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A SELECTIVE EXTRACTION TECHNIQUE FOR HYDROXYLATED FATTY ACIDS IN MAMMARY TISSUE BASED ON ANALYSIS BY GAS CHROMATOGRAPHY - MASS SPECTROMETRY

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By

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ABSTRACT

A SELECTIVE EXTRACTION TECHNIQUE FOR HYDROXYLATED FATTY ACIDS IN MAMMARY TISSUE BASED ON ANALYSIS BY GAS CHROMATOGRAPHY - MASS SPECTROMETRY

By

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Dietary polyunsaturated fatty acids have been shown to increase the rate and incidence of mammary tumorigenesis in animal and epidemiological studies. One possible mechanism involves formation of oxygenated metabolites of the polyunsaturated fatty acids, yet no direct evidence of such oxygenated fatty acids has been reported. Published methodologies for the extraction of oxygenated fatty acids with subsequent analysis by gas chromatography with flame ionization or mass spectral detection were determined to be unsuitable for analysis of the mammary gland matrix. In this study, a new procedure for the isolation of hydroxylated fatty acids from mammary tissue was developed. Analysis by gas chromatography - mass spectrometry detected some hydroxylated C18 fatty acids at the parts per billion level in many of the tissue extracts, indicating that the developed methodology is appropriate for the determination of hydroxylated fatty acids in mammary glands from mice fed diets of different fatty acid content.

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LIST OF ABBREVIATIONS

DMBA	dimethylbenzanthracene
BHT	butylated hydroxytoluene
ODC	ornithine decarboxylase
DNA	deoxyribonucleic acid
BHA	butylated hydroxyanisole
FAMEs	fatty acid methyl esters
BF_3	boron trifluoride
HCl	hydrochloric acid
TMS	trimethylsilyl
tBDMS	t-butyl dimethyl silyl
ECD	electron capture detection
OTMS	trimethylsilyl ester
EI/MS	electron ionization mass spectrometry
m/z	mass to charge ratio
u	atomic mass units
PGs	prostaglandins
NICI/MS	negative ion chemical ionization mass spectrometry
TXs	thromboxanes
HPLC	high performance liquid chromatography
PFB	pentafluorylbenzyl
KH_2PO_4	potassium phosphate, dibasic
NaOH	sodium hydroxide

d. H_2O	distilled water
ODS	octadecylsilyl
$CHCl_3$	chloroform
MeOH	methanol
v/v	volume/volume
TBA-HSO_4	tetrabutylammonium hydrogen sulfate
BSTFA	bis(trimethylsilyl)trifluoroacetamide
ECD	electron capture detection
i.d.	inner diameter
SPE	solid phase extraction
EtOH	ethanol
rpm	revolutions per minute
OH-FAMEs	hydroxylated fatty aicd methyl esters
SIM	selected ion monitoring
y_d	signal of the analyte at its limit of detection
y_b	the signal from the blank
s_s	standard deviation of the slope
C_d	concentration at the limit of detection
A	slope of the calibration curve
b	calculated y-intercept
Q_x	quantity injected
Q_d	quantity at the limit of detection
y_i	abundance of ion at m/z i
y_T	total ion signal
S/N	signal to noise ratio
TIC	total ion current
RSD	relative standard deviation

R_f	retention factor
dpm	disintegrations per minute
ppm	parts per million
MS/MS	tandem mass spectrometry
ME	methyl ester
min	minutes

I. Chapter One: Biochemical Significance of Oxygenated Fatty Acids

A. Introduction

The role of dietary polyunsaturated fatty acids in increasing the incidence of tumors, particularly in mammary glands, has been well documented in numerous animal and epidemiological studies. Several hypotheses have been proposed to explain the mechanism of this tumorigenic process (1). One of these theories proposes activation by oxidation at the sites of unsaturation in the fatty acid, and has been supported by reports from some laboratories that administration of antioxidants decreases or eliminates the enhancement in tumorigenesis usually realized with a diet high in polyunsaturated fatty acids. Yet no direct evidence of oxygenated metabolites in glands of animals fed diets high in polyunsaturated fatty acids has been reported. This thesis describes the development of analytical methodologies to extract and analyze one type of oxygenated metabolite, hydroxylated fatty acids, from mammary tissue.

B. Effects of Dietary Polyunsaturated Fat on Tumorigenesis

As early as 1942, Tannenbaum (2) reported an increase in the growth of spontaneous mammary tumors in mice fed a diet high in fat with respect to those mice fed a low-fat diet. This same effect of high fat diet on the incidence of mammary tumors in rats was observed by Engel and Copeland (3) when the tumors were chemically induced. Results from numerous laboratories have demonstrated a similar correlation of high dietary fat and an increase in mammary tumorigenesis in rats or mice when other carcinogens were employed (4-6).

Epidemiological evidence has been presented that suggests a similar correlation between the level of dietary fat and the incidence of breast cancer (as well as prostate, colon and other cancers) (5, 7). Although the trends are not strictly defined, a relationship has been observed between the age-adjusted mortality due to breast cancer and the amount of dietary fat available in numerous countries (8, 9). The

integrity of these assumptions and conclusions of these epidemiological studies has been disputed (10).

Further studies demonstrated that the degree of unsaturation in the fat is positively related to mammary tumorigenesis in experimental animals (11, 12). Those rodents fed a diet high in polyunsaturated fat had a higher rate of tumorigenesis than those rodents fed a similar diet high in saturated fat, which in turn exhibited a higher rate of tumorigenesis than rodents fed a same-calorie low-fat diet.

Animal models for the study of cancer in various organs have been developed. To investigate mammary tumorigenesis in experimental animals, rats and mice are often treated with a chemical carcinogen. Commonly used are lipophilic carcinogens such as 7,12-dimethylbenzanthracene (DMBA), but hydrophobic carcinogens such as N-nitroso-N-methylurea have also been utilized, with the same positive correlation of level of unsaturated fat to mammary carcinogenesis (13). These studies have focussed mostly on the effect of dietary fat on the number of tumors and rate of tumor development.

Supporting evidence of the role of dietary fat in mammary tumorigenesis also has been provided by studies of transplantable tumors (14). The rate of growth of transplantable mammary tumors was highest when the animals were fed a diet high in linoleic or linolenic acid, as compared to those animals fed diets high in oleic or other monounsaturated and saturated fats.

More recent studies with polyunsaturated fatty acids differing in the position of the double bonds have demonstrated that an increase in tumorigenesis is dependent upon the position of the double bonds (15). Those polyunsaturated fatty acids with double bonds starting at the sixth carbon atom from the methyl group (denoted n-6 or ω-6), such as linoleic and arachidonic acid, have been shown to cause an increase in tumorigenesis. Yet those animal fed diets high in n-3 polyunsaturated fatty acids, the so-called omega-3 fish oils, demonstrated no increase, or even a decrease, in mammary tumorigenesis, when compared to rodents fed diets high in n-6 fats (Figure 1-1).

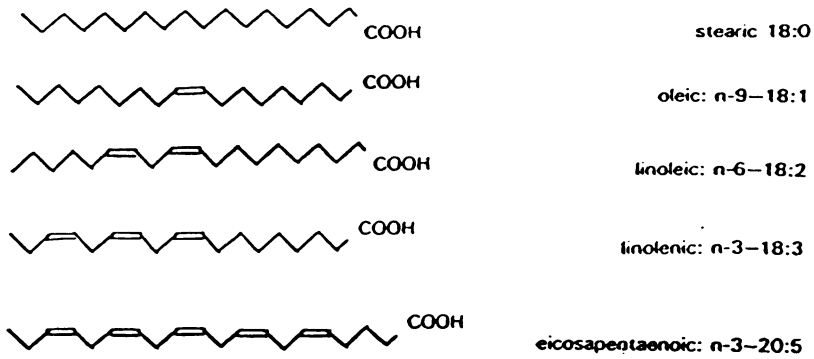


Figure 1-1: Structures of the different families of unsaturated fatty acids, demonstrating the different positional arrangements of the conjugated double bonds, counting from the methyl terminus. Source: Reference 16.

C. Proposed Mechanisms for the Effect of Dietary Fat on Mammary Tumorigenesis

1. Endocrine System

Numerous studies have been performed in the past twenty years to attempt to discover the mechanism(s) of increased mammary tumorigenesis by dietary fat. Several possible types of factors may be responsible for the carcinogenic pathway. One of the most researched areas involves the endocrine system. Initial studies which indicated an increase in secretion of prolactin and ovarian steroids in rats fed diets high in fat (17), were refuted by subsequent, more sensitive analyses of these hormones in the blood of animals fed high-fat diets (18). No increase in secretion of these hormones was observed for the animals fed high-fat diets when compared to those animals fed diets of moderate fat levels (19). The observance of increased tumorigenesis of organs not affected by the pituitary-ovarian system also casts serious doubts on possible mechanisms related to endocrine function.

2. Intercellular Considerations

It also has been proposed that high-fat diets may affect hormone and/or growth factor receptors (20), especially via a lipid-dependent enzyme system. The activation of protein kinase C and extracellular signal transduction seems to be enhanced by unsaturated diacyl glycerides with respect to saturated diacyl glycerides.

The findings that polyunsaturated fatty acids and prostaglandins can restrict some immune functions has suggested that the immune system is responsible for the increase in mammary tumorigenesis caused by high levels of dietary fat. Several studies have indicated that diets high in unsaturated fatty acids decreases lymphocyte levels or responsiveness (18). But the increase in mammary tumor cell growth *in vitro* observed when unsaturated fatty acids are added to the culture medium indicates a mechanism other than the immune is (also) involved.

Enhanced mammary tumorigenesis by high dietary levels of polyunsaturated fat could result in changes in the structure of the mammary gland or its sub-cellular components (14). A dietary increase in unsaturated fatty acids has been shown to increase the fluidity of cell membranes, which may increase cell mitosis (18). Cell membrane fluidity has been demonstrated to be increased in mammary tumors by certain hormonal agents which are known to promote mammary tumor growth. This increase in membrane fluidity may affect intercellular communication via a decrease in metabolic cooperation (18). Unsaturated medium-chain fatty acids have been shown to decrease metabolic cooperation, while their saturated analogues did not.

3. Dietary Factors

Another theory attempting to explain the positive relationship between mammary tumorigenesis and dietery fat involves the level of caloric consumption. The repeated observance that caloric restriction decreases mammary tumorigenesis (and tumorigenesis in other organs) suggests a mechanism relating caloric intake and the tumorigenic process (21, 22). But, many well controlled-diet studies in rodents utilizing isocaloric diets have not supported this theory (18). As an example, mice fed isocaloric high fat diets, which differed only in the amount of saturation or unsaturation exhibited different rates of mammary tumor development. The incidence of mammary tumors was significantly higher in the mice fed the unsaturated fat diet. These latter findings do not support the

concept that increased caloric intake is solely responsible for the increase in mammary tumor development documented in animals fed high-fat diets.

An alternative possible cause of decreased mammary tumorigenesis when a low-calorie diet is administered *ad libitum* (and animals consume increased amounts of food) is increased consumption of particular nutrients (22). Numerous studies have demonstrated an inverse relationship between the level of several nutrients and incidence of mammary tumors (23). Increased dietary levels of protein have been shown to suppress mammary tumorigenesis in rodents, possibly by increased activation of hepatic mixed function oxidases which metabolize, thus detoxify, chemical carcinogens (21). When administered in very high doses, vitamin A and other retinoids have prevented, or significantly reduced, chemically-induced mammary tumorigenesis in rats and mammary tumor cell growth *in vitro* (1, 17). Other nutrients have been shown to inhibit mammary tumor growth when fed at high levels, *e.g.*, selenium, vitamin E, and a combination of these two compounds (1, 23). Several mechanisms have been proposed for the anti-tumorigenic activity of these latter three chemicals including their roles as antioxidants, but such a mechanism has yet to be supported in a compelling manner.

D. Antioxidant Suppression of Fat-Enhanced Tumorigenesis

Besides the above-mentioned potential antioxidants, butylated hydroxytoluene (BHT) and propyl gallate - two more antioxidants, have been shown to inhibit DMBA-induced mammary tumorigenesis in rats fed high-fat diets (18, 24). These findings support the theory that oxygenated metabolites of fatty acids may be involved in this carcinogenic process. This mechanism is particularly attractive since diets high in unsaturated fatty acids enhance mammary tumorigenesis more so than do diets high in saturated fatty acids. Unsaturated fatty acids are susceptible to oxidation, possibly explaining the observed difference in tumor growth between the different levels of double bond character in the two high-fat diets (17).

The products and mechanisms of lipid oxidation, particularly of unsaturated fatty acids, have been extensively studied (25-27). Oxidation of fatty acids can be activated by enzymes (lipoxygenase reactions), free radicals (autoxidation and peroxidation), or light (photooxidation). Hydroperoxides are the initial products of lipid oxidation (25), but are unstable and would most likely be reduced to other oxygenated forms such as hydroxyl, carbonyl, or epoxy moieties.

To test this theory that oxygenated fatty acids play a role in tumorigenesis, Bull and coworkers have used rat colon tumorigenesis as a model (28). This group has shown that hydroperoxide and hydroxide derivatives of linoleic and arachidonic acids increase two indicators of tumor promotion - ornithine decarboxylase (ODC) and deoxyribonucleic acid (DNA) synthesis - in colonic mucosa. Subsequent analysis of the effect of structurally-similar fatty acids indicates specifically that an allylic oxygenated functional group is necessary for induction of ODC and stimulation of DNA sythesis, both indicators of mitogenic activity (29).

Additional studies investigating the roles of high-fat (saturated and unsaturated) diets and antioxidants in rodent mammary tumorigenesis support the theory that an oxygenated intermediate of unsaturated fatty acids is critical in the mechanism. One such study is the work by McCay *et.al.* (30) in which rats were fed purified diets of high polyunsaturated fatty acid content, high saturated fatty acid content, or low fat content. The ability of several antioxidants as inhibitors in DMBA-induced mammary tumorigenesis in these rats was assessed. Butylated hydroxytoluene was shown to be a strong inhibitor of induced mammary tumorigenesis in all three diet groups, but was more effective in the high saturated fat diet group than in the group fed high levels of unsaturated fat. But no inhibitory effect on mammary tumorigenesis was observed for those animals fed butylated hydroxyanisole (BHA), nor tocopheral acetate. These latter findings contradict other studies demonstrating an inhibitory effect of BHA and tocopherol on DMBA-induced mammary tumors in rats fed commercial rat diets. It is

suggested that the purified diets lack certain cofactor(s) which make these two antioxidants effective in the commercial diets, or that the purified diets contain a factor or factors which inactivate BHA and tocopheral acetate, but not BHT.

Another laboratory (31) later reported selective inhibition of DMBA-induced mammary tumorigenesis in rats by different antioxidants. Mammary carcinomas were inhibited by BHA or ethoxyquin, while development of mammary fibroadenomas was reduced by BHA, ethoxyquin, BHT and diaminodiphenylmethane (separately). In this study, tocopherol did not appear to inhibit mammary tumorigenesis.

Also supportive of the oxygenated polyunsaturated fatty acid mechanism is a more biochemically-oriented study by King's group (32) in which rats fed diets (iso-nutrient and iso-caloric) differing only in the amount of fatty acid or its degree of saturation were given DMBA. Select animals of each diet group were fed non-toxic levels of one of various antioxidants for different lengths of time before and after DMBA administration. Drug metabolism in the mammary gland and some hepatic microsomal parameters were analyzed. It was shown that those antioxidants which had the greatest inhibition of mammary tumorigenesis, as measured by incidence of tumors and by biochemical measures of mitogenic activity, were those which were incorporated into the mammary gland for the longest time. These results strongly support a mechanism for DMBA-induced mammary tumorigenesis involving an oxygenated intermediate of unsaturated fatty acids derived from dietary fat.

Reports from other laboratories have contradicted the oxidized metabolite theory (18, 24); one study found that a high-fat diet caused only a decrease in the latency period of mammary tumor appearance in mice, not an increase in the total number of tumors at long-term necropsy (33). Although there have been some studies which contradict the oxidized metabolite theory, this mechanism is still a promising, yet unproven, hypothesis.

E. Lack of Direct Evidence for Oxygenated Intermediate

Although conclusions from the above studies suggest that an oxygenated intermediate may play a role in a mechanism of the effect of dietary fatty acids on mammary tumorigenesis, no direct evidence has been reported in the literature. There are no known studies in which mammary glands were analyzed for possible oxygenated intermediates which may be involved in the tumorigenic process. It would be of great interest in attempting to test the proposed oxygenated intermediate theory to examine mammary glands or mammary tumors for any oxygenated intermediates, particularly as a function of the amount and type of dietary fat fed to the animal.

It is anticipated that any such oxygenated intermediate would be present in only trace quantities in the tissue, thus requiring a very sensitive detection technique. The exact nature of the intermediate(s) is unknown, but it is suspected that the most stable intermediates would be those which had been reduced to hydroxyl, ketone, and epoxide moieties. Since the exact oxidized fatty acids are unknown, the detection method must also be a fairly general one which could also provide structural identification.

Those oxygenated fatty acids proposed to be involved in this possible mechanism of tumorigenesis would most likely be metabolites of dietary fatty acids. This would indicate that the origin of the possible intermediates would have carbon chain lengths of 16 or 18, since palmitic, oleic, linoleic, and linolenic acids are the major fatty acid components of the common dietary fats and oils. These dietary fatty acids could be metabolized to form oxygenated intermediates of slightly different carbon length.

II. Chapter Two: Adaptation of Existing Analytical Techniques

Fatty acids, particularly methyl esters of fatty acids, have been well characterized by gas chromatographic techniques. There have been additional techniques for the separation and identification of oxygenated, particularly hydroxylated, fatty acids. After derivatization of the hydroxy fatty acids, separation is easily performed by gas chromatography with flame ionization or mass spectrometry as a detection method.

Several laboratories have utilized these gas chromatography techniques, as well as a few others, to identify oxygenated metabolites of fatty acids in body fluids and tissues. But, no reports have been found in the literature which describe analysis of mammary tissue for hydroxylated fatty acid content. Application of some techniques for extracting and analyzing oxygenated fatty acids in tissue for determination of hydroxylated fatty acids in mammary tissue was attempted, with unsatisfactory results.

A. Identification of Hydroxylated Fatty Acids

Analysis of fatty acids as their methyl esters has been and still is one of the most widely-used techniques. Traditionally, gas chromatography with flame ionization detection utilized packed, deactivated glass columns. The advent of fused silica open tubular columns improved the stability, resolution, and reproducibility of separation of fatty acid methyl esters (FAMEs) in numerous laboratories (34).

1. Esterification of Fatty Acids

Fatty acids are derivatized to make them more volatile and non-polar, thus more amenable to gas chromatography (35). The derivatized compound is more easily vaporized into the gas (mobile) phase and chromatographically behaves more ideally. As mentioned above, methyl esters are by far the most commonly used derivatives of fatty acids. They are easily prepared from the free acids by reaction with ethereal diazomethane in the presence of catalytic methanol (Figure 2-1). The esterification is rapid and the reactants are quickly evaporated. Fatty acid methyl esters also can be prepared by other methods such as with methanol-BF_3 or methanol-HCl (36).

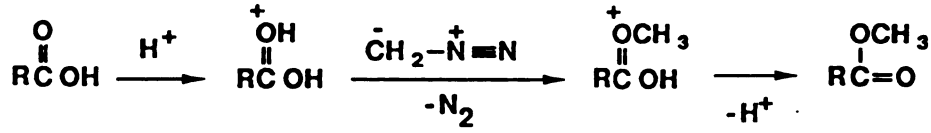


Figure 2-1: Acid catalyzed methylation of carboxylic acid by diazomethane.

Fatty acid methyl esters are resolved by polar and non-polar stationary phases according to their molecular weights (carbon number) and, if the stationary phase is correctly chosen, the number and position of the double bond(s) in the compound can be determined. The retention indices of the methyl esters of the naturally-occurring fatty acids are well characterized. Standards of many FAMEs are commercially available to characterize the individual gas chromatographic system. When the compound is an unknown, mass spectrometry is often used for identification.

Free fatty acids may also be derivatized into other esters, such as the trimethylsilyl (TMS) or t-butyl dimethyl silyl (tBDMS) esters. These derivatives are made when higher molecular weights are desired. This is often the case for short-chain fatty acids, where the methyl ester would have a shorter retention volume and resolution may be poor (36). Pentafluorobenzyl esters can be prepared to make the fatty acids suitable for electron capture detection (ECD). Pyrrolidine esters and picolinyl esters can be prepared if mass spectral analysis will be performed, as they have distinctive fragmentation patterns (35).

2. Trimethylsilyl Ethers from Hydroxylated FAMEs

Methyl esters of hydroxylated fatty acids are still polar and somewhat involatile since they contain the hydroxyl moiety. Thus, if a real fatty acid mixture is methylated, it is desirable to further derivatize the sample to convert any possible hydroxylated components to ethers. Preparation of the TMS ether (OTMS) of the fatty acid methyl esters provides very volatile and chromatographically well-behaved compounds (37).

Standards of methyl ester, TMS ethers of hydroxylated fatty acids are not commercially available, but are easily prepared from the free hydroxylated fatty acids or their methyl esters, some of which are available. Retention indices of the OTMS fatty acid methyl ester can then be determined experimentally, as these are not catalogued in the literature. On non-polar stationary phases, these derivatized compounds are separated by chain length, number of double bonds, number of OTMS moieties, and position of these functional groups. Positional isomers of OTMS-FAMEs will have similar retention indices, but are resolvable by the high-resolution capillary columns now so commonly used. Therefore, the approximate retention time of a TMS ether, FAME which is difficult to purchase or synthesize can be determined from a positional isomer which is more easily obtained.

