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Determination of Vegetable Tannin Fractions Using Solid Phase Extraction and UV-VIS Spectrophotometric Technique

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Summary

The correlation between low molecular mass catechins and catechin polymers in polyphenolic plant extracts was determined using the combination of solid phase extraction and UV-VIS technique. Grape seed and grape stem extracts of ten different grape cultivars were analysed during three vintages. Low molecular mass catechins were determined after solid phase extraction. Catechin polymers were calculated as the difference between catechins in raw plant extract and low molecular mass catechins. Low molecular mass catechins were present in higher amounts in the grape seed extracts (14.79 g/kg of grape seeds) than in the the grape stem extracts (4.36 g/kg of grape stems). The average relative amount of low molecular mass catechins was 56 % of total catechins in the grape seed, and 17 % in the grape stem extracts. Exceptionally high amounts of low molecular mass catechins were estimated in the grape seed extracts of Plavac mali cv. and grape stem extracts of Okatica white cv. The average mass fractions of catechin polymers, for analyzed cultivars were 11.78 g/kg of grape seeds, and 21.27 g/kg of grape stems. The ratio between low molecular masses of catechins and catechin polymers was about 1: 1 in the grape seed extracts, and 1:5 in the grape stem extracts.

Key words: grape, seeds, stems, tannins, catechins, solid phase extraction, UV-VIS spectrophotometry

Introduction

Vegetable tannins are found in high concentrations throughout the plant kingdom. Tannin extracts are often groups of hydrolyzable and condensed tannins with undefined degree of polymerization, molecular masses, monomer units composition and type of linkage (*1*). The condensed tannins, or more correctly proanthocyanidins, or catechin polyphenols almost invariably contain one or both of flavan-3-ols: (+)-catechin, or (–)-epicatechin. Catechin polyphenols exist as monomers, oligomers, and polymers. Adsorptive effects become prominent at the triflavonoid level, and after that increase exceedingly rapidly with molecular mass (*2–4*).

In most plant tissues the catechin polymers are of greatest quantitative significance but there is also a range of low molecular mass species – monomers, dimers, trimers, etc. The routine dietary intake of catechin polyphenols can cause a wide variety of effects, both harmful and beneficial (*5,6*). The wide range of physiological properties of flavan-3-ols stems from their affinity to proteins and their antioxygen effect. While the affinity to proteins increases with the length of polyphenolic polymer, the efficiency of low molecular mass flavans, as free radical scavengers, is well established (*3,7,8*). Catechins and their polymers are the common constituents of grapes (*7,9–11*).

The aim of this study was to determine the relationship of low molecular mass catechins and catechin polymers in the extracts of the solid parts of grape. The determination of condensed tannin fractions was done using the combination of UV-VIS spectrophotometric method and solid phase extraction (SPE).

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Materials and Methods

Preparation of plant material

The grape seeds and stems of ten different grape cultivars of *Vitis vinifera* L. (seven red cultivars: *Plavac mali, Trnjak, Vranac, Merlot, Cabernet Sauvignon* and *Game bojadiser,* and three white cultivars: *Kujundžuša, Okatica bijela* and *Rkaciteli),* grown in the Dalmatian part of Croatia, were analyzed in 1990, 1991 and 1992. For each grape cultivar, three rows of grapevines were selected and 20 stocks were marked. The grapes were harvested at the stage of technological maturity.

1. Grape stems: The stems of 1 kg of grapes (for each grape cultivar) were separated by hand, dried with filter paper, weighed and frozen till the time of analyses. Before the extraction, the stems were fragmented and crushed with silica adding.

2. Grape seeds: The sample size for each grape cultivar was 100 berries. The berries were dried with filter paper and weighed. The seeds were separated by hand, dried with filter paper, weighed and frozen till the time of analysis.

Extraction of polyphenolics

The polyphenolic constituents were extracted from the seeds and stems by a series of succesive extractions: 1) 100 % ethanol, 24 hours; 2) 80 % methanol, 4 hours; 3) 50 % methanol, 4 hours; 4) water, 15 hours at –18 °C; and 5) 75 % aceton, 2 hours, according to Bourzeix *et al.* (*9*). The extracts thus obtained were collected and filtered and the volume of total extract (T.E.) was determined. The total phenolics in T.E. were determined by offical AOAC spectrophotometric method with Folin-Ciocalteu reagens according to Singleton and Rossi (*12*).

