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Analyses of Food and Feed Compounds using Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry

by

Jian Wang 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

Food Science and Technology

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ABSTRACT

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) has several advantages over other techniques: speed of analysis, high sensitivity, wide mass range, mass determination of unfragmented molecules, tolerance to contaminants, and ability to analyze complex mixtures. This thesis presents the application of MALDI-MS to analyze fructooligosaccharides, debranched starch, anthocyanins, flavonol glycosides, and coccidiostats in food or feed samples. Important MALDI-MS conditions for these compounds are described, including the selection of matrices, the use of an internal standard or standard addition, and the determination of response factors.

Fructooligosaccharides demonstrated similar responses on a molar basis when 2',4',6'-trihydroxyacetophenone (THAP) was used as a matrix. Fructooligosaccharides in onions, shallots, and garlic were quantified using MALDI-MS and the results compared with high performance anion exchange chromatography with pulsed amperometric detection. Both techniques provided similar results, but MALDI-MS showed a higher tolerance to impurities. The use of MALDI-MS to study the molecular size profile of debranched starch was also achieved. 2,5-Dihydroxybenzoic acid (DHB) was the best matrix for the maltooligosaccharides from debranched starch, which exhibited similar response factors on a weight basis.

MALDI-MS analysis of anthocyanins was possible when THAP was used as the matrix. Anthocyanins fragment in the MALDI-MS ion source with loss of carbohydrate residues, but in a predictable manner. Chemically similar anthocyanins had similar responses. Anthocyanins in highbush blueberries were quantified using MALDI-MS and

the results compared with high performance liquid chromatography (HPLC). Both techniques provided similar quantitative anthocyanin profiles for the samples. HPLC could distinguish anthocyanin isomers, while MALDI-MS proved to be more rapid in accurate analysis of anthocyanins with different masses.

Details of ionization, response factors, and fragmentation were studied for flavonol glycosides in MALDI-MS using THAP as the matrix. This information allowed for proper interpretation of MALDI-MS spectrum for these analytes in food samples.

The use of MALDI-MS for quantification of coccidiostats in poultry feeds is also described. DHB was selected as the best matrix. The MALDI-MS limit of detection for lasalocid, monensin, salinomycin and narasin standards was 251, 22, 24 and 24 fmole, respectively. The method detection limit for salinomycin and narasin in poultry feeds was 2.4 μg/g.

This thesis is dedicated to my wife Min Liu,

to my daughter Rachel,

to my parents,

and

to everyone who has supported me with their love.

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List of Abbreviations

3-AQ	3-aminoquinoline
C18	octadecyl silane
DHB	2,5-dihydroxybenzoic acid
DP	degree of polymerization
ELISA	enzyme-linked immunosorbent assay
ESI	electrospray ionization
FAB	fast atom bombardment
FT-ICR	Fourier transform ion cyclotron
fwhm	full width at half maximum
GC	gas chromatography
GF_2	1-kestose
GF_3	nystose
GF_4	β-fructofuranosylnystose
HABA	[2-(4-hydroxyphenylazo)-benzoic acid]
HCCA	α-cyano-4-hydroxycinnamic acid
HIC	1-hydroxyisoquinoline
HN-APCI	heated nebulizer atmospheric pressure chemical ionization
HPAEC-PAD	high performance anion exchange chromatography with pulsed amperometric detection
HPLC	high performance liquid chromatography
HPLC-PCD	high performance liquid chromatography with post-column derivatization
IDA	*trans*-3-indoleacrylic acid
ISD	in-source decay
m/z	mass-to-charge ratio
MALDI	matrix-assisted laser desorption/ionization
MS	mass spectrometry
NMR	nuclear magnetic resonance
PSD	post-source decay
RSD	relative standard deviation
S/N	signal to noise ratio
SA	sinapinic acid
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
THAP	2',4',6'-trihydroxyacetophenone
TOF	time-of-flight
UV-Vis	Ultraviolet-visible

Chapter 1

Introduction

Mass spectrometry is perhaps the most widely applicable of all the analytical techniques available to scientists since it is capable of providing structural as well as quantitative information about an analyte of interest. It is based upon the *in vacuum* separation of ions, in the gas phase, according to their mass-to-charge (*m/z*) ratio. Samples may be introduced from gas, liquid or solid states. In the latter two cases volatilization must be accomplished either prior to, or accompanying ionization. Many ionization techniques are available to produce charged species from analytes in the gas phase and the most common are electron impact ionization and chemical ionization. In electron impact ionization, a neutral analyte molecule is hit by accelerated electrons, knocking free other electrons and resulting in a positively-charged ion. The ionization can either produce intact molecular ions, which will have the same molecular weight as the original molecule, or fragment ions (daughter ions), which correspond to a lower mass portion of the original analyte molecule. The molecular ions and fragment ions result in reproducible mass spectra (full ion scanning), which can be searched through detailed spectral libraries to determine the structure of the analyte of interest. However, instead of scanning and obtaining a full spectrum, the mass spectrometer can be employed as a detector and used simply to monitor one or more ions from the spectrum to achieve quantification of the analyte of interest. Chemical ionization relies on the interaction of analytes of interest with a reactive ionized reagent species. The chemical ionization process begins when a reagent gas, such as methane, is ionized by electron impact. The ionized reagent can react with the analytes to produce analyte ions in both positive and negative mode. Generally chemical ionization produces less fragmentation than electron impact. Chemical ionization is probably one of the most common procedures for producing ions for mass spectrometry. Both electron impact ionization and chemical ionization are applied only to thermally stable lower mass ($\leq$ 1000) volatile compounds, which limits their applications in some circumstances. A quadrupole mass

analyzer, with a practical *m/z* limit less than 2000, is often used for electron impact and chemical ionization to determine the masses of the ions formed.

The development of desorption ionization techniques allows analysis of nonvolatile or thermally unstable analytes. Fast atom bombardment (FAB), heated nebulizer atmospheric pressure chemical ionization (HN-APCI), electrospray ionization (ESI), and matrix assisted laser desorption/ionization (MALDI) are the most significant methods. In FAB, a sample is dissolved in a liquid matrix such as glycerol and then placed on a target. In a vacuum, the target is bombarded with a fast atom beam, for example xenon, that desorbs the analyte to produce molecular ions and fragment ions (Barber et al., 1981). FAB yields minimal fragmentation and performs well for polar and thermally-labile compounds. It is also applicable to masses up to 10000 daltons. However, it has high chemical background or noise, with moderate sensitivity. The heated nebulizer with atmospheric pressure chemical ionization is a robust interface for coupling HPLC at conventional flow rates (0.5 - 1.5 mL/min.) to a mass spectrometer (Horning et al., 1973; Sakairi and Kambara, 1988). In HN-APCI, a corona discharge is used to ionize the analyte in an atmospheric pressure region when the analyte is introduced into the ion source using a heated capillary tube. It is typically used to analyze molecules less than 1000 daltons using quadrupole or ion trap mass analyzers.

Electrospray ionization had a tremendous impact in the 1990's on the use of mass spectrometry. Dole et al. (1968) were first able to bring macromolecules into the gas-phase at atmospheric pressure by spraying a solution from the tip of an electrically charged capillary. However, it was in 1985 that Whitehouse et al. (1985) developed electrospray as a true interface for mass spectrometry. Electrospray ionization can produce multiply-charged ions, with the number of charges tending to increase as the molecular weight increases. Because mass spectrometers measure mass-to-charge ratios rather than mass itself, it is possible for higher mass molecules to carry sufficient numbers of charges to fall within a mass-to-charge ratio or mass range of 2000, which can be determined using a quadrupole mass analyzer. ESI can be used to analyze molecules up to 200000 daltons. However, the technique is very sensitive to contaminants such as alkali metals. Both electrospray and matrix assisted laser

desorption/ionization techniques (discussed below) have contributed dramatically to the progress in sensitivity and accessible mass range of mass spectrometry.

MATRIX-ASSISTED LASER DESORPTION/IONIZATION MASS SPECTROMETRY

Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) was developed in the middle to late 1980s (Karas et al., 1985, 1987; Karas and Hillenkamp, 1988; Tanaka et al., 1988). It is a "soft" ionization process that produces molecular ions from large nonvolatile molecules. Therefore, it provides a tool to analyze intact molecular ions for proteins, oligonucleotides, polysaccharides, and synthetic polymers, with minimal fragmentation. MALDI-MS is usually coupled to time-of-flight (TOF) mass analyzers, but several applications have been demonstrated using a Fourier transform ion cyclotron (FT-ICR) (Buchanan and Hettich, 1993; Castoro et al., 1993), magnetic sector analyzers (Harvey, 1996), and quadrupole ion trap mass spectrometers (Jonscher et al., 1993; Hall et al., 1999). For this thesis the acronym MALDI-MS will always represent MALDI TOF MS, that is the ionization technique coupled to time-of-flight mass analysers.

MALDI-MS has advantages over other methodologies, including speed of analysis, high sensitivity, wide applicability combined with a good tolerance towards contaminants, and the ability to analyze complex mixtures (Karas, 1996). However, simple MALDI-MS instruments can not tell the difference between isomers, which have identical mass. The potential application of MALDI-MS allows for analysis of most molecules. MALDI-MS analysis of any compounds consists of sample introduction, ionization, ion separation, ion detection and/or data analysis. Figure 1-1 shows the basic MALDI-MS components: ion source, time-of-flight mass analyzer, and detector.

A sample is placed in the MALDI-MS ion source directly after it has been cocrystallized with matrix on a probe. A laser ionizes analytes. Using a laser as a desorption/ionization method for organic molecules dates back to the 1970's, but all earlier experiments on laser desorption of organic ions were restricted to the analysis of molecular masses below 2000 (Bahr et al., 1994; Borman, 1995). The major breakthrough in laser desorption/ionization was achieved in 1988 when the matrix was

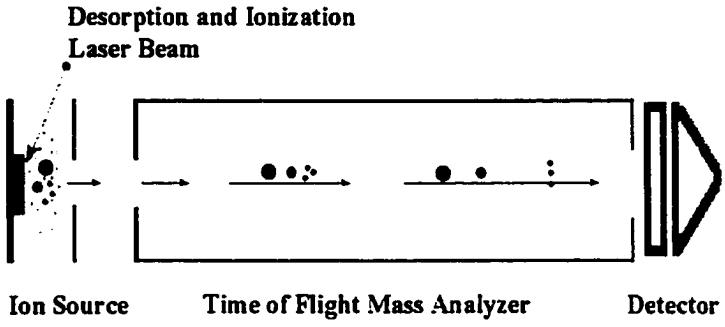
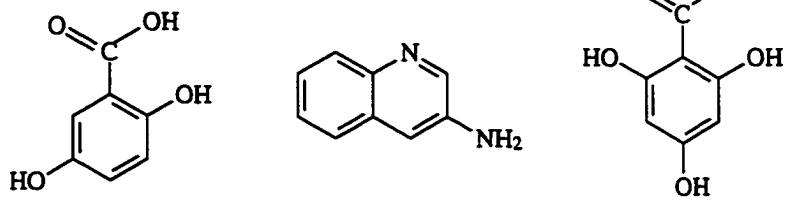


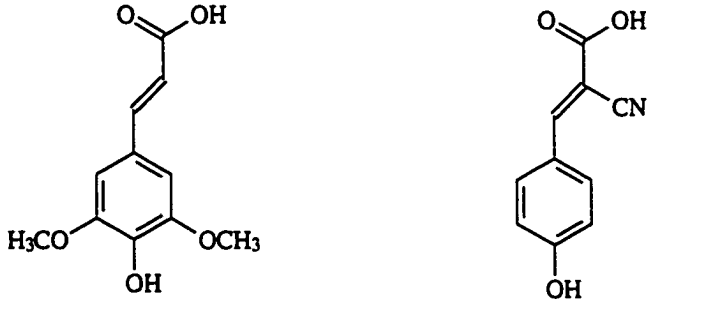
Figure 1-1. Schematic of the basic components of a linear MALDI-MS.

introduced to assist the desorption and ionization of large nonvolatile molecules to overcome the mass limitation of ionizing just the analyte with the laser (Karas and Hillenkamp, 1988; Tanaka et al., 1988). That is, to desorb large molecules, higher laser strength was needed for ion production and this inevitably destroyed larger molecules. A nitrogen (N_2) laser emitting at 337 nm is now almost universally employed for MALDI-MS analysis, and is supplied as standard on all commercial instruments (Harvey, 1999). The laser beam can be attenuated to reduce the strength of the initial beam. A typical laser strength is 10^6 - 10^7 Watts/cm^2 and occurs over a probe area of only 0.01 mm^2 (User's Guide to the Proflex™ III, undated).

The matrix (Figure 1-2) is a key aspect of MALDI-MS. Not only does the matrix help to introduce the sample into the ion source, but it also serves three other major functions: absorbing energy from the laser to desorb analytes and prevent decomposition of analytes; isolating analytes (specifically for large biomolecules) from each other to avoid aggregation; and ionizing analytes (Bahr et al., 1994). Generally, the matrix consists of small easily-crystallizable ultraviolet-absorbing molecules. Another important physical parameter for matrix compounds is that their crystals are stable under vacuum conditions. Compounds with very rapid sublimation rates are unsuitable. 2,5-dihydroxybenzoic acid (DHB) (Stahl et al., 1991; Harvey, 1999) is the most popular matrix for carbohydrates. 3-Aminoquinoline (3-AQ) is a good matrix for inulin or fructooligosaccharides (Metzger et al., 1994; Stahl et al., 1997). 2',4',6'-Trihydroxyacetophenone (THAP) has been successfully used for oligonucleotides (Pieles et al., 1993), inulin or fructooligosaccharides (Chapter 2, Wang et al., 1999a),



2,5-dihydroxybenzoic acid 3-aminoquinoline 2',4',6',-trihydroxyacetophenone



sinapinic acid α-cyano-4-hydroxycinnamic acid

Figure 1-2. Chemical structures of common MALDI-MS matrices.

glycoalkaloids (Abell and Sporns, 1996), anthocyanins (Chapter 4, Wang and Sporns, 1999), and flavonol glycosides (Chapter 6, Wang and Sporns, 2000). Sinapinic acid (SA) is often used for large molecular proteins (Kussmann et al., 1997), and α-cyano-4-hydroxycinnamic acid (HCCA) is a good matrix for peptides (Kussmann et al., 1997).

Proper preparation of the matrix and sample is important to obtain good MALDI-MS spectra. It is usually necessary to maintain a 500 or more fold molecular excess of matrix to analyte. There are two common methods to prepare matrix and sample, dried-droplet and fast evaporation. For the dried-droplet method, a 5-20 mg/mL solution, which is often a saturated solution, is prepared in either pure water or a mixture of water and organic solvent (acetonitrile, ethanol) or a mixture of water acidified with trifluoroacetic acid (0.1%) and acetonitrile (2:1). The analyte concentration is of the order of 10^{-3} to 10^{-7} M. Small amounts of both matrix solution and analyte solution (between 0.5 - 30 μL) are then mixed. The mixture (1 or 2 μL) of matrix and analyte is spotted on a metal target

wheel or probe. The solvent is evaporated and the cocrystalized sample is ready for MALDI-MS analysis (Karas and Hillenkamp, 1988; Bruker, 1995; Kussmann et al., 1997).

Fast evaporation was developed to improve MALDI-MS resolution and sensitivity (Vorm et al., 1994; Nicola et al., 1995). The matrix is dissolved in acetone (or acetone containing 1-2% water or 0.1% aqueous TFA), e.g. THAP in acetone. The matrix solution (0.3 - 0.5 μL) is spotted on the probe first. Because of the rapid evaporation of acetone, more homogeneous crystals are formed. Then the sample solution (0.5 - 1 μL) is spotted on top of the matrix crystals. The fast evaporation method results in very good spot-to-spot repeatability, which is important for MALDI-MS quantification (Abell and Sporns, 1996; Chapter 2, Wang et al., 1999a; Driedger and Sporns, 1999a, b; Chapter 4, Wang and Sporns, 1999). Usually, the amount of analytes, applied to MALDI-MS, is in the picomole or fentomole range (10^{-12} - 10^{-15} M).

Other techniques have also been developed to improve the preparation of matrix and sample. Nitrocellulose has been used to improve crystal homogeneity (Preston et al., 1993; Kussmann et al., 1997). Nitrocellulose solution (5 mg/mL in methanol) was spotted on the probe first, and then a mixture of matrix and sample was applied on top of the nitrocellulose. Preston et al. (1993) used this method to improve homogeneity of crystals and quantify bradykinin (a peptide) without using an internal standard. Electrospray deposition, traditionally used to prepare thin, uniform samples for plasma desorption and secondary ion mass spectrometry, has also been utilized to prepare MALDI-MS samples (Hensel et al., 1997). The small droplets formed during the electrospray process have been found to also significantly improve the homogeneity of the sample surface.

MALDI-MS is tolerant of impurities, but signal suppression caused by contaminants in a sample occurs through inhibition of analyte ionization in the ion source and samples, i.e. biologically-derived samples, must still be isolated and purified prior to analysis to obtain the best results. Purification removes salts and buffer components, which may interfere with the signal during data acquisition. Some simple cleanup procedures have been developed to remove impurities directly from the probe (on-probe cleanup). Rouse and Vath (1996) used an on-probe sample cleanup method involving placement *in situ* of amounts of chromatographic media such as cation- or anion-

exchange resins onto a probe containing a drop of matrix and sample. After air-drying, removal of the chromatographic media eliminated interference from salts or detergents in the samples. Brockman et al. (1997, 1998) derivatized the probe's surface with octadecanethiol (C18). The C18-derivatized probe could be used as a hydrophobic solid-phase extraction device to isolate and desalt biopolymers directly on the probe surface. On-probe affinity or immunoaffinity is a technique that combines affinity separations directly with MALDI-MS. In this approach, a binding molecule, such as an antibody, is attached to the surface of a MALDI probe. This permits the analyte of interest to be selectively captured and concentrated on the probe surface prior to MALDI-MS analysis (Brockman and Orlando, 1995, 1996; Liang et al., 1998).

The ionization mechanisms involved in MALDI are still largely unknown. Ions may be pre-formed in the solid state or may be formed in the gas phase by ion-molecule reactions immediately following desorption by the laser (Harvey, 1999). The most significant feature of MALDI ionization is the formation of singly-charged ions through protonation, alkali adducts (sodium and/or potassium), or deprotonation (Karas et al., 2000). After analytes have been ionized, they are accelerated in the ion source by a high voltage, typically 20 - 30 kV, to a fixed kinetic energy with variable velocity depending on their mass. Then the ions enter a time-of-flight mass analyzer or field-free drift tube (0.5 - 2 m, Figure 1-1). In time-of-flight (TOF), the mass-to-charge ratio (m/z) of an ion is determined by measuring its flight time. An equation relating the flight time of an ion with its m/z value can be expressed as follows:

$$\mathbf{t} = \mathbf{a}\,(m/z)^{1/2} + \mathbf{b}$$

where **a** and **b** are constants for a given instrument condition, and are determined experimentally from flight times of ions of known masses, e.g. calibrants. The flight time of an ion is **t**. Ionized species, including matrix ions and analyte ions, pass the time-of-flight mass analyzer, with a velocity proportional to $(m/z)^{1/2}$. Due to their m/z dependent velocities, ions are separated during their flight. A detector at the end of the flight tube produces a signal for each ion species. Typical flight times are between a few microseconds and several hundred microseconds.

The initial kinetic energy or velocity distribution of analytes during the desorption/ionization process causes a poor resolution, e.g. 500 fwhm (full width at half

maximum). For ions of the same *m/z*, those with higher axial velocity will move further from the repeller than the initially less-energetic ions. So far, two techniques have been used to increase the resolution, delayed extraction (Colby et al., 1994; Vestal et al., 1995; Guilhaus et al., 1997) and a reflectron (Bahr et al., 1994). In delayed extraction, ions, after desorption, are allowed to randomly distribute for hundreds of nanoseconds to several microseconds, followed by extraction of the ions into the flight tube. During the process, the closer ions are to the repeller (lower initial velocity), the more kinetic energy, resulting in ions of the same *m/z* reaching the detector at the same time if the delayed time is properly adjusted (Figure 1-1). Thus resolution is improved to at least 4000 fwhm. A reflectron (Figure 1-3), that is an ion mirror, also compensates for the difference in kinetic energy to improve the resolution. Ions of higher energy will penetrate deeper into an electrostatic repeller field (ion mirror) and will be turned around and arrive at the detector at the same time as ions of lower initial energy that penetrate the field less. Resolution improvement from a reflectron instrument of up to 6000 fwhm or more is possible.

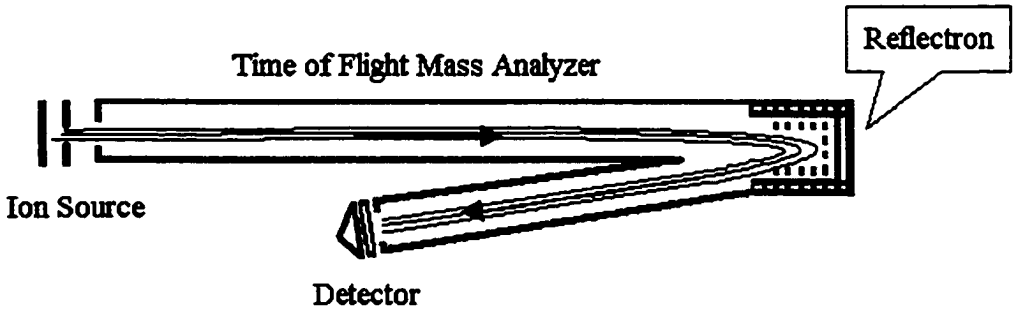


Figure 1-3. Schematic of a reflectron MALDI-MS.

Therefore, there are two types of instrumentation, a linear MALDI-MS (Figure 1-1) and a reflectron MALDI-MS (Figure 1-3). Each has advantages for its application in terms of fragmentation during in-source and post-source decay. The ions, formed after extraction from the ion source, are called post-source decay (PSD) ions, in contrast to ions formed rapidly within the ion source, which are know as in-source decay (ISD). In a

linear mode, PSD ions, such as neutral and charged fragments generated through fragmentation of molecular ions in the field-free drift tube, cannot be distinguished from the parent ions, because their velocity remains the same. Nevertheless, this is a great advantage for MALDI-MS quantification because parent ions and PSD ions from the same molecules will be observed as one single peak at the mass of the parent ions. For a reflectron mode, PSD ions generated prior to reflection have the same velocity as their parent ion, but can be separated by the reflectron. Using the reflectron mode, combining ISD and PSD information, can be a powerful tool for structural or sequencing determination of analytes that exhibit characteristic fragmentation, such as carbohydrates and peptides (Harvey, 1999; Pfeifer et al., 1999).

MALDI-MS QUANTIFICATION

The reasons for using MALDI-MS for quantification are related to previously noted advantages, such as speed of analysis, ease of use, high sensitivity, tolerance of contaminants, and ability to analyze complex mixtures. It is possible to use MALDI-MS to analyze most compounds in a sample after the analytes of interest have been extracted. The quantification is usually achieved using a linear MALDI-MS. MALDI-MS is known for its poor reproducibility, which means that for a series of MALDI-MS samples prepared under identical conditions, the analyte signal intensity or peak height intensity is remarkably variable. This variable analyte signal intensity or peak height intensity is related to the matrix used, the number of laser shots acquired, the laser strength attenuation, and spot-to-spot or sample-to-sample desorption. Therefore, key concerns for MALDI-MS quantification include the selection of matrices, preparation of matrix and sample, and the use of an internal standard. Significant MALDI-MS quantitative studies are summarized in Table 1-1.

MALDI-MS Quantification. Three strategies have been employed for MALDI-MS quantification: using an internal standard; using standard addition method; and without using any standard. Since 1993, there have been investigations into the quantitative aspects of MALDI-MS using pure carbohydrate or protein standards as samples. Abell and Sporns (1996) first used MALDI-MS to quantify glycoalkaloids in

Table 1-1. Summary of MALDI-MS Quantification

Analytes	methods of quantification	references
Benzoylecgonine (a cocaine metabolite form human urine)	internal standard: deuterated benzoylecgonine	Hall et al., 1999
Sulfatide (acidic glycosphingolipid)	internal standard: hydrogenated lysosulfatide	Sugiyama et al., 1999
Polyunsaturated diacylglycerols	internal standard: polyunsaturated diacylglycerols	Benard et al., 1999
Chaconine, solanine (glycoalkaloid)	internal standard: tomatine (glycoalkaloid)	Driedger and Sporns, 1999b
Chaconine, solanine (glycoalkaloid)	internal standard: tomatine (glycoalkaloid)	Driedger and Sporns, 1999a
Debranched starch	standard addition: maltoheptaose (DP7)	Wang et al., 1999b
Anthocyanins	internal standard: cyanidin 3-rutinoside (anthocyanin)	Chapter 4, Wang and Sporns, 1999
Fructooligosaccharides	standard addition: β-fructofuranosylnystose (DP 5)	Chapter 2, Wang et al., 1999a
Maltooligosaccharides	internal standard: N-acetylgalactosamine	Kazmaier et al., 1998
Dinoterb (pesticide) Aminotriazole (pesticide)	internal standard: dinitrophenol without internal standard	Benazouz et al., 1998
Sulfonamide (antibiotics)	internal standard: acetaminophen	Ling et al., 1998
Avenins	without internal standard	Camafeita and Mendez, 1998
bovine insulin	internal standard: des-pentapeptide human insulin	Wilkinson et al., 1997
Gliadins	without internal standard	Camafeita et al., 1997b
Gliadins	without internal standard	Camafeita et al., 1997a
Cocaine hydrochloride	internal standard: cocaine-d_3 (a deuterated analog of cocaine)	Nicola et al., 1996
21-base oligonucleotide (*m/z* 6361)	internal standard: 36-base oligonucleotide (*m/z* 11131)	Bruenner 1996
Chaconine, solanine (glycoalkaloid)	internal standard: tomatine (glycoalkaloid)	Abell and Sporns, 1996
Permethylated cyclodextrins β-cyclodextrin	internal standard: permethylated cyclodextrin internal standard: maltohexaose	Bartsch et al., 1996
Cyclosporin A (*m/z* 1202.2, oligopeptide) bovine insulin (*m/z* 5733.5) bovine insulin (*m/z* 5733.5)	internal standard: cyclosporin D (m/z 1216.2) internal standard: human Arg-insulin (*m/z* 5963.8) internal standard: cytochrome C (*m/z* 12384)	Gusev et al., 1996

Angiotensin II	internal standard: 1-SAR-8-Ile angiotensin II	Nicola et al., 1995
Peptides/proteins in mass range 1000-12000	internal standard: peptides/proteins in mass range 1000-12000	Jespersen et al., 1995
Cytochrome C	internal standard: myoglobin	Nelson and McLean, 1994
Lactoferrin	internal standard: human serum albumin	
3,4-dihydroxyphenylalanine	internal standard: [$^{13}C_6$] labeled 3,4-dihydroxyphenylalanine	Duncan et al., 1993
Acetylcholine	internal standard: [$^{2}H_{16}$] labeled acetylcholine	
H-Ser-Ala-Leu-Arg-His-Tyr-NH_2	internal standard: Ac-Ser-Ile-Arg-His-Tyr-NH_2	
bovine insulin	internal standard: cytochrome C or bovine insulin chain B	Gusev et al., 1993
Bradykinin	without internal standard	Preston et al., 1993
Lysozyme, myoglogin	internal standard: cytochrome C	Tang et al., 1993
3 or 4 base (mer) nucleotide	internal standard: 2 base (mer) nucleotide	
Oligosaccharide NA2	internal standard: fucosylated oligosaccharide NA2	Harvey, 1993
Oligosaccharide NA2	without internal standard	

crude food samples. A few earlier studies (Harvey, 1993; Tang et al., 1993; Preston et al., 1993) reported good correlation between analyte signal and concentration, but the linear response was significantly influenced by the laser strength, matrix, and the preparation of the matrix and sample. Generally, an internal standard was required to overcome this problem. Samples were spiked with known amounts of a molecule, which was similar to the analyte molecule (internal standard method); it was a structural analogue, or the same molecule, such as a stable isotope-labeled form (for instance with ^{13}C or ^{2}H) (Duncan et al., 1993). The internal standard should also be able to mimic the behavior of the analyte at all stages of sample preparation, desorption, and ionization, and give a similar response in MALDI-MS as the analyte. In theory, a stable isotope-labeled form (having the same chemical properties but distinguishable mass) of the same molecule would be the best internal standard. Duncan et al. (1993) first used isotope-labeled internal standards in MALDI-MS quantification. The analyte and its isotope-labeled form showed similar response and good linearity was achieved, but this method is likely only practical in the lower-mass region (below *m/z* 200) due to the cost of making large isotope-labeled molecules (Duncan et al., 1993). Therefore, using a structural analogue as an internal

standard is often a more simple and cheaper approach. For example, when quantifying oligosaccharide NA2, its fucosylated analogue was used as an internal standard (Harvey, 1993). Ac-Ser-Ile-Arg-His-Tyr-NH_2 was used as an internal standard for quantification of the peptide H-Ser-Ala-Leu-Arg-His-Tyr-NH_2 (Duncan et al., 1993). In fact, a structural analogue does not always give the same response as the analyte. The response of γ-cyclodextrin was only one twentieth the response of 2,3,6-tri-*O*-methyl-γ-cyclodextrin (Sporns and Wang, 1998) and was half the response of fructooligosaccharides (Chapter 2, Wang et al., 1999a). Gusev et al. (1993) investigated the use of an internal standard having chemical properties different from the analyte. For example, cytochrome C was used as an internal standard to analyze bovine insulin, and good linearity was achieved. However, Gusev et al. (1996) also indicated that the different chemical properties of the internal standard and analyte led to discrimination in the analyte/internal standard intensity ratios and might be caused by the changing of the analyte/matrix ratio or competition of analyte and internal standard with the matrix. If the concentration of the internal standard was kept constant and close to the highest analyte concentration, quantification was possible. In general, for a single analyte, a structural analogue has usually been chosen as the internal standard (Table 1-1).

However, an ideal internal standard is not always available. Specifically, when MALDI-MS is used to analyze a group of analytes or polymers in a mixture, effects between analytes, the internal standard, or the sample environment become complicated. Different responses of analytes and their internal standards may cause poor reproducibility for MALDI-MS quantification. One good example is the use of γ-cyclodextrin standard addition to quantify fructooligosaccharides in food extracts (Chapter 2, Wang et al., 1999a). Although the response of γ-cyclodextrin (internal standard) was consistent for pure standard fructooligosaccharides, its response changed in crude food extracts. Food extracts from red onions, for example, completely suppressed the production of ions from added γ-cyclodextrin. Therefore, a standard addition method was developed to quantify analytes in a mixture using MALDI-MS (Chapter 2, Wang et al., 1999a). A standard added is usually one of the analytes. The peak height increase is proportional to the amount of the standard added to the sample. Based on the increased peak height, the amount of other analytes can be calculated. The prerequisite or

assumption is that all analytes have the same responses as that of the standard added. Standard addition has the advantage to quantify a complex analyte mixture, for example oligosaccharides, but the analysis time is doubled.

Although it showed poor spot-to-spot repeatability, MALDI-MS is still often used to quantify analytes without using an internal standard. Camafeita et al. (1997a, 1997b) used MALDI-MS to quantify gliadins (about *m/z* 31 kDa) and extended the method to quantify avenins (*m/z* 18-33 kDa) for screening gluten in the diet of coeliac patients (Camafeita and Mendez, 1998). In these studies, absolute peak intensities or areas were used to plot a standard curve. It seemed that good linearity (R^2 not reported) was achieved in the range of 0.4 - 1.0 mg/100 g, with a detection limit 0.4 mg/100 g of food, which permitted the analysis of gluten-free foods below the toxic threshold (5 mg/100 g, Camafeita et al., 1997b). Furthermore, MALDI-MS results were well correlated to those of high sensitive ELISA (enzyme-linked immunosorbent assay) (Camafeita et al., 1997b). This indicates that MALDI-MS quantification without internal standard is feasible, and may apply to higher mass molecules if sufficient laser shots are collected.

In addition, quantification can also be achieved in MALDI-MS negative mode using an internal standard when analytes of interest are easily ionized through deprotonation to form negative ions. Sulfatide is one type of acidic glycosphingolipid, and MALDI-MS negative mode provided a sensitive, convenient, reliable technique to quantify sulfatide in serum, with linearity between 2 pmol and 1 nmol (Sugiyama et al., 1999).

Other Considerations. The selection of matrices is very important for MALDI-MS quantification of any analyte. The sensitivity of MALDI-MS for any analyte is matrix related. The selected matrix also needs to be applicable to crude sample extracts. Several matrices could produce flavonol glycoside ions from pure standards. Only 2',4',6'-trihydroxyacetophenone (THAP) worked for crude flavonol glycoside extracts (Chapter 6, Wang and Sporns, 2000). The response of a mixture of analytes in a sample in MALDI-MS is another key concern. First, for a group of chemically similar analytes, their responses with changing concentration should be linear. Secondly, all analytes in a mixture should show similar responses with addition of any single analyte for quantification (Chapter 2, Wang et al., 1999a; Chapter 3, Wang et al., 1999b).

Fragmentation of analytes in the ion source provides structural information but causes a problem for quantification. Fragment ions may overlap other molecular ions. Often fragmentation can be consistent, predictable and therefore quantitatively determined (Chapter 6, Wang and Sporns, 2000; Chapter 5, Wang et al., 2000).

Repeatability of MALDI-MS quantification. Driedger and Sporns (1999a) reported the repeatability of MALDI-MS quantification on two glycoalkaloids, chaconine and solanine. The same potato sample was analyzed in triplicate on seven separate days over a 4-month period. The relative standard deviations of triplicate measurements ranged from 1 to 16%, with an average of 9%. The day-to-day relative standard deviation for replicate determinations was 10% for chaconine and 12% for solanine. Recoveries of spiked samples were within 5% of their expected values. These results indicated that MALDI-MS is a reliable technique for quantification and can serve for routine analysis.

MALDI-MS in FOOD ANALYSES

Theoretically, MALDI-MS has the ability to analyze most molecules in a food matrix, yet it is even more applicable than competing techniques for nonvolatile compounds. Usually, MALDI-MS is used for determination of masses between 500 and 300,000. In practice the upper mass limit is about at *m/z* 1.5×10^6 (Schriemer and Li, 1996). Low mass determinations are also possible. For example, *m/z* at 84, aminotriazole, a pesticide (possible contaminant of water) was determined by Benazouz et al (1998). A summary of MALDI-MS food analyses is presented in Table 1-2. The compounds analyzed in various foods include carbohydrates, proteins, peptides, lipids, bacterial extracts, antioxidants or polyphenols, steroidal glycosides, pesticides, and many others. Since 1995, MALDI-MS has been used to analyze complex food matrices (Table 1-2). Most of the food analysis applications are in the area of protein or peptide analyses. The contribution of MALDI-MS to food analyses is significant and is discussed below.

