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USE OF EXTRACTION TECHNOLOGIES IN FOOD SAFETY STUDIES

BY

CHANGLING QIU

A Dissertation Submitted in Partial Fulfillment of the Requirements for the

Doctor of Philosophy

Major in Chemistry

South Dakota State University

2016

USE OF EXTRACTION TECHNOLOGIES IN FOOD SAFETY STUDIES

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 does not imply that the conclusions reached by the candidate are necessarily the conclusion of the major department.

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ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my advisor, Dr. Douglas E. Raynie, for his excellent guidance, continuous support, and persistent help of my Ph. D study. His encouragement and guidance helped me in all the time of research and writing of this dissertation. Without his encouragement, I would never have been able to finish my dissertation. I am so grateful for the numerous opportunities he provided me to develop my professional presentation skills at scientific conferences and his caring and enormous help for my career development.

I would like to thank the rest of my graduate committee: Drs. Jihong Cole-Dai, Brian Logue, Mathew Miller, and Christopher Schmit for their encouragement, insightful comments, and engagement through the entire learning process. Thank Dr. Jihong Cole-Dai for letting me experience the research of ice-core samples from Antarctic, patiently teaching me use of instrumentation, and his financially support for the first two semesters. I would like to thank Dr. Brian Logue for helping me develop background in analytical chemistry and his generosity of letting me use his analytical instruments. Thank Dr. Mathew Miller for developing my chemical communication skills. Special thanks go to Dr. Christopher Schmit, who was willing to participate in my committee.

Many thanks to David Ferris, Robert Oda, my labmates, and my fellow co-workers in the Department of Chem. & Biochem. for helping me and giving me valuable suggestions.

I would like to express my special appreciation and thanks to my family for their support and caring. They were always supporting me and encouraging me with their best wishes, and stood by me through the good times and bad.

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ABSTRACT

USE OF EXTRACTION TECHNOLOGIES IN FOOD SAFETY STUDIES

CHANGLING QIU

2016

Food safety has become a top concern in our society. The public in general is increasingly concerned about the safety of the food products they consume every day as more and more food contamination incidents and widespread recalls arise. It is necessary to trace any presence and/or the concentration levels of contaminants, pesticides, herbicides, or other harmful substances in food samples. Sample preparation is a crucial step in a food analytical method, as it takes up most of the total analysis time, contributing highly to the total cost of analysis and greatly influencing the results of the analysis. Traditional extraction methods for food samples such as liquid-liquid extraction and Soxhlet extraction are involved time-consuming and large solvent consumption steps. In recent years, some extraction techniques have been developing as the substitutions to the conventional sample preparation methods. Accelerated solvent extraction (ASE) and solid phase extraction (SPME), which are considered "green" sample preparation techniques, are among the most studied sample preparation techniques. They have advantages over traditional extraction methods, such as shortened extraction time, reduced solvent consumption, increased pollution prevention, and reduced cost. This dissertation reported the studies on method developments for food safety and quality analyses using these modern sample extraction techniques.

Perfluorooctanoic acid is an organofluorine compound that is synthetically produced and primarily used as an emulsifier in the production of polytetrafluoroethylene (PTFE or Teflon). These polymers provide oil and water repellency as well as stain resistance, which make them ideal coating materials for non-stick cookware. PFOA is bioaccumulative, persistent, and potentially harmful to humans. PFOA is not supposed to be found in the final products of non-stick cookware after processing. A method for determination of the leaching of PFOA from the cookware under simulated cooking conditions was presented. To simulate cooking conditions, PTFE-coated cookware was extracted with ethanol/water mixtures using accelerated solvent extraction (ASE). The extraction parameters such as pressure, cycle, and purge time were optimized. The resulting extracts were analyzed by liquid chromatography tandem mass spectrometry (HPLC-MS/MS). Good recoveries, precision, and linearity were obtained. Limits of detection (LOD) were as low as 0.03 and 0.02 μg/L, corresponding to 5.0 pg/cm^2 and 3.3 pg/cm^2, for PFOA analysis under watery- and fatty-food-simulation conditions, which are lower than the reported methods by approximately 80%. The method was successfully applied to analyze PFOA from used and new cookware under simulated cooking conditions. The results demonstrated that PFOA were detectable in all pan samples extracted with both watery-and fatty-food-simulation conditions. It is assumed that PFOA breaks down from fluoropolymer-coated cookware (new or used) may leach into foods under common cooking conditions (175 °C and 20 min). However, no attempt was made to correlate this data to PFOA levels found in fried foods or the average diet. Overall, the proposed method was an efficient, accurate, and precise method that can be applied to analyze contaminants and harmful substances from food contact materials and samples.

A headspace-solid-phase microextraction-gas chromatography-mass spectrometry (HS-SPME-GC-MS) method was developed to identify and quantify the flavor component allyl isothiocyanate (AITC) and related compounds in horseradish products. Solvent extraction, headspace sampling, and HS-SPME were compared, and HS-SPME gave acceptable accuracy and precision for the quantification of AITC and related compounds in horseradish. The optimized conditions for HS-SPME were 0.8 g sample size in a 4-mL vial at 30 °C for 20 min with one minute desorption in the GC injector at 250 °C. A calibration curve was generated in the concentration range of 50-3200 ppm of allyl isothiocyanate using the internal standard method. The validated method resulted in intraday and interday precision (% RSD) and accuracy (% recovery) of less than 10% and 80-120%, respectively. The method was applied to analyze allyl isothiocyanate in horseradish samples. Seven constituents were identified and the major constituents were allyl isothiocyanate (97.58%) and phenylethyl isothiocyanate (1.65%), representing 99.23% of the pungent components in prepared horseradish sample. The HS-SPME-GC-MS method presented is simple, accurate, and sensitive. Manufacturer, processors, and regulatory authorities can use this method to evaluate quality of flavored products before and after production.

Horseradish (*Armoracia rusticana*) is mostly used as a condiment in food due to its characteristic strong pungent smell and taste. Allyl isothiocyanate is responsible for the pungency of horseradish. In this study, a sensory analysis was carried out through the development of a method for studying the correlation between the level of allyl isothiocyanate and the perceived pungency in horseradish products. Sensory pungency analysis of 14 commercial horseradish products from 8 manufacturers was carried out by

a trained panel. The level of allyl isothiocyanate in horseradish products was quantified by the validated HS-SPME-GC-MS method. Differences due to water content are noted, but the impact of other sample ingredients is more complex. Both the sensory data and analytical results showed that there were differences in pungency among the 14 horseradish product samples. Panelists exhibited no significant difference in overall preferences among the 14 samples, with the average overall preference ratings ranging from 4.3-5.4. Some differences in terms of expectation, acceptability, and interpretation of sensory characteristics of horseradish might be present among the panelists. Due to the limited number of panel participants and samples, the information obtained from this study should be considered preliminary. For future study, a larger group of panelists is needed to better understand the links between sensory testing and instrumental analysis. Additionally, it will gain more insight if the influence of food components and masking effects are better understood.

CHAPTER 1. INTRODUCTION

1.1 Overall Significance

Food safety has become a top concern in our society. The public in general is increasingly concerned about the safety of the food products they consume every day as more and more food contamination incidents and widespread recalls arise. Melamine in milk products, the pesticide dichlorvos in Jinhua hams, high levels of persistent organic pollutants such as polychlorinated biphenyls (PCBs) and dichlorodiphenyltrichloroethane (DDT) in salmon, carbendazim in orange juice, and dioxin in eggs and meat are just a few food safety incidents[2]. These incidents have alerted the authorities and the public that more efforts and deeper investigations are needed on food safety regulations and analyses. It is absolute necessary to trace any presence and/or the concentration levels of contaminants, pesticides, herbicides, or other harmful substances in food samples[3]. As a result, reliable and efficient methods for food safety analyses are really needed. Sample preparation is the most important step in a food analytical method, as it takes up most of the total analysis time, contributing highly to the total cost of analysis and greatly influencing the precision and accuracy of the analysis[4,5]. Even with modern detection techniques, due to low concentrations of contaminants and complicated food matrices, efficient sample preparation is required[4,6,7]. Traditional extraction methods for food samples such as liquid-liquid extraction and Soxhlet extraction are often time-consuming and require large amounts of organic solvents. Therefore, one of the objectives of analytical food safety studies currently has been the development of new extraction

techniques that can improve the accuracy and precision of analytical results and simplify the whole analytical procedure[8].

1.2 Project Objectives

The objectives of this work are (1) to determine whether perfluoroctanoic acid (PFOA) leaches from frying pan under simulating cooking conditions using accelerated solvent extraction (ASE), (2) to quantify allyl isothiocyanate and related isothiocyanate compounds from horseradish products using solid phase microextraction (SPME), and (3) to perform sensory analysis of allyl isothiocyanate and correlate the levels of allyl isothiocyanate to perceived pungency.

1.3 Extraction Methods in Food Safety Analysis

Because of increased concerns for food safety, more attention is given to developing methods for determination of contaminants and other harmful substances from food samples. The analysis of food samples is usually a complicated procedure involving many steps. It requires extensive sample extraction prior to further analysis. Sample extraction is a crucial step in food sample analysis because it can affect the concentration of the analyte and the cleanliness of the sample[9]. Traditional sample extraction techniques used in food safety studies are based on the suitable choice of solvents and the use of heat and agitation to improve the solubility of the desired compounds and the mass transfer[10], like in Soxhlet extraction, liquid-liquid extraction, and solvent-shake extraction. Pedersen and Olsson performed Soxhlet extraction of acrylamide from potato chips[11]. It took 7 days to get a complete extraction. Frenich and

coworkers reported a method for the determination of residues of organochlorine (OCPs) and organophosphorus (OPPs) pesticides using Soxhlet extraction[12]. This extraction method involved laborious steps with the use of large amount of solvent. Analysis of polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs) in butter based on three different liquid-liquid extraction methods was studied by Ramos and his coworkers[13]. The reported methods involved time-consuming and large solvent consumption steps. These traditional extraction techniques are quite laborious, time consuming, and involve large quantities of organic solvents, which are flammable, expensive, and generate hazardous waste[14].
In recent years, several new extraction techniques have been developed as the substitutions to the conventional sample preparation methods, such as microwave-assisted extraction (MAE), ultrasound-assisted extraction (MAE), accelerated solvent extraction (ASE), supercritical fluid extraction (SFE), and solid phase extraction (SPME). These new extraction techniques have numerous advantages over traditional extraction methods, like shortened extraction time, reduced solvent consumption, increased pollution prevention, reduced cost, and improved automated operation[15,16].

1.4 Extraction from Liquids

1.4.1 Liquid-liquid Extraction

Traditionally in food safety tests, liquid-liquid extraction (LLE) is the most widely used method for the extraction of analytes from aqueous food samples. In LLE, the sample is distributed or partitioned between two immiscible solvents in which the analyte and matrix have different solubilities[17]. In LLE, the solution containing the

analyte must be immiscible with the solvent used to extract the analyte. The main advantages of this method are the wide availability of solvents and the use of low-cost apparatus[17]. However, due to the low recoveries, limited selectivity, and time-consuming procedures, applications of LLE as a sample preparation technique in food safety analysis are limited[18].

1.4.2 Solid Phase Extraction

Solid phase extraction (SPE) is an extraction technique that uses a solid phase and a liquid phase to isolate analytes from a solution[19]. In SPE, the sample passes over the stationary phase (solid phase), the analytes being separated according to the degree to which each component is partitioned or adsorbed by the stationary phase[20]. The analytes may favorably adsorb to the solid phase, or they may remain in the liquid phase. If the analytes are adsorbed on the solid phase, an eluting solvent or solvent mixture can be used to selectively desorb the analytes[21]. If the analytes remain in a liquid phase, they can be collected and prepared properly for further analysis[21].

Effective separation by SPE can be achieved by choosing suitably selective solid-phase sorbent and eluting solvents[22]. With proper selection of the sorbent and solvents, SPE is capable of being used for gases, solids, and liquids. However, the primary area of application of SPE is in the selective extraction and enrichment of liquids samples. SPE is used widely in the environmental, pharmaceutical, biological, clinical, forensic science, and food and beverage areas.

SPE is widely used for isolation, concentration, and cleanup. It can be used to extract compounds of interest from a sample. It is also used to concentrate and clean up a

sample before using a chromatographic or other analytical method. SPE has very extensive applications in food safety studies because of its low cost, good selectivity, small solvent consumption, and high recovery. However, long sample preparation times and multi-step procedures are also mentioned as its disadvantages[23].

1.4.3 Solid-phase Microextraction

Solid-phase microextraction (SPME) developed by Pawliszyn and co-workers[24] in 1989 is a new sample preparation technique. It involves the use of a fiber coated with suitable extracting material for the extraction of analyte(s) of interest from a sample matrix. The sample molecules are adsorbed onto the fiber and subsequently desorbed into the GC injection port for analysis. It is a simple, fast, inexpensive, and efficient extraction method that has been applied to both headspace and aqueous sample analysis with great sensitivity and selectivity[25].

SPME has been applied most effectively when coupled to gas chromatography. SPME has been used with high performance liquid chromatography (HPLC) separations too[26]. Figure 1.1 shows the SPME device[23]. It consists of a fiber bonded to a stainless steel plunger and installed in a holder. The fiber coated with suitable stationary phase for the analytes of interest is either immersed in the sample or exposed to the headspace above the sample. Analytes in aqueous samples can be extracted by direct immersion. In the direct-immersion extraction mode, analytes partition between the aqueous matrix and the fiber coating[27]. When equilibrium is reached the fiber is removed and exposed to the injection port of a gas chromatograph for analysis. Headspace analysis can be used for the extraction of volatile or semi-volatile analytes from solid, liquid or gaseous samples[23].

In the headspace extraction mode, the analytes first partition between the sample and the headspace, then the analytes are adsorbed by the fiber that is inserted directly into the injection port of a GC system[27].

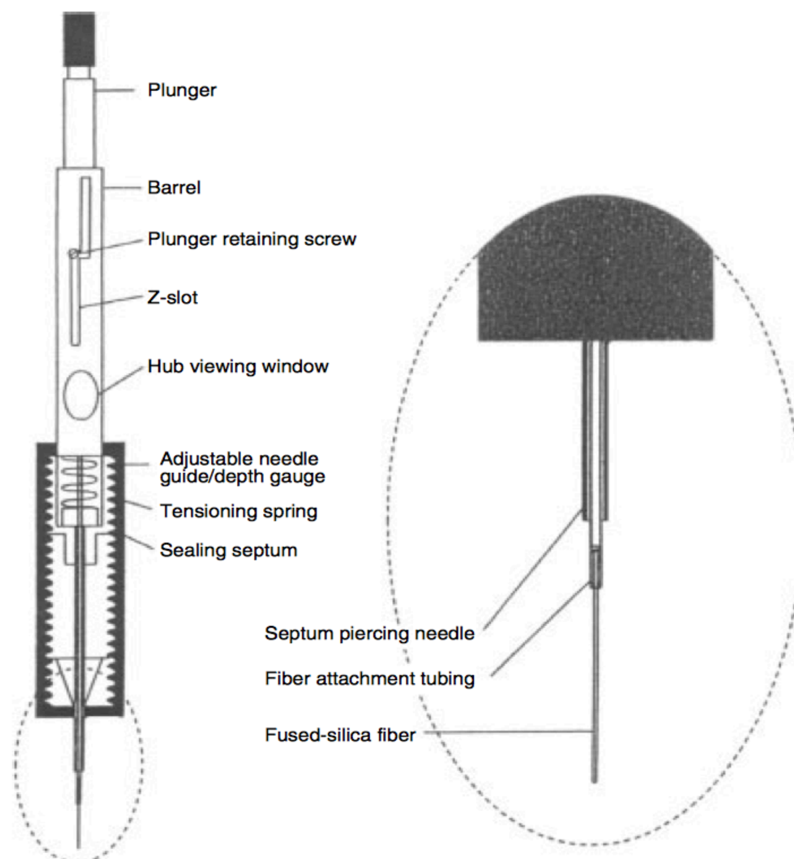


Figure 1.1 Components of solid phase microextraction (SPME)[23]. SPME involves the use of a fiber coated with suitable extracting material for the extraction of analyte(s) of interest from a sample matrix. The sample molecules are adsorbed onto the fiber and subsequently desorbed into the GC injection port for analysis.

SPME is an equilibrium extraction technique, several factors influence the extraction efficiency of analytes, such as fiber-coating thickness and characteristics, sample size, vial size, adsorption and desorption conditions (temperature and time)[27]. In order to perform quantitative analysis, it is vital that each of these variables is kept constant between analyses.

SPME method has become more and more popular in the analysis of volatile and semi-volatile compounds due to its superiorities over conventional extraction methods. It is simple, effective, and low cost. The extraction combines sampling, isolation, and concentration in one step[28]. SPME is also considered to be ‘environmentally friendly’ because of the elimination of organic solvents. The SPME technique has been widely applied to environmental, food, forensic, and pharmaceutical samples[23]. It can be used for food flavor and off-flavor analyses (vegetables, fruits, beverages, dairy products, oils) and food contaminants analyses[25].

1.5 Extraction from Solids

1.5.1 Shake-flask Extraction

The most common approach for extraction from solids is conventional liquid-solid extraction, in the form of shake flask extraction. Shake-flask extraction can be easily carried out by putting a sample into a flask, adding a solvent, and then agitating or shaking for a time period. After extraction, the solvent with extract(s) is separated from the solid matrix by means of centrifugation or filtration[29]. Shake-flask extraction requires minimal glassware, small amounts of organic solvent, and is comparatively fast (10-50 min). It is one of the oldest and most widely used extraction method. However, due to its poor recovery and low efficiency, the application is limited.

1.5.2 Soxhlet Extraction

Soxhlet extraction is a traditional extraction technique for many food samples. It was originally designed for the extraction of a lipid from a solid material by Franz von

Soxhlet in 1879[30]. However, Soxhlet extraction is not limited to the extraction of lipids. When a desired compound of low solubility needs to be extracted from a solid sample, a Soxhlet exatraction can be applied[31]. The technique utilizes a specialized piece of glass apparatus, called Soxhlet extractor, where the solid sample is placed in and is continuously extracted with a sub-boiling solvent[32]. Though Soxhlet extraction is simple, standard, and robust, there are disadvantages[33]. Soxhlet extraction usually requires long extraction times (8-12 h) and large amounts of solvent[33]. The extraction glassware is expensive and vulnerable to breakage. It requires a constant supply of water to cool the condenser of the Soxhlet apparatus. The operation is lack of automation. Due to these disadvantages, the applications of Soxhlet have been restricted.

1.5.3 QuEChERS

QuEChERS, standards for Quick, Easy, Cheap, Effective, Rugged, and Safe[34], has become a very attractive sample extraction method for various food samples. This method was developed by Steven Lehotay and Michelangelo Anastassiades in 2003 originally for the analysis of pesticides in vegetables and fruits[34]. Now, QuEChERS has also been widely used in pharmaceutical, clinical, and environmental analysis including steroids, hormones, acetaminophen, acrylamide, perfluorinated compounds, polycyclic aromatic hydrocarbons, alkaloids, mycotoxin, and other applications. Overall, this procedure has two main steps: (1) extraction with a solvent and partitioning salts (2) clean up with dispersive solid phase extraction (dSPE) using sorbent materials to remove interferences. The QuEChERS method has many advantages over traditionally used techniques. QuEChERS method provides accurate analytical results with high recoveries,

it saves time and labor, reduces hazardous solvent consumption and waste disposal, uses less laboratory glassware with a minimal number of steps.

1.5.4 Ultrasound Assisted Extraction (UAE)

UAE has been employed in food safety studies for the extraction of contaminants or bioactive components from food materials. The principle of UAE has been attributed to the propagation of ultrasound pressure waves and resulting cavitation phenomena[35]. Ultrasound waves are elastic waves that have a frequency above the threshold of human hearing, approximately 20 kHz. The extraction mechanism involves two steps, diffusion through the cell walls and releasing the cell content once the walls are disrupted[36]. The sample is immersed in an ultrasonic bath with a solvent and subjected to ultrasonic radiation for different time periods. Ultrasound waves create bubbles in the solvent and produce high local negative pressure that can cause the collapse of cavitation bubbles. The collapse of cavitation bubbles near cell walls produces cell disruption, as a result, solvent penetrates into the cells and causes the release of extractable compounds. The ultrasound waves can also facilitate the diffusion process and increase mass transfer. UAE can reduce extraction time and solvent consumption, thus resulting in higher extraction rates and good extraction efficiency. Compared to other extraction techniques, UAE is simple, fast, productive, low cost, and capable of operating with many samples at one time[37]. UAE usually provides good results for food samples. The benefits for using UAE for the food samples include: enhancement of extraction yield or rate, extraction of heat-sensitive bioactive and food components under lower processing temperature conditions[38].