Fractionation of low molecular mass catechins (LMM catechins)

The T.E. was concentrated under vacuum on rotary evaporator at 30 °C to remove the organic solvent. Redistilled water was added to the extract remaining in the evaporator until the initial volume was achieved, and the pH was corrected to pH = 7.0. The neutral and acidic phenolic constituents were separated using solid phase extraction (SPE) on C18 Sep Pak cartridges (Waters Associates, Milford, MA, USA). The adequate volume of the sample (1 mL of grape seed extract; 10 mL of grape stem extract) was passed (drop by drop) through two interconnected cartridges, previously conditioned with 10 mL of methanol and 10 mL of water (pH = 7.0). The acid phenol fraction was eluated with 10 mL of water (pH = 7.0). The cartridges were dried in the stream of nitrogen. The neutral phenolic fraction (LMM catechins) was eluated with 10 mL of ethyl acetate, evaporated to dryness under vacuum on a rotary evaporator at 35 °C, and the dry residue was dissolved in 1 mL methanol-water mixture (volume fraction (φ = 50 %).

Determination of catechins in T.E. and in neutral polyphenolic fraction

Catechins in total polyphenolic extract and catechins in the neutral polyphenolic fraction (LMM catechins) after SPE, were determined using the spectrophotometric method with vanillin (*5,13*). The following procedure is the Pompei-Peri variation of the Rebelein's method. Standard catechin solution was prepared dissolving 0.25 g of (+)-catechin in 96 % ethanol in a 100-mL volumetric flask and dilute to volume with this solvent. In a second 100-mL volumetric flask, 10 mL of first solution was diluted to volume with 96 % ethanol. This solution had a catechin concentration of 0.25 mg/mL.

To individual 2-mL volumetric flasks were added aliquots of the standard catechin solution (0, 1, 3, 5 and 10 mL), 10 mL of 11.5 M hydrocloric acid, and 5 mL of ethanolic vanillin 1 % solution, and diluted each to volume with 96 % ethanol. After mixing and allowing to stand for 20 min, absorbance was measured at 500 nm using a 1-cm cuvette. In a case of total polyphenolic extract and the neutral polyphenolic fraction (LMM) 1 mL of sample was used.

The samples were also injected into an HPLC using RP C18 column (250 mm × 4 mm, 10 μm) to control the effect of fractionation (*10*).

The average mass fractions of catechins, and catechins in neutral fraction were calculated for each of the cultivars. The results represent the average of three repetitions.

Analysis of variance with two variables, P1 = »cultivar«; P2 = »vintage«, was used for statistical processing of the results. The F-test at 0.05 level was used to establish the significance of the variable influence.

Results and Discussion

Flavonoid class of polyphenols with a reactive phloroglucinol moiety can react with vanillin or other similar aldehydes, resulting in colored compounds that can be quantitatively determined colorimetrically. The main reactants are flavan-3-ols and flavan-3,4-diols (*14*). Condensed tannin polymers also react but to a lesser degree (*7*). Even if the reaction is not strictly specific it can be used for the approximate evaluation of the amount of catechins in the polyphenolic plant extracts (*15*). Using the SPE technique on reverse phase the low molecular mass catechins (LMM catechins: mainly monomers, dimers, trimers, etc.) were eluted in the neutral polyphenolic fraction (C.N.F), while catechin polymers remain on the reverse phase. The efficiency of fractionation, controlled using HPLC on the reverse phase, was good (*10,11*). The increase of the base line caused by the presence of catechin polymers was not observed.

The results of the investigation of physical and chemical composition of grapes of 10 different grape cultivars are presented in Table 1, and Figs. 1–3. Generally, it can be said that the analysed grape seed and grape stem extracts were rich in phenolic compounds. Table 1 shows the range of total phenols, catechins and LMM catechins in ten grape cultivars analysed from the 1990, 1991 and 1992 vintages. The results confirmed the presence of LMM catechins and catechin polymers in all analysed plant extracts. The significant influence of the variable »different cultivar« (P1) on the concentration of these compounds was confirmed at the 0.01 level of significance.

