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Deuteration as an aid to the high-temperature gas chromatography–mass spectrometry of steryl fatty acyl esters

Richard P. Evershed*, Mark C. Prescott and L. John Goad

Department of Biochemistry, University of Liverpool, P.O. Box 147, Liverpool L69 3BX (UK)

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ABSTRACT

A method is presented for reducing degradative losses of steryl fatty acyl esters bearing polyunsaturated fatty acyl moieties during high-temperature gas chromatography (GC) and combined high-temperature GC–mass spectrometry (MS). The method employs selective deuteration of the double bonds in the fatty acyl moiety using a homogeneous catalyst (Wilkinson's catalyst). The Δ^5 double bond, which occurs in the most commonly occurring plant and animal sterols, is not deuterated under the conditions described. In addition to improving GC behaviour, the method has the advantage of preserving structure information by labelling each double bond present in the original unsaturated fatty acyl moiety. The carbon number and degree of unsaturation of the labelled fatty acyl moiety are readily revealed by combined GC–MS employing negative-ion ammonia chemical ionisation. Two applications of the method are demonstrated in the analysis of steryl fatty acyl esters isolated from a rape seed oil and ovary tissue of the marine prawn *Penaeus monodon*.

INTRODUCTION

Flexible fused-silica capillary columns coated with various high-temperature stable stationary phases are finding increasing use in the separation of high-molecular-weight acyl lipids, such as triacylglycerols, steryl esters and wax esters [1]. Although many impressive separations have been demonstrated, a number of workers have observed poor recoveries of certain high-molecular-weight and polyunsaturated compounds during gas chromatographic (GC) analyses [1–5]. Losses of apolar high-molecular-weight triacylglycerols have been attributed to their reversible saturation in the apolar immobilised dimethyl polysiloxane stationary phase employed for analysis [2]. Although this effect may also account for losses of polyunsaturated components, thermal decomposition or polymerisation may be a more important factor [1].

When performing high-temperature GC analyses of plasma steryl esters using a polar SP 2330 capillary column Kuksis *et al.* [3] observed a reduced abundance of steryl esters bearing an arachidonate (C20:4) moiety. Mareš [1] has also reported substantial losses of cholesteryl arachidonate and other polyenoic cholesteryl esters on capillary columns coated with a polarisable stationary phase. In our own work with authentic compounds, chromatographed on an immobilised dimethyl polysiloxane stationary phase, we observed only 40% recovery of cholesteryl arachidonate relative to cholesteryl palmitate [5]. Catalytic hydrogenation of this mixture, resulting in conversion of the cholesteryl arachidonate to cholesteryl arachidate, followed by GC, showed the similar recoveries of the palmitate and arachidate esters. Thus, the selective loss of ester that was occurring was due to the presence of the polyunsaturated arachidonate moiety. The wide oc-

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currence of highly unsaturated acyl moieties, particularly in marine organisms, would appear to limit the usefulness of high-temperature GC and GC–mass spectrometry (MS) in accurate steryl ester analyses.

In this paper we describe a technique for improving recoveries of steryl esters bearing polyunsaturated fatty acyl moieties during high-temperature GC and GC–MS analyses. The technique involves selective catalytic reduction of double bonds in unsaturated fatty acyl moieties using deuterium gas and Wilkinson's catalyst [tris(triphenylphosphine)–rhodium(I) chloride]. The resulting steryl esters display enhanced recoveries in GC analyses, with structure information (carbon number and degree of unsaturation) preserved through stable isotope labelling at the positions of the original double bonds in the fatty acyl moieties.

