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4-METHYL-7-METHOXYCOUMARIN AS A FLUORESCENT LABEL FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF DICARBOXYLIC ACIDS

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SUMMARY

Results of a study of the rates and products of the esterification of dicarboxylic acids with 4-bromomethyl-7-methoxycoumarin are presented. It was found that when tetrabutylammonium hydroxide was used instead of carbonate/crown ether, methylmethoxycoumarin (MMC) diesters were obtained in very high yield from C_5–C_{10} dicarboxylic acids. MMC monoesters were formed in significant amounts only with dicarboxylic acids smaller than C_5. The MMC diesters could be completely separated on an RP-18 reversed-phase column using gradient elution. The chromatographic peaks detected in earlier high-performance liquid chromatographic (HPLC) applications of this reaction for quantitative analysis of dicarboxylic acids (isocratic HPLC) were apparently formed by the respective MMC monoesters. MMC diesters elute from an RP-18 reversed-phase column only when more than 50% methanol is used in the mobile phase. However, HPLC with gradient elution is the preferred method for the separation of MMC diesters.

INTRODUCTION

As early as 1950, Baker *et al.*[1] proposed use of the 7-methoxycoumarin nucleus as a fluorescent label in liquid chromatography. More recently, Dünges introduced 4-bromomethyl-7-methoxycoumarin (BrMMC) as a labelling agent for fatty acids and several other organic acids; although he attempted to use it with dicarboxylic acids, he obtained no measurable yields with his method for the preparation of MMC esters[2,3]. Since then, various thin-layer chromatographic and high-performance liquid chromatographic (HPLC) applications have been described[4–9]. For dicarboxylic acids, Grushka *et al.*[5] reported the formation of MMC esters with yields that allowed the HPLC determination of trace amounts of C_2–C_{10} dicarboxylic acids. Although not explicitly stated, their paper implies that the respective MMC diesters were obtained. Gonnet *et al.*[8] studied the esterification reactions of BrMMC with various fatty acids and dicarboxylic acids using a procedure very similar to that of Grushka *et al.* and concluded that the esterification of dicarboxylic acids leads chiefly to MMC monoesters.

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Because, despite the uncertainty about the products of esterification with BrMMC, the method appeared well documented in the literature and relatively easy to handle, we decided to test its applicability to the analysis of the fraction of organic acids in precipitation. For calibration purposes, various MMC mono- and diesters were synthesized in order to establish whether the products obtained with BrMMC and dicarboxylic acids are mono- or diesters or a mixture.

EXPERIMENTAL

Materials

Commercially available analytical-reagent grade chemicals were used without further purification. Dicarboxylic acids from C_3 to C_{12} and 4-bromomethyl-7-methoxycoumarin (special batch) were obtained from Fluka (Buchs, Switzerland). Methanol, ethanol, acetone, ethyl acetate and triethylamine (TEA) were purchased from E. Merck (Darmstadt, F.R.G.). Because of its instability, aqueous tetrabutylammonium (TBA) hydroxide was prepared in the laboratory when needed from TBA bromide by means of ion exchange using AG 1-X8 anion-exchange resin. For cation-exchange operations, AG 50W-X8 cation exchange resin was utilized. Both resins were obtained from Bio-Rad (Munich, F.R.G.). HPLC-grade water was prepared using a Milli-Q water purification system from Millipore (Neu-Isenburg, F.R.G.).

Apparatus

For analytical separations and the detection of MMC esters, a Spectra-Physics Model SP 8100 HPLC instrument was employed. It was equipped with a 250 × 4 mm I.D. Hibar column, slurry packed with RP-18, particle size 10 μm (E. Merck), and two detectors, a Spectra-Physics Model SP 8400 UV detector and a Shimadzu Model RF 510 spectrofluorophotometer (with a 12-μl flow cell). Using the spectrofluorophotometer, the excitation wavelength was set at 335 nm and the emission wavelength at 395 nm, both with a window of 10 nm. Methanol and water–methanol mixtures were used as mobile phases.

