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ORIGINAL PAPER

Simple extraction procedure for free amino acids determination in selected gluten-free flour samples

Sylwia Kowalska[1] · Edward Szłyk[1] · Aneta Jastrzębska[1]

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Abstract

The simple extraction of flours samples followed by free amino acids determination procedures was studied and optimised. The conditions of amino acids derivatisation reaction with ninhydrin for chromatographic determination of free amino acids sum was discussed. The developed method was processed in terms of linearity, precision, accuracy, and limits of detection and quantification. Moreover, capillary isotachophoresis and HPLC methods were applied for individual free amino acids determination. The proposed extraction procedure is simple, fast and convenient for different flours samples. Studied procedures were used for free amino acids determination in twelve gluten-free flour samples (corn, oat, soy, rice, pumpkin, millet, peanut, hemp seed, buckwheat, amaranth, pea and chickpea) and the obtained results were compared with wheat flour.

Keywords Optimisation of extraction · Free amino acids · Gluten-free flours · Ninhydrin · Chromatographic analysis · Isotachophoretic analysis

Introduction

Due to the changes in consumer's preferences, the demand for gluten-free products is increasing [1]. Therefore, many different non-wheat cereals, pseudocereals and pulses have gained the increased attention of the food industry as an alternative to wheat products [2, 3]. Cereals such as corn, rice, barley, and legumes are known for their nutritional properties, hence they are consumed worldwide and used for gluten-free food production [1, 2]. Pseudocereals, such as buckwheat or amaranth, exhibit nutritional features which make them suitable for replacing, at least partly, traditional cereal-based products. They are recommended for celiac disease patients diet by the World Gastroenterology Organization and as ingredients for baby food formulation due to their low allergenicity [3–5].

The flours are essential and the most commonly used raw material for bakery products, however, due to the properties of proteins in gluten-free flours, their usage is limited. On the other hand, the nutritional properties of gluten-free flours are their main advantage due to the well-balanced composition of the amino acids with high biological value [5, 6].

Amino acids (AAs) are indispensable nutritional constituents for body growth and many biological activities, e.g. regulating gene expression, preventing tumorigenesis, suppressing obesity, and reducing blood pressure [7, 8]. In human organisms, amino acids take part in building the protein chain, but there is always a pool of free amino acids (FAAs). These compounds contribute significantly to the overall acceptability of food due to the impact on the flavour and taste. It is known that amino acids are substrates for microorganisms in the dough, hence the increase of FAAs content in a dough can improve the bread flavour. Many of them are considered as functional food quality control markers [7]. In living organisms, FAAs are precursors or substrates for several biological processes resulting in the formation of biogenic amines or different organic volatile compounds. Therefore, FAAs have an essential impact on food flavour, and variations of their concentration indicate proteolytic and hydrolytic activity in food processing [9]. In reaction with reducing sugars during high-temperature processing (Maillard reaction or non-enzymatic browning), FAAs have an impact on the formation of colour, aroma and flavour of food [10, 11]. However, the Maillard reaction also gives rise to some contaminants, including acrylamide or 5-hydroxymethylfurfural (HMF) [6, 12]. Acrylamide

✉ Aneta Jastrzębska
aj@umk.pl

[1] Chair of Analytical Chemistry and Applied Spectroscopy, Faculty of Chemistry, Nicolaus Copernicus University in Toruń, Gagarin 7 Str., 87-100 Toruń, Poland

can be formed in the reaction of free amino acids (mainly asparagine) with reducing sugars (fructose or glucose) at a temperature above 120 °C in food derived from wheat, maize, and other cereals. However, other possible routes of acrylamide formation were described and other amino acids can also contribute to acrylamide generation [6].

Despite the interest in the free amino acids in food and considering the possible contribution of these compounds to acrylamide formation reactions, the knowledge about these compounds in gluten-free flours is limiting. On the other hand, it is known that the level of free amino acids in plant matrices is low, hence their analysis in plant material is a challenging task [8]. The classical method is based on post-column ninhydrin derivatisation reaction, followed by ion-exchange chromatography [13–15]. In addition, separation techniques (LC, GC or CE) coupled with spectrophotometric, mass spectrometry, fluorescence or electrochemical detection techniques [3, 8, 16–21] were reported. Because amino acids can form cations, zwitterions or anions, depending on pH, the application of capillary isotachophoresis (ITP) technique for AAs determination is possible. It is worth noting that, despite many advantages and lack of the amino acids derivatisation stage, there is limited number of publications on amino acid determination in food matrices by ITP [22, 23].

The other challenge in the determination of free amino acids is the extraction stage. Several extraction procedures of FAAs from cereals have been described in the literature. In the case of plant products, FAAs were extracted with water [10] water/acetonitrile mixture [24], perchloric, trichloroacetic or hydrochloric acids [16, 25–28], formic acid/methanol mixture [20] and ethanol [8, 19, 21] using various techniques. Among many reports on the development and validation of new analytical methods of FAAs, extraction procedure has been less discussed and search for novel sample preparation method based on simple extraction is still valid.

The present work aimed to elaborate a simple, convenient and rapid extraction of free amino acids from flours samples. The selected conditions of the extraction procedure (solvent and time) were optimised and discussed. The developed sample preparation procedure was verified in the determination of free amino acids in commercially available gluten-free flours and discussed in terms of these compounds determination by different methods. The sum of FAAs determination based on ninhydrin reaction with amino acids and chromatographic analysis was proposed, and reactions conditions were developed. Additionally, the FAAs in flours samples were determined by column-coupling capillary isotachophoretic (ITP-ITP) and chromatographic (after reaction with 2-chloro-1,3-dinitro-5-(trifluoromethyl)benzene—CNBF) methods. Selected gluten-free flours: corn, rice, oat, millet, amaranth, buckwheat, soy, pea, peanut, hemp seed, pumpkin and chickpea were analysed. The obtained results were discussed regarding the contents of FAAs and related to the wheat flour.

