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Quali-Quantitative Analysis by LC/DAD and GPC of the Polyphenols of "Uva Di Troia Canosina" Grape Seeds for the Development of an Industrial Nutraceutical Product

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Abstract

The quali-quantitative determination of the principal components of "uva di Troia canosina" seed extracts by LC/DAD analysis and the optimization of the extraction and purification processes for the development of an industrial nutraceutical product, are described. Two different fractions of seeds collected at different stages of fermentation were compared: "Tesi" 2 when there is a spontaneous stratification of the seeds at the bottom of the recipient and "Tesi" 4 at the end of fermentation. Percolation was applied and compared to maceration and the purification step carefully evaluated to obtain extracts free of contaminant species endowed with polyphenolic content comparable to commercial preparations such as Leucoselect® (Indena, SpA, Italy), Vitis Vinifera extract 95% (seeds), Vitis Vinifera dry extract 95%, Biovin grape seed and vinasse extract. (Farmalabor, Italy). In particular, "Tesi" 2 extract obtained by percolation and purified with a LLE extraction with ethyl acetate showed a polyphenolic content similar to Leucoselect®.

From the quantitative analyses it was evident, as expected, that "Tesi"2 has a higher polyphenolic content compared to "Tesi" 4, because during vinification the must extracts polyphenols from the seeds. On the other hand, "Tesi" 4 is particularly convenient since it is easily obtained and very economical, being a waste product.

The residual content of organic solvents (ethanol and ethyl acetate) and water was assessed in the grape extracts according to ICH rules by means respectively of HS/GC and Karl Fisher titration in order to meet the requirements for commercialization. Furthermore, the high molecular weight polyphenolic fraction of our extracts was investigated through gel permeation chromatography (GPC) and compared to that of Leucoselect®.

Keywords: Uva di Troia canosina; Polyphenols; Extraction; Purification; Quantitative analysis; LC/DAD

Introduction

In a previous work we carried out the analysis of the polyphenolic content of the seed extracts coming from four different fractions of "uva di Troia canosina" grape seeds sampled at different stages of the fermentation process [1]. "Uva di Troia canosina" grape is a *Vitis Vinifera* variety characterized by a small berry and cultivated around the city of Canosa in Apulia, a region of southern Italy. This kind of grape, although considered unproductive from the oenological point of view, shows high polyphenolic content and a great wine ageing potential [2].

Polyphenols, especially flavonols, flavan-3-ols (monomeric catechins, proanthocyanidins) and anthocyanidins, are responsible for many organoleptic characteristics of wine and grapes; [3] their concentration and composition are influenced by viticultural and environmental factors, such as climate conditions, maturity stage and production area [4-7]. Phenolic compounds, due to their strong free radical scavenging and high antioxidant properties, display many pharmacological benefits such as cardio protective, vasodilatatory, anticarcinogenic, anti-inflammatory, anti-allergic, antibacterial, immune-stimulating, anti-viral and estrogenic activities [8-12].

The determination of polyphenolic composition is very important to characterize different grape varieties, moreover, analytical methods [13] have been improved throughout the years [14] and to this end different extraction procedures [15,16] involving a variety of solvents [17] have been widely studied.

From the data obtained it was evident that the polyphenolic content is lower in the seeds belonging to Tesi 4 than in the Tesi 2 seeds because the polyphenols are extracted from the seeds to the must during fermentation. Moreover, extraction conditions and purification were studied in order to obtain suitable extracts for the preparation of a nutraceutical product based on the antioxidant activity of polyphenols [1].

In this paper we describe the quantitative determination of the principal components of "uva di Troia canosina" seed extracts respect to catechin and the optimization of the extraction and purification processes for the development of an industrial nutraceutical product. The residual content of organic solvents (ethanol and ethyl acetate) and water was measured the grape extracts according to ICH rules by means respectively of HS/GC and Karl Fisher titration in order to meet the

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requirements for the commercialization of medicines and excipients. Moreover, the analysis of residual water and organic solvents was also useful for the determination of the title of the extracts in catechin + epicatechin, a parameter which allows the characterization and the comparison of products obtained with different methodologies, thus helping the optimization of extraction and purification procedures.

In particular, percolation was applied and compared to maceration and the purification step carefully evaluated to obtain extracts free of contaminant species such as high molecular weights polymers, fats, pectins and phlobaphenes. Seeds were not grounded, but extracted intact because the polyphenols are contained in the cuticle of the seed. Moreover intact seeds at the end of the extraction protocol can be used to obtain grape seed oil, thus exploiting in a more efficient way the waste products. Furthermore, the analytical method was optimized and validated and our extracts were compared to highly standardized grape seeds extracts such as Leucoselect® (Indena, SpA, Italy), Vitis Vinifera extract 95% (seeds), Vitis Vinifera dry extract 95% (procyanidins), Biovin grape seed and vinasse extract. (Farmalabor, Italy).

Materials and Methods

Fruit sampling

"Uva di Troia canosina" grape was cultivated in Canosa (Apulia, South Italy) in 2010. The crop was immediately divided into 4 fractions, called "Tesi"1-4, which were subjected to 4 different stages of the fermentation processes [1].

In this work "Tesi" 2 and Tesi "4", from the grape harvest in 2010, were compared with "Tesi" 2 deriving from the experimental cultivation "Dr. Sergio Fontana" by Farmalabor, harvested in 2012. These fractions were chosen since from our previous work we noticed that "Tesi" 2 is the richest in polyphenols which allows the concomitant production of wine. On the other hand, "Tesi" 4 is less abundant in polyphenolic content but it is a waste product of wine industry and for this reason it is particularly convenient and easy to obtain.