Accessing High Molecular Weight Compounds. MALDI-MS allows for analysis of higher mass compounds in foods. High molecular weight wheat glutenin subunits, wheat gliadin, barley hordeins, rye secalins, and oat avenins are groups of high molecular weight proteins, which have masses of *m/z* 20000 to 90000. Analyses of these

Table 1-2. Summary of Food Analyses using MALDI-MS

Group	specifics	references
Bacteria	fingerprints and biomarkers of *E. coli* and *B. atrophaeus*	Jarman et al., 1999
	fingerprints matching of *E. coli* strains	Arnold and Reilly, 1998
	fingerprints and biomarkers of *Enterobacteriaceae*	Lynn et al., 1999
	fingerprints and biomarkers of *E. coli* and *B. atrophaeus*	Saenz et al., 1999
Bacteriocin	identification of product formed by reaction of nisin and glutathione	Rose et al., 1999b
	identification of bacteriocin from *Lactococcus lactis*	Lee et al., 1999a
	detection of bacteriocins from culture supernatants	Rose et al., 1999a
Glycoalkaloid	quantification of potato glycoalkaloids	Driedger and Sporns, 1999a
	quantification of potato glycoalkaloids	Driedger and Sporns, 1999b
	quantification of potato glycoalkaloids	Abell and Sporns, 1996
Lipids	structural elucidation of fruit juice carotenol fatty acid esters and carotenoids	Wingerath et al., 1996
	triacyglycerol profile of flaxseed, walnut, sesame, grapeseed, and hazelnut oils	Ayorinde et al., 1999a
	triacyglycerol profile of cod liver oil	Ayorinde et al., 1999b
	triacyglycerol profile of canola, castor, and olive oils	Ayorinde et al., 1999c
	triacyglycerol profile of olive oil and onion seeds	Asbury et al., 1999
	quantification of saturated and polyunsaturated diacylglycerols	Benard et al., 1999
Maillard reaction	identification of melanoidin-like Maillard polymers or reaction	Tressl et al., 1998a
	identification of melanoidin-like Maillard polymers or reaction	Tressl et al., 1998b
	mass determination of protein glycosylation products	Kim et al., 1997
Naringin	identification of maltosylnaringin in citrus fruits	Lee et al., 1999b
Oligosaccharides	quantification of fructooligosaccharides in onions, shallots and garlic	Chapter 2, Wang et al., 1999a
	mass distribution of fructooligosaccharides in garlic	Losso and Nakai, 1997
	quantification aspects of maltooligo/polysaccharides from corn and barley debranched starch	Chapter 3, Wang et al., 1999b
	structural and branching pattern determination of oligosaccharides from beer	Vinogradov and Bock, 1998

	size distribution of oligosaccharides from enzymatic hydrolysis of barley starch	Chmelik et al., 1998
	structural characterization of cinnamoyl-oligosaccharides	Lequart et al., 1999
	size distribution of fructans	Stahl et al., 1997
Anthocyanins	identification and quantification of anthocyanins in red wine and fruits juice	Chapter 4, Wang and Sporns, 1999
	identification of catechin oligomers (condensed tannins) in apples	Ohnishi-Kameyama et al., 1997
	identification of anthocyanins in grape skins	Sugui et al., 1999
	analysis of 3-deoxyanthocyanidins and anthocyanins present in crude extracts from sorghum plant tissue	Sugui et al., 1998
Flavonols	qualitative and quantitative aspects of flavonol glycosides in tea and onion	Chapter 6, Wang and Sporns, 2000
Proteins or peptides	cheese flavor peptides identified and sequenced	Gouldsworthy et al., 1996
	mass of lysyl oxidase (a cuproenzyme) in chick tendons	Ruker et al., 1999
	mass of patatin and identification of protease inhibitors in potatoes	Pots et al., 1999
	mass of novel cysteine protease D3-alpha and beta in germinating soybean cotyledons	Asano et al., 1999
	mass of alkylated and unalkylated subunits in wheat glutenin	Masci et al., 1999
	mass changes of the chymotrypsin derivatives	Rawel et al., 1998
	identification of 60% (v/v) aqueous ethanol soluble proteins or gluten in food samples	Sorell et al., 1998
	identification of gluten avenins in foods	Camafeita and Mendez, 1998
	selective identification of gliadins, hordeins, scalins, and avenins in food	Camafeita et al., 1998
	identification of allergens, lysozyme and ovomucoid in egg white	Besler et al., 1998
	mass determination of beta-primeverosidase for black tea processing	Ijima et al., 1998
	mass determination of deglycosylated ovomucoid (allgergen in egg white)	Besler et al., 1997
	quantification of gliadins in food	Camafeita et al., 1997a
	mass determination of glutaredoxin (thioltransferase, catalyst of thio/disulfide exchange reaction) in rice	Sha et al., 1997
	identification of gliadins in food	Camafeita et al., 1997b
	mass determination of glutenin subunits	Hickman et al., 1995

	mass of a heparin-binding peptide derived from bovine lactoferrin	Shimazaki et al., 1998
	determination of the changes of milk protein profile in yogurt production	Fedele et al., 1999
	milk protein composition screening or quality control	Catinella et al., 1996
	identification of high molecular weight glutenin subunits from wheat	Garozzo et al., 1999
	identification of wheat varieties	Bloch et al., 1999
	protein profiles of infant formulae	Sabbadin et al., 1999
	mass determination of high molecular weight subunits of glutenin	Hickman et al., 1995
	protein profiles of water buffalo mozzarella cheese in quality control	Angeletti et al., 1998
	protein profiles of ewe cheese in quality control	Fanton et al., 1998
	protein profiles of human milk	Catinella et al., 1999
Pesticides	quantitative study of four pesticides, aldicarb, aminotriazole, dinoterb, and ioxynil	Benazouz et al., 1998

proteins are of significance, for example for wheat variety identification, wheat quality assessment, and gluten screening for coeliac disease. Traditionally, these proteins were analyzed by using SDS-PAGE or ELISA. The molecular weight determined using SDS-PAGE is commonly assumed to be within 5-10%, which is very inaccurate. ELISA antibodies can recognize mainly wheat gliadins, rye secalins, and to a much lesser extent barley hordeins, but they fail to detect avenins (Camafeita and Mendez, 1998). However, MALDI-MS provides a powerful tool to accurately analyze all these different high molecular weight proteins in foods. Applications of MALDI-MS on gluten proteins have been successfully established (Hickman et al., 1995; Mendez et al., 1995; Camafeita et al., 1997a, 1997b, 1998; Camafeita and Mendez, 1998; Sorell et al., 1998; Bloch et al., 1999; Garozzo et al., 1999).

Providing Authentic Fingerprints. MALDI-MS has the ability to analyze a complex mixture in a few minutes. The acquired MALDI-MS spectra provide authentic fingerprints of the samples of interest. The MALDI-MS fingerprints or biomarkers of bacteria, such as *E. coli*, have demonstrated this approach as a practical technique for rapid bacterial identification in the area of food safety. Assignment of specific biomarkers for several bacteria has also been reported (Arnold and Reilly, 1998; Saenz et

al., 1999; Jarman et al., 1999; Lynn et al., 1999). The principle behind the fingerprints or biomarkers is that, approximately, 50% of the bacterial cell is protein. MALDI-MS is ideal for the detection of high and low molecular proteins, i.e. bacterial proteins at *m/z* 2000 - 20000. MALDI-MS, with automated statistics-based data analysis algorithms (Arnold and Reilly, 1998; Jarman et al., 1999) or with an internet-accessible protein database (Demirev et al., 1999), showed good spot-to-spot and sample-to-sample repeatability for the acquisition of fingerprints. The accuracy and speed of MALDI-MS data make it a potentially important tool for screening food-borne pathogens.

Fingerprints can also serve as a tool for quality control. MALDI-MS has demonstrated its applicability in quality control or fraudulence in water buffalo mozzarella and ewe cheese (Angeletti et al., 1998; Fanton et al., 1998). MALDI-MS could generate fingerprints for bovine, buffalo, and ewe milk or cheese, thus verifying their authenticity. Other potential applications of MALDI-MS fingerprints have been reported. Anthocyanin profiles of various fruit juices, red wines, and grapes, and flavonol glycoside profiles of tea and onion have been reported and might also serve for quality control (Sugui et al., 1999; Chapter 4, Wang and Sporns, 1999; Chapter 6, Wang and Sporns, 2000).

High Sensitivity for Oligosaccharides. There are two competitive techniques for analysis of oligosaccharides, high performance anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) and MALDI-MS. Stahl et al. (1997) used HPAEC-PAD to resolve inulin with a degree of polymerization (DP) of approximately 70 single sugar units, but sensitivity and resolution decreased with increasing of DP. Wong and Jane (1997) used HPAEC-PAD and post-column enzymatic pad to convert debranched starch into glucose before detection to increase the sensitivity of HPAEC-PAD, and a resolution of DP 80 was achieved. However, Mohr et al. (1995) showed the potential of using MALDI-MS to resolve carbohydrates (dextran 40 kDa) of DP 100 or more. MALDI-MS showed higher or more constant sensitivity towards higher DP oligosaccharides than HPAEC-PAD (Kazmaier et al., 1998; Chapter 2, Wang et al., 1999a). MALDI-MS has been used to analyze fructooligosaccharides in onions, shallot, garlic (Stahl et al., 1997; Losso and Nakai, 1997; Chapter 2, Wang et al., 1999a), debranched starch in corn and barley (Chmelik et al., 1998; Chapter 3, Wang et al.,

1999b), and the branching pattern of oligosaccharides in beer (Vinogradov and Bock, 1998). It is believed that MALDI-MS can provide quantitative information for oligosaccharides with different DP in foods (Chapter 2, Wang et al., 1999a; Chapter 3, Wang et al., 1999b).

Applicability to Non-polar or Thermally Labile Molecules. One characteristic feature of MALDI-MS is high quasimolecular or intact ion yield with little or no fragmentation (Karas et al., 1987; Karas and Hillenkamp, 1988). As stated earlier, the matrix is believed to function to prevent decomposition of the analytes of interest by the laser. This is of significance for analysis of labile molecules in foods. For example, MALDI-MS has been successfully used to analyze intact triacylglycerols of polyunsaturated fatty acids in canola, castor, olive (Ayorinde et al., 1999a), flaxseed, walnut, sesame, grapeseed, hazelnut (Ayorinde et al., 1999b), and cod liver oils (Ayorinde et al., 1999c), and phospholipids in soybean and egg yolk (Schiller et al., 1999). MALDI-MS has the power to differentiate these intact lipids. Sixty-four triacylglycerols have been assigned in cod liver oils using MALDI-MS (Ayorinde et al., 1999c). Traditional gas chromatographic (GC) analysis is impractical for analysis of the non-volatile triacylglycerols and phospholipids unless they are saponified and the fatty acids liberated are further esterified to form volatile fatty acid methyl esters. HPLC, coupled with desorption chemical ionization or electrospray ionization mass spectrometry, has also been used to characterize these lipids, but the analytical protocols are tedious and time consuming, with complex instrumentation, and requiring specialized chromatographic columns (Ayorinde et al., 1999b, 1999c).

Another example for MALDI-MS determination of labile molecules is analysis of carotenoids and their fatty acid esters. Carotenoids and their fatty acid esters are complex non-volatile but thermally labile compounds, which makes them hard to analyze using other techniques. MALDI-MS provided both molecular ions and fragment ions of carotenoids and their fatty acid esters, from which structural information was easily obtained, with high sensitivity (Kaufman et al., 1996; Wingerath et al., 1996).

Tolerance of Impurity and High Speed Analysis. MALDI-MS is a technique showing high tolerance towards contaminants since crude sample extracts can be determined by MALDI-MS directly without purification. This may be due to the

selectivity of the matrix for certain analytes. When crude methanol/water (1:1) extract of potato was applied to MALDI-MS, only glycoalkaloids were detected in this crude complex extract mixture using THAP as matrix (Abell and Sporns, 1996; Driedger and Sporns, 1999a, 1999b). Fructooligosaccharides, in onions, shallots, and garlic, could be determined when hot water crude extracts were spotted directly for MALDI-MS analysis without any purification, using THAP as matrix (Chapter 2, Wang et al., 1999a). Since foods contain many compounds, partial purification is sometimes necessary. Solid-phase-extraction has demonstrated its importance in preparation for MALDI-MS food analysis. After extraction using a Sep-pak C18 cartridge, anthocyanins and flavonol glycosides were successfully analyzed using MALDI-MS (Chapter 4, Wang and Sporns, 1999; Chapter 6, Wang and Sporns, 2000).

The speed of MALDI-MS is also attractive to food scientists for routine analysis. The analysis or MALDI-MS run time depends on the number of laser shots collected. One hundred laser shots require about 1 minute to collect, and normally around 200 laser shots are collected for one spectrum. For triplicate analysis of glycoalkaloids, MALDI-MS used 20 min/sample, while 2 hr/sample was required for HPLC analysis (Abell and Sporns, 1996).

Other Applications. MALDI-MS can readily detect the mass change of a molecule. It can monitor chemical reactions in foods, such as interactions of α-chymotrypsin (a serine protease) with benzyl isothiocyanate (Rawel et al., 1998), nisin (a bacteriocin) with glutathione (Rose et al., 1999b), or the Maillard reaction (Kim et al., 1997; Tressl et al., 1998a, 1998b).

THESIS OBJECTIVES

The overall objectives of this thesis were to explore MALDI-MS applicability in food science. Food and feed compounds examined included fructooligosaccharides, debranched starch, anthocyanins, flavonol glycosides, and coccidiostats. Standards of each group of compounds were used to model analytes, to study their performances in MALDI-MS, including ionization and fragmentation, and to determine response factors (in a mixture) for quantification. Methodologies were developed to quantify these

compounds in food and feed samples using MALDI-MS. For each group of compounds, the research objectives were:

MALDI-MS of Oligosaccharides. To select proper matrices for fructooligosaccharides and maltooligosaccharide or debranched starch; to study their responses in MALDI-MS; to compare HPAEC-PAD and MALDI-MS quantitative analysis results of fructooligosaccharides in food samples (Chapter 2 and 3).

MALDI-MS Quantification of Anthocyanins. To study the responses of the monoglycosides and diglycosides in MALDI-MS; to analyze anthocyanins from various food sources using MALDI-MS; to define quantitative fragmentation of anthocyanins in MALDI-MS; to compare HPLC and MALDI-MS quantitative analysis results of anthocyanins in highbush blue berries (Chapter 4 and 5).

MALDI-MS Analysis of Flavonol Glycosides. To understand the performance of flavonol glycosides in MALDI-MS both positive and negative mode; to study the responses and fragmentation of flavonol glycosides, i.e. kaempferol and quercetin glycosides, in MALDI-MS; to analyze flavonol glycosides in food samples (Chapter 6).

MALDI-MS Quantification of Coccidiostats. To select a proper matrix for lasalocid, monensin, salinomycin and narasin; to develop a methodology to quantify these coccidiostats with simple sample preparation; to study the limit of detection for lasalocid, monensin, salinomycin or narasin using MALDI-MS (Chapter 7).

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Chapter 2

Analysis of Food Oligosaccharides using MALDI-MS: Quantification of Fructooligosaccharides[1]

INTRODUCTION

Fructans are nonreducing water-soluble carbohydrates formed in higher plants composed of fructosyl units but usually containing one terminal glucose moiety per molecule. They occur as linear, branched, or, less frequently, as cyclic molecules (Darbyshire and Henry, 1978, 1981; Bancal and Gaudillere, 1991; Stahl et al., 1997). Natural β-fructans have a degree of polymerization (DP) ranging from 2 to 55 or more. Lower mass (DP of 2 to 20) fructans are also called fructooligosaccharides (Figure 2-1), while higher mass polymers are known as inulin. It has been found that these nondigestible carbohydrates are effective in improving intestinal flora and increasing calcium and magnesium absorption (Ohta et al., 1995, 1998). Fructans have also been examined for their biological role in plant osmoregulation, adaptation to low temperature photosynthesis, protection from freezing stress (Darbyshire and Henry, 1978; Pollock, 1984; Nelson and Smith, 1986; Chatterton et al., 1990; Livingston, 1990), and storage life of bulbs (Darbyshire and Henry, 1981; Suzuki and Cutcliffe, 1989).

Traditionally a variety of techniques have been used to analyze fructooligosaccharides. Gel permeation chromatography (Darbyshire and Henry, 1978) has been used, but detection and identification of the separated fructooligosaccharides requires extensive additional methodology involving acid hydrolysis and various enzymatic and colorimetric procedures to identify the carbohydrates. The method of Manghi et al. (1995) detects fructooligosaccharides after various enzyme treatments and identification of the carbohydrates produced by high performance liquid chromatography

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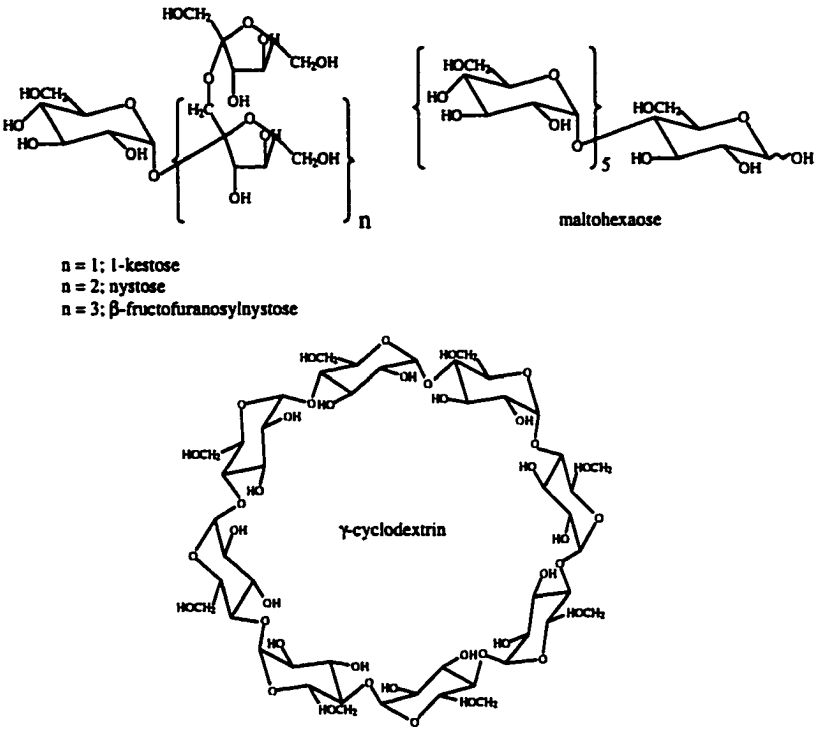

Figure 2-1. Chemical structures of fructooligosaccharides, maltohexaose, and γ-cyclodextrin.

(HPLC). However, the procedure is also lengthy and gives minimal information about which polymers are present. Loo et al. (1995) describe a number of analytical procedures for determination of fructooligosaccharides including HPLC for low DP fructooligosaccharides and a gas chromatography (GC) procedure. While the latter can be used for sensitive determination of fructooligosaccharides up to about DP of 12, extensive purification and derivatization with water sensitive reagents are required. In addition GC conditions of very high temperatures are required to volatilize the derivatized fructooligosaccharides. By far the most commonly used procedure for analysis of fructooligosaccharides is high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (Chatterton et al., 1989, 1993; Shiomi et al., 1991; Timmermans et al., 1994; Loo et al., 1995). The fructooligosaccharide response with HPAEC-PAD does vary (Timmermans et al., 1994) and the analyses often require significant sample purification.

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) was originally developed for measuring the mass of large molecules such as proteins. MALDI-MS has also been applied to carbohydrates since about 1991 (Mock et al., 1991; Stahl et al., 1991). Fructooligosaccharides or inulin in some plants have been qualitatively analyzed using MALDI-MS (Metzger et al., 1994; Stahl et al., 1997; Losso and Nakai, 1997) but there have been no reports on quantitative analysis. Our purpose in this study was to develop methodology for both qualitative and quantitative analysis of fructooligosaccharides in selected food samples.

MATERIALS AND METHODS

Materials and Reagents. Red onion bulbs (*Allium cepa* L.), shallot bulbs (*Allium cepa* L. *var. ascalonicum*), and elephant garlic (*Allium ampeloprasum*) were purchased from local markets in Edmonton, Alberta, Canada. Inulin from Jerusalem artichokes, γ-cyclodextrin, maltohexaose, 4-hydroxy-α-cyanocinnamic acid (HCCA), and sinapinic acid were purchased from Sigma Chem. Co. (St. Louis, MO). A mixture of 1-kestose (GF_2, DP = 3), nystose (GF_3, DP = 4), and β-fructofuranosylnystose (GF_4, DP = 5) (34%, 53%, and 10%) and these individual fructooligosaccharides in pure form were a gift from Dr. A. Ohta, Nutritional Science Center, Meiji Seika Kaisha, Japan. 2',4',6'-Trihydroxyacetophenone monohydrate (THAP), [2-(4-hydroxyphenylazo)-benzoic acid] (HABA), 3-aminoquinoline (3-AQ), 1-hydroxyisoquinoline (HIC), and 2,5-dihydroxybenzoic acid (DHB) were obtained from Aldrich Chem. Co. (Milwaukee, WI).

Extraction of Fructooligosaccharides from Onion, Shallot, and Garlic Samples. Fresh samples were peeled to remove the dry outer layers and then chopped using a food processor (Braun, UK 100, Type 4259, Germany) for 5 min. The chopped samples were freeze-dried. Samples were extracted according to Stahl et al. (1997) using water instead of 80% of ethanol as extraction solvent. Each freeze-dried sample (1 g) was extracted with two portions of 40 mL doubly deionized water heated to reflux for 1 hr. The cooled sample was centrifuged for 15 min. at 10 000 rpm after each water extraction; the supernatants were combined and made up to 100 mL with water. The aqueous extract

was then filtered using a Millipore HA 0.45 μm membrane (Chromatography Division/Millipore Corp., Milford, MA) and the extract was kept frozen at -20 C until needed. Fructooligosaccharides might undergo enzymatic hydrolysis and chemical degradation during extraction, however, this research was less concerned with extraction methodology and more interested in analytical comparison of the extract.

Moisture Content. Sample moisture content was determined according to AOAC Official Method of Analysis (1990) using a vacuum oven (National Appliance Co., USA) overnight *in vacuo* at 70 C.

MALDI-MS. MALDI-MS was performed using the Proflex™ III, Bruker Analytical Systems Inc. (Billerica, MA). Analytes cocrystallized with matrices on the probe were ionized using a nitrogen laser pulse (337 nm) and accelerated under 20 kV using pulsed ion extraction before entering the time-of-flight mass spectrometer. The preparation of matrices and samples is shown in Table 2-1. Laser strength was selected to obtain the best signal-to-noise ratios. The number of laser pulses collected was determined as needed to obtain good responses of all oligosaccharides.

Table 2-1. Performance of Matrices for Desorption and Ionization of Maltohexaose and γ-Cyclodextrin

matrix	references	matrix concentration	preparation of matrix and sample	spectra quality	repeatability	matrix peaks	analyte molar ratio
2,5-dihydroxybenzoic acid (DHB)	Bruker, 1995; Mohr et al., 1995; Losso et al., 1997	1. 12.3 mg/mL in ethanol/water=1:1	mix matrix and sample together in a ratio of 1 :1	excellent	good	medium	normal
		2. 10.1 mg/mL in double deionized water	as above	good	good	medium	normal
2',4',6'-trihydroxyacetophenenone monohydrate (THAP)	Pieles et al., 1993	1. saturated in acetone	put matrix on the probe first and then sample on top of matrix	excellent	excellent	few	normal
		2. 12.5 mg/mL in acetonitrile/water=1:1	mix matrix and sample together in a ratio of 1 :1	excellent	excellent	few	normal
3-aminoquinoline	Metzger et al., 1994; Stahl et al., 1997	10.1 mg/mL in 10 % ethanol	as above	good	poor	few	normal
4-hydroxy-α-cyanocinnamic acid (HCCA)	Bartsch et al., 1996	13.3 mg/ml in 50 % ethanol or matrix saturated in ethanol	as above	good	poor	medium	normal
sinapinic acid	Bruker, 1995	14.2 mg/mL in acetonitrile/ water=1:1	as above	no	no	medium	
2-(4-hydroxyphenylazo)-benzoic acid (HABA)	Mohr et al., 1995	saturated in acetone	put matrix on the probe first and then sample on top of matrix	poor	good	Lots	abnormal
2,5-dihydroxybenzoic acid (DHB)/1-hydroxyisoquinoline (HIC)	Mohr et al., 1995	0.2 M DHB/0.06M HIC in acetonitrile/water=1:1	mix matrix and sample together in a ratio of 1 :1	good	poor	few	normal

Quantification of fructooligosaccharides using MALDI-MS was achieved using standard addition. The frozen sample extracts were allowed to warm to room temperature. Samples were prepared by taking 50 μL of extract and mixing this with 50 μL of aqueous 0.01 M potassium chloride solution. The standard addition samples had GF_4 (7.9×10^{-4} M in 50 μL of aqueous 0.01 M potassium chloride) added to 50 μL of sample extracts. Samples and standard addition samples were each spotted in five separate positions on the probe. A single spectrum was then generated for each position on the probe (10 spectra in all, five for each sample and five for each standard addition sample) by random selection of three different spots for each probe position and collecting 60 laser pulses for each spot. That is, one spectrum represented the sum of 3×60 or 180 laser pulses. Peak heights (for potassium adducts) were determined for each fructooligosaccharide from each spectrum. These peak heights were then scaled relative to the GF_3 peak, which was arbitrarily set at a value of 1.0. Each of the five spectra for the sample was compared to a different spectrum from the five standard addition samples and the average increase in scaled relative peak height for GF_4 (standard fructooligosaccharide added) determined. This gave a value for the average increased response due to the addition of GF_4. This response factor was then used to determine the average amount of each fructooligosaccharide in the five sample spectra. The acquisition of the MALDI-MS data took about 20 minutes. All samples were analyzed in duplicate. That is, an entirely new 10 spots (five samples and five standard addition samples) were analyzed (see Appendix I for experimental design).

HPAEC-PAD.[2] The frozen red onion, shallot and garlic sample extracts were allowed to come to room temperature and diluted 1:5 (in 5-mL volumetric flask) with water. Fructooligosaccharide standards (10.0 mg) were prepared in a 50-mL volumetric flask with HPLC grade water. Each sample was passed through a 0.2-μm syringe filter (25 mm; Chromatographic Specialties, Brockville, ON). Filtered samples were analyzed on a Waters 625 metal free gradient HPLC (Waters Chromatography, Milford, MA). All samples and standards were injected (50 μL) with a Waters 712 Wisp autosampler.

[2] HPAEC-PAD was conducted by Dr. Low in Department of Applied Microbiology and Food Science at the University of Saskatchewan, Saskatoon, Saskatchewan.

Carbohydrates were separated on a Carbo Pac PA1 column (250 mm ×4 mm; Dionex, Sunnyvale, CA) coupled with a Carbo Pac PA1 guard column (50 mm × 4 mm). The solvents used were 100 mM sodium hydroxide (solvent A), 100 mM sodium hydroxide/400 mM sodium acetate (solvent B), and 300 mM sodium hydroxide (solvent C). The mobile phase flow rate was maintained at 1 mL/min, with a linear gradient profile consisting of solvent A with the following proportions (v/v) of solvent B or C: 0-8 min, maintain 0% B and 0% C; 8-60 min, 100% B; 60-61 min, 100% C; 61-90 min, maintain 100% C; 90-91 min, 0% B and 0% C. Sodium hydroxide (300 mM) was added post column (Waters Chromatography) at a flow rate of 0.70 mL/min to minimize baseline drift. Detection was achieved employing a Waters 464 pulsed amperometric detector (PAD) with a dual gold electrode and triple pulsed amperometry at a sensitivity of 50 μA. The electrode was maintained at the following potentials and durations: E_1= 0.05V (T_1 = 0.299 s); E_2 = 0.60V (T_2 = 0.299 s); E_3 = -0.80V (T_3 = 0.499 s). Chromatograms were plotted employing Millenium 2010 Chromatography manager software (Waters Chromatography). All samples were analyzed in duplicate. Quantification of fructooligosaccharides was determined from peak areas using sucrose as the external standard (the peak area of sucrose was used as the reference value to calculate all other response factors as in Timmermans et al., 1994).

Statistics. Linear regressions, exponential distributions, means and standard deviations were analyzed using Microsoft Excel 97 (Microsoft Office 97).

RESULTS AND DISCUSSION

For quantification of carbohydrates using MALDI-MS, several factors must be examined individually, including the selection of matrices, matrix and sample preparation, and the selection of an appropriate internal standard for quantification (Harvey 1993; Jespersen et al., 1995; Gusev et al., 1995; Abell and Sporns, 1996; Bartsch et al., 1996; Wilkinson et al., 1997). The best matrix offers spot-to-spot and sample-to-sample repeatability and reproducibility, which makes quantitative analysis of the analytes of interest possible.

Selection of Matrices. The main problems associated with matrix-assisted laser desorption/ionization (MALDI) quantitative analysis are poor spot-to-spot repeatability or crystal inhomogeneity (Gusev et al., 1995). Proper homogeneous crystallization over the entire probe area and a homogeneous embedding of analyte molecules in the matrix are the prime criteria for high repeatability and quantification. The selection of matrices is usually based on a comparison of spot-to-spot or sample-to-sample repeatability and the ability to obtain a good quality spectrum with reasonable signal-to-noise ratios with the best possible resolution. 2,5-Dihydroxybenzoic acid (DHB) (Bruker, 1995; Mohr et al., 1995; Losso and Nakai, 1997), 3-aminoquinoline (3-AQ) (Metzger et al., 1994), 4-hydroxy-α-cyanocinnamic acid (HCCA) (Bartsch et al., 1996), and 2,5-dihydrooxybenzoic acid (DHB)/1-hydroxyisoquinoline (HIC) (Mohr et al., 1995) have all been recommended as matrices for carbohydrate analysis using MALDI-MS. These matrices were tested for their suitability to ionize the oligosaccharides, maltohexaose and γ-cyclodextrin (Figure 2-1). The preparation of matrices and their performance are listed in Table 2-1. DHB, 3-AQ, HCCA, and DHB/HIC matrices all gave good quality spectra (Figure 2-2). The repeatability with DHB was acceptable, whereas the matrices 3-AQ, HCCA and DHB/HIC could not meet the need for high repeatability. DHB showed many matrix peaks in the low mass region, which could interfere with low molecular weight analytes of interest, such as kestose with a mass of 504. Metzger et al. (1994) first introduced 3-AQ as matrix for inulin using MALDI-MS. Compared to DHB, 3-AQ showed sharper peaks (that is better resolution) and a lower background, but the high quality spectra could not be repeatedly obtained in our experiments. Mohr et al. (1995) noted that DHB crystals formed only near the rim of the probe, complicating the location of suitable laser ionization positions because only a few crystals could be found in the center of the probe. Stahl et al. (1997) found 3-AQ to be more sensitive to contaminants, such as salts. Naven et al. (1997) found the spectra acquired from DHB exhibited the most abundant fragmentation and that those using 3-AQ exhibited the least. Mohr et al. (1995) pointed out that DHB/HIC crystallized equally over the entire probe as a fine powder using vacuum-drying for a few seconds. This resulted in high quality spectra with few matrix peaks and intense analyte peaks. However, this procedure was not suitable for

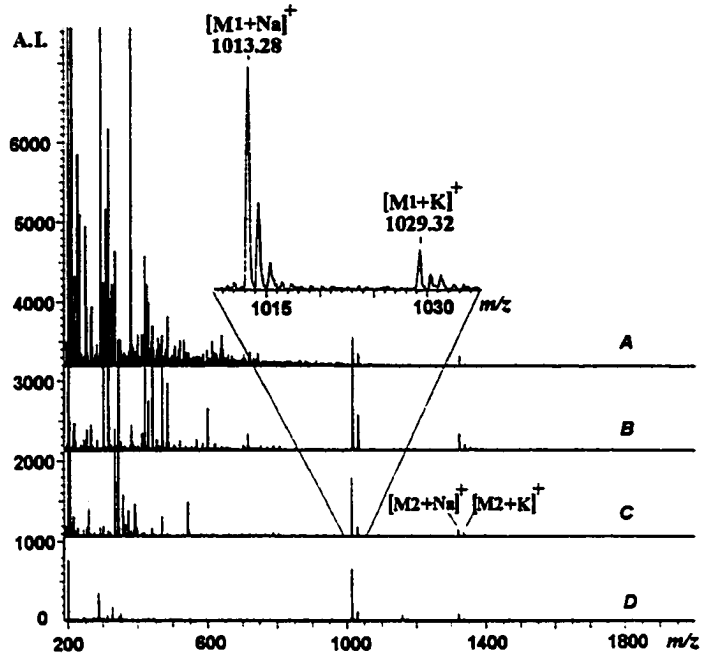

Figure 2-2. MALDI-MS positive ion spectra of γ-cyclodextrin and maltohexaose in various matrices. A: 4-Hydroxy-α-cyanocinnamic acid, 13.3 mg/mL in ethanol/water (1:1). A 20 μL of sample mixture containing γ-cyclodextrin (2.5×10^{-5}M, marked M2) and maltohexaose (1.1×10^{-4}M, marked M1) in double deionized water was mixed with 20 μL of matrix in solution and vortexed for 30 s. Then 0.5-μL mixture of matrix and sample was applied to the probe. Laser strength was set at an attenuation of 44. Twenty shots were accumulated for the final spectrum. B: 2,5-Dihydroxybenzoic acid, 12.3 mg/mL in ethanol/water (1:1). Other parameters were the same as in A except the laser strength was set at an attenuation of 32. C: 2',4',6'-Trihydroxyacetophonenone monohydrate saturated in acetone. A 0.3-μL aliquot of matrix was applied to the probe first and air-dried. Then, 0.5 μL of sample was put on the top of matrix. Other parameters were the same as in B. D: 3-Aminoquinoline (10.1 mg/mL in 10% ethanol). Other parameters were the same as in B.

a multiple-position probe, because samples could not be spotted and vacuum dried simultaneously so irregular crystals are formed, leading to poor spot-to-spot repeatability.

The laser strength used for the matrices 3-AQ, DHB, and DHB/HIC was almost the same, with an attenuation around 30 to 33, which was just above laser strength threshold values required to desorb and ionize analytes (Note that for the MALDI-MS Proflex™ III, attenuation is opposite to laser strength; that is, the higher the attenuation the lower the laser strength). At the same laser strength, DHB produced more matrix peaks than 3-AQ, or DHB/HIC. Whereas a much lower laser strength (attenuation of 44 or 49) was used for HCCA or HABA to desorb and ionize maltohexaose and γ-cyclodextrin, both of these matrices showed numerous matrix peaks making analysis of

masses below 600 difficult. Furthermore, the peak ratio (0.30) of maltohexaose to γ-cyclodextrin obtained from HABA was quite far from the actual molar ratio (0.63).

The rate of the evaporation of the solvent affects the cocrystallization of matrix and sample. Fast evaporation leads to fine crystals and more homogeneous incorporation of sample. Improvement in sample homogeneity using the fast-evaporation method enhanced both shot-to-shot repeatability and sample-to-sample reproducibility (Nicola et al., 1995). Fast evaporation could be enhanced with THAP using acetone as the solvent. THAP is very soluble in acetone, which then evaporates rapidly giving small homogeneous crystals. Previously, THAP was successfully used as a matrix for peptides and oligonucleotides (Kussmann et al., 1997; Pieles et al., 1993). Mohr et al. (1995) indicated that THAP was not a good matrix for carbohydrates such as DHB/HIC due to irregular crystallization and relative signal-to-noise ratios. However, they used water as solvent for this matrix. With water both the lower solubility of THAP and slower evaporation rate likely led to the noted problems. To get an appropriate excess of matrix to analyte the THAP was first crystallized from acetone and then the aqueous sample applied on top of the formed crystals. The water redissolved some of the matrix and the remaining undissolved THAP acted as seed crystals for rapid recrystallization of analyte and matrix as the water evaporated. This technique resulted in high quality MALDI-MS spectra (Figure 2-2) with high spot-to-spot repeatability. The technique can be used to resolve the oligosaccharides in inulin up to a mass of 9000 (DP of about 55, Figure 2-3). Another advantage of this technique was its tolerance to small amounts of protein or other impurities in samples, with few interfering matrix peaks from THAP and reasonable signal-to-noise ratio at an attenuation of between 30 and 32. Therefore, THAP with acetone prepared in a two step procedure was chosen as the matrix for later studies.

