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Note

Gas–liquid chromatographic method for the rapid analysis of the epicuticular wax composition of plants

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Detailed analysis of plant epicuticular waxes is usually carried out by gas–liquid chromatography (GLC) of the wax lipid classes (fatty acid, alcohol, aldehyde, ketol, ketone, ester, hydrocarbon, etc.) separated by thin-layer (TLC)[1–3] or column chromatography[4,5]. GLC procedures, however, are complicated by the need for lengthy derivatization processes (for example, alcohols acetylated[4] or trimethylsilylated[6] overnight, esters transmethylated[4] for up to 48 h, aldehydes reduced to alcohols and acetylated overnight[1]), and the analysis of the different derivatives may require different GLC conditions[1,2,4].

The analysis described in this paper [and illustrated using epicuticular wax of corn (*Zea mays* L.)] is based on the convenient GLC determination of fatty alcohol trifluoroacetate esters obtained by the rapid and simple derivatization of components of different wax classes. These derivatizations have not yet been used in wax analyses, and involve 4-dimethylaminopyridine (DAP)-catalysed acylation[7] (alcohols), reduction with lithium aluminium hydride (LAH)[8] followed by a novel, direct acylation of the lithium aluminate product with carboxylic acid anhydride (fatty acids, aldehydes, ketones, ketols, esters) and transmethylation with tetramethylammonium hydroxide (TMAH) reagent[9] (esters).

EXPERIMENTAL

Wax extraction and separation of wax lipid classes

Corn plants (hybrid JX-SC 92, Martonvásár, Hungary) were grown in sand culture[10]. Epicuticular wax of 14-day-old plants was extracted by dipping the leaves (5 g) in chloroform (50 ml) for 20 sec. Wax lipid classes were separated by TLC on silica gel using benzene as eluent and their amounts determined by gravimetry as described elsewhere[2]. Hydrocarbons were taken up in 0.2 ml of *n*-hexane, alcohols in 0.5 ml of tetrahydrofuran (THF) and other classes in 0.5 ml of toluene.

GLC

GLC was carried out using a Chrom 4 gas chromatograph (Laboratorni Pristroje, Prague, Czechoslovakia) with a flame-ionization detector, attached to a Digint 60 μ integrator (Chinoin, Budapest, Hungary). The glass column (2.5 m × 2.6 mm

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I.D.) was packed with 80–90-mesh Diatomite C AW (Pye Unicam, Cambridge, U.K.) coated with 3% SE-30 (Analabs, Karlsruhe, F.R.G.). The column temperature was programmed from 220 to 280°C at 6°C/min and the injector and detector temperatures were 290°C. The carrier gas (nitrogen) flow-rate was 46 ml/min.

Unknown compounds were identified by comparing the retention times with those of known standards. Calibration graphs were constructed using C_{21}, C_{23}, C_{27} and C_{31} *n*-alkanes, C_{22}, C_{24}, C_{28} and C_{30} primary alcohols and C_{18}, C_{20}, C_{24} and C_{28} fatty acid methyl esters (Analabs, North Haven, CT, U.S.A.). Peak areas in the gas chromatograms were converted into mass units by assuming a uniform response factor within a wax derivative class. Each analysis was repeated five times.

Derivatization for GLC

Hydrocarbons were analysed directly (1–2 μl samples taken for GLC).

To the fatty alcohol fraction 10 μl of DAP (EGA-Chemie, Steinheim, F.R.G.) solution (5%, w/v, in THF) and 20 μl of trifluoroacetic anhydride (TFA) (EGA-Chemie) were added. Esterification was complete in 2 min and 1–2-μl samples were taken for GLC.

To the fatty acid, aldehyde and ester fractions were added 20-μl aliquots of LAH solution [1 *M* in THF, or 1 *M* LAH bis (THF) in toluene; EGA-Chemie], the mixture was kept at 100–105°C for 5 min, cooled to room temperature and treated with 20 μl of TFA. After 2 min the solvent was evaporated under a stream of nitrogen and the residue taken up in 0.5 ml of THF; 1–2 μl samples were taken for GLC.