If electron ionization mass spectrometry (EI/MS) is to be used, this TMS ether derivatization provides additional an benefit in its abundant fragment at m/z 73 from McLafferty rearrangement, which can be a marker to indicate the presence of derivatized hydroxylated fatty acids (Figure 2-2). Other characteristic fragment ions include minor peaks corresponding to $[M\text{-}CH_3]^+$ and $[M\text{-}CH_3OH]^+$, allowing determination of the carbon chain length. As a further aid in identification of positional isomers, α-cleavage about the TMS ether provides two characteristic fragment ions, the mass sum of which equals the molecular weight plus 102 u.

B. Isolation Techniques for Hydroxylated Fatty Acids from Tissue

1. General Lipid Methods

Before identifying the hydroxylated fatty acids in a real sample, such as a bodily fluid or tissue, the interfering components of the matrix must be removed. This is usually done by extraction of the fatty acids, and subsequent cleanup to obtain the desired compounds to be analyzed. The objective of this research is to isolate and identify any hydroxylated fatty acids in mammary glands using the mouse as a model.

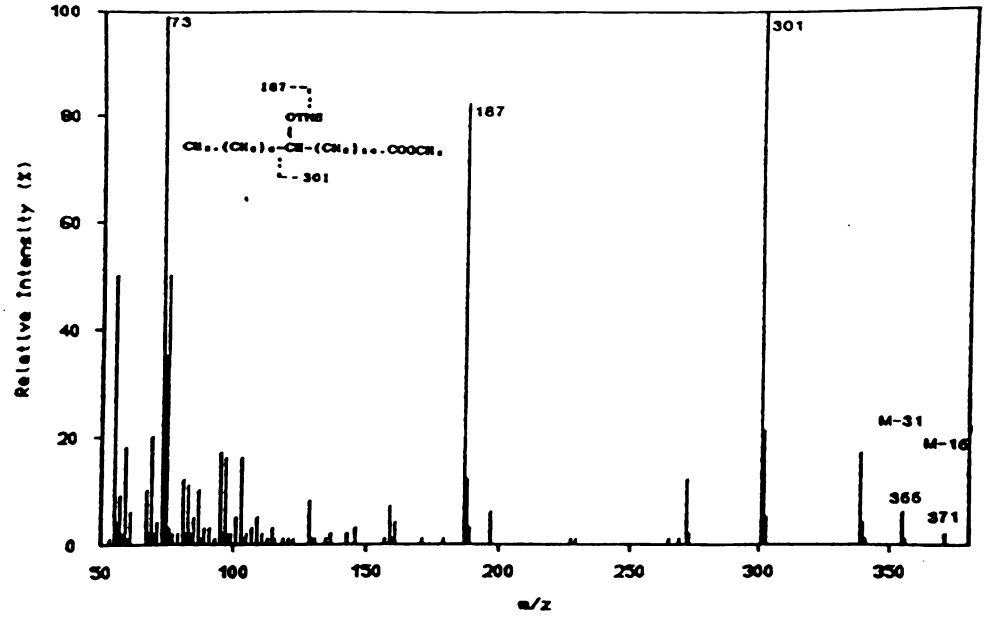


Figure 2-2: The electron ionization mass spectrum of the TMS ether derivative of the methyl easter of 12-hydroxy stearic acid. Source: Reference 37.

No literature procedures were found for the isolation of oxygenated fatty acids, that is, hydroxy-, keto-, or epoxy- fatty acids, from mammary tissue, yet there are many laboratories which have analyzed body fluids for oxygenated fatty acids. Pace-Asciak and Micallef used negative ion chemical ionization to analyze rat blood for prostaglandins (PGs), their keto catabolites, and their keto, hydroxy catabolites (38). Negative ion chemical ionization mass spectrometry (NICI/MS) was also used to analyze saliva, urine, cerebrospinal fluid and platelets for PGs, thromboxanes (TXs) and hydroxy C_{20} and C_{17} fatty acids (39), and to determine hydroxylated C_{20} (40). After derivatization to a fluorescent compound, high performance liquid chromatography (HPLC) was used to detect PGs, TXs, keto metabolites of prostaglandins, and hydroxy C_{20} fatty acids in rat plasma (41). These oxygenated fatty acid determinations were from fluid matrices, which differ greatly from the mammary gland that is to be examined in this application. Therefore, these extraction techniques were not considered applicable to the dissimilar matrix of the mammary gland.

Some laboratories report the isolation and analysis of oxygenated fatty acids, mostly arachidonic acid metabolites, from tissues. Electron ionization mass spectrometry was used to analyze TMS ether, methyl ester derivatives of hydroxy arachidonic metabolites from monkey seminal vesicles (42); TMS ether, methyl ester derivatives of hydroxylated lipoxygenase metabolites of C_{20} and C_{18} fatty acids from rabbit peritoneal tissue (43); TMS or tBDMS ether, methyl ester derivatives of hydroxylated metabolites of C_{20} and C_{22} fatty acid in trout gill tissue (44); TMS ether, methyl ester derivatives of PGs and hydroxylated arachidonic and linoleic fatty acids from rat and rabbit aortae (45,46); and hydroxylated C_{20} fatty acids and PGs in brain cortical tissue (47). In these studies the fragmentation provided by EI/MS allowed more definitive identification of oxygenated metabolites than the high performance liquid chromatography with spectrophotometric or radiological analysis concurrently performed.

The pentafluorylbenzyl (PFB) ester, trimethylsilyl ether derivatives of these oxygenated fatty acid metabolites have been prepared for negative ion chemical ionization, sometimes in addition to the methyl ether, TMS ethers for EI/MS analysis. The detection limits with NICI/MS can be lower than those with EI/MS by as much as 1000 fold (48). One reason for this is the ions formed by chemical ionization are primarily the characteristic anion $[M\text{-}PFB]^-$ (Figure 2-3), and the subsequent loss of the ether and hydrogen $[M\text{-}PFB\text{-}HOTMS]^-$ (49).

Gleispach and co-workers (48) utilized both EI/MS and positive ion chemical ionization of methyl ester, TMS ethers and NICI/MS of PFB ester, TMS ethers of hydroxy fatty acids, TXs, and PGs to determine the arachidonic acid metabolites in skin fibroblast and saliva. Qualitative data were provided by the fragmentation of these compounds, while quantitative determinations were made by the more sensitive chemical ionization techniques and isotope dilution. Murphy has used the PFB ester, TMS ethers with NICI-MS to analyze lipoxygenase products (50) particularly hydroxy arachidonic acids and leukotrienes, as well as the methyl ester, TMS ethers with EI/MS in lung tissue

(49). Keto PGs and keto, hydroxy catabolites of PGs in rat kidney were determined by NICI/MS of the PFB ester, methoxime, TMS ester derivatives (51).

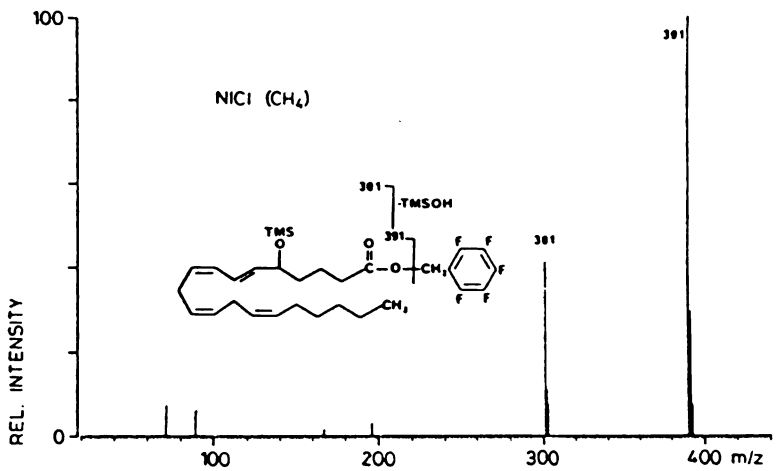


Figure 2-3: Negative ion chemical ionization mass spectrum of the TMS ether, pentafluorobenzyl ester of 5-HETE using methane as the auxiliary gas. Source: Reference 49.

So it was from these procedures, and general lipid extraction methods described in the literature, that preliminary attempts to extract hydroxylated fatty acids from mouse mammary tissue were based. In the fifties, early extraction techniques for the extraction of lipids from tissue were published. Chloroform-methanol was the conventional organic solvent system for homogenization of tissues, followed by an aqueous phase extraction (52, 53). These early extraction procedures are still widely used today, as they are rapid and fairly quantitative (54, 55). Further sample cleanup is usually achieved with chromatographic methods (56).

The samples used for testing and further development of the existing analytical methodology to isolate and identify hydroxylated fatty acids were obtained from three month old female Balb/c mice under the care of Dr. C.W. Welsch's laboratory. The inquinal (number four and five) mammary glands were removed from the animals by trained laboratory personnel with particular care to excise only mammary tissue.

2. Pace-Asciak Isolation of Keto-FAs from Kidney

The procedure for extraction of keto- and keto, hydroxy- prostaglandins from rat kidney used by Pace-Asciak and Micallef (38) was applied to the mouse mammary tissues. This procedure was selected due to the complex nature of both matrices, for which the sample cleanup procedure may prove to be appropriate. After extraction, the Canadian workers prepared the PFB ester, TMS ether derivatives of the oxygenated PG metabolites for analysis by GC-ECD and NICI/MS. Since GC-FID and EI/MS are the preferred analysis methods for the unknown oxygenated fatty acids possibly present in the mammary glands, the methyl ester, TMS ether derivatives were also prepared in this study.

The extraction procedure is outlined in Figure 2-4. Each mammary gland was approximately 0.1 g. The tissue was rinsed with 0.05 M KH_2PO_4 - NaOH buffer (pH 7.4), then homogenized with 10 volumes of buffer (10 mL) in a silanized homogenizer with a Teflon pestle. Five volumes of ethanol (5 mL) was added and the mixture was filtered with suction on No. 42 Whatman paper. These latter steps were to stop the incubation that Pace-Asciak and Micallef desired, and to filter precipitated protein, respectively.

The filtrate was evaporated *in vacuo* and 1 mL of distilled water (d. H_2O) acidified to pH 3 with HCl. This mixture was passed through an octadecylsilyl (ODS) C18 Sep Pak solid phase extraction column (Waters) which had been pretreated with 20 mL each of methanol and d. H_2O. The column was washed sequentially with 8 mL of d. H_2O, hexane and methanol. The methanol fraction was collected and dried by rotary evaporation. The residue was dissolved in 200 µL chloroform and applied to a 325 mesh silicic acid (Sigma) column in a small pasteur pipet. This column was washed with 8 mL chloroform, and 8 mL $CHCl_3$-MeOH (9:1, v/v). The eluent was dried *in vacuo* and reconstituted in 2.00 mL methylene chloride. The sample was divided into 1.00 mL

Rinse tissue in 0.05M KH_2PO_4-NaOH buffer (pH 7.4)
Homogenize in 10 volumes of buffer
Add 5 volumes EtOH
Filter from precipitated protein on Whatman No. 42 paper
Evaporate filtrate *in vacuo*
Dissolve in 1 mL d. H_2O (pH 3)
Pass through C18 Sep-Pak
Wash Sep-Pak with 8 mL each of:
    d.H_2O
    hexane
    MeOH
Collect MeOH eluent
Dry *in vacuo*
Dissolve in 200 µL $CHCl_3$
Deposit on 325 mesh silicic acid column
Wash column with 8 mL each of:
    $CHCl_3$
    $CHCl_3$:MeOH (9:1)
Collect $CHCL_3$: MeOH eluent
Dry *in vacuo*
Prepare methyl ester (CH_2N_2), then TMS ether (BSTFA)

Figure 2-4: Pace-Asciak procedure for the extraction of oxygenated fatty acids from rat kidney.

portions, each of which was placed in silanized vials. From these two aliquots the PFB ester, TMS ether or methyl ester, TMS ether was prepared.

For electron capture detection, the PFB ester, TMS ether was prepared by adding another 1 mL of methylene chloride and 2 mL of 0.1 M TBA-HSO_4 (tetrabutylammonium hydrogen sulfate) in 0.2 M NaOH to the vial. Twenty-five microliters of pentafluorobenzyl bromide was added. The vial was vortexed at five minute intervals for 30 minutes. The methylene chloride layer (top) was placed in another silanized vial. The aqueous layer was washed two times with methylene chloride. Solvent was removed by nitrogen evaporation from the combined organic fractions.

To form the TMS ether, 25 µl of dry pyridine and 100 µl bis(trimethylsilyl)trifluoroacetamide (BSTFA) were added to the dried residue. The vial was tightly capped and incubated at 80 °C for one hour. The reactants were dried by nitrogen evaporation and the sample was dissolved in an exact volume (usually 200 µl) of hexane-ethyl acetate (1:1, v/v).

The methylene chloride from the other aliquot of silicic acid column eluent was dried by nitrogen evaporation. About 200 µL and an excess of ethereal diazomethane (ca. 4 mL) were added to the vial and mixed. The solution was allowed to sit for 20 minutes at room temperature, then dried by nitrogen evaporation. The TMS ether of the methylated mixture was prepared and reconstituted as described above.

The samples were analyzed by gas chromatography with electron capture detection (ECD) (Varian 3740) or flame ionization detection (FID) (HP5890). For the ECD analysis, a 15-m megabore (0.52 mm i.d., 1.0 um thick film) fused silica column (DB-1, J&W Scientific) was used; a 60-m capillary (0.25 mm i.d., 0.25 um thick film) column of the same stationary phase and manufacturer was utilized. Results of these analyses are presented in Figures 2-5 and 2-6. Aliquots of the PFB ester, TMS ether derivatives produced chromatograms with at most a few major peaks, corresponding to

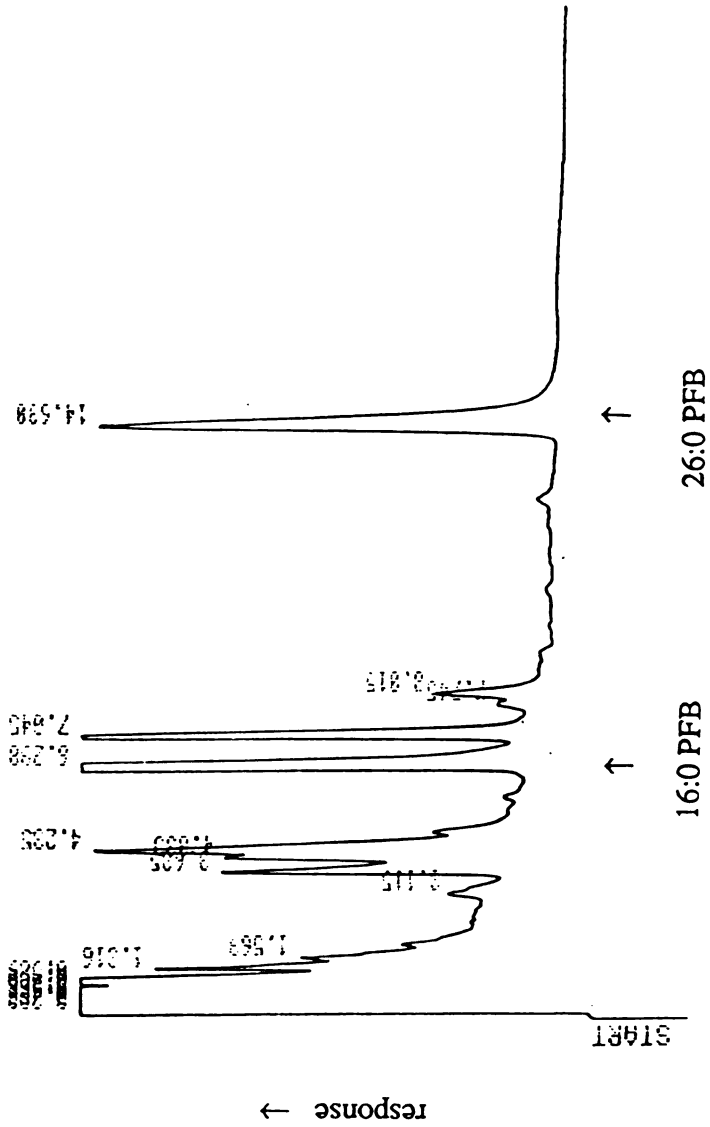


Figure 2-5: ECD chromatogram of PFB ester, TMS ether of Pace-Asciak extract of mammary tissue. 15 m DB-1 megabore (0.52 mm i.d., 1.0 µm film) column, 180° - 280°, 8°/min. 16:0 PFB ester and 26:0 PFB ester were added as internal standards.

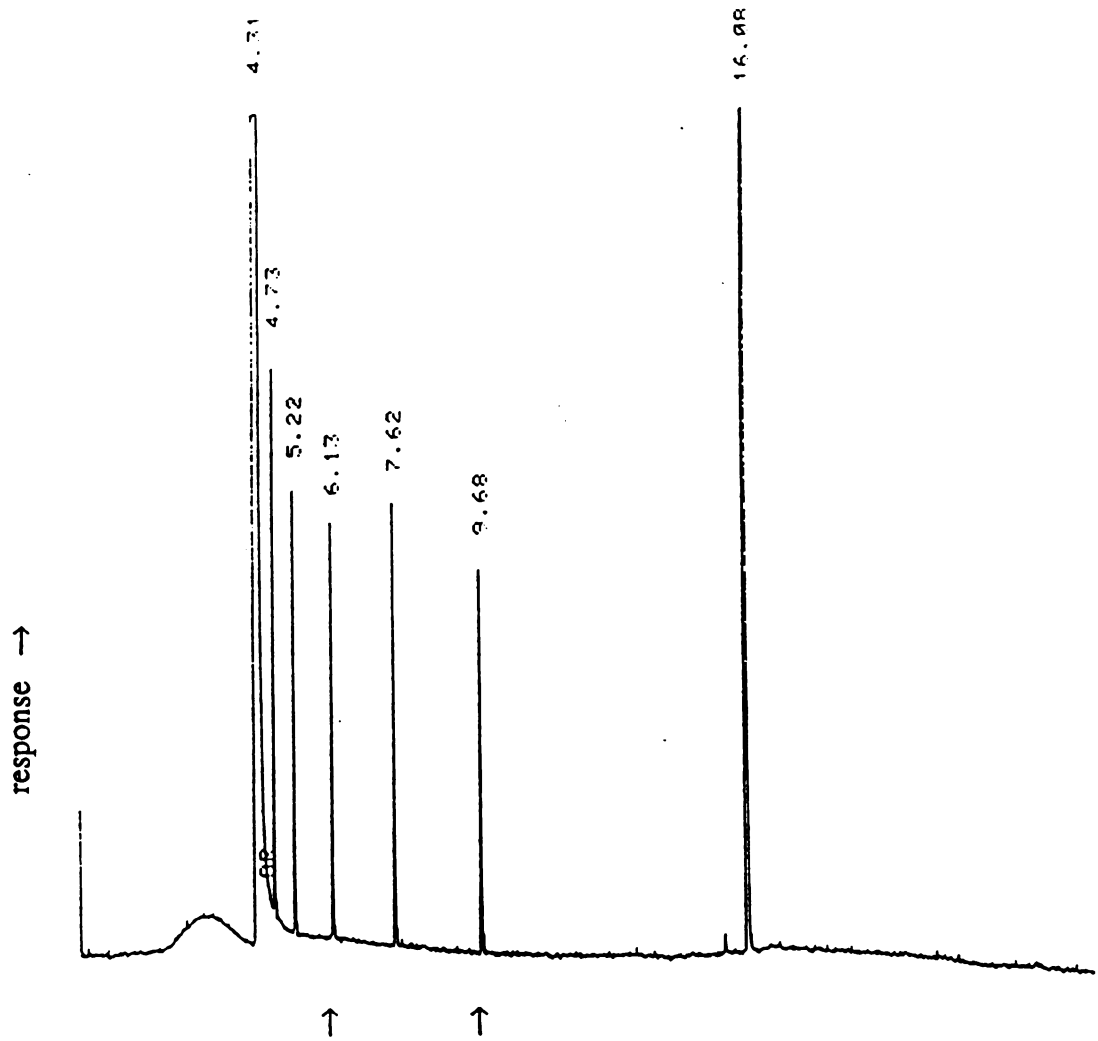


Figure 2-6: FID chromatogram of methyl ester, TMS ether of extract of mammary tissue processed by Pace-Asciak method. 60 m DB-1 capillary (0.25 mm i.d., 0.25 μm film) column, 180° - 280°, 4°/min. Hydrocarbons HC 10, HC 12, HC 14, HC 16, HC 18 and HC 20 were added for retention indexing.

residue from the derivatizing agents as determined by blanks. The other, minor components were not well resolved by the megabore column. Flame ionization detection of the methyl ester, TMS ether aliquots produced chromatograms with only one consistent major peak, which was identified as plasticizer by GC-EI/MS.

Several different trials using this extraction protocol were attempted, but the methyl ester, TMS ether derivative showed only plasticizer in its GC-FID chromatograms. Gas chromatography with flame ionization and electron capture detection of the PFB ester, TMS ether of several trials showed no biological components. (The plasticizer impurity was reduced by removal of a tygon tubing connection in the column set-up.)

Not only did this extraction procedure not extract any detectable levels of biologically-significant compounds, but it was very time consuming. Elution through the silicic acid column was extremely slow, due to the need to tightly pack the glass wool plug so the fine stationary material would not fall through the column. Thus, it was desired to investigate another extraction technique for this application.