Materials and methods

Reagents and apparatus

The analytical grade: L-tryptophan (Trp), L-tyrosine (Tyr), L-threonine (Thr), L-methionine (Met), L-valine (Val), L-isoleucine (Ile); L-histidine (His), L-phenylalanine (Phe); L-arginine (Arg), L-leucine (Leu), L-proline (Pro), L-lysine (Lys), β-alanine (Ala), L-glutamic acid (Glu), L-glycine (Gly), L-aspartic acid (Asp), L-asparagine (Asn), L-cysteine, L-glutamine (Gln), L-serine (Ser), 2-chloro-1,3-dinitro-5-(trifluoromethyl)benzene (CNBF), methanol (HPLC grade), acetonitrile (HPLC grade), triethylamine, ethanamine, 1,3-bis[tris(hydroxymethyl)methylamino]propane (BTP), 6-aminocaproic acid (EACA), 2-amino-2-methyl-1,3-propanediol (AMPD), hydroxyethylcellulose (HEC, average $M_v \sim 90.000$) were purchased from Sigma Aldrich (Poland). Hydrochloric acid, boric acid, perchloric acid, nitric acid, sodium chloride, sodium tetraborate, potassium dihydrogen phosphate, trichloroacetic acid, acetic aicd, sodium acetate anhydrous, tin(II) chloride dihydrate, ethanol 96% were purchased from Alchem Grupa Sp. z o.o. (Toruń, Poland). Deionised water was used for all solutions.

HPLC system (Shimadzu Corp, Kyoto, Japan), equipped with an autosampler SIL-20AC HT and a photodiode multiwavelength detector (SPD-M20A Prominence Diode Array Detector) was applied. Analyses were carried out on OptimaPak (OP) C-18, (5 µm particle size, 150 × 4.6 mm) and C18 (5 µm particle size, 250 × 4.6 mm) columns. The chromatographic data were recorded and processed by the LC solution program version 1.23 SP. Isotachophoretic separations were performed using a Villa Labeco EA 102 isotachophoretic analyser (Villa Labeco, Spišská Nová Ves, Slovakia) equipped with two columns employing capillaries made of fluorinated ethylene-propylene copolymer and a contact conductivity detectors. The isotachopherograms were evaluated using the software supplied by the analyser producer. Deionised water was obtained from a demineralised water supplier (Hydrolab, Poland).

Elaboration of extraction conditions

The following extracting solutions were prepared and tested: water, nitric acid (0.01–0.1 M), hydrochloric acid (0.01–0.1 M), perchloric acid (0.01–0.1 M), trichloroacetic acid, TCA (1%-5%) and ethanol (50–70%) [10, 16, 18, 19, 25–27]. The solvent for this study was chosen by analysis of wheat (sample 13) and pea flours (sample 11). The samples

(5 ± 0.01 g) were extracted with tested reagents using an orbital shaker for 60 min followed by centrifuged (9000 rpm; 15 min) and filtrated. All extracts were transferred into a 25 mL volumetric flask, made up to the mark with distilled water. The effectiveness of extraction was measured by determining the sum of free amino acids using the ninhydrin procedure. The best solvent was selected, and the ratio of solutions volume and time of extraction process was tested. The samples of wheat and pea flours (5 ± 0.01 g) were extracted as described above for 30, 60 or 90 min, centrifuged, filtrated and analysed by HPLC after reaction with ninhydrin.

RP-HPLC procedures

For the sum of free amino acids determination (ninhydrin procedure), a mixture of acetic acid (2%) + methanol (20:80 v/v) and isocratic elution was applied. The flow rate was 1.0 mL min^{-1}, the temperature set was 25 °C, and the detection wavelength 570 nm. Analyses were carried out on OP C-18, (5 µm particle size, 150 × 4.6 mm) column. HPLC measurements of amino acids derivative with CNBF were performed as described by Jastrzębska et al. [22] and Li et al. [29] using the solvent system: acetonitrile (A) and mixture of acetate buffer (0.05 mol L^{-1}) + acetonitrile + triethylamine (82.8:17:0.2 v/v/v) (B). The flow rate was 0.32 mL min^{-1}, the detection wavelength was 260 nm and the temperature was set at 30 °C.

The procedure of amino acids derivatisation with ninhydrin

The ninhydrin reagent was prepared by dissolving 2 g of ninhydrin in 50 mL ethanol/water (50:50 v/v) and mixed with sodium acetate buffer (pH = 5.5; 25 mL) and tin(II) chloride dihydrate solution (0.08 g in 25 mL of water) and stored refrigerated in a dark bottle. Determination of FAAs (total amount) was performed with standard calibration procedure using glycine (Gly) solutions. The appropriate volume of the Gly stock solution (400 mg·L^{-1}) was added to the flask, mixed with ninhydrin colour reagent (1 mL), sodium acetate buffer (pH = 5.5; 1 mL) and incubated in boiling water at 80^0C for 30 min. After cooling, the obtained solution was adjusted to 10 mL with a mixture of ethanol/water (50:50 v/v) and measured by HPLC (λ = 570 nm). The calibration curve was constructed using eight calibration solutions of Gly in the range 10–100 mg L^{-1}.

The same procedure was used for the samples after extraction (0.5 mL). For each tested flour, three independent samples were used for all procedures, and each sample was analysed in triplicate.

Procedure of amino acids derivatisation with 2-chloro-1,3-dinitro-5-(trifluoromethyl)benzene (CNBF).

