BENCH-SCALE EXTRACTION OF STILBENOIDS AND OTHER PHENOLICS FROM STORED GRAPE CANES (*VITIS VINIFERA*): OPTIMIZATION PROCESS, CHEMICAL CHARACTERIZATION, AND POTENTIAL PROTECTION AGAINST OXIDATIVE DAMAGE

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ABSTRACT

Dietary supplements have become the key to complement deficiencies in the occidental diet and therefore to reduce the incidence of oxidative stress related diseases. A bench-scale extraction procedure was studied to obtain a valuable product rich in phenolic compounds and antioxidant capacity from Pinot Noir grape cane enhanced by storage. Extraction solvent, cane-size, solid:liquid ratio, temperature, and extraction time, were systematically evaluated in order to obtain a natural functional product. Complete chemical characterization of a Pinot Noir grape cane extract produced under bench scale process is presented for the first time. Phenolic profiles of the extracts were characterized by HPLC-PDA-MS/MS and minerals by ICP-OES. Proteins, carbohydrates and lignins were also evaluated. The main phenolic compounds in the final product were stilbenoids, flavan-3-ols, procyanidins, and flavonols, with 6.53%, 4.84%, 2.11%, and 0.25%, respectively on a dry matter basis. Other chemical constituents were carbohydrates (27%), minerals (1%) and lignins (38.7%). The antioxidant capacity of the product was demonstrated using chemical assays ($TEAC_{ABTS}$/$_{CUPRAC}$ and ORAC-FL) and endothelial cells model. The extract produced under the described bench scale process using grape cane enhanced by storage have a chemical composition and protecting capacities to be used in functional foods industry.

Keywords: Stilbenoids, Procyanidins, Grape canes, Bench-scale extraction, Antioxidant capacity.

INTRODUCTION

Research on phytoalexins as nutritional components to promote health benefits have triggered the interest to obtain them at industrial scale with an ecofriendly process[1].

Health-promoting properties of many stilbenoids have been reported, including antioxidant, anti-inflammatory, anticarcinogenic, and neuroprotective effects[2]. Procyanidins are also known for their antioxidants, anti-inflammatory and antimicrobial properties, beside cardioprotective, hepatoprotective and neuroprotective effects[3-6].

Viticulture by-products have been extensively studied because of their interesting stilbenoids profiles and levels[7]. The levels of stilbenoids and the storage conditions of cane after the pruning in several *V. vinifera* varieties cultivated in Chile have also been reported [8,9].

Stilbenoid extraction from grape canes and other lignocellulosic matrices has also been investigated. Karacabey et al.[10]optimized the solid–liquid extraction conditions for obtaining stilbenoids from grape canes, using 54% of ethanol in water at 83.6 °C. They also modeled the supercritical solid–liquid extraction kinetics of *E*-resveratrol and *E*- *ε*-viniferin from Pinot Noir grape canes[11]. An evaluation of laboratory scale ultrasound-extraction of stilbenoids from grape stems concluded that the most significant variables of the process were solvent composition and sample−solvent ratio, where the optimal extraction conditions were solvent composition of 80% ethanol and 75°C during 15 min of sonication [12].

The main aim of this study is to develop a bench scale extraction process for the scale-up production of a functional crude ingredient containing stilbenoids and procyanidins, from stored grape canes. The scaling up of a solid–liquid analytical extraction method to a bench scale process was optimized for this purpose. The complete chemical characterization and determination of the antioxidant properties of the final dry extract, with emphasis on the phenolic constituents, was carried out in order to promote the use of this viticulture residue toward the functional food industry.

Materials and methods

2.1 Sample material

The study was performed using *V. vinifera* canes cv Pinot Noir obtained during commercial pruning of healthy plants from an organic vineyard, "Viña de Neira" located in Biobio region, south Chile (36°36'50.33" S, 72°39'40.63" W at an altitude of 279 m). The vines were pruned in August 2012 and the canes were collected from the soil after one week, which were immediately transported for storage and analysis. Gorena et al. [9] previously described that the stilbenoids level in grape canes is subjected to changes in time. Due to that, in order to compensate this increase in the concentration during the optimization of the extraction process, the stilbenoids levels were normalized using the results obtained by the analytical extraction carried out in parallel with the same material. The samples were stored over 3 months at 19°C±5 and 70% humidity-(patent 201403417), to obtain the lyophilized bench-scale extract which significantly increased the levels of stilbenoids in the raw material before the definitive extraction. The raw stored material was milled using hammer mill (Condux-Werk LS 10M, Wolfgang/Hanau, Germany).

