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Study of the extraction of proanthocyanidins from grape seeds

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The extraction of proanthocyanidins was studied in the system ethyl acetate–water–grape seeds. It was found that, in the absence of water, proanthocyanidins could not be practically extracted, and the increase in water content in the system up to the saturation level resulted in a substantial yield enhancement. It was also shown that ethyl acetate, with 10% of water, is capable of selectively extracting proanthocyanidins, which has not been the case with solvents used previously. This solvent mixture extracts mainly the proanthocyanidins of lower molecular masses, which are more important from a therapeutic point of view. A further increase in water content in the extraction system yielded a small increase in the yield of proanthocyanidins, but caused a decrease in selectivity of the extraction.

INTRODUCTION

Because of their wide spectrum of pharmacological action, plant extracts containing proanthocyanidins attract the increasing attention of both pharmacists and physicians. An important event in this respect was the discovery that these substances exhibit marked free-radical scavenger capacities (Uchida *et al.*, 1987; Zhao *et al.*, 1989), and that they show good bioavailability (Laparra *et al.*, 1977). Also, there are reports of their possible use in preventing and treating atherosclerosis (Masquelier *et al.*, 1981; Gavinet-Jeannin *et al.*, 1988; Kovač and Pekic, 1991) and some cancerous diseases (Okuda, 1993).

Included under the term 'proanthocyanidins' are the polymerization products of flavan-3-ols to oligomers. The flavan-3-ol monomers, (+)-catechin and (−)-epicatechin, as well as their dimers (Table 1) are found in grape and wine, along with the trimer (C_1) containing three molecules of (−)-epicatechin bound by interflavan C_4—C_8 bonds (Bourzeix *et al.*, 1986).

The determination of the above catechin monomers, dimers and trimer in grape and wine has recently been the subject of several reports (Boukharta, 1988; Kovač *et al.*, 1990; Kovač *et al.*, 1995), as they are apparently responsible for the favourable action that wine exhibits on the cardiovascular system, known as the 'French paradox' (Masquelier, 1982; Renaud and De Lorgenil, 1992).

Proanthocyanidins from plant material for analytical and preparative purposes have been extracted with methanol (Gupta and Haslam, 1981; Stafford and Cheng, 1980), ethanol (Tsai Su and Singleton, 1969; Romeyer, 1984), acetone (Michaud *et al.*, 1971; Nonaka *et al.*, 1982) and their mixtures with water in different proportions. The results of the application of these solvents cannot be correlated because of the diversity of plant material and analytical methods employed. Nevertheless, it can be concluded that the use of an acetone–water mixture as the extractant gives a better effect in proanthocyanidin extraction than other extractants used for this purpose (Dumon, 1990). However, the use of all these extractants generally yields a significant co-extraction of the concomitant substances, which makes the procedure of extract purification more difficult and decreases the yield of proanthocyanidins. As far as the authors are aware, there are no literature data on the use of selective solvents for extraction of proanthocyanidins.

It is known that proanthocyanidins are well soluble in ethyl acetate and that this solvent exhibits significant selectivity in respect of natural products, which has been confirmed by the example of the extraction of cardiotonic glycosides (Pekic and Stamenković, 1972). Hence,

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Table 1. Catechin dimers

Dimer designation	Structural formula		Interflavan bond	Configuration at C_4–atom
	Upper part	Lower part		
B_1	(−)-epicatechin	(+)-catechin	C_4—C_8	R
B_2	(−)-epicatechin	(−)-epicatechin	C_4—C_8	R
B_3	(+)-catechin	(+)-catechin	C_4—C_8	S
B_4	(+)-catechin	(−)-catechin	C_4—C_8	S

it is anticipated that the use of this solvent as extractant of proanthocyanidins from grape seeds could improve selectivity of the extraction process.

