Journal of Chromatography, 174 (1979) 234–238
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CHROM. 11,793

Note

Interference by phytol derivatives in the gas chromatographic analysis of fatty acids in the lipids of plant shoots

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(First received December 28th, 1978; revised manuscript received February 13th, 1979)

In the course of the isolation of lipids from photosynthetic tissue of plant shoots, appreciable amounts of non-lipid substances, such as chlorophyll and other pigments, are often coextracted with the lipids by organic solvents.

It has been widely recognized that the coextraction of chlorophyll with lipids is a serious and unsolved problem in the gas–liquid chromatographic (GLC) determination of fatty acids (FAs). Such coextractives are responsible for the occurrence of unassigned peaks in the GLC of FA methyl esters prepared by heating with anhydrous methanol in the presence of an acidic catalyst.

Usually, FA methyl esters of leaf lipids are separated by thin-layer chromatography (TLC) before GLC analysis[1]. However, some workers have reported the determination of the FA composition of leaf lipids by GLC without such a pretreatment[2–6]. In some of these reports, the assignment of peaks to certain FAs seems unreliable. As such unavoidable errors are found in FA compositions calculated from GLC peak area if the pre-treatment is not carried out, Farkas *et al.*[7] excluded short-chain FAs (from myristic acid downwards) in the calculation of the FA composition of leaf lipids.

This paper reports the elucidation of the precursors of unassigned peaks and the identification of these compounds.

EXPERIMENTAL

The shoot sample analysed was red clover. Phytol was obtained commercially and was purified by column chromatography over silicic acid (Mallinckrodt, St. Louis, Mo., U.S.A.; 100 mesh) with *n*-hexane–diethyl ether (50:50) as eluent. Pure chlorophyll was obtained from a lipid extract of the red clover shoot, and was separated on a silicic acid column with *n*-hexane–diethyl ether (80:20) as eluent.

^{1}H Nuclear magnetic resonance (NMR) spectra were recorded on a JEOL JNM-MN-60 (60-MHz) spectrometer with tetramethylsilane as the internal standard. Combined gas chromatography–mass spectrometry (GC–MS) was carried out on a JEOL-JMS-06 instrument operating at 75 eV. Infrared (IR) and ultraviolet (UV) spectra were recorded on JASCO IR-S and Hitachi–Perkin-Elmer 139 spectrophotometers, respectively. GLC analyses were performed using a Hitachi 063 instrument equipped with dual flame-ionization detectors and a stainless-steel column (2 m × 3

mm I.D.) containing 8% DEGS on 80–100-mesh Diasolid L at 180°. TLC separations were carried out on 20 × 20 cm TLC plates coated with silica gel G and developed with *n*-hexane–diethyl ether (80:20).

Extraction of shoot lipids and preparation of samples

The lipids of red clover shoot were extracted by homogenizing the plant material in chloroform–methanol (2:1). The lipids were transesterified with 3% HCl–, 2% H_2SO_4– and 14% BF_3–methanol at 80° for 2 h in a PTFE-lined screw-capped tube. Chlorophyll and phytol were treated with the same reagents. Saponification was carried out with an excess of 5% KOH–methanol at 80° for 3 h. Hydrogenation was carried out in methanol with a palladium black catalyst at atmospheric pressure.

RESULTS AND DISCUSSION

Origin of unassigned peaks

Fig. 1 shows the chromatograms of methyl ester preparations of total lipids obtained by treatment with HCl–methanol and of its unsaponifiable compounds. Fig. 2 showes the chromatograms of phytol derivatives obtained by treatment with HCl–methanol of phytol and of its hydrogenated compounds. Eleven peaks (a–k) were obtained and correspond well with those from the unsaponifiable compounds. No compounds other than phytol derivatives was detected in the GLC of the compounds formed from chlorophyll by treatment with HCl–methanol. The treatment of phytol with both H_2SO_4–methanol and BF_3–methanol gave the same chromatograms as did the HCl–methanol treatment.

