PROFILING OF BIOACTIVE LIPIDS USING ULTRAHIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY

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ABSTRACT

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Plants and microbes accumulate a diverse assortment of bioactive lipid metabolites, yet the genetic and environmental factors that influence lipid composition remain uncertain. Discoveries of these factors will rely on rapid methodologies to detect, identify, and quantify these important compounds. In this dissertation, new mass spectrometry based protocols were developed and applied to serve as platforms for future studies of the role of genetics in lipid composition. Methodology for identification, detection and quantification of diterpene glycoside sweeteners in Stevia leaf extracts using ultrahigh performance liquid chromatography/tandem mass spectrometry (LC/MS/MS) is presented and evaluated. This approach employed a QTRAP mass analyzer in multiple reaction monitoring (MRM) mode for selective and sensitive identification of sweeteners. Prior to this selective MS/MS detection, a rapid ultra-high performance liquid chromatographic separation was performed using a fused-core C18 column to elute and resolve sweeteners and their isomers. In some cases, the presence of unexpected diterpene glycoside isomers was revealed. Levels of stevioside, different Rebaudiosides and related natural sweeteners differed significantly across a population of more than 1200 Stevia leaf extracts.

The detection of multiple isomers of Stevia glycosides suggested a need to distinguish these isomers using mass spectrometry. Using flow injection and selective collision induced dissociation (CID) on different anionic forms of the various sweeteners, breakdown curves were generated at various collision energies. These breakdown curves exhibited dramatic differences in ion abundances as a function of collision energies for isomeric sweeteners differing in position of sugar attachment.

A combination of ultrahigh performance liquid chromatography and quasi-simultaneous acquisition of mass spectra at multiple collision energies yields a technique that provides fast spectrum acquisition using a time-flight mass analyzer. Non-selective collision induced dissociation facilitated identification of lipids based on their accurate molecular and fragment ion masses. This approach, called multiplexed collision induced dissociation (mux-CID), generates molecular and fragmentation mass spectra at different collision energies for abundant and low-abundance lipids in a single analysis. This methodology coupled to fast chromatography (less than 5 minutes per sample) was used for high-throughput screening of lipid species. Using gradients as long as 26 minutes with appropriate mobile phases allowed partial resolution of lipids that coeluted in the fast 5-minute screens. Different classes of lipids including sulfo-, galacto-, and phospholipids were resolved and identified using mux-CID approach.

The research presented in this dissertation has developed mass spectrometry-based protocols that greatly enhance the rate at which new bioactive lipids can be detected, identified, and quantified. Such methodologies can be applied for analysis of lipid species in a wide range of biological tissues including plants and microbes.

Copyright by BEHNAZ SHAFII 2012 I dedicate my thesis to my dad Dr. Ahmad Shafii, and my mom Zahra (Behnoosh) Akhzarmehr. Your unconditional love has not only always made my heart warm, but it also made me question the desire of having a child of my own someday because I feel that I can never be as great a parent as you have been to me, or be able to give the child all that was given to me. It is one thing to wish to be a parent, but it is a completely different thing to become a great parent. I also dedicate my thesis to my two beautiful sisters Dr. Mahnaz Shafii and Dr. Negah Shafii, and my unique brother Dr. Behshad Shafii with whom I could share my happiness and sorrow without being judged. Thank you all for loving me unconditionally.

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"even after all this time, the sun never says to the earth "you owe me"-look what happens with a love like that, it lights the whole sky".

Hafiz, an Iranian Poet of the 1300

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LIST OF ABBREVIATIONS

AA	Arachidonic Acid
AhR	Aryl hydrocarbon Receptor
BPI	Base Peak Intensity Chromatogram
CID	Collision Induced Dissociation
DESI	Desorption Electrospray Ionization
DGDG	Digalactosyl Diacylglycerol
DMAPP	Dimethylallylpyrophosphate
DP	Declustering Potential
EETs	Epoxyeicosatrienoic Acids
EPI	Enhanced Product Ion
ESI	Electrospray Ionization
FAME	Fatty Acid Methyl Ester
GC-MS	Gas Chromatography Mass Spectrometry
HETEs	Hydroxyeicosatetraenoic Acids
HILIC	Hydrophilic Interaction Liquid Chromatography
HPLC	High Performance Liquid Chromatography
ID	Inner Diameter
IPP	Isopentylpyrophosphate
JA	Jasmonic Acid
LC	Liquid Chromatography
MEP	Methylerythritol-4-phosphate

MGDG	Monogalactosyl Diacylglycerol
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
mux-CID	Multiplexed Collision Induced Dissociation
MVA	Mevalonate
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PKS	Polyketide Synthase
PPAR	Peroxisome Proliferator-activated Receptors
PUFA	Polyunsaturated Fatty Acid
PS	Phosphatidylserine
RP	Reversed Phase
Rt	Retention time
SQDG	Sulfoquinovosyldiacylglycerol
TIC	Total Ion Chromatogram
TLC	Thin-Layer-Chromatography
TOF	Time of Flight
UHPLC	Ultrahigh Performance Liquid Chromatography
UV	Ultraviolet

Chapter One: Introduction

1.1 The Need for Discovery and Improvement of Bioactive Chemicals

Biologically active substances have profound economic importance worldwide owing to their use as medicines, food constituents, and various other consumer products. For example, worldwide use of medicines accounted for 1.2% of the global gross domestic product in the year 1999, or about 320 billion US dollars annually [1]. About 61% of new drugs developed between 1981 and 2002 were based on natural products, and these natural product derivatives have been successful, especially in the treatment of infectious disease and cancer [2]. In addition to pharmaceutical use of natural product analogs, industry produces about 18 billion US dollars worth of food and flavor chemicals annually [3]. Production costs for many of these substances are a central factor driving economic competitiveness, and extensive resources are devoted across the globe for research and development to improve production and product quality.

Among biologically derived products, lipids have great economic value as biomembranes, skin care formulations, in cosmetics, detergents, and in drug delivery systems including liposome formulations [4,5]. Also, world reserves of petroleum and other fossil fuels are finite, and lipids offer potential as renewable sources of liquid transportation fuels. For example, algal neutral lipids have been suggested as a potential diesel fuel substitute [6,7] and algae farming has newly-appreciated potential to produce an alternative source of oil. Furthermore, different classes of lipids, most notably fatty acid derivatives, are important sources of nutrients [8-10]. For instance, various glycolipids (GL) are reported for their diverse biological activities including immunosuppressive activities, antitumor promoting, inhibition and promotion of cell growth [11], inhibition of number of viruses including SVF and HSV-1 [12].

Despite the economic importance of lipids, efforts to engineer improved lipid production

have enjoyed limited success. Most of the recent advances have involved manipulation of the degree of unsaturation in seed oils from crops such as canola and soybeans [13]. Future improvements will rely on enhancing our understanding about how living things synthesize and transport these compounds in various organisms including plants, yeast, and bacteria. For this matter, accurate methods are needed for identification, characterization, and quantification of lipids to accelerate research advances.

1.2 The Importance of Lipophilic Characteristics in Bioactive Substances

It has been more than a century since it was first proposed,[as reported in a review][14], that the biological potency of a series of compounds varied inversely with their water solubilities. Further research into quantitative relationships between structure and biological activity followed in the late 20^{th} century, as reviewed [15], is now widely accepted that many bioactive substances have lipophilic properties that are important to their pharmacological and biological activities because lipophilicity influences transport across cell membranes and ligand affinity to receptor binding sites. These findings led to a proposal, in 1997, by Christopher Lipinski, who formulated a series of rules for drug design known as Lipinski's rule of five [16] based on the observation that most successful drugs are relatively small and lipophilic molecules limited in heteroatom content with octanol-water partition coefficients (*P*) less than 10^5 . Though later examination of the rules has spawned many extentions [17], the challenges of delivering drugs to target tissues with desirable pharmacokinetic properties often focuses efforts to develop bioactive substances with log *P* values between 0 and 5. A role of lipophilicity in potency has been suggested to explain the greater sweetness of sucralose, a disaccharide with chlorine

substituted for hydroxyl groups, relative to sucrose [18].

Many biologically potent molecules have ionized functional groups that present energy barriers to their transport between subcellular compartments. In addition, some are sequestered in cells and biological fluids owing to their binding to proteins. These factors combine to affect the sequestration and solubility of bioactive substances *in vivo* [19]. For example, in plants, lipophilic metabolites are often conjugated to carbohydrates and other carboxylic acid containing groups. These structural features provide for their transport, often into vacuoles, for storage. Many of these substances serve the plant as chemical defense compounds, and such sequestration ensures that they will not be washed away or degraded within plant tissues.

1.3 Structural Diversity of Natural Lipids

The diverse group of compounds traditionally defined as lipids encompasses all compounds that are soluble in organic solvents [20]. Within this broad classification, subgroups are often defined as neutral lipids, largely consisting of steroids and glycerolipids, and ionic lipids containing functional groups that can be ionized under certain physiological conditions (e.g. fatty acids, sphingolipids, and phospholipids) [1]. In another classification, biological lipids are organized based on their origin entirely or partially from two different types of building blocks: isoprenoid and ketoacyl groups [21]. Using this definition, lipids can be divided into eight categories: fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids, and polyketides (derived from condensation of ketoacyl subunits), and sterol lipids, and prenyl lipids (derived from condensation of isoprene subunits). Structures of some examples of these different lipid classes are presented in Figure 1.1.



Figure 1.1 Structures of various lipid classes.

1.3.1 Lipids Derived from Fatty Acids

Lipids have many biological functions that are determined by their chemical structures. Some lipids, including many glycerophospholipids, perform structural functions, serving as constituents of membrane bilayers that sequester different domains of cells, whereas others play roles as signaling molecules that trigger or suppress specific signaling pathways. Furthermore, some lipids, such as the plant glycoalkaloid tomatine also act as chemical defenses to protect organisms from predation [22]. Lipids also serve important biological functions as energy storage compounds, as is the case for triacylglycerols. While the glycerophospholipids are the main structural component of biological membranes, other non-glyceride lipid components such as sphingomyelin, and sterols (mainly cholesterol in animal cell membranes) are also found in biological membranes [23]. algae and plants, galactosyldiacylglycerol In and sulfoquinovosyldiacylglycerol lipids are important components of membranes of chloroplasts and related organelles, and are the most abundant lipids in photosynthetic cells or organelles, including those of higher plants, algae and certain photosynthetic bacteria [24]. In recent years, evidence has shown that lipid signaling is a vital part of how cells sense and respond to changing environments, and members of several different lipid categories have been identified as cellular messengers and signaling molecules [25,26]. In plants, some lipids regulate defense mechanisms. For example, the plant oxylipin jasmonic acid (JA) is derived from fatty acid linolenic acid. JA has different functions in the plant including regulating plant biochemical responses to stresses and also in regulating plant growth, development, and reproduction [27]. Fatty acid structure affects cellular activities through changes in membrane lipid composition and the generation of a diverse suite of bioactive derivatives. In this regard, arachidonic acid (AA) produced by herbivores and pathogens, but not by plants, acts as a signaling molecule that

elicits plant stress and defense signaling cascades [28].

The oxygenation of lipids, particularly of unsaturated fatty acids released from cell membranes, produces an array of important oxylipin mediators of inflammation and vascular health, and these exhibit emerging roles in atherosclerosis development and progression. These lipids are commonly thought to act in or near cells and tissues where they are produced. Eicosanoids are oxygenated 20-carbon polyunsaturated fatty acids (PUFAs), and oxygenation of PUFAs is based on the activation and transfer of molecular oxygen to an unsaturated carbon. For example, the epoxyeicosatrienoic acids (EETs) are formed from arachidonic acid (AA) and have anti-inflammatory and cardioprotective actions [29,30]. The epoxides of other long-chain PUFAs are also bioactive but their impact on atherogenesis has not been clarified [31]. Another class of oxylipins is the hydroxyeicosatetraenoic acids (HETEs), which are eicosatetraenoic acids, substituted in any position by one or more hydroxyl groups. They are important intermediates in a series of biosynthetic processes leading from arachidonic acid to numerous biologically active compounds.

Lipid metabolites also regulate functions of assorted nuclear receptors that have broad biological importance. For example, the aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor, and genes transcriptionally regulated by the AhR are primarily involved in foreign chemical metabolism, including the xenobiotic-metabolizing cytochrome P450 enzymes from the 1A and 1B families [32,33]. Many types of compounds, ranging from widespread synthetic environmental contaminants to dietary metabolites formed in the acidic environment of the stomach, can serve as regulators of the AhR. More recently, strong evidence supporting the existence of a putative high-affinity endogenous ligand(s) that activate(s) AhR by directly binding and transforming the receptor to its DNA-binding form has also been provided [34].

Another nuclear receptor with broad functions in regulation of gene expression is the peroxisome proliferator-activated receptors (PPARs). PPARs regulate several biological processes including differentiation, development, neoplastic conversion, inflammation and wound healing in addition to their critical roles in energy (lipid and carbohydrate) metabolism in which PPARs play central roles in regulating the metabolic oxidation and storage of dietary lipids, essentially by serving as sensors for fatty acids and their metabolic intermediates [35]. A wide variety of natural or synthetic compounds have been identified as PPAR ligands. Among the synthetic ligands, the lipid-lowering drugs, fibrates, and the insulin sensitizers, thiazolidinediones, are PPAR alpha and PPAR gamma agonists, respectively, which highlights the important role of PPARs as therapeutic targets [36]. Also, endogenous ligands for the PPARs include free fatty acids and eicosanoids, some of which exert control of biological functions at trace levels [37].

1.3.2 Terpenes and Terpenoid Lipids

While the fatty acid-derived lipid constituents of membrane bilayers are well known, it is a different class of compounds, the terpenes and terpenoids, that are the largest and most diverse class of specialized lipid metabolites. More than 20,000 terpenoid compounds had been described by the mid-1990s, [38] making this perhaps the most diverse class of natural metabolites. Terpenoids are made up from isoprenoid subunits, which are branched five-carbon substructures. Oxidative modifications of terpene hydrocarbons generate various oxygen based functional groups (e.g. -COOH,-OH,-C-O-C-, -C=O), and form terpenoids. Terpenes and their metabolites exhibit a wide variety of biological functions. Some terpenoid lipids display as flavor or fragrance compounds that attract pollinators or repel predators [39]. Others have

insecticidal [40], bactericidal [41], antifungal [41], antiviral activity [28]. One group of diterpenoid glycosides from the plant genus Stevia have been used as natural non-caloric sweeteners for decades, and research improve glycoside profiles continues to this day [42].

Not all lipids are made from linear substructures; some, including diterpenes, cholesterol, and β -carotene are cyclic compounds. The biosynthesis of terpenes is derived from different precursors using two main pathways [43]. The mevalonate pathway (MVA pathway) was the pathway discovered first, and it takes place in the cytosol of the cell, at least in plants. Accumulation of experimental results from various ¹³C labeling experiments yielded findings that could not be explained by the MVA pathway and led scientists to research alternative biosynthetic pathways and terpene precursors. In the early–mid 1990s, the methylerythritol-4-phosphate pathway (MEP) pathway was first proposed. Further research has shown that plant plastids and most bacteria utilize the MEP pathway to accomplish terpene synthesis [44]. Isopentenyl pyrophosphate and dimethylallyl pyrophosphate are the two main precursors of terpene synthesis and both are the products of both pathways. These two pathways combine to produce certain terpenes that have origins from both pathways despite formation of the precursors in different locations of the cell.

1.3.2.1 Methyl Erythritol Phosphate (MEP) Pathway of Terpene Synthesis

The five-carbon metabolite methylerythritol phosphate (MEP) is produced in the nonmevalonate pathway (Figure 1.2) from pyruvate condensation with glyceraldehyde diphosphate, using thiamine diphosphate as a cofactor. MEP serves as the precursor of various terpenes since it is a precursor of isopentenylpyrophosphate (IPP) and dimethylallylpyrophosphate (DMAPP). Some mechanisms have been proposed that explain the conversion of MEP to IPP and DMAPP [44,45]. Once the pyrophosphate esters of precursor terpene alcohols (IPP, DMAPP, GPP, FPP and GGPP) are formed, a family of enzymes called terpene synthases (TPS) catalyzes the formation of hemiterpenes (C5), monoterpenes(C10), sesquiterpenes(C15) diterpenes(C20), and some yet larger terpenoid metabolites.



Figure 1.2 Biosynthetic origin of 2-methyl-D-erythritol-4-phosphate, an important precursor of terpenoid lipid metabolites.

1.3.2.2 Mevalonate (MVA) Pathway of Terpene Synthesis

In the mevalonate (MVA) pathway of terpene synthesis [39,43] (Figure 1.3), mevalonate is derived from condensation of three acetyl CoA molecules. A series of subsequent metabolic reactions form isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). These five-carbon pyrophosphates can undergo subsequent condensations to yield geranyldiphosphate (10 carbons), farnesyldiphosphate (15 carbons), and geranylgeranyldiphosphate (20 carbons). A family of terpene synthase genes can convert these pyrophosphate metabolites to an assortment of cyclized and rearranged products of enormous diversity owing to numerous double bond configurations, stereochemistry, and ring cyclizations (Figure 1.4).



