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From Grain By-products to Functional Food Through Innovative Processing:

"Effect of micronization (ultra-fine grinding) with and without application of cryogenic cooling on bioactive compounds of millet bran and buckwheat hulls"

Receiving organisation: University of Zagreb

Laboratory for Cereal Chemistry and Technology

Internship supervisor

Nikolina Čukelj
Assistant Professor
ncukelj@pbf.hr

Author: Victor Rodrigo Victoria González

Table of contents

List of tables

List of figures

List of annexes

List of abbreviations

ABTS	2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
CVD	Cardiovascular diseases
DPPH	2, 2-diphenyl-1-picryhydrazyl radical
FRAP	Ferric Reducing Antioxidant Power
GAE	Gallic Acid Equivalents
g.d.w.	Gram of dry weight
HSD	Honestly Significant Difference
IBS	Irritable Bowel Syndrome
TE	Trolox Equivalents
TPC	Total Phenolic Content

About University of Zagreb

The University of Zagreb, established in 1669, is the Croatia's flagship university and one of the largest higher education institutions in South-Eastern Europe with over 70,000 students and nearly 8000 teachers. As a public institution, University of Zagreb offers a wide range of undergraduate and graduate programs covering all fields of knowledge, from arts to natural science, law, humanities, engineering and biotechnology. At the university, teaching and research are organized into 29 Faculties, plus the 3 Arts Academies and the University Centre for Croatian Studies, each of which comprises multiples committed departments to ensuring academic excellence.

Faculty of Food Technology and Biotechnology

The Faculty of Food Technology and Biotechnology at the University of Zagreb is the leading institution in Croatia in terms of science, research and education in the fields of biotechnology, food technology and nutrition science. This unit consists of 6 departments that have 27 laboratories and 7 sections responsible for the education at undergraduate, graduate and postgraduate level, besides having the Food Control Center, a certified laboratory for the control of food safety and quality and Objects of Common Use in the EU. The internship was conducted at the Laboratory for Cereal Chemistry and Technology, which is part of the Department of Food Engineering. Currently, laboratory team consists of one full professor, two assistant professors, one senior PhD researcher, and one technician.

The laboratory works in a disciplinary approach, with research activities focused on different aspects of cereal food products, mainly on gluten and non-gluten bread, with or without sourdough addition. Research in general includes: recipe development (laboratory baking), applying innovative technologies in production process (vacuum cooling, high pressure, ultrasound, cryomilling), combined traditional (sourdough) and modern technologies for bakery industry, determination of nutritional profile and bioactive compounds content of product, shelf-life trials, texture measurements, and sensory evaluation.

The current project: "From Grain Byproducts to Functional Food through Innovative Processing" (IP-2016-06-3) funded by the Croatian Science Foundation is aimed at researching, integrating and deducing information about the influence of application of modern processing technologies (cryomilling and high intensity ultrasound with/without enzyme hydrolysis) on grain industry byproducts nutritional value (digestibility of macro and micronutrients, content of free bioactive functional components) in chosen byproducts, that can be later used as is, or as an ingredient for enrichment of food products and foods for special dietary needs.

I. Introduction

Cereals are staple foods throughout the world by providing significant quantities of energy, carbohydrates, proteins, B vitamins, minerals and fibers to the human diet. Apart from being an important part of diet, whole-grain cereals contain a range of substances which may provide desirable health benefits to reduce the risk of chronic diseases, these substances are often referred to as bioactive compounds (Gani *et al*., 2012).

The bioactive compounds in whole-grain cereals are unevenly distributed. Some are present in significant quantities in the endosperm, but most are in the bran (especially the aleurone layer) and germ fractions (Singh *et al*., 2016). However, they have very low bioavailability because the complex bran matrix hinders their access to the digestive enzymes which contribute to their release in the human gastrointestinal tract (Fardet, 2010).

Aside from cereal brans, bioactive compounds are widely present in other grain crop by-products: oilseed cakes. It is known that hulls (bran), remaining after extraction of the oil from oilseeds, contain appreciable amount of bioactive compounds like lignans, polyphenols, and phytosterols, nevertheless their bioavailability still remains low (Nagaraj, 2009).

Because of the high potential of cereal brans and oilseed cakes, recent scientific research is focused on liberating these bioactive compounds from bran matrices, in order to increase their accessibility, which has been demonstrated to be effective in enhancing their bioavailability (Wang *et al*., 2014). For this purpose, modern technologies in by-products processing (micronization with or without cryo-cooling, high intensity ultrasound treatment, and spontaneous fermentation with or without specific enzymes addition) have been developed (Saleh *et al*., 2013).

Considering the growing interest on grain industry by-products and their potential usability as functional ingredients for the nutritional improvement of traditional bakery products (Martins *et al*., 2017), this section of the research project "From Grain By-products to Functional Food through Innovative Processing" aims to add value to two chosen underutilized by-products (millet bran and buckwheat hulls) by providing accurate information of their contents of bioactive compounds.

Within this framework, the objective of this study was to investigate and compare the influence of micronization (ultra-fine grinding) with and without application of cryogenic cooling on the amount of bioactive compounds of millet bran and buckwheat hulls. Besides innovative cryo-grinding technology, tool for accomplishment of this goal has been application of assay methods 2, 2-diphenyl-1-picryhydrazyl radical (DPPH), ferric reducing antioxidant power (FRAP) and 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) to determine the antioxidant activities as well as total phenolic contents (TPC).

II. Goal

To investigate the effect of micronization (ultra-fine grinding) with and without application of cryogenic cooling on the amount of bioactive compounds of millet bran and buckwheat hulls.

2.1 Objective

To examine how cryogenic (ultra-fine) grinding influence the antioxidant activities and total phenolic contents of millet bran and buckwheat hulls.

III. Background

3.1 Cereal bran fractionation technologies and processing

The milling process of grains is generally associated with the disintegration and breakdown of the outer layers from the starchy endosperm generating two main product streams: the white flour and the bran. The bran fractionation is among the most important processing practices in the cereal industry for producing dietary fibers or antioxidant enriched flours which could be incorporated in conventional or whole grain bakery products as functional ingredients. Generally, the fractionation process of cereal bran can be classified in two main categories: wet and dry (Soukoulis & Aprea, 2012).

