Method Development for Quantification of Vitamins in Spinach using HPLC-MS²

by

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Abstract

Vitamin consumption is essential to human health as they are not synthesized in the body, meaning they must be obtained from external sources, such as vegetables. The vitamin content of each vegetable varies, and previous efforts to quantify vitamins have predominantly been focused on UV detection methods. The aim of this study was to design an efficient and robust method to extract 10 vitamins of interest, and subsequently quantify them using HPLC-MS². These vitamins included: α -tocopherol, β -carotene, phyloquinone, retinol, thiamine, pantothenic acid, niacin, choline, and folic acid. Further studies were performed to compare varying levels of pesticide exposure, and to analyze concentration of vitamins over time. Ultimately the vitamin content remained relatively constant over time despite matrix degradation, with the exception of the statistically significant increase of phyloquinone. Further studies were performed with organic spinach to compare varying levels of pesticide exposure and it was found that the concentration of vitamins was not significantly affected by pesticides. However, concentrations of β -carotene and choline were an anomaly as they were observed to increase with statistical significance in organic spinach samples.

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List of Abbreviations

CID = Collision-induced Dissociation

ESI = Electrospray Ionization

HPLC = High-performance Liquid Chromatography

LC-MS/MS = Liquid-chromatography tandem mass spectrometry

MeOH = Methanol

MRM = Multiple Reaction Monitoring

MS = Mass Spectrometry

MS/MS or MS² = Tandem Mass Spectrometry

S/N = Signal-to-noise ratio

SIR = Single Ion Reaction

SIM = Single Ion Monitoring

SM = Sphingomyelin

THF = Tetrahydrofuran

TIC = Total Ion Chromatogram

TrEnDi = Trimethylation Enhancement using Diazomethane

UV = Ultraviolet

XIC = Extracted Ion Chromatogram

1. Introduction

1.1 *Spinacia oleracea* (Spinach)

Spinach is a leafy green vegetable, widely regarded as important in the human diet.¹ Its production has increased consistently since the 1970s, with China and the United States being the top two producers worldwide.² It is a versatile vegetable as it can be consumed either raw or cooked without compromising flavour, however the nutrient content is dependent on the method of cooking (e.g. baking or boiling).

When spinach is purchased for consumption, visual appeal tends to be the most important component for selection, however there is no indication of whether or not this visual quality is a reflection of its nutrient value. The vitamin content of spinach has previously been investigated,^{3,4,5} though lacking in-depth analysis as it is a broad topic affected by a wide range of factors, including the method of growth, exposure to light, storage, and other environmental influences.

New agricultural techniques are now emerging that permit complete control over growth factors. Indoor farming has become an increasingly popular topic as the effects of climate change are observed more frequently.⁶ These systems provide a method of growing crops in climates where the natural materials, such as sunlight and soil, are lacking. In experimenting with various elements, nutrient content may be optimized, and crops may be grown in adverse climates.

Indoor farming not only allows for regulation of fundamental growth aspects, it also has a significant economic effect as it reduces crop loss and shipping costs, and provides consumers with fresh locally grown product year-round. A local company in the National Capital Region has recently begun production of spinach via indoor farming, and was interested in investigating the vitamin content that current product consumers are purchasing, which is imported from California, U.S.A. Methods to ex-

tract and quantifying these vitamins using HPLC-MS/MS have since been developed to achieve this goal.

1.2 Vitamins and Current Detection Methods

Vitamins are essential to human growth and development as they play key roles in various cellular processes, and ultimately lower the risk of chronic illnesses. Vitamins are defined as organic compounds that are not synthesized by the body but are required to maintain health. Their metabolic functions include: coenzymes, proton and electron donors/acceptors, antioxidants, and hormones. A lack of vitamins can lead to vitamin deficiency diseases such as scurvy and beriberi.⁷ Research that focuses on vitamin quantitation has become more important with the increased prevalence of vitamin-fortified foods, which tend to supplement an unbalanced human diet.

As deficiency diseases became more prominent, Casimir Funk identified the cause of these deficiencies to be ‘vital amines’, which was abbreviated to ‘vitamines’. Despite the discovery that the nutrients causing these deficiency diseases are not necessarily amines the name remained — though the spelling changed.⁸ These organic compounds can be divided into two categories: fat-soluble, and water-soluble. Fat-soluble vitamins are generally hydrophobic and non-polar; these include: vitamin A (retinol), vitamin E (α -tocopherol), and vitamin K₁ (phylloquinone). β -carotene also falls into the fat-soluble class; however, it is considered a pro-vitamin as it is a precursor to vitamin A.⁹ Water-soluble vitamins have more polar functional groups, and are not lipophilic; these include: vitamin B₁ (thiamine), vitamin B₃ (niacin), vitamin B₅ (pantothenic acid), vitamin B₉ (folic acid), and the essential nutrient choline. A vitamin class may consist of a variety of compounds holding similar structural and functional properties, these are called ‘vitamers’, for example vitamin K₁ is a vitamer of vitamin K.

1.2.1 Fat-soluble Vitamins

1.2.1.1 α -tocopherol (Vitamin E)

Vitamin E is a class of vitamins containing eight vitamers, including both tocopherol and tocotrienol species. α -tocopherol ($C_{29}H_{50}O_2$) is the only biologically active form and is a widely known antioxidant, functioning in prevention of damaging radical species.⁴ As it is a fat-soluble vitamin, its antioxidant activity is mainly directed at lipid species, specifically membrane lipids.

α -tocopherol's antioxidant activity originates from the donation of its phenolic hydrogen to peroxy radicals it encounters, effectively transforming itself into a radical species.⁴ The newly created radical is stabilized by the aromatic ring, and upon fulfillment of its antioxidant activity, α -tocopherol is regenerated via interaction with ascorbic acid.¹⁰

As plants experience stress, such as environmental stress, the level of α -tocopherol initially increases to raise the overall antioxidant level in an attempt to boost protection.¹¹ Eventually, as degradation becomes faster than synthesis, the level of α -tocopherol is reduced. Additionally, the concentration of ascorbic acid is reduced in a stressed state, contributing to degradation of α -tocopherol as it will no longer protonate the α -tocopherol.^{11,12}

Figure 1.1: Chemical structure of α -tocopherol.

Despite the fact that vitamin E exists in various forms including β -tocopherol and γ -tocopherol, α -tocopherol is the only form that will elicit a symptomatic response

if insufficient amounts are absorbed.^{13, 14, 15} Vitamin E deficiency is an uncommon condition as it is generally induced by genetic abnormalities, though it has been characterized by neuromuscular damage caused by free radical nerve damage.¹⁶

Detection of vitamin E has been successful *in vivo* using fluorescence detection as it has innate fluorescence properties.¹⁷ However, determination of vitamin E in foods has seen methods such as gas chromatography and high-performance liquid chromatography employed.¹² Vitamin E does not require derivatization prior to analysis via gas chromatography, however HPLC analysis grants a higher level of flexibility in terms of sample matrices.¹³

1.2.1.2 β -carotene

Over 60 species of carotenoids have been identified, defined as having eight isoprenoid units arranged such that the units are reversed in the centre of the molecule. The bioavailability of each carotenoid is determined by nine factors: carotenoid species, molecular linkage, intake, matrix, absorption and bioconversion influences, host nutrient status, genetics, and other host-related interactions.^{18, 19, 20}

β -carotene ($C_{40}H_{56}$) is considered a pro-vitamin meaning, that it is transformed into a vitamin. In this case transformation is induced by the enzyme β -carotene 15,15'-dioxygenase (BCO).²¹ β -carotene in particular is a precursor to vitamin A as it contains an unsubstituted β -ionone ring, and central cleavage of the molecule yields two molecules of retinal. This retinal is subsequently reduced by retinaldehyde reductase to produce retinol.²² The presence of two unsubstituted β -ionone rings, combined with its symmetrical structure, make β -carotene significantly more bioactive than other carotenoids such as α -carotene.

This pro-vitamin has been identified and quantified using its characteristic UV absorption spectrum, yielded by its many conjugated alkenes.¹³ Quantification has proven challenging as β -carotene must be extracted using an efficient procedure as

Figure 1.2: Chemical structure of β -carotene.

it decomposes upon exposure to oxygen, high temperature, and light.⁵ Historically, carotenoid assays were completed using thin-layer chromatography, or spectrophotometry in combination with open column chromatography. Presently, HPLC is a more advantageous method as it can be conducted at room temperature and lacks laborious sample preparation.²³ More recently, a compilation of mass spectrometry fragmentation data has been assembled using various ionization sources, including electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), and more.²⁴ In combination with liquid chromatography, mass spectrometry has the ability to differentiate between carotenoid species and co-eluting carotenoid species, making it an ideal analysis technique.²⁵

1.2.1.3 Retinol (Vitamin A)

Stored in the liver, vitamin A can exist in many forms including retinol, retinal and retinyl esters. When vitamin A intake is inadequate these can then be released into plasma for use by the Retinol Binding Protein (RBP), or conversion to 11-*cis*-retinoids to aid in ocular processes.²⁶

One of the most prominent roles of vitamin A is in the retina. Normal retina function in lighted conditions involves the conversion of 11-*cis*-retinal to all-*trans*-retinal after light absorption by rhodopsin, and the photoisomerization induces a signal cascade affecting the visual cortex. When the retina enters darkness after exposure to light, all-*trans*-retinal is converted to 11-*cis*-retinal and subsequently bound to opsin to resume full function in low light conditions. As it plays a significant

role in retina function, vitamin A deficiency leads directly to xerophthalmia, whose symptoms begin as night blindness and can eventually extend to irreversible blindness. In the absence of vitamin A, the conversion to 11-*cis*-retinal is insufficient.²⁷

Figure 1.3: Chemical structure of retinol.

Discovered in the early 1900s, retinol ($C_{20}H_{30}O$) is a member of the retinoid class, defined as having four head-to-tail isoprenoid units in its structure. A retinol molecule, in particular, has a trimethylated cyclohexane ring, a conjugated tetraene hydrocarbon chain, and a polar alcohol group. It is this conjugation that gives retinol a distinct UV spectrum, which has previously been used for identification and quantification.²⁸

Other analysis methods have been thin-layer chromatography and gas chromatography, however derivatization is required prior to gas chromatography allowing the molecule to withstand high temperatures. The overall consensus for chromatographic analysis of vitamin A is via liquid chromatography, as it is a room-temperature method that requires minimal sample preparation.

1.2.1.4 Phylloquinone (Vitamin K₁)

Henrik Dam was first to discover the existence of vitamin K, an antihemorrhagic vitamin, in 1935, and was supported by subsequent reports later that same year.^{29,30} As characterization studies of the vitamin unfolded it became clear that there are two forms of the vitamin, differing only slightly in structure: K₁ and K₂. Dam's discovery ultimately won him a Nobel Prize in Physiology and Medicine in 1943.

The defining feature of vitamin K is its 2-methyl-3-phytyl-1,4-naphthoquinone structure (Figure 1.4), all derivatives of this that elicit antihemorrhagic effects fall into the vitamin K class. Phylloquinone is designated as vitamin K₁ (C₃₁H₄₆O₂), and has an additional phytyl group on the third carbon.

Figure 1.4: Chemical structure of phylloquinone.

Quantification of vitamin K has proven quite difficult due to the presence of its vitamers. Separation is required in order to obtain an accurate value from a complicated sample matrix, such as serum. This separation was first completed using thin-layer chromatography,³¹ and eventually evolved to HPLC.³² Detection methods began with UV detectors, compromising sensitivity, and have since moved on to electrochemical or fluorescence detection following derivatization.^{33,34}

1.2.2 Water-soluble Vitamins

1.2.2.1 Choline

Although choline (C₅H₁₄NO⁺) is not a vitamin by definition, it is an essential nutrient found in both water-soluble and fat-soluble metabolites. Upon discovery, choline was defined as a lipotrope, meaning it functions in the liver to prevent build-up of neutral lipids. As a result, choline deficiency tends to result in fatty liver disease.

Many choline derivatives have been discovered, and these can be metabolically

Figure 1.5: Chemical structure of choline.

converted into free choline. These derivatives include: phosphocholine, phosphatidylcholine, sphingomyelin, dimethylglycine, and acetylcholine to name a few. Humans do not have a method of storing excess choline, when there is an excess it is oxidized in the mitochondria to yield energy.¹³

Detection of choline has proven to be challenging as it does not have UV absorbance or fluorescence properties.³⁵ Quantitation of choline has been successfully achieved using HPLC in combination with fluorescence detection after derivatization.³⁶ Liquid chromatography-tandem mass spectrometry has also emerged as a viable technique whereby derivatization is not required.^{37, 38, 39} More recently a database has been created to reflect choline content in various foods using HPLC in combination with isotope dilution mass spectrometry.¹³

1.2.2.2 Folic Acid (Vitamin B₉)

Folic acid (C₁₉H₁₉N₇O₆) falls into the folate class of compounds alongside tetrahydrofolate, and the monoglutamate form. Here, the number of glutamic acid molecules can vary to create a polyglutamate species. The oxidized form of the monoglutamate vitamer is known as folic acid, which is not often naturally occurring.¹³ When folate is to be stored within the cell, enzymatic conversion to a polyglutamate species is required, and when folate is to be released enzymatic conversion to the monoglutamate form is necessary.

The bioavailability of folates are dependent on a number of factors, including: folate species, matrix, intestinal tract environment, and postabsorption factors affecting metabolism. Generally, folic acid yields a higher level of folate in blood compared to

Figure 1.6: Chemical structure of folic acid.

folate obtained from food.¹³ Additionally, excessive alcohol consumption prevents folate absorption, and can lead to folate deficiency due to interference with the proteins involved in absorption.⁴⁰

Folic acid is commonly known for its requirement in pregnant women as its levels are correlated with negative outcomes, such as preterm delivery and low birth weight.⁴¹ However, it is uncertain if low folate is the cause, or if low folate is an indicator of these potential outcomes. Studies have also shown that folic acid leads to a significant decrease in congenital heart defects in unborn children.^{42, 43, 44}

The level of folate in various foods has been measured using methods such as microbiological assays, spectrophotometry, and HPLC-MS, with the microbiological assay being most popular amongst food analysis.¹³ The microbiological assay, however, only provides data on the sum of folates, the vitamers are indistinguishable.^{45, 46} HPLC resolves this issue by efficiently separating the vitamers, and coupled with mass spectrometry, sensitivity is not compromised.⁴⁵

1.2.2.3 Niacin (Vitamin B₃)

The structure of niacin (C₆H₅O₂) is fairly simple, consisting of a pyridine ring attached to a carboxylic acid. Upon ingestion of niacin it is converted to nicotinamide adenine dinucleotide (NAD), a co-factor involved in several biochemical processes

including the Citric Acid Cycle. During the late 1800's and early 1900's the prevalence of pellagra was at its peak due to low niacin intake, especially in regions whose staple food was corn.⁴⁷ Symptoms of this disease include diarrhea, sun-induced dermatitis, dementia, and death, historically known as the four D's, though pellagra is curable if treated with increased niacin intake.⁴⁸

Figure 1.7: Chemical structure of niacin.

