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Note

High-performance liquid chromatographic analysis of the pungent principles of pepper and pepper extracts

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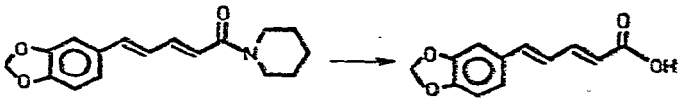
Piperine (I), first isolated from black pepper in 1820, was later also isolated from other pepper varieties[1]. The alkaline hydrolysis of piperine yields piperinic acid (II) and piperidine.



I II

The *trans* configuration of piperine was proved by Doebner[2] and Ladenburg and Scholtz[3]. It was soon postulated that the geometrical isomers of piperine could also exist in pepper extracts. When still unknown, these were given the names chavicine (*cis–cis* isomer), isochavicine (*trans–cis*) and isopiperine (*cis–trans*). In this nomenclature, assignment of the configuration to the double bonds starts from the amide function in piperine (*trans–trans*).

In 1876, Buchheim[4] claimed to have isolated chavicine, but later research[5,6] showed that this was not so. Buchheim[4] also advanced the idea that chavicine had a sharper taste than piperine and was even solely responsible for the pungency of pepper. As piperine can be photoisomerized into a mixture containing a relatively large percentage of chavicine, the possible economic importance of this prompted us to take up research in this area. Grewe *et al.*[5] described the synthesis of the four isomeric acids (II) and also the corresponding piperidine amides. In our hands, however, this synthetic pathway over the acid chloride easily led to mixtures of the isomers. We have described the separation of the isomers by preparative high-performance liquid chromatography (HPLC)[7]. The four isomers were characterized by NMR spectroscopy and their physical constants were recorded[8,9]. Once available in pure form, it was easy to ascertain that piperine is the pungent principle of pepper and that the other isomers have little taste. The pungency of piperine depends considerably, however, on its degree of dispersion. In crystalline form the taste is weak and it takes on its real force only in solution.

We have also described a chromatographic analysis of piperine and its isomers

and quantified a number of peppers and pepper extracts in terms of their piperine content with this method[7]. The chromatography involved the use of a 1-m column filled with 10–40-μm aluminium oxide and took approximately 2 h.

The recent development of HPLC, with the introduction of routine analyses on 25–30-cm columns, prompted us to reconsider this pepper analysis. To our confusion, however, we were unable to repeat the complete separation on aluminium oxide columns and this led to experimentation on a wide range of HPLC stationary phases. Because of the interest of a large number of laboratories in this separation, we reproduce the optimized results on some stationary phases in Fig. 1.

As shown, complete separation is obtained only on a nitro-silica gel stationary phase (No. 6 in Fig. 1). This nitro-silica gel phase can be synthesized by treating phenyl-silica gel with a nitric acid–sulphuric acid mixture. On this stationary phase, the separation of piperine and isomers is easy. The elution sequence is different to that on aluminium oxide as given earlier[7]. On nitro-silica gel the peaks are eluted in the order chavicine, isochavicine, isopiperine and piperine. On following the formation of the photoisomerization mixture by HPLC, peak No. 1 was found to be formed last.

EXPERIMENTAL

Pepper extraction

Pepper was ground in a coffee mill and extracted for 12 h with methylene chloride in a Soxhlet extractor. Repeated recrystallization from methanol afforded pure piperine with m.p. 127°.

Photoisomerization of piperine

Piperine (1 g) dissolved in methanol (100 ml) was irradiated at 350 nm in a photochemical reactor. Chromatography at repeated intervals showed that the photostationary state was reached after about 4 h. Photoisomerization also occurs in daylight and takes several days to reach completion.

p-Bromoacetanilide

Commercial material (Aldrich, Beerse, Belgium) was recrystallised until the m.p. was 163°.

Nitro-silica gel

Phenyl-silica gel was obtained in the usual way be reaction of silica gel with phenyltrichlorosilane. The total organic content of the material was 9 % as determined by thermogravimetric analysis. Phenyl-silica gel (10 g) with a mean particle size of 10 μm was mixed with sufficient fuming sulphuric acid to obtain a slurry (15 ml). The slurry was heated to 40° and nitric acid of specific gravity 1.5 (5 ml) was slowly added, keeping the temperature below 50°. After 5 h the slurry was poured into 1 l of cold water. The nitro-silica gel stationary phase was washed until acid free and then dried at 110° in an oven. The total organic (heat removable at 700°) content was 8–9 %.

