DEVELOPMENT OF ANTI-INFLAMMATORY AGENTS FROM THE AROMATIC PLANTS, *ORIGANUM* SPP. AND *MENTHA* SPP., AND ANALYTICAL METHODS ON THE QUALITY CONTROL OF BIOACTIVE PHENOLIC COMPOUNDS

by

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ABSTRACT OF THE DISSERTATION

DEVELOPMENT OF ANTI-INFLAMMATORY AGENTS FROM THE AROMATIC PLANTS, *ORIGANUM* SPP. AND *MENTHA* SPP., AND ANALYTICAL METHODS ON THE QUALITY CONTROL OF BIOACTIVE PHENOLIC COMPOUNDS

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Dr. James E. Simon

The development of new anti-inflammatory drugs comes at high cost and risk, as evidenced by the fact that several novel prescription non-steroidal anti-inflammatory drugs (NSAIDs) have been withdrawn from the market due to potential health risks. Meanwhile, natural dietary supplements with anti-inflammatory activities are gaining increased interest by consumers and industry as they are perceived to be of lower cost, with fewer adverse effects and yet function as complementary and alternative medicines. We successfully identified the anti-inflammatory constituents from post-distillation material of oregano, an aromatic plant long prized as a food seasoning and spice in western and Middle Eastern cuisine and a rich source of essential oil. The anti-inflammatory compounds in oregano were identified as rosmarinic acid, oleanolic acid and ursolic acid by using bioactivity-guided isolation, LC/MS and NMR techniques. We subsequently elucidated their possible inhibitory mechanisms during the inflammatory cell signaling pathways by screening different bioassays, and a synergistic action for the combination of these three compounds was observed on the LPS-induced

nitrite production assay. An LC/MS (SIM mode) method employing a tandem column system was developed for the simultaneous quantitation of these three compounds in oregano. Validation for this method showed a precision (relative standard deviation) ranging from 4.84% to 6.41%, and the recoveries varied from 92.2% to 100.8% for the three analytes. In order to investigate genetic diversity between species and within a species relative to the anti-inflammatory compounds, different oregano species, varieties and breeding lines were collected from around the world, vegetatively field transplanted into a Rutgers Agricultural Research Experiment Station farm, where the plants were field grown, harvested and quantitated for their anti-inflammatory contents based upon the LC/MS method developed in this study. We next examed mint, another aromatic plant in the Lamiaceae family, and proved that these three anti-inflammatory compounds also accumulated in this plant. An analytical survey was further performed to determine anti-inflammatory agent-rich sources from eight commercial mint species as well as many specialty varieties within the different species. To facilitate the frequent quality control requirement and material pre-evaluation, we developed and validated a near-infrared spectroscopy (NIR) method for the fast quantitation of anti-inflammatory constituents in oregano and mint. Finally, we investigated the anti-inflammatory compound distribution and related chemical properties of the oregano post-distillation materials.

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I acknowledge Dr. Arthur Tucker for providing the species authentication and taxonomy for all the mints; Dayton Jones and Dan Shiley for NIR spectra collection and multivariate analysis; and Dr. Lenore Rasmussen for her assistance and instruction on the 2D NMR experiment.

I would like to offer my special thanks to my wife, Xihao Li, for her love and support.

DEDICATION

I dedicate this work to my wife Xihao Li and my daughter Lindsay Shen.

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LIST OF ABBREVIATIONS

AA	arachidonic acid
COSY	homonuclear correlated spectroscopy
COX	cyclooxygenase
CD_3OD	deuterated methanol
DMSO	dimethyl sulfoxide
DPPH	1,1-diphenyl-2-picrylhydrazyl
ESI	electrospray ionization
EtOAc	ethyl acetate
EtOH	ethanol
HMBC	heteronuclear multiple bond correlation
HPLC	high performance liquid chromatography
iNOS	inducible nitric oxide synthase
LC	liquid chromatography
LOX	lipoxygenase
LPS	lipopolysaccharide
MeCN	acetonitrile
MeOH	methanol
MRM	multiple reaction monitoring
MS	mass spectroscopy
NIR	near-infrared spectroscopy
NMR	nuclear magnetic resonance spectroscopy
NO	nitric oxide
NOE	nuclear overhauser effect
NSAID	non-steroidal anti-inflammatory drug
OA	oleanolic acid
PAD	photodiode array detector
PLS	partial least-squares regression model
QC	quality control
RDA	retro Diels-Alder
RMSEC	root mean square error of calibration
RMSEP	root mean squire error of prdiction
ROS	reactive oxygen species
RosA	rosmarinic acid
RSD	relative standard deviation
SIM	selected ion monitoring
TIC	total ion chromatogram
UA	ursolic acid
UV	ultraviolet

CHAPTER 1. INTRODUCTION

1.1. ANTI-INFLAMMATION: BACKGROUND ON THE DEFINITION, CAUSES AND CURRENT TREATMENTS INCLUDING THE TYPES AND UNDERLYING MECHANISMS OF ACTION OF CURRENT ANTI-INFLAMMATORY DRUGS

Inflammation is a complex immune response to vascular tissues injury or infection caused by pathogens, clinically characterized by signs of swelling, redness, pain, warmth and loss of function (Issa, Volate & Wargovich, 2006). The process itself is not deemed as a disease, while failure to recover in a timely fashion results in exacerbation of tissue damage and function of cell signaling pathways. The mechanism of inflammatory pathways can be broadly classified as arachidonic acid (AA)-dependent and AA-independent pathways (Yoon & Baek, 2005). Arachidonic acid-dependent pathways may include cyclooxygenase (COX), lipoxygenase (LOX) and phospholipase A_2 (PLA_2) as mediators (**Fig. 1.1**). In contrast, the second mechanism of inflammation involves nitric oxide synthase (NOS), NF-*k*B, peroxisome proliferator activated receptor (PPAR) and NSAID activated gen-1 (NAG-1) in the AA-independent pathways.

In the first mechanism that leads to inflammation, AA, the fatty acid released by phospholipases from the phospholipid layers of the plasma membrane, is metabolized via either the COX pathway to generate thromboxane A_2 and prostaglandins (PGs), or through the LOX mechanism to produce hydroperoxyeicosateraenoic acids (HETEs) and

leukotrienes (LTs). The products from both pathways are considered to be important mediators in the control of inflammation. COXs can be further divided into three isomers, COX-1, COX-2 and more recently identified COX-3, depending on their tissue distributions, expressions and pathological properties. Similarly, several LOX isomers have been identified so far. 15-LOX synthesizes 15-HETE during the process of inflammation, while 12-LOX and 5-LOX produce their respective pro-inflammatory mediator 12-HETE and 5-HETE. The latter two LOXs are believed to be promising targets for anti-inflammatory agent development. Up-regulation of iNOS (inducible nitric oxide synthase) is an AA-independent pathway, which results in production of NO by oxidation of a terminal nitrogen on the guanidine of the amino acid L-arginine (Snyder & Bredt, 1992; Feldman et al., 1993). Its expression is induced by inflammatory stimuli, and the oxidation reaction requires calmodulin, NADPH, tetrahydrobiopterin, FMN and FAD as cofactors. Transcription factors such as NF-*k*B and AP-1 that regulate the expression of COX-2 usually co-regulate the expression of iNOS (Posadas et al., 2000). Endogenous or exogenous NO activates soluble guanylate cyclase (sGC) and the resulting increase of cGMP induces a signaling that up-regulates the COX-2 expression in head and neck squamous cell carcinoma (HNSCC) cell lines (Park et al., 2003). Nitric oxide, known as the product synthesized by iNOS protein, was reported to also stimulate COX-2 expression in cultured collecting duct cells through MAP kinases and superoxide (Yang et al., 2006).

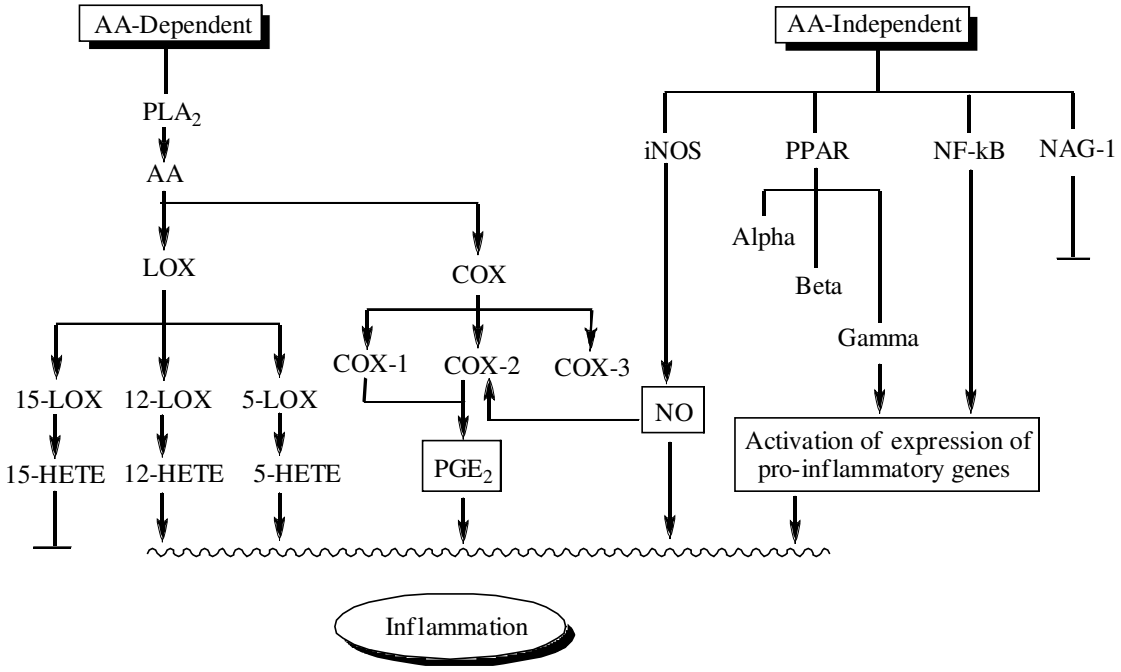

Figure 1.1. Proposed pathways of inflammation (Modified from Issa, Volate & Wargovich, 2006)

Anti-inflammatory drugs are the most frequently prescribed drug class in the world for the treatment of acute or chronic conditions where pain and inflammation are present (Asero, 2007; Chan, 2006; Cheng & Harris, 2005). These drugs are generally used for the symptomatic relief of conditions such as rheumatiod arthritis, osteoarthritis,

inflammatory arthropathies, acute gout, dysmenorrhoea, metastatic bone pain, postoperative pain, pyrexia, renal colic headache and migraine (Wolfe, Lichtenstein & Singh, 1999; Rossi, 2006; Chan, 2006). Anti-inflammatory drugs can be classified into several categories depending on their mechanisms of action such as glucocorticoids and NSAIDs (non-steroidal anti-inflammatory drugs). There is a long history in the use of glucocorticoids in the treatment of inflammatory and autoimmune disorders (Sullivan, 1982; Wolfe, Lichtenstein & Singh, 1999; Flower, 2003). In 1855, Addison first observed adrenocorticoids, as a type of glucocorticoids, exhibiting anti-inflammatory activities (Sullivan, 1982). In 1929, the Hench research group was awarded the Nobel Prize for the discovery that cortisone was effective in treatment of rheumatoid arthritis. This observation had prompted cortisone and other natural and semi-synthetic corticosteroids to be widely used in 1940s (Wolfe, Lichtenstein & Singh, 1999). Subsequently, many potent synthetic anti-inflammatory glucocorticoids, and very potent semi-synthetic corticosteroids with minimal salt-retaining activity were introduced to the market. The introduction of fluorine at the 9α-position to increase the binding affinity to corticosteroid receptors and oxidation on 11-hydroxy group is one such example (Khan, Park & Lee, 2005). The modified corticosteroids such as flurandrenolone, fluorometholone and flucinolone are potent anti-inflammatory drugs while mainly used as topical agents due to their systemic toxicities including hypertension, hypokalemia, hypernatremia and central serous retinopathy (Khan, Park & Lee, 2005). In the 1980s and 1990s, the development of anti-inflammatory glucocorticoids was focused on the increase of the therapeutic index

by reducing the drug systemic adverse effects. Cortisone and hydrocortisone are among the most important anti-inflammatory glucocorticoids due to their powerful and versatile therapeutic roles in different inflammatory diseases such as rheumatoid arthritis, bronchial asthma, malignancies, cerebral edema, and infectious, skin and gastrointestinal diseases (Khan, Park & Lee, 2005). Mechanism of glucocorticoids action is complex and not completely understood. It has been postulated that the glucocorticoids interact with specific receptor proteins (glucocorticoid receptors) to regulate the expression of glucocorticoid responsive genes, incorporating with transcriptional cofactors to change the level and array of proteins synthesized by the target tissues (Mangelsdorf et al., 1995). Protein-protein interaction between glucocorticoid receptors and the transcription factors NF-*k*B and AP-1 has been reported to repress the expression of genes encoding a number of cytokines and enzymes such as collagenase and stromelysin. The clinical use of glucocorticoids are complicated by a number of serious side effects e.g., acute adrenal insufficiency due to the suppression of the hypothalamic-adrenal axis, fluid and electrolyte abnormalities, increased susceptibility to hypertension, hyperglycemia, osteoporosis, myopathy, and habitus of steroid overdose including fat redistribution, striae, ecchumoses and acne (Mangelsdorf et al., 1995; Schimmer & Parker, 2001; McKay & Cidlowski, 1999). A major drawback of glucocorticoids was that these compounds could exert nonspecific reactions and they exerted actions in almost very organ system.

Non-steroidal anti-inflammatory drugs (NSAIDs) are another major class of anti-inflammatory drugs used to treat pain, fever and inflammation. The most prominent members of this group are aspirin, ibuprofen and naproxen, being over-the-counter (OTC) drugs in many areas (Rossi, 2006). More than 70 million prescription and 30 billion OTC non-aspirin non-steroidal anti-inflammatory drugs are sold annually in the US (Wolfe, Lichtenstein & Singh, 1999). Most NSAIDs inhibit cyclooxygenase, which catalyzes the oxygenation of arachidonic acid to prostaglandin H2 in the synthesis of prostaglandins, prostacyclins and thromboxanes (Issa, Volate & Wargovich, 2006). Although phenylbutazone was developed in the 1940s, the fenamates appeared in the 1950s, indomethacin was introduced in the 1960s, and the proprionates were commercialized in the 1970s, it was not until the 1970s that cyclooxygenase had been know as the target of acetyl salicylic acid (ASA) and NSAIDs (Flower, 2003). In the early 1990's, the cyclooxygenase enzyme was recognized to express in mammalian cells as isozymes, designated COX-1 and COX-2, which show about 60% amino acid sequence similarity (Xie et al., 1991). These two isozymes are similar in their catalytic properties, affinities for arachidonic acid and structures of their active sites, with minor differences regarding the requirements of hydroperoxides for activation and preferences for substrates (Smith, Garavito & DeWitt, 1996). COX-1 is expressed in healthy tissues and takes effect in thrombogenesis and in the homeostasis of the gastrointestinal tract and the kidney, whereas COX-2 was shown to be inducible by bacterial endotoxin, cytokines, including IL-1 and TNF, mitognes and growth factors (Xie et al., 1991). The observations that

COX-2 is associated with inflammatory condition and COX-1 acts as a constitutive enzyme have in part revitalized the field and for new and selective isoform inhibitors, as evidenced by the introduction of celecoxib (Celebrex), rofecoxib (Vioxx) and valdecoxib (Bextra) in Europe and North America (Gajraj, 2007). Encouraged by the concept of COX2/COX1 selectivity and the recognition of enzymatic binding site, the development of the "coxibs" was rapid, and many new selective COX-2 drug candidates were under reviewing by FDA (Black, 2004). However, the adverse effects of these drugs have also been recognized. In 2004, rofecoxib was voluntarily removed from the market by the manufacturer due to a potential risk of myocardial infraction and stroke (Gajraj, 2007). Valdecoxib was also withdrawn from the market in 2005, because of an increased risk of adverse cardiovascular events in coronary artery surgery trials (Gajraj, 2007).

1.2. SAFETY ISSUES AS TO ANTI-INFLAMMATORY DRUGS

The widespread use of NSAIDs for chronic disease management has meant that safety is of prime importance, while the adverse effects of these drugs have become increasingly prevalent. About 103,000 hospitalizations due to gastrointestinal adverse events and 16,500 deaths each year related to NSAIDs use have been reported in the United States (Singh & Triadafilopoulos, 1999). The two major adverse drug effects associated with

NSAIDs are gastrointestinal effects and renal effects. About 10-20% NSAID patients experience dyspepsia (Singh & Triadafilopoulos, 1999). Some severe side effects may cause the risk of ulcer perforation, upper gastrointestinal bleeding or death. The combinational risk has also been noticed when taking a COX-2 inhibiter with a traditional NSAID concomitantly (FDA Alert for Practitioners, 2005). Recent research revealed that the use of celecoxib and other COX–2 inhibitors have induced additional serious side effects, including stomach-ache, diarrhea, headache, liver problems, aseptic meningitis and anaphylaxis (Cox2 Inhibitors, 2007; Gajraj, 2007; Asero, 2007).

1.3. OPPORTUNITIES FOR THEN NEXT GENERATION OF NEWER ANTI-INFLAMMATORY AGENTS AND DIETARY SUPPLEMENTS

Recent surveys show that approximately 50% of the US population use dietary supplements at one time or another, and that annual sales in the dietary supplement industry were over $18 billion (Radimer et al., 2004; Supplement Business Report). The Food and Drug Administration (FDA) defines a dietary supplement as “a product (other than tobacco) that in intended to supplement the diet and bears or contains one or more of the following dietary ingredient: a vitamin, a mineral, a herb or other botanical, an amino acid, a dietary substance for use by man to supplement the diet by increasing the total

daily intake, or a concentrate, metabolite, constituent, extract, or combinations of these ingredients" (Issa, Volate & Wargovich, 2006). The Dietary Supplement Health and Education of Act (DSHEA) was signed by President Clinton and passed by the U. S. Congress in the year of 1994, assuring the US consumers access to dietary supplement products in a positive way. This legislature treats dietary supplements as food, not as drugs, no longer subjected to the pre-market safety evaluations for other new food ingredients or for new uses of old food ingredients (Issa, Volate & Wargovich, 2006). The label on a dietary supplement product may contain one of three types of claims: health claim, nutrient content claim, or structure/function claims. Under the DSHEA, it is the manufacturer's responsibility to notify FDA of its intent to market a new dietary ingredient in the product and provide information on how it determined that reasonable evidence exists for safety. Once the dietary supplement product is marketed, the FDA has the authority to prove that the product is not safe, restrict its use, or remove it from the market (Rapaka & Coates, 2006). Although it is important to determine the quality of a dietary supplement, no legal or regulatory definition is currently available specifically for dietary supplement products. The degree of quality control depends on the manufacturers, and FDA is authorized to issue Good Manufacturing Practice (GMP) regulations the dietary supplements must comply with, although these food GMPs are primarily concerned with safety and manufacturing sanitation rather than dietary supplement quality. FDA issued a proposed rule in March 2003 intending to ensure that the manufacturing practices of dietary supplements are accurately labeled and unadulterated

(Rapaka & Coates, 2006). The DSHEA authorized the establishment of the Office of Dietary Supplements (ODS) at the National Institutes of Health (NIH), dedicating to strengthen knowledge and understanding of dietary supplements by evaluation scientific information, creating databases of dietary supplement ingredients, and developing analytical methods and reference materials (Rapaka & Coates, 2006). More recently the FDA set forth legislation to ensure all dietary supplements are manufactured using GMP (FDA posts, 2007)

In addition to dietary supplements used by people, there is a significant market for supplement and feed additives for the livestock, poultry and pet industries. The global feed additive market was estimated as 6 billion dollars in 2000, covering the products containing vitamins, amino acids, growth enhancers, carotenoids, antioxidants and enzymes (Feed Additive: A Global Market Survey, 2001). The worldwide animal feed additives market is expected to reach US$15.4 billion by 2010, of which the disease-preventing agents are valued as $485 million (The Poultry Site Latest News, 2007). With the antibiotics widely used in feed additives, the strict regulatory environment is one of the biggest challenges facing the animal feed additives markets, and the development of natural plant-based feed additive containing phytopharmaceuticals are gaining increased interest by general public owing to their “natural” property. Horse arthritis is a major concern of horse owners and is becoming more common as more horses are living longer lives. The current treatments include

allowing the animal to rest, pursuing physical therapy such as ice or heat treatments, and receiving anti-inflammatories injected into the muscles or the affected joint (Horse Arthritis Relief, 1997). The development of anti-inflammatory feed additives from natural plants is expected to be an emerging market for animal health. Given the increasing interest in natural products to improve health and nutrition and the availability, the search for new natural anti-inflammatory agents that could have application for humans, animals and livestock is of interest.

1.4. BACKGROUND AS TO ANTI-INFLAMMATORY PROPERTIES OF OREGANO AND HYPOTHESIS

The chemical components in oregano volatile oils have long interested researchers and have been a focus of many studies which examined the aromatic, flavoring, antioxidative, antibacterial and antiseptic properties (Kulisic et al., 2004; Rodrigues et al., 2004; Velluti et al., 2004). Thymol and carvacrol are two of the major aromatic volatile components in the essential oil of oregano and as such have been used to characterize the two main chemotypes in the chemical composition of oregano essential oil (Dogan, Arslan & Ozen, 2005). While the main focus has historically been on the essential oils and the compounds contributing to the aroma and flavor of oregano, far less attention has focused

on the non-volatile bioactive components of this and other aromatic plants which contribute to the health and functional attributes of the plant and its extracts. In addition, the biomass following essential oil distillation of oregano and other aromatic plants, such as mints, has long been viewed as waste products used as soil amendments, animal feed or in some regions even dried and later used as fuel to fire and charge the next distillation. The uses of Lamiaceae family plants for treatment of infections of humans and animals as well as dental care compositions have been patented for commercial products (Ninkov, 2001; Worrell et al., 2006). More recently, the water-soluble extract of oregano has been described to exhibit strong anti-inflammatory activity by inhibiting COX-2 secretion (Lemay, 2006). Yoshino et al. (2006) found that oregano extract exhibited anti-inflammatory activities in mouse models of stress-induced gastritis and contact hypersensitivity. Moreover, the effect of aqueous methanol extract of *Origanum vulgare* ssp. *hirtum* on soybean lipoxygenase was described, revealing a promising potential of oregano for anti-inflammatory efficacy (Koukoulitsa, 2006). However, these anti-inflammatory studies were all performed on oregano crude extracts, and no research had identified compound(s) responsible for this activity, thus linking the bioactivities to specific compounds from oregano extracts. Few studies reported chemicals from the water-soluble extract of oregano. The main non-volatile compounds identified were galangin, quercetin, carnosol, caffeic acid and rosmarinic acid (Kato & Shimio, 1987; Kulisic et al., 2004). Of these, carnosol and rosmarinic acid has received the majority of attention in oregano due to their high antioxidant activity as measured by ROS activity.

Matsuura et al. (2003)reported the isolation of two phenolic glycosides from the extract of dried leaves of oregano. The two compounds, 4'-O-β-D-glucopyranosyl-3',4'-dihydroxybenzyl protocatechuate and 4'-O-β-D- glucopyranosyl-3',4'-dihydroxybenzyl 4-O-methylprotocatechuate, showed comparable DPPH radical scavenging activities to quercetin and rosmarinic acid. Such antioxidative and anti-inflammatory activities in oregano is also of particular interest because their long established historical use would suggest that whole plants and even extracts, or concentrated enriched extracts would likely be safe for consumption. As safety is always a prime consideration, drugs intended for chronic disease treatment through oral delivery require greater and longer-term testing. This in part has stimulated the search for natural plant-based anti-inflammatory "phytopharmaceuticals" to be used in the form of dietary supplements. As consumers perceive that such products will be lower cost and safe, those in the industry also see opportunity in that such products are easier to introduce into this marketplace than the traditional pharmaceutical marketplace. When reviewing adverse reports on herbs, particularly common culinary herbs and spices, few such adverse reports have been recorded.

We therefore hypothesized that the anti-inflammatory compounds in the non-volatile fraction of oregano would come from one or more polyphenols, which would be ideal for the dietary supplement development of a low cost, low toxicity and functioned as complementary and alternative medicine. Depending on the different potencies on the

preliminary anti-inflammatory tests, among *Origanum* spp. as well as other related species within the same family of Lamiaceae, we also hypothesized that the genetic influence would exert impact on the distribution of these anti-inflammatory constituents, and this can be characterized and qualitatively controlled by modern analytical techniques.

1.5. RESEARCH OUTLINE

This research covered a broad range on the areas of medicinal chemistry and the development of new plant-based anti-inflammatory agents. The medicinal chemistry research focused on natural products isolation, identification, biological activity tests including synergistic action studies, analytical method development, newer instrument application, quality control and manufacturing method development. Starting from the identification of the anti-inflammatory constituents as rosmarinic acid, oleanolic acid and ursolic acid, directed by bioactivity-guided isolation, we screened their anti-inflammatory activities through different bioassays involving the possible mechanisms as well as the synergistic action investigation (**Fig. 1.2**). We also evaluated pharmacokinetic features, bioavailability and safety issues for these bioactive ingredients. We then developed and validated a LC/MS (SIM mode) to simultaneously co-quantitate rosmarinic acid,

oleanolic acid and ursolic acid in oregano samples. Using this method, we then conducted a quantitative survey of these anti-inflammatory constituents in different oreganos (30 sources including three *Origanum* species, varieties and breeding lines). Our results showed oregano as a significantly rich source of rosmarinic acid, oleanolic acid and ursolic acid. As hypothesized, we observed significant variation observed by the genetics of the plant. This phase of the research demonstrated the critical importance of the genetics of the plant, as we observed significant genetic diversity between species and within a species both in content and composition of the bioactive compounds.

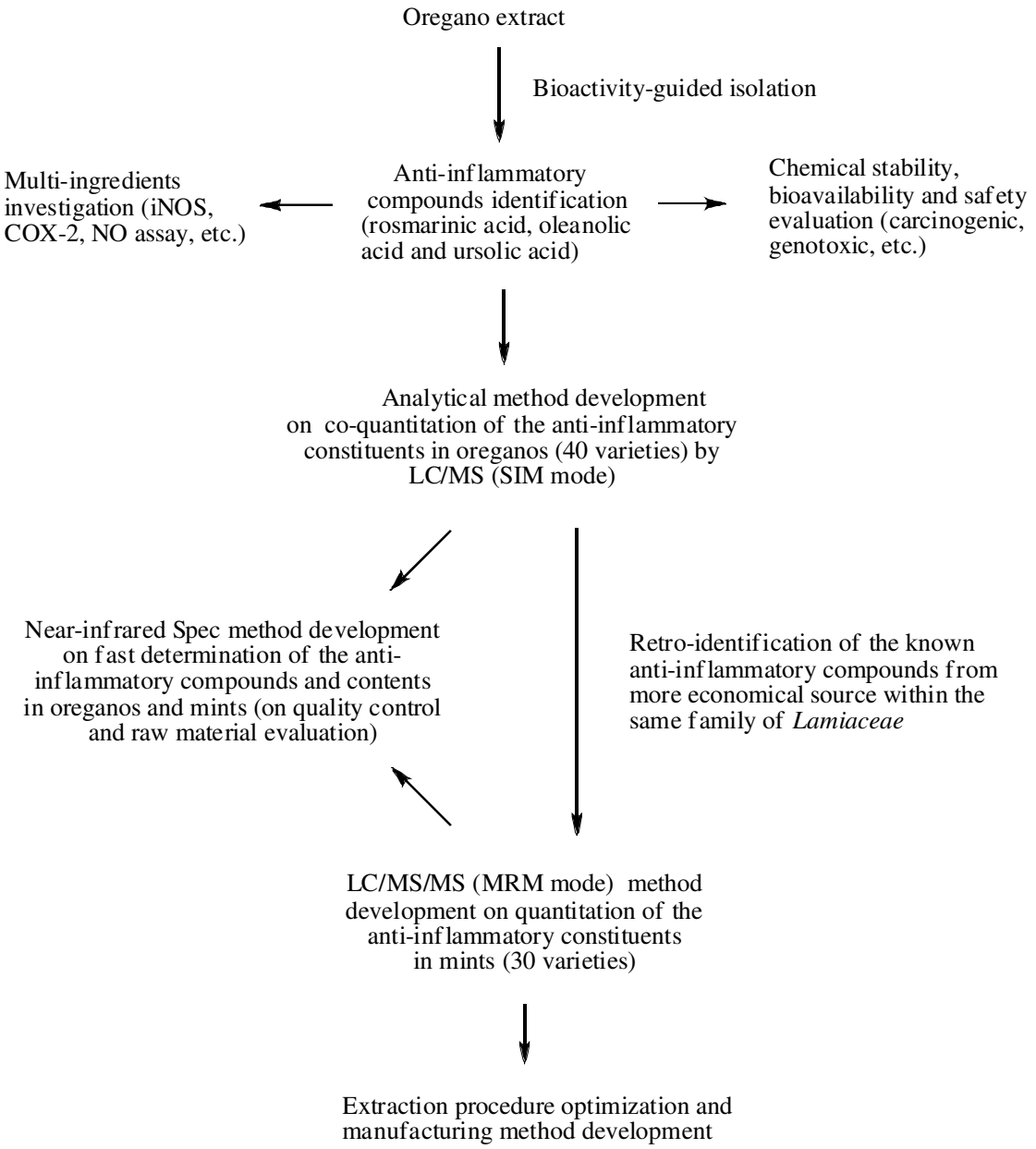

Figure 1.2. The proposed research scheme employed in this research in the development of a new phytopharmaceutical dietary supplement product

The discovery of the compounds in oregano that was responsible for the plants' anti-inflammatory action, and the research that now shows this species to be a rich source of rosmarinic acid, oleanolic acid and ursolic acid inspired us to look for additional source of these natural anti-inflammatory agents within the same family of Lamiaceae. Our final focus turned to mint (*Mentha* spp.), which we later also proved to contain these three anti-inflammatory organic acids identified by LC/MS. Thirty-five *Mentha* spp. and varieties, including all commercial species used in the production of essential oil distillation as well as many specialty varieties, were collected around the world, vegetatively propagated and both grown in our greenhouses for authentication and field transplanted onto our research farm. We then developed and validated an LC/MS (MRM mode) method to co-quantitate these three organic acids, and compared their distributions in different mints.

To facilitate the frequent quality control requirement and material pre-evaluation, a NIR (near-infrared spectroscopy) was developed for the fast quantitation of anti-inflammatory constituents in oregano and mint. NIR as a novel analytical technique has gained wide acceptance in recent years for material testing, product quality control, and non-destructive and non-invasive quantitation. Our validations (full cross validation and true test set) confirmed the predictive ability to fast determination of these three anti-inflammatory contents in varied samples.

Finally, we developed an extraction system that permitted the procurement of both the essential oil and the anti-inflammatories of interest. In doing this, we then modified the extraction systems to optimization the recovery of the targeted natural products and manufacturing method from the post-distillation material of mint and oregano as well as performed compound stability tests, which would facilitate to introduction into commercialization of a new phytopharmaceutical product for the dietary supplement and animal health care feed supplement product industries.

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CHAPTER 2. IDENTIFICATION OF THE ANTI-INFLAMMATORY CONSTITUENTS IN OREGANO AND THEIR BIOACTIVE PROPERTIES

2.1. INTRODUCTION

Several papers reported the non-volatile components of oregano showing anti-inflammatory activities as described in Chapter 1, however, these anti-inflammatory studies were all performed on oregano crude extracts, and no research to date has brought these into a molecular level. We assumed that the anti-inflammatory compounds in the non-volatile fractions of oregano would be several polyphenols including but not limited to rosmarinic acid. To facilitate the bioactive compound identification, we utilized a bioactivity-guided fractionation approach whereby LPS-induced nitrite assay has employed as a guide to evaluate the anti-inflammatory effect of each fraction during the isolation process. The fractions continuing to show activity were carried through further isolation until the pure compound was obtained. In this way, we focused on the isolation and purification of only these compounds that were responsible for the tested bioactivity. LC/PAD/MSD was applied for chemical screening at each stage, and thus enabled recognition of common constituents with known biological activity. We also hypothesized that a single phytochemical may affect more than one anti-inflammatory cell signaling pathway. Thus, the discovery of other possible anti-inflammatory mechanisms for the identified compounds was also part of our goal.

2.2. MATERIALS AND METHODS

2.2.1. Folin-Ciocalteu's Method on Total Phenolic Content Determination.

To evaluate a phenolic content level as the preliminary data, the Folin-Ciocalteu's method was utilized to test a representative Greek oregano (*Origanum vulgare* ssp. *hirtum*) sample. This method is based upon a chemical reaction leading to the formation of a blue colored product due to reduction of tungstate and/or molybdate in Folin-Ciocalteu's reagent by the phenolic functional groups in samples (Singh, Srivastava & Sahu, 2003). In this research, the air-dried botanical was used in all studies. The aerial part of the Greek oregano sample GO-36 (*O. vulgare* ssp. *hirtum*) was first finely ground with a coffee grinder. Around 200 mg of oregano powder was accurately weighted and placed into a 25 mL volumetric flask, and 15 mL of extraction solvent (40% ethanol) was added. After sonicating for 30 min following vortexing and inverting the flask about 10 min, the extract was allowed to cool down to ambient room temperature and filled to full volume with extraction solvent. The mixed extract solution was filtered through a 0.45 μm filter to get 10 mL of clear solution for UV analysis. Then 1 mL of clear extraction solution prepared from sample preparation was transferred to 100 mL volumetric flask with 70 mL HPLC-grade water. After swirling contents to mix, 5 mL of Folin-Ciocalteu's phenol reagent (Sigma Chemical Co., St. Louis, MO) was added into the solution and mixed again. Next, 15 mL of sodium carbonate solution (20 g in 100 mL) was added

within 8 min prior to making the final volume exactly to 100 mL using HPLC-grade water. After 2 hours, the UV absorption at 760 nm was recorded by UV spectrometer. Gallic acid (Sigma Chemical Co., St. Louis, MO), 20 mg, was next dissolved in 25 ml volumetric flask and diluted to 2, 4, 8, 16 and 32 times for use in preparing the standard solutions for generating a calibration curve. Analyses of the standard solutions were conducted in the same manner as described with the plant extract for UV analysis. A blank solution was also prepared without the extraction solution or standards. The percent phenolic content was calculated based on the calibration curves of gallic acid.

2.2.2. DPPH Radical Scavenging Assay.

The DPPH• (1,1-diphenyl-2-picrylhydrazyl free radical) assay was used to evaluate the free radical scavenging capacity of pure compounds isolated (Cos, Rajan & Vedernikova, 2002; Shahat, Cos & Hermans, 2003). The stable free radical DPPH• was dissolved in EtOH to yield a 100 μM solution, and 0.5 mL of the targeted compound in EtOH or extract that is to be tested was added to 3.0 mL of the ethanol DPPH• solution. For each test compound, different concentrations are normally used to capture a concentration range of ROS activity. The mixture was shaken and left in dark for 20 min. The decrease in DPPH• absorption was measured at 517 nm (purple color) and the inhibition percentage of the radical scavenging activity was calculated using the equation: inhibition (%) = 100 – 100 ($A_S \div A_0$), where A_0 is absorbance of the blank and A_S is absorbance of

the sample at 517 nm. The antioxidative activity of each sample was tested in triplicate and expressed as an IC_{50} value calculated by linear regression analysis. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2- carboxylic acid) is used as control.

2.2.3. Isolation and Identification of Rosmarinic, Oleanolic and Ursolic Acids.

All solvents used for chromatographic isolation were analytical grade and purchased from Fisher Scientific (Springfield, NJ). Silica gel (130-270 mesh). TLC plates (250 μm thickness, 2-25 μm particle size, Sigma Chemical Co., St. Louis, MO) were used for column chromatography, with compounds visualized by spraying with 5% (v/v) H_2SO_4 in an ethanol solution. ^{1}H NMR and ^{13}C NMR spectra were obtained on a 200 MHz instrument (Varian Inc. Palo Alto, CA). For isolation and identification purposes, the dried aerial part of oregano sample GO-36 (*O. vulgare* ssp. *hirtum*) (400 g) was extracted three times with ethanol and concentrated to dryness under reduced pressure to get around 100 g of extract. The extract residue was loaded no a silica gel (130-270 mesh, 1,500 g) column and eluted by hexane, hexane-EtOAc (1:1), EtOAc, EtOAc-acetone (1:1), acetone, acetone-methanol (9:1), acetone-methanol (3:1) and methanol in sequence. A total of 16 fractions were collected, and the second fraction containing oleanolic acid and ursolic acid was further subjected to a preparative HPLC separation. The compounds oleanolic acid and ursolic acid were then purified using a Varian C18 preparative column (250 × 41.4 mm, 8 μm) eluted with methanol-water (8:2). The structures of the two

triterpenoid acids were identified and elucidated by NMR and MS analysis. Rosmarinic acid was isolated from the seventh fraction by using preparative HPLC on a Luna phenyl-hexyl column (250 × 30.0 mm, 10 μm, Phenomenex Inc., Torrace, CA) with methanol-water (9:1) as the mobile phase. The identification of rosmarinic acid was determined by comparing the mass spectra, the UV spectra and the retention time on HPLC to the authentic standard (Sigma Chemical Co., St. Louis, MO).

2.2.4. Anti-inflammatory Assays for Plant Extract, Pure Compound and Synergistic Action Study.

LPS-induced nitrite assay was used in this research to facilitate bioactivity-guided isolation as well as pure compound and synergistic action study. Since a single phytochemical may affect more than one anti-inflammatory cell signaling pathway, we also screened many other anti-inflammatory assays to elucidate other possible mechanisms, either through cyclooxygenase, lipoxygenase or iNOS pathway. The bioassays from section 2.2.4 to 2.2.6 were conducted in collaboration with the research lab of Dr. Min-Hsiung Pan, National Kaohsiung Marine University.

2.2.5. Cell Culture.

RAW 264.7 cells, derived from murine macrophages, were procured from the American

Type Culture Collection (Rockville, MD). RAW 264.7 cells were cultured in RPMI-1640 (without phenol red) supplemented with 10% endotoxin-free, heat-inactivated fetal calf serum (GIBCO, Grand Island, NY), 100 units/mL penicillin, and 100 mg/mL streptomycin. Once the cells reached a density of 2–3 $\times$ 10^6 cells/mL, they were activated by incubation in a medium containing *E. coli* LPS (lipopolysaccharide) (100 ng/mL). Various concentrations of test compounds dissolved in DMSO (dimethyl sulfoxide) were added together with LPS. Cells were treated with 0.05% DMSO as vehicle control (Pan et al., 2006).

2.2.6. Nitrite Assay.

The RAW264.7 cells, cultured as described above, were then treated with test compounds and LPS or LPS only. The supernatants were harvested and the amount of nitrite, an indicator of NO synthesis, was measured by the Griess reaction (4). Briefly, supernatants (100 μL) were mixed with the same volume of Griess reagent (1% sulphanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water) in duplicate on 96-well plates. After incubation at room temperature for 10 min, absorbance was measured at 570 nm with the ELISA reader (Thermo Labsystems Multiskan Ascent, Finland).

2.2.7. Western Blotting.

After treatment with test compounds for 24 h, total proteins were extracted via addition 200 μL of gold lysis buffer (50 mM Tris-HCl, pH 7.4; 1 mM NaF; 150 mM NaCl; 1 mM EGTA; 1 mM phenylmethanesulfonyl fluoride; 1% NP-40; and 10 μg/mL leupeptin) to the cell pellets on ice for 30 min, followed by centrifugation at 10,000 rpm for 30 min at 4 ℃. The cytosolic fraction (supernatant) proteins were determined by Bio-Rad Protein Assay (Bio-Rad Laboratories, Munich, Germany). The samples (50 μg of protein) were mixed with 1 mL of sample buffer containing 0.3 M Tris-HCl (pH 6.8), 25% 2-mercaptoethanol, 12% sodium dodecyl sulfate (SDS), 25mM EDTA, 20% glycerol, and 0.1% bromophenol blue. The mixtures were boiled at 100 ℃ for 5 min and subjected to 12% SDS-polyacrylamide mini-gels at a constant current of 20 mA. Following electrophoresis was carried out on SDS-polyacrylamide gels. Proteins on the gel were then electrotransferred onto an immobile membrane (PVDF; Millipore Corp., Bedford, MA) with transfer buffer composed of 25 mM Tris-HCl (pH 8.9), 192 mM glycine, and 20% methanol. The membranes were blocked with blocking solution containing 20 mM Tris-HCl, followed by immunoblotting with primary antibodies including iNOS, β-actin, and COX-2 (Transduction Laboratories, Lexington, KY). The blots were rinsed three times with PBST buffer for 10 min each, and then incubated with 1:5000 dilution of the horseradish peroxide (HRP)-conjugated secondary antibody (Zymed Laboratories, San Francisco, CA) before finally washing three times with PBST buffer. The transferred

proteins were visualized with an enhanced chemiluminescence detection kit (ECL; Amersham Pharmacia Biotech, Buckinghamshire, UK). Quantification of iNOS and COX-2 expression was normalized to β-actin using a densitometer (AlphaImagerTM 2200 System) as described (Pan et al., 2006).

