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QUANTITATIVE GAS CHROMATOGRAPHIC ANALYSIS OF METHYL ESTERS OF HYDROXY FATTY ACIDS DERIVED FROM PLANT CUTIN

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SUMMARY

A capillary gas chromatographic method is described for the quantitative analysis of cutin acid methyl esters (CAMEs) obtained by methanolytic depolymerization of plant cutins. The errors resulting from differential losses during diethyl ether extraction of four major CAMEs from the reaction mixture are evaluated using partition coefficients (K). K values for the system diethyl ether–water strongly depend on the polarity of the CAMEs and the amounts of methanol present. The losses during sample work-up can be corrected for by factors calculated from K values and phase ratios. 2,2-Dimethoxypropane is used instead of solid desiccants for drying diethyl ether saturated with water. Correction factors for the differential response of the gas–liquid chromatographic system to the four CAMEs investigated are determined. It is shown that without correction the actual amounts of CAMEs would be underestimated by 6.2–21.3%.

INTRODUCTION

The cuticle is the protective skin of all above-ground primary parts of terrestial plants. It contains as a unique component the biopolymer cutin. Cutin is insoluble in any organic solvent, and in order to study its composition it has to be cleaved into its monomers. Comprehensive monographs treat the qualitative composition of the mixtures of monomers obtained by base or boron trifluoride-catalysed methanolysis[1,2] or by reductive cleavage of ester bonds by lithium aluminium hydride[3] from the cutins of various plant species[4,5].

When we studied the covalent binding of chemicals to epoxy groups of cutin[6] we needed a precise estimate of the epoxide content of cuticles. After critically studying the methods used so far, we realized that in no instance had gas chromatographic (GC) data been corrected for losses during work-up and for differential detector response. These corrections are considered necessary in quantitative gas–liquid chromatography (GLC)[7,8]. In order to test the errors inherent in the data on cutin composition derived from uncorrected peak areas, we developed a method for the quantitative analysis of the composition of small (less than 1 mg) cutin samples.

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EXPERIMENTAL

Preparation of cutin monomer samples

Adaxial cuticles of fully expanded leaves of greenhouse-grown rubber plants (*Ficus elastica* Roxb. var. *decora*) were isolated enzymatically as described previously[9]. The cuticular membranes obtained were exhaustively extracted with chloroform–methanol (1:1, v/v) in a Soxhlet apparatus. Subsequently, the epoxy groups present in *Ficus* cutin were transformed into their chlorohydrin derivatives with 0.2 *M* hydrochloric acid in dioxane[10]. The cuticles were washed several times with chloroform–methanol and dried. Cuticles from different leaves were finely cut and mixed to obtain homogeneous samples for analysis.

Accurately weighed amounts of cuticular material (0.1–1.0 mg) were placed in 2-ml glass vials with PTFE-lined septum caps, and a precisely known amount of *n*-eicosane (99.5%, Serva, Heidelberg, F.R.G.) in *n*-hexane solution was added as internal standard together with 200 μl of boron trifluoride–methanol complex (Merck, Darmstadt, F.R.G.). Care was taken that the cuticles were totally immersed in the liquid. The closed vials were heated to 70°C for 10–12 h. No increase in yield could be obtained by longer treatment or by the addition of larger amounts of boron trifluoride–methanol. No effect of boron trifluoride–methanol on the qualitative or quantitative composition of cutin depolymerizates has been detected when standard compounds (see below) were subjected to this treatment. The samples containing the cutin acid methyl esters (CAMEs) resulting from methanolytic depolymerization of cutin were cooled to room temperature, and 800 μl of deionized water and 1 ml of diethyl ether were added. After vigorous shaking for *ca.* 1 min the diethyl ether phase was removed and transferred to a 1-ml Reactivial (Wheaton, Millville, NJ, U.S.A.).

The volume of the diethyl ether phase was reduced to 500 μl under a gentle stream of nitrogen and washed with three 500-μl portions of water to remove polar contaminants, which interfere with the GLC determination of cutin monomers. The diethyl ether solution was transferred to a fresh vial and dried using 2,2-dimethoxypropane (98%, Merck-Schuchardt, Hohenbrunn, F.R.G.). The vial was heated rapidly to 50°C and cooled, and the diethyl ether was removed by a stream of nitrogen. Potential losses during the removal of the solvent could be excluded by the use of CAME standards (see below).

