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Journal of Chromatography A, 760 (1997) 309–313

JOURNAL OF CHROMATOGRAPHY A

Short communication

Rapid procedure for the isolation and analysis of fatty acid and fatty alcohol fractions from wax esters of marine zooplankton

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Received 18 June 1996; revised 11 September 1996; accepted 11 September 1996

Abstract

A rapid and simple method for the isolation of fatty acid methyl esters and fatty alcohols from the lipid fraction of marine zooplankton is described. Wax esters are the dominant lipid class in most calanoid copepods and *trans*-esterification results in a high fatty alcohol content in the analytical extract. Current procedures for the separation and purification of lipid classes by preparative thin-layer chromatography are time-consuming and are subject to low recovery of the analytes. In this method, fatty acid methyl esters and fatty alcohols were separated by liquid chromatography using silica or bonded amino-silica as the stationary phase. The procedure is equally applicable to the analysis of zooplankton with low wax ester (and hence fatty alcohol) content, for example, a number of species of euphausiid and, generally, for samples of low mass.

Keywords: Marine zooplankton; Fatty acid methyl esters; Fatty alcohols; Lipids

1. Introduction

The lipid fractions of many marine zooplankton are dominated by wax esters [1]. These are compounds composed of long chain fatty acids esterified to long chain fatty alcohols. In calanoid copepods, the lipids are composed predominantly of wax esters [2]; up to 90% of the total lipid in some species from polar regions [3]. In general, the major group of lipids in plants and animals is the triacylglycerols, which are accumulated and stored as metabolic energy reserves. This is also the role of wax esters in the majority of calanoid copepods. Exceptionally, there are a small number of polar species with low levels of wax ester, for example, *Calanus propinquus* [4] and *C. simillimus* [5], which appear to utilise triacylglycerols for energy storage. Other zooplankton, such as a number of species of euphausiid [6], generally have low proportions of wax ester in their lipid. The distributions of fatty acids and alcohols derived from these wax esters are significant dietary and trophic markers in amphipods, copepods and euphausiids from the Antarctic [7,8] and from temperate regions [9–11]. Hence the importance of the analysis of these compounds when investigating carbon pathways and interactions of zooplankton in oceanic ecosystems.

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PII S0021-9673(96)00780-7

The analysis of wax esters as a lipid class has been well documented [12]. The current procedure for the isolation of fatty acids and alcohols entails homogenisation and solvent extraction, which is usually a variation on the procedure of Bligh and Dyer [13]. This is followed by preparative thin-layer chromatography and derivatisation. Achieving a good recovery of the analytes with this procedure is inherently difficult. This is particularly important when analysing zooplankton, such as *Euphausia vallentini*, *E. lucens* and *Thysanoessa gregaria*, which have a low wax ester content [6] and low mass samples such as a certain copepodite stages that may form only a small proportion of the total population. For example, copepodite stages CI and CII in winter [14] or stages CI, CII and CIII of smaller species such as *C. simillimus* in spring [15]. Thus, there is a requirement for a quick and reliable method with a good recovery of the analytes.

Kattner and Fricke [16] have described an efficient and simple procedure for the simultaneous determination of fatty acids and alcohols from the wax esters of marine copepods by gas–liquid chromatography. Whilst this method was entirely applicable to the samples these workers were investigating, there are some situations where it would be less appropriate. For example, calanoid copepods with a high wax ester content will produce a high proportion of fatty alcohols during the preparation of the fatty acid methyl esters (FAMEs). On analysing the resulting chromatography data, it may be found that the response of the alcohol components of the analytical mixture has been obscured by that of the FAMEs. The converse may occur for animals with low wax ester content. The minor (nevertheless important) components will be particularly susceptible. The following procedure was devised with special attention given to these problems.

The use of proprietary solid-phase extraction (SPE) cartridges has also been assessed. Joh et al. [17] recently used Bond-Elut® NH_2 SPE cartridges (Analytichem International) to separate fatty acid methyl esters and fatty alcohols in roe oil prior to silver ion high-performance liguid chromatography and Van Horne [18] has described a procedure for isolating cholesterol from serum and tissue. Method (b) (see Section 2.2) was developed from the latter of these two.

