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QUANTITATIVE MICROSCALE LIQUID CHROMATOGRAPHY OF PIPERINE IN PEPPER AND PEPPER EXTRACTS

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SUMMARY

Conventional and microscale liquid chromatographic (micro-LC) systems were compared for the determination of piperine in pepper and pepper extracts. A polyphenol-derivatized silica gel was used in normal-phase adsorption chromatography and with anthraquinone as an internal standard. Differences in capacity ratios (k') and in selectivities (α) were noted and are discussed. The standard deviation of the analysis is smaller for micro-LC, but the reasons are not obvious. Advantages claimed for micro-LC (better efficiency, permeability and quantitation) are illustrated.

INTRODUCTION

Piperine (4 in Fig. 1) occurs in concentrations of *ca.* 3–5% in ground pepper used for culinary purposes. Freshly picked pepper berries are "green pepper", becoming "black pepper" on sun drying and "white pepper" when the dried outer shell of the berries is removed from the black variety. The geometric isomers of piperine, *i.e.*, chavicine, isopiperine and isochavicine (1, 2 and 3 in Fig. 1), also occur in pepper, but in very small amounts, together with other minor constituents of similar chemistry. We have shown that piperine is almost the only contributor to pepper pungency[1] and, therefore, that its quantitation is important. This analysis is not as easy as it may seem. For a long time, determination of the total nitrogen content was the preferred method for the quality evaluation of pepper. Hydrolysis and quantitation of the piperidine so formed has also been used. These methods do not determine piperine specifically, but rather a mixture of the stereoisomers and some other undefined compounds all together. Today it is obvious to turn to liquid chromatography (LC) for this analysis. The LC of pepper constituents and in particular the quantitation of piperine in peppers and the separation of the four possible piperine stereoisomers have been investigated by one of the laboratories involved in the present work[2–4].

Piperine is readily available by extraction of ground pepper with, *e.g.*, methylene chloride, and repeated crystallization of the extract residue from methanol–water until the melting point of the crystals is 131°C. The four stereoisomers are also easily

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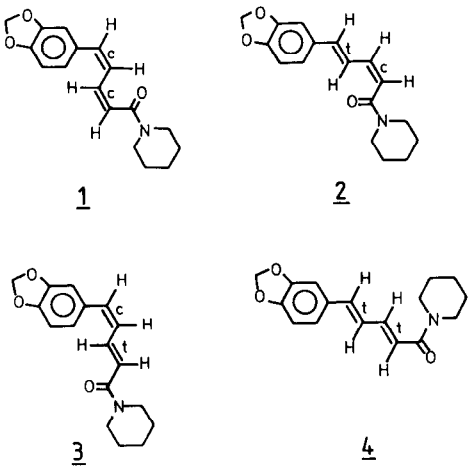

Fig. 1. Structural formulae of the four stereoisomers: 1, chavicine; 2, isopiperine; 3, isochavicine; 4, piperine.

accessible, as a mixture, since they are formed on irradiation of a methanolic piperine solution, eventually with sunlight. A 1-g amount of piperine dissolved in 100 ml of methanol and irradiated at 350 nm for 18 h affords a photostationary-state mixture containing the four stereoisomers (see Fig. 1) piperine (4, *trans–trans*), chavicine (1, *cis–cis*), isopiperine (2, *cis–trans*) and isochavicine (3, *trans–cis*). In this nomenclature for the diene system, the double bond closest to the piperidine ring is named first. These compounds are thus amides and as such can be hydrolysed. The four isomeric acids so obtained were separated by preparative chromatography and by countercurrent distribution. Their structures were elucidated and assigned by NMR spectrometry[5]. Piperine or the *trans–trans* isomer has the highest UV molar absorptivity, the longest wavelength of maximum absorption and the highest melting point. The opposite is true for chavicine (the *cis–cis* isomer), as expected. The data are summarized in Table I.

The photostationary-state mixture is best suited for studying LC conditions for the quantitation of piperine. In our earlier investigations of the analytical chromatography of piperine, many stationary phases were evaluated. Although several phase systems were discovered that yielded partial or complete separation of the four stereoisomers, none was completely satisfactory. Either the phase was not characterized accurately enough and the reproducibility was therefore questionable, or the column stability was deficient or the peaks showed annoying tailing, rendering electronic integration difficult. The preparative chromatography mentioned above was carried out, for example, on alumina E (Merck, Darmstadt, F.R.G.) on a 1 m × 0.85 cm I.D. column. The resolution was good[2] but later we had considerable difficulty in reproducing the chromatogram or could not do so.

