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Convenient preparation of picolinyl derivatives from fatty acid esters

It is essential to have simple rapid methods for the determination of fatty acid structures. Traditionally, fatty acids are analysed by gas chromatography using their methyl ester derivatives (FAME). However, their corresponding mass spectra exhibit molecular ions but are usually devoid of ions indicative of structural features and, notably, the position of double bounds on the aliphatic chains [1]. In the most useful approach to structure determination, the carboxyl group is derivatised with a reagent containing a nitrogen atom. Recently, a convenient method for preparing picolinyl esters from intact lipids has been published [2]. However, some problems occurred in our laboratory when this method was used, leading to some modifications and optimisation. Thus, hexane and water have been added while sodium bicarbonate has been removed in order to lower contamination. Temperature and length of the reaction have then been optimised in order to get 100% derivatisation for different kinds of lipids (45 °C and 45 min for FAME). Finally, a comparison of the response factors has confirmed the better sensitivity of the picolinyl derivative against FAME (five times more).

Keywords: Picolinyl esters, mass spectrometry, gas chromatography, fatty acid methyl esters, sterol esters, sensitivity.

1 Introduction

Fatty acids are the basic building blocks of all lipids. They are generally constituted of a linear chain of 16 to 22 carbon atoms, with zero to six double bonds of *cis* or *trans* configuration. The fatty acid components of a lipid determine, to a large extent, its physical and often its biological properties. In addition, fatty acids formed as by-products in industrial or related processes may have biological effects on consumers. Therefore, it is important that we have rapid unequivocal methods for the determination of fatty acid structures [3].

Fatty acids are traditionally analysed as methyl esters (FAME) by gas chromatography (GC), but it is usually considered that such derivatives are not suitable for locating double bonds or other centres of unsaturation [4]. Thus, when FAME are submitted to electron impact mass spectrometry (MS), the charged aliphatic chain breaks up into an indeterminate number of fragments, double bonds become mobile and move up and down the chain, so their original position cannot be determined [5]. However, in some cases, particular ions could be helpful for getting additional information, such as on the fatty acid family. Indeed, the mass spectra of *n*-3 fatty acids present an ion at m/z = 108, which is formed by cleavage between carbons 10 and 11, while *n*-6 fatty acids present an ion at m/z = 150 presumed to be formed from the terminal part of the molecule by cleavage between carbons 7 and 8 [6]. In addition, with FAME the location of methyl branch points and the determination of the type of oxygen (hydroxyl, epoxyl, keto, *etc.*) in the chain is also possible with GC-MS [4].

To obtain useful mass spectra, the carboxyl group is best derivatised with a reagent containing a nitrogen atom. In this way, during molecular ionisation in the mass spectrometer, it is the nitrogen atom rather than the alkyl chain which carries the charge; thus, the double bond ionisation and migration is minimised [3]. Pyrrolidides were the first derivatives commonly used. However, a comparative study of the utility of pyrrolidides and picolinyl esters for the recognition of unsaturated fatty acids in natural mixtures of animal and marine origin has indicated without doubt that picolinyl esters are to be preferred and give spectra from which both the number and the position of double bonds can be deduced [7]. Nowadays, analysts prefer either picolinyl ester or 4,4-dimethyloxazoline (DMOX) derivatives. Both have their merits in MS terms, and each has advantages for particular types of fatty acids; they are best considered as complementary. DMOX derivatives are commonly used because of their ease of preparation and because they have similar GC properties to FAME, so that they are readily analysed on polar columns. Nevertheless, they present some dis-

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advantages such as high temperature and long reaction time during the derivatisation procedure [8]. Moreover, incomplete reaction and appearance of an intermediate that elutes later from the GC columns (and gives a mass spectrum almost identical to that of the required derivative) have sometimes been observed [9]. For these reasons, picolinyl esters are generally preferred over DMOX derivatives for identifying fatty acids.

Until recently, picolinyl esters were prepared from free fatty acids (FFA), so intact lipid or methyl ester samples were first hydrolysed [10]. It has now been shown that picolinyl esters can be easily prepared directly from intact lipids or methyl esters by transesterification with 3-hydroxymethylpyridine and potassium *tert*-butoxide in tetrahydrofuran (THF) [2]. Following this methodology, we have optimised different parameters such as time and temperature of reaction and solvent in use. In addition, we have also determined the sensitivity of this method and the response factors of the resulting derivatives.

