Short Communication

Identification of flavan-3-ols and procyanidins by high-performance liquid chromatography and chemical reaction detection

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Abstract

A method for characterizing flavan-3-ols and procyanidins by HPLC separation in connection with on-line UV detection and chemical reaction detection (CRD) is described. The post-column derivatization of the polyphenols with 4-dimethylaminocinnamaldehyde in the presence of sulphuric acid improves the sensitivity as compared to UV detection. The extent of improvement strongly depends on the structure of the procyanidin, hence resulting in characteristic absorbance ratios (CRD/UV) for the individual compounds under given reaction conditions. The ratios are further influenced by the solvent composition, by reaction time and by temperature.

1. Introduction

High-performance liquid chromatography (HPLC) is a valuable tool for the analytical separation of complex mixtures of naturally occurring phenolic compounds [1–4]. However, peak identification is often critical, even when the compounds are described in the literature to occur in a given plant or tissue. In many cases, HPLC analysis comprise more peaks than is expected from previous results obtained by TLC

or column chromatography. Accordingly, photo-diode array detection is frequently employed both for characterizing phenolic compounds and for checking the peak purity by their UV-absorbance spectra [5]. This approach appears convenient for the identification of simple phenols, hydroxycinnamic acids and most of the flavonoids, and even permits differentiation between flavan-3-ols possessing o-di- and trihydroxylated B-rings [6]. However, the UV-absorbance spectra of catechin, epicatechin and their oligomers, the variform procyanidins, do not exhibit any differences with respect to the usual UV-range from 250 to 400 nm.

This article deals with the characterization of flavan-3-ols and procyanidins using the selective post-column derivatization of flavan-3-ols by

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dimethylaminocinnamaldehyde as previously described [7] in combination with UV detection.

2. Experimental

The HPLC equipment consisted of two pumps T-414 (Kontron) and the gradient programmer 205 (Kontron). The column (250 × 4 mm I.D.) was prepacked in our laboratory with Shandon Hypersil ODS, 3 μm. The solvents were 5% formic acid (A) and gradient grade methanol (B) with a flow-rate of 0.5 ml/min.

The gradient profile used was: 0–5 min, isocratic, 5% B in A; 5–15 min, 5–10% B in A; 15–30 min, isocratic, 10% B in A; 30–50 min, 10–15% B in A; 50–70 min, isocratic, 15% B in A; 70–85 min, 15–20% B in A; 85–95 min, isocratic, 20% B in A; 95–110 min, 20–25% B in A; 110–140 min, 25–30% B in A; 140–160 min, 30–40% B in A; 160–175 min, 40–50% B in A; 175–190 min, 50–90% B in A.

Directly behind the column a Kontron filter detector (Uvikon 740 LC) was used for detection at 280 nm. Thereafter the eluent containing the phenols was mixed with the reagent in a simple T-connection. A Gynkotek HPLC pump (Model 300 C) moves the reagent at a flow-rate of 0.5 ml/min. For both the T-connection and the pump heads stainless steel is used. The reactors were knitted PTFE capillaries (0.5 mm I.D.) with different lengths. The PTFE capillaries have to be replaced after 4–5 months due to the occurrence of insoluble, blue to violet precipitations which can absorb phenolic compounds leading to peak tailing.

The blue reaction products were measured at 640 nm by a VIS-detector (Model SP6V, Gynkotek, Germany). The data of both chromatograms were evaluated simultaneously by a computer equipped with Gynkosoft chromatography software (Gynkotek).

For the heating experiments, a stainless-steel capillary (50 cm × 0.5 mm I.D.) was inserted between the T-connection and the PTFE-reactor. This short capillary was clamped between the open ends of the secondary coil of a labora-

tory-made low voltage/high current transformer and heated directly by an alternating current of approximately 20–30 A. The temperature was controlled electronically using a micro temperature probe attached to the capillary.