This sample preparation procedure was conceived as a standardized method allowing for the correction of the differential losses of CAMEs that occur during the work-up of cutin depolymerizates. Correction procedures are described under Results and discussion.

Cutin acid standards

From experiments on cutin synthesis in *Clivia miniata* Reg.[11] methyl 9,10-dihydroxy[^{14}C]hexadecanoate (specific activity 51.0 TBq/mol, analytical purity 81%), a mixture of methyl 9-chloro-10,18-dihydroxy- and methyl 10-chloro-9,18-dihydroxy[^{14}C]octadecanoate (13.9 TBq/mol, 91%), methyl 18-hydroxy-9[^{14}C]-octadecenoate (2.9 TBq/mol, 75%) and methyl 9,10,18-trihydroxy-[^{14}C]octadecanoate (27.4 TBq/mol, 86%) were available. Radiochemical purities were better than 95% as determined by radio thin-layer chromatography.

The analytical concentrations were determined gravimetrically, and purities

were calculated from GLC chromatograms assuming equal flame ionization detection responses for both the main components and the contaminants of the standard solutions. The chlorohydrin derivatives of 9,10-epoxy-18-hydroxyoctadecanoic acid were obtained by *in situ* treatment of the cuticles with hydrochloric acid–dioxane[10]. 9,10,18-Trihydroxyoctadecanoate was produced from 9,10-epoxy-18-hydroxyoctadecanoate by treating cuticles *in situ* with sulphuric acid (30%, 30 min)[2].

Partition coefficients

The molal partition coefficients K of the ^{14}C-labelled CAME standards in diethyl ether–water were determined at 25 ± 2°C using 2-ml glass vials closed with PTFE-lined septum caps (Wheaton). The standard compounds were dissolved in 800 µl diethyl ether. After the addition of 400 µl of water and various amounts of methanol, the vials were vigorously shaken for 1 min. After phase separation, samples were drawn from both phases and the radioactivity was determined by liquid scintillation counting at a 2σ error of 1%. Repeated or prolonged shaking had no influence on K.

The molal partition coefficient K was calculated according to

$$K = \frac{C_o}{C_w} \cdot \frac{\rho_w}{\rho_o} \tag{1}$$

where C_o and C_w are the molar equilibrium concentrations of the compound in the diethyl ether (o) and water (w) phases, respectively. The second term on the righthand side is the ratio of the specific masses ρ of the two phases.

Methanol also partitioned between the diethyl ether and water phases. The amounts lost from the aqueous phase into the organic phase could be determined from the differences in the concentration of ^{3}H-labelled water in the aqueous phase (see below) before and after equilibration. The partitioning of methanol into the diethyl ether phase lead to an increase of the concentration of ^{3}H-labelled water in the aqueous phase. From this concentration change, both the amounts lost and remaining in the aqueous phase and thus the methanol concentrations in both phases could be estimated. Thus, an approximate value of K for methanol in the system used could be calculated.

The use of a 5 mM sodium chloride solution instead of water lead to precipitations of solid sodium chloride when methanol was present. This phenomenon affected the partition coefficients irreproducibly.

Water content of diethyl ether

The water content of diethyl ether saturated with water at 25 ± 2°C was determined with ^{3}HOH (specific activity 145 MBq/mol). The influence of methanol on the water content of the diethyl ether phase was also studied. Equal volumes of ^{3}H-labelled aqueous phase and diethyl ether together with various amounts of methanol were agitated for 1 min in 2-ml glass vials closed with PTFE-lined septum caps (Wheaton). After phase separation, samples were drawn from the organic phases and their radioactivity determined by liquid scintillation counting at a 2σ error of 1%. For each methanol concentration five replications were made.

Drying of diethyl ether solutions

The effectiveness of sodium sulphate, calcium chloride and copper sulphate (dried, Merck, Darmstadt, F.R.G.) as desiccants was tested with diethyl ether saturated with ^{3}HOH. The desiccants had been stored at 130°C in the oven. Various amounts of desiccant were added to 5 ml of diethyl ether saturated with ^{3}H-labelled water and the samples were periodically agitated. After a defined period of time, the water content of the diethyl ether was estimated from the amount of radioactivity remaining in the supernatant. The effectiveness of 2,2-dimethoxypropane (98%, Merck-Schuchardt) for drying diethyl ether was also studied: it was added in amounts stoichiometrically equivalent to the water content of diethyl ether and the mixture was heated to 50°C or left for 15 min at 25°C. Afterwards, the residual water content was determined semiquantitatively by tetrapropylorthotitanate (Merck).

