Journal of Chromatography, 133 (1977) 303–310

CHROM. 9725

MICRO-DETERMINATION OF TOTAL PHTHALATE ESTERS IN BIOLOGICAL SAMPLES BY GAS–LIQUID CHROMATOGRAPHY

RYUZO TAKESHITA and EIGO TAKABATAKE

Department of Public Health Pharmaceutics, Institute of Public Health, 4-6-11, Shirokanedai, Minato-Ku, Tokyo (Japan)

and

KOEI MINAGAWA and YUKIO TAKIZAWA

School of Medicine, University of Akita, Hondo, Akita (Japan)

(Received September 10th, 1976)

SUMMARY

A method was investigated in which all of the phthalate esters in biological samples were determined as phthalic acid by gas–liquid chromatography. The method is based on the separation of phthalate esters from the sample with *n*-hexane, saponification of the esters with an alkaline ethanolic solution to give phthalic acid, purification of the acid by extraction with diethyl ether and column chromatography using silica gel, and conversion of the acid into bis(2,2,2-trifluoroethyl) phthalate with a 2,2,2-trifluoroethanol solution containing boron trifluoride.

The derivative obtained is highly sensitive to an electron-capture detector, giving a sensitivity of 0.1 pg. Biological samples fortified with di(2-ethylhexyl) phthalate at levels of 5–100 ppb were analyzed, with recoveries of 70–110%.

INTRODUCTION

In Japan, phthalate esters are produced industrially at a level of more than 3×10^5 tons per year, mostly for use as plasticizers. The increase in the utilization of these esters has resulted in widespread contamination of the natural and human environment and therefore the concern of investigators has been focused on residues of the esters in water[1], foodstuffs[2,3] and biological samples[4,5].

Several attempts have been made to determine the esters by gas–liquid chromatography (GLC) with flame-ionization detection (FID)[3,6] or electron-capture detection (ECD)[7–9] and combined with mass spectrometry (GC–MS)[10]. Confirmation of the identification of these esters based only on the retention times of the peaks obtained often leads to errors due to the occurrence of peaks of interfering materials. However, it is difficult to find a procedure for eliminating impurities that interfere in the determination. In addition, the levels of residual esters in many samples are often too low for the GC–MS technique to be applied.

Although there have been many reports concerning residues of di(2-ethylhexyl) phthalate and its effect on organisms[11–16], when monitoring the environment the analyses should not be limited to this ester alone, as the toxicities of other phthalate esters

are not been sufficiently well known. An approach to the monitoring of the total residues of phthalate esters is to measure all of the esters in the samples as phthalic acid.

This paper describes a method for the identification and determination of trace amounts of phthalate esters present in biological samples based on alkaline hydrolysis of the separated esters, extraction of the phthalic acid formed, clean-up of the acid by column chromatography, conversion of the acid into a derivative sensitive to an ECD and GLC of this derivative.

EXPERIMENTAL

Reagents

Bis(2,2,2-trifluoroethyl) phthalate (DTFEP) was synthesized as follows, using reagents obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan). A solution of 2,2,2-trifluoroethanol (99%) and pyridine [20 g (0.24 mole) and 15 g (0.2 mole), respectively] in 50 ml of benzene was added dropwise to phthaloyl chloride [20 g (0.1 mole)] in 150 ml of benzene with vigorous stirring during 30 min while maintaining the temperature at 5–10°. Subsequently the mixture was refluxed on a boiling water-bath for 1 h. After cooling to room temperature, the mixture was filtered to remove the pyridine hydrochloride formed. The solution was washed twice with 50-ml volumes of water, dried over anhydrous sodium sulphate and then concentrated at 40° under reduced pressure. The residue obtained was dissolved in 100 ml of *n*-hexane. The solution was washed with three 20-ml volumes of a 1% sodium hydrogen carbonate solution and then with two 50-ml volumes of water. The solution was dried over anhydrous sodium sulphate and then concentrated at 40° under reduced pressure. The residue was purified by distillation under reduced pressure (b.p. 134–134.5°, 13 mm Hg) and, on crystallization from *n*-hexane, pure DTFEP was obtained as fine, colourless needles. The structure was confirmed by infrared spectroscopy, nuclear magnetic resonance spectroscopy and mass spectrometry.