Niacin detection began with microbiological and chemical assays, however these proved to be laborious, and in some cases employed toxic unstable reagents.⁴⁹ Quantification of niacin has since moved to LC-MS/MS⁵⁰ and HPLC in combination with UV detection,⁵¹ and through these innovations the separation of nicotinic acid and nicotinamide has been achieved, a task that was previously challenging due to structural similarities of both compounds.

1.2.2.4 Pantothenic Acid (Vitamin B₅)

Pantothenic acid (C₉H₁₇NO₅) came into existence as a vitamin when it was discovered that its structure is the backbone of coenzyme A (CoA).¹³ This coenzyme, in turn, plays an important role in the Citric Acid Cycle. Synthesis of this vitamin is limited to plants, eubacteria, archaea and bacteria, and is completed via linkage of β -alanine and pantoic acid.⁵²

Pantothenic acid is taken up in the small intestine, and enters the blood stream for subsequent cellular uptake. In excess quantities pantothenic acid is excreted in urine after cleavage of β -mercaptoethylamine and phosphate from CoA.¹³ Upon deficiency,

Figure 1.8: Chemical structure of pantothenic acid.

weakness is usually the primary symptom due to low levels of CoA, followed by neurological defects as acetylcholine synthesis is also affected.

The level of pantothenic acid has previously been measured via microbiological assay,⁵³ which then moved to enzyme-linked immunosorbent assays (ELISAs) as they are less time consuming, however sensitivity was compromised.^{54,55} Chromatographic methods have been tested, though pantothenic acid often requires derivatization for gas chromatography due to lack of volatility. Additionally, derivatization has proven useful in UV detection methods as pantothenic acid has weak UV absorbance.⁵⁶ Stable isotope dilution methods have also been reported in combination with gas chromatography, and it was proven more sensitive than an ELISA, though cumbersome derivatization remained.⁵⁷

1.2.2.5 Thiamine (Vitamin B₁)

Thiamine (C₁₂H₁₇N₄OS⁺) consists of a hydroxyethyl side chain, an unsubstituted carbon serving as a connection between pyrimidine and thiazole rings, and an amino group on the pyrimidine ring. The hydroxyethyl side chain is of note as it becomes phosphorylated inside a cell. The innate positive charge is advantageous during mass spectrometry analysis as the molecule will not be protonated or deprotonated, and additional agents are unnecessary.¹³

The most bioactive version of thiamine is thiamine diphosphate. This derivative plays an active role in cells as a cofactor in the Citric Acid Cycle, and for many enzymes such as pyruvate dehydrogenase, which is also involved in the Citric Acid

Figure 1.9: Chemical structure of thiamine.

Cycle.⁵⁸ When humans experience thiamine deficiency, a condition known as beriberi, the resulting symptoms are exhibited in the peripheral nervous system or cardiovascular system.

Past years have seen the quantification of thiamine using a transketolase activity assay. However, these methods have proven to be imprecise and may not only be influenced by lack of thiamine intake, as well as isozymes of the enzyme.⁵⁹ As a result, determination of thiamine is now commonly accomplished using HPLC in combination with a fluorescence detector following derivatization.⁶⁰ Alternatively, thiamine may be quantified by HPLC coupled with a photodiode array detector at 243 nm.⁶¹

1.3 High-Performance Liquid Chromatography (HPLC)

High-Performance Liquid Chromatography (HPLC) is a separation technique often used on complex samples in a liquid state.⁶² HPLC employs a closed-column system containing a stationary phase, in combination with a high pressure system to force solvent into the system and achieve separation of analytes. These small molecule stationary phases may include materials such as silica bonded with a hydrophobic or

hydrophilic ligand, depending on the chromatography method to be used.⁶³

A sample is injected into the system and loaded on to the column. A small guard column may be appended to the column such that the sample enters the guard column prior to its attachment on the column.⁶⁴ The guard column is intended to prevent column exposure to any contaminants that may bind irreversibly. The analytes in solution will adsorb onto the stationary phase based on their affinity.⁶⁵ As solvent flows through the system analytes in the sample detach from the column based on their affinity to the stationary phase, thus separation of the analytes is dependent on their polarity.

Two common types of include normal phase and reversed phase. Normal phase chromatography exploits a polar stationary phase, and a non-polar solvent; whereas reversed phase chromatography applies a polar solvent to a non-polar stationary phase.⁶⁵ For example, when using a reversed-phase system, large non-polar analytes will bind to the stationary phase with a high affinity, thus be retained longer than polar analytes.

Analyte separations can be done isocratically or via gradient elution. An isocratic separation maintains constant solvent conditions from start to finish. In contrast, a gradient separation varies the solvent composition throughout. Gradients are commonly used in separation of complex samples containing analytes of varying polarity because the solvent composition can be changed to target specific analytes at certain times for optimal separation. The solvent system selected for gradient elution is based on solvent strength, also known as the elutropic series. In a reversed phase chromatography setting, as the polarity of the solvent decreases, the eluent strength increases.

As analytes are eluted from the column they are detected, resulting in a chromatogram of peaks; each peak representing a single analyte.⁶⁶ HPLC methods can be coupled to a mass spectrometer for detection to improve sensitivity of the analysis.⁶⁷

This can be advantageous when analyzing a complex sample as the mass spectrometer will detect the analytes consecutively, rather than simultaneously, making ion suppression less of a concern.⁶⁸ Additionally, in combination with HPLC, mass spectrometry can provide qualitative as well as quantitative data.

1.4 Mass Spectrometry (MS)

Dating back to the early 1900s, mass spectrometry has continuously been improved, and it is now known as one of the most powerful analytical tools. It has been gaining momentum in recent years as advances have solidified it as a reliable and robust technique. Its versatility provides a substantial benefit to laboratories across the globe, as its applications range from general structure determination and characterization, to drug development and discovery.⁶⁹

Despite the innovation of instrumentation, the fundamental concepts of mass spectrometry have remained constant. A mass spectrometer is designed to detect ions based on their mass-to-charge ratio (m/z , with units called Thomson (Th)), and produce a spectrum of abundance vs. m/z . The instrument itself is composed of three basic components: an ion source, a mass analyzer, a detector. A sample enters the mass spectrometer through a sample inlet, is transported to the ionization source where it becomes positively or negatively charged, and it then moves through a mass analyzer based on its m/z , and the ions are detected.⁶⁹ A high vacuum is required in a mass spectrometer to prevent ion-molecule collisions, which can affect the ion trajectories and lead to a loss of sensitivity in the instrument.

The key requirements for an analyte to be compatible with a mass spectrometer are that it is charged and in the gas phase.⁷⁰ In order to achieve this it must undergo ionization. There are many ionization techniques that a mass spectrometer may be equipped with, some eliciting more fragmentation than others. Methods of ionization

include: electrospray ionization (ESI) a soft ionization technique and used for non-volatile analytes and large biomolecules, electron ionization (EI) a hard ionization technique exploited in analysis of small organic molecules, and matrix-assisted laser desorption ionization (MALDI) used in analyzing proteins from cell extracts and other biomolecules.⁶⁹

Following ionization, the analyte is transferred into the mass analyzer. A variety of mass analyzers are capable of separating the ions according to their m/z , including quadrupoles, time-of-flight, and ion traps. The resolution of these mass analyzers differs, and this becomes important when there are two analytes of very similar m/z being analyzed simultaneously. Additionally, the upper mass limit varies for different types of mass analyzers.⁷¹

The analyte ions are subsequently detected to generate a mass spectrum. To do so, the ions exit the mass analyzer and are transformed into a detectable signal representative of the abundance of ions.⁷² A number of detectors exist for this purpose, including an electron multiplier, a Faraday cup, an ion-to-photon detector, and a microchannel plate detector. Mass spectrometers are equipped with an appropriate detector based on the required response time, sensitivity, and dynamic range.

1.4.1 Electrospray Ionization (ESI)

Electrospray Ionization (ESI) is a widely available and commonly used ionization technique in mass spectrometry. Initially developed by Dole et al.,⁷³ ESI is a soft ionization technique applicable to large, non-volatile analytes. Further innovation to the method was continued by Fenn et al.,⁷⁴ and it has since been accepted in the mass spectrometry community.

One of the key components to this system is the use of a high voltage stainless steel capillary. The sample flows through this capillary at atmospheric pressure, and charged particles are expelled into the ionization chamber via formation of a Taylor

cone. Coulombic forces allow small droplets to emerge from the tip of the Taylor cone, and the solvent evaporates to leave behind molecular ions. The ions are propelled by the electric field into the mass analyzer.⁷⁰ These principles are illustrated in Figure 1.10, whereby the analyte is assumed to have acquired a positive charge from the acidic solution it is constituted in, or from excess ions such as Na^+ and NH_4^+ . The presence of these ions allows for adducts to be formed with molecule that may not have an ionizable site otherwise.⁷¹ If the polarity of the voltages is reversed, a negative charge will be imparted on the analyte from the basic solution it is constituted in.⁷⁵

Figure 1.10: Schematic of electrospray ionization (ESI) in positive ion mode.

In comparison to other soft ionization methods, such as MALDI, the noise present in the low mass range of the generated spectrum is significantly reduced, allowing for analysis of small m/z analytes.⁷⁶ Additionally, ESI supports the acquisition of more than one charge on an analyte, as opposed to singly charged species as observed with MALDI.⁷⁵

Based on molecular dynamics simulations, three models exist to describe how molecules are transferred into the gas phase during ESI: the ion evaporation model (IEM), the charged residue model (CRM), and the chain ejection model (CEM).⁷⁶ The IEM is applicable to low molecular weight species, and states that as an ion is released from its solvent shell, a solvent bridge is formed between the droplet and

the ion. The ion is then released upon severance of the solvent bridge. The CRM is generally applied to large globular species, and displays the release of the solvent shell by layers as the radius of the droplet steadily decreases until the charge from the final shell is transferred to the analyte. The CEM is noted for non-polar polymer chains of proteins, and describes the phenomenon that occurs when proteins unfold in solution during ESI. As proteins become unfolded their hydrophobic chains begin interacting with the solvent present within the droplet. As this is unfavourable for the non-polar chains, they move to the surface of the droplet where it is ejected.

1.4.2 Triple Quadrupole Instruments

A quadrupole mass analyzer is composed of four round metal rods set in parallel with one another with an applied radio frequency (RF) potential and direct current (DC) potential equal and opposite. A positive potential is applied to rods opposite each other in the x-direction, and a negative potential is applied to the two rods opposite each other in the y-direction. As ions enter the quadrupole they experience an electric field in the xz and yz planes, and are propelled in the z-direction.⁷⁷

Figure 1.11: Schematic diagram of a triple quadrupole mass spectrometer equipped with a photomultiplier detection system.

The quadrupole mass analyzer can behave in one of two ways. The first having only A/C applied to the rods such that ions of every m/z are stabilized and transmitted, RF-only mode. The second operating mode is resolving mode, and allows the quadrupole to act as a mass filter, whereby A/C and D/C currents are applied to the

rods.

Resolving mode results in the production of a mass spectrum, and has the ability to provide a wide range of data. A Total Ion Chromatogram (TIC) yields m/z values whereby all peaks intensities in the spectrum are displayed and summed. From this an Extracted Ion Chromatogram (XIC) can be produced by selecting a single mass to display in the software. This mode also allows for tandem MS (MS/MS or MS²) scans to be performed, which then yield more in-depth data, including fragmentation. A single-ion monitoring (SIM) scan may also be performed whereby Q1 scans for a specified m/z similar to an XIC, but with increased sensitivity.

A triple quadrupole mass analyzer consists of three consecutive quadrupoles: Q1, q2 and Q3. Q1 and Q3 are mass selective quadrupoles that can operate in resolving mode, whereas q2 is not mass selective and operates in RF-only mode as the collision cell, or linear accelerator collision cell (LINAC). The selected ions undergo collision-induced dissociation (CID) in q2 whereby they are accelerated into a chamber of inert gas, such as argon, and interactions between the ions and the gas molecules cause the analyte to dissociate into fragments. Though low pressure in the cell was initially hypothesized to prevent ion scattering, it has been proven to compromise ion transmission through the cell.⁷⁸ Elevated pressure in the collision cell, combined with gas molecule collisions, evidently lowers the energy of the ions and focuses them to the center, a process known as collisional cooling.⁷⁹

One of the key advantages of using a triple quadrupole mass analyzer is the ability to perform MS/MS scans, including: product ion scans, precursor ion scans, and Multiple Reaction Monitoring (MRM). In a product ion scan, Q1 is set to select for a single m/z , meaning all other m/z will not be transmitted. This ion is fragmented in q2, and the fragments are transmitted and analyzed in Q3. The spectrum then displays fragment ions (also known as daughter ions) of the parent ion.

A precursor ion scan scans a range of masses using Q1 and as each m/z is trans-

Figure 1.12: MS/MS scan modes on a triple quadrupole mass analyzer, including: a) Product ion scan; b) Precursor Ion Scan; c) MRM scan.

mitted it is then fragmented in q2; Q3 selects an ion of a specified m/z to proceed to the detector. The spectrum then displays the parent ions that yielded the specified fragment. This type of scan provides confirmation that the fragment ion was dissociated from the analyte of interest.

An MRM scan has Q1 set to a known specified parent m/z value and Q3 set to a known specified fragment m/z value that was previously determined using a product ion scan. The resulting chromatogram displays the abundance of the analyte over time. MRM scans are particularly advantageous when quantifying analytes in a complex sample because there is an added level of certainty that the peak displayed on the spectrum is the desired analyte, rather than another species that possesses the same parent m/z . In contrast, an SIM scan is similar to an MRM scan in that the quadrupole searches for a single mass, however it is not a type of MS² thus fragment ions are not involved and the scan is generally performed in a single quadrupole.

1.4.3 Photomultiplier Ion Detector

A variety of ion detectors may be employed in a mass spectrometer. The ideal ion detector has a fast response time, high efficiency, and low noise. The mass spectrometer employed in this dissertation utilizes a conversion dynode in combination with phosphor and a photomultiplier detection system.

