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SEPARATION, IDENTIFICATION, AND QUANTITATION OF MONOHYDROXYLATED POLYUNSATURATED FATTY ACIDS UTILIZING GC/MS AND CHIRAL HPLC

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By

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ABSTRACT

SEPARATION, IDENTIFICATION, AND QUANTITATION OF MONOHYDROXYLATED POLYUNSATURATED FATTY ACIDS UTILIZING GC/MS AND CHIRAL HPLC

By

Jennifer A. Johnson

While a number of mechanisms have been reported to explain the stimulatory effect of high levels of dietary fat on mammary (breast) tumorigenic processes in experimental animals, to date, none of these mechanisms has proven to be entirely satisfactory. We are proposing that oxygenated metabolites (products) of linoleic acid, via free radical and/or enzymatic processes, are the key products of fat hyperalimentation that stimulate the mammary tumorigenic process. The oxygenated products of primary interest are 9- and 13-hydroxyoctadecadienoic acid (9-HODE, 13-HODE) as these products have been shown to have a growth stimulatory effect in experimental animals in an array of organ sites including the mammary gland.

We have developed an analytical methodology for the isolation, identification, and quantitation by GC/MS and chiral HPLC of 9- and 13-HODE in mouse mammary gland tissue. The mammary gland tissue is pulverized in liquid nitrogen, internal standards consisting of $^{18}O_2$-labeled analogs of the 9- and 13-HODEs are added, and the tissue is extracted twice in ethanol. Non-lipid materials are removed in a chloroform:

methanol:water extraction step. The lipid material remaining is methylated with diazomethane, and much of the non-oxygenated fatty acids are removed via solid phase extraction on silica columns. The sample is derivatized with BSTFA to form the TMS ethers prior to quantitation by GC/MS. Those samples for HPLC analysis are reconstituted in hexane and eluted isocratically with a hexane:methanol mobile phase on a Chiralcel OD chiral stationary phase.

The concentrations of 9- and 13-HODE were highest ($P < 0.01$) in the mammary glands of mice fed high levels of polyunsaturated fatty acids (20% corn oil). Enantiomeric distributions of the HODEs in the tissues show that 13(S)-HODE and 9(R)-HODE are the more abundant isomers in the tissues.

A positive correlation between the mammary gland concentrations of 9-HODE and 13-HODE and the reported mammary gland tumorigenic activities of these diets in rodents (20% corn oil > 5% corn oil > 20% beef tallow) is observed in these studies. These results provide evidence that 9- and 13-HODE are produced in significat quantities in animals fed diets rich in polyunsaturated fatty acids.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

13-HODE	13-hydroxyoctadecadienoic acid
9-HODE	9-hydroxyoctadecadienoic acid
ANOVA	analysis of variance
BSTFA	bis(trimethysilyl)trifluoroacetamide
DNA	deoxyribonucleic acid
DNBPG	dinitrobenzoyl phenylglycine
EGF	epidermal growth factor
EI	electron impact ionization
GC	gas chromatography
GC/MS	gas chromatography/mass spectrometry
HEDE	hydroxyeicosadienoic acid
HETE	hydroxyeicosatetraenoic acid
HPLC	high-performance liquid chromatography
IPA	2-propanol
$M^{+\bullet}$	molecular ion
m/z	mass to charge ratio
ME	methyl ester

ME/TMS	methyl ester/trimethylsilyl
PUFA	polyunsaturated fatty acid
RSD	relative standard deviation
RTIC	reconstructed total ion chromatogram
SHE	Syrian hamster embryo
SIM	selected ion monitoring
TFA	trifluoroacetic acid
TLC	thin-layer chromatography
TMS	trimethylsilyl
UV	ultraviolet

Chapter One: Dietary Fat and Breast Cancer

I. Introduction:

Human and animal studies have shown that there is a positive association between mammary (breast) cancer and total fat intake. Animal experiments on dietary fat and mammary cancer have shown that increasing the amount of dietary fat increases mammary tumorigenesis, whether measured in terms of incidence or multiplicity of tumors. These studies have also shown that polyunsaturated fats stimulate tumorigenesis whereas saturated fats do not, unless the saturated fat is supplemented with a minimal amount of linoleic acid. It has been postulated that oxidized unsaturated fatty acids may play a role in stimulating the tumorigenic process. This dissertation describes the development of analytical methodology to isolate, identify, and quantitate hydroxylated fatty acid metabolites of linoleic acid in mouse mammary glands.

II. Fatty acids

The fatty acids of plant, animal, and microbial origin generally contain even numbers of carbon atoms in straight chains, with a carboxyl group at one end and with double bonds in the *cis* configuration. In animal tissues, the common fatty acids vary in chain-length from 14 to 22 carbons, and may have one to six double bonds. The most abundant saturated fatty acids (no double bonds) are straight-chain compounds with 14, 16, and 18 carbon atoms. The fatty acid with 18 carbon atoms and the structural formula, $CH_3(CH_2)_{16}COOH$, is systematically named octadecanoic acid, having the common name stearic acid. This acid may also be termed a C_{18} fatty acid, or more specifically an 18:0

fatty acid where the first number refers to the number of carbon atoms in the chain. The number following the colon depicts the number of double bonds.

Polyunsaturated fatty acids (PUFA), those fatty acids with more than one double bond, can be subdivided into families according to their derivation from specific biosynthetic precursors. Each of the families contain from two up to a maximum of six *cis*-double bonds, separated by single methylene groups (methylene-interrupted unsaturation). Some of the more important PUFA are listed in Table 1.1.

Table 1.1 Important polyunsaturated fatty acids.

Systematic name	Common name	Shorthand notation
9,12-octadecadienoic acid	linoleic acid	18:2
6,9,12-octadecatrienoic acid	γ-linolenic acid	18:3
8,11,14-eicosatrienoic acid	homo-γ-linolenic acid	20:3
5,8,11,14-eicosatetraenoic acid	arachidonic acid	20:4
9,12,15-octadecatrienoic acid	α-linolenic acid	18:3

Linoleic acid is the most wide-spread PUFA. It is found in most animal and plant tissues. Linoleic acid is an essential fatty acid in animal diets, as it cannot be synthesized in animal tissues yet is required for normal growth, reproduction, and healthy development. This is because enzymes in animals are only able to insert new double bonds between an existing double bond and the carboxyl group. Therefore, linoleic acid serves as a precursor to a family of fatty acids that are formed by desaturation and chain elongation.

III. Diet and breast cancer

A. Human population studies

Women in the United States develop cancer of the breast more frequently than at any other anatomical site (1). A tremendous variability in breast cancer incidence is seen among countries (see Figure 1.1 (2)). Those countries with the highest incidence are ones with a "Western" culture such as northern Europe and North America. Figure 1.1 demonstrates that there is a more than fivefold range between those countries with the highest and lowest rates of breast cancer. A wide international variation in per capita fat consumption also exists. The fat consumption-breast cancer relationship in international population studies is direct and linear. As can be seen, those countries with high fat consumption rates have high rates of breast cancer, those with low fat consumption have low breast cancer rates. Prentice et al. (3) have reported that the strong international correlation with breast cancer holds for total calories from fat but not from nonfat calories.

Cohort population studies have also shown that there is a positive association between fat consumption and breast cancer. Howe et al. (4) reported in a Canadian National Screening study consisting of a cohort of 56,837 women there was evidence of a positive association between breast cancer and total fat intake. An Italian case-control study reported that multivariate analyses of the data showed that the women in the high-fat group (46% of calories from fat) had a greater breast cancer risk than those in the control group (26% of calories from fat) (5). Studies of population migration from countries with low breast cancer incidence to countries with high breast cancer incidence have provided evidence implicating lifestyle, especially diet. For those individuals that

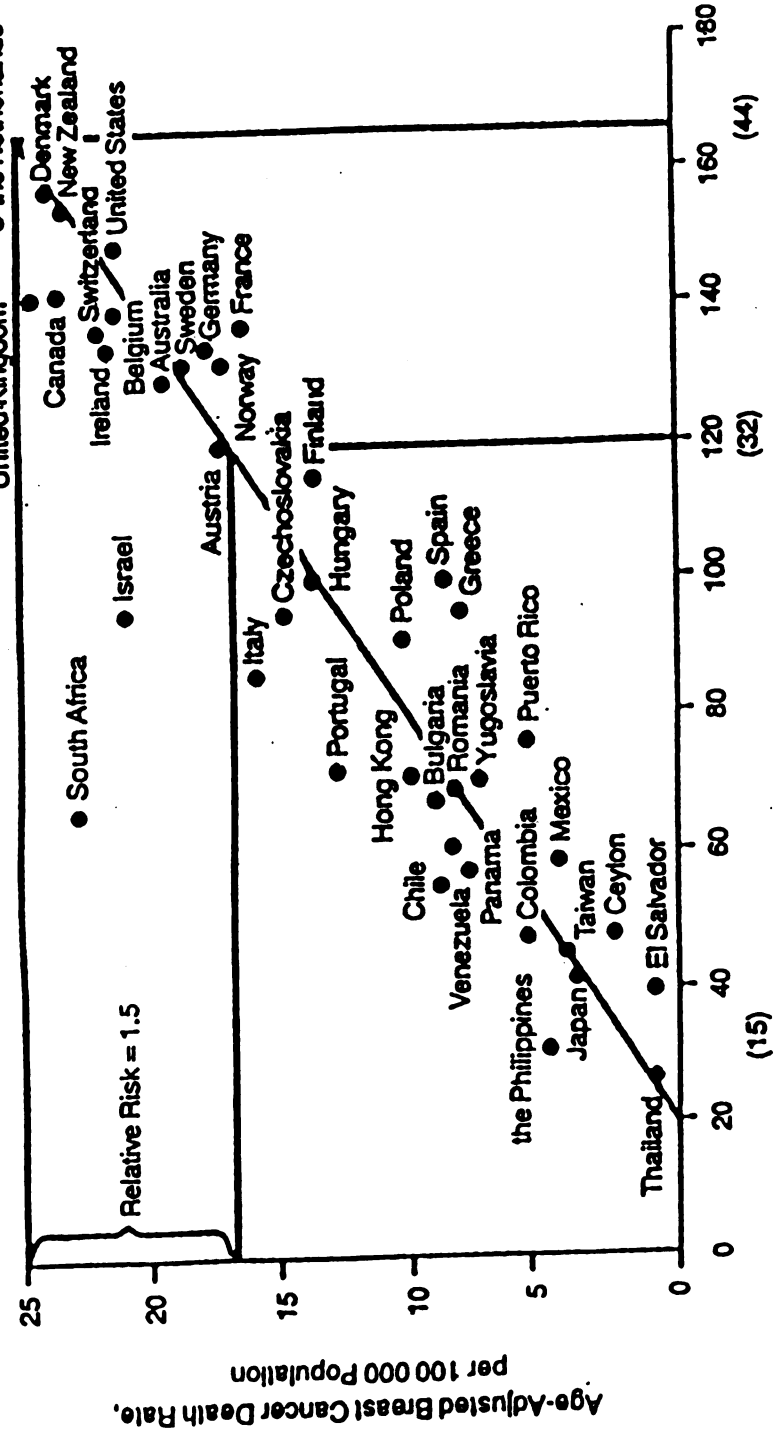


Figure 1.1: Trend in breast cancer incidence vs. dietary fat consumption among countries (2).

migrated from Poland or Italy to either the United States, the United Kingdom, or Australia it was reported that their age adjusted breast mortality rates were comparable to those of the host country after 10-30 years (3).

B. Animal studies:

1. Amount of fat

Because it is difficult to control many variables in human studies, the use of animal studies to model human breast cancer has allowed dietary factors to be controlled independently. In 1942, Tannenbaum reported a high incidence of mammary carcinomas in mice fed high-fat diets (6). Since 1942 numerous laboratories have confirmed and extended this observation (7). It has been seen that increased amounts of ingested fat were reported to increase the development of mammary tumors in experimental animal models. Caloric consumption also increased mammary tumor development. An as yet unresolved question is whether the enhancement of mammary tumorigenesis by hyperalimentation with fat results from specific metabolic activity of the fat, *per se*, or is due to excessive energy intake (8).

2. Type of fat

It has been seen that not only the amount of fat but also the type of fat influences the incidence of mammary tumors. High levels of saturated fatty acids from animals (beef tallow) or plants (coconut oil or palm oil) suppress the development of mammary tumors in experimental animals compared to high levels of polyunsaturated fatty acids from vegetables (reviewed in 8). If saturated fatty acid diets, which are low in essential fatty acids (i.e., linoleic acid, linolenic acid, and arachidonic acid), are supplemented with a

small amount of unsaturated fatty acid (linoleic acid), the inhibitory effect of the saturated fat diet is often reversed (9, 10). Mammary tumor development is as high in rats fed a diet containing 20% sunflower seed oil (high linoleic acid content) as it is in rats fed a diet containing 17% beef tallow and 3% sunflower seed oil (9) or 17% coconut oil and 3% ethyl linoleate (10). These experiments show that the amount and the type of fat in the diet is important in influencing mammary tumorigenesis in experimental animal models.

3. Proposed mechanisms

The mechanism for the stimulatory effect of the dietary fat on mammary tumorigenesis has not as yet been determined. Some mechanisms that have been proposed to explain the effect include (11):

- changes in the immune system
- alterations of the endocrine system
- modifications of cell membrane lipids
- changes in prostaglandin activity
- direct effects on tumor cell metabolism
- changes in the activity of carcinogen-metabolizing enzymes
- production of oxidized products from unsaturated fats

IV. Lipid oxidation

In recent years, interest in the role of lipid peroxidation and the production of oxidized products in tissues has increased. Lipid hydroperoxides and their breakdown products display a variety of toxic effects such as impairment of membrane function, decreased cell membrane fluidity, inactivation of membrane bound receptors and enzymes,

and increased non-specific permeability of ions such as Ca^{2+}. These lipid products have also been proposed to contribute to inflammation, reoxygenation injury, and parasitic infections, as well as contributing to degenerative diseases such as cancer, atherosclerosis, rheumatoid arthritis, and aging (12). The oxidation of polyunsaturated fatty acids *in vitro* has been well described, and both the products and the kinetics are well understood (13, 14).

Naturally occurring polyunsaturated fatty acids contain two to six methylene-interrupted double bonds within the hydrocarbon portion of the molecule. The greater the number of double bonds the greater the oxidizability. The peroxidation reaction is triggered by the abstraction of a weakly bonded allylic hydrogen by a strong oxidant. The alkyl radical of the fatty acid is stabilized by resonating to a conjugated diene system. The addition of molecular oxygen to an alkyl radical produces a conjugated peroxy radical which, upon abstracting a hydrogen from another polyunsaturated fatty acid, produces a hydroperoxide and an alkyl radical (see Figure 1.2). Several mechanisms involving one-electron transfer from a metal ion, radiation, photolysis, active oxygens, chlorinated hydrocarbons, and radicals generated from enzymatic processes are thought to be responsible for initiation of the radical reaction in biological systems (15).

There has been no direct evidence to support the idea that lipid peroxidation is influencing the incidence of mammary cancer in mice fed a high-fat diet. Slaga et. al., (16) however, reported that benzoyl peroxide, a free radical generating compound, is an *in vivo* promoter in the dimethylbenzanthracene-induced mouse skin model of tumorigenesis. Bull et. al. (17) provided evidence that oxidized fatty acids containing a site of unsaturation adjacent to the oxygen-containing functionality are mitogenic to the rat colon

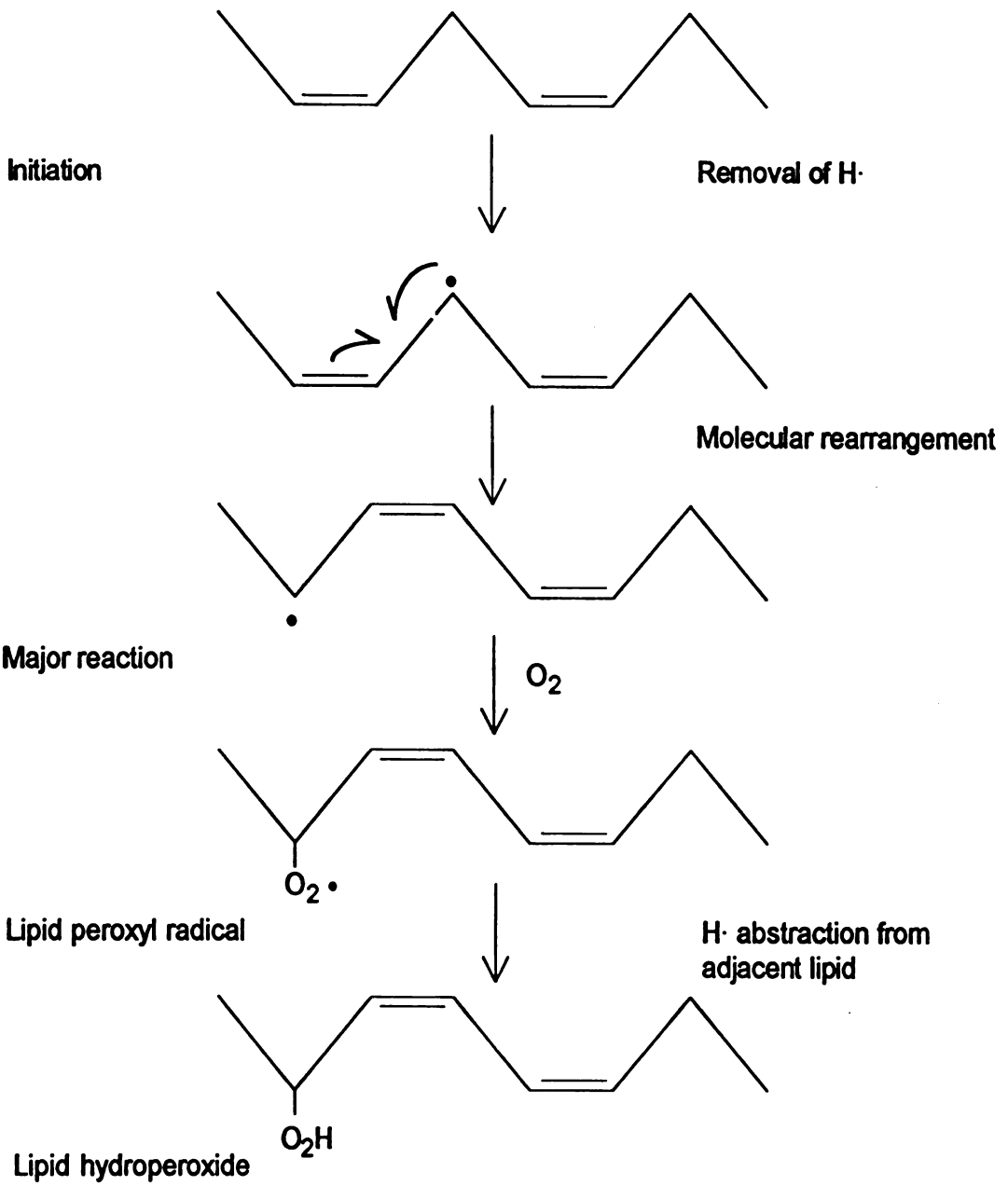


Figure 1.2. Free radical oxidation of fatty acids.

in vivo at the same concentrations as tumor-promoting bile acids. The mitogenic properties of these compounds suggests a role in the enhancement of tumorigenesis by high levels of dietary fat.

