Short communication

High-performance liquid chromatographic determination of flavan fingerprints in plant extracts [1]

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Abstract

Flavonoids are a group of natural products widely distributed in vascular plants. The isolation and quantification of flavonoids in plant material is difficult due to their chemical complexity. The flavan fingerprints were determined in the extracts from plants that can be used as raw material for the production of low molecular mass flavans. The method involves extraction of phenolics from plant material, sample clean-up using solid-phase extraction on reversed-phase cartridges, and HPLC separation of flavans in the neutral phenolic fraction. The flavan fingerprints obtained are specific and may serve for the detection of the origin of plant extracts. © 1997 Elsevier Science B.V.

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1. Introduction

One of the most interesting and fast developing fields in phytochemistry is chemotaxonomy, also called biochemical systematics, chemosystematics and chemical taxonomy [1–3]. Some of the most useful chemical markers are the secondary metabolites, e.g. phenols and phenolic acids [4] as well as well as flavonoids, which remain amongst the markers of choice [3,5–7].

Over the last 30 years flavonoids proved to be very useful markers at all levels of plant classification. They are very important at the ordinary level, e.g. for deciding what families should be included or excluded from a certain order, although it is also possible to define a flavonoid pattern at the family and species level. Moreover, they are also very useful for identifying natural plant hybrids, and for the recognition of plant cultivars [5].

The great progress made in chemotaxonomy is also due to better techniques used in sampling of plant material, improved preparation of extracts with neutral solvents and under nitrogen, improved chromatographic and computer techniques, and the identification of new flavonoids. Flavonoids constitute a powerful adjunct for the identification of a wide variety of plants and plant cultivars. The production of flower (or bark) flavonoid RP-HPLC fingerprints has proven to be of great value for cultivar recognition [6,7].

The description and classification of plant cultivars, especially economically valuable cultivars such as ornamentals (e.g. roses), plants of importance for the food industry (e.g. hops, barley), medicinal plants, etc., frequently pose serious problems. Very

often a distinct recognition of a cultivar is of great importance [7].

The aim of this work was to identify the flavan fingerprints of different plant extracts (grape seed extracts of different cultivars, grape stem extracts and pine bark extracts) which can be used as raw material for the production of low molecular mass flavans.

2. Experimental

2.1. Reagents

Standards of (+)-catechin and (−)-epicatechin were purchased from Aldrich (Milwaukee, WI, USA). All solvents used for extraction and HPLC were obtained from Merck (Darmstadt, Germany), and Kemika (Zagreb, Croatia). HPLC-grade water was prepared by redistillation.

2.2. Preparation of samples

The flavan fingerprints were determined for different plant extracts.

2.2.1. Grape seed extracts

Grape seeds of 10 different cultivars of *Vitis vinifera* L., grown in the Dalmatian (coastal) 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. The sample size for each grape cultivar was 100 berries (in three repetition). The berries were dried with filter paper and weighed. The pulp, skin and seeds were separated by hand. The skin and seeds were dried with filter paper, weighed and frozen till the time of analysis. The pulp was pressed and the juice thus obtained was used to determine the pH, total acids and total sugar content. The mass fraction of polyphenols in the seed extract was determined. The basic chemical composition and the physical parameters were determined according to Amerine and Ough [8].

2.2.2. Grape stem extracts

The stems of 1 kg of grapes were separated by hand, dried with filter paper, and frozen till the time of analysis. Before the extraction, the stems were fragmented and crushed with silica.

2.2.3. Pine bark extracts

10 g of black pine bark was collected in May 1994, in Dalmatia (coastal part of Croatia).

2.3. Extraction of polyphenolics and fractionation of neutral phenolics using SPE

The polyphenolic constituents were extracted from plant material according to Bourzeix et al. [9]. The extracts thus obtained were collected and filtered, and the volume of the total extract (T.E.) was determined. The T.E. was concentrated under vacuum on a rotary evaporator at 30°C, to remove the organic solvent without destroying the phenolic composition. Redistilled water was added to the extract remaining in the evaporator until the initial volume was achieved, and pH was corrected to pH 7.0. The neutral and acidic phenolic constituents were separated using solid-phase extraction (SPE), on C_{18} Sep Pak cartridges (Waters Associates, Milford, MA, USA). An adequate volume of the sample (1 ml of grape seed extract, 10 ml of grape stem or pine bark extracts) was passed (drop by drop) through two interconnected cartridges, previously conditioned with 10 ml of methanol and 10 ml of H_2O. The acidic phenol fraction was eluted with 10 ml of H_2O, pH 7.0. The cartridges were dried in the stream of nitrogen.The neutral phenol fraction was eluted with 10 ml of ethyl acetate, evaporated under vacuum on a rotary evaporator at 35°C, and the dry residue dissolved in 1 ml methanol–water (50:50, v/v).