Following analysis, ions exit the mass analyzer (i.e. the quadrupole) and contact the conversion dynode, causing the release of secondary particles.⁷¹ When performing analysis in positive ion mode, the conversion dynode is negatively charged; analysis in negative ion mode sees a positively charged conversion dynode. Secondary electrons generated from contact with the conversion dynode are accelerated toward phosphor, where they are converted into photons and subsequently detected by the photomultiplier.

2. Materials and Methods

2.1 Vitamin Sample Preparation

β -carotene, α -tocopherol, retinol palmitate, phyloquinone, thiamine hydrochloride, choline chloride, D-pantothenic acid hemicalcium salt, folic acid, nicotinic acid, and ascorbic acid standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). Vitamers were selected based on biological relevance, and prevalence in other vitamin research.

2.1.1 Fat-soluble Vitamin Preparation

β -carotene, α -tocopherol, retinol palmitate, and phyloquinone were each prepared in anhydrous ethanol and diluted to 50 μ M for HPLC-MS analysis. A mixture containing 50 μ M of each of these vitamins was also prepared in anhydrous ethanol.

2.1.2 Water-soluble Vitamin Preparation

Thiamine hydrochloride, choline chloride, D-pantothenic acid, folic acid, nicotinic acid, and ascorbic acid were prepared in deionized water (Millipore Sigma, Etobicoke, ON, CAN) and diluted to 50 μ M for HPLC-MS analysis. A mixture containing 50 μ M of each of the aforementioned vitamins was also prepared in deionized water.

2.2 Development of Mass Spectrometry Methods

Mass spectrometry analysis was performed on a Waters Micromass Quattro Ultima (Waters Corporation, Milford, MA, USA) triple quadrupole mass spectrometer. Vitamin standards were prepared as per section 2.1, with additional 10mM ammonium

acetate in solution. Each sample was directly injected into the instrument via syringe, and MS parameters were adjusted accordingly on MassLynx software to optimize observation of the analyte. A solution of fat-soluble vitamins, and a solution of water-soluble vitamins, were then injected consecutively to verify and optimize the simultaneous observation of the analytes.

Limits of detection (LOD) were defined when the signal-to-noise (S/N) ratio was less than 3:1, and limits of quantification (LOQ) were reached when the S/N was less than 10:1.

2.2.1 Multiple Reaction Monitoring (MRM)

Each vitamin standard (50 μ M) was directly injected in a solution of 10 mM ammonium acetate, and collision energies were tested to optimize observation of the fragment ions. The daughter ions for each analyte were then used to create MRM scans, one scan for all fat-soluble vitamins with fragments as follows: α -tocopherol 431 $\rightarrow$ 137, 165 (30 eV); β -carotene 536 $\rightarrow$ 444 (20 eV); 537 $\rightarrow$ 444 (20 eV); retinol 269 $\rightarrow$ 91, 128 (50 eV); phyloquinone 451 $\rightarrow$ 171, 187 (35 eV). A separate scan was performed for analysis of all water-soluble vitamins. The fragment ions are as follows: thiamine 265 $\rightarrow$ 81, 122 (30 eV); pantothenic acid 220 $\rightarrow$ 72, 90 (11 eV); niacin 124 $\rightarrow$ 78, 80 (35 eV); choline 104 $\rightarrow$ 45 (22 eV), 58 (45 eV), 60 (22 eV); folic acid 442 $\rightarrow$ 176, 295 (30 eV).

2.3 HPLC Analysis

HPLC analysis of the vitamins of interest was completed on a Waters 2695 (Waters Corporation, Milford, MA, USA) instrument equipped with a Zorbax SB-C18 reversed-phase column (5 μ m, 2.1 x 50 mm) and an Eclipse XDB-C8 guard column (5 μ m, 2.1 x 12.5 mm) (Agilent Technologies, Inc., Santa Clara, CA, USA). Two blank samples were run prior to each batch, and in between each sample to prevent

carry-over.

2.3.1 Fat-soluble Vitamins

A 10 μ L volume of each standard solution mixture was injected on the column, and the analytes were separated for a total of 20 minutes using a gradient of acetonitrile (0.1% formic acid) (A) and MeOH (10% IPA, 2 mM ammonium acetate) (B) at 500 μ L/minute. The analytes were initially subject to 2% A, and this linearly increased to 98% A over 10 minutes. The gradient was then reversed back to 2% A over five minutes, and held at this composition for five minutes.

2.3.2 Water-soluble Vitamins

The water-soluble vitamins were separated for a total of 15 minutes using a gradient of H₂O(0.1% formic acid) (A) and MeOH (B) at 200 μ L/minute. Beginning with 99% A, the gradient was held for 2.5 minutes before linearly decreasing to 8% A. After 3.5 minutes the solvent composition increased linearly to 90% A and was maintained for five minutes before increasing further to 99% A. The run was complete after four minutes.

2.4 Calibration Curves

Vitamin standards of α -tocopherol, β -carotene, phyloquinone, retinol, choline, folic acid, niacin, pantothenic acid, and thiamine were prepared at 1, 5, 10, 15, 20, 30, 40, and 50 μ M concentrations as described in section 2.1. The chromatography methods described in sections 2.3.1 and 2.3.2 were run in tandem with the MS/MS scans designed in section 2.2.1 in triplicates. The area under each chromatogram was plotted against concentration (μ M) to create the calibration curve, consisting of at least six points for each vitamin standard.

2.5 Extraction of Vitamins from Spinach

Spinach samples were stored at -80°C at least 24 hours prior to extraction. Approximately 3 g of frozen spinach was transferred into a mortar and pestle and ground for approximately two minutes. The sample was then extracted using 10 mL of 1:1 MeOH/THF and 10 mL of hexanes. Following a two minute incubation on a vortexer, the sample was centrifuged for 15 minutes at 3000 rpm. The hexanes were partitioned off, and 1.5 mL of each solvent layer was filtered through a 0.45 μM PTFE syringe filter into separate 1.5 mL HPLC vials. Both solutions were dried separately using N_2 gas. The hexane fraction, containing fat-soluble vitamins, was re-suspended in 1.5 mL of anhydrous ethanol; and the MeOH/THF fraction, containing water-soluble vitamins was re-suspended in 1.5 mL of deionized water. Approximately 60-80 μg of each sample was loaded on to the column for HPLC-MS/MS analysis.

2.6 Method Validation

A student's t-test was performed on the area under each peak for comparison of various conditions, and the methods yielding maximum vitamin content were determined. Changes were made to procedures if the results were found to be statistically significant (95% confidence interval). Additionally, this statistical analysis was used to compare two different types of spinach, and verify the change in vitamin content over time.

3. Results and Discussion

Previous analysis of water-soluble and fat-soluble vitamin mixtures have been reported, however most are performed on pharmaceuticals,⁶¹ or milk products⁸⁰ including infant formula.⁸¹ Additionally, some of these methods neglect to quantify an entire class of vitamins, such as fat-soluble vitamins.^{80,81} The methods created and employed in this dissertation reflect the need for versatility, sensitivity and specificity in analysis and quantification of analytes in complex matrices.

The method development process consisted of designing a chromatography method for each vitamin standard, followed by amalgamation into two methods, one for fat-soluble vitamins and one for water-soluble vitamins. An extraction procedure was then designed to obtain the vitamins from the spinach matrix. At the end of the method development process, subsequent experiments were performed using these procedures. Spinach samples were extracted (3 g) using a 1:1 solution of MeOH/THF and hexanes.

Figure 3.1: Extraction vessel after centrifugation. The fat-soluble vitamins reside in the hexanes (top), and the water-soluble vitamins reside in the MeOH (bottom).

This was followed by HPLC-MS² analysis using the final chromatography methods. Water-soluble vitamins were analyzed using a gradient beginning with 99% H₂O (0.1% formic acid) (A) and 1% MeOH (B), this was then decreased to 8% A before increasing to 90% A, and then further to 99% A. Fat-soluble vitamins were separated on a gradient consisting of 2% acetonitrile (0.1% formic acid) (A) and 98% MeOH (10% IPA, 2 mM ammonium acetate) (B), the gradient was then changed to 98% A, followed by reversal to 2% A.

Figure 3.2: Analysis of fat-soluble vitamin standards on the final gradient method consisting of acetonitrile (0.1% formic acid) and MeOH (10% IPA, 2 mM ammonium acetate).

Figure 3.3: Analysis of water-soluble vitamin standards on the final gradient method consisting of H₂O (0.1% formic acid) and MeOH.

3.1 Method Development

3.1.1 HPLC-MS²

Mass spectrometry methods were created by direct injection as per section 2.2. This step was used to optimize MS parameters and obtain optimal signal for each vitamin. A solution of vitamins was then directly injected into the mass spectrometer to consolidate the parameter values, and ensure each vitamin was observed at its highest possible level.

SIM scans and XIC's were used to verify the presence of the vitamins prior to

creation of MRM scans. The MRM scans became particularly advantageous when analyzing the complex mixture produced by the solvent-extraction method as other molecules holding the same mass as the analytes were not detected.

This process was applied to the development of HPLC methods, whereby each vitamin was analyzed individually prior to creation of a complex mixture of analytes. This was to ensure chromatographic properties and requirements of each vitamin were first established in order to facilitate facile design of a chromatography method that would accommodate a complex solution. Eventually the analytes were combined and chromatography methods were created to analyze the vitamins simultaneously. Relatively high concentrations of the analytes were applied throughout the method development process to ease troubleshooting.

3.1.1.1 Retinol

The standard of retinol palmitate (m/z 525) was first analyzed using an isocratic method of 92% MeOH and 8% H₂O (0.1% formic acid) in tandem with a TIC scan in positive ion mode. The parent ion of m/z 525 was not observed in the spectrum, rather a parent ion of m/z 269 [MH-256]⁺ was present (Figure 3.4).

To verify that this was the parent ion of the standard two SIR scans were performed for m/z 269 and m/z 525 revealing predominance of m/z 269 ions as expected. This is consistent with literature findings whereby retinol palmitate loses its palmitic acid moiety and forms m/z 269 in the ion source.⁸² The SIR scan was also successful in terms of chromatography as a single peak corresponding to retinol was observed at 1.79 minutes (Figure 3.5).

To increase compatibility with other fat-soluble vitamins, retinol was also tested using various gradient methods as a gradient method provides more options for separation of various analytes by changing the elution strength of the solvent. Beginning with 100% H₂O (0.1% formic acid) (A) and 0% MeOH (B), the gradient was directly

Figure 3.4: Mass spectrum produced by the analysis of retinol palmitate (10 μ M) on an isocratic method of 92% MeOH and 8% H₂O. The parent ion was observed at m/z 269.

converted to 90% A after seven minutes and held here for ten minutes. The gradient was then linearly changed to 0% A and maintained for another ten minutes before changing to 10% A. A final conversion to 100% A was completed after 50 minutes to re-equilibrate the column. This method was unsuccessful as it did not yield a clear observation of retinol in the convoluted chromatogram (Figure 3.6).

As retinol is fat-soluble it was assumed to bind with high affinity to the C18 column, as a result a gradient composed of two organic solvents—MeOH (A) and acetonitrile (0.1% formic acid) (B)—was investigated. This method began with 100% A for three minutes, and proceeded to linearly decrease to 30% over four minutes. After a further eight minutes, the composition had linearly increased to 50% A,

Figure 3.5: SIM analysis of retinol (10 μM) on an isocratic method of 92% MeOH and 8% H_2O .

and then to 100% A after five minutes, for a total run time of 20 minutes. The chromatogram yielded two distinct retinol signals at 2.00 and 10.47 minutes (Figure 3.7).

In order to facilitate consolidation of these peaks, a linear decrease to 0% A was implemented at the 11 minute mark and maintained for four minutes in the gradient method. Overall, this method began with 100% A for three minutes, and proceeded to linearly decrease to 30% over four minutes. This was followed by another linear decrease to 0% A over four minutes. After a further four minutes, the composition had linearly increased to 50% A, and then to 100% A after five minutes, for a total run time of 20 minutes. This gradient was successful to a degree as it produced a relatively well-defined peak in the SIR chromatogram at 5.70 minutes (Figure 3.8),

Figure 3.6: SIM scan of retinol (40 μM) on a gradient composed of H_2O (0.1% formic acid) and MeOH.

indicating the analyte was bound to the column and separation from other analytes may be achieved.

A daughter ion scan was performed (Figure 3.9) to identify the fragment ions at various collision energies. The resulting fragments with the highest intensities (m/z 91 and 128) were found at a collision energy of 50 eV, and were then used to construct an MRM scan (Figure 3.10). The MRM scan was tested with isocratic chromatography conditions of 92% MeOH and 8% H_2O (0.1% formic acid), and yielded a distinct retinol signal at 2.08 minutes.

Figure 3.7: SIM scan of 10 μ M retinol on a gradient composed of MeOH and acetonitrile (0.1% formic acid).

Figure 3.8: SIM analysis of 10 µM retinol on a gradient composed of MeOH (A) and acetonitrile (0.1% formic acid) (B). The method extended from 100% A to 30% A to 0% A over eight minutes, and was then reversed to 100% A over nine minutes.

Figure 3.9: Daughter ion scan of a 45 μ M solution of retinol palmitate in EtOH performed in positive ion mode at a collision energy of 50eV.

Figure 3.10: MRM test scan of retinol (45 μ M) in EtOH with fragment ions m/z 91 and 128.

3.1.1.2 Phylloquinone

The chromatography method for vitamin K₁ was initially based on the isocratic method used for retinol (Section 3.1.1.1) as they fall into the same fat-soluble vitamin class. The isocratic method employed was composed of 92% MeOH and 8% H₂O (0.1% formic acid), which yielded elution of phylloquinone at 15.72 minutes in an MRM scan (Figure 3.11).

Figure 3.11: MRM test scan of phylloquinone (50 μ M) using an isocratic method consisting of 92% MeOH and 8% H₂O (0.1% formic acid).

The development of the MRM scan was done by first investigating the daughter ions at various collision energies to confirm the collision energy that yields fragment ions of the highest intensity. A collision energy of 35 eV was selected as the signal of the fragment ions was optimal. The daughter ions selected for the MRM scans

were m/z 187 and 171 (Figure 3.12). Although m/z 171 is not one of the top two fragment ions holding the highest intensity in the mass spectrum, when the MRM was tested against m/z 187, 171, and 199, the resulting chromatogram yielded a higher area under the chromatogram for fragment ion m/z 171 compared to m/z 199 (Figure 3.13).

