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Commercial Dietary Ingredients from *Vitis vinifera* L. Leaves and Grape Skins: Antioxidant and Chemical Characterization

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This paper reports an attempt to functionally and chemically characterize commercial ingredients from *Vitis vinifera* L. grape skins, grape pomace, and leaves, which are used in the formulation of dietary antioxidant supplements. The antioxidant capacity of these ingredients was assessed for the first time by the oxygen radical absorbance capacity (ORAC) methodology. Ingredients from grape skins and pomace ($n = 17$) showed ORAC values from 1.38 to 21.4 μmol Trolox equivalents/mg whereas ingredients from leaves ($n = 4$) showed ORAC values from 1.52 to 2.55 μmol Trolox equivalents/mg. The high-performance liquid chromatography−diode array detection/electrospray ionization−mass sprectrometry analysis of anthocyanins and flavonols revealed the authenticity of the ingredients as derived from *V. vinifera* L. and confirmed large differences in their phenolic content and distribution. A progressive decline in both antioxidant capacity and total anthocyanin content of a grape skin ingredient (43 and 40% decrease, respectively) was observed over a 60 day storage period (45 °C and 75% relative humidity), demonstrating its poor stability under these conditions.

KEYWORDS: ORAC; anthocyanins; flavonols; commercial ingredients; *Vitis vinifera* L. grape skins; grape pomace; leaves

INTRODUCTION

Since the first observations of the "French paradox" (*1*), numerous studies have demonstrated the antioxidant and health-promoting effects of phenolic compounds present in grapes and wine, particularly in relation to cardiovascular diseases (*2*). These findings have led to a considerable interest in the evaluation of winery byproducts as a potential source of phenolic compounds to be used as functional food ingredients (*3*−*5*). It also gives a way of solving the disposal problems arising from the large amounts of residues generated by the wine and juice industries. The grape pomace obtained after fermentation and subsequent pressing is the main winery byproduct and consists of grape skins and seeds, and occasionally stems, and may undergo further distillation to produce spirits. Besides the grape pomace, two other byproducts are the unfermented seeds and skins discarded from wine (i.e., white wine) and juice processing (*6*). Currently, *Vitis vinifera* L. leaves and red wine are also being employed to produce dietary ingredients.

Grape skins and leaves are composed of anthocyanins and flavonols. The anthocyanins identified in *V. vinifera* spp. correspond to the 3-*O*-monoglucosides and the 3-*O*-acylated monoglucosides of the five main anthocyanidins: delphinidin, cyanidin, petunidin, peonidin, and malvidin. Acylation occurs at the C-6 position of the glucose molecule by esterification with acetic, *p*-coumaric, and caffeic acids (*7*). In *V. vinifera* spp., flavonols exist as the 3-*O*-glycosides of myricetin, quercetin, kaempherol, and isorhamnetin. Glucose, galactose, and glucuronide acid are the main sugar units (*8*). Grape seeds, skins, and stems are also an important source of proanthocyanidins (PROs). While seeds contain procyanidins [oligomers and polymers of (+)-catechin, (−)-epicatechin, and (−)-epicatechin gallate] (*9*), skins and stems also contain prodelphinidins [oligomers and polymers of (−)-epigallocatechin and trace amounts of (+)-gallocatechin and (−)-epigallocatechin gallate] (*10*, *11*). Anthocyanin-derived pigments that originate during alcoholic fermentation may also be present in grape pomace extracts. These result from the direct and acetaldehyde-mediated anthocyanin−flavanol condensation reactions, as well as the products originating from the C-4/C-5 cycloaddition reactions of anthocyanins with yeast secondary metabolites and other phenols (pyruvic acid, 4-vinylphenols, hydroxycinnamic acids, vinylflavanol, acetaldehyde, and acetone, among others), giving rise to the so-called pyranoanthocyanins (*8*). The phenolic composition and the extractability of grape byproducts largely depend on the grape variety and the processing conditions (*4*).

Over the past few years, considerable effort has been devoted to optimizing the extraction of phenolic compounds from winery byproducts and to their subsequent fractionation. Although polymeric adsorber resins are being introduced to isolate phenolic antioxidants from crude extracts (*12*), direct solvent extraction is more frequently used, and both extraction yield and antioxidant activity are largely dependent on the solvent (*13*−*15*). The extraction of anthocyanins from grape pomace

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with sulfurous water or acidified alcohols, which has long been used to prepare natural colorants, is also used to prepare anthocyanin dietary ingredients derived from grape pomace and unfermented grape skins (*16*, *17*).

Extracts from *V. vinifera* L. are commonly used to formulate dietary antioxidant supplements together with synthetic vitamins (E and C), minerals (selenium), soy isoflavones, tomato concentrate, rosemary extract, citrus flavonoids, and others (*18*). The supplement manufacturer selects the best brand for each ingredient based on its content of active compound/s (i.e., anthocyanins for grape skins, flavonols for leaves), physicochemical properties, and economic considerations. Although the dietary industry based on wine byproducts is rapidly growing, practically no scientific research (*6*, *19*) has been conducted on the wide range of ingredients currently available on the market. The aim of the present work was to study the antioxidant capacity and polyphenolic composition of commercial dietary ingredients derived from *V. vinifera* L. grape skins, pomace, and leaves. Commercial ingredients have been randomly selected from the market, and different production batches were supplied for some brands. Controlled storage of some ingredients was carried out in order to determine their stability.

MATERIALS AND METHODS

Chemicals. Fluorescein (FL) disodium was purchased from Sigma Chemical (St. Louis, MO). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and 2,2′-azobis (2-methylpropionamide)-dihydrochloride (AAPH) were obtained from Aldrich (Milwaukee, WI).

Commercial Dietary Ingredients. A total of 13 different brands of commercial dietary ingredients used in the elaboration of antioxidant supplements were directly supplied by the manufacturers. The commercial ingredients studied were derived from *V. vinifera* L. grape skins (nonprocessed) (ingredients #1−4), grape pomace (ingredients #5−10), and leaves (ingredients #11−13). Different production batches of ingredients #1 (batches #1−5), #2 (batches #1−4), and #13 (batches #1−2) were also studied. The ingredients were supplied in solid form, except ingredients #7−10, which were in the form of liquid concentrates or syrups. As indicated by the manufacturer, the syrup ingredients had been standardized to 38 °Brix. Both #7 and 8 ingredients were derived from winemaking pomace whereas ingredients #9 and 10 were from juicemaking pomace. **Table 2** reports source, appearance, and information about the obtention procedure of these ingredients, most of them under patent protection. In the solid ingredients, the water activity was determined at 25 °C using Novasina A_w Sprint TH-500 (Pfäffikon, Switzerland) equipment previously calibrated with saturated solutions of different salts. A_w determinations were performed in duplicate.