On nitrated–sulphonated phenyl silica gel, excellent selectivity with complete separation of the isomers has been achieved[3]. Later, however, we could not reproduce this phase with exactly the same selectivity characteristics. It is well known that the chromatographic separation of the stereoisomers of diene systems is difficult. Many

TABLE I

DATA FOR THE FOUR ISOMERS

Numbering as in Fig. 1.

Peak No.	*Compound*	*M.p. (°C)*	λ_{max} *(nm)*	ε $(lmol^{-1}cm^{-1}$	E^a	Fa^b
1	Chavicine	(75)	321	10 900	0.266	1.83
2	Isopiperine	86	335	13 200	0.207	2.00
3	Isochavicine	103	336	12 500	0.309	1.35
4	Piperine	131	343	34 100	0.418	1.00

[a] *E* is the absorbance measured for a 1 mg per 100 ml solution at 252 nm.

[b] *Fa* is the correction factor for quantitation of the photoisomers via peak areas *versus* piperine for measurements at 252 nm.

efforts were directed at finding solutions to this problem. High-surface-area silica gel chromatography can help and argentation of silica gel is often a positive approach, but it cannot be said that these solutions are efficient, clean, reproducible, etc. Anyone with experience of argentation chromatography knows how messy and capricious it can be.

We report here on a chromatographic system, with a polyphenol bonded to silica gel as the stationary phase, which produces acceptable peak shapes for piperine and its stereoisomers, good column efficiency and sufficient resolution of the mixture to allow quantitation, and that can be synthesized reproducibly. With the current interest in the miniaturization of LC, conventional and micro-LC were compared for the determination of piperine.

Micro-LC, or chromatography with packed fused-silica capillary columns, has several important advantages over conventional LC[6–8]. To date, micro-LC has mostly been applied to generate very large plate numbers in long columns and consequently with very long analysis times. We believe, however, that micro-LC with more usual column lengths of 10–30 cm has so many attractive features that it may well become the normal routine mode of chromatography in the near future. This paper illustrates some of the advantages of micro-LC over conventional LC.

EXPERIMENTAL

The chromatographic system for conventional LC consisted of a Model LC 5500 chromatograph, a Model 2050 UV detector and a Model CDS-401 integrator (Varian, Walnut Creek, CA, U.S.A.). The columns were 25 × 0.46 cm I.D. Lichroma tubes provided with a 10-μl sample loop injector (Valco, Houston, TX, U.S.A.). Polyphenol-RSiL (10 μm) (RSL, Eke, Belgium) was packed downwards at 500 bar in a sonicated water–methanol (10:90) slurry. The columns were rinsed thoroughly with acetone, methanol–THF and hexane–methanol–THF (in that order) before equilibration with the mobile phase used for the chromatography. Alternatively, columns were packed with a 10% acetone slurry and with acetone as a follow-up solvent. All conventional LC was carried out with the columns in a thermostat.

The micro-LC system consisted of a Model LC 5020 chromatograph, a Model 2050 UV detector with a modified miniaturized cell (obtained from RSL) and a

Model CDS-401 integrator (Varian). The micro-LC columns (250 × 0.32 mm I.D., polyimide-coated fused silica) packed with 10-μm Polyphenol-RSiL and the split tee were obtained from RSL. The split tee was connected to a 100-nl injector (Valco C1 4W) and to an old spent column with conventional dimensions. The tee, columns and injector were placed in a water-bath thermostat.

Peppers and pepper extracts were obtained from the local market. Anthraquinone (internal standard) was purified by repeated crystallization from ethanol. Solvents were of LC grade.

RESULTS AND DISCUSSION

Chromatography of piperine and its stereoisomers

Polyphenol-RSiL is intended to be used as a strongly polar normal-phase stationary phase. It is synthesized by bonding a tannin on to silica gel. It is more polar than silica gel itself[9,10]. A fairly large amount of modifier can therefore be used in the normal-phase solvent mixture, which is beneficial for the speed of phase equilibration and for the general reproducibility of the chromatography. Hexane mixed with various amounts of methanol, THF and dioxane was tested for the optimization of the separation of the photostationary-state mixture of the piperine stereoisomers. The best result was obtained with hexane–THF (60:40).

