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SEPARATION OF PHENOLIC COMPOUNDS FROM FOODS BY REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Key words: HPLC-DAD, reversed-phase, phenolic compounds.

ABSTRACT

As a part of a study on the content of antioxidants and phenolic compounds in Bolivian foods, a rapid high performance liquid chromatographic method using a reversed-phase column and a diode array detector (HPLC-DAD) method was established. It was used for the determination of phenolic compounds in foods and a good resolution of different standard compounds was achieved.

RESUMEN

Como parte del estudio sobre el contenido de antioxidantes y compuestos fenólicos en alimentos bolivianos fue establecido un método rápido de cromatografía líquida de alta resolución usando una columna de fase reversa con detector por arreglo de diodos (HPLC-DAD). El método fue utilizado para la determinación de compuestos fenólicos en alimentos y fue lograda una buena resolución en diferentes compuestos estándares.

INTRODUCTION

In the last years an increasing interest in natural antioxidants has been developed (1-3). Flavonoids and other phenolic compounds are widely distributed in foods and are important constituents of the human diet. The phenolic compounds may be protective against cardiovascular disease and have certain potential anticarcinogenic properties due to their antioxidant activity or other properties (4). Natural flavonoids and polyphenols encompass more than 4000 individual compounds., Considering this high number and the complexity of the mixtures, it is fortunate that only a limited number of the flavonoids occur in significant amounts in the human diet (5). The content of the antioxidants, phenols and flavonoids in foods has been evaluated by different methods (6-9). The most often used method to measure the individual flavonoids and phenolic compounds is reversed-phase high performance liquid chromatography (RP-HPLC) (10). HPLC allows a high resolution and a rapid and reproducible determination, even of trace amounts of these compounds. Several methods have been developed using the RP-HPLC technique (11-13). However, it is necessary to design tailor-made methods depending on the nature of the samples. In the present work a rapid RP-HPLC method for the determination of different phenolic substances was established based on the method of Cristea et al. (14).

RESULTS AND DISCUSSION

Separation of phenolic standards by HPLC

Thirty-five standards of phenolic compounds were chromatographed by HPLC using a UV-vis diode array detector recording at 280, 360 and 530 nm. The method showed a good separation of most reference compounds. The retention time and UV maxima of the thirty-five standards are shown in Table 1. These data were used for the identification of the substances found in food samples.

Phenolic acids, catechins and other substances

Different amounts of phenolic acids, catechins, and other phenolic substances were injected separately and in mixtures into the HPLC. Standard curves were created and Figure 1 shows a calibration curve of quercetin obtained on two different days.

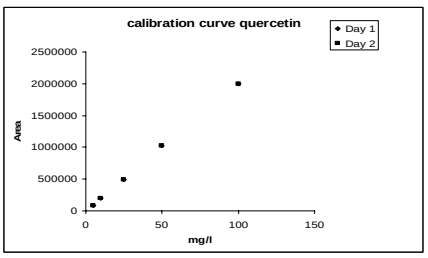

Figure 1. Calibration curve of quercetin standards

For this subgroup a good separation was obtained using a mixture of cinnamic acid, chlorogenic acid, ellagic acid, ferulic acid, benzoic acid, gallic acid, caffeic acid and vanillic acid as shown in Figure 2A.

Flavonoids

The HPLC method showed as well a good separation of a mixture of flavonoids. Six flavonoids were routinely used as reference compounds and a chromatogram using the measurement of the absorbance at 360 nm is shown in Figure 2B.

Anthocyanins

The maximum absorbance of anthocyanins is around 530 nm. This was used to measure anthocyanins which were injected into the HPLC either separately or in mixtures as shown in Figure 2C.

Concluding remarks

In conclusion the present work presents a convenient HPLC method for the separation of phenolic acids, flavonoids, anthocyanins and other phenolic substances from foods. Using methanol and acetic acid (1%) in water as solvents, good separations were obtained rapidly. The method showed a good reproducibility for the separation of different standard compounds. Absorbance data from the diode array detector were obtained at different wavelengths for the reference substances. The absorbance spectra and the retention times were used for the identification of the substances. In future studies additional confirmation of the identity of different compounds in foods is necessary using mass spectrometry and other techniques. Quantification of the substances in food was performed using external standard curves. The present method can be considered as a valuable alternative and complementary method to different spectrophotometric methods (15) for the study of the content of different phenolic substances in foods and other natural sources.

EXPERIMENTAL

Chemicals

Gallic acid was purchased from Merck (Darmstadt, Germany), baicalein (98%), 3,4-dihydroxybenzaldehyde (99%), benzoic acid (99%), catechin (99%), caffeic acid (99%), catechin gallate (99%), catechol (99%), 4-methylcatechol (99%), 5-methylcatechol (99%), tert.butylcatechol (99%), chlorogenic acid (99%), ellagic acid (99)%, epicatechin (99%), guiachol (99%), hydroquinone (99%), kaempferol (99%), morin (99%), myricetin (99%), naringenin (99%), pyrogallol (99%), quercetin (99%), resorcinol (99%), 4-methylresorcinol (99%), 5-methylresorcinol (99%), rutin (99%), vanillic acid (99%), and vanillin (99%) were obtained from Sigma-Aldrich (St. Louis, USA), acetic acid (glacial p.a.), ferulic acid, delphininCl, cyanidinCl, malvidinCl, pelargonidinCl and petunidinCl were obtained from Extrasynthèse (Genay, France), and methanol HPLC grade from Laboratory supplies (Poole, U.K.).

