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Analysis of betaine levels in cereals, pseudocereals and their products

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ABSTRACT

Betaine has a range of health benefits and therefore has been recommended as a functional ingredient in dietary supplements. The main dietary sources of betaine are processed grains such as bread, biscuits, cereals, pasta and similar products. This study describes analysis of 54 samples of cereals and pseudocereals for betaine content by using HPLC – ELSD method. By using this approach betaine levels were identified, quantified and compared. Analysis of variance showed significant differences between analyzed samples (from < LOQ mostly in gluten free products to 328.5 mg/100g DM in enriched plain biscuit with molasses). PCA analysis gave two large clusters, one for gluten-free samples and the second cluster containing all of the remaining samples. As a final result the average betaine levels in analyzed food samples were in the following order: buckwheat < millet < wheat < oats < rye < barley < amaranth < spelt.

KEYWORDS: betaine, HPLC-ELSD, cereals, pseudocereals

Chemical compound studied in this article:

Betaine (PubChem CID: 247)

Abbreviations used: HPLC-ELSD, high performance liquid chromatography- with evaporative light scattering detector; HPLC-UV, high-performance liquid chromatography with ultraviolet spectrometric detection; HILIC, Hydrophilic Interaction Chromatography; NP, normal-phase; RP-LC, reversed-phase liquid chromatography; ANOVA, Analysis of variance; PCA, Principal component analysis; SS, standard score; LOQ, the limit of quantitation; LOD, the limit of detection

1. Introduction

Although betaine is known as a non-essential nutrient, numerous studies in recent years reported a wide range of its health benefits (Craig, 2004; Schwahn, et al., 2003). For these reasons betaine is used as functional ingredient and dietary supplement (Filipčev et al., 2016). Betaine is considered as GRAS ingredient in the US, while in Europe, it has an approval for use in foods by the European Commission (Commision Regulation EU 432, 2012). Chemically, betaine (N, N, N -trimethylglycine) is a zwitterionic compound at neutral pH with dual function in the human organism: as an osmolyte and as a methyl donor. Betaine participates in the methionine cycle primarily in the human liver and kidneys by acting as methyl group donor required for the formation of methionine and S-adenosylmethionine (SAM) (Craig, 2004; Schwahn, et al., 2003; Zwart et al., 2003; Ross et al., 2014). Choline and betaine are important sources of one-carbon units, in particular, during folate deficiency (Ueland, 2011). By providing the one-carbon units, betaine also enables the conversion of homocysteine to methionine, conserves methionine, detoxifies homocysteine, and produces S-adenosylmethionine which is currently used successfully to treat liver disease (Craig, 2004; Lever et al., 2010; Barak et al., 1996). Zhang et al. (2016a) reported that betaine inhibits hepatitis B virus (HBV) and the antioxidant activity of betaine was confirmed by the same author (Zhang et al., 2016b). Recently, experiments in rats fed high fat diet and supplemented with 1% betaine resulted in anti-steatotic activity of betaine (Ahn et al., 2015).

As a dietary component of many foods, betaine is present at different concentrations, depending on the source and processing conditions. It has primarily been isolated from sugar beet, nowadays the major source of betaine in the Western diet are cereal based foods (Zwart et al, 2003; Likes et al., 2007; Ross et al., 2014; Gao et al., 2016). Slow et al. (2005) found high

68 levels of betaine in grain products such as bread, pasta and flour, ranged from 360 μg/g in white
69 bread to 7200 μg/g in cereal bran. Other sources of betaine are shellfish, shrimps, chicken, as
70 well as plant sources such as beetroot and spinach (members of the beet family) (Zwart et al.,
71 2003; Filipčev et al., 2015). A US Department of Agriculture database of the choline and betaine
72 content of food has been developed by Zeisel et al. (2003). They found highest betaine
73 concentration (mg/100 g) in: wheat bran (1339), wheat germ (1241), spinach (645), pretzels
74 (237), shrimp (218) and wheat bread (201). Since most of betaine sources have rather complex
75 matrices, the isolation and characterization of betaine could be a promising area of research.

76 Different extraction procedures have been performed for extraction of betaine from
77 different food matrices. The most commonly used solvent for extraction of betaine is water
78 (Zwart et al., 2003; Ross et al., 2014; Slow et al., 2005; Bruce et al., 2010; Hefni et al., 2016).
79 Hefni et al. (2016) reported strong impact of extraction conditions on the quantified betaine
80 content in different foods, demonstrating the necessity of repeating the extraction procedure to
81 obtain reliable results. In order to determine the betaine content in food, different methods have
82 been developed. The most of them are based on using liquid chromatography. However, there is
83 no a universal method which could be applied to all food matrices. Saarinen et al. analyzed
84 betaine in chicken liver using a cation exchange column of Ca^{2+} type and refractive index
85 detector although quantification is limited because of poor detection sensitivity (Saarinen et al.,
86 2001). Considering its physicochemical properties, this quaternary amine could not be analyzed
87 by the conventional reversed-phase high performance liquid chromatography, and could not be
88 detected with UV detector without derivatization. Zwart et al. (2003) derivatized wide range of
89 foods commonly found in the western diet and betaine analysis was performed by high-
90 performance liquid chromatography with standard ultraviolet spectrometric detection (HPLC-

91 UV) using different columns. Slow et al. (2005) extracted betaine in different products grouped
92 into 10 food categories: grains, fruit, vegetables, beverages-nonalcoholic, beverages-alcoholic,
93 meat, seafood, dairy products, nuts and miscellaneous using water and dichloromethane, and the
94 extracts were derivatized with 2-naphthacyl trifluoromethanesulfonate. Hefni et al. (2016)
95 developed a simple HPLC-UV method for betaine determination in several different food
96 matrices such as fresh spinach, whole wheat flour, wheat (*Triticum aestivum*), beet (*Beta*
97 *vulgaris*), etc. after derivatization on strong cation exchange column. Bruce et al. (2010) and
98 Ross et al. (2014) performed the analysis using LC-MS/MS coupled with an HILIC column.
99 Bruce et al. (2010) developed LC-MS/MS method for the analysis of 47 plasma samples, 32
100 cereal flours and cereal fractions, and 51 cereal products. Additionally, Ross et al. (2014)
101 analyzed betaine by liquid-chromatography with tandem mass spectrometry in a wide range of
102 commercially available cereal foods and cereal fractions. Du Shin et al. (2012) proposed HILIC
103 column in combination with evaporative light scattering detector (ELSD) for betaine analysis in
104 *Fructus* Lycii. Recently, Hydrophilic Interaction Chromatography (HILIC) is an alternative to
105 reversed-phase liquid chromatography (RP-LC). HILIC is a type of normal-phase (NP)
106 chromatography because it has the same polar stationary phase, but can use large amounts of
107 organic solvent (> 80%) as the mobile phase compared to NP. As such, the HILIC column was
108 more effective for the chromatographic separation of betaine (Buszewski et al., 2012).