Standard solutions for GLC were prepared by dissolving DTFEP in *n*-hexane to give concentrations in the range 0.01–0.10 $\mu g \cdot ml^{-1}$.

n-Hexane was of the highest purity grade so that the 300-fold concentrated solution was guaranteed for pesticide analysis. Benzene, ethyl acetate, ethanol and anhydrous sodium sulphate were of pesticide grade. Acetic and formic acid were of reagent grade (99.0 and 90.0%, respectively). The formic acid being used as a constituent of a solvent system for column chromatography, was washed with *n*-hexane. All other solvents were of reagent grade. The solvents used were obtained from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). The water used was tap water washed with *n*-hexane. Sodium hydrogen carbonate solution (1%) (freshly prepared) and 10 *N* potassium hydroxide solution were washed prior to use.

Di(2-ethylhexyl) phthalate was of standard grade (purity > 99.5%) for analyzing phthalate esters from Wako Pure Chemical Ind. Phthalic acid (obtained from Tokyo Kasei Kogyo Co.) was recrystallized from ethanol and then water and the crystals were dried for 2 h at 110°. The solutions of phthalic acid for testing its reactivity with 2,2,2-trifluoroethanol, its extraction with some solvents and its behaviour on silica gel were prepared by dissolving the acid in acetone, ethanol and ethyl acetate to give concentrations of 1.0 $\mu g \cdot ml^{-1}$, 1.0 $mg \cdot ml^{-1}$ and 1.0 $mg \cdot ml^{-1}$, respectively. Kieselgel 60 (0.063–0.200 mm) for column chromatography (Merck, Darmstadt,

G.F.R.) was packed into a column washed with ethanol and then dried for 3 h at 130° before use.

Apparatus

A 20 × 1 cm I.D. glass column equipped with a stopcock and a cotton plug was used for column chromatography. A 2.0 m × 3 mm I.D. glass tube was packed with 2% (w/w) of silicone OV-225 on Gas-Chrom Q (80–100 mesh), being pre-conditioned with nitrogen for 24 h at 240° before use. The column was installed on a Shimadzu EF gas chromatograph equipped with a ^{63}Ni ECD.

Gas–liquid chromatography of DTFEP

The column, inlet and detector temperatures were maintained at 180°, 230° and 250°, respectively. Nitrogen was used as the carrier gas at a flow-rate of 60 $cm^3 \cdot min^{-1}$.

A series of 2–5-μl volumes of the standard solutions of DTFEP were introduced into the gas chromatograph with a microsyringe. The peak heights obtained were measured and a calibration graph was constructed.

Reaction of phthalic acid with 2,2,2-trifluoroethanol

Each 2-ml volume of phthalic acid solution (1.0 $\mu g \cdot ml^{-1}$) was transferred into a 20-ml test-tube, the solvent being removed from the tubes at room temperature using a rotary evaporator. To each test-tube was added 1 ml of a 15% solution of boron trifluoride in 2,2,2-trifluoroethanol (BF_3–TFE) (obtained from Tokyo Kasei Kogyo Co.), the mixtures being heated for 1 h at several temperatures using Alline condensors equipped with potassium hydroxide tubes. To each reaction mixture were added 5 ml of water and 10 ml of *n*-hexane, the mixture was shaken in the capped tube for 2 min and the *n*-hexane layer obtained was transferred into a calibrated flask with a pipette. The aqueous solution was extracted repeatedly with *n*-hexane and the extracts were combined in the flask and made up to volume with *n*-hexane. The solution was then subjected to GLC.

Extraction of phthalic acid

Volumes of 1 ml of phthalic acid solution (1.0 $mg \cdot ml^{-1}$) were added to 100-ml separating funnels containing 50-ml volumes of 1 *N* sodium hydroxide solution. After adjustment of the pH to 2.0 with 6 *N* hydrochloric acid, the solutions were saturated with sodium chloride and extracted successively with 50 ml each of *n*-hexane, diethyl ether and ethyl acetate. Each extract was washed with three 5-ml volumes of the saturated sodium chloride solution, dried over anhydrous sodium sulphate for 15 min and evaporated to dryness at 40° under reduced pressure. The residue obtained was dissolved in ethanol to give a volume of solution of 10 ml, the phthalic acid in the solution being determined by measuring the absorbance at $\lambda_{max.}$ = 275 nm using ethanol as a blank, relative to the phthalic acid solution diluted 10-fold with ethanol. Two extractions using each solvent were also carried out.