EXPERIMENTAL

Samples

The authentic compounds listed in Table I were purchased from Sigma. Rapeseed oil steryl fatty acyl esters were isolated by column chromatography (Al_2O_3; Brockmann, Grade III) of the whole oil eluting with hexane, followed by 2% diethyl ether in hexane [6]. The latter fraction containing the steryl fatty acyl esters was further purified by preparative thin-layer chromatography (TLC; 20 × 20 cm; 0.5 mm silica gel layer). Elution with 2% (v/v) diethyl ether in cyclohexane and visualisation under UV light after spraying with berberine (0.005% in ethanol) revealed a band eluting adjacent to authentic cholesteryl oleate (R_F 0.35). Steryl fatty acyl esters of the ovaries of the marine prawn, *Penaeus monodon*, were obtained as above by preparative TLC of the hexane extract of freeze-dried ovary tissue [7].

Catalytic reductions

Heterogeneous catalyst. Deuterium reduction was carried out by adding catalytic amounts of platinum on activated carbon (Aldrich) to 100 μg–1 mg amounts of steryl fatty acyl esters dissolved in ethyl acetate (100 μl) in screw-capped vials (5 ml). Each of the vials was purged with deuterium gas (99.95%; Air Products) tightly capped, and allowed to stand at room temperature (2 h).

Homogeneous catalyst. Catalytic reductions using Wilkinson's catalyst (Aldrich Chemical) were based on the method used by Dickens *et al.* [8] to deuterate diacylglycerols released enzymatically from phospholipids. Reactions were again performed in screw-capped vials (5 ml) by heating (60°C, 2 h) steryl fatty acyl esters (100 μl to 1 mg), dissolved in 1,4-dioxane (300 μl) in the presence of Wilkinson's catalyst (0.5 mg) in the presence of deuterium gas.

Sample purification. The products of reductions using both heterogeneous and homogeneous catalysts were purified by TLC (SiO_2, 2% (v/v) diethyl ether in cyclohexane; R_F 0.35) prior to GC–MS analysis. Visualisation was by UV illumination following spraying with berberine as described above.

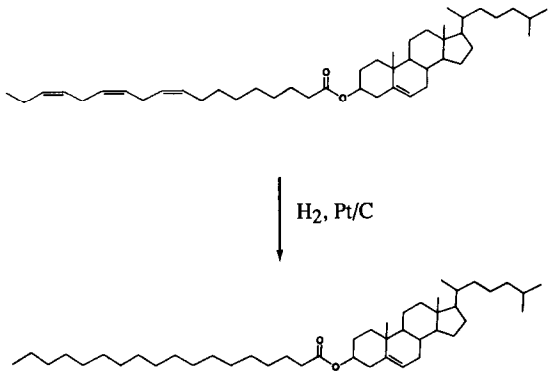
High-temperature GC–MS

The GC–MS instrumentation comprised a Pye 204 GC linked to a VG7070H double-focussing magnetic sector mass spectrometer *via* an interface oven modified for high-temperature operation (> 300°C) [9,10]. Samples were dissolved in hexane for injection *via* an SGE OCI III on-column injector at a GC oven temperature of 50°C. A 0.5-m "retention gap" was used to protect the analytical column and eliminate undesirable injection phenomena [11]. The analytical column was an SGE 12 m × 0.22 mm I.D. aluminium clad BP-1 coated capillary (immobilised dimethyl polysiloxane; 0.1 μm film thickness) connected directly into the MS ion source [12]. Temperature programming of the GC oven temperature was from 50 to 330°C at 8°C min^{-1}.

The mass spectrometer was operated in the full scan mode at an accelerating voltage of 4 kV. In analyses employing electron ionisation (EI; 70 eV) the m/z range was scanned from 40 to 700 in a total cycle time of 3.25 s. When conducting negative ion chemical ionisation (NICI) using ammonia as reagent gas [9,10,13] the m/z range was scanned from 150 to 450 in a total cycle time of 2.75 s. Instrument operation, data acquisition and processing were under the control of a Finnigan INCOS 2300 data system.

