Total Polyphenols Content, Antioxidant Activity and Stability of a Grape Pomace Incorporated in Animal Feed

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Abstract

Grape pomace (GP), resulting from wine making, is rich in antioxidant polyphenols originating from the input material, the grapes. Because of the high production volumes of grape pomace, environmental impact and nutritional content, new ways for its valorization are experimented. In order to incorporate it in animal feed (cow and pig), we determined its content in total polyphenols by the Folin Ciocalteu method, the antioxidant activity by the DPPH assay and its stability using the UV-Vis spectroscopy for different extraction media. In terms of total polyphenols the acetone extraction was the best (4667.1 mg GAE/100g sample) comparing with ethanol and water (2140.4 mg GAE/100g sample respectively 2083.9 mg GAE/100g sample), and the ruminal fluid (732.9 mg GAE/100g sample) and pepsin (712.2 mg GAE/100g sample). The highest antioxidant activity expressed as an antiradical activity against the DPPH radical, was registered for the acetone extract (32.8 μM Trolox Equivalents(TE)), followed by the ethanol (6.5 μM TE) and water extraction equal with pepsin extraction (4 μM TE), and the mixture of ruminal fluid (0.5 μM TE). The results show that even though the extraction of polyphenols in organic solvents is the best, when applied to the digestive media the situation changes. In both ruminal fluid and pepsin were measured almost the same amount of total polyphenols but the antioxidant activity was much lower in the ruminal fluid-8 times lower. The UV-Vis spectroscopy shows that the acetone extract is stable over time when kept at–20 °C.

Keywords: animal feed, antioxidant activity, grape pomace, stability, total polyphenols.

1. Introduction

Feeding is the major factor affecting the quality of animal farm products (milk and meat). The feeding strategies useful for increasing the levels of healthy fatty acids (FA), such as conjugated linoleic acid and omega-3 FA, in milk and meat are reported [1-2]. In this way, food industry by-products could help to improve milk and meat quality [1]. Grape pomace (GP) is rich in a wide range of polyphenols especially anthocyanins [3] and catechin, epicatechin and procyanidins. Formerly known as tannins, these polyphenols were considered to be anti-nutritional factors as their presence in certain ingredients, such as legumes, sunflowers and sorghum had negative effects on animal nutrition [4]. The major limitations of using GP in feed are the high level of lignified cell wall fraction and the high tannin content [4]. However, studies carried out *in vivo* and *in vitro* over the last few years have shown the beneficial effects of administering these bioactive compounds because of their antioxidant and antimicrobial activity [4-7]. Grape pomace improved oxidative stability of lamb meat and the use of polyphenols is recommended to limit lipoperoxidation and preserve animal health and product quality [1].

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In this context we measured the content in total polyphenols by the Folin Ciocalteu method and the antioxidant activity as a antiradical (1,1-Diphenyl-2-picrylhydrazyl, DPPH) activity (A_{AR}) of a GP and its stability using the UV-Vis spectroscopy. For this we used different extraction media (acetone, ethanol and boiling water) as well as the incubation of GP sample first with ruminal fluid and then with pepsin. This is an in vitro method used for estimation of organic matter disappearance/digestibility of feeds and reproduce ruminant digestion by incubating feed sample first with ruminal fluid and then with pepsin [8].

2. Materials and methods

The GP was provided by Dionis Ltd., a Romanian producer of grape seed oil and derived from red varieties of grapes from Valea Calugareasca, a Romanian winery. The pomace was dried in a heated airflow and contained skin, pulp, seeds and stalks, and kept in dark at room temperature until analysis.

Polyphenols extraction and determination of the total phenolic content

The polyphenols from GP were extracted in acetone 80%, ethanol and boiled water (ratio, sample: solvent being 1:7 w/v) for 24 hours at 37^{o}C under continuous shaking.

The GP sample was tested using a similar protocol of Tilley-Terry method [8]. One gram of GP was weighed in a glass tube and 7 ml of diluted rumen fluid was added. The rumen fluid was collected from fistulated cows and diluted 1:4 with McDougall buffer, kept at 39^{O}C and saturated with carbon dioxide for pH around 6.9. After capping the tubes they were incubated 48 h at 39^{O}C. Next, the tubes were centrifuged at 3000 g for 5 minutes and supernatants were collected. 7 ml of pepsin solution 2% in HCl 0.1N was added on retained sediments and also incubated 48 h at 39^{O}C. After centrifugation 5 minutes at 3000 g the supernatants were collected. For each incubation step duplicate blank tubes (without sample) were used.