Antioxidants can act as effective scavengers of lipid peroxy radicals and/or oxygen radicals. A number of laboratories have treated rats bearing mammary tumors with an array of antioxidants during the ingestion of diets rich in unsaturated fatty acids and found a decrease in tumorigenesis (18, 19). There also exists evidence to the contrary (20). It is possible that the effects seen by antioxidants are the result of altered carcinogen metabolism. King et al. (20) found that an antioxidant administered with a non-metabolized carcinogen did not reduce the promotional effect of the high-fat diet. Even though there exists conflicting evidence for the effect of lipid peroxidation on mammary tumor promotion, the idea remains an attractive concept.

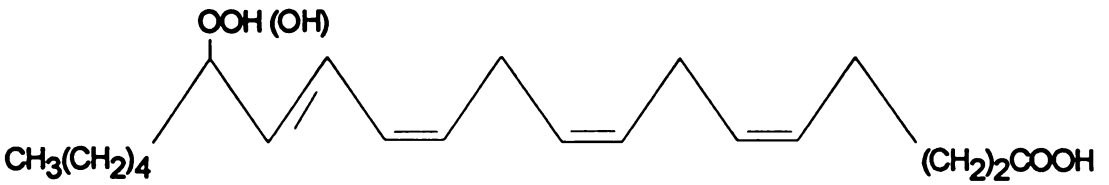
V. Structural requirements for biological activity

Bull et al. (17) reported that primary autooxidation products of major dietary polyunsaturated fatty acids, arachidonic and linoleic acids, were capable of stimulating rat colonic mucosal deoxyribonucleic acid (DNA) synthesis and ornithine decarboxylase activity *in vivo*, two early events in the activation of mitogenesis. This group carried the work further to determine the structural features of the fatty acid molecule that are responsible for the biological activity. A site of unsaturation adjacent to an oxidized moiety was found to be the minimal requirement. No significant differences in biological activity were seen if the oxidized functionality was a hydroperoxide, hydroxide, or carbonyl (21). Loss of mitogenic activity was seen with saturation of the hydrocarbon

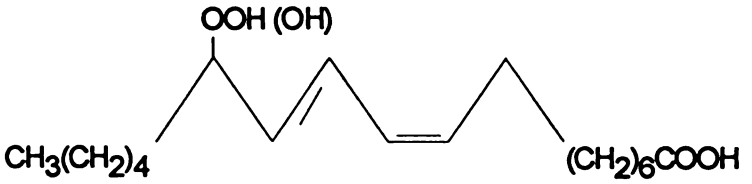
backbone, separation of the double bond from the oxidized moiety by a methylene group, or the absence of an oxidized group. Structures of the compounds found to be active are shown in Figure 1.3. Structures of the inactive compounds are shown in Figure 1.4.

Recently, Glasgow et al. (22) determined that enzymatically derived linoleate metabolites were observed to enhance [^{3}H]thymidine incorporation in epidermal growth factor (EGF)-activated Syrian hamster embryo (SHE) cells. Autoradiographic studies confirmed the mitogenic potential of the linoleate derivatives. Investigations into structure-activity relationships of analogous lipid compounds found that metabolites of arachidonic acid and linolenic acid were much less active than linoleic acid metabolites, and the lack of activity by ricinoleic acid suggested either a requirement for an allylic oxygenated functionality or a requirement for a diene. The structure-activity revealed that the primary derivative of linoleic acid, 13-hydroperoxyoctadecadienoic acid, was the most effective lipid compound in stimulating EGF-regulated DNA synthesis in SHE cells. The carbon chain length, degree of unsaturation, type and position of oxygenated functionality, double-bond geometry, and chirality are all factors that modulated the mitogenic activity of related compounds (23).

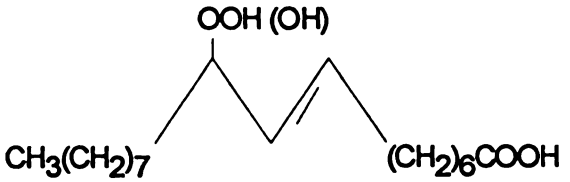
Welsch (24) assessed the potential of 9-hydroperoxyoctadecadienoic acid and 13-hydroperoxyoctadecadienoic acid for their ability to stimulate proliferation of primary mouse (Balb/c) mammary gland epithelial cells in a collagen gel cell culture system. 13-hydroperoxyoctadecadienoic acid stimulated cell proliferation more than did the parent fatty acid, linoleic acid. In contrast, 9-hydroperoxyoctadecadienoic acid inhibited proliferation of these cells. It was concluded that specific oxidation products of linoleic



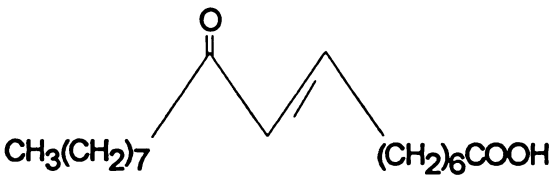
15-hydroperoxyeicosatetraenoic acid
15-hydroxyeicosatetraenoic acid



13-hydroperoxyoctadecadienoic acid
13-hydroxyoctadecadienoic acid



10-hydroperoxyoctadecenoic acid
10-hydroxyoctadecenoic acid



10-ketooctadecenoic acid

Figure 1.3: Structures of representative biologically active oxidized fatty acids.

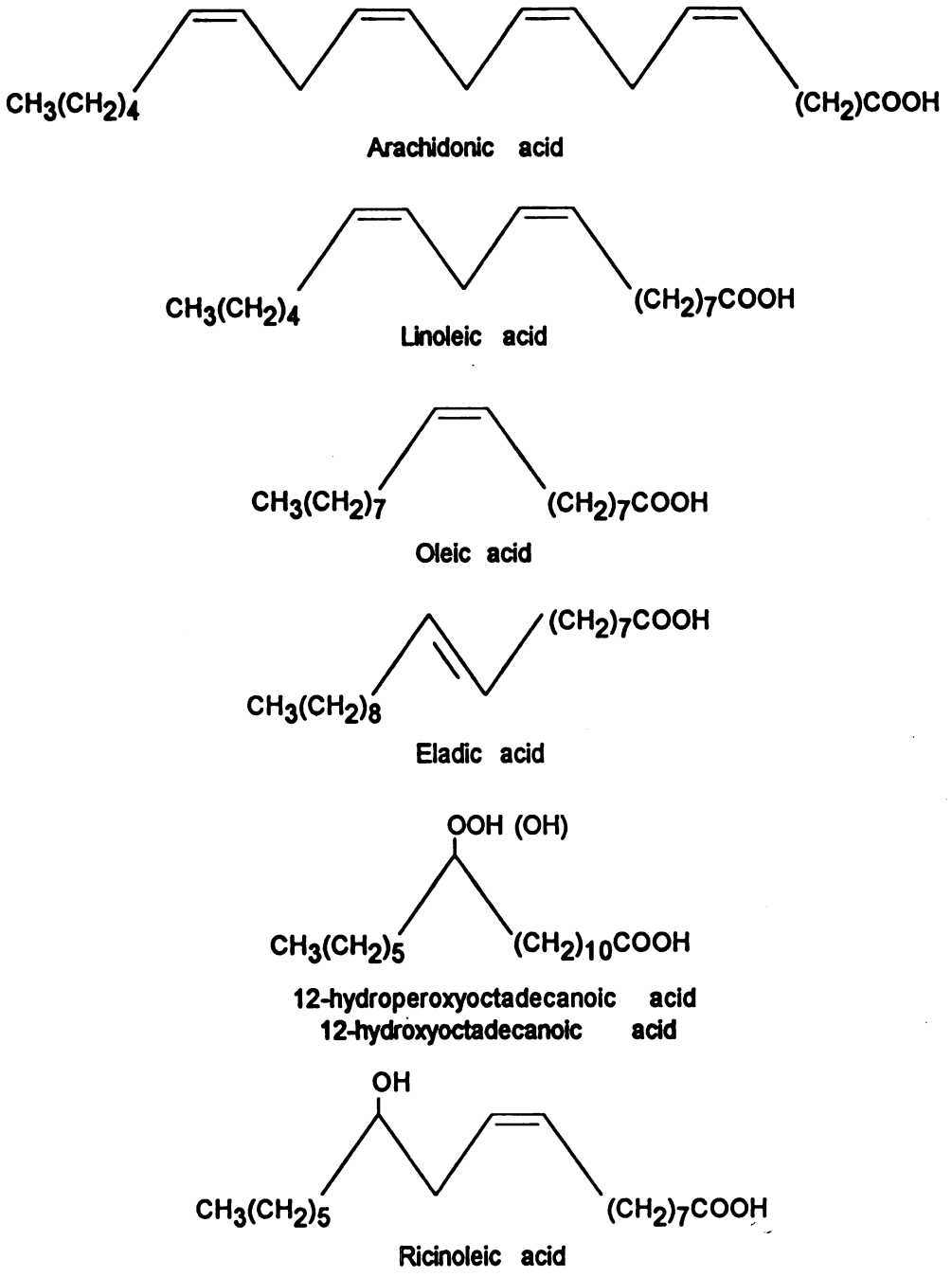


Figure 1.4: Structures of representative biologically inactive fatty acids.

acid could provide critical mitogenic stimuli to normal and neoplastic mammary gland epithelial cells, particularly in animals fed a high-fat, high linoleic acid containing diet.

All of the active compounds studied above can arise from autooxidation of the major dietary unsaturated fatty acids. The primary products of fatty acid autooxidation are allylic hydroperoxides containing *trans* double bonds (oleic acid derived) or a *cis-trans* conjugated diene system (linoleic and arachidonic acid derived) (14). The hydroxy fatty acids can arise from reduction of the hydroperoxides by either tissue or bacterial peroxidase activity. These types of hydroperoxide reductions are well precedented both *in vivo* and *in vitro* (25, 26, 27). Fatty acid hydroperoxides may also be produced by *in situ* generation in various tissues. Funk and Powell (28) have shown that linoleic acid can be metabolized, via a hydroperoxide intermediate, to 9-hydroxyoctadecadienoic acid (9-HODE) and 13-hydroxyoctadecadienoic acid (13-HODE) by the prostaglandin H synthase activity present in particulate fractions of the aorta. Similar reactions catalyzed by prostaglandin synthase or lipoxygenases have been reported in VX2 carcinoma cells (29), rabbit peritoneal tissue (30), human (31) and porcine leukocytes (32), and SHE fibroblast cells (33). The lipoxygenase pathway of linoleic metabolism may be seen in Figure 1.5.

VI. Goals of this research

As stated earlier in this chapter, linoleic acid seems to play an important role in the enhancement of mammary gland tumorigenesis in experimental animals. Unsaturated fat diets that are high in linoleic acid stimulate tumorigenesis. Furthermore, the addition of linoleic acid to a saturated fat diet returns the stimulatory effect. It is possible that the hydroxylated fatty acid metabolites, 9- and 13-HODE, are playing some role in the

tumorigenic process. The goals of this research are 1) to develop a methodology for the separation, isolation, and detection of 9- and 13-HODE in mammary gland tissue, and 2) to apply this methodology to quantitating the levels of 9- and 13-HODE from mammary glands in experimental animals fed diets that differ in the amount and the type of fat.

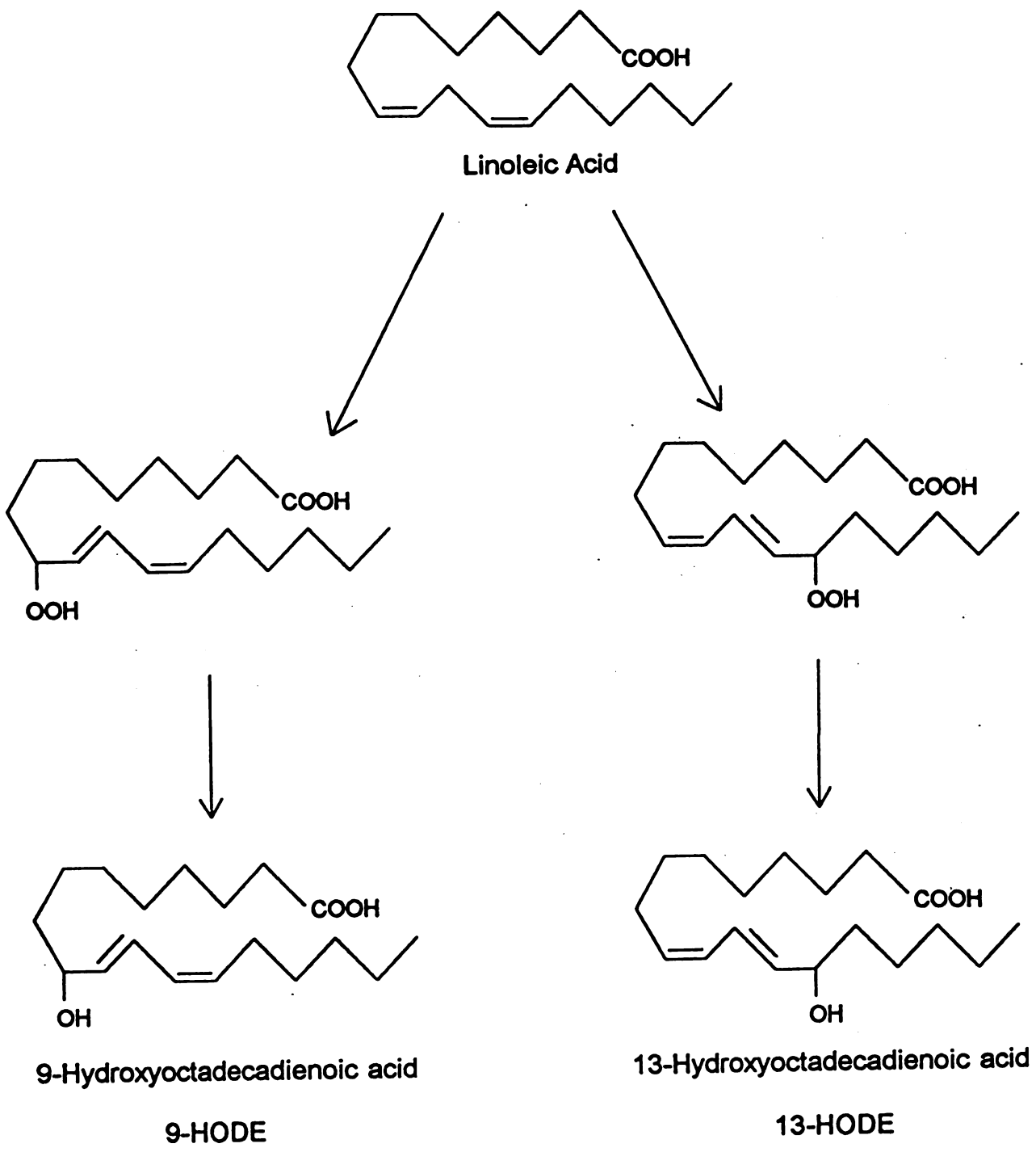


Figure 1.5: Lipoxygenase pathway of linoleic acid metabolism.

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Chapter 2: Development of Analytical Methodology

Oxygenated products (hydroxy- (1, 2), keto- (3, 4), epoxy- (5), and aldehydes (6)) have been isolated from many different body fluids and tissues. In general, each tissue studied requires its own methodology for extraction and/or removal of matrix interferents. There also exists a wide array of detection methods utilized to analyze the tissue extracts, yet none have been specifically developed for mammary gland tissue. Since it is the goal of this research to analyze hydroxylated fatty acids from mammary gland tissue, a method for the quantitative extraction and isolation of the analytes of interest had to be developed.

I. Characterization of fatty acids by GC/MS

Gas chromatography (GC) is a widely used and popular technique for the analysis of fatty acids after they have been converted to esters in most cases. However, fatty acids may only tentatively be identified by gas chromatographic retention times alone, but GC combined with derivatization and chemical degraditive or spectroscopic procedures, especially mass spectrometry, is an extremely powerful means of characterization of fatty acids (7).

Mass spectrometry has become a valuable tool for the identification of lipids and fatty acids. There are many ionization techniques that may be utilized for the analyses of lipids, but one that is most common is electron impact (EI) ionization. The principle of this technique is that organic molecules in the gas phase are bombarded by a beam of electrons in an ion source of a mass spectrometer producing positively-charged, radical ions. The positively-charged intact molecule is known as the molecular ion ($M^{+\bullet}$). This ion may then

fragment to form other smaller positively-charged ions. All of the ions are then accelerated out of the ion source, separated according to their mass to charge (*m/z*) ratios, and detected. A histogram plot of abundance vs. *m/z* is a mass spectrum. Often it is possible to deduce the structure of a fatty acid by its mass spectrum, i.e. by its fragmentation pattern. The combination of mass spectral and gas chromatographic retention time data may aid in eliminating alternative molecular structures.

Before gas chromatography/mass spectrometry (GC/MS) may be utilized, the fatty acids must be vaporized. Free fatty acids contain a polar functional group and typically are derivatized to make them more volatile and non-polar, improving peak shape and resolution simultaneously. A popular and widely used derivative for the analysis of fatty acids is the formation of the methyl esters. Methyl esters are easily prepared by reaction with ethereal diazomethane with a catalytic amount of methanol, or by reaction with methanol-BF_3 or methanol-HCl (8). The methyl ester derivatives of long-chain saturated fatty acids are easily characterized by electron ionization mass spectrometry. Their spectra are characterized by a prominent molecular ion and other significant ions consisting of *m/z* = $M^{+\bullet}$ – 31 and $M^{+\bullet}$ – 43. There also exists a series of ions of the general formula $[CH_3OOC(CH_2)_n]^+$ with peaks located at *m/z* = 87, 143, and 199 (9). The base peak, *m/z* = 74, represents the "McLafferty rearrangement ion", which is formed after hydrogen rearrangement and cleavage of the parent molecule *beta* to the carboxyl group (10).

Free fatty acids may also be derivatized into other esters to increase their volatility, or to improve their chromatographic or mass spectrometric characteristics. When a higher molecular weight is desired, trimethylsilyl (TMS) or t-butyldimethylsilyl (t-BDMS) esters

may be formed. Pentafluorobenzyl esters make fatty acids suitable for electron capture detection or negative ion mass spectrometry. The determination of the position of functionalities such as double and triple bonds, branches, or cyclopropane rings have been described using pyrrolidides (11), picolinyl (12), piperidyl and morpholinyl esters (13), 2-alkenylbenzoxazoles (14), and 4,4-dimethyloxazoline derivatives (15).

II. Characterization of hydroxylated fatty acids by GC/MS

Because of the hydroxyl functionality, the esters of hydroxylated fatty acids are still quite polar and somewhat involatile. It is, therefore, desirable to convert the hydroxyl group to an ether to improve the gas chromatographic and mass spectrometric characteristics of the compound. The choice of the derivative will depend on the compound, and it may be necessary to prepare several types of derivatives to confirm identification. Some common hydroxyl group derivatizations include acetylation (16), trifluoroacetylation (17), and trimethylsilylation (18, 19, 20).

Trimethylsilyl (TMS) ether derivatives are especially useful with EI mass spectrometry. The fragmentation of these molecules can be diagnostic for locating the position of the hydroxyl functionality in the carbon chain as major fragmentation occurs *alpha* to the carbon containing the functional group. A discernible peak for the molecular ion is not usually present, but an $[M - 15]^+$ ion is formed allowing molecular weight and carbon chain length to be determined.