Table 1. Physical and chemical parameters of grapes in technological ripeness (10 different grape cultivars; vintage 1990, 1991, 1992)

Grape cultivar		Components							
		Seeds				Stems			
		m (seed) / N (berries)	w (catechins)	w (catechins in neutral fraction)	w (catechin polymers)	w (stem)	w (catechins)	w (catechins in neutral fraction)	w (catechin polymers)
		g	g kg^{-1}	g kg^{-1}	g kg^{-1}	g kg^{-1}	g kg^{-1}	g kg^{-1}	g kg^{-1}
Plavac	90	4.9	37.84	33.60	4.24	17.8	9.87	5.79	4.07
mali	91	3.6	62.28	48.16	14.12	23.0	13.39	2.18	11.21
	92	3.1	57.98	50.32	7.66	21.0	29.91	7.13	22.78
Trnjak	90	7.2	15.28	4.87	10.42	32.7	27.38	-	-
	91	3.6	24.63	14.11	10.52	32.9	45.18	4.03	41.15
	92	8.6	15.88	8.84	7.04	34.0	49.22	8.52	40.71
Vranac	90	13.7	17.58	7.82	9.76	29.9	35.51	8.89	26.62
	91	9.8	25.22	13.68	11.54	32.4	26.05	6.96	19.10
	92	10.4	17.34	7.62	9.72	35.0	36.90	5.97	30.93
Merlot	90	9.2	12.36	4.11	8.26	25.3	10.29	0.20	10.10
	91	7.0	19.46	14.60	4.85	22.1	9.64	1.09	8.55
	92	5.4	13.54	7.82	5.72	30.8	7.51	1.50	6.02
Cabernet	90	7.0	13.65	6.47	7.18	42.3	8.83	0.46	8.37
Sauvignon	92	4.5	20.41	6.35	14.06	28.9	10.82	2.10	8.72
Plavina	90	9.6	16.02	13.39	2.62	28.9	16.79	0.86	15.93
	91	7.4	35.05	24.12	10.93	31.9	5.76	0.59	5.17
	92	5.5	51.30	21.63	29.67	35.3	13.20	1.01	12.18
Game	90	8.7	16.02	13.39	2.62	24.3	26.19	1.03	25.17
Bojadiser	91	7.2	57.31	30.99	26.32	-	-	-	-
	92	5.0	60.54	27.98	32.57	22.3	34.96	4.17	30.79
Kujun-	90	11.6	17.81	7.44	10.37	19.0	44.67	2.51	42.16
džuša	91	7.6	21.13	11.22	9.91	17.2	48.27	3.78	44.49
	92	10.3	21.85	10.26	11.59	20.3	26.15	1.43	24.72
Okatica	90	8.2	20.63	7.30	13.34	30.8	38.83	18.52	20.32
bijela	92	9.6	8.38	3.52	4.86	40.4	37.94	12.01	25.92
Rkaciteli	90	7.5	33.42	10.77	22.65	28.1	23.89	0.59	23.30
	91	6.9	31.22	15.24	15.99	23.7	22.14	3.29	18.85
	92	8.7	20.43	6.39	14.04	24.5	30.75	0.72	30.04
P1 = »different cv.«		0.01	0.01	0.01	-	0.01	0.01	0.01	-
P2 = »vintage«		n.s	n.s.	0.01	-	n.s.	n.s.	n.s.	-

Reproducibility of method for catechins: relative standard deviation was 7.3 %.
To evaluate the statistical significance of influence, F-test has been used at 0.05 significance level (n. s. = not significant).