2.2 Reagents and solvents

HPLC-grade acetonitrile, and water, ethanol, piceatannol (95%), cupric chloride dihydrate, ammonium acetate, calcium carbonate, sulfuric and formic acids were obtained from Merck (Darmstadt, Germany). Potable ethanol (98%) was obtained from Oxiquim (Concepción, Chile) for bench-scale extractions. *E*-resveratrol (99%), *E*-*ε*-viniferin (98%) and Procyanidin B2 (100%) were obtained from Phytolab (Vestenbergsgreuth, Germany). Hopeaphenol, *E*-vitisin-B, and vitisin-A were obtained from TU Braunschweig, Germany. An Oxiselect™ Kit for ORAC-FL method was obtained from Cell Biolabs, Inc. (San Diego, USA). (+)-Catechin hydrate (>98%), (-)-epicatechin(>90%), quercetin-3-L–rhamnoside (85%), quercetin-3-rutinoside (<94%), quercetin, kaempferol (90%), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), neocuproine hemihydrate, 3-ethylbenzothiazoline-6-sulfonic acid (ABTS), potassium persulfate, monosaccharide standards, namely l-(+)-arabinose (99%), l-(−)-rhamnose monohydrate (99%), *d*-(+)-glucose (99.5%), *d*-(+)-galactose (99%), l-(−)-mannose (99%), *d*-(−)-xylose (99%), *d*-(−)-fructose (99%) were purchased from the Sigma-Aldrich Chemical Co. (St. Louis, USA).

2.3 Extractions

Extraction procedures were performed at analytical and bench scale level, taking the first as a reference for the maximum amount of stilbenoids that can be extracted from each grape cane sample using ethanol/water 80:20 v/v [8-11].

2.4 Analytical-scale extractions

The analytical extractions were performed using ultrasonic bar homogenizer (Cole Palmer Series 4710, Chicago, USA), 80% v/v of ethanol/water mixture and milled grape cane material, the procedure described by our research group[8,9]. All portions were collected and before chromatographic analysis, the samples were diluted in mobile phase and filtered through a PES filter 0.22 μm.

The stilbenoids concentration of the stored grape canes were monitored between 2 and 9 months after commercial pruning, in order to reach the highest level of stilbenoids for the definitive bench scale extraction[9].

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2.5 Bench-scale extractions

Ultrasonic assistance was avoided in all bench-scale extractions. The study was carried out in stainless-steel hermetic reactors of 1 and 7 L, equipped with temperature and pressure sensor, in order to develop a better optimization of the scaling-up process[13]. A preliminary study of solid: liquid (S:L) ratio and temperature was carried out in the 1L reactor. S:L ratio of 1:15, 1:10 and 1:3 at 20°C, 80°C and 150°C were studied.

-Grape cane size, time and extraction temperature on the stilbenoids yield were studied as follows:

-Grape canes chopped to 0.5, 1.0, and 2.0 cm were extracted at 80°C for 65 min in the 1L stainless-steel reactor using a S:L ratio of 1:10.

-Temperature and extraction time were evaluated in a 7L reactor. A first extraction was carried out using ethanol 80% and S:L ratio 1:10 and 1 cm long canes pieces.

Considering the time required to heat 7 L of solvent, the extraction process was divided in two stages, the first one, a heating period where the temperature of the solvent in contact with the biomass was raised to 108°C; and the second one where the extraction temperature was kept constant at around 108°C, reaching a total time of 150 min. During the heating and extraction steps the concentration of each stilbenoid was determined and normalized as the extraction yield obtained, considering the analytical extraction.

A second extraction was carried out under the same conditions except for temperature which was set at 80°C. The whole process was monitored carefully. In order to evaluate the effect of consecutive extractions over the accumulated concentration, three consecutive extraction steps using "fresh" solvent were applied and evaluated. The extraction was performed under the best condition obtained (80% ethanol, chopped canes of 1 cm, S:L ratio1:10 and 80 °C for 100 min). Finally, under these conditions the definitive bench scale extraction was carried out using the grape cane enhanced by storage.

2.6 Lyophilized bench-scale extract (LBSE)

After the extraction procedure was established, 5L of the obtained crude extract was filtered and ethanol was removed using a rotavapor at 37 °C. The aqueous extract was lyophilized (Freeze dryer system, Alpha 2-4 LD plus, Christ, Osterode, Germany) to give 71.135 ± 0.001 g of solid extract for characterization. This product is referred to as lyophilized bench-scale extract (LBSE).

