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Identification of Soybean Lipoxygenase Products by Gas Chromatography-Mass Spectrometry (GC-MS)

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Dr. James Rife, *Faculty Mentor (Chemistry & Geology)*
Date: May 24, 2005

Abstract:

Lipoxygenases (LOXs) are enzymes which catalyze peroxidation of polyunsaturated fatty acids containing at least one cis, cis 1, 4-pentadiene moiety to form conjugated diene-hydroperoxides. Soybean seeds contain three LOX isoenzymes while at least five different isoenzymes are in the vegetative tissue. Lipoxygenases have been related to several functions i.e. plant growth, defense mechanisms against pathogens and pests, and lipid metabolism. Given the proposed roles of LOX isoenzymes and the presence of multiple LOX isoenzymes in soybean vegetative tissue, it is likely that individual isoenzymes play specific functions. Do the isoenzymes show differences in which products they form? The basic goal of this project was to develop a GC-MS assay to determine the product formed by soybean LOX, and determine the preferred products formed by the different isoenzymes. The complete characterization and comparison of products can give clues about the physiological roles of specific isoenzymes. In this research, linoleic acid and linolenic acid were used as substrates for LOX enzymes to analyze the primary reaction products of LOX in order to see which end of the 1, 4-pentadiene section is used in the reaction. The peroxidation of linoleic acid can take place either at Carbon atom 9 or the carbon atom 13 to form 9-hydroperoxylinoleic acid (9-HPOD) and 13-hydroperoxylinoleic acid (13 HPOD), respectively. Other reactants such as linolenic acid, which has three double bonds, can yield even more products. A GC-MS assay was developed and implemented for analysis of reaction products of LOX.

Abbreviations:

LOX = Lipoxygenase; GC-MS= Gas Chromatography-Mass Spectrometry; 9-HPOD = 9-Hydroperoxyoctadecadienoic acid; 13-HPOD = 13-hydroperoxyoctadecadienoic acid.

Introduction:

Lipoxygenases (LOXs) are non-heme iron containing dioxygenases which catalyze the regio- and stereospecific dioxygenation of polyunsaturated acids containing a cis, cis 1,4-pentadiene moiety (i.e. Linoleic acid and Linolenic acid), to form conjugated diene-hydroperoxides as seen in the reaction below (Fig. 01) [1].

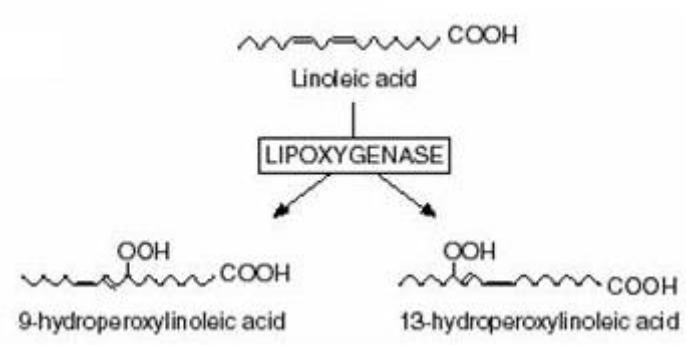


Fig. 01: ***Reaction of Lipoxygenase***

Soybean plants have several LOX isoenzymes or different proteins that catalyze the same reaction. Soybean seeds contain at least three LOX isoenzymes while at least five different isoenzymes are in the vegetative tissue [1, 2]. Lipoxygenases have been related to plant growth and development, lipid metabolism and senescence. In addition, LOX has been proposed to function as storage protein for nitrogen. LOX enzymes in soybean plants are involved in pathways that produce several volatile molecules like jasmonic acid and trans-2- hexenal [2]. Some products from the lipoxygenase pathways contribute to the characteristic, fresh, green odor emitted by leaves and play a role in defense against pests and pathogens. In soybean leaves, LOX has been intensively examined but the characteristics of the different forms are not well established. Given the proposed roles of LOX isoenzymes and the presence of multiple LOX isoenzymes in soybean vegetative tissue, it is likely that individual isoenzymes play specific functions.

As seen in the reaction above (Fig. 01), the substrate, linoleic acid, can be converted into two different products. The peroxidation of substrate can take place either at carbon atom 9 or the carbon atom 13 to form 9-hydroperoxylinoleic acid (9-HPOD or 9-hydroperoxyoctadecadienoic acid) and 13-hydroperoxylinoleic acid (13 HPOD or 13-hydroperoxyoctadecadienoic acid), respectively (Fig 2) [3].