1.5.5 Microwave Assisted Extraction (MAE)

MAE is an extraction technique that combines microwave and traditional solvent extraction. The use of MAE in food safety analysis has become one of the most common and low-cost extraction methods today. Typically, a microwave system includes a microwave power generator, waveguide for transmission, resonant cavity, and a power supply[39]. The microwave power generator is a magnetron, at the common microwave frequency of 2.45 GHz, electromagnetic energy is conducted from the magnetron to the cavity using a waveguide[39]. The sample and solvent placed inside the resonant cavity is therefore subjected to microwave energy. After typically 5-30 min the extraction is complete, the extract can be filtered and prepared for analysis.

Compared to traditional extraction methods and other extraction techniques, an important advantage of MAE is the extraction rate acceleration due to microwave energy, resulting in an immediate heating to high temperature. Therefore, short extraction times (a few minutes) can be obtained. Other advantages includes reduced solvent consumption, higher extraction rate, and improved extraction yield and product quality[40]. On the other hand, its disadvantages include an additional clean-up step is needed to remove the solid residue after the extraction, the efficiency of microwaves can be poor when the solvents are nonpolar and volatile, and the use of high temperatures that might degrade heat-sensitive bioactive compounds[40].

MAE has been applied to a diverse range of sample types (soils, sediments, sewage sludge, plants, food). MAE is employed extensively in the extraction of pesticides, pigments, bioactive compounds from vegetables, plants, and natural products as an alternative to traditional techniques of extraction[41,42,43,44].

1.5.6 Supercritical Fluid Extraction (SFE)

SFE is one of the widely used extraction technique that utilizes a fluid phase having unique properties between a gas and a liquid to effect the solubilization of solutes[45]. Compared to traditional solvents, supercritical fluids have lower viscosities and high diffusivities, thus allowing more efficient mass transfer of solutes from sample matrices[46]. SFE can be operated in two modes, off-line and on-line[47]. In on-line mode, the SFE instrument is coupled directly to the analytical instrument, such as SFE-gas chromatography. The off-line SFE focuses on the sample preparation only which can be used as a sample preparation step for analytical purposes or on a larger scale to either remove unwanted components from a product or collect desired components[48].

A scheme for a SFE unit is presented in Figure 1.2[45]. The system contains a reservoir of supercritical fluid, a reservoir of cosolvent, an extraction cell, and a collection vial. Typically, the supercritical fluid is pumped to a heating zone, where it is heated to supercritical conditions. It then passes into the extraction cell, where it rapidly diffuses into the sample and dissolves the components to be extracted. The dissolved components are pumped from the extraction cell into a collection vial, the supercritical fluid can then be condensed and recycled, or discharged to atmosphere.

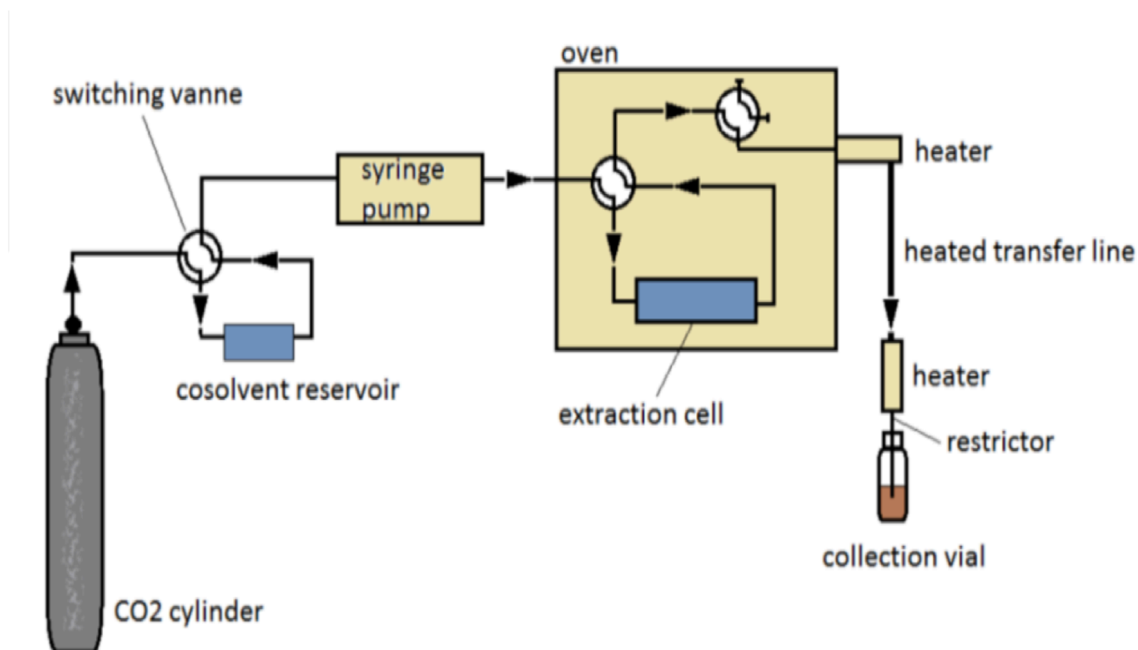


Figure 1.2 Supercritical fluid extraction apparatus[45]. The system contains a reservoir of supercritical fluid, a reservoir of cosolvent, an extraction cell, and a collection vial.

The most commonly used supercritical fluid is carbon dioxide which has a critical point of 31.3 °C and 72.8 bar[49]. This fluid has low critical temperature and pressure, which allows extraction to occurr near room temperature and mild pressure. Carbon dioxide is inexpensive, nontoxic, nonflammable, inert, and a good solvent for nonpolar molecules[49]. In general, supercritical carbon dioxide extraction has a very wide range of applications, such as in food, cosmetics, pharmaceutical, environmental, and other related industries. Pesticides, organic pollutants, fats and lipids, flavors, and natural bioactive components are all classes of compounds that can be separated and extracted from food sample[50].

1.5.7 Accelerated Solvent Extraction (ASE)

ASE is a fast and automatic sample extraction technique that utilizes elevated temperatures and pressures with liquid solvents to obtain fast and efficient extractions. ASE is similar in principle to Soxhlet extraction, except the use of elevated temperature and pressure[51]. ASE allows a high extraction efficiency with a small volume of solvent (10-40 ml) and a short extraction time (5-20 min).

ASE is mostly applicable to solid or semi-solid samples that can be held in the extraction cell during extraction. A schematic of the ASE apparatus is presented in Figure 1.3[45]. With ASE, a solvent or a mixture of solvents is pumped into an extraction cell containing the sample, which is then brought to an elevated pressure and temperature conditions for extraction[52]. The sample extract is then purged by compressed gas from the extraction cell into a collection vessel and prepared for analysis. The entire extraction process is fully automated and carried out in a short time period for fast and easy extraction with low solvent consumption. Application of ASE in food safety studies has been reported for the extraction of various compounds and contaminants like residual pesticides, fats and lipids, food additives, and microbial contaminants in food samples[53,54,55,56].

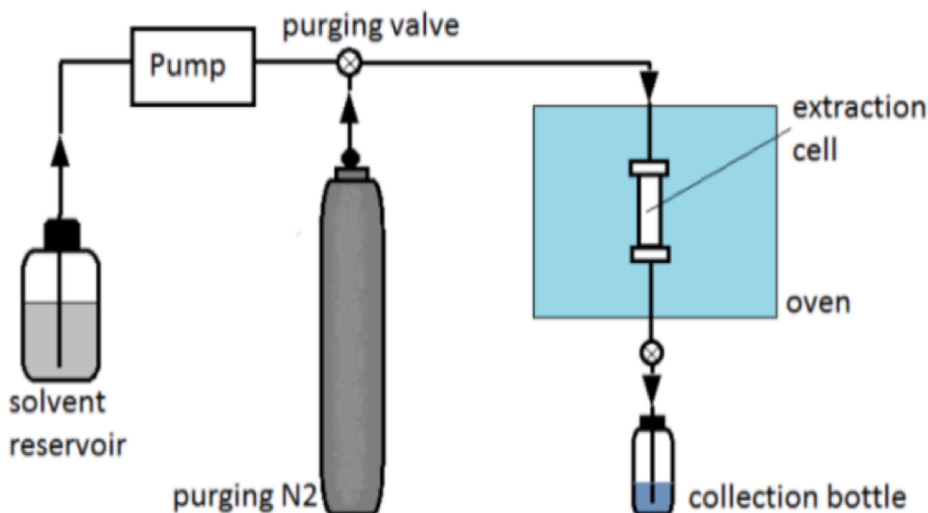


Figure 1.3 Accelerated solvent extraction apparatus[45]. In ASE, a solvent or a mixture of solvents is pumped into an extraction cell containing the sample, which is then brought to an elevated pressure and temperature conditions for extraction. The sample extract is then purged by compressed gas from the extraction cell into a collection vessel and prepared for analysis.

Optimization of various extraction parameters in ASE, including solvent, temperature, pressure, static cycles, and time is considered in order to achieve good efficiency, quantification, and reproducibility. For an efficient extraction, the solvent must be able to solubilize the desired analyte while keeping the sample matrix intact[57]. Most organic solvents and buffered aqueous solutions can be used in ASE, so the need for extraction and the cost of the solvent should be considered when developing a method. ASE uses high temperatures to accelerate the extraction processes. As the temperature is increased, the viscosity of the solvent decreases, thus increasing the solubility of the analytes in the solvent. This enables high diffusion rate of analyte in the solvent. Most ASE applications perform in the 50 to 200 °C range. Changing pressure has little impact on ASE extraction, as the main effect of pressure is to maintain the solvent in its liquid

state. Most accelerated solvent extractions are performed at 1500 psi as the standard operating pressure. Static extraction cycles are used to introduce fresh solvent during the extraction process, which assists to maintain a favorable extraction equilibrium[57]. Extraction time also needs to be optimized in order to obtain a complete and efficient extraction. Increasing the extraction time at an elevated temperature permits a better diffusivity of the analyte into the solvent.

1.6 Extraction of Volatile Compounds

1.6.1 Thermal Desorption

Thermal desorption is a well known sample introduction technique for GC for determination of volatile or semi-volatile organic compounds in gaseous and solid samples. For gaseous samples, volatile organic compounds are collected onto a sorbent first, and then thermally desorbed from the sorbent for GC analysis, while volatile or semi-volatile analytes in solid samples can be determined directly by thermal desorption. Thermal desorption has numerous benefits for analysis of trace-level volatile and semi-volatile organic compounds. Thermal desorption performs sample collection and concentration at same time. The use of sorbents enables accurate and efficient analyses of volatile organic compounds in large sample volumes (such as pollutants in air or residual components from solids) even when analytes levels are very low[58]. Thermal desorption uses heat instead of solvent to desorb analytes from the sorbent and transfer the entire collected analytes to a GC system for analysis[59]. This enables a complete, fast and solvent-free desorption of the analytes. Thermal desorption is a flexible, efficient, and

convenient sample introduction method. It has very wide applications, such as in water, air, fragrances, flavors, and forensic investigation analyses[60,61,62].

1.6.2 Static Headspace

Headspace extraction is usually defined as a vapor-phase extraction, involving the partitioning of analytes between a nonvolatile liquid or solid phase and the vapor phase above the liquid or solid[63]. In this process, the sample is placed in a sealed glass vial with a septum in the cap, the vial is then heated to a specific temperature so that the volatile compounds diffuse into the headspace above the sample[64]. Once the equilibrium between vapor phase and sample phase is reached, the analytes in the headspace is collected and then injected into a gas chromatography for analysis.

The extraction of volatile and semi-volatile organic compounds in solid, liquid, and gas samples can be achieved by headspace analysis. This extraction technique is simple, fast and can provide acceptable sensitivity. Common applications include analyses of organic volatile impurities in pharmaceuticals, flavor compounds in beverages and food products, and fragrance ingredients in perfumes products and cosmetics[65,66].

1.6.3 Purge and Trap

Purge and trap is a dynamic headspace technique that involves the purging of inert gas through a liquid or solid sample, followed by trapping of the volatile analytes on a sorbent and desorption into a GC for separation and identification[67]. This method uses the inert gas to strip the volatile analytes from the sample matrix and concentrate them on

a sorbent[68].

Purge and trap reduces matrix effects and increases sensitivity. This sampling method has been used extensively in different areas, like drinking water, air pollutants, environmental contaminants, and food flavors[69,70,71,72].

1.7 Conclusions

The various extraction methods described here provide an overview of methods that can be used in preparing samples for food safety analysis. Conventional methods such as Soxhlet extraction, liquid-liquid extraction, and solvent-shake extraction are laborious, require the use of large amount of solvents and tedious extraction steps, their applications in food safety studies are limited. Modern extraction methods such as SFE, ASE, MAE, UAE, and SPME have numerous advantages when compared to the traditional methods, such as shortened extraction time, reduced solvent and energy consumption, and improved extraction efficiency. They are considered as “green” sample preparation techniques and have been used extensively for determination of various contaminants and harmful substances in food samples. As a concluding remark, modern green extraction methods are promising sample preparation techniques for food safety studies because of the advantages (high efficiency, high reliability, and “green” features) over the conventional extraction methods, their development should be proceeded further.

CHAPTER 2. DETERMINATION OF PERFLUOROOCTANOIC ACID FROM THE SURFACE OF COOKWARE UNDER SIMULATED COOKING CONDITIONS USING ACCELERATED SOLVENT EXTRACTION (ASE) AND HPLC-MS/MS

2.1 Abstract

Perfluorooctanoic acid (PFOA) is used as a polymerization aid in the production of fluoropolymers. These polymers provide oil and water repellency as well as stain resistance, which make them ideal coating materials for non-stick cookware. PFOA is bioaccumulative and potentially harmful to humans. PFOA is not supposed to be found in the final products of non-stick cookware after processing. This study presents a method to determine the potential leaching of PFOA from the cookware under simulated cooking conditions. Fluoropolymer-coated cookware was extracted with ethanol/water mixtures using accelerated solvent extraction (ASE), and the extraction parameters such as pressure, cycle, and purge time were optimized. The resulting extracts were analyzed by liquid chromatography tandem mass spectrometry (HPLC-MS/MS).

The linearity of the method was good, with regression coefficients of 0.99961 and 0.99984 for watery- and fatty-food simulations. The recoveries and relative standard deviations of the method ranged from 81.4% to 118.0% and 0.9% to 14.9%, respectively. Limits of detection (LOD) were 0.03 and 0.02 μg/L, corresponding to 5.0 pg/cm^2 and 3.3 pg/cm^2, for PFOA analysis under watery- and fatty-food simulation conditions. The method was applied to analyze PFOA from used and new cookware under simulated cooking conditions. The study demonstrated that PFOA was detectable in all samples

under watery- and fatty-food simulation conditions. The highest concentration detected was 395 pg/cm^2. It is assumed that PFOA breaks down from fluoropolymer-coated cookware (new or used) may leach into foods under common cooking conditions.

2.2 Introduction

Perfluorooctanoic acid is an organofluorine compound that is synthetically produced and primarily used as an emulsifier in the production of fluoropolymers. The compound consists of chains of eight carbons with fluorine atoms bonded to each carbon and a carboxyl group at the end of the chain, the structure is shown in Fig. 2.1[73]. PFOA is a solid at room temperature with low vapor pressure, 4.2 Pa at 25 °C. The melting point and boiling point for PFOA are reported as 45-50 °C and 189- 192 °C. PFOA is highly soluble in water, having a solubility of 9.5 g/L. The pKa of PFOA reported as approximately 2.5 in the literature. PFOA typically presents as an anion (conjugate base) in solution. Ammonium perfluorooctanoate is the most common form. The physical and chemical properties for PFOA are shown in Table 2.1[74].

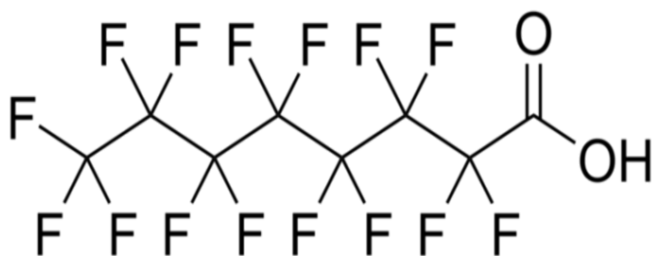


Figure 2.1 Structure of perfluorooctanoic acid[73]. Perfluorooctanoic acid is an organofluorine compound that consists of chains of eight carbons with fluorine atoms bonded to each carbon and a carboxyl group at the end of the chain.

Table 2.1 Physical and chemical properties of PFOA[74].

Property	Value
Physical state	Solid (at 20 °C)
Density	1.7921 g/cm at 20 °C
Molecular weight	414.07 g/mol
Boiling point	188 °C
Melting point	54.3 °C
Vapor pressure	4.2 Pa
Water solubility	9.5 g/L (at 25 °C)
pK_a	2.5

PFOA is very stable. The distinctive stability is mainly attributed to the strength of the carbon-fluorine bonds, the presence of the three electron pairs surrounding each fluorine atom, and the shielding of the carbon atoms by the fluorine atoms[75]. PFOA possesses a hydrophilic functional group and hydrophobic alkyl side chain. Overall it is hydrophilic, with hydrophobic and oleophobic character[76].

PFOA has been synthesized and used in commercial and industrial productions for more than 60 years. PFOA is primarily manufactured via the Simons electro-chemical fluorination (ECF)[77] and telomerization reactions[78]. In the ECF process, the carbon-hydrogen bonds on molecules of the organic feedstock (commonly 1-heptanecarbonyl fluoride) are replaced with carbon-fluorine bonds when an electric current is passed through a mixed solution of anhydrous hydrogen fluoride and the organic feedstock. This process produces a complex combination of molecules including branched, linear, and

cyclic isomers of various chain lengths of perfluoroalkyl fluorides, along with other byproducts and impurities. After removal of the byproducts and impurities, the acid fluoride is base hydrolyzed in batch reactors to yield PFOA. The process is inexpensive but generates perfluorochemicals with homologous series of even- and odd-number perfluorocarbons[79]. In the telomerization process, tetrafluoroethylene is reacted with fluorine-bearing chemicals to produce fluorinated intermediates that are then converted into PFOA[80]. Telomerization produces predominatly straight chain (linear) compounds with an even number of carbons, like PFOA. After telomerization, distillation is used to obtain pure components.

PFOA is primarily used as an emulsifier in the production of polytetrafluoroethylene (PTFE or Teflon), and other fluoropolymers. It can suspend and emulsify polymers during the manufacture. Since PTFE has properties such as strong water and oil repellency, chemical stability, thermal stability, chemical resistance, and non-adherence[81], it is used in homes and industries as oil-, stain-, and water-resistant-coating agents for clothing, food packaging papers, leather products, carpets, semiconductor materials, and nonstick cookware[82,83]. Electroplating, electronic etching bath surfactants, aviation hydraulic fluids, aqueous fire-fighting foams, paints, adhesives, waxes, polishes, and floor polishes also contain PFOA as a component [81,82].

PFOA has received a lot of attention recently due to its wide distribution and persistence in the environment. PFOA has been detected in a number of U.S. cities in surface waters, sediments, wastewater treatment plants, sewage sludge, and landfill leachate[84,85]. Industrial products such as stain-resistant carpets and furniture, paper bags

for food, carpet cleaning liquids, household dust, water, and food were also found to have detectable levels of PFOA[86]. Based on recent human biomonitoring data provided by industry, PFOA was found in the blood of workers and more than 98% of the general population in all geographic regions of the United States[87]. Exposure to PFOA is potentially nationwide. Recently, studies and assessments have reported that PFOA has been detected in fishes, surface waters, and foods in developed and developing countries around the world including in North America, Europe, and Asia[88, 89,82]. PFOA has become a global environment problem. Because of the presence of strong carbon-fluorine bonds, PFOA is stable and resistant to breakdown under environmental conditions. PFOA is thermally, chemically and biologically stable, does not hydrolyze, photolyze, or biodegrade[90]. As a result, PFOA is extremely persistent in the environment and can lead to bioaccumulation in fish, animals, humans, and environment. The current EPA standard for PFOA in drinking water is 0.4 ppb. EPA has recently proposed a permanent safe level for PFOA of 0.1 ppb[91].