2.2.8. Secretory Phospholipase A_2 Screening Assay.

The enzyme used in this research was the human type V secretory phospholipase A_2 ($sPLA_2$), with binding efficiently to phosphatidylcholine (PC) membranes and hydrolyze PC substrates. Type V $sPLA_2$ has been shown to be involved in eicosanoid formation in inflammatory cells including macrophages and mast cells, and suitable for acting on the outer plasma membrane (Han et al., 1999; Han, Yoon & Cho, 1998). This assay, available as a commercial $sPLA_2$ inhibitor screening assay kit (Cayman Chemical Co., Ann Arbor, MI), utilized 10 μL human Type V $sPLA_2$ enzyme dissolved in the assay buffer solution (25 mM Tris-HCl, 10 mM $CaCl_2$, 100 mM KCl, 0.3 mM Triton X-100 and 1 mg/mL BSA, pH 7.5) to a 96-well plate. 10 μL of the inhibitor in DMSO or blank DMSO solution as 100% initial activity were combined to the enzyme. The reactions were initiated by adding 200 μL of substrate, 1,2-dithio analog of diheptanoyl phosphatidylcholine (1.66 mM in the assay buffer) to each well. After incubation of 15 min at 25 ℃, Ellman's reagent (5,5'-dithio-bis-2-nitrobenzoic acid) was used to detect the free thiols hydrolyzed by $sPLA_2$ from the thio ester bond at the substrate's *sn*-2 position (**Fig. 2.1**). The UV

absorption at 410 nm was measured by UV spectrometer (Agilent Technologies, Waldbronn, Germany), and the percent initial activity was calculated by dividing the inhibitor sample value form the 100% initial activity sample value.

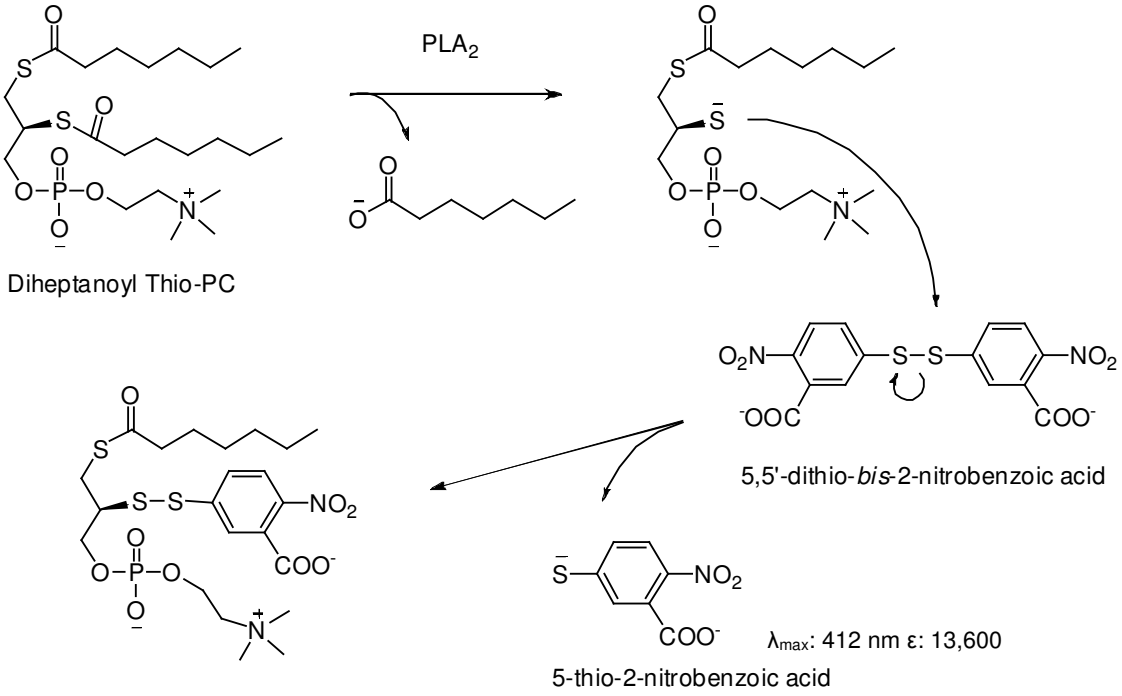

Figure 2.1. The reaction scheme of the $sPLA_2$ assay (Cayman Chemical Co., Ann Arbor, MI)

2.2.9. 5-Lipoxygenase Screening Assay.

Lipoxygenases (LOXs) are non-heme iron-containing dioxygenases that catalyzed the addition of oxygen molecular to fatty acids having a cis,cis-1,4-pentadien system (Yamamoto, 1992). The assay was carried out by using the commercial lipoxygenase inhibitor screening kit (Cayman Chemical Co., Ann Arbor, MI). 90 µL of potato 5-lipoxygenase dissolved in the assay buffer (0.1 M Tris-HCl, pH 7.4) was added to the wells of a 96-well plate. 10 µL of the inhibitors in DMSO or blank DMSO solution as 100% initial activity were combined to the enzyme. The reactions were initiated by adding 10 µL of substrate linoleic acid (1 mM) prior to shaking for 5 min. The product of the reaction, 4-hydroperoxy cis-trans-1,3-conjugated phtadienyl moiety within the unsaturated fatty acid, was detected at 410 nm by UV spectrometer (Agilent Technologies, Waldbronn, Germany) after adding 100 µL of Chromgen to each well. The percent initial activity was calculated by dividing the inhibitor sample value form the 100% initial activity sample value.

2.3. RESULTS AND DISCUSSION

2.3.1. Preliminary Tests on Oregano Extract.

The main non-volatile compounds identified in *Origanum* spp. to date are galangin, quercetin, carnosol, caffeic acid, rosmarinic acid (Kato & Shimo, 1987; Kulisic et al., 2004) and two phenolic glycosides 4'-*O*-β-D-glucopyranosyl-3',4'-dihydroxybenzyl protocatechuate and 4'-*O*-β-D-glucopyranosyl-3',4'-dihydroxybenzyl 4-*O*-methylprotocatechuate isolated from *O. vulgare* (Matsuura et al., 2003). There are few reports on additional bioactive polyphenols in oregano that are different and distinct from the well known essential oils (volatile aromatic compounds), the latter which has been well studied. No studies have yet shown that oregano has anti-inflammatory activity and thus which compounds may induce such activity *in vitro*. The species we first investigated was Greek oregano (*O. vulgare* ssp. *hirtum*), which Rutgers had been breeding and which we found to exhibit a high phenolic content level (5.3% gallic acid equivalent on dry weight basis), as determined by the Folin-Ciocalteu's method. Results from the DPPH free radical scavenging assay showed the whole oregano extract possessing a comparable antioxidative activity to trolox, a recognized free radical scavenger. These preliminary data suggested that this Greek oregano sample might contain high concentrations of phenolic compounds, either phenolic acids, flavonoids or tannins. The LPS-induced nitrite test suggested both crude plant extracts of Greek

oregano (*O. vulgare* ssp. *hirtum*) and Syrian oregano (*O. Syriacum*) showed significant anti-inflammatory effect (**Fig. 2.2**), however, the compounds responsible for the anti-inflammatory activities were unknown and therefore of interest. Starting from the whole plant extract, we applied chromatographic isolation on silica gel and separated the alcoholic oregano extract into sixteen fractions eluted by solvent system with different polarities including hexane, hexane-EtOAc, EtOAc, EtOAc-acetone, acetone, acetone-methanol and methanol. We also applied nitrite generation assay as the bioactivity-guided isolation at each separation stage to track the potential anti-inflammatory compounds in oregano.

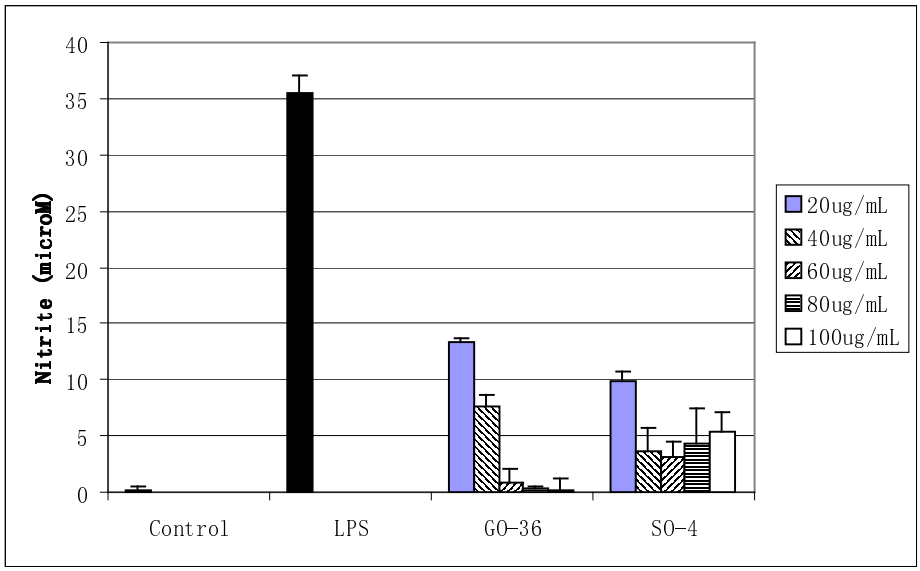

Figure 2.2. Anti-inflammatory effect of oregano crude extract GO-36 (*Origanum vulgare* ssp. *hirtum*) and SO-4 (*O. syriacum*) on LPS-induced nitrite production in RAW 264.7 macrophages. The cells were treated with 100 ng/mL of LPS only or with different concentrations (20-100 μg/mL) of oregano extracts for 24 h. At the end of incubation time, 100 μL of the culture medium was collected for nitrite assay. The values are expressed as means of triplicate tests.

2.3.2. LC/UV/MS Guided Preparative HPLC Separation.

Preparative HPLC was used for further compound isolation from each fraction. Currently, preparative HPLC is the most powerful and versatile method for purification tasks in the natural products research and pharmaceutical industry (Nuengchamnong et al., 2005).

Coupled with UV detection and fraction collector, this analytical approach provides large scale and automated separation. Traditionally, researchers use analytical HPLC/UV as the initial method development, and then scale-up to preparative HPLC for the production-scale work. The criterion is that the stationary phase and column length should be the same between analytical HPLC column and preparative column. However, the chemical profiles in natural plants are complex, and many natural products such as sugars, steroids and some saponins exhibit no UV absorption. Therefore, these compounds are invisible on a UV detector and cause the failure of purification. To address this limitation, here, we created a novel methodology: LC/UV/MS guided preparative HPLC separation, since the MS chromatogram provides an alternative and sensitive detection. With the HPLC/UV/MS as an initial method development, interference of "invisibles" can be avoided and a real separation was achieved. This approach improved the efficiency of the whole compound isolation process.

2.3.3. Identification of Rosmarinic Acid, Oleanolic Acid and Ursolic Acid.

The data from the nitrite assay suggested that Fraction 2 and Fraction 7 of the oregano extract possessed potent anti-inflammatory activities (**Fig. 2.3**). From Faction 2, we isolated two triterpenoid acids showing a potent anti-inflammatory activity, oleanolic acid and ursolic acid, by using preparative HPLC isolation. Upon mass spectra, they showed identical molecular ions and fragmentation patterns. The ion peaks at *m/z* 479 were the

sodium adduct molecular ions $[M + Na]^+$ of oleanolic acid and ursolic acid. Dehydration (*m/z* 439) and decarboxylic acid (*m/z* 411) products were observed as MS fragments. The fragmentation ions at *m/z* 191 and *m/z* 203 were due to RDA reactions, the characteristic MS fragmentation of Δ^{12}-unsaturated triterpenoids. The structures of oleanolic acid and ursolic acid were further elucidated by NMR data (**Table 2.1**), and compared with literature (Seto et al., 1986; Ahmad et al., 1993; Huang, Wang & Lin, 1999; Alves et al., 2000). From Oregano Fraction 7, we obtained rosmarinic acid from HPLC/UV/MS guided preparative HPLC isolation, which also plays an important role in the anti-inflammatory effect of oregano extract. Rosmarinic acid provided molecular ion at *m/z* 359 ($[M - H]^-$) on mass spectra under negative ion mode, and fragmentation ions at *m/z* 197 ([salvianic acid A - H] $^-$), *m/z* 179 ([M - H - caffeic acid]$^-$) and *m/z* 161 ([M - H - salvianic acid A]$^-$) for rosmarinic acid. The comparison of the retention time with the commercial reference compound on HPLC further supported the identification of rosmarinic acid in our oregano sample.

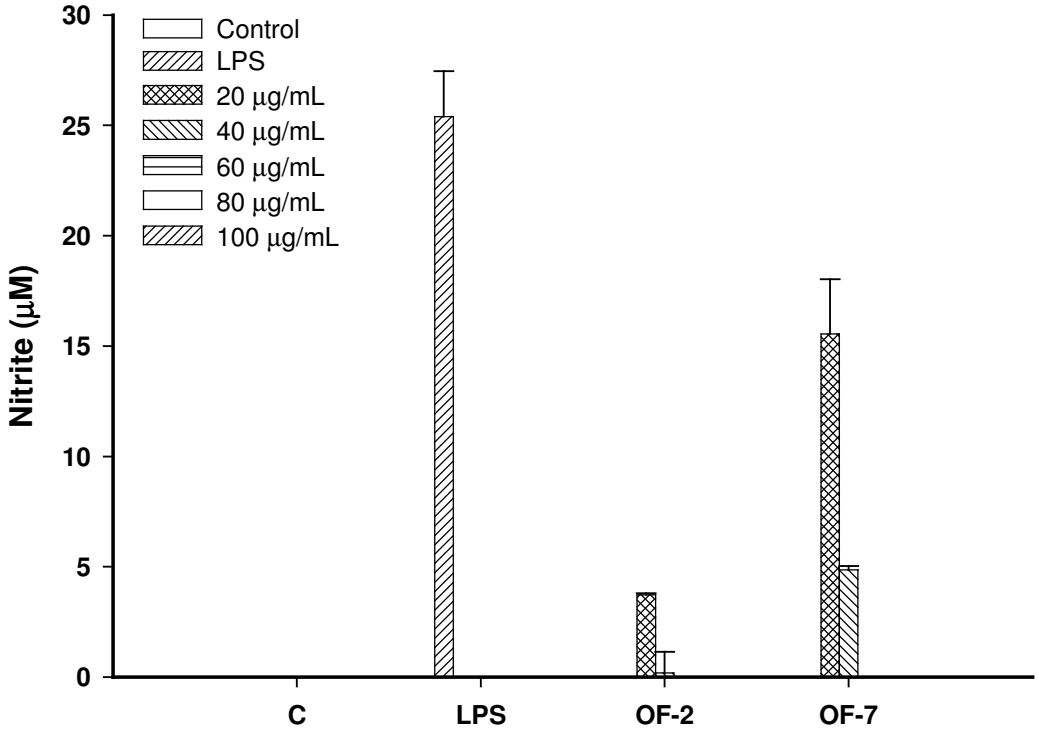

Figure 2.3. Anti-inflammatory effect of Oregano Fraction 2 (OF-2) and Fraction 7 (OF-7) on LPS-induced nitrite production in RAW 264.7 macrophages. The cells were treated with 100 ng/mL of LPS only or with different concentrations (20-100 µg/mL) of oregano extracts for 24 h. At the end of incubation time, 100 µL of the culture medium was collected for nitrite assay. The values are expressed as means of triplicate tests.

Table 2.1. ^{13}C and ^{1}H NMR Assignments for Oleanolic Acid and Ursolic Acid (in Pyridine-d_5)

	oleanolic acid		ursolic acid	
position	$\delta^{13}C$	$\delta^{1}H$	$\delta^{13}C$	$\delta^{1}H$
1	37.8		38.3	
2	27.0		27.1	
3	77.0	3.44 m	77.1	3.46 m
4	38.3		38.0	
5	54.7	0.83 brs	54.8	0.83 brs
6	17.7		17.7	
7	32.1		32.5	
8	38.7		38.9	
9	47.0		47.0	
10	36.3		36.2	
11	22.6		22.6	
12	122.9	5.49 brs	124.6	5.49 brs
13	143.6		138.2	
14	41.1		41.5	
15	27.6		27.6	
16	22.7		23.9	
17	45.5		47.0	
18	40.9		52.5	2.64 d (J = 11.2 Hz)
19	45.4		38.3	
20	29.8		38.4	
21	33.1		30.0	
22	32.1		36.4	
23	27.2	1.27 s	27.8	1.24 s
24	15.4	1.02 s	15.5	1.02 s
25	14.4	0.88 s	14.6	0.89 s
26	16.3	1.02 s	16.4	1.05 s
27	25.0	1.23 s	22.9	1.22 s
28	179.3		178.9	
29	32.2	0.93 s	16.5	1.00 d (J = 7.2 Hz)
30	22.6	1.00 s	20.4	0.94 d (J = 5.6 Hz)

2.3.4. Anti-inflammatory Activities of Rosmarinic Acid, Oleanolic Acid and Ursolic Acid on Nitrite Assay and Synergistic Study.

We first applied a nitrite assay to the individual pure compounds rosmarinic acid, oleanolic acid and ursolic acid, as well as to combinations of the three acids. In the combination of compounds, we used mixture of two compounds as well as the combination of the three compounds at the percentage of 40% rosmarinic acid + 20% oleanolic acid + 40% ursolic acid (This ratio reflects the natural distribution of theses compounds in *O. syriacum*) (**Fig. 2.4**). Results showed that each of these organic acids exhibited strong anti-inflammatory effect on the nitrite generation assay (**Table 2.2**). Moreover, we found a synergistic action from the combination of these three compounds based upon this bioassay. The combination of the three compounds, rosmarinc acid, oleanolic acid and ursolic acid, in a particular ratio (40% rosmarinic acid + 20% oleanolic acid + 40% ursolic acid) may lead to an even higher level of potency (IC_{50} = 17.7 μg/mL) on nitrite production assay than any single compound (**Fig. 2.5**).

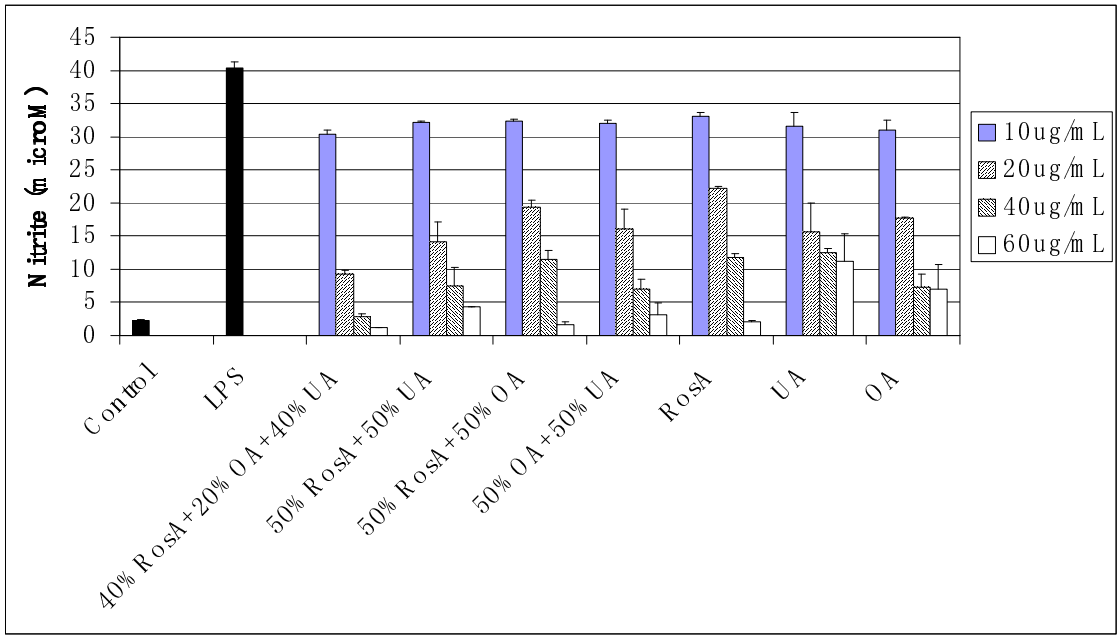

Figure 2.4. Anti-inflammatory effect of rosmarinic acid (RosA), ursolic acid (UA), oleanolic acid (OA) and combinations of these compounds on LPS-induced nitrite production in RAW 264.7 macrophages. At the end of incubation time, 100 µL of the culture medium was collected for nitrite assay. The values are expressed as means of triplicate tests.

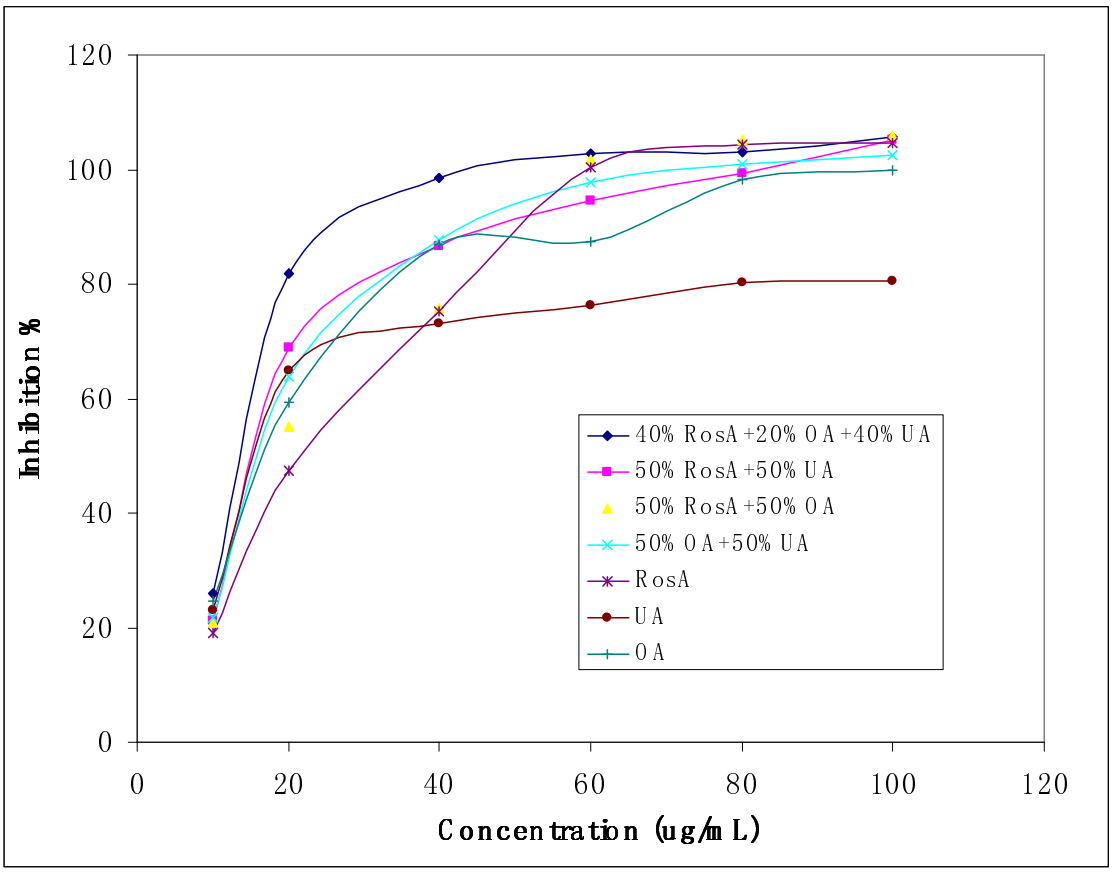

Figure 2.5. Inhibition of rosmarinic acid (RosA), ursolic acid (UA), oleanolic acid (OA) and combinations of these compounds on LPS-induced nitrite production in RAW 264.7 macrophages

Table 2.2. Effect of Rosmarinic Acid, Ursolic Acid, Oleanolic Acid and the Combinations of Theses Compounds on LPS-induced Nitrite Production in RAW 264.7 Macrophages[a]

Conc. (μg/mL)	LPS (100ng/mL)	40%RosA +20%OA+ 40%UA	50%RosA +50%UA	50%RosA +50%OA	50%UA+ 50%OA	RosA	UA	OA	Indometha-cin
control	-	2.27±0.07	2.27±0.07	2.27±0.07	2.27±0.07	2.27±0.07	2.27±0.07	2.27±0.07	2.27±0.07
	+	40.33±0.89	40.33±0.89	40.33±0.89	40.33±0.89	40.33±0.89	40.33±0.89	40.33±0.89	40.33±0.89
10	+	30.47±0.45	32.25±0.02	32.38±0.19	32.11±0.44	33.06±0.64	31.56±2.12	30.97±1.54	39.02±2.38
20	+	9.19±0.69	14.11±3.02	19.40±0.99	16.08±3.02	22.27±0.17	15.59±4.44	17.72±0.23	25.67±3.13
40	+	2.84±0.40	7.39±2.86	11.49±1.39	6.94±1.56	11.74±0.69	12.52±0.52	7.23±2.08	17.60±1.68
60	+	1.20±0.06	4.32±0.06	1.57±0.57	3.13±1.73	2.07±0.23	11.25±4.17	7.07±3.59	9.24±1.80
80	+	1.16±0.58	2.48±1.04	0.30±0.05	1.90±1.04	0.63±0.40	9.81±2.49	2.93±0.75	7.64±0.93
100	+	0.14±0.06	0.26±0.14	0±0.11	1.28±0.28	0.47±0.28	9.64±4.34	2.35±0.17	6.33±1.28

[a]The values are expressed as means of triplicate tests.

2.3.5. Western Blotting on iNOS and COX-2 Protein Levels.

RAW 264.7 cells were further treated with the eight samples, DS-1 (40% rosmarinic acid + 20% oleanolic acid + 40% ursolic acid), DS-2 (50% rosmarinic acid + 50% ursolic acid), DS-3 (50% rosmarinic acid + 50% oleanolic acid), DS-4 (50% ursolic acid + 50% oleanolic acid), DS-5 (rosmarinic acid), DS-6 (ursolic acid), DS-7 (oleanolic acid) and DS-8 (idomethacin). Whole-cell lysates were prepared to analyze iNOS and COX-2 protein expression by western blotting. The β-actin protein level was analyzed as a loading control. Equal amounts of total proteins (50 μg) were subjected to 10% SDS-PAGE. The expression of iNOS, COX-2 and β-actin protein was detected by

Western blot using specific antibodies. Quantification of iNOS and COX-2 protein expression was performed by densitometric analysis of the immunoblot. Results showed both iNOS and COX-2 proteins were inhibited by rosmarinic acid, ursolic acid, oleanolic acid and the combinations of these compounds (**Fig. 2.6**). These findings are consistent with the literature that they possessed COX-2 inhibition (Ringbom et al., 1998; Kelm et al., 2000). A previous study compared the COX-2/COX-1 selectivity ratios of ursolic acid and oleanolic acid to those of the traditional NSAIDs, reporting the following rank order: ursolic acid > oleanolic acid > ibuprofen > naproxen > indomethacin (Ringbom et al., 1998). Rosmarinic acid was described as possessing a slightly higher level of COX-2 inhibition than that of COX-1 (Kelm et al., 2000). As often the case that a single phytochemical may affect more than one cellular process involved in anti-inflammatory cell signaling pathway (Yoon & Park, 2005), we sought to examine whether additional anti-inflammatory mechanisms of rosmarinic acid, oleanolic acid and ursolic acid were active.

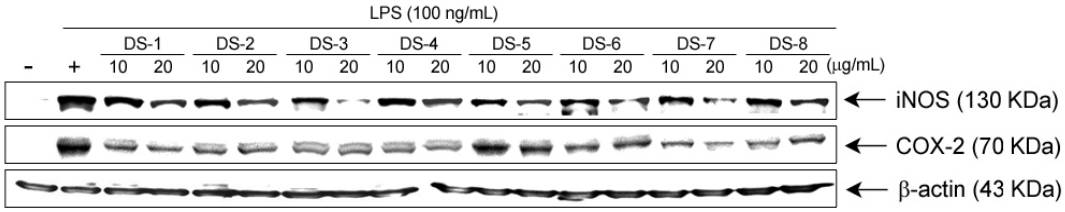

Figure 2.6. Effect of rosmarinic acid (RosA), ursolic acid (UA), oleanolic acid (OA) and their combinations on LPS-induced iNOS and COX-2 protein levels in RAW 264.7 cells.

DS-1: 40% rosmarinic acid + 20% oleanolic acid + 40% ursolic acid;
DS-2: 50% rosmarinic acid + 50% ursolic acid;
DS-3: 50% rosmarinic acid + 50% oleanolic acid;
DS-4: 50% ursolic acid + 50% oleanolic acid;
DS-5: rosmarinic acid; DS-6: ursolic acid; DS-7: oleanolic acid;
DS-8: indomethacin

2.3.6. Secretory Phospholipase A_2 Inhibitor Screening.

Phospholipase A_2 catalyzes the hydrolysis of phospholipids and yields free fatty acid such as arachidonic acid (AA) from the phospholipid layers of the plasma membrane (Dennis, 1983). Arachidonic acid is then metabolized through either the cyclooxygenase pathway to produce prostaglandins (PGs) and thromboxane A2 or the lipoxygenase pathway to generate hydroperoxyeicosateraenoic acids (HETEs) and leukotrienes (LTs) (Dennis, 1983). PLA_2 enzymes can be further classified into secretory PLA_2 ($sPLA_2$), intracellular

PLA_2 ($iPLA_2$) and cystolic PLA_2 ($cPLA_2$) depending on the properties on tissue distribution, expression, calcium requirement and substrate specificity (Chakraborti, 2003). The release of AA from membrane phospholipids by PLA2 is believed to be a key step in the control of eicosanoid production within the cell and as a means to induce the inflammation mediator synthesis (Dennis, 1994). $sPLA_2$s are proteins of relatively low molecular mass, highly enriched in disulfide bonds, and require millimolar amounts of calcium for activity. In the screening for potential inhibitors of $sPLA_2$, we did not find that any of these three organic acids rosmarinic acid, ursolic acid and oleanolic acid possessed comparable activities to the comtrol thioetheramide-PC (**Fig. 2.7**). In comparison, other phytochemicals including glucocorticoids and curcumin have been reported as $sPLA_2$ inhibitors (Lee et al., 1998; Hong et al., 2004).

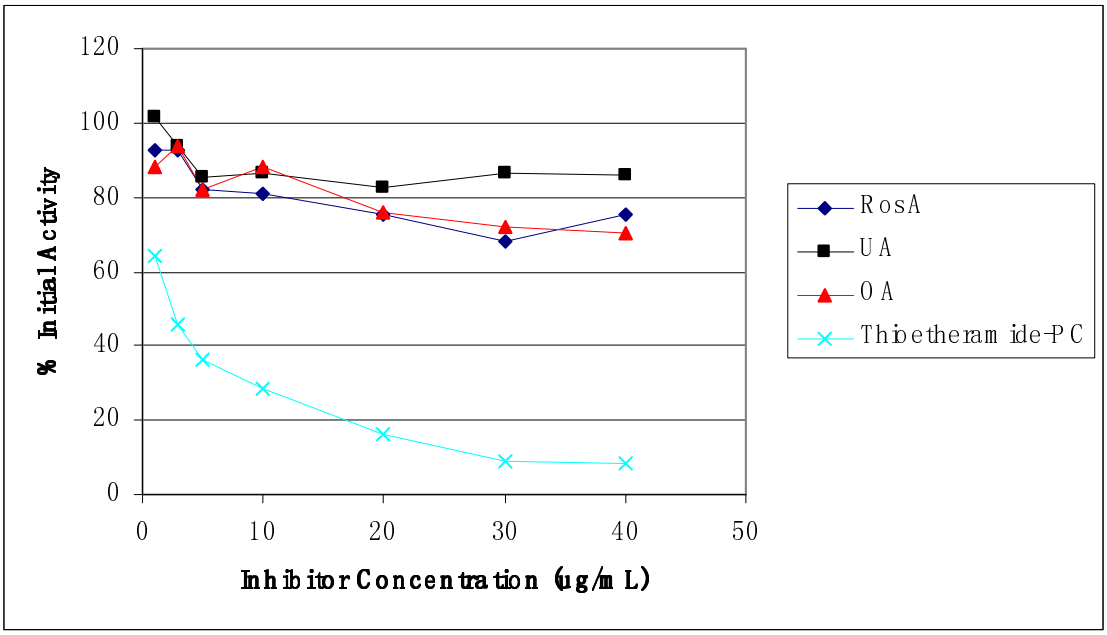

Figure 2.7. Inhibition of secretory phospholipase A_2 by rosmarinic acid (RosA), ursolic acid (UA), oleanolic acid (OA) and thioetheramide-PC (control) at different concentrations. The substrate concentration of diheptanoyl-thio PC in the assay was 1.44 mM.

2.3.7. 5-Lipoxygenase Inhibitor Screening.

Products from the lipoxygenase pathway have also been shown to be the key players in the inflammation process. Much research has been aimed to discover selective inhibitors of the enzymes involved in the biosynthetic pathway deriving from arachidonic acid to

the leukotrienes (Drazen, Israel & O'Byrne, 1999; Poff & Balazy, 2004). The inhibition of 5-lipoxygenase holds promise for therapeutic intervention in diseases characterized by leukotriene mediated inflammation, and several triterpenoid acids including boswellic acid were reported as potent 5-LOX inhibitors (Safayhi et al., 2000; Sailer et al., 1998). Thus we applied a 5-LOX assay to screen for the potential inhibitory activities of rosmarinic acid, oleanolic acid and ursolic acid on the leukotriene pathway. Quercetin, a reported 5-LOX inhibitor, was used as control on the bioassay (Yoon & Beak, 2005). Rosmarinic acid and oleanolic acid exhibited comparable 5-lipoxygenase inhibitory activities to quercetin (**Fig. 2.8**) (**Table 2.3**). Interestingly, ursolic acid showed no activity in this bioassay, though chemically this compound is very close to oleanolic acid, a position isomer of oleanolic acid with only a single methyl group difference.

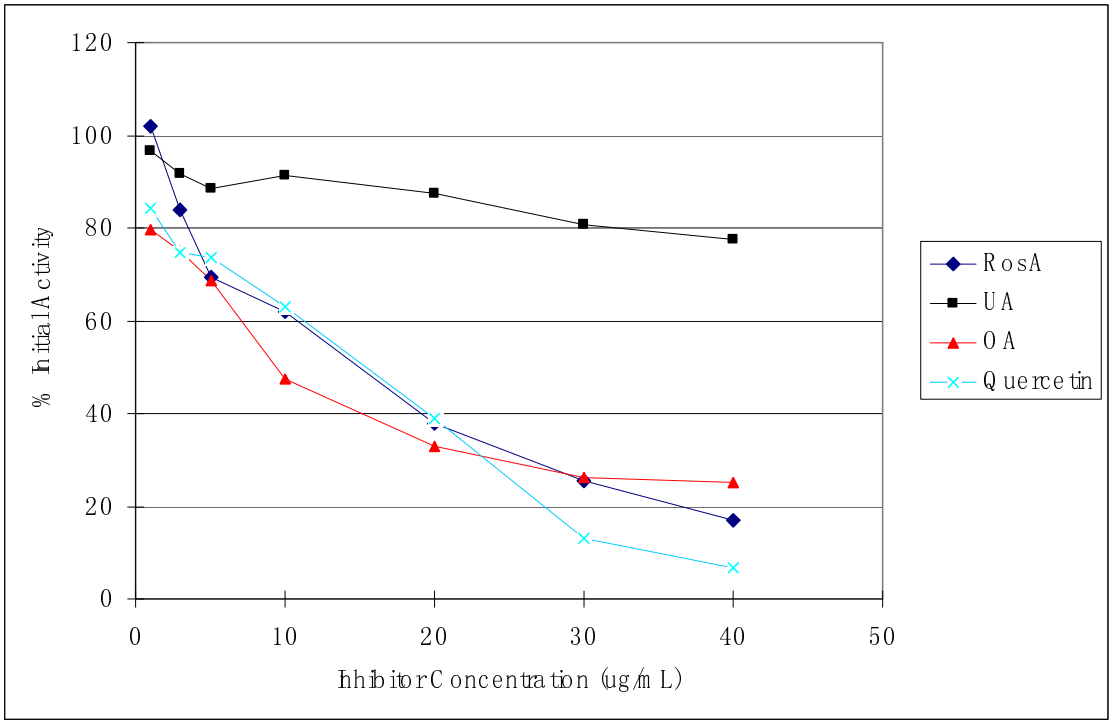

Figure 2.8. Inhibition of 5-lipoxygenase by rosmarinic acid (RosA), ursolic acid (UA), oleanolic acid (OA) and quercetin (control) at different concentration levels. The substrate concentration of linoleic acid in the assay was 100 µM.

Table 2.3. Inhibition of 5-Lipoxygenase by Rosmarinic Acid (RosA), Ursolic Acid (UA), Oleanolic Acid (OA) and Quercetin (Control) at Different Concentration Levels

Conc.	% initial activity			
(µg/mL)	RosA	UA	OA	Quercetin
20	102.1	96.6	79.5	84.1
60	83.8	91.6	75.1	74.6
100	69.3	88.3	68.8	73.5
200	62.0	91.4	47.3	63.0
400	37.8	87.3	33.0	39.0
600	25.3	80.7	26.2	13.0
800	17.0	77.6	25.1	6.6

2.3.8. The *in vivo* Anti-inflammatory Activities of Rosmarinic Acid Reported in Literature.

An early study reported an inhibitory effect of rosmarinic acid on complement-dependent stimulation of prostacyclin biosynthesis in human blood serum model (Rampart et al., 1986). Rosmarinic acid has been described to have antiviral and anti-inflammatory effects in the treatment of Japanese encephalitis in mice (Swarup et al., 2007), and thus significantly decreased in viral loads ($p < 0.001$) and proinflammatory cytokine levels ($p < 0.01$) in JEV-infected animals. Hur et al. (2007) observed apoptotic activity of rosmarinic acid through mitochondrial pathway, by inducing the release of cytochrome C from mitochondrial and the blockage of mitochondrial depolarization inhibited apoptosis from rheumatoid arthritis patients. Rosmarinic acid in *Perilla fructescens* extract has been reported to inhibit allergic inflammation in a mouse model (Sanbongi et al., 2004). A possible mechanism was suggested by the researchers that oral administration of perilla had an intervention effect through the amelioration of increases in cytokines, chemokines and allergen-specific antibody. The therapeutic potential of rosmarinic acid was assessed in an inflammatory autoimmune arthritis model, showing repeated administration of rosmarinic acid dramatically reduced the arthritic index and retaining nearly normal architecture of synovial tissues in mice (Youn et al., 2003). The author also reported that the synovial tissues from rosmarinic acid treated mice displayed reduced frequency of

COX-2-expressing cells compared with the control.

2.3.9. The *in vivo* and *in vitro* Anti-inflammatory Activities of Oleanolic Acid and Ursolic Acid Reported in Literature. An anti-inflammatory effect is found in many natural triterpenoids. Gupta et al. (1969) first reported the anti-inflammatory activity of oleanolic acid on formaldehyde-induced arthritis in 1960s. Ursolic acid is a position isomer of oleanolic acid, showing similar bioactivity as oleanolic acid. Ursolic acid was identified as an inflammatory component of *Pyrola rotundifolia* in inhibiting carrageen-induced paw edema in rats in 1980s (Kosuge et al., 1985). Subsequent research on the mechanisms of anti-inflammatory effects of oleanolic acid and ursolic acid showed the inhibition of histamine release from mast cells, inhibition of lipoxygenase and cyclooxygenase proteins, inhibition of elastase in tissue inflammatory response in rheumatic diseases, and inhibition of cytochrome P450 activities in human liver microsomes (Liu, 1995; Kim et al., 2004). More recently, oleanolic acid was described to attenuate capsaicin-induced nociception in mice due to mechanisms possibly involving endogenous opioids, nitric oxide and K_{ATP}-channel opening (Maia et al., 2006). In one study on the measurement of the cytokines in adjuvant inflammatory arthritis induced mice, researchers found ursolic acid inhibited arthritis through the mechanism of Th1/Th2 cytokine alteration (Ahmad et al., 2006). Although oleanolic acid and ursolic acid are structurally similar, research was reported that showed oleanolic acid and ursolic acid regulated differently in the process of epidermal keratinocytes via PPAR-gamma

pathway (Lee et al., 2006). Oleanolic acid was shown to be more active than ursolic acid in stimulating PPAR-gamma activity in CV-1 cells (Lee et al., 2006).

2.4. PHARMACOKINETIC FEATURES OF ROSMARINIC ACID, OLEANOLIC ACID AND URSOLIC ACID

Studies in rats have shown that orally administered rosmarinic acid is present in plasma as intact and conjugated forms such as methylated rosmarinic acid, glucuronide and sulfate (Nakazawa & Ohsawa, 1998; Baba et al., 2004). Rosmarinic acid, methyl-rosmarinic acid, caffeic acid, ferulic acid and *m*-coumaric acid were detected being excreted in the urine after oral administration. These metabolites in plasma and urine of rats were predominantly present as conjugated forms. Topical administration of rosmarinic acid to the skin of rats showed percutaneously absorption and distribution in skin, blood, bone and muscle (Ritschel et al., 1989). Intravenous administration of rosmarinic acid led to the distribution in various tissues such as lung, spleen, heart and liver. Absorption and metabolism of rosmarinic acid after intake of *Perilla frutescens* extract was recently investigated in humans (Baba et al., 2005). Briefly this study involved six healthy men that were orally administered with *Perilla frutescens* containing 200 mg rosmarinic acid and placebo with a 10 day interval between treatments. Blood

and urine samples were then collected at designated intervals and analyzed for rosmarinic acid and related metabolites by LC/MS. Results showed total rosmarinic acid (free and conjugated) in plasma reached the maximum level of 1.15±0.28 μmol/L within 0.5 h. Total methyl-rosmarinic acid reached a maximum level of 0.65±0.07 μmol/L after 2 h of intake. The major degraded metabolite was conjugated ferulic acid with a maximum level of 0.36±0.17 μmol/L within 0.5 h in plasma. The main metabolites found in the urine were free and sulfoglucuronide conjugates of rosmarinic acid and methyl-rosmarinic acid, and sulfate conjugates of caffeic acid and ferulic acid. The sum of the excreted rosmarinic acid and its related metabolites was 6.3±2.2% of the total dose.