Figure 1.3 Conversion of acetyl CoA into monoterpene and sesquiterpene precursors through the mevalonate (MVA) pathway.

1.3.3 Polyketide Lipids

The wide spectrum of activity of polyketides makes them economically, clinically and industrially some of the most sought after molecules. Almost two thirds of all drugs currently in use are at least in part derived from a natural source, [46] and many polyketide products are

successful pharmaceuticals including erythromycin A and oleandomycin (macrolide antibiotics), rapamycin (immunosuppressant), rifamycin (ansamycin antibiotic), lovastatin (anticholesterol drug), epothilone B (anticancer drug), oxytetracycline (tetracycline antibiotic), and resveratrol (polyphenol) are a few of the thousands of polyketides discovered so far.

Nature produces a wide assortment of bioactive lipids that are not derived from either fatty acids or terpenes. Polyketides are such a group of secondary metabolites, exhibiting phenomenal diversity in terms of their structures and functions. This class of metabolites is derived from malonyl CoA, which undergoes repeated condensation to yield intermediates with ketone groups on alternating carbon atoms in a linear chain. This biochemistry shares striking similarities with fatty acid biosynthesis [47,48], and subsequent Claisen condensations yield a diverse group of cyclic metabolites. The enzymes responsible for these condensations, the polyketide synthases (PKSs), are a family of enzymes or enzyme complexes that are known in bacteria, fungi, plants, and a few animal lineages.



Figure 1.4 Conversion of isopentenylpyrophosphate and dimethylallylpyrophosphate into an assortment of terpenoid metabolites via catalysis by terpene synthases [49].

1.4 Lipid Profiling and the "omics" Era

As was pointed out in the previous pages, there is a range of important natural products derived from lipid biosynthetic pathways. Lipids have been implicated in a wide range of disorders, including heart disease [50], stroke [51], arthritis [52], cancer [53], diabetes [54] and Alzheimer's disease [55], however, our understanding of the exact functions and biological mechanisms involved in lipid modulation of disease is still at an early stage. To address gaps in our knowledge of lipid functions, the U.S. National Institutes of Health established a research consortium known as "LIPID MAPS (Metabolites and Pathways Strategy)," One of the early activities of the LIPID MAPS group was to standardize lipid nomenclature in a manner consistent with modern databases. Additional research has focused on development of advanced analytical protocols for comprehensive lipid profiling. This global approach has been termed lipidomics, and represents a subset of metabolomics, the global profiling of all metabolites. The driving force behind the comprehensive "omics" approach has been the desire to propel biological research faster than can be achieved by studying a single compound at a time.

1.5 Analytical Technologies for Lipid Profiling

Lipids comprise a chemically diverse set of compounds that challenge standard analytical procedures. A portion of this challenge arises from the lack of distinguishing chromophores that would allow for selective detection and quantification of lipids. Particularly in the case of oxylipins, numerous isomers are possible that differ in position of substituents, double bond configurations, and stereochemistry. Most spectroscopic methods are not capable of

distinguishing these isomeric forms at the low levels at which they occur in biological tissues and fluids.

Physical separation of individual lipid forms often is required before detection because organisms produce complex mixtures of lipids and these compounds lack unique chromophores or other characteristics that common detectors can measure. Owing to the possibility that a single organism may accumulate thousands of lipid metabolites with similar physical properties, no single separation procedure can resolve all lipids present in biological tissues or fluids.

The partitioning of analytes between a mobile phase and a stationary phase has become a method of choice for separating lipids and other compounds. The origins of this approach were first explored by Mikhail Tswett in 1890, when he separated plant pigments using a packed column of calcium carbonate, eluting them with a liquid mobile phase. In 1903, he published his findings, but these were dismissed by the scientific community and did not resurface until 1931 when Kuhn and coworkers performed carotenoid separations and re-introduced the scientific community to column chromatography (liquid chromatography). In the 1970s, some improvements including pressurization of the mobile phase, use of smaller particle sizes for packing material, and shorter columns gave liquid chromatography a new life. This new technology, termed high performance liquid chromatography (HPLC), improved chromatographic resolution and shortened separation times. Furthermore, recent improvements such as use of sub-2 µm particles and shorter columns, introduced the technology of ultra high performance liquid chromatography (UHPLC), which is becoming the standard technique for separating a wide range of compounds.

Long-standing methods including thin-layer-chromatography (TLC) still find widespread use for separation of lipids [20, 56-58, 59-62]. Improvements to TLC separation are known as

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high performance TLC (HPTLC) which employ smaller particles and finer grades of silica than traditional separations [58]. Further improvements in chromatographic resolution are achieved through addition of various organic and inorganic compounds to the stationary and mobile phases [63-65]. However, even HPTLC separations yield limited resolving power, poor reproducibility in retention, and the potential for analyte oxidation during separations. Since lipid analytes are frequently exposed to atmospheric oxygen during TLC separations, oxidation and hydrolysis of unsaturated lipids can occur, particularly if the TLC plate is stored for long periods [66]. Perhaps the most limiting aspect of TLC separations derives from the common use of polar stationary phases such as silica with less polar solvents, in modes known as normal phase chromatographic separation. Such separations resolve lipids based almost entirely on their polar head groups, and do not provide information about structural variations within a head group class such as fatty acid composition.

The limitations of TLC can often be overcome by using alternative separations such as gas chromatography (GC), which offers a substantial increase in the number of theoretical plates of separation. Gas chromatography separates volatile compounds based on their partitioning between a carrier gas and a stationary phase, and modern capillary GC columns are long (typically about 30 meters) and offer more than 10⁵ theoretical plates of separation. This represents a more than 10-fold improvement relative to HPTLC. The high separation efficiency of capillary GC has made separations of fatty acids, usually as their methyl esters, a routine procedure that can distinguish number of double bonds and fatty acid chain lengths. However, polar complex lipids lack the volatility needed for GC analysis, and as a consequence, complex lipids are usually separated by head group class first, followed by conversion of their fatty acids to volatile esters for GC analysis. Without prior separation of lipids by head group class [67],

conversion to fatty acid methyl esters (FAMEs) [20] loses structural information because the presence of fatty acids in specific complex lipids is destroyed upon transesterification.

When GC separations are coupled to mass spectrometry (MS) detectors, this combination offers the advantage of using libraries of mass spectra to aid structural identification of the compounds. However, mass spectra often fail to distinguish isomeric FAMEs that differ in double bond positions or configurations. The combination of mass spectra with GC retention times usually does allow double bond locations and configurations to be distinguished, and this approach has found widespread use for fatty acid analysis [69].

Since TLC separations fail to resolve lipids differing in fatty acid content, and GC separations are limited to analyses of volatile fatty acid esters, comprehensive profiling of lipids requires separation methods capable of resolving large numbers of intact lipids, including resolution of fatty acid composition. In recent years, HPLC separations have been developed for lipid separations, but successful HPLC lipid profiling has depended on development of detectors capable of detecting a wide range of lipids. Nonselective light-scattering detectors [70] proved to be capable of detecting virtually all eluting lipids, but the chromatographic peak capacities of HPLC remained inadequate to resolve all lipid forms. Only when HPLC separations were coupled with mass spectrometric detection was the true complexity of the lipidome (total complement of lipids) evident. Research demonstrating use of HPLC for lipid separation by class [71-73] established that HPLC is the preferred method of separation when complex lipid analyses are to be performed.

The limitations of chromatographic resolution continue to be addressed by improvements in chromatographic column technologies. Chromatographic separation efficiency, usually measured in terms of the number of theoretical plates of separation, is directly linked to the size, stability, and structures of the stationary phase particles. Separation efficiency in chromatography is described by the Van Deemter model [74], which relates the height of a theoretical plate of separation (H) as a function of the mobile phase velocity (u). The Van Deemter equation proposes that efficiency is determined by: (a) the diversity of paths an analyte molecule can take through chromatographic media, (b) longitudinal diffusion through the media, and (c) rates of analyte mass transport during the separation [75]. Therefore, a reduction in stationary phase particle sizes and particle heterogeneity makes paths of analyte flow through the column more homogeneous. Mass transport between mobile and stationary phases is improved through the use of nonporous or superficially porous chromatographic media. These advances combine such that reducing particle sizes leads to three predominant effects: the minimal plate height is reduced (leading to more plates in a given column), the optimal linear velocity of the mobile phase for achieving the minimum of the H/u curve shifts to a higher flow rate, and the loss in efficiency at analysis speed far beyond the optimum is much less pronounced because hindered mass transfer becomes less distinct with shorter intra-particle path length. Today, particle sizes of less than 3 µm are typically considered as ultra high performance liquid chromatography (UHPLC) materials, which can be used for fast separations suitable for analyses of large numbers of samples [76]. UHPLC separations also yield another beneficial side-effect when columns with inner diameters (IDs) of 2 mm or less are used, as analytes elute in smaller peak volumes. Since many LC detectors including ultraviolet-visible and mass spectrometric detectors respond to analyte concentration (and not absolute analyte mass), these narrow columns afford lower limits of detection as well as large savings in solvent consumption and waste disposal costs.

As mentioned above, normal phase chromatographic separations of lipids rely on attractive

forces between the polar stationary phase and polar groups on the lipids. Because these interactions are strong, normal phase LC separations are efficient for separating different lipid classes, but often fail to resolve subtle structural differences between lipids that share similar polar groups. In contrast, reversed phase (RP) separations employ nonpolar columns and polar mobile phases, and separations are largely governed by solubilities of lipids in the mobile phase. Since solubilities in polar solvents show strong dependence on molecular size, shape, and solute-solvent interactions, RP separations are usually preferred for separations of compounds within a specific lipid class. As a result, reversed-phase HPLC has become the dominant approach to liquid chromatographic separations of lipids and many other analytes [77].

Lipids include a diverse group of chemicals from different structural classes. While they share common physical properties, there are no structural features common to all lipids. In view of this structural diversity, not all lipids exhibit useful ultraviolet (UV) or visible chromophores that can be used for universal lipid detection. Even though UV-visible spectroscopy has been used to detect some lipids [71,72], the inability of this approach to detect non-chromophoric lipids and the commonality of many lipid chromophores limit the utility of spectroscopic detection of lipids. Though nuclear magnetic resonance (NMR) spectroscopy has also been used as a powerful technique for elucidation of lipid structures [78,79], spectral overlap limits the ability of NMR to resolve lipids in mixtures [80]. Furthermore, NMR requires high lipid concentrations (about 1 mg/ml) and relatively high purity. NMR spectra of mixtures yield complex spectra that usually preclude quantitative analysis of individual lipid species [66].

Because natural lipids encompass a chemically diverse group of substances, comprehensive profiling of lipids requires analytical techniques that can detect as many lipids as possible. In addition, it is useful when the analytical method yields information about lipid

identity, such as molecular mass, that helps distinguish one lipid from another. Molecular mass alone may not distinguish isomeric lipids, so additional information about structure is also desirable. Modern mass spectrometry techniques go a long way toward achieving these analytical goals. However, until the late 20th century, the use of mass spectrometry for lipid analysis was largely confined to analysis of fatty acid methyl esters (FAMEs) because these were sufficiently volatile to be analyzed after GC separations.

The quest for more useful mass spectrometric analyses found great successes with the development of new "soft ionization" techniques that more reliably generated information about molecular masses of a wide variety of substances. An important aspect of these advances arose from newfound ability to evaporate and ionize substances that previously had decomposed upon heating. An assortment of soft ionization techniques including field desorption, fast atom bombardment, and chemical ionization found some success, but were not easily interfaced to liquid chromatographic separations which were necessary for numerous complex lipids. The development of electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) in the late 1980s led to easy coupling of LC separations with mass spectrometric detection for a vast array of analytes. Direct application of ESI/MS to analysis of lipid mixtures usually needs no derivatization, offers subpicomole detection limits, and yields molecular mass information and reproducible quantitative results [81].

The successes achieved by many laboratories through the application of mass spectrometry (MS) for lipid profiling has had a profound impact on establishing an area of research termed lipidomics, which aims to achieve comprehensive lipid profiling [82]. Mass spectrometry is the method of choice for global lipid profiling because of its low limits of detection, wide mass range, and richness of structure information [83]. Research increasingly employs two approaches toward for lipid profiling. One approach is known as global lipidomics, and the
focus of this method is identification and quantification of all lipids in a cell or tissue sample. The other approach is called targeted lipid profiling, and the main goal here is identifying and quantifying a large number of lipids within a single class [82].

About a decade ago, Han and Gross were pioneers in developing a now widely-used method for profiling cellular lipids using ESI-MS [84]. In this method, lipids are extracted in chloroform and the diluted extracts are introduced into a quadrupole mass spectrometer without any chromatographic separation. This approach yields profiles of abundant membrane lipids. However, since some analytes present at low concentrations will not be detected because more abundant compounds suppress their ionization, it is necessary to use chromatography as a physical separation method before introduction of analytes into the mass spectrometer ion source.

Following the formation of pseudomolecular ions in the mass spectrometer source, a mass filter can select ions of a specific mass, and ions of all other masses are removed from the instrument. The selected pseudomolecular ion can then be converted into fragment ions using one of several ion activation methods such as tandem or multistage mass spectrometry. The most prevalent ion activation method is called collision induced dissociation (CID). When an ion collides with a neutral atom or molecule, the collision converts some of the ion's kinetic energy into internal, usually vibrational, energy. When there is enough excess internal energy, the chemical bonds of the analyte can break and the ion will undergo a variety of unimolecular reactions that generate fragment ions of lower mass. By generating MS/MS fragment ion spectra derived from only one isolated mass, it is possible to track functional groups and connectivity of fragments to elucidate the structures of targeted pseudomolecular ions [85].

While MS/MS is a very powerful approach, it suffers from barriers to performing MS/MS

experiments on all of the observed ions in spectra of complex mixtures. An alternative approach involves in-source collision induced dissociation (CID) which is also called MS^E [86-88]. In this method, collisional activation is performed without pre-selecting a specific ion for fragmentation, and ions of all masses are activated and fragmented. The MS^E approach switches collision energies between high energy and low energy collisions, with the former leading to fragment generation. Spectra are obtained both with, and without, fragmentation, so molecular mass and fragment masses are determined in a single analysis. In many applications, accurate high resolution mass measurements are generated for fragments and molecular ions. When coupled with LC separation, MS^E allows fragments to be associated with molecular ions based on their common chromatographic retention times.

When collision induced dissociation mass spectra are generated, the masses of fragment ions often yield important information about molecular structures. However, one of the unfortunate limitations of using mass spectrometry for structure elucidation is its inability to distinguish isomeric compounds based only on fragment ion masses. One approach that provides additional structure information involves generating CID spectra at various different collision energies because isomeric compounds frequently have different activation energy barriers in fragmentation reactions. Fragmentation reaction activation energies can be probed by determining the collision energy thresholds for appearance of specific fragment ions. More detailed understanding usually requires that CID spectra be generated for multiple compounds in order to generalize whether appearance thresholds are characteristic of specific substructures or functional groups. However, simple interpretation of these results requires that the experimenter normalize collision energies in the center-of-mass reference frame, where the system includes the precursor ion as well as the target collision gas. This inertial reference frame allows calculation of the maximum amount of energy (E_{cm}) that can be deposited in the precursor ion as a result of a collision. Calculation of E_{cm} is straightforward as long as the mass of the ion, mass of the target gas, and the collision energy in the laboratory reference frame (E_{lab}) are known. This fraction can be calculated as follows:

$$E_{cm} = E_{lab} \left(\frac{m_{gas}}{m_{ion} + m_{gas}}\right)$$

Inert gases such as argon and nitrogen are often used as target gases for collision induced dissociation, and typical values of E_{lab} range from 0-100 volts. Increasing the ion kinetic energy or using a more massive target gas can increase the amount of kinetic energy that can be deposited in the ion as internal energy, usually in the form of vibrational energy. Where the precursor ions have kinetic energies in the range of few eV to a few hundred eV (E_{lab}), the collision is called a low-energy collision, and typically deposits less than 1 eV into the ion's vibrational modes. This energy undergoes rapid distribution into all of the vibrational degrees of freedom of the ion. On the time scale of picoseconds to microseconds, the excited ions undergo unimolecular decomposition reactions to form fragment, or product ions. The diversity of product ions that are observed upon low-energy collision depends strongly on the internal energy distribution. Increasing the collision energy shifts the center of internal energy distribution to a higher value and changes the observed product ions, yielding more products via higher activation energy reactions. Therefore, a product ion mass spectrum obtained from 10 eV collisions (E_{lab}) can show profound difference relative to a spectrum obtained from 25 eV collisions. The target mass has a dramatic influence on the MS/MS spectrum for low-energy collision, and when the yields of product ions are low, increasing the mass of the target gas usually increases yields of

product ions.

