VOLATILE AND NON-VOLATILE COMPONENTS OF BEEF MARROW BONE STOCKS

by

BELAYET H. CHOUDHURY

A Dissertation submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

Graduate Program in Food Science

Written under the direction of

Professor Henryk Daun and Professor Chi-Tang Ho

and approved by

_____

_____

_____

_____

New Brunswick, New Jersey

October, 2008

ABSTRACT OF THE DISSERTATION

Volatile and Non-volatile Components of Beef Marrow Bone Stocks

By BELAYET H. CHOUDHURY

Dissertation Directors:
Professor Henryk Daun and Professor Chi-Tang Ho

Beef bone marrow has been part of the human diet since prehistoric times. Marrow bone stock is important culinary base used by gourmet chefs. It is well known for its distinct savory character in foods. While there has been a great deal published on flavor active components in cooked meats, the flavor composition of bone marrow is still relatively unstudied.

For this study, commercial chopped fresh beef marrow bones were simmered in water for seven hours at 90ºC. Three batches of cooked marrow bone mixtures were prepared. First batch was not enzyme treated. The second and third batch was enzyme treated with papain and umamizyme and heated for one hour at 65ºC and 50ºC respectively. All three batches (untreated and enzyme treated) were defatted by microfiltration. Samples from all three batches were heated under pressure at 120ºC or 160ºC for one hour. In another series of experiments, the defatted stock samples of three batches (one untreated and two

treated with papain or umamizyme) were heated for one hour with ribose, xylose or methyglyoxal. Head space volatiles of all above samples were analyzed using Gas Chromatography-Mass Spectrometry (GC-MS) by Solid Phase Microextraction (SPME).

Stock sample prepared at 90ºC without further treatment showed presence of lipid oxidation products including diacetyl, alcohols, aldehydes and ketones. Stock samples both untreated and treated with enzymes and heated at 120ºC for one hour showed additionally Strecker aldehydes, dimethyl sulfides and furans. Stock samples treated with enzymes showed in addition pyrazines. Stock samples both untreated and treated with enzymes and heated at 160ºC for one hour showed all of the compounds identified at 120ºC heating at higher concentration and fatty acids, thiazoles and alkenals. Samples with addition of methylglyoxal showed significantly higher levels of pyrazines and alkenals. The results of our research showed that after heating and especially after treatment with enzymes and addition of ribose, xylose, and methylglyoxal a number of novel flavor alkenals and interesting volatiles are formed that were not previously identified in bone marrow stocks.

All three stock samples were analyzed using Liquid Chromatography-Mass Spectrometry (LC-MS) for non-volatiles mainly amino acids, di- and tri peptides. 20 peptides are identified in the Papain and Umamizyme digested samples. Umamizyme treated stock yielded the most peptides followed by papain treated stock. These non-volatiles act as volatile precursors and generated numerous volatiles when the stocks were treated with ribose, xylose or methylglyoxal.

Preface

Marrow bones are part of our food chain. Marrow bones stock is of culinary importance. There is a great need for research that bridges the gap between volatile and non-volatile understanding of the flavor chemistry of marrow bones. The dissertation provides volatile and non-volatile studies of beef marrow bone stocks. I hope this study will spur further research interest in this area.

Belayet H. Choudhury

Acknowledgement

I acknowledge the Rutgers food science department to accept me as a part-time student and appreciative of the cooperation and assistance given to me over the years in fulfilling my dream to obtain the Ph.D. I wish to express my deepest appreciation and gratitude to my dissertation advisors, Dr. Henryk Daun and Dr. Chi-Tang Ho for their support, encouragement, patience, guidance and their confidence in my capabilities which has enabled me to achieve this goal.

I am thankful to my other committee members Dr. Thomas Hartman and Dr. Sree Raghavan, whose suggestions, criticism and valuable time has helped me prepare this work.

My acknowledgement goes to my employer ConAgra Foods for supporting this research. I would like to thank Mr. Indarpal Singh and Mr. Ed Fedorczyk, both of ConAgra Foods for their assistance in the LC-MS and microfiltration support.

I am grateful to my wife Rehana, whose love, encouragements and understanding helped me complete this project. Without the cooperation and sacrifice of my daughters Maliha and Samantha; sons Noah and Jaffrey this dissertation would not have been possible. Jaffrey was born in the middle of this research. He had been a constant inspiration to me.

I thank God Almighty to give me patience, perseverance and faith to complete this task.

Dedication

I dedicate this dissertation to my wife Rehana and our children, Maliha, Samantha, Noah and Jaffrey. I would not have progressed to this point without their support and sacrifices.

I also dedicate this dissertation in memory of my parents whose unconditional love and sacrifice kindled inspiration and motivated me in every steps of my life.

Table of Contents

Lists of tables

Page

List of illustrations

Figure

Page

1. INTRODUCTION

Beef bone marrow is part of the human and animal diet since prehistoric time. It is part of the culinary practices and has been used in gourmet cooking, soup, broth and stock preparation. Meat industry today uses marrow bones as an industrial by-product and salvages as much as possible. Fat is extracted and prepared as tallow while the protein is salvaged as meat extracts. Thus bone marrow is part of our food chain prepared from by products of mechanically deboned meat (Field et al., 1981; Field et al., 1999). Bone marrow stock is well known for its unique savory flavor. There are many published literature on the immunological role of beef bone marrow. However, the knowledge of the flavor active compounds in beef bone marrow is very limited. Volatile and non-volatile flavor active compounds in bone marrow flavor have not been studied.

The volatiles and non-volatiles in beef meat have been extensively studied. As the flavor chemistry of beef marrow bones is little understood, it is assumed that beef marrow bone proteins will behave similar to meat proteins. The lipid-derived compounds are the most abundant compounds in meat products rich in fat. On the other hand, Maillard reaction, which occurs between amino compounds and reducing sugars, is one of the most important routes of flavor compounds in meat products. The Maillard reaction involving peptides, amino acids and reducing sugars generates volatile compounds that contribute the final cooked meat flavor. A cascade of Maillard reactions follows to generate further volatiles.

Meat flavor is generated through the complex interactions among amino acids, peptides, sugars, thiamine, nucleotides, lipids and products of lipid oxidation (Imafidon and Spanier, 1994). In the past, it has been proven that the water soluble non-volatile components mainly amino acids, peptides, organic acids contribute to a distinct savory broth character in meat products. Most of the flavor precursors of meat are amino acids and peptides. These precursors react with lipids or lipid degradation products during cooking. During heating and enzyme hydrolysis, the proteins are degraded to yield peptides of various length and amino acids. The size of the peptides, their amino acid contents and sequences play an important role in meat flavor development. The short chain low molecular weight peptides participate as flavor precursors and contribute to the taste. The major components of the water soluble components of bone marrow are peptides, amino acids, nucleotides, organic acids, minerals, vitamins, breakdown products of lipids, collagen, and glycogen. Therefore, bone marrow stock is an ideal candidate for Maillard reaction generating volatile components.

Beef marrow stock is cooked in water at simmering temperature. The beef marrow bones contain very high amount of fat and small amount of protein. The investigation of protein and its break down products are a major focus of the studies. It is a challenging task to separate the protein from the fat. Enzyme treatment is needed as heating alone may not effectively hydrolyze the peptide bonds that link the protein molecule. Heating followed by enzyme treatment and fat separation using microfiltration increases the protein yield of the stock and increases the palatability. The degree of hydrolysis of protein increases with enzyme treatment. The protein

molecules are broken further into smaller peptides and they are available in the aqueous phase. Without the enzyme treatment, bulky protein molecules are lost in the filtrate. Besides, the compounds from the Maillard reaction can also react with other volatiles such as aldehydes and other carbonyls formed during lipid oxidation, which react readily with Maillard intermediates. Such interaction contributes to the generation of characteristic flavor of marrow bones stock. The stocks possess volatiles mainly of lipid derived aldehydes. When treated with various temperatures they participate in Maillard reaction and generate numerous volatiles besides lipid derived aldehydes. The stocks in reaction with ribose, xylose and methylglyoxal generate further more volatiles. The volatiles yield will depend upon the degree of hydrolysis. A profound knowledge of these volatiles will better understand the flavor chemistry of beef marrow bone. Therefore, the main objectives of this research are the characterization of the flavor compounds of beef marrow bones stock.

2. LITERATURE REVIEW

2.1 Background Information

2.1.1 Bone Marrow Interest

Beef bone marrows have been of culinary interest for centuries. Beef marrow bones are considered a food industrial by-product. They have been used to make soup, broth and stock. The bone marrow stock may be defined as a clear, thin liquid flavored by soluble substances extracted from the bones. This stock can be utilized in meat dishes, soups, vegetable dishes, sauces or gravies (Ockerman, 2000).

Stock made from the bones of animals, has been consumed as a source of nourishment for humankind throughout the ages. Muscle based food products derived from advanced meat recovery system may contain bone marrow. Lipid oxidation is a major issue with bone marrow generating flavor and off flavors (Miller, 1982; Mancini, 2004).

2.1.2 Beef Bone Marrow Stock Preparation

Beef stock also known as broth or bouillon. The gradual heating of the liquid is of the highest importance for the clarity as well as for the flavorfulness of the stock. Famous French chef Marie-Antoine Careme proposed an explanation in L'Art de la cuisine francaise au XIXe siecle in 1833 that the stock must come to a boil very

slowly, otherwise the albumin coagulates and hardens, the water do not penetrate the protein. Justus von Liebig is universally known for his pioneering studies in organic chemistry and for broths and meat extracts. Using vacuum to evaporate the stock produced by cooking minced meat in cold water, Liebig obtained a “beef extract” that he sold throughout the world (Harve This, 2006). This concentrated stock is known as broth. A patent procedure illustrated beef bone marrow broth preparation at simmering temperature (Vollmer and Riney, 1979).

2.2 Review of Beef Bone Marrow Chemistry and Biology

Although bone marrow non-volatiles influences the thermal aroma generation and contribute to taste, no research has assessed the role of bone marrow non-volatiles mainly peptides and amino acids in taste and flavor generation. There is an abundance of peer-reviewed literature focused on the mechanism of meat flavor generation from muscle meat. However, there is a lack of published research evaluating flavor generation by bone marrow peptides or lipids. Because the amount of bone marrow research in published literature is limited, the literature review will focus on bone marrow from a biological standpoint. Bone marrow components that may have a role in flavor generation will be reviewed.

2.2.1 Bone Marrow Types and Composition

Bone marrow is housed in a hollow central cavity. Beef bone marrow is found in vertebrae, sternum, ribs and long bones. Long bones such femur bones are composed of "fatty marrow" while he rests are abundant in "red marrow" that is composed of hematopoietic cells (Agar, 1983). It is estimated that marrow is very high in fat, low in protein and carbohydrates. Average bone marrow composition of femur bone was determined to be: protein 2.6%, fat 79.5%, moisture 17.9% (Vollmer, 1979).

2.2.1.1 Bone Marrow Lipids

The lipid content of beef bone marrow tends to change with animal age (Field, 1980). The fat content of marrow from young calves is relatively low compared to steers. The nonpolar lipids are increased with age while polar lipids are decreased. Within the polar lipid fraction of bone marrow, palmitic and oleic acid are increased whereas stearic and linoleic acids are decreased as the cattle is matured. Anatomical location affects the lipid content. The femur bones are composed of fatty white marrow, whereas ribs and vertebrae are abundant in red marrow. Beef femur bones contain 85% lipid compared with 26-56% in vertebrae (Kunsman, 1981). Beef bone marrow lipids contain small amount of phospholipids. **Table 1** represents the bone marrow fatty acids composition of a two years old cow.

Table 1: Fatty acid composition of bone marrow of two years old cow (Mello et al., 1976)

Fatty Acids %	%
C14:0 Myristic acid	1.2
C15:0 Pentadecanoic acid	0.6
C16:0 Palmitic acid	19.2
C16:1 Palmitoleic acid	3.6
C17:0 Heptadecanoic acid	1.7
C18:0 Stearic acid	18.9
C18:1 Oleic acid	47.2
C18:2 Linoleic acid	3.6
C18:3 Linolenic acid	0.2
C20:4 Arachidonic acid	1.2
C22:0 Docosanic acid	0.1

2.2.1.2 Bone Marrow Protein and Peptides

Bone marrow peptides were unknown up until the middle of 1970's. They were analyzed by Russian scientists from porcine bone marrow and named myelopeptides (MPs) (Petrov et al., 1997; Akhmedov et al., 2004; Karelin et al., 1998). **Table 2** represents these myelopeptides.

Table 2: Myelopeptides

Phe-Leu-Gly-Phe-Pro-Thr (MP-1)
Leu-Val-Val-Tyr-Pro-Trp (MP-2)
Leu-Val-Cys-Tyr-Pro-Gln (MP-3)
Phe-Arg-Pro-Arg-Ile-Met-Thr-Pro (MP-4)
Val-Val-Tyr-Pro-Asp (MP-5)
Val-Asp-Pro-Pro (MP-6)

Table 3: Peptides isolated from beef bone marrow hemoglobin (Ivanov et al., 1991)

Segments of the α-chain of the hemoglobin:

^{1}VLSAADKGNVKAAWGK^{16} (val-leu-ser-ala-ala-asp-lys-gly-asn-val-lys-ala-ala-trp-gly-lys)

^{16}KVGGHAAEYGAEA^{28} (lys-val-gly-gly-his-ala-ala-glu-tyr-gly-ala-glu-ala)

^{27}AEALERM^{32} (ala-glu-ala-leu-glu-arg-met)

^{76} LPGALSELS ^{84} (leu-pro-gly-ala-leu-ser-glu-leu-ser)

^{109}LASHLPSDFTPAV^{121} (leu-ala-ser-his-leu-pro-ser-asp-phe-thr-pro-ala-val)

Segments of the β-chain of the hemoglobin:

^{1}MLTAEEKAAVT^{11} (met-leu-thr-ala-glu-glu-lys-ala-ala-val-thr)

^{15}GKVKVDEVGGEALGRL^{30} (gly-lys-val-lys-val-asp-glu-val-gly-gly-glu-ala-leu-gly-arg-leu)

^{71}SNGMKGLDDLKG ^{82} (ser-asn-gly-met-lys-gly-leu-asp-asp-leu-lys-gly)

^{94}KLHVDPE^{100} (lys-leu-his-val-asp-pro-glu)

ARNFGKFF (ala-arg-asn-phe-gly-lys-phe-phe)

NFGKFFTPV (asn-phe-gly-lys-phe-phe-thr-pro-val)

These above endogenous peptides **(Table 3)** were isolated from beef bone marrow hemoglobin. These peptides have shown biological and immunological activity. However, no flavor activity has been reported on these peptides.

2.2.1.3 Beef Bone Marrow Amino Acids

Beef marrow contains most of the amino acids. The predominant amino acids are illustrated in the **Table 4** below.

Table 4: Amino acids percentages in acid digested cervical vertebrae marrow protein (Field et al., 1978)

Amino Acids	%
Leucine	13.2
Lysine	9.4
Glutamic acid	9.2
Aspartic acid	8.6
Alanine	7.3
Valine	6.5
Arginine	5.8
Glycine	5.4
Serine	5.3
Threonine	4.9
Phenylalanine	4.7
Histidine	4.6
Proline	4.0
Cysteine	2.8
Methionine	2.7
Tyrosine	2.6
Isoleucine	2.5

2.2.1.4 Marrow Extracellular Matrix

Extracellular matrix consists of fibronectin, laminin, hyaluronic acid, chondroitin sulfate, keratin sulfate and collagen (Lee et al., 1999). Attached to a core protein are long strands of glycosaminoglycans (GAGs) also called mucopolysaccharides. These structures are naturally jellylike and upon heating forms gel similar to collagen. Hyaluronic acid is a linear polysaccharide consisting of linked disaccharide units of glucuronic acid and N-acetylglucosamine. Chondroitin sulfate is the most abundant

mucopolysaccharide comprised of alternating units of β-1, 4 linked glucuronic acid and β-1,3-N-acetyl galactosamine and is sulfated on either the 4, or 6 position of galactosamine residue.

Collagen is a major connective tissue protein. Fibers of collagen run throughout the bone matrix, which are created by stringing together amino acids, the building blocks of protein. About one third of collagen is composed of glycine, the smallest amino acid while another one third of collagen is composed of proline and hydroxyproline (Kaufman, 2001). Collagen is a source of hydroxyl amino acids and sugars. Collagen is denatured and the peptide bond breakage occurs during prolonged heating of meat above 70ºC. However, collagen fragments were confirmed after cooking which indicates the heat stability of these peptides possibly contributed by high proportion of proline resdues (Bauchart et al, 2006).

2.2.1.5 Bone Marrow Nucleotides:

Marrow from steers contained 15.7 and 4.8 times more DNA and RNA than muscle respectively (Arasu, 1981) and is influenced by vertebrae locations. Cervical marrow contained greater amounts of DNA and RNA than lumbar marrow.

2.2.1.6 Beef Bone Marrow Minerals

Bone and cartilage are both classified as connective tissue and contains mineral deposited in an organic matrix. The minerals include calcium, phosphorus, sodium, magnesium, potassium, fluorides, and chlorides.

2.3 Volatile Components Contributing to Aroma

Lipid oxidation and the Maillard reaction are possibly the two most important reactions in food chemistry. Both reactions include a network of reactions yielding extraordinary complex mixture of compounds and follow parallel reaction pathways, producing a number of reactive intermediates which are responsible for the later formation of highly colored polymers in both reactions by aldol condensations and/or carbonyl-amine polymerization (Hodge, 1953; Hidalgo and Zamora, 2004).

2.3.1 Lipid Oxidation

Lipids upon autoxidation produce carbonyl compounds to form brown, high molecular weight products. “Lipid-derived” volatile compounds dominate the flavor profile of pork cooked at temperatures below 100ºC (Chen and Ho, 1998). Fats play an important role in the development of flavors and off-flavors through autoxidation and produce aldehydes and ketones. At certain parts per million (ppm) level these carbonyl compounds are desirable while in excess may cause off-taste. The off taste may include waxy, fatty, painty undesirable notes in food and food products. Alkane dienals such as 2,4 decadienals possess roasty, nutty notes. Lipid oxidation and Maillard reaction are interrelated. Strecker-type degradation of amino acids occur at as low as 37ºC by some lipid oxidation products (Hidalgo et al., 2004).

Table 5: Some volatile aldehydes obtained from autoxidation of unsaturated fatty acids. (Ho and Chen, 1994)

Fatty Acids	Hydroperoxides	Aldehydes
Oleic acid	8-OOH	2-Undecenal
		Decanal
	9-OOH	2-Decenal
		Nonanal
	10-OOH	Nonanal
	11-OOH	Octanal
Linoleic Acid	9-OOH	2,4-Decadienal
		3-Nonenal
	13-OOH	Hexanal
Linolenic Acid	9-OOH	2,4,7-Decatrienal
		3,6-Nonadienal
	12-OOH	2,4 Heptadienal
		3-Hexenal
	13-OOH	3-Hexenal
	16-OOH	Propanal
Arachidonic Acid	8-OOH	2,4,7-Tridecatrienal
		3,6-Dodecadienal
	9-OOH	3,6-Dodecadienal
	11-OOH	2,4-Decadienal
		3-Nonenal
	12-OOH	3-Nonenal
	15-OOH	Hexanal

Lipid oxidation gives a wide range of aliphatic products, including both saturated and unsaturated hydrocarbons, alcohols, aldehydes **(Table 5),** ketones, acids, and esters as well as cyclic compounds (such as furans, lactones and cyclic ketones). Many of

these contribute intense aroma and taste in foods (Farmer, 1994). Some long chain alkyl substituted heterocyclic compounds have been identified in meat flavors. These may originate from the reaction of aldehydes from lipid degradation with heterocyclic compounds formed from the Maillard reaction. Long chain aliphatic aldehydes possess fatty notes.

Phospholipids are constituents of bone marrow fat and have been shown to contribute to the animal specific meaty character. They contribute through lipid-derived volatiles generated by thermally induced lipid oxidation and interaction of lipid intermediates with the Maillard reaction, both of which modify the overall aroma of the cooked meat. Hexanal, nonanal, 2-octenal, 2-decenal, 1-octen-3-ol, 2-pentylfuran are the major odor-active volatiles degradation product of heated phospholipids as this can be generated from various fatty acids, C18:2 and C20:4 (Lin and Blank, 2003).

The volatiles in meat and bone meal by-products was hexanal, heptanal, octanal, 3-octene-2-one, nonanal, pentanal, 3,5-octadien-2-one, 1-octen-3-ol, 2-pentyl furan, trans-2-alkenals, trans, trans-2,4-alkadienals, and several pyrazines. These compounds can be formed by autoxidative degradation of fatty acids such as arachidonic acid, linoleic or linolenic acids. 3,5-octadien-2-one and 3,5-undecadien-2-one may be derived from linolenic and arachidonic acids, respectively, via enzymatic oxidative reactions. 2-pentyl furan has been identified to be responsible for reversion flavor in soybean oil (Smouse and Chang, 1967). It was postulated that 2-pentyl furan originates from linoleic acid (Greenberg, 1981). The high fat content usually greater than 14% can accelerate autoxidation.

2.3.2 The Maillard reaction

The raw bone marrow has little aroma with bloody metallic taste while cooked bone marrow has a unique umami and delicious taste. Like in all meat components, a complex series of thermally induced reactions occur between nonvolatile components of fatty tissues, protein and degraded polysaccharides. The major source of volatile compounds in heated foods is the Maillard reaction between amino acids and reducing sugar, and thermal degradation of lipids (Gerard, 2006).

A number of factors such as pH, water content, temperatures are very important in generating volatile carbonyl compounds (Mottram, 1995). In heated foods, the main source of the aldehydes is Strecker degradation of amino acids, while in high fat containing foods is lipid oxidation. Acidic pH favors furan and furan derivatives. Pyrazines is increased with the increasing reaction pH. Both pH 6.0 and 8.0 are favorable conditions for sulfur-containing compound formation (Tai and Ho, 1998).

2.3.2.1 Amadori reaction

Maillard reaction undergoes in three stages: initial, intermediate and final. At the initial stage, sugar-amine condensation, instable Schiff base formation, isomerization occurs. Glycosylamine as an intermediate occurs with sugar-amine condensation which kicks off a series of chain reactions forming an unstable Schiff base. This triggers isomerization, forms sugar dehydration and fragmentation, flavor compounds,

precursors and pigments. In the final stage, products of the intermediate stage further reacts with amino components or amino acid degradation, compounds produces heterocyclic compounds. Glycosylamine further rearranged into aldosamine (Heynes compounds) and ketosamine (Amadori compounds)**.** Maillard reaction enolization is pH mediated. At an acidic pH, the Amadori compound produces 3-deoxyhexosone via 1,2-enolization and at basic pH, the Amadori compound produces to 1-deoxyhexosone via 2,3- enolization.

2.3.2.2 Strecker degradation

Strecker degradation refers to the degradation of α-amino acids (by a degrading agent especially carbonyl compounds) to aldehydes and ketones containing one less carbon atom than the original amino acid **(Table 6)**. Strecker degradation plays an important part in Maillard reaction to produce aldehydes and ketones that form pyrazines which are important part in Maillard reaction.

In the Maillard reaction system, there are plenty of α-dicarbonyl intermediates originating through the fragmentation of sugars. These dicarbonyls are very active in degrading the amino acids. The aldehydes generated from the amino acids can either undergo further reactions or stay in the mixture imparting a characteristic Maillard product aroma. The dicarbonyls, after taking the amino group from the amino acids, readily condenses into pyrazines, an important type of aroma compound in roasted cocoa bean. A number of carbonyl compounds have been found to have the capability

of degrading α-amino acids. The most accepted mechanism of pyrazines formation is from α-amino acids and reducing sugar is based on the Maillard reaction and Strecker degradation **(Figure 1)**. The reaction of α-amino acids and reducing sugars initially generates Amadori/Heyns compounds, rearrangement of which leads to the formation of reductones including α-dicarbonyls and α-aminocarbonyls, which in turn are condensed to pyrazines. Because sugar degradation also provides α-dicabonyls, pyrazines can also be formed directly from the Strecker degradation alone.

Table 6: Volatile aldehydes from the Strecker degradation (Whitefield, 1992)

Amino acids	Aldehydes
Glycine	Formaldehyde
Alanine	Acetaldehyde
α-Aminobutyric acid	Propanal
Valine	2-Methylpropanal (Isobutyraldehyde)
Leucine	3-Methylbutanal (Isovaleraldehyde)
Isoleucine	2-Methylbutanal
Norvaline	Butyraldehyde
Norleucine	Pentanal
Serine	2-hydroxyethanal
Threonine	2-hydroxypropanal
Methionine	2-Methylthiopropanal (Methional)
Cysteine	2-Mercaptoacetaldehyde or acetaldehyde
Phenylglycine	Benzaldehyde
Phenylalanine	Phenylacetaldehyde
Tyrosine	2-(p-Hydroxyphenyl)ethanal

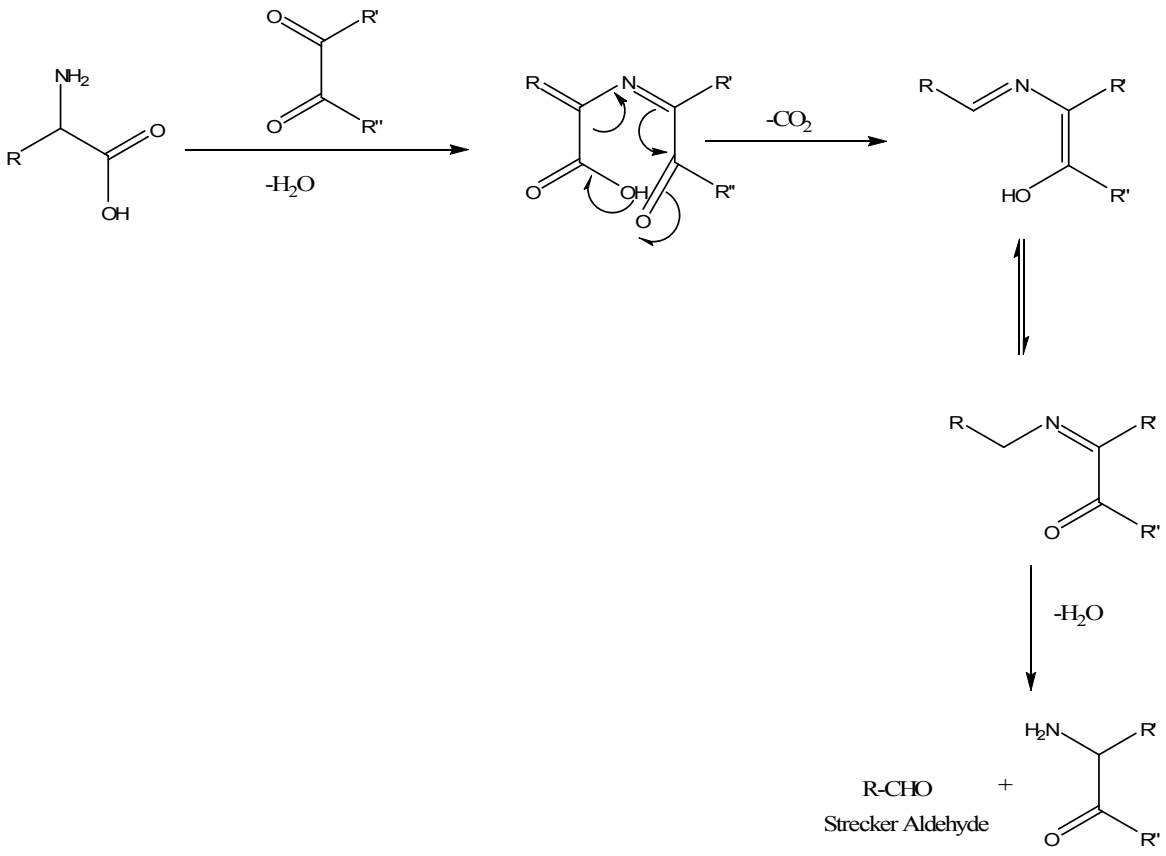
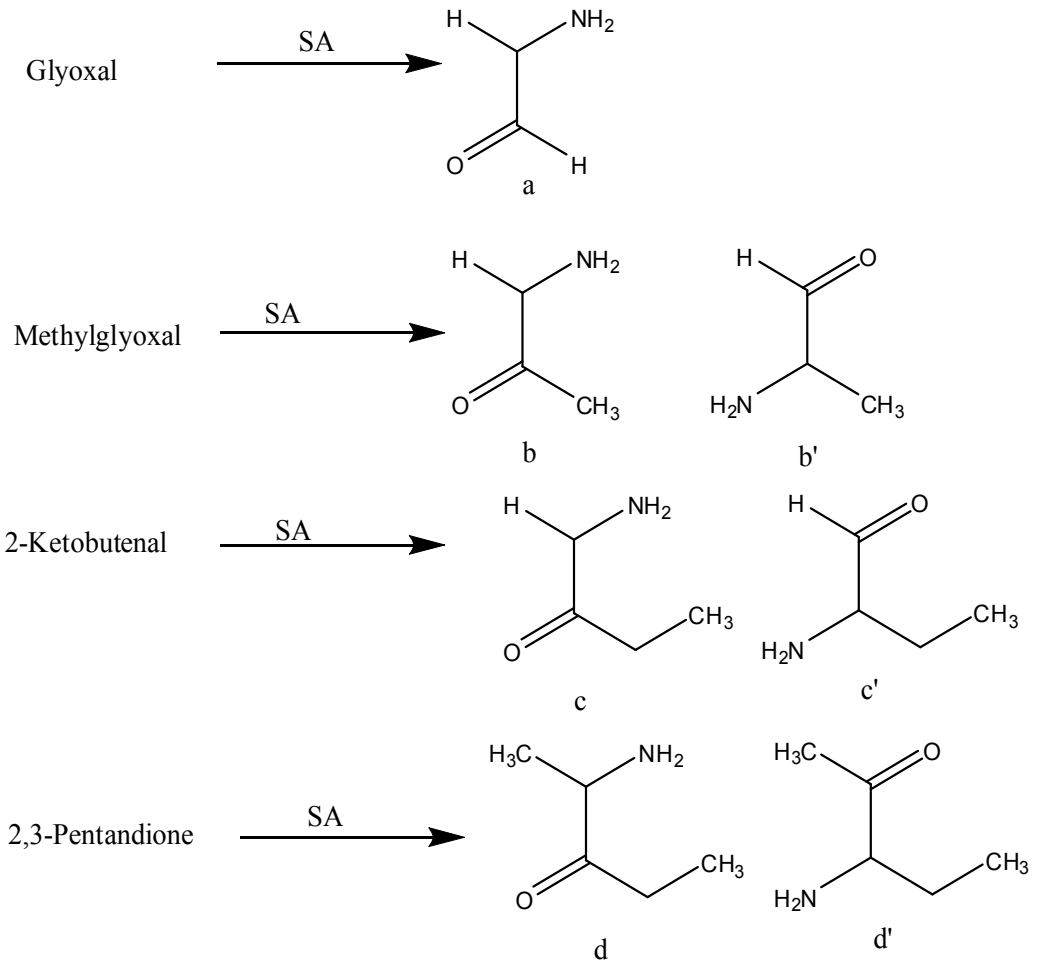


Figure 1: Strecker degradation of α-amino acids (Hidalgo and Zamora, 2004)

2.3.2.3 Mechanisms of pyrazine formation



SA=Strecker aldehydes

Figure 2: α-aminoketones and α-amoinoaldehydes produced upon Strecker degradation of selected dicarbonyls (Keyhani et al., 1996)

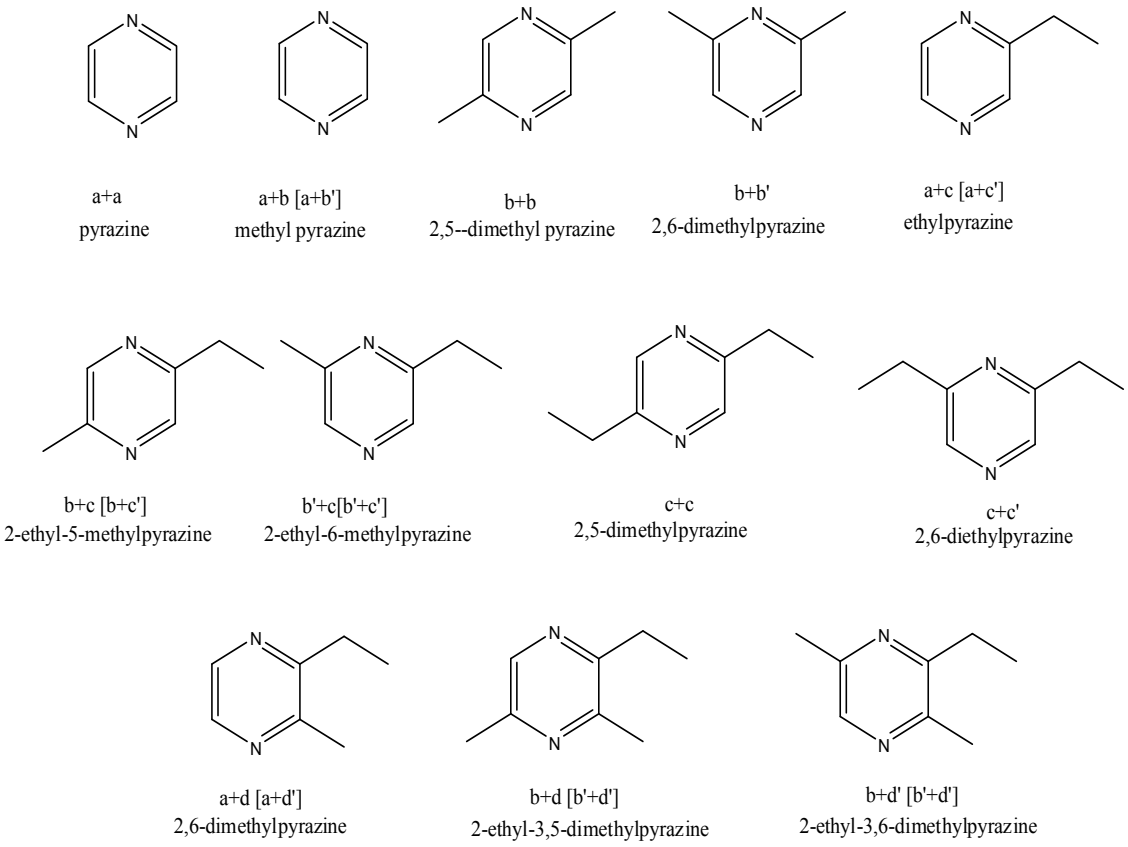


Figure 3: Pyrazines formed by dimerization or condensation and oxidation of the aminoketones and aminoaldehydes as formed in Figure 2 (Keyhani and Yaylayan, 1996)

Reaction of dicarbonyl compounds with amino acids through Strecker degradation, which involves a condensation reaction to initially form an imino ketone and subsequently, amino ketone accompanied by release of CO_2 and Strecker aldehyde. The final amino ketone can condense with other aminoketones to form the unstable dihydropyrazines **(Figure 2)**. These intermediates can undergo oxidation to form pyrazines or react with other aldehydes for the corresponding alkyl solutions. Based on the pyrazines identified in this study, it seems that Strecker degradation of

amino acids indeed occur resulting in the formation of aldehydes such as formaldehyde and acetaldehyde. The Strecker aldehydes reacted with dihydropyrazines to form the corresponding methyl and ethyl substituted pyrazines **(Figure 3)**. The greater possibility of forming 2,5-dimethylpyrazine rather than 2,6–dimethylpyrazine was reported in a model system of glycine peptides and glucose (Oh et al., 1991; Weenen, 1998). Alanine and phenylalanine is strong pyrazine producer. Methyl pyrazine production in valine reaction was the strongest, followed by phenyl alanine and methionine reaction. 2.5-dimethylprazine is most abundant in phenylalanine reaction followed by methionine reaction, alanine and valine. Glycine, leucine and isoleucine had the least pyrazine production (Li, 2005). Acetaldehyde and formaldehydes are Strecker aldehydes and also could be degradation products from glucose and other amino acids.

2.3.2.4 Other important volatiles

The boiled beef volatiles consists mainly of aliphatic aldehydes, alcohols and ketones with small quantities of pyrazines mainly methyl and dimethyl pyrazines and other heterocyclics (Mottram, 1994). Beef compounds identified were 3-methyl butanal, benzaldehyde, and 3,5-dimethyl-1,2,4-trithiolane (cis and trans). Other identified compounds in beef are: 2,3-pentanedione, methional, 1-methylthio-ethanethiol, and 2,4,6-trimethyl-perhydro-1,3,5-dithiazine (thialdine) (Brinkman et al., 1972).

2,5-dimethyl-4-hydroxy-3(2H)-furanone was identified in beef broth (Tonsebeek et al., 1968). 2,4,5-trimethyl-3-oxazoline was first found in the volatiles of boiled beef Thiazoles are found in roast, grilled and fried products. The thiophenes with the long alkyl chains arise from lipid sources, possibly by the interaction of hydrogen sulfide with unsaturated fatty acids or their oxidation products (Mottram, 1994).

3,5-dimethyl-1,2,4-trithiolne was first isolated from the volatiles of boiled beef (Mottram et al., 1994) and in beef broth (Brinkman et al., 1972). This was also identified in pork, lamb and chicken. This has a characteristic boiled beef aroma. They have also identified methanethioethanethiol, ethylthiomethanethiol, and 2-methylthioethanethiol in the beef broth. These compounds were reported to be alliaceous in character. Thialdine has been reported to have a roast-beef-like aroma. Trithioacetaldehyde and trithioacetone possesses meaty characters. 2,4,5–trimethyl-1,2-thiazoline was identified in beef broth. Methyldisulfides are formed from methional **(Figure 9).**

2.3.2.5 Peptides-specific Maillard compounds

The generation of peptide-specific aroma compounds1-alkyl-2(1H)-pyrazinones has been reported in the reaction of glucose with diglycine, triglycine, tetraglycine, gly-leu, leu-gly (Oh et al., 1992). The pyrazinones are generated by the direct condensation of dicarbonyl with dipeptide, followed by the decarboxylation reaction. The reactivity of free amino acids may not be the same when they present as bonded form in a peptide. Peptides between 2-5 kD contribute to meat flavors (Lieske, 1994).

2.4 Non-volatile components

Most non-volatiles are odorless compounds and extremely hydrophilic compounds such as table salt, citric acid, sugar. However, they exert physiological actions on the taste-buds and thus are considered flavor-substances. Non volatile components such as amino acids, peptides, fats, carbohydrates, organic acids, and collagen provide and enhance tastes in food. These non-volatiles also act as aroma precursors and generate characteristic volatiles. Upon boiling the meat products, hydrolytic cleavage of the precursors occurs (Hartman, 1982) leading to the formation of the species specific aroma and taste. The fat plays a significant role. The fatty tissues provide species specific characteristics (Chen and Ho, 1998).

Omission studies indicated that the sensory deficiency caused by peptides omission in the Maillard reaction can not be replaced by adding mixtures of free amino acids (Kochhar et al., 2004). The peptides directly contribute to the Maillard reaction flavors by forming two flavor compounds, cyclic dipeptides, also known as diketopiperazines, DKP's (Rizzi, 1989) and pyrazinones. The diketopiperazines are formed from tripeptides through degradation are well known for their characteristic bitterness. Peptides have also been described as being responsible for the undesirable bitter tastes of cheese and enzymatically hydrolyzed fish, soy, and wheat proteins. The bitterness is due to low molecular weight peptides composed of hydrophobic amino acid.

2.4.1 Basic Taste

Flavor is a complex response to a combination of receptors: salt, sweet, bitter, sour and umami (Japanese for 'tastes good'), odor, and trigeminal (heat, cooling, pain, temperature, tingling, astringency, and pungency (Prescot, 1995). Five basic tastes are: sweet, bitter, sour, salty and umami. Umami taste is of particular interest in the food industry and is universally recognized as the most important savory taste and included as a basic taste since late 1990's (Schlichtherle-Cerny, 2004). Mouthfulness and taste continuity known as 'kokumi' is another important attribute beside the five basic taste and has been reported recently in the miso paste (Ogasawara, 2006a).