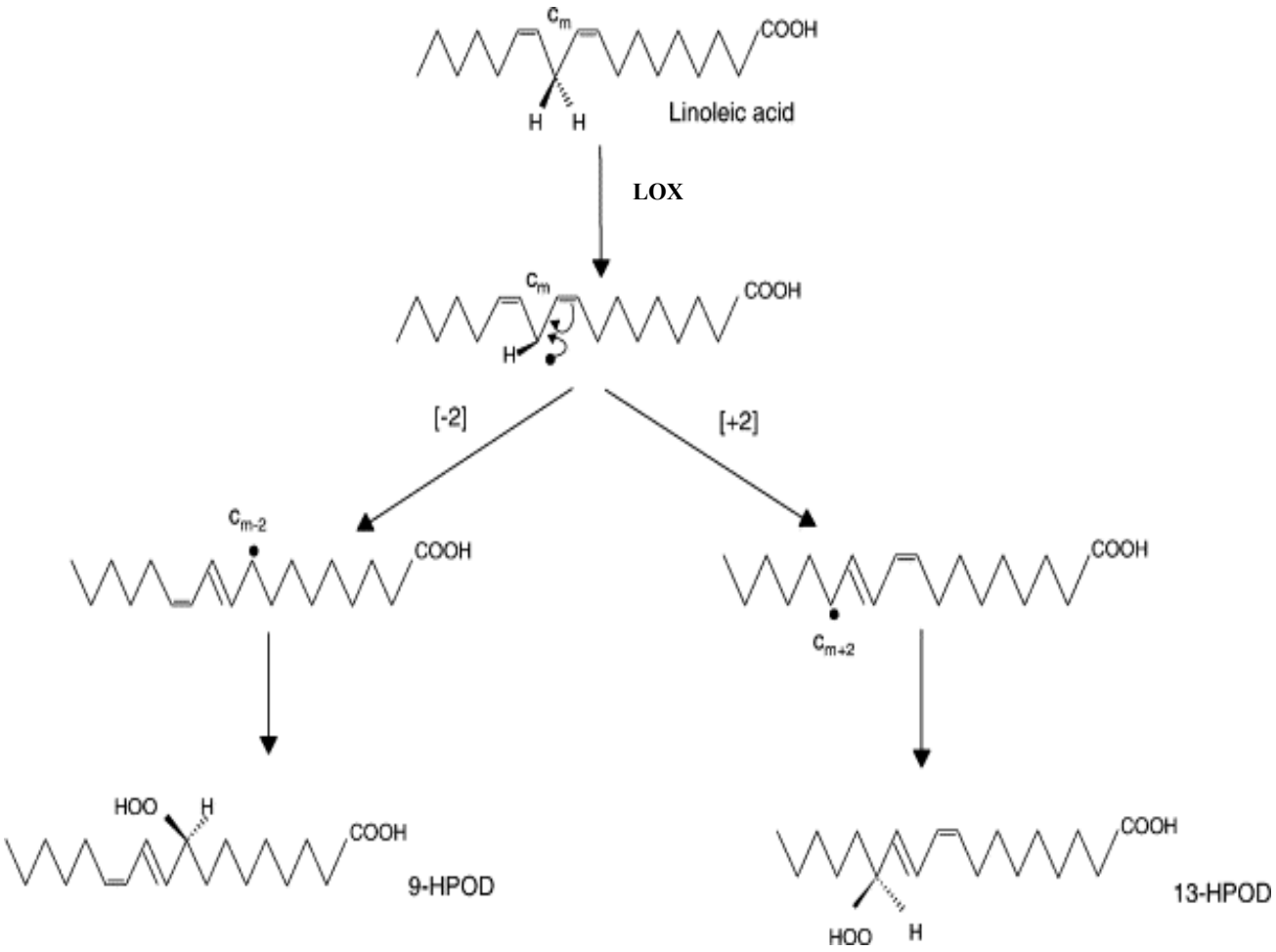


Fig.02: ***Mechanism of the dioxygenation reaction catalyzed by lipoxygenases with linoleic acid as substrate***

(Small arrows indicate electron shifts during the (−2) radical rearrangement of the fatty acid)

Depending on the number of double bonds of the substrate, two or more possibilities exist for hydrogen removal and, as a result for each removal of hydrogen, two products can be formed. Other reactants such as linolenic acid, which has three double bounds, can yield even more products.

The complete characterization and comparison of products can give clues about the physiological roles of specific LOX isoenzymes since the biological activity of LOX products and their roles as precursor to different end products are determined by the positional nature of hydroperoxides produced.

Do the isoenzymes show a preference for which product they form? Do different isoenzymes produce different products? The basic goal of this project is to determine the preferred products formed by the different isoenzymes.

Different methods have been used to determine the structure of LOX products [1, 3, 4, 5, 6, and 7]. In this research project, we are using linoleic acid and linolenic acid as substrates for LOX enzymes to analyze the primary reaction products of LOX in order to see which 1, 4-pentadiene section is used in the substrate and which end of the 1, 4-pentadiene is oxidized, and in other words which product is preferably formed. For this purpose, we have developed and implemented a GC-MS assay for analysis of reaction products of LOX.