Total polyphenols (TP) determination (Folin-Ciocalteu method)

The content in total phenolic compounds of the extracts was determined by the Folin–Ciocalteu method, adapted to a microscale [9]. The results were expressed as gallic acid equivalents (GAE)/L [10].

Evaluation of the antioxidant activity using the DPPH method

1,1-Diphenyl-2-picrylhydrazyl (DPPH) is a stable free radical and has been commonly used to screen phenolic compounds containing high free radical scavenging ability [11]. The antioxidant activity was determined using the DPPH test according to Arnous et al. [9] with slight modifications. An aliquot of 20 μL sample was added to 980 μL of DPPH solution (60 μM in methanol), vortexed, and the absorbance was read at t=0($A_{515}(0)$) and t=30($A_{515}(30)$) min using a Specord 250 (Analytik Jena, Jena, Germany) array spectrophotometer. The A_{AR} was determined as follows: $\%\Delta A_{515}$(μM Trolox Equivalents TE)=$0.0921\times A_{AR}+2.3146$ as determined from linear regression, after plotting %ΔA515 of known solutions of Trolox against concentration (85–800 μM, R^2=0.995) where:

$\%\Delta A_{515}=[(A_{515}(0)-A_{515}(30))/A_{515}(0)]\times 100$

UV-Vis spectra measurement

The spectra were recorded at room temperature using a spectrophotometer (a UV-visible diode array spectrophotometer Specord 250, Analytik Jena, Jena, Germany), Scan Mode module, in the UV–Vis range 190 nm–1100 nm and 200 nm-500 nm, a 4 nm slit, delta lambda 1 nm and speed 50 nm/s.

Statistical analysis

The results were presented as the mean percentages of control±standard errors of the mean (SEM) from at least three independent measurements. Experimental data were analyzed with the program Stat View 5.0, performing one-way analysis of variance (ANOVA), followed by a Fisher protected least significant difference (PSLD) test: P values lower than 0.05 were considered significant.

3. Results and discussion

For the determination of TP, as a first step in polyphenol analysis in any extract considered to contain phenolic compounds, even though different methods are used, Folin Ciocalteu is the

one used the most and it relies on the transfer of electrons in alkaline medium from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes to form blue complexes that are determined spectroscopically at approximately 760 nm [12-15]. In our case, TP content of the acetone extract was the best (4667.1 mg GAE/100g sample) comparing with ethanol and water (2140.4 mg GAE/100g sample respectively 2083.9 mg GAE/100g sample), and the ruminal fluid (732.9 mg GAE/100g sample) and pepsin (712.2 mg GAE/100g sample) ones (Table 1). GP extraction in ruminal fluid and in pepsin gave no statistically different yields; all others are different (Table 1).

We have chosen, besides the classical extraction solvents, also the ruminal fluid and pepsin in order to assess the TP extraction in the digestive media of ruminants (especially cow). The ruminants, digestive system has four stomachs: rumen, reticulum, omasum and abomasum. The most important for the digestion process are the rumen with the ruminal fluid and the abomasum where the pepsin is found.

For TP expressed as mg GAE/L extract, the GP extracted with ruminal fluid (1033.4 mg GAE/L extract) was not statistically different from the pepsin extract (1017.4 mg GAE/L extract) and pepsin alone (980.3 mg GAE/L pepsin). Pepsin extract (1017.4 mg GAE/L extract) is also not different than the pepsin alone (980.3 mg GAE/L pepsin) and ruminal fluid alone (953.3 mg GAE/L ruminal fluid), and no difference between ruminal fluid (953.3 mg GAE/L ruminal fluid) and pepsin (980.3 mg GAE/L pepsin) both without GP (Table 1).

The major shortcoming of Folin Ciocalteu assay is the fact that it measures besides the total polyphenols other oxidation substrates also [12-15]. This might be the reason for which we obtain high values of TP content for the ruminal fluid and pepsin alone.

