ISSN (Print 0974-2115) (Online 2349-8552)
www.jchps.com Journal of Chemical and Pharmaceutical Sciences

Usage of Phenolic Extract of Grape Waste as Natural Antioxidant for Milk Proteins

Dima Aldiab
Department of Analytical and Food chemistry, Faculty of Pharmacy, Tishreen University, Lattakia, Syria
***Corresponding author: E-Mail: dyabdima@yahoo.com**

ABSTRACT

Phenolic compounds are known for their different positive effects on health like antioxidant properties, therefore plants containing high level of phenolic compounds are important as natural antioxidants. Agro industries generate many byproducts like grape waste. Grape waste can be used to extract the phenolic compounds in order to use them as natural antioxidants in pharmaceutical, cosmetic and nutritional system. In the present study, ethanol was selected as a solvent to extract the phenolic compounds from grape waste. The moisture of grape waste was 55%. The total phenolic content was 4.6% (w/w) of dried waste. Protein carbonyls (PCs) content is used as indicator for protein oxidation. The content of PCs in boiled milk was 51.8 and 18.52 nmol/mg protein versus 13.64 nmol/mg protein in pasteurised milk. In the samples of milk with phenolic extracts PCs content in boiled milk was 29.46 and 12.73 nmol/mg protein versus 8.98 nmol/mg protein in pasteurised milk. The addition of phenolic extract of grape waste has led to decrease the formation of PCs about 35-45% ($p<0.05$). These results enable phenolic extract of grape waste to play a role in food preservation as natural antioxidant for proteins of milk and dairy products.

KEY WORDS: Grape Waste, Phenolic Extract, Oxidation, Protein Carbonyls, Milk Proteins.

1. INTRODUCTION

Phenolic compounds are plant secondary metabolites, which are widely studied due to their beneficial effects on human health. Phenolic compounds are known as health-promoting agents (Nick, 2007). This effect is based on the different positive effects, such as the association of polyphenol intake with lower risk of coronary heart disease, some types of cancer (Kaur and Kapoor, 2007), inflammation, in addition to the inhibition of platelet-activating factor activity (Nick, 2007). The antioxidant properties of plant extracts have been attributed to their polyphenol contents. Therefore, plants containing high level of phenolic compounds are important as natural antioxidants.

Agro industries generate many byproducts and wastes containing polyphenols, which may serve as food antioxidants and preventive agents against some diseases. One of these important wastes are grape byproducts (Baydar, 2007). The grape is one of the major fruit crops worldwide. About 80% of grape harvest is utilized for winemaking (Lafka, 2007). The phenolic composition of wines depends on grapes variety and vinification conditions (Perez-Magarino and Gonzalez-San Jose, 2006), where these compounds are responsible for many organoleptic properties of wines like color, astringency and flavor (Minussi, 2003). However, during wine and grape juice processing grape wastes products are generated (Murthy, 2002) in large quantities up to 9 million tons per year worldwide (Lafka, 2007). The amount of pomace produced from winemaking is dependent upon the species of grape (cultivar) as well as the pressing equipment used (chand, 2009). Many studies have determined grape waste to be approximately 20% of the original grapes weight. Grape waste consists mainly of grape skins, seeds and stems (murthy, 2002). Grape wastes contain many compounds like cellulose, hemicelluloses and proteins (Mendes, 2013). Additionally, grape wastes products are rich in polyphenols (Dwyer, 2014).

Generally, grape waste are used for animal feed without any pre-treatment. However, large amounts are produced during a short period of harvesting which could be detrimental to the environment, since the phenolic compounds decrease the pH and increase the resistance to biological degradation. Other environmental problems include pollution of surface and ground water; oxygen depletion in the soil and ground water, which affect surrounding flora and fauna, foul odors; attraction of flies and pests (Chand, 2009).

Grape waste forms a good source for many natural compounds, especially with decreasing consumer demand for the use of synthetic compounds and increasing the utilization of waste by-products as natural compounds for alternative uses, which enable this waste to be under focus of research.

