Determination of free amino acids in cheeses from the Czech market

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Abstract

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High performance liquid chromatography (HPLC) method with the pre-column derivatisation by AccQ•Tag agent and following determination of these derivates after their separation in reverse phase column followed by fluorescent detection was used for the determination of amino acids in cheeses. The contents of sixteen free amino acids in twenty five cheeses commercially available in the Czech Republic were measured. The total content of free amino acids in the studied cheeses varied in the range from 27 g/kg to 160 g/kg. Among individual amino acids, seven amino acids were more concentrated in all cheese samples and came from three distinctive taste groups: bitter tasting amino acids (leucine, lysine, and phenylalanine), bitter sweet amino acids (proline and valine), and salty-umami amino acids (glutamic acid and aspartic acid). The differences in the contents of the total and individual free amino acids were influenced by the kind of cheese and mainly by the duration and intensity of proteolysis.

Keywords: amino acids; cheese; HPLC; pre-column derivatisation; 6-aminoquinolyl-*N*-hydroxy-succinimidyl carbamate (AQC); fluorescent detection

For a significant section of the Czech population, cheese constitutes an important part of the diet. Cheese is a food with very important nutritional aspects, contributing to high efficiency in the metabolism. The substances are quickly and easily absorbed by the body. Cheese is easily digestible and constitutes an important source of vitamins, but also calcium, magnesium, phosphor, and other trace elements as well as amino acids and proteins (Fox 1993). The presence of amino acids, vitamin B (esp. B_6 and folic acid) and vitamins A and E, minerals (in particular calcium and magnesium), and protein derivates makes cheese a unique food. The amounts and patterns of free amino acids in cheeses are dependent on many factors. These comprise the quantities of proteins in the raw materials used for the production, activity of proteolytic enzymes in the dairy procedures, and microorganisms involved in this process (Yvon & Rijnen 2001). The release of amino acids during cheese ripening was shown already 100 years ago. Proteolysis in cheese during ripening plays a vital role in the development of texture as well as flavour and has been the subject of several reviews (Fox 1993; Fox & McSweeney 1996; Sousa *et al.* 2001; Čurda & Kukačková 2002). Proteolysis contributes to the textural changes of the cheese matrix, due to the breakdown of the protein network, decrease of a_w through water binding by the liberated carboxyl and

amino groups, and increase in pH (in particular in surface mould-ripened varieties), which facilitates the release of sapid compounds during mastication. It contributes directly to flavour and to off-flavour (e.g. bitterness) of cheese through the formation of peptides and free amino acids as well as liberation of substrates (amino acids) for secondary catabolic changes, i.e., transamination, deamination, decarboxylation, desuphuration, catabolism of aromatic amino acids, and reactions of amino acids with other compounds (Sousa *et al.* 2001).

The determination of free amino acids plays an important role in assessing the nutritional quality of foods (Erbe *et al.* 1999, 2000; Casella & Contursi 2003). In addition, amino acids determination also gives an indication of the possible adulteration and transformation occurring during the processing and storage procedures (Bűtikofer & Ardö 1999). The first method used for the analysis of individual amino acids was paper chromatography (Lindqvist & Storgards 1953). Gas chromatography methods were subsequently developed (Wood *et al.* 1985; Bertacco *et al.* 1992). Recent progress in amino acid determinations can be attributed to technological advantages in liquid chromatography and analytical detectors (Erbe *et al.* 1999, 2000). The separation of these molecules and their derivatives are readily achieved by using reverse–stationary phases mixtures and ion exchangers. Direct underivatised amino acid determination is not always simple because these compounds lack natural strong chromophore or fluorophore groups for photometric or flurometric detection (Casella & Contursi 2003). Consequently, a sensitive photometric detection requires some form of pre- or post-column derivatisation procedures in order to introduce strong chromophore groups. Various derivatisation procedures using derivative molecules such as dansyl chloride, ninhydrin, phenyl isothiocyanate, dimethylaminoazobenzenesulphonyl chloride, fluorescent derivatives (i.e. *ortho*-phthalaldehyde, fluorescein), etc. have been proposed for a sensitive determination (i.e. picomole level) of amino acids in chromatographic systems (Bartóak *et al.* 1994; Kutlán *et al.* 2003; You *et al.* 2003). The method using precolumn derivatisation with by 6-aminoquinolyl-*N*-hydroxy-succinimidyl carbamate (AQC) and fluorescent detection is one of the most sensitive and selective methods for the determination of free amino acids and was used for the analysis of cheese (Giraudo *et al.* 2002). This method was also used for the determination of free amino acid content in beer (Čížková *et al.* 2005; Kabelová *et al.* 2008).