Mass spectra were obtained using a VG Analytical Micromass 7035 GC–MS combination (direct probe and electron impact ionization).

IR and NMR spectra were recorded in the Microanalytical and NMR Laboratories of the Institute for Organic Chemistry, Johannes Gutenberg University, Mainz (F.R.G.) on a Beckman IR 4220 spectrophotometer and on a Bruker WH 90 NMR spectrometer, respectively. Data on elemental compositions were obtained in the same laboratories.

Preparation of MMC mono- and diesters of dicarboxylic acids $\geqslant C_3$

As opposed to earlier work on the esterification of organic acids with BrMMC, we used TEA instead of carbonate and crown ethers as a catalyst because with dicarboxylic acids $\geqslant C_3$, TEA appeared to be a more effective catalyst.

Method A. MMC monoesters are obtained with high yields by dissolving 10 mmol of a dicarboxylic acid, 1 mmol of BrMMC, and 8 mmol of TEA in 100 ml of acetone. The mixture is refluxed for 5–8 h in the dark, then concentrated in a rotary evaporator to a volume of about 50 ml and reaction products are precipitated by adding water. The precipitate is separated, washed several times with water, then

dried at 110°C. Finally, the dry precipitate is dissolved in ethanol or ethyl acetate and allowed to recrystallize. In this way, white crystalline MMC monoesters are obtained in yields of 70–80%.

Method B. MMC diesters are synthesized in a similar way by dissolving 1 mmol of a dicarboxylic acid, 4 mmol of BrMMC and 8 mmol of TEA in 100 ml of acetone.

HPLC determination of organic acids

Fluorescent labeling (TBA method). To a solution of organic acids in an aprotic solvent (*e.g.*, acetone or acetonitrile), freshly prepared TBA hydroxide is added until a pH of 7.5–8.5 is reached. Subsequently, the solution is evaporated to dryness using a rotary evaporator. The dry TBA salts are then dissolved in about 1 ml of a 0.004 *M* BrMMC solution in acetone such that the molar ratio of BrMMC to total organic acids is at least 3:1. Esterification is accomplished by refluxing the solution for 15 min. The labelling method is also applicable to organic acids in aqueous solution. The BrMMC reagent solution is not stable and decomposes after a few days.

A major difficulty when applying the TBA method to fluorescent labelling of organic acids from atmospheric particulate matter (aerosol particles) or from precipitation samples is the complex matrix in which they come[10]. When added to the acids of such samples, BrMMC not only reacts with the TBA salts of mono- and dicarboxylic acids but also with those of sulphuric and phosphoric acids and with other organic compounds, *e.g.*, phenols.

Because the concentration levels of the lower fatty acids (C_1–C_3) are usually much higher than those of all other organic acids that may be analysed by the TBA labelling/HPLC method, it is advantageous to collect rain or snow samples of 250 ml and more and to divide the samples into 10- and 100-ml fractions[10]. The 10-ml fractions are percolated through a strong cation-exchange column to substitute all cations by H^+, subsequently adjusted with TBA hydroxide to a pH of 8, and then derivatized with BrMMC as described above.

The 100-ml fractions are concentrated to a volume of 5 ml in a rotary evaporator. In order to remove sulphuric and phosphoric acids, $Ba(OH)_2$ is added until a pH of 10 is attained. The precipitated $BaSO_4$ and $Ba_3(PO_4)_2$ are then removed by centrifugation. The sulphate- and phosphate-free samples are percolated through a cation-exchange column in the same way as the untreated 10-ml fractions. After each sample, the column is rinsed with 10–15 ml of HPLC-grade water. The combined percolates (sample plus wash water) are concentrated to a volume of 2–3 ml. The pH of the resulting aqueous solution is then adjusted to 9–9.5. Subsequently, TBA hydroxide and BrMMC are added, and the esterification is carried out as above.