The reaction of amino acids with CNBF was performed by the method of Jastrzębska et al. [22]. The solution of each amino acid was mixed with CNBF in the presence of borate buffer and incubated at 60 °C for 30 min. Next, the reaction was stopped, and solutions were made up to 25 mL by phosphate buffer. The parameters of the calibration curves are listed in Table S1 (Supplementary Materials), while the chromatogram of standard solutions is presented at Figure S1 (Supplementary Materials). In the case of food samples, 1.0 mL of food extracts solution was used for the derivatisation procedure.

The determination of glutamic acid and tryptophan by ITP-ITP method

The isotachophoretic analysis of tryptophan (Trp) was performed as described earlier [22] with the leading electrolyte (LE): 10 mM HCl + 1% hydroxyethylcellulose (HEC) + 1,3-bis[tris(hydroxymethyl)methylamino]propane (BTP), pH = 9.0 and terminating electrolyte (TE): 10 mM 6-aminocaproic acid (EACA) + 10 mM 6-aminocaproic acid (AMPD), pH = 9.0. The glutamic acid (Glu) was determined with the same LE, whereas 10 mM histidine + AMPD, pH = 9.4 was applied as TE. The amino acids were identified by the relative step height parameter (RSH), which was calculated from the relation: RSH = (HX − HL)/(HT − HL), where HX—zone height of tested ion, HL and HT—zone step height of leading and terminating ion, respectively. The calibration curve statistical parameters are listed in Table S1 (Supplementary Materials), while isotachopherograms of standard solutions are presented in Figure S2 (Supplementary Materials). Samples of tested flour were analysed after the extraction procedure using 9 mL of sample in 10 mL flask.

Samples and samples preparation

Flour samples purchased from the local shops were marked as gluten-free: corn flour (sample 1), oat flour (sample 2), soy flour (sample 3), rice flour (sample 4), pumpkin flour (sample 5), millet flour (sample 6), peanut flour (sample 7), hemp seed flour (sample 8), buckwheat flour (sample 9), amaranth flour (sample 10), yellow-pea (*Pisum sativum*) flour (sample 11), chickpea flour (sample 12) and one sample of wheat flour with gluten (sample 13).

Samples (5 ± 0.01 g) were extracted with the mixture of 20 mL TCA (1%) + 5 mL EtOH solution using an orbital shaker for 60 min. The isolated extracts were centrifuged (9000 rpm; 15 min), filtered on the disc filter (125 mm) and PTFE syringe filter (0.45 µm), transferred into a 25 mL volumetric flask and made up to the mark with water.

Statistical analysis

All procedures were validated, and parameters such as linearity (coefficient of determination, R^2), accuracy and precision, detection and quantification limits (DL and QL) were determined. The DL and QL were calculated on the basis of the standard deviation of the incept and the slope of the calibration curves according to the following equations: $DL = 3.3 \times (S_{y/x} \cdot a^{-1})$ and $QL = 10 \times (S_{y/x} \cdot a^{-1})$, where $S_{y/x}$ is the standard deviation of the incept of the calibration curve, and a is the slope of the calibration curve. For determination of calibration curves accuracy, three concentrations of the tested amino acid (within the range of the appropriate calibration curve) were prepared, analysed and presented as recovery.

For each tested flour, three samples were used and analysed in triplicate for all procedures. The results for flour samples were presented as an average (X, mg 100 g^{-1}) ± standard deviation (SD). A comparison of the means was ascertained by a Tukey's test to a 5% level of significance using an analysis of the variance (ANOVA). The within-day precision of the obtained results was expressed as the coefficient of variation (CV, %). The accuracy of the proposed sample preparation method was determined using recovery tests. The wheat and pea flour samples (three independent samples) were mixed with 50 mg of Gly, one day stored at room temperature. Thus, blank and spiked flours samples were extracted with tested reagents, and FAAs sum determined by HPLC method after reaction with ninhydrin. The results were calculated using the measured signals intensity and plotted versus concentration of the added AAs standards.

Results and discussion

Analytical parameters of ninhydrin procedure

Majority of amino acids exhibit high polarity, low volatilisation, and lack of chromophore group, resulting in difficult separation and detection. These compounds were initially analysed by ion-exchange chromatography followed by post-column derivatisation with ninhydrin and ultraviolet detection. This method was replaced by faster, more sensitive, and versatile chromatographic methods. However, ninhydrin is still frequently used as a derivatisation reagent for spectrophotometric or chromatographic determination of amino acids [7, 13, 30]. Moreover, when it came to measure total content of free amino acids, the ninhydrin spectrophotometric method is still adopted [31]. The main advantages of the procedure with ninhydrin are: reactivity, reaction rate, simplicity of derivatisation with a pre-, and post-column procedure.

Ninhydrin causes oxidative decarboxylation of primary amino acids, releasing CO_2, NH_3, and an aldehyde with one carbon atom less than the parent amino acid. In the next step, reduced ninhydrin reacts with ammonia forming Ruhemann's purple (RP), with the absorption maximum at 570 nm [14, 30]. In a weak acidic medium, ninhydrin reacts with amino acids forming Ruhemann's purple due to nucleophilic-type displacement of the OH groups by a nonprotonated amino group. Only cysteine, proline and hydroxyproline with ninhydrin exhibit the absorption band at 440 nm due to their distinct structures [30]. According to Friedman [14], high temperature is required to accelerate the reaction of AAs with ninhydrin, while the buffer increases the stability of the obtained derivatives, whereas an organic solvent stabilise the hydrindantin and the new purple chromophores in solution. Different ninhydrin reaction conditions, including organic solvents, temperature, heating time, the buffer used (phosphate, sodium acetate, lithium acetate, potassium acetate, sodium citrate, etc.) were described in the literature [14, 31]. We proposed a derivatisation procedure based on the application of acetate buffer and heating for 30 min. at 80 °C. The obtained ninhydrin derivative was determined by HPLC method, and a typical chromatogram is presented in Figure S3 (Supplementary Materials). The correlation coefficient of the calibration curve for Gly was 0.9989, whereas DL = 2.95 mg L^{-1} and QL = 9.83 mg L^{-1}. The recovery for standard solutions of Gly ranged between 98.9 and 101% indicated satisfactory accuracy of the proposed calibration curve procedure. The retention time (1.90 min) and lack of interferences on chromatograms indicated that the proposed procedure is suitable for the rapid and accurate determination of free amino acids in food samples.

