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THE CHEMICAL MODIFICATION OF BIOLOGICALLY ACTIVE ALLYLIC HYDROXY FATTY ACIDS FOR THEIR SELECTIVE DETERMINATION BY ELECTRON CAPTURE

presented by

LINDA CAROL DOHERTY

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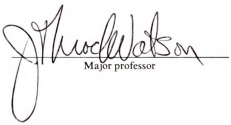

Major professor

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THE CHEMICAL MODIFICATION OF BIOLOGICALLY ACTIVE ALLYLIC HYDROXY FATTY ACIDS FOR THEIR SELECTIVE DETERMINATION BY ELECTRON CAPTURE

By

Linda Carol Doherty

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ABSTRACT

THE CHEMICAL MODIFICATION OF BIOLOGICALLY ACTIVE ALLYLIC HYDROXY FATTY ACIDS FOR THEIR SELECTIVE DETERMINATION BY ELECTRON CAPTURE

By

Linda Carol Doherty

Increases in certain allylic hydroxy fatty acids have been associated with diseases such as psoriasis, asthma and cancer. These acids are present in very low concentrations in biological samples so that sensitive analytical methods are required for detection. Some of the most important allylic hydroxy fatty acids are the metabolites of the lipoxygenase enzyme oxidation of eicosatetraenoic (arachidonic) acid. The hydroxy acids formed are called hydroxyeicosatetraenoic acids or HETEs. The HETEs have a characteristic allylic hydroxy moiety; it is this structural feature that has been exploited in this research project.

The objective of this work was to investigate whether these lipoxygenase metabolites of arachidonic acid could be selectively modified, by chemical means, to a compounds with improved chromatographic properties over those of the parent compound. The allylic alcohols of the lipoxygenase pathway were readily oxidized to unsaturated ketones, a distinguishing structural feature that was exploited for analytical advantage. Also, the chemical modification was designed to add electrophilic character to the analyte so the highly sensitive technique of electron capture negative ionization mass spectrometry could be used. The modification strategy selectively converted the analyte, by virtue of its structural features, to a compound with greatly improved stability thereby avoiding some of the problems of degradation and isomerization associated with the analysis of lipoxygenase metabolites.

The allylic hydroxyl was oxidized with a mild oxidizing agent to the α, β–unsaturated ketone. Reagents such as manganese dioxide, dichlorodicyanobenzoquinone and pyridinium dichromate favored the oxidation of allylic alcohols over compounds containing isolated hydroxyl groups. This oxidative specificity discriminated against the chemical background which could not be volatilized or transformed into an electrophilic species. This methodology was developed as an alternative to procedures involving the general derivatization scheme typically employed for hydroxy acids.

The production of the allylic alcohols and the refinement of the oxidation are discussed in detail. The improved oxidation procedure was applied to a sample of islet cells and the results compared with a method that employed conventional derivatization techniques to illustrate the potential usefulness of the proposed chemical modification procedure. Finally, other lipoxygenase metabolites are introduced and their determinations by conventional gas chromatographic-mass spectrometric and high performance liquid chromatographic techniques demonstrated.

To my family:

Dave and Doris, my parents;

Lesley, my sister and Tom, my loving husband

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Finally, I want to extend my deepest appreciation to those people that made it possible for me to attain this goal. To my parents Dave and Doris, thank you for letting me determine my own career and for always showing your love and approval when it was most important to me. To my sister, Lesley, thank you for helping me to grow into a responsible person and keeping me in touch with the latest fashions. To my husband Tom, thank you for helping me realize that I am a capable scientist and for showing your love and patience when it was most needed.

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KEY TO ABBREVIATIONS

AA	arachidonic acid
20:4	eicosatetraenoic acid
HPETE	hydroperoxyeicostetraenoic acid
HETEs	hydroxyeicosatetraenoic acids
5-HETE	5-hydroxy-6, 8, 11, 14-eicosatetraenoic acid
12-HETE	12-hydroxy-5, 8, 10, 14-eicosatetraenoic acid
15-HETE	15-hydroxy-5, 8, 11, 13-eicosatetraenoic acid
oxo-ETEs	oxo-eicosatetraenoic acids
15-oxo-ETE-ME	15-oxo-5, 8, 11, 13-eicosatetraenoic methyl ester
15-HEA	15-hydroxyeicosanoic acid
ME	methyl ester
TMS	trimethylsilyl ether
PFB	pentafluorobenzyl ester
DDQ	dichlorodicyanobenzoquinone
PDC	pyridinium dichromate
HODDs	hydroxyoctadecadienoic acids
BSTFA	N, O-bis-trimethylsilyl-trifluoroacetamide

18:2	octadecadienoic acid
13-HODD	13-hydroxy-9, 11-octadecadienoic acid
oxo-ODDs	oxo-octadecadienoic acids
13-oxo-ODD-ME	13-oxo-9, 11-octadecadienoic methyl ester
PGB_1	prostaglandin B_1
15-oxo-PGB_1	15-oxo-prostaglandin B_1
RIA	radioimmunoassay
HPLC	high performance liquid chromatography
GC-MS	gas chromatography-mass spectrometry
AUFS	absorbance units full scale
EI	electron impact
CI	chemical ionization
ECNI	electron capture negative ionization
ECD	electron capture detector
FID	flame ionization detector

CHAPTER I: INTRODUCTION

The objective of this research project was to develop an assay for allylic hydroxy unsaturated fatty acids; specifically those formed from the reaction of arachidonic acid with lipoxygenase enzyme (Figure 1-1). This reaction produces hydroxyeicosatetraenoic acids (HETEs). The assay incorporated the knowledge that allylic hydroxy moieties can be selectively oxidized to α, β–unsaturated ketones, and that α, β–unsaturated ketones are electrophilic. It was expected that the oxidized species would be responsive to electron capture negative ionization (ECNI) mass spectrometry (MS) which had shown success previously for other α, β–unsaturated ketones (1). Also, it was anticipated that the oxidation reaction would be a discriminating alternative to sample derivatization, greatly reducing background interferences.

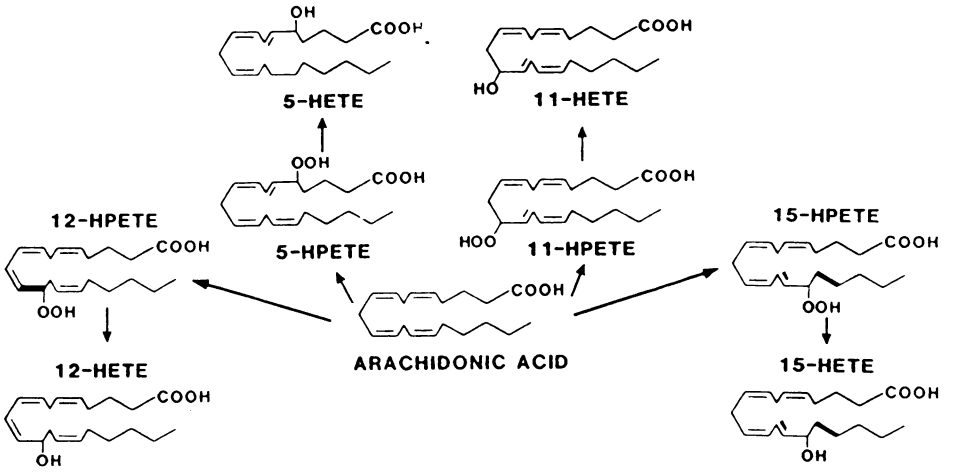

Figure 1-1. The various lipoxygenases that metabolize arachidonic acid. (source: *The Leukotrienes; chemistry and biology* **1984**, L. W. Chakrin and D. M. Bailey eds., Academic Press: Orlando, p. 198).

The reason for the development of this assay was to have a simple, easy method for determining the concentration of HETEs in biological samples. It was imperative that the assay be sensitive, due to the low concentration (high potency) of the analyte. Also,

the technique had to be applicable to a variety of biological fluids. It was hoped that the prepared oxidized compound would increase the stability of the analyte. Finally, the technique would have to be reliable, allowing for reasonable, reproducible recoveries (>80%) of the analyte.

As can be seen in Figure 1-2 the release of arachidonic acid, from the phospholipids, leads to its transformation by two enzymes, cyclooxygenase and lipoxygenase. Specifically, the reaction of 5-lipoxygenase with arachidonic acid produces 5-hydroperoxyeicosatetraenoic acid (5-HPETE) that is reduced to 5-HETE (Figure 1-3). 5-HPETE is primarily shunted to form the epoxide leukotriene A_4 (LTA_4) which is converted to the peptido-leukotrienes (peptido-LTs) LTC_4, D_4 and E_4 or the dihydroxy eicosanoids- the LTB_4s. The basis for this project was that if the presence of 5-HETE was known, the presence of leukotrienes could be assumed. The validity of this stems from the fact that 5-HPETE is a precursor to both compounds and although most of the 5-HPETE would be shunted to LTA_4 some would be non-enzymatically reduced to 5-HETE. Thus monitoring for 5-HETE would give some preliminary information about whether the 5-lipoxygenase pathway was activated. If results from a quick test were positive; a more rigorous analysis of a sample could be done to determine the exact leukotrienes present. Other samples might contain only HETEs (12-HETE, 15-HETE, etc.), but the same oxidative methodology could be used for their analysis. The molecule 15-HETE was used in these experiments to determine the feasibility of methodology developed.

The ability to analyze biological fluids for HETEs and LTs is important for the determination of the etiologies of many inflammatory and hypersensitivity diseases (e.g., asthma (2, 3), psoriasis (4, 5), acute arthritis (6) and bowel inflammatory disease (7)). One of the physiological effects of asthma (6, 8, 9), bronchoconstriction, has been associated with the presence of the peptido-LTs: LTC_4, D_4 and E_4. In another example,

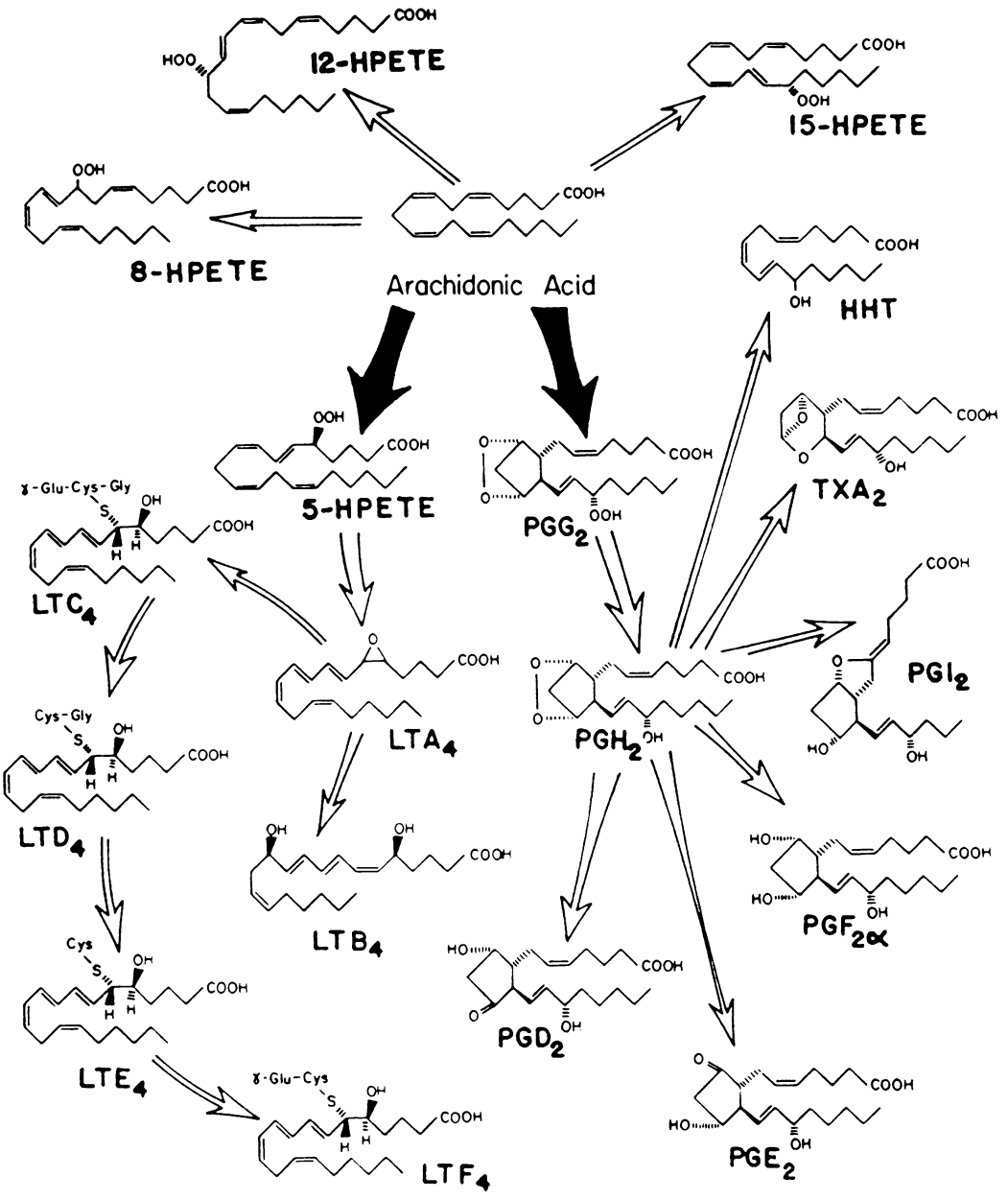

Figure 1-2. The arachidonic acid cascade. The large arrows are the enzymes cyclooxygenase (right) and 5-lipoxygenase (left). (source: *The Biochemistry of Arachidonic Acid Metabolism* **1985**, W. E. M. Lands ed., Martinus Nijhoff Publishing: Boston).

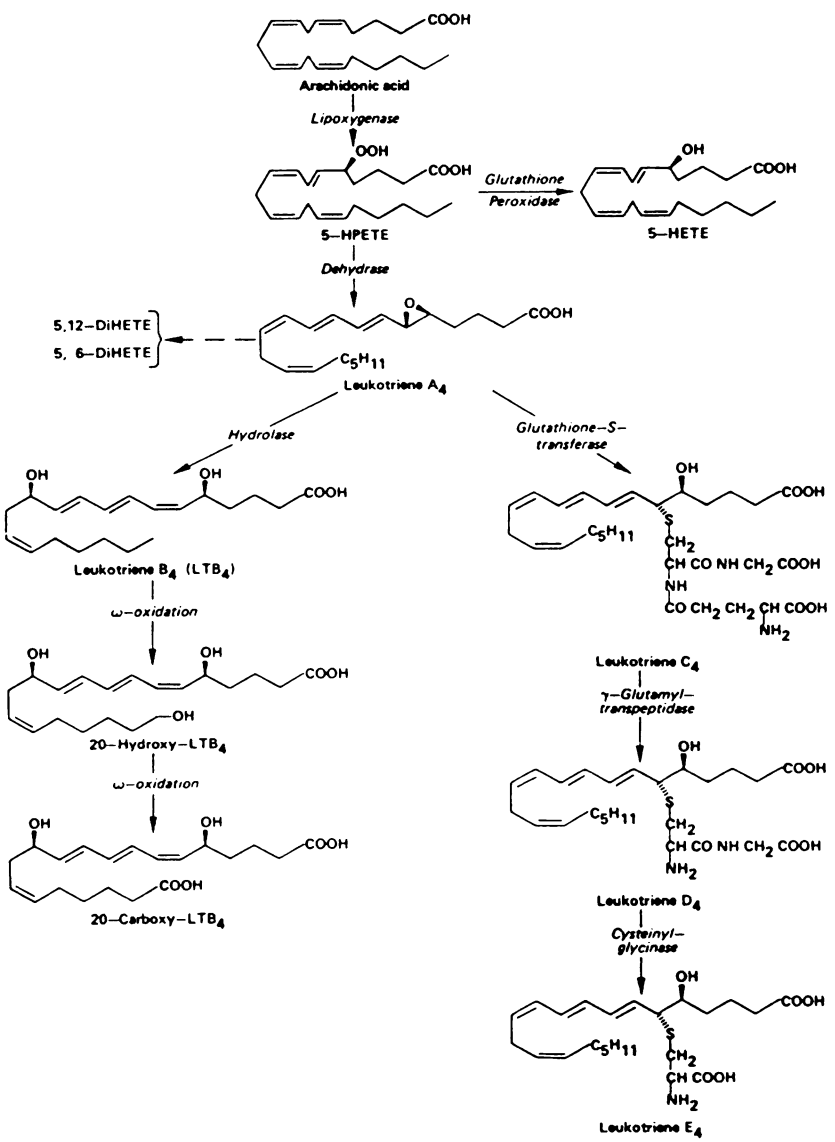

Figure 1-3. The 5-lipoxygenase pathway showing the formation of the various leukotrienes. source: *The Leukotrienes; chemistry and biology* **1984**, L. W. Chakrin and D. M. Bailey eds., Academic Press: Orlando, p. 199).

infiltration of neutrophils (granular leukocytes) in the fluid of psoriatic lesions is associated with a high concentration of LTB_4, as is the fluid from acutely inflamed arthritic joints (6, 8, 9, 10). Psoriatic lesions are also known to contain high concentrations of 12-HETE and 13-hydroxyoctadecadienoic acid (13-HODD) (5). Figure 1-4 shows the effects of selective lipoxygense eicosanoids on a polymorphonuclear (PMN) leukocyte. [The leukocyte is responsible for the removal of foreign substances (e.g., bacteria) by ingesting them (phagocytosis)].

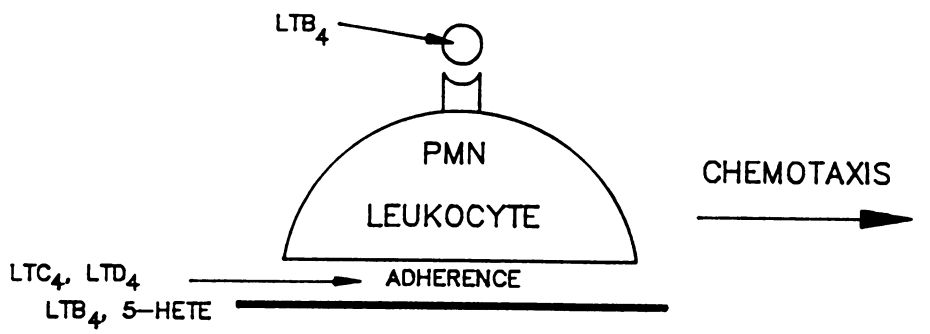

Figure 1-4. The effects of assorted arachidonic acid lipoxygenase products on a polymorphonuclear (PMN) leukocyte (a white blood cell).

By having analytical methods available to ascertain the concentration of eicosanoids, not only can certain eicosanoids be associated with diseases, but the efficacy of drugs or inhibitors developed against these diseases can be improved. In order to determine whether lipoxygenase products of arachidonic acid play a role in the mediation of the effects of any disease, inhibitors of the enzymes involved in the production of the lipoxygenase products had to be designed and evaluated (11, 12). By selectively blocking the production of arachidonic acid metabolites (from both cyclooxygenase and lipoxygenase), changes in the condition of the diseased tissue may be linked to certain eicosanoids. The amount of the eicosanoid is measured before and after the inhibitors are added to see how the inhibitor affected the enzyme it was supposed to block. The

determination of which cells promote the production of inflammatory mediators and which enzymes are activated in a certain disease, would lead to a better understanding of an inflammatory reaction. The increased knowledge could possibly lead to the design of drugs that more effectively eliminate inflammation. Some inhibitors of the arachidonic acid cascade are shown in Figure 1-5.

A problem with the identification of the lipoxygenase eicosanoids is that they are potent mediators of inflammation that are considered to be autacoids (compounds produced endogenously with a short half-life and local biological stimulus) (13). Thus, they are are labile and of low concentration in normal amounts of biological fluids (50-100 pmoles). It was necessary that analytical methods be developed for the analysis of each of these molecules taking into consideration their potency. Originally, assays of muscle tissues were used to detect eicosanoids, because eicosanoids have the ability to induce muscle contraction. As will be presented in chapter II, muscular bioassays are not specific for a certain analyte and do not give reliable information for any samples except standards. Radioimmunoassay (RIA) has also proven ineffective in reliably determining the presence of lipoxygenase metabolites of arachidonic acid in biological fluids. Cross-reactivity of the antiserum with structurally-similar molecules will cause responses not representative of the true analyte concentration. Only when HPLC has been used as a preliminary purification step, has RIA data agreed with gas chromatography-mass spectrometry (GC-MS) data. Because GC-MS is a reliable technique, many current eicosanoid analytical methodologies have been developed to be used with this tool. The differences in eicosanoid structure, as can be seen in Figure 1-3, make the development of GC-MS methodology for one eicosanoid not adequate for another compound. These structural differences require derivatizations and modifications unique to each analyte. Thus, the concerted effort of this research was to develop a GC-MS technique primarily for the mono-HETEs and possibly LTB_4. The peptido-LTs were not considered because they lack the structural moiety that this research was based on, the allylic alcohol.

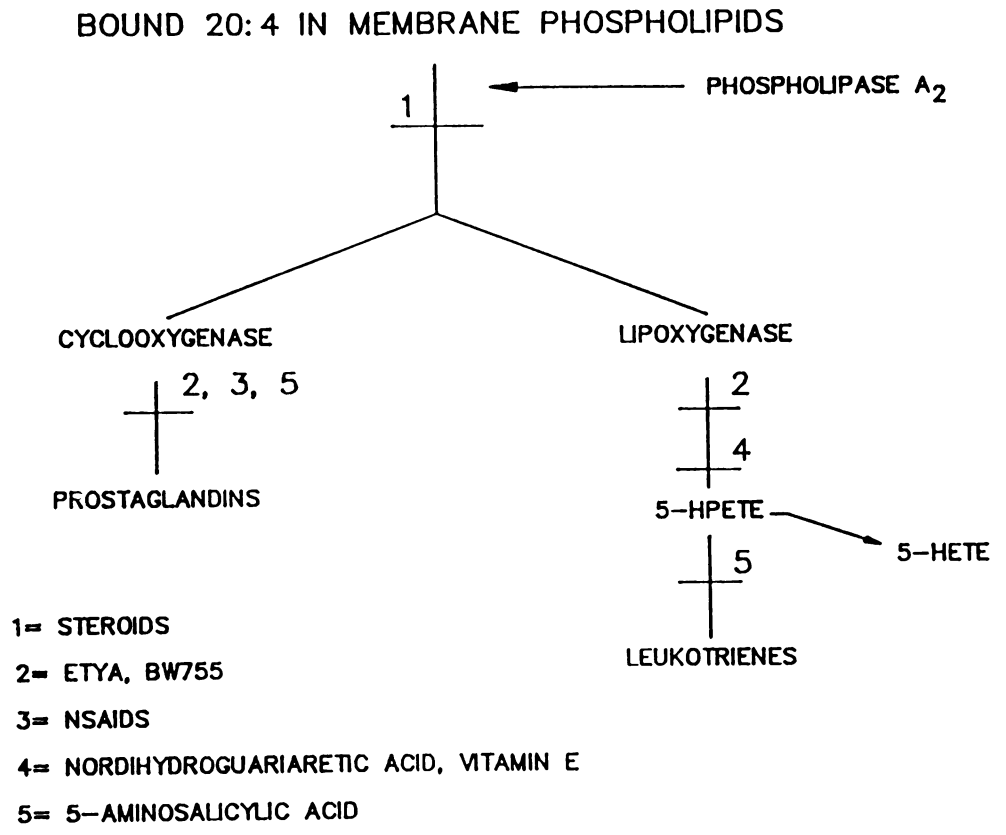

Figure 1-5. Inhibitors of the arachidonic acid cascade. The horizontal lines are where the inhibitor works. (20:4= arachidonic acid, ETYA= eicosatetraynoic acid, BW775= a Burroughs-Welcome competitive inhibitor, NSAIDS= nonsteroidal antiinflammatory drugs).

In order to develop a realistic GC-MS method, the fact that the analyte concentration would be low and the biological matrix complex had to be considered . Also, the analyte would have to be converted to a more stable structure. So, the metabolism of the analyte would have to be well understood to assure that the determination of the chosen metabolite was representative of the *in vivo* concentration (14). Possible degradation of the analyte *ex vivo* would cause it to be an undesirable choice as a product to be measured. This is one of the reasons why we chose to monitor the compound 5-HETE as a measure of the activity of 5-lipoxygenase. Although the LTs are usually of more interest, because they are more potent, only LTB_4 shows little degradation *ex vivo*. The peptido-LTs degrade quickly by loosing amino acid residues (15). Monitoring LTC_4 for the peptido-LT concentration may not be valid if most of the LTC_4 has actually degraded to LTE_4. Recently, methodology developed for the analysis of peptido-LTs has taken this degradation into account by converting all of the peptido-LTs to the same molecule (16). The actual procedure is discussed in chapter V.

Another consideration in the analysis of inflammatory fluids is the way in which they were obtained. Many methods of obtaining a sample may actually cause the concentration of inflammatory mediators to increase. If cells are injured during sample collection, lipoxygenase products of arachidonic acid may be formed. This obviously leads to incorrect determinations of *in vivo* concentration of the analyte. Preferable techniques would involve collection of fluids local to the site of inflammation without scraping or puncturing the skin or cell membranes.

Finally, the analysis must exhibit sufficient sensitivity, specificity and selectivity. Although bioassay and RIA are quite sensitive, specificity and selectivity are poor. However, with GC-MS selectivity is obtained in the form of molecular weight, structural fragmentation and retention time information for the analyte. Sensitivity can be increased by changing the ionization technique. Thus, GC-MS is a flexible instrumental

technique. The details of GC-MS are covered in chapter II.

The methodology that was developed, taking into account many of the above points, was a selective oxidation of the allylic alcohols of the lipoxygenase pathway from the substrates octadecadienoic acid, 18:2 (18 carbons, two double bonds) and eicosatetraenoic acid, 20:4 (20 carbons, four double bonds). The compounds formed were the hydroxyoctadecadienoic acids (HODDs) and HETEs, respectively. Since the alcohols are allylic, selective, mild oxidizing reagents were used for the transformation of the HETEs and HODDs to oxo-ETEs and oxo-ODDs, which are α, β–unsaturated ketones. The benefit of this method was that the ketones formed were electrophilic; thus, electron capture detection for GC or electron capture ionization for MS could be used. These techniques are known for their high sensitivity. The low concentration of analyte would be balanced by the high sensitivity of the detection method.

This methodology has been applied to the standards of 5- and 15-HETE. The improvement of volatility of the the oxidized compound over the original molecule made it unnecessary to derivatize the ketone moiety. The induced electrophilicity through chemical modification made the procedure quite selective because no general electrophilic derivative was required. Also, since the bulk of the biological matrix would not contain allylic alcohols, it should not be transformed into electrophilic α, β–unsaturated ketone molecules. This made the methodology specific for the analyte by decreasing the interference from the sample matrix. Unfortunately, by choosing this structure modification over general derivatization, we have decreased the potential detection limits for the analyte. The oxidized HETEs and HODDs are not as electrophilic as halogen-containing derivatives. The reduction of background interference tailors the use of this technique for samples that contain potentially interfering compounds with concentrations that are much larger than that of the analyte. Although they might be modified, it would be unlikely that they would oxidized to α,

β–unsaturated ketones and, thus, would not be responsive to electron capture.

It was crucial to discern the role that the oxo-ETEs played in the metabolism of arachidonic acid so that we could be sure that using them as a representation of the HETE concentration was valid. Skoog *et al* (17) reported the presence of 5-oxo-ETE in samples in an anaerobic environment. This was confirmed through earlier reports of the formation of 13-oxo-ODD (18) in an anaerobic atmosphere. Bryant *et al* have found 5-oxo-ETE is produced in monoclonal (MC)-9 mast cell cytosol (19). Fruteau de Laclos *et al* have recently reported the presence of 12-oxo-ETE in human platelets (20) They believe the transformation to be the heme-catalyzed degradation product of 12-HPETE and not necessarily related to presence of oxygen. Skoog and Fruteau de Laclos both determined the degradation of the HETE to oxo-ETE to be approximately 10% of the total HETE concentration. By oxidizing the remaining 90% of the HETE present, we would be able to assess the total HETE concentration regardless of the oxygen content in the atmosphere or existence of heme.

The success of the developed methodology in a biological matrix has been examined in samples of 12-HETE from islet cells. The preliminary results of these experiments determined this oxidation to be a reasonable alternative to the current derivatization methods for the HETEs (16). Future experiments by other graduate students will offer more insight into the feasibility of this methodology for general biological samples with complex matrices, e.g., lipid extracts from mammary tumors.

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CHAPTER II: CONVENTIONAL METHODS OF ANALYSIS

Eicosanoids have been determined by four analytical techniques. They are bioassay, radioimmmunoassay (RIA), high performance liquid chromatography (HPLC) and mass spectrometry (sometimes accompanied by gas chromatography). These methods are quite different in their sensitivity and specificity. The most desirable technique would have both a low detection limit and high specificity. As will be pointed out each method has its advantages, but a combination of analytical techniques usually affords the researcher with both of the desirable qualities.

This chapter will briefly address the methods of bioassay and RIA since they were not used in this research. The important development of specific HPLC methods will be considered in the chapters on model compound preparation and collaborative projects, so only general aspects of eicosanoid HPLC will be discussed. The thrust of the chapter will be in the area of mass spectrometry, where the figures of merit of the available ionization methods and their derivatization requirements will be compared with the procedures of sensitivity and selectivity.

The early finding that spurred interest in lipoxygenase eicosanoid analysis was the reaction of guinea pig ileum to the infusion of snake venom (1). The unusual muscular response from the unknown compounds in snake venom, as compared to histamine, gave them the label of slow reacting substances (SRS). After discovering the existence of SRS compounds, great effort was made to determine their structure. In 1979, Murphy, Hammarstrom and Samuelsson determined the structure of leukotriene C_4 (LTC_4) (2, 3). Further discoveries that the precursor of leukotrienes, 5-HPETE and other HETEs were present in many mammalian tissues, has given researchers new insights into diseases that thus far have had unknown origin.

Many quantitation procedures for the various lipoxygenase eicosanoids have been

modelled after methods originally developed for prostaglandins. The structures of the molecules have similar features, and sensitivity requirements for analysis are approximately the same. Since the lipoxygenase eicosanoids are potent mediators of the inflammatory response, their concentrations in biological fluids are very small. This requires analytical techniques to be sensitive at concentrations that are much less than one microgram/milliter.

BIOASSAY

The technique of bioassay is the most commonly used method for eicosanoid determination. The assay uses a muscle tissue to determine the biological response (activity) of a compound. Different types of muscle tissue are used for eicosanoid bioassays. SRS compounds are determined with guinea pig ileum strips. Lung parenchymal strips of guinea pigs are used for the measurement of LTB_4 because its contractile effects are much smaller than the peptido-LTs. The tissue is kept viable in a buffered solution and the leukotriene is periodically pumped into the buffer. The contraction of the muscle is determined through a strain gauge which is connected to a transducer. The response of the transducer is recorded in mV per minute. Each eicosanoid gives a unique response with respect to concentration and time. Histamine is used as a standard for comparison.

Bioassay cannot be used to determine the concentrations of specific analytes in bulk unknown samples, but it can be used to determine picomolar concentrations of pure, synthetic LTs and HPLC purified LTs (5). The temporal response of the tissue will only confirm the presence of a lipoxygenase eicosanoid. Bioassay is non-specific; i.e., non-eicosanoid compounds may give similar responses. The technique also requires some knowledge of tissue preparation. The benefits of bioassay lie in its ability to determine the presence of labile compounds in real time. The immediate degradation of compounds can be determined by a series of tissue baths. It can also be used to determine the chiral

centers of new compounds that have been synthetically prepared because only one enantiomer will have the proper conformation to elicit biological activity.

RIA

The technique of RIA has been used quite successfully in the area of eicosanoid measurement. Detection limits as low as 1 picogram are reported, thus RIA is a sensitive method for analyte measurement. Unfortunately, it does have selectivity problems due to the cross-reactivity between molecules of similar structures. This problem is common for RIAs developed for peptido-leukotrienes (LTC_4, LTD_4) and the specificity of antiserum will vary.

Antiserum is prepared by covalently bonding the eicosanoid to a large protein like bovine serum albumin (BSA) (6). The eicosanoids are not, by themselves, antigenic and are called haptens. In eicosanoid RIA, the most common linkage is between the carboxyl group of the eicosanoid and amino groups on the protein. The physical location of the covalent bond of the hapten to the BSA is critical. Different locations of bonds may inactivate the hapten, and antiserum may not be raised against the antagonist. The type of bonding may also affect the cross-reactivity of the antiserum if specific groups on the molecule are bound. If a chemical group that makes related molecules differing from one another is hidden, these compounds will take on structural similarity.

The problem of cross-reactivity is best understood by the example of the development of an antiserum for LTC_4. There are many compounds that have very similar structures to LTC_4 such as LTD_4, E_4, F_4, as well as some degradation products, the leukotriene sulfones. Coupling the peptido-LT to BSA with the fatty acid carboxyl group results in antiserum which does not differentiate between the peptido-LTs. However, coupling the BSA to the LT through the amino group from the peptide chain in the LT increases specificity. Surprisingly, a most successful antiserum was made by

coupling polyamino-bovine serum albumin to acetylated LTC_4 (7). This antiserum gave cross-reactivities to other peptido-LTs of <0.5%. As a comparison many other antisera have cross-reactivities of more than 20%.

After the antiserum is prepared, the radiolabelled antigen must be made. In most LT and HETE procedures tritiated standards are used. This labelling is accomplished by ^3H_2O exchange and the standards are not completely stable. Other radiolabelling techniques have been based on the incorporation of ^{125}I or preparation of ^3H-methyl esters (8). RIA determines the inhibition of the binding of the radiolabelled species to the antiserum. This is caused primarily by the presence of the unlabelled species. However, the binding of the radiolabelled compounds may be hindered by pH differences and protein concentrations. In the special case of eicosanoids, high concentrations of other fatty acids may cause cross-reactivity lowering the binding ability of the radiolabelled species and yielding an incorrect, high concentration response for the unlabelled analyte. These possibilities make RIA less reliable for complex samples like plasma and serum (6). RIA does have the advantages of low detection limits and high sample throughput. It is most useful when samples have been purified by HPLC (9,10). This eliminates ambiguous results. Quantitation by mass spectrometry can be used to confirm RIA results.

HPLC

HPLC has been the most widely used method of analysis for the lipoxygenase eicosanoids. This is probably due to, in part, the fact that most laboratories are equipped and familiar with a liquid chromatograph. The development of a selective and sensitive method for detection of HETEs and LTs has been primarily accomplished with UV absorbance detectors. Both normal phase (NP) and reverse phase (RP) techniques may be used for the HETEs as well as LTB_4 but the sulfidopeptidyl LTs can only be analyzed by RP-HPLC because of their solubility requirements. A recent review by W.S. Powell

of general HPLC techniques for eicosanoids is found in *Biochemistry of Arachidonic Acid Metabolism* (11).

HPLC has been used as a subsequent purification step in the preparation of all of the eicosanoids. The isomers of the different photooxidation products of linoleic and arachidonic acids have been purified by NP-HPLC which is discussed in Chapter III. The sulfidopeptidyl LTs need continuous purification prior to use in any experiments due to their chemical instabilites. The decomposition of LTC_4 is well-known (12) to occur within hours of biological production if precautions are not taken. Special additives to the mobile phase decrease the rate of decomposition. The development of a suitable aqueous mobile phase and the decomposition of peptidyl LTs is covered extensively in chapter V.

To reduce the complexity of the liquid chromatographic analysis of biological fluids, extensive sample preparation is required. The most widely used procedures are those employing C_{18} Sep-Pak cartridges. Powell (11) has developed a sequential elution system which utilizes a C_{18} Sep-Pak and separates all eicosanoids into their specific groups by changing the polarities of the flushing solvents. These cartridges also remove salts and denatured proteins that might cause interferences in the HPLC analysis. It is also important that all samples and standard solutions are passed through 0.45 μm pore filters. These steps will remove fine particulate and proteins that may clog frits in the column and decrease the its lifetime.

As already stated, the evaluation of the selectivity and sensitivity of HPLC as an analytical method for eicosanoids is based on the use of UV detector. The mobile phase may be optimized for the best separation but the lowest obtainable detection limits are determined by the quality of the HPLC system and the molar absorptivities (ε) of each LT or HETE at their λ_{max}. The HETEs have a λ_{max} at 235 nm (ε=30,000), LTB_4 at 270 nm (ε=50,000) and the peptido-LTs at 280 nm (ε=40,000). Since each compound

has its own λ_{max}, the selectively of HPLC is relatively high. Most mobile phase programs are designed to elute LTs and HETEs from biological samples sequentially according to their polarity so there is no overlap of the components and the wavelength of the detector may be changed during the analysis from 280 nm to 235 nm. Detection limits for each of the compounds varies and is greatly dependent on noise from the reciprocating solvent pumps. For example, experimental data showed that the analysis of LTB_4 and the peptido-LTs at 280 nm produced detection limits of 2 ng and 4 ng, respectively. Detection limits of less than one ng could have been achieved with a different system that had quieter pumps (more pulse dampening). Details of these findings are highlighted in chapter V.

A recent publication (13) gave a new RP-HPLC separation which employs fluorescence as a detection method. Fluorescence has been shown to improve the detection limits of compounds over those achieved by conventional UV methods. In this procedure fluorescent derivatives of the peptido-LTs were prepared. Detection limits for these fluorescent compounds were reported to be 1 nanogram. Other fluorescent derivatives of eicosanoids have been reported to have detection limits of less than 1 ng (11). Fluorescence is more sensitive, but non-fluorescing molecules must be derivatized. If the HPLC technique is being used as a purification step, it is unlikely that a fluorescent derivative is desired. Thus, its usefulness is limited to detection only.

The use of HPLC as the only method of analysis has its drawbacks. The confirmation of an analyte is accomplished by comparing the retention times of the standards to the samples. Slight differences in the composition of the solvent media of the standards and the samples may change retention times by as much as 5 minutes. This reduces the confidence in the peak identification. Also, samples may contain many other compounds that may have absorption maxima close to the wavelength used for analysis. If a contaminant is present in a large amount, extraneous peaks in the chromatogram are

likely to lead to an incorrect peak assignment for the analyte. However, a benefit of HPLC is that it is non-destructive. Samples can be collected, pooled and evaluated by some other technique. The most useful instrumental method for unambiguous determination is mass spectrometry.

Bioassay, RIA and HPLC cannot be used for the undeniable identification of the analyte. However, these methods are usually more sensitive than MS, and HPLC purification increases the reliability of the two assay techniques. HPLC is also useful for mass spectrometry. Mass spectrometric analysis requires high picogram amounts of analyte to be present. If one samples does not contain enough analyte, HPLC fractions can be pooled from many samples. The combined fractions then may contain sufficient amounts of analyte for mass spectral analysis.

MASS SPECTROMETRY

Once a sample has been purified for mass spectrometric analyte determination, the choice of ionization method has to be made. Of the available methods, each one gives different information and has different derivatization requirements as well as detection limits. Eicosanoids (fatty acids in general) have been examined by positive ion electron impact (PEI), positive and negative chemical ionization (PCI and NCI) and both positive and negative ion fast atom bombardment (FAB). FAB is the only ionization technique of those mentioned, that does not require the analyte to have appreciable volatility. The other methods require the analyte to be modified somehow to increase volatility. Since eicosanoids have low vapor pressures, derivatization or structural modification is necessary. The chemical derivatives of analytes for MS analysis will be described in detail after the types of ionization methods are explained.

Mass spectrometry of eicosanoids has been most recently reviewed by Murphy (14) in *Biochemistry of Arachidonic Acid Metabolism.* The different modes of ionization

useful for eicosanoid analysis are discussed with emphasis on the type of information that is obtained. The choice of ionization mode for eicosanoid analysis is dependent on the type of information desired (e.g., structure, concentration). It is helpful to know the approximate concentration of the analyte (μg, ng, pg, etc.). This can be determined preliminarily by HPLC or RIA. Low nanogram range samples are below the detection limit of GC-PEI-MS, but samples properly derivatized for GC-NCI-MS will provide much information.

