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Research Article

Methods for the Evaluation of Polyphenolic Content in "Uva Di Troia Canosina" Grape and Seeds at the Different Maceration Stages

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Seeds and skins derived from the small berries of "uva di Troia canosina" grape, a *Vitis vinifera* variety autochthonous of Apulia region (South Italy), collected at four different stages ("Tesi" 1–4) of the fermentation process were extracted by means of a maceration. The extracts were purified and analyzed, in order to study the influence of fermentation over grape seed and skin polyphenolic content. Seed extraction was performed by a multistep maceration with two solvents: ethanol and acetone, the former giving the best results; moreover, the extracts were purified with pure ethyl acetate in order to enrich their polyphenolic content. On the other hand, skin extraction was achieved by a single-step maceration in methanol and a purification with a brominated synthetic adsorbent resin. The evaluation of the extraction yield and polyphenolic content was carried out by TLC, UV/VIS, and LC/DAD analyses. In the seed extracts, the characteristic polyphenols (catechin, epicatechin, and procyanidin B1 and B2) useful for the development of a nutraceutical product, endowed with antioxidant properties, were present, while no resveratrol was detected in "uva di Troia canosina" grape skin extracts, even in an LC/MS-MS analysis.

1. Introduction

"Uva di Troia" is an autochthonous *Vitis Vinifera* grape variety native of Apulia, a region of southern Italy, and it can be classified into two main biotypes in relation to its berry size. The small berry biotype is called "uva di Troia canosina" because it takes its origins and is still cultivated in the area around the city of Canosa. Previous studies evidenced a high polyphenolic content in this grape variety and a great wine aging potential [1]. On the other hand, the cultivation of this grape biotype is going to be replaced by other varieties with a big berry because it is considered unproductive from the oenological point of view.

Phenolic compounds, including stilbenes (e.g., *trans*-Resveratrol), hydroxybenzoic and hydroxycinnamic acids, flavonols, flavan-3-ols (monomeric catechins, proanthocyanidins), and anthocyanidins, constitute a very important class of secondary metabolites ubiquitous in the plant kingdom, where they are synthesized to accomplish diverse biological and biochemical activities [2] and are responsible for many organoleptic characteristics of wine and grape. Their concentration and composition in grapes depends on the cultivar and so it is influenced by viticultural and environmental factors, such as climate conditions, maturity stage, and production area [3–6]. Polyphenols exhibit beneficial effects on human health thanks to the strong free radical scavenging and antioxidant activity, as well as cardioprotective, vasodilatatory, anticarcinogenic, anti-inflammatory, antiallergic, antibacterial, immune-stimulating, antiviral, and

estrogenic properties [7–9]. Therefore, in recent years the development of new dietary supplements based on catechins and proanthocyanidins has been deeply studied [10, 11].

The determination of polyphenols and the investigation of the factors affecting their composition using robust, sensitive, and reliable analytical methods are very important to characterize different grape varieties. Some common structures (catechin, proanthocyanidin, anthocyanins, etc.) have been generally identified and quantified in wines but other ones such as high molecular mass phenolics or new formed compounds during wine ageing still remain to study. Many different methods have been improved through years. General approaches have been developed allowing the determination of a global index (e.g., "total polyphenols") mainly achieved by spectrophotometric detection and are opposed to more specific analyses based on separation of the individual polyphenolic species typically by high-performance liquid chromatography or capillary electrophoresis. For a recent exhaustive review on the methods for detecting grape polyphenols, see [12].

Different extraction procedures and grape phenolic characterization methods have been widely investigated [13, 14] as well as the role of ripening and viticultural factors over polyphenolic composition and concentration [15].

Furthermore the role of the vinification process and storage in red wine phenolic composition was evaluated [16].

Methanol, ethanol, and acetone variously mixed with water represented the most common solvents used for polyphenol extraction from grape seeds and skins [17] while liquid chromatography equipped with a UV detector (LC/UV) constituted the analytic technique of choice [18].

However, to the best of our knowledge, there are no exhaustive studies concerning the "uva di Troia canosina" grape biotype and the direct influence of fermentation on its polyphenolic content.

Thus, the aim of this work was the evaluation of polyphenolic composition of seeds and skins of this kind of grape, depending on the fermentation process, in order to select the best fermentative, extraction, and purification conditions for the development of a new nutraceutical product based on polyphenol benefits, thus leading to the valorisation of a particular grape cultivar typical of Apulia region, of its territory and viticultural establishments.

2. Materials and Methods

2.1. Fruit Sampling. We extracted, purified, and analysed separately by thin layer chromatography (TLC), UV/VIS spectroscopy, and liquid chromatography equipped with a diode array detector (LC/DAD) four different fractions of grape seeds and skins, called "Tesi," collected at four different fermentation stages (from the beginning to complete fermentation).