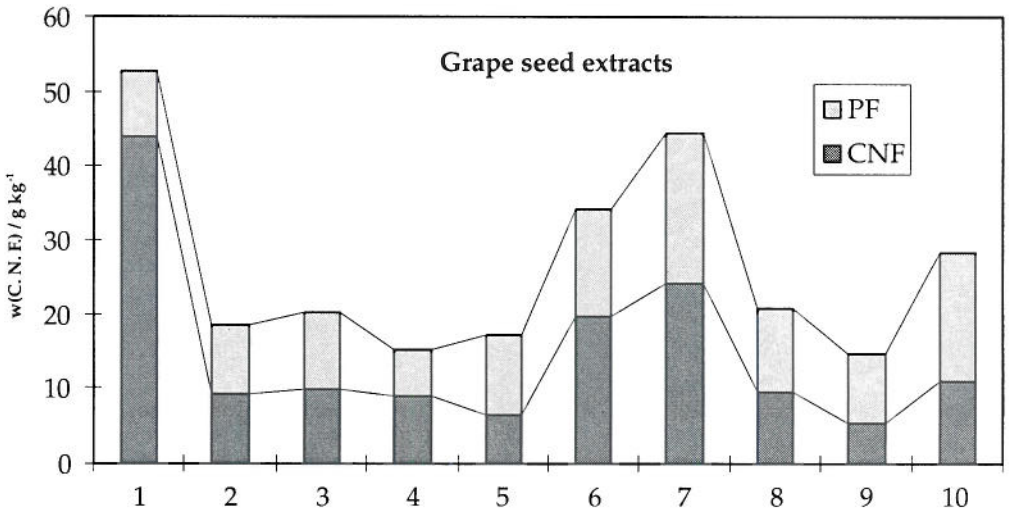

Fig. 1. The average mass fraction of low molecular mass catechins (C.N.F.) and catechin polymers (P.F.) in the grape seed extracts of 10 different grape cultivars (vintages: 1990, 1991, 1992)

Catechin polyphenols were present in considerable mass fractions both in the grape seed, and in the grape stem extracts. The average mass fraction of catechins was 26.57 g/kg of grape seeds, and 25.60 g/kg of grape stems, successively given for the grape seeds and grape stems. The highest amounts of catechins were estimated in the grape seed extracts of *Plavac mali cv.*, and the least in the grape seed extracts of *Okatica bijela cv.* The order of cultivars according to the average content of catechins in the grape seeds were as follows: *Plavac mali cv.* 52.70, *Game bojadiser cv.* 44.62, *Plavina cv.* 34.12, *Rkaciteli* cv. 28.36, *Kujundžuša cv.* 20.63, *Vranac cv.* 20.05, *Trnjak cv.* 18.60, *Cabernet Sauvignon cv.* 17.03, *Merlot cv.* 15.12, and *Okatica bijela cv.* 14.51 g/kg of grape seeds.

The average mass fractions of LMM catechins and catechin polymers in the grape seed and stem extracts are presented in Figs. 1 and 2.

According to the obtained results the mass fractions of LMM catechins in the grape seed extracts were cultivar dependent, and relatively high compared to the catechin polymers. The average mass fraction of LMM catechins was 14.79 g/kg of grape seeds (average for 10 different grape cultivars; vintages 90–92). Extremely high amounts of these compounds were found in the grape seed extracts of *Plavac mali cv.*: cca 44.03 g/kg of grape seeds, or 83.55 % of total catechins.

The presence of LMM catechins was confirmed in all analysed grape stem extracts. Generally, the amount of LMM catechins in the grape stem extracts was not as high as in the grape seed extracts. The average content of these compounds in the neutral polyphenolic fraction of the grape stems, was 4.36 g/kg of grape stems. Unusually high amounts of LMM catechins were present in the grape stem extracts of *Okatica bijela cv.* (cca 15 g/kg

