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# EVALUATION OF SOME BIOACTIVE NUTRACEUTICAL COMPOUNDS IN AGRO-INDUSTRIAL WASTE USED AS ANIMAL FEED ADDITIVES[1]

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**ABSTRACT.** Some parts of agro-wastes (pomace meal) are used for animal food containing variability in composition (proteins, dietary fibres, carbohydrates, polyphenols, minerals). Paper aim was to evaluate some nutraceutical bioactive compounds from pomace meal for use as animal feed additives. Studied meals are pomace obtained from solid remains of grapes and sea buckthorn after juice pressing, as well as flaxseed and rapeseed after oil pressing. HPLC methods were used to determine some carbohydrates (glucose, fructose, sucrose, maltose), organic acids (oxalic, citric, tartaric, malic), flavonoids (catechin, epicatechin, rutin, quercetin, luteolin) and phenolic acids (gallic, vanillic, caffeic, *p*-coumaric, ferulic). The content of total polyphenolic compounds and the antioxidant activity (DPPH and ABTS assays) of the pomace meals were evaluated by spectrophotometry. The results obtained show that the carbohydrates quantities (mg/100 g) in pomace meals were between 2943.31 (grapeseed) and 3210.11 (rapeseed). Sea buckthorn contains the most important amount of total organic acids of 8078.89 mg/100 g. Also, the highest quantities (mg/100 g) of total polyphenolic compounds were found in grapeseed (10789) and flaxseed (8537), respectively. These findings indicate a good source of carbohydrates, organic acids and polyphenols (phenolic acids and flavonoids) therefore these meals can be used as animal feed additives.

***Keywords:*** *bioactive compounds, animal feed additives, HPLC, total phenolic compounds, antioxidant activity*

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## INTRODUCTION

Fruit wastes cause a huge economic impact to environmental and the reduction and recycling of these waste need urgent attention since it would enhance food security, reduce the environmental footprint of food production chain, decrease waste management costs and open opportunities for production of novel products including animal feed [1].

To meet the high demand for animal feed, the efficient use of available feed resources, the enlargement of the feed resource base, and the search for new animal feed resources, particularly those not competing with human food, are pivotal for sustainable development of the livestock sector [2].

Some fruit wastes (apple, apricot, banana, citrus and raspberry) and by-products were used as animal feed and were evaluated by the nutritional value, conservation methods, feeding management and guidance on the levels at which these unconventional feed resources can be used in the diets of farm animal species [3]. Grape pomace contains up to 15% sugars, 0.9% phenolics and pigments (red grape pomace), 5.0–7.5% tartrates, 30–40% fibres, 9–12% crude proteins. The feed efficiency and growth rate of pigs declined with the increase in the level of dried, ground winery pomace, 10–15% level of incorporation in the diet [3, 4].

Sea buckthorn pomace as a coproduct of juice processing from the fleshy tissue of berries, contains many valuable vitamins, tocopherols, flavonoids, special fatty acids, and abundant amino acids [5, 6]. Sea-buckthorn flavonoids are beneficial for growth performance, rumen fermentation, and serum antioxidant activity in lambs and could be used as a natural feed additive in lamb production [7].

After extraction of oil from flaxseed, the by-product obtained, the flaxseed meal, still has good nutritional value. Flaxseed meal is rich in proteins, α-linolenic acid, dietary fibres, flaxseed gum, and other bioactive substances. Like many other feed ingredients, flaxseed meal has many excellent functions and can be used as a high-quality non-conventional protein feed for livestock and poultry [8–10].

Rapeseed meal was generated as a side stream of rapeseed oil production. It is commonly used as feed due to its high nutritional value. Rapeseed meal contains approximately 40% of proteins with balanced amino acid composition, phenolic compounds, fibres and minerals such as calcium, magnesium, zinc, and copper, a number of vitamins, tocopherols, B vitamins, and choline [11]. The effects of fermentation on individual polyphenolic compounds, carbohydrates, organic acids, fatty acids and minerals were evaluated on the fermented rapeseed meal [12]. Rapeseed meal could be supplemented up to 9% in growing-finishing pig diets without any detrimental effect on growth performance [13]. At the same time, the fermentation of rapeseed meal is an

effective way to reduce anti-nutrients [12,14] and to increase the level of lactic acid in the diet. It also stimulates the immune system, which improves piglet health, reducing the severity of diarrhoea and mortality [15].

