Eng. ANCA CORINA FĂRCAȘ

PhD THESIS

RESEARCH REGARDING THE IDENTIFICATION AND EXPLOITATION OF BIOLOGICALLY ACTIVE COMPOUNDS FROM BREWERS' SPENT GRAIN BY-PRODUCT

(SUMMARY OF PhD THESIS)

Scientific coordinator:
Prof. dr. MARIA TOFANĂ

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INTRODUCTION: AIM AND OBJECTIVES

Agro-industrial waste is the most abundant and renewable resource on our planet. The accumulation of huge amounts of this biomass every year leads to environmental degradation and especially to significant loss of valuable material that cold otherwise be exploited as food, fuels and a great variety of additives.

Recent research shows that modern technologies used for the exploitation of vegetal resources as by-products of the food industry could also be used in some competitive biotechnological fields, thus enabling the extraction of valuable ingredients such as antioxidants, proteins, fibers, sugars etc.

The brewing process will inevitably produce substantial amounts of by-products, especially spent grain, spent hop and spent yeast. These accumulate in tremendous amounts throughout the year but their use is relatively small – most of the times they are either discarded as waste or mixed with other ingredients as compost or food for animals.

Brewers’ spent grain is the insoluble residue generated from the production of wort in the brewing industry. This plant-derived by-product is known to contain significant amounts of valuable compounds that remain unexploited in the brewing processes. It is therefore essential to establish a more detailed description of brewers’ spent grain in order to highlight its potential in developing new value-added products. It is estimated that worldwide the annual output is around 30 million tons, about 200 t of wet spent grain (70 to 80 % water content) being produced per 10.000 hl of beer. It primarily consists of grain husks and other residual compounds not converted to fermentable sugars by the mashing process. Traditionally, this material is either sold as animal feed or discarded. On the other hand, the food industry is seeking to find new added-value solutions that will change the traditional view on ‘waste’ products and reclassify them as ‘co-products’. Using the brewers’ spent grain by-product - which has a low monetary value - as a high-nutrient functional ingredient may enhance the economic potential of breweries and improve the dietary attributes of food formulations.

The research conducted through this thesis aims to identify the means by which brewers' spent grain can be exploited as well as to quantify some biologically active compounds which can be used in the food industry.
In order to evaluate the various ways in which spent grain can be exploited on, two major research paths were approached:

I. Analyzing the composition of brewers' spent grain and raw materials it is derived from as well as monitoring the traceability of some biologically active compounds of interest for this research.

II. Evaluating the usability potential of brewers' spent grain in the development of an food product with functional properties.

Starting from these two main research paths, the following objectives were traced:

- Evaluation of main nutritional parameters of brewers' spent grain, as well as of the raw materials it is derived from, by applying various physicochemical methods and the NIRS technique.
- Identification and evaluation of some biologically active compounds with antioxidant potential, through UV-VIS spectroscopy analysis.
- Identification and quantification of fatty acids and of flavor compounds in brewers' spent grain and raw materials, by using the GC-MS and respectively the HS/ITEX/GC-MS methods.
- Sample discrimination and highlighting specific compounds by applying the PCA procedure.
- Obtaining of an innovative product based on brewers' spent grain and evaluation of its quality from a nutritional, technological, microbiological and psychosensorial point of view, in order to optimize the production technology.
- Statistical evaluation of the data specific to the functional food, by applying cluster analysis (CA), principal component analysis procedure (PCA) and establishing Pearson correlations.
THESIS STRUCTURE

The thesis has to main parts. The first part includes a literature study and the second, personal research, results, discussions and finally, conclusions.

PART I: LITERATURE STUDY, has two chapters:

Chapter 1. Presentation and characterisation of brewing by-products, describes the chemical composition of by-products resulted in the beer production process and the main classes of biologically active compounds.

Chapter 2. Pathways for processing and superior valorization of by-products generated by the brewing industry, includes nine subchapters showcasing the main ways in which brewers' spent grains can be exploited.

PART II: PERSONAL CONTRIBUTIONS, includes five chapters:

Chapter 3. Aim, objectives and experimental design, includes data related both to presenting the experimental material and work protocols.

Chapter 4. Biochemical compounds classes determination, includes three subchapters regarding the methods applied and results achieved by identifying, separating and quantifying the main biochemical compounds of brewers' spent grain raw materials.

Chapter 5. Evaluation of antioxidant activity and quantification of total polyphenols and flavonoids by UV-VIS spectroscopy, describes the spectrophotometric techniques used for identifying and quantifying some biologically active compounds with antioxidant potential, both from brewers' spent grain samples and from the raw materials.

Chapter 6. Traceability study of some targeted compounds by chromatographic methods, presents, in three subchapters, the techniques and results for identifying, quantifying and monitoring the traceability of volatile compounds and fatty acids, as well as the chemometric interpretation by principal component analysis (PCA).

Chapter 7. Design and characterization of a functional product based on brewers' spent grain flour, describes the technology for obtaining the functional bread, methods used to assess its quality as well as the chemometric analysis of data.
PERSONAL CONTRIBUTIONS

1. THE EXPERIMENTAL MATERIAL AND SAMPLE CODING

All the materials were supplied by the Microbrewery of the Faculty of Food Science and Technology of University of Agricultural Sciences and Veterinary Medicine from Cluj-Napoca. The BSG used in this work was obtained as a by-product from the mashing process of dark lager beer with 100% whole grain malted barley (Weyermann Specialty Malting Company, Bamberg - Germany). Beside Pilsner malt (MP) which imparts a malty-sweet and gentle notes of honey to the beer, Caramunich (MCm) and Carafa (MCf) malts were added in small amount (5-10%) to obtain a dark colour and to enhance the flavour characteristics with notes of caramel, coffee, cacao, dark chocolate and roast. A commercial wheat flour (WF) and wholemeal wheat flour (WWF) used for bread making was purchased from SC Dobrogrea Grup SA, România.