The phenolic compounds in grape pomaces are recognized for their beneficial effects on human health like its effects on cancer and inflammatory (Mendes, 2013), where it has many applications in different pharmaceutical, cosmetic and nutritional fields. A current application of grape pomace is its utilization as a dietary supplement. Bioflavia consists of dried and ground, red grape skin powder. The Bioflavia can be added to beverages or during cooking (Dwyer, 2014). In cosmetic industry, the topical application of oils extracted from grape pomace helps protect skin and reverses any UV-induced free radicals (Burke, 2004).

In food industry grape pomace is a possible input to biosurfactant and thus to use in food processing as emulsifying factor. Biosurfactants from grape pomace have lower toxicities and are more biodegradable than synthetic biosurfactants. Consumer demand for natural additives over synthetic additives has increased the importance of natural biosurfactants (Rivera, 2007). Grape pomace can be used in functional food due to its high

fiber and phenolic content (Gonzalez-Centeno, 2010). Depending on literature data grape wastes can be used to extract the polyphenols in order to use them as natural antioxidants instead of synthetic antioxidants like butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), especially after the growing the evidence of the potential toxic and carcinogenic effects of the later synthetic antioxidants (Loo, 2008).

Milk is a complex food contains about 87% water. The other constitutes are proteins, carbohydrates, saturated and unsaturated fat, minerals, and vitamins. During heat treatment of milk, many reactions take place (belitz, 2009) which involve many macronutrients in milk. For example, Lysinoalanine formed by the reaction of lysine with dehydroalanine (Mauron, 1990), unsaturated lipids are oxidized resulting in hydroperoxide as primary products, in addition to aldehyde and ketone as secondary products (Nielsen and Loliger, 1985). Milk proteins are also subjected to various oxidation reactions under processing conditions (Mauron, 1990) which can affect protein functionality and nutritional properties (Baxter, 2007). Some articles mentioned that phenolic compounds could inhibit protein oxidation in food systems (Estevez, 2008) or inhibit oxidation of some separate amino acids (Salminen and Heinonen, 2008). However, no studies investigate the antioxidant effect of phenolic compounds on milk proteins. Therefore, the objective of this study was to determine the total phenolic content of grape waste extract, and then to investigate the effect of this extracts on milk proteins as a natural antioxidant.

2. MATERIALS AND METHODS

Chemicals: Bovine serum albumin (Tifan Biotech LTD, India), Sodium Phosphate (Riedel-De Haen AG, Germany), Di Sodium Phosphate (Merck, Germany), Urea, glacial acetic acid, tricholoracetic acid, ethyl acetate (Merck, Germany), Dinitro phenylhydrazine (Hemedia Laboratory, India), Folin-Denis' reagent (Fluka, Sigma-Aldrich), Gallic Acid (Biotech LTD), Sodium Carbonate (BDH, England).

Apparatus: Spectrophotometer (Jasco v-530 UV), Analytical balance (RADWAG, AS 220/C/2), centrifuges (Labofuge 200 Heraeus, REMI Labotatory centrifuge R4C), water bath (Bandalin soronex Digitec), Oven (Carbolite), Micropipette (Labkit, Chemelex, Spain).

Extraction of phenolic compounds: Grape waste was dried before extraction at 60°C, and then extracted with a mixture of ethanol to water (1:1v/v) (Lafka, 2007). Briefly, 1 gram of grape waste was mixed with 20 ml of the extract mixture and left in an ultrasound bath for 45 min at 25 °C. After that, the mixture was filtered for the removal of particles. The phenolic content was determined directly in the ethanolic extract. Later, the extract was dried at 60°C and then reconstituted in distilled water before it was added to milk samples. The experiments were carried out in duplicate.

Determination of total phenolic content: Total phenolic content was determined using Folin-Ciocalteu reagent. First, 0.1 ml of the extract was mixed with two ml of freshly prepared sodium carbonate solution 2% (w/v) and allowed to stand for 5 min. 0.1 ml of Folin-Denis' reagent (1:1 diluted with distilled water) was added to the mixture and left at room temperature for 30 min. A blank solution was prepared in the same way like samples using 0.1 ml of distilled water instead of the sample. A standard curve was obtained using Gallic acid in concentrations range (0.1-0.7 g/L); the absorbance was read at 750 nm for both Gallic acid and extract. The equation was $y=1.1115x+0.0834$ and R^2 was 0.997. The total phenolic content was expressed as grams of Gallic acid equivalents (GAE) per 100g grape waste. The experiments were carried out in duplicate.