Electron impact was the first widely used ionization technique. Electrons are emitted from a tungsten filament with 70 eV of energy. In the gas phase, the electrons bombard the analyte and ionize it by removing an electron. The 70 eV electrons impart so much energy into the analyte that it will fragment in pieces indicative of its structure. Thus, PEI is very useful for structural assignment. Fatty acids and hydroxy fatty acids have been analyzed by GC-PEI-MS most commonly as the methyl esters (ME), trimethylsilyl (TMS) ethers. A representative mass spectrum of 13-OTMS 18:2 ME is shown in Figure 2-1. It is apparent from the mass spectrum that the abundance of the molecular ion is small. The fragment ion, m/z= 311, confirms the identity of the hydroxy group to be in the 13-position. The fragment is the α-cleavage product from the trimethylsilyl ether. Another isomer would have a different set of α-cleavage products, which would confirm the position of the hydroxy group. It is the molecular weight plus the GC retention time that give a firm identity to peaks eluting from a GC column. The problem with electron impact is that the total number of ions produced is spread over many mass units, and the higher abundance ions are usually of low mass, and lack important structural and molecular weight information. A way to produce more higher mass ions is to use a softer ionization technique which does not impart as much energy into a molecule. Examples are chemical ionization and fast atom bombardment .

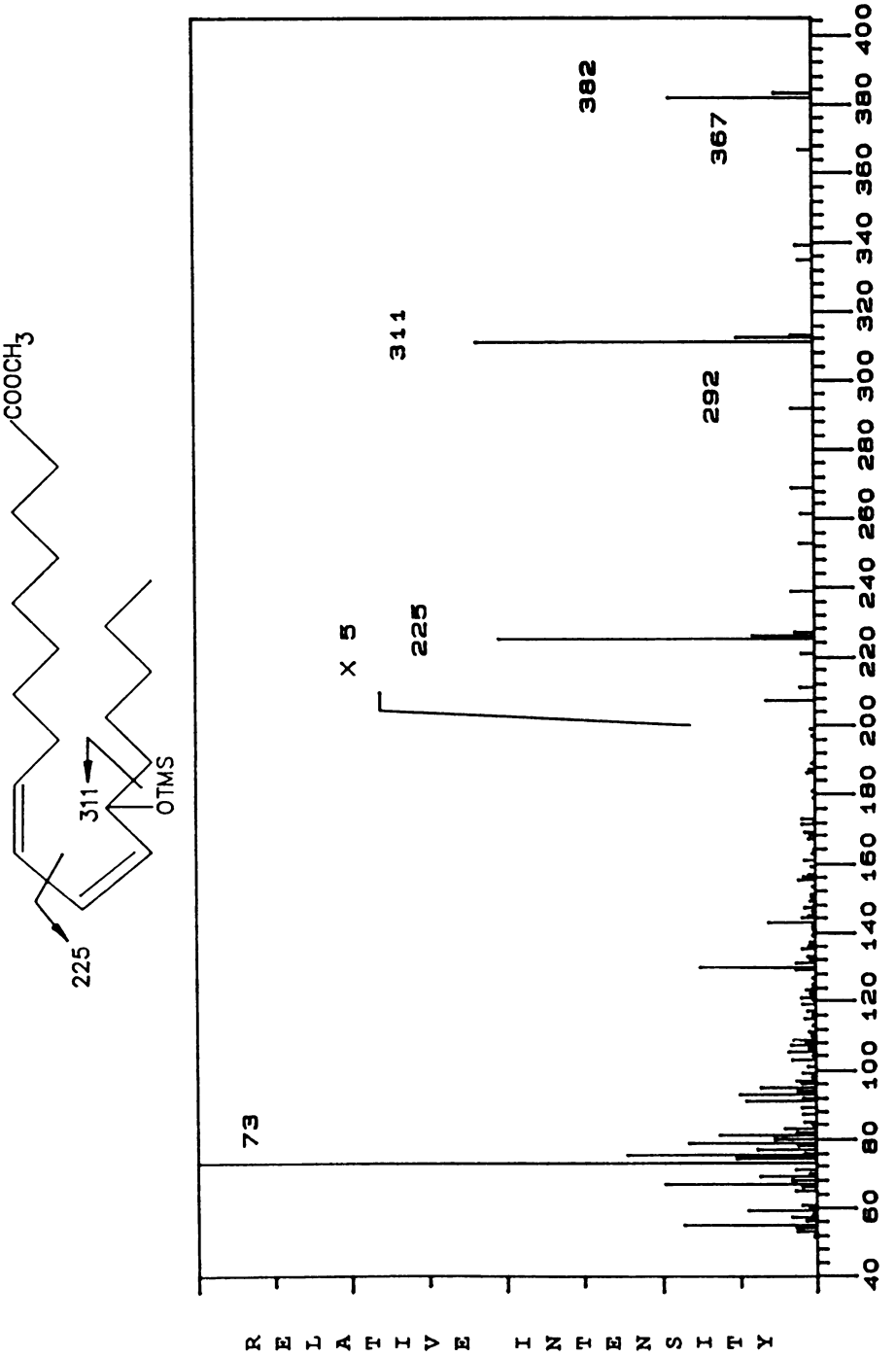

Figure 2-1. A typical 70 eV PEI mass spectrum of a hydroxy unsaturated fatty acid, 13-OTMS 18:2 ME (MW= 382). The fragments at 225 and 311 are confirmation ions for this molecule. m/z= 367 and 292 are $(M-15)^+$ and $(M-90)^+$ respectively.

Chemical ionization requires the analyte to be in the gas phase when it reacts with the reagent gas. Typical reagent gases are methane, ammonia and isobutane. The reagent gas is ionized by energetic primary electrons which form odd electron (radical), ionized species and secondary electrons (reaction 1). In PCI, the ionized gas reacts with other gas molecules and protonated gas ions are formed (reaction 2). These protonated ions react with analyte molecules (M) and proton transfer occurs (reaction 3). The more basic a reagent gas is the more selective the protonation. The basicity (proton affinity) of the analyte must always be higher than the reagent gas for proton transfer to occur (reaction 3).

Reaction 1. $CH_4 + e^- \text{----------} > CH_4^{+\bullet} + 2e^-$

Reaction 2. $CH_4 + CH_4^{+\bullet} \text{----------} > CH_5^+ + CH_3^\bullet$

Reaction 3. $CH_5^+ + M \text{----------} > CH_4 + (M+H)^+$

$^\bullet$ = odd electron (radical) species

Positive chemical ionization has been widely used for structure determinations. One important outcome has been the location of double bonds positions in fatty acids. This is outlined in Tables 2-2 and 2-3 later in this chapter. Recently, it was reported that branched and straight chain fatty acid methyl esters have been distinguished by methyl ether CI (15). Because it does not provide enough ion abundance at high masses; methane PCI has not been used extensively in eicosanoid quantitation. Instead, the fragmentation from PCI (14) proves most useful for structure determination.

Ammonia and isobutane PCI have proven successful for the determination of pure 9- and 13- hydroperoxy isomers of linoleic acid, HPETEs, LTA_4 and LTB_4. This method has yielded quantitative results for pure analyte. The underivatized hydroperoxyoctadecadienoic acids are placed on a direct insertion probe (16). A large

$(M+NH_4)^+$ ion is formed with ammonia CI, but isobutane CI causes fragmentation that yields primarily $(M+H-H_2O)^+$ and $(M+H-H_2O_2)^+$. Large quantities of $(M+H-H_2O)^+$ and $(M+H-H_2O_2)^+$ have also been recorded for ammonia PCI of 5- and 15- HPETE as well as $(M+H-H_2O)^+$ ions for ammonia and isobutane PCI of LTB_4 and LTA_4 (17). It is these fragmentation ions that were used for quantitation.

Negative Chemical Ionization has proven to be the most successful method for determining the concentration of eicosanoids in biological matrices. It is a soft ionization technique which creates mostly $M^{-\bullet}$ (molecular analyte anions). The name negative chemical ionization (NCI) implies that negative reagent gas ions react with the analyte ions. Hydroxide ion NCI is one example (18). The reagent gases produce stable hydroxide ions that then participate in acid/base ion-molecule reactions with the analyte. The reactions of OH^- NCI are demostrated below:

A. $N_2O + e^- \longrightarrow N_2 + O^{-\bullet}$

B. $O^{-\bullet} + i\text{-}C_4H_{10} \longrightarrow t\text{-}C_4H_9^{\bullet} + OH^-$

C. $OH^- + RCOOR' \longrightarrow RCOO^- + HOR'$

in general:

D. $OR^- + MH \longrightarrow (M-1)^- + ROH$

$R = H, CH_3$

OH^- NCI is much different than the method that is used most frequently in eicosanoid analysis, electron capture negative ionization (ECNI). ECNI employs a reagent gas (CH_4, i-C_4H_9 or NH_3) that when bombarded with high energy electrons >100 eV produces secondary electrons. These secondary electrons have their energies reduced to that of a thermal electron (approximately 0 eV) through a series of inelastic collisions with the high pressure reagent gas in the mass spectrometer source. These thermal

electrons may be captured by analytes which have a reasonable electron affinity. The electron affinity is based on the energy level of vacant low lying orbitals in the molecule (19). Thus the requirement for ECNI response is that the analyte be electrophilic. Although biological matrices contain many components, most are not be electrophilic. If the analyte is preferentially ionized over the matrix, the sensitivity and specificity of the ionization technique is enhanced. Some analytes are inherently electrophilic, but the eicosanoids are not. Appropriate derivatization or chemical modification is necessary.

The sensitivity of ECNI is attributed to the selective ionization of those species that are electrophilic. The rate constant of the electron capture reaction is also important and compound dependent. Thus, the ECNI response will vary for different molecules. It has been concluded that the ratio of positive to negative ion current, under equal chemical ionization conditions, is equal to the ratio of the rate constants for the ion-molecule reaction in PCI to the electron capture reaction in ECNI (20). The rate constants of PCI are 10^{-9} cm^3 mol^{-3} s^{-1} but ECNI rate constants may be much higher due to greater mobility of the electron compared to the positive reagent ions.

Ion formation by electron capture is possible by three mechanisms:

A. associative resonance capture (0 eV)

$$AB + e^- \text{----------} > AB^{-\bullet}$$

B. dissociative resonance capture (0-15 eV)

$$AB + e^- \text{----------} > A^- + B^\bullet$$

C. ion pair formation (>10 eV)

$$AB + e^- \text{----------} > A^+ + B^- + e^-$$

Thermal electrons produce analyte ions from both mechanisms (A) and (B). The

structure of the analyte will dictate whether or not dissociative resonance capture will occur. In the case of pentafluorobenzyl ester derivatives of eicosanoids, the major ion in the ECNI spectrum is the $(M-181)^-$ ion. The loss of 181 is the cleavage of the pentafluorobenzyl group $[(M-PFB)^-]$ leaving behind a highly stable carboxylate anion (see Figure 2-2. below). It has been postulated that the cleaved pentafluorobenzyl group does not compete for thermal electrons because an extra electron would make the anion formed less stable. The pi system in the benzene ring would no longer be aromatic (21). In comparison, chemically modified compounds, such as the ones prepared in this research (α, β-unsaturated ketones), primarily follow associative capture.

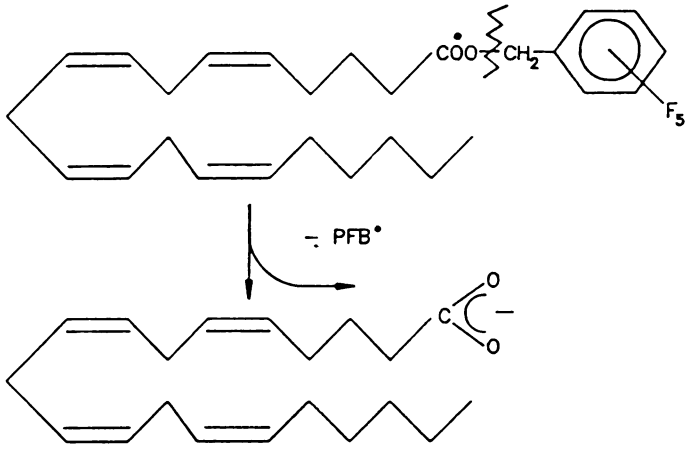

Figure 2-2. The fragmentation of a PFB ester under ECNI mass spectrometric conditions. The stable carboxylate anion is formed.

The final ionization method that will be discussed in this chapter is fast atom bombarbment (FAB). This technique does not require the analyte to have appreciable vapor pressure. Thus, derivatization is unnecessary. The technique has been used in eicosanoid analysis primarily for the structure determination of pure peptido-LTs. The peptido-LTs are good candidates for FAB because they form ionic solutions in the FAB matrix prior to ionization. This increases analyte ion current.

Fast atoms are produced from Xe or Ar gas. The gas is ionized and accelerated through an electric field into a plasma of electrons where the positive ion (Ar^+ or Xe^+)

picks up an electron but does not lose significant energy to slow down. Then the fast atoms bombard the surface of a probe covered with glycerol (a FAB matrix) and the analyte. Both glycerol and the analyte are desorbed from the probe as ions . Some ions receive excess energy and fragment. FAB can be used with both the positive and negative ion detection. Negative ions produced by FAB are mostly $(M-H)^-$ ions (loss of a proton) for molecules that, in solution, are not charged like LTB_4 (14). Positive ions produced by FAB are protonated $(M+H)^+$ ions. Also, Na^+ and K^+ adducts are commonly formed. FAB is not a selective ionization technique such as ECNI. Significant interference from the glycerol background makes this technique less sensitive than other mass spectrometric methods and not analytically useful for biological samples without prior purification.

SAMPLE INTRODUCTION

Methods of sample introduction to the source of a mass spectrometer for EI and CI include a direct insertion probe or a gas chromatograph. The direct probe allows for rapid analysis of an analyte. The probe is heated and compounds escape into the vapor phase. There is little separation of components of a complex sample. The separation of different components of the sample will be observed when reconstructed profiles of selected ions representative for each component are visually displayed. Usually eicosanoid biological samples are too complex to be analyzed on a direct probe.

Normally, biological fluids are analyzed for eicosanoids by gas chromatography-mass spectrometry. Sample components are separated chromatographically that so only one compound enters the mass spectrometer source at a time. If other compounds, that may be in the sample, were present in the source simultaneously with the analyte, they would compete with the analyte for ionization. This would result in less analyte being ionized and higher background, lowering the signal-to-background ratio.

Gas chromatography does require the analyzed compounds to be in the gas phase. Eicosanoids may be derivatized or chemically modified to make them more volatile. Lipoxygenase eicosanoids are derivatized by reagents that remove reactive hydrogens from the hydroxy and carboxyl groups. The choice of derivative is dependent on the mode of ionization. The most commonly used derivatization reagents are presented in Table 2-1.

In many cases, especially for the peptido-LTs, derivatization greatly increases the total mass of the analyte; it can make the compound impossible to determine by MS. Also some derivatives do not always improve the volatility of the analyte enough and poor chromatographic peak shape results. Extreme GC conditions (very high temperatures) may be necessary to improve the chromatographic properties. The problem of poor chromatographic peak shape is evident in the case of 15-HETE. Preparing 15-HETE for analysis by ECNI requires the 15-OH group and the COOH group to be derivatized to a TMS ether and pentafluorobenzyl ester (PFB) ester, respectively. These two derivatives increase the mass of the parent compound by 252 mass units. The GC-ECNI-MS profile of the derivatized 15-HETE is shown in Figure 2-3A. The excessive fronting of the peak is caused by the compound condensing in the GC. This can be eliminated by increasing GC temperatures to 300°C, but a more realistic approach is to reduce the double bonds of 15-HETE with hydrogen and 5% Rh/Al prior to TMS, PFB derivatization (22). The resulting 15-hydroxyeicosanoic acid (15-HEA) is then derivatized and the chromatographic peak shape is improved greatly (Figure 2-3B). (The split peak in (B) is caused by the injection technique (on column injection) and does not depict the presence of more than one compound). The reason for the improvement of the peak shape after the reduction of the double bonds is not known. It may be the relationship between the saturation of double bonds and the PFB derivative. The peptido-LTs have a related problem. Derivatization of all the hydroxy, amino and carboxylic acid groups would greatly increase the total mass of the compound. The extra mass may make the

TABLE 2-1. DERIVATIVES FOR THE ANALYSIS OF HYDROXY FATTY ACIDS BY GC

TYPE OF GROUP	IONIZATION MODE	DERIVATIZATION REAGENT	DERIVATIVE FORMED
-COOH	PEI, PCI	Diazomethane	Methyl Ester $-COO-CH_3$ (ME)
		ROH/H+	-COOR (ester)
		BSTFA	Trimethylsilyl ester (TMS) $-COO-Si(CH_3)_3$
	NCI	Pentafluorobenzyl Bromide	Pentafluorobenzyl ester (PFB) $-COO-CH_2-C_6F_5$
-OH	PEI, PCI, NCI	BSTFA	TMS ether $-O-Si(CH_3)_3$
$-NH_2$, -NHR (peptido-LTs)	PEI, PCI	Acetic acid anhydride	acetamide $-NH-COCH_3$, $-N-COCH_3$
	NCI	Trifluoroacetic Acid Anhydride (TFAA)	Trifluoro-acetamide (TFA) $-NH-COCF_3$, $-N-COCF_3$
		Pentafluoropropionyl Anhydride (PFPA)	Pentafluoro-propionamide (PFP) $-NH-COC_2F_5$

PEI= positive electron ionization
PCI= positive chemical ionization
NCI= negative chemical ionization

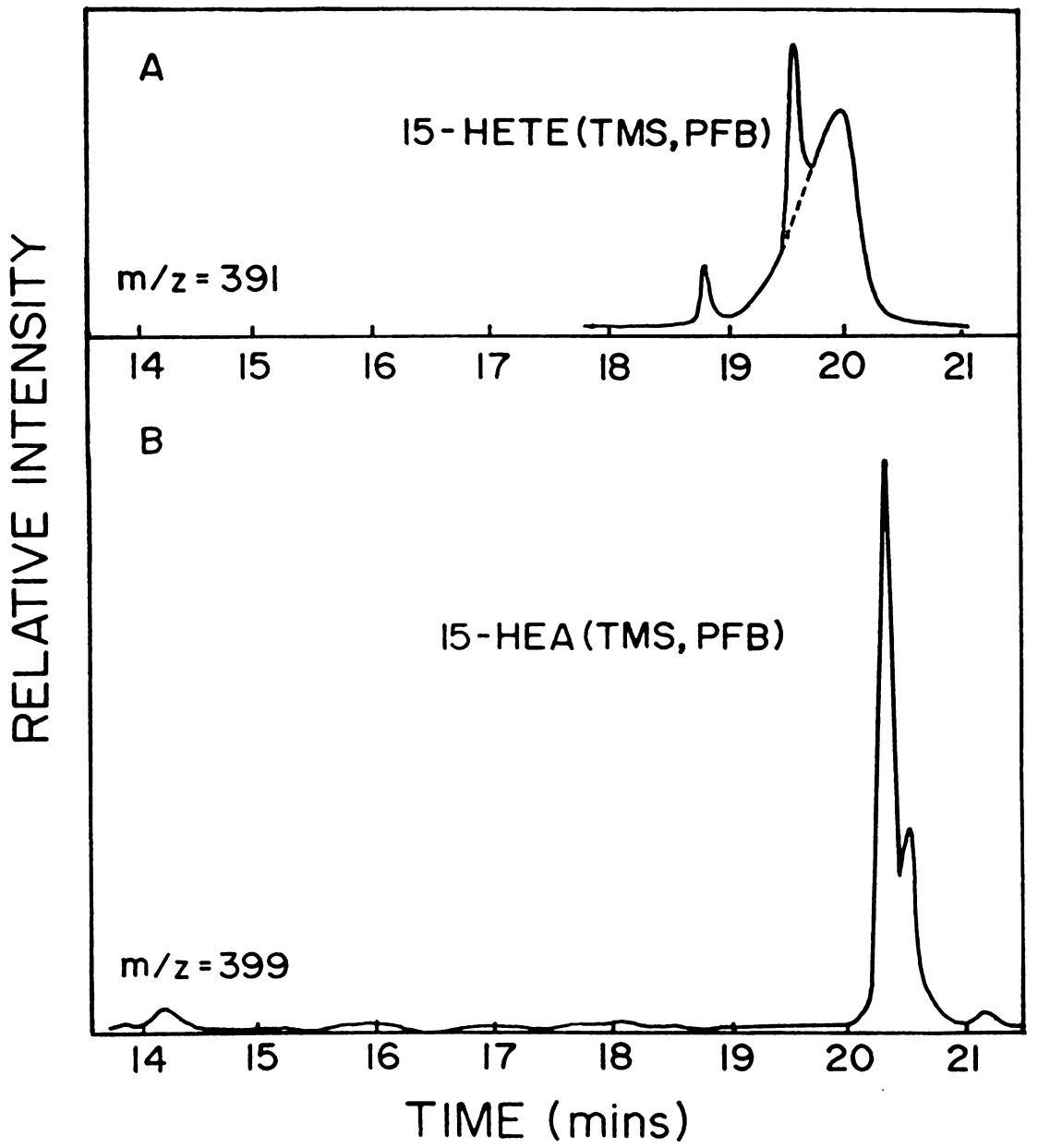

Figure 2-3. Selected ion current profiles of 15-HETE before, (A) and after, (B) hydrogenation. The PFB, TMS derivatives were subsequently prepared. Ions monitored were A: m/z= 391 and B: m/z= 399. The split peak in (B) is described in the text.

compound difficult to elute from a GC column. Also, with a molecular weight over 1000 the mass range of the mass spectrometer may be at its limit, and unit resolution between sequential masses may not be possible. It is advantageous to cleave off the peptide side chain and reduce the double bonds prior to derivatization. A detailed procedure for the analysis of sulfidopeptidyl-LTs is found in chapter V.

Another way of improving the gas phase properties of the analyte is structure modification. Oxidation of hydroxy groups to ketones will improve vapor phase properties and chromatographic peak shape. Oxidations employed in our laboratory also increase the electrophilic character of the analyte. The nascent electrophilic analyte can then be analyzed by ECNI. The oxidations are also more selective than the general derivatizations. This adds more specificity to the methodology and analysis. The benefits of oxidation over derivatization are covered in Chapter IV.

Derivatives can be used in structural assignments. One important example of this is a derivative of fatty acids that helps with the determination of double bond positions. Early work in fatty acid determinations required the knowledge of structural features, most commonly the location and geometric configuration of double bonds. The location of double bonds has not been easy to assign by mass spectrometry. During ionization double bonds tend to migrate. Many reactions that are geometrically specific (OsO_4) can be used to determine cis and trans double bonds. OsO_4 oxidizes the double bonds to cis diols and eliminates bond migration, and hence, ambiguity. Derivatives are then made of the diols and the compounds are analyzed by various mass spectrometric techniques. The fragmentation is driven by the heteroatoms now at the site of the original double bond, which reveals the double bond location. Other derivatives are made of the carboxylic group and a unique fragmentation pattern discloses the positions of the double bonds. Recently tandem mass spectrometry has been used for the determination of double bond positions in underivatized fatty acids.

All of these methods have drawbacks, which are explained in the comments in the tables. Most frequently double bond positions of monoenoic and dienoic acids are easy to assign from the mass spectra, but polyenoic acid mass spectra give no useful information. Another problem has been the assignment of double bonds in hydroxy polyenoic acids. The hydroxy group will change the fragmentation of the molecule. Tables 2-2 and 2-3 summarize the types of derivatives, the ionization mode used, and the information obtained from the determination which have been published. Work in our laboratory is being conducted to explore new methods for the determination of the double bond positions is fatty acids, especially those containing hydroxy groups.

It has been shown that a variety of analytical techniques is needed for the determination of structure, bioactivity and concentration of lipoxygenase eicosanoids and in general, all fatty acids. The analysis of biological samples generally requires more than one analytical tool. Although bioassay is important, the most regularly used techniques are HPLC with RIA, HPLC with mass spectrometry or both. The available amounts of biological fluids and an approximate analyte concentration will determine whether mass spectrometry is feasible.

Mass spectrometry does confirm the identity of the analyte more accurately than any other technique. The information obtained from a mass spectral analysis is not questionable unlike RIA data. Eventually it will become the most widely used method for eicosanoid analysis. It is now important that easier derivatization techniques be developed for the facile analysis of HETEs and LTs. Many existing methods require highly trained persons to assess the presence and concentration of these elusive compounds. New methodology is addressed in Chapter IV.

TABLE 2-2. DOUBLE BOND DERIVATIVES OF FATTY ACIDS FOR ELECTRON IMPACT

Reagent	Derivative (reference)	Analyte	Comments
OsO_4/ cis vicinal diols			
	methyl ether (23)	monoenoic polyenoic acids	much fragmentation: $4n^2 + 10n + 2$ fragments, where n= the # of double bonds few structurally important ions.
	TMS ether (24)	monoenoic polyenoic acids	distinctive fragmentation
	isopropylidene (25)	monoenoic	cannot determine polyenoic double bond locations; lack of structurally important ions
	alkylboronate (26, 27)	monoenoic	acts like isopropylidene derivative
	dimethyl disulfide (DMDS) thiol ether (28)	dienoic	distinctive fragmentation from number of $-CH_2-$ units separating double bonds
carboxylic acid derivatives			
	pyrrolidide ester (29, 30)	monoenoic polyenoic	easy location of double bonds will not work when hydroxy groups are present

TABLE 2-3. DOUBLE BOND DERIVATIVES OF FATTY ACIDS FOR CHEMICAL IONIZATION

Positive CI (PCI)

Reagent	Derivative (reference)	Analyte	Reagent Gas	Comments
OsO_4/cis vicinal diol				
	methyl ether (31)	polyenoic monoenoic acids	isobutane	structurally important ions easy to recognize
	(32)	"	NH_3	cleaner gas low pressure increases abundance of structurally important ions
	TMS ether (33)	polyenoic monoenoic acids	isobutane	< 3 double bonds increase weight of molecule; structurally important peaks small (M+H)+ small or absent
underivatized acid				
	(34)	polyenoic	NO	prominent fragment ions
	(35)	monoenoic	VME/CS_2/N_2 VME= vinyl methyl ether	small structural fragments not useful for polyenoic acids; no characteristic ions observed

TABLE 2-3. CONT'D

Sample Introduction (reference)	Analyte	Reagent Gas	Comments
MS/MS			
Negative Ionization			
fatty acids and esters			
batch inlet (36)	monoenoic polyenoic acids	OH^- ($N_2O/CH_4/He/N_2$)	CAD spectra of $(M-H)^-$ shows diagnostic ions for location of double bonds
FAB (37)	monoenoic polyenoic acids		CAD spectra of $(M-H)^-$

CAD= collisionally activated decomposition

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CHAPTER III: AUTOXIDATION, PHOTOOXIDATION AND LIPOXYGENATION OF FATTY ACIDS

The preparation of model compounds for this project was a challenging part of the research. The allylic hydroxy fatty acids are unstable, and they were not commercially available until recently. The formation of oxidized fatty acids in vegetable oil and other products has been a problem in the fats and lipids industry. The oxidation of fatty acids produces species that add unwanted flavors and odors (i.e., rancidity) to oils (1). The odors and flavors are linked to the thermal decomposition of the hydroperoxy species. The earliest information on the oxidation of fatty acids is found in journals which pertain to the chemistry of fats and oils. Lipid chemists have done exhaustive research to determine the causes of fatty acid oxidation. The fatty acid that has been used most frequently as a model compound in studies is linoleic acid (18:2). It is a major component in plant lipids. Researchers became interested in making allylic alcohols to determine the mechanism of production. Thus, the methodology for preparing allylic hydroxy fatty acids from parent acids is well documented.

The oxidation of fatty acids is known to occur by three different mechanisms. Each mechanism results in different products that have been oxidized at a specific carbon (regiospecificity). This chapter presents the mechanisms of the three oxidation methods. It includes the considerations necessary in model compound preparation in order to obtain maximum yield and purity of the isolated products from the starting compounds. The preparations are described in detail for the four methods that were evaulated; each procedure and its potential pitfalls are regarded with insight. It was observed that many problems can arise from incomplete descriptions in published papers. The published procedures were altered during this research many times. In order to minimize future problems with contamination the final step-by-step syntheses are presented here. Some of the more specific reactions and the glassware silanization procedure are found in other

chapters of this thesis.

The first step in oxidation is the formation of the hydroperoxy acid. The most selective way of preparing the hydroperoxy unsaturated fatty acids is by enzyme oxidation. This method insures regiospecificity that is not possible with more general oxidations such as photooxidation and autoxidation. Because the enzyme reaction was not initially fulfilling the needs for milligram quantity yields, the processes of photooxidation and autoxidation were utilized for gross productions of isomeric allylic alcohols which were then used for the optimization of the selective oxidation reactions found in chapter four. The reactions that were tried, their subtrates and the results obtained are found in Table 3-1.

TABLE 3-1. OVERVIEW OF THE SUCCESS OF HETE AND HODD REACTIONS

HETE= hydroxyeicosatetraenoic acid, HODD= hydroxyoctadecadienoic acid

REACTION TYPE	SUBSTRATE	% YIELD (gram amounts)/ # OF ISOMERS FORMED
lipoxygenase method:		
Sigma Chemical	18:2	0.06% (.05 mg)/ two isomers
"	20:4	0%/ one isomer
Graff	20:4	0%/ one isomer
Lands	20:4	6% (3 mg)/ one isomer
photooxidation	18:2	70% (70 mg)/ four isomers
"	20:4	20% (10 mg)/ eight isomers
autoxidation	20:4	0%/ six isomers

In each oxidation method the hydroperoxy acid, not the hydroxy acid, is formed. The hydroperoxy species is very unstable and may isomerize. Therefore, to prevent a mixture of products, it is very important to reduce the hydroperoxy group to the hydroxy group as soon as possible.

There are many reducting agents (enzymatic and non-enzymatic) that can be used for this purpose. The three utilized in this research were $NaBH_4$ (Aldrich), triphenylphosphine (TPP) (Aldrich), and glutathione/glutathione peroxidase (Sigma Chemical). Each of these reducing agents has its own procedure and, in some cases, such as with TPP, severe problems. $NaBH_4$ is the least expensive reagent and the easiest to use. The reaction procedure is explained in the autoxidation section. The only "trick" to using $NaBH_4$ successfully is to add water after the reduction step to neutralize the excess reagent. This makes the final extraction much easier.

TPP is a reagent specific for hydroperoxy groups. It is very easy to use, but can produce severe contamination problems. It also complicated the HPLC purification of 15-HETE. The complete procedure and drawbacks are explained later in the chapter in the Lands lipoxygenase method for the preparation of 15-HETE. The reduction may be accomplished enzymatically by glutathione (GSH) and glutathione peroxidase (GSH-Px). The reaction for this reduction method is shown below:

$$2\,GSH + HPETE \xrightarrow{GSH\text{-}Px} GSSG + HETE + H_2$$

This is the way that the hydroperoxy compounds are reduced in cells. This enzymatic method does not produce compounds that could potentially coelute with the HETE because the GSH, GSSG (oxidized GSH) and the GSH-Px are water soluble and are not extracted into the organic phase with the model compounds. The only disadvantage of this method is that the GSH-Px is very expensive and not a realistic choice for reducing large quantities of oxidized fatty acid.

AUTOXIDATION

Autoxidation is a free radical process with initiation, propagation and termination steps. Fatty acids such as arachidonic, linoleic and linolenic with methylene-interrupted double bonds are the best substrates. The initiation step is least understood but it is thought to be due to decomposition of hydroperoxides formed by photooxidation in the presence of trace iron and copper. This is explained later in detail in this chapter. One way to control autoxidation is to add a chelating agent to remove metal ions or a compound with which free radicals preferentially react. For example, BHT (butylated hydroxytoluene) is widely used for this purpose in the food industry.

Autoxidation was briefly examined as a method of preparation for hydroxyoctadecadienoic acids (HODDs) and hydroxyeicosatetraenoic acids (HETEs). The rate of autoxidation is about a thousand-fold slower than photooxidation (2). Most photooxidation reactions that have been conducted in our laboratory were at least forty hours long. The procedure was not expected to give great yields unless reaction times were weeks long. Autoxidation also produces many by-products such as dihydroperoxides and cyclic products. Other unwanted by-products are trans, trans-conjugated dienes instead of the desired cis, trans. It is only the cis, trans isomers that are of biological importance in the arachidonic acid cascade. Porter has found that the amount of trans, trans versus cis, trans isomers formed is directly connected to the thermodynamic/ kinetic control of the reaction. The concentration of the trans, trans species may be minimized by the use of low temperature during the oxidation (3) (thermodynamic control). Also high concentrations of fatty acids favor the cis, trans isomers (kinetic control). The addition of Vitamin E (α-tocopherol) greatly increases the amount of the cis, trans isomers. Vitamin E increases the chances of hydrogen abstraction (kinetic control) instead of β-scission (thermodynamic control). The latter is the cleavage of oxygen from the carbon β to the site where radical hydrogen abstraction

occurred (Figure 3-1). A drawback of using Vitamin E is that it causes a decrease in the formation of hydroperoxy products. Great care should be exercised when α-tocopherol is added due to the possibility of completely consuming all the peroxide radicals and forming no product (hydroperoxyeicosatetraenoic acid, HPETE). Only trace amounts of α-tocopherol should be added in an autoxidation reaction.

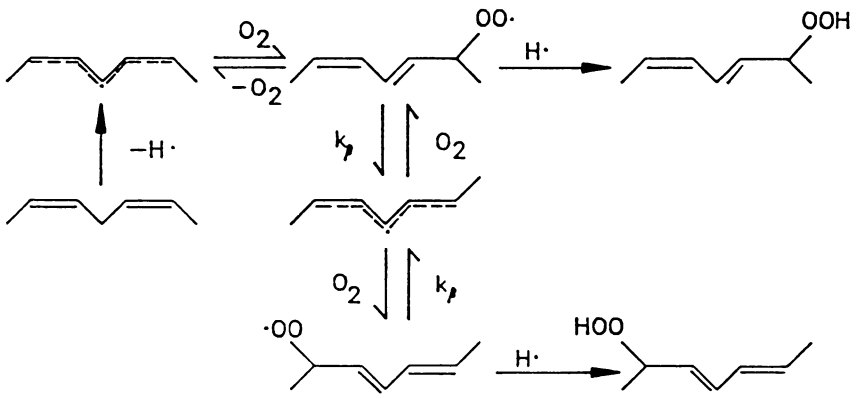


Figure 3-1. Competition between radical hydrogen abstraction and β-scission, also known as cis, trans to trans, trans isomerism.

Porter (4) has described the autoxidation mechanism in detail for linoleic and arachidonic acids. The possible products for the reactions are shown below in Figure 3-2. The autoxidation of arachidonic acid to HETEs was investigated by Brash, Porter and Maas (5). The mechanism of the propagation step was of interest to see if the reaction was a purely random process. This was found to be the case. The abstraction of a hydrogen radical and addition of the oxygen radical was not stereoselective unlike those in enzyme oxidations.

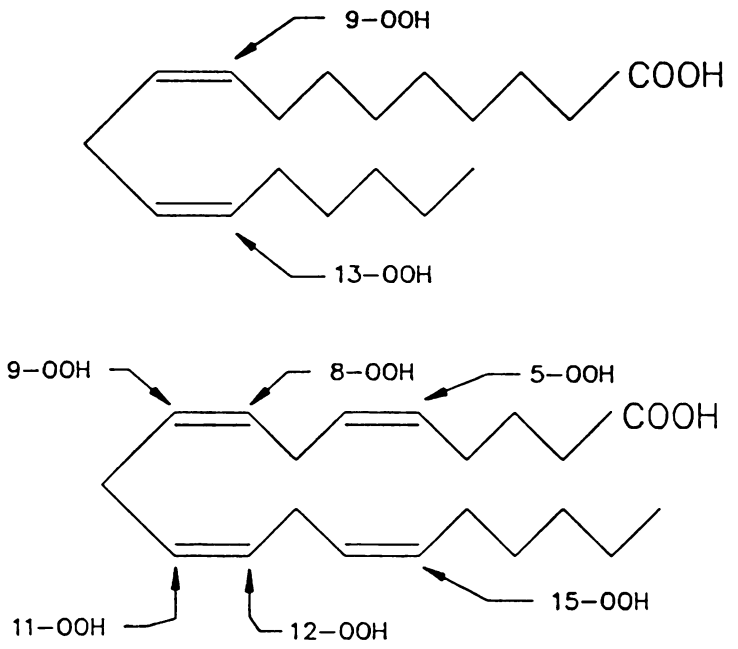


Figure 3-2. Autoxidation products of linoleic acid and arachidonic acid.

DIRECTIONS FOR THE AUTOXIDATION OF ARACHIDONIC ACID

Autoxidation of arachidonic acid was suggested by an employee of Cayman Chemical because previous trials at HETE production had not been successful. The autoxidation of AA produces only six isomers. The production of HETEs by autoxidation was accomplished in the following manner:

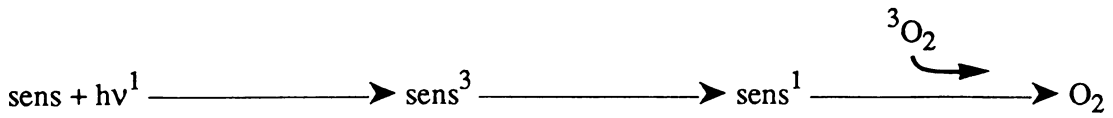
A known amount of AA (Nu-Chek Prep, Elysian, MN) (ca. 50 mg) is placed in a silylated flask. To the flask is added a small amount of α-tocopherol. The flask is filled with ca. 100 ml of ethanol or toluene and oxygen is bubbled into the solvent. The reaction is terminated by treating the solution with $NaBH_4$ for one hour (20 minutes at 0°C and 40 minutes at room temperature); water is added until a white cloudy solution remains. This solution is acidified to pH 3 with 6 N HCl and then extracted with ether, hexane or hexane/ethyl acetate (1:1). The solvent is removed under a stream of nitrogen and the reduced residue is again dissolved in methanol. It is this final solution that may be

purified by normal phase (NP) HPLC.

After an autoxidation reaction was conducted with arachidonic acid, the reaction mixture was methylated with diazomethane for GC-FID analysis. The resulting chromatogram is displayed in Figure 3-3. The largest peak is phthalate ester possibly originating as a contaminant from the ethanol, glass-distilled petroleum ether or the HPLC grade ethyl acetate all of which were used in ca. 100 ml volumes and concentrated to dryness. The problem of phthalate ester contamination will be addressed at length in the lipoxygenase section.

PHOTOOXIDATION

Photooxidation was also examined as a way of preparing large amounts of HODDs from linoleic acid and HETEs from arachidonic acid. The mechanism of photooxidation is much different from that of autoxidation. The reaction is initiated by singlet oxygen instead of an alkyl radical. Molecular oxygen is promoted to the singlet state by a sensitizer and light. Common sensitizers are chlorophyll and methylene blue. The reaction mechanism is shown below. The ground state sensitizer is promoted to the singlet state by absorption of a photon. Through intersystem crossing the sensitizer decays to the triplet state. When ground state triplet oxygen reacts with the triplet state sensitizer, it is promoted to the reactive singlet state. All reactions occur with the singlet state oxygen.