Therefore, the aim of this study was to assess some nutraceutical bioactive compounds in pomace meal for use as additives in animal feed. Four types of pomaces such as grape seeds, sea buckthorn, flaxseed and rapeseed were evaluated regarding the content of some individual carbohydrates, organic acids, flavonoids and phenolic acids, as well as the total polyphenolic compounds (polyphenols). The antioxidant activity was also investigated.

## RESULTS AND DISCUSSION

The use of natural products as food supplements has received increasing attention in recent years [1, 12, 16] due to their rich content in bioactive nutraceutical compounds.

### *HPLC of carbohydrates in pomace meals*

The residual carbohydrates that remain in the pomace after the disintegration and pressing of grapes, are mainly water soluble. The content of the carbohydrates (glucose, fructose, sucrose and maltose) in pomace meals is shown in Figure 1.

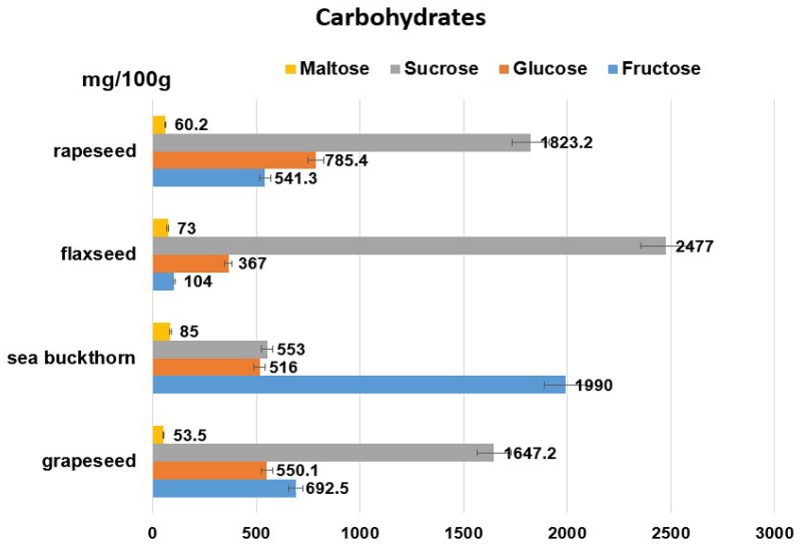


**Figure 1.** Concentrations of soluble carbohydrates (Mean ± SD) in the studied pomace meals

Thus, sucrose was found in the highest amount (mg/100 g meal) in 3 of the studied pomace meals: flaxseed (2477), rapeseed (1823.2) and grape seed (1647.2). The most important glucose amount of 785.4 mg/100 g was found in rapeseed meal while that of fructose of 1990 mg/100 g in sea buckthorn. In all studied pomace meals, maltose was found in the smallest amounts between 53.5 and 85 mg/100 g.

The total amount of carbohydrates in the studied meal samples (mg/100 g) were of 3210.1 for rapeseed meal, 3144 for sea buckthorn meal, 3021 for flaxseed meal, and 2943 for grapeseed meal, respectively.

Our results are in agreement with those reported in the references [12, 17, 18] for different pomace samples.

### *HPLC of organic acids in pomace meals*

Figure 2 shows the content of organic acids found in the studied meals.