There are several papers dealing with the levels of free amino acids in cheeses (Law 2001; Ardö *et al.* 2002; Skeie *et al.* 2006; Picon *et al.* 2007). There are only a few studies concerning cheeses of Czech origin (Dráb *et al.* 1999; Buňka *et al.* 2004), and comparisons of free amino acid contents in Czech cheeses with those in imported cheeses are also missing. Cheeses made in the Czech Republic and trade cheese samples from Slovakia, Poland, the Netherlands, France and Germany have been studied and compared. Free amino acid composition of cheese was evaluated in order to determine whether or not there was uniformity in the contents of these substances.

MATERIALS AND METHODS

***Chemicals*.** Acetonitrile (HPLC super gradient grade) and methanol (HPLC super gradient grade) were purchased from Lab-Scan (Dublin, Ireland). Hydrochloric acid p.a. (36.5%) and trichloroacetic acid were products of Penta (Chrudim, Czech Republic) and α-aminobutyric acid (p.a.) was purchased from Sigma Aldrich (Germany). Ultrapure water was produced by a Milli-Q Plus system (Millipore Corporation, USA). AccQ•Tag Reagent Kit was purchased from Waters (Milford, Massachusetts, USA). The reagent kit consists of Waters AccQ.Fluor Borate Buffer, Waters AccQ•Fluor Reagent Powder (6-aminoquinolyl-*N*-hydroxy-succinimidyl carbamate – AQC), Waters AccQ•Fluor Reagent Diluent, Waters AccQ.Tag Amino Acid Analysing Column (Nova-Pak C_{18}, 4 µl, 150 × 3.9 mm), and Waters Amino Acid Hydrolysate Standard (each ampoule contains a 2.5mM mixture of the 17 hydrolysate amino acids with the exception of cystine – 1.25mM – aspartic acid (Asp), serine (Ser), glutamic acid (Glu), glycin (Gly), histidine (His), arginine (Arg), threonine (Thr), alanine (Ala), proline (Pro), cysteine (Cys), tyrosine (Tyr), valine (Val), methionine (Met), lysine (Lys), isoleucine (Ile), leucine (Leu), and phenylalanine (Phe)).

***Materials*.** Twenty five commercial cheeses, which represented all principle brands available on the Czech market, were analysed for free amino acids contents. We tested sixteen different types of Czech cheese, produced by different manufacturers in the Czech Republic, four cheeses came

from Slovakia, two from Poland, one each from the Netherlands, Germany, and France. We purchased the samples at local markets. The samples were stored in the dark at low temperature (5°C).

Amino acid analysis of cheese – derivatisation procedure. *Reconstituting AccQ•Fluor Reagent.* 1.0 ml of AccQ•Fluor Reagent Diluent was transferred into a vial containing Waters AccQ•Fluor Reagent Powder. This closed vial was mixed with Vortex (IKA Works, Germany) for 10 s and heated on a heating block (55°C) until dissolving, but not longer than 10 minutes. Reconstituted reagent may be stored in a refrigerator for up to two weeks.

Preparing a calibration standard: Internal standard method – For the preparation, the calibration standard was combined with an internal standard – 40 µl Amino Acid Hydrolysate + 40 µl internal standard stock solution (6.45 mg α-aminobutyric acid to 25 ml 0.1M HCl) + 920 µl Milli-Q water.

