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RAPID AND SIMULTANEOUS METHYLATION OF FATTY AND HYDROXY FATTY ACIDS FOR GAS–LIQUID CHROMATOGRAPHIC ANALYSIS

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SUMMARY

Rapid and exhaustive methylation of fatty acids and hydroxy fatty acids may be achieved using methyl iodide in polar aprotic organic solvents such as dimethylacetamide and in the presence of sodium hydroxide or potassium hydroxide. The optimal proportions between the lipid sample, methyl iodide, alkali metal hydroxide and solvent were established empirically. The method is suitable for the gas–liquid chromatographic determination of the total fatty acid composition of lipid mixtures.

INTRODUCTION

One of the most important applications of gas–liquid chromatography (GLC) is in the determination of the fatty acid composition of lipids. For this purpose the fatty acids (FAs) must be converted into volatile derivatives such as their methyl esters.

We reported recently[1] a convenient method for the selective methylation of the free fatty acids from lipid mixtures, by means of methyl iodide in dimethylformamide and pyridine with a strong anion-exchange resin as a heterogeneous catalyst. A convenient method is further needed for the rapid methylation of all the fatty acids (free + bound) in a lipid mixture, in order to establish the total FA and HFA composition.

The usual saponification and methylation techniques[2] are satisfactory but hydroxy fatty acids (HFAs) cause several difficulties. A direct method of overcoming these problems, caused by their lower volatility, is to apply a special, high-temperature stationary phase such as SP-2100 DOH (Supelco)[3].

Suitable derivatizations of the hydroxyl groups in addition to that of carboxyl groups have also been proposed. Acetylation[4,5] improves the separation of components but long retention times result. The O-heptafluorobutyryl[6] and O-trimethylsilyl[7] derivatives are very sensitive to moisture, similarly to *n*-butylboronates[8], which are obtainable only for α- and β-hydroxy acids. In terms of volatility and stability the methyl derivatives obtained by with methyliodide–silver[9,10], diazomethane–boron trifluoride[11] or methyl iodide–dimethyl sulphoxide[12] treatments are the best, but

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their low yields (50%) are severe disadvantages for quantitative application.

The best solution for total FA analysis was a rapid simultaneous and quantitative methylation of the FAs and HFAs from lipid mixtures. This paper describes our studies, using methyl iodide and powdered sodium hydroxide in polar organic solvents.

EXPERIMENTAL

Materials

Analytical-reagent grade dimethyl sulphoxide (DMSO), dimethylacetamide (DMAA) and dimethylformamide (DMF) from Merck were used without further purification. Sodium and potasium hydroxide were obtained from Chemapol. Saturated (C_8–C_{20}) and unsaturated ($C_{18:1}$, $C_{18:2}$ and $C_{18:3}$) fatty acid standards were obtained from Supelco. Cholesteryl esters of the C_{14}–C_{18} acids and α-hydroxypalmitic, 12-hydroxyoleic and 9,10-dihydroxystearic acid standards were obtained from Serva.

A Chromatron GCHF-18.3 chromatograph modified for on-column injection with a flame-ionization detector was used. A silanized glass column (1.0 m × 3 mm I.D.) was packed with 2% SE-30 on Gas-Chrom Q (80–100 mesh). Peak areas were integrated with a Minigrator (Spectra-Physics).

Derivatizations were performed in mini-vials with a magnetic stirrer and thermostated in a Pierce Reacti-Therm module.

Method

To a lipid sample containing 0.05–0.1 mmol of FAs and HFAs, dissolved in 0.4–1.0 ml of dimethylacetamide (DMAA), 80–160 mg freshly powdered sodium hydroxide are added. The mixture is stirred for 15 min at 70°C, then cooled to 35°C. About 0.14–0.28 ml of methyl iodide is added, followed by stirring for 15 min at 35°C. Water (1 ml) and hexane (1 ml) are added and the hexane layer is washed with 3 × 10 ml of water and dried with anhydrous sodium sulphate.

RESULTS AND DISCUSSION

General scheme of reactions

The main problem was to establish the optimal reaction parameters (solvent, concentrations, temperature, reaction time, etc.) for the methylation of HFAs, together with FAs. These may be considered according to the following general scheme of the reactions:

$$NaOH + Solv. \rightleftarrows Na^+(Solv)_i + OH^-(Solv)_j \quad (1)$$

$$H_3C\text{-}(CH_2)_n\text{-}\underset{\underset{OH}{|}}{CH}\text{-}(CH_2)_m\text{-}COOR(H) + OH^- \rightarrow CH_3\text{-}(CH_2)_n\text{-}\underset{\underset{OH}{|}}{CH}\text{-}(CH_2)_m\text{-}COO^- + R(H)OH \quad (2)$$

$$H_3C\text{-}(CH_2)_n\text{-}\underset{\underset{OH}{|}}{CH}\text{-}(CH_2)_m\text{-}COO^- + OH^- \rightleftarrows CH_3\text{-}(CH_2)_n\text{-}\underset{\underset{O^-}{|}}{CH}\text{-}(CH_2)_m\text{-}COO^- + H_2O \quad (3)$$