Metabolite structure identification remains one of the most challenging aspects of global metabolite profiling, and the overwhelming majority of metabolites detected in analysis of biological extracts remain unidentified. Determination of molecular structure by mass spectrometry is analogous to solving a jigsaw puzzle, as a molecule or a mixture of molecules is ionized with sufficient amount of internal energy to fragment it into several smaller pieces which are then individually identified and reassembled using valence rules to determine the structure of the original ionized molecule [89]. As mentioned above, knowing the molecular and fragment masses is often still not sufficient for unambiguous structure elucidation. When proof of structure by synthesis is not practical, the final proof for identification of any compound usually relies on comparing LC retention times, accurate molecular mass measurements, and MS/MS spectra with an authentic standard [90]. However, most of the time, standard compounds are not available for such comparison; therefore, for identification of compounds (novel or non-novel) for which standards are not available, investigators should use combinations of physicochemical properties, such as accurate mass measurement, UV/IR spectra, elemental analysis, crystal structures, and NMR spectra [90-91].

As explained in previous pages, mass spectrometry has emerged as a method of choice for lipid identification and quantification due to figures of merit for such analyses. For lipid analyses described in Chapter Four, a Waters LCT PremierTM mass spectrometer was used which employs an orthogonal acceleration time-of-flight (TOF) mass analyzer. TOF mass analysis offers high mass resolution and accuracy, fast spectrum acquisition (~20,000 spectrum transients per second), and adequate dynamic range [92,93]. Due to these features, TOF analyses have been broadly applied for metabolic profiling, and are particularly powerful for metabolite

discovery research [94-96].

1.6 Sweeteners and Efforts to Discover Natural Sweet-tasting Substances

The fondness of human for sweet substances is inborn and mankind has always added sweeteners in their food. Honey, the first sweetener used by people in ancient cultures of China and Greece [97] was later replaced by sucrose (common sugar) extracted from sugar cane. In today's world, due to dietary and health issues, the market for sweeteners as alternatives to carbohydrate-based sweeteners has expanded. Consumption of calorigenic sweetening compounds is associated with medical and nutritional concerns including obesity [69,98,99], diabetes mellitus [100], and hyperlipidemia [101]. Therefore, great effort has been expended to substitute sugars with new sweet-tasting low caloric sweeteners [102], but it is also important to consider that alternative sweeteners are successful if only they can perfectly replace the taste quality of sucrose. Therefore, sucrose substitutes are required to have a clean sweet taste, a quick onset of sensation, and minimum persistence and aftertaste [103]. Also, these sugar substitute compounds are required to exhibit properties such as non-toxicity, non-cariogenicity, and exhibit satisfactory water solubility and hydrolytic and thermal stability [104]. However, most common alternative sweeteners on the diabetic and dietetic today's market are synthetic compounds such as aspartame, cyclamate, saccharin, and sucralose [105] which frequently exhibit metallic and bitter aftertastes that do not provide the appealing taste of sugar. In some cases artificial sweeteners cannot be consumed by certain subpopulations (e.g. aspartame) or are associated with increased risk of cancer (saccharin). For example, people with the metabolic disease phenylketonuria should avoid consumption of aspartame because they lack the ability to convert phenylalanine, a constituent group in aspartame, to tyrosine [102]. Also, high doses of saccharin have been reported to increase incidence of bladder cancer [106,107]. Reports such as these have raised concerns among people about the safety of artificial or synthetic sweeteners, and many consumers have shifted from consuming synthetic sweeteners to natural sweeteners. The dramatic increase in attention toward natural extracts is not only for their sweetening effects but also for anticipated additional health benefits attributed to the plant of interests.

One natural source of sweet chemicals is the plant Stevia rebaudiana Bertoni (Stevia), which has been of interest for many years. This plant species is one of the 154 members of the genus Stevia and one of the only two genera that accumulate sweet Stevia glycosides [108,109]. This plant, which grows to 65 cm tall is native to Brazil and northeastern Paraguay (known as sweet herb of Paraguay) and now is cultivated in countries across Asia, Europe, and North America [110] to sweeten a variety of food products. The leaves of this plant contain several different components including flavonoids, sterols, triterpenoids, organic acids, and inorganic salts, but the phytochemicals that attract greatest interest are diterpenoid glycosides known as steviol glycosides [111] (See Figure 2.1 in chapter 2). Among steviol glycosides, the most abundant is stevioside, with a sweetening power of 300 times that of sucrose. A related compound, rebaudioside A, is the second most abundant reported diterpene glycoside and has a sweetening potency 400 times of sucrose [112]. Result of studies have demonstrated that the glycosides of Stevia not only have different sweet potency, but also exhibit different organoleptic properties [113] for example, stevioside imparts a significant bitter aftertaste [114]. Therefore, since flavor of the individual glycosides has a significant effect on their economic value and sensory quality, knowledge of the enzymes and the genes involved in their synthesis is essential to controlling flavor [115] and such discoveries may lead to production of novel glycosides

which have the potential for novel sensory characteristics can be of value.

Above all, for efficient, large scale, and mechanized production of these sweet diterpenes, rapid and accurate analytical methodology of these substances is necessary to guide breeding of plants with desirable flavor and product yields. The analytical methodology should also be capable of distinguishing isomeric glycosides that exhibit similar physical properties with different degree of sweet taste. For analysis of Steviol glycosides described in the following chapters, a triple quadrupole (QQQ) mass spectrometer was employed which enable multiple reaction monitoring (MRM) experiments and generation of tandem mass spectra (MS/MS). The MRM approach fixes two mass analyzers to transmit only ions of preselected mass, with a collision event between the analyzers that converts pseudomolecular ions to compoundcharacteristic fragment ions. For one of the analytical protocols, pseudomolecular ions and their major fragments were used as transition channels for selective and sensitive detection of multiple natural sweeteners in a high-throughput mode designed to discover plants with desirable metabolite accumulation. These findings are reported in Chapter Two. In Chapter Three, product ion MS/MS spectra were generated to better understand the fundamentals of steviol glycoside fragmentation for structural elucidation and prediction of sugar moiety positions on known glycosides.

1.7 Challenges in Identification and Quantification of Sweeteners

One important aspect of profiling and quantifying natural sweeteners, or many phytochemicals, lies in the remarkable structural diversity of plant constituents. As a result, many plant tissues accumulate a variety of metabolites that are isomers, often varying only in the positions of substitution of functional groups. When such isomers are encountered, as is the case with Steviol glycosides, mass spectrometry alone may not be sufficient to distinguish isomeric metabolites. When these situations are encountered, a powerful separation method is necessary for separation of these to resolve isomers before introducing them to the mass spectrometer. However, isomers often have similar physical properties, and the separations are often challenging, so adequate chromatographic separation method frequently requires lengthy separation protocols, which are not ideal for large scale high throughput analysis. Fortunately, the advent of ultrahigh performance LC separations coupled with selective mass spectrometry protocols hold promise that rapid quantitative measurements can be made for numerous bioactive lipid metabolites in a single analysis. The primary goals of the research described in this dissertation have aimed to develop fast and powerful analytical protocols and develop an improved understanding of the factors that accelerate the profiling of bioactive lipids in extracts of biological tissues.

Chapter Two: Large-scale Profiling of Diterpenoid Glycosides from *Stevia rebaudiana* using Ultrahigh Performance Liquid Chromatography/Tandem Mass Spectrometry

2.1 Abstract

The plant Stevia rebaudiana accumulates a suite of diterpenoid metabolites that are natural sweeteners finding, increased use as sugar substitutes. To guide breeding of Stevia plants that accumulate substances with desirable flavor in high yield, rapid and accurate methods are needed to profile these substances in plant populations. We report an 8-minute ultrahigh performance liquid chromatography-tandem mass spectrometry (UHPLC/MS/MS) method for separation and quantification of seven stevia glycosides including steviolbioside, stevioside, Rebaudiosides A, B, and C, rubusoside and dulcoside as well as aglycones steviol and isosteviol. This negative mode electrospray ionization/multiple reaction monitoring (MRM) method yielded low limits of detection (LLOD) <1 ng/ml for steviol, 6 ng/ml for isosteviol, and <15 ng/ml for all stevia glycosides. Stevioside and Reb A, B, and C were quantified in more than 1100 extracts from Stevia leaves as part of a large scale profiling exercise. Leaf tissue levels in this population spanned about two orders of magnitude for stevioside (2-125 mg/g dry weight), Reb A (2.5-164 mg/g), Reb B (0.5-50 mg/g), and Reb C (1.5-125 mg/g), but levels of individual metabolites exhibited independent variation. The wide spread of metabolite levels highlights the utility and importance of performing targeted metabolic profiling for large plant populations.

2.2 Introduction

The search for improved natural non-caloric sweeteners

Stevia rebaudiana Bertoni is a sweet herb from the family *Compositae* native to Brazil and Paraguay, and is cultivated in countries from almost all continents. Natural non-caloric sweeteners called stevia glycosides are glycoconjugates of the diterpenoid aglycone steviol and

can be extracted from the leaves of Stevia plant. These sweeteners have been commercially available in much of East Asia, and are used to sweeten a variety of food products. Stevia leaf and extracts are also used as dietary supplements and for skin care in the United States [110,112,116].

Stevia glycosides consist of a diverse group of metabolites that differ in sugar composition and positions of substitution. Structures of steviol, its isomer isosteviol, and important stevia glycosides are depicted in Figure 2.1. Due to the variation in the structures of these diterpene glycosides, the quality of their flavors varies from one sweetener to another. For instance, Rebaudioside A (simply termed Reb A from here on) imparts a sweet taste whereas stevioside exhibits a significant bitter aftertaste [114,117]. This difference in biological activity is due to the presence of an extra glucose moiety in the Reb A structure. Manipulation of the glycoside moieties of stevia glycosides has been achieved in efforts to alter the sensory characteristics of these natural products.

Anticipated worldwide growth in usage of stevia glycosides as natural sweeteners is driving a need for improved plant breeding to enhance yields of those specialized metabolites with desirable flavor qualities and decrease levels of less desirable phytochemicals. To achieve these goals, rapid analytical methods are needed for quantitative profiling of an assortment of stevia glycosides in large numbers (thousands) of Stevia tissue extracts. Such analytical methodologies should be capable of distinguishing isomeric glycosides that exhibit similar physical properties. Chromatographic separations of stevia glycosides have been performed dating back to just after the first structure elucidations of these phytochemicals, but these frequently involved long separations of about 1 hour in duration per sample [118-122]. More recently, liquid chromatography (LC) coupled to ultraviolet (UV) or mass spectrometry (MS) detection has emerged as the dominant approach used for analysis of stevia glycosides. A recent report by Gardana and co-workers [123] measured levels of several stevia glycosides in leaf extract using a solid phase extraction cleanup followed by ultrahigh-performance liquid chromatography-electrospray ionization mass spectrometry (UHPLC-MS), using selected ion monitoring of chloride adduct ions. Although the method is rapid, two different LC gradients were used to quantify stevia glycosides and the aglycone steviol in separate analyses.



(1)		(2)	[M-H] ⁻	
Compound	R ₁	R ₂	(m/z)	
Steviol	Н	Н	317	
Isosteviol	Structure # 2		317	
Steviolbioside	н	β-Glc-β-Glc(2-1)	641	
Rubusoside	β-Glc	β-Glc	641	
Dulcoside	β-Glc	β-Glc-α-Rha(2-1)	787	
Stevioside	β-Glc	β-Glc-β-Glc(2-1)	803	
Reb B	Н	β-Glc-β-Glc(2-1) β-Glc(3-1)	803	
Reb F	β-Glc	β-Glc-β-Xyl(2-1) β-Glc(3-1)	935	
Reb C	β-Glc	β-Glc-α-Rha(2-1) β-Glc(3-1)	949	
Reb A	β-Glc	β-Glc-β-Glc(2-1) β-Glc(3-1)	965	
Reb D	β-Glc-β-Glc(2-1)	β-Glc-β-Glc(2-1) β-Glc(3-1)	1127	

Figure 2.1 Structures of (1) steviol metabolites including glycosides and (2) isosteviol, showing nominal masses of [M-H]⁻ ions observed in negative mode electrospray ionization mass spectra. Abbreviations: Glc: Glucose, Rha: Rhamnose, Xyl: xylose.

In efforts to accelerate analytical throughput, Cooks et al. employed another rapid approach for analysis of stevia sweeteners, desorption electrospray ionization (DESI) mass spectrometry. This approach yields direct and semiquantitative analysis of Stevia leaves without need for metabolite extraction ¹²⁴. While these analyses were indeed rapid, the lack of a separation method capable of distinguishing isomers such as stevioside and Reb B that yield fragment ions at the same masses limits the utility of this approach for comprehensive profiling of the desirable phytochemicals. To improve resolution of isomeric stevia glycosides, Pól and Hyötyläinen used a 50-minute two-dimensional (LC x LC) separation and electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS) to separate nine stevia glycosides [125]. Another two dimensional LC attempt for analysis of stevia glycosides was made recently by Cacciola et al [126]. In this work, a polyamine column was coupled to a UHPLC C18 column and UV detection for two dimensional separations that resolved five stevia glycosides and several flavonol glycosides. Although the method clearly resolved several stevia glycosides, the long separation times (~ 60 min) are not optimal for large-scale profiling of hundreds or thousands of tissues.

Despite these recent advances in stevia glycoside analyses, more traditional liquid chromatographic methods still play a workhorse role in these analyses. Stability of stevioside and Reb A in soft drinks was investigated using HPLC with a HILIC column and UV detection, and the identities of degradation products were confirmed using LC-ESI-MSⁿ [127]. Improved extraction efficiencies were reported for stevioside and Reb A using rapid microwave assisted extraction, using an amino column for HPLC separation using isocratic elution and photodiode array detection [128]. This approach afforded quantitative targeted measurements of stevioside and Reb A using a 30-minute separation, but did not aim to profile minor stevia glycosides.

Although several methods have been developed for identification and quantification of stevia glycosides, our need to profile more than 1100 plant tissues within a few months time required development of a reproducible and rapid method for large-scale screening. It was desired to quantify both the abundant stevia metabolites as well as several expected to be present in tissues at much lower levels. Here we present a 8 minute LC gradient using a C18 column packed with fused core particles for separation of 10 known stevia glycosides including steviol, isosteviol, steviolbioside, stevioside, Reb A, B, and C, rubusoside and dulcoside A. To minimize chemical interferences, multiple reaction monitoring (MRM) tandem mass spectrometry was optimized and performed. We have applied this method for analysis of more than 1100 extracts from Stevia plants to select plant lines with desirable biochemical phenotypes.

2.3 Experimental

2.3.1 Plant Growth Conditions

Stevia rebaudiana 'Eirete' and 'Criolla' plants (77 and 59 plants, respectively) were grown in a climate-controlled greenhouse. Dormant crowns were planted into 13-cm square plastic containers (1.1-L volume) filled with a commercial soilless medium (Suremix Perlite blend, Michigan Grower Products, Galesburg, MI) and grown under a constant temperature of 20 °C. Photoperiod was maintained at 16 h by supplementing ambient irradiance with ca. 60 µmol m⁻² s⁻¹ photosynthetically active radiation provided by high-pressure sodium lamps from 0600-2200 HR daily. Plants were irrigated as needed with reverse osmosis-treated well water supplemented with nutrients at a rate of 125 mg·L⁻¹ N, 12 mg·L⁻¹ P, 100 mg·L⁻¹ K, 65 mg·L⁻¹ Ca, 12 mg·L⁻¹ Mg, 1 mg·L⁻¹ Fe and Cu, 0.5 mg·L⁻¹ Mn and Zn, 0.3 mg·L⁻¹ B, and 0.1 mg·L⁻¹ Mo (MSU RO Water Special; GreenCare Fertilizers, Kankakee, Ill.). Three replicate samples were harvested from each plant at weekly intervals. A separate population of 984 Stevia plants derived from an improved seed lot was grown outdoors, and single leaf tissue collections were performed for these plants. Plants were cultivated under the supervision of Professor Ryan Warner of Michigan State University.

2.3.2 Metabolite Extraction

When outdoor-grown plants reached 15 cm in height, the second youngest fully expanded leaf was harvested for diterpene glycoside profiling. For greenhouse-grown plants, the two most apical, fully-expanded leaves were collected. Leaf tissues were dried and pulverized, and 5-10 mg of the dried powder were extracted in a 1.5 mL vial with 1.0 mL of ethanol/water (60%/40%) containing 1 μ M of digitoxin as an internal standard. The vial was vortexed for 30 min and centrifuged ($8,000 \times g$) for 3 minutes to pelletize cellular debris. Extract supernatant (225 μ L) was diluted 6-fold and filtered using Millipore deep well filter plates (0.45μ m) by centrifugation ($1000 \times g$) for 10 minutes into deep well 96-well receiving plates (VWR Scientific), which were stored at -20°C until analyzed.

2.3.3 LC/MS/MS Instrumentation

Analyses were conducted using a QTRAP 3200 mass spectrometer (AB/Sciex) equipped with binary LC-20AD pumps (Shimadzu), a SIL-HTc autosampler, and column oven. All mass spectrometric analyses, including data processing, were performed using Analyst v. 1.4.2 software (AB/Sciex).