Chemical and reagents [1]

Acetonitrile and Orthophosphoric acid 85% of HPLC grade quality were purchased from Sigma-Aldrich (St. Louis, USA) and Merck (Whitehouse station, USA), respectively. Absolute ethanol (Carlo Erba, Milano, Italy) and Acetone (VWR, Pennsylvania, USA) were of reagent grade. The resin SEPABEADS SP-207 was obtained by Resindion (Milan, Italy). Standards of Gallic acid, (+)-Catechin hydrate, (−)-Epicatechin were supplied by Sigma (Milano, Italy); Procyanidin B1 and Procyanidin B2 were obtained from Fluka (Milano, Italy). All standards were of purity >90%. Leucoselect was supplied by Indena SpA (Settala, Italy). Vitis vinifera 95% extract was obtained from Farmalabor Srl (Canosa, Italy). Milli-Q quality water was obtained with a Milli-Q (H2O) system by Millipore (Bedford, MA, USA).

Seed extract preparation

Loss on drying: Before starting the extraction procedure, the humidity percentage of each "Tesi" was evaluated, by drying about 10 g of seeds at 60°C for 5 days. The calculated humidity is reported in Table 1.

Maceration: Ethanol or acetone was added to intact frozen seeds (50 g) to cover them and to reach a final concentration of 70%(v/v), taking into account the amount of water contained in the vegetable material, in glass beakers protected from lights and air by aluminium foils; the maceration was carried out under magnetic stirring and the first 3 h extraction started when the seeds reached room temperature; seeds were filtered under vacuum and were subjected to the second 3h extraction adding fresh solvent (50 mL); at the end of the second 3 h extraction, seeds were filtered and extracted again with fresh solvent (50 mL) during the whole night; the steps of extractions were repeated until the sixth and last extraction, that is, the 2nd overnight extraction; then, the solvent of the combined extracts was completely removed at 40°C and the extracts were dried in oven for at least 12 h at 60°C.

The extraction procedure was evaluated by comparison of the recovery percentages (%REC) of each extract, calculated as W_{fin} / W_{init} x 100, where W_{fin} is the final weight and the initial weight W_{init} considers the intrinsic water content of seeds. % recoveries are reported in Table 2. 100.0 mg of each dry extract were then dissolved in 10 mL of a 1:1 0.3% H_3PO_4/Acetonitrile mixture, thus obtaining a 10 mg/mL solution. These solutions were filtered on 0.45 µm nylon filters before HPLC analysis.

Percolation: A comparison between "Tesi" 2 and "Tesi" 4, from the grape harvest of 2010, was carried out. Percolation was performed for each "Tesi" on 400 g of frozen seeds with a 70/30 ethanol/water (v/v) mixture, taking into account the humidity percentage of each "Tesi". A multi-step extraction was carried out, consisting in two extractions per day with a contact time of 3 hours plus 2 overnight extractions, with a total number of 6 extractions in 48 hours. At the end of the protocol the solvent was evaporated and a gummy dark violet extract was obtained as in the case of maceration. Percentage recoveries were calculated as described in the previous paragraph and the values obtained are reported in Table 3.

Purification with ethyl acetate: 500 mg of each ethanol extract were dissolved in 2.5 mL of distilled water and extracted five times with 2.5 mL of ethyl acetate previously saturated with water. The organic solutions were combined and evaporated to dryness. Each residue (20.0 mg) was then dissolved with a 1:1 mixture of 0.3% H_3PO_4/Acetonitrile, obtaining a 4 mg/mL solution. These solutions were filtered on 0.45 µm nylon filters before HPLC analysis.

Purification with ethyl acetate after salting: Extracts (500 mg) were dissolved in 2.5 mL of distilled water and 420 mg of NaCl was added; the mixture was extracted five times with 2.5 mL of ethyl acetate previously saturated with water. The organic solutions were combined and evaporated to dryness. Each residue (20.0 mg) was then dissolved with a 1:1 mixture of 0.3% H_3PO_4/Acetonitrile, obtaining a 4 mg/mL solution. These solutions were filtered on 0.45 µm nylon filters before HPLC analysis.

Purification with adsorbent resins: The resin (SEPABEADS® SP-207, Resindion, Milan, Italy) was suspended in absolute ethanol overnight. The extracts (10 g) were dissolved in 75 mL of water and filtered on cotton before loading into the column containing 100 mL of the activated adsorbent resin. The resin was abundantly washed with water in order to eliminate interfering substances. Potential loss of phenolic compounds was monitored by UV/VIS spectrophotometry between 200 and 700 nm and TLC; the washing water collected in four different fractions was analysed and no absorption was detected in the selected wavelengths. Finally, the analytes of interest, i.e. anthocyanins, were desorbed from the resin by dripping into the column about 500 mL of ethanol 95% with 0.01% of citric acid. The elution solvent was fractionated into two parts and analysed by spectrophotometry and TLC to control the complete elution of the analytes from the column.