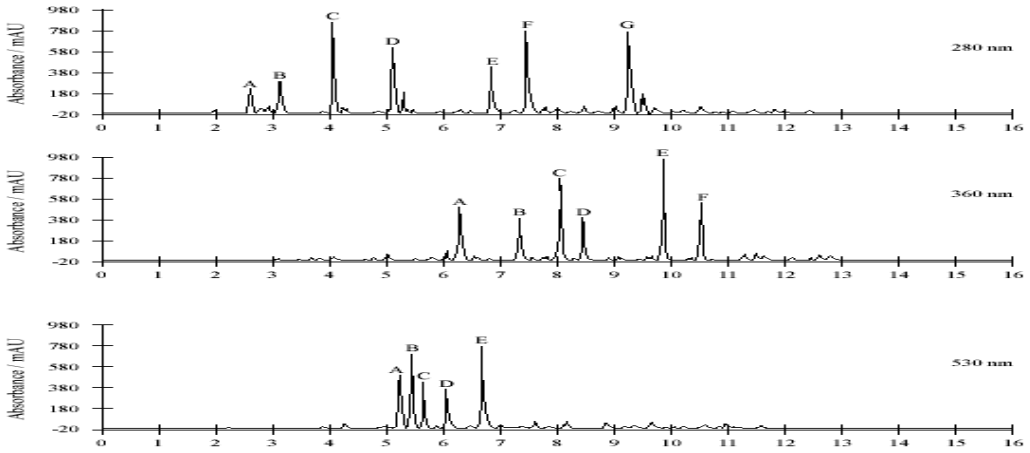

Figure 2, Chromatograms of different phenolic substances obtained by the HPLC method.
Panel A (upper). The detector was set at 280 nm mainly for phenolic acids. A: gallic acid; B: resorcinol; C: vanillic acid; D: 4-methylresorcinol; E: ellagic acid; F: benzoic acid; G: cinnamic acid.
Panel B (middle). The detector was set at 360 nm for flavonoids. A: rutin; B: myricetin; C: morin; D: quercetin; E: kaempferol; F: baicalein (used as an internal standard).
Panel C (lower). The detector was set at 530 nm for anthocyanins. A: delphinidin; B: cyanidin; C: petunidin; D: pelargonidin; E: malvidin

Sample Preparation

The standards were dissolved in ethanol HPLC grade at different concentration and filtered by 0.22 µm sterile Millex filters purchased from Millipore (Bedford,U.K.) before the injection.

High performance liquid chromatography

Before the HPLC analysis, the water-insoluble fractions were evaporated in a nitrogen stream and reconstituted in methanol and after that both the water-soluble and water-insoluble fractions were refluxed in 1.5 M HCl in methanol for 90 min for the hydrolysis of glycosides. Baicalein was added as an internal standard before the hydrolysis. Phenolic compounds were separated using a Shimadzu liquid chromatograph system (LC 10ADVP), comprising a vacuum degasser (DGU 14-A), a solvent delivery module (FCV-10ALVP), an auto-injector (SIL-10ADVP), a column oven (CTO-10ASVP) and a diode-array detector (SPD-M10AVP). The column was a 3.5 mm Kromasil reversed-phase column 150 mm x 4 mm protected by a Kromasil C 18 10 mm pre-column (Scantec Lab, Sävedalen, Sweden). The flow rate was 0.8 ml/min and the injection volume was 20 μl. The mobile phase was a binary solvent system consisting of (A) methanol and (B) 1% acetic acid/water and the gradient used was 0 min 40% B, 5 min 65% B, 10 min 90% B, 15 min 40% B until 17 min as modified from (14). The UV absorbance at 280, 360 and 530 nm was recorded in the eluate. The compounds were identified by comparing with standards of each identified compound using the retention time, the absorbance spectrum profile and also by running the samples after the addition of pure standards. The chromatographic and spectral features of the standards are shown in Table 1. The concentrations were calculated from the peak heights of the internal standard and each compound in the samples and in reference solutions.

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Table 1. Chromatographic and spectral features of phenolic substances
R, retention time.

Substance	R_t (min)	Maxima in UV-vis spectrum (nm)	Substance	R_t (min)	Maxima in UV-vis spectrum (nm)
Gallic acid	2.44	233-272	Ferulic acid	5.73	238 – 323
Catechin	2.65	231 – 278	4-Methylcatechol	5.78	234 – 281
Pyrogallol	2.84	230 – 265	5-Methylcatechol	5.98	234 – 278
Chlorogenic acid	2.9	225 – 329	Rutin	6.28	254 – 355
Hydroquinone	2.99	227 – 289	Guiachol	6.38	228 - 275
Epicatechin	3.2	227 - 278	Pelargonidin Cl	6.4	260 - 525
Resorcinol	3.3	278	Malvidin Cl	6.7	275 – 541
3,4 Dihydroxybenzaldehyde	3.5	233 – 276 – 311	Ellagic acid	6.88	254 – 367
Caffeic acid	3.8	231 – 323	Myricetin	7.38	230 – 254 – 375
Catechin gallate	3.9	278	Benzoic acid	7.48	232 – 275
Catechol	3.94	227 – 276	Morin	8.02	252 – 355
Vanillic acid	4.3	260 – 292	Quercetin	8.7	255 – 371
5-Methylresorcinol	4.53	233 – 269	Naringenin	8.93	232 – 289
Vanillin	4.8	239- 279 – 310	Cinnamic acid	9.27	295
Delphinin Cl	5.24	226 – 275 – 536	Kaempferol	9.92	256 – 236
4-Methylresorcinol	5.28	229 – 278	4–tert.Butylcatechol	10.42	234 – 276
Cyanidin Cl	5.4	255 – 530	Baicalein	10.52	275 – 323
Petunidin Cl	5.69	230 – 272 – 539			