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CHEMICAL REACTION DETECTION OF CATECHINS AND PROANTHOCYANIDINS WITH 4-DIMETHYLAMINOCINNAMALDEHYDE

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SUMMARY

An high-performance liquid chromatographic method with post-column derivatization is described which allows the specific detection of catechins and proanthocyanidins in crude extracts from plants and beverages. In the presence of concentrated sulphuric acid, 4-dimethylaminocinnamaldehyde can be employed as a selective reagent. The advantage of the reagent is that its condensation products with flavanols show maximum absorbance at about 640 nm. Other phenols, indoles and terpenes give reaction products with different absorbances or react very weakly. A 200–40 000 fold sensitivity was found for (−)-epicatechin as compared to other phenols and substituted indoles. Concerning the terpenes, this factor ranges from 4000 (for the aromatic thymol) to $2 \cdot 10^6$.

INTRODUCTION

Catechins and their oligomeric forms, namely the proanthocyanidins (condensed tannins), are an heterogeneous group of secondary compounds[1,2] which are widespread in the plant kingdom[3–5]. The astringency of these flavanols is well known in fruits[6–8]. The content of catechins is an important factor in determining the quality of juices[9] and wines[10–12]. The oligomeric proanthocyanidins play a rôle in the durability of beers[13–15]. All the above mentioned observations are related to the ability of flavanols to precipitate proteins[16]. This precipitation reaction is probably also responsible for the participation of catechins in plant defence mechanisms[17,18].

For these reasons it is often necessary to know the composition of the various catechins and condensed tannins in plant tissues and to monitor their structural variation during beverage processing[19–23] or during the wound response of plants[24,25]. In the latter case, oxidation processes often lead to oligomerization and polymerization[1] and diseased plant tissues sometimes show an enhanced synthesis of flavanols[26].

The analytical method normally used to estimate the amount of catechin and its derivatives is the colorimetric measurement of their total content after reaction with aromatic aldehydes[27–29] in a test-tube. The qualitative pattern of these phenols can be determined by thin-layer chromatography using the known aromatic aldehydes[30–32]

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or the trinitrophenol–potassium hydroxide reagent[33] for visualization. However, the quantification of each compound normally requires its purification from other phenolic compounds. A prepurification has been carried out by several authors[34,35]. In high-performance liquid chromatographic (HPLC) analysis of flavanols extracted from plants, which are rich in phenols, the main problem is the rather low molar extinction of catechins as compared to phenolic acids. The cinnamic acids also show similar retention behaviours and often occur in plants in a more concentrated form than the flavanols. Lea[36] solved this problem using a pH-shift technique during the HPLC separation of apple juices. A chemical reaction detection of flavanols by using 4-dimethylaminocinnamaldehyde (DMACA) after their preparative separation on a Sephadex column has been described by McMurrough and McDowell[37] and McMurrough[38].

This paper deals with the post-column derivatization of catechins and proanthocyanidins for their selective detection following analytical HPLC separation of crude plant extracts and beverages.

EXPERIMENTAL

The HPLC equipment consisted of two pumps T-414 (Kontron) and the gradient programmer 205 (Kontron). The column (250 mm × 4 mm I.D.) was prepacked with Shandon Hypersil ODS, 3 μm. The solvents were 5% acetic acid (A) and methanol (B).

Gradient range: 0–5 min, isocratic, 5% B in A; 5–10 min, 5–10% B in A; 15–25 min, 10–15% B in A; 25–35 min, isocratic, 15% B in A; 35–37 min, 15–20% B in A; 37–45 min, isocratic, 20% B in A; 45–55 min, 20–30% B in A; 55–70 min, 30–45% B in A; 70–90 min, 45–90% B in A.

Because of the corrosive reagent, an inert HPLC pump (Gynkotek, F.R.G.) was used. It was equipped with titanium pump heads. Capillaries and screws were both made of PTFE. The reactor was a knitted PTFE capillary (9 m × 0.5 mm I.D.) as described by Engelhardt and Klinkner[39]. The substrate–reagent mixing was performed by a simple T-connection (titanium). The compounds were detected with an inert UV–VIS detector (Gynkotek, F.R.G.).