Figure 3.12: Daughter ion scan of a 10 μ M solution of phylloquinone performed in positive ion mode at a collision energy of 35eV.

Figure 3.13: Chromatogram of MRM scan of phylloquinone with fragment ions m/z 187 (green), 199 (violet), 171 (red) and 213 (black).

3.1.1.3 β -carotene

The standard of β -carotene (m/z 536) was analyzed using the same isocratic method as retinol and phylloquinone, consisting of 92% MeOH 8% H₂O (0.1% formic acid) (Figure 3.14). An SIR scan was created for m/z 536 and 537, and both were observed abundantly. The peak at m/z 537 arises from the isotopic abundance of carbon and hydrogen as the structure of β -carotene is made up of a significant amount of both elements, some being ²H and ¹³C which is consistent with the literature.²⁴

The chromatography produced by this method was in need of improvement as the peak was unresolved, thus an isocratic method of 30% MeOH and 70% acetonitrile (0.1% formic acid) was tested for 25 minutes. Again, based on the structure of β -carotene it is extremely hydrophobic and has high affinity toward the C18 column, hence two organic solvents were employed. The results of this method were significantly enhanced in comparison to the previous isocratic method, a well-defined peak

Figure 3.14: Chromatogram of an SIR scan for β -carotene using an isocratic method of 92% MeOH and 8% H₂O (0.1% formic acid).

was observed at 7.82 minutes in the SIR chromatogram (Figure 3.15). However, a peak at 1.58 minutes was also observed.

Because this trend is undesirable, a gradient method was then investigated to consolidate the β -carotene signal. The gradient consisted of MeOH (A) and acetonitrile (0.1% formic acid) (B) solvent system. Beginning at 100% A, after three minutes the gradient was linearly decreased to 30% A over four minutes. It was then increased to 50% A over eight minutes, and held for five minutes before increasing to 100% A for a total run time of 20 minutes. The chromatography yielded by this method was improved in comparison to the isocratic methods, and β -carotene was observed at 2.32 minutes (Figure 3.16).

Figure 3.15: SIR analysis of β -carotene (20 μ M) using an isocratic method of 30% MeOH and 70% acetonitrile (0.1% formic acid).

Figure 3.16: SIM analysis of β -carotene (10 μ M) using an gradient method composed of MeOH and acetonitrile (0.1% formic acid).

3.1.1.4 α -tocopherol

Analysis of α -tocopherol began with gas chromatography mass spectrometry as it involved minimal sample preparation, and this analyte would not require derivatization. This was successfully completed, however the remaining fat-soluble vitamins required derivatization to maintain stability at high temperatures for GC-MS, thus HPLC-MS analysis was investigated.

The HPLC method for α -tocopherol was established whilst combined in a solution with the remaining fat-soluble vitamins. Chromatography methods for the retinol, phyloquinone and β -carotene had previously been established and it was likely that α -tocopherol would be harmonious throughout the method development process.

Figure 3.17: Daughter ion scan of a 10 μ M solution of α -tocopherol performed in positive ion mode at a collision energy of 30eV.

Prior to the development of a chromatography method suitable for all of the fat-soluble vitamins, the MRM scan for α -tocopherol was to be instituted. A daughter ion scan was performed (Figure 3.17), and the fragments of m/z 165 and 137 were used to create an MRM scan whereby Q1 was scanning for m/z 431 and Q3 was scanning for the fragment ions. The MRM scan was then applied throughout the creation of a chromatography method for simultaneous fat-soluble vitamin analysis.

3.1.1.5 Thiamine

Rationalizing that water-soluble vitamins have opposite properties of fat-soluble vitamins, the water-soluble chromatography methods began as the exact opposite of the most popular fat-soluble vitamin method. This consisted of 92% H_2O (0.1% formic acid) and 8% MeOH, and thiamine was successfully observed at 2.39 minutes (Figure 3.18). Despite the fact that a single analyte was injected into the HPLC-MS system, other molecules may have been present, for example those remaining on the column. To confirm the identity of thiamine, an XIC of m/z 265 was taken from the TIC. There is some peak tailing observed in the XIC of m/z 265, however this was likely caused by the high concentration of the standard in combination with the isocratic nature of the method. Had a gradient been explored it would be possible to discontinue elution of the analyte at a specific time point by changing the solvent composition.

The MRM parameters were discovered by performing a daughter ion scan to determine the fragment ions (Figure 3.19). The highest intensity fragments were observed at m/z 144, 122 at a collision energy of 30 eV, and 81, however m/z 122 and 81 were selected for MRM analysis as they produced peaks in the chromatogram with the largest area (Figure 3.20).

Figure 3.18: XIC scan of thiamine (50 μ M) analyzed isocratically using 92% H_2O (0.1% formic acid) 8% MeOH.

Figure 3.19: Product ion scan of thiamine, m/z 265 analyzed isocratically using 92% H_2O (0.1% formic acid) 8% MeOH.

Figure 3.20: MRM scan of thiamine analyzed isocratically using 92% H₂O (0.1% formic acid) depicting the abundance of the fragment ions m/z 144 (violet), 122 (green), and 81 (red).

3.1.1.6 Niacin

As the method created for thiamine in section 3.1.1.5 was successful, it was applied to analysis of nicotinic acid. This led to the observation of vitamin B₃ at 0.75 minutes (Figure 3.21); a significant amount of peak tailing was observed, however this is caused by a high concentration of sample in combination with isocratic analysis. Although it did not bind to the column sufficiently, this method was finalized for niacin as MRM scans would be used in the future for accurate identification of the vitamin.

Figure 3.21: XIC of niacin, m/z 124 extracted from a 50 μ M sample of nicotinic acid in EtOH, analyzed isocratically using 92% H₂O (0.1% formic acid) 8% MeOH.

The creation of the MRM method was done as described in previous sections, whereby a daughter ion scan of m/z 124 was executed for determination of the fragment ions. As seen in Figure 3.22, the highest intensity fragment ions used were m/z

78 and 80, observed at a collision energy of 35 eV. This MRM scan is depicted in Figure 3.23, whereby nicotinic acid was observed at 0.48 minutes.

Figure 3.22: Product ion scan of niacin, m/z 124 at a collision energy of 35 eV, analyzed isocratically using 92% H₂O (0.1% formic acid) 8% MeOH.

Figure 3.23: MRM scan of a 50 μM sample of nicotinic acid in H_2O at a collision energy of 35 eV, analyzed isocratically using 92% H_2O (0.1% formic acid).

3.1.1.7 Pantothenic Acid

The isocratic chromatography method designed for thiamine and applied to niacin in previous sections, consisting of 92% H_2O and 8% MeOH, was also successfully applied to pantothenic acid. The chromatogram depicted a resolved peak corresponding to pantothenic acid at 0.75 minutes (Figure 3.24). The chromatography signal has significant amount of tailing, however this is a result of the isocratic analysis in combination with a highly concentrated sample.

A product ion scan of m/z 220 was carried out to determine the fragment ions that result from CID in order to construct an MRM method. The highest intensity fragment ions were observed at m/z 124, 90, and 72 at a collision energy of 11 eV

Figure 3.24: XIC of m/z 220 from a 50 μM sample of pantothenic acid analyzed isocratically using 92% H_2O (0.1% formic acid) 8% MeOH.

(Figure 3.25). These were tested against an MRM scan of pantothenic acid to verify accuracy, and it was found that the area under the peak corresponding to m/z 90 was larger than that of m/z 124 (Figure 3.26) thus it was selected for inclusion in the finalized method.

Figure 3.25: Product ion scan of m/z 220 from a 50 μ M sample of pantothenic acid at a collision energy of 11 eV, analyzed isocratically using 92% H_2O (0.1% formic acid) 8% MeOH.

Figure 3.26: Overlay of pantothenic acid MRM chromatograms of overlaid daughter ions scans of m/z 124 (bottom), 90 (middle), and 72 (top) analyzed isocratically using 92% H₂O (0.1% formic acid) 8% MeOH.

3.1.1.8 Choline

When the isocratic method employed in analysis of thiamine, niacin, and pantothenic acid was applied to choline, a well-defined peak in the XIC chromatogram at 0.41 minutes was successfully observed (Figure 3.27). Despite the fact that the analyte did not bind to the HPLC column to facilitate separation, the method was accepted as independent MRM scans were to be designed for accurate identification of choline in a complex sample.

Figure 3.27: XIC of m/z 104 from a 50 μ M sample of choline, analyzed isocratically using 92% H_2O (0.1% formic acid) 8% MeOH.

A daughter ion scan was conducted to ascertain the fragment ions produced from dissociation of choline. The fragments holding the highest intensities were observed at a collision energy of 45 eV and 22 eV. At 22 eV the fragment ions were depicted at

m/z 45 and 60 (Figure 3.28), and at 45 eV the high intensity fragment ion was m/z 58 (Figure 3.29).

These fragment ions were used to create an MRM method whereby Q1 was scanning for m/z 104 and Q3 was scanning for the fragment ions at the specified collision energies. The MRM produced a distinct peak at 0.69 minutes with a significant amount of resolution (Figure 3.30).

Figure 3.28: Daughter ion scan of choline, m/z 104 at a collision energy of 22 eV.

Figure 3.29: Daughter ion scan of choline, m/z 104 at a collision energy of 45 eV.

Figure 3.30: XIC of m/z 104 from a 50 μ M sample of choline, analyzed isocratically using 92% H₂O (0.1% formic acid) 8% MeOH.

3.1.1.9 Folic Acid

Creation of a chromatography method for analysis of folic acid proved to be more challenging. An isocratic chromatography method similar to the isocratic method previously described for water-soluble vitamins was tested because further studies would not be required to analyze a complex sample of water-soluble vitamins. However, the method composed of 90% H₂O (0.1% formic acid) and 10% MeOH proved to be unsuccessful (Figure 3.31). The XIC of m/z 442 did not reveal the presence of folic acid at any specific time point in the method, however the intensity of the constant signal was at e⁶ suggesting folic acid was consistently eluting.

Figure 3.31: XIC of m/z 442 in an HPLC-MS analysis of folic acid using 90% H₂O (0.1% formic acid) and 10% MeOH isocratically.

The ineffective isocratic method lead to the investigation of a gradient method for

analysis of folic acid, as a gradient method would likely be required for analysis of the mixture of water-soluble vitamins in a complex matrix. Branching from the isocratic method, a gradient composed of H₂O (0.1% formic acid) (A) and MeOH (B) was tested. Beginning with 10% A, the gradient linearly progressed to 99% A after two minutes and was maintained for eight minutes. The chromatogram produced by this gradient yielded a more defined signal for folic acid compared to the isocratic method, however the peak is significantly distorted and thus unsuitable for quantitative studies (Figure 3.32).

Figure 3.32: XIC of folic acid, m/z 442 in an HPLC-MS analysis of folic acid using a gradient from 10% H₂O (0.1% formic acid) and 90% MeOH to 99% H₂O (0.1% formic acid) and 1% MeOH.

The gradient was then reversed to begin at a composition of 99% H₂O (0.1% formic acid) (A) and 1% MeOH (B). This was continued for 90 seconds before linearly

decreasing to 92% A, followed by a subsequent linear decrease to 10% A after seven minutes. After three and a half minutes a direct increase to 99% A occurred, and was held here for seven minutes, adding up to a run time of 24 minutes. The chromatogram produced by this gradient was similar in shape to the previous chromatogram, however folic acid eluted as a single peak and much later in the method (Figure 3.33).

Figure 3.33: XIC of an HPLC-MS analysis of folic acid using a gradient from 99% H₂O (0.1% formic acid) and 1% MeOH to 10% H₂O (0.1% formic acid) and 90% MeOH over eight minutes.

The large peak produced from the previous gradient indicated that there was potential in that gradient method as peak splitting was no longer present. As the method was 24 minutes in total, by shortening each step in the gradient it was hypothesized that further resolution of the peak would be achieved. Figure 3.35 represents an MRM scan of the gradient composed of H₂O (0.1% formic acid) (A)

and MeOH (B). The composition of 99% A and 1% B was held for 30 second before linearly decreasing to 92% A, followed by a further linear decrease to 10% A after two and a half minutes. This was held for a minute and a half before linearly increasing to 99% A for two minutes to re-equilibrate the column. The MRM scan was created using the highest intensity daughter ions which were found to be m/z 176 and 295 at a collision energy of 30 eV (Figure 3.34). This was selected as the final gradient for folic acid as resolution was significantly increased, and a well-defined peak was observed at 7.61 minutes (Figure 3.35).

Figure 3.34: Product ion scan of folic acid (m/z 442) at a collision energy of 30 eV.

Figure 3.35: MRM of an HPLC-MS analysis of folic acid using a gradient from 99% H₂O (0.1% formic acid) and 1% MeOH to 10% H₂O (0.1% formic acid) and 90% MeOH over five minutes.

3.1.1.10 Fat-soluble Vitamin Mixture

After verifying that each vitamin could be observed effectively via HPLC-MS and the chromatographic requirements for each vitamin had been established, the standard fat-soluble vitamin solution was used to study the chromatographic requirements of the vitamins when analyzed simultaneously. As β -carotene is the most hydrophobic and had the greatest need for organic solvents, other vitamins were tested on the method consisting of 30% MeOH, 70% acetonitrile. A mixture of phyloquinone, retinol and β -carotene was the first to be tested against this method, and the method successfully eluted phyloquinone (Figure 3.36) and β -carotene at 3.36 and 1.71 minutes, respectively (Figure 3.38). Unfortunately retinol elicited two distinct peaks

of similar intensities at 1.00 and 8.06 minutes (Figure 3.37), thus the method was deemed unfit.

Due to the structural versatility of the analytes it was clear that an isocratic method would be unsuccessful, as such gradient methods were then investigated. As the solvent composition for retinol and phylloquinone differed from that of β -carotene in individual studies, a gradient composed of three solvents was tested on a solution of phylloquinone, β -carotene, and retinol; as β -carotene is the most hydrophobic the goal of this gradient was to increase its retention time. The gradient began at 92% MeOH and 8% H₂O (0.1% formic acid), and was held for 17 minutes before directly switching to a composition of 70% acetonitrile (0.1% formic acid) and 30% MeOH for a total run time of 30 minutes. Unfortunately, the gradient produced a cluttered retinol chromatogram as a second peak began formation at approximately 15 minutes, and an increased number of peaks were produced upon the conversion to 70% acetonitrile in place of H₂O (Figure 3.39).