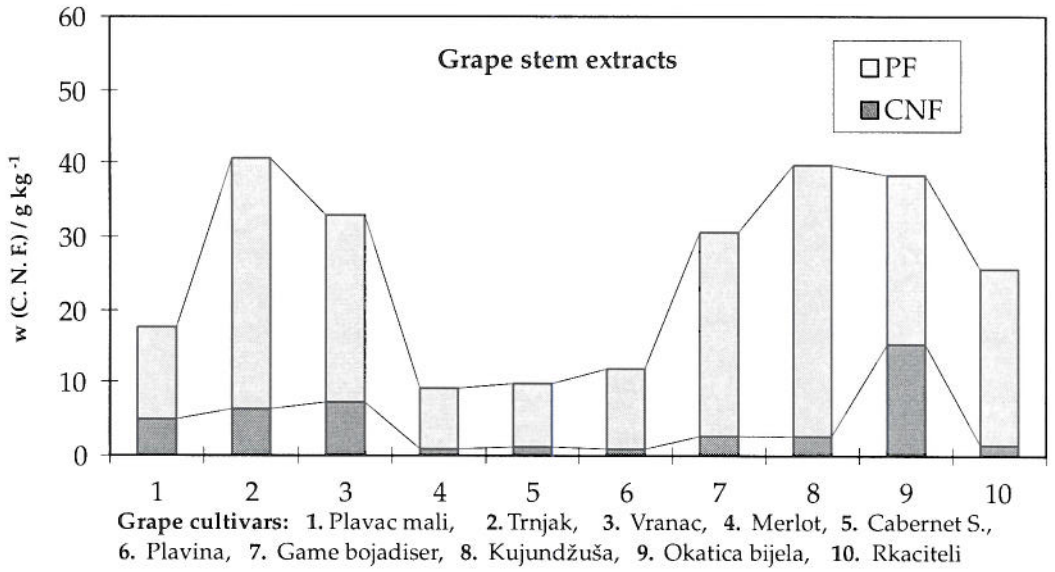

Fig. 2. The average mass fractions of low molecular mass catechins (C.N.F.) and catechin polymers (P.F.) in the grape stem extracts of 10 different grape cultivars (vintages: 1990, 1991, 1992)

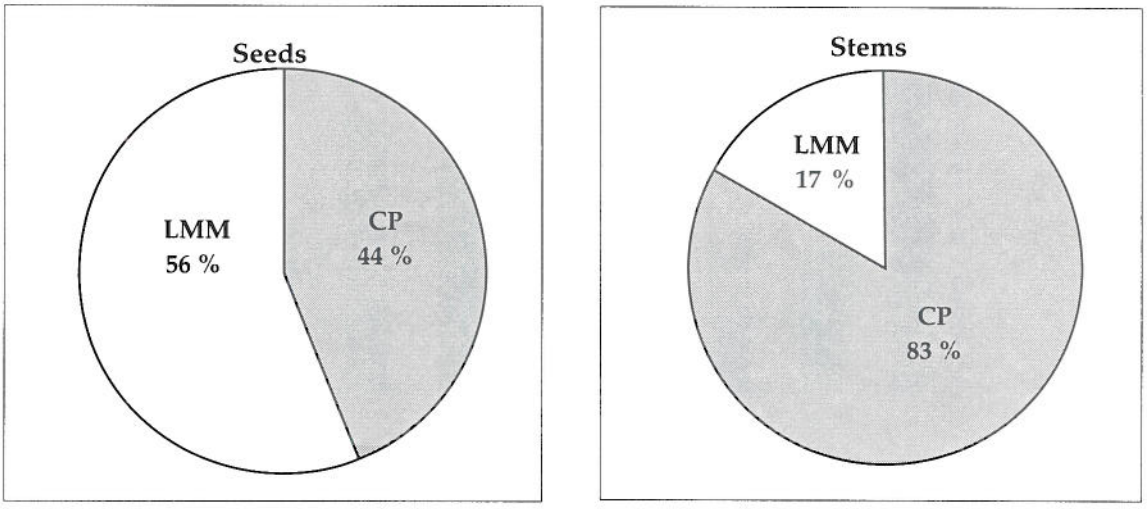

Fig. 3. The relationship between low molecular mass catechins and catechin polymers in the grape seed and grape stem extracts (average for 10 different grape cultivars; vintage 1990, 1991, 1992)

stems). It is interesting that grape seed extracts of *Okatica bijela cv.* were very poor in LMM catechins.