Determination of water content by Karl Fisher titration

Analyses were performed by a volumetric titrator Karl-Fisher

	Gallic acid		(+)-Catechin		(-)-Epicatechin		Procyanidin B1		Procyanidin B2		(-)-Epicatechin gallate	
Analisi	**t_R (min)**	**Area (mAU)**	**t_R (min)**	**Area (mAU)**	**t_R (min)**	**Area (mAU)**	**t_R (min)**	**Area (mAU)**	**t_R (min)**	**Area (mAU)**	**t_R (min)**	**Area (mAU)**
1	4.27	6654.7	13.17	11364.9	19-25	11728.6	9.23	2939.3	16.32	4678.9	38.21	15276.9
2	4.24	5215.8	13.25	10810.2	19.31	11891.4	9.07	2520.3	16.53	4530.2	38.34	15273.7
3	4.24	5004.6	13.36	11192.5	19.41	11818.9	9.41	2315.7	16.51	4149.9	38.40	15061.1
4	4.24	4945.6	13.55	10796.4	19.65	11821.6	9.36	2934.3	16.24	4914.6	37.28	13915.2
5	4.24	4972.6	13.36	10755.8	19.81	11927.7	9.04	2615.9	16.72	3743	38.66	14200.7
Mean	4.246	5358.66	13.338	10983.96	19.486	11837.64	9.222	2665.1	16.46	4403.3	19.48	14745.5
St.dev.	0.01	732.32	0.14	276.60	0.24	76.65	0.17	270.70	0.19	461.98	0.53	641.71
%CV	**0.32**	**13.67**	**1.07**	**2.52**	**1.22**	**0.65**	**1.80**	**10.16**	**1.15**	**10.49**	**2.71**	**4.35**

Table 1: Reproducibility of gallic acid, (+)-catechin, (-)-epicatechin, procyanidin B1, B2 and (-)-epicatechin gallate

Sample	**% Water content**	**Maceration Total %Recovery**		**Percolation Total %Recovery**			
		Simple	**LLE**	**Simple**	**LLE**	**LLE salting**	**Resin**
Tesi 2 (2010)	38.68	**24.48**	**8.08**	**16.74**	**5.05**	**4.02**	**16.88**
Tesi 4 (2010)	44.30	**13.64**	**9.72**	**10.51**	**4.76**	**3.36**	**17.47**
Tesi 2 (2012)	60.31	**26.91**	**10.68**				

Table 2: % Recovery of the extracts calculated on the dry weight before and after purification; LLE = liquid-liquid extraction with ethyl acetate.

Tesi	**Purification**	**%H_2O**	**Ethanol**		**Ethyl acetate**	
			Ppm Residual solvent	**% Residual solvent**	**ppm Residual solvent**	**% Residual solvent**
2	resin	13.1	12558,66	1.26		
	LLE	11.9			33238.43	3.32
	LE salting	14.9			39754.27	3.98
4	resin	15.1	308.97	0.03		
	LLE	13.5			4772.91	0.48
	LLE salting	12.8			677.63	0.07

Table 3: Amount of water and residual solvents in the purified grape extracts.

V20 (Mettler Toledo AG – Analytical; Switzerland) as described in the chapter 2.5.12 of the European Pharmacopoeia (method A). The extracts and Leucoselect® (5 mg) were dissolved in 1.5 mL of methanol and titrated with Hydranal® – Composite 5, methanol-free titrating agent (Fluka, Italy).

Determination of residual solvents by HS/GC

Apparatus and conditions: Analyses were carried out on an Ultra Thermo Electron Trace GC (Thermo Fisher, Waltham, MA, United States) with a split-splitless injection system and a HS 2000 Thermo Electron auto-sampler, coupled with a FID detector. The system was managed by a Thermo Electron Chrom Card 2.3 software. The GC was equipped with a "VF-624 ms" capillary column (30 m x 0,32 mm I.D., thickness 1.8 µm). The GC-FID system was operated under the following conditions: 50°C (10 min) – 250°C, 10°C/min; final isotherm 3 min. Temperatures: inlet 180°C; detector 250°C; split flow 30 mL/min; split ratio 15; carrier constant flow 1.3 mL/min (helium); detector gas flow: hydrogen 35 mL/min, air: 350 mL/min; make-up: 30 mL/min; signal range: 1. HS auto-sampler: syringe temperature: 100°C; incubation temperature: 100°C with alternate stirring; incubation time: 60 min; injection volume: 0.5 mL.

Preparation of standard solutions and samples: Standard solutions of propanol (IS), ethanol and ethyl acetate (200 µg/mL; 200 ppm) were prepared in DMSO and working standards samples were obtained mixing 1 mL of the standard solution of ethanol and 1 mL of the standard solution of propanol (IS). Blank samples were prepared mixing 1 mL DMSO with 1 mL IS standard solution. Samples were obtained dissolving 10.00 mg of purified extracts in 1 mL of DMSO, adding 1 mL of IS standard solution.

Calculation of the concentration of residual solvents: The calculation of the concentration of residual solvents was carried out determining the Response Ratio RR as follows.

Three standards solutions were analysed to determine RA_{std} defined as:

$$RA_{std} = A_{std}/A_{IS}$$

Where A_{std} is the area of peak of the standard solution; A_{IS} is the area of the peak of the internal standard. Then for each solution the response ratio RR was calculated as:

$$RR = RA_{std} / C_{std}$$

Where C_{std} is the concentration of the standard solution.

The RR_{mean} was then determined as the mean of the three RR obtained for the single standard solutions.

The concentration of residual solvents was expressed in ppm. The calculation was performed applying the following formula:

$$ppm = RA_{sample}/RR_{mean}$$

where RA_{sample} = ratio between the area of the peak corresponding to the solvent in the sample and the area of the peak corresponding to the IS; RR_{mean} = mean response ratio calculated from the analysis of

three working standards.

LC analyses

LC analyses were performed on a Varian™ Pro Star equipped with an auto-sampler mod. 410, two pumps mod. 210 and detector DAD mod. 335. The instrument was controlled by Software Galaxie.