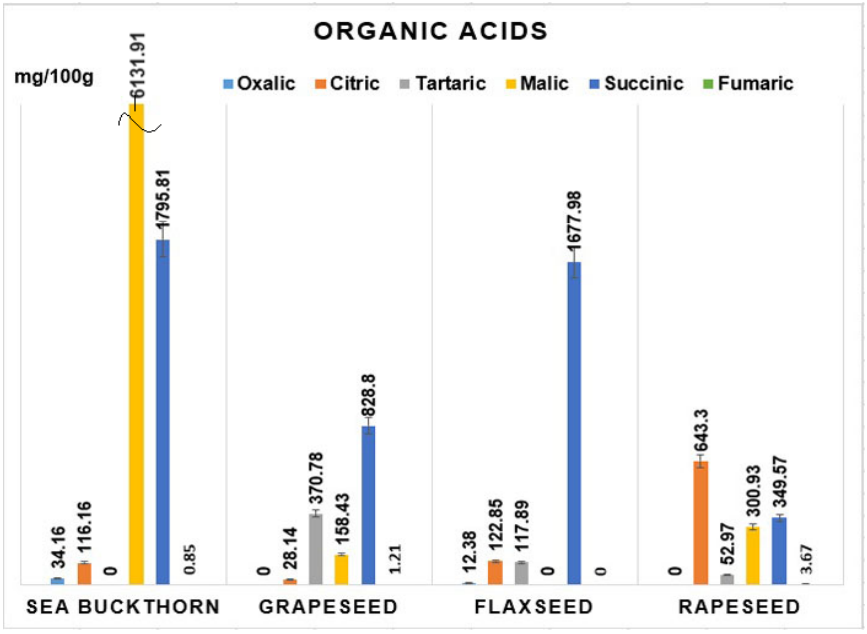


**Figure 2.** Concentrations of organic acids (Mean ± SD) in the studied pomace meals

The obtained results show that the largest content of organic acids was present in sea buckthorn meal, that also contains a highest amount of malic and succinic acids of 6131.91 and 1795.81 mg/100 g, respectively.

Also, the important amounts (mg/100 g) of succinic acid were found in grapeseed (828.8) and flaxseed (1677.98) meals, respectively.

Thus, the amount of total organic acids (mg/100 g) found in meal samples were as follows: 8078.89 in sea buckthorn, 1387.36 in grapeseed, 1931.1 in flaxseed, and 1350.44 in rapeseed, respectively.

Organic acids and their salts have been established as potential alternatives to prophylactic use of in-feed antibiotics in order to improve the performance of weaned piglets, fattening pigs and reproductive sows. Organic acids may also influence gut morphology, increasing the absorption capacity of proteins, energy and/or mineral [19].

### *HPLC of flavonoids and phenolic acids in pomace meals*

The amounts of some individual flavonoids and phenolic acids present in studied pomace meals, namely grapeseed, sea buckthorn, and flaxseed and rapeseed are shown in Figure 3.

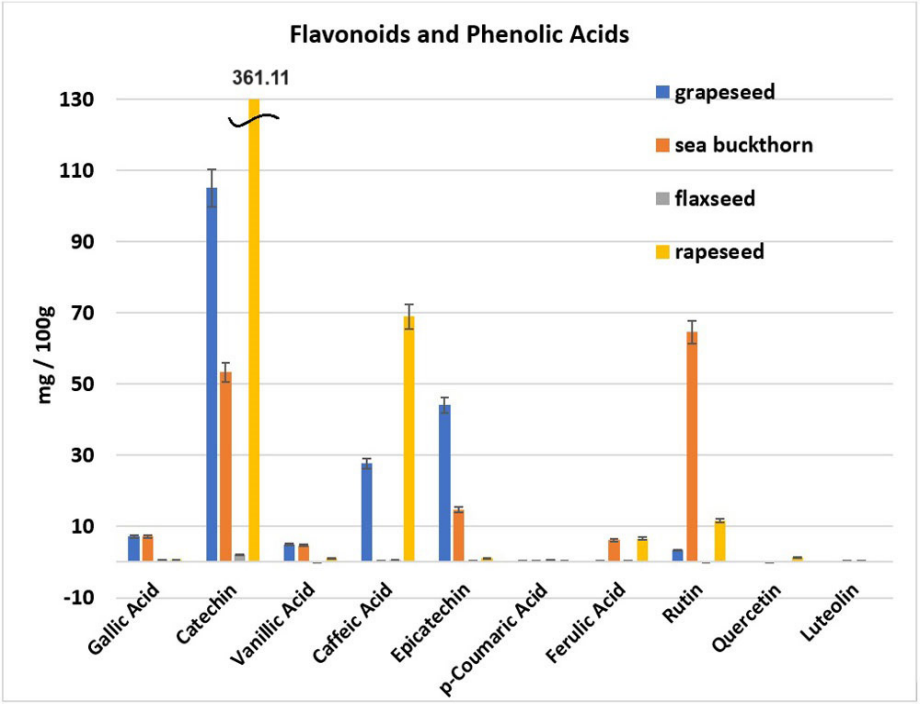


**Figure 3.** Concentrations (Mean ± SD) of individual phenolic compounds (flavonoids and phenolic acids) in the studied pomace meals (grapeseed, sea buckthorn, flaxseed and rapeseed.