109 The main aim of this study was to establish betaine content in 54 samples of cereals and
110 pseudocereals.In order to analyze betaine levels we have modified a method previously used by
111 Du Shin et al. (2012). This included a change from gradient to isocratic mode which resulted in
112 shortened sample elution time. Additionally, optimization of pH value of mobile phase and

vortex extraction time has been performed. By using this methodology betaine levels were analyzed and compared.

2. Materials and methods

2.1. Chemicals and reagents

Anhydrous betaine was used as an internal standard (98% purity, AlfaAesar GmbH&KG, Karlsruhe, Germany). Acetonitrile and methanol UHPLC grade were purchased from PanReac AppliChem (Barcelona, Spain). 10 mM ammonium acetate buffer was prepared using ammonium acetate (99% purity, Lach-Ner, Neratovice, Czech Republic) and the ultrapure water, which was produced by a Simplicity UV system from Millipore (Bedford, MA, USA). The pH was adjusted to target value by using concentrated acetic acid or a diluted ammonium hydroxide solution and finally buffer was filtered through a membrane of 0.45 μm (Millipore) into a measuring flask.

2.2. Sample collection and preparation

The majority of the food samples analyzed in this study have been obtained from the local market and food stores in Novi Sad (Serbia). Wheat grain (*Triticum aestivum*), all durum wheat (*T. durum*), triticale (*Triticosecale)*, barley (*Hordeum vulgare*), and rye (*Secale cereale*) samples were obtained from the collection of samples of the Laboratory of the Institute of Food Technology. Amaranth grain and related samples were provided from the local producer. Ground and homogenized sample (2 g) was weighted and suspended in methanol (25 mL) and vortexed for 10 min. After a 30 min of ultrasonic extraction in an ultrasonic bath (ATU Ultra-sonidos, Valencia, Spain), the sample was vigorously shaken and centrifuged for 10 min at 5000 r/min (Eppendorf Centrifuge 5804R, Eppendorf, Wien, Austria). Upper methanol layer (3 mL) was evaporated to dryness. Afterwards, the residue was reconstituted in 2 mL of water and filtered

through a membrane filter (regenerated cellulose, pore size 0.22 μm, diameter 25 mm, Agilent Technologies, Santa Clara, USA).

2.3. Optimal chromatographic conditions

Betaine analysis was performed using a HPLC system (Agilent Technologies Inc., USA) equipped with a Kinetex®HILIC (Phenomenex, Aschaffenburg, Germany) column (2.6μm, 100 × 2.1 mm) and ELSD detector (1290 Infinity ELSD, Agilent Technologies, USA). Separation was performed at a flow-rate 0.5 mL/min with a mixture of acetonitrile and 10 mM acetate buffer at pH 3.7 (80:20, v/v) following isocratic regime. Total run time was 10 min. Injection volume was 5 μL using autosampler injection mode. The injector was at room temperature. Detector parameters were as follows: evaporator temperature 40 °C; nebulizer temperature 55 °C; gas flow rate 1.60 standard liter per minute (SLM), a photomultiplier tube (PMT) gain 3.0.

2.4. Method performance

2.4.1. Calibration curve and linearity

A test for the general matrix effect is performed by means of 'standard additions' or the method of analyte additions according to guidelines for validation of analytical methods (Huber et al., 2010). A calibration curve is prepared in the same biological matrix as the samples by spiking the matrix with known concentrations of the analyte. A calibration curve consists of a zero sample and five non-zero samples covering the expected range (0.05, 0.075, 0.1, 0.15, 0.20 mg/mL). The curve was constructed by plotting the peak area against the of six different concentration values. The linearity of calibration curves was expressed by the coefficient of determination (r^2).

156 *2.4.2. Limit of quantitation and detection*

157 The limit of quantitation (LOQ) is quantitatively determined by the analysis of same
158 samples as calibration curve with known concentrations of analyte and by establishing the
159 minimum level at which the analyte can be quantified with acceptable accuracy and precision.
160 Samples with increasing amounts of the analyte (0.01; 0.03; 0.05; 0.075; 0.1; 0.125; 0.15; 0.1875;
161 0.2 mg /mL) are injected six times and relative standard deviation (RSD) is calculated and plotted
162 against analyte amount. The LOQ thus corresponds to that concentration or amount of analyte,
163 quantifiable with a coefficient of variance not higher than 10% (Taverniers et al., 2004).The limit
164 of detection (LOD) was estimated by increasing same amounts of the analyte are injected six
165 times and measuring the response at a signal-to-noise ratio (S/N) of ≥3.

166 *2.4.3. Intra-and Inter-day Precision and Accurancy*

167 The intra-day precision and accuracy was assessed by analyzing six replicates of the same
168 samples at seven different concentration points (0.050, 0.075, 0.10, 0.125, 0.15, 0.175, 0.20
169 mg/mL) within one day, whereas the inter-day precision and accuracy were estimated by
170 analyzing one measurement at each of seven concentrations for six consecutive days. Accuracy
171 was expressed as the observed value's percentage of the true value. Precision was expressed as
172 the relative standard deviation (coefficient of variance, CV).

173 *2.4.4. Recovery*

174 The recovery assay was performed at three different concentration levels (0.02, 0.03, and
175 0.05 mg/mL). Each 2g of spelt wheat flour and wheat flour were spiked with three concentrations
176 of betaine standard and prepared as described above. The recovery (R) was calculated according
177 to the following equation:

178 $$R=(C_{found} - C_{sample})/C_{added} \quad (1)$$

179 where C_{found} is the measured content in the spiked sample, C_{sample} is the measured content
180 in the sample before spiking, and C_{added} is the added concentration.

181 *2.5. Statistical analysis*

182 Analysis of variance (ANOVA) and Tukey's HSD test were used to differentiate the
183 samples according to betaine content and the content of the unknowns in the HPLC
184 chromatographic area between 2.3 and 3.1minutes of retention time. Binary system was applied
185 in data processing of the experimentally obtained HPLC-ELSD chromatograms; the presence or
186 the absence of a particular peak was coded with either (1) or (0), in each sample. In order to
187 enable optimization of HPLC parameters between investigated runs, standard score (SS) has been
188 introduced. Principal component analysis (PCA) was used to find the possible similarities
189 between observed samples. Descriptive analysis of the data, as well as PCA, were performed
190 using the software package STATISTICA 10.0 (StatSoft Inc., Tulsa, OK, USA).