![Fig. 1 Raw materials codification](image)

**Acknowledgments:** The research was financially funded by the program Innovation-subprogram Innovation Checks – PNII, CCCDI-UEFISCDI, project number PN-II-IN-ICI-2013-1-0018.
2. PHYSICO-CHEMICAL CHARACTERISATION OF BREWERS' SPENT GRAIN AND RAW MATERIALS

2.1 MATERIAL AND METHOD

In the brewery, malted barley is milled, mixed with water in the mash tun and the mash temperature then slowly increased from 37ºC to 78ºC to promote the enzymatic hydrolysis of malt constituents. The sweet liquid produced during this mashing stage is known as wort. The insoluble, un-degraded part of the malted barley grain is allowed to settle to form a bed in the mash tun and the sweet wort filtered through it (Linko et al., 1998). After saccharification is finished, the clear sweet wort is separated from the solid components - the brewers’ spent grain. Then, the wort is transferred to the wort kettle, while the spent grain is removed from the lauter tun.

Figure 1 shows a simplified schematic representation of the process by which the brewers’ spent grain is obtained.

Fig. 2 Schematic representation of the process to obtain BSG from malt

- Determination of moisture by oven drying.
- Determination of protein content by Kjeldahl method.
- Determination of lipids content by Soxhlet method.
- Determination of minerals content.
- Determination of starch by Ewers polarimetric method.
- Determination of fibre and sugars by Near Infrared Reflectance Spectroscopy.
2.2 RESULTS AND DISCUSSION

2.2.1 Protein content

The high nutritive potential of brewers’ spent grain is mainly due to the high amount of proteins, especially essential amino acids found in significant amounts (Waters et al., 2012). It is also estimated that about 65% of malt proteins remain in the brewers' spent grain (Celus et al., 2006).

The results presented in figure 3 show that the average protein concentration of brewers' spent grain is of 18%, a much higher value compared to the raw materials it results from, namely barley (11.65%), Pilsner malt (11.31%), Caramunich malt (10.81%) Carafa malt (11.26%).

![Graph of protein content in barley, malt, brewers' spent grain and flour samples.]

Fig. 3 Variation of protein content in barley, malt, brewers' spent grain and flour samples.

2.2.2 Fibre content

A literature study shows that the total amount of fiber in BSG is of approximately 48% (d.w.), mostly insoluble fibers (Waters et al., 2012). Together, cellulose and hemicellulose reach a value of almost 50% of BSG (Mussatto and Roberto, 2006). Our data, presented in figure 4, is in accordance with literature studies, and shows that dried and lyophilized brewers' spent grain has a fiber content of 41.28%, and 40.12% respectively – almost four times higher compared to malt samples.
2.2.3 Starch content

By analysing the data in figure 5 we notice that the malt samples have a starch content of about 60%, while dry spent malt has approximately 10.1%. We can therefore draw the conclusion that brewers' spent grain resulted after the mash-saccharification stage contains only residual amounts of starch. The data obtained are in agreement with the results obtained in similar studies (Waters et al., 2012; Makowska et al., 2013).

2.2.4 Lipids and minerals content

As we can see from figure 6, the lipids content of barley and malt varies between 2.31-2.96 %. During the mashing process, the endosperm is solubilized almost entirely and fermentable substances useful for the nutrition of yeast are extracted in the mash.
Lipids therefore accumulate in the brewers’ spent grain where they reach a concentration of up to 6.61% (reported on a dry weight basis). Also, the mineral content of brewers’ spent grain is about 50% higher than barley and malt samples.

![Fig. 6 Variation of lipids and minerals content in the barley, malt, brewers' spent grain and flour samples](image)

### 2.2.5 Sugar content

Surprisingly, brewers' spent grain has a relatively high concentration of simple sugars (approx. 15% on a dry weight basis) compared to barley and malt (maximum 3%, respectively 6%) (Waters et al., 2012). Of these, xylose, glucose and arabinose predominates (Mussatto, 2009). As can be seen in figure 7, the brewers' spent grain samples has a higher content of simple sugars (17.78% and 17.11% respectively) compared to malt (MP 5.16%, MCm 10.40%, MCF 10.33%), barley (4.69%), white and whole wheat flour respectively (0.32%, 3.16%).

![Fig. 7 Variation of sugars content in barley, malt, brewers' spent grain and flour samples](image)
3. EVALUATION OF ANTIOXIDANT ACTIVITY AND QUANTIFICATION OF TOTAL POLYPHENOLS AND FLAVONOIDS BY UV-VIS SPECTROSCOPY

3.1 MATERIAL AND METHODS

All the samples analysed were homogenized in methanol containing HCl (0.3%) and then were centrifuged at 4000 rpm for 10 min. Extracts were concentrated at 35°C under reduced pressure (Rotavap Laborata 4010 Digital, Heidolph, Germany) and stored at −18 °C for further analysis. The total phenolic content was estimated with Folin-Ciocalteu method (Singleton et al., 1999). Flavonoid content of the extracts was determined using aluminium chloride colorimetric method (Zhishen et al., 1999). The antioxidant activity assay was performed according to a method reported by Brand-Williams et al. (1995).

3.2 RESULTS AND DISCUSSION

Cereals, especially barley and malt, were shown to contain more phytochemicals than previously considered. The polyphenols of barley play an important role during the malting process as well as preventing the significant enzymatic oxidation of polyunsaturated fatty acid (Guido et al., 2005). Because the most of the phenolic compounds of the barley grain are contained in the husk, BSG represents a not only a rich source of natural antioxidants but also an inexpensive alternative to synthetic antioxidants. Flavonoids, as a class of phenolic compounds are also present in BSG.