The reference compounds were either commercially available (catechin, Roth, Karlsruhe, Germany; epigallocatechin, epicatechin-3-O-gallate, epigallocatechin-3-O-gallate, Extrasynthèse, Lyon, France) or isolated from horse chestnut (epicatechin, procyanidins B2, B5, A2, C1, epicatechin-(4$\beta \rightarrow$ 8)-epicatechin-(4$\beta \rightarrow$ 6)-epicatechin, epicatechin-(4$\beta \rightarrow$ 8)-epicatechin-(4$\beta \rightarrow$ 8)-epicatechin-(4$\beta \rightarrow$ 8)-epicatechin, epicatechin-(4$\beta \rightarrow$ 8;2$\beta \rightarrow$ 7)-epicatechin-(4$\beta \rightarrow$ 8)-ent-epicatechin) after [8,9] and from *Prunus spinosa* (ent-epicatechin-(4$\alpha \rightarrow$ 8; 2$\alpha \rightarrow$ 7)-epicatechin, epicatechin-(4$\beta \rightarrow$ 8; 2$\beta \rightarrow$ 7)-epicatechin-(4$\beta \rightarrow$ 8)-ent-epicatechin) [10]. The procyanidins B3, B6 and C2 were synthesized according to ref. 11.

3. Results and discussion

3.1. Sensitivity of chemical reaction detection vs. UV-detection

Besides the advantage of high selectivity [7] which overcomes separation problems, the sensitivity after derivatization is generally better than that of UV-detection as can be seen in Fig. 1. This was verified for both peak area and peak height calculation. The maximal noise of 0.2 mAU for UV and 0.5 mAU for visible light detection gives an idea of the differences concerning the respective detection limits.

The calibration curves (Fig. 1) indicate the influence of the flavanoid structure on the absorbance of both UV (280 nm) and visible light (640 nm), before and after derivatization with 4-dimethylaminocinnamaldehyde (DMACA), respectively. At 280 nm, the molar extinction of epigallocatechin is much lower than that of catechin and epicatechin. This can be explained by the weak molar extinction of the pyrogallol-type B-ring of the former molecule, as compared to the analogous brenzcatechin element of the
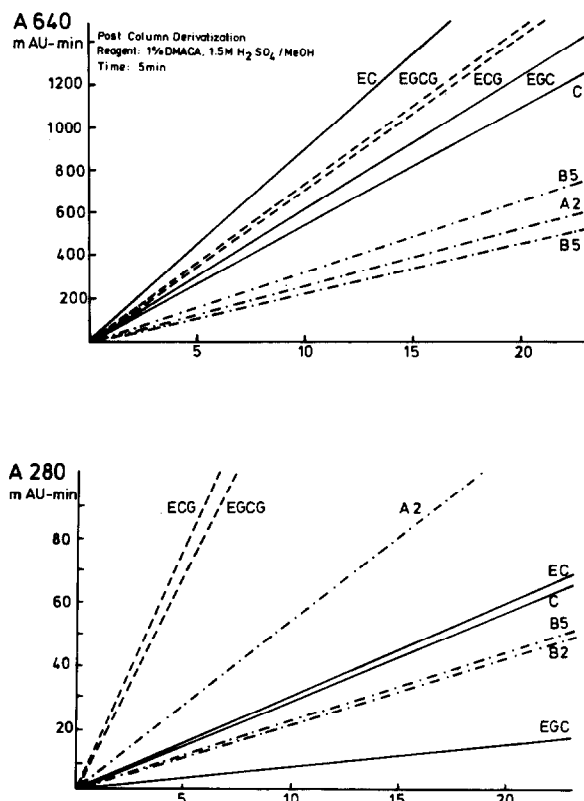
Fig. 1. Calibration curves of peak areas obtained at 280 nm and after derivatization with p-dimethylaminocinnamaldehyde at 640 nm. EC = epicatechin; EGCG = epigallocatechin-3-O-gallate; ECG = epicatechin-3-O-gallate; EGC = epigallocatechin; C = catechin. Units for x-axis: nM.

latter, exhibiting an absorbance about 4 times higher [12]. After derivatization, however, the absorbances of the reaction products of both phenols are quite similar.