Due to the wide distribution and persistence of PFOA in the environment, human and wild animals can continually be exposed to PFOA. Studies indicate continued exposure to PFOA could result in adverse health effects[81]. Tests using rats have demonstrated that the chronic exposure to PFOA can lead to the development of cancers, such as hepatic tumors and pancreatic tumors, as well as hepatic disorder, lipid metabolic disorder and developmental disorder[92,93]. PFOA has been shown to induce tumors of the liver, testis and pancreas (tumor triad) in rats following chronic dietary administration[94]. Toxicological studies have shown that exposure to PFOA can result in developmental/reproductive toxicity, liver damage, and possibly cancer[95]. Studies have

also revealed that PFOA accumulates primarily in the kidney, liver, and plasma after oral exposure[96]. The modes of action for PFOA are not fully described. However, according to a number of studies, PFOA activates the nuclear receptor peroxisome proliferator-activated receptor α (PPARα) and other nuclear receptors such as CAR (constitutive androstane receptor) and PXR (pregnane X receptor)[97,98,99]. The activation of PPAR α results in the up regulation of specific subsets of genes involved in peroxisome proliferation, lipid metabolism, and cell cycle control/apoptosis. This induces increased cell proliferation, leading to the formation of preneoplastic cells or the induction of new focal lesions. PFOA is not readily eliminated and excreted from humans and animals. PFOA has a long half-life of about 4.37 years in humans[100].

Because of the wide distribution, persistence, toxicity, and bioaccumulation of PFOA, the U.S. EPA has been investigating PFOA and requesting more information regarding sources and potential routes of human exposure to PFOA. Generally, the main routes of general population exposure to PFOA are likely via oral and inhalation exposures[101]. Food, food-packaging materials, drinking water, outdoor and indoor air, house dust, consumer and industrial products are all implicated as sources of PFOA to people. Since PFOA is essentially non-volatile, it is not likely that the general population get exposed via the inhalation route. The general population exposure to PFOA is most likely via the oral route by digestion of contaminated food or water[102]. PFOA has been detected in a number of food samples, including fish, meat, milk, eggs, potato, canned vegetables, bread, and other foods[103,104]. PFOA has also been found in food-contact materials, like non-stick cookware and food-packaging papers[105,106]. Food might get

contaminated during cooking and production processes due to contact with PFOA-based products like cookware and food packaging bags that can leach PFOA.

PFOA is used as a polymerization aid during the manufacture of polytetrafluoroethylene (PTFE). The particular physical and chemical properties (ability to resist flame, water, oil, and grease) of PTFE make them ideal coating materials for non-stick cookware. PTFE is well known for its use in coating non-stick cookware. Manufacturers of non-stick cookware claim that PFOA used for producing non-stick pan coatings is entirely destroyed in the process in manufacturing (conducted at >300 °C), and not present in the finished non-stick cookware[107]. PTFE has a high degradation point (327 °C) and is extremely chemically resistant to a lot of chemicals, temperatures greater than 327 °C are required for chemical decomposition of PTFE to occur[108]. While use of non-stick cookware is stable at lower temperature, it is found that the PTFE coated pan evolved lethally toxic agents at high temperatures (>280 °C)[109,110]. At higher temperatures PTFE-coated pans generate heavier highly toxic fluorinated compounds (hexafluoropropylene and perfluoroisobutylene) [111, 110]. It is recognized that PTFE-coated pans left on the heat to reach high temperatures (>280 °C) will result in the release of toxic fluorinated compounds[109,112]. However, few data has been reported for analysis of potential leaching of PFOA from non-stick cookware into food during cooking process. Bradley and coworkers investigated the migration potential of coating materials from cookware products, they reported there was no evidence of fluorinated substances released from the coatings[113]. However, due to the undefined perfluorinated chemicals analysis and the limited method detection limit, the results should be interpreted with care. According to a report by Environmental Working Group (EWG)[91], PFOA used in non-

stick pans might be unsafe at any level. Due to the lack of suitable analytical data, it is still suspicious that if PTFE coated cookware is safe for use under typical cooking conditions. Therefore, it is necessary to determine if PFOA is still present in the finished product, or if PFOA could be leaching into food under typical cooking conditions.

Accelerated solvent extraction (ASE) uses elevated temperature and pressure to achieve an efficient extraction which is suitable to simulate cooking conditions. Larson and coworkers compared ASE and reflux extraction for the determination of PFOA in polytetrafluroethylene polymers, ASE proved to be the more efficient extraction method[114]. The objective of this study is to develop a method for determination of the potential leaching of PFOA from cookware under simulated cooking conditions by ASE and UPLC-MS/MS. There are mainly three parts for this study: (1) optimization of ASE extraction, (2) development of a method for determination of PFOA by UPLC-MS/MS, and (3) investigation of potential leaching of PFOA from new and used cookware under simulated cooking conditions.

2.3 Experimental

2.3.1 Reagents and Materials

Perfluorooctanoic acid standard (98% purity) and internal standard octanoic acid (≥ 99% chemical purity) were purchased from Sigma-Aldrich Company (Milwaukee, WI, USA). HPLC-grade methanol and LC/MS-grade water were obtained from Fisher Scientific (Fairlawn, NJ, USA). Ammonium acetate (99%) was purchased from Fluka (Buchs, Switzerland). Standard Ottawa sand was obtained from EMD Chemicals, Inc. (Darmstadt, Germany). Polyethylene or polypropylene tubes, volumetric flasks,

autosampler vials, and pipettes tips were obtained from Fisher Scientific (Fairlawn, NJ, USA).

2.3.2 Fluoropolymer Coated Cookware

New and used frying pans coated with fluoropolymer materials were purchased from local retailers. These coated pans were cut into rectangular pieces measuring approximately 1 cm ×2 cm using a water jet by Industrial Machine & Engineering, LLC (Brookings, SD, USA), as shown in Fig. 2.2.

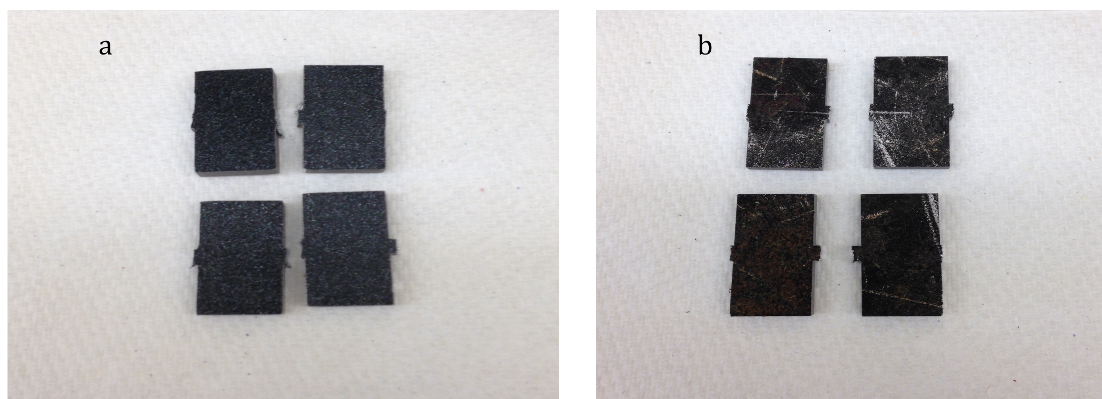


Figure 2.2 Frying pan samples. New (a) and used (b) frying pans coated with fluoropolymer materials were cut into rectangular pieces measuring 1 cm ×2 cm using a water jet.

2.3.3 Standard Preparation

Stock solutions of the standard and internal standard were prepared in methanol at a concentration of 1000 ppb and 40,000 ppb and stored in polypropylene flasks in a refrigerator (4 °C). Five calibration standards (0.1, 0.5, 2.0, 5.0, and 10.0 ppb) were prepared from the stock standard solution in 90:10 (v/v) methanol and water. Octanoic acid was added as an internal standard. Fifty microliter of 40,000 ppb internal standard

were spiked in each calibration standard and sample. In order to avoid contamination by perfluorinated compounds, all the tubes, autosampler vials, and pipette tips were composed of disposable polypropylene.

2.3.4 Sample Extraction

To simulate cooking conditions, accelerated solvent extraction (ASE) with a Dionex ASE 200 system (Dionex Corporation, Sunnyvale, CA) was used to perform extractions of PFOA using ethanol/water mixtures as food-simulating liquids, which is consistent with FDA guidelines[115]. An ethanol and water mixture of 1: 9 (v/v) was used to simulate watery and acidic foods, and 9: 1 (v/v) ethanol and water was used to simulate fatty or oily foods. To perform extraction, six pieces of frying pan were placed in a 11 mL ASE vessel. The ASE vessels and extraction system were preconditioned each time before use. Extraction parameters such as pressure, preheat time, flush volume, and cycles were optimized. Extraction temperature (100, 125, 150, 175, and 200 °C) and extraction time (14, 20, and 29 min) were investigated to evaluate the effects of temperature and time on PFOA analysis in foods under simulated cooking conditions. The extract collected from ASE was concentrated to dryness under nitrogen and then reconstituted with ethanol/water. *n*-Octanoic acid was added as an internal standard to all the samples. The sample was centrifuged for 20 min at 14000 RCF before transferred into an autosampler vial for analysis. Each sample was prepared in triplicate.

2.3.5 HPLC-MS/MS Analysis

The analysis of PFOA was performed using ultrahigh performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) on a Shimadzu UHPLC (Kyoto, Japan) and an AB Sciex Q-trap 5500 MS (Applied Biosystems, Foster City, CA, USA) mass spectrometer. Fifteen-microliter aliquots of the sample were injected on a Fusion RP column (2.0 mm × 50 mm, 4 µm d_p) (Phenomenex, Torrance, CA, USA). The mobile phase was consisted of 10% 10 mM ammonium acetate and 90% methanol. The flow of mobile phase was set at 0.2 mL/min and the column was maintained at 35 °C. The total analysis time was 3 min.

Electrospray in the negative ionization mode was used in the mass spectrometer source. N_2 (50 psi) was used as the curtain gas. Nebulizer gas GS1 and GS2 were set at 45 and 60 psi, respectively. Transitions for all ions were observed using multiple reaction monitoring (MRM). The most intense ion transition was used for the quantitative analysis while the second was used to confirm the identification. The parent to daughter ion transition at 413 > 369 was selected for quantitative analysis, while 413 > 169 is used for identification of PFOA. The parent to daughter ion transition at 143 > 125 was monitored for quantitation and the transition at 143 > 45 was monitored for identification of internal standard (*n*-octanoic acid). Optimized mass spectrometry detection parameters are presented in Table 2.2.

Table 2.2 Parent to daughter ion transition states.

MRM Transition (Q1 > Q3)	Collision Energy (CE)/V	Declustering Potential (DP)/V	Entrance potential (EP)/V	Collision cell exit potential (CXP)/V
413 > 369	-14.43	-56.78	-10.27	-15.90
413 > 169	-24.55	-63.34	-11.54	-11.31
143 > 125	-24.43	-75.45	-11.09	-8.28
143 > 45	-28.01	-27.40	-11.53	-7.62

2.3.6 Method Development

2.3.6.1 Quality Control

A quality-control program was developed to eliminate potential contamination during the extraction and analysis. All tubes, autosampler vials, and pipette tips used were polypropylene and disposable. The entire HPLC system was flushed extensively with 100% methanol to eliminate background contamination before each analysis. Solvent blanks, ASE blanks, and instrumental background checks were investigated each analysis.

2.3.6.2 Extraction Optimization

A Dionex ASE 200 was used for PFOA extraction. For better extraction efficiency, extraction parameters such as pressure, flush volume, purge time, and cycles were optimized before analysis. The extraction efficiency was evaluated in terms of the recovery of the spiked recovery check standards.

2.3.6.3 Method Validation

The method was validated by assessing limit of detection, limit of quantification, linearity, accuracy, and precision. The limit of detection (LOD) and limit of quantification (LOQ) were determined based on a signal to noise ratio of 3 and 10, respectively. Linearity was assessed over a spike concentration range from the LOQ up to 10 ppb. Accuracies were determined in triplicate at concentrations of 0.5, 2.0 and 10.0 ppb on three different days. The precision of the method was determined by calculating the average relative standard deviation of the replicate analysis of the recovery standard.

2.4 Results and Discussion

2.4.1 HPLC-MS/MS Performance

The identification and quantification of PFOA was performed by HPLC-MS/MS. Mass spectrometer detection conditions, including collision energy (CE), declustering potential (DP), entrance potential (EP), and collision cell exit potential for parent to daughter ion transitions of analyte were optimized. Spectra of parent ion and daughter ions of a PFOA standard are shown in Fig. 2.3.

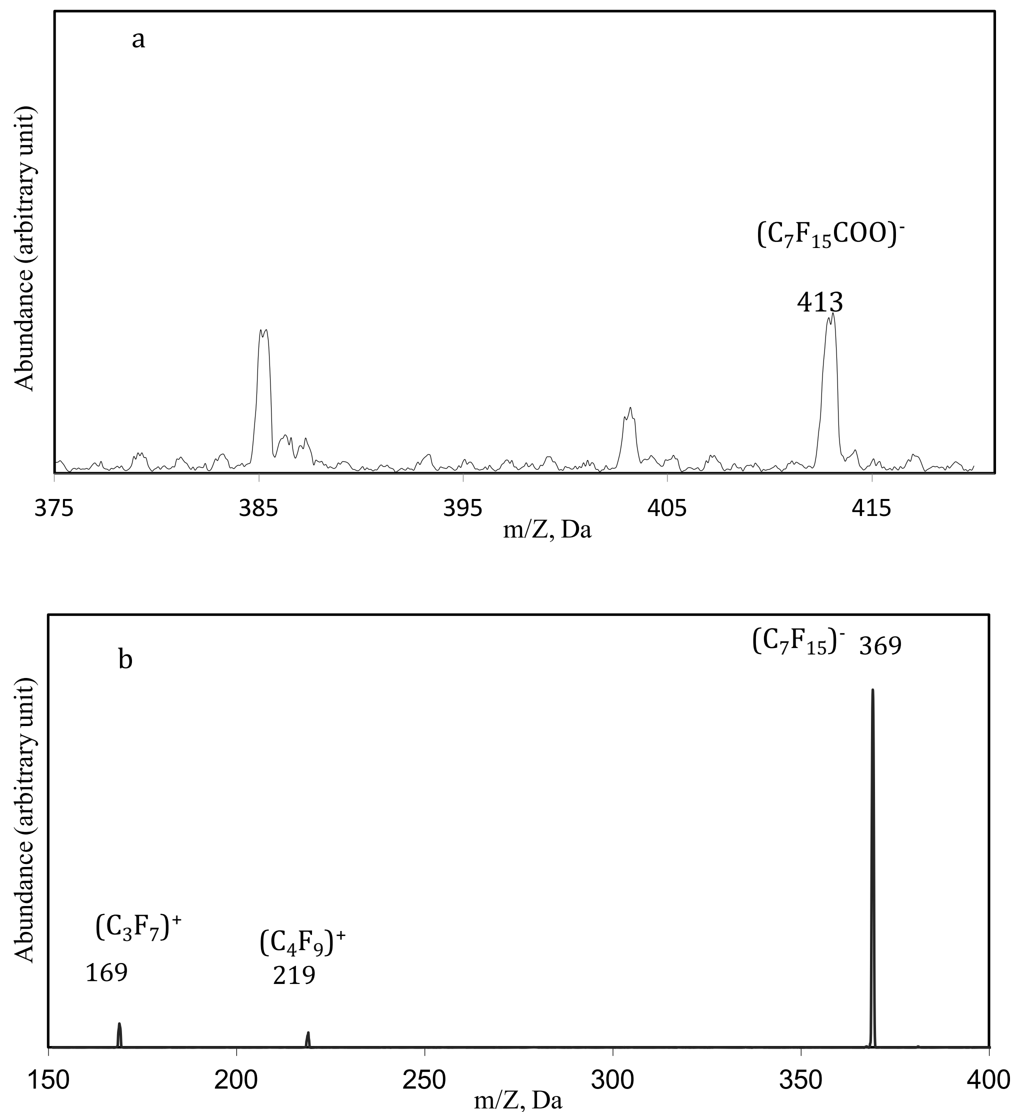


Figure 2.3 Mass spectra of PFOA. (a) Parent ion and (b) daughter ions. The parent to daughter ion transition at 413 > 369 was selected for quantitative analysis, while 413 > 169 is used for identification of PFOA.

PFOA eluted at approximately 1.2 min with a total running time of 3.0 min (Fig. 2.4). All chromatographic separations were achieved using isocratic elution (90% MeOH and 10% 2 mM ammonium acetate).

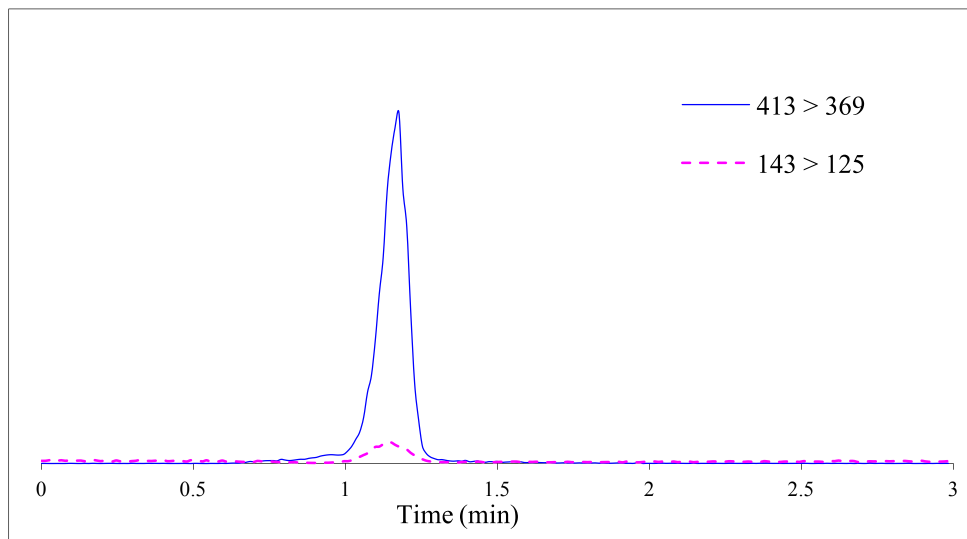


Figure 2.4 Representative chromatogram of PFOA with internal standard. Chromatographic conditions: a Fusion RP column (2.0 mm × 50 mm, 4 µm dp), 10% 2 mM ammonium acetate and 90% methanol isocratic elution, 0.2 mL/min flow rate, and 35 °C column temperature.

2.4.2 Elimination of Blank Contamination

Procedural and instrumental blank contamination is a major challenge in most PFOA analysis. The analyte can be found in many common laboratory supplies and equipment such as polytetrafluoroethylene products, sample bottles and caps, aluminum foil, and sample transfer lines. To identify the background contamination, solvent blanks and method blanks were investigated. No PFOA was observed in the solvent blank (methanol), as shown in Fig. 2.5. ASE blank contamination was reduced by avoiding the use of fluoropolymer materials and by completely rinsing all equipment with methanol before use (Fig. 2.5).