2.4. High-performance liquid chromatography

The HPLC system used was from Varian, with a 9010 pump, detector UV–VIS 9050, and integrator 4400. Separation of polyphenolics was carried out on a reversed-phase octadecylsilyl (LiChrosorb RP C18,) column (10 μm, 250×4 mm I.D.) from Merck, at room temperature. Separation was performed by gradient elution using acetic acid (0% 10 min; 0–5% in 10–60 min) with continuous inflow of methanol (10%), the solvent system recommended by Dumon [10]. The flow-rate was 1.2–2.0 ml in 60 min. The detection wavelength was set at 280 nm. Injection
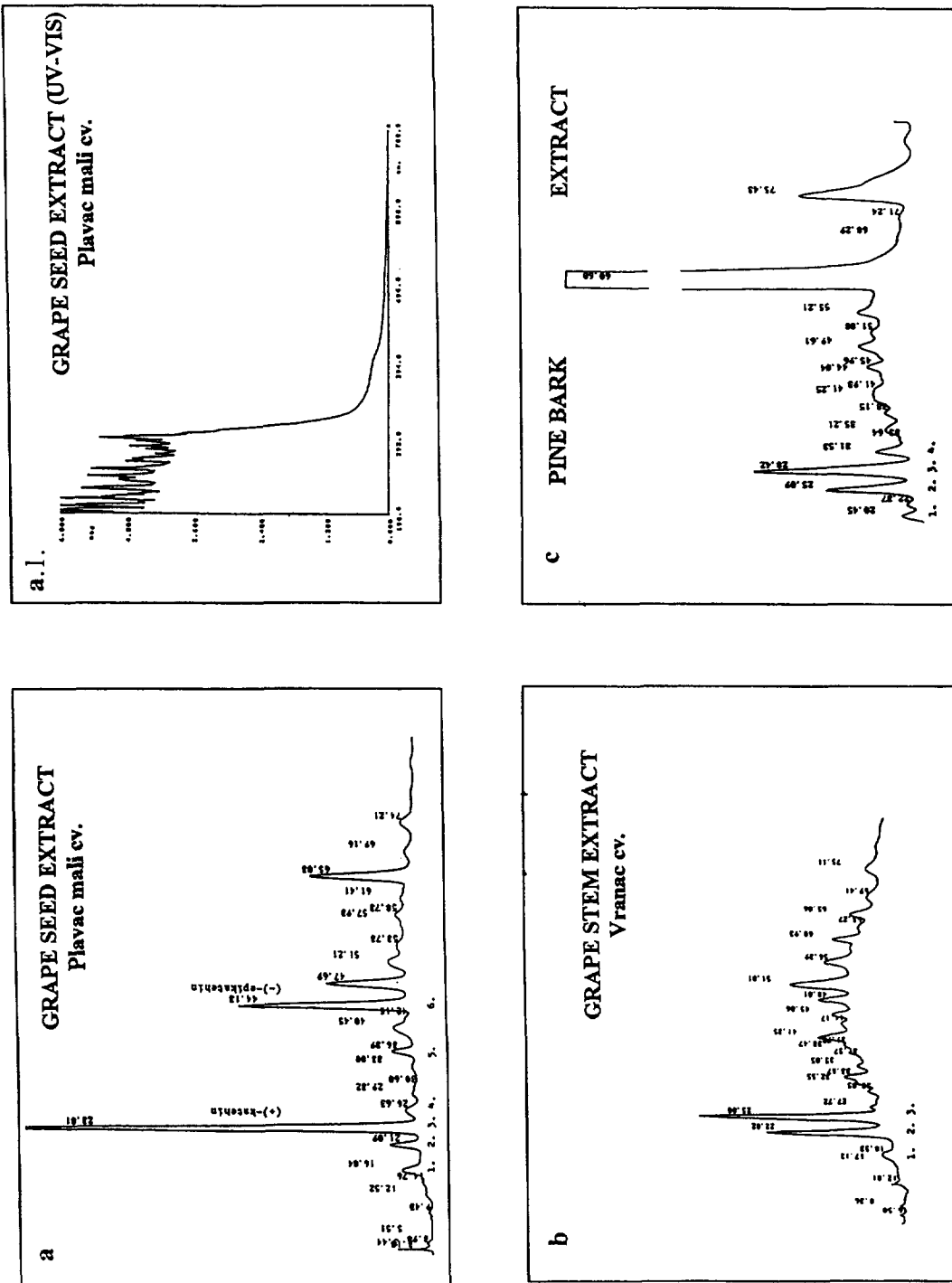
Fig. 1. RP-HPLC chromatogram of the neutral fraction of plant extracts: (a) grape seed extract; (b) grape stem extract; (c) pine bark extract. The elution order is given by peak numbers: 1, dimer B3; 2, dimer B1; 3, (+)-catechin; 4, dimer B4; 5, dimer B2; 6, (−)-epicatechin. *a-1*) UV–Vis absorption spectra of the same extract of *Plavac mali* cv., vintage 1991, with (a-1) UV–Vis absorption spectra of grape seed extract of *Plavac mali* cv.

