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Note

Quantitative recovery of polyunsaturated fatty acids on pyrolytic methylation of their trimethylphenylammonium salts

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We previously demonstrated the superiority of trimethyl(α,α,α-trifluoro-*m*-tolyl)ammonium hydroxide (TMTFTH) over trimethylphenylammonium hydroxide (TMPH) and tetramethylammonium hydroxide (TMH) in the recovery of polyunsaturated fatty acids (PUFAs) as their methyl esters in gas–liquid chromatographic (GLC) analyses[1] and this has been confirmed[2,3]. Ours was the first practical application of the powerful tool of flash alkylation of PUFAs from biological specimens. In order to achieve quantitative recoveries of PUFAs it was necessary to inject the TMTFTH solution of the fatty acids as their TMTFT salts after mixing the solution with methanolic methyl propionate in the injection syringe.

The major function of the short-chain fatty acid methyl ester in the reaction that takes place in the injector of the GLC unit is as a neutralizing reagent. The reaction appears to be an alkaline hydrolysis of the short-chain fatty acid methyl ester to yield methanol and the corresponding short-chain fatty acid. The excess quaternary ammonium hydroxide is neutralized by the released short-chain acid resulting in a sufficiently diminished alkalinity to reduce any alkaline degradation of the PUFAs to a non-detectable level. A minor function of the short-chain fatty acid ester appears to be an alkaline-catalyzed transalkylation, because when ethyl acetate was tested in the process, small amounts of ethyl esters of the long-chain fatty acids were detected.

The methyl propionate, which is 10.4 *M*, was diluted with two volumes of methyl alcohol, making the solution 3.5 *M*, in order to obtain a homogenous solution with the aqueous TMTFTH extract, because of the limited aqueous solubility of methyl propionate. Substitution of neat methyl acetate, which is 12.5 *M* and is soluble in water, for methanolic methyl propionate allows an increase in the concentration of the neutralizing ester by 3.6-fold —a sufficient increase in the neutralizing ability of the system to protect the PUFAs from the alkaline degradation in the hot injector inlet caused by the slightly stronger alkalinity of TMPH over TMTFTH.

Among the advantages of TMPH over TMTFTH are those of ready availability, lower cost, and greater stability. TMPH is easily generated in aqueous solution simply by mixing the appropriate amounts of trimethylphenyl ammonium iodide (TMPI) with silver oxide and water. Hydrolysis of the silver oxide yields silver hydroxide. The silver ion readily precipitates the iodide ion as silver iodide leaving

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trimethylphenylammonium and hydroxide ions in solution. TMPI in high purity is relatively inexpensive and commercially available. Although TMTFTH is generated just as readily from its iodide, TMTFTI is not commercially available. We described a procedure for the preparation of TMTFTI by reaction of α,α,α-trifluoro-*m*-toluidine with methyl iodide, but the yield is low and the carcinogenicity of methyl iodide has probably inhibited the use of TMTFTH with its obvious advantages.

Aqueous solutions of TMTFTH are offered by at least two suppliers, but the solution offered by one supplier, 0.2 *M* in concentration, is too dilute in our opinion. The other supplier offers 0.5 *M* TMTFTH.

Solutions of TMPH are more stable than TMTFTH solutions. This difference is undoubtedly related to the fact that the dimethyltrifluorotoluidine is the better leaving group.

EXPERIMENTAL

Reagents and methods

Trimethyl(α,α,α-trifluoro-*m*-tolyl)ammonium hydroxide (TMTFTH), 0.5 *M*, was prepared as described by MacGee and Allen[1] and stored in a stoppered test tube at 4°C in the dark.

Trimethylphenylammonium hydroxide (TMPH), 0.5 *M*, was prepared by mixing 1.3 g of trimethylphenylammonium iodide (Eastman-Kodak, Rochester, NY. U.S.A.) and 1.3 g silver oxide (Fisher Scientific, Fair Lawn, NJ, U.S.A.) with 10 ml of distilled water in a stoppered centrifuge tube. After vigorous mixing by hand and on a vortex mixer to achieve a uniform suspension, the preparation was centrifuged and one drop of the clear supernatant solution was diluted with 10 drops of water and tested for halide with one drop of 0.1 *M* silver nitrate in 6 *M* nitric acid. If a positive halide test resulted, the mixing and centrifugation steps were repeated until a negative halide test was achieved. The TMPH solution was stored in stoppered test tubes at 4°C in the dark.

Methanol–methyl propionate (2:1) was prepared as described previously[1].

Methyl acetate (certified, Fisher Scientific) was shaken in a stoppered test tube with granular anhydrous sodium carbonate (Mallinckrodt, St. Louis, MO. U.S.A.) to remove any water or free acetic acid and was stored at room temperature over the sodium carbonate. Occasional shaking of the mixture is recommended to keep it acid free.

PUFA-rich fatty acids were obtained in hexane by subjecting a human blood clot to a scaled-up modification of the procedure described by MacGee and Allen[1] up to and including the separation of the hexane extract from the neutralized saponified specimen.

GLC analyses were performed on two GLC units, a Bendix Model 2600 (Bendix Corp., Ronceverte, WV, U.S.A.) and a Perkin-Elmer Sigma I (Perkin-Elmer Corp., Norwalk, CT, U.S.A.). Both instruments were used with flame ionization detectors and 6 ft. × $\frac{1}{4}$ in. (4 mm I.D.) coiled glass columns containing pretested 10% Silar 10C on Gas-Chrom Q, 100–120 mesh (Applied Science Labs., State College, PA, U.S.A.).