2.7 Analytical methods

2.7.1 Determination of stilbenoids and other phenolic compounds

The extracts were analyzed using a Shimadzu Nexera UHPLC/HPLC system (Kyoto, Japan), coupled in tandem with mass spectrometry detector (QTrap 3200, AB Sciex, Dublin,USA) for identification and Shimadzu Nexera diode array detector (DAD) (Kyoto, Japan) for quantification. Instrument control and data collection were performed using a Class-VP DAD software and Analyst software (Version 1.5.2, Shimadzu Co., Kyoto, Japan). The separation and electrospray ionization conditions for qualitative analysis were those described by Gorena et al. [9]. Quantifications were performed using DAD detector by external calibration curves at 280 nm for flavan-3-ols and procyanidins, 306 nm for stilbenoids, and 360 nm for flavonols, using the standards of *E*-resveratrol, *E*-ε-viniferin and *E*-piceatannol for stilbenoids, (+)-catechin for flavan-3-ols and procyanidins, and quercetin for flavonols. The analytical parameters of the method are summarized in Table 1.

Table 1. Analytical parameters for phenolic compound quantification using HPLC-DAD.

Compound	Standard	Response (mAU)	R^2	Linear range (μmol g^{-1})	LOD (μmol g^{-1})	LOQ (μmol g^{-1})
Stilbenoids	*(E)*-Resveratrol	y = 44079207x − 34452	0.9999	0.009–0.491	0.003	0.009
	(E)-Viniferin	y = 76894x – 48492	0.9983	0.005–0.136	0.002	0.005
	(E)-Piceatannol	y= 138884x – 129139	0.9984	0.013–0.205	0.004	0.013
Flavonols	Quercetin 3-glucoside	$y = 455776x - 419.91$	0.9996	0.020–0.323	0.006	0.020
Procyanidins	Catechin	$y = 17815x - 1792$	0.9996	0.008–0.134	0.003	0.008
	Epicatechin	$y = 17619x - 2414$	0.9997	0.007–0.134	0.002	0.007

R^2: correlation coefficient; LOD: limit of detection; LOQ: limit of quantification.

2.7.2 Determination of carbohydrates and lignins in LBSE.

The LBSE was processed using the procedure described by Sluiter et al.[14].The hydrolyzed carbohydrates were analyzed using an HPLC with refractive index detector (RID) Prominence (Shimadzu Co., Kyoto, Japan). The chromatographic column was an Aminex HPX-87H (Biorad, Hercules, CA, USA). The compounds were identified by comparison of their retention times with standards, and quantified by external calibration.

Insoluble lignins were analyzed using the procedure described by Sluiter et al.[14]. The absorptivity used for the calculation was 110 cm^2mg^{-1}.

The average molecular weight (MW) of the insoluble lignins was determined by gel permeation chromatography (GPC) under the condition described by Ku et al. [15], using a Prominence HPLC with UV-Vis detector (Shimadzu, Japan). Two 5 μm Phenogel columns coupled in series were used. The pore size of the first and the second columns were 1×10^6Å and 1×10^3Å respectively. All columns were provided by Phenomenex (Torrance, CA, USA). The calibration curve was constructed using polystyrene standards (GPC/SEC Calibration Kit, Agilent Technologies, Santa Clara, USA).

2.7.3 Determination of metals in LBSE

The samples were analyzed using inductively coupled plasma-optical emission spectroscopy (Optima 7000 DV, PerkinElmer, Waltham, MA, USA), as described in UNE-CEN 15290 for determination of Ca, Fe, Mg, and K, and in UNE-CEN 15297 for As, Cu, Pb, and Zn. The LBSE was hydrolyzed using a microwave digester (Mars 240/50, CEM Corporation, Matthews, NC, USA) [16].

2.7.4 LBSE in vitro antioxidant capacity assays

The antioxidant assays were performed using a micro-volume spectrophotometer (Epoch Biotek System, Winooski, VT, USA). Standards solutions of these main compounds were prepared individually and mixed, at the same concentration level found in the extract, with the aim to compare their antioxidant capacities.