The content in total phenols, flavonoids and overall antioxidant capacity of the analyzed barley, malt, BSG and flour samples is presented in Table 1. The concentration in total phenolics varied between 21.12 mg GAE/100g fw (WF) and 335.88 mg GAE/100g fw (MCf). As expected the lowest values for the total phenolics content were obtained for the flour samples, while the highest content was retrieved in MCf sample, followed by the BSGd sample (284.20 mg GAE/100g fw) and BSGl sample (291.47 mg GAE/100g fw). The increase in phenolics concentration from barley to malt samples and also in BSG samples may be associated with the enzymatic release of bound phenolic compounds during different stages of the malting process (Dvořáková et al., 2008). The
determined flavonoid concentrations followed a similar pattern with phenolic compounds, MCm, MCf, BSGd and BSGl samples containing the larger amounts (8.97 – 13.16 mg QE/100g fw), while SB and MP had smaller levels (5.28 – 6.17 mg QE/ 100g fw). Our data roughly agrees with other authors findings. Depending on the solvent used for extraction, Meneses et al. (2013) reported for BSG values for total phenols ranging from 2.14 – 9.90 mg GAE/g and a flavonoid content varying between 0.02 and 4.61 mg QE/g. It is already known that the content in polyphenols is influenced not only by the extraction technique but also by factors such as barley cultivar and presence or absence of hull.

According to the results obtained for total phenols and flavonoid content, the greatest antioxidant capacity was found for the MCm (57.87%) followed by BSGd and BSGl samples (55.95% and 53.78% respectively). The increase of antioxidant activity of MCm and BSG samples compared to the barley sample is attributed to the formation during the malting process of non-enzymatic browning compounds (e.g. Maillard products) which may behave as antioxidants (Dvořáková et al., 2008). Although MCf has recorded the highest content of total polyphenols, the antioxidant activity is significantly reduced because it is obtained by roasting at temperatures above 250ºC. Above this temperature, the insoluble compounds formed by polymerization significantly reduce the antioxidant activity.

Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total phenols (mg GAE/100 g fw)</th>
<th>Flavonoids (mg QE/100 g fw)</th>
<th>DPPH inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spring barley (SB)</td>
<td>133.93±2.45</td>
<td>6.17±0.11</td>
<td>43.17±0.07</td>
</tr>
<tr>
<td>Pilsner malt (MP)</td>
<td>148.42±0.51</td>
<td>5.28±0.13</td>
<td>46.36±0.1</td>
</tr>
<tr>
<td>Caramunich malt (MCm)</td>
<td>256.42±6.18</td>
<td>10.72±0.18</td>
<td>57.87±0.07</td>
</tr>
<tr>
<td>Carafa malt (MCf)</td>
<td>335.88±4.41</td>
<td>8.97±0.16</td>
<td>42.07±0.02</td>
</tr>
<tr>
<td>Dried BSG (BSGd)</td>
<td>284.20±13.07</td>
<td>13.16±0.27</td>
<td>55.95±0.28</td>
</tr>
<tr>
<td>Lyophilized BSG (BSGl)</td>
<td>291.47±2.89</td>
<td>10.35±0.16</td>
<td>53.78±0.07</td>
</tr>
<tr>
<td>Wheat flour (WF)</td>
<td>21.12±1.42</td>
<td>2.85±0.10</td>
<td>32.74±0.24</td>
</tr>
<tr>
<td>Wholemeal wheat flour (WWF)</td>
<td>64.68±3.48</td>
<td>3.18±0.15</td>
<td>37.54±0.36</td>
</tr>
</tbody>
</table>
4. TRACEABILITY STUDY OF SOME TARGETED COMPOUNDS
BY CHROMATOGRAPHIC METHODS

4.1 THE ANALYSIS OF AROMA COMPOUNDS BY ITEX/GC-MS TECHNIQUE

4.1.1 Material and method

The volatile fingerprint of BSG samples and those of spring barley (SB), MP, MCm, MCf, WF, WWF samples were determined using the ITEX/GC-MS technique, as described in our previous work (Socaci et al., 2014). The analysis of volatile compounds was carried out on a GCMS QP-2010 (Shimadzu Scientific Instruments, Kyoto, Japan) model gas chromatograph - mass spectrometer. The volatile compounds were identified using the spectra of reference compounds from NIST27 and NIST147 mass spectra libraries and verified by comparison with retention indices drawn from www.pherobase.com or www.flavornet.org. All peaks found in at least two of the three total ion chromatograms (TIC) were taken into account when calculating the total area of peaks (100%) and the relative areas of the volatile compounds.

4.1.2 Results and discussion

A total of 37 compounds were separated and identified in the analysed samples. The volatile constituents found in the analysed samples include alcohols, aldehydes, ketones as well as furans and other classes of compounds. In the case of SB the main volatile compounds identified were hexanal (23.16%), 2-methyl-3-buten-2-ol (16.59%) and 2-pentyl-furan (16.72%). In relative high amounts were also detected 3-methyl-butanal (8.46%), 1-pentanol (8.11%), 2-methyl-1-propanol (4.29%), 1-penten-3-ol (3.98%) and toluene (4.15%). Aldehydes were the predominant chemical family in the SB samples, accounting 43.62% of total volatiles and imparting, based on their sensorial attributes a fresh, green and almond like notes. Four alcohols (2-methyl-3-buten-2-ol, 2-methyl-1-propanol, 1-penten-3-ol and 3-methyl-1-butanol) were identified in the SB samples, representing 32.97% of total volatiles.

Even though aldehydes represented the main class of aroma compounds found in malt samples (MP, MCm and MCf), their abundances was greater than in SB sample and similar with that detected in BSG and wheat flour samples. The predominant aldehydes
found in all malt samples were 3-methyl-butanal, 2-methyl-butanal, hexanal and 2-methyl-propanal. These compounds arise from the enzymatic oxidation of unsaturated or polyunsaturated fatty acids of barley or from Maillard reactions that occur during the roasting process (Ktenioudaki et al., 2013). The intense roasting process to which MCf is subjected lead to the formation of a larger variety of aldehydes in this type of malt. Furfural, heptanal, 2-heptanal were identified only in MCf, while benzaldehyde and 2-octenal were found in both MCm and MCf. Among the aldehyde detected only in MCf, furfural was present in high concentration (7.39%) having an almond-like aroma. Benzeneacetaldehyde, identified only in MCm sample, together with benzaldehyde contribute to the green, cacao, almond and burnt sugar notes. Benzaldehyde and benzenacetaldehyde were also present in the analysed BSG samples.