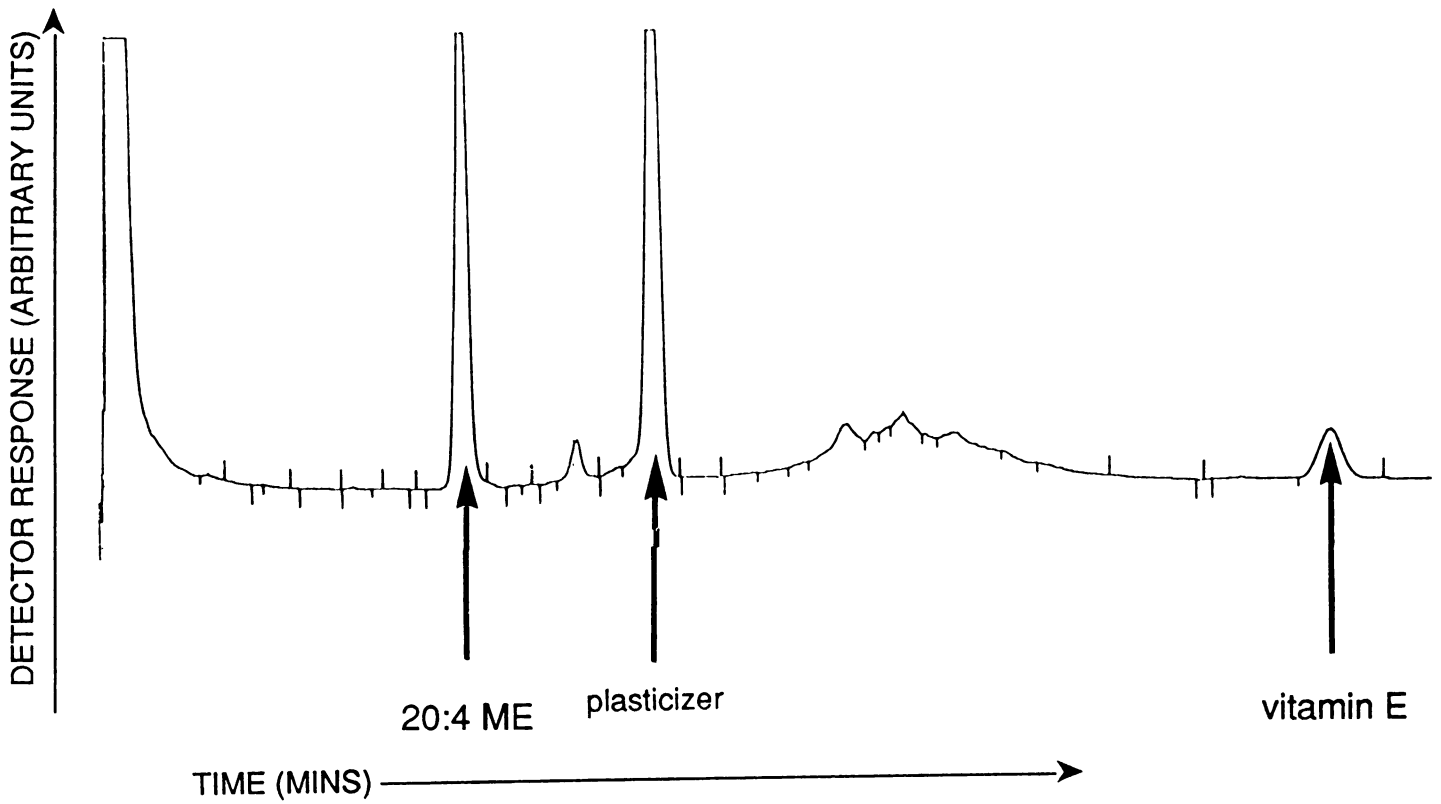


Figure 3-3. The GC-FID chromatogram of the overnight autoxidation of arachidonic acid after methylation. The production of HETE isomers was not detected by mass spectrometry. The actual retention time for the HETE isomers is approximately the same as the plasticizer.

The oxidation proceeds through an "ene" reaction which is presented in Figure 3-4.

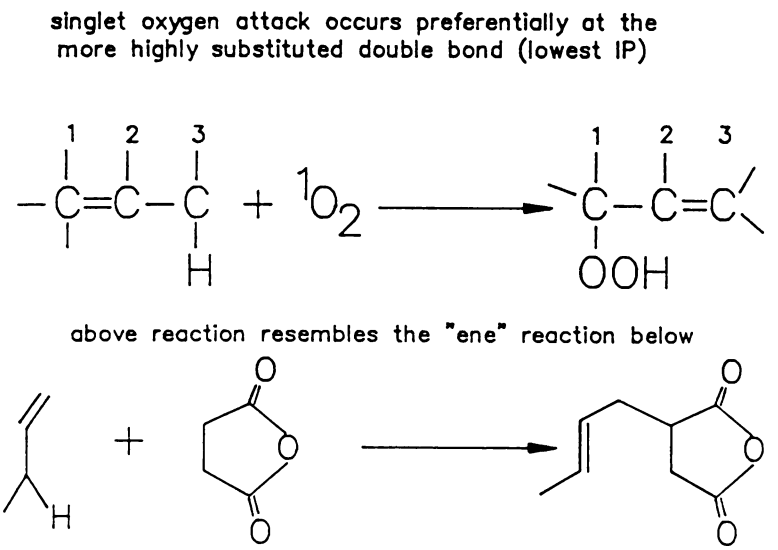


Figure 3-4. The "ene" mechanism. IP is the ionization potential.

Photooxidation is not affected by the introduction of antioxidants but may be terminated by singlet oxygen quenchers. It is thought that autoxidation in fats and lipids is initiated from the hydroperoxides produced by photooxidation. (6,7) The sensitizers necessary for photooxidation are present in the fats and lipids at very low concentrations even after bleaching. Sunlight excites the sensitizers and the unwanted hydroperoxy products are formed.

It was necessary to assemble a photooxidation system in our laboratory. The method which seemed to be easily implemented was that of Thomas and Pryor (8) involving the reaction of methyl linoleate (18:2 ME) with methylene blue. The light source used in the photooxidations must produce light at the λ_{max} of the sensitizer used. The λ_{max} of methylene blue is 653 nm; thus high intensity visible light was needed for excitation. The most easily accessible source of high intensity visible light was a slide projector. All photooxidations were accomplished at temperatures less than 10°C to minimize cis, trans to trans, trans conversions of the double bonds in the fatty acid.

DIRECTIONS FOR PHOTOOXIDATION

Approximately 50 mg of an unsaturated fatty acid is added to a vial. The methyl ester of the acid is prepared with diazomethane [prepared by the non-alcoholic instructions on the Diazald bottle (Aldrich)]. The methylated product is transferred to a 125 ml filter flask (an Erlenmeyer flask with a sidearm). Approximately 25 ml of methanol is added to the flask. There should be enough methanol in the flask to submerge the pipet which will be used to bubble in the oxygen. A small amount of methylene blue (Aldrich) is added to the flask containing a stir bar. The reaction is conducted in the cold room (5°C). The flask is placed on a magnetic stir box and plugged with a rubber stopper that has a pipet in it through which the oxygen flows. Excess oxygen escapes through the sidearm. When the oxygen starts to bubble, the magnetic stir box is turned on. Finally, the projector is set at the highest intensity and focused on the solution. The schematic for the irradiation system is shown in Figure 3-5.

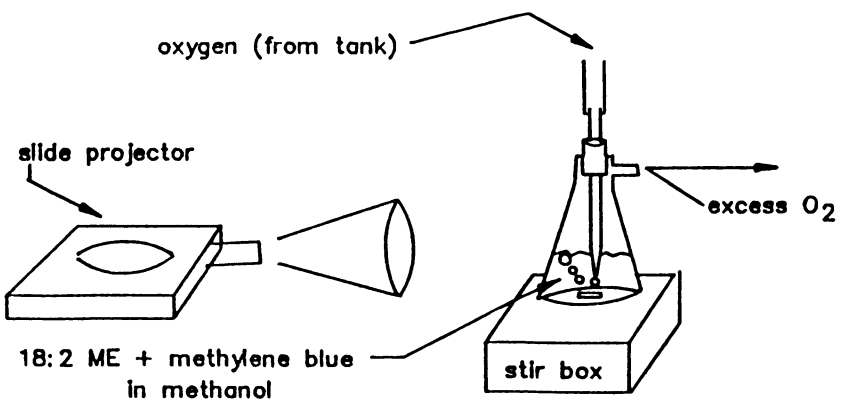


Figure 3-5. Photooxidation Apparatus.

The apparatus is periodically checked to make sure that the oxygen is bubbling, the stir bar is rotating, and that there is plenty of methanol in the flask. One milliliter aliquots are removed from the reaction vessel every 6 hours. After photooxidation, the

compounds are in the very reactive hydroperoxy form. They are initially reduced with sodium borohydride ($NaBH_4$) to the hydroxy form. The aliquot is transferred to a vial and about 2 milligrams of $NaBH_4$ is added. The vial is placed in the freezer for 20 minutes and then left at room temperature for an additional 40 minutes.

At the end of the hour, water is added to the vial with the reduced photooxidation product to double the volume. A pH electrode is used to monitor the pH of the solution as it is adjusted to 3.0 with 6 N HCl. A cloudy white solution will result. The sample is transferred to a separatory funnel for extraction. Three 10 ml portions of 50% ether in hexane are used as the organic phase. The ether layer from each extraction is collected. They are pooled and then washed with water two times. The combined, washed ether layers are dried with anhydrous Na_2SO_4.

The photooxidation reaction was conducted for 28 hours and the chromatograms of aliquots removed at 6 1/2 and 21 hours are presented in Figure 3-6. The concentration of photooxidized products with time is shown in Figure 3-7. The light source was of low intensity (30 watts), as compared to the type of source used by Thomas and Pryor (8) (650 watts), making it necessary to irradiate the reaction mixture longer.

There should be four cis, trans isomers made (# of possible isomers = 2n where n is the number of double bonds); the yields of these isomers (2,8) and the structures are presented below in Figure 3-8. There were more than four compounds made in the synthesis reported here; some of the cis bonds must have been converted to trans. Thus instead of having cis, trans isomers exclusively, the trans, trans isomers were produced; the trans, trans isomers cannot convert back to cis,trans.

To determine the identity of the peaks in the chromatograms, the products were analyzed by GC-EI-MS. Initially, the sample was analyzed after treatment with only diazomethane; this would leave a free hydroxyl group on any alcohol. If 13-OH 18:2

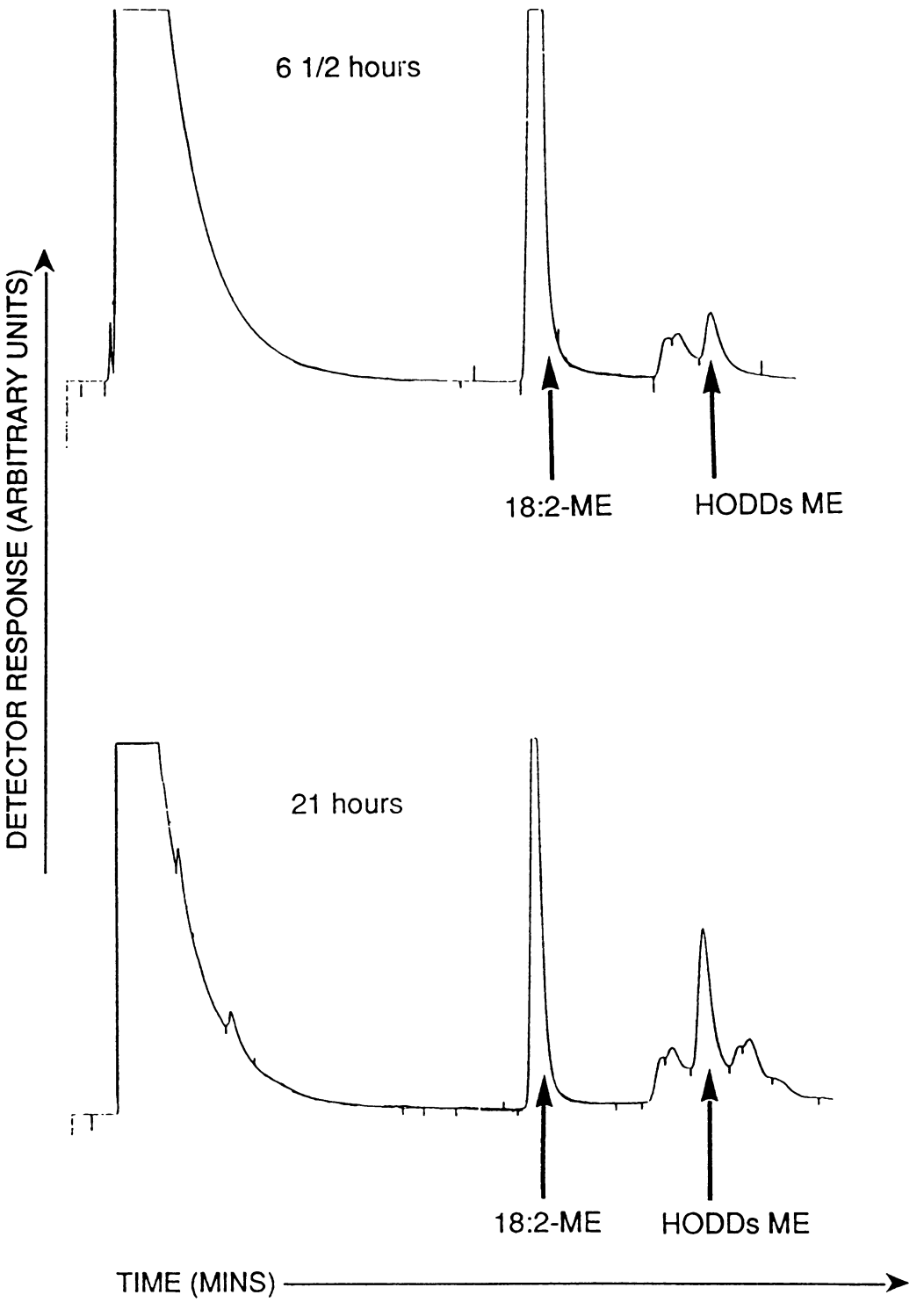


Figure 3-6. The chromatograms from a GC-FID of the methylated photooxidation products (HODDs-ME) of methyl linoleate (18:2-ME) after (A) 6 1/2 hours of irradiation and (B) 21 hours of irradiation.

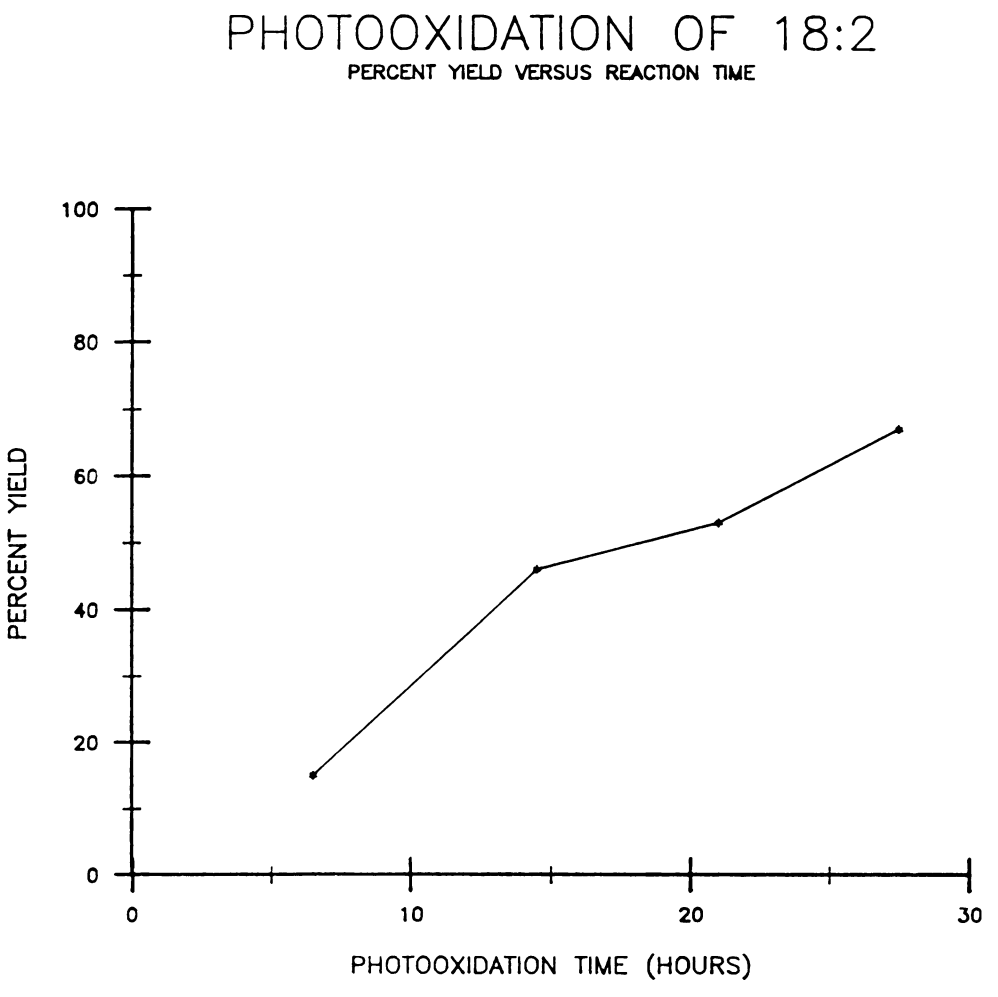


Figure 3-7. A graph of the percent yield of the photooxidation products of 18:2-ME verses irradiation time. The reaction was terminated after 27 hours.

were present the underivatized hydroxy acid methyl ester (13-OH 18:2 ME) would be

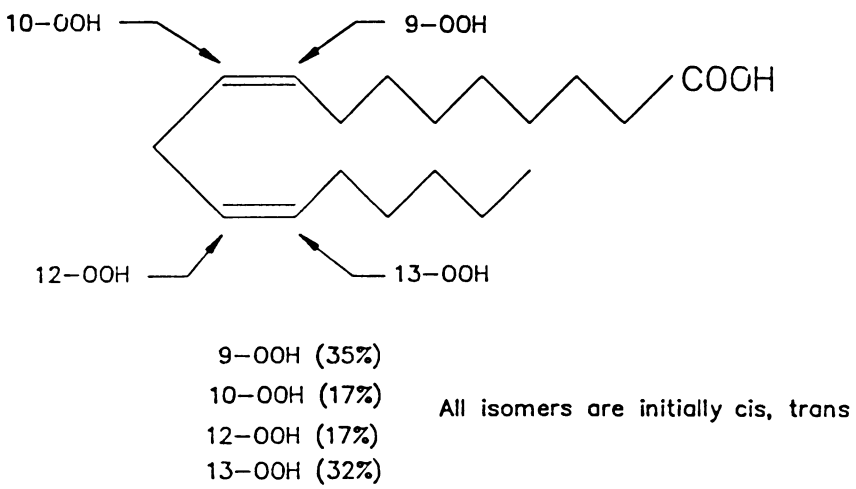


Figure 3-8. The photooxidation isomers of linoleic acid.

produced. This compound, if present in the sample, would have a molecular weight of 310 daltons. When the sample was analyzed by GC-EI-MS, the mass spectrum did not indicate a molecular ion but instead a peak at m/z= 292 was present. This peak represents an ion corresponding to $(M - 18)^+$ (loss of water). The loss of water is very common when free hydroxyl groups are exposed to EI conditions. Another aliquot of the compound was derivatized with BSTFA (Pierce) as well as diazomethane. The resulting compound would now have a molecular weight of 382 (13-OTMS 18:2 ME). The corresponding spectra for the two differently derivatized compounds are shown in Figure 3-9. It can be seen that when only the carboxylic acid group is derivatized, there is little structural information. Only low mass ions are present. However, when the trimethylsilyl ether is made, higher mass ions with structural information are of large abundance and can be used for determining the location of the hydroxy group in the molecule.

As can be seen in Figure 3-6., the mixture of photoxidized products is complex. It was necessary that the sample be purified. HPLC had proven successful for separation

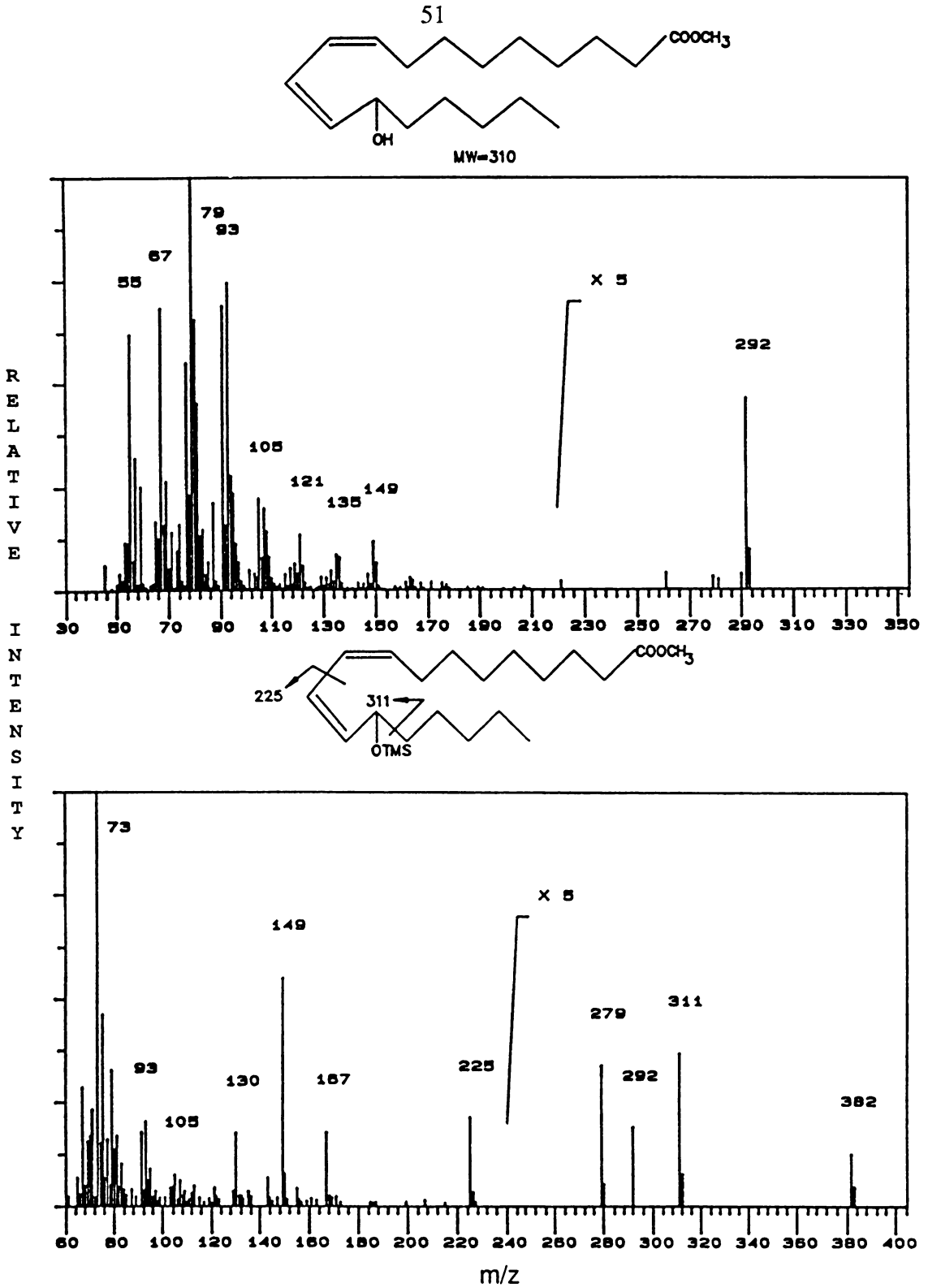


Figure 3-9. The GC-EI-MS mass spectra of 13-OH 18:2. (A) Only the methyl ester was prepared, m/z= 310. (B) The methyl ester, TMS ether was prepared m/z= 382.

of the hydroxy linoleate isomers.(8) Normal phase HPLC was used because the products were methyl esters with methyl linoleate as the starting compound. The molecules formed are conjugated dienes with a maximum absorption at 235 nm, so the HPLC detector conventional wavelength of 254 nm was not appropriate. It was discovered that the conventional wavelength of 254 nm was normally obtained with a low pressure mercury lamp. The Hg lamp did not have a strong emission around 235 nm. However, the Biochemistry departmental Beckman HPLC detector had a cadmium lamp. The Cd lamp did have an emission line near the desired wavelength and with a filter, produced an intense line at 229 nm.

The separation of the the photooxidized linoleic acid isomers by HPLC was completed by an undergraduate student. The separation was optimized by varying the percentage of isopropanol in the mobile phase (hexane) so that the isomers would be separated properly. The collection of fractions was also optimized by determining the time between each injection and the number of injections possible without peak overlap from previous injections. The best liquid chromatogram is shown below in Figure 3-10.

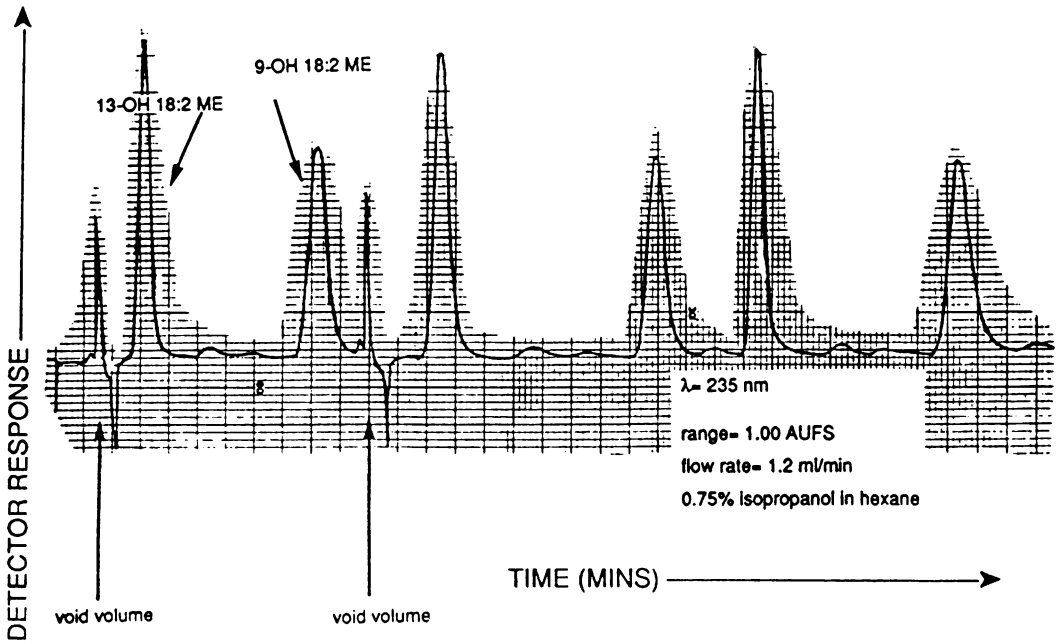


Figure 3-10. The normal phase HPLC chromatogram of separated photooxidized linoleic acid isomers. AUFS= absorbance units full scale

The first samples that were collected were examined by GC-FID and found to be contaminated. This contamination was due to plasticizers. The source of contamination was believed to be the use of plastic pump priming syringes. The syringes were changed to glass and this eliminated the problem. Other samples were collected and the purified allylic alcohol isomers were stored for later use.

Photooxidation proved to be successful in producing monohydroxy isomers of linoleic acid (18:2). It was not as successful for arachidonic acid. The larger number of double bonds increases the number of possible isomers from four to eight; this does not include the possibility of cis to trans isomerization. Also the HPLC separation of the monohydroxy isomers of arachidonic acid is much more difficult than that of the monohydroxy isomers of linoleic acid. Even Porter *et al* (9) do not get complete separation of their arachidonic acid photooxidized products.

LIPOXYGENASE

The lipoxygenase enzyme catalyzes oxygen uptake by polyunsaturated fatty acids. The enzyme requires the substrate to contain a 1,4-cis-pentadiene unit. The enzymatic oxidation is initiated by removal of a hydrogen radical from the methylene group in the pentadiene unit. The fatty acid radical reacts with oxygen to form a triplet state peroxy radical and is finally transformed into a hydroperoxy 1,3-cis,trans-diene. The lipoxygenase mechanism is found in Figure 3-11.

Preparation of allylic hydroxy model compounds for feasibility studies of their oxidation to α, β–unsaturated ketones required the use of the lipoxygenase enzyme. This preparation gives only one or two isomers (the enzymes may not be pure) as compared to the other mechanisms of oxidation. The lipoxygenase enzymes are a group of oxygenases that vary in their position of oxygenation. The most commonly used lipoxygenases are plant enzymes from tomato, potato or soybean. The soybean

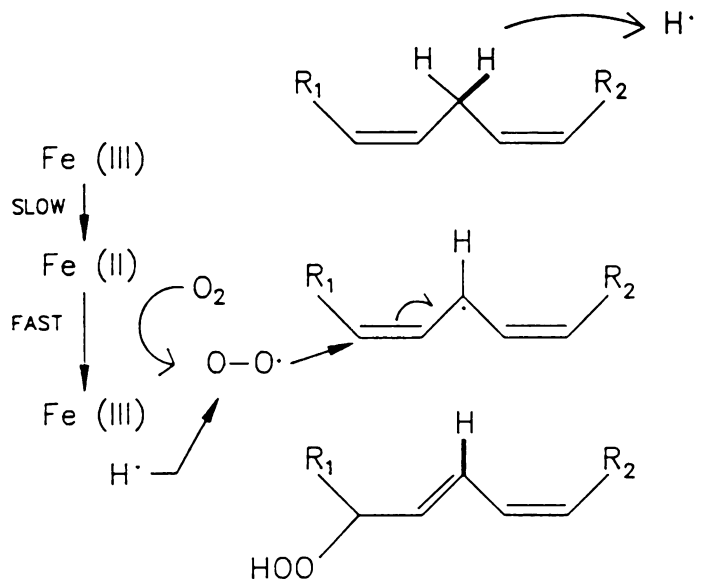


Figure 3-11. The lipoxygenase mechanism.

lipoxygenase is used most frequently because it is commercially available from Sigma or ICN Biochemicals. (I prefer the ICN product due to its high activity and white color.) The important reason for choosing a certain plant enzyme is its positional specificity. Different plant enzymes will mimic the specificity of animal enzymes and are easier to obtain in large quantities. The tomato and potato lipoxygenase enzymes primarily oxidize linoleic acid at the ω-10 position(ω-16 for arachidonic acid), which yields 9-hydroperoxyoctadecadienoic acid (9-HPODD) or 5-hydroperoxyeicosatetraenoic acid (5-HPETE). The soybean lipoxygenase-1 produces an oxidized product at the ω-6 position which yields primarily (13-HODD) 13-hydroxyoctadecadienoic acid or 15-hydroxyeicosatetraenoic acid (15-HETE). A list of plant enzymes is presented in Table 3-2 (10) on the following page.

TABLE 3-2. SPECIFICITY OF PLANT ENZYMES

ENZYME SOURCE	pH	REGIOSPECIFICITY*
		ω-6: ω-10 for 18:2
combined soybean isoenzymes	9.0	100:0 (0°C)
"	"	44:56
"	"	100:0 for 20:4
soybean-1	6.6	77:23
soybean-2	6.6	25:75
"	9.0	35:65
potato tuber	5.5	5:95
"	6.3	90% (5-HETE) 20:4
tomato	6.5	4:96

* T= 25°C unless otherwise specified.

It is important to note that the regiospecificity of the plant enzymes is pH dependent and substrate dependent. The substrate dependence is evident with soybean lipoxygenase. The specificity for linoleic acid differs with pH and temperature, but it does not with arachidonic acid. The specificity is shown in Figure 3-12. The pH dependence will be examined later in this chapter.

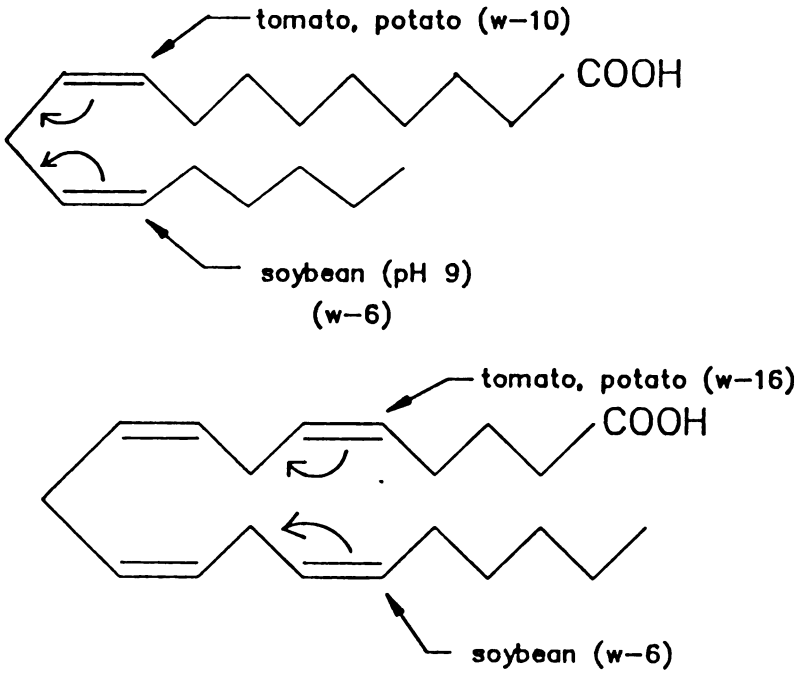


Figure 3-12. The specificity of the most common plant enzymes with (A) 18:2 and (B) 20:4 as substrates.

The kinetics of the soybean lipoxygenase enzyme have been studied extensively. A recent review can be found in *Biochemistry of Arachidonic Acid Metabolism* (10). Interest in the lipoxygenase kinetics helped determine how to obtain the best yields of HODDs and HETEs. Since the enzyme had never been used in our laboratory, it was important that the reaction and its mechanism be well understood. Initially, it was difficult to prepare large amounts of the desired hydroxy compounds by enzyme oxidation and this may have been due to a lack of knowledge about the characteristics of the enzyme. A possible problem could have been the age of the enzyme and its loss of

activity. This can be determined spectrophotometrically. The conversion by lipoxygenase of the diene unit into a conjugated diene allows for the spectrophotometric monitoring of product formation and hence the enzyme's activity. The conjugated diene formed has an absorption maximum at 235 nm. This is the wavelength that is also used in ultraviolet detection by HPLC.

The studies of the kinetics reveal that the hydroperoxide product concentration is important in the activation of the enzyme. The oxygen concentration is important due to the possibility of product decomposition. There is also some evidence (11) [which has been disputed by other laboratories (10)] of enzyme inactivation from adhesion th the walls of the reaction vessel. Other considerations include pH, addition of Ca^{2+}, removal of iron from the water used, the stability of the formed hydroperoxide, temperature of the reaction, the concentration of substrate verses enzyme and finally the extraction, reduction and purification of the hydroperoxide product. All of these parameters will be introduced and described in detail.

The concentration of hydroperoxide has been shown to be responsible for the lag time in the production of oxygenated species (11). Also the reduced hydroperoxy species, the hydroxy unsaturated acid, greatly inhibits the formation of the hydroperoxy products giving an extremely long lag time. This same time lag was seen by Smith and Lands (10) by supplying the reaction vessel with ample amounts of glutathione and glutathione peroxidase which immediately reduced the hydroperoxide formed. This shows that hydroperoxide is needed for activation throughout oxidation. This same phenomenon is true for animal-based enzymes such as those found in leukocytes (10). Addition of antioxidants or radical trapping agents shut down the supply of lipid hydroperoxide and limit the degree to which the lipid is oxidized. This can also be expressed initially as a time lag.

The lipoxygenase enzyme (formation of hydroperoxides) may be inhibited by free

radicals. This agrees with the reaction mechanism that was developed by deGroot (12). The participation of iron in this mechanism makes it important that the water used for the buffer solution is free of any iron (II) ion that might deactivate the enzyme. This can be accomplished by passing distilled water through an ion-exchange resin (Chelex-100 Bio-Rad).

Addition of Ca^{2+} ion was not tried in our laboratory. For future work the addition of 0.5 mM Ca^{2+} would be advantageous. According to Galpin and Allen (13), the addition of Ca^{2+} increases the amount of non-micellar fatty acid in solution. The formation of micelles is a great concern when preparing the substrate (linoleic or arachidonic acid) for the lipoxygenase reaction.

Since the enzyme reaction takes place in aqueous solution, direct addition of the fatty acid is impossible. Linoleic and arachidonic acid are not water soluble. The two choices for substrate addition are the preparation of ammonium or sodium salts or the "low-ethanol method". Both methods were tried and the low ethanol method was more successful. The preparation of the fatty acid salts is achieved by adding a dilute solution of ammonium or sodium hydroxide to the neat acid. The salt is then added to a buffered enzyme solution. The pH for the buffering solution is chosen to be nine because it promotes mainly ω-6 peroxidation. The low-ethanol method is accomplished by dissolving the fatty acid in pure ethanol and then adding it to the buffered enzyme solution. The reason it is called the low-ethanol method is because the percentage of ethanol in the buffered enzyme solution should not exceed 3%. It has been found that concentrations above 3% hinder the production of hydroxperoxide products. (11, 14). The high concentration of ethanol may yield homogeneous solutions of enzyme-substrate but the enzyme can be inhibited. This inhibition could be caused by either competition for the hydrophobic site of the enzyme or a change in the enzyme conformation. Both of these methods allows the addition of a large amount of substrate without inhibition of the

lipoxygenase enzyme. This so-called substrate inhibition is thought to be due to micellar formation of the fatty acid.

Another critical parameter is temperature. This reaction may be performed successfully at both 0°C and 25°C. Determining the appropriate temperature to run the experiments is really substrate dependent. The hydroperoxides formed from linoleic acid are much more unstable than those of arachidonic acid. This trend can be seen in Table 3-2 on the next page (15).

The production of hydroperoxides of linoleic acid was (Expt. 1) 100% ω-6 after a five-minute reaction. The major component was the hydroperoxide. After changing the pH to 7 (Expt. 2), the major product was still hydroperoxides, but now the regiospecificity had changed. Changing the temperature (Expt. 4) to 25°C now produced major components that were by-products. However, (Expt. 7) showed no by-product formation at the higher temperature.

Two final considerations from the previous tables are the length of time of the experiment and the difference in the method of substrate addition. Depending on whether the low-ethanol or salt method was used, the temperature dependence as well as the substrate concentration varied. The change in substrate concentration may be connected to each method's ability to increase the critical micellar concentration (CMC) of the substrate. The other consideration has to do with reaction time. Long reaction times tend to increase the by-products as well as isomerization. As mentioned earlier, the hydroperoxides (especially those of 18:2) are thermally unstable and isomerization is a problem. Isomerization may not be simply positional, but geometric rearrangement of double bonds may also occur and this is most unfavorable since separation is difficult. (16). Low temperatures have been suggested for the reaction, extraction and other stages of the purification process. (17) The isomerization can be minimized by reducing the hydroperoxide to its hydroxy species immediately after extraction. This is the procedure

TABLE 3-3. PRODUCT ANALYSIS OF SOYBEAN LIPOXYGENASE

Expt.	Substrate	pH	Temperature °C	Time (minutes)	TLC determination of: Hydroperoxides	Byproducts	Isomers: ω-10	ω-6
1	EtOH, 18:2	9	0	5	++	0	0	100
2	EtOH, 18:2	7	0	5	++	0	11	89
3	EtOH, 18:2	7	0	45	+	++	80	20
4	EtOH, 18:2	7	25	5	++	++	55	45
5	salt, 18:2	9	0	30	++	0	0	100
6	salt, 18:2	9	25	90	+	++	56	44
7	EtOH, 20:4	9	20	5	++	0	0	100

++ = major product + = minor product

that has been used in our laboratory. A high concentration of hydroperoxides in solution is a source of collisionally-induced isomerization. Light may produce by-products as well as unwanted isomers through photooxidation. Therefore, it is most important that reduction be accomplished swiftly after extraction or that the hydroperoxides be stored in a dilute solution in amber vials.

The thermal degradation of the hydroperoxides is also a problem. This is most likely to occur at longer reaction times and high temperatures (>0°C). It is these decomposition products that yield the odors mainly associated with rancidity. The products and mechanisms of formation are described in detail by Chan *et al* (1, 18). The immediate reduction of the hydroperoxides directly to hydroxy acids by glutathione and glutathione peroxidase is a possible solution to this problem but it has already been discussed that this would actually inhibit the enzyme. This isomerization is more easily eliminated by using a low temperature and short reaction times.