2.4.1.1 Umami taste

Cooked meat is known for its savory character. Savory flavor refers to the savory 'umami' taste typically induced by Monosodiumglutamate (MSG) which activates a T1R1/T1R3 receptor, also known as umami receptor or G protein coupled receptor (Tachdjian, 2005). Besides MSG, umami taste is also contributed by amino acids such as aspartic acid, glutamic acid, glutamine, isoleucine, leucine and by nucleotides such as: ionosine mono phosphate (IMP), guanisine mono phosphate (GMP) and adenosine mono phosphate (AMP). Peptides, inorganic salts, organic acids also a major contributor to umami character in meat products.

Non-volatile precursors are mainly water-soluble components with low molecular weight that are essential in the beef broth or stock flavor development. These non-volatiles are precursors for Maillard reaction products. Amino acids that contribute sweet flavor of meat are: alanine, glycine, while valine, tyrosine, isoleucine, leucine, phenylalanine and tryptophan produce bitter flavors. Aspartic acid and glutamic acids, histidine, and asparagine possess characteristic acidity (Pereira-Lima et al., 2000). L-amino acid structure with five carbon atoms similar to 5'-monophosphate ribonucleotides such as glutamic acid (glu) contribute salty, umami and savory notes. A range of amino acids such as glycine, alanine, lysine, threonine, methionine, phenylalanine, tyrosine is reported in the generation of beef broth flavor while hydroxyproline and taurine produces serum-like flavors (Pereira-Lima et al., 2000). The dipeptides carnosine and anserine have been detected in relatively high in beef broths. Increased amount of anserine content produces a greater increase in the intensity of beef flavor than would be produced by similar increase in carnosine content. Free amino acids are significantly lower in broths cooked above 75ºC.

Aspartic acid, glutamic acid containing peptides are known as glutamyl peptides. They are hydrophilic di-,tri-, and tetrapeptides containing polar side chains (Arai, 1972, 1973, Noguchi, 1975). These glutamyl peptides in particular glu-ser, ala-glu, glu-asp, and glu-glu have described as eliciting umami and brothy sensory properties. Amino acids and peptides individually demonstrate no taste effect, but in combination impart savory enhancement. Glutamyl peptides contribute to umami taste (Schlichtherle-Cerny, 2002). Eleven umami peptides were isolated from bromealin

digested chicken protein hydrolysate. They are: asp-ala, asp-val, glu-glu, glu-val, ala-asp-glu, ala-glu-asp, asp-glu-glu, asp-glu-ser, glu-glu-asn, ser-pro-glu, and glu-pro-ala-asp (Maehashi, 1999). **Table 7** illustrates glutamyl peptides reported in the literature.

Table 7: Dipeptides and tripeptides to evoke the umami taste (Grigorov, 2003)

asp-asp, asp-glu, glu-asp, glu-glu, glu-leu, glu-lys, glu-ser, glu-thr, lys-gly, thr-glu, ala-asp-ala, ala-glu-ala, asp-glu-leu, asp-glu-ser, glu-asp-glu, glu-asp-phe, glu-asp-val, glu-glu-glu, glu-gu-ile, glu-glu-leu, glu-gly-ala, glu-gly-ser, glu-leu-glu, gly-asp-gly, gly-glu-gly, ile-glu-glu, leu-asp-leu, leu-glu-glu, ser-glu-glu, val-asp-val, val-glu-val

2.4.1.2 Amadori compounds

The Amadori and Heyns intermediates are unstable and are nonvolatile flavor precursors. Amadori and Heynes rearrangement products and/or N-Glycosides such as N-(D-glucos-1-yl)-L-glutamic acid and salts impart umami or savory taste to food products. These compounds represent an alternative to impart umami or savory taste (Schlichtherle-Cerny, 2002a). Two glycoconjugates of glutamic acid, *N*-glycoside dipotassium *N*-(D-glucos-1-yl L-glutamate and corresponding Amadori compound *N*-(1-deoxy-D-fructos-1-yl)-L-glutamic acid were reported to exhibit pronounced umami, bouillon like taste, close to that of MSG (Beksan,2003). The non peptide novel compound responsible for the beef broth taste was identified as alanine [*N*-(1-methyl-4-hydroxy-3-imidazolin-2,2-ylidene)] (Shima et al., 1998). Alapyridaine generated from the heated aqueous solution of glucose and L-alanine was found to be a tasteless compound enhancing sweet and umami taste (Ottinger and Hofmann, 2003).

2.4.1.3 Maillard peptides

Xylose reacted Maillard peptide fraction with a molecular weight of 1000-5000 appeared to give Kokumi taste in long-ripened miso and also work as a flavor enhancer (Ogasara et al., 2006, 2006a).

2.4.1.4 Organic acids

Lactic acid, succinic acid, disodium succinate and tartaric acid demonstrated umami character. In goat cheese non-volatiles such as mineral salts and lactic acid are the main taste-active compounds, whereas lipids, the volatile fraction, lactose, amino acids, and peptides did not have any significant impact (Engel et al., 2000).

2.4.1.5 Taste enhancers

Taste enhancers are substances that directly increase the pleasantness of the flavor of another substance. First flavor synergism was reported forty years ago between MSG, and ribotide. An octapeptide, lys-gly-asp-glu-glu-ser-leu-ala also known as delicious peptide extracted from papain digested beef provided savory enhancement in broth (Yamasaki, 1978, Wang 1996). The savory flavor of protein hydrolysates is assumed to be caused by a high content of free amino acids, especially glutamic acid, low molecular weight peptides, salt, and organic acids (Asalyng, 1998). Besides amino acids and peptides, Amadri compounds, Maillard peptides, organic acids play a key role in taste enhancement.

2.5 Enzymatic Hydrolysis

The enzymatic hydrolysis of proteins is widely used in the food industry to improve the functional properties of proteins, such as taste and aroma. Enzyme hydrolysis is an effective way to break down proteins into smaller peptides, and amino acids. The extent of hydrolysis is determined by the degree of hydrolysis which measures the percentage of peptide bonds cleaved in the protein during a reaction and is expressed as:

$$Degree\ of\ \ Hydrolysis = \frac{Number\ of\ \ peptide\ bonds\ cleaved}{Number\ of\ \ peptide\ bonds\ available}\ X\ 100$$

Papain is a cysteine protease of the peptidase C-1 family and consists of a single polypeptide chain with three disulfide bridges and a sulfhydryl group necessary for activity of the enzyme. Optimal pH: 6.0-7.0. Optimal temperature: 65ºC (Kilara, 1977). Endopeptidase such as papain hydrolyzes internal, α-peptide bonds in a polypeptide chain, tending to act away from N-terminus or C-terminus. Exopeptidases require a free N-terminal amino group, C-terminal carboxy group or both, hydrolyse a bond not more than three residues from the terminus.

Umamizyme is a proteolytic enzyme which is manufactured by a fermentation process with a selected strain of *Aspergillus Oryzae*. Umamizyme has high peptidase and proteinase activity in contrast to other fungal enzymes. Endoprotease cleaves the

peptide bonds in the interior of protein while exopeptidase releases the amino acid located at the terminal end of the chain. It is reported that significantly more of the flavor compounds were generated in the umamizyme system than that of papain. This suggests that umamizyme is more efficient in cleaving off the peptide bonds of marrow to produce smaller fragments, resulting in more amino acids being available to participate in the complex pathway of Maillard reaction. Umamizyme hydrolyzes the protein linkage better than papain (Romero, 2006). The hydrolyzed fragments primarily amino acids and smaller peptides are invaluable flavors for savory flavor development.

2.6 Fat Separation

2.6.1 Membrane Filtration

Microfiltration (MF) has been widely used in the dairy industry for clarification of fermentation products. The liquid flows parallel to the membrane at high speed and under pressure so that the feed stream splits into two, one passes through the membrane. The liquid that passes through the membrane is known as the permeate, and the material that does not pass through is called the retentate. The membranes are long cylindrical pipes made of a variety of materials, including polymers, ceramics and metals. The pores of the membranes are so small (pore size of 0.3 microns) generates higher flux rates than its polymeric analog. The pressure is required to drive the liquid through them. MF is a low pressure (as low as 10psi), cross-flow membrane process for separating colloidal and suspended micrometer size particles. The pump supplies hydraulic pressure on one side of the membrane, while the other is at atmospheric pressure. The pressure difference across the membrane allows the separation to take place. The membranes are available with different molecular-weight cut-offs. A molecular weight cut-off is defined as the molecular weight at which the membrane rejects 90% of solute molecules. In the membrane filtration, permeation across a membrane is largely a function of the size and chemical nature of molecules. The mirofiltration process is designed to retain the permeate with a molecular weight cutoff of approximately 20,000Da.

It is a challenging task to remove the fat in food preparations that contain high amount of fat. There are many processes available such as: centrifugation, solvent extraction, salt precipitation, high pressure extraction, supercritical extraction. These techniques all have limitations such as high cost, long preparation times, artifact generation, thermal abuse or food safety issues. A patented filtration system known as Microfiltration system was modified and was employed to obtain a concentrated stock from beef marrow bones (Raghavan, 2005). Proteolytic enzymes such as papain and umamizyme are used to hydrolyze the non MF permeable, bulky protein polymers into monomeric and oligomeric fragments. Enzymatic hydrolysis enhances the permeation and recovery of non-fat solids as they are relatively small in size and hydrophilic in character. The hydrolyzed protein fragments readily pass through the MF membrane and are retained in the final product as non fat solids. Without the enzyme hydrolysis, these non-fat solids would be lost in the MF concentrate along with fat globules.

2.7 Flavor Analysis:

2.7.1 Volatile Analysis:

Because of the complexity of the Maillard reaction, mass spectrometry (MS), coupled with separation techniques, is a key tool in this research area. Vast majority of MS techniques used in food research were developed for drug discovery, pharmaceutical applications and natural molecules five years earlier (Fay and Brevard, 2005). The chemistry of Amadori compounds has been reviewed. Because of the thermal instability, early researchers investigated Amadori compounds by indirect methodologies. Following the development of Gas Chromatographic (GC) techniques and other instrumental methods (MS, NMR, GC-MS), more information concerning volatile constituents began to appear. In the late 1960's the introduction of the capillary column to gas chromatographic analysis made it possible to make more comprehensive analysis of cooked meat volatiles. Headspace sample analysis, GCO, and Gas Chromatography-Mass Spectrometry (GC-MS) has been extensively used to analyze volatile aroma components.

2.7.1.1 Solid Phase Micro Extraction (SPME)

Solid Phase Micro Extraction (SPME) was developed in the early 1990's and gaining popularity since because of its rapid, inexpensive and solvent free extraction technique. SPME combines sampling and pre-concentration in one single step. SPME head space volatiles. The coated fused silica SPME fiber is directly exposed to the

headspace of or immersed in a sample in a closed vial. Once equilibrium has been reached the fiber is introduced into a GC-MS for separation, identification and quantification. The fibers are: CAR, PDMS, CAR-PDMS. SPME uses a short fused silica fiber coated with a polymer stationary phase to adsorb the analytes from the headspace or aqueous sample. This is bonded to a stainless steel plunger and installed in a syringe holder. Coating materials for SPME fiber can be categorized according to their polarity: non-polar, bipolar, and polar. The non-polar polydimethylsiloxane (PDMS) is commonly used. Examples of bipolar coatings are polydimethylsiloxane-divinylbenzene (PDMS-DVB), carboxen-polydimethylsiloxane (CAR-PDMS), divinylbenzene-carboxen-polydimethylsiloxane (DVB-CAR-PDMS). Polar coatings include polyacrylate and carbowax. Adsorption of target components to the fiber is based on the principle "like adsorbs like." The best extraction performance was obtained with CAR-PDMS as indicated by increase area for almost all peaks (Romero, 2006). The worst performer was the PDMS by itself. These results suggest that the samples mostly contained bipolar compounds with relatively low molecular weights. SPME fiber with CAR-PDMS is suitable for volatile low molecular weight compounds. The incorporated porous carbon enables the migration of analytes between layers for increased capacity. As a result, even volatile flavor compounds can be adsorbed. As the extraction temperature increases, the peak areas also are increased. This is because of increased mass transfer and as a consequence, more analytes being adsorbed to the fiber. A complete analysis of every volatile and semi-volatile compound contained within a flavor mixture might not be possible when SPME is the only isolation technique. SPME will not provide a complete profile for every sample.

2.7.2 Non-volatile Analysis

The identification of non-volatile components is challenging. Liquid Chromatography (LC) in combination with Mass Spectrometry (MS), High performance liquid chromatography (HPLC) has facilitated the identification of compounds through different detectors (UV, fluorescence, electrochemical). The development of sophisticated LC/MS, LC/MS/MS has further the knowledge of the non-volatile Maillard reaction products. Nuclear Magnetic Resonance (NMR) instrument has been used in mechanistic studies. Researchers like the German scientist, Thomas Hofmann has identified and characterized many flavor compounds using novel techniques that combined instrumental analysis with human sensory evaluation, LC-MS, 1D and 2D NMR experiments, as well as ^{13}C labeling techniques. Advanced methodology that includes taste dilution analysis, omission experiments, odor activity value identified tasteless compounds that modify or enhances flavors (Hofmann, 2005). Indonesian soy sauce of various fractions (500, 3,000 and 10,000Da) was separated by ultrafiltration followed by electrophoresis. The peptides were analyzed by a photoiode array UV-Vis detector (Apriyantono, 2004). Photoiodes is used because most amino acids lack a strong chromophore for detection by UV-Vis detector. ESI-MS, and ^{1}H NMR was employed to identify umami components in Indonesian soy sauce fraction of less than 500Da (Lioe, H., et al., 2006). HPLC with a diode array detector was used to analyze non volatile Maillard reaction products (Ames et al., 1998). Oligopeptides from water-soluble extract of goat cheese was isolated and identified by MS. Peptides were isolated by size-exclusion chromatography, followed by anion exchange

chromatography, and RP-HPLC. The identification was performed by combined MS methods including electrospray ionization (Sommerer et al., 2001).

Separation of low molecular weight acidic oligopeptides from fish hydrolysates were chromatogrammed on a column containing Amberlite CG-120 by means of gradient elution. Aspartic and Glutamic acid were analyzed by amino acid analyzer, and derivatization mass spectrometry (Noguchi, 1975). Umami intensity of sodium L-glutamate is enhanced by the green tea components, l-theanine, succinic acid, 3,4,5-trihydroxybenzoic acid (gallic acid), and (1R,2R,3R,5S)-5-carboxy-trihydroxycyclohexyl-3,4,5-trihydroxybenzoate (theagallin). They were separated by HPLC and were identified by liquid chromatography-tandem mass spectrometry and one-/two-dimensional NMR studies. Non-volatile new Maillard reaction products, 7,8a-dihydroxy-4a ethyl-8-(α-D-glucopyranosyloxy) hexahydro-5-oxa-4-thia-1-azanapthalene-2-carboxylic acid was produced between the reaction of Amadori compounds, 1-deoxymaltulosyl glycine (Mal-Gly) and cysteine at 100ºC. LC-[Electrospray Ionization (ESI)], MS analysis and NMR analysis was employed to analyze the non-volatiles (Ota et al., 2006). N-terminally pyrazinone-modified peptides I (N-[2-(2-oxo-2H-pyrazin-1-yl)-propyl]-phenylalanine) and II (N-[2-(5-methyl-2-oxo-2H-pyrazin-1-yl)-propionyl]-phenylalanine) which were formed through reactions with peptide gly-ala-phe and α-dicarbonyl compounds such as glyoxal and methylglyoxal were identified by ESI-MS and NMR spectroscopy (Keyhani, 1996).

Water soluble cheese extract was fractionated with a molecular weight cut-off of 1000Da followed by separation in a G-10 gel permeation chromatograms of the water soluble extracts <1000 Da. Separation of peptides was performed on a C-18 Nucleosil. Free amino acids were analyzed by HPLC of the *o*-pthaldehyde (OPA) (Molina et al., 1999). The meaty flavor enhancer was fractionated from beef extract upon dialysis and was separated by anion-exchange chromatography, copper chelate chromatography, and gel filtration chromatography (Kuroda, 2004).

2.7.3 Protein Identification

2.7.3.1 Analysis and Separation of Sample Ions

Tandem mass spectrometry (MS/MS) plays an important role in protein identification due to its fastness and high sensitivity. The main function of the mass analyzer is to separate, or resolve the ions formed in the ionization source of the mass spectrometer according to their mass-to-charge (m/z) ratios. There are a number of mass analyzers available including quadrupoles**,** time-of-flight (TOF) analyzers, magnetic sectors, Fourier transform and quadrupole ion traps**.** Tandem (MS-MS) mass spectrometers are instruments that have more than one analyzer and can be used for structural and sequencing studies. The analyzers do not necessarily have to be of the same type, in which case the instrument is a hybrid one. The extent of side-chain fragmentation detected depends on the type of analyzers used in the mass spectrometer (Fay et al., 2005). A magnetic sector instrument will give rise to high energy collisions resulting in many different types of side-chain cleavages. Quadrupole – quadrupole and

quadrupole time-of-flight mass spectrometers generate low energy fragmentations with fewer types of side-chain fragmentations. The MALDI-TOF instrument produces data that is used for peptide identification and characterization. It accurately measures the molecular weight of peptides by peptide mass fingerprinting (PMF) and determines peptide sequence by post source decay (PSD) analysis. Electrospray ionization mass spectrometry (ESI-MS) has been widely used in the sequencing peptides. Various methods, including change of activation methods, chemical derivatization and isotopic labeling have been used to simplify the spectra and facilitate the interpretation (Chen et al., 2004).

2.7.3.2 The peptide sequencing

Several well-established chemical methods for the sequencing of peptides are known including the Adman degradation. ESI-MS and MS-MS are capable of sequencing peptides in a mixture without any chemical purification or separation. In a typical MS-MS sequencing experiment, molecular ions of particular peptides are selected by the first mass analyzer and fragmented by collisions with neutral gas molecules in a collision cell. The second mass analyzer is then used to record the fragment ion spectrum that generally contains enough information to allow at least a partial, and often the complete, sequence to be determined. The derivation without the help from a protein data base is called the *de novo* sequencing which is important in the identification of unknown protein. Accurate determination of the molecular mass of peptide is useful for its identification. The correct mass often is not sufficient for

identification but it does facilitate the search. Two different approaches are followed. The first approach is the *de novo* spectral interpretation that involves automatically interpreting the spectra using the table of amino acid masses. The second approach searches the data base to find the best sequence that matches the spectrum. However, this method is not able to interpret the spectra. First, the algorithms like SEQUEST or MASCOT, looks for all the peptides in the data base. Then the algorithm looks for the similarity between the predicted fragments of the sequence obtained from the data base and fragments present in the spectrum of the sample (Hoffmann et. al., 2007).

The proteins are digested with an enzyme to produce peptides. The peptides are charged (ionized) and separated according to their different mass to charge (m/z) ratios. Each peptide is fragmented into fragment ions and the m/z values of the fragment ions are measured. For each possible fragment ion there could a peak at the corresponding m/z value. The height of the peak is proportional to the frequency of the m/z value. In general, proteins consist of 20 different amino acids of unique masses, except for leucine and isoleucine. Consequently, different peptides usually produce different spectra. It is therefore possible to use the spectrum of a peptide to determine the sequence (Kinter and Sherman, 2000). Fragmentation of peptides (amino acid chains) typically occurs along the peptide backbone. Each residue of the peptide chain successively fragments off, both in the amino and carboxyl end. The fragmentation results in various ions such as a, b, c and x, y, or z ions. The most commonly observed ions are a, b, and y ions **(Figure 4)**.

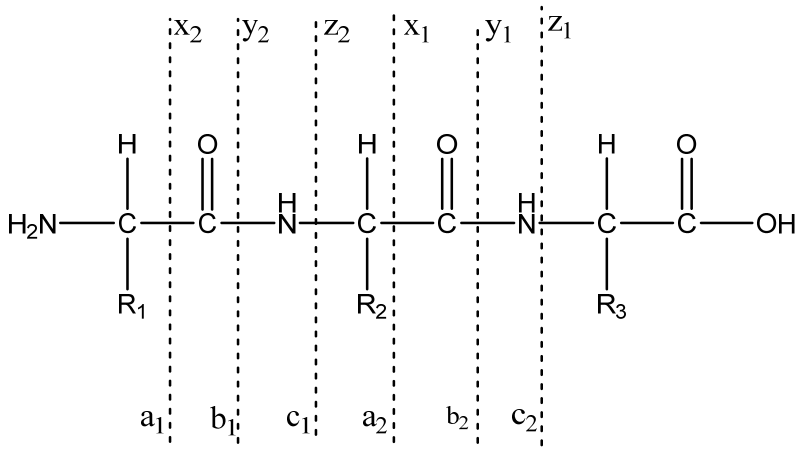


Figure 4: Formation of b and y ions during the fragmentation of a three amino acids residue peptide chain

An immonium ion is an internal fragment of a peptide with just single side chain formed by the combination of 'a' and 'y' type cleavage during MS/MS analysis. The immonium ion has been often served as an indicator for the presence or absence of a specific amino acid residue in the peptide sequences. The immonium ions are useful for detecting and confirming many of the amino acid residues in a peptide, although these immonium ions do no provide information regarding the position of these amino acid residues in the peptide sequence. **Table 8** illustrates common immonium ions from amino acids fragmentation. MS fragmentation of peptides primarily occurs at the amide bond (-CO-NH-) between two amino acid residues. Immonium ions appear in the very low m/z range of the MS-MS spectrum. Each amino acid residue leads to a unique immonium ion, with the exception of leucine (leu) and isoleucine (ile), or lysine (lys) and glutamine (glu), which produce immonium ions with the same m/z ratio, i.e. m/z 86 for ile and leu, m/z 101 for lys and gln.

Differentiating leu from Ile is achieved by low energy by fragmenting the immonium ions from these amino acids at m/z 86. It can also be performed by derivatizing the peptide to localize a positive charge on the N-terminal side in order to favor the formation of dn fragments, or to localize a positive charge on the C-terminal side in order to favor the formation of w_n fragments (Hoffmann et al., 2007).

Table 8: Immonium and related ion characteristics of amino acids (Medziharadszky, 2005)

Amino Acids	Residual mass in Da	Immonium and related ions		Ion strength
ala	71	44		
arg	156	129	59, 70, 73, 87, 100, 112	129, 73 usually weak
asn	114	87	70	87 often weak, 70 weak
asp	115	88		usually weak
cys	103	76		usually weak
gln	128	101	84, 129	129 weak
glu	129	102	84, 129	often weak if C-terminal
gly	57	30		
his	137	110	82, 121, 123, 138, 166	110 very strong and 82,121, 123, 138 weak
ile=leu	113	86		
lys	128	101	84, 112, 129	101 can be weak
met	131	104	61	104 often weak
phe	147	120	91	120 strong, 91 weak
pro	97	70		strong
ser	87	60		
thr	101	74		
trp	186	159	130, 170, 171	strong
tyr	163	136	91, 107	136 strong, 107 and 91 weak
val	99	72		fairly strong

3. HYPOTHESIS

The marrow bones stock is rich in non-volatile compounds including peptides and amino acids. The stocks will generate unique compounds at various temperatures via Maillard reaction and lipid oxidation. Enzyme hydrolysis improves the yield of these components. Addition of ribose and xylose will enhance and lead to the formation of additional volatile compounds, addition of methylglyoxal will result in the formation of novel volatiles.

4. OBJECTIVES

- To prepare stocks by heating the marrow bones at high moisture conditions and with the aid of proteases followed by fat removal.
- To identify selected volatile and non-volatile constituents of cooked beef marrow bones.
- To determine the effect of temperature, enzyme hydrolysis, reducing sugars and methylglyoxal on the volatiles composition of cooked beef marrow bones.

5. EXPERIMENTAL METHODS

5.1 Experimental design

5.1.1 Stock preparation

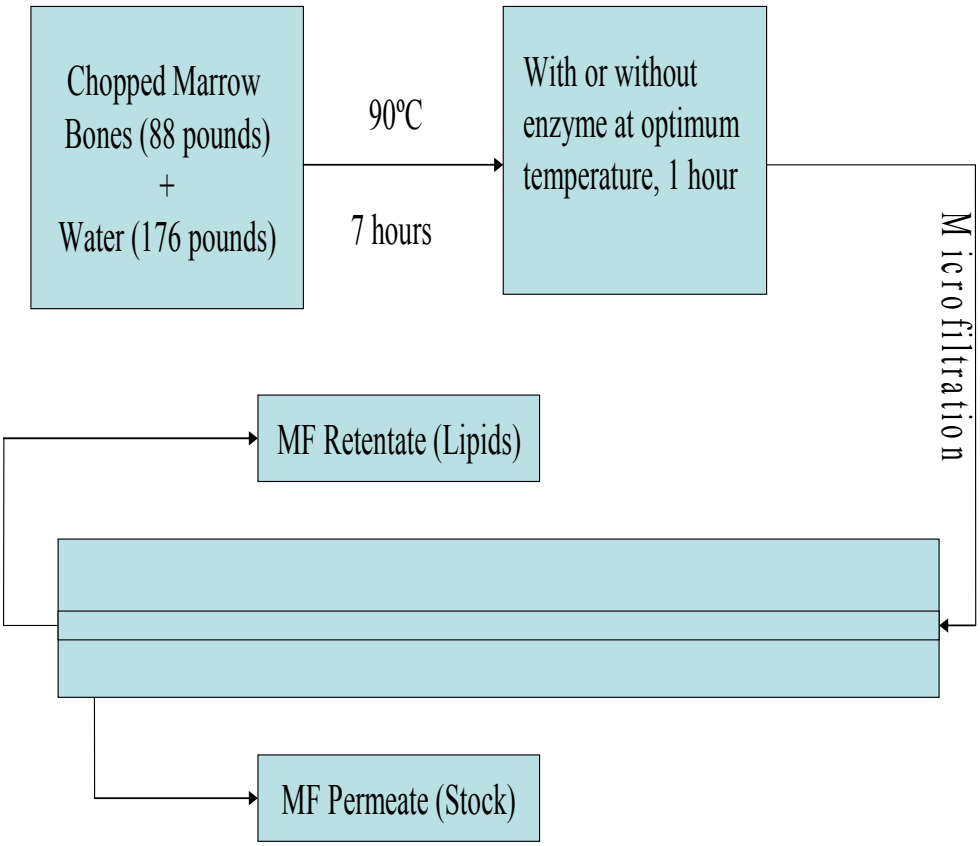


Figure 5: Experimental design of fat separation using microfiltration and stock preparation

5.1.2 Sample preparation

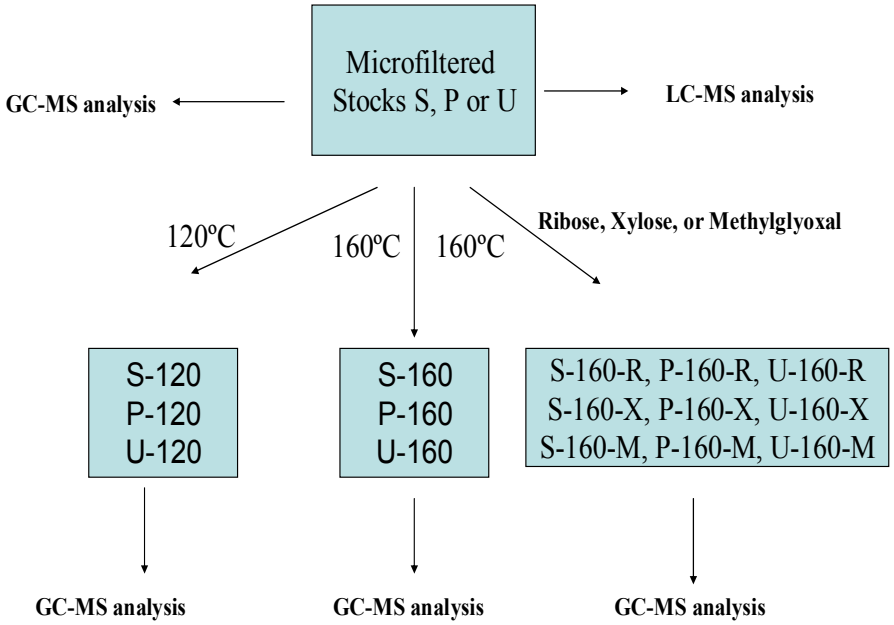


Figure 6: Flow Chart of samples investigated. Sample S (untreated), Sample P (Papain treated) and Sample U (Umamizyme treated)

Table 9: Stock samples

Untreated stock: S
Papain treated stock: P
Umizyme treated stock: U

Untreated stock heated at 120ºC: S-120
Papain treated stock at 120ºC: P-120
Umamizyme treated stock at 120ºC: U-120

Untreated stock heated at 160ºC: S-160
Papain treated stock at 160ºC: P-160
Umamizyme treated stock at 160ºC: U-160

Untreated stock heated at 160ºC with ribose: S-160-R
Untreated stock heated at 160ºC with xylose: S-160-X
Untreated stock heated at 160ºC with methylgloxal: S-160-M

Papain treated stock at 160ºC with ribose: P-160-R
Papain treated stock heated at 160ºC with xylose: P-160-X
Papain treated stock heated at 160ºC with methylgloxal: P-160-M

Umamizyme treated stock at 160ºC with ribose: U-160-R
Umamizyme treated stock heated at 160ºC with xylose: U-160-X
Umamizyme treated stock heated at 160ºC with methylgloxal: U-160-M

5.2 Sample Preparation

The chopped ground beef marrow bones were obtained from ConAgra Food Ingredients, Hyrum, Utah. The bones were collected from different locations of the animal and free of specified risk materials (SRM) such as spinal chord, eye, bowel, lymph nodes, spleen, pineal gland, endocrinium, placenta, pituitary gland, thymuses, super renal glands and tonsillitis. The bones are grounded to ½ inches. In beef femur bones, the marrow weight was calculated as 16-22%. The weight varies depending on the locations of the cattle.

CEM Smart Trac system, rapid fat and moisture analysis was employed to determine moisture and fat while LECO FP-2000 protein analyzer, St, Joseph, MI was used to analyze protein content. Protein: 0.20-0.30 gm sample was placed in a ceramic holder and protein % was calculated with nitrogen conversion factor 6.25 for meat **(Table 10).**

Table 10: Bone marrow composition

	Beef femur bone marrow %	Marrow by-products %
Moisture	8.0-12.0	30.0-90.0
Fat	80.0-92.0	40.0-60.0
Protein	0.60-0.90	5.0-15.0

5.2.1 Stock preparation:

The ground marrow bones include bones that are by-products and usually discarded after the meat was collected from marrow bones. The ground marrow bones were kept frozen until used. 264 pounds ground marrow bones were obtained and divided into three equal batches. Each 88 pounds batch of marrow bones were cooked in 176 pounds purified water at simmering temperature (90ºC) for 7 hours in a stainless steel vessel with constant mixing. The pH stayed around 7.0. The heating is carried out in steam jacketed open kettle. The enzyme hydrolyzes and solubilizes the protein and also help separate fats from the bone (Vollmer, 1979) and thus the protein yields is improved. The remaining two batches were treated with enzymes papain and umamizyme respectively at 0.10% of the total marrow bones weight. Fat was removed by microfiltration. The aqueous phase obtained is known as stock. The enzymes yieded more protein rich stock which otherwise would have been discarded in the filtrate (lipid phase). We performed degree of hydrolysis (DH) experiment using OPA method (Nielsen et al., 2001) and found the umamizyme digested stock to have the highest DH (76.35%) while the papain digested and undigested stock had 51% and 29% respectively. The high degree of hydrolysis is due to the fact that umamizyme has both endoprotease and exopeptidase activities. The sample treated with papain was heated at 65ºC for 60 minutes while the sample treated with umamizyme was heated at 50ºC for 60 minutes. Both the samples were heated to 90ºC for 15 minutes to inactivate the enzymes.

5.3 Fat Separation:

5.3.1 Membrane Filtration:

The microfiltration system consisted of a feed pump, a retentate, circulation pump, and permeate recirculation pump. The inlet and outlet pressure are approximately (10-15 psi and (11-15) psi respectively. A stainless steel vat with steam heat is connected to the MF unit. The metallic membranes can tolerate a wide range of pH, heat and pressure. Stainless steel membrane with pore size of 0.3 micron was used.

We prepared three different stocks from the ground marrow bones.

a) stock made from cooked ground marrow bones (Sample S)

b) stock made from cooked ground marrow bones-papain treated (sample P)

c) stock made from cooked ground marrow bones -umamizyme treated (sample U)

Figure 5 and 6 illustrates the experimental design. **Table 11** illustrates the pH and protein content of the three samples S, P and U.

5.4 Degree of Hydrolysis:

Degree of hydrolysis was determined according to the OPA (*o*-phthalaldialdehyde) method (Nielson et al, 2001). The following reagents and chemicals were obtained from Sigma-Aldrich chemical company. They are: disodium tetraborate, Sodium dodecyl sulfate (SDS), deionized water, OPA solution, dithiothreitol, serine (standard) and ethanol. Perkin Elmer uv/vis spectrometer Lambda 20 was employed. All spectrophotometer readings were performed at 340 nm using deionized water as the blank. The following equation was used to determine the degree of hydrolysis (DH) in terms of percentages.

$$Degree\ \ of\ \ Hydrolysis,\ DH = \frac{Absorbance_{sample} - Absorbance_{blank}}{Absorbance_{s\ \tan\ dard} - Absorbance_{blank}}\ X\ 100$$

Table 11: pH and protein comparison of untreated, papain and umamizyme treated stocks

Samples	pH	Protein %
Stock S (Untreated)	7.90	0.8
Stock P (Papain treated)	7.85	2.5
Stock U (Umamizyme treated)	7.52	4.0

5.5 Flavor analysis

The stock samples (S, P, and U) were heated to 120°C and 160°C to generate volatiles. The samples were labeled as S-120, S-160, P-120, P-160, U-120 and U-160. The samples were heated in a Parr reactor under 20 PSI for 60 minutes. The stock samples (S, P and U) were treated with d-Ribose, d-Xylose and Methylglyoxal 40% solution in an equal percentage of protein. The chemicals were obtained from Sigma-Aldrich, St. Louis. The samples were heated in Parr reactor at 40-50 PSI at 160ºC for 60 minutes. The samples were labeled as S-160-R, S-160-X, S-160-M. All samples were kept frozen (-20ºC) until used.

5.5.1 Volatile Analysis

5.5.1.1 Solid Phase Microextraction (SPME)

2 grams of Maillard reaction products were placed in a vial and then sealed. An incubation temperature of 60ºC for 20 minutes using carboxene polydimethylsiloxane (CAR-PDMS) purchased from Supelco (Bellfonte, PA). The SPME fiber was desorbed in a Shimadzu gas Chromatograph-17A (Shimadzu Scientific Instruments, Columbia, MD) equipped with DB-5 column with 60 meter length, 0.25 mm internal diameter, and 1.0µm phase thickness (J&W Scientific, Folsom, CA). The column outlet was directly coupled to the EI source of a QP 5000 mass spectrometer. Mass spectra were generated at 70 eV in the electronic ionization mode. A scan range of 35-350m/z was employed. The injector and detector temperatures were both 280ºC. The oven

temperature program was 2 min isothermal at 40ºC, increased to 280ºC at 4C/min rate, then held at the final temperature for 4 min.

$$Conc_{sample}\ in\ ppb = \frac{(Area_{sample})\ (Conc_{IS}\ in\ ppb)}{Area_{IS}}$$

Identification was performed by comparison of mass spectrometric data with authentic reference compounds available in the Wiley library. Quantification was made by using 4-Heptanone (Sigma-Aldrich, Milwaukee) as internal standard (IS). Identification of peaks was done by background subtraction and peaks which were not well separated or peaks that coeluted gave mixed spectra could not be identified.

5.5.2 Nonvolatile analysis:

Three samples of beef bone marrow stock (Sample S, Sample P and Sample U) were analyzed. Sample Preparation**:** Samples were thawed to ambient and filtered using 0.45um glass microfiber filters into autosampler vials.

5.5.2.1 Non-volatile mapping

Data Acquisition**:** Analysis was performed on Agilent 1200 LC coupled to Agilent 6210 TOFMS using Cosmosil C18 Reversed Phase Column (5um, 2mm x 150mm) with the following parameters:

Table 12: LC Parameters

Injection Volume: 5ul

Column Temperature: 30 °C

Mobile phase A: 0.1% formic Acid in water

Mobile phase B: 0.1% formic Acid in acetonitrile

Flow rate: 250ul/min.

Gradient: Time (min.)	%A	%B
0	95	5
10	5	95
12	5	95
13	95	5
20	5	95

TOF Parameters

Source Conditions: +ESI mode, Gas Temp 350 °C, Drying Gas 10L/min., Nebulizer 20psi

Voltages: Fragmentor 215V, Skimmer 60V, OCT RF 250V, Capillary 4000V

Data Acquired: Profile mode, 0-12min.

Data Range: 50-3200m/z

Data Processed: 50-1500m/z

5.5.2.2 Amino Acid Analysis

By pre-column derivitization with *o*-Phthalaldehyde (OPA) and 9-Fluorenylmethyl chloroformate (FMOC-Cl) separated by RP chromatography with gradient elution. Detection is done by Fluorscence at EX=340nm and EM=450nm.

Table 13: Chromatography Parameters

Column: ZORBAX Eclipse-AAA 4.6 x 150 mm, 5 µm

Temperature: 40°C

Flow: 2 mL/min

Mobile Phase A: 40 mM Na2HPO4, pH 7.8

Mobile Phase B: ACN: MeOH: water (45:45:10, v/v/v)

Gradient:

Time (min)	% B
0	0
1.9	0
18.1	57
18.6	100
22.3	100
23.2	0
26.0	0

5.5.2.3 Peptide fragmentation

LC-MS using hybrid quadropole linear ion trap.

Sample Preparation**:** Samples were thawed to ambient and filtered using 0.45um glass microfiber filters into autosampler vials.

Data Acquisition**:** Analysis was performed on Agilent 1200 LC coupled to Applied Biosystems 3200 QTrapMS using Cosmosil C18 Reversed Phase Column (5um, 2mm x 150mm) with the following parameters:

Table 14: LC-MS using hybrid quadropole linear ion trap.

LC Parameters

Injection Volume: 5ul

Column Temperature: 30 °C

Mobile Phase A: 0.1% Formic Acid in Water

Mobile phase B: 0.1% formic acid in acetonitrile

Flow rate: 250ul/min.

Gradient: Time (min.)	%A	%B
0	95	5
10	5	95
12	5	95
13	95	5
20	5	95

LC retention times were adjusted to match TOF data collected previously.

QTrap Parameters

Source Conditions: +ESI mode, Gas Temp 450^{o}C

CUR 30, CAD medium, GS1 60, GS2 40, IS 5500

Q1: unit resolution, scan 50-400amu

Q3: linear ion trap, dynamic fill time,

Data Acquired: Profile mode, 0-10min.

Data Range: 50-400m/z

5.5.2.4 Mass accuracy determination:

$$Mass\ accurracy\ in\ ppm = \frac{Theoretical\ mass - Measured\ mass}{Theoretical\ mass}\ X\ 1000000$$

Example: True mass = 400.0000

Measured mass = 400.0020

Difference = 0.0020

Error = (0.0020/400) X 1000000 = 5ppm

Typically 5ppm or less mass error is considered adequate for an unequivocal assignment, this is accomplished by a targeted analysis (where the detector range is narrowed around the mass of interest with known references). Peptide mass calculator was used to calculate the monoisotopic mass of peptides.

6. RESULTS AND DISCUSSION

6.1 Stock Preparation

The stocks were prepared by simmering at 90ºC for seven hours. This temperature is optimum in creating an intense brothy flavor (Cambero et al., 2000; Vollmer, 1979. As fat is a major constituents of marrow bones, it was a challenging task to remove the fat once the heating process was completed and to obtain a low fat stock that will be well liked by the consumers. The protein yield is a major factor in creating rich stock. Enzyme treatment was considered to increase the protein yield as the stock prepared without any enzyme do not pass through microfiltration due to high molecular weight and it's bulky nature. Therefore, the protein along with fat goes into the retentate phase. Two proteases: papain and umamizymes were selected based on their wide industry applications and a higher degree of hydrolysis of the later due to high endoprotese and exopeptidase activities. The samples were enzyme treated followed by microfiltration to remove fat. Microfiltration was considered due its acceptance in working with samples of large scale and its efficient performance in removing fat. The three samples yielded stock of different protein level. Umamizyme digested stock had the protein level of 4.0%, while papain digested had 2.5% and undigested only 0.8%. The degree of hydrolysis received was higher for the umamizyme treated stock (76%) than the papain treated ones (51%). The undigested stock had the degree of hydrolysis of only 29%.