As in the case of aldehydes, in the MCf sample was identified a greater number of ketones, as products of lipid oxidation or Maillard reactions (Ktenioudaki et al., 2013). The peroxidation of lipids during the malting process leads to the formation of furan compound. Beside 2-pentyl-furan, high amounts of 2-methyl-furan (9.97%) and 2,5-dimethyl-furan were also found in MCf sample contributing to the sweet, chocolate like flavour. BSG samples (BSGd and BSGI) were characterized by significant levels of aldehydes (95.02-98.45%), the representative ones being the same four as in the case of malt samples (3-methyl-butanal, 2-methyl-propanal, 2-methyl-butanal and hexanal). The concentrations of 2-methyl-propanal and 3-methyl-butanal were higher in BSG samples than in malt sample, being responsible for the malty, almond-like aroma. From ketones’ group, only acetophenone was found in BSG samples, while the furan compounds were represented by 2-pentyl-furan. Other compounds, among which toluene, propyl-benzene and 1,3,5-trimethyl-benzene were identified in BSG samples, imparting a sweet, fruity, balsamic or green notes. Regarding the flour samples, both WF and WWF, were characterized by a small number of detected aroma compounds, the major ones being 2-methyl-propanal and hexanal.
4.2 DETERMINATION OF FATTY ACIDS METHYL ESTERS BY GC-MS
TECHNIQUE

4.2.1 Materials and method

The total lipids (TLs) of the samples were extracted using a chloroform/methanol mixture (Dulf et al., 2013). Fatty acid methyl esters (FAMEs) of the total lipids were derivate by acid–catalyzed transesterification using 1% sulphuric acid in methanol (Christie, 1989).

The FAMEs were determined by gas chromatography-mass spectrometry (GC-MS), using a PerkinElmer Clarus 600 T GC-MS (PerkinElmer, Inc., Shelton, CT, USA) (Dulf et al., 2013). The identification of FAMEs was accomplished by comparing their retention times with those of known standards and the resulting mass spectra to those in the database (NIST MS Search 2.0). The amount of each fatty acid was expressed as percent of total fatty acids content.

4.2.2 Results and discussion on FAMEs determination by GC-MS

A total of 26 fatty acids were identified in the analysed samples, the most abundant of them being linoleic (18:2(z,z)n-6), palmitic (16:0) and oleic (18:1n-9) acids. This data agrees with previously published work that also found these three fatty acids as the major ones in BSG (Niemi et al., 2012). Amounts of α-linolenic (18:3n-3) and stearic (18:0) were also present in all samples as well as small levels of myristic (14:0), vaccenic (18:1n-7), arachidic (20:0), 11-eicosenoic (20:1n-9), behenic (22:0), lignoceric (24:0) acids. Only minor changes in fatty acid composition occur during malting and mashing and therefore, the fatty acid composition of BSG is similar to that of barley (Becker, 2007). Nevertheless, in barley, MP and MCM, the linoleic acid concentration is slightly higher than in BSG, while the palmitic acid is found in a greater proportion in BSG compared with the barley and malt samples. Figure 8 shows the GC-MS chromatogram of FAMEs in the TLs of dried BSG sample. The fatty acids profiles of barley, malt and BSG samples were qualitatively similar but different from those of wheat flour samples. Both WWF and WF samples contained a smaller number of fatty acids, only 16 and respectively 11 fatty acids being detected.
Fig. 8 Variation of saturated, monounsaturated and polyunsaturated fatty acids in samples

![Table showing the variation of fatty acids in different samples with columns for BSGs, BSGc, BSGi, BSGu, FA, Fl, MCF, MCM, MP, and O.]

Peaks: (1) Caproic, (6:0); (2) Caprylic, (8:0); (3) Capric, (10:0); (4) Lauric, (12:0); (5) Myristic, (14:0); (6) Pentadecanoic, (15:0); (7) Azelaic, (AzA); (8) Palmitic, (16:0); (9) Z-7-Hexadecenoic, [C16:1(n-9)]; (10) Palmitoleic, [16:1(n-7)]; (11) Margaric, (C17:0); (12) Heptadecenoic, [17:1(n-9)]; (13) Stearic, (18:0); (14) Oleic, [18:1(n-9)]; (15) Vaccenic, [18:1(n-7)]; (16) Linoleic, [18:2(Z,Z)(n-6)]; (17) Linoleaidic, [18:2(E,E)(n-6)]; (18) α-Linolenic, [18:3(n-3)]; (19) Arachidic, (20:0); (20) 11-Eicosenoic, [20:1(n-9)]; (21) Eicosadienoic, [20:2 (n-6)]; (22) Henicosanoic, (21:0); (23) Behenic, (22:0); (24) Erucic, [22:1(n-9)]; (25) Tricosanoic, (23:0); (26) Lignoceric, (24:0); (27) Nervonic, [24:1(n-9)] acids.

Fig. 9 GC-MS chromatogram of FAMEs in the TLs of dried BSG analyzed on a SUPELCOWAX 10 capillary column.

