The Cupuacu (Theobroma Grandiflorum) fruit. High performance liquid chromatographic determination of antioxidant phenolic substances in cupuacu

THE CUPUACU (THEOBROMA GRANDIFLORUM) FRUIT. HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF ANTIOXIDANT PHENOLIC SUBSTANCES IN CUPUACU SEED POWDER

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Abstract

A method for the qualitative analysis of antioxidant phenolic substances in cupuacu seed powder by high performance liquid chromatographic determination is described. We have used n-exhane to degrease the cupuacu seed powder and methanol-water (80:20) solution for the extraction of the analytes. HPLC separation was done using a binary gradient elution utilizing methanol-acetonitrile 50% (v/v) and 0.5% (w/v) phosphoric acid. Spectral scans were contiuously collected in the range 210-370 nm and the spectrophotometric chromatogram was plotted at 280 nm. Spectrofluorimetric detection was carried out with excitation at 280 nm and emission at 330 nm. Epicatechin and quercetin were identified comparing the chromatographic behaviour and the UV spectrum of the extracted components with those of pure standards, while the spectrofluorimetric detection, by stopped flow technique, has allowed the identification of catechin and has confirmed the spectrophotometric identification of epicatechin.

Keywords: High Performance Liquid Chromatography; Antioxidant Phenolic Substances; Cupuacu.

1. Introduction

The "cupuacu" (Theobroma Grandiflorum) is a tree of the same family of cocoa (Theobroma cacao) indigenous from Amazonia, Brazil. Today is also cultivated in plantations. The tree reaches a height of up to 20 metres (in plantations only up to 6 or 8 metres). Only about 20 fruits ripen on each tree, but they can achieve weights between 200 g and 5 Kg each. The fruit has a high economic potential due to its exotic aroma. The fruit is oval in form and possesses a brownish, hard, several millimetre thick woody shell, which surrounds the creamy-white fruit flesh and the from 30 to 50 walnut-sized seeds. The husk constitutes approximately 40 %, the seeds 15 % and the pulp 45 % of the fruit (1-4). After separating the seeds from the strong fibres, the plentiful flesh can be used

directly for food principally eaten fresh as a dessert, for instance with cream or yoghurt or else as a juice; it is also used as a cake coating or, with the addition of sugar, as a conserve. The seeds are subjected to a slight fermentation by piling them up and mixing well from time to time; then they are sun dried. Cracked seeds are grounded and pressed to give a paste. The fatty material (butter) is obtained by crushing the paste; the degreased material is called powder. Like the cocoa fruits, whose seeds are used in the production of cocoa butter and cocoa powder, it is possible also to obtain cupuacu butter and cupuacu powder from cupuacu seeds. The cupuacu seeds may be regarded as natural substitutes of those of cocoa. Its seeds are employed to prepare a chocolate like product (cupulate) (5-7). For this reason, the utilisation of cupuacu might be foreseen if its import would be favoured.

The frequent intake of substantial amounts of foods derived from plants such as vegetables and fruits may be useful to human health (8-10). Epidemiological studies suggested that chemopreventive substances existing in plants derived foods and their use in the diet may delay or prevent the diseases that are believed to be caused or at last enhanced by oxidative stress (11-14). Phenolic compounds constitute one of the most numerous and widely distributed groups of substances in the plant kingdom and have become an intense focus of research interest owing to their antioxidant capacity scavenging free radicals and chelating metals (9). Phisiological effects of phenolic substances on the human organism such as anti-oxidative, anti-atherogenic, anti-carcinogenic, anti-inflammatory, anti-dental caries, anti-microbial, anti-ulcer, in the treatment and prevention of cardiovascular, cerebrovascular, thrombosic diseases and immune modulating have been subjects of a number of both in vitro and in vivo studies (9, 15).

Cocoa products are particularly rich in phenolic substances and their regular consumption has the potential to improve an individual's oxidant defence system. Like cocoa products, also cupuacu products may be utilised for its good preservation properties.

The methods commonly used for the determination of phenolic compounds are high performance liquid chromatography (HPLC) coupled with photodiode array detector (PAD) and/or fluorescence detector (16). Other techniques such as thin-layer chromatography and capillary electrophoresis have been used as well (17-18). With the recent developments of interfacing and ionisation technologies for liquid chromatography, mass spectrometry coupled to HPLC has become a powerful tool in on-line detection and identification (19-20).

In this paper we describe a reversed phase (RP) HPLC method capable of separating phenolic substances and their tentatively identifying by utilizing PAD coupled with fluorescence detector.