GC-MS is an effective combination of two techniques; Gas chromatography and mass spectrometry. Gas Chromatography is used for the separation and detection of organic compounds. A GC system utilizes a carrier gas to transport the sample molecules from an injection port, through a long capillary tube or column which is located in a temperature-programmable oven where separation occurs and on to the detector where the specific compounds are observed. Separation of multiple compounds is done by taking advantage of the differences in boiling points of the compounds, as well as differences in their affinities toward the column through which they pass.

The mass spectrometer, which functions as the detector, receives the compounds as they exit the column. The mass spectrometer splits the compounds into charged

fragments, which are then separated according to mass. The structure of a sample can be deduced by careful analysis of the fragment pattern. Since most organic compounds have unique fragmentation patterns, we can distinguish between the different compounds in the original sample using the structural information obtained from the mass spectra.

Materials and Methods:

- ### Materials and Reagents:

The following reagents were used in this research.

0.2 M sodium phosphate buffer was prepared using monosodium phosphate and disodium phosphate. Linoleic acid, linolenic acid and BSTFA (Bis (Trimethylsilyl)-Trifluoroacetamide) were obtained from Sigma. $NaBH_4$ and Rhodium on alumina powder were obtained from Aldrich. Diazomethane was prepared from diazald (obtained from Sigma) using the procedure of P. Lombardi (obtained through internet [8]). Silica gel and Octadecyl solid phase extraction columns were obtained from Supelco. The Shimadzu GCMS-GP5050A GC-MS column was used.

- ### Experimental (Fig. 03 & 4)

Preparation of LOX Products:

The products of the reaction between lipoxygenases and the fatty acid were formed by incubation of approximately 1mg enzyme with 1.29 mM Linoleic acid and 1.29 mM Linolenic acid in 20mL of 0.2M sodium phosphate buffer, pH 6.5, with compressed air bubbling for 15 minutes [1].

Reduction of Products:

The unstable peroxide products were immediately reduced by adding 100 mg of $NaBH_4$ to the reaction mixture and incubating for 3 minutes. Then the reaction mixture was acidified with HCl to pH 2. The resulting solution was extracted twice with 5 ml ether. The ether fractions were dried over anhydrous Sodium Sulfate [1].

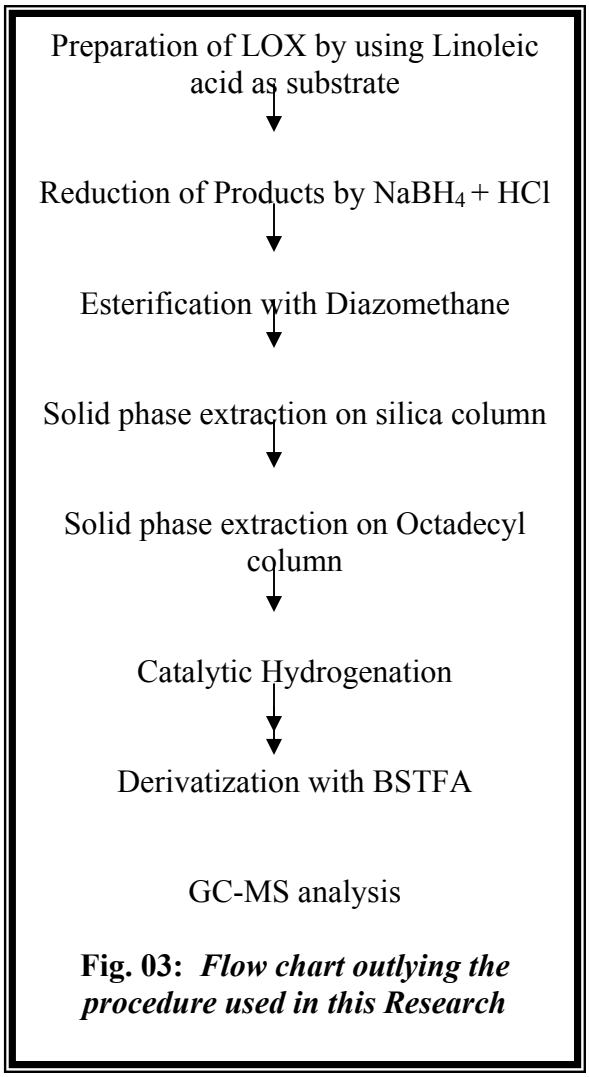


Fig. 03: ***Flow chart outlying the procedure used in this Research***

Esterification:

First of all diazomethane was prepared. For this purpose, 0.04975 g diazald (p-toluenesulphonylmethyl-nitrosamide) was suspended in 5 times its weight of ethanol in a vacuum flask. This flask was connected by tubing to a side arm test tube which contained the reduced product from the previous step. The side arm test tube was connected by tubing to a final flask containing acetic acid to trap un-reacted diazomethane. Nitrogen gas entered the first flask, flowed into the side arm test tube and finally flowed into the flask containing acetic acid. Both flasks were kept in an ice bath .The mixture of the first flask was stirred while N_2 was allowed to flow into the system. Diazomethane was produced by injecting 1.0 M sodium hydroxide into the diazald using a syringe. By the help of this setup, freshly prepared diazomethane was bubbled through to the dried residue from the previous step and left at room temperature for 10 minutes to form the methyl esters. Then the solvent and reagent were evaporated and residue was dissolved in 1 mL of hexane [4].