The positions of hydroperoxy groups formed during the oxidative deterioration or degradation of lipids are most commonly determined by reduction of the hydroperoxide to a hydroxide and hydrogenation of any double bonds, followed by methylation and

conversion to the TMS ether prior to analysis by GC/MS. This approach has been of use in the identification of eicosanoids derived from arachidonic acid (21, 22, 23, 24).

If the hydroxylated fatty acid is unsaturated, it is essential to prepare the methyl ester TMS ether if the mass spectra are to be interpretable (25, 26). The position of the double bond(s) relative to the TMS ether affects the fragmentation pattern. With esters in which the hydroxyl group is allylic to the double bond(s), no fragmentation occurs between them. Cleavage occurs on either side of the hydroxyl and double bonds. When there is one methylene group between the double bond and the carbon linked to the TMS ether, the ions caused by fragmentation *alpha* to the TMS carbon on the side of the double bond are most abundant. If the functional groups are separated by two methylene groups, the two peaks in the mass spectrum representing *alpha* cleavage on either side of the TMS carbon are of equal intensity (7).

III. Methods other than GC/MS for the analysis of fatty acids

Thin-layer chromatography (TLC) has been used in lipid analysis for many years on both an analytical and preparative scale. TLC procedures with silica gel G (with a calcium sulfate binder) have been employed most frequently for lipid class separations. A common solvent elution system consists of hexane-diethyl ether-formic acid (80:20:2 by volume). Bands on the TLC plate may be detected by spraying with an 0.1% (w/v) solution of 2',7'-dichlorofluorescein in 95% methanol and viewing under ultraviolet (UV) light. The lipids appear as yellow spots against a dark background, and the lipid fractions may be recovered from the plate by elution with solvents for further analysis.

High-performance liquid chromatography (HPLC) is increasingly being utilized for separation and detection of fatty acids and other lipid classes (27). Most lipids lack chromophores for spectrophotometric detection, but the absorbance of double bonds in the low ultraviolet (UV) range can be used successfully if care is taken in the choice of solvents for the mobile phase. Only a few solvents are transparent (methanol, hexane, acetonitrile, isopropanol, and water) in the low UV range, and the molar extinction coefficients are low so that impurities in the solvent can interfere with the signal of the compounds of interest. However, many different separations have been reported using both normal and reverse phase, and well as with isocratic and gradient elution (27).

IV. Initial attempts to extract mammary gland tissue

The high fatty acid content of the mammary gland extract was shown to produce a high chemical background when published isolation procedures for other tissues were applied to the mammary gland (28). As a starting point to learn practical methodology, the initial attempts at extraction of 9- and 13-HODE from mammary gland tissue were begun at the laboratory of Dr. Arthur Bull, Assistant Professor of Chemistry at Oakland University, Rochester, Michigan. Dr. Bull's laboratory has developed an extraction procedure to isolate 13-HODE from rat colon tissue using HPLC with UV detection as a means of analysis.

A. Extraction

A one-gram mammary gland tissue sample was stirred with 10 mL of a 2:1 chloroform:methanol (29) mixture, the supernatant was saved and the extraction was repeated with 5 mL of solvent; the two extracts were combined and the final volume

determined prior to the addition of 1/4 volume of water for an aqueous phase extraction (30, 31), resulting in a biphasic system. The upper phase was removed with a pipet and discarded. Solvent from the lower phase was removed *in vacuo*, prior to methylation with diazomethane and methanol for 30 minutes at room temperature. The sample was dried under nitrogen, reconstituted in 100 μL of 20 % acetonitrile, 0.1 % trifluoroacetic acid (TFA) in water for analysis by HPLC.

B. High-performance liquid chromatography

The entire 100 μL was injected onto a 25-cm octadecasilica column. Detection of the analytes was performed with a Perkin-Elmer LC-235 diode array detector at a wavelength of 235 nm to detect the absorption of the conjugated diene in the 13-HODE methyl ester (ME). The chromatographic conditions were as follows: solvent A was 20 % acetonitrile, 0.1 % TFA in water; solvent B was 100 % acetonitrile with 0.1 % TFA. The HPLC run consisted of a linear gradient from 50% A to 100% B over 30 minutes with a 20-minute hold. The flow rate was 1 mL/minute. Figure 2.1a is a chromatogram of a standard of 13-HODE methyl ester which elutes at 25.45 minutes. Figure 2.1b is a chromatogram of the methylated mammary gland extract. A spectrum of the peak eluting at 25.97 min. (Figure 2.2) shows possible conjugated diene features, but the absorbance at 235 nm is too intense to give a definitive result, and the elution time is not the same as that for the standard. From this work it was concluded that the extraction procedure may be applicable to mammary gland tissue, but a detection method with greater selectivity was necessary.

13-HODE Standard

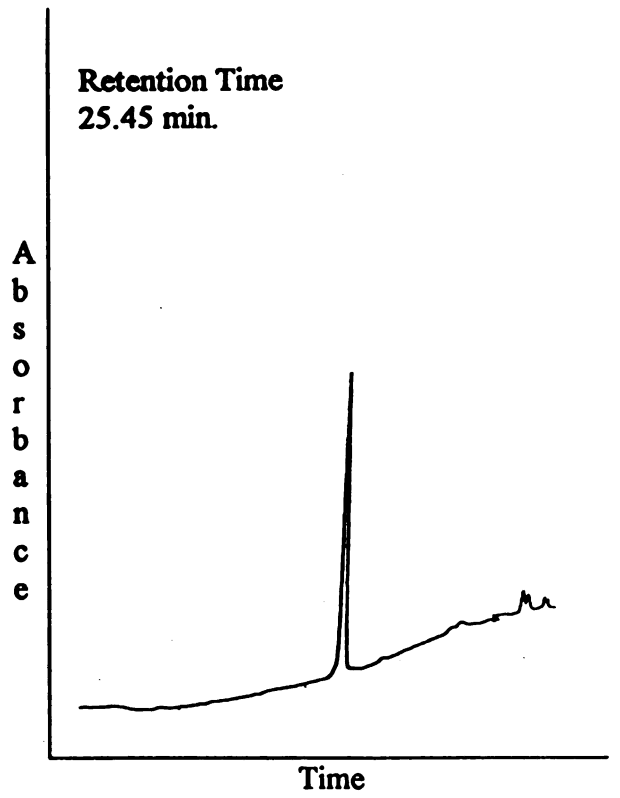


Figure 2.1a: HPLC chromatogram of a 13-HODE ME standard
$\lambda = 235$ nm.

Mammary Gland Extract

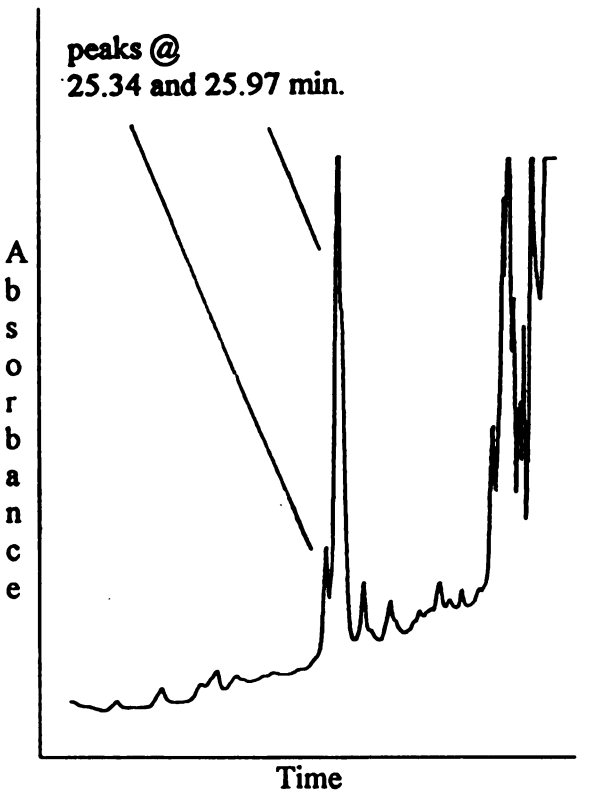


Figure 2.1b: HPLC chromatogram of a methylated mammary gland extract
$\lambda = 235$ nm.

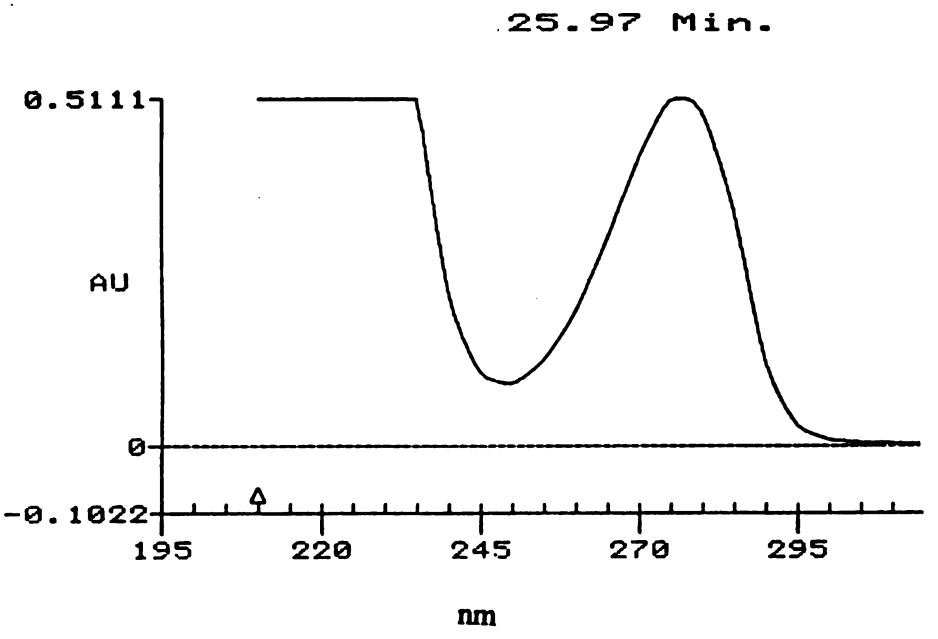


Figure 2.2: UV absorption spectrum of the peak at 25.97 min. in the chromatogram from the mammary gland extract.

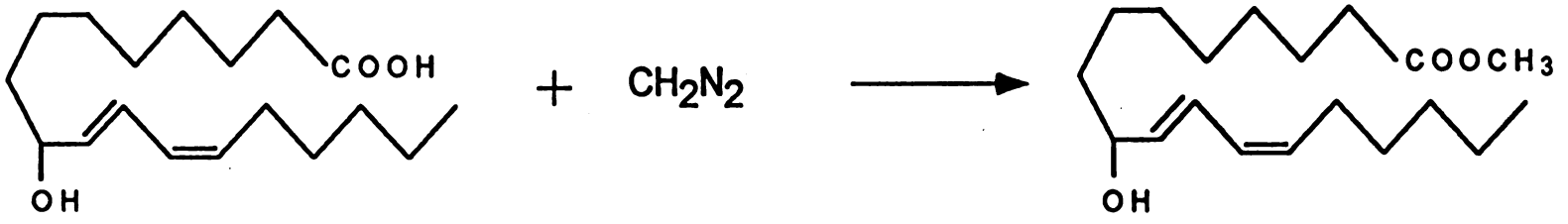
C. The use of GC/MS for detection

Because the use of UV detection was too general for this application, gas chromatography/mass spectrometry was investigated as a method of detection. As previously discussed, GC/MS has a long history in the analysis of lipids. This method of detection is advantageous in this case because the analytes may be identified by retention times and mass spectra.

Since the analytes of interest are both hydroxylated and contain points of unsaturation, the free fatty acids were derivatized to methyl ester/TMS (ME/TMS) ethers. Figure 2.3a shows the reaction of 9-HODE with diazomethane to form the methyl ester and Figure 2.3b shows the subsequent reaction with bis(trimethylsilyl)trifluoracetamide (BSTFA) to form the TMS ether. This reaction was carried out in pyridine at 60° for 30 minutes. This reaction mixture may be injected directly into the gas chromatograph.

The gas chromatographic mass spectrometric characteristics of 9- and 13-HODE ME/TMS are shown in Figure 2.4. The fragmentation of these compounds was first characterized by Hubbard et al. (32). The mass spectrum of 13-HODE ME/TMS has characteristic peaks at *m/z* 382 ($M^{+\bullet}$), 311 $[M - 71]^+$, and 225 $[M - 157]^+$. The mass spectrum of 9-HODE ME/TMS also has characteristic peaks at *m/z* 382 ($M^{+\bullet}$), 311 $[M - 71]^+$, and 225 $[M - 157]^+$. The major difference between the mass spectra of these two isomers is the relative intensities of the peaks.

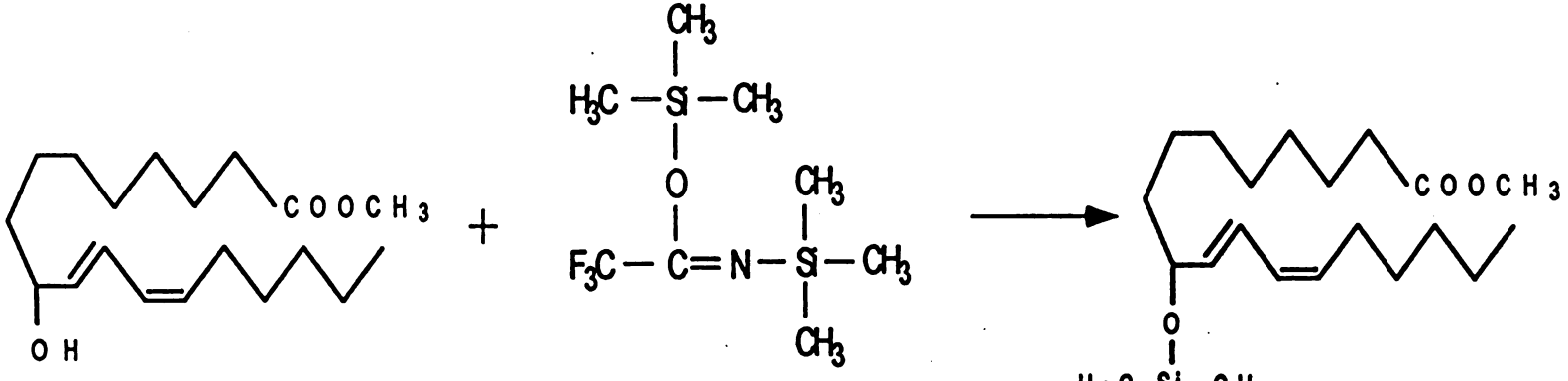
A reconstructed total ion chromatogram (RTIC) with mass chromatograms representing the analysis of a mammary gland extract with GC/MS detection is shown in Figure 2.5. There is evidence of the presence of both 9- and 13-HODE ME/TMS,



9-HODE

9-HODE ME

Figure 2.3a: Reaction of 9-HODE with diazomethane to form 9-HODE methyl ester.



9-HODE ME

bis(trimethylsilyl)trifluoroacetamide
BSTFA

9-HODE ME/TMS

Figure 2.3b: Reaction of 9-HODE ME with BSTFA to form the methyl ester/TMS ether.

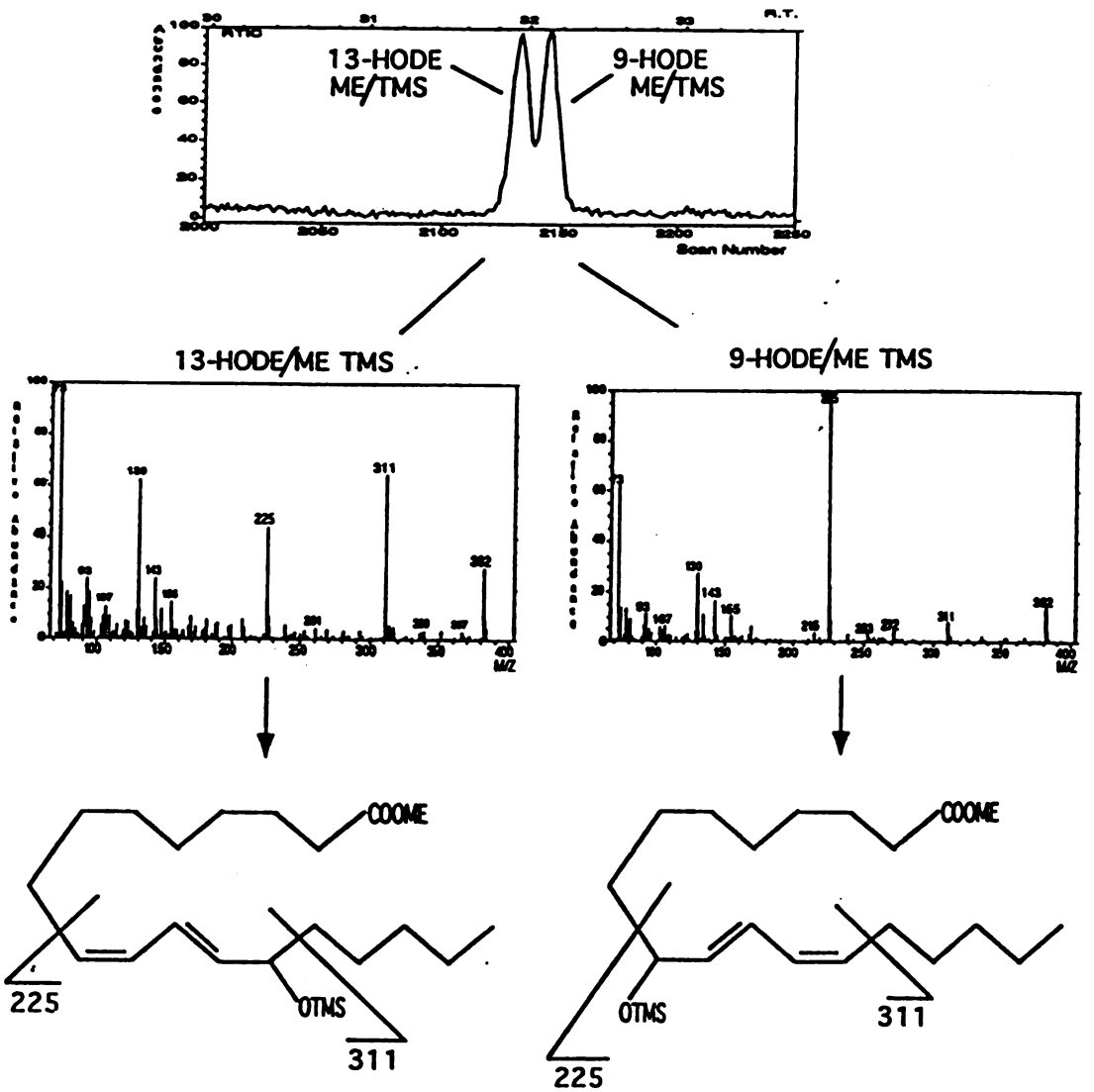


Figure 2.4: GC/MS characteristics of 13- and 9-HODE ME/TMS.