The wet fractionation is based on the partial hydrolysis of the insoluble material of the bran and the physical separation of both soluble and insoluble compounds. Wet fractionation could be classified according to the type of the hydrolysis: soluble matter extraction process (enzymatic hydrolysis) or extraction using aqueous, alkali or polar organic solvents (Kvist *et al*., 2010). Although wet fractionation is a quite popular processing practice for the recovery of the functional components from cereal bran, its high water and energy demands have led to the development of alternative processes based on dry fractionation (Teeter *et al*., 2011). Over the last years much research has been conducted for the expansion of dry fractionation, from the conventional air classification and milling techniques to the innovative electrostatic separation, micro-milling and ultra-fine grinding methods (Hemery *et al*., 2011).

Dry fractionation can be divided in two major techniques: the histological and the macromolecular fractionation. The goal of the histological fractionation is to classify the cereal bran material according to the tissues: aleurone, testa and pericarp particles. Macromolecular fractionation aims both to separate and fractionate the obtained bran tissues according to their subcellular constituents (Hemery *et al*., 2010). For achieving this, a small particle size is required, thus specific milling techniques can be implemented such as jet milling, ultra-fine grinding or pin milling (Antoine *et al*., 2004; Hemery *et al*., 2011).

3.1.1 Micronization (ultra-fine grinding)

Micronization is the mechanical process of reducing the average diameter of a solid material's particles (Zhu *et al*., 2014). Ultra-fine grinding means an innovative alternative to this process which reduce a sample material into extremely fine particles by combination of high friction and impact. Generally, grinding equipment can be operated at room temperature or under cryogenic conditions (where an integrated cooling system ensures that the sample is continually cooled with liquid nitrogen at –196°C before and during the grinding process) (Retsch, 2017). Ultra-fine grinding has recently gained importance in connection with the development of new functional materials in various industrial application including the foodstuff sector (Zhu *et al*., 2010).

3.2 Bran fractions: bioactive compounds

Depending on the fractionation process and conditions, the type of bran and its pre-treatment, a great number of bran fractions differing in their compositional, chemical, functional, physiological and technological properties can be produced. Nutritionally, bran fractions produced by milling are rich in fiber, minerals, vitamin B6, thiamine, folate and vitamin E and some phytochemicals, in particular antioxidants such as phenolic compounds (Shewry, 2009). The physiological effects of bran can be split into nutritional effects (from the nutrients present), mechanical effects (mainly on the gastrointestinal tract, due to the fiber content) and antioxidant effects (arising from the phytonutrients present such as phenolic acid) (Stevenson et al., 2012).

Studies have shown that cereal brans may have a beneficial effect on the prevention of diseases, including some cancers (in particular colorectal cancer), CVD, obesity and some gastrointestinal diseases, including diverticular disease, constipation and irritable bowel syndrome (IBS) (Fardet, 2010). However, bioavailability of bioactive compounds is affected by the food matrix as well as processing conditions. According to a study of Zhou *et al*. (2004), micronization changed the physical structure of the bran matrix, increasing thus the particle surface area and therefore the availability of compounds involved in the antioxidant capacity of bran. Since the reduction of particle sizes might cause the release of some antioxidant compounds, it is valuable to find out the antioxidant properties of bran affected by ultra-fine grinding in both ambient and cryogenic conditions (Zhu *et al*., 2010).

3.3 Applications in food industry

The diverse functional and technological properties of brans together with their particular physiological and nutritional aspects have led to a great interest on their incorporation as main or secondary components in different groups of food products including bakery and confectionery products, breakfast cereals and extruded foodstuffs, emulsions and functional dairy products (including ice cream), pasta products, sauces and dressings. Functional and technological properties of brans have been applied in the production of beverages as well (Soukoulis & Aprea, 2012).

3.3.1 Bakery Products

In a recent study, Noort *et al*. (2010), have investigated the impact of different wheat bran fractions (a coarse and an aleurone enriched one) produced by three successive milling processes (rotor, impact and jet) on bread making quality (dough mixing properties, gluten aggregation, and loaf volume). Although, the addition of both wheat bran fractions had a deteriorative effect on the bread making properties due to the gluten-fibre interactions, the researchers reported that the size reduction of the bran particles enhanced the adverse effects. The latter effect was attributed to two mechanisms: a) increase of the interaction surface for the more reactive components (ferulic acid monomers with gluten proteins) and b) liberation of reactive components due to cell breakage.

IV. Materials and methods

4.1 Bran samples

Two grain by-products were assayed for antioxidant activity and phenolic content: Proso millet (variety Sonček) bran and buckwheat (variety Ljiljana) hulls. Proso millet bran, from the 2017 crop year was collected after industrial decortication with abrasive disks (Mlinopek, Murska Sobota, Slovenia). The grain was not hydrothermally processed before decortication. The bran was size graded using screens of 1000 μm and 500 μm openings. The inner bran sizing <0.5mm acted as the "Control native" (control<500) and was kept frozen in tinted, air-tight containers for no more than six months prior to analysis. Likewise, buckwheat seeds were obtained from the 2017 harvest year (Semenarna Ljubljana, Slovenia) and dehulled at mill Pukanić (Velika Gorica, Croatia). The assays were carried out on samples from the micronization (ultra-fine grinding) of millet bran and buckwheat hulls with and without application of cryogenic cooling, during 2, 4, 8, and 12 minutes.

4.2 Grinding process

In order to obtain powdered material, each sample was ground in eight batches of about 8 g by using a Cryogenic Mixer Mill (CryoMill; Retsch GmbH, Haan, Germany) (Annex n° 2) in two different grinding modes. Half of these batches was ground at room temperature, while the other half was ground in cryogenic conditions by continuous supply of liquid nitrogen (−196 °C) which is stored in an APOLLO® 50 Dewar vessel (Retsch GmbH, Haan, Germany). All device parts were cleaned carefully before each sample processing and the operating conditions of the grinding are presented in Table 1. Finally, resulting powders were collected in plastic bottles and frozen at -18 °C until used for analyses.