Sample Preparation. Five milligrams (5 mg) of each ingredient was combined with 10 mL of methanol:HCL (1000:1, v/v), vortexed, and sonicated for 2 min. After a 15 min incubation period at room temperature, the samples were centrifuged (485*g*, 10 min), and the supernatants were collected and submitted to the antioxidant capacity assay [oxygen radical absorbance capacity (ORAC) assay] and to phenolic content determinations [total polyphenols (TPs), total anthocyanins (TAs), and PROs]. Sample extraction was performed in triplicate. To determine the influence of the extraction solvent on the ORAC value and TP content, a series of extractions was first carried out on ingredient #1 (batch #1) using the following solvents: methanol, methanol:water (50:50, v/v), methanol:HCl (1000:1, v/v), methanol:HCl (1000:1)/water (50:50, v/v), acetone:water (50:50, v/v), acetone:HCl (1000:1)/water (50:50), phosphate-citric buffer (pH 3.5), and water:HCl (1000:1, v/v).

Radical Scavenging Activity. Among the methods proposed for evaluating the in vitro antioxidant capacity of food products, the ORAC is perhaps one of the most suitable methods (*20*). The procedure used was based on that proposed by Ou et al. (*21*) and modified by Dávalos et al. (*22*). Briefly, the reaction was carried out at 37 °C in 75 mM phosphate buffer (pH 7.4) and the final assay mixture (200 μL) contained FL (70 nM), AAPH (12 mM), and antioxidant [Trolox (1−8 μM) or sample (at different concentrations)]. The plate was automatically shaken before the first reading, and the fluorescence was recorded every minute for 97 min. A Polarstar Galaxy plate reader (BMG Labtechnologies GmbH, Offenburg, Germany) with 485-P excitation and 520-P emission filters was used. The equipment was controlled by the Fluostar Galaxy software version (4.11-0) for fluorescence measurement. Black 96 well microplates (96F untreated, Nunc, Denmark) were used. AAPH and Trolox solutions were prepared daily, and FL was diluted from a stock solution (1.17 mM) in 75 mM phosphate buffer (pH 7.4).

Fluorescence measurements were normalized to the curve of the blank (no antioxidant). From the normalized curves, the area under the fluorescence decay curve (AUC) was calculated as:

$$\mathrm{AUC} = 1 + \sum_{i=1}^{i=80} f_i/f_0$$

where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time *i*. The net AUC corresponding to a sample was calculated as follows:

$$\text{net AUC} = \mathrm{AUC}_{\text{antioxidant}} - \mathrm{AUC}_{\text{blank}}$$

The regression equation between net AUC and antioxidant concentration was calculated. The ORAC value was calculated by dividing the slope of the latter equation by the slope of the Trolox curve obtained for the same assay. Final ORAC values were expressed as μmol of Trolox equivalent (TE)/mg of ingredient.

Phenolic Content Determinations. TPs were determined using the Folin−Ciocalteu method, as modified by Singlenton and Rossi (*23*). TAs were determined as described by Paronetto (*24*), and PROs were determined according to the method of Ribéreau-Gayon and Stonestreet (*25*).

Analysis of Anthocyanins by High-Performance Liquid Chromatography−Electrospray Mass Spectrometry (HPLC/ESI-MS). Commercial dietary ingredients (0.15−0.80 g for solid grape skin/pomace ingredients and 0.5−2.0 g for leaf ingredients) were extracted with methanol:HCl (1000:1, v/v) (25 mL for grape skin/pomace ingredients and 10 mL for grape leaf ingredients) following the protocol described above under Sample Preparation. Grape pomace ingredients presented as syrups (#7−10) were diluted (1:10) with distilled water. Samples were filtered through a 0.45 μm membrane before analysis. HPLC/ESI-MS analysis of anthocyanins was carried out as described by Monagas et al. (*26*). Quantification was carried out by area measurements at 530 nm, and the anthocyanin content was expressed as malvidin-3-glucoside (Estrasynthese, France) by a standard calibration curve.

Analysis of Nonanthocyanin Phenolic Compounds by HPLC/ESI-MS. Commercial dietary ingredients (0.5 g for leaf ingredients) were extracted with 10 mL of methanol:HCl (1000:1, v/v) following the protocol described above under Sample Preparation. Samples were filtered through a 0.45 μm membrane before analysis. HPLC/ESI-MS analysis of nonanthocyanin phenolic compounds was carried out as described by Monagas et al. (*27*). Quantification was carried out at 340 nm by external standard calibration curves. Flavonol glycosides and hydroxycinnamic acid derivatives were quantified by their respective free forms.

Stability Study. Ingredients #1 (batch #1) and #2 (batch #1) were submitted to a stability test. Each ingredient (5.4−5.8 mg) was encapsulated in cartilage capsules (Shionogi Qualicaps S. A., Alcobendas, Madrid, Spain). A total of 10 capsules of each ingredient were then introduced in screw-top amber bottles (250 mL) and stored in a humidity chamber (176 L of volume) (Lab-Line Instruments Inc., Melrose Park, IL) for 60 days at 45 °C and 75% relative humidity. Bottles were removed after 15, 30, 45, and 60 days of storage, and samples were submitted to the ORAC assay, phenolic content determinations, and HPLC analysis. To compare samples with different storage times, the sample moisture was determined and results were expressed in dry weight. The moisture content was determined from the weight difference before and after heating at 100 °C for 3 h.

Table 1. Influence of the Solvent on the Antioxidant Capacity (ORAC Values) and Phenolic Content (TPs) of the Dietary Ingredient #1

solvent	ORAC value[a]	total polyphenols[b]
methanol	2.74 ± 0.17	112 ± 18
methanol:water (50:50; v/v)	2.66 ± 0.08	101 ± 30
methanol:HCl (1000:1; v/v)	4.14 ± 0.02	147 ± 2
methanol:HCl (1000:1)/water (50:50)	2.50 ± 0.24	118 ± 5
acetone:water (50:50; v/v)	2.42 ± 0.09	137 ± 10
acetone:HCl (1000:1)/water (50:50)	3.19 ± 0.20	159 ± 9
phosphate-citric buffer (pH 3.5)	2.30 ± 0.01	101 ± 9
water:HCl (1000:1; v/v)	2.44 ± 0.12	107 ± 6

[a] Expressed as μmol of TE/mg of ingredient. Results are presented as the means ($n = 3$) ± SD. [b] Expressed as mg of gallic acid equivalent (GAE)/g of ingredient. Results are presented as the means ($n = 3$) ± SD.