Analyses of the grape extracts were carried out under conditions similar to those employed by Gabetta et al. [18] optimized for improving reproducibility.

Chromatographic column: Zorbax SB C18 250 x 4.6 mm i.d. particle size 5 µm (Agilent Technologies™); pre-column: Security Guard Cartridges C18 4 x 2.0 mm (Phenomenex™); column temperature: R.T.; detection wavelength: 278 nm; flow rate: 1.0 mL/min; Injection volume: 10 µL; solvent A: 0.3% H_3PO_4 in water; Solvent B: acetonitrile; mobile phase: solvents were filtered under vacuum on 0.45 µm membrane filters and degassed by immersion in ultrasonic bath for 15 minutes before column conditioning; linear gradients: 0-45 min, 10-20% B; 45-65 min, 20-60% B; 65-68 min 60% B; 68-69 min 60-10%B; 69-85 min, 10 % B.

All analyses were carried out in triplicate. The phenolic compounds in the samples were identified according to their elution order, comparing their retention times and spectroscopic spectra with those of the pure commercial standards and by means of sum tests.

Reproducibility of the LC analytical method: Due to the complexity of the vegetable matrix (Figure 1) reproducibility was assessed evaluating the areas and retention times (t_R) obtained analysing (+)-catechin, (-)-epicatechin, gallic acid, procyanidin B1, B2 and (-)-epicatechin gallate standard solutions five consecutive times in the same day (Figure 2). Reproducibility was expressed as %CV. The %CV calculated on the areas of the standards of the active principles and on the retention times show that the method is endowed with an adequate reproducibility (Table 1).

Linearity of the response of catechin: To evaluate the linearity of the analytical method (+)-catechin was taking into account in a range of concentrations from 0.1 mg/mL and 0.03 mg/mL. In this range six solutions of non-sequential concentrations (0.1, 0.07, 0.06, 0.05, 0.04, 0.03 mg/mL) were analysed (n=5). The linearity equation was y = 43258x + 214.99 with a good correlation coefficient (R^2 = 0.9962).

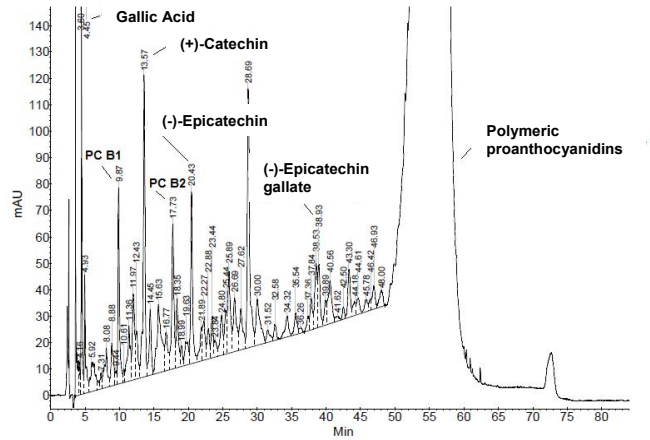

Figure 1: HPLC-DAD chromatogram of the crude extract obtained from Tesi 2 (2010) by maceration.

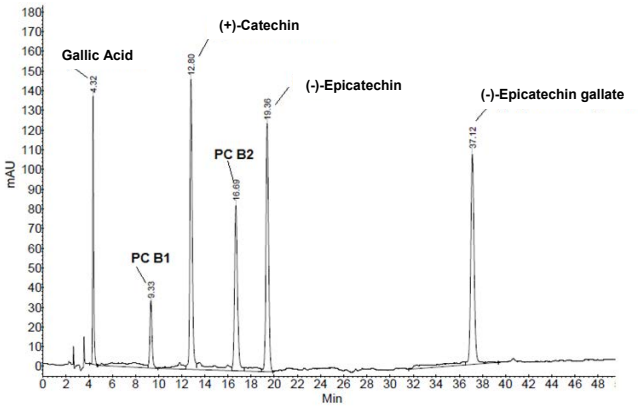

Figure 2: HPLC-DAD Chromatogram of the standard solution (100 µL Gallic Acid 0.025 mg/mL; 100 µL (+)-Catechin 0.25 mg/mL; 100 µL (-)-Epicatechin 0.25 mg/mL; 100 µL PCB1 0.09 mg/mL; 100 µL PCB2 0.09 mg/mL; 100 µL di (-)-Epicatechina gallato 0.1 mg/mL).

Quantification of the analytes

RR_{mean} (see 2.5.3) for catechin was calculated as the mean of three determinations of RR obtained analyzing three standard solutions of catechin (0.25 mg/mL) taking into account the purity (98%) of catechin.

The % title of the analytes was calculated respect to catechin according to the following formula:

$$\% T_{analyte} = [(A_{analyte} \times V_{sample}) / (RR_{mean} \times W_{sample})] \times 100$$

Where $A_{analyte}$ is the area of the analyte peak, V_{sample} is the volume of the sample solution (mL) and W_{sample} is the weight of the sample (mg). The title of the purified extracts was expressed as the sum of the title in catechin and epicatechin referred to the dry and solvent free preparation as follows:

$$\% T_{tot\ dry} = \% (\%T_{cat} + \%T_{epi}) \times [100 / (100 - \%H_2O - \%SR)]$$

Where % SR is the total amount of residual solvents determined by HS/GC and $\%H_2O$ is water content determined by Karl Fisher titration.