2. Experimental

2.1. Extraction

Samples of the copepods, *Rhincalanus gigas* and *Calanus simillimus*, from the Southern Atlantic Ocean in the region of the Sub-Antarctic island of South Georgia, were used to demonstrate this procedure. The lipid extraction and *trans*-esterification followed well-established procedures [12]. Briefly, animals that had been stored at −60°C were homogenised in a chloroform–methanol (2:1, v/v) mixture. The resulting liquors were filtered and the chloroform layer was separated by the addition of 1 *M* potassium chloride (AnalaR, BDH). The chloroform extract was dried over anhydrous sodium sulphate (AnalaR, BDH) and evaporated to dryness under a stream of nitrogen. *n*-Nonadecanoic acid and *n*-heptanol were added as internal standards to the lipid residue, which was redissolved in 10% hydrochloric acid in methanol–chloroform (9:1, v/v) for *trans*-esterification and refluxed for 3 h. The product was partitioned into hexane, dried over anhydrous sodium sulphate and concentrated to ca. 250 μl under a stream of nitrogen.

2.2. Isolation of fatty acid methyl esters and fatty alcohols

The FAME and fatty alcohol fractions were isolated by liquid chromatography on either (a) 5% deactivated silica (silica gel 60, Merck) or (b) an Isolute® NH_2 SPE cartridge (Jones Chromatography).

Method (a): the total hexane extract (≈100 μl) was loaded onto a glass column (10×0.5 cm) that was filled with 5% deactivated silica (0.6 g). Nonpolar aliphatic hydrocarbons were eluted with hexane (3 ml), followed by FAMEs eluted with pentane–dichloromethane (2:1, v/v; 6 ml) and fatty alcohols with pentane–methanol (2:1, v/v; 6 ml).

Method (b): the total hexane extract was loaded onto a cartridge (a 6-ml syringe barrel) containing 0.5 g of Isolute NH_2. The column was then eluted with hexane (2 ml), to remove non-polar components that may interfere with the alcohol fraction, which was eluted with hexane–ethyl acetate (9:1, v/v; 2 ml). The FAME fraction was then eluted with

pentane–dichlorormethane (2:1, v/v; 2 ml). The separation of FAMEs and fatty alcohols was checked by high-performance thin-layer chromatography (HPTLC) following the method by Olsen and Henderson [19].

The individual fractions were evaporated to dryness under vacuum and redissolved in hexane prior to analysis by capillary gas chromatography. FAMEs and fatty alcohols were quantified against a suite of known standards and the methyl esters of cod liver oil (Sigma) on a HP-Innowax® wall-coated open tubular capillary column (30 m×0.32 mm; film thickness, 0.5 μm). The column was installed in a Hewlett-Packard 5890A gas chromatograph with splitless injection and flame ionisation detection at 200°C and 220°C, respectively. The temperature programme was as follows: after an initial time of 1 min at 40°C, from 40 to 150°C at 55°C min^{-1}, then to 220°C at 1.75°C min^{-1} and held at 220°C for a further 15 min.

3. Results and discussion

Application of the above procedure gave good recovery (>90%) and separation of the FAMEs and fatty alcohols from the *trans*-esterified product of the lipids from marine zooplankton (N.B. the recovery for the complete procedure including the extraction and *trans*-esterification was typically 75–80%). Separation of a mixture of *n*-nonadecanoic acid and *n*-heptanol gave fractions that were 96.8 and 98.3% pure, respectively. Figs. 1 and 2 illustrate the separation of these fractions from the lipid extracts of adult stages of copepods *R. gigas* and *C. simillimus*, respectively. Table 1 lists typical concentrations of individual FAMEs and fatty alcohols in *R. gigas* and *C. simillimus*. The high proportion of fatty alcohol in the lipid extract of *R. gigas* can be seen in Fig. 1a, with the $C_{14:0}$ and $C_{16:0}$ alcohols dominating the total lipid fraction. The fatty alcohols have longer retention times than the FAMEs with the same carbon chain length and, if analysed simultaneously, they interfered with the methyl esters of important dietary marker fatty acids. The response of the dominant $C_{16:0}$ alcohol and the less common $C_{16:1}$ alcohol obscured the signal of the methyl ester of the diatom marker $C_{16:4}$ fatty acid. It was also difficult to