In Figure 3 it can be observed that the pomace meals with the highest amount of catechin (mg/100 g) were rapeseed (361.11 mg), grapeseed (105.06 mg), and sea buckthorn (53.27 mg). Also, significant amounts of caffeic acid (mg/100 g) were found in grapeseed (27.67 mg) and rapeseed (68.01 mg) meals, as well as epicatechin in grapeseed (44.15 mg) and sea buckthorn (14.74 mg) meals. Moreover, the highest content of rutin was present in sea buckthorn meal (64.56 mg).

The total of individual phenolic compounds in the studied meals (mg/100 g meal) were 192.96 mg for grapeseed, 151.91 mg for sea buckthorn, 4.82 mg for flaxseed and 453.03 mg for rapeseed, respectively.

For comparison, similar results on the concentrations of caffeic, ferulic, and *p*-coumaric acids in different rapeseed varieties were reported by other authors [4, 20]. Grapeseed is also appreciated due to their content of phenolic compounds such as gallic acid, catechin and epicatechin, and a wide variety of procyanidins (mainly condensed tannin) [16] and resveratrol [21].

In Figure 4, the concentration of some individual phenolic compounds (flavonoids and phenolic acids) was studied for the grapeseed and sea buckthorn mixture prepared under different ratios (from 3 to 27 parts from each of them) to see the influence of the mixture composition over the concentration of each phenolic compound.

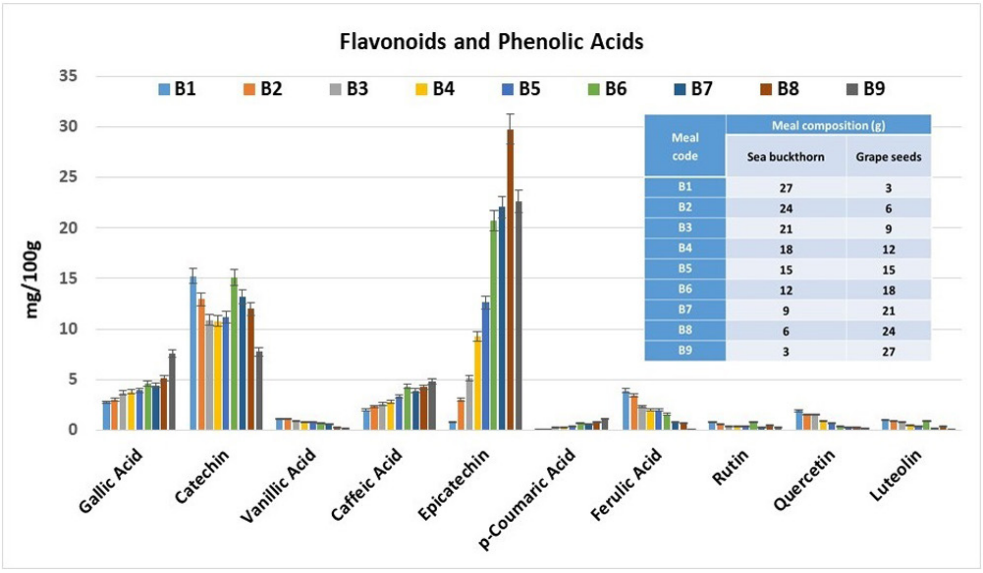


**Figure 4.** Concentrations (Mean ± SD) of individual phenolic compounds (flavonoids and phenolic acids) in meal samples containing mixtures of grapeseed and sea buckthorn in different ratios.

