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Note

High-performance liquid chromatographic determination of theobromine, theophylline and caffeine in food products

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Foodstuffs contain many physiologically active compounds. Methylxanthines such as theobromine (TB), theophylline (TP) and caffeine (CA) are typical compounds present in coffee, tea, chocolate and products made from them. There is increasing interest among consumers in the methylxantine content of food products.

A great variety of techniques for the analysis of methylxanthines in food products has been reported, ranging from the Kjeldal method[1], UV spectrophotometry[2], thin-layer chromatography[3,4], gas chromatography[5] and potentiometric titrations[6] to high-performance liquid chromatography (HPLC)[7–10]. However, these methods require tedious pretreatments or do not allow the separation and quantitation of a number of methylxanthines in the same sample.

The purpose of the present study was to establish a rapid and reproducible method for the determination of TB, TP and CA in food products by combining a simple pretreatment with the use of a Sep-Pak C_{18} cartridge.

EXPERIMENTAL

Apparatus and reagents

The HPLC equipment consisted of a Jasco (Tokyo, Japan) Uniflow 211 pump, a VL611 variable-loop injector with a 100-μl sample loop, a Uvidec 100II UV detector operating at 275 nm and a Nippon Denshi Kagaku (Kyoto, Japan) U-125M recorder.

Separations were carried out by using a stainless-steel column, 15 cm × 4.0 mm I.D. (Umetani, Osaka, Japan), packed by the balanced slurry technique with LiChrosorb RP-8 (5 μm) (E. Merck, Darmstadt, F.R.G.) and a mixture of methanol–water–0.2 *M* phosphate buffer pH 5.0 (9:36:5) as the mobile phase. The column was water-jacketed for temperature control (45°C).

A Sep-Pak C_{18} cartridge was obtained from Waters Assoc. (Milford, MA, U.S.A.) and a GF/B glass filter from Whatman (Maidstone, U.K.). TB, TP and CA were purchased from Tokyo Organic Chemicals (Tokyo, Japan) and methanol (HPLC grade) was obtained from Wako (Osaka, Japan). The phosphate buffer was prepared from 0.2 *M* potassium dihydrogen phosphate by titration to the required pH with 0.2 *M* phosphoric acid or 0.2 *M* sodium monohydrogen phosphate.

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PROCEDURE

Liquid sample

To an accurately weighed *ca.* 2-g quantity of sample in a 100-ml volumetric flask, 10 ml of 0.2 *M* phosphate buffer pH 4.0 were added and the volume was adjusted with water. After vigorous mixing, the mixture was filtered through a glass filter. The filtrate (10 ml) was poured into a Sep-Pak C_{18} cartridge at a rate of 2 ml/min. The resin in the cartridge was washed with 20 ml of methanol and 20 ml of water before use; subsequently 10 ml of water were passed through the cartridge to eliminate sugars and strongly polar compounds. The methylxanthines were eluted with 10 ml of a mixture of methanol–water–0.2 *M* phosphate buffer, pH 5.0 (5:13:2). The effluent was diluted with water to 20 ml. Aliquots (100 μl) of the solution were subjected to HPLC.

Quantitation was carried out using calibration graphs obtained from the standard solution which were treated with a Sep-Pak C_{18} cartridge according to the above analytical procedure.

Solid sample

To an accurately weighed, *ca.* 1-g quantity of ground sample in a 100-ml beaker, 80 ml of hot water (80°C) were added and the solution heated on a steam-bath for 60 min. The sample solution was filtered through a glass filter. The beaker was washed with hot water (80°C) and the resulting solution filtered through the same filter. The filtrates were combined, cooled to room temperature and transferred to a 200-ml of volumetric flask. Phosphate buffer (0.2 *M*, pH 4.0, 20 ml) was added to

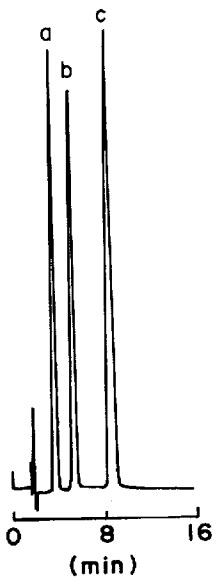

Fig. 1. Typical chromatogram of TB, TP and CA; peaks: a = TB, b = TP, c = CA. Operating conditions: column: LiChrosorb RP-8, 5 μm (15 cm × 4.0 mm I.D.); mobile phase: methanol–water–0.2 *M* phosphate buffer pH 5.0 (9:36:5); column temperature: 45°C; flow-rate: 1.0 ml/min; detector: UV photometer (275 nm, 0.16 a.u.f.s.).