Elaboration of extraction conditions

The flour extraction depends on complex interactions in matrices. The experiments of rapid extraction procedure resulted in cloudy solution in the case of distilled water, nitric acid and hydrochloric acid, and these reagents were postponed. The results of extraction with perchloric acid (0.01–0.1 M), trichloroacetic acid, TCA (1–5%), ethanol (70%) followed by the determination of free amino acids sum by ninhydrin procedure are listed in Table S2 (Supplementary Materials).

The extraction of flour samples with perchloric acid showed a significantly lower sum of the free amino acids. In contrast, the ethanol and trichloroacetic acid extraction resulted in stable solutions with a similar level of the free amino acids sum. For this reason, the TCA and 70% ethanol solutions were selected for efficient extraction. Moreover, results for TCA extraction did not significantly differ (Table S2, $\alpha = 0.05$), hence 1% TCA was selected for further testing. As a next step, two independent variables: the

ratio of solutions volume and extraction time (Table 1), were tested. Results of extraction procedures, followed by the determination of FAAs sum by ninhydrin procedure listed in Table 2 and presented at Fig. S4 (Supplementary Materials).

The highest amount of FAAs was extracted using the mixture of 20 mL (1%) TCA + 5 mL of EtOH (experiment 3, 6 and 9). Moreover, the obtained data of recovery test indicated that the best results of extraction, in regards of accuracy, were obtained for the discussed mixture. For comparison, the significantly lower contents of FAAs and recovery values were determined when the mixture of TCA/EtOH (1/1 v/v; in experiments 1, 4 and 7) was applied. The latter can be related to the different solubility of amino acids in aqueous and alcoholic solvents. It seems evident that presence of ethanol resulted in better solubilisation of polysaccharides, and other viscous polymers resulting in reduced starch gelatinisation process [19]. Discussed solvents were applied for extraction of FAAs from food samples, including cereals products, by many authors [16, 21, 27, 28, 32–34], however, a mixture of both reagents has not been tested yet.

Table 1 Extraction optimization plan

Experiment	Time of extraction (min)	Volume ratio of reagents	
		1% TCA (mL)	70% EtOH (mL)
1	30	12.5	12.5
2	30	15.0	10.0
3	30	20.0	5.0
4	60	12.5	12.5
5	60	15.0	10.0
6	60	20.0	5.0
7	90	12.5	12.5
8	90	15.0	10.0
9	90	20.0	5.0

TCA trichloroacetic acid, *EtOH* ethanol

The second variable of the extraction stage was time. The results indicate the lower values for all tested compounds for 30 min extraction time, while comparable values were obtained for 60 and 90 min. Furthermore, the data for FAAs in wheat flour after 60 and 90 min of extraction varied non-significantly. Comparing accuracy presented as recovery, noticeably lower values were obtained for 30 min, whereas comparable recoveries were observed for 60 and 90 min of extraction. In the case of precision of obtained data, no dependence of CV values on time or composition of the tested mixture was observed. Notwithstanding, the obtained CV value (< 5.2%) for the examined flour samples extraction exhibits satisfactory precision.

Summarising the discussion, one can suggest that the optimal sample extraction was reached with 20 mL (1%) TCA + 5 mL of EtOH, during 60 min.

Sum of FAAs determination in flour samples

The developed procedure was applied for the analysis of FAAs sum in complex food samples. The gluten-free flours available in Polish food stores were analysed to assess the quality of the FAAs content. The results are listed in Table 3.

The contents of FAAs sum varied depending on the type of tested flours. The pumpkin flour was characterised by the highest FAAs concentration (227 mg 100 g^{-1}) while the amaranth flour showed the lowest ones (59.9 mg 100 g^{-1}). The pumpkin flour is a rich source of carotenoids, for this reason, is used as a functional ingredient in the food processing industry. Due to the intense yellow-orange colour it improves the colour of food products increasing its acceptance by the consumer [35]. The high level of free amino acids may be considered another advantage of this flour.

Pulse flour (such as pea and chickpea) is naturally gluten-free and is a source of protein for healthy food innovation [36]. The sum of free amino acids in the pea and chickpea flours (Table 3) is noticeably higher in the first one. In the case of chickpea (sample 12), the determined value

Table 2 The experimental results of sum of FAAs ($X \pm SD$) determination in pea and wheat flours