191 **3. Results and discussion**

192 *3.1. Optimization of sample extraction*

193 The most frequently used extraction solvent for betaine is water (Zwart et al., 2003; Ross
194 et al., 2014; Slow et al., 2005; Bruce et al., 2010; Saarinen et al., 2001). Thus, by using water as a
195 solvent for the extraction and subsequent HPLC analysis of betaine, other water soluble
196 compounds are released into the extract, causing a complex chromatogram. Chromatographic
197 peaks of carbohydrates often overlap and hide betaine peaks which makes the characterization
198 and quantification of the betaine impossible or difficult (Supplementary Fig. 1b). In order to

199 overcome problems with impurity and betaine peaks overlapping, pure methanol was used in this
200 study as extraction solvent and the obtained chromatogram was shown in Supplementary Fig. 1a.
201 Betaine has a low molar absorptivity in UV-visible region and therefore it is necessary to use
202 derivatization reagents. Instead of a conventional UV detector for the quantitative HPLC analysis
203 of betaine in order to avoid derivatization, evaporative light scattering detector (ELSD) detector
204 was chosen in this study, as universal detector which provides stable baseline even with a
205 gradient elution and can detect most of non-volatile analytes. All this reduces the complexity of
206 the overlapping chromatogram peaks, making betaine visible and its characterization and
207 quantification significantly easier and more accurate.

208 *3.2. Optimization of HPLC-ELSD method*

209 *3.2.1. Optimization of pH value of mobile phase and gradient using normalized standard scores*

210 In order to enable optimization of HPLC parameters, standard scores (SS) were evaluated
211 using chemometric approach by integrating the measured values of resolution and signal-to-noise
212 ratio during HPLC runs for pH and gradient optimization. Min-max normalization is one of the
213 most widely used technique in standard score evaluation to compare various characteristics of
214 HPLC runs which are ranked based on the ratio of raw data and extreme values of the
215 measurements (Monzón et al., 2016; Klein et al., 2014). The data in each data set used for pH
216 value optimization should be transformed into normalized scores, dimensionless quantity derived
217 by subtracting the minimum value from the raw data, and divided by the subtraction of maximum
218 and minimum value, according to following equation:

219 $$\overline{x}_i = DPI \cdot \left(\frac{x_i - \min\limits_i x_i}{\max\limits_i x_i - \min\limits_i x_i} \right), \quad \forall i \ , \tag{2}$$

220 which is “the higher, the better” criteria (used for resolution parameter and signal-to-noise ratio).

221 $\bar{x}_i$ - the normalized score for the ith pH measurement (xi); min and max - the extreme pH values;

222 $\forall i$ - for every ith pH measurement; DPI - double peak identification (O or 1);

223 If the double peak (DPI) of betaine is observed during the HPLC run, (due to the different ionic

224 forms) DPI is equal to 0, otherwise DPI is equal to 1.

225 The similar equation could be used for the optimization of the gradient:

226 $$\bar{x}_i = \frac{x_i - \min\limits_i x_i}{\max\limits_i x_i - \min\limits_i x_i}, \quad \forall i \qquad (3)$$

227 (also used for resolution parameter and signal-to-noise ratio).

228 $\bar{x}_i$ - the normalized score for the ith gradient (xi); min and max - the extreme gradient values; $\forall i$

229 - for every ith gradient.

230 The average of normalized scores of different HPLC parameters (resolution and signal-to-

231 noise) give a single unitless value termed as a standard score (SS), which is a specific

232 combination of data from different measuring methods with no unit limitation. Standard scores

233 for observed HPLC runs are calculated and the results are summarized in Supplementary Table 1

234 (for optimization of pH) and Table 1 (for optimization of gradient).

235 Standard score analysis used for optimization of pH mobile phase showed that the

236 optimum resolution and signal-to-noise ratio was obtained in Run 4, in which pH value of the

237 mobile phase was 3.7. The value of the resolution parameter (3.58) and signal-to-noise ratio

238 (10.5) indicated that a standard score for the optimal run was 0.58. Using this pH value, both

239 important parameters (S/N and resolution) simultaneously gained satisfactory values, without

240 double peaks. Run 6 was optimal for the gradient adjustment, with SS equal to 0.81, resolution of

241 3.22 and signal-to-noise ratio of 58.6. Using isocratic regime (80% ACN and 20% 10 mM acetate

242 buffer, performed in Run 6), the maximum resolution and S/N is achieved, and also the optimal
243 peak separation, Fig. 1). Fig. 2 showed separately betaine peak at optimal chromatographic
244 conditions which are applied in this work. Optimal function for gradient and mobile phase pH
245 value adjusting is shown in Supplementary Fig. 2.

246 *3.21.2. Optimization of vortex extraction time*

247 Optimization of vortex extraction time is achieved through the maximum value of the
248 peak area. Optimal vortex extraction time was obtained in run 14, with peak area of 2491.2
249 (isocratic regime: 80% ACN and 20% 10 mM acetate buffer; flow 0.5 mL/min; pH mobile phase
250 3.7, non-controlled T column and injection volume 5 μl) Optimal vortex time was achieved after
251 10 minutes, after which no increase of the area was observed, Supplementary Fig. 3.

252 *3.3. Method validation parameters*

253 *3.3.1. Calibration curve*

254 The betaine calibration curve was constructed ranging from 0.05-0.2 mg/mL. Linearity
255 range was established based on determination by a series of three injections of six standard
256 additions mixtures whose concentrations covered the expected concentration range of betaine in
257 the samples. Each analyte showed an excellent linear behavior over the set concentration range,
258 with correlation coefficient (r^2=0.9958, n=6). The prepared samples were analyzed and the peak
259 area from the HILIC-ELSD chromatograms were applied to the calibration curve to calculate
260 betaine contents,

261 (4)

262 where *y* is the peak area and *x* is the concentration of betaine (mg/mL).

263

264 *3.3.2. LOQ i LOD*

265 Exponential function best fits our experimental data. Coefficient of determination was
266 0.99. From Supplementary Fig. 4 it can be seen that good correlation between experimental and
267 calculated data.

268 (5)

269 where y is precision (%) and x is concentration of betaine (mg/mL).

270 Supplementary Fig. 4 shows LOQ value which can be calculated from equation
271 (LOQ=0.038 mg/mL). The detection limit (S/N= 3) was 0.01mg/mL.