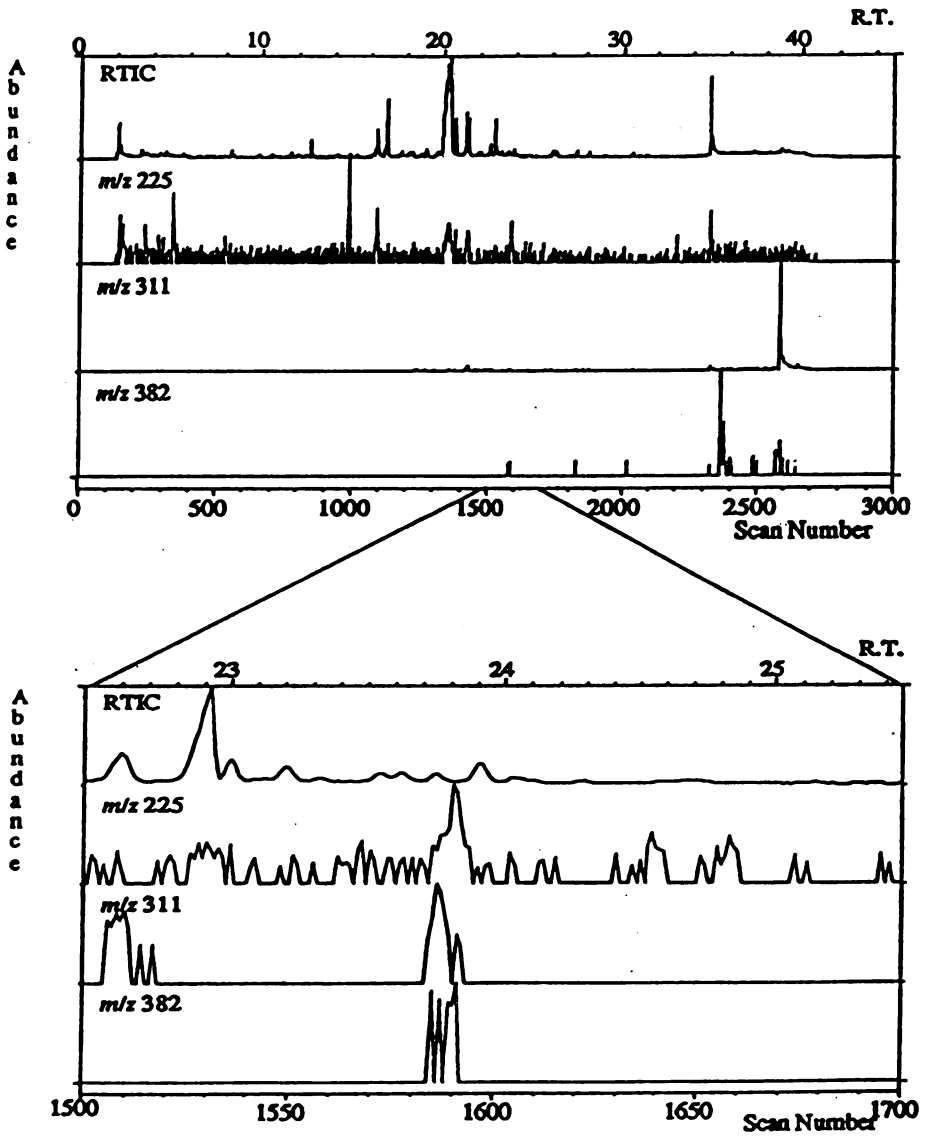


Figure 2.5: RTIC and mass chromatograms of a mammary gland extract.

however, interferences also exist so that peaks representing these compounds are barely detectable in the RTIC making it impossible to quantitate these compounds. From this result it was decided that this extraction procedure was not directly applicable to mammary gland tissue.

V. An extraction procedure for mammary glands

The first step taken to develop an extraction procedure for mammary glands was to use ethanol as an extraction solvent. Ethanol was tried for two reasons: 1) all of the hydroxylated fatty acid standards purchased were dissolved in ethanol, and 2) Lehmann et al. (33) utilized ethanol as an extraction solvent for the extraction of HODEs and hydroxyeicosatetraenoic acids (HETEs) from epidermal tissue. This proved to give similar results to the chloroform:methanol extraction, i.e. much interference. A silica solid-phase extraction step was added after methylation to remove interferences (34).

The 200 mg silica columns are preconditioned by washing with 2 mL hexane, 1 mL 50% ether/hexane, and 2 mL hexane. The methylated extract is reconstituted in 1 mL hexane and applied to the silica column which is then washed with 2 mL 5% ether/hexane and eluted with 5 mL 50% ether/hexane and 2 mL ethyl acetate. Results with either the ethanol or the chloroform:methanol extractions with the silica column cleanup were still not satisfactory.

We observed that mammary glands extracted with ethanol would produce a pellet upon centrifugation, whereas the chloroform:methanol:water extractions resulted in a suspension where the densities of the tissue and the solvent were similar so the tissue would not form a pellet making it difficult to obtain a clean extract.

The use of the two solvent extractions was attempted; ethanol was used first to extract the lipids and to pellet out the tissue residue. After evaporation of the ethanol *in vacuo*, a chloroform:methanol:water extraction was used to remove any non-lipid material remaining. A clean extract resulted following silica gel cartridge cleanup.

The extraction procedure in its final format may be seen in Figure 2.6. A mammary gland tissue sample (100-200 mg) is pulverized in liquid nitrogen prior to extractions in 10 mL, then 5 mL of ethanol. The ethanol extracts are combined and are centrifuged, the supernatant transferred to a pear-shaped flask, and the ethanol is evaporated *in vacuo*. A second extraction in dichloromethane:methanol (2:1) and water removes the remaining non-lipid material. The switch was made to dicholoromethane from chloroform as it has been reported that dichloromethane is less likely to promote oxidation of the lipids in the sample (35, 36). The mixture of dichloromethane:methanol:water separates into two phases; the upper phase is pipetted off and discarded. The remaining solvent is evaporated *in vacuo*, and the sample is methylated with diazomethane and methanol. After 30 minutes the solvent is evaporated under a stream of nitrogen, the residue is reconstituted in 1 mL of hexane and applied to silica columns according to the above procedure. Figure 2.7 shows the RTIC and selected mass spectra obtained during GC/MS analysis of a mammary gland extract processed according to the new procedure. As can be seen, the interferences have been removed.

A. Recovery experiments

Recovery experiments were performed in the laboratory of Dr. Arthur Bull at Oakland University. Tritium-labeled 13-HODE was prepared by soybean lipoxygenase

Tissue (100-200 mg)

Pulverize the tissue in liquid nitrogen

|

Extract in ethanol and centrifuge

|

Extract in CH_2Cl_2:MeOH (2:1)
Water
to form a biphasic system

|

Methylate with diazomethane and methanol

|

Solid phase extraction on silica columns
dissolve sample in hexane and apply sample to column
wash with hexane
5% ether/hexane
elute with 50% ether:hexane
ethyl acetate

|

Derivatize with BSTFA

|

Analyze by GC/MS

Figure 2.6: Extraction procedure for mammary glands.

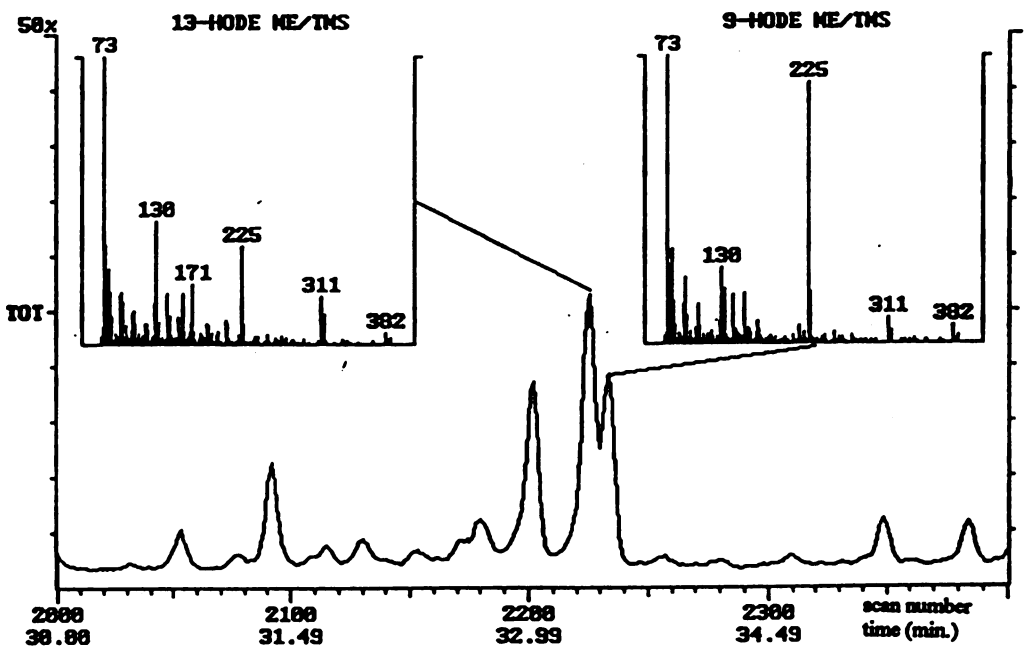


Figure 2.7: RTIC and selected mass spectra recorded during the GC/MS analysis of a mammary gland extracted utilizing the new procedure.

catalyzed oxygenation of ^{3}H-labeled linoleic acid followed by sodium borohydride reduction of the resulting 13-hydroperoxyoctadecadienoic acid (37, 38, 39). The labeled HODE was dissolved in ethanol, and the standard purity was determined by HPLC. The concentration of the standard solution was 3.1 mM with a specific activity of 2.68 μCi/μmol.

The recovery experiments were performed by adding the labeled standard to 150 mg rat mammary gland tissue sample prior to extraction with ethanol. An aliquot of a known volume of solvent was removed in duplicate after the initial ethanol extraction, following methylation, and after elution from silica columns to monitor losses throughout the extraction procedure. Radioactivity was measured by scintillation counting. The average recovery was 72% with a 5% relative standard deviation (RSD). Recoveries are summarized in Table 2.1.

Table 2.1: Percent recovery for ^{3}H-13-HODE from rat mammary gland tissue extract.

	Recovery (%)			mean (RSD)
	Extract #1	Extract #2	Extract #3	
after ethanol extraction	100	100	97	99 (1.7)
after methylation	74	79	70	73 (4.9)
final	**72**	**76**	**69**	**72 (4.8)**

B. Internal standards

Standards are essential for calibration of the instrument and for quantitation of the analyte. The addition of an internal standard increases the accuracy of the experiment especially if the internal standard has the same or similar chemical and physical characteristics as the analyte(s) of interest. If the internal standard of similar chemical and

physical properties is added at the beginning of the assay, it will undergo the same losses during chemical reactions (derivatizations) and physical manipulations such as extractions and transfers from flask to flask. Because the internal standard experiences the same losses as the analyte, the quantity of the internal standard relative to the analyte accounts for losses of the analyte. Quantification of the analyte can be carried out with the use of standard curves.

An ideal internal standard for GC/MS is a stable isotope-labeled analog of the analyte of interest. Biochemical synthesis from deuterium-labeled arachidonate has been described for isotopically labeled leukotrienes and HETEs, but dilution with arachidonic acid endogenous to the biological system reduces the isotopic purity of the labeled products (40). Chemical synthesis of deuterated lipoxygenase products has also been described (41). An alternative for eicosanoid internal standards is to incorporate stable isotope oxygen-18 [^{18}O] atoms into the carboxyl moiety of the fatty acid. The exchange of the carboxyl oxygen atoms with oxygen-18 water can be accomplished with acid catalysis, basic ester hydrolysis, or enzymatic means (42, 43).

For the preparation of oxygen-18 labeled HODEs as internal standards in this project, the procedure of cholinesterase-catalyzed carboxyl oxygen exchange was utilized (44, 45). Oxygen-18 labeled HODEs were prepared by the addition of 50 mg of 9- or 13-HODE dissolved in 20 μL methanol to 500 μL $H_2{}^{18}O$ (97-98% ^{18}O) containing 30 units of butyrylcholine esterase. After incubation for 24 hours at 37°, the reaction mixture was extracted four times with ethyl acetate, combined, dried under nitrogen, and reconstituted in ethanol. The concentration of the solution was determined by measuring the absorbance

at a wavelength of 234 nm and using a value of 23000 mol L^{-1} cm^{-1} for the molar absorptivity constant (ε). Standards were stored at -20°.

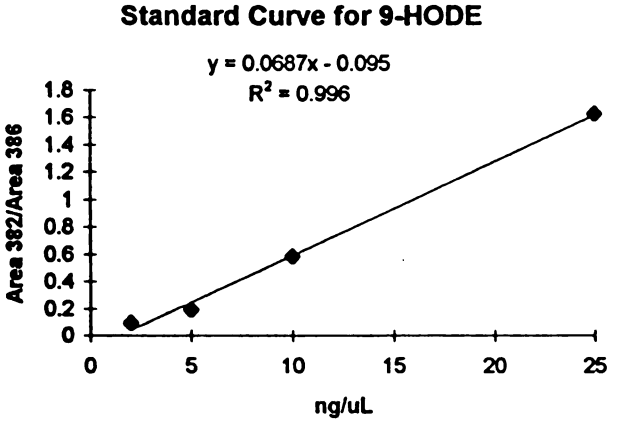
Oxygen-18 labeled HODEs were selected for internal standards for this research because of the simplicity of preparation and a four mass unit increase in molecular weight. The internal standards are added to the sample prior to extraction with ethanol. The mass spectrum of the labeled $^{18}O_2$-HODE has a peak at *m/z* 386 for $M^{+\bullet}$, and a peak at *m/z* 315 representing the fragment ion containing the label. The use of either selected ion monitoring (SIM) or repetitive scanning (mass chromatograms) to monitor the ion current at *m/z* 386 and *m/z* 315 gives a quantitative index of the internal standard, and monitoring the ion current at *m/z* 382 and *m/z* 311 gives a quantitative index of the amount of analyte introduced into the mass spectrometer. A plot of the ratios of the peak areas of the internal standard to the analyte vs. the analyte concentration is linear as can be seen in Figure 2.8.

Concentrations of unknowns may be calculated in the following manner: the peak areas of the unknown analyte and the added internal standard are acquired from the mass spectrometer response. The ratio of these two numbers gives the ratio of analyte to internal standard. Since the amount of internal standard added and the tissue sample size are known, the concentration of analyte can be calculated by the following equation (46):

$$\frac{\text{Area } m/z\ 382}{\text{Area } m/z\ 386} \times \frac{\text{amount of int. stand. added}}{\text{milligrams tissue}} = \frac{\text{amount HODE}}{\text{mg. tissue}}$$

VI. Preliminary application of the extraction protocol to glands from mice fed diets differing in the amount and type of fat

Female athymic nude mice (Balb/c) were divided into three groups to be fed three different diets. The diets were isocaloric and chemically purified differing only in the



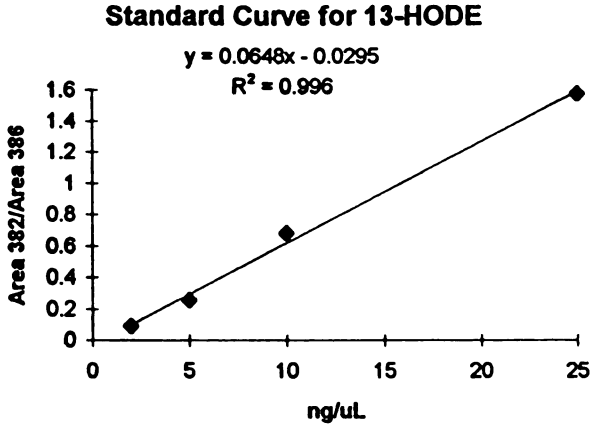


Figure 2.8: Calibration curves for 9- and 13-HODE ME/TMS.

amount and type of fat. One diet group was fed a high-saturated-fat diet (20% beef tallow by weight), one was fed a low-unsaturated-fat diet (5% corn oil by weight), and one was fed a high-unsaturated-fat diet (20% corn oil by weight). Corn oil was used not only because it is high in unsaturated fat, but also because it is high in linoleic acid, the precursor to 9- and 13-HODE (see Figure 1.5). The mice in each group were fed these diets beginning at three weeks of age. They were fed these diets for 6 weeks before being sacrificed. Three pairs of mammary glands from each mouse were excised, placed in polypropylene vials, and stored at -20° for approximately one month prior to being extracted.

Mammary glands (100-200 mg) were extracted following the methodology outlined above. Internal standards were added to the sample prior to the addition of ethanol. One sample from each of the diet groups was extracted per day. The amounts of internal standards added to each sample were 862 ng of oxygen-18 labeled 9-HODE and 412 ng of oxygen-18 labeled 13-HODE. The extracts were analyzed on a JEOL AX505 magnetic sector mass spectrometer in the selected ion monitoring mode (SIM) acquiring ion currents for *m/z* 386, 382, 315, and 311. The extract was separated gas chromatographically on a DB-5MS 30-m, 0.25-mm × 0.25-μm, column. Five samples, each representing a mammary gland sample from a different animal from each of the diet groups, were extracted and analyzed except for the group on the beef tallow diet as one sample was lost when a flask broke. An example of the chromatographic data may be seen in Figure 2.9. Table 2.2 contains the calculated concentrations of 9- and 13- HODE in the mouse mammary gland tissue.

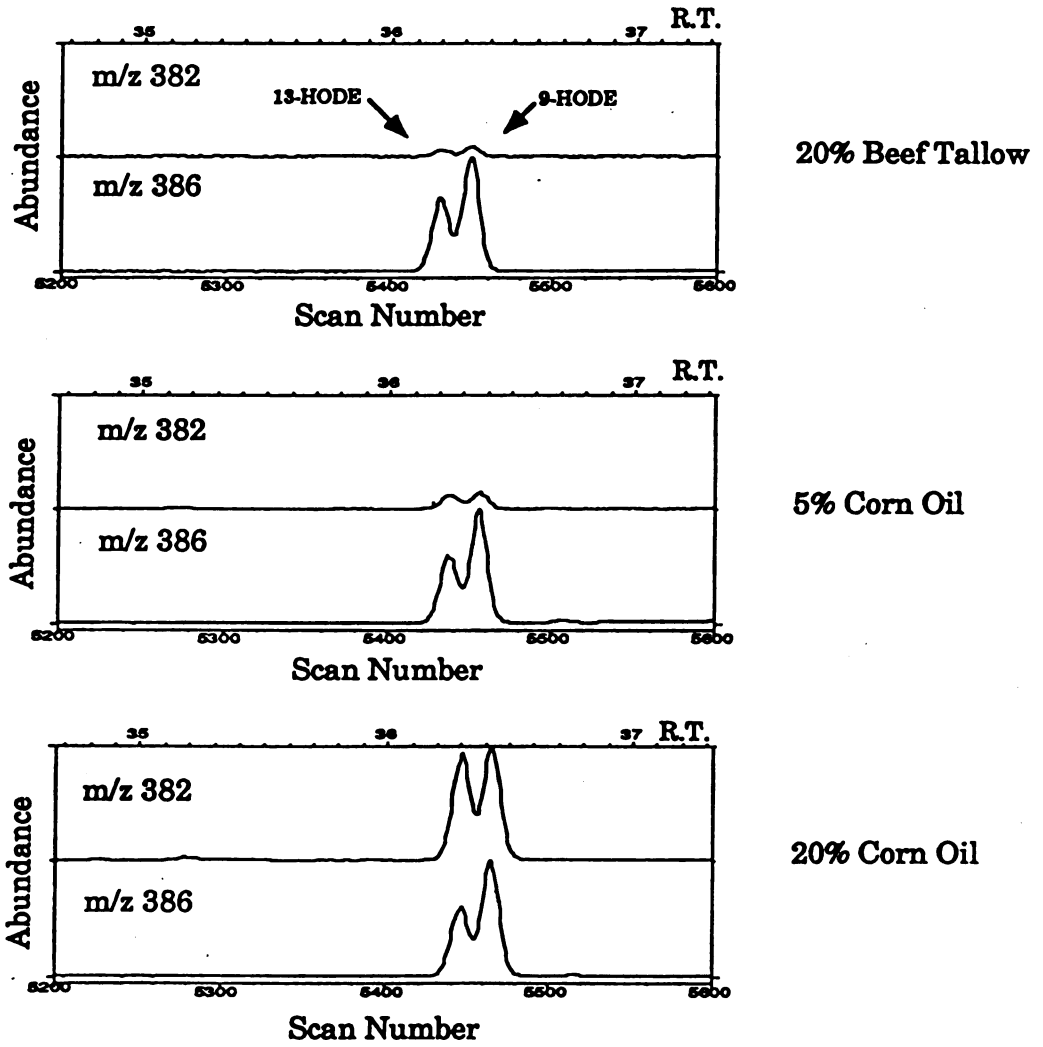


Figure 2.9: SIM chromatograms of mammary gland extracts: *m/z* 382 represents the analyte, and *m/z* 386 represents the oxygen-18 labeled internal standards.