Experiment	1	2	3	4	5	6	7	8	9
Wheat flour (mg 100 g^{-1})	$48.4^{a} \pm 2.48$	$51.7^{a} \pm 2.04$	$55.9^{b} \pm 1.05$	$68.0^{c} \pm 1.88$	$75.7^{d} \pm 3.91$	$93.1^{f} \pm 2.93$	$67.9^{c} \pm 2.36$	$86.3^{e} \pm 1.90$	$96.0^{f} \pm 1.96$
CV (%)	5.12	3.94	1.88	2.76	5.16	3.14	3.47	2.20	2.04
Recovery (%)	85.6	94.2	96.6	87.2	95.4	99.2	89.0	96.8	99.4
Pea flour (mg 100 g^{-1})	$151.8^{a} \pm 4.44$	$159.5^{b} \pm 4.25$	$166.8^{c} \pm 3.72$	$171.2^{c} \pm 4.63$	$197.1^{e} \pm 3.81$	$216.9^{g} \pm 3.31$	$185.4^{d} \pm 3.95$	$201.7^{e} \pm 4.15$	$208.2^{f} \pm 3.22$
CV (%)	2.92	2.67	2.23	2.70	1.93	1.52	2.13	2.05	1.55
Recovery (%)	83.2	93.7	95.1	89.9	97.4	99.6	93.3	98.8	99.6

Recovery was calculated as $[((X_2 - X_0)/X_1) \cdot 100\%]$, where X_0—concentration found in the blank sample, X_2—concentration found in spiked sample, X_1—added concentration of amino acids

$X \pm SD$ mean value ± standard deviation, *CV* coefficient of variation

Table 3 The sum of FAAs in tested flours by HPLC with ninhydrin procedure

Sample	1	2	3	4	5	6	7	8	9	10	11	12	13
$X \pm SD$ (mg 100 g^{-1})	$80.9^{b,c} \pm 2.10$	$104^{f} \pm 3.03$	$170^{h} \pm 1.19$	$76.4^{b} \pm 1.01$	$227^{j} \pm 4.50$	$87.6^{d} \pm 1.30$	$162^{g} \pm 4.10$	$168^{h} \pm 2.45$	$82.6^{c} \pm 2.63$	$59.9^{a} \pm 1.30$	$216^{i} \pm 3.31$	$167^{h} \pm 1.68$	$93.1^{e} \pm 2.95$
CV (%)	2.56	2.91	0.70	1.32	1.98	1.48	2.53	1.46	3.18	2.17	1.53	1.00	3.17

$X \pm SD$ mean value ± standard deviation, *CV* coefficient of variation

Sample 1—corn flour, sample 2—oat flour, sample 3—soy flour, sample 4—rice flour, sample 5—pumpkin flour, sample 6—millet flour, sample, 7—peanut flour, sample 8—hemp seed flour, sample 9—buckwheat flour, sample 10—amaranth flour, sample 11—pea flour, sample 12—chickpea flour, sample 13—wheat flour

Different letters (a–m) within the same column indicate significant differences (one-way ANOVA and Duncan test, $p < 0.05$); sorted from the lowest to highest values, where "a" was the lowest

was similar to the one obtained for legumes flours: peanut (sample 7), soy (sample 3), and hemp (sample 8). However, all discussed flours were good sources of free amino acids when compared to wheat flour. Moreover, legume seeds are rich in carbohydrates, proteins, and amino acids, while some are exhibit specific colours, which increases their attractiveness for consumers [2]. Due to the poor solubility of native hempseed protein and sensory characteristics, this product is mainly used in mixtures with other flours. However, the obtained results suggest that this flour can provide a comparable amount of FAAs sum as legumes ones.

The corn, rice, millet, buckwheat flours were characterized by FAAs sum contents lower than wheat. However, the observed level of FAAs sum in these flours were similar and varied from 76.4 mg 100 g^{-1} (rice flour) to 87.6 mg 100 g^{-1} (millet flour). A slightly higher level of total free amino acids was reported for oat flour. This flour is attractive to the consumer due to their nutritional profile, lack of allergenicity and palatable flavour. In the case of discussed compounds, it was characterized by a higher level of FAAs sum than wheat, while significantly lower than pumpkin or legumes flours.

The precision of the proposed procedure, expressed as CV values, varied from 0.70% (soy flour, sample 3) to 2.91% (oat flour, sample 2) what pointed satisfactory value of this statistical parameter for the tested procedure. Based on the obtained results, one can conclude, the proposed procedure allows for a quick and simple analysis of the sum of free amino acids in flours samples.

Determination of FAAs in tested flours

The individual FAAs were determined by RP-HPLC after reaction with 2-chloro-1,3-dinitro-5-(trifluoromethyl)benzene (CNBF). According to Guo et al. [37] CNBF as a derivatisation agent exhibits satisfactory ultraviolet absorption. Moreover, multiple derivatives or by-products, during the reaction are not formed, except CNBF hydrolysis compound. Presented at Fig. S1 (Supplementary Materials) chromatogram revealed that Glu and Trp peaks were not satisfactorily resolved. Because this problem could disturb the separation of tested FAAs in flour samples, we have decided to analyse these two flour essential amino acids by capillary isotachophoretic method (ITP-ITP). This method is efficient in the analysis of amino acids in food samples due to the lack of a derivatisation stage and a simpler procedure of analysis than HPLC. In our previous paper, we have described the procedure of tryptophan determination in beer samples by ITP-ITP method [22]. The composition of the electrolytes (LE: 10 mM HCl + 1% HEC + BTP; TE: 10 mM EACA + AMPD), and the pH value were discussed in detail. The proposed electrolytes system permitted the determination of both amino acids (Fig S2A, Supplementary Materials) with good quality parameters. In the case

of flour samples, the glutamic acid zone was located close to other unknown sample components zone (Fig S2B, Supplementary Materials), and quantitative analysis was not favourable. To solve the problem, the same LE composition was applied, whereas 6-aminocaproic acid (EACA) in the TE was replaced by histidine. The latter resulted in selective separation of Glu with sufficient precision of RSH value (Fig S2C, Table S1, Supplementary Materials). This amino acid (pI = 3.11, $pK_{\alpha\text{-carboxyl group}} = 2.19$, $pK_{\alpha\text{-ammonium ion}} = 9.67$, $pK_{\text{side chain group}} = 4.25$) is easily separated by ITP, but described in the literature conditions indicated the necessity of electrolytes at an acidic or slightly acidic pH during separation process [38]. Determination of Glu in flour samples at pH ~ 9 was possible with good analytical parameters for the first time.

