Antioxidant activity and storage regime of defatted grape seeds flour

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Abstract

In the present paper, we examined the antioxidant activity of a defatted grape seeds flour of different grape varieties locally grown in Bulgaria. The seeds are retrieved after alcoholic fermentation and the antioxidant activity of the flour was assessed by using four different methods, namely DPPH, ABTS, FRAP and CUPRAC. The results are presented in mM TE/g extract. The values are 586,08 (±41,55); 945,41 (±90,97); 553,39 (±45,57) and 667,73 (±64,30), respectively. The results are also showed in mM TE/g flour. The values are 58,67 (±4,16); 94,64 (±9,11); 55,40 (±4,56) and 66,85 (±6,44), respectively. During three-month storage of the flour in plastic bags (temperature 25 ºC and relative humidity 75%), no living cells of pathogenic organisms (*Escherichia coli*, *Staphylococcus aureus* and *Salmonella* spp.) or apparent molding were detected. The flour particle size has not changed either.

Introduction

Consumers have increasingly become aware of eating healthy and maintaining good health. In order to satisfy their needs, it is extremely important for food producers to develop and produce foods containing a wide range of biologically active substances with disease-preventing and prophylactic effect.[1-3] The interest in antioxidants has been constantly growing.[4,5] Antioxidants are low molecular weight substances, which prevent the oxidation of other chemical agents, absorbing to some extent the ionizing radiation from the environment and toxic pollutants. In biological systems, metabolic processes take place all the time, which results in the release of free radicals. These are highly chemically reactive particles, which oxidize various biomolecules, such as enzymes, proteins, DNA and lipids. This may lead to a number of serious diseases in humans, for instance, cancer, down syndrome, arthritis, atherosclerosis, neurodegenerative disorders, cardio-vascular disease, *etc*.[6] Antioxidants have the ability to capture free radicals before to influence on human health. According to Henning *et al.*,[7] the consumption of fruits and vegetables high in antioxidants was prevention of the effect of free radicals.

Grapes and grape seeds are very rich in substances characterized by antioxidant activity: resveratrol, catechin, epicatechin, gallic acid, quercetin, rutin, antocyanidins, *etc*.[8,9] A number of studies prove the health effects of grapes for the heart, kidneys, liver, and stomach and especially their role in neutralizing free radicals which degenerate human health.[10,11] Grapes contain calcium, magnesium, sodium, iron, aluminium, manganese, sulphur, phosphorus, copper, iodine, bromine, zinc, and silicon.[12]

In the past, the French used grape seeds to cure scurvy, thrombophlebitis, and injuries, which were difficult to treat.[13] This has made the current French nutritionists to become among the leading scientists in the world to study grapes and their products.

In contemporary times, grape seeds flour is gaining ground in the food industry as an ingredient in the manufacture of various food products.[14-16] Grape seeds flour is gluten-free and can be included as a biologically active substance in foods consumed by people who suffer from coeliac.[17]

The literature review provided the results for the antioxidant activity of grape seeds flour but the data published was not uniform.[2,10,18] There is no data on post-fermentation grape seeds flour produced from different grape varieties locally grown in Bulgaria. The literature also gives no information on the storage of post-fermentation grape seeds flour from Bulgarian grape cultivars. The conditions and length of storage of foods are very important in the preservation of their nutritive value and health effects. That is why we analyzed in detail the antioxidant activity and storage of defatted flour from post-fermentation grape seeds belonging to Bulgarian grape cultivars. The present study aims at determining the antioxidant activity and storage regimes of defatted flour from grape seeds (a post-fermentation by-product in wine production).

