

ANTIOXIDANT ACTIVITY AND STORAGE REGIME OF GRAPE SEEDS FLAKES – A WASTE PRODUCT IN WINE ELABORATION

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Abstract

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The current scientific research is focused on the grape seeds – a waste product retrieved after alcoholic fermentation of wine, in the form of grape seeds flakes. Recently, the grape residue has been considered as a functional food product, a source of bioactive compounds (BAC) including antioxidants. The present study presents data about the antioxidant activity of grape seeds flakes of different grape varieties of Bulgarian origin, analysed via four analytical methods differ in mechanism and reaction conditions, namely DPPH, ABTS, FRAP and CUPRAC. The obtained results were expressed as mM TE/g extract (842.50 ± 34.75 ; 959.63 ± 65.05 ; 94.73 ± 10.42 and 200.24 ± 48.60 , respectively) and mM TE/g flakes (as follows 141.40 ± 5.83 ; 161.06 ± 10.92 ; 94.89 ± 10.44 and 200.56 ± 48.68). The grape seeds flakes were stored at room temperature – 25°C and relative air humidity of 75%. The storage regime was optimized to one month by means of packaging the product in a co-extruded barrier film with copolymer covering for heat sealing. During the storage of grape seeds flakes, analysis of their microbial load, granulometric composition and moisture content were performed.

Key words: grape; grape seeds flakes; grape seeds; antioxidants; winery by-products; bioactive compounds

Introduction

Nowadays, contemporary society and living conditions require the use of bioactive compounds (BAC) – supplements with a beneficial effect on human health. Thus scientists and companies in food industry are interested to find and use new sources of BAC, and to offer new enriched products with a wide range of applications, as well. BAC is founded in small quantities in foods and they are considered such as natural sources of extranutrition compounds. Numerous studies reported for a protective and prevention effects of BAC against cardiovascular diseases and cancer. These compounds differ

in their chemical structure and function. They are naturally presented in fruits, vegetables, whole grains, legumes, oils, nuts and plants (Kris-Etherton et al., 2002; Choi et al., 2008; Kappagoda et al., 2017; Cádiz-Gurrea et al., 2017).

Reusing of waste products from food production offers a wide assortment for scientific research and new products for commercialization. They contain variety of beneficial ingredients that at regulated intake maintain certain favorable effects on human health. Nowadays they become more popular and take a place in the modern healthy diet. The benefits of grape products – grapes, grape juice and wine are proved by various researches across the world. Current interests are

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focused on the consumption of by-products obtained after primary processing in wine elaboration – grape oil, extracts and flour (Sausa et al., 2014). The grape contains BAC that has a protective role against chronic degenerative diseases also known as disorders associated with metabolism (Charradi et al., 2017).

Grape seeds in the form of flour are associated with a high content of antioxidants, in particular, flavonoids that are contributed to decrease cholesterol (Kim et al., 2014). The harmful impact of free radicals, presented in human organism, could be overcome by absorption and detoxification action of antioxidant compounds. Consumption of food, rich in antioxidant substances had an important role in preventing cancer and neurodegenerative diseases (Fan et al., 2006).

Due to the increasing consumption of wine and development of the new wine cellars in Bulgaria – scientists and industry are focused on finding and improve more areas of recycling the organic residue in the wine elaboration. A few years ago, the most frequent use of grape seeds (one of the waste enological product) was for fat extraction, and the defatted residue – as a food intended for livestock breeding (Song et al., 2017).

However, the trend of last years is an increasing use and consumption of grape seeds flour, in the form of defatted and full fatted flour in the daily menu. Grape seeds flour is considered as a functional alternative ingredient, which can be used in food products such as bread, biscuits, pasta, frankfurters, and others. In general, the interest in this sub-product from wine industry is due to its antioxidant, antiproliferative, anti-viral, neuroprotective properties, as well as its high content of dietary fiber (Özvural and Vural, 2011). In our previous study on the physico-chemical parameters and granulometric composition of defatted and full fatted grape seeds flour, we found that the both flours are appropriated for incorporation in whole grain flour (Bogoeva, 2016).

