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Rapid simultaneous lipid extraction and transesterification for fatty acid analyses

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An improved adaptation of the direct transesterification method of Lepage and Roy (*J. Lipid Res.* 25, 1391–96, 1984) for the preparation of fatty acid methyl esters allows notable saving of time and reagents. The material being analysed is heated for 10 minutes with methanol, acetyl chloride and hexane.

Key words: fatty acid, GLC, fatty acid methyl esters, transesterification

Introduction

Long chain polyunsaturated fatty acids (LCPUFAs), e.g., eicosapentaenoic acid (20:5n3, EPA), arachidonic acid (20:4n6, ARA), and docosahexaenoic acid (22:6n3, DHA), are precursors of prostaglandins, thromboxanes, and leukotrienes. Consequently, LCPUFAs are important to human health. LCPUFAs are essential to development of foetus and infants and their intake in diet reduces incidence of coronary desease and some cancers (Innis, 1991; Simopoulos, 1991; Iacono and Dougherty, 1993; Nettleton, 1993; Burns and Spector, 1994). Because of their impact on health, the pharmaceutical industry has shown interest in LCPUFAs.

Many biotechnology programs are oriented to the production of special fatty acids compositions derived from high producers of LCPUFAs. Mutants with altered fatty acid biosynthetic pathways have been obtained from plants such as sunflower (Mancha *et al.*, 1994), *Arabidopsis* (Somerville and Browse, 1991) and from the microalga *Porphyridium cruentum* (Khozin *et al.*, 1997). This type of research requires analysing a large number of samples; thus, simple, rapid, and reliable methods for fatty acid analysis are needed.

The most extensively used technique for fatty acids analysis is GLC, which requires an esterified sample of fatty acids, usually the methyl esters (FAMEs). Many FAMEs preparation methods require prior extraction and saponification of the lipid fraction followed by derivatization to the final FAMEs (García Camacho *et al.*, 1990). Because these require many manipulations, they are not suited to rapid processing of a large number of samples. Some procedures that allow direct transesterification of the sample, avoiding the lipid fraction extraction, have also been developed (Lepage and Roy, 1984; Garcés and Mancha, 1993). In the Garcés and Mancha (1993) method, transmethylation and extraction of the sample lipids take place in one step in the same vial. The modified Lepage and Roy method used in our laboratory (García Sánchez *et al.*, 1993) requires more manipulation because transmethylation and extraction occur in two steps; however, the Lepage and Roy methylation mixture is simple and easily prepared and it does not require antioxidants for protection of the unsaturated lipids (Lepage and Roy, 1984). We have improved the original Lepage and Roy method so that transesterification and FAMEs extraction are carried out simultaneously. This avoids many manipulations and allows rapid analysis of many samples.

Materials and methods

Commercial cod-liver oil (Acofarma) and dry biomass (lyophilised) of the microalgae *Porphyridium cruentum* and *Phaeodactylum tricornutum* grown in our laboratory, were used for fatty acids analyses. Determination of fatty acid profiles was carried out using a HP5890 series II gas chromatograph equipped with an automatic injector (HP 6890) and a flame ionization detector (FID). A Supelco Omegawax 250 (30 m × 0.25 mm × 0.25 μm) fused silica capillary column was used. The oven time-temperature program was the following:

> 205°C (10 min.) → 6°C/min. → 240°C (9 min.), giving a total heating time of 24 min.

Fatty acid quantitation was done using nonadecanoic acid (19:0) as an internal standard. The amounts of individual fatty acids was calculated using the expression: $C_i = C_p \cdot (A_i/A_p)$, where A is the chromatographic area units and C is

the amount of fatty acid. Subscript p stands for the internal standard, and i refers to any fatty acid.

Standards for fatty acids calibration were supplied by Supelco. Solutions of 19:0 were prepared by diluting 25 mg nonadecanoic acid (19:0) in 1 ml methanol/benzene (3:2 v/v). Methyl ester standard solution was prepared by diluting 25 mg 19:0-methyl ester in 1 ml of hexane. Statistical analyses of the data were conducted using Statgraphics ver. 7.0 software.

A comparison of the actual method used in our laboratory (adapted Lepage and Roy) and the proposed improved procedure is detailed below:

Procedure A: (adapted from Lepage and Roy, 1984)

Samples and 5 μl 19:0 solution were placed in test tubes. One ml of freshly prepared transesterification reagent (methanol/acetyl chloride, 20:1 v/v) was added to each tube. The tubes were heated at 100°C for 1 h for the transmethylation, being shaken every 10–15 min. The mixture was cooled to room temperature, and 1 ml each of water and hexane were added. The tubes were then shaken and centrifuged. Two phases were formed: the upper one (hexane) was transferred to another tube. This operation was repeated twice, to optimize sample lipid extraction (García Sánchez *et al.*, 1993). Hexanic phase (about 3 ml) was dried under N_2 atmosphere and FAMEs were resuspended in 0.5 ml of hexane and injected into the chromatograph.

Procedure B: (proposed procedure)

Samples are put in test tubes with 1 ml of the methylation mixture (methanol/acetyl chloride, 20:1 v/v) and 0.5 ml hexane. Now the samples were heated at 100°C for 10 minutes. A single methanol/hexane phase was formed. After cooling to room temperature, 1 ml distilled water was added. Two phases established very rapidly: the upper one (hexanic) was extracted and placed into the chromatograph vial for injection.

