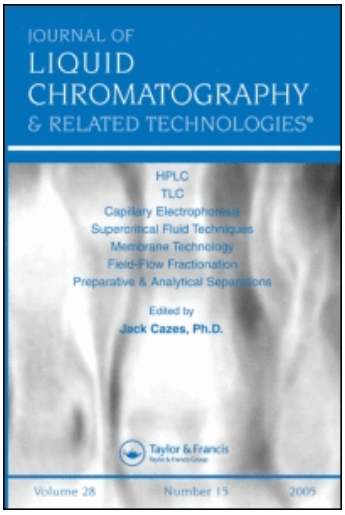

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LIQUID CHROMATOGRAPHIC DETERMINATION OF APPLE PULP PROCYANIDINS

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ABSTRACT:

Apple pulp procyanidins are separated by means of liquid column chromatography on Sephadex LH-20 and analysed by RP-HPLC. Three dimers and three trimers are identified by acid hydrolysis and formation of derivatives with phloroglucinol.

INTRODUCTION:

Proanthocyanidins are polymers of flavan-3-ols. They are susceptible to transform into anthocyanidins by heating in acidic solution and in the presence of oxygen. This has been the traditional method of estimating proanthocyanidins (1). Nevertheless, the recent developments in HPLC techniques allow their separation and itemized measure from different plant products (2-10).

For identification purposes, it is frequent to perform a previous fractionation based in molecular size, by means of liquid column chromatography with different packings, being the most commonly used: polyamide (7,11), Sephadex LH-20 (12-20), and Fractogel TSK HW-40(S) (21,22). After this purification, proanthocyanidins can be identified by acid hydrolysis and formation of derivatives with a nucleophyllic reagent, such as sulphite ion (23), phloroglucinol (20,24) or toluene-α-thiol (12-14,25-28).

Proanthocyanidins are compounds of great pharmacological interest due to their free radical scavenging properties (29,30) and their protective action against cardiovascular diseases (31,32).

They are also of importance in the field of Food Technology because of their influence on organoleptic characteristics of vegetable products, mainly bitterness and astringency (33-36) and their participation in technological processes leading to quality products (37,38), and in possible alterations (browning, formation of hazes and deposits...) (22,39-42).

One of the major phenolics in apple fruit is procyanidin B2 (43-46). However, the occurrence in apples of procyanidins up to heptameric forms is known by means of TLC (47). In this work we present the conditions for the separation of procyanidins by liquid column chromatography and high performance liquid chromatography, and a simple and rapid method of hydrolysis and derivatization for their identification.

MATERIALS AND METHODS:

1. Extraction:

Granny Smith apple pulp (15 g) was homogenized in 150 mL of methanol with a blender. After filtration, methanol was removed *in*

vacuo and the residue was redissolved with distilled water and applied to a Sephadex LH-20 column (8x2.2 cm I.D.), previously swollen with water. The column was washed with distilled water (100 mL) in order to eliminate sugars, acids and phenolics other than procyanidins. The column was finally eluted with 100 mL of acetone/water (70:30).

The aqueous fraction was concentrated to 25 mL and extracted with ethyl acetate (48) to be analysed by HPLC. It was proven that no catechins nor procyanidins, but only hydroxycinnamic acid esters passed into this fraction. The latter were identified as in (46).

The acetone/water fraction containing the procyanidins was taken to dryness in a rotatory evaporator and collected with 1 mL of ethanol 96% to be fractionated on the LH-20 column, as follows.

2. Liquid Column Chromatography:

A Sephadex LH-20 column (25x1.8 cm I.D.) swollen in ethanol 96% was used. The sample was first eluted with ethanol 96% (300 mL) and then with methanol/ethanol 96% (1:1) (100 mL). Fractions of 10 mL were collected, evaporated to dryness and redissolved in 1 mL of methanol/water (1:1). They were analysed by HPLC, and TLC by the monodimensional technique (13), using silica gel plates and toluene/acetone/formic acid (3:1:1) as developing solvent. Spots were visualized by spraying with vanillin-HCl reagent (40). The column was finally washed with acetone/water (70:30), and equilibrated with ethanol 96% to be re-utilised.