volume was 10 μl. The methanolic extracts were injected twice in order to check reproducibility.

2.5. Determination and quantification of catechin monomers and procyanidin dimers

Catechin monomers: (+)-catechin (C) and (−)-epicatechin (E) were identified on the basis of their retention times. Different concentrations of standards were prepared (10–500 mg/l). The calibration factors (CF) for C and E were determined. The quantification of (+)-catechin and (−)-epicatechin was done using the external standard method.

The procyanidin dimers B1, B2, B3, and B4 were not commercially available. The retention times for these phenolics were confirmed using literature

information. The samples were separated using two different methods, one by Dumon [10], and the second was the method described by Bourzeix et al. [11].

Gradient elution was done using the solvent system recommended by Dumon [10]. Using the calibration factors (CF) for (+)-catechin and procyanidin dimers B, given by Dumon [10], the ratios (K) between CF for procyanidin dimers (B1, B2, B3, and B4) and CF for (+)-catechin, were calculated $(K=\text{CFB}/\text{CFC})$:

$$K_{B1} = 1.1817; \quad K_{B2} = 1.2985; \quad K_{B3}$$
$$= 1.0648; \quad K_{B4} = 1.1599.$$

Using these K values for procyanidins of the group

Table 1
Mass amount of catechin monomers and procyanidin dimers in grape seed extracts of vintages 1990, 1991, and 1992

Vitis species	Year	Seed compounds (mg/kg grape seeds)						Sum monomers	Sum dimers
		(+)-Catechin	(−)-Epicatechin	Procyanidin dimers					
				B1	B2	B3	B4		
Plavac Mali	90	2885	1839	290	380	342	294	4724	1306
	91	5031	3359	568	953	529	429	8390	2479
	92	5042	3046	447	867	404	380	8088	2098
Trnjak	90	240	507	38	160	54	68	747	320
	91	347	1168	100	280	52	69	1515	501
	92	191	414	132	210	89	69	605	500
Vranac	90	569	624	82	139	163	121	1193	505
	91	1512	1100	118	260	235	143	2612	756
	92	928	806	188	232	258	85	1734	763
Merlot	90	486	773	151	416	124	172	1259	863
	91	724	1281	154	470	115	174	2005	913
	92	662	1191	308	506	401	171	1853	1386
Cabernet Sauvign.	90	852	760	69	110	47	24	1612	250
	92	1197	993	170	258	207	169	2190	804
Palvina	90	2205	2256	111	329	182	84	4461	706
	91	3192	2037	171	331	158	109	5229	769
	92	6647	2267	449	931	318	269	8914	1967
Game Bojadiser	90	951	1056	98	251	137	283	2007	769
	91	1593	1967	186	583	245	225	3560	1239
	92	1004	1652	163	555	150	227	3656	1095
Kujundzusa	90	473	1585	49	479	99	115	2058	742
	91	536	1803	99	336	133	159	2339	727
	92	895	1734	224	1100	101	269	2629	1694
Okatica bijela	90	1127	1239	31	227	171	0	2366	429
	92	215	305	75	142	97	0	520	314
Rkaciteli	90	2598	706	194	243	359	0	3304	796
	91	1845	656	265	181	364	60	2501	870
	92	628	389	114	66	173	23	1017	376
P1 = 'different cultivar'		0.01	0.01	0.0-1	0.01	0.0-1	–	0.01	0.01
P2 = 'vintage'		n.s.	n.s.	n.s.	n.s.	n.s.	–	n.s.	n.s.