Peaks: (1) Caproic, (6:0); (2) Caprylic, (8:0); (3) Capric, (10:0); (4) Lauric, (12:0); (5) Myristic, (14:0); (6) Pentadecanoic, (15:0); (7) Azelaic, (AzA); (8) Palmitic, (16:0); (9) Z-7-Hexadecenoic, [C16:1(n-9)]; (10) Palmitoleic, [16:1(n-7)]; (11) Margaric, (C17:0); (12) Heptadecenoic, [17:1(n-9)]; (13) Stearic, (18:0); (14) Oleic, [18:1(n-9)]; (15) Vaccenic, [18:1(n-7)]; (16) Linoleic, [18:2(Z,Z)(n-6)]; (17) Linoleaidic, [18:2(E,E)(n-6)]; (18) α-Linolenic, [18:3(n-3)]; (19) Arachidic, (20:0); (20) 11-Eicosenoic, [20:1(n-9)]; (21) Eicosadienoic, [20:2 (n-6)]; (22) Henicosanoic, (21:0); (23) Behenic, (22:0); (24) Erucic, [22:1(n-9)]; (25) Tricosanoic, (23:0); (26) Lignoceric, (24:0); (27) Nervonic, [24:1(n-9)] acids.
4.3 CHEMOMETRIC EVALUATION OF THE EXPERIMENTAL DATA BY PRINCIPAL COMPONENTS ANALYSIS

The discrimination between the barley, malt, BSG, whole-wheat flour and wheat flour samples was achieved by subjecting the chromatographic data obtained (volatile and fatty acids profile) together with the results obtained for total phenolic content, total flavonoids content and antioxidant capacity to principal component analysis (PCA) with cross validation (full model size and center data). All the statistical analyses were performed using Unscrambler X software version 10.1 (CAMO Software AS Norway).

As shown in figure 10A, the first two components explained 98% of the variance of data, leading to a very good discrimination of samples, even between the two types of BSG samples (dried and lyophilized). The contribution of each variable to the differentiation of samples can be assessed by computing the correlation loadings plot (fig. 10B). The antioxidant compounds, especially the polyphenols content significantly contributes to the discrimination of samples, higher concentrations being specific for MCm, MCf, BSGd and BSGl samples. The volatile profiles as well as the fatty acids composition of the samples also play an important role in their characterization. Among the volatile compounds that may be considered as key constituents it can be mentioned 2-methyl-propanal, 3-methyl-butanal, hexanal, 1-pente-3-ol, 1-pentanol, pentanal. From the fatty acids group, along with the total content in PUFAs, n-6PUFAs and PUFAs/SFAs, linoleic acid, palmitic acid and also fatty acids found in smaller amounts (caproic, caprylic, nervonic, Z-7-hexadecenoic or erucic acid) contribute to sample discrimination.

Fig. 10 Principal component analysis bi-plot of (A) and (B) correlation loading bi-plot
5. DESIGN AND CHARACTERIZATION OF A FUNCTIONAL PRODUCT BASED ON BREWER'S SPENT GRAIN FLOUR

The brewers' spent grain is a low-cost by-product of the brewing process but at the same time it is a valuable source of dietary fibre, protein and essential amino acids, minerals, polyphenols, vitamins and lipids. The aim of this research was to incorporate the brewers' spent grain, provided from black beer production, into a simple bread formulation and to evaluate its contribution to the nutritional composition, volatile profile as well as on the sensorial properties of the enriched bread.

The high initial moisture content of fresh BSG (75-80%) and the presence of considerable levels of polysaccharide and protein makes it particularly susceptible to microbial degradation within a few days (Stojceska et al., 2008). Therefore it is necessary to apply a method of preservation shortly after its production.

Fresh BSG samples were preserved by oven-drying at 78 ºC for 12 hours. The samples kilned to 6% moisture content were then grounded into flour using a laboratory milling machine, packed in sealed polyethylene bags and stored at room temperature until used.

Experimental breads were produced from wheat flour blends containing 0% (100% wheat flour), 5%, 10%, 15% and 20% of BSG (wheat flour replacement). The bread prepared from wheat flour without BSG substitution served as control.

5.1 THE CHEMICAL COMPOSITION OF BSG SUPPLEMENTED BREAD BY NEAR INFRARED REFLECTANCE SPECTROSCOPY TECHNIQUE (NIRS)

5.1.1 Material and method

The nutritive value of bread samples was investigated using Near Infrared Reflectance Spectroscopy technique. Bread samples spectra were collected in the NIR regions in reflectance (1100-2500 nm) at 2 nm intervals using a NIR FOSS 5000 system (Denmark). The parameters investigated for each sample were: moisture, ash, crude protein, crude fat, total fibre, carbohydrate and caloric energy.
5.1.2 Results and discussion

The moisture, total fibre, protein, fat, minerals and carbohydrate content as well as the caloric energy of the five tested bread formulations were determined using NIRS technique and are presented in table 2. As expected, the moisture, total fibre, protein, fat and minerals levels increased proportional to the quantity of added BSG, while carbohydrates and energy values decreased.

<table>
<thead>
<tr>
<th>BSG, (%)</th>
<th>Moisture (%)</th>
<th>Total fiber (%)</th>
<th>Protein (%)</th>
<th>Lipids (%)</th>
<th>Minerals (%)</th>
<th>Carbohydrates (%)</th>
<th>Energy (kcal/100g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>37.43±2.12</td>
<td>0.81±0.06</td>
<td>6.64±0.3</td>
<td>0.39±0.02</td>
<td>0.44±0.02</td>
<td>53.69±3.75</td>
<td>244.83±6.12</td>
</tr>
<tr>
<td>5</td>
<td>38.51±1.23</td>
<td>1.91±0.08</td>
<td>7.49±0.86</td>
<td>0.48±0.09</td>
<td>0.68±0.01</td>
<td>49.16±1.96</td>
<td>230.92±4.27</td>
</tr>
<tr>
<td>10</td>
<td>40.02±0.91</td>
<td>2.8±0.21</td>
<td>8.28±0.44</td>
<td>0.61±0.01</td>
<td>0.92±0.06</td>
<td>46.22±2.14</td>
<td>223.49±5.09</td>
</tr>
<tr>
<td>15</td>
<td>41.14±1.02</td>
<td>3.76±0.34</td>
<td>9.13±0.67</td>
<td>0.72±0.01</td>
<td>1.17±0.21</td>
<td>43.82±1.82</td>
<td>218.28±2.98</td>
</tr>
<tr>
<td>20</td>
<td>42.09±0.83</td>
<td>4.52±0.18</td>
<td>10.03±1.12</td>
<td>0.97±0.00</td>
<td>1.29±0.07</td>
<td>40.46±2.03</td>
<td>210.69±3.56</td>
</tr>
</tbody>
</table>