Alkali Metal Adducts. In general, carbohydrates ionize in a MALDI-MS source only after cationization with alkali ions (Börnsen et al., 1995). For quantification it was desirable that the investigated carbohydrate sample contained predominately one kind of alkali metal, resulting in a single molecular ion peak. With no modification the matrix and sample contain both sodium and potassium ions (Figure 2-2), resulting in multiple carbohydrate peaks. The peak intensity of carbohydrate alkali-metal ion adducts in an

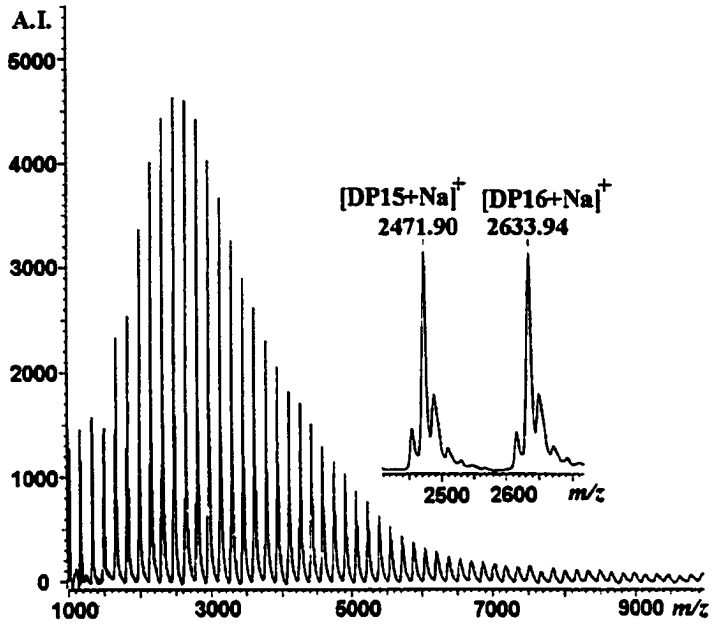

Figure 2-3. MALDI-MS positive ion spectrum of inulin from Jerusalem artichokes. Inulin was dissolved in double deionized water to give a final concentration of 4 mg/mL. A 0.3 µL of saturated THAP in acetone was first placed on the probe and a 0.5-µL inulin sample was put on top of the crystallized matrix to dry. Sixty laser pulses at an attenuation of 22 were accumulated for the final spectrum.

unmodified sample is dependent on the concentration of the alkali metal ions in final solution applied to the probe and the affinity between the metal and the carbohydrate. It has been shown that the affinity of alkali metals to carbohydrates follows the order of H<Li<Na<K<Cs (Mohr et al., 1995; Börnsen et al., 1995). Ion exchange and purification of carbohydrates on a Nafion membrane has been successfully used as a sample pretreatment for MALDI-MS producing a single alkali ion adduct (Börnsen et al., 1995). However, there is another simpler method to obtain a single alkali ion adduct peak. By dissolving carbohydrates in a 0.01 M solution of the alkali ion salt (e.g., potassium chloride) we were able obtain a single alkali ion adduct peak. The concentration of alkali ions was crucial, since too high a concentration of salts would also suppress the molecular ions. Often food samples, such as onions, shallots, and garlic, naturally contain a high concentration of potassium ions, and could be analyzed without further addition of

salts. The molecular ions seen in MALDI-MS for these food samples were almost entirely potassium adducts (Figure 2-4).

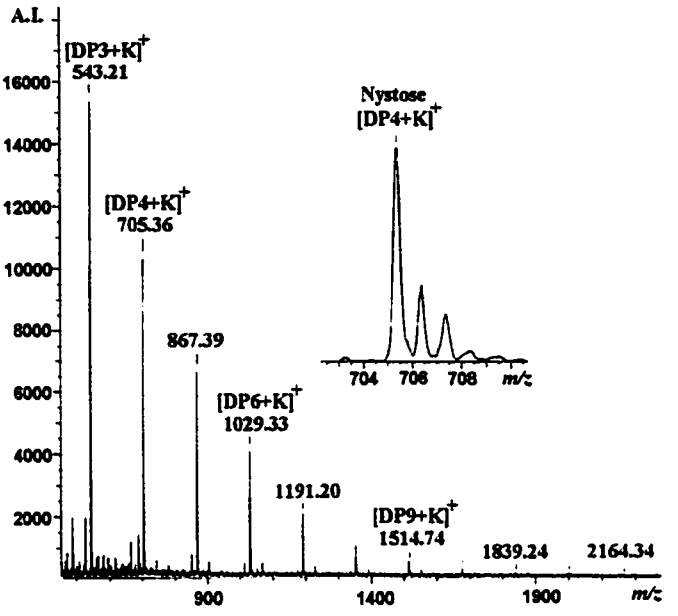

Figure 2-4. MALDI-MS positive ion spectra of fructooligosaccharides from shallots. The sample was prepared by first applying 0.3 μL saturated THAP in acetone on the probe, air-drying and then applying 0.5 μL of aqueous sample solution followed by further air-drying. Laser strength was set at attenuation of 31 and 180 laser pulses were accumulated in three random positions for the final spectrum.

Laser Strength. Laser strength determines the degree of the desorption and ionization of analytes in MALDI-MS. Usually, with increases of laser strength, more ions, including both matrix and molecular ions, are generated. Also higher laser strengths can lead to more fragmentation. For a high quality spectrum and quantitation, the ideal laser strength is very important. Generally, laser strength has been chosen based on the signal-to-noise ratios (Bartsch et al., 1996; Naven et al., 1997). However, the relationships between laser strength, resolution, and the analyte response have not been thoroughly investigated for carbohydrates. We found that laser strength played a very important role in obtaining quality spectra. As the laser strength increased over a certain amount, the resolution deteriorated rapidly. Figure 2-5 indicates the trend of the resolution of maltohexaose sodium adduct peak with the variation in laser strength. At the

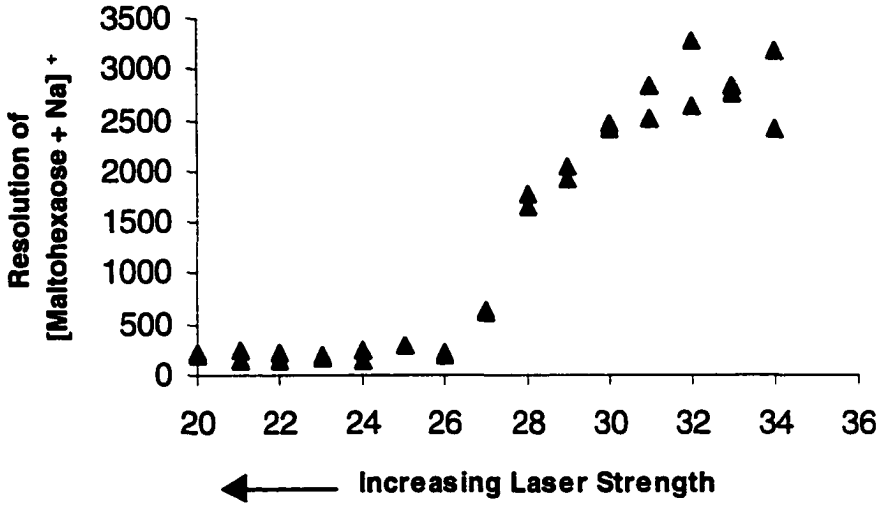

Figure 2-5. Relationship between laser strength and resolution. The sample was prepared by first applying 0.3 μL of saturated 2',4',6'-trihydroxyacetophonenone monohydrate saturated in acetone, air-drying and then applying 0.5 μL of maltohexaose (1.1×10^{-4} M) as described in Figure 2-4. The laser strength was changed from an attenuation of 34 down to 20 (*x* axis). At each attenuation, two spots were randomly chosen to collect two spectra (resolution shown for each spectrum) with 20 laser pulses totaled for each spot. The resolution of the molecular ion peaks was obtained from sodium adduct ion peak of maltohexaose (*y* axis).

attenuation between 30 and 33, high quality spectra were obtained with well resolved isotopic mass peaks having resolutions close to 3000 (full width at half maximum, fwhm), and exact isotopic mass could be determined with an accuracy 100 ppm or less using internal calibration. However, when the attenuation was decreased to 27, the resolution deteriorated rapidly to 500 fwhm. This effect can be easily seen in spectra since isotopic resolution is lost. These isotopic peaks include the main peak plus one unit mass (M+1) and (M+2) peaks due mainly to ^{13}C isotopes. At the same time, because of the loss of isotopic resolution, molecular ion peaks became broad and the measured masses of peaks were shifted to high masses with increasing laser strength (data not shown). More important, the molar peak ratio between maltohexaose and γ-cyclodextrin changed, making quantitative analysis impossible using one as the internal standard for the other, even though chemically these two molecules are very similar oligosaccharides. In Figure 2-6 the actual molar ratio of maltohexaose to γ-cyclodextrin was 4.4 and within the region of attenuation between 28 and 34, the peak ratio of maltohexaose and γ-

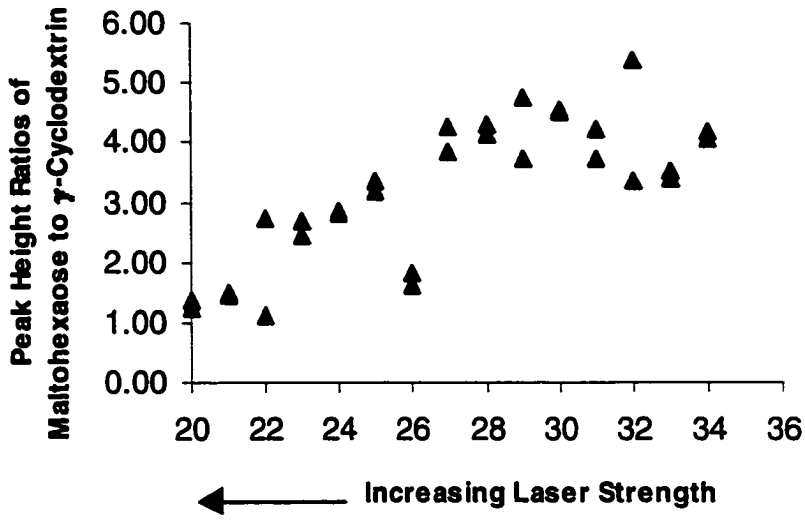

Figure 2-6. Relationship between laser strength and analyte peak ratios. The sample, preparation of matrix and sample, and other MALDI-MS parameters are the same as described in Figure 2-5. γ-Cyclodextrin was present at a concentration of 2.5×10^{-5} M. Total peak height of both sodium and potassium adduct peaks were used to plot this trend.

cyclodextrin was very close to this molar ratio (4.1± 0.53) even though both sodium and potassium adduct peaks were used. However, with the increase in laser strength, the peak ratio decreased. This indicated that relatively more γ-cyclodextrin was desorbed and ionized during the ionization than maltohexaose. It can be concluded that the behavior of molecules, even when they are only slightly different in their molecular structure, can be quite different during the ionization process. While higher laser strength does result in a significant increase in ions formed, another advantage of lower laser strength is to limit any molecular fragmentation.

Oligosaccharide Response in MALDI-MS. The response of the analytes in MALDI-MS plays a very important role in quantifying analytes of interest. In theory, the intensity or response of an analyte should be linearly correlated to its molar ratios in the MALDI-MS sample. Figure 2-7 indicates the linearity between the concentration of individual fructooligosaccharides and their response in MALDI-MS using γ-cyclodextrin as an internal standard. The slopes or the relative response factors of the fructooligosaccharides using MALDI-MS, were 2.1 for kestose, 2.1 for nystose, and 2.3 for GF_4. This indicated that the response of the individual fructooligosaccharides in

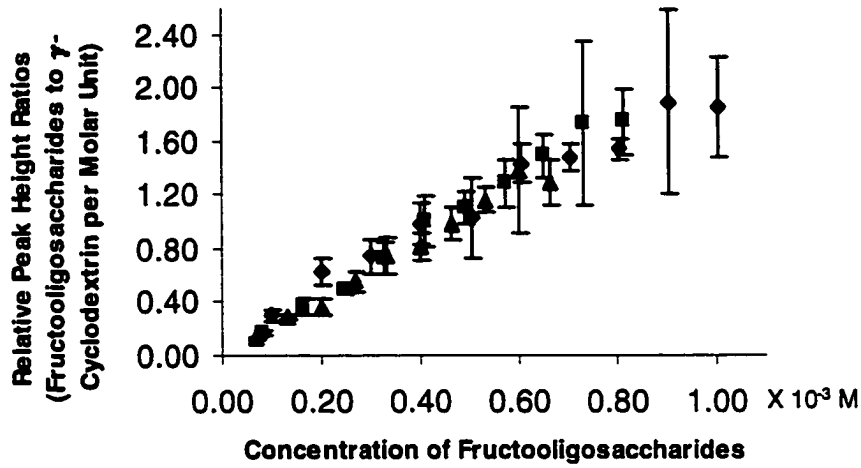

Figure 2-7. Responses of individual fructooligosaccharides in MALDI-MS. Diamond shapes: kestose (slope = 2.1, $R^2 = 0.93$). Squares: nystose (slope = 2.3, $R^2 = 0.99$). Triangles: GF4 (slope = 2.1, $R^2 = 0.98$). Internal standard γ-cyclodextrin (4.0×10^{-5} M) was dissolved in 0.01 M potassium chloride solution. The concentration of individual fructooligosaccharide ranged from 1.0×10^{-4} to 1.0×10^{-3} M for kestose, 8.2×10^{-5} M to 8.2×10^{-4} M for nystose and 6.7×10^{-5} M to 6.7×10^{-4} M for GF_4 in 0.01 M aqueous potassium chloride. Other MALDI-MS parameters were the same as in Figure 2-4. Each data point was the mean of three random positions for a total of 180 laser pulses. Each spectrum from a single sample position was collected from three random spots for a total three of the 180 laser pulses. Peak heights were used as for quantification. Each error bar stands for the standard deviation from the mean of three different spectra.

MALDI-MS was very similar and was more than twice that of γ-cyclodextrin. Although the responses of γ-cyclodextrin and the fructooligosaccharides were different on a molar basis, the molar ratio of fructooligosaccharides to γ-cyclodextrin seemed to be consistent, making it possible to use γ-cyclodextrin as an internal standard. γ-Cyclodextrin was a useful internal standard for fructooligosaccharides since it is readily available in pure form, was likely similar in chemical stability to fructooligosaccharides since all compounds are nonreducing sugars, and had a unique mass that would not overlap with any other fructooligosaccharide (18 mass units less than the corresponding DP fructooligosaccharide because of its cyclic structure).

Analysis of Fructooligosaccharides in Food Samples. When γ-cyclodextrin was added as an internal standard to extracted food samples, the relative responses noted above (about a 2:1 molar ratio) for pure standards changed. It became obvious that in the different food extract environments γ-cyclodextrin responded differently than fructooligosaccharides. Food extracts from red onions, for example, completely

suppressed the production of ions from added γ-cyclodextrin, even though the fructooligosaccharides could be seen. For this reason γ-cyclodextrin was abandoned as an internal standard.

Table 2-2. The Repeatability of MALDI-MS Analysis Data

concentration of GF_4 (DP = 5) added standard, mg/mL	degree of polymerization	fructooligosaccharides standard A[a]		fructooligosaccharides standard B[b]	
		actual value, mg/mL	MALDI-MS data[c]	actual value, mg/mL	MALDI-MS data[c]
0.72	DP = 3 (kestose)	0.23	0.21 (0.01)	0.37	0.41 (0.09)
	DP = 4[d] (nystose)	0.35	0.40	0.46	0.50
	DP = 5 (GF_4)	0.070	0.072 (0.01)	0.37	0.36 (0.03)
	Total	0.67	0.68 (0.01)	1.20	1.26 (0.07)
0.33	DP = 3 (kestose)	0.23	0.20 (0.03)	0.37	0.36 (0.04)
	DP = 4[d] (nystose)	0.35	0.37	0.46	0.50
	DP = 5 (GF_4)	0.070	0.073 (0.01)	0.37	0.38 (0.04)
	Total	0.67	0.65 (0.03)	1.20	1.23 (0.02)

[a] A mixture of 1-kestose (34%), nystose (53%) and GF4 (10%) made in Nutritional Science Center, Meiji Seika Kaisha, Japan.
[b] A mixture of 1-kestose, nystose, and GF_4 prepared in our experiment.
[c] Mean of a duplicate with five replicates, each replicate from three of 60 laser pulses and standard deviations (n = 2).
[d] The peak of fructooligosaccharide with DP = 4 (GF_3) was taken as the reference peak.

While working with food extracts, however, one feature seemed very consistent and that was the relative ratios of the individual fructooligosaccharides. For this reason it was decided to attempt the use of a single purified fructooligosaccharide in a standard addition method to quantitate all of the fructooligosaccharides.

Table 2-2 shows the results for standard addition using nystose (GF_3) as a reference peak (that is all other peak heights are compared to the peak height of this peak) and GF_4 as the standard added. Comparisons were carried out for two samples of known concentration, a supplied oligosaccharide mixture with known composition (standard A) and another mixture prepared from pure fructooligosaccharide standards (standard B). Even though differing amounts of internal standard (GF_4 added at both 0.72 and 0.33 mg/mL levels) were added, quantitation of three oligosaccharide compounds compared nicely with known values.

Of course the ultimate test was to examine food extracts. Table 2-3 shows the MALDI-MS results for determining the fructooligosaccharide content in red onions, shallots, and garlic analyzed using the standard addition method.

Table 2-3. Fructooligosaccharide Content Using HPAEC-PAD and MALDI-MS

	fructooligosaccharide standard, %			red onions, mg/g fresh; water content = 88.3 (0.063) (fresh), 5.2 (0.58)[a] (freeze dried)		shallots, mg/g fresh; water content = 84.2 (0.15) (fresh), 5.9 (0.22)[a] (freeze dried)		garlic, mg/g fresh; water content = 63.1 (0.85) (fresh), 2.1 (0.25)[a] (freeze dried)
degree of polymerization	actual value	HPAEC-PAD mean[b]	MALDI-MS mean[c]	HPAEC-PAD mean[b]	MALDI-MS mean[c]	HPAEC-PAD mean[b]	MALDI-MS mean[c]	MALDI-MS mean[c]
3	34	37.06 (0.31)	30.83 (0.36)	3.92 (0.17)	6.81 (0.41)	8.43 (0.06)	14.88 (0.33)	14.69 (3.14)
4	53	49.98 (0.21)	58.01 (0.66)	3.99 (0.00)	3.88 (0.15)	8.27 (0.03)	11.37 (0.15)	10.44 (2.02)
5	10	12.96 (0.53)	11.24 (0.94)	2.67 (0.01)	2.19 (0.15)	8.15 (0.09)	7.69 (0.47)	9.34 (1.87)
6				3.42 (0.02)	1.29 (0.16)	10.86 (0.08)	4.84 (0.53)	8.67 (1.78)
7				1.96 (0.06)	0.82 (0.18)	7.86 (0.52)	3.24 (0.48)	7.92 (1.53)
8				1.25 (0.02)	0.52 (0.11)	5.73 (0.08)	2.07 (0.19)	6.81 (1.14)
9				0.96 (0.05)	0.43 (0.15)	4.37 (0.10)	1.43 (0.13)	5.55 (0.89)
10				0.40 (0.01)		2.73 (0.03)	1.01 (0.05)	4.64 (0.61)
11				0.30 (0.02)		2.02 (0.01)	0.87 (0.07)	4.07 (0.54)
12				0.18 (0.03)		1.33 (0.00)	0.90 (0.00)	3.54 (0.53)
13						0.78 (0.04)		3.11 (0.30)
14								2.80 (0.43)
15								2.37 (0.23)
16								2.14 (0.29)
17								1.87 (0.12)
18								1.64 (0.05)
19								1.53 (0.15)
total				19.06 (0.13)	15.93 (1.32)	60.55 (0.71)	48.3 (1.52)	91.74 (15.56)

[a] Numbers in bracket indicate the standard deviation of triplicates (n = 3).
[b] Mean of a duplicate and standard deviations (n = 2)
[c] Mean of a duplicate (same extract) with five replicates, each replicate from three of 60 laser pulses and standard deviations (n = 2)

Comparison of MALDI-MS and HPAEC-PAD Results. High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) has been used to quantitatively analyze fructooligosaccharides or inulin (Chatterton et al., 1989, 1993; Timmermans et al., 1994). Table 2-3 shows the results of analysis for fructooligosaccharides contained in onion and shallot with both HPAEC-PAD and MALDI-MS and garlic with MALDI-MS. In general, the sensitivity of PAD detector decreases rapidly from DP = 2 to DP = 6, while for longer oligomers (DP = 7-17), the sensitivity of detector decreases only slightly (Timmermans et al., 1994). The calculation of fructooligosaccharides in food using HPAEC-PAD was based on the response factors and linear relationship reported by Timmermans et al. (1994). Peak areas were integrated and compared to an external standard, sucrose (Figure 2-10) for quantification. Onion bulbs contain various isomeric fructans (Darbyshire et al., 1981; Bancal et al., 1989, 1991; Stahl et al., 1997). These isomer ions yielded a more complex chromatogram pattern than for MALDI-MS (Figure 2-8 is the HPAEC-PAD chromatogram of shallots,

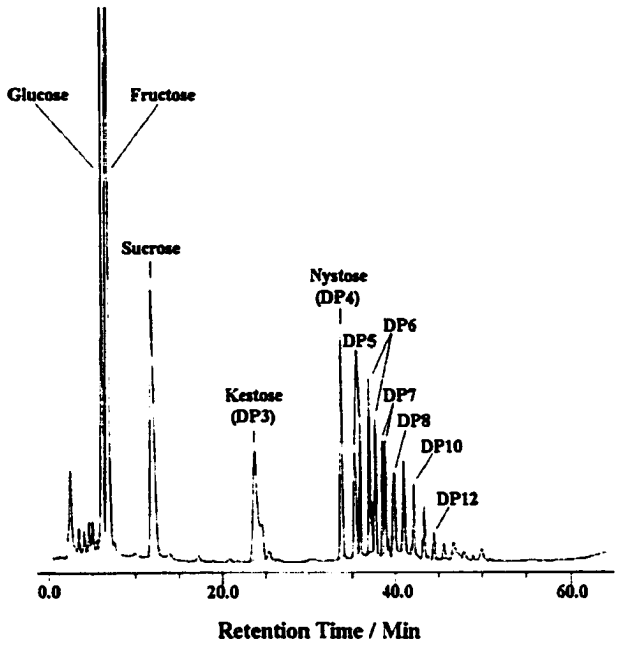

Figure 2-8. HPAEC-PAD chromatogram of shallots. Peaks are identified by comparison of the retention time with standards. The higher DP (DP = 6 or more) of fructooligosaccharides are labeled by retention time compared to DP = 5 and two adjacent peaks are treated as isomers.

while Figure 2-4 is the MALDI-MS spectrum for this sample). The retention times of individual peaks from both red onions and shallots were very comparable. Kestose (DP=3), nystose (DP=4) and GF_4 (DP=5) in red onion and shallot samples were determined in comparison to the retention times of fructooligosaccharide standards. With higher DP fructooligosaccharides, because of lack of individual standards, we assumed that adjacent peaks (Figure 2-8) were isomers to calculate the amount of individual fructooligosaccharide content. The HPAEC-PAD technique was more sensitive in terms of detection limit than MALDI-MS. The small amounts of higher DP fructooligosaccharides in onion or shallot samples were detected by HPAEC-PAD (Table 2-3) and could only be seen with MALDI-MS using higher laser strength where resolution and therefore quantitation suffered.

Both Loo et al. (1995) and Stahl et al. (1997) stated that for onion bulbs the major fructooligosaccharide had a DP = 5. However, using either analytical method, fructooligosaccharides other than DP = 5 were the major fructooligosaccharides in our onion sample. The distribution of fructooligosaccharides in red onions, shallots, and

garlic seen by MALDI-MS followed a definite pattern and the amounts of individual fructooligosaccharides could be correlated to different exponential distributions (correlation coefficients ranged from 0.97-099, Figure 2-9). The natural symmetry of these exponential distributions for fructooligosaccharides in red onions, shallots, and garlic seems to support the relative amounts of fructooligosaccharides assigned by MALDI-MS. Also this distribution may be useful in predicating the amounts of fructooligosaccharides with higher DP in a sample or elucidating the changes of fructooligosaccharides in onions, shallots, and garlic during storage.

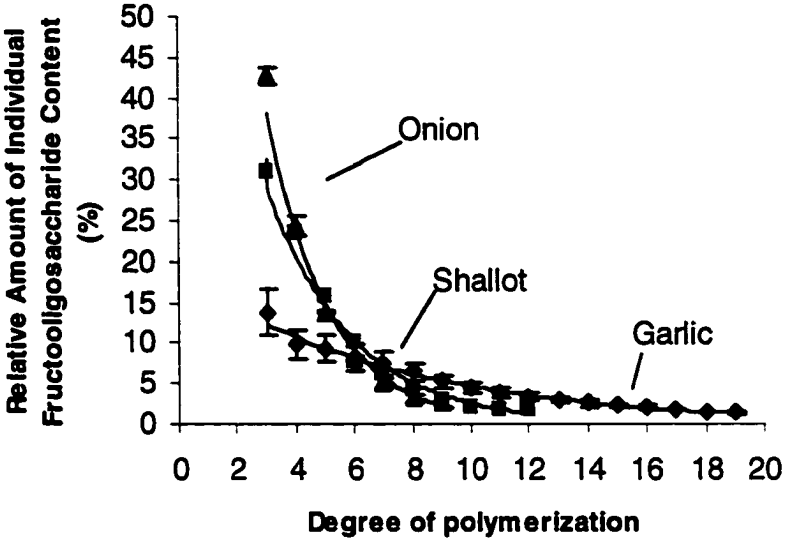

Figure 2-9. Distribution of individual fructooligosaccharides in red onions ($Y = 159.5e^{-0.48X}$, $R^2 = 0.99$), shallots ($Y = 82.8e^{-0.35X}$, $R^2 = 0.97$), and garlic ($Y = 18.73e^{-0.14X}$, $R^2 = 0.99$). Each data point and the error bars were determined from duplicate analyses.

The fructooligosaccharides in the garlic sample, however, could not be analyzed using HPAEC-PAD because of problems with baseline drift, but they could be analyzed using MALDI-MS with a slightly higher laser strength at an attenuation of 28 or 29. We are uncertain if the lack of sample purity affected the quantification of the other HPAEC-PAD samples. It was clear that the best correlation between the two analysis methods was for the pure fructooligosaccharide standard mixture.

While there are obvious differences in the quantitation of fructooligosaccharides using HPAEC-PAD and MALDI-MS, we feel that the MALDI-MS results more

accurately reflect the true amounts of individual fructooligosaccharides in these food samples. As has already been noted the response of a pulsed amperometric detector (PAD) is different for different DP fructooligosaccharides (Timmermans et al., 1994). However, while we tried to account for this changing response, this is only the detector response for linear fructooligosaccharides. Onion bulbs contained various isomeric fructooligosaccharides, including branched fructooligosaccharides (Darbyshire et al., 1981; Bancal et al., 1989, 1991; Stahl et al., 1997), and nothing is known about the differing responses of the PAD detector to these differently linked (2→6 links) fructooligosaccharides. However, standards for similar mass branched and linear glucose oligosaccharides are available and their PAD responses vary a great deal (Figure 2-10).

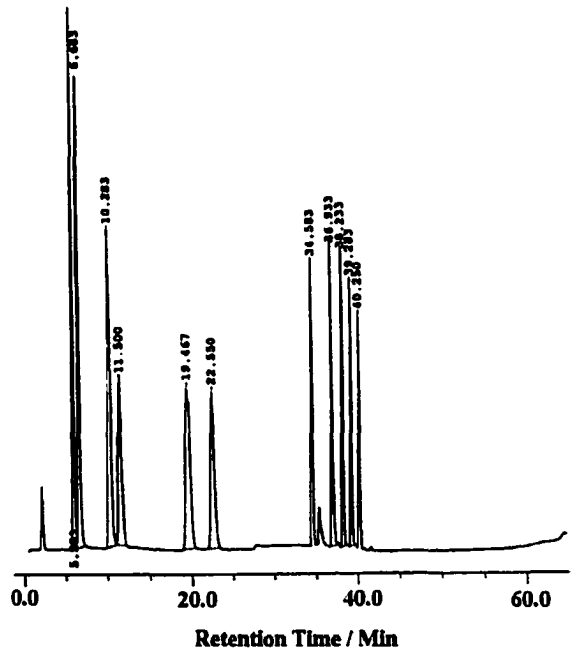

Figure 2-10. HPAEC-PAD chromatogram of carbohydrate standards. Glucose, 98 ppm/5.98 min; fructose, 104 ppm/6.68 min; isomaltose, 98 ppm/10.28 min; sucrose, 98 ppm/11.50 min; isomaltotriose, 108 ppm/19.47 min; maltose, 102 ppm/22.55 min; maltotriose 100 ppm/34.58 min; maltotetraose, 98 ppm/36.93 min; maltopentaose, 96 ppm/38.23 min; maltohexaose, 98 ppm/39.28 min; maltoheptaose, 102 ppm/40.25.

In fact the branched glucose oligosaccharides (that is 1→6 linked isomaltose and isomaltotriose; measured peak areas of 12.0 and 10.6 million, respectively) have significantly greater PAD responses than their linear mass equivalents (maltose and

maltotriose; measured peak areas of 8.1 million and 6.8 million, respectively). It would therefore be expected that using linear oligosaccharide PAD response factors, one would overestimate fructooligosaccharides with branched forms present. As can be seen from Table 2-3, HPAEC-PAD values are generally higher than corresponding MALDI-MS values.

MALDI-MS is a somewhat faster analysis method than HPAEC-PAD, taking about 20 minutes (for determining peak heights for 10 probe positions) rather than an hour for each analysis and MALDI-MS is more tolerant to impurities (as noted for our garlic sample). Further reduction in MALDI-MS analysis time can be obtained by a less rigorous quantitation procedure (i.e. less laser pulses, fewer spots), but even with the numerous replicates that were performed in our experiments standard deviations for MALDI-MS were still higher than for the HPAEC-PAD method. Finally, MALDI-MS gives better assurance of correct molecular assignment since the isotopic mass of each peak is available, although because of similar masses branched and linear isomers can not be distinguished. In fact with MALDI-MS the assignments can be further checked by substituting a different alkali metal in the sample preparation procedure to see expected mass shifts for each oligosaccharide adduct.

While we have concentrated on the analysis of fructooligosaccharides in this study, our feeling is that MALDI-MS can also be used to quantitate other oligosaccharides found in food by developing similar analytical methodology. To our knowledge this paper represents the first use of standard addition to quantitate using MALDI-MS.

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Chapter 3

MALDI-MS Characterization of Maltooligo/Polysaccharides from Debranched Starch Amylopectin of Corn and Barley[1]

INTRODUCTION

Starch, an α-D-glucan biopolymer, is composed of two molecular entities, a linear fraction, amylose, and its branched counterpart, amylopectin. Physicochemical properties of isolated starches differ widely due to plant origin, and the information on properties is vital in choosing starches for various food and nonfood (paper, textiles, mining, pharmaceutical, etc.) industrial applications. These properties depend on the starch molecular structure, composition and morphology of the starch granule itself. The structure refers to both fine structure (molecular size, degree of branching, chain length, etc.) of the component molecules (amylose and amylopectin) and the supra-molecular order, i.e. the manner in which amylose and amylopectin are arranged within the granule and the degree of intermolecular interaction among them.

Amylopectin is a highly branched starch molecule composed of many linear chains (α-1,4-D-glucan) with about 4–6% of the total glucosidic bonds branching from the linear polymers via α-1,6-D-glucan linkages. The presence of these branching points gives rise to a number of possible arrangements of the linear chains. Because of its general dominance in the composition of a starch granule (usually > 75%), the structure and properties of amylopectin have been the subject of many investigations on molecular size, branching, inner/outer chain lengths, etc. (Robin et al., 1974; Hizukuri et al., 1983; Hizukuri, 1985; Jane and Chen, 1992). These studies suggest that the molecular structural features of amylopectin highly influence starch physicochemical properties and functionality.

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[1] A version of this chapter has been published. Wang, J.; Jiang, G. S.; Vasanthan, T.; Sporns, P. MALDI-MS characterization of maltooligo/polysaccharides from debranched starch amylopectin of corn and barley. *Starch.* **1999**, 51, 243-248.

Structural investigation of amylopectin has relied upon the progress in methodological development, both enzymatic and instrumental. Enzymatic debranching of amylopectin using pullulanase or isoamylase, followed by the separation of linear chains using liquid chromatography, has been the most commonly used technique in the analysis and interpretation of the amylopectin fine structure. Various types of columns packed with size exclusion and anion exchange gels have been used for the separations. The analysis generally gives a bi- or tri-modal elution profile, indicating the presence of chains with different chain length or molecular weight (Hizukuri et al., 1983; Hizukuri, 1985).

The use of high performance anion exchange liquid chromatography (HPAEC) connected to a pulsed amperometric detector (PAD) has shown the highest resolution of molecular weights of the debranched chains. Usually in the application of this technique, after elution from the HPAEC column but prior to detection with PAD, the linear starch chain is converted to glucose using an enzyme pad. This conversion is carried out with the assumption that the conversion of linear debranched starch chains to glucose is quantitative. This is a requirement since different saccharides, such as glucose and maltose for example, can have different responses with PAD detection. The efficiency of enzymatic conversion is usually established using low molecular weight maltooligosaccharides as standard (with degree of polymerization, or DP, of 1–7) and then extrapolated to higher molecular weight debranched starch polysaccharides.

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) was originally developed for determining the mass of large molecules such as proteins. MALDI-MS has also been applied to carbohydrate molecular sizing and quantification (Mock et al., 1991; Stahl et al., 1997; Chapter 2, Wang et al., 1999). The measurement is quick and the technique often requires little sample purification. The objective of this study was to develop a methodology to determine the chain length profile of debranched amylopectin using MALDI-MS.

MATERIALS AND METHODS

Materials and Reagents. A small sample of waxy corn starch was obtained from Prof. Jay Lin Jane, Department of Food Science and Human Nutrition, Iowa State University, IA. Waxy barley grains (Candle) were obtained from Agricore, Calgary, Alberta. Regular barley grains (Phoenix) were obtained from Dr. Jim Helm, Alberta Agriculture, Food and Rural Development, Lacombe, Alberta. Isoamylase (E.C 3.2.1.68), Sepharose CL-6B, maltotriose (DP 3), maltotetraose (DP 4), maltopentaose (DP 5) maltohexaose (DP 6) and maltoheptaose (DP 7) were purchased from Sigma Chem. Co. (St. Louis, MO). Sep-pak C18 cartridges was from Waters Corp. (Milford, MA). Macro-sep centrifuge concentrator (30 K), from Filtron Tech. Corp. (Northborough, MA); and the Sephadex G10 desalting columns, from Amersham Pharmacia Biotech AB, (Uppsala, Sweden). 2',4',6'-Trihydroxyacetophenone (THAP) monohydrate, 3-aminoquinoline (3-AQ) and 2,5-dihydroxy-benzoic acid (DHB) were obtained from Aldrich Chem. Co. (Milwaukee, WI).

