Methyl Esterification of Fatty Acids and Eicosanoids With a Novel Reagent Trimethylsilyldiazomethane for Analysis by Gas Chromatography-Mass Spectrometry

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Methyl Esterification of Fatty Acids and Eicosanoids With a Novel Reagent

Trimethylsilyldiazomethane for Analysis by Gas Chromatography-Mass Spectrometry

By

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Thesis

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To my grandparents
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ABSTRACT

Fatty acids are compounds with limited water solubility that belong to the lipid class of bio-organic compounds and can be utilized as a source of energy by the body or as precursors to oxidized compounds that have roles in inflammation and in the regulation of vascular tone. The prostaglandins are naturally occurring 20-carbon cyclopentano fatty acid derivatives produced in mammalian tissue from polyunsaturated fatty acids (PUFAs) and are generated from endoperoxide precursors of arachidonic acid and related polyunsaturated fatty acids by the enzyme cyclo-oxygenase (COX). The lipoxygenase (LOX) enzymes convert arachidonic acid into hydroperoxy derivatives of PUFAs termed leukotrienes, which play a role in asthma and allergies. The reagent trimethyl silyl diazomethane (TMSD) which is less toxic and explosive than other reagents such as diazomethane, has been developed for use in the methyl esterification of drugs such as penicillin and some fatty acids. The objectives of the research were to (1) optimize the TMSD reaction conditions, (2) determine whether the TMSD reaction could support methylation of prostaglandins and other oxidized products of arachidonic acid, such as prostaglandins PGE\textsubscript{2} and PGF\textsubscript{2a}, (3) develop calibration curves for the fatty acids and prostaglandins with appropriate internal standards for use with GC-MS, and (4) determine whether this method could be used to characterize by GC-MS these lipids in cell culture and in other cell-free systems, such as those containing enzymes like COX.
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<td>AA</td>
<td>Arachidonic acid (C20:4n-6)</td>
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<tr>
<td>BSTFA</td>
<td>Bistrimethylsilylfluoroacetamide</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
</tr>
<tr>
<td>EFA</td>
<td>Essential Fatty Acids</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Endothelial growth factor</td>
</tr>
<tr>
<td>FA</td>
<td>Fatty acid</td>
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<tr>
<td>GLA</td>
<td>Gamma Linolenic Acid (C18:3n-6)</td>
</tr>
<tr>
<td>HPETE</td>
<td>Hydro-peroxy–eicosatetraenoic acid</td>
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<td>IL</td>
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<td>LA</td>
<td>Linoleic acid (C18:3n-3)</td>
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<td>LNA</td>
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<td>LOX</td>
<td>Lipoxygenase</td>
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<tr>
<td>LTB</td>
<td>Leukotrienes</td>
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<tr>
<td>PGE</td>
<td>Prostaglandin E</td>
</tr>
<tr>
<td>PLA₂</td>
<td>Phospholipase A₂</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acid</td>
</tr>
<tr>
<td>TMCS</td>
<td>Trimethylchlorosilane</td>
</tr>
<tr>
<td>TMSD</td>
<td>Trimethyl silyl diazomethane</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Tumor growth factor</td>
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INTRODUCTION

1.1 Polyunsaturated Fatty Acids (PUFAs)

Ingestion of polyunsaturated fatty acids (PUFA) will lead to their distribution to virtually every cell in the body with effects on membrane composition and function, eicosanoid biosynthesis, and signaling as well as in the regulation of gene expression (1). Dietary PUFAs have effects on diverse physiological processes impacting normal health and chronic disease, such as the regulation of plasma lipid levels, cardiovascular and immune function, insulin action, and neuronal development and visual function (2-7). Cell-specific lipid metabolism and the expression of fatty acid regulated transcription factors likely play important roles in determining how cells respond to changes in PUFA composition.

1.2 Structural Characterization of PUFAs

Chemically, PUFA belong to the class of simple lipids and are categorized as such due to the fact that they have two or more cis double bonds. The location of the first double bond, counted from the methyl end of the fatty acid, is designated by the omega- or n- number. For example, linoleic acid, in the n-6 family, is designated as C18:2 n-6 to indicate that it has 18 carbons and two double bonds, with the first double bond at the sixth carbon (Figure 1).
There are two main families of PUFA, the n-3 and the n-6 PUFA. These fatty acid families are not interconvertible and have very different biochemical roles. Linoleic acid (n-6) (LA) and α-linolenic acid (n-3) (LNA), two of the main representative compounds, are considered to be essential fatty acids (EFA); they must be provided in the diet because they cannot be synthesized in the body and their absence from the diet has pathological consequences.

1.3 Sources of PUFA

Vegetable oils, such as canola oil, are the major sources of the n-3 fatty acid, LNA. In particular, LNA is found in the chloroplast of green leafy vegetables, such as purslane and spinach, and in flax seed, linseed, walnuts, and so on (8). Purslane (*Portulaca oleracea*), a vegetable used in soups and salads along the Mediterranean basin and in Middle East, is the richest source of LNA of any green leafy vegetable examined to date (Figure 2). Moreover, it is one of the few plants known to be a source of eicosapentaenoic acid (C20:5 n-3, EPA) (8). Other
sources of n-3 fatty acids include nuts and seeds, vegetables and some fruits, egg yolk, poultry, and meat. Fish is the main source of EPA and of docosahexaenoic acid (C22:6 n-3, DHA, also known as Cervonic acid) (9).


Vegetables are the main source of n-6 fatty acids. The most important n-6 fatty acid, LA, is found in large amounts in western diets in corn oil, safflower oil, sunflower oil (Figure 3), and soybean oil (10). It is plentiful in nature and found in practically all plant seeds, with the exception of palm and cocoa.

Figure 3. Safflower plant (CARTHAMUS TINCTORIUS) (from http://www.unigraz.at/~katzer/engl/Cart_tin.html)
1.4 METABOLISM OF PUFA

Plants can insert additional double bonds into oleic acid, a monounsaturated fatty acid that has a double bond, to form LA (with two double bonds) and LNA (with three double bonds). Vertebrate animals cannot insert double bonds more proximal to the methyl end than the seventh carbon atom. Furthermore, all metabolic conversion occurs without altering the methyl end of the molecule that contains the n-3 and n-6 double bonds. Therefore once ingested, n-3 and n-6 fatty acids are not interconvertible.

The diet primarily contains EFA in the form of LA and LNA, which are synthesized by plants. Within the human organism these 18-carbon precursors can be elongated and desaturated to produce more highly unsaturated members of their family, principally, arachidonic acid and docosahexaenoic acid. The liver is the primary site for EFA metabolism, although it does take place in other tissues as well (11).

The metabolic pathways of LN and LNA are depicted in Figure 4. The first part of this pathway takes place in the endoplasmic reticulum and involves the formation of AA (20:4) and docosapentaenoic acid (C22:5 n-3, DPA), which are produced by alternating elongation and desaturation steps that are catalyzed by fatty acid elongase δ6- and δ5- desaturases. The mechanism of the final conversion to 22:5 n-6 and DHA is not completely understood. Traditionally this final step has been thought to occur via a δ4-desaturase (12), but no proof of the existence of this enzyme has been ever found (13). Most lipid biochemists are convinced that the last step occurs primarily via chain elongation and desaturation followed by a retro-conversion step involving peroxismal beta-oxidation, via the “Sprecher pathway”(14).
Figure 4. Metabolic pathways for formation of AA, DHA, and EPA from the precursors LN and LNA.

For each fatty acid, two alternate pathways are shown, both of which are comprised of a δ-6 desaturase and a retro-conversion step involving δ-4 desaturase.