The average mass fraction of catechin polymers in the analysed grape seed and grape stem extracts of ten different cultivars, was 11.74 g/kg of grape seeds, and 21.27 g/kg of grape stems.

The average relative amounts of LMM catechins and catechin polymers, in the grape seed and stem extracts for 10 different grape cultivars, are presented in Fig. 3. The average relative amount of LMM catechins was 56 % of total catechins in the grape seed extracts, and 17 % in the grape stem extracts. The average relative amount of catechin polymers in the grape stem extracts was cca 83 % of total catechins, which is relatively high compared with the grape seed extracts. The ratio between the average relative amounts of catechin polymers and LMM catechins was cca 1:1 for grape seeds, and 5 : 1 for grape stems (Fig. 3).

Catechin polymers are the class of polyphenols of particular significance for man. They form complexes with proteins, and this association may reduce the nutritive value of foodstuffs and the interaction, when it is with proteins of the palate, gives rise to a characteristic astringent taste (*16*). According to the results of several studies, herbivores tend to consume less of the polyphenol-containing diets. Feeding trials indicate much more efficient uptake of low molecular mass components than of polymeric tannins. Digestibility and index of palatability decrease with the increase of polyphenolic content (*6,17*). It is important to know the correlation between LMM catechins and catechin polymers in the raw plant material if it is used in animal diet. The average mass fraction of catechin polymers in the analysed grape stems was almost twice as high as in the grape seeds. So, a much more efficient uptake of grape seeds than grape stems, in animal feeding, can be expected. Grape seeds and stems can be used as a part of diet for animals with tannin binding proteins in saliva. Because the amount of catechin polymers in grape stems and seeds is significantly cultivar dependent, preliminary analysis is recommended (*10*). With considerable high amounts of LMM catechins, of cca 15 g/kg of the grape seeds, and stems of some cultivars too, these vinification by-products can also be considered to be rich and cheap raw material for the production of these biochemicals.

Conclusions

Using a combination of SPE and UV-VIS spectrophotometric techniques it was possible to determine, simply and rapidly, the aproximative correlation between LMM catechins and catechin polymers in polyphenolic plant extracts.

The grape extracts, and grape stem extracts contained considerable amounts of catechin polyphenols. LMM catechins were found in higher amounts in the grape seed than in the grape stem extracts. The grape stem extracts were rich with catechin polymers. Correlation between catechin polymers and LMM catechins was about 1:1 in grape seeds, and 5:1 in the grape stem extracts. Compared with the other cultivars, unusually high amounts of LMM catechins were present in the grape stem extracts of *Okatica bijela cv.*

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Određivanje vegetabilnih taninskih frakcija primjenom ekstrakcije na čvrstoj fazi i UV-VIS spektrofotometrijskog postupka

Sažetak

Povezujući ekstrakciju na čvrstoj fazi i spektrofotometrijski postupak, utvrđen je odnos između niskomolekularnih katehina i katehinskih polimera u polifenolnim biljnim ekstraktima. Analizirani su ekstrakti sjemenki i peteljki grožđa deset različitih sorta tijekom triju berba. Niskomolekularni katehini određeni su nakon ekstrakcije na čvrstoj fazi. Katehinski polimeri su izračunani na temelju razlike između katehina u sirovom biljnom ekstraktu i niskomolekularnih katehina. U ekstraktima sjemenki nalazilo se više niskomolekularnih katehina (14,79 g/kg sjemenki) nego u ekstraktima peteljki (4,36 g/kg peteljki). Prosječna relativna količina niskomolekularnih katehina iznosila je 56 % od ukupnih katehina u sjemenkama grožđa i 17 % u ekstraktima peteljki. Neobično velika količina niskomolekularnih katehina nađena je u ekstraktima sjemenki iz grožđa Plavac mali te u ekstraktima peteljki iz grožđa Okatica bijela. Prosječna je količina katehinskih polimera u svim sortama grožđa bila 11,78 g/kg u sjemenkama i 21,27 g/kg u peteljkama. Odnos količine niskomolekularnih katehina i katehinskih polimera bio je oko 1:1 u ekstraktima sjemenki, te 1:5 u ekstraktima peteljki grožđa.