Milk sample preparation: The analysis involved pasteurized milk as example of mildly heat-treated milk and boiled milk as examples for severely heat-treated milk. Milk samples were divided into six 100 ml portions. Phenolic extract were added to three portions to achieve a final concentration of 200 ppm. For pasteurisation, two 100 ml portions (one with phenolic extract) were heated at 60°C for 30 min and then cooled in ice. For severe heat treatment, two 100 ml portions (one with phenolic extract) were heated until boiling (boiled seconds), and then cooled directly in ice The latest two 100 ml portions (one with phenolic extract) were boiled for 2 minutes, and then cooled directly in ice. The experiments were carried out in triplicate.

Measurement of protein oxidation using carbonyls by spectrophotometer at 370 nm: The trial was carried out according to literatures (Fenaille, 2005). Briefly, an aliquot of bovine milk corresponding to 2 mg proteins was precipitated with TCA, where the final concentration was 10%. As second step, 2 ml of 10 mM DNPH in 2M HCl were added to the precipitants and left for incubation for 30 min at room temperature. The mixture was centrifuged at 3000 rpm for 5 min, and then the precipitants were washed three times with 1 ml of a mixture of ethanol/ethyl acetate (50:50 v/v %) to remove free DNPH. Finally, the precipitants were dissolved in 4 ml of 6M urea. The measurement of absorbance was at 370 nm. The PC_S amount was expressed as mille mole of carbonyl per kilogram of protein using an absorption coefficient of $22.000M^{-1}cm^{-1}$ at 370 nm (Zrekah, 2015). The protein content was determined spectrophotometric at 280 nm. The experiments were carried out in duplicate.

Determination of protein content by spectrophotometer at 280 nm: The trial was carried out at the same time of PC_S determination where 2 ml of 2M HCl was added to each precipitate instead of 10 mM DNPH in 2M HCl, additionally, the precipitates were dissolved in 4 ml of neutral phosphate buffer in the final step instead of 6M urea (Zrekah, 2015). Serial concentrations of bovine serum albumin was prepared in phosphate buffer in range of

(0.2- 1 mg/1 ml). The protein amount in the final precipitates was determined spectrophotometric by measuring the absorbance at 280 nm depending on the equation y=0.823x+0.009, since R^2 was 0.9998. Protein content was determined before and after the measurement of PCs. The experiments were carried out in duplicate.

Statistical Analysis: All the results were presented as means ± standard deviations. The differences between PCs formation with/or without the addition of grape waste phenolic extract were determined by applying the Student's t-test.

3. RESULTS AND DISCUSSION

The moisture of grape waste was 55% and the total phenolic content was 4.6 % (w/w) of dried waste (table.1). Grape waste was dried at 60°C before extraction, because increasing the temperature above 60°C can reduce the yield of extracted phenols (AL-As'aad and Aldiab, 2015). Additionally, the antioxidant activity of grape waste is significantly affected by the temperature of drying, for example drying at 80°C or 100°C can reduce the antioxidant activity by 21% and 33%, respectively (Lafka, 2007). Ethanol was selected as a solvent for extraction, since literatures describe ethanol as the most appropriate solvent for the extraction of phenolic compounds from grape waste. The extraction with ethanol yields high phenol content and high antioxidant activity of grape waste extracts. Generally, the major phenolic compounds in grape waste are anthocyanins, catechins, glycosides of flavonols and phenolic acids (Lafka, 2007).