Table 1 Operating conditions for the grinding of bran grain samples.

	Bran grain samples	
Grinding mode	Cryogenic	Ambient
Grinding jar (mL)	50	50
Grinding balls (mm)	1 ball x 25	1 ball x 25
Sample quantity (g)	8	8
Precooling time (min)	4	0
Grinding time (min)	2, 4, 8, 12	2, 4, 8, 12
Frequency (Hz)	30	30

4.3 Moisture content analysis

The moisture content of the ground brans was analyzed by oven drying a 2 g sample at 130°C for 90 min. After exactly 90 min, the sample was weighted and re-dried once again at 130°C for 30 min. Analysis was made in duplicate.

4.4 Extraction of Free polar compounds

Free polar compounds were extracted from ground hulls of millet and buckwheat, according to Li *et al.* (2009), with slight modifications. 250 mg of each powdered material were transferred to 2 mL Eppendorf safe-lock tubes and then extracted with 1 mL of 80% ethanol (v/v) on a MS 3 Basic Vortex Mixer (IKA) for 10 min at room temperature. After shaking, the tubes were placed in an ultrasonic bath (Bandelin Sonorex Super RK100H) maintained at 20°C for 10 min. The resultant slurry was centrifuged for 15 min at 8,000 rpm (Thermo Fisher Scientific Micro CL21 Centrifuge) and supernatant was collected in new safe-lock tubes, which were then placed under continuous nitrogen gas flush (MICROVAP 118 nitrogen evaporator) with gentle heating (40 °C) for evaporation.

The ethanol extraction procedure was repeated for two times and combined supernatants were evaporated to dryness. Evaporated extracts were kept in a freezer at −20°C until analyses. On the day of analyses, before being used, 500 µL of methanol were added to each tube and vortexed for a few seconds, followed by centrifugation for 10 min at 14000 rpm and transfer of the extracts into new tubes. Both extracts were employed without dilution for the determination of Total Phenolic Content (TPC), while for all the antioxidant activity methods, extracts were diluted with methanol as follows:

- DPPH assay: millet (1:5 dilution), buckwheat (1:20 dilution).
- ABTS assay: millet (1:5 dilution), buckwheat (1:10 dilution).
- FRAP assay: millet (1:5 dilution), buckwheat (1:10 dilution).

4.5 Antioxidant activity

4.5.1 DPPH radical scavenging activity assay

The DPPH radical scavenging activity assay was carried out according to Belščak *et al.* (2009), with some modifications. The stock solution (0.06 mM) was freshly prepared by dissolving 0.00236 g of DPPH with 100 mL anhydrous methanol. In each microcuvette, 20 µL of the appropriate extract dilution and 950 µL of DPPH reagent were mixed and kept in the dark at room temperature to react. After 30 min, the absorbance was measured at 517 nm on a Specord 50 ANALYTIKJENA® UV/Vis spectrophotometer against a control obtained by replacing the extract with the same volume of methanol. All measurements were performed in three parallels by extraction (one spectrophotometric measurement per sample).

The calibration curve (Annex n° 1) was made from a freshly solution of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) in methanol at different concentrations (0.050–0.250 mg/mL). DPPH radical scavenging activity of extracts was calculated according to the following equation:

$$DPPH\ radical\ scavenging\ activity\ (\%) = \left[\frac{Abs_{control} - Abs_{sample}}{Abs_{control}}\right] x\ 100$$

Where $Abs_{control}$ and Abs_{sample} are absorbance values for control and sample, respectively, after 30 min. The results were expressed as mmol Trolox equivalents (TE) per gram of dry weight.

4.5.2 ABTS radical scavenging activity assay

ABTS radical scavenging activity was measured following a reported procedure (Belščak *et al*., 2009) with some modifications. The ABTS reagent was prepared by reacting 4912 µL of 7 mmol/L ABTS aqueous solution with 88 µL of 140 mmol/L potassium persulfate ($K_2S_2O_8$). Thus, the final potassium persulfate concentration was 2.45 mmol/L. The mixture was kept in the dark at room temperature for 12 to 16 hours before use. The ABTS reagent was further diluted with 96% ethanol (v/v) to obtain a 1% solution, which was measured at 734 nm in order to get an absorbance of 0.700 ± 0.02.

In each regular cuvette, 20 µL of the appropriate extract dilution and 2 mL of 1% ABTS solution were mixed. The Absorbance at 734 nm was measured after a reaction time of 6 min at room temperature in the dark, together with a control (methanol instead of extract), by using the aforementioned UV/Vis spectrophotometer. The percent antioxidant activity of the sample was determined using the following formula:

$$Antioxidant\ activity\ (\%) = \left[\frac{Abs_{control} - Abs_{sample}}{Abs_{control}}\right] x\ 100$$

Where $Abs_{control}$ and Abs_{sample} are absorbance values for control and sample, respectively, after 30 min.

The analyses were carried out in triplicate for each sample. A calibration curve (Annex n° 1) was freshly prepared with different concentrations of Trolox in methanol (ranging from 0.075 to 0.450 mg/mL) and the results were expressed as mmol Trolox equivalents (TE) per gram of dry weight.

4.5.3 FRAP (Ferric Reducing Antioxidant Power Assay)

The FRAP assay was carried out according to the procedure of Belščak *et al*. (2009), with some modifications. The FRAP reagent was freshly prepared by mixing 25 mL of acetate buffer (300 mmol/L, pH 3.6), 2.5 mL of 10 mmol/L TPTZ solution (in 40 mmol/L HCl) and 2.5 mL of 20 mmol/L $FeCl3{\cdot}6H_2O$ solution in a ratio of 10:1:1 (v/v), respectively. The mixture was heated at 37°C in a Stuart® SBS40 shaking water bath prior to use and kept at this temperature. In each microcuvette, 1 mL of FRAP reagent and 10 µL of the appropriate extract dilution were mixed. The absorbance readings of the mixtures were started after 4 min of reaction and they were performed at 593 nm in the aforementioned UV/Vis spectrophotometer. The control was consisted of methanol.

