DETERMINATION OF THE PRESENCE OF BUCKWHEAT FLOUR IN BREAD BY THE ANALYSIS OF MINOR FATTY ACID METHYL ESTERS*

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Five samples of bread were prepared by mixing wheat and buckwheat flour. The buckwheat flour content was raised from 0, 20, 40, 50 to 60%. Fatty acids were extracted with hexane from crusts and crumbs of bread, and afterwards derivatised with TMSH (trimethylsulfonium hydroxide in methanol) into corresponding methyl esters and subjected to the analysis on a GC-MS device. Only the areas of methyl esters of the minor fatty acids: eicosenoic (C20:1), eicosanoic (C20:0) and docosanoic (C22:0) acid, were integrated. Multivariate analysis was applied for data processing. The results show that it is possible to determine the presence of buckwheat flour in crusts and crumbs of bread, successfully avoiding the need for quantitative determinations.

Keywords: buckwheat, bread, methyl esters of minor fatty acids, GC-MS, multivariate analysis

Introduction

In healthy people nutrition bread should comprise 25% of the energy required per day, which indicates that bread is the most common and most abundant quantity of foods in the daily diet of people, and that it should have good nutritional properties.

The development of food technology has enabled the production of a large number of different types of bread and bakery products. Under a multitude of different types of bread and bakery products the question raises - what is the best choice for the maintenance and promotion of the consumers' good health? Some manufacturers of bread and bakery products labeled certain types of bread and bakery products as functional food.

Bakery industry most commonly uses wheat flour. The dough produced from wheat flour is a viscoelastic system in which the protein net plays a crucial role, which further influences the properties of the mixing procedure and the textural characteristics of the final product as well. The main protein component responsible for the resistance of the protein network of the wheat flour mixture is gluten. Gluten mainly consists of gliadin and glutenin [1].

Buckwheat is a pseudocereal which does not contain gluten, a protein essential for the development of the gluten network in dough, which provides the structure, texture and volume of baking products. Therefore, the gluten-free buckwheat flour cannot produce a dough that has the elasticity and plasticity, but it can be used for the production of baked goods in a mixture with one or more types of cereal flour.

The level of incorporation of buckwheat flour in a bread formulation depends on the type of product and is limited by, among other things, rheological properties of the dough that is produced, and the sensory profile of the final product. Acceptability of buckwheat based products by the consumers is influenced by the texture and taste of these products.

Buckwheat seed is very rich in nutrients. Most of them are carbohydrates (about 73%), protein (11.7%) and vegetable oil (2.4%). The energy value of 100 g of edible part of the grain is 1407 kJ or 335 kcal. Until recently, this valuable food was neglected in the diet. However, nowadays it is becoming more and more important because it does not contain gluten, which is an integral part of most cereals i.e. bread, primarily [2,3].

Current literature reveals restrictions on the proportion of buckwheat flour in the formulation of bakery products, because it is affecting sensory and functional properties of the final product. Some authors [4] state that enrichment of white flour bread with whole grain buckwheat flour to the level of 15% leads to the reduction in a specific volume and some bread sensory characteristics (taste and feeling that the product produces in the mouth), and that the loaf volume containing 50% of buckwheat flour decreases as compared to control bread. Therefore, in determining the optimal level of buckwheat flour in bread formulation, it is necessary to consider rheological properties of the mixture containing buckwheat flour and sensory characteristics of

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the final product. It is also necessary to take into account the level of enrichment of the final product with functional components from buckwheat flour.

Buckwheat seed contains 1.5-4% of total lipids, and the fat content in buckwheat flour is greater than 3%. Free lipids isolated from buckwheat seeds make up 2.5%, and bound lipids around 1.3% of the dry matter. The exact compounds that constitute free and bound fraction of lipids of buckwheat seeds can be found in Smietana et al. [5]. Bound lipids in the buckwheat seeds and lipids in starch differ in the content of dominant and minor fatty acids. Linoleic (18:2), oleic (18:1) and palmitic acid (16:0) account for 88% of the total fatty acids of buckwheat seeds [6-8].