2 Materials and methods

2.1 General procedure for picolinyl ester preparation

Lipid standards [monoacylglycerol (MAG), diacylglycerol (DAG), triacylglycerol (TAG), phospholipids, galactosyl-diglyceride (MGDG), digalactosyl-diglyceride (DGDG), sterol esters and FAME] were purchased from Sigma Chemical (St. Louis, MO, USA). Potassium *tert*-butoxide (1.0 M in THF) and 3-hydroxymethylpyridine were purchased from Aldrich Chemicals (Milwaukee, WI, USA). The dichloromethane was dried on anhydrous sodium sulphate for one night before storage on a molecular sieve.

A solution of potassium *tert*-butoxide in THF was added to 3-hydroxymethylpyridine in the proportion 1 : 2 (vol/vol) to form the reagent. After homogenisation, the lipid sample (up to 10 mg) in dry dichloromethane (2 mL) was added to 0.5 mL of reagent, and the mixture was held at 45 °C for 45 min in a closed vial. After cooling at room temperature, water (2 mL) and hexane (4 mL) were added. The unit was then vortexed and the organic phase (the upper one) was collected. Water (2 mL) was added, and the organic phases were collected, dried over anhydrous sodium sulphate and evaporated. The sample was finally dissolved in hexane (1 mL) for GC-MS analysis.

2.2 GC-MS analysis of picolinyl esters

Analysis of the derivatives were performed by GC-MS on a Hewlett-Packard model 6890 series II GC attached to an Agilent model 5973N selective quadrupole mass detector. GC-MS was operated in the electron impact mode at 70 eV and was connected to a computer from Hewlett-Packard. The temperatures of injector and interface were maintained at 250 °C. Separation was achieved with a capillary column CP-Sil 5 CB low bleed MS (60 m × 0.25 mm i.d., 0.25 μm film thickness; Chrompack, Middelburg, The Netherlands) with an oven temperature linearly increasing from 220 to 270 °C at 0.4 °C/min. Helium was employed as the carrier gas at a constant flow rate of 1 mL/min.

All samples tested were prepared and injected in triplicate.

3 Results and discussion

According to the described procedure [2], FAME were derivatised and injected in GC-MS. Surprisingly, despite great care in cleaning reagents and glassware, the resulting total ionic current (TIC) chromatograms were contaminated by non-picolinyl ester compounds (Fig. 1A). These contaminations have been observed for all our standard derivatives. Major peaks were identified as residues of a reagent which should normally be located only in the aqueous phase (characteristic ions of a pyridin ring such as m/z = 92 and 108 were noticed). One hypothesis formulated was that for collecting derivatives, the aqueous phase has to be crossed (the organic phase containing the derivatives, *i.e.* dichloromethane, is the lower one) which can lead to small contamination. The other suggestion was that dichloromethane may solubilise these contaminants.

These problems were resolved by using hexane and water instead of sodium bicarbonate aqueous solution. Following the initial derivatisation procedure (lipid extract, 3-hydroxymethylpyridine and potassium *tert*-butoxide), hexane (4 mL) and water (2 mL) were added after cooling to room temperature. The solution was then vortexed and the upper organic phase was collected, dried on anhydrous sodium sulphate and injected. The resulting chromatograms were all devoid of such contaminations (Fig. 1B). With this protocol there is no more any risk of pollution by the aqueous phase (hexane is the upper phase) and the possibility of solubilisation of contaminants is reduced due to the lower polarity of hexane.

However, even with this improved procedure, non-picolinyl derivatives were observed. Their analysis revealed that they were FAME that were not completely derivatised. Thus, the reaction was carried out with increasing time (from 2 to 60 min) and increasing temperature (room temperature to 45 °C). Higher temperatures was not assayed in order to avoid artefact formation [2]. The best