$TEAC_{CUPRAC}$ assays were performed as described by Ribeiro et al. 2011 [17]. $TEAC_{ABTS}$ and the ORAC-FL assay was conducted according to the methodology described by Karacabey et al. 2010[18]. Reactive oxygen species (ROS) detection was performed using a dichloro dihydro fluorescein diacetate (DCF-DA) probe: ECV-304 endothelial cells were cultivated using 199 medium supplemented with 10% fetal bovine serum (Sigma-Aldrich) in a 5% CO_2 atmosphere. The cells were pre-incubated with a DCF-DA (5 μM) solution probe for 30 min. Several samples dilutions were added and incubated for additional 24 h. The fluorescence emission was measured using an excitation wavelength of 485 nm and emission wavelength of 540 nm. The results were expressed as DCF fluorescence intensity per cell protein content[19]. The samples and standards were diluted in order to fit the linear range and were analyzed by triplicate.

RESULTS AND DISCUSSION

3.1 Operating condition for bench-scale extractions

Stilbenoid extraction yields were determined for each experiment to identify the best conditions for bench-scale cane extraction. In the preliminary 1L study, the S:L ratio and temperature were evaluated. S:L ratio of 1:15 yielded 76±2% of stilbenoids, while for 1:10 and 1:3, the yields were 68±1% and 39±1%, respectively. The optimum condition was considered to have the highest extraction yield with a lowest possible amount of solvents that later must be evaporated and thus, a proportion of 1:10 was selected. The influence of temperature indicated that at 20°C and 150°C there was a considerable decrease in the extraction yield, while at 80°C the highest stilbenoid yield was reached (68%). These results are in accordance with other authors as Karacabey & Mazza[10].

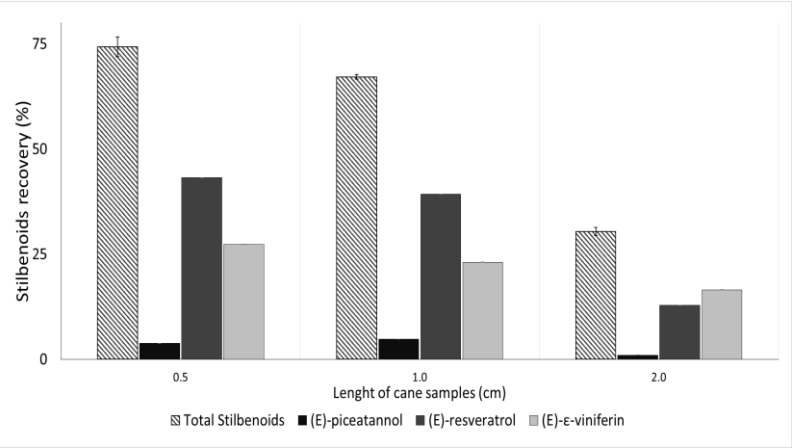

Figure 1. Recovery of stilbenoids in bench-scale extraction effect of cane sample length.

Figure 1 shows the extraction recovery obtained when cane samples of different lengths were subjected to the extraction procedure, the results were normalized through analytical extraction yield and expressed in average ± standard deviation. The extraction yield increased with decreasing sample size, in agreement with the results reported for phenolic compounds[20,21]. The reduction of sample size from 2.0 to 1.0 cm, increased the extraction yield in 37±1%, while the reduction from 1.0 to 0.5 cm increased only 7±1% the extraction. Therefore, subsequent experiments were performed with cane pieces of 1.0 cm; this represents a good compromise between cost (energy required for the mill step[22]) and efficiency in the scope of industrial scale-up.

The influence of the heating process from the beginning of the extraction, and the effect of temperature on stilbenoid stability in the extract were evaluated in a 7 L extraction in two steps. Four samples were collected during the heating step, and another four during the final extraction, spaced roughly every 25 min. The results are presented in Figure 2A. The highest concentrations were obtained at around 165 min from the beginning of the extraction process; this is 50 min after the extraction temperature (108°C) was reached. At this point the extraction yield was approximately 72% of the stilbenoids from the grape cane matrix. As expected, high temperature results in better solvent penetration into the grape cane matrix, by reducing the solvent viscosity and surface tension, which increases the stilbenoid diffusion from the matrix. All these processes may have helped to increase the stilbenoid concentration in the extract up to 165 min, after which it decreased to 53% yield, probably due to thermal degradation of the compounds.

