Determination of Piperine in Pepper *(Piper nigrum L)*

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Abstract: This paper introduces a new technique of piperine assay based on tlc-uv densitometry which produces nearly identical results to the already known tlc-uv spectrophotometric method. Results of this study confirms that Sri Lanka pepper has very high piperine content (7-15%); 2-6 fold that of commercial Indian, Malaysian and other varieties. This is the first report containing comprehensive data verifying the high piperine content of Sri Lanka pepper using reliable analytical techniques.

1. Introduction

Pepper *(Piper nigrum L.)* is one of the most important spices grown in Sri Lanka. Traditionally it has been exported as the primary product-black pepper. Sri Lanka pepper has had a reputation for containing high levels of volatile and pungent principles.[6] However, market prices are at present not always dictated by these quality factors. It is clear that proving the presence of these qualities in our pepper is a step in the direction of establishing premium prices for the product. The detailing of acceptable analytical techniques and their application is of special importance in the case of the piperine because this compound is present in our pepper at levels 2-6 fold than found in pepper from other sources judging from our studies and reports contained in the literature.[5,6]

Piperine is responsible for the pungency of black pepper - the contribution from other compounds to the pungency of this material being negligible.[6] The earliest method for determination of piperine in pepper was the Kjeldhal method[20] (for N) which obviously resulted in exaggerated values. An early method established was the spectroscopic method[3] (termed "direct uv method" hereafter) by which quantitation was achieved by measuring optical density at 345 nm. Here again (coloured) impurities could result in exaggerated values.

Several colorimetric methods have been reported eg. using chromatropic acid[11], nitric acid[7], phosphoric acid[8] but generally reproducibility of these methods was considered inadequate.[17]

Labruyere[10] proposed a hydrolytic method to liberate piperidine (from piperine) followed by steam distillation and titrimetric analysis to estimate the former. This method was later modified by substituting a colorimetric step for the titration eg. in methods by Shankaranarayana *et al*[18] and Kap.[9] However, the direct uv method

was still preferred over all these methods and until recently still advocated mainly due to its simplicity and the claim that its error is relatively small - over estimating piperine by only 5-10%. [6,12,16]

Notwithstanding this, analysis of piperine moved in the direction of methods based on thin-layer-chromatography (tlc) and piperine was analysed by Wijesekara *et al* using the tlc-spot area method.[19] here spot area was correlated to concentration. This trend was continued by Mori *et al*[12] advocating the separation of piperine by tlc before estimating it by uv spectrophotometry.

Govindarajan[6] in an exhaustive review stated that a method based on tlc and spectrophotometry at 342 nm (of the eluted spot) gave the best correlation between pungency (by sensory evaluation) and piperine content. However to our knowledge the details of this method (termed "tlc-uv method" hereafter) were not reported. Further, according to this author the error of the "direct uv" method caused by coloured substances was only of the order of 5-10%.[6]

Piperine has also been successfully separated by gas-liquid chromatography but the technique has not been sufficiently studied to merit the status of a good quantitative analytical technique[14,21] for the assay of piperine. HPLC, however has been used successfully to quantify piperine using a variety of systems.[2,4,13]

In this paper we report:—

(a)   A "tlc-uv" method which expands on Govindarajan's[6] report and also shows that the error of the "direct-uv" method is not merely 5-10% but varies markedly depending on nature of solvent used for extraction and extraction time rendering the results of the method very suspect.

(b)   Data on the tlc-spot area method providing further evidence of its inherent inaccuracies; first pointed out by Govindarajan.[6]

(c)   A new method based on tlc-uv densitometry the results of which tally very closely with the tlc-uv spectroscopic method.

2. Materials and Methods

2.1 Standard piperine

Pure piperine (m. pt. 129°C) was purchased from Sigma Chemical Company. Standard curves were prepared using this specimen and also using piperine extracted from pepper (with CH_2Cl_2) recrystallised repeatedly from ethanol. The latter standard had a m. pt.of 129-130°C and purity was verified using mixed melting points.

2.2. Pepper oleoresin

Pepper was sampled from commercial stocks at export dealing points(G.S.Chatoor & Co. Ltd.) as well as at estates (Mahavela and Wariyapola Estates) and other small holdings in Matale and Kandy. The pepper was ground in a mortar and a sample (50g) was extracted in a soxhlet apparatus for 4h using the selected solvent (250 ml). On evaporation of the solvent under reduced pressure, the oleoresin was obtained.