The assays were performed in triplicate and the final absorbance of each sample was compared with those obtained from the calibration curve (Annex n° 1) constructed from freshly solution of Trolox in methanol (0.050–0.250 mg/mL). Results were expressed as the FRAP mmol Trolox equivalents (TE) per gram of dry weight.

4.6 Total Phenolic Content (TPC)

The total phenolic content (TPC) of each extract was determined using the Folin-Ciocalteu colorimetric method as described by Yu *et al*. (2002), with slight modifications. Briefly, 15 µl of extract were added to a regular cuvette which already contained 400 µl of distilled water. After the sample, 100 µl of Folin–Ciocalteau reagent were added, followed after 3 min by 300 µl of 20% Na_2CO_3 solution. Finally, the mixture was brought up to 2 ml by adding 1185 µL of distilled water and vortex-mixed. Subsequently, the reaction mixture was allowed to stand 2 hours in the dark at room temperature and then the absorbance was measured at 765 nm in the aforementioned UV-VIS Spectrophotometer against a control (methanol instead of sample).

The TPC was assessed by plotting a Gallic acid calibration curve (from 0.10 to 1.50 mg/mL) (Annex n° 1) and expressed as mg Gallic Acid Equivalents (GAE) per gram of dry weight, which was calculated using the formula, $y = 0.8132\ x + 0.0148$, where, y is the absorbance at 765 nm and x is the amount of gallic acid equivalent (mg/mL). Data are reported as a mean value ± standard deviation (SD) for three replications.

4.7 Statistical analysis

The results obtained in this study were reported as mean ± standard deviation of triplicate determinations. The differences of mean values among grinding times were determined by one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) multiple rank tests ($P \leq 0.05$), significance level. Statistical comparisons were analyzed by STATISTICA, v. 13 (StatSoft Inc).

V. Results and discussion

5.1 Millet bran

The antioxidant activities and total phenolic contents of millet bran after grinding at room temperature (ambient) and under cryogenic conditions are shown in Table 3. These grinding processes had significant differences ($P \leq 0.05$) in the antioxidant activities measured by DPPH, FRAP, and ABTS assays.

The values of DPPH-measured antioxidant activity were ranged from 5.76 to 6.58 mmol TE/g.d.w. for cryo-grinding and 3.88 to 5.24 mmol TE/g.d.w. for ambient grinding. These results showed that after cryogenic grinding, millet bran possess significantly stronger ($p \leq 0.05$) antioxidant activity than control<500 (4.87 mmol TE/g.d.w.) and ground bran in conventional conditions (Figure 1). This could be explained by the fact that cryogenic grinding efficiently decrease the particle size of bran, increasing thus the particle surface area, which can result in a higher release of bioactive compounds from the bran matrix due to higher solvent compounds interactions, and can therefore increase the bioaccessibility and/or bioavailability of these compounds (Hemery *et al.*, 2011).

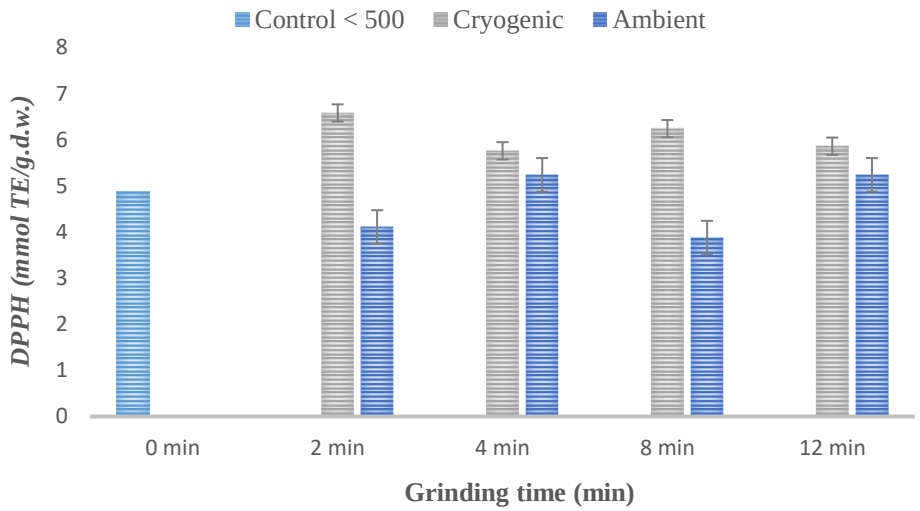


Figure 1 Effects of cryogenic and ambient ultra-fine grinding through different grinding times on radical DPPH scavenging activity of millet bran.

Regarding the Ferric reducing antioxidant power (FRAP), ambient grinding had higher antioxidant activity after 2 and 8 min (12.15 and 13.35 mmol TE/g.d.w, respectively) than control<500 (8.10 mmol TE/g.d.w). However, the proportion of fine particles decreased significantly ($P \leq 0.05$) for both processes after 4 and 12 min. These values of FRAP-measured antioxidant activity might be due to the slight variations in the moisture content of the samples with respect to control<500 (10.44%) (Table 2), which can produce more medium size particles and possibly leading to a lower contact between particles and free radicals (Balasubramanian *et al.*, 2011).

Zhou *et al*. (2004), demonstrated that micronization increased the antioxidant activity of aleurone layer of bran which was measured by ABTS assay. In this study, results revealed that the antioxidant activity varied significantly ($P \leq 0.05$) between both milling processes as well as grinding times. On one hand, ambient grinding had higher values of ABTS-measured antioxidant activity after 2 and 12 min (19.01 and 14.75 mmol TE/g.d.w., respectively) than cryogenic grinding. On the other hand, the obtained value from bran ground 8 min in cryogenic conditions (14.42 mmol TE/g.d.w.) was significantly stronger ($P \leq 0.05$) than ambient process. Although all these values were greater than control<500 (14.01 mmol TE/g.d.w.), it can be deduced that the grinding time, had slight more influence on the particle size during ambient grinding than during cryogenic grinding (Hemery *et al*., 2011).