Epicatechin and catechin show nearly identical curves at 280 nm. In contrast, the difference in absorbance at 640 nm is pronounced. This peculiar observation is only explicable in terms of the stereochemical difference of the 3-epimers despite of the fact that the chiral centre is relatively far removed from the site of reaction with the aldehyde.

The relatively high values of the epicatechin-3-O-gallate and the epigallocatechin-3-O-gallate at 280 nm are due to the gallic acid moiety which significantly contributes to the overall absorbances. This coincides with the observation of the

strong UV-absorbance of both benzoic and hydroxycinnamic acids [13].

For the dimers, the doubly linked procyanidin A2 differs from the singly linked representatives B2 and B5 with respect to UV-absorption (Fig. 1). By contrast, all these procyanidins with their 2,3-cis constituent units show similar behaviour following post column derivatization with DMACA.

From these data it can be concluded that the ratio of the absorbance before and after derivatization is structurally related and is constant for each single structure under given conditions.

3.2. Influence of reaction time and solvent composition

As shown in Fig. 2, the reaction kinetics of various structures obtained in a conventional colorimetric assay are different and depend on the acid concentration. This may partially contribute to the results shown in Fig. 1. In order to transfer the reaction kinetics to HPLC-conditions, the flow injection mode was chosen using several reactors each with a different length (Table 1). Previously it was shown that the presence of methanol in the mobile phase accelerates the formation of the coloured product [7] resulting in higher ratios after 1.5 min reaction time. Replacement by a longer reactor chamber permitting 5 min reaction time leads to higher ratios only in instances of eluents containing 5% methanol, whereas a 15% methanol content already favours discoloration resulting in reduced ratios for all three compounds tested. However, if methanol in the reagent was replaced by glacial acetic acid, as proposed in ref. 14 for the vanillin reagent, the procyanidin A2 exhibits its maximum values after 5 min.