6.2 Volatile Analysis

6.2.1 Volatiles comparison of stock samples S, P and U

Although, fat was removed by microfiltration, there was approximately 0.50% fat in the stocks S, P and U. The fatty acid components of lipids are prone to autoxidation. The volatiles dicarbonyls, aldehydes, alcohols, ketones were abundant in the stocks. Diacetyl (2,3-butanedione was identified in all three stock samples). Lipid derived aldehydes hexanal, pentanal, octanal and heptanal dominated as major volatiles **(Table 15)** using SPME GC-MS. The phospholipids contain a higher proportion of unsaturated fatty acids such as arachidonic acid (20:4).

Table 15: Volatiles comparison of stocks (S, P and U)

Volatiles	S	P	U
	in ppb		
Aldehydes			
Pentanal	1	66	2
Hexanal	9	159	10
Heptanal	10	80	0.2
Octanal	10	18	
Nonanal	1		
2-Methylpropanal		2	
Alcohols			
Pentanol	30		0.3
Heptanol	0.8	0.20	
Ketones			
Heptanone		0.5	
Dicarbonyls			
2,3-Butanedione (Diacetyl)	6	4.5	10

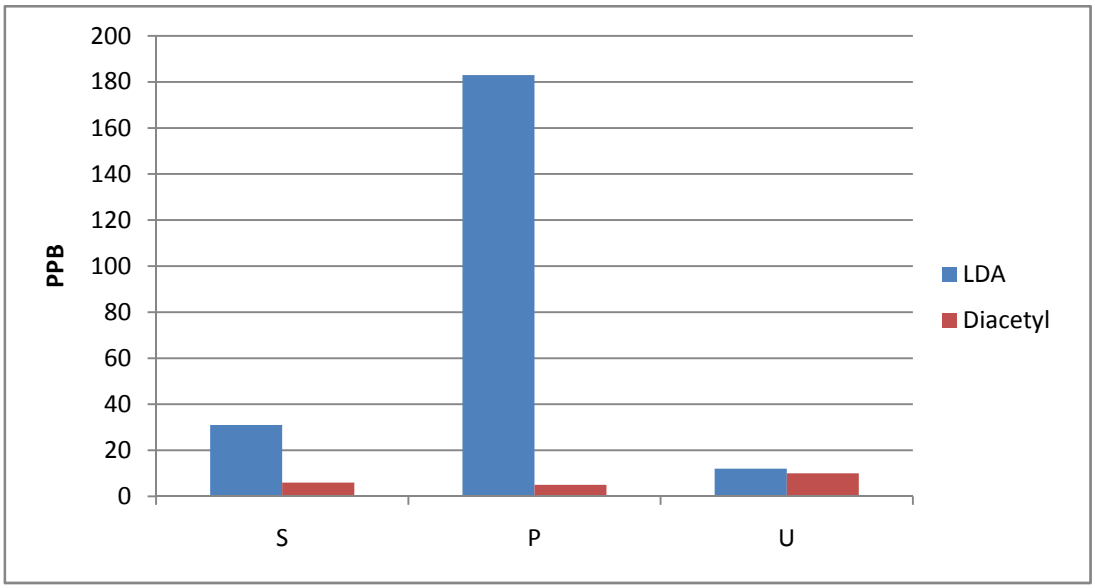


Figure 7: Comparison of 2,3-butanedione (diacetyl) and lipid derived aldehydes (LDA) in samples S, P and U

.

Stock samples S, P and U is dominated by lipid derived aldehydes (LDA) and small amounts of diacetyl **(Figure 7).** Stock sample P contain high amounts of LDA, followed by sample S and sample U contains the lowest. On the other hand, sample U contains the highest amount of diacetyl. Sample P contain the least amount of diacetyl.

6.2.2 Stock samples heated at 120ºC and 160ºC

Table 15 illustrates the comparison of stock sample S, P and U at 120ºC and 160ºC.

6.2.2.1 Untreated (Stock samples S-120 and S-160):

The untreated samples generated Strecker aldehydes along with dicarbonyl compounds and methyl sulfides. Lipid derived aldehydes was reduced **(Figure 8).** Higher amounts of Strecker aldehydes and methylsulfides were observed in S-160

6.2.2.2 Papain treated (Stock samples P-120 and P-160):

Papain digested stock generated more Strecker aldehydes, pyrazines, methyl sulfides and ketones at increasing temperatures. Furans, pyrrole, and thiazoles were detected at 160ºC, which was not identified at 120ºC.

6.2.2.3 Umamizyme treated: U-120 and U-160

U-160 generated high amount of Strecker aldehydes, methylsulfides, pyrroles and pyrazines at higher temperatures. Furans were only generated in P-160 and U-120 **(Figure 9).** The aldehydes are oxidative products of unsaturated fatty acids, which can be generated at temperatures as low as 60ºC (King, 1993). Hexanal is a major compound produced from the thermal decomposition of linoleic acid. Nonanal is the decomposition product of the 10-hydroperoxide, produced from the oxidation of the oleic acid, which is the dominant fatty acids in marrow.

Table 16: Volatile comparison of all three stocks at 120ºC and 160ºC

Volatiles	S-120	S-160	P-120	P-160	U-120	U-160
Aldehydes						
Lipid Derived:						
Pentanal		2	50	20	24	
Hexanal	6	4	137	967	115	0.75
Heptanal	7	0.4	63	49		
Octanal	5	2	18	24	39	
Nonanal				26	21	
t-2-Hexenal			2		1	
t-2-Heptenal					5	
t-2-Octenal					5	
2,4-Hexadienal			46	41		
2,4-Nonadienal				10		
Strecker:						
2-Methylpropanal		1.2		2.6		1
3-Methylbutanal	2	30	1	30	9	54
2-Methylbutanal	0.4	1	14	4		2
2-Methylpentanal				6		
Benzaldehyde		1.8	39	87	72	7
Phenylacetaldehyde				2	7	2
Alcohols						
Pentanol	1		19	6	12	
Hexanol			6	1	14	
Heptanol					24	
1-Penten-3-ol			0.2			
1-Hepten-3-ol				2.3		
Ketones						
Acetone		28	71	80	16	2.5
2-Butanone		2	0.6	12		2.6
2-Heptanone			14	30	13	
2-Octanone				13	6	
Dicarbonyls						

2,3-Butanedione (Diacetyl)	8	14	20	17	6	8
2,3-Pentanedione	3	10				
2,3-Octanedione				2.3		
Furans						
3-Methylfuran				26		
2-Ethylfuran				45	33	
Furfural		1				6
2-Pentyl furan				10	19	
Pyrroles						
1H-Pyrrole		0.8		9		6
Methylsulfides						
Dimethyldisulfide	2	6		39		4
Dimethyltrisulfide				2.4		
Pyrazines						
2,5-Dimethylpyrazine			0.1	56	9	12
2,3,5-Trimethylpyrazine				8		
3-Ethyl-2,5-dimethylpyrazine				83	6	12
2-Ethyl-5-methylpyrazine						0.2
3-Butyl-2,5-dimethylpyrazine						1.4
Thiazoles						
2-Acetylthiazole				4.4		
Hydrocarbons						
Ocimene				8		

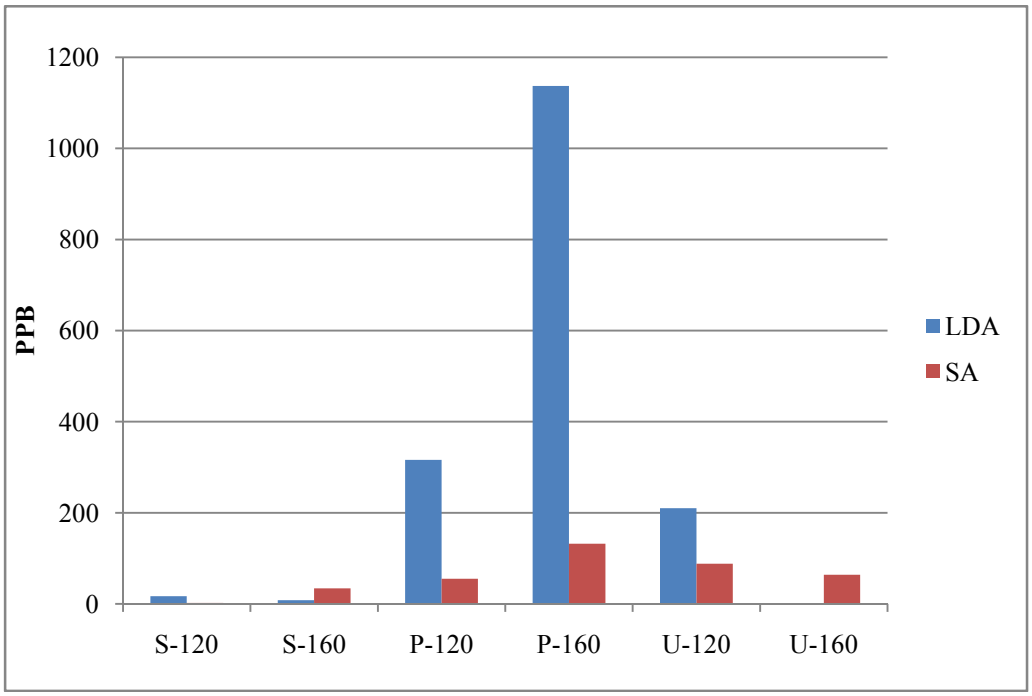


Figure 8: Comparison of Strecker aldehydes and lipid derived aldehydes in sample S, P, and U (LDA=lipid derived aldehydes, SA=Strecker aldehydes)

Lipid derived aldehydes (LDA) were reduced at higher temperature in both stock samples S and U. In stock sample P, LDA were increased at higher termperature. Strecker aldehydes (SA) were increased at higher temperatures in both stock S and P. In stock sample U, SA was decreased with higher temperature **(Figure 8).** Straight-chain aliphatic aldehydes are typical products of lipid oxidation. They have low odor and taste threshold values and play an important role in the flavor of marrow bones stock. Hexanal is the most abundant compound detected in the stocks. It is considered the main volatile derived from the oxidation of *n*-6 fatty acids such as linoleic and arachidonic acids.

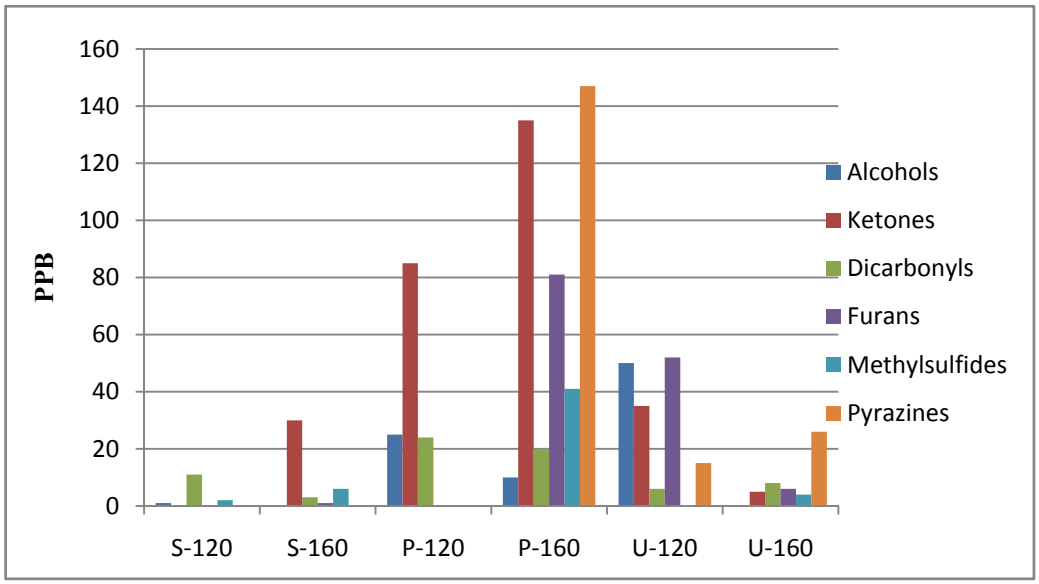


Figure 9: Comparison of volatiles in samples S, P and U at 120°C and 160°C

In both stock samples S and P, ketones, pyrazines and methyl sulfides increased with increasing temperature. However, dicarbonyl compounds decreased. The reverse occurred with stock sample U, where the dicarbonyls increased and ketones increased. In all cases, methyl sulfides and and pyrazines increased **(Figure 9)**. 2-Ketones may rise from fatty acids by chemical (autoxidation) or enzymatic (β-oxidation) oxidation of free fatty acids. Straight chain aliphatic alcohols probably resulted from the degradation of lipid hydroperoxides.

6.2.3 The effect of d-ribose, d-xylose and methylglyoxal at 160ºC

6.2.3.1 Untreated Stocks

(a) Untreated stock reacted with ribose at 160ºC

S-160-R generated high amounts of Strecker aldehydes which includes 2-methylpropanal (isovaleraldehyde), 2-methylbutanal and phenalacetaldehyde **(Table 17).** Dicarbonyls such as 2,3-butanedione was significant. Fufural, possibly a degradation product of d-ribose is present. No dimethyldisulfide is observed.

(b) Untreated stock reacted with xylose at 160ºC

S-160-X generated high amounts of pyrazines, 2-acetyl furan, benzaldehydes, 2-acetyl-5-methyl furan and nonetheless significant amount of furfural and 2-acetyl furan. No dimethyldisulfide is observed, however small amounts of dimethyltrisulfide was present. Very high amount of benzaldehyde is observed.

(c) Untreated stock reacted with methylglyoxal at 160ºC

Methylglyoxal occurs in food system naturally and produced via its precursors, acetoacetate and β-hydroxybutyrate which are known products of ketosis (Gerrard, 2006). S-160-M generated high amount of acetone, 2-methylbutanal, 2-3-pentanedione, 2,3-butanedione (diacetyl) and acetoin. Dimethyldisulfide and dimethyltrisulfide is present in significant amount and the mechanism of formation is described in **Figures 17 and 18**. Pyrazines, furans, thiopense have been identified. All three reactions showe very low amount of lipid derived aldehydes.

Table 17: Volatile comparisons of samples S-160-R, S-160-X, and S-160-M

	S-160-R	S-160-X	S-160-M
Volatiles		in ppb	
Aldehydes			
Lipid derived:			
Hexanal	2	1.6	1.7
Heptanal			19
Strecker:			
2-Methylpropanal	1.6		6
3-Methylbutanal	22	3	18
2-Methylbutanal	10		64
Benzaldehyde	3.7	242	12
Phenylactaldehyde			45
Alcohols			
Isopropylalcohol	10		
Pentanol		0.2	
Acetol			90
Ketones			
Acetone	15	1	56
1-(2,4-Dimethylfuran-3-ylethanone			20
1-(5-Methyl-2-furanyl-1H-propanone			2
6-Methyl-3,5-heptadien-2one			14
1,3-Dioxolan-2-yl-2-propanone			21
1-Phenylpropanone			13
Carbonyls/dicarbonyls			
2,3-Butanedione (diacetyl)	50	26	238
2,3-Pentanedione			82
2,4-Hexanedione	2		17
2,3-Hexanedione			7

Acetoin	1	2	28
Esters			
Methyl acetate			1
Ethyl acetate	18	1.5	55
Pentylacetate (amylacetate)			11
Acids			
Acetic acid	0.4		148
Acetoxyacetic acid			29
Hexanoic acid	2.5		37
Octanoic acid	1	0.3	58
2-Methylbutyric acid			17
Decanoic acid			35
Furans			
3-Methyl furan		12	
Furfural	3700	2352	9
2,5-Dimethyl-3(2H)-furanone			4
3,5-Dimethyl-2(5H)-furanone			4.5
2-Acetylfuran	34	242	
5 Methyl furfural	2.3		28
3-Acetyl 2,5-dimethylfuran			127
2-Acetyl dimethylfuran			110
2(1,1-Dimethylethyl)-4-methylfuran	24		
Oxazoles			
4,5-Dimethyl oxazole			6
2,3,5-Trimethyloxazole			22
Sulfides			
Dimethyldisufide			6
Dimethyltrisulfide	2	3	7
Pyrazines			
2-Methylpyrazine	8	37	

2,5 Dimethylpyrazine			5
2,3,5-Trimethylpyrazine			4
3-Ethyl-2,5-dimethylpyrazine			14
2-Acetoxy-5-methylpyrazine			32
Hydrocarbons			
Sabinene			4
β-Myrcene	2	0.6	
α-Pinene	2		
Limonene	0.4		12
Thiazoles			
2-Acetylthiazole	2.5	46	
Thiophene			
Thiophenecarboxaldehyde	1		
Phenols			
4-Methylphenol			10

The lipid derived compounds were the main group volatiles in the stocks. In general, acids are generated by lipid oxidation reactions. Acetic acid is originated by Maillard reaction and by the fermentation of sugars by microorganisms.

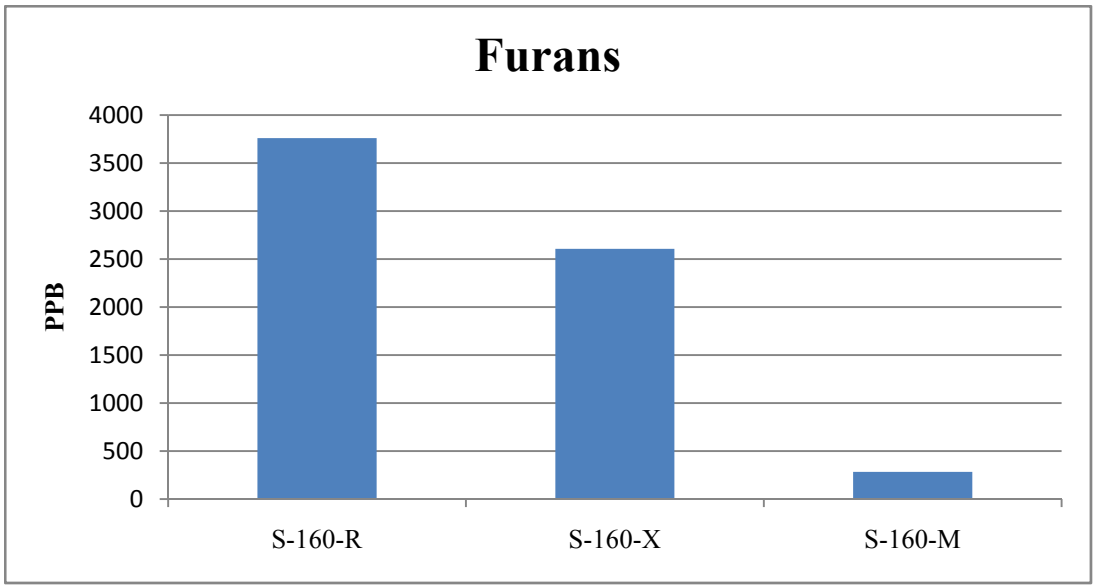


Figure 10: Comparison of furans in samples S-160-R, S-160-X, and S-160-M

Sample S-160-R generated most furans followed by S-160-X and S-160-M **(Figure 10).** Furans were produced generously from the Maillard reaction between enzyme digested stock and ribose or xylose. They are produced commonly from degradation of reducing sugars. Sugars and amino compounds react to form a Schiff base which is eventually converted to an Amadori compound. Furan compounds are produced after dehydration and cyclization. Furans provide sweet, caramelic notes to foods. Pyrroles are degradation products of furans and provide toast and burnt character

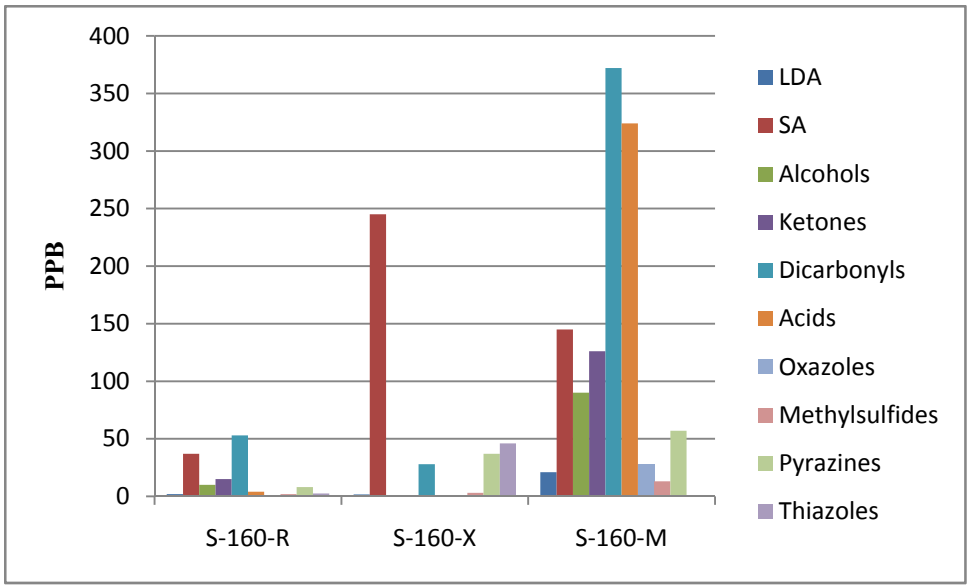


Figure 11: Volatiles comparison of S-160-R, S-160-X and S-160-M (LDA=lipid derived aldehydes, SA=Strecker aldehydes)

Untreated stocks with methyl glyoxal reaction generated higher volatiles **(Figure 11)**. In particular, higher pyrazines, dicarbonyls, dimethyl sulfides, alcohols, acids, oxazoles were observed. Strecker aldehydes were high in sample S-160-X. High amount of benzaldehyde was observed in sample S-160-X. Alcohols, ketones, dicarbonyls, acids, oxazoles, methylsulfides are comparatively hig in the sample S-160-M as sample S-160-R was dominated by furan compounds. Phenylacetaldehyde is a Strecker aldehyde of phenylalanine generated in the methyl glyoxal reaction (in sample S-160-M). A detailed mechanism is illustrated in **Figure 12**.

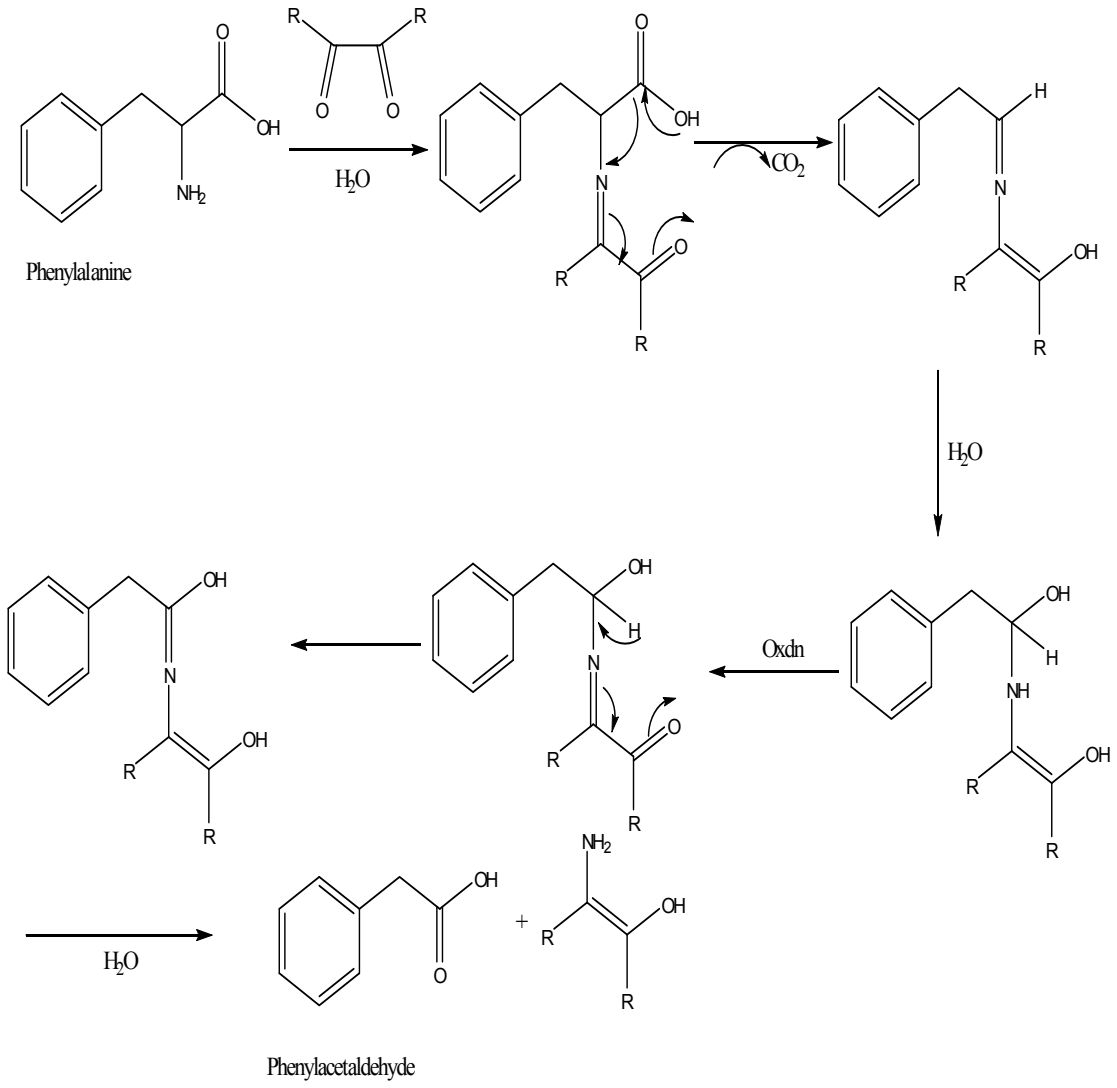


Figure 12: Strecker degradation of Phenylalanine to Phenylacetaldehyde (Schiberle et al., 2001)

6.2.3.2 Papain digested samples

(a) Sample P-160-R (Papain digested stock heated with ribose at 160ºC

P-160-R generated higher amount of Strecker aldehydes mainly 3-Methylbutanal, 2-methylbutanal, benzaldehyde, phenylacetaldehyde, Furan derivatives such as furfural, acetyl furans, 5-ethyl furaldehyde, bifuran, benzofuran is present in huge amounts. 2-acetyl thiazole was identified. Aldol condensation product, 3(2-furanyl)-2-phenyl-2-propenal was identified. Unsaturated aldehyde, 2,4-undecadienal was identified **(Table 18)**.

(b) Sample P-160-X (Papain digested stock heated with xylose at 160ºC)

Xylose reaction generated Strecker aldehydes 3-methylbutanal and 2-methyl butanal, and benzaldehyde. No phenylacetaldehyde is present. 2-acetylthiazole level is considered high compared to ribose and methyglyoxal reaction. The level of furfural is significant. Not identified any other furan derivatives such as acetyl furans.

(c) Sample P-160-M (Papain digested stock heated with methylglyoxal at 160ºC)

Methylglyoxal reaction generated high amount of acetone, acetol, diacetyl, dimethylsufides, and pyrazines than the xylose and ribose reactions. Pyrazines identified as 2,5-dimethylpyrazine, 3-butyl-2,5-dimethyl pyrazine, 2-(3-methylbutyl)-3,5-dimethyl pyrazine, trimethyl pyrazine, 3-ethyl-2,5-dimethyl pyrazine, furfurylpyrrole, 2-acetyl- 2,5-dimethyl furan, 3-acetyl-2,5-dimethyl furan was identified. Methyl sulfides were identified in high amount. Dicarbonyls such as

diacetyl, 2-3-pentanedione was present significantly. 2,4,5-trimethylthiazole, 4,5-dimethyloxazole, 3-5-dimethyl-2-(5H)-furanone, acetoin, 4-methylphenol was present only in the methylglyoxal reactions. Aldol condensation product, 2-methyl-2-butenal was identified.

Table 18: Volatile comparison of samples P-160-R, P-160-X, and P-160-M

	P-160-R	P-160-X	P-160-M
Volatiles		in ppb	
Aldehydes			
Lipid derived aldehydes			
Pyruvic aldehyde			3
Butanal			2.5
Pentanal			85
2-Hexenal		2.35	
Hexanal	3.4	20	29
Heptanal		8.5	38
Octanal			10
Nonanal	24		
2,4-Undecadienal	58		
2,4-Heptadienal	32		
Strecker Aldehydes			
3-Methylbutanal	62	44	90
2-Methylbutanal	9	14	83
2-Methyl-2-butenal			8
Benzaldehyde		4	94
Phenylactaldehyde	134		43
3(2-Furanyl)-2-phenyl-2-propenal	11		
Alcohols			
Hexanol	10		
Acetol			326
Ketones			
Acetone			143
2-Butanone		0.6	23
6-Methyl-3,5-heptadien-2one			22
1,3-Dioxolan-2-yl-2-propanopne			11
1-Phenylpropanone			19
Carbonyls/dicarbonyls			
2,3-Butanedione (Diacetyl)	56	20	255
2,3-Pentanedione			169
2,3-Hexanedione			3
Acetoin			24
3,4-Hexanedione			25
2,3-Octanedione			29
2,5-Hexanedione			13

Esters			
Methyl acetate			174
Ethyl acetate	1.58	116	
Furans			
3-Methylfuran	7		112
Furfural	31694	15697	22
2,5-Dimethyl-3(2H)-furanone			9
3,5-Dimethyl-2(5H)-furanone			22
5 Methyl furfural			31
Benzofuran	58		
2-Acetyl-5-methylfuran			18
Bifuran	117		
5-Ethylfuraldehyde	656		50
2-Acetyl-2,5-dimethylfuran			436
3-Acetyl 2,5-dimethylfuran			45
Oxazoles			
4,5-Dimethyloxazole			11
2,3,5-Trimethyloxazole			80
Sulfides			
Dimethyldisufide	1.5		60
Dimethyltrisulfide			61
Pyrazines			
2-Methylpyrazine	1.4		
2,5 Dimethylpyrazine			498
Trimethylpyrazine			53
3-Ethyl-2,5-dimethylpyrazine			311
2-(3-Methyl butyl)-3,5-dimethylpyrazine			13
2-(3-Methylbutyl)-3,5-dimethylpyrazine			8
3-Butyl-2,5-dimethylpyrazine			15
Acids			
Acetic acid	7		156
Hexanoic acid			29
Octanoic acid	191		61
Nonanoic acid	170		13
Decanoic acid	475		7
Hydrocarbons			
Sabinene		8	7.2
Ocimene		13	
α-Pinene			19

Limonene		27	28
Napthalenol	32		
Thiazoles			
2-Acetylthiazole	10	39	
Pyrroles			
Furfurylpyrrole	53		
Phenols			
4-Methylphenol			24

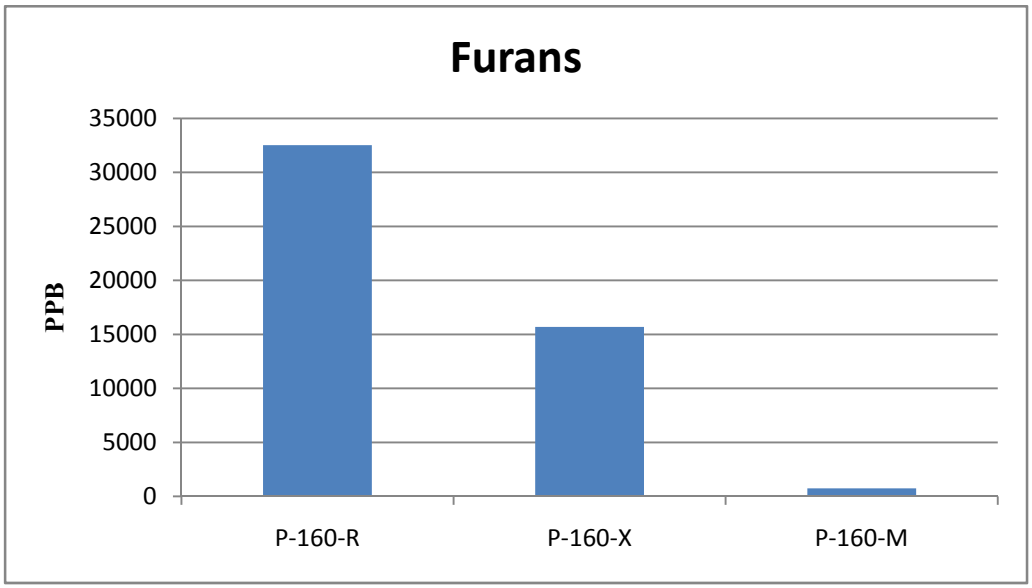


Figure 13: Comparison of furans generated samples P-160-R, P-160-X, and P-160-M

Furans dominated as major volatiles in sample P-160-R **(Figure 13)**. P-160-X generated almost half the amount of furans present in P-160-R. P-160-M generated the least amount of furans. The sugars in the collagen may be involved in the formation of furans, furanones, and pyrans the rough Amadori rearrangement, and or are present in meat flavor volatiles (Schutte, 1976). Furans are mainly produced through dehydration, fragmentation and cyclization of sugars (Nursten, 1980). 2-Acetylfuran is a degradation product of glucose and possibly formed through cyclization and dehydration of 1-deoxyhexosone. The higher yield of furan derivatives at a low pH in methylglyoxal reactions, on the other hand, reduced the amount of carbonyls formed. This may be due to the cyclization reaction, which stabilizes the deoxyglucasones and prevents further fragmentation to small carbonyls. Furthermore, the higher concentration of hydroxyl ion at higher pH values may facilitate fragmentation.

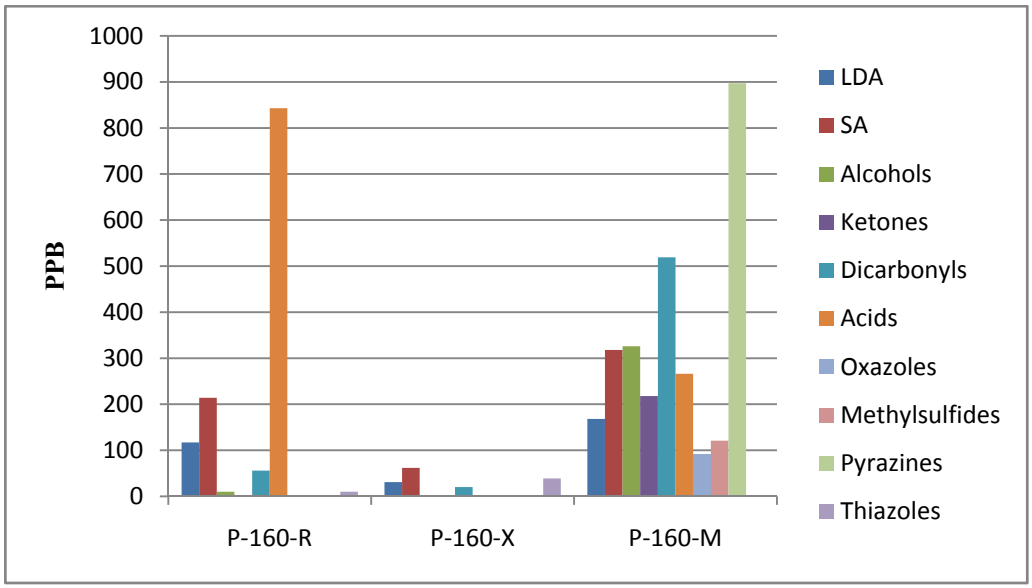


Figure 14: Comparison of volatile compounds in P-160-R, P-160-X, P-160-M LDA=lipid derived aldehydes, SA=Strecker aldehydes

Acids dominated the volatiles in sample P-160-R. Less Strecker aldehydes was observed in the sample P-160-X. P-160-M was populated with very high amounts of pyrazines. Other groups of volatile compound that dominates the sample are dicarbonyls, alcohols, ketones, oxazoles and methylsulfides. Thiazole is absent in the methylglyoxal reaction. Both sample P-160-X and P-160-R contains small amounts of thiazoles **(Figure 14)**. A detailed mechanism of thiazole formation is illustrated in the **Figure 26**.

6.2.3.3 Umimizyme digested stock samples

(a) Sample U-160-R (Umamizyme treated stock reacted with ribose at 160ºC)

U-160-R yielded Strecker aldehydes, 3-methylbutanal, 2-methylbutanal, benzaldehyde, phenylacetaldehyde. Furfural was the largest peak. 2-acetytl furan is identified. Dimethylsulfides amount was significant. Dicarbonyls such as diacetyl, 2-3-pentanedione, acetyl butyryl, was not identified. However, 2,3-octanedione was present **(Table 19)**.

(b) Sample U-160-X (Umamizyme treated stock reacted with xylose at 160ºC)

U-160-X reaction identified more Strecker aldehydes like 3-methylbutanal, 2-methylbutanal and less amounts of benzaldehyde and phenylacetaldehyde than the d-Ribose reaction. 2-acetylthiazole, 4-methyl phenol. Furan derivatives such as 2-acetylfuran, 1-(furyl)-3-butanone, 1-phenylpropanone, furfurilideneacetone and bicycle (3,3) octan-one-7-isopropilidene, 2-propyl furan, 3(2-furanyl)-2-phenyl-2-propenal was identified only in the d-Xylose reaction. No oxazoles were identified. Only pyrazines identified 2,5-dimethylpyrazine, 3-methylpyridazine. Aldol condensation products, 2-phenyl-2-butenal and 3(2-furanyl)-2-phenyl-2-propenal, 5-methyl-2-phenyl-2-hexenal is identified. Napthalolenol is identified.

(c) Sample U-160-M (Umamizyme treated stock reacted with methylglyoxal at 160ºC)

U-160-M yielded huge amounts of Strecker aldehydes, dicarbonyls, pyrazines, and significant amount of oxazoles, pyrazines, furans, aldol condensation products. The Strecker aldehydes include: 2-methyl propanal (isovaleraldehyde), 3-methyl butanal, 2-methylbutanal, methional, benzaldehyde, phenylacetaldehyde. Methional is not identified in the other two reaction samples. The dicarbonyls include: diacetyl, 2,3-pentanedione, acetylbutyryl, 2,6-hexaneione, 2,3-octanedione. The oxazoles include: 4,5-dimethylisoxazole, 2,4,5-trimethyloxazole, 4,5-dimethylpropyloxazole, 3-(3-butenyl)-5-methylisooxazole. Pyrazines include: 2,5-dimethylpyrazine, 3-ethyl 2,5-dimethylpyrazine, 2(3-methylbutyl)-3,5-dimethylpyrazine, 2(2-methylpropyl)-3,6-dimethylpyrazine, 2,5-dimethyl-3(2-methylpropyl)pyrazine, 3-butyl-2,5-dimethylpyrazine, trimethylpropylpyrazine, 2,5-dibutyl-3,6-dimethylpyrazine. Condensation products include: 2-methyl-2-butenal, isopropyl-5-methyl-hex-2-enal, 2-phenyl-2-butenal, 5-methyl-2-phenyl-2-hexenal. Furan derivatives include: furfural, 5-methl furfural, 3,5-dimethyl-2-(5H)-furanone, 2,5-dimethyl-3-2(2H)-furanone, 2-acetyl-5-methylfuran, 3-acetyl-2,5-dimethylfuran, 4,7-dimethylbenzofuran. Methylglyoxal seems like reacted vigorously with the nonvolatile precursors system in the umamizyme digested stock. The precursors may include peptides, amino acids, and organic acids.