272 *3.3.3. Intra-and Inter-day Precision and Accurancy*

273 Table 2 shows the results for intra- and inter-day precision and accuracy. Intra- and inter-
274 day precision ranged from 1.34 to 9.07% and from 0.95 to 4.39%, respectively. Intra- and inter-
275 day accuracy ranged from 87.44 to 110.85% and from 94.29 to 106.08%, respectively. All values
276 were within the acceptable range. Bruce et al. (2010) obtained the inter-day repeatability of 5 and
277 11%, for white and brown wheat flour samples, respectively. Intra-day repeatability for white
278 wheat flour had a coefficient of variation (CV) of 1-8% and for brown wheat flour were 3-9%.
279 Similar results to our study for intra- and inter-day precision and accuracy were obtained in a
280 study of Du Shin et al. (2012).

281 *3.3.4. Recovery*

282 Obtained results of recovery assay and spelt wheat flour and wheat flour were within the
283 range of 90.9-97.8% and 96.3-101.2%, respectively (Supplementary Table 2). Reported
284 recoveries were 102-119%, for refined white flour and brown flour extracts, respectively (Bruce

et al., 2010). Hefni et al. (2016) obtained the average recovery of 92-109% for whole wheat flour. All recoveries were within the same range, regardless the extraction procedure.

3.4. Analysis of betaine levels in samples

In Supplementary Table 3 are presented betaine content in examined samples. All values were reported on a dry matter (DM) basis.

In this study, betaine in 54 samples of cereals and pseudocereals were identified and quantified (Supplementary Table 3). The highest content of betaine is found in wheat bran (sample 46) and enriched plain biscuit with molasses (sample 14). Mixed grain products such as breakfast cereals (10) extensively varied in betaine content, due to variant ingredient composition which is based on various cereal flakes, dry fruits and bran fractions. Ross et al. (2014) reported low betaine content in muesli products (<150µg/g) because they are mainly based on oats and dried fruit which have low content of betaine. Two to four times higher content of betaine was found in wholegrain wheat products compared to betaine found in refined wheat by the same authors. They explained that differences in betaine content among samples may have caused by variation of betaine in the field or due to differences in losses of the bran fraction during milling. In our study, we have found that spelt wheat grain and wholegrain spelt flour have more betaine than common wheat flour (56.52-81.46 mg/100g DM and 125.640 mg/100g DM, and 31.00, respectively). Spelt wheat grain seems to be a richer source of betaine in comparison to common wheat as well as amaranth grain among gluten-free grains. It has been established that climate conditions and stress level of the crop influence betaine content in grains, thus drought conditions can lead to higher betaine levels in grains (Slow et al., 2005). Different varieties can also have different contents of betaine (Corol et al., 2012). Similar level can be noticed between durum

307 flour and semolina, where lower levels were noted for durum flour (31.0mg/100g DM), while
308 semolina had 48.27 mg/100g DM of betaine. High betaine content was found in other cereals
309 such as triticale, barley and rye, all in comparison to common wheat. In our study, we got similar
310 betaine levels to investigation of De Zwart et al. (2003) for oats (200-1000 μg/g). Gluten-free
311 products often have lower levels of betaine (Ross et al., 2014; Bruce et al., 2010). They noted less
312 than 150 μg/g of betaine in most of the commercially available gluten-free (GF) products.
313 Content of betaine in samples used in this investigation (starch, expanded grain, pasta, flakes
314 based on maize and rice) was below limit of detection. Likewise, in commercially available GF
315 products (bread mix, biscuits, crackers, pasta) betaine content was found to bellow LOD, which
316 is consistent with the literature (Ross et al., 2014). Moderate levels of betaine were found in
317 millet grain and buckwheat pasta. Analyses showed that addition of beet molasses affected
318 betaine content in plain GF biscuits, causing an increase to 328 mg/100g DM. Higginbotham and
319 McCarthy (1998) reported that beet molasses is an abundant source of betaine (5- 6%), therefore
320 it is used for industrial betaine extraction. Inclusion of amaranth, beet molasses, millet into GF
321 products formulations could improve the diet of those who follow gluten-free or vegan diet.

322 PCA was performed to classify the observed samples of cereals and pseudocereals
323 according to betaine content. HPLC chromatographic area under the identified peak, in the range
324 between 2.3 and 3.1 minutes of retention time were used as descriptors to differentiate between
325 analyzed samples. The orientation of the vector describing the variable in factor space indicates
326 an increasing trend of these variables, and the length of the vector is proportional to the square of
327 the correlation values between the fitting value for the variable and the variable itself. The angles
328 between corresponding variables indicate the degree of their correlations (small angles
329 corresponding to high correlations) as presented in the Figure 3

330 The results obtained by PCA demonstrate that the differences between samples are due to
331 betaine content. . The points shown in the PCA graph (Figure 3), which are geometrically close
332 to each other, indicate the similarity of samples, which are grouped into two large clusters (C_1
333 and C_2).

334 A large group of samples, in which no betaine content was found, forms a cluster C_1
335 which contains the following samples: 8, 12, 13, 16, 19, 32, 11, 15, 22, 23, 26, 25, 31, 33, 34, 44,
336 17, 18, 21. The betaine content was identified in C_2 cluster which includes samples; 2, 3, 4, 6,
337 20, 28, 29, 37, 39, 40, 41, 42, 47, 48, 49, 50, 51, 52, 53, 24, 10, 30, 5, 9, 1, 7, 27, 36, 38, 43, 45,
338 46, 54, 14, 35. All gluten-free samples are present within the cluster C1. According to PCA
339 analysis, the two major trends of samples can be observed: 1. gluten-free samples, which are
340 characterized by no betaine content (except for the samples of amaranth and millet) - marked as
341 cluster C1, and 2. all other samples with confirmed betaine content - labeled as cluster C2 in Fig.
342 3.

343 **4. Conclusions**

344 Betaine from samples of cereals and pseudocereals has been extracted using methanol
345 which does not extract undesirable hydrophilic compounds, and thus improves sample purity
346 andincreases HPLC column life. A simple isocratic HPLC–ELSD method has been applied for
347 quantification of betaine content. Proposed method accomplishes the requirements for the method
348 linearity, precision, accuracy, and limits of detection and quantitation for determination of betaine
349 in cereals and pseudocereals. Obtained data for betaine levels in 54 samples could be grouped in
350 two large groups: gluten-free samples with no betaine, and the remaining samples containing
351 betaine. PCA has confirmed this general trend among the samples. The average betaine content

obtained in food samples is in the following order: buckwheat < millet < wheat < oats < rye < barley < amaranth < spelt... This data could be used in design of new functional products.

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References

Ahn, C. W., Choi, Y. J., Hong, S. H., Jun, D. S., Na, J. D., Choi, Y. J., & Kim, Y. C. (2015). Involvement of multiple pathways in the protection of liver against high-fat diet-induced steatosis by betaine. Journal of Functional Foods, 17, 66–72.