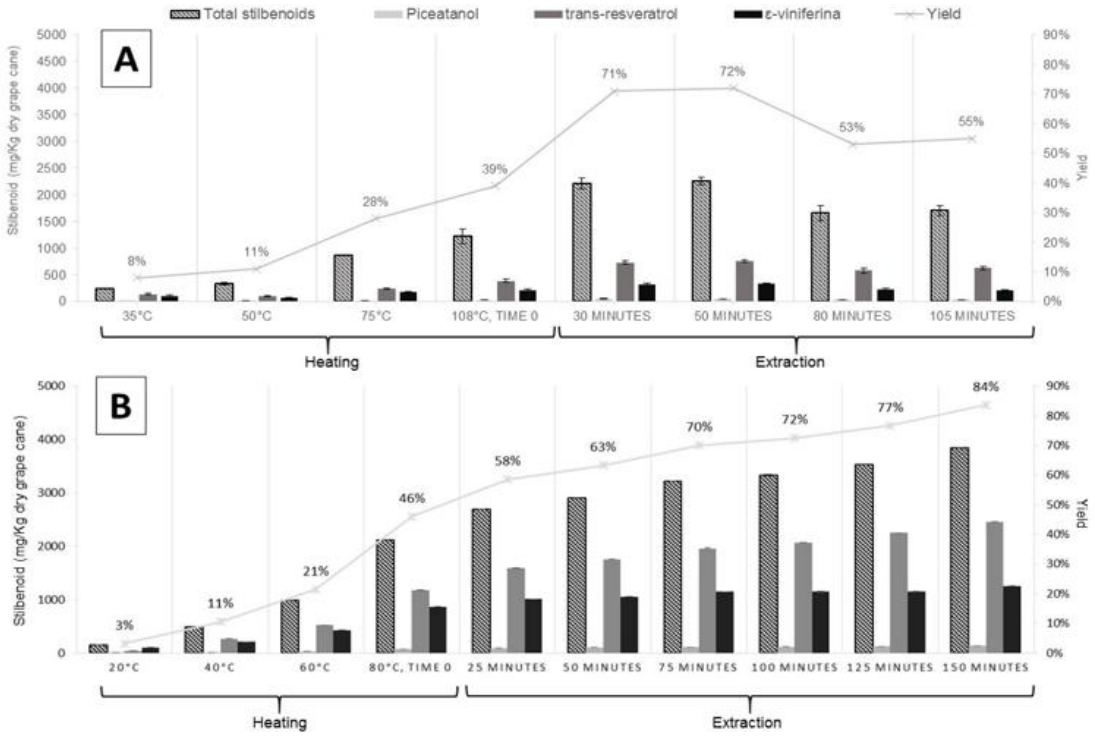

Figure 2. Extraction of stilbenoids from grape canes (A) with heating to 108°C and (B) heating at 80 °C.

Another unwanted effect produced at this temperature and time was a high pressure in the reactor caused by ethanol evaporation (pressure was monitored but not controlled). Considering as final objective the industrial scale up of the process, the use of these conditions requires a more complex infrastructure and higher energy input, which would increase the costs. Therefore, a second experiment was performed under the same conditions, except that the temperature was lowered to 80°C, to avoid an increase in pressure, and to compensate the extraction time was extended to 150 min. As Figure 2B shows, the stilbenoid concentration did not decrease at this temperature, despite the increase in the extraction time; moreover, the stilbenoid levels increased to 84% in 150 min at 80°C.

Considering these results, and to avoid thermal degradation of stilbenoids and maximize the extraction yield, the use of consecutive extraction steps for the same sample portion was evaluated in order to maximize the depletion of stilbenoids from grape canes. With a second extraction, it was possible to increase the amount of stilbenoids extracted in the first step by 39%, while a third extraction, only 4% of increase was produced. It was observed that the proportion of each stilbenoid in each extraction step did not change; the extracted stilbenoids of both accumulative extractions were 2868 mg Kg^{-1} dry grape cane, 3466 mg Kg^{-1} dry grape cane and 114 mg Kg^{-1} dry grape cane for *E*-resveratrol, *E*-ε-viniferin, and *E*-piceatannol, respectively, representing an extraction percentage of 100 ± 4%, 87 ± 4%, and 47 ± 3% respect to the concentration obtained applying analytical extraction. These results indicate that an increase in the extraction time and the use of successive batch steps can improve the extraction efficiency of stilbenoids from grape canes.

The final extraction was conducted as follows: 0.504±0.001 Kg of grape canes (dry basis) with a particle length of 1 cm was extracted with ethanol (80%), at a S:L ratio of 1:10, at 80 °C for 100 min at a pressure between 100 and 200 KPa (vapor pressure of ethanol/water mixture at this temperature) in a 7 L stainless-steel batch reactor in two successive extractions.

3.2 Characterization of final grape cane extract obtained at bench scale

Based on the preceding results, a grape cane extract was produced at the bench scale (7L) under the final conditions described in the previous section. The LBSE was characterized to evaluate the potential of grape canes as an industrial source of stilbenoids for bench scale extraction. The chemical properties of LBSE are summarized in Table 2.