In view of the finding that the seeds have a higher content of proanthocyanidins than the rest of the grape (Bourzeix *et al.*, 1986; Kovač *et al.*, 1990), we have undertaken a study of their extraction with ethyl acetate (Pekić and Kovač, 1993), with the aim of obtaining proanthocyanidins on a preparative scale.

MATERIALS AND METHODS

The experiments were carried out using the seeds of Italian Riesling grapes grown at Sremski Karlovci (Yugoslavia), harvested in 1992.

Samples

- *Sample I*. After wine making, the seeds were separated from the pomace immediately after grape pressing, and dried in the air in a thin-layer at room temperature. The moisture content was 5.5%.
- *Sample II*. The seeds were separated manually from the integral grapes slightly dried on filter paper. The moisture content was 33.2%.
- *Standard*. (+)-catechin hydrate (Fluka, Chimie AG, CH, Buchs).
- *Moisture content* was determined by heating grape seeds at 105°C to constant mass.

Spectrophotometric analysis

Reagent with vanillin: Solution A, 30% v/v H_2SO_4 in methanol; Solution B, 1% m/v vanillin in methanol.

Immediately before the use, solutions A and B were mixed in a ratio 1 : 1.

Procedure

The preparation or dry extract obtained in the study of extraction kinetics was dissolved in methanol to obtain a solution of concentration of 1 mg ml^{-1}; to 0.2 ml of this solution were added 6.0 ml of the vanillin reagent. After 5 min the absorbance was measured at 510 nm in a 10-mm cuvette. When colour intensity was outside the measurement range, a correction was made by varying the measured volumes of the methanolic solution of proanthocyanidins. Total content of proanthocyanidins was determined on the basis of the measured absorbance and a standard graph, obtained using (+)-catechin.

HPLC analysis

For this purpose, a solution of proanthocyanidins containing 1 mg ml^{-1} was prepared in 50% methanol (v/v). A chromatograph equipped with M501 and M510 pumps (Waters Associates), a 720 gradient controller (Waters Associates), a Rheodyne 7125 injection valve furnished with a 20 μl sample loop, a Linear UVIS 200 variable wavelength visible-ultraviolet detector set at 280 nm, and a Spectra Physics SP4290 integrator were used. The separation of catechins and proanthocyanidins was carried out on a reversed phase Nova-Pak C_{18} iron cartridge, 3.9/mm i.d. ×150 mm long (Waters Associates), placed in a water bath at 32°C, using a linear gradient of 10% acetic acid (solvent A) in water (solvent B), as shown in Table 2. The quantitation of catechins and proanthocyanidins was achieved by an external standard procedure, using multiple-point calibration. Results were expressed as (+)-catechin equivalents.

Catechins and proanthocyanidins (dimers and trimer) were identified on the basis of their retention times, determined previously (Revilla *et al.*, 1995).

Extraction of proanthocyanidins

Whole grape seeds were extracted on a horizontal shaker with 70 oscillations per minute.

Extraction kinetics

In each of eight 100-ml bottles, grape seeds (10 g) were placed and the solvent (40 ml), and after hermetically sealing them the bottles were mounted onto the shaker. At different time intervals during 24 h, the extracts were separated by decantation from particular bottles, dried with anhydrous Na_2SO_4, and an aliquot (20 ml) corresponding to one half of the starting amount of seeds, was taken, and the solvent removed under reduced pressure. The dry extract was dissolved in methanol and the content of proanthocyanidins was determined by spectrophotometry and, eventually, by HPLC.

Table 2. Linear gradient used in HPLC analysis of catechins and proanthocyanidins

Time (min)	Flow rate (ml min^{1-})	Solvent A (%)	Solvent B (%)
0	0.8	10	90
47	0.8	82	18
55	0.8	100	0
65	0.8	100	0
70	0.8	10	90

Preparative extraction

Extraction with ethyl acetate

Grape seeds (100 g) were extracted with ethyl acetate containing 10% of water (400 ml) in hermetically sealed glass vessels of 1 litre, during 24 h. Subsequently, the extract was separated by decantation, dried with anhydrous Na_2SO_4 (20 g), and about 90% of the solvent removed by evaporation under reduced pressure. Upon mixing the concentrated ethyl acetate solution with a five-fold volume of petroleum ether, the precipitate formed was separated by filtration through a nutsch filter B-4 and dried in a vacuum desiccator. The result was a crude preparation of proanthocyanidins (CP-PRO).