Debranching of starch.[2] Starch from barley grains (waxy Candle and regular Phoenix) was extracted according to Vasanthan and Bhatty (1995). The method of debranching of starch was a modification of that used by Jane and Chen (1992) and Yuan et al. (1993). The use of dimethyl sulfoxide (DMSO) was avoided as it interfered with MALDI-MS analysis and led to difficulties in the freeze-drying of the debranched amylopectin hydrolysate. Purified starch (100 mg) was dispersed in 9 mL distilled water and heated in a boiling water bath for 1 h. After being cooled to room temperature, 1 mL sodium acetate buffer (0.5 M, pH 3.5) and 1 mL isoamylase solution (30000 U) were added to the above starch solution in sequence. The mixture was incubated in a shaker bath at 40 C for 48 hr to complete the debranching reaction. The debranched starch solution was boiled for 5 min to inactivate the enzyme, cooled, and 10 mL of solution filtered through a Sep-pak C18 cartridge at a flow rate of 1 mL/min.

Desalting and Macro-sep procedure for MALDI-MS. The filtrate from the Sep-pak C18 cartridge after debranching was then put into a 30 K Macro-sep

[2] Debranching of starch was conducted by Dr. Jiang in Department of Agricultural, Food and Nutritional Science at the University of Alberta, Edmonton, Alberta.

concentrator and centrifuged at 5000 × g for 90 min. The 30 K Macro-sep filtrate (2 mL) was loaded on a Sephadex G10 desalting column (1.8 cm × 13 cm). A flow rate of 0.7 mL/min was maintained and fractions (1 mL/fraction) were collected. Fractions 10 to 17 were combined and made up to 10 mL. This sample solution was ready for MALDI-MS analysis.

Gel permeation column chromatography.[3] The debranched starch solution was also concentrated using a rotary evaporator under vacuum at 50 C and fractionated on a Sepharose CL-6B column. The column (1.5 cm × 80 cm) was run in the descending mode with degassed and deionized distilled water as the eluent. The flow rate was about 0.33 mL/min, and fractions of 2.31 mL each (7 min.) were collected and analyzed for total carbohydrate content using the phenol sulfuric acid method (Dubois et al., 1956). The gel permeation chromatography profile showed two peaks. One narrow peak (Fraction I) followed by a broad peak (Fraction II). Fraction II was used for MALDI-MS analysis.

MALDI-MS. MALDI-MS was performed using the Bruker Proflex™ III linear mode, Bruker Analytical Systems Inc. (Billerica, MA). Maltooligosaccharides, that cocrystallized with matrices on the probe using solvent evaporation, were desorbed and ionized by a nitrogen laser pulse (337 nm), and then accelerated under 20 kV with time-delayed extraction before entering the time of flight mass spectrometer. THAP, 3-AQ, and DHB were used as matrices. The preparation of matrices and samples followed Wang et al. (1999, Chapter 2), but 10% ethanol was used as a solvent for DHB. Laser strength was selected based on the degree of polymerization of oligosaccharides to obtain good signal-to-noise ratios. The number of laser pulses was acquired as 3 × 60 or 180 laser pulses for a final MALDI-MS spectrum.

Quantification of standard maltooligosaccharides (DP 3 to DP 7) using MALDI-MS was achieved using the standard addition method (Chapter2, Wang et al., 1999). Concentration of individual maltooligosaccharides in the mixture was as follows: DP 3 = 1.62×10^{-3} M, DP 4 = 1.40×10^{-3} M, DP 5 = 1.47×10^{-3} M, DP 6 = 1.65×10^{-3} M and DP 7 = 1.69×10^{-3} M. The mixture was put through the entire sample preparation

[3] Gel permeation column chromatography was conducted by Dr. Jiang in Department of Agricultural, Food and Nutritional Science at the University of Alberta, Edmonton, Alberta.

procedure (desalting and Macro-sep) before MALDI-MS analysis. DP 7 (2.8×10^{-4} M) was used as an added standard.

Statistics. Linear regressions, means and standard deviations were analyzed using Microsoft Excel 97 (Microsoft Office 97).

RESULTS AND DISCUSSION

For successful quantification of oligosaccharides using MALDI-MS there are several concerns that must be addressed. Key concerns are selection of the appropriate matrix and sample preparation conditions. The matrix should allow for good spot-to-spot repeatability and a linear response for the series of oligosaccharides of interest (discussed later). Fragmentation of the oligosaccharides or their reaction with the matrix should be avoided if possible, and there should be no discrimination for either high or low mass ions at a single run/scan.

Choice of Matrix and Sample Conditions. The performance of different matrices with oligosaccharides has been studied (Mohr et al., 1995; Pfenninger et al., 1999). This study investigated the suitability of three commonly used matrices, 2',4',6'-trihydroxyacetophenone (THAP), 3-aminoquinoline (3-AQ), and 2,5-dihydroxybenzoic acid (DHB). THAP gave very good spot-to-spot repeatability and produced abundant molecular ions in the case of fructooligosaccharides (Chapter 2, Wang et al., 1999). 3-AQ generated sharp ion peaks with little fragmentation to give good quality spectra. However 3-AQ was sensitive to contaminants, such as salts (Stahl et al., 1997; Naven et al., 1997). DHB was the most commonly used matrix for a variety of different oligosaccharides (Harvey, 1993; Garrozzo et al., 1995; Mohr et al., 1995; Kazmaier et al., 1998; Hao et al., 1998; Vinogradov and Bock, 1998). Quantification of carbohydrates using MALDI-MS is possible when response factors are determined in comparison to an internal standard. For single oligosaccharides, linear responses were reported by Harvey (1993) and Bartsch et al. (1996). However, the objectives of the present study were to analyze and quantify a mixture of oligosaccharides in a single sample. Wang et al. (1999, Chapter 2) have reported similar linear responses for fructooligosaccharides with different degrees of

polymerization and successfully used these response factors for quantification of these oligosaccharides in a number of different food samples.

For maltooligosaccharides (DP 3 to 7), the responses were influenced by the matrix (Figure 3-1). While the maltooligosaccharides depicted in Figure 3-1 had very similar molar concentrations, using THAP as matrix (Figure 3-1B) their response decreased with increasing mass/degree of polymerization, while for 3-AQ and DHB (Figure 3-1A and C) the reverse was true. Kazmaier et al. (1998) also found that when

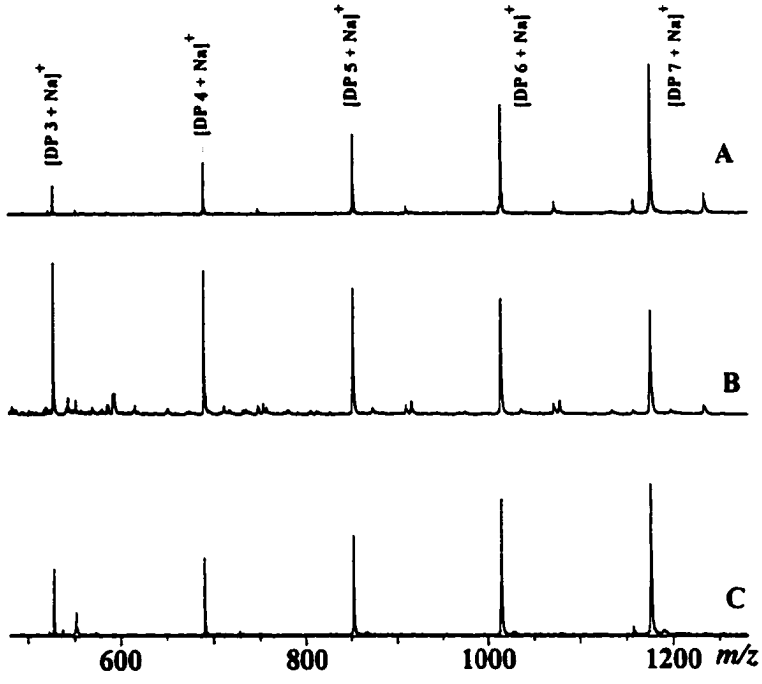

Figure 3-1. MALDI-MS spectra of standard maltooligosaccharides. Individual maltooligosaccharides were dissolved in 0.01 M NaCl solution at a concentration of DP 3 = 1.62×10^{-3} M, DP 4 = 1.40×10^{-3} M, DP 5 = 1.47×10^{-3} M, DP 6 = 1.65×10^{-3} M and DP 7 = 1.69×10^{-3} M. Matrices used: A. 3-AQ, B. THAP, and C. DHB.

DHB was used as the matrix, the responses of maltose (DP 2) and maltotriose (DP 3) were much lower than those of maltohexaose (DP 6) and maltoheptaose (DP 7), although the exact response relationships were not described. Further investigation of matrices focused on THAP and DHB since these gave the best spot-to-spot repeatability, also noted by Wang et al. (1999, Chapter 2) for fructooligosaccharides.

Using DHB as the matrix and an excess of sodium chloride (0.01M in sample solution), the concentrations of maltooligosaccharide standards, DP 4 to DP 7, were

varied and their responses compared to an identical amount of maltotriose (DP 3). The responses for the predominant ionic form (molecular mass of positive ion plus one sodium atom, $[M+Na]^+$) for each standard were linear with almost the same slope but different intercepts (the parallel lines were in the order DP4<DP5 ~ DP 7 < DP 6). However, from preliminary experiments with debranched starch samples, it was obvious that the ionic forms of maltooligo/polysaccharides in these samples would be difficult to control. Attempts to preferentially form a single ion using Nafion membrane ion exchange (Börnsen et al., 1995) or by adding excess alkali salt (Chapter 2, Wang et al., 1999; Pfenninger et al., 1999) could not be used since both methods suppressed the formation of high DP maltooligo/polysaccharide ions, especially for DP > 15. A further complication that had to be addressed in the MALDI-MS of maltooligo/polysaccharides was the formation of fragmentation peaks.

Using a linear MALDI-MS, post-source decay, that is fragmentation of ions after extraction, is not a concern. However in-source decay can lead to multiple peaks. Fragmentation of complex carbohydrates is known to occur by both glycosidic and cross-ring cleavage (Naven et al., 1997). It became clear that THAP (Figure 3-1 B) gave more fragmentation peaks than DHB (Figure 3-1 C). For this reason, and improved sensitivity for maltooligo/polysaccharides, DHB was chosen for further investigations. The major peaks noted with standards and debranched starch samples using DHB were a sodium adduct ion ($[M+Na]^+$), a potassium adduct ion ($[M+K]^+$), and a fragment ion with a loss of water from its parent sodium adduct ion ($[M-H_2O+Na]^+$). While the affinity of alkali metals to carbohydrates has been described in the following order H<Li<Na<K<Cs (Börnsen et al., 1995; Naven et al., 1997), the ion relationship was more complex in the debranched starch samples. Generally loss of water occurred more frequently with low molecular mass maltooligo/polysaccharides while the amount of potassium adduct ions increased with the increasing mass of the maltooligo/polysaccharides (Figure 3-2), until (around DP 26) the potassium adduct ion became the predominant peak rather than the sodium adduct ion. However, a detailed examination of the ratio of these peaks in debranched starch samples indicated an even more complex relationship (Figure 3-3). This general trend was also noted in the debranched waxy barley starch sample.

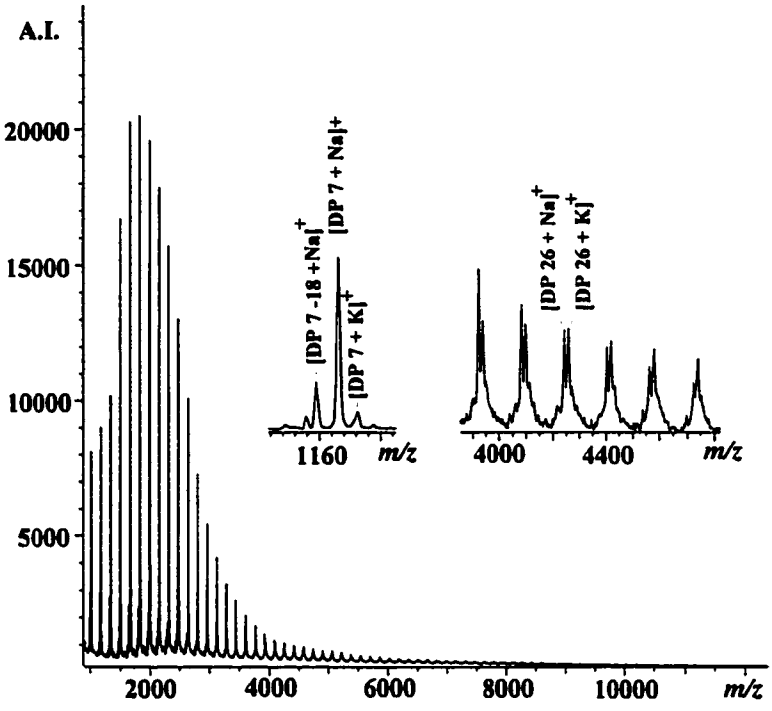

Figure 3-2. MALDI-MS spectrum of a debranched waxy corn starch sample passed through the Macro-sep and desalting procedure. DHB was used as the matrix.

Quantification of maltooligo/polysaccharides in debranched starch samples. Initially the experiments were carried out with debranched waxy corn starch. Considering all factors the matrix of choice was DHB. Increased laser strength resulted in some increase in fragmentation so the laser power was attenuated as much as possible and kept constant from sample to sample. The fragmentation of greatest concern with debranched starch samples would be glycosidic cleavage since this would overestimate lower DP maltooligo/polysaccharides, however when these samples were subjected to increasing laser strength there was no large shift in the distribution of maltooligo/polysaccharides (Figure 3-2 and Figure 3-4, bottom spectrum). Note that the DP 11 peak had the highest response in both spectra. Since dimethyl sulfoxide interferes with MALDI-MS response, this solvent was avoided in the starch debranching step. Sample preparation after debranching of the starch with isoamylase involved filtration of the solution through a reversed phase cartridge, ultracentrifugation using a centrifuge concentrator to remove high molecular weight starch (amylose or undebranched amylopectin) and desalting to remove ionic suppression from excess alkali ions.

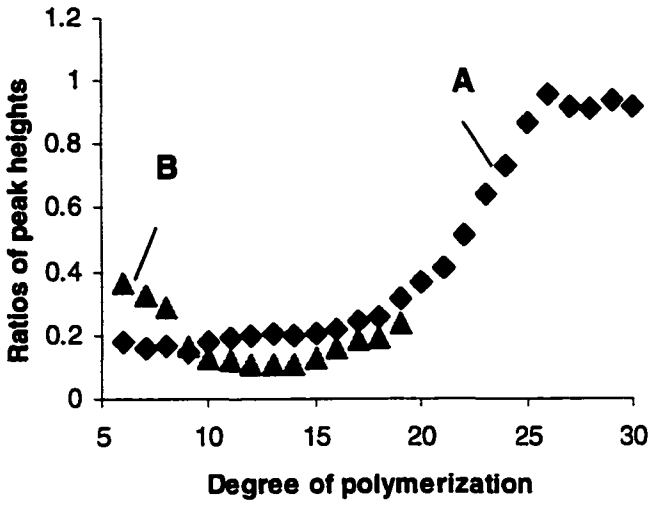

Figure 3-3. The trend of peak ratios of $[M+K]^+$ or $[M-H_2O+Na]^+$ over $[M+Na]^+$ changing with the degree of polymerization in a debranched waxy corn starch sample. Curve A and diamonds: Peak ratios of $[M+K]^+$ over $[M+Na]^+$. Curve B and triangles: Peak ratios of $[M-H_2O+Na]^+$ over $[M+Na]^+$.

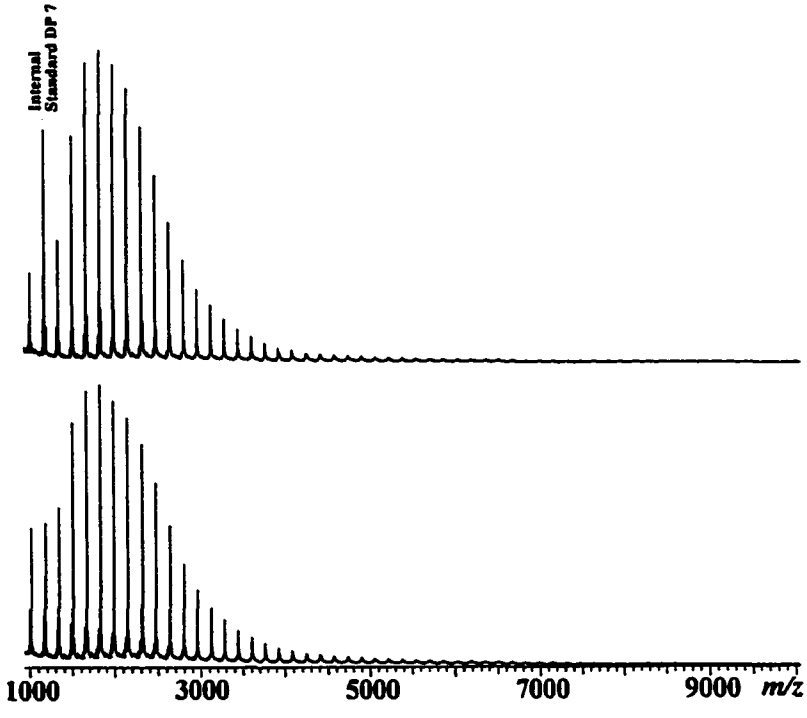

Figure 3-4. MALDI-MS spectra of the same sample as in Figure 3-2. DHB was used as the matrix. The top spectrum was produced at the same laser strength as in Figure 3-2 with the internal standard maltoheptaose (DP 7) added and the bottom spectrum, at a higher laser strength and without the internal standard.

When standard maltooligosaccharide (i.e. using DP 7 with DP 6 as the internal standard) responses were evaluated with DHB using different concentrations, it was found that summation of the various ion responses ($[M+Na]^+$ plus $[M+K]^+$ plus $[M-H_2O+Na]^+$) gave an excellent linear correlation ($R^2 = 0.99$) on a weight basis. It should be noted that for individual maltooligo/polysaccharides all ion responses exhibited a linear correlation, but as noted above and also indicated in Figure 3-3, lower DP maltooligo/polysaccharides had very different ion ratios than higher DP maltooligo/polysaccharides. The linear relationship for maltooligo/polysaccharides was different from that found for fructooligosaccharides (Chapter 2, Wang et al., 1999) since the linearity for fructooligosaccharides was for molar concentrations not weight concentrations.

When maltooligosaccharide standards at known concentrations were subjected to the entire debranched starch purification sequence (desalting and Macro-sep), the recoveries of DP4 to 7 were within 5% of their expected values using MALDI-MS quantification. This indicated that there were no significant losses of material in the purification scheme and that the MALDI-MS quantification, at least for these maltooligosaccharides, was valid. Finally, as can be seen in Figure 3-4 (upper spectrum) addition of a pure maltooligosaccharide internal standard (DP 7) to the debranched starch mixture did not have a marked effect on the relative ion ratios. This meant that a standard addition method could be used (as first shown by Wang et al., 1999, Chapter 2) to determine both the relative and absolute amounts of each maltooligo/polysaccharide using the weight concentration relationship.

In order to compare both protocols for sample preparation (after debranching by enzyme hydrolysis), the maltooligo/polysaccharides pattern of debranched waxy corn amylopectin was determined by the developed method, that is desalting and Macro-sep procedure, and the traditional column elution method. The patterns obtained (not presented) through both methods were found to be identical. To test the use of the developed methodology to examine the maltooligo/polysaccharide pattern from some other debranched starch samples, MALDI-MS was carried out for debranched starch amylopectin from waxy and regular barley (Figure 3-5).

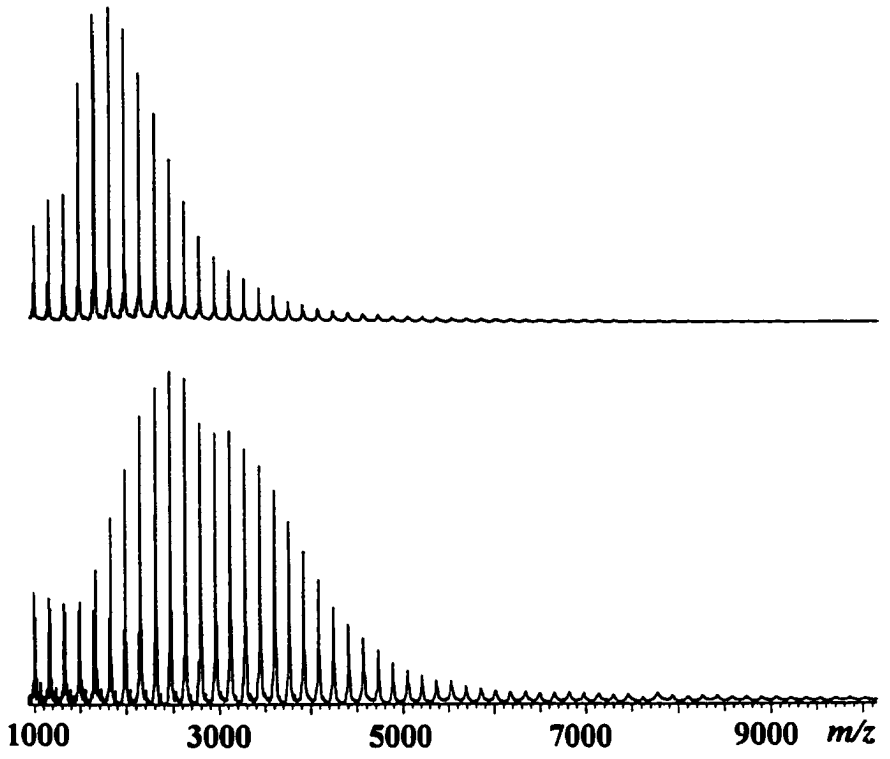

Figure 3-5. MALDI-MS spectrum of debranched. Waxy Candle barley starch passed through desalting and Macro-sep procedure (top spectrum) and regular Phoenix barley starch prepared by traditional method, freeze dried, 6.4 mg/mL in double deionized water (bottom spectrum).

This protocol/methodology can be used to obtain maltooligo/polysaccharide patterns in less than two hours after the debranching step and therefore has advantages in speed as well as detail over other methods presently used for starch molecular characterization.

While our methodology could be shown to apply to the quantification of all pure maltooligosaccharide standards available, there might still be doubt that the higher DP maltooligo/polysaccharides (higher than DP 7) in debranched amylopectin behaved in a similar manner. As a final check, the average DP of the maltooligo/polysaccharides in the investigated debranched waxy corn sample (Figure 3-2) was determined using MALDI-MS quantification. The average DP was found to be 14.5 ± 0.4 (n = 3, RSD = 2.7%). One of the most accurate assessments of the degree of branching (and therefore the average DP in debranched starch) is a recent ^{13}C NMR study, which indicated that the branching degree of amylopectin from waxy corn was 6.0 ± 0.7% (Falk et al., 1996). From this

value, the derived average DP would range from 14.9 to 18.9, very close to the value found using the MALDI-MS method.

In conclusion, MALDI-MS is a very rapid and accurate technique with high sensitivity for analysis of maltooligo/polysaccharide mixtures. The responses of maltooligo/polysaccharides in MALDI-MS were matrix related, and DHB was found to be most effective for quantification of the maltooligo/polysaccharides that resulted from debranched amylopectin using a weight to summed ion response relationship. Along with the new rapid purification procedure this methodology can be used to determine the relative pattern of debranched starch maltooligo/polysaccharides faster and with greater accuracy than any present analytical methodology.

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Chapter 4

Analysis of Anthocyanins in Red Wine and Fruit Juice using MALDI-MS[1]

INTRODUCTION

Anthocyanins are an important family of flavonoid compounds and have been thoroughly studied. They are widely distributed in nature, occurring in most higher plants, and are responsible for most of the red or purple colors in red wine and fruit juice. The anthocyanin profile for any given plant is distinctive, and the amount and composition of anthocyanins are important to red wine and fruit juice color (Gao et al., 1997; Hong and Wrolstad 1990a). Anthocyanin analysis has been very useful in studying the changes in anthocyanins in red wine or fruit juice and in distinguishing among different species.

Anthocyanins from many plants, including fruits, have been separated and analyzed by paper chromatography (Dekazos, 1970; Camire and Clydesdale, 1979; Francis, 1985; Mazza and Velioglu, 1992; Gao and Cahoon, 1995), thin-layer chromatography (Wrolstad and Struthers, 1971; Barritt and Torre, 1973; Pouget et al., 1990; Dussi et al.,1995), and high performance liquid chromatography (HPLC) (Wulf and Nagel, 1978; Camire and Clydesdale, 1979; Goiffon et al., 1991; Mazza and Velioglu, 1992; Gao and Mazza, 1994; Gao et al., 1997). HPLC coupled with photodiode array detection has often been used to qualitatively and quantitatively analyze anthocyanins and their glycosylated or acylated groups utilizing relative retention times and UV-vis spectra (Williams et al., 1978; Hong and Wrolstad, 1990a, b; Goiffon et al., 1991; Dallas et al., 1996). Fast atom bombardment mass spectrometry (FAB-MS) and nuclear magnetic resonance (NMR) have proven to be powerful supporting techniques for detailed structural determination of anthocyanins (Terahara and Yamaguchi, 1986;

[1] A version of this chapter has been published. Wang, J.; Sporns, P. Analysis of anthocyanins in red wine and fruit juice using MALDI-MS. *J. Agric. Food Chem.* **1999**, 47, 2009-2015.

Baubils and Berber Jiménez, 1995; Farina et al., 1995; Escribano et al., 1996; Bakker et al., 1997a, b; Takeoka et al., 1997). HPLC coupled with a mass spectrometer (HPLC/MS) using an atmospheric pressure ionization (API) interface has also been successfully applied to identify anthocyanins of *Vitis vinifera* L. (Baldi et al., 1995). Recently, electrospray ionization (ESI) mass spectrometry and ion trap multiple mass spectrometry (MS/MS) have also been used as another tool for detection and identification of anthocyanins (Takeoka et al., 1997; Piovan et al., 1998).

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) was first introduced in 1987 and originally developed for large biomolecules (Karas et al., 1987). MALDI-MS has advantages over other methodologies including the ease of use, speed of analysis, high sensitivity, wide applicability combined with a good tolerance towards contaminants, and the ability to analyze complex mixtures (Karas, 1996). However, simple MALDI-MS instruments can not tell the difference between isomers, which have the same mass. The potential application of MALDI-MS in food systems allows for the analysis of most molecules. Presently MALDI-MS food applications are limited and reviewed by Sporns and Wang (1998). While this manuscript was under review, Sugui et al. (1998) reported using MALDI to analyze 3-deoxyanthocyanidins and anthocyanins in sorghum plant tissue. This study focused on qualitative aspects and detection limits for some anthocyanins. However, our study represents the first application of a rapid MALDI-MS procedure for both qualitative and quantitative analysis of anthocyanins in several important foods.

MATERIALS AND METHODS

Materials and Reagents. Merlot '96, Pinot Noir '96, and Cabernet Sauvignon '97 were purchased from local markets in Edmonton, Alberta, Canada. Zinfandel '98 was homemade wine from a kit. Concord grape juice, cranberry juice, and raspberry syrup were also from local markets. Malvidin 3,5-glucoside was purchased from Indofine Chem. Co. (St. Louis, MO). Pelargonidin 3-glucoside, cyanidin 3-glucoside, cyanidin 3-rutinoside, peonidin 3-glucoside and malvidin 3-glucoside were obtained from Extrasynthese S.A. (Genay Cedex, France). 1-Kestose and nystose were a gift from Dr.

A. Ohta, Nutritional Science Center, Meiji Seika Kaisha, Japan. 2',4',6'-Trihydroxyacetophenone monohydrate (THAP) was obtained from Aldrich Chem. Co. (Milwaukee, WI).

Extraction of Anthocyanins from Red Wine, Fruit Juice and Syrup. A 200 mL sample of red wine was first prepared by removal of ethanol (about ¼ volume) using a Rotavopor *in vacuo* with a bath temperature maintained at 35 C, and then made up to 200 mL with doubly deionized water in a volumetric flask. Fruit syrup was diluted with an equal volume of water because of its high viscosity. Either 10 mL of ethanol free red wine or fruit juice or 20 mL of diluted syrup sample was loaded onto a Sep-pak C18 cartridge (Waters Associates, Milford, MA) with a flow rate of 1 mL/min, washed with 10 mL of doubly deionized water three times, and eluted with 2 mL of methanol/formic acid/water in a ratio of 70:2:28 (v/v/v). The reddish purple anthocyanin extracts were kept in a freezer at -20 C until used.

MALDI-MS. MALDI-MS was performed using the Proflex^{TM} III linear mode, Bruker Analytical Systems Inc. (Billerica, MA). Anthocyanins cocrystallized with matrices on the probe were ionized by a nitrogen laser pulse (337 nm) and accelerated under 20 kV with time-delayed extraction before entering the time of flight mass spectrometer. 2',4',6'-Trihydroxyacetophenone (THAP) in acetone (0.5 μL) was applied on the probe first, air-dried, and then 1 μL of extracted anthocyanin solution or 2 μL of preparative HPLC fraction spotted directly over the THAP crystals and further air dried. MALDI-MS was performed at an attenuation of 31 (the lower the attenuation, the higher the laser strength) to obtain the best signal-to-noise ratios and the best possible isotopic resolution with two-point external calibration using $[kestose + K]^+$ (exact isotopic mass = 543.1327) and $[nystose + K]^+$ (exact isotopic mass = 705.1856) as calibrants, resulting in a mass accuracy of ≤ 400 ppm. Each spectrum represents the sum of 30 laser pulses.

The responses of individual anthocyanins in MALDI-MS were recorded using cyanidin 3-rutinoside as the internal standard. The response of malvidin 3-glucoside in a food sample using MALDI-MS was obtained by adding malvidin 3-glucoside to Concord grape juice extracts, diluted with an equal volume of appropriate solution from the original anthocyanin extracts. The preparation of matrix and samples was as described above. In this study, the peak with a mass of 449 (cyanidin 3-glucoside) was used as a

reference peak and therefore was arbitrarily set to a value of 1.0 to study the response of added malvidin 3-glucoside in the juice extract. Each data point represented the mean of spectra collected from three sample positions. Therefore, each spectrum was collected from three randomly chosen spots per sample position for a total of 3 × 30 laser pulses, or 90 laser pulses in total. The major isotopic ^{12}C peak height was used for quantification.

Preparative HPLC. The preparative HPLC system consisted of a Varian VISTA 5500 high performance liquid chromatography, a Varian 9090 autosampler, and a Spectro Monitor III UV detector. The chromatography system was equipped with a 75 mm × 4.5 mm pre-injection C18 saturator column containing silica-based packing (12 μm) and a 50 mm × 4.6 mm guard column containing Supelco LC-18 reverse-phase packing (20-40 μm). Anthocyanins were separated on a SPLC-18-DB 250 mm × 10 mm (5 μm) preparative reverse-phase column (SUPELCOSIL, SUPELCO, Bellofonte, PA). The solvents used were 5% (v/v) aqueous formic acid (solvent A) and formic acid/water/methanol in a ratio of 5:5:90 (v/v/v) (solvent B). The flow rate was maintained at 4.5 mL/min, with a linear gradient profile consisting of solvent A with the following proportions (v/v) of solvent B: 0 min, 5% B; 0-1 min, 5-20% B; 1-12 min, 20-25% B; 12-32 min, 25-32% B; 32-38 min, 32-55% B; 38-44 min, 100% B; 44-46 min, 100% B; 46-47 min, 100-5% B. Detection was at 525 nm. The total run time was 55 min. Sample extracts (500 μL) were injected into the HPLC for preparative collection. A Shimadzu CLASS-VP chromatography data system was used to monitor the eluted peaks, and the anthocyanin fractions of interest were collected for direct UV-vis spectrometry and MALDI-MS analysis.

The chromatography of individual standard anthocyanins was achieved using the same conditions as above except employing a linear gradient profile as follows: 0 min, 5% B; 0-1 min, 5% B; 1-2 min, 5-17% B; 2-7 min, 17-19% B; 7-9 min, 19-28% B; 9-17 min, 28-34% B; 17-21 min, 34-70% B; 21-22 min, 70-100% B; 22-23 min, 100% B; 23-24 min, 5% B. Total run time was 26 min. The flow rate was set at 5.5 mL/min. For these analyses, an injection volume of 140 μL was used.

Statistics. Linear regressions were analyzed using Microsoft Excel 97 (Microsoft Office 97).

RESULTS AND DISCUSSION

Analytes are usually desorbed and ionized in the MALDI-MS source, forming protonated or alkali adduct ions. However, because anthocyanins under acidic condition are predominately in the aromatic oxonium ion form, they easily ionize in MALDI-MS to form molecular weight cations M^+ in the positive ion mode (Figure 4-1).

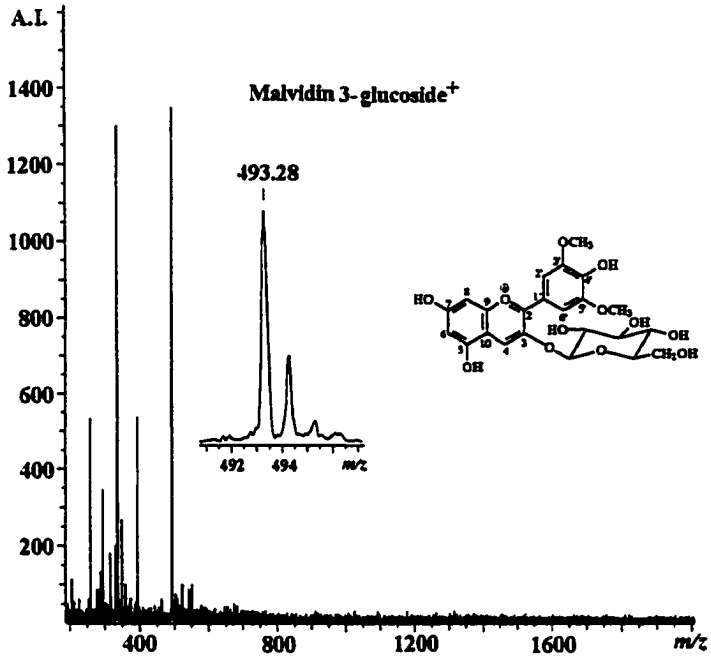

Figure 4-1. MALDI-MS spectrum of standard malvidin 3-glucoside (3.90×10^{-5} M). Note that matrix peaks are all at masses less than 400.