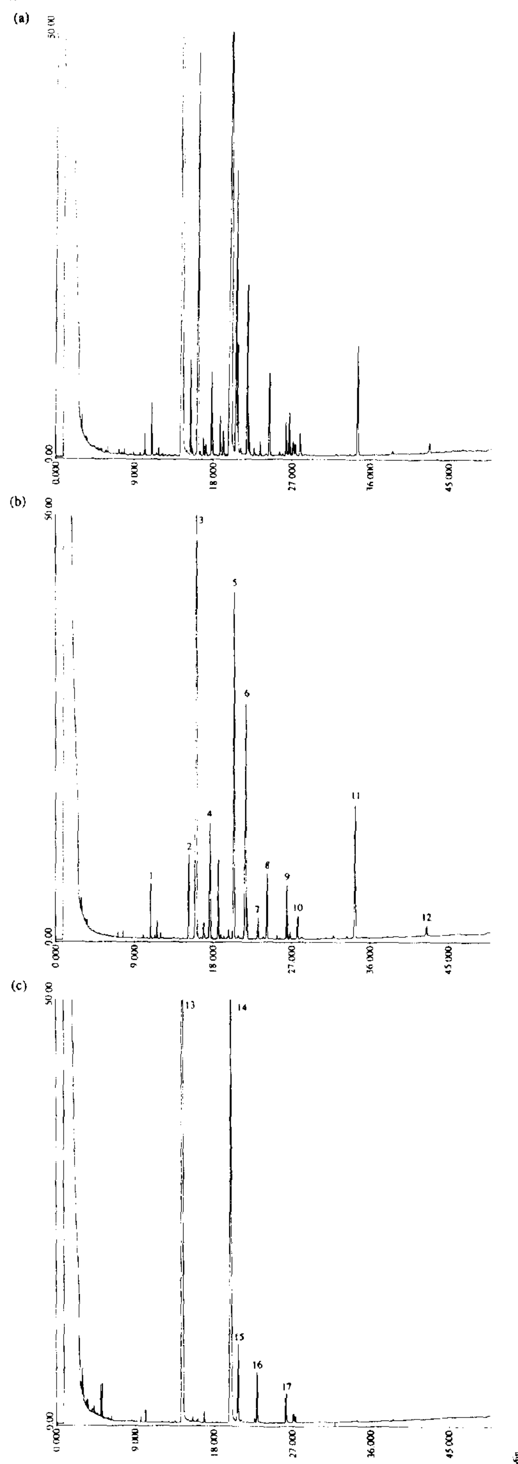

Fig. 1. Chromatograms of (a) the total lipid fraction after *trans*-esterification, (b) the fatty acid methyl esters and (c) the fatty alcohols of *Rhincalanus gigas*.

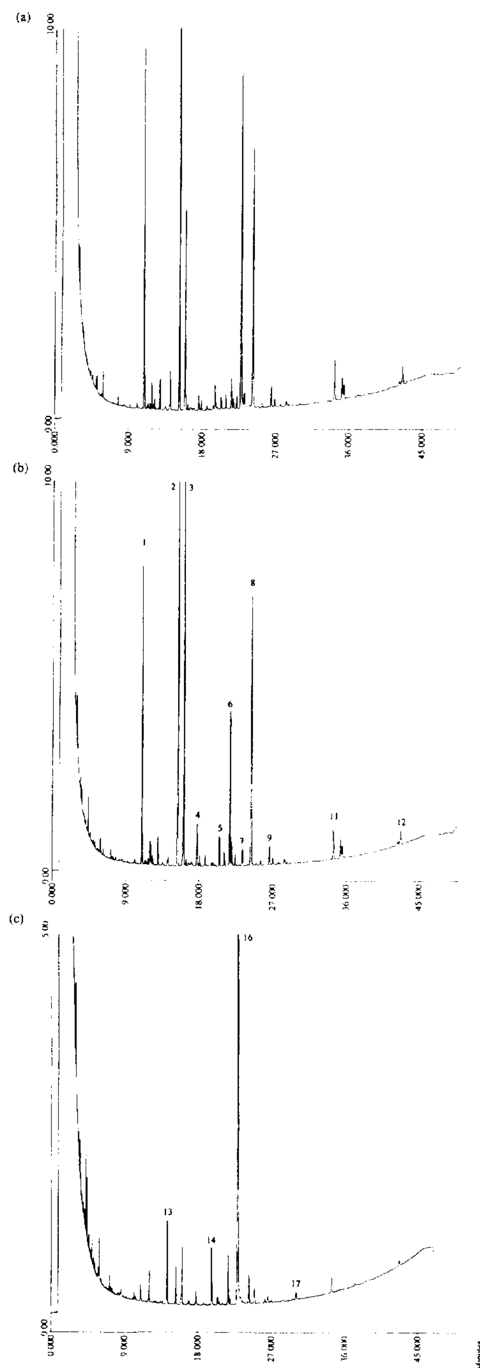

Fig. 2. Chromatograms of (a) the total lipid fraction after *trans*-esterification, (b) the fatty acid methyl esters and (c) the fatty alcohols of *Calanus simillimus*.