Extraction with acetone–water mixture

Grape seeds were extracted in the same way as in the previous case but with an acetone–water mixture (2:3) as extractant. After 24 h of shaking the extract was separated by decantation and acetone removed by distillation under reduced pressure. The aqueous solution was cooled (3 h at $+4°C$) and separated from the precipitate formed by centrifugation. Then, NaCl was added (40 g 100 ml^{-1}), which was dissolved by stirring the solution, and, after cooling (3 h at $+4°C$), the clear solution was separated from the precipitate by centrifuging. After the extraction with three successive portions of ethyl acetate (the first volume being equal to that of the solution and the subsequent two to one half of that volume), the organic solvent was dried with anhydrous Na_2SO_4 (20 g), and further procedure was identical to the previous one. This resulted in a purified preparation of proanthocyanidins (PP-PRO)

RESULTS AND DISCUSSION

Preliminary investigations showed that grinding of grape seeds could shorten the extraction time but did not increase the yield of proanthocyanidins which is in agreement with the findings of other authors (Dumon, 1990). However, the grinding caused a significant increase in the extraction of undesired concomitant components, which hindered obtaining of a preparation with an increased content of proanthocyanidins. For these reasons, in all the experiments presented in this work we used the whole grape seeds.

The extraction of dried grape seeds with ethyl acetate showed that this solvent is not practically capable of extracting proanthocyanidins. This is a consequence of a low permeability of the seeds tissue to ethyl acetate, which is a non-polar aprotic solvent. The use of ethyl acetate with addition of water resulted in a significantly increased yield of extracted proanthocyanidins. It is obvious that the presence of water increases permeability of seeds tissue and thus enables a better mass transport by molecular diffusion. For this reason, an experiment was undertaken to study how the moisture degree influences the yield of proanthocyanidins. As a precisely defined moisture degree is experimentally difficult to achieve, the amount of water added to ethyl acetate was varied. The results obtained in studying the kinetics of extraction of proanthocyanidins from dry grape seeds (sample I) using ethyl acetate with different contents of water as extractant are presented in Fig. 1.

As can be seen from the diagram in Fig. 1, the increase in yield of proanthocyanidins in the extraction with ethyl acetate saturated with water (3.3% of water) and with ethyl acetate:water (95 : 5) is linear. After 24 h of extraction, the yield obtained using ethyl acetate:water (95 : 5) was 2.3 times higher than that obtained with ethyl acetate saturated with water. The kinetic curves obtained using ethyl acetate with 10, 15 and 20% water are of parabolic shape, with the initial part being linear (extraction up to 8 h), thus reflecting a strong increase in the yield of proanthocyanidins, whereas their second parts show a slower increase and an asymptotic ending.

The yield of proanthocyanidins obtained after 24 h of extraction with ethyl acetate:water (90 : 10) was 73 times higher than that achieved with the extractant containing 3.3% of water. On the other hand, the yield increases obtained with ethyl acetate:water (85 : 15) and ethyl acetate:water (80 : 20) were quite similar, 7.5 and 7.6 times, respectively. An abrupt increase in proanthocyanidins yield obtained with ethyl acetate with increased water contents can be explained by increased permeability of grape seeds, which increases until the extractant