3.3. Influence of temperature

Instead of extending the reaction time, an increase in temperature appears to be an alternative for obtaining values characteristic for individual procyanidins (Table 2). The effect of temperature elevation on the CRD/UV ratio is most pronounced for the procyanidin A2. This
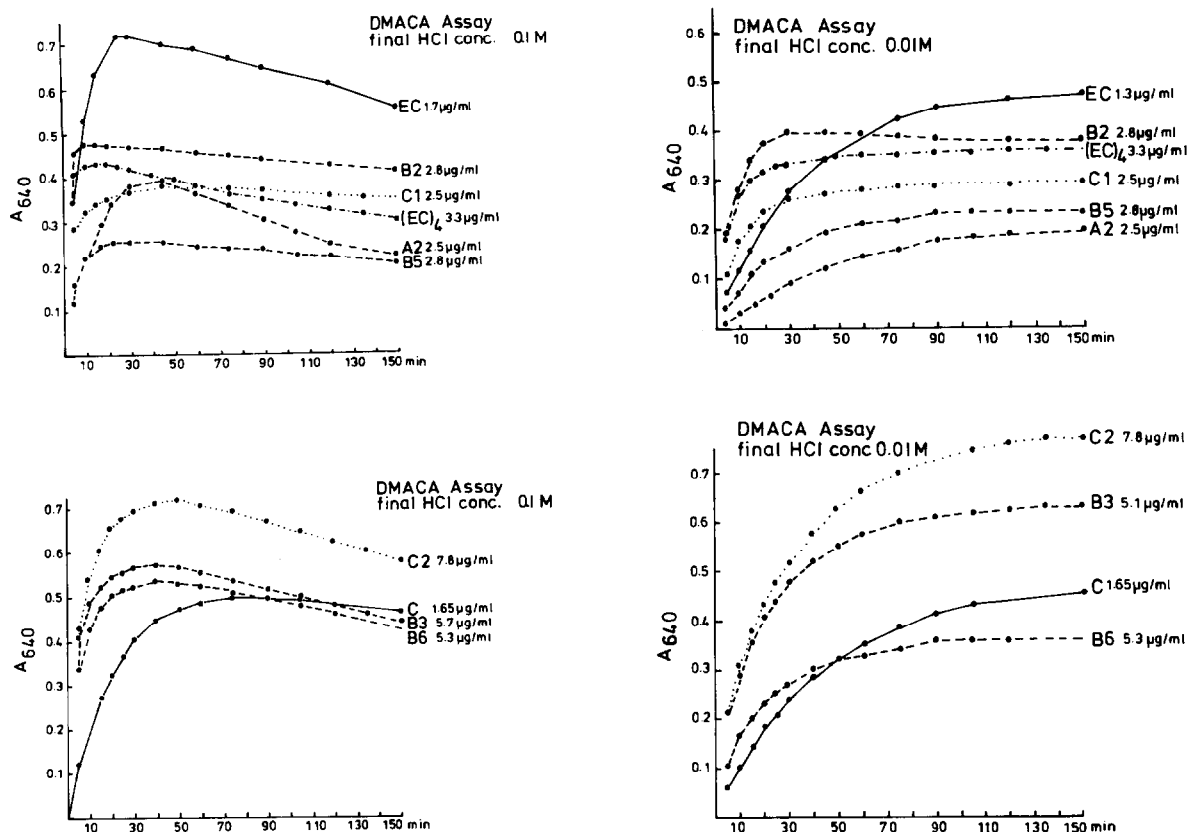
Fig. 2. Structure dependent kinetics of the aldehyde reaction at two acid concentrations using a colorimetric test. Reaction mixture: 1500 μl MeOH containing the flavanol, 300 μl reagent (2 mg DMACA per ml EtOH–6 M HCl, 9:1 or 99:1, respectively). Measurement at 640 nm in a Kontron spectrophotometer.

remains even true for the long reaction time of 5 min, i.e., conditions under which the ratio for epicatechin already decreased. These observations can be interpreted as a synergistic effect of both prolonged reaction time and increased temperature. In general: the shorter the reaction time, the more significant is the effect of heating on the CRD/UV ratio.

3.4. Structural dependence of the CRD/UV ratio under HPLC conditions

Table 3 shows the ratio of the peak areas of a series of monomeric flavan-3-ols and oligomeric procyanidins obtained after and before derivatization. As mentioned above, the ratio for epigallocatechin is very high, due to its low UV-

absorbance. On the other hand, galloylation enhances the UV-absorption resulting in reduced ratios for the 3-O-gallates (Table 3).

The degree of oligomerization generally decreases the ratio within a series of 4→8-linked oligoflavanoids, e.g. 2,3-cis, 2,3-trans and A-type procyanidins, respectively.

As compared to procyanidins with 4→8-linkages, those with 4→6-interflavanyl bonds show enhanced ratios, whereas doubly linked procyanidins such as A2 exhibit very low values.

The observation that the chain extending unit of a given procyanidin greatly influences the ratio is evident from procyanidins of mixed stereochemistry (B1 and B7) consisting of epicatechin and catechin constituent units. Here, the lower terminal catechin unit apparently does

Table 1
Influence of reaction time and solvent composition on the peak height ratio (CRD/UV)