3. High Performance Liquid Chromatography:

Samples (5 µL) of each fraction obtained from the Sephadex LH-20 column were injected into the chromatograph. The equipment was from

Waters Associates and consisted of two pumps M-6000A, Universal Injector U6K, absorbance detector M-440V, and Maxima 820 Chromatography Workstation. The column used was a Nova Pak C_{18} (30x3.9 mm), eluting with an acetonitrile/water gradient described elsewhere (46). Detection was performed at 280 nm.

4. Hydrolysis of Procyanidins:

The method of Rigaud *et al.* (28) was used, with some modifications. The procyanidins were obtained by injecting 100 µL of the desired fraction in the HPLC apparatus. The peaks corresponding to pure procyanidins were collected in heart-shaped flasks, and 1 mL of phloroglucinol reactive (10 mg of phoroglucinol in 20 mL of ethanol/acetic acid, 1:1) was added to the compound to be identified. Flasks were closed with ground stoppers under nitrogen to prevent from oxidations, and heated in a stove at 90°C for 30 min. Afterwards, the reaction product was taken to dryness in a rotatory evaporator and in the same flask dissolved with 0.2 mL of methanol/water (1:1). 20 µL of each were injected into the chromatograph.

RESULTS:

1. Identification of Procyanidins:

The HPLC analysis of the hydrolysates permits to identify the subunits forming the procyanidins. By this way, three dimers are found, the procyanidins B1 [epicatechin-(4ß→8)-catechin], B2 [epicatechin-(4ß→8)-epicatechin] and B5 [epicatechin-(4ß→6)-epicatechin]. The latter does not completely hydrolysate, since the C_4-C_6 linkage is more resistant to hydrolysis (26).

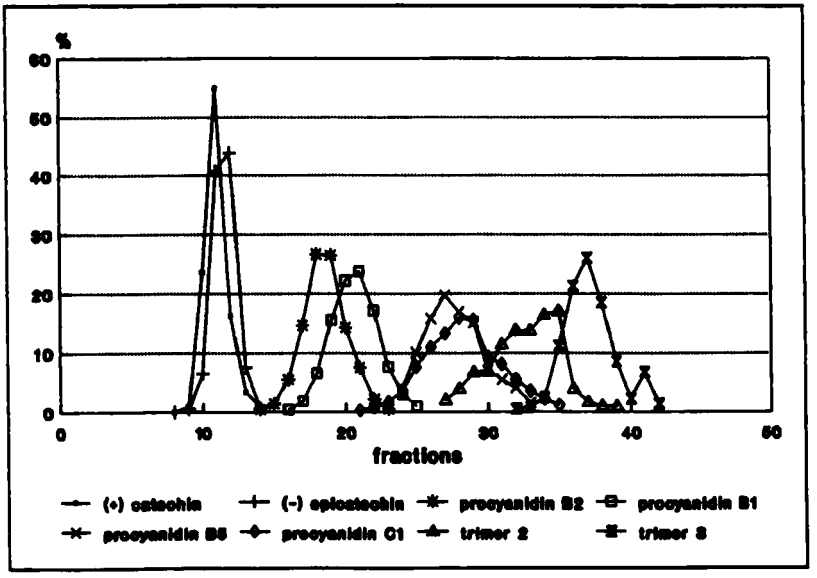

FIGURE 1. Elution profile of the identified compounds.

Three trimers are found, named procyanidin C1 [epicatechin-(4ß→8)-epicatechin-(4ß→8)-epicatechin], trimer 2 [epicatechin-(4ß→8)-epicatechin-(4ß→8)-catechin] and trimer 3 [epicatechin-(4ß→6)-epicatechin-(4ß→8)-epicatechin], following the terminology used in (22).