A second gradient method, consisting of MeOH (A) and acetonitrile (0.1% formic acid) (B) was tested, this one containing more gradual solvent conversions. Starting at 2% A, the gradient linearly increased to 10% A after five minutes. Three minutes later a further linear increase was completed to 45% A, and then again to 55% A after six minutes. After another minute the level of A increased to 70%, and subsequently declined to 2% one minute later. The total run time for this method was 17 minutes. Figures 3.40-3.42 depict the resulting chromatography for phylloquinone, retinol and β -carotene in an SIR scan. Well-defined peaks were observed for all three of the analytes, however additional peaks of smaller intensities were also observed in each spectrum which are not ideal for quantification.

Because the MeOH/acetonitrile solvent combination yielded more success than a solvent combination containing H₂O, variants of this gradient were tested beginning with the addition of 10% IPA into the MeOH solvent. This gradient began at 98%

MeOH (10% IPA) (A) 2% acetonitrile (0.1% formic acid) (B) and was held for 10 minutes before linearly decreasing to 2% A. After two minutes the gradient was reversed back to 98% A linearly. Sufficient success was observed using this gradient, however the observation of β -carotene was in need of improvement as there were multiple peaks toward the end of the chromatogram (Figure 3.43-3.46).

As the previous gradient had been the most successful thus far in terms of column retention and chromatographic separation, this gradient was maintained with the addition of 2 mM ammonium acetate to the MeOH (10% IPA) solvent. Ammonium acetate is a common buffer used in reverse-phase HPLC-MS analysis as it is known to improve separation and is able to form ion pairs with analytes to improve ionization.⁸³ This was tested using the same gradient, and chromatography of all analytes was drastically improved (Figure 3.47-3.50). β -carotene was observed as a resolved peak at 7.77 minutes, and α -tocopherol was consolidated into a single peak in the chromatogram as well. Due to the success of this method it was selected for HPLC-MS/MS analysis of all samples henceforth.

Figure 3.36: SIR analysis of phyloquinone using 70% acetonitrile (0.1% formic acid) and 30% MeOH isocratically.

Figure 3.37: SIR analysis of retinol using 70% acetonitrile (0.1% formic acid) and 30% MeOH isocratically.

Figure 3.38: SIR analysis of β -carotene using 70% acetonitrile (0.1% formic acid) and 30% MeOH isocratically.

Figure 3.39: MRM analysis of retinol using a gradient from 8% H₂O (0.1% formic acid) and 92% MeOH to 70% acetonitrile (0.1% formic acid) and 30% MeOH over 30 minutes.

Figure 3.40: SIR analysis of phyloquinone using a gradient from 98% acetonitrile (0.1% formic acid) and 2% MeOH, to 30% acetonitrile (0.1% formic acid) and 70% MeOH linearly.

Figure 3.41: SIR analysis of retinol using a gradient from 98% acetonitrile (0.1% formic acid) and 2% MeOH, to 30% acetonitrile (0.1% formic acid) and 70% MeOH linearly.

Figure 3.42: SIR analysis of β -carotene using a gradient from 98% acetonitrile (0.1% formic acid) and 2% MeOH, to 30% acetonitrile (0.1% formic acid) and 70% MeOH linearly.

Figure 3.43: MRM analysis of phylloquinone on a gradient from 2% acetonitrile (0.1% formic acid) and 98% MeOH (10% IPA) to the reverse over 10 minutes.

Figure 3.44: MRM analysis of retinol on a gradient from 2% acetonitrile (0.1% formic acid) and 98% MeOH (10% IPA) to the reverse over 10 minutes.

Figure 3.45: MRM analysis of β -carotene on a gradient from 2% acetonitrile (0.1% formic acid) and 98% MeOH (10% IPA) to the reverse over 10 minutes.

Figure 3.46: MRM analysis of α -tocopherol on a gradient from 2% acetonitrile (0.1% formic acid) and 98% MeOH (10% IPA) to the reverse over 10 minutes.

Figure 3.47: MRM analysis of phylloquinone on a gradient from 2% acetonitrile (0.1% formic acid) and 98% MeOH (10% IPA, 2 mM ammonium acetate) to the reverse over 10 minutes.

Figure 3.48: MRM analysis of retinol on a gradient from 2% acetonitrile (0.1% formic acid) and 98% MeOH (10% IPA, 2 mM ammonium acetate) to the reverse over 10 minutes.

Figure 3.49: MRM analysis of β -carotene on a gradient from 2% acetonitrile (0.1% formic acid) and 98% MeOH (10% IPA, 2 mM ammonium acetate) to the reverse over 10 minutes.

Figure 3.50: MRM analysis of α -tocopherol on a gradient from 2% acetonitrile (0.1% formic acid) and 98% MeOH (10% IPA, 2 mM ammonium acetate) to the reverse over 10 minutes.

3.1.1.11 Water-soluble Vitamin Mixture

In section 3.1.1.9 it was found that folic acid had the most complicated chromatography gradient of all the water-soluble vitamins, thus analysis of a complex mixture began with the final 8 minute gradient described in section 3.1.1.9. Unfortunately this was proven unsuccessful as the chromatograms corresponding to thiamine and folic acid were divided into two peaks, and pantothenic acid's signal had a significant amount of tailing. Additionally, all analytes eluted within one minute, indicating insufficient column retention (Figure 3.51-3.55).

Figure 3.51: MRM analysis of thiamine on a gradient from 99% H₂O (10 mM ammonium acetate) and 1% MeOH to 10% H₂O over five minutes, followed by the reverse.

The split peaks observed in the chromatograms may be caused by overloading the column. If there are not enough binding sites available the excess analyte will elute

Figure 3.52: MRM analysis of pantothenic acid on a gradient from 99% H₂O (10 mM ammonium acetate) and 1% MeOH to 10% H₂O over five minutes, followed by the reverse.

without binding, regardless of its affinity to the stationary phase. Thus the standard vitamin solution was diluted and the method was tested again to no avail (Figure 3.56-3.60). Folic acid held two peaks in its chromatogram, though the intensity of the early eluting peak (0.50 minutes) had significantly decreased. Pantothenic acid yielded a more complicated chromatogram as its signal was a split peak with poor resolution.

Because choline, niacin, and pantothenic acid were observed to elute from the column within one minute, it was assumed this was due to the high water content and their high water solubility. Hence the previous gradient was slightly modified to begin with 80% H₂O (10 mM ammonium acetate) and 20% MeOH for the first

Figure 3.53: MRM analysis of niacin on a gradient from 99% H₂O (10 mM ammonium acetate) and 1% MeOH to 10% H₂O over five minutes, followed by the reverse.

2.5 minutes. This gradient provided the most success thus far as all chromatograms had a high level of resolution, although the signal corresponding to pantothenic acid remained split. Additionally, all of the analytes were observed within two minutes, which is an improvement compared to prior gradients tested, however lack of column binding with this method remained a challenge (Figure 3.61-3.65).

The peak splitting of pantothenic acid was not ideal for quantification purposes as it significantly affects the area under the curve. The initial folic acid method was modified to increase the total analysis time, extend the gradient conversions, and replace 10 mM ammonium acetate with 0.1% formic acid. The derived gradient began with 99% H₂O (0.1% formic acid) (A) and 1% MeOH, and was maintained for three minutes prior to conversion to 92% A. After three minutes a decrease to

Figure 3.54: MRM analysis of folic acid on a gradient from 99% H₂O (10 mM ammonium acetate) and 1% MeOH to 10% H₂O over five minutes, followed by the reverse.

10% A was implemented and retained for two minutes. The column was then re-equilibrated with the initial conditions for two minutes, making a total run time of 10 minutes. The resulting chromatograms are depicted in Figures 3.66-3.70, whereby all of the water-soluble vitamins yielded a single peak holding a high degree of resolution. Additionally, an increased level of separation between the analytes was observed, in comparison to gradients previously tested. Due to its high performance and efficiency, this gradient was applied to all subsequent analyses of water-soluble vitamins.

Figure 3.55: MRM analysis of choline on a gradient from 99% H₂O (10 mM ammonium acetate) and 1% MeOH to 10% H₂O over five minutes, followed by the reverse.

Figure 3.56: MRM analysis of a diluted standard mixture of thiamine on a gradient from 99% H₂O (10 mM ammonium acetate) and 1% MeOH to 10% H₂O over five minutes, followed by the reverse.

Figure 3.57: MRM analysis of a diluted standard mixture of pantothenic acid on a gradient from 99% H₂O (10 mM ammonium acetate) and 1% MeOH to 10% H₂O over five minutes, followed by the reverse.

Figure 3.58: MRM analysis of a diluted standard mixture of niacin on a gradient from 99% H₂O (10 mM ammonium acetate) and 1% MeOH to 10% H₂O over five minutes, followed by the reverse.

Figure 3.59: MRM analysis of a diluted standard mixture of folic acid on a gradient from 99% H₂O (10 mM ammonium acetate) and 1% MeOH to 10% H₂O over five minutes, followed by the reverse.

Figure 3.60: MRM analysis of a diluted standard mixture of choline on a gradient from 99% H₂O (10 mM ammonium acetate) and 1% MeOH to 10% H₂O over five minutes, followed by the reverse.

Figure 3.61: MRM analysis of a standard mixture of thiamine on a gradient from 80% H₂O (10 mM ammonium acetate) and 20% MeOH to 10% H₂O over five minutes, followed by the reverse.

Figure 3.62: MRM analysis of a standard mixture of pantothenic acid on a gradient from 80% H₂O (10 mM ammonium acetate) and 20% MeOH to 10% H₂O over five minutes, followed by the reverse.

Figure 3.63: MRM analysis of a standard mixture of nicotinic acid on a gradient from 80% H₂O (10 mM ammonium acetate) and 20% MeOH to 10% H₂O over five minutes, followed by the reverse.

Figure 3.64: MRM analysis of a standard mixture of folic acid on a gradient from 80% H₂O (10 mM ammonium acetate) and 20% MeOH to 10% H₂O over five minutes, followed by the reverse.

Figure 3.65: MRM analysis of a standard mixture of choline on a gradient from 80% H₂O (10 mM ammonium acetate) and 20% MeOH to 10% H₂O over five minutes, followed by the reverse.

Figure 3.66: MRM analysis of a standard mixture of thiamine on a gradient derived from the gradient used to analyze folic acid. The gradient extended from 99% H₂O (0.1% formic acid) and 1% MeOH to 10% H₂O (0.1% formic acid) over six minutes, followed by the reverse.

Figure 3.67: MRM analysis of a standard mixture of pantothenic acid on a gradient derived from the gradient used to analyze folic acid. The gradient extended from 99% H₂O (0.1% formic acid) and 1% MeOH to 10% H₂O (0.1% formic acid) over six minutes, followed by the reverse.

Figure 3.68: MRM analysis of a standard mixture of niacin on a gradient derived from the gradient used to analyze folic acid. The gradient extended from 99% H₂O (0.1% formic acid) and 1% MeOH to 10% H₂O (0.1% formic acid) over six minutes, followed by the reverse.

Figure 3.69: MRM analysis of a standard mixture of folic acid on a gradient derived from the gradient used to analyze folic acid. The gradient extended from 99% H₂O (0.1% formic acid) and 1% MeOH to 10% H₂O (0.1% formic acid) over six minutes, followed by the reverse.

Figure 3.70: MRM analysis of a standard mixture of choline on a gradient derived from the gradient used to analyze folic acid. The gradient extended from 99% H₂O (0.1% formic acid) and 1% MeOH to 10% H₂O (0.1% formic acid) over six minutes, followed by the reverse.

3.1.2 Extraction of Vitamins from Spinach

The established methods, as described in previous sections, were then applied in a quantitative manner to the determination of the vitamin content in spinach. This was completed in a variety of ways, including vitamin content in the stems, vitamin content without the stems, vitamin content when frozen, and vitamin content when raw.

The procedure created for extraction of vitamins from spinach represents a fast, robust approach as both water-soluble and fat-soluble vitamins were extracted simultaneously. The development of this procedure was launched in the extraction of fat-soluble vitamins. Various solvents were tested with a 1.5 g portion of spinach, and all were unsuccessful at extracting the fat-soluble vitamins on their own. These experiments included incubation in hexanes, chloroform, dichloromethane, hexanes and isopropyl alcohol, and chloroform and methanol. Chromatograms of these results can be found in Appendix II, the inconsistent levels of phyloquinone and α -tocopherol specifically were clearly evident, and it was then determined that a combination of solvents may be required. This approach added the potential to extract the water-soluble vitamins simultaneously, which would be more accommodating of a high-throughput process.

As a result of preliminary solvent extraction tests (Appendix II), it was found that dichloromethane yielded the highest intensity peaks for retinol and β -carotene, thus in combination with methanol it was hypothesized that levels of phyloquinone and α -tocopherol may increase due to the cellular penetration effect of methanol. According to Yu and coworkers,⁸⁴ methanol is known as one of the most potent solvents in relation to penetration of botanical samples. It has the ability to solubilize both organic and aqueous components, making it ideal for penetration of an amphipathic cell membrane. The solvent-extraction method composed of dichloromethane and methanol (1:1) was then investigated, whereby the fat-soluble vitamins resided

in dichloromethane, and the water-soluble vitamins resided in methanol upon extraction. This was completed on a 1.5 g portion of spinach combined with 5 mL of each solvent. The fat-soluble vitamins were analyzed and quantified with some success. The levels of α -tocopherol and phylloquinone were below the LOQ, and the level of phylloquinone was also below the LOD. This method was modified to include grinding of the spinach sample, and as a result levels of β -carotene, phylloquinone, and α -tocopherol increased. However, the abundances of phylloquinone and α -tocopherol remained un-quantifiable, and the level of retinol decreased below quantification limits (Figure 3.71). The advantage in using a mortar and pestle to break down the spinach was clear in that three of the four fat-soluble vitamins experienced an increase in abundance. However, the decrease in retinol was undesirable, and because the abundance of phylloquinone and α -tocopherol did not increase above the LOQ, other solvent combinations were investigated, accompanied by the mortar and pestle technique. Additionally, the use of chlorinated solvents proved an inconvenience as there is an increased potential for observation of chlorinated species and isotopes in the spectrum.