Table 1
Compound key to Figs. 1 and 2 and sample analyses of FAMEs and fatty alcohols from the lipids of adult copepods *R. gigas* and *C. simillimus* (μg g^{-1} dry mass)

Figure codes	Compound	*R. gigas* (n=5)	*C. simillimus* (n=15)
FAMEs			
1	14:0	0.44	1.76
2	16:0	2.61	8.24
3	16:1	20.94	6.76
4	16:2	4.67	2.47
5	16:4	13.15	0.55
6	18:1	22.20	6.79
7	18:2	1.30	0.70
8	19:0 (I.S.)		
9	18:4	3.83	0.83
10	20:1	4.85	0.60
11	20:5	23.74	0.76
12	22:6	1.97	0.54
Fatty alcohols			
13	14:0	22.44	0.31
14	16:0	27.00	0.51
15	16:1	2.42	0.07
16	17:0 (I.S.)		
17	18:0	3.1	0.09

The dry mass data is from Ward et al., unpublished results.
I.S.=Internal standard.

resolve smaller components such as the C_{18} alcohols from polyunsaturated C_{18} FAMEs (which are also significant dietary markers) that had similar retention times. The chromatograms demonstrate the complete separation of FAMEs (Fig. 1c) and fatty alcohols (Fig. 1b) using method (a).

In zooplankton with low wax ester content, the fatty alcohols were minor components of the total lipid fraction and it was difficult to identify the fatty alcohols when they were analysed simultaneously with the FAMEs (Fig. 2a). FAMEs and fatty alcohols were effectively separated by method (b) (Fig. 2b,c). There appeared to be a trace breakthrough of long chain FAMEs in the alcohol fraction. The efficiency of commercially produced columns can be slightly less than that of laboratory-tailored columns in which a higher grade stationary phase was employed.

Method (a) produced clean discrete fractions of fatty alcohols and FAMEs without interference from hydrocarbons, phthalate esters and polar lipid components. Sterols are a minor, but commonly observed, component class in lipid extracts of most

biological material and might be expected to elute with the alcohol fraction. The chromatograms in Figs. 1 and 2 clearly demonstrate that this was not the case. It was possible that any sterols present in our zooplankton extracts were not sufficiently volatile to cause interference under the chromatographic conditions used. These advantages outweighed the disadvantages of a relatively long preparation time with re-usable glass chromatography columns. A material with higher purity and a smaller particle size could be used as the stationary phase instead of that normally available in proprietary SPE cartridges. The procedure also confirmed that the analysis of fatty alcohols can be carried out without the requirement to derivatise prior to chromatography (see also Ref. [16]).

In the case of limited facilities, such as when working in the field, method (b), using Isolute NH_2 (only available in cartridges at the time of analysis), may be more appropriate. However it would be necessary to flush the cartridges thoroughly with suitable solvents, such as hexane and methanol, before use, to reduce the occurrence of ghost peaks from plasticisers and baseline noise in the final chromatogram. The method would be equally applicable to the isolation of free alcohols in the lipid fraction (e.g. phytol) prior to *trans*-esterification. Kaluzny et al. [20] have described the use of similar amino-propyl bonded phase SPE cartridges (Bond-Elut) for the rapid separation of lipid classes with high yield and purity. However, despite reporting on a range of lipid classes, free aliphatic alcohols and fatty alcohol originating from wax esters were not included in their work.

The above method represents a significant improvement over the isolation of wax esters by preparative TLC. It is rapid and of low cost and is particularly applicable to samples of low mass.

Acknowledgments

The authors would like to thank Andrew Clarke and Julian Priddle for their helpful comments on the manuscript. G.A. Tarling was supported by a N.E.R.C. grant.

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