Finally, results also indicated that significant difference ($P \leq 0.05$) in total phenolic contents was observed. According to Zielinski *et al*. (2006), the hydrothermal treatment of foods may affect their content of phytochemicals. Because of the whole proso millet was not hydrothermally processed before decortication, these kind of losses were discarded. Thus, as it can be seen from Figure 2, cryogenic grinding showed higher TPC than control<500 (3.00 mg GAE/g.d.w.) and ambient grinding at 4, 8 and 12 min, having this latter tested time the highest concentration (4.05 mg GAE/g.d.w.).

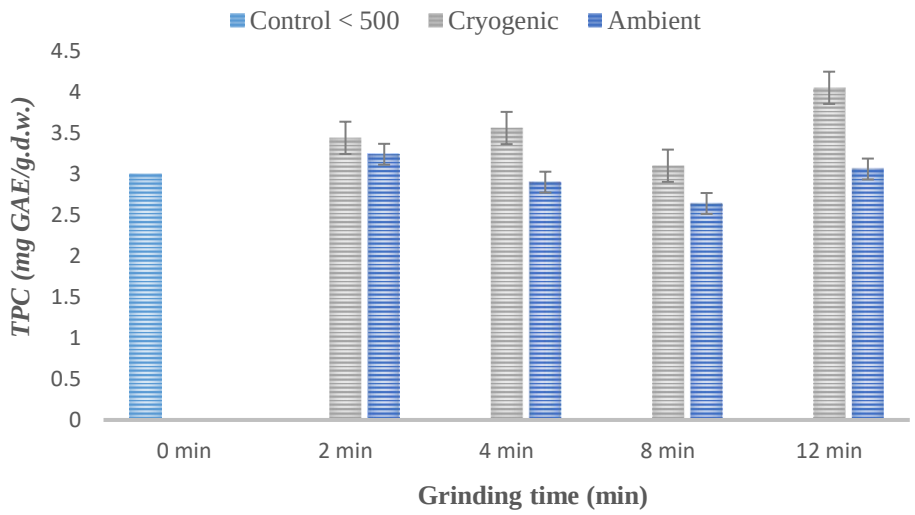


Figure 2 Effects of cryogenic and ambient ultra-fine grinding through different grinding times on Total Phenolic Content of millet bran.

In general, the amounts detected in millet bran fraction were significantly different ($P \leq 0.05$) to the one published in study by Abozed *et al*. (2014), for bran of Gemiza-9 (common wheat), where it was found TPC of 1.99 mg GAE/g.d.w. Those differences could be due to the effect of micronization with cryo-cooling. As stated by Rosa *et al*. (2013), increasing the brittleness of regular coarse bran by the use of very low temperatures positively affect the TPC. According to Wilczek *et al*. (2004), cryogenic grinding is also said to limit the re-agglomeration of particles and the destruction of thermo-sensitive compounds. Differences could be also due to the solvent used for the extraction process (Sulaiman *et al*., 2011).

In contrast with cryogenic grinding, the outcomes from the ambient grinding showed that TPC-measured decreased slightly after 4 and 8 min of milling (2.90 and 2.64 mg GAE/g.d.w., respectively). This decrease of TPC could be explained by high-energy impacts from grinding balls, the considerable development of heat in the milling jar or even by the extended milling times (Craeyveld *et al*., 2009).

5.2 Buckwheat hulls

The values of antioxidant activities (DPPH, ABTS and FRAP) and total phenolic contents (TPC) of buckwheat hulls after cryogenic and ambient ultrafine grinding are given in Table 4. As expected, cryogenic treatment had, in general, a greater content of bioactive compounds than ambient treatment. In fact, application of cryogenic cooling increased significantly ($P \leq 0.05$) DPPH antioxidant activities with respect to conventional grinding, except for 12 min tested time (12.57 mmol TE/g.d.w.). These results also suggest that with the increase in moisture level (Table 2), buckwheat could become slightly tougher, resulting in more consumption of energy (Balasubramanian *et al*., 2011).

Moreover, ABTS varied greatly among different grinding processes but also between grinding times. From results summarized in Figure 3, it may be concluded that, regardless of the chosen grinding process, a longer grinding time can significantly increase ($P \leq 0.05$) the antioxidant activity of the sample. Despite that, the values of cryogenic grinding demonstrated to be significantly higher ($P \leq 0.05$) than ambient grinding. For example, it can be assumed that the proportion of fine particles was higher after 2 min of cryogenic grinding (52.54 mmol TE/g.d.w.) than after 2 min of ambient grinding (39.26 mmol TE/g.d.w.), which means that the hulls ground for 2 min at ambient temperature still contained a high proportion of coarse particles (Hemery *et al*., 2010). In addition, the increase in moisture [control<500: (11.10%)] did not seem to influence the antioxidant activity measured.

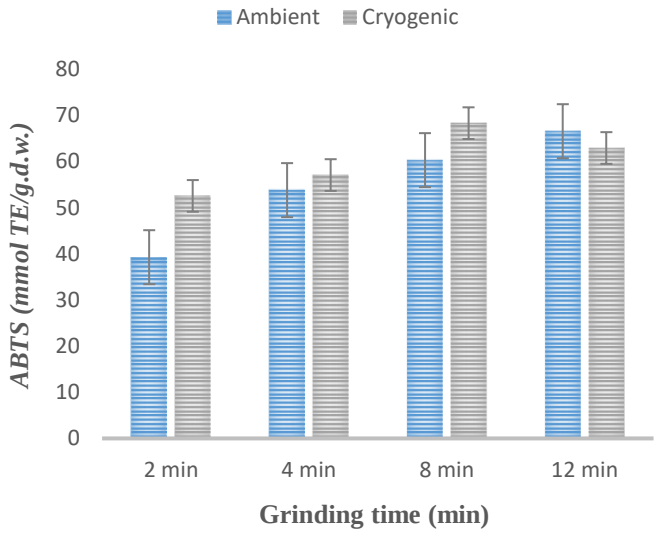


Figure 3 Effects of cryogenic and ambient ultra-fine grinding through different grinding times on ABTS radical scavenging activity of buckwheat hulls.

