during the shopping decisions, respondents showed price-sensitive attitude – mainly the members of the "price sensitive" cluster – and this has to be taken into consideration during the product development.

The "applied technology" was not connected to the tasted samples; thus, the judgment of the technology can be evaluated separately from the sensorial characteristics. Based on this, it can be stated that the "applied technology" has a remarkable importance. Results of the present study pointed out that the MW vacuum-dried apple piece product possesses a strong supporting group: the "gourmets," who showed high emotional preferences and low price sensitivity, as well as the "innovatives," who could be – after a better recognition of the favourable characteristics – potential purchasers of this new product family.

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