ACTA SCIENTIARUM POLONORUM *Acta Sci. Pol., Technol. Aliment. 6(2) 2007, 5-18*

APPLICATION OF HPLC AND GC/MS TO QUANTIFICATION OF PHENYLALANINE IN CHOSEN KINDS OF FOOD FOR PARTICULAR NUTRITIONAL USES

Małgorzata Piecyk, Agnieszka Śrama, Anna Bzducha, Mieczysław Obiedziński

Warsaw Agricultural University SGGW

Abstract. The aim of the work was the estimation of HPLC and GC/MS methods usefulness for the quantitative determination of phenylalanine in low protein cereal products (LPP). LPP products from two different producers (A and B), and related traditional products were investigated. The protein content was analysed with Kiejdahl method and the phenylalanine concentration was measured with high performance liquid chromatography (HPLC) coupled with UV detector (UV = 214 nm) and by the method of gas chromatography connected with mass spectrometry (GC/MS) using the 'EZ:faast' amino acid test (Phenomenex). The content of the amino acid was analysed after acid hydrolysis (6 M HCl, time: 24 and 48 h, temperature 110°C). The content of protein was determined higher than the producer's declaration for all products of the producer B and some of the producer A. The higher protein content did not influence the concentration of phenylalanine detected with HPLC. Phenylalanine level was found below the declaration of the producer in most of the products. In most cases, there was no significant influence of the hydrolysis time on the quantity of the analysed phenylalanine.

Key words: phenylalanine, low protein products, HPLC, GC/MS

INTRODUCTION

The delivery of energy in the form of food is one of the basic physiological needs of living organisms. There are many diseases connected with disfunction of metabolic pathways (eg. phenylketonuria, albinism, alkaptonuria) or disfunction of the alimentary tract. It extorts from the ill persons to use special diet that is poor in the component that causes undesirable reactions. This situation results in the development of the food market section interested in production of special food, addressed for these groups of con-

Corresponding author – Adres do korespondencji: Dr inż. Małgorzata Piecyk, Department of Biotechnology and Microbiology of Warsaw Agricultural University SGGW, Nowoursynowska 169 C, 02-778 Warsaw, Poland, e-mail: malgorzata_piecyk@sggw.pl

sumers with particular needs. On the market products similar to traditional ones appeared (flour, sweets, bread-stuffs, pasta etc.), but of lowered content of the factor causing disfunctions of the organism or completely free from such a component.

Phenylketonuria is a serious illness of genetic background conditioned by a recessive gene that is hereditary and by anenzymia. There is lack of the phenylalanine hydroxylase (1-of phenylalanine oxidases) which is responsible for the transformation of phenylalanine into tyrosine. As a result phenylalanine hydroxylase of deficiency there is a blockade of the metabolic pathway of phenylalanine and an accumulation of this amino acid and its improper metabolites (o-hydroksyfenylacetic acid, fenylpyruvate acid, fenyllactic acid) in excessive level in blood and urine occurs. Their elevated concentration in organism causes mental handicap neurological symptoms- convulsions, boosted and muscular trembles or behaviour disorders [Pustkowski 1999]. The diet of the person suffering from the phenylketonuria should deliver at maximum 10-40 mg of this amino acid per kilogramme of the body mass per day [Clemente 2000] and the individual tolerance should be taken into account. Because of the mentioned fact the diet of people afflicted with such a disfunction mainly consists of low protein products. The order of the Minister of Health from 26 April 2004, in the matter of food with special nutritional purpose does not specify detailed qualitative requirements referring to the products intended for people suffering from disfunction of phenylalanine metabolism or analogous illnesses. One can suppose that this fact is a result of small frequency in occurrence of such diseases in human population. In the world this is one per ten thousand of births [Pustkowski 1999] when in Poland – one per seven thousand of births [Markiewicz-Wujec and Kozłowska-Wojciechowska 2000].

Nevertheless, the presence on the market of the products designated for special groups of consumers and provided with producer declaration about the content of specific components, demands verification by suitable research and analytical methodology. The content of phenylalanine is low so instrumental techniques of high sensitivity (low detection limit) should be used. For determination of amino acids the amino acids automatic analyser is mostly used, wherein the sample after hydrolysis is separated on the ion exchange-column following derivatization. In the reaction of the I-amines with ninhydrin a blue product comes into being absorbing at 570 nm. In case of II-amines the yellowish derivative is formed demonstrating the absorption at 440 nm [Creighton 1984, Jakubke and Jeschket 1989, Holme and Pech 1993]. The sensivity of this method is on a level from about 500 pmol to 1 nmol. A disadvantage of the automatic analyser is that it is not an all-purpose instrument.