The pharmacokinetic studies of ursolic acid orally administered on rats were reported at a dose level of 80.32 mg/kg (Liao et al., 2005). The peak plasma concentration (C_{max} = 294.8 ng/mL) was reached at 1.0 h, and the half-life ($t_{1/2}$) of ursolic acid was 4.3 h. A similar *in vivo* study was performed on oleanolic acid to rats (Jeong et al., 2007). The main pharmacokinetic parameters through oral administration at 50 mg/kg were obtained as C_{max} = 132.0 ng/mL, T_{max} = 21.0 min and $t_{1/2}$ = 65.3 min. The research on determination of oleanolic acid in human plasma was recently reported in Chinese healthy male volunteers (Song et al., 2006). At an oral dose level of 40 mg, oleanolic acid reached a maximum plasma concentration at 12.12 ng/mL within 5.2 h. The half-life ($t_{1/2}$) was calculated as 8.73 h.

The challenge is to recognize the differences in direct ingestion of food and spice versus the consumption of a concentrated extract or pure compounds. In the normal consumption of a food stuff and spice/condiment (as is oregano), the "phytopharmaceuticals" are consumed in a significantly dilute concentration and in a complex food matrix. The use of a concentrated extract, the pure single compound or blended pure compounds could have significantly different bioactivity responses relative to safety and efficacy. Drugs, administrated in concentrated dose, can readily saturate the metabolic pathways. In contrast, food ingredients would be expected to be largely conjugated (Karakaya, 2004). When these ingredients are administered at pharmacological doses, they are usually found in the free form in the blood. The dose may also determine the primary site of metabolism, as evidence by the fact that large doses are metabolized mainly in the liver and small doses may be metabolized in the intestinal mucosa (Karakaya, 2004).

Many studies revealed that acidic anti-inflammatory drugs would accumulate in certain body compartments and reach high concentration in the inflamed tissue (Dencker, Lindquist & Ullberg, 1975; Waser & Nickel, 1969). Tissue selectivity was achieved by an acidic COX inhibitor Phenylbutazone while the non-acidic control Propylphenazone was distributed equally throughout the body (Brune, Gubler & Schweitzer, 1979; Rainsford, Schweitzer & Brune, 1981). Similar observations on a COX-2 inhibitor lumiracoxib (acidic) concentrated in inflamed tissue were reported compared to the homogeneous

distribution of a non-acidic inhibitor Celecoxib (Weaver et al., 2003). The anti-inflammatory ingredients in oregano (rosmarinic acid, oleanolic acid and ursolic acid) fulfill these criteria (acids), being the preferred chemical forms in inflammatory pain. The mechanism of tissue selectivity was proposed that weakly acidic drugs tend to highly bind to plasma proteins, resulting in a negative charge in the neutral pH blood (Brune & Furst, 2007). This property limits the exit of the drug from the plasma into most normal tissues by a closed endothelial layer of blood vessels. However, the endothelium becomes porous at the inflamed tissue, allowing oedema to form and protein-bound or unbound drugs to exit to the tissue. Moreover, the inflamed tissue showed a mildly acidic extracellular pH, reducing the plasma binding and increasing the free form of the drug. The non-ionic diffusion facilitates the drug into the cell interior and subsequently increases the intracellular drug concentration due to ion trapping.

2.5. COMPOUND SAFETY EVALUATION

Pereira et al. (2005) investigated and reported on the neurobehavioral aspect of rosmarinic acid on elevated plus-maze, step-down inhibitory avoidance and open field task in rats. The genotoxic effect on brain tissue was evaluated using the comet assay. Rosmarinic acid was found to increase the number of entries in the open arms without

affecting the short-term and long-term memories in lower doses, indicating this compound can generate anxiolytic-like effect without exerting locomotor alterations of DNA damage in brain tissue. The results from comet assay suggested that rosmarinic acid did not exhibit genotoxic activities in both the brain tissue and peripheral blood.

Oleanolic acid and ursolic acid are believed to be relatively non-toxic (Liu 1995). Oleanolic acid has been patented in Japan as a health-promoting additive to drinks, and also been marked in China as a non-prescription drug for the treatment of liver disorders (Liu 1995). A short-term safety evaluation (> 3 months) was conducted on 188 patients during the treatment of chronic hepatitis with oleanolic acid, indicating the absence of apparent side effects on this compound (Xu, 1985). A recent study performed on micronucleus test in peripheral blood and bone marrow of Balb/c mice showed both oleanolic acid and ursolic acid had protective effects on DNA mutation. This evidence would further support the fact that they are free from carcinogeneticity according to the compound safety (Resende et al., 2006).

Based upon the findings reported in the literature, rosmarinic acid, oleanolic acid and ursolic acid appear to be low-toxicity agents, and oregano is known as a food seasoning in cuisine. However, the safety issues for oregano extract at concentrated level still need to be investigated in order to develop anti-inflammatory dietary supplement products.

2.6. CONCLUSIONS

We successfully identified the anti-inflammatory constituents in oregano as romarinic acid, oleanolic acid and ursolic acid. Oleanolic acid and ursolic acid exist in many other plant species (Liu, 1995), but we are the first to demonstrate their their presence in the genus *Origanum*, and that these compounds are responsible for the anti-inflammatory property of oregano. The three organic acids identified in oregano were tested on the LPS-induced nitrite production assay and the Western Blotting of LPS-induced iNOS and COX-2 protein levels in murine cells and all showing stronger or comparable anti-inflammatory activities compared to the control indomethacin. Subsequently, we found the combination of the three or two ingredients at certain percentage may bring the activity to an even higher level of potency on nitrite production assay.

From these observations, we can conclude that oregano, particular those chemotypes with a high concentration of these bioactive phenolic acids, could be suitable candidates from which to develop a dietary supplement product as a potential complimentary and alternative Phytomedicine. Given the preliminary evidence of the plants safety, its current uses, and now our discovery as to the confirmation of the plant extracts anti-inflammatory activities and the actual compounds responsible for this activity, further work is needed on developing a process of extracting the bioactive water-soluble compounds while still recovering the essential oil using steam or hydro-distillation

material of oregano.

Lastly, in this study we provide the results of using a high bioactive oregano. However, we also recognized that oreganos varied significantly in the expression and concentration in such compounds as influenced by both the genetics of the plant and the environment under which the plant grows. Thus, further work is also needed to conduct an analytical survey and investigation on the genetic diversity relative to the presence and concentration of these three anti-inflammatory compounds distributed between *Origanum* species and within a species.

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CHAPTER 3. DEVELOPMENT OF ANALYTICAL METHOD (LC/MS-SIM MODE) ON SIMULTANEOUS DETERMINATION OF ANTI-INFLAMMATORY CONSTITUENTS IN ORIGANUM SPP.

3.1. INTRODUCTION

The chemical components in oregano volatile oils have long been to interest researchers for their aromatic flavoring, antioxidative, antibacterial and antiseptic properties. This genus is well recognized for its great diversity in aroma and flavor from the myriad of chemotypes available and from which many new varieties have been bred (Kulisic et al., 2004; Rodrigues et al., 2004; Velluti et al., 2004). Recently, the water-soluble extract of oregano was reported to inhibit COX-2 secretion showing anti-inflammatory activity in human epithelial carcinoma cells (Lemay, 2006). Yoshino et al. (2006) found that oregano extract exhibited anti-inflammatory activities in mouse models of stress-induced gastritis and contact hypersensitivity. Moreover, the effect of methanol and aqueous methanol extract of *O. vulgare* ssp. *hirtum* on soybean lipoxygenase was noted, revealing a promising potential of oregano for anti-inflammatory efficacy (Koudoulitsa et al., 2006). However, these anti-inflammatory studies were all performed on oregano crude extracts without any information as to what compounds may be responsible, and no research to date has brought these into a molecular level, linking the bioactivities to specific compounds from oregano extracts to the reported anti-inflammatory activity.

In the prior chapter, we described successful identification of the anti-inflammatory constituents in oregano as rosmarinic acid, oleanolic acid and ursolic acid by bioactivity-guided isolation. These three organic acids were tested on the LPS-induced

nitrite production assay and the Western Blotting of LPS-induced iNOS and COX-2 protein levels in murine cells and all showing stronger or comparable anti-inflammatory activities compared to the control indomethacin, a recognized anti-inflammatory agent (Ismaili et al., 2001). Rosmarinic acid is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid typically found in Lamiaceae plants such as basil (*Ocimum* spp.), rosemary (*Rosmarinus* spp.), thyme (*Thymus* spp.) and mint (*Mentha* spp.) (Petersen & Simmonds, 2003). This phenolic compound may function as an antioxidant, scavenging superoxide and hydroxyl radicals, and inhibiting oxidation of low–density lipoproteins (Nakamura et al., 1998; Fuhrman et al., 2000). The anti-inflammatory properties were described by the inhibition of lipoxygenases and cyclooxygenases (Parnham & Kesselring, 1985; Yamamoto et al., 1998). Oleanolic acid and its isomer, ursolic acid, are triterpenoids which exist in the plant kingdom as free acids or in the conjugated form known as triterpenoid saponins (Liu, 1995). The compound oleanolic acid has been patented in Japan as a health-promoting additive to drinks, and marketed in China as a safe non-prescription drug for treatment of liver disorders (Chen et al., 2007). Many triterpenoids possess anti-inflammatory effects, and oleanolic acid and ursolic acid are among the most notable bioactive triterpenoids (Price, Johnson & Renwick, 1987; Mahato, Sarkar & Poddar, 1988). The anti-inflammatory mechanism of oleanolic acid and ursolic acid is postulated as simultaneously affecting multiple targets in one or more signaling pathways (Dai et al., 1989; Tsuruga et al., 1991; Simon et al., 1992; Najid et al., 1992; Zhou et al., 1993; Ying et al., 1991; Dai, Hang & Tan, 1989; Kapil & Shanna,

1994).

Few papers have described the simultaneous quantitation of oleanolic acid and ursolic acid (Chen, Xia & Tan, 2003; Altinier et al., 2007), since these two organic acids are position isomers, and the only difference between the two is a single methyl group on ring E (Me-30 versus Me-29), which makes their separation for analytical purposes challenging. As we know, the selectivity of MS detection is based on the molecular ion and/or the fragments of analytes, while collision induced dissociation of MS detection provides identical molecular ion and fragmentation patterns for these two isomers, so that the MS separation under selected ion monitoring (SIM) or multiple reaction monitoring (MRM) mode can not be achieved unless they are base line separated on the HPLC column. Rosmarinic acid differs largely from these two triterpenoid acids relative to chemical polarity, and to date no HPLC method has been reported for the quantification of these three organic acids together in a single run. Therefore, the present work aimed to develop an analytical method to simultaneously quantitate these anti-inflammatory organic acids which was finally achieved by using a tandem column system coupled with MS detector.

We collected and grew in a single location a wide germplasm collection of different oregano species and varieties, purchased commercially available oregano as well that originated in other countries, and quantitatively analyzed the anti-inflammatory

constituents by the analytical method developed. Results show that *Origanum* spp. may contain significantly high concentration levels of rosmarinic acid, oleanolic acid and ursolic acid, and that significant differences in the accumulation of each compound was noted among the accessions and commercial products evaluated.

3.2. MATERIALS AND METHODS

3.2.1. Materials.

The solvents methanol (HPLC-grade), hexane, ethyl acetate (EtOAc) and acetone (HPLC-grade) used for extraction and chromatography were purchased from Fisher Scientific Co. (Fair Lawn, NJ). HPLC-grade water was prepared using a Millipore Milli-Q purification system (Millipore Corp., Bedford, MA) and used for preparing all solutions. The standard compound rosmarinic acid, HPLC buffers formic acid and ammonium hydroxide were procured from Sigma Chemical Co. (St. Louis, MO). The Syrian oregano (*O. syriacum*) varieties (SO1 – SO5 and SO7) were purchased from Lebanon as dry aerial parts and directly used for HPLC analysis. GO2 (*O. vulgare* ssp. *hirtum*), SO6 (*O. syriacum*) and a Cuban oregano (*Plectranthus amboinicus*) variety were procured as seeds from Richters Herbs (Goodwood, Ontario, Canada) and propagated in

the Department of Plant Biology and Pathology greenhouses, School of Environmental and Biological Sciences, Rutgers University. The rest of Greek oregano (*O. vulgare* ssp. *hirtum*) and European oregano (*O. vulgare*) varieties were collected as part of our ongoing plant breeding program in the New Use Agriculture and Natural Plant Products Program at Rutgers University and vegetatively transplanted into the Rutgers Snyder Research and Extension Farm in Pittstown, NJ (**Fig. 3.1**), with parent plants being maintained and grown in the Rutgers greenhouse (**Fig. 3.2**). This larger germplasm and breeding collection had been growing as a perennial crops with the plots being maintained using drip irrigation, and grown on raised beds, harvested once/year at full flowering. The plant aerial part was manually collected from all live plants at full flowering, and placed into a large-scale forced air drier at 40 °C for 2 weeks before any analytical study. All oregano varieties were microscopically authenticated by Dr. James Simon in our research team and deposited in Rutgers botanical products library for future reference.

Figure 3.1. Photograph of selected oregano breeding lines growing in the field at the Rutgers Snyder Research and Extension Farm in Pittstown, NJ

Figure 3.2. Photograph of some of the oregano varieties and breeding lines growing in the Rutgers Greenhouse

3.2.2. Isolation and Identification of Oleanolic Acid and Ursolic Acid.

The standard compounds oleanolic acid and ursolic acid were isolated from oregano samples (*O. vulgare* ssp. *hirtum*) and used for preparation of calibration standards. The dried oregano leaves (400 g) were extracted three times with ethanol and concentrated to dryness under reduced pressure. The residue was loaded to a silica gel (130-270 mesh)

column and eluted by hexane-EtOAc (1:1), EtOAc, EtOAc-acetone (1:1) and acetone in sequence. A total of 16 fractions were collected, and the second fraction containing oleanolic acid and ursolic acid was further subjected to a preparative HPLC separation. The compounds oleanolic acid and ursolic acid were then purified using a Varian C18 preparative column (250 × 41.4 mm, 8 μm) eluted with methanol-water (8:2). The structures of the two triterpenoid acids were elucidated by NMR and MS analysis.

3.2.3. Preparation of Calibration Standards for HPLC Analysis.

The stock solution was prepared by dissolving ~ 25 mg of each standard, rosmarinic acid, oleanolic acid and ursolic acid, in 45 mL methanol in a 50 mL volumetric flask. After sonication for 20 min, the flask was allowed to cool to room temperature and filled to volume with the diluent. Calibration curves were established on 15 data points by diluting the stock solution to cover the expected concentration range for rosmarinic acid, oleanolic acid and ursolic acid across all the oregano samples. The linearity range of the calibration curves was found to be 390 ng/mL - 100 μg/mL for rosmarinic acid, 49 ng/mL – 25 μg/mL for oleanolic acid, and 24 ng/mL – 25 μg/mL for ursolic acid.

3.2.4. Analytical Instruments.

Chromatographic analysis was performed on a Waters 2695 HPLC system (Waters Corp.,

Milford, MA) equipped with an auto-sampler, quaternary pump system, thermostated column compartment, degasser and Millenium 3.2 software. Separation was achieved by using a tandem column system: a Synergi 50 × 4.6 mm, i.d. 4 μm, Polar-RP column (Phenomenex Inc., Torrace, CA) and downstream a Microsorb 100 × 4.6 mm, i.d. 3 μm, C18 column (Varian Inc., Palo Alto, CA) (**Fig. 3.3**). Mass spectrometer used in this research was a triple stage quadrupole Quattro II (Micromass Co., Altrincham, UK) equipped with the orthogonal Z-spray electrospray ionization (ESI) interface and the acquisition data processor Masslynx 3.4 software.

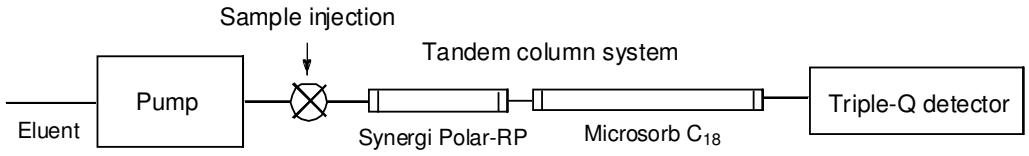

Figure 3.3. A scheme of the LC/MS system

3.2.5. Sample Preparation.

All dried oregano samples were first finely ground with a coffee grinder. About 25 mg

powder was accurately weighed from each sample and placed into a 50 mL volumetric flask, and ~ 45 mL of methanol was added. Each sample was sonicated for 20 min and allowed to cool to room temperature, and then filled to volume with the diluent. The extract was transferred to a centrifuge tube and centrifuged at 12,000 rpm for 2 min to obtain a clear solution and filtered through a 0.45 μm filter for HPLC analysis. The recoveries were validated by spiking each sample with known quantities of the standard compounds, rosmarinic acid, oleanolic acid and ursolic acid to approximately 100%, 75% and 50% of the expected values in the oregano samples and then extracting according the same extraction method described above.

3.2.6. Mass Spectrometry Conditions.

The ESI source of the Quattro II was operated with nitrogen serving as the nebulizing gas (10 L/h) and curtain gas (500 L/h). The source temperature was set at 120 °C and the desolvation temperature was held at 350 °C. Full-scan mass spectra were obtained with a scan time of 2 sec and inter-scan delay of 0.1 sec, operating in negative ion mode for rosmarinic acid and positive ion mode for oleanolic acid and ursolic acid. For quantitation, a selection of *m/z* values corresponding to rosmarinic acid (*m/z* 359, $[M - H]^-$), oleanolic acid (*m/z* 479, $[M + Na]^+$) and ursolic acid (*m/z* 479, $[M + Na]^+$) were monitored by using the instrument in the SIM mode with a dwell time of 1 sec and inter-channel delay of 0.03 sec. The mass spectrometer was set for two time segments:

0-10 min for the detection of rosmarinic acid, where the SIM was carried out in the negative ion mode with the capillary voltage at 3.0 V, cone voltage at 40 V and extractor voltage at 5 V; and 10-40 min for the detection of oleanolic acid and ursolic acid, and the instrument was set in positive mode with the capillary voltage, cone voltage and extractor voltage at 3.2 V, 45 V and 8 V, respectively.

3.2.7. HPLC Analysis.

The mobile phase for chromatographic separation consisted of solvent A (5 mM ammonium formate in water, pH 7.4, adjusted with ammonium hydroxide) and solvent B (5 mM ammonium formate in 90% methanol, pH 7.4) under an isocratic condition (13.5% solvent A and 86.5% solvent B) at a flow rate of 0.8 mL/min. One-fifth of the total effluent was split and injected into the electrospray LC/MS interface. The column compartment temperature was kept at 25 °C and the injection volume was 10 μL. Calibration curves were plotted using 1/x-weighted quadratic model for the regressing of peak area versus analyte concentration, resulting in equation of $y = 2.1655x + 1157.9$ ($r^2 = 0.999$) for rosmarinic acid; $y = 183.14x - 14038$ ($r^2 = 1$) for oleanolic acid; and $y = 270.22x + 22627$ ($r^2 = 0.999$) for ursolic acid. All samples were run in duplicate.

3.3. RESULTS AND DISCUSSION

3.3.1. Identification of Rosmarinic Acid, Oleanolic Acid and Ursolic Acid in Oregano by LC/MS and NMR Techniques.

Rosmarinic acid is a phenolic compound found in many culinary herbs within the Lamiaceae family, and several papers reported its presence in *Origanum* spp. (Exarchou et al., 2002; Hideyuki et al., 2003). In this study, full-scan LC/MS spectra under negative ion mode provided molecular ion at *m/z* 359 ($[M - H]^-$), and fragmentation ions at *m/z* 197 ([salvianic acid A - H] $^-$), *m/z* 179 ($[M - H -$ caffeic acid$]^-$) and *m/z* 161 ($[M - H -$ salvianic acid A$]^-$) for rosmarinic acid (**Fig. 3.4**). The comparison of the retention time with the commercial reference compound on HPLC further supported the identification of rosmarinic acid in our oregano samples. Oleanolic acid and ursolic acid have been isolated from more than 120 plant species (Liu, 1995), but never before have been reported in *Origanum* spp. Oleanolic acid and ursolic acid both produce identical molecular ions and fragmentations on mass spectra (**Fig. 3.4**). The ion peaks at *m/z* 479 were the sodium adduct molecular ions $[M + Na]^+$ of oleanolic acid and ursolic acid. Dehydration (*m/z* 439) and decarboxylic acid (*m/z* 411) products were observed as MS fragments. The fragmentation ions at *m/z* 191 and *m/z* 203 were due to RDA reactions, the characteristic MS fragmentation of Δ^{12}-unsaturated triterpenoids. The structures of oleanolic acid and ursolic acid were further elucidated by NMR data at the stage of

isolated pure compounds, and compared with literature (Seto et al., 1986; Ahmad et al., 1993; Huang, Wang & Lin, 1999; Alves et al., 2000).

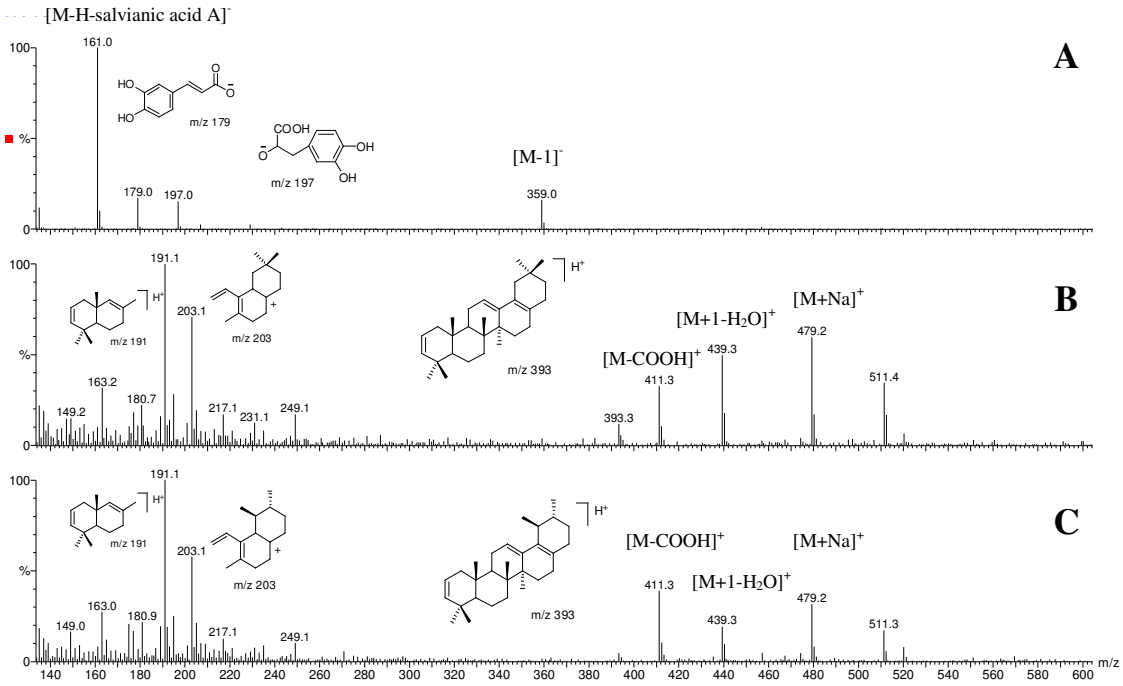

Figure 3.4. Negative (**A**, rosmarinic acid) and positive (**B**, oleanolic acid; **C**, ursolic acid) ESI mass spectra, obtained from the LC/MS TIC of oregano extract

3.3.2. Analytical Method Optimization.

Separation of organic acids can be difficult due to the retention time shift, peak broadening, and inability to achieve adequate resolution on the chromatogram. The challenge of co-quantitation for this research resides on the chemical similarity of oleanolic acid and ursolic acid, and the drastic difference of polarity between rosmarinic acid and the two triterpenoid acids. We found an older type ODS column (Microsorb C18) provided improved peak resolution between these two position isomers probably due to the silanol interactions present on the stationary phase. Ammonium formate as the buffer in mobile phase at slightly basic condition (pH 7.4) was optimal to achieve sharp peaks, a baseline separation of oleanolic acid and ursolic acid, and ideal retention-time stability. Additionally, the use of ammonium formate enhanced the signal response and improved the sensitivity for MS detection. Finally, the co-quantitation of rosmarinic acid with oleanolic acid and ursolic acid was achieved by employing a tandem column system using two different stationary phases: first a Synergi Polar-RP column from Phenomenex (50 × 4.6 mm) and downstream a Microsorb ODS column from Varian (100 × 4.6 mm). Polar-RP column is an ether-linked phenyl phase with polar endcapping, used to increase retention times of highly polar compounds and offer selective retention on aromatic compounds by π-π interactions between the aromatic rings of the analyte and the phenyl functional group of Synergy polar-RP (**Fig. 3.5**). Methanol, a protic solvent with pronounced hydrogen bonding ability, was found to be superior for chromatographic

resolution compared to acetonitrile when combining with water as the mobile phase. Also, aromatic selectivity is further enhanced by the presence of methanol in the mobile phase on Synergy polar-RP column due to the ability of methanol to facilitate π-π interactions between the aromatic rings of rosmarinic acid and the phenol functional group of the stationary phase. In contrast, the π electrons of the "CN" bond in acetonitrile are presumed to compete for the phenyl binding sites on the stationary phase (Yang et al., 2005). Although rosmarinic acid, oleanolic acid and ursolic acid are all organic acids, they perform differently under electrospray ionization. Rosmarinic acid is more sensitive for ionization under negative mode, whereas the optimal ionization condition for oleanolic acid and ursolic acid is under positive ion mode. The polarity switch as attained by creating two time segments in a single run where the polarity mode changed from negative (0-10 min) to positive (10-40 min).

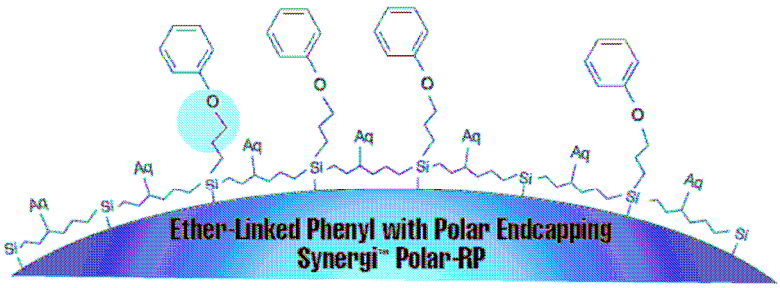

Figure 3.5. The stationary phase of Polar-RP column

3.3.3. Validation of the LC/MS Method.

The precision of this method was validated by carrying out six replicate determinations of a single oregano sample on the same day (intra-assay) and six analytical batches on three different days (inter-assay). The relative standard deviations (RSDs) of intra-assay were 6.16%, 5.37% and 4.84% for rosmarinic acid, oleanolic acid and ursolic acid, respectively. Inter-assay RSDs were 6.22%, 6.41% and 5.31% for those same analytes. The recoveries of this method were evaluated by the addition of known concentrations of the standards, rosmarinic acid, oleanolic acid and ursolic acid at three concentration levels, approximately 100%, 75% and 50% of the expected values in the oregano sample GO12 (*O. vulgare* ssp. *hirtum*). No considerable difference was found among the recoveries at different concentration levels, with RSDs being 3.0%, 3.4% and 2.0% for 100%, 75% and 50% spiked levels respectively (**Table 3.1**). The mean recoveries were calculated as 98.2% for rosmarinic acid, 97.4% for oleanolic acid, and 94.2% for ursolic acid. These validation studies showed that our newly developed analytical method was reliable, precise and sensitive for the simultaneous quantitation of rosmarinic acid, oleanolic acid and ursolic acid in oregano samples.

Table 3.1. Recoveries of Rosmarinic Acid, Oleanolic Acid and Ursolic Acid at Different Spiking Levels

Analyte	Concentration (g/kg)	Added (g/kg)	Found (g/kg)	Recovery (%)	Mean (%)	RSD (%)
Rosmarinic	48.63	44.40	93.36	100.7		
acid		33.30	80.24	94.9	98.2	3.0
		22.20	70.57	98.8		
Oleanolic	2.50	2.07	4.59	100.8		
acid		1.55	4.01	97.2	97.4	3.4
		1.04	3.47	94.1		
Ursolic	8.48	9.32	17.30	94.6		
acid		6.99	14.92	92.2	94.3	2.0
		4.66	12.95	95.9		

3.3.4. Quantitative Survey of Rosmarinic Acid, Oleanolic Acid and Ursolic Acid Contents in Different Oregano Varieties.

Different sources, varieties and even species of oregano were compared for the accumulation of these three anti-inflammatory constituents. Several oregano sources were commercially purchased as dry aerial parts directly for analysis; others were vegetatively transplanted to our field research station, with parent plants being maintained and grown in greenhouses. The first part of the analysis was not to definitively compare growing conditions or ‘sources’ per se but to first ask whether chemical differences in these three

bioactive acids were found in oregano, and if so, then comparison could be more strongly examined within the sub-groups of oregano from each breeding lines. Most of the varieties belonged to *O. vulgare* ssp. *hirtum*, *O. vulgare* and *O. syriacum*, which are among the major species of *Origanum* that enter into the global trade (Dogan, Arslan & Ozen, 2005). Based on the LC/MS (SIM mode) method developed (**Fig. 3.6**), different oregano varieties were quantitatively analyzed for rosmarinic acid, oleanolic acid and ursolic acid content (**Table 3.2**). Many *Origanum* varieties were found to be extraordinarily rich sources of the anti-inflammatory constituents, although the variation was large within the same species. Romarinic acid was the predominant compound in the varieties of *O. vulgare* ssp. *hirtum* and *O. vulgare*, ranging from 13.73 mg/g to 58.32 mg/g on dry weight basis. The average levels of oleanolic acid and ursolic acid in these two species were 2.28 mg/g and 7.35 mg/g, as calculated from twelve different species and varieties. The sources of *O. syriacum* showed a distinct feature of a high content level of triterpenoid acids, with oleanolic acid averaging 9.40 mg/g in seven different sources and ursolic acid averaging 24.07 mg/g. Cuban oregano (*Plectranthus amboinicus*), a Caribbean native and while having the same common name belonging to another genus in Lamiaceae family, was also collected and analyzed by the same LC/MS method as a comparison and control for analytical purposes. Results showed that, as expected, none of the three analytes were detected in the aerial part of Cuban oregano, demonstrating again it is distinct from the true members of *Origanum*. Results of our data allow us to conclude that rosmarinic acid, oleanolic acid and ursolic acid are major

nonvolatile second metabolites found in *Origanum* spp. These compounds accumulated in the aerial part and the distribution varied significantly by species and source. We also conclude that oregano can serve as a rich source of these bioactive anti-inflammatory compounds.

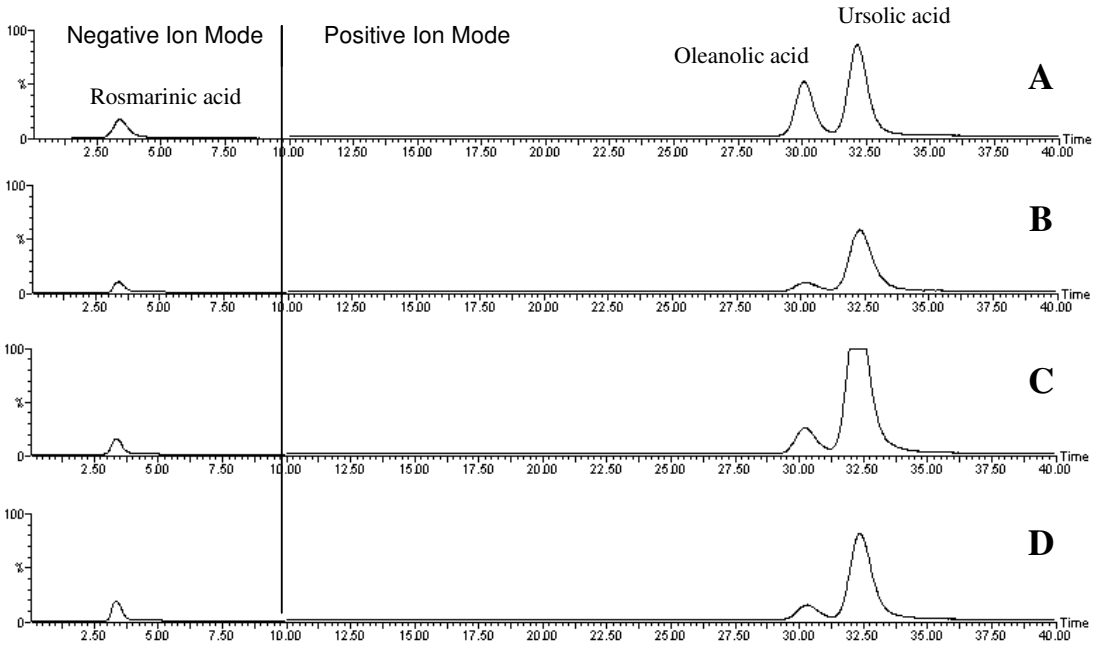

Figure 3.6. SIM (selected ion monitoring) chromatogram of (**A**) standards and representative chromatograms of (**B**) sample EO1 (*Origanum vulgare*), (**C**) sample SO6 (*O. syriacum*), and (**D**) sample GO2 (*O. vulgare* ssp. *hirtum*); the two time segments were set as 0-10 min at *m/z* 359 (rosmarinic acid), and 10-40 min at *m/z* 479 (oleanolic acid and ursolic acid).

Table 3.2. Contents of the Anti-inflammatory Compounds in Different Oregano Varieties (Milligrams per Gram of Dry Matter)

sample code[a]	rosmarinic acid[b]	oleanolic acid[b]	ursolic acid[b]
O. vulgare ssp. *hirtum*			
GO1	53.24±0.32	1.57±0.12	5.33±0.19
GO2	58.32±0.47	3.70±0.23	8.59±0.36
GO3	29.44±1.05	4.51±0.21	11.35±0.21
GO4	35.61±0.38	1.33±0.01	4.97±0.07
GO5	29.96±1.46	1.22±0.00	5.64±0.04
GO6	15.86±0.99	2.16±0.07	9.00±0.29
O. vulgare			
EO1	19.97±0.99	2.89±0.13	7.78±0.56
EO2	14.65±0.79	1.99±0.14	6.35±0.12
EO3	14.49±1.29	1.82±0.10	7.72±0.23
EO4	32.41±2.10	1.75±0.11	6.66±0.21
EO5	13.73±1.17	2.96±0.18	10.60±0.35
EO6	14.17±0.93	1.47±0.09	4.28±0.13
O. syriacum			
SO1	9.13±0.18	9.84±0.11	23.84±0.74
SO2	35.73±3.42	9.87±0.57	24.65±0.87
SO3	27.65±1.21	11.03±0.52	34.42±0.91
SO4	30.60±1.13	12.72±0.05	29.47±0.71
SO5	24.57±0.30	8.30±0.08	22.04±0.38
SO6	40.37±2.03	6.99±0.38	17.94±0.21
SO7	16.02±0.27	7.08±0.32	16.12±0.21

[a] GO 1-6 are the varieties of Greek oregano (*Origanum vulgare* ssp. *hirtum*), EO 1-6 are the varieties of European oregano (*O. vulgare*) and SO 1-7 are the varieties of Syrian oregano (*O. syriacum*). [b] Mean value ± SD in duplicate.

We further investigated the anti-inflammatory contents in different breeding lines from a Greek oregano variety GO1. All the daughter lines were propagated and grown on the Rutgers Snyder Research and Extension Farm in Pittstown, NJ, with parent plants being maintained in the Rutgers greenhouse. The plant aerial part was collected from all live plants at full flowering and air dried for analysis. The breeding lines all accumulated high concentration of rosmarinic acid (**Table 3.3**), with RSD = 28%, The RSDs for oleanolic acid and ursolic acid was both around 20%. We also found that although oleanolic acid and ursolic acid content levels may vary among different lines, the ratios of oleanolic acid to ursolic acid seemed to be a constant with very small variations, so we concluded that this variety was considered to be an advanced variety with good genetic stability and high anti-inflammatory contents, and an advanced breeding line could be selected and field developed.

Table 3.3. Contents of the Anti-inflammatory Compounds in Different Breeding Lines from a Greek Oregano (*Origanum vulgare* ssp. *hirtum*) Variety (Milligrams per Gram of Dry Matter)

sample code	rosmarinic acid	oleanolic acid	ursolic acid
OE-04-S3	43.53	1.61	5.09
OE-04-S41	48.63	2.50	8.48
OE-04-S37	45.77	1.44	6.41
OE-04-S8	40.59	1.42	4.91
OE-04-S12	42.77	2.16	7.08
OE-04-S1	36.14	1.80	6.88
OE-04-S49	24.80	2.30	7.98
OE-04-S50	54.12	1.77	6.95
OE-04-S10	41.72	2.49	9.10
OE-04-S11	84.71	2.03	6.97
OE-04-S28	45.12	1.70	5.81
OE-04-S13	30.89	1.67	6.26
OE-04-S10	40.20	1.95	8.03
OE-04-S14	66.90	1.14	4.66
OE-04-S5	33.73	1.45	5.58
OE-04-S3	52.42	1.94	7.27
OE-04-S33	35.13	2.23	8.60
OE-04-S4	45.74	1.57	5.66
OE-04-S2	50.43	1.79	7.06
OE-04-S6	52.32	1.47	5.21
OE-04-S9	42.35	1.36	4.58
OE-04-S7	66.00	2.17	7.90

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CHAPTER 4. RETRO-IDENTIFICATION OF THE KNOWN ANTI-INFLAMMATORY COMPOUNDS FROM A MORE ECONOMICAL SOURCE (MENTHA SPP.) WITHIN THE SAME FAMILY OF LAMIACEAE AND DEVELOPMENT OF ANALYTICAL METHOD (LC/MS-MRM MODE) ON SIMULTANEOUS QUANTITATION OF ANTI-INFLAMMATORY CONSTITUENTS IN MINTS

4.1. INTRODUCTION

Since ancient times, mints have been the popular aromatic plants used for food flavoring, teas, liqueur and medicines (Regina et al., 2007). As perennial herbs, mints may be strikingly variable with respect to morphology and chemical composition, notably in their essential oils, and relative to other natural products. Traditionally, mints are used to treat common cold, functional dyspepsia, and skin itching (Bruneton, 1995). Recent research reported their antioxidant, anticancer and anti-inflammatory properties, and being clinically effective in alleviating the nasal symptoms of allergic rhinitis (Thiagarajan et al., 2001; Triantaphyllou, Blekas & Boskou, 2001; Inoue et al., 2001; Takahashi & Nakata, 1995). The most frequently reported *Mentha* spp. and consequently the most widely used as traditional medicines include four taxa: *M.* × *piperita* and *M. spicata*, *M. arvensis*, followed by others such as *M. pulegium*, and *M. longifolia*. The musty smelling mints (*M. spicata* and *M. suaveolens*) were described restrictedly used for common cold and cough, while the medicinal uses proposed for the pungent smelling herbs may include the treatment of stomach, digestion and respiratory ailments (Regina et al., 2007). Mint oil distilled from leaves is extensively used in the cosmetics and food industries for its antifungal, antimicrobial, insecticidal and antioxidant properties (Bruneton, 1995).

In addition to the commonly recognized bioactivities, some other biological effects may also present in mint. The anti-inflammatory activity of mints are not found in all *Mentha*

spp., but appear to be limited to several taxa including *M. piperita*, *M. suaveolens* and *M. aquatica*. *M. piperita* was reported to show anti-inflammatory effects against acute (xylene-induced ear oedema in mice) and chronic (cotton-pellet granuloma in rats) inflammation (Atta & Alkofahi, 1998). Moreno et al. (2002) found the methanol extract from *M. suaveolens* possessed an anti-inflammatory action inhibiting the carrageenin-induced rat paw oedema. In a research of pharmacological screening of Mediterranean diets, the alcoholic extract of mint (*M. aquatica*) was described to exhibit inhibition on cytokine-induced cell activation on nitrite assays (Anon., 2005). More recently, Conforti et al. (2008) have described that the alcoholic extract of *M. aquatica* showed topical anti-inflammatory activity on oedema reduction. From these studies, we hypothesized that mint possessed anti-inflammatory activity and that the natural products that exhibit such activity in *Mentha* species, might be due to both the well known bioactive essential oils and non-volatile bioactive constituents such as the triterpenoids and phenolic acids. The relation between the anti-inflammatory activity and those compounds that may be responsible had not been explored in depth, nor have their been reports on the genetic effluence both between and within *Mentha* genus, an aromatic herb that is so well know for a diverse range of chemotypes, particularly with the essential oils.

The discovery of the compounds in oregano that were responsible largely for the plants antioxidant activity as well as anti-inflammatory action, and our research that now shows

this species to be a rich source of rosmarinic acid, oleanolic acid and ursolic acid inspired us to look for additional source of these natural anti-inflammatory agents within the same family of Lamiaceae. Rosmarinic acid has been reported in *Mentha* species as well as other plants in Lamiaceae family including basil (*Ocimum* spp.), rosemary (*Rosmarinus* spp.) and thyme (*Thymus* spp.) (Peterson & Simmonds, 2003). This phenolic compound was described a free radical scavenger and inhibitor of low-density lipoprotein oxidation (Nakamura et al., 1998; Fuhrman et al., 2000), while no one related this compound to the anti-inflammatory activities of mint. Oleanolic acid and ursolic acid were reported to present in several *Mentha* species including *M. arvensis*, *M. spicata*, *M. rotundifolia* and *M. vulgare* (Karasawa & Shimizu, 1980; Perva et al., 2001; Hadolin et al., 2001), however, the analytical survey of these triterpenoid acids in *Mentha* spp. and how much these contents contributing to the hydroalcholic extract of mints are still unclear. The anti-inflammatory mechanism of oleanolic acid and ursolic acid is believed to affect multiple targets through one or more signaling pathways (Dai et al., 1989; Tsuruga et al., 1991; Simon et al., 1992; Najid et al., 1992; Zhuo et al., 1993, Ying et al., 1991; Dai, Hang & Tan, 1989; Kapil & Shanna, 1994).

