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Note

Analysis of catechin and epicatechin by high-performance liquid chromatography after benzoylation

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The flavan-3-ols catechin and epicatechin are widespread in nature, for example, as naturally occurring monomers of tannins in wood plants[1,2]. The analysis of catechin and epicatechin from plant material has recently been carried out by means of reversed-phase high-performance liquid chromatography (HPLC) after extraction with methanol or methanol–water and purification on polyamide. The HPLC separation is effected by gradient elution with 2% acetic acid–methanol and detection at 280 nm[3–5]. The method of sample clean up on polyamide was optimal for catechins as well as for the ubiquitous hydroxycinnamic acid derivatives[5]. To confirm the results obtained, a second method of similar quality is required for the determination of catechins. To separate catechins quantitatively from aqueous solutions RP-18 cartridges have been tested as has the possibility of catechin detection as their benzoate derivatives. RP-18 cartridges are often used for rapid and selective clean up and/or accumulation procedures[3,6–12]. The isolation and detection of different polyhydroxy compounds from food after benzoylation have been described in earlier publications[9–12].

EXPERIMENTAL

Chemicals

Catechin and epicatechin are commercially available substances. The solvents used for HPLC were of analytical reagent grade purified with Extrelut® (Merck, Darmstadt, F.R.G.). All evaporations were performed in a rotary vacuum evaporator at a temperature not higher than 40°C.

Sample preparation

An 100-g amount of the fruit material without stones (cores) was cut into small pieces and frozen. The frozen or freeze-dried tissue was homogenized using an household blender and extracted with 400 ml of methanol. The residue was extracted twice more with 400 ml of 80% aqueous methanol. The pooled extracts were evaporated. The residual aqueous solution was made up to 100 ml with water. The resulting concentrations are 1.0 g fruit/ml solution. In the case of pome fruit, freeze drying helps to avoid enzymatic browning and allows more rapid extractions and separations of the pulp through a P-4 glass frit.

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Clean up on polyamide

A methanolic suspension of polyamide (MN-SC-6, 0.05–0.16 mm; Machery & Nagel, Düren, F.R.G., without traces of iron), was poured into glass columns (250 mm × 27 mm I.D.) used for separation. For preliminary treatment, the column was flushed with water. A 25-ml volume of the aqueous extract was applied to the column, washed with 150 ml of water to remove carbohydrates, acids and salts.

Catechins besides hydroxycinnamoylglucoses and glucosides were eluted from the column with 300 ml of methanol, concentrated and diluted to 25 ml of aqueous solution. These solutions (microfiltered, Millipore-Filter 0.2 μm; Sartorius, Göttingen, F.R.G.) were used for direct HPLC analysis or for separation and concentration with RP-18 cartridges. For quantitation of catechins it was necessary to insert the aqueous solutions immediately.

RP-18 cartridges

Extract solutions of pome and stone fruit in concentrations of g/ml were applied to Sep-Pak RP-18 cartridges (Waters Assoc., Milford, MA, U.S.A.). The cartridges were conditioned with 2 ml of methanol and 8 ml of water by removing them from each solvent by pressing with a syringe. The separation potential was not influenced by repeated use (tested five times). Plastic syringes with a capacity of 10 ml were used.

Benzoylation

The benzoylation procedure is described in several publications[9–12]. The reagents used were those for chemical reaction with 50 mg of solids or 30 μl of liquids. The eluate containing catechins obtained with or without cartridges was carefully evaporated to dryness before benzoylation. Reagents and conditions for benzoylation: dissolution in 4 ml of pyridine addition of 0.5 ml of benzoyl chloride, reaction in an ultrasonic apparatus at 60°C for 1 h; addition of 0.5 ml of methanol, precipitation of benzoates with 50 ml of water, separation with RP-18 cartridges; flushing with water in portions (4 × 5 ml); final elution of the benzoates with isooctane–diethyl ether–acetonitrile (150:80:20) made up to 50 ml in a graduated flask. This solution is used for HPLC.