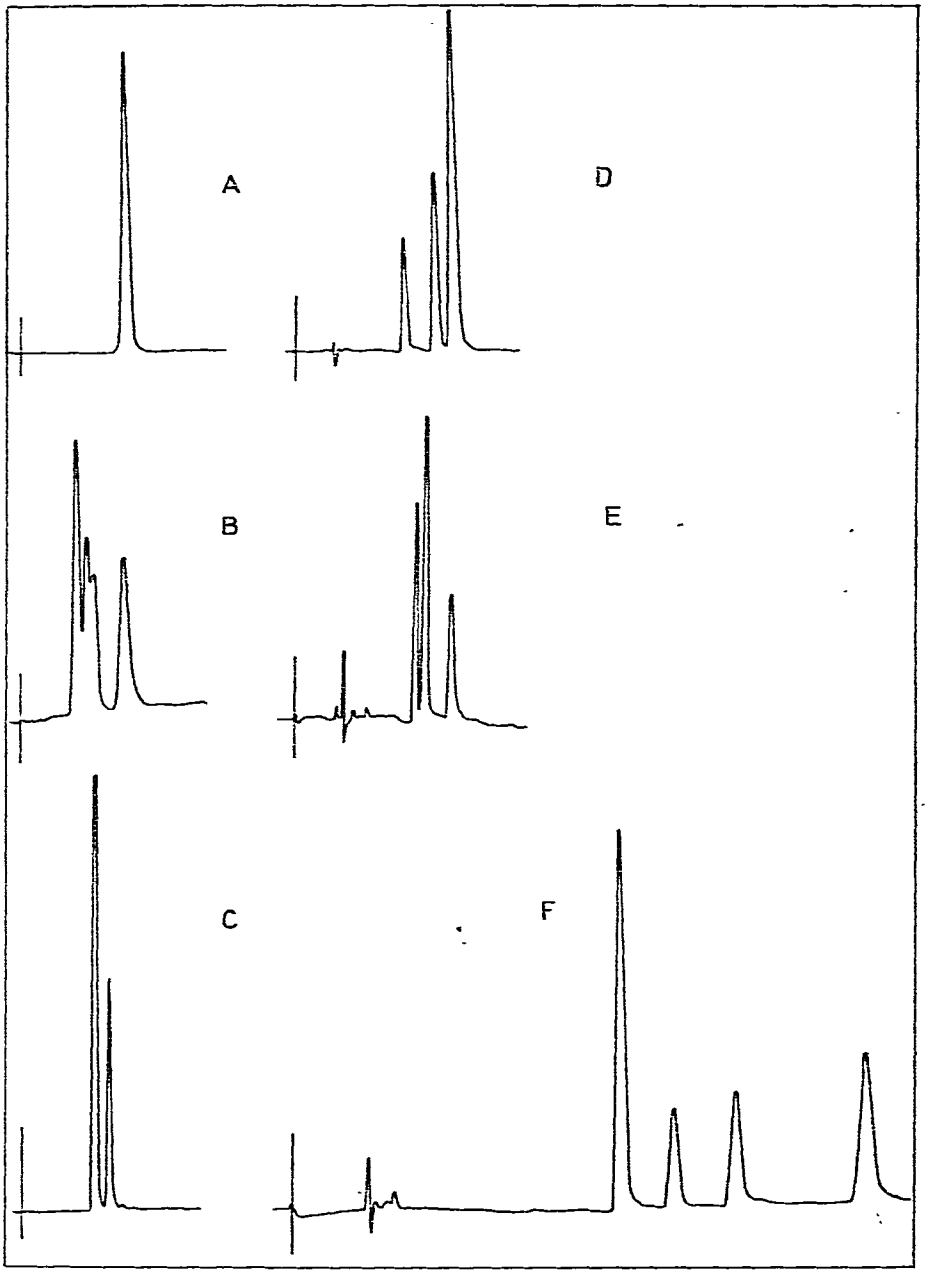


Fig. 1. HPLC of piperine photostationary state mixture on different 10-μm silica gel phases. Column, 25 × 0.46 cm. Isocratic at 60, 90 or 120 ml/h. Pressure, *ca.* 50–100 bar. Varian 8500 chromatograph with Valco 7000 p.s.i. injector. UV detection at 280 nm. A, Octadecyl-silica gel with methanol–water (80:20) with Ag^+ as complexing ion; B, Al_2O_3 60 (Merck, Darmstadt, G.F.R.) with methyl chloride–isooctane (45:55); C, diol-silica gel with methylene chloride–isooctane (80:20); D, strong cation exchanger, sulphonic acid-silica gel with chloroform–isooctane (150:20); E, cyano-silica gel with isooctane–methylene chloride–methanol (17.5:17.5:8.5); F, nitro-silica gel with dry methylene chloride–methanol (100:4.5). Peaks in elution sequence: chavicine, isochavicine, isopiperine and piperine.