The estimation of the absorbance maximum and the wavelength ratio (640:620 nm) was performed with a Beckman Model 24 spectrophotometer.

RESULTS AND DISCUSSION

Application possibilities of aldehyde reagents

The reactivity of aldehydes in solutions containing strong mineral acids as well as the colour reactions of aromatic aldehyde have long been used to detect many different substances. Unsaturated compounds such as phenols[40–46], pyrroles and indoles[41,44] as well as some terpenes[42,43,45,47,48] were reported to react with aldehydes. Additionally, aliphatics, *i.e.*, alcohols, ketones may be converted into olefins under the influence of mineral acids and may then be sensitive to the aldehyde reaction[43,47,49].

In spite of these findings, colour reactions of aromatic aldehydes have often been employed specifically, such as for flavanols[29,38,45,50–57] or for indoles[58–61].

TABLE I

ABSORBANCE OF THE CONDENSATION PRODUCTS OF DMACA WITH (−)-EPICATECHIN IN THE PRESENCE OF VARIOUS ALCOHOLS

Concentration of sulphuric acid was 1.5 *M* in the corresponding alcohol.

Alcohol	*Wavelength of maximum absorbance (nm)*
Methanol	632
Ethanol	636
Propanol	638
Butanol	640

Mode of action

Principally, when dissolved in strong acids, aldehydes become electrophilic and therefore very reactive. The reaction mechanism with formaldehyde and phenols has been clarified by Finn and James[62] and by Hillis and Urbach[63]. However, such aromatic aldehydes, which are substituted, show a reduced reactivity, as compared to formaldehyde, because of the possible delocalization of the positive charge[64,65]. This requires an activated aromatic ring of the substrate, *i.e.*, of the phloroglucinol type in order to obtain optimum condensation reaction with phenols.

DMACA has the advantage that its reaction product with catechin shows an absorbance maximum between 632 and 640 nm depending on the solvent (Table I). Other aldehydes, commonly used, lead to absorption at a shorter wavelength (Table II), so that anthocyanidins or other substances which yield a red colour in the presence of acid may interfere. Moreover, the molar extinction of the products yielded with DMACA is about 1000 times higher than that with 4-dimethylaminobenzaldehyde[51].

Optimization of the derivatization system

As shown by several authors[51,60], the reaction with DMACA depends on the concentration of acid and alcohol. Since in the HPLC separation of flavanols a gradient system is necessary, the reaction conditions change during an experiment. Methanol both accelerates the reaction and increases the extinction value. However, after reaching the maximum absorbance, the extinction declined, which may be

TABLE II

ABSORBANCE OF THE CONDENSATION PRODUCTS OF VARIOUS ALDEHYDES WITH (−)-EPICATECHIN IN THE PRESENCE OF 0.075 *M* SULPHURIC ACID IN METHANOL

Aldehyde	*Wavelength of maximum absorbance (nm)*
Anisaldehyde	455
Vanillin	490
4-Dimethylaminobenzaldehyde	510
Syringaldehyde	515
4-Dimethylaminocinnamaldehyde	632

explained by a superimposed decomposition of the condensation product. The latter is also influenced by the acid and the alcohol concentration (Fig. 1). Water, acetonitrile and acetone inhibit the formation of the coloured product (Fig. 2).

In order to obtain a good sensitivity, 1% DMACA in 1.5 *M* methanolic sulphuric acid was used. The length of the knitted capillary reactor was 9 m, resulting in a reaction time of 90 s.

Selectivity and sensitivity

The use of the DMACA reagent for the specific detection of catechins demands knowledge of the relative sensitivity of other substances which are also known to give coloured products with aromatic aldehydes.

For this purpose the method of flow injection analysis[39] was used. Except for omission of the column, the system was the same as that described for the separation procedure. The solvent normally consisted of 40% methanol in 1% aqueous acetic acid. Only for some phenols and terpenes, butanol–methanol (1:5, v/v) was used as the solvent and the reagent was dissolved in butanol (containing 1.5 *M* sulphuric acid) to prevent demixing. For each compound a calibration graph was constructed to estimate the relative sensitivity.

