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Application of Analytical Techniques in Food Product Development

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APPLICATION OF ANALYTICAL TECHNIQUES IN FOOD PRODUCT DEVELOPMENT

BY

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This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy in Chemistry degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this dissertation does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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THIS DISSERTATION IS DEDICATED TO MY MOTHER AND SISTER

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LIST OF ABBREVIATIONS

AC	Allyl cyanide
AITC	Allyl isothiocyanate
ASE	Accelerated solvent extraction
ATC	Allyl thiocyanate
BSTFA	Bis-(trimethylsilyl)-trifluoroacetamide
CAR	Carboxen
CE	Capillary electrophoresis
CE	Collision energy
CETP	1-Cyano-2,3-epithiopropane
CW	Carbowax
CXP	Collision cell exit potential
DA	Decanoic Acid
DAD	Diode array detection
DLLME	Dispersive liquid-liquid microextraction
DSC	Differential scanning calorimetry
d-SPE	Dispersive solid-phase extraction
DVB	Divinylbenzene
DVB	Divinylbenzene
EMR	Enhanced matrix removal
ESI-MS	Electron spray ionization- mass spectrometer
ESP	Epithiospecifier protein

FD	Florescence detector
FTIR	Fourier-transform infrared spectroscopy
GC	Gas chromatography
GCB	Graphitized carbon black
GRAS	Generally recognized as safe
HLB	Hydrophilic-lipophilic-balanced
HPLC	High-pressure liquid chromatography
HS-SPME-GC-MS	Headspace-solid-phase microextraction-gas chromatography-mass spectrometry
IFST	Institute of Food Science and Technology
ITC	Isothiocyanate
ITC	Isothermal Titration Calorimetry
LC	Liquid chromatography
LLE	Liquid-liquid extraction
LOD	Limit of detections
LOQ	Limit of quantification
MAE	Microwave-assisted extraction
MCX	Mixed mode cationic exchange
MEPS	Microextraction by packed sorbent
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MSPD	Matrix solid-phase dispersion

NP-HPLC	Normal-phase high-performance liquid chromatography
OA	Oleic Acid
PA	Polyacrylate
PA	Palmitic Acid
PDMS	Polydimethylsiloxane
PE	Pulsed-electric field extraction
PEITC	2-Phenylethylisothiocyanate
PITC	Phenyl-ITC
PLE	Pressurized liquid extraction
PPM	Parts per million
PSA	Primary, secondary amine
PTFE	Polytetrafluoroethylene
PXRD	Powder X-ray diffraction spectrum
RP-HPLC	Reverse-phase high-performance liquid chromatography
SA	Stearic Acid
SAX	Strata strong anion exchange
SBSE	Stir-bar sorptive extraction
SFE	Supercritical fluid extraction
SPE	Solid-phase extraction
SPME	Solid-phase micro-extraction

UHPLC	Ultra-high-pressure liquid chromatography
USDA	United States Department of Agriculture
UV	Ultraviolet
ΔH	Enthalpy
ΔS	Entropy

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ABSTRACT

APPLICATION OF ANALYTICAL TECHNIQUES IN FOOD PRODUCT DEVELOPMENT

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Products are analyzed in major sectors of food industry to meet regulatory standards, food safety, quality control, and research and development. Analytical and extraction techniques have been applied for simultaneous investigation of multiple product characteristics. Food analysis faces critical challenges due to the complex composition of food, leading to matrix effects that result in loss of accuracy and reliability. The analytical techniques require an efficient sample preparation to avoid or minimize matrix effects. Solid-phase extraction has been widely used to address this issue.

In this dissertation, the aim is to apply analytical techniques in food product development.

To accomplish this, the objectives of this work are: 1) to investigate the factors that influence the shelf life of horseradish products. 2) study the interaction between lipid classes and Enhanced Matrix Removal (EMR-Lipid), thereby developing a fundamental understanding of lipid-binding properties, and 3) to determine the concentration of resveratrol in wines from grapes grown in colder climates.

In **chapter II**, we utilize headspace-solid-phase microextraction-gas chromatography-mass spectrometry method to determine the AITC concentration in

horseradish products and study the influence of various factors on product shelf life. The samples stored at room temperature possess a shorter shelf life compared to the refrigerated samples. Fall-harvest horseradish possesses higher concertation of AITC compared to spring harvest, and the change in ingredient affects the shelf life of the product (stabilizer, salt, water).

In **chapter III**, we study the molecular interactions in host/guest complexes. The combined results from various physicochemical techniques depicts complex formation between the polymer and lipids evaluated. The results from differential scanning calorimetry (DSC) and isothermal titration calorimetry (ITC) analysis further confirms complexation between the polymer and the lipids. The ITC indicates involvement of hydrogen bonding with hydrophobic interaction driving the complex formation.

In **chapter IV**, we have developed a reversed-phase high-performance liquid chromatography (RP-HPLC) method for the determination of *trans*-resveratrol in red wines by direct injection without sample pre-treatment. The study depicts that the concertation of resveratrol in wine from grapes grown in South Dakota were found to be in line with the resveratrol concentration in wine grown in other climates.

CHAPTER I

INTRODUCTION

1.1. Food Analysis

Food analysis deals with the development, application, and study of the analytical procedure(s) characterizing the food properties, with regards to their constituents, sensory attributes, physicochemical properties, and contaminants. This information helps to understand the factors that affect the quality of the food product(s). Furthermore, it provides information that helps us to develop safe, nutritious, and consumer desired food product(s). Chemists from government agencies, food industries and universities perform the analysis to ascertain food product quality, implementing compliance with national and international food standards, safety, quality, regulatory enforcements, ingredient, pricing, nutrient labeling requirements, etc. Food analysis is performed at various stages, starting from the raw materials (post-harvest handling and storage) through production until the end product.

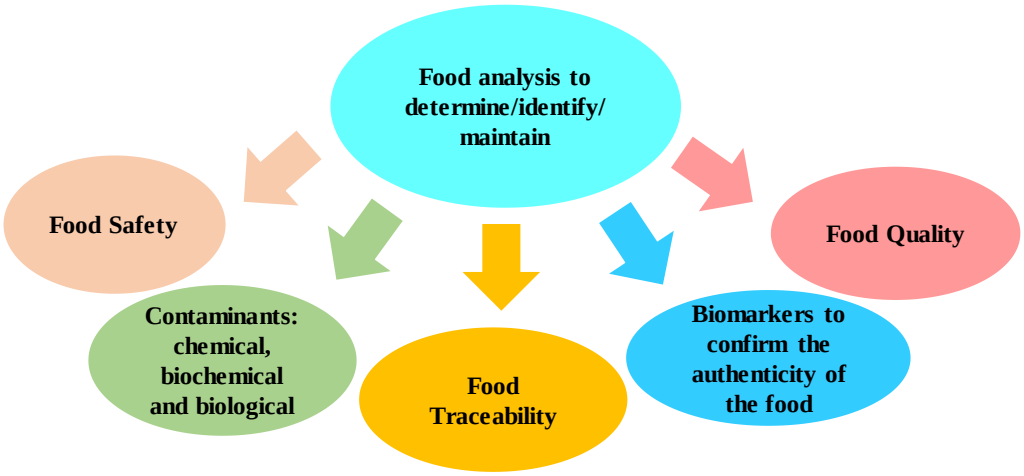

Figure 1.1: The qualitative and quantitative analysis of food products.

The food properties that need to be studied depends on the source of food, (animal and plant-based), where sensory analysis such as appearance, taste, texture, color, and flavor are very critical aspects of sensory quality followed by the food component, shelf life, stability, etc. Though the available separation and modern techniques such as gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) are highly sensitive and effective. However, due to the increasing food diversity, food chemists are facing a challenge to develop more effective and sophisticated analytical techniques to offer improved qualitative and quantitative results. The idea is to provide with enhanced sensitivity, accuracy, precision, selectivity, and speed of analysis[1].

There are large number of food properties that are analyzed to determine the factors involved in the food production, such as processing, addition of active ingredient, contamination, and inactive toxins, to ensure compliance with the food and trade laws ensuring food safety and traceability of adulteration, contaminants and pesticides residues, the characterization of foods (sensory characteristics, rheology, morphology, structure, physicochemical, or microbial content, etc.). These characteristics influence food quality, safety, and consumer acceptance[2, 3]. Food being a complex matrix with a range of chemical substances it is a challenging task to isolate and analyze individual components from the food matrix. It is essential to select a suitable sample preparation method for the analysis of components from the food matrix.

1.2. Present Challenges and Approaches in Food Analysis

The conventional analytical methods are time-consuming, tedious, requires a large amount of organic solvent, creates organic waste, contamination, and loss of sample,

eventually contributing to the cost. The complexity of the food samples requires a multistep extraction technique for effective analysis. Additionally, the extraction and purification technique depend on the, sample composition, analyte type, and concentration. In food safety, the persistent analytical challenge is to present results regarding the official guidelines without affecting the method properties such as accuracy, precision, sensitivity, specificity, selectivity, and recovery. This is because of increased food industry expansion, new agricultural practices, pollution, and growing consumer preference. LC-MS and GC-MS are frequently used for the separation, identification and quantification of contaminants in food[4, 5]. The advancement is leading to the development of more sensitive, and rapid analytical techniques to be able to detect compounds of interest in food. Despite these advancements, alternatives are required to recognize and solve the associated drawbacks, such as matrix effect, which may not influence the analyte but might impair detection[6]. In general, the efficiency of an analytical method depends on the technique used, the sample matrix type, and the analyte type[2].

1.3. Matrix Effect

Sample matrix interference brings limitations in the analysis by affecting the accuracy, precision, reproducibility, and linearity of the methods. This effect from co-eluting residual matrix component from the sample is termed as the matrix effect[7]. The most common matrix effect occurs in mass spectrometric detection as suppressed or enhanced ionization of the target analytes[8]. Matrix effect can be classified as absolute matrix effect and the relative matrix effect. The absolute matrix effect is the difference between the equally concentrated analyte in solvent and matrix extracts response whereas

the relative matrix effect could be defined as the variation of absolute matrix effect between different batches of the same matrix[9]. Matrix effect could be, endogenous (caused by compounds inherently present in the sample such as lipids, protein, phospholipids) or exogenous (caused by excipients, reagents utilized, and analyte stabilizer)[10]. Matrix effect could either lead to an increase or decrease in the sensitivity over time, increased baseline, alter the precision of the result, retention time change, or even tailing of the chromatographic peak[11].

One of the ways to avoid matrix effect is to achieve as clean as possible, and matrix-free sample before it is introduced to the analytical instrument. Therefore, sample preparation procedures play a crucial role in reducing the matrix effect. The sample preparation method type to minimize the matrix effect is governed by the analyte and the sample matrix type[11, 12]. However, it is a tedious procedure and might have the risk of sample loss. For example; the matrix interference from lipids could cause problems when analyzing trace residues in fat-containing samples[13]. However, the methods currently employed for matrix removal, often make the sacrifice between analyte recovery and the extraction of target analytes along with the lipids.

1.4. Sample Preparation and Matrix Effect

In most cases, food samples need some preliminary sample preparation. Sample preparation is a critical step in food analysis. Sample preparation, such as extraction, concentration, and sample clean-up, affects the reliability and accuracy of the analysis. A typical step in food analysis is mentioned in Figure 1.2.

Consequently, the sample preparation affects the data obtained, that may not be corrected or modified. Sample preparation method could be a significant bias step in an analytical method for food analysis as the experimental data accuracy, reproducibility, and confidence primarily depend on the accuracy of the analyte separation and clean-up technique[14]. Advanced analytical instruments have become more sensitive, demanding matrix-free samples that require multi-step sample preparation method. In case of the determination of analytes present in trace-level in complex matrices, there is often need of extensive sample-preparation protocols before the analysis that includes, extraction, clean-up followed by pre-concentration step (to achieve adequate sensitivity). Simpler sample-preparation routines are adapted to decrease the sample processing time and to eliminate or minimize the errors associated with each stage of the process. Automation in analytical procedures, including sample preparation, improves reproducibility, and decreases the labor[15, 16]. In recent years, the advancement in the analytical processes has been achieved, to prepare food and environmental samples for extraction and investigation of contaminants and residues. Modern techniques that are faster, efficient, less expensive, compared to the conventional methods have replaced the classical method.

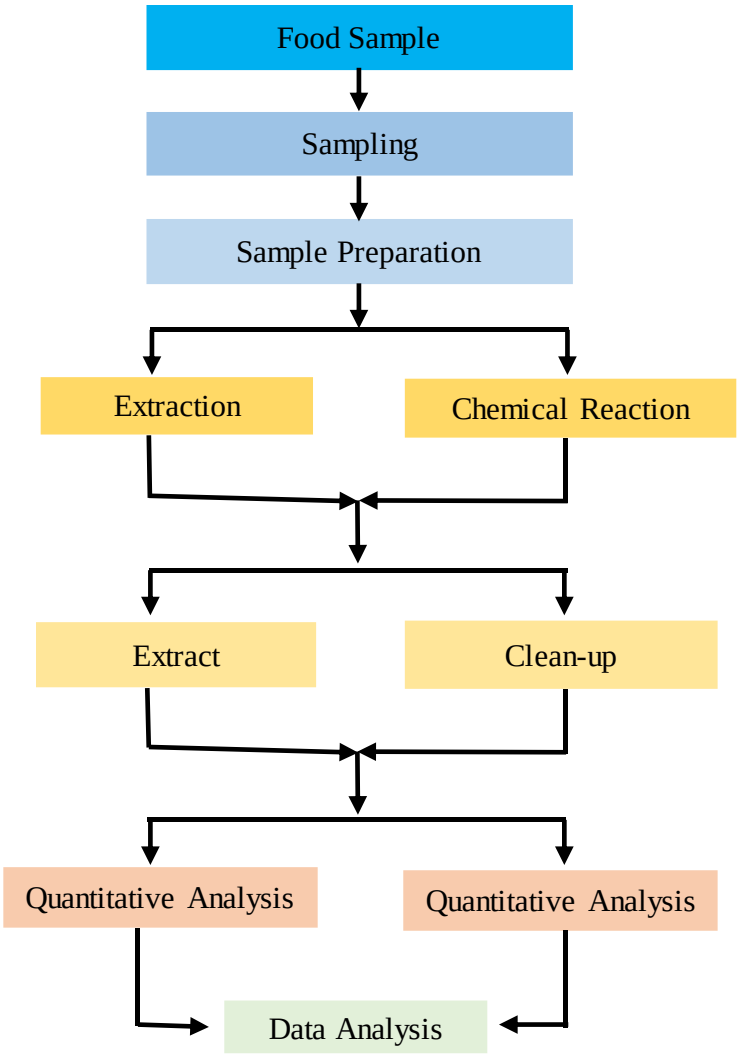

Figure 1.2: A flowchart depicting the steps in food analysis from sampling to data interpretation[17].

Conventional Extraction Techniques

Conventional extraction methods include two major types: solid-liquid extraction and liquid-liquid extraction[18]. High solvent and energy consumption, the risk of thermal degradation of heat-labile components, and prolonged extraction time are main disadvantage associated with these methods[19-21]. The cons of traditional extraction can now be replaced with better techniques in terms of speed and cost. Recent developments

have introduced novel processes of extraction, including microwave-assisted extraction (MAE), pressurized liquid extraction (PLE), ultra-sonication, supercritical fluid extraction (SFE), solvent, pulsed-electric field extraction (PE), and ionic-liquid extraction[22, 23]. The modification from conventional organic solvent-based processes to the novel, minimal or zero-waste processes showcases the development of greener methods without compromising the efficiency of the method[24]. These extraction techniques overcome the drawbacks associated with traditional extraction methods. However, modern techniques have their disadvantages when it comes to the sample preparation method[25]. The modern techniques are expensive, require highly pure solvent, and often need high energy, pressure, and more of an instrument set-up. Additionally, it still requires clean-up, and purification steps before the sample are introduced to the analytical instrument[26].

Table 1.1: Modern Extraction Techniques in Food Sample Preparation[23].

Food	Extraction Method	Extraction Solvent
Apple, green bean and carrot	SFE*	CO_2
Vegetables (cabbage, carrots, tomatoes,	MAE**	Acetone
chilies, potatoes, and beetroot)	PLE***	Methanol/Acetone
Oranges	MAE	Acetone/hexane
Pork and chicken meat	ASE****	Hexane
Honey	SFE	CO_2
Fresh vegetable	ASE	Ethyl acetate/acetone
Fish	SFE	CO_2
Sesame seeds	MAE	Water/acetonitrile

*Super-critical extraction
**Microwave-assisted extraction
***Pressurized liquid extraction
****Accelerated solvent extraction

The analytical techniques based on green chemistry principles are being widely considered, that includes, no or reduced solvents, reagents, and utilization of eco-friendly chemicals. The chemists are working towards the advancement of greener analytical techniques that are energy-efficient and techniques without compromising the efficiency. The new approaches include the use of green sample preparation techniques, such as the use of green solvents, solvent-less technique or miniaturization, green analytical technique, and the combination of separation techniques[2, 27]. Solid adsorbent material for the extraction of analytes has been applied as a solution for more than thirty years and is widely applied to many complex matrices. A sorbent with a strong affinity for compounds of interest will concentrate those compounds from the matrix[28]. In many cases, sorbents are specific for the extraction of various compounds with various degrees of selectivity[29]. Solid-phase extraction (SPE) has been developed as an alternative and widely adopted. SPE has gained popularity due to its usage simplicity and time and solvent economic, and small sample size, that is generally the drawbacks of modern extraction techniques[30].

1.5. Solid-phase Extraction in Sample Preparation

Solid-phase extraction is an extensively used sorbent-based technique and has become one of the best, rapid, and selective sample preparation method[31]. It offers high precision and throughput, with the less consumption of solvent. SPE involves the use of disposable sorbent bed that traps analytes or matrix, separating analyte from the matrix. In the procedure, the prepared sample solution is passed through the sorbent bed where analyte concentrates on the sorbent surface, while the other matrix components pass through the sorbent bed. In another case, when the sample solution is passed through the

sorbent bed, the analyte passes through the sorbent bed while the matrix retains in the sorbent bed followed by cleanup if required[32]. Commercially available sorbent are alumina, magnesium silicate, and graphitized carbon, where silica is the most common material being used for sample preparation[33]. Due to the reactivity of silica, it is modified on the surface by chemical reaction allowing extraction for a wide range of compounds. Polymer-based sorbent beds are in demand and lately being modified to offer SPE materials with higher stability and advanced extraction for food analysis[34]. The chemistry of the sorbent bed (physio-chemical) and elution solvent (pH, ionic strength, polarity flow rate) need to be considered for the method development in SPE.

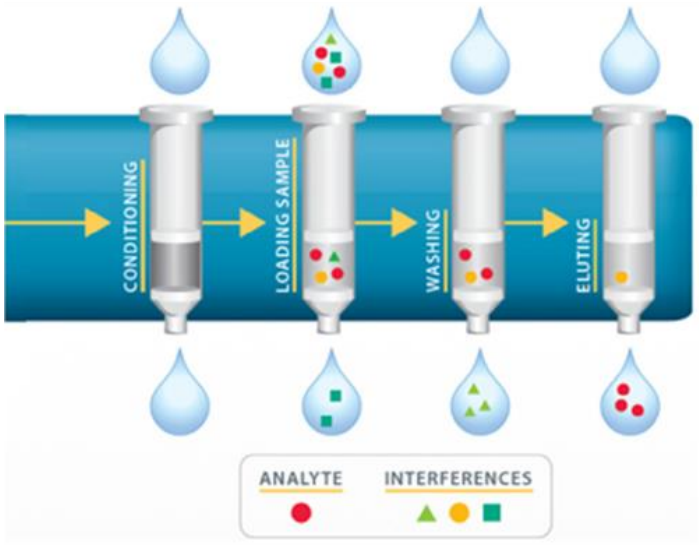

Figure 1.3: Typical four-step SPE procedure involving conditioning, loading sample, washing, and eluting[35].

The SPE method is operated in four steps:

i) *Conditioning*, the sorbent bed is activated by solvation, allowing the functional group of sorbents to interact with the matrix component,

ii) *Retention/Loading*, the analytes or the sample matrix component are retained on the sorbent bed,

iii) *Washing* allows the removal of undesired compounds from the sorbent,

iv) *Elution* allows the collection of compounds of interest.

1.5.1. The Formats in SPE

There are several approaches made to achieve innovation in sample preparation method using SPE. By combining standard tools and techniques, the goals of sample preparation can be achieved. The common and important protocols that need to be considered are to reduce the number of steps, to eliminate sample transfer during the sample preparation process and to reduce the sample scale (keeping the processing time, labor and cost-effective)

In the SPE technique for sample preparation, mostly four types of sorbent formats are available:

1. *Cartridges:* This format is typically 40-60 µm of particle diameter (dP) packing material. The restricted flow-rates and clogging of the surface for samples with suspended solid are the drawbacks of this type of SPE[36].
2. *Discs:* In this format type, the adsorbent is immobilized in a web of microfibrils (~ 0.5 mm thickness). The polymer or silica-based sorbent is embedded in a polytetrafluoroethylene (PTFE) network or glass fiber. Glass fiber provides rigidity and allows higher flow-rate than PTFE membranes. The sorbent particle size is smaller (8 µm) compared to the sorbent in cartridges[37].

3. *SPE Pipette Tips:* In this type of format the solid-phase sorbent is placed inside the pipette The SPE pipette offers faster extraction time ~1-2 min, small sample volume (200 µL), cleaner extracts, and requires less solvent volume[38].
4. *96-well SPE Plates:* This format is the standard 96-well microtiter plate that allows parallel sample processing in less than one hour. The parallel sample processing reduces handling errors and limits labor-input. The volume of each well is 1-2 mL and 3-10 mg of packing material[39].

These formats could be used for extraction, clean-up, or both. SPE formats in comparison to sample preparation process provides various benefits such as lower cost by reducing the solvent and reagent consumption and simple apparatus, better recoveries by minimal sample transfer and fewer processing steps resulting in rapid processing[40, 41]. SPE can be coupled with automated analytical procedures resulting in higher laboratory throughput with enhanced accuracy and precision. The key factor in the method development of SPE that need to be considered is the sorbent material and the elution protocol. Typical sorbents for SPE are shown in Table 1.2.