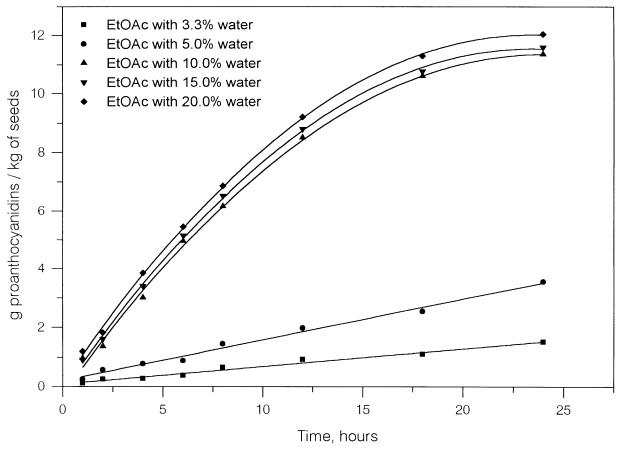

Fig. 1. Dependence of the yield of proanthocyanidins on extraction time and water content in ethyl acetate.

is completely saturated with water. Starting from the fact that ethyl acetate is saturated with water at its content of 3.3%, and that water-saturated seeds contain 33–35% of water, we arrive at a conclusion that the extraction system ethyl acetate–water–grape seeds is saturated with water when the extractant is ethyl acetate:water (90 : 10). A further increase in water content in the extraction system (15 and 20% of water) results only in a small increase in the yield of proanthocyanidins (by 2–3%), but the extraction selectivity is substantially lowered. Namely, in the presence of 'free water' which is a markedly polar solvent, significant amounts of the concomitant substances are extracted.

In order to check the assumptions on the effect of water on the yield of proanthocyanidins, we have investigated the extraction of grape seeds with the different moisture contents, using ethyl acetate without addition of water. To obtain the seeds with different moisture contents, fresh seeds (Sample II) were dried in a thin-layer at room temperature (22°C). Moisture content in seeds was determined before drying, and each day of the four subsequent days of drying, at the same time taking the samples for the extraction. The results of the experiments lasting 8 h are presented in Table 3.

It is evident from the results given in Table 3 that the yield of proanthocyanidins from the seeds with maximal moisture content, using ethyl acetate with no water added, is substantially lower than the yield achieved using ethyl acetate:water (90 : 10). Furthermore, the yield of proanthocyanidins shows an abrupt decrease with decreasing moisture content in the grape seeds extracted with ethyl acetate without addition of water. On the other hand, the extraction with ethyl acetate:water (90 : 10) gave practically the same yield. These findings support the above conclusion that an optimal extraction of proanthocyanidins is achieved when the extraction system ethyl acetate-water-grape seeds is saturated with water.

On a preparative scale, the grape seeds (sample I) were extracted with ethyl acetate:water (90 : 10) and, after the decantation, the major part of the solvent was removed by evaporation at a reduced pressure. Precipitation with petroleum ether yielded a crude preparation of proanthocyanidins (CP-PRO).

For the sake of comparison, the grape seeds were extracted under the same conditions using an acetone–water mixture (2 : 3), and a complex procedure yielded a purified preparation of proanthocyanidins (PP-PRO). A crude preparation could not be prepared in this way because the extractant was extremely non-selective. The yields of proanthocyanidins, as well as their qualitative and quantitative compositions determined by spectrophotometric and HPLC methods, are presented in Table 4.

The yield of proanthocyanidins in the form of powder using ethyl acetate as extracting agent (CP-PRO) is 2.3 times higher than the yield of the preparation obtained using the acetone–water mixture (PP-PRO). Furthermore, the yield of total proanthocyanidins is 1.9 time higher when extraction was carried out with ethyl acetate. It is obvious that the complex procedure involved in the purification of acetone–water extract of grape seeds resulted in substantial losses of proanthocyanidins. However, the purity of the resulting preparation (PP-PRO) was not higher than that of the crude preparation (CP-PRO) obtained by extraction with ethyl acetate. This conclusion is based on the HPLC analysis of the obtained preparations. The chromatograms presented in Fig. 2 differ only in their first part (to RT of 9 min), and especially in their final part (after RT of 37 min), i.e. in respect of contents of flavan-3-ols of higher molecular masses (tetra- and pentamers), which are found in the PP-PRO, but not in the CP-PRO preparation. The relative contents of the particular flavan-3-ols in these preparations are also different