Most of the above discussion was concerned with linoleic acid as a substrate. The important model compounds formed from arachidonic acid are much more stable and thermal degradation is not as important. A method that stresses the use of cold temperatures for HETE production is the Graff Procedure (19). The extraction of the HPETEs is accomplished with solvents kept on dry ice. The Graff procedure was tried but yields were small and contamination from phthalates a problem. The source of contamination was discovered to be in the diethyl ether which was used during the extraction step. Plasticizers in the plastic cap on the can were extracted by the ether fumes. Since a large amount of the ether was used and then evaporated to dryness the phthalate contaminant was concentrated. This problem may be eliminated by covering the can opening with aluminum foil before capping.

The phthalate contamination of the prepared HETE is just one of a series of minor problems that led to poor yields and incorrect concentration determination of the 15-

HETE. Phthalates are quite a problem in the analyses that were completed. Their λ_{max}, which is close to the λ_{max} of the HETEs, may make the collection of the wrong component a problem, but they complicate analyses much more. The phthalates make it impossible to determine the concentration of a solution of HETEs by GC. They elute from the GC at approximately the same time as the derivatized (TMS, ME) HETEs and the oxidized HETEs, too. The phthalates are electrophilic and show up in ECD chromatograms. Also they are responsive to electron-capture negative ionization (ECNI). Thus, they cannot be tolerated in a sample and, if found, the source must be immediately defined. It is important that these problems be explained for those who intend to use the various procedures available in the literature.

PRODUCTION OF 15-HETE BY GRAFF METHOD

The preparation of 15-HPETE was tried using the preparation scheme found in Methods in Enzymology, volume 86 (19). The only difference between this procedure and many others is the extraction of the 15-HPETE from the aqueous enzymatic solution. 15-HPETE is not stable and may isomerize. This was prevented by keeping the extraction medium extremely cold (-70°C).

Arachidonic acid in ethanol is added to Tris-HCl buffer (Sigma Chemical) adjusted to pH 9. This solution is sonicated before adding it to more Tris buffer which has been equilibrated at 30°C. Oxygen is bubbled in at a rate of at least 30 ml/min. The soybean lipoxidase is added and the reaction is terminated after 5 minutes by pouring the reaction mixture into a separatory funnel with 100 ml of cooled (-25°C) ethyl acetate/petroleum ether (1:1) and 20 ml of 0.2 N HCl (adjusts the pH to 3). The organic phase is collected twice, anhydrous Na_2SO_4 is added and the solution is placed on dry ice.

The final extraction of the organic phase occurs after the aqueous phase is frozen

solid. The organic layer (ethyl acetate/petroleum ether) is poured into and passed through a fritted glass funnel and then evaporated to dryness on a rotary evaporator. The residue (15-HPETE plus other reaction by-products) is dissolved in methanol. The methanolic solution is cooled and $NaBH_4$ is added to reduce the 15-HPETE to 15-HETE. The procedure is followed as previously described. It is this final solution that is purified by reverse phase (RP) HPLC.

The above synthesis was completed many times with one minor adjustment. Instead of using ethyl acetate/petroleum ether (1:1), ethyl ether/hexane (1:1) was incorporated. This mixture is a little less polar. Unfortunately, as will be pointed out later, this change caused many problems.

The RP-HPLC of 15-HETE was accomplished using methanol/water/acetic acid 65:35:0.03 with 0.5 mM oxalic acid adjusted to pH 5.6 with NH_4OH (20). This is a very common mobile phase for the analysis of lipoxygenase products of arachidonic acid by HPLC. Other RP-HPLC mobile phases involve acetonitrile and/or trifluoroacetic acid (TFA) instead of the methanol and acetic acid. TFA is a volatile acid that can be removed easily under vacuum. It is a better choice over acetic acid if fractions are to be collected for further analysis. The wavelength used was 235 nm, as this is the λ_{max} for conjugated diene moieties. Most recent HPLC work has been accomplished using non-polar mobile phases with a preparatory silica column (normal phase). Normal phase has proven to be more successful and will be covered in the final lipoxygenase section explaining the most useful synthesis for 15-HETE.

The determination of the retention time of 15-HETE was the next important step in the RP-HPLC analysis. Since commercially prepared (+,-) 5-HETE ME (Cayman Chemical) was readily available, an aliquot of this standard was sufficiently diluted with methanol for detection by HPLC (1-10 ng/ml). Analysis by HPLC gave the chromatogram presented in Figure 3-13; note the large symmetrical peak at 15 minutes.

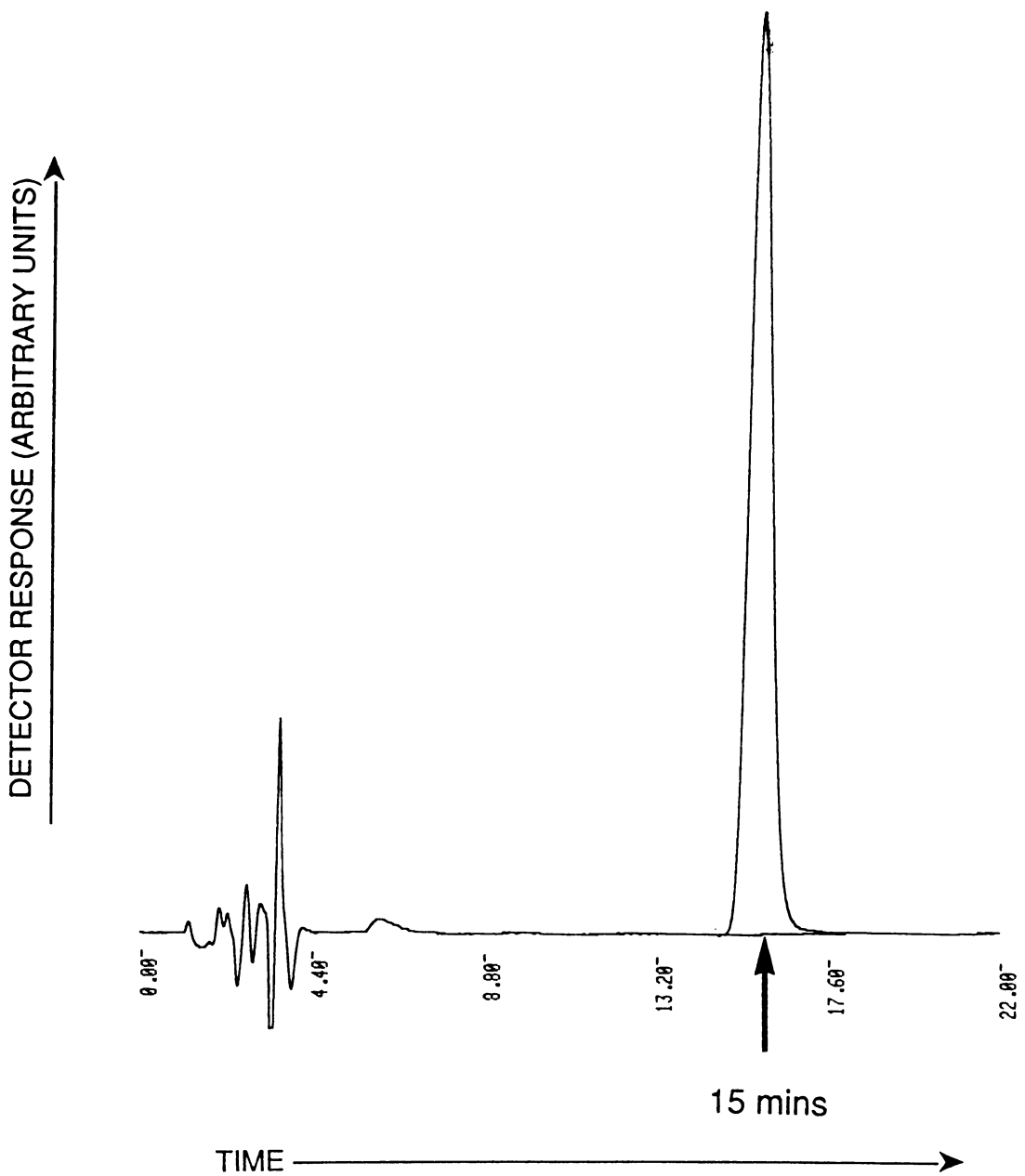


Figure 3-13. The RP-HPLC chromatogram of a 50 µl injection of (+,-) 5-HETE-ME (Cayman Chemicals). The HPLC flow rate was 1.0 ml/min; λ_{max}= 235 nm; 65:35:0.03 MeOH/H_2O/HOAc with 0.5 m<u>M</u> oxalic acid adjusted to pH 5.6 with NH_4OH

After successive trials, it was concluded that this peak was probably the (+,-) 5-HETE ME. This was assumed because, as seen in Figure 3-13, there were no other large peaks in the chromatogram. A methylated sample prepared by the Graff synthesis was then injected into the chromatograph and a representative chromatogram is displayed in Figure 3-14. It can be seen by the labelled peak that there is a compound with the approximate retention time of the standard (+,-) 5-HETE ME. This peak was collected and determined by GC to contain one compound but it was not immediately verified by EI-GC-MS to be the actual 15-HETE ME.

The Graff synthesis was tried again with a larger amount (100 mg) of arachidonic acid. The extraction procedure remained the same; analysis of the reaction mixture produced a RP-HPLC chromatogram similar to the one in Figure 3-14. Once again a fraction corresponding to the largest peak in the chromatographic run was collected. It was assumed that it must be 15-HETE because it absorbed at 235 nm. This fraction was analyzed by GC after DDQ oxidation (see oxidation chapter) and again only one peak eluted Figure 3-15.

Due to various problems with the Hewlett Packard GC-MS systems, the identity of this component was not determined immediately. Before using the GC-MS, the sample was also chromatographed by GC-ECD. The chromatogram is shown in Figure 3-16. The peak at eleven minutes is the same compound which eluted at seven minutes in the GC-FID trace. Thus, the component present in the collected HPLC fraction that had absorbed at 235 nm, showed good chromatographic properties when oxidized with DDQ and was proven to be electrophilic by GC-ECD. This happened to be only a coincidence.

When the sample was analyzed by GC-EI-MS the mass spectrum obtained proved this compound to be a phthalate ester. The most intense peak was m/z= 149.

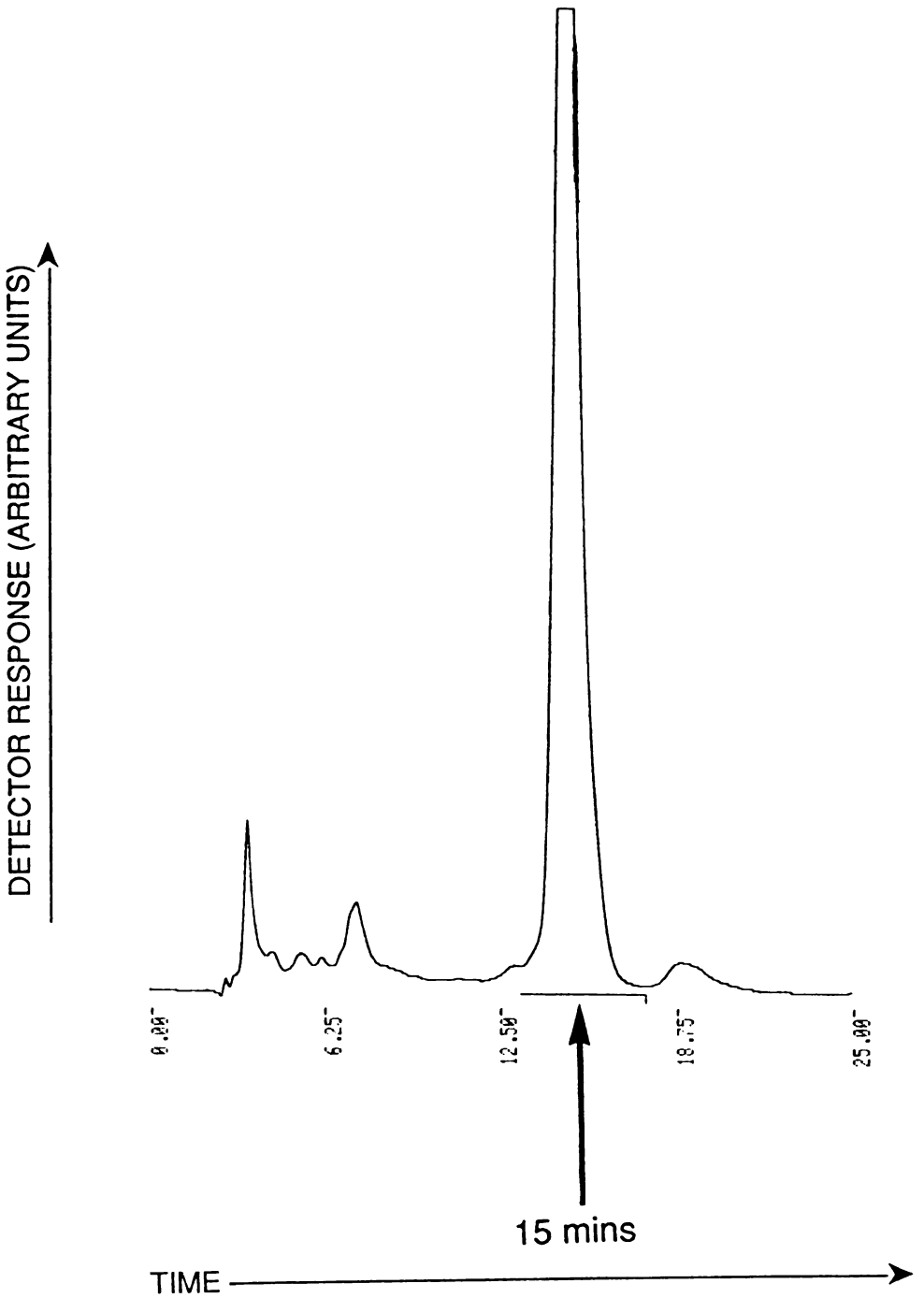


Figure 3-14. The RP-HPLC chromatogram of a 50 µl injection of 15-HETE ME made by the Graff procedure. The HPLC flow rate was 1.0 ml/min; λ_{max}= 235 nm; 65:35:0.03 MeOH/H_2O/HOAc with 0.5 mM oxalic acid adjusted to pH 5.6 with NH_4OH

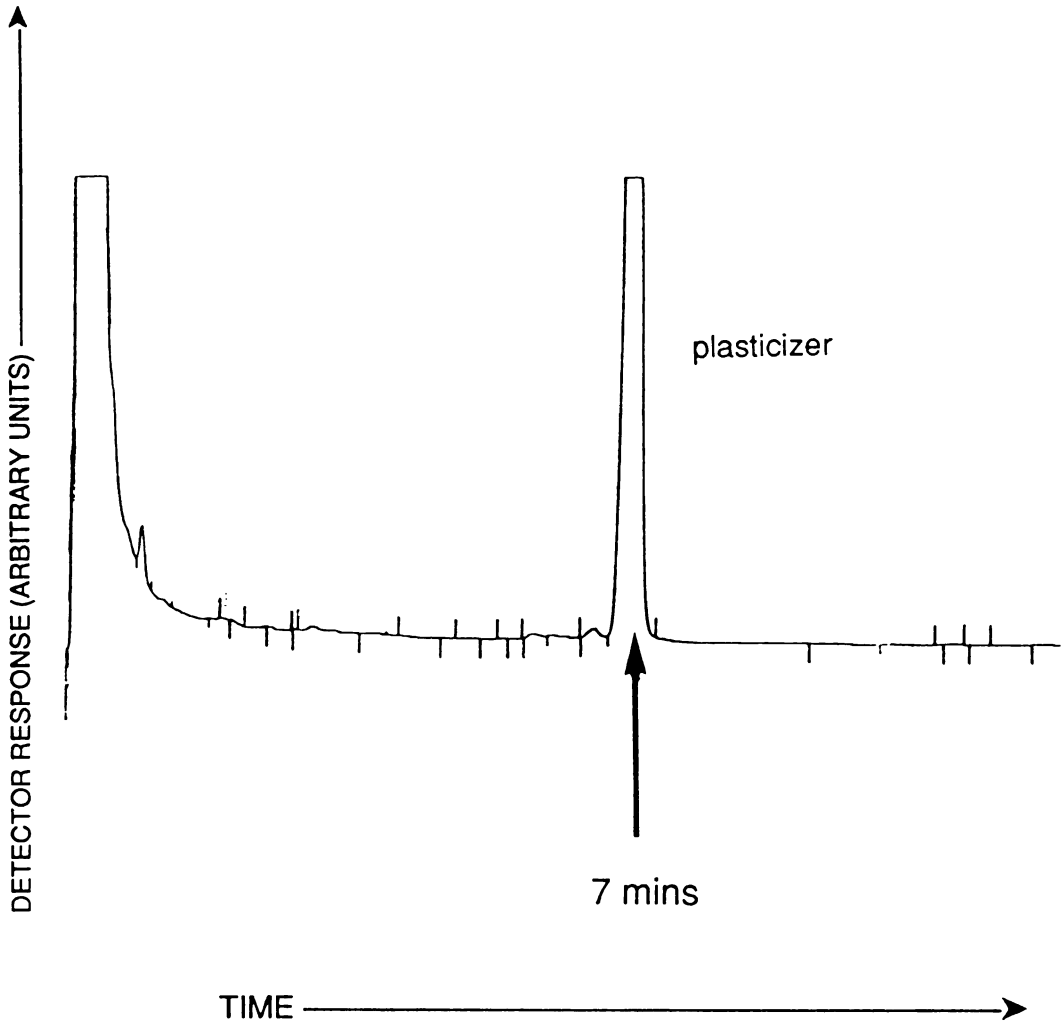


Figure 3-15. The GC-FID chromatogram of 15-HETE-ME made by the Graff procedure. The temperature program was 180-280°C at 5°C/minute, 15 m DB-1 megabore column.

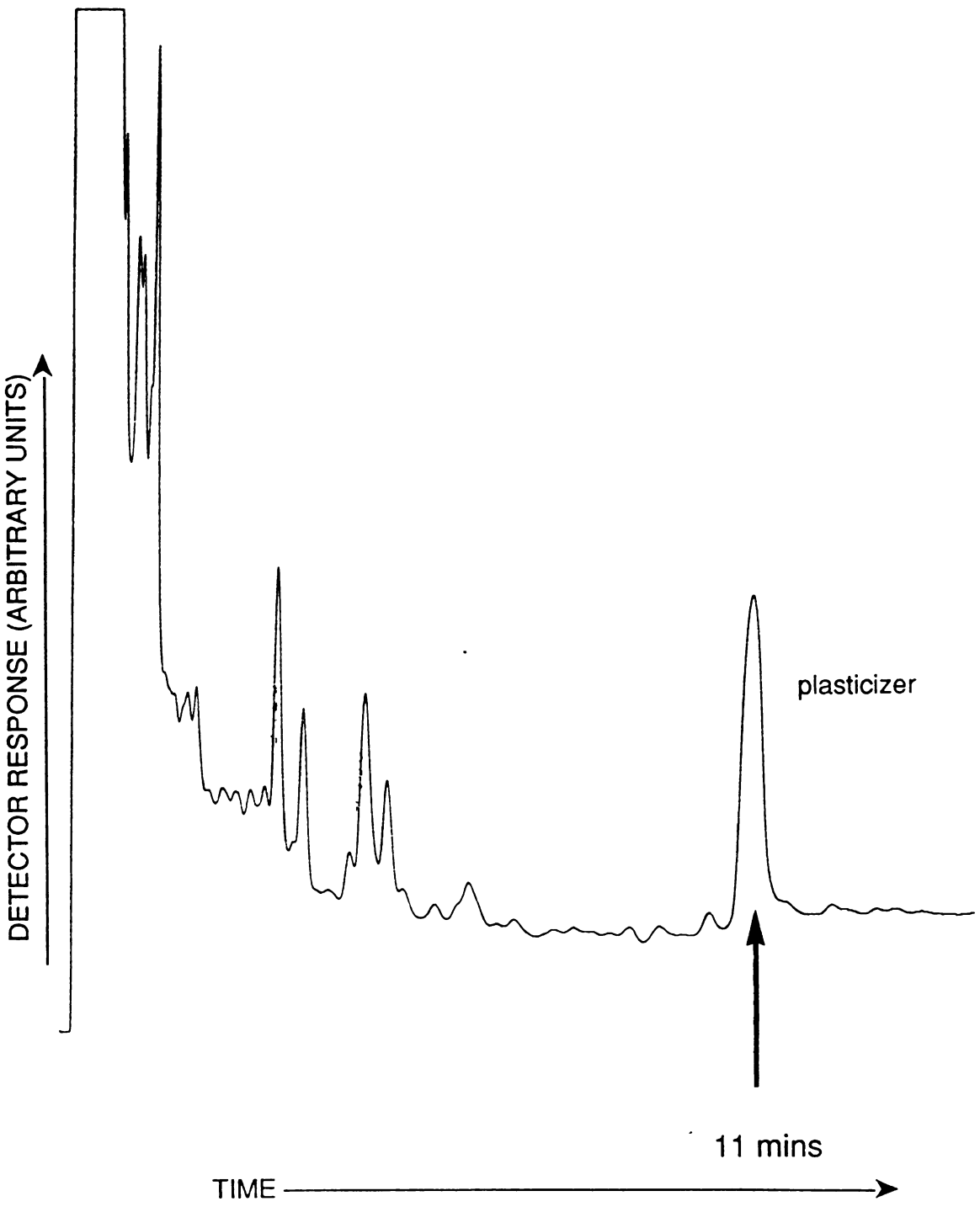


Figure 3-16. The Sigma 3B GC-ECD chromatogram of 15-HETE-ME made by the Graff procedure after DDQ oxidation. The temperature program was 180-280 5°C/min, 6 ft. OV-1 packed column.

There were no ions that would confirm the presence of the oxidized 15-HETE ME anywhere in the GC-MS data recorded for the sample. Thus, the fraction that had been collected by HPLC was, in fact, a phthalate ester, and not 15-HETE. Since the phthalate species absorbed at 235 nm, the λ_{max} for phthalate esters was researched. According to the Handbook of Chemistry and Physics the maximum absorbance for n-butyl phthalate is 225 nm. Since the concentration of the phthalate in this sample was so large the absorbance at 235 nm was still large.

LANDS PREPARATION OF 15-HETE

The Graff procedure never produced worthwhile yields of 15-HETE. A method by Lands *et al* (21) has proven to be the most successful of all those tried previously. The actual method has been modified from this paper for the best yields. The differences in the Lands method over all the others are that the reaction is conducted at 30°C and phenol is added as a radical scavenger. Precaution was paid to silanize all the glassware used. The reaction worked well because the paper was carefully written. The experience gained from the previous failures was also helpful in determining the success of the procedure.

In a one dram vial, a solution of 50 mg of arachidonic acid is prepared in 3.0 ml of ethanol. In a silanized 250 ml Erlenmeyer flask, 100 ml of 0.1 M Tris-HCl buffer (pH=8.5-9.0), and 6.3 mg of phenol (antioxidant), (Fischer) is added with 3.6 mg (260,000 units/mg) soybean lipoxidase (ICN Biochemicals). The enzyme solution is stirred vigorously at room temperature while oxygen is bubbled in at a rate of at least 30 ml/min. The arachidonic acid solution is added to the enzyme solution over a 5 minute period. The substrate/enzyme solution is stirred with oxygen for an additional 5 minutes and then 5.0 ml of 1M citric acid is added along with 50 ml of diethyl ether. (The diethyl ether is capped with aluminum foil liner.)

This mixture is transferred to a silanized 500 ml separatory funnel and the ether phase collected. The water phase is washed an additional two times with 50 ml of ether. The ether fractions are pooled and washed three times with water. The ether fraction is then placed in a silanized 250 ml Erlenmeyer flask with anhydrous sodium sulfate. After drying, the ether solution is transferred to a silanized 500 ml round bottom flask and evaporated to dryness on a rotary evaporator. The residue is dissolved in a solution of $NaBH_4$ in methanol and reduced as previously described. The 15-HETE is extracted and prepared for HPLC purification by finally dissolving it in the HPLC mobile phase (found in the next paragraph) or pure HPLC hexane.

An aliquot of the prepared 15-HETE is methylated with diazomethane and the TMS ether is made with BSTFA/pyridine. The GC-EI-MS spectrum and the reconstructed mass chromatograms are obtained to determine (1) existence and (2) purity of the nascent 15-HETE. If only one compound is present in the GC profile and that compound is 15-HETE, further purification is unnecessary. However, if there is more than one compound in the GC trace, HPLC is highly recommended.

It can be seen by the reconstructed total ion current (TIC) in Figure 3-17, that there is more than one compound in the newly made 15-HETE. The upper trace of the molecular ion m/z= 406 identifies which peak is actually an HETE. The mass spectrum in Figure 3-18 show the fragmentation that is expected for the 15-HETE isomer. Thus the presence of other compounds makes it necessary for purification.

The HPLC purification procedure is from Porter *et al* (9). The purification is accomplished on a normal phase (NP) preparative column (EM Science LiChrosorb Si-60, 10 mm, 250mm X 10mm). The mobile phase used, hexane/isopropanol/acetic acid (989:10:1), elutes 15-HETE in less than 20 minutes. The flow rate is set at 5 ml/min. The Beckman HPLC system used was a with the UV detector configured for 229 nm. (The cadmium lamp and the 229 filter must be placed in the detector.) The 15-HETE is

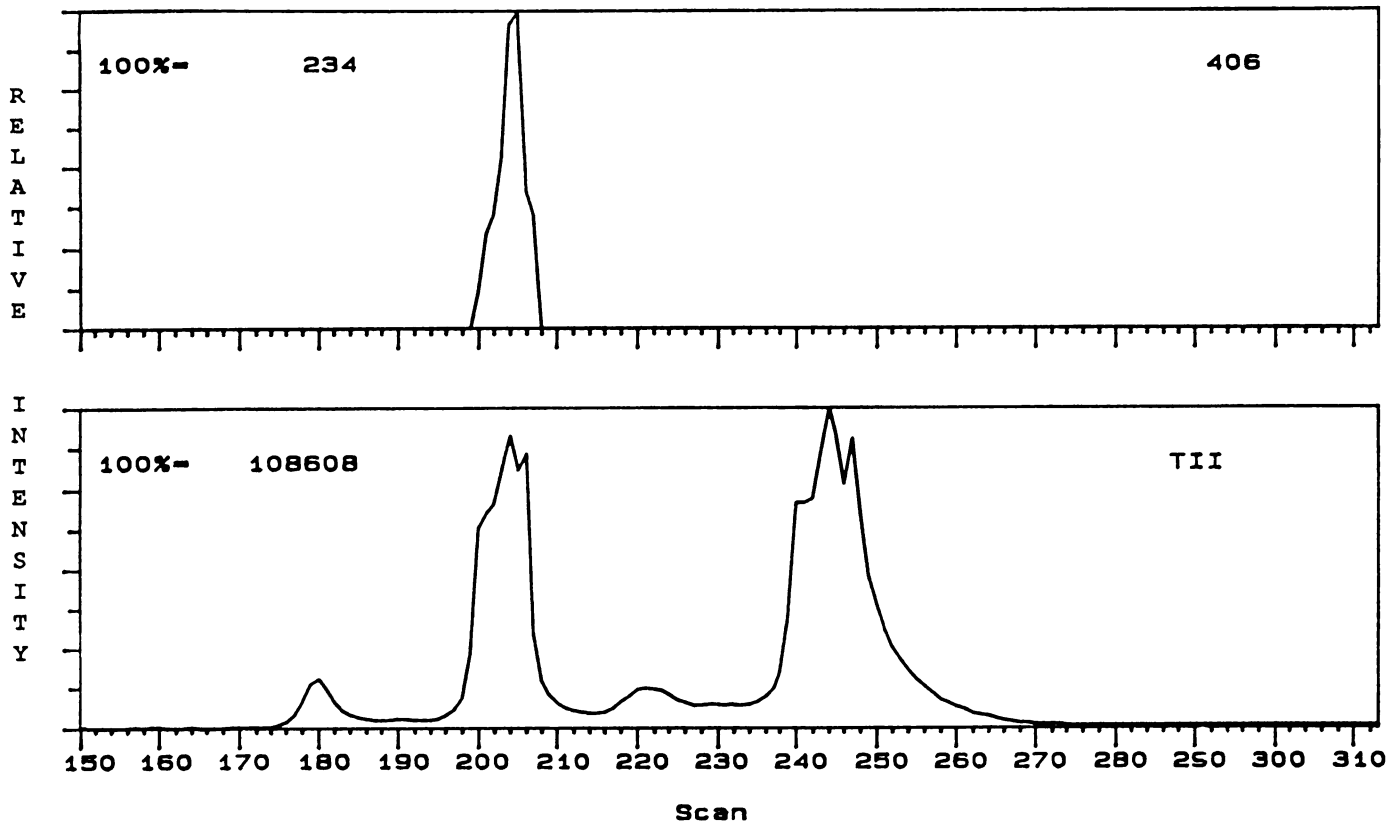


Figure 3-17. The GC-EI-MS reconstructed mass chromatograms of synthesized 15-HETE as the methyl ester, TMS ether. The lower trace is the total ion current (TIC) profile, showing all the eluting compounds. The upper trace at m/z= 406, the molecular weight of 15-OTMS ETE-ME, shows which peak is the desired compound.

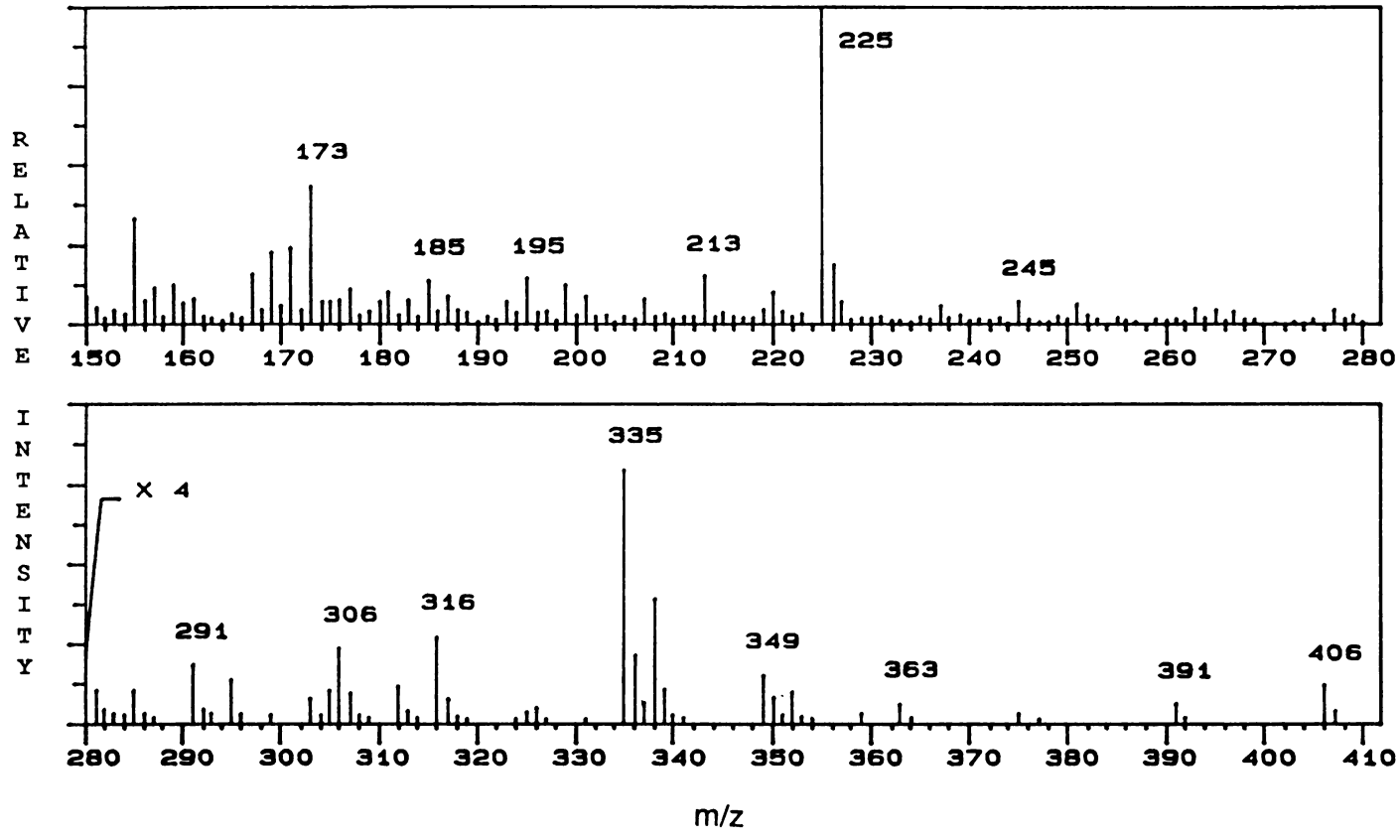


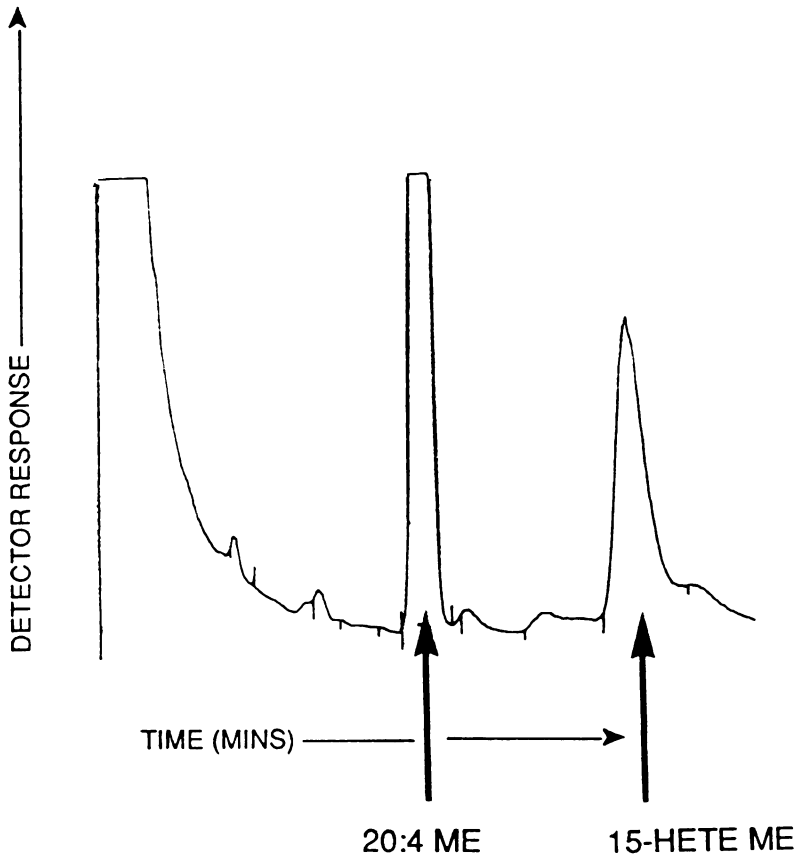
Figure 3-18. The EI mass spectrum of the peak in Figure 3-17 that corresponded to the derivatized 15-HETE. The fragment ion at m/z= 335 confirms the position of the hydroxy group to be on the ω-6 carbon.

injected into a 100 µl loop and the detector range is set at 2.0 AUFS. The 15-HETE should be in a reasonable amount of mobile phase- ca. 1-2 ml; this decreases the amount of injections needed and the preparative column can handle a large amount of material without showing overload.

The 15-HETE is collected in a silanized-glass, screw-top bottle (125 ml) with a teflon-lined cap. The fractions are pooled and placed in the 500 ml silanized round bottom flask and the mobile phase is removed *in vacuo*. The residue is finally reconstituted in hexane/ethyl acetate 1:1 (2 ml) and stored in the freezer in the dark.

An aliquot of the normal phase (NP) HPLC purified 15-HETE is methylated with diazomethane. It is co-injected into a GC-FID with a known amount of 20:4-ME (Figure 3-19). The area response of the compounds is directly related to their weights and thus the mg/ml for 15-HETE-ME (15-HETE) can be calculated by ratio. 15-HETE can be quantified by comparing the ratios of areas generated by GC-FID of 15-HETE-ME to standard solutions 20:4-ME. This is accurate because the FID response is based primarily on the number of carbons present in a compound. The response increases as the number of carbons increase. The only difference between 15-HETE-ME and 20:4-ME is one oxygen so the ratios of the areas can be related to the concentration.

The above method has produced milligram quantities of the 15-HETE for our laboratory. When starting with 50 mg of arachidonic acid approximately 6% yield is expected. The HPLC purification gives reasonably pure product with no arachidonic acid contamination without using the silicic acid column. The $NaBH_4$ is not a problem unlike the triphenylphosphine. Triphenylphosphine and the silicic acid column which are used in the Lands' article produced problems that could not be tolerated. These problems are explained in the following paragraphs.



1.25 µg of 20:4-ME y µg of 15-HETE-ME

1.25 µg/2.3 x 10^{+6} area units= y µg/ 1.0 x 10^{+6} area units

y= 0.35 µg

Figure 3-19. The GC-FID response comparison of 1 µl each of 20:4-ME and 15-HETE-ME. The area for 1.25 µg of 20:4-ME was compared to the area for an unknown amount of 15-HETE-ME. This procedure was used to determine the percent yield for the lipoxygenase reaction.

Initially, the residue from the enzyme reaction was applied to a 2 g silicic acid column that had been equilibrated with 90:10 pet ether/diethyl ether. The column was connected to a Milton-Roy LC pump to increase the flow rate. Forty ml of 90:10 pet ether/diethyl ether eluted through the column. This removed unreacted arachidonic acid. The 15-HPETE then eluted with 50 ml of 80:20 pet ether/diethyl ether. The eluent was transferred to the 500 ml round bottom and the mobile phase evaporated. This procedure was eliminated because the 15-HPETE was coeluting with the arachidonic acid and much of it was being discarded. Then the residue from the round bottom flask was dissolved in diethyl ether and triphenylphosphine (TPP) added to reduce the 15-HPETE to 15-HETE. The molar ratio of HPETE:TPP was 1:1.5. Since there was no way of assessing the amount of 15-HPETE present, 1.5 times the initial molar amount of arachidonic acid should provide adequate excess of TPP. This reaction continued for 30 minutes at 0°C. The TPP was removed by placing the reaction mixture onto the silicic acid column again and repeating the elution procedure. The TPP was to be removed in the 90:10 mobile phase and the 15-HETE eluted in the 80:20 solvent. The solvent is again removed *in vacuo* and the 15-HETE is stored in hexane in the dark at 0°C. Unfortunately, the use of the silicic acid column greatly reduced the yield of the 15-HETE. For this reason the use of this column was completely removed from the procedure.

The TPP used proved to be a source of contamination during HPLC purification. The reaction for the reduction by TPP is shown below:

$$\text{TPP} + \text{HPETE} \longrightarrow \text{TPP-oxide} + \text{HETE}$$

The oxidized TPP or TPP-oxide absorbs at λ_{max}= 228 nm. It is very soluble in alcohol. The TPP-oxide was supposed to be removed by the silicic acid column, but it was not. The TPP-oxide was so concentrated that some of it was collected from the HPLC instead of 15-HETE. In a gas chromatographic analysis of the collected TPP-oxide, it was also found that the TPP-oxide elutes from a DB-1 column at approximately the same retention

time as 15-HETE ME. This makes it thoroughly impossible to use. This is why the method was changed to employ $NaBH_4$ as the reducing agent.

OTHER IMPORTANT ISOMERIC HETES

The enzymatic preparation of 5-HETE cannot be accomplished with soybean lipoxygenase; only the ω-6 position of arachidonic acid is oxygenated with soybean lipoxygenase. The plant enzyme which yields 5-HETE is potato or tomato lipoxygenase. These enzymes are not commercially available. However, there are documented procedures for the production of 5-HETE. They involve the use of plant enzymes which have to be purified, animal enzyme or chemical synthesis for the generation of the physiologically important HETE. Corey has found the potato lipoxygenase to be cleaner than tomato lipoxygenase (22). These enzymes must be purified from the vegetables directly. This was not attempted here. 5-HETE may also be generated through synthetic means. Corey has developed preparation schemes for both racemic and stereochemically pure 5-HETE (23). They are larger scale preparations that are quite difficult.