To evaluate the statistical significance of influence, the F-test has been used at the 0.05 level of significance.

B, and CF for (+)-catechin (determined with standard samples in this experimental work), the mass amounts of procyanidin B1, B2, B3 and B4 were calculated using the following equation:

$$\text{Amount}_B = \text{Detector response} \times CF_C \times K_B$$

The K value for E calculated using CF values (for C and E) given by Dumon, corresponds to the experimentally determined ratio between CF-s for C and E.

Thus obtained mass amounts of catechin monomers and procyanidin dimers in the grape seed extracts, were used to define the differences between cultivars. The method of variance analysis with two variables ($P1$ = vintage $P2$ = cultivar), was used for the statistical processing of the results. The F test at level 0.05 was used to establish the significance.

3. Results and discussion

Using reversed-phase HPLC, a good separation of

catechin monomers C and E, and procyanidin dimers B1, B2, B3, and B4 was achieved (Fig. 1). The retention times for catechin monomers C and E (enantiomers) differ by approximately 20 min. The selectivity was: $\alpha = 1.32$; 1.54; 1.14; 1.44; and 1.23, successively given for B1/B3; C/B1, B4/C; B2/B4; and E-B2.

The mass amounts of catechin monomers and procyanidin dimers in the grape seed extracts of 10 different grape cultivars are given in Table 1. The results confirmed the presence of catechin monomers (C and E) and procyanidin dimers B1, B2, B3, and B4, in all grape seed extracts which were analyzed from the vintages 1990, 1991, and 1992, except Okatica white cv. The grape seed extracts of Okatica white cv. were pure with dimers, and the presence of B4 was not confirmed. Generally, the analysed grape seed extracts were rich in catechin monomers (C and E), and contained considerable amounts of procyanidin dimers B. The mass amounts of these phenolic constituents were significantly dependent on the cultivar. The average total mass amount of catechin monomers (C and E) and procyanidin
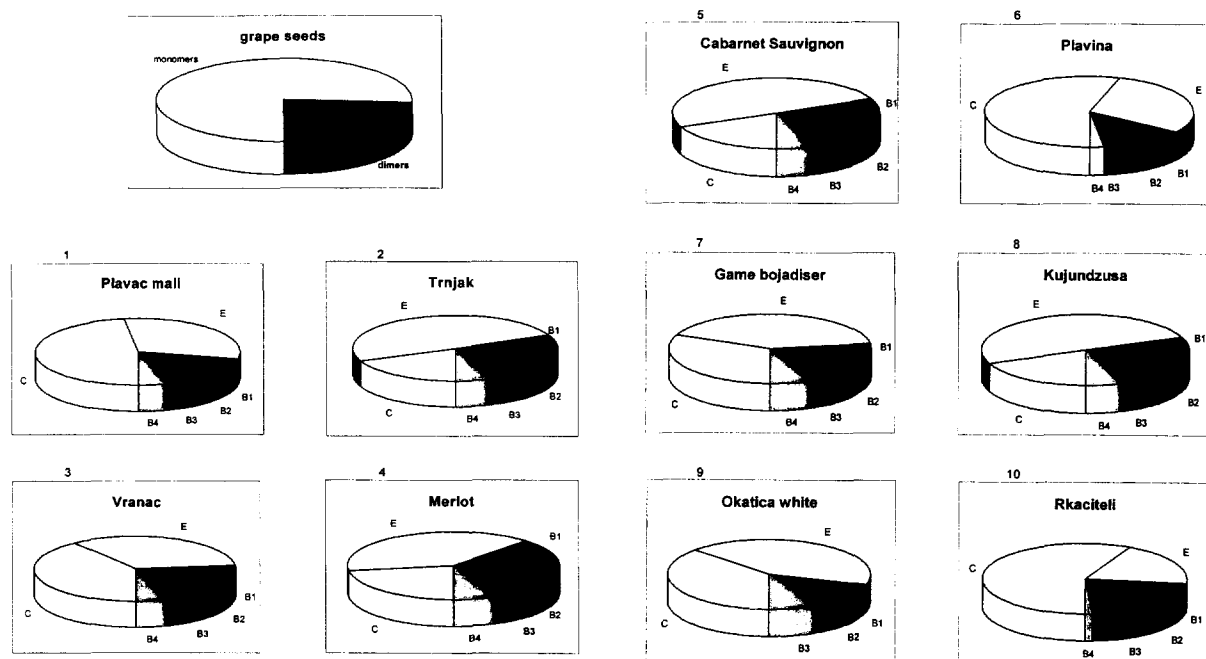