Table 2.2: Concentration of 9- and 13-HODE in mammary gland tissue from mice fed three different diets.

	Concentration (ng HODE/mg tissue)		
	20% Beef Tallow	5% Corn Oil	20% Corn Oil
13-HODE	0.018	0.19	1.0
	------	0.09	1.1
	0.000	0.33	0.7
	0.18	0.39	0.9
	0.068	0.37	2.5
9-HODE	0.03	0.25	1.5
	------	0.14	1.4
	0.00	0.46	0.8
	0.21	0.54	1.2
	0.13	0.55	3.1

To test for significance a one-way analysis of variance (ANOVA) was performed. The ANOVA values for 9-HODE are found in Table 2.3, and the ANOVA values for 13-HODE are found in Table 2.4. These data show that at a 95% confidence level there are significant differences among the levels of both 9- and 13-HODE in mammary gland tissue from mice fed isocaloric diets differing only in the amount and type of fat.

Analysis of variance tests for the main effects of independent variables and potential interactions among them; it does not report which of the pairwise comparisons are statistically different from each other. To find out which groups were different, Tukey's test for making paired comparisons was employed. This test allows for 100(1-α)% confidence interval, where $\alpha = 0.05$, for all comparisons. The method is based on a studentized range distribution. The appropriate percentile point is a function of α, κ, and ν, where α is the significance level, κ is the number of treatments, and ν is the number of

Table 2.3: Analysis of variance for 9-HODE

ANOVA: Single factor for 9-HODE

Summary

Groups	Count	Sum	Average	Variance
20% beef tallow	4	0.38	0.09 ± 0.05	0.01
5% corn oil	5	1.9	0.39 ± 0.08	0.3
20% corn oil	5	7.9	1.5 ± 0.4	0.8

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	5.9	2	2.9	10.1	0.003	7.2
Within Groups	3.2	11	0.29			
Total	9.1	13				

Table 2.4: Analysis of variance for 13-HODE.

ANOVA: Single factor for 13-HODE

Summary

Groups	Count	Sum	Average	Variance
20% beef tallow	4	0.265	0.07 ± 0.04	0.006
5% corn oil	5	1.4	0.27 ± 0.06	0.02
20% corn oil	5	6.3	1.2 ± 0.3	0.5

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	3.8	2	1.9	10.5	0.003	7.2
Within Groups	2.0	11	0.18			
Total	5.8	13				

degrees of freedom for s^2. The method of paired comparisons by Tukey's test involves finding a significant difference between means i and j ($i \neq j$) if the difference in means exceeds $q[\alpha, \kappa, \nu]s\sqrt{1/n}$, where n is the number of samples per group and q is a tabulated value based on α, κ, and ν (47). The standard deviation (s) is obtained from the ANOVA Table. The results of Tukey's test for this diet study are shown in Table 2.5.

Table 2.5: Tukey's test results for differences among the levels of 9- and 13-HODE among the different diet groups.

	13-HODE	test statistic	9-HODE	test statistic
$\bar{X}_{5\% \text{ corn}} - \bar{X}_{20\% \text{ beef tallow}}$	0.21	0.73	0.30	0.93
$\bar{X}_{20\% \text{ corn}} - \bar{X}_{20\% \text{ beef tallow}}$	1.19	0.73	1.50	0.93
$\bar{X}_{20\% \text{ corn}} - \bar{X}_{5\% \text{ corn}}$	0.98	0.73	1.20	0.93

The results of Tukey's test indicate that the levels of 9- and 13-HODE in the mice fed a high-unsaturated-fat diet (20% corn oil) are significantly different from those levels in the group fed the low-unsaturated-fat diet (5% corn oil) and the group fed the high-saturated-fat diet (20% beef tallow) at a 95% confidence level. The groups fed the low-unsaturated-fat diet and the high-saturated-fat diet were not significantly different from each other at a 95% confidence level.

A closer look at the numbers show that the levels of 9-HODE in the tissues were almost equal to the levels of 13-HODE. Enzymatic synthesis of 9- and 13-HODE would favor the production of more of one or the other isomer, not production of equal amounts of both. Baer et al. (48) states that "if the 13-HODE had been derived from non-enzymatic

(auto)oxidation, then the amount of 13-HODE would have equaled that of 9-HODE, and the 13-HODE would have been racemic". There was some concern that the results seen here are artifactual (due to autooxidation during storage in the freezer or during sample workup) rather than an indication of what had occurred biologically.

VII. Validation of the extraction methodology

Because there was concern that the results obtained may not reflect a biological distribution of the HODEs, it was decided to approach answering the question of autooxidation with two methods: 1) test the precision of the extraction methodology as a function of storage time and 2) determine the enantiomeric distribution of the HODEs by chiral chromatography.

A. Method precision as a function of storage time

A large variance from sample to sample (from the same animal) may indicate that autooxidation is occurring during sample workup. To test this idea the precision of the extraction methodology was tested using mammary glands from one animal instead of multiple animals. Concurrently, precision was measured as a function of storage time. The logic here is that if autooxidation is occurring only during sample workup, the variance should be large in each case, but the levels of 9- and/or 13-HODE should be similar to each other. If autooxidation is occurring during sample storage, the levels of 9- and/or 13-HODE should increase, the levels of 9- and 13-HODE should start out being different then become more equal, and the variance should increase the longer the tissue is stored.

For these experiments a male rat that had been fed a low-fat diet was sacrificed, and two pairs of mammary glands were excised and immediately placed into liquid

nitrogen. The glands were pulverized in liquid nitrogen, thoroughly mixed, and divided into replicate samples ranging from 100-200 mg. These samples were randomly assigned to one of four test groups: one day (-80°), one week (-20°), one month (-20°), and one month (-80°). Each group was comprised of three samples. The glands were extracted, and data obtained are shown in tables 2.6 (9-HODE) and 2.7 (13-HODE).

There was one extra sample from the rat mammary glands used in this study. It was stored in a -4° freezer for three weeks. When it was extracted and analyzed the level of 13-HODE was 15.3 ng/mg of tissue. The level of 9-HODE was 14.8 ng/mg tissue.

Three points are evident from this data: 1) the concentration of 9-HODE is initially less than the concentration of 13-HODE, 2) the concentrations of 9- and 13-HODE are elevated in the samples stored for one month at -20°, 3) the variance of the one month (-20°) group has increased, 4) if samples are stored in an oxidizing environment (-4°) the concentration of HODEs in the tissues are high and are equal to each other.

The relative standard deviation for the intra-animal tests range from 5-35% for 13-HODE with the highest deviation in the one month (-20°) group. Similar results for 9-HODE were obtained with RSD ranging from 10-33%. The interanimal variation (from the mouse diet study), for comparison, was greater than 50% for both 9- and 13-HODE in each of the diet groups.

Table 2.6: Concentration of 9-HODE in aliquots of a common pool of mammary gland tissue as a function of storage time and temperature.

	Concentration (ng HODE/mg tissue)			
	One day (-80)	One week (-20)	One month (-20)	One month (-80)
9-HODE				
	0.53	0.92	1.4	0.75
	0.26	1.1	0.9	0.71
	0.50	0.93	1.8	0.48

Summary

Groups	Count	Sum	Average	Variance
one day (-80°)	3	1.3	0.43 ± 0.08	0.02
one week (-20°)	3	2.9	0.98 ± 0.06	0.01
one month (-20°)	3	4.0	1.3 ± 0.3	0.2
one month (-80°)	3	1.9	0.64 ± 0.08	0.02

Table 2.7: Concentration of 13-HODE in aliquots of a common pool of mammary gland tissue as a function of storage time and temperature.

	Concentration (ng HODE/mg tissue)			
	One day (-80)	One week (-20)	One month (-20)	One month (-80)
13-HODE				
	1.19	1.12	1.6	0.99
	0.95	1.22	1.4	1.11
	1.29	1.24	2.6	0.74

Summary

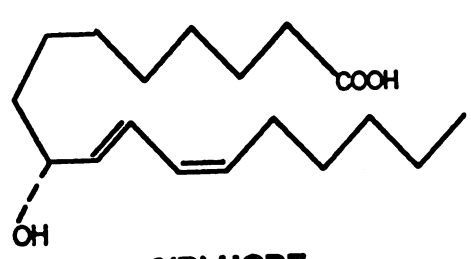
Groups	Count	Sum	Average	Variance
one day (-80°)	3	3.43	1.1 ± 0.1	0.03
one week (-20°)	3	3.58	1.19 ± 0.04	0.004
one month (-20°)	3	5.6	1.9 ± 0.4	0.4
one month (-80°)	3	2.85	0.9 ± 0.1	0.03

The above data suggest that mammary gland tissue is oxidized during storage. The levels of 9- and 13-HODE increase with increasing storage time, and the variance is largest for a long storage time at the higher temperatures. The variance is consistent in the one day, one week, and one month (-80°) groups, suggesting that autooxidation is not occurring during sample workup.

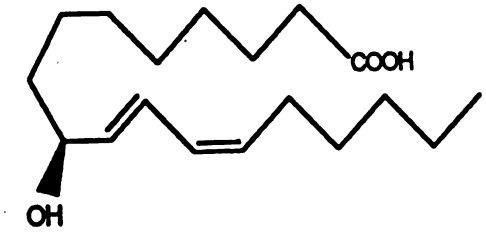
B. Chiral analysis of HODE enantiomers by HPLC

The most accurate test for autooxidation is to determine the enantiomeric distribution of the HODEs in the mammary gland tissue. Each of the HODEs has a center of chirality at the carbon containing the hydroxyl moiety. Each HODE therefore, has the possibility of forming two enantiomers, an R form and an S form (see Figure 2.10). Enzymatic oxidation of linoleic acid produces one enantiomeric form in mammalian systems. Autooxidation, however, produces racemic (1:1) mixtures. If autooxidation is occurring, racemic mixtures of 9- and/or 13-HODE should be detected with chiral chromatographic analysis of the mammary gland tissues.

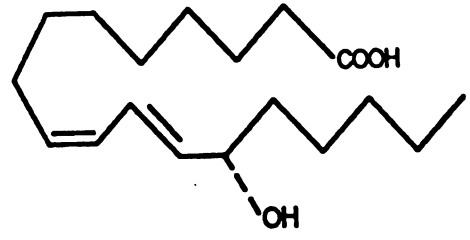
Enantiomeric separation by HPLC has taken various approaches over the last 25 years, with the most recent progress focused on the design and manufacture of synthetic chiral stationary phases. The three-point interactive rule of chiral recognition first proposed by Dagliesh (see Figure 2.11) for paper chromatographic separation (49), was extended to HPLC and verified by Baczuk et al. (50). Pirkle and coworkers began the first systematic approach to the design of chiral stationary phases for HPLC. They utilized various optically active π-acids and π-bases (see Figure 2.12) and proved their utility (51, 52, 53, 54). Conformational rigidity was found to be important, and resolution is achieved



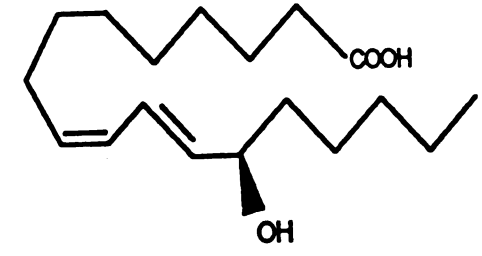
9(R)-HODE



9(S)-HODE



13(R)-HODE



13(S)-HODE

Figure 2.10: 9- and 13-HODE enantiomers.

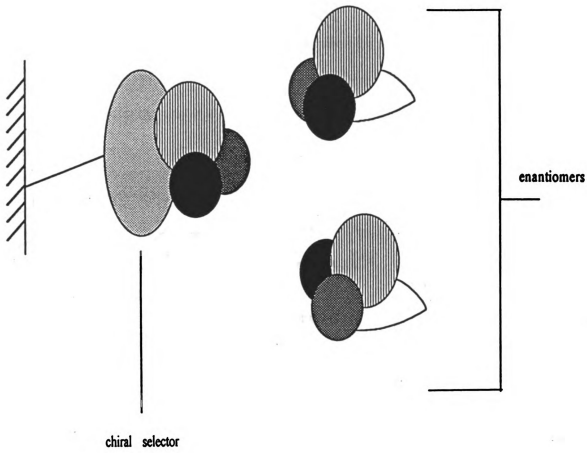


Figure 2.11: Representation of three point interaction necessary for chiral recognition.

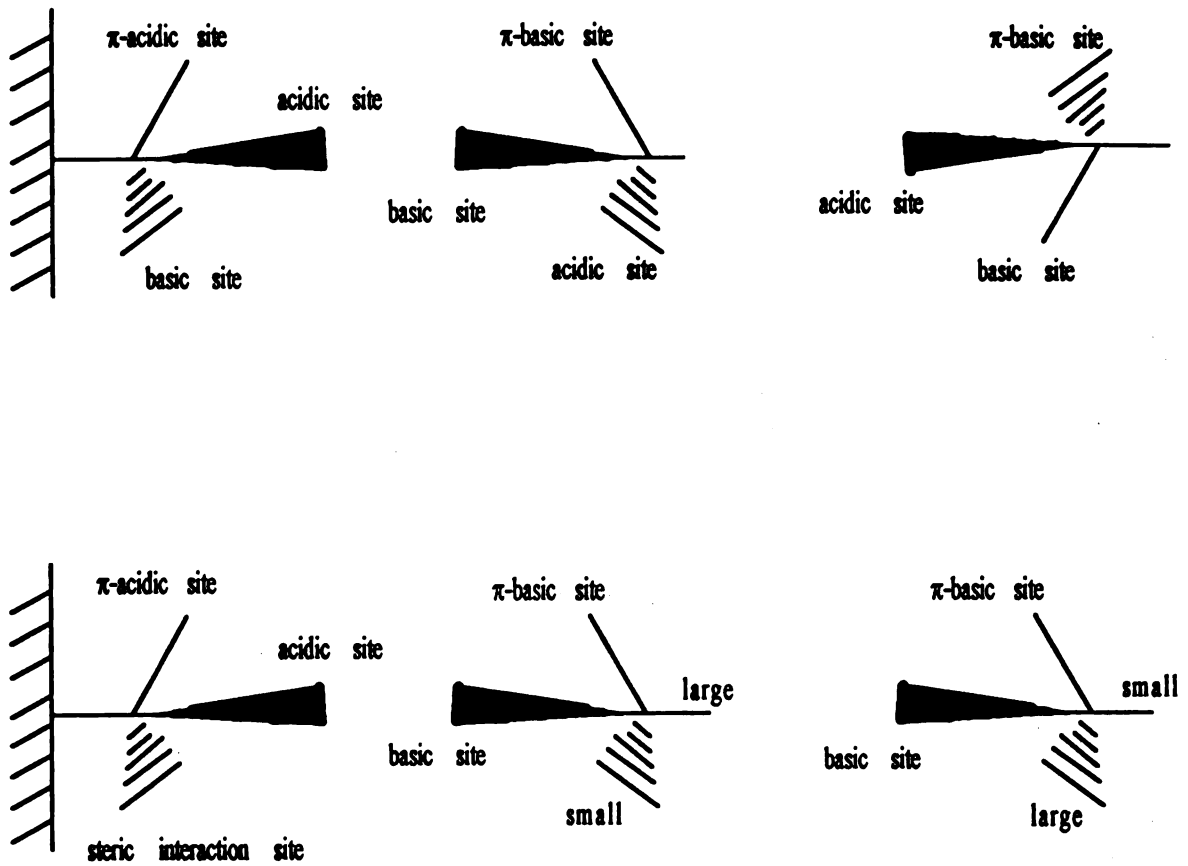


Figure 2.12: Model used to interpret the stereoselectivity obtained with charge-transfer based chiral selectors (51).

by a variety of diastereomeric interactions, including π-π bonding, hydrogen bonding, dipole-dipole interactions, and steric hinderence. Three sets of combinations of these interactions are common. Each set of the interactions entails the approach of the analyte enantiomers to the most accessible face of the immobilized chiral selector. The enantiomer that forms the most stable complex with the stationary phase is retained on the column the longest. In the more retained enantiomer, the complementary sites on the stationary phase are arrayed more favorably for interaction than in the less retained enantiomer (55).

The methodology that has been developed for the separation of HODE methyl ester enantiomers uses a Pirkle concept chiral stationary phase consisting of a dinitrobenzoyl phenylglycine (DNBPG) phase coupled either covalently or ionically over aminopropyl residues on silica gel with a mobile phase composition of hexane:2-propanol (IPA) in mixtures from 100:2 to 100:0.5 (hexane:IPA). Flow rates vary from 2 mL/min. to 0.5 mL/minute.

Pirkle phase columns have been used for the analysis of polyenoic fatty acids formed by the lipoxygenases of soybeans, reticulocytes, pea seeds, and tomato fruits (56), to the analysis of oxidative products of linoleic metabolism in human leukocytes (57), to the analysis of the stereospecificity of the products of fatty acid oxygenases from psoriatic scales (58, 59, 60), and to the analysis of structure-activity relationships in the potentiation of mitogenesis by oxygenated metabolites of linoleic acid (61). In each case the investigators used this approach to determine the enantiomeric composition in order to see if autooxidation was occurring. The investigators also wanted to determine which one of

the enantiomers was in a greater abundance to be able to correlate the enantiomer to biological activity.

1. Chiral chromatography with a DNBPG HPLC column.

Initial attempts at reproducing the chiral chromatographic analysis of the HODE MEs that had been reported in the literature was unsuccessful. A Pirkle column with a covalently bonded dinitrobenzoyl phenylglycine to an aminopropyl silica stationary phase was purchased from Supelco, Inc.. Mobile phase mixtures of hexane:isopropanol (IPA) ranging from 100% hexane to a 100:5 hexane:IPA were attempted along with varying the flow rate from 0.5 to 2 mL/min.. All samples are chromatographed as the methyl esters. Resolution of the enantiomers was not obtained. It was suggested to try lengthening the column to increase the number of theoretical plates. Increasing the number of theoretical plates by using two columns in tandem resulted in no separation of the 13(R,S)-HODE and only a slight separation of the 9(R,S)-HODE.