Some chemicals such as aromatic amino acids can be determined using HPLC without derivatization. It is possible thanks to the natural fluorescence of these chemicals, or thanks to the ability to absorb the electromagnetic radiation. To determine the phenylalanine content the measurement is carried out at the excitation wavelength of 205 nm and the emission wavelength of 284 nm [Wróbel and Wróbel 1997]. When natural absorption of light is used the measurement is performed at $\lambda = 214$ nm [Wróbel and Wróbel 1997, Prodolliet and Bruelhart 1993]. The use of spectrofluorimetric detectors enables the detection of the compound on the level of 5×10^{-10} mol/dm^3, while the sensivity of spectrophotometric detectors oscillates at about at 1×10^{-7} mol/dm^3 [Kołodziejczyk 2004].

For amino acids analysis gaschromatography coupled with mass spectrometry can be used. “EZ: faast” is a kit-test (Phenomenex) for free amino acids analysis. The test

contains reagents for cleaning the hydrolysates, extraction and derivatization of amino acids. Producer declared the limit of detection of amino acids of 1 nmol/ml, what in case of phenylalanine relates to 1.65×10^{-7} g/ml [Instruction... 2004], and the time of sample preparation is about 15 minutes.

The aim of the work was the estimation of HPLC and GC/MS methods usefulness for quantitative determination of phenylalanine in low protein cereal products (LPP).

MATERIAL AND METHODS

The low protein products from the producer A and B available on Polish market were used as a material for investigation. Those products were offered to the consumer suffering from phenylketonuria and celiakia. Besides, traditional products corresponding to chosen products of special purpose were investigated. The characterization of the products is showed in Table 1.

In all samples the content of protein was measured with the Kjeldahl method. The content of phenylalanine was analysed with high performance liquid chromatography (HPLC) and gas chromatography (GC/MS) after acid hydrolysis [Weiss et al. 1998, Adebiyi et al. 2005]. To the glass-phial (10 cm^3) approx. 0.50 g of the sample (low protein products) or approx. 0.25 g in case of traditional products was weighed. 8 cm^3 of 6M HCl was then added. Samples were deaerated and closed under nitrogen over the gas burner. Then samples were placed in a laboratory dryer and hydrolysed at 110°C for 24 and 48 h. For each product the samples were prepared in three parallel repetitions.

Hydrolisates for HPLC analysis [Prodolliet and Bruelhart 1993] were clarified with Carrez solution, centrifuged for 10 min (5000/rot. per min) and then supernatants were filtered using Acrodisc filters with 0.45 µm GHP membranes. The filtrate was chromatographed on the RP C18 column (LiChrosorb 5 µm, 250 × 4 mm) in duplicate for each hydrolizate. The injection volume was 20 µl. The time of the chromatographic resolution of samples was 15 min (Fig. 1) using the mobile phase flow of 0.8 ml/min at the pressure 107-109 kgf/cm^2. As the mobile phase the mixture of phosphate buffer (pH = 3.5) and the acetononitrile (98:2) was used. The measurement was carried out at λ = 214 nm. For the preparation of calibration curve the standard L-phenylalanines (99%, FLUKA) in different concentrations was used. The correlation coefficient of the calibration curve obtained was 0.9998. For samples preparation prior to GC/MS analysis "EZ:faast" kit-test (Phenomenex) was used. The entire time of sample analysis was 12 min. Samples were prepared in duplicate for each hydrolysate. Injected sample (1 µl) was chromatographed on ZB-AAA (10 × 0.25 mm) column. The splitless injection mode was applied. The GC oven temperature program was as follows: initial temperature 65°C for 1 min then temperature rise in the rate 4°C per minute to the final isotherm 300°C (5 min). As a mobile phase helium about the flow of 1.65 ml/min was used. Employed conditions of the mass spectrometer were: the temperature of the ion source – 200°C, the interface temperature 250°C, detector voltage 1.0 kV. The quadrupole analyser measured the abundance of ions of m/z from 100 to 360. The calibration curve was prepared using solutions of amino acids mixtures included in the "EZ:faast" test (Phenomenex). The standard curve was prepared basing on the phenylalanine peak surface to the area peak of norvaline (internal standard) ratio depending on the concentration of phenylalanine (expressed in nmol/µl). The determination coefficient was 0.984.

Table 1. Characterization of the investigated material
Tabela 2. Charakterystyka materiału badawczego