Animal lipoxygenases are much more specific as compared to the plant enzymes, probably because the available plant enzymes are not 100% pure. The first discovered animal lipoxygenase was 12-lipoxygenase in blood platelets. The product that had been isolated from the tissue was 12-HETE. The enzyme produces 12-HPETE but it is highly probable that glutathione and glutathione peroxidase or some specialized enzyme (24) are present so the HPETE is easily reduced. See Table 3-4 for a summary of cell specific lipoxygenases and their products.

Since there are so many different sources of the HETEs the methodology developed here will be useful for many applications. It will be especially useful for preliminary GC-ECD screening where other general derivatization techniques have proven be be useless due to the high background from the matrix.

TABLE 3-4. SPECIFICITY OF ANIMAL LIPOXYGENASES

CELL	(species generated) species isolated
rabbit polymorphonuclear leukocytes	(5-HPETE) 5-HETE
rat mast cells	5-, 11- and 15-HETE
VX_2 carcinoma	11- and 15-HETE
guinea pig lung	11-, 12- and 15-HETE
human T lymphocytes	5-, 11-, 12-, and 15-HETE
rabbit aveolar macrophages	12- and 15-HETE
platelets	8-, 10- and 12-HETE
eosinophils	15-HETE
mouse peritoneal macrophages	12-HETE
rat pituitary and vascular tissue	"
human epidermis	"
peritoneal, blood and lung monocytes	5-, 12- and 15-HETE
neutrophils	5-HETE, 15-HETE
endothelial cells	15-HETE, 11-HETE

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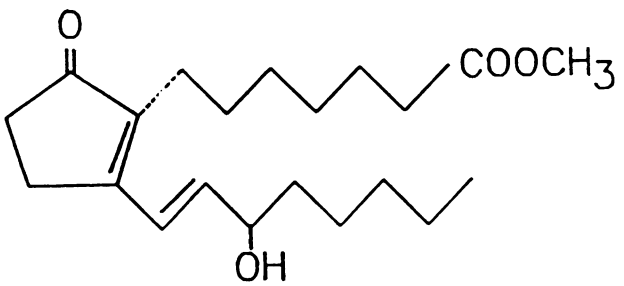
CHAPTER IV: DEVELOPMENT OF METHODOLOGY

This chapter highlights the main goal of this research project which involved developing a new strategy for the analysis of allylic alcohols by GC-MS. As is presented in chapter II, the most amenable mass spectrometric ionization technique, ECNI, requires the analyte to be electrophilic. This chapter discusses the progress of an alternative procedure which leads to the formation of an analyte with inherent electrophilicity. The stepwise manner in which reaction conditions were optimized for the various reagents used is illustrated. The importance of refining the methodology for samples containing biological concentrations of analyte is addressed. The optimized scheme is compared to the existing approach for the analysis of the allylic alcohols. Finally, suggestions for the continued improvement of the new method are stated.

When the allylic hydroxy unsaturated acids were prepared from arachidonic acid and linoleic acid, they were oxidized to α, β–unsaturated ketones. The labile hydroxyoctadecadienoic acids (HODDs) and hydroxyeicosatetraenoic acids (HETEs), which were not electrophilic, were converted to the unsaturated ketones. In general, many compounds containing α, β–unsaturated ketones had already shown electrophilic character (1, 2) especially those in steroids (3, 4). A goal of this research was to determine if oxidizing these eicosanoids would make them electrophilic. A specific example of an eicosanoid that has been analyzed by detectors that base their response on the electrophilicity of the analyte are the B prostaglandins (5). The structure of prostaglandin B_1, PGB_1, is shown in Figure 4-1. Later in this chapter, PGB_1 is discussed in detail because it was used in methodology studies.

The interest in selectively oxidizing HETEs and HODDs had originated with the successful experiments conducted in the area of oxidation of exogenous steroids. The improvement in volatility and electrophilicity of the steroids after oxidation, made them

amenable to analysis by ECNI-MS. For example, the corticosteroid dexamethasone becomes electrophilic when it is oxidized eliminating the need for a general electron-capturing derivative. The use of a selective oxidation reagent over a general derivative sometimes even eliminates tedious sample preparation.



PGB_1 ME

Figure 4-1. The structure of PGB_1-ME, MW= 348.

The success of the steroid project inspired the interest in oxidation as an alternative to derivatization. The physiological interest in eicosanoids as well as their structure made them an excellent choice as an analyte that had the potential for selective oxidation. The derivatization procedure for hydroxy unsaturated acids had been well established by others, but the derivatives did not always lead to a compound that had improved vapor phase properties over the original analyte. An example of this is found in the chapter II discussion of the derivatization of 15-HETE.

Another problem with a general derivative is the likelihood that other endogenous compounds may be derivatized along with the analyte. If these species are of a higher concentration than the analyte, severe interferences may result. Electrophilic derivatives of eicosanoids that were originally designed to be used in gas chromatography with an electron capture detector (ECD) are a good example of this problem. In a biological sample, the pentafluorobenzyl ester (PFB) derivative greatly enhances the response of the

matrix as well as the analyte. If the background is high, the sample cannot even be qualitatively evaluated without the aid of GC-MS, since GC-MS can selectively monitor for the analyte. An ECD chromatogram of a cell extract after PFB, TMS derivatization is shown in Figure 4-2A. It can be seen that little information can be obtained. An ECD chromatogram of the same sample selectively oxidized (Figure 4-2B) illustrates that, in this case, there is the ability to determine the presence of the analyte immediately.

For this project, the lipoxygenase eicosanoids of interest were those compounds that contained an allylic alcohol (HETEs and leukotriene B_4, LTB_4). The allylic alcohol has the advantage of being selectively converted to an α, β–unsaturated ketone by mild oxidizing reagents. The specific reagents that were tried in this research were manganese dioxide (MnO_2), pyridinium dichromate (PDC) and dichlorodicyano-benzoquinone (DDQ), all purchased from Aldrich. Initially MnO_2 was used successfully to oxidize the 13-HODD to 13-oxo-ODD. All three reagents were researched as possibilities for the oxidation of the HETEs. Each converted 15-HETE to 15-oxo-ETE but the advantages of one reagent over another had to be evaluated. It is felt that PDC or MnO_2 are the most worthwhile reagents to use. Dichlorodicyanobenzoquinone has caused many problems and it should not be used. This chapter presents the data and the methodology developed for each reagent. Also, the sensitivity and chromatographic properties of 15-oxo-ETE compared to the presently established methodology for HETE analysis (6).

THE SELECTIVE OXIDATION OF THE HODDS

The mildest reagent which has been used extensively in this research is MnO_2. Manganese dioxide is a fine black powder which oxidizes the analyte in a heterogenous manner. The reaction mechanism is thought to be triphasic (7); the substrate adsorbs to the MnO_2, is oxidized, and then desorbs from the MnO_2. There is an equilibrium on the MnO_2 surface between the substrate and its oxidized product, and reactions never reach

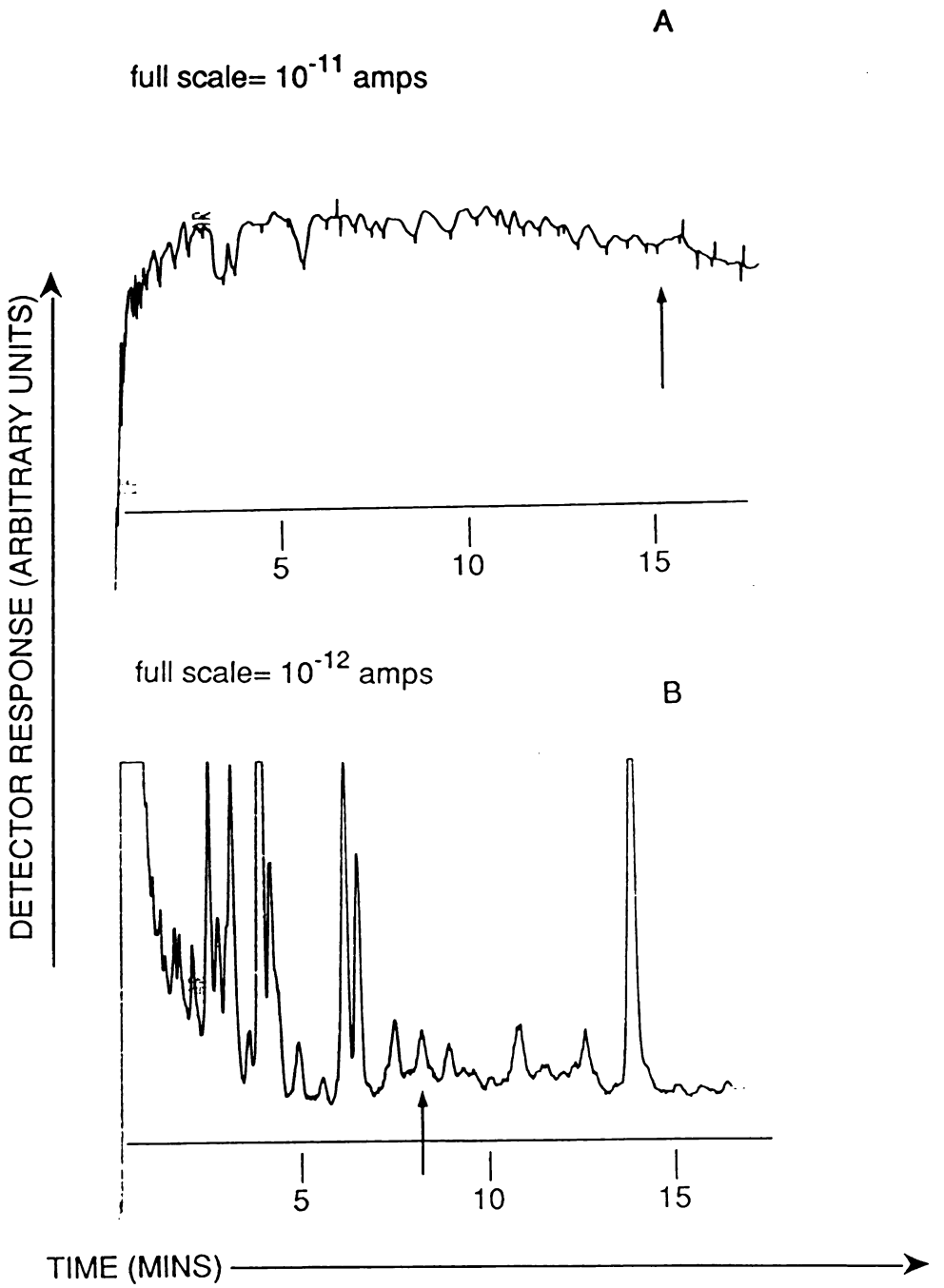


Figure 4-2. The ECD chromatograms of a fraction from cell wash that should contain 12-HETE. (A) The wash derivatized as the PFB ester, TMS ether. (B) The same fraction was selectively oxidized and then methylated with diazomethane. The arrows point to the appropriate elution time of the 12-HEA, PFB ester, TMS ether (A) or the 12-oxo-ETE-ME (B).

100% completion (7). The more readily oxidized compounds are those that form a stable carbonium ion, which is why the allylic alcohol is selectively oxidized. The reaction mechanism for the MnO_2 oxidation of benzyl alcohol has been postulated (8).

The oxidation is generally completed in CH_2Cl_2 or petroleum ether (pet ether). It has been demonstrated that the MnO_2 oxidation proceeds at a much higher rate in pet ether (9) than in CH_2Cl_2. Thus, it is the preferred solvent. The difficulty in using MnO_2 as the oxidizing reagent is realized when an attempt is made to extract the analyte from the reagent. The fine black powder is not easily removed. In preliminary studies, exhaustive filtering with a 0.45 μm pore filter proved to be the most facile way of removing the MnO_2. Most recently, MnO_2 has been removed from the pet ether by centrifugation. The solid MnO_2 is then washed 2-3 times with excess pet ether to completely remove the analyte from the MnO_2 precipitate in the centrifuge tube. The development and optimization of the MnO_2 oxidation are presented in the following paragraphs.

The initial preparation of an α, β–unsaturated ketone from an allylic alcohol was accomplished with enzymatically prepared 13-HODD. The MnO_2 reaction and its selectivity are shown in Figure 4-3. The oxidation of the 13-HODD was accomplished with MnO_2 in petroleum ether. The reactions was conducted for 3 hours. The reaction was stirred constantly to expose fresh MnO_2 to the analyte. The MnO_2 was removed by passing the solution through a filter. The EI mass spectrum of the 13-oxo-ODD-ME from the reaction is presented in Figure 4-4A. The fragmentation pattern confirmed that the α, β–unsaturated ketone had been prepared. The methodology had to be refined to yield reproducible and complete oxidations. This also involved perfecting the extraction protocol so that the analyte would be separated from the oxidizing reagent. The refined methodology would then be applied to the oxidation of the HETEs.

The MnO_2 oxidation was optimized by an exhaustive study where the reaction

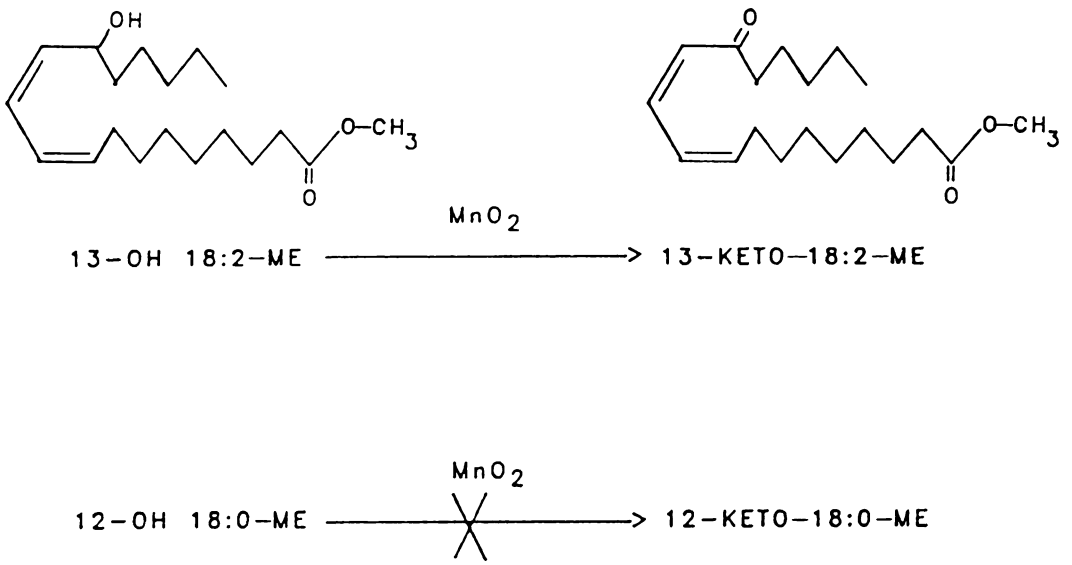


Figure 4-3. MnO_2 can oxidize only allylic alcohols under most reaction conditions.

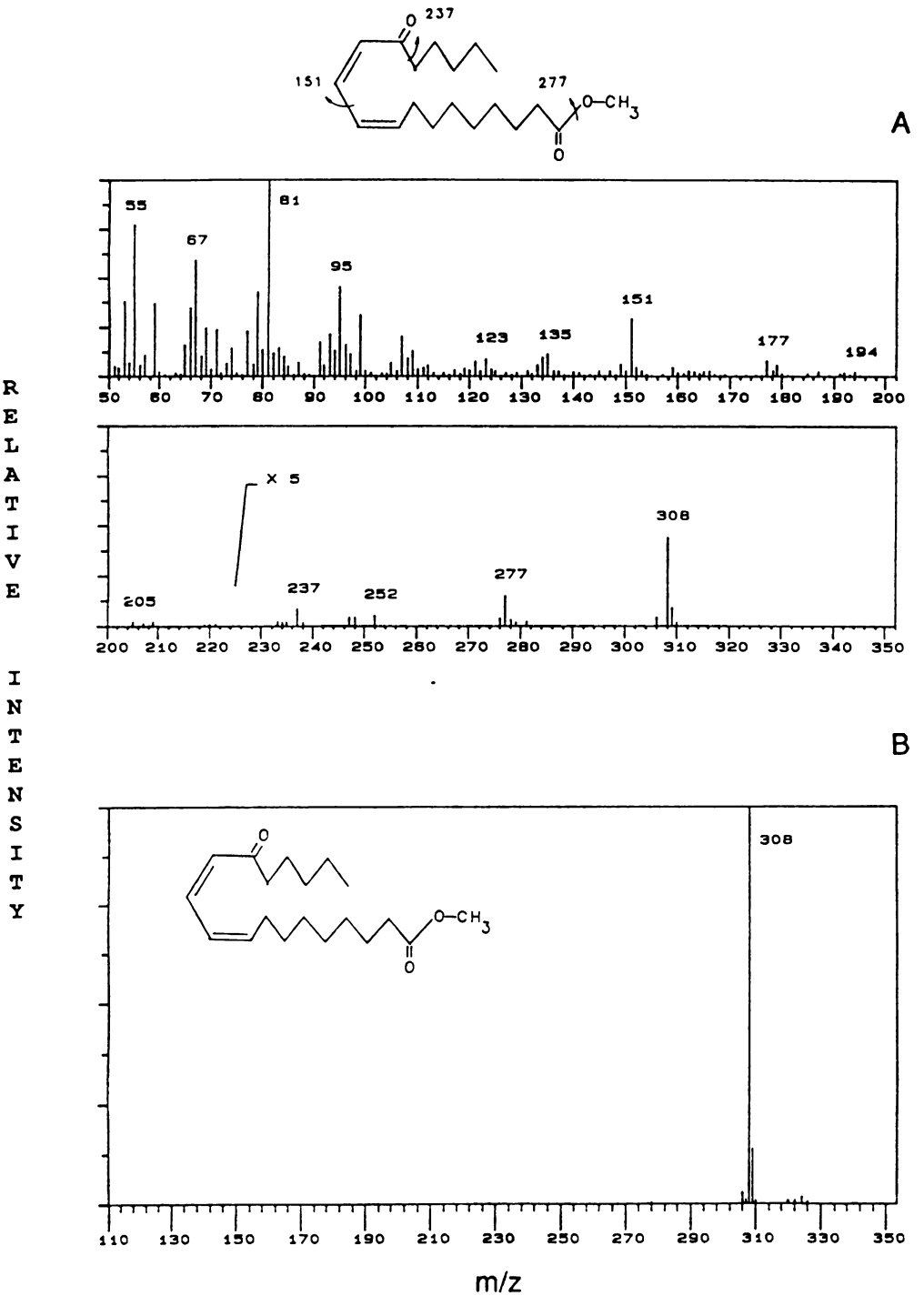


Figure 4-4. The (A) EI and (B) ECNI mass spectra of 13-oxo-ODD-ME (MW= 308), the MnO_2 oxidized 13-HODD.

time, MnO_2 stoichiometry and extraction method were varied. The conversion of the analyte to the desired product was measured. An optimum reaction would yield at least an 80 percent conversion of the analyte to the desired product in a reproducible manner. The analyte used for the study was a photooxidized product of linoleic acid. It was found that the optimal conditions included a 6-hour reaction time with a 25-fold excess of MnO_2 by weight which was extracted with a series of 0.45 μm pore filters. The percent conversion of 13-HODD to 13-oxo-ODD is presented in Table 4-1.

TABLE 4-1. THE OPTIMIZATION OF THE MANGANESE DIOXIDE REACTION

HOURS FOR REACTION	PERCENT CONVERSION
3	85
6	97

After their preparation, it was important to determine the electrophilicity of the α, β–unsaturated ketones by gas chromatography with an electron capture detector (ECD). The conjugated carbonyl compounds capture a thermal electron by resonance capture and a negative ion is formed. In terms of signal response FID chromatograms of the α, β–unsaturated ketones could be compared to the electron impact (EI) total ion current (TIC) profiles, but the electron-capture negative ionization (ECNI) TIC profiles would resemble the ECD chromatograms. A problem that arose when initially trying to analyze the α, β–unsaturated ketones was that there was no way to correlate when they would elute from a gas chromatograph with electron capture detection as compared to a gas chromatograph with flame ionization detection because the columns installed in the two detectors were different. In order to relate retention times of the ECD chromatograph (Perkin-Elmer Sigma 3B) to that of the FID chromatograph (Varian 2100), marker compounds were needed. The column in the Sigma chromatograph was

much shorter and the chromatograms looked entirely different than those examined by FID. Hydrocarbon markers normally used with FIDs could not be used; they were not electrophilic. Markers were needed that were FID/ECD compatible, i.e., electrophilic and contained more than 12 carbons.

The importance of marker compounds must be stressed. Typically, when a FID is used, hydrocarbon standards are coinjected to allow for the calculation of retention indices (RIs). The "picket fence" produced by the hydrocarbons is used for this purpose. Changes in flow rate and column packing result in retention times that are variable; with absolute numbers that may vary by a half a minute or more. Changes in temperature programming, column length or instrument will also greatly affect the retention time. It is more advantageous to calculate the retention indices because the number is a ratio and does not vary as much.

A series of compounds that would mimic the picket fence effect of the hydrocarbon standards was desired. It was eventually concluded that pentafluorobenzyl (PFB) esters of fatty acids would work. Since they had to be prepared, it was advantageous that the procedure was relatively simple. Initially monobrominated hydrocarbon standards with 14,16, and 18 carbons were tried but they eluted too quickly. Pentafluoropropionyl (PFP) derivatives of fatty alcohols were not retained long enough either. Finally 2-bromohexadecanoic acid was examined. It was commercially available from Aldrich. When methylated it could be used as the early eluting goalpost. (Because only two markers were actually needed, the use of the word goalpost reflects the appearance of the markers in the chromatogram.) The later eluting goalpost was more difficult to find. Monobrominated 18- and 20-carbon acids unfortunately were not commercially available. An undergraduate student was assigned the task of determining a useful compound. The compounds tried were 20:4, 12-OH 18:0, 16:0 and 18:0. The PFB esters were made of each of these acids as was the TMS ether of the free hydroxyl

group in 12-OH 18:0. Both 20:4 and 12-OH 18:0 were found to be ineffective markers. 20:4 PFB had too long of an elution time and the hydroxy acid took too long to make. However, the PFB esters of 16:0 and 18:0 eluted well after the 13-oxo-ODD ME. The PFB ester of 18:0 has been employed as the ending marker in this research. Other students in our laboratory have used the series of PFB esters as markers for ECD and ECNI-MS to determine when compounds elute from a gas chromatograph as compared to a gas chromatograph-mass spectrometer. Chromatograms of the markers used in this research obtained with both an ECD and a FID are presented in Figure 4-5.

At first the electrophilic nature of the analyte could be tested by GC with the ECD only. The technique of ECNI-MS had not been learned. The electrophilic character of the 13-oxo-ODD-ME was determined with the aid of marker compounds. First, the oxidized sample was coinjected into a chromatograph with the markers and detected by a FID. The retention index of the analyte was determined and the identity of the 13-oxo-ODD-ME was confirmed by GC-EI-MS. The same markers were then coinjected with the analyte into the ECD gas chromatograph. The retention index was calculated, and it was found that the 13-oxo-ODD did indeed have electrophilic characteristics. The 13-oxo-ODD-ME was then analyzed by GC-MS with methane ECNI conditions. The mass spectrum showed only one ion, the molecular ion. The mass spectrum is shown in Figure 4-4B

The response of the α, β–unsaturated ketone was compared to 12-OH 18:0 as the TMS ether, PFB ester derivative. The relative response of these two electrophilic molecules was 1:9 ketone: PFB ester. Figure 4-6 shows the comparison of various standard fatty acids analyzed by two methods, FID and EC-NCI (ECNI). The top trace shows the response to 60 ng of each compound by a FID. They are essentially the same because the FID is a universal, mass sensitive detector where the response is dependent on the number of carbons in the molecule. However, the selective detection by EC-NCI-

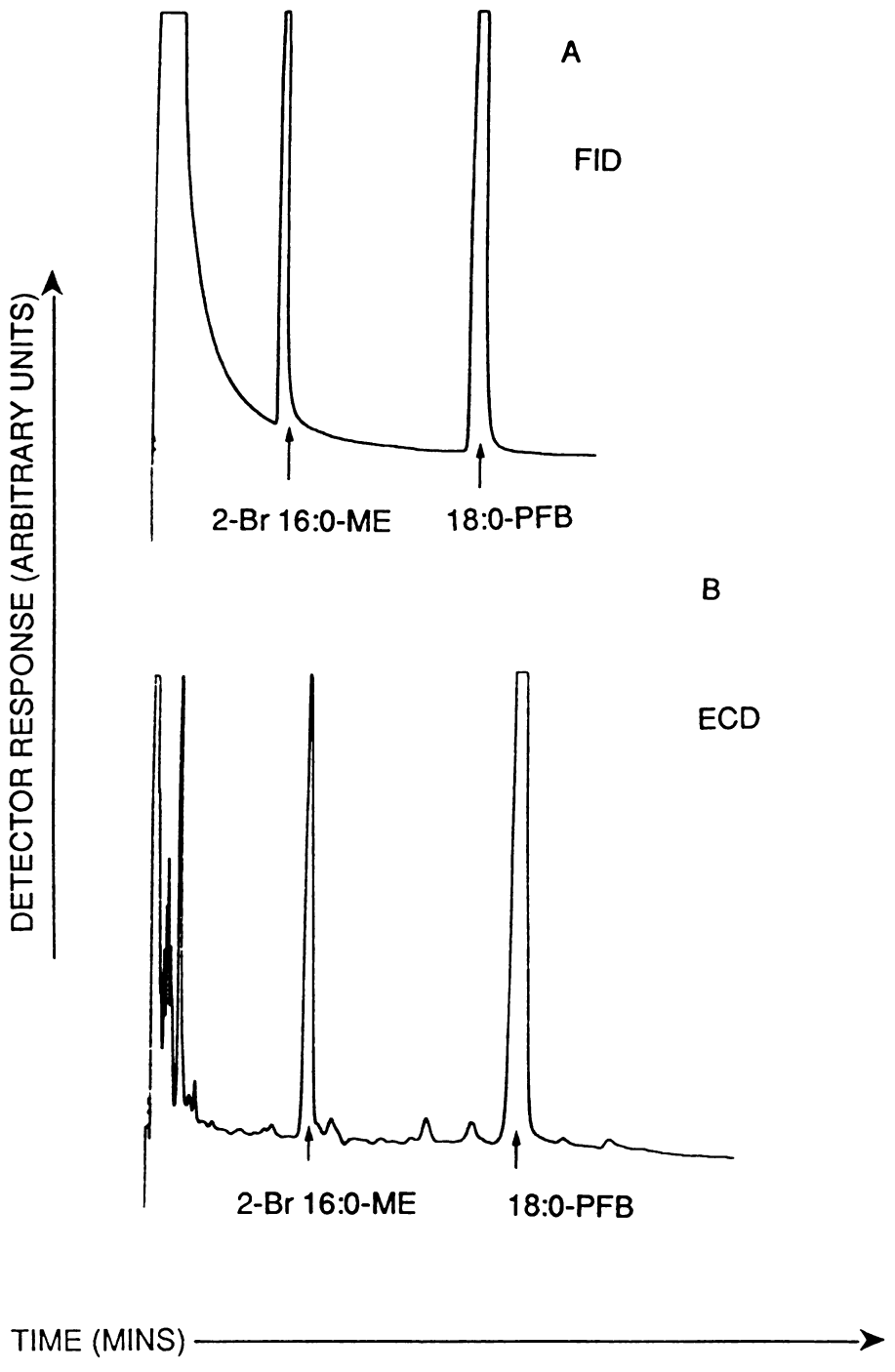


Figure 4-5. The (A) FID and (B) ECD chromatograms of the marker compounds 2-Br 16:0-ME and 18:0-PFB. The column was a J&W 15 m megabore DB-1. The flow rate was 15 ml/min. The temperature program was 180-280°C at 5°C/min.

MS shows a response only for 13-keto (oxo)-18:2-ME and 12-OTMS 18:0-PFB. For each compound approximately 100 ng of sample was injected on-column as compared to the 60 ng injected into the gas chromatograph. Another important feature of the oxidized 13-HODD over the 12-OTMS 18:0 PFB is the retention time. The PFB ester greatly increases the retention time of a compound as compared to a methyl ester. Also it has been determined experimentally in our research and by others (6) that the carbon-carbon double bonds of the allylic hydroxy acids have to be reduced before the PFB ester of the acid is made. This oxidation of the carbon-carbon double bonds improved the vapor phase properties of the original unsaturated compound as was explained in chapter II.

The success of the MnO_2 oxidation of 13-HODD to the 13-oxo-ODD made it reasonable to try its oxidation capabilities on 15-HETE. The preparation of copious amounts of 15-HETE was not easy; thus, many of the initial MnO_2 oxidations were conducted with small amounts of the substrate. Because MnO_2 was a heterogenous catalyst, it was possible that the oxidized analyte was not extracted from the solid reagent. Many preliminary reactions were tried, but the 15-oxo-ETE was never successfully detected by EI or ECNI GC-MS. At the time, it was felt that the lack of oxidized product was due to the mildness of the reagent. It was discovered later that MnO_2 does successfully oxidize 15-HETE to 15-oxo-ETE. The application of MnO_2 as an oxidizing reagent is recommended because of its mild properties, but extremely long reaction times (>10 hours) are necessary. This limits its usefulness.

THE SELECTIVE OXIDATION OF THE HETES

Although the original preparation of α, β–unsaturated ketones from lipoxygenase products of linoleic acid was accomplished using the reagent MnO_2, as discussed above, this had proved unsuccessful at first for the oxidation of the lipoxygenase products of arachidonic acid, the HETEs. Thus, it was necessary to explore other selective oxidizing reagents. The reagent had to have mild oxidative properties like

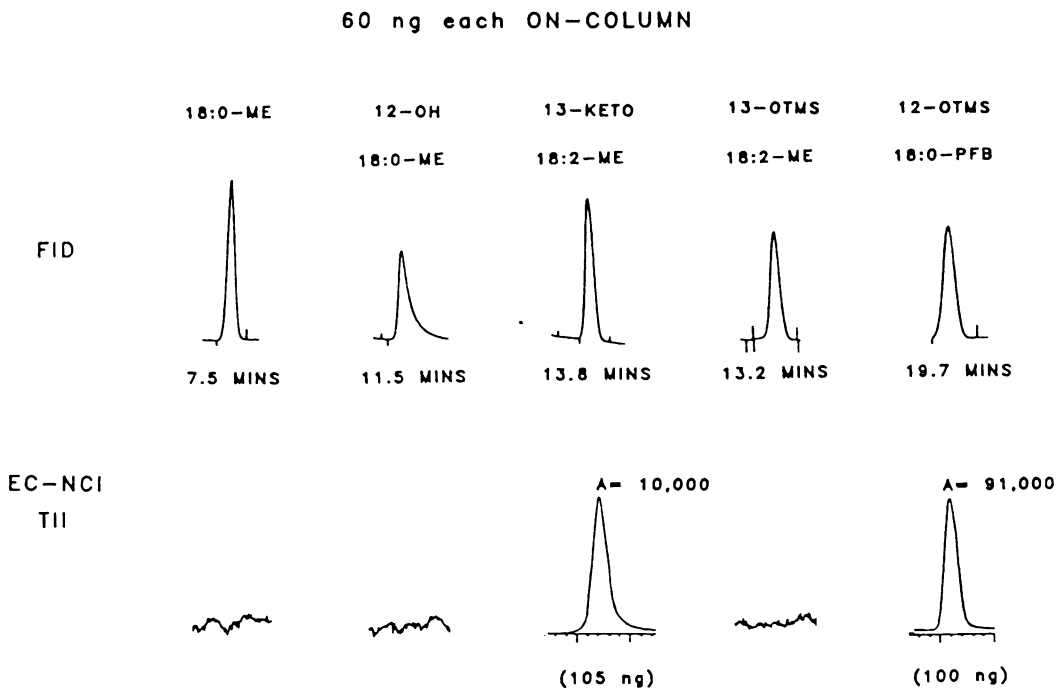


Figure 4-6. The chromatographic profiles of various compounds as detected by FID and ECNI (EC-NCI).

those of MnO_2. Most importantly, it had to oxidize allylic alcohols faster than isolated alcohols. It was necessary to know if the selectivity would be compromised if reaction conditions were modified from those presented in the literature. For example, the preferential oxidation by MnO_2 for allylic alcohols is decreased when higher temperatures are used during oxidation (7). This would lead to the production of unwanted secondary oxidation products. If the selectively was not compromised, would the oxidation form only α, β–unsaturated ketones? Some reagents may be strong enough to form carbon-carbon double bonds as well as carbonyl groups. The oxidation had to stop at the formation of the α, β–unsaturated ketone. Formation of various secondary products would yield compounds with different molecular weights and chromatographic retention properties. It was important that the analyte be converted to only one product.

Other beneficial qualities of a reagent had to be defined; the two most important were reaction time and sample clean-up. Short reaction times were beneficial because reactions that required 24 hours for completion would not lead to high sample throughput, making sample processing long and tedious. This would certainly outweigh the advantages of a new technique. If the reagent could not be removed easily or the percent extraction of the analyte was poor, the reagent was not desirable. The contamination of the analyte with unused reagent could produce high background during analysis or might further oxidize the analyte with time. Also, the inability to extract the analyte from the reaction mixture could make further analysis impossible. It was possible to choose the best reagent for the HETEs by assessing these attributes for a given reagent. Each allylic alcohol might require a slightly different reagent or a different reaction condition for the greatest success.

DDQ AND PDC

The oxidation reagent dichlorodicyanobenzoquinone (DDQ) was evaluated because publications (10, 11, 12) showed its selective oxidation of the allylic alcohol in the 3-position of gangliosides and sphingolipids. Other literature on DDQ disclosed its vast use in the oxidation of allylic alcohols of steroids (13). The preferential oxidation of allylic alcohols over saturated alcohols is shown in Figure 4-7. In both cases preferential oxidation of the allylic hydroxy moiety is demonstrated. Thus, DDQ had satisfied the major criterion of selectively oxidizing the allylic alcohols to α, β–unsaturated ketones. Unfortunately, another reaction which might occur along with the dehydrogenation of the alcohol is dehydrogenation to form a carbon-carbon double bond (13). Figure 4-8 shows how this occurs with steroids and how it might affect an isolated allylic alcohol.

The DDQ oxidation was found to be a kinetically favorable reaction with successful formation of the ketone. The reaction is shown in Figure 4-9. The HETEs were oxidized by reacting them with a 5:1 molar excess of DDQ for 2 hours. These parameters were determined to be the most successful for the primary production of the oxo-ETEs. The oxo-ETEs were purified by removing the reaction solvent under N_2, adding 0.1 M NaOH, and then extracting with diethyl ether. Unfortunately, there were problems with using DDQ as a reagent. First, it seemed to produce a multitude of secondary oxidation compounds. The appearance of dehydrogenation (loss of H_2) and dehydration (loss of H_2O) compounds, confirmed by EI and ECNI-MS, showed the reagent to be a much stronger oxidant than MnO_2. These compounds were present when the effectiveness of DDQ was initially tested on photooxidized arachidonic acid samples. The photooxidation had yielded the eight isomeric HETEs and possibly dehydration products. Since the initial mixture was complex, many of the compounds that were thought to be secondary oxidation products actually could have been products of the

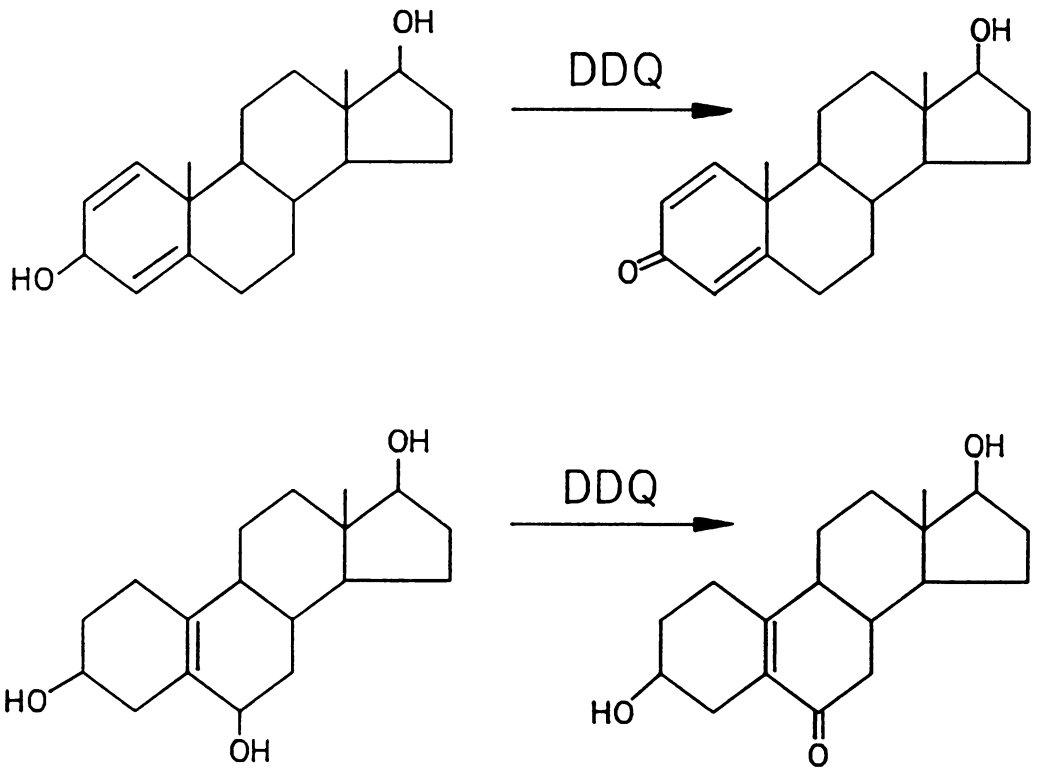


Figure 4-7. The selectivity of DDQ for allylic alcohols in steroids (from ref. 13).

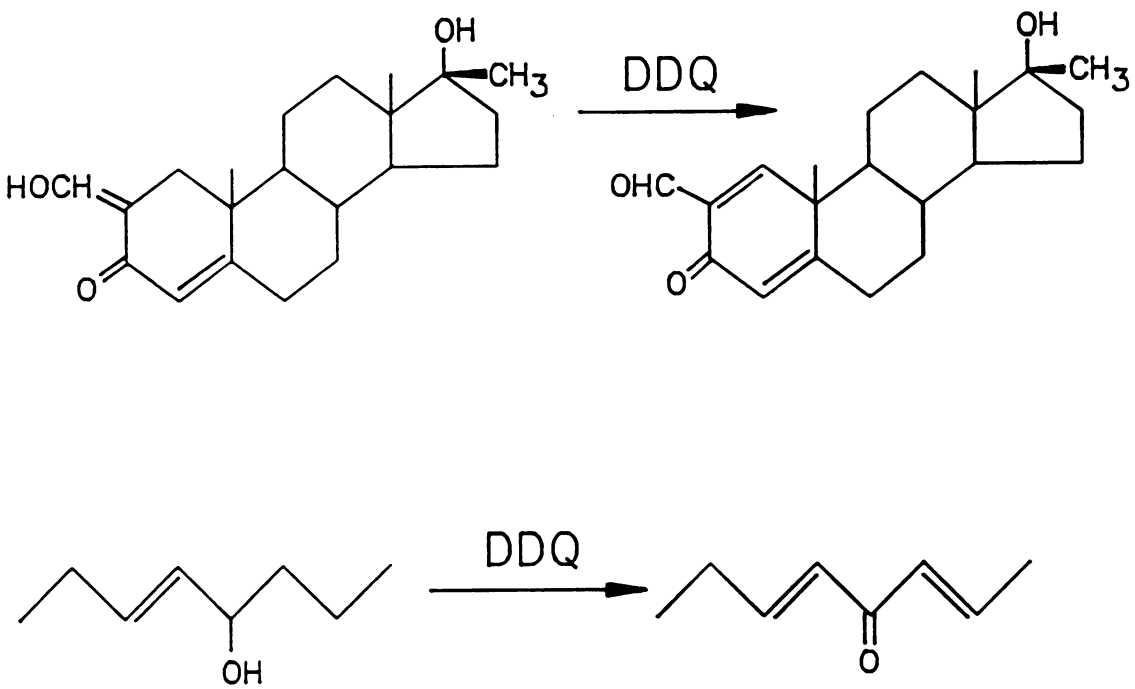


Figure 4-8. The ability of DDQ to oxidize carbon-carbon bonds and the possible effects on an allylic alcohol (from ref. 13).