When hexanes was analyzed in extraction of fat-soluble vitamins the abundance of β -carotene and retinol were marginally lower than those yielded by dichloromethane (Appendix II), thus further work was completed using hexanes. Derived from a method employed by Hao et al.⁸⁴ to extract α -tocopherol and other carotenoids from wet botanical tissues, the extraction method created consisted of incubation of the spinach in equal quantities of MeOH and THF (1:1), and hexanes for approximately two minutes. This was directly followed by centrifugation at 3000 rpm for 15 minutes at 4°C. THF was intended to provide the fat-soluble vitamins with a more suitable place to reside prior to the addition of hexanes as methanol is significantly polar. When spinach was exposed to the 1:1 mixture of MeOH and THF the cells were immediately lysed, as evidenced by the release of chlorophyll. The water released by

Figure 3.71: Areas under each MRM chromatogram for the fat-soluble vitamins of interest extracted from spinach using dichloromethane and methanol.

the spinach allowed the solvents to separate and prevented emulsion formation. The green solution then moved to the top layer upon addition of hexanes as chlorophyll is more hydrophobic. The volume in the top layer of the extraction was approximately 1.5x larger than the volume in the yellow bottom layer, indicating that the THF had migrated from the MeOH into the hexanes as expected. The quantification of the fat-soluble vitamins was greatly improved as α -tocopherol was above the LOQ, however improvements were required to observe phyloquinone at quantifiable levels. Improvements to the fat-soluble vitamins lead to analysis of the methanol layer, and it was found that niacin, pantothenic acid, and choline were also detected above the LOQ. Because phyloquinone, folic acid, and niacin were not present in quantifiable amounts further advancements of this method were investigated.

To increase the levels of phyloquinone, thiamine and folic acid, several variables were considered, and all samples were ground to increase surface area exposure of

the spinach to the solvents. The sample size was the first to be investigated. Initial extraction tests were performed on a 1.5 g portion of spinach as this was a reasonable sample size, and this was then compared to a 3 g sample size. Figure 3.72 depicts the difference in vitamin content extracted from each sample, and it was found that the increase in portion of spinach was statistically significant for α -tocopherol and β -carotene. The levels of retinol and phyloquinone increased, but still remained below the LOQ. As the vitamin content increased when the sample size increased, as expected, the remaining spinach extractions were carried out using 3 g of spinach.

Figure 3.72: Comparison of sample size using areas under each MRM chromatogram for the fat-soluble vitamins extracted using MeOH/THF.

It was then hypothesized that the length of time the spinach was exposed to the solvents could play a role in extraction of the vitamins. Specifically, the incubation time in MeOH/THF, as methanol penetrates the cells. This was tested on a spinach sample incubated in MeOH/THF overnight on a stir plate, the sample was also in ice to reduce the effect of degradation of the vitamins at room temperature. In compari-

son to a sample incubated for approximately two minutes, the levels of α -tocopherol, β -carotene, retinol, phyloquinone and thiamine decreased, such that retinol, phyloquinone, and thiamine fell below the LOQ. Meanwhile, the levels of pantothenic acid, niacin, and choline increased (Figure 3.73). The level of folic acid was below quantifiable limits for both extraction tests. This trend may be due to degradation of the vitamins throughout the increased incubation time, and the increased level of some water-soluble vitamins was likely due to increased water loss of the matrix that occurred as a result of the increased incubation time, as well as lack of degradation.

Figure 3.73: Comparison of incubation time in MeOH/THF using areas under each MRM chromatogram.

All of the experiments performed to this point had been completed using frozen spinach as it provided a relatively equal sample distribution in relation to leaves and stems. After freezing, the spinach was efficiently broken inside the bag prior to weighing the sample in order to protect against bias between the amount of leaves and the amount of stems present. The effect of freezing was assumed to be negligible, and using this method would allow for the vitamin content at a specific period in

the degradation process to be frozen in time. This hypothesis was confirmed upon comparison of frozen spinach and raw spinach. In Figure 3.74 it was observed that the vitamin abundance decreased for most vitamins, and increased only for retinol and phyloquinone. This decline may have been caused by increased difficulty of sample manipulation. The frozen matrix was ground more fluently with the mortar and pestle in comparison to the raw sample as it broke apart frozen pieces, and also released an increased level of water in the process. The caliber of decrease in vitamin content outweighed the level of increase, thus the spinach samples continued to be frozen prior to analysis.

Figure 3.74: Comparison of frozen and raw spinach analysis using areas under each MRM chromatogram for vitamins extracted with MeOH/THF and hexanes.

The degree to which the sample was ground also may have had an effect on the extracted vitamin content. The samples were previously ground for approximately two minutes in a mortar and pestle, however it was hypothesized that using a food blender may increase efficiency. This is because the hand blender would deliver a sharp metal surface at a high speed and would likely break open more cells, in comparison

to a dull ceramic club employed at a speed and strength that is user-dependent. This hypothesis proved to be false as the abundance of most vitamins ground using the blender was lower than the abundance of vitamins ground using the mortar and pestle. The only exceptions being phyloquinone, which increased in abundance, and niacin and folic acid which remained below the quantification limit (Figure 3.75). The decline in vitamin levels may have been caused by the increased air exposure as oxygen causes most of the vitamins to begin deteriorating. The hand blender was more tedious as spinach is a soft matrix and was easily funneled above the blade, free from mutilation.

Figure 3.75: Comparison of areas under each MRM chromatogram for the vitamins of interest when the sample was ground with a mortar and pestle versus a blender.

This result, combined with the observed decrease in retinol content when the mortar and pestle was used in the extraction with DCM/MeOH, it was hypothesized that there could be some ion suppression due to the increased level of molecules being released by the grinding process. The sample produced as a result of the solvent-extraction method is an extract from a complicated matrix, meaning the

extract samples also consist of proteins, lipids, and other molecules capable of ion suppression. The investigation into the vitamin content in the stems compared to the leaves was a result of potentially creating a sample that is less complex, yet provides the necessary quantitative data. Prior to freezing at -80°C the stems were separated from the leaves manually, and the two samples were frozen separately. These samples were analyzed as per the established protocol, and compared to a ground sample containing both stems and leaves. As per Figure 3.76, it was found that the vitamin level in the sample composed of both stems and leaves was significantly larger for almost all of the vitamins of interest. Retinol and phyloquinone were the outliers in this trend as their concentrations were largest in the stems. In fact, the abundance of phyloquinone was only quantifiable in the stem sample. The level of α -tocopherol and pantothenic acid was also increased in the stems compared to the leaves, however the abundance of these vitamins in the sample consisting of both stems and leaves was much larger overall. From here it was concluded that the stems do not cause ion suppression in the MS analysis, and significantly contribute to the quantity of vitamins in most cases, thus inclusion of the stems was maintained throughout the quantification studies.

Overall, the prevailing method applied MeOH/THF (1:1) to spinach ground with a mortar and pestle, and was then extracted with hexanes. This was enforced in further studies as it was found to be efficient in extraction of most of the vitamins of interest, the exceptions being niacin, folic acid and thiamine. Further studies into extraction of these water-soluble vitamins are recommended.

Figure 3.76: Comparison of areas under each MRM chromatogram for all 9 vitamins of interest in the leaves and stems of spinach.

3.2 Quantification of Vitamins in Spinach

3.2.1 Degradation of Spinach Over Time

After spinach is purchased, it is commonly placed in the fridge at 4°C and consumed as needed. Over time degradation of the spinach is observed, however stability of the vitamin content is unknown. A study was conducted over period of 23 days in order to investigate how the vitamin content would change over time. This was completed using non-organic spinach and the trends were applied to all spinach types as an assumption can be made that vitamin degradation patterns remain constant in the same matrix. Niacin, thiamine and folic acid were not included in these studies as they were found to be extracted inconsistently in the previous study, and at levels below limits of quantification. Figure 3.77 depicts the relative concentration levels of α -tocopherol, retinol, pantothenic acid, choline, phyloquinone, and β -carotene at day

3, day 4, day 14, day 22, and day 23, day 0 being the day the spinach was purchased.

Figure 3.77: Relative concentrations of α -tocopherol, retinol, pantothenic acid, choline, phylloquinone, and β -carotene in non-organic spinach measured intermittently over a period of 23 days.

The level of vitamin E remained relatively stable, with a slight increase observed between day 14 and day 22. This could have been due to the previously mentioned cellular response, whereby the concentration of α -tocopherol increases to increase the overall level of antioxidants and enhance cell survival. However, the statistical difference between the concentration at day 3 and the concentration on day 23 was insignificant.

The trend observed in levels of vitamin A was fairly inconsistent as a dramatic decrease was observed at day 14, and this was followed by an increase on day 22 to levels above that of day 3. The difference between the concentration observed on day 14 and on day 23 was statistically significant, whereas the concentration difference between day 3 and day 23 was not statistically significant. However, the relative error of the day 14 sample was extremely high. In comparison this error is almost

double the error observed on day 22, meaning that there may have been other factors contributing to analysis of that sample.

Pantothenic acid was found to have relatively constant concentrations throughout the spinach degradation process, aside from day 22. The increase in concentration observed at day 22 was statistically significant, however it does not follow the trend observed in values for days 3, 4, and 23 whose differences are not statistically significant from one another. The error on this value was noted to be approximately double the relative error of the concentration value found for the spinach on day 3. It is unlikely that the concentration of pantothenic acid would experience such a drastic change over a single day, as such this may have been caused by varied degradation levels of the spinach sample.

The relative concentration of choline was observed to consistently decrease from day 3 to 23, and the difference between the concentration values was statistically significant. This indicated that as the matrix degraded the choline molecules were degrading as well. This may have been as a cellular response to matrix decline. As choline possesses cell signalling functions, it may have been used to induce a type of survival process, thus concentrations were observed to decrease as the choline in the cell was depleted.

The only vitamin that complied with an increasing trend in terms of relative concentration was vitamin K. In fact, the values obtained for day 3 and day 23 were identified as statistically significant, and the final concentration was observed to be the highest relative to the remaining vitamins of interest. This may be because as the matrix and cell walls became destabilized over time an increased amount of vitamin K was released from the cells and thus extracted.

Finally, the level of β -carotene was observed to fluctuate moderately, though not at a statistically significant rate. This is consistent with the literature as previous studies on levels of β -carotene and other carotenoids show that the level of the provitamin

remains relatively consistent throughout degradation of the spinach matrix.³

3.2.2 Organic vs. Non-Organic Spinach

A study of the comparison between organic and non-organic spinach was also performed. Organic spinach purchased in a grocery store is typically more expensive than the non-organic spinach, however the difference in vitamin content is relatively unclear to the consumer. This was performed on α -tocopherol, retinol, pantothenic acid, choline, and β -carotene.

Figure 3.78: Comparison of the vitamin concentrations in organic spinach vs non-organic spinach.

It was discovered that the trend in vitamin concentration varies depending on the vitamin species (Figure 3.78). α -tocopherol was observed to decrease in concentration for organic spinach, however this difference was not statistically significant. Pantothenic acid and β -carotene also had statistically insignificant differences, meaning that the levels of those vitamins remain constant in organic spinach, compared to

non-organic spinach. Retinol and choline, on the other hand, exhibited statistically significant increases in the organic spinach. The retinol content in organic spinach was approximately double that of non-organic spinach, and the choline content was approximately four times larger in the organic spinach samples. With this information in mind it is up to the consumer to decide whether or not the price increase is valid for a higher concentration of only two vitamins.

The lack of divergence in vitamin content indicates that pesticide use does not have a direct effect on the stability of α -tocopherol, β -carotene, and pantothenic acid. However, choline and retinol may be destabilized or broken down by pesticides used to optimize growth of non-organic spinach.

4. Conclusions

Overall, two distinct methods were successfully created to analyze various fat-soluble and water-soluble vitamins using HPLC-MS², including: α -tocopherol, β -carotene, retinol, phyloquinone, pantothenic acid, thiamine, niacin, folic acid, and choline. The fat-soluble gradient was composed of acetonitrile (0.1% formic acid) and MeOH (10% IPA, 2 mM ammonium acetate), and the water-soluble gradient consisted of MeOH and H₂O (0.1% formic acid). MRM scans were designed for each vitamin in order to increase sensitivity and reliability of the analysis.

A relatively successful protocol was also designed to extract these vitamins of interest from spinach samples. Many factors were considered, such as the employed solvents, incubation times, the stem-to-leaf-ratio in the samples, whether or not the spinach was frozen, and the grinding method. Ultimately, the extraction procedure took into account both leaves and stems of frozen spinach that was ground via mortar and pestle, and was incubated for approximately two minutes in MeOH/THF (1:1) followed by extraction with hexanes.

The methods were applied to analysis of the vitamin content in common spinach samples purchased at the supermarket. It was determined that the level of phyloquinone increases, and the level of choline decreases as spinach degrades at 4°C. The concentration values of other vitamins remained relatively constant over time. Additionally, when non-organic spinach was compared to organic spinach it was discovered that levels of β -carotene and choline increased in the organic samples, indicating their sensitivity to pesticides.

5. Other Projects and Future Works

This section contains information and data related to unfinished research projects.

5.1 Trimethylation Enhancement using Diazomethane (TrEnDi)

Trimethylation Enhancement using Diazomethane (TrEnDi) is a derivatization technique that increases the sensitivity of lipids and peptides in mass spectrometry analysis. The method applies a permanent positive charge to the analyte via the addition of methyl group(s) on a functional group with a pKa value of approximately 11 or less (Figure 5.1). This effectively decreases the variety of ionization species (e.g. $[M+Na^+]$) thus enhancing sensitivity.

Figure 5.1: Schematic of the Trimethylation Enhancement using Diazomethane (TrEnDi) mechanism.

The number of methyl groups is dependent on the structure of the analyte, for example phosphatidylethanolamine acquires four methyl groups on its head group (Figure 5.2a), three on the amine, and one on the phosphate; whereas phosphatidylcholine only gains one methyl group on the phosphate moiety (Figure 5.2b).

The key component of TrEnDi is the use of $HB\dot{F}_4$. This is a strong acid that yields a non-coordinating counterion and protonates the phosphate moiety of the lipid

Figure 5.2: Schematic of Trimethylation Enhancement using Diazomethane (TrEnDi) on a) a phosphoethanolamine head group, and b) on a phosphocholine head group.

headgroup. These properties ensure that the conjugate base of the acid is unaffected by diazomethane, thus all methylation is directed at the lipid to achieve maximum yield.

5.1.1 Methods

5.1.1.1 In-solution Derivatization

N-Methyl-*N*-nitroso-*p*-toluenesulfonamide (diazald) and diazomethane reagents were synthesized as previously reported.⁸⁵ The remainder of the chemical derivatization was also performed as previously described;⁸⁶ however ethanol was added to the HBF₄ solution until a homogenous solution was observed. Following all derivatizations the modified samples were dried under N₂ and re-constituted in 100 μ L of ethanol for subsequent analysis, unless otherwise stated.