Low protein products from producer A Produkty niskobiałkowe firmy A		Low protein products from producer B Produkty niskobiałkowe firmy B		Traditional products Produkty tradycyjne	
product produkt	declared content of protein and phenylalanine in 100 g of product deklarowana zawartość białka i fenyloalaniny w 100 g produktu	product produkt	declared content of protein and phenylalanine in 100 g of product deklarowana zawartość białka i fenyloalaniny w 100 g produktu	product produkt	declared content of protein in 100 g of product deklarowana zawartość białka w 100 g produktu
EXTRA universal low protein flour concentrate EXTRA uniwersalny koncentrat mąki niskobiałkowej PKU	protein – białko: 0.36 g phenylalanine fenyloalanina: 3.30 mg	low protein flour mąka nisko- białkowa	protein – białko: 0.41 g phenylalanine fenyloalanina: 19.38 mg	flour mąka Szy- manowska	protein – białko: 9.2 g
Low protein bread- crumbs Bułka tarta nisko- białkowa PKU	protein – białko: 0.85 g phenylalanine fenyloalanina: 40.7 mg	breadcrumbs bułka tarta PKU	protein – białko: 1.0 g phenylalanine fenyloalanina: 46.0 mg	breadcrumbs bułka tarta	–
Pasta without gluten Makaron wstążki krótkie i krótkie kolanka bezglute- nowe	protein – białko: 0.6 g phenylalanine fenyloalanina: 20.0 mg	low protein pasta makaron skrobiowy niskobiałko- wy – wstążki PKU	protein – białko: 0.84 g phenylalanine fenyloalanina: 40.25 mg	pasta makaron nitki domowe	–
Fragile spicy cakes Ciasteczka kruche korzenne bezglute- nowe PKU	protein – białko: 0.14 g phenylalanine fenyloalanina: 18.0 mg	cookies herbatniki PKU	protein – białko: 0.62 g phenylalanine fenyloalanina: 28.70 mg	butter cook- ies herbatniki o smaku ma- ślanym	protein – białko: 7.2 g
Cakes with jam Ciastka kruche z marmoladą	protein – białko: 0.77 g phenylalanine fenyloalanina: 13.5 mg	cookies mix mieszanka herbatnikowa PKU	protein – białko: 0.9 g phenylalanine fenyloalanina: 40.0 mg		

Results were processed statistically (StatGraphics 4.1). The following calculation were performed: arithmetical means, standard deviations, coefficients of the variability and the significance of statistical differences between the results obtained with different methods.

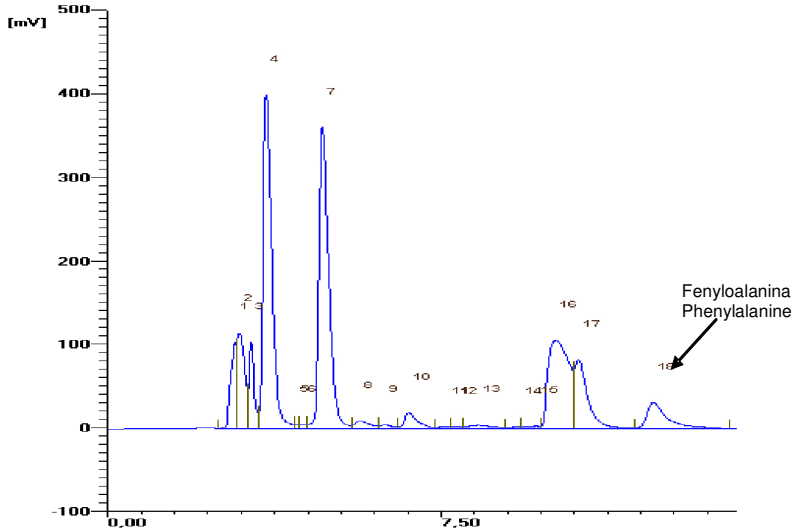

Fig. 1. Chromatographic separation of amino acids of breadcrumbs hydrolysate from producer B
Rys. 1. Rozdział chromatograficzny aminokwasów z hydrolizatu bułki tartej firmy B

RESULTS AND DISCUSSION

The results of protein content analysis in the investigated products are presented in Table 2. Our results show that in case of low protein products from the firm A, in most of the analysed products (except cakes with jam), the producer declared a lower protein content. Differences between the declared content and our results varied within the range from 7% (breadcrumbs) to 271% for fragile spicy cakes. Firm B also declared lower protein content. Differences between the values declared and our results were from 5% for flour to 64% for breadcrumbs.

Analysis of phenylalanine content with HPLC showed (Table 3) that in 1/3 of the investigated products the content of this amino acid was higher comparing with the producer declaration. In products of producer A the concentration declared was lower in 50% of products. The content of phenylalanine in flour and fragile cakes with jam offered by this firm exceeds the declared value by approx. 30%. In case of the pasta and breadcrumbs the content of phenylalanine was lower from the declared by approx. 30%, while in cakes by approx. by 60%. When regarding producer B only in breadcrumbs the content of phenylalanine was higher by 20% in relation to the producer declaration. In offered flour the content of phenylalanine was lower from the declared by approx. 60 %. In pasta and two kinds of cakes the determinated content of this amino acid was lower by 20% and 10-25%, resp. In traditional products the content of phenylalanine was higher from the average table data [Kunachowicz et al. 2005], in breadcrumbs approx. by 12%, in flour phenylalanine content was close to the table value, while pasta and butter biscuits had lower phenylalanine content taking into account table average level of this

Table 2. Protein content in 100 g of products, g
Tabela 2. Zawartość białka w 100 g produktu, g