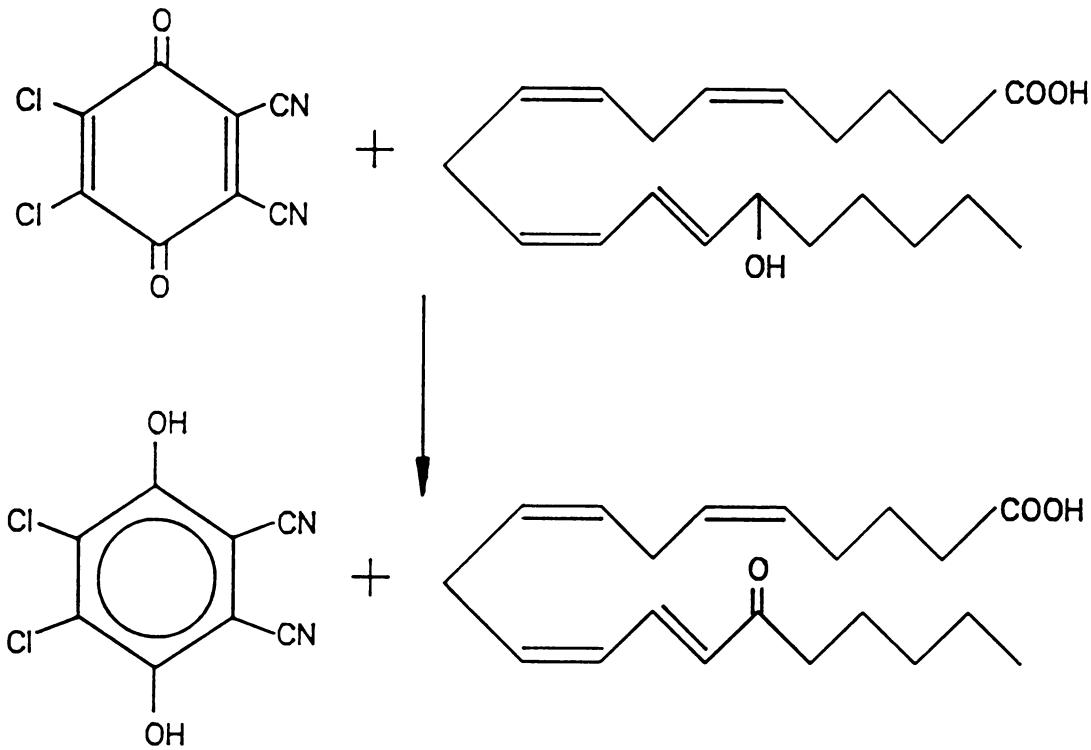


Figure 4-9. The reaction between HETEs and DDQ.

photooxidation. When purer standards became available it was discovered that the same types of products were formed when pure 15-HETE or 5-HETE was oxidized with DDQ. The presence of $(M - H_2)^+$ and $(M - H_2O)^+$ were concluded to be from the DDQ oxidation and not from the initial photooxidation.

Because of its complexity, the photooxidized HETE solution hindered determinations of the FID chromatographic properties of the newly formed α, β–unsaturated ketone. There was such an overlap of structurally similar compounds in one area of the gas chromatogram that the ketone products were not adequately separated from the initial reactants. This can be seen in the TIC profile in Figure 4-10A. Thus the gas phase properties of the oxo-ETE could not be determined at first. This problem was magnified by the fact that the electron impact mass spectrometry (EI-MS) reconstructed mass chromatograms of the oxidized HETEs showed the formation of compound which had a molecular weight two mass units less than expected which was consistent with the formation of a ketone and a carbon-carbon double bond . This phenomenon was reproducible and characterized by a series of fragment ion peaks appearing two mass units below those corresponding to the expected ketone. These compounds coeluted and made it impossible to accurately assess the vapor phase properties of the ketone. The GC-EI-MS molecular ion mass chromatographic profiles for the molecules are shown in Figure 4-10B and C. One of the reasons for oxidizing the allylic hydroxy group to the ketone was to improve the chromatographic properties of the allylic alcohol. The convoluted mass chromatograms had not produced conclusive results about the chromatographic characteristics of the HETEs. Also, the complexity of the sample had made it impossible to determine the optiminal oxidation conditions.

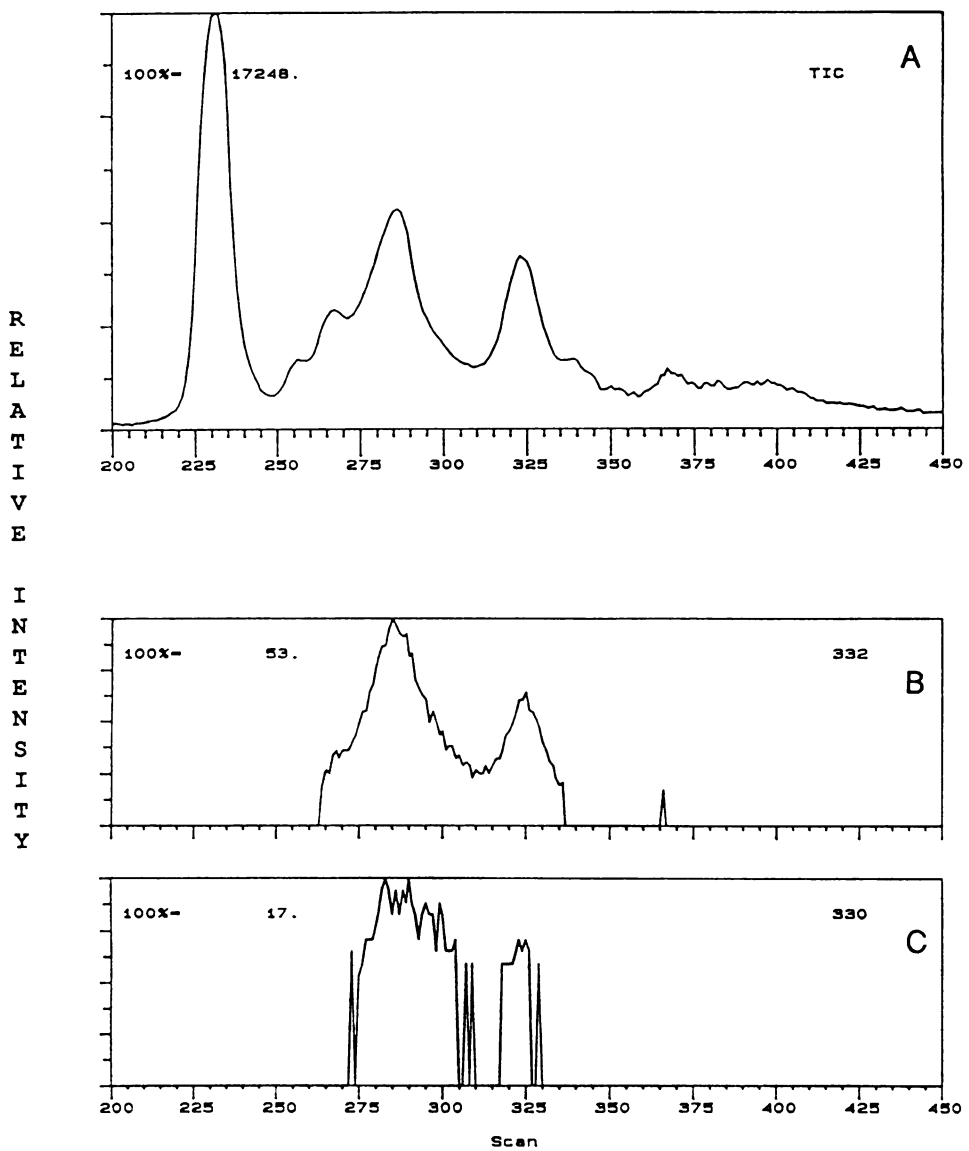


Figure 4-10. The TIC profile (A) and reconstructed mass chromatograms (B & C) for the oxidation reaction of DDQ with photooxidized HETEs. The molecular weight of the oxo-ETE-ME is 332; 330 is the molecular weight of the overoxidized unwanted secondary oxidation product.

Dichlorodicyanobenzoquinone and Prostaglandin B_1

Because of the confusing results obtained from using complex mixtures in the preliminary studies, it became necessary to chose a purer starting material. The reaction could be evaluated with 15-HETE, 5-HETE, or prostaglandins (PGs). The 15-HETE had not yet been prepared in sufficient quantities due to problems that were discussed in chapter III. The 5-HETE compounds had to be purchased and was quite expensive, therefore, experimental reactions with this compound were not economically feasible. It was then decided that prostaglandins be tried. These were used because they contain an allylic alcohol which is in the same position as that found in 15-HETE. They are pure, commercially available and reasonably priced. Reactions with dichlorodicyano-benzoquinone could be executed using a simple prostaglandin and then, when purified 15-HETE was available, approximate reaction conditions already would have been established. Prostaglandin B_1 (PGB_1), Sigma Chemical, was chosen due its structural simplicity. It had no isolated hydroxy groups in the 9 or 11 position like many of the other PGs. The structure of PGB_1 is found in Figure 4-1.

The solvent used in the DDQ oxidation greatly influenced the products generated. For example, the PGB_1 was initially reacted with DDQ in dioxane for 10 minutes, 1, 2 and 3 hours. The molar ratios of DDQ:PGB_1 were 18:1. In each case the reaction mixture contained four different compounds in different ratios. At first the most predominant compound was the unoxidized PGB_1. However, after 3 hours the most predominant compound was the dehydration product of PGB_1. They were identified by GC-ECNI-MS and reasonable structures that were deduced from the data are presented in Figure 4-11.

Originally it was not known if the various products were due to the action of DDQ, the solvent employed or the age of the PGB_1 which was about two months old and may have degraded. The choice of an appropriate reaction solvent had been based on the

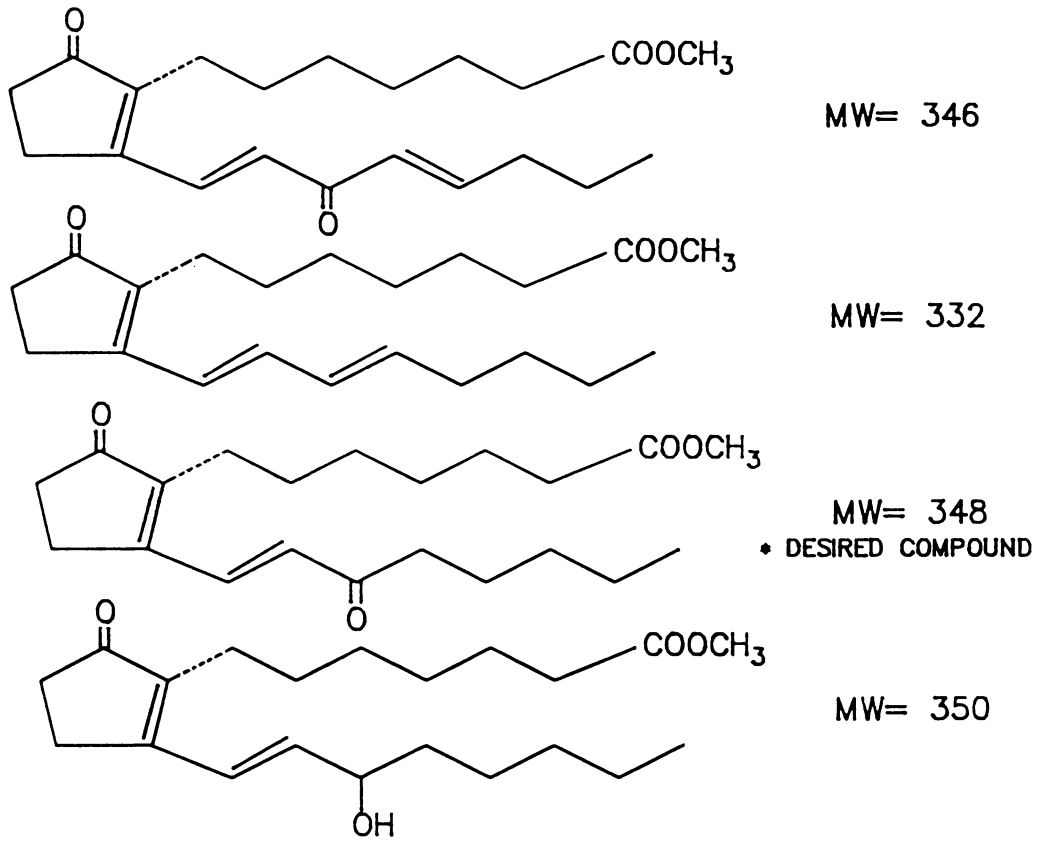


Figure 4-11. The possible products of the reaction of PGB_1 with DDQ/dioxane which are consistent with the ECNI mass spectrometric data and comments from ref. 16 which pertain to the formation of secondary oxidation products.

solubility of DDQ (13). Many of the steroid reactions had been conducted in dioxane. Dichlorodicyanobenzoquinone is most soluble in dioxane. It was necessary to compare dioxane to another solvent to determine if the PGB_1 reactions discussed above were a result of the choice of reagent, solvent or analyte. New PGB_1 was purchased and the oxidation experiments were conducted again. The two PGB_1 reactions were conducted with different solvents: dioxane and dichloromethane. The solubility of DDQ in CH_2Cl_2 is slightly less than in dioxane. The reaction times were 2 hours for each solvent and the DDQ molar ratio to PGB_1 was 5:1. Chromatograms examined immediately after oxidation and 18 hours later showed differences. The 18 hour sample disclosed the formation of only one new compound. Thus the presence of four compounds was due to both the dioxane and the old PGB_1. It was then decided that CH_2Cl_2 would be used as the solvent. To show the difference between the original and final reactions, the ECNI TIC profiles for a one hour DDQ/dioxane from the original reactions and for a 15 minute DDQ/CH_2Cl_2 reaction are presented in Figure 4-12. The experiment with DDQ in dioxane showed four compounds but CH_2Cl_2 gave only the desired 15-oxo-PGB_1. The EI and ECNI mass spectra of 15-oxo-PGB_1-ME are shown in Figure 4-13. The EI mass spectrum was obtained to confirm that the desired molecule had been made. The fragments at (a) m/z= 317 $(M-31)^+$ and (b) m/z= 249 $(M-99)^+$ show that the molecule is (a) a methyl ester and (b) a ketone. Loss of $-OCH_3$ is common for methyl esters and loss of 99 is from an α-cleavage at the 15-ketone.

The first oxidation procedure for prostaglandin B_1 began with methylation of 100 μg of PGB_1 with diazomethane. The excess diazomethane and ether were removed under N_2. The analyte was dissolved in 100 μl of dioxane or CH_2Cl_2. Approximately 1 mg of DDQ was added and the reaction mixture stirred for 0-3 hours. The various reaction times were used to establish the optimum reaction time. It was found that the DDQ reaction was 90% complete within 30 minutes (see Table 4-2). The solvent was removed under N_2 at the end of the desired time. One hundred microliters of 0.1 M

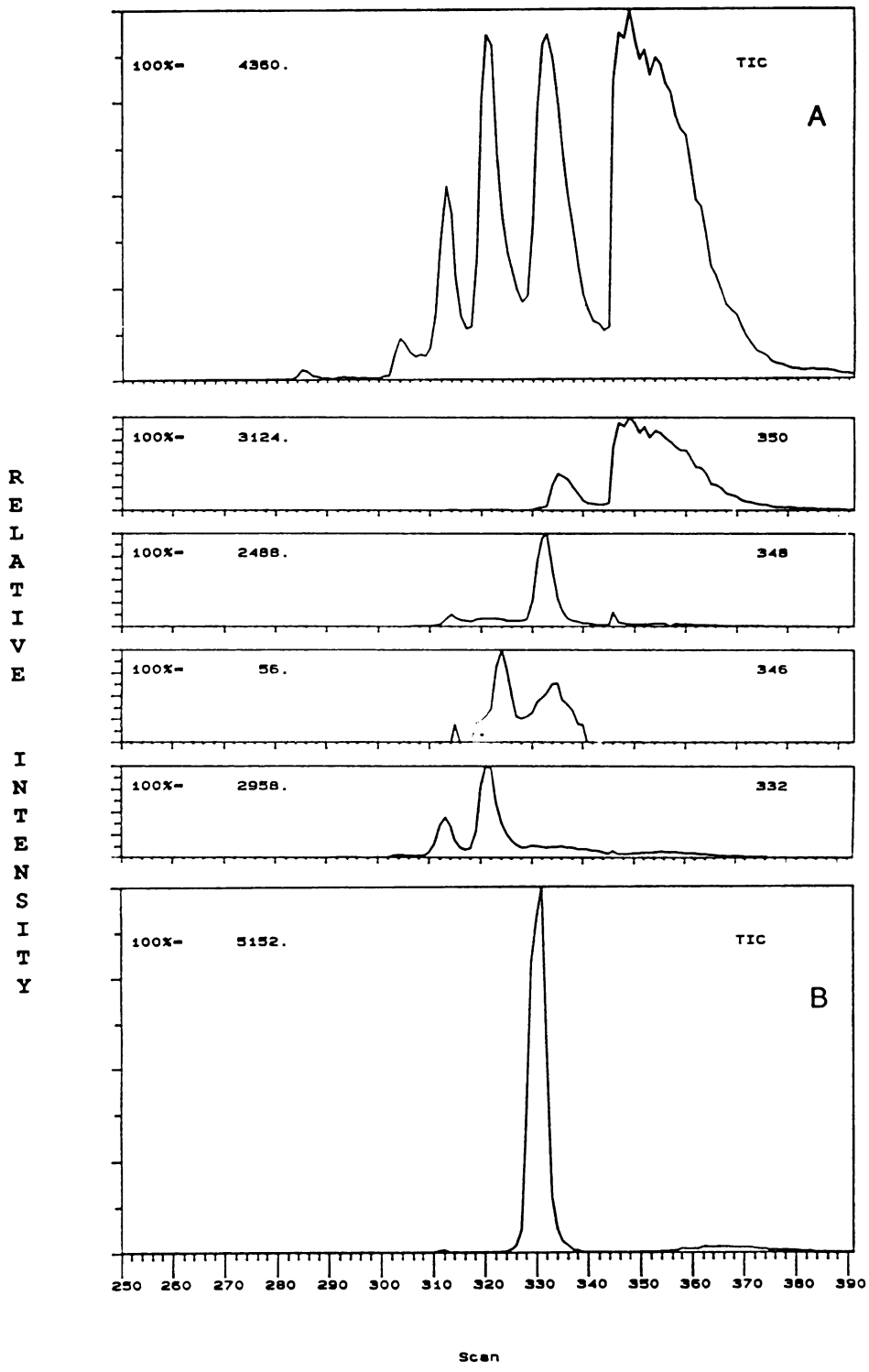


Figure 4-12. The ECNI TIC and reconstructed mass chromatograms of PGB_1 after DDQ oxidation in dioxane (A). The TIC of PGB_1 after DDQ oxidation in CH_2Cl_2 (B). The structures corresponding to the various masses in (A) are found in Figure 4-11.

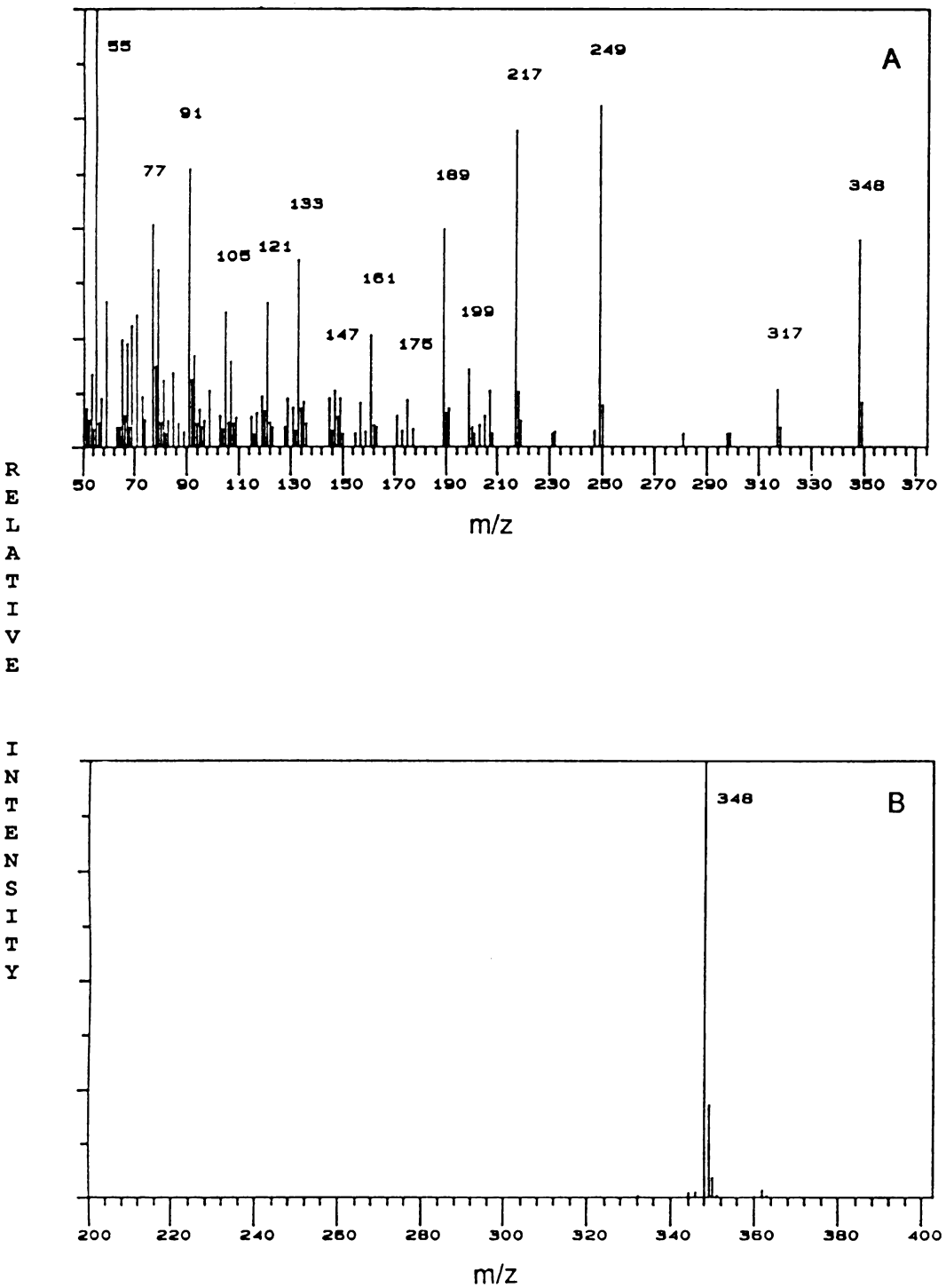


Figure 4-13. The EI (A) and ECNI (B) mass spectra of oxidized PGB_1, the 15-oxo-PGB_1-ME (MW= 348). The EI fragments at m/z= 317 and m/z= 249 are explained in the text.

NaOH was then added. This minimized the extraction of the DDQ but it did not prove to be consistent from sample to sample. The oxidized reaction mixture was extracted with diethyl ether/hexane in a 1:1 ratio. The solvent was again removed with N_2 and the residue dissolved in a measured amount of suitable solvent such as hexane/ethyl acetate 1:1. The extracted solution always contained residual DDQ that made the solution appear yellow. Dichlorodicyanobenzoquinone had electrophilic character and would lead to high background during analysis by ECD or ECNI-MS. It was also possible that undesirable side products would be produced over extended periods of time.

When the parameters for PGB_1 were optimized, the addition of the appropriate stoichiometric amount of solid DDQ was not possible for small amounts of the analyte. Thus, it became necessary to make solutions of DDQ in CH_2Cl_2. These solutions allowed for the controlled addition of a 5-fold molar excess of DDQ regardless of the analyte concentration. It was discovered that the DDQ solutions should be freshly prepared. Such solutions lasted approximately two months if stored in the dark at 0°C. The degradation of DDQ was obvious when the solution changed color (bright yellow to dark yellow or orange). It has been found that the best way to evaluate the color change is to prepare a solution and expose it to room temperature and room light. A color change will occur within a few hours.

Pyridinium dichromate and Prostaglandin B_1

The many original problems associated with the use of DDQ led to further research about other possible oxidation reagents. Articles that had described the attractive feature of short reaction times of the oxidation of prostaglandins with pyridinium dichromate (PDC) (14, 15, 16) were encouraging. The oxidation of the allylic 15-OH group of a PG to a ketone had produced the α, β–unsaturated ketone. This reaction had proved helpful for the authors because the ketoprostaglandins absorbed at a higher wavelength than the initial hydroxy prostaglandins (190 nm to 230 nm). The

ketoprostaglandins could be determined by HPLC with an UV detector without interference of solvent absorption. Normally a general derivative had been prepared to shift the prostaglandin absorption maximum to longer wavelengths.

The PDC procedure was evaluated in our laboratory with PGB_1. The reaction was a success after forty minutes and the separation of the PGB_1 was quite facile since PDC is not soluble in nonpolar organic solvents. Initial PDC oxidations of PGB_1 started with the methylation of PGB_1. The excess diazomethane was removed under a N_2 stream and 10 equivalents of PDC/equivalent of analyte were added in dry acetonitrile (stored in 4Å molecular sieves) . The mixture was left to react for forty minutes or less, and the reaction was terminated by the addition of enough water to make the acetonitrile concentration 90% by volume. Then the mixture was diluted with sufficient 10 mM phosphoric acid to make the acetonitrile 10% by volume. This enhanced the extraction of the analyte (16) from the aqueous phase, which was accomplished by repetitive washings with ethyl acetate or hexane/ethyl acetate 1:1. Pyridinium dichromate has no appreciable solubility in these solvents (14). The ethyl acetate fractions were pooled and dried. The residue was then dissolved in a measured amount of ethyl acetate or hexane. The PDC reaction produced only one compound, the oxidized PGB_1. The strength of the separate oxidizing compounds (PDC and DDQ) suggested that PDC was a milder reagent than DDQ. Also, the rate of the PDC reaction could be increased by adding acetic acid and/or molecular sieves (17). It was found that molecular sieves complicated the extraction of the analyte. However, in studies with HETEs, acetic acid increased the reaction rate considerably.

Optimization of pyridinium dichromate and 15-HETE

After preparing ample amounts of 15-HETE, the PDC and MnO_2 reagents were reevaluated. The MnO_2 oxidation was accomplished by placing a known amount of 15-HETE from the stock solution in a silanized vial. A small amount of MnO_2 was added

(the tip of a spatula) and pet ether added. The reaction was conducted at room temperature for 6 1/2 hours stirring constantly. The MnO_2 was removed by centrifugation. The residue was methylated with diazomethane. The FID and ECD chromatograms from this reaction are presented in Figure 4-14A. It can be seen in the FID chromatogram that there are at least two compounds in the sample. The first compound is 15-HETE-ME and the second, hidden component is 15-oxo-ETE-ME. This demonstrates that the MnO_2 oxidation did not come close to reaching completion (12%) and a much longer reaction time was required.

The PDC reaction of five separate aliquots of 300 μg of 15-HETE was monitored for 1, 2, 3, 4 and 7 1/2 hours. It was found that the reaction reached completion after seven hours (see Table 4-2). The chromatogram in Figure 4-14B shows the presence of two compounds in the FID trace at 4 hours. Only one compound was found in the 7 1/2 hour sample (Figure 4-14C). It is interesting to note that the ECD chromatograms do not show the presence of the allylic alcohol because it is not electrophilic. Thus, anyone completing these oxidations for the first time should be careful to analyze the reactions by GC with a FID before concluding that the oxidation was 100% complete.

It has been established that the most promising method is the PDC oxidation. It fulfills all the criteria for an appropriate oxidation reagent. It is a selective oxidation reagent that is easily removed and reaction times are reasonable. The procedure has been modified from the above PGB_1 oxidation. A 1 mg/ml solution of PDC in acetonitrile is freshly prepared. These solutions have the same degradations problems that DDQ solutions have, they last approximately 2 weeks in the dark at 0°C. It is important that the color be bright yellow and not golden yellow. It is not known if the color change affects the PDC potency. These shade differences are best tested by the researcher by leaving a fresh solution exposed to room temperature and light.

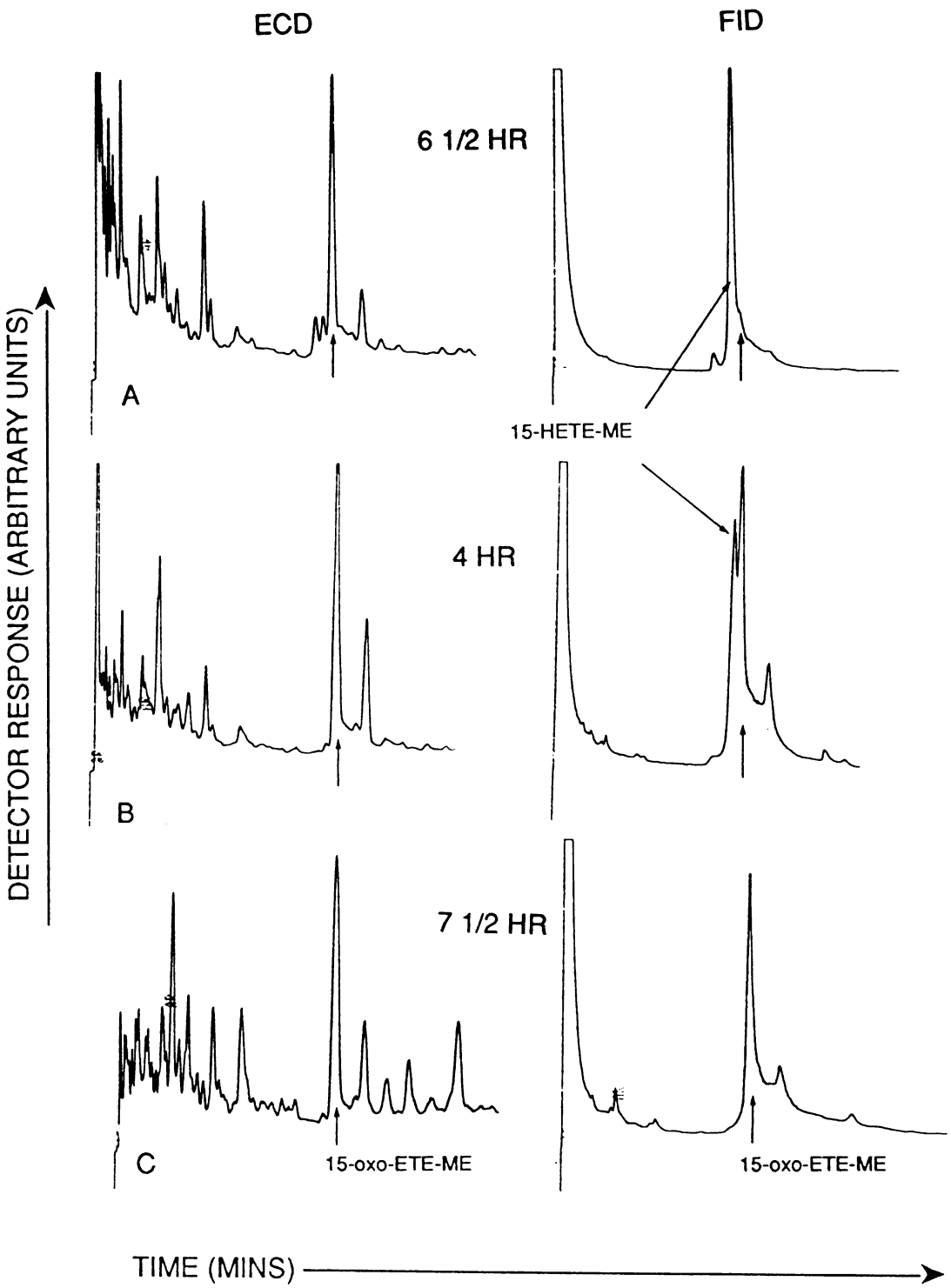


Figure 4-14. The ECD and FID chromatograms showing 15-oxo-ETE-ME after oxidation of 15-HETE with (A) MnO_2, 6 1/2 hours; (B) PDC, 4 hours; and (C) PDC, 7 1/2 hours.

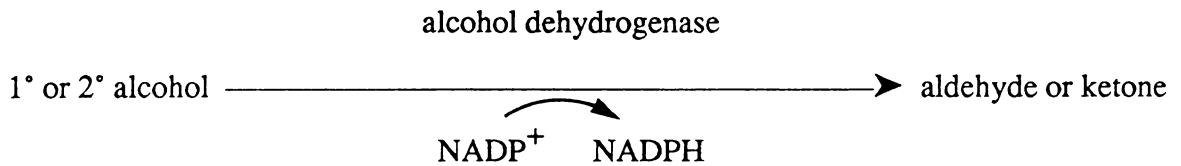
TABLE 4-2. THE COMPARISON OF THE SUCCESS OF THE VARIOUS OXIDATION REAGENTS WITH HETES

REAGENT	REACTION TIME IN HOURS	PERCENT CONVERSION OF HETE TO OXO-ETE
MnO_2/pet ether		
	6.5	12
DDQ/dioxane (before PGB_1 optimization)		
	0.66	23
	2	50
DDQ/CH_2Cl_2 and PGB_1		
	0.25	86
	0.5	92
	2	100
DDQ/CH_2Cl_2 (after PGB_1 optimization)		
	1	40
PDC/acetonitrile		
	1	40
	2	50
	3	51
	4	50
	7.5	100

The recommended sample processing procedure is as follows: the analyte is placed in a silanized vial and the solvent removed with N_2. Approximately 200 µl of the PDC solution is added to the vial. A stir bar is added and the vial capped. The reaction is conducted at room temperature constantly stirring. After 7 hours, the reaction is stopped by adding enough 10mM phosphoric acid to make the acetonitrile 10% by volume. The oxidized product is removed with hexane/ethyl acetate 1:1. The aqueous layer is washed three times and the organic layers are pooled. The solvent is removed under N_2. At this time, the oxidized analyte may be derivatized to the methyl ester or pentafluorobenzyl ester.

It was discovered accidently that having some hexane/ethyl acetate present during the oxidation increased the extraction efficiency of the analyte by a factor of four. Unfortunately, it also made the PDC more difficult to remove. This was apparent by the color of the organic phase. The organic phase is normally colorless, but in the sampleswhere the hexane/ethyl acetate was present during the oxidation, the organic layers were orange. Organic solvents may decompose PDC. This should be determined before continuing the use of the nonpolar organic solvents during the oxidation. Also, it is not known how the residual PDC affects the analyte's stability with time.

Another possibility for the oxidation of allylic alcohols to α, β–unsaturated ketones is enzymatic oxidation with alcohol dehydrogenase (EC 1.1.1.2). The reaction incorporates NADP as an hydride acceptor. The specificity of alcohol dehydrogenase varies from source to source; so it is important that the enzyme source is known because many enzymes oxidize only primary alcohols.



The reaction is not as specific as the chemical reagents but it may work. This

reaction was tried on 15-HETE that had been prepared with soybean lipoxygenase and subsequently reduced with glutathione and glutathione peroxidase. The reaction mixture initially at pH 9 was acidified to pH 7.8, and alcohol dehydrogenase and NADP (both from Sigma Chemical) were added. Five minutes were allowed to elapse and the solution acidified to pH 3 and extracted into an organic solvent. After analysis by GC with a FID, it was apparent that initial experiments were not successful. It should be tried again because, there may be benefits to enzymatic oxidation such as a short reaction time and an easy sample extraction.

COMPARISON OF OXIDATION TO CONVENTIONAL DERIVATIZATION

Most mass spectrometric analyses of HETEs in biological fluids have been accomplished by general derivatization of the analyte and subsequent quantitation by ECNI-MS. This procedure involves the production of a electrophilic pentafluorobenzyl ester and derivatization of the hydroxy group to a trimethylsilyl ether. Unfortunately, this derivative has poor chromatographic properties so, prior to derivatization all the double bonds in the molecule are reduced. Since this procedure is the accepted method, it was necessary to compare the electrophilic character of the oxidized HETE and the reduced, derivatized HETE. The substrate was 15-HETE. One aliquot of the 5-HETE was reduced with Rh and H_2, then derivatized with pentafluorobenzyl bromide (Aldrich) and N, O-bistrimethylsilyltrifluoroacetamide (Pierce). Another aliquot was oxidized with PDC and half of this aliquot was esterified with pentafluorobenzyl bromide and the other half with diazomethane [prepared according to non-alcoholic directions on Diazald bottle (Aldrich)]. Each compound was injected into a JEOL HX-110 mass spectrometer and the molecular ion current areas for each molecule were obtained. The response of the ketone as the methyl ester was compared to the 15-HEA-PFB, TMS and the 15-oxo-ETE-PFB. The areas were acquired under ECNI conditions by both selected ion monitoring (SIM) of the most intense ion and regular scanning (50-500 amu). Figure 4-15 shows the ECNI

spectra for the three compounds. Figure 4-16 shows the comparison of the peak shape of the three compounds. Table 4-3 gives the comparison of the responses of the three compounds to ECD and ECNI and their retention times. It is obvious that the PFB ester is more sensitive, however, in a real sample the gain in sensitivity for the PFB ester can be reduced by matrix interference. This was demonstrated in Figure 4-2. One of the benefits of using the oxidation instead of the derivatization is that the compound created has a short analysis time. The methylated ketone elutes quickly in a reasonable temperature program. Another benefit is that it is not necessary to reduce the double bonds of the α, β–unsaturated ketone methyl ester. The only shortcoming of this methodology is that the detectability is much less than that for a PFB ester, but for some samples it should be adequate.

A final comparison of the oxidation procedure to the published derivatization methodology was completed with islet cell-generated 12-HETE. A sample donated by Dr. Pek at the University of Michigan was divided into two aliquots. Each half was prepared for ECNI experiments, one derivatized as outlined above, the other oxidized and methylated. The SIM profiles for the two samples are presented in Figure 4-17. The reduced, derivatized aliquot (Figure 4-17A) shows the existence of two peaks at m/z= 399. The earlier peak is 12-HEA-PFB, TMS and the later eluting peak is probably 5-HEA-PFB, TMS. The arrow in the oxidized sample mass chromatogram (Figure 4-17B) points to the approximate location of the 12-oxo-ETE-ME. The signal to background ratio (S/B= 2.4) is not acceptable for positive confirmation of the presence of 12-HETE even though it looks likely. It is obvious that the signal/background is much more acceptable (S/B= 14) in Figure 4-17A. This comparison of S/B response demonstrates the difference in electrophilicity of the two compounds, since each aliquot contained the same amount of 12- and 5- HETE (approximately 10 ng of 12-HETE).