Solid phase extraction:

The methyl ester products of fatty acid were applied to silica solid phase extraction column that had been pre-conditioned with 2mL hexane. The column was washed with 2 mL 5% ether in hexane and then samples were eluted with 50% ether in hexane (1/1, v/v). The solvents were evaporated under N_2 and then dissolved in 50 % methanol in water (1/1, v/v). Then the samples were again purified using Octadecyl solid phase extraction column that had been preconditioned with 2 mL methanol followed by 2 mL water. The column was washed with 2mL 50% methanol and elution of samples was carried out with 2mL of 100% methanol [4].

Catalytic Hydrogenation:

Following this purification, samples were evaporated and reconstituted in 1 mL methanol and then combined with 5mg rhodium on alumina powder. Then samples were placed on ice and hydrogen gas was bubbled through them for 30 minutes. The samples were filtered and washed with 1 mL methanol and then evaporated to dryness [4].

Derivatization:

After drying, derivatization of samples was carried out with Bis (Trimethylsilyl)-Trifluoroacetamide (BSTFA) for 30 minutes at 60 ^{o}C. Excess BSTFA was removed by evaporation under N_2 [4].

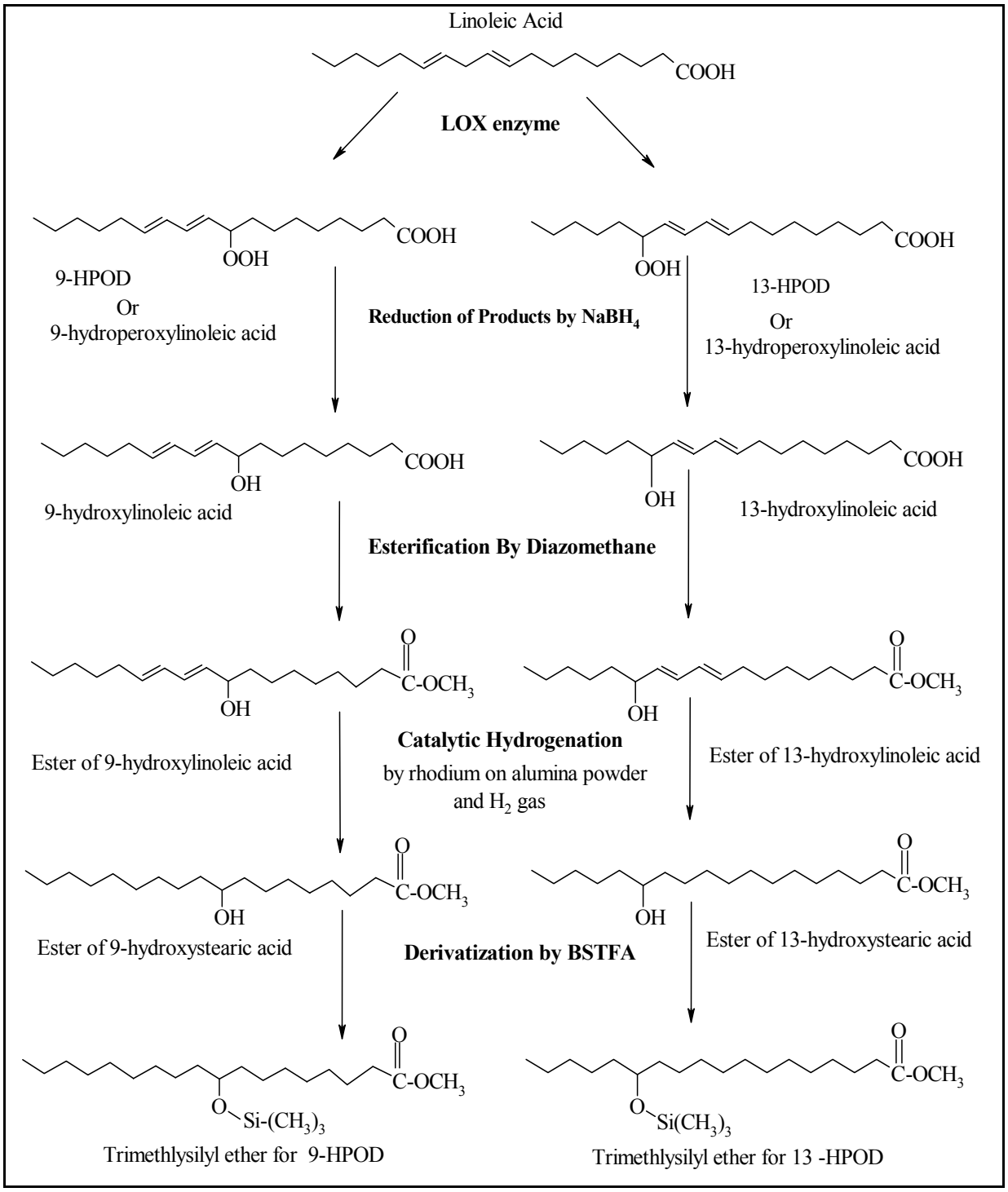


Fig. 04: ***Reactions used throughout experiments prior to GC-MS***

GC-MS analysis

For GC-MS technique, 50µL hexane was added to the samples. Then the samples were analyzed on a Shimadzu GC-MS-GP5050A gas chromatograph mass spectrometer. 0.5 µL samples were injected. The injection temperature was 250 ^{o}C, while interface temperature was also 250 ^{o}C. The GC was used on a split mode with split ratio = 20. Column flow rate was 1 mL/min. The GC temperature was programmed from 100 ^{o}C (hold 5 min.) to 280 (hold for 25 min.) with 6 ^{o}C/min increment. The carrier gas was helium. The Mass spectrometer was used as follows: solvent cutoff, 3 min; detector volt, 1.05 kV; interval, 0.42 second; lower cutoff, 44 m/z; higher cutoff, 500 m/z.

RESULT:

First, LOX products were prepared by using linoleic acid and linolenic acid as substrates. Since analysis of these products was not possible due to their thermal instability, they were reduced by $NaBH_4$ and then hydrogenated to yield hydroxy product. After methylation and hydrogenation these products were converted into trimethylsilyl ethers by using BSTFA (Fig. 04).

The determination of the products obtained from linoleic acid and linolenic acid was performed by GC-MS. There were no peaks observed with linolenic acid. With linoleic acid, two major peaks were observed in the chromatogram (spectrum 1 & spectrum 2). Their retention times were 30.174 and 29.957 min., respectively.

The mass spectrum of peak 1 showed the characteristic peaks. In the spectra peak at m/z 229 due to the (CH_3 $(CH_2)_8$ -CH-OSi-$(CH_3)_3$) and at m/z 259 due to the (($(CH_3)_3$-Si-O-CH-$(CH_2)_7$COO CH_3) were obtained. Based on the basis of fragmentation pattern (Fig. 05) the peak with retention time 29.957 min was identified as 9-HPOD or 9- hydroperoxylinoleic acid. These peaks were obtained due to the α–α'- fragmentation of the TMSi group, while peak at m/z 73 showed (Si-$(CH_3)_3$), this peak was also observed in the mass spectra of 2nd peak. (Fig. 05)

Due to the α–α'- fragmentation of the TMSi group, 2 characteristics peaks were obtained from mass spectra of 2nd peak; one at m/z 173 for (CH_3 $(CH_2)_4$ CH-OSi-$(CH_3)_3$), 2nd at m/z 315 for (($(CH_3)_3$-Si-O-CH-$(CH_2)_{11}$COO CH_3). Based on the mass spectra the peak with retention time 30.174 min was identified 13-hydroperoxylinoleic acid (13-HPOD). (Fig. 05)

