# A RAPID MICRO-METHOD FOR THE SEPARATION, IDENTIFICATION AND ESTIMATION OF THE PURINE BASES: CAFFEINE, THEOBROMINE AND THEOPHYLLINE

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The work now reported was done in connection with the development of new analytical techniques for studies on the Ceylon-grown cocoas (*Theobroma cacao*). The problem of studying the relative amounts of caffeine and theobromine present at various stages of growth of the cocoa bean seemed to warrant the development of a rapid and simple micro-method for quantitative estimation of these bases. Various methods are available for the determination of caffeine, theobromine and theophylline. They are based on spectrophotometry[1,2], titrimetry[3,4], gravimetric analysis[5,6], or the Kjeldahl method of nitrogen determination[1]. When the three bases or any two of them are present together, a prior separation has been found to be necessary. In the methods for determination of caffeine in tea[1] where a micro-Kjeldahl determination gives the total "basic nitrogen", the quantities of theobromine and theophylline present are also included in the values obtained. In cocoa products where caffeine and theobromine occur together, the former is separated by preferential extraction into ether[1]. Many of these methods are quite unsuitable for purposes of repeated assays over a small period of time. Methods based on thin-layer chromatography on buffered silica gel plates, for the qualitative detection of caffeine and theobromine as well as the base theophylline, have been described[7–9]. A rapid quantitative method based on thin-layer chromatography by which the three bases could be conveniently assayed even when they occur together, has been developed and is described in this paper.

## EXPERIMENTAL

### *Preparation of the plates*

A slurry of 25 g of Kieselgel G nach STAHL (E. Merck A.G., Darmstadt, Germany) in 50 ml of distilled water was applied to clean glass plates (20 × 20 × 0.3 cm) at a predetermined thickness (250 $\mu$); a Desaga adjustable spreader was used. The plates, prepared in batches of five, were allowed to stand at room temperature for 90 min and dried in an oven at 110° for 1 h. The coated plates were cooled in a Shandon desiccator cabinet over silica gel and used as described.

### *Standard solutions*

Standard solutions of the three bases were made from samples kindly provided by British Drug Houses, Ltd., Poole, England. Theobromine and caffeine were also