With samples of atmospheric particulate matter, loaded glass-fibre filters are acidified with 0.1 *N* sulphuric acid and extracted under a purified nitrogen atmosphere with diethyl ether in an ultrasonic bath after a known amount of suberic or azealic acid has been added as an internal standard. Subsequently, the two layers of the extract are separated and the aqueous sulphuric acid fraction is extracted a second time with diethyl ether in order to ensure that the organic acids have been completely transferred to the diethyl ether fraction. The combined diethyl ether fractions are then extracted twice by shaking with 0.1 *N* sodium hydroxide solution. The combined alkaline extracts are percolated through a cation-exchange column as described

above. After each sample, the column is rinsed with 10–15 ml of HPLC-grade water. The combined percolates (sample plus wash water) are concentrated to a volume of 5 ml in a rotary evaporator. $Ba(OH)_2$ is then added to the concentrate until a pH of 10 is attained. The subsequent steps are identical with the procedure described above for the processing of 100-ml rain water samples. While the recovery rate for dicarboxylic acids $\geqslant C_4$ is 80–90%, about 90% of the monocarboxylic acids $< C_7$ are lost during the concentration of the acidic percolates.

HPLC separations. The obtained MMC ester mixtures in acetone are separated using the described HPLC arrangement in the gradient elution mode. For each run, 10 μl of an ester mixture are injected on to the RP-18 column, which is maintained at room temperature. Methanol and water are utilized for the mobile phase with decreasing relative proportions of water. At a flow-rate of 1.5 ml min^{-1}, a linear gradient is applied so that starting with 40% water in methanol, pure methanol is reached after 30 min. Unless stated otherwise, these HPLC conditions were utilized throughout this study.

RESULTS AND DISCUSSION

For both methods of preparation of ester standards (methods A and B), the progress of esterification was monitored by taking samples from the reaction mixture as the reaction proceeded. The samples were diluted 1:10 with acetone and subsequently analysed by HPLC. As illustrated in Fig. 1 for the esterification of malonic acid using method B, the MMC monoester attains maximum concentration in the reaction mixture after 45 min (curve 4). The MMC diester, however, does not reach maximum concentration until after 5 h (curve 1). Concurrently, a by-product is formed which, using UV detection, produces a signal of about 20% of that obtained

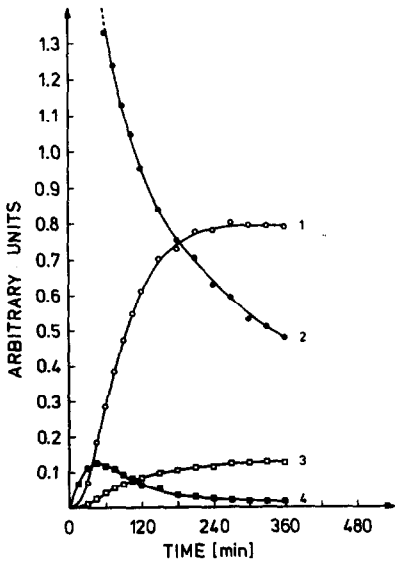


Fig. 1. Esterification of malonic acid with 4-bromomethyl-7-methoxycoumarin. 1 = Malonic acid MMC diester; 2 = BrMMC; 3 = by-product; 4 = malonic acid MMC monoester.

for the MMC diester (curve 3). After 5 h, most of the monoester has disappeared again.

