# Selection of Internal Standards for Determining Quantitative Recovery of Isoprenoid Acids After Urea Complexing

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## ABSTRACT AND SUMMARY

Various types of esters are considered as internal standards for the quantitative gas liquid chromatographic determination of isoprenoid fatty acids after urea complex treatment of esters of total or saturated fatty acids. It is shown that aromatic esters are potentially unsuitable because of on-column losses with polyester columns. Two classes of aliphatic esters, either of 2-ethyl-1-hexanol, or of 3-cyclohexylpropionic acid, have been evaluated as satisfactory and certain gas liquid chromatographic retention data is given to show which ester might be selected for a particular application.

## INTRODUCTION

The presence of three isoprenoid fatty acids derived from phytol in fats of animals and in marine oils has been thoroughly documented (1-5) and studied in humans because of Refsum's syndrome, a rare hereditary neurological condition in which phytanic (3,7,11,15-tetramethylhexadecanoic) acid accumulates in body tissues (6,7). The other two acids are respectively 4,8,12-TMTD (4,8,12-trimethyltridecanoic) and pristanic (2,6,10,14-tetramethylpentadecanoic). The presence of these acids can be used by food chemists to indicate or confirm any admixture of animal fats into ostensibly all-vegetable margarines or cooking fats (8,9.10) and they also have potential use in food web research in marine biochemistry (2,4,11).

The total of these acids in oils and fats of commercial interest (2,8) is normally of the order of 0.01-1.0%. It is therefore usually desirable or necessary to concentrate the acids (as methyl esters) prior to GLC (gas liquid chromatography) analysis by either packed (12) or open-tubular columns (2,12). The preferred general method is treatment with urea (2,3,10,13) since isoprenoid acids do not form complexes and esters of $C_{18}$ and higher straight chain fatty acids which do are thus conveniently removed. As a simplifying step TLC (thin layer chromatography) with silica gel impregnated with silver nitrate was adopted to eliminate all esters except those of saturated fatty acids. The urea treatment did not always remove all shorter chain fatty acids or foreign materials and it was therefore decided that GLC analyses, especially on packed columns, should be carried out on both nonpolar and polar columns (12).

In order to quantify analyses for isoprenoid acids carried out on a small scale, an internal standard that could be added to the methyl esters of fatty acids prior to urea treatment was deemed useful (9). It is not possible to use a compound which might be found in animal fats or which might be removed by urea complexing (e.g., the 15:0 or 17:0 sometimes used for blood fatty acids are ruled out on both counts). This report indicates some possible pitfalls in the use of esters of otherwise suitable aromatic acids and evaluates some alternative aliphatic esters suitable for GLC on both nonpolar and polar liquid phases.

## EXPERIMENTAL PROCEDURES

Esters to be evaluated as internal standards were purchased or prepared by conventional means. For example the decyl ester of 3-cychohexylpropionic acid was made by refluxing *n*-decanol (0.025 mole) (British Drug Houses, Poole, England) and 3-cyclohexylpropionic acid (0.05 mole) (Aldrich Chem. Co.; Milwaukee, WI) for 10 hr in 20 ml of dry benzene with 1 ml of concentrated sulfuric acid. The cooled product was poured into a separatory funnel containing 100 ml water, the top layer was separated and the aqueous layer extracted with diethyl ether. The combined extracts were washed with saturated sodium bicarbonate solution until effervescence ceased, then they were washed twice with water and dried with anhydrous sodium sulphate. The solution was filtered and the solvent evaporated on the rotary evaporator. TLC (thin layer chromatography) showed no free alcohol in the preparation.

An official procedure for recovery of fat from edible food products such as margarine, the recovery of fatty acids, and their conversion to methyl esters is detailed elsewhere (9). Other techniques can be used provided that they are chosen to help eliminate or at least minimize any minor foreign impurities which might interfere with GLC, for example phthalates (14).