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Materials and Methods

The grape seeds flour was delivered by a scientific institute in the town of Parvenets, Bulgaria. Briefly, the flour was produced as follows. After the alcoholic fermentation of wine, the grape seeds were extracted, then dried at atmospheric conditions and subjected to oil extraction using a screw press. Different grape varieties were blended in order to obtain sufficient amount of samples for analysis. After de-oiling phase, the grape seeds were milled to a flour. All the samples were packed in plastic bags, then thermo-sealed and stored for three months at temperature 25 ºC and relative humidity 75%. The plastic bags were provided by *Ilko*

Tyanev - Itaplast ET company, Asenovgrad, Bulgaria. It was prepared extracts from defatted grape seed flour to analyze antioxidant activity. The analyzed sample was subjected to triple extraction with 10 mL 70% ethanol using a reflux condenser and a water bath at a temperature of 80 °C. The mixed extracts were filtered through filter paper and vapourized till dry in a rotary vacuum evaporator. The dry extract was dissolved in the necessary volume of 70% ethanol before the analysis.

The antioxidant activity of defatted flour from post-fermentation grape seeds was determined on the basis of the following methods.

DPPH assay

Each analyzed extract (0.15 mL) was mixed with 2.85 mL freshly prepared 0.1 mM solution of 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) in methanol. The reaction was at 37 °C in darkness and the absorbance at 517 nm were recorded after 15 min against methanol.

ABTS assay

ABTS radical was generated by mixing aliquot parts of 7.0 mM 2, 2'azinobis (3)-ethylbenzthiazoline-6-sulfonic acid (ABTS) in distilled H_2O and 2.45 mM potassium persulfate in distilled H_2O. The reaction was performed for 16 h at ambient temperature in darkness and the generated ABTS radical is stable for several days. Before analyses, 2.0 mL of generated ABTS.+ solution was diluted with methanol at proportions 1:30 (v/v), so the obtained final absorbance of the working solution was about 1.0÷1.1 at 734 nm. For the assay, 2.85 mL of this ABTS.+ solution was mixed with 0.15 mL of obtained extracts. After 15 min at 37 °C in darkness the absorbance was measured at 734 nm against methanol.

Ferric reducing antioxidant power (FRAP) assay

The FRAP reagent was freshly prepared before analyzes by mixing 10 parts 0.3 M acetate buffer (pH 3.6), 1 part 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ, Fluka) in 40 mM HCl (Merck) and 1 part 20 mM $FeCl_3.6H_2O$ (Merck) in distilled H_2O. The reaction was started by mixing 3.0 mL FRAP reagent with 0.1 mL of investigated extract. Blank sample, prepared with ethanol instead of extract was developed as well. The reaction time was 10 min at 37 °C in darkness and the absorbance at 593 nm of sample against blank was recorded.

Cupric reducing antioxidant capacity(CUPRAC) assay

Reaction was started by mixing 1.0 mL 10 mM $CuCl_2.2H_2O$ (Sigma) in distilled H2O, 1.0 mL 7.5 mM Neocuproine (Sigma) in methanol, 1.0 mL 0.1 M ammonium acetate buffer (pH 7.0), 0.1 mL of investigated extract and 1.0 mL distilled H2O. Blank sample, with ethanol instead of extract was developed as well. The reaction was carried out for 20 min at 50°C in darkness and the sample absorption at 450 nm was recorded against the blank.

The antioxidant activity defined by all of the tested methods was expressed as mM Trolox equivalents (TE) per g dry weight (DW) and g extract by using calibration curve, build in range of 0.05-0.5 mM 6-hydroxy-2,5,7,8-tetramethylchroman-2-carb oxylic acid (Trolox®) dissolved in methanol.