(http://www.eunutroll.org/calvani.htm)

Infante and Huszangh (15,16) believe that the current evidence supports their proposal that the biosynthesis of 22:5n-6 and DHA occur via separate channeled carnitine-dependent mitochondrial pathways as shown in Figure 5. According to their view, the outer mitochondrial membrane may well be the sole site for DHA, whereas EPA and AA could be synthesized in the endoplasmic reticulum. They suggest that the mitochondrial and microsomal systems could be interregulated, redundant systems. The essential features of this pathway include the transport of acetate and long chain fatty acid substrates into mitochondria as carnitine esters (17), the use of
D-α-tocopherol or its quinone metabolites as specific cofactors in this pathway (18), a much greater incorporation of mitochondrial acetate when 22-carbon PUFA are present (19), and an intra-mitochondrial source of acetyl units, such as acetyl-carnitine, as the acetyl donor.

Several in vivo and in vitro studies on rats and other animals have shown that LNA is a strong suppressor of n-6 fatty acid metabolism, whereas ten times as much LN is required to give an equal suppression of n-3 metabolism (12). In vitro studies on rat liver microsomes has confirmed that the n-6 and n-3 substrate competition occur at several steps in the microsomal pathway (20). These effects may not only be mediated by direct competitive mechanisms, but also very likely via regulation of the activity of desaturation and elongation enzymes at the level of gene expression (21).
1.5 BIOLOGICAL AND FUNCTIONAL EFFECTS OF PUFA

Both LNA and LA are now regarded as nutritionally essential fatty acids (EFA). However, all the classic symptoms of essential fatty acid deficiency (dermatitis, growth retardation, and infertility) can be completely cured by administration of the n-6 fatty acids alone. These symptoms relate to the biological function of n-6 fatty acids (22) examples of which include LA, the structural component in the ceramides that provides a water barrier of the skin and is involved in energy nutrition and fat absorption; AA, a precursor of the eicosanoids, which are local hormones that participate in a number of physiological as well as pathophysiological conditions (e.g. parturition initiation, platelet aggregation, renal electrolyte regulation, blastocyte implantation, and activation of immune cells); and n-6 fatty acids, which possibly also play a role as second messengers in signal transduction across cell membranes.

There is insufficient understanding of the essentiality of the n-3 fatty acids. The n-3 fatty acids can partially substitute for the n-6 fatty acids, which may comprise a sparing effect to ameliorate some of the EFA deficiency symptoms (e.g. growth retardation), but are now considered also to have their distinct role.

The biological functions of dietary n-3 fatty acids in the organism involve a carbon source for energy production and as a precursor to synthesis of other molecules (20). For example, EPA and DHA serve as a precursor for “n-3 eicosanoids,” which have a much lower potency than those that are derived from n-6 fatty acids and are only formed in considerable amounts in tissues at fairly high dietary intakes of EPA and DHA. For this reason the effects of n-3 fatty acids on the synthesis, bioactivity, and metabolic clearance of eicosanoid products accounts partly for their anti-inflammatory properties. There is increasing evidence for a specific role of DHA in membrane function, especially in retinal and other neuronal tissues. Deficiencies
of n-3 PUFA lead to a loss of DHA from brain and retina rod outer segment phospholipids with a compensatory replacement by 22:5 n-6 fatty acids. The minor change in membrane phospholipid structure is sufficient to lead to memory loss, learning disabilities, and impaired visual acuity.

1.6 EICOSANOID METABOLISM

EFA in the plasma membrane serve as substrates for the enzyme cyclooxygenase (COX) and lipoxygenase (LOX) and are converted into a number of important, very active, short-lived, hormone-like compounds referred to as “eicosanoids.” The families of prostaglandins, leukotrienes, and related compounds are called eicosanoids because they are derived from 20-carbon EFA that contain three, four, or five double bonds (AA, EPA, and di-homo-γ-linolenic acid or C20:3 n-6, DGLA) and include prostaglandins (PGE), prostacyclins (PGI), thromboxanes (TXB) and leukotrienes (LTB).

In human beings, AA is the most abundant precursor; it is either derived from dietary LA or ingested in the diet. Arachidonate is esterified in phospholipids of the cell membrane or other complex lipids. The concentration of free AA in the cell is very low, so the biosynthesis of eicosanoids depends primarily on its hydrolytic release from phospholipids in the cell membrane by phospholipase A2 (PLA2). Eicosanoids influence numerous metabolic activities including platelet aggregation, inflammation, hemorrhage, vasoconstriction, vasodilatation, blood pressure, and immune functions. The overall synthesis of the prostaglandins, leukotrienes, and thromboxanes from AA and EPA are shown in Figure 6. AA is the substrate for the “series 2” prostaglandins, the prostaeyclins, the thromboxanes and the “series 4” leukotrienes, whereas EPA is the substrate for the “series 3” prostanoids and the “series 5” leukotrienes. The PUFA-
mediated immune response may be altered by changes in the production of immunologic mediators known as cytokines.

The synthesis of prostaglandins is accomplished by COX, a microsomal enzyme (also known as endoperoxide synthase or fatty acid cyclooxygenase) that uses AA or EPA as a substrate. There are two isoforms of this enzyme, COX-1 and COX-2. The former is constitutively expressed in most cells (i.e. gastric mucosa, vasculature, glomeruli, and collecting ducts of the kidney), and is concentrated in the endoplasmic reticulum. In contrast, COX-2 is normally not present but may be induced, both in the endoplasmic reticulum and over the surface

Figure 6. PUFA Oxidation Metabolic Route: AA- and EPA-derived eicosanoids (http://www.eunutroll.org/calvani.htm)
of the nucleus, by certain serum factors that serve as inflammatory cytokines (IL-11, IL-1β, IL-6, TNFα, ), by growth factors (TGFβ, EGF), by tumor promoters, and by cAMP. Induction of COX is inhibited by treatment with glucocorticoids such as dexamethasone (23, 15, 16). Moreover, non-steroidal anti-inflammatory drugs compete directly with arachidonate for binding to both COX-1 and COX-2 sites thus inhibiting their activity (16).

More recently, an “unorthodox” metabolic route for PUFA oxidation has been revealed. In particular, PUFA derived from membrane phospholipids can undergo auto-oxidation in vivo, generating a complex mixture of hydroperoxides, epoxides, and cyclic peroxides (24). Of particular medical interest are the isoprostanes and epoxides of AA and other PUFA. Isoprostanes of PUFA constitute a family of prostaglandin-related compounds that act as autacoids. The urinary isoprostane index offers a method to estimate lipid peroxidation in various diseases. Epoxides of PUFA can be formed by autooxidation, by cytochrome P450, and possibly by the oxidative burst of inflammatory cells (24). Epoxides of LNA are toxic, whereas epoxides of AA have a wide range of biological effects. The 5,6-epoxide of AA is an excellent substrate for COX and thromboxane synthase and has vascular and renal effects (25,26).

1.7 ANALYSIS OF FATTY ACIDS AND PROSTAGLANDINS BY GAS CHROMATOGRAPHY

The preparation of the methyl ester derivatives of fatty acids for analysis by gas chromatography is by far the most common chemical reaction performed by lipid analysts. In acid-catalyzed methylation or transesterification procedures, the most common and mildest reagent is 5% (w/v) anhydrous hydrogen chloride in methanol. This method is best suited to bulk preparation of the reagent. A simpler and safer procedure that is more suited to small-scale
preparations involves the slow addition of a solution of acetyl chloride to cooled, dry methanol. Some methyl acetate is formed as a by-product, but it does not generally interfere with methylation. As an alternative to methanolic hydrogen chloride, a solution of concentrated sulphuric acid in methanol transesterifies lipids in the same manner and at much the same rate. It is very easy to prepare, although high temperatures are required for the reaction to occur and the yield of methyl ester product is generally low.

In base-catalyzed methods, triglycerides and phospholipids are transesterified very rapidly in anhydrous methanol in the presence of a basic catalyst, usually sodium methoxide, which facilitates the exchange between glycerol and methanol, and the methyl esters required are quickly obtained in quantitative yield. Free fatty acids are not esterified under such conditions, so care must be taken to exclude water from the reaction medium to prevent the occurrence of this unwanted and irreversible hydrolytic reaction. Sodium methoxide in anhydrous methanol, prepared simply by dissolving fresh clean sodium in dry methanol, is the most popular reagent. Boron trifluoride-methanol is very popular as a methylating reagent, but it has a number of disadvantages. Inconsistent results are obtained with this method, which are thought to be due to the age of the reagent, probably as a result of the formation of further fluoroboron compounds by reaction with atmospheric oxygen. Apart from the undesirable reaction with unsaturated fatty acids, boron trifluoride/methanol will also cleave the rings in cyclopropane fatty acids (commonly encountered in microorganisms); and it reacts with the other reagents to produce spurious peaks in chromatograms (27).

