Study of the feeding value and antioxidant capacity of winery by-products, potential natural antioxidants for farm animal diet formulations

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SUMMARY

The need to use antioxidants in animal diet formulations, particularly natural antioxidants, whose role is to block oxidation by their reaction with the free radicals, appeared particularly because of the use of high polyunsaturated fatty acids dietary ingredients, with the purpose to obtain animal products rich in omega 3 fatty acids, which are beneficial to human health. Some of the sources rich in polyphenols are the winery by-products: grape marc, grape seeds and peels. The abundant bioactive polyphenols from these by-products are of real interest for researchers and for the feed industry companies. The purpose of our work was to determine the basal nutrients and the antioxidant capacity of the winery by-products: dry grape marc, seed-less grape marc and grape seeds cakes, obtained from a Romanian winery in order to evaluate the potential of these by-products to be used as feed additives. We determined in the laboratory the concentration of dry matter, protein, amino acids, fat, fatty acids, fibre, ash and minerals, as indicators of the feeding value, as well as the concentration of polyphenols a and the antioxidant capacity, using the proper methodologies according to the acting standards. the dry matter concentration ranged between 80.37% in the dry grape mark with seeds and 88.44% in the grape seeds cakes, wile the highest protein concentration (12,64%) was determined in the dry grape mark with seeds. The lysine concentration ranged between 0.421% (grape seeds cakes) and 0.544% (dry grape mark with seeds), while methionine concentration ranged between 0.09% (dry grape mark with seeds) and 0.1% (grape seeds cakes). The highest concentration of linolenic acid (C18:3n3) was determined in the dry grape mark with seeds (6.43 g/100 g fat). The antioxidant capacity determined in the grape seeds cakes (6.24 mM

trolox equivalent/g sample) was lower than in the dry grape mark with seeds (11.21 mM trolox equivalent/g sample). These results show that the dry grape mark with seeds or without seeds, or the grape seeds cakes, are potential natural feed additives with antioxidant properties and good feeding value.

Keywords: winery by-products, additives, antioxidants, feeding value

INTRODUCTION

The increased use of fats and oils in animal diet formulations provide an adequate environment for rancidity, which deteriorates the feeding value of the forages and, therefore, impedes on animal performance and health. As the dietary concentration of fatty acids increase, the susceptibility of the cell membranes to induce the oxidative stress in animal organism also increases (Miret et al., 2003), as well as the susceptibility of the animal products (meat, eggs) to lipid oxidation, which depresses their acceptance by the consumer (Sparks, 2006). This prompted the necessity to supplement the diets, particularly those high in polyunsaturated fatty acids, with antioxidants whose role is to inhibit oxidation (Criste et al., 2009).

Antioxidants are substances able to slow/block the oxidative processes. The feeding practice uses mostly the synthetic antioxidants based on phenolic structures (Mukai et al., 2005; Andjelkovic et al, 2006). The consumers became, however, reticent to the use of synthetic products in animal feeding and as feed additives, preferring the natural products and the foods that have high feeding value and no synthetic ingredients.

The natural antioxidants too can protect biologically the cell components involved in the oxidative processes caused by the reactive oxygen species (Su et al., 2008). Thus, extracts or dry leaves of oregano, officinal rosemary and savory have been added to poultry diets in order to determine their performance and the oxidative stability of the poultry eggs or meat. (Giannenas et al., 2005; Florou-Paneri et al., 2006; Botsoglou et al., 2007). Winery by-products have also been evaluated as sources of natural antioxidants rich in polyphenols (Novaka et al., 2008; Ky et al., 2014). Most of the total amount of polyphenols from the grapes are in the seeds (60-70%), followed by the peals (28-35%) and pulp (less than 10%) (Shi et al., 2003). Surai (2014) shows that extracts obtained from grape seeds and pomace are complex in composition and contain monomeric phenolic compounds such as (+)-catechins, (_)-epicatechin and (_)-epicatechin-3-O-gallate, and dimeric, trimeric, and tetrameric proanthocyanidins.

The grape polyphenols undergo chemical reactions during the processing and storage period, including polymerization and depolymerisation, enzyme reaction and copigmentation. Hatzidimitrioua et al. (2007) showed that the

total concentration of phenols in the grape seeds decreases during storage. The changes are minor for the samples stored at less than 55% humidity, but a high humidity (75%) accelerated degradation, decreasing the phenols content by 50%. Using the indicator of the continuous release of gallic acid, the authors determined that this degradation was caused by the hydrolytic reactions. Gollücke et al., (2008a), however, showed that these processes of transformation don't necessarily influence the total polyphenols content or the *in vitro* antioxidant activity.