2.3.4 Optimization of Instrumental Parameters

Mass spectrometric analysis parameters were optimized by performing flow injection analyses of standard stevia glycosides (ChromaDex, Irvine, CA, USA) using isocratic elution of 50% solvent A (10 mM aqueous ammonium acetate) and 50% solvent B (acetonitrile) with a total flow rate of 250 μ L/min. Enhanced product ion (EPI) scans were generated for the [M-H]⁻ ions for each metabolite using electrospray ionization in negative ion mode to identify abundant product ions. Precursor ions were the deprotonated molecular species for each stevia glycoside using the following initial conditions to establish the masses of product ions used for parameter optimization: curtain gas: 10 (arbitrary units), ion spray voltage: 4500 V, temperature: 500°C, gas 1: 25; gas 2: 25, declustering potential (DP): 20 V and entrance potential: 10 V. EPI spectra were initially generated using three different collision cell potentials (30, 50, and 70 V) to adequately survey a range of product ions. These parameters were used for selecting suitable product ions for MRM transitions. Source and gas parameters were optimized for all the standards including the internal standard digitoxin. Parameters that were optimized for individual compounds and their optimal values are presented in Table 2.1.

2.3.5 LC/MS/MS Analyses

Analytes were separated using an Ascentis Express C18 column (5 cm \times 2.1 mm \times 2.7 µm) using a reversed phase binary gradient. Solvent A was 10 mM aqueous ammonium acetate and solvent B was acetonitrile. Total solvent flow was maintained at 0.4 mL/min and gradient elution was performed using the following solvent compositions: Initial: 93%A/7%B, held for 0.2 min; linear gradient to 80%A/20%B at 0.3 min and then to 52%A/48%B at 5 min; sudden

increase to 1%A/99%B at 5.01 min and to 100%B at 7 min; followed by a linear gradient to initial condition at 7.01 min and a final hold at this composition until 8 minutes. Injection volume and column temperature were 5 µL and 40°C respectively. Instrumental conditions were optimized for MRM detection of each stevia glycoside ion as described below, and the optimized MRM conditions are presented in Table 2.1. For each set of 50 plant extracts that were analyzed, three technical replicates were analyzed for quality control purposes, and a set of calibration standards was analyzed at least three times for each set of two 96-well plates.

2.3.6 Standard Calibration Curves and Quantification

Standard solutions of steviol, isosteviol, and various stevia glycosides were prepared at 0.1, 1, 5, 10 and 20 μ M in 50/50 ethanol/water. A cocktail of all standards was prepared for each concentration to reduce the analysis time. Digitoxin was added to each standard solution at 1 μ M to match the concentration in the plant tissue extracts. Standards were analyzed in triplicate (n=3) at the beginning of each batch of samples, halfway through the batch, and at the end of the batch. Leaf extract samples were analyzed in random order using Microsoft Excel to randomize the order. Technical replicate injections of 3-5 plant tissue extracts were analyzed in each batch to assess instrument performance and method reproducibility.

Calibration curves were generated by integrating MRM chromatogram peaks for each target analyte, and the ratio ($A_{analyte}/A_{internal \ standard}$) was graphed as a function of analyte concentration. Linear regressions were performed using 1/X weighting, and by forcing the calibration line through the origin.

	MRM Transition								
Compound	(m/z)	Rt [*]	DP [*]	EP*	CE*	LLOD [*]	LLOQ [*]	R^2	Slope [*]
Steviol	317 > 317	5.6	-65	-5	-30	0.7	2.3	0.992	28.4
Isosteviol	317 > 273	6.4	-20	-10	-50	6.0	20.0	0.993	0.176
Steviolbioside	641 > 479	3.7	-60	-10	-40	13.0	43.3	0.990	2.47
Rubusoside	641 > 479	3.9	-40	-7	-35	2.7	9.2	0.990	14.1
Digitoxin	763 > 633	5.3	-50	-10	-50	ND [*]	ND	ND	ND
(IS) [*]									
Dulcoside	787 > 625	3.7	-70	-10	-40	3.7	12.5	0.989	2.78
Stevioside	803 > 641	3.4	-45	-7	-40	5.7	19.1	0.988	3.27
Reb B	803 > 641	3.6	-55	-5	-50	14.3	47.6	0.988	1.79
Reb C	949 > 787	3.6	-60	-10	-70	7.5	24.8	0.991	0.997
Reb A	965 > 803	3.3	-70	-10	-65	5.0	16.8	0.995	2.12

Table 2.1 MRM transitions and optimized instrumental parameters LC/MS/MS measurements

 of steviol, isosteviol, and stevia glycosides.

Rt: Retention time (min), DP: Declustering potential (V); EP: Entrance potential (V); CE: Collision potential (V); LLOD: Low limit of detection (ng/mL) for an injection of 5 μ L; LLOQ: Low limit of quantification (ng/mL) for an injection of 5 μ L; Slope: Slope of the calibration curve, all calibration curves were forced through zero with 1/X weighting; IS: Internal Standard; ND: not determined.

2.3.7 Limits of Detection and Quantification

Low limit of detection (LLOD) was defined as the lowest concentration that yielded a signal-to-noise ratio equal to 3 (S/N=3). Instrumental low limit of quantification (LLOQ) was defined as the level giving a signal-to-noise ratio equal to 10 (S/N=10) for each using authentic standards. Background regions of each chromatogram used for S/N calculations were defined

manually.

2.4 Results and Discussion

2.4.1 Ionization and Collision Induced Dissociation of Stevia Glycosides

All of the stevia glycosides, as well as steviol and isosteviol, yielded abundant [M-H]⁻ ions in preliminary investigations of their ionization behavior. Such behavior was expected for the aglycones steviol and isosteviol as well as Reb B and steviolbioside, as these substances have carboxylic acid groups that readily undergo deprotonation. More surprising was the dominance of deprotonated ions for stevia glycosides with acylglucoside moieties, since their most acidic groups are the sugar hydroxyls. Since deprotonated ions were of such high abundance, addition of substances such as CH₂Cl₂ to form chloride adduct ions was deemed unnecessary [123]. The internal standard digitoxin, a steroidal glycoside, also yielded abundant [M-H]⁻, and was judged to exhibit ionization behavior similar to the stevia glycosides.

Plant tissues often accumulate complex mixtures of specialized metabolites. To develop a selective method for quantification of stevia glycosides, tandem mass spectrometry, in multiple reaction monitoring (MRM) mode, was selected to minimize interference by other phytochemicals. In order to maximize the ion current for each MRM transition, collision induced dissociation (CID) was performed on each [M-H]⁻ ion using multiple collision potentials, and the potential that yielded the strongest signal was chosen for the MRM method. As was expected, losses of glucose units were the dominant fragmentation occurred upon CID. Figure 2.2 presents CID enhanced product ion spectra for [M-H]⁻ from Reb A and isosteviol as representatives of all analytes of interest. For all stevia glycosides, transition from [M-H]⁻ to the

fragment corresponding to loss of one anhydroglucose was selected for MRM. Yields of fragment ions derived from $[M-H]^{-}$ of steviol were formed only at negligible abundances at collision cell potentials up to 70 V. Increasing the collision potential further yielded numerous product ions, but all exhibited low abundance. As a consequence of this behavior, the method incorporated a selected ion monitoring mode, transmitting the steviol $[M-H]^{-}$ ion (m/z 317) through both mass analyzers. Isosteviol, however, showed loss of 44 (-CO₂) upon CID, and this transition was used for the MRM method.

2.4.2 Rapid Profiling of Stevia Metabolites Using LC/MS/MS

Profiling of specialized metabolites in large numbers of complex biological samples has become more practical in recent years following improvements in chromatographic and mass spectrometric technologies. Fast ultrahigh performance LC separations have been useful for exploring the biochemical diversity of plant introgression lines [129] and establishing unanticipated connections between levels of primary and specialized metabolites that have led to gene function discoveries [130]. In the current study, our goal has focused on profiling bioactive stevia metabolites extracted from leaves of more than 1100 Stevia plants to guide breeding of plants with desirable flavor and product yields.

The chemical complexity of plant tissues presents a substantial challenge to the goal of rapid metabolite profiling in large numbers of tissue samples. While it is often the case that the



Figure 2.2 Enhanced product ion (EPI) mass spectra for products of [M-H] for (a) Reb A (m/z 965) and (b) isosteviol (m/z 317).

selectivity of tandem mass spectrometry avoids problems associated with interference by other compounds, LC/MS/MS analyses still encounter situations where nontarget metabolites interfere with measurements of target compounds. In the case of stevia glycosides, this issue arises owing to in-source fragmentation of metabolites containing acylglycoside groups. For example, (Figure 2.3), mass spectra of the isomeric compounds stevioside (Fig. 2.3a) and Reb B (Fig. 2.3b) show different ionization chemistry, with the acylglucoside isomer (stevioside) showing extensive neutral loss of the sugar moiety from the ester, whereas Reb B, which lacks acylglucoside groups, underwent minimal fragmentation before mass analysis. The mass spectra shown in Figure 2.3 were generated before parameters such as the declustering potential were optimized. All of the acylglycosides examined in this study underwent facile in-source fragmentation even after parameter optimization. Because the group of stevia glycosides includes numerous members with acylglucoside groups, in-source fragmentation has the potential to form fragment



Figure 2.3 Negative mode electrospray ionization enhanced mass spectra of (a) stevioside and (b) its isomer Reb B showing different amounts of in-source fragmentation, as evidenced by the abundance of the ion at m/z 641. Source parameters used to generate these spectra differ from optimized source parameters used for multiple reaction monitoring analyses.

ions that have the same masses as [M-H]⁻ ions of less glycosylated metabolites. In source fragmentation may not pose a serious problem for targeted LC/MRM analyses, but should be taken into account during efforts to identify stevia glycosides present in plant extracts.

The structural similarity of stevia glycosides, coupled with the wide range of concentrations at which they occur in extracts of plant tissues, presents a special set of considerations during efforts for large-scale metabolite screening, in which speed and reproducibility from sample to sample are desirable. As in the study described below, plant breeding can be directed to identify individual plants that have desirable metabolic traits, but such efforts usually require analyses of hundreds to thousands of extracts in a single study. For this reason, short analysis times are essential. Because the mass spectrometer does a good job of discriminating compounds of different masses, the need for chromatographic separation largely arises to distinguish isomers and compounds that yield fragment ions at the same masses as other target metabolites. After extensive evaluation of various chromatographic parameters, a protocol was settled upon based on an 8-minute gradient UHPLC separation that yielded valleys between isomer peaks less than 10% of the tallest peak (Figure 2.4) for the stevia metabolite standards. Chromatographic peaks for all stevia glycosides were symmetrical, with widths at half height of approximately 4 seconds.

2.4.3 Profiling of Diterpenoid Glycosides in Stevia Leaves

Owing to high concentrations of the most abundant stevia glycosides, tissue extracts were diluted from 5- to 10-fold before LC/MS/MS analyses. To achieve quantification of low level glycosides, extracts were not diluted sufficiently to keep all measurements within the calibrated range of concentrations that gave a demonstrated linear response. After dilution, LLOQ values for all of the stevia glycosides corresponded to less than 1 μ g/g dry weight, or more than three orders of magnitude below the observed levels. Extraction of smaller quantities of plant tissue would have involved greater uncertainties in weighing the tissue, and we settled on weighing



Figure 2.4 Extracted ion LC/MS/MS chromatograms generated from a mixture of stevia glycoside standards, showing (a) Reb A, (b) stevioside and Reb B, (c) Reb C, (d) dulcoside A, and (e) steviolbioside and Rubusoside. Additional standards are not shown because they were not measured as part of the targeted LC/MRM method.

about 10 mg for each plant tissue sample. Standard stevia glycoside concentrations of 20 μ M, corresponding to approximately 10 mg/g dry weight, fell into the linear range for all glycoside metabolites. As a result, we anticipate that measured levels above 50 mg/g dry weight may slightly underestimate tissue levels owing to nonlinear electrospray ionization responses at high concentrations. About half of the Reb A measurements exceed this level, and we anticipate that these measurements may underestimate Reb A quantities. Since only about 1% of measurements entailed such high concentrations, these outlier samples, which are often displaying a desirable trait, were readily recognized from the LC/MS/MS results.

The targeted LC/MRM method detected signals for 9 target metabolites plus digitoxin, a steroidal glycoside used as internal standard. Our primary aim was to report the desirable sweeteners stevioside, Reb A, B, and C in more than 1100 Stevia leaf extracts, and use this information to guide subsequent plant breeding efforts. The diversity of the content of the four metabolites of interest across this large set of tissue samples is depicted in the histograms displayed in Figure 2.5. Our findings demonstrate that stevia glycoside abundances, range over about two orders of magnitude as follows (normalized to dry weights): stevioside (2-125 mg/g), Reb A (2.5-164 mg/g), Reb B (0.5-50 mg/g), and Reb C (1.5-125 mg/g). A few plant extracts had high contents of specific stevia glycosides. For example, 12 tissue extracts (approximately 1% of the population) contained more than 40 mg/g dry weight of Reb C, with two tissue extracts exceeding 100 mg/g DW.



Figure 2.5 Distribution of leaf levels of (a) stevioside, (b) Reb A, (c) Reb B, and (d) Reb C in more than 1100 extracts from Stevia leaves. Inset in (c) shows the magnified frequencies of Reb B for the lower amounts. Frequencies for histogram bars too small to be seen are labeled.

2.4.4 Separation of Stevia Glycosides Isomers Present in Stevia Leaves

The LC/MRM method resolved chromatographic peaks for the target glycosides, based on characteristic chromatographic retention times and MRM mass transitions. However, additional peaks were frequently observed, and these did not correspond to signals that could be attributed to any of the stevia glycosides available as standards. These features are illustrated in Figure 2.6. In the first instance (Fig. 2.6a) that reports results for a single Stevia leaf extract, at least six chromatographic peaks are evident for the MRM transition of m/z 641>479, which targets detection of steviolbioside (peak a2) and rubusoside (peak a3). For this particular extract, the greatest signal (peak a1) does not correspond to any metabolite for which we had standards. Figure 2.6b displays signal for an additional channel that was added for this specific analysis, targeting detection of Reb F using MRM transition m/z 935>773. The surprising evidence in this chromatogram suggests that the Reb F channel detects one major and one minor isomer. To our knowledge, this extent of diversity in isomers among the stevia glycosides has not previously been demonstrated, though a recent report of a UHPLC/MS method reported a second isomer of Dulcoside A [123] Such findings highlight the dilemma faced in large-scale metabolite profiling, in that increased throughput usually comes at a cost of decreased resolution. The 8-minute gradient described in this study, when coupled to MRM detection, provides a robust compromise between resolution and throughput.



Figure 2.6 Extracted ion UHPLC/MS/MS chromatograms of Stevia leaf extracts showing two MRM channels on Stevia leaf extract sample. a) m/z 641>479 (steviolbioside and isomers) and b) m/z 935>773 (Reb F and isomer).

Two explanations are proposed for the apparent detection of additional stevia glycoside isomers. Since some enzymes involved in specialized metabolite biosynthesis exhibit substrate promiscuity, more than one isomer may be formed from a specific substrate. In such cases, numerous products, often including isomers, may form from common biosynthetic catalysts [131]. We cannot rule out that some of these peaks arise from diversity in attached sugars or sites or orientations of attachment. An alternative explanation suggests that some of the extra peaks arise from metabolites that are more extensively glycosylated, and in-source fragmentation of acylglycosides could generate ions of the same masses as the target glycosides. Most of the peaks attributed to isomers elute later than the target metabolites, and we anticipate that more glycosylation would usually shorten retention times on the C18 column. For these reasons, we anticipate that variable glycosylation may be more extensive than has previously been resolved using chromatographic methods.

2.5 Conclusions

Profiling of bioactive metabolites in large numbers of plant tissue samples invariably encounters the conflict between throughput and depth of coverage of the target metabolites. As chromatographic systems improve in resolution and selectivity, it is frequently the case that metabolites that had previously been missed are now resolved and detected, yet the throughput needed for large-scale profiling often mandates a sacrifice of analytical resolution. The LC-MS/MS method used in this study for quantifying stevia glycosides and aglycones achieved a functional compromise between these pressures, and delivered analysis times of 8 min/sample, plus about 1 minute interval for injection of the next extract. Limits of detection were far lower than needed for characterization of plant extracts, yielding low ng/ml limits of quantification. We anticipate that this methodology should be readily adapted to measure stevia glycosides and their degradation products in beverages and other food products. This method was used for quantification of stevia glycosides in more than 1100 extracts from Stevia leaves from a large plant population, usually in batches of two 96-well plates at a time. The broad range of levels measured in this study serves to indicate the chemical diversity among Stevia leaf tissues, and the small fraction of hyperaccumulating plants instructs us regarding the need to analyze large numbers of samples when developing plants with desirable biochemical traits.