Statistical Analysis. Standard deviation was calculated for all data. Pearson's correlation and stepwise multiple lineal regression analysis ($F_{enter} = 4.00$; $F_{remove} = 3.99$) were performed to describe the relationship between the ORAC values and the phenolic composition using SPSS software (version 12.0; SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Antioxidant Capacity and Phenolic Content of Commercial Dietary Ingredients. A series of extractions was first carried out on ingredient #1 using eight different solvents (**Table 1**). The extractions were evaluated in terms of ORAC and TPs. Although the mixture acetone:HCl (1000:1)/water (50:50) yielded slightly higher TP levels than methanol:HCl (1000:1, v/v), this latter rendered the highest ORAC value and was, therefore, selected for extraction of the commercial ingredients studied. Acidified methanol has been proven to be a suitable solvent to extract anthocyanins from other materials (*12*, *14*). In the ORAC test, antioxidants present in the methanol:HCl (1000:1, v/v) extracts of the ingredients delayed the decay in the fluorescence curve until a certain time proportional to the antioxidant concentration (**Figure 1**). In the range of concentrations studied, all of the ingredients showed a linear response between the net AUC and the concentration in the assay (**Figure 1**).

Table 2 shows the antioxidant capacity and the phenolic content of the methanol:HCl (1000:1, v/v) extracts of the commercial dietary ingredients studied. In all of the solid samples, the water acitivity (A_w) values were below 0.44, indicating a low risk of microbial contamination. The ingredients derived from grape pomace and supplied as solids (ingredients #5 and 6) showed the highest ORAC values (13.3 and 21.4 μmol TE/mg, respectively) (**Table 2**). This was expected from their high TP and PRO contents (**Table 2**), possibly resulting from the contribution of grape seeds, which are abundant in procyanidins. On the other hand, grape pomace ingredients supplied as syrups (ingredients #7−10) presented ORAC values between 1.38 and 3.32 μmol TE/mg, which were also in accordance with their low TP and PRO contents (**Table 2**). In fact, for all of the ingredients derived from grape pomace ($n = 6$), a good correlation ($p \leq 0.01$) was found between ORAC and TP ($r = 0.993$) and between ORAC and PRO ($r = 0.993$) but not between ORAC and TAs ($r = 0.460$). For the group of ingredients derived from grape skins (ingredients #1−4), although the ORAC values were very similar, a good correlation ($p \leq 0.01$) was observed between ORAC and TA ($r = 0.853$), but no correlation was found with TP ($r = -0.396$). Finally, ORAC values recorded for the ingredients derived from grape leaves (ingredients #11−13) were among the lowest recorded for solid ingredients (between 1.52 and 2.55 μmol TE/mg), in accordance with their phenolic content (**Table 2**).

These differences in the ORAC and phenolic content among ingredients from different manufacturers may partly explain the variability observed in the antioxidant capacity of commercial dietary supplements containing *V. vinifera* L. ingredients

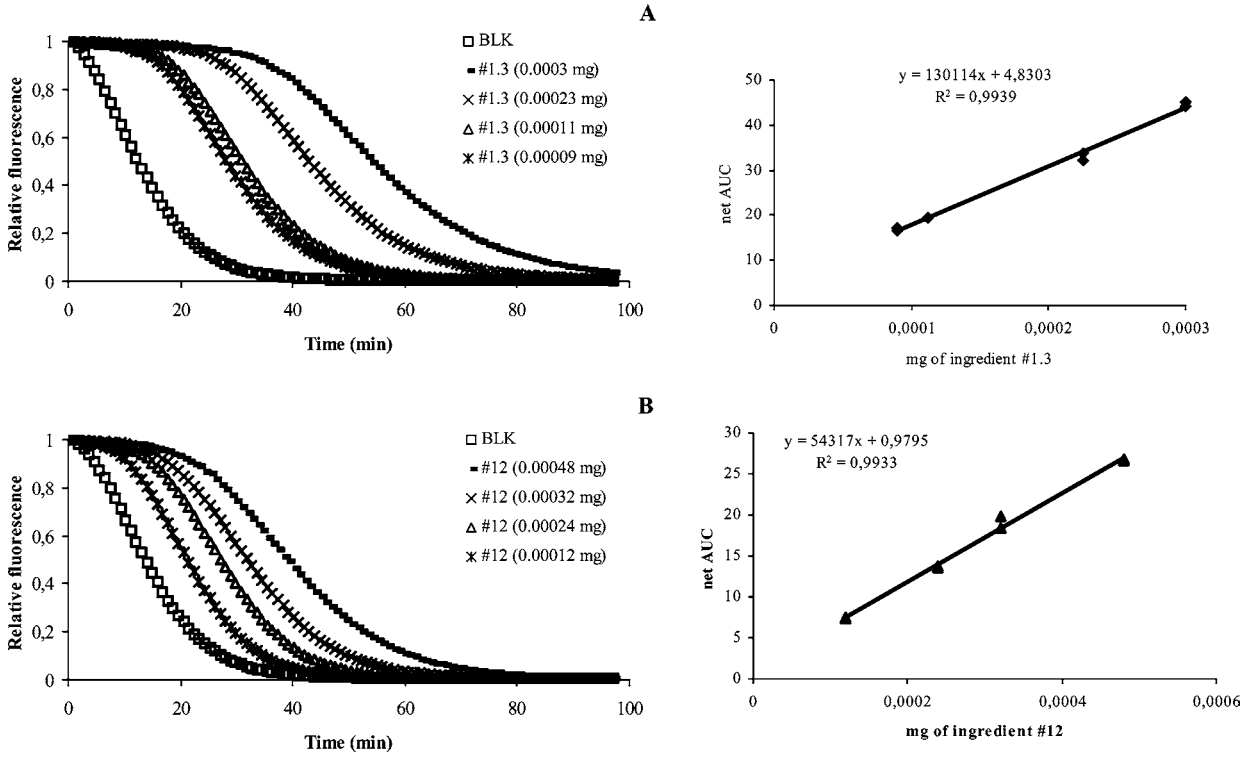

Figure 1. Time course of the reaction of FL with AAPH in the absence (BLK) and in the presence of dietary ingredients from *V. vinifera* L. grape skins (**A**) and leaves (**B**). The regression analysis of the net AUC vs ingredient concentration is also included.