2. Liquid Column Chromatography:

The fractions obtained from the Sephadex LH-20 column were analysed by HPLC, and the procyanidins quantitated by using the standard curve of (-) epicatechin. The concentrations in µg/g of apple pulp are: (+) catechin: 17.68; (-) epicatechin: 83.05; procyanidin B2: 113.44; procyanidin B1: 24.60; procyanidin B5: 6.12; procyanidin C1: 42.43; trimer 2: 7.35; trimer 3: 17.29.

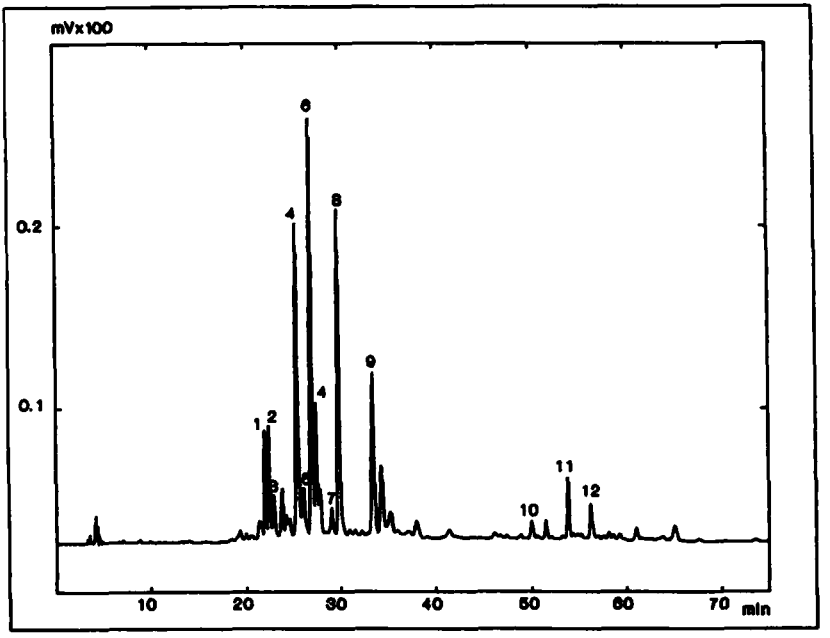

FIGURE 2. Chromatogram of apple pulp phenolic compounds. Peaks: 1: procyanidin B1; 2: (+) catechin; 3: trimer 2; 4: chlorogenic acid; 5: trimer 3; 6: procyanidin B2; 7: *p*-coumaroylquinic acid; 8: (-) epicatechin; 9: procyanidin C1; 10: procyanidin B5; 11: phloretin derivative; 12: phloridzin.

Owing to the wide variation in the quantities of the different compounds, in fig.1 we represent the percentages of the total collected for each one. There is a progressive enlargement of the bands as the time of permanence in the column increases. That is what we tried to avoid by changing the solvent, for methanol provides a faster elution but with less selectivity, according to our previous experience.

3. High Performance Liquid Chromatography:

The gradient used not only allows the separation of the procyanidins, but also of other compounds present in apples, as can be seen in fig. 2, that shows a chromatogram of an apple pulp polyphenol extract obtained as described in (46).

DISCUSSION:

Liquid column chromatography on Sephadex LH-20 with the proposed solvents permits to separate the major procyanidins present in apple pulp, which elute according to their degree of poly-merisation. However, it should be noted that procyanidins with C_4-C_8 linkages (dimer B5 and trimer 3) elute later than expected by their degree of condensation. That is why, when identificating them, it is convenient to employ TLC to check the polymerisation.

The method of procyanidin hydrolysis is useful for their identification even from very small quantities, as are those collected from an analytical column. This may be of maximum interest to assure peak identity when the chromatographic conditions or the column are changed.

The HPLC gradient used in this work is valid for the analysis of procyanidins without the need to separate them from the rest of phenolics present in apple pulp. It is expected to be equally useful for analyzing procyanidins from other fruit matterial.

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