Barak, A. J., Beckenhauer, H. C., & Tuma, D. J. (1996). Betaine, ethanol, and the liver: a review. Alcohol, 13, 395–398.

Bruce, S. J., Guy, P. A., Rezzi, S., & Ross, A. B. (2010). Quantitative Measurement of Betaine and Free Choline in Plasma, Cereals and Cereal Products by Isotope Dilution LC-MS/MS. Journal of Agricultural and Food Chemistry, 58, 2055–2061.

Buszewski, B., & Noga, S. (2012). Hydrophilic interaction liquid chromatography (HILIC) — a powerful separation technique. Analytical and Bioanalytical Chemistry, 402, 231 – 247.

Corol, D. I., Ravel, C., Raksegi, M., Bedo, Z., Charmet, G., Beale, M. H., Shewry, P. R., & Ward, J. L. (2012). Effects of genotype and environment on the contents of betaine,

373 choline, and trigonelline in cereal grains. Journal of agricultural and food chemistry,
374 60, 5471-5481.

375 Craig, S. A. (2004). Betaine in human nutrition. The American journal of clinical nutrition,
376 80, 539-549.

377 De Zwart, F. J., Slow, S., Payne, R. J., Lever, M., George, P. M., Gerrard, J. A., & Chambers,
378 S. T. (2003). Glycine betaine and glycine betaine analogues in common foods. Food
379 Chemistry, 83, 197-204.

380 Filipčev, B. V, Brkljača, J. S., Krulj, J. A., & Bodroža-Solarov, M. I. (2015). The betaine
381 content in common cereal-based and gluten-free food from local origin. Food & Feed
382 Research, 42, 129-138.

383 Filipčev, B., Krulj, J., Kojić, J., Šimurina, O., Bodroža Solarov, M., & Pestorić, M. (2016).
384 Quality attributes of cookies enriched with betaine. III International Congress "Food
385 Technology, Quality and Safety", 25.-27.10.2016., Novi Sad, 46-51.

386 Gao, X., Wang, Y., Randell, E., Pedram, P., Yi, Y., Gulliver, W., & Sun, G. (2016). Higher
387 dietary choline and betaine intakes are associated with better body composition in the
388 adult population of Newfoundland, Canada. PloS one, 11, e0155403.

389 Hefni, M., McEntyre, C., Lever, M., & Slow, S. (2016). Validation of HPLC-UV Methods for
390 the Quantification of Betaine in Foods by Comparison with LC-MS. Food analytical
391 methods, 9, 292-299.

392 Higginbotham, J. D., & McCarthy, J. (1998). Quality and storage of molasses. In P. W. van
393 der Poel; Schiweck, H.; Schwartz T. (Eds.), Sugar Technology-Beet and Cane
394 Manufacture (pp. 973-992). Berlin, Germany: Bartens.

395 Huber L. (2010). Parameters and Tests for Method Validation. In Validation of Analytical
396 Methods (Eds.) (pp. 14-28). Germany: Agilent Technologies.

397 Klein J. (2014). Assessing university students' achievements by means of standard score (Z
398 score) and its effect on the learning climate. Studies in Educational Evaluation, 40,
399 63-68.

400 Lever, M., & Slow, S. (2010). The clinical significance of betaine, an osmolyte with a key
401 role in methyl group metabolism. Clinical biochemistry, 43, 732-744.

402 Likes, R., Madl, R. L., Zeisel, S. H., & Craig, S. A. (2007). The betaine and choline content
403 of a whole wheat flour compared to other mill streams. Journal of cereal science, 46,
404 93.

405 Monzón, C. M., Teglia, C. M., Delfino, M. R., & Goicoechea, H. C. (2016). Chemometric
406 optimization and validation of a novel dispersive liquid–liquid microextraction–HPLC
407 method for gliclazide, glibenclamide and glimepiride quantitation in serum samples.
408 Microchemical Journal, 127, 113-119.

409

410 Ross, A. B., Zangger, A., & Guiraud, S. P. (2014). Cereal foods are the major source of
411 betaine in the Western diet–analysis of betaine and free choline in cereal foods and
412 updated assessments of betaine intake. Food chemistry, 145, 859-865.

413 Saarinen, M. T., Kettunen, H., Pulliainen, K., Peuranen, S., Tiihonen, K., & Remus, J. (2001).
414 A novel method to analyze betaine in chicken liver: effect of dietary betaine and
415 choline supplementation on the hepatic betaine concentration in broiler chicks. Journal
416 of agricultural and food chemistry, 49, 559-563.

417 Schwahn, B. C., Hafner, D., Hohlfeld, T., Balkenhol, N., Laryea, M. D., & Wendel, U.
418 (2003). Pharmacokinetics of oral betaine in healthy subjects and patients with
419 homocystinuria. British journal of clinical pharmacology, 55, 6-13.

420 Shin, H. D., Suh, J. H., Kim, J. H., Lee, H. Y., Eom, H. Y., Kim, U. Y., Yang, D. H., Han, S.
421 B. & Youm, J. R. (2012). Determination of betaine in Fructus Lycii using hydrophilic
422 interaction liquid chromatography with evaporative light scattering detection. Bulletin
423 of the Korean Chemical Society, 33, 553-558.

424 Slow, S., Donaggio, M., Cressey, P. J., Lever, M., George, P. M., & Chambers, S. T. (2005).
425 The betaine content of New Zealand foods and estimated intake in the New Zealand
426 diet. Journal of Food Composition and Analysis, 18, 473-485.

427 Taverniers, I., Loose, M. D., & Bockstaele, E. V. (2004). Trends in quality in the analytical
428 laboratory. II. Analytical method validation and quality assurance. Trends in
429 Analytical Chemistry, 23, 535- 552.

430 Ueland, P. M. (2011). Choline and betaine in health and disease. Journal of inherited
431 metabolic disease, 34, 3-15.

432

433 Zeisel, S. H., Mar, M. H., Howe, J. C., & Holden, J. M. (2003). Concentrations of choline-
434 containing compounds and betaine in common foods. The Journal of nutrition, 133,
435 1302-1307.

436 Zhang, M., Wu, X., Lai, F., Zhang, X., Wu, H., & Min, T. (2016a). Betaine inhibits hepatitis
437 B virus with an advantage of decreasing resistance to lamivudine and interferon α.
438 Journal of agricultural and food chemistry, 64, 4068-4077.

439 Zhang, M., Zhang, H., Li, H., Lai, F., Li, X., Tang, Y., Min, T., & Wu, H. (2016b).
440 Antioxidant mechanism of betaine without free radical scavenging ability. Journal of
441 Agricultural and Food Chemistry, 64, 7921−7930.