Table 2. Source, Appearance, Obtention Procedure, Excipients Added, Water Activity (A_w), and Mean ± Standard Deviation Values ($n = 3$) of the Antioxidant Capacity (ORAC Value) and Phenolic Content (TPs, TAs, and PROs) of the Commercial Dietary Ingredients Studied[a]

ingredient no.	source	appearance	obtention procedure	excipients	batch no.	A_w	ORAC value[b]	TPs[c]	TAs[d]	PROs[e]
1	fresh grape skins	dark violet powder	water extraction	no	1	0.23	4.14 ± 0.02	147 ± 2	39.7 ± 2.5	
					2	0.30	2.91 ± 0.01	230 ± 8	17.5 ± 1.2	
					3	0.28	4.30 ± 0.24	201 ± 4	28.4 ± 1.5	
					4	0.27	3.06 ± 0.08	258 ± 13	9.68 ± 0.62	
					5	0.38	2.23 ± 0.04	171 ± 4	9.60 ± 0.49	
2	fresh grape skins	dark violet powder	hydroalcoholic extraction	NR	1	0.27	5.76 ± 0.36	155 ± 1	52.4 ± 3.4	
					2	0.27	5.49 ± 0.45	161 ± 7	51.8 ± 4.1	
					3	0.27	5.69 ± 0.28	174 ± 4	52.4 ± 2.5	
					4	0.27	6.32 ± 0.18	173 ± 2	54.6 ± 2.3	
3	fresh grape skins	black powder	NR	no	1	0.19	6.16 ± 0.50	210 ± 13	31.7 ± 4.0	
4	fresh grape skins	violet powder	alcoholic extraction	maltodextrin	1	0.21	5.02 ± 0.41	130 ± 12	54.2 ± 1.2	
5	grape pomace	dark purple powder	NR	maltodextrin	1	0.23	13.3 ± 1.3	374 ± 51	49.7 ± 2.9	649 ± 28
6	grape pomace	violet powder	aqueous ethanol extraction	NR	1	0.20	21.4 ± 0.3	508 ± 12	9.24 ± 0.61	659 ± 18
7	grape pomace from winemaking	syrup	water extraction	no	1		2.23 ± 0.13	59.9 ± 3.6	3.26 ± 0.11	126 ± 3
8	grape pomace from winemaking	syrup	water extraction and further purification	no	1		1.38 ± 0.10	50.3 ± 3.8	2.74 ± 0.11	97.0 ± 0.7
9	grape pomace from juicemaking	syrup	water extraction	no	1		1.71 ± 0.26	42.7 ± 2.0	3.36 ± 0.15	106 ± 5
10	grape pomace from juicemaking	syrup	water extraction and further fermentation	no	1		3.32 ± 0.22	119 ± 11	8.81 ± 1.21	321 ± 1
11	leaves	dark brown powder	water extraction	glucose, silica	1	0.19	2.55 ± 0.21	84.0 ± 3.1	ND	60.5 ± 3.0
12	leaves	light brown powder	alcoholic extraction	NR	1	0.23	2.19 ± 0.07	60.4 ± 0.4	ND	46.6 ± 4.5
13	leaves	brown powder	pulverization	no	1	0.44	1.52 ± 0.14	48.4 ± 5.8	ND	65.3 ± 5.9
					2	0.32	2.48 ± 0.13	64.7 ± 1.7	ND	107 ± 5

[a] A blank cell means not determined; NR, not reported; ND, not detected. [b] Expressed as μmol of TE/mg of ingredient. In the case of ingredients #7–10, results are expressed as μmol of TE/mg of soluble solids. [c] TPs, expressed as mg of gallic acid equivalent (GAE)/g of ingredient. In the case of ingredients #7–10, results are expressed as mg of GAE/g of soluble solids. [d] TAs, expressed as mg of malvidin-3-glucoside equivalent/g of ingredient. In the case of ingredients #7–10, results are expressed as mg of malvidin-3-glucoside equivalent/g of soluble solids. [e] PROs, expressed as mg of cyanidin equivalent/g of ingredient. In the case of ingredients #7–10, results are expressed as mg of cyanidin equivalent/g of soluble solids.

(0.018−3.18 μmol TE/mg) (*22*). On the other hand, it is important to highlight that the ingredients studied presented a higher antioxidant capacity as compared to the grape fruit (ORAC value = 0.0126 μmol TE/mg of fresh weight) (*28*) and to fresh grape skins (ORAC value = 0.428 μmol TE/mg of dry weight) (unpublished results).

When studying different production batches of ingredients #1 and 2, a high variability was found between batches of ingredient #1 (% coefficient of variation, ORAC = 26.2%; PT = 22.2%; TA = 61.9%) as compared to ingredient #2 (% coefficient of variation, ORAC = 6.1%; PT = 5.5%; TA = 2.3%) (**Table 2**). A similar situation was found when comparing batches #1 and 2 of leaf ingredient #13 (% coefficient of variation, ORAC = 33.7%; PT = 20.4%; PRO = 33.9%). This can be explained by the biological variability intrinsic in the raw materials, which may be attributed to the grape variety and to other factors that affect the berry development, such as soil, geographical location, and weather conditions. However, other factors related to the production process of the ingredient (i.e, extraction, fractionation, drying, etc.) may also influence the antioxidant capacity and the phenolic content of the final product.