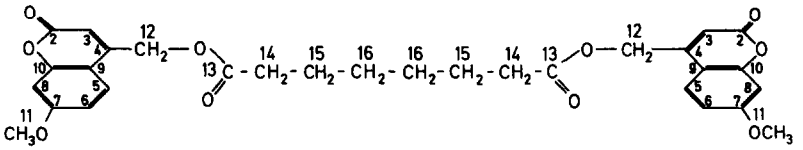
Using method A, the MMC monoester reaches maximum concentration after about 6 hs. There is very little MMC diester formed during this time. With both methods, increasing amounts of TEA lead to decreasing MMC ester yields. Instead, a fluorescent by-product (which was not further characterized) is formed in increasing amounts. This shows that both methods employing TEA as a catalyst and phase transfer agent may be used for the preparation of individual MMC esters but are less suitable for the fluorescent labelling of mixtures of organic acids for subsequent analysis.

Instead, TBA hydroxide is used for analytical applications as described above. The TBA method yields MMC esters of fatty acids up to C_{10} and higher in yields of more than 90%. From the C_5–C_{10} dicarboxylic acids, MMC diesters are obtained in yields of 80–90%. MMC monoesters are not formed to any significant extent. Dicarboxylic acids $< C_5$ were found to form both mono- and diesters in varying amounts so that the TBA method appears less applicable for these acids. A further complication with the MMC diesters of the various C_4 dicarboxylic acids is their low solubility in acetone. The TBA method is also applicable to the fluorescent labelling of aromatic acids[10]. With benzoic acid, complete conversion to the respective MMC ester is obtained. For phthalic and terephthalic acid, the yields of the respective MMC diesters are low, of the order of 25%, but they are reproducible.

Verification of purity and structure of synthesized MMC esters

All synthesized MMC esters (chiefly diesters) were analysed for elemental composition and structure. The data obtained on elemental composition of MMC diesters were within 1.5% of the theoretical values. For succinic and adipic MMC diesters, chromatographic analysis showed the presence of at least one other compound. This and the melting points of the latter (see Table I) were taken as evidence for the presence of appreciable amounts of impurities (it is not easy to purify the MMC diesters of succinic and adipic acid because of their poor solubility in organic solvents).

The structures of the synthesized MMC esters were verified by their IR, NMR and mass spectra. The spectroscopic characteristics of MMC diesters are exemplified in Tables II and III and Figs. 2–4 by the various spectra of the MMC diesters of pimelic and suberic acid.



Suberic acid 4-methyl-7-methoxycoumarin diester

Fig. 2 shows the IR spectrum of the MMC diester of pimelic acid. The ^1H NMR spectrum of suberic acid MMC diester is depicted in Fig. 3. The peak assignment is given in Table II. Fig. 4 shows the low-resolution electron impact mass spectrum of suberic acid MMC diester. As in the mass spectra of all other MMC

TABLE I

MELTING POINTS OF MMC DIESTERS OF VARIOUS DICARBOXYLIC ACIDS

Acid	*M.p. (°C)*	*Acid*	*M.p. (°C)*
Malonic	212–214	Suberic	167–168
Succinic*	260	Acelaic	140
Glutaric	183–184	Sebacic	155–156
Adipic*	245–247	Undecanedioic	126–128
Pimelic	118–120	Dodecanedioic	138–140

* See text.

TABLE II

^{1}H NMR SPECTRAL DATA FOR SUBERIC ACID MMC DIESTER

Assignment	*Position**	*Chemical shift (ppm)*	*Signal*	*Number of protons*
CH_3–O–Ar	11	3.88	s	2 × 3
–C–CH_2–C–	15, 16	1.25–1.95	m	4 × 2
–C–CH_2–O–CO–R	12	5.30	s	2 × 2
–C–CH_2–CO–O–R	14	2.38–2.60	t	2 × 2
H in coumarin	3	6.30	s	2 × 1
H in coumarin	6, 8	6.75–6.95	m	4 × 1
H in coumarin	5	7.25–7.50	m	2 × 1

* See structure.

diesters synthesized, the base peak occurs at *m/e* 206. Further characteristic fragments are an ion at *m/e* 248 (MMC−OOC−CH_3^+) and ions of the series MMC−OOC−$(CH_2)_n$–CO^+ and MMC−OOC−$(CH_2)_n^+$ (n = 1, 2, ..., 10) due to the loss of –O–MMC and –COO–MMC, respectively (see Table III). In the mass spectra of MMC monoesters, a peak occurs at *m/e* 60 [$CH_2C(OH)OH^+$], originating from the non-esterified carboxylic group after McLafferty rearrangement. The NMR and mass spectral data are in good agreement with the results obtained for MMC esters of monocarboxylic acids by Meyer[11].