$$H_3C\text{-}(CH_2)_n\text{-}\underset{\underset{O^-}{|}}{CH}\text{-}(CH_2)_m\text{-}COO^- + 2CH_3I \rightarrow CH_3\text{-}(CH_2)_n\text{-}\underset{\underset{OCH_3}{|}}{CH}\text{-}(CH_2)_m\text{-}COOCH_3 + 2I^- \quad (4)$$

$$H_3C\text{-}(CH_2)_n\text{-}\underset{\underset{OCH_3}{|}}{CH}\text{-}(CH_2)_m\text{-}COOCH_3 + OH^- \rightarrow CH_3\text{-}(CH_2)_n\text{-}\underset{\underset{OCH_3}{|}}{CH}\text{-}(CH_2)_m\text{-}COO^- + CH_3OH \quad (5)$$

$$CH_3I + OH^- \rightarrow CH_3OH + I^- \quad (6)$$

The use of a very strong base such as sodium hydroxide or potassium hydroxide is necessary because of the extremely low acidity of the hydroxyl group. Only a small amount of this base (added in large excess) is dissolved in the polar organic solvent (DMSO, DMAA and DMF) according to eqn. 1. The dissolved base consumed in reactions 2 and 3 is continuously replaced from the finely dispersed solid phase. The large excess of solid base serves also to retain water (reaction 3) but may be consumed in side reactions (6) also.

Methylation of the carboxyl group

In lipid mixtures the carboxyl group of FAs may be free or esterified. The formation of alkaline salts is instantaneous in the first instance. For esters the strong base in a polar organic solvent will first cause saponification, followed by methylation.

Table I gives the experimentally obtained reaction times needed for the saponification of some FA ester standards.

These data show that in lipid samples without cholesteryl esters the saponification is very fast. In this instance methyl iodide may be added immediately after the admixture of solid sodium hydroxide. For samples that contain cholesteryl esters, heating for 15 min at 75°C in DMAA is necessary before adding the methylation agent. The carboxylate anions of the FAs form some reversed micelles[13] in the polar organic solvents. The fine structure of these micelles can play an important role in the reaction with methyl iodide but detailed information is lacking.

Methyl iodide proved to be the most convenient methylation agent; a 10% molar excess calculated on the added sodium hydroxide seems to be the optimal amount. The very large molar excesses of these reagents relative to the amount of

TABLE I

SAPONIFICATION RATE OF FATTY ACID ESTERS

For 1 mmole of FA, 40 mmole of sodium hydroxide and 45 mmole of methyl iodide were added.

Compound	*Solvent*	*Temperature (°C)*	*Reaction time (min)*
Phosphoglycerides	DMAA	35	0.5
Glycerides	DMAA	35	1.0
Cholesteryl esters	DMAA	75	15.0
	DMSO	75	30.0
Methyl esters	DMAA	35	35.0

FA (40:1 for sodium hydroxide and 45:1 for methyl iodide) is necessary for exhaustive and rapid methylation. If dimethyl sulphate was used instead of methyl iodide the non-reacted agent interferes with some FA methyl esters in the gas–liquid chromatogram.

The reaction time and the methylation yield depend on the solvent and the base to solvent ratio. Table II gives the results obtained by methylation of palmitic acid. An increase in the reaction rate with increasing polarity of the solvents (DMSO > DMAA > DMF)[14,15] is observed. The decrease in the reaction rate at higher base to solvent ratios may be connected with the existence of reverse micelles. Potassium hydroxide favours the methylation of FA with methyl iodide. The higher solubility of potassium iodide compared with sodium iodide and the influence of this salt on micelle formation may account for this effect.

Methylation of the hydroxyl group

The strong base applied will remove the proton from the hydroxyl group of the hydroxy fatty acid according to eqn. 3. The large excess of sodium hydroxide (40 ×) shifts equilibrium 3 to the right. The influence of the excess applied was investigated for several HFAs. Fig. 1 shows the results obtained for the methylation of 12-hydroxyoleic acid (12-HOA) using various sodium hydroxide to HOA ratios (*n*).

The 100% methylation of the hydroxyl group in 12-HOA needs molar ratios *n* higher than 20. At *n* ratios equal to or higher than 40, full methylation is achieved in 10–12 minutes.

The influence of the solvent and base was investigated with 12-HOA (Table III).