RESULTS AND DISCUSSION

The aim of this work was to develop a technique for improving the GC behaviour of intact steryl fat-



Scheme 1.

ty acyl esters bearing polyunsaturated acyl moieties that would also allow structure investigations to be performed by mass spectrometry. The approach which was investigated involved: (i) Catalytic reduction of the double bonds in the fatty acyl moieties in order to enhance thermal stability of the intact steryl esters in high-temperature GC–MS analyses. (ii) Labelling the double bond with deuterium in order that the carbon number and degree of unsaturation of the original polyunsaturated fatty acyl moiety could be deduced by GC–MS employing NICI using ammonia as reagent gas [9,10,13].

Heterogeneous catalyst

Hydrogenation, using heterogeneous catalysts, has been used previously to enhance recoveries of

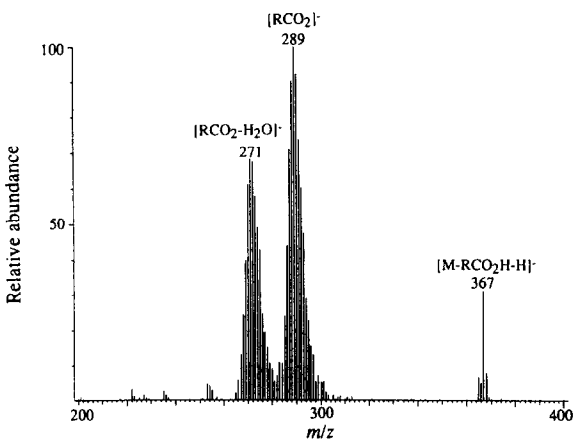


Fig. 1. NICI mass spectrum of cholesteryl linolenate reduced with deuterium gas in the presence of platinum on activated charcoal.

polyunsaturated lipids in GC and GC–MS analyses. The recovery of steryl ester in GC analyses is greatly improved in the case of cholesteryl linolenate, following its catalytic reduction to cholesteryl arachidate using hydrogen and a heterogeneous catalyst, such as platinum on activated carbon. Although this latter technique is useful for revealing the presence of polyunsaturated lipid species which display poor GC performance, all structural information concerning the degree of unsaturation of the original unsaturated fatty acyl moiety is lost (Scheme 1). Simply replacing hydrogen with deuterium gas in the catalytic reduction when using a heterogeneous catalyst is ineffective, as the addition of deuterium atoms to the double bonds in alkyl compounds, in the presence of this type of catalyst is not discrete but rather non-specific with hydrogen exchange for deuterium at other methylene groups. This lack of specificity in the catalytic reduction is clearly revealled by the ammonia NICI mass spectrum shown in Fig. 1 recorded for cholesteryl linolenate reduced with $^2H_2$ over Pt/C. The mass spectrum shows two complex envelopes of fragment ion peaks centering on *m/z* 289 and 271, respectively. The former envelope represents a cluster of ions corresponding to the $[RCO_2]^-$ moiety while the group at *m/z* arise from $[RCO_2-H_2O]^-$ and $[RCO_2-H_2O-H]^-$ ions bearing varying numbers of deuterium atoms in the fatty acyl moiety. This lack of specificity in the labelling precludes any deductions concerning the degree of unsaturation in an unknown compound, particularly if a mixture of compounds is present. The ion at *m/z* 367 corresponds to $[M-RCO_2H-H]^-$, (*i.e.* the steryl moiety) and the lack of deuterium and consequent mass change shows that the Δ^5 double bond in the sterol moiety was not catalytically reduced under the reaction conditions employed.

Homogeneous catalyst

Homogeneous catalysts are preferred for more specific labelling of double bonds by catalytic deuterium reduction. Among the catalysts that were considered, bis(triphenylphosphine)rhodium(I) chloride (Wilkinson's catalyst) was favoured on account of its ease of use and previously demonstrated ability to catalyse the deuteration of unsaturated diacylglycerols with a high degree of specificity [8].