Table 19: Volatile comparison of samples U-160-R, U-160-X, and U-160-M

	U-160-R	U-160-X	U-160-M
Volatiles		in ppb	
Aldehydes			
Lipid derived:			
Butanal			5
Hexanal			28
Heptanal			14
Octanal			4.4
Nonanal			28
Strecker:			
2-Methylpropanal			81
3-Methylbutanal	405	775	530
2-Methylbutanal	41	34	209
2-Methyl-2-butenal			2.5
2-Methyl-2-hexenal			110
Benzaldehyde	88	13	321
Phenylactaldehyde	162	32	73
Isopropyl-5-methyl-hex-2-enal			28
2-Phenyl-2-butenal		82	33
5-Methyl-2-phenyl-2-hexenal		111	326
3(2-Furanyl)-2-phenyl-2-propenal		505	
Alcohols			
Isopropylalcohol	205		
Acetol			452
Ketones			
Acetone			11
2-Butanone			30
1-Phenylpropanone		10	280
3-Methylhexen-3-one			12
Carbonyls/dicarbonyls			
Diacetyl			448
2,3-Pentanedione			211
2,3-Hexanedione			2.6

2,5-Hexanedione		4	43
2,3-Octanedione		45	485
Thiazoles			
2-Acetyl thiazole		2.4	
Esters			
Methyl acetate			262
Ethyl acetate			20
Furans			
3-Methyl furan			
Furfural	55564	114481	69
2,5-Dimethyl-3(2H)-furanone			28
2-Propylfuran		591	
5 Methylfurfural		25	83
5-Ethyl-2-furancarboxaldehyde	7		
2-Acetyl-5-methylfuran			421
Bifuran	456	169	
5-Ethylfuraldehyde			58
3-Acetyl 2,5-dimethylfuran			263
4,7-Dimethylbenzofuran			53
2-Acetyl-3,5-dimethylfuran			115
Furancarboxaldehyde	6.55		
1-(2-Furyl)-propanone	113		
Oxazoles			
4,5-Dimethyl-2-propyloxazole			56
4,5-Dimethyloxazole			1.75
2,3,5-Trimethyloxazole			11
3-(3-Butenyl)-5-methylisoxazole		11	
Sulfides			
Dimethyldisufide		5.3	469
Dimethyltrisulfide	75	45	296
Pyrazines			
2-Methylpyrazine			
2,5 Dimethylpyrazine	9	33	967
2,3,5-Trimethylpyrazine			54
Acetoxy-5-methylpyrazine	498		

3-Ethyl-2,5-dimethylpyrazine			573
2-(3-Methyl butyl)-3,5-dimethylpyrazine			28
2,5-Dimethyl-3(2-methylpropyl)pyrazine			44
2-(3-Methypropyl)-3,6-dimethylpyrazine			37
3-Butyl-2,5-dimethylpyrazine			2892
Trimethylpropylpyrazine			10
2,5-Dibutyl-3,6-dimethylpyrazine			125
Acids			
Acetic acid			243
Pyruvic acid			5.7
Butyric acid			5.8
Valeric acid			14
Hexanoic acid		58	
Octanoic acid	39	39	40
Nonanoic acid	93	75	21
Decanoic acid			105
Hydrocarbons			
Myrcene	35		
Napthalenol	115	273	
7-Isopropylidene-bicyclo[3.3.0]octanone		9383	
Phenols			
Methylphenol			19

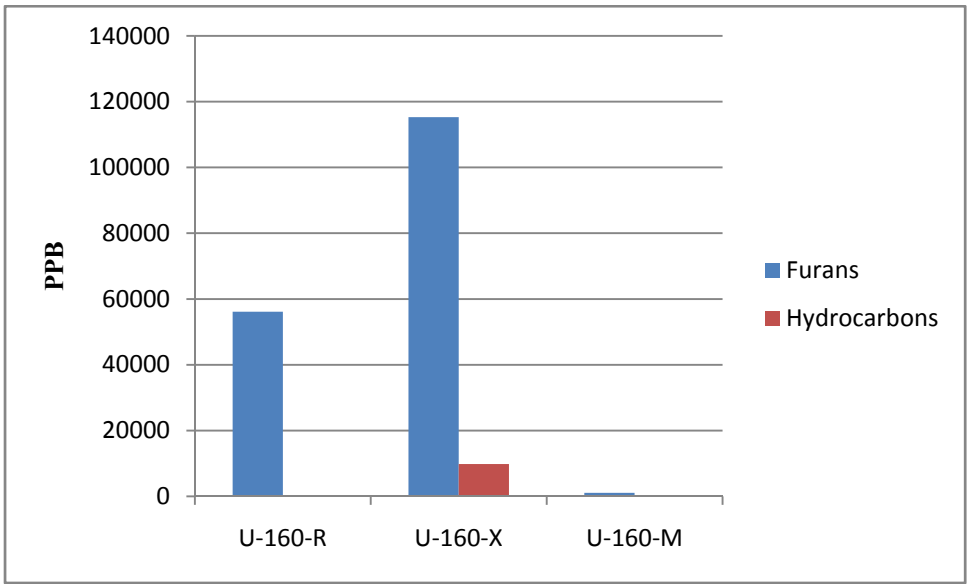


Figure 15: Comparison of furans and hydrocarbons in samples U-160-R, U-160-X, and U-160-M

Furans and hydrocarbons dominated the sample U-160-X. Furan derivatives such as 2-acetylfuran, 1-(furyl)-3-butanone, 1-phenylpropanone, furfurilideneacetone 2-acetylthiazole, 4-methyl phenol. and bicycle (3,3) octan-one-7-isopropilidene, 2-propyl furan, 3(2-furanyl)-2-phenyl-2-propenal was identified only in the d-Xylose reaction **(Figure 15).** The presence of high terpenes in many samples indicates that they might have originated from the plant feed which is a rich source of hydrocarbons and terpenes. These hydrocarbons may have formed via lipid oxidation process through alkyl radicals and further rearrangement.

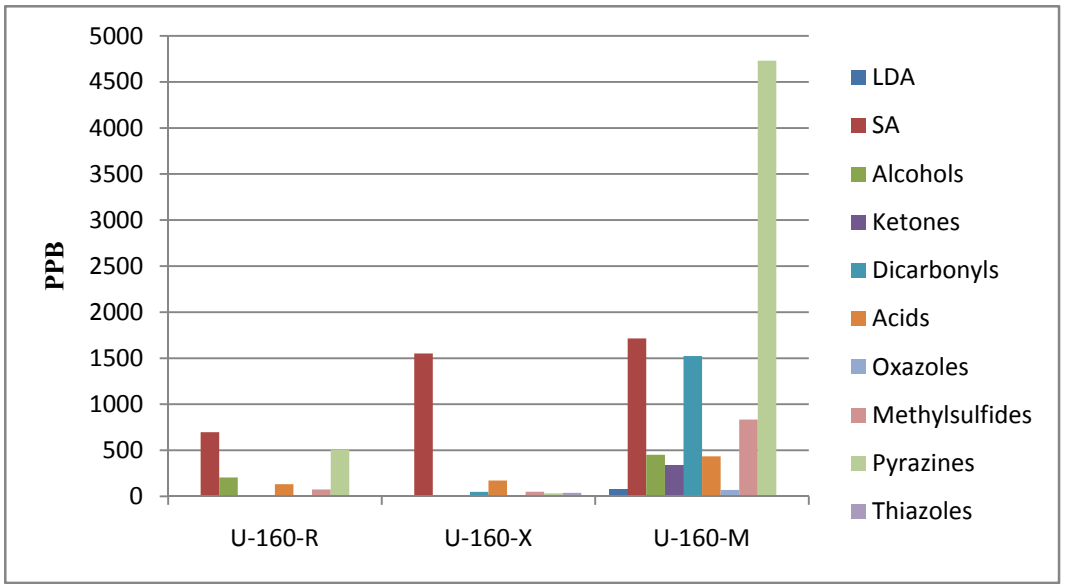


Figure 16: Comparison of volatiles in samples U-160-R, U-160-X and U-160-M

Pyrazine compounds, Dicarbonyls, carboxyl acids, methylsulfides dominated the volatiles in the sample U-160-M. Strecker aldehydes, dicarbonyl compounds and alcohols are the next major groups. Strecker aldehydes dominate the sample U-160-X. Both U-160-R and U-160-X is dominated by furan compounds **(Figure 16).**

Pyrazines formation: In general, the dimethylpyrazines are formed from the dimerization of α-aminoacetone **(Figure 1-3)**. Ethylpyrazines are formed from the condensation of α-aminoacetaldehyde and α-aminobutanal (or α-aminobutanone). Ethylmethylpyrazines are formed from the condensation of α-aminoacetone and α-aminobutanal (or α-aminobutanone). Diethylpyrazines are formed from the condensation of α-aminobutanal. A detailed mechanism of dimethyl butyl pyrazine which dominates the sample U-160-M is illustrated in the **Figure 25**.

Pressure also affects the Maillard reactions. In glucose-lysine model systems it was studied that high pressure promote aldol condensations of carbonyl compounds and reduce volatile productions (Hill, 1999). 5-Methyl-2-phenyl-2-hexenal characteristic of chocolate, cocoa is the aldol reaction product from phenylacetaldehyde and 3-methylbutanal, both Strecker aldehydes has deep bitter cocoa note. 5-methyl-2-phenyl-2-hexenal provides a deep bitter, harsh cocoa note and is the aldol reaction product from phenylacetaldehyde and 3-methylbutanal, both of which are Strecker aldehydes (Marsilli, 2006). 2-phenyl-2-butenal, 5-methyl-2-phenyl-2-hexenal, 2-methyl-2-butenal was identified in cooked liver at 325ºF (Mussinan, 1974). 2-methyl-2-butenal provides sweet and fruity character. Isopropyl-5-methyl-hex-2-enal is fruity and blueberry like. 2-Phenyl-2-butenal is green, vegetative, floral, cocoa, while 3(2-furanyl)-2-phenyl-2-propenal is fruity and caramelic. **Figures 19-24** illustrates the reaction mechanism of the alkenal formation.

Proline and hydroxyproline are imino acids and clearly differ from amino acids. They do not generate ammonia in Strecker degradation and therefore do not give rise to pyrazines. Addition of gelatin as a glycoprotein with reducing sugars and amino acids provide “mouthfeel.” Gelatin is hydrolyzed. Collagen contains large amount of glycine, alanine, proline, and hydroxy proline while deficient in fewer sulfur-containing amino acids. Dimethylsulfide, dimethyltrisulfide, pyrroles, and alkyl pyrazines reduced during thermal degradation of collagen may be significant contributors to meat aroma (Hartman, 1982). Some of the most important components

of meat flavors are derived from the thermal break down of fats, proteins and carbohydrates. Aldehydes, acids, and esters are derived from the break down of fats.

The marrow has a pH between 7 and 8. This relatively high pH is conducive to producing nitrogen containing heterocyclic compounds. The shortage of ammonia inhibits the interaction between ammonia and α-hydroxyketones, minimizing the formation of pyrazines and other nitrogen containing compounds. The deficiency of thiazole compounds in the sample U-160-M (methylglyoxal reactions) is due to the shortage of ammonia, which is provided by amide and amine group containing amino acids. Most of the heterocyclic compounds reported in Maillard reaction involvs α-dicarbonyl in the formation of pyrroles, pyrazines, oxazoles and α-hydroxycarbonyl intermediates in the formation of furans. The formation of isoxazoles requires β-dicarbonyl moieties and is generated from reducing sugars (Yaylayan and Haffenden, 2003). At higher pH, more heterocyclic compounds are formed compared to an acidic environment. This is probably due to the fact that the carbonyl compounds react to form other compounds (Tai and Ho, 1998).

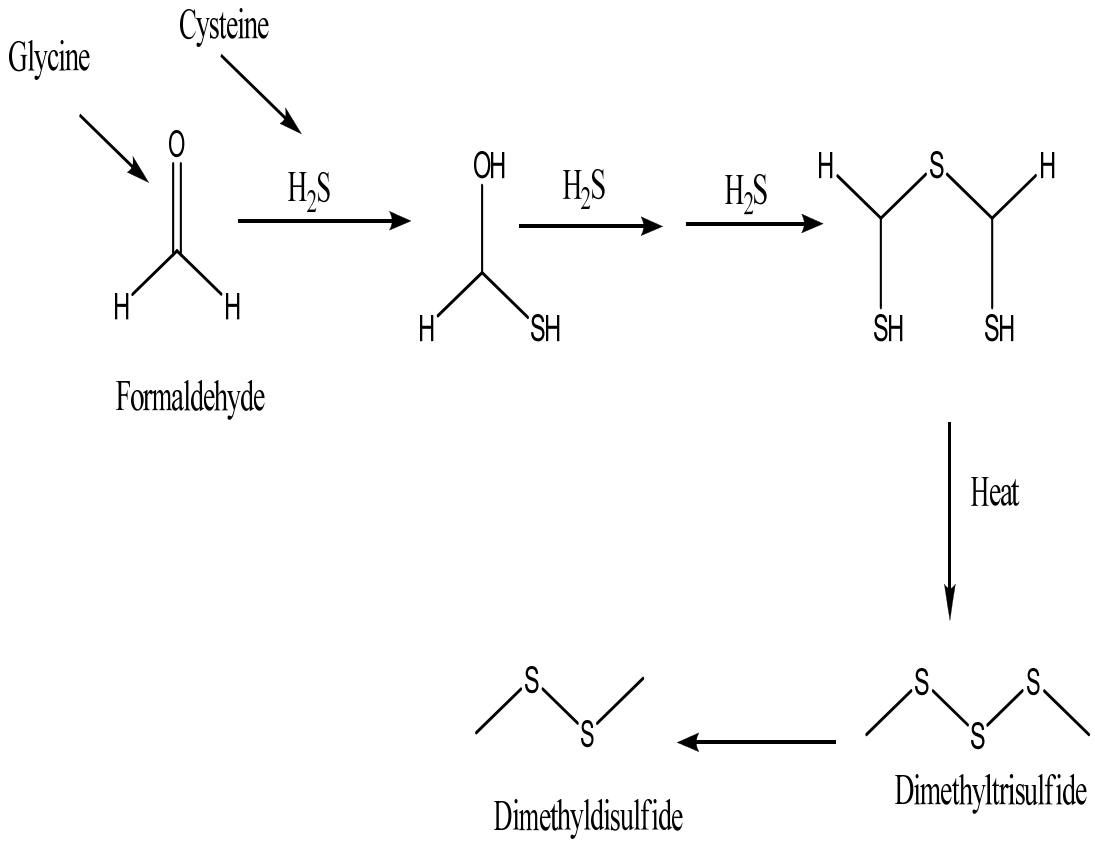


Figure 17: Formation mechanisms of methylsulfides from glycine and cysteine

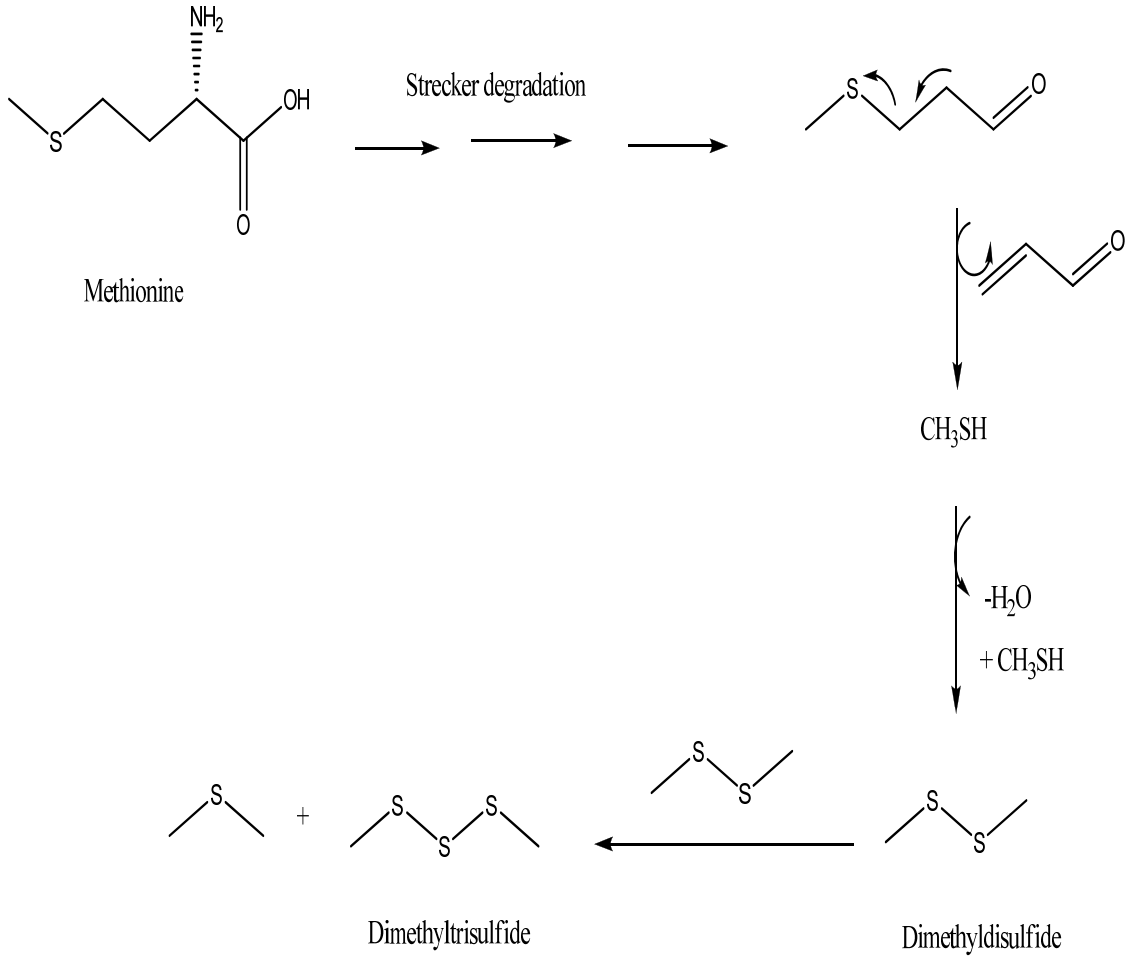


Figure 18: Formation of methylsulfides from methionine (Belitz et al., 2004)

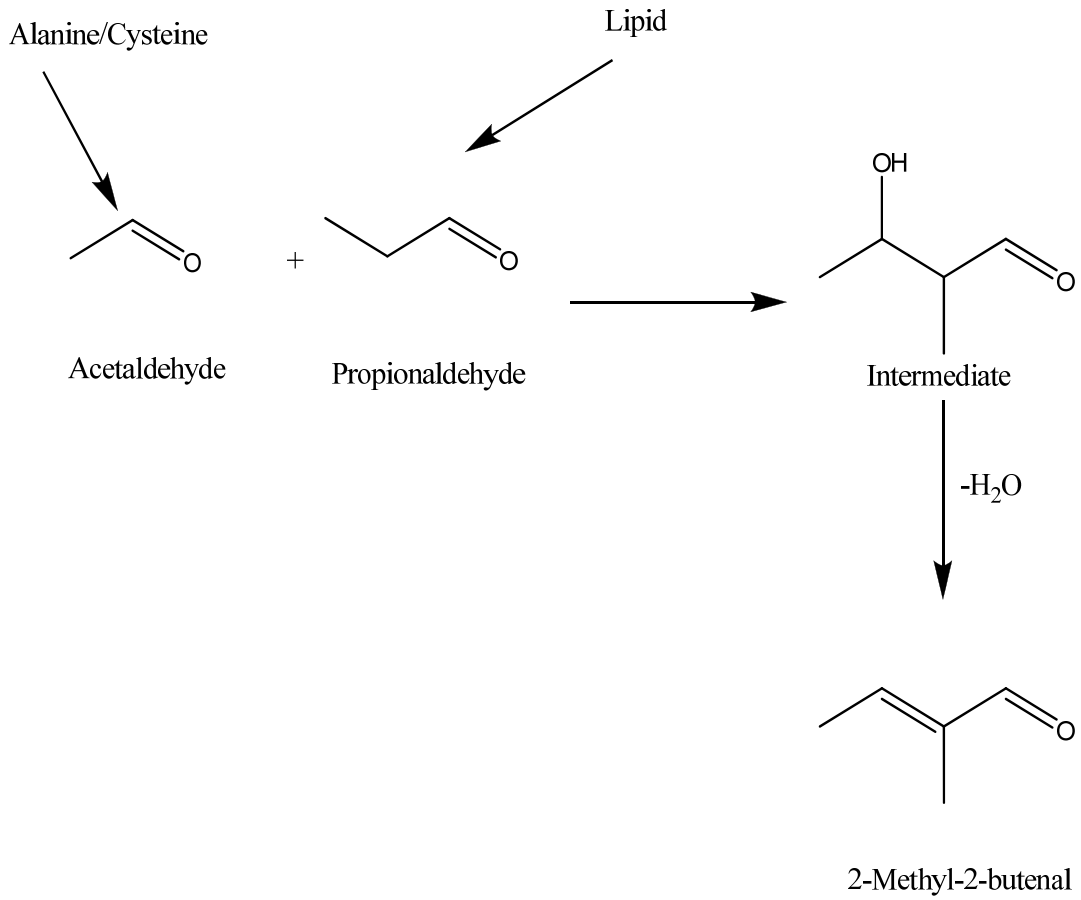


Figure 19: Proposed mechanisms of the formation of 2-Methyl-2-butenal

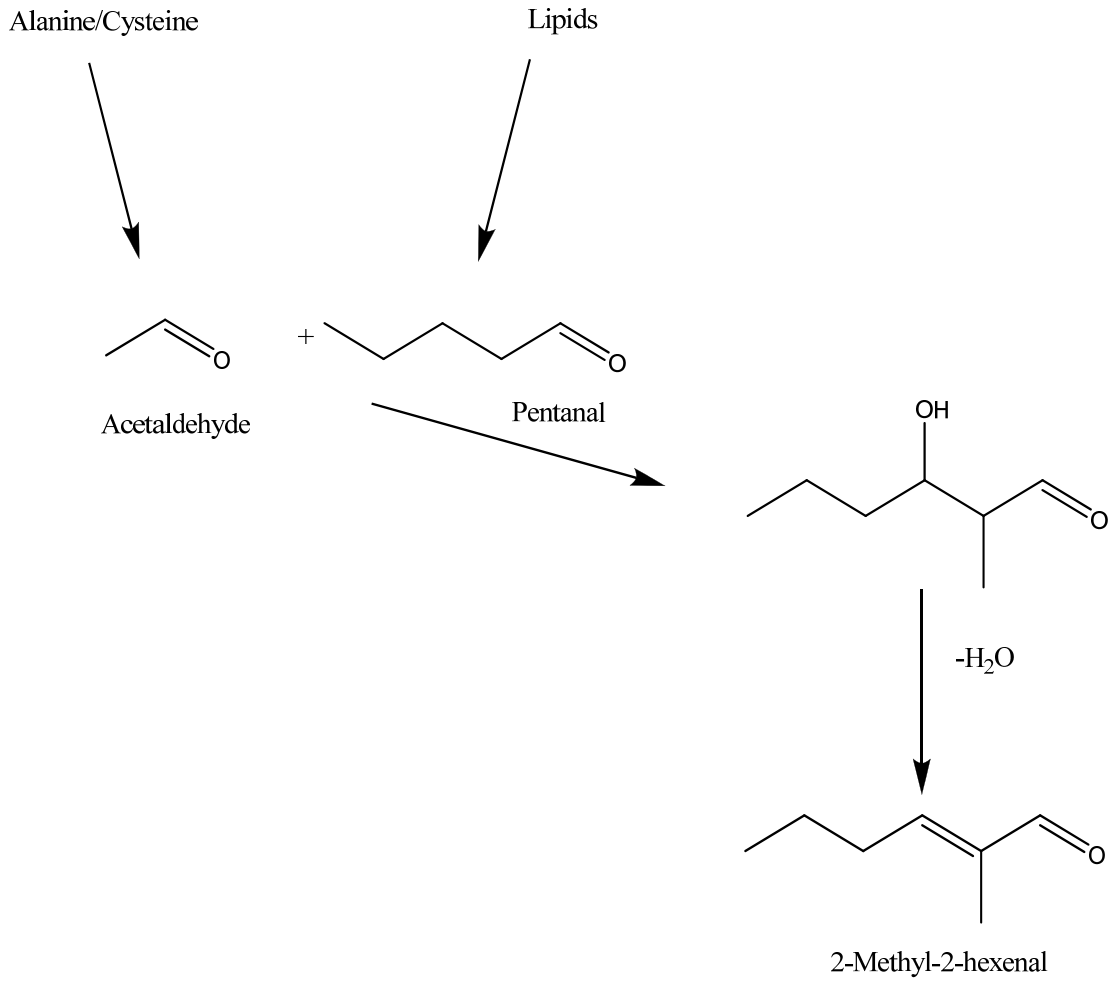


Figure 20: Proposed formation of 2-Methyl-2-hexenal

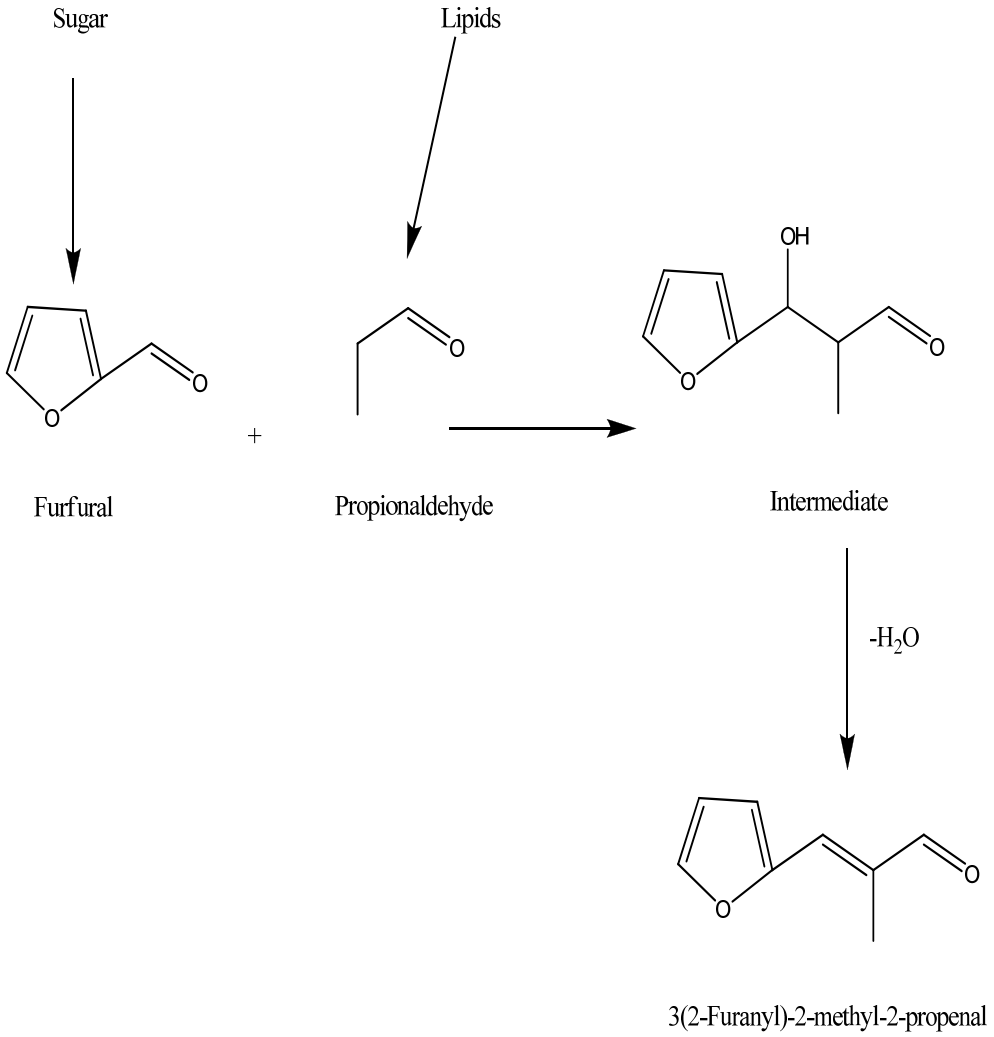


Figure 21: Proposed mechanisms of the formation of 3(2-Furanyl)-2-methyl-2-propenal

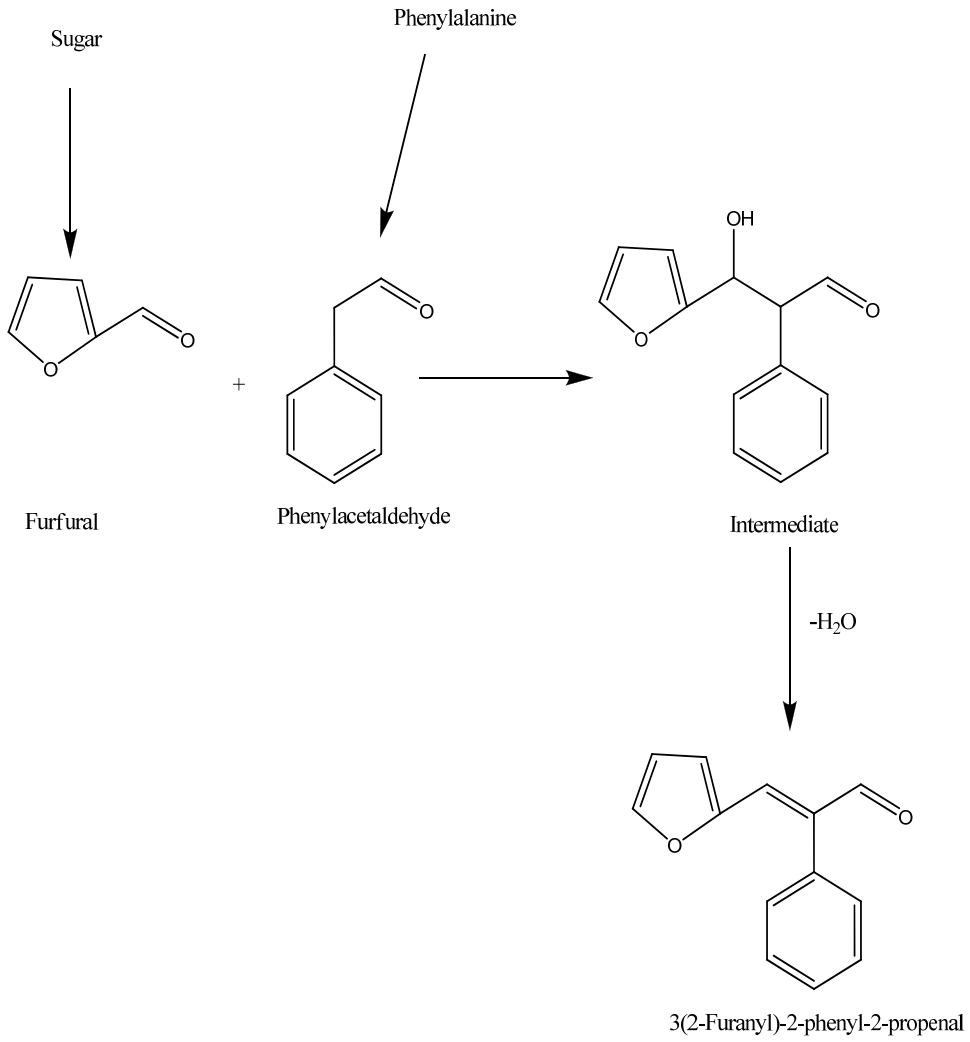


Figure 22: Proposed mechanisms of the formation 5-Methyl-2-phenyl-2-hexenal

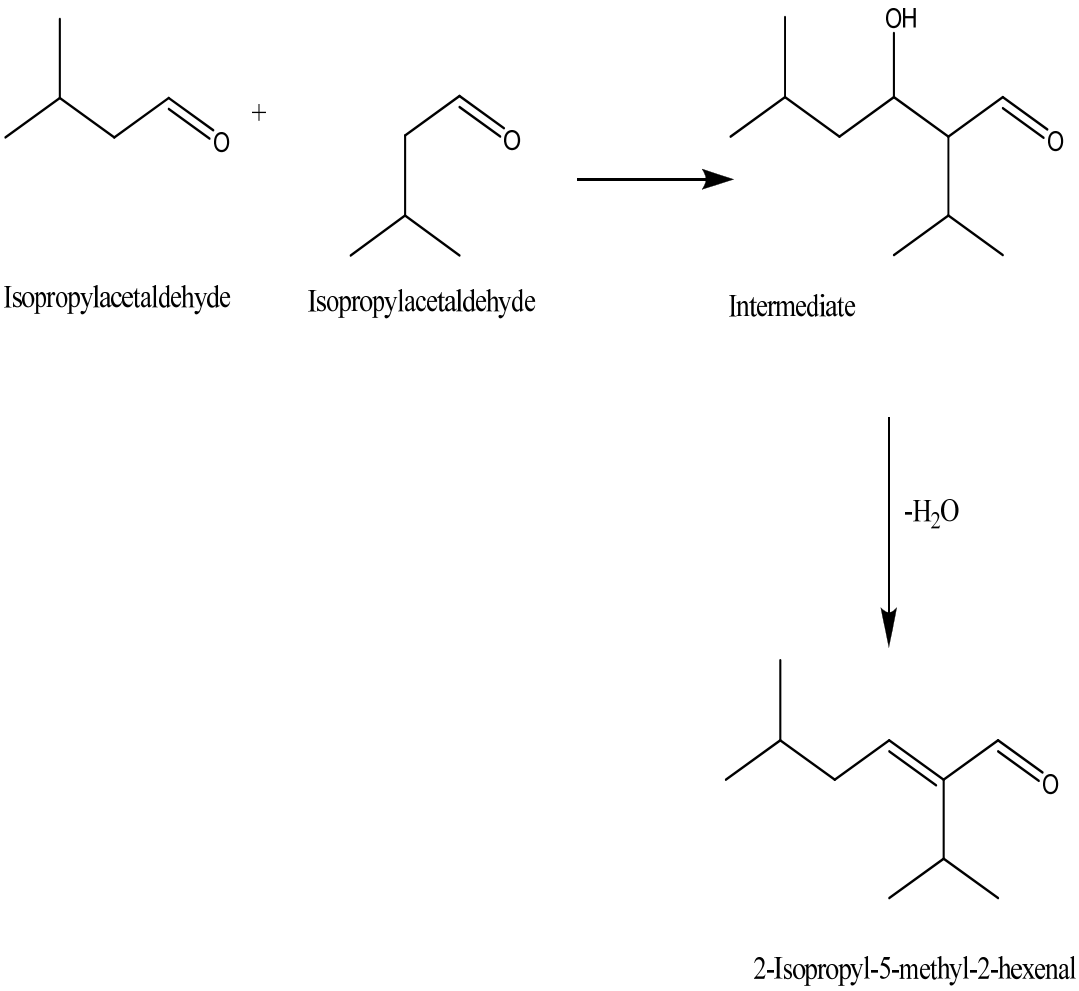


Figure 23: Proposed mechanisms of the formation of 2-Isopropyl-5-methyl-2-hexenal

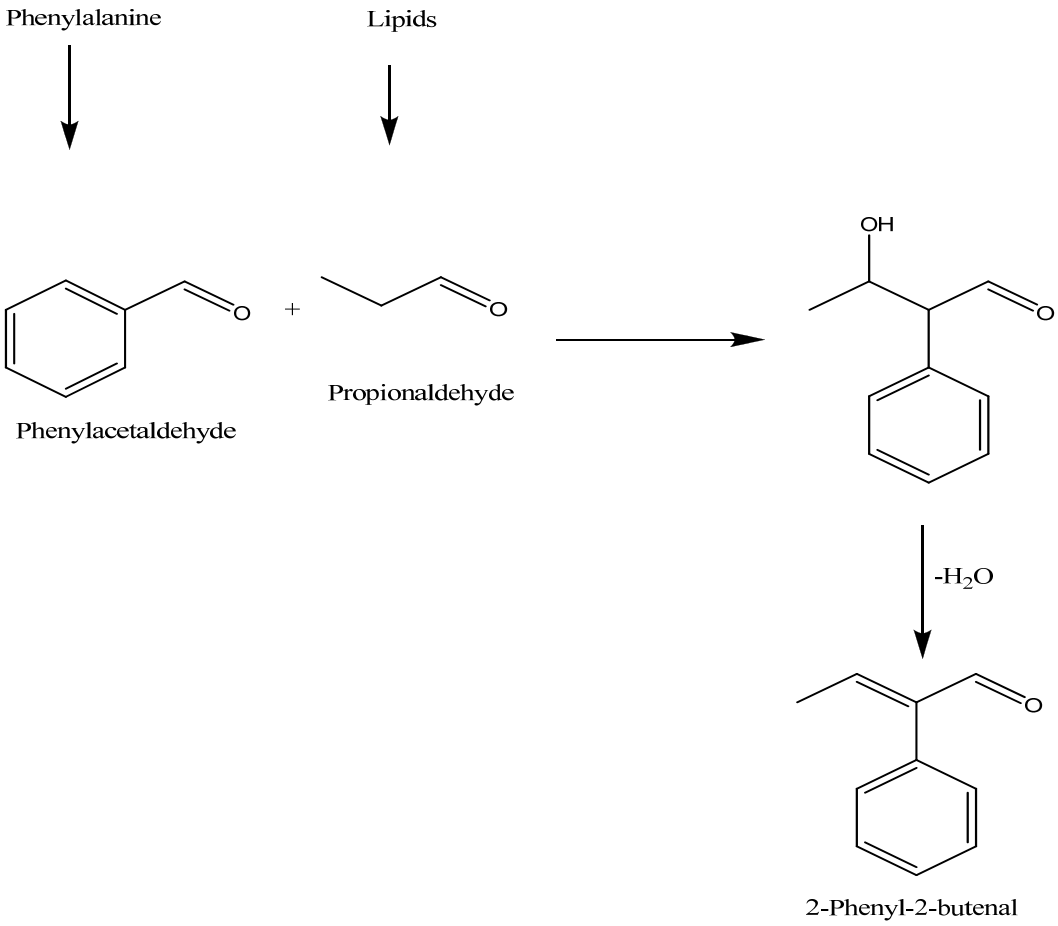


Figure 24: Proposed mechanisms of the formation of 2-Phenyl-2-butenal

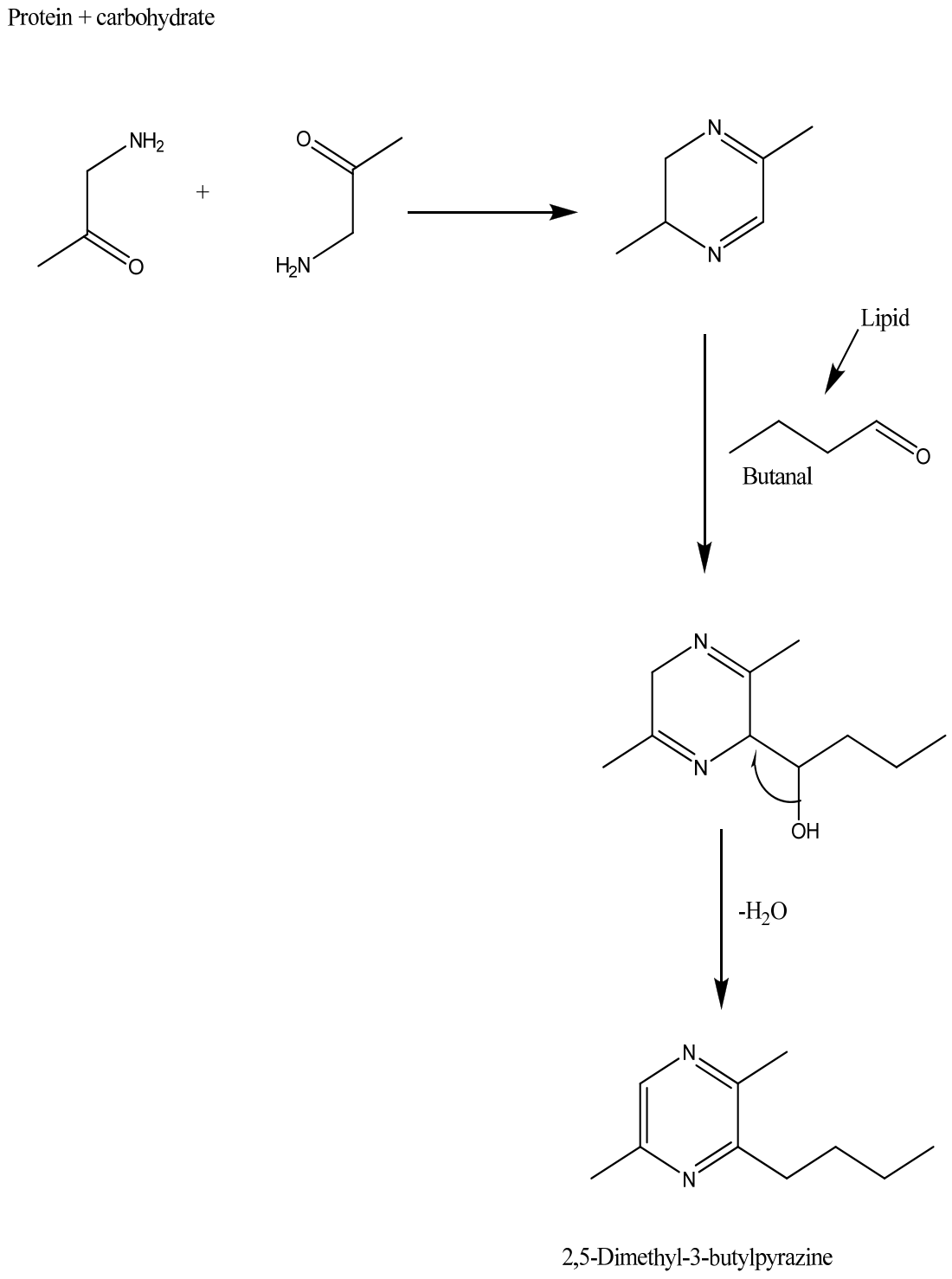


Figure 25: Formation of 2,5-dimethyl-3-butylpyrazine from their precursors (based on the formation pathway of 2,5-dimethyl-3-pentylpyrazine as proposed by Chiu et al., 1990)

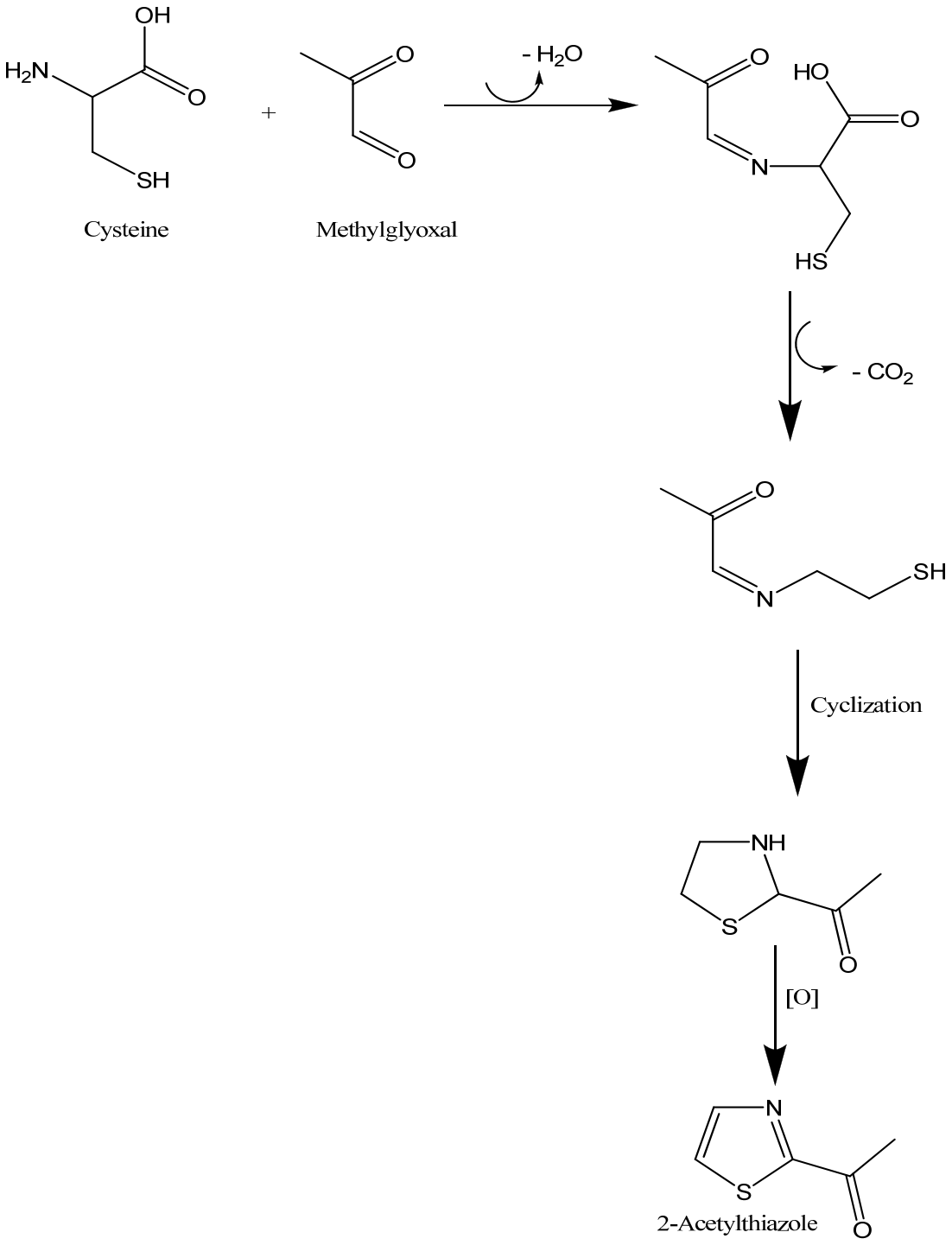


Figure 26: 2-Acetylthiazole formation from cysteine and methylglyoxal (Griffith and Hammond, 1989)

In Maillard reaction, amino acids are decarboxylated and deaminated, forming corresponding aldehydes, while dicarbonyls formed in the Maillard reaction are converted to amino ketones or amino alcohols, which can react with themselves or with other compounds, providing a variety of aromatic compounds. 2-and 3-methylbutanal are products of the Strecker degradation of the amino acids isoleucine and leucine, while phenylacetaldehyde comes from the amino acid phenylalanine **(Figure 12)**. 2-Methylbutyric acid **(Table 17)** may be formed by the oxidation of 2-methylbutanal (Ramirez et al., 2007). Generation of dimethyldisulfide occurs from sulfur containing amino acids, such as methionine, cysteine and cystine. Thiols are readily oxidized to dimethyldisulfide, which can disproportionate to dimethyltrisulfide **(Figure17 and 18)**. 2,4,5-trimethyl-3-oxazoline was identified in boiled beef (Chang et al., 1968). Pyrroles are the degradation products of proline.