3. Use of Funk and Powell Extraction Methodology

Funk and Powell isolated and analyzed prostaglandins and hydroxylated linoleic and arachidonic acid metabolites from rat and rabbit aortae (45). Their isolation procedure employed classical chloroform-methanol extraction and sample clean-up on ODS cartridge columns as outlined in Figure 2-7. All glassware used in these experiments was silanized prior to use. The sample was sliced into several pieces and then hand-homogenized in a glass homogenizer with a Teflon pestle in a dry ice-acetone bath in $CHCl_3$-MeOH (2:1) containing an internal standard and 0.05% butylated hydroxy toluene (BHT) as an antioxidant.

Cut up tissue on ice bath

Homogenize @ -20° in$CHCl_3$MeOH (2:1), 0.05% BHT, int. std.

Filter rapidly through sintered glass

Concentrate *in vacuo*

Divide into two equal aliquots

Free Lipid Fraction	**Free + Esterified Lipid Fraction**
C18 Solid Phase Extraction	Saponification
Evaporate *in vacuo*	Evaporate under Ar
Add 15% aq. MeOH	Dissolve in 0.28N KOH in 95% MeOH
Apply to C18 column	Incubate @ 55°for 45 min under Ar
Wash column with: (20 mL each)	Follow extraction as for Free Lipid Fraction
15% MeOH	
Distilled H_2O	
petroleum ether	
pet. ether:$CHCl_3$ (65:35)	
Collect pet. ether:$CHCl_3$ eluent	

Prepare methyl ester (CH_2N_2)

Make TMS ether (BSTFA)

Figure 2-7: Funk and Powell procedure for the extraction of oxygenated fatty acids from rat and rabbit aortae, as modified in this laboratory.

The homogenate was rapidly filtered through scintered glass under vacuum. The filtrate was dried by rotary evaporation, then 6.00 mL $CHCl_3$-MeOH was added. One-half of the sample was used to determine the free fatty acids, while the other aliquot was hydrolyzed to provide analysis of total (free and esterified) lipids.

The solvent in the free fatty acid fraction was removed by rotary evaporation and reconstituted in 15% aqueous ethanol. An ethanolic solvent was used in the literature procedure (45, 57), but was changed to 15% aqueous methanol after the first trials to prevent any ethyl ester formation. Once ethylated, fatty esters will not be converted to methyl esters during diazomethane methylation. The diluted homogenate portion was applied to a C18 Sep-Pak solid phase extraction (SPE) cartridge (Waters) prewashed as described in the preceding section. The column was washed with 20 mL each of 15% EtOH (or MeOH), d. H_2O, petroleum ether, and petroleum ether-chloroform (65:35). The water removes alcohol from the column; the petroleum ether wash dries the column of the water. The petroleum ether-$CHCl_3$ fraction contains fatty acids and monohydroxy fatty acids, and was collected and dried *in vacuo*. These mobile phases were pushed through the SPE cartridge with a syringe to achieve a flow rate of about 1-2 drops per second.

The aliquot designated for saponification was dried by nitrogen evaporation. The residue was dissolved in several milliliters of degassed 0.28 M NaOH in 95% ethanol (or methanol) and the vial was capped under nitrogen. The reaction mixture was incubated at 55 °C for 45 minutes. It was determined that polypropylene vials were the most appropriate vessels for the hydrogenation as the basicity of the solution desilylates and etches glassware, thus causing retention of the polar hydroxylated fatty acids (determined by retention of radio-labeled hydroxy fatty acid). The alcohol in the solution will leach phthalates from plastic vials, so these are unacceptable for hydrogenation. Upon completion of the incubation time, the sample was acidified slightly with HCl and dried under nitrogen. This step was time-consuming since the vial contained some water, but

rotary evaporation would require yet another transfer of the sample. The residue contained considerable particulate matter, presumably salts, and was dissolved in 3-4 mL of 15% aqueous ethanol (or methanol). The hydrolyzed aliquot was extracted by the same ODS silica column procedure described above.

The methyl ester of each fraction of the isolated mammary gland was prepared as described above. Derivatization to the TMS ether was achieved by addition of 50 µL dry pyridine and 100 µL BSTFA to vial containing the dried methyl esters. This reaction mixture was incubated for 45 minutes at 80 °C. The reactants were evaporated under nitrogen and the sample was dissolved in an exact amount of degassed toluene containing 0.05% BHT. These extracts were analyzed by GC-FID and by GC-EI/MS.

The complexity of the extract required use of 60-m long, capillary (0.25 mm i.d., 0.25 um thick film) non-polar (DB-1, J&W Scientific) columns. Initial FID and EI/MS (LKB 2091 mass spectrometer) analyses with similar megabore (DB-1, 0.52 mm i.d., 1.0 um thick film) columns demonstrated that the numerous components were not resolved (Figure 2-8). Capillary column GC-FID (HP 5890) chromatograms revealed approximately 120 components in the free fatty acid fraction (Figure 2-9) and over 150 components in the total lipid extract (Figure 2-10).

By comparison of retention indices and fragmentation from EI/MS with reference compounds, several of the major components of these extracts were identified as fatty acid methyl esters. Table 2-1 lists the FAMEs and their approximate amount in the free fatty acid fraction. Methyl esters of the even-carbon saturated fatty acids C14 through C24 and/or their unsaturated analogues were the most abundant components. Lower levels of odd-carbon saturated fatty acid methyl esters (C15, C17, C19) were detected in the extracts. Also present were phosphate esters and cholesterol. The identities of these compounds were confirmed by their mass spectra.

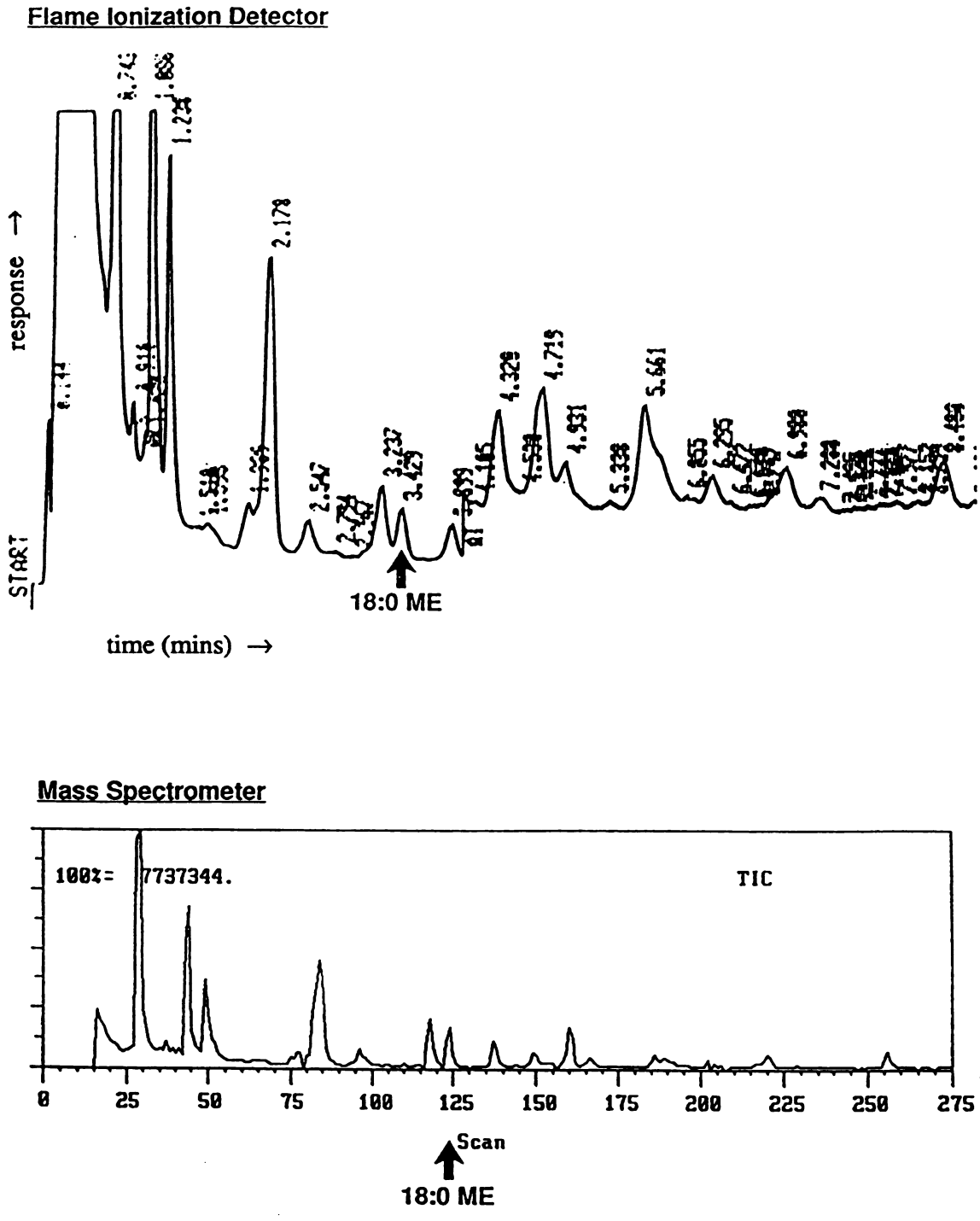


Figure 2-8: FID chromatogram (top) and reconstructed total ion chromatogram (bottom) of the methyl ester, TMS ether of a free fatty acid extract processed by the modified Funk and Powell method. Both analyses - 15-m DB-1 fused silica megabore (0.52 mm i.d., 1.0 μm thick film) column .

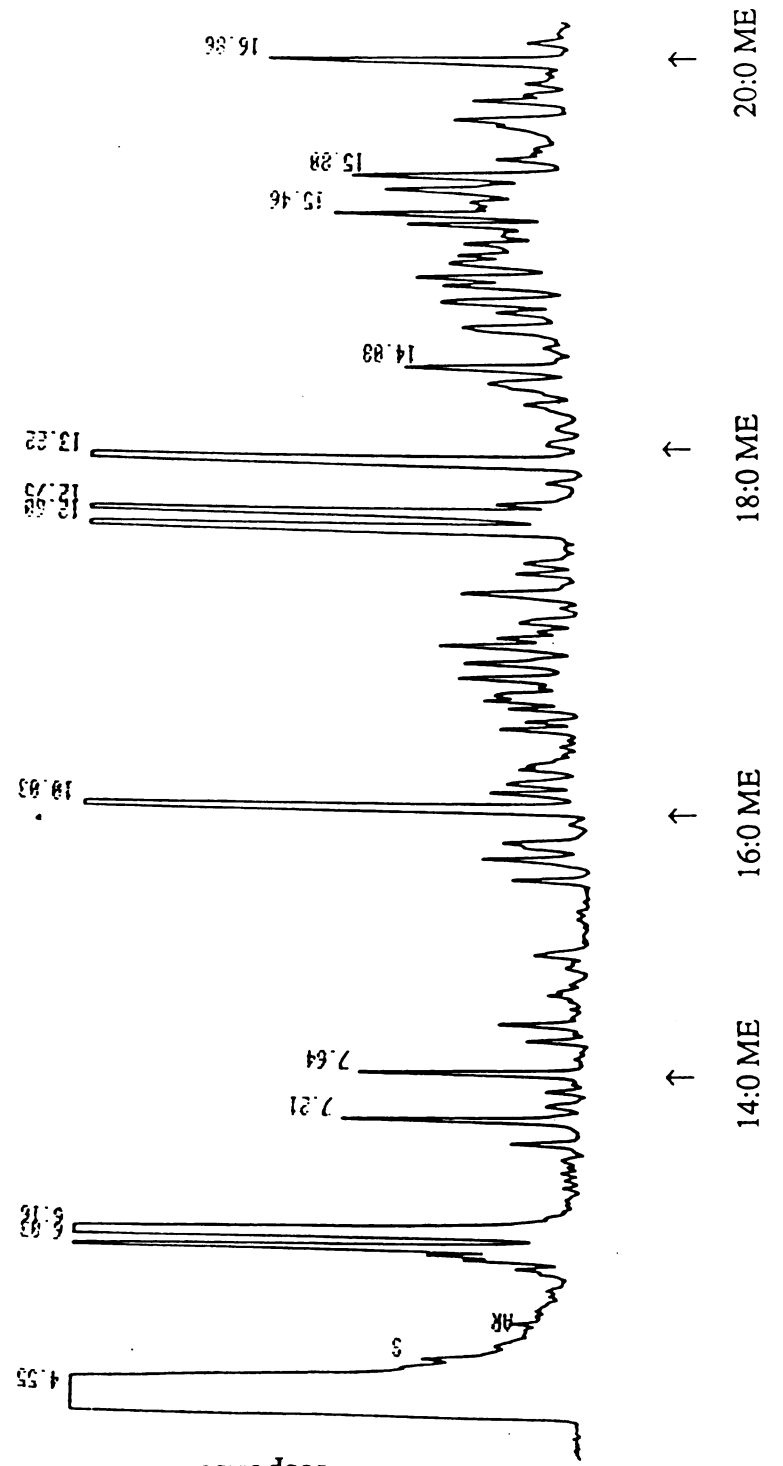


Figure 2-9: FID chromatogram of the methyl ester, TMS ether of a free fatty acid extract processed by the modified Funk and Powell method. 60-m DB-1 capillary (0.25 mm i.d., 0.1 µm thick film) column, 180° -325°, 4°/min, attentuation=1.

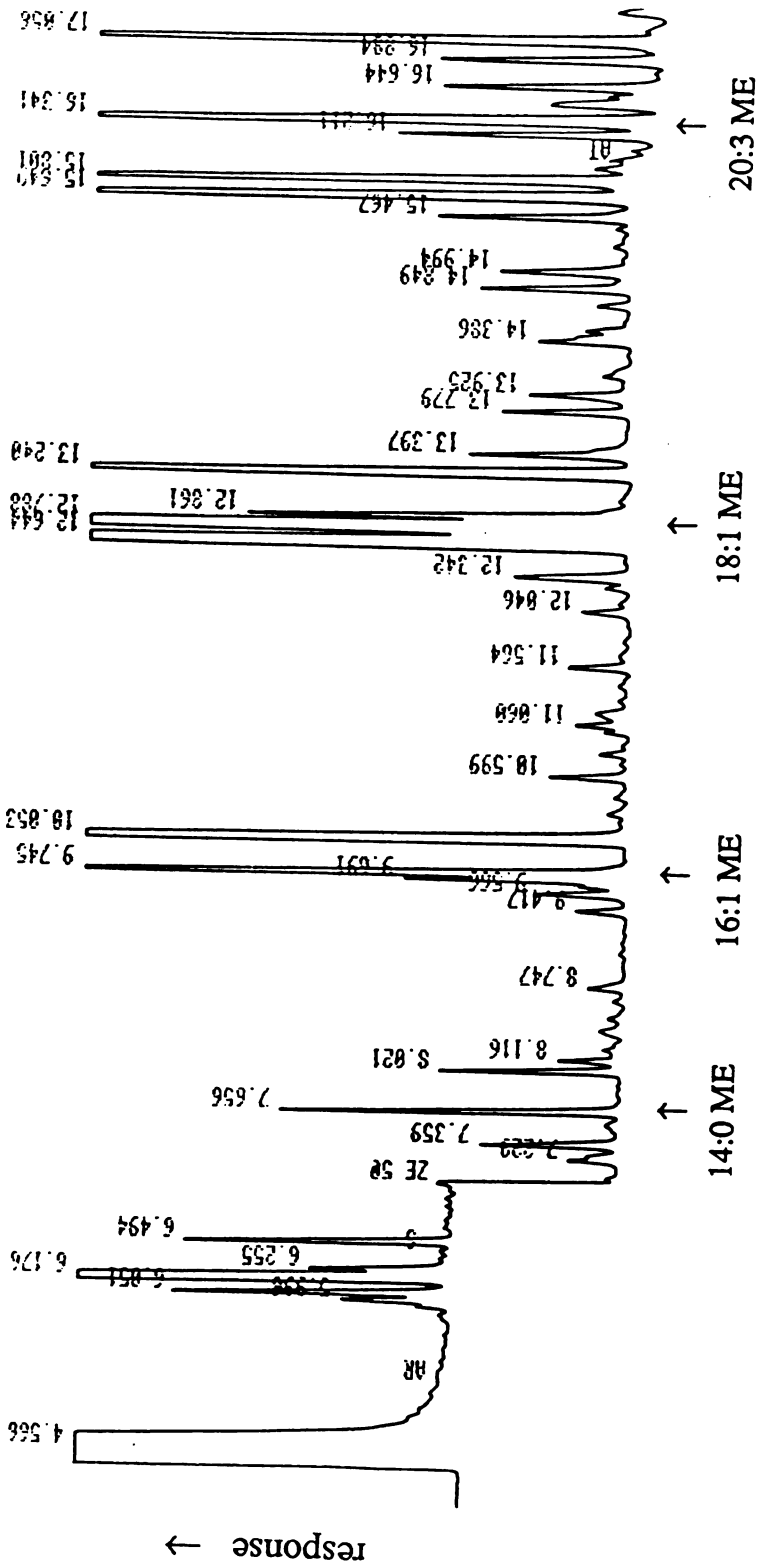


Figure 2-10: FID chromatogram of the methyl ester, TMS ether of a total lipid extract processed by the modified Funk and Powell method. 60-m DB-1 capillary (0.25 mm i.d., 0.1 µm thick film) column, 180° -325°, 4°/min, attentuation=3.

Table 2-1: Fatty Acid Composition of Free Fatty Acid Extract

FAME	Amount (ng)	% of Free Fatty Acid Extract
14:0	420	0.2
$14:1^{9}$	87	0.04
15:0	440	0.2
16:0	5200	3
$16:1^{9}$	230	0.1
17:0	1010	0.4
18:0	9120	5
$18:1^{9}$	700	0.4
$18:2^{9,12}$	510	0.3
19:0	700	0.4
20:0	330	0.2
$20:3^{8,11,14}$	880	0.5
$20:3^{11,14,17}$	440	0.2
$20:4^{5,8,11,14}$	350	0.2
22:0	430	0.2
$22:1^{9}$	410	0.2
$22:6^{4,7,10,13,16,19}$	360	0.2
24:0	170	0.1
24:1	330	0.2
unidentified components		88%

There were numerous other peaks in the FID chromatograms of the extracts which did not have retention indices corresponding to a derivative of a common fatty acid. Mass spectral analysis did not allow identification of numerous components, either due to inability to interpret the mass spectrum (no characteristic fragmentation) or the inability of the detector to accurately record the true mass spectra of the eluents.

High levels of unsubstituted saturated and unsaturated fatty acids in the mammary gland could provide interferences in the detection of any oxygenated fatty acids possibly present. Any oxygenated fatty acid would be expected to be a minor component, in the parts per million or less range. For a 100 mg tissue, this would correspond to 100 ng in the combined extracts (assuming 100% recovery). Thus, if there were any overlap in the elution of a the methyl ester, TMS ether of an oxygenated fatty acid and the methyl ester of one of the unsubstituted fatty acids so abundant in the mammary gland, the FID or ion signals from the oxygenated component would be dwarfed by the signals from the latter. This overlap is very possible, as illustrated by the retention times of the methyl ester of arachidic acid and the methyl ester, TMS ether of 13-OH 18:0 (Figure 2-11).

4. Hydrogenation of Extracts

One way of correcting for the possible chromatographic overlap is to simplify the extract. Hydrogenation of the extract will saturate the unsaturated fatty acids, decreasing the possibility of chromatographic overlap of the high-level unsubstituted fatty acids with any oxygenated fatty acids. After reduction, any unsaturated hydroxylated fatty acids of the same carbon number and same hydroxy position will coelute, merging the signals. This would possibly increase the signals from any low-level oxygenated fatty acids.