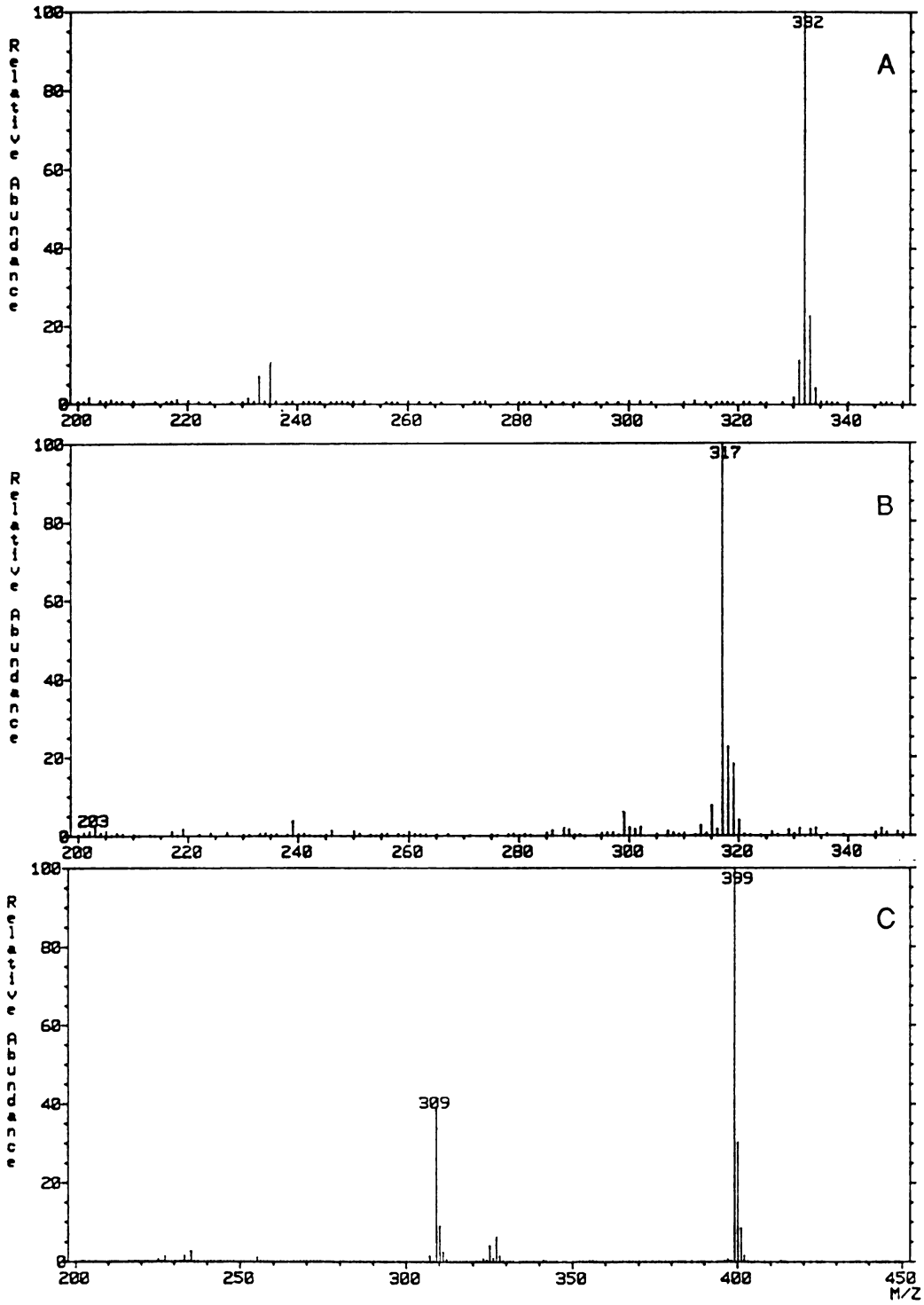


Figure 4-15. The ECNI mass spectra of (A) 15-oxo-ETE-ME, (B) 15-oxo-ETE-PFB and (C) 15-HEA-PFB, TMS. The column used was a DB-1 15m megabore, flow rate 12ml/min. The CH_4 pressure was 7 X 10^{-6} torr.

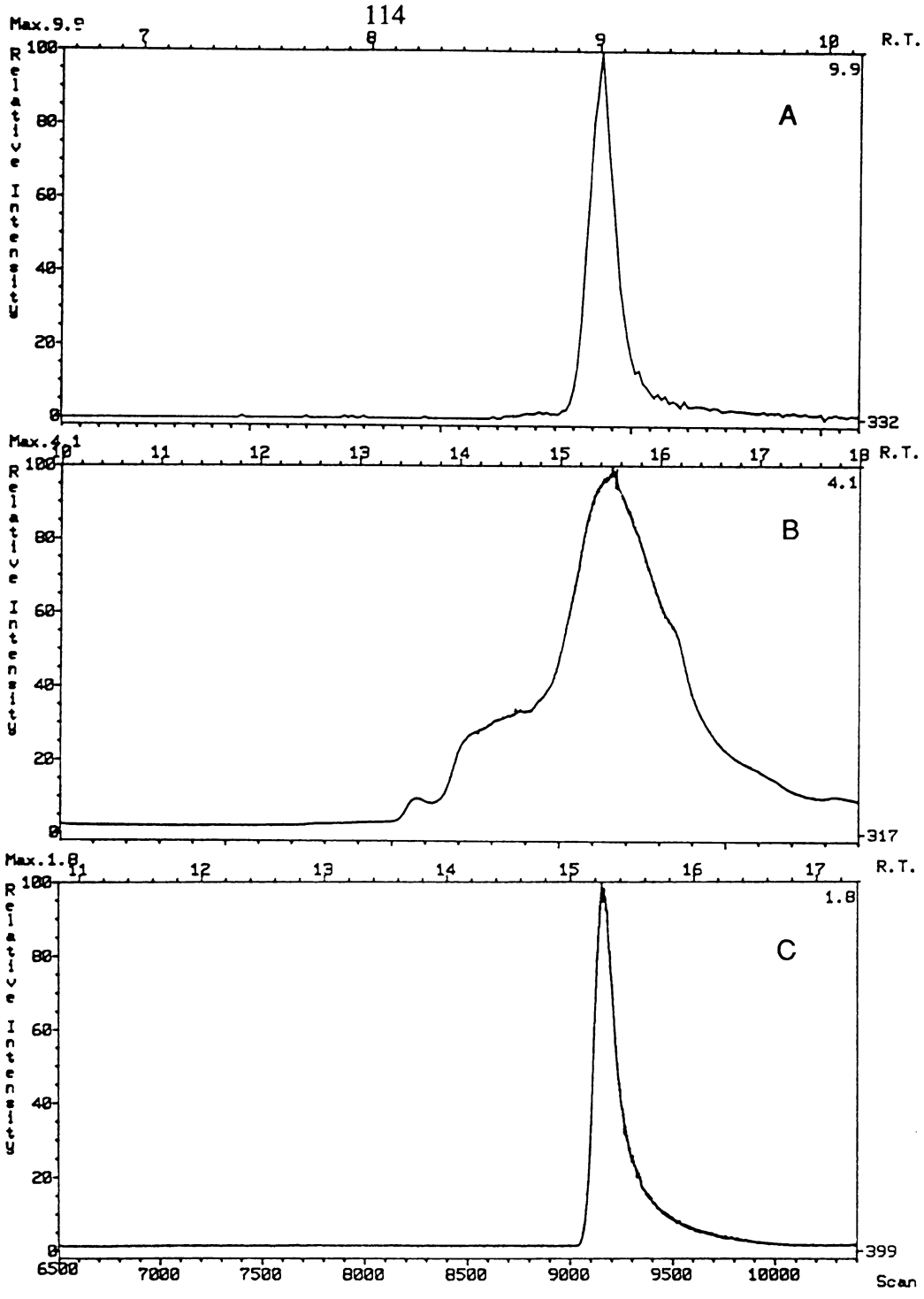


Figure 4-16. The ECNI SIM profiles of (A) 15-oxo-ETE-ME, (B) 15-oxo-ETE-PFB and (C) 15-HEA-PFB, TMS. The profiles were obtained on the JEOL HX-110 mass spectrometer with a DB-1 column. Note the poor chromatographic properties of the 15-oxo-ETE-PFB. The peak shape is similar to that of the 15-HETE-PFB, TMS.

TABLE 4-3. PROPERTIES OF VARIOUS PRODUCTS OF CHEMICALLY MODIFIED 15-HETE

COMPOUND	RETENTION TIME[a] (mins)	MOLAR RESPONSE: ECD (area)	ECNI (height)
15-oxo-ETE-ME	8.4	1	1
15-HEA-PFB, TMS	15.4	110	400
15-oxo-ETE-PFB	15.0	70[b]	30[b]

a= The retention time recorded is from the Varian gas chromatograph.

b= Theoretically, the response for the 15-oxo-ETE-PFB should be 85% (gravimetric conversion) of the 15-HEA-PFB, TMS. The poor peak shape of the 15-oxo-ETE-PFB makes the area and height calculations uncertain.

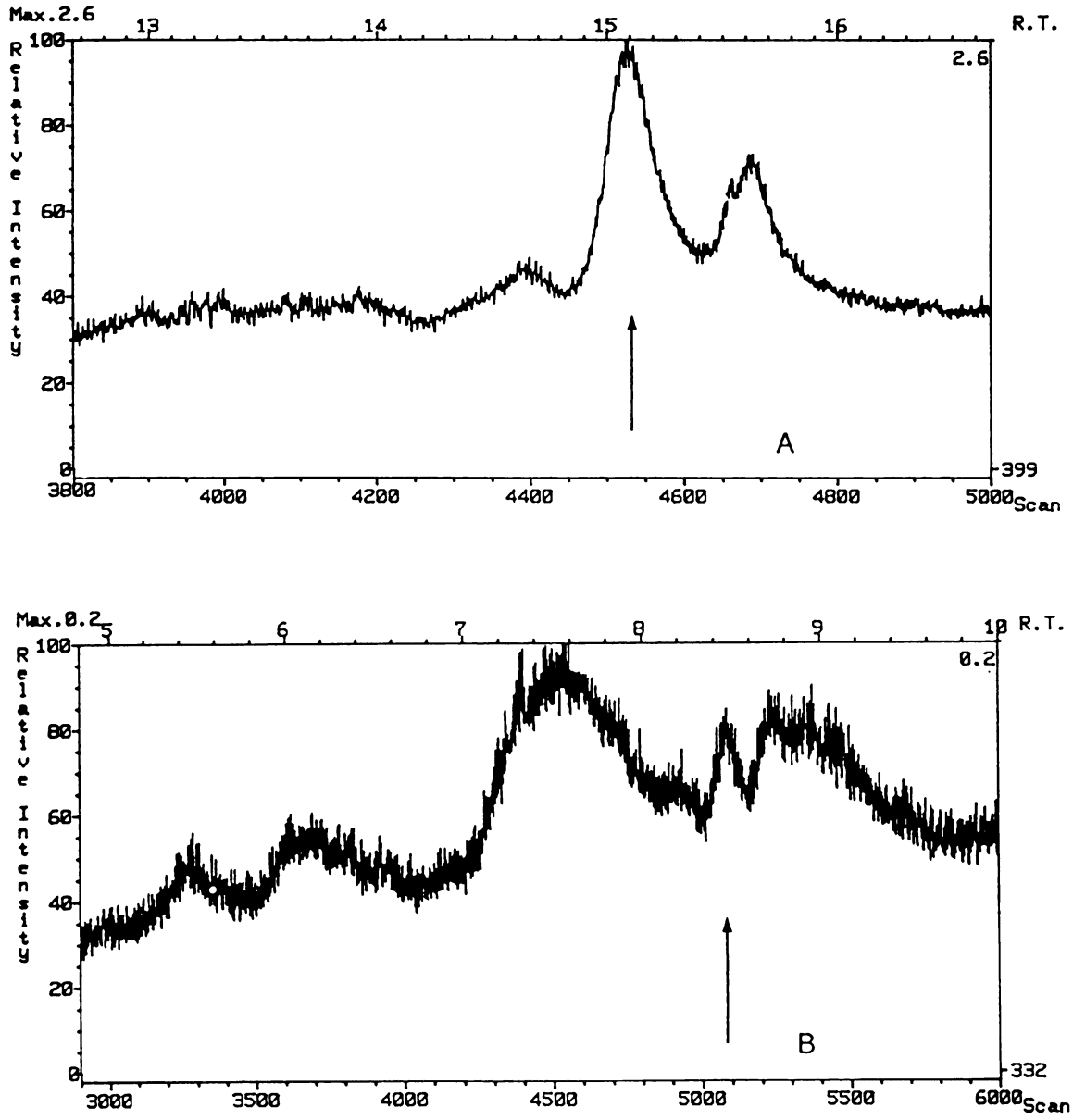


Figure 4-17. The ECNI SIM profiles of the HETE HPLC fraction from islet cells: (A) the reduced, derivatized portion, and (B) the oxidized, methylated portion. The arrows point to the appropriate elution time of the 12-HEA, PFB ester, TMS ether (A) or the 12-oxo-ETE-ME (B). The same sample is also presented in Figure 4-2 as the ECD chromatogram.

In conclusion, the oxidation procedure is as easy to use as the reduction, derivatization technique. Application of this methodology to samples where the primary interest is the presence of allylic hydroxy fatty acids in a matrix of many other higher concentration fatty acids will prove its usefulness as an analytical method. Improvements in the oxidation methodology lie in the areas of decreasing the reaction times, increasing the number of potential substrates and successfully oxidizing and extracting small quantities of analyte. As the costs of leukotrienes decrease, it will become feasible to use LTB_4 in experiments. As with the first experiments with 15-HETE and MnO_2, there has been no success with the oxidation of LTB_4. Since the sample size of LTB_4 has always been restricted to high nanogram- low microgram amounts, the extraction from the reaction mixture has probably been insufficient. No analyte was discovered when the samples were analyzed by selectively monitoring the molecular ion of the oxidized LTB_4 under ECNI conditions. If the reaction had not worked, the unoxidized LTB_4 would not have been responsive to ECNI. Since the original concentration of LTB_4 was small analysis by conventional EI was not possible. It was confirmed by ECNI-MS that the standard LTB_4 was present in the starting material after derivatization to the PFB ester, di-TMS ethers. We hope that recent improvements in the PDC and MnO_2 oxidations will make the the oxidation of LTB_4 a reality. In order to determine compounds such as LTB_4 in biological fluids it will also be important to consider the extraction of very small amounts of the analyte after oxidation. This can be determined by slowly decreasing the amount of the analyte (15-HETE) in a series of experiments. Although many biological samples may contain a much higher concentration of hydroxyeicosatetraenoic acids compared to leukotrienes, the ability to oxidize and efficiently extract small quantities of hydroxyeicosatetraenoic acids will help with the extraction of the potent, expensive inflammatory mediators.

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CHAPTER V: COLLABORATIVE PROJECTS

This chapter explores the three collaborative projects that expanded our knowledge of eicosanoid analysis to include leukotrienes (LTs). Each project involved the analysis of a biological sample. These samples included epithelial cell and islet cell washes as well as equine plasma and bronchial lavage. An explanation of of each project is given. A brief summary of the objectives of each project is presented, and a detailed report of how the samples from each study were prepared for analysis appears in the experimental protocol section at the end of the chapter. The current, accepted methodology for LTs was employed for each sample. However, islet cell wash was also analyzed by the oxidation methodology discussed in chapter IV.

In two of the projects, our interest in leukotrienes was derived from the fact that LTB_4 is present in fluids from cells that have been challenged by an allergen. The goals of these projects involved determining the presence of LTB_4 in the biological fluid. LTB_4 is known for its chemotactic properties. Chemotaxis causes the movement of immune cells toward the site of inflammation. LTB_4 is a mediator of neutrophil (white blood cell) infiltration to inflamed areas.

The final study, in which islet cells were employed , involved determining the potential of eicosanoids as regulators of pancreatic islet hormone (insulin and glucagon) secretion. Results of this study could possibly provide information explaining a cause of diabetes. It is known that shifts in arachidonic acid metabolism result in regulation of insulin or glucagon. By determining what types of arachidonic acid metabolites are present in healthy islet cells and how they affect insulin production, diabetic cells could be compared. The goal of this project was to determine if peptido-LTs are formed in stimulated islet cells.

It should be stated here that all glassware used, that the analyte might come in

contact with in these experiments, was silanized prior to use. In each study, the analyte contained a least one hydroxy group and these molecules tend to adhere to the active sites on the glass surface. Since the concentration of the analyte is so small, it is possible that all of the analyte could become permanently adhered to the glassware. To prevent this problem, silanization of the glassware is required. The process of silanization can be found in the experimental section.

PROJECT I

The first project completed was the analysis of rabbit tracheal epithelial cell wash. This wash was removed after the cells had been exposed to ozone. The presence of chemotactic factors (LTB_4) due to cell injury was to be examined. It had been shown previously that canine tracheal epithelial cells had produced LTB_4 after ozone exposure (1, 2, 3). Ozone is a potent inflammatory agent (4, 5, 6) which causes lung hyperresponsiveness.

The methodology for cell injury developed by Stephen Alpert, M. D., was to be a model system that closely resembled *in vivo* ozone exposure. Dr. Alpert felt that previous studies may have been too harsh and cells producing chemotactic agents were not injured, but were dying (not viable). Dying cells might have their membranes ruptured and membrane-bound phospholipids would be released. This could cause an abnormally high pool of arachidonic acid to be released, and lipoxygenase enzymes might be activated. This could cause the formation of arachidonic acid metabolites, but it would not be due to inflammation.

If it was found that epithelial cells produce chemotactic factors that lead to neutrophil infiltration, the contribution of these cells to the physiological effects of diseases such as asthma and cystic fibrosis would be known. Currently only endothelial cells are thought to be involved in these diseases; thus the drugs used for treatment affect

only those cells.

Preliminary experiments with cell wash samples showed no formation of LTB_4 (see Figure 5-1.). The samples did show the presence of peptido-LTs. This was confirmed by radioimmunoassays conducted by Michael Bach's laboratory at Upjohn, Kalamazoo, MI. Studies were concluded at this point.

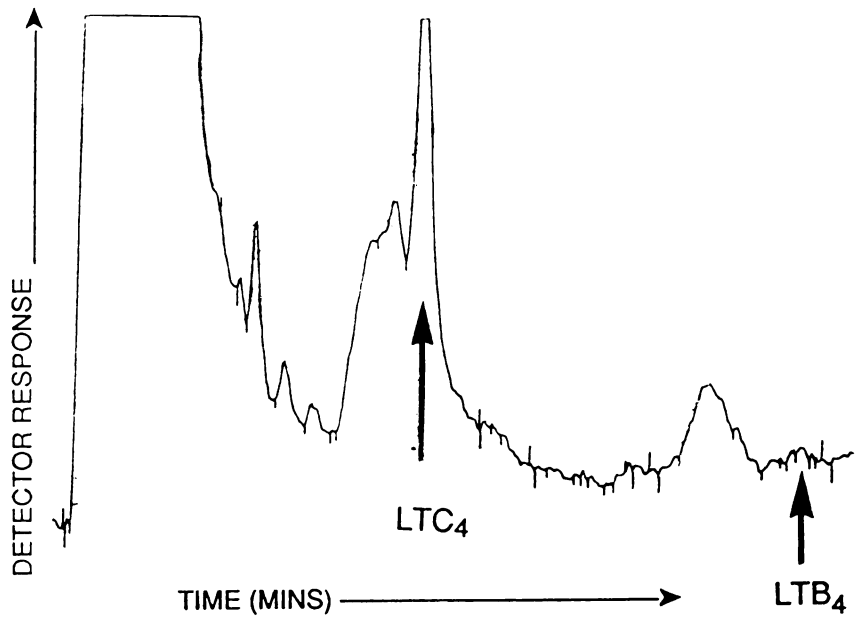

Figure 5-1. The reverse phase HPLC chromatogram of 30 µl of epithelial cell wash after ozone exposure. The flow rate was 1.0 ml/min, range: 0.01 AUFS and λ= 280 nm. The arrows show where LTC_4 and LTB_4 standards would elute. The area corresponding to LTC_4 is equivalent to the injection of 20 ng of standard LTC_4.

PROJECT II

The second collaborative effort was a preliminary study of LTB_4 in equine plasma and bronchial lavage. This study was not completed and will be continued by other students in our laboratory.

The study of plasma and lavage for arachidonate metabolites is being conducted on ponies which are challenged with an allergen which produces airway

hyperresponsiveness commonly called heaves. Heaves are the equine equivalent to asthma. Thus, it is an excellent animal model system for human asthma. Although it has already been shown that heaves are accompanied by neutrophil infiltration (suggesting the presence of LTB_4), arachidonate metabolites have not yet been detected.

Lavage and plasma from two ponies (Frisky and Augie) were spiked with LTB_4. These samples were used to test the extraction protocol which was developed by the collaborating scientists. Also it was important that spiked and unspiked biological fluids be compared to determine possible basal amounts of LTB_4. The actual determination of the percent recovery of the spiked samples was also of interest. However, this could only be accomplished by spiking the collected samples with radiolabelled or ^{18}O-labelled LTB_4. Radiolabelled LTB_4 recoveries could be determined by liquid scintillation counting and ^{18}O-labelled LTB_4 recoveries by mass spectrometry. Although radiolabelled LTB_4 is available, ^{18}O-labelled LTB_4 must be prepared. The preparation of ^{18}O-labelled LTB_4 is described at the end of the chapter.

Preliminary results showed that LTB_4 had been extracted from all four of the spiked plasma samples examined. In the two lavage samples analyzed, one that had been spiked with LTB_4, LTB_4 was not found. Also a plasma sample that was not spiked did give positive LTB_4 response that was approximately one-fourth the response of a spiked sample. This could be the basal level of LTB_4. The percent extraction of LTB_4 was not determined with the first group of samples analyzed because ^{18}O-labelled LTB_4 was not prepared. Recently, ^{18}O-labelled LTB_4 was successfully made and future samples will have their [LTB_4] determined.

PROJECT III

The final collaborative project involved the determination of peptido-LTs produced from islet cells. Islet cells are known to contain 12-lipoxygenase (12-HETE)

but it is not known whether 5-lipoxygenase is present. In order for LTs to be present this would have be the case. The cells had been stimulated with glucose and calcium ionphore to produce arachidonate metabolites. HPLC analysis of the cell wash from the stimulated cells showed peaks which corresponded in time to standard LTs. Fractions were collected and sent to our laboratory from the University of Michigan to be analyzed. A representative HPLC chromatogram is presented in Figure 5-2. Fraction II was thought to contain the peptido-LTs and Fraction IV contained 12-HETE. These fractions were to be analyzed by mass spectrometry.

Intact sulfidopeptide leukotrienes are very difficult to analyze by mass spectrometry. They are soluble only in water; thus, only fast atom bombardment (FAB) ionization is appropriate. FAB, however, is not very sensitive, which makes it an unrealistic ionization mode for the analysis of biological samples containing only nanogram amounts of leukotrienes in the presence of microgram quantities of other compounds in the matrix. The technique which affords the researcher better sensitivity is GC-MS.

Gas chromatography requires the analyte to have appreciable volatility. The labile sulfidopeptide leukotrienes have poor vapor phase properties. Possibilities for gas phase analysis require the derivatization of carboxylic, amino and hydroxyl groups. Although the derivatization itself is easy the derivatives have higher mass. For example an isopropyl ester, pentafluoropropionyl amido and an ether derivative of LTC_4 (MW=623), is 418 mass units heavier than the underivatized LTC_4. This takes the molecular weight well over 1000 amu. Many mass spectrometers that are used routinely do not have unit resolution at such a large mass.

An alternative proposed by Murphy and Balazy (7) is the reduction of sulfidopeptide leukotrienes by cleavage of the cysteinyl sulfur bond (see Figure 5-3). This is accomplished by using the Birch reduction; this reaction originally was used to

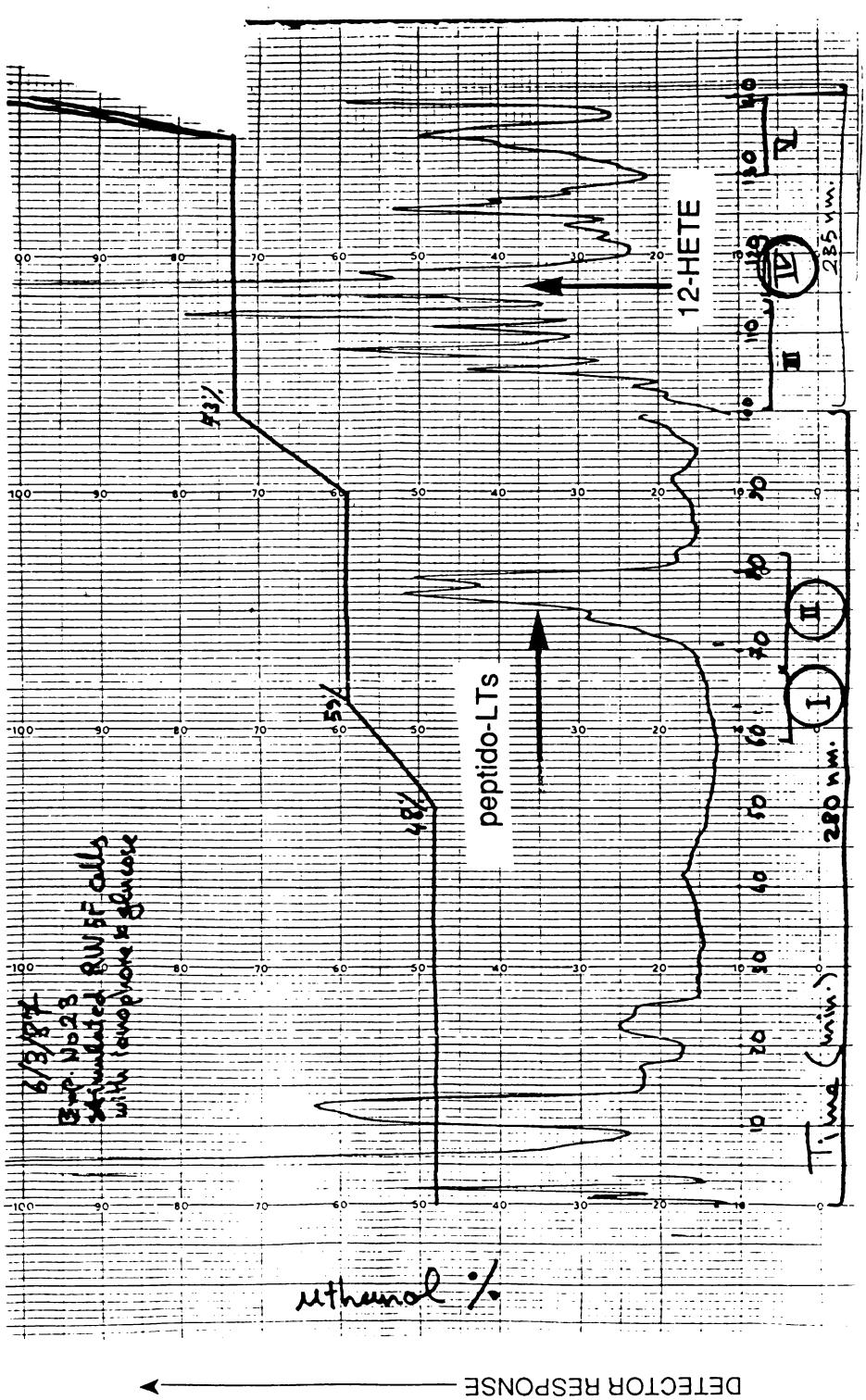

Figure 5-2. The reverse-phase HPLC chromatogram of the islet cell wash. The fractions collected are noted at the bottom. Fractions I and II had the same retention times as standard peptido-LTs and fraction IV contained 12-HETE. The chromatogram was sent by Dr. Parviz Hayat, from the lab of Dr. Pek, University of Michigan.

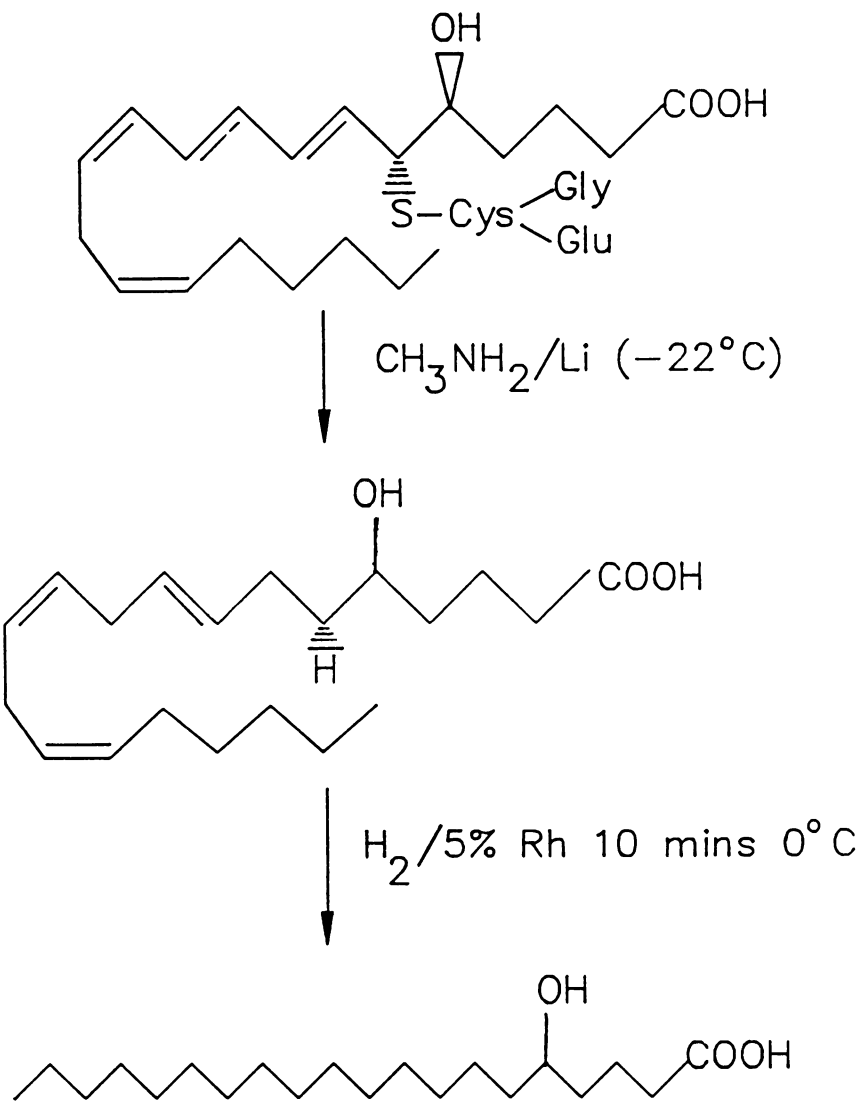

Figure 5-3. The modification of LTC_4 for analysis by GC-ECNI-MS. The Birch reduction and the hydrogenation of LTC_4 are shown.

reduce conjugated unsaturated systems such as α, β–unsaturated ketones to alcohols (8, 9). It has also been used in the preparation of peptides due to its capacity to cleave sulfide bonds (10, 11, 12). The specific reagent used for the cleavage of most sulfur-carbon bonds is lithium metal in liquid methylamine. In the case of peptido-LTs, the peptide is cleaved from the leukotriene skeleton, and the residual double bonds are hydrogenated. The resulting compound is then derivatized to the pentafluorobenzyl (PFB) ester, trimethylsilyl (TMS) ether and analyzed by GC-MS. The reduction of double bonds of the molecule is accomplished simply with hydrogen gas and a rhodium catalyst. Fission of the sulfur-carbon bond is a much more sophisticated procedure, but it can be accomplished quite simply if the correct equipment is obtained.

Two different batches of islet cell samples and leukotriene standards were prepared for GC-MS analysis. In both batches the LT standards were used to confirm that the Birch reaction and hydrogenolysis had worked successfully. An internal standard, linolenic acid (18:3) was added to each sample to approximately determine the losses of the analyte through the sample preparation. Although 18:3 was not a substrate for the Birch reduction, it was reduced during hydrogenolysis. By comparing the areas of the $(M-181)^-$ ions for 18:0 (m/z=283, hydrogenated 18:3) and coinjected 20:0 (m/z=311), approximate recoveries for the Birch reduction/ H_2 reduction were 50%. Figure 5-4 shows the profile of the two compounds and their areas.

The sample fractions (labelled II in Figure 5-2) which were thought to contain LTC_4, D_4 and E_4 were compared to a blank sample. The blank was from an HPLC "analysis" in which no sample was injected. The effluent was collected during the mobile phase gradient at the same time that the peptido-LTs would elute. Since peptido-LTs have a tendency to adhere to the column, if standards were initially injected to determine retention times, some of the standard might be retained. If it eluted in a subsequent analysis, a response that would not be completely due to the sample would be

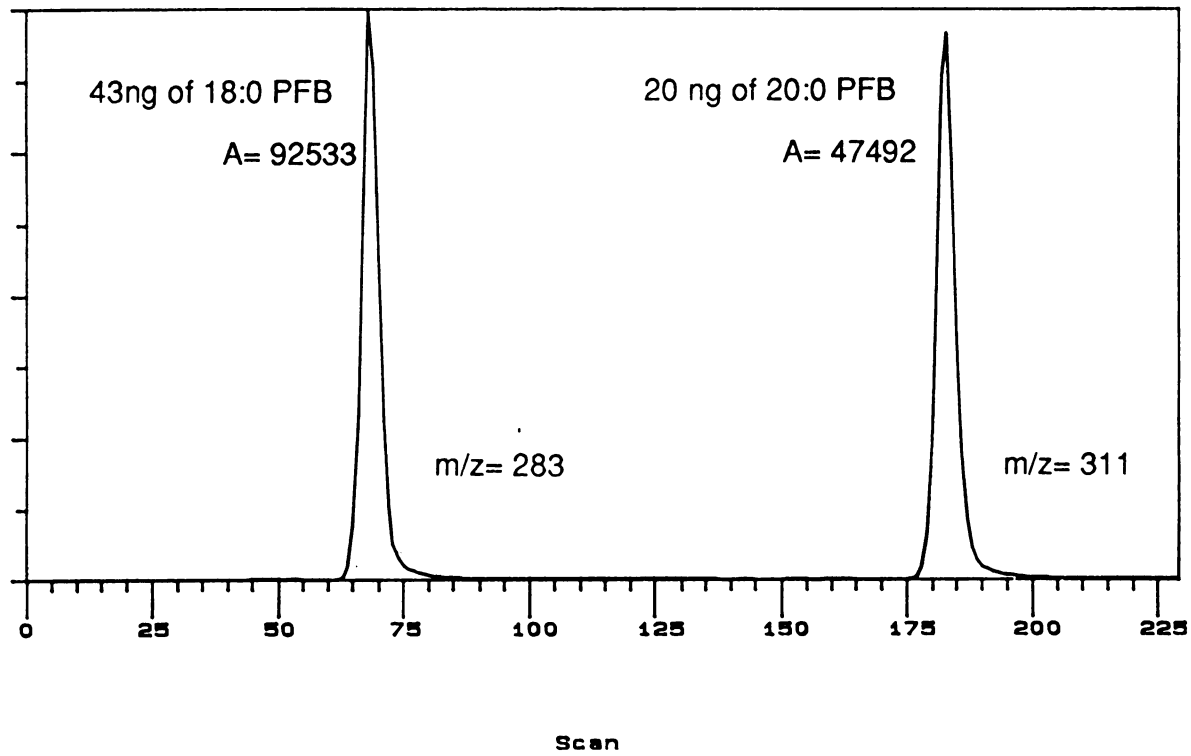

Figure 5-4. The superimposed mass chromatograms of 18:0 PFB and 20:0 PFB. The areas were compared for the crude approximation of the percent recovery from the Birch reduction and the hydrogenation reactions. The peak heights are not relative to each other but are normalized to themselves.

recorded. This was found to be true. In Figure 5-5 comparison between a standard, a blank and a sample is shown. Comparison of the areas showed the samples to have only twice as much signal as the blank at m/z=399.

The second set of experiments showed an area unit increase of three for the sample over the blank (Figure 5-6). It is clear from Figures 5-5 and 5-6 that experiments must be continued and the concentration of metabolites in the cell wash increased before a definite conclusions can be made about the presence of 5-lipoxygenase in islet cells. This will require larger colonies of cells or pooled experiments. Hopefully this is feasible.

The fraction containing 12-HETE (indicated by IV in Figure 5-2) was not treated with the Birch reagent; it was not required. Instead it was split and half was oxidized with pyridinium dichromate. The other half was reduced with H_2 and 5% Rh/Al then derivatized to the PFB ester, TMS ether. The nascent 12-HEA PFB, TMS compound was also monitored by ECNI-MS at m/z= 399. The 12-HETE was oxidized to 12-oxo-ETE which was then methylated and selectively monitored at m/z= 332. The comparison of the two derivatives is explained in detail in Chapter IV.

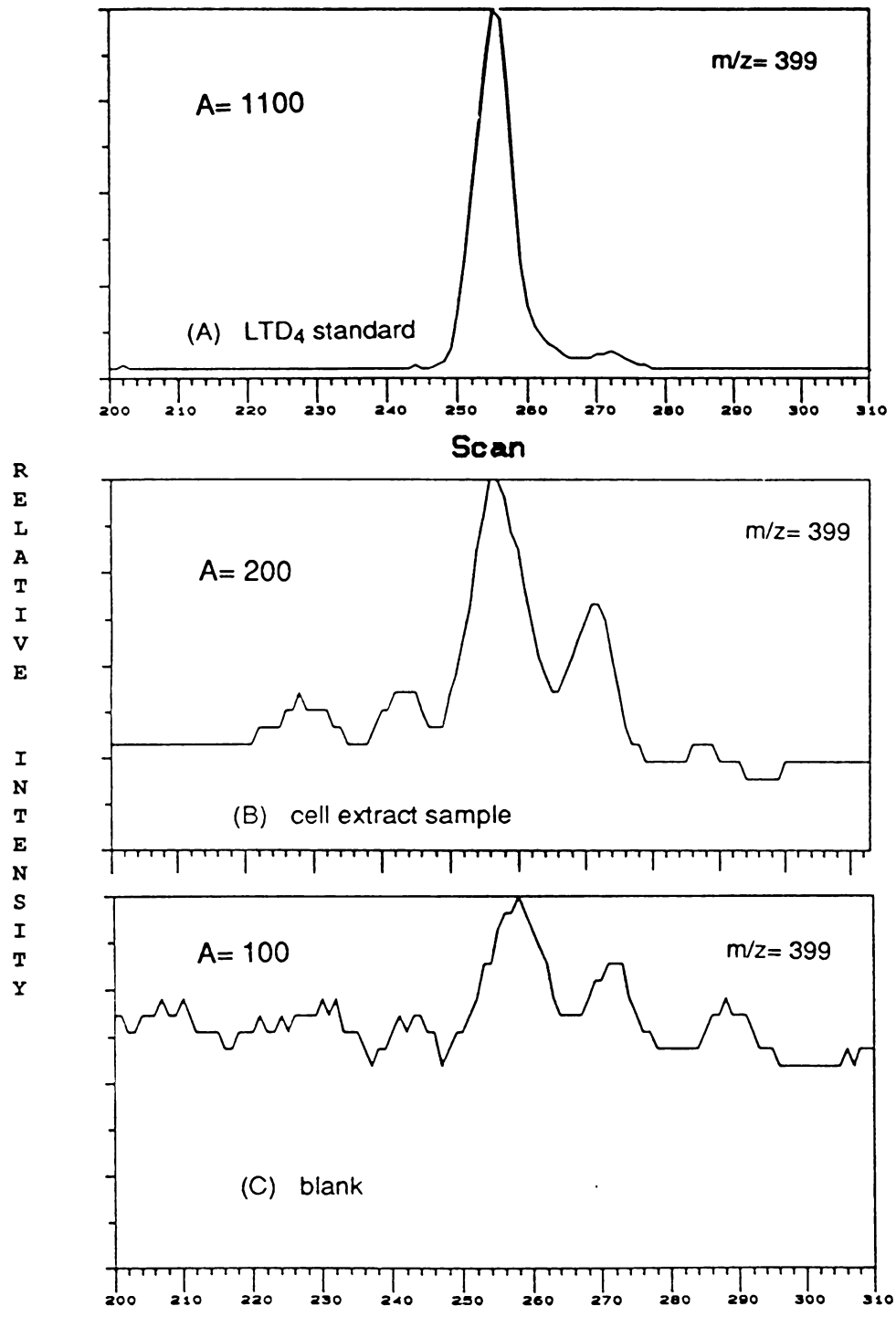

Figure 5-5. The HP 5985A GC-ECNI-MS SIM profiles of (A) LTD_4 standard, modified according to the text, TMS, PFB, (B) islet cell wash sample and (C) a blank derivatized similarly. The temperature program was 60-200°C at 20°C/min (30 m DB-5 megabore, on-column injector), 200-300°C at 7°C/min, source temperature= 200°C, EM= 3000 V and CH_4 pressure= 2.2 X 10^{-4} torr.