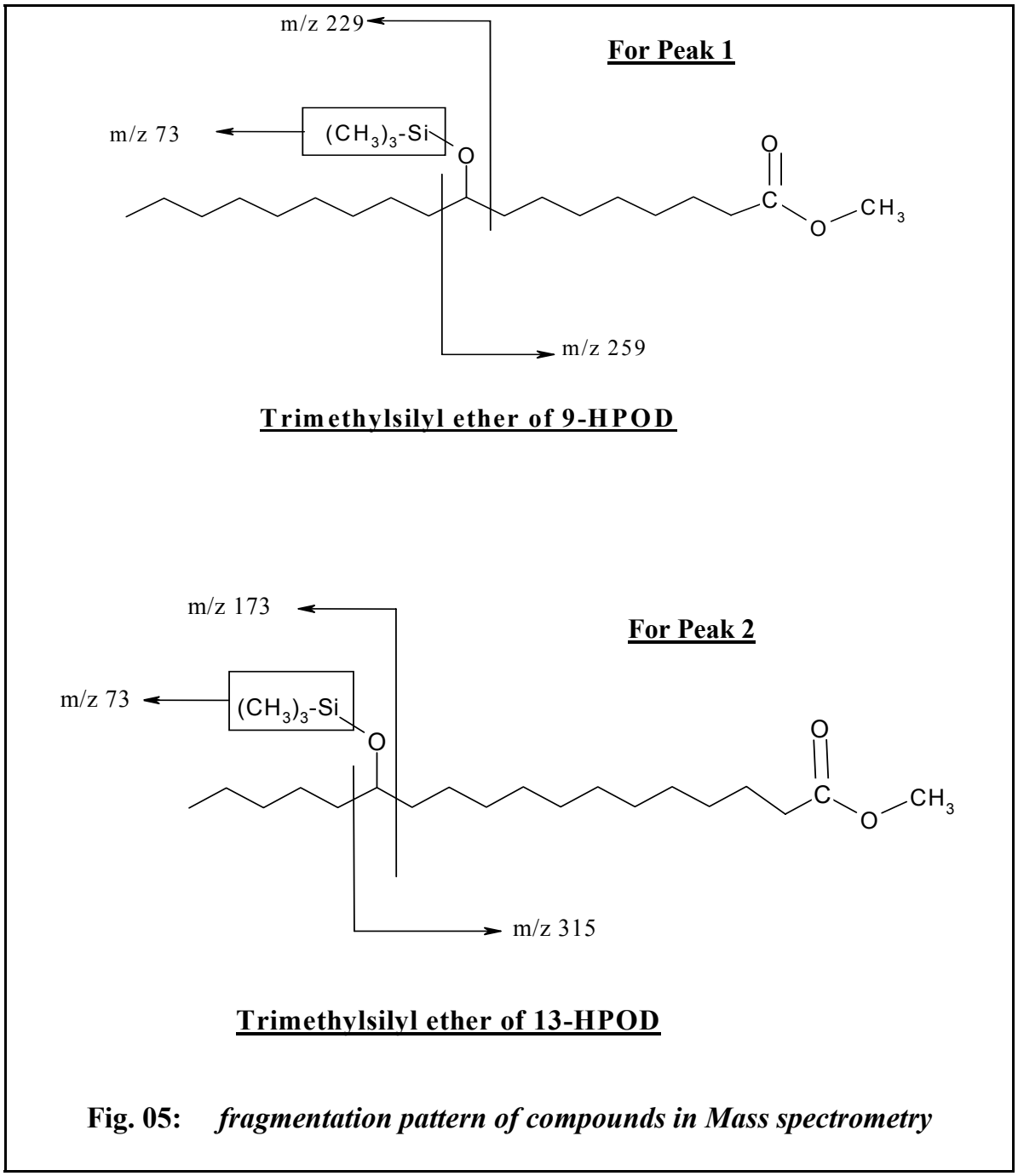