Mints are one of the most cultivated plants for essential oils, including peppermint and spearmint as the two major crops followed by Japanese Mint (*M. arvensis*) (Simon, 1990). The volatile oil of peppermint is the main reason for its popular use and the uses of natural mints oils or synthetic mint flavoring for the flavoring oral care, confectionery

and chewing gum are of growing importance (Guntert et al., 2001). The estimated production of peppermint oil in the United States was about 5,000 metric tons (Guntert et al., 2001). The essential oil is typically found in concentrations from 0.3 to 0.4% in peppermint, while for some varieties it may reach as high as 1.5%. Menthol is the main active constituent for peppermint, followed by menthone and menthyl acetate as well as other 100 different compounds in the oil (Guntert et al., 2001). The essential oil profiles of other mints can be different from peppermint. For example the typical spearmint oil usually contains carvone as the major ingredient in the oil (55-67%) and also contains limonene (2-25%), while the other constituents (menthone, menthol, menthofuran, menthyl acetate and cineole) are expected to be less than 2%. Japanese mint is the natural source of commercial menthol, as that compound is the primary constituent found in its essential oil (Simon, 1990).

Flavonoids, widely distributed in the plant kingdom, have been recognized as important nutrients for human health (Merken & Beecher, 2002). These compounds are considered antioxidants, scavenging free radicals by donating hydrogen, and agents preventing low-density lipoproteins oxidation. Numerous health benefits have been reported for flavonoids including heart disease prevention, anti-AIDS, anti-arthritic, anticancer, anti-hypertensive, anti-inflammatory and antiviral activities (Arpentine et al., 1992; Frankel, Waterhouse & Kinsella, 1993; Hu et al., 1995; Khokhar et al., 1997; Terencio, Sanz & Paya, 1991; Meunier et al., 1987; Kuo, 1997). Rosmarinic acid, an ester of caffeic

acid found in *Mentha* species as well as in other Lamiaceae family plants, has been noticed exhibiting antioxidative, anti-inflammatory and anti-depressive activities (Exarchou et al., 2002; Takeda et al., 2002). Flavonoid glycosides from peppermint have been previously identified as luteolin-7-*O*-glucoside, luteolin-7-*O*-rutinoside, apigenin-7-*O*-glucoside, isorhoifolin, hesperidin, eriocitrin, piperitoside, menthoside diosmetin and diosmin (Guedon & Pasquier, 1994; Areias et al., 2001; Hoffmann & Lunder, 1984; Yamanura et al., 1998; Subramanian & Nair, 1972). Free flavonoid aglycones including luteolin, apigenin, acacetin were found in peppermint (Justesen, 2000; Voirin, Saunois & Bayet, 1994; Voirin & Bayet, 1992; Zakharov, Zakharova & Smirnova, 1987; Voirin et al. 1999), and exhibited anti-allergic, anti-inflammatory and choleretic activities (Malialal & Wanwimolruk, 2001; Takahashi & Nakata, 1995; Nair et al., 2001). The phenolic profiles in members of this genus, outside of the popular main essential oil varieties or sources of peppermint and spearmint, have not been systematically explored and surveyed. Several papers described the qualitative or quantitative determination of water-soluble counterpart in mint species (Kosar et al., 2004). Guedon et al. developed an HPLC method to analyze plant samples by using a Nucleosil C18 column with water (pH = 2.5) and acetonitrile as gradient eluent, resulting in flavonoid glycosides and rosmarinic acid quantitation (Guedon & Pasquier, 1994). The screening of free radical scavenging compounds in water extracts of *Mentha* samples was performed by using a postcolumn DPPH assay (Kosar et al., 2004). In our work, seven flavonoids plus caffeic acid and rosmarinic acid were quantitated in mint samples.

However, many of the flavonoids determined in theses samples were flavonoid aglycones. The more complex flavonoid glycosides such as luteolin-7-*O*-rutinoside, narirutin, hesperidin and diosmin, naturally present as the predominant flavonoid forms, still remain to be further examined and approved through different *Mentha* spp.. More recently, four phenolic compounds luteolin-7-*O*-glucuronide, caffeic acid, rosmarinic acid, and lithospermic acid were quantitatively determined in medicinal herbs including mint by using HPLC/UV with a C18 column and acetonitrile-water-formic acid mobile phase (Fecka, Raj & Krauze-Baranowska, 2007).

The primary aim of this research was to develop and validate an HPLC method for co-quantitation of the anti-inflammatory constituents in mints. Based on the LC/MS method on oreganos, we further developed a LC/MS/MS method for the simultaneous quantitation of the three anti-inflammatory acids in mints. The investigation of flavonoid contents in different mints was also part of our goal, since flavonoids contribute to a major part of phytochemicals in mints and possess numerous health-promoting properties. Thirty-five different mint varieties and accessions (coming from 9 *Mentha* species collected from around the world) were examined for their chemical profiles. An HPLC/UV method on the determination of the major flavonoid contents in mints was developed and validated herein.

4.2. MATERIALS AND METHODS

4.2.1. Plant Samples.

A wide collection of mint germplasm as maintained by Prof. Steve Weller was procured from Purdue University and shipped over to Rutgers to be vegetatively propagated both in the greenhouse and later for field planting. This collection of 35 mints consisted of 9 species including the main species used in the commercial production of traditional peppermint, spearment and Japanese mint oils, plus a wide range of specialty mints grown as horticultural ornamentals and novelty varieties due to their unusual aromas and flavors. Several clonal materials of mints, not completely identified but found in commercial grower fields were also included for comparative purposes. All the mints once received at Rutgers (in 2001) were immediately rooted and grown in greenhouses under controlled conditions. Plant materials were then purposely increased by clonal or vegetative propagation until sufficient numbers of new plants were regenerated for field planting. In 2001, field plots were established for the entire mint collection and transplanted into the Rutgers Cooperative Research and Extension Center (Upper Deerfield, Cumberland, NJ) in June, 2001 (**Fig. 4.1**), with parents plants being maintained and grown in the departmental greenhouse, School of Environmental and Biological Sciences, Rutgers University (**Fig. 4.2**). The plants were cultivated under

identical field conditions in single rows with 60 cm between plants and 2 m between beds. Field plots were maintained and harvested as needed during full flowering. Plots were hand weeded twice during the growing season and a Roterra cultivator maintained. No insecticides or fungicides were applied, and additional water was applied with a reel type irrigator. Harvesting was carried out by hand, and plant samples were dried at 40°C for 2 weeks prior to chemical analysis. Given the wide array of mints and the difficulty in establishing and authenticating the varietal names and species, herbarium voucher specimens were collected for each mint in this study. Herbarium voucher specimens of all 35 mints were then submitted for authentication to Dr. Arthur O. Tucker in Delaware State University, who microscopically identified and authenticed each accession in this study. Voucher specimens of all entries were deposited into and are being stored at the Claude E. Phillips Herbarium in Delaware State University, Dover, DE

Figure 4.1. Field-grown spearmint at Mr. Mint, Dalponte Family Farm, Richland, NJ

Figure 4.2. Photograph of selected mint varieties growing in the Rutgers University Greenhouse

4.2.2. Chemicals.

Acetonitrile (HPLC grade), methanol (HPLC grade), isopropanol (HPLC grade), formic acid and phosphoric acid used for chromatographic analysis were purchased from Fisher Scientific Co. (Fair Lawn, NJ). Distilled water was prepared using a Millipore Milli-Q purification system (Millipore Corp., Bedford, MA) and used for HPLC analysis. The

standard compounds (rosmarinic acid, hesperidin and diosmin), and HPLC buffers (formic acid and ammonium hydroxide) were purchased from Sigma Chemical Co. (St. Louis, MO), luteolin 7-*O*-glucoside, luteolin 7-*O*-rutinoside, narirutin, isorhoifolin were purified from artichoke described before (*53*). Silica gel (130-270 mesh), RP-18 silica gel and Sephadex LH-20 (Sigma Chemical Co., St. Louis, MO) were used for column chromatography. Oleanolic acid and ursolic acid used as standards were isolated from oregano (*Origanum vulgare* spp. *hirtum*), another member of the Lamiaceae family plant, and structurally elucidated by NMR and MS analysis. The liquid nitrogen used for LC/MS analysis was high-purity nitrogen (99.999%) and from Airgas Co. (Salem, NH). ^{1}H-NMR and ^{13}C-NMR spectra were obtained on the 200 MHz NMR spectroscopy (Varian Inc., Palo Alto, CA).

4.2.3. Analytical Instruments.

HPLC analysis was applied on a Waters 2695 system (Waters Corp., Milford, MA) equipped with an auto-sampler, quaternary pump system, thermostated column compartment, degasser and Millenium 3.2 software. Chromatographic separation was achieved by using a tandem column system: a Synergi 50 × 4.6 mm, i.d. 4 μm, Polar-RP column (Phenomenex Inc., Torrace, CA) and downstream a Microsorb 100 × 4.6 mm, i.d. 3 μm, C18 column (Varian Inc., Palo Alto, CA). Mass spectrometer used in this research was a triple stage quadrupole Quattro II instrument (Micromass Co., Altrincham, UK)

equipped with the orthogonal Z-spray electrospray ionization (ESI) interface and the acquisition data processor Masslynx 3.4 software.

4.2.4. Qualitative Analysis of Flavonoids by HPLC/UV/MS.

The quantitative analysis utilized an Agilent 1100 Series LC/UV/MSD system (Agilent Technologies, Waldbronn, Germany) equipped with a quaternary pump, diode array and multiple wavelength detector, thermostatted column compartment, degasser, and electrospray source. The software was HP ChemStation, Bruker Daltonics 4.0 and Data analysis 4.0. The mobile phase used the same gradient procedure as the HPLC method described above except mobile phase A was 0.1% formic acid solution instead of 0.1% phosphoric acid. The eluent flow was a 2 to 1 stream splitting for the MSD detector. The electrospray-ionization mass spectrometer (ESI-MS) was operated under positive ion mode and optimized collision energy level of 60%, scanned from *m/z* 100 to 1000. ESI was conducted using a needle voltage of 3.5 kV. High-purity nitrogen (99.999%) was applied as drying gas with flow rate at 9 L/min. Capillary temperature was 325 °C, and nebulizer was set at 45 psi. The ESI interface and mass spectrometer parameters were optimized to obtain maximum sensitivity. The UV absorption spectra were recorded from 200 to 400 nm for all peaks

4.2.5. Calibration Standards for Anti-inflammatory Content Analysis.

The stock solution was prepared by dissolving approximate amount of 25 mg each standard, rosmarinic acid, oleanolic acid and ursolic acid, with 45 mL methanol in a 50 mL volumetric flask. The solution was sonicated for 20 min and allowed to cool to room temperature before filling to volume with the diluent. Calibration curves were established on 15 data points by diluting the stock solution to cover the expected concentration range for rosmarinic acid, oleanolic acid and ursolic acid in all mint samples. The linearity of the calibration curves was determined as 3.13 µg /mL - 400 µg/mL for rosmarinic acid, 98 ng/mL – 25 µg/mL for oleanolic acid, and 391 ng/mL – 25 µg/mL for ursolic acid. 10 µL aliquot was used for HPLC analysis.

4.2.6. Calibration Standards for Flavonoid Analysis.

About 5 mg of each compound was accurately weighed into a 25 mL volumetric flask. 5 mL DMSO and 15 mL of 70% methanol was added and the flask was sonicated for 15 min. The flask was allowed to cool to room temperature and filled to full volume with 70% methanol solution. 5 mL of the above solution was transferred to a new 50 mL volumetric flask and diluted to the full volume with 70% methanol. Calibration curves were established on six data points covering a concentration range of 0.25-250 µg/mL for eriocitrin, 0.224-224 µg/mL for luteolin-7-*O*-rutinoside, 0.172-172 µg/mL for luteolin-7-*O*-glucoside, 0.184-184 µg/mL for narirutin, 0.212-212 µg/mL for isorhoifolin,

and 0.21-210 μg/mL for hesperidin, and 0.315-315 μg/mL for diosmin, with correlation coefficients of 1 for all seven compounds. 10 μL aliquot was used for HPLC analysis.

4.2.7. Plant Sample Preparation for Anti-inflammatory Content Analysis.

Air-dried mint samples (aerial part without flowers) were finely ground with a coffee grinder. About 25 mg plant powder was accurately weighed from each sample and placed into a 50 mL volumetric flask with ~ 45 mL of methanol added. The sample was sonicated for 20 min and allowed to cool to room temperature before filling to volume with the diluent. The extract was transferred to a centrifuge tube and centrifuged at 12,000 rpm for 2 min to obtain a clear solution, which was then filtered through a 0.45 μm filter and placed into sample vials for HPLC analysis. The validation on recoveries were carried out by spiking each sample with known quantities of standard compounds, rosmarinic acid, oleanolic acid and ursolic acid to approximately 200%, 100% and 50% of the expected values in the mint samples and then extracting together according to the same extraction method.

4.2.8. Plant Sample Preparation for Flavonoid Analysis.

The dried mint aerial part was finely ground with a coffee grinder. About 100 mg of powder was accurately weighed into a 25 mL volumetric flask. 20 mL of 70% methanol

solution was added and the samples were sonicated for 30 min. The flasks were allowed to cool to room temperature and filled to the final volume with 70% methanol. Using a disposable syringe and 0.45 μm filter, the samples were filtered into HPLC vials for HPLC analysis.

4.2.9. Quantitative Determination of Anti-inflammatory Constituents by LC/MS/MS Method.

The mobile phase for chromatographic separation was composed of solvent A (5 mM ammonium formate in water, pH 7.4, adjusted with ammonium hydroxide) and solvent B (5 mM ammonium formate in 90% methanol, pH 7.4) under an isocratic condition (13.5% solvent A and 86.5% solvent B). The flow rate was set at 0.8 mL/min, and four-fifth of the total effluent was split before injecting into the electrospray LC/MS interface. The injection volume was 10 μL, and the column compartment temperature was maintained at 25 °C. A plot of the area response ratio versus concentration resulted in calibration equation of $y = 0.196x – 481.9$ ($r^2 = 0.999$) for rosmarinic acid; $y = 0.9444x – 46.776$ ($r^2 = 0.998$) for oleanolic acid; and $y = 0.3776x - 118.05$ ($r^2 = 0.999$) for ursolic acid. All samples were run in duplicate. The ESI source of the Quattro II uses nitrogen as the nebulizing gas (10 L/h) and curtain gas (500 L/h). The ion source temperature was kept at 120 °C and the desolvation temperature was set at 350 °C. Full-scan mass spectra were operated with a scan time of 2 sec and inter-scan delay of 0.1 sec, under negative

ion mode for rosmarinic acid and positive ion mode for oleanolic acid and ursolic acid. Quantitation was performed by LC/MS/MS in the multiple reaction monitoring (MRM) mode. Rosmarinic acid was monitored with a selection of *m/z* 359 as the parent ion and *m/z* 161 as the daughter ion; oleanolic acid and ursolic acid were detected by choosing *m/z* 439 as the parent ion and *m/z* 203 as the daughter ion. The instrument was set with a dwell time of 1 sec and inter-channel delay of 0.03 sec. The mass chromatographic monitoring was divided into two time segments: 0-10 min for the detection of rosmarinic acid, where the MRM was carried out in the negative ion mode with the capillary voltage at 3.0 V, cone voltage at 40 V and extractor voltage at 5 V; and 10-40 min for the detection of oleanolic acid and ursolic acid, where the instrument was set in positive mode with the capillary voltage, cone voltage and extractor voltage at 3.2 V, 45 V and 8 V, respectively.

4.2.10. Quantitative Determination of Flavonoids and Rosmarinic Acid by HPLC/UV Method.

Quantitative HPLC analysis was performed on a Waters HPLC system 2695 equipped with an auto-sampler, a quaternary pump system, a photodiode array detector 2996 and Millennium 3.2 software. Separation used a Luna C18 (2) column, 5 μm, 250 × 4.6 mm i.d. (Phenomenex Inc., Torrace, CA). The column temperature was set to 30 °C and the mobile phase included water (containing 0.1% phosphoric acid, solvent A), acetonitrile

(solvent B), and isopropanol (solvent C) in a gradient system. The gradient ran as follows: 0 min, 88% solvent A, 10% B and 2% C; 30 minutes, 68% A, 30% B and 2% C; 50 minutes, 38% A, 60% B and 2% C; 60 minutes, 38% A, 60% B and 2% C; the post running time is 10 min. The flow rate was 1.0 mL/min, injection volume was 10 μL and detection wavelength was set at 280 nm.

4.2.11. Qualitative Analysis by HPLC/UV/MS.

Qualitative analysis utilized an Agilent 1100 Series LC/MSD system (Agilent Technologies, Waldbronn, Germany) equipped with a quaternary pump, diode array and multiple wavelength detector, thermostatted column compartment, degasser, and electrospray source. The software was HP ChemStation, Bruker Daltonics 4.0 and Data analysis 4.0. The mobile phase was the same as the HPLC method above except mobile phase A was 0.1% formic acid solution instead of 0.1% phosphoric acid. The flow was a 2 to 1 stream splitting for the MSD detector. The electrospray-ionization mass spectrometer (ESI-MS) was operated under positive ion mode and optimized collision energy level of 60%, scanned from *m/z* 100 to 1000. ESI was conducted using a needle voltage of 3.5 kV. High-purity nitrogen (99.999%) was applied as drying gas with flow rate at 9 L/min. Capillary temperature was 325 °C, and nebulizer was set at 45 psi. The ESI interface and mass spectrometer parameters were optimized to obtain maximum sensitivity. The UV absorption spectra were recorded from 200 to 400 nm for all peaks

4.3. RESULTS AND DISCUSSION

4.3.1. Optimization of Chromatographic Conditions and ESI-MS Parameters for Determination of Rosmarinic Acid, Oleanolic Acid and Ursolic Acid.

The co-quantitation of rosmarinic acid, oleanolic acid and ursolic acid is difficult due to the chemical similarity of oleanolic acid and ursolic acid, and the drastic difference of polarity between rosmarinic acid and the two triterpenoid acids. The retention time shift and peak broadening also cause failure to quantitate on the chromatogram, especially for the separation of theses organic acids. To achieve simultaneous quantitation of rosmarinic acid with oleanolic acid and ursolic acid, we utilized a tandem column system composed of two different stationary phases: first a Synergi Polar-RP column from Phenomenex (50 × 4.6 mm) and downstream a Microsorb ODS column from Varian (100 × 4.6 mm). Polar-RP column, an ether-linked phenyl phase with polar endcapping, applied as the upstream column to selectively increase the retention time of rosmarinic acid by π-π interactions between the aromatic rings of the analyte and the phenyl functional group of Synergy polar-RP. Ammonium formate was finally chosen as the buffer in mobile phase because it provided sharper peaks and baseline separation of oleanolic acid and ursolic

acid, ideal retention-time stability, and enhanced sensitivity under MS detection. ESI mass spectra in both negative and positive modes were examined for the detection of analytes. Under negative ion mode, rosmarinic acid presented a better signal-to-noise ratio on mass spectra. The peak at *m/z* 359 was designated as the molecular ion $[M - H]^-$ (**Fig. 4.3**). The fragmentation ions at *m/z* 197 ([salvianic acid A - H] $^-$) and *m/z* 179 ([M - H - caffeic acid]$^-$) were also observed (**Fig. 4.4**). The base peak at *m/z* 161 on the spectrum was the fragmentation ion $[M - H - \text{salvianic acid A}]^-$, which was subsequently selected as the daughter ion being monitored in the third quadrupole. Along with the deprotonated molecule at *m/z* 359 chosen as the parent ion, the MRM mode provided sensitive detection for rosmarinic acid and good linearity ($r^2 = 0.999$) covering the expected concentration range in all mint samples. Oleanolic acid and ursolic acid showed almost identical mass spectra under positive ion mode, with peaks at *m/z* 479 designated as the sodium adduct molecular ions $[M + Na]^+$ and same fragmentation patterns. The $[M + H]^+$ at *m/z* 457 was hardly observed, while the dehydration ion at *m/z* 439 was abundant and being an important intermediate for the subsequent RDA fragmentations such as product ions at *m/z* 191 and *m/z* 203, the characteristic MS fragmentations of Δ^{12}-unsaturated triterpenoids (**Fig. 4.5**). During the optimization of tandem mass conditions, when ion peak at *m/z* 439 was utilized as the precursor ion with the downstream fragmentation ion at *m/z* 203 being the daughter ion, it showed to be sensitive and specific for the detection of oleanolic acid and ursolic acid in the mint samples. The simultaneous quantitation of rosmarinic acid under negative ion mode and

triterpenoid acids under positive ion mode was accomplished by creating two time segments in a single run where the polarity mode was changed from negative (0-10 min) to positive (10-40 min).

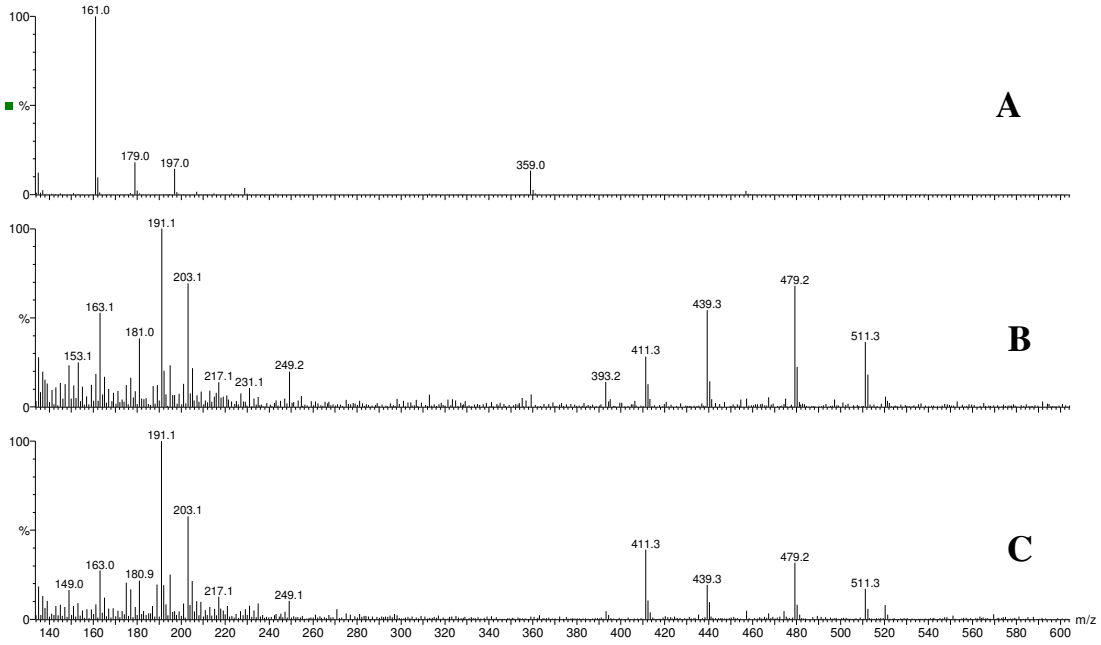


Figure 4.3. Negative (**A**, rosmarinic acid) and positive (**B**, oleanolic acid; **C**, ursolic acid) ESI mass spectra, obtained from the LC/MS TIC of mint extract (*Mentha* × *piperita*)

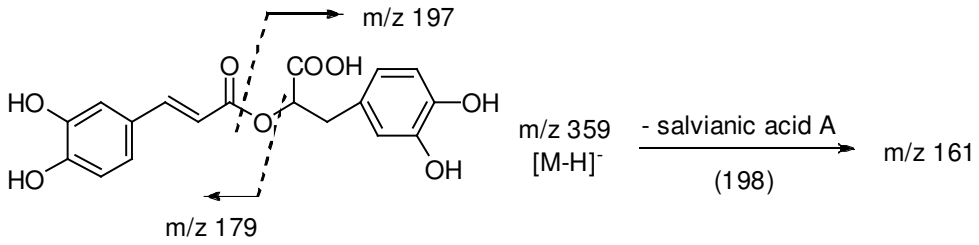


Figure 4.4. Our proposed MS fragmentation pathway of rosmarinic acid

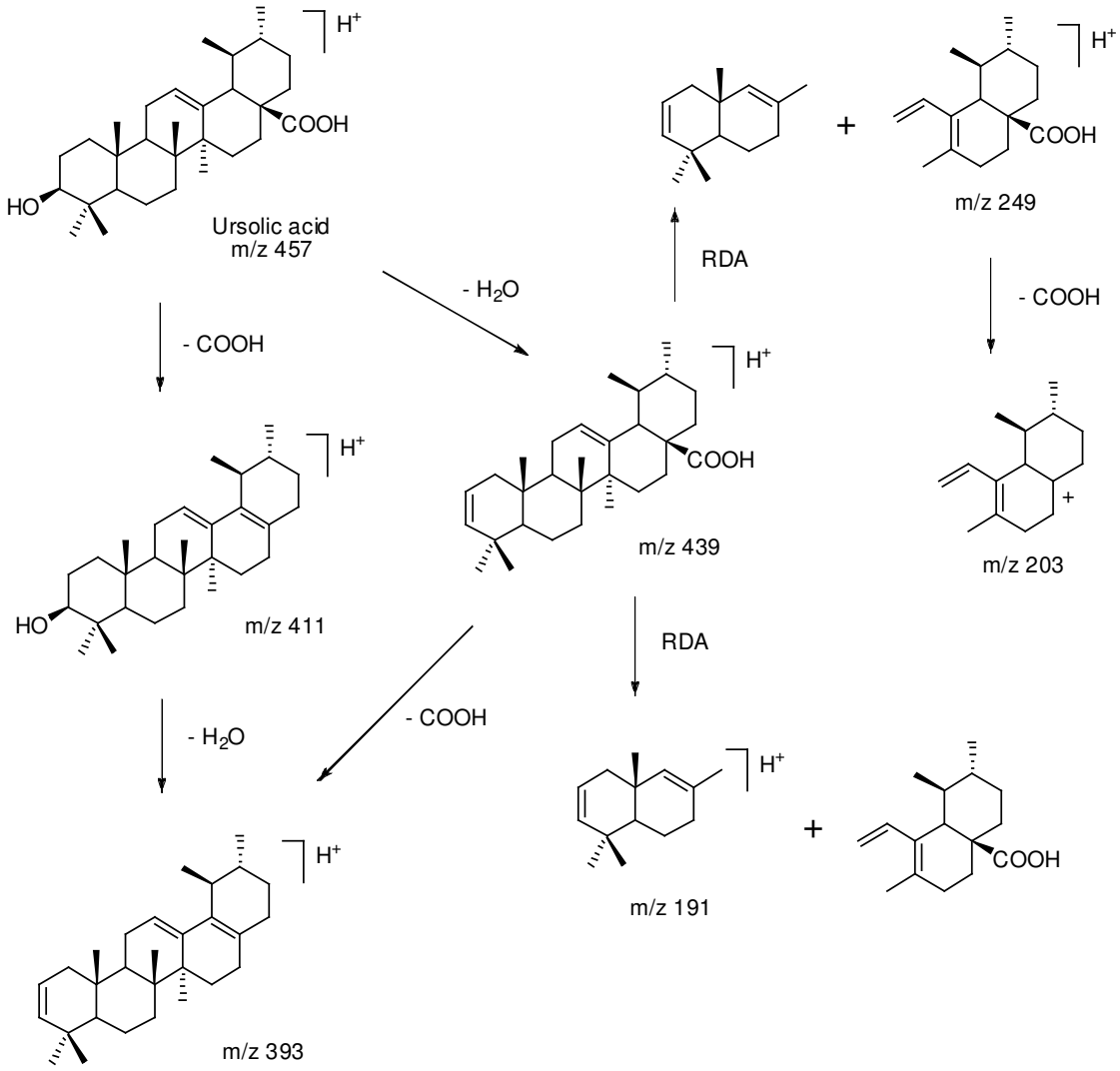


Figure 4.5. Our proposed MS fragmentation pathway of ursolic acid

4.3.2. LC/MS/MS Method Validation.

The precision of this method was validated by intra-assay and inter-assay evaluations. The relative standard deviations (RSDs) of intra-assay were calculated as 4.68%, 2.36% and 4.89% for rosmarinic acid, oleanolic acid and ursolic acid, respectively, conducted by carrying out six replicate injections of a single mint sample within the same day (**Table 4.1**). The inter-assay validation was applied with six replicate determinations of the same mint sample on three different days, and the RSDs were 5.51%, 4.21% and 5.34% for these three analytes. The recoveries of this method were performed by addition of known concentration levels, approximate 200%, 100% and 50% of the expected values in a spearmint sample (*M. spicata*). The mean recovery rates were 98.7%, 99.3% and 97.8% for rosmarinic acid, oleanolic acid and ursolic acid, respectively, without considerable difference at different spiking levels (RSD < 4.6%). This validation study suggested that the developed LC/MS/MS method was reliable, precise and sensitive for the simultaneous quantitation of rosmarinic acid, oleanolic acid and ursolic acid in mint samples.

Table 4.1. Recoveries of Rosmarinic Acid, Oleanolic Acid and Ursolic Acid at Different Spiking Levels

Analyte	Concentration (mg/g)	Added (mg/g)	Found (mg/g)	Recovery (%)	Mean (%)	RSD (%)
Rosmarinic	23.51	51.00	73.58	98.2		
acid		25.50	49.38	101.5	98.7	2.6
		12.75	35.81	96.5		
Oleanolic	0.75	1.80	2.47	95.7		
acid		0.90	1.65	99.7	99.3	3.5
		0.45	1.21	102.6		
Ursolic	1.36	3.00	4.24	96.1		
acid		1.50	2.77	94.3	97.8	4.6
		0.75	2.13	102.9		

4.3.3. Distribution of Rosmarinic Acid, Oleanolic Acid and Ursolic Acid in Different *Mentha* Species.

The use of species or varieties that display superior phytochemical content relative to the bioactivity can help to insure the quality of botanicals or raw materials to the dietary supplement industry. In this study, we comparatively examined a wide range of mint species and sources of mints within many of the species in order to first ask whether there is genetic variation in the accumulation or expression of the polyphenols in mint; and if

so, to then identify which sources may be the highest accumulators. Using this approach, we hypothesized that several species would be significantly higher than others. Thus, such results could help to guide further collections made to develop improved sources of specific phenolic acids. We also hypothesized that variation would be noted and that plant sources may vary in the specific targeted anti-inflammatory constituents while as an aggregate group or sum total we may not observe such differences. By sampling the mint collection when all grown under the identical environmental conditions, the differential genetic expression (under the defined environmental condition) is easier to observe. As such, all plants were cultivated under identical field conditions. During the harvest season, 35 mints belonging to 9 different *Mentha* species were collected and dried under low temperature (32 °C), of which 25 mints were then quantitatively analyzed for rosmarinic acid, oleanolic acid and ursolic acid contents based on the LC/MS/MS method which we developed (**Fig. 4.6**). The average rosmarinic acid content was 10.34 mg/g on dry weight basis across the 25 mints, with RSD = 58.6%. The most rosmarinic acid-rich sample was Green Curly Mint (*M.* × *piperita*), followed by Spearmint (*M. spicata*). The contents of oleanolic acid and ursolic acid were much lower, both within the range of 0.12-5.11 mg/g during all samples (**Table 4.2**). The mean content levels of oleanolic acid and ursolic acid were calculated as 0.84 mg/g (RSD = 79.6%) and 2.02 mg/g (RSD = 78.6%), respectively. For most of the mints, the concentration of ursolic acid was 2 to 3 times higher than that of oleanolic acid, while this trend was inversed for Japanese Field Mint (*M. Canadensis*). In Japanese Field Mint, the oleanolic acid content (2.41 mg/g) was much higher than

ursolic acid (0.54 mg/g). The Australian Mint (*M.* × *gracilis*) was characterized as that these two triterpenoid acids were almost at the same concentration level (0.43 mg/g versus 0.42 mg/g), whereas the concentration ratio of ursolic acid to oleanolic acid may reach to as high as 5.7 for Hajek Mint (*M. longifolia*). The high triterpenoid acid-rich samples include Grapefruit Mint (*M.* × *piperita*), Egyptian Mint (*M.* × *villosa*), Hillary's Sweet Mint (*M. aquatica*× *M. suaveolens*) and Apple Mint (*M.* × *villosa*). We also noticed that the concentration variation of these three compounds was not limited among different species, but also observed striking differences, as expected within the species between the varieties and accessions. The genetic expression and influence in the different *Mentha* species as well as variety are critical components to consider in the standardization of anti-inflammatory agents from mint.

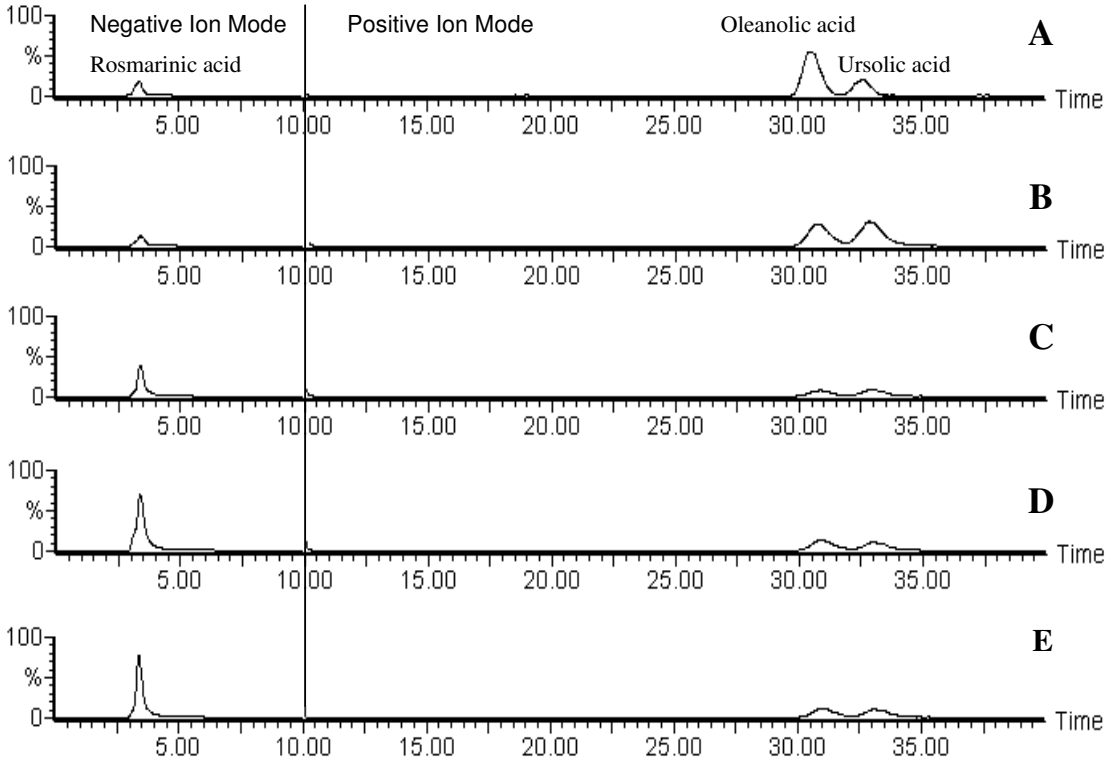


Figure 4.6. MRM (multiple reaction monitoring) chromatogram of (**A**) standards and representative chromatograms of (**B**) Peppermint (*Mentha* × *piperita*), (**C**) Lavender Mint (*M. aquatica*), (**D**) Persian Mint (*M.* × *piperita*), and (**E**) Orange Mint (*M. aquatica*); The two time segments were set as 0-10 min at *m/z* 359→161 (rosmarinic acid), and 10-40 min at *m/z* 439→203 (oleanolic acid and ursolic acid).

Table 4.2. Anti-inflammatory Acid Contents in Different Sources of *Mentha* spp. (Milligrams per Gram of Dry Matter)

sample name	Latin name	rosmarinic acid	oleanolic acid	ursolic acid
Peppermint	*M.* × *piperita*	6.69±0.38	0.79±0.06	2.37±0.09
Spearmint	*M. spicata*	23.51±0.69	0.75±0.05	1.36±0.09
Lavender Mint	*M. aquatica*	9.52±0.64	0.12±0.01	0.54±0.03
Persian Mint[a]	*M.* × *piperita*	14.56±0.52	0.27±0.01	0.67±0.02
Chewing Gum Mint	*M.* × *piperita*	15.55±0.21	0.60±0.01	2.01±0.08
Orange Mint	*M. aquatica*	14.60±0.40	0.30±0.00	0.85±0.01
Apple Mint	*M.* × *villosa*	12.21±0.61	2.02±0.03	4.78±0.04
Austrian Mint	*M.* × *gracilis*	11.37±0.62	0.43±0.03	0.42±0.04
Balsam Tea Mint	*M.* × *piperita*	3.73±0.26	0.45±0.02	1.39±0.03
Chocolate Mint	*M.* × *piperita*	6.44±0.20	0.58±0.02	1.54±0.03
Curly Mint	*M. spicata*	9.33±0.23	0.67±0.01	1.74±0.00
Egyptian Mint	*M.* × *villosa*	7.66±0.38	1.99±0.03	5.11±0.04
Fuzzy Spearmint	*M. spicata*	19.76±0.71	1.07±0.04	2.93±0.14
Grapefruit Mint	*M.* × *piperita*	5.60±0.26	2.00±0.16	5.72±0.36
Green Curly Mint	*M.* × *piperita*	23.80±0.42	0.72±0.02	1.89±0.06
Hajek Mint[b]	*M. longifolia*	6.99±0.42	0.45±0.03	2.57±0.18
Hillary's Sweet Mint	*M. aquatica*× *M. suaveolens*	5.79±0.12	1.87±0.03	5.04±0.01
Hypocalyx Mint	*M. canadensis*	4.88±0.35	1.25±0.04	2.88±0.08
Japanese Field Mint	*M. canadensis*	1.75±0.00	2.41±0.03	0.54±0.02
Lime Mint	*M. aquatica*× *M. suaveolens*	2.99±0.12	0.54±0.02	1.78±0.03
Regular Mint	*M. spicata*	10.14±0.18	0.51±0.01	1.41±0.08
Scotch Mint	*M.* × *gracilis*	16.91±0.30	0.21±0.00	0.39±0.04
Todd Mitcham Mint	*M.* × *piperita*	7.09±0.26	0.45±0.01	1.23±0.08
Variegated Mint	*M.* × *piperita*	9.94±0.50	0.19±0.00	0.50±0.04
Water Mint	*M.* × *smithiana*	7.18±0.40	0.53±0.02	0.92±0.02

[a] Persian Mint was named by us at Rutgers to give respect to the purported origin of this unusual mint found in San Francisco by those of Persian background and used in cooking and salads. The mint was found and originally sent to Michigan State University where university researchers had evaluated it in the field and found the essential oils to be unacceptable relative to commercial traditional mint. They graciously gave this un-named mint to Prof. Simon and since then Rutgers has been working with it as a potential new culinary herb for those consumers seeking a more exotic less known mint with unusal aroma and flavor. This line has been named informally as Persian Mint and is also not commercially available.

[b] Hajek mint refers to the commercial farm from which the materials was sourced from the commercial farm of Bob Hajek, Indiana. This material is also not commercially available.

4.3.4. Characterization of the Flavonoids in Mints.

HPLC/MS coupled with a photodiode array detector (PAD) enabled us to obtain the UV and MS spectra for flavonoids on chromatograms. Wherever possible, we compared the data with reported literature, and sometimes with the spectra of reference standards to check the identification and purity of each peak. Under positive mode, at the energy level of 60%, the flavonoids showed strong molecular ions $[M+1]^+$ and/or $[M+Na]^+$, and fragment peaks including $[M\text{-glucose}+1]^+$, $[M\text{-rhamnose}+1]^+$ and $[M\text{-rhamnose-glucose}+1]^+$, offering useful information to identify the sugar moieties. The neutral loss of *m/z* 146 (rhamnose) along with *m/z* 308 (rhamnose+glucose) is a typical MS fragmentation pattern for rutinosides. We observed this fragmentation pattern for many peaks of chromatograms. According to the identification of rutinsides in *Mentha* species in literature, these compounds were tentatively determined as eriocitrin (peak 1), luteolin 7-*O*-rutinoside (peak 2), narirutin (peak 4), isorhoifolin (peak 5), hesperidin (peak 6), and diosmin (peak 7) (**Fig. 4.7** and **Fig. 4.8**). The comparison with the UV spectra and retention times of the reference compounds on HPLC further confirmed our identification (**Table 4.3**). Peak 3 with molecular ion $[M+1]^+$ at *m/z* 449 and fragmentation $[M\text{-glucose}+1]^+$ at *m/z* 287 was identified as luteolin 7-*O*-glucoside, which was also reported by other researchers from mint (Guedon & Pasquier, 1994; Kosar et al., 2004). By screening 29 *Mentha* spp. and sources within 8 species (some of mint samples

were not screened due to the unavailability of harvesting from the research field), we concluded that the flavonoids in mints were mostly glycolated with rutinoside. Some researchers reported the presence of free flavones such as eriodictyol and apigenin in *Mentha* species (Kosar et al., 2004); however, they were hardly detected in our mint samples or detected at very low concentration levels. Acacetin 7-*O*-rutinoside was reported present in *M. haplocalyx* and *M. arvensis* (Lin et al., 2006; Oinone et al., 2006). We detected this compound by HPLC/MS with fragmentation ion $[M+1]^+$ at *m/z* 593 and fragmentations [M-rhamnose+1]$^+$ at *m/z* 447 and [M-rhamnose-glucose+1]$^+$ at *m/z* 285 in some *M. piperita* and *M. aquatica* varieties. Since our mint samples contain low concentration levels of acacetin 7-*O*-rutinoside, and no pure standard compound was commercially available, the quantification of this constituent was not performed and work with this compound was not continued.