The somewhat simplified approach adopted in

TABLE I

MASS SPECTRA OF AUTHENTIC CHOLESTERYL FATTY ACYL ESTERS OBTAINED BY GC–MS WITH NICI BEFORE AND AFTER TREATMENT WITH DEUTERIUM GAS IN THE PRESENCE OF WILKINSON'S CATALYST

Compounds	Characteristic fragment ions *m/z* (relative abundance)	
	Before	After
Saturated		
Cholesteryl myristate (14:0)[a]	367(10), 227(65), 209(100)	367(10), 227(60), 209(100)
Cholesteryl palmitate (16:0)	367(9), 255(65), 237(100)	367(13), 255(63), 237(100)
Cholesteryl stearate (18:0)	367(15), 283(71), 263(100)	367(11), 283(58), 263(100)
Unsaturated		
Cholesteryl palmitoleate (16:1)	367(23), 253(59) 235(100)	367(15), 257(60), 239(100)
Cholesteryl oleate (18:1)	367(27), 281(58), 263(100)	367(14), 285(54), 267(100)
Cholesteryl linoleate (18:2)	367(21), 279(38), 261(100)	367(31) 287(48), 269(100)
Cholesteryl linolenate (18:3)	367(28), 277(47), 259(100)	367(21), 289(43), 271(100)

[a] (Carbon number/number of double bonds) in the fatty acyl moiety.

this investigation was very economical in terms of the amounts of deuterium gas required for each reaction. Preliminary investigations with the commercially available authentic cholesteryl esters showed a high degree of specificity was attainable in deuterium reductions performed using Wilkinson's catalyst according to the conditions described in the Experimental. Table I summarises the results obtained for the range of authentic compounds tested. Fig. 2 shows the NICI spectrum for cholesteryl linolenate reduced with deuterium gas in the presence of Wilkinson's catalyst. The absence of excessive clustering in the $[RCO_2]^-$, and $[RCO_2-H_2O]^-$ and $[RCO_2-H_2O-H]^-$ ion regions confirms that a high degree of specificity was attained in the reduction. It can be assumed from this that each double bond in the fatty acyl moiety has been labelled with two deuterium atoms and minimal exchange has occurred between deuterium and hydrogen atoms at other sites in the molecule (Scheme 2). The *m/z* val-

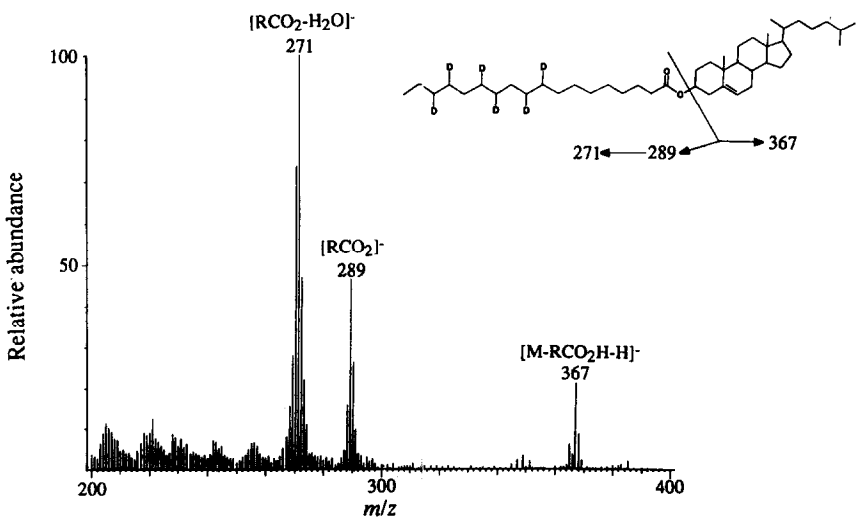
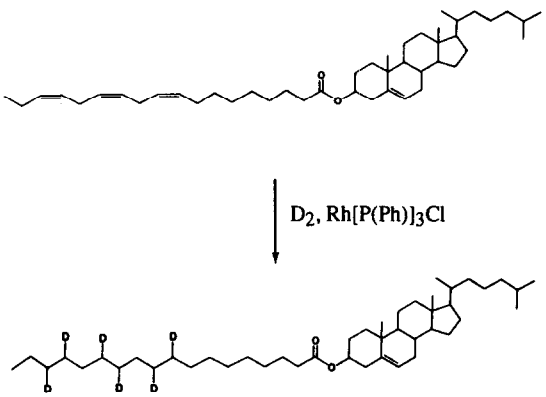


Fig. 2. NICI mass spectrum of cholesteryl linolenate reduced with deuterium gas in the presence of Wilkinson's catalyst.