Column chromatography of phthalic acid

Volumes of 1 ml of phthalic acid solution (1.0 $mg \cdot ml^{-1}$) for column chromatography were loaded on to a column that had been prepared with a 10-cm bed of a slurry of activated Kieselgel 60 in ethyl acetate. The elution was carried out with ethyl

acetate and ethyl acetate–acetic acid (150:1) at a flow-rate of *ca.* 1 ml·min^{-1}. The behaviour of the acid was also tested by stepwise elution using 50 ml of ethyl acetate, 40 ml of ethyl acetate–acetic acid (150:1) and then 30 ml of ethyl acetate–formic acid (150:1) at the same flow-rate. Volumes of 2 ml of the effluent were collected in small test-tubes. The concentration of phthalic acid in each fraction was determined by measuring the absorbance at $\lambda_{max.} = 275$ nm using the eluting solvent as a blank.

Determination of phthalate esters in biological samples

Extraction of phthalate esters. In a Wareing blender, a 10–20-g weighed sample was blended with 100 g of anhydrous sodium sulphate and then extracted with two 200-ml volumes of *n*-hexane. The combined extracts were washed with 50 ml of 1% sodium hydrogen carbonate solution, followed by two 50-ml volumes of water, and dried over anhydrous sodium sulphate. The extract was concentrated to about 5 ml using a Kuderna-Danish condenser.

Hydrolysis of phthalate esters and extraction of phthalic acid formed. The concentrated solution was transferred into a 100-ml round-bottomed flask using 30 ml of ethanol. After the addition of 2 ml of 10 *N* potassium hydroxide solution, the mixture was refluxed for 30 min. After cooling, the solution was concentrated to remove ethanol and the residue obtained was dissolved in 50 ml of water, the solution being neutralized with 10% hydrochloric acid using cresol red reagent as the indicator. The solution was transferred into a separating funnel, the flask being rinsed with a small volume of water. The combined solution was extracted with 50 ml of *n*-hexane and the organic layer was transferred into another separating funnel and washed with 10 ml of water. All of the aqueous layers obtained were combined in the funnel. After adjustment of the pH to 2.0 with 10% hydrochloric acid the solution was saturated with sodium chloride and then extracted with two 50-ml volumes of diethyl ether. The extract was washed with four 5-ml volumes of saturated sodium chloride solution in order to remove excess of hydrochloric acid. After drying over anhydrous sodium sulphate, the solution was concentrated under reduced pressure at 40°.

Clean-up of phthalic acid by column chromatography. The inside of the flask was washed with 30 ml each of *n*-hexane, benzene and ethyl acetate, the solution obtained being loaded stepwise on to the column. After washing the column with a further 50 ml of ethyl acetate and then with 30 ml of ethyl acetate–acetic acid (150:1), phthalic acid was eluted with 25 ml of ethyl acetate–formic acid (150:1). The effluent was evaporated to dryness at 40° using a rotary evaporator. After the addition of a few millilitres of benzene to the flask, the solvent was re-evaporated in order to remove residual formic acid.

Esterification of phthalic acid. A 1-ml volume of BF_3–TFE was added to the flask, the contents then being esterified at 80° according to the procedure described above.

Determination of DTFEP by gas–liquid chromatography. The *n*-hexane solution obtained by extraction after the esterification was subjected to GLC as described under Experimental.

The phthalate esters in the sample were calibrated as phthalic acid from the expression

$$C = \frac{166}{330} \cdot \frac{V_1}{V_2} \cdot \frac{1}{W} \cdot [\text{DTFEP}]$$

where C (ppm) = concentration of phthalic acid, V_1 (ml) = total volume of the test solution for gas chromatography, V_2 (μl) = volume of the test solution injected, W (g) = sample weight and [DTFEP] (ng) = content of DTFEP in V_2.

RESULTS AND DISCUSSION

When phthalate esters are determined by GLC using either FID or ECD, the detection sensitivities are generally low (of the order of nanograms). A method for determining trace amounts of the esters with high sensitivities is to induce them to form a compound that is more sensitive to an ECD. Although an attempt had made to esterify them with 2-chloroethanol, it was not suitable for the determination because the yields were too low[17].