Table.1. Moisture and phenolic content of grape waste extract

	Moisture	Phenolic content of dried grape waste
Ethanolic extract	55 %	4.6 %

The industrial production of dairy products requires thermal treatment in order to ensure microbiological safety (Fenaille, 2006), as well as to increase the shelf life of the products (Stanciuc, 2009). However, the exposure of milk to heat treatment can promote protein oxidation and glycation reactions (Mauron, 1990) leading to the formation of different compounds. Some amino acids are particularly sensitive to oxidative modification. For example, tryptophan can be oxidized to form a number of derivatives generated by opening of the indole ring. Oxidation converts methionine to methionine sulfoxide and methionine sulphone. Other amino acids, such as lysine and arginine, can also be subjected to oxidation, which is often characterized with the formation of carbonyl groups (Guy and Fenaille, 2006). Protein carbonyls (PCs) content is used as an indicator for protein oxidation (Zrekah, 2015) and thus for heat treatment.

Studies mentioned that protein is lost during the preparation of samples for the measurement of PCs, which leads to underestimation. One way to avoid this problem was to measure the final protein amount in the final precipitates by spectrophotometric method at 280 nm (Estevez, 2009). In this study, about 20% of protein was lost from milk samples during the procedure of PCs determination (table.2).

Table.2. Protein content of milk samples

	Boiled milk	Pasteurized milk
Protein content before PCs measurement (mg/ml)	33	33
Protein content after PCs procedure (mg/ml)	26	27

The highest contents of PCs were found in milk boiled for 2 min (51.8 nmol/mg protein) (figure.1), while pasteurization - as a mild heat treatment- induced lower level of oxidation (13.64 nmol/mg protein) (figure.2). Even if PCs are formed only in little amount in boiled and pasteurized milk, the nutritional value is hardly affected, especially when taking into account that some essential amino acids like lysine and arginine are of the main contributor of PCs formation (Guy and Fenaille, 2006). On the other hand any decrease of PCs can improve milk quality.

The formation of PCs was noticed in all milk samples regardless the addition of phenolic extract, in the way that PCs content was increasing as the thermal treatment increased. This can be explained by the high temperature applied to boil milk which leads to the degradation of the Amadori product (as the first stable product from glycation in milk), and thus promoting the generation of hydroxyl radicals and enhancing the oxidation reaction (Mossine and Linetsky, 1999). PCs are also resulted by the reaction between the amino group and the reactive dicarbonyl compounds generated from the Maillard reaction and/or lipid oxidation (studied milk was high fat milk). For example lysine reacts with the secondary products of lipid oxidation to form lysine aldehyde (Nielsen and Loliger, 1985). Lysine aldehyde is an example of carbonyl products formed during heat treatment of milk proteins. Additionally, free radical produced by metal-catalyzed oxidation can lead to the formation of carbonyl groups (Meltretter, 2007).

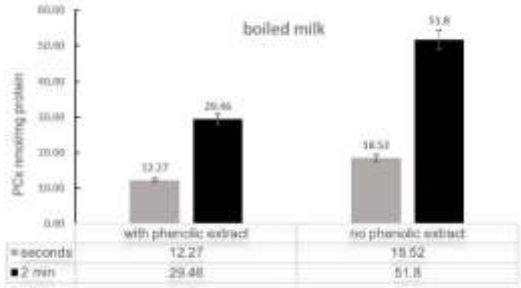

Figure.1. Decrease of PCs after the addition of grape waste phenolic extract to boiled milk

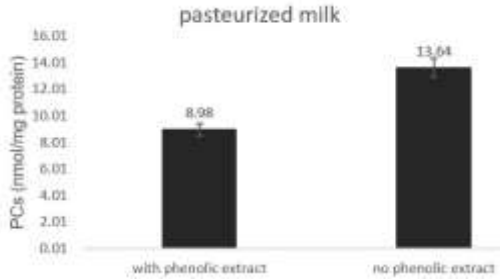

Figure.2. Decrease of PCs after the addition of grape waste phenolic extract to pasteurized milk