Fats are a major component in grape seeds. According to Girard and Mazza (1998), they contain from 11 to 15% fat (basis of dry weight), of which 61 – 73% are linoleic acid (18:2), from 14 to 28% – oleic acid (18:1), and 10 to 14% saturated fat: from 7 to 13% – palmitic acid (C16:0), from 3 to 6% – stearic acid (C18:0), 0 – 9% palmitoleic acid (C16:1), 0 – 6% linolenic acid (C18:3), 0 – 22% myristic acid (C14:0). The oil contains from 0.03 to 0.07% tocopherols, which protect the oil quality during storage.

The high content of fats provides a reduced shelf life of grape seeds flour compared with different types of wheat flour. This fact gives us a reason to determine a storage regime. In a previous our study of defatted grape seeds flour, we were confirmed the high purity during the storage regime of three months at 25°C and relative humidity 75% in co-ex-

truded barrier film with copolymer covering for heat sealing designed for food industry (Bogoeva et al., 2017).

After reference research, we didn't find information on antioxidant activity and storage regime of grape seeds flakes. Thus, the aim of the present study was to determine antioxidant activity and storage regime of grape seeds flakes – a waste product after alcoholic fermentation in wine elaboration.

Materials and Methods

Materials

Grape seeds flakes (of different grape varieties of Bulgarian origin, namely Mavrud, Cabernet Sauvignon, Syrah, Merlot, Dimyat, Sauvignon Blanc) were delivered by experimental institute located in Parvenets, Bulgaria. Grape seeds were extracted after alcoholic fermentation of wine as a sub-product. They were dried under atmospheric conditions and milled with a reconstructed mill roll [using 2 smooth shafts with a differential difference of 0.05/each roller rotates at different speeds, the difference between them for 1 rpm (rotation per minute) is 1.5 mm to 5 mm]. The applied pressure was between 200 and 280 tons. Grape seeds flakes were full fat, and their high fat content was confirmed by Soxhlet extractor at the Department of Technology of Grain, Fodder, Bread and Confectionery Products at the University of Food Technologies, Plovdiv, Bulgaria.

In order to optimize the storage period, the product was packaged in a co-extruded barrier film with copolymer covering for heat sealing designed for food industry, produced by Itaplast “ET – Ilko Tyanevita Plast”, Assenovgrad, Bulgaria.

Methods

Sample extraction for analysis of antioxidant activity

The analyzed samples were subjected to triple extraction with 10 mL 70% ethanol using a reflux condenser and a water bath at a temperature of 70°C. The combined extracts were filtered through filter paper and vapourized till dry in a rotary vacuum evaporator (at a temperature of 55 ÷ 60°C). The dry extracts were dissolved in the necessary volume of 70 % ethanol before the analysis. The antioxidant activity of flakes from post-fermentation grape seeds was determined based on the following methods (9):

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 was 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 H₂O and 2.45 mM potassium persulfate in distilled H₂O. 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 FeCl₃·6H₂O (Merck) in distilled H₂O. 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 CuCl₂·2H₂O (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-carboxylic acid (Trolox®) dissolved in methanol.

The microbial load

The microbial load of the product was determined during the one-month storage via:

Mesophilic aerobic and facultative anaerobic bacteria, according to Bulgarian State Standard (BSS EN ISO 4833-2, 2014); Yeasts and fungi, according to BSS EN ISO 21527-2, 2011; *Escherichia coli*, according to BSS EN ISO 16649-2, 2014; *Salmonella* spp., according to BSS EN ISO 6579, 2003; *Coagulase-positive staphylococci*, according to BSS EN ISO 6888-1, 2005.

Flour particle size and moisture content

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, 1990.

All tests were run in triplicate. Data presented are mean values and standard deviations.