Two TLC techniques were used. In one, 0.5 mm silica gel plates were prepared from silica gel containing silver nitrate (Supelcosil 12D; Supelco Inc., Bellefonte, PA). Details of the preparation and use are given elsewhere (9), but generally development in a benzene-*n*-hexane 1:1 solution gave a clean saturate band at $R_f$ 0.68, well separated from methyl esters of monoethylenic fatty acids. As an alternative commercial silica gel TLC plates (0.25 mm; Adsorbosil-5 Prekotes, Applied Science Laboratories, State College, PA) were dipped in silver nitrate (10%) in acetonitrile, (See applied Science Laboratories Information Sheet, "Procedure for Silver Nitrate-Impregnated PREKOTES"), dried, and developed in benzene-hexane (2:1). The $R_f$ of methyl esters of saturated acids was 0.65. A No. 17700 streaker (Applied Science Laboratories) was used. Recovery procedures for bands visualized with iodine vapor (9), or 2,4-dichlorofluorescein, usually involved diethyl ether as one solvent and sometimes chloroform-methanol as another, but were not critical. Full details of the use of a suitable combined suction/washing apparatus for recovery of TLC bands have been described (9).

Gas liquid chromatography in packed columns was conducted with stainless steel columns 6 ft in length and 1/8 in. OD packed with 15.5% EGSS-Y (an organosilicone polymer, Applied Science Laboratories) on 100-120 mesh Gas-Chrom P; 6% DEGS (diethylene glycol polyester) on 80-90 mesh Anakrom ABS; 3% OV-17 (50% methyl, 50% phenyl silicone) on 80-100 mesh Gas-Chrom Q; 5% SE-30

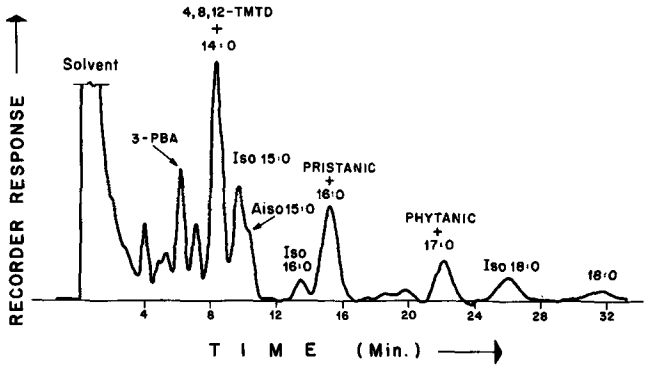


FIG. 1. Gas liquid chromatogram (packed column, EGSS-Y liquid phase) of fraction of methyl esters of saturated fatty acids isolated from a margarine known to contain marine oil, after two-stage urea treatment, and with the addition of the methyl ester of 3-phenylbutyric acid.

or 3% JXR (100% methyl silicone) on 80-100 mesh Gas Chrom Q; or 10% UCW 98 (vinyl, methyl silicone) on 80-100 mesh Diatoport S. GLC units were either a Varian Hi-FY model 600 or Hewlett-Packard 5750B (both with flame ionization detectors). Component areas on recorder charts were compared on some charts by the peak height x retention time to apex system (15,16), or on other charts by triangulation.

A Perkin-Elmer model 3920 apparatus was used for wall-coated open-tubular (capillary) stainless-steel columns, 150 ft (46 m) in length and 0.01 in. (0.25 mm) ID. Liquid phases were BDS (butanediolsuccinate polyester): SILAR-5CP (50% cyanopropyl, 50% methyl silicone); SILAR-7CP (70% cyanopropyl, 30% methyl silicone); or Apiezon-L. Peak areas were determined with a ball and disc type integrator on the recorder.

## RESULTS AND DISCUSSION

The problem of separating the three isoprenoid fatty acids from all other components with simple routine technology and packed-column GLC is probably insoluble (9,12). At best elimination of most of the saturated and *iso* acids is feasible, but esters of *anteiso* acids, among others, resist urea complexing to a considerable degree (2). Accordingly on a medium polarity liquid phase such as EGSS-Y (Figure 1), 4,8,12-TMTD tends to coincide with residual 14:0, pristanic coincides with any 16:1, and phytanic with 17:0. On highly polar columns pristanic can merge with *iso* 16:0 (or acetal products) ahead of 16:0 and phytanic acid with *anteiso* 17:0. On SE-30, 4,8,12-TMTD elutes later in the *iso/anteiso* 15:0 region, pristanic in the *anteiso* 17:0 region, and phytanic acid between *iso* 18:0 and 18:0 as illustrated elsewhere (12). Basically, if urea complexing is incomplete, both a polar and a nonpolar GLC analysis are needed to permit corrections for coincident components on one or the other type of packed column. In the Figure 1 analysis there is some residual 14:0 and a trace (≈ *iso* 16:0) of 16:0, but the 17:0 is reduced to negligible proportions.