Pentafluorodiazooalkanes have been used to prepare derivatives from unesterified fatty acids that are suitable for GC with sensitive electron-capture detection(28, 29, 30). Diazomethane (31) reacts rapidly with unesterified fatty acids to give methyl esters but does not affect
transesterification of other lipids. Solutions of diazomethane in diethyl ether (with a little alcohol) are stable for short periods if stored in the dark over potassium hydroxide pellets at refrigeration temperatures. However, if they are kept too long, polymeric by-products are formed, which interfere with GC analyses (32-34). Diazomethane is potentially explosive and great care must be exercised in its preparation; in particular, apparatus with ground-glass joints and strong light must be avoided. The reagent is toxic and is liable to cause development of specific sensitivity; nitrosamides used in the preparation of diazomethane must be handled with care, as they are potential carcinogens. Small-scale procedures are recommended, because if sensible precautions are taken, the risks to health are slight, while methyl esters are prepared rapidly with virtually no formation of artifacts. Suitable micro-equipment is available from commercial sources (34-36).

From the previous discussion, it is evident that there are a number of compounds with highly similar structures, which play important roles and need to be measured in food materials and in the body. High-performance liquid chromatography is a popular means by which scientists resolve and quantify fatty acid and fatty acid-derived compounds, mainly because the compounds that are measured do not have to be modified for analysis (37). However, in gas chromatography, the molecules need to be made thermally stable to withstand vaporization for

\[
\begin{align*}
\text{CH}_3\text{CH}_2\text{C} &\text{O} \\
\text{CH}_3\text{CH}_{2n}\text{C} &\text{OH} \\
\text{CH}_3\text{C} &\text{O}\text{CH}_3
\end{align*}
\]

Figure 7. Preparation of methyl ester of fatty acids
entrance into the gas phase, usually to more apolar derivatives, that are volatile at GC-friendly
temperatures and thus require chemical modification (38). One moiety that is common to these
compounds is the carboxylic acid, which is commonly converted to the methyl ester prior to
analysis. Fatty acids are converted to the methyl esters (Figure 7) by a number of different
methods, including treatment with an acid catalyst (hydrochloric acid or sulfuric acid) in the
presence of methanol, tetramethyl ammonium hydroxide, borontrifluoride, IRA-400 and
methanolic hydrochloride, or diazomethane (27). Many of these reagents are unsuitable for
formation of methyl esters of prostaglandins due to conditions that may degrade these unstable
species, although diazomethane has enjoyed widespread use as an appropriate reagent for this
application (28). Unfortunately, there are a number of drawbacks to the use of diazomethane
that include a lengthy preparation of the reagent, a potential to explode, and precursors used for
preparation of diazomethane that are mutagenic and carcinogenic (35, 36, 34).

Trimethyl silyl diazomethane (TMSD) was originally used with methanol as the
methylating reagent in the preparation of drugs, organic compounds, and some fatty acids (39).
The mechanism for formation of the methyl ester with TMSD is shown in Figure 8. Three-point
GC calibration curves were demonstrated by Yokota et al. (40) to be linear over a three- to four-
fold range, and the reagent was shown to be as capable as diazomethane in promoting the
formation of methyl esters as shown in Figure 9. Moreover, TMSD is stable, and so it does not
need to be prepared from a precursor compound, is less hazardous, and provides an excellent
yield at room temperature and thus does not require heat for efficient catalysis (40).
Several procedures have been described to recover lipids from organisms, mainly bacteria, tissues, or cell types. The first study on the dissolution of lipid materials was done by Chevreul (41). In 1879, Franz Von Soxhlet described the first method based on dissolution of
milk lipids in diethyl ether (42). A further improvement was made in 1914 with a mixture of ethanol:ether (3:1) for lipid extraction (43). Chloroform was used in extraction of lipids from bacteria (44) or plant material (45). Folsch developed mild procedures for quantitative extraction of brain lipids leading to the classical use of a chloroform-methanol mixture. The factors affecting the extractability of lipids by solvents have been reviewed comprehensively by Zahler and Niggli (46). Hexane is quite popular in the extraction of a wide range of lipid oxidation products from biological media such as plasma, rat liver, and microsomes for quantitative determination (47).

Adiposomes store ether lipids inside the cells for better packaging and distribution inside the cell, particularly in the dynamic organelle system composed of neutral lipids surrounded by a monolayer of phospholipids and associated proteins. Diethyl ether would seem to be an appropriate solvent for extraction of these lipids since it has similar physical/chemical properties to the molecules that require extraction (48). A mixture of hexane:diethyl ether was used in the extraction of oxysterols and other lipid oxidation products from normal human arteries, fatty streaks and other lipid oxidation products (49). Whereas petroleum ether has been chosen as an appropriate reagent for lipid extraction, hexane has been widely used in quantitative determination of lipids and the lipid metabolites.
1.8 OBJECTIVES OF THE RESEARCH PROJECT

The first objective of the research project is to study the TMSD reaction to determine [1] the optimal amount of TMSD for methylation and [2] to attempt to develop the reaction for use in safer solvents than the benzene solvent that is usually used. The second objective is to determine whether the TMSD reaction could support methylation of C18 to C22 polyunsaturated fatty acids and the prostaglandins, PGE$_2$ and PGF$_{2\alpha}$. The third objective is to develop GC calibration curves for these fatty acids and prostaglandins using appropriate internal standards. The final objective involves an attempt to determine whether this method could be used as a part of the chemical derivatization scheme to characterize these lipids by GC-MS in cell culture and other cell-free systems, such as those containing enzymes like cyclooxygenase.
EXPERIMENTAL PROCEDURES

2.1 Fatty acid preparation

All of the fatty acids, arachidonic acid, linoleic acid, γ-linolenic acid, α-linolenic acid, eicosapentanoic acid, docosahexanoic acid were shipped in an ethanol stock (Cayman Chemical, Ann Arbor). A stock concentration of 1 mg/mL in ethanol was prepared for all of the fatty acids. A sample calculation to determine the number of moles of arachidonic acid is shown below for 50 μL of the stock solution, which contained 1 mg/mL of arachidonic acid (MW = 304.5 g/mol).

\[ \text{1 mg/mL} \times \frac{0.050 \text{ mL}}{304.5 \text{ g/mol}} = 1.642 \times 10^{-7} \text{ mol}. \]

In some experiments, the volume of fatty acid preparation used was kept constant, and therefore, the concentration of the stock solution was varied to allow different concentrations of fatty acids (preparation #1). In other experiments, stock concentrations of fatty acids were prepared in ethanol as shown in Table 1 (preparation #2).

Table 1   Molecular weight and stock concentrations of fatty acids used for the experiments

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Molecular weight (g/mol)</th>
<th>Stock conc. prepared (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arachidonic acid</td>
<td>318</td>
<td>2.5</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>294</td>
<td>2.3</td>
</tr>
<tr>
<td>Gamma- linolenic acid</td>
<td>292</td>
<td>2.3</td>
</tr>
<tr>
<td>Alpha-linolenic acid</td>
<td>292</td>
<td>2.3</td>
</tr>
<tr>
<td>Eicosapentanoic acid</td>
<td>302</td>
<td>2.5</td>
</tr>
<tr>
<td>Docosahexanoic acid</td>
<td>342</td>
<td>2.6</td>
</tr>
</tbody>
</table>
The internal standard used in the experiments was heptadecanoic acid. Heptadecanoic acid (MW 270.45 g/mol) was provided as a powder, and a stock solution of 2.3 mg/mL ethanol was prepared.