Alternatively, after TLC, wax esters were taken up in 0.5 ml of THF and treated with 10 μl of TMAH solution (20%, w/v, in methanol; EGA-Chemie). After complete transesterification (2 min) the solvent was evaporated under a stream of nitrogen and the residue partitioned between 1 ml of benzene and 1 ml of water. The organic phase was layered on a Kieselgel 60 mini-column (1 g in a 5 mm I.D. column; Merck, Darmstadt, F.R.G.). Fatty acid methyl esters were eluted with 5 ml of benzene and fatty alcohols with 5 ml of THF. The solvents were evaporated and the residues taken up in 0.2 ml of THF. Fatty acid methyl esters were analysed directly and alcohols after DAP-catalysed trifluoroacetylation as above. Samples of 1–2 μl were taken for analysis.

RESULTS

Under the above conditions the retention times of C_{18}–C_{32} hydrocarbons, trifluoroacetate esters of C_{18}–C_{32} alcohols and methyl esters of C_{18}–C_{32} fatty acids were 1.0–14.5, 2.0–17.6 and 2.5–18.5 min, respectively. To illustrate the GLC separation of fatty alcohol trifluoroacetate ester derivatives, a profile of the wax ester fraction of corn leaf after reduction–trifluoroacetylation is shown in Fig. 1.

Replacement of TFA with acetic anhydride in the above derivatization processes led to *ca.* 50% higher retention times and slower formation of the derivatives. Acetylation of fatty alcohols and their lithium aluminates was complete in 5 and 15 min (2 min for the latter reaction at 100–105°C), respectively.

We found that corn leaf wax consists of [wax class (percent of total wax ± S.D.), main homologue (percent of the wax class ± S.D.)]: alcohol (59.4 ± 5.1), C_{32} (96.6 ± 1.5); aldehyde (19.5 ± 1.8), C_{32} (81.5 ± 2.4); ester (12.8 ± 2.1), C_{24} (23.5

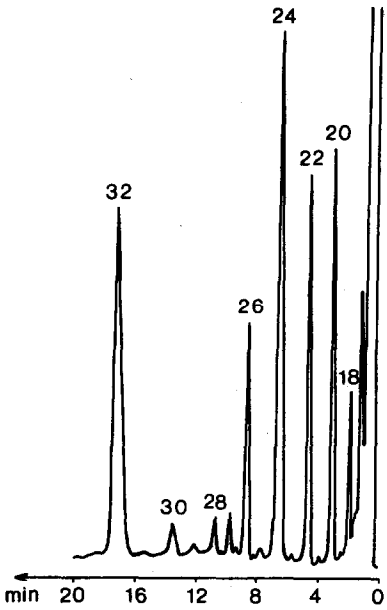

Fig. 1. Gas chromatographic profile of fatty alcohol trifluoroacetates obtained from corn leaf wax esters by reduction–trifluoroacetylation. The numbers above the peaks represent the chain lengths of the alcohol portions. For conditions see Experimental.

± 1.1) for its fatty acid portion and C_{32} (54.1 ± 1.8) for the fatty alcohol portion; fatty acid (4.8 ± 1.2), C_{24} (28.2 ± 1.8); and hydrocarbon (3.5 ± 0.9), C_{31} (42.0 ± 3.6). These values are in good agreement with literature data[1,4] for corn inbred WF9: alcohol (53.1), C_{32} (96.8); aldehyde (20.4), C_{32} (83.6); ester (23.3), C_{24} (27.1) for its fatty acid portion and C_{32} (53.4) for the fatty alcohol portion; fatty acid (not published), C_{24} (23.4); and hydrocarbon (9.5), C_{31} (42.0).

Preliminary experiments with cabbage leaf wax (extracted and separated by TLC as described)[2] indicate that our methods are not restricted to corn leaf wax classes. Secondary alcohols were analysed by GLC as above after DAP-catalysed trifluoroacetylation, and ketones and ketols after LAH reduction–trifluoroacetylation.

DISCUSSION

The method presented here for the analysis of plant epicuticular waxes has several advantages over those published in the literature. It is rapid and, as common derivatives are formed from different wax classes, derivatization and GLC are very simple. Moreover, a smaller number of reagents and standards is needed and quantitation is easier (signals from components of the same carbon chain are directly comparable). The method allows the rapid determination of the fatty acid and alcohol composition of esters by GLC–mass spectrometry, using lithium aluminium deuteride in the reduction step, and deuteration as the basis to distinguish between derivatives formed from the acyl and alkyl portions.

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