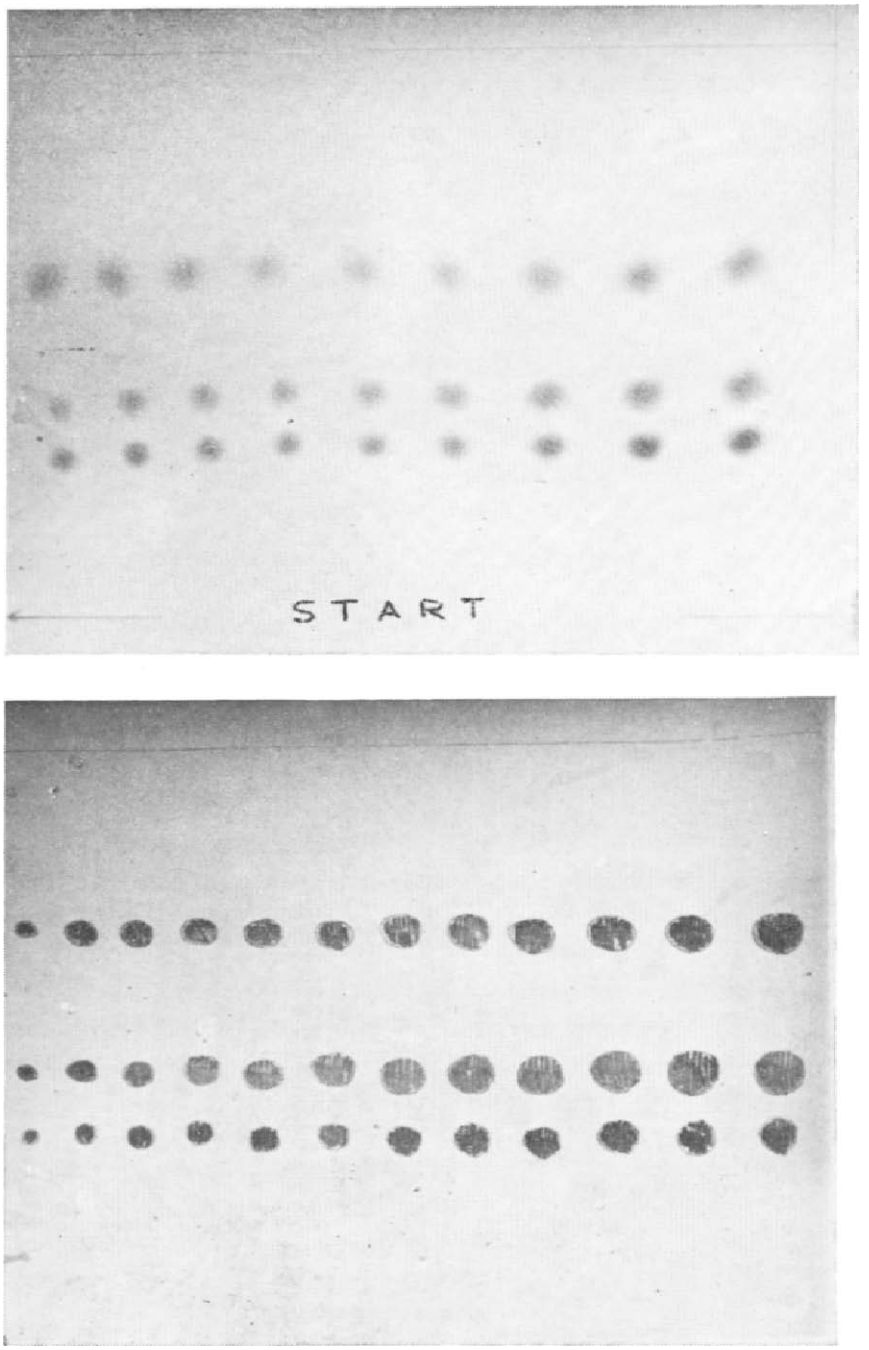



Fig. 1. (a) Photograph of a typical chromatogram of a mixture of caffeine, theobromine and theophylline. The spots have been visualised by the reported spray reagent. (b) A photograph of a similar chromatogram, where the matrix within the spot has been removed.

extracted by us from cocoa bean shells and cocoa powder and were purified by repeated crystallisation. The concentrations of the standard solutions were: theobromine and theophylline 2.0 mg/ml; caffeine 4.0 mg/ml. The solvent was *n*-butanol–acetic acid (3:1). In the case of theobromine and theophylline the solid material tended to precipitate overnight and consequently the solutions had to be warmed prior to use. The high boiling *n*-butanol was used as solvent to minimise loss by evaporation. The standard solutions were stored at room temperature.

*Development*

Several solvent systems[8–10] were tried and the most satisfactory one for development was chloroform–carbon tetrachloride–methanol (8:5:1). This system gave a clear separation of the three bases in a developing time of 53 min approximately for a 15 cm run. (Fig. 1). The $R_F$ values were reproducible within satisfactory limits (Table I) and the spots were nearly circular, a feature that is important for quantitative analysis[11]. The developing tanks used (dimensions: 22 × 22 × 5 cm)* were lined with filter paper as usual for better equilibration. It was found that a single charge of solvent could be satisfactorily used to develop not more than five chromatoplates.

*Technique of spotting*

Self-filling "Camag" micropipettes (2–5 $\mu$l) were used to spot the solutions on to the chromaplates. The spotting had to be carefully done as any damage to the matrix resulted in undesirable distortion of the shape of the spot. It was also necessary to ensure that the areas of the spots on application were equal and so it was necessary to dry each spot completely before another application was made over it. Thorough drying was also ensured before development to avoid trailing. The *n*-butanol–acetic acid mixture proved to be an ideal solvent for quantitative application of the substances on to the chromatoplates; more volatile solvents like chloroform and carbon tetrachloride tended to cause trouble by vapourising in the pipette during application.