TABLE I

EFFECT OF THE DEFATTING TREATMENT ON THE RECOVERIES OF TB, TP AND CA

Sample: 1 g of chocolate spiked with 0.2 mg of TB, 0.2 mg of TP and 3.0 mg of CA. Procedure: the sample was shaken with 30 ml of hexane or petroleum ether, centrifuged at 560 *g* for 10 min and the solvent decanted. The residue was treated according to the procedure described.

	Recovery (%)		
	TB	*TP*	*CA*
Hexane	99.0	93.5	59.2
Light petroleum	97.2	94.0	60.8
Treatment omitted	99.3	94.7	98.3

the flask and the volume was adjusted with water. After vigorous mixing, 10-ml aliquots of the solution were poured into the washed Sep-Pak C_{18} cartridge at a rate of 2 ml/min. In cases where the methylxanthine content was too high to calibrate, the solution above was appropriately diluted with 0.02 *M* phosphate buffer, pH 4.0, before being passed through the Sep-Pak C_{18} cartridge.

The subsequent procedure was the same as with the liquid sample.

RESULTS AND DISCUSSION

Fig. 1 shows a typical chromatogram obtained from a standard mixture of TB, TP and CA. A baseline separation is attained in only 10 min by isocratic resolution.

The detector wavelength of 275 nm used corresponds to maximum absorption of the methylxanthines in the mobile phase.

Using hot water (80°C) as the extraction solvent, good extraction efficiency could be achieved. Table I shows the effect of the defatting treatment for fatty foods such as chocolate on the recoveries of the methylxanthines. The good TP and TB

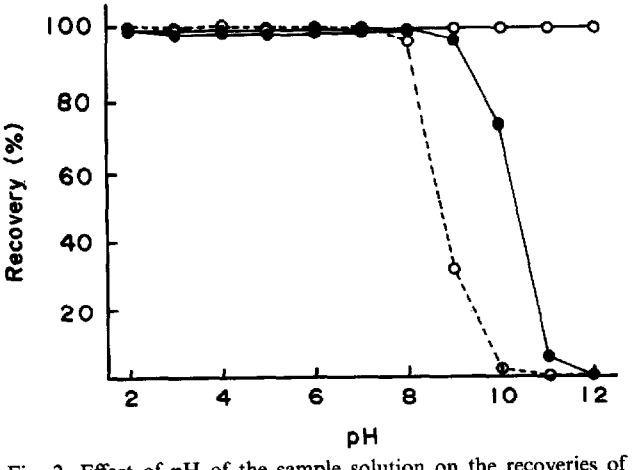

Fig. 2. Effect of pH of the sample solution on the recoveries of TB, TP and CA from Sep-Pak C_{18} cartridge, (●), TB; (O---O), TP; (O—O), CA.

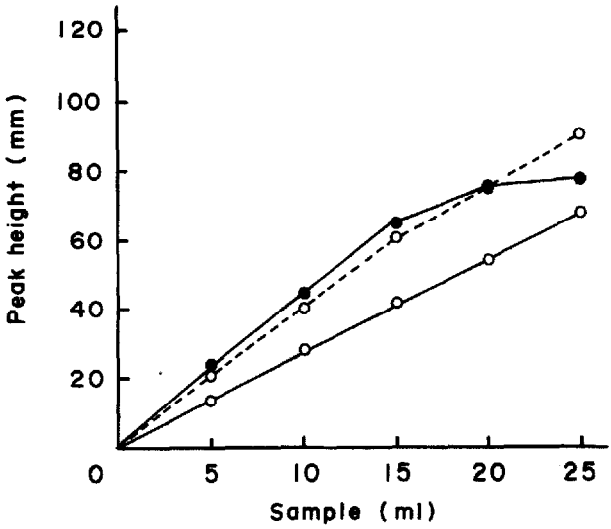

Fig. 3. Relationship between the sample solution volume and the peak height on the chromatogram. (●), TB; (O---O), TP; (O—O), CA.

recovery figures were unaffected by the treatment but the CA figure was reduced 40% by the defatting treatment. The present method, therefore, omits the defatting step.