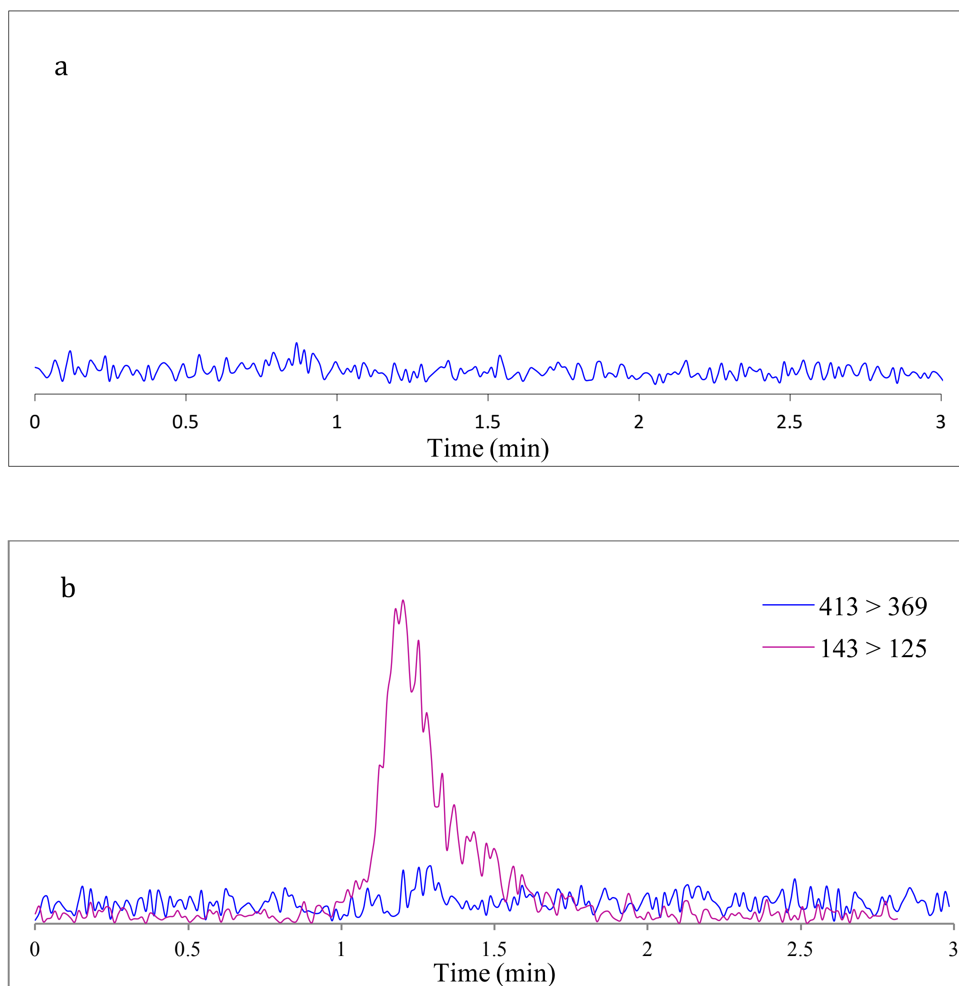


Figure 2.5 Chromatogram of a methanol blank (a) and an ASE blank with internal standard (b)

2.4.3 ASE Optimization

ASE conditions such as pressure (1500 and 1000 psi), flush volume (50, 100, 150 %), purge time (30, 60, 90 sec), and cycles (1, 2, 3) were optimized to obtain high extraction efficiency. To simulate cooking conditions, ethanol and water mixtures of 1: 9 (v/v) and 9: 1(v/v) were used to simulate watery or acidic foods and fatty or oily foods, respectively. The optimization was carried out at an extraction temperature of 175 °C for 20 min, corresponding to a frying temperature of about 350 °F, which are the most

common cooking conditions. The extraction efficiencies of PFOA were calculated and compared based on the recovery of the spiked standard solution.

For the extraction pressure, 1000 psi was found to have lower extraction efficiencies than 1500 psi in both watery-and fatty-food simulating solvent extractions. Compared to using a 50% flush volume, 100 and 150% flush volumes had higher extraction efficiencies for PFOA with no significant difference between the two. Purge times of 60 and 90 s obtained higher extraction efficiencies than a 30 s purge, with no significant difference between these two. It was also found that the three-cycle extraction process yielded the highest extraction efficiencies in watery-and fatty-food simulating solvent extractions. The results suggest that the optimized conditions for watery and fatty food simulation extractions are comparable. Thus, 1500 psi, 100% flush volume, 60 s purge, and three cycles were chosen as the conditions for both watery- and fatty-food-simulation extractions.

2.4.4 Limit of Detection, Limit of Quantification, and Linearity

The method limit of detection (LOD) and limit of quantification (LOQ) were determined by analysis of PFOA with the complete analytical method (ASE extraction and HPLC-MS/MS). The method linearity was evaluated with spiked samples at five different concentrations between the limit of quantification and 10 ng/mL. A calibration curve was obtained using response ratios of PFOA to internal standard. LODs for watery- and fatty-food simulation extractions were found to be as low as 0.03 and 0.02 ng/mL, corresponding to 5.0 and 3.3 pg/cm^2 surface area, respectively. Both the watery- and

fatty-food-simulation methods were found to have LOQs of 0.1 ng/mL and linearity ranges of 0.1-10 ng/mL with excellent R^2 values. The data is summarized in Table 2.3.

Table 2.3 Validation data

Condition	LOD		LOQ		R^2	Linearity range (μg/L)
	μg/L	pg/cm²	μg/L	pg/cm²		
Watery-food simulation	0.03	5.0	0.1	16.7	0.99961	0.1-10
Fatty-food simulation	0.02	3.3	0.1	16.7	0.99984	0.1-10

2.4.5 Recovery and Precision

It is shown that acceptable recovery data (80-120%) were obtained at levels of 0.5, 2.0, and 5.0 μg/L ranging between 81.4% and 118.0%. Relative standard deviations showed good precision of the method ranging from 0.9% to 14.9%. All recovery and precision data are presented in Table 2.4.

Table 2.4 Recovery results

Condition	Spiked level (μg/L)	Measured* (μg/L)	Recovery (%)	RSD (%)
Watery-food simulation	0.50	0.440±0.021	87.9	4.8
	2.00	1.63±0.24	81.4	14.9
	10.00	8.277±0.073	82.8	0.9
Fatty-food simulation	0.50	0.570±0.030	114.1	5.2
	2.00	2.392±0.039	118.0	1.7

	10.00	11.78±0.45	117.8	3.9

* Values are mean ± standard deviation.

2.4.6 Analysis of Fluoropolymer-coated Cookware

The method was applied in the analysis of four different samples, namely pan A, B, C, and D. Pans A, B, and C were cut from three different used frying pans coated with fluoropolymer materials, respectively, while pan D was cut from new pans coated with fluoropolymer materials. The pans were extracted using ASE at simulated-cooking conditions of 175 °C and 20 min, the extracts were then analyzed by HPLC-MS/MS.

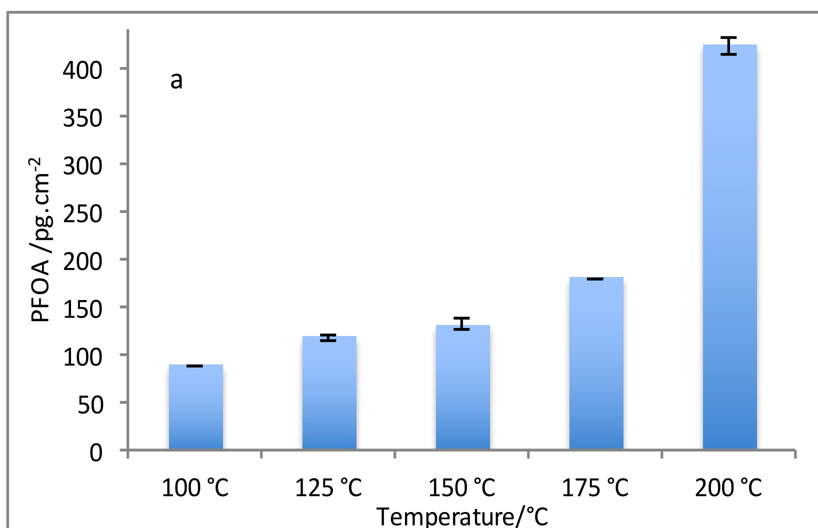
The amounts of PFOA detected from the pans are listed in Table 2.5. The data showed that PFOA were detectable in all pan samples that extracted at both watery-and fatty-food-simulation conditions. The detected PFOA levels were in the range of 113 and 290 pg/cm^2 surface area. There was no consistent trend observed in the results of the analysis of PFOA concentration regarding to the number of extraction repeated. The highest concentrations of PFOA, of 290 pg/cm^2 and 267 pg/cm^2, were detected in the extracts of pan C and pan B under fatty-food simulation condition. Results also showed that lower levels of PFOA were detected from pan A under watery-food simulation than from fatty-food simulation extraction. These results could therefore indicate that PFOA from fluoropolymer-coated cookware (new or used) may leach into watery-and fatty-foods under common cooking conditions (175 °C and 20 min). Since the new pan (D) was not significantly different than used pans of unknown history (A-C), we can conclude that the PFOA leaching from fluoropolymer-coated pans appears to be the result of fluoropolymer degradation rather than residual PFOA in the coating.

Table 2.5 PFOA detected levels in pans

Condition	Number of extraction repeated	Pan A		Pan B		Pan C		Pan D	
		PFOA pg/cm^2	RSD (%)	PFOA pg/cm^2	RSD (%)	PFOA pg/cm^2	RSD (%)	PFOA pg/cm^2	RSD (%)
	1	132	12.7	113	2.1	122	0.8	179	0.6
Watery-food simulation	2	113	6.5	143	12.8	163	12.2	118	10.6
	3	138	13.3	163	6.7	203	13.9	162	5.6
	1	170	11.8	267	8.1	258	13.5	127	5.6
Fatty-food simulation	2	162	11.3	222	15.0	252	7.3	152	0.5
	3	142	11.8	252	5.0	290	10.3	123	4.7

2.4.7 Effect of Cooking Temperature and Time on Potential Leaching of PFOA from Fluoropolymer Coated Cookware into Foods

To test the effect of cooking temperature on the migration of PFOA from cookware into foods, different simulated cooking temperatures (100, 125, 150, 175, and 200 °C, corresponding to a range from 212 to nearly 400 °F) were investigated and compared. The extractions of samples from a frying pan (D) were carried out at different temperatures for 20 min. The results are presented in Fig. 2.6. PFOA was detected in all samples except the fatty-food-simulation sample that extracted at a temperature of 100 °C. In general, lower amounts of PFOA were detected in fatty-food-simulation samples than watery-food simulation samples. As the extraction temperature increases, higher amount of extractable PFOA was detected. It was also shown that the detected amount of PFOA increased dramatically at an extraction temperature of 200 °C under watery-food simulation. It can be concluded that a higher cooking temperature results in greater PFOA leaching into food.



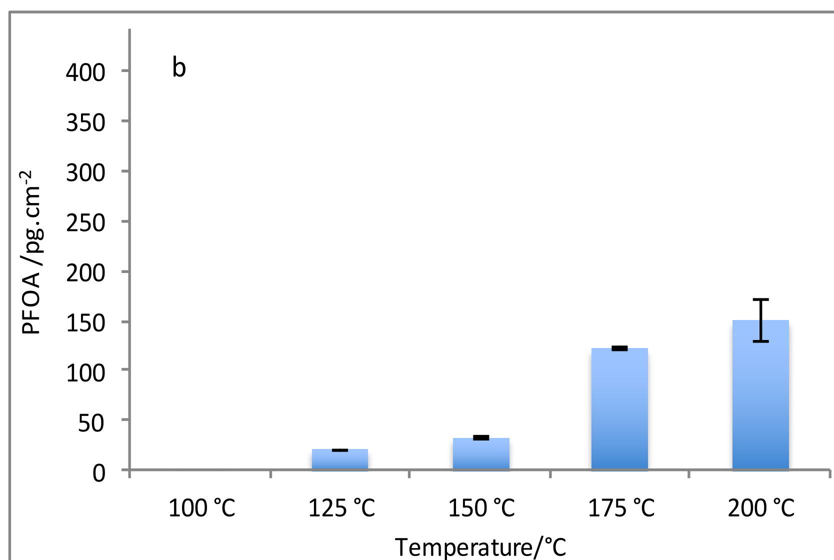


Figure 2.6 Extraction of pan D at different temperature, (a) watery-food simulation (b) fatty-food simulation. The extractions of pan D were carried out for 20 min at 100, 125, 150, 175, and 200 °C. The error bars represent the standard deviation of the measurements.

Investigations were also made to cooking time on the analysis of PFOA from cookware. Different simulated cooking times (14, 20, and 29 min) were investigated and compared. The extractions were performed at 175 °C. The detected PFOA levels from the extractions for different times are summarized in Fig. 2.7. As shown, longer extraction times, higher amounts of PFOA were observed, for both watery and fatty food simulations. Watery food simulation sample extracted for 29 min has the highest amount of PFOA, which was found to be 395 pg/cm^2. The data also suggested that the amount of PFOA detected in the fatty food simulation samples of 14 and 20 min extraction time were comparable. Therefore, one might expect the highest level of PFOA to be found in watery food rather than in fatty food if a long cooking time is needed.

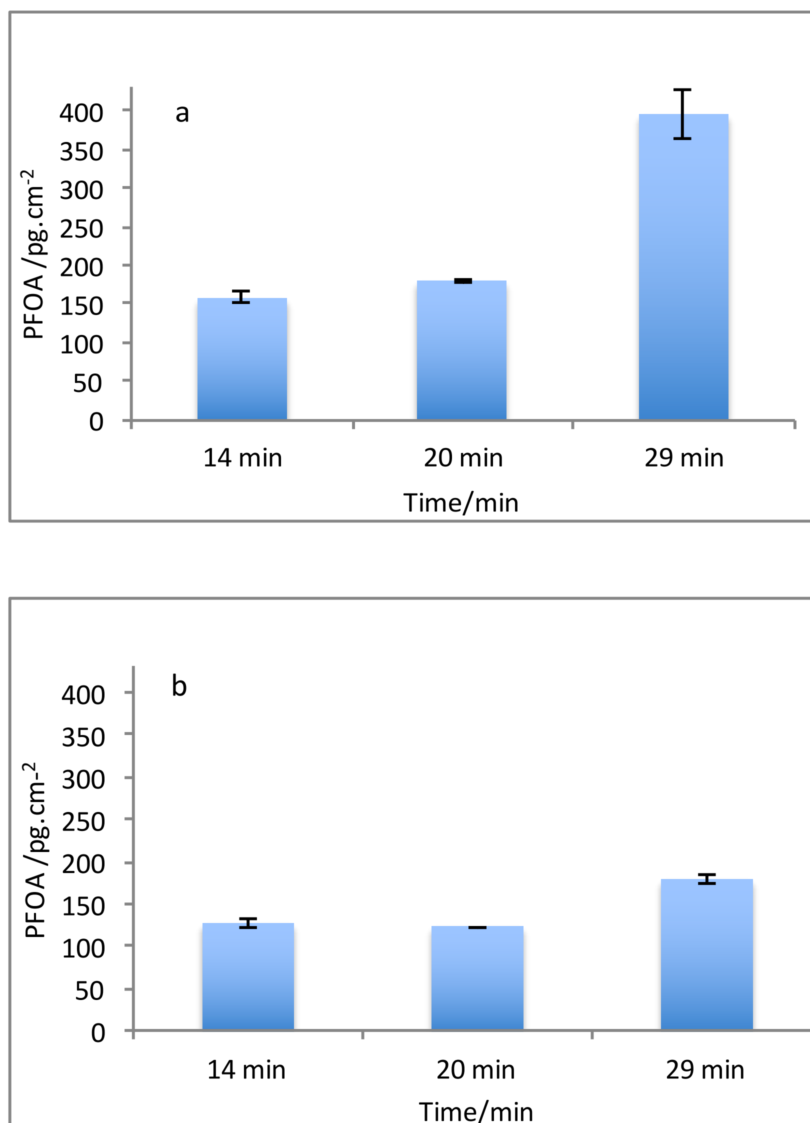


Figure 2.7 Extraction of pan D for different times, (a) watery-food simulation (b) fatty-food simulation. The extractions of pan D were carried out at 175 °C for 14, 20, and 29 min. The error bars represent the standard deviation of the measurements.

2.5 Conclusions

In this study, optimized chromatographic, extraction and sample preparation procedures, analytical recovery, method precision, method limit of detection, method limit of quantification, and linear range for analysis of PFOA are presented. The developed method was successfully applied to analyze PFOA from used and new

cookware under simulated cooking conditions. The study demonstrated that PFOA were detectable in all pan samples extracted with both watery-and fatty-food-simulation conditions, except water at 100 °C. It is assumed that PFOA from fluoropolymer-coated cookware (new or used) may leach into watery and fatty foods under common cooking conditions (175 °C and 20 min). The amount of PFOA detected appears to be related directly to the cooking temperature. PFOA level as high as 395 pg/cm^2 could be expected in watery food cooked at 175 °C for 29 min. Therefore, it can be concluded that higher cooking temperature and longer cooking time may result in a higher PFOA level in the food. However, no attempt was made to correlate this data to PFOA levels found in fried foods or the average diet.

2.6 Acknowledgments

The authors kindly thank the South Dakota State University Campus Mass Spectrometry Facility Center for use of the HPLC-MS/MS instrumentation.

CHAPTER 3. DETERMINATION OF ALLYL ISOTHIOCYANATE AND DERIVATIVE COMPOUNDS IN HORSERADISH EXTRACT BY HS-SPME-GC-MS

3.1 Abstract

A headspace-solid-phase microextraction-gas chromatography-mass spectrometry (HS-SPME-GC-MS) method was developed to quantify allyl isothiocyanate (AITC) and related compounds in horseradish products. Solvent extraction, headspace sampling, and HS-SPME were compared, and HS-SPME gave acceptable accuracy and precision for the quantification of AITC and related compounds in horseradish. The optimized conditions for HS-SPME were 0.8 g sample size in a 4-mL vial at 30 °C for 20 min with one minute desorption in the GC injector at 250 °C. A calibration curve was generated in the concentration range of 50-3200 ppm of allyl isothiocyanate using the internal standard method. The validated method resulted in intraday and interday precision (% RSD) and accuracy (% recovery) of less than 10% and 80-120%, respectively. Seven constituents were identified and the major constituents were allyl isothiocyanate (97.58%) and phenylethyl isothiocyanate (1.65%), representing 99.23% of the pungent components in prepared horseradish sample. The HS-SPME-GC-MS method presented is simple, accurate, and sensitive. Manufacturer and processors can use this method to evaluate quality of flavored products before and after production.

3.2 Introduction

Horseradish (*Armoracia rusticana*) is a perennial plant that belongs to the Brassicaceae family, which also includes mustard, wasabi, broccoli, and cabbage. Horseradish originated in Eastern Europe and the southern part of Russia[116]. Now, it has a wide-spread distribution throughout the world, and grows mostly in England, France, the United States, Canada, Austria, Japan, and China. Horseradish can be found in various environments, like fields, home gardens, weedy areas, farmland, roadsides, ditches and disturbed areas[116]. Horseradish is a hardy perennial plant with large lancet to heart-shaped basal leaves with long stalks[117]. The plant can grow up to 150 cm (five feet) tall. The root is long and thick. Horseradish has been primarily cultivated for its thick and fleshy root since ancient times[118].

Due to its characteristic strong smell and taste, horseradish has been used as a medicinal herb and a spice for almost 2,000 years[117]. In the past it has been used medicinally to treat everything from back aches to the common cold[119]. Horseradish was believed to relieve various forms of pain and even cure a range of diseases. The ancient Greeks used it as a rub to alleviate pain in the back[120]. A German abbess and founder of cloisters recommended horseradish as a treatment for lung and heart diseases[121]. Horseradish was also used as a stimulant and drug to cure heartache or heart diseases by Chinese[117]. Horseradish has a pungent smell and unique taste. The use of horseradish as food or condiment was established from the Europe and Mediterranean areas during the 5th century[122]. It was believed that the custom of using the root as a spice came from Germany and spread to England and later also to the Nordic countries[121]. It was common

that horseradish was served together with food in the northern parts of Europe. Horseradish was also used to preserve foods such as cucumber, red beet, and herring due to its antibacterial characteristics. Nowadays, the use of horseradish as a condiment is still popular in Europe and North America[122]. Horseradish sauce is usually served with beef, fish, chicken, and meat dishes, or on sandwiches.

Horseradish is a good source of a number of bioactive compounds such as glucosinolates and their derived products. Sinigrin is the dominant glucosinolate in the intact root of horseradish, accounting for about 90% of total glucosinolate content[123,124]. When horseradish tissues become disrupted by cutting or grating, the native enzyme myrosinase comes into contact with sinigrin, the hydrolysis reaction takes place, and isothiocyanate compounds are produced (Fig. 3.1)[125,126]. The pungent odor and unique flavor of horseradish are mainly attributed to allyl isothiocyanate (AITC). Since water is a substrate in the hydrolysis reaction, humidity from the air can be used to activate the release of AITC[127].

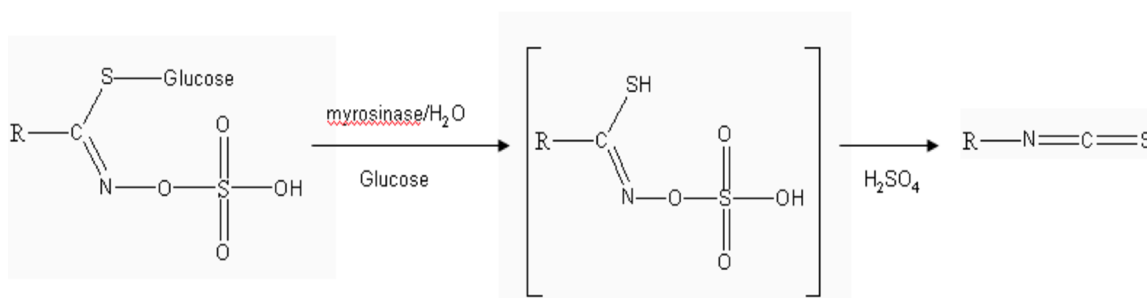


Figure 3.1 Hydrolysis of glucosinolates to isothiocaanates[125]. Glucosinolates are enzymatically hydrolyzed by myrosinase to isothiocaanates.