2. Experimental

2.1. Chemicals and Materials

Standards of (-)-epicatechin, (±)-catechin, quercetin, (-)-gallocatechin and (-)-epigallocatechin gallate (Sigma-Aldrich, Milan, Italy), acetonitrile, methanol and *n*-hexane HPLC grade (J.T. Baker, Deventer, The Netherlands) and ortho-phosphoric acid (Sigma-

Aldrich) were used. High-quality water (18 M) was obtained by a Milli-Q water purification system (Millipore Corporation, Bedford, MA, USA).

Cupuacu seed powder was imported from Amazonia, Brazil.

2.2. Preliminary sample processing

0.5-g of cupuacu seed powder was extracted with 2 mL n- hexane and 5 mL of methanol-water (80-20, v/v) in a bath at 50 ºC for 15 min under stirring. The cooled sample was centrifuged at 3000 g for 10 min; the hexanic phase was eliminated, while the methanolic phase, after filtration through a GV13 Millex filter (Millipore Products Division, Bedford, MA, USA) was ready for HPLC analysis.

2.3. Instrumentation

A Perkin Elmer Serie 4 liquid chromatograph (Norwalk, CT, USA), equipped with a Rheodyne valve 8125 (loop 20 mL) (Redwood Drive Cotati, California, USA) was used. The liquid chromatography was coupled to an Photo Diode Array detector Spectra System UV 6000 LP (Thermo Separation Products, Rodano, MI, Italy) connected in series to a Perkin Elmer LS-4 Spectrofluorimetric detector. Chromatographic data were processed with a Chrom Quest Chromatography Workstation (ThermoQuest Italia S.p.A., Rodano, MI, Italy) and a Chrom Card software (Fisons Instruments S.p.A., Rodano, MI, Italy).

2.4. Analytical procedures

RP-HPLC analyses were performed using a 5μm Supelcosil LC-18 column (250 x 4.6 mm; Supelco, Sigma-Aldrich, Milan, Italy) and a 5μm Supelguard LC-18 Cartridge pre-column (20 x 2,1 mm, Supelco, Sigma-Aldrich). Analyses were carried out at room temperature. The mobile phase was 50 % (v/v) methanol-acetonitrile (A) and 0.5 % (w/v) phosphoric acid in Milli-Q water (B). The gradient elution profile started with A-B (5:95), A was gradually increased to 30% at 25 min, to 40 % at 35 min, to 48 % at 40 min, to 70 % at 50 min, to 100 % at 55 min. Re-equilibration of the column was achieved with a linear gradient to 5% A (initial condition) in 3 min, followed by 10 min of isocratic elution before the next injection. The flow rate was 1.0 mL min^{-1}. The spectral scans were continuously collected in the range 210-370 nm and the spectrophotometric chromatogram was plotted at 280 nm. Spectrofluorimetric detection was carried out with excitation at 280 nm and emission at 330 nm. The qualitative analysis was carried out comparing the chromatographic behaviour, the spectrophotometric and the spectrofluorimetric spectra of the present components with those of the pure standards.

3. Results and discussion

In this paper a HPLC method for qualitative analysis of antioxidant phenolic substances in cupuacu seed powder is described. Preliminary sample processing was carried out as described in the section 2.2. We have used methanol-water (80:20) solution for the extraction because this mixture was recently found to quantitatively remove antioxidant phenolic compounds from vegetables and fruits (21). We have used n-exhane to degrease the cupuacu seed powder. HPLC was chosen as the separation technique owing its high

resolution and high efficiency; we have used a binary gradient elution because allows better separation as found in preceding works (22, 23). Spectral scans were continuously collected in the range 210-370 nm and the chromatogram UV plotted at 280 nm, the wavelength of the maximum absorption of a great number of the interest components, is reported in figure 1.