2.2 Preparation and optimization of the solvent system used in methyl esterification with TMSD

We used the acetone-methanol solvent system (20% methanol in acetone), which provided an optimal yield of methyl ester product relative to other solvent systems, with fewer side products. For the preparation of 20% methanol in acetone, 800 μL of acetone is taken in the test tube to which was added 200 μL of methanol, and the mixture was vortexed for 1 min prior to use. The solvent system was prepared fresh daily (40).

2.3 Preparation and standardization of the methylating agent

A stock solution of 2.0 M TMSD was provided by Aldrich. The optimum concentration used for all our reaction steps was 0.02 M TMSD. To make the stock solution, 10 μL of 2.0 M TMSD was added to 990 μL of heptane, and the mixture was subjected to vortexing for 1 min. Fresh TMSD reagent solutions were made on the day of their use (40).

2.4 Conversion of polyunsaturated fatty acids and phospholipids to their methyl esters by TMSD

The procedure for methyl esterification of fatty acids by TMSD was modified from a previously established procedure (40). A fixed quantity (10, 20, 50 μL) of each of the polyunsaturated fatty acids and 10 μL of the internal standard, heptadecanoic acid, was added to
a test tube, and the mixture was evaporated to dryness under a stream of nitrogen (N2). Addition
of 350 μL of 20% methanol-acetone solution was used to resuspend the fatty acid and provide a
source for the methyl group, after which an equal volume of 0.02 M TMSD was added, and the
mixture was vortexed for 1 min and left at room temperature for 20 to 30 min, vortexing
periodically (every 15 min). The mixture was evaporated under a stream of nitrogen or argon,
after which 100 μL of ethyl acetate was added to the lyophilized sample. The resulting products
were then subjected to analysis by gas chromatography-mass spectrometry.

An experiment was conducted in which varied amounts (50 μL to 500 μL) of TMSD
(with an equal volume of 20% methanol-acetone 1:1) were used with a constant amount of
arachidonic acid (20 μL of 1 mg/mL). The maximal production of arachidonic acid methyl ester
occurred at 100 μL of TMSD, and when a volume greater than 350 μL of TMSD was used, there
were additional, undesirable products formed. Fatty acids other than arachidonic acid required
amounts of TMSD up to 350 μL to give the methyl ester. The optimal amount required for the
reaction was eventually found to be 350 μL of 0.02 M TMSD (0.007 mol TMSD). For
convenience, we used equal volumes of the solvent system and the methylating agent. The
reactions were carried out at room temperature. The data for conversion of the fatty acids to their
methyl esters were confirmed by thin layer chromatography using silica as the stationary phase
and 0.1% glacial acetic acid as the mobile phase. TLC was also used to detect side products. The
products of the reaction in ethyl acetate were then subjected to gas chromatography and mass
spectrometry for analysis (50, 51).
2.5 Methyl esterification of fatty acids contained in phospholipid and egg yolk

We also used the methylation process to identify the constituent fatty acids from phosphatidylcholine containing palmitic acid and arachidonic acid. A 20 μL aliquot of the sample from the stock concentration of 1 mg/ml (in water) was delivered to a test tube and sonicated. Two mL of potassium hydroxide (10%) were added to the sample and heated at 50 °C for one hour, after which 1M HCl was added to bring the pH to 3. Extraction of the liberated fatty acids was accomplished with hexane in 10% (v:v) ether. The organic phase was collected in a clean test tube, and the process was repeated two times. A pinch of magnesium sulfate was added to the mixture, which was set aside for 1 hr to allow for the removal of water. The drying agent was then removed by centrifugation. The extracted hexane layer was then evaporated under nitrogen to bring it back to half of the original volume, which came to about 500 μL. The saponified sample was preserved in argon. A certain fixed amount (10, 20, and 50 μL) of the saponified sample was evaporated to dryness under N₂, and the lyophilized sample was resuspended in methanol/acetone solvent and subjected to methyl esterification by using TMSD using the procedure outlined above.

A 20 μL sample of egg yolk (Eggland’s Best) and 2 mL of potassium hydroxide (10%) were combined and heated at 50 °C for one hour, and fatty acid methyl esters were prepared in the same manner as for those prepared from phospholipid. Fatty acid methyl esters were prepared from fish oil capsules (Nature Made) in the same manner as those prepared from egg white. The same procedure was followed for derivatisation of the fatty acid capsules as that for the fortified egg yolk (52-54).
2.6 Preparation of the stock solutions of prostaglandins

Dilute solutions of the prostaglandins PGE$_2$ and PGF$_{2\alpha}$ were prepared from a stock of 1mg/mL in ethanol. The stock solution was prepared by dilution in neat ethyl alcohol. Several-fold dilution of the prostaglandin was done to make a stock concentration of 1 ng/ml. The deuterated sample of PGE$_2$-d$_4$ (Cayman Chemical) and PGF$_{2\alpha}$-d$_4$ (Cayman Chemical) came as a stock of 50 μg/ml in ethanol. The four deuterium atoms are located at the third and fourth carbons at the carboxylate end of the prostaglandin. The deuterated-PGE$_2$ and -PGF$_{2\alpha}$ stocks were diluted with 100% pure ethyl alcohol to a concentration of 1 ng/ml; 10 μL was used as an internal standard in the calibration curve for the respective prostaglandin (55,56).

2.7 Methyl esterification of prostaglandins by TMSD

PGE$_2$ derivatives for GC-MS analysis were prepared by first converting the carbonyl group to an oxime according to a modification of the method of Kristrer Green (57), methylating the carboxylic acid ester with TMSD, and then silylating the hydroxyl groups according to a modification of the method used for penicillins and cephalosporins (58). A sample of PGE$_2$ (1 ng/ml) was added to a test tube along with 10 μL of the deuterated sample (1 ng/ml), and solvent was removed by evaporation under nitrogen. An aliquot (1 mg) of methyloxime in 100 μL of pyridine was added to a test tube containing the lyophilized sample, vortexed for 1 min, and kept in a hot water bath at 60 °C for 2 to 3 hr. The methyl ester was formed by using trimethylsilyl diazomethane in a solvent system of 20% methanol in acetone using the same procedure as described above. To prepare the silylated compound, the lyophilized derivative was resuspended in 75 μL of bis-trimethylsilyl trifluoroacetamide (BSTFA) in 1% trimethyl chlorosilane (TMCS) and 25 μL of pyridine, and the reaction was allowed to proceed in a hot water bath (60°C) for 1
hr. The samples were then subjected to chromatography on a silica column and eluted from the column with ethyl acetate, and the volume of the preparation was reduced to one-half of the original volume prior to GC-MS analysis. PGF$_{2\alpha}$ has only alcohol groups that require silylation, and thus the methyl esterification was accomplished by the TMSD reaction, and then the three hydroxyl groups were silylated as described for preparation of the PGE$_2$ derivative.

2.8 Analysis of prostaglandins by methyl esterification in a cell-free system

To analyze prostaglandin production in cell culture, we have obtained cell culture media in which A-549 cells and 9HTE cell-free system were grown. A 1.5-mL aliquot of the media was mixed with 2 mL of distilled, deionized water that was acidified with HCl to pH 3.0, and the mixture was kept on ice for 15 minutes. A prostaglandin-containing fraction was obtained by purification on a C18 Sep-Pak cartridge column in the following manner. After application of the sample, the column was washed with 15% methanol in acidified water (pH 3.0), followed by 2 mL of water and 2 mL of acetonitrile, and then washed with distilled, deionized water. The prostaglandin fraction was eluted with 4 mL of methyl formate, which was evaporated under a stream of nitrogen and then resuspended in solvent for derivatization according to the procedure outlined for PGE$_2$, except that a silica column was used after the methylation reaction (59-63).