The use of a Sep-Pak C_{18} cartridge for the pretreatment had several advantages over the direct injection method[8,10] and the column chromatographic clean-up[9]. It provided chromatographically cleaner extracts because of the partial separation, it removed any materials which otherwise might have adsorbed irreversibly to the chromatographic column, and it effected a significant time saving per pretreatment compared with the column chromatographic clean-up because tedious preparations were unnecessary and a single operation sufficed.

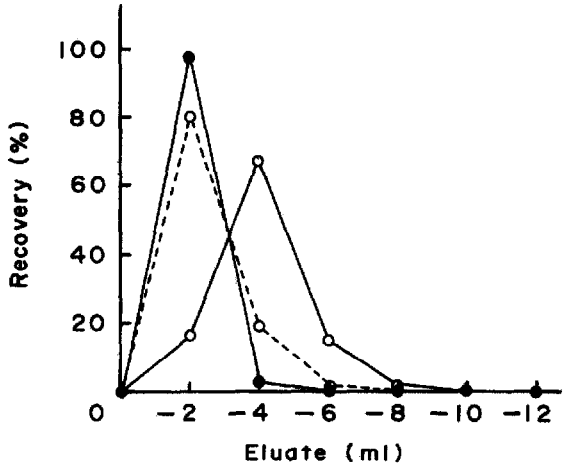

Fig. 4. Elution pattern of TB, TP and CA from Sep-Pak C_{18} cartridge. (●), TB; (O---O), TP; (O—O), CA.

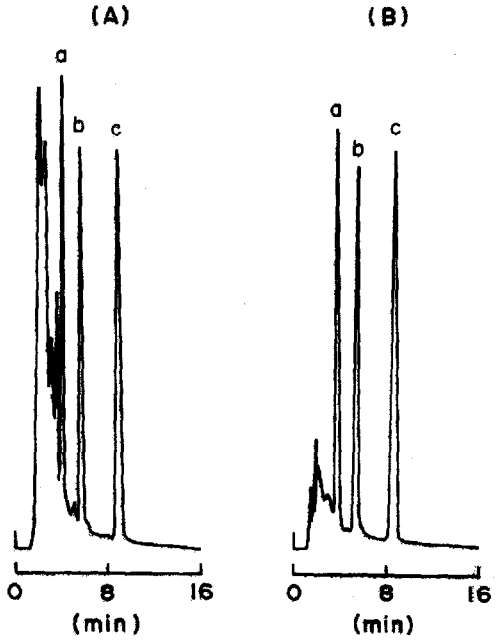

Fig. 5. Chromatograms obtained from coffee caramel. (A) before treatment with Sep-Pak C_{18} cartridge, (B) after treatment with Sep-Pak C_{18} cartridge. Sample was spiked with 0.5 mg/g of TB and 0.5 mg/g of TP. Operating conditions: see Fig. 1.

Fig. 2 shows the effect of the pH of the sample solution on the recoveries of the methylxanthines from the Sep-Pak C_{18} cartridge. In the pH range 2–7, the recoveries were a constant 100%, but in the pH range above 8 for TP and 9 for TB, the recoveries dropped sharply. On the basis of these results, 0.2 *M* phosphate buffer, pH 4.0, was added to the sample solution in order to maintain in a pH below 7.

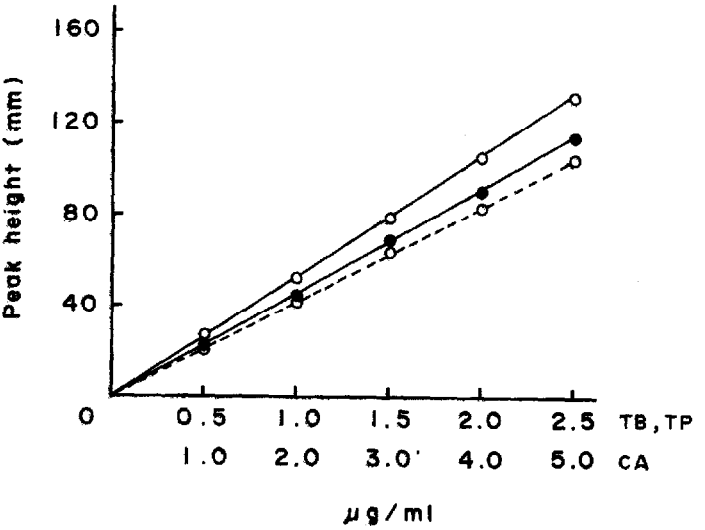

Fig. 6. Calibration curves of TB, TP and CA. (●), TB; (O---O), TP; (O—O), CA.