Low protein products from producer A Produkty niskobiałkowe firmy A		Low protein products from producer B Produkty niskobiałkowe firmy B		Traditional products Produkty tradycyjne	
product produkt	analysed protein content in 100 g of products oznaczona zawartość białka w 100 g produktu g	product produkt	analysed protein content in 100 g of products oznaczona zawartość białka w 100 g produktu g	product produkt	analysed protein content in 100 g of products oznaczona zawartość białka w 100 g produktu g
EXTRA universal low protein flour concentrate EXTRA uniwersalny koncentrat mąki niskobiałkowej PKU	0.91 +/-0.01	low protein flour mąka niskobiałkowa	1.64 +/-0.00	flour mąka Szymanowska	11.76 +/-0.08
Low protein breadcrumbs Bułka tarta niskobiałkowa PKU	0.44 +/-0.01	breadcrumbs bułka tarta PKU	0.43 +/-0.01	breadcrumbs bułka tarta	11.09 +/-0.01
Pasta without gluten Makaron wstążki krótkie i krótkie kolanka bezglutenowe	0.75 +/-0.00	low protein pasta makaron skrobiowy niskobiałkowy – wstążki PKU	0.91 +/-0.01	pasta makaron nitki domowe	11.46 +/-0.02
Fragile spicy cakes Ciasteczka kruche korzenne bezglutenowe PKU	0.72 +/-0.01	cookies herbatniki PKU	0.74 +/-0.02	butter cookies herbatniki o smaku maślanym	6.59 +/-0.02
Cakes with jam Ciastka kruche z marmoladą	0.52 +/-0.00	cookies mix mieszanka herbatnikowa PKU	1.16 +/-0.01		

amino acid in such products. The calculated differences were about 20 and 30%, resp. These oscillations came probably from the fact that the table values are averaged. Differences between experimental results and table data can be due to different content of protein in the raw material.

The use of the "EZ: faast" test, except the estimation of its usefulness in analysis of phenylalanine content in low protein products, was simultaneously an attempt at its adaptation to work under conditions of the gas-chromatograph coupled with mass spectrometer (GC/MS) type QP2010S (SHIMADZU). Earlier the set was used for instrumental analyses with Agilent 5973 and Varian Saturn 2000 GC/MS, where the main ions

Table 3. Phenylalanine content analysed with HPLC
Tabela 3. Zawartość fenyloalaniny oznaczona metodą HPLC

Low protein products from producer A Produkty niskobiałkowe firmy A			Low protein products from producer B Produkty niskobiałkowe firmy B			Traditional products Produkty tradycyjne		
product produkt	analysed phenylalanine content in 100 g of products after hydrolysis oznaczona zawartość fenyloalaniny w 100 g produktu po hydrolizie g		product produkt	analysed phenylalanine content in 100 g of products after hydrolysis oznaczona zawartość fenyloalaniny w 100 g produktu po hydrolizie g		product produkt	analysed phenylalanine content in 100 g of products after hydrolysis oznaczona zawartość fenyloalaniny w 100 g produktu po hydrolizie g	
	24 h	48 h		24 h	48 h		24 h	48 h
EXTRA universal low protein flour concentrate EXTRA uniwersalny koncentrat mąki niskobiałkowej PKU	26.0[a] +/-4.7	32.6[a] +/-0.7	low protein flour mąka niskobiałkowa	56.30[a] +/-2.85	55.18[a] +/-2.45	flour mąka Szymanowska	497.65[a] +/-8.67	497.60[a] +/-13.14
Low protein breadcrumbs Bułka tarta niskobiałkowa PKU	4.6[a] +/-0.3	4.2[a] +/-0.3	breadcrumbs bułka tarta PKU	6.13[a] +/-0.11	8.05[b] +/-0.19	breadcrumbs bułka tarta	465.71[a] +/-27.64	477.02[a] +/-12.55
Pasta without gluten Makaron wstążki krótkie i krótkie kolanka bezglutenowe	13.5[a] +/-2.6	15.2[a] +/-2.8	low protein pasta makaron skrobiowy niskobiałkowy – wstążki PKU	31.06[a] +/-0.87	34.61[b] +/-1.61	pasta makaron nitki domowe	502.41[a] +/-4.00	507.74[a] +/-2.74
Fragile spicy cakes Ciasteczka kruche korzenne bezglutenowe PKU	16.5[a] +/-2.0	18.1[a] +/-2.3	cookies herbatniki PKU	21.39[a] +/-0.35	21.72[a] +/-0.33	butter cookies herbatniki o smaku maślanym	248.36[a] +/-1.98	243.23[b] +/-1.03
Cakes with jam Ciastka kruche z marmoladą	7.9[a] +/-0.5	7.8[a] +/-0.4	cookies mix mieszanka herbatnikowa PKU	35.24[a] +/-2.40	35.58[a] +/-0.33			

Different letters indicate significance of statistical difference for $\alpha = 0.05$.
Wartości różniące się indeksami różnią się istotnie dla $\alpha = 0{,}05$.