Table 1.2: Extraction and clean-up for various analytes in food samples[42].

Sample	SPE	Purpose	Detection method
Honey[43]	C18, SPE cartridges	Extraction and clean-up	HPLC–FD
Milk samples[44]	Strata strong anion exchange (SAX), Strata C18-E, and Strata Phenyl	Extraction and preconcentration	Reverse phase-LC and amperometric detector
Fortified Beverages[45]	C18 Plus cartridges	Purification	HPLC-DAD
Apple juice concentrate[46]	Oasis® (mixed mode cationic exchange) MCX sorbent and C18	Extraction	HPLC-UV
Roasted coffee[47]	NH_2 column	Clean-up	HPLC
Cereal-based Foods[48]	Oasis HLB (hydrophilic-lipophilic-balanced) HLB cartridges	Clean-up	LC-MS/MS
Milk and dairy Products[49]	Strata C18-E, Strata CN and Strata Phenyl	Clean-up	GC-MS
Fish tissues[50]	Strata X cartridge	Clean-up	LC-FD, LC-MS/MS
Citrus essential Oil[51]	Mixed Florisil-C18 cartridge	Extraction	GC-MS, HPLC-MS
Fruits and Vegetables[52]	Graphitized carbon black (GCB), primary, secondary amine (PSA) aminopropyl, and C18, columns	Clean-up	GC/MS
Wine[53]	C18 bonded porous silica cartridges	Extraction and clean-up	GC-MS
Egg[54]	GCB, aminopropyl and Florisil SPE columns	Clean-up	GC-FD

1.6. SPE Principle and Types

SPE is based on the liquid chromatography principle, where the functional group interactions of the sample, solvent, and stationary phase (in the case of SPE, sorbent) are optimized to achieve sample fractionation. The sorbent type determines the extraction mechanism in SPE. Sorbents are of three major classes: polar, non-polar, and ion-exchange. The activity of these sorbent depends on the properties of the bonded phase. The nature of the food matrix and compound of interest determines the selection of the sorbent to be used for the extraction[55].

Reversed-Phase SPE

Reversed-phase separation is used for polar or moderately polar sample matrix where the analyte and the sorbent both are nonpolar. SPE material like silica with alkyl or aryl comes under the reversed-phase category (octadecyl (C18) and phenyl). Silica is functionalized at the surface with hydrophilic silanol groups, hydrophobic alkyl or aryl groups, with 60Å pore size and 40µm particle size. The retention of analytes is due to the attractive forces between the carbon-hydrogen bonds in the analyte and the functional groups on the silica surface. The retention is based on the attraction force, van der Waals forces or dispersion forces between the nonpolar-nonpolar molecule. For the elution step, a non-polar solvent is used, that disrupts the attraction forces between the analyte and sorbent bed[37]. Another type in reverse phase SPE is carbonaceous adsorption and polymeric adsorption, which provides an alternative when the bonded silica is not applicable. The carbon surface consists of hexagonal ring structures and is inter-connected forming layers in graphitic sheets allowing interaction with planar aromatic or hexagonal ring-shaped molecules and alkyl chains[56]. The retention ability of the molecule

is determined by the size and shape of the molecule. The elution solvent is nonpolar. Polymeric sorbent such as divinylbenzene (DVB) is applicable for the extraction of hydrophobic compounds. Polyphenols extraction under C18 sorbent is difficult due to less solubility in organic matrices. On the other hand, polymeric sorbent has been very effective in retaining polyphenols[37].

Normal Phase SPE

Normal phase separation is used for polar compound extraction, where the sample matrix is nonpolar (e.g., acetone and hexane), and the sorbent is polar. The stationary phase used in the type is silica with a bonded polar-functional group (kieselguhr and silica gel), and polar adsorption media (Florisil). The retention of analytes is based on the interactions between polar functional groups of the analyte and the sorbent. The interaction involves hydrogen bonding, pi-pi, dipole-dipole, and dipole-induced dipole interactions. The elution is achieved by using a more polar solvent that disrupts the binding mechanism. The hydroxyl groups on the surface of the silica particles result in adsorption of compounds from nonpolar matrices. The very common use of this kind of sorbent is applicable for the analyte that passes sorbent media, and the matrix remains in the sorbent bed. When an organic extract is applied to the sorbent bed, the analyte of interest passes through the sorbent bed unretained, and the unwanted compounds from the sample matrix are adsorbed onto sorbent bed. The technique is generally used as a sample cleanup procedure.

Ion Exchange SPE

Ion exchange separation is used for analytes that are charged in a solution. Negatively charged (anionic) analytes can be separated by positively charged silica. Positively charged (cationic) analyte are isolated by negatively charged silica sorbent. The retention mechanism is that the electrostatic attraction between the charged functional groups of the analyte and the sorbent. The retention of the analyte occurs when the pH is the same for the sample matrix and the sorbent. To achieve elution, solvent or solvent mixture with a pH that neutralizes either the functional group of the analyte or the sorbent material is used. The neutralization disrupts the electrostatic force between the analyte and the sorbent. The eluent in this method must be of high ionic strength or must contain anionic species that could replace the adsorbed analyte[55].

Table 1.3: Sorbents utilized for solid-phase extraction along with their SPE type and structure [37, 56, 57].

SPE Type	Sorbent	Structure
Reverse Phase	Octadecyl (C18)	$– (CH2)_{17}CH_3$
	Octyl (C8)	$–(CH_2)_7CH_3$
	Ethyl (C2)	$–CH_2CH_3$
	Cyclohexyl	$–CH_2CH_2\text{-}C_6H_{12}$
	Phenyl	$–CH_2CH_2\text{-}C_6H_6$
Normal Phase	Cyano (CN)	$\text{-}(CH_2)_3CN$
	Amino (NH_2)	$– (CH_2)_3NH_2$
	Diol (COHCOH)	$– (CH_2)_3OCH_2CHOHCH_2OH$
	Kieselguhr (Diatomaceous Earth)	–SiOH
	Silica gel	–SiOH
	Florisil	Mg_2SiO_3
	Alumina (neutral)	Al_2O_3
Ionic Exchange	Amino (NH2)	$(CH_2)_3nH^{2+}$
	1°, 2°- Amino (NH/NH_2)	$– (CH_2)_3NH^+ CH_2CH_2NH_2$
	Quaternary amine (N^+)	$– (CH_2)_3N^+ (CH_3)_3$
	Carboxylic acid (COOH)	$– (CH (CH_2)_2COO^-$
	Propyl sulfonic acid (SO_2OH)	$– (CH_2)_3SO_2O^-$
	Aromatic sulfonic acid ($ArSO_2OH$)	$– (CH_2)_3\text{-}C_6H_6\text{-}SO_2O^-$

Anion Exchange SPE: In this category, the example of the sorbent material is silica with an aliphatic quaternary amine group. A quaternary amine is positively charged and is a strong base. This type of sorbent attracts anionic species in the sample matrix; hence, the binding principle is called an anion-exchange mechanism. The quaternary amine is charged at all pHs due to its high pKa value (greater than 14), resulting in separation of analytes with very low pKa, <1 (strong anionic) or analytes with low pKa, >2 (weak anionic)[55].

Cation Exchange SPE: The sorbent material in this category, is silica with aliphatic sulfonic acid groups, negatively charged. The strong acidic nature of the sorbent with pKa <1, replaces cationic species in the sample matrix; hence, the binding principle is called a cation exchange mechanism. The sorbent is charged at all the pHs, allowing separation of the analyte with very high pKa, >14 (strong cationic) or analyte with moderately high pKa, <12 (weak cationic). Though the SPE technique has been broadly accepted as an efficient sample preparation technique, few limitations have encouraged researchers to have more advanced SPE with more analyte specificity. Another solid-phase extraction technique, a method that reduces or eliminate the use of solvents, efficiently extracts volatile compounds can be employed to prepare samples for chromatographic analysis. This includes, solid-phase micro-extraction (SPME), matrix solid-phase dispersion (MSPD), and stir-bar sorptive extraction (SBSE). Additionally, this sample preparation is easily adapted for fieldwork, employs less costly materials, and is very effective for portable GC analysis[30]. Extraction of analytes with volatile nature has been very challenging. Therefore, there was a need for a method to avoid the

potential destruction of volatile components, any loss of volatile analyte before the sample introduction to the analytical instrument. SPME is used where the volatile compounds are adsorbed on the sorbent bed followed by desorption in an analytical instrument such as gas chromatography. SPME operates under mild condition avoiding thermal degradation and other chemical and biochemical changes in the sample. This technique can be used by either direct immersion of fiber in the sample or by exposing the fiber in the headspace of the sample, where the adsorption of analyte occurs[58]. The sample preparation for analytes in lipid loaded sample matrix has led to the development of dispersive-SPE (d-SPE). D-SPE is widespread in the area of multi-residue pesticide analysis for food and agricultural samples[59]. In this method, samples are extracted with an aqueous solvent, acetonitrile followed by the addition of sorbent for removal of lipids by extraction and clean up.

Table 1.4: Common examples of SPE, SPME, and d-SPE in food analysis[2, 58-60].

Techniques	Sorbent	Sample	Analytes
SPE	C18	Wine	Anthocyanins
SPE	PS-DVB	Wine	Stilbenes
SPE	PS-DVB	Olive Oil	Phenolic compounds
SPME	DV-PDMS	Wine	Pyrazines
SPME	CAR-PDMS	Cheese	70 VOC
SPME	PDMS	Honey, Fruit juice	Organophosphorus pesticide residue
d-SPE	Captiva	Barley	Dicamba, MCPA, 2,4-D, MCPP
d-SPE	BondElut	Avocado	Pesticides

Later the mixture is centrifuged, and the supernatant is separated for further analysis by the direct introduction to the analytical instrument and/or if needed dilution and cleanup will be performed before sample introduction[60]. Table 1.4. is a list of some

of the SPE, SPME and d-SPE technique with the various sorbent and each sorbent specific for the compounds of interest from various food samples.

1.7. Summary, Scope, and Objectives

It is essential to study the composition of food products to assess its nutritional value, safety, quality, and authenticity, thereby develop products of the highest possible quality. Numerous conventional and modern analytical approaches are being utilized for food analysis. However, because of the complex nature of the food matrix coupled with the serious limitations of the analytical methods, it is imperative to look for alternative approaches or advancements in terms of sample preparation techniques and analytical methodologies.

Matrix effect is a serious limitation with the quantitative analysis that affects the reproducibility, accuracy, and linearity of the methods. SPE is one of the most common techniques to circumvent matrix effects. It is a sorbent-based technique that offers high precision and throughput using a disposable sorbent bed that separates the target analyte of interest and separates it from the bulk of the matrix. Based on the principle, SPE could be classified into three main categories, such as reversible phase, normal phase, and ion-exchange SPE. Various sorbent formats such as cartridges, discs, SPE pipette tips, and 96-well plate are utilized. Some of the common sorbents utilized are C18, silica, modified silica, and graphite carbon black for the matrix types such as wine, food products such as milk, and honey. SPE suffers from one of the limitations when it comes to sampling preparation of matrix with increased content of lipids, requiring multistep sample preparation method via emulsion formation, contributing to inefficient extraction.

With regard to volatile compound analysis using SPE, it requires stringent processing to avoid loss of the analyte. To address the efficient extraction of volatile compounds in a complex matrix, SPME, is a more advanced type of SPE. It has been popular for volatile compound extraction due to its ease, simplicity, avoidance of solvent use, automation and compatibility with GC-MS and LC-MS. d-SPE is another advanced form of SPE, that is employed for analytes in lipid dominating sample matrix. Thus, SPE, SPME, and d-SPE represent the state-of-the-art for overcoming concern related to matrix effects.

Recent advances in SPE method as a sample preparation technique has unfolded many opportunities to the analytical chemist by providing an easy, quick, greener, efficient approach in sample preparation. However, when it comes to the analyte-specific sorbent, there are certain limitation exists such as (i) Volatile analyte, what type of sorbent coating in combination with thickness would be the most effective for extraction (chapter II). (ii) Extraction of lipid class-specific using polymer-based sorbent (chapter III). (iii) Extraction of specific polyphenol from a complex matrix (chapter IV). In this dissertation, we demonstrate the applicability/extension of SPE and advanced forms of SPE for the analysis of matrix types such as food products (horseradish products-chapter II), advanced types of d-SPE sorbents (chapter III), and wine (chapter IV) and thereby avoid matrix effect in the investigation of compounds of interest from food samples. Subsequent sections below will describe a brief introduction about the matrix type, the problem being addressed, and the objectives of the study.

Horseradish *(Armoracia rusticana)* liberates isothiocyanates, which are utilized as flavor in the food industry due to their characteristic taste. Allyl isothiocyanate (AITC),

the predominant isothiocyanate in horseradish, is generated from allyl glucosinolate (sinigrin) by hydrolysis. The results from our previous study have demonstrated a strong correlation between the headspace levels of AITC and consumer-perceived pungency and product preference(s). However, the effect of various factors that influence the shelf-life of commercial horseradish products at different storage conditions is not studied. It is essential to study the impact of the factors mentioned above to develop products with optimum shelf life and stability that are of the highest possible quality for increased consumer acceptability. These are some of the essential things that need to be addressed and have been outlined above. On these lines, the objective of this study (chapter II) is to investigate the factors that influence the shelf life of horseradish products.

SPE is a widely used sorbent-based technique that has been utilized for a variety of sample types as mentioned above. However, driven by technological advancements, the need for analyte specificity, and reduction of solvent utilization, it is essential to develop next-generation of materials that aid in efficient sample preparation and removal of matrix effect. Over the past few years, Agilent Technologies has developed novel sorbent materials that are capable of selectively capturing hydrophobic compounds, leading to the product Enhanced Matrix Removal-Lipid (EMR-Lipid). This material-based product aids agricultural, food safety, and other laboratories to remove matrix-effect. The EMR-Lipid removes hydrophobic molecules utilizing a combination of size exclusion and hydrophobic interactions. This will provide minimal ion suppression of target analytes, prevents analyte loss, and leads to higher selectivity and efficient analysis. Although EMR-Lipid has demonstrated to be efficient in the removal of matrix effect from a variety of sample types, to be specific in terms of removal of hydrophobic

compounds, it is essential to develop sorbent materials that are specific to lipid class. Driven by this need, it is essential to develop next-generation materials for the specific separation of lipid compounds. Hence, the central objective of this work (chapter III) is to study the interaction between lipid classes and a newly developed sorbent, Enhanced Matrix Removal (EMR-Lipid), thereby develop a fundamental understanding of lipid-binding properties and guide the development of next-generation materials for removal of the *sample matrix*.

Resveratrol is a member of the stilbene family, has been an essential component of wines due to its associated health benefits. Research has demonstrated that climate conditions affect wine production and eventually alter the resveratrol concentration in red wines. Several studies report resveratrol concentration in wines; however, very limited information is available regarding the resveratrol concentration in red wines from grapes grown in extreme environments. Grapes grown in a northern climate, like Minnesota and Dakota's must contend with extremes of cold in the winter and heat in the summer. Based on this, the objective of chapter IV is to determine resveratrol concentration in wines from South Dakota grown grapes.

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CHAPTER II

INVESTIGATION OF THE FACTORS AFFECTING THE SHELF LIFE OF HORSERADISH PRODUCTS

ABSTRACT

Horseradish *(Armoracia rusticana)* liberates isothiocyanates, which are utilized as flavor in the food industry due to their characteristic taste. Allyl isothiocyanate (AITC), the predominant isothiocyanate in horseradish, is generated from allyl glucosinolate (sinigrin) by hydrolysis and is known to be utilized as an antimicrobial agent. It is highly volatile and possesses a strong odor and taste, imparting the characteristic pungency to horseradish products. The results from our previous study have demonstrated a strong correlation between the headspace levels of AITC and consumer-perceived pungency and product preference(s). We have shown that the degradation of sinigrin and the resulting AITC relates to product quality, stability, and consumer acceptance. To this end, the goal of the current study is to utilize a headspace-solid-phase microextraction-gas chromatography-mass spectrometry (HS-SPME-GC-MS) method to study the effect of various factors on the shelf-life of commercial horseradish products at different storage conditions. The study also includes the influence of SPME fiber coating for the extraction of AITC in horseradish using Taguchi factorial design. A calibration curve was generated in the concentration range of 50-4000 ppm of AITC using the internal standard method. We also studied the influence of sodium azide, a compound known to inactivate AITC-producing enzyme, at room temperature and under refrigeration. The result indicates that the fiber coating made of carboxen/polydimethylsiloxane with 85μm film thickness, has highest capacity for adsorption of AITC and the fiber coating made of polydimethylsiloxane/divinyl-benzene with 65μm demonstrated the lowest adsorption among the four fiber coatings evaluated.

The study shows that AITC levels in samples stored at room temperature decreased drastically below detection limits, while AITC levels in refrigerated samples demonstrated consistent loss but overall maintained high levels of AITC compared to samples stored at room temperature over the period of six months. These results demonstrate that sinigrin-derived AITC creates an overall increase in AITC concentration and loss of AITC from fresh-ground horseradish follows an identical trend regardless of storage condition. The rate of AITC degradation slowed down considerably for samples after three weeks.

2.1. Introduction

2.1.1. Horseradish, Origin, and History

Isothiocyanates (ITCs) are secondary plant metabolites produced as a system of defense against pathogen attack[1]. They arise from the hydrolysis of glucosinolates in several plants such as *Brassicaceae* (e.g., broccoli, cabbage, cauliflower, kale, etc.), *Capparaceae* (e.g., the mustard family), and *Caricaceae* (e.g., papaya.). ITCs are sulfur-containing compounds that are produced by members of *Brassicaceae* family[2]. Horseradish, scientifically named as *Armoracia rusticana, Cochlearia Armoracia, or Armoracia lapthifolia*, plants belonging to the *Brassicaceae* family. It is a well-known as perennial and horticultural root crop native to southeastern Europe. It is found mostly in the moderate climates in Northern, Middle, and Southeastern Europe and in Northern America. Horseradish possesses a very intense pungent flavor and odor. The taste could be described as *"immediate sweet turnip flavor with a harsh pungency and grassy after-taste"*[3]. In addition to employing it as a flavoring agent in sauces, pickled beets, and various other food products, it has been exclusively used for the production of products such as horseradish sauce, and cream.[4-6]. The United States Department of Agriculture (USDA) guideline states that horseradish roots are classified as U.S. Fancy, U.S. No. 1, and U.S. No. 2, based on the diameter and length of the root[7, 8]. The commercial production of horseradish occurs as an annual crop. The roots develop at ambient temperature (15–27 °C); hence planting occurs mostly in the early spring. On harvest, the horseradish roots are stored in a cold temperature before processing to maintain the freshness[9, 10].

2.1.2.Horseradish Culture

Currently, the use of horseradish as a condiment is popular in Europe and North America[11]. Horseradish sauce is usually served with beef, fish, chicken, and other meat-based dishes, or on sandwiches. Horseradish has been also utilized to preserve foods such as cucumber and red beet due to its antibacterial characteristics. The use of AITC as an additive for the preservation of food products has been really popular and widely adopted in Japan. In Japan, only naturally sourced AITC is allowed, as synthetically produced AITC might be contaminated with chloride compounds[12]. AITC is permitted for use as a flavor in the USA, however, its use as a preservative or antimicrobial agent is not approved. AITC vapor is considered to be GRAS (generally recognized as safe)[13, 14]. In Canada, the Food and Drug Regulations permit the use of mustard flour and mustard seed as spices[15]. It is estimated that the average daily intake from dietary sources is 130-1500 µg/day[16, 17]. The expert panel of the Flavor and Extract Manufacturers Association (FEMA) lists the daily per capita intake of 2.21 µg/kg body weight as a flavoring additive[18-22].

2.1.3. Shelf Life and Factors Affecting Shelf Life

Consumers are continuously demanding higher quality food and have expectations that the quality of food is maintained for its complete life cycle (from manufacturing to purchase and final consumption). The food quality must comply with the labeling requirements and must be met by the food manufacturers. It is crucial for manufacturers to predict the shelf life under a given set of conditions. The Institute of Food Science and Technology (IFST) definition raises the concern on storage conditions on food product shelf life. The measurement of storage characteristics of any food product, starting from

raw material to end product, takes place under carefully controlled environmental conditions. The understanding of the product characteristics is paramount for the evaluation of the mechanism of deterioration. The factors affecting shelf life are mainly categorized into *intrinsic* and *extrinsic* factors. The *intrinsic factors* include water activity, available oxygen, pH, total acidity, and use of the product formulation. These factors are affected by variables such as raw material, its quality, and product composition. The *extrinsic* factors are associated with the final product exposure such as time-temperature profile during processing, relative humidity, and exposure to light during processing, storage, and distribution. These intrinsic and extrinsic factors either inhibit or stimulate a number of processes which directly affects the shelf life.

2.1.4. Sinigrin Hydrolysis

Horseradish is rich in several bioactive compounds such as glucosinolates and its derivatives. Sinigrin is the dominant glucosinolate in the root of horseradish, accounting for ~ 90% of total glucosinolate content. When the integrity of the horseradish root is compromised by cutting or grating, the cell contents come in contact with water. When sinigrin is exposed to the enzyme myrosinase, it undergoes a hydrolysis reaction leading to the formation of ITCs. Studies indicated that the freshly grated horseradish root continuously releases cell contents, whereas, the hydrolysis reaction occurs at a higher rate due to the abundance of the enzyme and starting material. Since water is a substrate in the hydrolysis reaction, humidity from the air could be used to activate the release of ITCs[23-25]. Figure 2.1 explains the hydrolysis reaction of sinigrin in the presence of an enzyme (myrosinase). Upon injury of plant tissue, sinigrin is hydrolyzed by myrosinase in a reaction that produces four main compounds: allyl isothiocyanate (AITC) at pH 7,

allyl thiocyanate (ATC) and allyl cyanide (AC) at pH 4, and in the presence of epithiospecifier protein (ESP). The concentration of these hydrolysis products depends on the plant source. CETP will be more in cabbage compared to ATC, AC levels are higher in cauliflower compared to horseradish and ATC is more present in stinkweed compared to cabbage. But overall, AITC is most abundant in *Brassicaceae* species. Each compound contributes to the flavor and characteristic aroma of such plants[23, 26, 27].