Fig. 2. The ratio of catechin monomers to procyanidin dimers B in the grape seeds extracts of different *Vitis vinifera* L. cultivars (average of 10 different cultivars of three vintages 1990–1992). (1–10) The average relative mass amounts of (+)-catechin (C), (−)-epicatechin (E), and procyanidin dimers B1, B2, B3, and B4 in the grape seed extracts of: 1, Plavac mali; 2, Trnjak; 3, Vranac; 4, Merlot; 5, Cabernet Sauvignon; 6, Plavina; 7, Game bojadiser; 8, Kujundžuša; 9, Okatica white; 10, Rkaciteli cultivars (vintages 1990–1992).

dimers of group B was ca. 3742 mg/kg grape seeds. The cultivars Plavac mali and Plavina, which had the highest procyanidin content in their grape seed extracts, are indigenous to Dalmatia.

The order of cultivars according to the average content of catechin monomers (C and E), expressed in mg/kg grape seeds, was as follows: Plavac mali cv. 7067, Plavina cv. 6021, Game bojadiser cv. 2741, Kujundžuša cv. 2342, Rkaciteli cv. 2274, Cabernet Sauvignon cv. 1901, Vranac cv. 1846, Merlot cv. 1706, Okatica white cv. 1443 and Trnjak cv. 956 mg/kg grape seeds.

The order of cultivars according to the average content of procyanidin dimers (B1, B2, B3, and B4) expressed in mg/kg grape seeds, was as follows: Plavac mali cv. 1961, Plavina cv. 1147, Kujundžuša cv. 1054, Merlot cv. 1054, Game bojadiser cv. 1034, Rkaciteli cv. 681, Vranac cv. 675, Cabernet Sauvignon cv. 527, Trnjak cv. 440 and Okatica white 372 mg/kg grape seeds.

The average relative mass amount of catechin monomers and procyanidin dimers in the grape seed extracts (10 different grape cultivars, vintages 90–92) is shown in Fig. 2. Catechin monomers were present in higher amounts than procyanidin dimers of the group B.

The average relative mass amount of (+)catechin, (−)-epicatechin, procyanidin B1, B2, B3, and B4 in the grape seed extracts, for each cultivar, are also shown in Fig. 2 (2.1–2.10). The dominating catechin monomer was cultivar dependent. The dominating procyanidin dimer in the grape seed extracts was procyanidin dimer B2. The results correlate with the results of Czochanska et al. [12], Lee and Jaworski 1987 [13], and Kovaè et al. [14].

According to the obtained results grape cultivars can be divided in the three groups with regard to the predominant monomer: 1, domination of (+)-catechin (Plavac mali, Plavina, Rkaciteli); 2, domination of (−)-epicatechin (Trnjak, Merlot, Kujundžuša); 3, relatively similar mass amounts of (+)-catechin and (−)-epicatechin (Vranac, Game bojadiser, Cabernet S., Okatica white).