Pyrazines can be formed through a few pathways. The most basic pathway includes the Strecker degradation of amino acids in the presence of α-dicarbonyls, which forms a critical intermediate, i.e., α-aminoketone molecule, which can be also formed by the reaction of α-hydroxyketone with ammonia. Two molecules of α-dihydropyrazine can undergo further reactions such as oxidation, disproportionation, or dehydration forming pyrazines. Dihydropyrazine could also be subjected to Michael addition of a ketone or aldehyde forming pyrazines (Amrani-Hemaimi et al., 1995). The precursors as the carbon source or the pyrazines present in our reaction systems included proposedly α-hydroxyacetaldehyde, methylglyoxal (pyruvaldehyde), 3-hydroxy-1,2-butanedione, and 4-hydroxy-2,3-pentanedione.

The formation of the ethylated pyrazines such as 2-ethylpyrazine, 2-ethyl-6-methyl pyrazine, and 2-ethyl-5-methylpyrazine needs 2-amino-butyraldehyde or 1-amino-2-butanone. The intermediate could be generated by the reaction of 1-hydroxy-2-butanone or 2-hydroxy-1-butanal with ammonia or by the reaction of 1,2-butanedione with amino acid through Strecker degradation. Different amino acids possess different side chains and produces different Strecker aldehydes and eventually pyrazines. These pyrazines could also be formed by the reaction of dihydropyrazine with acetaldehyde through Michael addition. The formation of the ethylated and methylated pyrazines such as 2-ethyl-3,5-dimethylpyrazine, 3-ethyl-2,5-dimethylpyrazine, and 2-ethyl-3-methyl pyrazine is probably formed by the condensation of 2-amino-3-pentanone or 3-amino-2-pentanone with another aminoketone. Hydroxyacetaldehyde, 2,3-butanedione, and 2-hydroxy-3-butanone are the precursors of the carbon source for pyrazines. Most of these pyrazines were identified in the umamizyme digested sample reacted at 160ºC under pressure with methylglyoxal (U-160-M). Umamizyme is effective in hydrolyzing the peptide linkages to produce amino compounds which were essential substrates of Maillard reaction. Pyrazines are formed through the reaction of dicarbonyl compounds with amino acids through Strecker degradation. This involves condensation reaction to initially form an imino ketone and subsequently an amino ketone accompanied by release of CO_2 Strecker aldehydes. The final amino ketone self condense or condense with other amino ketones to form the unstable dihydropyrazines. These intermediates then undergo oxidation or dehydration to produce pyrazines or react with other aldehydes to form alkyl substituted pyrazines **(Figure 25).** In this case, 2,5-dibutyl-3-

butyl pyrazines were formed. Glycine peptides generates 2,5-dimethylpyrazine rather than 2,6-dimethylpyrazine (Oh et al., 1991; Weenen et al., 1998). Pyrazines are well known for their roasted, toasted, nutty character and posses low threshold value and yet provide high impact.

The Umamizyme (*Aspergilllus orzae*) contains peptidase activities. Umamizyme is capable of hydrolyzing food proteins at high level because of endoprotease and exoprotease activities. Endoprotese cleaves the peptide bonds in the interior of the protein while exopeptidase releases the amino acid located at the terminal end of the chain. It was evident that significantly more of the flavor compounds were generated in the umamizyme and papain digested stock than the stocks that was not treated with any enzymes. Umamizyme digested stock produced more Maillard reaction volatiles than the papain digested stocks especially when reacted with ribose, xylose or methylglyoxal. This suggests that umamizyme is more efficient in cleaving off the peptide bonds to produce fragments such as smaller protein, peptides and amino acids. Therefore, more amino compounds are available to participate in the complex Maillard reaction pathway and generate numerous volatiles. This was confirmed by a higher degree of hydrolysis for umamizyme digested stock than papain digested stock or untreated stock.

6.3 Non-volatile Studies:

6.3.1 LC-MS studies

The LC-MS studies find that the samples S, P and U posses peptides of different molecular weight, which may be serving as precursors to generate volatiles. Analysis of the stock digest by LC-TOFMS gives a large number of compounds in each sample. The complexity of each sample can be seen from the density maps of each digest **(Figure 27)**. The density maps provide a 3D visual representation in 2D space (hidden z-axis) of distribution of compounds in each digest. Each dash on the map represents a unique compound that is plotted retention time vs. m/z vs. response. The plot on the left shows the raw data prior to background subtraction, while the plot on the right shows the background subtracted data of interest. These maps indicate the presence of 264, 3422, and 5361 possible compounds in control digest, papain digest, and umamizyme digest, respectively **(Table 20)**. LC-MS-ESI TIC chromatogram of samples S, P, and U illustrates the relative abundance of non-volatile compounds vs. retention time **(Figure 28)**. The enzyme treated stocks have higher concentration of non-volatiles than the untreated stock. The stock U posses the highest concentration of non-volatile compounds followed by stock sample P.

Table 20: Comparison of peptides with mass ranges in undigested, papain digested and umamizyme digested stocks

Peptide Size (Amino Acids)	Mass Range (MW)	Number of possible peptides (sample S)	Number of Possible Peptides (sample P)	Number of Possible Peptides (sample U)
2	100 - 400	88	1054	1312
3	150 - 600	249	1923	2331
4	200 - 800	531	2457	2566
5	250 - 1000	602	2632	3942
6	300 - 1200	811	2662	4208
7	350 - 1400	1500	2538	4197

The beef bone marrow stock is a crude mixture of large proteins, which upon digestion would produce a great number of peptide fragments. The large degree of coelution observed in these chromatographic separations also increases the complexity of the analysis. The identification process is laborious. This may be due to the fact that number of possible peptides grows rapidly with the number of amino acids residues. If we consider the 20 natural amino acids, the dependence of the number (Nn) of possible peptides on the number of residues (n) is expressed by the formula, $N_n = 20^{n.}$ The total number of possible dipeptide sequences is 400 while the number of possible tripeptide sequences is 8000**.** Therefore, the difficulty of finding the correct sequence is enormous.

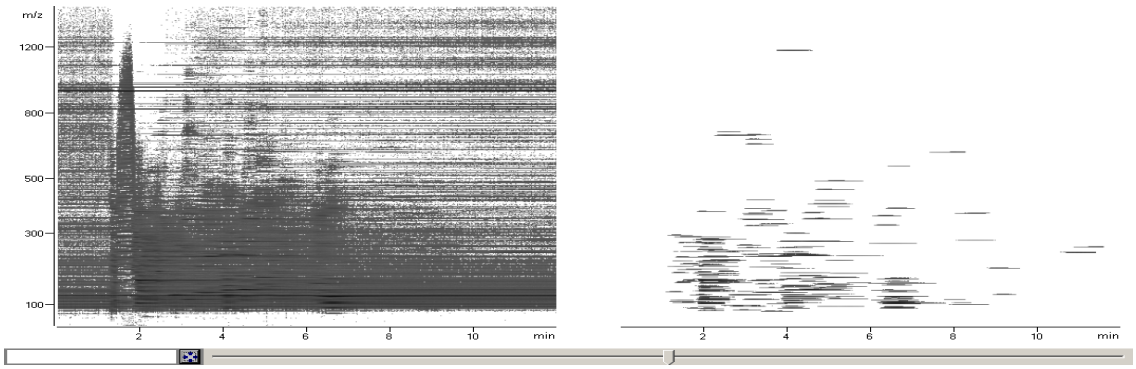
6.3.1.2 Amino acids analysis

In amino acid determination, derivatization technique is used to modify the peptides which make the distinction between C-and N-terminal fragments easier. Derivatization allows specific residues to be detected and located by comparing the spectra before and after derivatization. The derivatization forms ethyl esters from asp, glu and C-terminal carboxylic acids; acetylation of the N-terminal amino group and lys. The amino acids analysis revealed that that enzyme digested marrow bones stocks yielded more amino acids. **(Figures 29-31)** illustrates LC-MS chromatogram showing amino acids in all three stock samples (S, P and U). The highest two peaks in the stock samples S are norvaline (l-2-aminovaleric acid) and sarcosine (N-methylglycine) followed by alanine, threonine, glutamic acid. Norvaline participates in the Strecker degradation to produce butyraldehyde. Sarcosine is sweet in taste. The relative abundance of arginine, leucine, isoleucine, phenylalanine, methionine content was higher in stock sample U than stock sample P **(Table 21).** The second and third major peaks in stock sample P are norvaline and sarcosine respectively. However, stock sample P contained higher concentration of aspartic acid, glutamic acid, threonine, alanine and lysine than stock sample U **(Figure 32).** The amino acid analysis did not show any cysteine, which is probably degraded to volatiles of low molecular weight compounds. Strecker dgradation of cysteine yields mercaptoacetaldehyde and an amino ketone, which breaks down into hydrogen sulfide, ammonia and acetaldehyde. However, they probably participated in the Maillard reaction to generate volatile compounds.

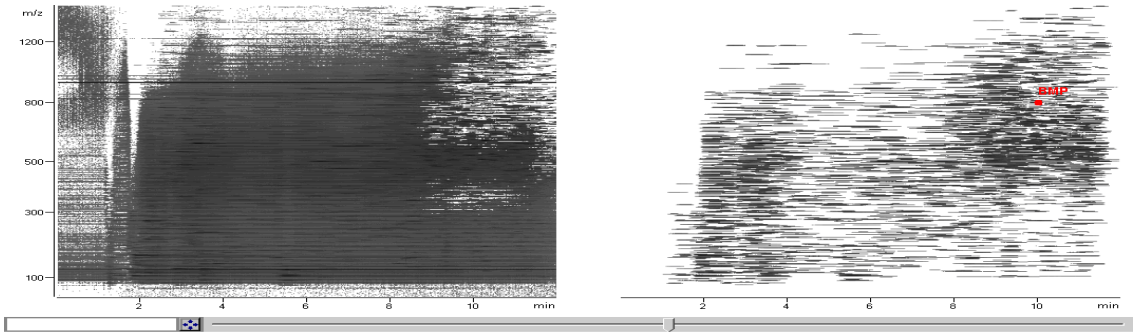
Table 21: Amino acids expressed in gms/100 gms of the stock samples

Amino Acids	Sample S	Sample S %	Sample P	Sample P %	Sample U	Sample U %
Aspartate	0.009	2.47	0.014	2.02	0.013	0.91
Glutamate	0.056	15.38	0.078	11.27	0.047	3.29
Serine	0.012	3.3	0.021	3.03	0.052	3.64
Histidine	0.009	2.47	0.018	2.6	0.05	3.5
Threonine	0.049	13.46	0.098	14.16	0.077	5.38
Glycine	0.004	1.1	0.006	0.87	0.037	2.59
Arginine	0.012	3.3	0.024	3.47	0.196	13.7
Alanine	0.1	27.47	0.162	23.41	0.127	8.88
Tyrosine	0.013	3.58	0.016	2.31	0.053	3.71
Valine	0.014	3.84	0.02	2.89	0.093	6.5
Methionine	0.01	2.75	0.014	2.02	0.054	3.78
Isoleucine	0.008	2.2	0.013	1.88	0.152	10.63
Phenylalanine	0.01	2.75	0.017	2.46	0.082	5.73
Leucine	0.02	5.5	0.026	3.76	0.248	17.34
Lysine	0.017	4.67	0.144	20.81	0.135	9.44
Proline	0.021	5.7	0.021	3.03	0.014	0.98
Total	**0.364**	**100**	**0.692**	**100**	**1.43**	**100**

(a) Undigested stock



(b) Papain digest



(c) Umamizyzme digest

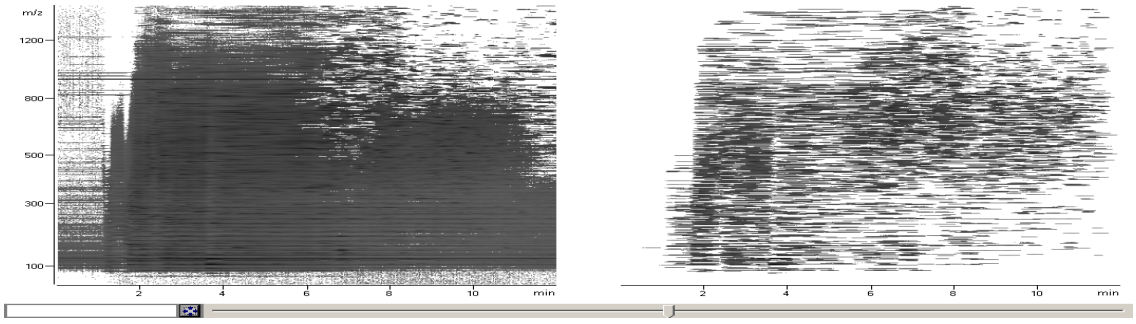
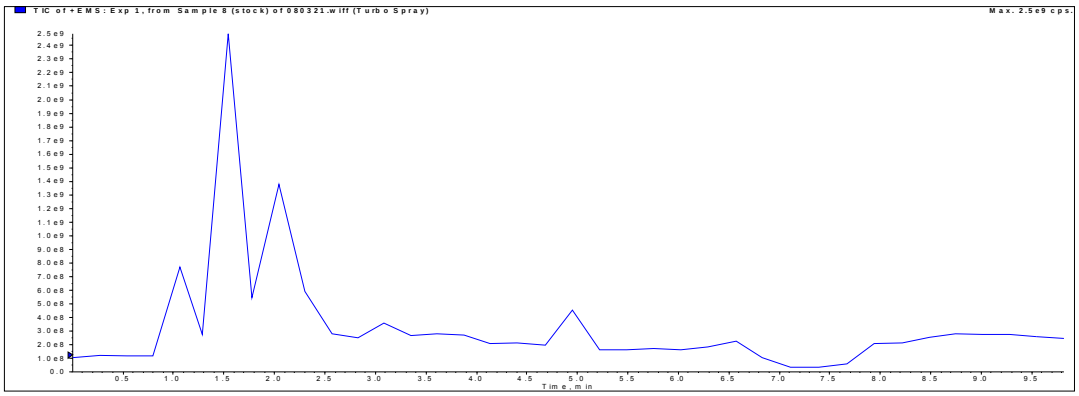
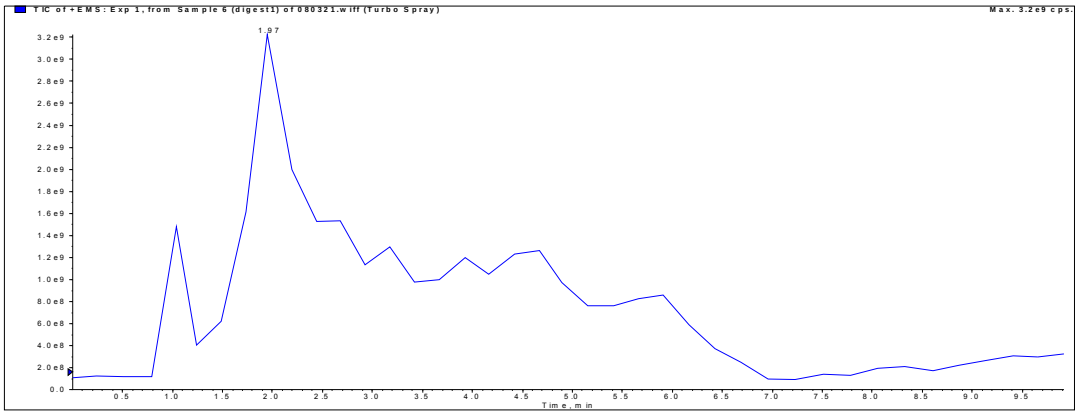


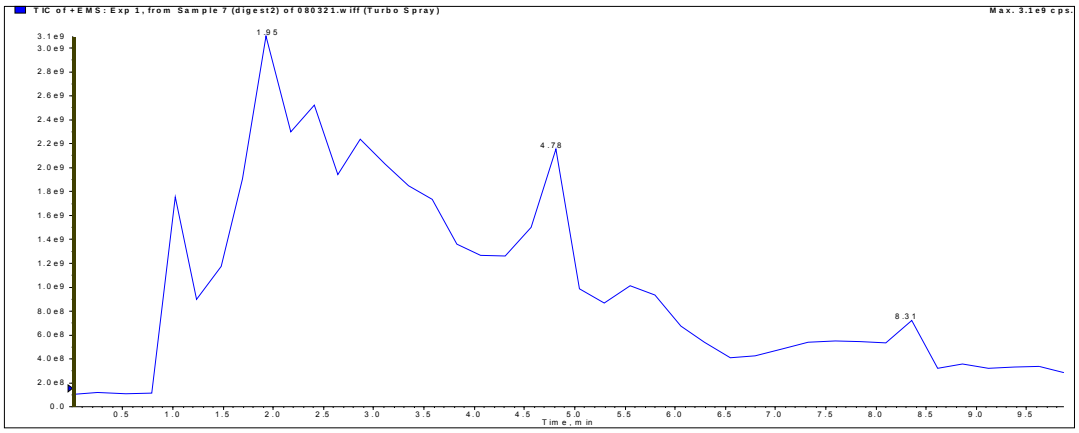
Figure 27: Comparison of distribution of non-volatiles in 3-D images of LC-MS TOF chromatogram of (a) Samples S (b) Sample P and (c) Sample U



(a) Sample S



(b) Sample P



(c) Sample U

Figure 28: LC-MS-ESI TIC chromatogram of Sample S, P and U

6.3.3 Peptide analysis

It is important to digest the protein samples as sequence determination by mass spectrometry is based on the mass of the amino acids. However, leu-ile and lys-gln have the same nominal masses. Several algorithms have been developed to interpret tandem mass spectra of peptides. Two different approaches are followed. The first approach is the *de novo* special interpretation that involves automatically interpreting the spectra using the table of amino acid masses. **Table 22** illustrates mass fragmentation of peptides identified in sample P and sample U.

6.3.3.1 Sample S

No di and tri peptides were identified.

6.3.3.2 Sample P:

In the **figure 33**, the fragment ions of m/z 86 and 72 indicate the presence of either leucine or isoleucine as they share the same molecular mass. The weak fragment of m/z 132 indicates the loss of 57Da, which is the molecular mass of glycine. The m/z at 132 is the y ion of ile/leu. The molecular mass of 188Da matches the mass of the peptide consists of gly and ile/leu. The b ion for glycine is missing. The peptide is identified as gly-ile/leu.

In the **figure 34**, the fragment at m/z 191 shows the loss of 18Da. The fragment at m/z 120 indicates the loss of 99Da, which is the molecular mass of valine and also the y ion for threonine. Although fragment of m/z 120 strongly indicates the presence of phenylalanine, it was not substantiated. The molecular mass of 219Da indicates the presence of valine and threonine. The b ion for valine is missing. The peptide is identified as val-thr.

In the **figure 35**, the fragments at m/z 110, 166, 138, 123 and the loss of 45Da at 182 strongly indicates the presence of a histidine molecule. The fragment at m/z 156 shows a loss of 71Da, which is the molecular mass of alanine. The y ion for histidine is m/z 156 indicates the molecule at the carboxy end of the peptide. The b ion for alanine is missing. The immonium ions at m/z 156 and 110 indicate the presence of histidine. The molecular mass of 226Da matches the molecular weight of peptide consist of alanine and histidine. The ions y(156) and z(139) suggest the peptide as ala-his.

In the **figure 36**, the fragment ion at m/z 211 shows the loss of 18Da. The fragment at m/z 116 indicates the loss of 113Da which is the molecular mass of ile/leu. The y ion of proline is also at the m/z 116. The fragment ion at m/z 86 indicate the presence of leu/ile. The molecular mass of 228Da indicates the peptide consists of ile/leu and proline. The ion 114 is the b ion for ile/leu. The ion m/z 86 is the a ion. The peptide is tentatively identified as leu-ile/pro.

In the **figure 37**, there is a loss of 17Da at m/z 243 indicates ammonia loss. Ammonia loss occurs due to the presence of amide (asparagines and glutamine) or amine containing amino acids (lysine or arginine) but may also occur from the N-terminus. The ion at m/z 147 indicates the presence of lysine. The loss of 113Da at fragment of m/z 147 matches the mass of ile/leu. The fragment at m/z indicates the presence of ile/leu. The molecular mass of 259 matches the dipeptide consists of leucine/isoleucine and lysine. The m/z 147 is also the y ion for lysine. The fragment ions at m/z b(114), a(86), y(147), z(130) tentatively identify the peptide is leu/ile-lys.

In the **figure 38**, the presence of ions m/z 120, 166, 157 indicates the presence of phenylalanine and the presence of m/z 175, 129, 73, 70 indicates the presence of arginine. At m/z 276 the loss of 46Da further confirms the presence of phenylalanine. Loss of 18Da indicates loss of water. At fragment 175 the loss of 147Da equals to the mass of phenylalanine. The molecular mass of 321 indicates the peptides to consist of phenylalanine and arginine. The b ion is missing while the ions a(130), z(175), and y (158) tentatively identify the peptide as phe-arg.

In the **figure 39**, the loss of 17Da at m/z 314 indicates the loss of ammonia which may occurs in the amino acids asparagines, glutamine, lysine and arginine. The fragment ion at m/z 175 indicates C-terminal arginine peptide. The ions m/z 70, 100, 129 indicates further the presence of arginine. The molecular mass of 330Da indicates that the peptide may consist of two arginine molecules. The ions b(157), a(129), y(175), and z(158) tentatively identify the peptide as arg-arg.

6.3.3.3 Sample U

In the **figure 40**, the fragment ion at m/z 116 indicate the loss of 87Da which is the mass of serine. The ion m/z 60 indicates the presence of serine. The fragment at m/z 116 is also the y ion for proline. The molecular mass of 202Da indicates the peptide consists of serine and proline. The b ion is missing and the ions y(106) and a (60) tentatively identified the peptide as ser-pro.

In the **figure 41**, the fragmented ion at m/z 72 and 86 indicates the presence of leucine/isoleucine. The fragment ion at m/z132 indicates the loss of 97Da which is the mass of valine. The m/z 113 is also the y ion for ile/leu at the C-terminal end of the peptide. The mass of 230Da matches the dipeptide consists of valine and leucine/isoleucine. The b ion is missing and the ions a(72), y(132) indicates the peptide as val-leu/ile.

In the **figure 42**, there is a loss of 17Da, which indicates the loss of an ammonia molecule. At the fragment ion m/z 116, there is a loss of 128Da which is equal to the mass of glutamine. The m/z 116 is also the y ion for proline at the C-terminal peptide. The ion at m/z 70 indicates the presence of proline. The molecular mass of 243Da matches dipeptide consists of proline and glutamine with b(129), a(101) and y(116) and tentatively identify the peptide as gln-pro.

In the **figure 43**, the fragment ion m/z 132 indicates a loss of 147Da which is the same as the mass of phenylalanine. The ions m/z 120, 91 is also indicative of phenylalanine while 86 for ile/leu. The molecular mass of 278Da matches the dipeptide consists of phenylalanine and isoleucine/leucine. The ions y(132) and a(120) tentatively identified the peptide as phe-ile/leu.

In the **figure 44**, the ions m/z 86 and m/z 136 are indicative of ile/leu and tyr respectively. The fragment ion at m/z 132 shows a loss of 163Da, which is the mass of tyrosine. The molecular mass of 294Da matches the dipeptide consists of leucine and tyrosine. The ions b(164), a(136), y(132) further suggest the peptide as tyr-leu/ile.

In the **figure 45**, the fragmentation ion of m/z 213 indicates the loss of 101Da, which is equal to the mass of threonine. 213Da is the molecular mass of the dipeptide gly-his. At m/z 56 indicates the loss of 57Da, which equals to the mass of glycine. The ions m/z 82, 166, 138 indicates the presence of histidine. The ions are b_1(102), b2(missing), a_1, and a_2 missing, y_1(156), y_2(213), z_1(missing), z_2(196) suggest the peptide as thr-gly-his.

In the **figure 46**, the fragmentation ion m/z 205 indicates the loss of 113Da, which matches the mass of ile/leu. The m/z 205 is the molecular mass of the dipeptide ser-val. The loss of 87Da at the fragment ion m/z 118 indicates the mass of serine. The fragment m/z 86 indicates the presence of ile/leu. The molecular mass of 317Da matches the molecular weight of the tripeptide consists of ile/leu, ser and val. There is

a loss of NH_3 molecule. The ions b_1 is missing, b_2(201), a_1(86), a_2 is missing, y_1(118), y_2(205), z_1(101), z_2(188) suggest the tripeptide as ile/leu-ser-val.

In the **figure 47**, the fragmentation ion of m/z 228 indicates the loss of 113Da, which equals to the mass of ile/leu.The ion at m/z 228 indicate the molecular mass of the dipeptide pro-leu. At m/z 115, the loss of 113Da indicates another molecule of ile/leu in the peptide. The mass fragment at m/z 86 suggests the presence of ile/leu. The fragment at m/z 70 indicates the presence of a proline in the peptide. The molecular mass of 340Da indicates the peptide consists of leucine/isoleucine, leucine/isoleucine and proline. The ions b_1(missing), b_2(missing), a1(86), a2(199), y_1(missing), z_1(99), y_2(229), z2(212) suggests the peptide as leu-leu-pro.

In the **figure 48**, the loss of 131Da at m/z 215 which equals the mass of methionine. Thereafter, the loss of 99Da at m/z 116 equals the mass of valine. The molecular mass of 345Da matches the tripeptide consists of methionine, valine and proline. The ions b_1(missing), b_2(missing), a_1(104), a_2(203), y_1(116), z_1(99), y_2(215), z2(198) suggests the peptide as met-val-pro.

In the **figure 49**, the fragment ions at m/z 159 and m/z 130 indicate the presence of tryptophan while m/z 120 shows the immonium ions of phenylalanine. The loss of 147Da at m/z 205 matches the mass of phenylalanine. The ion m/z 351 matches the mass of the dipeptide consists of phenylalanine and tryptophan. The ions b(187), a(159), y(166), z(188) suggests the peptide as phe-trp.

In the **figure 50**, there is loss of 18Da at 350Da indicates the loss of a water molecule. The fragment ion at m/z 136 indicates the presence of tyrosine. The loss of 18Da at m/z 350 indicates the loss of a water molecule. The loss of 129Da at m/z 239 equals the mass of glutamic acid and thereafter the loss of 57Da at 182 equals the mass of glycine. The molecular mass of 367Da matches the tripeptides with glu, gly and tyr and the b_1(129), a_1(102), b_2(187), a_2(159), y_1(182), z_1(165), y_2(239) suggests the peptide as glu-gly-tyr.

In the **figure 51**, the fragment ion m/z 70 strongly indicates the presence of praline. The loss of 97Da at m/z 297 equals to the mass of proline. Thereafter, the loss of 147Da at m/z150 equals to the mass of phenylalanine. The presence of an immonium ion of m/z 120 is a further indication of the presence of phenylalanine. The molecular mass of 393Da matches the tripeptide consists of proline, phenylalanine and methionine. The ions b_1(98), b_2(245), y_1(150), y_2(297), z_1(133), z_2(280) suggests the peptide as pro-phe-met.

In the **figure 52**, the loss of 18Da at m/z 376 indicates the loss of a water molecule. The loss of 113Da at m/z 281 and thereafter the loss of 115Da at m/z 166 indicate that the first two amino acids are isoleucine/leucine and aspartic acid. The fragment ions 86 and 120 represent the immonium ions of ile/leu and phe respectively. The molecular mass 393Da indicates the peptide consists of isoleucine/leucine, aspartic acid and phenylalanine and the ions b_1(114), b_2(229), a1(86), a2(200), y_1(166), y_2(281), b_2-CO_2(183) suggests the peptide as ile/leu-asp-phe.

6.3.4 Interpretation of spectra

Peptide mass finger printing is a very powerful and popular technique for peptide-protein identification. Once mass spectra are measured with high mass accuracy, the next step is to pick relevant monoisotopic mass peaks from spectra which can be used for data base search. However, the interpretation of the fragment spectra is not straight forward. Manual interpretation requires considerable experience and is time consuming.

All but two of the 20 amino acids from which most naturally occurring proteins are comprised have different masses. Therfore, it is possible to establish the sequence of amino acids from the difference in mass of peaks which correspond to the successive loss of an amino acid residue from the original peptide. The cleavage of amide bonds results b-ions (containing the N-terminal) and y-ions (containing the C-terminal). The interpretation of spectra is based upon b and y ions, neutral losses, internal fragment ions, immonium ions (low mass fragment ions from single amino acids) and the precursor ions (Ma, et al., 2005; Courchesne et al., 2002). The most common neutral losses in peptide fragmentation are water (18Da) and ammonia (17Da). The water loss typically occurs from the hydroxyl group containing amino acids such as serine and threonine or carboxylic acid containing amino acids such as aspartic acid and glutamic acid but may be lost from the C-terminal of y-ions. Ammonia losses are generally due to the presence of the amide group containing amino acids such as asparagines and glutamine or amine containing amino acids such as lysine and arginine but may also

occur from the N-terminus (Kinter and Sherman, 2000). **Table 22** shows mass fragment of the peptides. Other neutral losses are observed from ions with different amino acid composition such as methionine with a loss of 48Da. A loss of CO (28 Da) occurs at the 'a' ion. The ion is generally not of high relative abundance and in some cases may not be present. The dipeptide, tripeptide, tetrapeptide containing phenylalanine or histidine residues observes the loss of a neutral 45Da with an exception of gly-phe or leu-phe which did not give the 45Da losses. Glycine has no side chain to provide the addition hydrogen for the fragmentation and the stearic hindrance of the isopropyl group in leucine might make the rearrangement impossible (Chen et al., 2004).

The amino acid pairs may range from the smallest combination of peptide gly-gly (115Da) to the largest combination at trp-trp (373Da). Immonim ions is informative about the amino acid content. However, they do not provide any information about the amino acid sequence in the peptide. Lack of immonium ion is not conclusive. The presence of m/z 110 indicates high possibility of histidine somewhere in the peptide chain but the absence of m/z 110 does not preclude the presence of histidine in a peptide. If a b_2 ion is seen at m/z 145Da, then only combination of amino acid is ser-gly or gly-ser. The product ion spectrum would then be inspected to find a y-ion corresponding to the loss of 87Da, to assign a ser or the loss of 57Da to assign a gly. Other b_2 ions produce more amino acid combination. For example, a b_2-ion at m/z 228 could be leu-asp, ile-asp, val-gln, val-lys, or arg-ala, which limits the possible sequences to ten. The residues masses of the amino acids vary considerably from 57Da

for glycine to 186Da for tryptophan. There is some overlap among residue masses of several amino acids where amino acids combination equal to a single residue. Asparagine (Asn) mass (114Da) equals the mass of gly-gly; gln or lys mass (128Da) matches the mass of gly-ala; arg mass (156Da) matches gly-val and trp mass (186Da) matches the mass of gly-glu, ala-asp, or ser-val. It was observed that the y-ion at m/z 147 indicates C-terminal lysine peptides while m/z 175 indicates C-terminal arginine peptides(Kinter and Sherman, 2000).

The difficulties and ambiguities arise in assigning the peaks, particularly when certain peaks are either missing or unrecognized. Moreover, other peaks are typically present in a spectrum due to various more complicated fragmentation or rearrangement routes. Further electrospray ionization tends to produce multiply charged ions which further complicate the interpretation of the spectra (Skilling, 2006). This may be due to the MS analysis such as poor solubility, selective absorption, ion suppression, selective ionization, very short peptide length, proteolytic cleavage or other artifacts. Some of the minor peaks were highlighted when the sequence was studied. Many times minor immonium ions are absent because of the low mass cut-off of the ion trap mass analyzer (Kinter and Sherman, 2000).

7. CONCLUSIONS

Bone marrow stocks were prepared using Microfiltration (MF) system from beef marrow bones. Proteolytic enzymes were used to break down of protein molecules. The break down of these linkages generates smaller peptide fractions of amino acids units as well as individual amino acids monomers.

All three stock are not rich in volatile compounds, however they are densely populated with higher molecular weight non-volatiles such as peptides. Our initial studies of the volatile analysis showed only hand full of lipid oxidation products in all three stock sample, undigested (sample S), papain digested (sample P), and umamizyme digested (sample U). The same stock samples when heated to 120ºC and 160ºC generated numerous volatiles which include Strecker aldehydes, pyrazines, furans, methyl sulfides.

When these stock samples were reacted with ribose, xylose and methylglyoxal at 160ºC, the stock generates more volatiles such as Strecker aldehydes, methylsulfides, pyrazines, alcohols, and ketones. When reacted with ribose, xylose and methylglyoxal, yielded more volatiles including some novel alkenals that provides characteristic aroma and overall flavor. Ribose produces mostly furan based compounds. Pyrazines were the major volatile constituents observed in all enzyme digested stocks reacted with methylglyoxal at160ºC.

Our studies also showed that the enzyme digested marrow bones generated more volatiles than the undigested one. Umamizyme digested stock (Stock U) produced more volatiles than the papain digested (Stock P) and undigested stock. (Stock S). Both the lipid oxidation aldehydes and Strecker aldehydes dominated in the marrow bones stock.

Alkylated pyrazines were the major volatile constituents observed in all enzyme digested stocks reacted with methylglyoxal at 160ºC. More Strecker aldehydes, pyrazines, methyl sulfides are produced in umamizyme treated stock. Enzyme treated stock generates more Strecker aldehydes and less lipid oxidation aldehydes than the stock prepared without enzyme.

Novel alkenals including 5-methyl-2-phenyl-2-hexenal, 2-methyl-2-butenal, 2-methyl-2-hexenal, 2-phenyl-2-butenal, 2-isopropyl-5-methyl-2-hexenal, 3(2-furanyl)-2-phenyl-2-propenal were observed in the ribose, xylose, and methylglyoxal reactions of enzyme digested stock at 160ºC. They are results of aldol condensation and occurred at high temperature and pressure. These alkenals are well known for their characteristic aroma and taste.

The degree of hydrolysis (DH) of stocks improved with the enzyme treatment. Stock U had the highest DH (76%) followed by stock P (51%). Stock S had the lowest DH (29%).

Our non-volatile studies included mapping with TOFMS, amino acids analysis and peptide fragmentation using LC-MS quadropole linear trap.

Free amino acids study finds stock 'U' contained higher leucine, isoleucine, phenylalanine, methionine, valine, tyrosine, arginine, glycine, histidine and serine than stock S and stock P. Total free amino acids in stock U is 1.4 gm/100 grams of stock, while stock p contains amino acids 0.69 gm/100 gm stock and the stock S contains the lowest amount of amino acids, 0.69 gm/100 grams of stock.