Results and discussion

The internal standard recovery was checked in the two procedures to ensure validation of the calculated fatty acids profiles. Five μl (1.25 μg) of 19:0 solutions transmethylated with each method and 5 μl 19:0 methyl ester solution were injected in the chromatograph. The area under the chromatogram peaks was always in the same range: an area of 56,000 units (mean of the three injections) was obtained for the methyl ester, while the area was 55,017 and 54,613 units for the 19:0 fatty acid treated with A and B procedures, respectively, (data are averages of three replicates). Thus, the recovery of 19:0 was 98.24% and 97.52%, respectively; complete conversion of the internal standard fatty acid to the methyl ester could be assumed, giving consistent computations of the fatty acid profile by the two procedures.

Table 1 Comparison between fatty acid analyses of cod-liver oil using the two procedures.

Fatty acid	*Procedure A*	*Procedure B*	*F-ratio*
14:0	0.33 ± 0.021	0.36 ± 0.011	0.798 ns
16:0	0.99 ± 0.020	1.00 ± 0.031	0.024 ns
16:1n7	0.62 ± 0.027	0.62 ± 0.020	0.012 ns
18:0	0.23 ± 0.004	0.23 ± 0.007	0.177 ns
18:1n9	1.59 ± 0.030	1.63 ± 0.052	0.594 ns
18:1n7	0.38 ± 0.007	0.38 ± 0.013	0.069 ns
18:2n6	0.16 ± 0.002	0.15 ± 0.004	0.322 ns
18:3n3	0.15 ± 0.003	0.15 ± 0.005	0.426 ns
18:4n3	0.26 ± 0.005	0.27 ± 0.008	0.097 ns
20:5n3	0.90 ± 0.031	0.87 ± 0.030	0.326 ns
22:5n3	0.13 ± 0.005	0.13 ± 0.004	0.102 ns
22:6n3	1.20 ± 0.019	1.22 ± 0.037	0.137 ns
TFA	9.69 ± 0.240	9.44 ± 0.300	0.415 ns

Only the main fatty acids are shown. Figures are averages (mg of fatty acid in 10 mg cod liver oil) of three independent measurements ± the standard error. The F-ratio statistic is from ANOVA comparison between the two procedures (ns = not significant; TFA = total fatty acids).

The two procedures were compared using three materials: cod liver oil, *Phaeodactylum tricornutum* biomass and *Porphyridium cruentum* biomass.

The fatty acid compositions obtained by the two methods were virtually identical for cod-liver oil (Table 1). The differences between the two procedures was always less than 3% for any fatty acid. The analysis of variance (ANOVA) showed that the differences were not statistically significant for any fatty acid. Procedure B seemed to recover slightly greater quantities of almost all identified fatty acids.

Results of fatty acids analyses carried out with *Phaeodactylum* showed small differences between the two procedures (Table 2). ANOVA comparison between both procedures showed statistically significant differences in two cases: for palmitic acid (16:0) and DHA (Table 2). However, from a practical point of view, the difference among estimates of fatty acid contents were mostly irrelevant (Table 2). There was also little differences between the two methods for main fatty acids of *Porphyridium* (data not shown).

Significant differences between the two procedures were not detected in samples of cod-liver oil (CLO) probably due to the nature of the material. CLO is a clean (refined) liquid which is completely homogeneous. In contrast, solid

Table 2 Comparison between fatty acid analyses of *Phaeodactylum* using the two procedures.

Fatty acid	*Procedure A*	*Procedure B*	*F-ratio*
14:0	0.95 ± 0.032	0.91 ± 0.003	1.077 ns
16:0	1.30 ± 0.040	1.53 ± 0.014	28.115 *
16:1n7	2.30 ± 0.078	2.30 ± 0.014	0.000 ns
20:5n3	3.23 ± 0.115	2.93 ± 0.019	6.532 ns
22:5n3	0.22 ± 0.006	0.22 ± 0.004	0.147 ns
22:6n3	0.25 ± 0.004	0.21 ± 0.010	13.124 *
TFA	12.01 ± 0.441	11.00 ± 0.100	4.978 ns

Only the main fatty acids are shown. Figures are averages (percentage of dry matter) of three independent measurements ± the standard error. The F-ratio statistic is from ANOVA comparison between the two procedures (ns = not significant; * significant for $0.05 > P > 0.01$; TFA = total fatty acids).

samples (dry biomass) were more heterogeneous and contained more impurities which interfere with the analyses (whole dry biomass, and not the isolated lipid fraction, was transmethylated).

There was no apparent relationship between the statistically significant difference between the two procedures and the type of fatty acid (i.e., saturated or unsaturated, short chain or long chain). Procedure A extracted slightly higher quantities of the principal fatty acids when dry biomass was used (Table 2). At any rate, estimates of fatty acid contents were equivalent for both procedures; the differences, when present, were generally minors. Hence, one could take advantage using procedure B when it is planned to carried out a lot of GLC analyses. Main advantages of the proposed procedure are: the decreased number of needed manipulations (one hexanic phase extraction vs. three extractions and N_2 drying), and a shorter transesterification time (10 minutes vs. 60 minutes). Therefore, procedure B allows both time and reagents to be saved.

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