Gel Permeation Chromatography (GPC)

GPC analyses were carried out as described by Gabetta et al. [18] at room temperature on a PL Gel 5, particle size 5 µm, pore type 500 Å column (Agilent PL1110-6525) linked to a pre-column 0,5 µm (Supelco). The separation was performed with an isocratic elution mode using THF and an aqueous solution of LiBr 12 x 10^{-3} M (95:5 V/V) at a constant flow of 1,0 mL/min. The signal was detected at 280 nm and the injection volume was 10 µL. The samples were prepared dissolving 10.00 mg of extract in 10 mL of mobile phase (1 mg/mL).

Results and Discussion

The evaluation of the mass recovery and the analysis of the extracts were carried out. "Uva di Troia canosina" grape seeds underwent two different types of extraction procedures: maceration and percolation. The latter gave larger quantities of extracts in a shorter period of time and for this reason it can be applicable industrially, as it is cheaper and less time consuming compared to maceration. The vegetable material was maintained intact thus avoiding the extraction of oils, fats, pectins and mucilage contained inside the seeds. Furthermore, at the end of the extraction it is possible to recover the exhausted drug to obtain grape seed oil exploiting this waste material of the wine industry in the best

possible way. The extraction protocol was optimized for maceration in our previous work [1], the same multi step procedure was applied to percolation, thus obtaining six cycles of extraction in two days. The vegetable material was not completely exhausted, but at the end of the protocol the amount of the active principles extracted is so low that it is not convenient to go on. Either maceration or percolation yielded semi-solid dark violet preparations.

The percentage recovery on the dry weight of the seeds is reported in Table 2.

The extraction yield decreases from "Tesi" 2 to "Tesi" 4 because the polyphenols are extracted by the must during the fermentation process. Comparing "Tesi" 2 obtained in 2010 with the harvest in 2012 we can see that the recovery is similar, although it is slightly higher in 2012. As far as percolation is concerned, the extraction yield results lower than maceration and this is due to the fact that at the end of the process a huge amount of solvent remains in the seeds and in the tubes of the system linked to the peristaltic pump. Moreover, as the extract is semi-solid, it is difficult to eliminate the solvent completely during evaporation. The sixth extraction has a low yield compared to the previous five, consequently we decided to stop the process at the fifth extraction.

In order to enrich and standardize the polyphenol composition of the "uva di Troia canosina " grape seed extracts and to reduce the high molecular weight polymers, different purification methodologies were applied. In this way gums, fats, oils and pectins which give to the extract a soft consistency, were eliminated, yielding a dry dark orange powder which was easier to handle and standardize. The crude extracts obtained by maceration were purified by means of a liquid-liquid extraction (LLE) with ethyl acetate (Figure 3), while in the case of percolation three different purification protocols were applied to the crude extracts: LLE with ethyl acetate (Figure4), LLE with ethyl acetate after salting (Figure 5) and purification with an adsorbent resin (Figure 6). In Table 2 the percentage recovery for the different procedures are reported.

It is evident that the yield after treatment with ethyl acetate, with or without salting, is considerably lower than the recovery obtained operating with SEPABEADS SP-207. The purification with ethyl acetate gave a higher yield in the case of maceration probably because with percolation the component of the extract soluble in water is eliminated. This fraction could be responsible for the higher weight of the extract obtained with maceration.

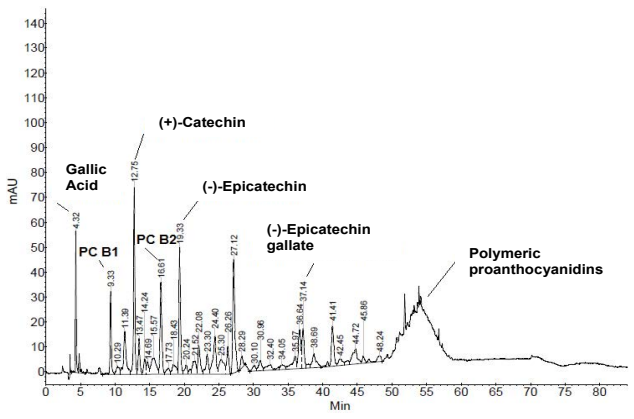

Figure 3: HPLC-DAD Chromatogram of the Tesi 2 (2010) extract purified with ethyl acetate obtained by maceration (0.5 mg/mL).

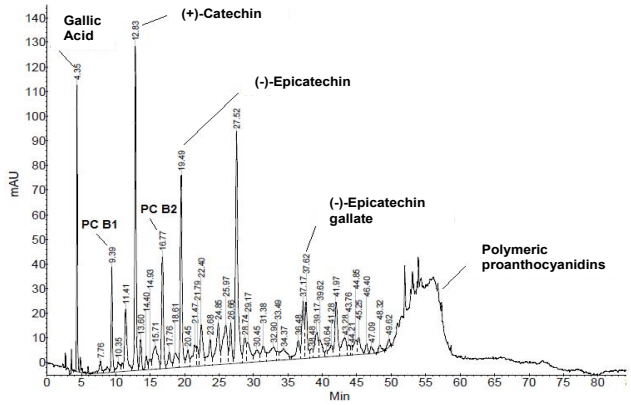

Figure 4: HPLC-DAD Chromatogram of the Tesi 2 (2010) extract purified with ethyl acetate obtained by percolation (0.5 mg/mL).