Derivatising the calibration standard – The derivatisation procedure involved preheating of the heating block to 55°C and tube mixing of 10 µl of the calibration standard + 70 µl AccQ•Fluor Borate Buffer in a tube, then mixeing the mixture with Vortex briefly, the addition of 20 µl, reconstituted AccQ•Fluor Reagent, and mixing immediately for several seconds with Vortex. The content was then transferred to the bottom of an autosampler vial which was heated in the heating block or an oven at 55°C for 10 minutes. A 10 µl injection of the derivatised standard contained 50 pmol of each amino acid derivative (except cystine, at 25 pmol).

Preparing cheese samples for AccQ•Tag method – The extraction and deproteination were carried out de by first dispersing 1.0 g of grated cheese in 100.0 ml 0.1 mol/l hydrochloric acid solution containing 0.4 µmol/ml α-amino butyric acid as an internal standard in a 250 ml centrifuge tube. The dispersion was homogenised on Ultra-Turrax (IKA Werke, Germany) at 20 000 rpm and kept in a covered tube (Para-Film) for a maximum of 30 min in an ultrasonic bath. The sample was then centrifuged at 3000 g and 4°C for 10 min, and 5 ml of the supernatant was added to 5 ml of 400 g/l trichloroacetic acid solution. The suspension was mixed on a Vortex mixer, kept in an ice bath for 10 min, and centrifuged at 20 000 g for 10 min (BŮTIKOFER & ARDÖ 1999). From this solution 10 µl was taken for the derivatisation procedure.

Derivatisation of samples – 10 µl of the treated sample + 70 µl AccQ•Fluor Borate Buffer were mixed in a sample tube and briefly homogenised with Vortex, 20 µl reconstituted AccQ•Fluor Reagent was added and the mixture was mixed immediately for several seconds with Vortex. The content was then transferred to the bottom of an autosampler vial, and the vial was heated in the heating block at 55°C for 10 minutes.

HPLC. A Waters Alliance 2695 HPLC system with a 2475 Multi λ Fluorescence detector (Waters, Milford, Massachusetts, USA) was used for the HPLC analyses (excitation at 250 nm and emission at 395 nm). AccQ•Tag amino acid column Nova-Pak C_{18}, 4 µm (150 × 3.9 mm) from Waters was used. The column was thermostated at 37°C. 10 µl was the injection volume (concentration of amino acids 5–200 pmol).

A gradient mobile phase was used for chromatography. The mobile phase consisted of eluent A (prepared from Waters AccQ•Tag Eluent A concentrate, by adding 200 ml of concentrate to 2 l of Milli-Q water and mixing), eluent B (acetonitrile, HPLC grade) and eluent C (Milli-Q water). The best gradient separation programme was as follow:

Time 0 min	A – 100%	B – 0%	C – 0%
Time 0.5 min	A – 99%	B – 1%	C – 0%
Time 18 min	A – 95%	B – 5%	C – 0%
Time 19 min	A – 91%	B – 9%	C – 0%
Time 29.5 min	A – 83%	B – 17%	C – 0%
Time 33 min	A – 0%	B – 60%	C – 40%
Time 36 min	A – 100%	B – 0%	C – 0%
Time 53 min	A – 100%	B – 0%	C – 0%

Statistical methods. Statistical evaluation of the results achieved was done employing the computer program Statgraphics plus for Windows 3.1 (Statistical Graphics Corp., USA).

RESULTS AND DISCUSSION

A total of twenty five cheese samples were characterised, out of which sixteen were of the Czech origin (samples 1–7, 10, 12, 15, 17, 19, 21, 23–25) and nine were imported cheeses (samples 8, 9, 11, 13, 14, 16, 18, 20, 22). The survey of the samples is shown in Table 1. We tested nine varieties of surface mould-ripened cheeses (samples 1–3, 7–9, 13, 20, 22), two blue cheeses (samples 4 and 25), four bacterial surface ripened cheeses (samples 5, 6, 21, 23), four semi-hard cheeses (samples 10, 16, 17, 19), one hard cheese (sample 12) and five pasta filata cheeses (samples 11, 14, 15, 18, 24). The contents of the amino acids determined in

Table 1. Total free amino acid content, description and land of origin of analysed cheeses