2.3 Direct-uv spectrophotometric method

The oleoresin was dissolved in $CHCl_3$ and an aliquot diluted so that absorbance at 342 nm lay within the limits of a linear standard curve of piperine concentration *vs* absorbance. All manipulations were carried out away from light. $CHCl_3$proved to be a better solvent than methanol as the use of the latter resulted in a marked decline in optical density with time.
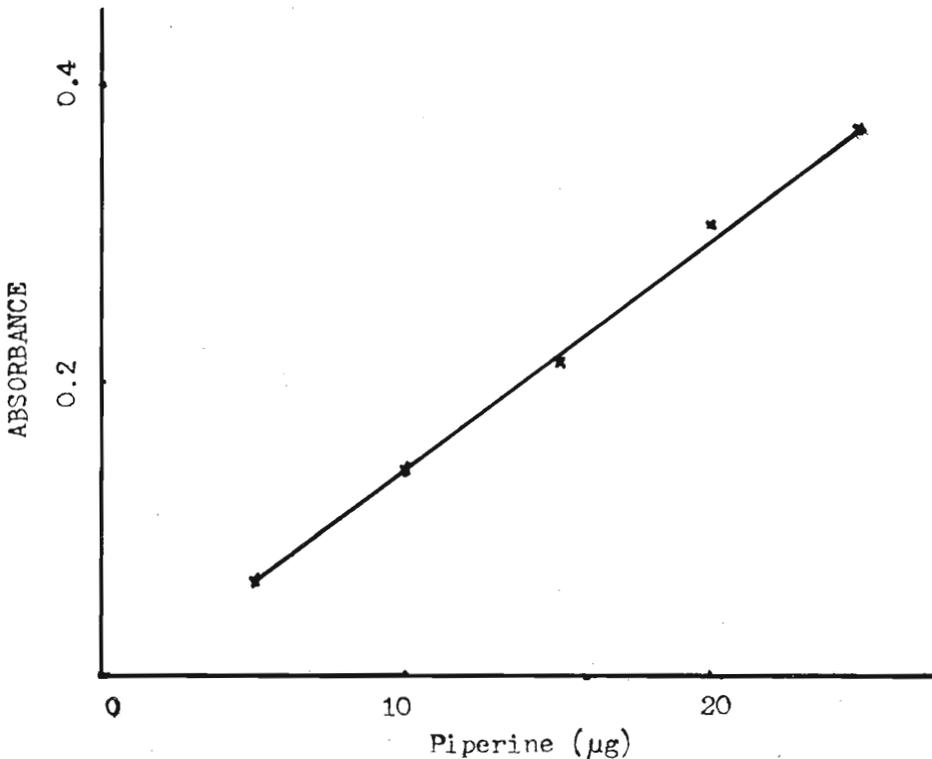

Figure 1 : Standard curve for piperine using "tlc-uv" method

Piperine (μg) is correlated with absorbance after thin-layer chromatography and uv-spectroscopic analysis at 342 nm.
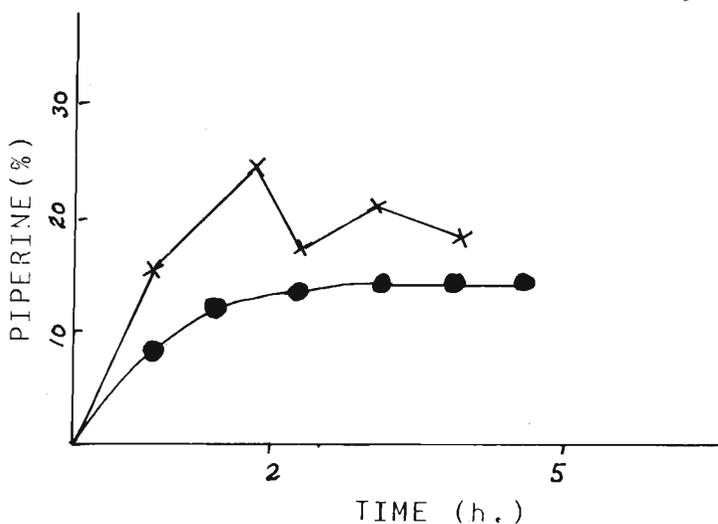
Figure 2:   Effect of extraction time on piperine content as determined by the 'direct-*uv*'
              and *tlc-uv*' methods.

Solvent, CH$_2$ Cl$_2$

O ————— O, tlc-uv;

X ————— X, direct uv.

2.4 Tlc-spot area method

This was carried out by the procedure of Wijesekara *et al*[17] using diluted oleoresin.

2.5 Tlc-uv spectrophotometric method[15]

A   10 μl aliquot of oleoresin in CHCl$_3$ containing approximately 15 μg was spotted on a *tlc* plate (silica gel G-60 300 μm) and developed in toluene : methanol (85 : 15). The position of the piperine spot (Rf = 0.45) located by fluorescence in *uv* light. The silica gel containing the spot was scraped off and extracted into chloroform (5 ml) using a vortex mixer for 2 min. The silica gel was separated by centrifugation for 10 min at 600g and *uv* absorbance determined within 10 min using a silica gel blank (0.D = 0.02) with a Varian 634S spectrophotometer. Piperine content was calculated using a standard curve (Figure 1). The coefficient of variation of the method was 1.4%.