The lipid content of buckwheat seeds varies depending on the variety of buckwheat, a site on which it is grown, but the differences may also occur due to a different growing season [9]. The content of fatty acids (dominant and minor) in different types of buckwheat (common, tartaric and new buckwheat) and wheat are shown in Table 1. The dominant fatty acids in buckwheat seeds are unsaturated fatty acids (18:1 and 18:2) and saturated fatty acids (16:0 and 18:0) [10,11]. Minor fatty acids are: eicosanoic (arachidic 20:0), eicosenoic (C20:1) and docosanoic (behenic, 22:0).

Table 1. Dominant and minor fatty acid content of buckwheat and wheat grains (g/100 g of total fatty acids)

Fatty Acids	Ref. [9] Common Buckwheat	Ref. [9] Tartaric Buckwheat	Ref. [6] New Buckwheat	Ref. [10] Wheat
Palmitic (C16:0)	15.6	19.7	17.7	31.2
Stearic (C18:0)	2.0	3.0	1.8	1.9
Oleic (C18:1)	37.0	35.2	36.8	10.7
Linoleic (C18:2)	39.0	36.6	38.1	25.6
Linolenic (C18:3)	1.0	0.7	2.7	2.0
Eicosanoic Arachidic (C20:0)	1.8	1.8	1.1	0.3
Eicosenoic (C20:1)	2.3	2.0	n.d.	0.9
Docosanoic Behenic (C22:0)	1.1	0.8	1.6	1.2

Ref.-reference; n.d. – no data

In previous studies [12,13] it has been proven that it is possible to unambiguously distinguish buckwheat flour from wheat flour, based on GC-MS and multivariate analysis of characteristic ions of (1) simple sugars in alcoholic extracts of flour samples, and (2) fatty acid methyl esters and non-saponifiable components in fat-soluble (hexane) extracts of flour samples. Previous research has also shown that it is possible to determine the content of buckwheat flour in crusts and crumbs of bread samples with addition of buckwheat flour, based on the content of simple sugars and methyl esters of dominant fatty acids [14,15].

The aim of this study was to determine whether the application of gas chromatography and multivariate analysis can differentiate wheat flour bread samples from the samples of bread made with the addition of buckwheat

flour, by forming a correlation dendrogram of methyl esters of the three minor fatty acids. Due to the processes that take place during bread baking, it is necessary to perform a chemical analysis of both crumbs and crusts of bread samples. The content of buckwheat flour in bread was raised from 0, 20, 40, 50 up to 60%.

Experimental

Five loaves of wheat flour bread were made three times each, wherein the proportion of buckwheat flour was raised from 0, 20, 40, 50 to 60%. The following raw materials were used for making five bread samples: dark buckwheat flour (ash content 2.1%, moisture 9.7%; Tehnohemija, Novi Sad, Serbia), wheat flour T-500 (ash content 0.52%, moisture 1.5%; DEM, Kulpin, Serbia), yeast, salt, and a bread dough improver (sodium bicarbonate, disodium dihydrogen pyrophosphate, monocalcium phosphate; Centropoizvod, Beograd, Serbia). Analyzed crusts of the bread samples are labeled: K-1 (crust of pure wheat flour bread); K-2 (crust of bread with 20% buckwheat flour); K-3 (crust of bread supplemented with 40% buckwheat flour); K-4 (crust of bread with 50% buckwheat flour); K-5 (crust of bread supplemented with 60% buckwheat flour). Analyzed crumbs of bread samples are labeled: S-1 (crumb of pure wheat flour bread); S-2 (crumb of bread supplemented with 20% buckwheat flour); S-3 (crumb of bread supplemented with 40% buckwheat flour); S-4 (crumb of bread supplemented with 50% buckwheat flour); S-5 (crumb of bread supplemented with 60% buckwheat flour).