Table 3. Results of extraction of grape seeds with different moisture contents (extraction time: 8 h)

Water content in ethyl acetate (%)	Moisture content in grape seeds (%)				
	33.2(0)[a]	19.2(1)	11.8(2)	7.5(3)	6.1(4)
	Yield of proanthocyanidins g kg^{-1} seeds				
0	3.196	1.146	0.160	0.123	0.113
10	5.360	5.226	5.210	5.228	4.980

[a]Numbers in the parentheses denote seeds drying time in days

Table 4. Yield and composition of proanthocyanidins (extraction time: 24 h)

Preparation	Yield, g kg^{-1} grape seeds		PRO g $100\,g^{-1}$ preparation	Relative content %						
	Preparation	Total PRO		C	E	B_1	B_2	B_3	B_4	C_1
CP-PRO	11.03	5.18	47.0	35.7	24.7	9.6	11.7	1.8	4.3	12.7
PP-PRO	4.77	2.67	56.0	29.1	21.8	3.3	13.0	4.5	2.9	25.5

PRO = proanthocyanidins; C = (+)-catechin; E = (−)-epicatechin; B = dimers; C_1-trimer.

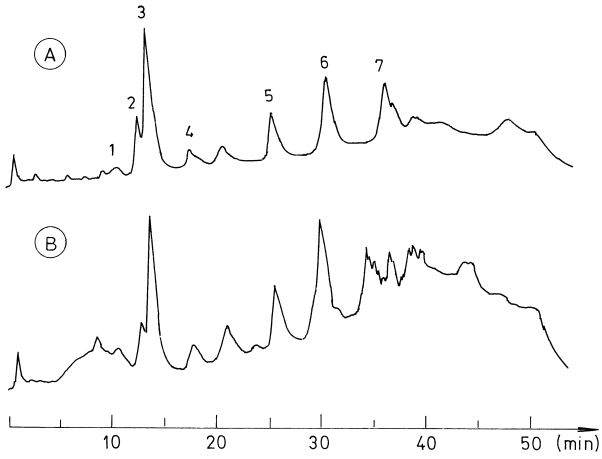

Fig. 2. Chromatograms of proanthocyanidin preparations. (A) CP-PRO;(B) PP-PRO; (1) procyanidin B_3; (2) procyanidin B_1; (3) (+) catechin; (4) procyanidin; B_4; (5) procyanidin B_2; (6) (−) epicatechin; (7) procyanidin C_1.

(Table 4): content of total monomers in the preparation CP-PRO is by 8.9% higher than that in PP- PRO; relative content of the dimer is higher by 3.7%, whereas the trimer content is lower by 12.3%. Therefore, it can be concluded that ethyl acetate:water (90 : 10) selectively extracts particular flavan-3-ols from grape seeds, primarily the monomers, dimers, and the trimer C_1. In view of these facts, a question may be posed. How did the PP-PRO preparation contain proanthocyanidins of higher molecular masses (tetra- and pentamers) when the final stage in obtaining them involved extraction of an aqueous solution with ethyl acetate? This can be answered by the fact that the aqueous solution of proanthocyanidins was saturated with sodium chloride, which caused a change in the distribution of proanthocyanidins between the aqueous and organic phase.

On the basis of all the above it can be concluded that ethyl acetate:water (90 : 10) selectively extracts proanthocyanidins, and the use of this extractant can significantly simplify the separation of proanthocyanidins on preparative and industrial scales. In this way the extraction of flavan-3-ols of lower molecular masses, which are more important from a therapeutic point of view, is favoured. In other words, it is known that an increase in the degree of polymerization of flavan-3-ols enhances their tanning character (Ribéreau-Gayon and Stonestreet, 1964), and thus decreases bioavailability (Masquelier, 1989)

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