The antioxidant activity A_{AR} decreases in the same order like for the TP in case of the classical solvents: acetone (32.8 μM TE)>ethanol (6.5 μM TE)>water (4 μM TE) (Table 1). For DPPH test there were statistically significant differences for all the compared samples but not for water and pepsin. Equal A_{AR} actions proved to have the water and pepsin extracts (4 μM TE). An 8 time lower activity was measured for the ruminal fluid extract (0.5 μM TE) even though that the TP content was higher than the one of pepsin extract (Table 1). Surprisingly pepsin alone gave a higher A_{AR} than ruminal fluid and pepsin extracts and the ruminal fluid alone. Interestingly is that GP transforms the pro-oxidant environment of the ruminal fluid alone (-0.9 μM TE) to an antioxidant tendency for the ruminal fluid extract (0.5 μM TE).

As the GP acetone extract proved to be the richest one in polyphenols with the highest A_{AR} activity, its stability when kept at -20^{o}C for 8 months was assessed. The UV-Vis spectrum of a fresh extract (b) was overlaid with the UV-Vis spectrum of an older one (a) kept in these conditions (Figure 1). As Figure 1 shows no bathochromic or hypsochromic shift was registered for the maximum absorption wavelength (λ_{max}) which in both cases is 260 nm.

Table 1. Total polyphenols (mg GAE/100g sample) and antioxidant activity expressed as antiradical activity against the DPPH radical (μM Trolox Equivalents) for dried grape pomace extracted with different media

Extraction media	Total Polyphenols (mg GAE/L extract)	Total Polyphenols (mg GAE/100g sample)	Antioxidant activity A_{AR} (μM Trolox Equivalents)
Acetone 80%	6710.9±84.7^{a}	4667.1±37.8^{a}	32.8±0.1^{a}
Ethanol	3057.7±61.0^{b}	2140.4±42.7^{b}	6.5±0.05^{b}
Water	2977.0±13.2^{c}	2083.9±9.2^{c}	4±0.04^{c}
Ruminal fluid	1033.4±1.5^{d}	732.9±12.3^{d}	0.5±0.03^{d}
Pepsin	1017.4±12.3^{d,e,f}	712.2±8.6^{d}	4±0.03^{c}
Ruminal fluid alone	953.3±8.7^{f}	-	-0.9±0.07^{e}
Pepsin alone	980.3±12. 8^{d,e}	-	5.3±0.09^{f}

$^{a, b, c, d, e, f}$ Different superscript letters indicate statistically significant differences within each column, for $p<0.05$.

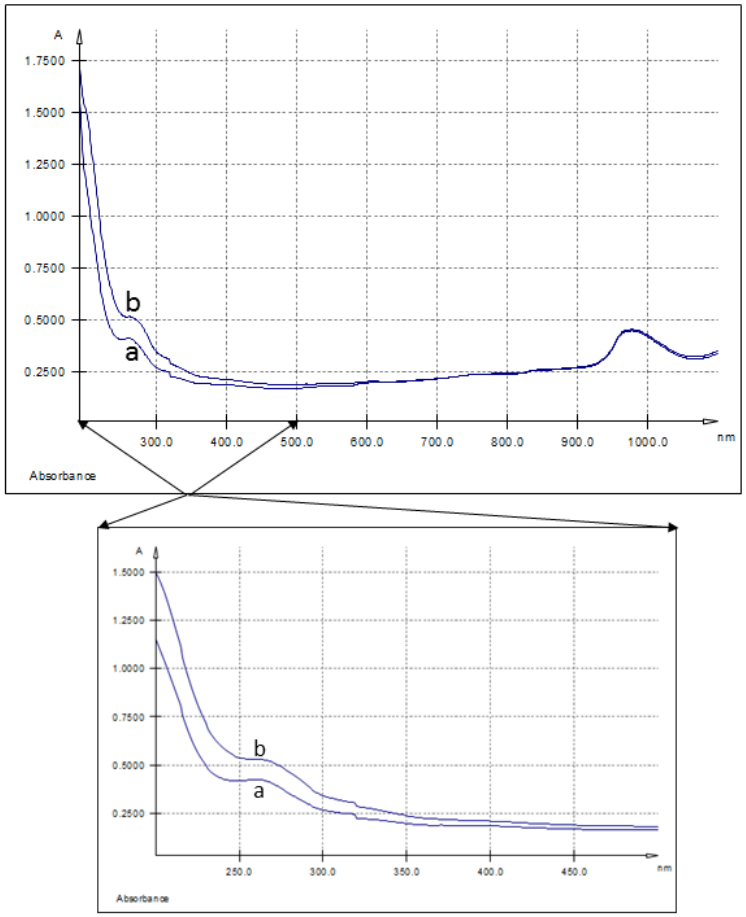

Figure 1. Overlay of UV-Vis spectra of a GP extract in 80% acetone kept at -20°C for 8 months (a) and of fresh 80% acetone GP extract (b)

4. Conclusions

Different extraction media (acetone, ethanol and boiling water) as well as the incubation of GP sample first with ruminal fluid and then with pepsin were used in order to assess the TP content and A_{AR} activity of GP. The GP acetone extract proved to be the richest one in polyphenols with the highest A_{AR} activity, keeping its stability at -20°C for 8 months.