*Visualisation of the spots*

Several spray reagents recommended for visualisation of purines were examined. WOOD's reagent[12] and the eosin–mercury reagent[13] failed to satisfactorily locate the spots. Dragendorff's reagent as prepared by SHUTE[14] and modified by dilution with a 4 % solution of iodine in potassium iodide and alcohol (50:50) proved satisfactory. A method[8,9] of visualisation which involves the use of two solutions; firstly an ethanolic solution of iodine in potassium iodide followed by ethanolic hydrochloric acid (25 %) was also successful. This is essentially the same as the above method using the modified Dragendorff reagent. The colours produced were similar and possibly the Dragendorff reagent itself plays little part in the colour development. However, it was important that a reagent which could be used as a single spray be developed, as on a second spraying the spots tended to diffuse. This was undesirable for quantitative estimation of the spot area. Accordingly, the reagent was modified for final use as follows: Iodine (2g)–potassium iodide (2g) was dissolved in 95 % ethanol (50 ml) and concentrated hydrochloric acid (50 ml of 25 %) was added. The colours generated were as given in Table I. The colours remained stable for about 2–3 h and slowly faded away possibly due to the volatilisation of iodine. The colours could always be regenerated by spraying again. However, the drawbacks of double-spraying make it imperative that measurement of spot area be done immediately after the first spraying.

*Measurement of spot area*

The spot area was measured as follows: After spraying the developed chromato-

* Obtained from: Shandon Scientific Company Limited, 65, Pound Lane, Willesden, London, England or Camag Ltd., Homburgerstrasse 24, Muttenz, Switzerland.

TABLE I

MEAN $R_F$ VALUES AND COLOUR RESPONSE TO CHROMOGENIC SPRAY REAGENTS

Colour charts: (1) A. KORNERUP AND J. H. WANSCHER, *Methuen Handbook of Colour*, Methuen & Co., London, 1963; (2) *British Standard 381 C: 1964. Colours for Specific Purposes*, British Standards Institution, London; (3) *"Derwent" Colour Chart*, The Cumberland Pencil Co. Ltd., Smethwick, Staffs., England.

| *Substance* | *Mean $R_F$ (mean of 16 readings)* | *Standard deviation* | *100 × $R_F$-Caffeine mean ± standard deviation* | | *Colour developed* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | *Modified Dragendorff* | | | *Present reagent* | | |
| | | | | | *1* | *2* | *3* | *1* | *2* | *3* |
| Caffeine | 0.54 | ± 0.01 | 100 | ± 1.0 | 8E7 | No. 435 Camouflage red | 63 Venetian red | 9D6 | No. 444 Terra-cotta | 62 Burnt-sienna |
| Theophylline | 0.46 | ± 0.01 | 80.4 | ± 1.0 | 8E5 | No. 435 Camouflage red | 63 Venetian red | 8D4 | No. 48 Leaf brown | 61 Copper beech |
| Theobromine | 0.31 | ± 0.02 | 57.4 | ± 2.0 | 15F2 | — | 69 Gun metal | 21E3 | No. 638 Dark sea grey | 69 Gun metal |

gram the spots were outlined by means of a metal scriber. The matrix within the outline was carefully scraped off and removed with a micro-spatula (Fig. 1). The plate was then placed, matrix downwards, on a sheet of millimetre graph paper. The area was estimated in square millimetres by counting the squares within each spot-outline, to the nearest half-square, using a magnifying lens.