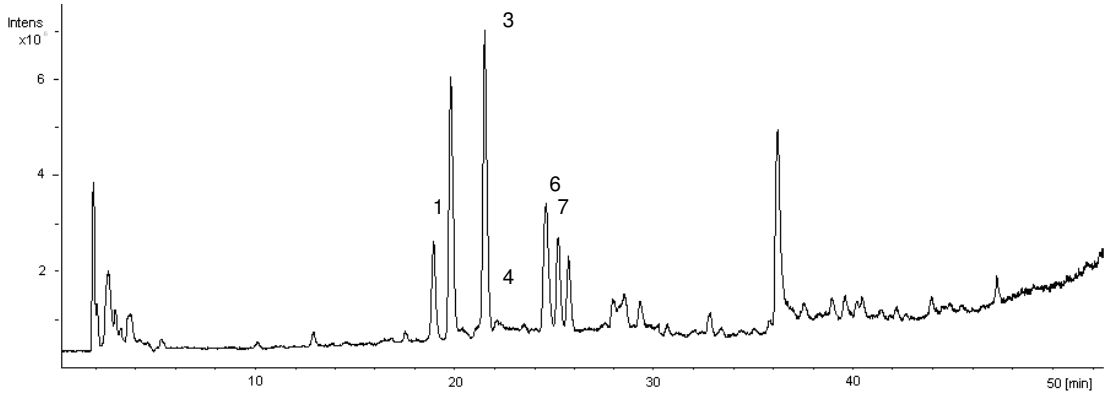
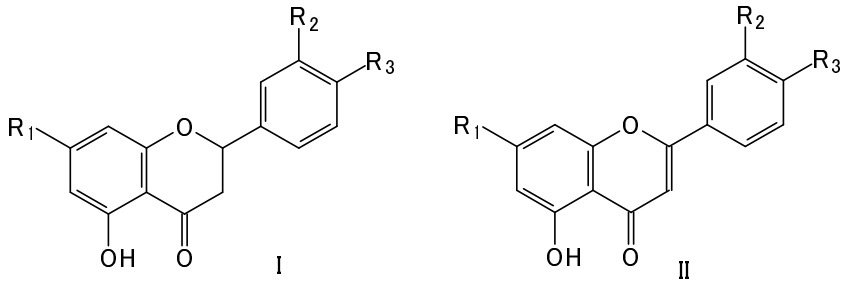


Figure 4.7. TIC (Total Ion Chromatogram) of a mint sample (Peppermint, *Mentha* × *piperita*). For peak identities, see **Table 4.3**.



Eriocitrin (ERC): aglycone = I, R_1 = *O*-rutinose, R_2 = OH, R_3 = OH

Luteolin 7-*O*-rutinoside (LR): aglycone = II, R_1 = *O*-rutinose, R_2 = OH, R_3 = OH

Luteolin 7-*O*-glucoside (LG): aglycone = II, R_1 = *O*-glucose, R_2 = OH, R_3 = OH

Narirutin (NRT): aglycone = I, R_1 = *O*-rutinose, R_2 = H, R_3 = OH

Isorhoifolin (IRF): aglycone = II, R_1 = *O*-rutinose, R_2 = H, R_3 = OH

Hesperidin (HSP): aglycone = I, R_1 = *O*-rutinose, R_2 = OH, R_3 = OCH_3

Diosmin (DSM): aglycone = II, R_1 = *O*-rutinose, R_2 = OH, R_3 = OCH_3

Figure 4.8. Chemical structures of the major phenolic compounds in mint (*Mentha* spp.)

Table 4.3. Peak Assignments of Flavonoids in Mint

peak no.	t_R (min)	$[M+1]^+$ MS (*m/z*)	$[M+Na]^+$ MS (*m/z*)	fragmentation ions (*m/z*)	UV λ_{max} (nm)	compound identification
1	18.5	597		289, 451	284, 328	eriocitrin
2	20.3	595		287, 449	255, 350	luteolin 7-*O*-rutinoside
3	21.5	449		287	254, 350	luteolin 7-*O*-glucoside
4	22.2	581		273, 435	284, 331	narirutin
5	23.4	579		271, 433	267, 338	isorhoifolin
6	23.8	611	633	303, 465	284, 328	hesperidin
7	24.4	609		301, 463	253, 347	diosmin

4.3.5. Quantitative Analysis of the Predominant Phenolic Constituents in Different *Mentha* Species.

Some research groups have developed HPLC method to separate the phenolic compounds from several *Mentha* species. The purpose of this investigation was to further develop and validate an HPLC/UV quantitative method applicable for extensive quantitation of different mint varieties including some rare species. We utilized a Luna C18 (2) column to achieve ideal resolution, particular with the separation of isorhoifolin, hesperidin and eriocitrin from other small peaks. A 2% isopropanol was added to the mobile phase to

provide better peak shapes. This method was also successfully used for qualitative analysis in the research except 0.1% phosphoric acid was changed to 0.1% formic acid (volatile buffer) on LC/MS. Mints from eight *Mentha* species (*M.* × *piperita*, *M. spicata*, *M.* × *gracilis*, *M. longifolia*, *M. Canadensis*, *M.* × *villosa*, *M. aquatica* and *M. aquatica* × *M. suaveolens*) including the commercial varieties used in the production of mint oils, as well as many specialty mints were vegetatively propagated and field transplanted at the research farm, and further applied for quantitative analysis based on this HPLC method. Seven predominant flavonoids were efficiently separated and quantitated for all mint samples (**Fig. 4.9**). The analytes quantitated in the investigation cover the major phenolic constituents normally found in mints.

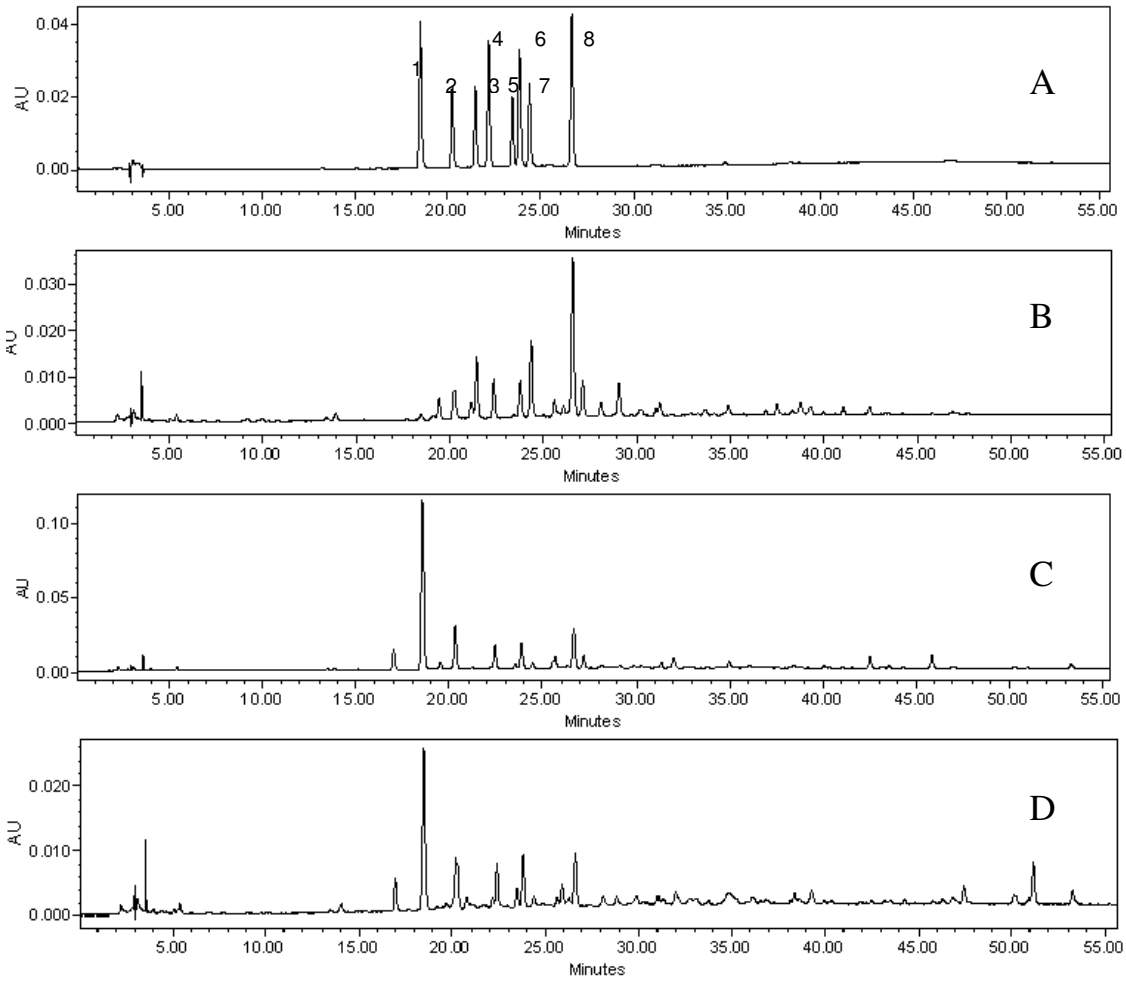


Figure 4.9. HPLC/UV chromatogram of the phenolic standards (**A**), and 3 representative chromatograms of mint samples, Fuzzy Spearmint (*Mentha spicata*) (**B**), Peppermint (*M.* × *piperita*) (**C**), and Lime Mint (*M. aquatica*× *M. suaveolens*) (**D**), monitored at wavelength of 280nm.

4.3.6. Validation on the HPLC/UV Quantitative Method.

System precision was validated by conducting six replicated injections of a Chewing Gum Mint (*M.* × *piperita*) sample within the same working day. The RSDs (Relative Standard Deviations) of the peak areas for eriocitrin, luteolin-7-*O*-rutinoside, narirutin, isorhoifolin, hesperidin and diosmin were 0.50%, 0.44%, 1.00%, 2.38%, 0.57% and 1.70%, respectively; the RSDs of the retention times for these six compounds were calculated as 0.13%, 0.10%, 0.11%, 0.11%, 0.10% and 0.09%, respectively. The precision of the extraction procedure was also validated on the chewing gum mint. Four sample replicates, weighing about 100 mg, were extracted as described above in 25 mL of 70% methanol. An aliquot of each sample was then injected and quantitated. The average amounts of eriocitrin, luteolin-7-rutinoside, narirutin, isorhoifolin, hesperidin and diosmin and rosmarinic acid were 3.62% with a RSD of 0.50%, 1.51% with a RSD 0.50%, 0.13% with a RSD of 1.20%, 0.11% with a RSD of 2.50%, 0.51% with a RSD of 2.10% and 0.22% with a RSD of 1.56%, respectively. The recovery was evaluated by the spiking of five analytes, eriocitrin, luteolin-7-*O*-rutinoside, narirutin, isorhoifolin and hesperidin approximately 100% of the expected values in a typical mint sample preparation. The recovered percentages were 98.9% for eriocitrin, 97.3% for luteolin-7-*O*-rutinoside, 100.4% for narirutin, 97.3% for isorhoifolin and 94.7% for hesperidin. In conclusion, the recommended method was reliable, consistent and precise for the quantitation of the predominant phenolic contents in varied mint samples.

4.3.7. Distribution of the Phenolic Constituents in *Mentha* Species.

Flavonoids, the major non-volatile phenolic compounds in mints, are distributed predominantly as glycolated forms, specifically flavonoid rutinosides. Rosmarinic acid, a typical phenolic acid present in many Lamiaceae family plants, is extensively present in all the tested mint samples, ranging from 0.22±0.16% to 1.41±0.36% through eight *Mentha* species on dry weight basis (**Table 4.4** and **Table 4.5**). Peppermint and spearmint are the most cultivated crops in the United States for essential oil production. The other commercial mints may include Black peppermint, Chinese mint, Japanese field mint, Todd Mitcham, and Scotch spearmint. Peppermint, black peppermint and Todd Mitcham are taxonomied as *M. piperita*. Along with other *M. piperita* varieties tested, namely variegate mint, chocolate mint, chewing gum mint, blue balsam mint, and green curly mint, we found this widely grown species contained notably high concentrations of phenolics compared to other *Mentha* species. Eriocitrin was quantitated at 2.49±0.94% on dry weight basis in these six *M. piperita* varieties, followed by luteolin 7-*O*-rutinoside at 1.00±0.33%, and rosmarinic acid at 1.03±0.61%. The common commercial mint varieties were compared by the flavonoids (the sum of eriocitrin, luteolin 7-*O*-rutinoside, luteolin 7-*O*-glucoside narirutin, isorhoifolin, hesperidin, and diosmin) concentrations. Todd Mitcham mint showed the highest flavonoid content (5.84%), followed by black spearmint (3.62%) and spearmint (3.36%), all of which belong to *M. piperita*. The

species *M. spicata* (spearmint, regular mint, Kentucky mint, fuzzy spearmint, curly mint and large-leaf spearmint) showed a different flavonoid profile, with significantly lower content of eriocitrin (0.06±0.09%) and a higher content of diosmin (0.49±0.20%) than that of *M. piperita* (0.15±0.07%). The other high phenolic content species noteworthy is *M. aquatica* (orange mint and lavender mint), with the eriocitrin content as 1.22±0.25%, luteolin 7-*O*-rutinoside as 0.71±0.15% and hesperidin as 0.66±0.21%. Based on this extensive survey of mint phenolic constituents, it can be concluded that many mint varieties particularly some commercial mints from *M. piperita* are rich source of bioactive phenolic compounds, and it would be worthwhile to consider the economical values of these non-volatile components from postdistillation materials.

Table 4.4. Flavonoids and Rosmarinic Acid Contents (g/100g d. w.) in Different Sources of Mint (*Mentha* spp.)[a]

Mint varieties[c]	ERC	LR	LG	NRT	IRF	HSP	DSM
Water Mint	1.10	0.99	---[b]	---	0.51	0.24	0.20
Variegated Mint	1.73	0.80	0	0.02	0.153	0.90	0.28
Todd Mitcham Mint	3.73	1.40	0.0	0.13	0.10	0.36	0.12
Peppermint	2.01	0.85	---	---	0.11	0.29	0.10
Orange Mint	1.39	0.81	0.04	0.15	0.46	0.80	0.35
Lime Mint	0.65	0.38	---	0.03	0.14	0.20	0.07
Lavender Mint	1.04	0.60	---	---	0.31	0.51	0.27
Green Curly Mint	1.20	0.57	---	0.03	0.08	0.55	0.16
Egyptian Mint	0.51	1.11	---	---	0.06	0.23	0.18
Eau de Cologne Mint	1.16	0.78	---	0.03	0.30	0.50	0.37
Chocolate Mint	3.28	1.17	---	0.03	0.13	0.31	0.12
Chewing Gum mint	3.62	1.51	---	0.13	0.11	0.51	0.22
Blue Balsam Tea Mint	1.94	0.97	---	---	0.12	0.21	0.08
Black Peppermint	2.40	0.74	---	0.02	0.09	0.28	0.09
Apple Mint	0.20	0.47	0.05	---	0.09	0.11	0.34
Pineapple Mint	---	0.12	0.05	---	---	---	---
Spearmint	0.03	0.23	---	---	---	0.19	0.69
Regular Mint	0.25	0.10	---	---	0.16	0.19	0.68
Kentucky Mint	0.03	0.48	0.06	---	0.17	0.29	0.47
Hyplocalyx Mint	---	0.25	0.05	---	---	0.22	0.23
Horse Mint	0.17	0.36	0.09	0.03	0.14	0.12	0.10
Himalayan Silver Mint	0.06	0.24	0.05	---	---	0.13	0.08
Hillary's Sweet Mint	0.35	0.40	---	---	0.31	0.21	0.18
Fuzzy Spearmint	0.03	0.20	0.30	---	---	0.16	0.56
Curly Mint	0.03	0.08	---	---	---	0.37	0.31
Scotch Spearmint	---	---	---	---	---	0.46	0.15
Persian Mint	---	---	---	---	---	1.67	0.49
Large Leaf Spearmint	---	0.10	---	---	---	0.13	0.21
Japanese Field Mint	---	---	---	---	---	0.42	0.17
Grapefruit Mint	0.05	0.10	---	---	0.04	0.62	0.41
Chinese Mint	---	---	---	---	---	0.18	0.61
Austrian Mint	0.02	0.09	---	---	0.08	0.86	0.73
Ginger Mint	---	---	---	---	---	0.08	0.93
Bergamot Mint	---	---	---	---	---		0.27
Hajek Mint	---	0.14	---	---	0.12	0.07	0.23

[a] Abbreviations: ERC, eriocitrin; LR, luteolin 7-*O*-rutinoside; LG, luteolin 7-*O*-glucoside; NRT, narirutin; IRF, isorhoifolin; HSP, hesperidin; DSM, diosmin. [b] Not detectable.
[c] *Mentha* species listed in Table 4.2 and also in Table 4.5.

Table 4.5. Flavonoids and Rosmarinic Acid Contents (g/100g d. w.) Summarized as Different Mint (*Mentha* spp.) Species[a]

Species[b]	ERC	LR	LG	NRT	IRF	HSP	DSM
Mentha × *piperita*	2.49±0.94	1.00±0.33	ND	0.05±0.05	0.11±0.02	0.43±0.22	0.15±0.07
Mentha spicata	0.06±0.09	0.20±0.15	0.06±0.12	ND	0.06±0.09	0.22±0.09	0.49±0.20
Mentha × *gracilis*	ND	0.03±0.05	ND	ND	0.03±0.05	0.44±0.43	0.38±0.31
Mentha longifolia	0.08±0.09	0.25±0.11	0.05±0.05	0.01±0.02	0.09±0.08	0.11±0.03	0.14±0.08
Mentha canadensis	ND	0.08±0.14	0.02±0.03	ND	ND	0.27±0.13	0.34±0.24
Mentha × *villosa*	0.36±0.22	0.79±0.45	0.03±0.04	ND	0.08±0.02	0.17±0.08	0.26±0.11
Mentha aquatica	1.22±0.25	0.71±0.15	0.02±0.03	0.08±0.11	0.39±0.11	0.66±0.21	0.31±0.06
Mentha aquatica× *M. suaveolens*	0.50±0.21	0.39±0.01	ND	0.02±0.02	0.23±0.12	0.21±0.01	0.13±0.08

[a] Abbreviations: ERC, eriocitrin; LR, luteolin 7-*O*-rutinoside; LG, luteolin 7-*O*-glucoside; NRT, narirutin; IRF, isorhoifolin; HSP, hesperidin; DSM, diosmin; RA, rosmarinic acid; ND, not detectable. [b] Values are expressed as mean±SD generated from different varieties for the same species. *M.* × *piperita* was composed of peppermint, variegated mint, Todd Mitcham mint, chocolate mint, chewing gum mint, blue balsam mint, black peppermint and green curly mint, n = 8; *M. spicata* was composed of spearmint, regular mint, Kentucky mint, fuzzy spearmint, curly mint and large-leaf spearmint, n = 6; *M.* × *gracilis* was composed of Scotch mint, Austrian mint and bergamot mint, n = 3; *M. longifolia* was composed of horse mint, Himalayan silver mint and Hajek mint, n = 3; *M. Canadensis* was composed of Hypocalyx mint, Japanese field mint and Chinese mint, n = 3; *M.* × *villosa* was composed of Egyptian mint and apple mint, n = 2; *M. aquatica* was composed of orange mint and lavender mint, n = 2; *M. aquatica*× *M. suaveolens* was composed of lime mint and sweet mint, n = 2. The other mints from Table 4.4 were not authenticated as to *Mentha* spp..

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CHAPTER 5. NIR ANALYTICAL METHOD DEVELOPMENT ON RAPID QUANTITATION AND RAW MATERIAL QUALITY CONTROL OF THE ANTI-INFLAMMATORY CONSTITUENTS IN OREGANO AND MINT

5.1. INTRODUCTION

Near-infrared spectroscopy (NIR) as a novel analytical technique has gained wide acceptance in the pharmaceutical and food industries in recent years for material testing, product quality control and as a rapid non-destructive and non-invasive quantitation screen. The most salient aspect of this technique is that it is rapid (readings recorded in seconds) without sample pretreatment, and even the ability to scan through plastic and glass. The major pharmacopoeias have generally adopted NIR techniques for use in pharmaceutical analysis (Reich, 2005). The European and United States Pharmacopoeias both contain sections addressing the suitability of NIR instrumentation for operational qualification and performance verification such as response repeatability, photometric linearity and photometric noise, however, these sections provide only an overview on developing and validating the methodology (Reich, 2005). With each new application, new models built specifically to first ascertain whether NIR technology can be sufficiently sensitive, robust and reliable is needed. The purpose of our research into the use of NIR as a quality control technology was precisely to examine whether such an approach can be used as a rapid screen to quantify or predict the concentration of specific anti-inflammatory constituents in extracts of herbs and if so, to conduct a feasibility study and method validation. Successful results would permit the incorporation of NIR as a component within a larger QC system. Dependent upon the sensitivity and robustness of

this approach, we also wanted to examine whether NIR could be used as a technique to support plant breeding and genetic studies and processing studies which seek to increase the level of these compounds in plants, or maximize the concentration and record compound stability in final extracts and product preparations with raw material pre-evaluation of the anti-inflammatory constituents in oregano and mint.

The NIR region spans the wavelength ranging from 780 to 2500 nm. The absorption bands correspond mainly to overtones and combinations of fundamental vibrations (Reich, 2005). The molecular vibration can be described using the anharmonic oscillator model, by which, the energy of the difference can be calculated from

$$\Delta E_{vib} = h\nu\,[1 - (2\upsilon + \Delta\upsilon + 1)\,y]$$

Where y is the anharmonicity factor, υ is the vibration quantum number, h is the Plank constant, and ν is the fundamental vibration frequency of the bond that yields an absorption band in the middle IR region. The anharmonicity can result in transitions between vibrational states at $\Delta\upsilon = \pm 2, \pm 3\ldots$. These transitions between non-contiguous vibrational energy states generate absorption bands called overtones at multiples of the fundamental vibrational frequency. These bands appear at 780-2000 nm, according to the overtone order and the bond nature and strength. In polyatomic molecules, multiple vibrational modes can interact in such a way as to cause simultaneous energy changes and give rise to absorption bands known as combination bands appearing at wavelength 1900-2500 nm. The main bonds typically observed in the NIR region correspond to the

bonds containing hydrogen atoms, namely C-H, N-H, O-H and S-H. The other weak absorptions may be caused by C=O, C-C and C-Cl vibrations. Interactions between atoms in different molecules alter vibrational energy states, allowing physical properties of the material (such as density, viscosity and particle size) to be determined. Therefore, the NIR technique not only provides chemical information and compositions, but also the physical properties of samples. (Blanco & Villarroya, 2002)

The analytical information contained in the NIR spectra is generally broad bands, extensively overlapped and influenced by a number of physical, chemical and structural variables. Additionally, spectral differences between samples may be very slight and hard to be distinguished with naked eyes. With the development of chemometrics techniques, the NIR is currently becoming available to provide vast amounts of data with speedy and efficient processing. Chemometrics help to extract as much relevant information as possible from the analytical data, and ignore the irrelevant information. The NIR spectra can be extracted by using various multivariate analysis techniques that relate analytical variables to analyte properties, and allow samples with similar characteristics to be grouped, in order to established classification method for the qualitative or quantitative prediction of unknown samples.

Chemometrics, known as factor analysis methods, derives form the use of abstract functions called factors to model variance in a data set (Reich, 2005). Each factor

attempts to span the maximum amount of variance in the data set. The challenge of using chemometrics is determining which factors to use in a calibration. Calibration of samples is desirable to have a wide range in composition of not only the current interest but also of all possible source of variation likely to be encountered in the future. Multivariate analyses such as partial least-squares regression analysis (PLS), principal component regression analysis (PCRA), principle component analysis (PCA), and soft independent modeling of class analogy (SIMCA) are the most frequently used methods for analyzing NIR spectra (Akikazu et al., 2006).

PLS finds the directions of the greatest variability by comparing the information of the spectral and target property with new axes, named PLS factors. The optimum number of factors used to build the calibration model represents the most relevant variations correlated with the target property values. Too many factors may lead to an “overfitting”, a high coefficient and a low standard error of prediction (Reich, 2005; Juliani et al., 2006; Liu et al., 2006).

5.2. MATERIALS AND METHODS

5.2.1. Instrument.

The LabSpec 5000 (ASD Inc., Boulder, CO) collects spectra from 350 to 2500nm with an increment of 1 nm. This portable instrumentation allows for a variety of sampling accessories to be used, including an ASD's Muglight attachment for scanning. The ground samples of the botanicals were manually placed into the Muglight cuvette at identical amount, and reflectance spectra for all three sample types were collected for using and an ASD Labspec 5000 spectrometer system with the Muglight high intensity source probe. Spectra were collected using a 100 scan average with two replications per sample. This number of scans corresponds to a collection time of 10 seconds. Spectra were then imported into the UNSCRAMBLER version 9.7 (CAMO, Woodbridge, NJ) for evaluation and modeling. These works were done by our partner at ASD Inc., Boulder, CO.

5.2.2. Pretreatment of Spectra.

The spectra of solid samples are influenced by their physical properties. Thus any spectral pretreatment should be used to minimize those contributions and develop more

simple and robust models. By derivation and smoothing of the spectra, baseline drifts are eliminated and small spectral differences are enhanced. Savitzky-Golay smoothing was used for pretreatment of spectra, being a moving window averaging method by which the data were fitted by a polynomial of a certain degree. The central point is the window replaced by the polynomial value (Reich, 2005).

5.2.3. Multivariate Analysis.

PLS1 models were developed for each sample type as well as for all sample types in the same calibration. PLS regression seeks to provide statistical models to reduce the space spanned by the often large number of correlated predictors to a lower dimensional space by generating derived PLS components. It is commonly expected that a small number of derived components will be finally used as regressors in the PLS model (Athanassios, www.agro-montpellier.fr). PLS has been particularly successful in developing multivariate calibration models because it uses the concentration information (Y variable) in determining how the regression factors are derived from the spectral data matrix (X), resulting a more information-rich data set and reducing the impact of irrelevant variations in the calibration model.

5.2.4. Method Validation.

Both full cross-validation and true test set validations were used for calibration model

validation. Samples were randomly assigned to either calibration or validation sets for creation of the true test set. In this study, 50 samples were used for the calibration set and 16 for the validation set. Test set validation was used only for the calibrations developed with all samples. Full cross validation is also called leave-one-out cross-validation. The left-out sample is predicted with the remaining spectra of the training set by the regression model, and the procedure is repeated with leaving out each of the samples from the training set. The root mean square error of calibration (RMSEC) is calculated each time as:

$$\text{RMSEC} = \sqrt{\frac{\sum(y_i - \hat{y}_i)^2}{n}}$$

Where y is the measured value, ý is the predicted value and n is the number of samples in the training set (Luypaert, Zhang & Massart, 2003). The PLS factor number is chosen depending on the lowest RMSEC. This procedure is calculated for the test data set as root mean squire error of prediction (RMSEP). The true test validation was preformed by dividing all spectra into a training set and a test set. PLS1 model was used to make the calibration on the training set. The number of PLS factors included in the model was optimized by the comparison of RMSEC for the training set. RMSEP of the test set was calculated for each analyte.

5.2.5. NIR Probe.

NIR spectroscopy instrumentation has developed rapidly to meet the requirement of rapid on-line real time analyses and flexibility in response to different sample states. The appropriate measuring mode of NIR probe is dictated by the optical properties of the samples. Transparent materials are generally measured in transmittance mode. Solid, semi-solid and turbid liquid may be in diffuse transmittance, diffuse reflectance or transflectance mode according to their different absorption and scattering characteristics. The measurement of absorbance (A) values relative to a standard reference material is required, with A representing to log 1/R and log 1/T for reflectance and transmittance spectra, respectively. In our study here, the ground oregano and mint samples were placed into the Muglight Cuvette and reflectance spectra were converted from Reflectance to Absorbance (Log 1/R) for model development.

5.2.6. Reference Analysis.

Chromatographic analysis was performed on a Waters 2695 HPLC system (Waters Corp., Milford, MA) equipped with an auto-sampler, quaternary pump system, thermostated column compartment, degasser and Millenium 3.2 software. Separation was achieved by using a tandem column system: a Synergi 50 × 4.6 mm, i.d. 4 μm, Polar-RP column (Phenomenex Inc., Torrace, CA) and downstream a Microsorb 100 × 4.6 mm, i.d. 3 μm, C18 column (Varian Inc., Palo Alto, CA). Mass spectrometer used in this research was a

triple stage quadrupole Quattro II (Micromass Co., Altrincham, UK) equipped with the orthogonal Z-spray electrospray ionization (ESI) interface and the acquisition data processor Masslynx 3.4 software. In this study, we randomly used 27 mint, 32 Greek oregano and 7 Syrian oregano samples where in addition to the NIR screens we first chemically profiled to quantitate their respective contents of rosmarinic acid, oleanolic acid, ursolic acid and total triterpenes, which was the addition of both oleanolic and ursolic acids (**Table 5.1** – **Table 5.3**).

Table 5.1. Reference Contents of Anti-inflammatory Agents in Greek Oregano (*Origanum vulgare* ssp. *hirtum*) Samples

Sample code	Description	Rosmarinic acid (g/100g)	Oleanolic acid (g/100g)	Ursolic acid (g/100g)	Total triterpenes[a] (g/100g)
G1	Greek oregano	4.35	0.16	0.51	0.67
G4	Greek oregano	4.06	0.14	0.49	0.63
G5	Greek oregano	4.28	0.22	0.71	0.92
G6	Greek oregano	3.61	0.18	0.69	0.87
G7	Greek oregano	2.48	0.23	0.80	1.03
G8	Greek oregano	5.41	0.18	0.69	0.87
G9	Greek oregano	4.17	0.25	0.91	1.16
G13	Greek oregano	4.02	0.19	0.80	1.00
G14	Greek oregano	1.41	0.19	0.63	0.82
G15	Greek oregano	6.69	0.11	0.47	0.58
G19	Greek oregano	4.57	0.16	0.57	0.72
G20	Greek oregano	5.04	0.18	0.71	0.88
G21	Greek oregano	5.23	0.15	0.52	0.67
G22	Greek oregano	4.24	0.14	0.46	0.59
G23	Greek oregano	6.60	0.22	0.79	1.01
G24	Greek oregano	7.70	0.17	0.77	0.94
G25	Greek oregano	1.36	0.17	0.76	0.93
G26	Greek oregano	3.09	0.17	0.65	0.82
G27	Greek oregano	1.29	0.28	1.03	1.32
G28	Greek oregano	1.35	0.14	0.42	0.56
G29	Greek oregano	5.35	0.15	0.52	0.67
G30	Greek oregano	3.22	0.15	0.59	0.74
G31	Greek oregano	3.59	0.13	0.49	0.62
G32	Greek oregano	3.01	0.19	0.88	1.08
G33	Greek oregano	3.53	0.13	0.50	0.64
G34	Greek oregano	3.57	0.13	0.54	0.66
G35	Greek oregano	3.10	0.12	0.57	0.69
G36	Greek oregano	3.13	0.14	0.50	0.64
G37	Greek oregano	2.89	0.12	0.56	0.68
G38	Greek oregano	1.52	0.21	0.88	1.09
G39	Greek oregano	1.66	0.22	0.92	1.14
G40	Greek oregano	2.30	0.22	0.93	1.15
Average	Mean ± SD	3.66 ± 1.65	0.17 ± 0.04	0.67 ± 0.17	0.84 ± 0.21

[a] Total triterpenes expressed as the addition of oleanolic acid and ursolic acid

Table 5.2. Reference Contents of Anti-inflammatory Agents in Syrian Oregano (*Origanum syriacum*) Samples

Sample code	Description	Rosmarinic acid (g/100g)	Oleanolic acid (g/100g)	Ursolic acid (g/100g)	Total triterpenes (g/100g)
S1	Syrian oregano	0.90	0.98	2.44	3.41
S2	Syrian oregano	3.82	1.03	2.53	3.55
S3	Syrian oregano	2.85	1.07	3.51	4.57
S4	Syrian oregano	3.14	1.28	3.00	4.27
S5	Syrian oregano	2.44	0.84	2.23	3.07
S6	Syrian oregano	1.31	0.08	0.08	0.16
S7	Syrian oregano	1.58	0.73	1.63	2.36
Average	Mean ± SD	2.29 ± 1.06	0.86 ± 0.39	2.20 ± 1.11	3.06 ± 1.47

[a] Total triterpenes expressed as the addition of oleanolic acid and ursolic acid

Table 5.3. Reference Contents of Anti-inflammatory Agents in Mint (*Mentha* spp.) Samples

Sample code	Description	Rosmarinic acid (g/100g)	Oleanolic acid (g/100g)	Ursolic acid (g/100g)	Total triterpenes (g/100g)
M1	Mint sample	0.64	0.08	0.24	0.33
M2	Mint sample	2.35	0.07	0.14	0.21
M3	Mint sample	0.91	0.01	0.06	0.07
M4	Mint sample	1.42	0.03	0.07	0.09
M5	Mint sample	1.57	0.06	0.20	0.26
M6	Mint sample	1.43	0.03	0.09	0.12
M7	Mint sample	1.26	0.20	0.48	0.68
M8	Mint sample	1.18	0.04	0.04	0.08
M9	Mint sample	0.35	0.04	0.14	0.18
M10	Mint sample	0.66	0.06	0.15	0.21
M11	Mint sample	0.92	0.07	0.17	0.24
M12	Mint sample	0.34	0.04	0.13	0.17
M13	Mint sample	0.74	0.20	0.51	0.71
M14	Mint sample	2.03	0.10	0.28	0.39
M15	Mint sample	0.58	0.21	0.60	0.81
M16	Mint sample	2.35	0.07	0.18	0.26
M17	Mint sample	0.73	0.04	0.27	0.31
M18	Mint sample	0.59	0.18	0.50	0.69
M19	Mint sample	0.51	0.13	0.29	0.42
M20	Mint sample	0.17	0.24	0.05	0.29
M21	Mint sample	0.29	0.05	0.18	0.23
M22	Mint sample	1.00	0.05	0.14	0.19
M23	Mint sample	1.67	0.02	0.04	0.06
M24	Mint sample	0.74	0.04	0.09	0.13
M25	Mint sample	0.69	0.04	0.12	0.16
M26	Mint sample	0.96	0.02	0.05	0.07
M27	Mint sample	0.69	0.05	0.09	0.15
Average	Mean ± SD	0.99 ± 0.60	0.08 ± 0.07	0.20 ± 0.16	0.28 ± 0.21

[a] Total triterpenes expressed as the addition of oleanolic acid and ursolic acid

5.3. RESULTS AND DISCUSSION

5.3.1. NIR Spectra and Calibration Model.

From our spectrum collection of 66 samples (32 Greek oreganos, 7 Syrian oreganos and 27 mints), they showed very similar NIR absorbance spectra (**Fig. 5.1**). Savitzky-Golay first derivative with 11 point second order smoothing was used as a pretreatment in all PLS1 models presented for this study (**Fig. 5.2**). Models created with SG1 pretreatment outperformed models created without this pretreatment. For clarity purpose only data from models with SG1 are presented in this chapter. Calibration modeling was performed on each sample type as well as the combination of all three types. Validations of full cross and true test for each model were compared to determine the feasibility of the model.

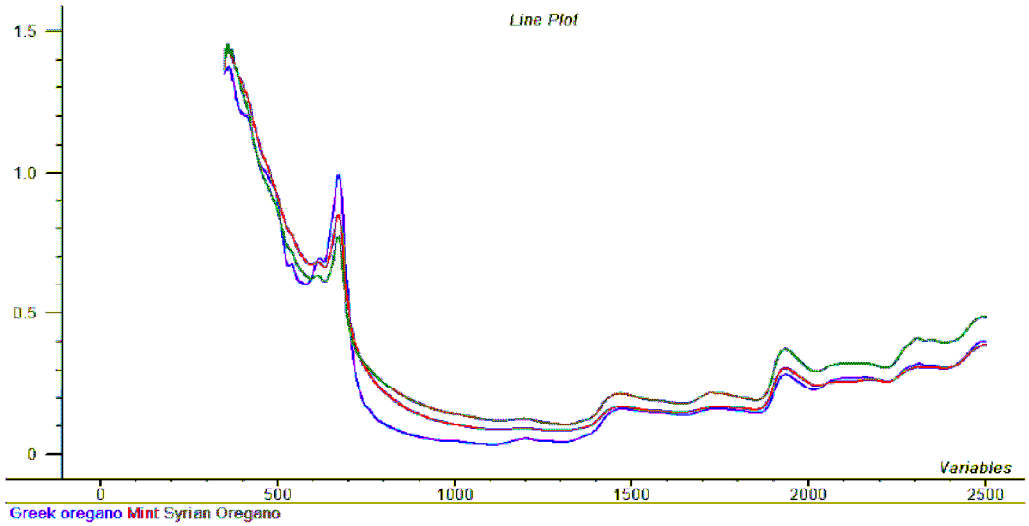

Figure 5.1. NIR absorbance spectral comparison of mint, Greek oregano and Syrian oregano

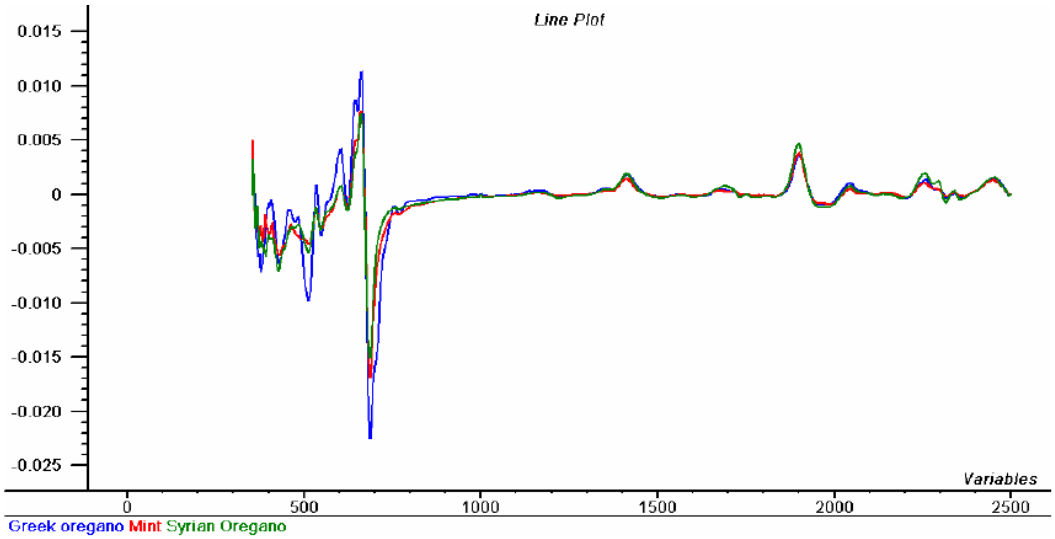

Figure 5.2. The 1st derivative Savitzky-Golay 11 pt 2nd order smoothed spectral comparison

5.3.2. Rosmarinic Acid.

The best model was obtained using the 50 sample calibration set with 16 sample true test set. Model used 6 factors (principle components), with the calibration had a Root Mean Square Error of calibration (RMSEC) of 0.76 with RSQ of 0.82. The validation set had RMSEP of 0.56 and RSQ of 0.88 (**Fig. 5.3**).

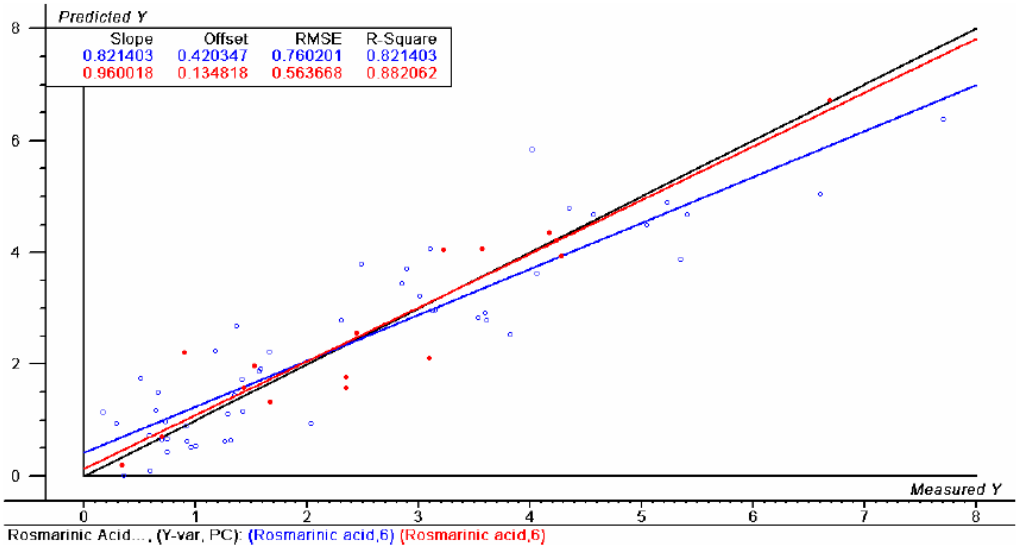

Figure 5.3. True test set on rosmarinic acid

5.3.3. Oleanolic Acid.

Since few of the total set included a wide range of oleanolic acid, the best model results were from a calibration created with all 66 samples together. One outlier was removed and wavelengths were reduced to create a model with 3 factors. The RMSEC was 0.08 and RSQ was 0.91. Full cross validation was used in place of a true test set for this equation. RMSEP was 0.09 and RSQ was 0.88 (**Fig. 5.4**).