In Figure 4 it can be observed that with the increase in the amount of grapeseed in the studied meal samples, the amounts of some polyphenols increase as follows: gallic acid, caffeic acid, epicatechin, and p-coumaric acid. The B8 sample containing 6 parts of sea buckthorn and 24 parts of grapeseed represents the meal sample with the highest amount of epicatechin. It can be also seen that the majority polyphenols are represented by the flavonoids catechin and epicatechin. Catechin occurs in 8 meal samples (B1–B8) in amounts greater than 10 mg/100g, while epicatechin occurs in 5 meal samples (B5–B9) in amounts greater than 10 mg/100g, and in 4 meal samples (B6–B9) in amounts greater than 20 mg/100g. As for phenolic acids, they are represented by gallic and caffeic acids with amounts in all meal samples close to 5 mg/100g, except for sample B9 which presents gallic acid around 7 mg/100g.

Sea-buckthorn pulp and flaxseed residues are rich in phenolic fractions and aroma substances, thus providing high antioxidant and antimicrobial activity [22]. Sea buckthorn pomace contains dietary fibres that are bioactive and health promoting, but due to the lack of suitable handling and/or processing facilities, this pomace is often either used as animal feed or discarded [6]. Grapes from the common grapevine (*Vitis vinifera L.*) are grown worldwide and are common food sources of phenolic compounds, particularly flavonoids [23].

Literature data showed that by including 5% grapeseed cake in the diet of fattening-finishing pigs, a modulatory effect on antioxidative status was observed, as well as anti-inflammatory and hypocholesterolic properties without effect on pig performance [24].

Grape seed, whether whole, ground or after oil extraction, has been used in livestock feeds, mainly for rabbit, ruminants and piglets with a content of up to10% grapeseed meal in the feed [25, 26].

### *Total phenolic compounds and antioxidant activity*

Total phenolic compound **(TPC)** and antioxidant activity of the studied pomace meals determined with DPPH and ABTS assays is shown in Table 1. TPC was determined spectrophotometrically at 760 nm with Folin-Ciocâlteu method and the results were expressed as mg GAE (gallic acid equivalent) / g dry weight (meal).

It is well known that polyphenolics can act as antioxidants due to their hydrogen atom and single electron donating capabilities. Velioglu et al. [27] reported in their study of several fruits and vegetables that there was a significant correlation among the total polyphenolic content and the antioxidant activity [27, 28].

TPC (mg GAE / g) was found in the studied pomace meals of 47.12 in sea buckthorn, 97.89 in grapeseed, 16.44 in flaxseed and 85.37 in rapeseed, respectively. Similar values of TPC were obtained by some authors [4, 18, 20].

The results of the antioxidant properties determined using DPPH and ABTS assays are expressed as mg TE (Trolox equivalent) / 100 g dry weight (meal).

**Table 1.** Total phenolic compounds and antioxidant activity (Mean ± SD) of pomace meals determined with DPPH and ABTS assays

| **Meal** | **TPC*** mg GAE/ g | **DPPH**** | **ABTS**** |
|---|---|---|---|
| | | mg TE/ 100 g | |
| sea buckthorn | 47.12 ± 1.23 | 3925 ± 26.85 | 3018 ± 19.46 |
| grapeseed | 97.89 ± 6.89 | 7938 ± 21.21 | 8272 ± 26.70 |
| flaxseed | 16.44 ± 1.02 | 1887 ± 20.39 | 1872 ± 18.91 |
| rapeseed | 85.37 ± 3.85 | 5641 ± 18.97 | 6319 ± 29.20 |

*TPC – expressed by mg GAE (gallic acid equivalent) / g dry weight;
**DPPH and ABTS – expressed by mg TE (Trolox equivalent) / 100 g dry weight

The DPPH• radical scavenging activity ranged from 1887 to 7938 mg TE / 100 g. By using ABTS•+ assay, the antioxidant activity values were calculated between 1872 and 8272 mg TE / 100g. By both assays, the highest values of antioxidant activity were found for grapeseed and rapeseed meals.

The results of this investigation were, however, in agreement with other researches concerning the content of total phenolic compounds and antioxidant activity that were found in the studied pomace meals [4, 12, 18, 20, 29–31].

## CONCLUSIONS

This study presents a modern approach regarding the evaluation of some bioactive nutraceutical compounds from pomace meals for use as additives in animal feed. Thus, pomace meals such as grapeseed and sea buckthorn, flaxseed and rapeseed were investigated to determine their content in carbohydrates, organic acids, individual polyphenolic compounds and total polyphenolic compounds, as well as to evaluate the antioxidant activity (DPPH and ABTS tests).