Possible interference with catechins was shown to depend on the activation of the phenol group which determines the sensitivity towards the reagent. For this reason,

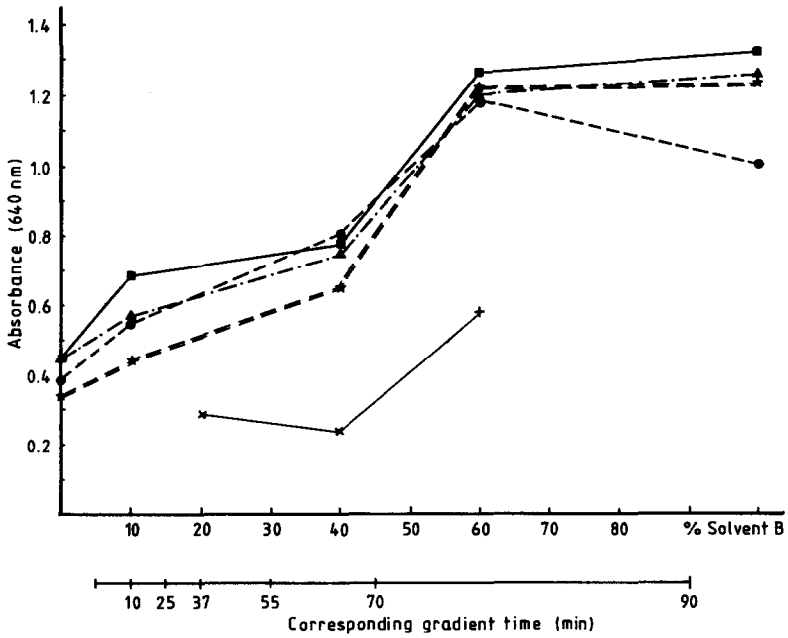

Fig. 1. Influence of the solvent composition and the reaction conditions (concentration of sulphuric acid in the 1% DMACA reagent; reaction time) on the detection sensitivity (640 nm) for (−)-epicatechin. Each data point represents the mean of four injections during flow injection analysis without the column. The injected flavanol was dissolved in that solvent which corresponded to the flow condition used at each point. (■) 0.65 *M*, 90 s; (▲) 1.15 *M*, 120 s; (●) 1.65 *M*, 60 s; (★) 5.30 *M*, 90 s; (×) 2.55 *M*, 90 s.

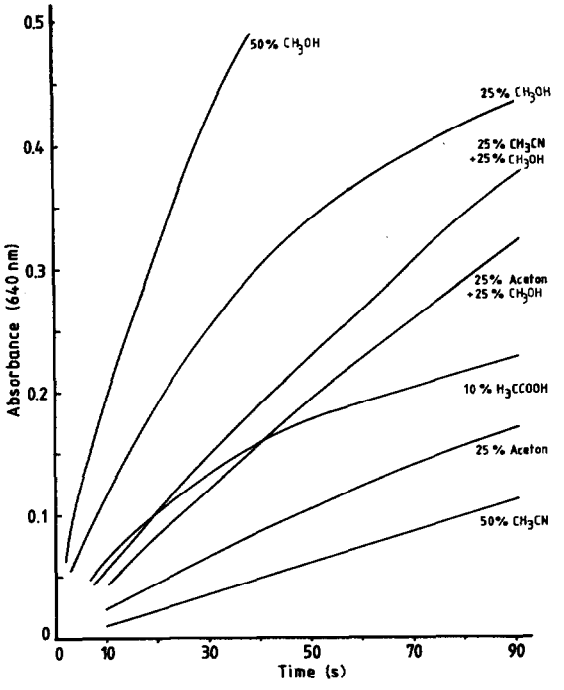

Fig. 2. Influence of the solvent on the reaction kinetics of DMACA (0.5%) with (+)-catechin (10 μg/ml) in the presence of 0.75 *M* H_2SO_4. The solvent was made up to 100% with water.

flavonoids with a carbonyl function at C4, *i.e.*, naringenin show a rather weak reaction (Table III) as already shown by Sarkar and Howarth[54]. The high sensitivity of indole was diminished by substitution at the pyrrole ring, *cf.*, tryptamine. Additionally, the relative sensitivity was affected by chromophoric groups and their binding sites.