The values of FRAP-measured antioxidant activity obtained after 2 and 4 min (24.71 and 30.46 mmol TE/g.d.w., respectively) of cryogenic ultra-fine grinding increased significantly ($P \leq 0.05$) compared to ambient grinding values. On the other hand, cryogenic treatment also displayed a low antioxidant activity after 8 min (18.07 mmol TE/g.d.w.), which was not significantly different ($P > 0.05$) from that of the ambient grinding. These results suggest that during the first 4 minutes, more fine particles were observed under cryogenic conditions than for grinding at ambient temperature, and that fine particles were obtained faster, in particular for the intermediate layers (Hemery *et al*., 2010).

The results of total phenolic content (TPC) showed, that values were variable between grinding processes. Thus, hulls had in general the highest TPC after the cryo-grinding (ranged from 4.16 to 6.06 mg GAE/g.d.w.). These results are significantly greater ($P \leq 0.05$) than those published by Ragaee *et al*. (2006), who determined TPC in unground wheat and rye to obtain 0.56 mg GAE/g.d.w. and 1.03 mg GAE/g.d.w. respectively, thus demonstrating the positive effect of the cryogenic fragmentation. Besides, it was demonstrated by Zielinski *et al*. (2006), that extrusion of buckwheat seeds increase the phenolic acids content primarily due to the increased release of phenolic compounds from their matrix.

Table 2 Moisture content (%) of millet bran and buckwheat hulls.

Moisture content (%)									
Sample	Control<500	Cryogenic				Ambient			
	0 min	2 min	4 min	8 min	12 min	2 min	4 min	8 min	12 min
Millet bran	10.44	10.49	10.28	10.12	10.68	9.89	9.81	9.27	9.09
Buckwheat hulls	11.10	12.86	12.66	12.71	13.24	12.94	12.75	11.89	11.45

Table 3 Effects of ultra-fine grinding on TPC, DPPH, ABTS and FRAP of millet bran.

Grinding time	**TPC** (mg GE/g.d.w)		**DPPH** (mmol TE/g d.w.)		**ABTS** (mmol TE/g d.w.)		**FRAP** (mmol TE/g d.w.)	
	Cryogenic	Ambient	Cryogenic	Ambient	Cryogenic	Ambient	Cryogenic	Ambient
0 min	3.00 ± 0.01		4.87 ± 0.01		14.01 ± 0.03		8.10 ± 0.02	
2 min	3.44 ± 0.06^{a1}	3.24 ± 0.05^{b1}	6.58 ± 0.01^{c2}	4.11 ± 0.10^{a1}	14.24 ± 0.02^{b1}	19.01 ± 0.00^{d2}	7.47 ± 0.01^{b1}	12.15 ± 0.03^{c2}
4 min	3.56 ± 0.06^{a2}	2.90 ± 0.06^{ab1}	5.76 ± 0.01^{a2}	5.24 ± 0.10^{b1}	13.27 ± 0.04^{a1}	13.61 ± 0.02^{b1}	6.35 ± 0.02^{a1}	6.25 ± 0.02^{a1}
8 min	3.10 ± 0.04^{a2}	2.64 ± 0.03^{a1}	6.24 ± 0.04^{bc2}	3.88 ± 0.10^{a1}	14.42 ± 0.05^{b2}	12.95 ± 0.01^{a1}	7.13 ± 0.03^{b1}	13.35 ± 0.03^{d2}
12 min	4.05 ± 0.00^{b2}	3.06 ± 0.07^{ab1}	5.86 ± 0.02^{ab2}	5.24 ± 0.00^{b1}	14.03 ± 0.02^{b1}	14.75 ± 0.00^{c2}	7.50 ± 0.00^{b1}	7.68 ± 0.03^{b1}

Same letters in each column and same numbers in each raw for different assays are not significantly different ($P > 0.05$).

Table 4 Effects of ultra-fine grinding on TPC, DPPH, ABTS and FRAP of buckwheat hulls.

Grinding time	**TPC** (mg GE/g.d.w)		**DPPH** (mmol TE/g d.w.)		**ABTS** (mmol TE/g d.w.)		**FRAP** (mmol TE/g d.w.)	
	Cryogenic	Ambient	Cryogenic	Ambient	Cryogenic	Ambient	Cryogenic	Ambient
2 min	4.34 ± 0.08^{a2}	3.71 ± 0.07^{a1}	10.06 ± 0.10^{b2}	6.35 ± 0.00^{b1}	52.54 ± 0.03^{a2}	39.26 ± 0.02^{a1}	24.71 ± 0.04^{b2}	14.34 ± 0.02^{a1}
4 min	5.47 ± 0.12^{b2}	3.88 ± 0.04^{a1}	8.17 ± 0.10^{a2}	4.59 ± 0.10^{a1}	57.06 ± 0.01^{b2}	53.79 ± 0.02^{b1}	30.46 ± 0.03^{c2}	18.65 ± 0.00^{b1}
8 min	4.16 ± 0.06^{a1}	5.75 ± 0.08^{c2}	11.15 ± 0.10^{c2}	9.93 ± 0.00^{c1}	68.30 ± 0.03^{d2}	60.29 ± 0.05^{c1}	18.07 ± 0.00^{a1}	18.45 ± 0.02^{b1}
12 min	6.06 ± 0.11^{c2}	4.36 ± 0.08^{b1}	10.37 ± 0.10^{b1}	12.57 ± 0.10^{d2}	62.92 ± 0.02^{c1}	66.56 ± 0.02^{d2}	18.97 ± 0.03^{a1}	20.64 ± 0.01^{c2}

Same letters in each column and same numbers in each raw for different assays are not significantly different ($P > 0.05$).

VI. Conclusions

In conclusion, the outcomes obtained about phenolic contents and antioxidant activities of ground millet bran and buckwheat hulls have demonstrated, in general, that ultra-fine grinding achieves a significantly higher performance under cryogenic conditions than performed at room temperature. Interestingly, the antioxidant activities and total phenolic contents of millet bran fraction were found to be highest than the control (<500 μm) after 2, 8 and 12 min of cryogenic grinding, whilst at 4 min they were lower.

Likewise, results of the analyses measured on buckwheat hulls were quite similar, demonstrating that the highest antioxidant activities and phenolic contents were achieved after 8 and 12 min of cryogenic grinding, whilst at 2 and 4 min they were lower. From these results it may be concluded that, depending on the bran grain, a short grinding time could lead to a fast production of fine particles, however, extended milling times are necessary to significantly increase the proportion of fine particles, which improve the release of bioactive compounds.