A sample of bread (1 g) was weighed and placed in the centrifuge tube of 12 mL. The volume of 5 mL of n-hexane was added to a vial and stirred using Vortex for 2 minutes. The content is centrifuged at 2000 r/min for 5 minutes. The clear supernatant was taken in the amount of 3 mL and transferred into a beaker of 10 mL and allowed to evaporate at room temperature. The oily residue was taken in the amount of 10 μ L, reconstituted in 400 μ L of methanol and afterwards 100 μ L of reagent for transesterification was added: trimethylsulfonium hydroxide (TMSH) in 0.2 M methanol (Macherey-Nagel). With this transesterification reagent fatty acids esterified from acyl-glycerols into corresponding methyl esters in the gas chromatograph injector [16].

All tests were conducted on the gas chromatography-mass spectrometry system (GC-MS) (Agilent Technologies 7890), coupled with the MSD 5975 equipment (Agilent Technologies, Palo Alto, CA, USA), working in EI mode at 70 eV. A DB-5 MS column was used (column dimensions: length 30 m, internal diameter 0.25 mm and film thickness 25 μ m). The temperature program was: 50 to 130 $^{\circ}$ C at 30 $^{\circ}$ C/min and 130 to 300 $^{\circ}$ C at 10 $^{\circ}$ C/min. Injector temperature was 250 $^{\circ}$ C. The carrier gas flow (helium) was 0.8 mL/min. Split ratio 1:50 was used for the injection of 1 μ L of each sample. Six replicates were performed for each sample, and the extreme deviating values were discarded. The elution order of analyzed

minor fatty acid methyl esters was as follows: first minor fatty acid methyl ester at 15.8 min (eicosenoic acid methyl ester, C20:1, match 99%), then eicosanoic acid methyl ester (C20:0, match 97%) at 15.999 min, and finally docosanoic acid methyl ester (C22:0, match 96%) at 18.312 min.

Agilent MSD Productivity ChemStation software with WILEY 275 mass spectra library was used for the mass spectra analysis and identifications of minor fatty acids by using a probability-based matching algorithm.

PAST program ver. 2.17c was used for statistical analysis of the mean values of the obtained data [17]. Hierarchical cluster analysis is a complementary, nonlinear, and widely used method for cluster analysis, with the result represented by a dendrogram. Cluster analysis of automatically integrated surface areas of methyl esters of detected minor fatty acids was performed [18]. Single linkage algorithm and correlation similarity measure gave the highest values of cophenetic correlation coefficient (0.9028) and thus present the dendrogram very faithfully.

Results and Discussion

The SIM (Selected Ion Monitoring) chromatograms of fatty acid methyl esters of crusts and crumbs of bread, in Figures 1 and 2, were separated from TIC (Total Ion Chromatogram) by selecting the characteristic fragmentation ion 74 Da. In Figures 1 and 2, the overlaid SIM chromatograms of crusts and crumbs hexane extracts of five samples of bread are shown. Figures 1A and 2A show the chromatograms of crusts and crumbs hexane extracts from 7 minutes. Methyl esters of the dominant fatty acids elute between 13 min and 15 min in the following order: C16:0 (Palmitic), C18:2 (Linoleic), C18:1 (Oleic) and C18:0 (Stearic) [15]. As it can be observed from the Figures, the elution order of minor fatty acid methyl esters is: C20:1 (Eicosenoic), C20:0 (Eicosanoic) and C22:0 (Docosanoic). Figures 1B and 2B show the enlarged parts of SIM chromatograms where minor fatty acid methyl esters, in hexane extracts of crusts and crumbs of bread, elute.