5.1.1.2 Gas-phase Derivatization

Approximately 0.1 g of *N*-Nitroso-*N*-methylurea was placed into a sealed headspace vial, along with 1 mL of diethyl ether and 3 mL of deionized H₂O. A solution of KOH

(50% m/v) was added dropwise (3 mL) through a 200 μm silica line which penetrated the cap of the headspace vial. A second silica line penetrated the opposite side of the cap and extended into the sample, contained in a 300 μL HPLC insert. After the KOH was added, the syringe was removed from the silica line, and the line was then attached to a stream of N_2 gas. Diazomethane gas produced in the headspace vial was then projected into the sample.

5.1.2 Optimization of TrEnDi for Sphingomyelin (SM) Species

Although sensitivity was increased for most phospholipid classes using methods described in section 5.1.1.1, sphingomyelin was found to be an anomaly in this trend as its sensitivity is neither increased nor decreased, despite the permanent positive charge.⁸⁷ Upon further investigation it was found that the signal is split by up to four methylation states. Based on reactivity, these are hypothesized to be applied to the phosphate moiety, the allylic alcohol of the *sn*-1 chain, the amine on the *sn*-2 chain, and the fourth may be on the alkene of the *sn*-1 chain or on the carbonyl of the *sn*-2 chain, respectively.

Figure 5.3: Unmodified sphingomyelin species (18:1/16:0).

Several variations of a dimethylglycine derivatization were tested as it was hypothesized that derivatization of the hydroxyl would also prevent the effect of diazomethane from extending beyond a single methylation state. The dimethylglycine derivatization has shown to be successful on diacylglycerol species,⁸⁸ and it was inferred that this chemistry could also be applied to phospholipids containing hydroxyl moieties. The

methods were completed according to Han and coworkers.⁸⁸ Briefly, the derivatization involves incubation of the sample with dimethylglycine, dimethylaminopyridine (DMAP) and *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide (EDC) followed by two Bligh and Dyer extractions.

Figure 5.4: Sphingomyelin (18:1/16:0) with an applied dimethylglycine derivatization.

Unfortunately, this hypothesis was not proven to be true as a number of experiments yielded unsuccessful results as the derivatization did not go to completion on SM species. An increased incubation time was tested whereby the reagents were incubated with the lipid 2x longer than in the published protocol. As a result the unmodified species exhibited an intensity higher than that of the modified SM species (Figure 5.5), but was not the most intense peak in the spectrum. An increased number of peaks above m/z 800 were observed, m/z 825 being the most intense, meaning the level of byproducts increased. This led to the consideration of product loss during the Bligh and Dyer extraction.

The Bligh and Dyer extraction was put in place to remove excess salts stemming from reaction byproducts, thus decreasing potential ion suppression. Variations of the Bligh and Dyer were tested in order to improve recovery. As high concentrations of dimethylglycine, dimethylaminopyridine, and *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide were listed in the protocol, it was noted that increased volumes of solvents may be required for this type of Bligh and Dyer extraction in comparison to a

Figure 5.5: Sphingomyelin (18:1/16:0) with an applied dimethylglycine derivatization whereby the reagents were incubated 2x longer.

typical Bligh and Dyer extraction employed for extraction of lipids from cell extracts. The ratio of solvents was kept constant and the volume of each increased, and it was found that the reaction was somewhat successful as the derivatized SM species was observed at m/z 788. However, a significant level of unmodified SM remained present at m/z 703.

Further experimentation with the Bligh and Dyer extraction was performed by increasing the volume of acidified methanol, and decreasing the level of sodium acetate. The resulting spectrum yielded a large amount of unmodified SM, and some dimethylglycine-derivatized SM. Additional peaks in the spectrum were observed above m/z 800, however these masses did not correspond to expected products of the reaction.

Figure 5.6: Precursor ion scan of sphingomyelin (18:1/16:0) with an applied dimethylglycine derivatization, whereby the volumes employed in the Bligh and Dyer solvent extraction were increased.

Figure 5.7: Precursor ion scan of sphingomyelin (18:1/16:0) with an applied dimethylglycine derivatization, whereby the volumes of acidified methanol employed in the Bligh and Dyer solvent extraction was increased, and in turn the volume of sodium acetate was decreased.

5.1.3 Stabilization of Ascorbic Acid

Famous for its deficiency disease, called 'scurvy', ascorbic acid ($C_6H_8O_6$) has many functions throughout the body. Its reducing properties make it vital in cell protection, protection of other antioxidants (such as α -tocopherol), intestinal absorption of iron, and production of amino acids.

Ascorbic acid deficiency has been noted as early as 1535, with the consequence being a condition called 'scurvy'.⁸ The deficiency became prominent on long sea voyages, men were found to be weak, in pain, with purple spots on their skin, and having loose teeth. In 1747, Dr. James Lind found that feeding the sailors oranges and lemons relived their symptoms. However, it was not until 1795 that Dr. Gilbert Blane influenced the Board of Sick and Hurt Seamen to have lemon juice issued to the British Navy.⁸ Further studies isolated ascorbic acid and elucidated the structure, leading to the 1937 Nobel prize for Medicine and the Nobel prize for Chemistry being granted to research done on vitamin C.

Figure 5.8: Chemical structure of ascorbic acid.

As it is easily oxidized, ascorbic acid often acts as a reducing agent. The pKa of the proton in the hydroxyl on C3 is approximately 3-4, and the pKa of the proton in the hydroxyl of C2 is approximately 11, allowing for facile removal of the hydroxyl proton on C3.⁸⁹ Subsequent removal of the hydroxyl proton on C2 transforms them into two carbonyl groups to the molecules, and ultimately yields the structure of dehydroascorbic acid in a reversible process.⁸⁹

Oxidation of ascorbic acid is dependent on several factors, such as the presence of oxygen, pH, and temperature causing it to lack stability, particularly in solution. Its degradation pathway has been previously reported, transforming irreversibly from dehydroascorbic acid into 2,3-diketogulonic acid, and subsequently into threonic acid and oxalic acid.^{89,90} In alkaline solution this degradation progresses rapidly, whereas in alkali solution it is much slower.

Ascorbic acid can be detected via spectrophotometric analysis in a pH-dependent manner. In acidic solution the wavelength of maximum absorbance decreases. The molar absorptivity of ascorbic acid has been debated due to its oxidation. The oxidation products do not absorb in the same region, and have a significantly smaller molar absorptivity value. Thus a low UV absorbance value will be consistently read, unless measured under anaerobic conditions. Additionally, complications arise when ascorbic acid is present in a matrix that has the same maximum absorbance. In order to mitigate this, colourimetric detection methods were developed for titration and spectrophotometric determination of ascorbic acid. More recently detection methods have moved toward chromatography, with the trimethylsilyl ether derivative allowing for analysis via gas chromatography. This was followed up by HPLC techniques as sample preparation is unnecessary, and high-performance capillary electrophoresis (HPCE) came along shortly thereafter with decreased sample time and sample quantity requirements.⁹¹

The focus of this study was to prevent oxidation and degradation of ascorbic acid using TrEnDi, as its oxidation prevented quantitative analysis. The standard sample of ascorbic acid was prepared as in section 2.1.2. After exploring published chromatography methods with limited success, ultimately a novel method was created. The procedure developed for ascorbic acid analysis via HPLC-MS/MS consisted of an isocratic elution using 70% MeOH and 30% H₂O (0.1% acetic acid) for 10 minutes, and MS analysis in negative ion mode. An MRM scan was created using the frag-

ment ions m/z 87 and 139, resulting from a daughter ion scan previously performed at a collision energy of 35 eV (Figure 5.9). The MRM scan revealed the presence of ascorbic acid at 1.01 minutes with sufficient resolution (Figure 5.10).

Figure 5.9: Daughter ion scan of a standard solution of ascorbic acid at a collision energy of 35 eV in negative ion mode using an isocratic method of 70% MeOH and 30% H₂O (0.1% acetic acid).

Throughout the method development process, it was noted that ascorbic acid transformed into dehydroascorbic acid (m/z 173) and threonic acid (m/z 135) fairly rapidly in solution. The caliber of this degradation was such that construction of a calibration curve using HPLC-MS/MS was impossible as the ascorbic acid would fall below the LOQ within 30 minutes (Figure 5.11-5.12), and the same trend was observed when the sample was suspended in methanol.

Figure 5.10: HPLC-MS/MS of a standard solution of ascorbic acid in negative ion mode using an isocratic method of 70%MeOH and 30% H₂O (0.1% acetic acid).

Additionally Figure 5.13-5.14 depict the spectra yielded immediately following sample preparation and then 90 minutes later. The intensity of m/z 175 clearly decreases from 6.24×10^7 to 2.39×10^7 , and the intensity of m/z 173 increases. It should be noted that the presence of m/z 135 is not immediately observed as the conversion from dehydroascorbic acid is at a slower rate than the conversion from ascorbic acid to dehydroascorbic acid, however it remains present and its intensity is ever increasing.⁹²

The limited success of some chromatography methods was likely due to the presence of ammonium hydroxide in the HPLC solvent system, a common proton acceptor when performing MS analysis in negative ion mode. The ammonium hydroxide would immediately oxidize ascorbic acid in solution at such a high pH. As oxidation was also observed in using the 0.1% acetic acid solvent system, it was hypothesized that

Figure 5.11: MRM scan of 50 μM ascorbic acid in H_2O , 20 minutes after solution preparation.

a reducing agent may prevent, or at least slow the rate of oxidation. Nevertheless, when β -mercaptoethanol was added to a sample of ascorbic acid, the analyte was no longer observed after 30 minutes (Figure 5.15-5.16).

It was hypothesized that applying a methyl group to at least one of the —OH moieties on C2 and/or C3 will prohibit degradation. Although ascorbic acid has the propensity to receive up to four methyl groups, the addition of a —CH_3 to only one of the C2 or C3 hydroxyl groups would prevent further oxidation and overall degradation because only a single proton would potentially be removed from the other hydroxyl. If two methyl groups are added via TrEnDi, oxidation of both hydroxyls is prevented. However if the methylation is directed at the hydroxyl groups on C5 and C6 this hypothesis will be proven untrue.

Figure 5.12: MRM scan of 40 μM ascorbic acid in H_2O , 30 minutes after solution preparation.

Research thus far has proven ascorbic acid receives a single methyl group using methods described in Section 5.1.1.2. Following purification on a TLC plate, this can be observed in negative ion mode at m/z 189 $[\text{M}+\text{CH}_3]^-$ (Figure 5.17), or in positive ion mode at m/z 191 $[\text{M}+\text{CH}_3]^+$ and 213 $[\text{M}+\text{CH}_3+\text{Na}]^+$ (Figure 5.18).

The chromatography trend of the methylated ascorbic acid was also briefly studied using the same isocratic method used to observe unmodified ascorbic acid. XIC's of m/z 191 and 213 in positive ion mode were fairly convoluted (Figure 5.19 and 5.21), however their MRM scans show improvement in comparison (Figure 5.20 and 5.22). This is likely due to the presence of other species in the system holding the same m/z as methylated ascorbic acid, the MRM scan eliminates these interferences and presents only the analyte of interest based on its fragment ions.

Figure 5.13: TIC scan of 50 μ M ascorbic acid in H_2O immediately following sample preparation.

Figure 5.14: TIC scan of 50 μ M ascorbic acid in H_2O analyzed 90 minutes after sample preparation.

Figure 5.15: MRM scan of ascorbic acid and β -mercaptoethanol immediately following sample preparation.

Figure 5.16: MRM scan of ascorbic acid and β -mercaptoethanol analyzed 30 minutes after sample preparation.

Figure 5.17: Mass spectrum of methylated ascorbic acid in negative ion mode (m/z 189).

Figure 5.18: Mass spectrum of methylated ascorbic acid in positive ion mode (m/z 191, 213).

Figure 5.19: XIC of m/z 191 in positive ion mode, corresponding to protonated methylated ascorbic acid.

Figure 5.20: MRM scan of protonated methylated ascorbic acid (m/z 191) in positive ion mode.

Figure 5.21: XIC of m/z 213 in positive ion mode, corresponding to sodiated methylated ascorbic acid.

Figure 5.22: MRM scan of sodiated methylated ascorbic acid (m/z 213) in positive ion mode.

5.1.4 Conclusions

Overall the dimethylglycine chemistry was deemed unfit for SM, and further experiments are currently being investigated to accommodate TrEnDi methods to SM species. Additionally, preliminary research on alternative charge tags is being conducted with 2-fluoro-1-methylpyridinium *p*-toluenesulfonate. As this charge tag has had success with various phenolic species,^{93,94} it is hypothesized that the allylic alcohol moiety is acidic enough for the reaction to proceed, and ultimately apply a positive charge on the lipid.

Furthermore, it can be concluded that TrEnDi was successfully applied to ascorbic acid. An ascorbic acid species that had acquired a single methylation was remarkably observed after purification on a TLC plate. Further investigation into other methylated species that would have separated on the TLC plate should be completed. Additionally, TrEnDi reaction conditions may be adjusted to better suit methylation of ascorbic acid, and potentially drive the reaction to two or four full methylation states.

References

- [1] Anjana, S.; Iqbal, M. *Agron. Sustain. Dev.* **2007**, *27*, 45–57.
- [2] Roberts, J. L.; Moreau, R. *Food & Function* **2016**, *7*.
- [3] Bergquist, S. Å.; Gertsson, U. E.; Olsson, M. E. *J. Sci. Food Agric.* **2006**, *86*, 346–355.
- [4] Bramley, P.; Elmadfa, I.; Kafatos, A.; Kelly, F.; Manios, Y.; Roxborough, H.; Schuch, W.; Sheehy, P.; Wagner, K.-H. *J. Sci. Food Agric.* **2000**, *80*, 913–938.
- [5] Van den Berg, H.; Faulks, R.; Granado, H.; Hirschberg, J.; Olmedilla, B.; Sandmann, G.; Southon, S.; Stahl, W. *J. Sci. Food Agric.* **2000**, *80*, 880–912.
- [6] Thomaier, S.; Specht, K.; Henckel, D.; Dierich, A.; Siebert, R.; Freisinger, U. B.; Sawicka, M. *Renewable Agriculture and Food Systems* **2015**,
- [7] Combs, G. F.; McClung, J. *The Vitamins: Fundamental Aspects of Nutrition and Health*, 5th ed.; Academic Press: London, UK, 2012; Chapter 3, pp 33–70.
- [8] Davies, M.; Austin, J.; Partridge, D. *Vitamin C: Its Chemistry and Biochemistry*; The Royal Society of Chemistry: Cambridge, 1991.
- [9] Thurnham, D. I. *J. Sci. Food Agric.* **2007**, *87*, 13–39.
- [10] Packer, J.; Slater, T.; Willson, R. *Nature* **1979**, *278*, 737–738.
- [11] Munne-Bosch, S. *J. Plant Physiol.* **2005**, *162*, 743–748.
- [12] Saini, R.; Keum, Y.-S. *Food Res. Int.* **2016**, *82*, 59–70.
- [13] Zempleni, J.; Rucker, R. B.; McCormick, D. B.; Suttie, J. W. *Handbook of Vitamins*, 4th ed.; CRC Press: Boca Raton, FL, 2007.