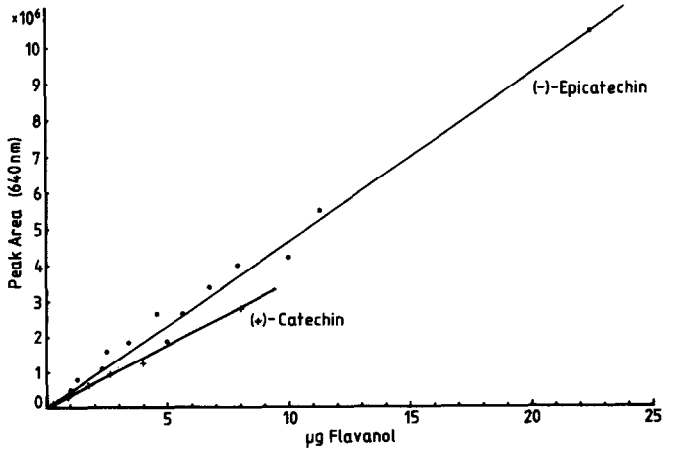

Fig. 3. Calibration graphs for (+)-catechin and (−)-epicatechin. Detection after chemical reaction with DMACA (1% in 1.5 *M* methanolic suphuric acid); for separation conditions see Experimental.

TABLE III

RELATIVE SENSITIVITY OF THE DMACA REAGENT TOWARDS VARIOUS SUBSTRATES

Flow injection conditions: solvent, 40% aqueous methanol; flow-rate 0.6 ml/min; reagent, 1% DMACA in 1.5 *M* sulphuric acid in methanol, flow-rate 0.6 ml/min.

Common name	*Structural name*	*Amount resulting in a peak of 0.04 a.u. at 640 nm (μM)*	*Relative sensitivity*[a]	*Absorbance maximum*	*Ratio 640/620 nm*
(−)-Epicatechin	3,3′,4′,5,7-Flavanpentol	$0.2 \cdot 10^{-3}$	1000 000	632	1.15
Indole		$0.4 \cdot 10^{-3}$	500 000	620	0.71
Orcinol	3,5-Dihydroxytoluol	$26 \cdot 10^{-3}$	7692	628	0.90
Phloroglucinol	1,3,5-Trihydroxybenzol	$35 \cdot 10^{-3}$	5714	618	0.51
Tryptamine	3-(2-Aminoethyl)indole	$38 \cdot 10^{-3}$	5263	576	0.72
Resorcinol	1,3-Dihydroxybenzol	$51 \cdot 10^{-3}$	3921	628	0.96
Pyrogallol	1,2,3-Trihydroxybenzol	$58 \cdot 10^{-3}$	3448	630	0.97
Catechol	1,2-Dihydroxybenzol	$60 \cdot 10^{-3}$	3333	617	0.42
Serotonine	5-Hydroxytryptamine	$160 \cdot 10^{-3}$	1250	594	0.67
Thymol[b]	2-Isopropyl-5-methylphenol	$800 \cdot 10^{-3}$	250	636	1.22
Naringenin	4′,5,7-Trihydroxyflavanone	1.8	111	570, 614	0.47
Naringin	Naringenin-7-glucosidorhamnoside	8.5	24	626	0.89
Terpinene[b]	1,3-*p*-Menthadiene	27	7.4	595	0.91
Citral[b]	3,7-Dimethyl-2,6-octadienal	54	3.7	575	0.76
Linalool	3,7-Dimethyl-1,6-octadien-3-ol	61	3.3	604	0.32
Camphene[b]	2,2-Dimethyl-3-methylenebicyclo[2.2.1]heptane	62	3.2	510	0.50
Geraniol[b]	*trans*-3,7-Dimethyl-2,6-octadienol	110	1.8	595	0.73
Guaiacol	*o*-Methylphenol	178	1.1	504	0.76
D-Limonene[b]	1,8(9)-*p*-Menthadiene	215	0.9	594	0.64
Menthol[b]	1-Methyl-4-isopropylcyclohexan-3-ol	384	0.5	600	0.53

[a] Calculated from the values in column 3 of the table as [epicatechin (μM)]/[other compound (μM)] · 10^6.

[b] Use of a butanol-containing solvent and reagent, to prevent demixing.