The main product of wine production is the grape marc, or pomace. Grape marc consists of all the vegetal parts composing the grapes used to produce the grape juice or wine by pressing, and which can be unfermented or in various degrees of alcohol fermentation. It consists of 55-65% peals, 20-25% stems and 18-25% seeds, depending in the grape processing technology, representing about 20% of the weight of the grapes transformed in wine (Llobera and Cañellas, 2007). This grape by-product is a rich source of polyphenols (Kammerer et al., 2004; Katalinić et al., 2010).

The phenols composition of the grape marc varies much with the variety of grapes, with the environmental and climate conditions, with the soil, level of ripening and with the technological wine-making process (Hatzidimitrioua et al., 2007; Lachman et al., 2007; Iacopini et al., 2008; Rababah et al., 2008; Xu et al., 2010). The seeds cakes are another by-product resulting from the cold pressing or chemical extraction of oil. The literature shows the beneficial effects of these by-products, such as improved layer performance and lower egg yolk cholesterol concentration following the use of grape seeds extract (Hu et al., 2013) or of grape seeds cakes (Su et al., 2008). On the other hand, Lau and King, (2003), reported lower average final weight of the animals fed grape marc extracts. These contradictory results can be explained by the impact of these by-products on nutrient bioavailability. The efficiency of the natural antioxidants depends mainly on their gross chemical composition and on their concentration in the forage (the low to moderate concentration are beneficial).

The recovery of the antioxidant compounds from these wastes is also environmentally important because large amounts of wastes are generated in the wine-making areas, which create ecologic and economic problems in terms of storage, transformation or disposal (Bustamante et al., 2008). This explains the increasing interest to use these winery by-products, particularly their antioxidant compounds. Romania also has extended vineyard areas which produce large amounts of winery by-products, hard to store or dispose of, which could be used for other purpose rather than the production of distilled beverages.

Within this context, this paper shows the results of a study on the feeding value and antioxidant capacity of winery by-products, which evaluated their potential use as natural sources of antioxidants in poultry diets.

MATERIAL AND METHODS

The studied by-products were the dry grape marc, seed-less grape marc and grape seeds cakes, obtained from a south-east Romanian winery. The grape marc consists of grape peals, pulp and seeds of red grapes, with no stems. The dry, seed-less grape marc consists of red grapes peals and pulp only, with no fermentation smell. The seed cakes, by-product of oil extraction, had no smell of fermentation or rancidity.

The feeding properties of these winery by-products were determined by assays of the dry matter, protein, amino acids, fat, fatty acids, fibre, ash and minerals. The polyphenols concentration (mg equivalent gallic acid/g sample) and the antioxidant capacity (mM equivalent Trolox/g sample) were also determined. The following standardized methods were used to determine the concentration of basic nutrients.

The dry matter (DM) was determined with the gravimetric method which involves determining the mass of the sample by drying it at 103^0C, according to Regulation (CE) nr. 152/2009 and standard SR ISO 6496:2001, using analytical scales Sartorius (Gottingen, Germany) and oven BMT model ECOCELL Blueline Comfort (Neuremberg, Germany).

The crude protein (CP) was determined by the Kjeldahl method which involves decomposing the sample by heating with sulphuric acid in the presence of a catalyst for the conversion of the protein nitrogen into ammonium sulphate. The reaction products are alkalinized with sodium hydroxide to release the captured ammonia, by distillation in a solution of boric aid in excess, followed by titration with hydrochloric acid solution. The method complies with Regulation (CE) nr. 152/2009 and standard SR EN ISO 5983-2:2009 and used a semiautomatic KJELTEC auto 2300 – Tecator (Sweden).

The amino acids were determined by liquid chromatography (HPLC) which involves breaking the amino acids chain from the protein molecule by acid hydrolysis with HCl 6N. The sulphur amino acids – cystine and methionine, are oxidized with performic acid to cysteic acid and methionine sulphone, before hydrolysis. The excess of performic acid decomposes by addition of sodium metabisulphite. The amino acids are determined by high performance liquid chromatography after sample derivatization with ortho-phthalaldehyde (OPA) and detection at 338 μm. The method complies with Regulation (CE) nr. 152/2009 and standard SR EN ISO 13903:2005 and used Surveyor Plus HPLC

fitted with PDA detector (Thermo Electron, USA), Hypersil BDS C18 column with silica gel (250 ×4.6 mm), particle size 5 µm, with reverse phase (Thermo Electron, USA), Rotavapour R-205 (Bűchi, Switzerland).