Sample No.	Land of origin	Content of total free amino acids (g/kg)	Cheese variety	Dry weight content (%)	Fat in dry weight (%)
Surface mould-ripened cheeses					
1	Czech Republic	56.3	Hermadur	50.5	61
2	Czech Republic	116.6	Brie	51	61
3	Czech Republic	56.4	Vltavín	53	57
7	Czech Republic	52.3	Camembert	45	44
8	Poland	46.2	Gérabon	51	60
9	Poland	43.3	Président	48	60
13	Germany	70.8	Bonifaz	68	55
20	France	105.9	Révérend	52	60
22	Slovakia	80.0	Plesnivec	43	43
Blue cheeses					
4	Czech Republic	128.5	Niva	52	50
25	Czech Republic	99.9	Caesar	52	50
Bacterial Surface Ripened Cheeses					
5	Czech Republic	70.6	Olomoucké tvarůžky	33	0.2
6	Czech Republic	124.2	Romadur	44	40
21	Czech Republic	123.2	Baladur	45	48
23	Czech Republic	159.5	Pivní sýr	40	40
Semi-hard cheeses					
10	Czech Republic	52.9	Madeland	57	45
16	Netherlands	96.9	Leerdammer	58	45
17	Czech Republic	73.1	Eidam	56	45
19	Czech Republic	84.8	Blaťácké zlato	51	48
Hard cheeses					
12	Czech Republic	71.6	Emmentaler	61	45
Pasta filata cheeses					
11	Slovakia	36.9	Koliba	50	40
14	Slovakia	30.1	Gazdovská pařenice	45	40
15	Czech Republic	23.0	Mozzarella	48	18
18	Slovakia	43.5	Tatranské nitě	–	25
24	Czech Republic	43.2	Jadel	54	37

cheeses are given in Table 2. The example of free amino acids chromatogram in cheese is shown in Figure 1. Individual groups of cheese showed a very variable and characteristic amino acid pattern. The highest amount of amino acids was determined in a Czech cheese Pivní sýr (Beer cheese) from the group of bacterial surface ripened cheeses (sample 23–159.5 g/kg) (Table 2). On the other hand, the lowest amino acid content was detected in the Czech cheese Mozzarella (sample 15) from the group of surface mould-ripened cheeses.

We found, in agreement with Fox (1993), that blue and bacterial surface rippened cheeses have higher free amino acid contents than surface mould-