Conditions	Peak height ratio		
	Epicatechin	Procyanidin B2 E(4β→8)E	Procyanidin A2 E(4β→8;2β→O→7)E
Reagent: 1% DMACA in 1.5 M sulphuric acid in methanol			
Reaction time: 1 min; solvent composition[a]: 5%	4.16	1.52	0.41
Reaction time: 1 min; solvent composition[a]: 15%	4.23	1.50	0.45
Reaction time: 1.5 min; solvent composition[a]: 5%	6.97	2.16	0.71
Reaction time: 1.5 min; solvent composition[a]: 15%	7.67	2.26	0.77
Reaction time: 5 min; solvent composition[a]: 5%	10.9	2.23	0.78
Reaction time: 5 min; solvent composition[a]: 15%	8.13	1.69	0.67
Reagent: 1% DMACA in 1.5 M sulphuric acid in glacial acetic acid			
Reaction time: 5 min; solvent composition[a]: 5%	10.3	1.83	0.91
Reaction time: 5 min; solvent composition[a]: 15%	9.5	1.4	0.9

The flow injection mode in combination with reactors of different tube lengths was employed. The apparatus and the reaction conditions are described in the Experimental section. Each value represents the mean of 3 to 5 replicates.
[a] Solvent composition: B (methanol) in A (5% formic acid).

Table 2
Influence of temperature on the peak height ratio after and before derivatization (640/280)

| | Reaction time | | | | | | | |
| | 1 min | | 1.5 min | | | | 5.0 min | |
	30°C	90°C	30°C	50°C	70°C	90°C	30°C	90°C
Epicatechin	4.1	7.0	8.1	8.7	9.7	10.2	7.2	6.5
Procyanidin B2	1.4	1.6	2.2	2.3	2.3	2.3	1.5	1.6
Procyanidin A2	0.3	0.7	0.7	1.0	1.1	1.1	0.4	0.6

The apparatus is described in the Experimental section. The flow injection mode was employed.

not influence the ratio, compared to their homogeneous 2,3-*cis* analogues B2 and B5, respectively (Table 3).

The relative low ratios of 2,3-*trans* compounds as compared to those with 2,3-*cis*-configuration may at least in part be rationalized on the basis of kinetic differences, *i.e.* the somewhat delayed reaction of 2,3-*trans* procyanidins with the alde-

Table 3
Influence of the structure on peak area ratio (CRD/UV) and retention time

Common name	Structure	Ratio 640/280, reaction time 2 min	Retention time without CRD	Elution order (peak number in Fig.3)
2,3-cis Series				
Epigallocatechin		98.8	31.3	4
Epigallocatechin-3-O-gallate		3.2	49.5	6
Epicatechin-3-O-gallate		2.8	85.4	11
Epicatechin		20.9	55.6	7
Procyanidin B2	E(4$\beta\to$8)E	10.9	41.2	5
Procyanidin C1	E(4$\beta\to$8)E(4$\beta\to$8)E	7.7	63.6	9
	E(4$\beta\to$8)E(4$\beta\to$8)E(4$\beta\to$8)E	3.8	67.9	
Procyanidin B5	E(4$\beta\to$6)E	14.5	112.0	14
	E(4$\beta\to$8)E(4$\beta\to$6)E	14.3	126.9	15
2,3-trans Series				
Catechin		12.4	28.5	3
Procyanidin B3	C(4$\alpha\to$8)C	5.5	20.7	1
Procyanidin C2	C(4$\alpha\to$8)C(4$\alpha\to$8)C	3.4	20.7	1
Procyanidin B6	C(4$\alpha\to$6)C	6.3	31.3	4
A-types				
Procyanidin A2	E(4$\beta\to$8;2$\to$O$\to$7)E	2.9	100.5	13
	entE(4$\alpha\to$8;2$\alpha\to$O$\to$7)E	2.0	94.2	12
	E(4$\beta\to$8;2$\beta\to$O$\to$7)E(4$\beta\to$8)entE	1.9	49.5	6
	entE(4$\alpha\to$8;2$\alpha\to$O$\to$7)C	1.8	70.9	10
Stereochemically mixed procyanidins				
Procyanidin B1	E(4$\beta\to$8)C	9.7	24.0	2
Procyanidin B7	E(4$\beta\to$6)C	11.9	57.5	8