Results and Discussion**Antioxidant activity**

After extraction of 1 g of grape seeds flakes, it was obtained 167.8 mg (16.8%) extract by the described procedure. The antioxidant activity of the obtained 70% ethanol extract was determined by four methods based on different mechanisms and reaction conditions – DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2'-azinobis (3)-ethylbenzothiazoline-6), FRAP (ferric reducing antioxidant power) and CUPRAC (cupric reducing antioxidant capacity). Methods based on single electron transfer (SET) and/or hydrogen atom transfer (HAT method) are DPPH and ABTS, and methods based on single electron transfer (SET method) are FRAP and CUPRAC. The obtained results (mean ± standard deviation) are presented in Table 1 expressed as mM TE/g extract of grape seeds flakes, and as mM TE/g grape seeds flakes.

According to the obtained results, the analyzed extract of grape seeds flakes possesses antioxidant activity by all of the

Table 1
Antioxidant activity of 70% ethanol extract of grape seeds flakes, expressed as mM TE/g extract and mM TE/g flakes

METHODS	mM TE/g extract	mM TE/g flakes
DPPH (2,2-diphenyl-1-picrylhydrazyl)	842.50 ± 34.75	141.40 ± 5.83
ABTS (2,2'-azinobis (3)-ethylbenzothiazoline-6-sulfonic acid)	959.63 ± 65.05	161.06 ± 10.92
FRAP (ferric reducing antioxidant power)	94.73 ± 10.42	94.89 ± 10.44
CUPRAC (cupric reducing antioxidant capacity)	200.24 ± 48.60	200.56 ± 48.68

tested methods. There was no information in the scientific studies about antioxidant activity of flakes from Bulgarian varieties of grape seeds. Similar results were reported by Bogoeva et al. (2017) for defatted grape seeds flour, retrieved after alcoholic fermentation in wine elaboration. The results obtained from other authors about the antioxidant activity of grape seeds flour are significantly lower than our results for the Bulgarian full fat grape seeds flakes. In a study of Binzer et al. (2011) the results obtained by the DPPH method for 70% ethanolic extract of grape seeds flour of different varieties (Chardonnay, Concord, Norton, Ruby Red, White) varied between 0.5-7.0 mM TE/g of flour. Lutterodt et al. (2011) also reported significantly lower antioxidant activity of grape seeds flours of different varieties (Muscadine, Concord, Ruby Red, Chardonnay, Soybean) defined by DPPH method (11.8 – 15.0 mM TE/g flour). The antioxidants composition of grape seeds depends largely on a heat treatment, according to Kim et al. (2006). Probably these differences in the results were due to the varietal and climatic conditions, as well as differences in the enological practice in wine production and flakes technology.

The microbial load

It was performed a one month storage of grape seeds flakes at 25 °C and 75 % relative humidity. During the storage, the microbiological parameters for *Escherichia coli*, *Salmonella* sp., coagulase-positive staphylococci, total numbers of mesophilic aerobic and facultative anaerobic bacteria, yeasts, fungi, as well as the granulometric composition and humidity were monitored.

Product storage conditions (relative humidity and temperature) are conformed in the conditions in the store network. The expiry date was considered with the high fat content (19.13%) in the product.

The results of microbiological tests for the whole period of storage are presented in Table 2.

Table 2
Microbiological parameters of post-fermentation grape seeds flakes for one-month storage at 25 °C and relative humidity 75%.

Sample	Total numbers of mesophilic aerobic and facultative anaerobic bacteria,	<i>Escherichia coli</i> ,	<i>Staphylococcus aureus</i> ,	<i>Salmonella</i> sp.	Yeast,	Fungi,
Day	CFU/g	CFU/g	CFU/g	/ 25 g	CFU/g	CFU/g
Day 1	1.2x10 ⁵	<10	<100	Not detected	<10	9.5x10 ⁴
Day 15	3.6x10 ⁵	<10	<100	Not detected	<10	2.5x10 ⁵
Day 30	2.1x10 ⁵	<10	<100	Not detected	<10	1.7x10 ⁵