Table 2. Contents of free amino acids in cheese samples

No	Concentration of amino acids (g/kg)																
	Asp	Ser	Glu	Gly	His	Arg	Thr	Ala	Pro	Tyr	Val	Met	Lys	Ile	Leu	Phe	Total AA
1	6.2	0.3	14.2	0.5	0.5	6.6	5.1	4.2	6.7	0.3	2.2	2.4	2.1	0.4	3.1	1.5	56.3
2	10.6	0.6	34.2	0.5	0.5	5.2	1.0	1.7	18.5	1.6	12.2	2.0	5.1	0.7	14.6	7.6	116.6
3	5.8	1.2	13.8	0.7	0.8	2.5	2.2	1.8	6.8	1.0	3.6	0.3	5.9	1.0	6.1	2.9	56.4
4	10.1	3.4	37.2	2.3	1.8	5.3	2.5	2.6	18.1	3.8	7.4	1.3	9.9	3.2	10.8	8.8	128.5
5	5.3	1.2	15.6	1.4	1.2	1.2	1.8	1.4	8.4	1.5	5.1	1.7	6.7	3.5	7.8	6.8	70.6
6	9.8	1.8	29.5	2.8	1.9	2.3	3.6	2.9	13.2	1.5	9.6	2.3	12.1	7.1	14.1	9.7	124.2
7	5.4	1.1	12.8	0.6	0.7	1.6	1.5	2.8	7.2	0.9	3.0	0.3	5.8	0.9	5.8	1.9	52.3
8	2.9	0.2	11.5	0.6	0.5	1.5	1.6	2.7	7.2	0.7	2.5	0.4	5.3	0.8	5.2	2.6	46.2
9	4.9	0.3	10.4	0.5	0.2	1.5	1.6	2.6	8.5	0.4	0.1	2.8	3.5	0.3	2.9	2.8	43.3
10	4.3	0.4	13.3	0.5	2.3	1.5	1.7	2.2	7.1	0.3	4.2	0.1	4.4	2.1	4.8	3.7	52.9
11	3.3	0.7	7.3	0.2	0.1	0.8	0.9	2.4	4.9	0.7	2.2	1.6	4.4	2.1	3.2	2.1	36.9
12	5.8	0.8	18.0	0.8	1.2	2.3	0.9	3.2	9.1	1.8	6.2	2.3	6.4	1.3	6.9	4.6	71.6
13	6.2	2.4	18.2	1.3	1.4	1.9	1.5	2.5	7.0	0.3	6.0	1.2	12.2	1.0	5.1	2.6	70.8
14	2.9	0.4	7.1	0.5	0.2	0.9	1.1	2.1	5.5	0.3	1.1	0.9	2.3	0.4	2.9	1.5	30.1
15	1.9	0.3	4.8	0.8	0.4	0.8	0.9	1.0	2.6	0.5	1.6	0.4	2.9	0.9	2.1	1.1	23.0
16	7.8	2.7	26.3	3.1	2.3	2.9	2.4	2.6	14.0	2.3	3.7	2.5	10.3	1.7	8.9	3.4	96.9
17	6.1	1.4	14.7	1.4	1.3	1.8	4.2	2.1	6.7	2.9	7.3	2.4	6.8	2.3	6.9	4.8	73.1
18	3.5	1.6	8.9	1.4	1.7	1.8	1.9	1.8	5.9	1.2	3.3	1.3	3.4	1.2	3.5	1.1	43.5
19	7.2	2.7	18.1	3.0	2.7	2.4	2.4	2.9	10.2	2.1	7.7	2.8	7.3	3.5	7.7	2.1	84.8
20	10.1	0.4	33.2	0.6	1.2	3.4	3.9	1.5	20.4	1.2	12.4	2.1	7.3	1.2	5.1	1.9	105.9
21	8.6	5.9	26.8	3.4	5.6	2.9	3.0	2.4	15.3	3.8	8.9	4.2	15.9	4.3	7.5	4.7	123.2
22	7.5	1.4	19.0	2.5	2.9	2.4	2.7	1.9	9.8	2.8	6.8	2.6	6.1	1.5	6.9	3.2	80.0
23	11.7	4.1	42.0	5.3	4.0	6.4	5.2	3.1	21.8	4.7	12.2	4.4	13.3	5.8	11.7	3.8	159.5
24	3.3	1.4	8.8	1.2	1.2	2.1	1.7	2.5	5.5	1.7	1.8	1.9	3.2	1.6	3.2	2.1	43.2
25	8.1	3.2	33.7	3.4	2.1	2.6	3.8	4.9	12.9	2.5	3.4	1.2	1.5	3.7	9.6	3.3	99.9

ripened cheeses. No general difference occurred between the Czech and imported cheeses in free amino acid content. Among individual amino acids, seven amino acids were more concentrated in all cheese samples, and came from three distinctive taste groups: bitter tasting amino acids (leucine, lysine, and phenylalanine), bitter sweet amino acids (proline and valine), and salty-umami amino acids (glutamic acid and aspartic acid).

We processed the amino acid data using statistical methods. The main objective was to find the possible hidden relations between the variables as well as between the individual objects. Multidimensional statistical methods (factor analysis and cluster analysis) are the most powerful tool for solving this problem (Čejka *et al.* 2004).

