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Note

Separation and determination of piperine in ground pepper by high-performance liquid chromatography

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Pepper, from the seeds of *Piper nigrum* L., contains a variety of closely related nitrogen containing compounds[1] among which piperine (2-*trans*-4-*trans*-N-piperoyl-piperidine) is responsible for the characteristic pungent taste of pepper[2,3]. Piperine in solution is readily isomerised by exposure to light to iso-piperine, chavicine and iso-chavicine[4]; these compounds, which may occur in low concentrations in pepper, have little taste and therefore any assessment of the quality of pepper, based on the determination of the piperine content, must be capable of separating piperine from the related isomers. Methods of estimating piperine based on Kjeldahl nitrogen, colorimetric methods based on the reaction of the methylenedioxy group or methods based on direct UV spectrophotometric measurements lack that capability but high-performance liquid chromatography (HPLC) has been shown to be capable of separating and quantitating piperine and its isomers. De Cleyn and Verzele[4] used a mobile phase of anhydrous ethyl acetate and hexane with an alumina column to separate and isolate the four isomers but were later unable to repeat this separation using a commercially available alumina column[3]. A stationary phase capable of separating the four isomers was prepared by nitrating a phenyl silica gel column packing with fuming nitric and sulphuric acids and was used with anhydrous dichloromethane and methanol as mobile phase; *p*-bromoacetanilide was used as an internal standard, with detection at 280 nm[3]. Galetto *et al.*[5] obtained a partial separation of piperine isomers with chloroform as mobile phase and a silica column. The determination of piperine in pepper using aqueous methanol as mobile phase with a cyano reversed-phase column has been reported[6] but no separation of isomers was described. Piperine has been separated from the related isomers by gas chromatography (GC)[7,8] and quantitative thin-layer chromatography (TLC)[9,10].

This note describes the separation and determination of piperine in pepper using an aqueous mobile phase with a C_8 stationary phase and phenazine as an internal standard.

EXPERIMENTAL

Chromatography

The apparatus used consisted of an Altex Model 321 liquid chromatograph

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with a Rheodyne 7125 sample injector fitted with a 20-μl loop and an Erma Model ERC-7210 variable-wavelength detector set at 345 nm and 0.08 absorbance units. A LiChrosorb RP-8 reversed-phase column, 250 × 7 mm O.D., 5 μm particle size, was used with a flow-rate of 2.0 ml/min. The mobile phase was a mixture of 345 ml of filtered deionised water, with 160 ml of acetonitrile and 40 ml of tetrahydrofuran, both of HPLC grade.

Reagents

Piperine (2-*trans*-4-*trans*-N-piperoyl-piperidine) and phenazine (2,3:5,6-dibenzopyrazine) were both purum grade (Fluka). The piperine gave a λ_{max} of 345 nm and a molecular extinction coefficient of 35 300 in ethanol (reported[4] 343 nm and 34 100). Standard piperine solution in ethanol, 20 mg/100 ml; standard phenazine solution in ethanol, 20 mg/100 ml. All solutions containing piperine should be protected from direct sunlight and stored in the dark when not in use.

Procedure

Add to a series of glass-stoppered test tubes 0.2, 0.4, 0.6, 0.8 and 1.0 ml of standard piperine solution and dilute the contents of each tube to 2.0 ml with ethanol; add to each tube 2.0 ml of phenazine solution and mix. Inject 20 μl of each solution and determine the ratios of the peak areas of piperine to phenazine. Weigh accurately about 0.5 g of ground sample and add to about 90 ml of ethanol. Boil gently on a steam-bath for 15 min, cool and transfer the mixture completely to a 100-ml volumetric flask with ethanol; dilute to 100 ml with ethanol. Protect this solution from light. Filter an aliquot of this solution through a Whatman 541 paper (or equivalent), rejecting the first 5 ml. Transfer 0.5 ml of the filtrate to a glass stoppered test tube, add 1.5 ml of ethanol and 2.0 ml of phenazine solution, mix and inject 20 μl of this solution. Protect this solution from light. From the peak area ratios of piperine to phenazine in the sample and standard solutions, calculate the percentage of piperine in the sample.