Interestingly, the samples of milk with phenolic extracts, boiled 2 min, boiled seconds and pasteurised milk (figure.1 and 2), showed lower levels of oxidation (29.46, 12.27 and 8.98 nmol/mg protein, respectively) compared to those with no phenolic additions (51.8, 18.52 and 13.64 nmol/mg protein, respectively), ($p<0.05$). This can be explained by the effect of phenolic compounds as natural antioxidants. The antioxidant activity of phenolic compounds are related to many mechanism like H•-donating activity, scavenge free radicals and chelate pro-oxidants (transition metals). The addition of phenolic extract of grape waste inhibited the formation of PCs about 35% to 45% in pasteurized and boiled milk, respectively. It should keep in mind that PCs are indicators for oxidation; consequently, the reduction of them may be concomitant with the reduction of oxidation in milk and thus the protection of other nutrients. Previously, the phenolic extract of grape pomace was used for meat and meat products and showed intense antioxidant effect in pork burgers at concentration of 0.06g/100 g (Garrido, 2011). The effect of phenolic compounds as food preservative results from preventing the formation of off-flavor and toxic compounds generating from lipid oxidation (Lafka, 2007). In addition to this, the preservative effect in our study resulted from the reduction of protein oxidation. Thus, grape pomace can be used for food preservation. However, more experiments should be done to investigate the effect of phenolic extract concentration on protein oxidation, because some studies mention that the phenol extracts acts as antioxidants in a narrow range of concentrations of 50–200 ppm. Outside this range, the phenolic extracts act as prooxidants, certainly at low concentrations (Lafka, 2007). Finally, more experiments should be done to investigate the effect of phenolic compounds on the functional properties of milk protein.

4. CONCLUSION

The phenolic compounds were extracted from grape waste and added to boiled and pasteurized milk. The phenolic extract inhibited the oxidation of milk proteins presenting by decrease the formation of protein carbonyls, which enable phenolic extract of grape waste to play a role in food preservation as natural antioxidant for proteins of milk and dairy products.

REFERENCES

AL-As'aad N, Aldiab D, Determination of Phenolic Compounds Levels and Their Antioxidant Activity in Some Local Functional Juices, Tishreen University Journal for Research and Scientific Studies, 2015, 37.

Baxter J.H, Si Lai C, Phillips R, Dowlati L, Dimler S.R, Johns P.W, Direct determination of methionine sulfoxide in milk proteins by enzyme hydrolysis/high-performance liquid chromatography, Journal of Chromatography A, 1157 (1-2), 2007.

Baydar G.N, Oezkan G, Yasar S, Evaluation of the antiradical and antioxidant potential of grape extracts, Journal of Food Control, 2007, 18.

Belitz HD, Grosch WS, Schieberle P, Food Chemistry, 4th edition, Springer, 2009.

Burke K.E, Photodamage of the skin, protection and reversal with topical antioxidant, Journal of Cosmetic Dermatology, 3, 2004, 3.

Chand R, Narimura K, Kawakita H, Ohto K, Watari T, Inoue K, Grape waste as a biosorbent for removing Cr (VI) from aqueous solution, Journal of Hazardous Materials, 163, 2009, 1.

Duh P.D, Yen G.C, Antioxidant efficacy of methanolic extracts of peanut bulls in soybean and peanut oil, Journal of American Oil Chemical Society, 1997, 74.

Dwyer K, Hosseinian F, Rod M, The Market Potential of Grape Waste Alternatives, Journal of Food Research, 2014, 2-3.

Estevez M, Kylli P, Puolanne E, Kivikari R, Heinonen M, Oxidation of skeletal muscle myofibrillar proteins in oil-in water emulsions, Interaction with lipids and effect of selected phenolic compounds, Journal of Agricultural and Food Chemistry, 2008, 56.

Estevez M, Ollilainen V, Heinonen M, Analysis of protein oxidation markersa–aminoadipic and γ-Glutamic semialdehydes in food proteins using liquid chromatography (LC)-electrospray ionization (ESI)–multistage tandem mass spectrometry (MS), Journal of Agricultural and Food Chemistry, 2009, 57.

Fenaille F, Paresiod V, Visan P, Populaire S, Tabet JC, Guy PA, Modification of milk constituents during processing, a preliminary benchmarking study, International Dairy Journal, 2006, 16.