Recent studies have shown that AITC in horseradish can strongly inhibit the growth of bacteria such as *Escherichia coli*, *Listeria monocytogenes*, *Salmonella*

typhimurium, *Serratia grimesii*, and *Staphylococcus aureus*[128,129]. It is believed that isothiocyanates can be an alternative to other preservatives. AITC also showed insecticidal activity against pests such as the book louse (*Liposcelis entmophilia*), lesser grain borer (*Rhizopertha dominica*), maize weevil (*Sitophilkus zaemais*), and *Tribolium ferrugineum*[130,120]. Research showed that AITC might inhibit different kinds of human prostate cancer, the induction of lung cancer, and the development of tumours in the liver and forestomach[126,131,132,133]. AITC is characterized by the presence of the N=C=S group, in which central carbon atom is highly electrophilic[134]. The antibacterial and insecticidal activity, and inhibition effects toward cancers can be attributed to this characteristic. The biological activities of AITC are mediated through the reaction of this carbon atom with nucleophilic reagents in the cells[131].

Horseradish root is now mostly used as a food condiment. Intact horseradish does not have pungency, but while cutting, grating it or water contact, a very strong pungent smell is released, which is mainly from allyl isothiocyanate and other isothiocyanante compounds[135]. To ensure a certain level of the pungent flavor in horseradish products, mustard oil (allyl isothiocyanate) is usually added to horseradish during processing. To optimize horseradish production and perform a quality control program, it is necessary to determine the amount of isothiocyanate compounds that contribute to the pungent flavor of horseradish, fresh and after the production. Little data has been reported for the quantification of pungent compounds in horseradish products. The only data reported was based on the relative amount and not the absolute concentrations present in the samples[135-136]. So, there is a need for a simple, sensitive, and reliable method for quantification of isothiocyanate compounds in horseradish. However, due to the complex

nature of the horseradish sample, the accurate quantification of pungent components is a difficult process. Previous analytical methods, such as thin-layer chromatography[137,138] and cyclocondensation assay[139,140], were mostly used for qualitative analysis, when more advanced methods were not available. Recently, methods like high-performance liquid chromatography (HPLC)[141,142,143,144] and gas chromatography (GC)[145,136,146] have been developed for analysis of isothiocyanate compounds. However, due to inefficient sample preparation steps, the limit of detection and sensitivity were poor using these methods. So, an efficient sample preparation is crucial for accurate analysis of isothiocyanate compounds in horseradish.

Solid-phase microextraction (SPME) has attracted a lot of attention due to its advantages over conventional extraction methods. Compared with other extraction methods like liquid-liquid extraction and Soxhlet extration, SPME combines sampling, extraction, and concentration into a single step, and thus avoids the loss of analytes during sample preparation[147]. It is a simple, low cost, and efficient extraction method that has been applied to both headspace and aqueous sample analysis with great sensitivity and selectivity. There is little data and information on the analysis of horseradish by SPME. The only data reported was by D'Auria and coworkers[148]. However, there is no detailed quantitation method information in the report.

The objectives of this study are to develop a method for the identification and quantification of allyl isothiocyanate and related compounds in horseradish root and prepared horseradish sauces. Allyl isothiocyanate and other related isothiocyanates were analyzed using the internal standard method with HS-SPME-GC-MS. This study will

help food processors to optimize production when they manufacture horseradish sauce products and gather important information for quality control.

3.3 Experimental

3.3.1 Horseradish Samples

Fresh horseradish root, prepared horseradish sample spiked with 0.13% mustard oil, and prepared horseradish sample were obtained from Spring Silver Foods (Eau Claire, WI, USA). All samples were kept refrigerated and analyzed within three months.

3.3.2 Chemicals and Reagents

All chemicals were of analytical reagent grade or better. Allyl isothiocyanate 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). Anhydrous sodium sulfate was purchased from Thermo Fisher Scientific (Fairlawn, NJ, USA). All solvents were of HPLC grade or better. Ethyl acetate and 1-propanol were purchased from Thermo Fisher Scientific (Fairlawn, NJ, USA).

3.3.3 Sample Preparation

3.3.3.1 Solvent Extraction

Ethyl acetate was used to perform solvent extraction. Fresh horseradish root sample was peeled and then crashed using a food processor. The horseradish root sample was then placed into a 50-mL centrifuge tube containing one milliliter of chilled distilled water and ethyl acetate solvent was added. The extraction was carried out in an incubator

at room temperature for different time periods. The extraction solvent was dried over anhydrous sodium sulfate and then filtered prior to GC/MS analysis. Prepared horseradish sample spiked with 0.13% mustard oil and prepared horseradish sample were extracted using the same procedures except without adding water.

3.3.3.2 Headspace Extraction

The headspace was generated from 0.8 grams sample (fresh horseradish root, prepared horseradish sample spiked with 0.13% mustard oil, and prepared horseradish sample) in a 4-mL glass vial with a polytetrafluoroethylene needle-pierceable septum screw cap. The vial was equilibrated for 20 min in a water bath at 40 °C prior to extraction. A gas-tight syringe was used to get the vapor from the vial and then introduced in the injection port of GC/MS.

3.3.3.3 SPME Extraction

0.8 grams of sample (fresh horseradish root, prepared horseradish sample spiked with 0.13% mustard oil, and prepared horseradish sample) were placed in a 4-mL glass vial with septum screw cap. The vial was put in a water bath and equilibrated for 20 min. A SPME fiber coated with 100 μm of polydimethylsiloxane (PDMS) (Supelco, Bellefonte, PA, USA) was used for extraction. Prior to use, the fiber was preconditioned at 250 °C for 0.5 h. The SPME fiber was exposed to the headspace of the sample for 20 min. The fiber then was introduced into the injection port of GC/MS.

3.3.4 Gas Chromatography Mass Spectrometry (GC/MS) Analyses

GC/MS analysis was performed on an Agilent 7890A gas chromatograph equipped with an Agilent 5975C mass selective detector. A DB-5 column (30-m × 0.25-mm ID × 0.25-μm film, Phenomenex, Torrance, Calif., U.S.A.) was used for the separation of the volatiles. The flow rate of the carrier gas H_2 was 1.0 mL/min. The oven temperature was programmed to hold at 35 °C for 2 min and then increased to 250 °C at 8 °C/min and held at 250 °C for 5 min. The injector temperature was maintained at 250 °C. A volume of 1.0 μL of sample from solvent extraction was injected in splitless mode. Sample extracted by headspace extraction and SPME was exposed to the injector inlet at 250 °C for 1 min under splitless or split mode. 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 70 eV.

3.3.5 Method Development

3.3.5.1 Optimization of Headspace-solid Phase Microextraction (HS-SPME) Conditions

Extraction and desorption conditions (e.g. extraction time, temperature, sample size, desorption time, and desorption temperature) were optimized to yield highest peak area ratio of allyl isothiocyante 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 4-mL vial for different times (5, 10, 20, 30, 40, and 60 min) at different temperatures (22, 30, and 40 °C). Sample size (0.1, 0.4, 0.8, 1.2, and 1.6 g) and SPME

fiber desorption time (0.5, 1, 2, and 4 min) and temperature (230 and 250 °C) were optimized.

3.3.5.2 Qualitative and Quantitative analyses

Identification of volatile compounds was achieved by comparing the mass spectra data with the National Institute Standards and Technology mass spectral library or published mass spectra data.

Quantification was obtained using extracted ion areas and a specific internal standard. A stock standard solution of allyl isothiocyanate of 5000 ppm was prepared in 1-propanol. The stock standard solution was further diluted with water to get concentrations of 50.0, 100.0, 200.0, 400.0, 800.0, 1600.0, and 3200.0 ppm. An internal standard solution containing 2000 ppm of propyl benzene was prepared in 1-propanol. An aliquot (0.1 g) of the internal standard solution was then added to 10 g of 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 gas chromatographic 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.

3.3.5.3 Method Validation

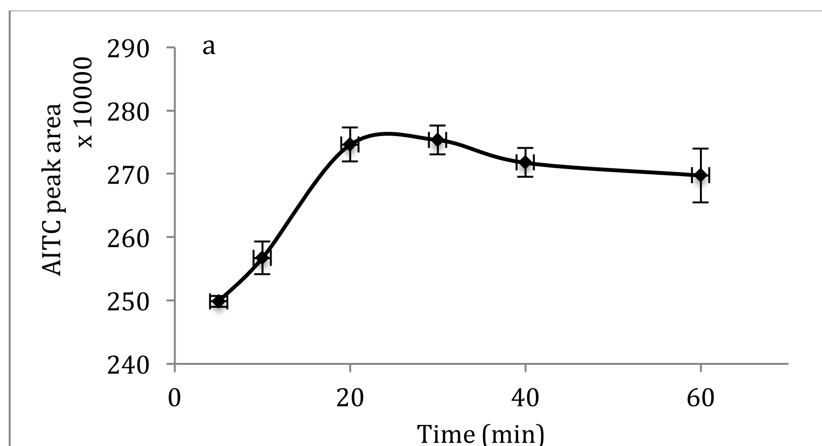
The method was validated over 3 days using two different concentrations of standard solution along the calibration curve. The method was evaluated for accuracy,

intraday precision, and interday precision. This was performed in triplicate for each concentration. Analyte concentrations were calculated from the calibration curve.

3.4 Results and Discussion

3.4.1 Optimization of Extraction Conditions

HS-SPME allows sampling, extraction, concentration, and sample introduction within one step. Adsorption of compounds of interest depends on the extraction conditions. Thus, optimization of extraction conditions is necessary. In this study, extraction conditions were studied and optimized in order to get good precision, high sensitivity, and better extraction efficiency. The extraction study was conducted using five extraction times (5, 10, 20, 30, 40, and 60 min), three extraction temperatures (22, 30, and 40 °C), and five sample sizes (0.1, 0.4, 0.8, 1.2, and 1.6 g). Desorption time (0.5, 1, 2, and 4 min) and desorption temperature (230 and 250 °C) were also evaluated. During the optimization, triplicate samples of 100 ppm of allyl isothiocyanate were analyzed under different extraction conditions and peak areas were optimized.



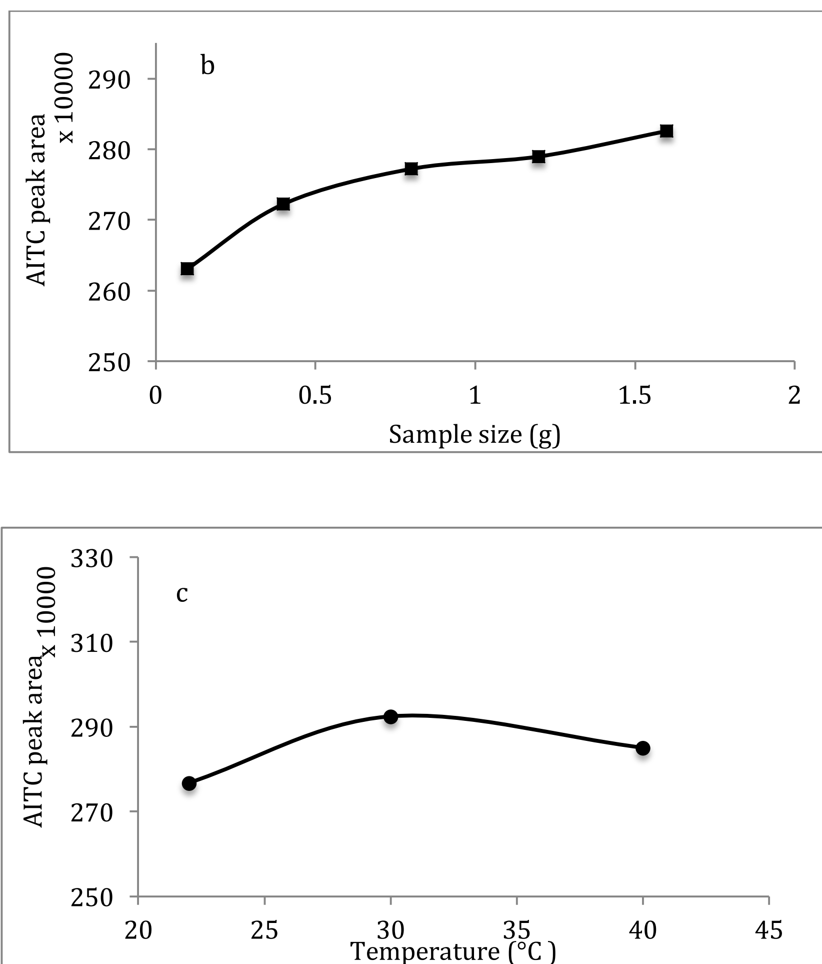


Figure 3.2 Optimization of SPME extraction conditions (a) time, (b) sample size, and (c) temperature. The extraction study was conducted using five extraction times (5, 10, 20, 30, 40, and 60 min), three extraction temperatures (22, 30, and 40 °C), and five sample sizes (0.1, 0.4, 0.8, 1.2, and 1.6 g).

The conditions optimized for SPME are shown in Fig.3.2. The highest peak area of allyl isothicyanate was achieved within 20 min without any major differences between 30, 40, and 60 min. The peak area of allyl isothiocyanate increases with a larger sample size. However, a sample size of 0.8 grams was selected for extraction instead of 1.0 gram due to the headspace volume limitation. Extraction temperature at 30 °C yielded highest amounts of allyl isothiocyanate when compared with extraction at 22 °C and 40 °C. At

lower temperature, the release of allyl isothiocyanate was insufficient. At higher temperature, the stability of allyl isothiocyanate was decreased. Desorption times evaluated including 0.5, 1, 2, and 4 min. Allyl isothiocyanate had a maximum peak area with a desorption time of 1 min. Desorption temperature was optimized using conditions of 230 and 250 °C. Possible desorption temperatures are limited by the capabilities of the instrument and SPME fiber. According to the analysis data, desorption temperature of 250 °C yielded higher peak area of allyl isothiocyanate.

Based on these results, the optimum analysis conditions for allyl isothiocyanate were: 20 min, 0.8 g sample size, 30 °C, 1 min desorption time, and 250 °C desorption temperature.

3.4.2 GC/MS Analysis

The gas chromatographic conditions of the GC were optimized. The chromatographic run time was 30 min. The total analysis time for each analytical run was 50 min because the extraction time was 20 min. The eluted compounds were identified by use of the NIST mass spectra library and literature mass spectra data. Fig 3.3 shows the chromatographic separation of prepared horseradish sample obtained by HS-SPME method. Based on the chromatogram and spectra obtained and literature data, seven isothiocyanate related compounds were identified, including allyl isothiocyanate, isobutyl isothiocyanate, 1-butene 4-isothiocyanate, benzene propanitrile, propyl isothiocyanate, benzyl isothocyanate, and phenylethyl isothiocyanate. These isothiocyanate compounds were also observed in fresh horseradish root sample and prepared horseradish sample spiked with 0.13% mustard oil.

Calculated as percent peak area of the gas chromatography analysis, allyl isothiocyanate (97.58%), phenylethyl isothiocyanate (1.65%), and others (<0.77%, combined) were found in the prepared horseradish sample. It showed that allyl isothiocyanate was the most abundant component in prepared horseradish sample, contributing as high as 98% of the total isothiocyanates found in this study. In addition, other components such as phenylethyl isothiocyanate, isobutyl isothiocyanate, propyl isothiocyanate, and benzyl isothiocyanate were also found in prepared horseradish sample.

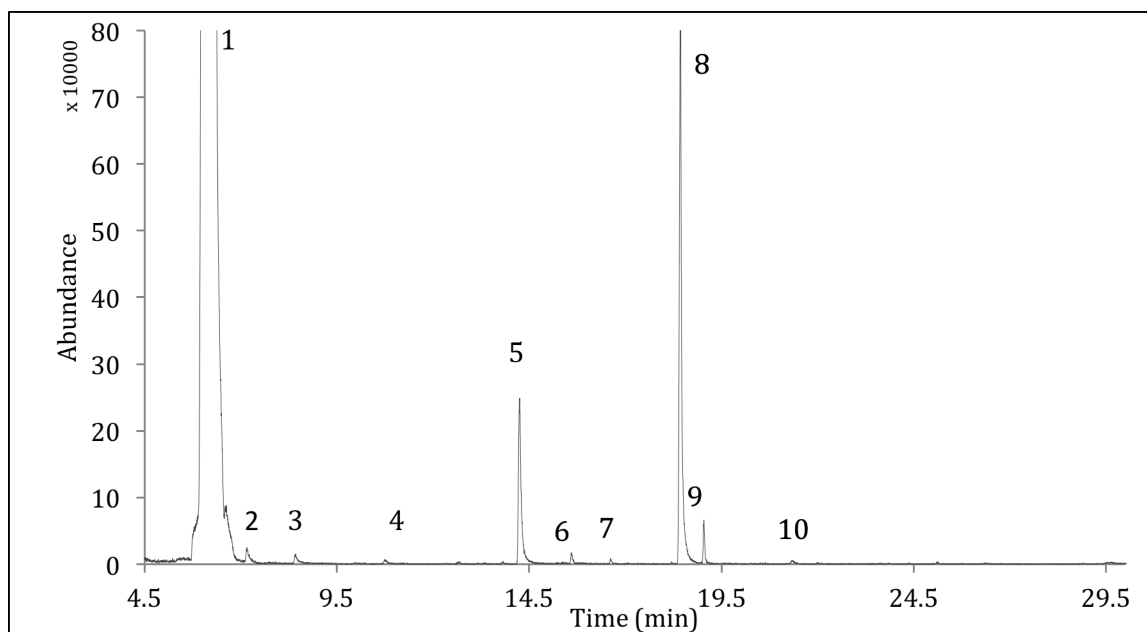


Figure 3.3 Gas chromatogram of the prepared horseradish sample obtained by HS-SPME method (peaks 1: allyl isothicyanate, 2: isobutyl isothiocyanate, 3: 1-butene 4-isothiocyanate, 4: not confirmed, 5: Benzene propanitrile, 6: propyl isothiocyanate, 7: benzyl isothocyanate, 8: phenylethyl isothiocyanate, 9: not confirmed, 10: not confirmed). 0.8 grams of the prepared horseradish sample were placed in a 4 ml glass vial with septum screw cap. A SPME fiber coated with 100 µm of polydimethylsiloxane was exposed to the headspace of the sample for 20 min. The fiber then was introduced into the injection port of GC/MS.

Table 3.1 Isothiocyanate compounds found in the prepared horseradish sample by HS-SPME

Peak no.	Compounds	R.T. (min)	Relative Amount (%)
1	Allyl isothicyanate	5.55	97.58%
2	Isobutyl isothiocyanate	7.16	< 0.05%
3	1-butene 4-isothiocyanate	8.43	< 0.04%
4	Not confirmed	10.75	< 0.02%
5	Benzene propanenitrile	14.26	0.56 %
6	Propyl isothiocyanate	15.60	< 0.03%
7	Benzyl isothocyanate	16.62	<0.01 %
8	Phenylethyl isothiocyanate	18.44	1.65%
9	Not confirmed	19.05	< 0.09%
10	Not confirmed	21.34	< 0.02%

Fig. 3.4 shows the chromatogram of the prepared horseradish sample extracted by ethyl acetate solvent followed by GC-MS analysis. The main compounds identified were allyl isothiocyanate, benzenepropanenitrile, and phenylethyl isothiocyanate. The level of allyl isothiocyanate observed from ethyl acetate solvent extraction was much smaller than using HS-SPME method, accounting for 37.5% of the size of the peak in HS-SPME.