The LBSE contained high levels of sugars, mainly glucose, which can be initially present in canes or can be a product of cellulose hydrolysis during the extraction[16]. A high lignin percentage was also observed which is typical of this type of biomass. The molecular weight distribution of the acetylated lignin was 1893 g mol^{-1} in number (Mw) and 770 g mol^{-1} in weight (MN), with a polydispersity index of 2.4. Taking into account that the main aim is the production of industrial phenolic compounds, all these compounds can be considered as impurities present in the crude extract, however, none of them shows toxicity to human health.

Table 2. Chemical composition of LBSE.

Parameter	**% w/w**
Moisture	5.0±0.1
Proteins	6.7±0.1
Total sugars	27.5±0.1
Glucose	21.4±0.2
Xylose	UD
Arabinose and rhamnose	UD
Mannose	6.1±0
Total lignins	38.7±1.4
Insoluble lignins	34.1±1.4
Soluble lignins	4.6±0.1
Number-average molecular weight (M_n; g/mol)	770
Weight-average molecular weight (M_w; g/mol)	1893
Polydispersity (M_w/M_n)	2.4
Ash	1.03
Mg	$5.53\pm0.03 \times 10^{-2}$
Ca	$2.34\pm0.05 \times 10^{-2}$
Fe	$2.1\pm0.5 \times 10^{-3}$
Cu	$2.4\pm0.3 \times 10^{-3}$
As	UD
Zn	$4.5\pm0.1 \times 10^{-3}$
Pb	UD
K	$8.6\pm0.1 \times 10^{-1}$
Na	$8.01\pm0.04 \times 10^{-2}$

UD: undetected

Toxic elements such as Pb and As were not detected in the LBSE, while significant amount of K and Na were found. Other elements as a Zn, Fe and Mg were also detected in the extract. All these minerals are considered micronutrients: potassium in diet reduces cardiovascular disease and decreases the risk of osteoporosis, Zn and Fe are involved in immune system and blood composition and Mg play an important function in living cells [16].

The identities and contents of stilbenoids and other phenolic compounds found in the LBSE are summarized in Table 3; the concentrations of the main compounds are also given.

Twelve stilbenoids were detected in the LBSE. Three of them are the most abundant: *E* –resveratrol, *E*- *ε*-viniferin, and (*E*)-piceatannol, followed by *E*-*ω*-viniferin, vitisin B and a tetramer. Some minor oligostilbenoids like ampelopsin A, (*E*)-piceid and hopeaphenol were also detected. The identities of two dimers at trace levels with maximum absorption at 280 nm were assigned tentatively as pallidol and isohopeaphenol by comparison with DAD and MS data published by Gabaston et al., 2017[23].

The total stilbenoid concentration was 65300±469 mg Kg^{-1} of crude extract [dry weight (DW)]. The stilbenoid profile of the crude extract was similar to that previously reported for Pinot Noir grape canes[9], despite the important differences between the extraction procedures. This is relevant because it shows that the proposed bench-scale process did not significantly alter the stilbenoid profile of the grape cane extract. The concentration of *E*-resveratrol found in LBSE were 33 times higher than the reported concentration for vine shoot of Melon of Burgundy patent EP2920160A1. For *E*-*ε*-viniferin concentration was only 3.7 time higher than the reported example. These differences could be produced by several factors in the process that consisted in a more dynamic extraction due to the extrusion with a smaller sample size (1 to 8 mm), lower extraction temperature (60°C) and a similar extraction time (2 h)[24]. However, the differences in the *E*-resveratrol concentration are too high to be explained only by the extraction process and must be highly influenced by the grape variety and especially by the post-pruning storage process, inducing an important increment of this stilbenoid, which is available for extraction. The post-pruning storage is a relevant aspect that must be considered in the industrial process, due to their effect in the initial concentration of stilbenoids present in the vegetal material.

Table 3. HPLC-DAD-MS/MS data for phenolic compounds in LBSE.