Chapter Three: Collision Induced Dissociation of Negative Ions from Steviol Glycosides Generated using Electrospray Ionization

3.1 Abstract

The use of mass spectrometry for profiling metabolites has grown as an important tool for discovery of functions of genes involved in metabolite biosynthesis and the regulation of these processes. Numerous metabolites accumulate in plant tissues in the form of various glycosides, and many are isomers that are not readily distinguished based on their mass spectra. To accelerate discoveries of metabolic genes such as glycosyltransferases, improved mass spectrometric technologies are needed to distinguish isomeric metabolites when authentic standards are not available. In this study, a series of diterpene glycosides that are non-caloric natural sweeteners from the leaves of Stevia rebaudiana were characterized using negative ion mode tandem mass spectrometry across a range of energies for collision induced dissociation. Specifically, a systematic comparative analysis of MS/MS product ion spectra derived from [M-H] and a series of adduct ions ([M+Cl], [M+formate] and [M+acetate]) of five steviol glycosides including stevioside, Rebaudiosides A, B, C, and D was conducted by varying collision energies ranging from 5 to 110 eV. Breakdown curves were generated to display the dependence of pseudomolecular and fragment ion abundances on collision energy. The primary findings of this work reveal that dissociation of steviol acylglycosides occurs at lower collision energies than for isomeric alkylglycosides. Differences between isomers and other steviol glycoside behavior points to important roles for charge localization, gas phase basicities, and configurations of functional groups in governing the energydependence of ion fragmentation. These findings have potential utility for distinguishing isomeric glycoconjugates of various terpenoid metabolites.

3.2 Introduction

The plant kingdom accumulates specialized metabolites of incredible structural diversity that may well exceed 10⁵ distinct substances. Many of these metabolites are processed in vivo by attachment of various sugars [132], and this process alters the physical and chemical properties as well as subcellular locations of many plant metabolites. Furthermore, glycosylation can have profound effects on the biological activities of plant metabolites.

During the past decade, mass spectrometry has assumed a more prominent role as the analytical tool of choice for profiling specialized metabolites, and improvements in mass spectrometer sensitivity and ease of use have led to a new age of metabolite profiling, leading to global metabolite profiling known as 'metabolomics'. These advances offer the potential to accelerate discoveries of gene functions across a wide range of organisms. One of the great remaining bottlenecks to this approach lies in the limited ability of mass spectrometric analyses to distinguish isomeric metabolites. This is particularly the case for glycosylated terpenoid metabolites that vary in core structure as well as the positions and stereochemistry of functional group attachments. Tens of thousands of terpenoid metabolites have been isolated from natural sources [133]. Understanding the functions of plant metabolic genes is expected to lead the development of improved production of agricultural crops, including enhancements in yield of high-value phytochemicals [134].

One important area where improvements of crop sources of high-value are desired lies in the areas of nutraceuticals, and other food additives such as natural sweeteners. The growing prevalence of obesity and diabetes mellitus in many parts of the world has stimulated interest in low-calorie sweeteners as alternatives to consumption of sugars [125,135]. Numerous synthetic sweeteners have been developed since the discovery of saccharin in the 19th century, and aspartame and sucralose have emerged as widely used alternatives. Owing, in part, to aspartame toxicity in individuals with phenylketonuria [136], interest remains in developing alternative and natural sugar substitutes, and the remarkable chemical diversity of the plant kingdom provides a rich resource in this regard. Leaves of the herbaceous perennial plant *Stevia rebaudiana* Bertoni, a native of Brazil and Paraguay, have been consumed as a food and medicine for years in many countries ranging from Japan to Paraguay [135,137,138]. Stevia leaves accumulate a suite of glycosides of the diterpenoid acid steviol, which exhibit varying degrees of sweetness. Stevia glycosides comprise a class of plant specialized metabolites that differ in the number and types of hexose or pentose sugars attached to the diterpenoid core, and exhibit variations in the sites of glycosylation. Discoveries of new stevia glycosides continue as more metabolites are isolated and structures are elucidated [139]. Structures of five major steviol glycosides are presented in Figure 3.1.

Since some steviol glycosides including stevioside and rebaudioside A (termed Reb A from here on) are 300-400 times sweeter than sucrose, they are prized worldwide as natural and non-caloric sweeteners [127,140]. These diterpene glycosides however have different sweetening properties from one another [117]. For example, Reb A, which has an extra glucose unit in its structure in compare to Stevioside, is sweeter and its quality of taste is better [114] while stevioside causes a significant bitter aftertaste. Therefore, the quality of the commercial products strongly depend on the proportion of each of the diterpene glycoside isomers in the used mixtures [117]. So, there is a great need in industry for isomer differentiation of steviol glycosides.



Figure 3.1 Chemical structures of five steviol glycosides: Rebaudiosides A, B, C, D and stevioside.

Electrospray ionization (ESI) of steviol glycosides has been reported before for LC/MS separation and quantification of known isomers. It was shown that these sweeteners are capable of forming $[M+H]^+$, $[M-H]^-$, and adduct ions as expected from sugars. Desorption electrospray ionization (DESI) which was introduced by Cooks et al. [135] was also used for direct screening

of steviol glycosides from Stevia leaves.

Mass spectra alone do not differentiate unknown isomers since isomers have the same molecular masses, and often form fragment ions whose masses fail to distinguish isomers. For example, the stevia glycosides Reb B and Stevioside differ only in the position of the glucose units. Both isomers yield deprotonated molecules observed as m/z 803 using electrospray ionization in negative mode. While collision induced dissociation (CID) yields fragment ions that often arise from loss of carbohydrate groups from glycosides, the formed fragments often have masses consistent with losses of neutral carbohydrates, again failing to distinguish most isomers.

Tandem mass spectrometry has been applied to help assign structures to steviol glycosides in earlier reports, but some features of the mass spectra required detailed investigation to explain the ion fragmentation chemistry. Prakash et al. reported observation of tetrasaccharide product ions upon CID of [M+H]⁺ from Reb A, even though the four sugar units are not attached except through the terpenoid core [141]. Using fully deuterated glucose units on Reb A, they established that intramolecular rearrangement followed by elimination of four glucose units resulted in neutral loss of 318 Da, corresponding to the neutral aglycone steviol, from protonated Reb A. Similar results were observed for Reb B, Stevioside and Reb F. Although this information helps build an understanding of the fundamentals that govern fragmentation pathways of cationized steviol glycosides, the product ion spectra did not differentiate isomers such as stevioside and Reb B.

Investigations of the dependence of ion fragmentation on collision energy have been used in several laboratories to understand the dynamics of ion fragmentation [142] and optimize MS/MS conditions for quantitative analysis. The incorporation of variable collision energy adds another dimension of information that has potential to distinguish isomeric ions. In recent years, interpretation of breakdown curves derived from soft ionization methods and collision induced dissociation has been extended to aid metabolite identification for metabolites including acylglycines [143] and chalcones[144], and to distinguish diastereomers of isoquinoline alkaloids [145].

In this study, by examining the relationship between collision energy and the appearance or disappearance of various ions derived from five different steviol glycosides, information about sites of attachment and linkage types that distinguishes steviol glycoside isomers can be determined by experiment.

3.3 Experimental

3.3.1 Chemicals

Standards of Reb A, Reb B, Reb C, Reb D and stevioside (ChromaDex, Irvine, CA) were kindly provided by Dr. Randy Beaudry and Dr. Ryan Warner from the Michigan State University Department of Horticulture. Stock solutions of 50 μ M of each standard were prepared individually in 50/50 water/ethanol and directly injected to the mass spectrometer through the pumps.

3.3.2 LC/MS Instrumentation

The LC/MS system used in this work consisted of a binary HPLC based on Prominence LC-20AD (Shimadzu Corp.) pumps connected to a QTRAP 3200 hybrid-linear ion trap mass
analyzer equipped with an electrospray ionization source (Applied Biosystems). This instrument was housed at the Michigan State University Mass Spectrometry Facility.

3.3.3 LC/MS Analyses

Flow injection analyses were carried out using isocratic elution of 1:1 (v/v) mixture solvent A (aqueous) and solvent B (acetonitrile) at a total flow rate of 250 μ L/min. The composition of solvent A was changed to generate different adduct ions. 0.3% aqueous formic acid was used to generate [M-H]⁻ and [M+formate]⁻ and 100 mM ammonium acetate and 10 mM ammonium chloride were used to generate, [M+acetate]⁻ and [M+Cl]⁻ adduct ions respectively. All flow injection analyses were performed in electrospray ionization negative mode.

Enhanced product ion (EPI) scan mode used for performing MS/MS on the deprotonated and adduct molecular ions of each steviol glycoside separately at the following conditions: curtain gas: 10, ion spray voltage: 4500 V, temperature: 500°C, ion source gases both 1 and 2: 25, declustering potential (DP): 20 V and entrance potential: 10 volts. Collision energy values were 5, 10, 30, 50, 70, 90 and 110 eV. 5 eV was the minimum collision energy allowed by the Analyst software and was used as the lowest collision energy. Analyst software v. 1.4.2 was used to operate the LC/MS/MS system and data analysis throughout the experiments.

3.4 Results and Discussion

Under conditions of negative mode electrospray ionization, all stevia glycosides studied yielded abundant [M-H]⁻ ions, which dominated the spectra unless a source of anion such as formate, acetate, or chloride was added to the analyte solution. Manipulation of solution

composition allowed collision induced dissociation (CID) spectra to be generated for deprotonated ions and acetate, formate, and chloride adducts without the need to add acids or nucleophiles to the stevia glycoside solutions. Ion intensities were normalized to the total ion intensity by eliminating the ion intensities below five percent.

3.4.1 Comparison of Stevioside and Reb B

Since the isomeric stevia glycosides are built from common substructures, masses of fragment ions generated using CID may not provide information capable of distinguishing isomers. Instead, the dependence of pseudomolecular and fragment ion abundances upon collision energy should be explored, particularly in cases where fragmentation dynamics might be expected to differ owing to charge localization or differential reactivity of isomeric ions. Figures 3.2a and 3.3a present breakdown curves for the deprotonated molecules of stevioside and Reb B, respectively. Examination of the collision energy dependence of $[M-H]^-$ abundances reveals that even at the lowest collision energy ($E_{lab} = 5 \text{ eV}$), more than 80% of $[M-H]^-$ of stevioside has already been converted to fragment ions, primarily loss of neutral anhydroglucose (designated as $[M-H-Glu]^-$). In contrast, nearly 100% of Reb B remains as $[M-H]^-$ at collision energies up to 30 eV, whereas no ions corresponding to deprotonated stevioside could be detected at this collision energy. This clearly demonstrates that deprotonated stevioside undergoes more facile loss of anhydroglucose than Reb B. The only difference between the structures of stevioside and Reb B lies in the position of attachment of one glucose unit, with the former containing an acylglycoside moiety, and the latter an alkylglycoside.



Figure 3.2 Breakdown curves for deprotonated and three adduct ions of stevioside at seven collision energies ranging from 5 to 110 eV. The Y-axis shows the % abundance of each fragment ion normalized to the total abundances of all observed fragment ions in the CID spectra for each parent ion. Error bars represent the standard deviation of three replicates.

(For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.)



Figure 3.3 Breakdown curves for deprotonated and three adduct ions of rebaudioside B at seven collision energies ranging from 5 to 110 eV. The Y-axis shows the % abundance of each fragment ion normalized to the total abundances of all observed fragment ions in the CID spectra for each parent ion. Error bars represent the standard deviation of three replicates.

These findings are consistent with the acylglucoside of stevioside undergoing loss of anhydroglucose via a lower activation energy pathway than cleavage of any of the glycosidic bonds in Reb B.

At collision energies greater than 30 eV, fragment ions corresponding to losses of up to three carbohydrate units are evident in the CID mass spectra of all four precursor ions for both stevioside and Reb B. Some additional fragments had masses 18 Da lower than the major fragments depicted in the breakdown curves, consistent with losses of the intact carbohydrate rather than the anhydrosugars. These are not presented in the Figures for the sake of clarity, but their abundances are included in calculating the total ion current at each collision energy. It is evident from the breakdown curves (Figs. 3.2a and 3.3a) that as collision energy was increased, abundances of lower mass ions, corresponding to losses of more than one sugar unit, also increased. Yields of fragments from the aglycone portion of each metabolite were found to be minimal.

Another difference between Reb B and Stevioside [M-H] breakdown curves which is less obvious is the threshold collision energy needed to form [M-H-2Glu]. Such energy is about 40 eV and 50 eV for stevioside and Reb B respectively. Such observations are consistent with lower activation energy for ion decomposition for the acylglycoside stevioside than for the alkylglycoside Reb B in gas phase.

Since other reports have proposed analyzing stevia glycosides as chloride ion adducts rather than deprotonated ions [123], the behavior of various adduct ions under CID conditions of different energies was also explored. Perhaps the most notable aspect of the behavior of chloride adduct ions is clear from Figures 3.2b and 3.3b, which again shows breakdown curves for the isomers stevioside and Reb B. Even at the lowest collision energy, no conversion of [M+Cl]⁻ to

[M-H][•] via loss of HCl was detected for stevioside, whereas Reb B yielded more than 30% at low collision energies, increasing to more than 80% from 30-50 eV, followed by a decrease as collision energy increased further. This difference in behavior is attributed to differences in gas phase acidities, as Reb B contains a carboxylic acid whereas the sugar hydroxyls are probably the most acidic groups of stevioside. Another feature of the behavior of stevioside worth mentioning is the formation of [M-H-Glu][•] appears to correspond with the disappearance of [M+Cl][•]. No [M-H][•] product was observed, therefore we propose that the loss of anhydroglucose is consistent with direct loss of chlorodeoxyglucose (198 Da) which yields the same mass as obtained by loss of anhydroglucose from [M-H][•]. Such a reaction would be the product of an intramolecular displacement reaction.

In similar fashion, breakdown curves were generated for formate and acetate adducts of both stevioside and Reb B (Figure 3.2c and 3.2d, 3.3c and 3.3d, respectively). For stevioside, both of these adducts generated [M-H][–] upon CID, and formation of [M-H-Glu][–] was observed at the lowest collision energies. Higher collision energies yielded a suite of product ions resembling the behavior of [M-H][–] for both acetate and formate adduct precursor ions. In contrast, CID spectra of formate and acetate adducts of Reb B gave >70% yields of [M-H][–] even at the lowest collision energies. No formate adducts of Reb B survived transport through the mass analyzers under gentlest conditions, but~ 30% of the observed ions for the acetate adduct survived under identical instrumental conditions.

The differences in behavior of the carboxylate (formate or acetate) and chloride adduct ions are attributed to two factors – their gas phase basicities and their reactivities as nucleophiles. The greater gas phase basicities of formate and acetate (1445 and 1459 kJ/mol respectively); [146] relative to chloride (1395 kJ/mol); [147] drive elimination of formic or acetic acids as neutrals, whereas the lower basicity of chloride reduces yields of HCl elimination. Instead, we propose that chloride may participate in nucleophilic displacement of acylglucoside groups, as is the case for stevioside. When acylglycosides are not present, and an acidic carboxylate exists as for Reb B, the dominant low energy pathway for these two isomers involves elimination of HX (acetic, formic or hydrochloric acids) as neutrals, followed by elimination of anhydrosugars.

3.4.2 Comparison of Reb A and Reb C

Fragmentation of ionized glycoconjugates is governed by rates of unimolecular reactions following collisional deposition of energy in the various vibrational modes of the ion. Most ions formed by electrospray ionization in negative ion mode are even-electron (non-radical) species such as deprotonated molecules or adducts with anions such as chloride or carboxylates. The reactions that take place following collisional activation usually involve S_N2-type displacement reactions or concerted eliminations that often require hydrogen migration. In either case, differences in rates of ion decomposition between isomers may be attributed to the accessibility of groups to participate in displacement or hydrogen migration reactions. In the case of steviol glycosides, these rates may depend on the positions and orientation of the sugar hydroxyl groups and, perhaps, to locations of charged groups. To explore the influence of these factors, the product ion spectra of two closely related stevia glycosides, Reb A and Reb C, which differ only in the substitution of either a glucose (Reb A) or rhamnose (Reb C) via a 1,2- linkage, in tetraglycosides of steviol were examined. CID spectra of the [M-H] ions from both metabolites (Figures 3.4 and 3.5) exhibited behavior similar to stevioside, showing facile loss of anhydroglucose at the lowest collision energies. The formate and acetate adducts also exhibit fragmentation behavior similar to stevioside, showing conversion to [M-H] at low collision

energies followed by more extensive losses of carbohydrate groups. In addition, the chloride adduct lost anhydroglucose at low collision energies without appreciable formation of [M-H]⁻ product, again in similar fashion as stevioside, which lacks the 3'-position glucose unit. These findings underscore minimal participation of the additional glucose unit in ion fragmentation.



Figure 3.4 Breakdown curves for deprotonated and three adduct ions of Reb A at seven collision energies ranging from 5 to 110 eV. The Y-axis shows the % abundance of each fragment ion normalized to the total abundances of all observed fragment ions in the CID spectra for each parent ion. Error bars represent the standard deviation of three replicates.