The factor analysis reduces the large number of variables to merely two or three so that the similarities between the objects can be recognised. Figure 2 shows that the factor analysis divided the cheeses into two groups. The first group (right side, horizontal scale 4 to 15) contained six Czech samples (well rippened with highcontent of free amino acids), the second group (left side, horizontal scale –10 to –3) contained all samples from group pasta filata cheeses (5 samples) and five samples with low content of free amino acids (low ripened), the third group (middle, horizontal scale –3 to 3)
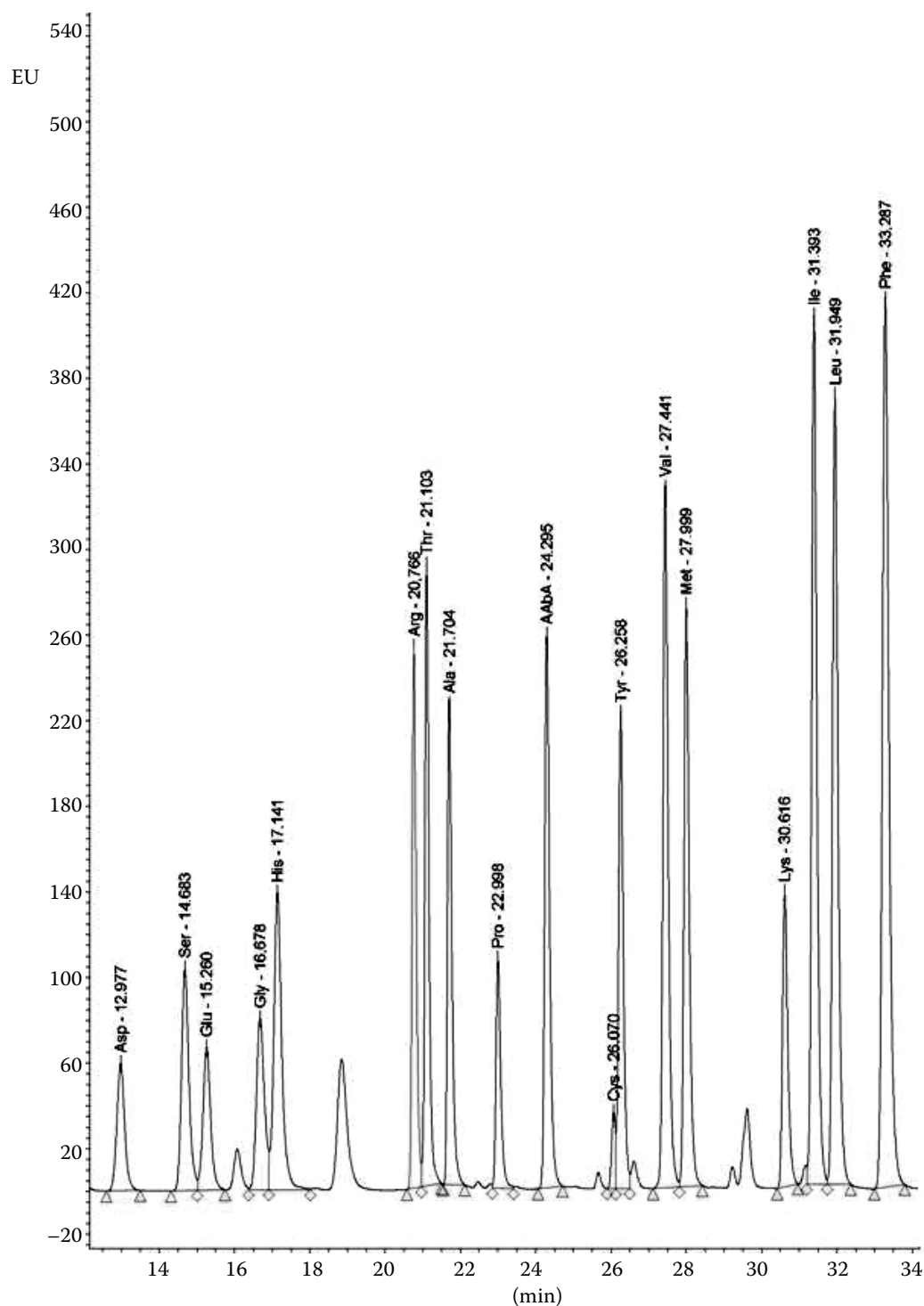
6-aminoquinoline (AMQ), aspartic acid (Asp), serine (Ser), glutamic acid (Glu), glycin (Gly), histidine (His), amonia (NH$_3$), arginine (Arg), threonine (Thr), alanine (Ala), proline (Pro), α-aminobutyric acid – internal standard (AAbA), cysteine (Cys), tyrosine (Tyr), valine (Val), methionine (Met), lysine (Lys), isoleucine (Ile), leucine (Leu), phenylalanine (Phe)