The incorporated BSG had greatly influenced the total fiber content of the fortified bread samples. Thus, a 5% BSG flour addition was shown to double the total fiber amount of the bread while a 20% BSG bread formulation had a total fiber level five times higher than the reference bread sample (100% wheat flour). Stojceska and Ainsworth, (2008) reported also high levels for fiber content of BSG supplemented bread (6.3-11.5%) in comparison with the control sample (2.3%). Although the consumption of dietary fiber has important implications in human health, the fiber intake is commonly lower than recommended. Thus, the BSG enriched breads may be considered a good source of dietary fiber in order to attain the necessary daily fiber intake (28-36g/day) required for a healthy nutrition.

The increase in moisture content from 37.43% for the control sample to 42.09% for 20% BSG containing sample can be explained by the increasing fiber content which leads to higher water absorption during dough preparation. Contrariwise, carbohydrates amount decreased (from 53.69% to 40.46%) as the content of BSG in the samples increased. The main carbohydrate in the wheat flour is represented by starch, while the BSG contains only residual amounts of starch, this compound being consumed by the extensive amylolysis during the mashing process (Waters et al., 2012; Makowska et al., 2013).
According to recent studies, the predominant lipids identified in BSG were triglyceride (55% to 67% of all identified lipid compounds), followed by a notable amount of free fatty acids (from 18% up to 30%) with beneficial properties for health (Niemi et al., 2012). An addition of BSG up to 20% increases the amount of total lipids by almost 3 times compared to control sample. The data obtained are compatible with previously published work which also found a 2.5 to 4 times increase of the fat content of baked snacks containing 25% BSG (Ktenioudaki et al., 2013).

Micronutrients as minerals are also important components when considering the nutritional characteristic of a potential food ingredient. The minerals content was 0.44% for the control sample and increased to 1.29% for the bread with 20% BSG supplementation. The level of minerals reported in BSG by Waters et al., 2012 is of 1.13% (w/w), including relatively high amounts of calcium, magnesium and phosphorus.

5.2 ANALYSIS OF BREAD AROMA COMPOUNDS BY ITEX / GC-MS TECHNIQUE

5.2.1 Material and method

The extraction of volatile compounds was performed using the ITEX technique as described in a previous study (Socaci et al., 2014). All samples were analysed in triplicate. The volatile compounds were identified by matching the obtained mass spectra with the spectra of reference compounds from NIST27 and NIST147 mass spectra libraries and verified by comparison with retention indices drawn from www.pherobase.com or www.flavornet.org. Relative peak areas, being expressed in arbitrary units (a.u.), were considered to quantitatively compare the bread samples, one a.u. corresponding to 100000 units of peak area (Dong et al., 2013).

5.2.2 Result and discussion

One of the main issues associated with the incorporation of BSG in food products is the effect on flavour. The compounds identified from the volatile profile of the bread samples and their concentrations expressed as relative peak areas arbitrary units are listed in table 3.
Table 3

Volatile compounds of bread containing different levels of BSG

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Odour perception</th>
<th>0% BSG</th>
<th>5% BSG</th>
<th>10% BSG</th>
<th>15% BSG</th>
<th>20% BSG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohols</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Methyl-1-propanol</td>
<td>Wine, solvent</td>
<td>37.83</td>
<td>43.02</td>
<td>75.70</td>
<td>104.71</td>
<td>137.83</td>
</tr>
<tr>
<td>2-Methyl-1-butanol</td>
<td>Alcoholic, green, malt</td>
<td>6.46</td>
<td>8.17</td>
<td>18.81</td>
<td>34.95</td>
<td>42.57</td>
</tr>
<tr>
<td>3-Methyl-1-butanol</td>
<td>Malty, alcoholic, fruity, whiskey, burnt</td>
<td>82.15</td>
<td>106.16</td>
<td>185.01</td>
<td>291.41</td>
<td>312.81</td>
</tr>
<tr>
<td>Aldehydes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Methyl-propanal</td>
<td>Wine, solvent, malty</td>
<td>10.32</td>
<td>7.68</td>
<td>10.22</td>
<td>82.96</td>
<td>79.61</td>
</tr>
<tr>
<td>2-Methyl-butanal</td>
<td>Malty, buttery, oily, cocoa</td>
<td>9.84</td>
<td>6.33</td>
<td>10.41</td>
<td>75.05</td>
<td>84.74</td>
</tr>
<tr>
<td>3-Methyl-butanal</td>
<td>Buttery, oily, dark chocolate, cocoa,</td>
<td>15.46</td>
<td>12.08</td>
<td>21.90</td>
<td>130.35</td>
<td>136.75</td>
</tr>
<tr>
<td></td>
<td>almond</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hexanal</td>
<td>Green, grass, fat</td>
<td>4.08</td>
<td>4.03</td>
<td>5.83</td>
<td>12.69</td>
<td>15.30</td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>Almond, burnt sugar</td>
<td>3.30</td>
<td>4.55</td>
<td>4.77</td>
<td>5.82</td>
<td>5.58</td>
</tr>
<tr>
<td>Nonanal</td>
<td>Fat, citrus, green</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>2.03</td>
<td>1.12</td>
</tr>
<tr>
<td>Ketones</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,3-Butanedione</td>
<td>Buttery, cheesy</td>
<td>5.48</td>
<td>3.53</td>
<td>6.61</td>
<td>7.71</td>
<td>9.06</td>
</tr>
<tr>
<td>2,3-Pentanedione</td>
<td>Cream, butter</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>3.58</td>
<td>4.27</td>
</tr>
<tr>
<td>Acetophenone</td>
<td>Must, flower, almond</td>
<td>2.39</td>
<td>2.89</td>
<td>1.68</td>
<td>3.46</td>
<td>1.44</td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Pentyl-furan</td>
<td>Green bean, butter</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>8.91</td>
<td>9.55</td>
</tr>
<tr>
<td>Limonene</td>
<td>Citrus, mint</td>
<td>0.00</td>
<td>2.46</td>
<td>6.00</td>
<td>19.02</td>
<td>18.20</td>
</tr>
<tr>
<td></td>
<td>Pungent, caramel, ethereal, fruity,</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>rubbery, solvent-like</td>
<td>10.35</td>
<td>10.37</td>
<td>9.08</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