for phenylalanine were of mass to charge ratio (m/z) 206 and 190 in the first case, while in second case: 147, 128 and 91 [Instruction... 2004]. For the phenylalanine identification under applied analysis conditions we took into account ions of the mass to charge ratio 148, 190, 206 (Fig. 2). The results received with GC/MS showed that 2/3 of the investigated products had a lowered declaration of phenylalanine content. The data obtained with HPLC also showed that more products of producer A had a lower declared value of phenylalanine content comparing to the results received in our analysis. Only in breadcrumbs there was no higher amount of this amino acid. Phenylalanine concentration, relating to the declaration, was smaller by 20%, what gives the value close to the HPLC results. In remaining products of producer A the level of phenylalanine, regarding the results obtained, is considerably higher than the declared. The highest content of phenylalanine was detected in flour, where the declaration was exceeded approx. 10 times. In cakes with jam, in pasta and fragile spicy cakes the determinated content of phenylalanine exceeded the declared level by: 30, 80 and 100%, resp.

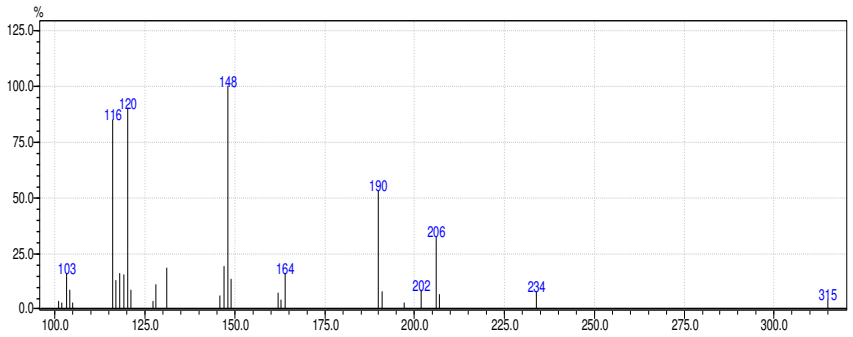

Fig. 2. Mass spectrum of phenylalanine for breadcrumbs from producer B (main ions: 148, 190, 206)
Rys. 2. Spektrum masowe fenyloalaniny pochodzącej z hydrolizatu bułki tartej firmy B (jony główne: 148, 190, 206)

More profitably, on the ground of analyses using the "EZ:faast" test, the food produced by firm B fell out. Only in flour this amino acid content was greater by approx. 25% from the declared one. In the remaining products the quantity of phenylalanine oscillated around the value declared on the label. Considering traditional products analysed with GC/MS in 75% of these articles we noticed that content of phenylalanine was on the higher level than the theoretical table data [Kunachowicz et al. 2005]. In flour, biscuits and breadcrumbs the appointed concentration of phenylalanine was higher by: 30, 40 and 60%, resp. The quantity of phenylalanine in the pasta oscillated round the table value.

On the ground of the results of phenylalanine quantity analysis with HPLC we observed that in 66% of the investigated products the content was lower from the declared value. Taking that into account, the influence of the sample preparation procedure on the received results should be checked. Special attention ought to be paid to the process of hydrolysis, which was performed under drastic conditions and could cause amino acids losses. The lack of any preservative in the phenolic form, thioglycolic acid

Table 4. Phenylalanine content analysed with GC/MS
Tabela 4. Zawartość fenyloalaniny oznaczona metodą GC/MS

Low protein products from producer A Produkty niskobiałkowe firmy A			Low protein products from producer B Produkty niskobiałkowe firmy B			Traditional products Produkty tradycyjne		
product produkt	analysed phenylalanine content in 100 g of products after hydrolysis oznaczona zawartość fenyloalaniny w 100 g produktu po hydrolizie g		product produkt	analysed phenylalanine content in 100 g of products after hydrolysis oznaczona zawartość fenyloalaniny w 100 g produktu po hydrolizie g		product produkt	analysed phenylalanine content in 100 g of products after hydrolysis oznaczona zawartość fenyloalaniny w 100 g produktu po hydrolizie g	
	24 h	48 h		24 h	48 h		24 h	48 h
EXTRA universal low protein flour concentrate EXTRA uniwersalny koncentrat mąki niskobiałkowej PKU	34.1[a] +/-6.4	33.4[a] +/-2.9	low protein flour mąka niskobiałkowa	38.18[a] +/-5.03	45.30[a] +/-0.40	flour mąka Szymanowska	793.29[a] +/-227.67	674.33[a] +/-130.79
Low protein breadcrumbs Bułka tarta niskobiałkowa PKU	46.7[a] +/-10.5	27.0[a] +/-6.7	breadcrumbs bułka tarta PKU	25.29[a] +/-0.48	23.95[a] +/-0.51	breadcrumbs bułka tarta	636.72[a] +/-214.67	740.33[a] +/-82.78
Pasta without gluten Makaron wstążki krótkie i krótkie kolanka bezglutenowe	31.9[a] +/-3.1	30.6[a] +/-5.1	low protein pasta makaron skrobiowy niskobiałkowy – wstążki PKU	39.15[a] +/-9.17	37.35[a] +/-2.06	pasta makaron nitki domowe	657.35[a] +/-76.63	675.93[a] +/-63.95
Fragile spicy cakes Ciasteczka kruche korzenne bezglutenowe PKU	24.3[a] +/-0.9	25.0[a] +/-2.3	cookies herbatniki PKU	24.14[a] +/-7.03	28.75[a] +/-7.23	butter cookies herbatniki o smaku maślanym	527.21[a] +/-34.62	468.10[a] +/-33.20
Cakes with jam Ciastka kruche z marmoladą	23.2[a] +/-3.0	23.0[a] +/-3.4	cookies mix mieszanka herbatnikowa PKU	35.27[a] +/-8.01	38.18[a] +/-0.66			