The injectors were held at 250°C, the detectors at 220°C and nitrogen was the carrier gas at 32 ml/min for the Bendix GLC unit and 50 ml/min for the Perkin-Elmer

GLC unit. The column ovens were temperature programmed from 160°C to 220°C at 2°C/min. Peak areas were measured with an Autolab System I computing integrator (Spectra Physics, Santa Clara, CA, U.S.A.) connected to the Bendix GLC unit and by the Perkin-Elmer Sigma I system.

Procedure

The fatty acids from the total human blood clot from a 7-ml whole blood specimen were obtained in 100 ml of hexane as described above. Aliquots of 5 ml were extracted with 10 μl of either 0.5 *M* TMTFTH or 0.5 *M* TMPH in conical centrifuge tubes by shaking vigorously by hand for 1 min followed by centrifugation at *ca.* 600 *g* for 1 min. The mixing of the TMPH or TMTFTH extract with the neutralizing ester in the injection syringe was as described previously[1], *i.e.* the syringe was prewetted with the short-chain fatty acid methyl ester and an aliquot of the extract was drawn up followed by 0.5 to 1 μl of the neutralizing ester. After drawing the syringe plunger back and forth to mix the contents of the syringe, the sample was injected into the GLC unit. A moderately slow injection, 3–5 sec, was used[1,4].

When more than 1 μl of the quaternary ammonia hydroxide extraction of the fatty acids was required for analysis, it was necessary to employ the following "club sandwich" technique to assure adequate contact between the extract and the neutralizing short-chain fatty acid methyl ester. A 10-μl No. 701SN Hamilton microsyringe with a 3-in. needle (Hamilton, Reno, NV, U.S.A.) was prewetted and filled to the 1-μl mark with methyl acetate (volume of needle plus 1 μl = *ca.* 2.4 μl) followed in turn by 1 μl of the TMPH extract, 1 μl of methyl acetate, 1 μl of the TMPH extract and finally 1 μl of methyl acetate, for a total of 2 μl of the TMPH extract and *ca.* 4.4 μl of methyl acetate. After pumping the syringe plunger back and forth a few times to mix the contents of the syringe, the sample was injected into the GLC unit.

RESULTS AND DISCUSSION

Human blood cells contain large quatities of PUFA, especially arachidonate (20:4) with significant amounts of docosahexaenoate (22:6), making a blood clot a convenient material for this study.

Table I compares the values of the major fatty acids in the blood clot obtained with TMTFTH and TMPH using the procedure described and the Perkin-Elmer Sigma I system. The values are comparable and as can be seen from the table, the amount of the PUFAs recovered by TMPH is the same as the amount recovered by TMTFTH.

In examining the reaction with the two quaternary ammonium hydroxide reagents and methyl acetate on two different GLC units, no differences were observed in the recoveries of the fatty acid methyl esters with the "club sandwich" injection technique. However, small, but significant, losses of PUFAs were noted when the TMPH extract was used with methanolic methyl propionate or when 2 μl of the TMPH extract was sandwiched between two plugs of methyl acetate by the technique originally described[1], but these losses were evident only in the Perkin-Elmer Sigma I instrument. We attribute this small loss to differences in the geometry or composition of the instrument's injector system. The Bendix instrument was configured in such a way that the sample was injected on-column, *i.e.* the sample was introduced into the

TABLE I

COMPARISON OF VALUES OBTAINED WITH TMTFTH AND TMPH AS PERCENT OF TOTAL

	Average value ± S.D. (n = 6)	
	TMTFTH	*TMPH*
16:0	22.43 ± 0.136	22.00 ± 0.493
16:1	2.22 ± 0.177	2.20 ± 0.159
18:0	13.68 ± 0.110	13.44 ± 0.114
18:1	22.29 ± 0.090	22.24 ± 0.101
18:2	14.67 ± 0.101	14.74 ± 0.064
20:3	1.75 ± 0.060	1.79 ± 0.085
20:4	14.48 ± 0.145	14.87 ± 0.275
22:5	1.44 ± 0.019	1.48 ± 0.026
22:6	1.81 ± 0.024	1.90 ± 0.026
Others	5.25 ± 0.175	5.40 ± 0.276

empty inlet end of the column in the heated injector zone. On the other hand, in the Perkin-Elmer instrument the sample was injected into a separate injection zone containing a glass liner and a short stainless steel connector between the injector and the inlet end of the column. There are two simple solutions to this problem, the "club sandwich" technique described in this report or a readily-available reconfiguration to allow on-column injection into the empty heated inlet end of the column.

While the "club sandwich" injection technique is apparently only necessary with TMPH extracts on non-on-column systems, it is simple enough that we recommend it be used with all specimens requiring 2 μl or larger.

CONCLUSION

With the substitution of methyl acetate for methyl propionate, TMPH can be used instead of TMTFTH for the flash alkylation production of fatty acid methyl esters for GLC analysis. The methyl acetate provides a 3.6-fold molar increase in the amount of neutralizing ester which allows the higher alkaline TMPH to be used without destruction to polyunsaturated fatty acids. The advantages of TMPH over TMTFTH include ready availability, lower cost, and greater stability.

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REFERENCES

1 J. MacGee and K. G. Allen, *J. Chromatogr.*, 100 (1974) 35.
2 W. C. Kossa, R. R. Kutz, D. K. McCreary and S. Ramachandran, *28th Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, Cleveland, OH, February 28–March 4, 1977.*
3 K. O. Gerhardt and C. W. Gehrke, *J. Chromatogr.*, 143 (1977) 335.
4 J. MacGee and M. G. Williams, *J. Chromatogr.*, 205 (1981) 281.