"Uva di Troia canosina" grape was cultivated in Canosa (Apulia, South Italy) in 2009. The crop was immediately divided into 4 fractions, called "Tesi," which were subjected to 4 different stages of the fermentation processes.

"Tesi" 1 was obtained from a white vinification process; the berries were trodden, destalked, and crushed in order to separate the pomace from the must and the solid residues were collected.

"Tesi" 2 was obtained crushing, destemming, and pressing the berries into open fermentation tanks. Once we obtained a spontaneous separation of the various components, sampling of skins and seeds was carried out.

"Tesi" 3 was obtained adding specific yeasts to the must and pomace to achieve a better fermentation. Once an alcohol content of 5%-6% was reached, sampling of skins and seeds was carried out.

"Tesi" 4 was obtained through sampling seeds and skins at complete fermentation before wine racking.

All skins and seeds were washed, drained, and kept refrigerated at −20°C until use.

Before extraction, seeds and skins were manually separated and the seeds washed with distilled water to eliminate skin residues.

2.2. Chemicals and Reagents. 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, Kuromanin Chloride, Oenin Chloride, Cyanidin Chloride, Quercetin, Quercetin-3-β-D-glucoside, Myricetin, t-Resveratrol, and Malvidin, Polydatin, and t-Piceid 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 (H_2O) system by Millipore (Bedford, MA, USA).

2.3. Apparatus and Conditions

2.3.1. TLC. TLC analyses were performed on Silica gel plates 60 F_{254} 20 × 20 cm (Merck, Germany) with a Butyl Acetate/Ethanol/Formic Acid/Water (8 : 1 : 1 : 1) mixture as eluent. Also, 2 μL of each extract were applied on the plate. A solution made of 5% sulphuric acid in ethanol and 1% vanillin in ethanol mixed in a 1 : 1 ratio was chosen as colour reagent for spot detection. Consider

$$\mathrm{R}_f \text{ catechin} = 0.70; \qquad R_f \text{ epicatechin} = 0.66. \qquad (1)$$

2.3.2. UV/VIS. Analyses were carried out with a Cary 50 Scan (Varian).

Seeds. The progress of the extraction was evaluated on "Tesi" 1 seeds, by studying the polyphenol release during a continuous extraction in 70/30 ethanol/water at room temperature (23°C). Thus, 50 mL of solvent were added to 50 g of seeds and the mixture was stirred. Also, 50 μL of extract were taken off every 30 min, diluted to 5 mL with 70/30 ethanol/water,

and analyzed by UV-VIS spectrophotometry at 280 nm, using 70/30 ethanol/water as blank.

Skins. The total amount of anthocyanins in "uva di Troia canosina" skin extracts was determined by measuring the absorbance between 200 and 700 nm, and particularly around 537 nm, against a blank of 2% HCl in methanol. In order to quantify total anthocyanins, the maximum of absorbance was compared to malvidin specific absorbance (Table 5) [19].

2.3.3. LC Analyses. LC analyses were performed on a Varian Pro Star equipped with an autosampler 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. [20] for the analysis of the standard Leucoselect.

Chromatographic column: Zorbax SB C18 250 × 4.6 mm i.d. particle size 5 μm (Agilent Technologies); precolumn: SecurityGuard Cartridges C18 4 × 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; 65-66 min 60%–10% B; 66–85 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.

LC analyses of grape skin extracts were performed on a 1220 Infinity LC (Agilent Technologies) equipped with two chromatographic pumps, a manual injector with a 20 μL loop, and a UV/VIS detector. The instrument was controlled by Software ChemStation.

Grape skin extracts were analyzed in the following chromatographic conditions: chromatographic column: Zorbax SB C18 250 × 4.6 mm i.d. particle size 5 μm (Agilent Technologies); precolumn: SecurityGuard Cartridges C18 4 × 2.0 mm (Phenomenex); column temperature: R.T.; detection wavelength: 254 nm; flow rate: 0.7 mL/min; Injection volume: 20 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: 0–15 min, 2%–20% B; 15–35 min, 20%–30% B; 35–50 min 30%–45% B; 50–58 min 45%–60% B; 58–65 min, 60%–2% B. All analyses were carried out in triplicate. The phenolic compounds in the samples were identified according to their elution order and comparing their retention times with those of a mix of pure commercial standards, prepared as follows: 100 μL Kuromanin-Cl 0.1 mg/mL + 250 μL Oenin-Cl 0.025 mg/mL + 150 μL Cyanidin-Cl 0.1 mg/mL + 150 μL Quercetin 0.025 mg/mL + 100 μL Quercetin-3-β-D-glucoside 0.1 mg/mL + 100 μL Myricetin 0.1 mg/mL + 100 μL *t*-Resveratrol 0.1 mg/mL. Sum tests were performed to guarantee the exact nature of the peaks eluted.

Table 1: Loss on drying of "uva di Troia canosina" grape seeds.