The majority of detected volatiles are typically found in wheat bread, being formed during fermentation, Maillard reaction or lipid oxidation. The predominant volatile constituents indicated by the peak areas obtained from GC-MS chromatograms are the alcohols (2-methyl-1-propanol, 3-methyl-1-butanol and 2-methyl-1-butanol) and the corresponding aldehydes (2-methyl-propanal, 3-methyl-butanal and 2-methyl-butanal). These compounds were found in three to eight times higher concentrations in the bread formulation containing 15% and 20% added BSG compare to the control sample. Their odour attribute has been generally described as malty flavour, suggesting that the differences in aroma of the bread formulations perceived by the panellists may be associated with the addition of BSG (Coghe et al., 2004).

Three volatiles, nonanal, 2-pentyl-furan and limonene, were identified only in the bread samples incorporating BSG flour. Nonanal and 2-pentyl-furan were detected only for bread formulations containing 15% and 20% BSG, while limone was found in all BSG bread samples, its concentration increasing with the amount of added BSG. Instead, toluene, a compound which odour attributes may vary from fruity, caramel to solvent-
like, was found in relative high amounts in the 0%, 5% and 10% BSG breads and absent in the samples with 15% and 20% BSG added.

Usually, the concentration of detected volatile compounds increases with the amount of incorporated BSG. Nevertheless, there are certain compounds, like 2-methylpropanal, 3-methylbutanal and 2-methylbutanal, which have a slightly lower concentration in 5% BSG formulation than in 0% BSG sample. Our findings corroborate with those of other authors and were explained by the factors that affect the release of the volatile compounds and by the changes in the bread samples microstructure induced by the addition of BSG (Dong et al., 2013).

The aromatic compounds listed in table 2 have a wide spectrum of pleasant and unpleasant odour descriptors, but the contribution of each compound to the aroma of the bread samples depends on its odour thresholds as well as on the product matrix. Also, the flavour imparted to the food by a certain volatile compound is directly related to its concentration, its release during mastication and the presence of other volatile compounds (Ktenioudaki et al., 2013).

5.3 METHODS TO HIGHLIGHT THE QUALITY OF BREAD

5.3.1 Material and method

- Determination of bread volume
- Determination of core porosity
- Determination of core elasticity
- Determination of height-diameter ratio (H/D)
- Determination of bread acidity

5.3.2 Results and discussion regarding BSG addition on bread characteristics

The bread aspect represents, for the great part of consumers, the main criterion by which they evaluate bread quality and it implicitly represents the main factor in the acquisition process. The influence of spent gain over bread quality parameters is shown in figures 11-15.
Fig. 11 Brewers' spent grains influence on bread volume

Fig. 12 Brewers' spent grains influence on bread H/D ratio

Fig. 13 Brewers' spent grains influence on bread porosity
The main consequence of adding fiber into bakery products is their decrease in volume. As we can see in figure 11, the relation between the percentage of BSG and the volume of bread can be described as a descending regression line where the correlation coefficient $r = -0.9853$ indicates a very strong relation between these two parameters. The bread volume decreased from $331.68 \, \text{cm}^3/100\text{g}$ for the reference sample (100% wheat flour) to $272.66 \, \text{cm}^3/100\text{g}$ for 20% BSG containing sample is caused by the reduction of gluten percentage in the dough and by decrease in the dough’s capacity to retain fermentation gases. Literature studies have shown that for an addition of up to 7% fiber-rich material, the volume of bread will decrease proportionally to the content of gluten proteins. Beyond this value, the volume of bread will decrease with a rate that is higher than the theoretical model (due to the decrease in gluten proteins) (Laurikainen et al.,
1998). Izzo and Franck assert that, when adding up to 20% fibers, the decrease tendency is insignificant while a 40-50% boost will make the bread unsatisfactory (Izzo and Franck, 1998). Another hypothesis suggests that, in the presence of fibers, due to the competition for the water, gluten proteins cannot receive enough hydration, hence rendering a weaker gluten network.

By observing the variation of the shape of the bread during baking, by comparison with the control sample, it was noticed that the fiber-rich dough establishes its shape and volume quicker, due to faster gelatinization of starch. Therefore, the correlation between the H/D ratio and the incorporated BSG percentage, graphically represented in figure 12 is described through a descending regression line. The determination coefficient ($R^2 = 0.885$) shows that in the case of the samples analyzed, the H/D ratio variation is owed in proportion of 88% to the variation of BSG content.

The influence of brewers’ spent grains over porosity and elasticity of the bread is represented in figures 13 and 14. The regression lines indicate in both cases very tight reverse relation, as both elasticity and porosity decrease when BSG percent increases. These phenomena are explained by the mechanical deterioration of gluten film by the fibers from brewers' spent grain. Also, microscopic investigation has shown that in bread with fiber content, the fine structure of the bread crumb marked by filaments and thin layers, specific to the white bread, is absent (Bordei, 2003).

As regards the acidity of bread containing BSG, graphically reproduced in figure 15 above, we can see a subtle increase, directly proportional to the percentage of BSG incorporated, a fact confirmed also by the positive regression coefficient we achieved ($r=0.9887$), which describes an intense positive relationship between these two parameters.