Anthocyanin Composition of Commercial Dietary Ingredients from Grape Skins. Grape anthocyanins as well as anthocyanin-derived pigments were detected in the grape skin ingredients by HPLC-diode array detection (DAD)/ESI-MS (**Table 3**). Grape anthocyanins identified included the following: anthocyanidin-3-glucosides [delphinidin (DG), cyanidin (CG), petunidin (PtG), peonidin (PnG), and malvidin (MG)], -3-(6-acetyl)glucosides [delphinidin (DAc), cyanidin (CAc), petunidin (PtAc), peonidin (PnAc), and malvidin (MAc)], -3-(6-*p*-coumaroyl)glucosides [delphinidin (DCum), petunidin (PtCum), peonidin (PnCum), and malvidin (MCum)], and -3-(6-caffeoyl)glucosides [peonidin (PnCaf) and malvidin (MCaf)]. Anthocyanin-derived pigments identified consisted of pyranoanthocyanin pigments resulting from the C-4/C-5 cycloaddition of anthocyanins with pyruvic acid [malvidin-3-glucoside pyruvate (Derv 2)], vinylflavanols [malvidin-3-(6-acetyl)glucoside-vinylepicatechin (Derv 4) and malvidin-3-glucoside-vinylepicatechin (Derv 5)], and 4-vinylphenols and/or hydroxycinnamic acids [malvidin-3-glucoside-vinylcatechol (Derv 3), malvidin-3-glucoside-vinylphenol (Derv 6), and malvidin-3-glucoside-vinylguaiacol (Derv 7)], as well as the dimer resulting from the direct condensation reaction of anthocyanins with (epi)catechin [malvidin-3-glucoside-(epi)catechin dimer (Derv1)] (**Table 3**). The absence of anthocyanidin-3,5-diglucosides from all of the ingredients was also confirmed by HPLC-DAD/ESI-MS, finally revealing the authenticity of the ingredients as derived from *V. vinifera* L. tissues (*29*). In accordance with their product label (grape pomace), both grape anthocyanins and anthocyanin-derived pigments were detected in ingredients #5−10 (**Table 3**).

Anthocyanidin-3-glucosides presented the largest concentration in all of the ingredients studied. As expected from the *V. vinifera* spp., malvidin-3-glucoside was the most abundant pigment (*29*) (**Table 3**). However, acylated anthocyanins, in particular the acetylated ones, were present in a relatively lower concentration than in the fresh tissue (*29*). Although the anthocyanin profile is a characteristic of the grape variety (*29*), the results indicate that the production process could enhance

Table 3. Anthocyanin Compounds Present in the Commercial Dietary Ingredients from *V. vinifera* L. Grape Skins

ingr no.	batch no.		Derv1[a]	DG	CG	PtG	PnG	MG	Dac + Derv2	CAc	PtAc	PnAc + MAc + DCum	PnCaf	MCaf	PtCum	PnCum	MCum	Derv3	Derv4 + Derv5	Derv6	Derv7
1	1	mean	tr[b]	385.3	138.3	785.7	2303	6584	tr	ND[b]	tr	1023	52.38	156.3	102.7	513.7	1150	tr	tr	tr	tr
		SD		27.7	10.6	50.2	162	490				98	4.56	10.3	14.1	52.0	85				
	2	mean	tr	104.6	40.69	250.4	355.2	1890	tr	ND	tr	353.7	14.22	52.78	136.5	201.7	720.7	tr	tr	tr	tr
		SD		1.8	0.44	5.7	18.9	17				14.0	0.58	4.84	14.2	13.2	23.4				
	3	mean	tr	231.8	90.71	659.6	824.4	4612	tr	ND	tr	661.5	36.44	157.2	266.5	419.7	1590	tr	tr	tr	tr
		SD		16.0	4.67	24.2	36.8	247				32.0	2.23	4.5	4.7	36.0	106				
	4	mean	tr	30.11	16.33	65.91	135.3	581.6	tr	ND	tr	220.4	6.179	20.75	53.57	97.83	276.1	tr	tr	tr	tr
		SD		2.88	1.53	2.34	9.9	17.8				22.8	0.325	2.40	3.24	7.73	23.6				
	5	mean	tr	22.0	12.6	46.6	91.1	488.0	tr	ND	tr	383.9	6.027	25.98	58.25	99.62	501.0	tr	tr	tr	tr
		SD		1.9	0.9	2.5	2.2	30.9				10.9	0.160	3.66	1.17	0.97	44.9				
2	1	mean	ND	504.0	224.1	1326	2701	9473	tr	tr	tr	1265	80.42	246.7	283.0	759.4	2222	ND	ND	ND	ND
		SD		37.2	17.4	97	212	614				85	1.65	23.2	14.5	70.6	87				
	2	mean	ND	514.7	229.1	1347	2744	10332	tr	tr	tr	1286	62.16	255.4	320.2	728.8	2407	ND	ND	ND	ND
		SD		40.7	14.7	86	183	721				119	2.44	22.6	19.4	87.9	227				
	3	mean	ND	451.5	204.0	1203	2451	9213	tr	tr	tr	1163	70.15	222.5	277.6	783.7	2171	ND	ND	ND	ND
		SD		31.4	7.8	47	66	253				65	4.94	0.9	2.8	69.7	34				
	4	mean	ND	465.2	203.4	1201	2427	9116	tr	tr	tr	1150	67.41	214.9	273.6	755.8	2137	ND	ND	ND	ND
		SD		13.1	6.5	29	43	227				60	7.41	4.3	6.0	68.7	43				
3	1	mean	ND	513.7	123.6	707.5	817.6	3595	467.9	tr	109.3	1234	tr	41.27	123.2	198.4	474.4	ND	ND	tr	ND
		SD		33.6	5.3	25.3	27.6	95	14.2		5.3	34		0.41	2.4	3.6	2.9				
4	1	mean	ND	571.3	199.6	1252	3905	10571	tr	tr	tr	1228	ND	211.5	141.1	711.3	1551	ND	ND	ND	ND
		SD		17.6	7.2	69	248	756				105		25.0	15.3	82.0	165				
5	1	mean	ND	373.1	79.34	747.8	896.2	7561	347.6	tr	167.1	3004	ND	205.3	125.4	305.4	975.8	ND	tr	tr	ND
		SD		11.8	7.35	43.5	46.9	409	12.9		6.2	273		17.6	13.5	20.5	96.5				
6	1	mean	34.8	48.76	28.54	63.49	105.0	1036	77.82	tr	tr	163.5	ND	14.46	108.9	38.33	318.9	9.041	14.51	ND	ND
		SD	1.9	1.02	1.16	0.12	1.2	4	1.37			3.9		1.24	4.9	0.48	5.5	0.754	0.75		
7	1	mean	tr	145.0	24.02	221.2	41.99	684.7	18.30	tr	tr	109.7	tr	57.19	104.8	25.85	304.1	ND	ND	ND	ND
		SD		2.0	0.65	4.4	1.67	16.2	2.33			15.1		4.06	4.3	0.70	4.4				
8	1	mean	tr	103.9	22.46	138.0	35.8	553.9	28.42	tr	tr	85.45	tr	12.71	54.28	10.52	162.1	ND	ND	ND	ND
		SD		9.3	0.62	5.2	4.3	24.5	1.15			5.44		0.38	0.10	1.58	12.4				
9	1	mean	6.408	190.6	45.51	169.4	328.2	898.5	tr	tr	tr	39.80	tr	9.558	37.23	47.67	170.8	ND	ND	ND	ND
		SD	0.223	5.7	1.21	5.9	8.9	50.0				2.27		1.227	5.91	0.65	18.5				
10	1	mean	tr	229.1	54.14	198.4	382.0	1032	tr	tr	tr	14.90	ND	32.70	50.83	31.21	88.69	ND	ND	ND	ND
		SD		10.2	1.05	7.6	6.9	32				1.47		1.18	4.66	0.92	4.43				