Values with different letters indicate significance of statistical difference for $\alpha = 0.05$.
Wartości dla danego produktu w wierszach różniące się indeksami różnią się istotnie dla $\alpha = 0{,}05$.

or tryptamine recommended by the methodology [Nollet 1996] additionally made conditions more drastic, however that should not influence the quantity of phenylalanine present in hydrolysates [Fountoulakis and Lahm 1998]. Similar hydrolysis conditions were used in quantitative analysis of amino acids in soya bean [Albin et al. 2000]. Another essential parameter, which can lead to the loss of amino acids, is the hydrolysis time that is chosen depending on the amino acid composition of protein. Bonds between phenylalanine and other amino acids are broken comparatively quickly, during first 20 hours of hydrolysis in standard conditions of the process [Rakowska et al. 1978]. Prolongation of the hydrolysis time can cause losses of this component.

Results of the analysis with HPLC showed that the hydrolysis time influenced the content of the amino acid only in case of four products: flour and pasta (producer B) and traditional products like butter biscuits. In producer's B products the extension of the hydrolysis time caused an increase of the phenylalanine quantity, while in biscuits decreased its quantity. The literature suggests prolongation of hydrolysis time by above 24 hours (48-72 h) when isoleucine and valine are quantified, because bonds between these amino acids are strong, which is not confirmed relating to phenylalanine [Rakowska et al. 1978, Fountoulakis and Lahm 1998]. The results received with GC/MS did not ascertain us of the essential influence of the hydrolysis time on the quantity of the detected amino acid.

The use of 6M HCl is usually recommended in different kinds of analytical procedures and accepted as the standard parameter of the hydrolysis [Dyrektywa... 1998, Weiss et al. 1998, Adebiyi et al. 2005]. The fact that this is an optimum concentration could be confirmed by research led on protein of the soya bean. The influence of the HCl concentration (1, 3, 6, 9 and 12 M) on the quantity of the released amino acids during hydrolysis was checked. According to the mentioned study an increase of the HCl concentration above 6 M does not change the content of phenylalanine in the investigated hydrolysates [Albin et al. 2000].

Table 5. Relative standard deviation for phenylalanine content analysed with HPLC and GC/MS after 48 hours of hydrolysis for products of producer A
Tabela 5. Współczynniki zmienności dla zawartości fenyloalaniny oznaczonej metodami HPLC i GC/MS po 48-godzinnej hydrolizie dla produktów firmy A

Product Produkt	HPLC RSD%	Acceptable RSD% Dopuszczalne RSD% (Horowitz)	GC/MS RSD%	Acceptable RSD% Dopuszczalne RSD% (Horowitz)
Low protein breadcrumbs Bułka tarta niskobiałkowa	2.3	8	8.6	8
Universal flour concentrate Koncentrat mąki uniwersalnej	6.4	11.3	25.0	11.3
Pasta Makaron	18.6	8	16.6	8
Fragile spicy cakes Ciasteczka kruche korzenne bezglutenowe PKU	12.7	8	9.2	8
Cakes with jam Ciastka kruche z marmoladą	4.9	11.3	14.8	11.3

To determine practical usefulness of the HPLC and GC/MS instrumental methods for the quantitative analysis of phenylalanine, the coefficients of relative standard deviation (RSD) were calculated (Tables 5-7). After their comparison it is clear that the results obtained with HPLC are characterized with a smaller scattering comparing with the GC/MS analysis. In case of liquid high performance chromatography admissible RSD% was exceeded only for two samples (pasta and fragile cakes with the jam – producer A), while for GC/MS this value was too high for almost 75% of the investigated samples. Basing on the calculated RSD% we could ascertain that for the quantitative determination of phenylalanine in cereal (wheat) products, both low protein products and the traditional ones, results were more repetitive.