- [14] Sokol, R. J.; Kayden, H. J.; Bettis, D. B.; Traber, M. G.; Neville, H.; Ringel, S.; Wilson, W. B.; Stumpf, D. A. *J. Lab. Clin. Med.* **1988**, *111*, 548–559.
- [15] Brin, M. F.; Pedley, T. A.; Emerson, R. G.; Lovelace, R. E.; Gouras, P.; MacKay, C.; Kayden, H. J.; Levy, J.; Baker, H. *Neurology* **1986**, *36*, 669–673.
- [16] Traber, M. G.; Sokol, R. J.; Ringel, S. P.; Neville, H. E.; Thellman, C. A.; Kayden, H. J. *N. Engl. J. Med* **1987**, *317*, 262–265.
- [17] Saha, S.; Walia, S.; Kundu, A.; Pathak, N. *Anal. and Bioanal. Chem* **2013**, *405*, 9285–9295.
- [18] Castenmiller, J.; West, C. *Annu. Rev. Nutr.* **1998**, *18*, 19–38.
- [19] Yeum, K.-J.; Russell, R. *Annu. Rev. Nutr.* **2002**, *22*, 483–504.
- [20] de Pee, S.; West, C. *Eur. J. Clin. Nutr.* **1996**, *50*, S38–53.
- [21] Goodman, D.; Huang, H.; Kanai, M.; Shiratory, T. *J. Biol. Chem.* **1967**, *242*, 3543–3544.
- [22] Fidge, N.; Goodman, D. *J. Biol. Chem.* **1968**, *243*, 4372–4379.
- [23] De Leenheer, A.; Lambert, W.; De Ruyter, M. *Modern Chromatographic Analysis of the Vitamins*, vol. 30 ed.; Marcel Dekker, Inc.: New York, 1985.
- [24] Rivera, S. M.; Christou, P.; Canela-Garayoa, R. *Mass Spectrom. Rev.* **2014**, *33*, 353–372.
- [25] Rivera, S.; Canela-Garayoa, R. *J. Chromatogr. A* **2012**, *1224*, 1–10.
- [26] Suthutvoravoot, S.; Olsen, J. *Am. J. Clin. Nutr.* **1974**, *27*, 883.
- [27] McBee, J. *Prog. Retin. Eye Res.* **2001**, *20*, 469.
- [28] Gunderson, T.; Blomhoff, R. *J. Chromatogr. A* **2001**, *935*, 13–43.

- [29] Almquist, H.; Stokstad, E. *Nature* **1935**, *136*, 31.
- [30] Almquist, H.; Stokstad, E. *J. Biol. Chem.* **1935**, *111*, 105–113.
- [31] Sommer, P.; Kofler, M. *Vitam. Horm.* **1966**, *24*, 349–399.
- [32] Shearer, M. *Adv. Chromatogr.* **1983**, *21*, 243–301.
- [33] Davidson, K.; Sadowski, J. *Meth. Enzymol.* **1997**, *282*, 408–421.
- [34] McCarthy, P.; Harrington, D.; Shearer, M. *Meth. Enzymol.* **1997**, *282*, 421–433.
- [35] McEntyre, C.; Slow, S.; Lever, M. *Anal. Chim. Acta* **2009**, *644*, 90–94.
- [36] Hefni, M.; McEntyre, C.; Lever, M.; Slow, S. *Food Anal. Methods* **2015**, *8*, 2401–2408.
- [37] Bruce, S.; Guy, P.; Rezzi, S.; Ross, A. *J. Agric. Food Chem.* **2010**, *58*, 2055–2061.
- [38] Xiong, Y.; Zhao, Y.-Y.; Goruk, S.; Oilund, K.; Field, C.; Jacobs, R.; Curtis, J. *J. Chromatogr. B* **2012**, *911*, 170–179.
- [39] Zhao, Y.-Y.; Xiong, Y.; Curtis, J. *J. Chromatogr. A* **2011**, *1218*, 5470–5479.
- [40] Halstead, C.; Villanueva, J.; Devlin, A.; Chandler, C. *J. Nutr.* **2002**, *132*, 2367S–2372S.
- [41] Scholl, T.; Johnson, W. *Am. J. Clin. Nutr.* **2000**, *71*, 1295S–1303S.
- [42] Shaw, G.; Schaffer, D.; Velie, E.; Morland, K.; Harris, J. *Epidemiology* **1995**, *6*, 219–226.
- [43] Botto, L.; Mulinare, J.; Erickson, J. *Am. J. Epidemiol.* **2000**, *151*, 878–884.
- [44] Czeizel, A. *Am. J. Med. Genet.* **1996**, *62*, 179–183.

- [45] Eitenmiller, R.; Landen, W. In *Vitamin analysis for the health and food sciences*; Eitenmiller, R. R., Landen, W. O., Eds.; CRC Press: Boca Raton, FL, 1999; pp 411–466.
- [46] Tamura, T. *J. Nutr. Biochem.* **1998**, *9*, 285–293.
- [47] Rajakumar, K. *South. Med. J.* **2000**, *93*, 272–7.
- [48] Hegyi, J.; Schwartz, R. A.; Hegyi, V. *Int. J. Dermatol.* **2004**, *43*, 1–5.
- [49] Fontaine, J.; Horr, J. *Agribiol. Res.* **1993**, *46*, 10–19.
- [50] Liu, M.; Zhang, D.; Wang, X.; Zhang, L.; Han, J.; Yang, M.; Xiao, X.; Zhang, Y.; Liu, H. *J. Chromatogr. B* **2012**, *904*, 107–114.
- [51] Valls, F.; Sancho, M. T.; Ferná Ndez-Muiñ, M. A.; Checa, M. A. *J. Agric. Food Chem.* **2000**, *48*, 3392–3395.
- [52] Webb, M.; Abeil, C. *Nat. Prod. Rep.* **2004**, *21*, 695–721.
- [53] Peknington, D.; Snell, E. E.; Williams, R. J. *J. Biol. Chem.* **1940**, *135*, 213–222.
- [54] Finglas, P. M.; Faulks, R. M.; Morris, H. C.; Scott, K. J.; Morgan, M. R. A. *J. Micronutr. Anal.* **1988**, *4*, 47–59.
- [55] Gonthier, A.; Fayol, V.; Viollet, J.; Hartmann, D. J. *Food Chem.* **1998**, *63*, 287–294.
- [56] Hudson, T. S.; Subramanian, S.; Allen, R. J. *J. AOAC Int.* **1984**, *67*, 994–998.
- [57] Rychlik, M. *J. Agric. Food Chem.* **2000**, *48*, 1175–1181.
- [58] Manzetti, S.; Zhang, J.; Van Der Spoel, D. *Biochemistry* **2014**, *53*, 821–835.
- [59] Kaczmarek, M.; Nixon, P. *Clin. Chim. Acta* **1983**, *130*, 249–256.

- [60] Losa, R.; Sierra, M.; Fernandez, A.; Blanco, D.; Buesa, J. *J. Pharm. Biomed. Anal.* **2005**, *37*, 1025–1029.
- [61] Li, H. B.; Chen, . E. *Chromatographia* **2001**, *54*, 270–273.
- [62] Berridge, J. C. *Trends Anal. Chem.* **1984**, *3*, 5–10.
- [63] Anderson, D. J. *Anal. Chem.* **1995**, *67*, 475R–486R.
- [64] Majors, R. *LCGC North America* **2003**, *21*, 19–26.
- [65] Bird, I. M. *British Medical Journal* **1989**, *299*, 783–787.
- [66] Murray, K. K. *J. Chromatogr. A* **2010**, *1217*, 3922–3928.
- [67] Baldwin, M.; McLafferty, F. *Org. Mass Spectrom.* **1973**, *7*, 1111–1112.
- [68] Gosetti, F.; Mazzucco, E.; Zampieri, D.; Gennaro, M. C. *J. Chromatogr. A* **2010**, *1217*, 3929–3937.
- [69] Glish, G. L.; Vachet, R. W. *Nat. Rev.* **2003**, *2*, 141–150.
- [70] Vestal, M. L. *Chem. Rev.* **2001**, *101*, 361–375.
- [71] De Hoffmann, E.; Charette, J.; Stroobant, V. *Mass Spectrometry Principles and Applications*; John Wiley & Sons, Ltd.: Paris, France, 1996.
- [72] Pavia, D.; Lampman, G.; Kriz, G.; Vyvyan, J. *Introduction to Spectroscopy*, 5th ed.; Brooks Cole: Belmont, 2015.
- [73] Dole, M.; Mack, L.; Hines, R.; Mobley, R.; Ferguson, L.; Alice, M. *J. Chem. Phys.* **1968**, *49*, 2240.
- [74] Fenn, J. B.; Mann, M.; Meng, C.; Wong, S.; Whitehouse, C. *Science* **1989**, *246*, 4926.

- [75] Konermann, L.; Rodriguez, A. D.; Liu, J. *Anal. Chem.* **2012**, *84*, 6798–6804.
- [76] Konermann, L.; Ahadi, E.; Rodriguez, A. D.; Vahidi, S. *Anal. Chem.* **2012**, *85*, 2–9.
- [77] Dawson, P. H. *Quadrupole Mass Spectrometry and its Applications*; Elsevier Scientific Publishing Company: New York, 1976.
- [78] Douglas, D. *Mass Spec. Rev.* **2009**, *28*, 937–960.
- [79] Douglas, D.; French, J. *J. Am. Mass Spectrom.* **1992**, *3*, 398–408.
- [80] Zafra-Gómez, A.; Garballo, A.; Morales, J. C.; García-Ayuso, L. E. *J. Agric. Food Chem.* **2006**, *54*, 4531–4536.
- [81] Cellar, N. A.; McClure, S. C.; Salvati, L. M.; Reddy, T. M. *Anal. Chim. Acta* **2016**, *934*, 180–185.
- [82] Van Breemen, R. B.; Nikolic, D.; Xu, X.; Xiong, Y.; Van B B C Lieshout, M.; West, C. E.; Schilling, A. B. *J. Chromatogr. A* **1998**, *794*, 245–251.
- [83] Lim, C. K.; Peters, T. J. *J. Chromatogr* **1984**, *316*, 397–406.
- [84] Hao, Z.; Parker, B.; Knapp, M.; Yu, L. *J. Chromatogr. A* **2005**, *1094*, 83–90.
- [85] Wasslen, K.; Tan, I.; Manthorpe, J.; Smith, J. *Anal. Chem.* **2014**, *86*, 3291–3299.
- [86] Wasslen, K.; Canez, C.; Lee, H.; Manthorpe, J. M.; Smith, J. *Anal. Chem.* **2014**, *86*.
- [87] Canez, C. R.; Shields, S. W. J.; Bugno, M.; Wasslen, K. V.; Weinert, H. P.; Willmore, W. G.; Manthorpe, J. M.; Smith, J. C. *Analytical Chemistry* **2016**, *88*, 6996–7004.
- [88] Wang, M.; Hayakawa, J.; Yang, K.; Han, X. *Anal. Chem.* **2014**, *86*, 2146–2155.

- [89] Szultka, M.; Buszewska-Forajta, M.; Kaliszan, R.; Buszewski, B. *Electrophoresis* **2014**, *35*, 585–592.
- [90] Deutsch, J. *Anal. Biochem.* **1998**, *260*, 223–229.
- [91] Davey, M. W.; Montagu, M. V.; Inze, D.; Sanmartin, M.; Kanellis, A.; Smirnoff, N.; Benzie, I. J.; Strain, J. J.; Favell, D.; Fletcher, J. *J. Sci. Food Agric.* **2000**, *80*, 825–860.
- [92] Munné-Bosch, S. *J. Plant Physiol.* **2005**, *162*, 743–748.
- [93] Thieme, D.; Sachs, H.; Thevis, M. *J. Mass Spectrom.* **2008**, *43*, 974–979.
- [94] Beasley, E.; Francese, S.; Bassindale, T. *Anal. Chem.* **2016**, *88*, 10328–10334.

Appendix I: Calibration Curves

Figure 5.23: Calibration curve created for thiamine.

Figure 5.24: Calibration curve created for pantothenic acid.

Figure 5.25: Calibration curve created for niacin.

Figure 5.26: Calibration curve created for choline.

Figure 5.27: Calibration curve created for folic acid.

Figure 5.28: Calibration curve created for α-tocopherol.

Figure 5.29: Calibration curve created for β -carotene.

Figure 5.30: Calibration curve created for retinol.

Figure 5.31: Calibration curve created for phylloquinone.

Appendix II: Chromatograms

Figure 5.32: Chromatogram of fat-soluble vitamins extracted using 10mL CHCl_3 incubated with 1.5 g of spinach for 10 minutes. Top to bottom: phyloquinone, retinol, β -carotene, α -tocopherol. The extraction did not yield any α -tocopherol or phyloquinone.

Figure 5.33: Chromatogram of fat-soluble vitamins extracted using a Bligh and Dyer style extraction consisting of 3 mL H₂O, 4 mL MeOH, and 2x 4 mL CHCl₃. Top to bottom: phylloquinone, retinol, β -carotene, α -tocopherol. The extraction did not yield any α -tocopherol or phylloquinone.

Figure 5.34: Chromatogram of fat-soluble vitamins extracted using 10 mL of dichloromethane. Top to bottom: phylloquinone, retinol, β -carotene, α -tocopherol. The extraction did not yield any α -tocopherol or phylloquinone.

Figure 5.35: Chromatogram of fat-soluble vitamins extracted using 10 mL of hexanes. Top to bottom: phyloquinone, retinol, β -carotene, α -tocopherol. The extraction did not yield any phyloquinone, and minimal amounts of α -tocopherol.

Figure 5.36: Chromatogram of fat-soluble vitamins extracted using 10mL of hexanes and 10 mL of isopropyl alcohol on 1.5 g of spinach. Top to bottom: phyloquinone, retinol, β -carotene, α -tocopherol. The extraction did not yield any α -tocopherol or phyloquinone.