The stock sample U had 1.43 grams amino acids/100 grams of sample followed by stock P (0.69gms/100 gm) while stock S had the lowest (0.36gms/100gms). Leucine, arginine, lysine, isoleucine, phenylalanine, threonine, methionine, valine, tyrosine, histidine, glycine and serine are major amino acids in stock U. Alanine, threo nine, lysine, glutamic acid, aspartic acid are major amino acids in stock P. These amino acids participated in forming Strecker aldehydes and generating volatile components.

Marrow bone stocks contain numerous non-volatile peptides. The papain digested stock was investigated and the following eight peptides were tentatively identified: gly-leu/ile, val-thr, ala-his, leu/ile-pro, leu/ile-lys, phe-arg, arg-arg, ser-pro.

Twelve peptides were tentatively identified in the umamizyme digest stock (stock U). They are: val-leu/ile, gln-pro, phe-leu/ile, tyr-leu/ile, thr-gly-his, leu/ile-ser-val, leu/ile-leu/ile-pro, met-val-pro, phe-trp, glu-gly-tyr, pro-phe-met, leu/ile-asp-phe.

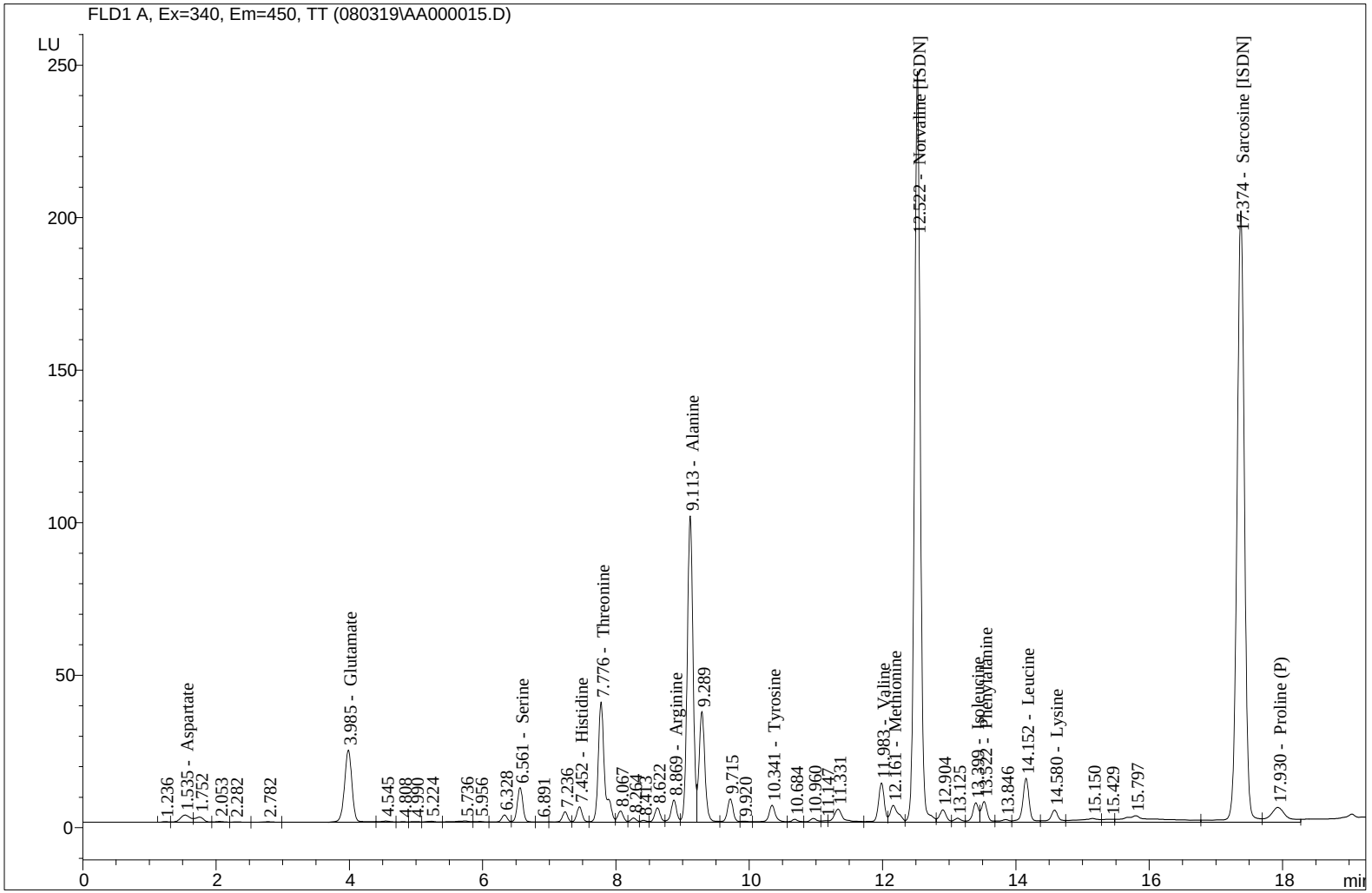


Figure 29: LC-MS chromatogram showing aminoacids in stock sample S

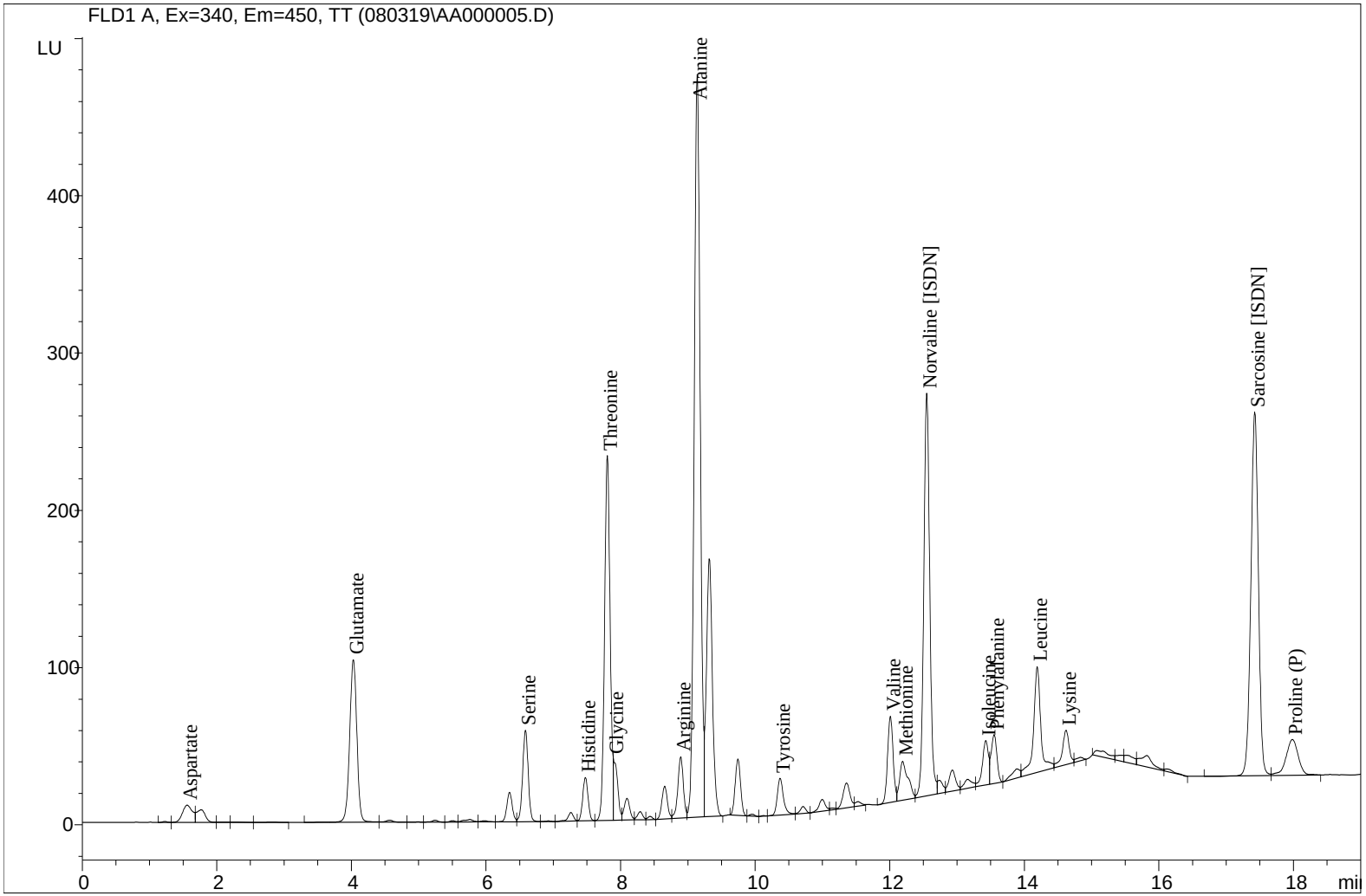


Figure 30: LC-MS chromatogram showing amino acids in stock sample P

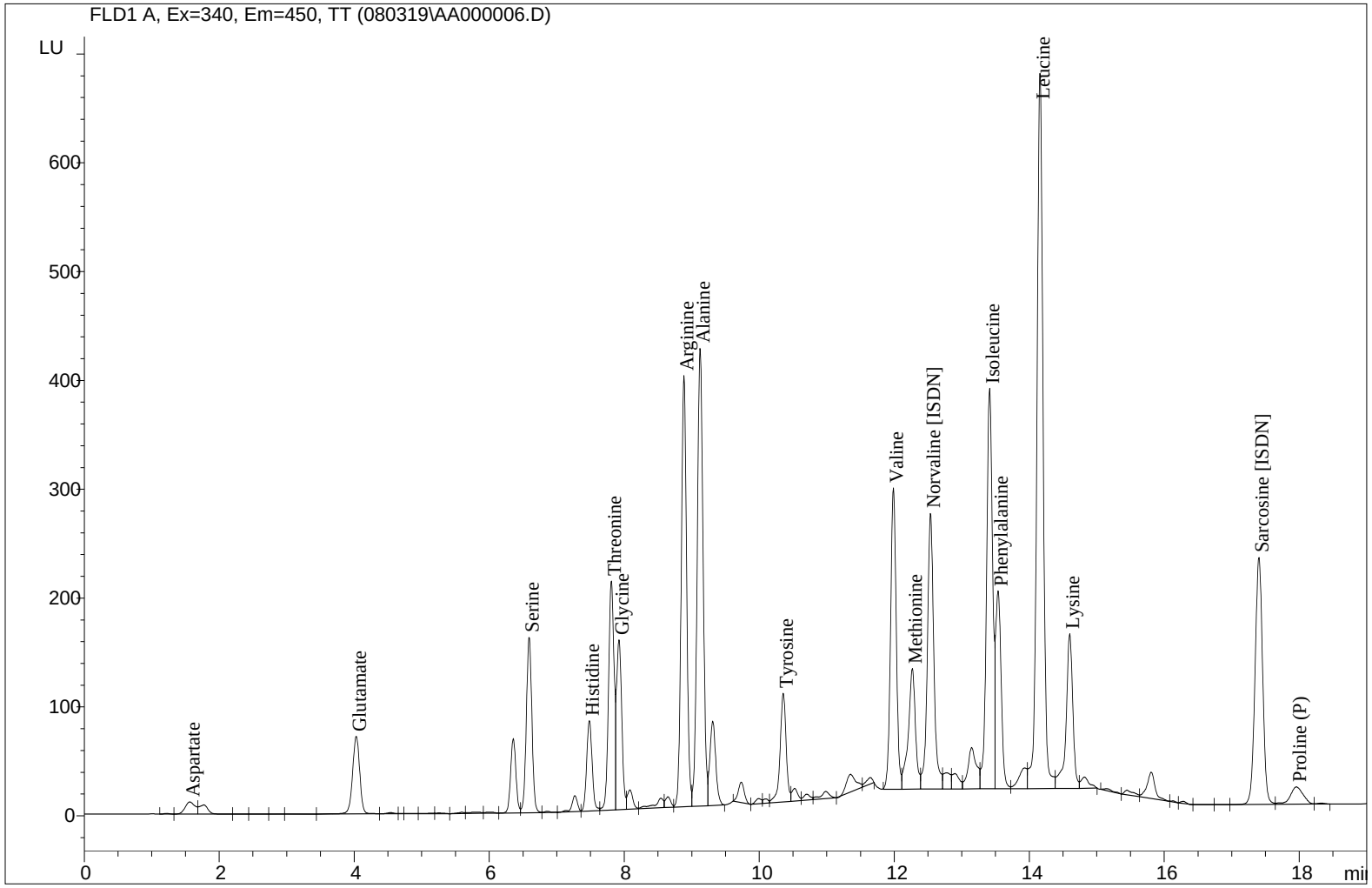


Figure 31: LC-MS chromatogram showing amino acids in stock sample U

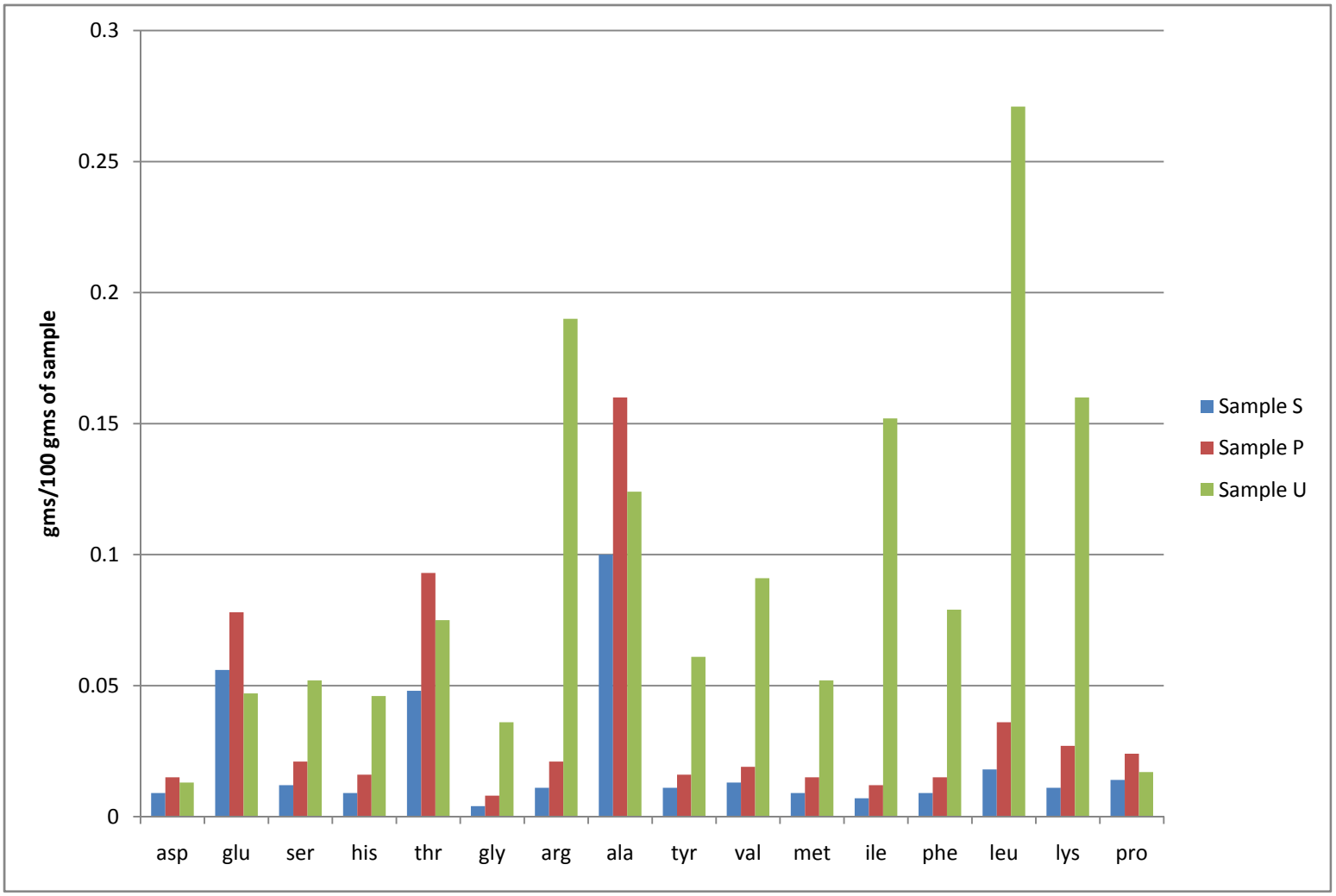


Figure 32: Amino acids comparison of stock sample S (untreated), P (papain treated) and U(umamizyme treated)