A J.T. Baker ionically bonded DNBPG column was purchased. With one column, a mobile phase composition of hexane:IPA 100:1, and a flow rate of 0.5 mL/min., an approximate 50% valley for both the 13(R,S)- and 9(R,S)-HODEs was obtained as seen in Figure 2.13a. Figure 2.13b shows the addition of 13(S)-HODE to confirm the retention times of the enantiomers. The R form of both the 13-HODE and the 9-HODE is more retained on this type of column and elutes later. These results were reproducible until some water was introduced on to the column destroying its selectivity. HPLC columns with this type of stationary phase have large lot to lot variability, and a second column from J.T. Baker did not perform under any chromatographic conditions.

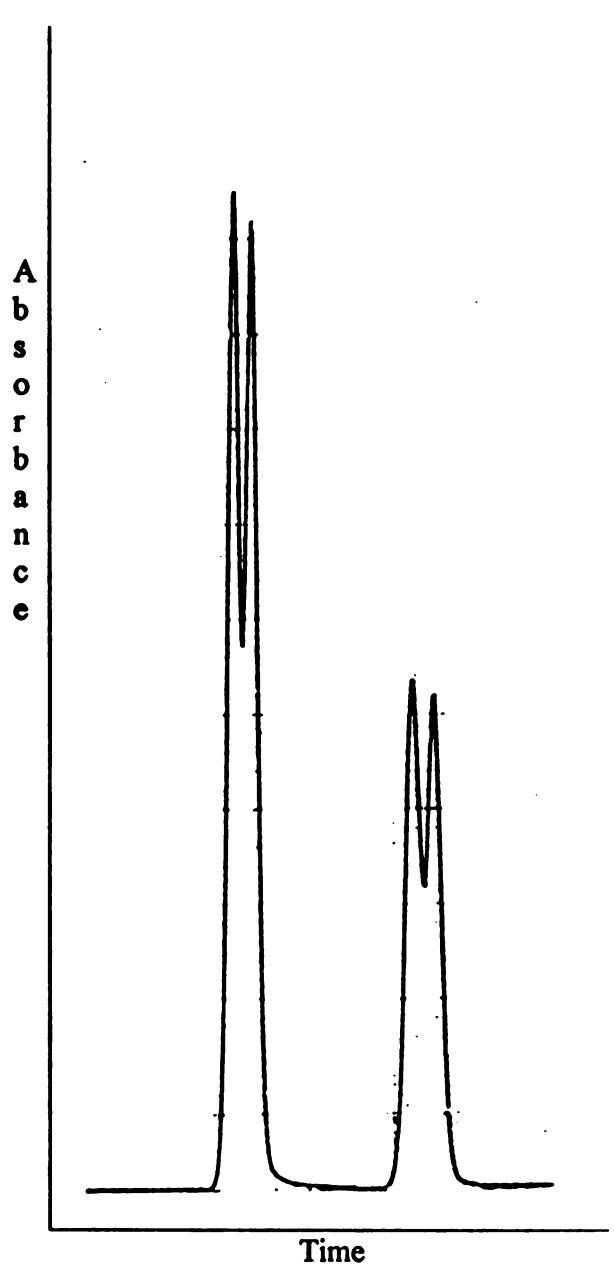


Figure 2.13a: HPLC chromatogram of 13(S,R)- and 9-(S,R)-HODE ME, J.T. Baker column λ = 234 nm.

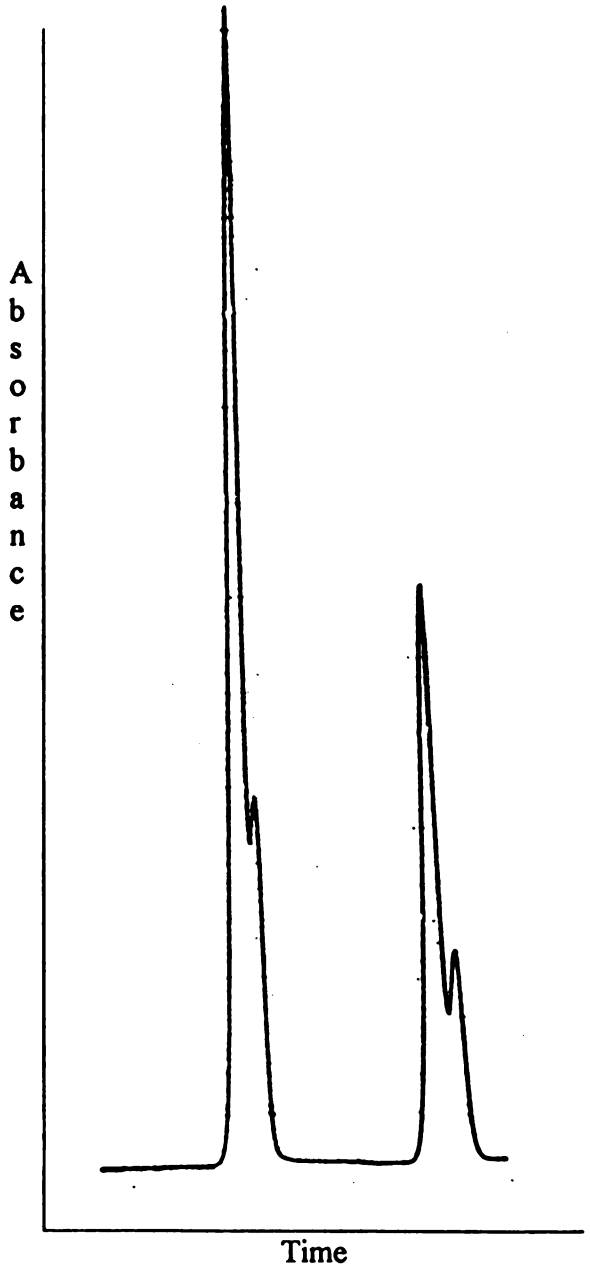


Figure 2.13b: HPLC chromatogram of 13(S,R)- and 9-(S,R)-HODE ME plus added S enantiomer of each, J.T. Baker column λ = 234 nm.

The use of two Regis ionically bonded DNBPG, 25-cm. × 4.6-mm., columns, a mobile phase composition of hexane:IPA 100:1, and flow rate of 1 mL/min., gave results similar to that of the previous J.T. Baker column as shown in Figure 2.14.

2. Chiral analysis of 13- and 9-HODE from tissue samples

Although the storage time experiments discussed above suggest that the tissues are not being autooxidized during the extraction procedure, chiral analysis of an extract provides more definitive results. Since an internal standard had been added to the samples for the storage and precision study, they could not be analyzed by chiral chromatography. Two mammary gland samples and one skin sample from the same animal as above were extracted after one day at -80°. One of the mammary gland samples was extracted with solvents containing butylated hydroxytoluene (an antioxidant). The samples were extracted, and the methyl esters chromatographed on a Waters μBondapack reverse phase C_{18} column 30-cm. × 3.9-mm. at a flow rate of 0.5 mL/min. with a mobile phase composition of methanol:water 80:20. Absorbance was measured at 234 nm. Under these conditions the 9- and 13-HODE MEs elute together and were collected for chiral chromatography.

Two Regis chiral phase columns with a mobile phase of hexane:IPA 100:1 and a flow rate of 1 mL/min. were used for the chiral analyses. Because the levels of the HODEs in these samples were so low, the chiral analyses of these samples were carried out by coinjection of racemic standards of 9- and 13-HODE ME. The 9-HODE standard had an S:R ratio of 1.02:1; the 13-HODE standard had an S:R ratio of 1:1 based on peak heights. The data from these experiments are found in Table 2.8.

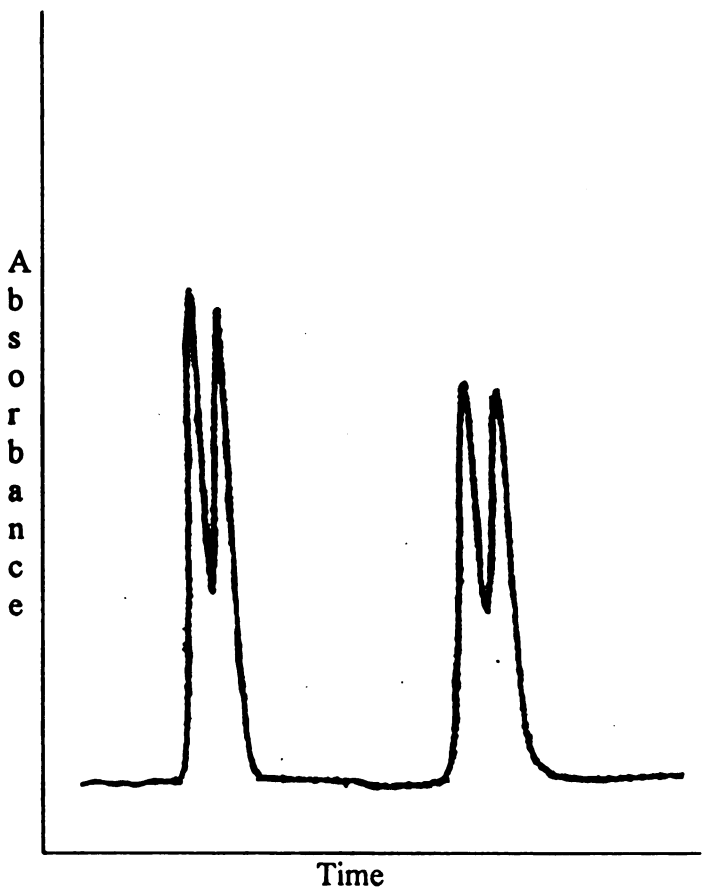


Figure 2.14: HPLC chromatogram of 13(S,R)- and 9-(S,R)-HODE ME, Regis columns $\lambda = 234$ nm.

Table 2.8: Chiral analysis of tissues for autooxidation during sample workup.

Sample	13-HODE S:R	9-HODE S:R
Mammary gland	1.27	1.00
Mammary gland + BHT	1.16	0.94
Skin	1.19	1.10

If autooxidation was occurring during sample workup, each of the values obtained for these experiments should have remained 1:1. The S enantiomer of the 13-HODE increased in each case supporting enzymatic formation rather than autooxidation. Also, the level of 13-HODE is greater than that of 9-HODE. It is more difficult to draw any conclusions for 9-HODE as the peaks were not detectable without coinjection of the racemic standards. These chiral results support the quantitative data from GC/MS.

From these experiments it can be concluded that the tissues are not being autooxidized during sample workup. However, the mammary gland samples will autooxidize after prolonged storage. This raises questions concerning the biological significance of the preliminary results.

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Chapter 3: Development of a New Methodology for Separation of HODE Enantiomers.

The use of chiral chromatography in the analysis of mammary gland tissue is important for two reasons: 1) the presence of racemic mixtures of HODEs in mammary gland tissue is the most reliable indicator of autooxidation, and 2) biological activity may be determined by the enantiomeric form of the hydroxylated fatty acids. Therefore it is necessary to determine if the distribution of optical isomers favors the biologically active enantiomer.

The published methods applied to the analysis of HODE enantiomers have resulted in unresolved enantiomeric peaks with a 50% valley (1-6). This analysis has also suffered from poor reproducibility and large lot to lot variation in column performance. Because an accurate assessment the distribution of optical isomers in a sample requires baseline separation of enantiomers, a new protocol has been developed for the chiral analysis of HODE enantiomers. This protocol is applicable to other monohydroxylated fatty acid enantiomers.

I. Selection of a different chiral column

The published procedures for the analysis of HODE enantiomers utilize a Pirkle column with a stationary phase of DNBPG ionically or covalently bonded to aminopropylsilica (1-6). Stationary phases comprised of cellulose derivatives on silica gel have also been applied to chiral chromatography. These types of stationary phases contains an assemblage of subunits acting together to achieve chiral recognition. The chiral recognition processes have not been determined because of the complex nature of

the substrate (7). Some cellulose derivatives that have been applied to chiral stationary phases include nitrates and aromatic esters such as benzoate, cinnamate, and phenyl carbamate. The chiral discriminatory capabilities of these columns are enhanced by the introduction of substituents on the phenyl groups of the chiral stationary phase (8, 9).

Capdevila et al. (10, 11) have reported the resolution of dihydroxyeicosanoates and dihydroxyeicosatrienoates by chiral chromatography on Chiralcel OC (cellulose tris(phenylcarbamate) on a silica-gel substrate) or OD (cellulose tris(3,5-dimethylphenyl carbamate) on a silica-gel substrate) columns with hexane:IPA mobile phases. The dihydroxy fatty acids were chromatographed as either methyl esters or pentafluorobenzyl esters. Optimal resolution was achieved after extensive equilibration with the mobile phase, and each compound studied required different chromatographic conditions (mobile phase composition and flow rate). When the published conditions for the analysis of the dihydroxy methyl esters on a Chiralcel OD column was applied to the analysis of HODE methyl esters, no resolution of the HODE enantiomers was obtained as seen in Figure 3.1.

II. Selection of a mobile phase

A. Background

In HPLC, the mobile phase is a dynamic part of the system. In normal phase and reversed phase chromatography, optimization of the mobile phase is the most important factor for improving separations (12). Optimization of the separation of racemates by manipulation of the mobile phase rests on three principles: 1) solvent selectivity, 2) solvent strength, and 3) adsorption of solvent on specific sites of the chiral stationary phase followed by displacement of solvent by the solute (13). The selectivity of a nonpolar

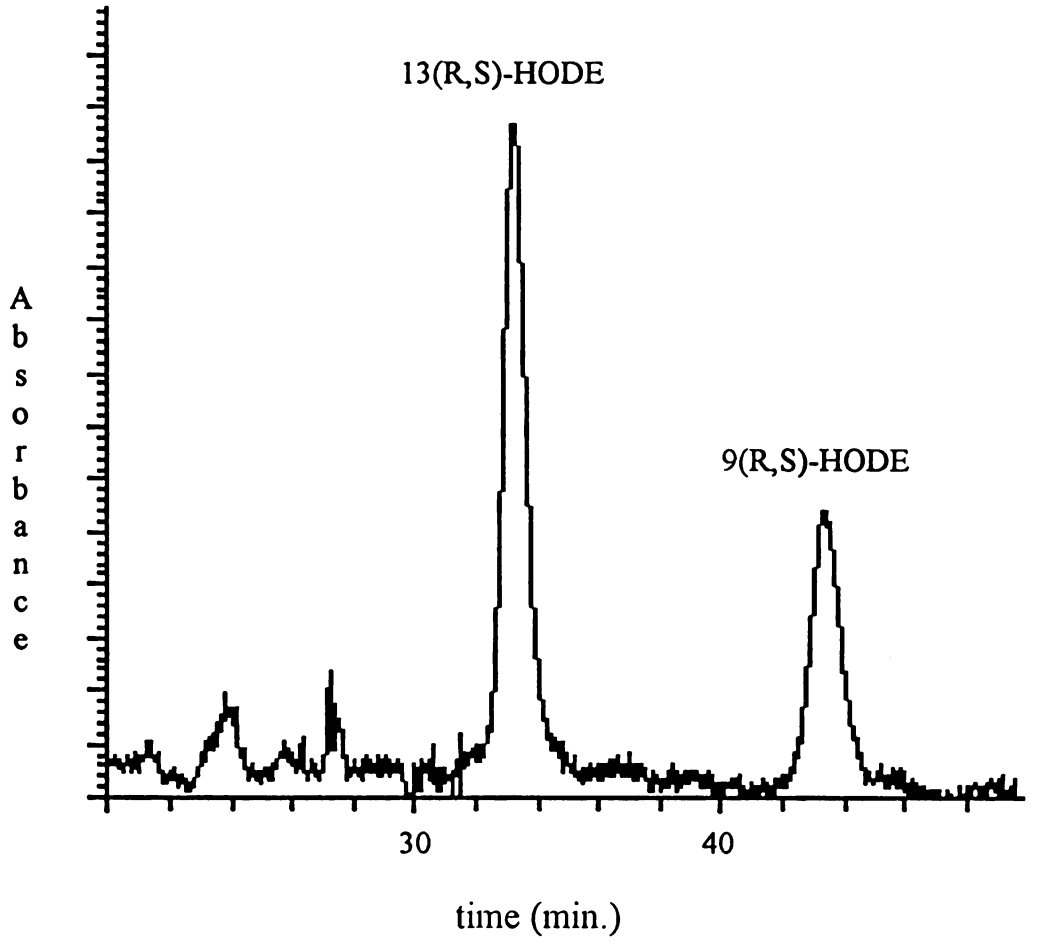

Figure 3.1. Chromatogram resulting from chiral analysis of a mixture of 13(R,S)- and 9(R,S)-HODE ME on a Chiralcel OD column, hexane:IPA 100:1, flow = 1 mL/min.. The enantiomers are not resolved under these conditions.

solvent like hexane can be modified by the addition of solvents that act as proton acceptors, proton donors, or strong dipoles. The strength of the modified solvent is affected by the quantity of the added modifier. A typical approach is to use a mobile phase that is as inert as possible but possesses sufficient solubilizing ability to move the enantiomers through the stationary phase. As has been discussed a typical solvent system for the analysis of HODE enantiomers is hexane modified by a small amount of isopropyl alcohol.

Snyder (14) has proposed that solute retention in liquid-solid chromatography on polar solvents can be explained by solvent adsorption onto and displacement from specific sites on the adsorbent by molecules of solute (14). On aminopropyl silica and silica, the amino and silanol groups are fully exposed sites for interaction with solvent. When chiral groups are bonded via a spacer to silica, the amide linkages adjacent to the chiral carbon are exposed for solvent interaction.

According to Snyder (14), the ease of displacement of solvent on the adsorbent by solute will largely determine the retention time of the solute. A decrease in elution time signifies that solvent molecules adsorbed onto active sites of the chiral phase are displaced with greater difficulty by solute molecules.

Care must be taken when selecting solvent systems for cellulose based stationary phases as these polymeric chiral phases are soluble in dichloromethane, chloroform, and tetrahydrofuran. These stationary phases are limited to mobile phases such as hexane, 2-propanol, methanol, and ethanol.

B. Selection of mobile phase

After conversations with Dr. Larry Nicholson, a scientist at The DOW Chemical Company (Midland, MI), the utility of a pentane:methanol mobile phase with either Chiralcel OD or OC chiral stationary phase was investigated. Alcohol modified pentane-based mobile phases were described recently (15, 16). Use of a pentane:methanol mobile phase with a Chrialpak AD chiral stationary phase often yielded improved resolution on enantiospecific separations that could not be obtained with conventional 2-propanol or ethanol modified hexane mobile phases. The mechanism of this enhancement has not been determined as yet. The racemic HODE methyl esters were not resolved on a Chiralcel OC column with this mobile phase, but were baseline resolved on a Chiralcel OD column. When racemic mixtures of the HODEs are spiked with the pure S enantiomer of each, the elution order of the HODEs may be characterized (see Figure 3.2). This order is 13(R), 13(S), 9(R), and 9(S) indicating that the S enantiomer is more retained by the column under these conditions (pentane:methanol 180:1, flow rate = 1 mL/min.).