*Typical procedure*

The bases are first extracted from the material using any suitable method[1]. We used defatted cocoa powder and the following method of extraction: A weighed amount of the powder (10 g) was ground with magnesium oxide (2.5 g) and moistened with water (20 ml). The material was dried over a steam bath and quantitatively transferred to a Soxhlet thimble of suitable size. The final traces of powder were removed by means of pieces of cotton wool which were also packed into the thimble. The thimble was placed in a Soxhlet extractor and extracted continuously with chloroform. Initially, the extraction process was monitored by withdrawing drops of solvent from the flask at 5 min intervals and examining them on thin-layer chromatograms in the manner indicated above. Thereby, it was noted that nearly all the caffeine was removed in about 30 min and all the theobromine in 8–10 h. The extract after removal of the chloroform by distillation is dissolved in butanol–acetic acid and diluted to suitable proportions in order to be within the limits for the assay. A single standard solution of the bases (all three or any two) is made and diluted to give three suitable standards for the standard curve. The standards are chromatographed on a single plate together with the unknown solution which is spotted in two dilutions. The respective spot areas are then measured as above and the weights calculated from a standard curve.

## RESULTS AND DISCUSSION

The methods available[11,15–17] for the quantitative determination of substances by thin layer chromatography fall into four categories:

(i) Gravimetric analysis.
(ii) Spectroscopic analysis.
(iii) Methods based on evaluation of spot size.
(iv) Optical densitometric methods.

The gravimetric method has several serious defects, the main one being that the quantities involved are generally too small for accurate weighing. Both this type of method and the methods based on spectroscopic analysis require the substances to be removed from the matrix by elution prior to estimation. The error involved in the recovery process can at times be as much as 50 %[18]. The other two techniques do not suffer from these drawbacks and they have about the same degree of accuracy of 3–5 %[11,17]. The method based on the determination of spot size has the added advantage of not requiring any expensive apparatus, in lieu of which, however, it does require a high degree of manipulative skill on the part of the experimenter. Competently handled, the method is reportedly[11,15,17] as good as the best spectroscopic methods.

In the present method the bases could be assayed even when the three of them occur together. On the thin-layer chromatograms using the chosen solvent system

TABLE II

COMPARISON OF THE MEAN SPOT AREAS (AT DIFFERENT CONCENTRATIONS) WHEN THE COMPOUNDS ARE DEVELOPED SINGLY AND AS A MIXTURE

| *Purine* | *Concentration in μg** | *4* | *8* | *12* | *16* | *20* | *24* |
|---|---|---|---|---|---|---|---|
| *Caffeine* | Mean spot area $mm^2$ | 27.8 | 48.7 | 68.3 | 83.6 | 97.3 | 103.0 |
| | Standard deviation | ± 1.47 | ± 0.57 | ± 1.00 | ± 1.14 | ± 0.70 | ± 5.84 |
| In tert. mixture | Mean spot area $mm^2$ | 26.7 | 47.7 | 67.2 | 82.2 | 98.0 | 105.5 |
| | Standard deviation | ± 0.43 | ± 0.43 | ± 0.43 | ± 0.43 | ± 0 | ± 0.50 |
| | *Concentration in μg** | *2* | *4* | *6* | *8* | *10* | *11* |
| *Theophylline* | Mean spot area $mm^2$ | 22.0 | 39.6 | 55.8 | 68.6 | 81.8 | 90.4 |
| | Standard deviation | ± 0.4 | ± 0.49 | ± 0.40 | ± 0.49 | ± 0.4 | ± 0.49 |
| In tert. mixture | Mean spot area $mm^2$ | 21.6 | 40.0 | 54.0 | 69.0 | 81.6 | |
| | Standard deviation | ± 0.48 | ± 0.8 | 0 | 0 | ± 0.47 | |
| *Theobromine* | Mean spot area $mm^2$ | 23.0 | 35.6 | 47.3 | 62.2 | 79.4 | 88.3 |
| | Standard deviation | ± 1.07 | ± 1.26 | ± 1.60 | ± 1.08 | ± 2.06 | ± 2.44 |
| In tert. mixture | Mean spot area $mm^2$ | 23.4 | 37.2 | 47.8 | 61.4 | 76.8 | 82.4 |
| | Standard deviation | ± 0.49 | ± 0.4 | ± 0.4 | ± 0.49 | ± 0.4 | ± 0.8 |

* Intermediate values are not given here. They are shown in the graph.