2.1.5. Bioactive Compounds in Horseradish and AITC

Allyl isothiocyanate, the predominant ITCs in horseradish, is generated from allyl glucosinolate (sinigrin). The pungency of AITC is due to hydrolysis of sinigrin by the enzyme myrosinase. AITC induces phase II enzymes and is a potent antimicrobial agent[28, 29]. It has been well-known that sinigrin in horseradish gets primarily hydrolyzed to bioactive AITC and 2-phenylethylisothiocyanate (PEITC)[10, 30]. It was reported that the level of AITC in roots of two different types of horseradish cultivated in Poland was 3-10 fold higher than PEITC[31-34].

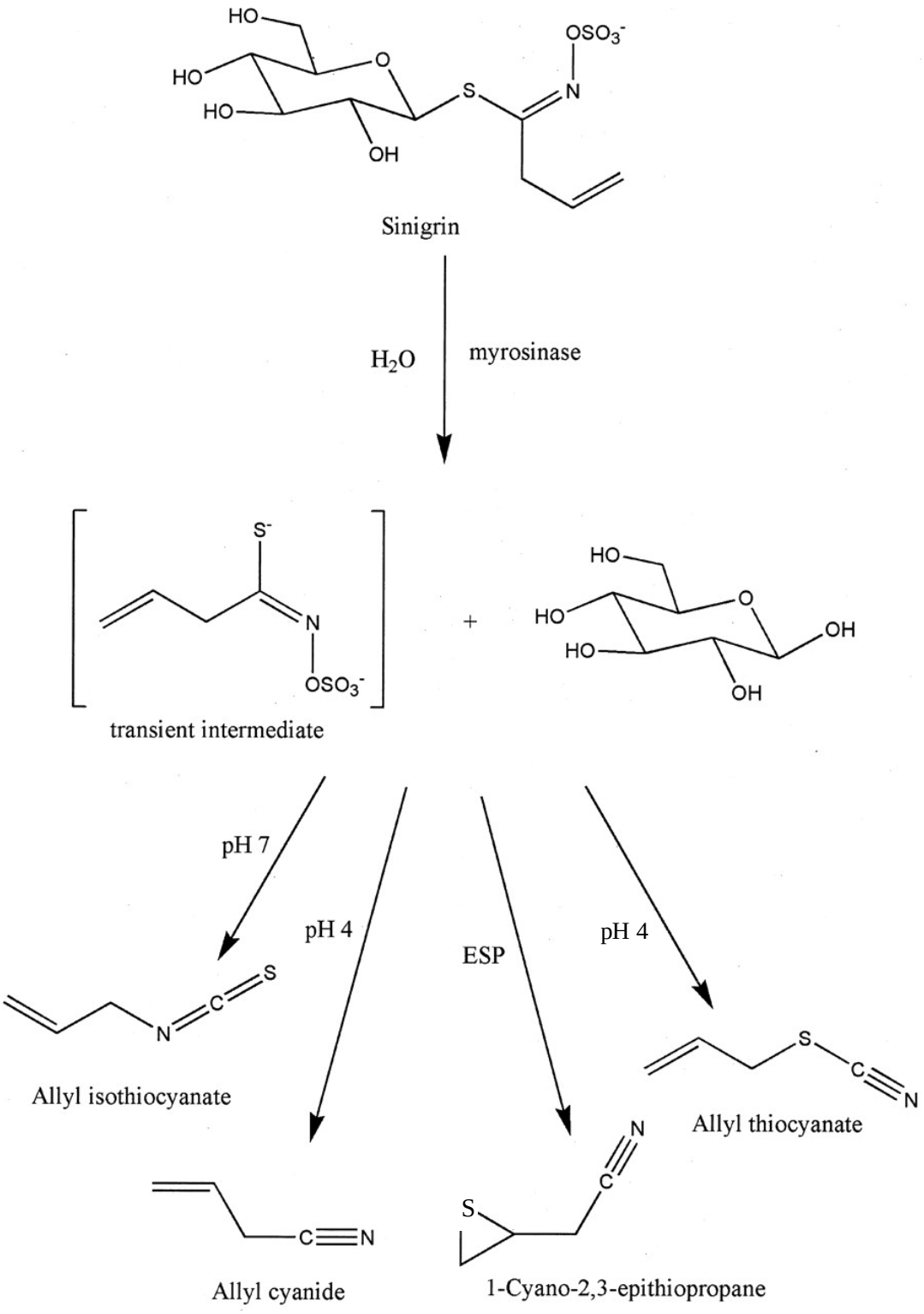

Figure 2.1: Sinigrin hydrolysis in the presence of the myrosinase enzyme into four different products. Modified and adopted from[35].

2.1.6. Isothiocyanate Derivatives

Gas chromatographic analysis indicated (Figure 2.2) allyl isothiocyanate (97.58%), phenylethyl isothiocyanate (1.65%), and others (<0.77%, combined) in the prepared horseradish sample represented as percent peak area[36].

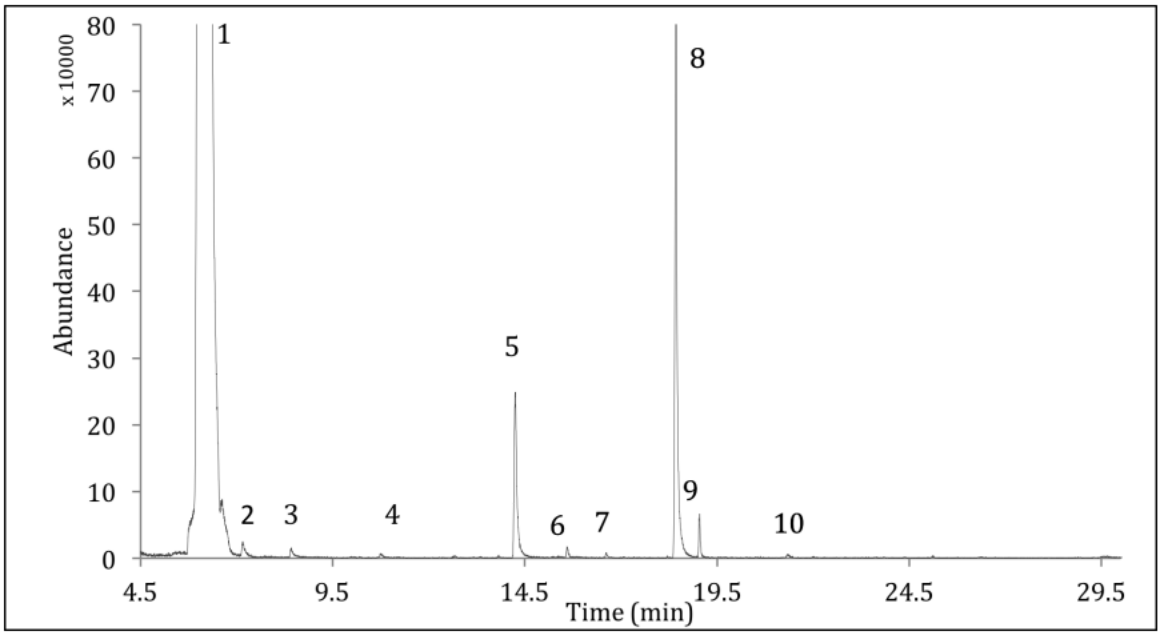

Figure 2.2: GC-MS chromatogram of the prepared horseradish sample. The peaks represent various ITC derivatives, peak 1: allyl isothiocyanate, 2: isobutyl isothiocyanate, 3: 1-butene 4-isothiocyanate, 4: unknown, 5: Benzene propanitrile, 6: propyl isothiocyanate, 7: benzyl isothiocyanate, 8: phenylethyl isothiocyanate, 9: unknown, 10: unknown. A SPME fiber (100 μm of polydimethylsiloxane) was exposed to the headspace of the sample for 20 min, followed by desorption into the injection port of GC/MS. Modified and adopted from[36].

Table 2.1: Chromatographic profile of isothiocyanates. Adopted from[36].

Compound	Relative amount(%)
Allyl isothiocyanate	97.58%
Isobutyl isothiocyanate	< 0.05%
1-butene 4-isothiocyanate	< 0.04%
Unknown	< 0.02%
Benzene propanenitrile	0.56 %
Propyl isothiocyanate	< 0.03%
Benzyl isothiocyanate	<0.01 %
Phenylethyl isothiocyanate	1.65%
Unknown	< 0.09%
Unknown	< 0.02%

2.1.7. Physical and Chemical Properties of AITC

AITC is highly volatile with a pungent odor. The volatility of AITC is such that it readily evaporates at room and refrigeration temperatures[37]. It has low solubility in water but is soluble in lipids, acetone, and hexane[38]. The physical and chemical properties of AITC are listed in Table 2.2. AITC is unstable and known to degrade in water. At 80°C, reaction products include allylamine, allyl dithiocarbamate, diallyl urea, diallyl sulfide, and carbon disulfide. The rate of reaction increases with temperature. AITC may completely react to form end products in ~80 min at 80°C, with low pH corresponding to slower reaction rates[37]. Reaction products in aqueous solution at 100 °C include di, tri, and tetraallyl sulfides, allyl thiocyanate, and N, N'-diallylthiourea[32]. AITC also reacts with proteins and is noted to cleave disulfide bonds[39, 40]

Table 2.2: Physical and chemical properties of AITC[41].

Physical and Chemical Property	Characteristics
Color	Pale yellow
Melting point	-102.5˚C
Boiling point	148 to 154 °C
Solubility in water	2 g/L
Density	1.017 g/L
Vapor pressure	0.493 kPa
Flashpoint	46°C
Molecular mass	99.15 $g \cdot mol^{-1}$
Molecular formula	C_4H_5NS
Linear formula	$CH_3=CHCH_2N=C=S$

AITC attacks the free amine group of both lysine and arginine, reducing the activity of some enzymes[40]. Products formed from reactions with amino groups include thiourea derivatives. Sulfhydryl side chains of proteins react with AITC to produce dithiocarbonate esters[42, 43].

2.1.8. Existing Analytical Method for AITC Analysis

Various analytical techniques such as gas chromatography (GC) and liquid chromatography (LC) have been used to investigate ITCs in variety of sample matrix. However, these techniques have been limited to the detection of only a few ITCs. This could be due to the physicochemical properties, especially in terms of polarity and volatility. To combat this, hyphenated techniques have been employed for the analysis of a range of compounds, e.g., GC for volatile ITCs, normal-phase high-performance liquid chromatography (NP-HPLC) for polar, and reverse-phase high-performance liquid chromatography (RP-HPLC) for nonpolar compounds[44]. One of the most common LC

technique for analyzing ITCs is RP-HPLC, based on C18-bonded silica phase. It has been commonly used for the determination of several analytes in *Brassicaceae* plants, sulforaphane in broccoli and cabbage, AITC in mustard, propyl-ITC, and phenyl-ITC (PITC) in rapeseeds with wide concentration range from g/mL to mg/mL[45, 46].

2.1.9. Comparison of Ethyl Acetate Extraction and HS-SPME for the Isolation of AITC

Prior to analysis, an efficient extraction technique is required to isolate ITCs from the sample matrix. Two different extraction methods were employed for AITC, followed by gas chromatography-mass spectrometry (GC-MS) analysis[36]. Freshly grated horseradish sample was utilized with extraction by ethyl acetate or HS-SPME. Extraction with ethyl acetate resulted in benzene propane nitrile and phenylethyl isothiocyanate in addition to AITC. Conversely, HS-SPME extraction was specific for AITC. The level of AITC observed from ethyl acetate extraction was much smaller than the HS-SPME method, accounting for 37.5% of that in HS-SPME g/L. AITC was the only compound observed in the chromatogram with higher sensitivity than previous extraction method. Increase in sample size and headspace volume showed no differences in the results. In conclusion, the HS-SPME method was found to be a more sensitive and accurate extraction method in comparison to solvent extraction. The HS-SPME method can provide excellent sensitivity for the qualitative analysis of flavor components in natural plants. It is a simple, easy, quick, green technique with good reproducibility. Thus, the analysis of horseradish sample was carried out using HS-SPME-GC-MS method[36].

2.2. Objectives

The objectives of this study are two-fold. First, is the selection of a SPME fiber for optimum extraction. We compared four types of fibers with different coating material and thickness. In addition, the objective is to investigate the application of a factorial design to elucidate the conditions that influence the efficiency of HS-SPME of the volatile compound AITC from horseradish. The second objective is to investigate the shelf life of food products, i.e. freshly grated horseradish, and factors affecting the shelf life such as ingredient changes, temperature, and harvest season.

2.3. Materials

Chemicals and Reagents

AITC standard was purchased from Sigma-Aldrich (Milwaukee, WI, USA). Propyl benzene was used as an internal standard and was obtained from Arcos Organics ((Morris Plains, NJ, USA). 1-propanol and all other chemicals and reagents of analytical grade were obtained from Thermo Fisher Scientific (Fairlawn, NJ, USA).

2.4. Methods

2.4.1.Sample Preparation

Fiber for Solid-Phase Microextraction

The SPME technique comprises a fiber holder and a fiber assembly. The fiber assembly consists of a 2 cm long retractable SPME fiber and a built-in 2-cm long, retractable, coated fiber[47]. The coating bonds a strong stationary phase to the flexible core, which results in a more stable coating and a fiber that is less prone to breakage. After the sample is placed in an airtight, septum cap sealed vial, the SPME needle is

pierced through the septum and the fiber is extended into the vial and exposed to the headspace. Extraction can be headspace or direct immersion of SPME fiber in the sample. The analyte partitions between the sample matrix and the headspace. The maximum adsorption is achieved and a proportional relationship is obtained between the amount of the extracted analyte by the SPME fiber and its initial concentration in the sample[47-51] .

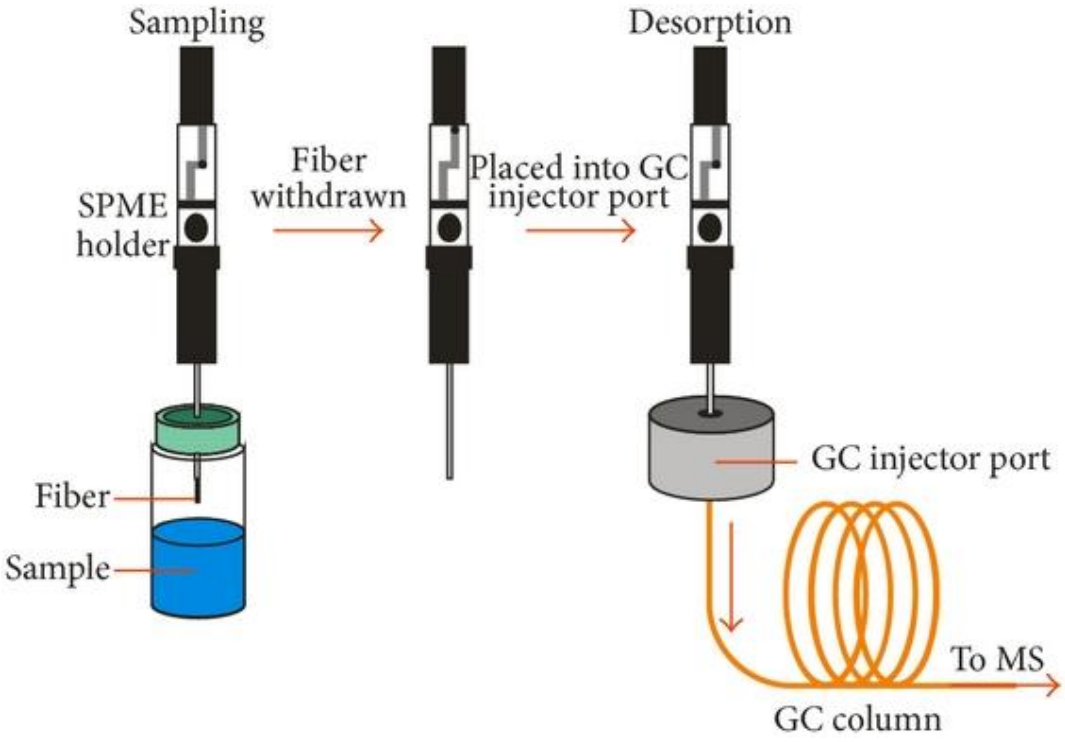

Figure 2.3: A typical solid-phase microextraction-gas chromatography-mass spectrometry (SPME-GC-MS) extraction procedure. Adopted from[51].

2.4.2. SPME Fibers for AITC Extraction

In this section, the optimization and comparison of SPME fibers for the extraction of AITC from horseradish samples were performed. Automated headspace solid-phase microextraction (HS-SPME) using four different coated fibers coupled to gas

chromatography-mass spectrometry (GC-MS) were used to analyze the AITC in horseradish sample. The extraction conditions were optimized using Taguchi (fractional factorial design) experimental design to investigate the maximum absorption of AITC on the fiber[52]. The fiber coating absorption capacity was studied with respect to extraction time and temperature by keeping the sample concentration constant[50, 53, 54]. Taguchi is factorial design that is based on well-defined guidelines using a special set of orthogonal arrays. Utilization of Taguchi design stipulates the way of conducting the minimal number of experiments that provide the full information of factors affecting the performance. It has been a widely utilized method for optimization of process parameters.

Currently, for the purposes of determining volatile compounds, the solid-phase microextraction (SPME) technique has been applied to overcome the problems associated with conventional sample preparation and extraction methodologies, such as cross contamination, loss of analyte, elevated costs, time consumption, and the use of large volumes of organic solvents[55, 56]. Among the commercially available SPME fibers, fibers with liquid polydimethylsiloxane (PDMS) and solid [carboxen (CAR) and/or divinylbenzene (DVB)] phases have been widely used for the extraction of volatile compounds due to their high sensitivity[57, 58]. Heating the sample increases the analyte release from the matrix resulting in their increased concentration in the headspace and, therefore, enhance the adsorption of the analyte on the fiber until the equilibrium is achieved[47, 59].

2.4.3. Selection of Fiber Coatings

The selection of the fiber coating mainly depends on the chemistry of the analyte.. Foremost, identify the molecular weight range of the analytes of interest that need to be

extracted from the sample matrix. Compounds with higher molecular weight desorb easier from the surface of the absorption fiber coatings (e.g., PDMS), compared to smaller molecules that tend to remain in the pores of the fibers with adsorbents in the coating (e.g., carboxen or divinylbenzene). Commonly utilized fiber coatings for food and environmental sample, are polydimethylsiloxane (PDMS), polyacrylate (PA), carboxen (CAR; a carbon molecular sieve), DVB, and carbowax (CW, polyethylene glycol). Fibers with more advanced features, such as high adsorption capacity and sensitivity towards the analytes, are available in various combinations and film thicknesses[60, 61]. Among the above mentioned coatings, the most common examples of liquid-fiber coatings are PDMS and PA. CAR/PDMS and DVB/CAR/PDMS fibers are widely used for a wide range of analytes with different polarities and molecular weights due to their best extraction capacities[62, 63, 64]. PDMS can withstand high temperatures up to 300 °C with great stability. PDMS fibers are appropriate for the analysis of nonpolar analytes, whereas PA fibers are used to extract polar analytes. DVB, with a polar porous solid coating, is efficient in the extraction of polar compounds such as disulfides and trisulfides[65, 66]. The fibers with a combination of coating provide extraction for the wide range of bipolar compounds like alcohols, aldehydes, ketones, ethers, and carboxylic acids, e.g., l (PDMS)/polar material (DVB), CARB/PDMS and DVB/CARB/PDMS[66, 67]. However, these coating combinations are accompanied by a disadvantage, the displacement effect of analytes with a lower affinity to the coating[60, 61, 68].

2.4.4. Instrumental Details

The samples used in this work consisted of freshly grated horseradish root provided by Silver Springs Food, Inc. (Eau Claire, WI). The HS-SPME procedures were

performed using a PAL autosampler (Zwingen, Switzerland). GC/MS analysis were performed on an Agilent 7890A gas chromatograph equipped with an Agilent 5975C mass selective detector. A DB-5 column (30m × 0.25mm ID × 0.2 μm film, Phenomenex, Torrance, Calif., U.S.A.) was utilized for the separation of volatiles. The autosampler was equipped with a SPME fiber/syringe holder, a temperature-controlled six-vial agitator tray, and a temperature-controlled needle heater port. The table 2.3 presents the four fibers used for screening the AITC from horseradish root. Prior to GC-MS analysis, each fiber was conditioned in the needle heater for 30 mins at 230˚C. Sampling by SPME for different fibers was done on the selected method as per the factorial design experiment.

Table 2.3: SPME fiber coating with description of film thickness and details about stationary phase.

Film Thickness	Stationary Phase
85μm	Carboxen/Polydimethylsiloxane (Carboxen/PDMS)
50/30μm	Divinylbenzene/Carboxen/PDMS (DVB/CAR/PDMS)
65μm	Polydimethylsiloxane/Divinylbenzene (PDMS/DVB)
85μm	Polyacrylate

2.4.5. Optimization Strategy

The optimization procedure was achieved with a three-level Taguchi (fractional factorial design). All experiments performed in multiples of triplicate. The three main variables (factors) in this study were temperature, time, and fiber type/coating. The results are represented as average AITC concentration in ppm calculated from the standard curve.

2.4.6. Solid-phase Microextraction

The headspace was generated from a 2.0g sample in a 10mL glass vial with a pierceable septum screw cap. The vial was equilibrated at respective temperature and time in the agitator prior to the extraction. A SPME fiber coated with a polymer (Supelco, Bellefonte, PA) was used for extraction via adsorption from the vial and then introduced in the injection port of GC/MS. Prior to use, the fiber was preconditioned at 250°C for 0.5h.

2.4.7. Headspace Extraction Time

Mass transfer process is time-dependent, and its rate directly affects the equilibrium conditions. To achieve distribution equilibrium between the sample and the fiber, the headspace (gaseous sample phase) where the fiber is exposed, is an important parameter. Hence the extraction procedure was carried out for 5-35 min for the determination of an optimum equilibration time and then optimum sampling time. Three consecutive samplings at pre-determined extraction time were taken and then the plot of peak area against sampling time was measured. The results depicted that with increasing sampling time the related peak size was increased until 15 min after exposing the SPME fiber to the standard.