Esters of aromatic acids were not expected to form urea complexes and certain commercially available acids (Table I) were selected for testing on the basis of their short retention times on nonpolar columns (i.e. methyl ester emergence earlier than 14:0). Unfortunately these were found to emerge later on polar columns (Table I). Generally the GLC retention times of these particular aromatic esters were too short for accurate quantitation with one or the other type of liquid phase since they tended to be on the solvent tail. As expected none of the internal standard materials of Table I formed urea complexes.

The basic reason for discarding esters including aromatic rings was, however, on-column losses of the test materials on the polyester liquid phases. For example, when methyl 3-phenylbutyrate was compared to methyl phytanate as a quantitative internal standard (area by triangulation), the peak area of the aromatic ester on EGSS-Y was only 43% of that from the same solution analyzed on OV-17. On the same basis the amount of *n*-butyl benzoate passing through the DEGS column was only 53% of that passing through either the JXR or UCW 98 nonpolar columns. By contrast, the recovery of 2-ethyl-1-hexyl butyrate from a DEGS column by this area estimation was 105% of that for the UCW 98 column, and the recovery of 2 ethyl-1-hexyl caproate was 117% on the same basis. These area determinations were subject to a systematic error (i.e. the 4,8,12-TMTD peaks were also proportionately higher on the polar liquid phases) suggesting that uncritical application of the peak height x retention distances formula (15,16) should not be used for comparisons between differing types of column and/or structurally differing classes of chemical compounds. The 2-ethyl-1-hexyl butyrate ($C_{12}$) ester had too short a retention time to be practical as it appeared on the petroleum hydrocarbon solvent tail, but 2-ethyl-1-hexyl caproate ($C_{14}$) ester had a very suitable retention time, appearing just before lauric (12:0) acid on DEGS, and since it had no aromatic characteristics, in essentially the same position on the nonpolar columns. The use of an early-eluting internal standard saves time in analyses, and it appears that due to the relatively unchanging relationships of retention times on different liquid phases for apolar structures (17,18) the 2-ethyl-1-hexyl ester of caprylic ($C_{16}$) acid would also be suitable as it should elute just ahead of 14:0 in a position not normally occupied by any fatty acid of interest, although sometimes acetal degradation products can appear ahead of corresponding methyl esters (17).

TABLE I

Relative Retention Data for Some Potentially Suitable Internal Standard Esters Evaluated on Packed Columns

| | Ester and Liquid Phase | | | | | | |
|---|---|---|---|---|---|---|---|
| | Methyl 3-Phenylbutyrate[a] | | | Methyl 5-Phenylvalerate[a] | *n*-Butyl benzoate[b] | | |
| Compound | EGSS-Y | DEGS | OV-17 | EGSS-Y | DEGS | JXR | UCW98 |
| Ester | 0.7 | 0.5 | 0.3 | 1.6 | 0.9 | 0.5 | 0.7 |
| 12:0 | 0.6 | – | – | – | – | – | – |
| 14:0 | 1.0 | – | – | 1.0 | – | – | – |
| 16:0 | 1.8 | 1.0 | – | 1.9 | – | – | – |
| 18:0 | 3.8 | – | – | 3.8 | – | – | – |
| 4,8,12-TMTD | 1.0 | – | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Pristanic | 1.9 | – | 3.0 | 1.9 | 1.4 | 1.6 | 1.5 |
| Phytanic | 2.7 | – | 3.5 | 2.7 | 1.9 | 2.2 | 2.3 |

[a]Acid purchased from Aldrich Chem. Co., Milwaukee, Wis; Esterification with diazomethane.

[b]Prepared as described in experimental section.