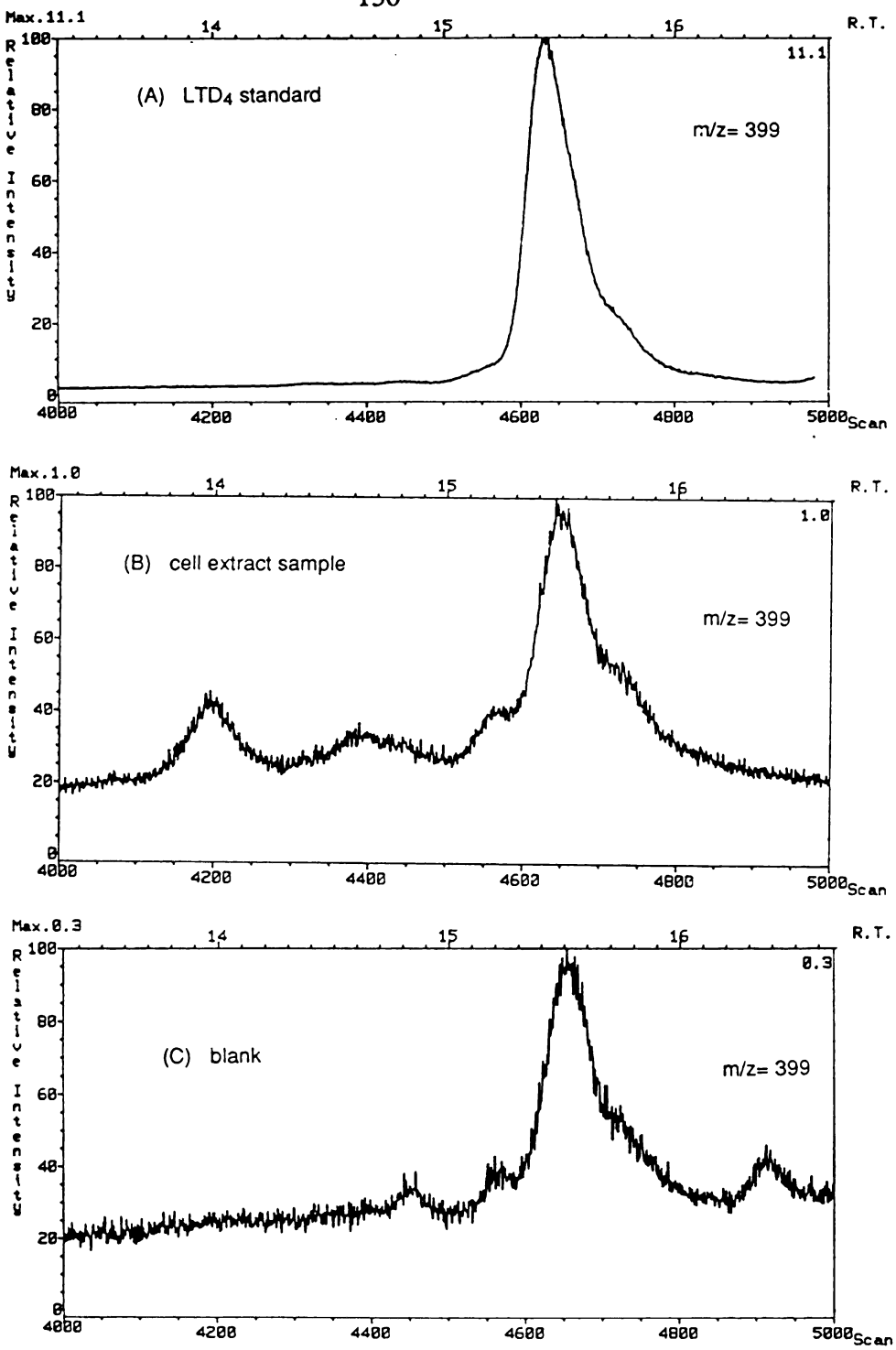

Figure 5-6. The JEOL HX-110 GC-ECNI-MS SIM profiles of another islet cell sample after the Birch reduction and derivatization (PFB ester, TMS ether): (A) standard LTD_4, (B) sample and (C) blank. The temperature program was 180-280°C at 5°C/min (DB-1 15m megabore), CH_4 pressure 5 X 10^{-5} torr, source temperature 200°C.

EXPERIMENTAL PROTOCOL

Silanization of Glassware

The process of glassware silanization inactivates the hydroxy groups on the glass surface by chemically converting them to methoxydimethylsilyl ethers. The glassware is prepared by soaking it in a solution of 5% dimethyldichlorosilane (DMCS), Sigma Chemical, in hexane or toluene for at least 20 minutes. The DMCS solution is toxic and should be handled with gloves while wearing goggles. The glassware is rinsed with hexane to remove the excess DMCS and then with methanol to exchange the residual chlorine with a methoxy group. The glassware is rinsed a final time with hexane and it is then ready for use. The glassware may be slippery so care should be taken during handling. The process of silanization is shown below in Figure 5-7.

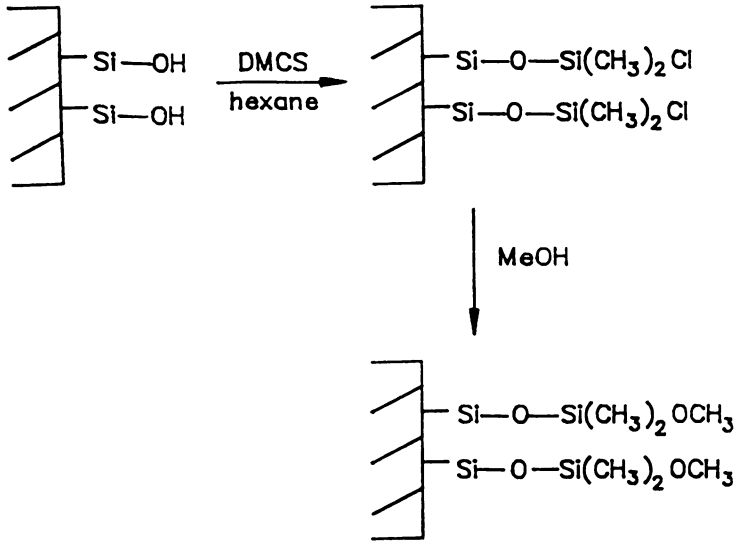

Figure 5-7. The silanization of glass to derivatize the active sites on the glass surface.

Project I

The analysis of the cell wash for leukotrienes (LTs) was accomplished by HPLC. Standard solutions of LTB_4, LTC_4, D_4 and E_4 were a gift from J. Rokach (Merck-Frosst,

Canada). Standards were necessary to determine the HPLC retention times and detection limits for the LTs. The development of an appropriate reverse-phase mobile phase was important. It had been shown that a fraction of peptido-LTs were permanently retained on a ODS (octadecylsilyl) C_{18} column. This led to poor concentration reproducibility and variable recovery (13). By using various EDTA sodium salts [Na_2EDTA (13) and Na_4EDTA (14)] this recovery problem is eliminated. EDTA also chelates the metal ions that may be the in the mobile phase. The additive is helpful in preventing the degradation of the peptido-LTs. LTC_4 decomposition is thought to be caused by the presence of transition metal ions (15). The problem with EDTA salts is that they precipitate in the presence of methanol. This will clog the chromatographic system. The system must be flushed with 100% water before and after the EDTA salts are added. The flushing is usually completed overnight. Unfortunately, constant changing of the mobile phases leads to long and frequent equilibration times. Long equilibration times made the use of EDTA tedious. Oxalic acid was added instead of EDTA. Oxalic acid can be added directly to the mobile phase; it is soluble in methanol. The oxalic acid works the same way EDTA does; both are chelating agents. The actual mobile phase employed was 70:30:0.03 MeOH/H_2O/HOAc with 0.5 mM oxalic acid adjusted to pH 5.6 with NH_4OH. In Figure 5-8, the chromatographic profile of the standard LTs is shown as they elute from a 10 μm particle 4.6 mm X 250 mm Ultrasil ODS C_{18} (Altex).

After the retention times of the LTs were known, it became necessary to determine their detection limits in the Beckman system that was used. The detection limit of LTB_4 was determined to be 2 ng and the peptido-LTs had a limit of 4 ng (range: 0.01 absorbance units full scale, λ_{max}= 280). The difference in detection limits is due to the different molar absorptivities for the two types of LTs.

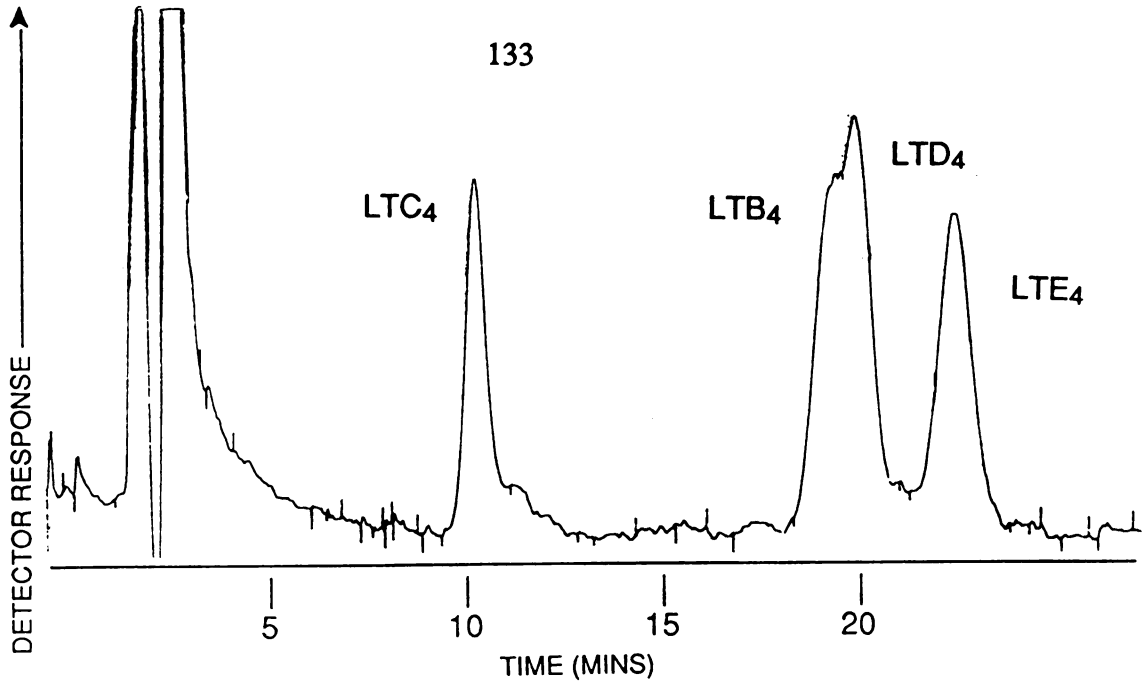

Figure 5-8. The reverse phase Ultrasil ODS C_{18} HPLC chromatogram of 50 ng of LTC_4, D_4, and E_4, and 25 ng LTB_4. The flow rate was 1.0 ml/min, range: 0.02 AUFS, and λ= 280 nm.

In order for the project to be successful, the extraction efficiency of a leukotriene standard had to be determined at each step of the sample processing. This required methodology which would preferentially extract the LTs from the cell wash media effectively. The recovery experiment was conducted by spiking cultured cells with LTB_4. The cells were exposed to ozone and the cell wash collected. The cell wash was added directly to methanol so the LTB_4 would not be degraded. The methanol denatured any proteins that might decompose the LTB_4.

The eicosanoid extraction was facilitated by the use of C_{18} Sep-Paks (Waters). The cell medium was initially acidified to pH 3 with 1M citric acid. It was then introduced onto a Sep-Pak which had been previously conditioned sequentially with 20 ml of ethanol and water. Methyl formate eluted the hydroxy eicosanoids (HETEs and diHETEs) preferentially (16). The methyl formate was evaporated and the residue dissolved in the mobile phase. Representative HPLC chromatograms of the cell media before and after LTB_4 spiking are shown in Figure 5-9. Recoveries of LTB_4 were

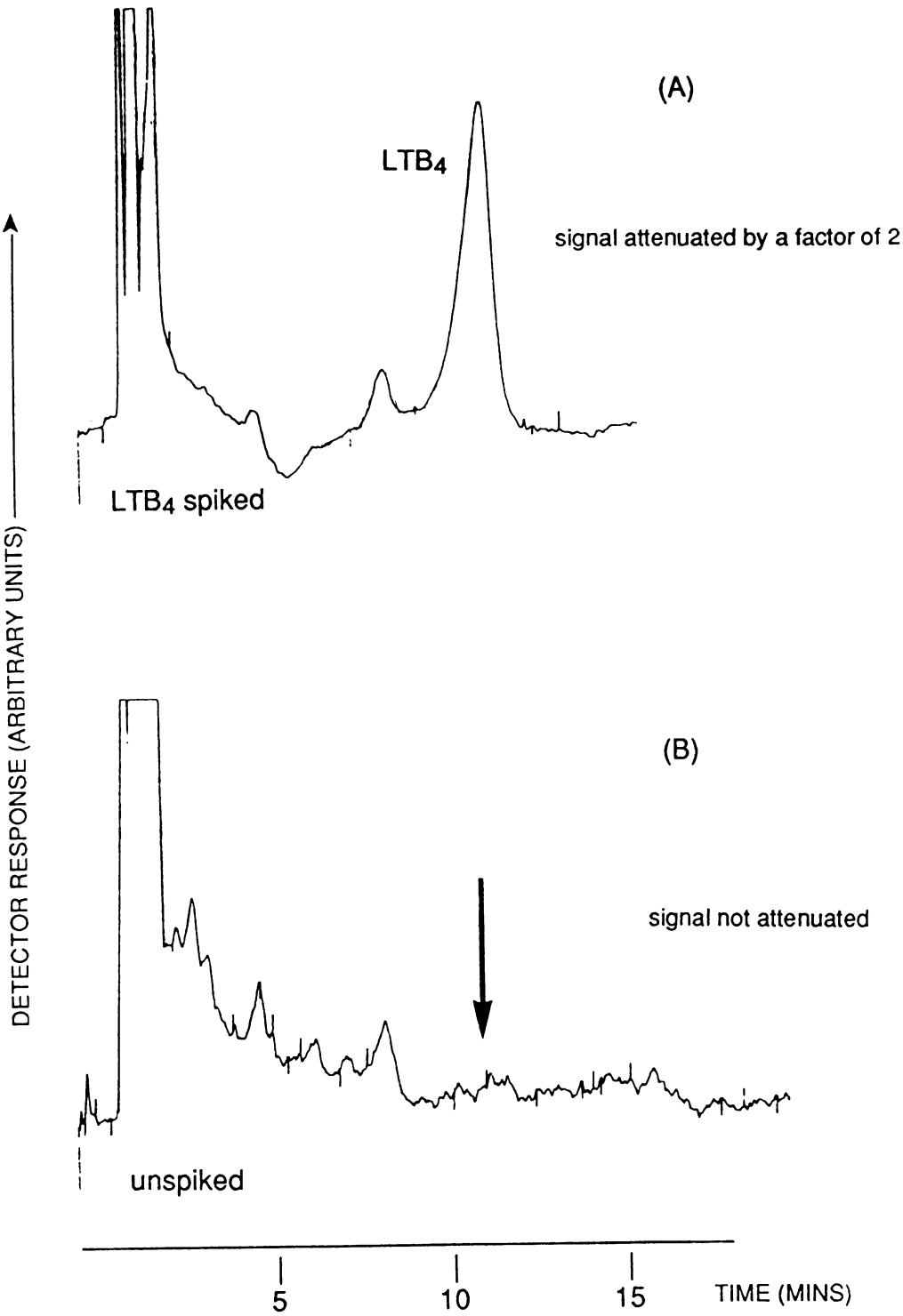

Figure 5-9. The reverse phase HPLC chromatograms of 100 µl of LTB_4-spiked (A) and unspiked (B) cell wash. The flow rate was 1.8 ml/min, range: 0.01 AUFS and λ= 280 nm. The area corresponding to LTB_4 in (A) is equivalent to the injection of 60 ng of standard LTB_4.

calculated to be >70%.

After determining the extraction efficiency for the methodology that would be used, samples that had been exposed to ozone were analyzed for LTB_4. Initially the levels of ozone were quite small, to mimic true *in vivo* concentrations. However, there was no formation of LTB_4 that could be detected by HPLC. More extreme conditions were tied and finally exogeneous arachidonic acid was added along with calcium ionphore. These final samples did not produce LTB_4, but peptido-LTs were detected. An example of an HPLC chromatogram showing LTC_4 was shown in Figure 5-1. Further progress in this project was discontinued by the collaborator because of instrumental availability conflicts.

Project II

To determine whether LTB_4 would be successfully extracted from the equine lavage and plasma samples, preliminary studies with standards were tried. These samples had been extracted through a detailed procedure and were received in methanol. The methanol was removed by nitrogen evaporation and the residue was derivatized to the pentafluorobenzyl (PFB) ester, trimethylsilyl (TMS) ether compounds (17). The plasma samples were easily derivatized but the lavage samples turned cloudy and thick during the PFB reaction. This may cause a subsequent extraction problem as well as incomplete formation of derivatives resulting in lavage samples that may not be correctly assessed for LTB_4. It is suggested that blank bronchial lavage be spiked with 12-OH 18:0 and tested for recovery by GC-ECD before other precious samples are inadvertently destroyed by just derivatizing the lavage as received.

A standard solution of LTB_4 was also derivatized to the PFB ester, TMS ether. The ECNI spectrum of the derivatized LTB_4 is shown in Figure 5-10. The samples were analyzed by GC-ECNI-MS. The $(M-181)^-$ ion (m/z=479) and the $(M-181-90)^-$ ion (m/z=389) were selectively monitored for confirmation of LTB_4 in the plasma and lavage. (Loss of 90 is common for TMS ethers. It corresponds to the loss of trimethylsilanol, $(CH_3)_3SiOH$.)

Selected ion monitoring (SIM) profiles of spiked and unspiked plasma and lavage are shown in Figure 5-11. The GC-MS parameters are also presented. The ions 389 and 479 were monitored for equal time intervals, but they may be monitored for different times because their intensities are different. The intensities for 479 and 389 vary with GC-MS conditions. Higher source temperatures and high primary electron voltages (>200 eV) will lead to more fragmentation, i.e., more m/z= 389. The daily fluctuation of the two ion intensities makes it necessary to establish their ratio initially with standard LTB_4. This should be the accomplished before the biological samples are analyzed. The

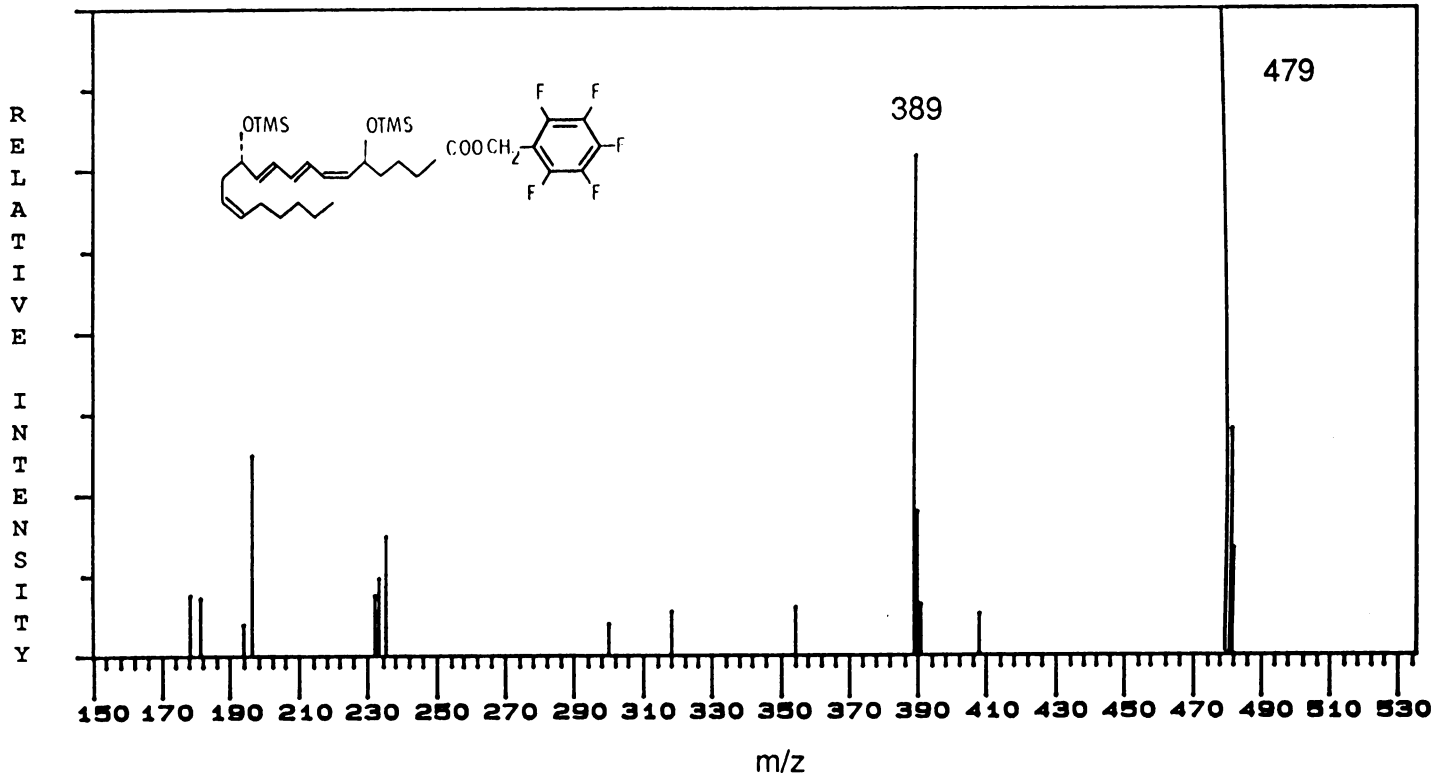

Figure 5-10. The ECNI spectrum of derivatized, standard LTB_4. The peak at m/z= 479 represents the $(M-181)^-$ ion and the peak at m/z= 389 represents the $(M-181-90)^-$ ion. MS parameters: source T= 200°C, CH_4 pressure= 2.1 X 10^{-4} torr. GC temperature program: 60-200°C at 20°C/min, 200-300°C at 7°C/min, on-column injection.

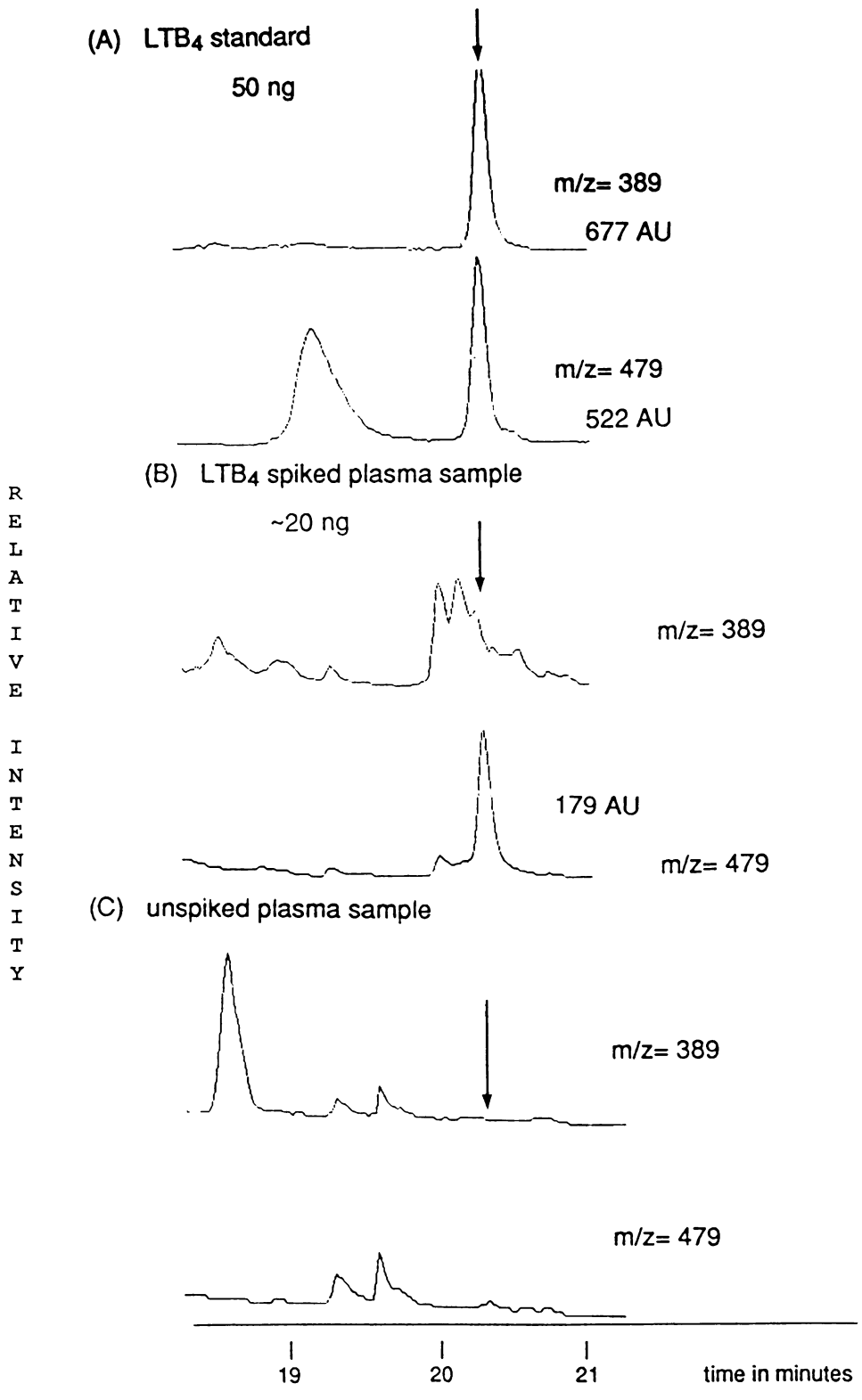

Figure 5-11. The HP 5985A GC-ECNI-MS SIM profiles of TMS, PFB derivatized: (A) standard LTB_4, (B) LTB_4 spiked plasma and (C) unspiked plasma. The GC-MS parameters are listed in Figure 5-5. AU= area units.

ion at 389 is used only as a confirmation ion. The retention time of the derivatized LTB_4 also can be used for confirmation. Thus, it is not always necessary to monitor both m/z= 389 and m/z= 479. When ^{18}O-labelled LTB_4 is incorporated into the samples, only the molecular ions 479 and 483 should be monitored simultaneously, for equal time intervals.

The exact amount of LTB_4 present in the lavage and plasma samples was not determined. The $^{18}O_2$ labelled LTB_4 had not been prepared. The first time it was prepared, the LTB_4 used was the same as the LTB_4 in the spiked samples. The chromatographic profile showed that the labelled LTB_4 was not suitable to be used as an internal standard. There was more than one compound present at the known retention time of LTB_4. The presence of more than one peak could have been caused by the degradation of the LTB_4 during the labelling process or the LTB_4 could have already been degraded when it was received. The labelling of LTB_4 requires at least 24 hours for 90% ^{18}O exchange. This reaction had proceeded for 52 hours.

Labelled LTB_4 is made in the following manner: $H_2{}^{18}O$ (Icon Services Inc., Summit, NJ) and LTB_4 (gift- J. Rokach) are stirred at 37°C for at least 24 hours with pseudocholinesterase XI as the catalyst (18). Both carboxylic oxygens are exchanged with the labelled oxygen in water. After 24 hours the reaction is acidified with HCl in $H_2{}^{18}O$. It is extracted with ethyl acetate. The % of ^{18}O incorporation is determined by monitoring the molecular ions of the labelled and unlabelled compounds. This reaction should increase the molecular weight of the LTB_4 by four. The reaction was conducted again using a different source of LTB_4 and was allowed to react for only 24 hours instead of 52 hours. The incorporation was successful. The selected ion profiles for the PFB ester, TMS ether derivatized LTB_4 are shown in Figure 5-12. As soon as adequate $^{18}O_2$-labelled LTB_4 is prepared, samples will be spiked before derivatization and the recovery of the LTB_4 in plasma and lavage determined.

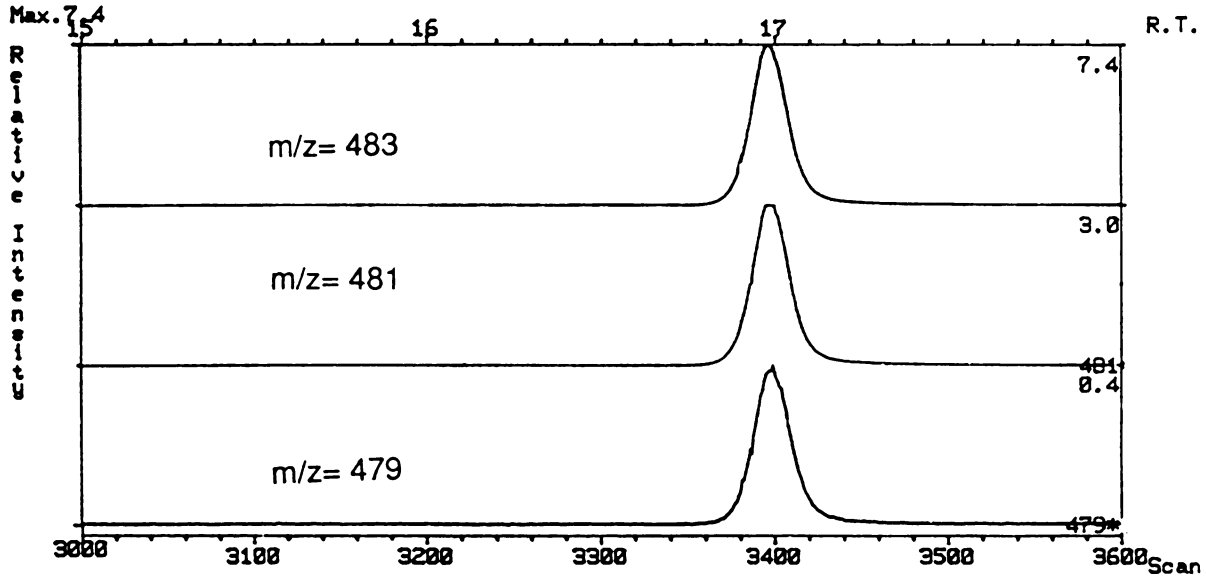

Figure 5-12. The SIM profiles of ^{18}O-labelled LTB_4. The 24-hour reaction incorporated 70% of the total LTB_4 with two ^{18}O atoms (m/z= 483) and 29% of the total LTB_4 with one ^{18}O atom (m/z= 481).

Project III- The Birch Reduction

In order to attempt this project much research went into determining just how to physically accomplish the organic reaction. Gratitude is expressed to Dr. William Reusch and his graduate student, Wuyi Wang for their assistance. The physical set-up for the reaction is shown in Figure 5-13. The apparatus was assembled in a fume hood. The reaction was performed in an argon atmosphere; thus argon must be available in the fume hood. The glassware used must be very dry. It is also preferable to do this reaction on a day when the humidity was low.

Glassware with ground glass joints was required. A 25 ml three-necked round bottom flask was used for the preparation of Li/CH_3NH_2 solution. All glassware was stored in a 150°C oven until use. The three-necked flask was fitted with a condenser. The condenser and three-necked flask are removed from the oven and allowed to cool slightly. All joints are greased and stoppers are placed in the open holes. The greasing was critical because it will prevent moisture from entering the flask when the condenser is filled with dry ice.

The system is purged with argon. The condenser and Dewar are then filled with dry ice and isopropanol (-22°C). A squeeze bottle allows for the controlled addition of isopropanol. It is also important that the dry ice be added to the condenser and Dewar before the glass reaches room temperature (i.e. while the glass is still hot). This prevents moisture from adhering to the glass surface that will condense when the dry ice is added.

The three-necked flask where the Li/CH_3NH_2 reagent is produced is cooled in the Dewar and methylamine (Aldrich) is added through a ground glass jointed flow controller. The argon is turned off momentarily to allow methylamine to bubble through the paraffin. After a few minutes methylamine should start to condense in the flask and refluxing will occur. The methylamine is collected until there are approximately 3 ml of

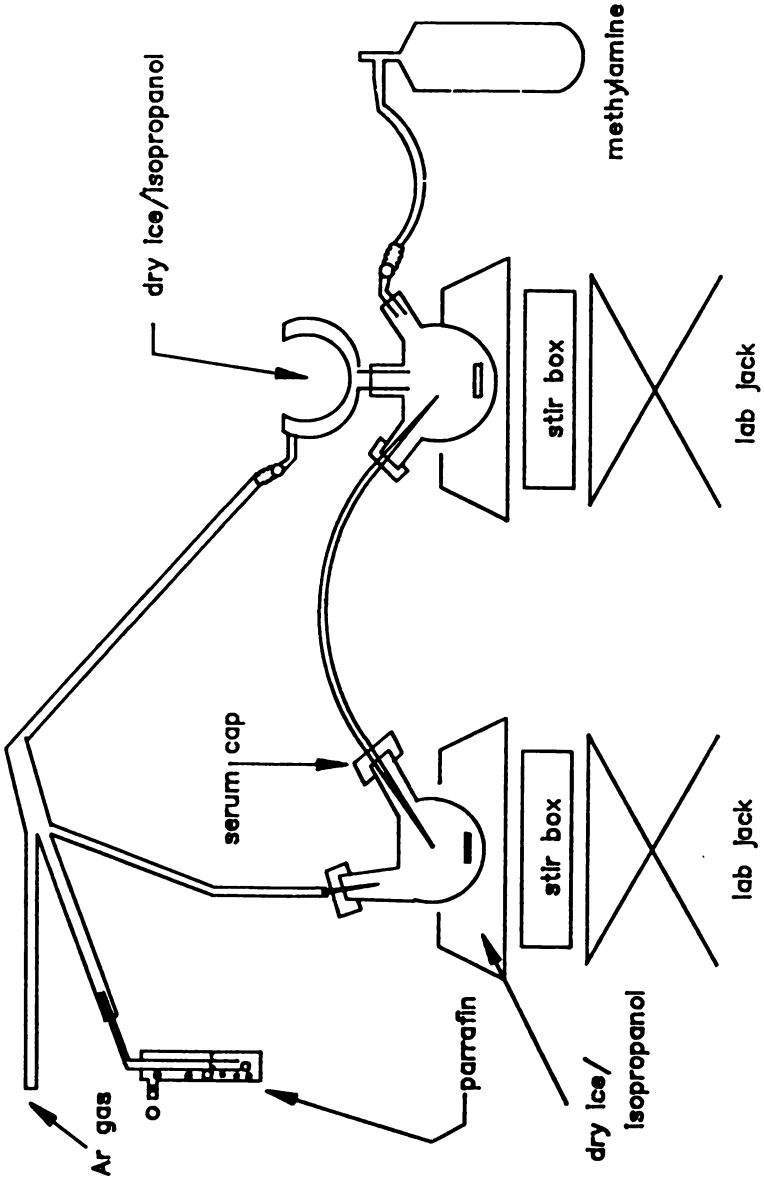

Figure 5-13. The physical set-up for the apparatus used in the Birch reduction.

methylamine for each sample. Then the methylamine is turned off but at the same time the argon flow is increased to assure that no air enters the system. The methylamine tank is turned off and the flow controller is closed.

Lithium metal (Aldrich) is cut and placed in the refluxing liquid methylamine. The procedure for cutting the lithium is as follows: using scissors cut a piece of lithium, while it is covered with mineral oil. The metal should not be handled with bare hands. (Since the sulfidopeptide leukotrienes are of very small concentration, approximately 5 mm of wire is cut; this will usually correspond to a molar excess.) The piece of Li metal that has been cut should be shiny and not dull (oxidized). Using tweezers, the metal is placed in hexane to remove the mineral oil. It is then quickly transferred to the liquid methylamine.

After the lithium dissolves in the liquid methylamine, the solution in the round bottom flask will turn blue. Failure to achieve a blue color may result from two causes: a) there is water present in the closed system, or b) the liquid methylamine is too cold and the metal has not dissolved. Case b is the more likely. If the metal does not dissolve then the dewar surrounding the round-bottom is removed to let the system warm slightly. Once the blue solution appears and remains for at least ten minutes it is ready to be transferred to the sample flasks.

The sample fractions are received in the mobile phase ($MeOH/H_2O/TFA$) in which they eluted. They are transferred to ground glass jointed flasks. Sample vessels require at least two necks and should be 10 ml in volume. The sample vessels should be silanized. The sample vials are rinsed twice with water to assure complete transfer of the sample. Methanol and trifluoroacetic acid (TFA) are removed first by purging with nitrogen. The sample is then lyophilized (freeze-dried) to remove the residual water. The Birch reaction should be completed soon after the lyophilization due to the stability of the leukotrienes. The internal standard is added at this time. The proper internal

standard is ^{18}O-labelled 5-HETE. The flask should contain a stir bar and the joints should be stoppered with the proper size serum caps.

Before the transfer of the Li/CH_3NH_2 reagent, the sample vessel is purged with argon and placed in a dry ice/isopropanol bath. The lithium solution is transferred through a piece of metal tubing. The two-necked sample flask will have an outlet that is at atmospheric pressure and in the other neck will hold the transfer line. The solution passes through the tubing because of pressure which builds up in the system. In order for this to occur, the argon pressure is increased at the tank and the stoppers on the solution vessel are held tightly. The outlet hole for the Ar on the paraffin flask is plugged. About 2-3 ml of the lithium solution are added per sample flask. The measurement is very crude due to the method of transfer. The sample vessels are left to react for ten minutes and the blue color may disappear. The reaction is quenched with a volume of methanol that is 10% of the Li/methylamine volume (0.2-0.3 ml). When the solution is colorless the reaction has been quenched. The sample vessels are allowed to warm to room temperature under the hood. The septa are removed to allow the escape of methylamine gas.

Once the methylamine has evaporated, which may be expedited with N_2 purging, 1 ml of water is added to each flask and the samples are treated with HCl to adjust the pH to 3. The analyte is extracted three times with hexane/ethyl acetate (1:1). The extracts are pooled and transferred to a silanized one dram vial. The solvent is evaporated. The residue is then redissolved in 0.2 ml of methanol. A small amount (tip of a spatula) of 5% Rh (Aldrich) on alumina is added. The vial is placed on ice. Hydrogen gas is bubbled into the methanol while it is constantly stirred for fifteen minutes. The hydrogen must be bubbled into the methanol. If it is allowed to just blow on the surface of the methanol, the methanol will evaporate quickly and a small fire will commence at the top of the vial. After fifteen minutes the hydrogen is stopped and the

catalyst is removed by centrifugation. Small silanized 6 X 50 mm test tubes containing the solution are placed in a microcentrifuge for ten seconds. The methanol can then be separated easily from the catalyst. The vial and catalyst are washed three times with methanol and the methanol washings are pooled. The methanol is removed with nitrogen.

At this stage the analyte has been converted to 5-hydroxyeicosanoic acid (MW=328). The compound is derivatized with both pentafluorobenzyl (PFB) bromide to form the PFB esters and BSTFA to prepare the 5-OTMS ethers. The derivatized species (MW=580) can then be analyzed by GC-MS following electron capture under methane chemical ionization conditions. The carboxylate anion fragment (m/z=399) is monitored.

The outcome of these reactions was quite positive. The success of finding the desired compound, which was present in ng to μg quantities, after such an extensive procedure was encouraging. The samples had been confirmed to have peptido-LTs by RIA, but the collaborating scientists wanted mass spectral confirmation. Unfortunately, GC-ECNI-MS cannot confirm that peptido-LTs are present in the islet cell wash samples that were sent. Since the S/N ratio was only equal to three, it cannot be stated that the islet cells produce peptido-LTs when challenged. A S/N ratio of at least five is required.

In conclusion, it is apparent from these last two discussions that much more work will be required to complete the two projects. They will be continued by other students in our laboratory. If the procedures are followed exactly, with forethought and careful planning, there should be no problem in passing these projects over to other people. It is hoped that successful experiments with definite positive answers will be completed within a year's time.

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