The crude fat (EE) was determined by extraction in organic solvents which involves the extraction of fat in petrol ether, removal of the solvent by distillation, drying and weighing the residue. The method complies with Regulation (CE) nr. 152/2009 and standard SR EN ISO 6492:2001 and used a SOXTEC-2055 FOSS – Tecator (Sweden).

The fatty acids were determined by gas chromatography which involves the transformation of the fatty acids from the sample in methyl esters and separation of the components in the chromatographic column, their identification by comparison with the standard chromatograms. The method complies with standard SR CEN ISO/TS 17764 -2: 2008 and used a Perkin Elmer-Clarus 500 chromatograph with capillary injection, high polarity stationary phase (BPX70: 60m x 0.25mm inner diameter and 0.25µm film thickness); or high polarity cyanopropyl phase which produce similar resolution for different geometric isomers (THERMO TR-Fame: 120m x 0.25mm ID x 0.25µm film).

The crude fibre was determined by the method with intermediary filtration, which involves the determination of sample mass after successive boiling with sulphuric acid and sodium hydroxide solutions. The resulting residue is then filtered, dried, burned and weighed. The method complies with Regulation (CE) nr. 152/2009 and standard SR EN ISO 6865:2002 and used a FIBERTEC 2010–Tecator.

The ash was determined with the gravimetric method, which involves sample decomposition by burning and weighing of the resulting ash. The method complies with Regulation (CE) nr. 152/2009 and standard SR EN ISO 2171:2010 and used a Caloris CL 1206 furnace.

The calcium, copper, iron, manganese and zinc were determined by atomic absorption spectrometry, which involves sample digestion under pressure using a microwave oven. The digested sample was absorbed in the flame of an atomic absorption spectrophotometer with graphite furnace (GF-AAS), with double beam and background correction. The absorption of the radiation was measured at the wavelength corresponding to the analysed element 422.7 nm (calcium), 324.8 nm (copper), 248.3 nm (iron), 279.5 nm (manganese) and 213.9 nm (zinc). The method complies with standard SR EN ISO 6869:2002 and used a SOLAAR M Thermo Electron atomic absorption spectrophotometer.

The phosphorus was determined by spectrophotometry, which involves its reaction with molybdovanadate reagent, which produces a yellow complex whose optical density, directly proportional to phosphorus concentration, is measured by photocolorimetry at 420 nm. The method complies with

Regulation (CE) nr. 152/2009 and used a molecular absorption spectrophotometer ABLE JASCO Romania V 530.

The gross energy was determined by calculation, using the dry matter, protein, fibre, fat, nitrogen-free extractives and ash, using the equations developed by Burlacu et al. (2002).

The polyphenols concentration and the antioxidant capacity of the samples involved first the extraction of the phenolic compounds in acidified methanol (methanol:HCl = 80:20). 10 ml acidified methanol were poured over 1 g of sample and stirred, at room temperature, for 48 h. The homogenate was centrifuged twice at 10,000 RCF, for 15 minutes, at room temperature, and the supernatant (methanolic extract) was stored at 4^0C until analysed. The following instruments were used: orbital stirrer Heidolph Unimax 1010, Microstirrer Vepl Scientific, Eppendorf 5810R centrifuge, RADWAG AS220/C/2 (10-220 mg) and PS600/C/2 (0.01-600 g) scales, WTW Senix-HW pH metre.

The polyphenols content of the methanolic extracts was determined according to the method described by Mihailovic et al. (2013), modified. The reaction mixture consisted of he methanolic extract diluted according to the analysed sample, the Folin-Ciocâlteu reagent and a solution of 7.5% Na_2CO_3. The reaction mixture was kept for 30 min. at room temperature and then absorbance was read at 765 nm. Three replicates of the same sample were used, and the average of the readings, representing the total content of polyphenols, was expressed as equivalents of gallic acid/g fresh matter (mg EAG/g sample). The determinations used a UV-VIS Thermo Scientific spectrophotometer.