VII. Bibliography

Abozed, S. S., El-Kalyoubi, M., Abdelrashid, A., & Salama, M. F. (2014). Total phenolic contents and antioxidant activities of various solvent extracts from whole wheat and bran. *Annals of Agricultural Sciences*, *59*(1), 63-67.

Antoine, C., Peyron, S., Lullien-Pellerin, V., Abecassis, J., & Rouau, X. (2004). Wheat bran tissue fractionation using biochemical markers. *Journal of Cereal Science*, *39*(3), 387-393.

Balasubramanian, S., Sharma, R., & Kumar, S. V. (2011). Effect of moisture content and feed rate on size reduction of pearl millet. *Journal of Food Science and Engineering*, *1*(2), 93.

Belščak, A., Komes, D., Horžić, D., Ganić, K. K., & Karlović, D. (2009). Comparative study of commercially available cocoa products in terms of their bioactive composition. *Food Research International*, *42*(5-6), 707-716.

Cryogenic Mixer Mill CryoMill, *version 0005*, Retsch, Jan. 23, 2017. Accessed on: May. 31, 2018. [Online]. Available: https://www.retsch.com/products/milling/ball-mills/cryomill/information-downloads/

Fardet, A. (2010). New hypotheses for the health-protective mechanisms of whole-grain cereals: what is beyond fibre?. *Nutrition research reviews*, *23*(1), 65-134.

Gani, A., Wani, S. M., Masoodi, F. A., & Hameed, G. (2012). Whole-grain cereal bioactive compounds and their health benefits: a review. *J Food Process Technol*, *3*(3), 146-56.

Hemery, Y., Chaurand, M., Holopainen, U., Lampi, A. M., Lehtinen, P., Piironen, V., & Rouau, X. (2011). Potential of dry fractionation of wheat bran for the development of food ingredients, part I: Influence of ultra-fine grinding. *Journal of Cereal Science*, *53*(1), 1-8.

Hemery, Y. M., Anson, N. M., Havenaar, R., Haenen, G. R., Noort, M. W., & Rouau, X. (2010). Dry-fractionation of wheat bran increases the bioaccessibility of phenolic acids in breads made from processed bran fractions. *Food Research International*, *43*(5), 1429-1438.

Kvist, S., Carlsson, T., Lawther, J. M., & Decastro, F. B. (2010). *U.S. Patent No. 7,709,033*. Washington, DC: U.S. Patent and Trademark Office.

Li, L., Harflett, C., Beale, M. H., Ward, J.L. (2009). *Healthgrain Methods: Analysis of Bioactive Components in Small Grain Cereals*. Rothamsted Research, Harpenden, UK, str. 41-52.

Martins, Z. E., Pinho, O., & Ferreira, I. M. P. L. V. O. (2017). Food industry by-products used as functional ingredients of bakery products. *Trends in Food Science & Technology*, *67*, 106-128.

Nagaraj, G. (2009). Oilseeds: properties, processing, products and procedures. New India Publishing.

Noort, M. W., van Haaster, D., Hemery, Y., Schols, H. A., & Hamer, R. J. (2010). The effect of particle size of wheat bran fractions on bread quality–Evidence for fibre–protein interactions. *Journal of Cereal Science*, *52*(1), 59-64.

Ragaee, S., Abdel-Aal, E. S. M., & Noaman, M. (2006). Antioxidant activity and nutrient composition of selected cereals for food use. *Food chemistry*, *98*(1), 32-38.

Rosa, N. N., Barron, C., Gaiani, C., Dufour, C., & Micard, V. (2013). Ultra-fine grinding increases the antioxidant capacity of wheat bran. *Journal of Cereal Science*, *57*(1), 84-90.

Saleh, A. S., Zhang, Q., Chen, J., & Shen, Q. (2013). Millet grains: nutritional quality, processing, and potential health benefits. *Comprehensive Reviews in Food Science and Food Safety*, *12*(3), 281-295.

Shewry, P. R. (2009). The HEALTHGRAIN programme opens new opportunities for improving wheat for nutrition and health. *Nutrition Bulletin*, *34*(2), 225-231.

Singh, A., Sharma, V., Banerjee, R., Sharma, S., & Kuila, A. (2016). Perspectives of cell-wall degrading enzymes in cereal polishing. *Food Bioscience*, *15*, 81-86.

Soukoulis, C., & Aprea, E. (2012). Cereal bran fractionation: processing techniques for the recovery of functional components and their applications to the food industry. *Recent patents on food, nutrition & agriculture*, *4*(1), 61-77.

Stevenson, L., Phillips, F., O'sullivan, K., & Walton, J. (2012). Wheat bran: its composition and benefits to health, a European perspective. *International journal of food sciences and nutrition*, *63*(8), 1001-1013.

Sulaiman, S. F., Sajak, A. A. B., Ooi, K. L., & Seow, E. M. (2011). Effect of solvents in extracting polyphenols and antioxidants of selected raw vegetables. *Journal of Food Composition and Analysis*, *24*(4-5), 506-515.

Teeter Jr, F. C., Haider, G. L., Scott, J. D., MacKinnon, B. D., & Devine, J. L. (2011). *U.S. Patent No. 7,938,345*. Washington, DC: U.S. Patent and Trademark Office.

Van Craeyveld, V., Holopainen, U., Selinheimo, E., Poutanen, K., Delcour, J. A., & Courtin, C. M. (2009). Extensive dry ball milling of wheat and rye bran leads to in situ production of

arabinoxylan oligosaccharides through nanoscale fragmentation. *Journal of agricultural and food chemistry*, *57*(18), 8467-8473.

Wang, T., He, F., & Chen, G. (2014). Improving bioaccessibility and bioavailability of phenolic compounds in cereal grains through processing technologies: A concise review. *Journal of Functional Foods*, *7*, 101-111.

Wilczek, M., Bertling, J., & Hintemann, D. (2004). Optimised technologies for cryogenic grinding. *International Journal of Mineral Processing*, *74*, S425-S434.