2.9 Prostaglandin formation in the cylooxygenase-1 reaction

An amount of 100 μL of phosphate buffer was added to 50 μL of 1% Tween-20, 100 μL of 50% glycerol, 100 μL of 2.5 mM phenol, and 8 μL of cylooxygenase-1 enzyme preparation (0.8kU/μL sample; Cayman Chemical) to produce a total of 500 μL. An aliquot of 50 μL (from 2.5 mg/mL stock) of the fatty acid (arachidonic acid, γ-linolenic acid, or EPA) was added to a
test tube and the solvent was removed under a stream of nitrogen. The 500 μL of the components listed above were added to the tube containing the lyophilized fatty acid, and the mixture was sonicated to resuspend the fatty acid. The whole reaction mixture was placed in a 37 °C water bath for 25 min, after which 200 μL of anhydrous methanol and 100 μL of 1 M citric acid were added to quench the reaction. Three successive extractions were done with hexane, and the sample was prepared for GC-MS analysis per the procedure described for PGE2 (64-66).

2.10 Gas Chromatography and Mass Spectrometry Conditions

Methoxime and silyl derivatives of prostaglandin methyl esters were analyzed (62) in chemical ionization mode and electron impact mode in GC-MS using the Saturn Varian 3800 GC and 2000 MS with 8410 autosampler. Helium was used as the carrier gas with a VF-5 ms factor four capillary column (30 m long, 0.25 mm i.d., 0.25 μM film thickness) (67). A 1 μL of the sample was injected onto the column with a speed of 5 μL/sec. Chromatographic separations were performed using a constant pressure injection of 10 psi. The GC column temperature, in general, was programmed from 240 to 300 °C at a rate of 40 °C/min. In constant pressure mode, the column flow rate would decrease as column temperature increased during the run. This would cause a slight shift in the mass axis for the collection of the GC/MS data. To assure correct mass assignments, the mass calibration was run with the GC column oven set at or near the high temperature for the column program in the GC/MS method. For analysis of prostaglandins in the chemical ionization and electron impact mode, in general, the injector temperature was set at 250 °C with a column oven end-time of 18.50 min. The column flow was maintained constant at 1.4 mL/min, with the stabilization time being 0.20 min. The detector
bunch rate during data acquisition is 4 Hz as compared to the maximum value of 10Hz. The noise monitor length is 64 sec, the bunched points being 6.4 sec.

The MS method was specified to have a 2.4 min solvent delay. As a rule, the first chromatographic peak eluted shortly after 2.0 min. In all our experiments the elution time was between 5 and 12 min, the full time length of running being 15 min. For CI method conditions, the injector temperature was set at 250 °C and the column oven temperature was set at a range between 200 to 300 °C, with a total holding time of 11 min. The initial signal noise ratio was five with a deviation tolerance of 100% and a range tolerance of 10%. The CI reagent was methanol.

In the EI method, the injector temperature was set at 250 °C. The column oven temperature was set at a range of 200 to 275 °C with a total hold time of 12 min. The tolerance range for the signal to noise detection was 20% as compared to CI mode, which was 10%. This ensured a better signaling for the specific mass ions of the elutants.

Calibrating with an internal standard accounted for the variation in sample volumes lost during sample preparation. A small amount of internal standard was added to sets of standards and samples. Any variation in the sample volume was reflected by a detectable variation in the ratio of the areas and amounts for the internal standard.
Table 2: Gas Chromatography conditions for fatty acid methyl esters (chemical ionization mode)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
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<tbody>
<tr>
<td>Emission current</td>
<td>10 microamps</td>
</tr>
<tr>
<td>Mass defect</td>
<td>0mmu/100u</td>
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<tr>
<td>Count threshold</td>
<td>1 counts</td>
</tr>
<tr>
<td>Multiplier offset</td>
<td>0 volts</td>
</tr>
<tr>
<td>Scan time</td>
<td>10.00 secs</td>
</tr>
<tr>
<td>Segment start time</td>
<td>3.00 mins</td>
</tr>
<tr>
<td>Segment end time</td>
<td>10.00 mins</td>
</tr>
<tr>
<td>Segment low mass</td>
<td>40m/z</td>
</tr>
<tr>
<td>Segment high mass</td>
<td>650m/z</td>
</tr>
<tr>
<td>Ionization mode</td>
<td>CI</td>
</tr>
</tbody>
</table>

Table 3: Gas Chromatography conditions for Fatty Acid Methyl Esters (Electron Impact Mode)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emission current</td>
<td>20 microamps</td>
</tr>
<tr>
<td>Mass defect</td>
<td>0 mmu/100u</td>
</tr>
<tr>
<td>Count threshold</td>
<td>1 counts</td>
</tr>
<tr>
<td>Multiplier offset</td>
<td>0 volts</td>
</tr>
<tr>
<td>Scan time</td>
<td>0.500 secs</td>
</tr>
<tr>
<td>Segment start time</td>
<td>3.00 mins</td>
</tr>
<tr>
<td>Segment end time</td>
<td>15.00 mins</td>
</tr>
<tr>
<td>Segment low mass</td>
<td>40m/z</td>
</tr>
<tr>
<td>Segment high mass</td>
<td>650m/z</td>
</tr>
<tr>
<td>Ionization mode</td>
<td>EI</td>
</tr>
</tbody>
</table>
Table 4: Gas Chromatography Conditions for Prostaglandin Methyl Esters (Chemical Ionization Mode)

<table>
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<th>Front injector type</th>
<th>1177</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>250</td>
</tr>
<tr>
<td>Column flow (ml/min)</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Table 5: Column oven temperature for the Chemical Ionization Mode

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Rate (°C/min)</th>
<th>Hold (min)</th>
<th>Total (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>-</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td>240</td>
<td>40.0</td>
<td>0.00</td>
<td>3.00</td>
</tr>
<tr>
<td>304</td>
<td>8.0</td>
<td>0.00</td>
<td>11.00</td>
</tr>
</tbody>
</table>

Table 6: Gas-Chromatography conditions for prostaglandin methyl esters (Electron Impact Mode)

<table>
<thead>
<tr>
<th>Front Injector type</th>
<th>1177</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>250</td>
</tr>
<tr>
<td>Column flow (ml/min)</td>
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</tr>
</tbody>
</table>

Table 7: Column oven temperature for the Electron Impact Mode

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Rate (°C/min)</th>
<th>Hold (min)</th>
<th>Total (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>-</td>
<td>2.00</td>
<td>2.00</td>
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<tr>
<td>240</td>
<td>40.0</td>
<td>0.00</td>
<td>3.00</td>
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<tr>
<td>275</td>
<td>8.0</td>
<td>4.50</td>
<td>11.88</td>
</tr>
</tbody>
</table>
RESULTS

3.1 Methylation of Fatty Acids/Optimization of the Methylation reaction for TMSD

In addition to defining the optimal amount of TMSD that was needed to form the methyl ester, in experiments that were explained in the Experimental Procedure, there was also an interest in evaluating several solvents for their ability to substitute for benzene as the solvent in a reaction to form the methyl ester of arachidonic acid. Figures 10, 11, 12, and 13 show the calibration curves for arachidonic acid methyl ester for benzene, ether, chloroform, and acetone, respectively.

![Calibration Curve](image)

Figure 10. Calibration curve for methyl ester formation with TMSD using benzene as the solvent. Each data point represents the average of two or more samples. The $R^2$ value is 0.9988 and the equation for the line is $y = 25.5x - 23$. 
Figure 11. Calibration curve for methyl ester formation with TMSD using ether as the solvent. Each data point represents the average of two or more samples. The $R^2$ value is 0.9492 and the equation for the line is $y = 58.16x - 54.25$.

Figure 12. Calibration curve for methyl ester formation with TMSD using chloroform as the solvent. Each data point represents the average of two samples. The $R^2$ value is 0.7764 and the equation for the line is $y = 568x - 734$. 
To aid in determining the optimal conditions for formation of the methylated fatty acids with TMSD, the extent of the methylation reaction had to be monitored. The first and more qualitative approach to determining the extent of the methylation involved thin layer chromatography. The free and methylated fatty acids were separated on silica plates using a solvent system containing an apolar solvent of hexane:diethyl ether:glacial acetic acid, with phosphomolybdic acid as the reagent used to visualize the two species. Retention factor values for the free and methyl esters of the fatty acids were 0.5 and 0.75, respectively, and TLC plates showing native and methyl esters in preliminary studies for methylation with TMSD of a linolenic acid is shown in Figure 14 (68, 69). There was originally a minimal conversion of
linolenic acid to the methyl ester as shown by the relatively small amount of material with $t_R$ of 0.75 in Figure 14, but nearly complete conversion to the methyl ester was obtained following optimization of the amount of TMSD used in the methylation (data not shown).