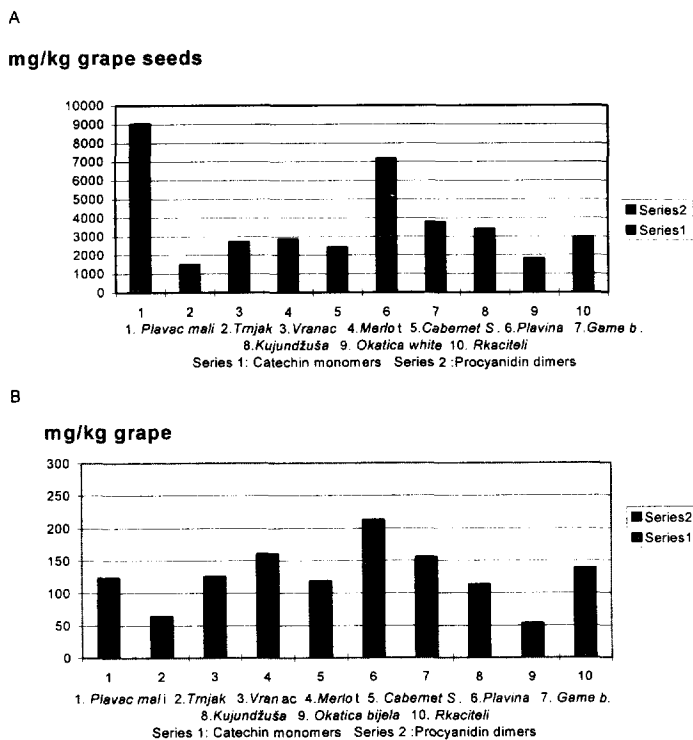Generally it can be said that cultivars with higher