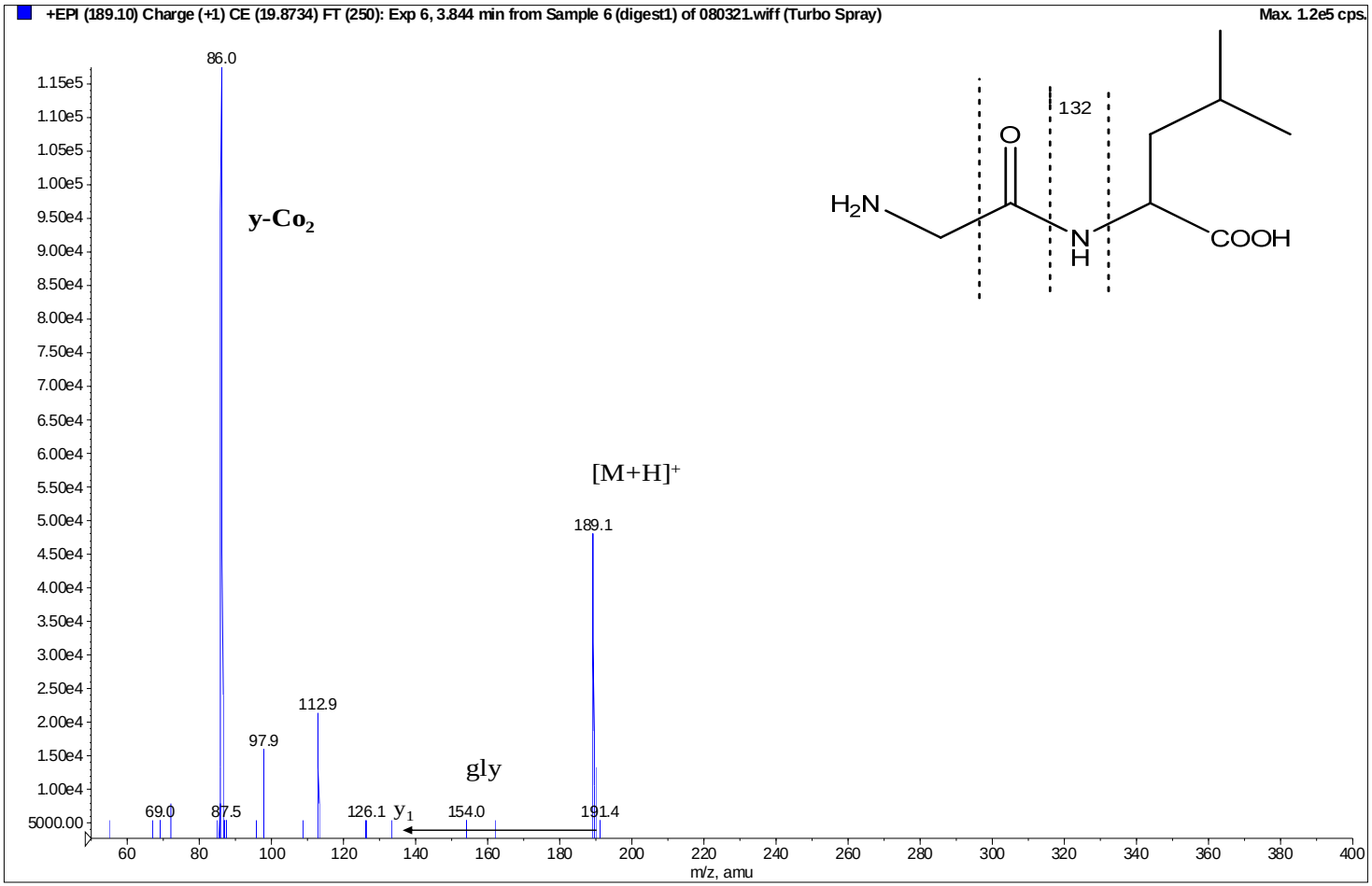


Figure 33: Sample P: LC-MS spectrum of gly-leu

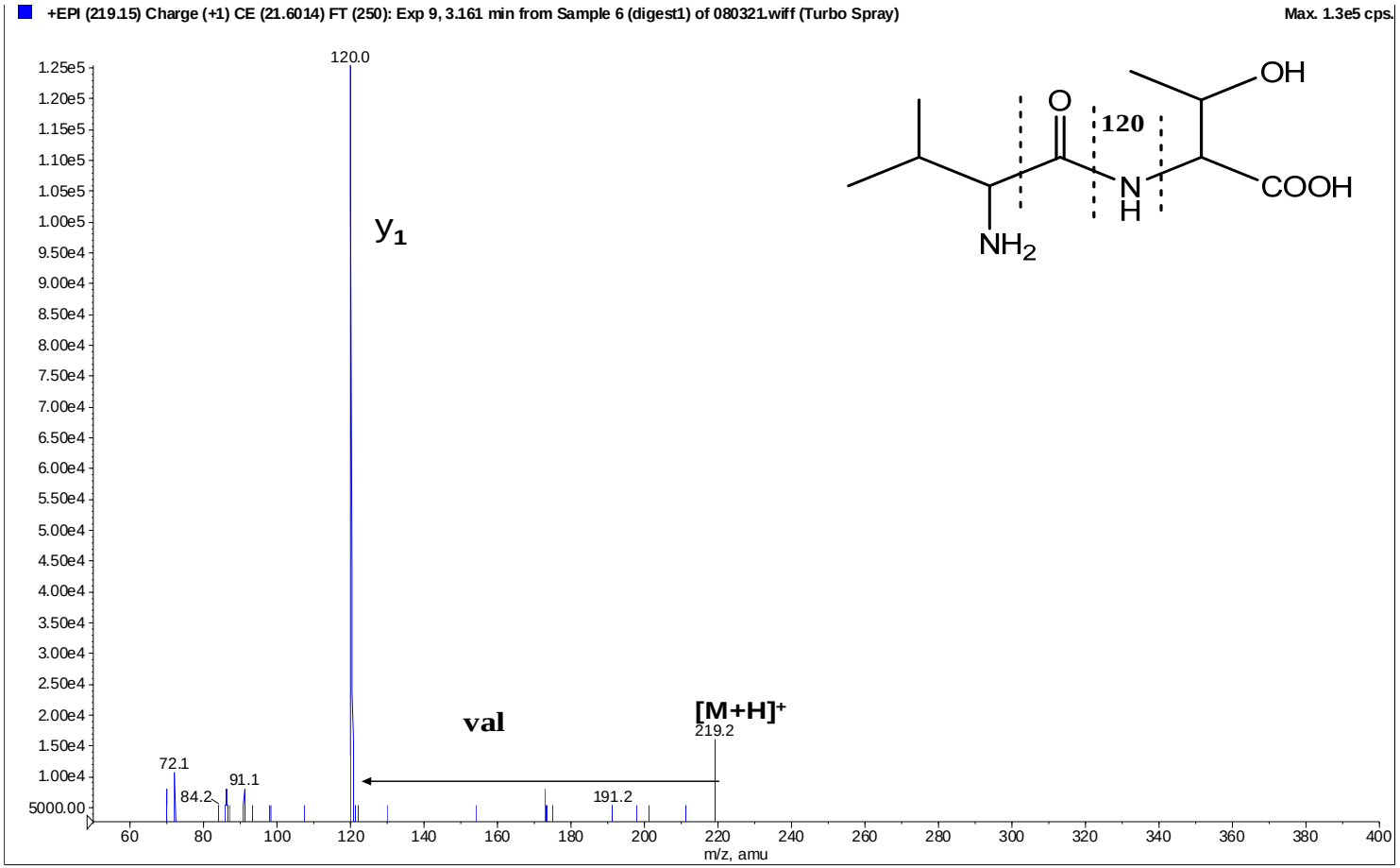


Figure 34: Sample P: LC-MS spectrum of val-thr

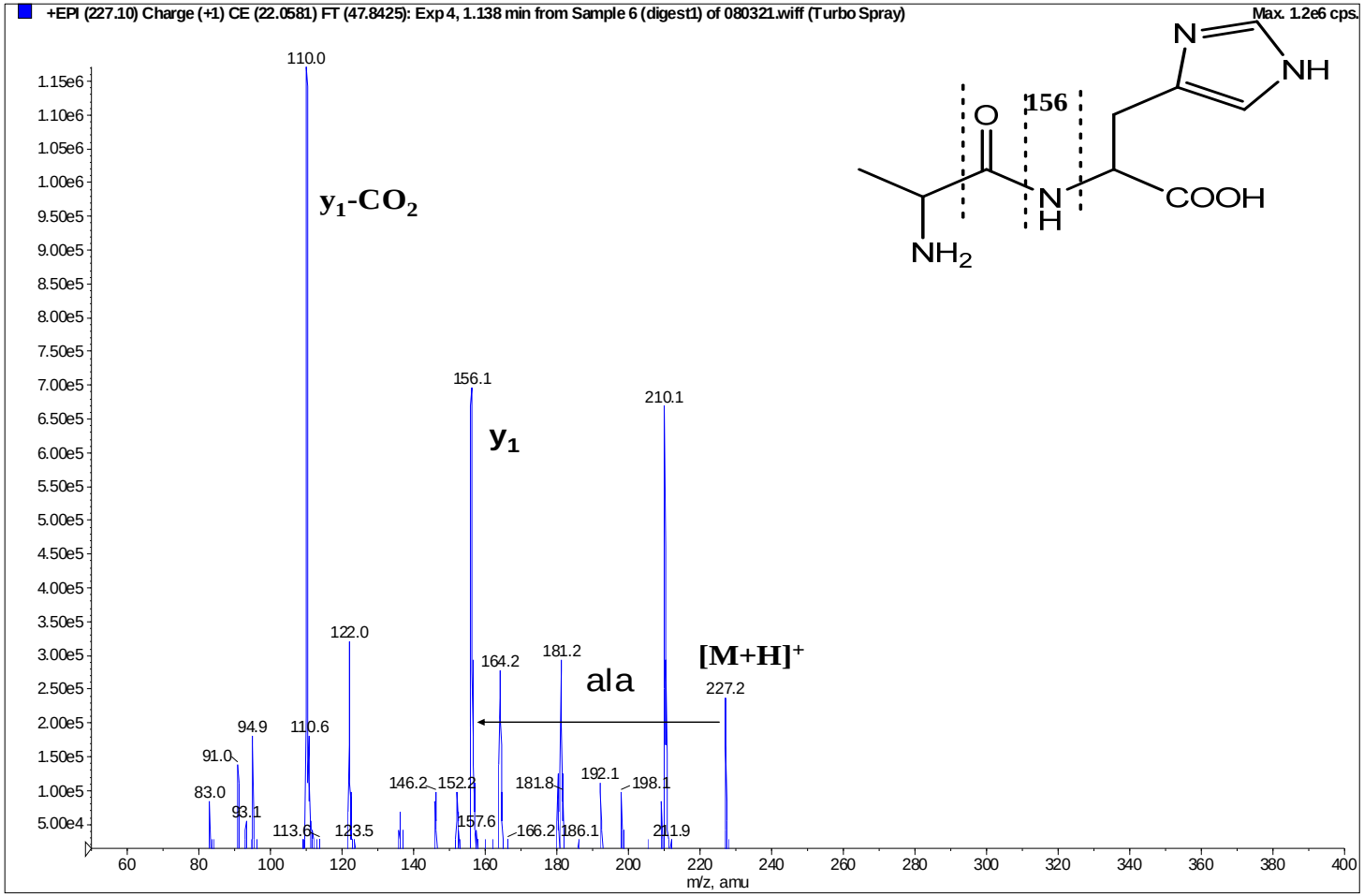


Figure: 35: Sample P: LC-MS spectrum of ala-his

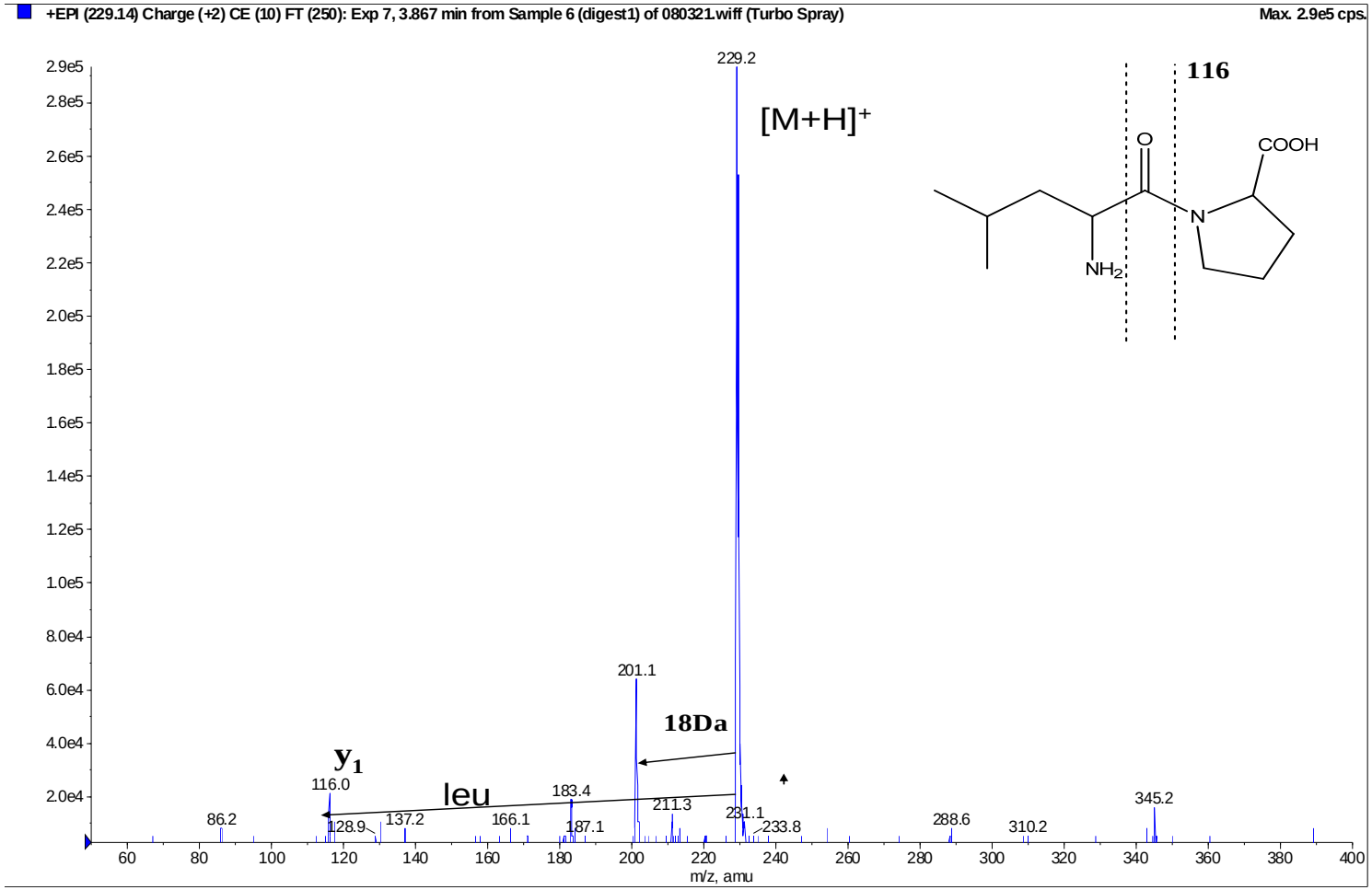


Figure 36: Sample P: LC-MS spectrum of leu-pro

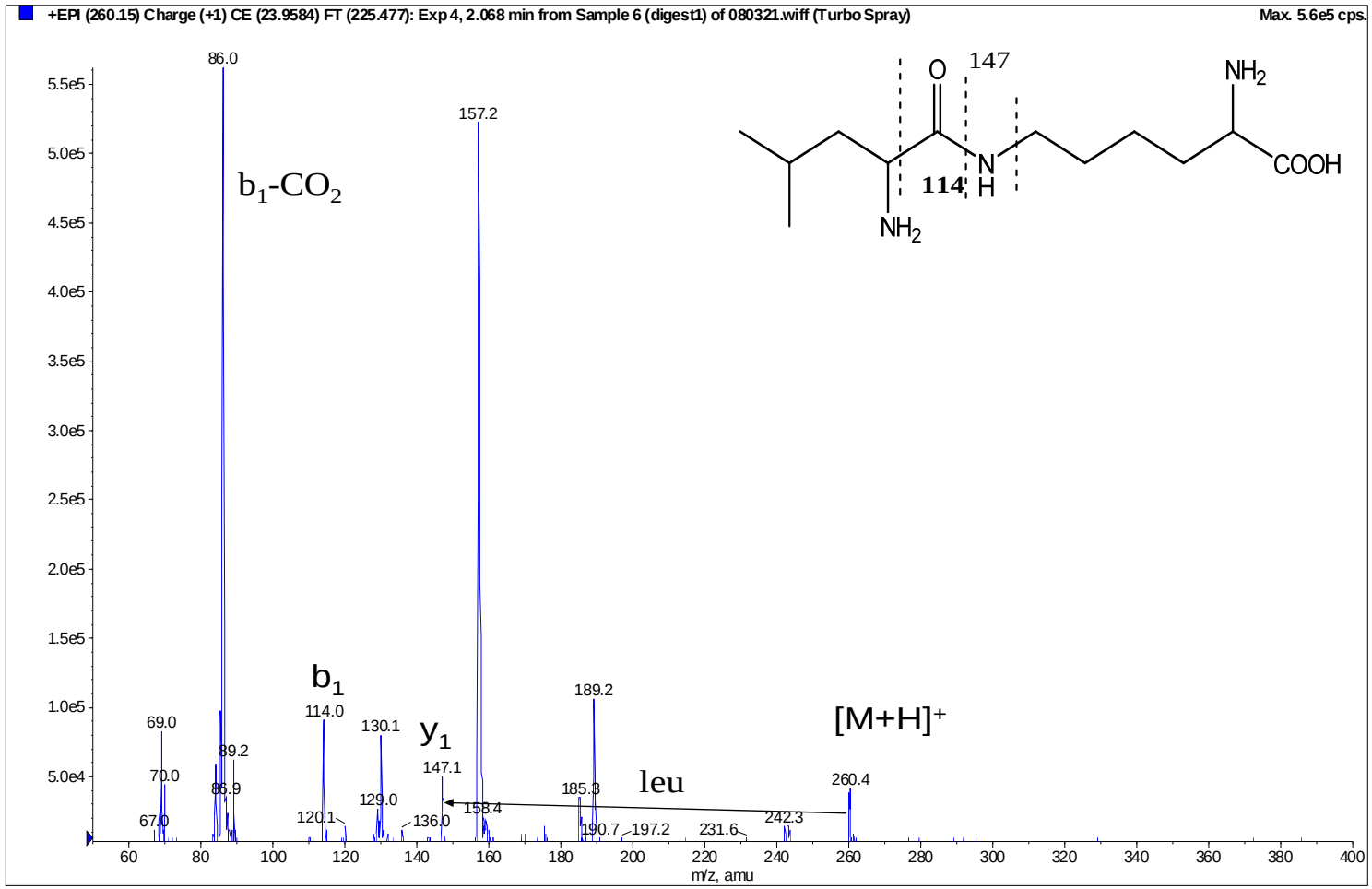


Figure 37: Sample P: LC-MS spectrum of leu-lys

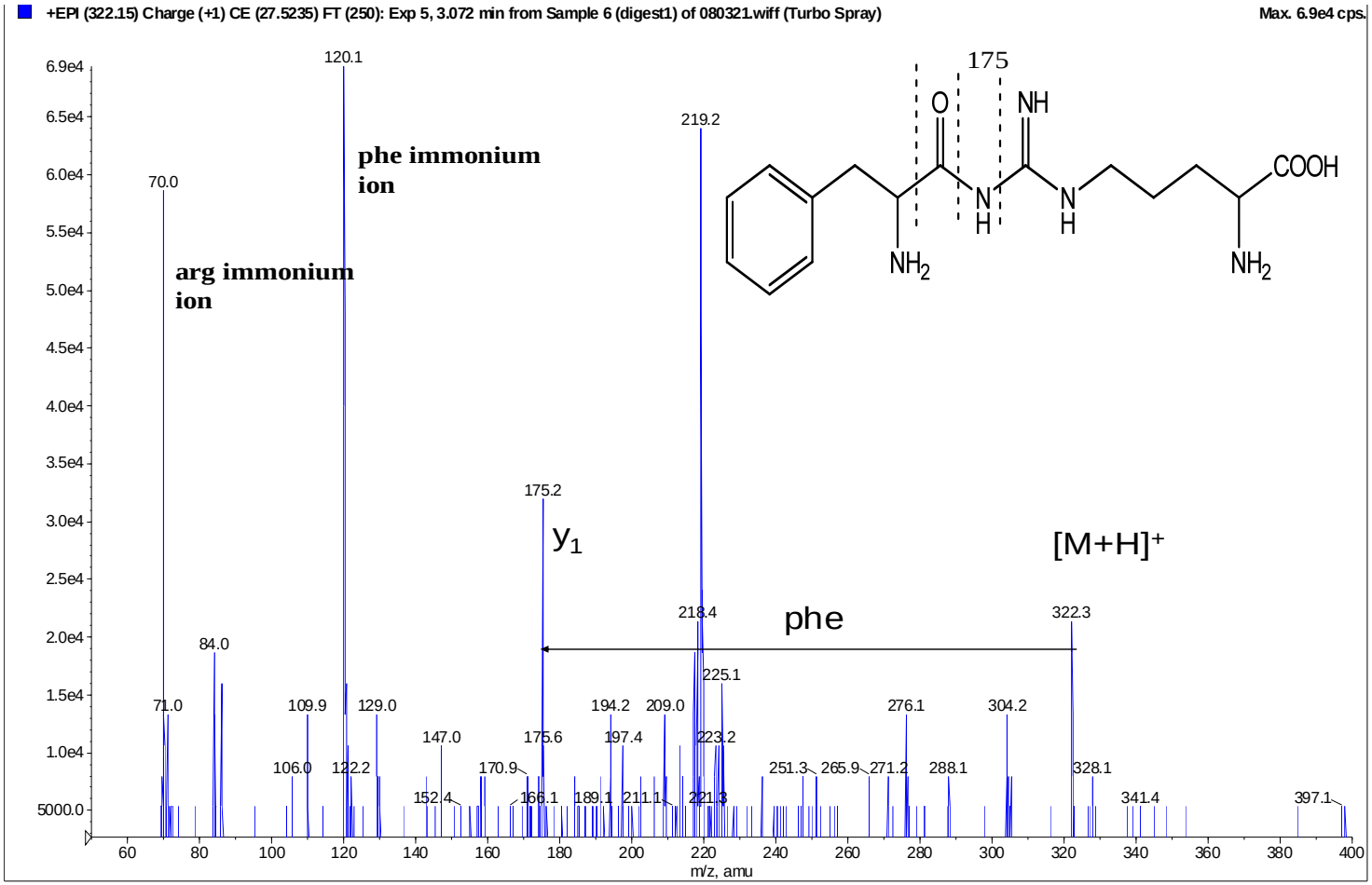


Figure 38: Sample P: LC-MS spectrum of phe-arg

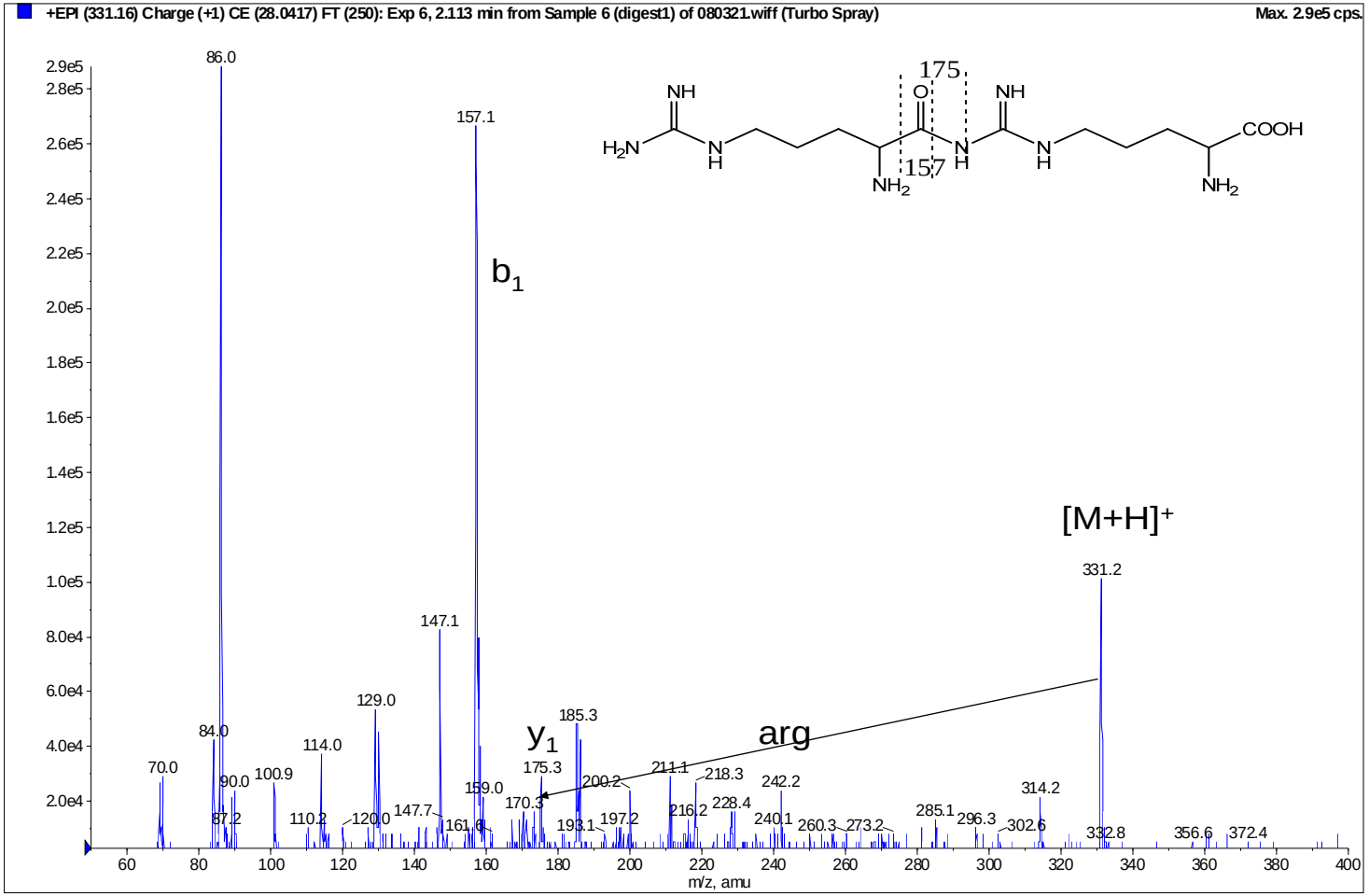


Figure 39: Sample P: LC-MS spectrum of arg-arg

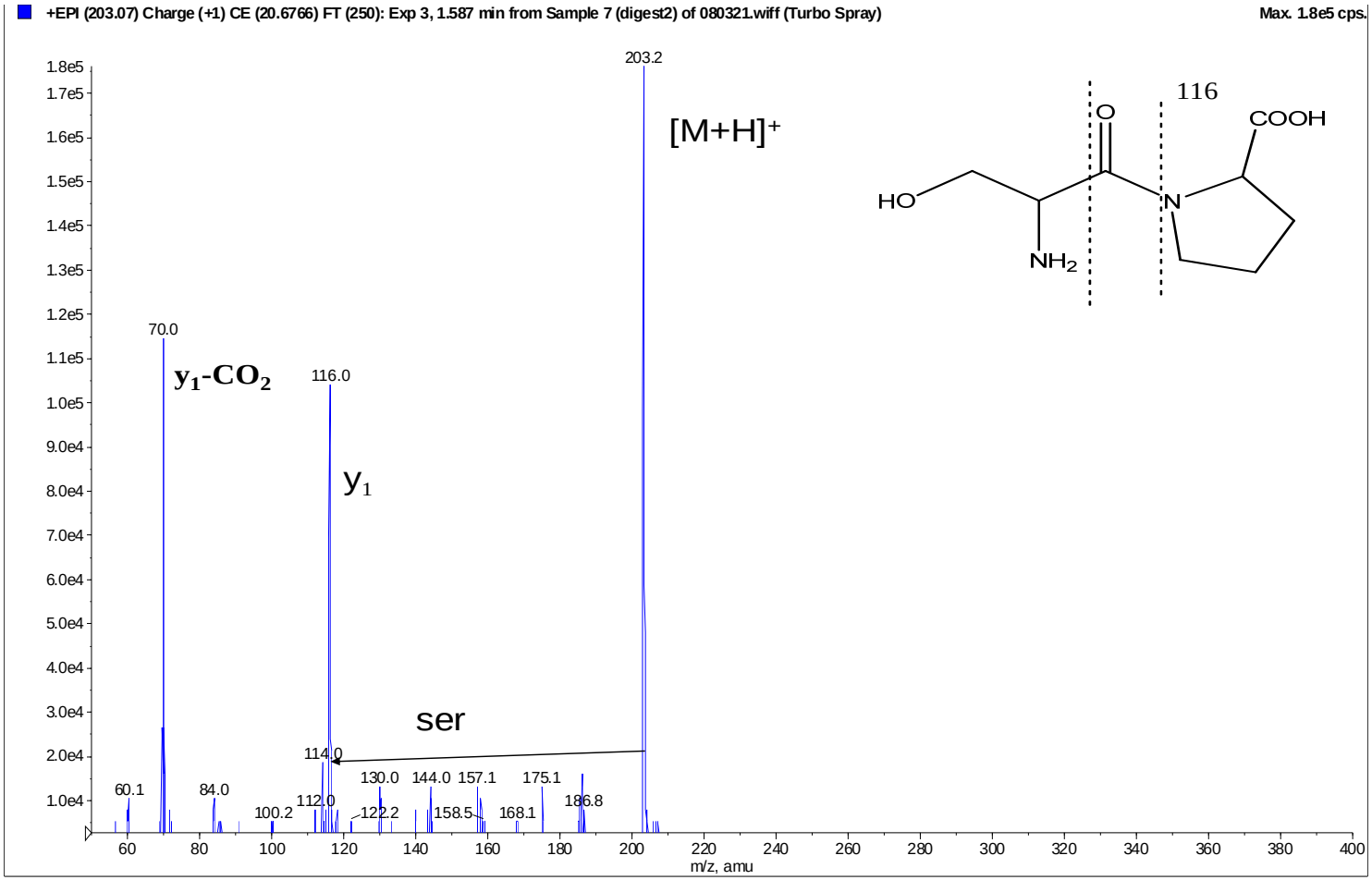


Figure 40: Sample P: LC-MS spectrum of ser-pro

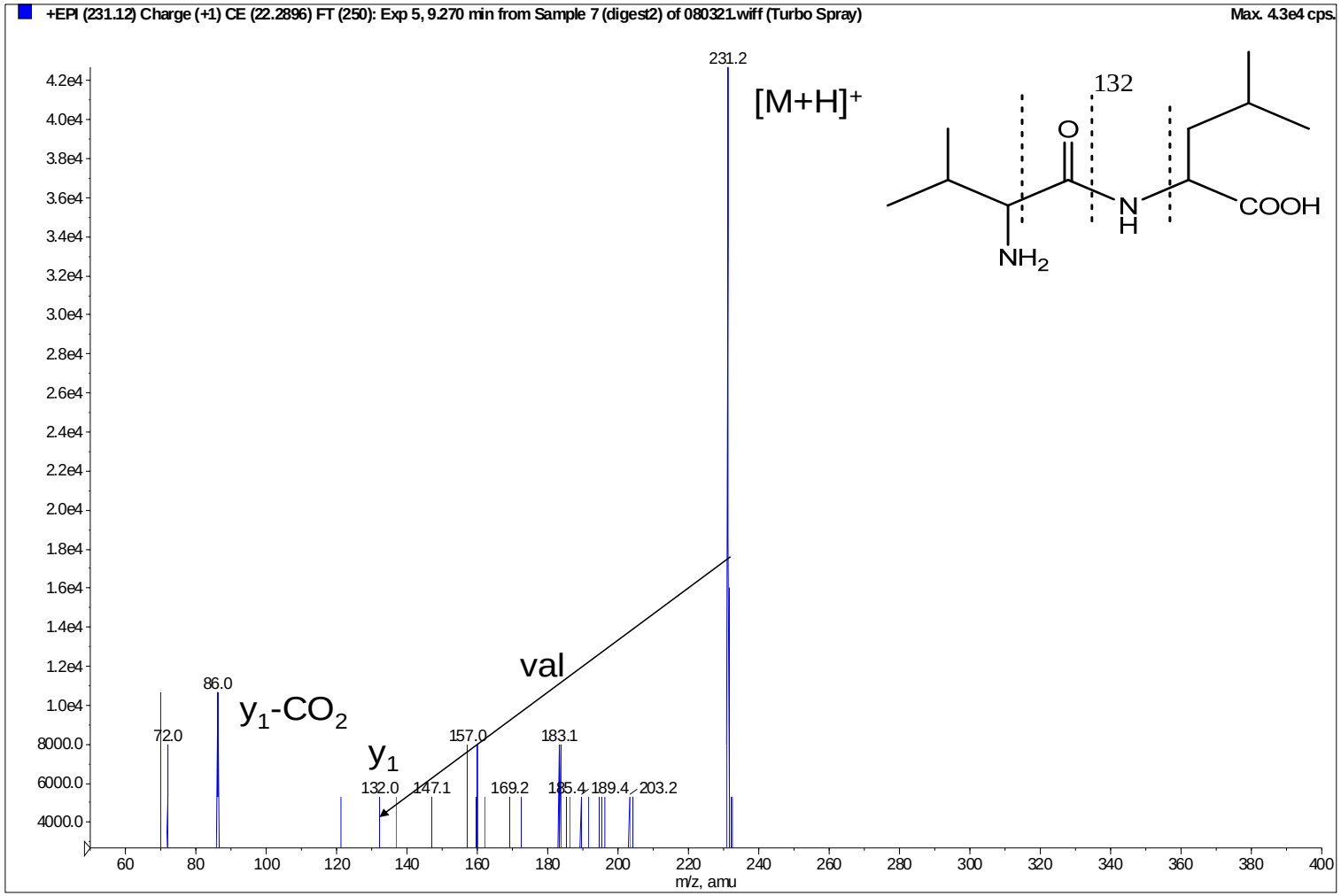


Figure 41: Sample U: LC-MS spectrum of val-leu

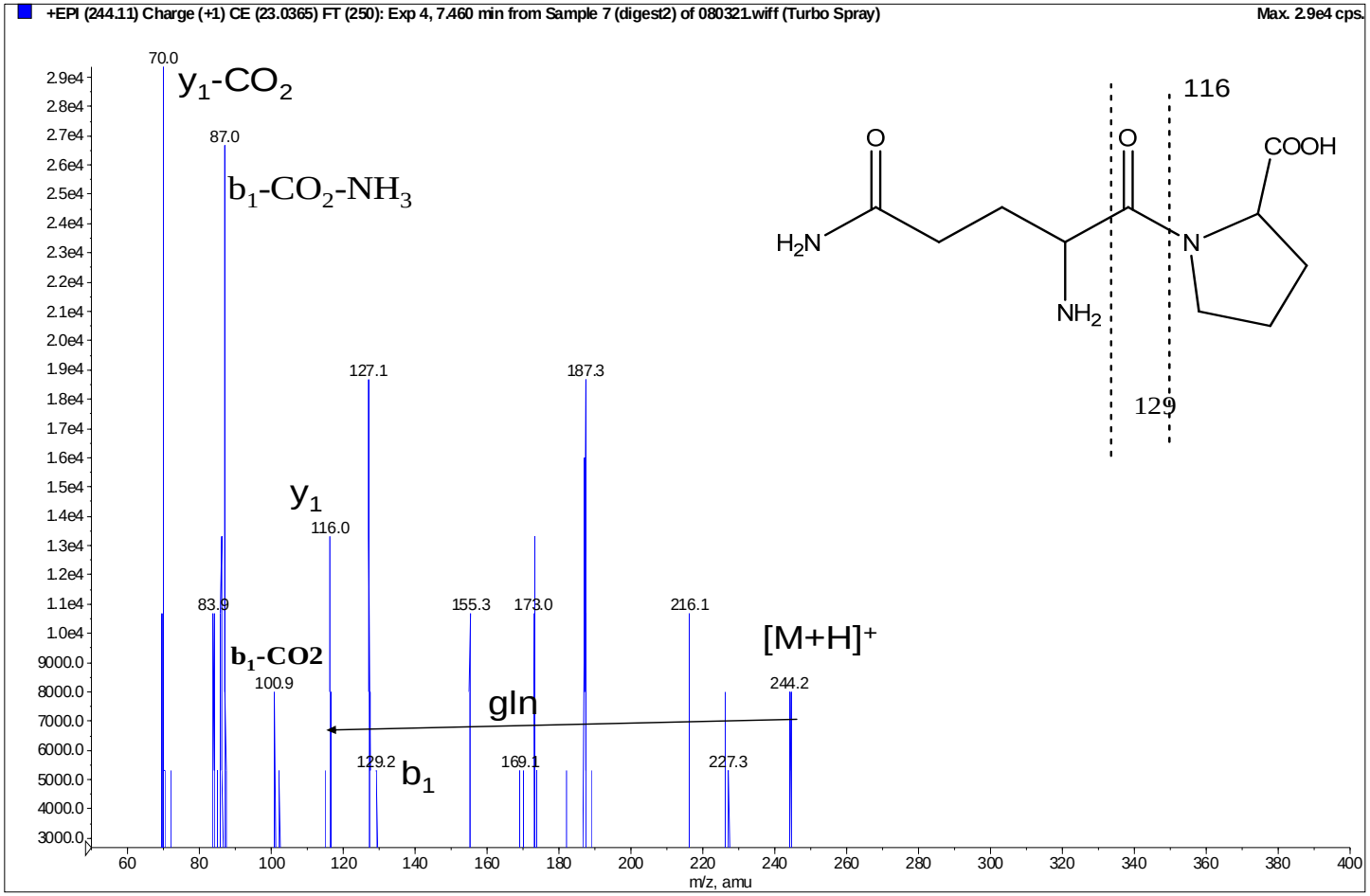


Figure 42: Sample U: LC-MS spectrum of gln-pro

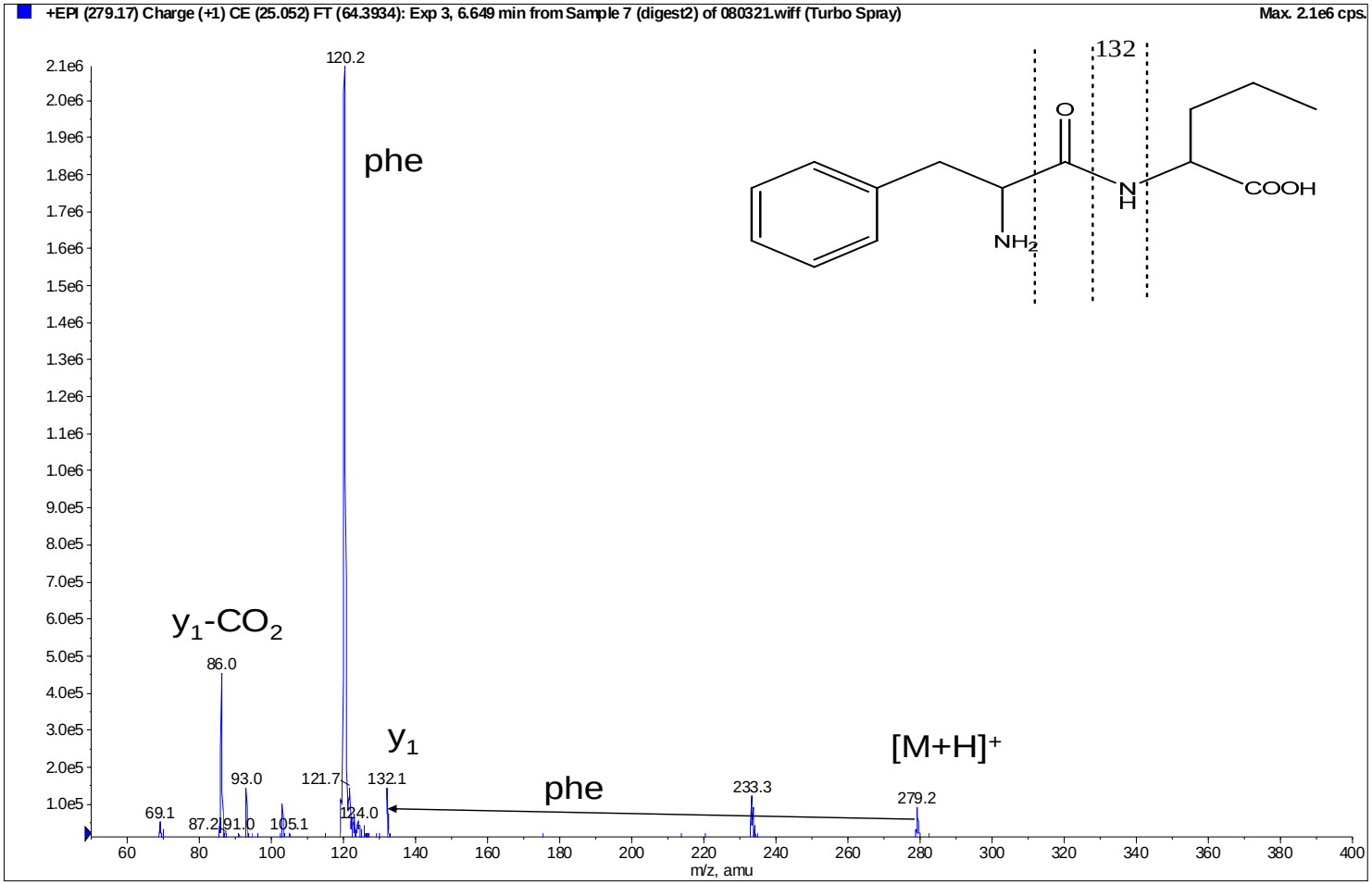


Figure 43: Sample P: LC-MS spectrum of phe-leu

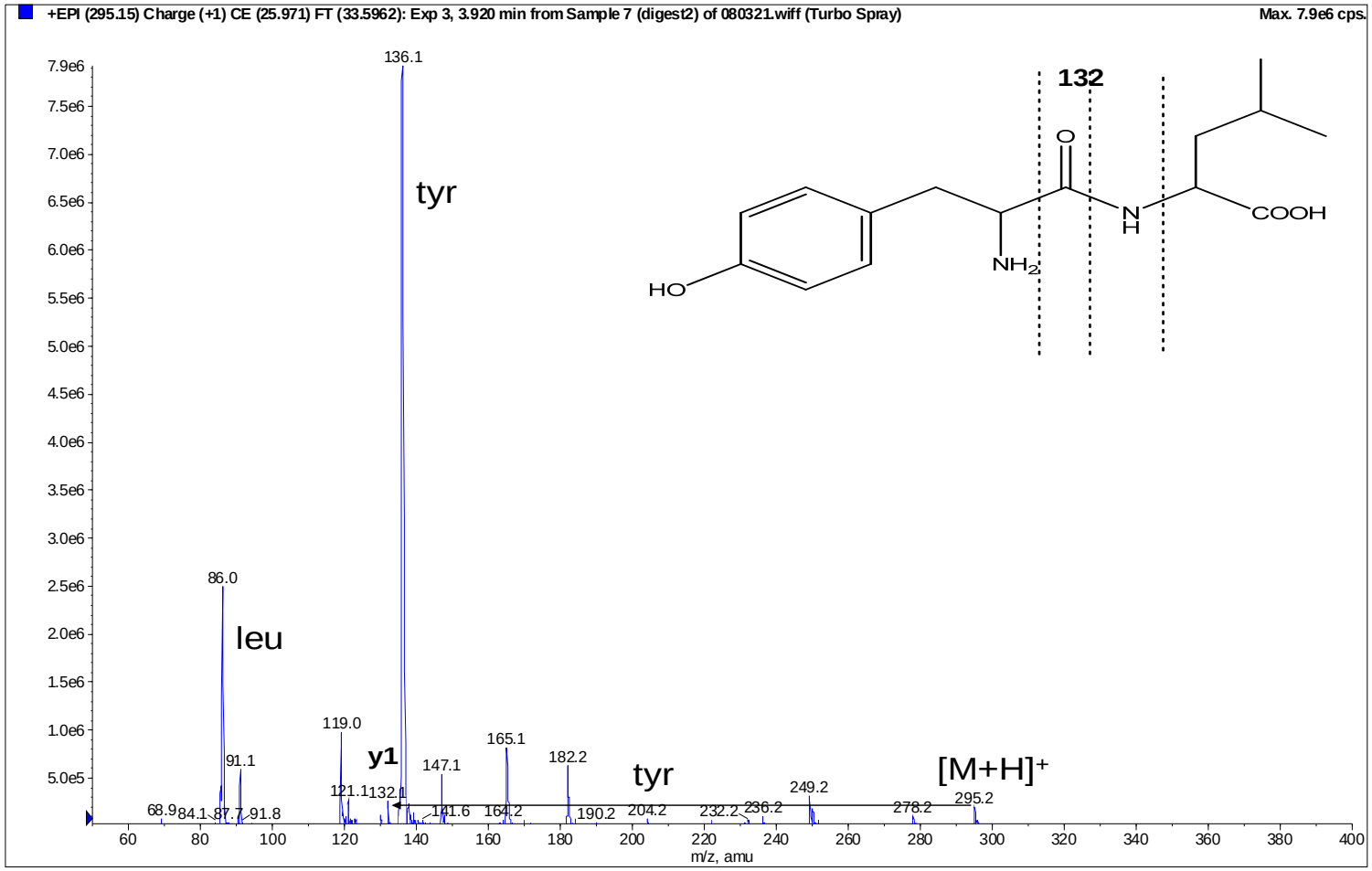


Figure 44: Sample P: LC-MS spectrum of tyr-leu

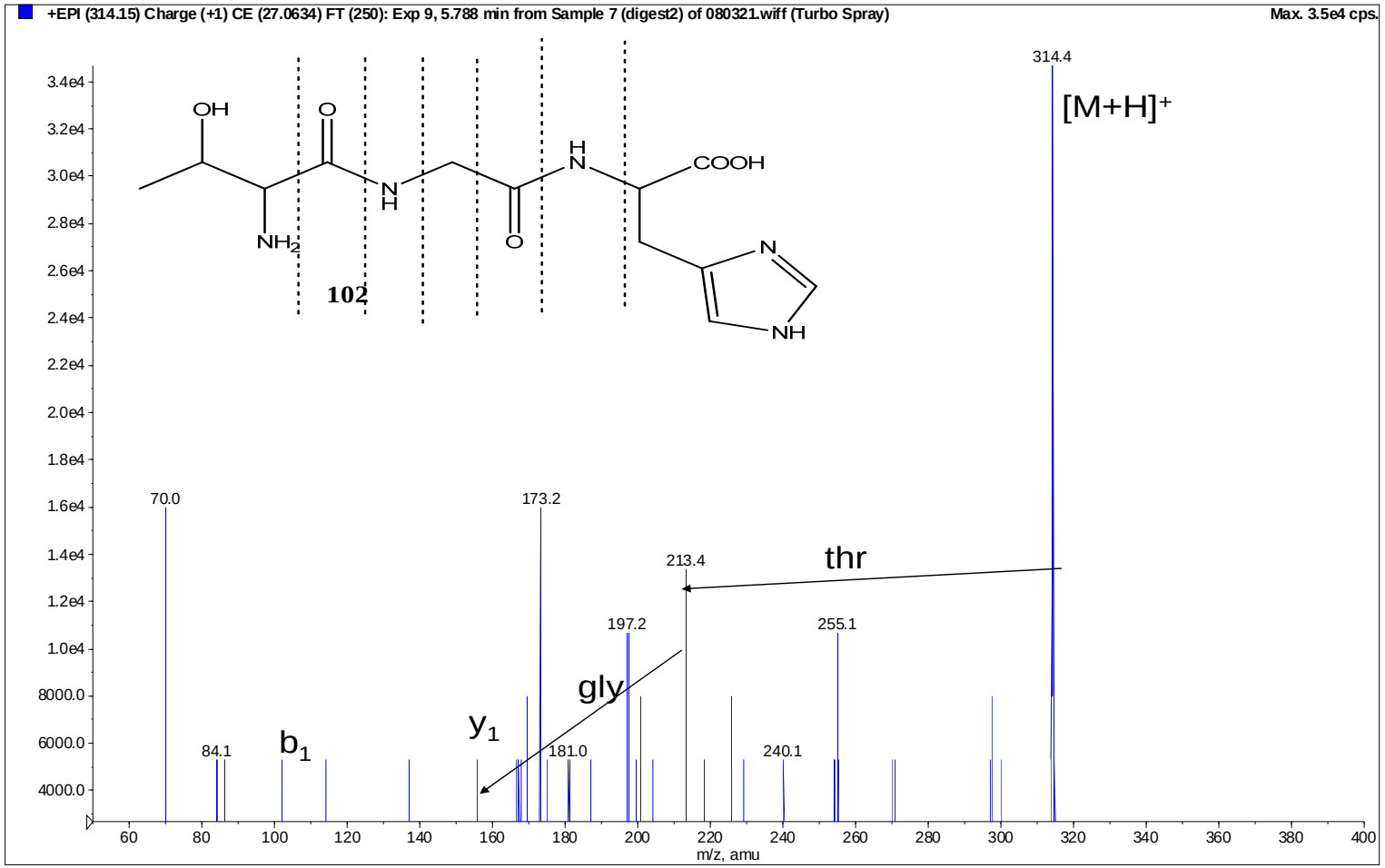


Figure 45: Sample U: LC-MS spectrum of thr-gly-his

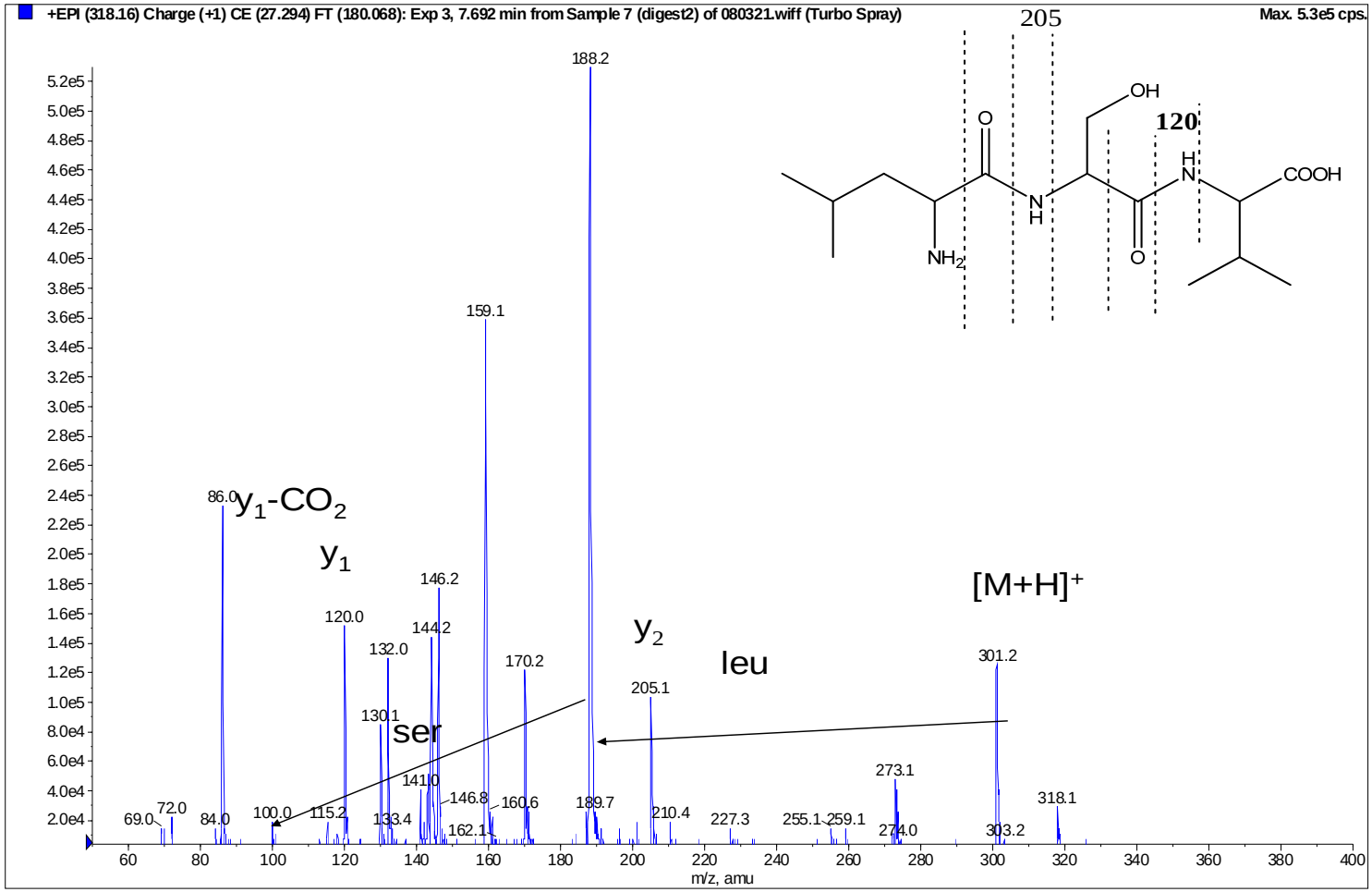


Figure 46: Sample U: LC-MS spectrum of leu-ser-val

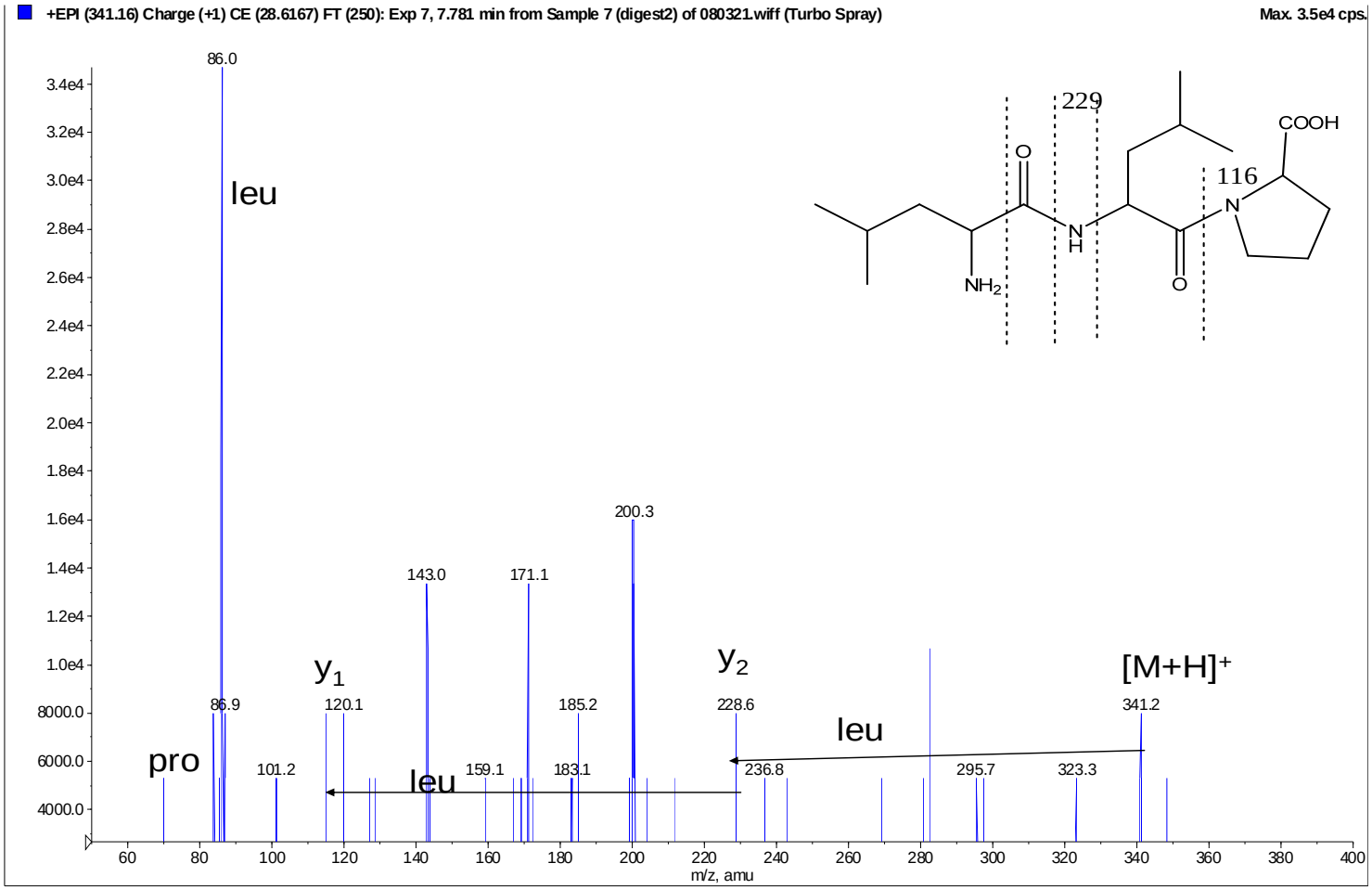

+EPI (341.16) Charge (+1) CE (28.6167) FT (250): Exp 7, 7.781 min from Sample 7 (digest2) of 080321.wiff (Turbo Spray)
Max. 3.5e4 cps.
86.0
leu
229
116
COOH
O
N
H
NH2
O
N
y1
y2
[M+H]+
pro
leu
leu
86.9
101.2
120.1
143.0
159.1
171.1
183.1
185.2
200.3
228.6
236.8
295.7
323.3
341.2
3.4e4
3.2e4
3.0e4
2.8e4
2.6e4
2.4e4
2.2e4
2.0e4
1.8e4
1.6e4
1.4e4
1.2e4
1.0e4
8000.0
6000.0
4000.0
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
400
m/z, amu