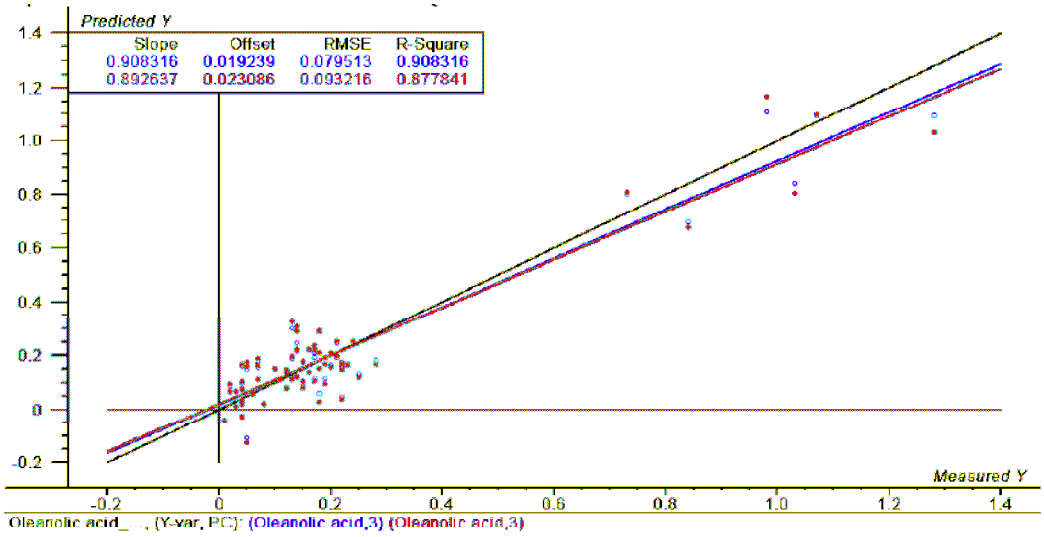

Figure 5.4. Full cross validation on oleanolic acid

The calibration model produced using the 50 sample calibration and 16 sample test set yielded a 6 factor calibration with an RMSEC of 0.09 and RSQ of 0.89. RMSEP of the true test set was 0.10 with an RSQ of 0.86 (**Fig. 5.5**).

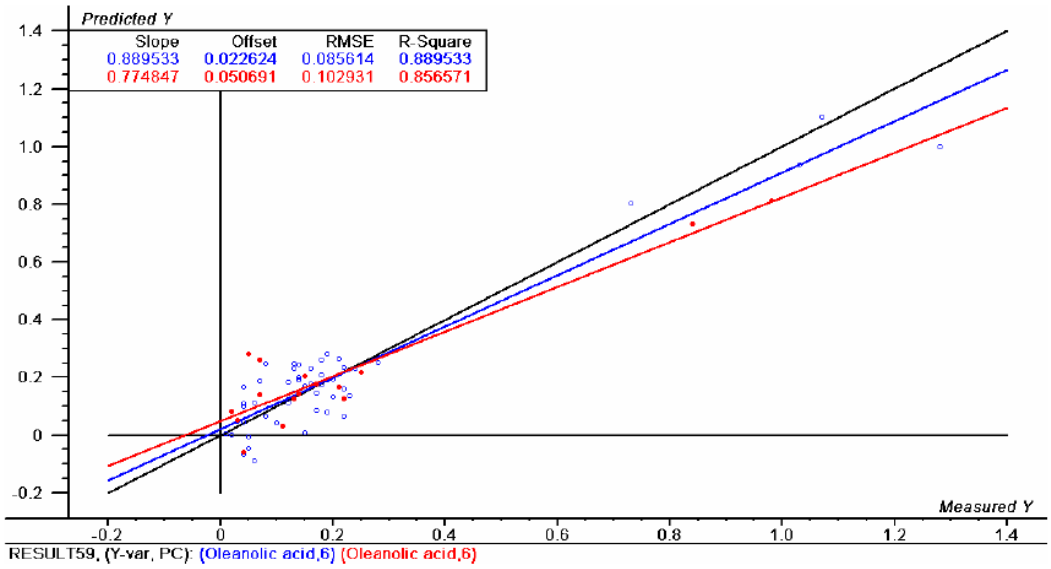

Figure 5.5. True test set on oleanolic acid

5.3.4. Ursolic Acid.

Calibration using 50 sample calibration set and 16 sample test set produced a 5 factor calibration with RMSEC of 0.29 and RSQ of 0.82. RMSEP of the true test set was 0.31 with RSQ of 0.81 (**Fig. 5.6**).

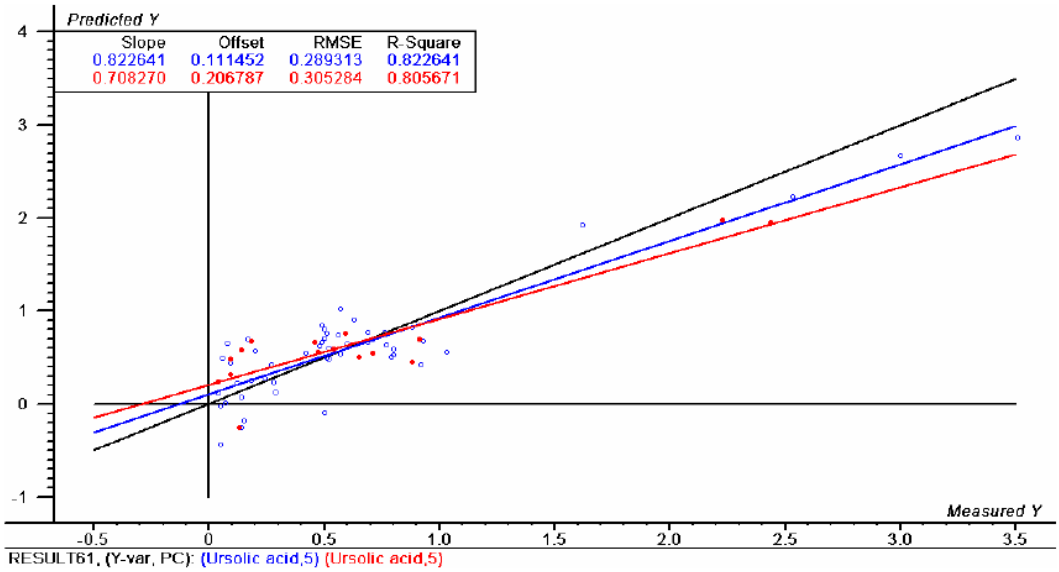

Figure 5.6. True test set on ursolic acid

5.3.5. Total Triterpenes.

As with oleanolic acid, the best model results were from a calibration created with all 66 samples together. One outlier was removed and wavelengths were reduced to create a model with 5 factors. The RMSEC was 0.23 and RSQ was 0.94. Full cross validation was used in place of a true test set for this equation. RMSEP was 0.34 and RSQ was 0.88 (**Fig. 5.7**).

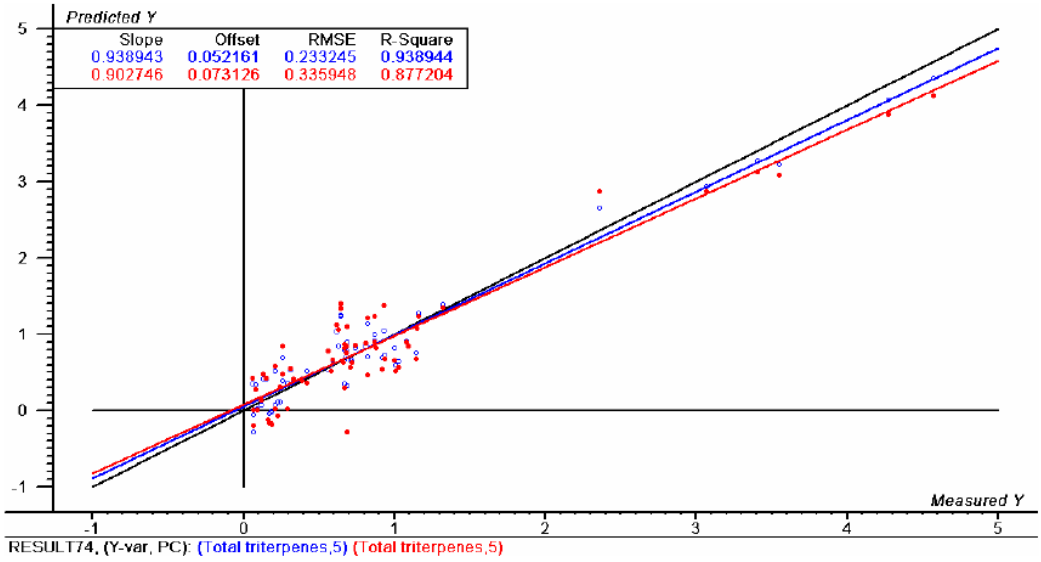

Figure 5.7. Full cross validation on total triterpenes (the addition of oleanolic acid and ursolic acid)

5.4. CONCLUSIONS

Near Infrared spectroscopy combined with a suitable pretreatment and with PLS1 regression showed to be a promising technique for predicting the individual anti-inflammatory compound in oregano and mint. Calibration for the test constituents appeared to be feasible, and different sample types could be combined into a single calibration model. Cross validation and true test set results confirmed the predictive ability for combining these three sample types into a single calibration, with r = 0.89 for rosmarinic acid, 0.94 for oleanolic acid, 0.95 for ursolic acid and 0.94 for total triterpenes (Full cross, **Table 5.4**); and r = 0.94 for rosmarinic acid, 0.93 for oleanolic acid, 0.90 for ursolic acid and 0.86 for total triterpenes (True test set, **Table 5.5**). Our results suggested that the combination of the three type samples into a single calibration possessed superior calibration and validation correlations than the single sample group performed. This is probably because a larger set of sample set with a greater range may improve the overall correlation. Calibration of samples is desirable to have a wide content range of not only the current interest but also of all possible source of variation likely to be encountered in the future. The availability of building a calibration model across different sample types makes it promising to develop a generic quantitation method for anti-inflammatory compound analysis in other plant species within the Lamiaceae family. The precision of this model would be acceptable for fast determination and on-field quality evaluation of rosmarinic acid, oleanolic acid and ursolic acid in oregano and mint.

Table 5.4. Best Results of the Multivariate Analyses for Each of Analytes (Full Cross Validation)

Analyte	PLS1 factors	RMSEC[a]	r (calibration)	RMSEP[b]	r (validation)
RosA	4	0.66	0.92	0.75	0.89
OA	3	0.08	0.95	0.09	0.94
UA	7	0.16	0.97	0.22	0.95
Triterpenes	5	0.23	0.97	0.33	0.94

[a] Root mean squire error of calibration
[b] Root mean squire error of prediction

Table 5.5. Best Results of the Multivariate Analyses for Each of Analytes (True Test Set Validation)

Analyte	PLS1 factors	No. of standards	RMSEC[b]	r (train)	No. of test set	RMSEP[b]	r (test)
RosA	6	50	0.76	0.91	16	0.56	0.94
OA	6	50	0.09	0.94	16	0.10	0.93
UA	5	50	0.29	0.91	16	0.31	0.90
Triterpenes	6	50	0.29	0.95	16	0.37	0.86

[a] Root mean squire error of calibration
[b] Root mean squire error of prediction

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CHAPTER 6. ISSUES RELATED TO MANUFACTURING OF ANTI-INFLAMMATORY DIETARY PRODUCTS FROM POST-DISTILLATION MATERIAL OF OREGANO AND MINT

6.1. BACKGROUND ON ESSENTIAL OIL DISTILLATION

6.1.1. Essential Oil Plants.

Essential oils are natural plant products that accumulate in specialized regions of plant structures such as cavities or ducts below the epidermis (outer skin), and glands or hairs originating from epidermal cells (Simon, 1990). Chemically, the essential oils are primarily composed of terpenes and aromatic polypropanoids, contributing to the aroma and flavor associated with herbs, spices and perfumes. The evolution of these unique secondary metabolites with promising bioactivities may be due to the ecological pressures in the natural environment including competition for space, defense of predation, symbiosis between different species.

The history of essential oils in particular use may date back thousands years (Price, 1993; Tisserand, 1977; Lawless, 1995). The Egyptians invented a rudimentary distillation unit that allowed for the crude extraction of essential oil (Tisserand, 1977; Lawless, 1995). Oils of cedarwood, clove, cinnamon, nutmeg and myrrh were used by the Egyptians for spiritual, medicinal, fragrant and cosmetic use. Other cultures including Persia (today, Iran) and India were among the first to invent systems to extract or distil essences from plants (Tisserand, 1977; Lawless, 1995). A major event for the essential oil distillation came with the invention of a coiled cooling pipe as condenser in the 11th century,

allowing the plant vapor and steam to cool down more effectively than previous distillers that used a straight cooling pipe (Lawless, 1995). In the 13th century, the appearance of pharmaceutical industry century encouraged great distillation of essential oils. During the 15th century, more plants were distilled for essential oils including frankincense, juniper, rose, sage and rosemary. From the late 20th century and the beginning of 21st century, there was an invigoration to utilize more natural products including essential oils for therapeutic, cosmetic and aromatic benefit (Price, 1993; Tisserand, 1977; Fiola et al., 2002). From this a rose industry was established in France. As a result of twentieth century distillation technology, the numbers and types of individual commercial oils have increased enormously, and international markets and industries have evolved and grown to rather sophisticated global trade and market on essential oils. Essential oil plants have been regarded as industrial raw materials for decades. The complex mixtures of volatile oils can be separated into individual compounds and used as building blocks to introduce particular flavors and aromas into products.

6.1.2. Essential Oil Distillation Apparatus.

Steam distillation involves the flow of steam or leaves of the plant into a chamber (Fig. 6.1). Pressurized steam is introduced into the lower chamber and passes through the botanical ingredients. The aromatic volatiles are then vaporized in the heat, swept away in the steam out of the chamber where the plant material resides (also called a distillation

tank, retort or alembic) and into a cooling condenser, where the steam once again turns into water. Droplets of the essential oils released from the plant matter are skimmed off the top. The oil and water are then separated in the separator.

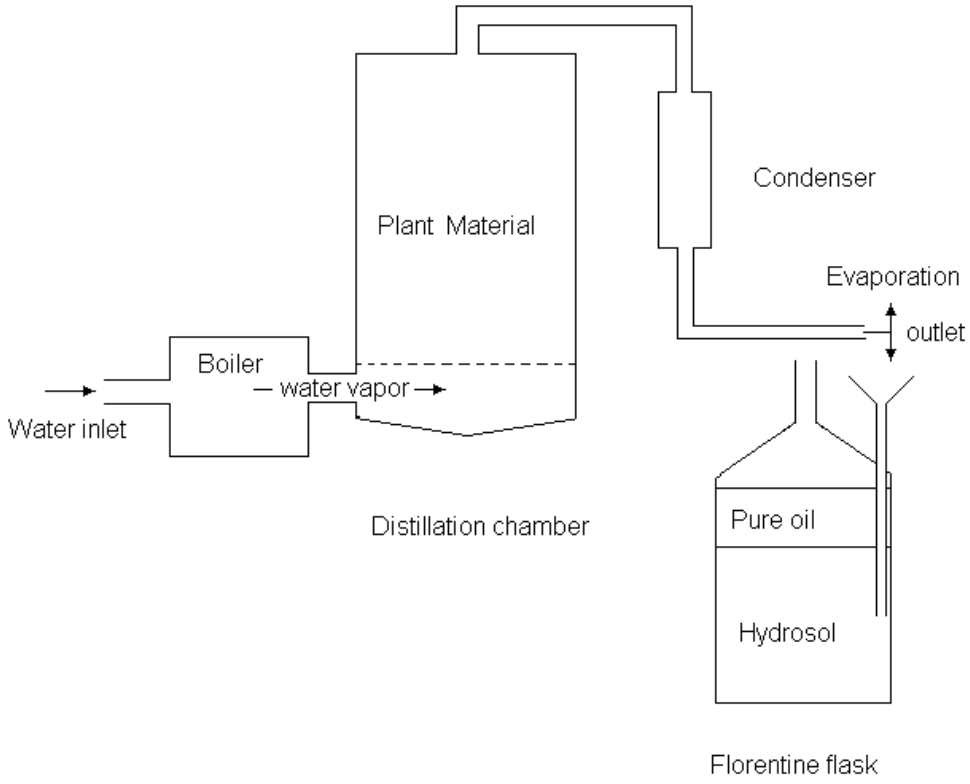

Figure 6.1. A general schematic of the steam distillation process (by R. Juliani & J. E. Simon, Rutgers)

6.1.3. Mint and Oregano as Essential Oil Plants.

The most important aromatic plants cultivated and produced for their essential oils in the USA includes mints (Simon, 1990), of which peppermint and spearmint are the two main crops cultivated in the largest-scale exclusively for essential oil production. Peppermint and spearmint are also produced in the USA for the fresh and dried markets (Bruneton, 1995). Essential oil contents and composition are known to be significantly impacted by the genetics of the plant, the environment where the plant is grown, and time of harvest. The chemical components of mints, especially for the essential oils from peppermint have been well studied and compared with other mints (Guntert et al., 2001). This oil is typically found in concentrations of 0.3 to 0.4% in peppermint, but may be as high as 1.5%. Menthol is the main constituent (**Fig. 6.2**), followed by menthone and menthyl acetate as well as other more than 100 different volatile compounds in the oil (Gunter et al., 2001). The essential oil profiles of other mints are different from peppermint, as evidenced by the fact that spearmint oil usually contains carvone as the major ingredient (55-67%) and limonene (2-25%). The contents of other constituents in spearmint (menthone, menthol, menthofuran, menthyl acetate and cineole) are less than 2% (Hethelyi et al., 2002).

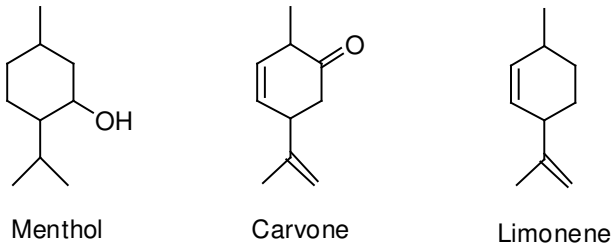

Figure 6.2. Representative terpenes in mint

There has been a significant increase in the consumption of oregano for both the fresh and essential oil market (Tucker & Maciarello, 1987). The European types of oregano are mainly composed of subspecies of *O. vulgare* L. including ssp. *hirtum* Ietswaart, ssp. *virens* Ietswaart and ssp. *viride* Hayek (Tucker & Rollins, 1989). Mexican oregano, also called Mexican sage, belongs to another genus, *Lippia graveolens* H.B.K. (Simon, Chadwick & Craker, 1984), Cuban oregano, a Caribbean native, is in fact taxonomized as *Plectranthus amboinicus*. The main essential oil components in European types of oreganos are carvacrol and thymol (**Fig. 6.3**), with variations between species and within a species both in contents and composition. The herb or the extracted oil is used in a variety of meat and sausage products, as well as salads, stews, and soups. Oregano is generally planted to a field and grown on light, dry well-drained soils for periods of 3 to 6

years. Multiple-time harvest could be applied each year depending upon the location and end-use.

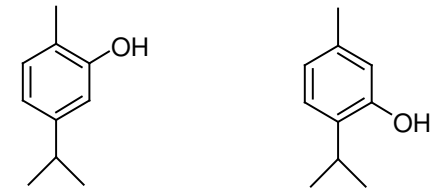

Figure 6.3. Representative terpenes in oregano

As the research focus has been largely on the aroma and flavor or oregano, far less attention has focused on the non-volatile bioactive components of this and other aromatic plants which contribute to the health and functional attributes of the plant and its extracts. In addition, the biomass following essential oil distillation of oregano and mint, have long been viewed as waste products used as soil amendments, animal feed or in some regions even dried and later used as fuel to fire and charge the next distillation. In this chapter, we started by using a number of advanced lines from the Rutgers breeding trial on an essential oil-rich oregano variety (*Origanum vulgare* spp. *hirtum*), then evaluated the distribution of the anti-inflammatory constituents in different parts of the

post-distillation materials, and investigated several issues related to the dietary supplement product manufacturing.

6.2. OREGANO BREEDING TRIAL

Four Greek oregano (*O. vulgare* ssp. *hirtum*) entries were included in the trials from 2005 to 2007 (all Rutgers selections, OS-00, OS-10, OS-14 and OS-37). These entries were selected because they represent advance lines of Rutgers and have been screened for a wide tolerance to environmental conditions including heat stress, high essential oil content and rich in polyphenols. Specifically, >60% of the total essential oil recovered was carvacrol, the main compound responsible for the characteristic flavor. Oregano seeds were greenhouse sown on May 13, 2005 into cell packs (72 cells per tray) at the Clifford and Melda E. Snyder Research and Extension Farm in Pittstown, NJ. Cell packs were grown in organic medium and given weekly liquid fertilizer treatments. Oregano lines were field transplanted on June 14, and straw mulch applied on June 29, 2005.

Lines were evaluated for winter survival on 6/14/2006. Field evaluation and first-harvest were conducted on 7/18/2006. A second evaluation was performed for the second growth on 9/20/2006. Live single plant selections were vegetatively propagated for the Rutgers

selections on 1/30/2007 at the Rutgers greenhouse (**Fig. 6.4**). Lines were transplanted for this summer season on 5/25/2007. Field evaluation and harvest were conducted on 8/9/2007 (**Fig. 6.5**). A second harvest in 2007 was not conducted due to limited plant growth. Each line was evaluated for plant height, spread, leaf color, flower color, spike color, uniformity (1 = low uniformity, 5 = high), vigor (1 = low growth vigor, 5 = high), insect damage (1 = low insect damage, 5 = high), disease (1 = low disease, 5 = high), flowering time, fresh weight, and dry weight (**Table 6.1**). Essential oils were further quantitatively analyzed for major constituents by using GC/MS, showing R-OS-00, R-OS-10, R-OS-14, and R-OS-37 were tolerant to environmental conditions and genetically stable relative to each individual volatile compound (**Table 6.2**). All lines accumulated carvacrol in high amounts (>60% of the total essential oil), exhibited high vigor, little disease and/or insects, and successfully withstood the winter condition.

Two months old

Four months old

Figure 6.4. First cuttings of oregano line R-OS-14 grown in Rutgers Greenhouse

Ave. height: 31.3 cm
Ave. spread: 52.2 cm
Leaf length: 2.7 cm
Leaf width: 1.9 cm
Leaf color: green
Flower color: white
Spike color: light green

Figure 6.5. Photograph of oregano field-grown breeding line R-OS-14 in year 2006

Table 6.1. Evaluation of Biomass and Oil Yield on Field-grown R-OS-14 (Rutgers Oregano Selection 14 during Year 2005 to 2007)

Tested year	Plant height (cm)	Plant spread (cm)	Vigor (1-5)	Uniformity (1-5)	Insect (1-5)	Disease (1-5)	Oil yield (mL/100g)	Fresh weight (kg/acre)	Dry weight (kg/acre)
2005	31.6	55.4	3.7	4.2	1	1	2.23	3,619	752
2007	31.3	52.2	4.8	4.3	1	1	3.13	2,452	563
1 year old	31.5	53.8	4.3	4.3	1	1	2.68	3,036	658
2006 summer	72.7	97.5	3.7	3	2	2	4.8	6,143	897
2006 fall	31.3	61.9	2.5	2.2	1.3	1.7			
2 years old	52	79.7	3.1	2.6	1.7	1.9	4.8	6,143	897
Average	41.3	66.8	3.7	3.4	1.3	1.4	3.39	4,071	737

Table 6.2. Major Constituents in the Essential Oil (EO) of Field-grown R-OS-14 (Rutgers Oregano Selection 14 during Year 2005 to 2007)

Retention time (min)	EO constituent	Relative content (as % total oil)		
		2005	2006	2007
6.1	α-Thujene	0.79	1.29	0.45
6.3	α-Pinene	0.72	0.95	0.8
7.8	Myroene	1.5	1.21	1.8
8.8	*para*-Cymene	12.58	19.85	12.03
9.9	γ-Terpinene	7.45	4.73	10.19
11.1	Lineol	0.34	0.33	ND[a]
13.4	Borneol	ND[a]	0.18	0.55
13.7	Terpinen-4-ol	0.43	0.48	0.36
17.8	Carvacrol	68.22	61.04	62.62
21.6	β-Caryophyllene	0.45	0.48	0.58
23.3	2-Nomulene	0.16	0.13	0.18
24.7	Germoluene	0.26	0.21	0.18
26.4	*d*-Cardinene	0.21	0.19	0.1

[a] Not detectable

6.3. DISTRIBUTION OF ANTI-INFLAMMATORY COMPOUNDS IN OREGANO POST-DISTILLATION MATERIAL

The breeding line R-OS-14-3 (year 2006) was selected herein to investigate the distribution of anti-inflammatory constituents in the non-volatile component after essential oil distillation. Total 20 g of air-dried aerial part of R-OS-14-3 were placed in a

lab-scale essential oil distillation apparatus. 400 mL of water was added to the flask boiling for 45 min. The yellow-brown boiling water was let cool down and filtered out (300 mL), from which 30 mL of boiling water was taken to evaporate under vacuum. The dried residue (0.38 g) was used for HPLC analysis. The waste plant material from distillation was air dried at 37 °C to get 16.2 g of dry matter.

Chromatographic analysis was performed on a Waters 2695 HPLC system (Waters Corp., Milford, MA) equipped with a Microsorb 100 × 4.6 mm, i.d. 3 μm, C18 column (Varian Inc., Palo Alto, CA). Mass spectrometer used in this research was a triple stage quadrupole Quattro II (Micromass Co., Altrincham, UK) equipped with the orthogonal Z-spray electrospray ionization (ESI) interface and the acquisition data processor Masslynx 3.4 software. The mobile phase for chromatographic separation consisted of solvent A (5 mM ammonium formate in water, pH 7.4, adjusted with ammonium hydroxide) and solvent B (5 mM ammonium formate in 90% methanol, pH 7.4) under an isocratic condition (13.5% solvent A and 86.5% solvent B) at a flow rate of 0.8 mL/min. For MS quantitation, a selection of *m/z* values corresponding to rosmarinic acid (*m/z* 359, $[M - H]^-$), oleanolic acid (*m/z* 479, $[M + Na]^+$) and ursolic acid (*m/z* 479, $[M + Na]^+$) were monitored by using the instrument in a SIM mode with a dwell time of 1 sec and inter-channel delay of 0.03 sec.

The air-dried R-OS-14-3 (year 2006) sample without essential oil distillation was

quantitatively analyzed by the same HPLC method, showing 20 g of original plant material contained 734 mg of rosmarinic acid, 25 mg of oleanolic acid and 80 mg of ursolic acid. After essential oil distillation, 575 mg of rosmarinic acid, 3 mg of oleanolic acid and 4 mg of ursolic aicd went into boiling water, and the anti-inflammatory compounds left in waste plant material were 162 mg, 22 mg and 71 mg for rosmarinic acid, oleanolic acid and ursolic acid, respectively (**Fig. 6.6**). These results suggested the boiling water extracted about 78% of the total rosmarinic acid from the oregano material that was distilled while only trace amounts of oleanolic acid and ursolic acid were recovered. Most oleanolic acid and ursolic acid were left in the waste plant material, from where still certain amount of rosmarinic acid can be extracted out as well.

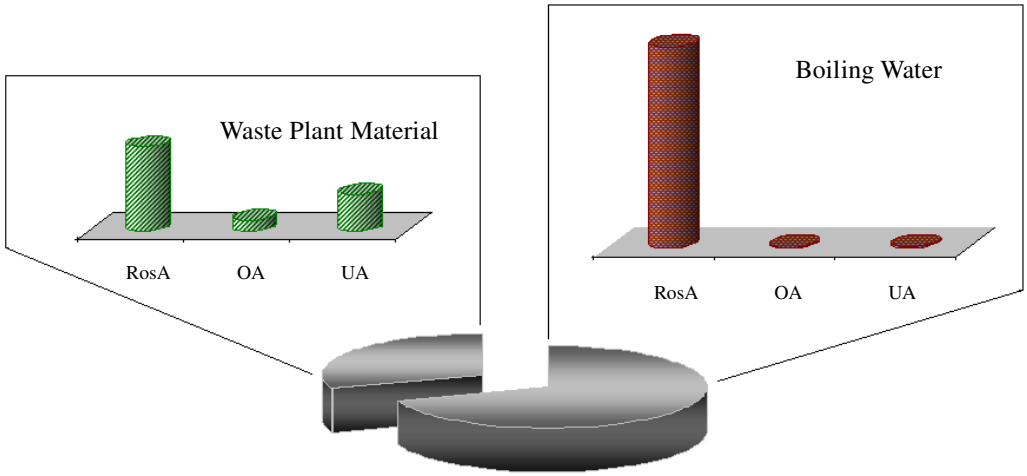

Figure 6.6. Distribution of anti-inflammatory compounds in R-OS-14-3 (*Origanum vulgare* ssp. *hirtum*) post-distillation material

6.4. ANTI-INFLAMMATORY EFFECTS OF OREGANO EXTRACTS FROM DIFFERENT PARTS OF POST-DISTILLATION MATERIAL

The oregano breeding line R-OS-14-3-W was defined as the boiling water extract obtained from the lab-scale distillation described above. The waste plant material was then air dried at 37 °C, and 400 mL of ethanol was extracted on this dried material to

provide 3.75 g of ethanol extract dryness (R-OS-14-3-E). R-OS-14-3 represents the total extract of R-OS-14-3-W and R-OS-14-3-E (mixed the two extracts thoroughly and evaporated to dryness). These three extracts were screened and compared for anti-inflammatory activities on the nitrite assay.

The RAW264.7 cells were treated with test compounds and LPS or LPS only. The supernatants were harvested and the amount of nitrite, an indicator of NO synthesis, was measured by Griess reaction. Briefly, supernatants (100 μL) were mixed with the same volume of Griess reagent (1% sulphanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water) in duplicate on 96-well plates. After incubation at room temperature for 10 min, absorbance was measured at 570 nm with the UV reader.

Water extract R-OS-14-3-W containing high concentration of rosmarinic acid showed a significant anti-inflammatory effect on the nitrite assay (**Fig. 6.7**, **Table 6.3**), however, this inhibition was weaker compared to the other two extracts due to lack of oleanolic acid and ursolic acid contribution (trace amount in R-OS-14-3-W). As we expected, ethanol extract R-OS-14-3-E exhibited even stronger activity on the nitrite assay when the concentration of oleanolic acid and ursolic acid reached to 5.3 mg/g and 18.7 mg/g, respectively, although its rosmarinic acid content was less than the water extract R-OS-14-3-W (**Table 6.4**). R-OS-14-3 was a mixture of R-OS-14-3-W and R-OS-14-3-E

with anti-inflammatory compound concentrations between the other two extracts, showing a medium activity among the three extracts, consistent with our hypothesis that the combination of three compound possesses a synergistic effect even though it may contain less amount of total weight than any of the single pure compounds.

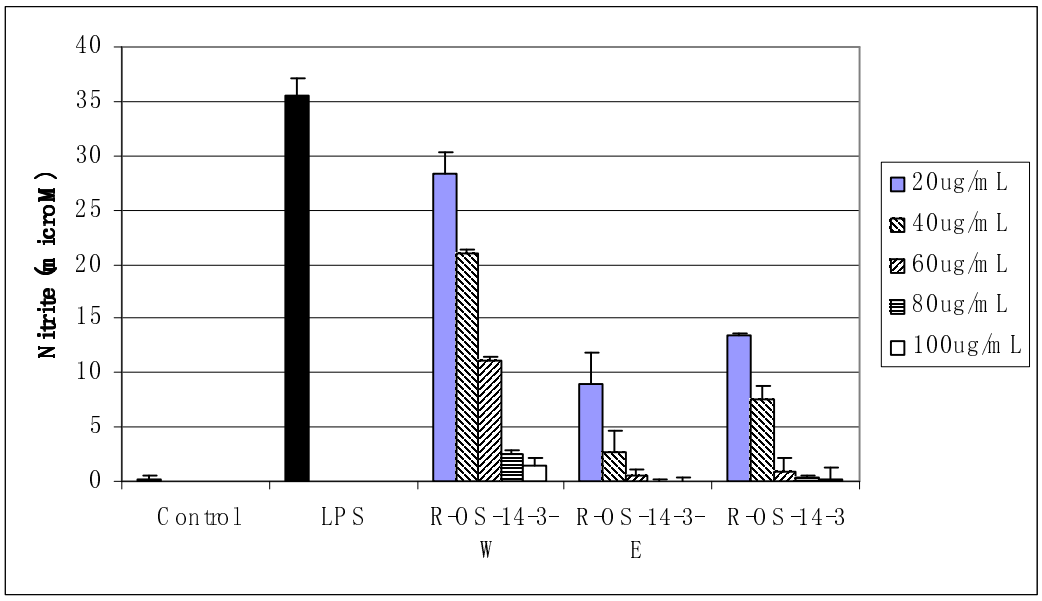

Figure 6.7. Anti-inflammatory effects of oregano extracts ROS-14-3-W (waste boiling water), R-OS-14-3-E (ethanol extract of the waste plant material) and R-OS-14-3 (total post-distillation material)

Table 6.3. Effect of Oregano Extracts on LPS-induced Nitrite Production in RAW 264.7 Macrophages[a]

Conc. (μg/mL)	LPS (100ng/mL)	R-OS-14-3	R-OS-14-3-W	R-OS-14-3-E
control	-	0.3 ± 0.5	0.1 ± 0.4	0.1 ± 0.4
	+	38.6 ± 1.1	35.5 ± 1.6	35.5 ± 1.6
20	+	13.4 ± 0.2	28.4 ± 2.0	8.9 ± 2.9
40	+	7.6 ± 1.1	20.9 ± 0.5	2.7 ± 1.9
60	+	0.9 ± 1.2	11.1 ± 0.3	0.6 ± 0.5
80	+	0.4 ± 0.1	2.6 ± 0.3	0.0 ± 0.1
100	+	0.1 ± 1.1	1.4 ± 0.8	0.0 ± 0.3

[a] ROS-14-3-W represents the waste boiling water extract; R-OS-14-3-E represents the ethanol extract of the waste plant material; R-OS-14-3 represents the total post-distillation material extract

Table 6.4. Concentrations of the Anti-inflammatory Constituents in Oregano Extracts (Milligrams per Gram of Dry Matter) [a]

sample code	rosmarinic acid	oleanolic acid	ursolic acid
R-OS-14-3-W	151.3	0.7	1.0
R-OS-14-3-E	48.0	5.3	18.7
R-OS-14-3	98.4	2.9	7.8

[a] ROS-14-3-W represents the waste boiling water extract; R-OS-14-3-E represents the ethanol extract of the waste plant material; R-OS-14-3 represents the total post-distillation material extract

6.5 CHEMICAL STABILITY OF ROSMARINIC ACID

As rosmarinic acid is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid and the chemical stability during essential oil distillation is an important issue we concerns, thus we designed a stability test to investigate the degradation of rosmarinic acid at different pH conditions. The stock solution was prepared by dissolving 20 mg of rosmarinic acid in ethanol (20 mL). 1 mL of stock solution was diluted by 9 mL of distilled water in a test tube. The pH of each solution in the respective test tube was adjusted by HCl and NaOH solution to different pH values (from pH 2 to 11). All the test tubes were placed in water bath at 100 °C for 1 hour and stayed at room temperature for 24 hours prior to HPLC analysis. The mobile phase for HPLC consisted of solvent A (0.1% formic acid in water, v/v) and B (0.1% formic acid in acetonitrile, v/v) in gradient. The total running time was 50 min. The gradients were 0 min, 15% solvent B; 35 min, 65% solvent B; 40 min, 95% solvent B; 50 min, 95% solvent B. Prior to the next injection, the column was equilibrated for 15 min with 15% B. The flow rate was set to 1.0 mL/min with the column compartment maintaining at 25 °C, and the injection volume was 20 µL. The wavelength of UV detection was set at 280 nm. The compound stability of rosmarinic acid in different pH conditions was evaluated by calculating the peak areas of the chromatograms.

The results showed rosmarinic acid was stable through pH 2 to pH 7 in boiling water (**Fig.**

6.8), while it degraded dramatically in basic condition (pH > 7). The boiling water after oregano distillation we tested was around pH 5, so rosmarinic acid would be quite stable during the essential oil distillation process. The results from the study of anti-inflammatory constituents in post-distillation material (R-OS-14-3) also suggested that the total amount of rosmarinic acid could be recovered from the boiling water and the waste plant material.

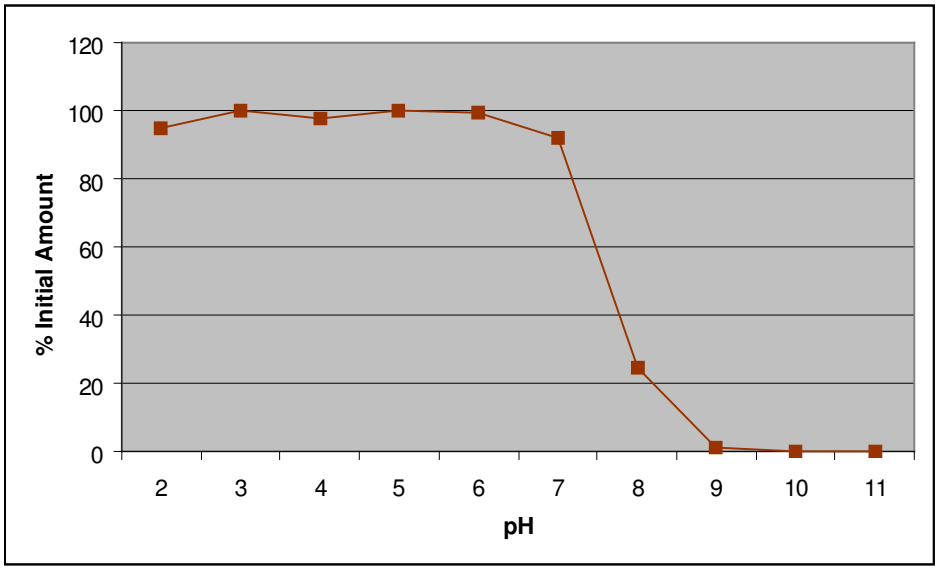

Figure 6.8. Chemical stability of rosmarinic acid under different pH values in boiling water

6.6. EXTRACTING METHODS ON MANUFACTURING

For *Origanum* species and many *Mentha* species, as the concentrations of anti-inflammatory constituents may not reach the effective content and to ensure standardized botanicals, an extraction or chromatographic procedure is needed to enrich the contents of rosmarinic acid, oleanolic acid and ursolic acid. This is done in part by simultaneously removing other inactive plant components. Since the solubility of non-ionic forms of organic acids was poor in water, we may take advantage of this property to precipitate the anti-inflammatory acids in acidic water solution to achieve this, or enrich the anti-inflammatory acids by solvent partition between organic solvent and water solution, depending on the property of compound solubility. Ion exchange chromatography relies on charge-charge interactions between the solutes and the charges immobilized on the resin. Our anti-inflammatory ingredients are all organic acids, so anion exchanger, of which the binding ions are negative and the immobilized functional group is positive, can be utilized to isolate the anti-inflammatory acids out of plant extract. Once solutes are bonded on the column, the acids are able to be washed out by acidic eleunt at low pH, and get the fraction containing the compounds with acidic functional groups. The details of procedures and data related to dietary supplement manufacturing were included elsewhere on our patent protection material.

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CHAPTER 7. ANTIMICROBIAL ACTIVITIES OF OREGANO AND THE USE OF BIO-DIRECTED FRACTIONATION TO IDENTIFY THE RESPONSIBLE CONSTITUENTS

7.1. INTRODUCTION

The antimicrobial activities from the essential oils of *Origanum* spp. have been described by researchers previously (Hersch-Martinez, Leanos-Miranda & Solorzano-Santos, 2005; Liao et al., 2004; El-Nakeeb, Fathy & Salama, 2006; Veres et al., 2007), however, seldom have these studies related to specific natural products responsible. Oxygenated monoterpenes and monoterpene hydrocarbons including carvacrol, γ-terpinene and *p*-cymene were characterized as the main chemotypes in the chemical composition of oregano essential oils (Salgueiro et al., 2003). Recently, Ozkan et al. (2007) investigated antibacterial activities of oregano water extract by using agar diffusion method against 15 species of bacteria, and reported that the extract of *Origanum sipyleum* was effective against all the bacteria tested, except for *Yersinia enterocolitica*. In another study focusing on the anti-*Staphylococcus aureus,* plant extacts revealed the water extract of oregano contained antibacterial activity (Kwon et al., 2007).

Identification of possible constituents responsible for antimicrobial activities from oregano was part of our interest, although not our prime focus relative to the identification and examination of the plants' anti-inflammatory agents. In addition, we sought to identify other natural products from this plant, apart from the bioactive compounds to reveal any novel or interesting structures.

7.2. ANTIMICROBIAL STUDIES ON OREGANO

7.2.1 Preparation of Microorganisms.

Bacterial cultures (*Escherichia coli* and *Staphylococcus aureus*) grown on solid agar media (LB Agar, Miller, Fisher Scientific) were transferred into liquid media in 125 mL flasks and cultivated overnight at 37 °C on a gyratory shaker (model G10, New Brunswick Scientific Co.) at 120 rpm. *Saccharomyces cerevisiae* was treated in the same manner as previous described except that the initial culture was cultivated on a potato dextrose agar media (Difco Laboratories) and transferred to potato dextrose liquid media one day prior to screening (Poulev et al., 2003). The optical densities of all microorganism suspensions were measured at 560 nm by using a Beckman DU 640 spectrophotometer (Poulev et al., 2003). Suspensions of microorganisms used in the screens reached the optical densities of 0.015-0.030 for *E. coli*, 0.01-0.02 for *S. aureus*, and 0.5-0.7 for *S. cerevisiae*.