Figure 3.5 Breakdown curves for deprotonated and three adduct ions of Reb C at seven collision energies ranging from 5 to 110 eV. The Y-axis shows the % abundance of each fragment ion normalized to the total abundances of all observed fragment ions in the CID spectra for each parent ion. Error bars represent the standard deviation of three replicates.

However, substitution of rhamnose for glucose (Reb C vs. Reb A) allows elucidation of the involvement of the remaining individual sugar units, as rhamnose-containing fragments are 16 Da lighter than analogs containing glucose instead. Comparison of the [M-H] breakdown curves for Reb A and Reb C shows that loss of a second anhydroglucose unit ([M-H-2Glu]) occurs with almost identical yields for these two metabolites, despite there being two potential pathways for loss of an anhydroglucose in Reb A. This result suggests that cleavage of the 1,3-glucose moiety occurs more efficiently than for the 1,2- substituted group. This finding is further supported by the observation of analogous loss of anhydrorhamnose ([M-H-Glu-Rha]) at almost 30% of the yield of anhydroglucose ([M-H-2Glu]) in Reb C. We consider three factors with potential contributions to this outcome: (1) differences in reactivity of the 1,2- relative to 1,3-linkages, (2) differences in hydroxyl group orientations in rhamnose and glucose units, and (3) involvement of the 6-position hydroxyl (or its anion) as a nucleophile in displacement of the 1,2-linked carbohydrate. Differences in behavior of the chloride, formate, and acetate adducts of Reb A and Reb C were minimal, probably owing to the common intermediacy of [M-H-Glu] in their formation.

3.4.3 Reb D

Further effects of carbohydrate substitution on fragmentation chemistry are evident in comparisons of Reb D, which has an additional glucose unit attached through a 1,3-linkage to the acylglucose moiety, with analogs that contain only a single carbohydrate esterified to the diterpenoid core (Figure 3.6) Three striking differences between the behavior of Reb D and its analogs stevioside, Reb A, and Reb C are evident. First, at low collision energies, the [M-H]⁻ yield is greater for Reb D than for its analogs. Second, the most facile reaction leads to loss of

an anhydrodisaccharide rather than a monosaccharide, consistent with loss of the acylated disaccharide group via cleavage of the ester bond. Third, CID spectra of [M+CI][–] yield [M-H][–] as the dominant fragment ion upon 50 eV collisions, whereas the analogous fragment was not formed in significant yield under any conditions for stevioside, Reb A, or Reb C. Taken together, these findings suggest that loss of the acyl disaccharide, presumably as the neutral anhydrodisaccharide, has a higher activation energy than corresponding losses of anhydroglucose from the related compounds. Such behavior would be in keeping with a greater distance (or decreased accessibility) between the negative charge site and the reactive ester group, though we cannot exclude the possibility that increased gas-phase acidity and slightly lower center-of-mass collision energies may also play roles in increasing yields of [M-H][–].



Figure 3.6 Breakdown curves for deprotonated and three adduct ions of Reb D at seven collision energies ranging from 5 to 110 eV. The Y-axis shows the % abundance of each fragment ion normalized to the total abundances of all observed fragment ions in the CID spectra for each parent ion. Error bars represent the standard deviation of three replicates.

3.5 Conclusions

This comparative investigation of the fragmentation behavior of negative ions derived from a collection of diterpene glycosides illustrates two findings relevant to specialized metabolite identification. These compounds exhibited differences in energy-dependent fragmentation that allow discrimination of acyl and alkyl glycosides, with the former undergoing elimination of anhydrosugar units at lower collision energies than the latter. Furthermore, differences in energy-dependence of fragmentation of rhamnose- and glucose-substituted analogs suggest that negative charge localization, gas phase acidities, and functional group configurations are important determinants of rates of specific ion fragmentation reactions. Variation in the structures of acylglycosides yielded different fragmentation dynamics again suggestive that the proximity of negative charge to the ester group influences rates of anhydrosugar elimination. The presence of acyl monoglycosides is further supported when chloride adducts do not generate [M-H] as product ions upon CID. Combined, these findings extend earlier studies that distinguished oligosaccharide isomers from their CID spectra, and demonstrate that similar considerations have utility in elucidation of metabolite structures from CID spectra. It is evident that fragment masses alone are not sufficient to distinguish many glycosylated metabolites, and given the potential for substantial chemical diversity in glycosylated metabolites across the plant kingdom, it is recommended that the collision energy dependence of CID spectra be more widely employed for distinguishing glycoconjugate isomers and analogs varying in glycosylation.

Chapter Four: Profiling of Lipids in the Red Alga *Porphyridium cruentum* using Ultrahigh Performance Liquid Chromatography-Mass Spectrometry with Nonselective Collision Induced Dissociation

4.1 Introduction

Renewable sources of liquid transportation fuels are finding increasing attention owing to anticipation of increasing petroleum scarcity in years to come. Additional concerns regarding the environmental ramifications of fossil fuel combustion including global climate change have led to interest in developing biodiesel derived from lipids of photosynthetic organisms as reusable and renewable source that emits lower levels of greenhouse gas. However, many liquid biofuels are derived from plant seeds and seed oils that are also used to feed humans and animals. Elevated demand for grains has generated shortages in food supplies in underdeveloped countries, leading public opinion to recognize that production of biofuels from grains including corn and beans to create as many problems as it solves [18]. Therefore, since the mid-1980s, studies have examined whether microalgae might be cultured for production of biodiesel derived from algal lipids [148-150].

In addition to their value as precursors of biofuels, many lipids have potential economic value because numerous biological functions are regulated by oxidized metabolites of polyunsaturated fatty acids (PUFAs), known as oxylipins. Epoxyeicosatrienoic acids (EETs) are epoxides of arachidonic acid (C20:4) and are important chemical compounds that regulate blood pressure in animal models. These epoxides are generated in the vascular endothelium cells by cytochrome P450 epoxygenases from their precursor, arachidonic acid [29, 151] and could be of greater value if production costs could be decreased. In addition, many lipids also are used as food and dietary supplements. Microalgae and marine fungi can be used as an alternative for fish oils in marine food chain or oil supplements with high PUFAs, as fish also accumulate persistent organic pollutants such as polychlorinated biphenyls that are undesirable contaminants.

It is thought that lipids extracted from marine fish that are rich in omega-3 polyunsaturated fatty acids (PUFAs) originate from the diet of fish [152], and this diet is rooted in algae and other phytoplankton at its base. Many algae are considered as promising sources for production of PUFAs as they can synthesize and accumulate substantial amounts of omega-3 fatty acids [153]. While their PUFAs composition and the amount of PUFAs that they can accumulate varies from one species to another one [154], algal lipids are considered to have nutritional values. To better understand the genetic and environmental factors that influence algal lipid composition, it is useful to have rapid analytical methods that can support lipid identification. In addition, environmental factors including temperature and nutrient composition affect the lipid composition of microorganisms [155,156], and efficient lipid screening methods are helpful for recognizing culture conditions that promote accumulation of desirable lipids.

As was explained in Chapter One of this dissertation, GC/MS has long been the most widely used analytical method for lipid identification and quantification, but it relies on conversion of lipids to volatile derivatives of fatty acids, most often methyl esters. With this approach, prior separation of lipids by class, often using thin layer chromatography, is necessary, followed by transesterification to yield fatty acid esters, However, with this method, information about the structures of individual lipids within a class is lost. A more recent approach takes advantage of modern soft ionization techniques for mass spectrometry to perform lipid identification on intact complex lipids. In this technique, the molecular mass of the lipid is established, and fragment ions yield information about the lipid class and the composition of the fatty acids for each lipid. In addition, some structural information can be gained about the positions of the attached fatty acids, usually from relative abundances of fatty acid anion fragments. When these mass spectrometric methods are coupled with HPLC, suppression of ionization by coeluting compounds is reduced and can yield improved quantitative accuracy. Mass spectrometry in combination with LC can be designed to be a fast method for rapid screening of lipids in large sample sets such as profiling of multiple genotypes or environmental conditions. In this chapter, the performance of two relatively short LC separations (a 5-minute gradient and 26-minute gradient) for lipid separation is compared. To maximize structural information from fragment ions, two mass spectrometric methods, MS^E and multiplexed CID were employed to generate fragment ions to aid lipid identification of lipid. The red microalga *P. cruentum* was used to demonstrate since this alga is rich in PUFAs including longer chain fatty acids such as arachidonic acid [157].

4.2 Experimental

4.2.1 Chemicals and Reagents

Concentrated hydrochloric acid, sodium chloride, magnesium chloride hexahydrate, calcium chloride dihydrate, potassium phosphate monobasic, sodium bicarbonate, and were purchased from VWR Scientific. Zinc chloride, boric acid, cobalt (II) chloride hexahydrate, copper (II) chloride dihydrate, manganese chloride tetrahydrate, ammonium molybdate tetrahydrate, and iron (III) chloride hexahydrate, magnesium sulfate heptahydrate, vitamin B₁₂, biotin, and thiamine hydrochloride were obtained from Sigma-Aldrich. Tris hydrochloride was purchased from Invitrogen. EDTA-disodium salt (disodium ethylenediaminetetraacetate) was procured from Fisher Scientific.

4.2.2 Preparation of Solution

Nalgene[®] bottles were washed by soaking for 24-48 hours in 1% aqueous hydrochloric acid. Then, bottles were rinsed five times with distilled water and twice with Milli-Q water.

4.2.3 Preparation of Stock Solutions for Algal Growth Media

Stock solutions of calcium chloride (15.0 g/100 mL), potassium nitrate (10.0 g/100 mL), potassium phosphate monobasic (700 mg/100 mL) sodium bicarbonate (400 mg/100 mL), Tris-HCl buffer (1 M) trace metal stock solution for artificial seawater, chelated iron (1.2 g FeCl₃•6H₂O and 9.3 g disodium EDTA in 500 mL, pH 7.6 and vitamin B₁₂ (14 mg in 100 mL of 10 Mm Tris-HCl pH 7.6) were prepared in Nalgene bottles using MilliQ water. Stock solutions of biotin (5 mg/100 mL of 10 mM Tris-HCl, pH 7.6) and thiamine (1.2 mg/mL) were prepared by filtering through 0.45 µm sterile filters.

4.2.4 Algae Growth

The alga used in this research was obtained from the University of Texas Algal Culture Collection in the form of agar slants. Growth of algae *P. cruentum* on plates and selection of a single colony for overproduction of this algal has been done. The red alga *P. cruentum (UTEX # LB 2757)* was streaked on agar plates made with modified Jones' medium [158] and 1.5% agar (Physiol Plant)[®] to select a colony with no bacterial contamination (Figure 4.1). The selected colony was transferred into 250 mL Erlenmeyer flask containing 100 mL of modified Jones medium and was set at room temperature under "cool white" fluorescent lamps.





Figure 4.1 Colonies of *P. cruentum* streaked in agar plate and agar slant.

4.2.5 Extraction of Lipids

Lipids were extracted from 1 mL of dense algal culture $(10^{6} \text{ to } 10^{7} \text{ cells/mL})$ using 4 ml of a mixture of chloroform, isopropanol, and MilliQ water saturated with sodium chloride (2:1:1 v/v/v) in order to extract lipids from both algal cells and secreted lipids in the media. The mixtures of liquid from the culture flask and the extraction solvent were centrifuged at 10000 × g for 5 minutes to separate the pellet of cells from supernatant. Centrifugation resulted in formation of three layers. From the bottom of the tube to the top, the first layer was the pellet of cells, the second layer was the organic solvent layer (mostly chloroform), and the third layer was largely aqueous. With the use of a Pasteur pipette, the second layer was transferred to a 13 x 100 mm borosilicate glass tube, and the tube was placed in a SpeedVac[®] to evaporate solvent to dryness at room temperature. The residues were later redissolved in 1-2 mL of isopropanol, transferred to amber autosampler vials, and placed in a -20[°]C freezer until analysis by LC/MS.

4.2.6 LC/MS and LC/MS/MS Instrumentation

The LC-MS system used in this work consisted of three Shimadzu LC-20AD HPLC pumps coupled through a low volume mixer to a Waters LCT Premier Time-of-Flight Mass Spectrometer (TOF-MS). For further confirmation of specific lipid structures, analyses were performed using two Shimadzu LC-20AD pumps coupled to a QTRAP 3200 mass spectrometer (Applied Biosystems).

4.2.7 LC/MS and LC/MS/MS Analyses

In all LC/MS methods, lipid extracts were separated on an Ascentis Express C18 reverse phase (5 cm x 2.1 mm x 2.7 μ m) column (Supelco, USA) before mass spectrometric detection. Three different gradient elutions were performed over 5, 20, and 26 minutes based on experiment needs at different stages of the research.

The LC conditions for the 5 minute gradient were as follows: Solvent A: 10 mM aqueous ammonium acetate, Solvent B: isopropanol. Total solvent flow was maintained at 0.25 mL/min, and gradient elution was performed using the following solvent compositions: initial 90%A/10%B with linear gradient to 60%A/40%B at 1.0 min and then to 5%A/95%B at 2.5 min then held at this composition until 4.5 min followed by sudden change to initial condition at 4.51 min and the final hold at this composition until 5 minutes. Negative mode electrospray ionization was employed, along with multiplexed collision induced dissociation (CID) by switching between two different aperture 1 voltages (15 and 85 V) for non-selective fragmentation and detection of ions. For MS/MS analyses performed on the QTRAP mass

spectrometer, parameters were as follows: capillary voltage: -2500 V, sample cone: 15 V, Desolvation temperature: 350° C, source temperature: 100° C, cone gas flow: 40 L/h, desolvation gas flow: 350 L/h.

The LC conditions for the 26 minute gradient were as follows: Solvent A: 10 mM aqueous ammonium acetate, solvent B: isopropanol, and solvent C: acetonitrile. Total solvent flow was maintained at 0.25 mL/min, and gradient elution was performed using the following solvent compositions: Initial: 95% A/0% B/5% C, held for 0.5 min; linear gradient to 25% A/0% B/75% C at 2.0 min and then to 10% A/0% B/90% C at 15 min; then 10% A/90% B/0% C at 20 min; held for 3 min; followed by a linear gradient to initial condition at 23.01 min and a final hold at this composition until 26 minutes. Negative electrospray ionization along with multiplexed collision induced dissociation (CID) in the TOF analyzer was performed by switching among 6 different aperture 1 voltages ranging from 15 to 85 V (15, 30, 45, 60, 75, and 85 V). Injection volume and column temperature were 10 μ L and 55 °C respectively. Other mass spectrometer parameters were as follows: capillary voltage: -3000 V, sample cone: 15 V, desolvation temperature: 350 °C, source temperature: 100 °C, cone gas flow: 40 L/h, desolvation gas flow: 400 L/h.

The LC conditions for 20 minute gradient were as follows: solvent A: 10 mM aqueous ammonium acetate, solvent B: isopropanol. Total solvent flow was maintained at 0.25 mL/min, and gradient elution was performed using the following solvent compositions: Initial: 95%A/5%B; linear gradient to 25%A/75%B at 2.0 min, and then to 10%A/90%B at 8 min; then 2%A/98%B at 18 min; followed by sudden change to initial condition at 18.01 min and a final hold at this composition until 20 minutes. Injection volume and column temperature were 5 μ L and 50 °C respectively. Mass spectrometer parameters for the QTRAP instrument were as follows: electrospray voltage: -4500V, Temperature: 500 °C, Curtain gas 1 and 2: 15, declustering

potential: -20 V, Entrance potential: -10 V, collision energy: 30-70 eV. Processing of LC/MS data was accomplished using MassLynx and Analyst software.

4.3 Results and Discussion

The vast majority of naturally occurring lipids are small molecules with molecular mass below 2,000 Da [159]. Therefore, the screening of lipids in our MS method was performed by acquiring mass spectra over a range of m/z 100-2500. Electrospray ionization (ESI) was chosen for these analyses, as ESI is particularly useful for the analysis of polar lipids including phospholipids and sphingolipids because this gentle ionization approach yields strong pseudomolecular ions and minimal yields of fragment ions under normal experimental parameters. In addition, nonpolar lipids that lack easily ionized groups including diacylglycerols (DAG) and triacylglycerols (TAG) have been also successfully measured by ESI [159], usually by employing positive mode ionization and mobile phases that incorporate cations such as NH4⁺ that form adduct ions. One major drawback to the use of ESI arises from suppression of analyte ionization by other constituents in complex extracts, and such suppression results from competition for ionization by the various substances that enter the mass spectrometer ion source at the same time. Though many investigators perform lipid profiling by direct infusion of complex mixtures, prior separation of lipids using liquid chromatography (LC) helps minimize ionization suppression and may improve quantitative accuracy.

Two different LC gradients were performed to separate lipids in algae extracts in line with mass spectrometric detection. The first gradient, developed for rapid screening of lipids, employed a short 5-minute gradient. The second, designed for more extensive resolution of lipid components, employed a 26-minute gradient. Base Peak Intensity (BPI) chromatograms, which

plot the signal for the most abundant ion in each spectrum are presented because, weak background signals do not contribute to the features observed in the chromatogram (Figure 4.2).