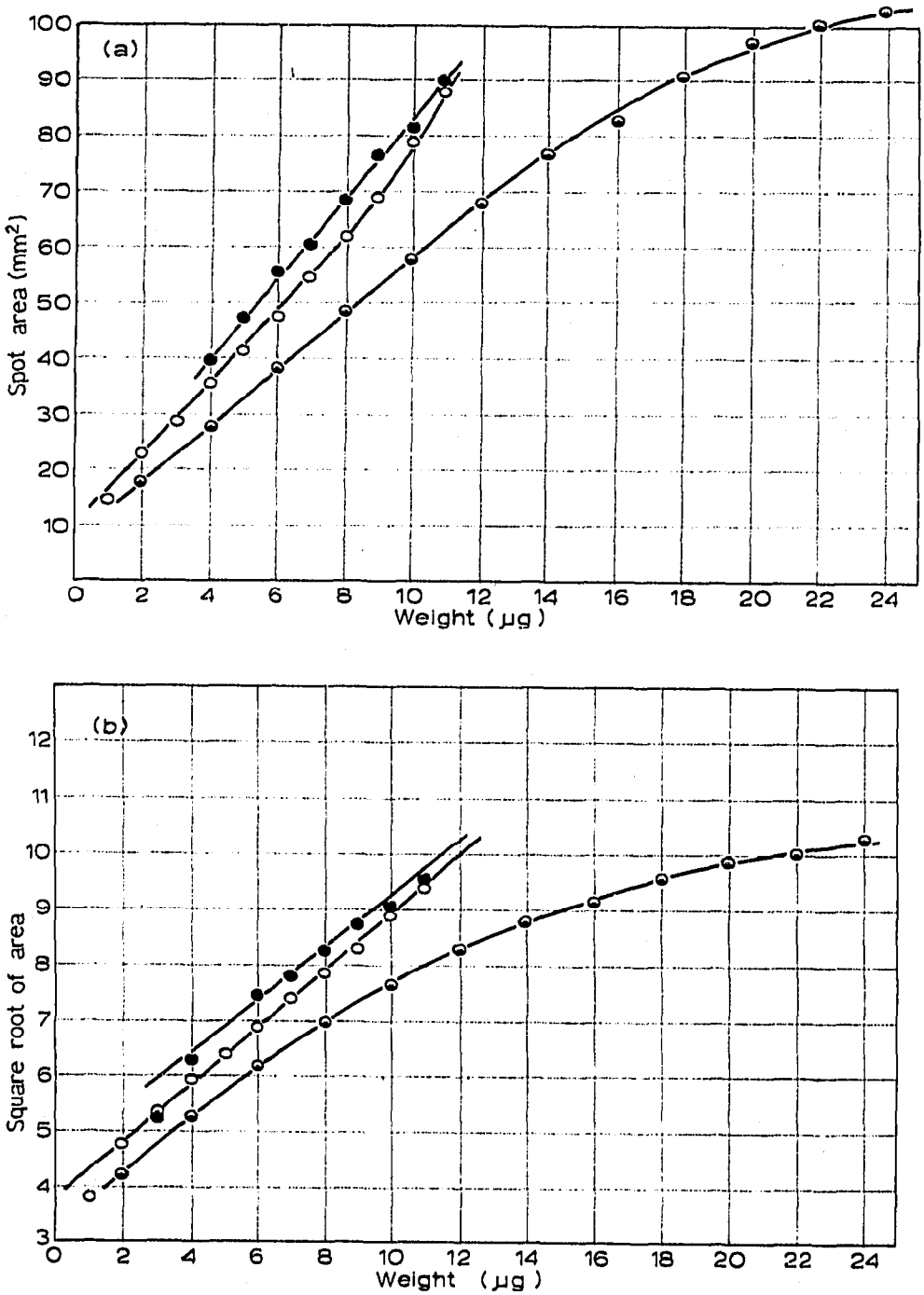
TABLE III

COMPARISON OF RESULTS ON TEST MIXTURES

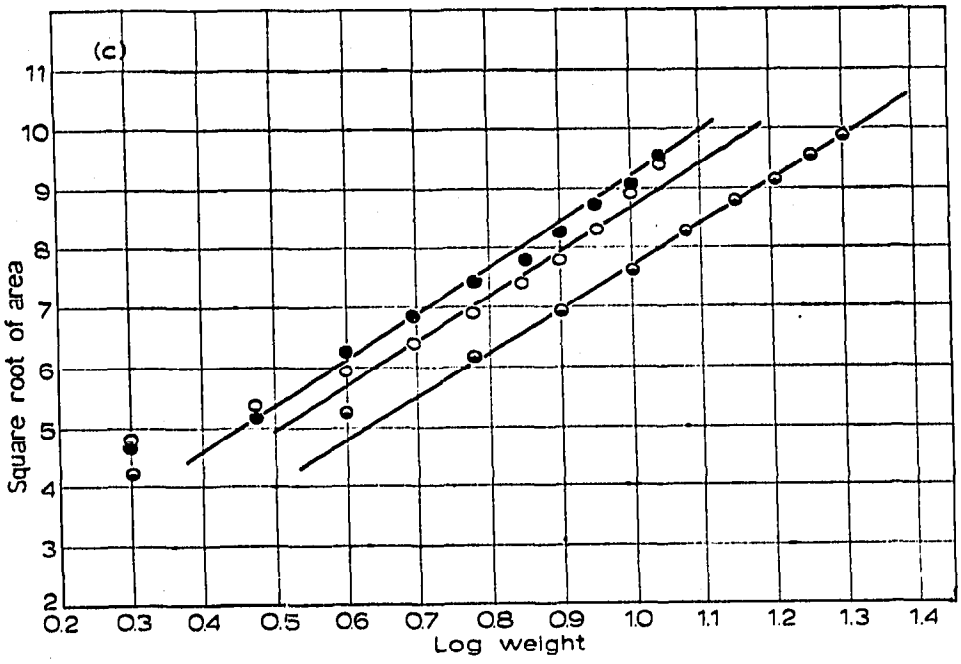
Amounts are given in mg/10 ml solution

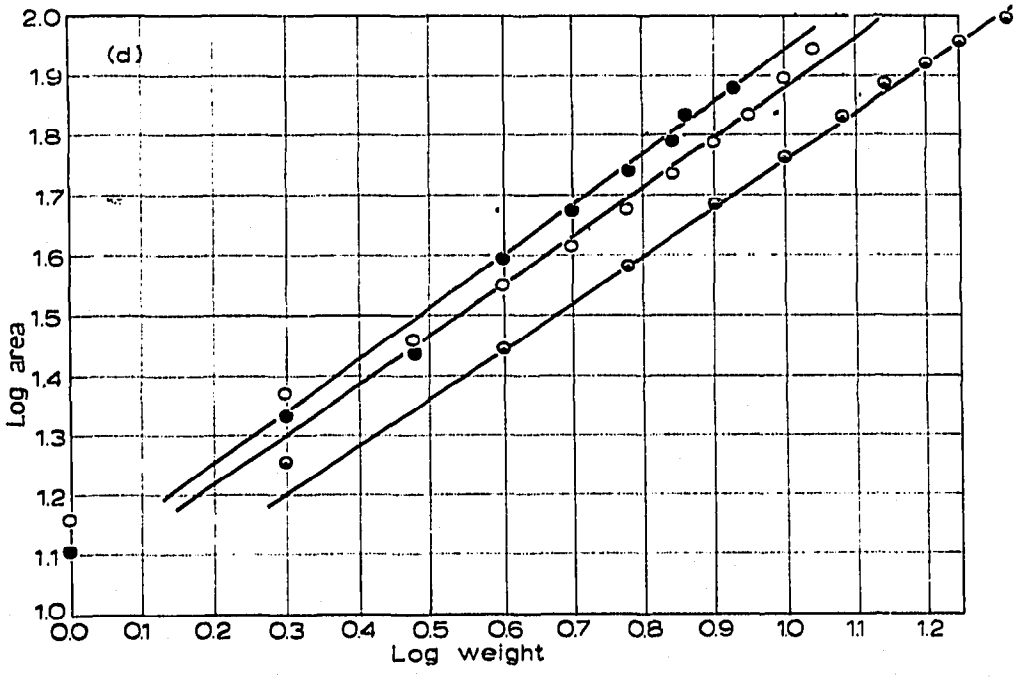
| | *Weight of caffeine* | *Weight of theobromine* | *Weight of caffeine by TLC method* | *Weight of caffeine by iodimetric titration* | *Weight of theobromine by TLC method* | *Total by TLC method* | *Total gravimetric method* | *Percentage difference from gravimetric method* |
|---|---|---|---|---|---|---|---|---|
| Test mixture I | 15 | 20 | 14.0 | 14.8 | 19.7 | 33.7 | 34.2 | −1.46 |
| Test mixture II | 25 | 20 | 24.2 | 26.0 | 19.0 | 43.2 | 44.6 | −3.14 |
| Test mixture III | 35 | 20 | 35.0 | 37.1 | 19.5 | 54.5 | 53.5 | +1.87 |
| Cocoa powder extract (fat free) | Unknown | Unknown | 21.0 | 22.2 | 32.0 | 52.0 | 51.5 | +0.97 |

caffeine, theophylline and theobromine arrange themselves in that order with the latter nearest the origin. (Fig. 1; Table I). An aliquot of a solution or extract containing all three bases could be conveniently applied on to a single chromatogram and the amount of each base assayed simultaneously (Table II). Table III gives the results of comparative assays on "test mixtures", and a cocoa powder extract, using titrimetric[3]




Figs. 2a and b. Graphs showing the relationships between weight and spot area. (a) Plot of mean values for spot area against weight. (b) Plot of root square of mean spot area against weight. (●) Theophylline; (○) theobromine; (◒) caffeine.




Figs. 2c and d. Graphs showing the relationships between weight and spot area. (c) Plot of square root of mean spot area against log weight (PURDY–TRUTER relationship). (d) Plot of log mean spot area against log weight. (●) Theophylline; (○) theobromine; (◒) caffeine.