The extracts were hydrogenated after the methyl ester was prepared by a classical, platinum-catalyzed reaction (58). The methylated extracts were dissolved in methanol in a silanized vessel. Initially, a side arm flask fitted with a ground glass connection to a gas buret was utilized. Later a gas manifold was constructed to permit simultaneous reaction in four screw cap vials. Hydrogen from the manifold was leaked into the

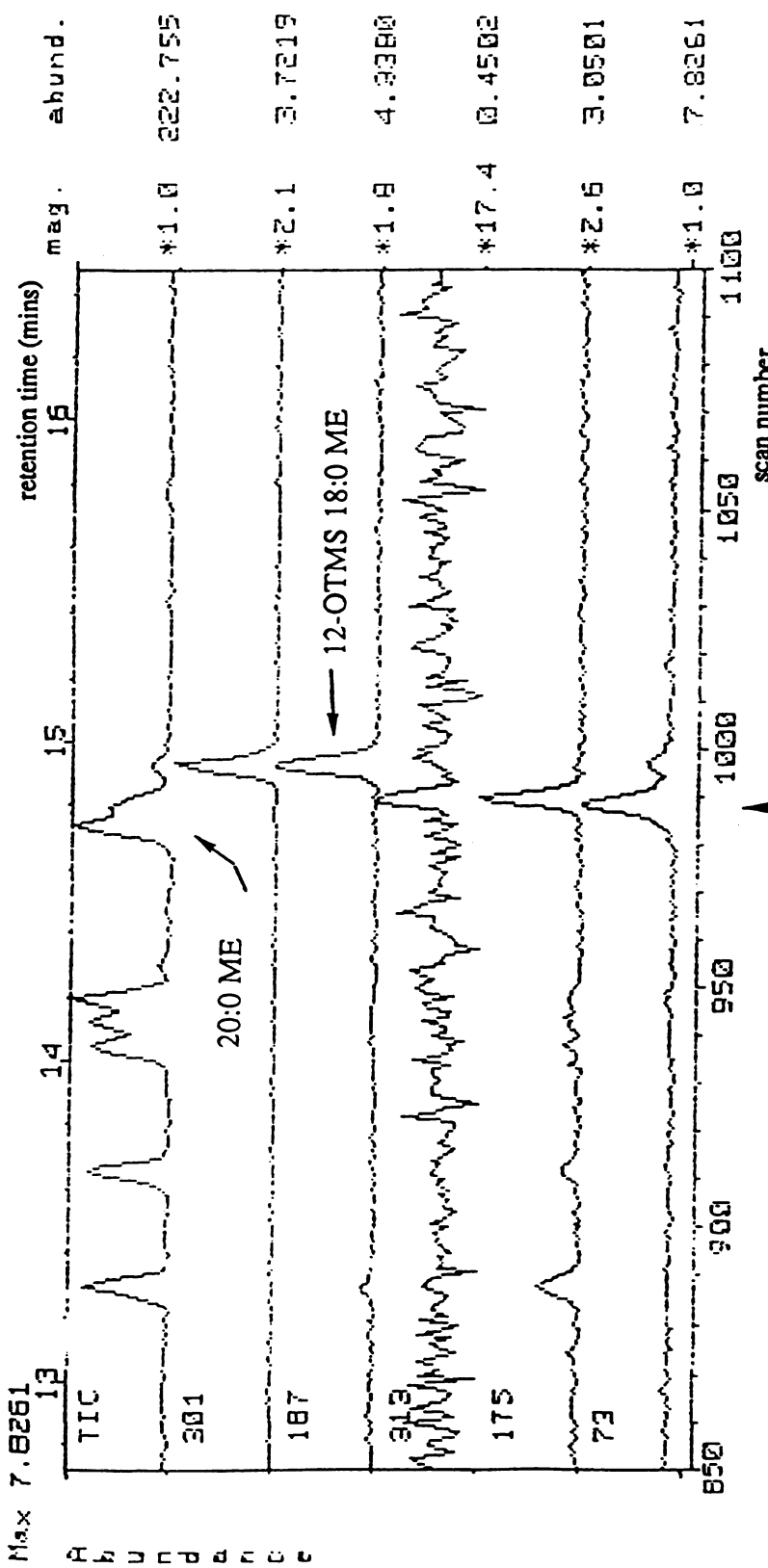


Figure 2-11: Reconstructed total ion and mass chromatograms at m/z 301, 187, 313, 175 and 73 from GC-EI/MS (scanning m/z 50-500), illustrating potential chromatographic overlap of 13-OTMS 18:0 ME (10 ng inj.) and 20:0 ME (50 ng inj.). Other peaks in the TIC are unsaturated C20 methyl esters.

reaction vial by a needle through a Teflon-lined septum. A stirbar, a very small amount of platinum dioxide (Aldrich), and one drop of acidified methanol was added to the vessel before it was sealed. The vessel was evacuated and magnetically stirred. The vessel was purged with hydrogen and evacuated, and the cycle was repeated. If the reaction vessel atmosphere is hydrogen and the oxygen has been replaced, the platinum oxide reduces to platinum black. A positive hydrogen pressure was left on the vessel for 20 minutes. The reaction mixture was filtered on Whatman No. 1 paper, and rinsed with methanol three times. Care was taken to keep the paper wet, as the platinum could spark. After rinsing was complete, the paper was doused with water. The filtrate was dried under nitrogen evaporation, and the TMS ether was prepared as described above.

The FID chromatograms (Figure 2-12) of hydrogenated and non-hydrogenated portions of a total lipid extract show the extent of reduction. The chromatograms of the extracts were greatly simplified. Noting the shift in retention time of certain peaks signals the presence of unsaturated components.

The efficiency of the hydrogenation was verified with standards of high levels (20 and 5 mg) of unsubstituted fatty acid methyl ester and 10 and 0.5 ppm, respectively, hydroxylated FAME (Table 2-2). Analysis by GC-FID showed that under these conditions of sample level, hydrogenation of these fatty acid methyl esters was complete. It should be noted that these conditions were not robust enough to reduce all of the unsaturated fatty acids in some of the extracts. In particular, methyl esters of oleic and linoleic acid were detected in some of these extracts. This is due to the high amounts of unsubstituted fatty acids in the mammary gland, a fatty tissue. It is this high content of unsubstituted fatty acid that made analysis of hydroxylated fatty acids with these extraction procedures very difficult. There were no mass spectral data that indicated any derivatized hydroxylated fatty acids were present in the fatty acid extracts.

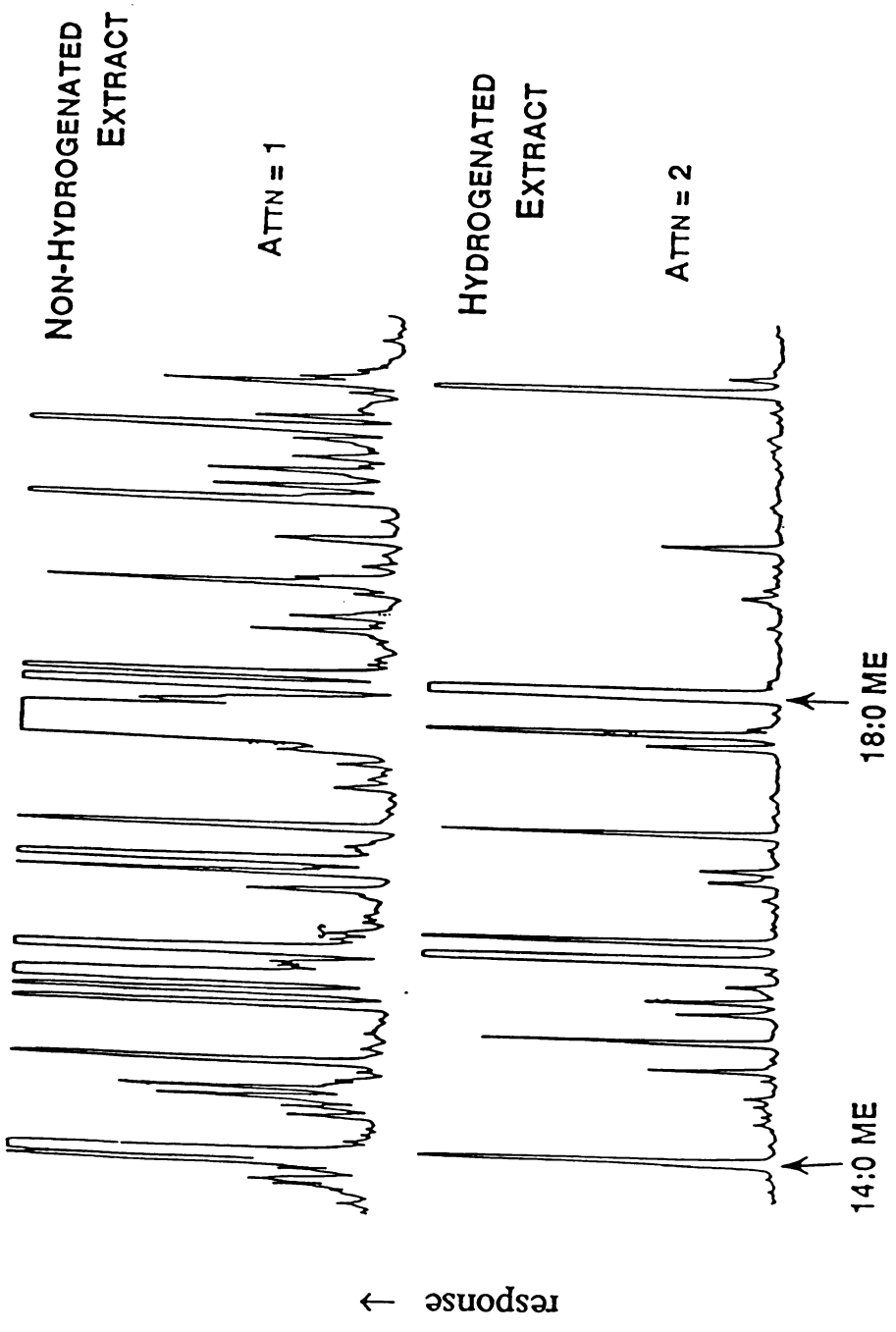


Figure 2-12: FID chromatograms of non-hydrogenated (top) and hydrogenated (bottom) aliquots of total lipid extract. 60 m DB-1 capillary (0.25 mm i.d., 0.1 μm film) column, 180°-325°, 4°/min.

Table 2-2: Efficiency of Hydrogenation Reaction

Vial No.	18:2 ME		12-OH 18:1 ME	
	(mg)	Efficiency	(ng)	Efficiency
1	20	99.6%	100	100.0%
2	20	100.0%	100	100.0%
3	20	100.0%	100	100.0%
4	5	100.0%	50	100.0%
5	5	100.0%	50	100.0%

III. Chapter Three: Development of New Isolation Procedure

The high fatty acid content of the mammary gland provided a high chemical background in the determination of any possible trace hydroxylated fatty acids when the isolation procedures described above were employed. These isolation procedures were developed in other laboratories for the analysis of tissues with much lower lipid content, and have been found in this study to be unsuitable for the analysis of mammary tissue. After isolation by the above-mentioned techniques, the extract has found to contain many components, many of which coeluted, even with high-resolution chromatography. Thus a new extraction technique needed to be developed to separate any possible hydroxylated fatty acids, which would be expected to be present only in trace amounts, from the unsubstituted fatty acids, which constitute a large portion of the mammary gland.

The original intent of the new extraction procedure was to perform a class separation of the oxygenated fatty acids in the mammary tissue from the non-lipids and the unsubstituted fatty acids. Thus, the extract would become greatly simplified and some potentially coeluting compounds would be eliminated. This goal was restricted somewhat by the use of only hydroxylated fatty acids as model compounds during the development and assessment of the new isolation procedure. This decision was made because some standards for these hydroxylated compounds are readily available and relatively temperature- and light-insensitive (with respect to the analogue keto- and epoxy- fatty acids).

A. Development and Description of New Isolation Procedure

1. Solvent Extractions

The first steps of the mammary gland extraction are to homogenize the tissue and to separate the lipids from the non-lipid matter. To achieve this latter goal an aqueous phase extraction (52, 53) was performed after the initial chloroform-methanol (2:1) solvent extraction (45). (All glassware was silanized before use). For a tissue of 0.1 gram, two milliliters of $CHCl_3$:MeOH (2:1) was used during homogenization in a dry

ice:isopropanol bath. After the homogenization, which was mechanically driven by a drill motor, the sample was quickly filtered through sintered glass under vacuum and an additional 0.5 mL of the solvent mixture was used to quantitatively rinse the glassware. The filtrate was collected into an inert vessel, and 20% by volume (0.5 mL) deionized water was added.

The mixture was capped and vortexed, then separated in a bench-top centrifuge at about 500 rpm for one minute. A polypropylene test tube was found to be the inert vessel easiest to use and prepare . No phthalates were found by gas chromatography-flame ionization detection (GC-FID) in blank solvent systems ($CHCl_3$, MeOH, and water) which were allowed to sit in the polypropylene test tubes for 15 minutes. The test tubes readily fit into the centrifuge and into the side arm flask used for vacuum filtration. The polypropylene test tubes required no preparation, unlike glass test tubes, which must be cleaned and silanized before each use. There was visible material in the aqueous wash which separated from the organic layer. It was determined by GC-FID that no lipids were extracted into the aqueous layer, even when the mammary gland was spiked with a mixture of unsubstituted and hydroxylated fatty acids just before homogenization.

2. Methylation

The organic layer was transferred to a one dram, screw-top vial and dried under nitrogen. The lipids were methylated with diazomethane and methanol at room temperature for 20 minutes, as described in the previous chapter. At this point the extract contained methyl esters of all of the lipids originally present in the homogenate (tissue plus any internal standard added). As described in the previous chapter, a portion of the extract can be removed at this time for acidic, platinum-catalyzed hydrogenation.

3. Hydrogenation

This hydrogenation step was originally performed in individual vials and was redesigned to a simultaneous and less labor-intensive process. A gas manifold was designed in this laboratory and crafted in the Chemistry Department Glassblowing Shop

with four outlet ports, each with an individual Teflon vacuum stopcock (Figure 3-1). Each outlet was connected to an 18-gauge needle which easily introduced hydrogen into the vial through a Teflon-lined septum in the screw cap. To rid the vial of oxygen, a vacuum connected to the manifold could be used or the vial could be purged via a 22-gauge needle through the septum during the initial three to five minutes. Contents of the vial were stirred by magnetic stirrers below the vials. A small positive hydrogen pressure was applied to the manifold to replenish any hydrogen consumed or leaked. After 20 minutes, the hydrogenated extracts were filtered and dried as previously described.

4. Normal-Phase Chromatography

A column chromatography step was developed to separate the methyl esters of the unsubstituted fatty acids from the extract, leaving the hydroxylated (and possibly other oxygenated) fatty acid methyl esters (FAMEs). Since separation by published methods with octadecylsilyl C18 (non-polar) columns did not appear to fulfill this goal, normal phase chromatography with silica (polar) columns was investigated.

In the 1950s, early work with silica columns utilized different proportions of diethyl ether-petroleum ether as solvents for elution of lipid classes. But Horning and coworkers (59) utilized benzene-hexane mobile phases to elute various lipid classes from silicic acid columns. The latter mobile phases were easier to use, partially due to its lower volatility. The increase in greater differences in percent composition with the benzene-hexane versus the diethyl ether-petroleum ether also makes the benzene-hexane system more desirable.

Initial studies in this laboratory were conducted with a standard mixture of unsubstituted and hydroxylated FAMEs, both alone and in the presence of mammary tissue extract to determine the percentage of benzene in hexane needed to remove the unsubstituted FAMEs from the column, while keeping the hydroxylated FAMEs (OH-FAMEs) adsorbed to the stationary phase for subsequent elution. The first trials were performed with silicic acid (Sigma 325 mesh) in a 2.5 mm i.d. column, slurry (hexane)

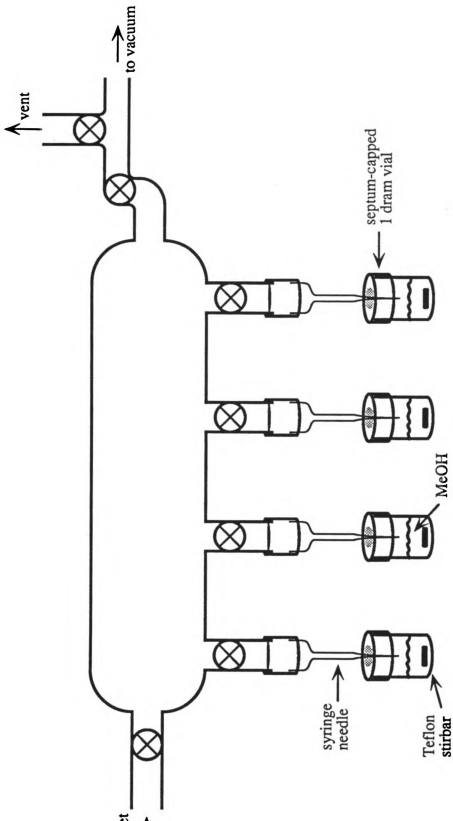


Figure 3-1: Schematic representation of the gas manifold used to hydrogenate four samples simultaneously.

packed by gravity to a bed height of about 40 cm. After dilution in hexane, the standard or spiked tissue was applied to the column, which was rinsed with one column volume of hexane. Mobile phases of 6%, 20%, 30%, 40%, 50%, and 60% benzene in hexane; $CHCl_3$:MeOH (1:1); and 100% MeOH were prepared and applied to the column sequentially. Fractions of eluent from each mobile phase composition were collected, dried under rotary evaporation, and analyzed by GC-FID (DB-1, 15-m, 0.52 mm i.d., 1.5 μm thick film). The results of these analyses for a mammary gland spiked with the standard mixture are given in Figure 3-2. The unsubstituted FAMEs began to elute when the 6% benzene in hexane mobile phase was applied, and 20% benzene in hexane appeared to be the mobile phase in which the last of the unsubstituted FAMEs were removed from the stationary phase. The hydroxylated FAMEs began to elute with the 50% benzene in hexane and were not completely removed from the column until $CHCl_3$:MeOH (1:1) was used as a mobile phase.

This large gravity-fed column probably contained an excess of theoretical plates and made the procedure very time-consuming, even when a slight head pressure of argon was applied to the column to achieve a flow rate of about 2 drops per second. The process was further improved by the use of solid phase extraction columns and a vacuum manifold with Teflon stopcocks (Burdick & Jackson). Silica SPE cartridges (Burdick & Jackson, 500 mg) were pretreated by rinsing with two to three column volumes of $CHCl_3$:MeOH (1:1) and then with enough hexane to fully saturate the stationary phase. The vacuum manifold provided simultaneous pretreatment of twelve columns and elution of up to three columns with a constant, controllable flow. With the addition of adapters and 50 mL reservoirs, the mobile phase could be loaded onto the apparatus, requiring much less attention from the operator. The claims of inertness of adapters and reservoirs from different manufacturers were tested with washes of $CHCl_3$ and MeOH and megabore GC-FID, and the Burdick & Jackson parts were found to be the best.

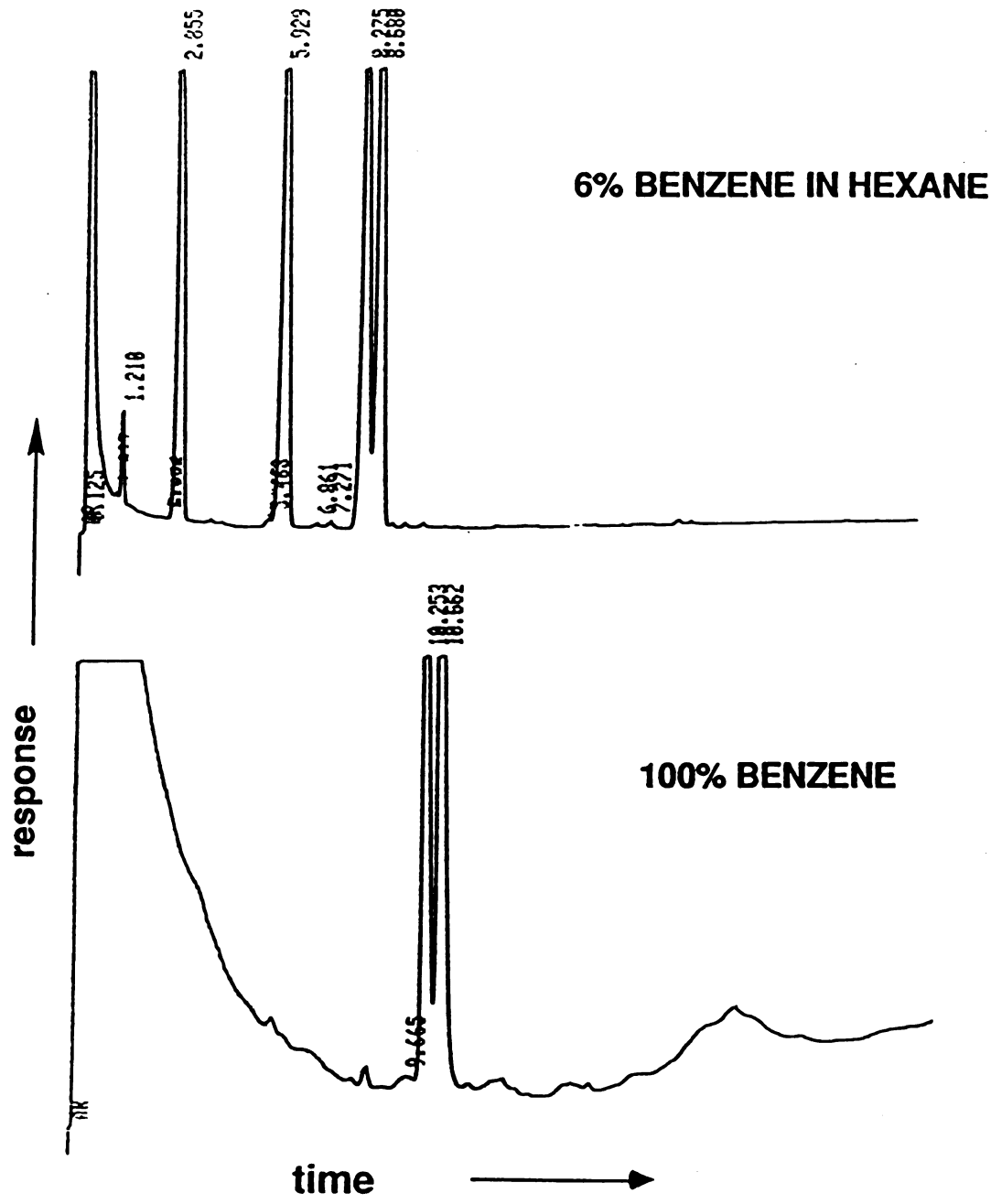


Figure 3-2: Megabore column (DB-1, 15-m long, 0.52 mm i.d., 1.5 μm thick film) GC-FID chromatograms resulting from analysis of spiked mammary gland extract eluents from selected mobile phases through silica SPE columns. The bottom chromatogram is 12-OH 18:1 ME and 12-OH 18:0 ME in the eluent from 100% benzene (1.2% of the eluent injected; attenuation=3). The top chromatogram represents a mixture of 14:0, 16:0, 18:1, and 18:0 MEs (1.7% of the eluent injected; attentuation=8). GC conditions: N_2 flow rate 15.0 mL/min; temperature program-160° for 4 min, then 8°/min to 280°.