In this study, the esterification of phthalic acid with 2,2,2-trifluoroethanol was investigated. As shown in Fig. 1, the compound obtained gave a sharp and symmetrical peak, with the same retention time as that of the derivative synthesized from phthaloyl chloride and 2,2,2-trifluoroethanol, and the structure was confirmed to be DTFEP by GC–MS. The spectrum obtained is illustrated in Fig. 2.

As shown in Table I, the reaction of phthalic acid with BF_3–TFE for 1 h at 80° gave quantitative results; heating the reaction mixture at temperatures higher and lower than 80° produced significantly lower yields. In particular, the compound could be detected at a level of about 0.1 pg, representing 100 times the sensitivities generally obtained when phthalate esters are determined using an ECD.

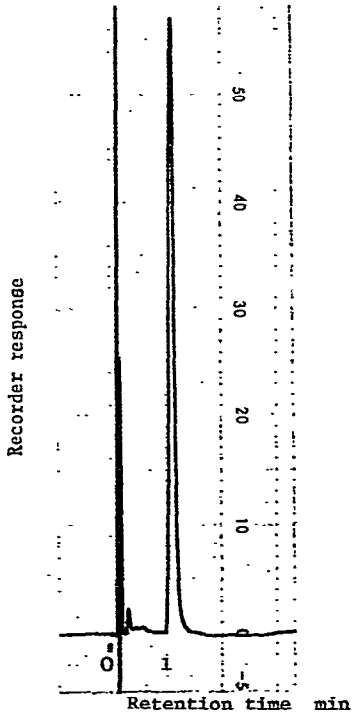

Fig. 1. Gas chromatogram of bis(2,2,2-trifluoroethyl) phthalate.

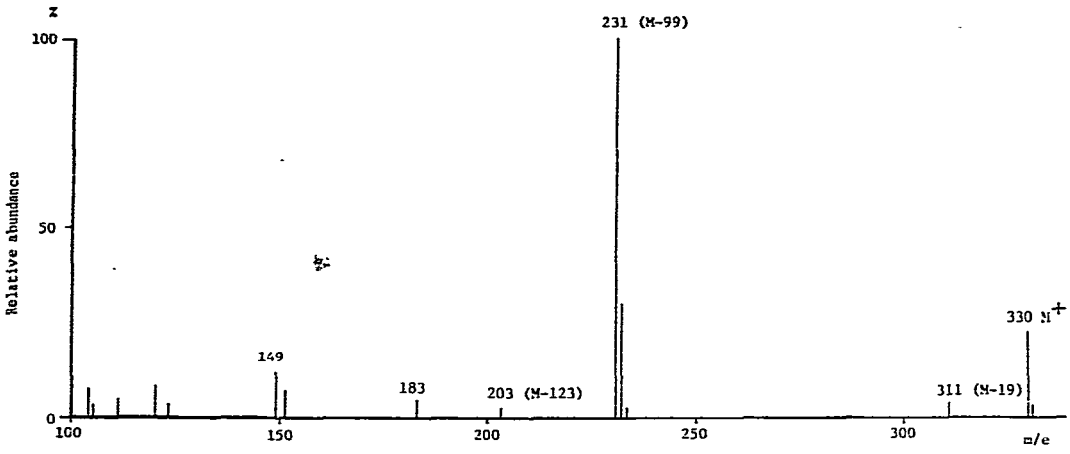

Fig. 2. Mass spectrum of bis(2,2,2-trifluoroethyl) phthalate.

In order to establish an effective separation of phthalic acid produced from phthalate esters by alkaline hydrolysis, the most suitable solvent for the extraction was sought. The extraction of phthalic acid using either ethyl acetate or diethyl ether gave good results recoveries, as shown in Table II. Although it was recovered quantitatively from the acidic solution saturated with sodium chloride by one extraction with 50 ml of ethyl acetate, the use of diethyl ether was superior to ethyl acetate because the latter led to the distribution of more polar impurities.

As phthalic acid was hardly extracted with *n*-hexane, extraction with *n*-hexane under both alkaline and acidic conditions prior to the extraction with diethyl ether was used in order to eliminate non-polar substances.