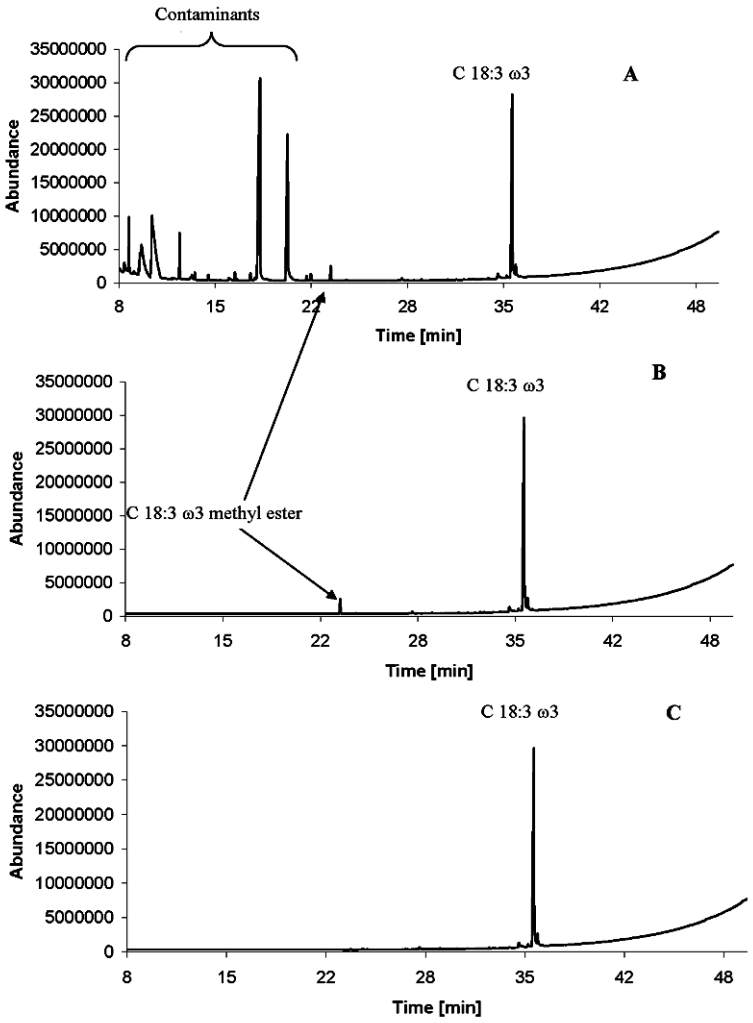

Fig. 1. TIC of picolinyl ester derivative of *cis*-9,*cis*-12,*cis*-15 18:3 fatty acid obtained by various procedures on CP-Sil 5 CB low bleed MS (Chrompack). (**A**) Procedure described by Destaillats and Angers [2] with sodium bicarbonate; (**B**) hexane and water instead of sodium bicarbonate; (**C**) same as (B) but with increasing time (45 min) and temperature (45 °C).

compromise appeared to be 45 min at 45 °C. Under these conditions, no more residual FAME were found, indicating that they had all been converted into picolinyl esters (Fig. 1C).

Under these optimal conditions, the transformation of octadecanoic acid (C18:0) methyl ester into the resulting picolinyl ester was followed (Fig. 2). In contradiction to Destaillats and Angers [2], the derivatisation of this FAME is not complete after 15 min. Indeed, even after 30 min of reaction, traces of residual FAME can be found, which was not the case after 45 min (Fig. 2). In Fig. 2, it should also be pointed out that the response area obtained for the picolinyl derivative appeared higher than the one obtained for the corresponding FAME. This is well illustrated by Fig. 3 where the calibration curves of the octadecanoic acid in methyl ester and picolinyl ester form have been consigned. As expected, these curves are both linear; thus, a comparison factor can be estimated, which is the ratio between the area of the picolinyl derivative divided by the one of the corresponding FAME. In our case, and for octadecanoic acid, this ratio was estimated to be equivalent to about 5. Such a level was also found for other fatty acids (hexanoic acid, palmitic and dodecanoic acid; data not shown), which confirms the better response of the picolinyl derivative under electron impact by comparison to the FAME.

In order to validate this modified protocol, different kinds of lipids [glycerolipids (TAG, DAG, MAG), glycoglycerolipids (MGDG, DGDG), phosphoglycerolipids (phosphatidylcholine (PC)), and sterol esters (cholesterol ester (CE))] were derivatised under the same conditions and the resulting derivation curves were established (Fig. 4). As previously indicated [2], 2 min at room temperature were found sufficient to completely derivatise TAG. Moreover,

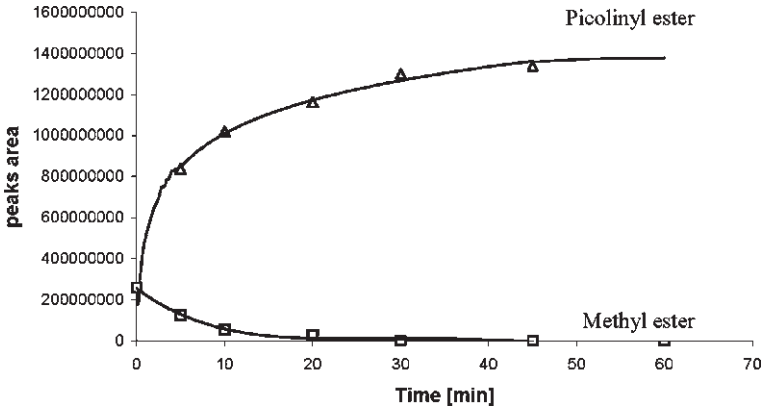

Fig. 2. Time course of conversion of stearic acid methyl ester to picolinyl ester.