Sample	Initial weight (g)	Final weight (g)	% H_2O
"Tesi" 1	25.0	16.2	35.2
"Tesi" 2	50.0	32.0	36.0
"Tesi" 3	50.0	28.9	42.2
"Tesi" 4	50.0	28.0	44.0

Moreover, 1 mL of the crude methanol extract, previously filtered on 0.45 μm filters, was evaporated to dryness under a stream of nitrogen at 40°C and recovered with 1 mL of a mixture 70/30 of Solvent A/Solvent B.

Experiments carried out with a liquid chromatography system coupled to a mass detector (LC/MS-MS) were performed on a Varian LC-320 with an electron spray ionization (ESI) source and a 320-MS triple quadrupole mass spectrometer, equipped with two Varian 212 LC chromatographic pumps and a Varian 410 tray cooled autosampler. The system was managed by Varian MS Workstation software (Version 6.9.1). The ESI-triple quadrupole mass spectrometer was set to perform collision induced dissociation experiments in positive ionization mode, using argon as collision gas. Particularly, multiple reaction monitoring (MRM) experiments were conducted by the continuous injection at a rate of 20 μL/min of the standards of interest (10 μg/mL) into the mass spectrometer set in positive ionization mode, in either aqueous or methanolic solutions. In order to enhance ion formation and to improve conductivity, the solvent for electrospray ionization was prepared by mixing formate buffer 3 mM pH 3 with 0.1% formic acid in acetonitrile. The method was optimized for the research of the ions of interest, for example, the specific MRM transitions of each standard. In detail, once recognized the molecular ion of each standard $(M + H)^+$, MRM experiments were performed to study the characteristic fragmentation pattern of the analytes Kuromanin chloride, Cyanidin chloride, Oenin chloride, Quercetin, Myricetin, and *t*-Resveratrol. ESI source settings and mass spectrometer parameters used for compound identification were Needle Voltage: +5000 V; Shield Voltage: +600 V; Nebulizing Gas (N_2) Pressure: 40.00 psi; Drying Gas (N_2) Pressure: 15.00 psi; Drying Gas (N_2) Temperature: 400°C; Q_0 Offset: +3.261 V; L_4 Offset: +2.000 V; Housing Temperature: 50°C; CID Gas (Ar); Pressure: 2.00 mTorr; Electron multiplier: 1650.0 V; Scan time: 4.0 sec; Dwell time: 0.1 sec.

2.4. Seed Extract Preparation

Loss on Drying. Before starting the extraction procedure, the humidity percentage of each "Tesi" was evaluated, by drying 50 g of seeds at 60°C for 5 days. The calculated humidity is reported in Table 1.

Extraction. Ethanol or acetone was added to 50 g of frozen seeds 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

Table 2: Seed extraction yield.

Sample	Solvent (v/v)	Initial weight (g)	Final weight	% REC
"Tesi" 1	EtOH 70%	25.0093	2.941	18.15
	Acetone 70%	25.0032	3.508	21.65
"Tesi" 2	EtOH 70%	25.0009	2.667	16.67
	Acetone 70%	25.0051	2.969	18.55
"Tesi" 3	EtOH 70%	50.0210	3.838	13.27
	Acetone 70%	50.0072	4.297	14.87
"Tesi" 4	EtOH 70%	50.0160	2.994	10.69
	Acetone 70%	50.0210	3.530	12.60

Table 3: Loss on drying of "uva di Troia canosina" grape skins.

Sample	Initial weight (g)	Final weight (g)	% H_2O
"Tesi" 1	5.367	1.492	72.20
"Tesi" 2	5.715	1.536	73.12
"Tesi" 3	5.068	1.504	70.32
"Tesi" 4	5.019	1.413	71.85

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 3 h 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 $W_{fin}/W_{init} \times 100$, where W_{fin} is the final weight and the initial weight W_{init} considers the intrinsic water content of seeds. Percentages of recoveries are reported in Table 2.

Also, 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.

Purification. Five hundred milligrams of each ethanol extract were solved 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. Furthermore, 20.0 mg of each residue were 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.

2.5. Skin Extract Preparation

Loss on Drying. Five grams of grape skins were desiccated in oven at 100°C and weighted, once cooled in the dry atmosphere of a desiccator, twice a day during 5 days until a constant weight was reached. The calculated humidity is reported in Table 3.

Table 4: Skin extraction yield (maceration in methanol).

Sample	Initial weight (g)	Final weight	% REC
"Tesi" 1	10.033	2.221	22.14
"Tesi" 2	10.035	1.586	15.80
"Tesi" 3	10.029	0.579	5.77
"Tesi" 4	10.010	0.490	4.90

Extraction. Frozen skins of "Tesi" 1, 2, 3, and 4 were independently subjected to the following extraction protocol; separated frozen skins were milled with an electronic grinder; 100.0 g of the drug were accurately weighted and suspended in a beaker, protected from light and air exposure by aluminium foils, with about 250 mL of methanol; once reaching the room temperature, extraction was carried out for 45 min and the extract was filtered under vacuum before further analyses.