The new silica SPE system was implemented with the following mobile phases:

a) application of sample dissolved in ca. 2 mL hexane

b) 7 mL hexane to rinse SPE column

c) 50 mL 35% benzene in hexane to elute the unsubstituted FAMEs

d) 50 mL $CHCl_3$ to elute the OH-FAMEs

e) 50 mL $CHCl_3$:MeOH (1:1) to determine if all OH-FAMEs eluted.

The last column washing step was eliminated after several trials of mammary gland spiked with the standard fatty acid mixture demonstrated that no hydroxylated FAMEs remained on the column after elution with 100% chloroform. The volumes of the benzene:hexane and chloroform mobile phases were reduced to 20 mL, with no loss in efficiency of elution of the desired type of fatty acid methyl ester.

5. Trimethylsilylation of Alcohols

The eluent from the 100% chloroform mobile phase was collected in a round bottom flask and dried under rotary evaporation. The residue was quantitatively transferred to a conical reaction vial with methanol and dried under nitrogen. The trimethylsilyl ether was prepared with bis(trimethylsilyl)trifluoroacetamide as described in the previous chapter. The derivatized extract was reconstituted in an exact amount (usually 100 μL) of dry hexane:ethyl acetate (1:1) containing 0.05% BHT and 5% BSTFA. The new isolation procedure is summarized in Figure 3-3.

6. Reduction of Contaminants

Throughout the development of the new isolation procedure, care was taken to minimize the amount of blank interferences from the apparatus and chemicals. The selection of Teflon and inert plastic (polypropylene) parts for the vacuum manifold and the choice of polypropylene test tubes for centrifugation were described earlier in this chapter. All solvents used were analytical or HPLC grade. High boiling impurities were discovered by GC-FID in HPLC grade chloroform. After unsuccessful attempts to dry or chromatographically clean the chloroform, distillation removed the impurities. To

1. Mechanically Homogenize Tissue in $CHCl_3$/MeOH (2:1) with 0.05% BHT (2 mL).

2. Filter through Sintered glass.

3. Aqueous Wash (0.2 Volumes).

4. Separate Organic Layer and Remove solvents with Nitrogen.

5. Methylate with ethereal CH_2N_2/MeOH.

6. Remove excess reagent and solvent with Nitrogen.

7. (Optional Hydrogenation - Pt catalyst, positive H_2 pressure).

8. Silica Column Clean-Up:

    a. Apply Sample in Hexane.

    b. Wash with 35% Benzene in Hexane to Elute Non-Substituted Fatty Acids.

    c. Wash with 100% $CHCl_3$ and Collect Eluate of Oxygenated Fatty Acids.

9. Remove solvents by rotary evaporation.

10. (Optional Conversion of Ketones to Methoximes.)

11. Trimethylsilylate Hydroxyl Groups with BSTFA/Pridine

12. Dry excess reagents with Nitrogen.

13. Add 50 or 100 µL 0.05% BHT in Solvent/BSFTA (95:5), Solvent is Hexane:EtOAc or Toluene.

Figure 3-3: Summary of new procedure to isolate hydroxylated fatty acids from mammary glands. See text for detailed description.

minimize chlorine formation, 0.05% ethanol was added to the pure $CHCl_3$;fortunately, this did not appear to cause reintroduction of the high boiling impurities.

Selection of the best commercially available SPE silica column was based upon a determination of which generated the lower amount of background contaminants as analyzed by GC-FID analysis of column washes with distilled chloroform. Two 500-mg silica SPE columns from each of Burdick & Jackson and J.T. Baker were pretreated with $CHCl_3$:MeOH (1:1) and hexane as previously described. Then 40 mL of distilled $CHCl_3$ were run through each column and collected in individual round bottom flasks. The flasks were dried *in vacuo* and the residue was transferred with methanol to a one-dram vial. The contents were dried under nitrogen, and 200 μL of hexane:ethyl acetate were added. The reconstituted eluents were analyzed by megabore column (DB-1, 15-m long, 0.52 mm i.d., 1.5 μm thick film) GC-FID. The gas chromatogram of the eluents from the SPE column manufactured by Burdick & Jackson had fewer impurities, especially in the area of retention of the higher boiling FAMEs (Figure 3-4).

B. Detection Techniques for Analysis of Extracts

A summary of historical and current procedures for gas chromatography with flame ionization detection or electron ionization mass spectrometry is presented in Chapter Two. In order to resolve the large number of components in the mixture of methyl ester, TMS ethers from the mammary gland extract that was processed by adaptation of the Funk and Powell method described in Chapter Two, a very long (60-m) high-resolution capillary column (DB-1, 0.25 mm i.d., 0.1 μm thick film) was needed. The final derivatized mammary gland extract from the new isolation procedure described above contained many fewer components, particularly in the region of elution of the methyl ester, TMS ethers of the C_{18} hydroxylated fatty acids, due to the absence of the unsubstituted FAMEs (Figure 3-5). The GC-FID chromatograms of extracts from the new isolation procedure did not contain any peaks which were thought to represent any of the unsubstituted FAMEs which were detected in the extracts processed by the Funk

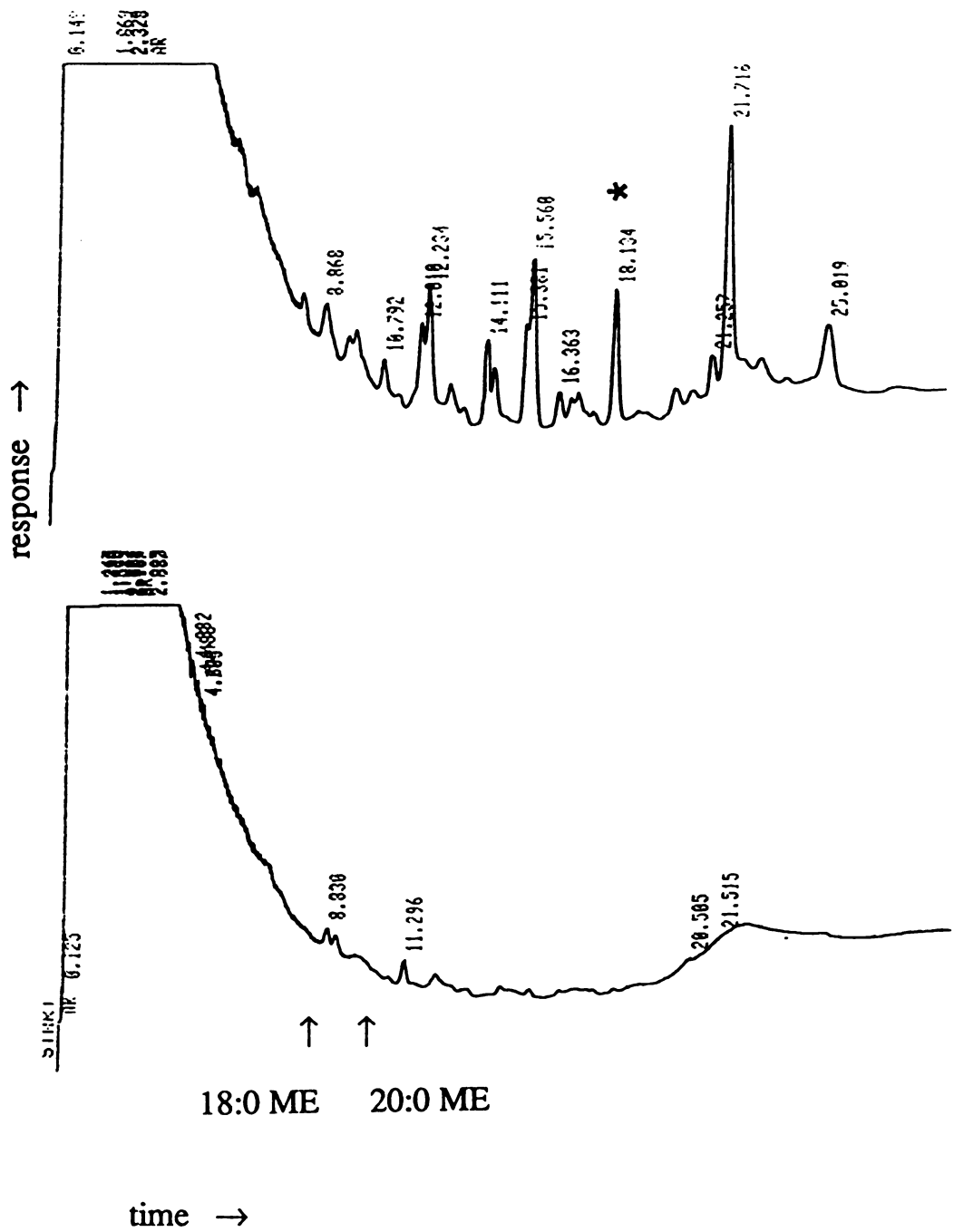


Figure 3-4: Megabore column (DB-1, 15-m long, 0.52 mm i.d., 1.5 µm thick film) GC-FID chromatograms of chloroform solvent blanks through silica SPE columns from Burdick & Jackson (bottom) and J.T. Baker (top) with elution times of some FAMEs designated. GC conditions: N_2 flow rate 15.0 mL/min; temperature program-140° for 4 min, then 8°/min to 280°; 2 mL injection volumes. Attenuation=5. The marked peak has an area equivalent to the area of the signal from 0.1 µg 18:0 methyl ester.

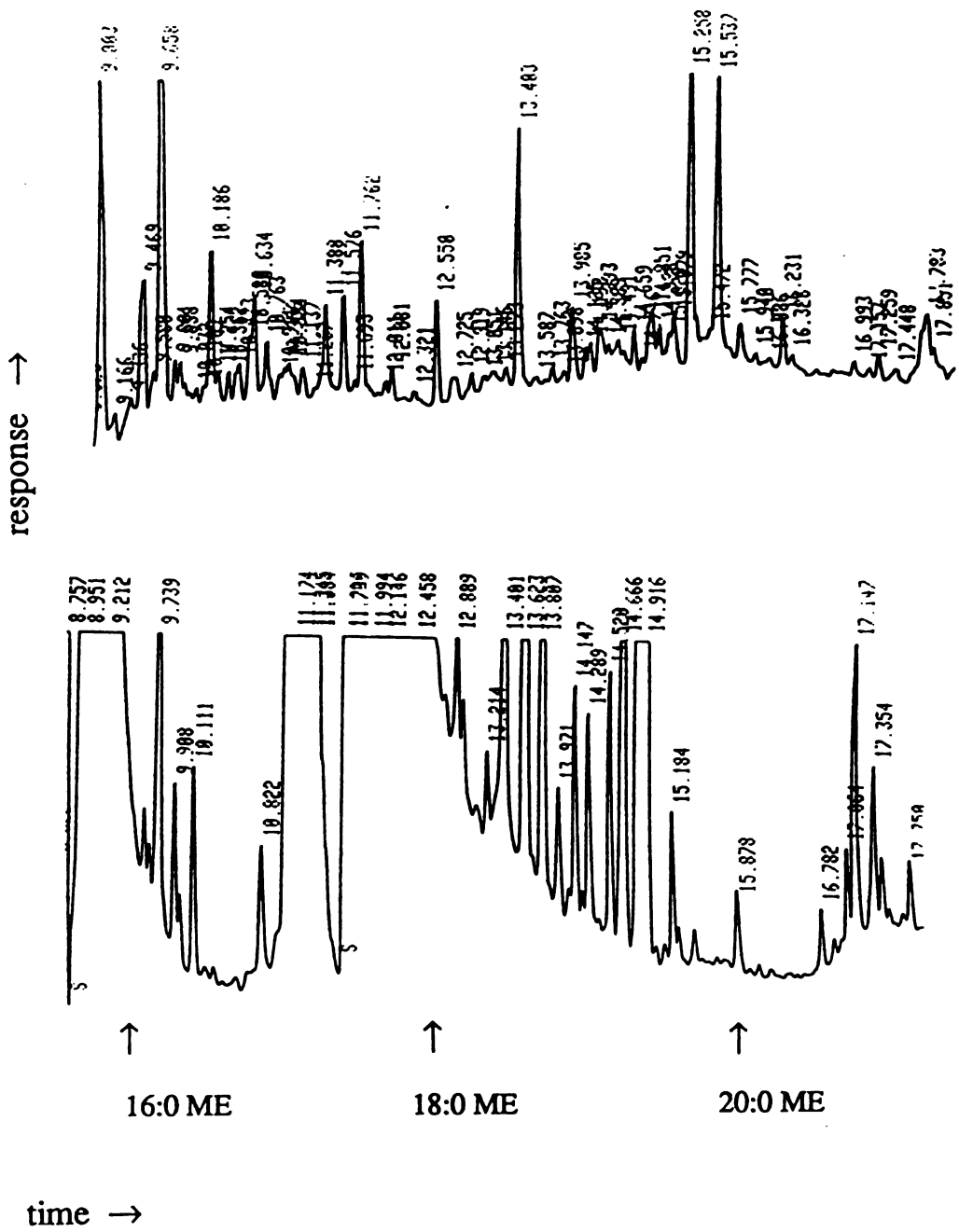


Figure 3-5: Capillary column (DB-1, 60-m long, 0.25 mm i.d., 0.1 μm thick film) GC-FID chromatograms of 4.0% of extract from new isolation procedure (top) and 0.5% of total lipid extract from modified Funk and Powell Method (bottom) with elution times of some FAMEs designated. GC conditions: He flow rate 1 mL/min; temperature program-180°, 4°/min to 320°; attentuation=3.

and Powell method (Table 2-1). Mass spectral analysis confirmed that no C_{16} nor C_{18} unsubstituted FAMEs, nor phosphate esters, were present in the extracts processed by the new isolation method. The TMS ether of cholesterol was identified by GC-FID and GC-EI/MS to be a very late eluting component of extracts from both procedures. Many of the extracts processed by the new isolation procedure contained minor components which were confirmed by GC-EI/MS to be TMS ether, methyl esters of C_{18} hydroxylated fatty acids (see Chapter Four for details). These OTMS C_{18} methyl esters (nor any other OTMS-FAMEs) were not observed by the same mass spectral or flame ionization detection methods in the extracts from the Funk and Powell extraction method, possibly due to the high level of other lipids, particularly unsubstituted FAMEs, in the region in which the former compounds elute. Thus, only a 30-m capillary column (0.25 or 0.314 mm i.d., 0.25 μm film) was needed to resolve the components near the retention indices of the C_{18}- and C_{20}-hydroxylated FAMEs (Figure 3-6).

Flame ionization detection provides a sensitive and continuous method of analysis of the column eluent, but its capacity to identify components is limited, since retention indices must be known from standards, which are not always available. Mass spectrometry, EI/MS, can provide additional structural information to assist in the identification of compounds which are not resolved chromatographically or are not readily identified by their retention indices. Specific fragmentation characteristics of the methyl ester, TMS ethers of hydroxylated fatty acids were described in the previous chapter.

1. Electron Ionization/Scanning Mass Spectrometry

The methyl ester, TMS ethers of the monohydroxylated C_{18} and C_{20} fatty acids have molecular weights in the 380-420 u range. Although a molecular ion $[M]^{+\cdot}$ is not always observed with EI/MS, loss of a methyl group $[M-15]^{+}$ is a common fragment ion, strongly suggesting that the mass range of the mass spectrometer be extended to at least m/z 430. Characteristic α-cleavage fragments give intense peaks in the mass spectra of

Figure 3-6: Capillary column (DB-1, 30-m long, 0.314 mm i.d., 0.25 µm thick film) GC-FID chromatogram of 1.5% of hydrogenated extract from new isolation procedure with elution times of some FAMEs designated. GC conditions: He flow rate 1 mL/min; temperature program-180°, 4°/min to 325°; attenuation=2.

these compounds and they provide important structural information, therefore it is critical to monitor these ions. A list of these α-cleavage ions from the methyl ester, TMS ethers of the possible monohydroxy isomers of stearic acid is given in Table 3-1. The range of these ions is large (m/z 103-327) and the instrument's mass range must be extended to encompass all of the fragments. At the lower m/z values, other characteristic fragments, especially those for the trimethylsilyl fragment (m/z 73) and the McLafferty rearrangement fragment at m/z 74, are observed. Thus, it is ideal to analyze all of the ions in the range m/z 70-430 in order to record all of the ions which may give structural information and help identify the methyl ester, TMS ethers of hydroxylated fatty acids.

With sector mass spectrometers, the range of ions to be analyzed is scanned repetitively in a finite time. The time of ion sampling and the rest time can restrict the instrument to only one to a few scans of a large mass range, such as m/z 50-500, per second. This causes possible distortions in the mass spectra for compounds that are eluting from the high-resolution column in a very short time span (60). If the duration of elution of a component is only two to four seconds, then the partial pressure of that compound would change significantly during a typical 0.9 second scan time. The relative peak intensities would be distorted from those observed when the compound is at a constant pressure in the mass spectrometer source (Figure 3-7). When the repetitive scan rate is low, another problem encountered is that the reconstructed mass chromatogram is composed of only a relatively few data acquisition points during the short elution time of the compound, thus distorting the apparent elution profile.

The highest scan repetition rate possible for the particular instrument is desired when high resolution chromatography is utilized. However, conventional magnetic sector instruments are severely limited by the rest time needed to correct for the hysteresis of the magnetic field, limiting scan rates for the m/z 50-500 range to less than two sweeps per second. On the instrument available for this project, the greatest scan speed requires 0.9 second per scan of m/z 50-500.

Table 3-1: List of m/z Values at which Peaks for α-cleavage Ions Would Occur for Isomers of OTMS-18:0 ME

Isomer	Expected Peaks	
	m/z	m/z
(α)2-OTMS	161	327
(β)3-OTMS	175	313
4-OTMS	189	299
5-OTMS	203	285
6-OTMS	217	271
7-OTMS	231	257
8-OTMS	245	243
9-OTMS	259	229
10-OTMS	273	215
11-OTMS	287	201
12-OTMS	301	187
13-OTMS	315	173
14-OTMS	329	159
15-OTMS	343	145
16-OTMS	357	131
17-OTMS	371	117
18-OTMS	385	103

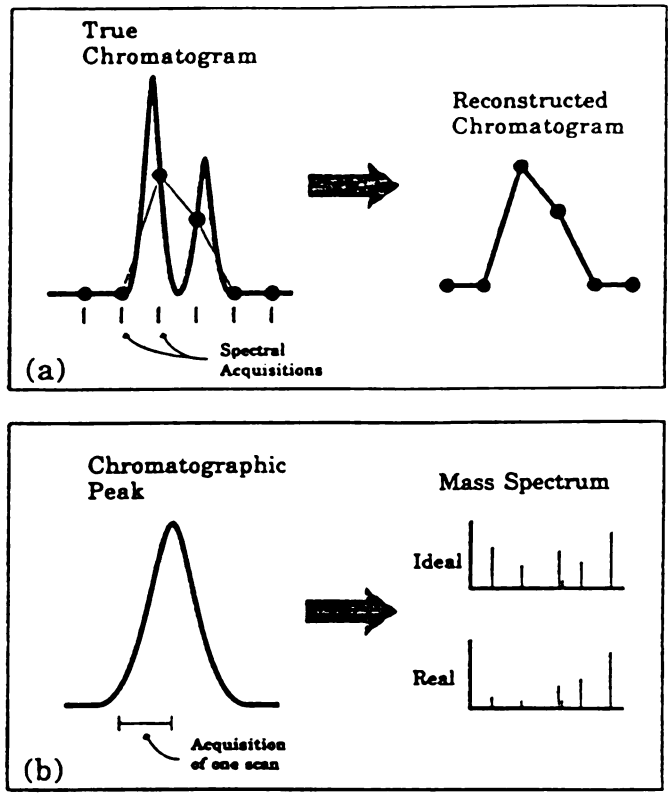


Figure 3-7: Two problems caused by inadequate spectral generation rates in GC-MS. Top: True chromatographic profile is poorly represented due to an insufficient number of TIC points. Bottom: Mass spectral peak intensities are skewed due to the change in partial pressure of the analyte which occurs during the scan acquisition time. Source: Reference 61.

A disadvantage to such rapid scanning is the decrease in ion counting statistics, lowering the signal to noise ratio. This signal to noise ratio will be improved only if the time allowed to detect a particular ion current is increased (Nyquist theory). Therefore, limiting the mass range scanned will allow a higher scanning rate since the magnetic field will not have to change as much and the hysteresis will not be as great, allowing a shortened rest time. With a shorter rest time and decreased scanning time, the scan repetition rate will be increased for a limited mass range. This will tend to correct for some of the inaccuracies in recorded relative peak intensity and simultaneously allow for more scans while a given compound elutes, thus improving ion counting statistics and increasing the signal to noise ratio.

2. EI/MS with Selected Ion Monitoring

Selected ion monitoring (SIM), where only a few ions are analyzed and recorded, has been used quite often when specific ions of interest are known and a more sensitive technique is desired. But the limitations of this technique for analysis of numerous possible compounds are great; usually only ten or fewer ions can be monitored during a single chromatographic run. If the abundant, characteristic α-cleavage ions are selected to identify the methyl ester, TMS ethers of hydroxylated fatty acids, then only five or fewer compounds may be analyzed during a given interval following each injection. Since these chromatographic runs last 40-45 minutes each, searching for several isomers of different compounds could be very time-consuming and require several injections of the extract, which is available only in limited quantity. Another disadvantage found with SIM is that should the selected ions have m/z values which are too widespread, the scan repetition rate is set by the instrument to be the same or lower than during scanning over the same range.