By hydrogenation of the phytol derivatives, three main compounds were formed, as shown in Fig. 2. Fig. 3 shows the TLC of phytol derivatives and other related compounds. Phytol, with an R_F value of *ca.* 0.15, disappeared completely and

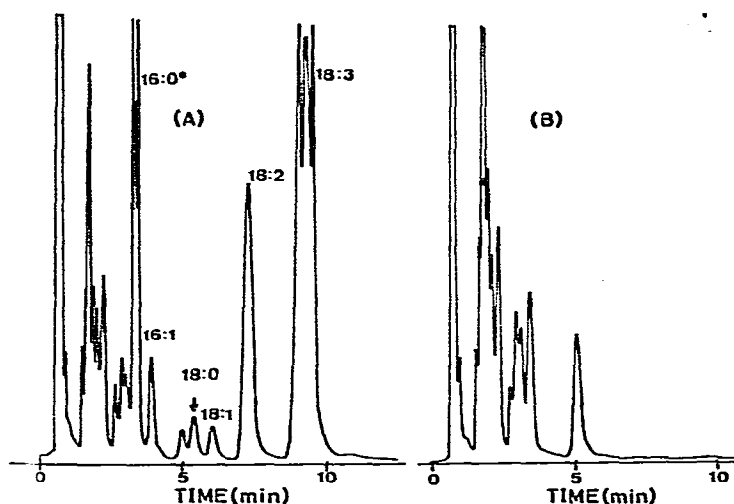

Fig. 1. GLC of methyl ester prepared from (A) the total lipid and (B) its unsaponifiable compounds in red clover shoot. Abbreviations for fatty acids, representing carbon chain length:number of double bonds.

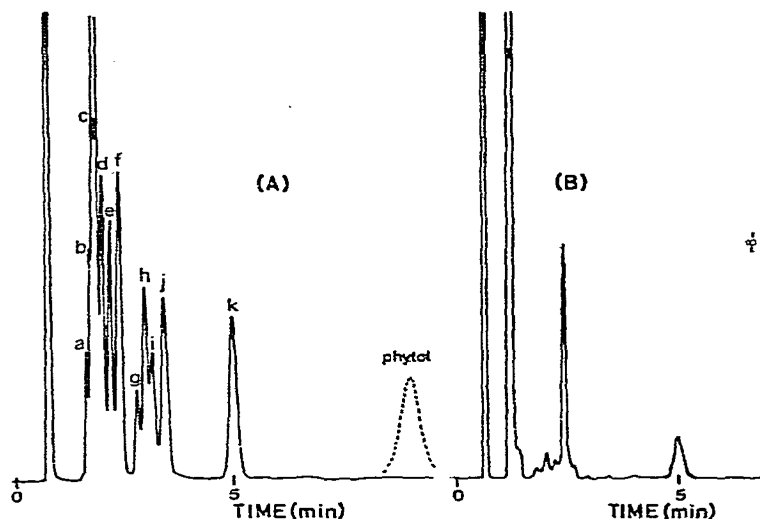

Fig. 2. GLC of (A) phytol derivatives and (B) its hydrogenated compounds. Peaks a–k as in Table I.

four new compounds (A–D) were formed, although C and D were not separated clearly from the methyl esters on TLC.

Table I gives GLC and TLC data for the phytol derivatives. The equivalent chain lengths (ECL) of phytol derivatives relative to straight-chain saturated FA methyl esters are also given. The number of GLC peaks is identical with the number of TLC spots.

Identification of phytol derivatives

Each phytol derivative in TLC spots A–D was extracted and analysed by GC–MS, ^{1}H NMR, UV and IR spectroscopy.