Scheme 2.

ues of the $[RCO_2]^-$ and $[RCO_2-H_2O]^-$ ions, 289 and 271, respectively, determined from the NICI spectrum allow confident assignment of the carbon number and degree of unsaturation of the fatty acyl moiety in the case of an unknown compound. The ion at *m/z* 367 corresponds to $[M-RCO_2H-H]^-$ and shows that the Δ^5 double bond in the sterol moiety is not reduced under the reaction conditions employed; this is confirmed by the EI spectrum shown in Fig. 3. The EI spectrum shown in Fig. 3 is typical for this class of compounds and shows the paucity of ions containing information concerning the fatty acyl moiety [13–15]. Table I also shows NICI data for cholesteryl esters bearing saturated fatty acyl moieties, which again confirms that deuterium exchange does not occur at other sites in the steryl ester molecule in the presence of Wilkinson's catalyst.

Analysis of biological extracts

Rape seed oil steryl esters. Application of the deuteration technique is demonstrated first through the analysis of steryl fatty acyl esters from rape seed oil. Previous work in this laboratory [6] has shown that the steryl fatty acyl esters of this sample of rape seed oil are composed principally of sitosteryl esters of oleate (C18:1), linoleate (C18:2) and linolenate (C18:3). A partial total ion current (TIC) chromatogram resulting from the GC–MS analysis using NICI of rape seed oil steryl esters, following deuterium reduction is shown in Fig. 4. The mass spectrum for the major peak in the chromatogram is shown in Fig. 5. The mass spectrum confirmed that the reduction had proceeded to completion. The $[RCO_2]^-$ ions at *m/z* 285, 287 and 289, separated by 2 a.m.u. increments, (see inset partial mass spectrum in Fig. 5) corresponds to the fully deuterium-reduced oleate, linoleate, and linolenate, respectively. The presence of an ion at *m/z* 395 corresponds to $[M-RCO_2H-H]^-$ and confirms that sitosterol is the major sterol moiety associated with each of these acyl groups and it has not been reduced. The oleate, linoleate and linolenate content of these sitosteryl esters deduced from the deuterated products is in accord with the amounts observed in our

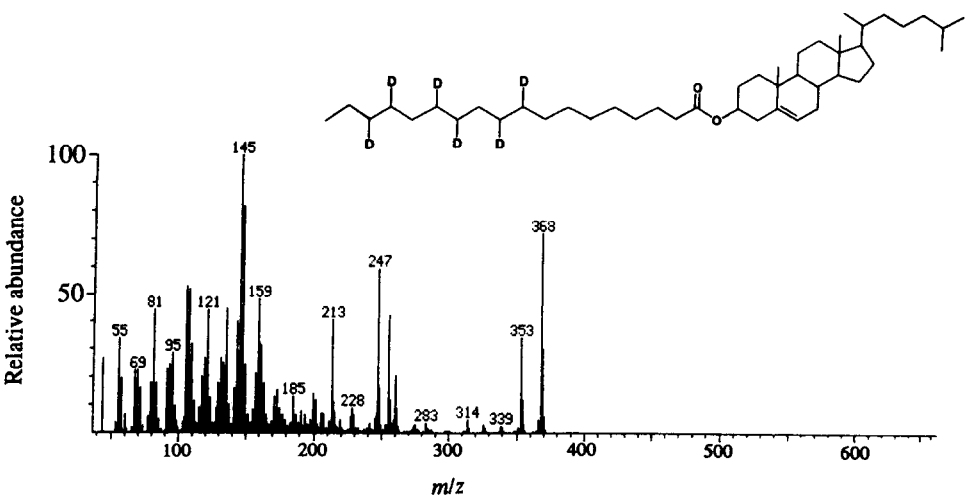


Fig. 3. EI (70 eV) mass spectrum of cholesteryl linolenate reduced with deuterium gas in the presence of Wilkinson's catalyst.