Chromatography

The chromatography of CAMEs was carried out on a Perkin-Elmer (Norwalk, CT, U.S.A.) Sigma 1B gas chromatograph fitted with a flame ionization detector and an on-column injector (SGE, Melbourne, Australia). The cutin monomers were separated on a 25 m × 0.32 mm I.D. fused-silica wall-coated open-tubular (WCOT) column coated with CP-Sil 5 CB (polymethylsiloxane equivalent to OV-1 or SE-30, d_f = 0.13 μm) from Chrompack (Middelburg, The Netherlands). The hydrogen carrier gas flow-rate was adjusted to 2.1 ml/min at 150°C oven temperature. The temperature programme was: injection at 50°C, 2 min at 50°C, 40°/min up to 150°C, 1 min at 150°C, 6°/min up to 280°C, 2 min at 280°C. Peak areas were integrated by a Perkin-Elmer Sigma 1 gas chromatographic system.

For GLC analysis CAMEs were transformed into the corresponding trimethylsilyl ethers. Derivatization was performed for 30 min at 70°C in pyridine (99.5%, less than 0.01% water, Merck) using N,N-bis-trimethylsilyltrifluoroacetamide (Macherey-Nagel, Düren, F.R.G.). After cooling to room temperature, the reaction mixture was diluted with *n*-hexane (99.5%, Merck).

Specific correction factors[8] were determined for the CAME standards mentioned above with *n*-eicosane as reference. A standard solution with precisely known concentrations of the four hydroxy fatty acid methyl esters together with the reference standard was prepared in methanol. In the standard mixture, the five compounds were present in approximately equal concentrations. Samples from this mixture were transformed into trimethylsilyl ethers and diluted with *n*-hexane to concentrations of *ca.* 10 and 300 ng/μl, respectively. Usually, 1 μl from each sample was injected. The specific correction factors were found to be independent of concentration in the range investigated.

In calculating the specific correction factors, a factor of 1.000 was assigned to the unknown contaminants in the CAME standard solutions. This leads to an error that is within the limits of accuracy attainable during the determination of correction factors[8].

RESULTS AND DISCUSSION

Diethyl ether–water partition coefficients of CAMEs

Almost all methods for the (trans)esterification of fatty acids require an ex-

traction step that recovers the methyl esters from the reaction mixture. Bannon *et al.*[12] have shown that this step is crucial for the recovery of short-chain esters. The problem is equally severe with CAMEs because this type of compounds strongly differs in polarity due to different numbers of hydroxy groups per molecule.

Partition coefficients are a universal quantitative measure for the distribution of sample components under the conditions of the extraction step[13]. From partition coefficients the losses occurring during the consecutive distribution steps of the work-up can be estimated separately. Thus partition gives a rationale for the selection of optimum conditions for each distribution step. Generally, partition coefficients give more information than correction factors estimated only by running standard compounds through the sample preparation procedure.

Molal partition coefficients K in diethyl ether–water were determined for the methyl esters of the following major cutin acids: 9,16-dihydroxyhexadecanoate, 18-hydroxy-9-octadecenoate, and 9,10,18-trihydroxyoctadecanoate, as well as the chlorohydrin derivatives of 9,10-epoxy-18-hydroxyoctadecanoate, namely 9-chloro-10,18-dihydroxy- and 10-chloro-9,18-dihydroxyoctadecanoate. In the absence of methanol, partition coefficients ranged from 420 to 994 (Fig. 1). The methyl ester of the trihydroxyoctadecanoic acid was the most soluble in the aqueous phase (lowest K), whereas the monosubstituted unsaturated compound had the highest partition coefficient (Fig. 1).