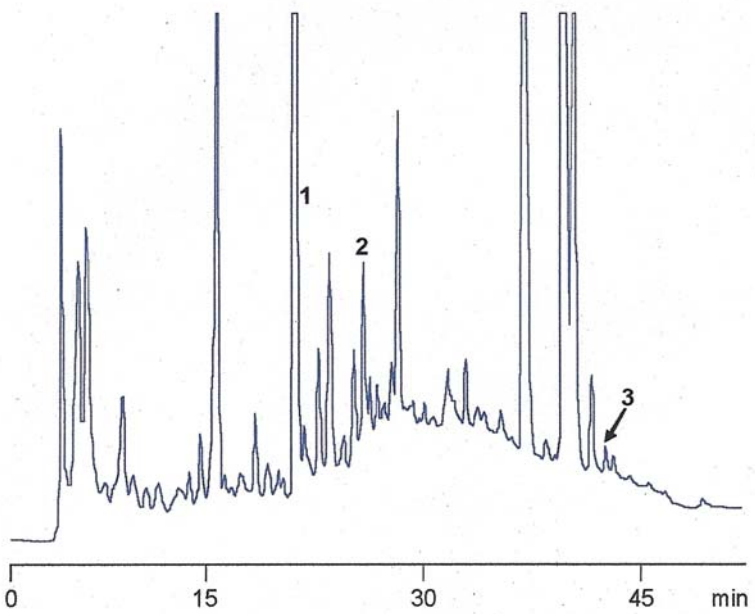

Figure 1. High performance liquid chromatographic separation of the antioxidant phenolic substances present in cupuacu seed powder with spectrophotometric detection at 280 nm. For conditions of analysis see Experimental. Peak identification: 1) catechin and not identified component; 2) epicatechin; 3) quercitin.

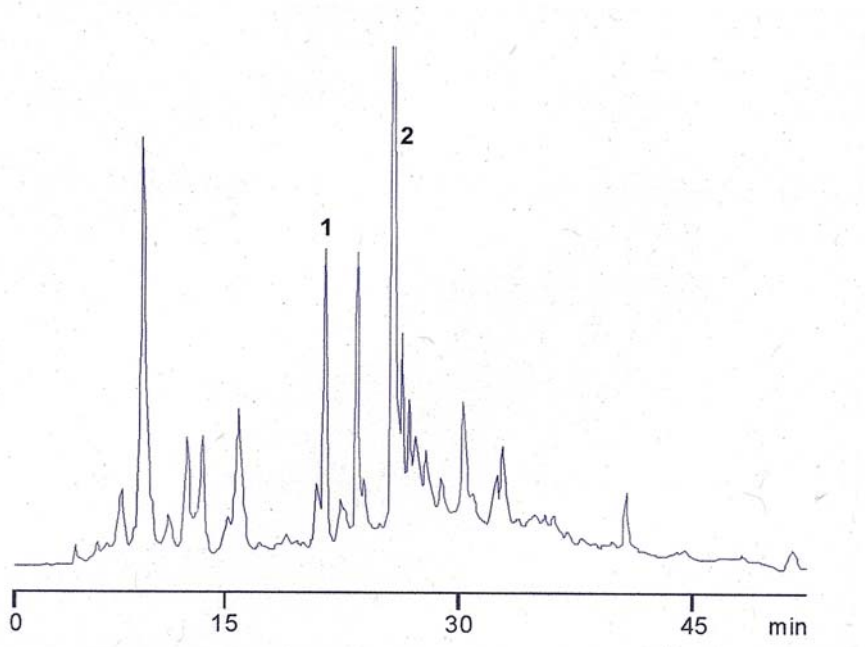

Figure 2. High performance liquid chromatographic separation of the antioxidant phenolic substances present in cupuacu seed powder with spectrofluorimetric detection (excitation at 280 nm and emission at 330 nm). For conditions of analysis see Experimental. Peak identification: 1) catechin; 2) epicatechin.

As may be seen many components, about which in the literature there aren't notices, are well separated. We have concentrated our study only on some components as catechins that are the major components identified in cocoa products (20). The study done with the assistance of adequate purity standards, has allowed, for the moment, to identify three components: catechin, epicatechin and quercetin. Epicatechin and quercetin were identified comparing the chromatographic behaviour and the UV spectrum of the extracted components with those of pure standards. In addition, the application of the peak purity software to the photo-diode array data indicated no impurities in these peaks. UV identification of catechin was prevented by an unidentified, eluted at the same retention time, compound. The problem was solved by spectrofluorimetric detection comparing the excitation and the emission spectrum of the standard of catechin with that directly registered, by stopped-flow technique, of the peak at the retention time of catechin. In figure 2 the spectrofluorimetric chromatogram with the excitation at 280 nm and the emission at 330 nm is reported. As may be seen also epicatechin shows fluorescence and this confirmes the spectrophotometric identification.

4. Conclusion

The HPLC method provides a sensitive procedure for the identification of catechin, epicatechin and quercetin in cupuacu seed powder.

Preliminary obtained results could be useful for the characterisation of cupuacu seed powder, but it is necessary a further qualitative and quantitative investigation to identify other phenolic components, since the nutritional importance that these compounds assumed because of their possible anti-oxidant behaviour.

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