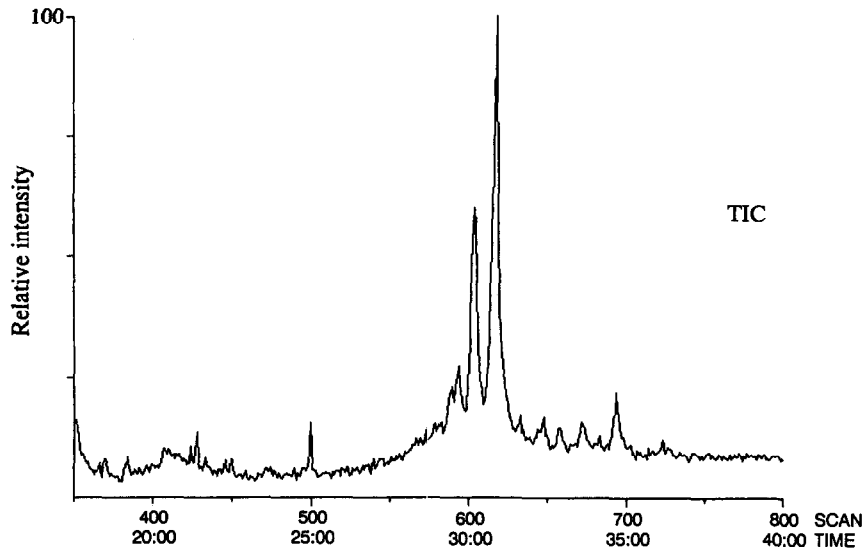


Fig. 4. TIC chromatogram for rape seed oil steryl fatty acyl esters reduced with deuterium gas using Wilkinson's catalyst det capillary GC–MS using NICI.

earlier studies [6]. Interpretation of the relative proportions of the fatty acyl moieties from the $[RCO_2 - H_2O]^-$ ions is complicated by the clustering that results from the attendant $[RCO_2 - H_2O - H]^-$ ions.

Penaeus monodon ovary steryl fatty ac
Application of this technique to the analys steryl fatty acyl ester mixture from the ova marine prawn, *P. monodon* provides an ex the examination of a sample in which poc