The elution sequence of the four stereoisomers can be established by following the changes in the composition of a pure piperine solution subjected to a photoisomerization experiment. Isochavicine appears first in the chromatogram, next to the piperine peak, then isopiperine becomes visible and finally chavicine. In the final photostationary-state mixture, chavicine is the largest peak. The elution sequence for the four stereoisomers on Polyphenol-RSiL is first chavicine, then isopiperine, isochavicine and finally piperine. On nitrated–sulphonated phenyl silica gel[3] the LC elution sequence is chavicine, isochavicine, isopiperine and piperine. On acid-buffered (pH 2.5) silica gel[4] the order of elution is chavicine, isopiperine, isochavicine and piperine. On alumina[2] the elution sequence is isochavicine, isopiperine, chavicine and piperine. On Polyphenol-RSiL the elution sequence is the same as on acid-buffered silica gel. The reason for these selectivity differences is unknown to us. Alumina is basic, whereas nitrated–sulphonated and polyphenol phases are fairly acidic, but this does not explain the results. Hydrogen bond formation is always important in adsorption chromatography but nothing can be deduced in this respect from the structures in Fig. 1. Rotation around the single bonds of the conjugated system is free. The conformations shown in Fig. 1. are therefore not the only important ones, although conformational equilibration is probably slow.

An increased temperature proved to have a positive effect on the chromatographic separation of the photoisomers. Fig. 2 shows the chromatograms obtained with the micro-LC system at 25, 40 and 50°C. Some data deduced from these chromatograms are given in Table II.

Similar results for the conventional LC system are shown in Fig. 3 and Table III. It is obvious that an LC determination of piperine can be based on both systems.

Effiency (plate number) of the columns

The efficiency of the conventional LC column measured for piperine at the

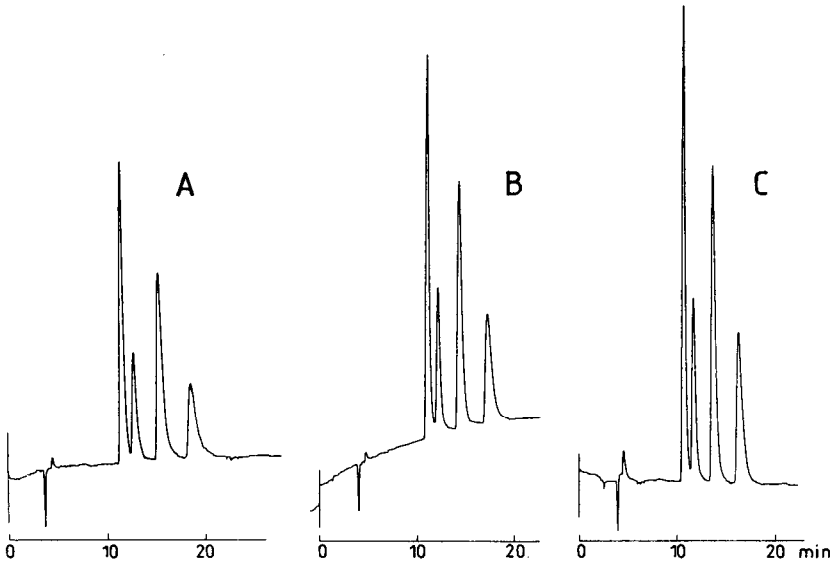

Fig. 2. Chromatograms obtained with a 250 × 0.32 mm I.D. fused-silica capillary column packed with 10-μm Polyphenol-RSiL. Mobile phase, hexane–THF (60:40) at 4 μl/min. UV detection at 252 nm. Peaks in order of appearance: 1, chavicine; 2, isopiperine; 3, isochavicine; 4, piperine. Temperature: (A) 25°C; (B) 40°C; (C) 50°C. Back-pressures at the split tee (A) 24; (B) 20; (C) 18 bar. Sample: 100 nl of the photostationary-state mixture obtained after irradiation for 18 h in a Rayonet photoreactor. Solution in hexane–dioxane.