2.4.8. *Headspace Extraction Temperature*

In extraction, temperature is a crucial parameter governing the efficiency of the process. The temperature increase results in an improved rates of mass transfer between the phases while adversely affecting the partition coefficient(s)[69]. According to SPME theory, during an extraction process the fiber equilibration process is exothermic where an increased temperature of the sample will decrease both, the analyte recovery and the

extraction time[70, 71]. To obtain on optimum extraction temperature profile, the analytical procedure was performed at temperatures of 30-60°C.

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

GC/MS analysis was performed on an Agilent GC/MS, as previously described. The flow rate of the H_2 carrier was 1.0mL/min. The oven temperature was programmed to hold at 40°C for two min and then increased to 250°C at a rate of 8°C/min and held at 250°C for 5 min. Sample extracted by SPME was exposed to the injector at 250°C for one minute with the split ratio of 1:10. Mass spectra were obtained by electron-impact ionization. The temperature of the ion source was set at 230°C. Mass spectrometric data from m/z 45 to 300 were collected using a scan rate of 5.36/s, with an ionization voltage of 70eV. Quantification was obtained using extracted ion areas and a propyl benzene internal standard.

A stock solution of allyl isothiocyanate of 5000 ppm was prepared in 1-propanol. The stock solution was further diluted with 1-propanol to get a final concentration in the range of 50-4000 ppm (50, 100, 250, 500, 1000, 2000, and 4000 ppm). An internal standard solution containing 2000 ppm of propyl benzene was prepared in 1-propanol. An aliquot of the internal standard solution was then added to the standard solutions and sample to yield a final concentration of 20 ppm. Volatile compounds were extracted using the same SPME fiber under the same conditions as those used for the horseradish sample. The GC running conditions were the same as those used for the sample. Three replicates were carried out for each run. The calibration curve was used to calculate allyl isothiocyanate in horseradish.

2.4.9. Method Development of Headspace-Solid Phase Microextraction (HS-SPME)

Extraction and desorption conditions (e.g., extraction time, temperature, sample size, desorption time, and temperature) were optimized to yield the highest peak area ratio of AITC to internal standard propyl benzene. The extraction profiles were determined with a PDMS fiber that was exposed to the headspace of a sample in a 10mL vial for 10 min at 40°C. Sample size (2 g) and SPME fiber desorption time (5 min) and temperature (250°C) were optimized.

2.4.10. Method Validation

A calibration curve was produced using the concentration of standard solution against the peak area ratios of standard solution to an internal standard. The linearity was determined by evaluation of the regression curve and correlation coefficient (R^2) > 0.99 was considered precise. The limit of detection (LOD) was calculated as the minimum concentration that generates a peak signal at least three times higher than the signal from adjacent noise. A chromatogram of AITC and internal standard is presented in the figure. The peak area ratios for triplicate runs were averaged, and relative standard deviations were calculated for the analyte. The LOD was found to be 0.75 ppm, indicating its good sensitivity. The dynamic range studied was from 50 to 4000 ppm, which was suitable for the analysis of allyl isothiocyanate in horseradish samples.

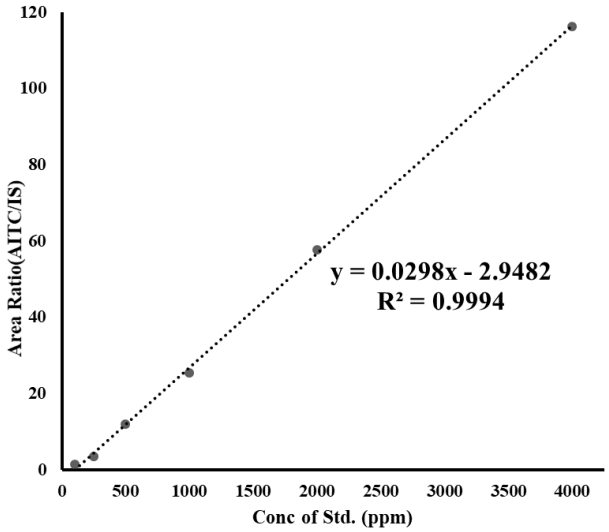

Figure 2.4: Calibration curve for AITC calculated in the concentration range of 50-4000 ppm obtained using HS-SPME-GC-MS.

Table 2.4: The Accuracy and precision of Allyl Isothiocyanate analysis by SPME-GC-MS.

Concentration (ppm)	**Intra-assay**		**Inter-assay**	
	% Accuracy	**% Precision**	**% Accuracy**	**% Precision**
750	94.3-101.1	<5.3	93.0-99.2	<6.0
1500	91.7-96.4	<4.4	97.0-111.4	<5.2
3000	88.3-96.1	<4.2	90.8-94.3	<8.9

The method was validated for intra- and inter-day accuracy and precision over three days using two different concentrations along the calibration curve. Validation was tested at 750-3000 ppm. The accuracy was calculated from the calibration curve against the concentration added to the sample. The precision was expressed as the relative standard deviation. The data for accuracy and precision were determined at each validation level. All intra- and inter-day precisions gave satisfactory results, which were less than 10%.

The accuracies were ranged between 88-112%. Based on the above results, it suggests that the proposed method is simple, accurate, and sensitive.

2.4.11. Influence of SPME Fiber Coating for the Extraction of AITC in Horseradish

We evaluated the influence of SPME fiber coating for the extraction of AITC in horseradish. The combination of various factors is depicted in Table. 2.6, along with the average AITC concentration determined. The 3D-surface plot of each stationary phase coating with respect to time, the temperature on the AITC concentration (response factor) is shown in Figure 2.5. The order of AITC concentration among the four different tested followed the order carboxen/PDMS, DVB/CAR/PDMS, PDMS/DVB, and polyacrylate. The carboxen/PDMS fiber demonstrated the highest average AITC concentration (~1550-1750 ppm), followed by DVB/CAR/PDMS, polyacrylate, and PDMS/DVB among the four different fiber tested. PDMS/DVB showed the lowest average AITC concentration (125-250 ppm). The fiber coating made of carboxen/polydimethylsiloxane (85μm film) showed the highest adsorption of AITC at all the different times and temperatures among the four fiber coatings evaluated. The fiber coating made of polydimethylsiloxane/divinyl benzene with 65μm demonstrated the lowest adsorption of AITC at all different times and temperatures.

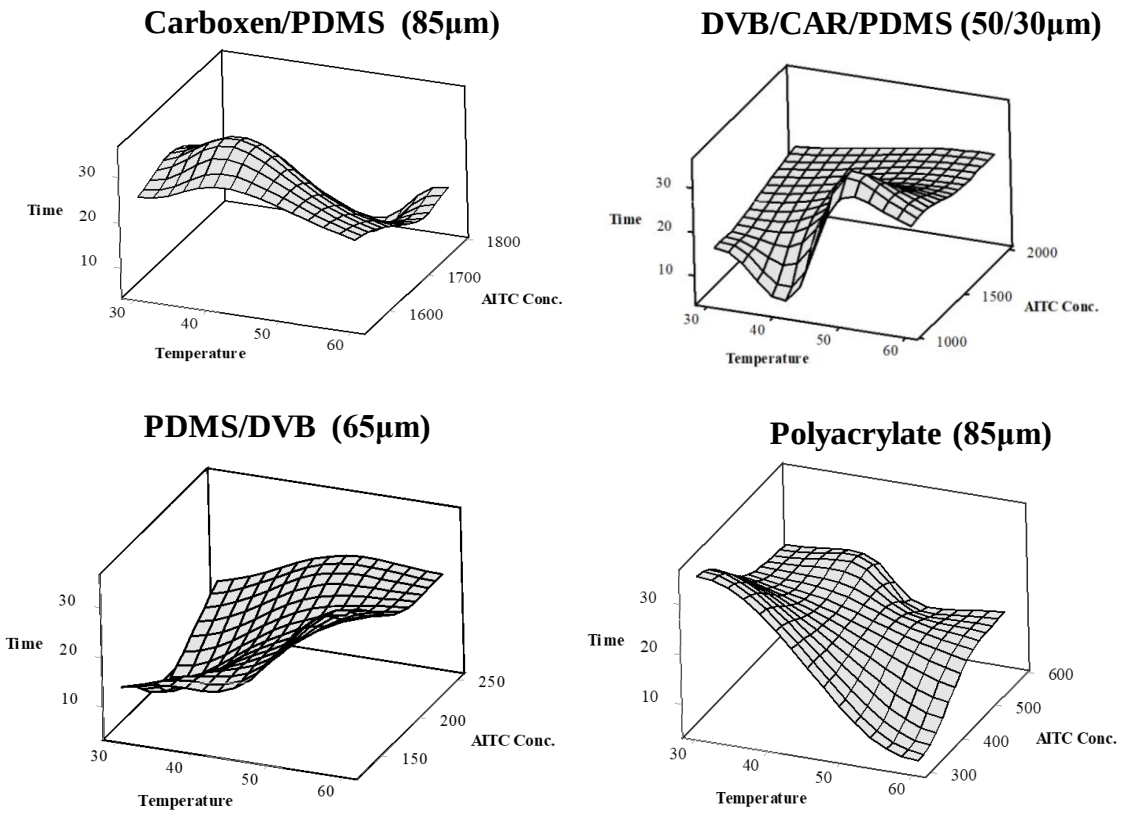

Figure 2.5: Taguchi factorial design to optimize the effect of experimental variables (extraction time and temperature) on the AITC extraction using four different fiber coatings. The data represents average AITC concentration (ppm) under the influence of time and temperature (experimental variables) in presence of four different fiber coatings.

Table 2.5: Extracted AITC average concentration (ppm) using Taguchi factorial design at different temperatures and times with four different fiber coatings.

Fiber	Time (min)	Temp. (˚C)	Avg. AITC Conc. (ppm)
Carboxen/PDMS	25	30	1549.75 ± 53.96
Carboxen/PDMS	35	40	1613.78 ± 50.46
Carboxen/PDMS	5	50	1770.55 ± 43.92
Carboxen/PDMS	15	60	1789.79 ± 56.70
DVB/CAR/PDMS	15	30	1120.629 ± 52.14
DVB/CAR/PDMS	5	40	1075.708 ± 48.71
DVB/CAR/PDMS	35	50	1178.194 ± 48.11
DVB/CAR/PDMS	25	60	1983.194 ± 57.04
PDMS/DVB	5	30	178.8316 ± 14.14
PDMS/DVB	15	40	127.599 ± 11.80
PDMS/DVB	25	50	251.2528 ± 34.60
PDMS/DVB	35	60	125.9698 ± 7.75
Polyacrylate	35	30	299.6526 ± 20.36
Polyacrylate	25	40	543.5213 ± 22.01
Polyacrylate	15	50	572.2037 ± 14.01
Polyacrylate	5	60	309.5141 ± 20.19

2.4.13. Sample Analysis

The investigation of the factors affecting AITC concentration in horseradish was performed on six batches of samples. Table 2.6 below lists the factors studied, storage conditions and the objective for each factor. The study was performed over 24 weeks for samples stored at room temperature and under refrigeration.

Table 2.6: Study design of parameters/factors along with the sample duration and reason(s) for analysis at room and refrigerated temperatures.

Parameters/ Factors	Sample Analysis Duration	Reason(s)
Jar-jar variation	24 jars analyzed	To investigate the variability in AITC concentration within a batch of horseradish sauce.
Influence of storage condition	24 weeks	To study the effect of storage condition temperature on the shelf life of horseradish sauce by analyzing the AITC concentration.
Opened and sealed sample jar	26 weeks	To study the trend in the loss of AITC concentration with time by comparing the freshly opened jars and in-use jar sample at two storage conditions.
Influence of harvest season	24 weeks	To evaluate the effect of harvest season, fall and spring on the shelf life of horseradish sample by monitoring the AITC concentration.
Influence of modifiers/ ingredients	24 weeks	To evaluate the effect of ingredient, rosemary oil and mustard oil on the shelf life of horseradish sample by monitoring the AITC concentration.
Influence of increased salt concentration as an ingredient	24 weeks	To evaluate the effect of increased salt concentration on the shelf life of horseradish sample by monitoring the AITC concentration.

2.5.Results and Discussions

We hypothesized that hydrolysis of sinigrin in the presence of myrosinase enzyme adds to the AITC concentration and is one of the main factors contributing to AITC concentration. Being volatile, AITC continuously diffuses from the sample leading to the loss in concentration, and further, AITC hydrolyzes into smaller ITC molecules.

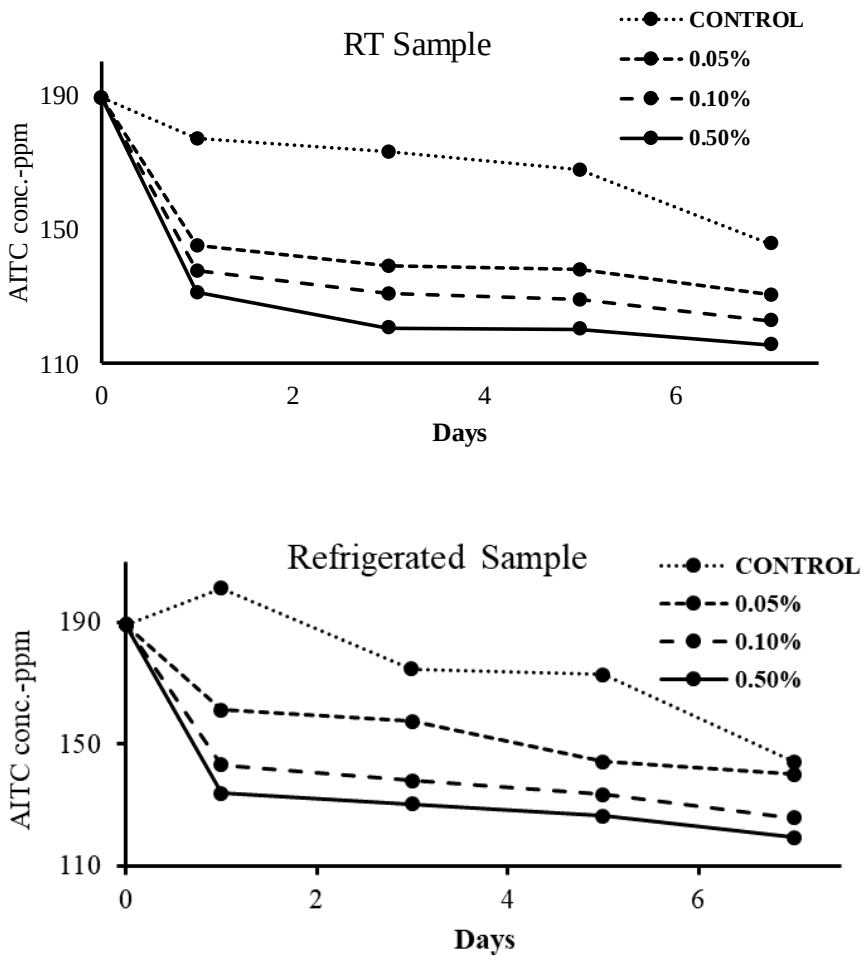

Figure 2.6: Change in the concentration of AITC in the presence of sodium azide for sample stored under room temperature (A) and refrigerated temperature over the course of 7 days.

To prove the hypothesis, continuous production of AITC via hydrolysis reaction, we performed the analysis by inactivating the enzyme in a given sample and investigating the change in AITC concentration with time.

The samples were prepared by addition of 0.05-0.5% sodium azide to the samples. Prepared samples were analyzed at predetermined time points over seven days. The sample mixtures were stored for 24h at the respective storage conditions before analysis to allow the enzyme inactivation. The analysis was done in triplicates for control and test samples.

2.5.1.Jar-Jar variation in the AITC Concentration

One of the essential factors to be considered for shelf life testing via analytical methods is the sensitivity of the method and the robustness of the instrumental technique. To address the reliability of the method and the instrument, we evaluated the AITC concentration in thirty jars (Jar 1-24). All the samples jars under testing were analyzed within 72h of manufacturing. Prior to the analysis, samples were refrigerated, and sample analysis was performed in triplicates for appropriate statistical analysis as previously described. The concentration of AITC in each jar was calculated from the calibration curve previously performed. The concentration of AITC within a batch of samples ranged from 747.75 ppm (lowest) to 1094.45 ppm (highest) with the average concentration being 868.71 ± 112.30 ppm. The results from this preliminary experiment provide an indication that the variability in the concentration of AITC when evaluated under these conditions was not significantly altered. There was minimal variation due to the instrument providing the reliability of the method for further analysis.

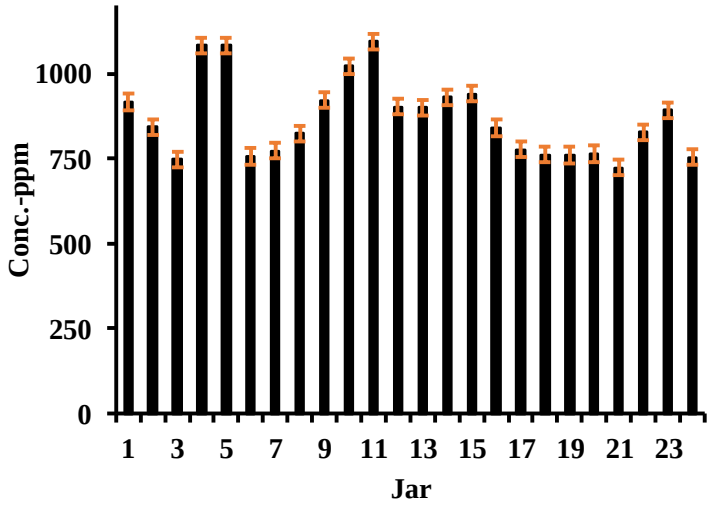

Figure 2.7: Variation in the AITC concentration (ppm) in different jars (jar 1-24) from the same batch of prepared horseradish. Data represents mean ± std.dev. (n=3-4).

2.5.2. Room Temperature versus Refrigerated Temperature (Storage Condition)

To study the effect of temperature on the AITC concentration, we evaluated the AITC concentrations over 24 weeks at two different temperatures, room temperature (23˚C-25˚C) and under refrigeration (4˚C). The concentration of AITC in each jar was calculated from the calibration curve, and samples were analyzed in triplicates or more for statistical analysis. The average AITC concentration for samples stored at room and under refrigeration were found to be 267.53 ± 170.25 ppm and 563.30 ± 224.32 ppm respectively.

One possible reason for the loss of AITC might be due to the storage temperature. At higher temperature, the enzymes are more active and could promote the conversion of AITC from sinigrin via enzymatic hydrolysis. Simultaneously, AITC being volatile diffuses from the sample. However, at lower temperature, the enzymes are less active and subsequent hydrolysis is negligible and the diffusion rate of AITC is decreased. Hence,

we do not see the similar pattern of decrease in AITC concentration from weeks 6-24 for samples stored under refrigerated temperature. The rate of degradation followed a similar trend for both the temperature conditions; however, the samples stored under refrigerated condition demonstrated higher AITC concentration.

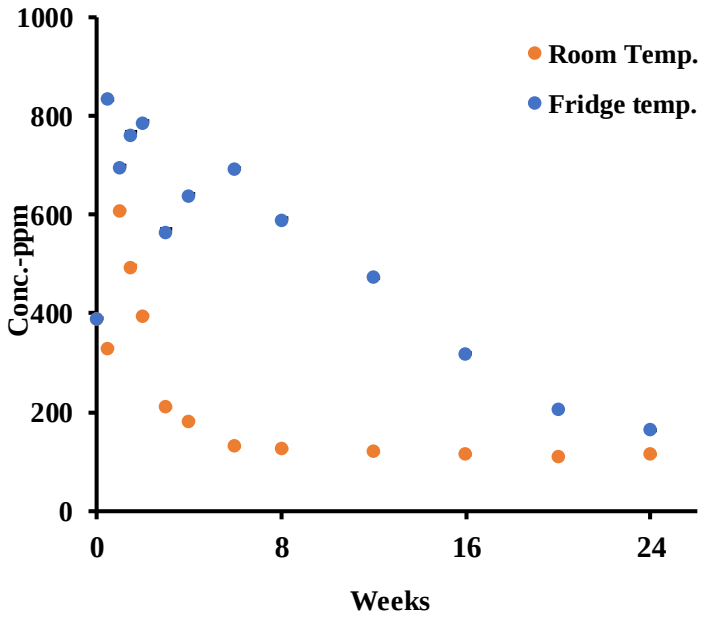

Figure 2.8: Variation in AITC concentration concerning temperature (room and refrigerated temperature). Samples were stored at room and refrigerated temperature over 24 weeks, and the AITC concentration was analyzed. Data represents mean ± std.dev. (n=3-4).

The results of this study demonstrate that storage at a lower temperature could potentially increase the stability of the product. The samples stored at room temperature showed loss of AITC concentration after two weeks, and this continues over 24 weeks, whereas the refrigerated samples were preserved up to week 12.Based on the results

obtained from this experiment, we have studied the influence of various parameters/factors at refrigerated temperature.

2.5.2. Stability of Unopened Jars

To further study the shelf-life stability of AITC in horseradish, we evaluated two different set of samples, continual sampling from the same jar or a new jar for each sampling point. Both the sample groups were refrigerated, and the analysis was performed for 24 weeks. The concentration of AITC in each jar was calculated from the calibration curve.

Table 2.7: Variation in AITC concentration with respect to a new jar and opened jar over six months at refrigerated temperature. Data represents mean ± std.dev. (n=3-4).