2.6 Tlc-uv densitometric method

Approximately 1.5 μg (in 10 μl) of piperine was spotted on a *tlc* plate (300 μm) and chromatography carried out as in 2.4. The plate was scanned using a Camag automatic scanning variable wavelength densitometer   model 7650 (monochromater model).

Scanning was carried out at 342 nm (band width 30 nm) with a slit width of 12 mm and a sensitivity setting of 6 using a Deuterium lamp. Scan speed was 2mm / sec.

A linear standard curve was obtained for aliquots containing 0.5 μg to 2.5 μg when plotting peak area against mass of sample applied. However this standard curve itself was not used for the calculation of the concentration of the unknown as peak area varied slightly from one tlc plate to another. Therefore calculations were made from peak area after running standards on the same plate as the unknown. The coefficient of variation of the technique was 2.4%.

3. Results

3.1 Comparison of analytical techniques

Table 1 shows an analysis of a sample of pepper using different analytical techniques. The solvent used for extraction of oleoresin was also varied.

The results led to the following conclusions:

(i)   The three methods yielded vastly different results.

(ii)   In general the "direct uv" method gave the highest values.

(iii)   The enhanced values obtained with the "direct uv" method were far greater than the 5-10% reported previously.[6]

(iv)   Values using the spot area method do not have a fixed trend with respect to the results of other methods.

(v)   As 6a, 6b, 6c and 6d were from the same specimen of pepper it was clear that the results of the tlc-uv method has the least dependence on the solvent used.

(vi)   Sri Lanka pepper appears to have a relatively high piperine content.

3.2 Errors of the "direct-uv method"

Errors arose as a result of the presence of 342 nm absorbing impurities in the extract. These impurities in the oleoresin (solvent extract) vary depending on the solvent used (Table 2). It is envisaged that the 5-10% enhancement reported previously was possibly due to the fact that methylene and ethylene dichloride were used for extraction in previous studies. The ban on the use of chlorinated hydrocarbons in oleoresin manufacture in some parts of the world will result in other solvents being used for the purpose and therefore the exaggeration of piperine content as determined by the "direct uv" method will be variable and could be very high. Further, an additional problem would be posed in the analysis of an oleoresin where the history of extraction is unknown.

A comparison of the effect of extraction time using CH_2Cl_2 on apparent piperine content by the "direct uv" method and the "tlc-uv method" is shown in Figure 2. It is seen that while the piperine content as estimated by the "uv-tlc method" falls on a smooth hyperbolic curve, the readings of the direct uv method are erratic. This is interpreted as being due to the uv-reading of the extract being a function of not only the extent of extraction of uv absorbing material but also its rate of decomposition during extraction. From this it is inferred that the determination of piperine by the direct-uv method will be subject to errors connected not only with the solvent used but also with extraction time.

Table 1.— Apparent piperine content of pepper with different analytical techniques

Sample	Extraction Solvent	Oleoresin (% dry wt)	Piperine (% dry wt pepper)		
			Spot area	direct-uv	tlc-uv
1. FAQ	CH_2Cl_2	10.6	7.3	—	6.4
2. FAQ	Methanol	27.3	14.1	17.2	11.7
3. Dried green pepper	Methanol	31.7	7.3	13.8	11.4
4. Fresh pepper	Methanol	40.5	12.3	20.3	13.4
5. FAQ	CH_2Cl_2	17.8	—	12.9	8.6
6. Grade I	(a) CH_2Cl_2	14.4	6.9	9.8	7.7
	(b) Methanol	17.3	8.2	14.1	7.3
	(c) Methanol	17.4	10.6	14.9	7.3
	(d) Acetone	11.6	—	8.4	6.3
7. Light berries	CH_2Cl_2	14.4	6.9	9.8	7.7
8. Light berries	CH_2Cl_2	16.3	—	12.5	9.2
9. Off-grade black	CH_2Cl_2	18.0	12.3	14.6	9.1

Graded berries were obtained from the Export trade (G.S.Chatoor & Co,.Ltd) in the course of 1980. Fresh pepper was obtained from Palapathwela, Matale and dried green pepper prepared from fresh pepper in the laboratory.

FAQ — Fair average quality grade.

Extraction time — 4 h.