Other types of aromatic esters suitable for standards, since they did not form urea complexes, were tested and rejected for reasons similar to those for the aromatic esters of Table I. Among these were phthalates, which could also be present in samples (14) for reasons of contamination, and cinnamoyl esters although with the latter esters a methoxy derivative forms readily during alkali treatment (19), suggesting unwelcome instability in an internal standard. Retention data for methyl esters of several of these acids has recently been published elsewhere (19). To our knowledge the problem of failure of quantitative analysis of aromatic esters on polyester liquid phases has not been reported elsewhere. Methyl esters of benzoic and terephthalic acids were apparently more stable on columns of 12% FFAP (a terephthalate-modified Carbowax 20M) on Chromosorb W than were dimethyl esters of acids such as azelaic, unless water vapor was added to the carrier gas (20).

The natural occurrence in certain lipids of 11-cyclohexylundecanoic acid (13,21-23) suggested another class of compounds suitable for analyses of materials resistant to urea complexing. We prepared both decyl and nonyl esters of 3-cyclohexylpropionic acid for investigation. In comparing retention properties with capillary columns (Table II) we found that the decyl ester of 3-cyclohexyl-propionic acid ($C_{19}$) had almost the same equivalent chain length (ECL) on BDS (18.35) as that (18.40) for methyl 11-cyclohexylundecanoate ($C_{18}$) recorded previously (21) on BDS at the same temperature of 150 C. The more centralized ester position (17,18,24) and polarity change in the ester linkage due to the proximity of the ring to the ester linkage results in a difference of 1 ECL carbon on BDS and 0.5 ECL carbon on AP-L. The other data given in Table II shows, through the effect of different conditions and polarities of columns on retention times for the more polar liquid phases such as DEGS and SILAR-7CP and the two esters, that esters of 3-cyclohexylpropionic acid are not strictly apolar, thus offering some flexibility in choice of GLC operating conditions. The data offer a guide to the choice of aliphatic ester materials for inclusion in mixtures such as the isoprenoid acids or monomethyl-branched aliphatic esters (21,25,26) where one might be inconvenient due to coincidence with a known compound such as methyl 11-cyclohexylundecanoic acid or methyl 9-cyclohexylnonanoic acid (13).

In a test on an open-tubular BDS column of proportionation of internal standard and methyl ester of an isoprenoid fatty acid during urea complexing, the average recovery of decyl cyclohexylpropionate relative to methyl phytanate (three analyses, ball and disc integration) differed by only 1.6% from the same solution analysed before treatment. This result shows that both esters totally resist urea complexing. The same solutions were analyzed on the SE-30 packed column and the recovery of decyl cyclohexylpropionate relative to methyl phytanate differed by only 1.1% from the BDS result. This shows that both esters pass through the open-tubular column with BDS polyester liquid phase with little or no differential reaction with the polyester or other loss on the column, or fractionation in the split system used with the open-tubular columns.

The esters of cyclohexylpropionic acid thus appear to be suitable for quantitative internal standards in analyses where a synthetic and analytically stable ester which does not form urea complexes is required to determine esters of naturally-occurring isoprenoid or other bulky natural fatty acids.

TABLE II

Retention Data (Equivalent Chain Lengths) for Two Reference Esters of Cyclohexylpropionic Acid and for Methyl Phytanate

| Liquid phase | Temp. | Decyl ECL[a] | Nonyl ECL | Phytanate ECL |
|---|---|---|---|---|
| BDS (capillary) | 150 | 18.35 | 17.36 | 17.15 |
| | 170 | 18.48 | 17.50 | – |
| DEGS (capillary) | 150 | 18.57 | 17.61 | 16.74 |
| | 170 | 18.78 | 17.80 | – |
| SILAR-5CP (capillary) | 165 | 18.72 | 17.75 | – |
| | 185 | 18.90 | 17.94 | 17.00 |
| SILAR-7CP (capillary) | 150 | 18.78 | 17.83 | – |
| | 170 | 19.00 | 18.01 | 16.83 |
| SE-30 (packed) | 200 | 18.19 | – | 17.71 |
| AP-L (capillary) | 160 | 18.21 | 17.22 | – |
| | 180 | 18.27 | 17.30 | 17.48[b] |

[a]ECL = equivalent chain length.

[b]At 170 C-not very temperature dependent on AP-L (26)

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