Time (weeks)	**Average concentration (ppm)**	
	Same jar	**New jar**
0	469.56 ± 1.46	473.91 ± 1.29
0.5	376.99 ± 1.40	465.24 ± 1.21
1	506.35 ± 3.73	628.03 ± 2.26
1.5	446.64 ± 1.08	467.49 ± 2.34
2.5	352.66 ± 0.74	374.17 ± 0.80
3	277.83 ± 0.26	298.59 ± 0.49
4	274.72 ± 0.51	300.88 ± 0.17
6	252.70 ± 0.42	299.58 ± 0.44
8	333.64 ± 0.92	307.20 ± 0.56
10	282.11 ± 0.49	250.37 ± 0.67
12	281.58 ± 0.77	499.71 ± 2.75
16	324.08 ± 1.86	305.71 ± 0.31
22	177.42 ± 0.39	234.93 ± 0.78
26	135.11 ± 0.11	173.56 0.27

There was little difference in the AITC concentration over 24 weeks. The concentration of AITC in both sample groups in the initial weeks was higher as compared to the same groups at approximately four weeks (Table 2.7). One possible reason for this

decrease in the AITC concentration is enzyme hydrolysis, as well as loss due to the volatilization, as open jars are continuously being exposed to the environment.

2.5.3. Harvest Season

The influence of harvest season on the AITC concentration was studied over 24 weeks. The manufacturing method and the ingredients were kept the same. Horseradish from different seasons, spring and fall, were used for the manufacturing of the horseradish sample. Samples were refrigerated, and a new jar was used at each sampling point. The average AITC concentration in the horseradish samples harvested in the spring was 213.03 ± 111.40 ppm, whereas that harvested in the fall was 321.43 ± 101.87 ppm. Figure 2.9 shows that spring-harvest samples showed a decrease in AITC following week three, which slowed down after eight weeks.

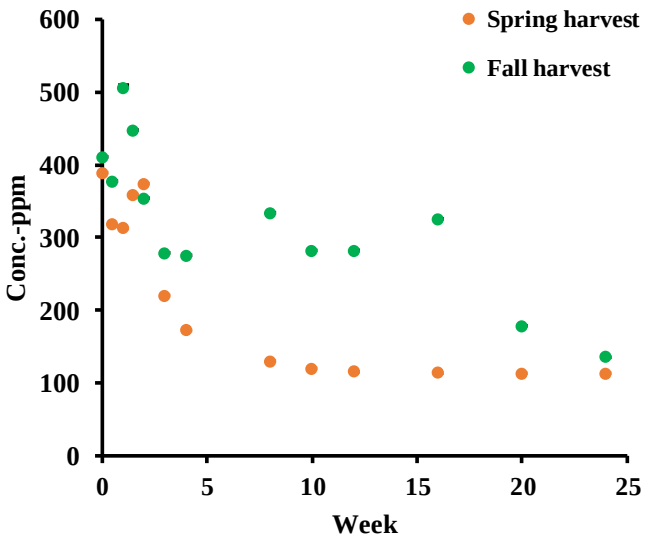

Figure 2.9: Variation in AITC concentration for the harvest season (spring and fall) for six months. Data represents mean ± std.dev. (n=3).

Fall-harvest samples maintained high levels of AITC up to sixteen weeks and then showed a decrease by week twenty. The results from this study indicate that the fall harvest horseradish contains higher levels of AITC, and a possible reason could be the plant and roots are more mature by fall compared to spring.

2.5.4. Addition of Antioxidants

Mustard oil or rosemary oil were added to the samples by the manufacturer to minimize oxidative loss of AITC. All samples were analyzed in replicates of three or more, and the concentration was calculated from the calibration curve. The average AITC concentration for mustard oil-modified samples was found to be 1518.18 ± 844.80 ppm and for rosemary oil-modified samples, 1362.12 ± 896.65 ppm. The variation in the AITC concentration was observed due to the addition of the stabilizer (antioxidant) in the horseradish product. Figure 2.10 demonstrates consistency in the sample with mustard oil until week 10, which starts to decrease from week 10 to week 24. However, the rosemary containing sample demonstrated a consistent decrease in the AITC concentration over the 24 weeks analyzed. Traditionally, herbs and spices have been recognized and utilized for food preservation and medicines. Rosemary and mustard are among a few herbs that are being used as a food preservative. Both the oils are rich in antioxidant property, supporting the longer shelf life[72, 73].

Rosemary (*Rosmarinus Officinalis*) belongs to the Lamiaceae family and is an aromatic plant with medicinal peoperties[74]. Rosemary extract is well known for its activity due to phenolic components and some important antioxidant oil, that prevents oxidative degradation of food, hence, widely used in food industry[75, 76]. The rosemary oil is rich in α-pinene, borneol, β-pinene, camphor, bornyl acetate, camphene, 1,8-cineole,

limonene, borneol, myrcene, terpineol, and caryophyllene[75]. Mustard seeds, belonging to the Brassicaceae family, is a very popular oilseed crop in Asian countries. Whereas, the mustard oil contains omega-3 alpha-linolenic acid and the omega-6 linoleic acid in addition to monounsaturated fatty acids and polyunsaturated fats. In light of consumer preferences, the antioxidant properties of plant-based phenolic compounds offer natural options to food manufacturers as an antioxidant to enhance the shelf life and overall stability of the food product.[77, 78].

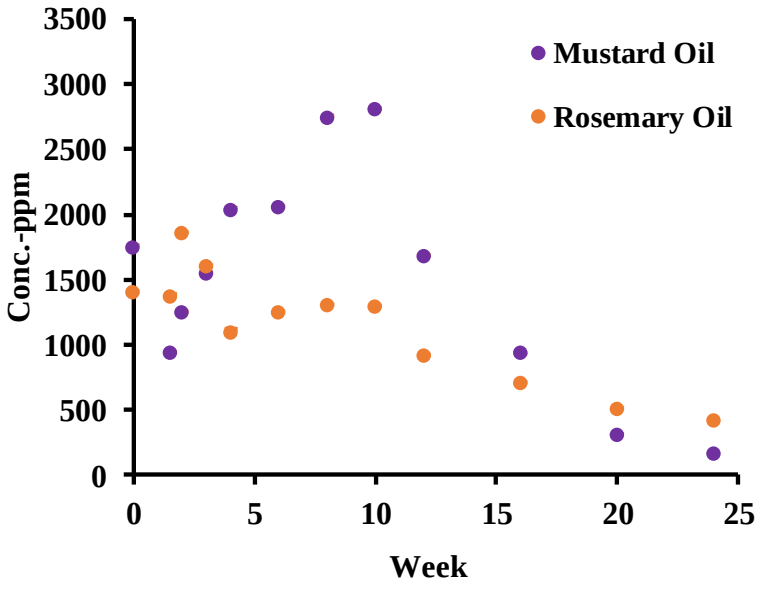

Figure 2.10. Effect of antioxidant (mustard and rosemary oil) on the AITC concentration (ppm) for 24 weeks. Data represents mean ± std.dev. (n=3-4).

The change in AITC concentration was investigated on two batches of refrigerated samples, containing mustard and rosemary oil. Figure 2.10 indicates the addition of rosemary oil as an antioxidant preserves the horseradish sample better compared to the samples containing mustard oil as a preservative. One possible reason could be because rosemary is highly rich in polyphenols that are known for their

antioxidant property; hence, it has maintained the AITC concentration. Whereas, mustard oil is rich in AITC mainly incorporated AITC and resulted in an increased level of AITC levels up to ten weeks in the sample.

2.5.5. Modified Horseradish Sauce (Salt Addition)

Since ancient time, salt has been utilized as a preservative and known to prolong the shelf life of food products. With salt, civilizations were able to store food and build up reserves that would last in times of scarcity. Salt concentration up to 20% in food preservation has been optimal[79, 80]. Salt potentially preserves food in two ways: (i) *High salt concentration is toxic to microbes:* High salt concentration kills and prevents most of the microbes in food product because of the osmolarity effect. Osmolarity cause water to diffuse from the cells in the environment to achieve solute equilibrium in the system the cell and the enviroment[81, 82]. (ii) *Salt dries out food*: Salt dries out food by drawing water out from food resulting in dehydration. This process forbids bacterial growth that can cause food posioning[82, 83].

The influence of salt concentration on the AITC concentration for the fall harvest horseradish samples was studied over 24 weeks. All samples were refrigerated, and a new jar was opened each week. The average AITC concentration was found to be 316.02 ± 72.07 ppm. The variation in the AITC concentration could be due to the differences in the salt concentration in the final product. The trend in the loss of AITC concentration was found the same as the control sample, though the samples overall showed lower concentration (below 500 ppm) of the AITC throughout the analysis period. Figure 2.11 indicates an overall low concentration of AITC throughout the six months of analysis. The addition of salt might have helped to avoid food spoilage, but as salt draws out water

from the cells, it resulted in excess of water which might have interrupted the continuous hydrolysis process and overall decrease the AITC level in the sample. Hence the addition of increased salt concentration did not preserve the horseradish sample.

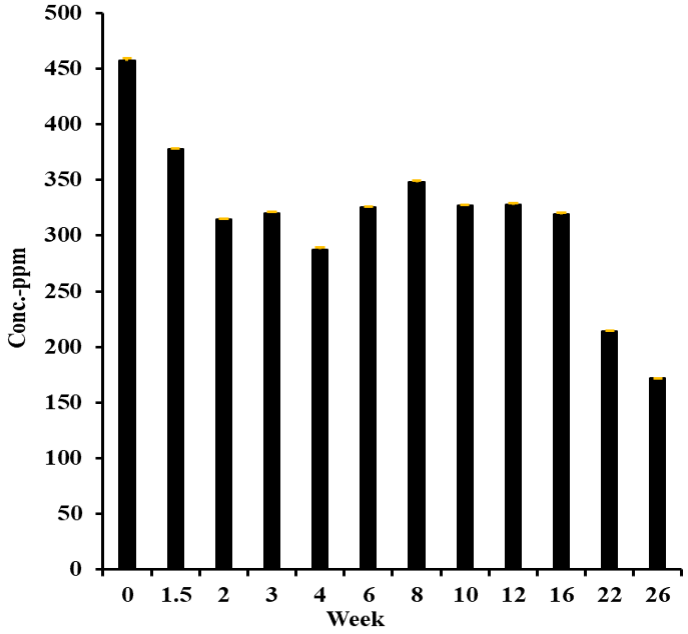

Figure 2.101: Effect of salt on AITC concentration (ppm) for 6 months. Data represents mean ± std.dev. (n=3).

2.6. Overall Summary

Upon receipt of the samples, one set was kept on the open laboratory bench (room temperature). The other set was refrigerated upon receipt, and the initial analysis was performed the following day. In all cases, the concentration of AITC dropped significantly during the first two weeks, then stabilized. However, the amount of AITC loss was much less in the refrigerated samples. The sealed sample jar (new jar) has maintained the AITC level on both storage conditions, but the refrigerated samples have indicated prolonged shelf life compared to the samples stored at room temperature. Fall-

harvest horseradish roots indicated high levels of AITC. This could be due to the maturity of the horseradish roots compared to the spring-harvest horseradish roots. Addition of antioxidant (rosemary oil and mustard oil) has increased the shelf life of horseradish sample. Increased salt concentration indicates an adverse effect on the shelf life of horseradish sauce.

For each factor, the samples were studied for both the storage conditions. There are three processes taking place once the food processing begins, starting with the cutting or grating of horseradish roots. 1. There is continuous hydrolysis reaction taking place which produces AITC, 2. Continuous diffusion of volatile compounds (ITCs) into the environment, and 3. Further degradation of AITC into smaller ITCs. The sample stored under room temperature conditions, especially, exhibited an exponential decay of the AITC concentration, stabilizing at about one-and-a-half to two weeks where the above-mentioned process is still going on. Whereas, the apparent rise in AITC levels with the refrigerated sample was observed. It is because processes 2 and three are slowed down at lower temperatures. Subsequently, the refrigerated sample appears to degrade similarly as the room temperature sample, except it has maintained the higher levels of AITC throughout the storage period of 26 weeks.

2.7. Conclusions

This work has demonstrated the effect of various parameters on the shelf life of horseradish product. The study includes complete optimization, development, and validation of a simple, quick, robust, and effective method for analysis of AITC in horseradish sauce. The analytical method utilizes a popular and fast-growing technology of microextraction, SPME, for sample preparation. The sample preparation is easy, quick,

fast, environmentally friendly with high precision and accuracy. The method presented can be used to assess the change in volatile components during the storage and processing of food flavor products. A sample stored at room temperature possesses a shorter shelf life compared to the refrigerated samples. There was no significant difference in AITC concentration for a new jar and opened jar when stored under refrigeration. The fall-harvest horseradish possesses higher concertation of AITC compared to spring harvest, and the change in ingredient affects the shelf life of the product (stabilizer, salt, water). Table 2.8 is the summary of the factors studied and their effects on the shelf life of the horseradish product. The effects are summarized as a positive effect or as an adverse effect.

Table 2.8: Summary of the effect of factors on the shelf life of Horseradish products.

Parameters	Effect on shelf life
Jar-Jar variation in AITC concentration	NA
Room temp. v/s Fridge temp	++
New Jar v/s same jar	+
Fall harvest v/s spring harvest	+
Modified horseradish sauce (ingredient -oil)	++
Modified horseradish sauce (ingredient -salt)	_ _

Adverse (-), low (+), medium (++).

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CHAPTER III

INVESTIGATION OF MOLECULAR INTERACTIONS OF HOST/GUEST COMPLEXES

ABSTRACT

The binding strength or the energy of the intermolecular interaction, between the solute and the sorbent determines the retention of analyte species in chromatography and sorbent-based extraction, such as solid-phase extraction (SPE). This binding energy depends on the identity of the sorbent phase, amount of stationary phase (e.g., carbon loading), degree of endcapping, temperature, solvent environment, and other sorbent properties which influence the solute-sorbent interactions. The EMR (Enhanced Matrix Removal)-Lipid is a novel technology that removes lipid utilizing a combination of size exclusion and hydrophobic interactions. The efficient lipid removal could provide minimal ion suppression of target analytes, preventing analyte loss and leads to higher selectivity and efficient lipid/matrix removal. The current study aims to evaluate the binding capacity utilizing a model system of a lipid mixture with a commercially available sorbent, EMR-Lipid. The extent of complex formation was studied using various ratios of sorbent/lipid (1:1, 1:2, and 2:1) employing various analytical and physicochemical techniques. Fourier-transform infrared spectroscopy (FTIR), differential scanning calorimetry (DSC), and powder X-ray diffraction spectrum (PXRD) of the complexes were studied. The difference in FTIR spectra was observed by changes in the peak intensity and shift in bands of the sorbent-lipid. The data from XRD indicates the absence of crystalline behavior. The DSC thermogram for the complex showed characteristic peaks indicating complex formation. The results from ITC reveals that the complex formation between the polymer and the lipid is driven by the negative enthalpy and entropy factor. Taken together, the combined results from FTIR, DSC, PXRD, and ITC confirm complex formation. Furthermore, the results of this study will provide a

fundamental understanding of lipid-binding properties and guide the development of next-generation materials for the separation of lipid classes.

3.1. Introduction

Liquid chromatography-mass spectrometry (LC-MS) has become a popular method of choice for the analysis due to its advantages such as high specificity, sensitivity, and throughput[5]. However, the high selectivity of LC-MS does not promise the efficient elimination of interferences due to co-eluting matrix components. These are not detected in the chromatogram but can have an unfavorable effect on the analysis. This happens as the co-eluents cause ion suppression or enhancement of the analyte in spectrometric detection. Hence, an efficient sample preparation is designed to achieve maximum extraction of analytes with minimum matrix component in the final extracts.

Food matrix complexity is a result of various components. These components have their own physical and chemical properties. Food such as avocado, nuts, dairy products, seafood, etc. are termed as fatty food as the fat composition >2% . Table 3.1 is a list of few fatty food samples with fat, water, and carbohydrate percentage. The fat in the matrix could be of both lipophilic and hydrophilic nature. To meet consumer's demand, the agricultural industry uses the pesticide and fertilizer at different stages of cultivation to provide the protection from the pest and to have a higher yield respectively. The persistence and contamination of this pesticide continue to the end product. Therefore, there are strict government regulations to limit and monitor the residue in the food product as they are harmful to the environment and human health.

Table 3.1: Fatty vegetables with respect to their composition. Modified and adapted[6].

Sample	Water %	Carbohydrate %	Fat content %
Avocado	73.2	-	14.66
Oats	8.2	66	6.9
Olives	-	-	18-25
Almond, nuts	4.7	21.7	49.4
Coconut	46.9	15.2	33.49
Hazelnut	5.3	16.7	60.7
Walnut	4.0	13.7	65
Soybean green	67	11.0	6.8
Sesame	-	23.4	49.7
Acorns, nuts, raw	27.9	40.7	23.86

The sample preparation is an essential step in the process of food analysis, but it becomes much more critical with complex matrices. It can be cumbersome, time-consuming, and labor-intensive. Especially, with lipid dominated samples, it becomes highly challenging to extract analytes from the sample matrix. If not, this could lead to analytical error and variability in results and further reducing the instrument and column lifetime. Several approaches have been made to achieve efficient sample preparation and cleanup for food samples, such as solid-phase extraction.

QuEChERS: Enhanced Matrix Removal-Lipids

QuEChERS ("quick, easy, cheap, effective, rugged, and safe") is a sample preparation mechanism that employs acetonitrile solvent for the extraction step followed by dispersive solid-phase extraction (d-SPE) for cleanup. The application involves the extraction of multi-residue analytes, (pesticides in fruit and vegetables)[7, 8]. The method was developed by Anastassiades, Lehotay, Stajnbaher, and Schenck in 2003. Its simplicity and high throughput has led to an increased considerable interest and acceptance, in the analysis of residues of pesticides[9], veterinary drugs[10], environmental

contaminants[11], and mycotoxins[12] in diverse food and environmental samples. It is applied to fruit and vegetables[9], and other hydrophobic matrices that are rich in fat such as avocado, olives and olive oil. This is essential as it enables the analytical laboratories to meet the increasing requirement cost-economic and high sample throughput operations[13]. Some of the reasons that make the application of QuEChERS for the analysis of a variety of sample types is its ability to perform analysis with small sample size, amount of laboratory glassware, fewer processing steps.

Extract with a small amount of lipid might cause damage to chromatographic columns and its coating, in gas chromatography – (tandem) mass spectrometry [GC–MS(/MS)][14]. Though several QuEChERS cleanup steps are available in sample preparation methods, each method has some limitation in effectiveness and/or efficiency[15]. To address the above limitations with the sorbents currently employed, Agilent Technologies Inc. has developed the Bond Elut Enhanced Matrix Removal—Lipid (EMR—Lipid)[16, 17]. EMR-Lipid is a novel sorbent that permanently removes major lipids from the sample matrix. It is highly selective, doesn't cause analyte loss and is employed for fat samples with > 5% fat content[9]. Additionally, it provides fast, accurate, robust, and effective sample preparation for preparation of samples with high-fat content employing LC/MS or GC/MS. It is highly effective in matrix removal and provides better analyte recoveries which substantially reduce instrument maintenance and downtime[18, 19].

EMR-Lipid Binding Principle

The make-up of EMR-Lipid is proprietary. EMR-Lipid mechanism of action is unlike solid adsorbent in (d-)SPE, though it does dissolve to saturation in matrix solution.

The separation principle based on hydrophobic interactions. EMR-Lipid is selective to all lipid classes[20]. Lipids with long-chain hydrocarbons fit within the EMR-Lipid structure and are trapped. The complex formed, the lipid-EMR-Lipid complex is separated in the solution either it precipitates out of the solution or stays in the aqueous phase (salting-outstep)[21, 22]. EMR-Lipid selectively extracts out lipids from QuEChERS extracts of fatty food samples without compromising analyte loss, such as pesticide or drugs[23, 24].

Lipids in Food

Lipids are of three major class, glycerides (mono, di, and tri), phospholipids and fatty acids. These classes are further classified. Table 3.2 is a list of few oils with saturated and unsaturated fatty acid and total fat content[25]. Edible fats and oils are a complex mixture of triglycerides (~95%), diglycerides (~2%), free fatty acids (~0.5%), phospholipids (Table 2)[26].

Table 3.2: Vegetable oils with respect to their fat composition. Modified and adapted[6].

	Composition		
Sample	**Saturated %**	**Unsaturated %**	**Fat content %**
Corn oil	12.9	27.6	100
Mustard oil	-	59	100
Olive oil	13.8	72.9	100
Peanut oil	16.9	46.2	100
Sesame oil	14.2	39.7	100
Soybean oil	15.6	22.8	100
Sunflower oil	9	57	100

Fats and oils play major role in human nutrition and also contributes to the texture and flavor to the food[27]. Therefore, it would be advantageous to have an advanced SPE

method in the form of QuEChERS that is lipid class-specific. This will bring an advanced approach in the analysis of a particular lipid class in a sample of lipid mixture.

Sorbent Chemistry and Binding Mechanism

The SPE process can provide analyte separation in the sample, with minimal matrix components and analyte with enough concentration to be detected. Solid-phase extraction process involves the interaction of three components: the sorbent, analyte, and the eluent[28, 29]. For an efficient extraction the analyte must have stronger interaction to the sorbent than to the sample matrix[30]. The chemical properties of analyte and sample matrix are the basis for the sorbent selection. Although the principle "like dissolves like" is the basic principle underlies for all experimental work, the chemistry of the solvent is of more importance in the separation science[30, 31]. By varying the chemistry of solvent an analyte can be extracted from the sample matrix by a sorbent. The analyte and the other matrix components separation in the SPE procedure could be achieved in three ways:[32]

a. Selective extraction, where sorbent bed retains selected components allowing the remaining matrix components to passed through the bed.
b. Selective washing, where the sorbent retains both, the analyte and the sample matrix . The washing step removes the unwanted components from the matrix while allowing the analyte trapped in the sorbent bed.
c. Selective elution, only the trapped analytes are allowed to pass through the sorbent bed using an appropriate elution solvent leaving the matrix retained in the sorbent bed.

Hydrophobic Interaction

In presence of an aqueous solution, the apolar sorptive material and water molecules show polar-apolar interaction that can be readily substituted by an appropriate "guest molecules" with less polarity than water[33, 34]. In this case the dissolved sorptive material is the "host" molecule, and the complex formation is driven by the substitution of the high enthalpy water molecules by an appropriate molecule, a "guest" [35]. The mechanism might vary depending on the nature of the lipid chemistry. In the case of lipid removal from the aqueous sample matrix, the sorbent material has both hydrophobic and hydrophilic characteristics, where it gets solubilized in aqueous media and provides a hydrophobic environment for binding of lipids. The foremost common complex type is inclusion complexes, that can be separated as a stable component, crystalline form. The dissolution of these complexes is followed by the equilibrium between dissociated and associated forms resulting in the complex stability constant. The association and the dissociation of the host/guest complex is driven by thermodynamic equilibrium[37, 38].