TABLE III

MASS SPECTRAL DATA FOR SUBERIC ACID MMC DIESTER

m/e	*Fragment(s)*
550	Molecular ion (M^+)
345	MMC−$OOC(CH_2)_6CO^+$
317, 303, 289, 275, 261	MMC−$OOC(CH_2)_n^+$ (n = 2, 3, ..., 6)
248	MMC−$OOCCH_3^+$
206	MMC−OH^+
190, 178, 161, 145, 133, 118, 102	Fragments of MMC
60*	$CH_2C(OH)OH^+$

* Only with MMC monoesters.

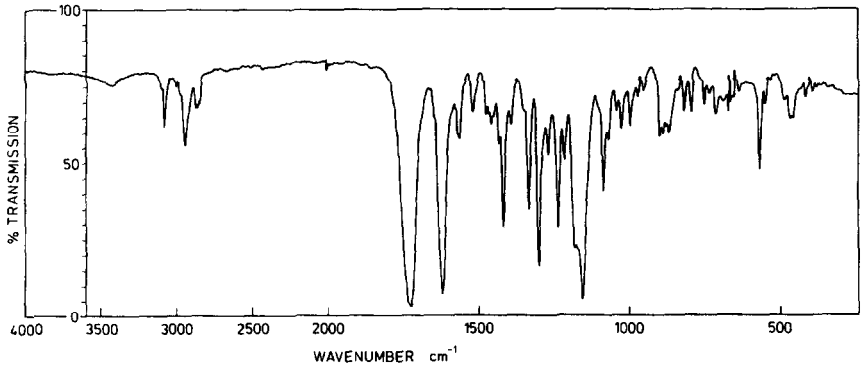


Fig. 2. IR spectrum of pimelic acid MMC diester (KBr pellets).

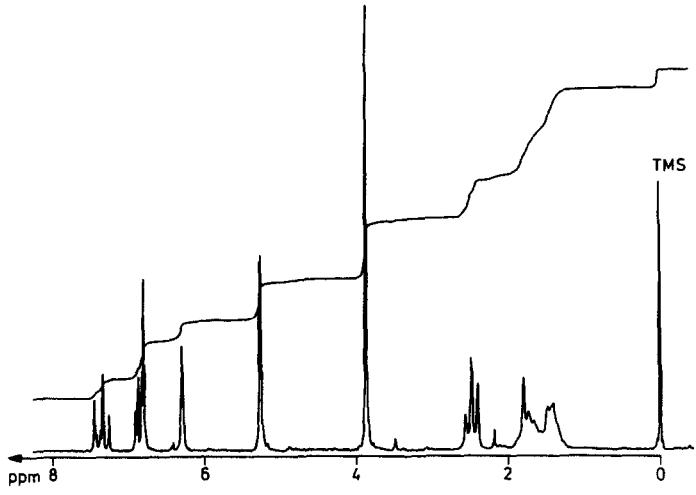


Fig. 3. 90 MHz ^{1}H NMR spectrum of suberic acid MMC diester.