An alternative, compromising method is to scan rapidly over a narrow mass range, particularly a higher mass range. The sweep of the magnetic field in most mass spectrometers is not linear, but is an exponential function, scanning at an increasingly

faster rate as the magnetic field, and thus the mass/charge ratio being measured, increases. Thus, the mass range m/z 200-300 is scanned more quickly than the range m/z 50-150. With the magnetic sector instrument used in this study, the range m/z 170-330 can be scanned in 0.6 seconds per cycle. The signals observed for α-cleavage ions of standard ME, TMS ether hydroxy fatty acids under the limited scanning method were comparable in intensity to those for the same ions in SIM mode when only ten ions in the range m/z 173-315 were observed. The 0.6 second per cycle scan frequency over the m/z 170-320 range allows more frequent scans than does SIM of these ten ions (1.0 second per scan file). The short duration of elution of the compound means that the additional scan(s) during analyte elution can provide peak enhancement approaching that achieved with the limited ion sampling SIM provides.

3. Determination of Limit of Detection

The limit of detection of an analyte in a real sample can be determined from the linear expression for its calibration curve (62,63). The signal of the analyte at its limit of detection, y_d, is equal to at least the sum of the signal from the blank, y_b, plus three times the variability in the blank's signal, which can be approximated by the standard deviation of the slope, s_s. ($y_d = y_b + 3s_s$) The value of three as the factor for the variability is liberal, giving only a 7% probability of false positives. Since this is an acceptable level, this study used three as the variability factor. The concentration at the limit of detection, C_d, is derived from the slope, A, of the calibration curve (Figure 3-8). Thus, $C_d \geq (y_d - b)/A \geq (y_b + 3s_s - b)/A$, where b is the calculated y-intercept. When no sample blank is available, the intercept is an acceptable estimate of y_b, and the equation simplifies to $C_d \geq (y_b + 3s_s - y_b)/A = 3s_s/A$. Since it is not known if these hydroxylated fatty acids exist in these tissues, no blanks were possible. Two isomers, 12- and β-, of OTMS 18:0 methyl ester was chosen as the model compound on which to base the estimate of the limit of detection. In this study, the calibration curves were plotted as quantity injected, Q_x, vs. signal, not concentration vs. signal, but the equation is easily adapted to

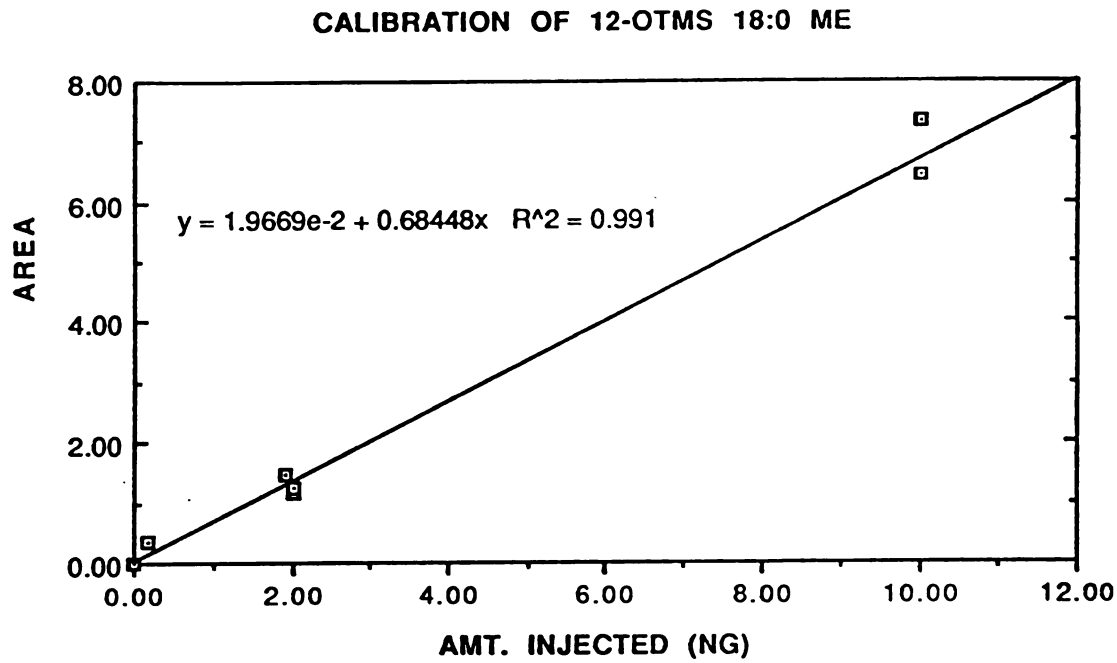


Figure 3-8: Calibration curve and equation for the determination of 12-OTMS 18:0 ME by GC-EI/MS with SIM at m/z 187.

$Q_d \geq 3s_s/A$. A sample calibration curve is shown in Figure 3-8 and the results for this definition of limit of detection for two isomers (12- and β-) of OTMS 18:0 ME under the different mass spectral scanning modes described above are given in Table 3-2. A Q_d of 0.10 ng corresponds to 67 parts per billion (ppb), based on a typical 2 µl injection of 100 µl total extract from a tissue weighing 75 mg.

These estimates of the limits of detection are a function of the ratio of the abundance of the ion used for detection to the analyte's total ion signal, that is y_i/y_T. Thus even if the ion of highest abundance (base peak) is chosen for detection for each of two analytes, the limits of detection may not be similar if the abundance of one base peak is of a different proportion to the sum of all of the ions from that analyte. It is seen that the lower limit of detection must be evaluated separately for each analyte and its most abundant ion(s), unless these ions have the same y_i/y_T.

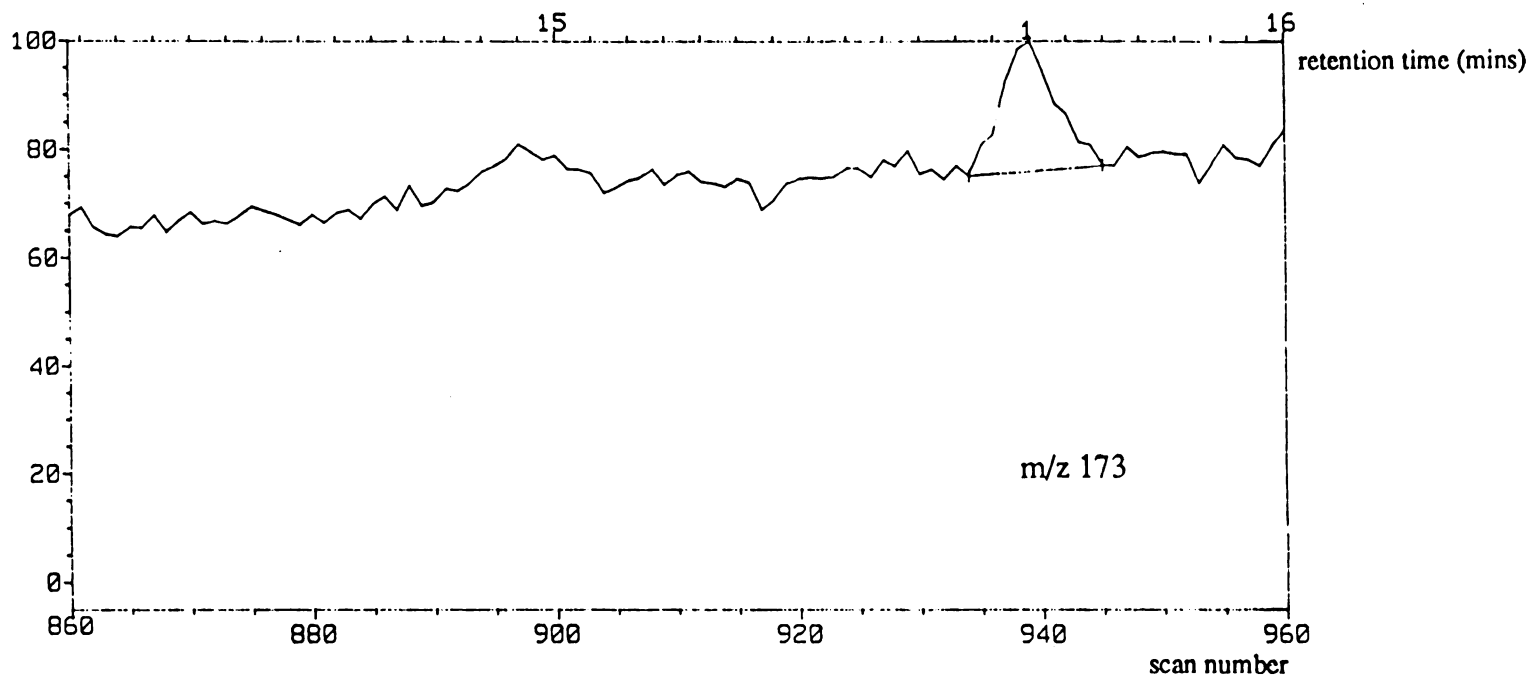
Other factors which greatly influence the estimation of limit of detection are the slope of the calibration curve (the sensitivity) and the variability in the slope. A larger slope for the calibration line (a more sensitive method), will lower the limit of detection. This is usually caused by a higher analyte signal and has been achieved with the use of an internal standard for the calibration curves while scanning from m/z 170-320. The variability of the slope, that is the precision of the method, is a function of the number of points used to calculate the regression line. As the number of points increases, the method should become more precise, decreasing the standard deviation of the slope (and the signal of the blank), therefore lowering the limit of detection.

These statistical estimates of the limit of detection can be compared to another, but less rigorous, method of determining the minimum signal that can be considered a positive measure of the analyte. That method is to calculate the signal to noise ratio (S/N) for the suspected analyte response, with three being the lowest value generally accepted. Figure 3-9 shows a sample selected ion current profile in which SIM of ten ions in the range m/z 173-315 were analyzed and the S/N was calculated to be five. This

Table 3-2: Limit of Detection for OTMS 18:0 ME Isomers Based on Injection of Standards

Scanning mode	Analyte	Ion (m/z)	Regression Values intercept	slope	s_s	N	Q_d (ng)
m/z 50-500 0.9s/cycle	12-OTMS	187	-2.75	5.33	0.19	18	0.11
SIM 10 ions[2]	12-OTMS	187	0.230	2.18	0.42	3	0.57
SIM 10 Ions[2]	β-OTMS	175	0.122	0.470	0.097	3	0.62
SIM 187 Only	12-OTMS	187	0.0232	0.684	0.026	8	0.12
m/z 170-320 0.6s/cycle	β-OTMS	175	0.0706	0.467	0.015	7	0.099/ 2.00 ng IS[2]
m/z 170-320 0.6s/cycle	12-OTMS	187	0.0692	1.788	0.064	8	0.11/ 2.00 ng IS
m/z 170-320 0.6s/cycle	12-OTMS	187	-0.048	2.771	0.051	18	0.055/ 2.00 ng IS
m/z 50-500 0.9s/cycle	12-OTMS avg, normalized	187	-2.55	5.27	0.35	5	0.20
SIM 187 only avg	12-OTMS	187	0.0775	0.679	0.019	4	0.086
m/z 170-320 0.6s/cycle	12-OTMS avg	187	0.0596	1.772	0.070	4	0.12/ 2.00 ng IS
m/z 170-320 0.6s/cycle	12-OTMS avg	187	-0.0405	2.776	0.037	6	0.040/ 2.00 ng IS

[1]Ten ions analyzed - m/z 173, 175, 187, 229.2, 243.2, 259.2, 287.2, 301.2, 313.2, 315.2 [2]IS = internal standard, 2-OTMS 12:0 ME



Scan# 860 to 960 (960) RT 14'19" to 15'59" (15'59") EI (Pos.) Lv 0.00
Mass : 173.000 Group# 1 Channel# 1
Total Area: 2.535823E-01 page 1/1

No.	R.Time	Top	Start	End	Type	Area	Rel	%	Height	Rel	%
1	15'38"	939	934	945	IN	0.2536	100.00	100.0	0.0484	100.00	100.0

Figure 3-9: Selected ion current profile at m/z 175 from GC-EI/MS analysis with SIM of the ten ions listed in Table 3-2, representing an α-cleavage ion of 13-OTMS 18:0 ME. The signal represents injection of 11 pg of the analyte, or 10 ng analyte/g tissue (10 ppb).

peak was determined to be 13-OTMS 18:0 ME from its retention index and the observance of a coeluting peak at m/z 301, the mass of the other α-cleavage ion. This signal represents injection of 0.011 ng corresponding to 9.9 ng/g tissue or 9.9 ppb, based on methods described in the next chapter. It can be seen that the statistically calculated criterion is more rigorous than the S/N criterion for determination of the limit of detection.

C. Recovery of Hydroxylated Fatty Acids

1. 12-OH 18:0 as the Model Compound

In order to determine the recovery of hydroxylated fatty acids in the tissue, a known amount of 12-hydroxy stearic acid, the standard selected as the model hydroxylated fatty acid, was added in the homogenizing tube just before tissue homogenization. After sample work-up, the extract was analyzed for the derivatized standard. In the initial stages of development, a large amount (10.0 μg) of the standard was added to the tissue and excellent recovery was obtained (Table 3-3). With this indication of the capability of the procedure to isolate hydroxylated fatty acids, a smaller amount (80.0 or 60.0 ng) of the standard, closer to the amount expected to be present naturally in the tissue, was added to fresh tissue before homogenization. With this lower quantity of the polar fatty acid, a more accurate assessment of the recovery was possible, since some standard was expected to be lost due to adherence, etc.

The high sensitivity, low limit of detection, and excellent resolution provided by capillary GC-FID would make this detection technique ideal for detection of the internal standard. But there were severe reproducibility problems with the instrument at the time of these analyses. The deviation of replicate injections was unusually high and a typical standard calibration curve from GC-FID illustrates the high imprecision (RSD = 6%-46%) in replicate analyses of standards (Figure 3-10). The signal for later injections of similar volumes of the same extract would usually be considerably higher than the initial response. It was thought that the sample itself may be contaminating the column or

Table 3-3: Recovery of Hydroxylated Fatty Acids

Amt. OH-FA Per 80 mg Tissue	OH-FA Used	Detection Method	No. Samples	No. Data Pts.	Recovery
10.0 ug	12-OH 18:0	scanning m/z 50-500	2	4	111 ± 5%
80.0 or 60.0 ng	12-OH 18:0	scanning m/z 50-500	4 4	5 4	78% ± 8% 82% ± 5%
80.0 or 60.0 ng	12-OH 18:0	SIM m/z 187.2	4	8	increased 119% - 376%
80.0 or 60.0 ng	12-OH 18:0	SIM m/z 187	4	6	93% ± 31%
80.0 or 60.0 ng	12-OH 18:0	scanning m/z 50-500	4	4	200% ± 40%
100.0 ng	2-OH 12:0	scanning m/z 170-320	1	2	93% ± 22%
100.0 ng	2-OH 12:0	scanning m/z 170-320	8	12	150% ± 80%
< 1pg	3H-5-HETE	scintillation counting	8	8	31%±4%

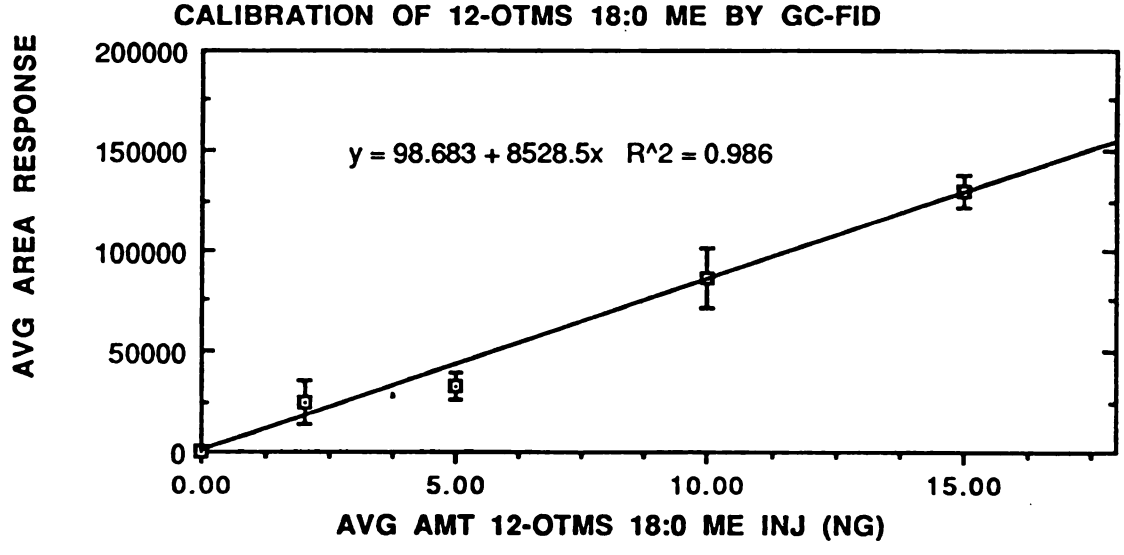


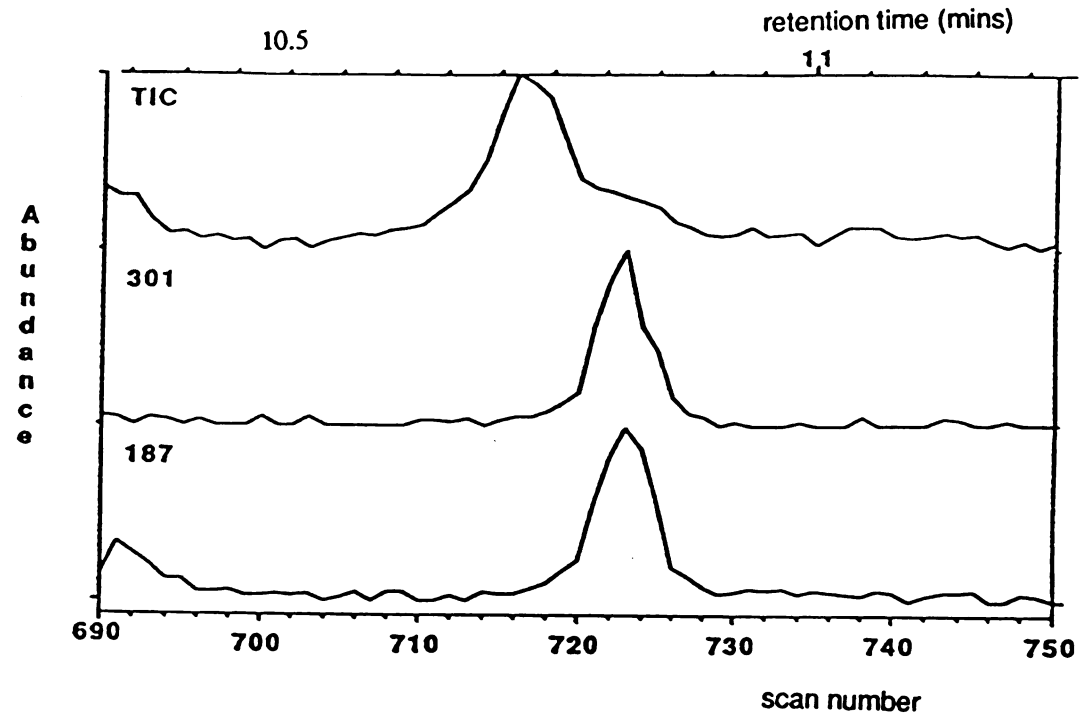
Figure 3-10: Calibration curve for determination of 12-OTMS 18:0 ME by GC-FID. The average area response of replicate injections (N=2, 3, or 4) is plotted. Error bars represent ±1 standard deviation.

detector, thus causing fluctuations in response. Some, but not all, randomly-spaced injections of blank solvent did indicate a high and variable background.

One possible way to eliminate these inconsistencies is to utilize the mass discrimination provided by reconstructed mass chromatograms with GC-EI/MS. Figure 3-11 demonstrates how the selection of major fragment ions (in this case, α-cleavage ions), can produce a cleaner chromatogram than the total ion current (TIC) chromatogram. Some other component, either one in this spiked extract or a "ghost" compound from the column, eluted in the region of the derivatized standard, making quantification from the TIC impossible. Therefore, quantitation was henceforth calculated from the area obtained from reconstructed mass chromatograms of characteristic fragment ions.

The recovery of 60-80 ng of 12-OH 18:0 was calculated from GC-scanning EI/MS with a new column was good (78%), but the standard deviation was fair, possibly due to the small number (four) of spiked extracts analyzed (Table 3-3). A saturated fatty acid was selected as the model hydroxylated fatty acid so that the efficiency of hydrogenation would not influence the recovery estimation. The column appeared to be clean of contaminants, as indicated by a very low signal for the characteristic fragment ions after injections of solvent. In an effort to improve the sensitivity and reproducibility of the analysis, SIM of ten or only one ion was performed.

Subsequent analysis of the same extracts by GC-EI/MS with SIM and the same column just ten days later, gave recovery results which increased dramatically with each subsequent run. Recovery was calculated to be 119% from the first injection, and 376% from the sixth extract injection. With the next two injections, the response did tend to level off. Even duplicates of the same extract gave quite different responses (RSD = 11-15%). Immediately after these extract analyses, another standard injection was performed, and its value agreed with the calibration line.



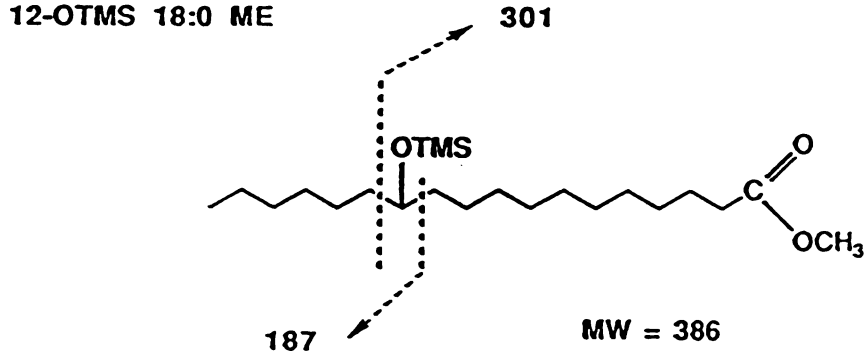


Figure 3-11: Reconstructed total ion current chromatogram and mass chromatograms at m/z 301 and m/z 187 from GC-EI/MS (scanning m/z 50-500) analysis of tissue spiked with 12-OH 18:0 and extracted by the new isolation procedure. Structure indicates the origin of these α-cleavage fragment ions.