The antioxidant capacity of the methanolic extracts was determined by the DPPH method proposed by Marxen et al. (2007), and the antioxidant capacity was estimated by calculating the difference between the control and the sample, versus a standard curve which used Trolox (synthetic antioxidant analogue to α-tocopherol), as standard antioxidant. Three replicates of the same sample were used, and the average of the readings, representing the antioxidant capacity was expressed as Trolox equivalents /g fresh matter (mM Trolox/g sample). The determinations used a UV-VIS Analytik Jena Specord 250 Plus spectrophotometer.

Origin 5 software was used for the statistical processing of the data.

RESULTS AND DISCUSSION

Table 1 shows the concentration of basic nutrients (gross chemical composition) of the studied winery by-products. The table shows that dry grape marc with seeds had the highest level of protein (12.64 g/ 100 g), followed closely by the other two by-products. Because the grape seeds cakes

are a by-product from oil extraction, their crude fat content was just 1.56%, 67% lower than in the other two by-products. On the other hand, the 40.66% fibre is 71.78% higher than in grape marc with seeds and 91.25% higher than in the seed-less grape marc.

Table 1 data are in agreement with the data on grape seeds reported by Razavi and Fathi, 2009; Mironeasa et al., 2010; Elagamey et al., 2013, for dry matter (86.74 – 89.17%), crude protein (6.26-9.01%) and ash (2.14-8.28%). The differences are largely due to the different varieties of grapes from which the seeds were obtained.

Table 1. Gross chemical composition of the winery by-products

Specification	DM %	OM %	CP %	CF %	Fibre %	NFE %	Ash %	GE Mj/kg
Dry grape marc with seeds	80.37	75.35	12.64	4.87	23.67	34.17	5.02	15.45
Seed-less grape marc	86.73	80.67	10.52	4.56	21.26	44.33	6.06	16.09
Grape seeds cakes	88.44	85.34	10.64	1.56	40.66	32.48	3.10	16.71

DM - Dry matter; OM – Organic matter; CP – Crude protein; CF – Crude fat; Fibre – Crude fibre; NFE – nitrogen-free extractives; GE – gross energy; NDF - Neutral Detergent Fibre; ADF - Acid Detergent Fibre.

Table 2. Amino acids concentration in the analysed samples (%)

Amino acids	Dry grape marc with seeds	Seed-less grape marc	Grape seeds cakes
Aspartic acid	0.683	0.759	0.797
Glutamic acid	2.386	2.276	3.894
Serine	0.197	0.204	0.213
Glycine	0.625	0.583	0.875
Threonine	0.386	0.422	0.377
Arginine	0.546	0.524	0.702
Alanine	0.487	0.533	0.543
Tyrosine	0.193	0.204	0.118
Valine	0.421	0.462	0.432
Phenylalanine	0.367	0.393	0.355
Isoleucine	0.411	0.487	0.467
Leucine	0.620	0.667	0.700
Lysine	0.544	0.616	0.421
Cystine	0.152	0.158	0.200
Methionine	0.090	0.100	0.102

The amino acids concentration (Table 2) shows that the levels of essential amino acids are similar to those from the cereals. Lysine, for instance, ranges between 0.421% in cakes and 0.544% in the seed-less grape marc, higher than the values reported by Burlacu et al., (2002) for corn (0.261-0.399%) and wheat (0.352-0.395%).

Threonine ranged between 0.377% in cakes and 0.422% in the grape marc with seeds, higher than in corn (0.276-0.358%), and wheat (0.361-0.376%) as reported by Burlacu et al., 2002. On the other hand, methionine ranged between 0.09% in the grape marc with seeds and 0.1% in cakes, values which are lower than in the corn 0.19% (Larbier M. and Leclercq B., 1992).

Table 3. Fatty acids concentration in the analysed samples (g/100g fat)

Amino acids		Dry grape marc with seeds	Seed-less grape marc	Grape seeds cakes
Capric	C 10:0	0.26	0.26	0.05
Lauric	C 12:0	0.07	0.05	0.11
Myristic	C 14:0	0.39	0.41	0.16
Pentadecanoic	C 15:0	0.18	0.37	0.43
Pentadecenoic	C 15:1	0.12	0.23	0.29
Palmitic	C 16:0	14.36	15.45	10.05
Palmitoleic	C 16:1	0.53	0.61	0.28
Heptadecanoic	C 17:0	0.11	0.12	0.07
Stearic	C 18:0	3.89	4.07	3.67
Oleic	C18:1n9	16.86	17.58	17.05
Linoleic	C18:2n6	58.99	48.24	62.26
Linolenic	C18:3n3	2.19	6.43	2.32
Arachidic	C20:0	0.39	0.75	0.26
Eicosenoic	C 20:1n9	0.30	2.43	1.78
Eicosadienoic	C20:2n6	0.03	0.18	0.10
Eicosatrienoic	C20:3n6	0.37	0.49	0.13
Heneicosanoic	C 21:0	0.38	0.00	0.25
Behenic	C 22:0	0.06	0.09	0.05
Docosadienoic	C22:2n6	0.49	0.00	0.20
Docosatetraenoic	C22: 4n6	0.26	0.31	0.04
Other fatty acids		0.00	1.91	0.45
Total fatty acids		*100*	*100*	*100*