Both procedures were used to assess the quality of the tested flours regarding the content of selected amino acids. The flour samples were extracted according to the elaborated in this work conditions, and results are listed in Table 4. Exemplary chromatograms and isotachopherograms are presented in Supplementary Materials (Fig S1B and S2D).

In the case of isotachophoretic determination, the separation of tryptophan and glutamic acid in flour samples was satisfactory. The calculated coefficient of variation (CV) for Trp and Glu analysis in flours samples varied from 0.62% to 3.85% and from 0.21% to 4.98%, respectively, indicating reasonable repeatability of applied procedures.

Out of the studied free amino acids only selected: Met, Phe, Lys, Gly, His, Tyr, Ser, Val were determined in flours samples by HPLC-CNBF procedure. Moreover, due to the insufficient separation of Glu from Asp and Trp from Leu and Ile, these amino acids were determined, and concentration was expressed as the sum (Table S3, Supplementary Materials). Discussing obtained results, one can conclude that the proposed sample preparation method can be applied to determine selected free amino acids by HPLC and ITP-ITP. The main advantages of the proposed extraction procedure are: extraction yield, simplicity, flexibility and applicability in every laboratory.

Data in Table 4 indicate glutamic acid as the most abundant free amino acid in all tested flours (except chickpea flour). Glu is a non-essential amino acid however, at pH 7, dietary glutamic acid is transformed into glutamate and acts as an excitatory neurotransmitter [39]. The highest concentration was observed in pea (55.7 mg 100 g^{-1}), pumpkin (47.5 mg 100 g^{-1}), and soy (42.5 mg 100 g^{-1}) flours. In contrast, some amino acids such as Ala, Asn, Arg, Cys, Gln, Pro and Thr were not detected in the tested samples. The second most abundant was methionine which was found in corn, rice, pumpkin, millet, peanut, hemp seed, pea and wheat flours and its contents varied from 2.99 mg 100 g^{-1} for amaranth flour to 36.7 mg 100 g^{-1} for pea flour.

Noticeable differences between tested flours were observed for His: 2.97 mg 100 g^{-1} in millet flour and 28.3 mg 100 g^{-1} in pea flour, whereas significant amounts of this amino acid have been detected in pumpkin, hemp and soy flours. A similarly high level of free Val was found in peanut (12.3 mg 100 g^{-1}), pea (11.6 mg 100 g^{-1}), and chickpea (11.4 mg 100 g^{-1}) flours. The highest content of free Lys was noted for pea flour (3.76 mg 100 g^{-1}), whereas in wheat, millet and rice flours, this AA was not detected.

Among tested free amino acids, special attention should be paid to tryptophan. It is a precursor of serotonin, and the synthesis of this neurotransmitter in the brain depends on the availability of the respective dietary precursor [40, 41]. Obtained results indicate the presence of this amino acid in all flours. The most significant amount was found for soy, pumpkin and hemp seed flours (8.36, 7.86 and 6.01 mg 100 g^{-1}, respectively). In contrast, corn flour revealed the lowest content (1.33 mg 100 g^{-1}). In the case of free Phe, it was detected only in five flour samples; but, its significant amount was found in peanut and pumpkin flours (10.5 and 9.41 mg 100 g^{-1}, respectively). Statistically noticeable differences of Tyr content were noted for corn flour (0.94 mg 100 g^{-1}) and pumpkin flour (24.5 mg 100 g^{-1}), whereas the significant amounts of this amino acid were determined in chickpea and soy flours (23.5 and 22.8 mg 100 g^{-1}). Among the tested samples, hemp seed and pumpkin flours were characterised by the highest content of Gly, on the contrary to corn and rice flours. Analysis of Ser revealed a high level in peanut flour and a noticeable amount in soy and chickpea flours, which are acknowledged as good sources of this amino acid. In our study, we have noted similar amounts of Ser in millet and hemp flours. It is worth noting that this important AA was found in all gluten-free flours, except wheat flour.

The aspartic acid level was calculated as the difference between the sum of Glu + Asp (HPLC, Table S3, Supplementary Materials) and Glu concentration determined by ITP-ITP, and results are presented at Fig. 1.

The Asp content varied from 1.7 mg 100 g^{-1} (hemp seed flour) to 19.9 mg 100 g^{-1} (pea flour). This amino acid was the third most abundant FAAs in the oat flour, whereas it was the fourth in pea and pumpkin flours. In comparison, Ciesarová et al. [6] determined free amino acids in selected non-wheat flours, and the levels of aspartic and glutamic acids were as follows: 32–792 and 39–429 mg kg^{-1}, respectively. In the case of Leu and Ile, their calculated levels were low, whereas the highest content was observed for pea flour (Fig S5, Supplemenatry Materials).

Comparison of the tested gluten-free flours with wheat flour

Due to its unique baking performance, wheat flour is one of the most important raw materials in bread making [42].