Thus, the studied pomace meals possess an important amount of carbohydrates with values between 2943.3 mg/100 g for grapeseed and 3210.1 mg/100 g for rapeseed. The organic acids (mg/100g) were present in the highest amount in sea buckthorn (8078.89) and in smaller amounts in rapeseed (1350.44), in grapeseed of 1387.36 and in flaxseed of 1931.1, respectively. Also, the highest quantities of TPC (mg GAE / g) were found in grapeseed of 97.89 and in rapeseed of 85.37, respectively. The antioxidant activity is correlated with TPC content and shows important values for grapeseed and rapeseed meals.

In conclusion, these pomace meals are a good and promising source of nutritionally bioactive compounds and can be used as additives in animal feed.

## EXPERIMENTAL SECTION

### *Chemicals and materials*

Acetonitrile and methanol with of HPLC-grade, sulphuric acid p.a., formic acid p.a., and standards of organic acids (tartaric, citric, malic, succinic and oxalic) were purchased from Merck (Darmstadt, Germany). The standards of flavonoids (catechin, epicatechin, rutin, quercetin and luteolin), phenolic acids (gallic, vanillic, caffeic, p-coumaric, ferulic), and carbohydrates (glucose, fructose, sucrose, maltose) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Folin-Ciocâlteu reagent, 2,2′-diphenyl-1-picrylhydrazyl radical (DPPH, 95%) and 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, 98%) were purchased from Alpha Aesar – Thermo Fisher Scientific, Lab Chemicals (Kandel, Germany). 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, 98%) was purchased from TCI America (Portland, OR, USA).

### *Pomace meals samples*

Pomace by-products: grapeseed, sea buckthorn, flaxseed and rapeseed from local producers were dried in an industrial automated forced hot air dryer (Blue Spark Systems SRL, Bucharest, Romania) at 60ºC. Then, the dried pomaces were finely ground (1 mm mesh) using an IKA M 20 Universal Mill (7.5 kW) (IKA-Werke GmbH & Co. KG, Staufen, Germany).

### *Extraction procedure*

Extraction of flavonoids and phenolic compounds from the studied samples was performed in 80% methanol solution, while carbohydrates and

organic acids were extracted in ultrapure water (18.2 MΩ cm ionic purity at 25ºC) produced in the laboratory by means of a Simplicity system (Millipore; Bedford, MA, USA).

A quantity of 1 g sample with 10 mL of extraction solvent was well stirred, sonicated for 60 minutes at ultrasonic power 100% and 80 KHz ultrasonic frequency (Ultrasonic bath Sonorex, Bandelin, Germany), centrifuged at 4500 rpm for 20 minutes, and then the supernatant was filtered through a 0.45 µm PTFE syringe filter (Chromafil Xtra PTFE, 25 mm, 0.45 µm; Macherey-Nagel, Düren, Germany) and injected into HPLC equipment.

### *Equipment and method*

The analyses were performed by high-performance liquid chromatography (HPLC) on a Jasco Chromatograph (Jasco Corporation, Tokyo, Japan) equipped with an ultraviolet-visible (UV/Vis) detector and a refractive index (RI) detector, and an injection valve with a 20 µL sample loop (Rheodyne, Thermo Scientific). The ChromPass software (Varian Jasco, Tokio, Japan) was used to control the HPLC system and to collect and process data.

Total phenolic compounds and antioxidant activity was determined spectrophotometrically using an UV-Vis Spectrophotometer Specord 205 (Analytik Jena, GMbH, Germany).

### *HPLC methods*

*HPLC-RI analysis of carbohydrates* was adapted from [32]. Separation was carried out on a Kromasil-$NH_2$ column (5 µm; 250 × 4.6 mm) (Nouryon AB, Göteborg, Sweden), set at 25ºC and eluted with the acetonitrile–water (70:30, v/v) mobile phase at a flow rate of 1 mL/min.

*HPLC quantification of organic acids* was carried out by the method described in [33]. Separation was done on a CarboSep Coregel 87H3 column (300 × 7.8 mm) (Phenomenex, Aschaffenburg, Germany) at a column temperature of 35ºC, with a 0.005 M sulfuric acid solution as the mobile phase at a flow rate of 1 mL/min. UV detection was at 214 nm.