Yu, L., Perret, J., Davy, B., Wilson, J., & Melby, C. L. (2002). Antioxidant properties of cereal products. *Journal of Food Science*, *67*(7), 2600-2603.

Zhou, K., Laux, J. J., & Yu, L. (2004). Comparison of Swiss red wheat grain and fractions for their antioxidant properties. *Journal of Agricultural and Food Chemistry*, *52*(5), 1118-1123.

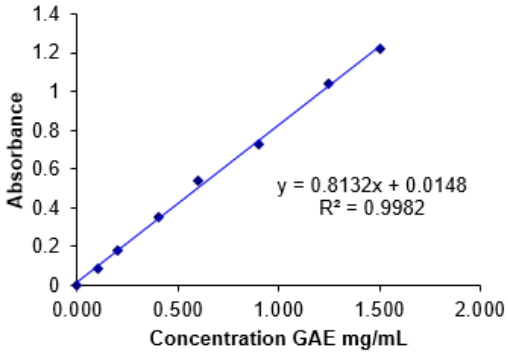
Zhu, F., Du, B., Li, R., & Li, J. (2014). Effect of micronization technology on physicochemical and antioxidant properties of dietary fiber from buckwheat hulls. *Biocatalysis and Agricultural Biotechnology*, *3*(3), 30-34.

Zhu, K., Huang, S., Peng, W., Qian, H., & Zhou, H. (2010). Effect of ultrafine grinding on hydration and antioxidant properties of wheat bran dietary fiber. *Food Research International*, *43*(4), 943-948.

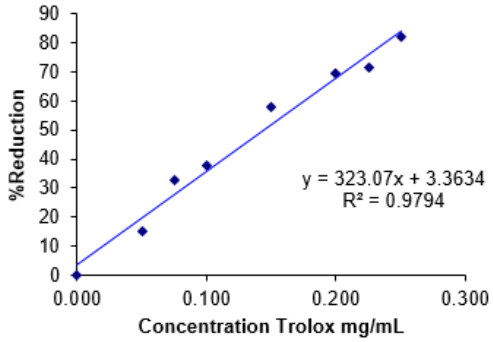
Zieliński, H., Michalska, A., Piskuła, M. K., & Kozłowska, H. (2006). Antioxidants in thermally treated buckwheat groats. *Molecular Nutrition & Food Research*, *50*(9), 824-832.

Annexes

Annex 1 Calibration curves of TPC, DPPH, ABTS and FRAP.



TPC



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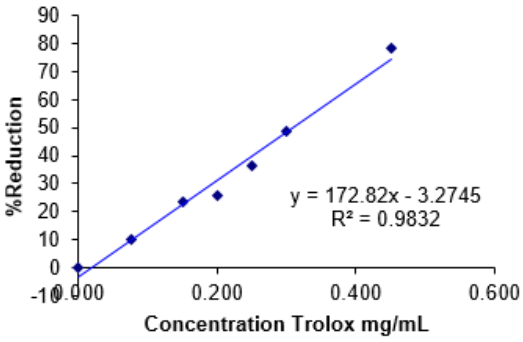
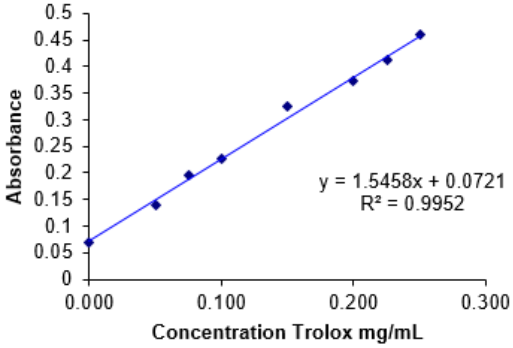


ABTS



FRAP

Annex 2 Quotation for the Retsch CryoMill

N° client :

Votre référence CRYOMILL

Date : 31/07/18

Monsieur VICTORIA
Téléphone
4 rue Artaud
Fax
84000 Avignon

A l'attention de : Monsieur VICTORIA

OFFRE N° DV18+04924

Madame, Monsieur,
Suite à votre demande, nous avons le plaisir de vous adresser notre offre pour :

N°	Désignation	Qté	P.U.H.T.	%	Montant H.T.
20.749.0001	Broyeur Cryogénique CRYOMILL	1	10 179,00	7	9 466,47
	100-240 V, 50/60 Hz				
01.462.0332	Bol de broyage inox 50 ml	1	1 030,00	7	957,90
	Cryomill				
05.368.0063	Bille 10 mm acier inox	14	2,60	6,93	33,88

01.462.0336	Bol CryoMill, Oxyde Zr, 25 ml	1	2 055,00	7	1 911,15
05.368.0094	Bille 10 mm oxyde de zirconium	14	36,00	7	468,72

02.480.0002	Autofill avec récipient de LN2, 50 litres	1	7 044,00	7	6 550,92
	et valve de sécurité				
	GARANTIE 2 ANS				
	TRANSPORT EN SUS				
	DELAI 4/5 SEMAINES				

MISE EN ROUTE ET FORMATION OFFERTES

N° client :

Votre référence CRYOMILL

Date : 31/07/18

Monsieur VICTORIA
Téléphone 4 rue Artaud
Fax 84000 Avignon

A l'attention de : Monsieur VICTORIA

OFFRE N° DV18+04924

N°	Désignation	Qté	P.U.H.T.	%	Montant H.T.

Total EUR HT	**19 389,04**
TVA 20%	3 877,81
Total EUR TTC	**23 266,85**

Votre contact commercial:

Magalie ABSALON : 06.20.05.15.12

Nous restons à votre disposition pour tout renseignement complémentaire, et vous prions d'agréer, nos sincères salutations.

Conditions générales de vente :

- Minimum de commande : 100€
- prix départ usine, hors emballage.
- validité de l'offre : 4 semaines.
- conditions de paiement : 45 jours fin de mois
- Termes des conditions de vente disponibles sur le site: www.verder.fr.
- Le client déclare avoir pris connaissance des Conditions générales de vente et les accepter comme partie du contrat.

Adresse destinataire
4 rue Artaud 84000 Avignon