Figure 14. Thin layer chromatography of (A) free and (B) esterified after reaction of linolenic acid with TMSD. The $t_R$ values of the free and esterified linolenic acid are 0.5 and 0.75, respectively

The TLC was also used as the screening procedure to identify undesirable side products. A more quantitative measurement to determine the percent conversion of the fatty acid to its methyl ester was taken from the literature (70). The extent of conversion for a fatty acid in the presence of the internal standard was determined by independently running the methylation reaction for the fatty acid alone and then, in a second reaction, conducting the methylation reaction with the fatty acid and the internal standard in the same reaction tube. The total number of moles for the individual reactions was set equal to the total number of moles for the fatty acid and internal standard together for the second reaction. The peak area of the fatty acid obtained from the chromatographic data when assayed alone was divided by the peak area for the fatty
acid in the reaction with the combined fatty acid and internal standard and multiplied by 100 to obtain the percent conversion. The percent conversions for several fatty acids are given below in Table 8, which reveals that all of the fatty acids tested were quantitatively converted to their methyl esters except for linoleic acid. In addition to evaluation of solvents for the methylation reaction supported by TMSD, other experiments were conducted to determine the optimal amount of TMSD to use in the reaction (see Experimental Procedures).

Table 8: The extent of conversion of fatty acids in the TMSD reaction to the methyl ester

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Desgination</th>
<th>% Conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linoleic acid</td>
<td>18:2</td>
<td>70</td>
</tr>
<tr>
<td>α-Linolenic acid</td>
<td>18:3</td>
<td>92</td>
</tr>
<tr>
<td>γ-Linolenic acid</td>
<td>18:3</td>
<td>90</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>20:4</td>
<td>95</td>
</tr>
<tr>
<td>Eicosapentenoic acid</td>
<td>20:5</td>
<td>99</td>
</tr>
<tr>
<td>Docosahexenoic acid</td>
<td>22:6</td>
<td>100</td>
</tr>
</tbody>
</table>

3.2 Chromatograms and Mass Spectra of Some Fatty Acids Methyl Esters

The reaction with TMSD was carried out with a number of polyunsaturated fatty acids, and the methyl esters were subjected to gas chromatography and ion trap mass spectrometry. The chromatogram and mass spectra of the arachidonic acid methyl ester are shown in Figure 15. Interestingly, the methyl ester elutes from the column in a non-Gaussian fashion, with a considerable tail on the backside of the peak; the retention time for the apex of the peak occurs at 12.9 min, with most (about 90%) of the compound eluting from 12.8 to 13.2 min. The apex was assayed for the mass spectrum, which possessed a prominent ion at 319 m/z, and other prominent ions above 200 m/z of 203, 207, 221, 269, and 287. Three ions with an m/z of 97, 137 and 177
m/z have been reported as prominent ions in the mass spectrum for arachidonic acid methyl ester (71). These ions are envisioned to be generated by cleavage of the carbon-carbon bond on the ester side of the double bonds that start at carbons 8, 11, and 14.

The chromatogram of the methyl ester of eicosapentanoic acid and its mass spectrum are shown in Figure 16. The compound eluted from 13.0 to 13.5 min, its apex was at 13.1 min, and, like arachidonic acid, was not Gaussian. One distinguishing ion had an m/z of 319, with other ions above 200.

Figure 15. Chromatogram (above) and mass spectrum (below) of arachidonic acid methyl ester generated by the TMSD methylation reaction.

The chromatogram of docosahexanoic acid methyl ester and its mass spectrum are shown in Figure 17. The compound elutes in a range of 14.2 to 14.3 min and has a peak shape that is
not Gaussian. The molecular ion is expected to have an m/z of 343, but it is not a prominent ion. The other ions of m/z greater than 200 are those of 293, 310, and 213. Literature values for the fragmentation products include m/z of 201, 274, and 299 (72). A similar pattern of ions below m/z of 200 for all of the methyl esters generated thus far indicates that the regular structures of all these polyunsaturated fatty acids are cleaved at similar carbon-carbon bonds in all these molecules.

Figure 16. Chromatogram (above) and mass spectrum (below) of eicosapentanoic acid methyl ester generated by the TMSD methylation reaction
Figure 17. Chromatogram (above) and mass spectrum (below) of docosahexanoic acid methyl ester.
Figure 18. Chromatogram (above) and mass spectrum (below) were obtained for the methyl ester of linoleic acid.

Chromatograms and mass spectra were obtained for the methyl esters of linoleic acid, $\alpha$-linolenic acid, and $\gamma$-linolenic acid, which have prominent ions of 291, 294, and 293, respectively, as shown in Figures 18, 19, and 20. These prominent ions presumably represent the molecular ions.
Figure 19. Chromatogram (above) and mass spectra (below) were obtained for the methyl esters of α-linolenic acid.
Figure 20. Chromatogram (above) and mass spectra (below) were obtained for the methyl esters of γ-linolenic acid.

The oleic acid methyl ester was generated by the TMSD reaction, and its chromatogram and mass spectrum are shown in Figure 21. Oleic acid methyl ester elutes as a symmetrical peak, with an apex at about 12.2 min. The molecular ion at m/z 297 is the prominent ion in the mass spectrum, with a secondary peak at m/z 263 (73).
Figure 21. Chromatogram (above) and mass spectrum (below) for oleic acid methyl ester.

The compound that we wished to use as an internal standard, heptadecanoic acid, a 17-carbon, saturated fatty acid, and heptadecanoic acid methyl ester chromatogram and mass spectrum is shown in Figure 22 (74). Heptadecanoic acid methyl ester elutes from 11.8 to 11.9 min, with an apex at 11.85 min. The molecular ion with m/z of 285 is well represented in the mass spectrum, and the other ion that is generated has an m/z of 251.
3.3 Calibration Curves for Selected Polyunsaturated Fatty Acids

The calibration curves for arachidonic acid, eicosapentanoic acid, and docosahexanoic acid methyl esters are shown in Figures 23, 24, and 25, with heptadecanoic acid used as the internal standard. The standards were linear with concentration over four-, five-, and six-fold range for AA, EPA, and DHA methyl esters, respectively. A range of values from one greater to one less than the value for the molecular ion were used to calculate the peak area for each compound and for the internal standard.
Figure 23. Calibration curve for arachidonic acid methyl ester with heptadecanoic acid (HDA) as the internal standard. The x-axis values represent the amount of arachidonic acid in µL (concentration being 2.5 mg/mL).

Figure 24. Calibration curve for eicosapentaenoic acid methyl ester with heptadecanoic acid (HDA) as the internal standard. The x-axis values represent the mole ratio of EPA to HDA, with a constant amount of HDA used for each standard.
Figure 25. Calibration curve for docosahexanoic acid methyl ester with heptadecanoic acid (HDA) as the internal standard. The x-axis values represent the mole ratio of Docosahexaenoic acid to HDA, with a constant amount of HDA used for each standard.

3.4 Characterization of Chromatography and Mass Spectra of Prostaglandins PGE2 and PGF$_2$α

For TMSD to be truly useful as a broad-spectrum reagent for ester formation, experiments were conducted to determine whether it could be used with prostaglandins, PGE$_2$ and PGF$_2$α, in a synthetic regime to produce derivatives that could be characterized by GC-MS. The synthetic scheme for PGE$_2$ involved, in successive reactions, the methoximation of the carbonyl group, formation of the methyl ester with TMSD, and silylation of the hydroxyl group,
as shown in Figure 26. The prostaglandins were subjected to gas chromatography that promoted
the elution of prostaglandins PGE<sub>2</sub> and PGF<sub>2α</sub> at about 7.2 min. The chromatogram of the
derivative of PGE<sub>2</sub> and its mass spectrum are shown in Figure 27. The peak shape was not
symmetrical, and assay of the mass spectrum of the compound at the apex revealed several
prominent ions, including a major ion with an m/z of 513.