Anthocyanins in Red Wine. Figure 4-2 is the MALDI-MS anthocyanin spectra from red wine extracts. Anthocyanins listed in HPLC elution order from important wine varieties are shown in Table 4-1. Malvidin 3-glucoside, as expected, was the major anthocyanin (Wulf and Nagel, 1978; Nagel and Wulf, 1979; Gao et al., 1997). Table 4-1 also shows some of the anthocyanin masses obtained using MALDI-MS, which are in excellent agreement to their theoretical values. The peak with mass of 493 (malvidin 3-glucoside) is the major peak. Three major anthocyanins were successfully isolated as preparative HPLC fractions from Zinfandel wine extract and then were applied to

Table 4-1. Anthocyanins in *Vitis vinifera* L. Grapes

anthocyanins and their derivatives		exact mass	MALDI-MS mass[a]	Cabernet Sauvignon (Wulf and Nagel, 1978)	Merlot (Nagel and Wulf, 1979)	Pinot Noir Gao et al., 1997
delphinidin	3-glucoside	465.10	465.17	•	•	•
cyanidin	3-glucoside	449.11		•	•	•
petunidin	3-glucoside	479.12	479.23	•	•	•
peonidin	3-glucoside	463.12	463.13	•	•	•
malvidin	3-glucoside	493.13	493.23	•	•	•
delphinidin	3-glucoside-acetate	507.11		•	•	
cyanidin	3-glucoside-acetate	491.12		•	•	
petunidin	3-glucoside-acetate	521.13		•	•	
malvidin		331.08		•		
peonidin	3-glucoside-acetate	505.13		•	•	
malvidin	3-glucoside-acetate	535.15	535.33	•	•	
delphinidin	3-glucoside-coumarate	611.14		•		
cyanidin	3-glucoside-coumarate	595.15		•		
malvidin	3-glucoside-caffeoate	655.17	655.45	•	•	
petunidin	3-glucoside-*p*-coumarate	625.15	625.39	•	•	
peonidin	3-glucoside-*p*-coumarate	609.16	609.38	•	•	
malvidin	3-glucoside-*p*-coumarate	639.17	639.42	•	•	

[a] Masses from Zinfandel wine extracts

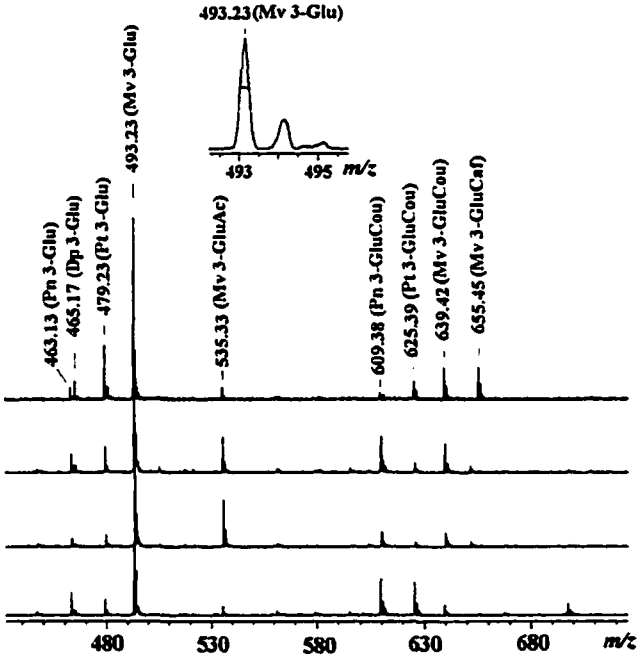

Figure 4-2. MALDI-MS natural cation spectra of anthocyanins from red wine extracts. The spectra from top to bottom are from Zinfandel '98, Merlot '96, Cabernet Sauvignon '97 and Pinot Noir '96 red wine extracts. Pn 3-Glu = peonidin 3-glucoside; Dp 3-Glu = delphinidin 3-glucoside; Pt 3-Glu = petunidin 3-glucoside; Mv 3-Glu = malvidin 3-glucoside; Mv 3-GluAc = malvidin 3-glucoside-acetate; Pn 3-GluCou = peonidin 3-glucoside-coumarate; Pt 3-GluCou = petunidin 3-glucoside-coumarate; Mv 3-GluCou = malvidin 3-glucoside-coumarate; Mv 3-GluCaf = malvidin 3-glucoside-caffeoate.

MALDI-MS giving the correct masses for petunidin 3-glucoside, peonidin 3-glucoside and malvidin 3-glucoside (Figure 4-3). The UV-vis spectra patterns and λ_{max} absorbances of these three preparative HPLC anthocyanin fractions are almost the same as reported before (Wulf and Nagel, 1978). On the basis of the C18 cartridge selectivity, the HPLC relative retention time, the general UV-vis spectra pattern and λ_{max} absorbances, and the MALDI-MS determined masses, the reddish purple pigments adsorbed and eluted from the Sep-pak C18 cartridge were confirmed to be the anthocyanins assigned in Figure 4-2.

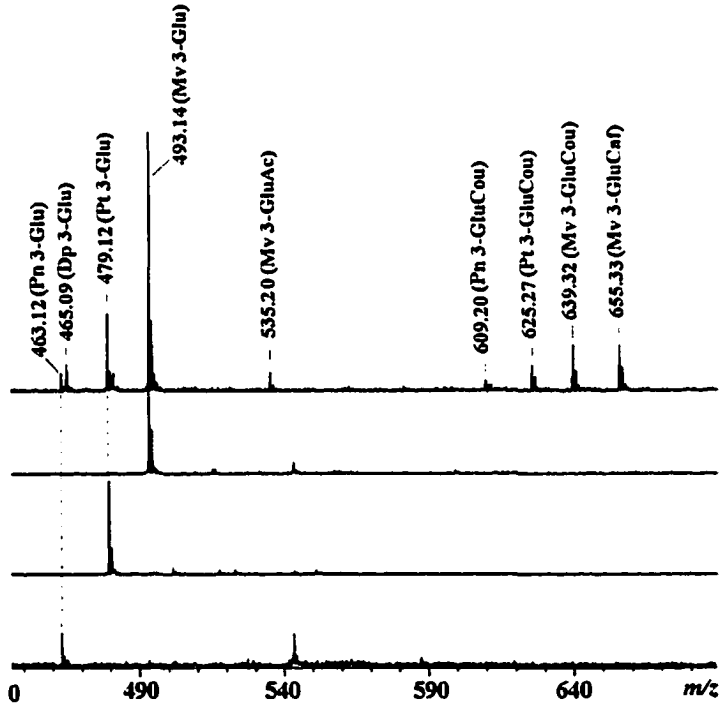

Figure 4-3. MALDI-MS natural cation spectra of anthocyanins from preparative HPLC fractions. The spectra from top to bottom are from Zinfandel '98 red wine extracts, HPLC the third fraction (malvidin 3-glucoside), the first fraction (petunidin 3-glucoside), and the second fraction (peonidin 3-glucoside). Pn 3-Glu = peonidin 3-glucoside; Dp 3-Glu = delphinidin 3-glucoside; Pt 3-Glu = petunidin 3-glucoside; Mv 3-Glu = malvidin 3-glucoside; Mv 3-GluAc = malvidin 3-glucoside-acetate; Pn 3-GluCou = peonidin 3-glucoside-coumarate; Pt 3-GluCou = petunidin 3-glucoside-coumarate; Mv 3-GluCou = malvidin 3-glucoside-coumarate; Mv 3-GluCaf = malvidin 3-glucoside-caffeoate.

Anthocyanins in Concord Grape Juice, Cranberry Juice, and Raspberry Syrup. Concord grape juice contains the 3-glucosides, 3-glucoside-*p*-coumarate, 3,5-diglucosides and 3-glucoside-*p*-coumarate-5-glucosides of cyanidin, peonidin,

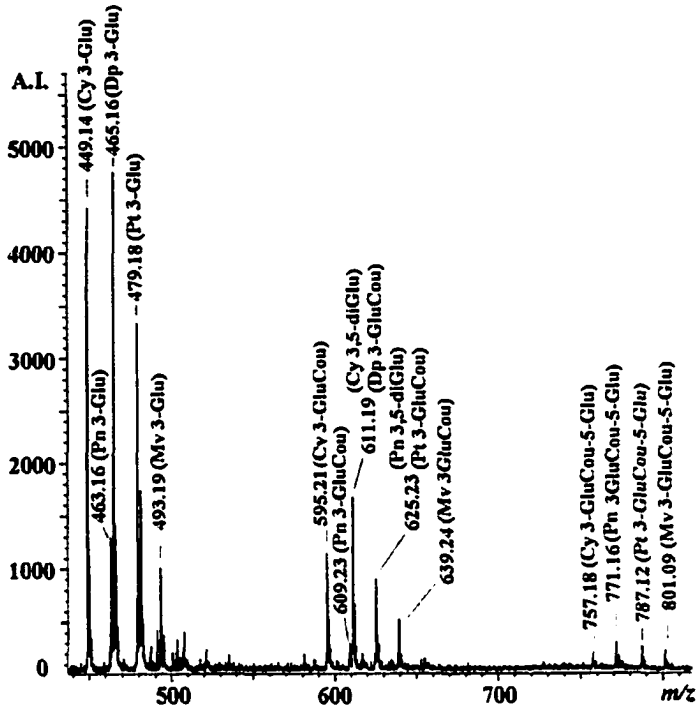

Figure 4-4. MALDI-MS natural cation spectrum of anthocyanins from Concord grape juice extracts. Cy 3-Glu = cyanidin 3-glucoside; Pn 3-Glu = peonidin 3-glucoside; Dp 3-Glu = delphinidin 3-glucoside; Pt 3-Glu = petunidin 3-glucoside; Mv 3-Glu = malvidin 3-glucoside; Cy 3-GluCou = cyanidin 3-glucoside-coumarate; Pn 3-GluCou = peonidin 3-glucoside-coumarate; Cy 3,5-diGlu = cyanidin 3,5-diglucoside; Dp 3-GluCou = delphinidin 3-glucoside-coumarate; Pn 3,5-diGlu = peonidin 3,5-diglucoside; Pt 3-GluCou = petunidin 3-glucoside-coumarate; Mv 3-GluCou = malvidin 3-glucoside-coumarate; Cy 3-GluCou-5-Glu = cyanidin 3-glucoside-coumarate-5-glucoside; Pn 3-GluCou-5-Glu = peonidin 3-glucoside-coumarate-5-glucoside; Pt 3-GluCou-5-Glu = petunidin 3-glucoside-coumarate-5-glucoside; Mv 3-GluCou-5-Glu = malvidin 3-glucoside-coumarate-5-glucoside.

delphinidin, petunidin and malvidin with cyanidin 3-glucoside and delphinidin 3-glucoside as the major pigment constituents (Hrazdina 1975; Williams and Hrazdina 1978). MALDI-MS results confirmed the presence and relative amounts of these anthocyanin and their derivative profiles, in good agreement with the literature. The peaks with masses 449 (cyanidin 3-glucoside) and 465 (delphinidin 3-glucoside) are the major peaks (Figure 4-4; Table 4-2). Because delphinidin 3-glucoside-*p*-coumarate and cyanidin 3,5-diglucosides have almost the same mass of 611, these anthocyanins came out at the same *m/z* of 611 in MALDI-MS spectra, as did peonidin 3,5-diglucosides and petunidin 3-glucoside-*p*-coumarate at *m/z* 625.

Table 4-2. Anthocyanins from Concord Grape Juice

anthocyanins		exact mass	MALDI-MS mass
delphinidin	3,5-diglucoside	627.16	
cyanidin	3,5-diglucoside	611.16	611.19
petunidin	3,5-diglucoside	641.17	
peonidin	3,5-diglucoside	625.18	625.23
malvidin	3,5-diglucoside	655.19	
delphinidin	3-glucoside	465.10	465.16
cyanidin	3-glucoside	449.11	449.14
petunidin	3-glucoside	479.12	479.18
peonidin	3-glucoside	463.12	463.16
malvidin	3-glucoside	493.13	493.17
delphinidin	3-glucoside-coumarate	611.14	611.19
cyanidin	3-glucoside-coumarate	595.15	595.21
petunidin	3-glucoside-coumarate	625.15	625.23
peonidin	3-glucoside-coumarate	609.16	609.23
malvidin	3-glucoside-coumarate	639.17	639.24
delphinidin	3-glucoside-coumarate-5-glucoside	773.19	
cyanidin	3-glucoside-coumarate-5-glucoside	757.19	757.18
petunidin	3-glucoside-coumarate-5-glucoside	787.21	787.12
peonidin	3-glucoside-coumarate-5-glucoside	771.21	771.16
malvidin	3-glucoside-coumarate-5-glucoside	801.22	801.09

Previous research has confirmed the presence of the 3-galactoside and 3-arabinoside of cyanidin and peonidin as the four major anthocyanins in cranberries with the additional small amount of cyanidin 3-glucoside or peonidin 3-glucoside (Camire and Clydesdale, 1979; Hong and Wrolstad, 1990a). Figure 4-5 shows the anthocyanin spectra from cranberry juice, closely matching the exact isotopic masses of cyanidin 3-arabinoside (theoretical value, 419.10), cyanidin 3-galactoside (449.11), peonidin 3-arabinoside (433.11), and peonidin 3-galactoside (463.12).

Anthocyanins expected in raspberries included cyanidin 3-sophoroside (mass: 611; the major anthocyanin), cyanidin 3-rutinoside, cyanidin 3-(2^G-glucosylrutinose), cyanidin 3-glucoside, pelargonidin 3-sophoroside, and pelargonidin 3-(2^G-glucosylrutinose) (Goiffon et al., 1991; Boyles and Wrolstad, 1993). The anthocyanin MALDI-MS spectrum from raspberry syrup extracts shows the molecular cations M^+ at *m/z* 449, 595, 611, and 757 corresponding to the exact isotopic masses of cyanidin 3-glucoside (449.11), cyanidin 3-rutinoside (595.17) or pelargonidin 3-sophoroside (595.17), cyanidin 3-sophoroside (611.16), and cyanidin 3-(2^G-glucosylrutinose) (757.22) (Figure 4-6).

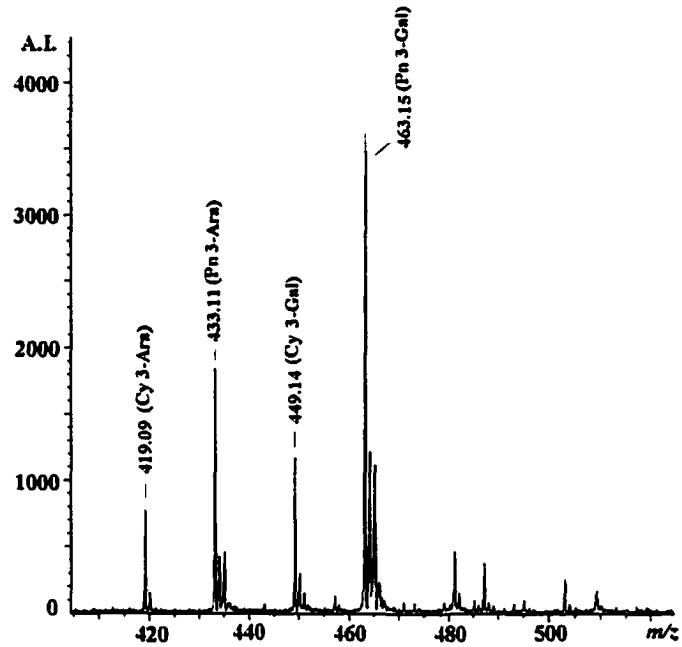

Figure 4-5. MALDI-MS natural cation spectrum of anthocyanins from cranberry juice extracts. Cy 3-Ara = cyanidin 3-arabinoside; Pn 3-Ara = peonidin 3-arabinoside; Cy 3-Gal = cyanidin 3-galactoside; Pn 3-Gal = peonidin 3-galactoside.

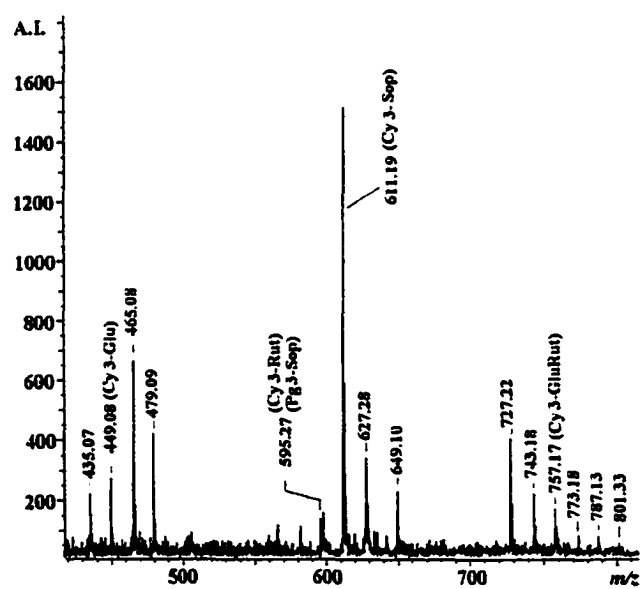

Figure 4-6. MALDI-MS natural cation spectrum of anthocyanins from raspberry syrup extracts. Cy 3-Glu = cyanidin 3-glucoside; Cy 3-Glu = cyanidin 3-rutinoside; Pg 3-Sop = pelargonidin 3-sophoroside; Cy 3-Sop = cyanidin 3-sophoroside; Cy 3-GluRut = cyanidin 3-(2^G-glucosylrutinoside).

Responses of Anthocyanin 3-Glucosides. For quantification of anthocyanins using MALDI-MS, the relative responses of the analytes are a key factor. Ideally, the intensity or response of an analyte should be linearly correlated to its relative molar ratios in a MALDI-MS sample. The relative responses of known amounts of individual anthocyanins in MALDI-MS are shown in Figure 4-7. For monoglucoside anthocyanins, the responses were only slightly different, but for a diglucoside anthocyanin, e.g., malvidin 3,5-diglucosides, or an anthocyanin with a second carbohydrate moiety to a 3-glucoside, e.g., cyanidin 3-rutinoside, the relative molar response was only about one fourth of that noted for monoglucoside anthocyanins. Figure 4-8 indicates the exact

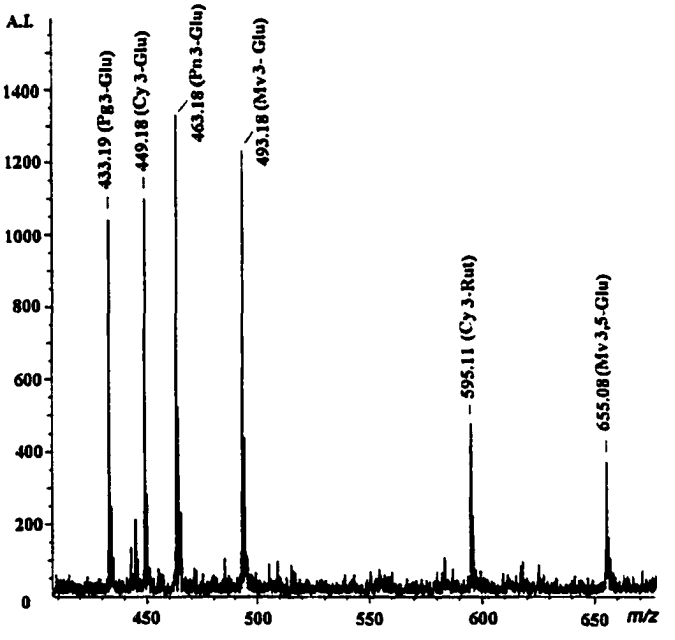

Figure 4-7. MALDI-MS natural cation spectrum of standard individual anthocyanins. Pg 3-Glu = pelargonidin 3-glucoside (1.60×10^{-5} M, peak height 1060.8); Cy 3-Glu = cyanidin 3-glucoside (1.58×10^{-5} M, peak height 1122.0); Pn 3-Glu = peonidin 3-glucoside (1.42×10^{-5} M, peak height 1357.6); Mv 3-Glu = malvidin 3-glucoside (1.47×10^{-5} M, peak height 1256.6); Cy 3-Glu = cyanidin 3-rutinoside (2.21×10^{-5} M, peak height 489.6); Mv 3,5-Glu = malvidin 3,5-diglucosides (2.03×10^{-5} M, peak height 381.5).

responses of different concentration of monoglucoside anthocyanin for MALDI-MS using cyanidin 3-rutinoside as the internal standard. The linear models were fit to the responses of all monoglucoside anthocyanins ($R^2 = 0.94$). Other evidence that the

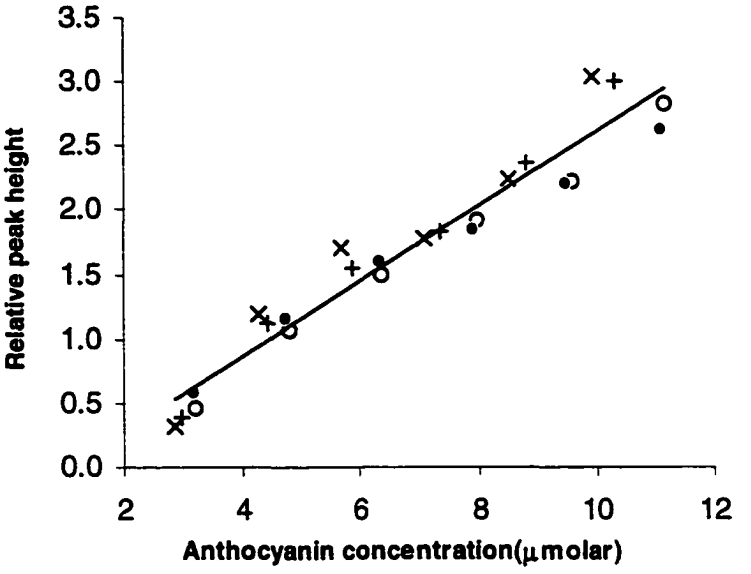

Figure 4-8. Linear responses of individual anthocyanins in MALDI-MS. o, pelargonidin 3-glucoside; ●, cyanidin 3-glucoside; ×, peonidin 3-glucoside; +, malvidin 3-glucoside. For the total data points, $Y = 0.29X - 0.29$ ($R^2 = 0.94$). The concentration of individual anthocyanins ranged from 3.19×10^{-6} M to 1.12×10^{-5} M for pelargonidin 3-glucoside, 3.16×10^{-6} M to 1.11×10^{-5} M for cyanidin 3-glucoside, 2.83×10^{-6} M to 1.00×10^{-5} M for peonidin 3-glucoside, and 2.94×10^{-6} M to 1.03×10^{-5} M for malvidin 3-glucoside, and internal standard cyanidin 3-rutinoside (4.42×10^{-5} M) in methanol/formic acid/water = 70:2:28 (v/v/v).

responses of anthocyanins fit a linear model was the response of malvidin 3-glucoside added to concord grape juice extracts. The addition of this anthocyanin to food extracts also showed a linear response with R^2=0.99 (Figure 4-9). Also, the relative responses of anthocyanins, for example, anthocyanins in red wine extracts, were not affected in MALDI-MS by an addition of internal standard cyanidin 3-rutinoside as shown in Figure 4-10. All these factors indicate that the responses of individual anthocyanins in MALDI-MS are predictable and unaffected by each other. When the individual anthocyanin standard curves are plotted using an appropriate internal standard or using the standard addition method (Abell and Sporns, 1996; Chapter 2, Wang et al., 1999), all the anthocyanins in food can be also quantitatively analyzed.

For comparison, Figure 4-11 indicates the relative responses of standard individual anthocyanins in HPLC system with detection at 525 nm. The peak areas of individual anthocyanins with almost the same molar amount of anthocyanins were quite different. This was because the maximum absorbance of anthocyanins was slightly

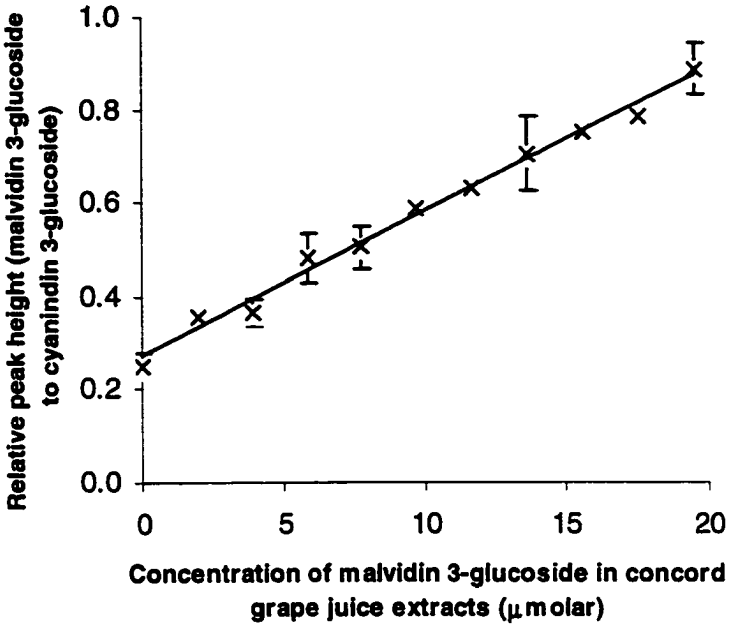

Figure 4-9. Linear MALDI-MS response of malvidin -3-glucoside (Y= 0.031X + 0.27, R^2=0.99) in Concord grape juice extracts. The concentration malvidin 3-glucoside ranged from 1.95×10^{-6} M to 1.95×10^{-5} M.

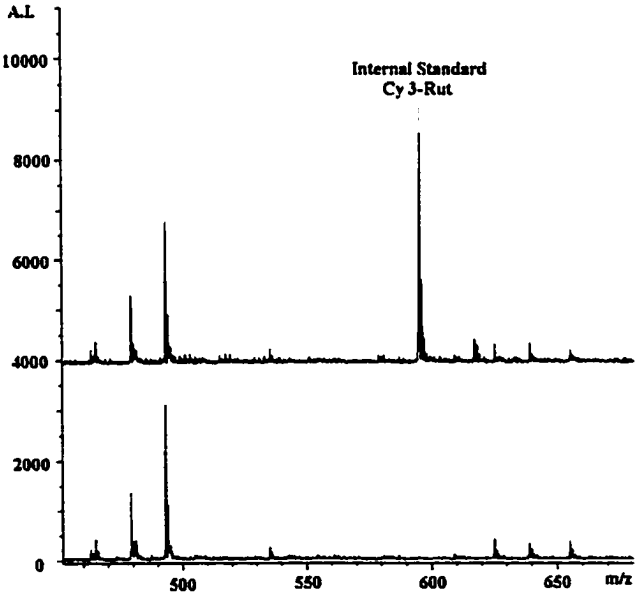

Figure 4-10. MALDI-MS natural cation spectra of anthocyanins with and without internal standard. The spectra are from Zinfandel '98 red wine extracts, which were diluted 20 times from original extracts. Top: with internal standard cyanidin 3-rutinoside (4.42×10^{-5} M); Bottom: without internal standard. Note that the addition of the internal standard has no effect on the relative responses of the other anthocyanins.

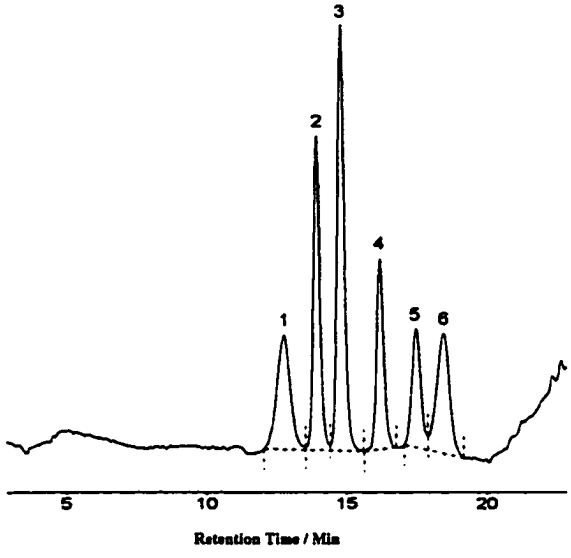

Figure 4-11. HPLC chromatography of standard anthocyanins in Figure 4-7. Integration values are (1) malvidin 3,5-diglucoside (peak area 227 729), (2) cyanidin 3-glucoside (peak area 295 507), (3) cyanidin 3-rutinoside (peak area 439 029), (4) pelargonidin 3-glucoside (peak area 206 928), (5) peonidin 3-glucoside (peak area 157 198), (6) malvidin 3-glucoside (peak area 225 614).

different and the various isomeric forms that can be formed were very dependent on pH and the solvent variation. For quantification using HPLC, delphinidin 3-glucoside or cyanidin 3-glucoside are often used as external standards and the amount of individual anthocyanins in samples expressed in terms of these standard anthocyanins (Takeoka et al., 1997; Gao et al., 1997). Because responses vary in different solvent systems, the absolute amount of anthocyanins in samples may be misinterpreted by comparing to a single external standard. Compared to HPLC, MALDI-MS shows a more consistent response for a group of anthocyanins. Certainly for at least mono-glucoside anthocyanins, it seems that any individual anthocyanin can be used as a standard for others.

In conclusion, MALDI-MS is a very valuable technique for the rapid analysis of anthocyanins. MALDI-MS can be used to obtain the exact masses of various anthocyanins in food and can provide the anthocyanin profile in a food sample within a

few minutes. Also, the use of MALDI-MS to quantify anthocyanins has been demonstrated with the use of appropriate internal standards. Finally, we feel that MALDI-MS can make a significant contribution to the use of anthocyanin "fingerprints" to determine the authenticity of samples and to explore varietal and growth differences.

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Chapter 5

Comparison between HPLC and MALDI-MS Analysis of Anthocyanins in Highbush Blueberries[1]

INTRODUCTION

Highbush blueberries (*Vaccinium corymbosum*) are one of several *Vaccinium* species native to North America. Highbush blueberries are grown commercially in North America, and more recently in other parts of the world, primarily for use in processed food products. Highbush blueberries, like some other commercial *Vaccinium* species (e.g. lowbush blueberry, bilberry) are noted for their high content of anthocyanin pigments. For example, highbush blueberries may contain more than 15 times the anthocyanin content of strawberries, and more than 3 times the pigment level of raspberries (Kalt et al., 1999). Anthocyanins have been of great interest to the food industry because of their important contribution to food color. An extract of bilberry (*V. Myrtillus*) called Myrtocyan® is used for various health applications, based on the reported biological activities of its anthocyanins (Marazzoni and Bombardelli, 1996).

Rapid accumulation of anthocyanins is a characteristic event during ripening of many fruit species. In highbush blueberries, the extent of color change from white/green to blue serves as an important visual indicator of fruit maturity. Under ripe fruit will accumulate anthocyanins when either attached to, or detached from, the plant (Woodruff etal., 1960; Suomalainen and Keranen, 1961). Anthocyanin analysis has been an important element in characterizing the changes that occur in fruit during ripening and after harvest.

There are fifteen major anthocyanins in highbush blueberries (Sapers et al., 1984; Mazza and Miniati, 1993; Kader et al., 1996); these are the 3-monoarabinosides, 3-

[1] A version of this chapter has been accepted for publication. Wang, J.; Kalt, W.; Sporns, P. Comparison between HPLC and MALDI-TOF MS analysis of anthocyanins in highbush blueberries. *J. Agric. Food Chem.*

monogalactosides, and 3-monoglucosides of cyanidin, delphinidin, peonidin, petunidin and malvidin (Figure 5-1). Gao and Mazza (1994a) have identified small amounts of acetylated anthocyanidin monoglycosides in the highbush variety Bluecrop. Detailed anthocyanin analysis has been very useful in studying the changes that occur in the maturation and storage of fruits. Ultra Violet-visible spectroscopy (UV-Vis) (260-600 nm) has been widely used to study anthocyanins (Harborne, 1958, 1967; Gao and Mazza, 1994b; Baldi et al., 1995; Bakker et al., 1997). High performance liquid chromatography

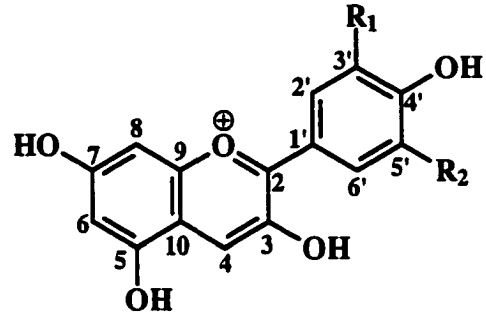

Figure 5-1. Structures of anthocyanidins. Cyanidin: R_1 = H, R_2 = OH. Delphinidin: R_1 = R_2 = OH. Peonidin: R_1 = OCH_3, R_2 = H. Petunidin: R_1 = OCH_3, R_2 = OH. Malvidin: R_1 = R_2 = OCH_3.

(HPLC) is the main technique used to quantify anthocyanins (Wulf and Nagel, 1978; Camire and Clydesdale, 1979; Goiffon et al., 1991; Mazza and Velioglu, 1992; Gao and Mazza, 1994a; Gao et al., 1997). The coupling of HPLC and UV-Vis spectroscopy (HPLC / photodiode array detection) has been the most common method to determine anthocyanin identity and quantification (Andersen, 1985, 1987; Hong and Wrolstad, 1990a, 1990b; Goiffon et al., 1991; Dallas et al., 1996; Garcia-Viguera et al., 1997).

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-MS) was first introduced in 1987 and originally developed for large biomolecules (Karas et al., 1987). MALDI-MS has several advantages over other methodologies, including speed of analysis, high sensitivity, wide applicability with a good tolerance towards contaminants, and the ability to analyze complex mixtures (Karas, 1996). However, simple MALDI-MS instruments can not tell the difference between isomers, which have identical molecular weight. Recent research on applications of

MALDI-MS has been extensive. In food analysis, MALDI-MS has been used to identify anthocyanins (Sugui et al., 1998; Sporns and Wang, 1998; Chapter 4, Wang and Sporns, 1999; Sugui et al., 1999). Wang and Sporns (1999, Chapter 4) reported the potential quantitative analysis of anthocyanins in foods using MALDI-MS. The objective of this study was to compare HPLC and MALDI-MS analysis of anthocyanins in blueberry samples.

MATERIALS AND METHODS[2]

Materials and Reagents. Highbush blueberries (*V. corymbosum*, cv. Bergitta) were harvested when the fruit surface color was between 5 and 50% fully blue. The remaining surface area of the fruit was pink; the stem end of the fruit was largely white. Three replicate samples were collected from three different shrubs at the same production site. Fruit samples were stored in the dark in a controlled temperature room set at 20 ± 0.5°C. A constant vapor pressure deficit of 0.212 kPa was maintained in the chambers using solutions of glycerol-water as described by Forney and Brandl (1992). The three replicate samples were removed after 0, 2, 4, and 8 days of storage. Pelargonidin 3-glucoside, cyanidin 3-glucoside, peonidin 3-glucoside and malvidin 3-glucoside were obtained from Extrasynthese S.A. (Genay Cedex, France). Maltotriose and maltotetraose were purchased from Sigma Chem. Co. (St. Louis, MO). 2',4',6'-Trihydroxyacetophenone monohydrate (THAP) was obtained from Aldrich Chem. Co. (Milwaukee, WI). All water used was double deionized (Milli-Q® water purification system, Millipore Corp., Bedford, MA).

Extraction of Anthocyanins from Blueberries. The samples were prepared by grinding 30 g of frozen blueberries in a small food processor. Approximately 15 g of these ground samples was added with a half teaspoon of Celite 545 (J.T. Baker Inc. Phillipsburg, NJ) to 30 mL of mixed solvent (acetone:methanol:water:formic acid, 40:40:20:0.1, v/v/v/v). This sample was blended for 2 min. on a Virtis homogenizer (The

[2] Sample collection, anthocyanin extraction, and anthocyanin HPLC analyses were conducted under the supervision of Dr. Kalt in Atlantic Food and Horticulture Research Centre, Agriculture and Agri-Food Canada, Kentville, Nova Scotia.

Virtis Company Inc., Gardiner, NY) at a speed setting of 4. The solid material was removed from the extract using vacuum and a Buchner funnel lined with two sheets of Whatman No. 4 filter paper (Whatman International Ltd., Maidstone, UK). The residue was rinsed with the solvent and the filtrate was made up to 50 mL. Some samples (7.5 g), which were less than 15 g, were extracted with only 25 mL total volume of solvent. The solvent was removed under vacuum using a centrifugal evaporator (Savant Instruments Inc., Hicksville, NY) and then dried extracts were resolubilized in an equivalent volume (i.e. 50 or 25 mL) of water. Extract (8 mL) was passed through a Sep-Pak C18 cartridge (Waters Scientific, Mississauga, ON) which had been prerinsed with 2 mL of 100% methanol and 5 mL of water. Once loaded, the column was rinsed with 5 mL of water to remove sugars and organic acids. Anthocyanins were eluted with 10 mL of 0.1% formic acid in methanol, and stored at -20 C until analyzed.