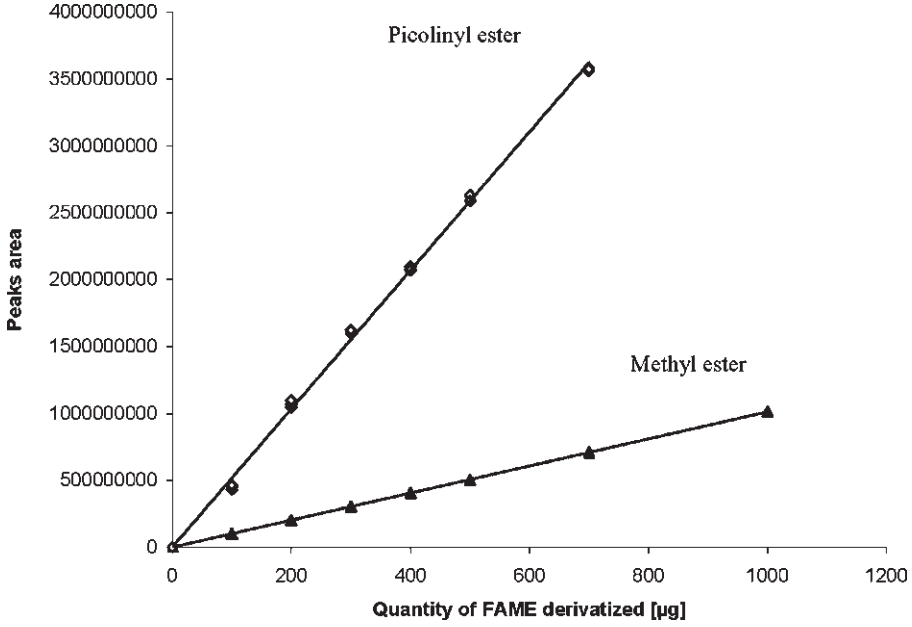

Fig. 3. Calibration curve of stearic acid (C18:0).

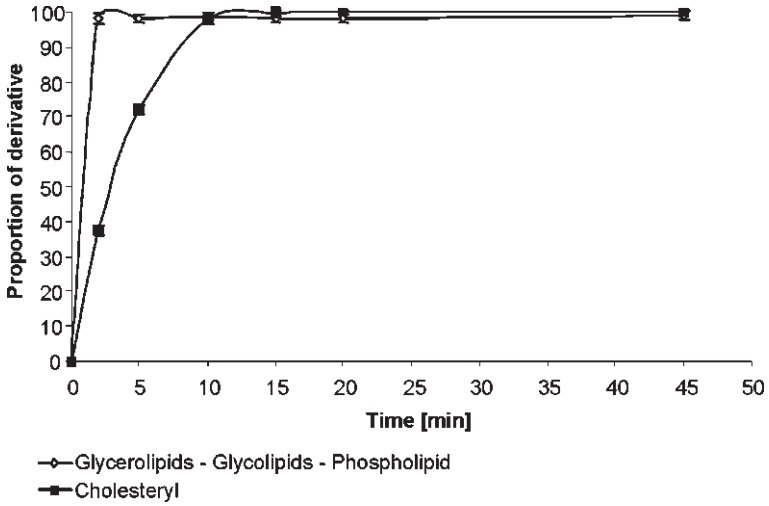

Fig. 4. Picolinyl ester formation from glycerolipids (MAG, DAG and TAG), glycolipids (MGDG and DGDG), phospholipids (PC) and sterol esters (CE).

this was also the case for all the glycerolipids tested, including those with a polar head group (glycoglycerolipids and phosphoglycerolipids), indicating that the polar head did not influence the derivatisation process. For sterol esters, the best conditions were found to be 15 min at 45 °C. Additional assays were also conduced on sphingolipid (amide linkage between fatty acid and serine backbone) and on FFA. As expected, no picolinyl esters were detected (which is notably in accordance with the basic principle of chemistry that it is impossible to esterify an FFA in an alkaline solution where salts are formed in the presence of an alcohol).

The results presented here confirm the advantage of using picolinyl esters instead of FAME for GC-MS analysis, not only because of their useful resulting mass spectra but also due to their better sensitivity (about five times more). Moreover, the preparation of such derivatives is quite easy and rapid (no more than 45 min under our conditions) and may be milder than some conditions currently in use for methylation (such as BF3/methanol) or the DMOX derivatisation procedure (lipids react with 2-amino-2-methyl-1-propanol in a nitrogen atmosphere at 180 °C – 2 h for free acids, or 18 h for methyl esters and intact lipids [8]) which can lead to partial denaturing of fatty acids (methoxy artefact [11], destruction of labile functional groups [12, 13], *etc.*).

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