The differences in the number of lipids resolved by the 5- and 26-minute LC gradients were assessed by integrating the total ion chromatograms generated by analysis of a *P. cruentum* extract using negative mode electrospray ionization. The 5-minute gradient resolved 10 chromatographic peaks (Table 4.1). In contrast, the 26-minute gradient yielded 55 chromatographic peaks (Table 4.2). However, neither of these values reflects the true chemical diversity of the lipids present in the extract. The diversity of lipids was estimated by combining all mass spectra generated using each LC gradient, using a bin window of 0.01 Da. The number of distinct masses observed with a minimum threshold of 500 ion counts was 794 and 7781 from the 5- and 26-min gradients respectively. While these values overestimate the number of lipids observed because a single compound will exhibit multiple isotopolog peaks and will often form multiple ions such as various pseudomolecular adduct ions, noncovalent dimer ions, and occasionally, fragment ions. Based on a rough assumption that each lipid generates 5-10 resolved ions above the minimum threshold, it is estimated that the 5-minute gradient detects about 100 different lipids, and the 26-minute gradient detects 500-1000 lipid species. While many of the abundant lipids were identified as described below, the large number of less abundant lipids have yet to be conclusively identified owing to their low abundances and weak ion signals.

The increase in the number of chromatographic peaks observed using the longer gradient might be attributed to several factors. The improved chromatographic resolution is expected to decrease suppression of ionization because of decreased chances for chromatographic overlap and coelution. Avoidance of ion suppression yields more efficient ionization, with more lipids generating ions above the 500-count threshold.

Table 4.1	Detected total ion	chromatogram	peaks from	negative n	node LC/MS	analysis o	of a <i>P</i> .
cruentum	extract using the 5-	minute gradien	ıt.				

Peak #	Rt [*] (min)	Ion counts
1	1.7	4594
2	1.9	8087
3	2.3	5708
4	2.4	5827
5	2.5	6048
6	2.9	15158
7	3.2	18144
8	3.3	11302
9	3.5	12954
10	3.6	7988

Rt: Retention time

ESI is a soft ionization method that provides pseudomolecular ions with minimal fragmentation, and this approach has become widely used for analysis of complex lipids because it yields clear evidence of molecular mass. This information alone is not sufficient to identify most lipids because there are often multiple isomeric lipids that share a common elemental formula and molecular mass. Most of these isomers are derived from combinations of fatty acid groups with different numbers of double bonds and sometimes, carbon atoms. To probe fatty acid composition of complex lipids, conversion of pseudomolecular anions to fragment ions via collision induced dissociation usually results in formation of fatty acid anion fragments. This CID process can be performed on a single ion mass, as is the case in MS/MS analyses, or can be

Peak #	Rt [*] (min)	Ion counts	Peak #	Rt [*] (min)	Ion counts
1	0.64	35850	29	15.91	24674
2	3.17	17807	30	16.08	24574
3	3.54	7434	31	16.38	45320
4	3.78	5201	32	17.15	55233
5	4.75	28868	33	17.76	64746
6	5.13	3342	34	18.30	29980
7	6.31	25366	35	18.50	88999
8	6.54	13832	36	18.76	63844
9	7.49	29094	37	18.90	195621
10	7.86	5391	38	19.20	135610
11	8.33	4508	39	19.40	89849
12	8.67	6339	40	19.56	83884
13	8.94	11668	41	19.83	91736
14	9.55	23538	42	19.96	88883
15	10.39	3326	43	20.16	113493
16	10.76	1966	44	20.46	61291
17	11.03	3229	45	20.76	38178
18	11.53	17661	46	21.03	51525
19	11.76	9589	47	21.60	35681
20	12.14	3321	48	21.80	32937
21	12.88	19899	49	22.10	30885
22	13.18	6920	50	22.37	29605
23	13.52	40860	51	22.64	27489
24	13.93	14058	52	22.88	26738
25	14.16	30788	53	23.28	26201
26	14.77	74234	54	23.52	25864
27	15.24	41478	55	24.09	27509
28	15.54	27463			

Table 4.2 Detected total ion chromatogram peaks from negative mode LC/MS analysis of a *P. cruentum* extract using the 26-minute gradient.

Rt: Retention time

performed by activating all ions at the same time. In the Waters LCT Premier time-of-flight mass spectrometer, increasing the aperture 1 voltage causes all ions to collide with background gas, presumed to be nitrogen at a pressure around 10^{-4} mbar, and these collisions may yield fragment ions in a nonselective process. Perhaps the most prominent description of nonselective



Figure 4.2 Base peak intensity chromatograms of *P. cruentum* lipid extracts from 5 and 26 minute gradients using electrospray ionization in negative mode at low Aperture 1 voltage (AP1=15 V).

collision induced dissociation was reported in 2006, when rapid alternating spectra were acquired at low and high collision energies [88]. These investigators described their method as MS^{E} to distinguish it from MS/MS methods that select a specific mass for collisional activation.

This approach, when extended to more than two collision energies, was pioneered in the Jones laboratory at Michigan State University [129] and given the name multiplexed CID. With multiplexed CID analysis, it is possible to generate fragments of all pseudomolecular ions nonselectively to generate information about the m/z of fragments to support structural elucidation analysis, even when multiple compounds elute at the same time.

The multiplexed CID approach addresses important challenges faced by investigators designing LC/MS protocols. First, there is no single set of ion source conditions that are optimized to yield only the desired pseudomolecular ions for all analytes because lens voltages that promote adequate desolvation during the ESI process for one compound may cause severe fragmentation in another. In view of this, the analyst may not always be able to assign each ion species as molecular, fragment, adduct or noncovalent dimer ion. By establishing the dependence of ion abundances on CID potential, this challenge can be overcome. For example, one can expect that the abundance of a pseudomolecular ion will decrease with increasing CID potential, whereas fragment ion abundances will often increase as CID voltage increases. One final consideration arises because algae and other organisms accumulate lipids and other metabolites with abundances that span a wide range of concentrations; some are very low and some are quite high in concentration, this range often exceeds the linear dynamic range of the mass spectrometer. For the instrument to yield information about the ion type and to generate quantitative measurements of molecular and fragment mass information for as many analytes as possible, extended dynamic range offers improved metabolome or lipidome coverage and quantification.

Comprehensive profiling of lipid molecular and fragment masses can be achieved through rapid switching among multiple CID voltages (on the time scale of about 100 ms) using parallel acquisition of the spectra obtained using each collision potential. This method yields spectra under gentle and harsh condition in addition to several conditions of intermediate severity. In the first chromatogram (Figure 4.2) that employed a 5-minute HPLC gradient, CID conditions were switched between only two different aperture 1 voltages (15 and 85 V; MS^E) while in the 26-minute gradient, CID conditions were applied by switching among six different aperture 1 voltages (ranging from 15 to 85 V). The combination of pseudomolecular and fragment masses observed in the major chromatographic peaks using the 5-minute method and MS^E are presented in Table 4.3.

Table 4.3 Prominent ions observed in the major chromatographic peaks from LC/MS analysis of a *P. cruentum* lipid extract using a 5-minute gradient, negative mode electrospray ionization, and MS^{E} .

	Retention		Most abundant ions detected using low AP1	Most abundant ions detected
Peak #	time (min)	Ion counts	voltage (m/z)	using high AP1 voltage (m/z)
1	1.7	4594	*	*
2	1.9	8087	*	*
3	2.3	5708	*	*
4	2.4	5827	*	*
5	2.5	6048	*	*
6	2.9	15158	767.39, 719.49, 839.40,	225.21, 255.23, 537.20,
			841.41, 843.41	767.32, 841.35
7	3.2	18144	671.20, 681.20, 695.23,	255.21, 319.20, 671.20,
			1005.57, 1307.33	681.20, 1127.57
8	3.3	11302	255.23, 283.26, 417.30,	255.23, 281.25, 283.27,
			671.19, 681.20	417.30, 671.19
9	3.5	12954	813.59, 859.40, 975.50,	255.23, 279.24, 301.23,
			985.47,1021.55	303.24, 397.18
10	3.6	7988	927.37, 937.40, 951.40,	526.15, 540.13, 927.37,
			1005.37, 631.44	937.40, 951.40

*Detected ions were judged to not arise from lipids based on the low relative mass defects.

Table 4.3 presents masses observed in 10 resolved peaks that were detected in the LC/MS total ion chromatogram using the 5-minute gradient. Columns 4 and 5 in the table show the most abundant ions within each peak at low and high Aperture 1 voltages respectively. No ions annotated as lipids were detected from the peaks eluting in the first 2.5 minutes of the gradient. Owing to the positive mass defect for hydrogen atoms and high hydrogen content in most lipids, the majority of lipids are expected to relative mass defects (RMD) from 400-1000 ppm [160]. Values of RMD correspond to the mass defect divided by the observed ion mass, and this value is highly correlated with percent hydrogen by weight. Most of the ions from peaks 6-10 have relative mass defects that lie in this range and are annotated as lipid ions. For example the lipid observed at m/z 841.41 has RMD of 487 ppm as calculated below:

$$[(841.41-841.00)/(841.41)] \times 10^{\circ} = 487 \text{ ppm}$$

Using similar calculations, ions with m/z 767.39, 937.40 and 1005.57 have RMDs of 508, 426 and 566 ppm. Fragment ions generated at high Aperture 1 voltage including m/z 255.23 and 303.24 have RMDs of 901 and 791 respectively, as expected for fragments with high hydrogen (and low heteroatom) content.

To illustrate how multiplexed CID mass spectra are used to identify lipids, Figure 4.3 shows negative ion ESI mass spectra of *P. cruentum* lipid at retention time 2.94 minutes at low aperture 1 voltage (15 V) in the top panel while the bottom panel shows the non-selective CID spectrum obtained at the same retention time using Aperture 1 = 85V. The prominent ion the low energy spectrum (m/z 841) was assigned as [M-H]⁻ because no obvious adduct ions 36, 46, or 60 Da heavier were observed. For some lipids, particularly those lacking an acidic functional group, these adducts, corresponding to chloride, formate, or acetate adducts often appear in negative mode ESI mass spectra. In the corresponding high CID voltage spectrum (bottom

panel; aperture 1= 85V), numerous ions appears that were insignificant in the low energy mass spectrum, and these are attributed to fragment ions generated upon collisional activation. The major observed fragment ions are as follows; m/z 585, 537, 303, 255, and 225. The signature fragment in this spectrum is m/z 225.0 which was reported before as one of the common fragment ions upon CID of sulfoquinovosyldiacylglycerol (SQDG) lipids, which are common in plant chloroplasts. This fragment is assigned as a dehydrated sulfonated glucose with chemical formula of $C_6H_9O_7S^{-161}$. Also, ions of m/z 255 (C16:0) and 303 (C20:4) correspond to deprotonated free fatty acid anions. Additional support for the presence of these fatty acids in the SQDG lipid comes from fragment ions (m/z 585 and 537) corresponding to losses of 256 Da (C16:0) and 304 Da (C20:4) fatty acids, respectively.

One concern that is encountered in rapid LC separations arises from coeluting substances. To assess whether these fragment ions share the same retention time as the SQDG [M-H]⁻ ion, extracted ion chromatograms (XIC) for these ions were generated from the high Aperture 1 data and these chromatograms confirm coelution with our pseudomolecular ion of interest m/z 841 (Figure 4.4). One notable feature of this figure demonstrates that the extracted ion chromatogram (XIC) peak for m/z 841 is narrower than all of the fragment ion peaks. This is attributed to the formation of the same fragment ions from other lipids that elute slightly earlier and later than the SQDG detected at m/z 841. Based on extracted ion chromatograms, fragmentation pattern, and the presence of signature fragment of m/z 225.0, the ion at m/z 841 is assigned as [M-H]⁻ of a sulfoquinovosyl diacylglycerol (SQDG) with C16:0 and C20:4 fatty acid esters. Figure 4.5 shows the proposed structure for this lipid (positions of double bonds are not evident from the mass spectra but are assigned based on the common arachidonic acid structure for C20:4). The putative assignments of the C20:4 attachment at the *sn*-1 position and C16:0 at

the *sn*-2 position are based on the greater abundance of the carboxylate anion for the *sn*-2 fatty acid group as reported for MS/MS spectra of phospholipids in negative ion mode [162] Further support for this assignment comes from the inverse selectivity for generating fragments from neutral losses of fatty acids. The fragments at m/z 535.2 and 585.2 correspond to losses of neutral fatty acids C20:4 and C16:0 respectively. A recent paper commented that neutral loss from the *sn*-1 position is favored, and the relative ion abundances provide additional support for the structure described above [163]. The possibility cannot be excluded that m/z 841 corresponds to multiple positional isomers that are not resolved by chromatography. Nevertheless, plant chloroplasts are known to selectively incorporate C16 fatty acids at the *sn*-2 position, which is consistent with the structure presented in Figure 4.5 [164]. Each SQDG lipid contains one acidic sulfonic acid group, which loses its acidic hydrogen easily to yield [M-H]⁻ pseudomolecular ion. This facile loss of proton explains why other adducts (chloride, formate, and acetate) are not observed appreciably in the MS spectrum, and why [M-H]⁻ is the ion that forms more efficiently for SQDGs compared with other adduct ions.



Figure 4.3 Negative mode electrospray ionization spectra at 2.94 minute showing abundant m/z 841 at 15 V (top) and fragment ions at 85 V (bottom) Aperture 1 potential.



Figure 4.4 Extracted ion LC/MS chromatograms of fragment ions at 2.94 minute coeluting with m/z 841 at 85 V Aperture 1 voltage for a *P. cruentum* extract.



Chemical Formula: C₄₅H₇₈O₁₂S

Figure 4.5 Proposed structure for the lipid with molecular mass of 842 Da.

Another abundant signal observed in the LC/MS analysis of the *P. cruentum* extract is assigned as a digalactosyl diacylglycerol (DGDG), a class of lipids that contains two galactose moieties. In contrast with SQDG, which contains an acidic sulfonic acid group and yields abundant [M-H] ions, this lipid lacks a strong acid functional group. Instead DGDG lipids most acidic functionality consists of the hydroxyl groups on the galactose moieties. While these are weak acids, the hydroxyl groups can form attachments with chloride, formate, and acetate, yielding adduct ions ([M+Cl], [M+formate] and [M+acetate]) rather than [M-H]. Figure 4.6 shows the extracted ion chromatograms for deprotonated molecule and the three adducts at low Aperture 1 voltage. One can observe from the chromatograms that all four ions have the same retention time at 3.51 minutes. Figure 4.7 shows negative ion ESI mass spectra of this *P. cruentum* lipid at low aperture 1 voltage (top panel) and high aperture 1 voltage (lower panel). At Aperture 1=15 V (top panel), chloride (m/z 973), formate (m/z 983), and acetate (m/z 997) adduct ions are observed along with the deprotonated molecule (m/z 937.5). The mass unit difference between [M-H] and [M+Cl] is 35 Da, which is consistent with chloride ion attachment to the neutral lipid. The mass difference between [M+Cl] and [M+formate] is 10 Da, and the difference between [M+formate] and [M+acetate] is 14 Da, and all three of these adducts are observed in the ESI mass spectra. This information along with extracted ion chromatograms provides the necessary information to assign the adduct peaks for a certain molecular mass (938.5 Da).



Figure 4.6 Extracted ion chromatograms for m/z 937, 973, 983 and 997 for an extract of *P*. *cruentum* at 3.54 minute using low Aperture 1 voltage from the 5 minute gradient.

By increasing the aperture 1 voltage, the relative abundances of adduct ions decrease, (Figure 4.7) though the abundance of the chloride adduct (m/z 973.5) remains high in the high collision energy spectrum. This behavior is consistent with a decreased reactivity of the chloride adduct relative to the carboxylate adducts, and is attributed to lower gas phase basicity of chloride and greater reach of the carboxylate adducts to abstract protons from elsewhere on the lipid, followed by elimination of the neutral acid (formic or acetic). Another interesting feature in the spectrum of DGDG that differs from the behavior of SQDG described above, is that fatty acids here are lost either as free fatty acids or as ketenes. Examination of the high collision energy spectrum in Figure 4.7 shows fragment ions at m/z 681 and 635, corresponding to neutral losses of C16:0 (256 Da) and C20:5 (302 Da) fatty acids from [M-H]⁻ respectively. At lower masses, a series of fragment ions at m/z 379, 397, and 415 are observed, and these are explained by losses of both neutral fatty acids, one fatty acid and one fatty ketene, and two fatty ketenes respectively. Losses of fatty acids as ketenes has been reported before for other lipids [157,165], but was not
observed the SQDG CID spectra in this study. Ketene loss must involve migration of a hydrogen from the fatty acid chain to the remaining portion of the precursor ion. While it is not yet clear why SQDG does not fragment by ketene loss, it is considered likely that other fragmentation reactions occur faster than the ketene elimination for SQDG. Fragment ions with m/z 681, 635, 415, 397, 379, 301 and 225 are observed at elevated collision energy, and extracted ion chromatograms of all these ions (Figure 4.8) suggest coincidence of elution of all of these fragments with m/z 937 ([M-H]⁻). Ions of m/z 255 (C16:0) and 301 (C20:5) are fatty acid anions lost from the deprotonated molecule m/z 937. From this information, Figure 4.9 shows the proposed structure and proposed fragmentation sites of this lipid (DGDG) with the molecular weight of 938 based on esters with C16:0 and C20:5 fatty acids. Because the abundances of the C16:0 and C20:5 carboxylate ion fragments are similar, there remains some uncertainty regarding the positions of substitution of these fatty acid groups on the MGDG base structure.