The recovery percentage (%REC) of each extract was calculated as $W_{fin}/W_{init} \times 100$, where the initial weight W_{init} considers the intrinsic water content of seeds. Percentages of recoveries are reported in Table 4.

One milliliter of each crude methanolic grape skin extract, previously filtered on 0.45 μm nylon filters, was evaporated to dryness under a stream of nitrogen at 40°C and recovered with 1 mL of a 70/30 mixture of 0.3% H_3PO_4 in water and acetonitrile. The solutions thus obtained were directly injected in the LC instrument.

Purification. The resin was suspended in absolute ethanol overnight and was employed on "Tesi" 1 grape skin extract. 16.016 g of extract were solved in 50 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. Eventual loss of phenolic compounds was monitored analyzing by UV/VIS spectrophotometry between 200 and 700 nm the washing water collected in four different fractions; no absorption was detected in the selected wavelengths. Finally, the analytes of interest, that is, anthocyanins, were desorbed from the resin by dripping into the column about 500 mL of EtOH 95% with 0.01% of citric acid. The elution solvent was fractionated into two parts and analyzed by spectrophotometry to control the complete elution of the analytes from the column.

3. Results and Discussion

The first step was the optimization of grape seeds extraction conditions. First of all the loss on drying was determined (Table 1).

Then, the ideal contact time between the seeds and the specific organic solvent was achieved by maceration under magnetic stirring, thanks to spectrophotometric analyses, sampling the extraction solvent every thirty minutes from the beginning of the extraction. Acetone did not represent

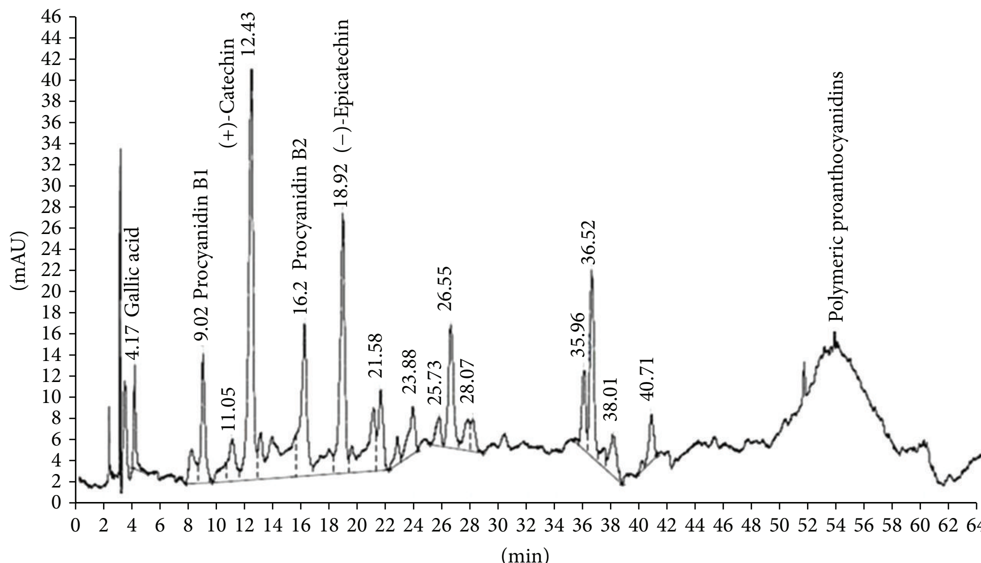

Figure 1: LC/DAD profile of Leucoselect 1 mg/mL.

a suitable solvent for the evaluation of contact time, since it interfered in the UV absorption, and consequently a mixture of 70/30 ethanol/water was chosen.

The progress of the extraction process was performed on "Tesi" 1 seeds at 280 nm, using 70/30 ethanol/water as blank. The polyphenol absorbance increased constantly with the extraction time, without reaching the saturation of the extraction medium during 24 h of maceration. Thus, in order to obtain the best phenolic recovering as possible, a multistep extraction was performed, 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. This protocol was subsequently applied to the seeds coming from each of the four "Tesi" of "uva di Troia canosina" grape with two different solvent mixtures: 70/30 ethanol/water and 70/30 acetone/water. The results showed how the % recovery decremented from "Tesi" 1 to "Tesi" 4 seeds. Even if these values are not absolutely related to the phenolic compounds present in our samples, it could be assumed that the % recovery represents a valid marker of the phenolic content in the vegetable drug. Evidently the vinification process leads to the extraction of phenolic compounds from the grapes to the must, thus depleting seed and skin polyphenols. The mixture acetone/water seemed to be the best solvent for seed extraction (Table 2).