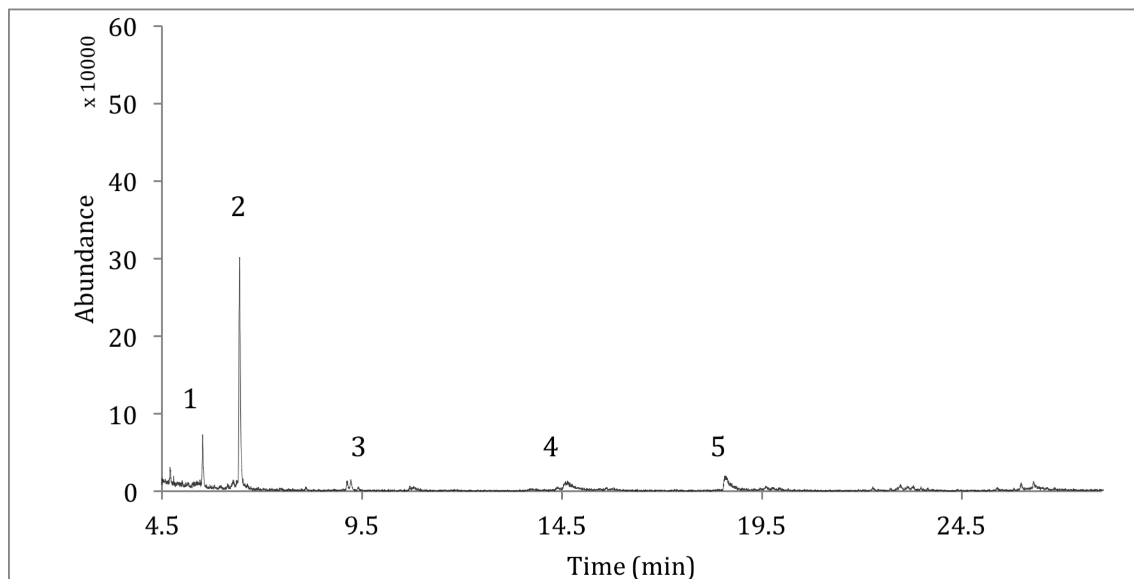


Figure 3.4 GC chromatogram of the prepared horseradish sample obtained by ethyl acetate extraction (peaks 1: not confirmed, 2: allyl isothicyanate, 3: not confirmed, 4: benzenepropanitrile, 5: phenylethyl isothiocyanate). The prepared horseradish sample was placed into a 50-mL centrifuge tube containing one milliliter of chilled distilled water and ethyl acetate solvent was added. The extraction was carried out in an incubator at room temperature. The extraction solvent was injected for GC/MS analysis.

Fig 3.5 represents chromatographic separation of a prepared horseradish sample obtained by headspace extraction followed by GC-MS analysis. The headspace extraction was performed at 40 °C. A gas-tight syringe was used for sampling to avoid undesirable loss of volatile compounds. Allyl isothiocyanate was the only compound observed in the chromatogram. Larger sample size and larger headspace samples were tried, did not alter the results. The recovery was poor when using headspace extraction.

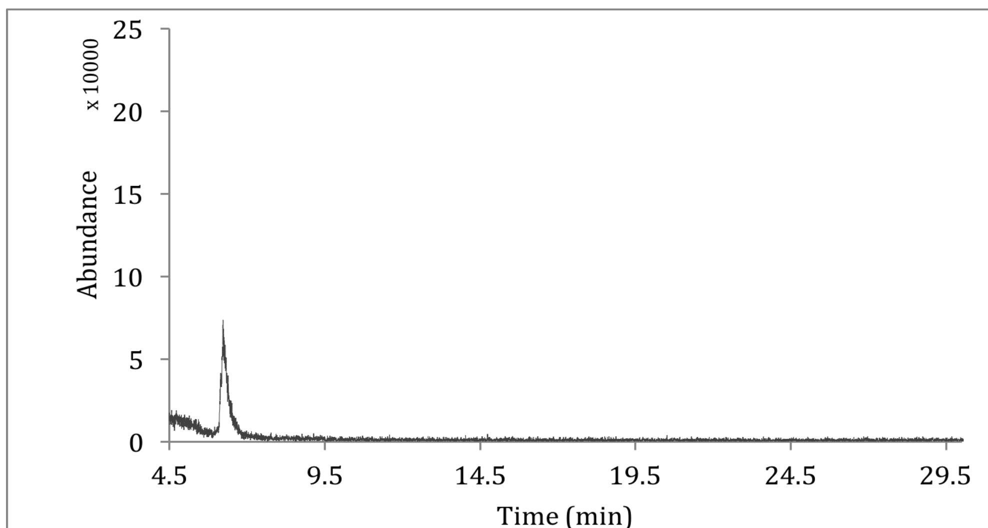


Figure 3.5 Gas chromatogram of the prepared horseradish sample by headspace extraction. The headspace was generated from 0.8 gram of the prepared horseradish sample in a 4-mL glass vial with a septum-lined screw cap. The vial was equilibrated for 20 min in a water bath at 40 °C prior to extraction. A gas-tight syringe was used to sample the vapor from the vial and then introduced in the injection port of the GC/MS.

As can be seen, the contents of volatile compounds varied significantly using different extraction methods. Based on the above results, HS-SPME method was the most sensitive and accurate extraction method. According to the literature data, HS-SPME method can provide excellent sensitivity for the qualitative determination of flavor components in natural plants. It is a very simple, quick technique and shows good reproducibility. Thus, the analysis of horseradish sample was carried out using HS-SPME-GC-MS method.

Based the results, among the isothiocyanate compounds observed in all three extracts, allyl isothiocyanate was the most abundant component. Furthermore, other compounds showed very low GC-MS signal responses and odor intensity, thus further

analysis of these compounds was not necessary. Allyl isothiocyanate was determined and subject to the following analysis.

3.4.3 Validation of the Method

The method was validated over 3 days. A seven-point calibration curve ranging from 50 to 3200 ppm was produced using the concentration of standard solution against the peak area ratios of standard solution to 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 3 times higher than the signal from adjacent noise.

A chromatogram of allyl isothiocyanate and internal standard is present as in Fig. 3.6. Peak area ratios for triplicate runs were averaged and relative standard deviations were calculated for the analyte. A wide linear range was achieved with a correlation coefficient (R^2) of 0.9992 (Fig. 3.7). The LOD was found to be 0.750 ppm, indicating its good sensitivity. The dynamic range studied was from 50 to 3200 ppm, which was suitable for the analysis of allyl isothiocyanate in horseradish samples.

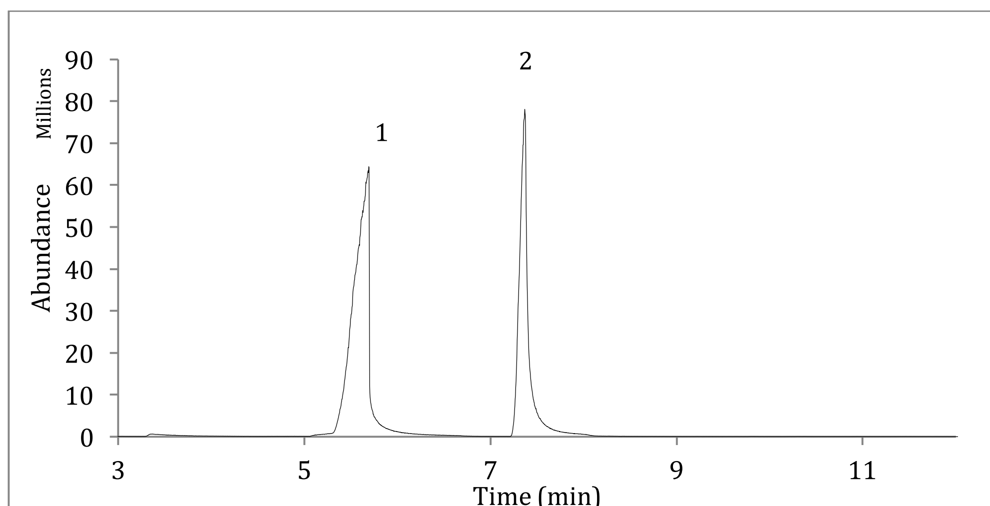


Figure 3.6 Gas chromatogram of allyl isothiocyanate (peak 1) and internal standard (peak 2) on Column: DB-5 column. 1.0 mL/min H2, oven temperature 35 °C for 2 min), then 8 °C/min to 250 °C, and held for 5 min, 250 °C splitless injector, and SPME sampling at 250 °C for 1 min, splitless mode.

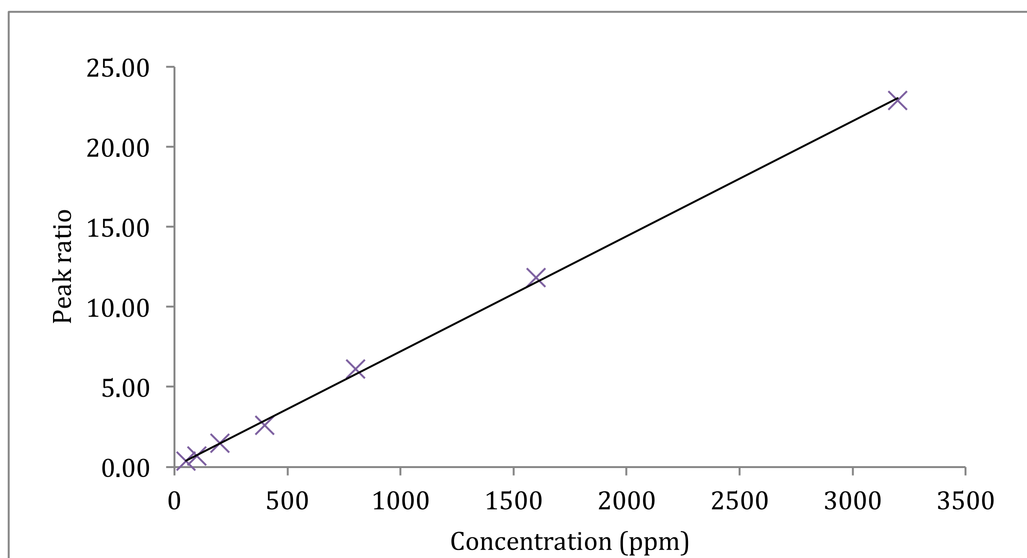


Figure 3.7 Calibration curve for allyl isothiocyanate. A seven-point calibration curve ranging from 50 to 3200 ppm was produced using the concentration of standard solution against the peak area ratios of standard solution to internal standard ($y = 0.0072x + 0.0178$, $R^2 = 0.9992$).

The method was validated for intra- and interday accuracy and precision over 3 days using two different concentrations along the calibration curve. Validation was tested

at 150.0 and 2000.0 ppm. The accuracy was calculated by the concentration determined from the calibration curve against the concentration added to the sample. The precision was expressed as relative standard deviation. Table 3.2 shows the data for accuracy and precision at each validation level. All intra- and interday precisions gave satisfactory results, which were less than 10%. The accuracies were ranged between 80%-120%. Based on the above results, it suggests that the method proposed is simple, accurate, and sensitive.

Table 3.2 The accuracy and precision of allyl isothiocyanate analysis by SPME-GC-MS

Concentration (ppm)	Intraday accuracy	Interday accuracy	Intraday precision	Interday precision
150.00	92.7-102.1	91.2-113.2	< 4.0	< 5.1
2000.00	89.1-94.6	88.4-95.6	< 3.6	< 9.9

3.4.4 Quantification in Horseradish Sample

The validated method was then applied to analyze allyl isothiocyanate in prepared horseradish samples. Table 3.4 presents the allyl isothiocyanate amounts determined in prepared horseradish sample spiked with 0.13% mustard oil, prepared horseradish, and horseradish root. It was found that the amounts of allyl isothiocyanate in prepared horseradish sample spiked with 0.13% mustard oil and horseradish root sample were higher than in prepared horseradish. 2514 ppm and 862 ppm, equivalent to 3.143 mg/g and 1.078 mg/g allyl isothiocyanate were found in prepared horseradish spiked with 0.13% mustard oil and horseradish root, while 110.6 ppm or 0.0383 mg/g of allyl isothiocyanate

was detected in the prepared horseradish sample. It was also shown that allyl isothiocyanate had the most intense peak areas in the gas chromatograms of all three samples. Since no noticeable odor was observed with other isothiocyanate compounds (phenylethyl isothiocyanate), it is suggested that the pungency of horseradish is mostly from allyl isothiocyanate. Based on these results, it is revealed that production of horseradish sauce products may be more efficient immediately after harvesting. Besides, the intensity of pungent flavor in horseradish samples can be enhanced by adding flavor agents, such as mustard oil.

Table 3.3 Analyses of horseradish samples

Sample	AITC level (ppm)	AITC level (mg/g)	RSD %
Prepared horseradish spiked with 0.13% mustard oil	2514 ± 53	3.143 ± 0.066	2.1
Prepared horseradish	110.6 ± 3.1	0.1383 ± 0.0039	2.8
Horseradish root	862 ± 13	1.078 ± 0.016	1.5

3.5 Conclusions

This work has demonstrated the complete optimization, development, and validation of a simple, robust, and effective method for determination of volatile compounds in horseradish. This method utilizes the fast-growing technology of SPME for sample preparation. The sample preparation is easy, fast, and environmentally friendly. Acceptable precision and accuracy were obtained. The method presented can be used to assess volatile components changes during the storage of food flavor products.

Method from this study will allow producers and processors to evaluate quality of flavored products before and after production. This method should also be applicable for determining potential links between the chromatographic profile and sensory profile examined by a panel of tasters.

3.6 Acknowledgements

The authors gratefully acknowledge the financial support from Spring Silver Food Company.

CHAPTER 4. SENSORY ANALYSIS OF THE PUNGENCY OF HORSERADISH PRODUCTS

4.1 Abstract

Horseradish (*Armoracia rusticana*) is mostly used as a condiment in food due to its characteristic strong pungent smell and taste. Allyl isothiocyanate is responsible for the pungency of horseradish. In this study, a sensory pungency analysis of fourteen commercial horseradish products from eight manufacturers was carried out by a trained panel. The correlation between the amount of allyl isothiocyanate and the perceived pungency of horseradish products was investigated. The level of allyl isothiocyanate in horseradish sample was quantified by a HS-SPME-GC-MS method. It was demonstrated that the sensory pungency ratings of the allyl isothiocyanate solutions were well correlated ($R^2 = 0.975$) with the concentrations of allyl isothiocyanate within the range of 0-3200 ppm. The results showed there were significant differences in the pungency ratings among the fourteen samples of horseradish products. Panelists exhibited no significant difference in overall preferences among the 14 samples, with the average overall preference ratings ranging from 4.3-5.4. In general, good correlations were obtained between the sensory data and the analytical data, suggesting positively correlated relationship between the amount of AITC and the perceived pungency in horseradish products. Some differences in terms of expectation, acceptability, and interpretation of sensory characteristics of horseradish might be present among the panelists. It will gain more insight if the influence of food components and masking effects are better understood.

4.2 Introduction

Horseradish (*Armoracia rusticana*) is a perennial plant that belongs to the Brassicaceae family, which includes cabbage, broccoli, and mustard. Due to its characteristic strong pungent smell and taste, horseradish is mostly used as a vegetable and a condiment in food. The pungent smell and taste is mainly attributed to allyl isothiocyanate (AITC). According to studies, AITC has shown anticarcinogenic[134], antibacterial[128], and insecticidal activities[130]. Research has showed that AITC can inhibit human prostate cancer, the induction of lung cancer, and the development of tumors in the liver and fore stomach[131,132,133]. Due to its health benefits and characteristic pungent smell, horseradish products have a great market opportunity. For consumers, pungency is an important index and often decisive in the purchase. Therefore, it is necessary to investigate and determine the amount of AITC that contributes to the perceived pungent flavor in horseradish during production and storage.

Sensory analysis, defined as "a scientific discipline used to evoke, measure, analyze and interpret reactions to those characteristics of foods and materials as they are perceived by the senses of sight, smell, taste, touch and hearing"[149] has been an essential and fast-growing method for assessment of flavors and smells in the food industry. Sensory analysis can be used in a number of applications, such as research and development for long-term studies, new product development, quality control, shelf-life evaluation, process change investigation, study of sensory changes over time, and competitor benchmarking[150,151,152,153]. This technique uses the human senses to evaluate a product. A group of trained assessors usually carries it out. This approach, based on the

collection of the sensations of a large numbers of persons, requires a rigorous environment and a suitable statistical analysis tool. Sensory analysis can be used to evaluate the sensory characteristics of the products and to develop products that have best delivery the consumer wants[154,155]. It can provide a wider understanding of the perception mechanisms of sensory stimuli and the acceptability of the products.

The sensory analysis of a product can be made through discrimination, descriptive, and affective tests[149]. Discrimination tests are used to determine if there is any sensory difference between samples. Triangle test, duo-tri test, and paired comparison test are the most three common ways for discrimination tests[149]. Descriptive tests involve detection and description of both qualitative and quantitative sensory attributes[156]. Descriptive tests are usually used to evaluate the nature and intensity of the differences of sensory components of a product. There are several different methods of descriptive tests, such as Flavor Profile, Texture Profile Analysis, and Quantitative Descriptive Analysis (QDA)[156]. Descriptive tests may be used to study the sensory properties of a new product, to track product changes over time, to define the characteristics for quality control purpose, to investigate the effects of ingredients or processing variables on the final sensory quality of a product, and to understand consumer sensory perceptions of products[157]. Descriptive tests can establish relationships between descriptive sensory and instrumental or consumer preference measurements[156]. Affective tests are commonly used to determine preference and/or acceptance of products. This test is based on a measurement of preference or a measure from which relative preference may be determined such as pleasure-displeasure, like-dislike[158]. There are three ways of affective tests, paired

preference, ranking, and rating. Generally, a large number of panelists are required to represent target or potential target populations.

There is no data and information on the sensory characteristics of horseradish. However, studies on sensory analyses of volatile components in other different food samples have been reported. Valli et al. evaluated the quality and consumer acceptance of extra virgin olive oils by sensory analysis, they found that different expectation and interpretation of sensory characteristics of extra virgin olive oils were mainly due to the unfamiliarity with positive sensorial attributes, such as bitterness and pungency[159]. Hatzidimitriou et al. carried out sensory analyses of balsamic vinegars and discussed the difficulties during the development for sensory evaluation of balsamic vinegars[160]. van Ruth and coworkers performed gas chromatography/sniff-port analysis and sensory evaluation of commercially dried bell peppers (Capsicum annuum) after rehydration by descriptive and hedonic panels[161]. The relationship between pyruvate analysis and flavor perception for onion pungency was examined by Wall and Corgan, they pointed out that pyruvate analysis could be used as a reliable selection technique for pungency in onion breeding programs[162]. Andreu-Sevilla and coworkers carried out a study on the determination of volatile compositions in pomegranate juice and wine using HS-SPME/GC-MS and sensory analysis[163]. Benn and Peppard reported methods for chemical characterization of tequila flavor using gas chromatography and sensory analysis, five constituents (isovaleraldehyde, isoamyl alcohol, β-damascenone, 2-phenylethanol, and vanillin) were determined to be the most powerful odorants of tequila[164]. However, Sensitization and desensitization to allyl Isothiocyanate in the Nasal Cavity has been investigated by Brand and Jacquot[165].

Horseradish root is usually manufactured as horseradish sauce and consumed with food as a condiment. Knowing consumers' preferences, expectations, and perceptions of the sensory characteristics of horseradish products is very important to horseradish manufacturers. Comparing perceived pungency with instrumentally determined allyl isothiocyanate levels could be beneficial to the quality management of pungent food products.

The objectives of this study were to determine the sensory characteristics of fourteen commercial horseradish products and their levels of allyl isothiocyanate. Another objective of this study was to evaluate the correlation between the level of AITC and the perceived pungency in these horseradish products. Sensory analysis of fourteen horseradish products was performed by a trained panel to determine pungency and overall preference. A method based on HS-SPME/GC-MS was used to determine the amount of AITC in horseradish products. The sensory data was analyzed statistically by one-way analysis of variance (ANOVA) and compared to the instrumental results. To our best knowledge, this is the first sensory evaluation of the pungency from horseradish and correlation between perceived pungency and AITC levels.

4.3 Experimental

4.3.1 Materials and Chemicals

All chemicals used for identification and quantification purpose were of analytical reagent grade of or better. Allyl isothiocyanate standard was purchased from Sigma-Aldrich (Milwaukee, WI, USA). Propyl benzene was used as an internal standard and obtained from Arcos Organics (Morris Plains, NJ, USA).

4.3.2 Horseradish Samples

Horseradish (*Armoracia rusticana*) samples were purchased from local stores (Brookings, SD, USA). Fourteen samples of horseradish products from different companies were studied. The samples are described in Table 4.1 All samples were kept refrigerated and analyzed within three months.