Fig. 3. Average content of catechin monomers [(+)-catechin and (−)-epicatechin], and procyanidin dimers B (B1+B2+B3+B4) in the grape seed extracts of different *Vitis vinifera* L. cultivars. (A) mg/kg grape seeds; (B) mg/kg grapes.
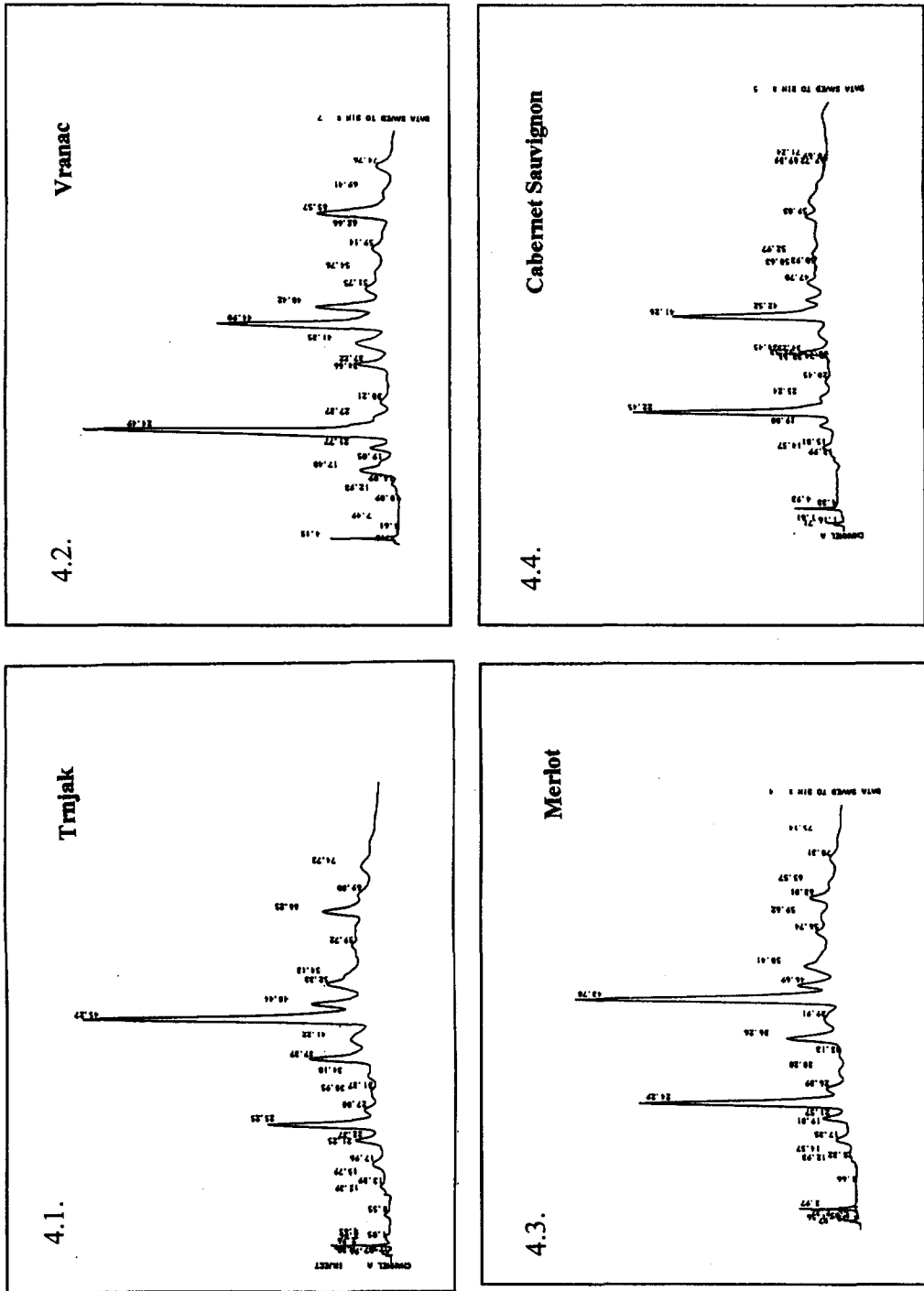
Fig. 4. RP-HPLC chromatograms of the neutral phenolic fraction of grape seed extracts of different *Vitis vinifera* L. cultivars.
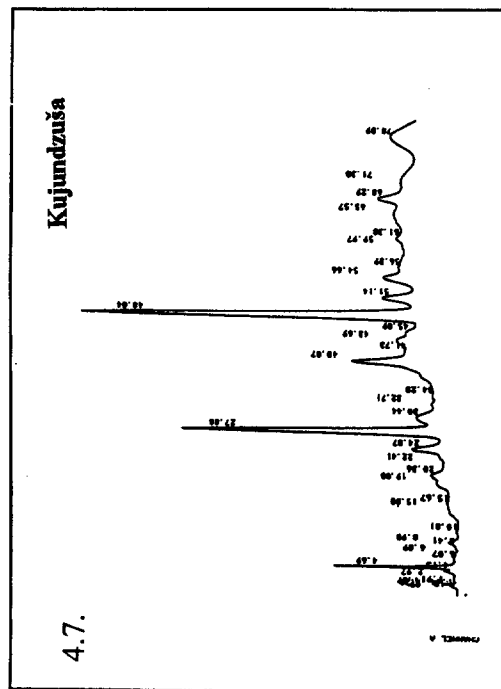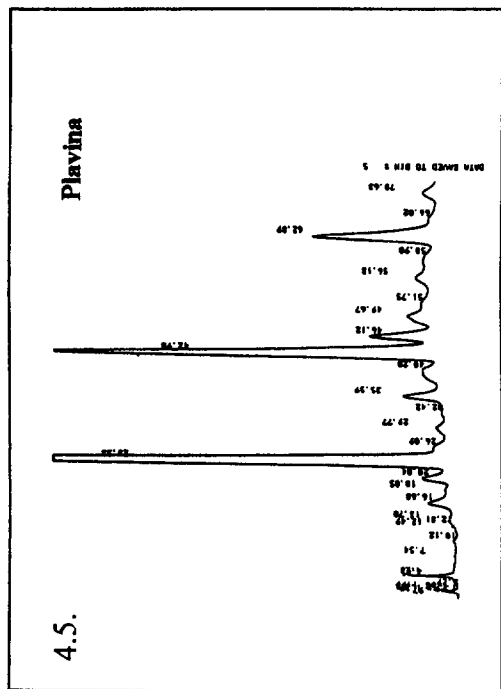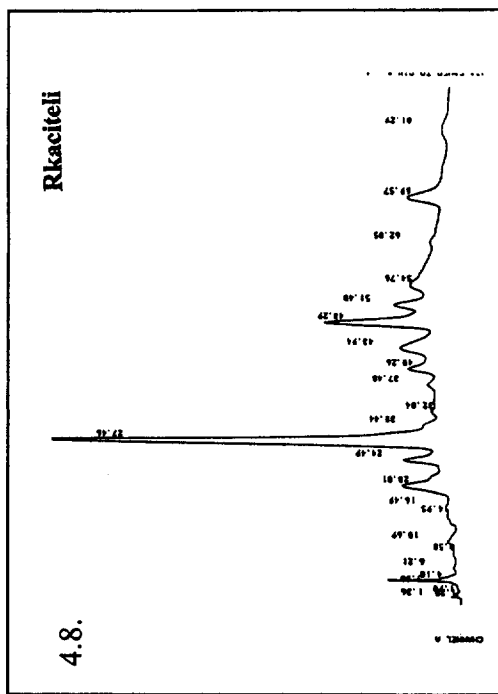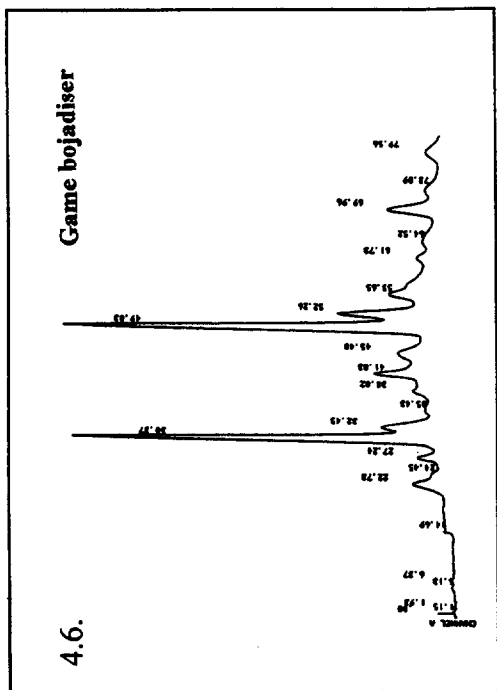
Fig. 4. (continued)