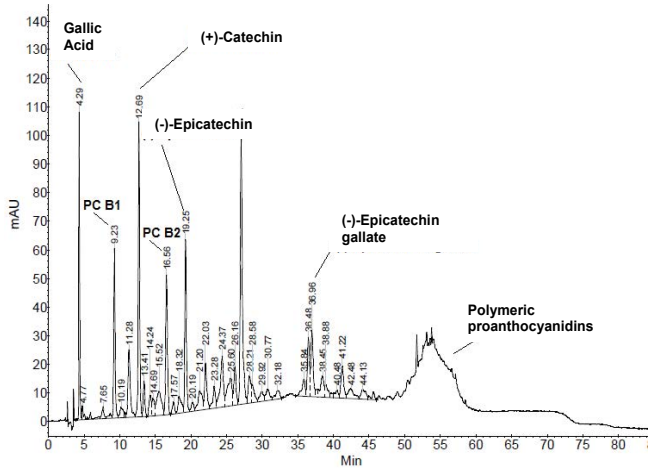

Figure 5: HPLC-DAD Chromatogram of the Tesi 2 (2010) extract purified with ethyl acetate and salting obtained by percolation (0.5 mg/mL).

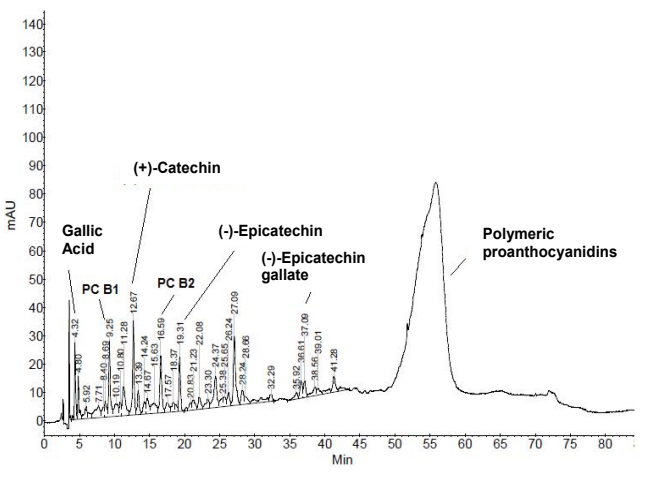

Figure 6: HPLC-DAD Chromatogram of the Tesi 2 (2010) extract purified with Sepabeads® SP-207
obtained by percolation (0.5 mg/mL).

The search for residual solvents in the purified extracts was carried out for two reasons: first of all we wanted to verify if the residual content of solvents was below the limits imposed by ICH rules, thus allowing us to calculate the percentage title of the extracts in terms of catechin and epicatechin referred to the dry preparation. The determination was performed on the extracts obtained by percolation and purified with different methodologies in order to compare these different protocols. The solvents taken into consideration were ethanol and ethyl acetate whose permitted day exposure (PDE) is 5000 ppm (0.5%). From Table 3 it is possible to notice that "Tesi" 2 extracts have a residual content of organic solvents which overcomes ICH limits, while in the case of "Tesi" 4 this does not happen. Ethanol is not present in the extracts purified by means of a LLE with ethyl acetate, even though it is used for percolation.

The total amount of water was calculated as the mean of three determinations, taking into account the water contained in the methanol used for the preparation of the extracts, which is different from that used by the instrument. The results are reported in Table 3. It is evident that there are not significant differences among the various preparations.

"Uva di Troia canosina" grape seeds extracts were compared with commercial extracts such as Leucoselect® (Indena SpA), Vitis Vinifera Extract 95% (seeds), Vitis Vinifera dry extract 95% (procyanidins) and Biovin seeds and vinasse dry extracts (Farmalabor). The results are reported in Table 4. In particular, Leucoselect® was used as reference for the purified extracts because it is a patented highly standardized preparation and as control of the efficiency of the chromatographic separation. In fact, it contains about 15% of catechin and epicatechin, 80% of epicatechin gallate and its dimers, trimers and tetramers and 5% of pentamers, hexamers and heptamers.

The percentage title (%T) of the analytes was calculated in comparison to catechin as described in paragraph 2.6.2; three different solutions of the analytes were analysed and the results were expressed as the mean of these determinations. The chromatographic method resulted adequate, as the %T calculated for Leucoselect® (15.14%) matched the result obtained by Gabetta et al. [18] (15-16%). This preparation has a significantly higher polyphenolic content than other commercial extracts.

From the data obtained in the analysis of the crude extracts, it is evident that the %T is higher in case of "Tesi" 2 respect to "Tesi" 4 in accordance with the fact that during fermentation polyphenols are extracted from the seeds to the must. "Tesi" 2 coming from the harvest in 2012 has higher polyphenolic content than the same "Tesi" of 2010. Moreover, the LC analysis confirmed the trends observed for the percentage recovery, indicating that this parameter is representative for polyphenolic content. From the comparison between the two extraction processes it is possible to notice that percolation gives better results, although comparable to those of maceration. The polyphenolic content of these crude extracts is dramatically lower compared with Leucoselect®.

The purification step produces an increase and an enrichment of the polyphenolic content, in fact %T of the purified extracts is significantly higher than the corresponding crude extracts.

In Table 5 the total content of catechin and epicatechin for the commercial, crude and purified extracts is reported.

The three purification protocols led to different results. The purification with the adsorbent resin gave the best results in terms of recovery but the extracts had a lower quality which was evident in the chromatographic profile in which the broad peak, due to the polymeric substances, was higher than the chromatograms obtained with the other purification protocols (Figures 3-6) [1].

Purification with ethyl acetate, with or without salting, allowed us to obtain extracts with high polyphenolic content. In particular, "Tesi" 2 extract purified with ethyl acetate had a %T comparable to that of Leucoselect®.