The fatty acids profile from the fat of the analysed winery by-products (Table 3) shows that the concentration of linoleic acid (C18:2n6), omega 6 acid, was highest in the seed cakes (62.26 g/100 g fat), followed by the dry grape marc with seeds (5.25% less than in the cakes) and by the seed-less grape marc (22.52% less than in the cakes). The concentration of linolenic acid (C18:3n3), omega 3 acid, was highest in the seed-less grape marc (6.43 g/100g fat),

followed by the seed cakes (63.91% less) and by the grape marc with seeds (65.94% less). These results are in agreement with the literature (Tangolar et al., 2009; Kikalishvili et al., 2012; Sabir et al., 2012), which shows that the linoleic acid (53.6-69.6%) was found in the highest concentrations in the fat of the grape seeds of different grape varieties, followed by the oleic acid (16.2-31.2%), palmitic acid (6.9-12.9%) and stearic acid (1.44-4.69%).

Table 4 shows the data on the total concentration of saturated and unsaturated fatty acids and on their ratio in the fat of the winery by-products. The total unsaturated fatty acids (UFA) amount to 84.90% in the seed cakes, which is 5.94% higher than in the seed-less grape marc and 8.26% higher than in the grape marc with seeds. The seed-less grape marc had the highest concentration of saturated fatty acids and the lowest concentration of UFA. These results fort UFA are lower than those reported by Elagamey et al., (2013), who found in a study on six grape varieties, 86-88% UFA and 12-14% SFA.

Table 4. Fatty acids categories and their ratio in the fat of the studied by-products (g/100g fat)

Amino acids	Dry grape marc with seeds	Seed-less grape marc	Grape seeds cakes
SFA	20.09	21.57	15.10
PUFA	62.33	55.66	65.05
UFA	80.14	78.42	84.90
PUFA /SFA	3.10	2.58	4.30

The same table shows that the ratio of the polyunsaturated fatty acids (PUFA) and the saturated fatty acids (SFA) ranged between 2.58 in the seed-less grape marc and 4.30 in the seed cakes, values in agreement with that of 3.17 reported by Canbay and Bardakçı (2011), who used GC/MS coupling to determine the fatty acids in the grape seeds.

The calcium concentration in the 3 by-products ranged between 0.80–0.86% (Table 5). On the other hand, Canbay and Bardakçı (2011) reported only 0.53 g Ca % in the grape seeds.

Table 5. Macro elements and trace elements concentration in the winery by-products

Specification	Calcium %	Phosphorus %	Copper ppm	Iron ppm	Manganese ppm	Zinc ppm
Dry grape marc with seeds	0.86	0.31	28.34	223.45	19.67	18.25
Seed-less grape marc	0.80	0.31	32.67	285.58	23.86	26.81
Grape seeds cakes	0.84	0.37	15.39	83.52	27.73	20.89

The concentration of trace elements varied in the three by-products (Table 5). Thus, copper concentration was lowest (15.39 ppm) in the grape seeds and 112.28% higher in the seed-less grape marc. The lowest concentration of iron (83.52 ppm) was also determined in the seed cakes and 241.93% higher in the seed-less grape marc.

The lowest manganese concentration (19.67 ppm) was determined in the grape marc with seeds and it was 40.97% higher in the seed cakes. The lowest zinc concentration (18.25 ppm) was lowest in the grape marc with seeds too, and it was 46.90% higher in the seed-less grape marc. These results are similar to those reported by Canbay and Bardakçı (2011) for grape seeds: manganese - 39 ppm; zinc – 12 ppm.