Table 4.1 Description of horseradish products used in this study

Sample ID	Sample Description	Manufacturer	Best By Date Given	Moisture Content (%)	RSD[b] (%)
A	Horseradish mayonaise	1	3/11/2016	21.6±1.2	5.75
B	Cream style horseradish	2	6/24/2015	84.45±0.48	0.57
C	Horseradish sauce	3	05/24/2015	46.1±2.3	4.99
D	Horseradish sauce	4	8/11/2015	51.60±0.85	1.65
E	Horseradish sauce	2	1/13/2016	60.60±0.75	1.23
F	Horseradish sauce	5	1/28/2016	29.16±0.98	3.37
G	Horseradish mustard	6	5/3/2016	78.6±1.3	1.64
H	Fresh ground horseradish	1	11/04/2015	85.02±1.3	1.51
I	Fresh ground horseradish	2	9/15/2015	85.9±1.1	1.25
J[a]	Prepared horseradish	2	8/28/2015	86.5±1.1	1.24
K[a]	Prepared horseradish	2	9/21/2015	87.1±1.7	1.95
L	Horseradish mustard	7	4/18/2015	79.0±2.7	3.39
M	Wasabi sauce	2	2/12/2016	61.38±0.81	1.33
N	Prepared grated horseradish roots	8	10/18/2015	78.1±1.4	1.79

[a] Sample J and K are two different bottles of the same sample manufactured on different dates.
[b] RSD represents relative standard deviation and was calculated by dividing the standard deviation by the mean value.

4.3.3 Moisture Content of Horseradish Samples

Moisture content of the horseradish samples was determined by oven-drying five grams of each horseradish sample. The sample was weighed accurately and subsequently dried in an oven at 70 °C until constant weight. Then the sample was weighed again and the moisture content was determined. Moisture content was carried out in triplicate. A mean value is reported as the moisture content of the horseradish sample in Table 4.1.

4.3.4 Sensory Analysis

4.3.4.1 Panelists

For the sensory evaluation, 21 panelists, 9 female and 12 male, were selected from volunteers from the Department of Chemistry and Biochemistry and the Department of Pharmaceutical Science at South Dakota State University (SDSU) for the sensory study. The sensory analysis study was exempted by SDSU Office of Research for evaluation by the Human Subject Committee.

4.3.4.2 Panel training

Two training sessions were conducted to enhance the ability of each volunteer to recognize and quantify the pungency. During the first training session, two sets of three mustard oil (allyl isothiocyanate) samples were prepared in water. In each set, two samples were prepared at the same concentration level (2500 ppm or 100 ppm) and the

third sample was at different concentration level (100 ppm or 2500 ppm). Only those who can distinguish by smell among these three samples in each set were chosen to participate the next training session.

In the second training session, the panelists were trained to rate the intensity of pungency on a continuous scale from 0 to 10. The sensory method developed by Gillette was modified and used in this study[166]. Level 0 (0 ppm (i.e., distilled water), no pungency), 1 (200 ppm, threshold pungency), 2 (800 ppm, slight pungency), 5 (1600 ppm, moderate pungency), 8 (3200 ppm, strong pungency), and 10 (5000 ppm, very strong pungency) were used to indicate perceived intensities of pungency (Table 4.2).

Table 4.2 Definition of pungency used in this study

Rating	AITC Concentration (ppm)	Pungency
0	0	No pungency
1	200	Threshold pungency
2	800	Slight pungency
5	1600	Moderate pungency
8	3200	Strong pungency
10	5000	Very strong pungency

4.3.4.3 Sensory Evaluation

Sensory analysis was used to evaluate the pungency in horseradish products. 14 commercial horseradish products were evaluated by the trained panel. The trained panel evaluated the commercial horseradish products.

Sensory evaluation of pungency in horseradish products was carried out following specific and standardized procedures. Samples, 0.8 grams of each horseradish product (as received, uncorrected for moisture content) were placed in a 4-mL amber glass vial with cap. The sample was then evaluated and rated by each panelist in individual bench. Each sample was randomly evaluated three times by the panelists. Coffee beans were provided to to cleanse for olfactory fatigue between samples. Panelists were asked to rate the intensity of the pungency of each horseradish product and the overall preference of each horseradish product based on 10-point continuous scales (note: these two ranking scales are not directly related). The ranking scale for pungency and overall preference are as presented in Table 4.2 and Table 4.3. All the results were analyzed statistically.

Table 4.3 Preference scale used in this study

Rate	Overall Preference
0	Extremely dislike
2	Dislike
4	Slightly liked
6	Moderately Liked
8	Like
10	Extremely like

4.3.4.4 Accuracy of the Sensory Analysis Method

To test the accuracy of the sensory evaluation method, a series of six AITC solutions of known concentration were prepared. Standard stock solution of AITC of 5000 ppm was prepared in 1-propanol. The stock standard solution was further diluted

with water to get a final concentration of 0, 200, 800, 2000, 3200, and 5000 ppm. Each AITC standard solution (0.8 grams) was provided in a 4-mL amber glass vial with cap. The standard sample was then tested and rated by each panelist. Each standard sample was evaluated three times by each panelist in randomized order.

4.3.5 Instrumental Analysis of Horseradish Samples

All 14 horseradish samples were extracted by SPME and then analyzed by GC/MS using the method presented in Chapter 3.

Samples (0.8 grams) were placed in a 4-mL glass vial with septum screw cap. The vial was kept in a water bath and equilibrated at 30 °C for 20 min. A SPME fiber coated with 100 μm of polydimethylsiloxane (Supelco, Bellefonte, PA, USA) was used for extraction. Prior to use, the fiber was preconditioned at 250 °C in the injection port of a GC for 0.5 h. The SPME fiber was exposed to the sample headspace. After sampling, the fiber was then introduced into the injection port of GC/MS. All samples were analyzed in triplicate.

GC/MS analysis was performed on an Agilent 7890A gas chromatograph equipped with an Agilent 5975C mass selective detector (Santa Clara, CA, USA). A DB-5 column (30 m × 0.25- mm ID × 0.25-μm film, Phenomenex, Torrance, Calif., U.S.A.) was used for the separation of the compounds. The flow rate of the H_2 carrier gas was 1.0 mL/min. The oven temperature was programmed to hold at 35 °C for 3 min and then increased to 250 °C at a rate of 8 °C/min and held at 250 °C for 5 min. The injector temperature was maintained at 250 °C. Sample extracted by SPME was exposed to the

injector inlet at 250 °C for 1 min under splitless or split mode (ratio = 1: 10). Mass spectra were obtained by electron impact ionization. The temperature of the ion source was set at 230 °C.

4.3.6 Statistical Analysis

Data from horseradish sensory analyses were statistically examined by one-way analysis of variance (ANOVA) using SPSS software (IBM, Armonk, NY, USA). The mean values were compared with significance defined at $p < 0.05$ using Duncan's multiple range test.

Correlations between samples were determined using Pearson's correlation coefficient. Significance of the correlation followed standard guidelines used in psychology studies as presented in Table 4.4.

Table 4.4 Significance of the correlation guidelines.

r value	Significance of Correlation
0.001-0.199	No or negligible correlation
0.200-0.299	Weak correlation
0.300-0.399	Moderate correlation
0.400-0.699	Strong correlation
0.700-0.999	Very strong correlation

4.4 Results

4.4.1 Moisture Content of Horseradish Sample

The moisture content of horseradish sample was determined by oven-drying. Results are presented in Table 4.1 It was found that the moisture content in all 14 horseradish samples was in the range of 26.61 and 87.1%, with relative standard deviations ranging from 0.57% to 5.75%. Horseradish mayonaise sandwich spread (sample A) has the lowest moisture content (21.6±1.2%), while prepared horseradish samples J and K have the highest moisture contents (87.1 and 86.5%, respectively). The horseradish sauce samples (C, D, and E) and wasabi sauce (sample M) have similar moisture content levels around 51.6-61.4%.

4.4.2 Test of the Sensory Method

In order to test the sensory analysis method, the sensory panel rated six AITC standard solutions. The panelists evaluated each standard sample three times in randomized order. There is a very strong positive correlation between the panelist pungency ratings and the AITC concentration (R^2 = 0.975) presented in Figure 4.1 and Table 4.5 up to an AITC concentration of 3200 ppm. The Pearson correlation coefficient value of 0.993 (r = 0.993) confirms there is also a very strong positive correlation between the panelist rankings and the pungency scale with some bias toward higher panelist ratings, as presented in Table 4.4.

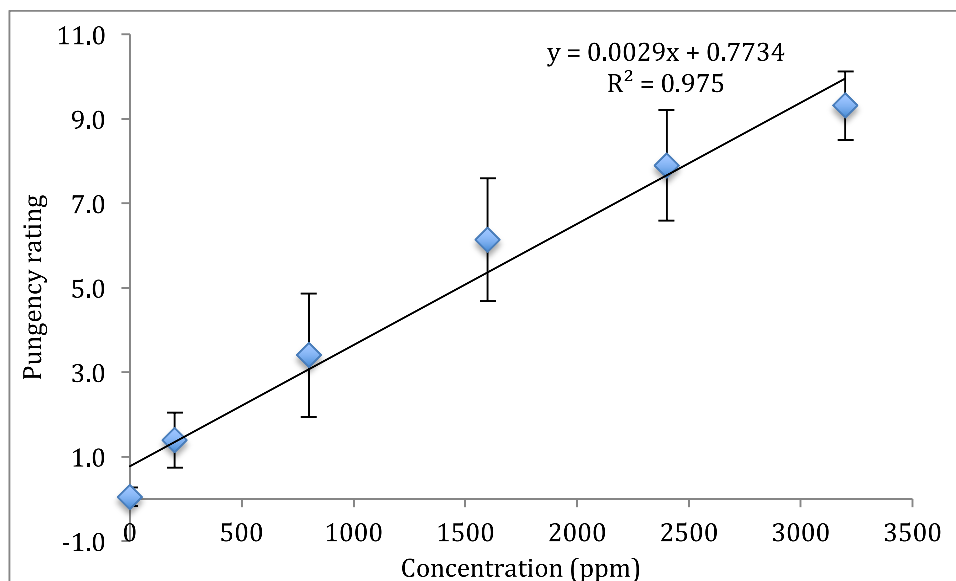


Figure 4.1 Pungency ratings as a function of concentration of allyl isothiocyanate. Allyl isothiocyanate solutions of known concentration were prepared in deionized water and 0.8 gram of each allyl isothiocyanate standard solution in a 4-mL amber glass vial was rated by each panelist based on the scale of 0-10 presented in Table 4.1.

Table 4.5 Results of sensory ratings of allyl isothiocyanate standard solutions

Concentration of AITC	Pungency Scale	Average Panelist Rating
0	0	0.0±0.2
200	1	1.4±0.6
800	2	3.4±1.5
1600	5	6.1±1.5
2400		7.9±1.3
3200	8	9.3±0.8

4.4.3 Sensory Evaluation of Horseradish Samples

The trained panel then evaluated the horseradish products. To investigate the reproducibility of the sensory evaluation and obtain accurate results, the sensory

evaluation of the horseradish samples was repeated on two consecutive days. Pungency evaluation results are presented in Table 4.6 and Figure 4.2. According to these pungency rating scores, there is a very strong positive correlation between the pungency scores for each day ($r = 0.958$) across all samples. It was suggested that the panelists were reacting in a similar manner to each sample. The sensory pungency data were analyzed using Duncan's multiple range test at $p<0.05$. For Day 1 test, the pungency ratings of sample H, I, J, K, N, and B did not differ significantly. There were no significant differences between sample A and F, sample C and D, and sample G and L. For Day 2 test, sample H, I, J, K, and B did not show significantly difference in pungency. Statistically, sample M and N, sample A and G, and sample D and F were rated as the same pungent levels. Thus, looking at individual sample types, there were very strong positive correlations for the day to day evaluation of horseradish sauce (samples C, D, E, and F; $r = 0.994$) and fresh ground horseradish (samples H, I, J, and K; $r = 0.883$). The differences in the ratings between samples could be due to horseradish cultivar, growth environment, production and storage conditions, or the stability of the pungent components (AITC). Of the different sample types, horseradish sauce tended to have the mildest pungency ratings (sample F had the lowest average score, 1.9, of all samples) and fresh ground horseradish had the strongest perceived pungency (sample J had the highest average score, 7.0, of all samples). Other sample types were intermediate between these, presumably based on horseradish content.

Table 4.6 Pungency ratings of the fourteen commercial horseradish products on a scale 0-10.

Sample ID	Day 1	Day 2	Average rating
	Pungency rating (n= 21)	Pungency rating (n=15)	
A	2.2 f*	2.5 def*	2.4
B	6.6 a	5.9 ab	6.2
C	2.8 ef	1.9 ef	2.4
D	2.7 ef	1.7 f	2.2
E	4.1 cde	3.9 cd	4.0
F	2.3 f	1.4 f	1.9
G	3.8 def	2.3 f	3.0
H	6.3 ab	6.9 a	6.6
I	5.6 abc	5.7 ab	5.6
J	7.1 a	7.0 a	7.0
K	6.0 ab	6.0 ab	6.0
L	3.9 def	3.5 cde	3.7
M	4.8 bcd	5.0 bc	4.9
N	5.8 ab	4.9 bc	5.3

* Values followed by the same letter, within the same column, were not significantly different according to Duncan's multiple range test at $p<0.05$.

To compare the pungency evaluation of all of the 14 horseradish samples, the data are displayed in Figure 4.2. The spread of the data is explained by the differences in

pungency perception by the panelists, differences in sample variability, and possible masking effects of sample components such as water, fat, and sugar.

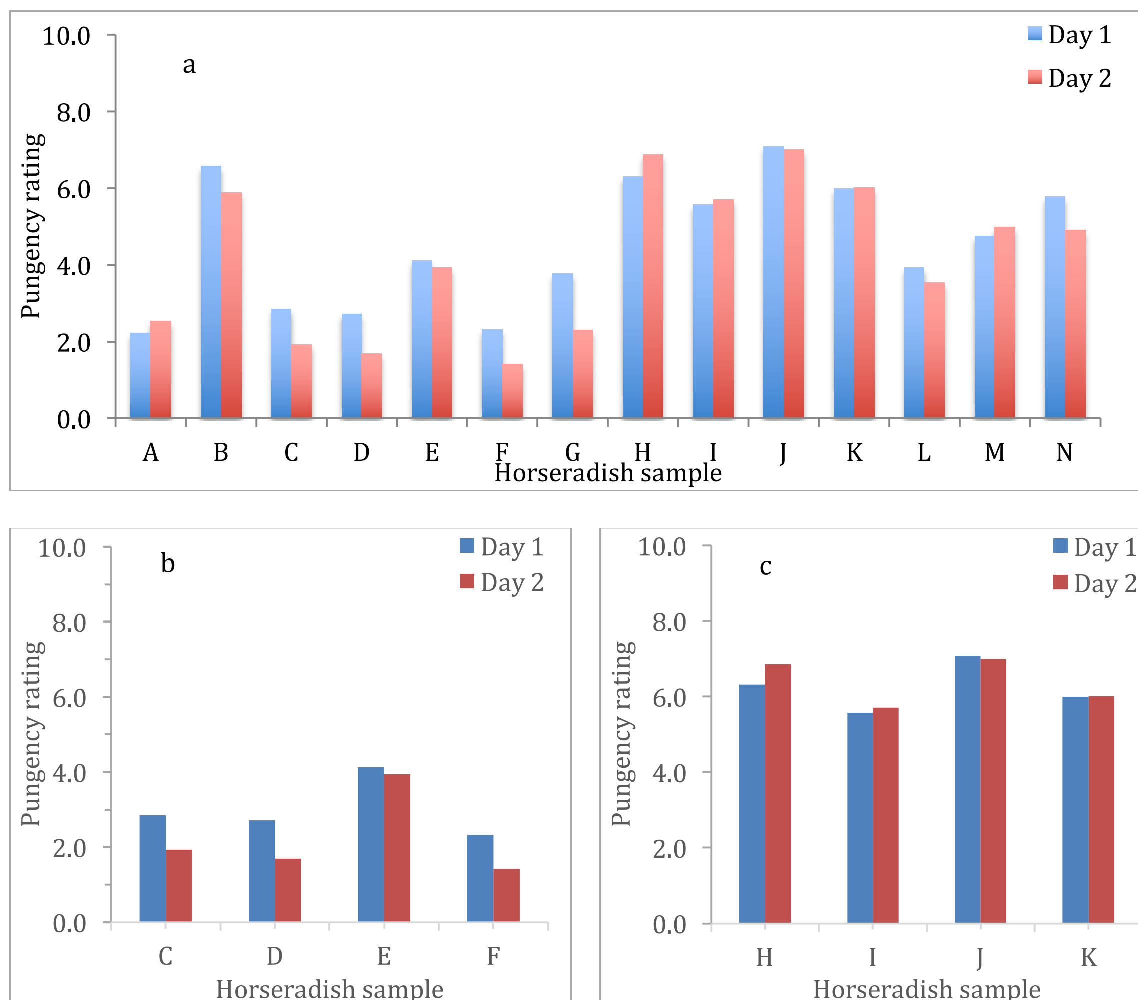


Figure 4.2 Sensory pungency evaluation of the horseradish samples (a)14 commercial horseradish samples, (b) horseradish sauce samples, and (c) fresh ground horseradish samples (Scale: 0-10, 0 = no pungency, 10 = extremely pungent).

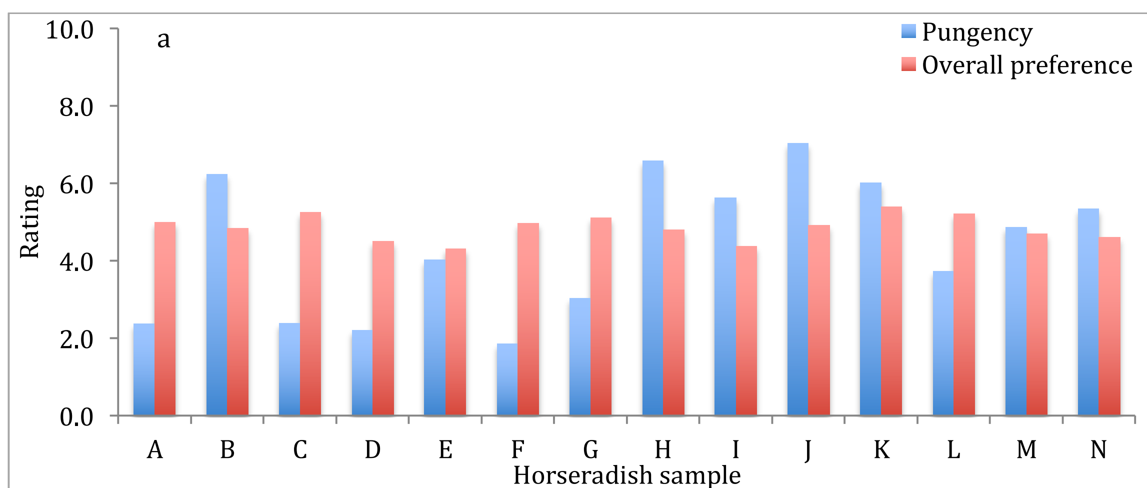
Besides pungency, the panelists expressed their overall preference of the 14 samples of horseradish products. Table 4.7 presents the overall preference ratings. All the ratings are in the range of 4.0 and 5.7 on the 0-10 scale. There was negligible correlation between days (r = 0.102). That is, for some samples, the Day 1 score was higher, while for others, the Day 2 score was higher. However, within a product type, a

strong positive correlation on the day to day preference was observed (r = 0.291 for horseradish sauce and r = 0.422 for fresh ground horseradish). Sample K is the most well-liked sample and sample E the least liked, with the average ratings of 5.4 and 4.3, respectively. In general, panelists showed overall preference among the 14 horseradish product samples, with the average ratings ranging from 4.3-5.4. The panelists slightly or moderately liked all samples.

Table 4.7 Overall preference data for the fourteen horseradish samples

Sample ID	Overall Preference Day 1 (n=21)	Overall Preference Day 2 (n=15)	Average rating
A	5.3	4.7	5.0
B	4.8	4.8	4.8
C	4.9	5.6	5.2
D	4.3	4.7	4.5
E	4.3	4.3	4.3
F	5.4	4.6	5.0
G	5.5	4.7	5.1
H	4.8	4.8	4.8
I	4.8	4	4.4
J	4.9	4.9	4.9
K	5.0	5.7	5.4
L	5.4	5.0	5.2
M	5	4.4	4.7
N	4.2	5	4.6

To explore the correlation between overall preference and pungency, Figure 4.3 presents the average ratings of pungency and overall preference from Days 2. Pungency had no relevant impact on the panelists' overall preference when all samples are considered in total (r = -0.134). However, for fresh ground horseradish (samples H, I, J, and K), there is a weak positive correlation (r = 0.221) between pungency and preference. This means that, to a small degree, panelists prefer samples with greater pungency. This is in contrast to horseradish sauce (samples C, D, E, and F), which exhibited a strong negative correlation (r = -0.671), meaning there is an inverse relationship between pungency and panelist preference. This might be due to matrix effects from other ingredients in horseradish sauce samples such as fat and sugar.