The presence of methanol drastically reduced partition coefficients of CAMEs (Fig. 1), and a striking dependence on polarity was again observed. The greatest

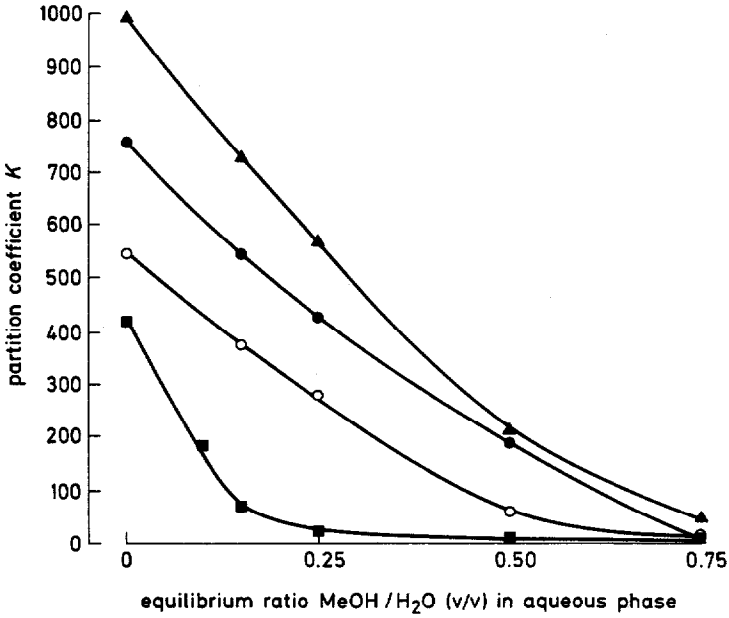

Fig. 1. Effect of methanol on the molal partition coefficients of four major CAMEs in the system diethyl ether–water at 25 ± 2°C. The equilibrium ratio methanol–water in the aqueous phase was calculated using the partition coefficient of methanol in the system diethyl ether–water ($K_{MeOH} \simeq 0.76$), Key: ▲ = methyl 18-hydroxy-9-octadecenoate; ● = methyl 9-chloro-10,18- and 10-chloro-9,18-dihydroxyoctadecanoate; ○ = methyl 9,16-dihydroxyhexadecanoate; ■ = methyl 9,10,18-trihydroxyoctadecanoate.

effect of methanol on K was found with methyl 9,10,18-trihydroxyoctadecanoate, whereas the distribution of methyl 18-hydroxy-9-octadecenoate was much less affected. As the yield of the diethyl ether extraction of CAMEs from water–methanol mixtures strongly depended on both the polarity of the cutin acids and the concentration of methanol, a correction for differential extraction as well as a standardized extraction procedure with constant volume and methanol content are mandatory.

The losses during extraction can be quantitatively estimated from K and the volumes of the organic and the aqueous phases. For one partitioning step, the amount of a substance lost relative to the amount intitially present is given by eqn. 2:

$$\frac{m_w}{m_o} = \left(\frac{\rho_w}{\rho_o} \cdot \frac{K}{\beta} + 1\right)^{-1} \tag{2}$$

where m_w and m_o are the amount lost into the aqueous phase and the amount initially dissolved in the organic phase, respectively, β is the phase ratio defined by the quotient of the volumes of the aqueous and the organic phase, and ρ_w and ρ_o are the specific masses of water (w) and the organic solvent (o) under the experimental conditions. For the diethyl ether–water system at 25°C this term can be substituted by 1.4. Eqn. 2 shows that losses during a partitioning step may be due to a low K and/or a high phase ratio β.

The substitution of the K values of Fig. 1 into eqn. 2 yields losses from 0.3% (methyl 9,10,18-trihydroxyoctadecanoate) to 0.1% (methyl 18-hydroxy-9-octadecenoate) assuming equal volumes of aqueous and diethyl ether phases ($\beta = 1$). A ten-fold excess of water ($\beta = 10$) would lead to losses from 3.2 to 1.4%.

The solutions of CAME mixtures obtained by boron trifluoride or base-catalysed methanolysis necessarily contain some methanol. The presence of methanol lowers the partition coefficients of CAMEs (Fig. 1). This effect can be attributed to an increased solubility of sample components in the aqueous phase since it is correlated with the polarity of the compound studied. This differential susceptibility to the effect of methanol can lead to pronounced discriminations between the methyl esters of higher substituted hydroxy fatty acids during partitioning.

By substitution into eqn. 2 of the appropriate values of K for an equilibrium methanol content of 25% in the aqueous phase (taken from Fig. 1) and a phase ratio $\beta = 1$, losses ranging from 5% (methyl 9,10,18-trihydroxyoctadecanoate) to 0.2% (methyl 18-hydroxy-9-octadecenoate) can be estimated for one distribution step. The selective loss of higher substituted CAMEs will be aggravated by repeated extractions. Thus a critical evaluation of the reductive depolymerization procedure using lithium aluminium hydride, which leads to alcohols[3] considerably more polar than the methyl esters used in this study, seems to be necessary.