TABLE II

MICRO-LC OF PIPERINE AND STEREOISOMERS AT VARIOUS TEMPERATURES (FIG. 2)

Parameter	*Compound*	*Temperature (°C)*		
		25	*40*	*50*
Retention time (min)	Chavicine	11.22	10.92	10.53
	Isopiperine	12.48	12.03	11.53
	Isochavicine	15.03	14.20	13.48
	Piperine	18.33	17.07	16.08
k' Values	Chavicine	1.95	1.87	1.77
	Isopiperine	2.28	2.16	2.03
	Isochavicine	2.95	2.74	2.53
	Piperine	3.82	3.49	3.23
Plates/m	Chavicine	18.833	23.124	26.686
	Isopiperine	17.799	21.553	25.333
	Isochavicine	14.299	19.808	22.304
	Piperine	11.979	15.095	18.220
Peak asymmetry factor	Chavicine	2.56	2.44	2.16
	Isopiperine	2.85	2.17	2.03
	Isochavicine	3.23	2.72	2.52
	Piperine	2.98	3.22	2.83

optimum flow-rate is only 8000–12 000 plates/m, whereas micro-LC columns under comparable conditions lead to 12 000–18 000 plates/m (Tables II and III). This difference is large enough to be important. Usually 10-μm RSiL materials produce much larger plate numbers. For the very best micro-LC and conventional columns packed with high-quality reversed-phase materials (5-μm ROSiL-C_{18}-D) and with a compact rigid molecule such as pyrene as a sample, the efficiency is about the same on the two systems. It is easier, or more readily possible, with micro-LC to achieve very good results (reduced plate height below 2), but this low h value (above 100 000 plates/m) can also be achieved with conventional column dimensions. In not such ideal situations (as in the present example with complex molecules such as piperine), the higher efficiency of micro-LC is more evident. The low plate numbers for the determination of piperine with both systems is ascribed to the complexity of the piperine molecule and to slow conformational equilibration. A temperature increase is important in these instances, as shown in Tables II and III. The present results therefore illustrate that micro-LC can be more efficient (*ca.* 50%?; see below) than conventional LC. Why this is so is not clear, but it is an important aspect of micro-LC that merits further investigation.

Mostly in LC, the plate numbers increase with increasing k' value because the contribution of the extra-column dead volume decreases with longer retention. This is the case, for example, for polycyclic aromatic hydrocarbons in reversed-phase LC. The effect is even greater for micro-LC where the dead volume is relatively more important because of the small total volume of the system. The plate numbers for the four piperine stereoisomers decrease, however, with increasing retention. In comparing the efficiency of micro-LC and conventional LC for the same compound, *e.g.*,

TABLE III

CONVENTIONAL LC OF PIPERINE AND STEREOISOMERS AT VARIOUS TEMPERATURES (FIG. 3)

Parameter	*Compound*	*Temperature (°C)*		
		30	*40*	*50*
Retention time (min)	Chavicine	13.75	12.88	12.27
	Isopiperine	15.26	14.23	13.48
	Isochavicine	17.33	16.08	15.14
	Piperine	21.44	19.77	18.52
k' Values	Chavicine	2.82	2.58	2.41
	Isopiperine	3.24	2.95	2.74
	Isochavicine	3.81	3.47	3.21
	Piperine	4.96	4.49	4.14
Plates/m	Chavicine	13.416	16.804	19.696
	Isopiperine	13.196	16.424	18.936
	Isochavicine	11.340	14.188	16.636
	Piperine	7.864	9.780	11.612
Peak asymmetry factor	Chavicine	2.47	2.28	2.09
	Isopiperine	–	–	–
	Isochavicine	2.77	2.47	2.28
	Piperine	3.11	2.84	2.60