In the determination of phthalate esters involving the conversion of phthalic acid into DTFEP, it was necessary to eliminate impurities that would exhibit an electron-capture response themselves or that would be esterified by the reagent to produce compounds with such properties. In order to find a procedure for the purification of phthalic acid by column chromatography, the behaviour on a silica gel column was investigated by stepwise elution with ethyl acetate and polar solvent systems containing a small amount of acetic or formic acid.

The phthalic acid loaded was not eluted entirely from the column with 100 ml of ethyl acetate. When eluted with the more polar solvent system ethyl acetate–acetic

TABLE I

EFFECT OF REACTION TEMPERATURE ON FORMATION OF BIS(2,2,2-TRIFLUOROETHYL) PHTHALATE

Amount of phthalic acid reacted = 2.0 μg. Reaction time = 60 min.

Temperature (°C)	*Amount formed (%)*			
	Expt. No. 1	*Expt. No. 2*	*Expt. No. 3*	*Average*
70	65.1	65.1	67.5	65.9
80	99.8	90.0	101.3	97.0
90	80.3	99.8	96.8	92.3
100	55.4	53.8	31.0	46.7

TABLE II

RECOVERY OF PHTHALIC ACID BY EXTRACTION WITH DIFFERENT SOLVENTS
Amount of phthalic acid added = 1.0 mg in each instance.

Solvent	*Extraction*	*Recovery of phthalic acid (%)*
Ethyl acetate	1×	98 ± 3
	2×	102 ± 4
Diethyl ether	1×	96 ± 3
	2×	103 ± 5
n-Hexane	1×	1
	2×	2

TABLE III

RECOVERY OF DI(2-ETHYLHEXYL) PHTHALATE FORTIFIED TO BREAST MUSCLE OF A BIRD

Expt. No.	*DEHP added (μg)*	*Phthalic acid found (μg)*	*DEHP calculated (μg)*	*Recovery (%)*
1	0.10	0.036	0.085	85
2	0.10	0.030	0.070	70
3	0.50	0.19	0.45	90
4	0.50	0.18	0.42	84
5	1.0	0.36	0.85	85
6	1.0	0.42	0.99	99
7	2.0	0.94	2.2	110
8	2.0	0.81	1.9	95

acid (150:1), the compound was not found in the first 40-ml fraction but was found in the next fraction at very low levels. Elution with the former solvent will thus eliminate non-polar substances, such as polychlorinated biphenyls and chlorinated pesticides, and most monocarboxylic acids. The elimination of more polar substances such as phenol carboxylic acids and chlorinated phenoxy herbicides will be also achieved by the initial elution with the latter solvent. The elution patterns of salicylic acid and 2-(2,4,5-trichlorophenoxy)propionic acid obtained by elution with the latter solvent system, are shown in Fig. 3. A sharp and steady elution of phthalic acid was obtained with ethyl acetate–formic acid (150:1), as shown in Fig. 4.

The activity of the silica gel used did not greately influence the reproducibility of the elution pattern unless the gel had adsorbed a large amount of moisture before use.

In the loading of the extract of the hydrolyzate on to the column, a large amount of fatty acids formed from fat might induce a more rapid elution of phthalic acid than that expected, owing to their competition on the silica gel. Therefore, the loading had to be started from the non-polar solvent system so that phthalic acid might be adsorbed completely on the top of the column.

Although residual formic acid remaining after evaporation of the required fraction would be esterified with the reagent, the ester formed could be easily hydrolyzed in the presence of water to give the original acid.