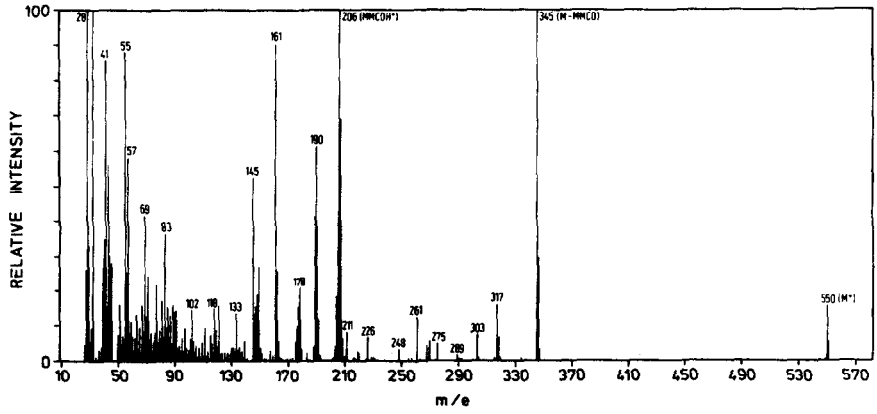


Fig. 4. Low-resolution mass spectrum of suberic acid MMC diester.

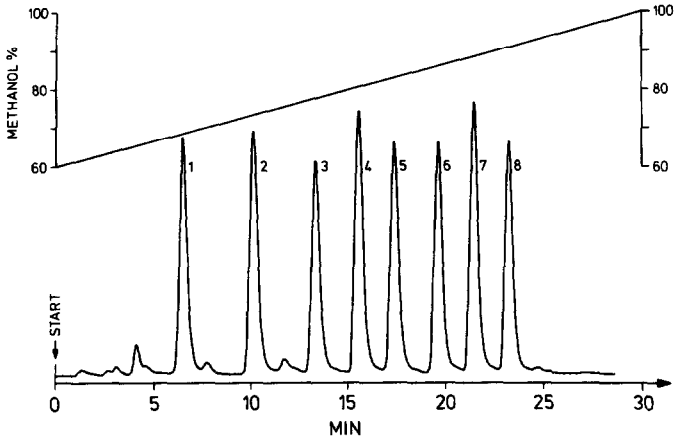


Fig. 5. Gradient elution HPLC separation of dicarboxylic acid MMC diesters. 250 × 4 mm I.D. Hibar column, slurry packed with RP-18, particle size 10 μm. Mobile phase: linear gradient from 40% water in methanol to 100% methanol. The gradient slope is shown in the upper part of the figure. Flow-rate: 1.50 ml min^{-1}. Temperature: ambient. Peaks: 1 = malonic; 2 = glutaric; 3 = pimelic; 4 = suberic; 5 = acelaic; 6 = sebacic; 7 = undecanedioic; 8 = dodecanedioic.

Separation of MMC diesters by HPLC

As shown in Fig. 5 for a standard mixture of various MMC diesters, complete separation can be achieved within 25 min on an RP-18 reversed-phase column using gradient elution. Comparable separations are obtained for the MMC diesters resulting from dicarboxylic acids when the TBA fluorescent labelling method is applied to real samples. The fluorescence internity obtained from a given concentration of MMC diester (about 5 μ*M*) was found to vary with eluent composition, *i.e.*, with the concentration of water in methanol, as exemplified in Fig. 6 by the variations observed for the MMC diesters of glutaric acid and dodecanedioic acid (1,10-decanedicarboxylic acid). A similar effect was observed for MMC esters of fatty acids by Lloyd[9]. It is, therefore, important for quantitative analysis that the HPLC instrument used operates with good reproducibility in the gradient elution mode.

According to Grushka *et al.*[5], dicarboxylic acids can be analysed by HPLC after fluorescent labelling with BrMMC using the carbonate–crown ether method. They showed chromatograms to demonstrate that sufficient separation of the resulting MMC ester mixture can be achieved by employing isocratic HPLC. However, Gonnet *et al.*[8] have shown that the carbonate–crown ether method yields two different esters for a given dicarboxylic acid and that the main product still has an ionic group, the MMC monoester in other words. This leaves serious doubts as to the reproducibility of fluorescent labelling using the carbonate–crown ether method and the reproducibility of the analytical method for dicarboxylic acids, based on this labelling technique.