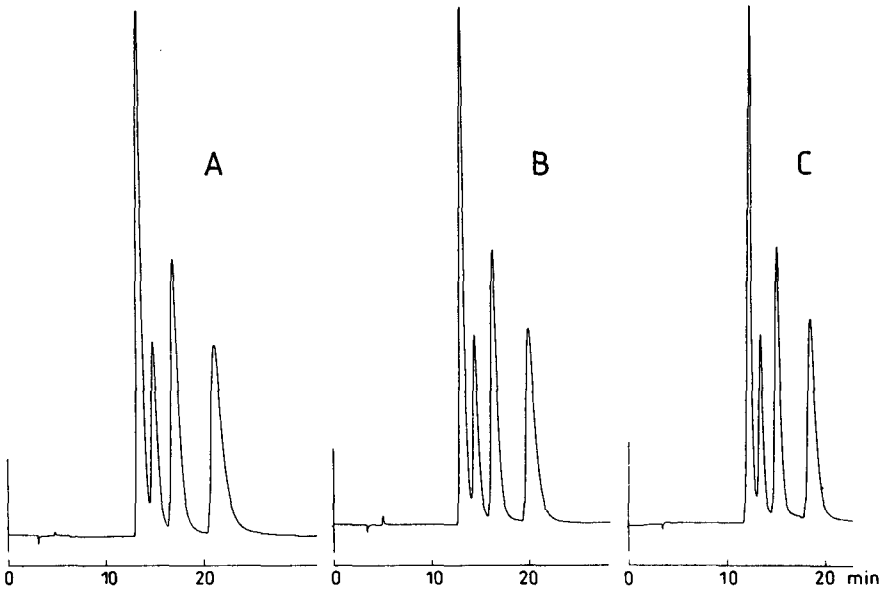

Fig. 3. Chromatograms obtained with a 25 × 0.46 cm I.D. column packed with 10-μm Polyphenol-RSiL. Mobile phase, hexane–THF (60:40) at 0.8 ml/min. UV detection at 252 nm.- Peaks in order of appearance: 1, chavicine, 2; isopiperine; 3, isochavicine; 4, piperine. Temperature: (A) 30°C; (B) 40°; (C) 50°C. Back-pressures: (A) 34; (B) 32; (C) 30 bar. Sample injected: 10 μl of the same solution as in Fig. 2.

piperine, this effect of the k' value should be considered. The piperine micro-LC peak at 50°C is 50% more efficient than the conventional piperine peak at 50°C, but its k' value is much lower. Even when almost identical k' values in Tables II and III are compared, micro-LC is found to be more efficient. With its lowest k' values, conventional LC does not achieve more than 20 000 plates/m in Table III. Micro-LC does better, even with k' values that are higher. Similar results were noted with a large number of columns over a long period of time. This study therefore illustrates an important advantage of micro-LC, which is clearly more efficient than conventional LC.

Retention (k' values) and relative retention (α values) in both systems

The differences in k' values, which are on average about 30% higher on the conventional system than on the micro-LC system (see Tables II and III), deserve attention. They are due to a relatively small difference in the total porosities of the columns. From the dead time (measured with hexane) and the calculated empty column volume, it can be deduced that this total porosity is 0.56 for the conventional and 0.62 for the micro-LC columns. The resulting phase ratios [the β values in gas chromatography (GC)?] are 1.27 and 1.63, respectively, or about 25% higher for the micro-LC system. The phase ratio for adsorption LC (the mobile phase is a liquid and the stationary phase an adsorbent) cannot really be compared to the phase ratio in GC, since the adsorbent surface area is not strictly related to its volume. That such a relative small difference in total porosity would have such a dramatic influence on the k' values was at first a surprise, although of course normal with hindsight. The Pol-

yphenol-RSiL stationary phase was the same in both chromatographic systems. The above observation leads us to believe that differences in k' values, often ascribed to different surface chemistries of derivatized silica gels (manufacturers are blamed!), might in fact often be due to such porosity differences. Differences in total porosity can thus, inversely, be deduced from differences in k' values. The smaller k' values in micro-LC illustrate the higher permeability of this form of chromatography. Higher permeability often means lower stability, but in the present instance it does not, because the columns are made from inner wall polymer-coated fused-silica capillary tubing. The stabilizing effects of "inner wall coating and other wall effects" were discussed recently[11]. Under these conditions, the better permeability of micro-LC must be considered an advantage.

The k' values in the two chromatographic systems do not change proportionally to the same extent and therefore the α values are also slightly different in the two systems. This is shown in Table IV.

Analysis of Table IV shows that the compounds with a double bond *trans* to the amide function are selectively more retarded on the micro-LC system. These are the more polar compounds. Piperine elutes last under normal-phase adsorption conditions and first, or is unseparated, from the other stereoisomers under reversed-phase conditions. The higher polarity of piperine and isochavicine also conforms with other physical parameters of the compounds (higher melting point and longer wavelength of maximum absorption; see Table I). This therefore means that the micro-LC column appears to be more polar than the conventional column. We have no rational explanation for this effect.