[a] Expressed as μg of malvidin-3-glucoside/g of ingredient. In the case of ingredients #7–10, results are expressed as μg of malvidin-3-glucoside/g of soluble solids. Results are presented as the means ($n = 3$) ± SD. Derv1, malvidin-3-glucoside-(epi) catechin dimer; DG, delphinidin-3-glucoside; CG, cyanidin-3-glucoside; PtG, petunidin-3-glucoside; PnG, peonidin-3-glucoside; MG, malvidin-3-glucoside; DAc + Derv2, delphinidin-3-(6-acetyl)glucoside + malvidin-3-glucoside-pyruvate; CAc, cyanidin-3-(6-acetyl)glucoside; PtAc, petunidin-3-(6-acetyl)glucoside; PnAc + MAc + DCum, peonidin-3-(6-acetyl)glucoside + malvidin-3-(6-acetyl)glucoside + delphinidin-3-(6-*p*-coumaroyl)glucoside; PnCaf, peonidin-3-(6-caffeoyl)glucoside; MCaf, malvidin-3-(6-caffeoyl)glucoside; PtCum, petunidin-3-(6-*p*-coumaroyl)glucoside; PnCum, peonidin-3-(6-*p*-coumaroyl)glucoside; MCum, malvidin-3-(6-*p*-coumaroyl)glucoside; Derv3, malvidin-3-glucoside-vinylcatechol; Derv4 + Derv5, malvidin-3-(6-acetyl)glucoside-vinylepicatechin + malvidin-3-glucoside-vinylepicatechin; Derv6, malvidin-3-glucoside-vinylphenol; and Derv7, malvidin-3-glucoside-vinylguaiacol. [b] tr, trace level; ND, not detected.

the degradation of acylated anthocyanins, which can easily be hydrolyzed to their respective nonacylated forms (anthocyanidin-3-glucosides).

In general, the concentration of individual anthocyanins varied considerably among the ingredients derived from the same source (**Table 3**). In the case of ingredients derived from nonprocessed grape skins, ingredients #2 and 4 presented a much higher anthocyanin concentration [9533 (mean value between batches) and 10571 μg/g for malvidin-3-glucoside, respectively] than ingredients #1 and 3 [2831 (mean value between batches) and 3595 μg/g for malvidin-3-glucoside, respectively] (**Table 3**). Considering the ingredients derived from grape pomace and supplied as solids, ingredients #5 and 6 showed marked differences in anthocyanin concentration (7561 and 1036 μg/g for malvidin-3-glucoside, respectively) whereas those supplied as syrups (ingredients # 7−10) presented less differences [from 553.9 (#8) to 1032 (#10) μg/g for malvidin-3-glucoside]. Anthocyanin-derived pigments identified in these ingredients were present at very low concentrations or trace levels. Grape pomace is a highly heterogeneous raw material (*4*, *30*). In addition to the varietal characteristics of the grape varieties used, factors arising from the fermentation process (i.e., yeast strains and fermentation temperature) that influence the formation of anthocyanin-derived pigments may also affect the composition of the raw material. Further variability is added if the grape pomace is submitted to a subsequent distillation after wine-making to produce spirits. Finally, the production process employed to transform these raw materials into the final commercial ingredients will also determine the composition of the final products.

The study of the anthocyanin composition of the different production batches of ingredient #1 revealed large differences in the concentration of the individual pigments between batches, the level of malvidin-3-glucoside, for example, ranging from 488.0 (batch #5) to 6584 (batch #1) μg/g (**Table 3**). In addition, differences were also found in terms of the percentage distribution of the different anthocyanins (for example, malvidin-3-glucoside represented from 28 to 50% of TAs), indicating that the anthocyanin profile was not maintained in each batch. For ingredient #2, small differences were found in the concentration and distribution of the different anthocyanin pigments between batches (9116−10332 μg/g for malvidin-3-glucoside, representing 50−51% of TA concentration) (**Table 3**). These findings agree with the results shown above (**Table 2**) for the antioxidant capacity and the total phenolic content of these batches.

In the case of ingredients derived from nonprocessed grape skins ($n = 11$), a good correlation ($p \leq 0.01$) was found between the ORAC values and the concentration of each of the individual anthocyanidin-3-glucosides present (DG, CG, PtG, PnG, and MG), but poorer ($p \leq 0.05$) in the case of acylated anthocyanins (MCaf, PtCum, and MCum) with the exception of PnCum, which presented a significance level of $p \leq 0.01$. Because glycosylation of anthocyanins reduces their antioxidant activity when compared to that of the corresponding aglycones (*31*), acylation of glycosides is also expected to have an influence on this property. This could explain the lower degree of significance found for the correlation ORAC vs caffeoyl and vs *p*-coumaroyl derivatives when compared to simple glucosides. In fact, when a stepwise linear regression analysis was performed to describe the ORAC values of nonprocessed grape skins ($n = 11$) in terms of their individual anthocyanin concentrations, the anthocyanidin-3-glucosides CG and MG were selected as the first and second best predictive variables,

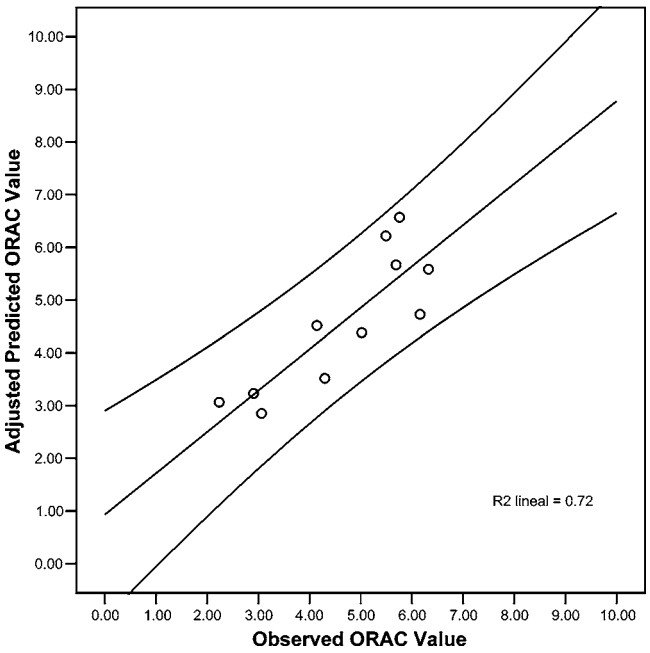

Figure 2. Plot of observed ORAC values vs adjusted predicted values obtained after application of the linear regression model for the grape skin ingredients.