Correction factors for losses due to partitioning

The errors caused by the selective loss of the more polar components during the preparation of CAME samples cannot be easily corrected for. Internal standards, although widely used in cutin analysis, are unsuitable for the detection and correction of discriminations occurring during partitioning steps. Correction factors can be estimated, however, in two ways: (1) from partition coefficients K and phase ratios β

TABLE I

LOSS OF METHYL 9,10,18-TRIHYDROXYOCTADECANOATE DURING SAMPLE WORK-UP

Step	*Methanol/water**	K_j**	*Loss (%)*
Extraction	0.154	70	1.57
First wash	0.103	182	0.76
Second wash	0.034	350	0.40
Third wash	0.011	420	0.33
Total loss			3.03

* Equilibrium ratio of methanol in the aqueous phase.
** Molal partition coefficient for the *j*th step.

or (2) by running standard CAMEs through the sensitive steps of the work-up procedure. In both cases cutin acid standards must be available and a standardized sample work-up has to be followed.

The factor f_c correcting for the loss of material from the diethyl ether phase is given by eqn. 3:

$$f_c = 1.4 \frac{\beta}{K} + 1 \tag{3}$$

where β is again the phase ratio. For n subsequent partitioning steps, where both β and K may vary, an overall correction factor can be estimated according to eqn. 4:

$$f_c = (1.4 \frac{\beta_j}{K_j} + 1)\,(1.4 \frac{\beta_k}{K_k} + 1) \ldots (1.4 \frac{\beta_n}{K_n} + 1) \tag{4}$$

where β_j and K_j are the phase ratio and the partition coefficient for the *j*th step, respectively.

The losses of methyl 9,10,18-trihydroxyoctadecanoate that occur in the standard procedure described in this paper were calculated from eqn. 2. The estimated total loss is 3.03% of the amount initially present (Table I). This compares well with the losses measured by running ^{14}C-labelled methyl 9,10,18-trihydroxyoctadecanoate through the same steps of the work-up: here a total loss of 3.73% (95% confidence interval: 2.70 to 4.76) was determined.

An analysis of the different partitioning steps of the work-up according to eqn. 2 shows that the largest loss takes place in the extraction step, where the methanol concentration is highest (Table I). The amount of boron trifluoride–methanol was thus kept as small as possible in the standard method. Owing to considerably higher partition coefficients, lower losses can be estimated for the remaining three CAME standards: for methyl 9,16-dihydroxyhexadecanoate a total loss of 1.15% has to be expected. Less than 1% of the initially present amounts of methyl 18-hydroxy-9-octadecenoate, methyl 9-chloro-10,18-dihydroxy- and methyl 10-chloro-9,18-dihydroxyoctadecanoate should be lost if the standard method is followed.

Drying of diethyl ether solutions of CAMEs

The methyl esters of hydroxy fatty acids as obtained by methanolysis of plant cutins usually are analysed as their corresponding trimethylsiloxy derivatives. Silylation must be performed under anhydrous conditions in order to be quantitative. The diethyl ether solution of CAMEs remaining after the last wash with water, however, is not water-free.

Diethyl ether saturated with water at 25°C was found to contain 0.9% (v/v) of water. The presence of methanol in diethyl ether–water increased the water content of the diethyl ether phase (Fig. 2). For instance, 25% of methanol in the aqueous phase resulted in an increase of the water content of the diethyl ether phase by a factor of 3 to *ca.* 3%.