However, the little difference of the recovery values could not demonstrate a difference in the phenolic extraction between the two solvents. This aspect was further investigated by chromatographic analyses.

Grape seed extracts were analyzed by thin layer chromatography (TLC) in order to control the extractive process and to have qualitative information about the phenolic content of the extracts. Samples were compared to reference standards of catechin and epicatechin and to commercialized products derived from the extraction of different grape seeds biotypes: Leucoselect and Vitis vinifera 95% extract. Grape seed polyphenols were well extracted with the protocol developed. Particularly, we could identify the presence of the monomers catechin and epicatechin. Furthermore, TLC separation gave us an idea of the differences in the phenolic content between the various "uva di Troia canosina" "Tesi." In fact, it was pretty clear that the color intensity of the spots decreased from "Tesi" 1 to "Tesi" 4 extracts, confirming the UV/VIS analysis and the recovery values. Moreover, a higher polyphenolic content in the ethanol extracts with respect to the acetone ones was evidenced.

The next step for the characterization of grape seed extracts was based on liquid chromatography (LC) analyses. Following the chromatographic conditions described in the literature [20] for Leucoselect (Figure 1), it was possible to detect and separate gallic acid, procyanidin B1, (+)-catechin, procyanidin B2, and (−)-epicatechin in all the extracts. The LC/DAD profile revealed that low molecular weight constituents, such as monomers and dimers, were well separated, while polymeric proanthocyanidins coeluted as a broad peak at approximately 60 minutes, confirming what is reported in the literature [12]. The same profile was observed in the analysis of Leucoselect, even if the polymeric procyanidins (PCs) in our extracts were definitely more abundant.

The qualitative comparison between the LC profiles obtained with the two solvent, demonstrated that ethanol better extracted the analytes in all the four "Tesi." Therefore, we could deduce that acetone represented a less selective solvent. For this reason ethanol was chosen as the solvent for the extraction of grape seeds.

The huge amount of high polymeric components strongly interfered with the elution of the low molecular weight constituents.

Thus, in order to purify grape seed extracts, a liquid-liquid extraction (LLE) using water presaturated ethyl acetate as organic solvent for the removal of interfering species, such as lipids, proteins, sugars, and highly polymerized

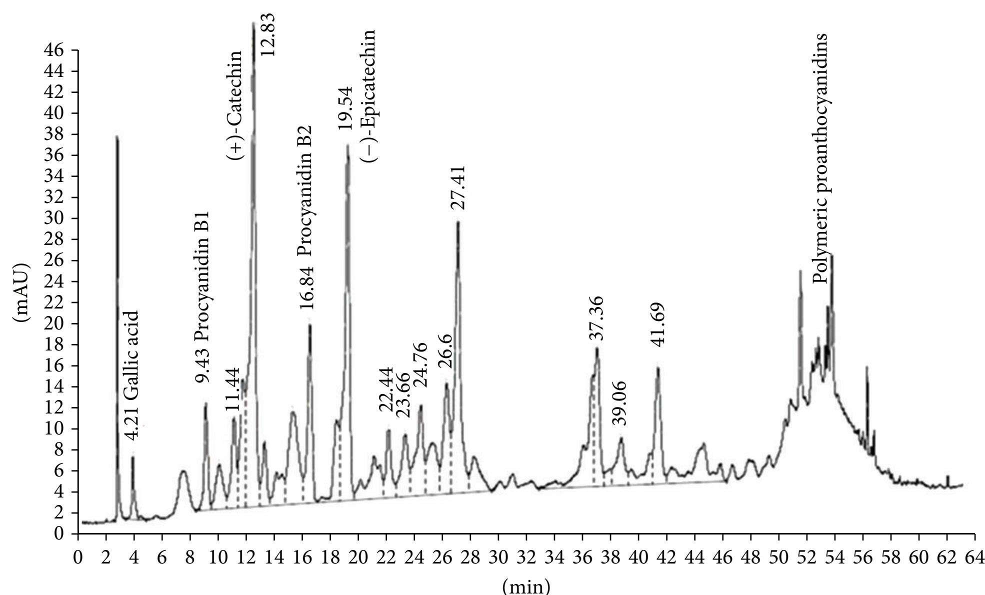

Figure 2: LC/DAD profile of "Tesi" 2 purified seed extract.

compounds, was performed. The purification strongly reduced the content of polymeric proanthocyanidins in our extracts (Figure 2) and a chromatographic profile similar to that of Leucoselect was obtained.

The second purpose of our research was to define the phenolic composition of "uva di Troia canosina" skins, focusing our attention on the characteristic polyphenols such as anthocyanins (anthocyanidins and their glycosides), flavonols, and, especially, resveratrol.

Loss on drying of grape skins was determined (Table 3).

Grape skins extraction was achieved by a one-step maceration with methanol under magnetic stirring. Methanol was chosen in order to maximize the recovery of polyphenols, thanks to its high extractive potential [21, 22].