After baking out the column again, these same extracts were re-analyzed by GC-EI/MS with SIM set only at m/z 187. Recovery values for the tissues spiked with 60.0 or 80.0 ng 12-OH 18:0 were more reproducible, having relative standard deviations of 5% or less for duplicate analyses. The average recovery from this day's analyses was 93% (Table 3-3), but the deviation of the recovery estimates between the samples was very large (RSD = 33% for six data points). The results from the two days which produced reasonably precise values for duplicate injections indicate the recovery of the hydroxylated C_{18} fatty acid was about 86%, an acceptable value for such a complex matrix.

2. 2-OH 12:0 as the Model Compound

It was decided that one possible contributor to the high recovery was endogenous 12-OH 18:0 or precursors which produce that compound. Thus, a shorter carbon-chain length compound, 2-OH 12:0, was used as an internal standard in a manner similar to that described for 12-OH 18:0. The earlier elution time of the shorter chain-length compound may reduce some of the possible interferences in the extract or from the column. Using the peak intensity at m/z 243, representing a major fragment ion for quantification, a preliminary estimation of the recovery of 100.0 ng from a mammary tissue with duplicate analyses of one extract was 93% ± 22%. Subsequent analysis of the recovery of additional spiked tissues under the same scanning conditions displayed a similar imprecision (150% ± 80%). The irreproducibility in the latter data set can be partly attributed to the scatter of the calibration curve, particularly in the region corresponding to the peak intensities observed for the internal standard in the spiked extracts. But again, the RSDs of duplicate analyses of the same extract (7.0% to 52%) indicated severe irreproducibility.

3. ^{3}H-5-HETE as the Model Compound

In order to separate the imprecision of the analytical method of detection from the imprecision of the extraction technique, an independent detection method was needed.

The sensitivity of radiation counting makes it an attractive quantitation technique. ^{3}H-5-hydroxyeicosatetraeneoic acid (^{3}H-5-HETE) (New England Nuclear) was purified by thin layer chromatography on silica plates (10 cm x 10 cm, Merck) with an ethyl acetate:isooctane:acetic acid:water mobile phase (64). Parallel elution of 1-2 μg of cold 5-HETE (Cayman Chemicals) and visualization by iodine vapor was utilized to determine its chromatographic mobility, which would be the same as any pure ^{3}H-5-HETE. The experimentally determined retention factor for the 5-HETE standard, R_f = 0.53, was close to the retention pictured in the literature (64). The corresponding ^{3}H-5-HETE band was quickly marked and scraped with suction through a plugged (silanized glass wool) pasteur pipet. For the purpose of determining the purity of the original ^{3}H-5-HETE as it existed after -4 °C storage, the entire band was placed in a scintillation vial with cocktail. The rest of the ^{3}H-5-HETE vertical channel was likewise scraped in 12 fractions, collected, and counted (LKB Wallac, courtesy of Dr. McConnell's research laboratory). Quenching corrections are automatically performed by the instrument. The band parallel to the cold 5-HETE contained 28% (82000 dpm of 291200 dpm) of the radioactivity. To prepare a working solution of ^{3}H-5-HETE, another silica plate was spotted with the original ^{3}H-5-HETE solution and with cold 5-HETE, and developed. Only the band parallel to the cold 5-HETE, as determined by iodine vapor visualization, was scraped and collected. The ^{3}H-5-HETE was removed from the silica stationary phase with $CHCl_3$:MeOH (1:1) rinses in a tightly-plugged pasteur pipet. The eluent was collected, solvents were removed by nitrogen evaporation, and an exact amount of ethanol was added. This entire procedure was carried out as quickly as possible to minimize degradation of the light- and air-sensitive compound.

Each of four tissues was spiked with 50.0 μL of the working ^{3}H-5-HETE solution (200 dpm/μL). Cold 5-HETE (50 μL, 5.0 μg) was added to each tissue before homogenization to act as a carrier for the low amount of labeled compound. Each sample

and a blank were extracted according to the above procedures, including hydrogenation of one-half of each tissue preparation.

A one-quarter portion of each extract was analyzed by a scintillation counting . Recoveries of ^{3}H-5-HETE from the spiked tissues, based on the ratio of the radioactivity in the final extracts to the radioactivity of another 50 μL aliquot of the working ^{3}H-5-HETE solution and corrected for the average dpm of scintillation cocktail blanks, were quite low and imprecise - 31% ± 4% (13% RSD) for the non-hydrogenated extracts and 24% ± 7% (27% RSD) for the hydrogenated extracts.

These results were considered quite suspect when the radioactivity of the filter paper used after hydrogenation and the discarded aqueous wash were examined. A most striking feature is the high amount of radioactivity in the filter paper, which had been rinsed three times with methanol (in which 5-HETE is very soluble). Each filter contained 23-34% of the counts in one-half of the volume of ^{3}H-5-HETE added to each tissue. There was also an unexpectedly high amount of radioactivity (5% - 10% of the total radioactivity) in the discarded phase from the aqueous wash. Earlier experiments with GC-FID in this laboratory supported literature statements that an aqueous wash does not extract lipids. Thus it is suspected that the "purified" ^{3}H-5-HETE was not very pure.

One possible explanation is that the ^{3}H-5-HETE had not been completely unbound from the silica stationary particles after filtration through tightly-plugged glass wool. If this were the case, ^{3}H-5-HETE irreversibly bound to the silica particles would be too large to pass through the filter paper. Such bound ^{3}H-5-HETE would also behave uncharacteristically in other separation processes, such as the aqueous wash. The imprecision in the calculated recovery can also be possibly explained by this theory. If some (about one quarter) of the ^{3}H-5-HETE was bound to silica particles, its solubility in ethanol, the working solution solvent, could be lowered. If so, not all of the ^{3}H-5-HETE was in solution, and some ^{3}H-5-HETE-silica particles were undissolved, making the aliquots of the working ^{3}H-5-HETE solution added to each tissue unequal in their tritium

content. It was concluded that these tritium-based recovery results are not reliable. These recovery results based on ^{3}H-5-HETE were also suspect because they were so low compared to the estimates obtained with GC-MS, when the measurements are reproducible.

D. Conclusions

The preceding chapter described the development of a new procedure to isolate hydroxylated fatty acids from mouse mammary glands. Estimates for the recovery of hydroxylated fatty acids by this technique were highly variable, probably due to chemical interferences in the case of analysis by GC-FID and GC-EI/MS and probably due to an impure radioactively-labeled standard in the case of radioactive counting. Nevertheless, the extraction procedure did effectively remove the large amount of unsubstituted fatty acids which possibly could interfere with the detection of any hydroxylated (or other oxygenated) fatty acids. Thus, it was decided to utilize this new extraction procedure on a preliminary number of mouse mammary glands to determine if they contain any hydroxylated fatty acids.

IV. Chapter Four: Analysis and Quantitation of Hydroxy Fatty Acids in Mammary Tissues

The preceding chapter described the development of a new procedure to isolate hydroxylated fatty acids from mouse mammary glands. This chapter describes the use of this extraction procedure to analyze several mouse mammary tissues for a select group of derivatized hydroxylated fatty acids. The purpose of these analyses was to provide a preliminary determination of the presence, if any, and concentration of C_{18} mono-hydroxylated fatty acids. Results and examples of two different methods of quantitation of the OTMS C_{18} methyl esters are given.

A. Analysis of Mammary Tissues for C_{18} OH-Fatty Acids

This new technique for the isolation of hydroxylated fatty acids from mammary glands was utilized on several tissues of mice which had been fed controlled-fat diets. The diets were similar, except in their fat composition. The mice were fed diets of high concentration in specific fats, either 20% corn oil, 20% butter, or 20% fish oil (Table 4-1).

The derivatized extracts from the tissues were analyzed by GC-EI/MS utilizing a 30-m, DB-1 capillary column (0.314 mm i.d., 0.25 μm thick film) at 10 psi head pressure, 1.5 mL/minute helium flow rate, and 260° injector temperature. The temperature program was 50°-180° at 35°/minute, then 180°-290° at 4°/minute. All mass spectrometric analyses were performed on a JEOL AX-505 magnetic sector instrument at 70 eV ionization voltage, 100 μA ionization current, and -10 kV post-acceleration voltage.

Standards were purchased or synthesized of the β- (Supelco), 9-, 12- (NuCheck Prep), and 13- (Oxford Biomedical Research Inc., Oxford, MI) isomers of OTMS 18:0 methyl ester, and their retention times and fragmentation spectra under these laboratory conditions were determined. As previously described, the methyl ester, TMS ethers of hydroxylated fatty acids produce characteristic, relatively intense ions from α-cleavage

Table 4-1: Fatty Acid Composition of Oils and Butter

Fatty Acid	Corn Oil	Menhaden Oil (Fish Oil)	Butter
Capric (10:0)	--	--	2.0%
Lauric (12:0)	--	--	2.3%
Myristic (14:0)	--	8.0%	8.2%
Palmitic (16:0)	10.1%	28.9%	21.3%
Palmitoleic (16:1)	--	7.9	1.8%
Stearic(18:0)	1.6%	4.0%	9.8%
Oleic (18:1)	31.4%	13.4%	20.4%
Linoleic (18:2)	56.3%	1.1%	1.8%
Linolenic (18:3)	--	1.0%	1.2%
Eicosapentaenoic (20:5)	--	10.2%	--
Docosahexaenoic (22:6)	--	12.8%	--

Fatty acid concentrations of < 1% are not included.

Sources: Dr. C.W. Welsch and Reference 65.

about the carbon atom with the OTMS moiety (Table 3-1). Agreement with the respective standard of the ratio of the areas of ion current for each ion in the pair of major α-cleavage ions, along with their retention indices, was used to identify these OTMS C_{18} FAMEs in the extracts, as analyzed by capillary GC-EI/MS with SIM of nine ions (m/z 173, 175, 187, 229.2, 259.2, 301.2, 313.2, 315.2, 339.2) (Figure 4-1). Since the response for many α-cleavage ions was near the limit of detection, especially for the ion with the lower abundance of each pair (Figure 4-2), the window of acceptable values for the ratio of peak intensities was liberal.

Quantitation of the 12- and β-OTMS 18:0 ME compounds was based upon ratios of the areas of m/z 187 and m/z 175, respectively, with 0-10 ng of analyte standard injected. The available quantities of the 9- and 13- isomers were too low to prepare nanogram concentrations for calibration curves based on the α-cleavage fragments at m/z 229 and m/z 173, respectively. The ratios of characteristic ion current area to total ion current area for these fragment ions were similar to the ratio of m/z 187 ion current area to TIC area for 12-OTMS 18:0 ME. Therefore, the calibration curve of 12-OTMS 18:0 ME was used as a working curve for the 9- and 13- isomers. A list of the hydrogenated and non-hydrogenated halves of the extracts analyzed by this method and the estimates of quantitation are given in Table 4-2.

The small number of samples analyzed by this method and the uncertainty inherent with calibration curves of only a few data points do not allow any definitive conclusions to be made regarding the OTMS 18:0 methyl ester content of mammary gland extracts. Some ion signal at the appropriate retention index was observed for each isomer in the non-hydrogenated tissues from the animals fed a 20% butter diet. Very low or zero concentrations of the 13-, 12-, or 9- isomers were detected in the tissues of mice fed 20% corn oil or 20% fish oil. It does seem that, when present, the β-OTMS 18:0 methyl ester is present in higher concentration (parts per million) than the other isomers (parts per billion).

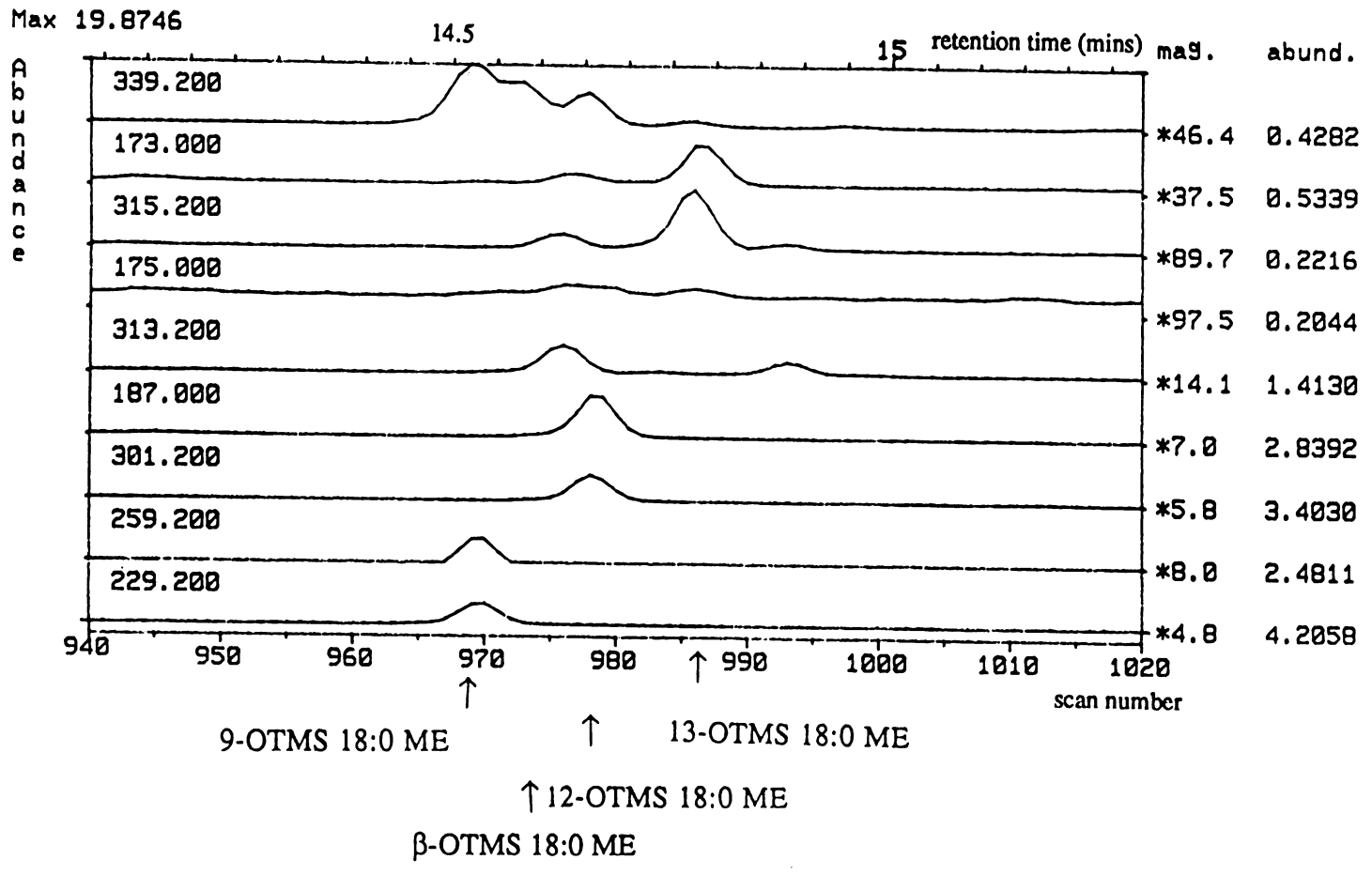


Figure 4-1: Selected ion-current profiles at m/z 229.2 & 259.2 (9-OTMS 18:0 ME), m/z 301.2 & 187 (12-OTMS 18:0 ME), m/z 313.2 & 175 (β-OTMS 18:0 ME) and m/z 315.2 & 173 (13-OTMS 18:0 ME),representing the α-cleavage fragment pairs, and m/z 339.2 $[M-47]^+$ from GC-EI/MS with SIM of these nine ions.

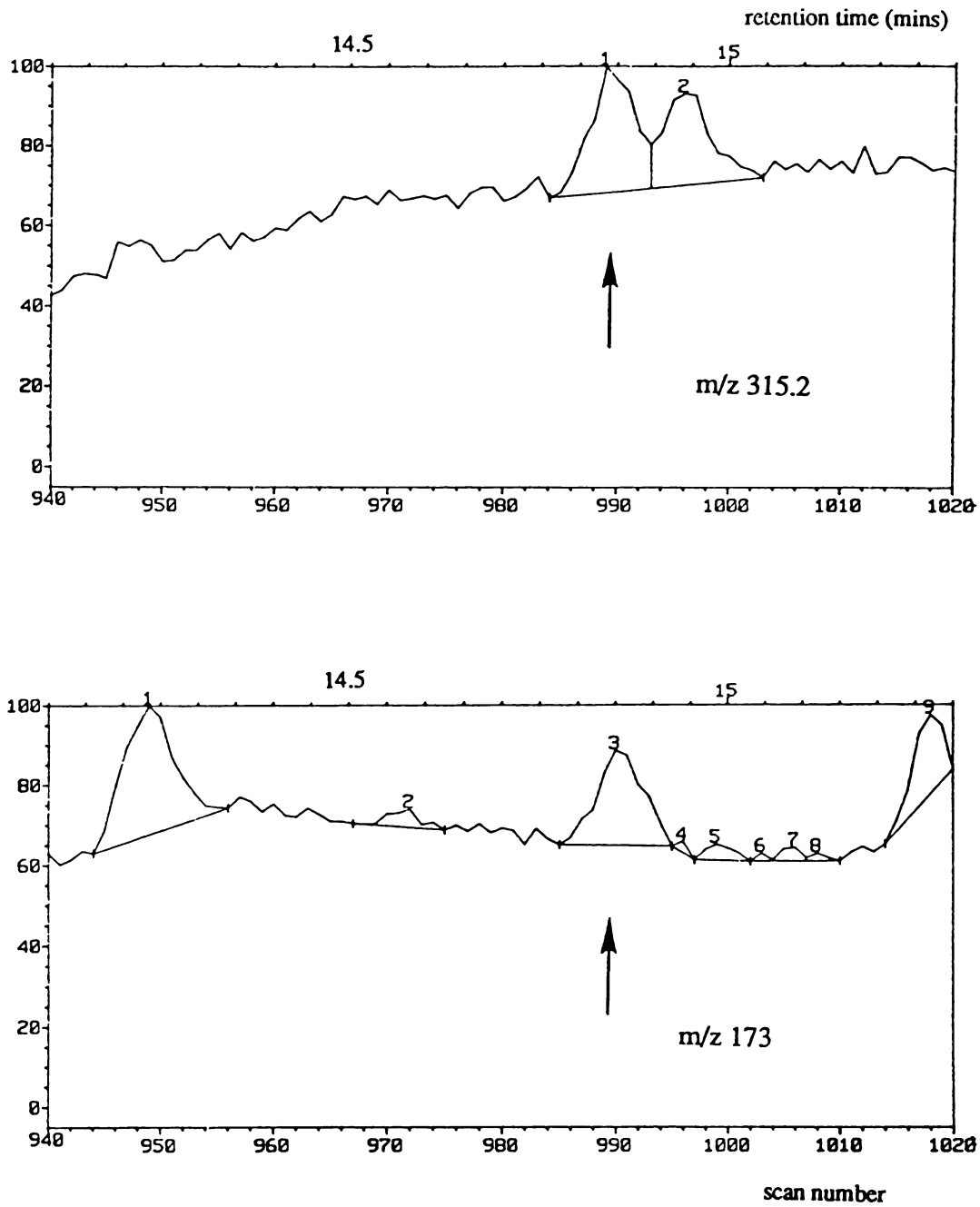


Figure 4-2: Selected ion-current profiles at m/z 315.2 (top) and m/z 173 (bottom) from GC-EI/MS with SIM of the nine ions displayed in Figure 4-1. The arrow indicates elution time of endogenous 13-OTMS 18:0 ME.