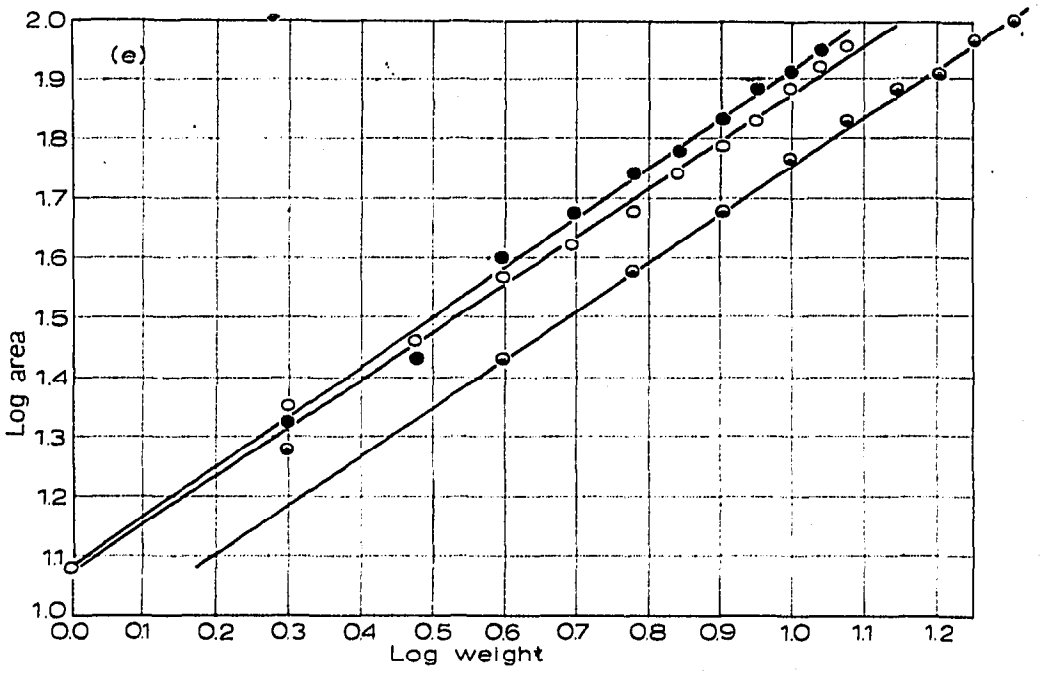



Fig. 2e. Graph showing the relationships between weight and spot area. Plot of log area against log weight in a typical single experiment. (●) Theophylline; (○) theobromine; (◒) caffeine.

and gravimetric[5,6] methods along with the TLC method. The results are comparable although quantities to suit the gravimetric and titrimetric methods have been employed with the test mixtures being diluted for assay with the TLC method.

PURDY AND TRUTER[19,20] have shown that in thin layer chromatography the square root of the area of a spot is a linear function of the logarithm of the weight of the material present. Quantitative methods of assay based on this relationship have given accuracies to within ± 2 % under controlled conditions, and the relationship has been proposed as a basis for a general method of analysis of compounds by TLC[15]. PURDY AND TRUTER have verified the validity of the relationship with respect to several types of compounds. We have examined this empirical relationship in the case of the three purine bases together with a few of the more obvious ones such as:

(i) Direct plot of area *vs.* weight.
(ii) Log area *vs.* log weight.
(iii) $\sqrt{}$Area *vs.* weight.

which have also been examined for selected compounds by PURDY AND TRUTER. Each empirical relationship seems to be satisfactory within limits (Fig. 2) and could be used to give a standard calibration line within the appropriate range (Table IV).

The range of the method could be extended either way by varying the layer thickness. Experiments showed that when the layer thickness was decreased to 200 $\mu$ the spots tended to be diffused and correspondingly large. If the layer thickness was increased to 300 $\mu$ the spots were correspondingly smaller and more clearly defined. Therefore, solutions more concentrated than those used in the present study could be effectively chromatographed on a matrix of thickness around 300–500 $\mu$. Thus the technique could be suitably adapted for use in a wider range of concentration. We have found layers of 250 $\mu$ adequate for purposes of determining the quantity of

TABLE IV

RANGE OF APPLICABILITY OF THE DIFFERENT STANDARD CURVES

| *Relationship between weight and area* | *Range of applicability (μg)* | | |
|---|---|---|---|
| | *Caffeine* | *Theobromine* | *Theophylline* |
| Weight *vs.* area | 2–16 | 2– 9 | 4–11 |
| Log weight *vs.* log area | 4–20 | 3–10 | 2–11 |
| Weight *vs.* √area | 2–10 | 2–11 | 4–10 |
| Log weight *vs.* √area | 6–24 | 4–10 | 3–11 |

caffeine and theobromine in various cocoa products. The method has proved to be simple, accurate, and convenient for repeated application. Furthermore, only small quantities of material are required for each determination.

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SUMMARY

A sensitive method for the quantitative detection of the purine bases caffeine, theobromine and theophylline is described. The method is based on thin layer chromatography and has the added advantage that it does not require any expensive apparatus.

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