Is also remarkable the fact that, although brewers' spent grain has a quiet negative influence over these quality parameters of the bread, all the results found fall within the quality frame established by SR 91:2007. We can therefore state that by adding brewers' spent grain in a proportion of up to 20% in the composition of bakery products will not raise significant technological issues and neither will it affect the quality of final products.
5.4 MICROBIOLOGICAL EVALUATION OF BREAD

5.4.1 Determination of the total number of yeasts and molds in bread by colony count technique at 25 °C

Nowadays, there is a continuous need to improve the microbiological, nutritional and organoleptic attributes of bread. Microbiological spoilage, in particular mold growth, is often the major factor limiting the shelf life of bread and is a serious and costly problem for bakeries.

Bakery products are classified as products of intermediate moisture content and the nutritional composition will influence fungal and yeast growth. The traditional method by counting of colony forming units (cfu) has been used to quantify the molds and yeast population, also the moisture and pH of bread samples has been monitored.

According to the Order no.435/22.06.2011, published in the Official Gazette of Romania, the microbiological conditions for bread are formed just from the number of yeasts and fungal that must not exceed 100cfu/g. All the analyzed samples were, from the microbiological point of view, in the predicted limits of the present legislation and it was concluded that the different brewers spent grain incorporation levels did not have any effect on the shelf-life of the bread samples.

5.5 SENSORIAL EVALUATION OF BREAD

5.5.1 Acceptance test

Hedonic testing of the bread samples was conducted within 24 h after the bread was prepared, in the sensory evaluation laboratory of the Faculty of Food Science and Technology, Cluj-Napoca. Sensory profiling of bread samples was performed by 80 panellists. The panellists evaluated all five bread formulations for colour, aroma, taste, texture and overall acceptability using a 9-point hedonic scale with 0 being “extreme dislike” and 9 being “extreme like”.

5.5.2 Results and discussion

It is very important that the organoleptic properties of bread enhanced with brewer’s spent grain remained acceptable to consumers and the quality level similar to
the current commercially available products. The results of sensorial evaluation of bread samples containing different level of BSG substitution compared to the control sample (100% wheat flour) are shown in the table 4.

Table 4

<table>
<thead>
<tr>
<th>Bread sample</th>
<th>Colour</th>
<th>Aroma</th>
<th>Taste</th>
<th>Texture</th>
<th>Acceptability</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 %</td>
<td>7.9</td>
<td>7.58</td>
<td>7.58</td>
<td>7.9</td>
<td>7.8</td>
</tr>
<tr>
<td>5 %</td>
<td>7.9</td>
<td>7.9</td>
<td>7.63</td>
<td>7.9</td>
<td>7.88</td>
</tr>
<tr>
<td>10 %</td>
<td>7.88</td>
<td>7.68</td>
<td>7.53</td>
<td>7.78</td>
<td>7.83</td>
</tr>
<tr>
<td>15 %</td>
<td>7.48</td>
<td>7.2</td>
<td>7.3</td>
<td>7</td>
<td>7.33</td>
</tr>
<tr>
<td>20 %</td>
<td>7.7</td>
<td>7.1</td>
<td>7.03</td>
<td>6.75</td>
<td>7.35</td>
</tr>
</tbody>
</table>

A decrease in acceptability was observed when the levels of BSG were higher than 10%. The sample with 5% BSG substitution had the highest acceptability score (7.88) as well as for the other organoleptic characteristics. Also, it can be observed that the bread samples with 5% and 10% added BSG showed similar results to the control sample obtained from wheat flour only. An increase % of added BSG (15% and 20%) resulted in a lower scores for the overall acceptability characteristics (7.33 respectively 7.35).

The attributes which influenced the panellist’s acceptability were predominantly taste and texture, with samples containing both 15% and 20% BSG receiving significantly lower scores when compared to the control samples, and again with the samples supplemented with 5% and 10% BSG. For all the bread samples the scores for texture decreased with increase in BSG substitution and simultaneously with the increase in fiber content. In general, the addition of rich fiber ingredients has led to an increase in the hardness of crumb by cross linking gluten proteins (Stojceska and Ainsworth, 2008).

The colour of the bread slices became visually darker as the level of BSG increased. Also, the darker colour of the crumbs was reported by Hu et al. (2007) to be directly related to the increase in fibre content.

The considerable increase in the amounts of volatile compounds with malty flavour in the case of 15% and 20% BSG formulations compare with 0%, 5% and 10% BSG samples, could be responsible for the poor scores that were obtained for those samples.

Finally, the sensorial evaluation revealed that breads with BSG substitution up to 10 % achieved higher score than the control white bread sample.
CONCLUSIONS

The general conclusions resulting from the research carried out in PhD thesis entitled "RESEARCH REGARDING THE IDENTIFICATION AND EXPLOITATION OF BIOLOGICALLY ACTIVE COMPOUNDS FROM BREWERS' SPENT GRAIN BY-PRODUCT" can be formulated briefly as follows:

During the mashing process, the endosperm is solubilized almost entirely and fermentable substances useful for the nutrition of yeast are extracted in the mash. Hence, brewers' spent grain has considerable levels of biologically active compounds with high exploitation potential. These quality characteristics, in addition to its low cost and high levels of availability, make BSG suitable as a food ingredient.

Four formulations of bread supplemented with BSG were developed and analyzed in order to assess the contribution of BSG to the nutritional value of the fortified end-product. The substitution of wheat flour with 5-20% BSG resulted in bread formulations with enhanced nutritional value (increased fiber, protein, fat and minerals content) and with pleasant flavor attributes imparted by the characteristic volatile compounds.

The overall acceptability of the BSG enriched breads was performed by sensorial analysis, revealing good organoleptic attributes for the samples up to 10% BSG flour.

All the analyzed samples were, from the microbiological point of view, in the predicted limits of the present legislation and it was concluded that the different brewers spent grain incorporation levels did not have any effect on the shelf-life of the bread samples.

The obtained results emphasized the importance and the opportunities of re-use this agro-industrial by-product.
REFERENCES