Figure 47: Sample U: LC-MS spectrum of leu-leu-pro

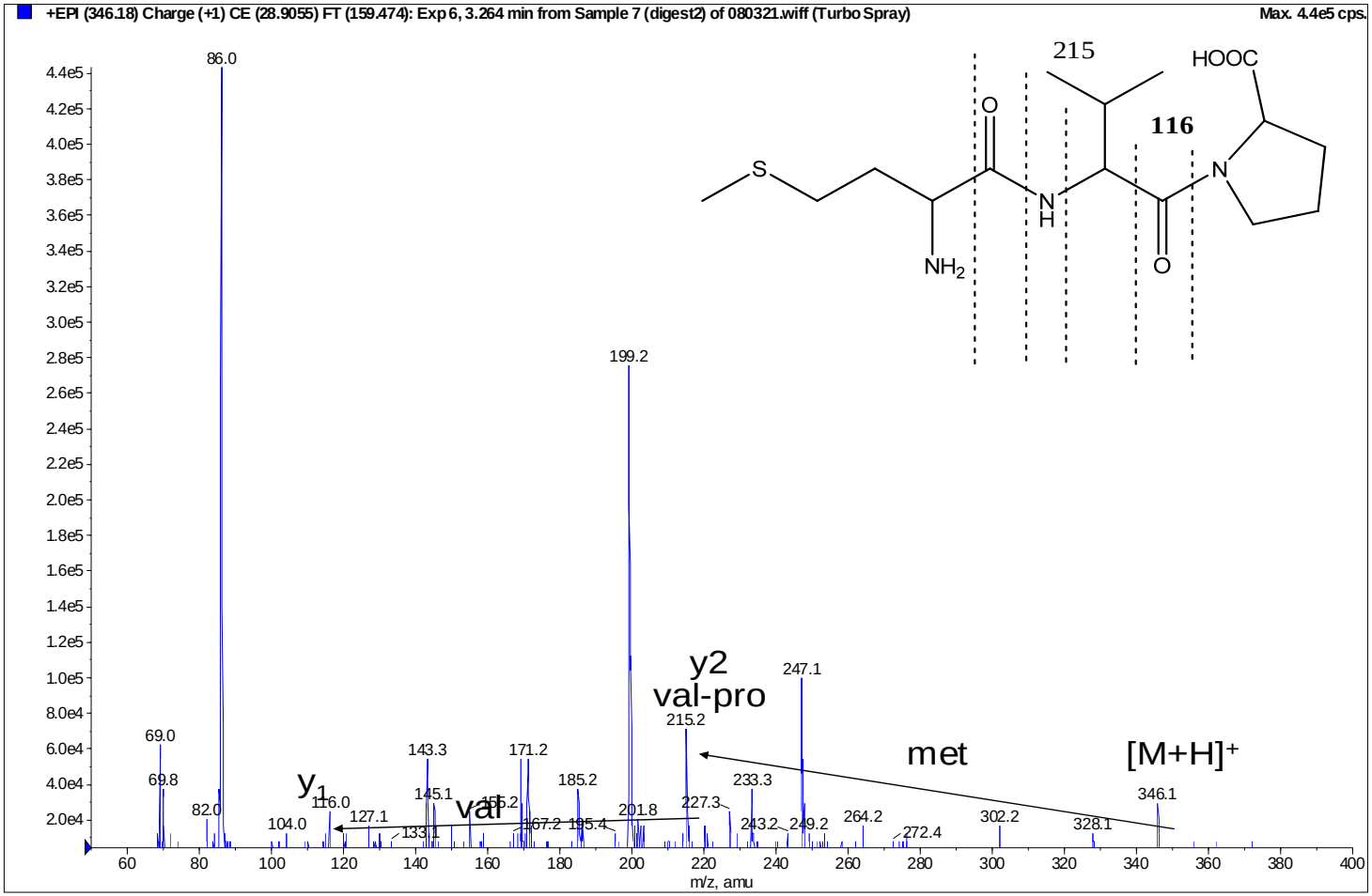


Figure 48: Sample U: LC-MS spectrum of met-val-pro

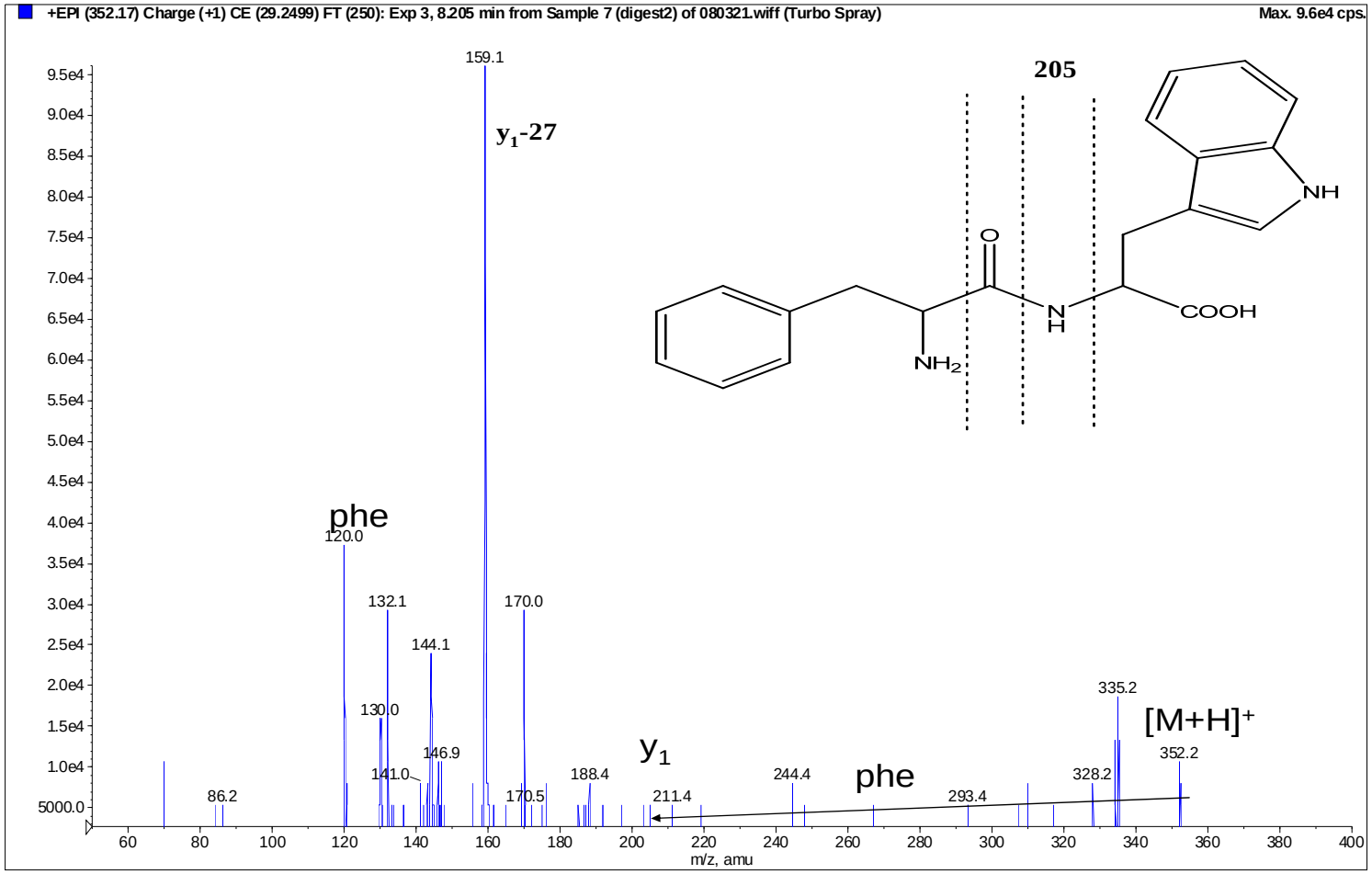


Figure 49: Sample U: LC-MS spectrum of phe-trp

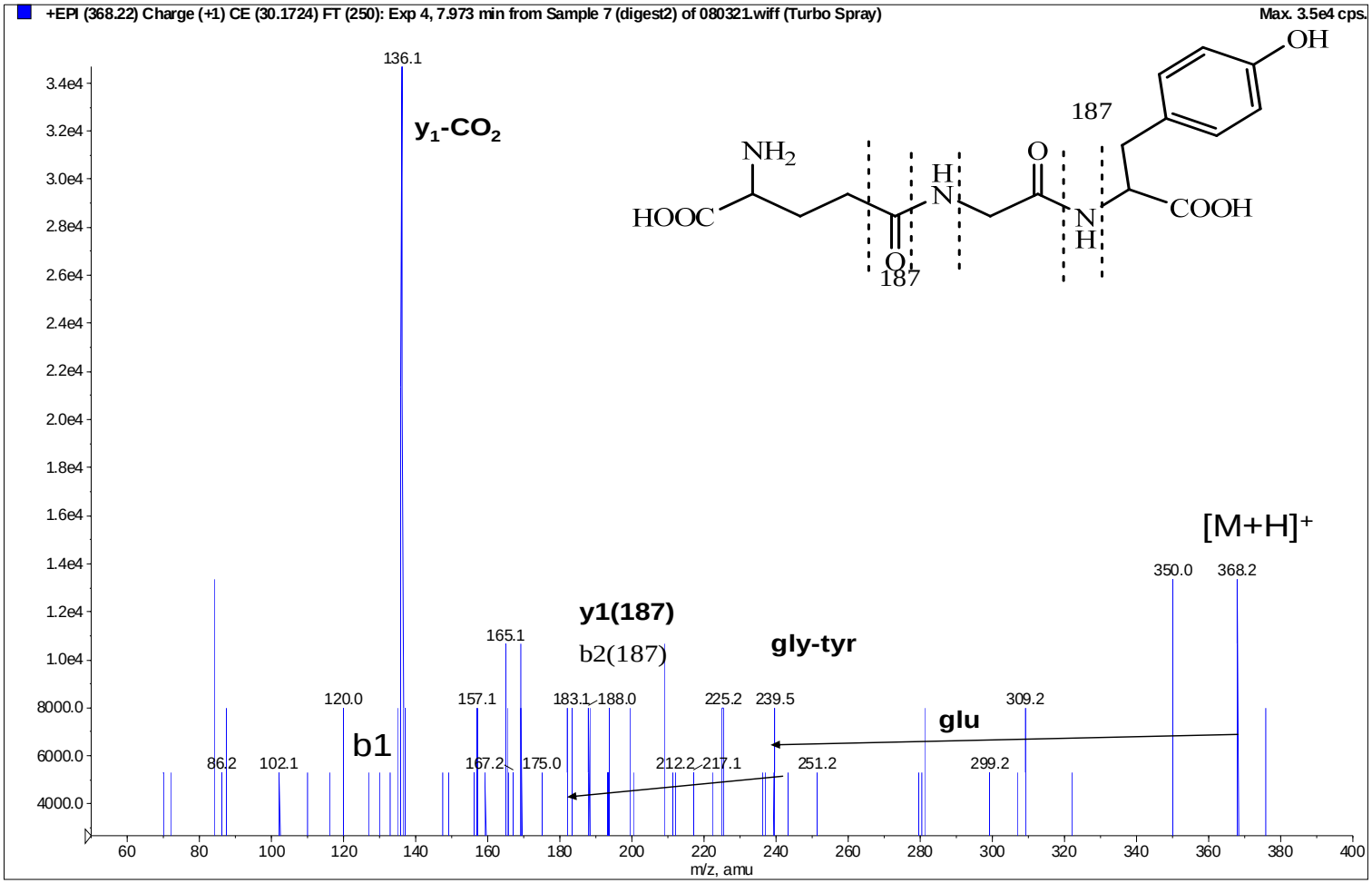


Figure 50: Sample U: LC-MS spectrum of glu-gly-tyr

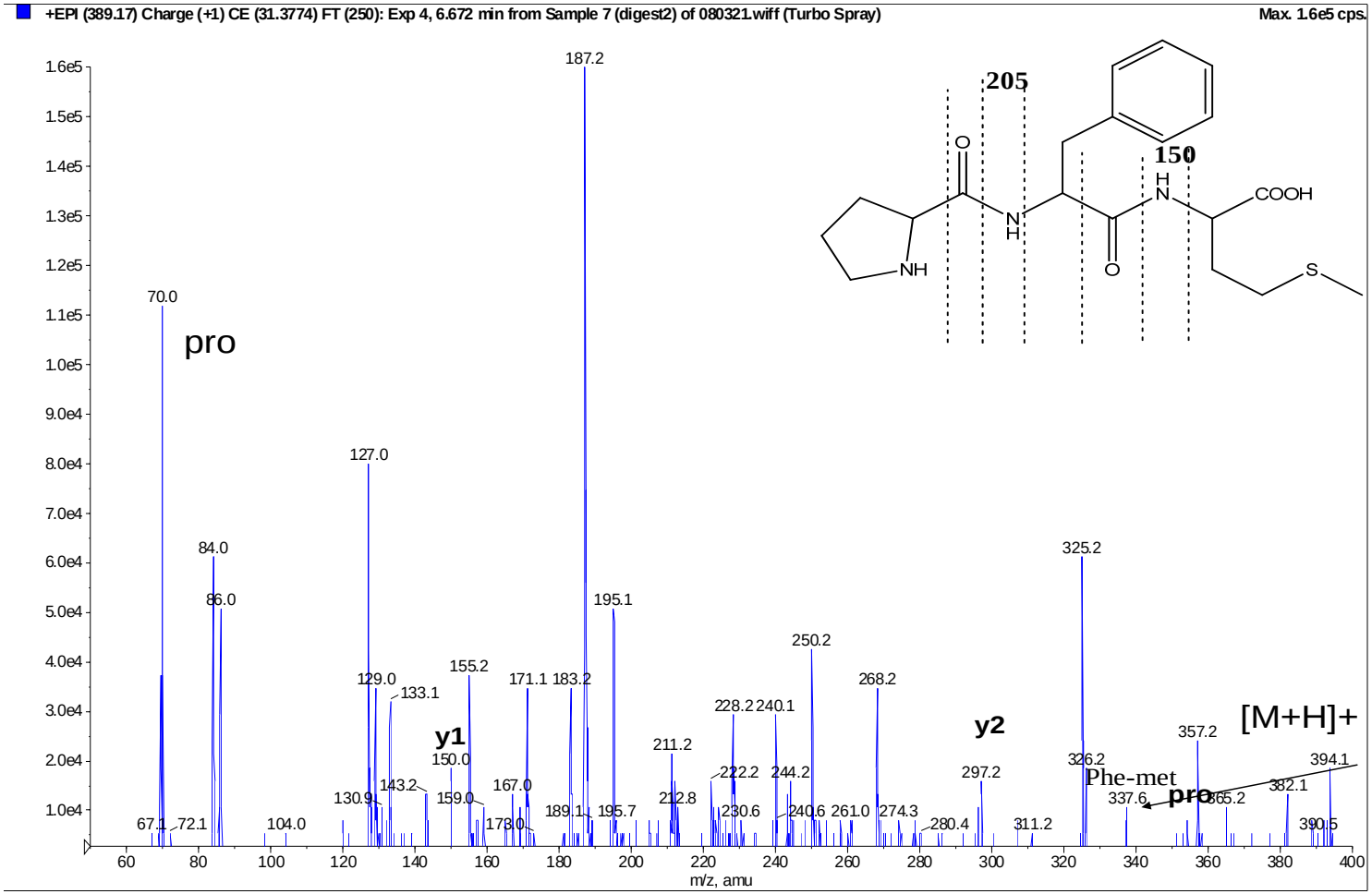


Figure 51: Sample U: LC-MS spectrum of pro-phe-met

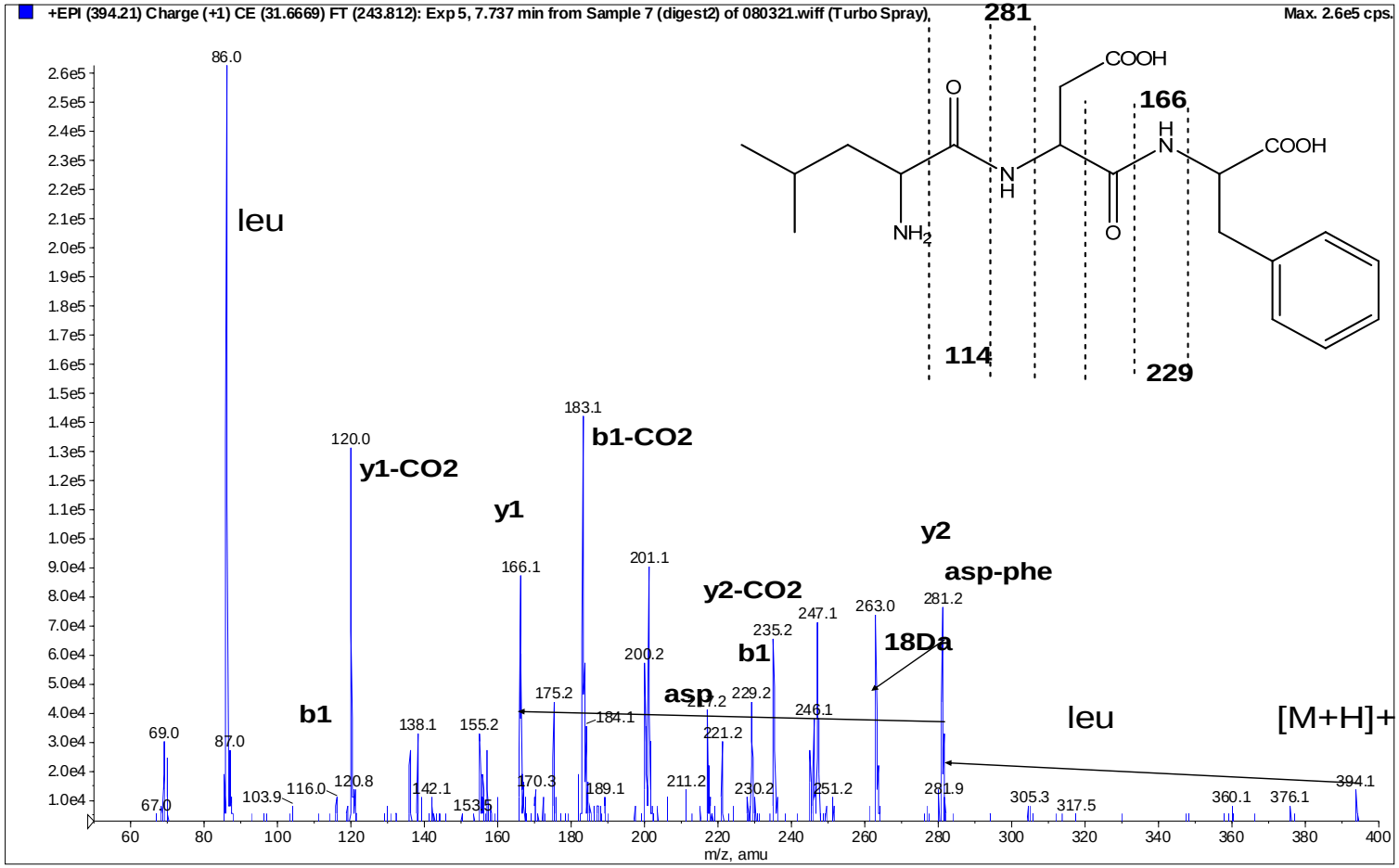


Figure 52: Sample U: LC-MS spectrum of leu-asp-phe

Table 22: Major MS fragments of peptides in beef marrow bone stocks

Sample P:	
gly-leu	86(100), 189(41), 113(18.2), 98(14), 72(7), 67(5), 69(5), 87.5(5), 108.5(5), 126(5), 133(5), 154(5)
val-thr	120(100), 219(13), 72(8.5), 70(6.4), 173(6), 91(6) 86(4), 98(4)
ala-his	110(100), 156(59), 210(57), 122(27), 181(25), 227(20), 94.9(15), 91(12), 83(7), 93.1(5), 180(11), 157(10), 182(8), 198(8), 152(8)
leu-pro	229.2(100), 201.1(22), 230(14), 116(7), 211(5), 86(2.7)
leu-lys	86(100), 157(93), 189(19), 85.4(17), 69(15), 114(14), 89(11), 147 (9), 70(8), 260.4(7), 67(2)
phe-arg	120(100), 219(87), 70(85), 175(46), 218.4(31), 322.3(30.7), 84(27), 225(23), 86(23), 71(19), 129(19), 304.2(19), 276(19), 110(19), 147(15), 328.1(12), 397.1(8), 69(4)
arg-arg	86(100), 157(93), 331(35), 147(29), 129(19), 130(16), 84(15), 114(13), 70(10), 69(9), 111(9)
Sample U:	
ser-pro	203(100), 70(65), 116(60), 114(11), 186(9),

	130(7.5), 175.1(7.58), 144(7.58), 84(6), 60(6)
val-leu	231(100), 231.6(37.5), 70(25), 86(25), 72(18.75), 183(18.75), 157(18.75), 132(12.5), 121(12.5), 232.6(12.5), 72(12.5)
gln-pro	70(100), 87(91), 187(64), 127(64), 116(45), 173(45),155(36), 84(36), 216(36), 244(27), 101(27), 102(18), 227(18)
phe-leu	120(100), 86(22), 93(6), 121.7(6), 132.1(6), 233(6), 279(4), 69(3), 70(1.48)
tyr-leu	136(100), 86(32), 119(12), 165(10), 182(8), 91(8), 249(4), 85.6(4), 295(2.5), 69(1)
thr-gly-his	314(100), 70(46), 173(46), 213.4(38), 255(35), 197(31), 255(31), 169.6(23), 201(23), 297(23), 226(23), 204(15), 199.4(15), 87(15), 181(15), 84(15), 86(15), 101(15), 114(15), 37(15), 156(15), 240(15)
leu-ser-val	188(100), 159(68), 86(44), 146(34), 120(29), 144(27), 132(24), 170(24), 301(23), 318(6), 273(5), 115(3.5), 72(4), 69(3), 70(3), 84(3)
lu-leu-pro	86(100), 200(46), 171(38), 143(38), 115(23), 228.6(23), 185(23), 70(15), 87(15), 159(15), 84(8), 120(8), 183(8)
met-val-pro	86(100), 199(62), 247(23), 215(16), 69(14),

	143(12), 171(12), 185(8), 116(6), 203(4), 104(3)
phe-trp	159(100), 120(39), 170(30), 144(25), 132(19), 335(19), 130(17), 70(11), 188(8), 141(8), 328(8), 86(6). 187(6), 205(6), 166(3)
glu-gly-tyr	136(100), 84(38), 350(38), 368(38), 169(31), 165(31), 209(31), 157(23), 376(23), 309(23), 87(23), 120(23), 135(23), 199(23), 70(15), 72(15), 86(15), 110(15), 116(15), 127(15), 130(15), 133(15), 217(15), 322(15), 309(15), 299(15), 280.6(15), 147.4(15), 149(15), 156(15), 157(15), 159(15)
pro-phe-met	187(100), 70(70), 127(50), 84(38), 325(38), 195(32), 250(27), 183(22), 129(22), 171(21), 155(21), 133(20), 240(18), 357.2(15), 150(12), 211(11), 222(10), 244(10), 382(8), 167(8), 260(3), 67(3), 72(3)
leu-asp-phe	86(100), 183(54), 120(50), 120(34), 166(33), 81(29), 263(28), 247(27), 235(25), 200(22), 175(17), 29(17), 217(16), 184(14), 246(14), 138.5(13),155(13), 221(11), 69(11), 394.1(5), 87(10), 67(3), 65(1)

8. REFERENCES

Agar, N.; Board, P. *Red Blood cells of domestic animals*. Elsevier Science Publishers, New York, NY, 1983.

Akhmedov, N.A.; Ismailova, L.I.; Abbasli, R.M.; Akhmedov, N.F.; Godjaev, N.M. Spatial structures of myelopeptides: 1. Conformational analysis of MP-1, MP-2 and MP-3. *Russian Journal of Bioorganic Chemistry*, 2005, 31, 31-38.

Ames, J.M.; Defae, A.B.; Bailey, R.G.; Bates, L. Analysis of the non-volatile Maillard reaction products formed in an extrusion-cooked model food system. *Food Chem.* 1998, 61, 521-524**.**

Amrani-Hemaimi, M.; Cerny C.; Fay, L. Mechanisms of formation of alkylpyrazines in the Maillard Reaction. *J. Agric. Food Chem*. 1995, 43, 2818-2822.

Apriyantono, A.; Setyaningsih, D.; Hariyadi, P.; Nuraida, L. Sensory and peptides characteristics of soy sauce in *Quality of fresh and processed foods* edited by Laszlo Vecsei, Abel Lajtha, Fereidoon Shahidi, Arthur M. Spanier, Chi-Tang Ho, Terry Braggins. 2004, 213-226.

Arai, S.; Yamashita, M.; Fujimakui, M. Glutamyl oligopeptides as factors responsible for tastes proteinase modified soybean protein. *Agric. Biol. Chem*. 1972, 36, 1253-1256.

Arai, S.; Yamashita, M.; Fujimakui, M. Tastes of oligopeptides in relation to their chromatographic properties. *Agric. Biol. Chem*. 1973, 37, 151-156.

Arasu, P.; Field, R.A.; Kruggel, W.G.; Miller, G.J. Nucleic acid content of bovine bone marrow, muscle and mechanically deboned meat. *J. Food Sci*. 1981, 46, 1114-1116.

Asalyng, M.D.; Martens, M.; Poll, L.; Nielsen, P.M.; Flyge, H.; Larsen, L.M. Chemical and sensory characterization of Hydrolyzed Vegetable Protein, a savory flavoring. *J. Agri. Food Chem*. 1998, 46, 481-489.

Bauchart, C.; Remond, D.; Chambon, C.; Mirand, P.; Savary-Auzeloux, I.; Reynes, C.; Morzel, M. Small peptides (<5 kDa) found in ready-to-eat beef meat. *Meat Science*, 2006, 74, 658-666.

Beksan, E.; Schiberle, P, Robert, F.; Blank, I.; Fay, L.; Schlichtherle-Cerney, H.; Hofmann, T. Synthesis and sensory characterization of novel umami tasting glutamate glycoconjugates. *J. Agri. Food Chem*. 2003, 51, 5428-5436.

Belitz, H.; Grosch, W.; Schiberle, P. *Food Chemistry;* Springer: New York, 2004.

Brinkman, H.W.; Copier, H.; de Leuw, J.J.; Tjan, S.B. Components contributing to beef flavor analysis of the headspace volatiles of beef broth. *J. Agri. Food Chem*. 1972, 20, 177-181.
Cambero, M.I.; Pereira-Lima, C.I.; Ordonez, J.A.; de Fernando, G.D.; Beef broth flavor: relation of components with the flavour developed at different cooking temperatures. *J.Sci Food Agric*. 2000, 80, 1519-1528.

Chang, S.; Hirai, C.; Reddy, B.; Herz, K.; Keto, A. Isolation and identification of 2,4,5-Trimethyl-3-oxazoline and 2,5-Dimethyl-1,2,4-trithiollane in the volatile flavor compounds of boiled beef. *Chem. Ind*. (London), 1968, 1639-1640.

Chen, J; Ho, C-T. The flavour of poultry meat in *Flavor of Meat, Meat products and seafoods* edited by F. Shahidi; Blackwell Publishers: London, 1998, 84-100.

Chen, Y.; Wang, J.; Liu, X-H.; Sun, M.; Chen, J.; Jiang, Y.; Zhao, Y-F. Novel arrangement of small peptides in electrospray ionization tandem mass spectrometry. *Chinese J. of Chem*. 2004, 22, 477-481.

Courchesne, P.L.; Patterson S.D. Matrix-assisted laser desorption/ionization mass spectrometry for protein identification using peptide and fragment ion masses In *The protein protocols handbook* edited by J.M. Walker, Humana Press Inc., Totowa, New Jersey, 2002, 711-732.

Chiu, E-M.; Kuo, M-C.; Bruechert, L.; Ho, C-T. Substitution of pyrazines by aldehydes in model systems. *J. Agri. Food Chem*. 1990, 38, 58-61.

Engel, E.; Nicklaus, S.; Septier, C.; Salles, C.; Le Quere, J. Taste active components in a goat cheese Water-Soluble Extract. 2. Determination of the relative impact of Water-Soluble Extract components on its taste using omission tests. *J.Agric Food Chem.* 2000, 48, 4260-4267.

Farmer, L.J. The role of nutrients in meat flavour formation. *Proceedings of the Nutrition Soc.* 1994, 53, 327-333.

Fay, L.; Brevard, H. Contribution of mass spectrometry to the study of the Maillard reaction in food. *Mass Spectrometry Reviews*. 2005, 24(4), 487-507.

Field, R.A. Mechanically deboned red meat. In: *Advances in Food Research,* Academic Press: New York, 1981, 27, p23.

Field, R.A. Bone marrow measurements for mechanically recovered products from machines that press bones. *Meat Sc.* 1999, 51, 205-214.

Field, R.A.; Sanchez, H.M.; Ji, T.H.; Chang, Y.O.; Smith, F.C. Amino acid analysis and acrylamide Gel Electrophoresis patterns of bovine hemopoietic marrow. *J. Agri. Food Chem.* 1978, 26(4), 851-854.

Gerard, J.A. The Maillard reaction in food: Progress made, challenges ahead-Conference report from the Eighth International Symposium on the Maillard Reaction. *Trends in Food Science & Technology* 2006, 17, 324-330.

Greenberg, M.J. Characterization of meat and bone meal flavor volatiles. *J. Agri. Food Chem.* 1981, 29, 1276-1280.

Grigorov, M.G.; Schlichtherle-Cerny, H.; Affolter, M.; Kochhar, S. Design of virtual libraries of Umami-tasting molecules. *J. Chem. Inf. Comput. Sci*. 2003, 43, 1248-1258.

Griffith, R.; Hammond, E.G.; Generation of Swiss cheese flavor components by the relactive amino acids with carbonyl compounds. *J. Dairy Sc*. 1989, 72, 604-613.

Hartman, G.J; Ho, C-T. 1982 Volatile compounds formed in the thermal degradation of hydrated collagen. *Lagensm. Wiss. Technology*. 15(5), 253.

Harve This, *Molecular gastronomy-exploring the science of flavor* translated by M.B. Debevoise. New York, 2006, pp 23-28.

Hidalgo, F.J.; Zamora, R. Strecker degradation produced by the lipid oxidation products 4,5-epoxy-2-alkenals. *J. Agic. Food Chem*. 2004, 52, 7126-7131

Hill, R. Hydrolysis of protein. *Adv. Protein Chem*. 1974, 20:37-107

Ho, C-T.; Chen, Q. *Lipids in food flavors in lipids in food flavors* edited by C-T Ho and TG Hartman. ACS symposium series 558, Washington D.C., 1994, p6.

Hodge JE. 1953. Chemistry of browning reactions in model systems. *J Agric Food Chem*. 1953, 1, 928-943.

Hoffmann, E.; Stroobant, V. Mass Spectrometry principles and applications John Wiley & Sons, Ltd., New Jersey USA, 2007

Hofmann, T. Taste-active Maillard reaction products: the ‘tasty’ word of non-volatile Maillard reaction products. *Annals of the New York Academy of sciences*, 2005, 1043, 20-29.

Imafidon, G.I.; Spanier, A.M. Unraveling the secret of meat flavor, *Trends in food science and technology*, 1994, 5, 315-321.

Ivanov, V.T; Karelin, A.A.; Karelina E.V.; Ul’yashin, V.V.; Mikhaleva, I.I.; Vaskovsky, B.V.; Nazimov, I.V.; Grishina, G.A.; Khavinson, V.K.; Morozov, V.G.; Mikhaltsov, A.N. Novel active peptides from bovine brain and bone marrow, In *Peptides* 1990, Proceedings of the Twenty-First European Peptide Symposium

September 2-8, 1990, Platja d'Aro, Spain, Edited by Ernest Giralt and Dvid Andreu, Escom, Leiden, 1991, 813-815.

Karelin, A. A.; Philippova, M.M.; Karelina , E.V.; Strizhkov, B.N.,; Grishina, G.A.; Nazimov, I.O.; Blishchenko, E .Y.; Ivanov, V.T. Peptides from bovine brain: Structure and biological role. *J. Peptide Sci*, 1998, 4, 211-225.

Kaufman, R. Meat composition. In *Meat science and Applications* edited by Hui Y.H.; Nip,Wai-Kit; Rogers, Robert; Young, Owen. Mercel Dekker, New York, 2001.

Keyhani, A.;Yaylayan, V. Elucidation of the mechanisms of pyrazine formation in glycine model systems using labeled sugars and amino acids. *J. Agri.Food Chem.* 1996, 44, 2511-2516.

Kilara, A.; et al, Biotechnology Bioeng, 14, 1703-1714, 1977 cited in *Sigma Aldrich Catalogue*, 2007).

Kinter, M.; Sherman, N.E. *Protein sequencing and identification using tandem mass spectrometry*, 2000, Wiley Interscience, Toronto, Canada, pp-64-116.

Kochhar, S; Hansen, E.; Juillerat, A. Flavor-active peptides, *US patent* 2004/0224077

Kunsman, J.E.; Collins, M.A.; Field, R.A.; Field, R.A.; Miller, G.J. Cholesterol content of beef bone marrow and mechanically deboned meat. *J. Food Sci*., 1981, 46, 1875-1878.

Kuroda, M.; Harada, T. Fractionation and characterization of the macromolecular meaty flavor enhancer from beef meat extract. *J. Food Sci*. 2004, 69, 542-547.

Lee, M.; Chistopherson, I.P.; Lehman,J.M.; Bennett, C.J.; Cheung, H.T. Comparison of bone marrow extracellular matrices. *Biochimica et Biophysica Acta*. 1999, 1428, 300-304.

Li, Jiangang.; Ho, C-T. Generation of aldehydes from Maillard reaction of glucose and amino acids. In *Food flavor and chemistry* edited by AM Spanier, F. Shahidi, TH Parliament, C. Mussinan, C-T Ho, ET Contis, The Royal Society of Chemistry, 2005, pp 213-218.

Lin, J.; Blank, I. Odorants generated by thermally Induced degradation of phospholipids. *J. Agri. Food Chem.* 2003, 51, 4364-4369.

Lioe, H.N.; Takara, K.; Yasuda, M. Evaluation of Peptide contribution to the intense Umami taste of Japanese soy sauces. *Journal of Food Sc*. 2006, 71 (3), S277–S283.

Lieske, B; Konrad, G. Protein hydrolysis-the key to meat flavoring systems, *Food Reviews International*. 1994, 10, 287-312.

Ma, B.; Zhang K.; Liang C. An effective algorithm for peptide *de novo* sequencing from MS/MS spectra, *J.Comp and Syst. Sc*. 2005, 70, 418-430

Mabrouk, A.; Jarboe, J.; O'Connor, E. Water soluble flavor precursors of beef: Extraction and fractionation. *J. Agri. Food Chem.* 1969, 17, 5-9.

Maehashi, K.; Matsuzaki, M.; Yamamoto, Y.; Shigezo, U. Isolation of peptides from an enzymatic hydrolysate of food proteins and characterization and their taste properties, *Biosci. Biotechnol. Biochem*. 1999, 63 (3), 555-559.

Mancini, R.A. Current research in meat color, *Meat sc.* 2005, 71, 100-121.

Marsili, R. Comparing sensory and analytical chemistry flavor analysis. In the *Sensory-directed flavor analysis* edited by Ray Marsili: Taylor and Francis, New York, 2006, p 11.

Medziharadszky, K.F. Peptide sequence analysis, *Methods in Enzymology*, 2005, 402, 209-238.

Mello, F.C.; Field, R.A.; Forenza, S.; Kunsman, J.E. Lipid characterization of bovine bone marrow. *J. Food Sci*. 1976, 41, 226-230.

Miller, G.J.; Frey, J.E.; Kunsman, J.E.; Field, R.A. Bovine bone marrow lipids. *J. Food Sci.*1982, 47, 657-665.

Moerck, K.E.; Ball, H.R. Lipids and fatty acids of chicken bone marrow. *J. Food Sci*. 1973, 38, 978-980.

Molina, E.; Ramos, M.; Alonso, L.; Lopez-Fandino, R. Contribution of low molecular weight water soluble compounds to the taste of cheeses made of cows', ewes' and goats milk. *Food Chem*, 199, 9, 613-621.

Mottram, D.S. Maillard-lipid interactions in nonaqueous systems: volatiles from the reaction of cysteine and ribose with phosphotidylcholine. *J. Agric. Food Chem*. 1995, 43, 1302-1306.

Mottram, D.S. Meat flavour in *Understanding natural flavors*, Edited by Piggott, J.R. and Paterson, A., Blackie Academic & Professional, 1994, Page-152-156.

Mussinan, C.J.; Walradt, J.P. Volatiles constituents of pressure cooked pork liver. *J. Agric. Food Chem*. 1974, 22, 827-831.

Nielsen, PM; Peterssen, D.; Dambmann, C. Improved method for determining food protein degree of hydrolysis. *J. Agric. Food Chem*. 2001, 6, 642-646.

Noguchi, M.; Arai, S.; Yamashita, M.; Kato, H.; Fajimaki, M. Isolation and identification of acidic oligopeptides occuring in a flavor potentiation fraction from a fish protein hydrolysate. *J. Agric. Food Chem*. 1975, 23, 49-53.

Nursten, H. Aroma compounds from the Maillard reaction. In *Developments in food flavours* by GG Birch and MG Lindley. Elsevier Applied Science, New York, 1986, 173-190.

Ockerman, H.W.; Hansen, C.L. Edible tissue from bone. In *Animal by-product processing & utilization*. Technomic publishing Co., Inc: Lancaster, Pennsylvania, 2000.

Ogasawara, M.; Katsumata, T.; Egi, M. Taste properties of Maillard reaction products prepared from 1000 to 5000 Da peptides. *Food Chem*. 2006, 99, 600-604.

Ogasawara, M.; Yamada, Y.;, Egi, M. Taste enhancer from the long-term ripening of miso (soybean paste). *Food Chem.* 2006a, 99, 736-741.

Oh, Y.C.; Shu, C.K.; Ho, C-T. Formation of novel 2(1H)-pyrazinones as peptide-specific Maillard reaction products. *J. Agric. Food Chem.* 1992, 40, 118-121.

Ota, M.; Kohmura, M.; Kawaguchi, H. Characterization of new Maillard type reaction product generated by heating 1-deoxymaltulosyl-glycine in the presence of cysteine. *J. Agric. Food Chem.* 2006, 54, 5127-5131.

Ottinger, H.; Hofmann, T. Identification of the taste enhancer alapyridaine in beef broth and evaluation of its sensory impact by taste reconstitution experiments. *J. Agric. Food Chem.* 2003, 51, 6791-6796.

Pereira-Lima, C.; Ordonez, J.; Garcia de Fernando, G.; Cambero, M. Influence of heat treatment of carnosine, anserine and free amino acid composition of beef broth and its role in flavor development. *Eur Food Res Technol*. 2000, 210: 165-172.

Petrov, Rem V.; Mikhailova, Augusta A.; Fonina, Larissa A. Bone marrow immunoregulatory peptides (Myelopeptides): Isolation, structure, and functional activity, *Biopolymers* 1997, 43, 139-146.

Prescott, J. Stevenson. R.J. Food Rev. Int. 1995, 11(4), 665-698 (as cited by Carl Frey, *Natural flavors and fragrances: Chemistry, analysis, and production,* ACS symposium series 908 edited by Carl Frey and Russell Rouseff.

Raghavan, S.; LaMarta, J.; Shah, P.; Holmes, J.; Chigurupati, S., Process for producing a low fat, concentrated meat broth from meat by-products, *US Patent 20050170060*, 2005.

Ramirez, Rosario, Cava, Ramon, Volatile profiles of dry-cured meat products from three different Iberian X Duroc genotypes, *J. Agric. Food Chem*, 2007, 55, 1923-1931.

Rizzi, G.P. Heat-induced flavor from peptides. In Thermal generation of aromas; Parliament, T.H., McGorin, T.H., Ho, C-T., ACS Symposium Series 409; American Chemical Society: Washington DC, 1989, pp. 285-301.

Romero, M. Enzymatic hydrolysis and deamidation of wheat proteins for flavor generation via Maillard reaction, *PhD. Dissertation*, Rutgers University, 2006.

Schieberle, P., Hofmann, T., and Munch, P. Studies on potent aroma compounds generated in Maillard-type reactions using the odor-activity-value concept. In *Flavor Chemistry: Industrial and Academic research* edited by Sara J. Risch and Ch-Tang Ho ACS symposium series 756, Amercican Chemical Society, Washington D.C., 2001, 133-150.

Schlichtherle-Cerny, H. and Amado R. Analysis of taste-Active compounds in an enzymatic hydrolysate of deamidated wheat gluten, *J. Agric. Food Chem*., 2002, 50. 1515-1522.

Schlichtherle-Cerny H.; Affolter, M.; Blank, I.; Cerny Christoph; Robert, F; Beksan, E.; Hoffmann, T.; Schieberle, P, *Flavouring compositions, WO 02/087361*, 2002.

Schlichtherle-Cerny, H.; Affolter, M.; Cerny, C. Taste-active glycoconjugates of glutamate: New Umami Compounds in *Challenges in taste chemistry and biology* edited by Thomas Hofmann, Chi-Tang Ho, and Wilhelm Pickenhagen ACS symposium series, American Chemical Society, 2004.

Schutte, 1976 Flavor precursors of food stuffs, in Charalambous and Katz. cited by Cramer (1983).

Shima, K., Yamada, N., Suzuki, E-I., Harada, T., Novel brothy taste modifier isolated from beef broth, *J. Agric. Food Chem,* 1988, 46, 1465-1468.

Skilling, John, Apparatus for identifying peptides and proteins by mass spectrometry US patent US2006/024865.

Smouse, T, Chang, S., Relationship between alkylfuran and the reversion flavor of soybean oil, *J. Am. Oil Chem. Soc.*, 1967, 44, 159.

Sommerer, N., Salles, C., Prome, D., Prome, J.C., Le Quere, J.L., Isolation of oligopeptides from the water-soluble extract of goat cheese and their identification by mass spectrometry, *J. Agric. Food Chem*, 2001, 49, 402-408.

Tachdjian, Catherine; Patron, Andrew P; Adamski-Werner, Sara L.; Bakir, Farid; Chen Quing; Darmohusodo, Vincent; Hobson, Stephen T.; Li, Xiaadong; Qi Ming; Rogers,

Harry; Rinnova, Marketa; Servant, Guy; Tang, Xiao-Qing; Zoller, Mark; Wallace, David; Xing, Amy; Gubernator, Klaus, Novel flavors, flavor modifiers, tastants, taste enhancers, umami or sweet testants, and/or enhancers and use thereof, *United States Patent*, US 2005/0084506 A1., 2005)

Tai, C-Y and Ho, C-T., Influence of glutathione oxidation and pH on thermal formation of Maillard-type volatile compounds, *J. Agric. Food Chem*, 1998, 46, 2260-2265.

Tamura, M., Nakatsuka, T., Tada, M., Kawasaki, Y., and Kikuchi, E., The relationship between taste and primary structure of "delicious peptide" from beef soup. *Agric. Biol. Chem.*, 1989, 53, 319-325

Tonsebeek, C.H.T., Plankcken, A.J. and Weerdhof, T.V.D., Component contributing to beef flavor. Isolation of 4-hydroxy-5-methyl-3-(2H)-furanone and its 2,5-dimethyl homolog from beef broth. *J. Agric. Food Chem*., 1968, 16, 1016-1021.

Vollmer, A. N. and Rainey, G. E. Extraction of protein from edible beef bones and product, *United States Patent 4,176,199* November 27, 1979.

Wang, J-S; Zhao, M-M, Zhao, Q-Z, Bao, Y., Jiang, Y-M, Characterization of hydrolysates derived from enzymatic hydrolysis of wheat gluen, *J. Food Sc.*, 2007, 72, 103-107.

Wang, K., Maga, J., and Bechtel, P, Taste properties and synergisms of beefy meaty peptide. *J. Food Sci*., 1996, 61, 837-839.

Weenen, H, Reactive intermediates and carbohydrate fragments in Maillard chemistry, 1998, 62(4), 393-401.

Whitfield, F.B., *Volatiles from interactions of Maillard reactions and lipids*, CRC Crit. Rev. Food Sci. Nutr. 1992, 31, 1-58.

Yai, Y., and Maekawa, K., A peptide with delicious taste. *Agric. Biol. Chem*., 1978, 42, 1761-1765.

Yamasiki, Y., and Maekawa, K., A peptide with delicious taste, *Agric. Biol. Chem*., 1978, 42, 1761-1765.

Yaylayan, V**.** and Haffenden, L. Mechanism of pyrazole formation in [13C-2]labeled glycine model systems: N-N bond formation during Maillard reaction. *Food Res. Int.* 2003, 36, 571-577.

Zhang, Y., *Thermal generation of food flavors from cysteine and glutathione*, Dissertation: Rutgers, the state university, 1991.

9. VITA

Belayet H. Choudhury

1981	BSc (Hons) in Biochemistry, University of Dhaka, Bangladesh
1985	BS in Chemistry, University of Central Oklahoma, Oklahoma
1986	MS in Nutrition and Foods, University of Central Oklahoma, Oklahoma
1986-1987	Department of Food science, Oklahoma State University, Oklahoma
1987-1990	Supervisor, Nutrition Services, West Hudson Hospital, Kerney, New Jersey
1990-1997	Flavor Chemist, Universal Flavors, Kerney, New Jersey
1997-2001	Senior Flavor Chemist, Takasago International, Rockleigh, New Jersey
2001-present	Principal Scientist, ConAgra Food Ingredients, Cranbury, New Jersey
2003-2008	Department of Food Science, Rutgers, The State University of New Jersey
October, 2008	Ph.D. in Food Science, Rutgers, The State University of New Jersey

Manley, C.H.; Choudhury, B.H.; Mazeiko, P. Thermal Process Flavorings In Food Flavorings edited by Philip R. Ashurt, Aspen Publication, 1999, 282-322.

Choudhury, B; Daun, H; Ho, C-T; Raghavan, S. Volatiles generation in beef marrow bone stocks American Chemical Society presentation, Boston, August 2007.