Techniques to Measure Binding Constant

The binding capacity of a polymeric sorbent with different lipids class will provide an insight about the host-guest interaction. There are several techniques available to determine the binding capacity (Table 3.3)[39-42]. The combination of techniques will be used for the investigation of binding capacity including the preliminary investigation such as DSC, FTIR and XRD to confirm the complex formation of polymer with different lipids. The information from the preliminary studies will determine the analytical technique to be used for the quantitative analysis or complexes.

Table 3.3: Techniques available to determine binding capacity.

Spectroscopic	Ultraviolet/Visible Circular Dichroism Fluorescence Nuclear Magnetic Resonance Electron Spin Resonance
Electroanalytical	Polarography Voltammetry Potentiometry Conductimetry
Separations	Chromatography Electrophoresis
Other[43]	Isothermal Titration Calorimetry

3.2. Materials

Chemicals and Reagents:

Agilent Technologies provided enhanced matrix removal-lipid (EMR-lipid (polymer)). Lipids (>98% pure), LC grade water and ethyl alcohol were purchased from Fisher Scientific (Pittsburgh, PA, USA).

3.3. Methods

Preparation of lipid-polymer Complexes:

The solid compounds (polymer and lipid/fatty acid) were accurately weighed and dissolved in ~15-20 ml of water and 1-2 ml of ethanol (to solubilize the complex), the total volume was ~ 20 ml. The solution appeared to be milky white with the absence of any detectable solid. The solution was vortexed for 40 to 70 sec to ensure homogeneity of the solution. Various complexes of the polymer to fatty acid/lipid were prepared in molar concentration ratios. The compounds prepared include the following: polymer to decanoic acid (DA) (1:1, 1:2 and 2:1), polymer to stearic acid (1:1), polymer to palmitic acid (1:1) and polymer to oleic acid (1:1). The prepared solutions were subsequently

freeze-dried and lyophilized for 48 h. Lyophilization was performed at a condenser temperature of -104˚C and less than ten mTor pressure. The scheme for subsequent analysis of the prepared complexes (Figure 3.1).

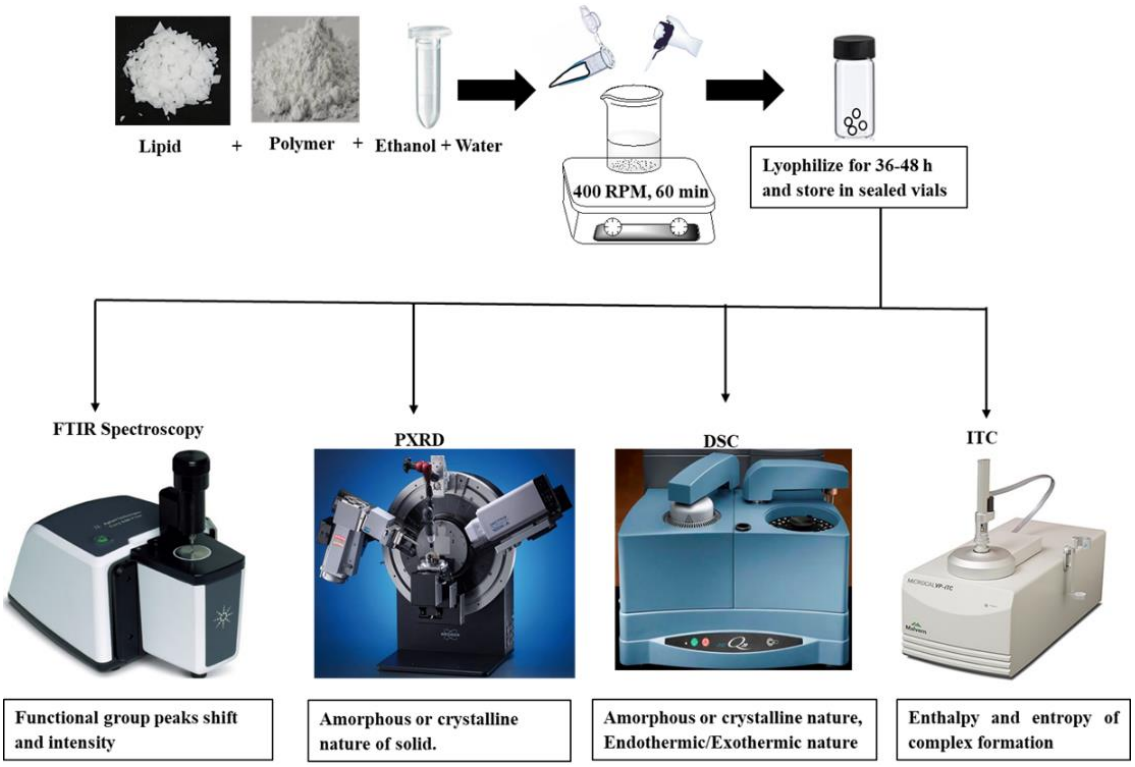
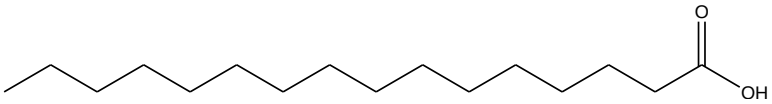
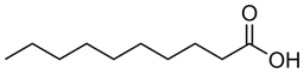


Figure 3.1: A diagram for the process employed for the preparation of lipid-polymer complexes and subsequent analytical methods for studying various physicochemical properties.

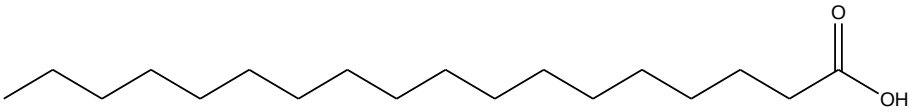
Chemical Structure of Lipids



(a) Palmitic acid



(b) Decanoic acid



(c) Stearic acid

Figure 3.2: Chemical Structure of Lipids: (a) Palmitic acid, (b) Decanoic acid, and (c) Stearic acid.

Fourier Transform Infrared (FTIR) Spectroscopy: FTIR spectra of polymer, decanoic acid (DA), and various complex ratios of polymer to decanoic acid (1:1, 1:2 and 2:1) were performed using Nicolet 380 ATR-FTIR spectrophotometer (Thermo Electron Corp., Madison, WI). The data were acquired between 400 cm^{-1} and 4000 cm^{-1} with the scanning speed of 4 cm^{-1} and 50 scans and the average of 50 scans data was presented.

Powder X-ray Diffraction Analysis: Powder X-ray diffraction measurements of polymer, decanoic acid (DA), the physical mixture of polymer and DA, and polymer to decanoic acid (1:1) were recorded using Rigaku powder X-ray diffractometer using Cu

radiation, running at 40 kV and 44 mA. For this study, samples were mounted on double-sided silicone tape and measurements were performed from 2 °C to 60 °C at a scan speed of 4 °C/min and increments of 0.02 °C.

Differential Scanning Calorimetry Analysis: The DSC analysis of polymer, fatty acid/lipid: decanoic acid (DA), stearic acid, palmitic acid, and oleic acid, the physical mixture of polymer and DA, and various ratios of polymer to the fatty acid. The complexes that were evaluated include: polymer to DA (1:1, 1:2 and 2:1), polymer to stearic acid (1:1), polymer to palmitic acid (1:1) and polymer to oleic acid (1:1) were performed using TA Instruments Q200 Differential Scanning Calorimeter (TA Instruments, New Castle, DE, USA). Samples were weighed (equivalent to curcumin) and placed in sealed Tzero aluminum hermetic pans. With liquid nitrogen as a coolant, samples were scanned at 10 °C/min from -20 °C to 300 °C and thermograms were recorded.

Isothermal Titration Calorimetry: The thermodynamics of binding between lipid and the polymer were studied using MicroCal ITC200 (GE Healthcare) with lipids (20 mM), and polymer (20 mM) respectively loaded in the cell and syringe at 25°C. Twenty 2 µl injections with a 3-minute injection-interval, with a syringe stirring speed of 1000 rpm were used for the titrations. ΔG° of binding at 25°C was computed as –RTln (Ka) where R, T, and Ka are respectively the gas constant, temperature, and association. All titrations were carried out in identical conditions of buffer and temperature. Binding measurements (titrations) for each complex was run in triplicate to confirm the data.

3.4. Results and Discussion

Fourier Transform Infrared (FTIR) Spectroscopy

We have performed FTIR spectroscopy analysis for our initial/preliminary studies using decanoic acid as the model lipid. The figure below presents the FTIR spectrum of DA (a), polymer (b), various ratios of polymer-DA: polymer-DA (c) (1:1), polymer-DA (d) (1:2) and polymer-DA (e) (2:1). The spectrum was recorded using a Nicolet 380 ATR-FTIR spectrophotometer (Thermo Electron Corp., Madison, WI) between 4000 cm^{-1} and 600 cm^{-1} at a resolution of 4 cm^{-1} and 50 scans. The average of 64 scans of data is presented. The FT-IR spectroscopy data can be used to evaluate the interaction between polymer (host) and the lipid (guest) molecules as that the characteristic bands of the guest can change upon complexation. IR analysis of decanoic acid resulted in a very sharp peak at ~1710.29 cm^{-1} and ~2920-2850 cm^{-1} (Figure 3.3a). A strong and complex band at 900–1180 cm^{-1} arising from the stretching of C-O bonds and a broad band at 3000–3600 cm^{-1} due to the symmetric and antisymmetric O-H stretching mode can be seen in case of the polymer (Figure 3.3b). The preliminary indication of complex formation for various ratios can be seen from the change in the interaction pattern and peak intensity. The FT-IR spectrum of the polymer-DA complex at various ratios shows the same characteristic peaks that can be assigned to the polymer and DA, however of weak intensity. In particular, the broad band at 3000–3600 cm^{-1} due to the symmetric and antisymmetric O--H stretching mode seen in case of the polymer reduced in terms of the intensity with various ratios of the polymer-DA from 1:1,1:2 and 2:1 (Figure 3.3c-e). This could be assigned to the increase in the amount of the host (polymer). These changes in the FT-IR spectrum potentially suggests the formation of inclusion complexes.

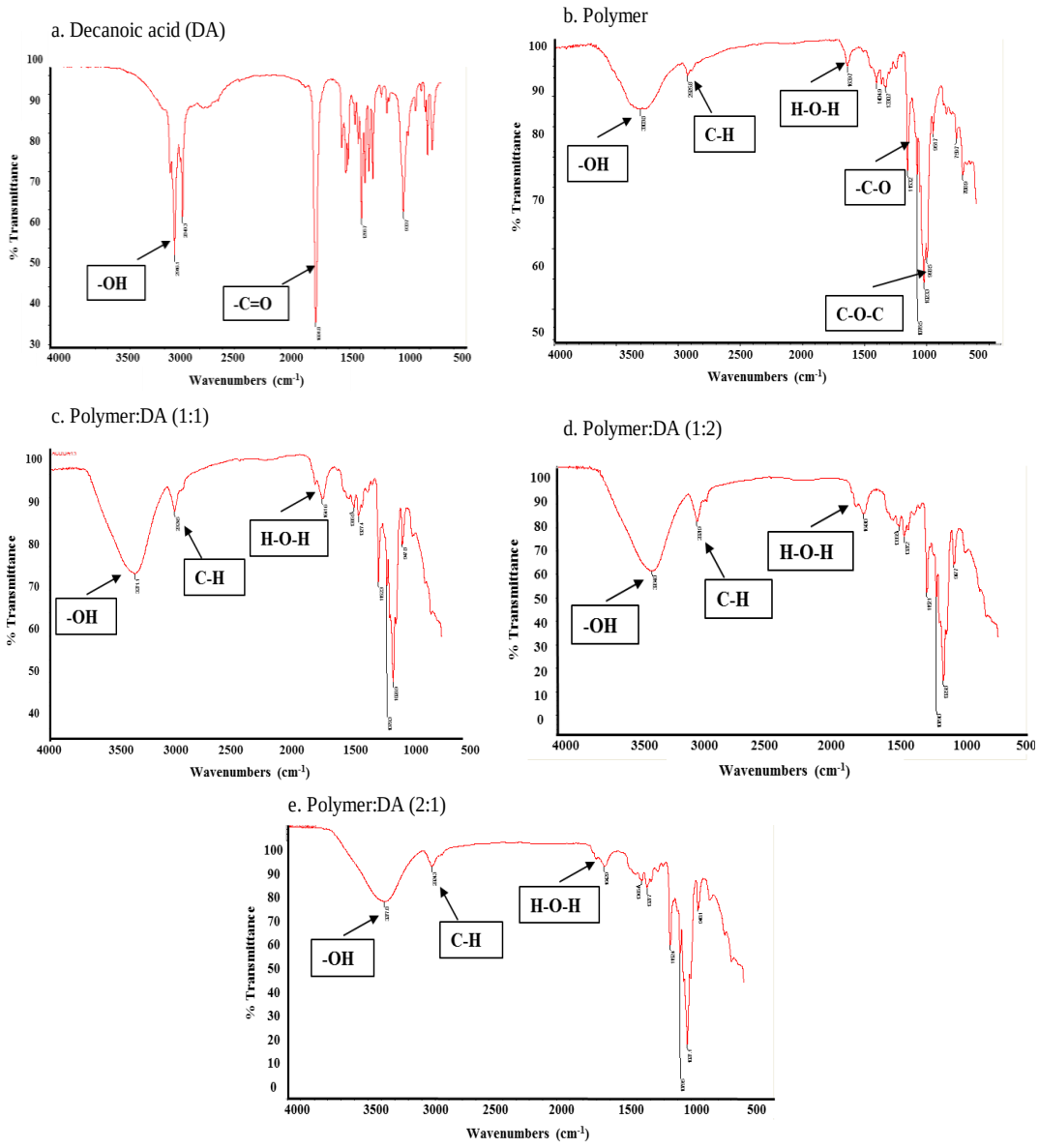


Figure 3.3: FTIR spectrum of DA (a), polymer (b), physical mixture of polymer-DA and various ratios of polymer-DA, polymer: DA (1:1), polymer: DA (1:2) and polymer: DA (2:1).

Powder X-ray Diffraction (XRD)

We have confirmed the results obtained from the differential scanning calorimetry of polymer-DA complex using powder x-ray diffraction. Also, PXRD studies helps to

evaluate the inclusion complex formation comparing the change in crystallinity or amorphicity with host-guest interaction. The figure below represents the PXRD spectrum of lipid (DA), polymer, physical mixture of the polymer-DA and the 1:1 complex of polymer-DA. The plot represents the intensity in CPS and 2θ. The lipid DA (Figure 3.4a) is crystalline and can be seen from the PXRD spectrum. The XRD patterns of the polymer revealed several diffraction peaks (Figure 3.4b), indicating its crystalline character. Comparing the diffraction patterns of the pure components with the physical mixture of the lipid and the polymer, it is observed that the characteristic peaks of the pure lipid and polymer are still present in the diffractograms of the physical mixture, though with reduced intensity (Figure 3.4c).

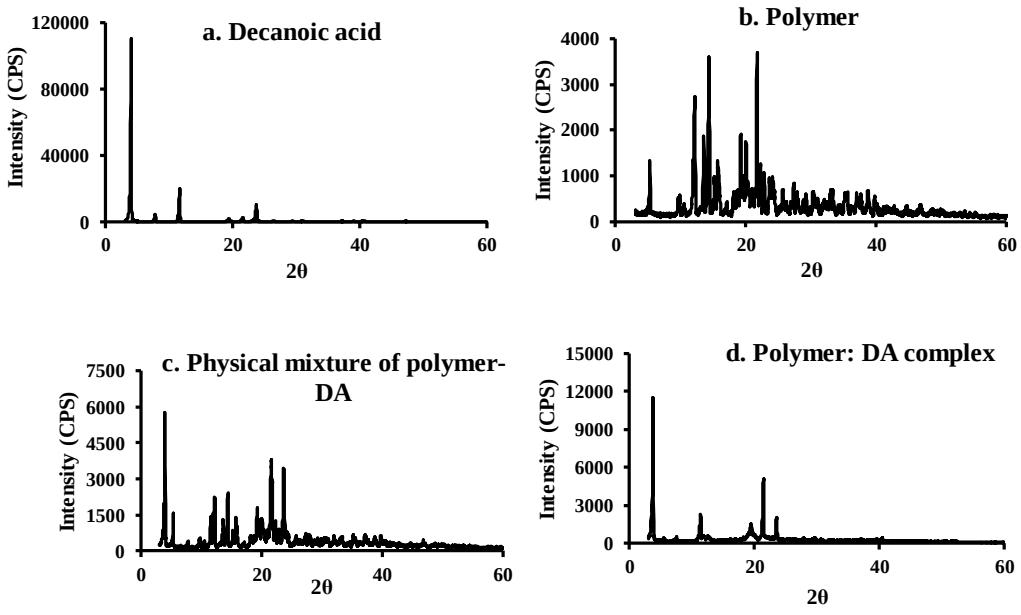


Figure 3.4: PXRD spectrum of DA (a), polymer (b), physical mixture of polymer-DA (c) and polymer-DA complex (d) obtained using Rigaku powder X-ray diffractometer using Cu radiation, running at 40 kV and 44 mA.

The prepared lyophilized complex shows absence of any detectable crystalline behavior and is predominantly non-crystalline/amorphous except in the initial part which might be due to the presence of certain crystalline impurities (Figure 3.4d).

Differential Scanning Calorimetry

The DSC thermogram for the isolated host (polymer) and guest (lipid), along with the physical mixture and the inclusion complexes at various ratios are present in the figure below. The DA show thermograms that is indicative of its characteristic melting point (34˚C) (Figure 3.5a). The DSC curve of the polymer shows endothermic peaks at 86˚C and 163˚C due to release of water molecules and a phase transition (Figure 3.5b). The physical mixture shows peaks that are inclusive of both the DA and polymer. For DA, the melting point is observed ~ at 32˚C at the same temperature as the free lipid indicating that it is present as an isolated solid (Figure 3.5c). In contrast, the polymer peaks are shifted to lower temperatures, potentially suggesting the occurrence of non-inclusion interactions. The inclusion complex formation for various ratios of the polymer could be seen either as a shift in the peaks or appearance of additional peaks. The DSC curves of the freeze-dried inclusion complex show only broad endothermic peak in the range of 30-130˚C, possibly due to water release. In case of polymer-DA complexes of 1:1 and 1:2 showed a weak intensity that can be ascribed to DA melting point; however, the absence of DA melting peak is at 2:1 polymer-DA complex is consistent to inclusion (Figure 3.5d-f). Finally, no DSC peaks assigned to crystal phase transitions can be observed, as the inclusion complex is non-crystalline, according to its XRD pattern.

Furthermore, we have extended the studied by using lipids with a higher carbon chain. Palmitic acid (16 carbon saturated fatty acid) and stearic acid (18 carbon saturated

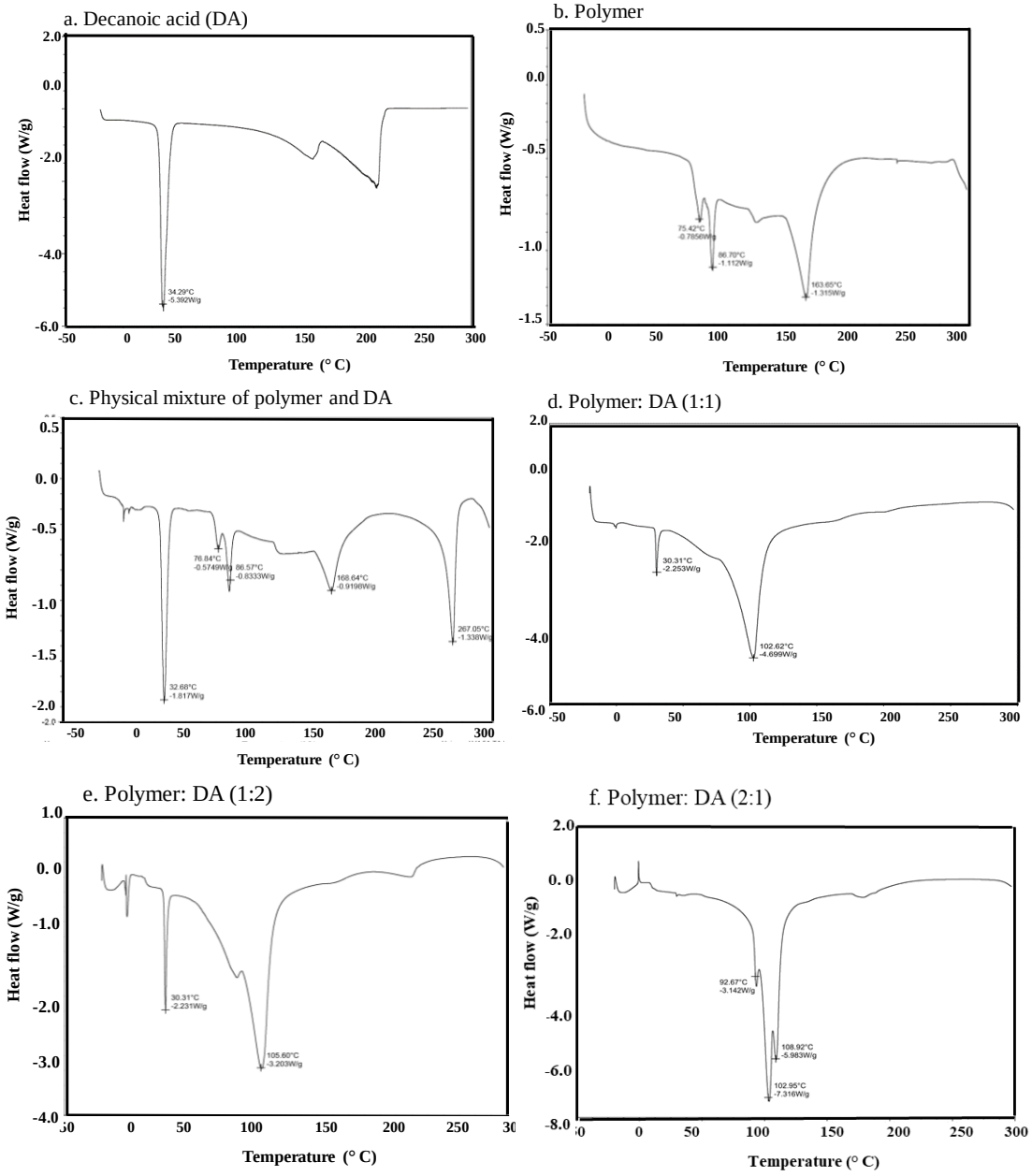


Figure 3.5: DSC spectrum of DA (a), polymer (b), physical mixture of polymer-DA (c) and various ratios of polymer-DA, polymer: DA (d) (1:1), polymer: DA (e) (1:2) and polymer: DA (f) (2:1).

fatty acid). The DSC curves of stearic acid and palmitic acid displayed characteristic peak that is indicative of their melting point, palmitic acid (~ 65 ˚C) and stearic acid (~ 70 ˚C) (Figure 3.6a and c). The DSC curves of the freeze-dried inclusion complex show only broad endothermic peak in the range of 80-220˚C, possibly due to water release (Figure 3.6b and d).