HPLC Analysis Blueberry Anthocyanins. Samples for HPLC analysis were dried, resolubilized in an equivalent volume (i.e. 10 mL) of 86% Solvent A and 14% Solvent B (see below) and then filtered through a 0.2 μm PVDF filter (Whatman International Ltd., Maidstone, UK). 20 μL of extract was injected onto the HPLC. The HPLC system consisted of a Hewlett-Packard 1100 Series HPLC (Hewlett-Packard [Canada], Mississauga, Ontario, Canada) coupled with an auto sampler and a photodiode array detector. Anthocyanins were separated on a Zorbax SBC18 150 × 4.6 mm (5 μm) reverse phase column (Hewlett-Packard (Canada), Mississauga, Ontario, Canada). The solvents used were 5% (v/v) aqueous formic acid (solvent A) and 100% HPLC grade methanol (solvent B). The flow rate was at 1 mL/min, with a linear gradient profile consisting of solvent A with the following proportions (v/v) of solvent B: 0 min, 14% B; 1-10.24 min, 14-17% B; 10.24-35.28 min, 17-23% B; 35.28-64.59 min, 23-47% B; 64.59-66.59 min, 47-14% B. The total run time was 70 min. The column temperature was maintained at 26 C. Quantification of anthocyanins was based on peaks areas determined at 520 nm and compared to the absorbance of cyanidin 3-glucoside, which was used as the standard. For each storage stage, three samples were analyzed.

MALDI-MS. MALDI-MS was performed using a Proflex™ III in linear mode (Bruker Analytical Systems Inc., Billerica, MA). Anthocyanins co-crystallized with

matrix on the probe were desorbed and ionized by a nitrogen laser pulse (337 nm) and accelerated under 20 kV with time-delayed extraction before entering the time of flight mass spectrometer. 2',4',6'-Trihydroxyacetophenone monohydrate (THAP) was used as matrix. THAP was saturated in acetone. Matrix solution (0.3 μL) was applied on the probe first and air dried. Then 1 μL of final anthocyanin extracts was spotted directly over THAP crystals and again air dried. Laser strength was attenuated (the lower the attenuation, the higher the laser strength) to obtain the best signal-to-noise ratios while isotopic resolution was maintained. Anthocyanin extracts were diluted to obtain about 0.35 A_{520} using methanol:formic acid:water (70:2:28, v/v/v) before MALDI-MS analysis so that the detector would not be saturated. When the UV-Vis absorbance of dilute extracts at 520 nm was 0.35 ± 0.05, anthocyanins would not cause detector saturation problems and fit in the linear MALDI-MS response range (Chapter 4, Wang and Sporns, 1999). MALDI-MS was calibrated with two point external calibration using [maltotriose + K]$^+$ (exact isotopic mass = 543.13) and [maltotetraose + K]$^+$ (exact isotopic mass = 705.19) as calibrants, resulting in a mass accuracy ≤ 500 ppm.

Quantification of anthocyanins using MALDI-MS was achieved using a standard addition method, as reported earlier by Wang et al. (1999, Chapter 2). MALDI-MS samples contained 30 μL of dilute extract and 30 μL of solvent (methanol:formic acid:water, 70:2:28, v/v/v), while the standard addition samples contained 30 μL of the extract plus 30 μL of cyanidin 3-glucoside (9.5×10^{-5} M in solvent). Samples and corresponding standard addition samples were applied to the ten positions on the probe; one probe could be used for the analysis of five samples and their standards. From each of the ten probe positions, a single spectrum was obtained by collecting 3 × 40 or total of 120 laser pulses from three randomly selected spots. Peak heights were determined for each anthocyanin from each spectrum. These peak heights were then scaled relative to the delphinidin 3-glycoside's peak, which was arbitrarily set at a value of 1.0. Each of the five spectra for the sample were compared to a different spectrum from the five standard addition samples and the average increase in scaled relative peak height for cyanidin 3-glucoside (standard anthocyanin added) was determined. This gave a value for the average increased response due to the addition of cyanidin 3-glucoside on molar basis.

This response factor was then used to determine the average amount of each anthocyanin in the five sample spectra. The acquisition of the MALDI-MS data (five runs, total of 10 spectra) took about 20 minutes. One single run represented acquiring two spectra (one from sample and another from standard added sample). The mean of five runs was used to calculate the average anthocyanin content of each sample. For each storage stage, three samples were analyzed (see Appendix II for experimental design).

Statistics. Means and standard deviations were analyzed using Microsoft Excel 97 (Microsoft Office 97).

RESULTS AND DISCUSSION

HPLC has been a very popular and widely used technique for analysis of anthocyanins in foods or plants (Andersen, 1985, 1987; Hong and Wrolstad, 1990a, 1990b; Goiffon et al., 1991; Dallas et al., 1996; Garcia-Viguera et al., 1997). Quantification of anthocyanins using HPLC was based mainly on peak areas determined at a certain wavelength such as 525 nm (Gao et al., 1997), which was usually close to the maximum absorbance wavelength (λ_{max}) of individual anthocyanins. Malvidin 3-glucoside or cyanidin 3-glucoside were usually selected as the standard for quantification (Wulf and Nagel, 1978; Boyles and Wrolstad, 1993; Gao et al., 1997). Studies have shown that anthocyanins with different chromophores have different λ_{max} even in the same solvent system (Harborne, 1958, 1967; Francis, 1982; Hong and Wrolstad, 1990b). For example, the λ_{max} of pelargonidin 3-glucoside, cyanidin 3-glucoside, delphinidin 3-glucoside, peonidin 3-glucoside, petunidin 3-glucoside and malvidin 3-glucoside were 506, 523, 534, 523, 534 and 534 nm respectively in 0.01% HCl in methanol (Francis, 1982). Gradient elution in HPLC likely also has an effect on the anthocyanin visible absorbance characteristics due to changes in solvent composition during spectral detection. Therefore, direct comparison of spectral characteristics with those published in the literature may be inappropriate and quantification of anthocyanins using HPLC at a fixed wavelength, e.g. 525 nm, may over- or under-estimate some anthocyanins. Wang and Sporns (1999, Chapter 4) have noted considerable variation in HPLC responses at

525 nm. In this study, if cyanidin 3-glucoside was selected as the standard and its peak area was arbitrarily set at a value of 1.0, the relative molar response factors of malvidin 3,5-diglucoside, cyanidin 3-rutinoside, pelargonidin 3-glucoside, peonidin 3-glucoside, malvidin 3-glucoside would be 0.60, 1.06, 0.69, 0.60, 0.82 respectively. This would indicate that the amount of most individual anthocyanins and the total amount of anthocyanins might be under-estimated if cyanidin 3-glucoside was used as standard for quantification.

In MALDI-MS, it has been shown that anthocyanin monoglucosides gave similar responses (Chapter 4, Wang and Sporns, 1999). Therefore, using a single anthocyanin monoglucoside standard one can determine the correct amounts of anthocyanins present (individually and in total).

A major disadvantage for MALDI-MS analysis is that it can not distinguish anthocyanin isomers. For example, the peak at *m/z* 449 (Figure 5-2) could be produced by

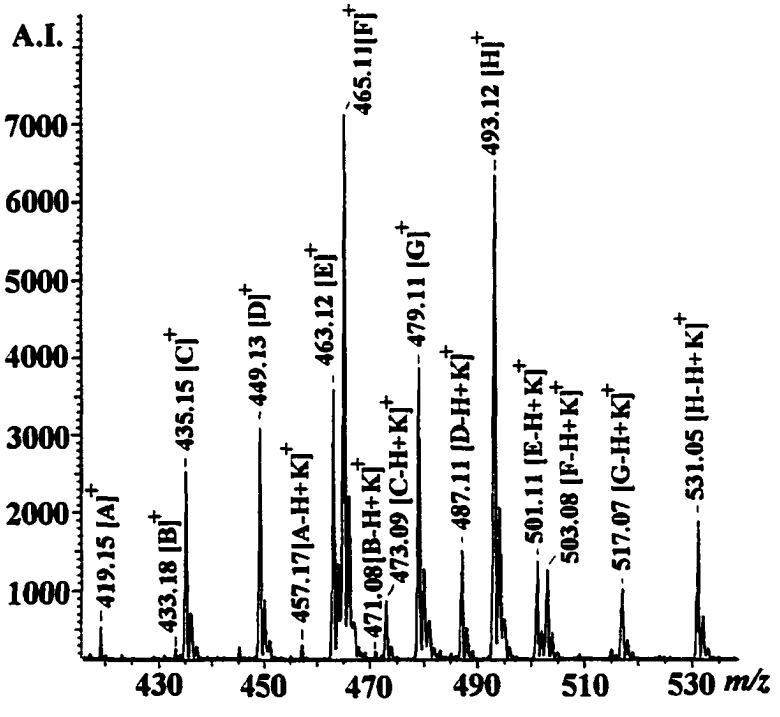

Figure 5-2. MALDI-MS positive ion spectrum of highbush blueberry anthocyanins (stored for 4 days). Number in parentheses indicates corresponding peak number measured by HPLC (Figure 5-3). A = cyanidin 3-arabinoside (7); B = peonidin 3-arabinoside; C = delphinidin 3-arabinoside (4); D = cyanidin 3-galactoside (3) + cyanidin 3-glucoside (5) + petunidin 3-arabinoside (10); E = peonidin 3-galactoside (9) + malvidin 3-arabinoside (13); F = delphinidin 3-galactoside (1) + delphinidin 3-glucoside (2); G = petunidin 3-galactoside (6) + petunidin 3-glucoside (8); H = malvidin 3-galactoside (11) + malvidin 3-glucoside (12). Note that any anthocyanin can also be ionized in forms of potassium adducts (K^+) combined with deprotonation.

cyanidin 3-galactoside, cyanidin 3-glucoside or petunidin 3-arabinoside. HPLC can differentiate these three anthocyanins (Figure 5-3, peaks 3, 5 and 10). However since MALDI-MS gives a mass weight, it can identify anthocyanin mass directly without the use of standards.

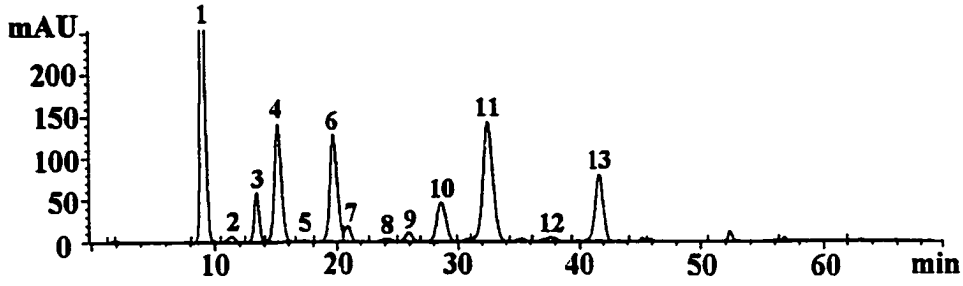

Figure 5-3. HPLC chromatography of anthocyanins (same sample as in Figure 5-2). 1 = delphinidin 3-galactoside; 2 = delphinidin 3-glucoside; 3 = cyanidin 3-galactoside; 4 = delphinidin 3-arabinoside; 5 = cyanidin 3-glucoside; 6 = petunidin 3-galactoside; 7 = cyanidin 3-arabinoside; 8 = petunidin 3-glucoside; 9 = peonidin 3-galactoside; 10 = petunidin 3-arabinoside; 11 = malvidin 3-galactoside; 12 = malvidin 3-glucoside; 13 = malvidin 3-arabinoside.

Another concern with MALDI-MS is fragmentation of anthocyanins. Using a linear MALDI-MS, post-source decay, or fragmentation of ions after extraction, is not a concern. However in-source decay can lead to the loss of a portion of the molecule such as loss of carbohydrate residues from anthocyanins forming different mass ions. Wang and Sporns (2000, Chapter 6) have found that fragmentation patterns and amounts of flavonol glycosides were predictable for any defined sample preparation method. Further research on anthocyanins has shown that the monoglucosides also fragment quantitatively. This fragmentation was examined by using a mixture of the monoglucosides (pelargonidin 3-glucoside, cyanidin 3-glucoside, peonidin 3-glucoside and malvidin 3-glucoside). These glucosides fragment with loss of their 3-glucoside moieties. The amount of fragmentation of individual anthocyanins was calculated based on the percentage of fragment ions (loss of 3-glucoside) to their unfragmented parent ions (aromatic oxonium ion form) in a single MALDI-MS sample or MALDI-MS spectrum (Table 5-1). The fragmentation of individual anthocyanins varied widely from MALDI-MS sample-to-sample as indicated by the large inter-standard deviation

Table 5-1. Quantitative fragmentation[a] (%) of anthocyanins in MALDI-MS

Anthocyanins[b]	MALDI-TOF MS samples					Inter-average[d]	Inter-standard deviation[d]
	1	2	3	4	5		
Pelargonidin 3-glucoside	12.4	25.5	18.4	14.3	16.9	17.5	5.0
Cyanidin 3-glucoside	13.3	22.8	19.7	14.5	13.1	16.7	4.4
Peonidin 3-glucoside	13.3	24.0	22.5	16.5	17.2	18.7	4.4
Malvidin 3-glucoside	15.2	27.7	16.9	15.6	14.8	18.0	5.4
Intra-average[c]	13.5	25.0	19.4	15.2	15.5		
Intra-standard deviation[c]	1.2	2.1	2.4	1.0	1.9		

[a] Quantitative fragmentation (%) was expressed as ratios of the fragment ions (loss of 3-glucoside) to their unfragmented parent ions (aromatic oxonium ion form). Number of laser pulses equals 3 × 40 or total of 120 for each MALDI-MS sample.

[b] Concentration of individual anthocyanins in the mixture was $9.0 \pm 0.5\ 10^{-5}$ M.

[c] Average or standard deviation of individual anthocyanins within one single MALDI-MS sample (in columns).

[d] Average or standard deviation of each anthocyanin from five MALDI-MS samples (in rows).

(Table 5-1). However, within any single MALDI-MS sample, all anthocyanins exhibited a similar fragmentation percentage with small intra-standard deviation (Table 5-1). That is, the ratios of fragment ions to parent ions were very consistent for the monoglucosides in a single MALDI-MS sample. In general, an internal standard, such as cyanidin 3-glucoside, will fragment with the same pattern or relative amount as other anthocyanins, and therefore can be confidently used as a reference for all other anthocyanin monoglycosides. Further quantitative fragmentation studies on di- or triglycosides present in other foods would be necessary to determine the applicability of MALDI-MS quantification of samples containing these anthocyanin derivatives.

Comparison between HPLC and MALDI-MS Quantification on Individual Anthocyanins. Figure 5-3 shows a HPLC chromatogram of anthocyanins in blueberries. There were thirteen anthocyanins. Based on retention time, they were assigned as delphinidin 3-galactoside (1), delphinidin 3-glucoside (2), cyanidin 3-galactoside (3), delphinidin 3-arabinoside (4), cyanidin 3-glucoside (5), petunidin 3-galactoside (6), cyanidin 3-arabinoside (7), petunidin 3-glucoside (8), peonidin 3-galactoside (9), petunidin 3-arabinoside (10), malvidin 3-galactoside (11), malvidin 3-glucoside (12), malvidin 3-arabinoside (13). Peonidin 3-arabinoside was not detected using HPLC, but it was identified in MALDI-MS (Figure 5-2).

When analyzed by MALDI-MS in positive mode, anthocyanins were detected in the aromatic oxonium ion form $[M]^+$ (Sugui et al., 1998; Sporns and Wang, 1998; Chapter 4, Wang and Sporns, 1999; Sugui et al., 1999). Our current study indicated that

anthocyanins may also form single alkali metal adducts $[M-H+Na]^+$ or $[M-H+K]^+$ ions, depending on the amount of alkali metal in MALDI-MS samples. Since most plants contain large amounts of potassium, anthocyanins from blueberries can also be detected as potassium adducts (Figure 5-2). Also anthocyanins showed higher affinity towards potassium than sodium (data not shown). MALDI-MS spectrum (Figure 5-2) shows eight anthocyanins at *m/z* 419, 433, 435, 449, 463, 465, 479 and 493 respectively. They were cyanidin 3-arabinoside (A, *m/z* 419.15), peonidin 3-arabinoside (B, *m/z* 433.18), delphinidin 3-arabinoside (C, *m/z* 435.15), cyanidin 3-galactoside or cyanidin 3-glucoside or petunidin 3-arabinoside (D, *m/z* 449.13), peonidin 3-galactoside or malvidin 3-arabinoside (E, *m/z* 463.12), delphinidin 3-galactoside or delphinidin 3-glucoside (F, *m/z* 465.11), petunidin 3-galactoside or petunidin 3-glucoside (G, *m/z* 479.11), malvidin 3-galactoside or malvidin 3-glucoside (H, *m/z* 493.12). The potassium adduct peaks were also detected with 38 mass shift (addition of potassium and loss of one proton). For quantification, peak heights of both aromatic oxonium ion $[M]^+$ and potassium adduct ion $[M-H+K]^+$ were totaled. Obviously, MALDI-MS can not differentiate anthocyanins with the same mass (Figure 5-2, 5-3 and 5-4). Generally, as expected the amount of individual anthocyanins determined by MALDI-MS was higher than that by HPLC (Figure 5-4 and 5-5), except for delphinidin 3-arabinoside. Also, total amount of anthocyanins determined by MALDI-MS was higher than that of HPLC (Figure 5-6).

Changes of total, individual anthocyanins at different storage stages are shown in Figure 5-6, 5-7 and 5-8. Both HPLC and MALDI-MS analyses indicated that the total amount of anthocyanins increased with increased storage (Figure 5-6). For individual anthocyanins, HPLC results (Figure 5-7) illustrated changes of 13 anthocyanins at each stage while MALDI-MS (Figure 5-8) showed changes of 8 anthocyanins (on molecular weight basis) at each stage. HPLC (Figure 5-7) showed that delphinidin 3-galactoside, delphinidin 3-arabinoside, petunidin 3-galactoside, malvidin 3-galactoside and malvidin 3-arabinoside were the major anthocyanins that changed during color development. MALDI-MS (Figure 5-8) indicated that anthocyanins, F (delphinidin 3-galactoside and delphinidin 3-glucoside), C (delphinidin 3-arabinoside), G (petunidin 3-galactoside and

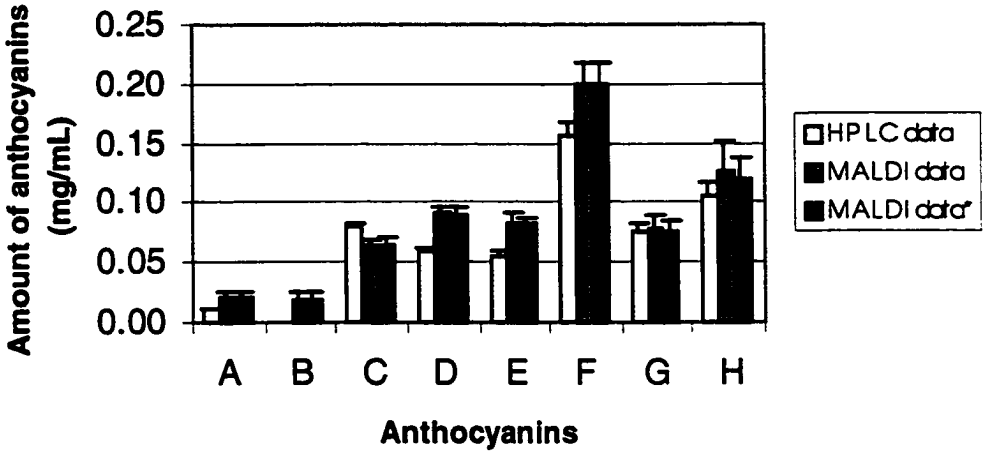

Figure 5-4. Comparison between HPLC and MALDI-MS analysis of highbush blueberry individual anthocyanins (stored for 4 days). Number in parentheses indicates corresponding peak number measured by HPLC (Figure 5-3). Percentages in parentheses represent the proportion of correspondent anthocyanins calculated from HPLC data (triplicates). A = cyanidin 3-arabinoside (7); B = peonidin 3-arabinoside; C = delphinidin 3-arabinoside (4); D = cyanidin 3-galactoside (3) (40%) + cyanidin 3-glucoside (5) (4%) + petunidin 3-arabinoside (10) (56%); E = peonidin 3-galactoside (9) (12%) + malvidin 3-arabinoside (13) (88%); F = delphinidin 3-galactoside (1) (97%) + delphinidin 3-glucoside (2) (3%); G = petunidin 3-galactoside (6) (95%) + petunidin 3-glucoside (8) (5%); H = malvidin 3-galactoside (11) (96%) + malvidin 3-glucoside (12) (4%). Each column represents the mean of three samples. Error bars indicate standard deviations (n=3). * Data calculated from one single MALDI-MS run.

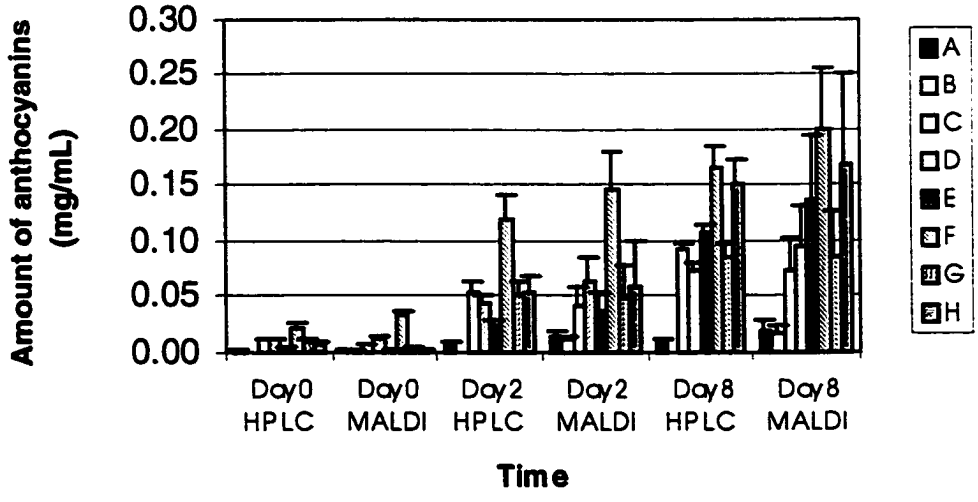

Figure 5-5. Comparison between HPLC and MALDI-MS anthocyanin profiles at three different stages of color development. A to H denotes the same anthocyanins as shown in Figure 5-2. Each column represents the mean of three samples. Error bars indicate standard deviations (n=3).

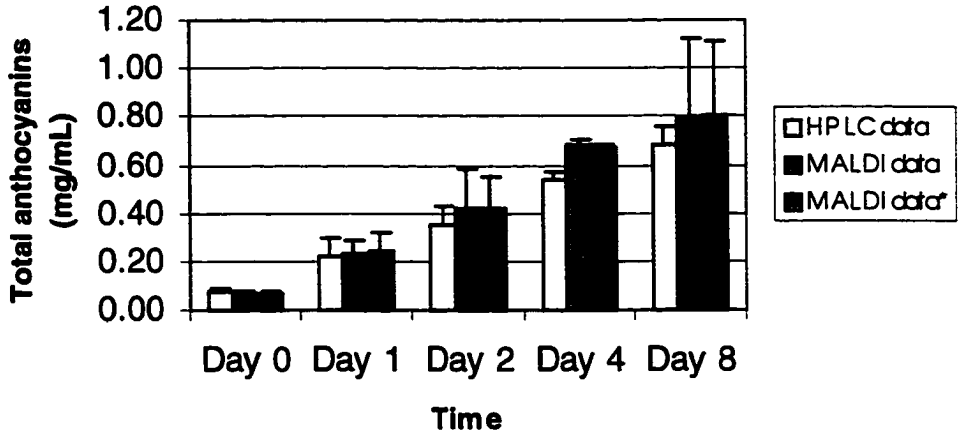

Figure 5-6. Comparison of total anthocyanin content determined by HPLC and MALDI-MS at different stages of color development. Each column represents the mean of three samples. Error bars indicate standard deviations (n=3). * Data were calculated from one single MALDI-MS run.

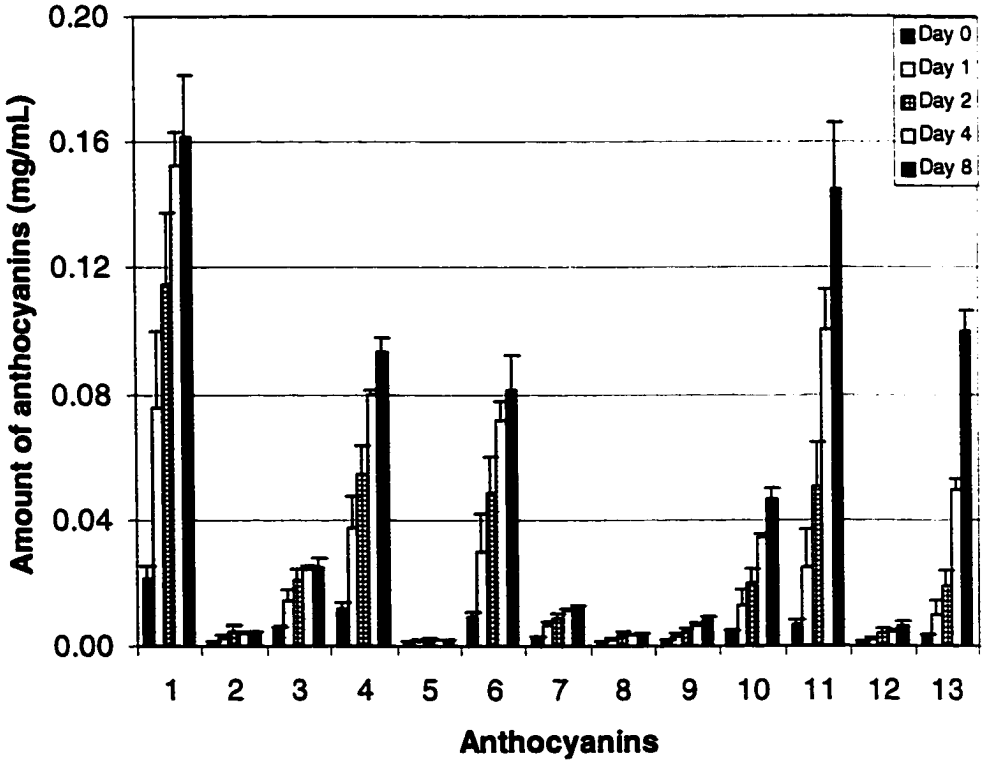

Figure 5-7. HPLC profile of anthocyanins in blueberries at different stages of color development. Numbers denote the same anthocyanins as shown in Figure 5-3. Each column represents the mean of three samples. Error bars indicate standard deviations (n=3).

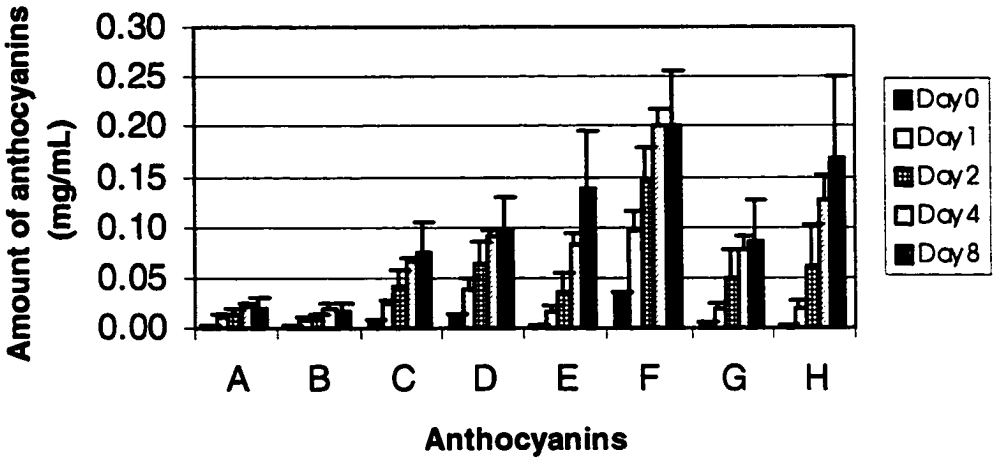

Figure 5-8. MALDI-MS profile of anthocyanins in blueberries at different stages of color development. A to H denotes the same anthocyanins as shown in Figure 5-2. Each column represents the mean of three samples. Error bars indicate standard deviations (n=3).

petunidin 3-glucoside), H (malvidin 3-galactoside and malvidin 3-glucoside), E (peonidin 3-galactoside and malvidin 3-arabinoside), followed the same trend as HPLC results. In general, HPLC and MALDI-MS provided similar quantitative profiles of anthocyanins in blueberries at different stages.

Comparison of Analysis Speed. Under our experimental conditions, it took 70 min. to complete one HPLC run. The total run time could be reduced, but some anthocyanins would not be resolved. The acquisition of the MALDI-MS data consisted of five runs per sample, and took about 20 minutes. It has been suggested that MALDI-MS shows poor repeatability from spot-to-spot due to crystal inhomogeneity. Therefore, increasing MALDI-MS runs for a sample can reduce sample standard deviations. However, as shown in Figure 5-4 and 5-6, there was no significant difference between five and one MALDI-MS run on both individual and total anthocyanins ($p \leq 0.05$). One MALDI-MS run took just 4 min.

In conclusion, HPLC and MALDI-MS are both valuable techniques for quantification of anthocyanins. HPLC has the power to differentiate anthocyanin isomers but may underestimate the amount of anthocyanins when cyanidin is used as the internal standard. MALDI-MS is a more rapid technique to identify and quantify a group of

anthocyanins with different masses. It is likely that MALDI-MS has the potential to rapidly identify and quantify anthocyanins in other foods, and could make a significant contribution in developing anthocyanin "fingerprints" to explore differences in fruit varieties and maturities.

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Chapter 6

MALDI-MS Analysis of Food Flavonol Glycosides[1]

INTRODUCTION

Flavonoids are polyphenolic compounds that occur ubiquitously in foods of plant origin. More than 2000 chemically distinct flavonoids have been reported, and each fits into one of several classes. In a dietary context, the most significant are the flavonols (Figure 6-1), especially quercetin and kaempferol, which are found at high concentrations in onions, tea and apples. The flavonols are almost entirely found in glycosylated forms, with one, two or three carbohydrates attached to flavonol hydroxyl groups (Williamson et al., 1998). Epidemiological studies have shown that flavonols have anticarcinogenic and antioxidant properties (Formica and Regelson, 1995; Rhodes and Price, 1996; Lean et al., 1999).

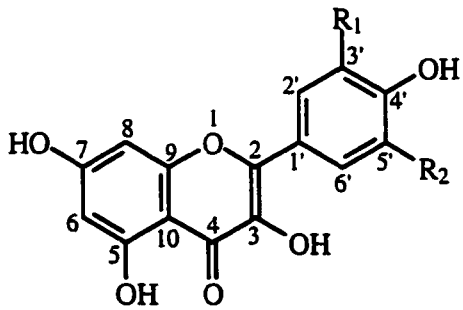

Figure 6-1. Structures of flavonols. Kaempferol: $R_1 = R_2 = H$. Quercetin: $R_1 = OH$, $R_2 = H$. Myricetin: $R_1 = R_2 = OH$. Isorhamnetin: $R_1 = OCH_3$, $R_2 = H$.

Because of the importance of flavonol glycosides, many techniques have been used to identify and quantify these compounds. Early investigations on flavonol glycosides were carried out using paper chromatography and thin-layer chromatography (Roberts et al., 1956; Oshima and Nakabayashi, 1953 a,b). In the early 1990's, high

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performance liquid chromatography (HPLC) with photodiode array detection was used to isolate and quantify flavonol glycosides in tea (McDowell et al., 1990; Bailey et al., 1990; Finger et al., 1991). ^{1}H and ^{13}C NMR spectroscopy were the most powerful techniques for determination of flavonol glycoside molecular structure (Finger et al., 1991). The coupling of HPLC and mass spectrometry methods, such as electrospray, thermospray or fast atom bombardment, has been widely used to provide molecular weight and characteristic fragment ions for structural elucidation of flavonol glycosides (Finger et al., 1991; Bailey et al., 1994; Kiehne and Engelhardt, 1996; Sägesser and Deinzer, 1996; Price et al., 1997).

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-MS) was first introduced in 1987 and originally developed for large biomolecules (Karas et al., 1987). MALDI-MS has advantages over other methodologies including speed of analysis, high sensitivity, wide applicability combined with a good tolerance towards contaminants, and the ability to analyze complex mixtures (Karas, 1996). However, simple MALDI-MS instruments can not tell the difference between isomers, which have identical mass. The potential application of MALDI-MS in food systems allows for analysis of most molecules. Presently, MALDI-MS food applications are limited (Sporns and Wang, 1998). MALDI-MS has been reported for both qualitative and quantitative analysis of anthocyanins, which are structurally very similar to flavonols, in several important foods (Chapter 4, Wang and Sporns, 1999). This study presents the use of MALDI-MS to study food flavonol glycosides.

MATERIALS AND METHODS

Materials and Reagents. Yellow onion bulbs (*Allium cepa* L.) and green tea were purchased from local markets in Edmonton, Alberta, Canada. Isoquercitrin (quercetin 3-glucoside), kaempferol 3-glucoside and kaempferol 3-rutinoside were obtained from Extrasynthese S.A. (Genay Cedex, France). Rutin (quercetin 3-rutinoside), 4-hydroxy-α-cyano-cinnamic acid (HCCA), N-t-Boc-Met-Asp-Phe Amide, N-t-Boc-Trp-Met-Asp-Phe Amide were purchased from Sigma Chem. Co. (St. Louis, MO). 1-Kestose

and nystose were a gift from Dr. A. Ohta (Nutritional Science Center, Meiji Seika Kaisha, Japan). 2',4',6'-Trihydroxyacetophenone monohydrate (THAP), 2-(4-hydroxyphenylazo)-benzoic acid (HABA), 3-aminoquinoline (3-AQ), *trans*-3-indoleacrylic acid (IDA) and 2,5-dihydroxybenzoic acid (DHB) were obtained from Aldrich Chem. Co. (Milwaukee, WI). All water used was double deionized (Milli-Q water purification system, Millipore Corp., Bedford, MA). A common solvent used was aqueous 70% methanol.

Extraction of Flavonol Glycosides from Yellow Onion and Green Tea. Fresh yellow onion was pealed to remove the dry outer layers and then chopped using a food processor (Braun, UK 100, Type 4259, Germany) for 5 min. The mixed sample was freeze dried. The dried sample (5 g) was extracted with 100 mL 70% methanol for 30 min. with stirring, and then filtered through Whatman No. 4 filter paper. Methanol was removed from the filtrate using a Rotavopor 461 water bath (Brinkmann Instruments Ltd., Mississauga, Ontario, Canada) *in vacuo* with a bath temperature of 35 C. Methanol-free filtrate (10 mL) was loaded onto a Sep-pak C18 cartridge (Waters Corp., Milford, MA) at a flow rate of about 1 mL/min, washed with 10 mL water three times, and eluted with 2 mL 70% methanol. Green tea (4 g) was extracted with 100 mL of hot water (80 C), stirred for 15 min, then filtered through glass wool. The purification of flavonol glycosides using a Sep-pak C18 cartridge was the same as the onion sample, except that 2 mL filtrate was loaded on the Sep-pak C18 cartridge and the final eluant was 2 mL 0.01 M NaCl in 70% methanol. The yellowish flavonol glycoside extracts were kept in a refrigerator until used.