Figure 1. Chromatogram of free amino acids in Czech cheese sample

contains samples with middle content of free amino acids. The results of the factor analysis confirmed that there is no difference between the Czech cheese varieties and foreign brands in respect to

their amino acid contents and differecences was due to cheese types and their ripeness. The factor analysis of the loading of individual amino acid contents in cheeses is shown in Figure 3. Glutamic
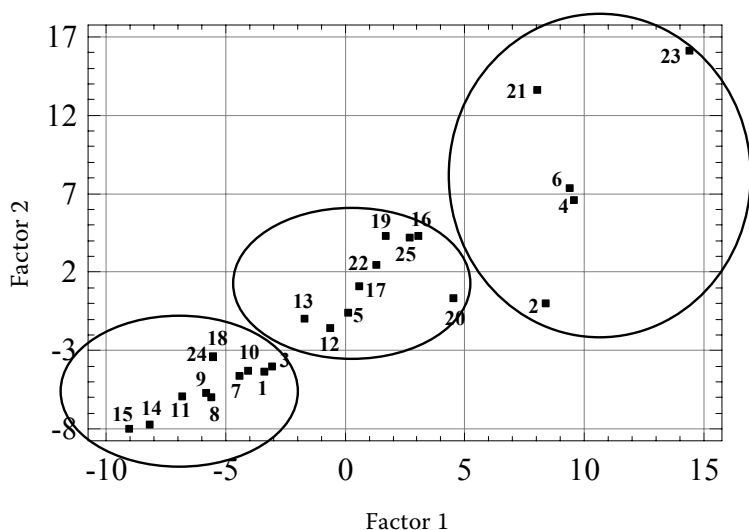
Figure 2. Factor analysis – scatterplot of cheeses sorted by their amino acid contents
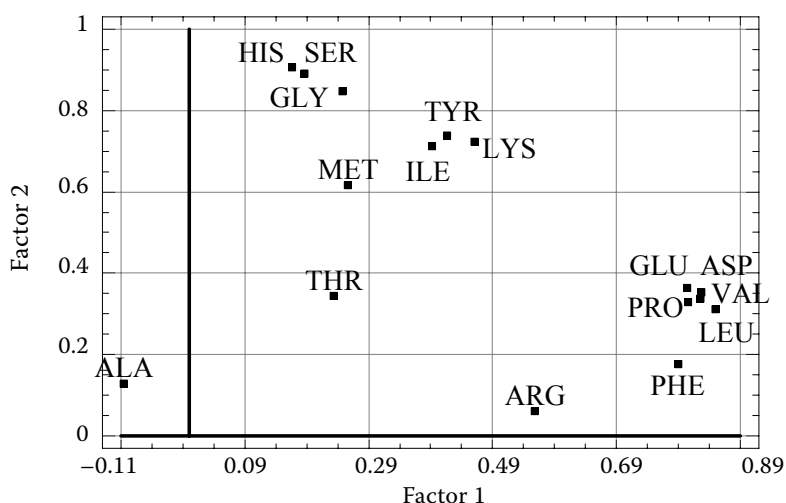

Figure 3. Factor analysis – plot of factor loadings of individual amino acid contents in cheeses

acid, proline, aspartic acid, leucine, valine, and phenylalanine are the amino acids which were described only by factor 1 and these amino acids, are present in higher concentrations. Other amino acids (lower concentrations) can be described by factor 2 or by the combination of both factors.

cheese and mainly by the duration and intensity of proteolysis.

CONCLUSIONS

In conclusion, we have presented herein an optimised analytical method for the determination of free amino acids in cheese. The total contents of free amino acids in the cheeses studied varied in the range of 23 g/kg to 160 g/kg. There is no general difference between Czech and imported cheeses in free amino acid contents. The differences in the contents of total and individual free amino acids were influenced by the kind of

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