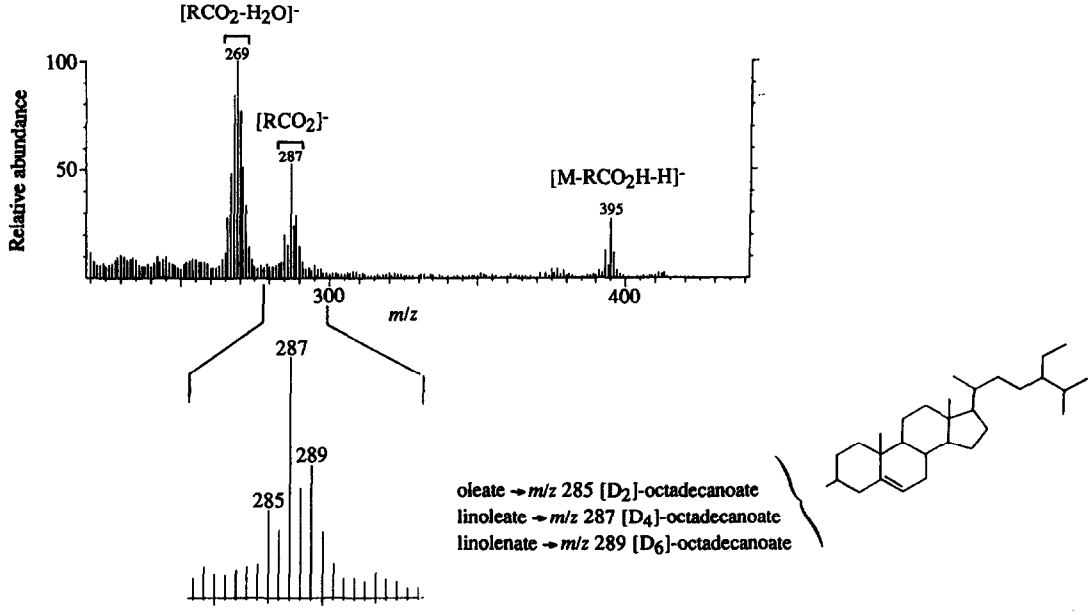


Fig. 5. NICI mass spectrum of the major peak in the TIC of the deuterated rape seed oil steryl fatty acyl esters shown in

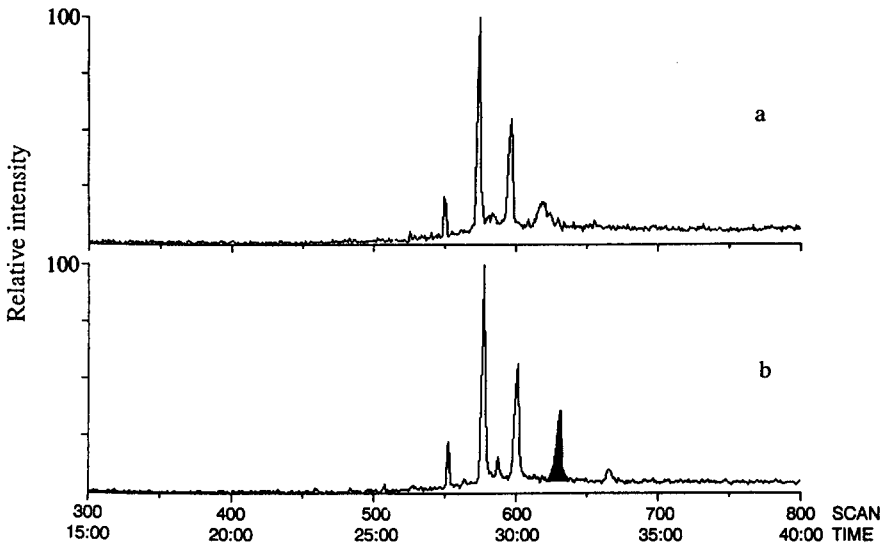


Fig. 6. TIC chromatograms obtained by GC–MS with NICI of the steryl fatty acyl esters from prawn ovaries (a) before and (b) after deuterium reduction using Wilkinson's catalyst.

eries were observed in high-temperature GC and GC–MS analyses. The partial TIC chromatogram for the intact steryl esters recorded before catalytic reduction, is shown in Fig. 6a. The chromatogram shows 3 well-shaped peaks eluting between scans 550 and 600, and a single much broader peak eluting at *ca.* scan 615. The TIC chromatogram shown in Fig. 6b was recorded after deuterium reduction using Wilkinson's catalyst. In addition to the three well-shaped peaks eluting between scans 550 and 600 two further later eluting peaks became clearly evident in the chromatogram. The inference was that these new peaks corresponded to steryl esters bearing polyunsaturated fatty acids that were being