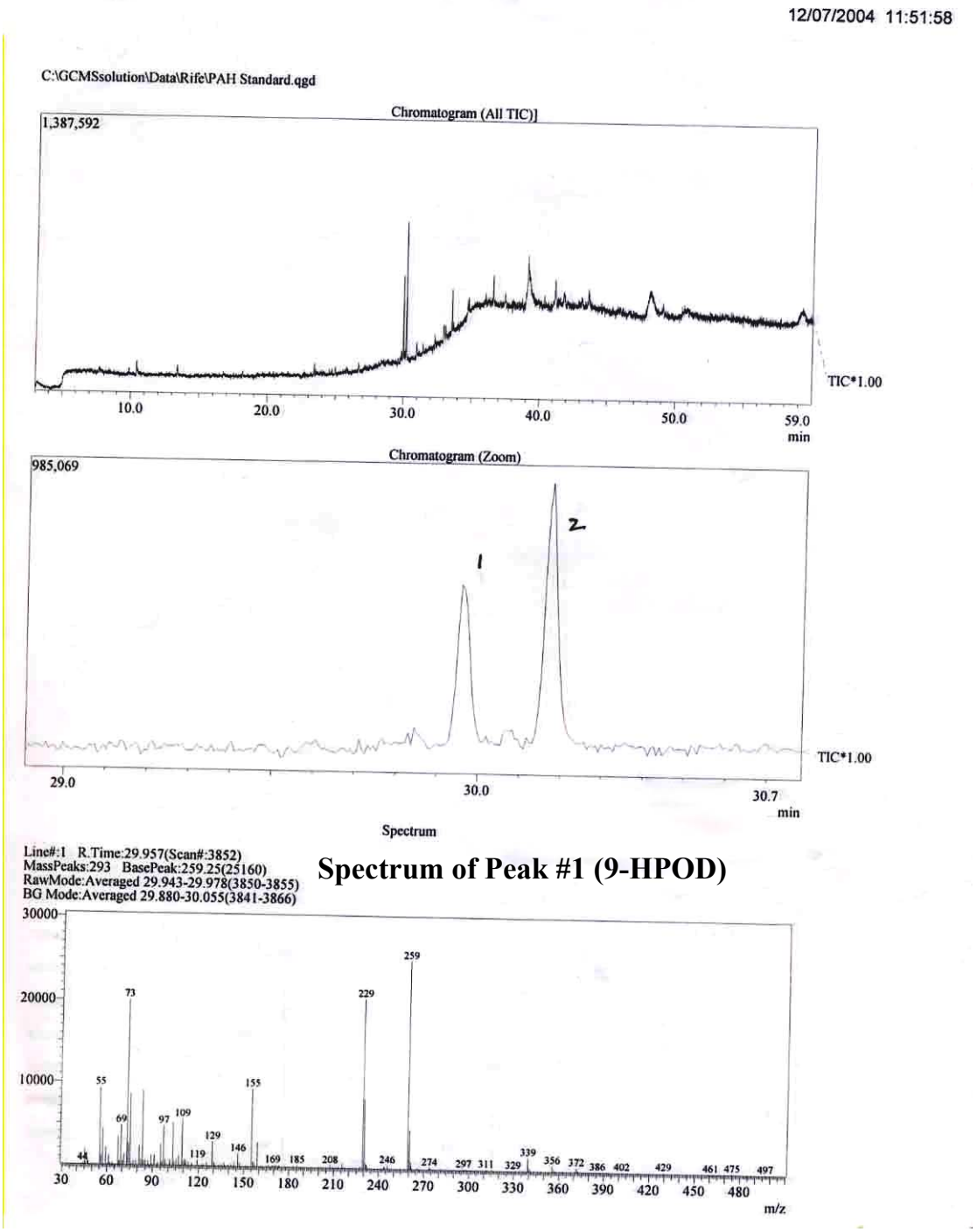