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References

1. Manso, T., Gallardo, T., Guerra-Rivas C., Modifying milk and meat fat quality through feed changes, Small Ruminant Research, 2016, in press.

2. Habeanu, M., Lefter, N. A, Ropota, M., Chedea, V. S, Gheorghe, A., Toma, S. M, Ciurescu G., Dragomir, C., Dried grape pomace influenced fatty acids composition of *Longissimus dorsi* muscle and plasma polyphenols spectrum in finishing pigs, Indian Journal of Animal Sciences, 2015, 85(7), 786–789.

3. Chedea, V. S., Palade L. M., Rotar, M. C., Călin, L. G., Dragomir, C. The anthocyanin composition of a red grape pomace in relation with the wine industry by-products-valorization in animal feed, Lucrări Ştiinţifice Seria Horticultură U.S.A.M.V. Iaşi, 2015, 58(1), 29-34.

4. Brenes, A., Viveros, A., Chamorro, S., Arija, I., Use of polyphenol-rich grape by-products in monogastric nutrition, A review, Animal Feed Science and Technology, 2016, 211, 1-17.

5. Alonso, A. M., Guillé, D. A., Barroso, C. G., Puertas, B., García, A., Determination of antioxidant activity of wine-byproducts and its correlation with polyphenolic content, J. Agric. Food Chem. 2002, 50, 5832–5836.

6. Torres, J. L., Varela, B., García, M. T., Carilla, J., Matito, C., Centelles, J. J., Cascante, M., Sort, X., Bobet, R., Valorization of grape (*Vitis vinifera*) byproducts. Antioxidant and biological properties of polyphenolic fractions differing in procyanidin

composition and flavonol content, J. Agric. Food Chem., 2002, 50, 7548–7555.
7. Viveros, A., Chamorro, S., Pizarro, M., Arija, I., Centeno, C., Brenes, A., Effects of dietary polyphenol-rich grape products on intestinal microflora and gut morphology in broiler chicks, Poultry Science 2011, 90, 566–578.
8. Tilley, J .M. A. and Terry, R. A. A two-stage technique for the in vitro digestion of forage crops. J. Brit. Grassl. Soc., 1963, 18, 104–111.
9. Arnous, A., Makris, D. and Kefalas, P. Effect of principal polyphenolic components in relation to antioxidant characteristics of aged wines, J Agric Food Chem., 2001, 49, 5736–5742.
10. Chedea, V. S., Pelmuş, R. Ş., Toma, S. , Ţăranu, I., Grosu, H., Dragomir, C. Evaluation of camelina meal as a dietary source of polyphenol for dairy cows, Bulletin UASVM Animal Science and Biotechnologies, 2014, 71(2), 279-280.
11. Wettasinghe, M. and Shahidi, F. Scavenging of reactive-oxygen species and DPPH free radicals by extracts of borage and evening primrose meals. Food Chemistry, 2000, 70, 7–26.
12. Singleton, V. L., Orthofer, R., and Lamuela-Raventós, R. M., Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. In Oxidants and Antioxidants Part A, in Enzymology, Ed. Academic Press, 1999, 299, pp. 152–178.
13. Dai, J., and Mumper, R. J., Plant phenolics: Extraction, analysis and their antioxidant and anticancer properties, Molecules, 2010, 15, 7313–7352.
14. Singleton, V. L., and Rossi, J. A., Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents, Am. J. Enol. Vitic., 1965, 16, 144–158.
15. Palade L. M. and Chedea V. S., Antioxidant/pro-oxidant action of polyphenols from grape seeds. In Grape Seeds: Nutrient Content, Antioxidant Properties and Health Benefits, J. M. L. Rodríguez, and D. F. Ruiz Eds. Ed. Nova Science Publ. 2016, in press.