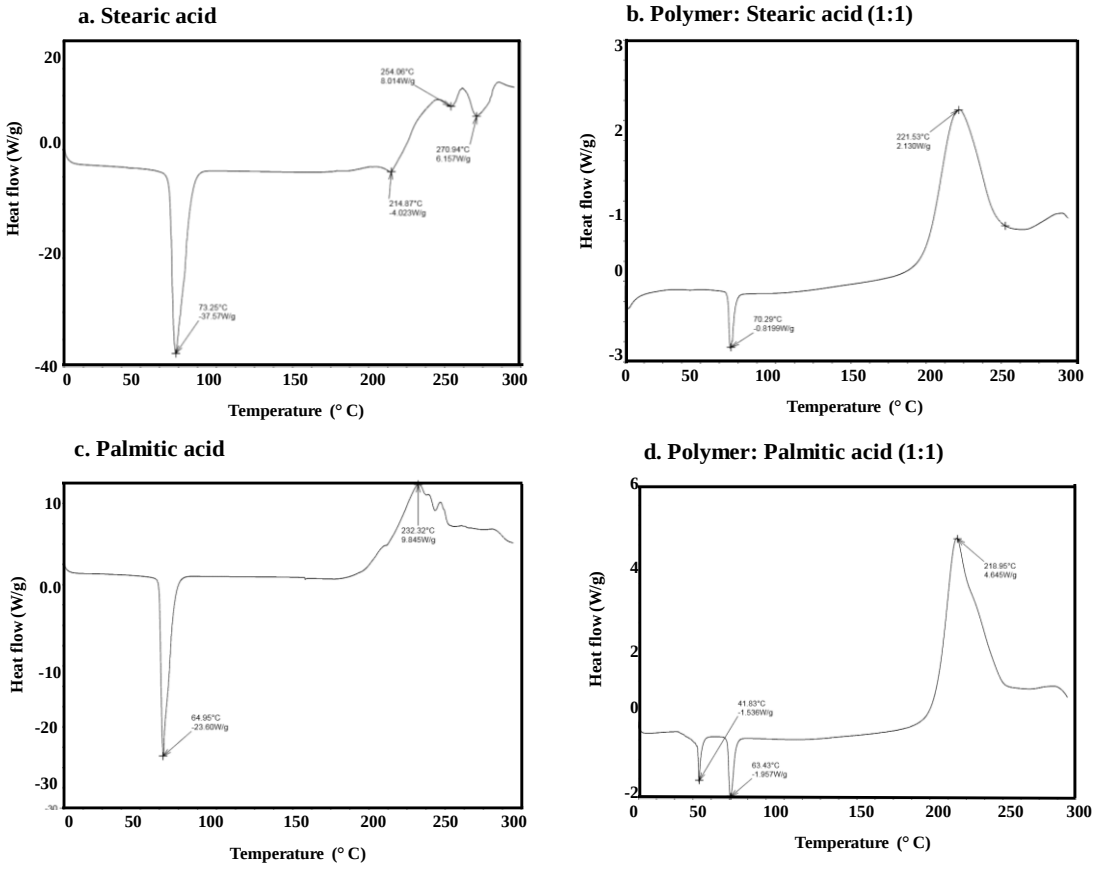


Figure 3.6: DSC spectrum of stearic acid (a) and polymer-stearic acid (b) (1:1) complex and palmitic acid (c) and polymer-palmitic acid (d) (1:1).

Isothermal Titration Calorimetry

We have performed isothermal titration calorimetry of three different polymer-DA complexes (1:1, 1:2, and 2:1) at 25°C. The obtained results of the ITC experiments are given in Figure. shows the equilibrium constants (K) and enthalpies of complex

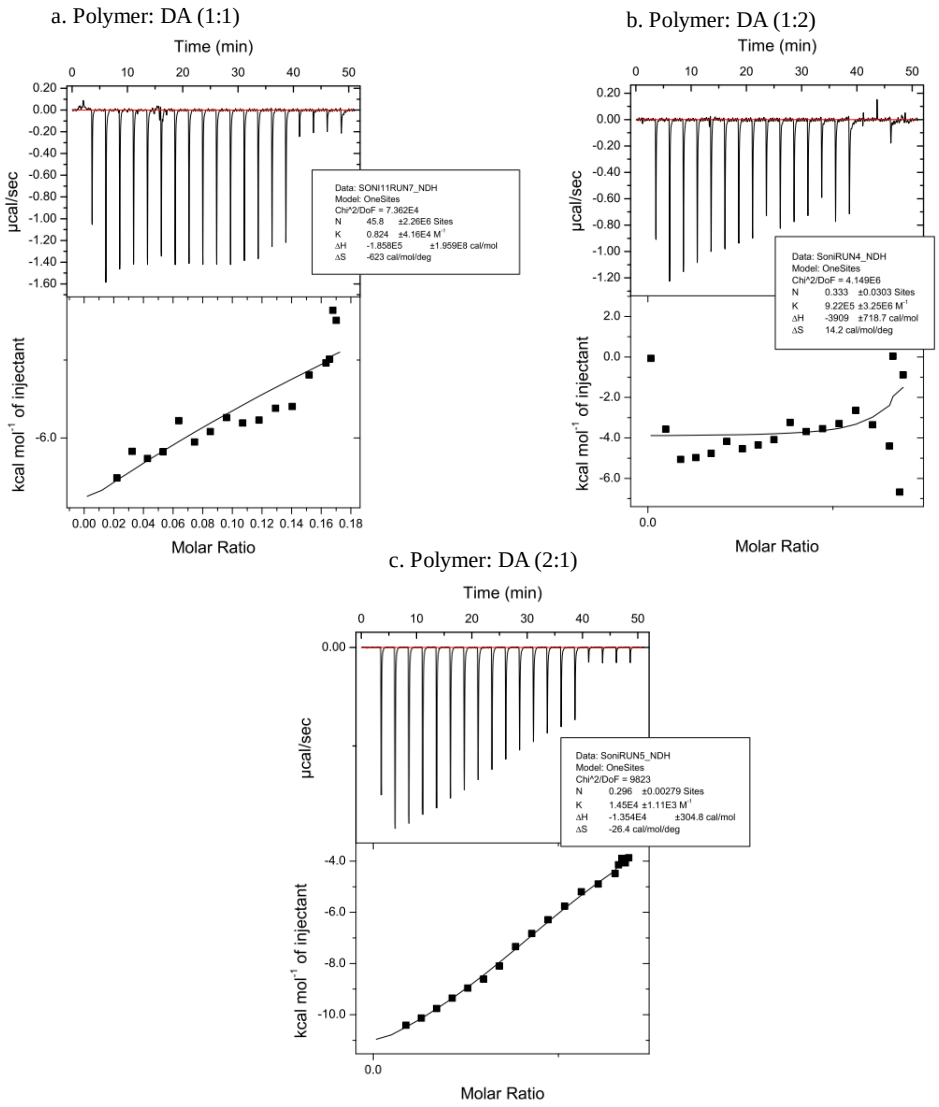


Figure 3.7: Isothermal titration calorimetry thermogram (top) and binding isotherm (bottom) of various ratios of polymer-DA: (a) polymer-DA (1:1), (b) polymer-DA (1:2) and (c) polymer-DA (2:1) using PBS pH 7.4 to achieve 1mM concentration.

formation (ΔH) for all the investigated inclusion complexes. Figure a, b and c represents the curve fitting of the enthalpy of complex formation for selected systems. The results depict that the complex formation between the polymer and lipid is mostly entropy-driven. The thermodynamic parameters are represented within the plot. These plots reveal that the binding of DA to polymer could be comprised to hydrogen bonding and

hydrophobic interactions as indicated by the negative or favorable binding enthalpy (ΔH) and entropy factor (ΔS) (Figure). An entropy-driven interaction tends to be more hydrophobic compared to an enthalpy-driven, which tends to be driven by hydrogen-bonding and van der Waals interactions (Figure 3.7a-c).

3.5. Conclusions

EMR—Lipid is a new generation sorbent material for selective lipid cleanup for multi-residue analysis and offers simple sample preparation with improved analytical method performance. The combined results from FTIR and PXRD provide a preliminary indication of complex formation between the polymer and lipids evaluated. The results from DSC and ITC analysis further confirm complexation between the polymer-lipids at various ratios evaluated. The ITC data indicate the involvement of hydrogen bonding with hydrophobic interaction responsible for driving the complex formation. Future studies will involve lipids with different carbon chain length and studying the complex formation using a combination of FTIR, PXRD, DSC, and ITC. The result of these studies would provide a fundamental understanding of lipid-binding properties and guide the development of next-generation materials for the separation of lipid classes.

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CHAPTER IV

DETERMINATION OF BIOLOGICALLY ACTIVE INGREDIENT (RESVERATROL) IN RED WINES FROM THE NORTHERN CLIMATE

ABSTRACT

Resveratrol (a phytoalexin) belongs to the stilbene family, is synthesized in response to external stress such as mechanical injury, infections (fungal or bacterial), extreme climate (temperature and humidity) and UV exposure. Resveratrol has been an essential component of wines due to its associated beneficial effects such as anticancer, cardioprotective, antioxidant, and anti-inflammatory activity. Research has demonstrated that climate conditions affect wine production (grape growing/planting/harvesting, winemaking, wine economics, and environmental factors), and eventually alter the resveratrol concentration in red wines. Several studies report resveratrol concentration in wines; however, very limited information is available regarding the resveratrol concentration in red wines from grapes grown in extreme environments. Grapes grown in a northern climate, like Minnesota and Dakota's must contend with extremes of cold in the winter and heat in the summer. It is important to study the influence of various factors (effect of geographical origin, variety, the procedure employed for winemaking and the growing methods) on resveratrol concentration. The overall goals of this study are to (1) develop and validate a method to determine resveratrol concentration in wines from northern climate and (2) compare the resveratrol concentration in red wine from South Dakota to other regions. The concentration of resveratrol in red wines from South Dakota was determined using ultra-high-pressure liquid chromatography-UV/Vis (UHPLC-UV/Vis), and the resveratrol level was found to be in the range of 0.29-7.99 mg/L for five different red wines. Furthermore, the results of this study will demonstrate that these red wines with functional properties are gaining increased attention in the eyes of consumers.

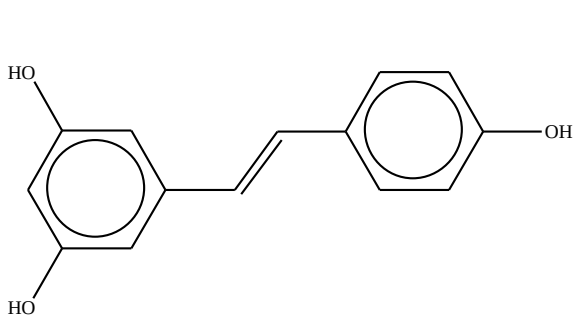
4.1. Introduction

Phytoalexin is a class of low-molecular-weight compounds that are synthesized and stored in plants in response to stress (heavy metal ions, extreme temperature, humidity, plant disease, and UV light). In grapevines, the stress response involves the synthesis of resveratrol, a phytoalexin. Resveratrol has gained interest because of its associated health benefits in humans such as antioxidant, anticancer, anti-inflammatory, etc. Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) is synthesized in plants in the presence of stilbene synthase enzyme[1]. Its synthesis occurs in response to environmental stress or infections (bacteria or fungi). Resveratrol was reported as a phytoalexin to reduce *Botrytis Cinerea* infection in grapes[2]. The skin of grapes, peanuts, raspberries, and blueberries are the main source of resveratrol[2-3,]

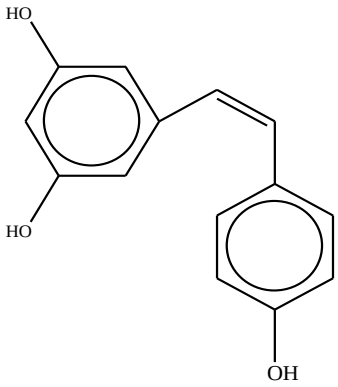
The wine produced from grapes is one of the essential sources of resveratrol for humans[4]. It is found in all the varieties of wines (white, red, and rose). However, the highest amounts are found in red wines[5]. The evidence from epidemiological studies raised considerable interest, demonstrating a relationship between moderate wine consumption and its health benefit specifically, risk of coronary heart disease, the so-*called "French Paradox*[6, 7]. However, there has been limited evidence on the health-associated benefits of red wine on human[8].

Resveratrol exists as both *trans*- and *cis*-isomers (Figure 4.1.). Research demonstrates that naturally, there is a high ratio of *trans*-to the *cis*-isomers. This indicates resveratrol synthesis as a *trans*-isomer and the formation of *cis*-isomer via isomerization of *trans*-isomer[9]. The conversion *trans*- to the *cis*-isomer occurs by the exposure to the UV-light. The *trans*-resveratrol is usually stable and undergo isomerization to *cis*-

resveratrol in buffers of high pH. *Cis*-resveratrol is stable at neutral pH. *Cis*-resveratrol gets converted to trans-resveratrol at low pH values[10].



trans-resveratrol



cis-resveratrol

Figure 4.1: Structure of trans- and cis-resveratrol isomers.

The resveratrol synthesis mainly occurs in grape skin; hence, the resveratrol levels are highest in the skin compared to any other parts of the plant and the concentration is high before the grape maturity. Stilbene synthase is an enzyme that is involved in the resveratrol biosynthesis, in response to stress factors such as ultraviolet radiation, environmental stress, injury, etc. The resveratrol level is highest at 24 h after the stress exposure and starts to decline after ~72 h due to the activation of stilbene oxidase enzyme[5, 11]. Since the resveratrol is produced in response to external stress factors, the region plays an important role in resveratrol levels in wine[12,13]. Furthermore, several factors involved in the winemaking process (temperature, levels of SO_2, pH, fermentation duration, and sugar content) affect the *trans*-resveratrol level in the final product, wine [14,15,10].

In ancient folk medicine of Japan and China, resveratrol was used as an active ingredient named as "Kojo-kon which has been utilized for the treatment of dermatitis,

gonorrhea, athlete's foot, hyperlipemia, arteriosclerosis, allergic, and inflammatory disease[4, 16, 17]. These ancient empirical studies can aid to correlate findings of resveratrol present in the red wine concerning its health benefits. The popular term, "French Paradox" was formulated due to lower death rates in the French residents even after consumption of a high-fat diet compared to the Americans. This is attributed to the higher consumption of wine in France that decreases the incidence of heart disease[18].

Resveratrol provides a list of beneficial health effects, such as anticancer, antiviral, antibacterial, neuroprotective, antioxidant, and anti-inflammatory effects, reported, but so far, the studies are *in-vitro* and only in species such as rat, bacteria, yeast, mice, fish, and fruit flies. It has been observed that, in the hyperlipidemic rats, the consumption of the resveratrol inhibited the accumulation of triacylglycerol and cholesterol in hepatic tissue[19]. The resveratrol levels in plasma and several organs in the rat was detected with the acute dose of 26 µg resveratrol over 15 days. Hence, in the long term, an average red wine drinker can absorb a sufficient quantity of resveratrol with beneficiary effects on human health[20]. However, there is still a need for investigation on the metabolic and physiological effects of resveratrol.

Major Biological Activities of Resveratrol:

Free radical scavenging and antioxidant activity: Resveratrol is a polyphenol is known for its antioxidant activity[19b]. In polyphenols, the total hydroxyl groups number, configuration and substitution affects several mechanisms of antioxidant activity, including radical scavenging and metal ion chelation capacities. The hydroxyl group at position 3′, 4′, and 5′ contribute to the antioxidant activity[21]. Researchers have used, density functional quantum chemistry and computational kinetics to study the antioxidant

effect against radicals such as hydroxyl (˙OH) and hydroperoxyl (˙OOH) in aqueous simulated media and found that *trans*-resveratrol may act as a radical scavenger[22]. Pharmaceutical industries are actively adopting the use of resveratrol in pharmaceutical products to minimize oxidation and enhancing shelf-life[23]. Resveratrol has been employed to protect cells against hydrogen peroxide-induced oxidative stress[24]. The chemo-preventive effects of the resveratrol are attributed to its intrinsic antioxidant property, showed an increased plasma antioxidant capacity and decreased lipid peroxidation associated with the risk of coronary heart disease and myocardial infarction[25].

Anticancer effects: Several *in-vitro* and *in-vivo* studies have indicated the anticancer properties of resveratrol, where resveratrol inhibits all carcinogenesis stages initiation, promotion, and progression[26]. Resveratrol acts as a chemo-preventive agent and possesses chemotherapeutic properties linked to its pro-apoptosis antioxidant, anti-proliferative and anti-inflammatory actions[26b, 27]. Various *in-vitro* and animal-based studies have indicated resveratrol effect as apoptotic and anti-proliferative effects on human cervical carcinoma, anticancer action in pancreatic cancer cells and enhance chemotherapy effects (breast, colon, prostate, and lungs cancer[26b, 28].

Cardioprotective effects: Resveratrol protective effect was studied in diabetic rats where, a significant reduction in blood glucose, body weight, triglyceride, heart rate, and increased insulin levels in plasma were observed[30]. Additionally, the data obtained showed that resveratrol treatment could improve cardiovascular function by reducing myocardial ischemia-reperfusion injury, vasodilation, and atherosclerosis[29a, 31].

Neuroprotective effects: Resveratrol effects also include neuroprotective effects in various neurodegenerative impairments such as Huntington′s, Parkinson′s diseases, and Alzheimer′s[32]. Resveratrol decreases cholinergic neurotransmission, oxidative stress, and promotes β-amyloid peptide clearance, and reduces neuronal apoptosis[33]. A meta-analysis showed that resveratrol could potentially decreases *Profile of Mood States* (POMS), vigor and fatigue with no significant effect on cognitive performance. Resveratrol tends to improve motor abilities and disable neuroinflammatory response in rats[34].

Anti-inflammatory activity: Studies indicated resveratrol ability to suppress the secretion and expression of inflammatory factors. Resveratrol inhibits the ear edema of mice, inflammation that might be an important factor for the treatment of arteriosclerosis. The investigation suggests that resveratrol activity can provide beneficial effect against inflammation and oxidative stress, and carcinogenesis hence, act as the potential bioactive agent to improve the quality of life.[35].

Antimicrobial activity: Resveratrol tends to inhibit the growth of some pathogenic microorganisms, such as Gram-positive and Gram-negative bacteria and fungi[36].Resveratrol also shows antiproliferative and androgen-lowering effects on theca-interstitial cells of ovary[38].

The difference in wines such as red and white wines is due to the grape varieties, enzyme addition, bacterial and fungal infection, and winemaking factors. Resveratrol concentration in red wines ranged from 2.86 to less than 0.003 mmol/L, whereas in white wines it ranged from 0.438 to less than 0.001 mmol/L (one mmol/L corresponds to 171 mg/750 mL bottle)[39]. The level of resveratrol in the red wine is higher primarily because red wine is fermented from skins as part of the winemaking process, and resveratrol level

is higher in the grape skin. Whereas, in the white wine production, the skins are removed before fermentation process[40].

Table 4.1: Average trans-resveratrol concentration (mg-L) in wines from a different region. Modified and adopted[39c, 41].

Variety	Region	*Trans*-resveratrol (mg-L)
Pinot Noir	Brazil	2.9 ± 1.6
	Czech Republic	3.4 ± 3.0
	France	5.4 ± 1.2
	Hungary	3.2 ± 0.5
	Italy	4.8 ± 1.4
	Japan	1.3 ± 0.3
	Spain	5.1 ± 4.0
	Switzerland	11.9
	USA	2.3 ± 1.3
Merlot	Australia	1.0
	Brazil	4.0 ± 1.0
	Czech Republic	1.3
	Hungary	3.9 ± 4.0
	Italy	3.4 ± 2.3
	Japan	1.5 ± 0.6
	Spain	4.0 ± 2.9
	USA	1.5 ± 1.0
Cabernet Sauvignon	Australia	0.9 ± 0.6
	Brazil	1.8 ± 0.5
	Czech Republic	3.7
	Greece	1.0
	Hungary	2.9 ± 2.5
	Italy	4.0 ± 3.1
	Japan	0.9
	Spain	1.2 ± 0.4
	USA	0.5 ±0.6
Zinfandel		
	Italy	9.6
	USA	0.4 ± 0.5

Since resveratrol is a secondary metabolite and produced in large amounts when there is an external attack to the plant, the research has indicated that there is a direct correlation the between resveratrol levels in wine and disease resistance of the grapevines. *Xarel-lo* is the most resistant and had the highest resveratrol level, and Chardonnay is the least resistant and has the lowest resveratrol level[39b].