respectively. The model obtained, ORAC = 2.574 + 0.42 (CG) − 0.001 (MG), presented a determination coefficient ($R^2 = 0.879$) and a residual standard deviation ($s = 0.551$) that indicated an acceptable error of the estimation. **Figure 2** shows the plot of observed ORAC values vs adjusted predicted values obtained after application of the linear regression model. In contrast to ingredients derived from nonprocessed grape skins, no correlation was found between the ORAC values and the concentration of individual anthocyanins for the ingredients derived from grape pomace ($n = 6$), in agreement with the results of the correlation ORAC vs TA found above.

Phenolic Composition of Commercial Dietary Ingredients from Leaves. The HPLC-DAD/ESI-MS analysis of leaf ingredients allowed the identification of anthocyanidin-3-glucosides, -3-(6-acetyl)glucosides, and -3-(6-*p*-coumaroyl)glucosides, characteristic of the *V. vinifera* spp. (**Table 4**). Nonanthocyanin phenolic compounds identified in leaf ingredients included the flavonols quercetin-3-*O*-galactoside, quercetin-3-*O*-glucuronide, quercetin-3-*O*-glucoside, kaempherol-3-*O*-galactoside, kaempherol-3-*O*-glucuronide, kaempherol-3-*O*-glucoside, and quercetin (**Table 4**). *trans*-Caffeoyltartaric acid (*trans*-caftaric acid) was the only hydroxycinnamic acid derivative identified in the leaf ingredients studied.

As in *V. vinifera* skins, anthocyanidin-3-glucosides were the most abundant pigments in the ingredients derived from leaves. However, the anthocyanin profile was different from that of the skins. Peonidin-3-glucoside and cyanidin-3-glucoside were quantitatively the most important anthocyanins, followed by malvidin-3-glucoside. Coumaroyl-glucosides were the second most abundant group of anthocyanins followed by acetyl-glucosides, which were present in very low concentrations. Ingredients #11 presented the lowest anthocyanin concentration followed by ingredients #12 and #13. The anthocyanin profile also differed among ingredients. For example, although ingredient #11 presented a lower content of simple glucoside than ingredient #12, the acetyl-glucoside content was higher in the former than in the latter.

Table 4. Phenolic Compounds Present in the Commercial Dietary Ingredients from *V. vinifera* L. Leaves[a]

compound	**11**	**12**	**13** (batch #1)	**13** (batch #2)
		anthocyanins (μg/g)		
delphinidin-3-glucoside	0.795 ± 0.034	3.05 ± 0.37	11.0 ± 0.2	121 ± 3
cyanidin-3-glucoside	7.70 ± 0.47	15.7 ± 1.0	56.8 ± 0.6	689 ± 14
petunidin-3-glucoside	0.990 ± 0.024	3.48 ± 0.39	8.37 ± 0.17	75.5 ± 1.5
peonidin-3-glucoside	14.9 ± 0.7	21.2 ± 2.1	85.8 ± 1.0	567 ± 9
malvidin-3-glucoside	3.40 ± 0.12	8.16 ± 1.19	31.2 ± 0.4	197 ± 3
cyanidin-3-(6-acetyl)glucoside	0.625 ± 0.052	tr[b]	0.548 ± 0.034	6.73 ± 0.01
peonidin-3-(6-acetyl)glucoside	0.924 ± 0.023	tr	0.889 ± 0.170	8.82 ± 0.28
delphinidin-3-(6-*p*-coumaroyl)glucoside	0.656 ± 0.012	tr	1.90 ± 0.14	14.8 ± 1.8
cyanidin-3-(6-*p*-coumaroyl)glucoside	1.21 ± 0.11	1.92 ± 0.15	6.23 ± 0.16	51.0 ± 1.8
petunidin-3-(6-*p*-coumaroyl)glucoside	tr	0.646 ± 0.040	1.58 ± 0.01	12.7 ± 0.4
peonidin-3-(6-*p*-coumaroyl)glucoside	1.01 ± 0.00	2.41 ± 0.20	10.4 ± 0.0	60.7 ± 0.7
malvidin-3-(6-*p*-coumaroyl)glucoside	1.12 ± 0.08	1.82 ± 0.14	6.27 ± 0.08	35.3 ± 1.0
		hydroxycinnamic acid derivatives (mg/g)		
trans-caftaric acid	1.83 ± 0.10	1.63 ± 0.02	0.729 ± 0.060	1.24 ± 0.01
		flavonols (mg/g)		
quercetin-3-*O*-galactoside	1.97 ± 0.10	2.68 ± 0.04	0.659 ± 0.068	0.991 ± 0.009
quercetin-3-*O*-glucuronide	18.7 ± 1.0	16.9 ± 0.3	5.78 ± 0.43	9.92 ± 0.08
quercetin-3-*O*-glucoside	10.4 ± 0.2	13.8 ± 0.4	3.81 ± 0.09	5.92 ± 0.14
kaempherol-3-*O*-galactoside	0.764 ± 0.098	1.38 ± 0.08	0.271 ± 0.025	0.429 ± 0.028
kaempherol-3-*O*-glucuronide	0.745 ± 0.101	1.27 ± 0.23	0.278 ± 0.018	0.569 ± 0.004
kaempherol-3-*O*-glucoside	2.35 ± 0.23	4.23 ± 0.09	1.03 ± 0.10	1.22 ± 0.03
quercetin	0.762 ± 0.033	0.642 ± 0.025	0.067 ± 0.009	0.111 ± 0.001

[a] Results are presented as the means ($n = 3$) ± SD. [b] tr, trace levels.