Table 4-2: Amounts of OTMS 18:0 ME in Extracts as Analyzed by GC-EI/MS with SIM

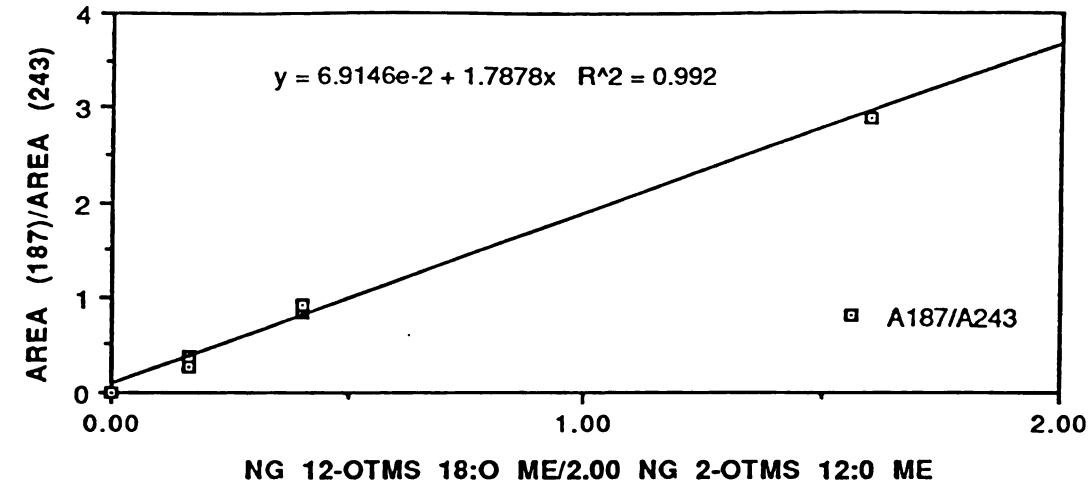
Sample	Tissue Wt. (mg)	Percent Injected	13-OTMS 18:0 ME Amt. Inj. (ng)	13-OTMS 18:0 ME ng/mg tissue (ppm)	β-OTMS 18:0 ME Amt. Inj. (ng)	β-OTMS 18:0 ME ng/mg tissue (ppm)	12-OTMS 18:0 ME Amt. Inj. (ng)	12-OTMS 18:0 ME ng/mg tissue (ppm)	9-OTMS 18:0 ME Amt. Inj. (ng)	9-OTMS 18:0 ME ng/mg tissue (ppm)
C-7	66.3	2.40	0.00	0.00	0.00	0.00	0.17	0.11	0.16	0.10
C-7 H[1]	66.3	2.36	0.00	0.00	0.00	0.00	0.15	0.98	0.064	0.041
C-5	88.5	2.24	0.00	0.00	0.23	0.12	0.00	0.00	0.019	0.010
C-5 H	88.5	2.40	0.00	0.00	0.00	0.00	0.055	0.026	0.078	0.037
F-1	61.8	2.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
F-1 H	61.8	1.95	0.051	0.042	4.4	3.6	0.10	0.087	0.00	0.00
B-4	54.7	2.40	0.068	0.052	1.9	1.4	0.27	0.20	0.12	0.94
B-4 H	54.7	2.40	0.00	0.00	0.00	0.00	0.51	0.039	0.00	0.00
B-1	68.5	2.48	0.087	0.51	4.1	2.4	0.42	0.25	0.33	0.19
B-1 H	68.5	2.40	0.00	0.00	0.00	0.00	0.042	0.026	0.00	0.00

[1]H - The hydrogenated half of the tissue extract.

One curiosity of the data is the observance of some saturated OTMS C_{18} methyl ester isomers in the non-hydrogenated portion of some tissue extracts, but no signal for the same isomer on the hydrogenated portion of that same tissue extract. This is quite unexpected, since any endogenous saturated hydroxylated C_{18} fatty acid should be observed whether the extract was hydrogenated or not. The hydrogenated portion of the extract is expected to have the same or an increased signal for each isomer of saturated OTMS C_{18} methyl ester with respect to the non-hydrogenated portion, since any unsaturated hydroxylated fatty acids of the same positional isomer possibly in the tissue would become saturated during hydrogenation.

B. Use of Internal Standard for Quantitation

In order to provide more accurate determinations of these OTMS 18:0 methyl esters in mammary tissue, a more rigorous calibration curve was needed. In addition, the use of an internal standard to make corrections for variation in recovery of hydroxylated fatty acids and fluctuations in analysis conditions and response (such as noise from column background) would improve the analysis greatly. New mammary tissues were spiked with 100.0 ng of 2-OH 12:0 (Matreya, Inc., Pleasant Gap, PA) just before homogenization. The extracts were analyzed by capillary column GC-EI/MS under the same conditions described above, but the mass range analyzed was only m/z 170-320 to increase the scan cycle rate and lower the limit of detection. α-Cleavage ions and retention indices were again used to determine the presence of an OTMS FAME. Specifically, peaks at m/z 243 and m/z 287 were used to confirm the elution of the TMS ether, methyl ester of the internal standard. For 12- and β- OTMS 18:0 ME, standard curves of the ion current areas at m/z 187 or m/z 175, respectively, for the analyte ion current area relative to the ion current area of the m/z 243 ion from the internal standard versus the amount of analyte per 2.00 ng 2-OTMS 12:0 ME were prepared (Figure 4-3). The quantities of the OTMS 18:0 methyl ester positional isomers measured in non-hydrogenated and hydrogenated portions of tissue extracts were calculated from these



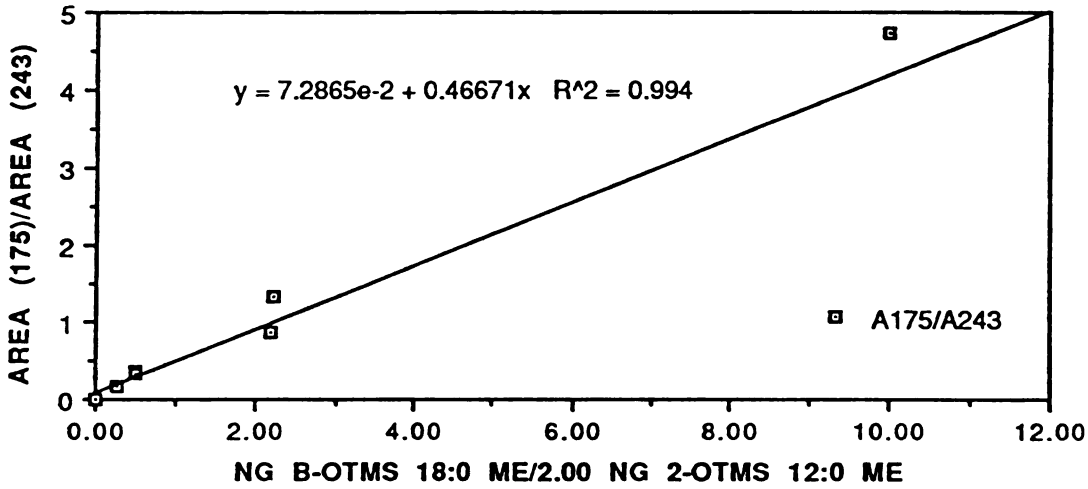


Figure 4-3: Calibration curves and linear regression equations for the determination of 12-OTMS 18:0 ME (top) and of b-OTMS 18:0 ME (bottom), using 2.00 ng 2-OTMS 12:O ME as an internal standard.

curves and are given in Table 4-3. Representative reconstructed total ion and mass chromatograms for the ions resulting from α-cleavage of selected OTMS 18:0 methyl esters are given in Figure 4-4.

The limited number of tissues extracted and analyzed by the internal standard calibration method prohibits absolute conclusions to be made, but some generalizations were noticed. Quantitation results from duplicate, same-day analyses of the hydrogenated portion of two of the three extracts (samples B-5 H and F-3 H) were in good agreement, with relative standard deviations of 0% to 17%. This does not include the one sample (B-5) in which no analyte signal was observed for 13-OTMS 18:0 ME during one trial analysis of the hydrogenated portion nor during the one trial of the non-hydrogenated half of the extract. Results from duplicate analyses of sample F-II H are suspect, however. The peak intensities, and thus the calculated OTMS 18:0 ME concentrations, were consistantly lower for the second analysis trial than for the first. This variation in calculated concentration was large, as evidenced by the high relative standard deviation - 28% - 110%. Analysis of the hydrogenated portion of the extract from sample F-II by GC-EI/MS scanning m/z 170-320 on another date supports the results from the trial where 1.98 μL was injected, that is, the estimates at higher concentrations of the OTMS 18:0 methyl esters.

As similarly observed in the tissues previously analyzed by GC-EI/MS with SIM, the β- isomer of OTMS 18:0 methyl ester was determined to be present in low parts per million (1-4 ppm) concentrations in all tissue extracts. The other isomers, 13-, 12-, and 9-OTMS 18:0 methyl esters, were detected at concentrations in the parts per billion range (2-140 ppb) in the hydrogenated portion of each extract. The sole exception is one of the duplicate runs of the extract from the mouse fed the 20% butter diet. No 13-, 12-, or 9-OTMS 18:0 methyl ester isomers were detectable in the non-hydrogenated portion of the tissue extract from the mouse fed the 20% butter diet.

Table 4-3: Amounts of OTMS 18:0 ME in Extracts as Analyzed by GC-EI/scanning MS with Internal Standard

Sample	Tissue Wt. (mg)	Percent Injected	13-OTMS 18:0 ME ng Inj. /2.00ng IS	13-OTMS 18:0 ME ng/mg tissue (ppm)	β-OTMS 18:0 ME ng Inj. /2.00ng IS	β-OTMS 18:0 ME ng/mg tissue (ppm)	12-OTMS 18:0 ME ng Inj. /2.00ng IS	12-OTMS 18:0 ME ng/mg tissue (ppm)	9-OTMS 18:0 ME ng Inj. /2.00ng IS	9-OTMS 18:0 ME ng/mg tissue (ppm)
B-5	76.7	2.00	0.00	0.00	1.4	0.94	0.00	0.00	0.00	0.00
B-5 H[1]	76.7	2.00	0.00	0.00	1.3	0.87	0.16	0.11	0.11	0.070
B-5 H	76.7	2.00	0.095	0.062	1.6	1.1	0.18	0.12	0.13	0.087
F-II	117.0	1.98	0.00	0.00	3.0	1.3	0.00	0.00	0.085	0.036
F-II H	117.0	1.98	0.16	0.067	11	4.8	0.040	0.017	0.16	0.070
F-II H	117.0	2.03	0.020	0.008	7.6	3.2	0.005	0.002	0.10	0.043
F-3	52.2	1.97	0.066	0.063	1.3	1.2	0.066	0.063	0.075	0.072
F-3 H	52.2	1.97	0.14	0.13	2.2	2.1	0.21	0.20	0.15	0.14
F-3 H	52.2	2.07	0.15	0.14	2.2	2.1	0.17	0.17	0.14	0.13
F-7	54.2	1.98	0.00	0.00	0.75	0.69	0.61	0.56	0.077	0.071

[1]H - The hydrogenated half of the tissue extract.

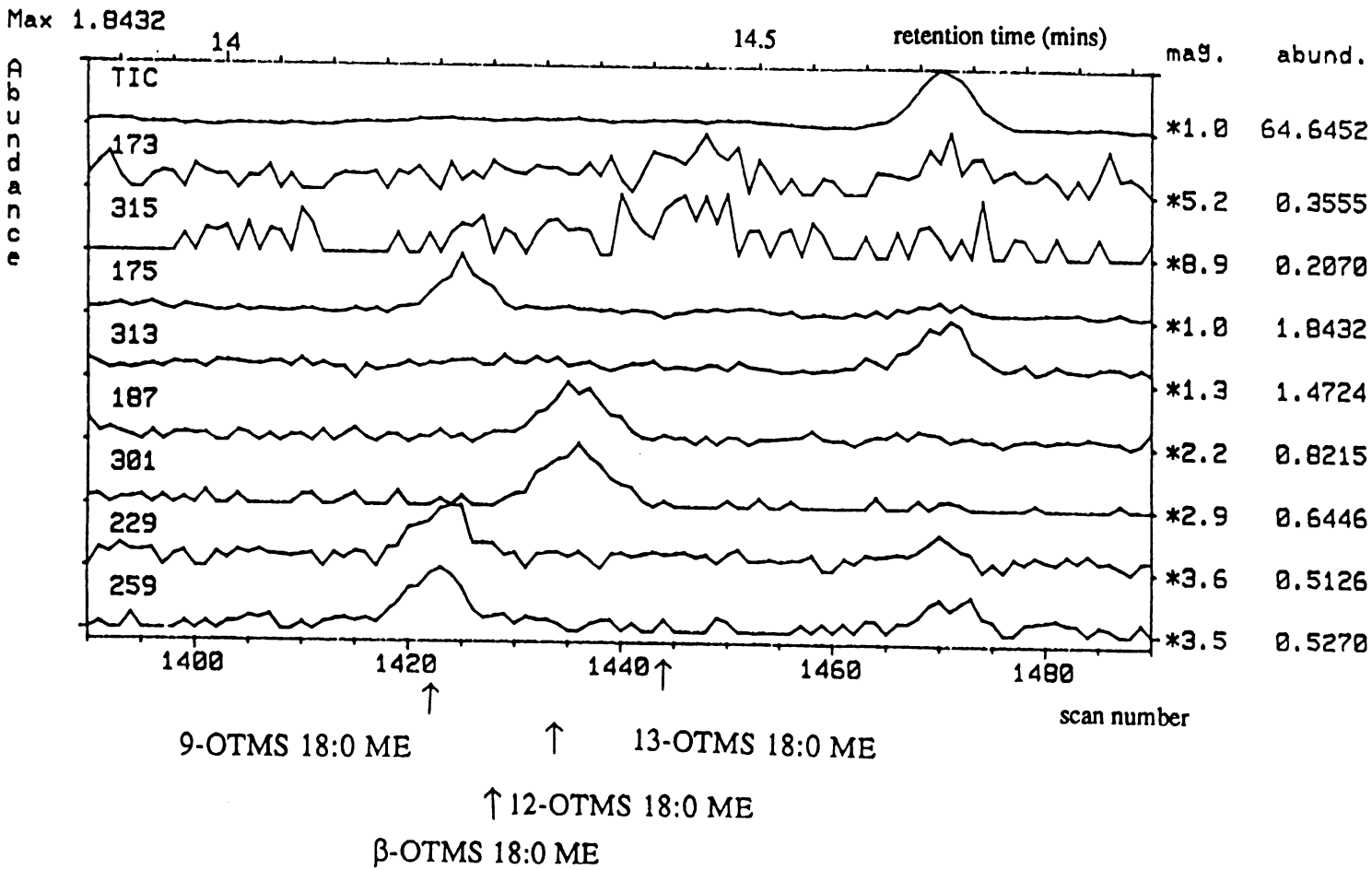


Figure 4-4: Reconstructed total ion chromatogram and mass chromatograms at m/z 259, 229, 301, 187, 313, 175, 315, 173, representing the OTMS 18:0 ME isomers listed in Figure 4-1, from GC-EI/ scanning MS (m/z 170-320). Injection of 2.0% of the hydrogenated portion of tissue B-5, results of quantitation are listed in Table 4-3.

It should be noted that for these samples, and with this quantification method, the hydrogenated portion of each extract was determined to possess a higher concentration of each OTMS 18:0 methyl ester isomer than the concentration of that isomer in the non-hydrogenated portion of the same tissue extract. Hydrogenated portions are expected to contain the same or higher levels of saturated fatty acid than the non-hydrogenated portion, as described above. This observation and the fact that the use of an internal standard can correct for many variations in sample preparation and analysis indicates that use of the internal standard for calibration is preferred.

These few estimates of the concentrations of C_{18} mono-hydroxylated fatty acids are only preliminary, but indicate that those compounds probably exist in some mammary glands at the parts per billion level. The results strongly suggest that this new extraction procedure is appropriate to isolate hydroxylated fatty acids from mammary glands and that there are possibly some isomers of mono-hydroxylated C_{18} fatty acids which are detectable by GC-EI/MS in the parts per billion range.

Further analysis of a statistical number of tissues from mice fed diets of different fatty acid content is needed to determine if there exists a relationship between the amount, if any, of hydroxylated fatty acid in the mammary gland and the fat content of the subject's diet. It is suggested that two or more glands from the same animal be pooled to provide increased levels of any hydroxylated fatty acids present. With the same final volume (100 μL) for the extract of pooled glands, the ratios of weight of analyte/weight of tissue will not be affected, but the amount of analyte introduced into the gas chromatograph-mass spectrometer will be increased for each 2 μL injection (2% of the extract). An alternative solution to increasing the amount of potential analyte in 100 μL total volume of extract is to use rats instead of mice as the study animal. The rat is a much larger animal and each mammary gland can be extracted individually, providing replicate trials for each subject.

The results from these GC-EI/MS analyses of mammary glands with SIM and with small mass range scanning and an internal standard indicate that further use of the isolation procedure is warranted. Some isomers of C_{18} mono-hydroxylated fatty acids do appear to be present in most of the tissues examined to date. β-Hydroxy stearic acid was observed to be the isomer in highest concentration (low ppm), but other isomers (13-, 12-, and 9-) are probably present in many tissues at the parts per billion level.

V. Chapter Five: Directions for Future Work

The need for a new extraction procedure to isolate oxygenated fatty acids from mouse mammary glands, to determine if there exists a relationship between dietary fat and the oxygenated fatty acid content of the tissue, was explained earlier. This thesis has described the development and preliminary evaluation of a new technique for the isolation of hydroxylated fatty acids from mammary glands, with analysis by GC-EI/MS. This chapter presents possible analytical directions for further application of the new extraction technique to probe the biochemical problem under investigation.

A. Determination of Other Hydroxylated Fatty Acids

The development and evaluation of the new isolation procedure used 12-hydroxy stearic acid as the model oxygenated fatty acid. The mammary glands extracted by the new method were analyzed only for a few isomers of mono-hydroxylated stearic acid. The recovery and concentration of other hydroxylated fatty acids should be determined. Those compounds of interest include unsaturated C_{18} hydroxylated fatty acids, hydroxylated fatty acids of 20, 22, and 16 carbon-chain lengths, and dihydroxylated fatty acids.

The non-hydrogenated portion of each extract can be analyzed for the presence of derivatives of those unsaturated hydroxylated fatty acids which would be expected to be oxidation products of dietary unsaturated fatty acids, such as 18:2, 18:3, 20:5 and 22:6 (65). There are other dietary and endogenous unsaturated fatty acids which may be oxidized to hydroxylated compounds, including 16:1, 18:1, 20:1, 20:3, and 20:4. If dihydroxylated fatty acids are present, their concentrations may not be sufficiently high to be observed by the current methods. The isolation procedure should be evaluated for recovery of these compounds, since they are more polar than their monohydroxy analogues, and their retention with the silica column clean-up procedure is unknown.

B. Analysis for Other Oxygenated Fatty Acids

Hydroxylated fatty acids are not the only form an oxygenated fatty acid can have, just one of the more stable, which is why it was selected as a model for the development of the new isolation procedure. The initial oxidation products are highly unstable hydroperoxides, which are quite easily reduced to alcohols, ketones or epoxides. The carbonyl groups are fairly stable and should be detectable if present in the tissue. Treatment of a ketone with BSTFA or other moderate to strong silylating reagents will produce a different positional isolmers of OTMS fatty acid methyl esters. If determination of the position of the carbonyl group is desired, methoximes can be prepared from the FAMEs just before the silylation. Then the extract can be analyzed by GC-EI/MS for characteristic fragments of methoximes. Again, the recovery of keto-fatty acids from tissue homogenate should be evaluated using the new isolation procedure. Epoxides across a carbon-carbon double bond can also be a product of oxidation. The extract can be analyzed by GC-EI/MS, where characteristic ions result from, α-cleavage on either side of the epoxide ring and β-cleavage on the far side of the ring with hydrogen atom rearrangement. The recovery of a representative epoxide fatty acid should be determined with the new extraction procedure.

C. Minimization of Background Contaminants

It is expected that any oxygenated fatty acids present will only exist in trace quantities, and in this study some hydroxylated fatty acids were determined to be present in the tissues at the parts per billion level. The analysis of such low levels of analytes requires low background from the instrumentation used for analysis and from the isolation procedure itself. During the development of the extraction procedure, care was taken to attempt to eliminate contaminants, especially phthalates and other high-boiling compounds. But not all background was eliminated, in particular, there was some background noise from concentrated solvent which had passed through the silica SPE column. This background could probably be reduced with the use of the recently-

available "inert" columns, which are made of silanized glass cartridges. But these columns are quite expensive, and the background reduction must be great in order to justify their cost.

Another source of background in the analysis of the extracts is from eluents from the GC column. On certain days of analysis, there were many problems with a high signal upon injection of solvent only, both with GC-FID and with GC-EI/MS using the same GC column. The baseline signal was particularly high and/or variable after several injections of extracts had been performed. Since this background was only observed on certain days, and not after numerous extract injections on other days, there could be another cause of the high background. A change to a new, slightly more polar GC column (DB-5, 5% phenylmethyl silicone) may reduce the amount of "ghost" peaks resulting from previously-injected compounds which adhered to the column. This change would require the redetermination of retention indices, so less dramatic methods, such as changing the temperature programming of the GC oven, should be attempted first. Using a lower final temperature (280°) for the GC oven after an overnight bake-out at a higher temperature appeared to reduce the signal from injection of a solvent blank, even after a dozen extract injections. Since it seemed to work, this possible solution was not rigorously evaluated.

Corrections for background due to the matrix can be made with the discrimination provided by tandem mass spectrometry (MS/MS) or triple quadrupole MS. The first sector is set to allow ionized GC effluent with only a specified m/z value to pass to the second sector. In triple quadrupole MS, the analyte reacts with an inert gas in this sector, causing dissociations. The daughter ions pass into the next sector where they are detected to give characteristic daughter spectra. The potential benefit of background elimination with MS/MS warrants an examination of the feasibility of application of this detection technique to the tissue extracts.

D. Separation of Extract by High Performance Liquid Chromatography

Recent developments in the use of HPLC-MS for analysis of low level components (66) and for lipids (67) have offered a potential method of fatty acid separation with on-line mass spectral detection which was not widely available at the inception of this project. The picomole detection limit obtained by Hemling and coworkers was for analysis of glycoproteins, not oxygenated fatty acids. Detection limits for HPLC-MS are not as low for fatty acids as for proteins, which have been investigated much more often by HPLC-MS.

The appropriate HPLC method for separation and the best interface to the mass spectrometer could be investigated. Implementation of an HPLC-MS system could serve to reduce some of the background noise, since the column in HPLC will have different interactions with the extract's components in the GC column. The different stationary phases and the different physical states of the extract for each separation method produce dissimilar interactions.

The use of HPLC-MS/MS to analyze the oxygenated fatty acid methyl esters could eliminate the use of a silica SPE clean-up column and a gas chromatographic column for additional resolution of the resolution of the extract, combining these steps into one process.

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