As Figure 1 shows, the highest concentration of polyphenols (3.22 mg equivalent gallic acid/g sample) was determined in the dry grape marc with seeds, followed by 3.18 mg equivalent gallic acid/g sample in the seed-less grape marc, while the lowest concentration, 0.64 mg equivalent gallic acid/g sample was determined in the seed cakes. Several other researchers analysed the polyphenols concentrations in grapes. Thus, Pastrana-Bonilla et al. (2003) cited by En-Qin Xian et al. (2010), or Ky et al., (2014) reported values of 23.8-44.5 mg equivalent gallic acid/g sample in the grape pulp, 31.6-374.6 mg equivalent gallic acid/g sample in the grape peals. Shi et al., (2003) showed that the polyphenols concentrations determined in winery by-products are comparable with those of the flax seeds (5.09 mg equivalent gallic acid/g sample), wheat germs (3.49 09 mg equivalent gallic acid/g sample) or buckwheat seeds (7.26 mg equivalent gallic acid/g sample).

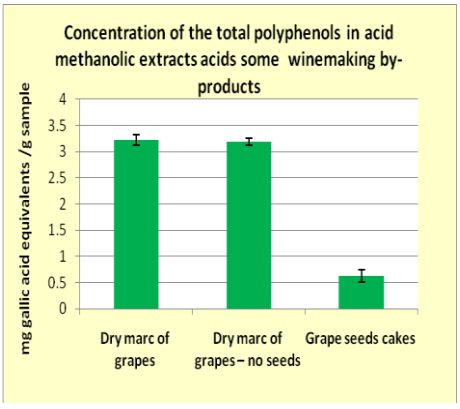

Figure 1

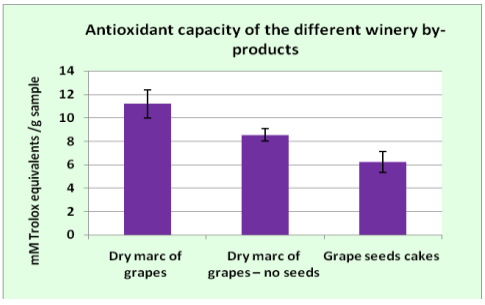

Figure 2

Figure 2 shows that the values of the antioxidant capacity of the studied winery by-products had the same evolution as the polyphenols concentrations. Thus, the highest value (11.21 mM equivalent Trolox/g sample) was

determined in the dry grape marc with seeds, followed by 8.55 in the seed-less grape marc and 6.24 in the grape seeds cakes. Poudel et al. (2008) cited by En-Qin Xian et al. (2010), showed that the antioxidant capacity of the grape seeds ranged between 16.8-92 mM equivalent Trolox/g sample, while that of the grape peals ranged between 15.7-113.3 mM equivalent Trolox/g sample.

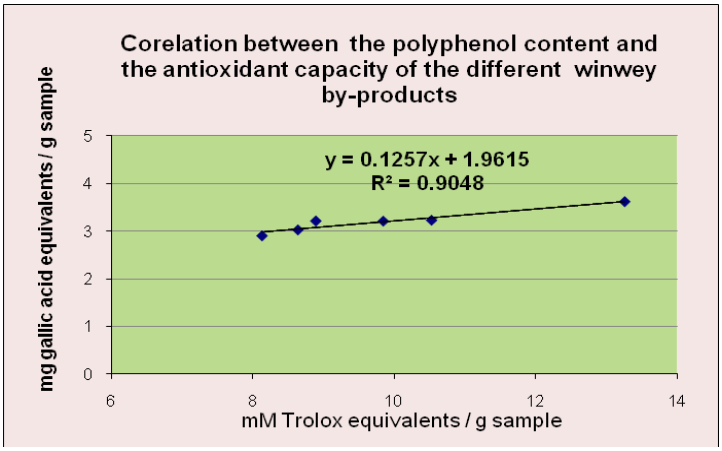

Figure 3

The higher concentrations of polyphenols in the dry grape marc produced the higher antioxidant character of this by-product. This is supported by Figure 3, which shows a strong correlation (0.9048) between the antioxidant capacity and the polyphenols concentration.

CONCLUSIONS

The feeding value and the antioxidant properties of the winery by-products (dry grape marc, seed-less grape marc and grape seeds cakes) show that they are potential natural feed additives with high feeding value and antioxidant properties. Their high concentration of polyphenols recommend their utilization particularly in diet formulations enriched in polyunsaturated fatty acids, diets used for the production of animal foods high in omega 3 fatty acids. The use of winery by-products, after thorough analyses, in animal feeding, also solves the problems of their storage, transformation or disposal, thus maintaining the environmental balance.

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