hyde reagent (Fig. 2). Additionally, the composition of the eluent has to be considered. For instance, the aqueous proportion of the reaction mixture decreases, while the amount of methanol increases with time and *vice versa*. Since the concentration of methanol strongly influences the reaction kinetics [7], all compounds which are retarded by the stationary phase find improved conditions for the derivatization as specified in the Experimental section.
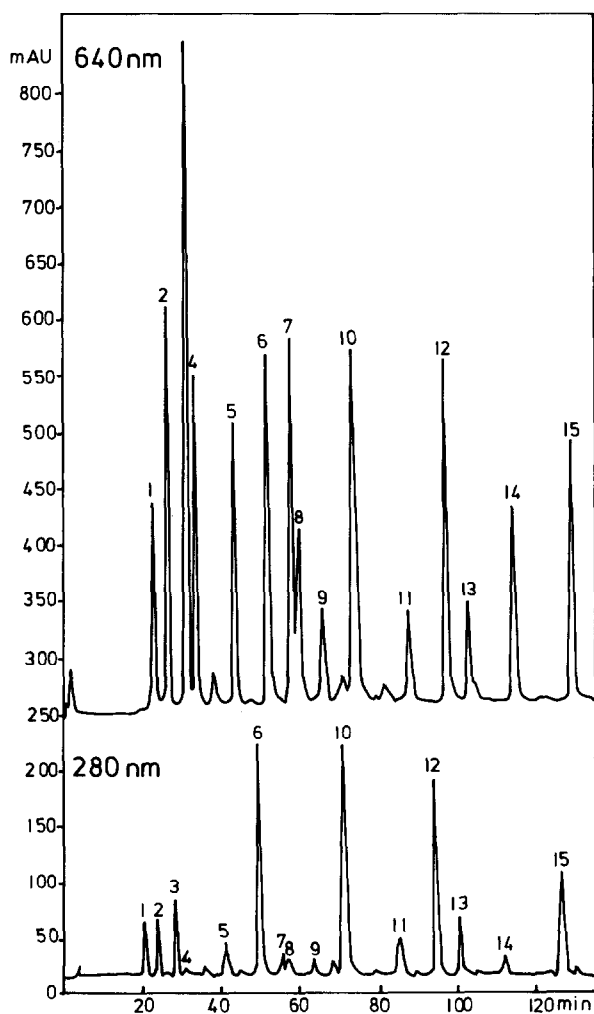

Fig. 3. HPLC separation of a synthetic mixture of catechins and procyanidins with UV-detection (280 nm) followed by derivatization with DMACA resulting in coloured products recorded at 640 nm. The method is described in the Experimental, for peak description *cf.* Table 3.

The presence of enantiomeric (2*S*) constituent units in A-type procyanidins has very weak influence on the CRD/UV ratio. Noteworthy is the marked effect on the retention time (Fig. 3). This may presumably be explained in terms of their overall configuration, associated with differences in the number of intramolecular hydrogen bridges. The elution sequence of the remaining oligomeric flavan-3-ols follows common rules observed by many authors [2,15–19]. Under the experimental conditions, flavan-3-ols and their oligomers possessing 2,3-*trans*-configuration elute earlier than their corresponding 2,3-*cis* analogues. Oligoflavanoids with 4→6 intramolecular linkages and doubly linked procyanidins (A-types) show relatively high t_R values when comparing structures with the same degree of oligomerization.

4. Conclusion

The method described assists in characterizing flavan-3-ol and procyanidin peaks in HPLC analysis, providing satisfactory resolution. Simultaneously, it may be applied for a purity check of compounds following various fractionation procedures. It should also prove helpful in unambiguous identification of procyanidins in plant extracts, even in complex mixtures.

5. Acknowledgements

6. References

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