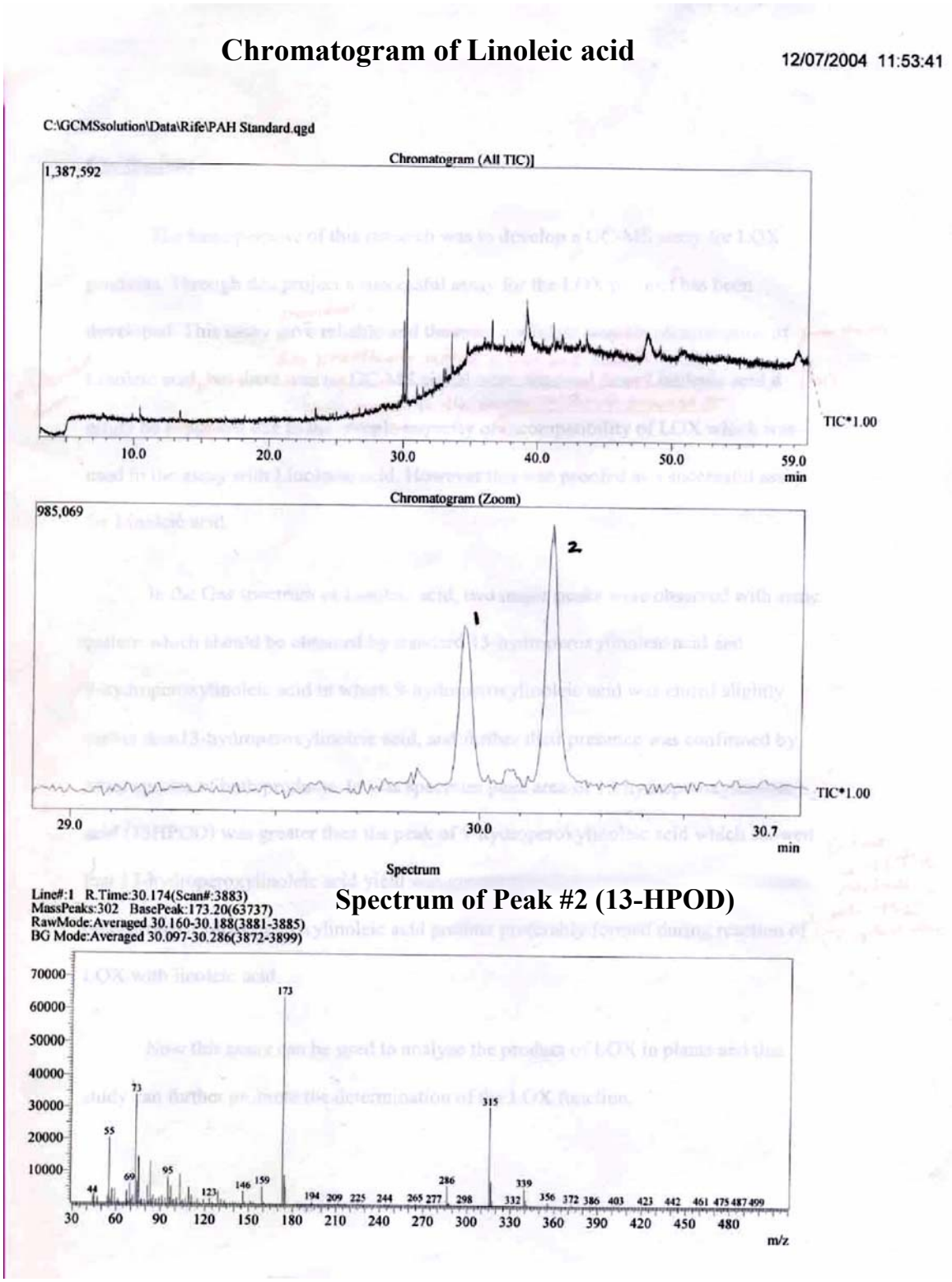
Fig. 05: ***fragmentation pattern of compounds in Mass spectrometry***