MALDI-MS. MALDI-MS was performed using a Proflex™ III in linear mode (Bruker Analytical Systems Inc., Billerica, MA). Flavonol glycosides co-crystallized with matrices on the probe were ionized by a nitrogen laser pulse (337 nm) and accelerated under 20 kV with time-delayed extraction before entering the time of flight mass spectrometer. For THAP, HABA, 3-AQ, DHB and HCCA, the preparation of matrix and sample was the same as previously reported (Chapter 2, Wang et al., 1999). For IDA, the matrix solution contained 10 mg/mL IDA in 1:1 acetone:water. The ratio of matrix solution and sample was 1:1. For all matrices either 1 μL sample solution or a 2 μL mixture of matrix and sample was applied to a MALDI-MS probe and air dried. When

flavonol glycosides were identified after HPLC separation, 0.5 μL THAP (saturated in acetone) was applied on the probe first and air dried. Then 2 μL of the preparative HPLC fraction was spotted on the THAP crystals and further air dried. MALDI-MS was attenuated (the lower the attenuation, the higher the laser strength) to obtain the best signal-to-noise ratio and isotopic resolution for the matrix used. Two point external calibrations were performed using $[kestose + K]^+$ (exact isotopic mass = 543.13) and $[nystose + K]^+$ (exact isotopic mass = 705.19) for positive MALDI-MS mode, and [N-t-Boc-Met-Asp-Phe Amide - H]$^-$ (exact isotopic mass = 509.21) and [N-t-Boc-Trp-Met-Asp-Phe Amide - H]$^-$ (exact isotopic mass = 695.29) for the negative mode. The mass accuracy was below 500 ppm. Each spectrum represents the sum of 60 laser pulses.

Preparative HPLC. The preparative HPLC system consisted of a Varian VISTA 5500 HPLC (Varian Canada Inc., Mississauga, Ontario, Canada), a Varian 9090 Auto sampler, and a Spectro Monitor III UV detector (LDC/MILTON ROY, Riviera Beach, FL). The system was equipped with a 75 × 4.5 mm pre-injection C18 saturator column containing silica-based packing (12 μm) and a 50 × 4.6 mm guard column containing Supelco LC-18 reverse phase packing (20-40 μm, Supelco, Bellefonte, PA). Flavonol glycosides were separated on a Supelcosil™ SPLC-18-DB 250 × 10 mm (5 μm) preparative reverse phase column (Supelco, Bellefonte, PA). The solvents used were 2% (v/v) aqueous acetic acid (solvent A) and acetonitrile (solvent B). The flow rate was at 5 mL/min, with a linear gradient profile consisting of solvent A with the following proportions (v/v) of solvent B: 0 min, 15% B; 0-26 min, 15-18% B; 26-30 min, 18-31% B; 30-32 min, 31% B; 32-33 min, 31-15% B; 33-34 min, 15% B. Detection was at 354 nm, and the total run time was 38 min. Sample extracts (500 μL) were injected into the HPLC for preparative collection. A Shimadzu CLASS-VP™ chromatography data system (Shimadzu Scientific Instruments Inc., Columbia, MD) was used to monitor the eluted peaks and the flavonol glycoside fractions of interest were collected for MALDI-MS analysis.

Statistics. Means and standard deviations were analyzed using Microsoft Excel 97 (Microsoft Office 97).

RESULTS AND DISCUSSION

For analysis of any compound in food samples using MALDI-MS, several factors, including the selection of matrices, preparation of matrix and sample, signal-to-noise ratio and spot-to-spot repeatability, must be considered.

Selection of MALDI-MS Matrices for Flavonol Glycosides. Six common matrices were examined for desorption and ionization of flavonol glycosides. Of these matrices, 4-hydroxy-α-cyano-cinnamic acid (HCCA), 2',4',6'-trihydroxyacetophenone monohydrate (THAP), 2-(4-hydroxyphenylazo)-benzoic acid (HABA), *trans*-3-indoleacrylic acid (IDA) and 2,5-dihydroxybenzoic acid (DHB) produced good quality spectra in MALDI-MS positive mode for flavonol glycosides. THAP and IDA were also good matrices for producing negative ions with flavonol glycosides. 3-Aminoquinoline (3-AQ) was not a suitable matrix since matrix and sample did not co-crystallize properly, although noticeable (but poor) analyte signal was still observed. Among all the matrices studied, THAP was chosen as ideal for flavonol glycosides for further study in terms of the ease of preparation of matrix and sample, tolerance to contaminants, good spot-to-spot repeatability, and ability to generate both positive and negative analyte ions. All figures presented in this paper are spectra using THAP as matrix.

Laser strength used for desorption and ionization of flavonol glycosides was matrix related. HCCA, HABA and IDA can desorb and ionize flavonol glycosides at very low laser strength, while THAP and DHB required higher laser strength. Generally, laser strength was selected based on good signal-to-noise ratio and the best resolution of analytes of interest (Chapter 2, Wang et al., 1999). In all experiments, isotopic resolution was achieved in both positive and negative modes for all matrices. The best spectra were those that reduced fragmentation to a minimum while maintaining isotopic resolution.

Ionization of Flavonol Glycosides. When analyzed by MALDI-MS in positive mode, flavonol glycosides showed ion forms of protonated $[M+H]^+$, single alkali metal adducts $[M+Na]^+$ or $[M+K]^+$ and double alkali metal adduct $[M-H+Na+K]^+$ ions (Figure 6-2). Ionization of flavonol glycosides was mainly influenced by matrices. HCCA and HABA generated alkali adduct ions only, whereas DHB, IDA and THAP produced

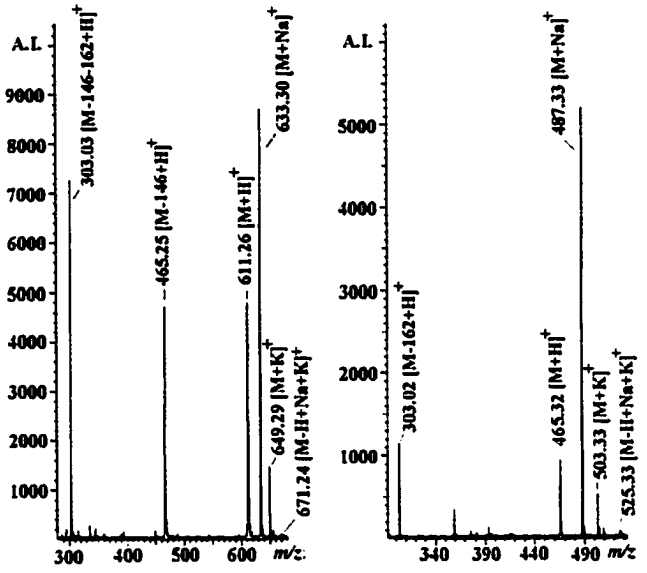

Figure 6-2. MALDI-MS positive ion spectra of flavonol glycoside standards. Left: rutin (1.5×10^{-3} M in 70% methanol). Right: isoquercitrin (1.5×10^{-3} M in70% methanol).

both protonated and alkali adduct ions. Compared to flavonol monoglycosides, the di- or triglycosides exhibited high affinity for alkali metals (Figure 6-3). Furthermore, the relative amount of $[M+Na]^+$ vs $[M+H]^+$ was correlated to the concentration of alkali metals in a sample. By addition of an alkali metal (0.01 M NaCl) in the sample solution, $[M+H]^+$ ion formation decreased while $[M+Na]^+$ ion intensity increased. Alkali adduct ions could be exchanged by adding a relatively high concentration (0.01 M) of various alkali metal salts (Figure 6-4).

In MALDI-MS negative mode, flavonol glycosides were ionized through deprotonation. Only one molecular negative ion form (Figure 6-5) was observed. Generally, the intensity or abundance of molecular ions in negative mode was much less (4 times or more) than in positive mode. The laser strength used for desorption and ionization was slightly higher in negative mode than in positive mode.

Fragmentation of Flavonol Glycosides. In MALDI-MS positive mode, all the flavonol glycosides exhibited fragmentation with loss of their carbohydrate residues.

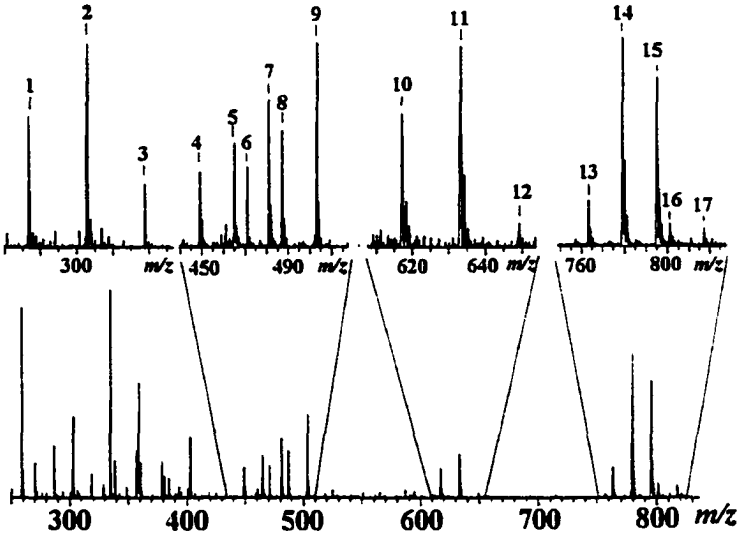

Figure 6-3. MALDI-MS positive ion spectrum of flavonol glycosides from green tea. 1: 286.83 [kaempferol+H]$^+$. 2: 302.88 [quercetin+H]$^+$. 3: 318.93 [myricetin+H]$^+$. 4: 449.15 [kaempferol 3-glycoside+H]$^+$. 5: 465.15 [quercetin 3-glycoside+H]$^+$. 6: 471.20 [kaempferol 3-glycoside+Na]$^+$. 7: 481.20 [myricetin 3-glycoside+H]$^+$. 8: 487.20 [quercetin 3-glycoside+Na]$^+$. 9: 503.20 [myricetin 3-glycoside+Na]$^+$. 10: 617.28 [kaempferol 3-rutinoside+Na]$^+$. 11: 633.33 [quercetin 3-rutinoside+Na]$^+$. 12: 649.25 [myricetin 3-rutinoside+Na]$^+$. 13: 763.31 unknown (possibly [kaempferol 3-glucosylrhamnosylrhamnoside+Na]$^+$). 14: 779.29 [kaempferol 3-glucosylrhamnosylglycoside+Na]$^+$. 15: 795.27 [quercetin 3-glucosylrhamnosylglycoside+Na]$^+$. 16: 801.27 [kaempferol 3-glucosylrhamnosylglycoside-H+2Na]$^+$. 17: 817.28 [quercetin 3-glucosylrhamnosylglycoside-H+2Na]$^+$.

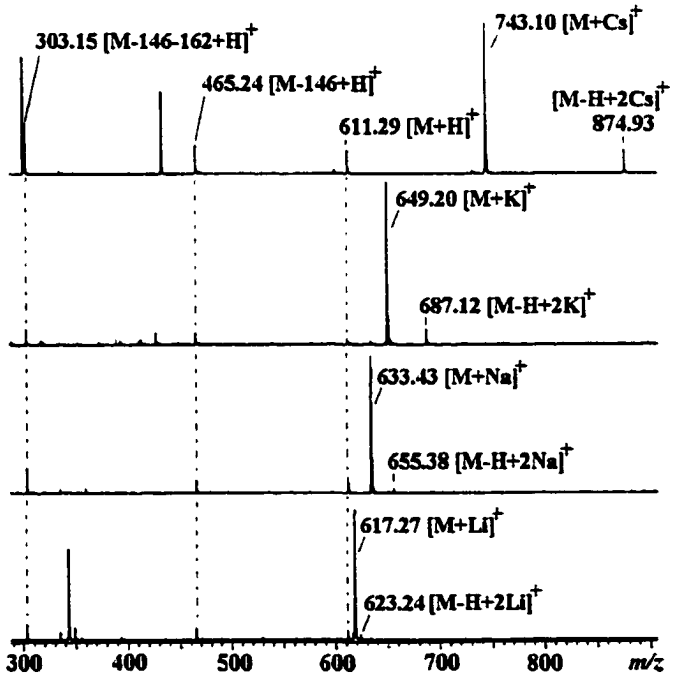

Figure 6-4. MALDI-MS positive ion spectra of rutin (1.5×10^{-3} M dissolved in 0.01 M alkali 70% methanol) with different alkali adduct ions (Li^+, Na^+, K^+ and Cs^+). Note that all the other peaks are matrix peaks by fragmentation.

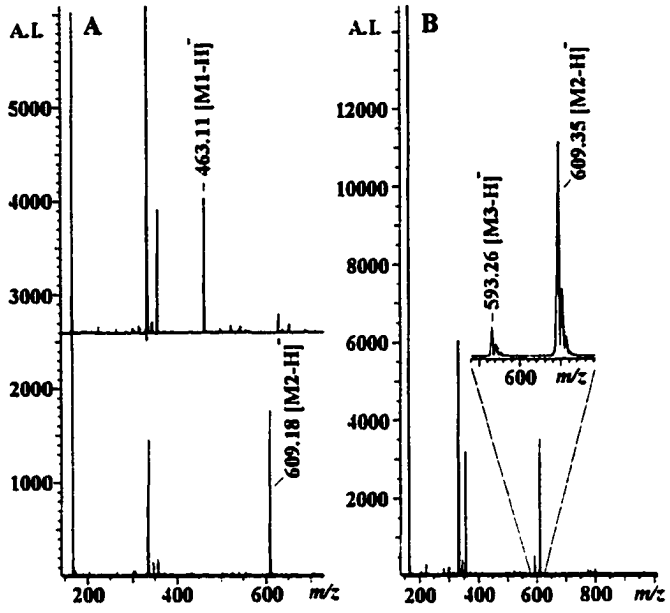

Figure 6-5. MALDI-MS negative ion spectra of flavonol glycoside standards. M1: isoquercitrin. M2: rutin. M3: kaempferol 3-rutinoside. A: the spectra from bottom to top are rutin (1.5×10^{-3} M in 70% methanol) and isoquercitrin (1.5×10^{-3} M in 70% methanol). B: a mixture of rutin (4.2×10^{-4} M in 0.01 M NaCl in 70% methanol) and kaempferol 3-rutinoside (4.2×10^{-4} M in 0.01 M NaCl in 70% methanol). Ions at masses less than 400 are from the THAP matrix.

Figure 6-2 shows that rutin fragmented and produced $[M-146+H]^+$ ions at *m/z* 465 with loss of the rhamnose residue, and $[M-146-162+H]^+$ ions at *m/z* 303 with loss of the rutinose residue (6-*O*-α-L-rhamnosyl-D-glucose). Isoquercitrin generated fragment ions $[M-162+H]^+$ at *m/z* 303 resulting from loss of the glucose residue. No noticeable negative mode fragment ions were observed (Figure 6-5). The ionization forms and fragmentation patterns of flavonol glycosides in MALDI-MS are similar to those observed in electrospray mass spectrometry, except for the low responses in the negative mode (Sägesser and Deinzer, 1996). The fragmentation patterns and ion forms of flavonol glycosides in MALDI-MS provide characteristic information for structural elucidation of flavonol glycosides, which is important for their identification.

The relative fragmentation of flavonol glycosides was examined in a quantitative manner (Table 6-1). Flavonol glycosides with a different aglycone, for example kaempferol or quercetin, were chosen to prepare the sample so that molecular or fragment

Table 6-1. Fragmentation and Responses of Flavonol Glycosides in MALDI-MS[a]

	A mixture of flavonol glycosides[b]	[Kae 3-Rut[c]-146+H]$^+$ /[Kae 3-Rut[c]+H]$^+$	[Rutin-146+H]$^+$ /[Rutin+H]$^+$	Ratios of fragment ions[e] over parent ions[f] % Kaempferol	Quercetin	Kaempferol/Quercetin	Response ratios of all ions Kaempferol/Quercetin
Data Column:		1	2	3	4	5	6
MALDI-MS	Kae 3-Rut[c]+Rutin	1.05 (0.24)	0.94 (0.14)	39.3 (14.7)	39.7 (11.6)	0.97 (0.08)	1.25 (0.08)
positive Mode	Kae 3-Glu[d]+Isoquercitrin			37.0 (8.0)	37.3 (9.6)	1.00 (0.07)	1.24 (0.06)
	Kae 3-Glu[d]+Rutin		0.96 (0.06)	29.5 (16.9)	29.3 (17.9)	1.03 (0.10)	1.02 (0.10)
	Kae 3-Rut[c]+Isoquercitrin	1.03 (0.15)		20.3 (7.4)	24.8 (9.8)	0.82 (0.10)	1.53 (0.05)
	Kae 3-Rut[c]	0.97 (0.14)		31.2 (14.8)			
	Rutin		1.01 (0.18)		16.4 (5.2)		
MALDI-MS	Kae 3-Glu[d]+Isoquercitrin						0.14 (0.02)
negative Mode	+Kae 3-Rut[c]+Rutin						0.17 (0.03)

[a] All data are means of five replicates and numbers in parentheses indicate the standard deviation of these five replicates (n=5).
[b] The concentration of individual flavonol glycoside standards was 1.5×10^{-3} M in methanol (stock solution). Any mixture of flavonol glycosides was made by mixing equal amounts (v/v) from stock solution. Final solution was made by mixing 70 µL flavonol standard solution and 30 µL 0.033 M NaCl. THAP as matrix
[c] Kaempferol 3-rutinoside.
[d] Kaempferol 3-glucoside.
[e] Ions from flavonol glycosides with loss of their carbohydrate residues.
[f] Total of unfragmented protonated and alkali adduct molecular ions

ions would not overlap. The fragmentation of individual flavonol glycosides was calculated based on all the fragment ions (loss of carbohydrates) to their parent ions (total of unfragmented protonated and alkali metal adduct ions) in terms of percentage observed in a single MALDI-MS sample or MALDI-MS spectrum (Table 6-1, data columns 3 and 4). The fragmentation of each flavonol glycoside varied widely from sample-to-sample as indicated by the large standard deviation (Table 6-1, data columns 3 and 4). However, within any single MALDI-MS sample, all the flavonol glycosides exhibited a similar fragmentation percentage. This similarity was shown by the ratios of fragment ions of kaempferol glycosides to those of quercetin glycosides, which were close to 1 (Table 6-1, data column 5). That is, for individual MALDI-MS spectra the ratios of fragment ions to parent ions were very consistent and similar for different flavonol glycosides. With further investigation on rutin, it was found that the formation of $[M-146+H]^+$ ions was linearly proportional to $[M+H]^+$ ions in an equal amount (slope = 0.95, $R^2 = 0.98$) for varying concentrations of rutin. This relationship was also evident since ratios of $[M-146+H]^+$ to $[M+H]^+$ were close to 1 even in a mixture of flavonol glycosides (Table 6-1, data columns 1 and 2). The above quantitative information about fragment ions is useful for proper evaluation of the amount of monoglycosides in a mixture, since fragment ions from the diglycosides, e.g. $[M-146+H]^+$ ions at *m/z* 465 from rutin (quercetin 3-rutinoside), would overlap the monoglycosides with the same aglycone, e.g. $[M+H]^+$ ions at *m/z* 465 of isoquercitrin (quercetin 3-glucoside). In general, it seems that fragmentation patterns and amounts are predictable for any defined sample preparation method.

Therefore, any flavonol glycoside MALDI-MS spectrum should provide both qualitative and quantitative information about the flavonol glycosides in a sample.

Responses of Flavonol Glycosides in MALDI-MS. Generally the intensity (total of all ions) or response of flavonol glycosides in MALDI-MS positive mode was linearly correlated to their molar ratios in the sample. This linearity (slope=1.24, R^2=0.98) was determined by choosing kaempferol 3-rutinoside (ranging from 8.4×10^{-5} M to 4.2×10^{-4} M) as an analyte while using rutin (quercetin 3-rutinoside, 4.2×10^{-4} M) as internal standard. Extensive studies showed that the responses of kaempferol and quercetin glycosides were all similar and the ratios of the intensity of kaempferol glycosides over quercetin glycosides were close to 1 (Table 6-1, data column 6). However, in MALDI-MS negative mode, kaempferol glycosides exhibited one fifth or less the responses of quercetin glycosides (Figure 6-5B; Table 6-1, data column 6).

In general, because MALDI-MS positive mode can generate abundant ions and the spectra provided more structural information, subsequent studies on flavonol glycosides in food samples were carried out in the positive mode.

MALDI-MS Identification of Flavonol Glycosides in Yellow Onion Extracts. Onions primarily contain two or three quercetin conjugates, quercetin 3,4'-diglucoside, the 4'-glucoside (Price et al., 1997) and isorhamnetin 4'-glucoside (Park and Lee, 1996). After HPLC separation, three major peaks, at 7.7, 25.9 and 32.1 min, were collected and analyzed by MALDI-MS. The characteristic ions, observed at *m/z* 465, 627, 649 and 665 ($[M-162+H]^+$, $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$, respectively), were assigned to quercetin 3,4'-diglucoside (HPLC peak at 7.7 min.), at *m/z* 465 and 487 ($[M+H]^+$ and $[M+Na]^+$, respectively) for 4'-glucoside (HPLC peak at 25.9 min), and at *m/z* 479 and 501 ($[M+H]^+$ and $[M+Na]^+$, respectively) for isorhamnetin 4'-glucoside (HPLC peak at 32.1 min) (Figure 6-6). The fragmentation patterns were as expected. The monoglycosides produced fragment ions at *m/z* 303 or 317 ($[M-162+H]^+$), and the diglucosides generated fragment ions at *m/z* 465 ($[M-162+H]^+$) and 303 ($[M-162-162+H]^+$). THAP also generated matrix ion peaks at *m/z* 303 and 287. The intensities of matrix ions were affected by the concentration of flavonol glycosides. High concentrations of flavonol glycosides suppressed the matrix ions in the 200 and 400 *m/z* region (Figure 6-2). Therefore, the

peak at *m/z* 303 (Figure 6-2) is definitely from the fragmentation of the flavonol glycosides. However, at lower flavonol glycoside concentrations, both flavonol glycoside and THAP fragmentation can contribute to ion peaks at *m/z* 303 and 287.

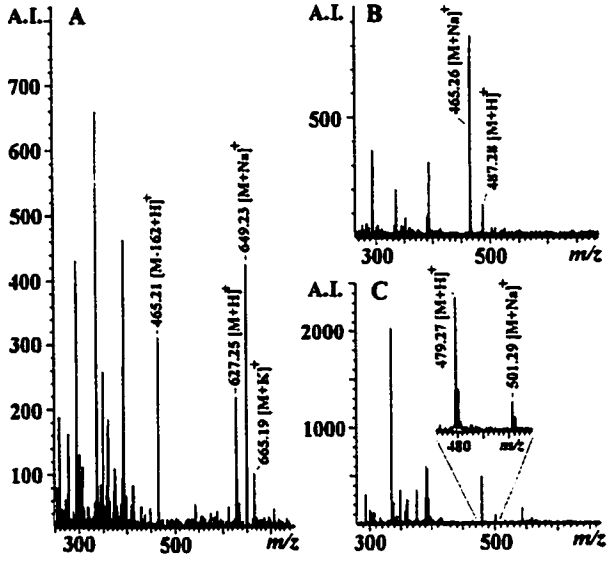

Figure 6-6. MALDI-MS positive ion spectra of flavonol glycosides (from yellow onion) after HPLC separation. A: quercetin 3,4'-diglucoside. B: quercetin 4'-glucoside. C: isorhamnetin 4'-glucoside.

MALDI-MS Identification of Flavonol Glycosides in Green Tea Extracts.

Flavonol glycosides present in green tea are kaempferol, quercetin and myricetin mono-, di-, and triglycosides. They are listed in Table 6-2 using the HPLC retention time order according to Bailey et al. (1990), Engelhardt et al. (1992) and McDowell et al. (1995). Figure 6-3 shows the MALDI-MS spectrum of flavonol glycosides in a green tea extract. Because flavonol glycosides were prepared with an excess of sodium ions (0.01 M NaCl in 70% methanol), the di- and triglycosides were predominately ionized in the form of sodium adduct ions, while monoglycosides were ionized as both protonated and sodium adduct ions. Since only very small amounts or no noticeable $[M+H]^+$ ions were observed for flavonol diglycoside (masses between 590 and 650) or triglycoside (masses between 740 and 820), the amounts of the fragment ions $[M\text{-glycoside}+H]^+$ at *m/z* 465 or 449 were assumed to be insignificant. The MALDI-MS profiles in the monoglycoside region

Table 6-2. MALDI-MS Identification of Flavonol Glycosides in Green Tea

Flavonol glycosides[a]	Theoretical mass	Mass of MALDI-MS detected ions after HPLC separation						
		$[M+H]^+$	$[M+Na]^+$	$[M+K]^+$	$[M-146+H]^+$	$[M-162+H]^+$	$[M-308+H]^+$	$[M-470+H]^+$
Myricetin 3-rhamnosylglucoside	626							
Myricetin 3-galactoside	480	481	503			319		
Myricetin 3-glucoside[b]	480	481	503			319		
Quercetin 3-glucosylrhamnosylgalactoside[c]	772	773	795	811			465	303[d]
Quercetin 3-glucosylrhamnosylglucoside	772	773	795	811			465	303[d]
Kaempferol 3-glucosylrhamnosylgalactoside	756	757	779	795			449	287[d]
Quercetin 3-rutinoside	610	611	633		465			303[d]
Quercetin 3-galactoside	464	465	487			303[d]		
Quercetin 3-glucoside	464	465	487			303[d]		
Kaempferol 3-glucosylrhamnosylglucoside	756	757	779	795			449	303[d]
Kaempferol 3-galactoside	448	449	471			287[d]		
Kaempferol 3-rhamnosylglucoside	594							
Quercetin 3-rhamnoside	448							
Kaempferol 3-glucoside	448	449	471			287[d]		

[a] Based on the HPLC retention time order
[b] Shown in Figure 6A
[c] Shown in Figure 6B
[d] THAP also produced fragment ions at *m/z* 287 and 303

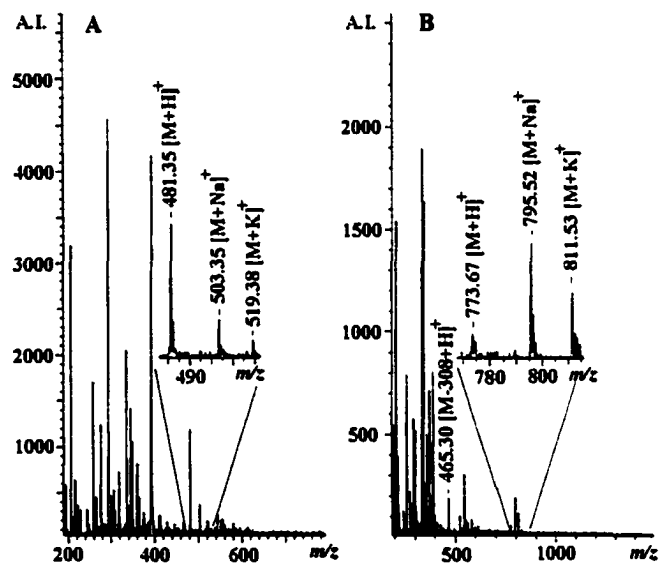

Figure 6-7. MALDI-TOF MS positive ion spectra of flavonol glycosides (from green tea) after HPLC separation. A: myricetin 3-glucoside. B: quercetin 3-glucosylrhamnosylgalactoside.

(masses between 400 and 500) should represent the monoglycosides in the original sample. The masses of flavonol glycosides were determined in eleven HPLC fractions by MALDI-MS (two of them given in Figure 6-7). The MALDI-MS spectra provided the characteristic molecular or fragment ions for all these HPLC fractions, which were then assigned as flavonol glycosides in green tea (Table 6-2). However, flavonol triglycosides, such as quercetin 3-glucosylrhamnosylgalactoside, did not exhibit fragment ions [M-

162+H]$^+$ at *m/z* 611 with loss of the glucose residue. This may be because of the low concentration of the triglycoside in the HPLC fraction or the easy cleavage of glycosidic linkage between rhamnose and galactose, since mass at *m/z* 465 (loss of glucosylrhamnose residue) was observed (Figure 6-7B).

In conclusion, MALDI-MS is a valuable rapid technique for identification of flavonol glycosides, even in complex mixtures. The fragment ions of flavonol glycosides provided characteristic information for structural elucidation of flavonol glycosides. Fragmentation and amounts of flavonol glycosides were predictable in a MALDI-MS sample. MALDI-MS spectra, therefore, could provide the flavonol glycoside profiles for food samples and also be used to identify flavonol glycosides in conjunction with other separation techniques such as HPLC. It is likely that MALDI has the potential to analyze flavonol glycosides in other foods and that these spectral ion patterns could be used for identification and quality control.

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Chapter 7

MALDI-MS Quantification of Coccidiostats in Poultry Feeds[1]

INTRODUCTION

Lasalocid, monensin, salinomycin and narasin (Figure 7-1) are polyether ionophores and serve as the four major coccidiostats for prevention of coccidiosis in the poultry industry (Elliott et al., 1998). Their mode of action is attributed to their ionophoric properties (forming complexes with polar cations, i.e. K^+, Na^+, Ca^{++}, and Mg^{++}) and effects on cell membrane function (Pressman and Fahim, 1982; Braunius, 1985). Typical fortification levels of feeds range from 20 to 125 mg/kg, depending on the species treated (Braunius, 1985; Rodewald et al., 1994; Moran et al., 1994; Muldoon et al., 1995; Blanchflower and Kennedy, 1995; Elliot et al., 1998). Toxicity studies have shown that coccidiostats exert marked cardiovascular effects in experimental animals (Novilla, 1992; Novilla and Owen, 1994; Elliott et al., 1998), and might poison poultry or other animals (Rollinson et al., 1987; Salles et al., 1994; Andreasen and Schleifer, 1995). Although withdrawal periods from medicated feeds are required prior to slaughter, there is still concern over possible toxicological problems for humans consuming poultry meat. Therefore reliable analytical techniques are required for these compounds.

Several analytical techniques for the identity and quantification of coccidiostats were reviewed by Elliot et al. (1998). Of chemical methods for detection and quantification of coccidiostats, high performance liquid chromatography (HPLC) with post-column derivatization (PCD) with vanillin (Rodewald et al., 1994; Moran et al., 1994, 1995) was the most common technique used. HPLC-PCD, with either a liquid-liquid or a silica gel solid-phase extraction, had a limit of detection of under 5 ng/g (Moran et al., 1994, 1995). HPLC electrospray mass spectrometry (MS) was another sensitive technique and offered the best possible confirmation of low levels of

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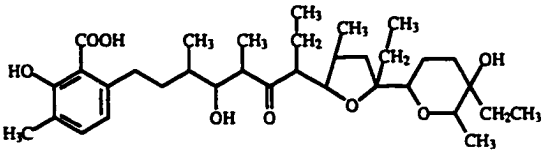
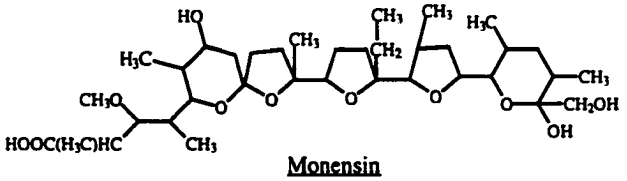
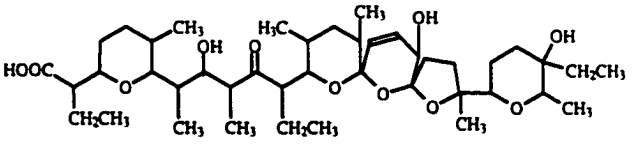


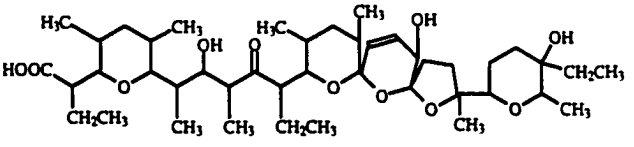
Lasalocid



Monensin



Salinomycin



Narasin

Figure 7-1. Structures of coccidiostats.

coccidiostats after a simple clean-up procedure (Blanchflower and Kennedy, 1995, 1996; Harris et al., 1998). The detection limit of this assay was 1 ng/g (Blanchflower and Kennedy, 1995, 1996).

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-MS) was first introduced in 1987 and originally developed for large biomolecules (Karas et al., 1987). The advantages MALDI-MS has over other methodologies include speed of analysis, high sensitivity, wide applicability combined with a good tolerance towards contaminants, and the ability to analyze complex mixtures (Karas, 1996). The performance of MALDI-MS allows for analysis of most molecules, except that simple linear instruments can not tell the difference between isomers, which

have identical mass. Presently, MALDI-MS food related applications are limited. The objective of this study was to explore and develop a simple fast protocol using MALDI-MS to quantify the commonly used coccidiostats in poultry feeds.

MATERIALS AND METHODS

Materials and Reagents. Narasin (70 mg/kg) or salinomycin (60 mg/kg) medicated poultry feeds were obtained from Unifeed Grain Growers Ltd. (Okotoks, Alberta). Blank poultry feeds were from the Poultry Center, University of Alberta (Edmonton, Alberta). Poultry feed samples were acquired under the coordination of Dr. Frank Robinson of the Department of Agricultural, Food and Nutritional Science, University of Alberta. Lasalocid (sodium salt), monensin (sodium salt), salinomycin, narasin, maltotriose and maltotetraose were purchased from Sigma Chem. Co. (St. Louis, MO). 2',4',6'-Trihydroxyacetophenone monohydrate (THAP), 3-aminoquinoline (3-AQ), and 2,5-dihydroxybenzoic acid (DHB) were obtained from Aldrich Chem. Co. (Milwaukee, WI). All water used was double deionized (Milli-Q water purification system, Millipore Corp., Bedford, MA). Individual stock solutions, lasalocid (1.34×10^{-4} M in methanol), monensin (1.16×10^{-4} M in acetonitrile), salinomycin (1.28×10^{-4} M in acetonitrile), and narasin (1.28×10^{-4} M in acetonitrile), were prepared and stored at 4 C until used.

Extraction of Coccidiostats from Poultry Feeds. The feed samples were pulverized using a coffee grinder (Braun Inc., Woburn, MA) to obtain a homogeneous powder. Ground feed (1 g) was added to 10 mL of acetonitrile containing the internal standard monensin (1.45×10^{-5} M). The mixture was shaken with a horizontal shaker for 1 hr., and then filtered through Whatman No. 4 filter paper. Filtrate (2 mL) was loaded onto a Sep-pak C18 cartridge (Waters Corp., Milford, MA) at a flow rate of about 1 mL/min. Filtrate from the Sep-pak C18 cartridge was collected in a 10 mL volumetric flask. A further 4 mL of methanol/water (94:6) was used to elute the retained coccidiostats on the Sep-pak C18 cartridge at the same flow rate. This eluent was also collected in the 10 mL volumetric flask. The volume was then made up to 10 mL with

methanol/water (94:6). This partially purified coccidiostat extract was kept at 4 C for MALDI-MS analysis.