Table 2.– Effect of using different solvents on piperine content as determined by the 'direct-uv' method

Solvent	Piperine (% dry wt pepper)
CH_2Cl_2	8.8
Acetone	7.6
Methanol (Experiment 1)	12.5
Methanol (Experiment 2)	13.4

Samples of the same lot of pepper were extracted for 4h. using the solvents indicated above.

3.3 Errors of the spot-area method[19]

As indicated by Govindarajan[6] (no details were published by him) the spot area method was found to be not reproducible.

In this study the same sample of oleoresin when applied on different plates led to vast discrepancies leading to a coefficient of variation of the order of 50%. This is largely due to small variations in the characteristics of different *tlc* plates. When the same tlc plate was used then a reduced coefficient of variation (12%) was observed for multiple determinations. Therefore if the "spot-area method" is used along with standard curves for each tlc plate, then the errors would be infinitely more tolerable.

However the importance of this tlc method lay in the fact that it introduced the concept of separating out 342 nm absorbing impurities by thin-layer chromatography.

The above point was confirmed by measuring the optical density at 342 nm contained in $CHCl_3$ extracts of the silica gel from different parts of the plate. Only approximately 70% of the *uv* absorbance was found to coincide with the piperine spot and a further 25% was found to occur in other parts of the tlc-plate.

3.4 The tlc-uv spectrophotometric method.

Next, an attempt was made to determine the recovery of piperine using the tlc-uv spectrophotometric method. For this purpose a low piperine substrate had to be obtained. The mother liquor of the oleoresin after piperine crystallisation was selected for this purpose. Experiments led to recovery of piperine to the extent of 105%. As the

Table 3.—Piperine content of black pepper by tlc-uv and densitometric methods

Sample No.	Piperine (% dry wt)	
	Tlc-uv	Tlc-densitometric
1. GRADE I	13.3	13.5
2. GRADE I	15.0	14.8
3. GRADE I	13.9	13.8
4. GRADE I	13.3	12.9
5. GRADE I	12.2	12.2
6. LIGHT BERRIES	12.5	12.5
7. LIGHT BERRIES	14.1	13.8
8. LIGHT BERRIES	14.1	14.2
9. ESTATE COLLECTION	13.9	13.8
10. ESTATE COLLECTION	13.5	13.5
11. ESTATE COLLECTION	14.1	14.1
12. ESTATE COLLECTION	13.4	13.3
13. ESTATE COLLECTION	13.8	13.8
14. ESTATE COLLECTION	14.2	14.1
15. ESTATE COLLECTION	13.9	13.9
16. ESTATE COLLECTION	13.8	13.8
17. ESTATE COLLECTION	12.0	11.9

Solvent, CH_2Cl_2; Extraction time, 4h.

Graded pepper (samples 1-8) were obtained from the export trade (G.S. Chatoor & Co., Ltd.) in the course of 1982. Estate collections were obtained from Wariyapola estate (sample 9-12) and small holdings in the Matale district (samples 13-17).

results are calculated from two separate determinations, viz; (i) mother liquor (containing small amounts of piperine) and (ii) mother liquor plus added piperine, this value for recovery is acceptable.

This confirmed the conclusions of Govindarajan[6] and taken together with his sensory evaluation data establishes the credibility of the method.

3.5 Comparison of results of tlc-uv spectrophotometer and tlc-uv densitometric methods

The great disadvantage of the tlc-uv spectrophotometric method is the tedious work involved in scraping out the silica gel, eluting piperine into $CHCl_3$ and centrifuging prior to spectrophotometry. The tlc-uv densitometer obviates the need for these operations and experimental requirements are limited to scanning of the tlc plate at the appropriate wavelength and determination of peak area from the recorded chart. Further the sensitivity of the densitometer method is a great advantage as the quantity of material required is one tenth that of the tlc-uv method thus enhancing separation. Results of both methods tally very closely (Table 3).

4. Discussion

Two methods are now available for the routine analysis of piperine in pepper. The methods are very similar with respect to the separation of the piperine spot but differ in the final quantitative step.

The paper also provides a bank of data illustrating the high levels of piperine in Sri Lanka pepper - generally in the range of 7-15% as against 2 -7% for the commercial Indian, Malaysian and other varieties. There appears to be a strong case for the inclusion of piperine content in our certificate of export in order to fully exploit this favourable natural quality of our pepper.

The paper is important at this juncture as the introduction of high yielding Kuching (Malaysian) and Panniyur (Indian) varieties to local plantations is now being advocated,[1] these varieties are not known for high piperine levels. In fact the literature reports the piperine content of the Panniyur variety to be in order of 3-5%.[6] It is foreseen that the replacement of the indigenous variety with the new cultivars although resulting in a significant increase in pepper yield will also result in a decline in oleoresin and piperine yield.

Acknowledgements

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