7.2.2. Antimicrobial Screens.

The growth inhibition assay was carried out according to the previous described method (Poulev et al., 2003). Briefly, a growth inhibition assay on solid medium was used to determine antibacterial and antifungal activity of plant root extracts. Sterile 24-well

culture plates (Greiner Labortechnik, Frickenhausen, Germany) were filled with 1 mL/well of nutrient LB agar media, which was used for bacterial bioassays to determine the inhibitory activities of extract fractions and isolated compounds. Then, 10 µL of the tested sample dissolved in DMSO was added to the surface of the agar media. After 5-10 min drying, 30 µL of microorganism suspension was uniformly added to each well. All samples and the control were placed in triplicate. After incubation at 30 °C for 24 h in an incubator (Isotemp, Fisher Scientific), plates were examined and the antimicrobial activity was visually scored using rating of grades 1 to 5, where 1 = 100% inhibition (no bact.); 2 = 70% inhibition; 3 = 50% inhibition; 4 = 20% inhibition; 5 = 0% inhibition (no inhibition).

7.2.3. Results and Discussion.

In the screening of oregano to assess their potential antimicrobial activity, we used an oregano water extract and tested this against three microbial species, *E. coli*, *S. aureus* and *S. cerevisiae.* We first tested the 16 oregano fractions eluted from silica gel column (**Table 7.1**) for potential antimicrobial activity again using a bioactivity-guided isolation approach. Fraction 2 and Fraction 6 showed 50% inhibition on *S. aureus* assay at the concentration of 33 µg/mL in DMSO. From Fraction 2, we isolated two triterpenoid acids, oleanolic acid and ursolic acid, possessing mild anti-*S. aureus* activities with 70% and 100% inhibitions respectively at the same concentration level as the crude extractions on

the assay (33 μg/mL). The phenolic constituents in oregano were also reported to be responsible for the activity against *S. aureus*, a Gram-positive organism related to numerous infections and syndromes involved in skin and soft tissue infections, endocarditis and septic arthritis (El-Ahdab et al., 2005; Kwon et al., 2007). Virulent strains of *E. coli* can be responsible for gastroenteritis, urinary tract infections and neonatal meningitis (Goldenberg, 1998). *Saccharomyces cerevisiae* is a species of budding yeast, and the antifungal activity of the essential oil of oregano was also reported (Salgueiro et al., 2003). Unfortunately, the compounds responsible for the anti-*S. aureus* activity of Fraction 6 were not identified due to the difficulty on chromatographic isolation and the small amount of material that was available in this fraction. The contribution of this fraction to the antimicrobial activity of total oregano extract should be minor, because Fraction 2 was over 100 times larger compared to Fraction 6 on a weight basis. We did not pursue further studies on this fraction also because it did not show stronger anti-*S. aureus* activity than Fraction 2 on the bioassay.

Table 7.1. Percent Inhibition of the Oregano (*Origanum vulgare* spp. *hirtum*) Fractions on the three Microorganisms, *Escherichia coli, Staphylococcus aureus* and *Saccharomyces cerevisiae*

Fraction number	% Inhibition [a]		
	Escherichia coli	*Staphylococcus aureus*	*Saccharomyces cerevisiae*
1	0	0	0
2	0	50	0
3	0	0	0
4	0	0	0
5	0	20	0
6	0	50	0
7	0	0	0
8	20	0	20
9	0	0	0
10	0	0	0
11	0	0	0
12	0	0	0
13	0	0	0
14	20	0	20
15	0	0	20
16	0	0	20

[a] the antimicrobial activity was visually scored form 1 to 5. 1 = 100% inhibition (no bacterial growth); 2 = 70% inhibition; 3 = 50% inhibition; 4 = 20% inhibition; 5 = 0% inhibition (no inhibition).

7.3. STRUCTURAL ELUCIDATIONS

7.3.1. 4α-Methyl-29-chloro-stigmast-9(11)-en-3β-ol-3β-D-glucopyranoside (1).

Amorphous white powder; melting point 253-255 °C; ESI MS (positive ion mode) *m/z* 649, 647, 627, 625, 607, 605, 591, 585, 465, 463, 445, 427, 409, 399, 395, 381; ESI MS (negative ion mode) *m/z* 625, 623, 293, 195; ^{1}H NMR (200 MHz, pyridine-d_5) δ 5.05 (1H, d, *J* = 7.6 Hz, H-1'), 4.56 (1H, dd, *J* = 2.0, 11.6 Hz, H-3'), 4.40 (1H, dd, *J* = 5.2, 12.2 Hz, H-4'), 4.29 (1H, dd, *J* = 2.2, 8.0 Hz, H-2'), 4.29 (1H, brs, H-6'a), 4.22 (1H, brs, H-6'b), 4.08 (1H, m, H-3), 3.99 (1H, m, H-5'), 0.98 (3H, d, *J* = 6.6 Hz, H-21), 0.93 (3H, s, H-19), 0.87 (3H, d, *J* = 6.6 Hz, H-26), 0.87 (3H, d, *J* = 6.6 Hz, H-27), 0.86 (3H, d, *J* = 6.6 Hz, H-30), 0.66 (3H, s, H-18); ^{13}C NMR, see **Table 7.2**; molecular formula calculated as $C_{36}H_{61}O_6Cl$.

The molecular formula of compound **1** was determined from its positive and negative MS spectra as well as ^{1}H NMR, ^{13}C NMR and DEPT analysis. The ^{1}H NMR revealed the presence of six methyl groups resonating from δ 0.66 to 0.98, of which two singlets of tertiary methyls and four three-proton doublets. Sugar-like signals ranging from δ 3.99 to 5.05 indicated that the molecule might contain a saccharide moiety. The DEPT spectrum suggests that there are 6 methyls, 12 methylenes, 15 methines and 3 quaternary carbons. The isotope peaks differentiated by 2 mass units were observed for molecular ions and

some fragmentations on both positive and negative ESI MS spectra, and the abundance ratio of the related isotope peaks was around 3:1, indicating the presence of a chlorine atom in the molecule (**Fig. 7.2** and **Fig. 7.3**). The unsaturation degree was calculated as 6 from the proposed molecular formula of $C_{36}H_{61}O_6Cl$. ^{13}C NMR showed the vinylic carbon signals appeared at δ 141.4 and 122.4, the carbonol carbon at δ 79.1, the anomeric carbon at δ 103.0, and the other sugar moiety carbons ranging from δ 78.9 to 63.3. From these evidences, we proposed the parent skeleton of compound **1** as a tetracyclotriterpene glycoside. Compared to a reported compound 14-methyl-stigmast-9(11)-en-3α-ol-3β-D-glucopyranoside (Hung et al., 2007), the NMR data of compound **1** were similar to that molecule, however, the number of methyl groups was less (six versus seven), suggesting the chloro substitution possibly on one of the methyl group of the reference compound. Upon MS fragmentation, the loss of ethyl chloride at *m/z* 585 $[M-62+Na]^+$ indicateted the chloro group attached to the C-29 position. The fragmentation at *m/z* 625 was due to the loss of a isopropyl group at the side chain, and the characteristic feature for containing hexose was observed at *m/z* 463 $[M-162+H]^+$. Acid hydrolysis of compound **1** afforded a free glucose, confirmed by comparing to the reference compound relative to the retention time on HPLC. The saccharide attached to position C-3 of the triterpene was determined due to the downfield shift of C-3 on ^{13}C NMR. The β conformation of the anomeric proton was identified by the fact that the coupling constant of H-1' was 7.6 Hz on ^1H NMR (**Fig. 7.1**).

7.3.2. Oleanolic acid (2) and ursolic acid (3).

Compounds **2** and **3** were identified as oleanolic acid and ursolic acid by ESI MS, ^{1}H NMR and ^{13}C NMR spectra compared to previous reported data in literature. The comparison of the retention time with commercial reference compounds on HPLC further supported the identification of oleanolic acid and ursolic acid (**Fig. 7.1**).

7.3.3. 3β,21α-Dihydroxy-olean-12-en-28-oic acid (4).

Amorphous white powder; ESI MS (positive ion mode) *m/z* 495, 471, 455, 437, 409, 391, 201, 191; ^{1}H NMR (200 MHz, methyl-d_3 alcohol-*d*) δ 5.26 (1H, brs, H-12), 3.43 (1H, m, H-3), 3.15 (1H, m, H-18), 2.92 (1H, dd, *J* = 3.0, 13.2 Hz, H-19a), 1.17 (3H, s, H-27), 0.97 (3H, s, H-29), 0.96 (3H, s, H-23), 0.94 (3H, s, H-30), 0.92 (3H, s, H-26), 0.81 (3H, s, H-24), 0.77 (3H, s, H-25); ^{13}C NMR, see **Table 7.2**; molecular formula calculated as $C_{30}H_{48}O_4$.

The positive ESI MS provided the sodium adduct molecular ion (*m/z* 495) of compound **4**. Dehydration (*m/z* 455 and *m/z* 437) and further decarboxylic acid (*m/z* 409 and *m/z* 391) products were observed as MS fragments. The fragmentation ions at *m/z* 191 and *m/z* 201 were due to RDA reactions, the characteristic MS fragmentation of Δ^{12}-unsaturated triterpenoids. The ^{1}HNMR spectrum showed signals for seven tertiary methyl groups at δ0.77 (s), 0.81 (s), 0.92 (s), 0.94 (s), 0.96 (s), 0.97 (s), 1.17 (s) and a trisubstitued olefinic

proton at δ 5.26, which is typical of the oleanolic acid skeleton. The ^{13}C NMR data was closely related to those of oleanolic acid, while the molecular formula of **4** ($C_{30}H_{48}O_4$) was higher by one oxygen atom compared to oleanolic acid, indicating the presence of one more hydroxyl group in addition to the C-3 hydroxyl group. The chemical shifts at δ 78.4 and 73.6 were assigned to the hydroxymethine carbons of C-3 and C-21. Compared to the reported ^{1}H NMR and ^{13}C NMR data on this compound (Mimaki et al., 2004), **4** was confirmed as 3β,21α-dihydroxy-olean-12-en-28-oic acid (**Fig. 7.1**).

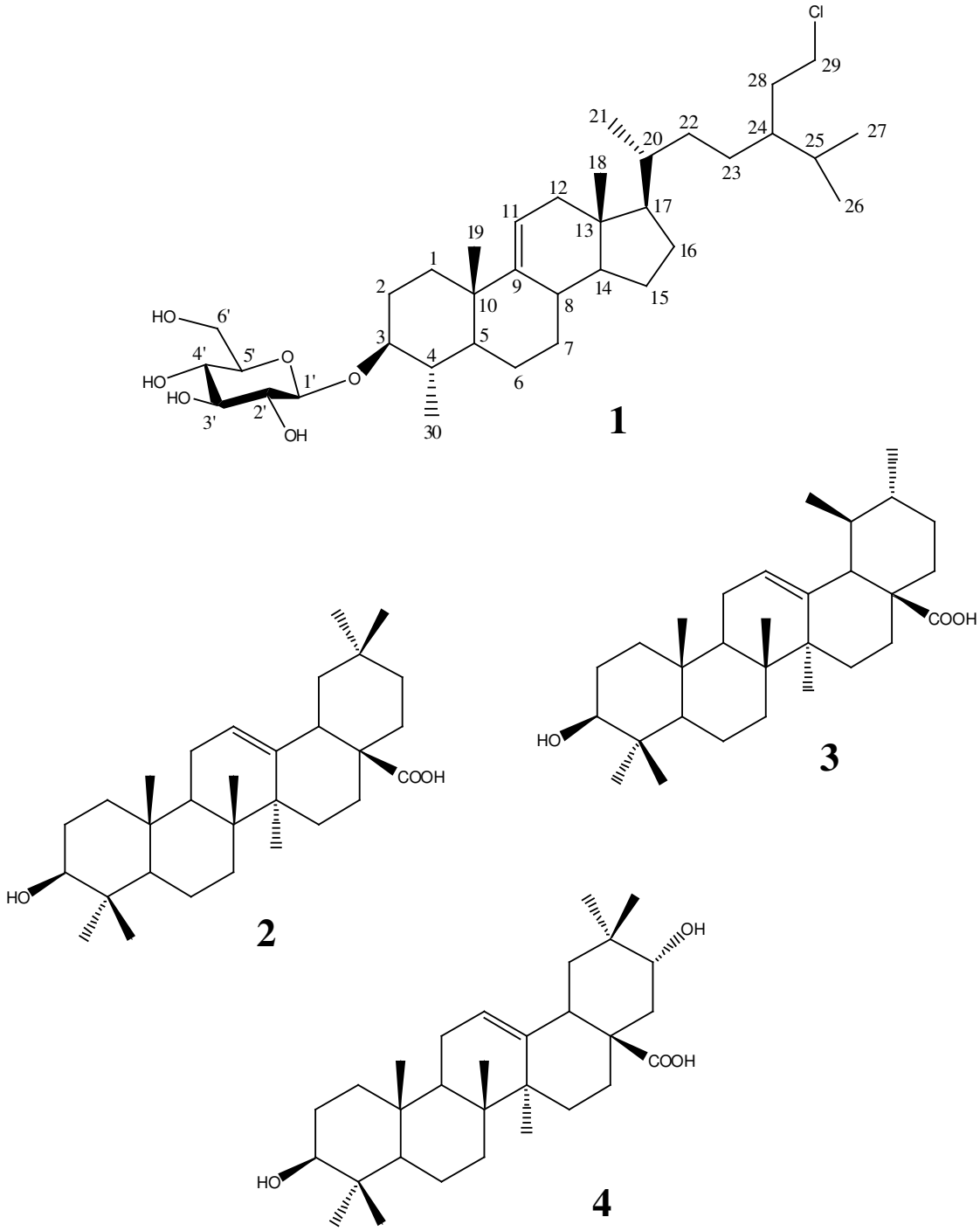

Figure 7.1. The structures of triterpenoids isolated from oregano (*Origanum. vulgare* spp. *hirtum*)

Table 7.2. ^{13}C NMR Assignments for Triterpenoids Isolated from Oregano (in Pyridine-d_5)

	compound[a]			
position	**1**	**2**	**3**	**4**[b]
1	37.9	37.8	38.3	38.5
2	30.7	27.0	27.1	26.2
3	79.1	77.0	77.1	78.4
4	32.5	38.3	38.0	38.6
5	50.8	54.7	54.8	55.5
6	21.7	17.7	17.7	18.1
7	29.0	32.1	32.5	32.9
8	36.9	38.7	38.9	39.1
9	141.4	47.0	47.0	---
10	37.4	36.3	36.2	36.8
11	122.4	22.6	22.6	23.3
12	39.8	122.9	124.6	122.3
13	42.9	143.6	138.2	143.8
14	56.7	41.1	41.5	41.8
15	25.0	27.6	27.6	27.8
16	26.8	22.7	23.9	26.5
17	57.3	45.5	47.0	---
18	12.4	40.9	52.5	41.2
19	19.7	45.4	38.3	41.0
20	36.9	29.8	38.4	34.8
21	19.4	33.1	30.0	73.6
22	32.6	32.1	36.4	38.9
23	23.8	27.2	27.8	26.6
24	46.5	15.4	15.5	14.9
25	29.9	14.4	14.6	14.6
26	20.5	16.3	16.4	16.4
27	19.9	25.0	22.9	24.4
28	34.7	179.3	178.9	180.1
29	40.4	32.2	16.5	27.4
30	12.6	22.6	20.4	24.0
1’	103.0			
2’	75.8			
3’	78.6			
4’	72.1			
5’	78.9			
6’	63.3			

[a] Chemical shift in ppm (δ). [b] Dissolved in methyl-d_3 alcohol-*d*.

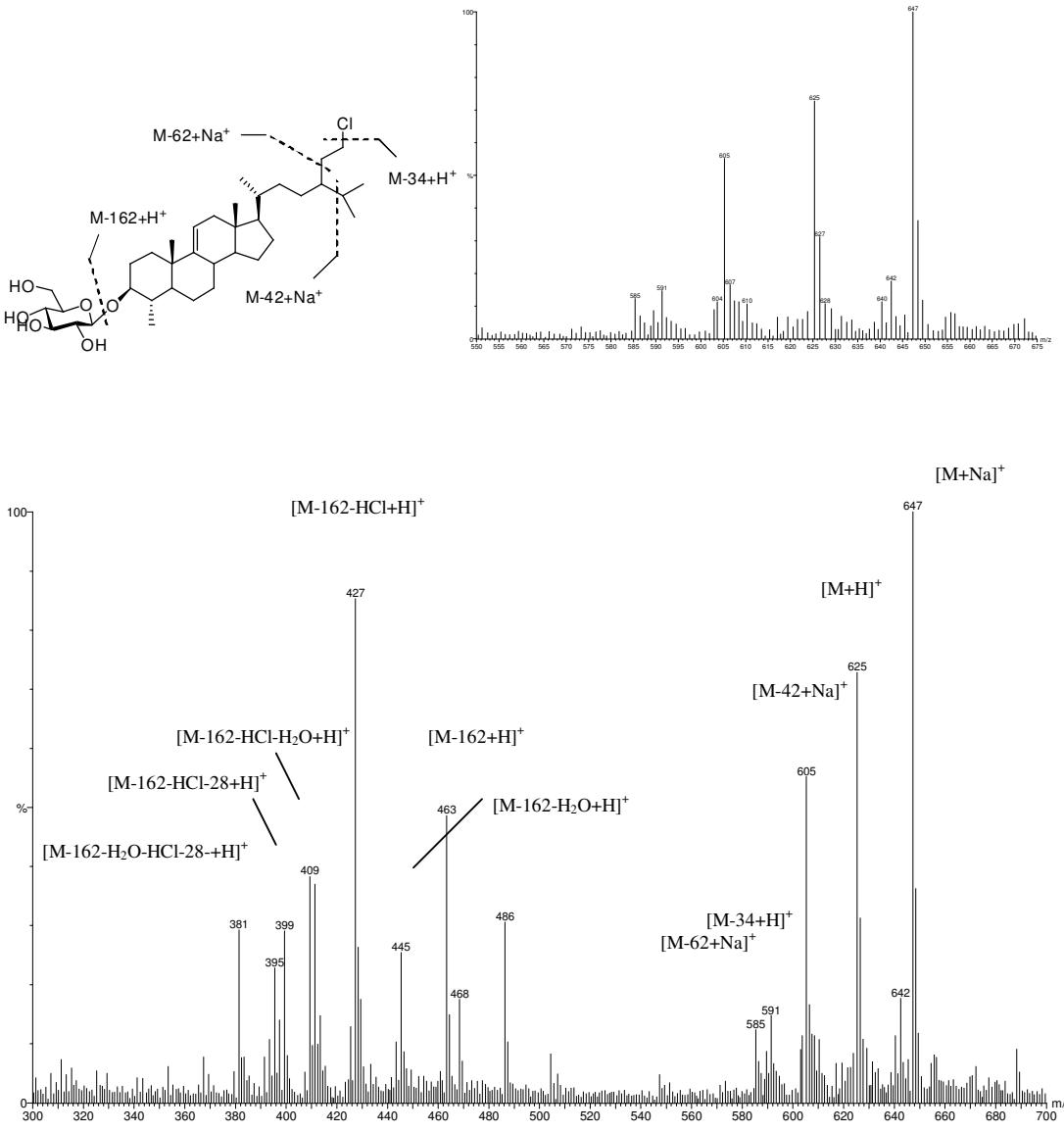

Figure 7.2. Proposed MS fragmentations of compound **1** under positive ESI

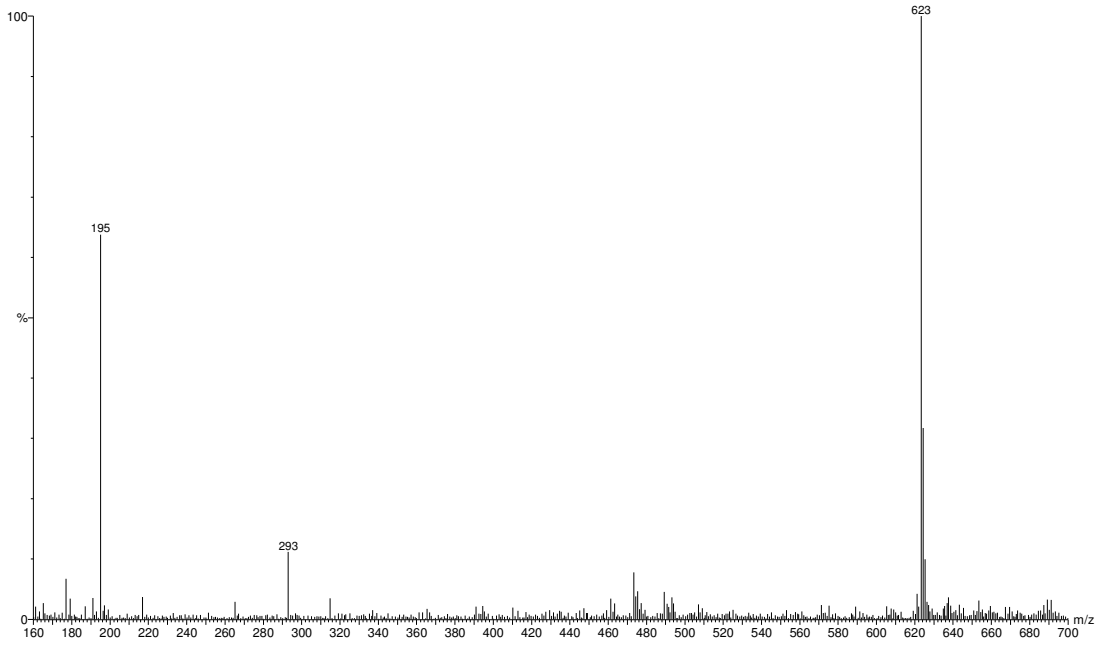

Figure 7.3. Negative ESI MS spectrum of compound **1**

7.3.4. H-1,5-Benzodioxepin (5).

The molecular formula of compound **5**, obtained as light yellow solid, was established as $C_{18}H_{14}O_7$ based on MS data (ESI MS m/z 343 $[M+1]^+$, 361 $[M+H_2O+1]^+$, 383 $[M+H_2O+Na]^+$), and the numbers of protons and carbons in ^1H and ^{13}C NMR spectra. The

proton decoupled ^{13}C NMR combined with proton coupled ^{13}C NMR indicated the presence of 18 carbons including two carbonyl groups, fourteen olefinic carbons, one methine group and one methylene group, suggesting a lignan-like skeleton. ^{1}H NMR spectrum exhibited two sets of 1,2,4-trisubstitued phenyl ring systems with chemical shifts at δ 6.70 (d, *J* = 8.0Hz), 6.62 (dd, *J* = 8.0, 2.4Hz), 6.76 (d, *J* = 2.4Hz); and δ 6.78 (d, *J* = 8.0Hz), 6.94 (dd, *J* = 8.0, 2.4Hz), 7.05 (d, *J* = 2.4Hz). The *trans* conformation at H-7' and H-8' on the double bond was identified by the coupling constant of 15.6Hz at δ 7.55 (H-7') and 6.27 (H-8'). The large difference of chemical shifts for H-7' and H-8' was caused by the electron withdrawing effect of carboxylic acid group at position 9'. The protons of methylene and methine groups were coupled, of which the two protons from methylene group were futher split by each other at δ 3.01 (dd, J = 14.4, 8.4Hz) and 3.10 (dd, J = 14.4, 8.4Hz) due to the stereo hindrance in a ring system. With the consideration of above information and the rationale of chemical shifts in ^{13}C NMR spectra (**Table 7.3**), compound **5** could be a dimer of two caffeic acids, which were connected to form a new seven-membered ring in between (**Fig. 7.4**).

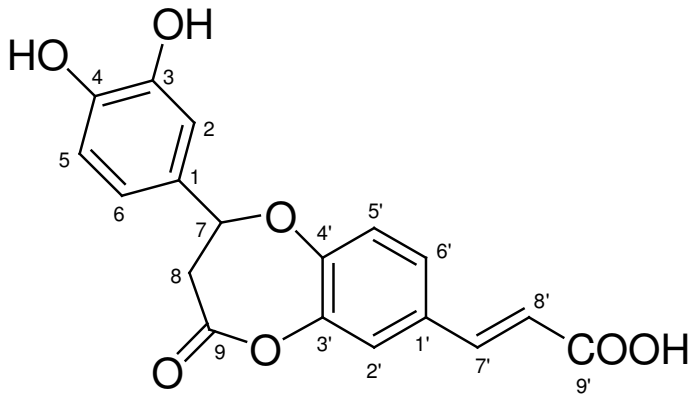

Figure 7.4. Chemical structure of compound **5**

Table 7.3. ^{1}H and ^{13}C NMR Data of Compound **5** (400MHz, CD_3OD)

position	^{1}H	^{13}C
1		128.0
2	6.76 (1H, d, *J* = 2.4Hz)	116.2
3		145.4
4		143.8
5	6.70 (1H, d, *J* = 8.0Hz)	114.9
6	6.62 (1H, dd, *J* = 8.0, 2.4Hz)	120.4
7	5.19 (1H, dd, *J* = 8.4, 4.4Hz)	73.5
8α	3.01 (1H, dd, *J* = 14.4, 8.4Hz)	36.6
8β	3.10 (1H, dd, *J* = 14.4, 4.4Hz)	
9		172.5
1'		126.3
2'	7.05 (1H, d, *J* = 2.4Hz)	113.8
3'		144.7
4'		148.3
5'	6.78 (1H, d, *J* = 8.0Hz)	115.1
6'	6.94 (1H, dd, *J* = 8.0, 2.4Hz)	122.7
7'	7.55 (1H, d, *J* = 15.6Hz)	146.3
8'	6.27 (1H, d, *J* = 15.6Hz)	113.1
9'		167.1

The structure of compound **5** was further conformed by its ^{1}H-^{1}H COSY spectra. The correlations between H-5 and H-6, and between H-5' and H-6' approved the two vicinal protons on each phenyl ring. H-7 was coupled with H-8α and H-8β individually. The correlation of H-8α and H-8β was also observed on the spectrum. H-7' and H-8' at the *trans* position was correlated clearly on COSY. H-2 and H-2' without any correlation to other protons on the phenyl rings, further conformed the substitution format on phenyl rings we postulated (**Fig. 7.5**). The NOEs were observed between H-5 and H-6, between H-5' and H-6', between H-7' and H-8', between H-7 and H-8α, between H-7 and H-8β, and the correlation between two geminal protons H-8α and H-8β was also present. Because compound **5** is a relatively flat molecule, hardly any NOEs between two phenyl rings were seen. In the HMBC experiment, the quaternary carbon at δ 148.3 assigned as C-4' showed correlations with C-2' and C-6', indicating the side chain 7'-8'-9' was on the *para* position of C-4' on the phenyl ring. The assignment of C-3 at δ 144.7 and C-4 at δ 143.8 was further confirmed by their correlations with the neighboring protons in HMBC.

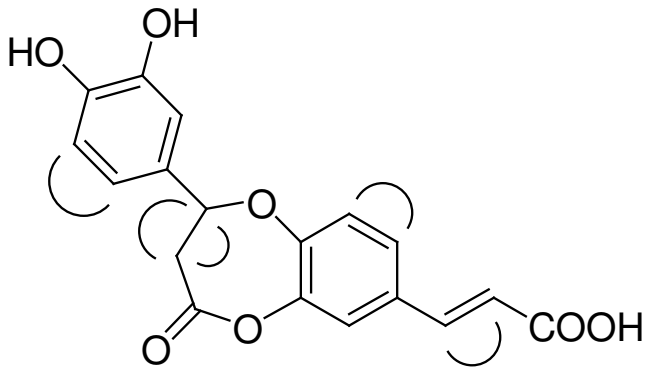

Figure 7.5. Observed ^{1}H-^{1}H COSY correlations in compound **5**

7.3.5. Results and Discussion.

Rosmarinic acid, oleanolic acid and ursolic acid are the predominant constituents in oregano, while the other compounds we identified herein are the very minor constituents in *O. vulgare* spp. *hirtum*. Compound **1**, containing the halogen chlorine, is a structurally novel compound. Natural organohalogen compounds are present in marine plants and animals, terrestrial plants, bacteria, fungi and mammals (Gribble, 2003). The formation of naturally occurring organohalogen compounds may be from several pathways and mechanisms. The enzymatic biosynthesis is a proposed pathway, which utilizes peroxidase enzymes, usually with hydrogen peroxide to oxidize halide to halogen, and

then biosynthesize the organohalogen compounds (Littlechild, 1999; Rorrer et al., 2001; Almeida et al., 2000). Chloroperoxidase, bromoperoxidase, iodoperoxidase and myeloperoxidase are among the extensively characterized halogenating enzymes that oxidize halide to halogen or enzyme-bound halogen. Chemical leading to organohalogens, such as oxidation of organic matter in the presence of halide salts or other halogen sources is another route (Keppler et al., 2000). This process may occur and contribute to the presence of atmospheric compounds CH_3Cl, CH_3Br and CH_3I (Keppler et al., 2000). More than 3800 organohalogen compounds, mainly containing chlorine or bromine while a few with iodine and fluorine, have been identified from living organisms (Gribble, 2003). The marine natural products are the largest source of biogenic organohalogen compounds, followed by terrestrial plants, fungi, lichen, bacteria, insects and some higher animals (Gribble, 2003). Many researchers reported the presence of these organohalogens. Chloromethane was described to present in potato tubers, and bromomethane, a commercial nematicide, was produced by broccoli, cabbage, mustard, radish turnip and rapeseed (Harper, 2000; Gan et al., 1998). The global annual production of bromomethane by rapeseed only was estimated to be 6600 tons (Gan et al., 1998). Scietists also found that some peas, lentil and vetch biosynthesized compounds such as 4-chloroindole-3-acetic acid and its methyl ester as the growth hormones (Gribble, 2003). Japanese researchers reported seven novel chlorophenol fungicides in edible Japanese lily (*Lilium maximowiczii*), and which functioned as the plant's self-defense chemicals in response to invasive microorganisms (Monde et al., 1998). We are the first to report this

novel chloro triterpenoid compound in oregano. We hypothesized that compound **1** was possibly produced through enzymatic biosynthesis in the oregano plant (*O. vulgare* spp. *hirtum*). In the absence of any pesticide, soil amentdments and historical use in the fields, an external chloro triterpenoid form another source is unlikely. The chlorination by microorganisms during the sample storage might be another possibility to form this organohalogen. Compound **1** is hardly soluble in water or organic solvent, so we postulated it would not contribute much to the bioactivities of the oregano extract.

Compounds **4** and **5** are new from the genus *Origanum*. Since we could not accumulate sufficient quantities of these compounds from the 100 g crude oregano extract due to their low natural abundance, further bioactivity screening was not performed. Compound **4** is structurally related to oleanolic acid, with one more hydroxyl group attached on C-21. This compound was previously reported as a glycoside form present in the roots of *Clematis chinensis* (Mimaki et al., 2004). Compound **5**, structurally related to caffeic acid derivatives, has been reported isolating from *Messerchmidia sibirica* (Song, Wang & Jia, 1996). We would expect Compound **5** to possess similar bioactivities as rosmarinic acid, while its contribution to the biological activities of oregano (*O. vulgare* spp. *hirtum*) extract should be minor due to its low natural abundance.

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CHAPTER 8. AN ANALYTICAL SURVEY OF ISOFLAVONES IN TOFU AND EDAMAME SOYBEANS CULTIVATED IN NORTH AMERICA

This chapter and the next chapter (Chapter 9) are supportive projects on HPLC method development of phenolic compound determination in botanicals other than the species involved in the dietary supplement product development. These projects were performed to strengthen and complement the LC/MS methodologies on bioactive phenolic compound analysis and served as part of my training on natural products analytical chemistry and instrumentation.

8.1. INTRODUCTION

Soybeans and soy-based food products long considered as a nutritional component of Asian diets, are now gaining acceptance for its potential health-promoting properties. Recent studies showed dietary consumption of soy could lead to the lowering of blood cholesterol, and provide prevention of cancers, osteoporosis, and other chronic diseases (Mahungu et al., 1999; Kuo, 1997). Soybeans are rich in proteins, minerals, vitamins and omega-3 fatty acids, which are each related to the prevention of heart diseases in humans (Nair et al., 1997). Evidence has shown that isoflavones from soybeans contribute to the properties to lower the risk of cardiovascular diseases (Chiang, Shih & Chu, 2001) and to prevent several cancers by acting as antiestrogens, antioxidants, and tyrosine protein kinase inhibitors (Wang & Murphy, 1994).

The major isoflavones in soybeans include daidzein, glycitein, genistein, their glycosides, glycoside malonates and glycoside acetates (**Fig. 8.1**), in which the predominant isoflavone forms in soybeans and non-fermented soy products are glycoside malonates, 6"-*O*-malonylgenistin and 6"-*O*-malonyldaidzin (Wu et al., 2004; Kudou et al., 1991). After consumption, aglycones daidzein, glycitein and genistein are enzymatically released in our small intestine (Bowey, Adlercreutz & Rowland, 2003). It appears that the hydrolysis to aglycone is for the absorption (Setchell et al., 2002). Recent studies showed that long-term storage and high temperature in processing could increase the amount of isoflavone aglycones. This may be due to the presence of functional enzymes and the effect of heat converting some isoflavone glycosides of daidzein, glycitein and genistein to daidzein, glycitein and genistein, respectively (Caldwell, Britz & Mirecki, 2005). Given these observations, the ability to apply a reliable method to quantify the total isoflavone content without the influence of other variables is needed. The most often method used to quantitate total isoflavones is to quantify all the individual isoflavone forms and calculate the total isoflavone contents on the basis of their molecular weights (Wang & Murphy, 1994; Tsukamoto et al., 1995). Hydrolysis of soybeans with HCl has been reported to transform isoflavone glycoside derivatives to aglycones (Chiang, Shih & Chu, 2001; Penalvo, Nurmi & Aldercreutz, 2004). This transformation was applied and further modified by our research group to hydrolyze all the glycosides to simple aglycones, daidzein, glycitein and genistein for the quantitation of total isoflavones.

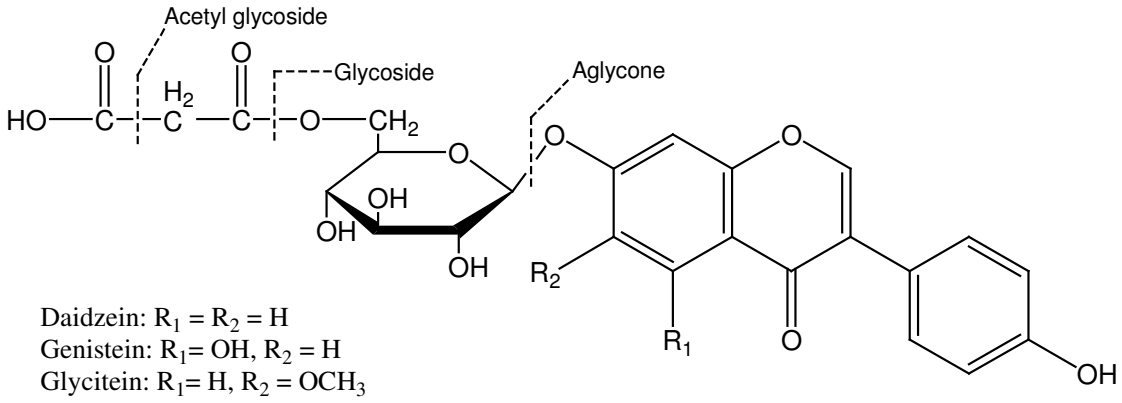

Figure 8.1. Chemical structures of isoflavones and the proposed MS fragmentation pathway of 6"-*O*-malonyl-β-glucoside.

The object of our research was to investigate whether isoflavone content differences could be found during different Tofu soybean varieties, to assess the variation within the same varieties, and to develop a practical analytical method to determine the total isoflavone content. The applied study is part of a larger program entitled "Human Food Soybean Systems" which seeks to provide information for farmers who are changing from conventional animal feed agronomic production to food grade production for Tofu and Edamame soybean market. Tofu soybeans can differ from conventional soy varieties in terms of seed size, seed composition and plant growth. At present the concentration of

nutritional components such as protein and oil content in soybeans are major considerations for growers in switching from traditional soybean varieties used for animal feed to human food. This study was conducted to evaluate the isoflavone content so that this factor may also be considered a component of quality in specialty soybeans. Tsukamoto et al. (Tsukamoto et al., 1995) reported that different parts in soybean seeds contain different isoflavone contents, and the isoflavone level in cotyledon exhibited a large response to temperature during seed fill, whereas the level in hypocotyl remained a relatively constant. By using HPLC coupled with MS and UV detection, the isoflavone profile and total isoflavone contents were examined from different Tofu soybean varieties in crop year 2002 and 2003. Under the optimized conditions in LC/MS, a total of 20 isoflavone derivatives were identified. The quantification of total isoflavone contents was achieved by acid hydrolysis of soybean to the three aglycones, and LC/UV as the analytical method. The information obtained will be valuable for the evaluation of the potential of dietary soybean as well as the effect of variety, location, and crop year on the isoflavone contents.

8.2. MATERIALS AND METHODS

8.2.1. Materials.

Standard compounds, daidzein, glycitein, genistein, and the internal standard formononetin were purchased from Sigma Chemical Co. (St. Louis, MO). Formic acid was obtained from Acros Organics (NJ). HPLC-grade methanol (MeOH), HPLC-grade acetonitrile (MeCN), 95% ethanol (EtOH) and concentrated hydrochloric acid (HCl) were purchased from Fisher Scientific Co. (Fair Lawn, NJ). HPLC-grade water (18MΩ) was prepared using a Millipore Milli-Q purification system (Millipore Corp., Bedford, MA) and was used for preparing all solutions.

The two 2002 Tofu soybean varieties (early ground II maturity type named 2F11 from Iowa and HP204 from Minnesota) were purchased from NJ Plus Organics (Lincoln, NE) and planted at Rutgers Research Farm in Pittstown, NJ. Five replications of 2F11 Early and HP204 Early were planted in the early of June, and five replications of 2F11 Late and HP204 Late were planted in the middle of June. The soil pH was 6.3 and the harvest dates were about 190 days after planting. In year 2003, three Tofu soybean varieties Vinton81, HP204 and Iowa1007 were obtained from N J Plus Organics (Lincoln, NE) and planted at seven sites in three different zones in New Jersey. The southern portion includes Rutgers Research Farm in Deerfield, NJ, the central zone includes Rutgers Research Farm in

Cream Ridge, NJ and the northern zone includes Rutgers Research Farm in Pittstown, NJ. A non-Tofu type conventional soybean as standard was planted at each site for comparative purposes. All the samples in 2003 were planted from middle to late June and harvested about 180 days after planting. All soybeans were harvested when the seeds were mature.

8.2.2. Equipment.

HPLC separation was performed on a Prodigy ODS(3) column, 5μm, 150×4.6mm i.d. (Phenomenex Inc., Torrace, CA). Agilent 1100 Series LC/MSD system (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler, quaternary pump system, diode array, multiple-wavelength detector, thermostated column compartment, degasser, MSD trap with an electrospray ion source (ESI), and HP ChemStation software. Bruker Daltonics 4.1 and DataAnalysis 4.1 software were used.

8.2.3. Preparation of Standards for HPLC Analysis.

The stock solution was prepared by dissolving the appropriate amounts of ~5.0 mg each standards daidzein, glycitein and genistein in 15 mL of diluent (70% methanol) with sonication for 25 min. The final volume of each solution was then diluted to 20 mL with the diluent at room temperature. Calibration standards were prepared by diluting the

stock solution with the diluent and then spiking the same amount of internal standard formononetin. The calibration curves were established on 10 data points covering a concentration range for daidzein, glycitein, and genistein in Tofu soybeans.

8.2.4. Sample preparation.

Air dried mature soybean whole seeds, separated seed coats, cotyledons or hypocotyls were finely grounded with a coffee grinder. For qualitative analysis, about 1 g powder was weighted to a 25 mL volumetric flask and ~20 mL of diluent (70% methanol) was added. The sample was sonicated at 0°C for 20 min and allowed to warm up to room temperature, and then filled to the full volume with the diluent. The extract was transferred to a centrifuge tube and centrifuged at 12,000 rpm for 2min to obtain a clear solution and filtered through a 0.45 μm filter. The sample (10 μL injected) was applied for LC/MS analysis right after the extraction in order to prevent possible decompositions in the solvent. The extraction procedure for quantitative analysis was modified from previous studies (Chiang, Shih & Chu, 2001; Wu et al., 2004; Tsukamoto et al., 1995). Approximately 2 g of soybean samples were placed into a 250mL flask along with 70 mL 95% ethanol, and 8 mL of concentrated HCl. The mixture was refluxed for 2h. The solution was then filtered to a 100 mL volumetric flask and carefully washed to final volume. Each hydrolyzed sample (20 μL injected), filtered through a 0.45 μm filter, was analyzed by duplicate injections. The recoveries were validated by spiking with known

quantities of isoflavone standards corresponding approximately to 100%, 75%, and 50% of the expected values in the soybean samples and then together extracting according to the same extraction method.

8.2.5. LC/MS Conditions for Identification.

The mobile phase for chromatographic separation consisted of solvent A (0.1% formic acid in water, v/v) and solvent B (0.1% formic acid in acetonitrile, v/v) in gradient. The linear gradient elution was performed from 10% B to 35% B in 40 min at flow rate 0.8 mL/min. Prior to next injection, the column was equilibrated for 10 min with 10% B. The column compartment was maintained at 25°C, and the injection volume was 10 µL. The eluent was monitored by electrospray ion mass spectrometer (ESI-MS) under positive ion mode, and the sample was scanned from *m/z* 100-600. ESI was conducted by using a needle voltage of 3.5kV under optimum collision energy level of 80%. The identity of isoflavones was performed based on their molecular and fragmentation ions in total ion chromatogram (TIC). Helium was used as Nebulizer at 40 psi, and dissolvation was achieved by using high-purity nitrogen (99.999%) heated to 325°C at a flow rate of 8 L/min.