Chromatogram of Linoleic acid



Spectrum of Peak #1 (9-HPOD)

Spectrum 1


Chromatogram of Linoleic acid
12/07/2004 11:53:41
C:\GCMSsolution\Data\Rife\PAH Standard.qgd
Chromatogram (All TIC)]
1,387,592
TIC*1.00
10.0
20.0
30.0
40.0
50.0
59.0
min
Chromatogram (Zoom)
985,069
2
1
TIC*1.00
29.0
30.0
30.7
min
Spectrum
Line#:1 R.Time:30.174(Scan#:3883)
MassPeaks:302 BasePeak:173.20(63737)
RawMode:Averaged 30.160-30.188(3881-3885)
BG Mode:Averaged 30.097-30.286(3872-3899)
Spectrum of Peak #2 (13-HPOD)
70000
60000
50000
40000
30000
20000
10000
173
73
315
55
95
69
286
339
44
123
146
159
194 209 225 244 265 277 298
332
356 372 386 403 423 442 461 475 487 499
30 60 90 120 150 180 210 240 270 300 330 360 390 420 450 480
m/z

Spectrum 2

Conclusion:

The basic purpose of this research was to develop a GC-MS assay for LOX products. Through this project a successful assay for the LOX products has been developed. This assay provided a reliable and confident identification of products obtained from linoleic acid. No products were observed with linolenic acid. This might be happened due to an error in the derivatization process or incompatibility of LOX which was used in the assay with linolenic acid.

In the Gas spectrum of linoleic acid, two major peaks were observed with same pattern which should be obtained by standard 13-hydroperoxylinoleic acid and 9-hydroperoxylinoleic acid in which 9-hydroperoxylinoleic acid was eluted slightly earlier than13-hydroperoxylinoleic acid, and further their presence was confirmed by Mass spectra of both products. In Gas spectrum peak area of 13-hydroperoxylinoleic acid (13HPOD) was greater than the peak of 9-hydroperoxylinoleic acid (9-HPOD) and their ratio was 1.6:1 which showed that 13-hydroperoxylinoleic acid yield was greater than 9-hydroperoxylinoleic acid. In other words, 13-hydroperoxylinoleic acid product preferably formed during reaction of LOX with linoleic acid.

Given the proposed roles of LOX isoenzymes and the presence of multiple LOX isoenzymes in soybean vegetative tissue, it is likely that individual isoenzymes play specific functions. The complete characterization and comparison of products can give clues about the physiological roles of specific isoenzymes. Now this assay can be used to analyze the product of LOX in plants and this study can further promote the determination of the LOX function.

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Rehan Ahmed Malik was born and grew up in Karachi, Pakistan. In December 2000, he received a BSc. degree (major in Chemistry and minors in Microbiology and Biochemistry) from University of Karachi in Karachi, Pakistan. After his Graduation he moved to Mankato, Minnesota, USA for further studies. Now he has recently graduated from Minnesota State University, Mankato on May 14, 2005 with BS degree in Biochemistry and minor Computer and Information Sciences. He conducted an undergraduate research project on "Identification of Soybean Lipoxygenase products by GC-MS assay" in fall 2004 under the supervision of Dr. James Rife. He was awarded a grant for this research by URC. He participated in the URC conference and presented his research work in the form of PowerPoint presentation on April 26, 2005. Now he is willing to earn a PhD in neural sciences from a reputable University.

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Faculty Mentor Biography:

James E. Rife obtained a B.S. in Chemistry from the University of Illinois (Champaign-Urbana) in 1972 and a Ph.D. in Biochemistry from the University of Wisconsin-Madison in 1978. He was a post-doctoral research associate in the Department of Chemistry at the University of Wisconsin-Madison from 1978 to 1980. From 1980 to 1986, he was an Associate Professor of Chemistry at Mundelein College in Chicago. He has been a faculty member in the Department of Chemistry and Geology at Minnesota State University, Mankato since 1986.

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