A flow rate of 1 mL/min. is used so that the 13(S)-HODE ME does not coelute with the 9(R)-HODE ME. Total analysis time for this separation is less that 35 minutes. Flow rates may be increased to shorten the analysis time if the 9- and 13-HODE are analyzed separately as seen in Figure 3.3. An analysis time of 16 minutes for the 9(R,S)-HODE ME results with a flow rate of 2 mL/min. with baseline separation of the enantiomers. For comparison, the data reported in the literature with the DNBPG columns

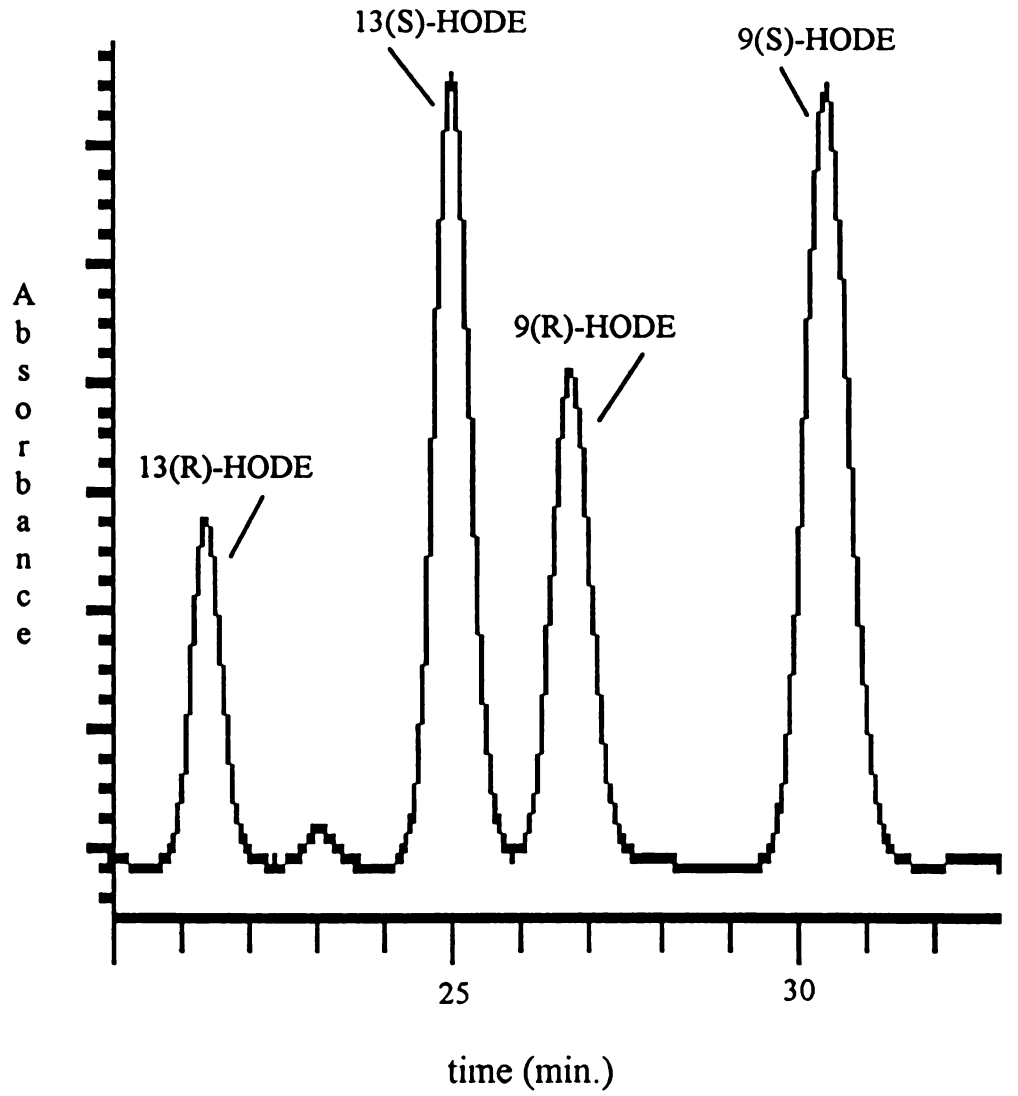

Figure 3.2. Chromatogram resulting from enantiomeric separation of a mixture of 13(R,S)- and 9(R,S)-HODE methyl esters with a Chiralcel OD chiral stationary phase, pentane:methanol 180:1, flow = 1 mL/min.. The methyl esters of 13(S)- and 9(S)-HODE have been added to determine the retention order of the enantiomers.

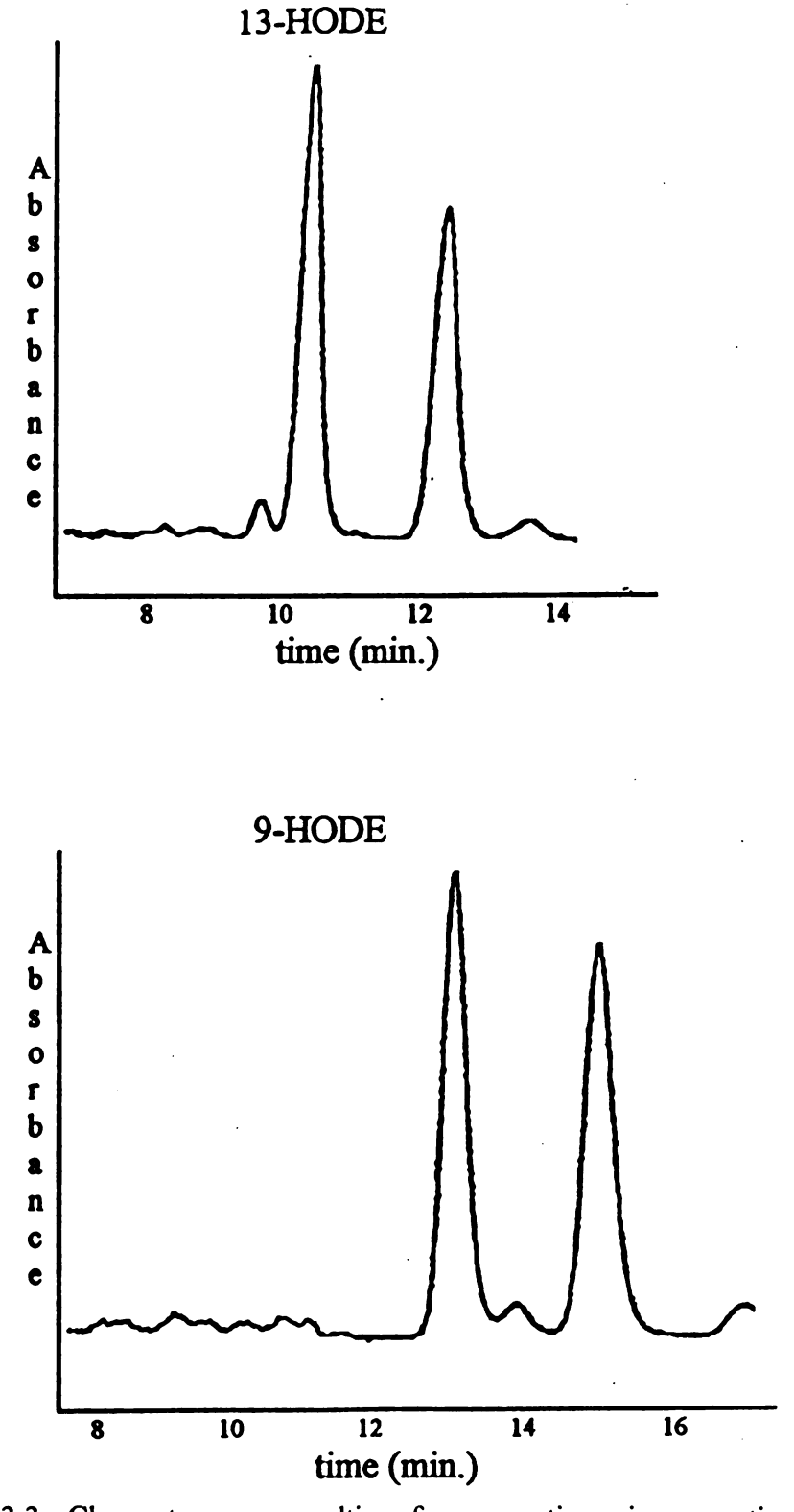

Figure 3.3. Chromatograms resulting from enantiomeric separation of 13(R,S)- and 9(R,S)-HODE methyl esters, Chiralcel OD CSP, pentane:methanol 180:1, flow rate = 2 mL/min..

and hexane:IPA mobile phases have analysis times of 40 to 60 minutes without baseline separation of the racemates (1, 5).

C. Alteration of the mobile phase

Because pentane is a volatile solvent, its use as a mobile phase is plagued with difficulties. The number one difficulty is the introduction of bubbles (even after degassing the solvents) into the HPLC system. In an attempt to circumvent this problem, the utility of a mobile phase consisting of hexane:methanol was investigated. The chromatograms of 13(R,S)- and 9(R,S)-HODE MEs are shown in Figure 3.4 with hexane:methanol 180:1 as a mobile phase and a flow rate of 1 mL/min.. Baseline separation of the racemates with the Chiralcel OD column is obtained. The analysis time is approximately thirty minutes, and the 13(S)- is resolved from the 9(R)-HODE ME.

III. Application of the HPLC conditions to enantiomer separation of other monohydroxylated polyunsaturated fatty acids

To determine the universality of this methodology to the general analysis of monohydroxylated polyunsaturated fatty acid enantiomers, several hydroxyeicosadienoic acid (HEDE) methyl esters; 8-HEDE, 11-HEDE, 15-HEDE (Figure 3.5), and several hydroxyeicosatetraenoic acid (HETE) methyl esters; 5-HETE, 8-HETE, 12-HETE and 15-HETE (Figure 3.6), were successfully analyzed under these chromatographic conditions (Chiralcel OD chiral stationary phase, hexane:methanol 180:1, flow rate = 1 mL/min.). A chromatogram of the photooxidation products of arachidonic acid (Kühn, 1987) is shown in Figure 3.7 as an example of published results utilizing a Pirkle DNBPG column, hexane:IPA 100:0.5, flow = 0.5 mL/min..

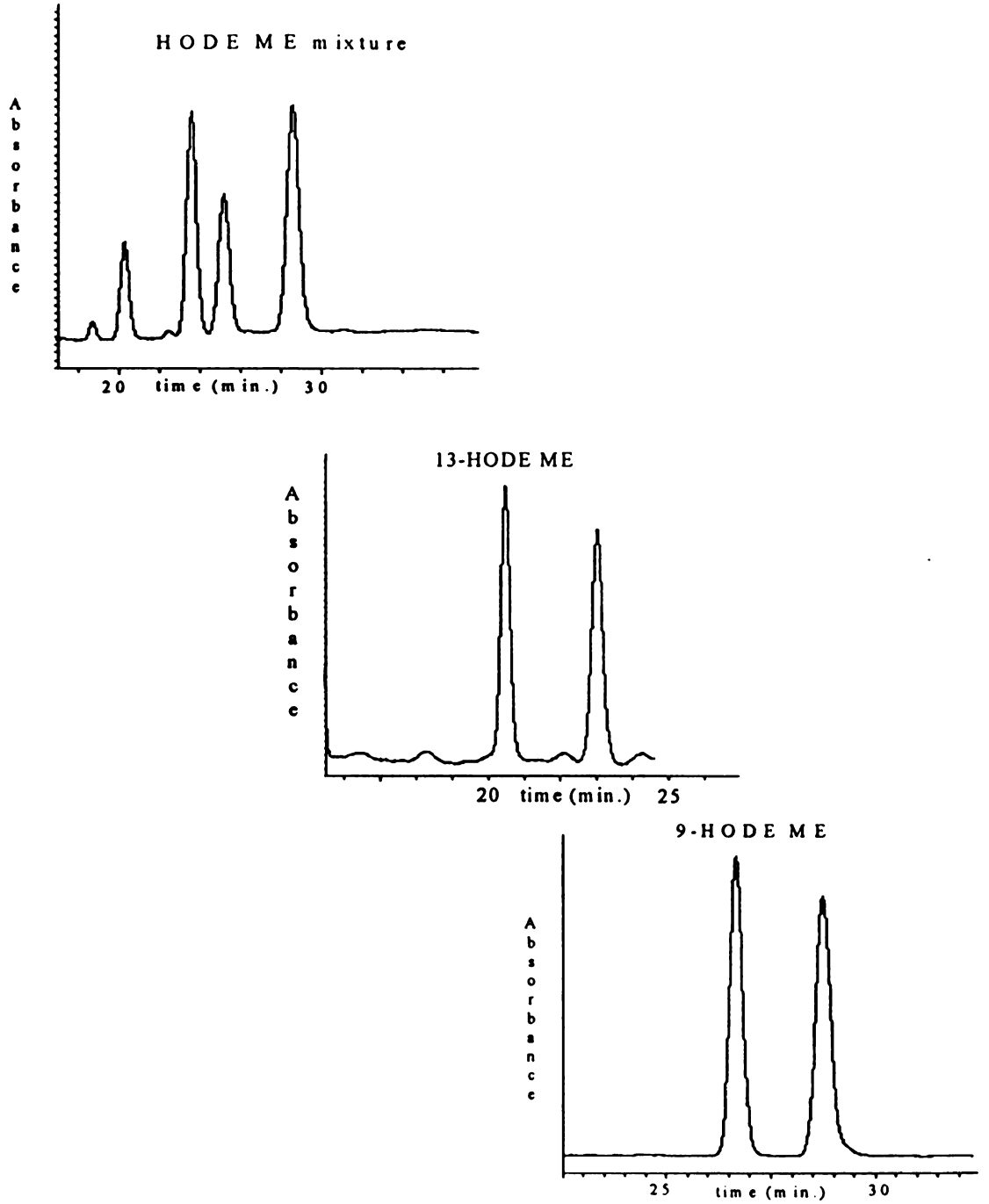

Figure 3.4: 13(R,S)- and 9(R,S)-HODE ME, hexane:methanol 180:1, flow = 1 mL/min.

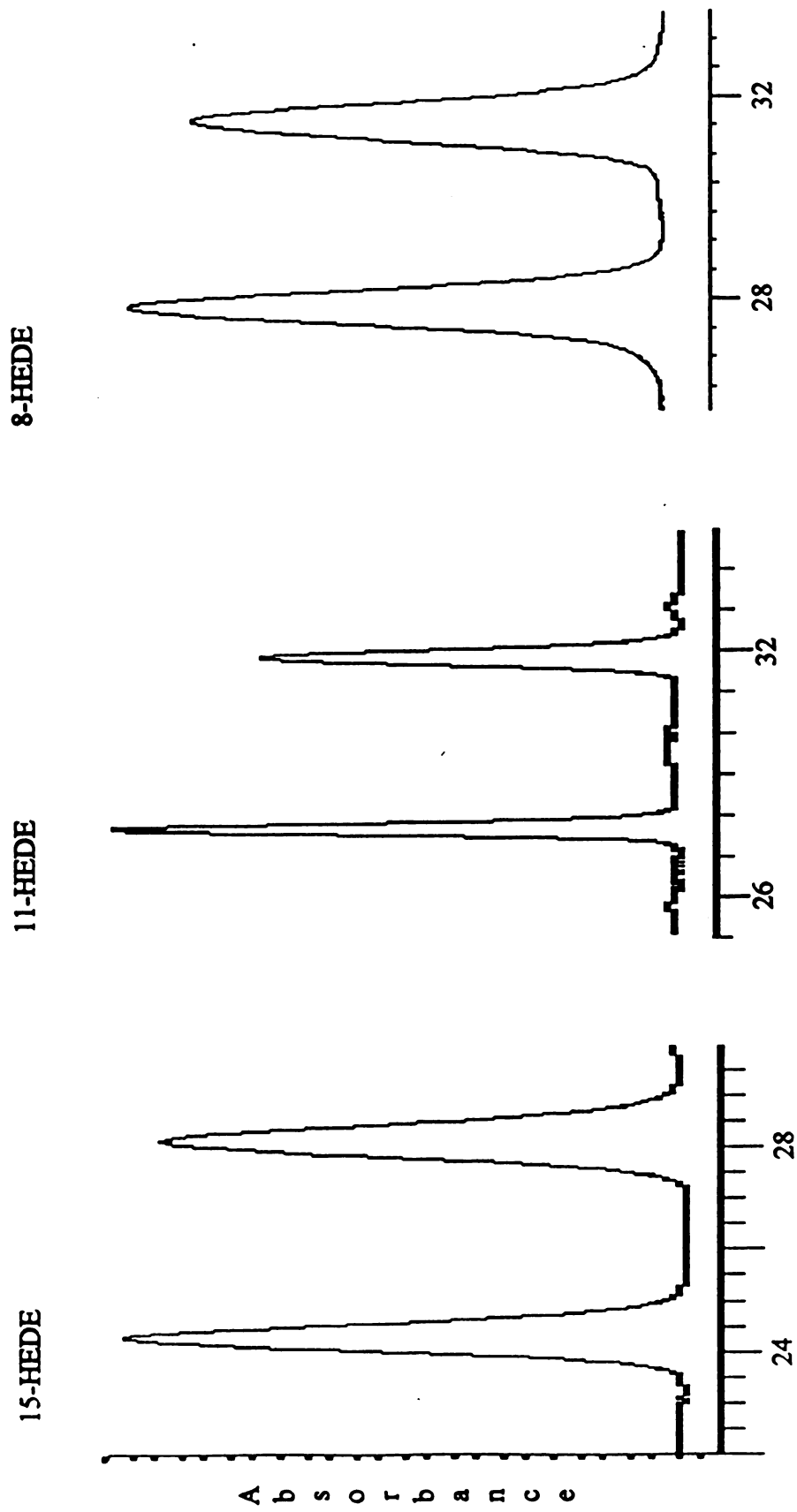

Figure 3.5: Chromatograms resulting from enantiomeric separation of HEDE methyl esters (hexane:methanol 180:1, flow = 1 mL/min.).