amounts of catechin monomers contain a higher amount of procyanidin dimers. If the content of catechin monomers and procyanidin dimers is expressed in mg/kg grape instead of mg/kg grape seeds these differences between cultivars are not so significant (Fig. 3). It seems that the cultivars with smaller amounts of seeds in the grapes (Plavac mali cv.) contain higher amounts of catechin monomers and procyanidin dimers.

The chromatograms of grape seed extracts for different grape cultivars are shown in Fig. 1a and Fig. 4. The grape seed extracts had a specific, similar flavan fingerprint. Predominant C, and procyanidin B1 are specific for the pine bark flavan fingerprint, Fig. 1(c), while high levels of catechin monomers (C and E) are specific for grape seed flavan fingerprints. Since grape seeds and grape stem vinification by-products, and pine bark, are the cheap raw material for the production of low molecular mass flavans, the obtained flavan fingerprints can be used as a useful tool for identifying the origin of plant extracts.

Can grape seed flavan fingerprints be used in chemotaxonomy for grape cultivar recognition? According to the Bourzeix et al. [11], for the grape seed extracts of Merolt cv., the predominance of C is characteristic. However, according to the results of this research the predominant catechin monomer in the grape seed extracts of Merlot cv. was E (vintages 1990, 1991, and 1992). Obviously, the flavan patterns, although they are specific for grape seed extracts, do not provide a definite answer with regard to grape cultivar recognition. Probably some more investigation has to be done to determine whether the soil or microclimate conditions can influence the mass amount of catechin monomers and their ratio.

4. Conclusions

The chromatographic analysis of a mixture of low molecular mass flavans in the neutral polyphenolic fraction allows a convenient separation of catechin monomers and procyanidin dimers B1, B2, B3, and

B4 in plant extracts. Plant bark and seed-coat samples can be analyzed by RP-HPLC. The method is relatively fast. The grape seed extracts were rich in catechin monomers [(+)-catechin and (−)-epicatechin] and contained a considerable amount of procyanidin dimers of the group B. The dominating catechin monomer was cultivar dependent. The predominant procyanidin dimer was B2. The obtained grape seed flavan fingerprints are specific and can serve in the control of the origin of plant extracts.

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