Identification and quantification using GC/MS is considered as very reliable and exact. The producer of the "EZ:faast" test declares the sensivity on its level of 1 nmol/ml (0.1 mg/l). Dilutions used in this study caused the decrease of the phenylalanine content in samples to the level of 0.1 nmol/ml (0.01 mg/l), what surely influenced the repeatability

Table 6. Relative standard deviation for phenylalanine content analysed with HPLC and GC/MS after 48 hours of hydrolysis for products of the producer B
Tabela 6. Współczynniki zmienności dla zawartości fenyloalaniny oznaczonej metodami HPLC i GC/MS po 48-godzinnej hydrolizie dla produktów firmy B

Product Produkt	HPLC RSD%	Acceptable RSD% Dopuszczalne RSD% (Horowitz)	GC/MS RSD%	Acceptable RSD% Dopuszczalne RSD% (Horowitz)
Breadcrumbs – Bułka tarta	4.4	8	0.9	8
Flour – Mąka	2.3	11.3	2.1	11.3
Pasta – Makaron	4.7	8	5.5	8
Cookies – Herbatniki	1.5	8	25.1	8
Cookies mix Mieszanka herbatników	2.3	8	1.7	8

Table 7. Relative standard deviation for phenylalanine content analysed with HPLC and GC/MS after 48 hours of hydrolysis for products of the producer B
Tabela 7. Współczynniki zmienności dla zawartości fenyloalaniny oznaczonej metodami HPLC i GC/MS po 48-godzinnej hydrolizie dla produktów tradycyjnych

Product Produkt	HPLC RSD%	Acceptable RSD% Dopuszczalne RSD% (Horowitz)	GC/MS RSD%	Acceptable RSD% Dopuszczalne RSD% (Horowitz)
Breadcrumbs Bułka tarta	2.6	5.7	19.4	5.7
Flour – Mąka	2.6	5.7	11.2	5.7
Pasta – Makaron	0.5	5.7	9.5	5.7
Cookies Herbatniki	0.4	5.7	7.1	5.7

of the results – relatively big relative standard deviation value (RSD). However comparing HPLC and GC/MS technique, the last one made it possible to measure phenylalanine in a very small concentration (ng/ml). This is confirmed by the research on beer or honey, where the same test was used without modification, as additional sample dilution. The results of those analysis realized with GCxGC-TOFM were more repeatable (RSD at the level of 7%), and the detection level was 0.01 mg/l [Mayadunne et al. 2005].

The method of modification by additional sample dilution was extored by the equipment requirements. The final volume of sample (100 µl) proposed in the Phenomenex procedure was too small to apply an autosampler, without special vials of smaller volume. Assuring the repeatability of injection conditions the volume of samples was enlarged by adding 1000 µl of isooctane. This additional dilution of the samples, containing low concentration of phenylalanine, influenced accuracy of our results.

Comparing our results and the literature data [Mayadunne et al. 2005] it seems that for the purpose of providing the repeatability of the received results, sample preparation should be modified or proper vials should be used. The increase of the phenylalanine concentration to the level assuring its proper detection could be obtained by the enlargement of the weight of samples used for hydrolysis with an unchanged volume of the acid (6 M HCl). Besides, the modification of GC/MS conditions could influence the accuracy of our results. It was an effect GC/MS QP2010S (SHIMADZU) requirements. The analysis parameters suggested by the producer of the test: injection – 2 µl, carrier gas flow – 1.1 ml/min, the temperature program: 30°C/min from 110°C to 320°C, interface temperature 180°C were adapted to requirements of other chromatographs – Agilent 5973 and Varian Saturn 2000 [Instruction... 2004].

However, it should be noticed that the analysis using "EZ: faast: test enables the determination of all amino acids in short time and at low level of concentration while HPLC has a limited application.

CONCLUSIONS

1. Higher than the declared protein content in all products from producer B and in some from firm A was observed.

2. GC/MS method allowed to analyse the phenylalanine content in injected samples at the level of 1 ng/ ml.

3. On the basis of the results received with HPLC it can be stated that in most (2/3) of the investigated products the level of phenylalanine was lower comparing with the producer declaration.

4. On the ground of the statistical analysis (significance of statistical differences) it can be concluded that the extension of hydrolysis time from 24 to 48 h did not influence the quantity of the released phenylalanine in case of 73% products.

5. Higher content of protein did not influence phenylalanine quantity measured with HPLC – its level in most of the products was found below the producer's declaration.

6. On the ground of differences between the theoretical content of phenylalanine in protein and with the content declared by the producer and our research we concluded that the content of the phenylalanine in cereal products should not be counted on the ground of the theoretical participation of this amino acid in protein.

REFERENCES

Adebiyi A.P., Jin D.H., Ogawa T., Muramoto K., 2005. Acid hydrolysis of protein in a micro-capillary tube for the recovery of tryptophan. Biosci. Biotechn. Biochem. 69 (1), 255-257.

Albin D.M., Wubben J.E., Gabert V.M., 2000. The influence of hydrochloric acid concentration and measurement method on the determination of amino acid levels in soya bean procucts. Anim. Feed Sci. Techn. 87, 173-186.

Clemente A., 2000. Enzymatic protein hydrolysates in human nutrition. Trends Food Sci Techn. 11, 251-262.

Creighton T.E., 1984. Proteins structures and molecular properties. W.H. Freedman New York, 25-27, 42-45, 191.