MALDI-MS. MALDI-MS was performed using a Proflex™ III in linear positive mode (Bruker Analytical Systems Inc., Billerica, MA). Coccidiostats co-crystallized with matrices on the probe were ionized by a nitrogen laser pulse (337 nm) and accelerated under 20 kV with time-delayed extraction before entering the time-of-flight mass spectrometer. When THAP and 3-AQ were used, the preparation of matrix and sample was the same as previously reported (Chapter 2, Wang et al., 1999). When DHB was used, two types of matrix solutions were made. One contained 10-15 mg/mL DHB in 10% ethanol and the other 10-15 mg/mL DHB in 0.01 M NaCl in10% ethanol. The ratio of matrix solution and sample was 1:1. For all matrices either 1 μL sample solution or 1.5 μL mixture of matrix and sample was applied to a probe. The MALDI-MS sample was air dried using a fan for 5 min. Laser strength was attenuated (the lower the attenuation, the higher the laser strength) to obtain the best signal-to-noise ratio and isotopic resolution. MALDI-MS was calibrated with two-point external calibration using $[\text{maltotriose} + K]^+$ (exact isotopic mass = 543.13) and $[\text{maltotetraose} + K]^+$ (exact isotopic mass = 705.19) as calibrants, resulting in a mass accuracy ≤ 500 ppm. Any one spectrum or one MALDI-MS run was acquired using 40 laser pulses at each of three randomly chosen spots per MALDI-MS sample, for a total of 120 laser pulses, which took about 2 min. The major isotopic ^{12}C peak height or intensity was used for quantification.

Statistics. Linear regressions, means and relative standard deviations (RSD) were analyzed using Microsoft Excel 97 (Microsoft Office 97).

RESULTS AND DISCUSSION

It is possible to use MALDI-MS to analyze most compounds in a sample after the analytes of interest have been extracted from a sample matrix. Key concerns include the selection of matrices, preparation of matrix and sample, signal-to-noise ratio, spot-to-spot repeatability and response factors. Because of spot-to-spot variation, an internal standard is a requirement for quantification of analytes using MALDI-MS.

Selection of Matrices and Internal Standard. Based on our previous research, we chose three common matrices, 2',4',6'-trihydroxyacetophenone monohydrate (THAP), 3-aminoquinoline (3-AQ), and 2,5-dihydroxybenzoic acid (DHB) to examine their applicability for desorption and ionization of coccidiostats using MALDI-MS. All three matrices produced good quality spectra for coccidiostats (Figure 7-2). Laser

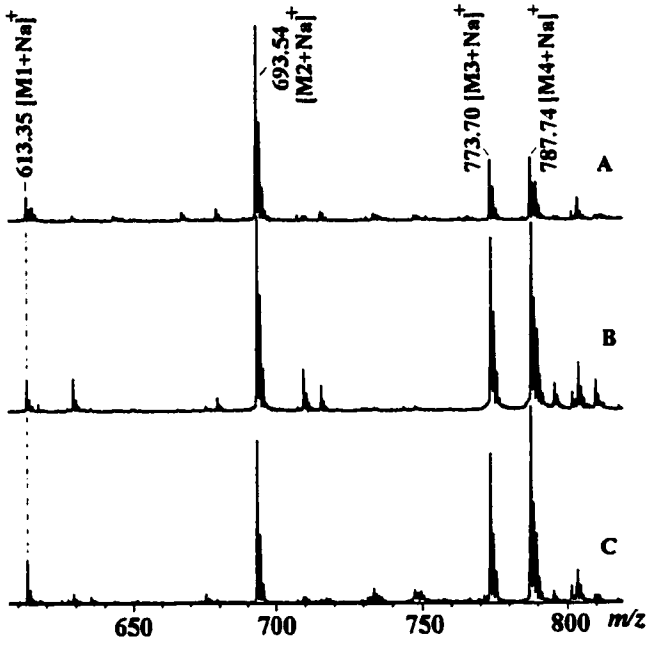


Figure 7-2. Performance of different matrices for coccidiostat standards in MALDI-MS. A: THAP. B: 3-AQ. C: DHB. A mixture of lasalocid (M1), monensin (M2), salinomycin (M3) and narasin (M4) was made by mixing equal amounts of stock solution (v/v). Note that only predominant sodium adduct peaks are labeled, while potassium adduct peaks (with 39 mass shift) and double sodium adduct peaks (loss of one proton, with 45 mass shift) are not labeled.

strength used for desorption and ionization of coccidiostats was matrix related. 3-AQ required higher laser strength than THAP and DHB. Laser strength was selected based on good signal-to-noise ratio and the best resolution of analytes of interest (Chapter 2, Wang et al., 1999). In all experiments, isotopic resolution was achieved for all matrices. Compared to 3-AQ, THAP and DHB are good matrices for coccidiostats since they produced good quality spectra with a relatively low laser strength. The response or peak intensity was another factor used to determine the best matrix. No matter which matrix was used, lasalocid exhibited only one fifth or less the response of monensin under

similar molar concentrations. Therefore, lasalocid was not the best internal standard to be selected for other coccidiostats in MALDI-MS quantification. When THAP was used as matrix (Figure 7-2A), salinomycin and narasin showed one third the response of monensin. In 3-AQ (Figure 7-2B) or DHB (Figure 7-2C), monensin, salinomycin and narasin displayed similar responses in MALDI-MS. Since DHB gave reasonable peak intensities for monensin, salinomycin and narasin under lower laser strength with good spot-to-spot repeatability and high sensitivity, it was selected as the best matrix for coccidiostats. All further research was carried out using DHB. Monensin, salinomycin or narasin could serve as internal standards for each other using DHB.

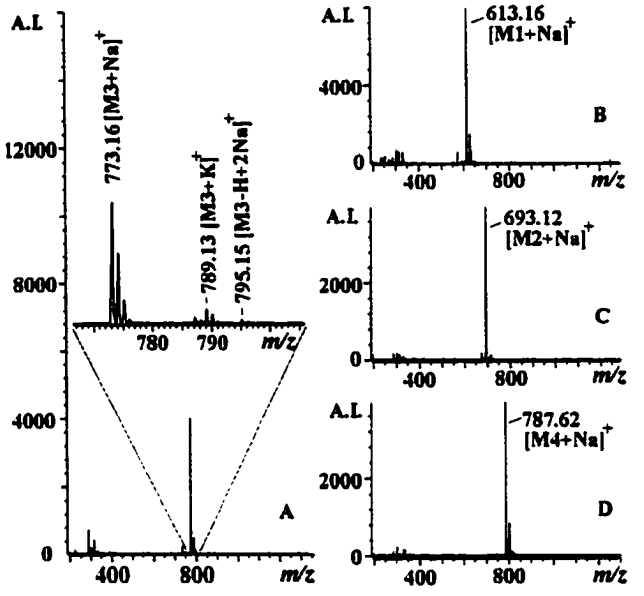


Figure 7-3. Ionization of coccidiostat standards in MALDI-MS positive mode with DHB as matrix. A: salinomycin (M3) (3.20×10^{-5} M). B: lasalocid (M1) (1.34×10^{-4} M). C: monensin (M2) (2.87×10^{-5} M). D: narasin (M4) (3.20×10^{-5} M). Detailed ionization for salinomycin is labeled in A. For lasalocid, monensin and narasin, only sodium adduct peaks are labeled.

Ionization and Linear Responses. In MALDI-MS, salinomycin (Figure 7-3A), lasalocid (Figure 7-3B), monensin (Figure 7-3C) and narasin (Figure 7-3D) were predominately ionized as $[M+Na]^+$ ions, with additional small amounts of $[M+K]^+$ and $[M-H+2Na]^+$ ions (Figure 7-3A). When an excess of sodium (0.01 M NaCl in DHB matrix solution) was applied to the preparation of MALDI-MS samples, only sodium adduct ions, $[M+Na]^+$ and $[M-H+2Na]^+$, were observed. For quantification, peak heights

of $[M+Na]^+$ and $[M-H+2Na]^+$ were summed if $[M-H+2Na]^+$ was observed in a spectrum. There were no observed fragmentation or fragment ions for coccidiostats in MALDI-MS. However, in HPLC electrospray MS, they do fragment, for example, salinomycin formed fragment ions at *m/z* 225 and 373 (Harris et al., 1998).

Coccidiostats, for example monensin or narasin, contain molecular variations known as coccidiostat factors. Some minor factors are often with 14 mass lower or higher, occasionally 2 mass lower, than the major coccidiostats (Coleman et al., 1994; Rodewald et al., 1994). MALDI-MS could easily detect all factors when the concentration of the total coccidiostats was high enough, e.g., in order of $\times 10^{-5}$ M. However, as expected, peak intensities from these minor factors were very low and 5% or in most cases much less than the peak height of the major coccidiostats. In this paper, MALDI-TOF MS quantification of coccidiostats focused on the major coccidiostats. For example for narasin, only narasin factor A was determined, and for monensin, only monensin factor A was studied. These minor factors account for the slightly reduced purity of standards noted in the Materials and Reagents section.

Since monensin, salinomycin and narasin showed similar responses (Figure 7-2C), and the sample to be analyzed contained salinomycin or narasin, monensin was selected as an internal standard for quantification. Generally, peak intensity (total of all ions) or response of coccidiostats in MALDI-MS was linearly correlated to their molar ratios in a MALDI-MS sample. The linearity was separately determined for salinomycin (slope=0.82, R^2=0.98) and narasin (slope=1.15, R^2=0.97) in a range from 3.20×10^{-7} M (0.24 μg/mL) to 3.20×10^{-6} M (2.40 μg/mL) using monensin as an internal standard (2.87×10^{-6} M). The slopes were used as response factors to quantify salinomycin or narasin in poultry feeds and spiked samples.

Stability of Coccidiostats in MALDI-MS Samples. Among the four coccidiostats, salinomycin and narasin tended to degrade in aqueous solutions. Davis et al. (1999) indicated that some coccidiostats, particularly salinomycin and narasin, were unstable in water/methanol (98:2) solution. However, no significant degradation was observed in pure acetonitrile or methanol for these compounds. When analyzed using MALDI-MS, we found the preparation method of matrix and sample could result in the degradation of salinomycin and narasin. This might have occurred because the mixture of

matrix (DHB) solution and coccidiostats was acidic, which could have caused salinomycin and narasin to dissociate. The stability of salinomycin and narasin was studied in two ways to determine a method to prepare stable MALDI-MS coccidiostat samples for quantification. The first experiment was performed by applying the mixture to the probe at 0, 30, 60 and 120 min. intervals after coccidiostats had been mixed with matrix solution before acquiring MALDI-MS data. The second experiment involved applying the mixture to the probe immediately after the coccidiostats had been mixed with matrix solution, followed by acquiring MALDI-MS data at 0, 30, 60 and 120 min. intervals. (Note that it took 5 min. for the mixture to dry on the probe.) Figure 7-4 shows the spectra of the four coccidiostats under these conditions. Peak intensities of lasalocid and monensin (Figure 7-4A and B) remained constant, which indicated they were stable under either condition. However, peak intensities of salinomycin and narasin (Figure 7-4A) decreased significantly at each interval under the condition of the first experiment.

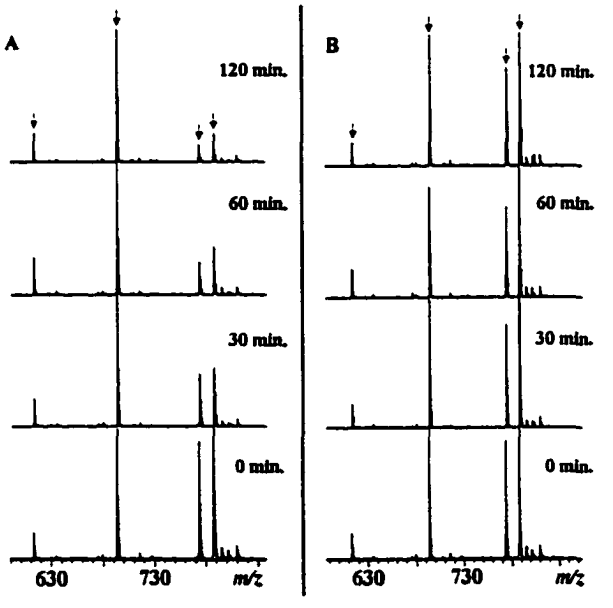


Figure 7-4. Stability of coccidiostats in MALDI-MS samples. A: spectra of coccidiostats by applying the mixture to probe at different intervals (0, 30, 60, 120 min.) after coccidiostats had been mixed with matrix solution and followed directly by acquisition of MALDI-TOF data. B: spectra of coccidiostats applied to the probe and dried immediately after coccidiostats had been mixed with matrix solution, followed by acquisition of MALDI-TOF data at different intervals (0, 30, 60, 120 min.). A mixture of lasalocid, monensin, salinomycin and narasin was made by mixing equal amounts of stock solutions (v/v). Arrows, from left to right, indicate lasalocid, monensin, salinomycin and narasin sodium adduct peaks.

An attempt was made to study details of the degradation since several peaks at masses between 490 and 590 were generated as the peak intensities of salinomycin and narasin decreased (spectra not shown). However, the degradation of salinomycin and narasin was complex and peaks between *m/z* 490 and *m/z* 590 tended to degrade further with increased time. Nevertheless, if salinomycin and narasin were dried on the probe immediately after mixing with matrix, the peak intensities (Figure 7-4B) stayed constant. All the linear responses and quantification of coccidiostats in this study were carried out in this manner with 0.01 M NaCl in the matrix solution. It also seemed that salinomycin and narasin were slightly more stable with addition of the sodium salt (data not shown).

MALDI-MS Quantification of Coccidiostats. One of the advantages of MALDI-MS is its tolerance of contaminants. Crude sample extracts can be applied to MALDI-MS directly without purification, and the analytes of interest can still be determined (Abell and Sporns, 1996; Chapter 2, Wang et al., 1999; Driedger and Sporns, 1999). Coccidiostats (Figure 7-5A3 and B3) in crude extracts or acetonitrile extracts were identified. The extracts also contained a group of unidentified compounds (at *m/z* 758, 780, 782 and 804; Figure 7-5A1, A3, B1 and B3). These compounds were close enough in mass to interfere with the detection of salinomycin (Figure 7-5A3) or narasin (Figure 7-5B3), or possibly suppress the coccidiostat molecular ions. Therefore, a simple fast protocol was developed to remove interfering compounds using a Sep-pak C18 cartridge. After crude extracts were loaded on the cartridge, interfering compounds were retained on the cartridge. Some coccidiostats passed through the cartridge and some were also retained. With elution of an additional 4 mL of methanol/water (94:6), all coccidiostats were eluted from the cartridge. Because of this selective elution, the use of other C18 cartridges could required a modified elution protocol. After the cartridge purification procedure, coccidiostats and interfering compounds were separated, and MALDI-MS could generate clean spectra for coccidiostats of poultry feed extracts (Figure 7-5A2 and B2). The interfering compounds, retained on the cartridge, were eluted with chloroform/methanol (95:5) and the spectra are shown in Figure 7-5A1 and B1. The recovery of the purification procedure for coccidiostats was studied with standards. A 2 mL mixture of coccidiostats (monensin = 2.1×10^{-5} M, salinomycin = 2.1×10^{-5} M, and narasin = 2.1×10^{-5} M) was loaded on the cartridge and carried through the entire

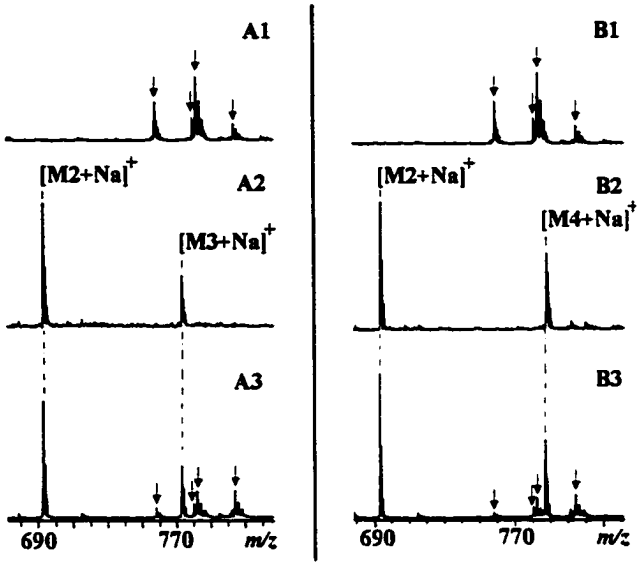


Figure 7-5. MALDI-MS spectra of coccidiostats from poultry feeds. A1 and B1: interfering compounds eluted with chloroform/methanol (95:5). Arrows, from left to right, indicate interfering compounds at *m/z* 758, 780, 782, and 804 respectively. A2 (salinomycin [M3]) and B2 (narasin [M4]): purified coccidiostats from poultry feed samples (using Sep-pak C18 cartridge purification procedure). M2: internal standard monensin. A3: salinomycin medicated poultry feed crude extracts. B3: narasin medicated poultry feed crude extracts.

procedure. After 3 mL of methanol/water (94:6) washing, no coccidiostat standards could be detected in the eluent by MALDI-MS. Because the concentration of coccidiostats in sample extracts was lower than that of the above standard mixture, it was assumed that all sample coccidiostats were recovered since the cartridge could not be overloaded.

All poultry feed samples and spiked samples were analyzed using the developed method and the results are presented in Table 7-1. Monensin (1.45×10^{-5} M) was used as an internal standard and added to the extraction solvent (acetonitrile). Medicated poultry feed contained 60 μg/g salinomycin or 70 μg/g narasin, and the MALDI-MS assay for salinomycin or narasin in medicated samples found 60.3 μg/g and 66.0 μg/g respectively (Table 7-1). The validation of the method was further confirmed by spiking experiments. Blank poultry feed was spiked at two concentrations and medicated samples were spiked at one concentration of salinomycin or narasin (Table 7-1). Recovery for salinomycin ranged from 99 to 102%, and recovery for narasin was from 92 to 96%. All these data

Table 7-1. MALDI-MS Quantification of Coccidiostats in Poultry Feeds

salinomycin assay				
sample	spike level (μg/g)	assay[a] (μg/g)	RSD[b] (%)	recovery (%)
blank sample	58.7	59.1	4.1	101
	39.2	40.0	5.0	102
medicated sample[c]	0.0	60.3	3.9	101
	23.7	83.4	7.0	99
narasin assay				
sample	spike level (μg/g)	assay[a] (μg/g)	RSD[b] (%)	recovery (%)
blank sample	56.8	52.3	2.6	92
	37.8	36.4	2.6	96
medicated sample[d]	0.0	66.0	4.3	94
	24.5	88.4	4.8	93

[a] mean of three extracts (n=3); each extract value was determined using three MALDI-MS runs, or a total of 3 x 3 or 9 total MALDI-MS runs.
[b] relative standard deviation of three extract values.
[c] contain salinomycin 60 μg/g.
[d] contain narasin 70 μg/g.

indicate that MALDI-MS coupled with Sep-pak C18 cartridge purification procedure can be used to accurately determine coccidiostat levels in poultry feeds.

The MALDI-MS limit of detection (S/N≥3) for standard lasalocid, monensin, salinomycin or narasin was 251 fmole (0.205 μg/mL), 22 fmole (0.020 μg/mL), 24 fmole (0.024 μg/mL), and 24 fmole (0.025 μg/mL), respectively. The low limit of detection indicates that MALDI-MS is a very sensitive technique for identification of coccidiostats. The method detection limit (S/N≥3) in this study for salinomycin and narasin was 2.4 μg/g or 2.4 μg/10 mL in the extraction solvent. This limit of detection was about the same level (2.5 μg/g) as HPLC-PCD with vanillin for poultry feeds (Rodewald et al., 1994). However, as stated earlier, the method detection limit could be 5 ng/g or 1 ng/g if coccidiostats extract were purified and concentrated before HPLC-PCD (Moran et al., 1994,1995) or HPLC electrospray MS analyses (Blanchflower and Kennedy, 1995, 1996). Of note, for both HPLC-PCD and HPLC electrospray MS, was the concentration of working standards in a range of about 0.10 to 1.0 μg/mL, which is about the same level as used in our study. Theoretically, the detection limit reported in this study could

also be improved by concentrating sample extracts and/or increasing sample size in the extraction procedure.

In conclusion, MALDI-MS is a simple technique to identify and quantify coccidiostats without derivatization, or without HPLC separation before use of a mass spectrometer. MALDI-MS can serve as a valuable rapid and sensitive technique for quantification of coccidiostats in poultry feeds with minimum sample purification. It is also likely that MALDI-MS has the potential to analyze coccidiostats in other complex sample matrices and to be used as an alternative to HPLC-PCD and HPLC electrospray mass spectrometry.

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Chapter 8

General Discussion and Conclusions

The introduction of MALDI-MS in the latter part of the 1980's has brought tremendous change and progress in the analyses of non-volatile large and/or thermally labile molecules (Karas et al., 1985, 1987; Karas and Hillenkamp, 1988; Tanaka et al., 1988). The applications of MALDI-MS in food science have exhibited advantages over other techniques, including speed of analysis, high sensitivity, wide mass range, mass determination of unfragmented molecules, tolerance to contaminants, and ability to analyze complex mixtures. This thesis explored the applicability of MALDI-MS for analyses of fructooligosaccharides, debranched starch, anthocyanins, flavonol glycosides, and coccidiostats, using an internal standard or standard addition.

MALDI-MS of Oligosaccharides. High performance anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) and MALDI-MS are currently the two most powerful techniques for analysis of oligosaccharides. However, the sensitivity and resolution of HPAEC-PAD decrease with increasing degree of polymerization (DP), therefore it does not provide accurate quantitative profiles for oligosaccharides (Timmermans et al., 1994; Wong and Jane, 1997; Stahl et al., 1997; Kazmaier et al., 1998). When analyzed by MALDI-MS, fructooligosaccharides showed similar responses on a molar basis (THAP as matrix, Chapter 2, Wang et al., 1999a) and maltooligosaccharides exhibited similar responses on a weight basis (DHB as matrix, Chapter 3, Wang et al., 1999b). Consequently, MALDI-MS provided accurate quantitative profiles for fructooligosaccharides or maltooligosaccharides in food samples. For higher DP, Mohr et al. (1995) showed the potential of using MALDI-MS to resolve carbohydrates (dextran 40 kDa) of DP 100 or more. MALDI-MS, so far, is the simplest, fastest and most accurate method to analyze fructooligosaccharides in foods such as onions, shallots, and garlic, and maltooligosaccharides from debranched starch. The sensitivity and accuracy of MALDI-MS for oligosaccharides open up opportunities for further understanding the structure of some very large molecular carbohydrates, such as β-glucans or branching patterns of amylopectin in various crops. Furthermore, since it

can determine molecular weight changes, MALDI-MS should have a great impact on the study of modified starch products.

MALDI-MS Quantification of Anthocyanins. Anthocyanins are a group of important antioxidants in food. Since under acidic conditions they are predominately in the aromatic oxonium ion form, anthocyanins easily ionize to form molecular weight cations (M^+) and occasionally potassium adducts in MALDI-MS (Chapter 4, Wang and Sporns, 1999; Chapter 5). Traditional HPLC has been widely employed for quantification of anthocyanins. The required gradient elution with HPLC has an effect on anthocyanin visible absorbance characteristics due to changes in solvent composition. Therefore, proper quantification can be very difficult. In MALDI-MS, a group of chemically similar anthocyanins, such as the monoglycosides, exhibited similar responses, which allowed for more accurate quantification with proper standard addition methods. Anthocyanins fragmented in the MALDI-MS ion source with the loss of carbohydrate residues and the monoglycoside anthocyanins fragmented in a predictable manner (Chapter 5). In any single MALDI-MS sample, all anthocyanins exhibited a similar percentage fragmentation (Chapter 5, Table 5-1). In fact, an internal standard, such as cyanidin 3-glucoside, will fragment with the same pattern or relative amount as all other anthocyanins, and therefore can be used as a reference for quantification. Further quantitative fragmentation studies on di- or triglycosides or a mixture of mono-, di-, and triglycoside anthocyanins will be necessary in order to use MALDI-MS to quantify these anthocyanins in food. MALDI-MS was rapid and accurate in identification and quantification of anthocyanins with different masses. Reliable anthocyanin profiles of food samples could be obtained in a few minutes. The methodology developed should be very useful for monitoring anthocyanin changes due to breeding, storage, or processing of fruits.

MALDI-MS Analysis of Flavonol Glycosides. The reason for MALDI-MS tolerance to contaminants may be the selectivity or preference of a matrix towards ionizing a particular analyte. Five common matrices, e.g. 4-hydroxy-α-cyano-cinnamic acid (HCCA), 2',4',6'-trihydroxyacetophenone (THAP), 2-(4-hydroxyphenylazo)-benzoic acid (HABA), *trans*-3-indoleacrylic acid (IDA), and 2,5-dihydroxybenzoic acid (DHB), could all produce good quality spectra in the MALDI-MS positive mode for flavonol glycoside standards. THAP and IDA were also good matrices for producing

negative ions with flavonol glycosides. However, only THAP worked for crude flavonol glycoside extracts from tea. Hence, selection of the proper matrix can be a key consideration for MALDI-MS analysis of analytes in food extracts. The flavonol glycoside ion responses in MALDI-MS positive and negative modes were different. The negative mode for all flavonol glycosides resulted in one $[M-H]^-$ ion peak, without detectable fragmentation. In the positive mode, multiple ion forms, along with further fragmentation through loss of glycosidic residues, provided more structural information for flavonol glycosides. Flavonol glycosides showed similar intensities or responses in the positive mode, while kaempferol glycosides exhibited much less response than quercetin glycosides in the negative mode. Although flavonol glycosides fragmented in the ion source, the mono- and di-glycosides fragmented in the same ratio and the amount of fragmentation could be determined with a defined MALDI-MS sample preparation (Chapter 6, Wang and Sporns, 2000). Therefore, any flavonol glycoside MALDI-MS spectrum was able to provide both qualitative and quantitative information on the flavonol glycosides in food samples.

The applications of MALDI-MS for anthocyanins and flavonol glycosides allow it to serve as a powerful tool to provide an authentic "fingerprint" for foods. This fingerprint can be used to determine differences in fruit varieties and maturity as well as changes due to storage or processing of fruits. It can also be used for quality control in functional foods or various herb extracts, for example *Ginkgo* extracts, which mainly contain flavonol glycosides (Mauri et al., 1999). Further research can extend the application of MALDI-MS to the entire flavonoid family (isoflavones in soy or soy products) or compounds with weak chromophores (terpenoids in *Ginkgo*).

MALDI-MS Quantification of Coccidiostats. Lasalocid, monensin, salinomycin and narasin have ionophoric properties (Pressman and Fahim, 1982; Braunius, 1985), which likely contribute to the ease of formation of coccidiostat alkali adduct ions in MALDI-MS and the resulting high sensitivity. The MALDI-MS limit of detection for lasalocid, monensin, salinomycin or narasin standards was 251, 22, 24 and 24 fmole, respectively. Proper matrix selection, which was DHB in this case, once again indicated its importance for proper MALDI-MS quantification. Monensin, salinomycin or narasin could serve as internal standards for each other when DHB was used as the matrix.

However, the preparation method of matrix and sample could result in the degradation of analytes, such as was seen with salinomycin and narasin, due to the acidic solvent environment produced by the high concentration of the DHB matrix. However, salinomycin and narasin were stable when dried on the MALDI-MS probe after mixing with matrix solution. Since a matrix mainly serves to ionize and desorb analytes in the MALDI-MS, it may be prudent that samples always be rapidly dried on the probe after the sample and matrix have been mixed.

Further Improvement. Most MALDI-MS commercial instruments have been designed for qualitative purposes instead of for quantification. The numbers of laser shots and desorption locations were quite often arbitrarily set, and peak heights or intensities were usually used for quantification. This may add error and lengthen the analysis. With the progress in instrumentation design, a fully automatic analysis system will likely improve MALDI-MS analytical speed, accuracy and precision. Currently, some automated machines are commercially available. For example, Bruker's Reflex™ III and Biflex™ III MALDI-MS systems have an automated sample and data acquiring system, and 1,536 samples can be loaded on a single target. An automated MALDI-MS system allows MALDI-MS to serve not only as a powerful tool for peptide or nucleotide sequencing or oligosaccharide structural studies but also as a routine rapid analytical technique for many food analyses.

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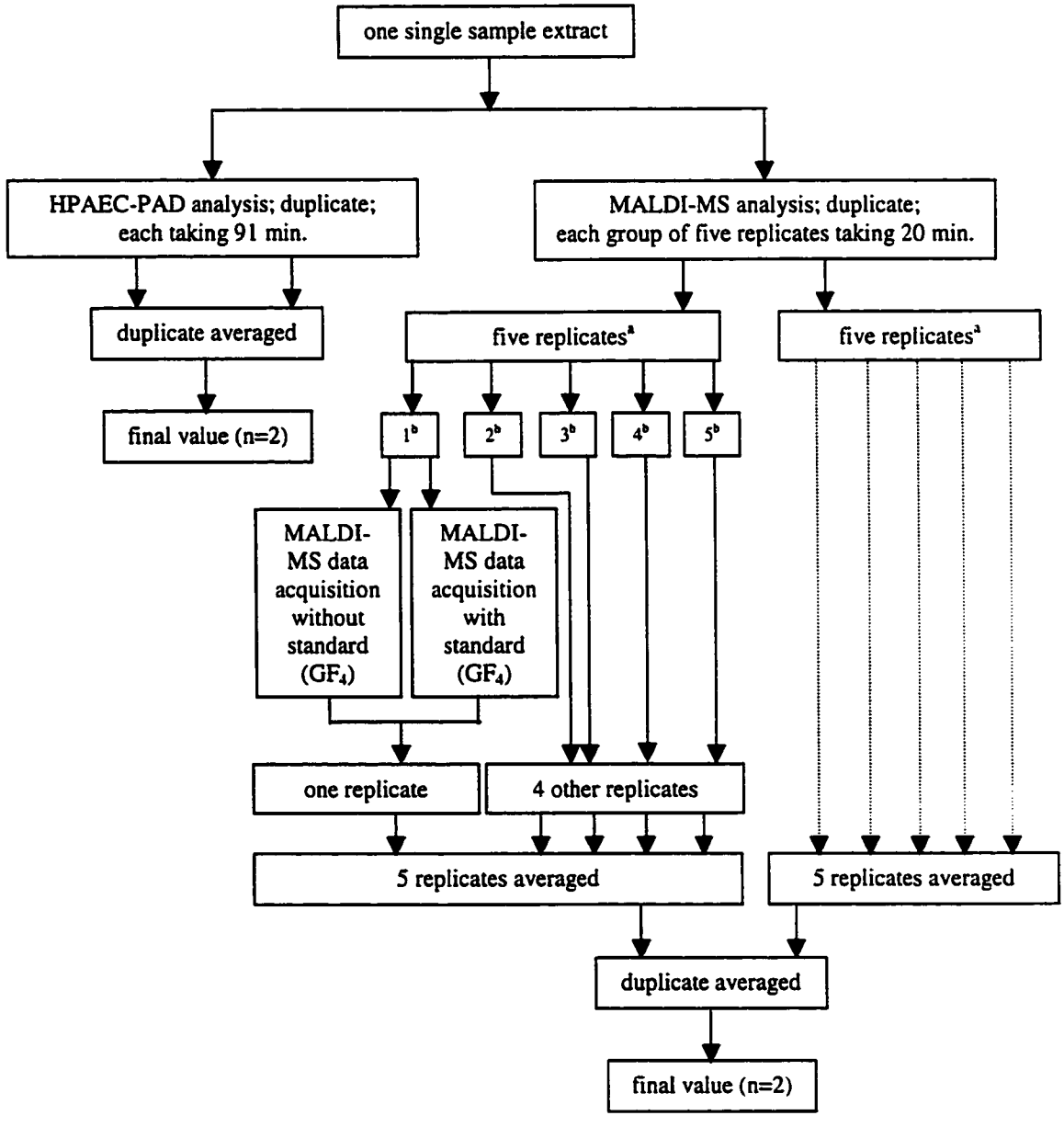
Appendix I

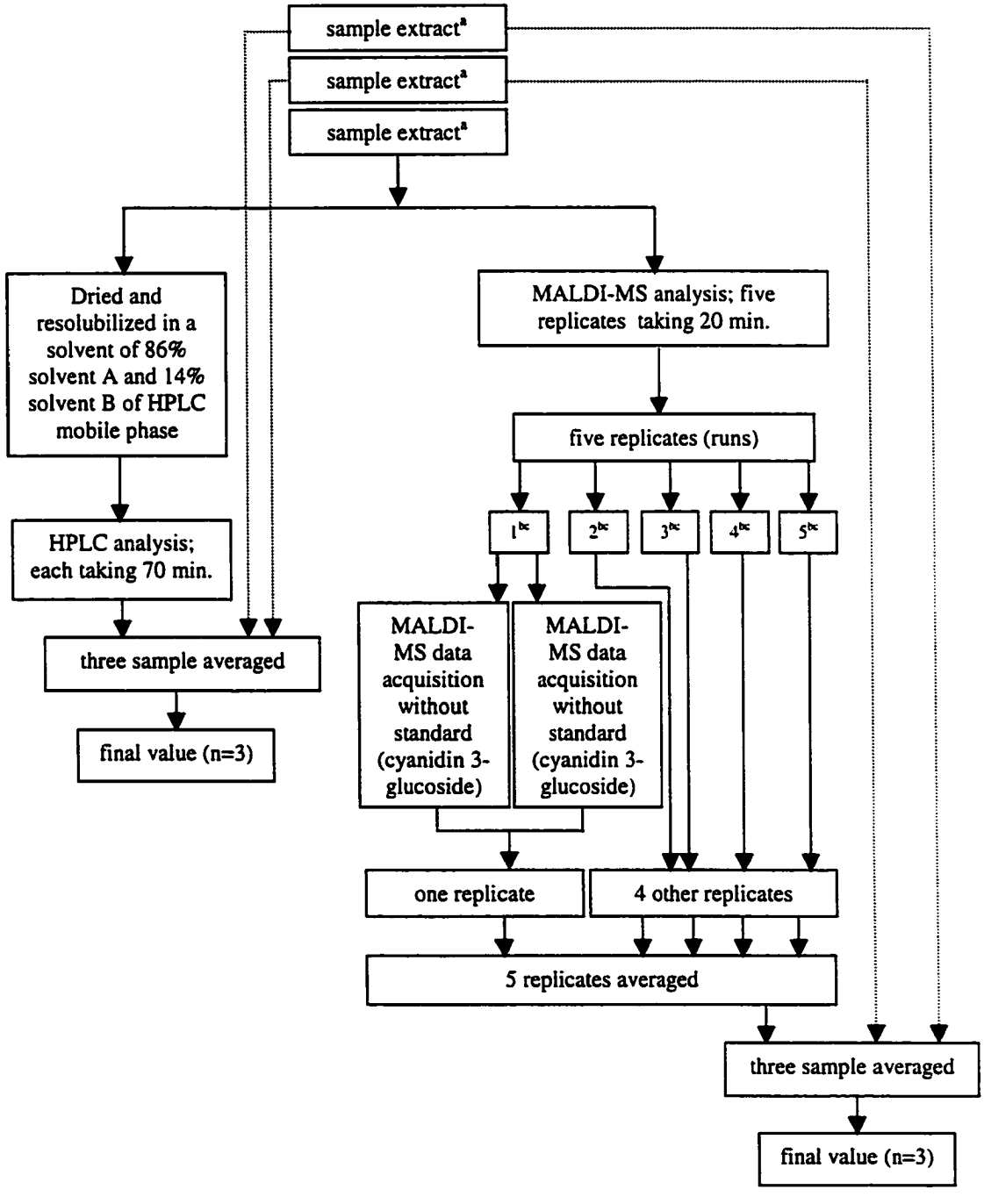
Experimental design for analysis of fructooligosaccharides using both HPAEC-PAD and MALDI-MS (Chapter 2)



Note: [a] two groups of five replicates were managed in the same manner. [b] experiments were conducted in the same way.

Appendix II

Experimental design for analysis of anthocyanins using both HPLC and MALDI-MS (Chapter 5)



Note: [a] three samples were analyzed in the same manner. [b] experiments were conducted in the same way. [c] represented one single MALDI-MS run. The first replicates from three samples were used to calculate the means of one single MALDI-MS run.