Desiccants often used for drying diethyl ether differ widely in their effectiveness (Fig. 3). Sodium sulphate which is frequently used, was found to perform very poorly: the addition of 200 mg to 1 ml of diethyl ether saturated with water reduced the water content after 30 min only by 22% (final water concentrations, 0.7%). Under the same conditions, calcium chloride and copper sulphate produced final water con-

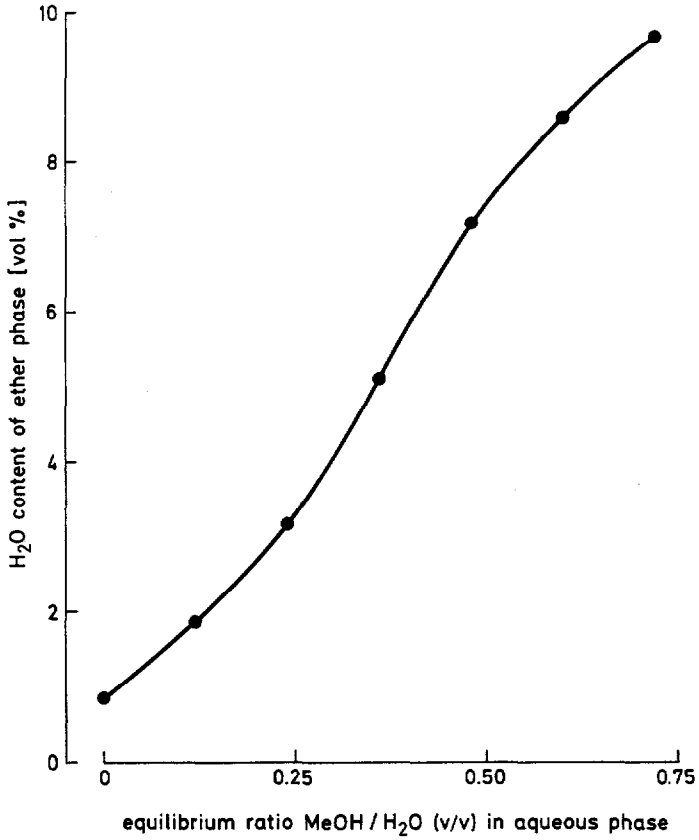

Fig. 2. Water content of the diethyl ether phase in the system diethyl ether–water at 25 ± 2°C as a function of the methanol concentration in the aqueous phase. The equilibrium ratio methanol/water in the aqueous phase was calculated using the diethyl ether–water partition coefficient of methanol ($K_{MeOH} \simeq 0.76$).

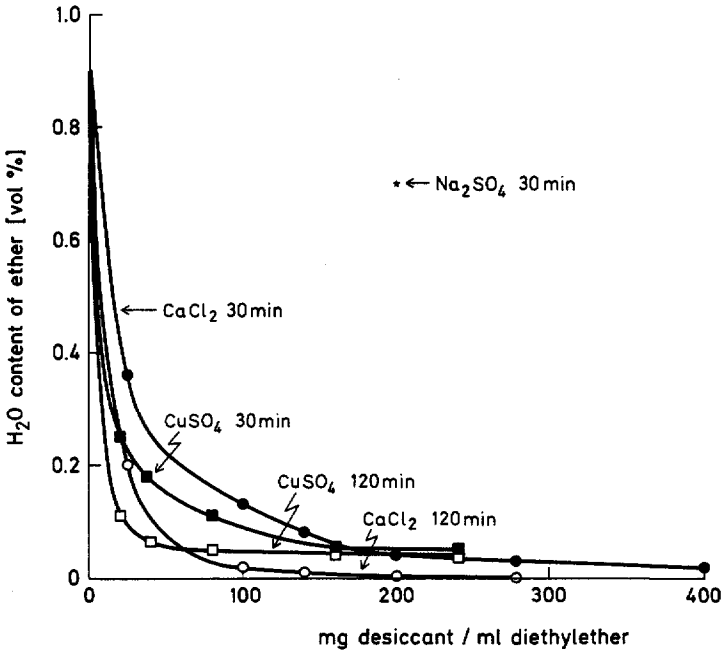

Fig. 3. The effectiveness of desiccants as a function of the amount added to diethyl ether saturated with water and the duration of treatment at 25 ± 2°C. The residual water content of diethyl ether was determined from the concentration of ^3H-labelled water remaining in the supernatant.

tents of *ca.* 0.05% (Fig. 3). When diethyl ether was exposed to the desiccant for 120 min, final water contents of 0.05% could be obtained by adding only 60 mg of calcium chloride or copper sulphate to 1 ml of diethyl ether saturated with water. Considerably larger amounts of desiccant would be needed to dry the diethyl ether phases resulting from the work-up of CAME samples since increased amounts of water would have to be removed owing to the presence of methanol. Diethyl ether in equilibrium with an aqueous phase containing 10% methanol would hold about twice as much water as a system free of methanol.