Concerning the flavonols, the concentration of quercetin derivatives in leaf ingredients was higher than that of kaempherol derivatives. Quercetin-3-*O*-glucuronide was the most abundant flavonol followed by the -3-*O*-glucoside derivative, whereas in the case of kaempherol glycosides, it corresponded to the -3-*O*-glucoside. In relation to the aglycones, quercetin was found at very low levels. An inverse relationship was found between the concentration of nonanthocyanin compounds (flavonols and *trans*-caftaric acid) and that of anthocyanins in the leaf ingredients studied. The content of nonanthocyanin compounds was higher in ingredients presenting low anthocyanin concentrations (#11 and 12), whereas the opposite was observed for ingredient #13. This could be due to the fact that both anthocyanins and flavonols are synthesized in plants from the same precursor (dihydroflavanols or flavanonols) but via different enzymatic pathways (*32*).

A large variability was found between batches of ingredient #13 in terms of their phenolic composition. Besides the marked differences observed in the concentration of individual anthocyanins, which was much higher in batch #2 than in batch #1, the distribution of anthocyanins in the two batches differed considerably, especially in relation to the levels of cyanidin and peonidin-3-glucosides: 26 and 39% of TAs for batch #1, respectively, and 38 and 30% for batch #2, respectively. However, in terms of nonanthocyanin phenolic compounds, differences between batches were less pronounced. Differences observed between ingredients and batches of the same ingredients may be attributed to the cultivar, color of leaves, period of growth cycle of the plant, and finally, to the processing conditions involved in making the ingredient.

Stability Study of Commercial Dietary Ingredients from Grape Skins. To evaluate the possible changes in the antioxidant capacity of the ingredients during their commercial life on the market, ingredients #1 (batch 1) and #2 (batch 1) were encapsulated and stored for 60 days under controlled conditions of temperature (45 °C) and relative humidity (75%). The stability test could not be completed for ingredient #1 because after 30 days the product crystallized and came out of the capsules.

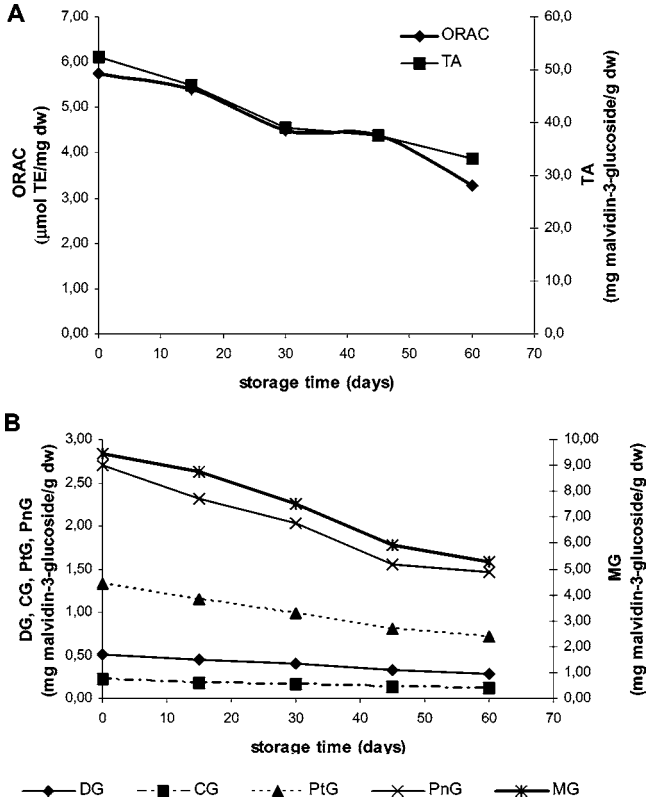

Figure 3. Antioxidant capacity (ORAC value) and TA concentration (**A**) and content of individual anthocyanidin-3-glucosides (**B**) of dietary ingredient #2 (batch #1) during storage at 45 °C and 75% relative humidity (dw, dry weight).

During this period, 21% of its initial antioxidant capacity was lost (data not shown).

Figure 3 shows the evolution of the ORAC and anthocyanin concentration (TA and individual anthocyanidin-3-glucosides) of ingredient #2 (batch #1) during the storage time studied. A progressive decrease in the antioxidant capacity and anthocyanin concentration occurred during storage, representing a 43 and

Table 5. Disappearance Rate of Anthocyanidin-3-glucosides in Ingredient #2 during Storage at 45 °C and 80% Relative Humidity during 60 Days[a]

	$t_{1/4}$ (days)	$k \times 10^{-3}$ (days^{-1})	R^2
delphinidin-3-glucoside	29.4	9.8	0.9860
cyanidin-3-glucoside	26.6	10.8	0.9804
petunidin-3-glucoside	27.4	10.5	0.9967
peonidin-3-glucoside	26.6	10.8	0.9727
malvidin-3-glucoside	27.7	10.4	0.9727

[a] $t_{1/4}$, time required for a 25% reduction of the initial anthocyanin concentration; k, constant rate.

40% decrease of the initial ORAC value and TA concentration, respectively, after 60 days of storage. Degradation of monomeric anthocyanins due to the effects of temperature in ingredient #2 followed a first-order kinetics ($\ln [A] = -kt + \ln [A]_0$, where $[A]$ is the pigment concentration, k is the reaction rate constant, and t is the time of storage). The reaction rate constant (k) for each pigment was determined by calculating the slope of the curve ln $[A]$ vs t by linear regression analysis. The reaction quarter-life ($t_{1/4}$), corresponding to the time required for a 25% reduction of the initial anthocyanin concentration, was also calculated by the equation $t_{1/x} = (\ln x - \ln (x - 1))/k$, $[A]_0/x$ corresponding to the reduced concentration. **Table 5** summarizes the disappearance kinetics data for the different anthocyanidin-3-glucosides. As expected, the different anthocyanin presented very similar k ($9.8-10.8 \times 10^{-3}$ days^{-1}) and $t_{1/4}$ (26−29 days of storage) values, indicating no influence of the chemical structure on the anthocyanin stability at 45 °C. This model also allows prediction of the reduction in anthocyanin concentration at a certain time of storage, which can be useful to establish the commercial lifetime of these ingredients. The fact that the losses in ORAC values were very close to the losses in anthocyanins once again suggests that the antioxidant capacity of ingredients derived from fresh grape skins was mainly due to this type of compound.

In conclusion, the suitability of the ORAC test to evaluate the antioxidant capacity of commercial dietary ingredients from *V. vinifera* L. byproducts is proven. Data reported in this paper could be of great value for the dietary industry dedicated to the formulation of dietary supplements containing *V. vinifera* L. ingredients as well as to develop new ingredients with improved antioxidant properties from other plant sources.

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