![Diagram of synthetic scheme]

Figure 26. Synthetic scheme for production of the derivative of PGE<sub>2</sub> that was subjected to gas
chromatography and mass spectral analysis.
Figure 27. Chromatogram (above) and mass spectrum (below) of the PGE$_2$ derivative. Several chromatograms are overlaid, which show a variety of peak sizes related to different amounts of PGE$_2$.

The chromatogram and mass spectrum of derivative of deuterated PGE$_2$ standard are shown in Figure 28. The mass spectrum is rather complex with no prominent ions that will not presently allow for the successful development of a quantitative assay for PGE$_2$. Nevertheless, a calibration curve for PGE$_2$ was constructed for a series of PGE$_2$ concentrations without the internal standard and is shown in Figure 29. The curve was linear over a 90-fold range, with a correlation coefficient of 0.9860. Work with the mass spec programming will need to be done in an attempt to preserve the molecular ion to allow the successful distinguishing of the two PGE$_2$ compounds within the same sample.
Figure 28. Chromatogram (above) and mass spectrum (below) of PGE$_2$-d4.
Figure 29. Calibration curve for PGE$_2$. Each data point represents the value for a single
determination and the total ion chromatogram was used to determine peak area. The
correlation coefficient is 0.9860 and the equation for the line is $y = 9.9994x + 480$

The derivatization of PGF$_{2\alpha}$ required only the formation of the methyl ester and silyl
ethers as shown in Figure 30.

Figure 30. Synthetic scheme for synthesis of a derivative of PGF$_{2\alpha}$. 
The chromatogram of the PGF$_{2\alpha}$ revealed an asymmetrical peak that, like the derivative for PGE$_2$ and the mass spectrum generated by the derivative in the chemical ionization mode, is shown in Figure 31.

Figure 31. The gas chromatogram (above) and the mass spectrum (below) of the standard PGF$_{2\alpha}$.

The mass spectrum for PGF$_{2\alpha}$ is virtually indistinguishable from that of the PGE$_2$ derivative, with the same prominent m/z 513 and 456 ions and identical minor ions of 150, 169, 249, 280, and 500. Both derivatives PGE$_2$ and PGF$_{2\alpha}$ possess similar molecular weight occasionally with different functional groups. The derivative for the deuterated form of PGF$_{2\alpha}$ did not give a substantial ion abundance in the chromatogram to be used successfully in a quantitative assay (data not shown). A calibration curve was determined by assaying the peak area for various
concentrations of PGF$_{2\alpha}$ without the deuterated-PGF$_{2\alpha}$, and is shown in Figure 32. As with PGF$_{2\alpha}$, the curve is linear over a 90-fold range with the lowest level evaluated 10 pg/mL.

Figure 32. Calibration curve for PGF$_{2\alpha}$. Each data point represents the value for a single determination, and the total ion chromatogram was used to determine peak area. The correlation coefficient is 0.9828 and the equation for the line is $y = 10220x + 112400$.

3.5 Attempts to characterize fatty acids in phospholipids, egg yolk, and fish oil

Preliminary attempts were made to determine whether the methyl esterification of fatty acids using the TMSD reagent could lead to their identification in biological samples. To determine whether the fatty acids AA and palmitic acid can be identified, the phospholipids phosphatidylcholine or phosphatidylethanolamine shown in Figure 33 were saponified to release the fatty acids, and then the free fatty acids were esterified using the TMSD reagent (75, 76).
Figure 33. Structures of phosphatidylcholine and phosphatidylethanolamine, which contain palmitic acid (18:0) and arachidonic acid (20:4) at the SN1 and SN2 positions, respectively.

The chromatogram of the resultant preparation shown in Figure 35 (panel A) contains only a single peak centered at about 6.9 min, which is significantly less than the retention time for arachidonic acid as shown in Figure 14. The GC conditions for this experiment were the same as were used for the prostaglandins, which leads to an earlier elution for the fatty acids relative to the conditions used for the fatty acids presented earlier in the Results. The mass spectrum of the species at the apex of the peak is shown in Figure 36 (panel B). An m/z of 391 was observed in a few experiments (not shown), which may be distinguishing for palmitic acid methyl ester (m/z 279), although palmitic acid methyl ester was not synthesized in this study.
In another experiment, the fatty acids that were esterified in egg yolk were saponified in potassium hydroxide, and the resultant fatty acids were then esterified with TMSD and resolved and identified by GC-MS. The chromatogram of the broadly eluting peak and the mass spectrum are shown in Figure 35. The mass spectrum at the peak of the chromatogram was complex and does not have ions that distinguish any of the fatty acids.
Figure 35. The chromatogram (above) and mass spectrum (below) of a sample of egg yolk that was saponified and treated with TMSD to identify hexane-extracted fatty acids.

Finally, fish oil capsules were treated with potassium hydroxide to liberate the esterified fatty acids, and the sample was treated with TMSD to make fatty acid methyl esters. The chromatogram and the mass spectrum of the broadly eluting peak centered around 7.0 min are
shown in Figure 36. The mass spectrum at the peak of the chromatogram was complex and does not have ions that distinguish any of the fatty acids.

Figure 36. Chromatogram and mass spectrum of 6.9-min eluting species after saponification of hexane-extractable fatty acids in fish oil and treatment with TMSD

3.6 Attempts to identify prostaglandins in media containing cultured cells

It is known that interleukin1β (IL1β) induces the secretion of PGE2 from a well-characterized alveolar cell line (A549), so the media in which this cell line was incubated was used to determine the chemical synthesis scheme including the novel TMSD reagent could be used to identify a PGE2 derivative in the media of cells treated for 0, 6, or 12 hours with IL1β. A prostaglandin fraction was obtained following hexane extraction and purification on a silica
column, after which the sample was treated with TMSD to form the methyl ester, the methyl oxime, and BSTFA as per the PGE$_2$ and PGF$_{2\alpha}$ standards.

Figure 37. Chromatogram of media containing A549 cells purified on C18 column and treated with reagents for preparation of the PGE$_2$ derivative. Cells were treated with interleukin1$\beta$ for 0 (top), 6 (middle), or 12 hr (bottom). Mass spectrum of 6 hrs treated A549 cells
The chromatograms for all the time periods had a prominent peak in the region where PGE$_2$ and PGF$_{2\alpha}$ standards elute are shown in Figure 39. Unfortunately, the mass spectrum was complex and was not distinguishing for PGE$_2$ or PGF$_{2\alpha}$.

Figure 38. Chromatogram of treated 9 HTE cells with IL-1β at an elution time of 7.00 mins for 0 hrs, 6 hrs and 12 hrs
The chromatograms for the defined time periods had a prominent peak in the region where the PGE\(_2\) and PGF\(_{2\alpha}\) standards elute are shown in Figure 41. Unfortunately, the mass spectrum was complex and was not distinguishing for PGE\(_2\) or PGF\(_{2\alpha}\) (data not shown).

3.7 Attempts to identify prostaglandins in reactions of cyclooxygenase-1 with arachidonic acid

The metabolism of arachidonic acid by cyclooxygenase-1 is known to yield PGE\(_2\), which is a decomposition product of PGH\(_2\), the product of the reaction with the enzyme. An attempt was made to determine whether PGE\(_2\) could be identified in the reaction mixture after incubation of arachidonic acid with the cyclooxygenase. The chromatogram for the reaction mixture that was treated with TMSD to make the methyl ester, methyl oxime to modify the carbonyl group, and the silylation of the alcohol groups is shown in Figure 40. A lipid specific fraction was obtained by using a C18 column for purification. A single peak in the chromatogram that occurs with the same retention time as for PGE\(_2\) is observed, and the mass spectrum of the species at the peak of the chromatogram did match that of the purified PGE\(_2\) derivative standard as shown in Figure 31. The ions with m/z of 513 and 456 that are distinguishing for PGE\(_2\) (and PGF\(_{2\alpha}\) for that matter) are found in the mass spectrum.
Figure 39. Chromatogram and mass spectrum of enzyme substrate reaction of cyclooxygenase-1 and arachidonic acid
DISCUSSION

Diazomethane has been long considered as a common methylation agent for derivatisation of mainly the carboxyl groups. However it has some major drawbacks, common among them are concerned with its preparation which is time-consuming and complicated. The precursors used in the preparation of Diazomethane are potent mutagens including diazomethane itself which is carcinogenic and explosive. Hence Trimethyl Silyl Diazomethane was proposed as a potential alternative to Diazomethane (Ref: Martina Preu).