After the addition of di(2-ethylhexyl) phthalate to 20-g portions of homogenized

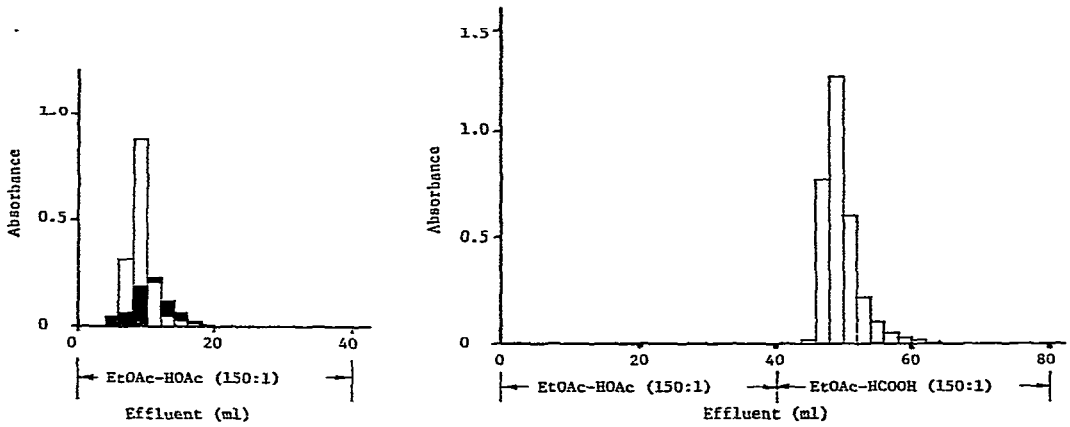

Fig. 3. Elution patterns of salicylic acid (□) and 2-(2,4,5-trichlorophenoxy)propionic acid (■) from silica gel columns. Amount loaded = 100 μg. Silica gel column: 10 × 1 cm I.D. Each fraction was measured at $\lambda_{max.}$ = 305 nm for salicylic acid and $\lambda_{max.}$ = 290 nm for 2-(2,4,5-trichlorophenoxy)-propionic acid.

Fig. 4. Elution pattern of phthalic acid from a silica gel column. Silica gel column: 10 × 1 cm I.D. Amount of phthalic acid = 1.0 mg.

breast muscle of a bird at levels of 5–100 ppb, recoveries of the phthalate ester were determined as described under Experimental. The washing of the initial *n*-hexane extract with 1% sodium hydrogen carbonate solution was an important step in determining only phthalate esters in the presence of the metabolite half-esters[17–20].

As shown in Table III, the addition at lower levels gave decreased recoveries. However, the method will give adequate recoveries for the determination of trace amounts of phthalate esters in biological samples, allowing background contamination to be kept at very low levels.

REFERENCES

1 R. A. Hites, *Environ. Health Perspect.*, Exp. Issue No. 3 (1973) 17.
2 J. Cerbulis and J. S. Ard, *J. Ass. Offic. Anal. Chem.*, 50 (1967) 646.
3 D. T. Williams, *J. Ass. Offic. Anal. Chem.*, 56 (1973) 181.
4 R. J. Jaeger and R. J. Rubin, *New Engl. J. Med.*, 287 (1972) 1114.
5 D. J. Nazir, M. Beroza and P. P. Nair, *Environ. Health Perspect.*, Exp. Issue No. 3 (1973) 141.
6 E. W. Godly and A. E. Mortlock, *Analyst (London)*, 98 (1973) 493.
7 F. D. Lee, J. Britton, B. Jeffcoat and R. F. Mitchell, *Nature (London)*, 211 (1966) 521.
8 G. H. Thomas, *Environ. Health Perspect.*, Exp. Issue No. 3 (1973) 23.
9 E. Weisenberg, Y. Schoenberg and N. Ayalon, *Analyst (London)*, 100 (1975) 857.
10 R. A. Hites, *J. Chromatogr. Sci.*, 11 (1973) 570.
11 D. D. McCollister, *Food Cosmet. Toxicol.*, 2 (1964) 23.
12 D. Calley, J. Autian and W. L. Guess, *J. Pharm. Sci.*, 55 (1966) 158.
13 R. L. DeHann, *Nature New Biol.*, 231 (1971) 85.
14 E. O. Dillingham and J. Autian, *Environ. Health Perspect.*, Exp. Issue No. 3 (1973) 81.
15 R. J. Rubin and R. J. Jaeger, *Environ. Health Perspect.*, Exp. Issue No. 3 (1973) 53.
16 A. R. Singh, H. H. Lawrence and J. Autian, *J. Pharm. Sci.*, 61 (1973) 51.
17 D. W. Woodham, W. G. Mitchell, C. D. Loftis and C. W. Collier, *J. Agr. Food Chem.*, 19 (1971) 186.
18 D. L. Stalling, J. W. Hogan and J. L. Johnson, *Environ. Health Perspect.*, No. 3 (1973) 159.
19 P. W. Albro, R. Thomas and L. Fishbein, *J. Chromatogr.*, 76 (1973) 321.
20 P. W. Albro and B. Moore, *J. Chromatogr.*, 94 (1974) 209.