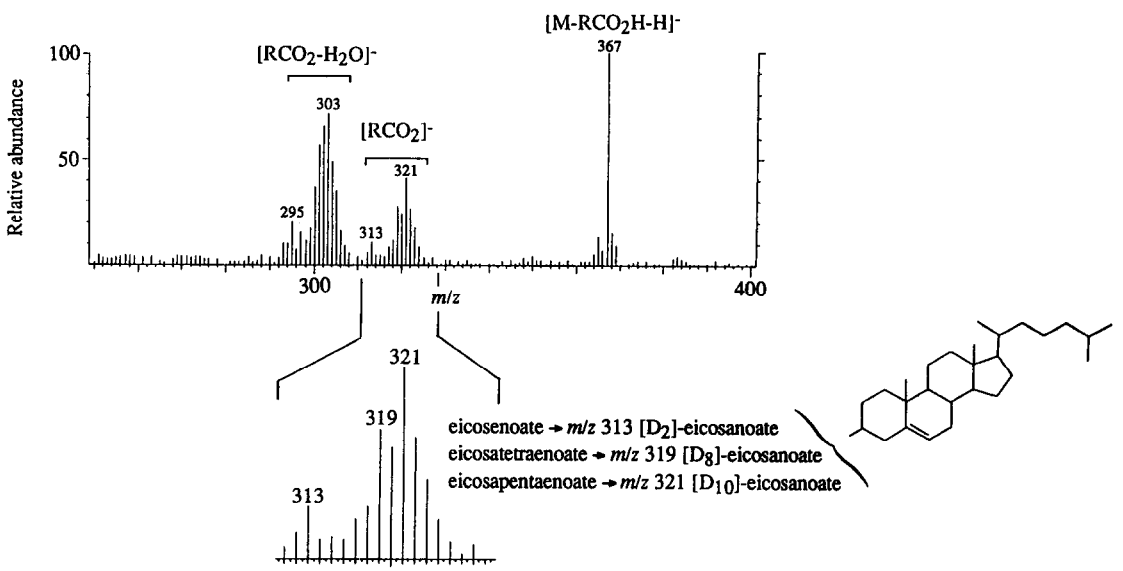


Fig. 7. NICI mass spectrum of the highlighted peak in the TIC shown in Fig. 6b.

lost through thermolysis during the analysis represented by the chromatogram shown in Fig. 6a. Subsequent deuterium reduction gave steryl esters bearing fully saturated fatty acyl moieties which exhibit the greatly improved GC behaviour, as shown in Fig. 6b. Examination of the NICI mass spectra of these later eluting peaks confirmed this interpretation. For example, the NICI mass spectrum of the highlighted peak in Fig. 6b is shown in Fig. 7. The complex cluster of the $[RCO_2]^-$ ion can be readily interpreted to reveal the nature of the original fatty acyl moieties present. The ion at *m/z* 367 corresponds to $[M-RCO_2H-H_2O]^-$, and confirmed that cholesteryl esters predominated. The cluster of $[RCO_2]^-$ ions indicates that this peak contains principally cholesteryl esters bearing twenty carbon fatty acyl moieties. As shown in Fig. 7 the fragment ions at *m/z* 313, 319 and 321 correspond respectively to $[RCO_2]^-$ ions resulting from the deuterium reduction of eicosenoate, eicosatetraenoate and eicosapentaenoate moieties in cholesteryl esters. The more complex clustering at lower mass is the result of overlap of the $[RCO_2-H_2O]^-$ and $[RCO_2-H_2O-H]^-$ ions resulting from these compounds. However, the ions present do corroborate the deductions made on the basis of the $[RCO_2]^-$ ions. The greatly improved chromatographic behaviour of the steryl esters bearing deuterated fatty acyl moieties, compared to that of their polyunsaturated precursors, clearly illustrates the usefulness of this technique in high temperature GC–MS analyses of steryl ester mixtures.

CONCLUSIONS

In summary, we have shown that deuterium reduction, using a homogeneous catalyst (Wilkinson's catalyst), of the widely occurring Δ^5-steryl esters bearing polyunsaturated fatty acyl moieties affords: (i) improved GC recovery through reduction of only those double bonds associated with the fatty acyl moiety; (ii) retention of structure information content in NICI spectra owing to the specificity of the reduction achieved using Wilkinson's catalyst. In addition to improving GC behaviour, the reduction and labelling provides a convenient means of preserving compounds that are particularly sensitive to autoxidation for structure investigation at a later date.

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