The microbial load of the product under study was determined at one-month intervals during the three-month storage via: Mesophilic aerobic and facultative anaerobic bacteria, according to Bulgarian State Standard (BSS) EN ISO 4833-2:2014;[19] Yeasts and moulds, according to (BSS) EN ISO 21527-2:2011;[20] *Escherichia coli*, according to ISO 16649-2:2014;[21] *Salmonella* spp., according to (BSS) EN ISO

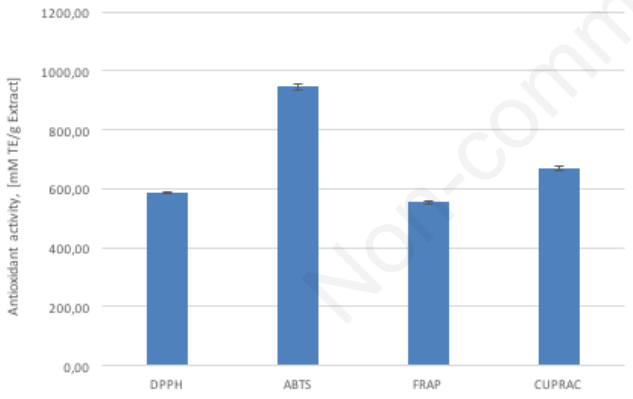

Figure 1. Antioxidant activity of defatted grape seeds flour expressed as mM TE/g extract.

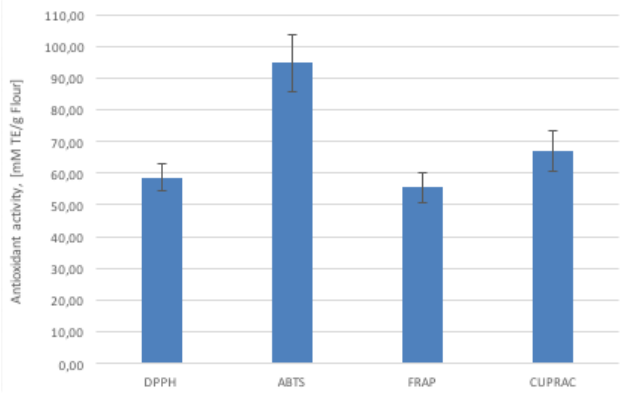

Figure 2. Antioxidant activity of defatted grape seeds flour expressed as mM TE/g flour.

Table 1. Microbiological parameters of defatted flour from post-fermentation grape seeds for three-month storage.

Sample/ Day	Total numbers of mesophilic aerobic and facultative anaerobic bacteria, CFU/g	*Escherichia coli*, CFU/g	*Staphylococcus aureus*, CFU/g	*Salmonella* spp. / 25 g	Yeasts and molds, CFU/g
Day 1	$1.5.10^4$	<10	<100	Not detected	5.10^2
Day 30	$2.5.10^4$	<10	<100	Not detected	2.10^3
Day 60	3.10^4	<10	<100	Not detected	2.10^3
Day 90	5.10^4	<10	<100	Not detected	2.10^3

6579:2003;[22] Coagulase-positive *staphylococci*, according to (BSS) EN ISO 6888-1:2005.[23]

Flour particle size was determined with *ProMel LP - 200* sieve analysis equipment. Based on preliminary analysis, the set of sieves was determined as well as their size. The sieving of the sample in the apparatus continues for ten minutes if it amounts to 100 g.

The moisture content [%] was determined according to AOAC 960.39.[24]

All tests were run in triplicate.

Results and Discussion

We obtained 100.1 mg (10.01%) of extract in order to determine antioxidant activity using the methods described via extracting 1g of defatted flour from post-fermentation grape seeds. The antioxidant activity of the extract was determined using four methods differing in mechanism and development conditions (DPPH, ABTS, FRAP and CUPRAC). DPPH and ABTS methods are based on the transfer of a single electron (SET method) and/or the transfer of a hydrogen atom (HAT method), whereas FRAP and CUPRAC methods are based solely on single electron transfer. The results (mean value ± standard deviation) are presented as mM TE/g extract in Figure 1 and mM TE/g flour in Figure 2.