Figure 1. A - Overlaid SIM chromatograms of bread crusts with different buckwheat flour contents (K-crust); B - Enlarged overlaid SIM chromatograms with methyl esters of minor fatty acids in bread crusts with different buckwheat flour contents

Figure 2. A - Overlaid SIM chromatograms of bread crumbs with different buckwheat flour contents (S-crumb); B - Enlarged overlaid SIM chromatograms with methyl esters of minor fatty acids in bread crumbs with different buckwheat flour contents

As shown in the obtained chromatograms, the similarities between both crusts and crumbs of the samples of bread are very high. The peaks of minor fatty acid methyl esters exclusively were integrated and the corresponding numeric values introduced into multivariate analysis. Multivariate analysis is suitable for quick identification of fundamental differences between the samples. In this way, the application of analytical standards and calibration curves was successfully avoided, thus simplifying the entire analysis procedure significantly. Many types of similarity measures were investigated such as Manhattan, Rho, Horn and Euclidean, but Correlation similarity measure gave the best distinction between analyzed crusts and crumbs of bread samples made from wheat and buckwheat flour mixtures in specified proportions.

Figure 3A shows the dendrogram of crusts and crumbs of all investigated bread samples. It is obvious that the crust and crumb samples of pure wheat bread (K1 and S1) can be clearly differentiated from crust and

crumb samples made of wheat and buckwheat flour mixtures, which present high similarities among each other (more than 97%).

The dendrogram presented in Figure 3B is obtained by eliminating crust and crumb samples of pure wheat flour, to show the separations between the rest of the samples more clear and noticeable. Both samples of crusts and crumbs are ordered according to the growing content of buckwheat flour in bread flour mixtures (from 20, 40, 50 to 60%). The crust sample K2 can be differentiated from other crust samples (K3, K4 and K5), which strong similarities present. On the other hand, crumb samples S2 and S3 can be separated from S4 and S5, which also present high values of similarity measures among each other.

Figure 3. Dendrogram of crusts and crumbs of bread: A - obtained by the analysis of each bread sample; B - obtained by the elimination of bread samples made of pure wheat flour (K1 and S1)

List of Symbols and Abbreviations

GC-MS Gas chromatography with mass spectrometry

TIC Total ion chromatogram

SIM Selected ion monitoring

TMSH Trimethylsulfonium hydroxide

K Bread crusts

S Bread crumbs

Conclusion

In this paper, it is shown that the analysis of methyl esters of minor fatty acids can quite reliably distinguish the samples of crusts and crumbs of pure wheat flour bread from the samples of bread with 20, 40, 50 and 60% of buckwheat flour, which in most cases show strong similarities among each other.

The preliminary results obtained in this study are in accordance with the results, which indicate that other small molecules (suitable for the analysis by GC-MS system, such as simple sugars) can be used for the determination of buckwheat flour in bread [14], with the application of multivariate analysis of GC-MS data, without performing quantitative determinations.

It should also be noted that there is another very important advantage of using GC-MS system in these investigations. The transesterification reaction of triglycerides into methyl esters of fatty acids occurs directly in the injector of the gas chromatograph. The laboratory preparation of methyl esters from corresponding fatty acids, beginning with triglycerides, is thus avoided.

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Izvod

ODREĐIVANJE PRISUSTVA HELJDE U HLEBU ANALIZOM METIL ESTARA MINORNIH MASNIH KISELINA

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Pet uzoraka hleba je pripremljeno mešanjem pšeničnog i heljdinog brašna. Udeo heljdinog brašna je povećavan od 0, 20, 40, 50 do 60%. Ekstrakcijom sa heksanom iz kore i sredine hleba su ekstrahovane masne kiseline, koje su derivatizovane sa TMSH (trimetilsulfonijum-hidroksid u metanolu) i podvrgnute analizi na GC-MS uređaju. Integrisana je površina metil estara isključivo minornih masnih kiselina: eikozenske (C20:1), eikozanske (C20:0) i dokožanske (C22:0). Pri obradi rezultata primenjena je multivarijantna analiza. Dobijeni rezultati pokazuju da je moguće ustanoviti prisustvo heljde u kori i sredini hleba, bez potrebe za primenom kvantitativne analize.

Ključne reči: heljda, hleb, metil estri minornih masnih kiselina, GC-MS, multivarijantna analiza