A microbiological analysis of the grape seeds flakes was performed on firstday, 15 days and 30 days, with limited storage for 30 days at 25°C and 75% relative humidity. During the period of the analysis in particular period a few measurements were taken of the following microbiological parameters: “Total number of mesophilic aerobic and facultative anaerobic microorganisms”, “Fungi and yeasts” and presence of pathogenic microorganisms (*Escherichia coli*, *Salmonella* sp., coagulase-positive staphylococci). The result of the analysis taken on the 1st, 15th and 30th day of the storage of the grape seeds flakes shows that *Salmonella* sp was not detected and the presence of *Escherichia coli* and coagulase-positive staphylococci is under the allowable limits from the first day of the storage and on the 30 day of the storage.

The results of «Total number of mesophilic aerobic and facultative anaerobic microorganisms» and «Fungi and yeasts» in the grape seeds flakes are retained from the first day to the 30th day of the storage. The detected higher amount of the microbial cells of the two parameters, which are below the allowable limits of this type of food, could be reduced by using heat treatment.

The results of the microbiological analysis show that the grape seed flakes, which are natural waste of the alcoholic fermentation in wine production, could be stored in the condition of the current experiment for the period of one month without disturbing their nutritional qualities and the microbiological safety of the product.

The experimental results show that the grape seed flakes can be added as a food supplement and ingredient in the production of food products.

Flour particle size and moisture content

In the selected conditions – temperature 25 °C and relative humidity 75 % for a one-month storage period, we analyzed the granulometric composition of grape seeds flakes,

as well. The results of the granulometric composition and the corresponding measured humidity for the same period are presented in Table 3.

Table 3
Granulometric composition and moisture content of post-fermentation grape seeds flakes during one-month storage

№	Particales size,	Quantity of break stock, %	
	µm	Day 1	Day 30
1	1000	11.33	11.22
2	800	24.59	21.22
3	670	17.39	17.45
4	560	12.4	12.38
5	450	12.87	12.81
6	355	19.91	23.44
7	280	0.65	0.45
8	less than 280	0.86	1.04
9	Moisture content, %	11,59	10.78

We found a change in moisture content results for the examined period that is a reason to imply that the product has desorbed moisture. Percentage distribution of the quantity of break stock is similar for particales size over 800 µm and 355 µm, as the results varied between 19.91% and 24.59% and at 1000, 670, 560 and 450 µm – 11.22% and 17.45%. On the first day of storage, the greatest amount of lumps is at 800 µm light and at the last day of storage – at a light aperture up to 355 µm. The distribution of particulate matter in this range largely depends on the storage regime and not on the relative humidity of the flakes (Charradi, 2017). Due to the decreased humidity at the end of the storage period of the grape seeds flakes, the amount of the agglomerated particles was distributed to the finer fractions. After analyzing the particle size distribution for a one-month storage period of grape seeds flakes we defined that the product is suitable for incorporation as a functional additive to base flours whose size is over 280 µm.

Conclusions

In the present study, the 70% ethanol extract of grape seeds flakes, a natural waste product after alcoholic fermentation in wine elaboration, showed a relatively high antioxidant activity. Results were established via four methods based on free radical capture (DPPH and ABTS methods) and the ability to reduce iron and copper ions (FRAP and CUPRAC methods).

For a one-month storage period, the total numbers of mesophilic aerobic and facultative anaerobic bacteria were retained. No visible growth of fungi was observed, as well as

presence of pathogenic microorganisms.

In the experimental one-month storage conditions – 25°C and 75% relative humidity for grape seeds flakes, the percentage distribution of particle size was not change considerably at 10.78% to 11.59% moisture content.

The present study proves that the analyzed grape seeds flakes – a natural waste product after alcoholic fermentation in wine production – were with high purity and can be stored for up to one month in a co-extruded barrier film with copolymer covering for heat-sealing at 25°C and relative humidity of 75%.

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