Analytical HPLC

The HPLC analysis was carried out with an isocratic system. A Beckman pump 114 M or an LC-XPD-pump (Pye Unicam, Kassel, F.R.G.) was used with a flow-rate of 0.8 ml/min. The 7125 injection valve (Rheodyne, Berkeley, CA, U.S.A.) was equipped with a 20-μl sample loop. Chromatograms, spectra and results were registered with a diode-array detector (Philips, Kassel, F.R.G.) consisting of a Pye Unicam PU 4021 multichannel detector and a PU 4850 video chromatography control center. Chromatograms were recorded at 231 nm, spectra from 190 to 390 nm. Three-dimensional presentations (chromascans) are possible. A stainless-steel column (125 mm × 5 mm I.D.) packed with SC-Hypersil 3 μm (Gynkotek, München, F.R.G.) was used for separations with the solvent isooctane–diethyl ether–acetonitrile (150:60:10).

RESULTS AND DISCUSSION

A second method of determination only for catechins was developed without the disadvantages of the method described in earlier publications[3–5]. The first method was harmonized for a simultaneous determination of catechins and hydroxycinnamic acid esters and therefore represents a compromise. In the process of separation and purification, catechins and some hydroxycinnamic acid derivatives are obtained by elution with methanol from polyamide. The previous flushing with water to remove sugars, salts and other undesired compounds is to limit so that the hydroxycinnamic acid derivatives are obtained quantitatively. Also separations by analytical HPLC with gradient elution take a longer time. The employment of aqueous extract solutions is required for HPLC on a RP-18 phase with the eluents used. Because of their lower stability in aqueous solutions, catechin should be detected immediately. These solutions cannot be stored for later quantitations. The change to benzoates as employed for the direct quantitation of several polyhydroxy compounds from food[9–12] makes it possible to keep the catechins for a longer time.

To compare the results obtained by direct HPLC, we used the same aqueous

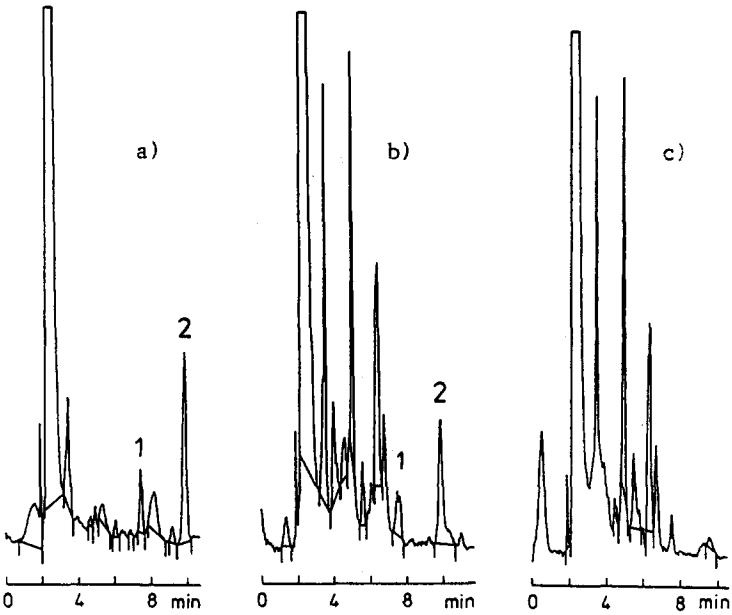

Fig. 1. Chromatograms of "Granny Smith" apple after benzoylation. HPLC separation of derivated catechin (1) and epicatechin (2) in the extracts without and with the use of RP-18 cartridges for clean up before benzoylation. A 2-ml volume of aqueous sample solution in concentrations of g/ml was used in each case. Column: SC-Hypersil 3 μm (125 mm × 5 mm I.D.). Detection: 231 nm. Flow-rate: 0.8 ml/min. Isocratic elution with isooctane–diethyl ether–acetonitrile (150:60:10). (a) Extract purified with a RP-18 cartridge before derivatization. Catechins were eluted with 5 ml of methanol after flushing with 2 ml of water. They can be detected without overlapping and measurable losses. (b) Extract benzoylated without use of RP-18 cartridge. Overlapping and losses can be seen. (c) Catechin and epicatechin retained on a RP-18 cartridge as shown by the absence in the chromatogram of the benzoylated extract and of the water used for flushing (2 ml) after separation of the catechins.