*HPLC analysis of flavonoids and phenolic compounds* was carried out by the HPLC method described in [34] using a Lichrosorb RP-C18 column (5 µm; 250 × 4.6 mm) (Merck, Darmstadt, Germany) at 22ºC column temperature, a flow rate of 1 mL/min and UV detection at 270 nm. Elution was done by gradient using as mobile phase methanol (A, HPLC-grade) and 0.1% formic acid solution according to the method: 0–10 min, linear gradient 10–25% A; 10–25 min, linear gradient 25–30% A; 25–50 min, linear gradient 35–50% A; 50–70 min, isocratic 50% A.

Also, the all HPLC determinations were made by triplicate.

### *Total Phenolic Compounds (TPC)*

TPC was determined spectrophotometrically using the Folin-Ciocâlteu method, according to our previous adapted procedure [12]. Briefly, 0.4 mL of methanolic meal extract and 2 mL of Folin-Ciocâlteu reagent (dilution 1:1) were shaken for 3 min and then 1.6 mL of sodium carbonate solution (7.5%) was added and brought to 10 mL, using water. After 10 minutes at 50ºC, the solutions were cooled, and the absorbance was measured at 760 nm against a reagent blank (0.4 mL water + 2 mL of Folin-Ciocâlteu reagent + 1.6 mL sodium carbonate solution). The absorbance of standard samples of gallic acid (GAE) were recorded. The TPC of each meal extract was quantified as mg gallic acid equivalent per 1 g dry weight (meal) (mg GAE/g). All determinations were performed in triplicates.

### *Determination of Antioxidant Activity*

To evaluate the antioxidant activity of the studied samples, two different chemical methods were applied, namely the DPPH (2,2′-diphenyl-1-picrylhydrazyl radical) and ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) assays. All determinations were performed in triplicate.

*DPPH• radical scavenging assay.* Our adapted DPPH method [12] was used for the spectrophotometrically determination (517 nm) against methanol, as the blank of the antioxidant capacity of the studied samples. The free radical scavenging activity of the extracts was measured by absorbance (Abs) with respect to the effect of standard solutions of methanolic Trolox (0.02–0.1 µmol/mL) or pomace meal extracts. An aliquot of 0.5 mL of each Trolox solution (or extract) was added to 2 mL methanol and 0.5 mL DPPH solution. The control sample was prepared by mixing 2.5 mL methanol with 0.5 mL of DPPH solution. The effective concentrations (DPPH) were expressed in µmol Trolox/ 100 g dry weight. The scavenging activity of DPPH was calculated with Equation (1):

$$\text{DPPH scavenging activity } (\%) = \frac{(\text{Abs control} - \text{Abs sample})}{\text{Abs control}} * 100 \qquad (1)$$

where: Abs control is the absorbance of DPPH• radical in methanol; Abs sample is the absorbance of DPPH• radical solution mixed with sample extract/ standard.

*ABTS•+ radical scavenging assay*. The spectrophotometric determination of the antioxidant activity of meal samples by the ABTS method is based on the percentage inhibition of peroxidation of this radical. The reaction was carried out according to our previously adapted method [12]. The radical cation ABTS•+

was generated by the persulphate oxidation of ABTS. The working solution (ABTS solution of 7 mM and potassium persulphate solution of 2.45 mM, 1:1, v/v) were left to react for 17 hours at room temperature in the dark. This solution was then diluted with methanol to obtain an absorbance between 0.700 to 0.800 units at 734 nm. Pomace meal extracts (0.5 mL) were allowed to react with 2.5 mL of the fresh ABTS solution for 6 min. The ABTS scavenging activity of the extracts was measured taking into account the effect of the Trolox standard solutions (2.5–12.5 µg/mL), regarding the discolouring capacity of the blue-green colour of the ABTS solution. The percentage inhibition was calculated using Equation (2):

$$\text{ABTS} \bullet + \text{radical scavenging activity } (\%) = \frac{(\text{Abs control} - \text{Abs sample})}{\text{Abs control}} * 100 \quad (2)$$

where: Abs control is the absorbance of ABTS• + radical in methanol; Abs sample is the absorbance of ABTS• + radical solution mixed with sample extract/standard.

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