The purified extracts obtained by maceration gave results in terms of %T lower than those obtained by percolation, probably because in the latter process the component of the insoluble extract in water, which interferes with purification, is eliminated.

The salting of the extracts resulted the more problematic protocol from a practical point of view and it gave results comparable to the simple LLE extraction with ethyl acetate. For this reason salting was excluded. The purification with ethyl acetate led to the best quality, while the purification with the resin gave the highest recovery, thus these two purification methodologies yielded two different kind of products in terms of costs and commercial characteristics.

The profile of high molecular weight compounds in the purified extracts obtained by percolation, was carried out by means of gel permeation chromatography (GPC). The GPC analysis of Leucoselect® (black trace) [18] compared with "Tesi" 2 and 4 extract obtained by percolation and purified with the resin (Bordeaux trace) and "Tesi" 2 and 4 extract obtained by percolation and purified with ethyl acetate (blue trace) gave the profiles reported in Figures 7 and 8. The region labelled A refers to epicatechin; region B corresponds to epicatechin gallate, dimers and their gallates, while region C is representative of trimers, tetramers, pentamers, hexamers, heptamers and their gallates.

"Tesi" 2 extract purified with ethyl acetate has a molecular weight distribution comparable to that of Leucoselect®, even if it has a reduced polymeric portion. The fine structure is therefore different, because different kinds of seeds were used (Figure 9). "Tesi" 4 extract purified with ethyl acetate shows a profile which is superimposable with "Tesi" 2 extract purified in the same way, but the signal intensity is lower, confirming the decrease in polyphenolic content passing from "Tesi" 2 to "Tesi" 4, observed in the LC analysis and in the percentage recovery. The profiles of the extracts purified with the resin are shifted towards the higher molecular weight region: the monomer content is lower as shown by the LC analysis, confirming the different features which the two purification methods give to grape extracts.

Conclusion

The aim of this work was the valorization of waste products of wine industries, in particular of the kind of grape called "Uva di Troia canosina" with a small berry, that is a variety considered not so convenient from an oenological point of view, because it has a higher skin/pulp ratio than other varieties. Previous studies [1] demonstrated that this grape biotype is particularly rich in polyphenols, compounds endowed with several beneficial properties. For this reason, we carried out a complete quali-quantitative characterization of the polyphenols contained in the seeds of this kind of grape, optimizing the extraction and purification protocol of the extracts. Two different fractions of seeds sampled at different stages of the fermentation were compared: "Tesi" 2 when there is a spontaneous stratification of the seeds at the bottom of the recipient and "Tesi" 4 at the end of fermentation. From the quantitative analyses it was evident that "Tesi"2 has a higher polyphenolic content compared to "Tesi" 4, as expected since during vinification the must extracts polyphenols from the seeds. On the

			%T					
			(1)	(2)	(3)	(4)	(5)	(6)
Commercial extracts		Leucoselect	8.91	6.23	3.39	4.50	4.57	3.86
		Vitis Vinifera seeds	2.96	4.40	0.37	0.36	1.03	0.43
		Vitis Vinifera procyanidins	2.18	2.98	0.71	0.31	0.67	0.70
		Biovin	2.37	2.54	0.42	2.46	3.06	0.47
Tesi 2 2010	Maceration	crude	0.44	0.30	0.27	0.26	0.29	0.13
		LLE	4.55	3.13	2.12	1.65	2.38	1.85
		enrichment	**10.3**	**10.4**	**7.8**	**6.3**	**8.2**	**14.2**
	Percolation	crude	0.49	0.28	0.23	0.33	0.34	0.16
		LLE	8.62	5.70	3.37	2.50	3.78	2.49
		enrichment	**17.6**	**20.4**	**14.7**	**7.6**	**11.1**	**15.6**
		LLE salting	6.49	4.31	3.04	3.53	3.78	2.72
		enrichment	**13.2**	**15.4**	**13.2**	**10.7**	**11.1**	**17.0**
		resin	2.47	1.45	1.14	1.65	1.80	0.76
		enrichment	**5.0**	**5.2**	**5.0**	**5.0**	**5.3**	**4.8**
Tesi 4 2010	Maceration	crude	0.41	0.24	0.28	0.24	0.32	0.09
		LLE	3.35	1.99	2.74	1.36	1.91	2.01
		enrichment	**8.02**	**8.3**	**9.8**	**5.7**	**6.0**	**22.3**
	Percolation	crude	0.44	0.25	0.29	0.27	0.29	0.12
		LLE	5.56	3.37	4.13	1.63	2.50	1.38
		enrichment	**12.6**	**13.5**	**14.2**	**6.0**	**8.6**	**11.5**
		LLE salting	4.94	2.96	4.38	2.26	2.96	1.19
		enrichment	**11.2**	**11.8**	**15.1**	**8.4**	**10.2**	**9.9**
		resin	1.97	1.10	1.40	1.33	1.32	0.53
		enrichment	**4.5**	**4.4**	**4.8**	**4.9**	**4.6**	**4.4**
Tesi 2 2012	Maceration	crude	0.69	0.35	0.27	0.41	0.44	0.21
		LLE	6.32	3.57	2.11	1.68	2.65	2.12
		enrichment	**9.20**	**10.2**	**7.8**	**4.1**	**6.0**	**10.1**

Table 4: Content (% Title) of the active principles contained in the commercial, crude and purified grape extracts. catechin=(1); epicatechin=(2); gallic acid=(3); Procyanidin B1=(4); Procyanidin B2=(5); epicatechin gallate=(6).