The extraction method (45 min, room temperature) was performed considering that the degradation rate of anthocyanins is time and temperature dependent. The extraction yields obtained are reported in Table 4.

The total amount of anthocyanins was determined by means of UV/VIS analyses (Table 5) and LC analyses, confirming the decrease in polyphenolic content from "Tesi" 1 to "Tesi" 4.

The presence of Oenin chloride and Quercetin-3-β-D-glucoside in each "Tesi" and of Quercetin in "Tesi" 2, 3, and 4 was attested, even if at the very low concentrations (Figure 3).

The extracts obtained from "uva di Troia canosina" skins had a soft consistency, probably due to the extraction of interfering species. Thus, a purification method based on the use of adsorbent resins was investigated (see Section 2.5). After the purification protocol, we noticed that the consistency of the purified extracts was completely different, appearing as fine violet powders, instead of semisolid dark violet gums. Therefore, the purification method tested provided a feasible procedure for the production of purified grape skin extracts with better handling characteristics.

Table 5: Concentration of anthocyans in grape skin extracts with respect to malvidin at 537 nm.

Sample	Mean Abs	Initial weight (g)	% concentration
"Tesi" 1	0.250	10.033	0.247
"Tesi" 2	0.117	10.035	0.115
"Tesi" 3	0.109	10.029	0.108
"Tesi" 4	0.087	10.010	0.086

Due to its strong antioxidant properties, which exert beneficial effects on human health, and to its previous detection in "uva di Troia" berries [2], the absence of t-resveratrol in our grape skin samples was immediately noticed, even in sum tests (Figure 4).

Consequently, we decided to further investigate this important phenolic compound. First of all, resveratrol can also be encountered in the 3-glycosylated form, which is referred to as polydatin or t-piceid, which could be present in quantities comparable or even superior to free resveratrol in grape berries and wines. So, a standard of t-piceid was analyzed but it could not be detected in our skin extracts. Thus, LC/MS-MS [14] technique was applied; the fragmentation pattern of selected polyphenol standards, representative of those present in grape skins, was preliminary characterized. Finally, in order to gain information on "uva di Troia canosina" grape phenolic content, skin crude and purified extracts were directly injected in the ESI source without performing any chromatographic separation. The identification of each analyte in the extract was based on the presence of all its specific MRM transitions. Figures 5 and 6 illustrate the positive matches between standard characteristic MRM transitions, that is, Oenin chloride and Quercetin and "Tesi" 2 grape skin crude extract.

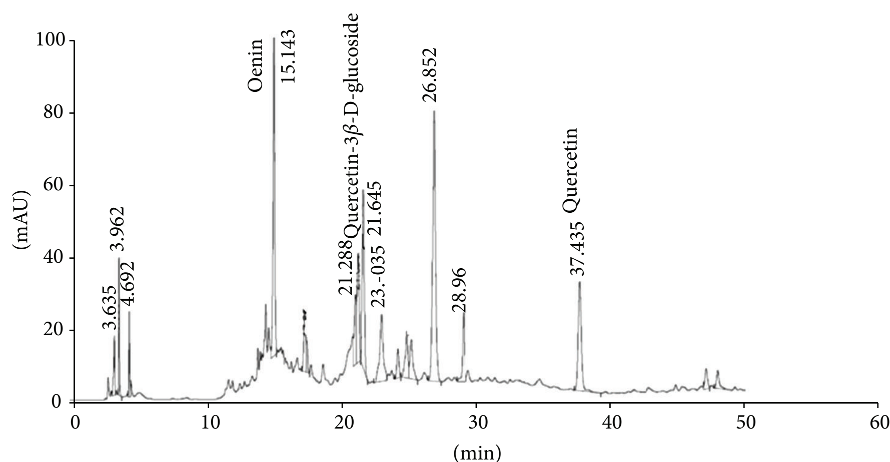

Figure 3: LC/DAD profile of "Tesi" 2 purified skin extract.