Another factor, which plays an important rôle in analyzing plant extracts, is the volatility of some compounds. During the concentration procedure with an evaporator, volatile substances such as indole and some terpenes disappear and do not disturb the catechin detection further. If any uncertainty about the catechin nature of a peak remains, an absorbance ratio between 640 and 620 nm for instance (Table III) may be helpful.

All these facts summarized in Table III led to the conclusion that the DMACA reagent described can be used for specific chemical reaction detection of catechin and oligomeric proanthocyanidins. It has been shown that the detector responded linearly to the signal (Fig. 3). For epicatechin the detection limit was 2.5 ng with a signal-to-noise ratio of 2.

Fig. 4 shows the separation and selective detection of the catechins and proanthocyanidins extracted from a chinese tea which is known to be rich in flavanols[66,67]. In Fig. 5 two chromatograms of the phenols of a bottled beer are compared with the detection at 280 nm (upper part) and 640 nm after chemical

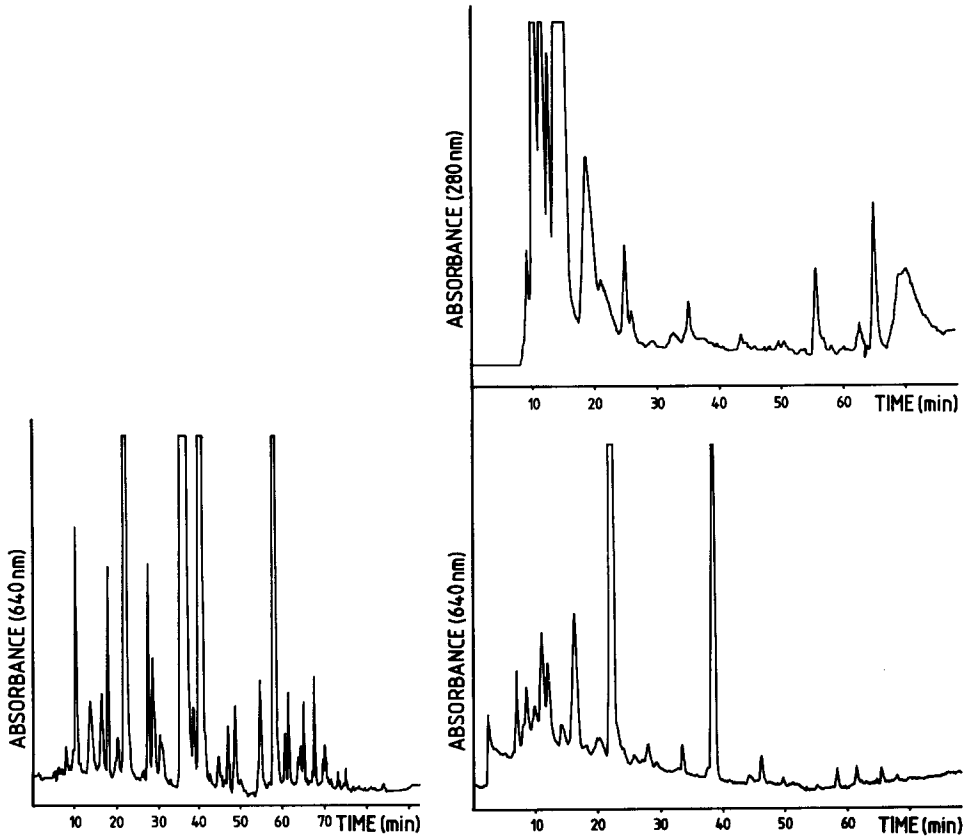

Fig. 4. HPLC separation and chemical reaction detection with DMACA of a phenolic extract from 1.5 mg dry chinese tea (*Camellia sinensis*).

Fig. 5. HPLC separation of the phenolic compounds of 0.5 ml bottled beer (concentrated to 10 μl) with detection at 280 nm (above) and after chemical reaction with DMACA at 640 nm (below).

reaction with DMACA. The UV-absorbance spectra of the main peaks of the 280-nm chromatogram (measured with a diode array detector) showed a maximum between 260 and 270 nm. From this one can conclude that the main peaks with short retention times are structurally not related to flavanols and that they overlap the catechins and proanthocyanidins. The latter were visualized with DMACA, resulting in the lower chromatogram (Fig. 5).

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