Trimethyl silyl diazomethane (TMSD) was originally used with methanol as the methylating reagent in the preparation of drugs, organic compounds, and some fatty acids (39). TMSD is stable, and so it does not need to be prepared from a precursor compound, is less hazardous, and provides an excellent yield at room temperature and thus does not require heat for efficient catalysis (40).

The advantages of TMSD as the methylation agent as compared to all the previous methods available is that it can be efficiently applied to analytical work by gas chromatography and mass spectrometry.

Initial experiments were conducted to determine whether the benzene used as the co-solvent during methyl esterification of arachidonic acid with methanol could be substituted with other solvents like chloroform, or acetone. The use of benzene-methanol as the solvent system was developed as an alternative method to those involving chloroform-methanol (82). In addition, the work presented here demonstrates that remarkably good results are obtained with acetone-methanol as the solvent system (83). The acetone-methanol system was used to promote
extraction of a lipid fraction, which was visualized by thin layer chromatography (84). Other work demonstrated the use of 20% methanol-acetone as the solvent system in isolation of a lipid fraction as revealed by thin layer chromatography (85). The widespread use of acetone-methanol in solubilizing and derivatizing lipids was a main factor that influenced its use in the present study. In the work presented here, a number of solvents were evaluated for use in the TMSD reaction, and all of them provided marginal-to-acceptable linearity in support of the calibration curve for arachidonic acid methyl ester. However, acetone has a relatively low toxicity and provided superior linearity over a four-fold range.

The formation of the methyl ester of each of the polyunsaturated fatty acids tested yielded a molecule with the expected molecular ions for arachidonic acid methyl ester (m/z 319), eicosapentanoic acid methyl ester (m/z 319), docosahexanoic acid methyl ester (m/z 343), oleic acid methyl ester (m/z 297), and heptadecanoic acid methyl ester (m/z 285), which was subsequently used as the internal standard to develop the calibration curves for the polyunsaturated fatty acids. The methyl ester for all of these compounds was confirmed by thin layer chromatography, and confirmation by GC/MS analysis of the prepared fatty acid methyl esters. The calibration curves for the PUFAs, AA, EPA, and DHA were linear over about a four- to five-fold range. These results extend the previously reported results to include PUFA of greater molecular weights than fatty acids containing more than 20 carbon atoms. For fatty acids with conjugated double bonds have attracted great interest because of their reported potent bioactivity. It has been seen interestingly in our experiments that as the number of double bonds increased the consumption of methylation agent increased accordingly. Hence the optimum level of TMSD required for maximal conversion of linoleic acid with two double
bonds was 60 μLs, for α and γ-linolenic acid with three double bonds is 70 μLs, for arachidonic acid with four double bonds is 100 μLs, for eicosapentaenoic acid with – double bonds 150 μLs, and docoahexaenoic acid with _ double bonds 300 μLs. Interestingly as predicted heptadecanoic acid, which is a saturated fatty acid consumed 40 μLs of TMSD for full conversion.

The synthesis of the prostaglandins was a bit more complex and involved methoximation (PGE2) and/or silylation of alcohol groups (PGE2 and PGF2α) and methyl esterification using the TMSD reaction. The mass spectrum obtained following fragmentation under chemical ionization conditions gave no evidence of the expected value for the molecular ion (m/z 538). The calibration curve for PGE2 was linear over a 90-fold range, with the lowest concentration evaluated at 10 pg/mL. Future experiments to find conditions to limit fragmentation will be used to characterize the PGE2-derivative and the deuterated PGE2 standard, which did not provide a substantial peak in the chromatogram. The calibration curve for the PGE2 and PGF2α compounds showed linearity over a wide concentration range, although a better characterization of the mass spectral properties of these compounds and those of the deuterated standards is required to obtain a useful calibration curve for quantitation.

In the present study, several attempts were made to determine whether TMSD could be used in an assay to detect fatty acids in biological samples. Saponification of the phospholipids, phosphatidyl choline or phosphatidyl ethanolamine, and subsequent hexane extraction were required prior to analysis of the palmitic acid and arachidonic acids that were contained in the phospholipids. The hexane extractions that were subjected to treatment with the TMSD reagent gave a single peak in the chromatogram. This single peak would suggest that palmitic acid is present in the phospholipid as expected. Unfortunately, palmitic acid was not characterized and so its presence in the phospholipid could not be confirmed. Moreover, the column conditions for
detection of the fatty acid methyl esters were different from those earlier used for the standards, and hence, the retention times could not be appropriately compared.

Experiments to identify PUFA in biological samples were performed with egg yolk and fish oil capsules, which also first involved a saponification of the sample to release esterified fatty acids prior to methyl esterification with TMSD in methanol-acetone. The chromatogram of the sample was broad, and the mass spectrum at the apex of the peak revealed a complex spectrum that did not allow characterization of the constituent fatty acids. Thus, much more work needs to be done to more effectively identify these and/or other fatty acids in these biological samples.

Attempts were also made to determine whether prostaglandins could be identified in biological samples. The results obtained with a very well characterized alveolar cell line (A549) did indicate that a peak in the chromatogram with a retention time of about 7.2 min was observed in the media containing native or interleukin1β-treated cells. Interleukin 1β stimulates the production of PGE2 in this cell line. Unfortunately, the chromatography is not good enough to distinguish either of these prostaglandins in the cell culture media. On the other hand, the results obtained with cyclooxygenase reaction with arachidonic acid gave the expected product, PGE2.

In summary, these experiments extend the number of polyunsaturated fatty acids that can be evaluated in the TMSD-catalyzed reaction to those containing up to 22 carbon atoms, and also to the class of eicosanoids known as the prostaglandins. The TMSD reaction is supported in acetone, which is as effective as the considerably more toxic benzene solvent that was previously used. Finally, the data suggest that it might be possible to identify fatty acids and prostaglandins in biological reactions, although considerably more work will need to be done under the same conditions for standards and for samples so that it is possible to make conclusions as to whether
these compounds can be identified in biological samples. Finally, if chromatographic resolution of the compounds cannot be achieved, selected ion monitoring or MS/MS methods may have to be employed to identify and quantify the prostaglandins in biological samples. The application of this procedure in biological systems detecting very minute amounts of prostaglandins increases the applicability of this process in detecting prostaglandin like products, in biological samples. This method can be extended for derivatizing thromboxanes and Leukotrienes. Hence this method can be of importance in detecting fatty acids and their oxidation products in the field of clinical chemistry.
REFERENCES


42. Die gewichtsanalytische Bestimmung des Milch fettes; (1879) *Dingler's polytechnisches J.*, 232, 461

43. Bloor, WR. (1914) *J Biol Chem*, 17, 377

44. Andersson RJ, (1927) *J Biol Chem* 74, 525


63. Ulberth, F.; Henninger, M. On-column injection of fatty acid methyl esters on to polar capillary columns without distortion of early eluting peaks. *J.of High Resolution Chromatography*, 15:1, 54-56


70. Ministry of Economy, Trade and Industry(1983). Determination of Fatty Acid Methyl Ester and Triglycerides in Diesel Fuels


78. Schreiner, M.; Moreira, R.G.; Hulan, H.W. Positional distribution of fatty acids In egg yolk lipids. *Journal of Food Lipids*, **13**:1, 36-56