RESULTS AND DISCUSSION

The aim of the present work was to develop an HPLC method, using an aqueous mobile phase with a commercially available column, which would permit the separation and quantitation of piperine in pepper samples with a suitable internal standard. Piperine has a UV absorption maximum at 343 nm[4] and 345 nm was chosen as the nearest detector wavelength to give maximum sensitivity and to reduce interference from other UV absorbing compounds present in pepper which absorb at lower wavelengths. Preliminary experiments with a 10-μm particle C_{18} column showed that binary solvent systems did not give adequate resolution of piperine from the related isomers but the addition of a third solvent[11] improved the separation. The best separation was obtained with a 5-μm particle size C_8 column and a mobile phase containing acetonitrile, water and tetrahydrofuran. Phenazine (2,3:5,6-dibenzopyrazine) was chosen as an internal standard; this compound has maximum UV absorption at 248 nm and a shoulder at 350 nm (log E 4.03) and has a suitable retention time under the chromatographic conditions used. Piperine is soluble in a variety of organic solvents and has been extracted from pepper for analysis with

TABLE I

EXTRACTION OF PIPERINE FROM PEPPER

Method	*Piperine found (%w/w)*
Boil with ethanol 15 min	3.47
30 min	3.44
45 min	3.52
Macerate in Ultra Turrax Mixer with ethanol	
cold	3.40
cold, followed by 30 min boiling	3.45
Allowed to stand with cold ethanol overnight	3.44
Extraction with dichloromethane:	
extracted with 3 × 25 ml hot solvent; filtered extract evaporated to dryness and the residue dissolved in 100 ml of ethanol; 0.5 ml taken for analysis.	3.40
extracted with 3 × 25 ml hot solvent; the extract diluted to 100 ml with solvent and 0.5 ml evaporated to dryness and the residue dissolved in 2 ml ethanol for analysis.	3.48

ether[6], dichloromethane[3,7,9], chloroform[10] and dichloroethane[5]. Ethanol was chosen as an extraction solvent and was found to be as effective as dichloromethane; the results of various extraction procedures on the same sample are shown in Table I. A chromatogram of a standard mixture of phenazine and piperine is shown in Fig. 1; typical retention times found for phenazine were 5.1–5.3 min and for piperine, 11.0–11.2 min. Fig. 1 also shows the chromatogram of a pepper extract before and after exposure to bright winter sunshine (latitude *ca.* 33°S) and demonstrates the rapid isomerisation of piperine and the separation of piperine from the isomeric compounds. The composition of the final equilibrium mixture, calculated from the extinction coefficients of De Cleyn and Verzele[4], was similar to the equilibrium composition reported by those authors. Recovery experiments were carried out by adding accurately weighed quantities of piperine to pepper in duplicate before extraction with ethanol; the amounts added and the percentages recovered were as follows:

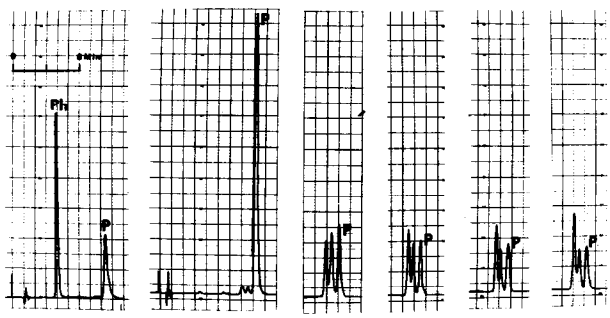

Fig. 1. HPLC chromatograms of (left to right): a standard solution of phenazine (Ph) and piperine (P); an ethanolic extract of pepper without phenazine; the same solution exposed to light and analysed at 30-min inervals. Conditions: mobile phase, acetonitrile–tetrahydrofuran–water (160:40:345); flow-rate 2.0 ml/min; detector wavelength, 345 nm.

TABLE II

PIPERINE CONTENT OF PEPPER SAMPLES DETERMINED BY HPLC

Sample	*Piperine content (%w/w)*
White pepper	
Reference sample, freshly ground	4.35
Reference sample, freshly ground + 20% rice starch	3.28
Reference sample, freshly ground + 50% rice starch	2.20
Commercial samples (9), range 3.72–4.46, mean:	4.01
Commercial sample A	3.16
Commercial sample B	3.17
Commercial sample C	2.20*
"Manufacturers pepper"	2.47*
"Aromatic pepper"	2.20*
Black pepper	
Commercial samples (2)	3.73, 5.03
Commercial samples (2)	2.10*, 2.31*

* These samples did not comply with the New South Wales Pure Food Act for alcohol or ether extract and/or total ash.

5 mg, 94%, 107%; 10 mg, 103%, 96%; 15 mg, 94%, 95%. Some results obtained using the method described are shown in Table II. The concentrations of piperine found are similar to those found by HPLC[4], GC[7,8] and TLC[9,10] where *trans,trans*-piperine has been separated from other piperine isomers.

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