Table 4 The mean value ± standard deviation (mg 100 g^{-1}) of individual AAs in tested flour by CNBF-HPLC and ITP-ITP procedures

Sample	Met*	Phe*	Lys*	Gly*	His*	Tyr*	Ser*	Val*	Trp**	Glu**
1	$10.1^{d} \pm 0.10$	nd	$1.53^{a} \pm 0.5 \cdot 10^{-2}$	$0.32^{a} \pm 0.8 \cdot 10^{-2}$	$4.81^{d} \pm 0.07$	$0.94^{a} \pm 0.04$	$1.83^{f} \pm 0.02$	$4.38^{b} \pm 0.08$	$1.33^{a} \pm 0.02$	$26.5^{e} \pm 0.15$
2	$7.88^{c} \pm 0.11$	nd	$1.81^{d} \pm 0.01$	$2.36^{e} \pm 0.09$	$12.8^{h} \pm 0.57$	$2.73^{c} \pm 0.09$	$0.89^{c} \pm 0.01$	$5.27^{c} \pm 0.10$	$3.21^{d} \pm 0.02$	$28.7^{f} \pm 0.06$
3	$18.0^{f} \pm 0.24$	$1.95^{c} \pm 0.04$	$2.54^{f} \pm 0.02$	$4.17^{g} \pm 0.09$	$21.6^{j} \pm 0.66$	$22.8^{h} \pm 0.62$	$3.68^{i} \pm 0.9 \cdot 10^{-2}$	$5.43^{c,d} \pm 0.11$	$8.38^{k} \pm 0.12$	$42.5^{h} \pm 0.69$
4	$8.29^{c} \pm 0.28$	nd	nd	$0.73^{b} \pm 0.01$	$4.46^{c} \pm 0.04$	$0.95^{a} \pm 0.7 \cdot 10^{-3}$	$0.44^{b} \pm 0.8 \cdot 10^{-2}$	$3.68^{a} \pm 0.2 \cdot 10^{-2}$	$2.83^{c} \pm 0.05$	$24.0^{c,d} \pm 0.93$
5	$34.8^{k} \pm 1.32$	$9.41^{d} \pm 0.24$	$1.79^{d} \pm 0.01$	$9.64^{j} \pm 0.11$	25.71 ± 0.35	$24.54^{j} \pm 0.26$	$1.57^{e} \pm 0.7 \cdot 10^{-2}$	$8.52^{g} \pm 0.03$	$7.86^{j} \pm 0.06$	$47.5^{i} \pm 1.00$
6	$15.5^{e} \pm 0.66$	nd	nd	$1.39^{c} \pm 0.9 \cdot 10^{-2}$	$2.97^{a} \pm 0.02$	$3.67^{d} \pm 0.07$	$3.24^{h} \pm 0.02$	$8.17^{f} \pm 0.03$	$2.83^{c} \pm 0.07$	$28.8^{f} \pm 0.53$
7	$25.3^{i} \pm 0.87$	$10.5^{e} \pm 0.04$	$1.83^{d} \pm 0.02$	$2.62^{f} \pm 0.8 \cdot 10^{-2}$	$13.2^{i} \pm 0.51$	$16.8^{g} \pm 0.60$	$10.6^{l} \pm 0.48$	$12.3^{j} \pm 0.16$	$2.34^{b} \pm 0.09$	$27.9^{f} \pm 0.45$
8	$20.8^{h} \pm 0.22$	$1.17^{b} \pm 0.06$	$1.66^{c} \pm 0.01$	$13.7^{k} \pm 0.16$	$24.7^{k} \pm 0.08$	$16.7^{g} \pm 0.13$	$4.31^{k} \pm 0.08$	$8.61^{g} \pm 0.06$	$6.01^{i} \pm 0.01$	$32.5^{g} \pm 0.98$
9	$4.38^{b} \pm 0.02$	nd	$1.91^{e} \pm 0.02$	$9.68^{j} \pm 0.13$	$11.8^{g} \pm 0.39$	$2.03^{b} \pm 0.07$	$1.51^{d} \pm 0.03$	$5.95^{d} \pm 0.03$	$4.22^{g} \pm 0.08$	$22.8^{b,c} \pm 0.83$
10	$2.99^{a} \pm 0.02$	nd	$1.78^{d} \pm 0.01$	$2.76^{f} \pm 0.02$	$3.44^{b} \pm 0.10$	$0.49^{a} \pm 0.6 \cdot 10^{-2}$	$0.40^{a} \pm 0.01$	$5.62^{d} \pm 0.05$	$3.52^{e} \pm 0.07$	$19.6^{a} \pm 0.47$
11	$36.7^{l} \pm 1.70$	nd	$3.76^{g} \pm 0.08$	$6.34^{i} \pm 0.8 \cdot 10^{-2}$	$28.3^{m} \pm 0.01$	$8.38^{f} \pm 0.7 \cdot 10^{-2}$	$2.68^{g} \pm 0.2 \cdot 10^{-2}$	$11.6^{i} \pm 0.5 \cdot 10^{-2}$	$4.23^{g} \pm 0.09$	$55.7^{j} \pm 1.61$
12	$31.2^{j} \pm 1.30$	$0.78^{a} \pm 0.6 \cdot 10^{-2}$	$1.58^{b} \pm 0.3 \cdot 10^{-2}$	$1.93^{d} \pm 0.7 \cdot 10^{-2}$	$9.88^{f} \pm 0.32$	$23.5^{i} \pm 0.91$	$3.89^{j} \pm 0.10$	$11.4^{h} \pm 0.07$	$4.78^{h} \pm 0.07$	$21.5^{b} \pm 0.29$
13	$19.6^{g} \pm 0.07$	nd	nd	$4.42^{h} \pm 0.10$	$7.52^{e} \pm 0.24$	$5.85^{e} \pm 0.10$	nd	$6.34^{e} \pm 0.07$	$3.84^{f} \pm 0.09$	$24.7^{d} \pm 1.23$

Different letters (a–k) within the same column indicate significant differences (one-way ANOVA and Duncan test, $p < 0.05$); sorted from the lowest to highest values, where "a" was the lowest