We found that when isocratic HPLC is employed as described by Grushka *et al.*[5], *i.e.*, a mobile phase of 55% water in methanol at a flow-rate of 2.33 ml min^{-1}, none of the MMC diesters that we synthesized eluted from an RP-18 reversed-phase column, even after several hours. The same holds for MMC monoesters of higher dicarboxylic acids such as sebacic acid. As shown in Fig. 7, the MMC monoesters

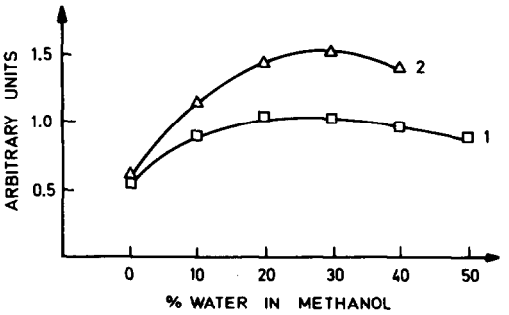


Fig. 6. Variation of fluorescence intensity of MMC diesters, excited at 335 nm and monitored at 395 nm, with mixing ratio (%, v/v) of water in methanol eluent. Isocratic conditions. Flow-rate: 1.50 ml min^{-1}. Temperature: ambient. Curve 1 = glutaric acid; curve 2 = dodecanedioic acid (diester concentrations about 5 μM).

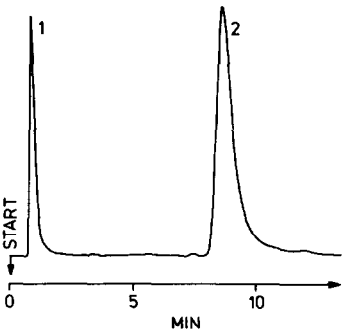


Fig. 7. Isocratic separation of dicarboxylic acid MMC monoesters. 250 × 4 mm I.D. Hibar column, slurry packed with RP-18, particle size 10 μm. Eluent: 45% water in methanol. Flow-rate: 2.33 ml min^{-1}. Temperature: ambient. Peaks: 1 = malonic acid; 2 = pimelic acid.

of malonic and pimelic acid, however, elute under these conditions after 1 and 8 min, respectively, which is in good agreement with the results obtained by Grushka *et al.* Using a mobile phase of 50% water in methanol at an increased flow-rate of 3.30 ml min^{-1}, the retention times of the MMC diesters of glutamic and adipic acid are still 32 and 52 min, respectively. Under these circumstances, the peaks are broadened to such an extent that such separation conditions are not useful for analytical purposes. Sebacic acid MMC monoester elutes after 13 min under the same conditions. This leads to the conclusion that the peaks in the chromatograms shown by Grushka *et al.*[5] were formed by MMC monoesters of dicarboxylic acids which, according to Gonnet *et al.*[8], are the main product of fluorescent labelling with BrMMC using the carbonate–crown ether method and that Grushka *et al.* did not see any MMC diesters in their chromatograms, as under their HPLC conditions MMC diesters elute either very late or not at all from an RP-18 reversed-phase column.

The TBA method for fluorescent labelling of organic acids with BrMMC gives reproducible results for the acids mentioned above and, with C_5–C_{10} dicarboxylic acids, only MMC diesters are obtained in high yields. The MMC diesters can be separated on an RP-18 reversed-phase column using gradient elution with water–methanol as the mobile phase.