Research has demonstrated that there is a high variance in the *trans*-resveratrol levels in monovarietal red wine[14]. The highest levels of trans-resveratrol were found in wines made from pinot noir that is grown in France, Spain, and Italy. The least amounts were found in wines made from zinfandel variety, USA. The highest levels of *trans*-resveratrol were found in wines made from pinot noir (3.6 ± 2.9 mg/l), St. Laurent (3.2 ± 1.8 mg/l), marzemino (3.0 ± 2.1 mg/L), merlot (2.8 ± 2.6 mg/l) and blaufrankish (2.6 ±1.3 mg/L). The least amount (0.6 ± 0.2 mg/L) was found in the agiorgitiko variety, predominantly grown in Greece[15, 42].

Grapes and resulting wine quality are generally determined based on phenolic compounds present. Phenolic compounds are the main class that affects product characteristics such as color, stability, and astringency (or sourness)[43]. Accumulating evidence from literature has shown that the concentration of resveratrol is affected by various factors such as grape variety, weather, location, soil type, and cultural practices. The soil is an essential factor that plays an essential role in the health of the plant since it promotes the biosynthesis of resveratrol. The nature of the soil promotes the biosynthesis process of resveratrol during elicitation. It has been found that calcareous and alkaline soil provides favorable conditions and promotes the resveratrol concentration in grapes as compared to non-calcareous and neutral soil[44]. The climate affects the ripening season,

with cooler weather being more favorable than warmer weather condition. It has been found that genotype and meteorological conditions affect the resveratrol concentration in grapes during harvest. Cluster thinning technique (removal of flower or fruiting clusters on the vine) has improved resveratrol concentration and its antioxidant capacity in Barbera wine[45]. However, it is challenging to study the biotic/abiotic and viticulture factors and their direct effect on resveratrol concentration in grapes as there are other factors involved such as type and severity of bacterial and fungal infection, infection, extraction methods, and harvest season and storage conditions[46]. Although several reviews on resveratrol and its health benefits have been published, very limited information regarding the levels of resveratrol in red wine from the northern climates is available.

The purpose of this work is to fill that gap, starting with method development and validation for the determination of resveratrol in red wine and by comparing the resveratrol content in red wines from a grape grown in different regions. Extraction techniques, such as solid-phase extraction (SPE) is employed for the sample preparation prior to analysis of wine using chromatographic techniques, such as gas chromatography (GC) or High-pressure liquid chromatography (HPLC). Depending on the technique of analysis, chemical conversion of analyte into a more stable form might be required for final determination[47]. The resveratrol occurs in very low concentration in wine, so a pre-concentration step must be conducted. For example, before performing the GC analysis of resveratrol, derivatization is required to enhance the volatility[48]. This is cumbersome and could potentially lead to *trans* to *cis* isomerization of resveratrol. The literature review on such technique reports the application of LC coupled with MS for the

analysis[49]. However, application of such hyphenated techniques is expensive and not employed in the routine laboratories in the wine industry. Hence, because of the above-mentioned challenges, the comparison between the resveratrol levels between different wine should take into consideration statistical parameters, method of sample preparation followed by sample analysis. The current percentage share of techniques used for the analysis of resveratrol from wine is shown in the figure 4.2.

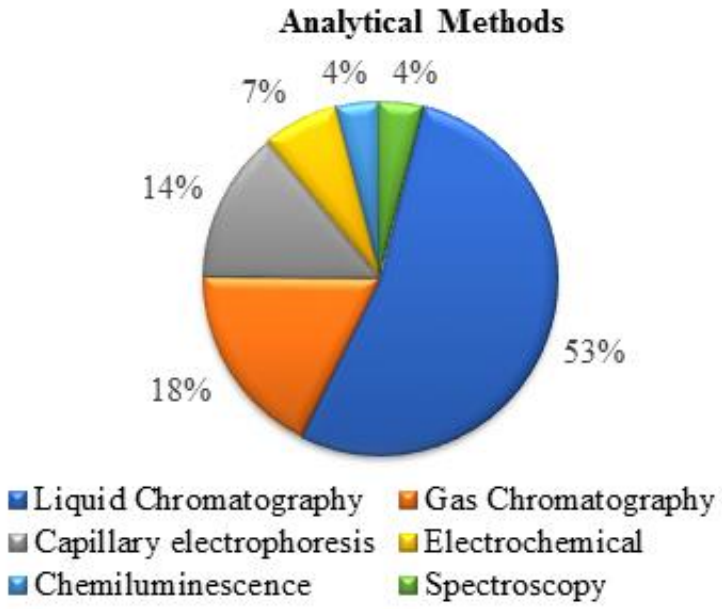


Figure 4.2: The details of various methodologies in percentage used for the determination and quantification of resveratrol from wines[47-49].

The complete procedure for the determination of resveratrol could be divided into three significant steps: (i) sample preparation, (ii) derivatization, and (iii) analysis. The subsequent portion will describe each of these approaches in some detail.

Sample preparation techniques are employed due to the complexity of the matrix. In most cases, extraction is performed. The techniques employed for this are solid-phase extraction (SPE), solid-phase microextraction (SPME), liquid-liquid extraction

(LLE), microextraction by packed sorbent (MEPS), stir-bar sorptive extraction (SBSE), and dispersive liquid-liquid microextraction (DLLME)[50].

Derivatization is a process where the sample is treated to modify the functional group of the analyte of interest. This results in analyte with increased volatility, thermal stability, fluorescence, sensitivity, and improved resolution[51]. This technique is mainly used for the analyte with high polarity, low volatility, and thermal low or instability[52]. Derivatization of the analyte is suitable for electrophoresis (causes ionization of the analyte), GC, and HPLC. Though different derivatizing agents are used for different analytical technique, few examples of derivatizing agents for resveratrol determination are bis-(trimethylsilyl)-trifluoroacetamide (BSTFA), acetic anhydride when GC is used and dansyl chloride when HPLC is used[52b, 53].

Common Analytical Methods to Determine Resveratrol in Wines

Wine is rich with polyphenols, is a complex matrix containing compounds such as sugars, tannins, minerals, vitamins, nitrogen compounds, organic acids, and compounds with esters and aldehydes characteristics. The components provide different characteristics to the wine, such as aroma and flavor. This complexity brings challenges for the wine analysis. Hence, sample preparation is a very crucial step in wine analysis that later determines the efficiency of the analytical technique. The commonly used techniques for wine analysis are liquid chromatography (LC), gas chromatography (GC), electrochemical method, spectrophotometry, and capillary electrophoresis.

Liquid chromatography: High-performance liquid chromatography (HPLC) is the most common technique used for the analysis of resveratrol, coupled with different detectors such as UV diode array detection (DAD), electrochemistry, fluorimetry, and

mass spectrometry[54]. Initially, LC technique was used for resolution, which also included multi-step extractions as a sample preparation step, whereas, modern LC technique offers improved LC instruments with more sensitive detectors providing the specific detection and quantification of resveratrol isomers. The commonly used mobile phase includes mixtures of electrolytes (acetic acid and formic acid), water, methanol and acetonitrile[50a, 55]. For resveratrol detection, the most common column used in LC is reverse-phase or C18. The advanced LC technique today that operates at higher pressure, ultra-performance liquid chromatography (UPLC) offers increased sensitivity, resolution, and analysis speed. A clean and filtered sample can be directly injected, though in some cases derivatization is required[50a, 56].

Table 4.2: Common analytical techniques for resveratrol determination in wine samples[80, 82, 86, 92].

Method	Matrix	Detector
Capillary electrophoresis	Wine	UV/Vis
Capillary electrophoresis	Red wine	Electrochemical detector
Gas chromatography	Peanuts and pistachios	Mass Spectrometer
HPLC	Peanuts and pistachios	Diode-array detector
GC-SPME	Wine and grapes	MS
HPLC	Grapes	Fluorescence
HPLC	Red grape skin	Electrochemical
LC	Grape, Cranberry Juice, and Wine	MS

Gas chromatography: Gas chromatography coupled with mass spectrometer has become another approach for resveratrol determination. Since the resveratrol is non-volatile, derivatization requirement in the analysis method is the major drawback of this technique. The common derivatizing agent used for resveratrol is acetic anhydride and BSTFA for the acetylation or sialylation reaction, modifying the functional group of the analyte[50b]. Derivatization reaction increases the volatility by decreasing the boiling point of the analyte, resulting in the mixture separation, detection, and quantitative determination. The derivatization process is an additional step before the sample is introduced the GC, though the with online extraction technique has provided an advantage where the derivatization can be performed similarly to online microextraction techniques. The most common microextraction technique used is dispersive liquid-liquid microextraction (DLLME), SBSE, and SPME. This technique, in combination, has been efficient and highly reproducible with accurate identification and quantification of resveratrol[52].

Capillary electrophoresis: Capillary electrophoresis (CE) offers a rapid and highly efficient separations technique tool for the determination of resveratrol in wine. In compare to LC and GC, CE provides major advantages, such as low sample and reagent requirement, suitable for thermally unstable compounds and more rapid separations than LC. CE associated drawbacks such as lower sensitivity and reproducibility may be overcome by using an appropriate sample preparation/pre-concentration method and sensitive detector. SPE has been the most popular solution for the sensitivity issue for CE[57].

4.2. Objectives

Literature suggests that wine produced from grapes grown in colder climates possess a higher concentration of resveratrol in response to environmental stress[58]. The goal of this study is to develop and validate the method for determination of resveratrol content in red wines from South Dakota and perform the comparative study with red wines from other regions. The information obtained from this study regarding the resveratrol concentration in red wines from South Dakota could support the local wine industry.

4.3. Materials

Chemical and Reagents

The analysis was conducted on wines produced in South Dakota from local wineries sourced from grapes grown in Minnesota and South Dakota (Dakota Falls, Schade, Homesteader, and Prairie Berry). *Trans*-resveratrol was purchased from Sigma Aldrich (St. Louis, MO). The other chemicals such as acetonitrile, methanol, and 2-propanol of (HPLC grade), formic acid (analytical grade), and HPLC-grade water were obtained from Fischer Scientific (Fairlawn, NJ, USA).

4.4. Methods

4.4.1. Standard Preparation

The stock solutions containing 10 mg/mL of *trans*-resveratrol in ethanol were prepared and stored at 4°C covered with aluminum foil. Before storage the stock solution was exposed to a nitrogen stream to eliminate the oxygen to avoid decomposition of phenolic compounds. For calibration, the standard solutions were prepared in the range 1-20 ppm for trans-resveratrol were prepared by diluting stock solutions with the mobile phase.

4.4.2.Method Development

4.4.2.1. Reverse Phase High-performance Liquid Chromatography (RP-HPLC) Analysis: The resveratrol levels in red wines were determined with an ultra-high-pressure liquid chromatography UHPLC (Ultimate 3000 Thermo Fischer UHPLC system) equipped with Zorbax Eclipse Plus C18, 2.1 x 150 mm, 1.8 µm, Agilent) and UV/VIS detector at 300 nm. The integration was accomplished utilizing the Thermo Scientific Chromeleon software, to generate the regression equation and the coefficient of regression (R^2). The elution step was performed using a mobile phase of 1% (v/v) aqueous formic acid: acetonitrile: 2-propanol (70:22:8) with the flow rate of 0.2 mL/min. A standard curve was obtained in the concentration range of 1-20 ppm. The calibration plot of signal ratio versus concentration was made, and the analytes in samples were identified by comparing their retention times and UV-vis spectra at 300 nm with those of the standard compound.

4.4.2.2. Liquid Chromatography-Electrospray Mass Spectrometry (LC-ESI-MS): The identification of trans-resveratrol was confirmed by analyzing the samples using a Shimadzu HPLC (LC-20AD, Shimadzu Corp., Kyoto, Japan) coupled to AB Sciex Q-trap 5500 MS-MS (Applied Biosystems, Foster City, CA, USA), using the same column and mobile phase previously described in above section, using electrospray ionization (ESI)-MS-MS. The optimal operating parameters of the ESI interface and quadrupole were achieved by infusing standard solutions of *trans*-resveratrol in the mobile phase (0.1µg/mL) at 5 µL/min using a syringe pump, isocratic flow of 0.2 mL/min at 30 °C. The mass spectrometer was operated in negative ion (NI) mode and was scanned over the m/z 100–300 range with 0.1 Da of step size and 2 ms per step of dwell time. The nitrogen gas at 30 psi was used as the curtain and nebulization gas. The spectrometric

detection was performed in multiple reaction monitoring (MRM) mode with collision energy (CE), declustering potential (DP), and collision cell exit potential (CXP) were optimized for each MRM transition. The parameters are mentioned in the table 4.3.

Table 4.3: Optimized parameters for MRM transitions for resveratrol.

Parameter	Value
CE	20 V
DP	50 V
CXP	10 V
Ion source	−4,500 V and 500 °C
Source Temperature	130 °C
De-solvation Temperature	150 °C
Electrospray Voltage	- 2.5 kV
Cone Voltage	- 40 V
Rf Lens	0.5 V
Nebulizer (GS1) Gas Pressure	40.0 psi
Heater (GS2) Gas Pressures	60.0 psi

The five-point calibration curves with a dynamic range of 1-20 ppm, was generated and the linearity was determined by evaluation of the regression curve and correlation coefficient (R^2) > 0.99 was considered precise ($y = 95.799x + 4.3536$, $R^2 = 0.9982$) for *trans*-resveratrol. The limits of detections (LODs) was calculated, 0.19 mg/L as the minimum concentration, generates a peak signal at least three times higher than the signal from noise and quantification limits (LOQs) were 0.48 mg/L for *trans*-resveratrol.

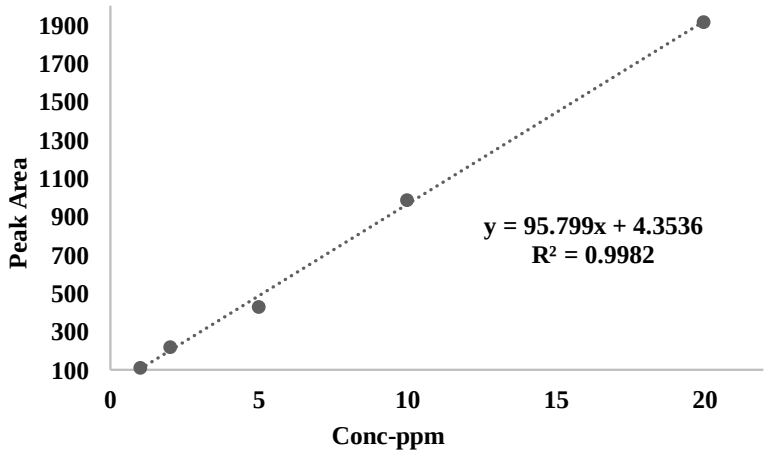


Figure 4.3: A calibration of trans-resveratrol in the concentration range of 1-20 ppm.

4.4.3. Sample Pretreatment

The seven red wines were analyzed for their resveratrol concentration immediately after opening the bottle. Samples were centrifuged for 5 mins at 2000 x g, and the supernatant was collected and filtered through a 0.45 μm membrane filter (Millipore, Milford, MA). SPE was used for the extraction step using Varian (Walnut Creek, USA) Bond Elut C18 cartridge (1 mL, 100 mg. Methanol was used to activate the sorbent bed by passing 1 mL of methanol through the cartridge for five times followed by conditioning step by passing ultrapure water for five times. The diluted wine sample (5 mL wine + 1 mL ultrapure water) was loaded onto a conditioned cartridge. The elution of analytes was done by passing 2 mL of methanol. The collected eluate was concentrated under vacuum and further dissolved with 1 mL of mobile phase [1% (v/v) aqueous formic acid: acetonitrile: 2-propanol (70:22:8)] and then directly injected to UHPLC system. For each wine, the analysis was done in triplicate.

4.4.4. Method Validation

The method was validated for intra- and inter-day accuracy and precision over three days using three different concentrations along the calibration curve. To study the precision of the method as the intra- and inter-day assay at three concentrations (3, 6 and 12 ppm) for *trans*-resveratrol was performed. The inter-day (day-to-day variation) was assay was performed by analyzing replicates of standards (same concentration) on three separate days. The precision was expressed as relative standard deviation. The data for accuracy and precision were determined at each validation level. All intra- and inter-day precisions gave satisfactory results, which were less than 10%. The accuracies were ranged between 91-103%. Based on the above results, it suggests that the proposed method is simple, accurate, and sensitive.

Table 4.4: The accuracy and precision of resveratrol analysis by UHPLC-UV/Vis.

Concentration	**Intra-assay**		**Inter-assay**	
(ppm)	**% Accuracy**	**% Precision**	**% Accuracy**	**% Precision**
3	91.3-99.1	< 4.8	92.6-100.1	< 4.1
6	95.5-99.8	< 3.0	95.9-103.1	< 6.3
12	92.8-96.1	< 5.3	95.0-99.8	< 7.6

4.5. Results and Discussion

Analysis of Wine

The optimized method was used to determine the *trans*-resveratrol in the seven red wines. Figure 4.4. shows the chromatograms at 300 nm for wine samples, and figure depicts the UV-vis spectra of *trans*-resveratrol in red wine. The identification of trans-resveratrol was confirmed by electron spray ionization- mass spectrometer (ESI-MS) data. The ESI-MS mass spectra of *trans*-resveratrol were characterized by the protonated molecules [M+2H] at m/z 229. The trans-resveratrol peak was well-resolved under

isocratic elution with a retention time of 5.13 ± 0.19 min. There was negligible interference observed from other present phenolic compounds in the samples. Table 4.4. summarizes the values of trans-resveratrol found in the in the seven different wines along with the winery and their names of red wine samples analyzed. The lowest resveratrol concentration was found in the Christmas pleasure wine (0.29 ± 0.09 mg/L) from the Schade winery, whereas the highest concertation was in Calamity Jane (7.99 ± 0.44 mg/L) from Prairie Berry winery. We could not detect the resveratrol concentration in the Signature red and Oakwood red wine from the Schade winery.

Table 4.5: Resveratrol concentration (mg/L) in wines from grapes grown in colder climate.

Winery	Name	Resv. Conc. (mg/L)
Dakota Falls	Ringneck Red	1.01 ± 0.13
Schade	Christmas Pleasure	0.29 ± 0.09
Homesteader	Red wine	0.98 ± 0.18
Schade	Dakota Red	5.81 ± 0.23
Prairie Berry	Calamity Jane,	7.99 ± 0.44
Schade	Signature red	-
Schade	Oakwood red	-

The average resveratrol concentration in mg/L in red wines from other regions was compared to the ones from South Dakota (Table 4.6). The lowest average concentration of resveratrol in red wine was found in Serbia, followed by Central Europe, France, and South America. Whereas, considerably higher average resveratrol concentration was found in Canada, Greece, Australia, South Dakota, and California from the United States. In summary, the average concentration of resveratrol in red wines from South Dakota fared well as compared to the red wines from other colder climates.

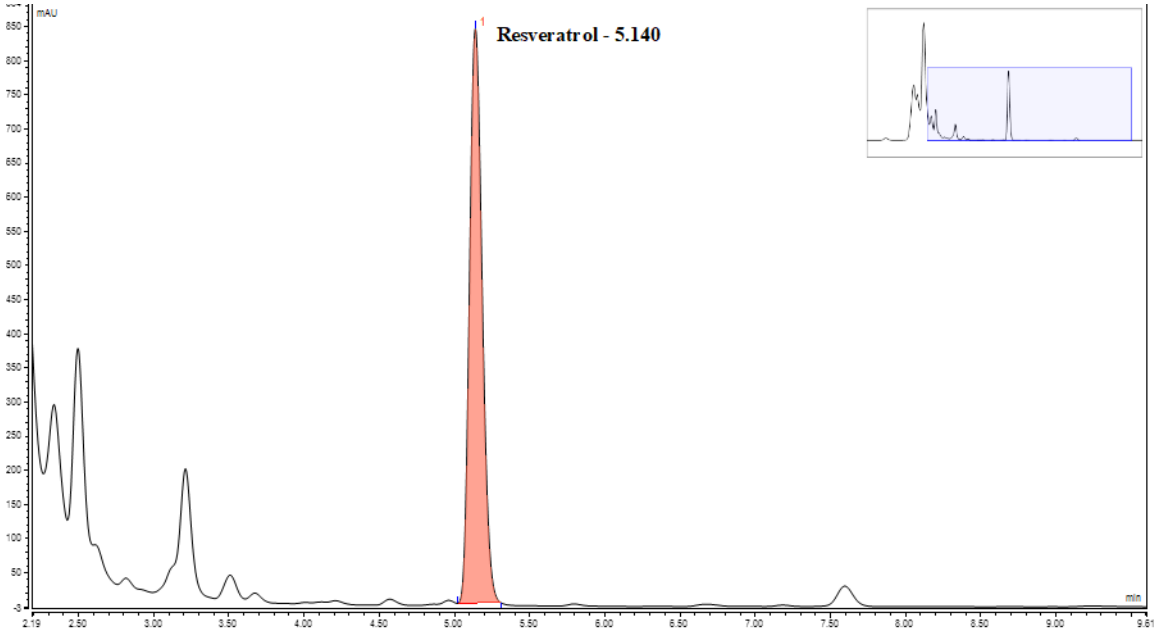


Figure 4.4: Chromatogram of wine sample depicting the resveratrol peak at 5.140 min.

Table 4.6: Comparative analysis of the average concentration of resveratrol (mg/L) in red wines from various regions.

Region	Resveratrol, Avg. Conc. Range (mg/L)
South Dakota, USA	0.29-7.99
Oregon	0.9 to 9.1
California, USA	1.47 - 6.02
Serbia	0.11-1.69
Australia	1.47-9.26
Canada	5.30-13.4
Italy	1.76-4.23
France	0.89-3.60
Central Europe	0.23-3.26
Greece	5.0 to 12.3
South America	0.54o 4.95

4.6. Conclusions

A simple, rapid, and reliable RP-HPLC method was developed for determination of *trans*-resveratrol levels in red wine. Optimum separation was achieved under isocratic conditions. Hence the method was described by excellent precision, linearity, and accuracy. The proposed method allowed the determination of *trans*-resveratrol in red wine by direct injection without sample pre-treatment. The concentration of resveratrol in red wines from South Dakota was compared with red wines from other regions. In general, the concertation of resveratrol in wine from grapes grown in South Dakota was found to be in line with the resveratrol concentration in wine grown in other cold climates.

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