Sample 1—corn flour, sample 2—oat flour, sample 3—soy flour, sample 4—rice flour, sample 5—pumpkin flour, sample 6—millet flour, sample 7—peanut flour, sample 8—hemp seed flour, sample 9—buckwheat flour, sample 10—amaranth flour, sample 11—pea flour, sample 12—chickpea flour, sample 13—wheat flour

*Amino acids determined by HPLC-CNBF method; **amino acids determined by ITP-ITP method

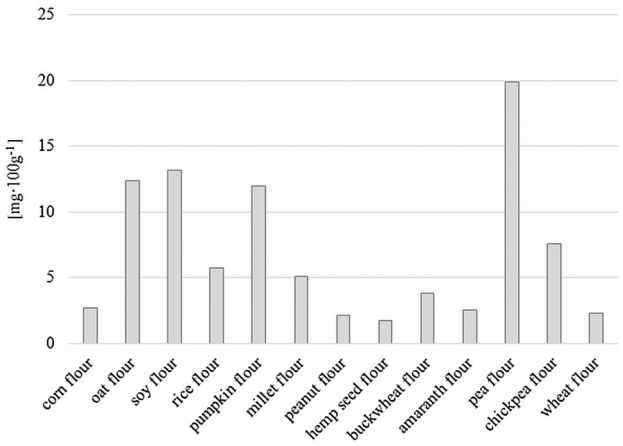

Fig. 1 The content of aspartic acid (expressed as mg 100 g^{-1}) in tested flours

The gluten-free flours are rarely used in bakeries due to their properties of proteins, and sensory characteristics (appearance, colour, smell, taste). Therefore, the majority of the discussed flours are used as an additive or supplementation component in bakery products. However, other components of gluten-free flours, such as vitamins, minerals, dietary fibre, and antioxidants, are nutritionally important, and there are bigger amounts than in wheat flour. In summary, one can conclude the significantly higher level of FAAs sum was determined for pumpkin, pea, soy, peanut, hemp seed, and chickpea flours than in wheat flour (Table 3). However, corn, rice, millet, buckwheat and amaranth flours exhibited similar or slightly lower contents of FAAs sum. These compounds are essential due to their many biological roles. Free amino acids are absorbed faster and assimilated more efficiently, are the building blocks of peptides and proteins, and are key precursors for the synthesis of low-molecular-weight nitrogenous molecules [43]. Surprisingly, these data for amaranth flour (sample 10) were the lowest among the tested samples. The latter suggests that despite the numerous health and functional benefits, amaranth flour is not a good source of free amino acids.

In the case of individual FAAs, significant diversity between tested gluten-free flours was observed. Usually, Lys and Trp are limiting amino acids in wheat and other cereals (such as corn, oats and rice) [4, 19, 36]. However, the determined free Trp value for wheat flour (3.84 mg 100 g^{-1}) was higher or similar to the one obtained for corn, oat, rice, millet, peanut and amaranth flours. Free Lys were found in almost all tested gluten-free flours (except rice and millet). An amino group of Lys is susceptible to the Maillard reaction; hence considerable content of this amino acid is lost during the baking processes.

The food taste determines customer selection decisions on food purchase. Free amino acids can contribute to the flavour and taste of protein-rich foods; however, the taste intensity of amino acids is dependent on the hydrophobicity of the side chain. Glu is taste-active amino acid contributing to umami taste, and it is one of the primary amino acids in wheat [19]. A noticeably higher amount of this amino acid, compared to wheat flour, was found in pea, pumpkin and soy flours. A similar situation was noted for the second umami amino acid (Asn), followed by oat flour. The noticeably higher level of Val and Phe (bitter amino acids) was detected in peanut flours, whereas Ile + Leu in pea one. In contrast, only four flours: pumpkin, hemp seed, buckwheat and pea, showed a higher Gly (sweet amino acid) content than wheat one. It should be noted that discussed data relate to the raw products, and their value may change significantly after heat treatment. Free amino acids play an essential role in the sweetness, sourness, bitterness and umami taste. They undergo important changes during processing and storage, and therefore the different food technologies also have an impact on AAs profiles. The FAAs participate in the Maillard reaction during high-temperature processing, resulting in the formation of colour, aroma, and flavour compounds as well as contaminants, including acrylamide. Moreover, the level of FAAs in food and their loss during the processing or storage is interesting in relation to nutritional aspects.

Conclusion

The sample preparation method elaborated in this study is based on the optimised conditions (20 mL of 1% TCA + 5 mL of EtOH, 60 min of stirring time) appeared to be effective for the determination of FAAs (sum and individual) in flours samples. The procedure is relatively simple and convenient with acceptable quality parameters. Moreover, a rapid and straightforward method for the determination of free amino acids sum using ninhydrin pre-column derivatisation was proposed. The method appeared to be simple, precise, and reliable with a satisfactory limit of quantification. Additionally, individual free amino acids were determined in selected flour samples using the proposed extraction procedure and HPLC and ITP methods. To the best of our knowledge, the determination of Trp and Glu in flours samples by ITP-ITP method was not reported.

The discussed procedures were applied for the determination of FAAs in selected gluten-free flours, and obtained results were compared to the wheat flour. Among the tested flours pea, pumpkin, soy, peanut, hemp seed, chickpea and oat flours were characterised by a noticeably higher content of FAAs sum in comparison to wheat one. The latter suggests the potential usage of flours as a good source of these compounds for food. However, the level of individual free

amino acids was varying and further studies are foreseen to study the relationship between FAAs in raw flours and after thermal treatment.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Consent to participate Not applicable.

Consent for publication All authors have read and approved this version of this article, and all authors consent to the publication of the-manuscript.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors. No ethical approval was required.

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