Figure 4.7 Negative mode electrospray ionization mass spectra of lipid eluting at 3.54 minutes from an extract of *P. cruentum* showing the putative [M-H]⁻ ion at m/z 937.5 using 15 V (top) and 85 V (bottom) Aperture 1 voltages.



Figure 4.8 Extracted ion chromatograms of an extract of *P. cruentum* at 3.54 minute showing fragment ions at high Aperture 1 voltage (Ap1=85 V).



Figure 4.9 Proposed structures of the 938 Da DGDG 36:5 lipid from *P. cruentum* with fragment ions and neutral losses consistent with this structure assignment.



Figure 4.9 (cont'd)



The short 5-minute gradient method performed well enough to enable identification of several abundant lipids, but when it came to less abundant lipids, coelution often resulted in fragments of the less abundant lipids being obscured by fragments of the more abundant coeluting compound. One example in this regard is for m/z 833.5, which appears as a minor peak in the mass spectrum shown in Figure 4.10. This signal coelutes with the more abundant m/z841 (SQDG 36:4 lipid described above). In the high collision energy spectrum (Aperture 1 = 85V) fragment ions that could be attributed to the minor lipid were not readily recognized owing to the higher abundances of fragments (m/z 225, 255, and 537) from the abundant lipid. Lengthening the gradient to 26 minutes (Figure 4.11) still resulted in coelution of m/z 833 and 841 lipids as evident from the mass spectra (Figure 4.12). In contrast to the spectra obtained using the 5-minute gradient, the high energy CID spectrum generated using the 26-minute gradient shows a low abundance ion at m/z 241. The difference in results is attributed to improved, though only partial resolution, of the two lipids using the longer gradient. Partial resolution of these two lipids allowed the m/z 241 fragment to appear larger owing to less overlap by the SQDG lipid.



Figure 4.10 Negative mode electrospray ionization mass spectra of a lipid eluting at 2.93 minutes from an extract of *P. cruentum* showing the putative [M-H]⁻ ion m/z 833.5 using 15 V (top) and 85 V (bottom) Aperture 1 voltages. The abundant ion at m/z 841 is assigned as the coeluting SQDG 36:4 lipid described in the text above.



Figure 4.11 LC/MS extracted ion chromatogram of ions with m/z 833.5 of an extract of *P. cruentum* using a 26-minute gradient and Aperture 1 voltage of 15 V). The prominent peak at 7.29 minutes corresponds to the lipid that eluted at 2.93 minutes using the 5-minute gradient.



Figure 4.12 Negative mode electrospray ionization mass spectra obtained at Aperture 1 = 15 V (top) and 80 V (bottom) showing coelution of m/z 833 and 841 lipids at 7.29 minutes using the 26-minute gradient.

To remove the effects of coeluting lipids on the CID spectra, an MS/MS enhanced product ion spectrum was generated (products of m/z 833) to assign the structure of this lipid by selective collisional activation of only m/z 833. The extract of *P. cruentum* was analyzed using a 20-

minute gradient similar to the 26-minute method described above, and enhanced product ion scans were acquired on a QTRAP triple quadrupole/linear trap hybrid mass spectrometer. Figure 4.13 shows the total ion chromatogram of products of m/z 833. The first major chromatographic peak, which is highlighted, is consistent with the first lipid detected as described above using the 26-minute gradient method. Figure 4.14 shows the MS/MS enhanced product ion spectrum of this lipid. The presence of m/z 241 confirms its assignment as a phosphatidylinositol [166,167], and the presence of the two fatty acid carboxylates at m/z 255 and 279 are consistent with C16:0 and C18:2 fatty acid groups. Using this information, the lipid is assigned as PI (16:0, 18:2), and the proposed structure is presented in Figure 4.15.

It is noted that the CID spectrum obtained using the LC/TOF mass spectrometer differs from the enhanced product ion spectrum generated on the QTRAP instrument, in that the fatty acid anion fragment at m/z 279 was not observed in the former. These differences are attributed to the different time scale between ion-molecule collisions and detection of fragment ions. In the case of the TOF mass analyzer, the time between ion activation and ion acceleration is on the order of 10^{-4} to 10^{-5} seconds, whereas the enhanced product ion scans on the QTRAP instrument are generated by trapping all product ions, then ejecting one mass at a time. The elapsed time between ion-molecule collision and ion detection is 10^{-1} to 10^{-2} seconds, which is about 1000fold greater elapsed time relative to the TOF instrument. This difference in time frame means that products of slower ion decomposition reactions are more likely to be observed in the QTRAP than in the TOF mass spectrometer.



Figure 4.13 LC/MS product ion chromatogram of m/z 833 for an extract of *P. cruentum*.



Figure 4.14 Enhanced product ion spectrum for m/z 833, detected using negative mode electrospray ionization, for the chromatographic peak highlighted in Figure 4.13.



Chemical Formula: C₄₃H₇₉O₁₃P

Figure 4.15 Proposed structure of the identified phosphatidylinositol lipid (PI 16:0; 20:2)

Resorting to the use of MS/MS scans was not always necessary for lipid structure elucidation, especially when the slower 26-minute chromatographic gradient was used. An example of the performance of LC/TOF MS with multiplexed CID is observed in the detection of monogalactosyl diacylglycerol (MGDG) lipids, which contain only one galactose substructure. Examination of the combined mass spectra over the entire chromatogram generated for a *P. cruentum* extract revealed numerous abundant ions. Two of these ions, at m/z 869 and 883, differed in mass by 14 Da. A less abundant peak at m/z 859 was also observed, and differences between these masses suggested that these ions were [M+formate], [M+acetate] and [M+Cl]. To locate the elution time of specific ions, extracted ion chromatograms were calculated and displayed for these masses as well as the anticipated mass of [M-H]. Figure 4.16 shows the chromatogram of these ions, and confirms the presence of the deprotonated molecule (m/z 823) that coelutes at 12.88 minutes.

The utility of multiplexing CID conditions is clear from mass spectra generated using collision potentials ranging from 15 to 85 V (Figure 4.17). At the lowest collision energies, the only observed ions are the acetate, formate, and chloride adducts and a minor ion corresponding to [M-H]⁻. When the collision potential is raised to 45 V, the yield of [M-H]⁻ has increased substantially, and minor fragment ions at m/z 301, 303, 537 and 539 appear. The former two

masses correspond to fatty acid anions of C20:5 and C20:4 respectively, and the fragments at m/z 537 and 539 correspond to losses of neutral C20:4 and C20:5 fatty acid ketenes respectively. Upon increasing collision potential to 85 V, only the fatty acid anions remain. The near-simultaneous acquisition of six different kinds of mass spectra for each eluting lipid allows the analyst to use whichever spectrum best provides structural information without need for optimizing collision conditions ahead of time.



Figure 4.16 Extracted ion chromatograms of deprotonated molecule (m/z 823) at 12.88 min and its adducts (m/z 859, m/z 869, and m/z 883) at low Aperture 1 voltage (Ap1=15 V).



Figure 4.17 Negative mode electrospray ionization mass spectra from a *P. cruentum* extract at retention time of 12.88 minutes obtained at six different Aperture 1 voltages ranging from 15 V (top) to 85 V (bottom).



Chemical Formula: C₄₉H₇₆O₁₀

Figure 4.18 Proposed structure of the 824 Da lipid (MGDG 20:4; 20:5) lipid from *P. cruentum* that eluted at a retention time of 12.88 minutes.

Based on accurate mass measurements, non-selective collision induced dissociation spectra, and in some cases selective MS/MS fragmentation spectra, the major lipids identified in extracts of *P. cruentum* using negative mode electrospray are from the following lipid classes: sulfoquinovosyl diacylglycerols (SQDG), digalactosyl diacylglycerols (DGDG), monogalactosyl diacylglycerols (MGDG) and phosphatidylinositol (PI). The individual lipids identified from each class are presented in Table 4.4. Lipids in all three classes primarily consist of one C16:0 fatty acid ester plus one polyunsaturated fatty acid ester with either 18 carbon atoms and 1-2 double bonds, or 20 carbon atoms and 2-5 double bonds. These findings are consistent with earlier reports of lipid composition of *P. cruentum* using TLC fractionation and GC/MS of fatty acid esters [168,169].

4.4 Conclusions

One of the initial factors that guided profiling of lipids in *P. cruentum* was the expectation that this red alga accumulates a large proportion of arachidonic acid (C20:4)-containing lipids, and the LC/MS results presented above are consistent with earlier observations from other *P. cruentum* strains [169,170]. It was hoped that bioactive lipid oxidation products might be formed from these unsaturated lipids, either *in vivo* in the algal cells, or *ex vivo* using other organisms or enzymes that could convert arachidonates to their anti-inflammatory epoxides. Lipid profiles generated during this study gave evidence of barely-detectable oxidized arachidonates in the form of ions of m/z 319, but levels were too low for more extensive quantitative analyses. Unfortunately, the strain used in this investigation (*P. cruentum* UTEX LB2757) proved recalcitrant to isolation as a pure axenic culture.

Table 4.4 Identified lipids from the *P. cruentum* extract using a 26-minute ultrahigh performance liquid chromatographic separation, negative mode electrospray ionization mass spectrometry, and multiplexed collision induced dissociation. Column three shows m/z of deprotonated molecular fatty acid anions.

				Fatty acid anion	
	Туре	m/z	Rt (min)	fragments (m/z)	Lipid annotation
1	SQDG	839.54	6.61	255,301	SQDG 16:0; 20:5
2	SQDG	841.55	7.52	255,303	SQDG 16:0; 20:4
3	SQDG	843.57	8.33	255,305	SQDG 16:0; 20:3
4	SQDG	819.56	9.30	255,281	SQDG 16:0; 18:1
5	SQDG	845.58	9.54	255,307	SQDG 16:0; 20:2
6	SQDG	817.56	7.89	253,281	SQDG 16:1; 18:1
7	SQDG	793.53	8.94	255,255	SQDG 16:0; 16:0
8	MGDG	753.57	17.76	255,279	MGDG 16:0; 18:2
9	MGDG	777.56	17.19	255,303	MGDG 16:0; 20:4
10	MGDG	775.55	15.17	255,301	MGDG 16:0; 20:5
11	MGDG	825.57	14.74	303,303	MGDG 20:4; 20:4
12	MGDG	801.56	15.27	279,303	MGDG 18:2; 20:4
13	MGDG	823.55	12.88	301,303	MGDG 20:5; 20:4
14	MGDG	827.57	16.35	303,305	MGDG 20:4; 20:5
15	DGDG	915.62	14.16	255,279	DGDG 16:0; 18:2
16	DGDG	937.61	11.53	255,301	DGDG 16:0; 20:5
17	DGDG	939.63	13.52	255,303	DGDG 16:0; 20:4
18	DGDG	983.60	8.06	301,301	DGDG 20:5; 20:5
19	DGDG	985.62	9.63	301,303	DGDG 20:5; 20:4
20	DGDG	987.63	11.35	301,305	DGDG 20:5; 20:3
21	DGDG	915.63	14.16	255,279	DGDG 16:0; 18:2
22	DGDG	917.63	16.53	255,281	DGDG 16:0; 18:1
23	DGDG	943.64	16.97	255,307	DGDG 16:0; 20:2
24	DGDG	963.62	11.71	279,303	DGDG 18:2; 20:4
25	DGDG	961.60	9.89	279,301	DGDG 18:2; 20:5
26	DGDG	987.62	11.36	303,303	DGDG 20:4; 20:4
27	PI	833.54	7.29	255,279	PI 16:0; 18:2
28	PI	857.54	8.33	255,303	PI 16:0; 20:4

culture despite repeated treatments with antibiotics, and further study was discontinued when fungal culture contaminants raised concerns about the source and stability of lipids extracted from culture.

Despite the challenges posed in sustaining pure algal cultures, the major findings of this study have shown that multiplexed CID mass spectra are useful for lipid profiling. This is evident from the ease with which fatty acid groups are observed in their anionic forms using elevated collision potentials, and the flexibility generated by acquisition of mass spectra at multiple extents of fragmentation. The multiplexed CID approach is user-friendly, as it does not require optimization of mass spectrometry parameters, and the mass accuracy provided by the TOF mass analyzer allows lipids to be distinguished from contaminants and other substances based on relative mass defects. This experimental approach is well suited for both rapid lipid screening as well as more comprehensive separations and lipidome characterization. **Chapter Five: Concluding Remarks**

Identification and quantification of bioactive lipids is important due to their use as medicines, food constituents and many other consumer products. Understanding the state of bioactive lipids is not an easy task since the types and amounts of individual lipids vary due to physiological changes of the organs containing those lipids. Comprehensive studying of such changes may generate enormous numbers of samples for analysis. Such demanding task requires fast and comprehensive analysis methods.

Modern analytical chemistry has moved toward improved high-throughput and comprehensive analysis techniques in recent years. Such methodologies can be used for analysis of many compounds more efficiently than one-at a time approaches. Other than time, cost is another important factor in large scale screening. To move toward such capabilities of cost and time effective large scale screening, research in this dissertation has developed mass spectrometry based techniques that enhance bioactive lipid identification and quantification. LC/MS with relatively inexpensive time-of-flight and quadrupole mass analyzers has been used in this research to achieve these goals.

In Chapter Two, LC/MS/MS methods were developed and applied to identify and quantify 10 natural sweeteners, including several not described in this document owing to a confidentiality agreement with the research sponsor, with RSDs less than 5%, using less than 8 minutes of instrument time per extract. The developed method was used to analyze over 1200 samples from Stevia leaves grown at different physiological conditions. Multiple reaction monitoring mode (MRM), is a selective and targeted MS/MS technique, was successfully optimized using a quadrupole ion-trap, and was applied for targeted identification and quantification. Although this technique was used for quantification of known analytes, additional peaks observed in the LC/MS chromatograms led to the discovery of additional isomeric diterpene glycosides in Stevia leaf extracts using the same MRM transitions. Our findings indicate the real need for powerful chromatography before mass spectrometry analysis. Adding one more mass transition to the original MRM transitions led us to identify Rebaudioside F as a compound that was not initially included in the list of target analytes. Finding Reb F and other previously unreported sweetener isomers demonstrates the importance of thinking beyond the target analysis list and think out of the box.

In Chapter Three, experiments were conducted to establish whether isomeric diterpenoid glycosides could be distinguished from their tandem mass spectra. This exercise was initiated with low expectations, since the isomers differed only in the position of sugar attachment. Applying collision induced dissociation at different energies led to breakdown curves for deprotonated and adduct molecular ions of Stevia glycosides. Comparisons of such breakdown curves resulted in unexpected and dramatic differences between isomers with regard to the ease of fragmentation upon collision induced dissociation. These findings are specifically helpful for identification of isomeric metabolites that have identical sugars attached at different positions with different linkages. Although this study was not used to identify unknown isomers from Stevia leaf extracts but applied to explain fragmentation behavior at specific collision energies.

Screening of lipids extracted from the red alga *Porphyridium cruentum* using two different approaches was discussed in Chapter Four. Although application of the time-of-flight mass analyzer did not yield true MS/MS spectra for lipid screening, the relatively new approach developed in our laboratory called multiplexed collision induced dissociation (mux-CID) generated non-selective fragmentation at multiple collision energies in a single analysis. This approach is beneficial when using a relatively inexpensive time-of-flight analyzer because both molecular and fragment masses can be obtained with high mass accuracy at the same time. Such information can be generated from more expensive analyzers such as quadrupole time-of-flight or Orbitraps also, but in the case of the latter, at the expense of longer mass analysis times.

One aspect of this comprehensive lipid identification was using different chromatography approaches. Fast screening with short gradients on ultrahigh performance liquid chromatographic columns allowed detection of lipid molecular masses as is useful for surveys of large numbers of samples. Although some eluted lipids from the short LC gradient were successfully identified, the complexity of overlapping spectra at some retention times presented an obstacle to identification of individual lipids. To address this shortcoming, a longer gradient was applied to obtain greater chromatographic resolution of individual lipids.

Although long gradients along with mux-CID were successfully applied for identification of many lipids, the complexity of the algal lipidome still prevented resolution of coeluting lipids without targeted MS/MS analysis. For such reasons, especially for identification of phosphatidylinositols, MS/MS spectra were generated using a QTRAP analyzer to associate fragment ions with a specific lipid molecular mass.

Results shown in Chapter Four identify compromises that must be made when choosing between short and longer analysis methods. While short and non-targeted methods are helpful for fast screening of couple of analytes, more comprehensive analyses need more resolution from both chromatographic and mass spectrometric protocols. Therefore, no single method is sufficient to resolve the entire lipidome. References

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