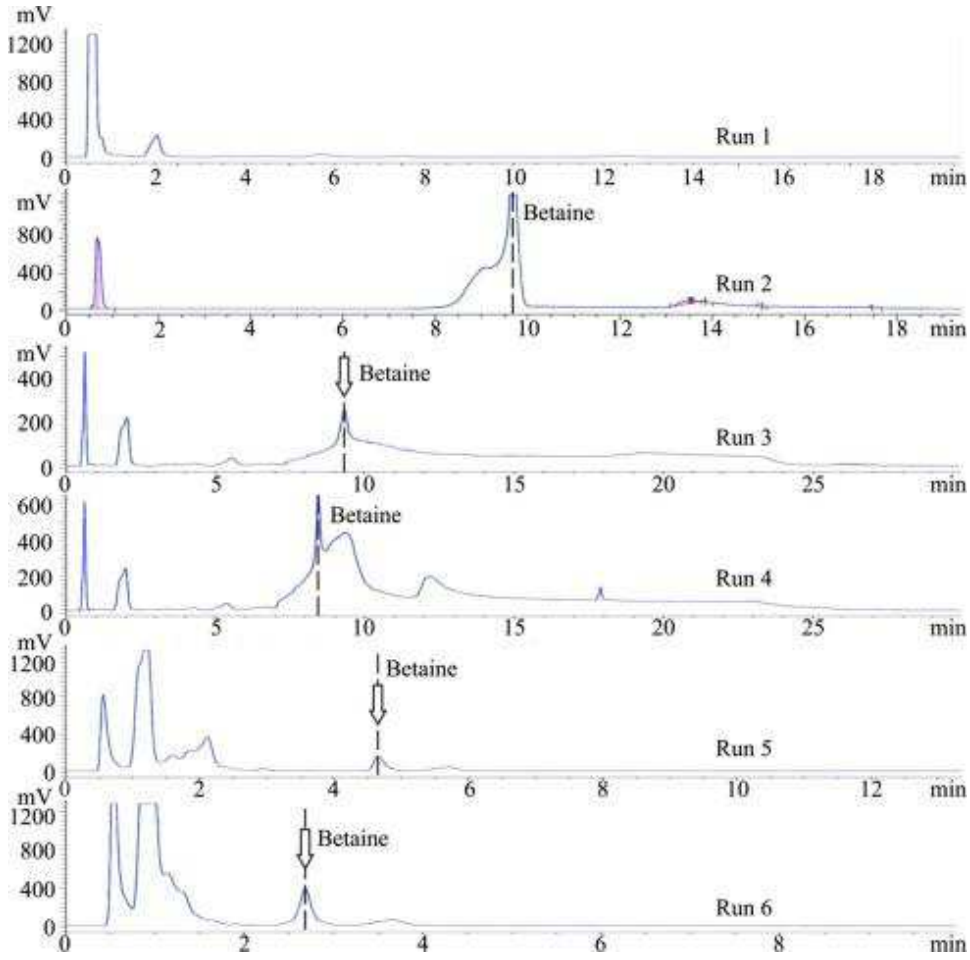
442

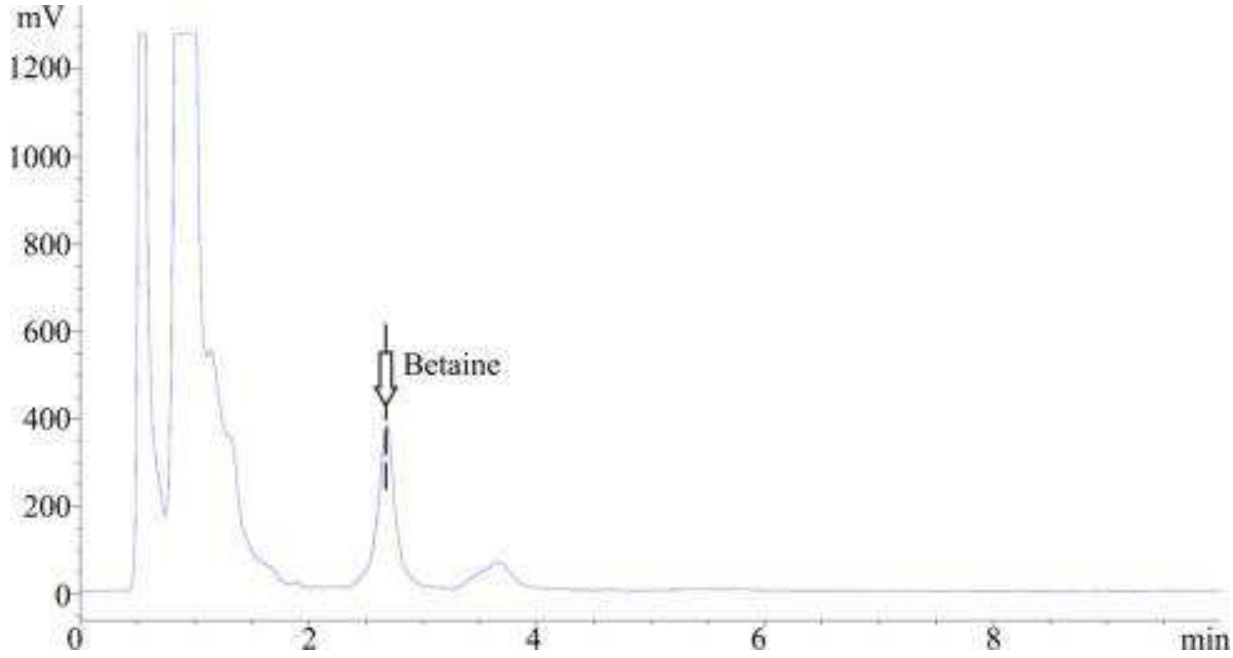
443 FIGURE CAPTIONS

444 Fig. 1. Betaine peak at different gradient or isocratic regime

445 Fig. 2. Betaine peak at optimal chromatographic conditions which are applied in this work

446 Fig. 3. PCA analysis of cereal and pseudocereal samples





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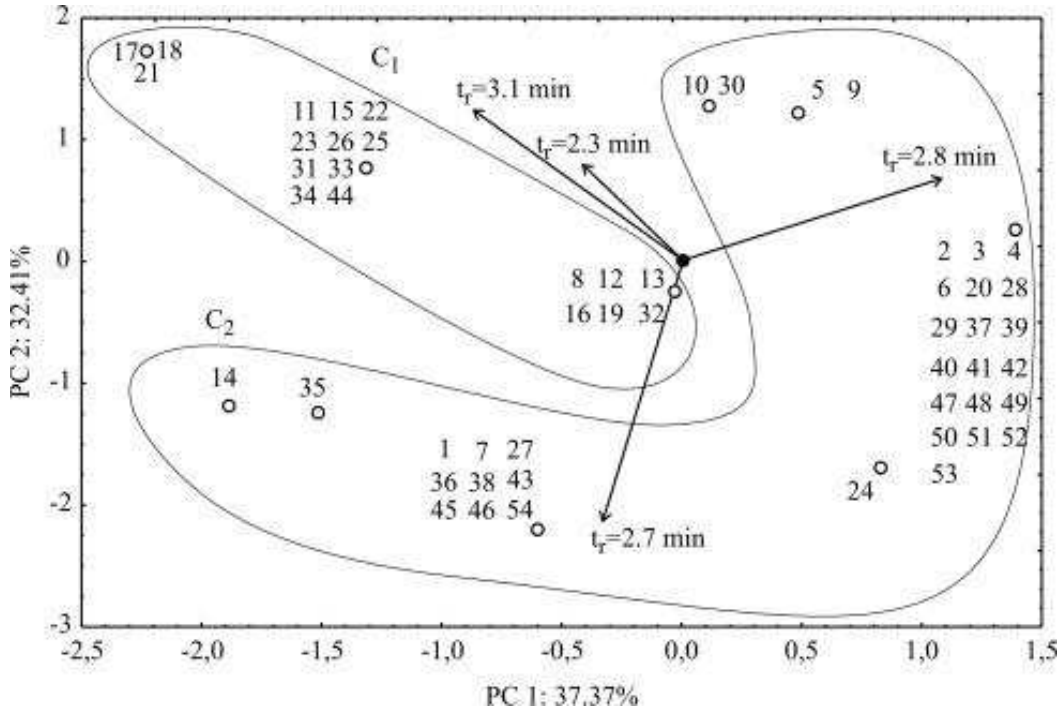

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452 Supplementary Fig. 1. Chromatograms of the wheat flour samples extracted using two different

453 solvents: a) methanol b) water

454 Supplementary Fig. 2. Optimal function for gradient and pH value adjusting

455 Supplementary Fig. 3. Optimization of vortex extraction time

456 Supplementary Fig. 4. Limit of quantitation based on selected precision

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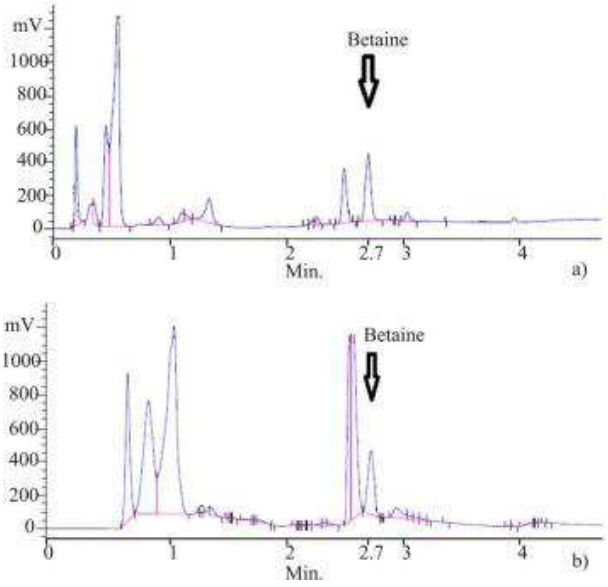

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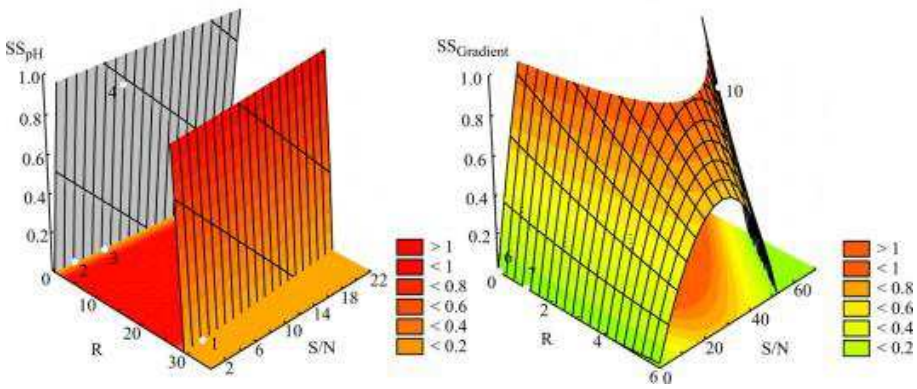

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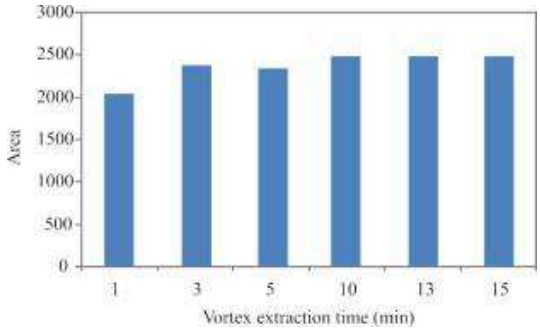