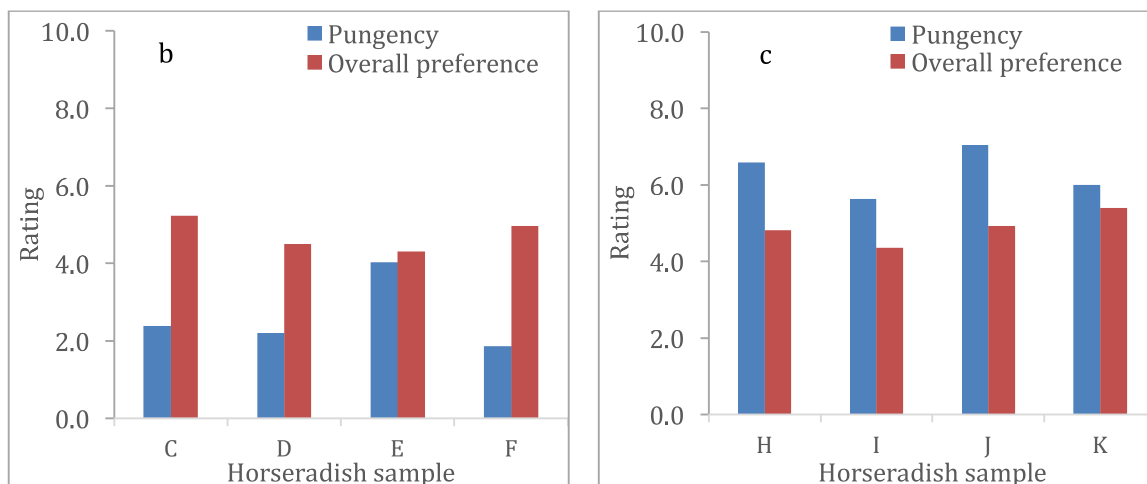


Figure 4.3 Comparison of pungency and overall preference ratings from (a) the 14 horseradish products, (b) horseradish sauce samples, and (c) fresh ground horseradish samples (Pungency scale: 0-10, 0 = no pungency, 10 = extremely pungent; overall preference scale: 0-10, 0 = extremely dislike, 10 = extremely like. These two ranking scales are not directly related.).

4.4.4 Analysis of Commercial Horseradish Products

The 14 samples of horseradish products were analyzed by GC-MS using the developed method as described in Chapter 3. A calibration curve was obtained by plotting the peak area ratio of AITC to the internal standard propyl benzene against the AITC concentration. The AITC in the different horseradish samples were quantified from the calibration curve based on these peak area ratios. Table 4.8 illustrates the results. The results revealed a high variation in the AITC level in the analyzed samples. The AITC level in sample L was below the limit of quantification, so there was no instrumental data for sample L. The AITC level in the samples ranged from 1134±17 to 5900±590 ppm in an as received basis, which is equivalent to 1.417±0.021 to 7.37±0.74 mg/g. Samples A and I were detected to have the highest and the lowest AITC level, 7.37±0.74 and 1.417±0.021 mg/g, respectively. When taking into account the moisture content of each horseradish sample, the sample K had the highest level of AITC, with a mean value of

28.4±1.3 mg/g and a relative standard deviation of 4.73%. Sample F had the lowest AITC level among all samples, 4.82±0.42 mg/g. Sample J and K were determined to have comparable AITC levels, as it was expected (Sample J and K are two different bottles of the same sample manufactured on different dates). Any real difference in the amount of AITC of sample J and K could be due to horseradish cultivar, grow environment, production and storage conditions.

Table 4.8 AITC concentrations determined in the commercial horseradish samples

Sample ID	AITC level (as received) (ppm)	AITC level (as received) (mg/g)	AITC level (dry basis) (mg/g)	RSD (%)
A	5900±590	7.37±0.74	9.41±0.94	9.98
B	2000±60	2.500±0.075	16.08±0.48	2.99
C	2680±14	3.350±0.017	6.216±0.032	0.52
D	2516±63	3.145±0.078	6.50±0.16	2.49
E	1596±42	1.995±0.052	5.06±0.13	0.77
F	2730±240	3.41±0.30	4.82±0.42	8.82
G	1136±18	1.420±0.022	6.64±0.10	1.55
H	2155±62	2.694±0.078	17.99±0.52	2.90
I	1134±17	1.417±0.021	10.04±0.15	1.50
J	2960±110	3.70±0.14	27.3±1.0	3.79
K	2940±140	3.67±0.17	28.4±1.3	4.73
L	n.a	n.a	n.a	n.a
M	5270±110	6.59±0.14	17.07±0.35	2.05

N	2910±170	3.63±0.22	16.58±0.99	5.96

Figure 4.4 and 4.5 chart the AITC levels and perceived pungency. Samples of horseradish products with nearly the same pungency ratings were found to have relatively similar AITC levels. Water content appears to greatly influence the relationship between AITC level and pungency. When considering all samples in total, there is no correlation (r = -0.167) between AITC concentration on an as received (i.e., moist) basis and pungency and a weak positive correlation (r = 0.230) between AITC level and panelist preference. However, when AITC concentrations are determined on a dry mass basis, there is a very strong positive correlation (r = 0.821) with pungency and a moderate positive correlation (r = 0.307) with preference. When evaluating specific product types, it becomes more complicated. For the fresh ground horseradish, there is a strong positive correlation between AITC concentration and perceived pungency on both an as received (r = 0.252) and dry mass (r = 0.575) basis. The relationship between AITC amount and preference for the fresh ground horseradish displays a very strongly positive correlation, essentially unchanged, on both an as received (r = 0.863) and dry mass (r = 0.889) basis. However, for horseradish sauce, which contains a more complex blend of ingredients, there is a negative correlation between AITC level and pungency, very strongly correlated with as received samples (r = -0.974) but only weakly correlated (r = -0.297) when AITC is reported on a dry mass basis. Preference, on the other hand, shows a very strong positive correlation (r = 0.809) with AITC amount when reported as received and negligible correlation (r = 0.095) on a dry mass basis. The difference between the AITC concentrations determined and the sensory data could be due to matrix effects from other sample ingredients, masking effects, sensory characteristics, or the interpretation of

panelists. It is believed that the masking effect could highly influence the perception of pungency if the sample matrix is complex. Samples may be perceived as less pungent if the matrix consists of complex mixtures of components such as fat, sugar, or starch.

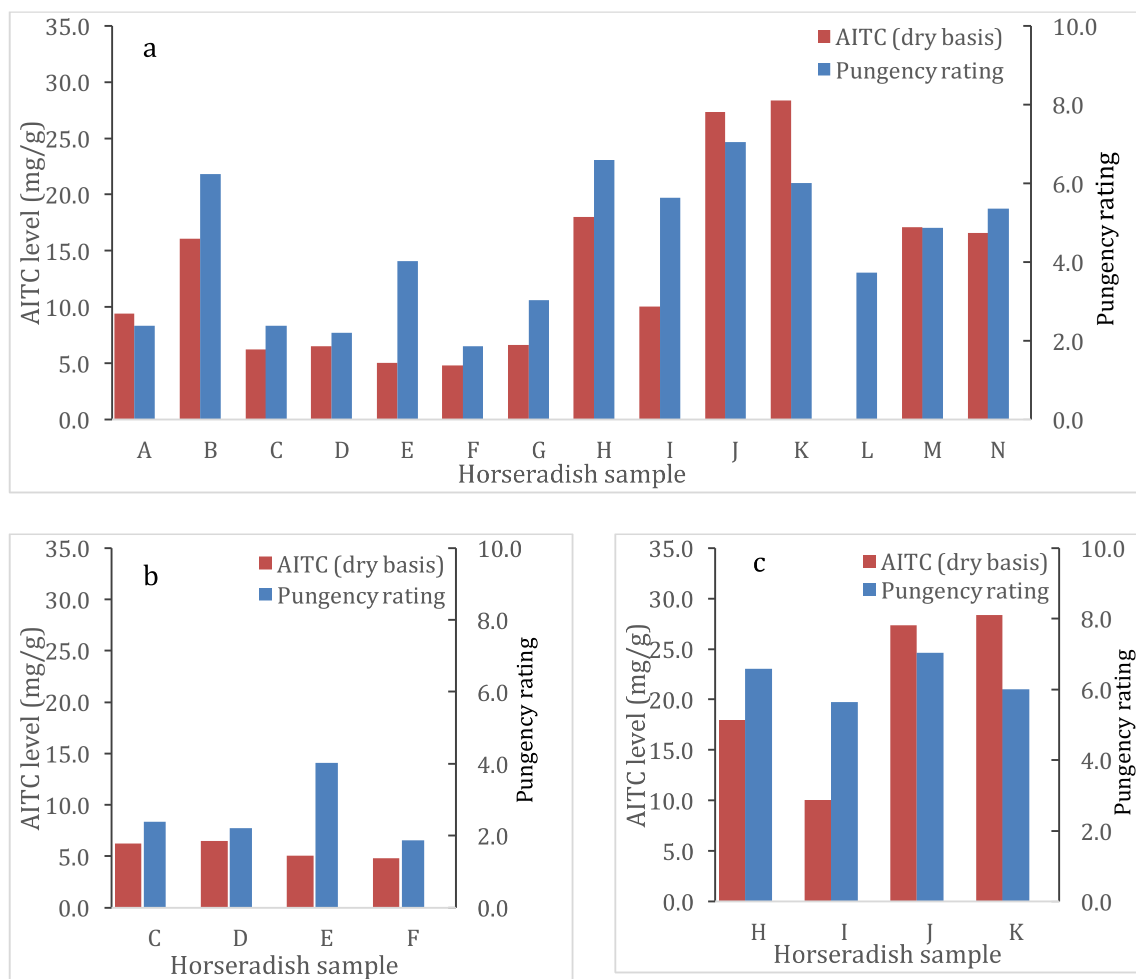


Figure 4.4 AITC levels and sensory ratings determined for the horseradish samples. (a) the 14 horseradish products, (b) horseradish sauce samples, and (c) fresh ground horseradish samples (Pungency scale: 0-10, 0 = no pungency, 10 = extremely pungent; Preference scale: 0-10, 0 = extremely dislike, 10 = extremely like. These two ranking scales are not directly related.).

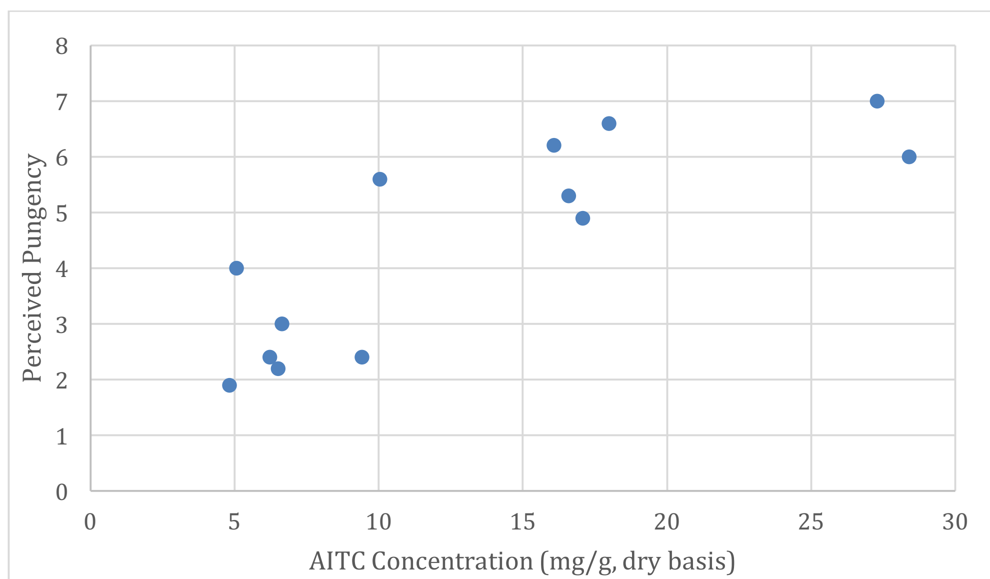


Figure 4.5 Scatter plot of AITC concentration and perceived pungency for the 14 horseradish products.

Based on the "best by date given" in Table 4.1, it does not appear that there were effects of time on pungency, preference, or AITC level. However, since manufacturing date is not known nor is amount of AITC at time of manufacturing, it would be preliminary to conclude that time has no effect. The one exception is for horseradish sauce samples C and D had higher levels of AITC than samples E and F (6.36 average AITC, dry basis vs. 4.94), about 22+ weeks separated these samples. Based on the fresh ground samples and also comparing creamy style to mayonaise, it appears that Manufacturer 2 has higher AITC levels than Manufacturer 1, but not enough data to make any real conclusions. For future study, the effect of time on pungency, preference, or AITC level could be further investigated. Additionally, it will gain more insight on the links between sensory testing and instrumental analysis if the influence of food components and masking effects are better understood.

4.5 Conclusions

A trained panel performed the sensory analysis of 14 commercial horseradish products and the sensory data were compared to the AITC content of the samples. Correlations were made between the AITC concentrations and perceived pungency and panelist preference. Differences due to water content are noted, but the impact of other sample ingredients is more complex. Both the sensory data and analytical results showed that there were differences in pungency among the 14 horseradish product samples. Panelists slightly or moderately liked all 14 samples, with the overall ratings ranging from 4.0 to 5.7. It was suggested that the panelists may have different expectations, acceptability, and interpretations of the sensory characteristics of horseradish samples.

This method could also be applied to other flavored food samples to determine potential relationships between the chromatographic profile and sensory profile by a trained panel. Due to the limited number of panel participants and samples, the information obtained from this study should be considered preliminary. For future study, a larger group of panelists is needed to better understand the links between sensory testing and instrumental analysis. Additionally, it will gain more insight if the influence of food components and masking effects are better understood.

4.6 Acknowledgements

This study was supported by a grant from Silver Spring Foods LLC (Eau Claire, WI, USA).

CHAPTER 5. CONCLUSIONS AND FUTURE WORK

The overall goal for this dissertation was to develop new extraction techniques that can improve the accuracy and precision of analytical results applied to selected food safety and quality analysis situations. This dissertation has described the method development for determination of the leaching of perfluorooctanoic acid (PFOA) from cookware under simulated cooking conditions, identification and quantification of allyl isothiocyanate and related compounds in horseradish products by HS-SPME-GC-MS, and sensory evaluation of horseradish products to correlate the level of allyl isothiocyanate and the perceived pungency.

Determination of the potential leaching of perfluorooctanoic acid (PFOA) from cookware under simulated cooking conditions was carried out with accelerated solvent extraction (ASE) and HPLC-MS/MS. To simulate cooking conditions, accelerated solvent extraction (ASE) was used to perform extractions of PFOA using ethanol/water mixtures as food-simulating liquids, which is consistent with FDA guidelines[115]. An ethanol and water mixture of 1: 9 (v/v) was used to simulate watery and acidic foods, and 9: 1 (v/v) ethanol and water was used to simulate fatty or oily foods. The extraction parameters such as conditions such as pressure (1500 and 1000 psi), flush volume (50, 100, 150 %), purge time (30, 60, 90 sec), and cycles (1, 2, 3) were optimized and carried out at an extraction temperature of 175 °C for 20 min, corresponding to a frying temperature of about 350 °F, which are the most common cooking conditions. It was found that 1500 psi, 100% flush volume, 60 s purge, and three cycles gave the maximum recoveries for both watery- and fatty-food-simulation extractions. The identification and

quantification of PFOA was performed by HPLC-MS/MS on a Fusion RP column (2.0 mm × 50 mm, 4 µm d_p) using 0.2 mL/min isocratic elution (90% MeOH and 10% 2 mM ammonium acetate) at 35 °C. The analytical method (ASE and HPLC-MS/MS) was validated. Good recoveries, precision, and linearity were obtained. Limits of detection (LOD) were as low as 0.03 and 0.02 µg/L, corresponding to 5.0 pg/cm^2 and 3.3 pg/cm^2, for PFOA analysis under watery- and fatty-food-simulation conditions, which are lower than the reported methods by approximately 80%. The method was successfully applied to analyze PFOA from used and new cookware under simulated cooking conditions. The results demonstrated that PFOA were detectable in all pan samples extracted with both watery-and fatty-food-simulation conditions, except water at 100 °C. It is assumed that PFOA breaks down from fluoropolymer-coated cookware (new or used) may leach into watery and fatty foods under common cooking conditions (175 °C and 20 min). However, no attempt was made to correlate this data to PFOA levels found in fried foods or the average diet. Overall, the proposed method was an efficient, accurate, and precise method that can be applied to analyze contaminants and harmful substances from food contact materials and samples.

A headspace-solid-phase microextraction-gas chromatography-mass spectrometry (HS-SPME-GC-MS) method was developed to identify and quantify the flavor component allyl isothiocyanate (AITC) and related compounds in horseradish products. The optimized conditions for HS-SPME were 0.8 g sample size in a 4-mL vial at 30 °C for 20 min with one minute desorption in the GC injector at 250 °C. The identification and quantification of allyl isothiocyanate and relative compounds was performed by GC/MS on a DB-5 column (30-m × 0.25-mm ID × 0.25-µm film). A calibration curve

was generated in the concentration range of 50-3200 ppm of allyl isothiocyanate using the internal standard method. The validated method resulted in intraday and interday precision (% RSD) and accuracy (% recovery) of less than 10% and 80-120%, respectively. The method was applied to analyze allyl isothiocyanate in horseradish samples. Seven constituents were identified and the major constituents were allyl isothiocyanate (97.58%) and phenylethyl isothiocyanate (1.65%), representing 99.23% of the pungent components in prepared horseradish samples. The HS-SPME-GC-MS method presented is a simple, accurate, and sensitive method for determination of volatile compounds in horseradish. This method utilizes the fast-growing technology of SPME for sample preparation, which is easy, fast, and environmentally friendly. Manufacturer, processors, and regulatory authorities can use this method to evaluate flavored products before and after production for quality control.

Sensory analysis was carried out through the development of a method for studying the correlation between the level of allyl isothiocyanate and the perceived pungency in horseradish products. Sensory pungency analysis of 14 commercial horseradish products was performed and carried out by a trained panel. The level of allyl isothiocyanate in horseradish sample was instrumentally determined by the validated HS-SPME-GC-MS method. Good correlation was found between the instrumentally determined allyl isothiocyanate levels and the sensory pungency ratings, suggesting positively correlated relationship between the allyl isothiocyanate levels and the perceived pungency in horseradish products. The sensory data and instrumental results showed there were significant differences in pungency among the 14 horseradish products. Panelists exhibited no significant difference in overall preference among the 14

samples, with the average overall ratings ranging from 4.3-5.4. Some differences in terms of expectation and interpretation of sensory characteristics of horseradish might be present for panelists. Due to the limited number of panelists, the information obtained from this study should be interpreted with care. For future study, a larger group of panelists is recommended in order to fully understand the links between sensory test and instrumental analysis.

Various food sample preparation techniques have been employed to obtain reliable analytical results. However, some of these methods are time-consuming, use large amounts of organic solvents, and often involve complicated procedures. Accelerated solvent extraction (ASE) and solid-phase microextraction (SPME), which are considered "green" sample preparation techniques, have been extensively studied as the substitution to this type of sample preparation processes. This dissertation reported the studies on food safety and quality analyses using these modern sample extraction techniques. ASE demonstrated high extraction efficiencies with small volumes of solvents and short extraction times. SPME showed the capabilities of sampling, isolation, and concentration in one step with great sensitivity and recovery. SPME is considered 'environmentally friendly' because of the elimination of organic solvents. In past few years, progress has been made in modern detection techniques and chromatographic technologies, and less devoted to sample preparation and clean-up. But still in many cases, due to low concentrations of analytes and complex mixtures and sample matrices, sample preparation remains the most important step in food safety study. More attention and effort should be given to the development of new and improved sample preparation techniques that can provide a more robust solution for complex samples.

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