The increase in the reaction time with decreasing polarity of the solvent is similar to that observed for simple (non-hydroxylated) fatty acids (Table II). However, the nature of the base here seems to have the opposite effects, with shorter reaction times with sodium hydroxide than with potassium hydroxide. This may be

TABLE II

METHYLATION DATA FOR PALMITIC ACID

Solvent	*Base*	*Base to solvent molar ratio*	*Reaction time (min)*	*Yield (%)*
DMSO	NaOH	5	6	100
DMSO	NaOH	3	4	100
DMSO	KOH	3	0.8	100
DMSO	NaOH	1	1.5	100
DMAA	NaOH	5	9	100
DMAA	NaOH	3	6	100
DMAA	KOH	3	1.5	100
DMAA	NaOH	1	2	100
DMF	NaOH	5	15	62
DMF	NaOH	3	9	81
DMF	KOH	3	2	100
DMF	NaOH	1	4	100

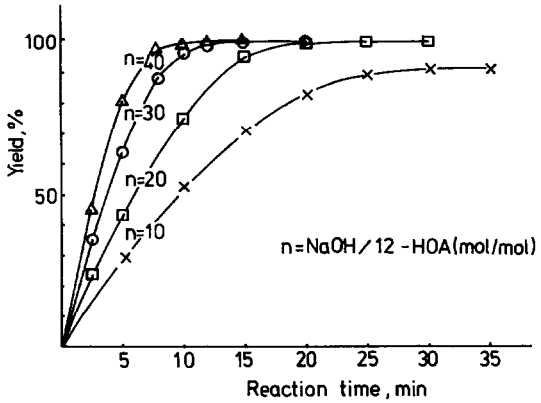

Fig. 1. Methylation of the hydroxyl group in 12-hydroxyoleic acid at different sodium hydroxide to 12-HOA molar ratios. Temperature, 35°C; 5 mol of sodium hydroxide per litre of DMAA.

due to the different organization of the hydroxy fatty acid micelles and a different influence of the more soluble potassium iodide[16] on the equilibria.

Influence of the base to solvent ratio

The base to solvent ratio acts in a different manner on the methylation of HFAs than that of simple fatty acids. This is illustrated in Fig. 2, where the methylation of oleic acid (OA) is compared with that of 12-hydroxyoleic acid (12-HOA).

The straight line obtained for oleic acid shows the decrease in the reaction rate with increasing base to solvent ratios owing to the modifications to the micelle structure. The hydroxy acid seems to have an increased tendency for methylation at lower concentrations. At higher base to solvent ratios they behave like common fatty acids.

The optimal ratio of base to solvent deduced from Fig. 2 is 4–6 mole of base per litre of solvent. Similar results were obtained for α-hydroxypalmitic acid and 9,10-dihydroxystearic acid. A composition of 5 mole of base per litre of solvent is recommended for general application.

Influence of temperature and stirring

Temperature and stirring have a favourable influence on the rate of methylation. An increase of 15°C doubles the reaction rate. The upper limit is the boiling point of methyl iodide. A magnetic stirrer at 100 rpm was applied.

TABLE III

REACTION TIMES FOR MAXIMAL CONVERSION OF 12-HOA WITH DIFFERENT SOLVENTS AND BASES

Base	*Base to 12-HOA molar ratio (n)*	*DMSO*		*DMAA*		*DMF*	
		Time (min)	*Yield (%)*	*Time (min)*	*Yield (%)*	*Time (min)*	*Yield (%)*
NaOH	40	8	99.6	12	99.9	20	99.8
KOH	40	9	99.2	15	99.0	45	97.2

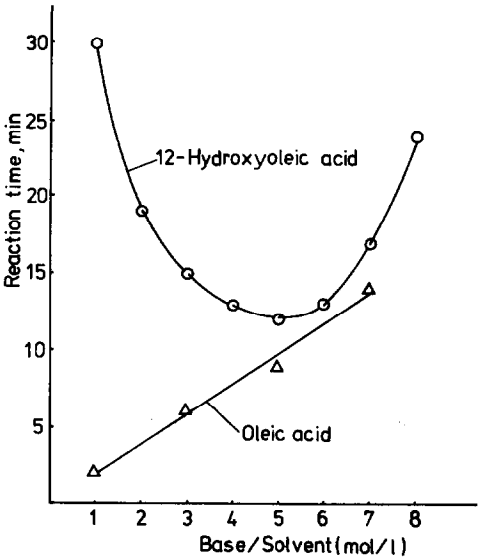

Fig. 2. Reaction time for methylation of oleic and 12-hydroxyoleic acids depending on the base to solvent molar ratio. Temperature, 35°C; solvent, DMAA; 40 mol of sodium hydroxide per mole of acid.

CONCLUSIONS

A simple and rapid method has been developed for the simultaneous methylation of fatty acids and hydroxy fatty acids. Owing to the large difference between the chemical behaviour of hydroxyl group and the carboxyl group, a multi-parameter optimization was afforded.

The following reagent amounts are recommended: fatty acid or hydroxy fatty acid, 0.1 mmol; sodium hydroxide (cor potassium hydroxide), 4.0 mmol; methyl iodide, 4.5 mmol; and solvent (DMAA), 0.8 ml.

For lipid mixtures that contain cholesteryl esters, heating of the alkaline solution of the sample for 15 min at 75°C is necessary. The usual reaction temperature is 35°C.

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