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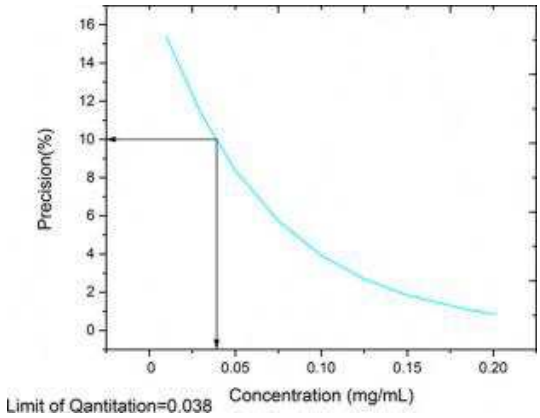

462 **Table 1.** Optimization of gradient

RUN	Flow rate (ml/min)	Gradient regime or isocratic (mobile phase of ACN and 10 mM acetate buffer)	Resolution 1	S/N ratio 1	SS
1	0.5	Isocratic: 90% ACN and 10% 10 mM acetate buffer	/	/	0.00
2	0.5	– 90% ACN for 5 min, – decrease to 70% ACN during 10 min – maintaining this proportion for 5 min – and then final increase to 90% ACN.	1.05	0.098	0.00
3	0.5	– 90% ACN for 3 min, – decrease to 70% ACN during 15 min – maintaining this proportion for 6 min – and then final increase to 90% ACN	2.46	2.3	0.16
4	0.5	– 90% ACN for 3 min, – decrease to 50% ACN during 15 min, – maintaining this proportion for 6 min – and then final increase to 90% ACN	4.38	7.2	0.40
5	0.5	– Isocratic: 85% ACN and 15% 10 mM acetate buffer	5.11	36.9	0.78

opt. 6	0.5	– –	Isocratic: 80% ACN and 20% 10 mM acetate buffer	3.22	58.6	0.81

*Temperature was not controlled, injection volume was 5 µl, pH mobile phase was 3.7, and double peaks were not observed