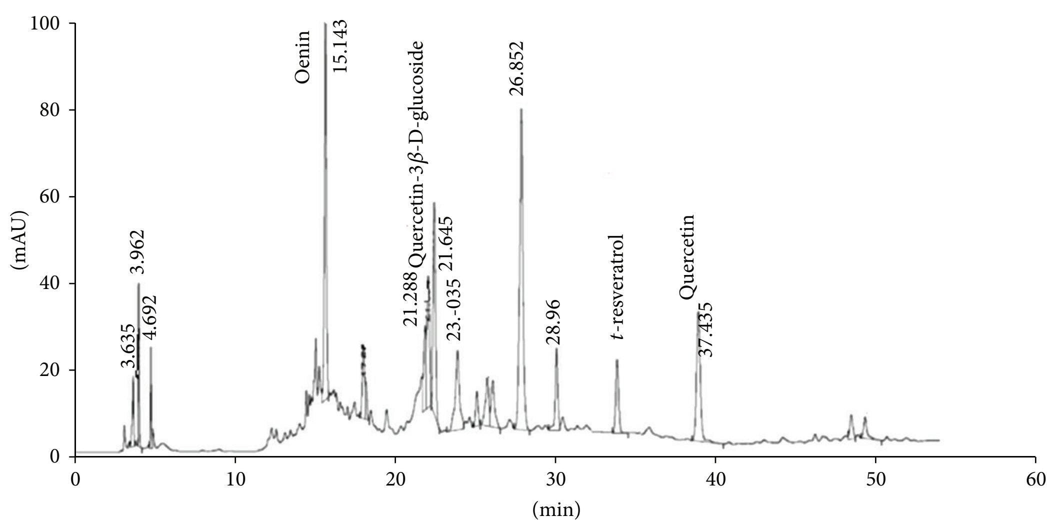

Figure 4: LC/DAD profile of "Tesi" 2 purified skin extract + resveratrol.

On the other hand, Figure 7 shows the mismatch between some MRM transitions relative to *t*-resveratrol and "Tesi" 2 grape skin crude extract.

Thus, considering also the scarce resolution and the low intensity on the peaks, we could confirm the absence of such important phenolic compound in our grape skin extracts. The extraction and characterization of skins involved many analytical problems, such as a complicated storage of frozen samples and a difficult chromatographic separation of the analytes. For these reasons and for the absence of resveratrol, in order to develop a new nutritional supplement, we decided to focus our attention on the grape seeds.

From the data obtained, we could also observe that the phenolic content of grape seeds decreased from "Tesi" 1 to "Tesi" 4. Thus to obtain a nutraceutical product based on grape polyphenol benefits, preserving the concomitant production of wine and leading to the total exploitation of the cultivar, among the "Tesi" investigated, "Tesi" 2 represented the most suitable fraction to achieve this purpose.

Another possibility from the industrial point of view should be the exploitation of "Tesi" 4, whose extracts were the less abundant in polyphenolic content, but which is particularly easy to obtain and cheap because it is a waste product in the vinification process.

4. Conclusion

The polyphenolic composition of seeds and skins of "uva di Troia canosina" grape, a variety autochthonous of Apulia region in Southern Italy, was studied depending on the fermentation process, in order to select the best fermentative, extraction, and purification conditions for the development of a new nutraceutical product based on polyphenol benefits, thus leading to the valorisation of a particular grape cultivar, of its territory and viticultural establishments. To achieve this goal, seeds and skins derived from the small berries of this kind of grape, collected at four different stages of the fermentation process, were purified and analysed separately by TLC, UV/VIS, and LC/DAD.

Thus, in order to obtain the best phenolic recovering as possible, a multistep extraction was performed on grape seeds, consisting in two extractions per day with a contact

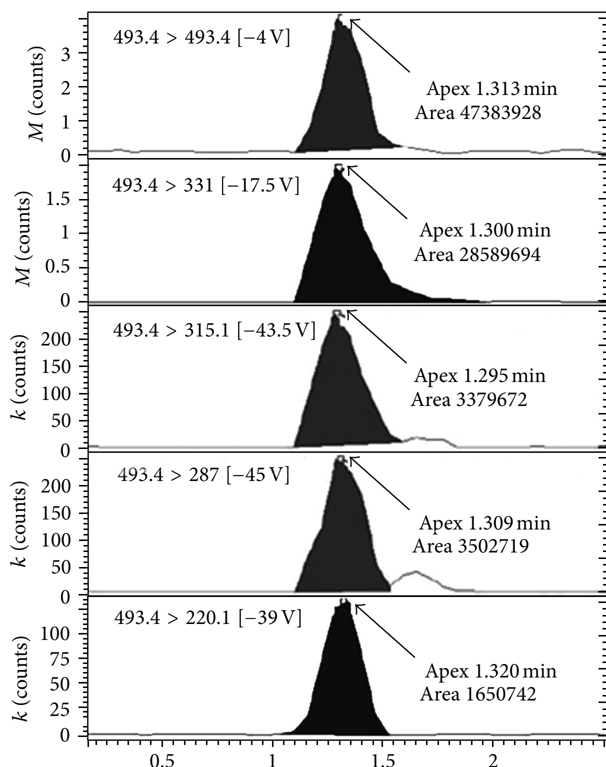

Figure 5: LC-MS/MS analysis of "Tesi" 2 grape skin extract: positive match with oenin fragmentation patterns.