Compound	t_R**(min)**	**[M − H]$^-$**	**Fragments**	λ_{max} **(nm)**	**mg Kg^{-1} (dry weight)**	**% (w/w)**
Stilbenoids						
(*E*)-Piceid*	19.6	389	227	306 (318)	Traces	
Ampelopsin A	23.0	469	451; 423; 375; 365; 345; 317; 241	282	Traces	
(*E*)-Piceatannol*	24.4	243	-	324 (300)	3 668	0.37
Pallidol	27.9	453	359; 265	280 (285)	Traces	
(*E*)-Resveratrol*	32.2	227	-	306 (318)	30230	3.02
Hopeaphenol*	38.3	906	358; 451; 360; 718; 345; 812; 265	282	Traces	
Isohopeaphenol	39.3	906	359; 265; 451; 317; 345; 330; 813	283	Traces	
(*E*)-ε-Viniferin*	43.3	453	359; 347; 225; 411; 279; 197; 145	323	30 082	3.01
Tetramer	44.9	906	680; 330; 341; 319; 452; 574	299	160	0.02
(E)-ω-Viniferin	46.9	453	411; 435; 385; 359; 347; 243; 225	321 (324)	720	0.07
Tetramer	48.2	906	347; 359; 451; 649; 705	318 (330)	Traces	
(*E*)-Vitisin B*	54.2	906	359; 347; 439; 800; 279	325	440	0.04
Flavan-3-ols / procyanidins						
Dimer	8.4	577	407;125;289;246;161;203	279	Traces	
(Epi)gallocatechin	9.3	305	219;178;125;164;204	272	Traces	
Dimer	10.2	577	407;289;125;161;245	283	9980	1.0
(+)-Catechin*	11.5	289	245;203;123;205;137;221	279	31700	3.17
Dimer	14.6	577	289;125;407;245;161	282	11100	1.11
(-)-Epicatechin*	13.6	289	245;203; 125; 137; 179; 221	279	16690	1.67
(Epi)catechin gallate	20.1	441	169; 125; 289; 245; 227; 151; 165	269 (350)	Traces	
Flavonols						
Quercetinpentoside	16.1	433	300	354	310	0.03
Quercetinpentoside	17.2	433	300	354	290	0.03
Quercetin-3- rutinoside*	17.7	609	300	354	160	0.02
Quercetinhexoside	19.3	463	300	-	350	0.04
Quercetinhexoside	19.7	463	300	-	580	0.06
Kaempherolglucoside	23.5	447	284; 255; 227; 327; 299; 241; 151	-	310	0.03
Quercetin-3- rhamnoside*	23.9	447	300	360	270	0.03
Quercetin*	37.4	301	151; 187; 255; 233	-	220	0.02
TOTAL					**137260**	**13.7**

* Identification confirmed with standards

The data in Table 3 show that other phenolic compounds were also extracted under the optimized extraction conditions. Flavan-3-ols and procyanidins were detected in the crude extract, mainly (+)-catechin, (-)-epicatechin, and two unidentified dimers of procyanidin. Other flavan-3-ols, (epi)gallocatechin, and (epi)catechin gallate were detected for the first time in a grape cane extract at trace levels. Sánchez-Gómez et al.[24] and Rajha et al.[25] reported the presence of (+)-catechin and (-)-epicatechin in vine shoots, but not other procyanidins. The concentrations of monomeric procyanidins in the LBSE were higher than those of oligomeric procyanidins. The total concentration of procyanidins, including monomers, was 69470 ±698 mg Kg^{-1} of crude extract (DW), which is the same order of magnitude as the level found for stilbenoids. Several quercetin derivatives were also detected the in LBSE, together with a kaempferol derivate. The total flavonol concentration in the extract was lower than those of the other studied families, reaching a level of 2490 ± 201 mg Kg^{-1} of crude extract (DW); however, the mixture of different families of phenolic compounds is interesting in terms of the bioactivities of these compounds. The total concentration of the three groups of phenolics in the LBSE was 13.7% w/w, based on dry matter. This is a significant level in a crude product, which could be used as ingredient in food industry.

The stability of stilbenoids in the LBSE was monitored during 3 months. The LBSE was dissolved in 80% ethanol and stored in darkness at -18°, 4°C and room temperature (20°C) and exposed to light at room temperature. The results showed no variation in stilbenoid concentrations at any of the studied temperatures, being only necessary its protection from light due to the isomerization of *E*-resveratrol.

3.3 Antioxidant and biological activities of LBSE

The extraction efficiency in terms of bioactive products was evaluated *in vitro* using validated antioxidant assays.

The total concentration of the main families of phenolic compound in the methanolic solution of LBSE, determined by HPLC, was 49.6, 21.6 and 43.2 mg L^{-1} of flavonols, procyanidins and stilbenoids respectively. Based on these concentration, antioxidant capacity was studied comparing with standards. The antioxidant capacities of LBSE and the mix solution prepared with standards of *E*- resveratrol (49.6mg L^{-1}), (+)-catechin (21.6 mg L^{-1}) and procyanidin B2 (43.2 mg L^{-1}) are summarized in Table 4. Both solutions were compared with the sum of the antioxidant capacities of the individual standards at these same concentrations.