Determination of piperine in peppers and pepper extracts

For the quantitative analysis of mixtures containing piperine and its stereoisomers, the detection wavelength is of course important, not only for the compounds to be measured, but also for the internal standard. We have previously used phloracetophenone[4] and *p*-bromoacetanilide[3] as internal standards (I.S.) for this analysis. The phase system with Polyphenol-RSiL is, however, different to that in the previous procedures. Therefore, a new internal standard had to be found. Anthraquinone proved to be a possibility as it can be purified thoroughly by recrystallization from ethanol, elutes before piperine in a relatively uncomplicated part of the chromatogram and has a UV absorption maximum at 252 nm, where the photoisomers of piperine have relatively flat and similar absorption characteristics. A calibration graph was established at this wavelength with various amounts of piperine and the

TABLE IV

α VALUES ON CONVENTIONAL AND MICRO-LC SYSTEMS AT 50°C

Compounds	*Conventional LC*	*Micro-LC*
Isopiperine/chavicine	1.14	1.15
Isochavicine/isopiperine	1.17	1.25
Piperine/isochavicine	1.29	1.28
Piperine/isopiperine	1.51	1.59
Piperine/chavicine	1.72	1.82

same concentration of anthraquinone. Each solution was analysed several times. The equation found for the micro-LC system was

$$Y = 0.190X - 0.108$$

where Y = surface ratio of piperine/I.S. and X = concentration ratio of piperine/I.S., with a correlation coefficient $r = 0.9999$. The same solutions were used to establish the calibration graph using the conventional LC system and the equation obtained was

$$Y = 0.196X - 0.160 \ (r = 0.9989)$$

These two equations should, of course, be the same but are in fact slightly different. Neither line (Fig. 4) passes through the origin, and this effect is slightly more pronounced for the conventional LC system. A similar observation has been made in the analysis of hop and beer bitter substances[12]. This effect implies that some of the material to be analysed disappears in the system, which was ascribed to the negative influence of metals (in the columns and frits and also in the packing material). This was to be expected for hop and beer bitter acids, as these compounds are notably sensitive to metal, but it was a surprise to find that piperine was also affected.

With the above-discussed possibilities, a method for the determination of piperine was developed. The extraction time for ground pepper was evaluated by analysis after extraction for 10, 30, 60, 90 and 1440 min. No difference in result was observed. The procedure is then as follows. A standard solution (0.06356 mg/ml) of anthraquinone in hexane–THF (60:40) is prepared and 25 ml are added to about 400 mg of ground pepper and stirred for 30 min in a vessel protected from light with aluminium foil. A 100-nl volume of the supernatant is injected on to a 250 × 0.32 mm I.D. micro-LC column packed with 10-μm Polyphenol-RSiL. The measuring wavelength

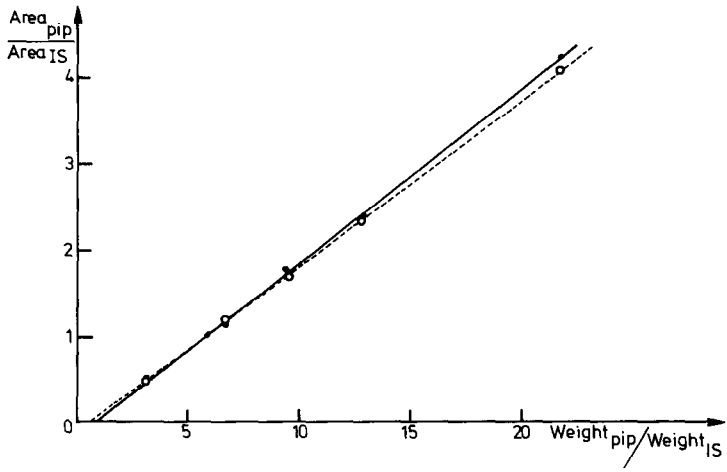

Fig. 4. Calibration graphs of peak surface area ratios *versus* weight ratios for piperine determination via micro-LC (○) and conventional LC (●). Internal standard, anthraquinone.

TABLE V

DETERMINATION OF PIPERINE BY MICRO-LC

The samples were obtained from local suppliers, except for the Zairese pepper and the extracts, which had been kept in the laboratory for more than 20 years without special precautions and apparently without much deterioration (see ref. 2). The Cayenne pepper is not supposed to contain piperine and its pungency is derived from other compounds.