The results obtained via all four methods demonstrate that defatted post-fermentation grape seeds flour shows antioxidant activity. The flour analyzed is characterized by a much greater antioxidant activity as compared to that reported by other authors. Using the DPPH method,[2] determined the antioxidant activity of 70% ethanol extract of grape seeds flour from different grape varieties (Chardonnay, Concord, Norton, Ruby Red, White) as varying between 0.5-7.0 mM TE/g flour. Lutterodt *et al.*[10] also arrived at a considerably lower antioxidant activity, as compared to the DPPH method, of various grape seeds flours (for the grape cultivars Muscadine, Concord, Ruby Red, Chardonnay, and Soybean), the values amounting to 11.8-15.0 mM TE/g flour. According to Kim *et al.*,[18] antioxidant content depends to a large extent on the treatment of grape seeds during thermal processing.

Regarding the antioxidant activity of defatted flour from post-fermentation grape seeds, the differences between the data in the present study and that of other authors[2,10,18] are attributable to the specificities of grape varieties, climatic conditions, and technological processes used in flour production.

During the three-month storage of the grape seeds flour at a temperature of 25 ºC and relative air humidity of 75%, we studied the microbiological parameters having to do with *E. coli*, *Salmonella* spp., coagulase-positive *staphylococci*, total amount of mesophilic aerobic and facultative anaerobic bacteria, yeasts and moulds, granulometric composition, and flour moisture (Table 1).

Storage conditions. Relative air humidity and temperature were selected in accordance with the storage conditions of the product in warehouses and markets.

Table 1 shows the results from the microbiological parameters analyzed for the whole period of storage.

The parameters *Total numbers of mesophilic aerobic and facultative anaerobic bacteria* and *Yeasts and molds* remain from Day 1 to Day 90 of storage. The higher microbial load concerning these two parameters, still below the threshold limits, can be reduced in subsequent heat processing. The presence of *Escherihia coli* and coagulase-positive *staphylococci* also falls below the threshold limit. *Salmonella* spp. was not detected.

The microbiological analysis demonstrated that the flour from post-fermentation grape seeds could be stored for three months without microbial deterioration, thus being safe for use.

The results from the granulometric analysis and the moisture values of the grape seeds flour during three-month storage at a temperature of 25 ºC and relative air humidity of 75% are presented in Table 2.

We find out that, during three-month storage, flour moisture changes. Greatest is the amount of flour particles sized 560/200 µm- starting at 57.7% on day 1 and reaching 73.5% after one-month and three-month storage. The distribution of flour particles in this range depends to a great extent on the duration of storage, not on the relative moisture of flour (only for the given moisture range). There is also decrease in the amount of smallest-size fractions below 180 µm, which explains the greater quantity of fractions sized between 200-560 µm. We believe that the finest fractions agglomerate on the first days of storage. During the subsequent two months, however, there are no considerable differences in the granulometric composition of post-fermentation grape seeds flour. The size of flour particles in grape seeds flour is suitable for incorporating the flour into food products if the base products are not flours with particles sized below 200 µm.

Table 2. Granulometric composition of post-fermentation grape seeds flour during three-month storage.

	Particles size, m	Quantity of break stock, %		
		1 day	1 month	3 months
1	670	4.3	9.2	10.1
2	560	8.9	7.3	8.7
3	450	14.3	13.1	15.4
4	355	40.1	43.3	40.6
5	280	0.3	3.1	2.4
6	200	3.1	15.9	15.1
7	180	4.2	2.3	2.0
8	150	13.1	2.3	2.9
9	132	2.1	1.6	1.1
10	less than 132	9.6	1.9	1.7
11	Flour moisture content, %	9.65	10.45	7.85

Conclusions

The analyzed 70% ethanol extract of post-fermentation grape seeds flour demonstrated a relatively high antioxidant activity as assessed through the capacity to interact with free radicals (DPPH and ABTS methods) as well as the capacity to reduce iron and copper ions (FRAP and CUPRAC methods).

During the three-month storage of the grape seeds flour in a plastic bags (at temperature 25 ºC and relative humidity 75%), no living cells of pathogenic organisms or apparent molding were detected. The flour particles size was not changed considerably.

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