Table 4. Antioxidant capacity of LBSE.

Concentration (mgL^{-1})	$TEAC_{ABTS}$[a] (mmol g^{-1})	$TEAC_{CUPRAC}$[a] (mmol g^{-1})	ORAC-FL[a] (mmol g^{-1})
LBSE	1.954±0.264	1.918±0.154	6.895±0.996
Synthetic mix of C+P+R (49.6+21.6+43.2)	1.891±0.119	2.144±0.068	19.698±2.566
Sum of result of C+P+R	1.387±0.044	1.691±0.017	9.275±0.310

[a]Expressed as Trolox equivalents. Where C: *(+)*-catechin; P: procyanidin B; R: *(E)*- resveratrol

No statistical differences were observed for A_{280} between the standard mix and the sum of antioxidant capacity of all standards, as is expected (there are no differences in the amount of phenolics). However, the antioxidant capacity test, especially ORAC, shows higher values for the standard mix solution than the sum of individual standard antioxidant capacities, indicating a probable synergistic effect of compounds when they are mixed. Other authors have reported synergistic effects on the antioxidant capacities of phenolic compounds, especially between *E*- resveratrol and flavan-3-ols[26].

The TEAC assay results showed the reducing capacity of the LBSE, while the protecting capacity of the LBSE was demonstrated using ORAC-FL.

The TEAC values obtained for LBSE were similar to the antioxidant levels observed for the standards mix but higher than the sum of individual antioxidant capacity of the standards. On the other hand, protecting capacity measured by ORAC-FL, was lower than the sum of the individual values for each standard and lower than the protecting capacity of the synthetic mixture of standards. These behaviors can be explained due to the presence of other compounds in the crude extract and show that there are important influences of the LBSE matrix on the antioxidant capacity of the extract. Finally, the antioxidant capacity of the product could be considered to be high having as a reference point the ORAC values ranked for different fresh fruits. From this comparison, it can be considered that 1 g of final product has an equivalent antioxidant capacity as commonly consumed fresh fruits, such us certain varieties of cherries, blueberries and quinces [27].

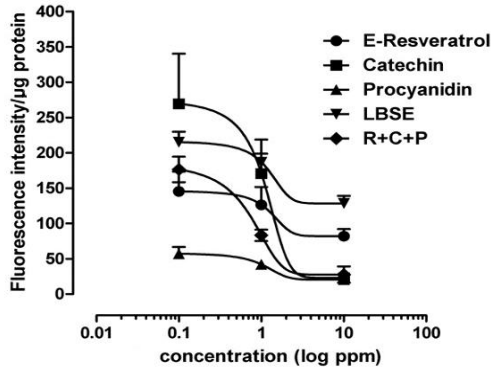

Figure 3. Protecting effect of LBSE, determined using ROS-DCF

The cell-based ROS-DCF assay also showed the protecting effect of LBSE, Figure 3 shows the results obtained for individual standards and a mixture of them at the same concentration found in LBSE, diluted to different levels (1, 10, and 100 times). The results suggest the protecting capacity of the LBSE, and show a concentration dependence to the individual standards and their mixture. Our results indicate that Procyanidin B had the highest protecting capacity at all dilution levels. The protecting capacity of the LBSE, at all the studied concentrations, was closer to that of the resveratrol standard, and was lower than the protecting capacity of the mixture of standards. This could be caused by competing or antagonistic behavior by the other compounds present in the LBSE.

CONCLUSIONS

The bench-scale extraction process using post pruning stored Pinot Noir grape canes, produced a natural extract with high levels of stilbenoids (6.5% w/w DW), flavan-3-ols/procyanidins (6.9 % w/w DW), lignins, carbohydrates, proteins and some micronutrients. The extraction process carried out with 80% of potable ethanol using 1:10 solid:liquid ratio at 80°C for 100 minutes, allowed to produce a natural and stable ingredient with high potential against oxidative damage. It can be used in the food industry, adding value and utilizing a residue from viticulture.

ABBREVIATIONS USED

ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); CUPRAC, cupric ion reducing antioxidant capacity; DCF-DA, dichlorodihydrofluorescein diacetate; LBSE, Lyophilized bench-scale extract; ORAC-FL, oxygen radical absorbance capacity-fluorescein; ROS, Reactive oxygen species; TEAC, Trolox equivalent antioxidant capacity.

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