Compound A, which gives five GLC peaks, was identified as phytadiene and its isomers (*m/e* 278). The ^{1}H NMR spectrum showed that no exchangeable proton existed in the molecule, and the multiplet assigned to the proton of the diene occurred

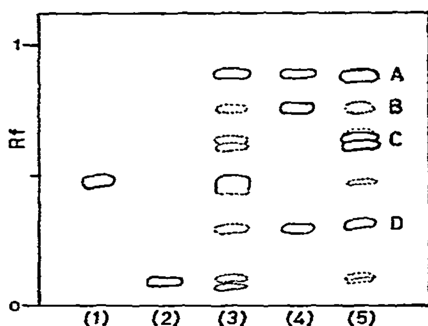

Fig. 3. TLC of phytol derivatives and related compounds. (1) Methyl linolate; (2) phytol; (3) methyl ester prepared from the total lipid of red clover shoot; (4) hydrogenated compounds of phytol derivatives; (5) phytol derivatives. A–D: Spots of phytol derivatives as in Table I. Solvent: *n*-hexane–diethyl ether (80:20).

TABLE I

EQUIVALENT CHAIN LENGTHS OF PHYTOL DERIVATIVES

Equivalent chain lengths relative to saturated fatty acid methyl esters.

GLC peak	*ECL*	*TLC spot*
a	12.16	A
b	13.00	A
c	13.05	A
d	13.40	A
e	14.00	B
f	14.45	B
g	15.00	C
h	15.32	C
i	15.60	C
j	15.95	C
k	17.75	D

at 5.0–5.8 ppm. In UV spectrometry (methanol), the λ_{max} value was 238 nm. The only other characteristic absorption observed in the IR spectrum was that of alkene, at 1665 and 1550 cm^{-1}. On hydrogenation, the derivatized compound gave GLC peak and showed a molecular ion at *m/e* 282. No UV absorption was observed at 220–250 nm. These results identify the hydrogenated derivative as phytan. It is concluded that the phytadienes were formed by dehydration of phytol.

Compounds B and C were 3-methyl and 1-methyl phytyl ether, respectively (both *m/e* 310). ^1H NMR spectroscopy of B ($CDCl_3$) gave δ = 1.50 (3H, s, $-O-\overset{|}{\underset{|}{C}}-CH_3$), 3.11 (2–3H, s, $-O-CH_3$), 5.1–5.7 (3H, m, $-CH{=}CH_2$). IR spectrometry of B (neat) gave 1105 cm^{-1} (ether). ^1H NMR spectroscopy of C ($CDCl_3$) gave δ = 1.62 (3H, s, $-CH = \underset{|}{C}CH_3$), 2.05 (2H, m, triplet pattern, $-CH{=}\underset{|}{C}CH_2-$), 3.30 (2–3H, s, $-O-CH_3$), 3.88 (2H, d, J = 6 Hz, $=CH-CH_2-O-$), 5.30 (1 H, m, triplet pattern, $=CH-$). IR spectrometry of C (neat) gave 1065 cm^{-1} (ether). On hydrogenation, both B and C gave one GLC peak and one TLC spot. It is clear that these compounds were formed by methylation of phytol and isophytol in HCl–methanol.

Compound D, which gave GLC peak k, was identified as 1,3-dimethyl phytyl ether (*m/e* 342). ^1H NMR spectroscopy of D ($CDCl_3$) gave δ = 1.12 (3H, s, $-O-\overset{|}{\underset{|}{C}}CH_3$), 1.75 (2H, t, J = 7 Hz, $-CH_2CH_2-O-$), 3.13 (3H, s, $-O-CH_3$), 3.30 (3H, s, $-CH_3$), 3.41 (2H, t, J = 7 Hz, $-CH_2CH_2-O-$). IR spectrometry of D (neat) gave 1085 cm^{-1} (ether) and 1120 cm^{-1} (ether).

From the above results, it is concluded that these compounds derived from chlorophyll and phytol may lead to the ambiguous assignment of peaks to FA in the GLC analysis of shoot lipids with the acid–methanol treatment. Phytol is also contained as a component of wax[8–10] and in the free state[9] in leaf lipids.

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