Table 2. Intra- and inter-day precision and accuracy (n=7)

Betaine				
Nominal conc. (mg/ml)		Measured conc. (mg/ml)	Precision (% RSD)	Accuracy (%)
Intra-day (n=7)	0.05	0.0554	9.07	110.85
	0.075	0.0737	4.77	98.28
	0.1	0.0950	2.74	94.96
	0.125	0.1093	5.37	87.44
	0.15	0.1470	1.68	97.99
	0.1875	0.1876	2.11	100.07
	0.2	0.2038	1.34	100.88
Inter-day (n=7)	0.05	0.0530	3.33	106.08
	0.075	0.0755	4.39	100.69
	0.1	0.0981	0.95	98.13
	0.125	0.1178	1.30	94.29
	0.15	0.1489	0.97	99.31
	0.1875	0.1876	2.11	100.06
	0.2	0.2037	1.34	101.89

468 **Supplementary Table 1.** Optimization of pH (flow=0.5 mL/min and linear gradient

469 conditions as in work Ross et al. (2014)

RUN	pH mobile phase	Resolution 1	Resolution 2	S/N ratio 1	S/N ratio 2	Duble peak index	SS
1	1.9	29.12	29.2	4.51	2.4	0	0.00
2	4	2.83	2.66	22.1	5.5	0	0.00
3	5	1.24		8.2		1	0.00
4	3.7	3.58		10.5		1	0.58

470

471

472 **Supplementary Table 2**. Recovery of methods for betaine determination in wheat flour

473 and spelt wheat (n=3)

Food matrices	C_{sample} (mg/mL)	C_{added}(mg /mL)	C_{found}(mg /mL)	Recovery (%)
Wheat flour	0.0996	0.02	0.1192	98.1±10
		0.03	0.1285	96.3±12
		0.05	0.1502	101.2±8
Spelt flour	0.1444	0.02	0.1637	96.7±12
		0.03	0.1737	97.8±12
		0.05	0.1899	90.9±4

474

475 **Supplementary Table 3**. Betaine content in the observed cereals and pseudocereals

No.	Samples	Betaine (mg/100g DM)
1	Amaranth (*Amarantuscruentus*) expanded grain	60.73 ± 0.54^{k}
2	Amaranth (*Amarantuscruentus*) flour 1	92.51 ± 0.32^{p}
3	Amaranth (*Amarantuscruentus)* flour 2	81.72 ± 0.66^{o}
4	Barley (*Hordeumvulgare*) bran	35.94 ± 0.29^{e}
5	Barley (*Hordeumvulgare*) flour from hullesvariates	42.36 ± 0.37^{fgh}
6	Barley (*Hordeumvulgare*) pearled	27.44 ± 0.21^{c}
7	Barley (*Hordeumvulgare*) wholegrain flour	77.87 ± 0.82^{l}
8	Bread mix	<LOQ
9	Breakfast cereals 1	29.98±0.22
10	Breakfast cereals 2	18.00±0.03
11	Buckwheat (30%) and wheat pasta, cooked	<LOQ
12	Buckwheat (*Fagopyrumesculentum*) pasta	17.53±0.01
13	Buckwheat (*Fagopyrumesculentum*) wholegrain flour	<LOQ
14	Enriched plain biscuit with mollases	328.45 ± 3.53^{t}
15	Gluten-free bread mix	<LOQ
16	Gluten-free cookie with almonds	<LOQ

17	Gluten-free cracker	<LOQ
18	Gluten-free salty sticks	<LOQ
19	Hull-less pumpkin seed pasta	<LOQ
20	Maize (*Zea mays*) bran	18.45 ± 0.05^{a}
21	Maize (Zea mays) expanded grain	<LOQ
22	Maize (Zea mays) flakes 1	<LOQ
23	Maize (Zea mays) flakes 2	<LOQ
24	Maize (Zea mays) grain	17.55 ± 0.17^{a}
25	Maize (Zea mays) starch	<LOQ
26	Maize and rice flour pasta	<LOQ
27	Oats (Avena sativa) grain 1	41.82 ± 0.45^{fg}
28	Oats (Oryza sativa) grain 2	35.80 ± 0.08^{e}
29	Pasta with added spinach	25.13 ± 0.20^{c}
30	Millet (*Panicummiliaceum*) grain, dehulled	22.65 ± 0.32^{b}
31	Rice (*Oryza sativa*) expanded grain	<LOQ
32	Rice (*Oryza sativa*) grain	<LOQ
33	Rice (*Oryza sativa*) pasta	<LOQ
34	Rice (*Oryza sativa*) starch	<LOQ
35	Rye (*Secalecereale*) grain	44.42 ± 0.28^{h}
36	Rye (*Secalecereale*) wholegrain flour	98.57 ± 0.67^{q}
37	Soy bran	18.16 ± 0.15^{a}
38	Spelt (*T.aestivumspp.spelt*) wheat grain 1	56.52±0.15
39	Spelt (*T.aestivumspp.spelt*) wheat grain 2	71.42 ± 0.46^{m}
40	Spelt (*T.aestivumspp.spelt*) extruded product	30.80 ± 0.23^{d}
41	Spelt (*T.aestivumspp.spelt*) refined flour	41.00 ± 0.34^{f}
42	Spelt (*T.aestivumspp.spelt*) wheat grain	82.46 ± 0.34^{o}
43	Spelt (*T.aestivum*) wholegrain flour	125.64 ± 0.23^{r}
44	Sweet biscuits gluteen free	<LOQ
45	Triticosecale grain	64.08 ± 0.48^{l}
46	Wheat (*T.aestivum*) bran	271.68 ± 3.27^{s}

47	Wheat (*T.aestivum*) grain	44.03±0.64^{gh}
48	Wheat (*T.aestivum*) pasta dry	25.33±0.31^{c}
49	Wheat (*T.aestivum*) refined flour 1	49.15±0.35
50	Wheat (*T.aestivum*) refined flour 2	41.55±0.29^{f}
51	Wheat (*T.aestivum*) wholegrain biscuits	42.49±0.29^{fgh}
52	Wheat (*T.durum*) flour	31.00±0.24^{d}
53	Wheat (*T.durum*) semolina pasta	18.83±0.09^{a}
54	Wheat (*T.durum*) semolina	48.27±0.68^{i}

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