Dyrektywa Komisji 98/64/WE z dnia 3 września 1998 r. ustanawiająca wspólnotowe metody analiz do oznaczania aminokwasów, surowych olejów i tłuszczów oraz olaquindoksu w paszach i zmieniająca dyrektywę 74/393/EWG. 1998. Dz. UE, 257/14.

Fountoulakis M., Lahm H.W., 1998. Hydrolysis and amino acid composition analysis of proteins. J. Chromatogr. A (826), 109-134.

Holme D.J., Pech H., 1993. Analytical biochemistry. Longman Sci. Tech. New York, 392-393.

Instruction "Ez:faast". 2004. Phenomenex.

Jakubke H.D., Jeschket H., 1989. Aminokwasy, peptydy, białka [Amino acid, peptides, proteins]. PWN Warszawa, 38-338 [in Polish].

Kołodziejczyk A., 2004. Naturalne związki organiczne [Natural organic compounds]. PWN Warszawa, 11-76, 175-228 [in Polish].

Kunachowicz H., Nadolna B., Iwanow K., 2005. Tabele wartości odżywczej produktów spożywczych [Tables of the nutritional value of food products]. IŻŻ Warszawa [in Polish].

Markiewicz-Wujec M., Kozłowska-Wojciechowska M., 2000. Dieta w fenyloketonurii [Diet in the phenylketonuria]. Żyjmy dłużej 6, 40-41 [in Polish].

Mayadunne R., Nguyen T.T., Marrriot P.J., 2005. Amino acid analysis by using comprehensive two-dimentional gas chromatography. Anal. Bioanal. Chem. 382, 836-847.

Molnár-Perl I., 2003. Quantitation of amino acids and amine in the same matrix by high-performance liquid chromatography, either simultaneously or separately. J. Chromatogr. A, 987, 291-309.

Nollet L.M.L., 1996: Handbook of food analysis. Part 1. Physical characterisation and nutrient analysis. Marcel Dekker New York, 206-207.

Prodolliet J., Bruelhart M., 1993. Determination of aspartame and its major decomposition products in foods. J. AOAC Int. 76 (2), 275-281.

Pustkowski M., 1999. Fenyloketonuria [Phenylketonuria]. Żyjmy dłużej 9, 32-33 [in Polish].

Rakowska M., Szkiłłądziowa W., Kunachowicz H., 1978. Biologiczna wartość białka żywności [Biological value of the food protein]. WNT Warszawa, 118-131 [in Polish].

Rozporządzenie Ministra Zdrowia z 26 kwietnia 2004 r. w sprawie środków spożywczych specjalnego przeznaczenia żywieniowego. 2004. Dz.U. nr 104, poz. 1084.

Weiss M., Mannenberg M., Juranville J.F, Lahm H.W., Fountoulakis M., 1998. Effect of the hydrolysis method on the determination of the amino acid composition of proteins. J Chromatogr. A, 795, 263-275.

Wróbel K., Wróbel K., 1997. Determination of aspartame and phenylalanine in diet soft drinks by high-performance liquid chromatography with direct spectrofluorimetric detection. J. Chromatogr. A, 773, 163-168.

ZASTOSOWANIE HPLC I GC/MS DO ILOŚCIOWEGO OZNACZANIA FENYLOALANINY W PRODUKTACH NISKOBIAŁKOWYCH

Streszczenie. Celem pracy była ocena przydatności metod HPLC i GC/MS do ilościowego oznaczania fenyloalaniny w zbożowych produktach niskobiałkowych (PKU). Materiałem badawczym były produkty PKU dwóch firm, A i B, oraz odpowiadające im produkty tradycyjne. Przeprowadzono oznaczenia zawartości białka metodą Kiejdahla i oznaczenie fenyloalaniny metodą RP-HPLC z detektorem UV (λ = 214 nm) oraz metodą GC/MS z użyciem zestawu testowego EZ:faast. Oznaczenie zawartości aminokwasu wykonano po hydrolizie kwasowej (6M HCl, czas: 24 i 48 h, temp. 110°C). Stwierdzono większą zawartość białka od zawartości deklarowanej we wszystkich produktach firmy B i niektórych firmy A. Większa zawartość białka nie wpłynęła na ilość fenyloalaniny wykrytej metodą HPLC. W większości produktów jej poziom był niższy niż deklarowany przez producenta. W większości wypadków nie stwierdzono istotnego wpływu czasu hydrolizy na ilość uwolnionej fenyloalaniny.

Słowa kluczowe: fenyloalanina, produkty niskobiałkowe, HPLC, GC/MS

Accepted for print – Zaakceptowano do druku: 29.05.2007

For citation – Do cytowania: Piecyk M., Śrama A., Bzducha A., Obiedziński M., 2007. Application of HPLC and GC/MS to quantification of phenylalanine in chosen kinds of food for particular nutritional uses. Acta Sci. Pol., Technol. Aliment. 6(2), 5-18.