solutions (purified by polyamide) for tests of separation and benzoylation. Catechin and epicatechin exist only in small amounts compared with the naturally occurring sugars in fruit, which are also UV-detectable after benzoylation. To achieve good conditions for a subsequent benzoylation of the dry residues, we developed an additional clean-up procedure. The use of aqueous solutions (without cartridges) did not yield reproduciable results. Also, direct benzoylation of apple tissue fresh or freeze dried did not give an acceptable proof of catechins. On the other hand, RP-18 cartridges conditioned as shown retain catechin and epicatechin quantitatively from aqueous extracts and standard solutions. They are eluted with methanol which can be removed quickly and carefully without losses of catechins. Additional clean-up steps with water before the elution with methanol seemed to be necessary for quantitation of epicatechin from some extract solutions as well as to reduce or avoid overlapping of catechins with undesired compounds in the HPLC chromatograms of the benzoates, as shown by the example of an apple extract (Fig. 1).

Test series with different kinds of fruit (apple, pear, peach, apricot, plum, cherry) and tea as well as with standard solutions of catechins with contents comparable to those in the fruit extracts showed that catechin and epicatechin could be detected quantitatively after application of Sep-Pak RP-18 cartridges to 1–3 g (ml) fruit extract, flushing with 2 ml of water and elution with 5 ml of methanol. Clean up with more than 4 ml of water gave losses of catechin of about 5%. The results obtained are independent of the aqueous sample solutions investigated and reproducible and agree with those obtained by analytical HPLC of the underivatized catechins. The contents of catechins in 57 fruit samples examined by direct HPLC varied from less than 10 to 180 mg/kg[5]. Samples tested five times under the same conditions using the same aqueous solutions from the polyamide column showed standard deviations of the average contents of benzoylated catechins of not more than ±4%.

Optimum separation with isocratic HPLC was carried out on silica gel with isooctane–diethyl ether–acetonitrile (150:60:10). The wavelength of detection is 231 nm. Catechins are eluted before most of the sugars with short retention times of less than 10 min. They can also be distinguished from sugars and other benzoylated compounds by their UV spectra. Fig. 2 shows the normalized and superimposed UV spectra of derivatized catechins and rhamnose.

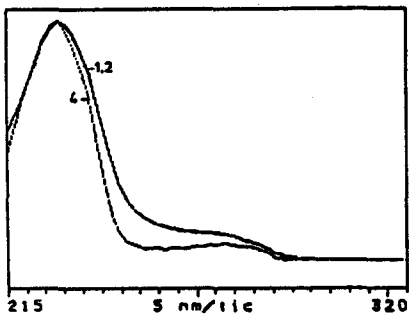

Fig. 2. Normalized and superimposed UV spectra of benzoylated catechin (1), epicatechin (2) and rhamnose (4).

Catechins were found with a reproducibility of 92 ± 3%. The detection limit of catechins as their benzoates increases by up to 10- or 20-fold compared with the underivatized compounds. Catechin and epicatechin in standard solutions of 0.2 mg/kg each can be detected after benzoylation. The linearity of detection was tested up to 100 mg/kg of derivatized catechins in the final solutions applied for HPLC. For samples with low contents of catechins it is also possible and useful to concentrate the final solutions of 50 ml as described here. The clean-up procedure allows the amount of sample used to be increased without changing the reagents used for benzoylation.

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