Sample	*Piperine (%)*	*Standard deviation*	*Relative standard deviation (%)*
Black Liebig	3.95	0.5774	1.46
White Liebig	4.52	0.0306	0.68
Liebig Cayenne	0.00	–	–
Black Delhaize	4.44	0.0231	0.52
White Delhaize	4.80	0.0231	0.48
Maille 1747	4.52	0.0306	0.68
Zairese pepper (older than 1955)	1.36	0.0200	1.47
Extract (Fritzche)	26.71	0.4065	1.52
Extract (Chiris)	39.83	0.4521	1.14
Extract (Lampong)	33.26	0.2303	0.69

is 252 nm and the flow-rate of hexane–THF (60:40) is 4 μl/min. The back-pressure (*ca.* 20 bar) is strongly dependent on temperature, but it is always lowest on the micro-LC columns (see the legends to Figs. 2 and 3). Results of the analyses were calculated with the calibration equations mentioned above. Table V shows some data for the determination of piperine in various peppers and pepper extracts following this procedure.

The standard deviations in Table V, obtained by running three analyses, are for the chromatographic run only. The mean relative standard deviation is 0.96%. The standard deviation for the total analysis (extraction and LC) was determined by running the same pepper analysis six times (x = 4.52%, s = 0.0346, s_{rel} = 0.77%).

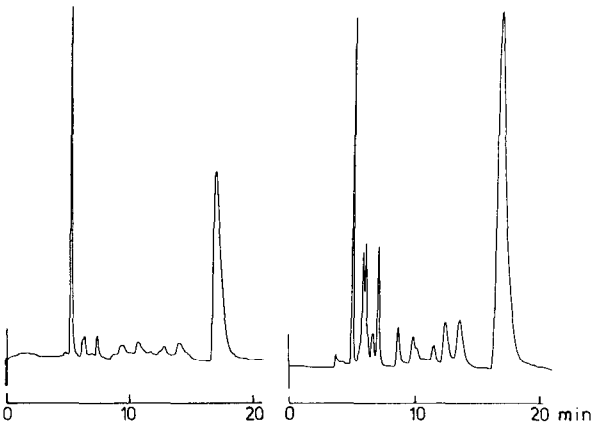

Fig. 5. Micro-LC traces for white Liebig pepper (left) and a Lampong pepper extract (right) with anthraquinone (first peak) as internal standard. Other conditions as in Fig. 2. The last peak is piperine.

TABLE VI

DETERMINATION OF PIPERINE BY CONVENTIONAL LC

Sample[a]	*Piperine (%)*	*Standard deviation*	*Relative standard deviation (%)*
Black Liebig	4.03	0.0608	1.51
White Liebig	4.55	0.0919	2.02
Liebig Cayenne	0.00	–	–
Black Delhaize	4.82	0.0611	1.27
White Delhaize	5.49	0.0636	1.16
Maille 1747	4.88	0.0636	1.31
Zairese pepper (older than 1955)	1.46	0.1556	10.66
Extract (Fritzche)	28.17	0.6450	2.29
Extract (Chiris)	39.92	1.3051	3.27
Extract (Lampong)	36.98	0.6689	1.81

[a] As in Table V.

An example of an analytical chromatogram for white Liebig pepper and for an extract is shown in Fig. 5.

The same analyses were run on a conventional system. The amount injected was 10 μl and the flow-rate 0.8 ml/min. The results are shown in Table VI. The mean relative standard deviation is 2.81%. Even if we exclude the outlayer of Zairese pepper, the relative standard deviation is still 1.83%, *i.e.*, considerably higher than for micro-LC. The micro-LC system was thermostated; this is more necessary with micro-LC than with conventional LC as the small micro-LC columns take up temperature fluctuations very easily. For strict comparison purposes the conventional chromatograms were also produced under thermostated conditions. Most of the results in Tables V and VI compare well, but there are differences that we cannot explain.

The above results indicate that better quantitation was achieved on micro-LC columns. A similar conclusion was drawn for the analysis of hop and beer bitter acids[1,2]. With simpler systems and sample molecules such as phthalates or polycyclic aromatic hydrocarbons such a difference in the results given by the two chromatographic systems is not observed. Obviously, this "quantitation advantage" and the better permeability and efficiency of micro-LC deserve further attention.

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