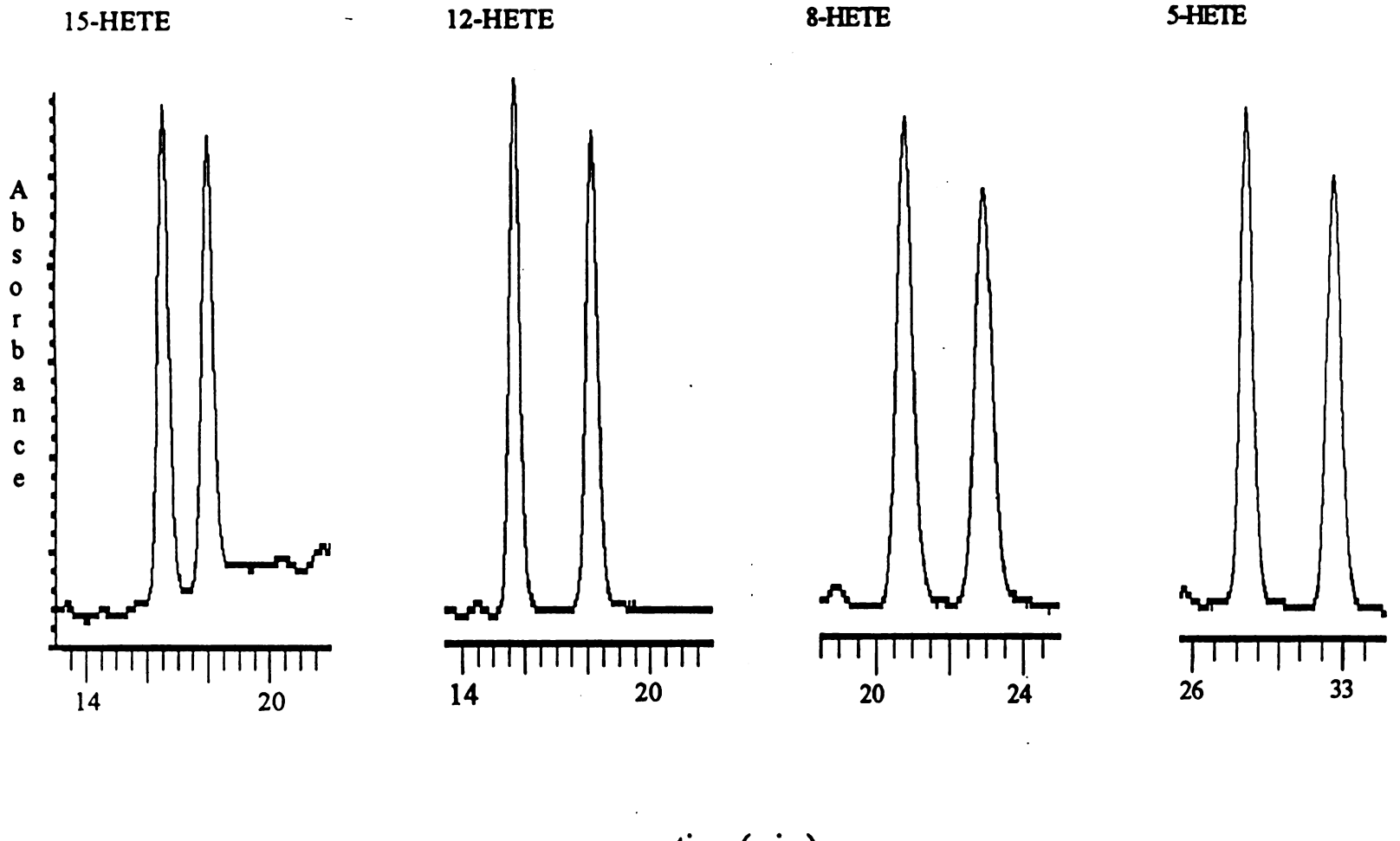

Figure 3.6. Chromatograms resulting from enantiomeric separation of HETE methyl esters (hexane:methanol 180:1, flow = 1 mL/min.).

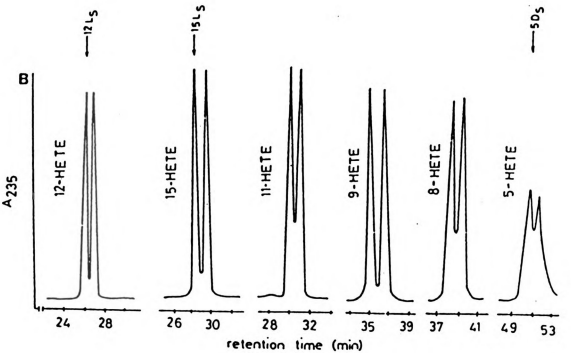

Figure 3.7: Published results for the analysis of HETE ME enantiomers with a hexane:IPA mobile phase and a Pirkle DNBPG chiral stationary phase (1).

This HPLC methodology for the analysis of the enantiomeric composition of monohydroxylated fatty acids is an improvement over published procedures both in enantiomeric resolution and in analysis time. Baseline separation of racemates result with this procedure, providing confidence that the enantiomeric composition of 9- and 13-HODE in mammary gland extracts will be reported accurately.

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Chapter 4: Application of the Extraction Methodology to the Analysis of Mammary Gland Tissue from Mice Fed Variously Controlled Fat Diets

The goals of this research are twofold: 1) develop an improved analytical methodology to isolate, identify, and quantitate 9- and 13-HODE in mammary gland tissue, and 2) to be able to apply this methodology to help to answer biological questions; specifically to determine if the levels of 9- and/or 13-HODE and their enantiomers are higher in the glands from mice fed a diet that is high in linoleic acid. This chapter describes the application of the analytical methodology to the analysis of mammary gland tissue from mice fed diets differing only in the amount and/or type of fat. The tissues were extracted and analyzed by GC/MS to quantitate the concentration of HODEs in the tissue. In addition the enantiomeric distribution of the (R,S)-HODEs was determined by peak area ratios.

I. Experimental

Female Balb/c mice were divided into three groups at three weeks of age. Each group was placed on one of three chemically purified isocaloric diets differing only in the amount and type of fat in the diet. These mice were fed either a high-saturated-fat diet (20% beef tallow by weight), a low-unsaturated-fat diet (5% corn oil by weight), or a high-unsaturated-fat diet (20% corn oil by weight) for five weeks. At the end of the five week period, one mouse from each of the diet groups was sacrificed on the day the tissue was to be extracted to eliminate the storage time variable. Three pairs of mammary glands were excised from the mouse and immediately frozen in liquid nitrogen. All of the glands from each mouse were pulverized in liquid nitrogen and divided into two samples; one for

GC/MS quantitation which was extracted in the morning, the other for chiral analysis which was extracted in the afternoon. Tissues that were not being extracted were stored at -80°.

II. Results

The results for this diet study are listed in Tables 4.1 and 4.2 for 13-HODE and Tables 4.3 and 4.4 for 9-HODE. The concentration of the HODEs in the mammary gland tissue are reported in ng HODE/mg. tissue. The chiral data are represented as a peak area ratio of S/R for 13-HODE, but as the reciprocal R/S for 9-HODE because the R enantiomer was in a greater abundance for most of the samples.

There was enough tissue from one sample from the high-unsaturated-fat diet to extract it in triplicate. The relative standard deviation for 13-HODE was 13%; 9-HODE was 24%.

III. Data analysis and discussion

A. Concentration of HODEs

An analysis of variance for the 13-HODE data from the second diet study shows significance at a 95% confidence level (F_{calc} = 6.4, F_{tab} = 3.5). An ANOVA for the 9-HODE data also indicates significance at a 95% confidence level (F_{calc} = 8.7, F_{tab} = 3.5). Tukey's test on the data from both the 9- and 13-HODE finds no differences between the HODE levels in the glands from mice fed the 20% beef tallow diet and those fed the 5% corn oil diet. However, there are significant differences between levels of 9- and 13-HODE in the 20% corn oil diet and the 20% beef tallow diet and between the 20% corn oil diet and the 5% corn oil diet at a 95% confidence level.

Table 4.1: Concentration and enantiomeric ratios for 13-HODE.

13-HODE

20% Beef Tallow diet		5% Corn Oil diet		20% Corn Oil diet	
ng/mg tissue	area S/area R	ng/mg tissue	area S/area R	ng/mg tissue	area S/area R
0.73	2.23	1.5	4.63	1.6	6.13
0.98	1.46	0.53	2.31	0.54	1.83
1.6	3.32	0.64	4.57	5.9	11.18
0.52	1.15	1.4	3.88	5.4	4.39
0.68	1.15	0.53	3.19	1.6	4.48
1.4		0.64	0.75	0.95	0.54
0.66	1.40	0.45	1.79	2.5	1.54
0.35	1.16	0.79	4.28	3.3	5.25
	2.15		2.01		1.93
	1.01		13.22		1.32

Table 4.2: Summary of concentration data for 13-HODE.

Summary

Groups	Count	Sum	Average
20% Beef Tallow Diet	8	6.9	0.9 ±0.1
5% Corn Oil Diet	8	6.5	0.8 ± 0.1
20% Corn Oil Diet	8	21.6	2.7 ± 0.7

Table 4.3: Concentration and enantiomeric ratios for 9-HODE.

9-HODE

20 % Beef Tallow diet		5% Corn Oil diet		20% Corn Oil diet	
ng/mg tissue	area S/area R	ng/mg tissue	area S/area R	ng/mg tissue	area S/area R
0.42	0.42	1.2	0.26	2.1	0.65
0.91	0.61	0.29	0.36	0.42	1.10
1.2	0.53	0.36	2.86	5.6	0.76
0.085	5.56	0.67	10	4.4	0.54
0.46	2.44	0.38	2	1.6	0.84
0.56		0.25	0.81	0.77	0.88
0.73	0.89	0.14	1.2	2.3	1.49
0.32	0.85	0.48	0.80	2.2	0.51
	0.36		0.73		0.74
	0.85		0.28		0.76

Table 4.4: Summary of concentration data for 9-HODE.

Summary

Groups	Count	Sum	Average
20% Beef Tallow Diet	8	4.7	0.6 ± 0.1
5% Corn Oil Diet	8	3.7	0.5 ± 0.1
20% Corn Oil Diet	8	19.4	2.4 ± 0.6

This study has shown that there are significant differences in the levels of 9- and 13-HODE in mammary gland tissue as a function of the amount and the type of fat in the diet. These numbers may now show biological significance as well as mathematical significance.

B. Chiral analysis

A representative chromatogram from the chiral analysis of the mammary gland tissue extracts may be found in Figure 4.1. The small peaks that are unlabeled have not been identified. The identity of the 9- and 13-HODE enantiomers has been confirmed by coinjection of racemic standards of 9- and 13-HODE. Average values for the enantiomer ratios for 13- and 9-HODE are listed in Table 4.5.

Table 4.5: Summary of chiral data for 9- and 13-HODE.

	20% Beef Tallow diet	5% Corn Oil diet	20% Corn Oil diet
13-HODE (S/R)			
average	1.6 ± 0.2	3 ± 0.5	3 ± 1
9-HODE (R/S)			
average	0.9 ± 0.2	1.0 ± 0.3	0.82 ± 0.09

In calculating the averages for the 13-HODE (S/R) ratios for the 5% corn oil diet and for the 20% corn oil diet and averages for 9-HODE (S/R) for 20% beef tallow diet and the 5% corn oil diet, the largest value in each data set was removed by a Q-test so the above numbers are based on n-1 data points.

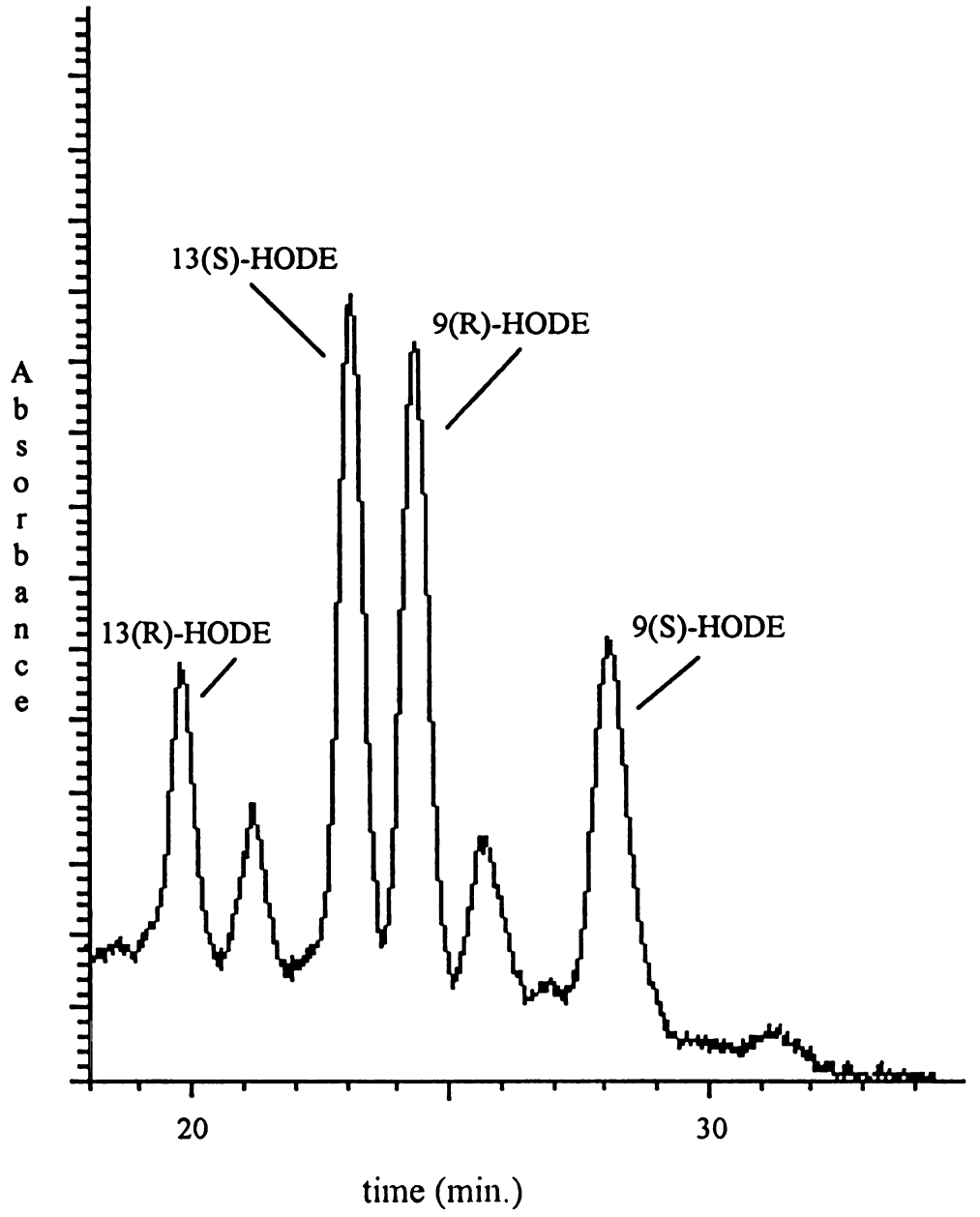

Figure 4.1. Chromatogram resulting from enantiomeric separation of 13(R,S)- and 9-(R,S)-HODE methyl esters from a mammary gland extract.

The data from the chiral analysis of the mammary gland extracts indicates that non-racemic mixtures of the HODEs exist in these tissue samples. The 13(S)-HODE and the 9(R)-HODE are the more abundant enantiomers in these tissues.

Most of the studies reported in the literature have focused on *in vitro* studies of enzymatic oxidation of linoleic acid by various lipoxygenase enzymes. Kühn (1987) investigated the positional and optical isomers formed by various lipoxygenases from linoleic acid. This investigation found that different lipoxygenases formed different ratios of 13:9 HODE as well as different S:R ratio. The results of this study are shown in Table 4.6.

Table 4.6: Composition of the positional and optical isomers formed by various lipoxygenases from linoleic acid (1).

Lipoxygenase	13/9 ratio (%)	13-HODE (S/R)	9-HODE (S:R)
Soybeans I	98:2	97:1	1:1
Reticulocytes	96:4	47:1	1:1
Wheat	15:85	2:1	41.5:1
Pea Seeds I	39:61	1.5:1	1.1:1
Pea Seeds II	89:11	44:1	1.2:1
Tomato fruit	15:85	6.5:1	84:1
Quasi-LOX of hemoglobin	50:50	1:1	1:1

Reinaud et al. (2) reported that the major product of the biotransformation of linoleic acid by human leukocytes is 13(S)-HODE. Glasgow and Eling reported that the oxygenation of Syrian hamster embryo fibroblasts produces the pure (S) enantiomer of 13-HODE with a minor product of 9-HODE (enantiomer not reported) in a 3:1 ratio (3). They also demonstrated that it is the 13(S)-HODE that was active in stimulating DNA synthesis.

Baer et al. has reported an *in vivo* experiment. In this study it was discovered that the principal ***in vivo*** oxygenase products of linoleic acid in psoriatic skin scales are 13-HODE (S/R = 1.9) and 9-HODE (R/S = 2.4) (4). Further ***in vitro*** investigations with cell cultures and radiolabeled linoleic acid produced an enantiopure 13(S)-[^{14}C]-hydroxyoctadecadienoic acid. The difference between the *in vivo* and *in vitro* results was explained as follows (5): the 13-HODE produced *in vivo* was not enantiopure, but was a mixture of stereoisomers with an S/R ratio averaging 1.9. The discrepancy between the pure (S) stereospecificity of the *in vitro*-produced 13-HODE and nonracemic stereospecificity of the *in vivo* compound suggests that there is autooxidation of linoleic acid in the epidermis resulting in a nonenzymatically derived racemic 13-HODE and an enzymatically produced 13(S)-HODE.

Baer's research on *in vivo* and *in vitro* production of HODEs in psoriatic skin may help to explain the results seen in the *in vivo* study with mouse mammary gland tissue. The data obtained with the mouse mammary glands follow the same trend as that for psoriatic skin. 13(S)-HODE was produced in a greater abundance than was the 13(R)-HODE, and the 9(R)-HODE is produced in a greater abundance than is the 9(S)-HODE in both the mouse mammary gland tissue and the human psoriatic skin tissue. The mixtures of enantiomers are non-racemic in the mammary gland tissue, but are not stereospecific for 13(S)-HODE suggesting that there may also be autooxidation in the cells of the mouse mammary gland as was seen in the psoriatic skin.

A study by Kühn (1990) on the oxygenation of biological membranes by reticulocyte lipoxygenase provided some interesting results (6). At low substrate

concentrations, reticulocyte lipoxygenase enzymes led to pure 13(S)-HODE formation. However, a high substrate concentration resulted in loss of the product enantiomeric specificity. A similar decrease in product enantiomeric specificity has been reported for the oxygenation of high concentrations of linoleic acid by the soybean lipoxygenase (7). Dissociation of radical intermediates from the enzyme and then subsequent non-enzymatic reactions with oxygen have been proposed as a mechanistic explanation of this lack of specificity (7, 8, 9).

IV. Summary

This diet study using the validated methodology (including chiral chromatography) has shown that mice fed a diet that is high in unsaturated fat show significantly higher levels of 9- and 13-HODE in their mammary glands (Data are summarized in Table 4.7.) Chiral chromatographic data indicate that 13(S)- and 9(R)-HODE are the more abundant enantiomers in the tissues.

Table 4.7: Summary of the quantitative data from the diet study. [b]All data reported as mean ± standard error of the mean. $P < 0.05$ for c vs. d, c vs. e, and d vs. e.

Diets	Number of Samples	9-HODE (ng/mg tissue)[b]	13-HODE (ng/mg tissue)[b]
20% beef tallow (very low linoleic acid)	8	0.6 ± 0.1[c]	0.9 ± 0.2[c]
5% corn oil (low linoleic acid)	8	0.5 ± 0.1[d]	0.8 ± 0.2[d]
20% corn oil (high linoleic acid)	8	2.4 ± 0.6[e]	2.7 ± 0.7[e]

The results obtained here indicate enzymatic production of the HODEs on top of autooxidation products as evidenced by the equivalent amounts of 9- and 13-HODE and

the non-racemic mixtures of the HODE enantiomers.. This autooxidation most likely occurs within cells primary prior to extraction.

V. Conclusion

An analytical methodology has been developed for the analysis of 9- and 13-HODE in rodent mammary gland tissue. This methodology utilizes gas chromatography mass spectrometry to quantitate the absolute amounts of these metabolites in the tissues. Lipoxygenase oxidation of linoleic acid produces 9-hydroxyoctadecadienoic acid and 13-hydroxyoctadecadienoic acid; these metabolites may also be formed by free radical oxidation of linoleic acid. Analysis of the mammary gland tissue extracts by enantiomeric separation with HPLC on a chiral stationary phase provides a qualitative assessment of the degree of enzymatic vs. non-enzymatic production of the HODEs.

This methodology has been applied successfully to analyses of mouse mammary gland tissues to assess the influence of diets consisting of different amounts and/or types of fat on the production of HODEs. Significant differences were seen among the levels of the HODEs in mammary gland tissue from mice in different diet groups. The enantiomeric distribution of 9(R,S)- and 13(R,S)-HODE in mammary gland tissue as a function of diet indicates that there is a combination of factors contributing to the significant difference seen.

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