Fenaille F, Parisod V, Tabet JC, Guy PA, Carbonylation of milk powder proteins as a consequence of processing conditions, Proteomics, 2005, 5.

Garrido MD, Auqui M, Marti N, Linares MB, Effect of two different red grape pomace extracts obtained under different extraction systems on meat quality of pork burgers, LWT-Food Science and Technology, 2011, 44.

Gonzalez-Centeno M.R, Rossello C, Simal S, Garau MC, Lopez F, Femenia A, Physicochemical properties of cell wall materials obtained from ten grape varieties and their byproducts, grape pomaces and stems, LWT-Food Science and Technology, 2010, 43.

Guy PA, Fenaille F, Contribution of mass spectrometry to assess quality of milk-based products, Mass Spectrometry Reviews, 25 (2), 2006.

Kaur C, Kapoor H, Antioxidants in fruits and vegetables and the millennium's health, International Journal of Food Science and Technology, 2007, 36.

Lafka T.L, Sinanoglou V, Lazos S.E, On the extraction and antioxidant activity of phenolic compounds from winery wastes, Journal of Food Chemistry, 2007, 104.

Loo A, Jain K, Darah I, Antioxidant activity of compounds isolated from the pyroligneous acid, Rhizophora apiculate, Journal of Food Chemistry, 2008, 107.

Mauron J, Influence of processing on protein quality, Journal of Nutritional Scince and Vitaminology (Tokyo), 1990, 36.

Meltretter J, Seeber S, Humeny A, Becker C-M, Pischetsrieder M, Site-specific formation of Maillard, oxidation, and condensation products from whey proteins during reaction with lactose, Journal of Agricultural and Food Chemistry, 2007, 55.

Mendes A.S.J, Prozil O.S, Evtuguina V.D, Cruz-Lopesb P.L, Towards comprehensive utilization of wine making residues: Characterization of grape skins from red grape pomaces of variety Touriga Nacional, Journal of Industrial Crops and Products, 2013, 43.

Minussi R.C, Rossi M, Bologna L, Cordi L, Rotilio D, Pastore G.M, Phenolic compounds and total antioxidant potential of commercial wines, Journal of Food Chemistry, 2003, 82.

Mossine V.V, Linetsky M, Superoxide free radical generation by Amadori compounds, the role of acyclic forms and metal ions, Journal of Chemcal Research in Toxicology, 12 (3), 1999.

Murthy K.N.C, Singh R.P, Jayaprakasha G.K, Antioxidant activity of grape (*Vitis vinifera*) pomace extracts, Journal of Agricultural and Food Chemistry, 2002, 50.

Nick K, Anastasia M, Antonia C, Maria S.I, Nikolaos K.A, Retention and distribution of natural antioxidants (a-tocopherol, polyphenols and terpenic acids) after shallow frying of vegetables in virgin olive oil, LWT-Food Science and Technology, 2007, 40.

Nielsen H.K, Loliger J, Reactions of Proteins with Oxidizing Lipids, Analytical Measurements of Lipid Oxidation and of Amino-Acid Losses in a Whey-Protein Methyl Linolenate Model System, British Journal of Nutrition, 53 (1), 1985.

Perez-Magarino S, Gonzalez-San Jose M.L, Polyphenols and color variability of red wines made from grapes harvested at different ripeness grade, Journal of Food Chemistry, 2006, 96.

Rivera O.M.P, Moldes A.B, Torrado A.M, Dominguez J.M, Lactic acid and biosurfactants production from hydrolyzed distilled grape marc, Process Biochemistry, 42 (6), 2007.

Salminen H, Heinonen M, Plant phenolics affect oxidation of tryptophan, Journal of Agricultural and Food Chemistry, 2008, 56.

Stanciuc N, Traceability indicators for heat treatments of Milk, Innovative Rom Food Biotechnology, 2009, 5.

Zrekah G, Aldiab D, Abboud A, Determination of Protein and fat oxidation levels in imported infant formula available in Syria, International Journal of Pharmacy and Pharmaceutical Sciences, 8, 2015, 2.