HPLC

A 25 × 0.46 cm Lichroma tube was filled in the normal way using a suspension of the nitro-silica gel in carbon tetrachloride. The column was rinsed with methanol and methylene chloride and then equilibrated with the eluting solvent (methylene chloride–methanol, 100:4.5). The methylene chloride was thoroughly dried on calcium chloride and the methanol was of UV quality. The analysis was isocratic and took about 15 min. The column was mounted in a Varian 8500 HPLC chromatograph equipped with a Valco 7000 p.s.i. 10-μl loop injector. The solvent flow-rate was 120 ml/h and the detection wavelength 280 nm. The pressure was typically around 80–100 bar.

Calibration graphs

The internal standard *p*-bromoacetanilide (*ca.* 50 mg) was dissolved in methylene chloride (50 ml) and different amounts of piperine between 5 to 20 mg were added. Details of the calibration graphs are given below. With other amounts of *p*-bromoacetanilide (*e.g.*, 100 mg per 50 ml) the calibration graph was different, indicating that linearity with the internal standard used is not good. A different (better) internal standard could probably improve the results. It was also ascertained that if the piperine content in the 50 ml of methylene chloride solution is not between 2 and 10 mg, the results are difficult to reproduce.

RESULTS AND DISCUSSION

With the availability of the nitro-silica gel stationary phase (R.S.L., St. Martens-Latem, Belgium), it became feasible to develop a method for the quantitative analysis of piperine in pepper and pepper extracts. *p*-Methoxy- and *p*-bromoacetanilide proved to be suitable as internal standards in experiments with the photostationary-state isomer mixture. The peak of the methoxy derivative seems to be preferable because its retention places it between peaks 3 and 4 in the chromatogram, while the *p*-bromo derivative elutes well before the four isomers. The *p*-methoxyacetanilide peak coincides, however, with a small unknown peak in pepper and pepper extracts and therefore *p*-bromoacetanilide was preferred as an internal standard. A chromatogram of a pepper extract with added *p*-bromoacetanilide is shown in Fig. 2.

A calibration graph of piperine to internal standard peak area ratio *versus* the ratio of the weight of piperine to the weight of internal standard was established with a Varian CDS-111 electronic integrator. The equation derived was $y = 0.1031x - -0.0165$ ($r = 0.9984$). The same chromatograms were also used to establish a calibration graph based on surface area measurement by epidiascopic enlargement on a screen and measuring retention time and peak width at half-height. This led to the equation $y = 0.0978x - 0.0109$ ($r = 0.9989$). Results for piperine content in pepper and commercial pepper extracts determined on nitro-silica gel were as follows: South African pepper (1962), 6.0; Muntok pepper, 5.6; white pepper extract, 24.4; black pepper extract, 20.8; and white Sarawak pepper extract, 33.0%.

The precision of this analysis is not very good. However, a high precision was not our aim, as we wanted only to show the feasibility of the analysis. The analysis could probably be improved, although in general we find that quantitation is more

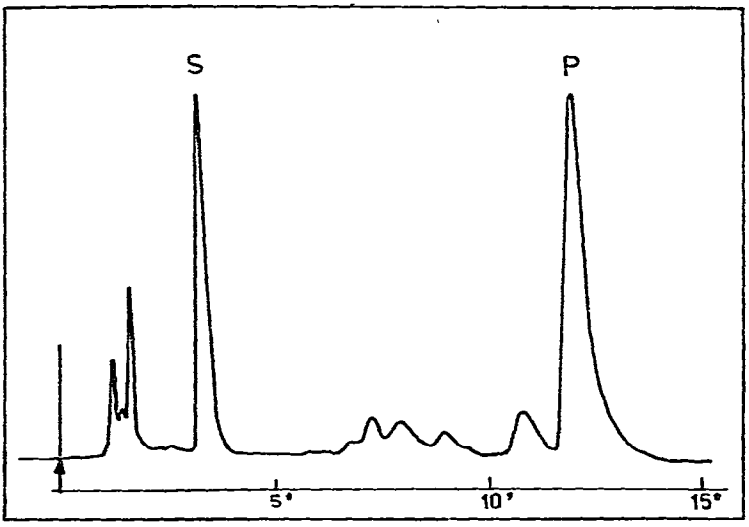


Fig. 2. HPLC of pepper extract. Same conditions as in Fig. 1F but with *p*-bromoacetanilide added as internal standard. S = Standard; P = piperine.

precise and easier on non-polar stationary phases. The main result of our work is that the described chromatography on the very polar nitro-silica gel phase separates piperine clearly and rapidly from its isomers. The results for piperine content are therefore representative, which is important as piperine is the pungent principle of pepper and pepper extracts and as the piperine content of these products can vary considerably.

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