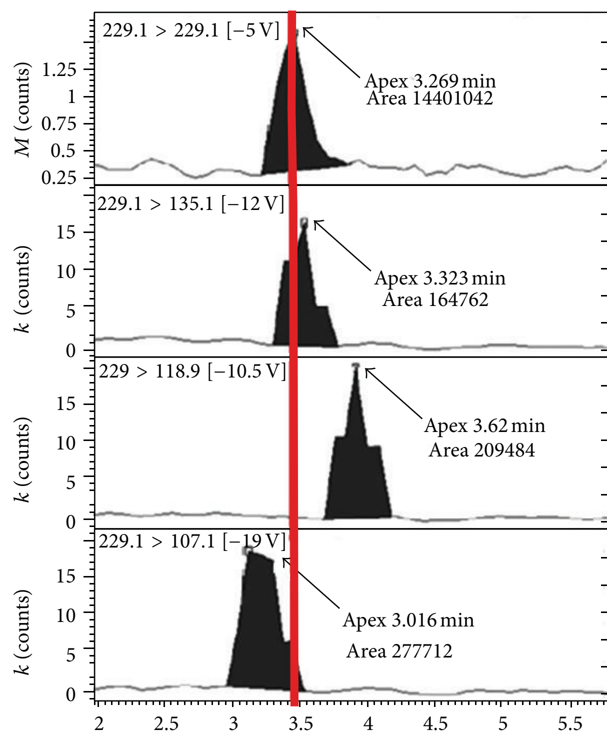

Figure 7: LC-MS/MS analysis of "Tesi" 2 grape skin extract: mismatch with t-resveratrol fragmentation patterns.

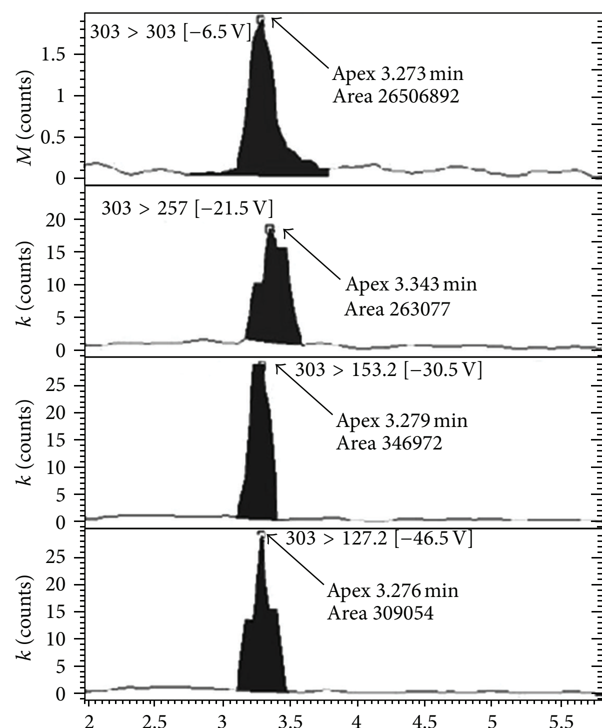

Figure 6: LC-MS/MS analysis of "Tesi" 2 grape skin extract: positive match with quercetin fragmentation pattern.

time of 3 hours plus 2 overnight extractions, with a total number of 6 extractions in 48 hours. The results showed how the % recovery decremented from "Tesi" 1 to "Tesi" 4 seeds. Evidently the vinification process leads to the extraction of phenolic compounds from the grapes to the must, thus depleting seed and skin polyphenols. The mixture acetone/water seemed to be the best solvent for seed extraction. Grape seed extracts were analyzed by thin layer chromatography (TLC) in which we could identify the presence of the monomers catechin and epicatechin. Moreover, a higher polyphenolic content in the ethanol extracts with respect to the acetone ones was evidenced. In the LC analyses, it was possible to detect and separate gallic acid, procyanidin B1, (+)-catechin, procyanidin B2, and (−)-epicatechin in all the extracts, polyphenols useful for the development of a nutraceutical product, endowed with antioxidant properties.

The same profile was observed in the analysis of Leucoselect, even if the polymeric procyanidins (PCs) in our extracts were definitely more abundant. In order to purify grape seed extracts, a liquid-liquid extraction (LLE) using water presaturated ethyl acetate as organic solvent for the removal of interfering species, such as lipids, proteins, sugars, and highly polymerized compounds, was performed. The purification strongly reduced the content of polymeric proanthocyanidins in our extracts and a chromatographic profile similar to that of Leucoselect was obtained.

On the other hand, skin extraction was achieved by a single-step maceration in methanol and a purification with a brominated synthetic adsorbent resin.

The presence of Oenin chloride and Quercetin-3-β-D-glucoside in each "Tesi" and of Quercetin in "Tesi" 2, 3, and 4 was attested, even if at the very low concentrations.

Surprisingly no resveratrol was detected in "uva di Troia canosina" grape skin extracts, even in an LC/MS-MS analysis.

Conflict of Interests

No author has a direct financial relation with the commercial identities mentioned in the paper that might lead to a conflict of interests.

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