As an example of the results obtained with real samples, a chromatogram of the MMC esters of organic acids from atmospheric particulate matter, collected in October 1984 on the campus of the University of Mainz, is shown in Fig. 8. The separation conditions utilized were the same as described above (see *HPLC separations*), except for a slight modification of the water–methanol gradient. Instead of a linear gradient starting at 40% water in methanol and reaching pure methanol after 30 min, the gradient utilized here began at 55% water in methanol and reached 40% water after 10 min and pure methanol after further 40 min. The MMC esters of valeric and caproic acid co-elute under these conditions together with the MMC diesters of glutaric acid (DC_5) and pimelic acid (DC_7), respectively. Co-elution of MMC esters of monocarboxylic acids and MMC diesters of dicarboxylic acids can

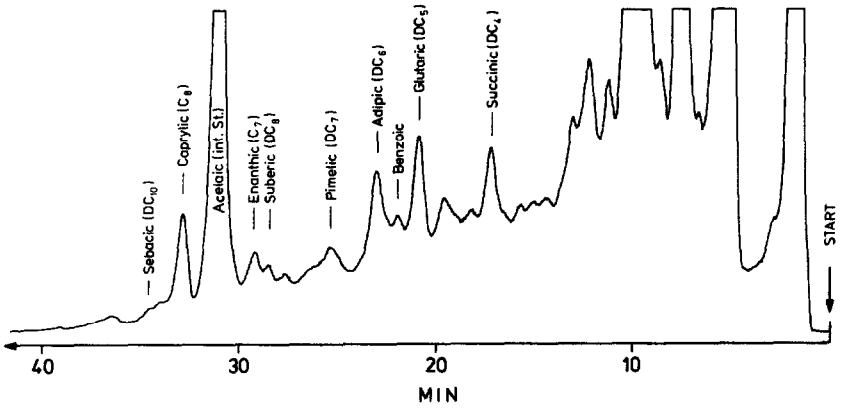


Fig. 8. HPLC separation of MMC esters of organic acids from atmospheric particulate matter, collected in Mainz in October 1984. 250 × 4 mm I.D. Hibar column, slurry packed with RP-18, particle size 10 μm. Mobile phase: linear gradient from 55% water to 40% water in methanol after 10 min and to 100% methanol after a further 40 min. Flow-rate: 1.50 ml min^{-1}. Temperature: ambient. Acelaic acid was added as an internal standard (int. St.).

be influenced by varying the water–methanol gradient. In this chromatogram, the peaks of DC_5 and DC_6, for example, correspond to 12.3 ng of glutaric acid [*i.e.*, 2.3 ng of glutaric acid per m^3 (STP) of air] and 10 ng of adipic acid [*i.e.*, 1.9 ng of adipic acid per m^3 (STP) of air], respectively.

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REFERENCES

1 W. Baker, C. N. Haksar and J. F. W. McOmie, *J. Chem. Soc.*, (1950) 170–173.
2 W. Dünges, *Anal. Chem.*, 49 (1977) 442–445.
3 W. Dünges, A. Meyer, K. E. Müller, M. Müller, R. Pietschmann, C. Plachetta, R. Sehr and H. Tuss, *Z. Anal. Chem.*, 288 (1977) 361–368.
4 W. Dünges and N. Seiler, *J. Chromatogr.*, 145 (1978) 483–488.
5 E. Grushka, S. Lam and J. Chassin, *Anal. Chem.*, 50 (1978) 1398–1399.
6 S. Lam and E. Grushka, *J. Chromatogr.*, 158 (1978) 207–214.
7 S. G. Zelenski and J. W. Huber, *Chromatographia*, 11 (1978) 645–646.
8 C. Gonnet, M. Marichy and N. Philippe, *Analusis*, 7 (1979) 370–375.
9 J. B. F. Lloyd, *J. Chromatogr.*, 178 (1979) 249–258.
10 A. Neftel, S. Breitenbach, W. Elbert and J. Hahn, *Proceedings of International Conference on Gas–Liquid Chemistry of Natural Waters, Brookhaven, April 1984*, Brookhaven National Laboratory Report No. BNL 51757.
11 H. D. Meyer, *Dissertation*, Eberhard-Karls-Universität, Tübingen, 1978.