The desiccants conventionally used are unsuitable if a fast and quantitative removal of water from diethyl ether is desirable: more than *ca.* 200 mg of desiccant per millilitre of diethyl ether would be necessary. Such large amounts of desiccant, however, would occupy up to 10% of the solvent volume and would considerably reduce the recovery of CAMEs owing to porosity. The effectiveness of drying could be enhanced by repeated use of fresh desiccant. This procedure, however, would lead to a further reduction in the yield of CAMEs. Additionally, selective adsorption of sample components by these desiccants may lead to specific discrimination.

2,2-Dimethoxypropane was found to dry diethyl ether quantitatively during short periods of time. The addition of stoichiometric amounts to diethyl ether saturated with water proved to be sufficient to produce residual water contents less than 0.004% within 15 min. This reagent removes water by a chemical reaction that leads to acetone and methanol. The reaction is endothermic and can be accelerated by heating. The vapour pressures of both the reaction products and 2,2-dimethoxy-

TABLE II

SPECIFIC GLC CORRECTION FACTORS (f_i) FOR CAMEs

Methyl ester of*	f_i
18-Hydroxy-9-octadecenoate	1.066
9,16-Dihydroxyhexadecanoate	1.069
9-Chloro-10,18-dihydroxyoctadecanoate**	1.157
9,10,18-Trihydroxyoctadecanoate	1.232

* Chromatographed as the corresponding trimethylsiloxy derivatives.
** Together with 10-chloro-9,18-dihydroxyoctadecanoate.

propane are high enough for them to be easily removed under a stream of nitrogen blown into the reaction vessel. No effect on the qualitative and quantitative composition of standard CAME samples could be detected when 2,2-dimethoxypropane was used as drying agent.

Correction factors for GLC analysis

The factors correcting for the specific differences in the sensitivity of the GLC system used were determined for the trimethylsiloxy derivatives of the four CAME standards also employed in the experiments described above. After silylation, these were methyl 9,16-bis-trimethylsiloxyhexadecanoate, methyl 9-chloro-10,18-bis-trimethylsiloxy- and methyl 10-chloro-9,18-bis-trimethylsiloxyoctadecanoate, methyl 18-trimethylsiloxy-9-octadecenoate and methyl 9,10,18-tris-trimethylsiloxyoctadecanoate. The specific correction factors related to *n*-eicosane had values between 1.066 and 1.232 (Table II). They were significantly different from 1.000, and differences between compounds were also statistically significant. The coefficients of variation for the means from five replications ranged from 0.5 to 1.3%.

The results show that specific differences in the sensitivity of the chromatographic system may be a further source of systematic error during the analysis of CAMEs. The need for correction has been stressed repeatedly for the analysis of fatty acid methyl esters[14–16] and generally for all other compounds[7,8].

TABLE III

COMPOSITION OF *FICUS* LEAF CUTIN DEPOLYMERIZATE

Monomer	*No correction (μg)**	*f***	*Corrected (μg)**
9,16-Dihydroxyhexadecanoate	59.4	1.079	64.1
18-Hydroxy-9-octadecenoate	35.3	1.066	37.6
9,10,18-Trihydroxyoctadecanoate	47.5	1.271	60.4
9,10-Epoxy-18-hydroxy-octadecanoate	168.0	1.157	194.4

* Amount recovered by boron trifluoride–methanol treatment of 1 mg of extracted *Ficus* leaf cuticle.
** Overall correction factor including correction for losses during sample work-up and differential GLC response.

CONCLUSION

Our evaluation of the procedure for the preparation of CAME samples revealed systematic errors at several steps that cannot be avoided by experimental modifications. They lead to erroneous results as far as the absolute amounts of several monomers are concerned. Correction is possible, however, by means of an overall correction factor that takes into account both the losses during sample work-up and the differential response of GLC. An overall correction factor has to be determined for each compound. This factor is calculated as the product of the factors correcting for partitioning loss (from eqn. 4 or from running a standard through the work-up procedure) and for GLC analysis (Table II). Without correction, the actual amounts of the four major CAMEs investigated here would be underestimated by 6.2–21.3% (Table III).

All the data on the composition of plant cutins published so far are uncorrected for either losses during work-up or the differential response of the GLC system. They are only qualitative and thus are of limited usefulness as to a deeper understanding of the structure of the biopolymer cutin.

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