			Extract	Catechin + epicatechin (%T)
Commercial extracts			Leucoselect	**15,14**
			Vitis Vinifera seeds	**7,36**
			Vitis Vinifera procyanidins	**5,16**
			Biovin	**4,91**
Crude extracts	maceration		"Tesi" 2 2010	**0.74**
			"Tesi" 4 2010	**0.65**
			"Tesi" 2 2012	**1.04**
	percolation		"Tesi" 2 2010	**0.77**
			"Tesi" 4 2010	**0.69**
Purified extracts	maceration	LLE AcOEt	"Tesi" 2 2010	**7.68**
			"Tesi" 4 2010	**5.34**
			"Tesi" 2 2012	**9.89**
	percolation	Resin	"Tesi" 2 2010	**3.92**
			"Tesi" 4 2010	**3.07**
		LLE AcOEt	"Tesi" 2 2010	**14.32**
			"Tesi" 4 2010	**8.92**
		LLE AcOEt salting	"Tesi" 2 2010	**10.80**
			"Tesi" 4 2010	**7.91**

Table 5: Content (% Title) catechin + epicatechin; of the commercial, crude and purified extracts

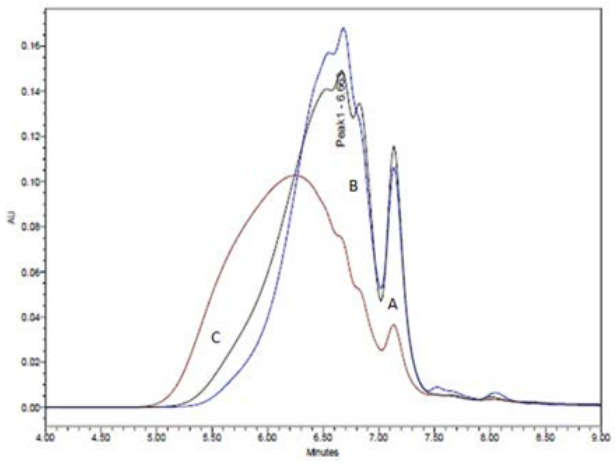

Figure 7: GPC profiles of Leucoselect® (black trace); "Tesi" 2 extract obtained by percolation and purified with the resin (Bordeaux trace) and "Tesi" 2 extract obtained by percolation and purified with ethyl acetate (blue trace).

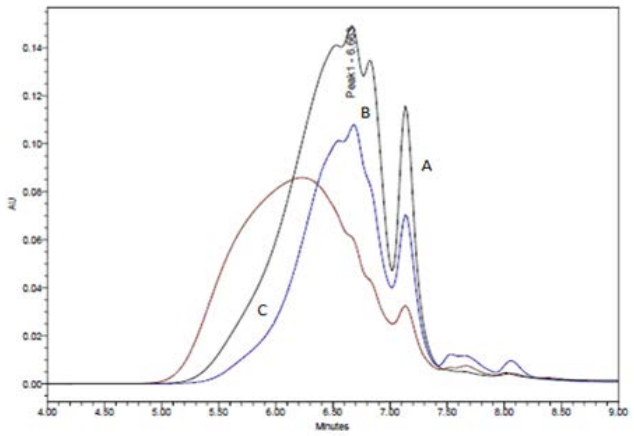

Figure 8: GPC profiles of Leucoselect® (black trace); "Tesi" 4 extract obtained by percolation and purified with the resin (Bordeaux trace) and "Tesi" 4 extract obtained by percolation and purified with ethyl acetate (blue trace).

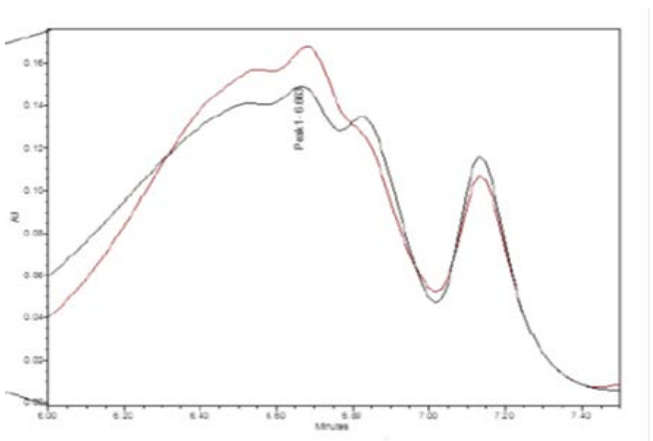

Figure 9: Comparison between Leucoselect® (Bordeaux trace) and "Tesi" 2 obtained by percolation and purified with ethyl acetate (black trace).

other hand, "Tesi" 4 is particularly convenient because it is very easy to obtain and very cheap, being a waste product. From an industrial point of view it could be more convenient to extract "Tesi" 4 seeds rather than "Tesi" 2 seeds. The purification process was carefully investigated in order to obtain the highest enrichment and a polyphenolic content comparable with commercial preparations sold on the market. In particular, "Tesi" 2 extract obtained by percolation and purified with a LLE extraction with ethyl acetate showed a polyphenolic content similar to Leucoselect®.

The residual content of organic solvents (ethanol and ethyl acetate) and water was determined on the grape extracts according to ICH rules by means respectively of HS/GC and Karl Fisher titration in order to meet the requirements for commercialization. Moreover, the high molecular weight polyphenolic fraction of our extracts was investigated by means of GPC and compared to Leucoselect®.

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