TABLE II

RECOVERIES OF TB, TP AND CA FROM FOOD PRODUCTS

Rv = recovery; C.V. = coefficient of variation.

Sample	*Theobromine*			*Theophylline*			*Caffeine*		
	Added (mg/g)	*Rv (%)*	*C.V. (%)*	*Added (mg/g)*	*Rv (%)*	*C.V. (%)*	*Added (mg/g)*	*Rv (%)*	*C.V. (%)*
Canned cocoa	0.20	102.9	4.46	0.20	99.2	1.93	0.20	103.3	2.93
Canned lemon tea	0.40	92.6	2.07	0.40	94.7	0.67	0.40	93.6	1.52
Cola	0.20	97.9	2.04	0.20	99.0	1.14	0.20	99.2	1.49
Coffee caramel	0.50	93.1	3.41	0.50	93.6	0.94	0.50	96.5	2.80
Chocolate	3.00	97.6	2.75	0.20	98.1	2.86	0.20	102.5	4.60
Chocolate cookie	1.00	94.0	2.21	1.00	101.4	1.38	1.00	102.6	1.67
Chocolate paste	0.60	97.8	2.13	0.60	98.5	2.08	0.60	97.5	2.99

Fig. 3 shows the relationship between the sample solution volume poured into the Sep-Pak C_{18} cartridge and the peak height on the chromatogram obtained by the overall procedure. It indicates that the sample should be less than 15 ml to ensure stable recoveries of TB and TP. Consequently, 10-ml volumes of sample solutions were used.

Fig. 4 shows the elution pattern of the methylxanthines from a Sep-Pak C_{18} cartridge. The methylxanthines were eluted completely with 10 ml of a mixture of methanol–water–0.2 *M* phosphate buffer, pH 5.0 (5:13:2).

Fig. 5 shows the chromatograms obtained from a coffee caramel spiked with TB and TP before the Sep-Pak C_{18} clean-up (A) and after the clean-up (B). The eluates from the Sep-Pak C_{18} cartridge were generally clean and free from interfering compounds such as salts, acids, sugars and pigments.

TABLE III

TB, TP AND CA CONTENTS OF COMMERCIAL FOOD PRODUCTS

Average of five determinations of the same sample ± standard deviation. – = not detected.

Sample	*Theobromine (mg/g)*	*Theophylline (mg/g)*	*Caffeine (mg/g)*
Ground coffee (Colombia)	–	–	12.28 ± 0.13
Instant coffee	–	–	26.38 ± 0.27
Instant coffee (decaffeinated)	–	–	0.776 ± 0.073
Green tea	–	–	22.38 ± 0.27
Black tea	1.07 ± 0.03	–	29.96 ± 0.11
Coffee chewing gum	–	–	0.609 ± 0.033
Chocolate	3.36 ± 0.04	–	0.316 ± 0.026
Chocolate cake	1.37 ± 0.02	–	0.158 ± 0.016
Chocolate paste	0.633 ± 0.021	–	0.029 ± 0.001
Cola	–	–	0.101 ± 0.015

A Sep-Pak C_{18} cartridge could be re-used at least eight times without any loss of performance if it was washed with 20 ml of methanol and 20 ml of water each time after use.

Sample solutions containing individual methylxanthines were measured according to the procedure described and linear relative response graphs passing through the origin were obtained with all of the methylxanthines studied for concentrations in the range 0.5–2.5 μg for TB and TP and 1.0–5.0 μg for CA in 1.0 ml of solution which was subjected to HPLC (Fig. 6).

In recovery tests, the proposed method was applied to samples of food products with spiked TB, TP and CA levels ranging from 0.2 mg/g to 3.0 mg/g each. The reproducibility was determined by carrying out five identical analyses, with the results which are summarized in Table II.

Table III shows the results of analysis for TB, TP and CA of typical food products containing methylxanthines; no interfering peaks were observed. The limits of detection were 5 μg/g for TB and TP and 10 μg/g for CA when 2 g of liquid sample were used and the detector sensitivity was 0.16 a.u.f.s.

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