8.2.6. LC/UV Conditions for Quantification.

The gradient elution was carried out at a flow rate of 1.0 mL/min with the solvent system containing solvent A and B in gradient from 20% B to 35% B in 30 min, where A was 0.1% formic acid (v/v) in water and B was 0.1% formic acid (v/v) in acetonitrile. The wavelength of UV detection was set at 254 nm. An internal standard formononetin was added to the analytes before injection, and all analytes were carried out in duplicate. A plot of the peak area ratio (analyte/IS) versus analyte concentration resulted in calibration equations of $y = 0.615x + 0.006$ ($r^2 = 0.9998$) for daidzein, $y = 0.58x + 0.0023$ ($r^2 = 0.9974$) for glycitein, and $y = 0.6134x + 0.0057$ ($r^2 = 0.9998$) for genistein. The concentrations of soybean samples were calculated from these linear equations.

8.2.7. Statistical Analysis.

The experimental results were analyzed using the SAS package (JMP IN version 4) developed by the SAS Institute Inc. (Cary, NC). Analyses of variance using the general linear models were conducted for interactions of variety, sowing date and location. Differences between the sample means were examined by least significant difference (LSD) test.

8.3. RESULTS AND DISCUSSION

8.3.1. Identification of Isoflavones Soybean Seeds.

It is generally recognized that there are twelve types of isoflavones in soybean seeds, which could be divided into three groups depending on the different aglycone moieties, namely daidzein, glycitein and genistein. Within each group, there are four types of isoflavones, the isoflavone aglycone, and its glycoside, 6"-*O*-malonyl glycoside and 6"-*O*-acetyl glycoside derivatives. Other groups have reported 14 to 16 isoflavones as detected by LC/MS (Wu et al., 2004; Gu & Gu, 2001; Fang, Yu & Badger, 2004). By separating the soybean seeds into their respective major parts, including the seed coats, cotyledons and hypocotyls, we identified or tentative identified 20 isoflavones (**Table 8.1**), including constituents which were not detectable in the whole seed extract, yet detectable in the seed parts when they were concentrated.

Table 8.1. Peak Assignments of Isoflavones in Tofu Soybeans

no. in Figure 2	t_R (min)	ESI/MS[a] positive (m/z)	compound identification[c]
1	8.3	433, **271**	genistein-G
2	9.0	417, **255**	daidzein-G (daidzin)[b]
3	10.0	447, **285**	glycitein-G (glycitin)[b]
4	13.8	503, **255**	daidzein-G-M
5	14.0	433, **271**	genistein-G (genistin)[b]
6a	14.6	519, **271**	genistein-G-M
6b	14.6	503, **255**	daidzein-G-M
7	14.8	533, **285**	glycitein-G-M
8	16.0	503, **255**	daidzein-G-M
9	16.5	533, **285**	glycitein-G-M
10	19.0	459, **255**	daidzein-G-A
11a	19.3	519, **271**	genistein-G-M
11b	19.3	533, **285**	glycitein-G-M
12	20.1	489, **285**	glycitein-G-A
13	20.2	519, **271**	genistein-G-M
14	20.9	519, **271**	genistein-G-M
15	22.7	255	daidzein[b]
16	24.1	285	glycitein[b]
17	25.0	475, **271**	genistein-G-A
18	30.7	271	genistein[b]

[a] Numbers in bold are base peaks in the MS spectrum. [b]Compared with the reference sample. [c]G = glucosyl/galactosyl moiety; M = malonate; A = acetate.

Positive LC/MS were used to obtain information about the molecular weights, the molecular masses of conjugates and the sugar moieties bonded to the aglycones. With the photodiode array detection (PAD), the comparisons of UV spectra and retention times with corresponding standards or their structure-related standards were also extensively applied to support the identification of peaks. Ion peaks at *m/z* 251, 285 and 271 are the characteristic positive ions of the three isoflavone aglycone moieties, daidzein, glycitein and genistein, which could be extracted out as the selective ions from total ion chromatogram (TIC) in the positive mode in order to screen out all components belonging to these three groups.

In addition to the qualitative examination of the whole soybean seed extract, the seed coats, cotyledons and hypocotyls were also analyzed by LC/MS (**Fig. 8.2**), which led to four more isomers of 6"-*O*-malonyl isoflavones detected from hypocotyl extract. Previous studies indicated that glycitein and its derivatives exclusively occurred in the hypocotyls, and only trace amount of isoflavones existed in the seed coats (Eldridge & Kwolek, 1983). This observation was confirmed in detailed chromatograms by LC/MS (**Table 8.2**). Twenty isoflavones were found and characterized from the soybean seed coats, cotyledons and hypocotyls, including 3 aglycones, 4 glycosides, 10 malonyl glycosides and 3 acetyl glycosides. As for many types of malonyl glycosides, 6"-*O*-malonyl isoflavones were the major components in soybean as has been previously described in detail (Kudou et al., 1991). The other forms of malonyl isoflavones in low concentration

levels could be the isomers in which the malonyl group is attached to a position other than the 6" position of the glycosyl moiety, position 2", 3" or 4". The existence of such minor isomers in soybeans was also implied by Gu et al. (Gu & Gu, 2001). A similar chemical profile was observed in LC/MS chromatograms from the seeds of all soybean varieties in our study, indicating that the qualitative composition can be characteristic of the Tofu soybeans evaluated (**Fig. 8.3**).

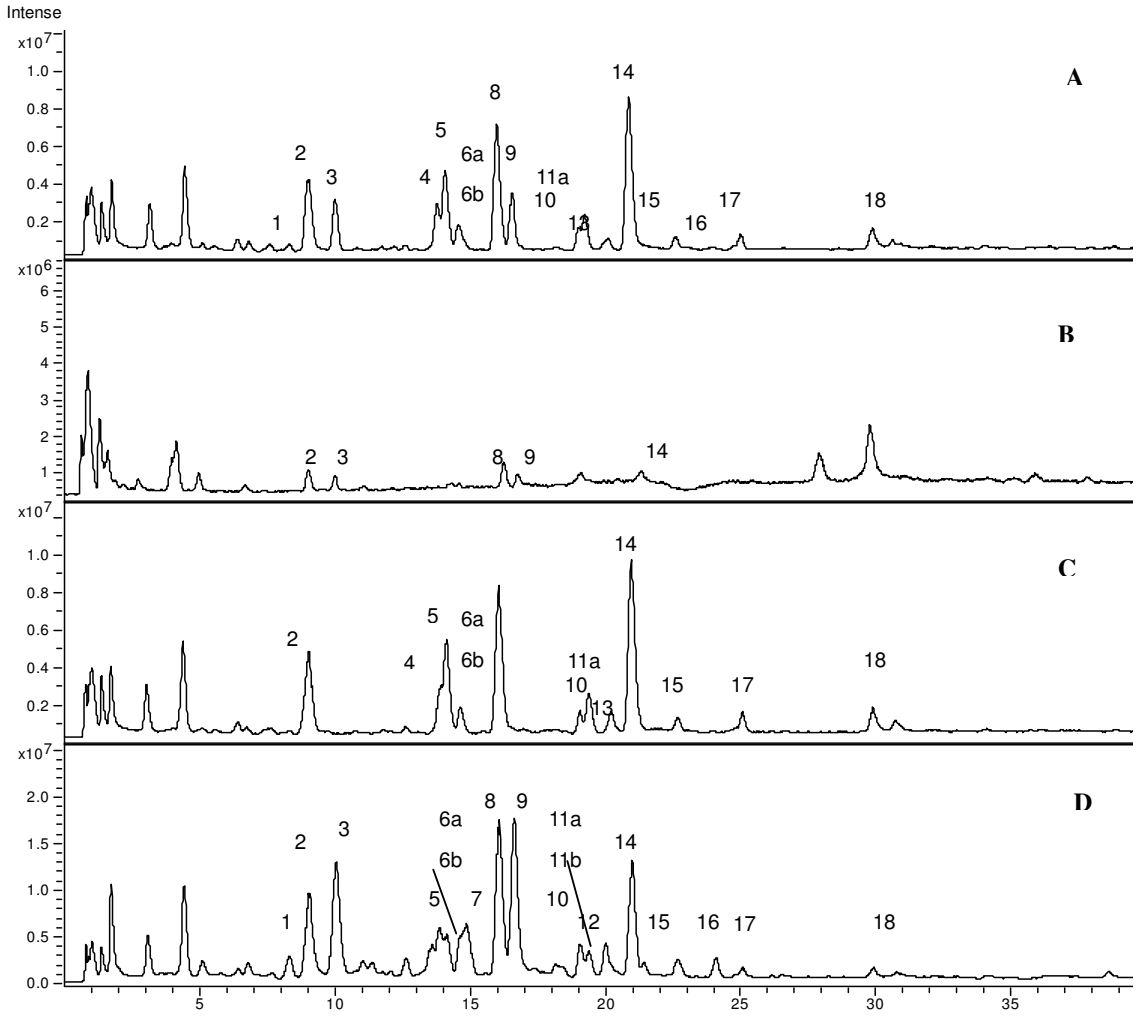

Figure 8.2. Comparison of different soybean parts (Vinton81A, 2003) by total ion chromatogram (TIC) of whole seed extract (**A**), seed coat extract (**B**), cotyledon extract (**C**) and hypocotyl extract (**D**), scanned from *m/z* 100 to 600. For identities, see Table 8.1.

Table 8.2. The Presence of Isoflavones in Tofu Soybean Whole Seeds and Seed Parts[a]

no. in Figure 2	compound	sample			
		whole seeds	seed coats	cotyledons	hypocotyls
1	genistein-G	+	-	-	+
2	daidzein-G	+	+	+	+
3	glycitein-G	+	+	-	+
4	daidzein-G-M	+	-	+	+
5	genistein-G	+	-	+	+
6a	genistein-G-M	+	-	+	+
6b	daidzein-G-M	+	-	+	+
7	glycitein-G-M	-	-	-	+
8	daidzein-G-M	+	+	+	+
9	glycitein-G-M	+	+	-	+
10	daidzein-G-A	+	-	+	+
11a	genistein-G-M	+	-	+	+
11b	glycitein-G-M	-	-	-	+
12	glycitein-G-A	-	-	-	+
13	genistein-G-M	+	-	+	-
14	genistein-G-M	+	+	+	+
15	daidzein	+	-	+	+
16	glycitein	+	-	-	+
17	genistein-G-A	+	-	+	+
18	genistein	+	-	+	+

[a](+) Present; (-) not detectable.

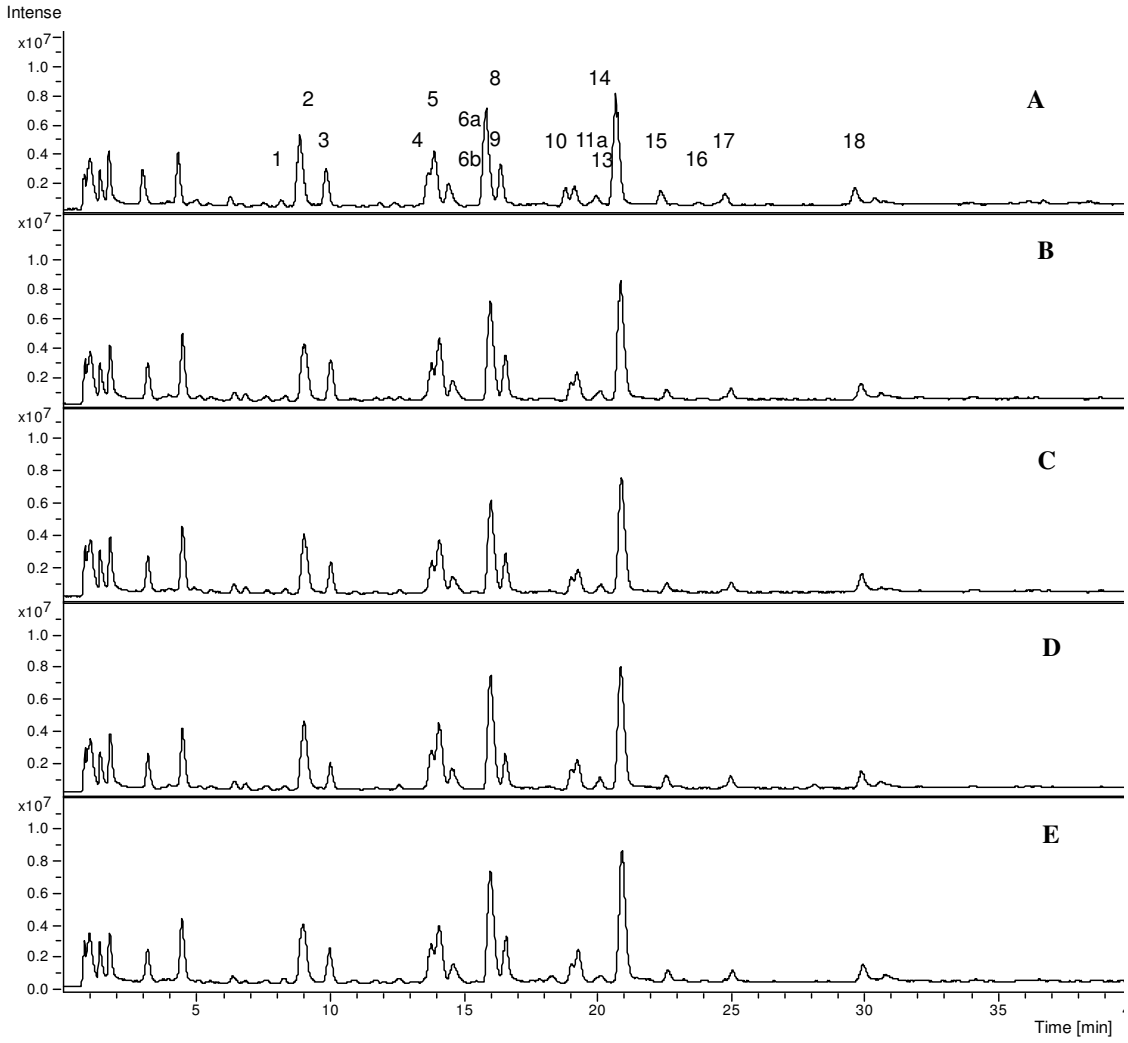

Figure 8.3. Representative total ion chromatograms (TIC) of soybean whole seed extract of 2F11A, 2002 (**A**); Vinton81A, 2003 (**B**); HP204A, 2003 (**C**); Iowa1007A, 2003 (**D**); and standardC, 2003 (**E**).

8.3.2. Quantification of Isoflavones by Acid Hydrolysis and LC/UV Detection.

Quantitative measurement of all individual isoflavones is difficult due to the complexity of the chemical profile and lack of available commercial standards for most of the soy isoflavones. Others have reported that long-term storage and high temperature in processing degraded isoflavones and cleaved malonyl groups, acetyl groups and glycosidic bonds (Mahungu et al., 1999; Caldwell, Britz & Mirecki, 2005), making the individual components variable to quantitate. Part of the purpose of this research was to confirm current methods or to develop a modified method allowing a simple, rapid, reliable and low-cost method for the regular quantification of total isoflavone levels that could be used in quality control and traceability back to a specific farm. Previous studies showed that aglycones, daidzein, glycitein and genistein can be enzymatically released in the small intestine in our digestion (Mahugu et al., 1999), suggesting that total amount of aglycones in soybeans can be quantitated as the total isoflavones related to bioavailability. Therefore, hydrolysis of all aglycoside derivatives to aglycones, daidzein, glycitein and genistein, and LC/UV detection could be a practical method for quantitative determination of total isoflavones. HCl hydrolysis of isoflavones has been reported by other researchers (Chiang, Shih & Chu, 2001; Penalvo, Nurmi & Aldercreutz, 2004), and this transformation was applied and further modified by our research group. Because in the soy-containing food industry, the cotyledons, hypocotyls and seed coats are not differentiated, we used whole intact soybean seeds for our quantitative analysis. Under

optimized HPLC conditions, the three target isoflavone aglycones, daidzein, glycitein, genistein and an internal standard formononetin were successfully separated within 25 min and monitored by UV detection (**Fig. 8.4**). The calibration curves were constructed by injecting the standard solution across 10 different concentrations (48-24500 ng/mL for daidzein, 53-27000 ng/mL for glycitein, and 53-27000 ng/mL for genistein), and regression for each was greater than 0.997.

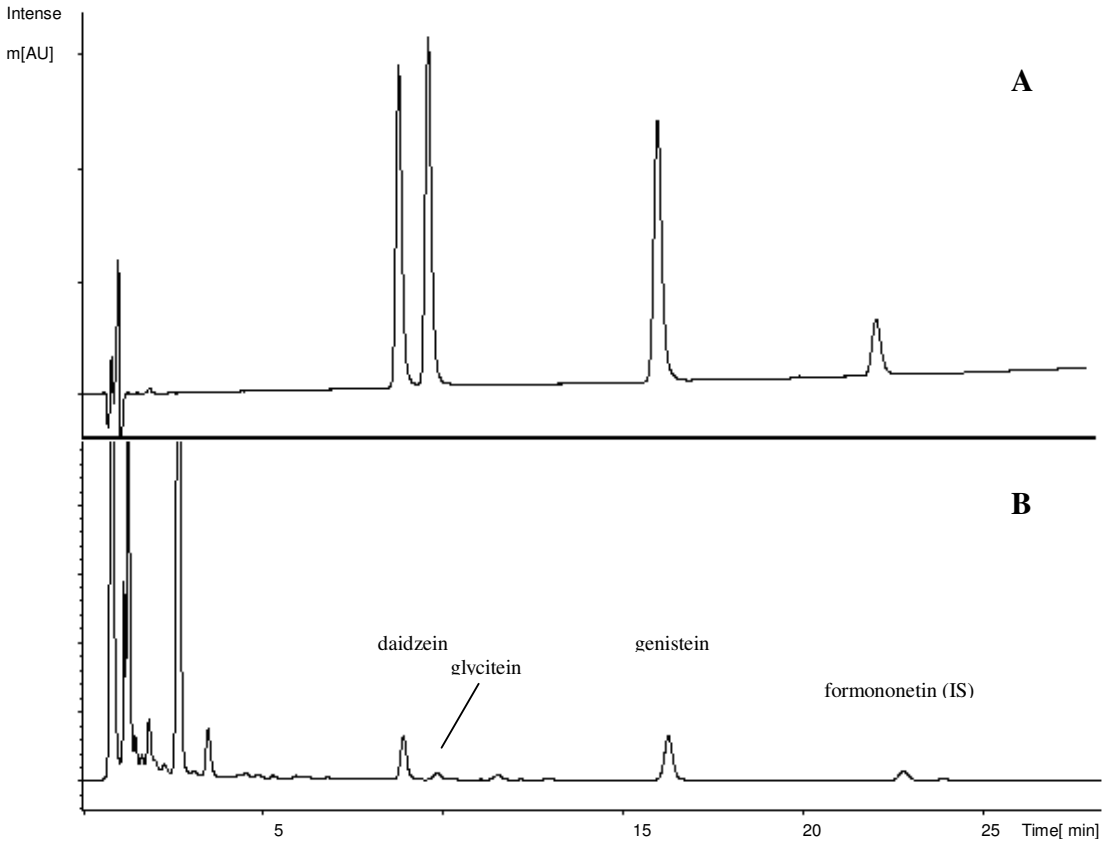

Figure 8.4. HPLC/UV Chromatogram of isoflavone standards spiked with formononetin as internal standard (**A**) and a representative chromatogram of hydrolyzed soybean seed extract (Iowa1007, 2003) spiked with formononetin for quantitative analysis (**B**).

The recovery of this method was tested by the addition of the three analytes, daidzein, glycitein and genistein at three different concentration levels. The samples were spiked with known concentrations of isoflavone standards corresponding approximately to 100%, 75%, and 50% of the expected values in the sample HP204b, 2002 (**Table 8.3**). No considerable differences were found among recoveries at different spiked levels (RSD < 3.2%). The mean recoveries were 95.5% for daidzein, 87.3% for glycitein, and 98.2% for genistein. The abundance of aglycone glycitein in soybeans is 5-10 times lower than daidzein and genistein on a weight basis, and thus the same amount of absorption from the matrix affects the recovery more for glycitein than of either daidzein or genistein according to the percentage of the compound in which it is present. That could partially explain why the recovery of glycitein was lower than the other two aglycones. The polarity difference of isoflavones could also account for this result and we used a polar solvent system for extraction. In conclusion, a consistent, precise and reproducible analytical method was accomplished.

Table 8.3. Recoveries of Isoflavones in Different Concentrations

isoflavone	concentration (μg/g)	added (μg/g)	found (μg/g)	recovery (%)	mean (%)	RSD (%)
daidzein	356.6	333.7	671.9	94.5		
		250.3	598.6	96.7	95.5	1.2
		166.8	515.4	95.2		
glycitein	52.0	62.6	107.8	89.1		
		46.9	93.6	88.7	87.3	3.2
		31.3	78.3	84.0		
genistein	506.3	434.5	923.7	96.1		
		325.9	826.0	98.1	98.2	2.2
		217.3	724.5	100.4		

8.3.3. Factors Affecting Isoflavone Content in Soybean Seeds: Genotype, Crop Year, Location and Sowing Date.

Based on the quantitative method developed, we further quantitated 48 samples from four different Tofu soybean varieties as well as some traditional non-Tofu soybean varieties at different growing locations during two crop years (**Table 8.4**, **Table 8.5**). While the study was not originally designed to test robustly the impact of season in the same location and with all the same varieties, our findings on genetic differences provide a stronger foundation than our preliminary observations relative to crop year and location. In year

2002, two varieties of Tofu soybeans 2F11 and HP204 were planted at the research farm in Pittstown, New Jersey, and five replications of early planted soybeans versus late planted soybeans were compared for each variety (**Fig. 8.5**). No significant difference of total isoflavone contents was observed between early and late planted soybeans for either variety 2F11 or HP204. Differences in the accumulation of total isoflavones by variety was significant ($p < 0.05$) for the soybean samples in 2002, indicating it could be a trait for which plant breeders could select. The mean isoflavone content of 2F11 was 1073.3 μg/g, significantly higher than that of HP204 (947.6 μg/g).

Table 8.4. Isoflavone Contents (μg/g) and Aglycone Ratios of Genistein to Daidzein in Crop Year 2002[a]

Sample code[b]	Daidzein	Glycitein	Genistein	Total	Genistein/ daidzein
2F11a	467.4	59.3	465.0	991.7	0.99
2F11b	499.7	60.9	517.5	1078.1	1.04
2F11c	571.6	65.4	598.2	1235.3	1.05
2F11d	484.1	57.3	514.3	1055.7	1.06
2F11e	415.5	47.0	455.8	918.4	1.10
Mean±SD	487.7±56.6	58.0±6.8	510.2±56.6	1055.8±118.0	1.05±0.04
2F11A	446.2	62.2	494.6	1002.9	1.11
2F11B	477.2	64.8	512.7	1054.7	1.07
2F11C	548.5	65.8	606.0	1220.3	1.10
2F11D	535.6	65.2	575.9	1176.6	1.08
2F11E	445.3	54.7	499.8	999.8	1.12
Mean±SD	490.5±48.9	62.5±4.6	537.8±50.1	1090.9±101.8	1.10±0.02
HP204a	345.5	46.0	476.5	868.0	1.38
HP204b	356.6	52.0	506.3	914.9	1.42
HP204c	422.9	54.4	578.8	1056.0	1.37
HP204d	343.3	56.5	506.1	906.0	1.47
HP204e	360.2	44.9	500.7	905.8	1.39
Mean±SD	365.6±32.8	50.7±5.1	513.7±38.4	930.0±72.7	1.41±0.04
HP204A	371.1	56.1	521.9	949.0	1.41
HP204B	359.2	60.8	544.4	964.4	1.52
HP204C	372.4	56.3	572.6	1001.3	1.54
HP204D	416.0	61.9	554.8	1032.6	1.33
HP204E	350.3	47.3	480.8	878.5	1.37
Mean±SD	373.8±25.2	56.5±5.7	534.9±35.4	965.2±58.4	1.43±0.09

[a] Sample measured in duplicate. [b] The letter followed by the variety name represents a location at the Research Farm in Pittstown, NJ. Lower case letters represent early-planted replications and upper case letters represents late-planted replications.

Table 8.5. Isoflavone Contents (μg/g) and Aglycone Ratios of Genistein to Daidzein in Crop Year 2003[a]

Sample code[b]	Daidzein	Glycitein	Genistein	Total	Genistein/ daidzein
Vinton81A	399.5	69.1	597.6	1066.2	1.50
Vinton81B	385.4	49.6	586.3	1021.2	1.52
Vinton81C	395.0	40.2	523.1	958.2	1.32
Vinton81D	250.3	43.8	343.9	637.9	1.37
Vinton81E	269.3	47.1	361.2	677.7	1.34
Vinton81F	265.5	48.0	366.6	680.1	1.38
Vinton81G	430.7	48.8	573.7	1053.2	1.33
Mean±SD	342.2±76.8	49.5±9.3	478.9±116.4	870.6±195.6	1.40±0.08
HP204A	294.6	44.0	446.5	785.0	1.52
HP204B	324.6	43.1	448.0	815.7	1.38
HP204C	340.6	36.3	510.6	887.5	1.50
HP204D	217.6	53.9	320.1	591.6	1.47
HP204E	217.0	54.3	322.7	594.0	1.49
HP204F	214.9	53.4	329.9	598.3	1.53
HP204G	280.9	64.2	405.3	750.5	1.44
Mean±SD	270.1±53.7	50.0±9.3	397.6±75.2	717.5±122.2	1.48±0.05
Iowa1007A	529.1	49.4	722.4	1300.9	1.37
Iowa1007B	453.5	37.8	642.3	1133.6	1.42
Iowa1007C	208.4	43.4	317.4	569.2	1.52
Iowa1007D	153.4	37.6	219.3	410.3	1.43
Iowa1007E	177.6	42.6	259.4	479.7	1.46
Iowa1007F	163.5	42.7	243.6	449.7	1.49
Iowa1007G	255.3	49.4	343.5	648.1	1.35
Mean±SD	277.3±151.6	43.3±4.8	392.5±203.8	713.1±356.5	1.43±0.06
StandardA[c]	427.3	62.5	583.0	1072.8	1.36
StandardB[c]	496.3	76.2	637.2	1209.6	1.28
StandardC[d]	422.8	57.6	560.1	1040.5	1.32
StandardD[d]	325.7	68.4	494.5	888.6	1.52
StandardE[d]	400.7	82.3	572.9	1055.8	1.43
StandardF[d]	184.2	44.3	279.1	507.5	1.52
StandardG[d]	236.8	57.0	334.2	627.9	1.41
Mean±SD	356.3±112.4	64.0±12.8	494.4±135.8	914.7±257.0	1.41±0.09

[a] Sample measured in duplicate. [b] The letter followed by the variety name represents a different growing site in New Jersey. [c] Traditional non-Tofu variety Asgrow2703.

[d] Traditional non-Tofu variety Syngenta STS.

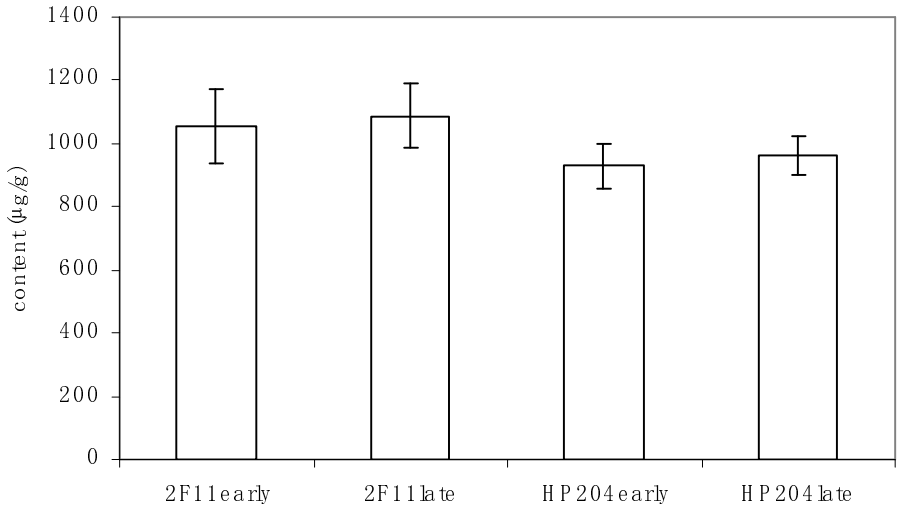

Figure 8.5. Total isoflavone contents of soybean seeds from early planted 2F11, late planted 2F11, early planted HP204 and late planted HP204 in crop year 2002.

In year 2003, three varieties of Tofu soybeans, Vinto81, HP204 and Iowa1007, with some non-Tofu type conventional soybeans as standard were planted (**Fig. 8.6**). Seven replications of each variety were extended to different sites in New Jersey. Total isoflavone concentration ranged from 410.3 µg/g to 1300.9 µg/g for all samples in 2003.

Statistical interactions of genotype of isoflavone contents were not significant due to the large variance among different growing sites. Iowa1007 exhibited the largest variance with RSD = 49.3% at all seven sites, reflecting it was the least stable variety across the changing environments. The average isoflavone contents for all three Tofu varieties were not higher than conventional non-Tofu type soybean. A significant ($p < 0.05$) location effect was observed for the total isoflavone contents in 2003, suggesting that the environment may play an important role over genotype in terms of isoflavone distribution.

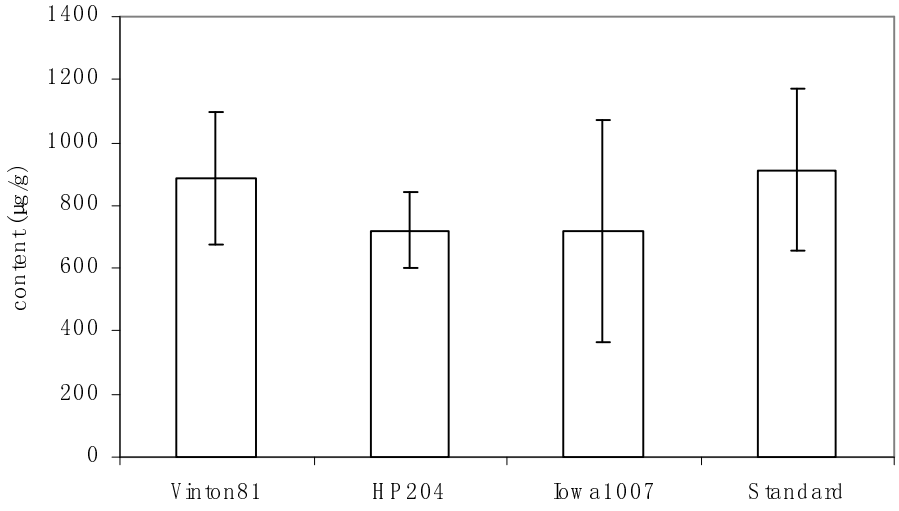

Figure 8.6. Total isoflavone contents of soybean seeds from Vinton81, HP204, Iowa1007 and standard in crop year 2003.

Others have reported that the crop year potentially affected the isoflavone contents (Wang & Murphy, 1994). This influence was also observed in our research in that the average isoflavone content of HP204 from 2002 (947.6 μg/g) was much higher than that from 2003 (717.5 μg/g). The general climatic conditions were different between 2002 and 2003 growing seasons, and the 2002 season could be described as having more heat units and drought during seed development, yet no conclusions can be drawn from this preliminary observation except that seasonal differences were found.

The significant variance among locations and the difference observed over the two growing seasons do suggest that climate and environment might be major factors that can impact the accumulation of isoflavones in soybeans.

8.3.4. The Study of Genistein and Daidzein Proportion in Soybeans.

The total isoflavone contents were calculated as the addition of three target isoflavone aglycones, daidzein, glycitein and genistein. Glycitein is found naturally at much lower concentrations in soy whole seeds than the other two aglycones, daidzein and genistein. The latter are the two isoflavones also known to link to many health-promoting benefits (Mebratu et al., 2004). We noticed the isoflavone aglycones, especially daidzein and genistein were distributed proportionally with very small variance of total contents for each soybean variety. The total isoflavone contents were positively related with daidzein

(each r > 0.93) and genistein (each r > 0.96) at different growing sites for the same variety. The aglycone ratios of genistein to daidzein were around 1.4 for most Tofu and non-Tofu soybean varieties with an exception of 2F11 (ratio of genistein/daidzein = 1.08) (**Fig. 8.7**), which showed a uniquely higher proportion of daidzein in the variety 2F11 compared to all other soybeans.

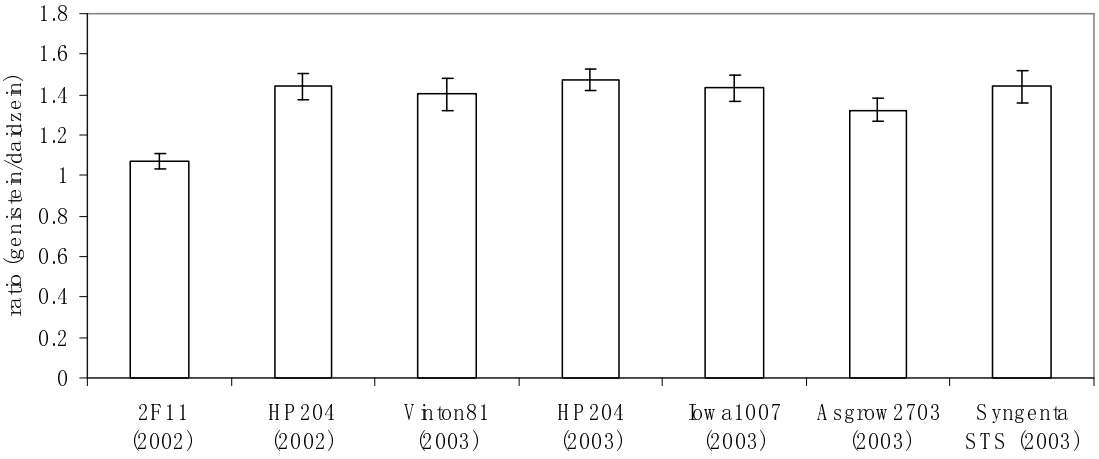

Figure 8.7. Aglycone ratios of genistein/daidzein in soybean seeds from different varieties. The ratios of 2F11 (2002) and HP204 (2002) were generated from 10 soybean replications; the ratios of Vinton81 (2003), HP204 (2003) and Iowa1007 (2003) were from 7 replications; the ratio of Asgrow2703 (2003) was from 2 replications; and the ratio of Syngenta STS (2003) was from 5 replications.

8.4. ANALYSIS OF EDAMAME SOYBEANS

Edamame as a type of edible soybeans has large seeds, a light colored hilum, green seed coat and high sugar content (Sciarappa et al., 2007). It is traditionally cultivated in China, Japan, and other Asian countries because it provides an excellent substitute for protein that avoids saturated fats and cholesterol. These beans contain 30%-40% protein and are rich in calcium, vitamin A, and phytoestrogens. The increase of soy products in the news, touting their health benefits, has increased the public's awareness of this traditional Asian vegetable (Sciarappa & Quinn, 2004). In order to develop production information for American growers of this new crop, Edamame cultivar trials were seed planted in New Jersey and Maryland to determine suitability in the Mid-Atlantic region. The New Jersey study site was located at the Rutgers Snyder Research and Extension Farm in Pittstown, New Jersey. In 2003 growing season, the isoflavone contents of 15 vegetable soybean cultivars were compared. Ten of these cultivars' seeds were received from the Asian Vegetable Research and Development Center in Taiwan and five were procured from Evergreen Seeds, an Asian seed company in Japan. The planting was made on July 11, 2003. All cultivars were pre-treated with *Rhizobium* inoculant prior to hand planting. Measurements were made on a daily basis such as time of first plant emergence, percent stand, first trifoliate leaf, first flower, plant height at flowering, number of days until maturity, plant height at maturity, number of nodes per plant, plant stand at harvest,

lodging, and total pod yield.

The isoflavone content analysis was performed by using the LC/UV/MS method developed for Tofu-type soybeans. The chromatographic conditions and MS parameters were the same as described above. The phytochemical profile showed much diversity compared to Tofu-soybeans. For example, the concentration of daidzein (peak 11) was higher than genistein-G-M (peak 10) for sample 1, 5, and 9, which was inversed from Tofu-soy samples and most other samples from this batch (**Fig. 8.8**). For sample 4 and 8, the concentration of daidzein (peak 11) is almost as high as genistein-G-M (peak 10). The minor acetyl derivatives of daidzein (peak 8) was only detected in mature (yellow color) soy sample 3, 6, 12 and 13. Qualitative study also revealed that the content of isoflavone aglycones, e.g. daidzein (Peak 11 in **Fig. 8.8**), is much higher in some samples (sample 4 and 8, see **Fig. 8.8F**), and even higher than the glycosides and glycoside malonic derivatives (sample 1, 5 and 9, in **Fig. 8.8C**, **D** and **E**). Furthermore, the quantitative study indicated that the total isoflavone content for those diversified samples (sample 2, 8 and 1, 5, 9) is relatively lower (**Table 8.6**, **Fig. 8.9**). It was probably because they were less mature than others. While for the Tofu-soybeans we analytically surveyed, the major isoflavone components were glycosides and glycoside molanates of daidzein and genistein, and the chemical profile was quite uniform.

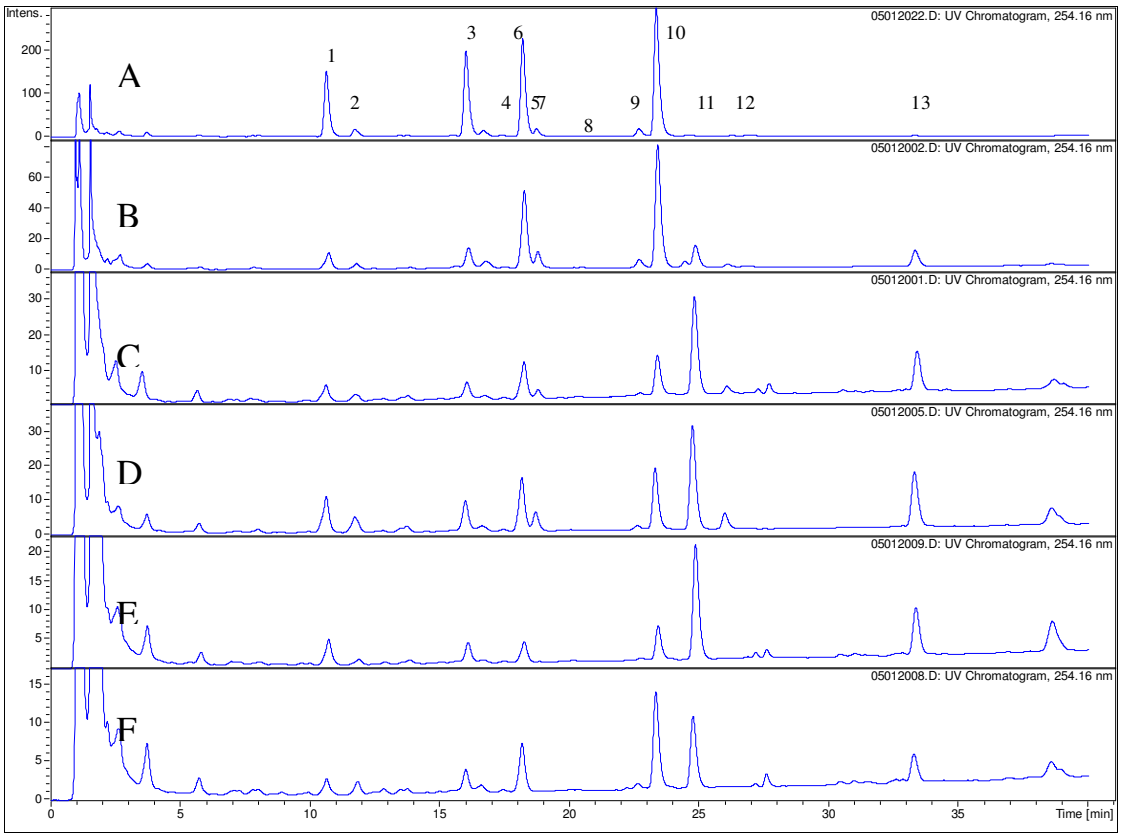

Figure 8.8. Representative LC/MS Chromatograms of Edamame samples in 2003 growing season. (**A**) sample10 from 2002 growing season, (**B**) sample 2, (**C**) sample 1, (**D**) sample 5, (**E**) sample 9, and (**F**) sample 8.

Table 8.6. Isoflavone Contents of 15 Edamame Soy Varieties Cultivated in 2003

Sample code	Commercial name	Content (%)			
		Daidzein	Glycitein	Genistein	Total
1	Taisho Shirage	0.0128	0.0034	0.0071	0.0233
2	Shironomai	0.0118	0.0025	0.0181	0.0324
3	Tzuzunoko	0.0639	0.0058	0.0618	0.1315
4	Emerald	0.0155	0.0029	0.0207	0.0391
5	Shirofumi	0.0082	0.0024	0.0088	0.0194
6	Neu Ta Pien	0.0788	0.0093	0.0932	0.1813
7	Ryokkoh	0.0338	0.0026	0.0379	0.0743
8	Neu Ta Pien Pi	0.0059	0.0001	0.0047	0.0107
9	Ryokkoh Pi	0.0072	0.0006	0.0039	0.0117
10	Ryokkoh Taisho	0.0772	0.0072	0.0814	0.1658
11	Lucky Lion	0.0597	0.0066	0.0594	0.1257
12	Early Hakucho	0.0503	0.0064	0.0498	0.1065
13	Be Sweet	0.0406	0.0039	0.0402	0.0847
14	Green Legend	0.0695	0.0059	0.0586	0.1340
16	Taiwame	0.0201	0.0024	0.0307	0.0531

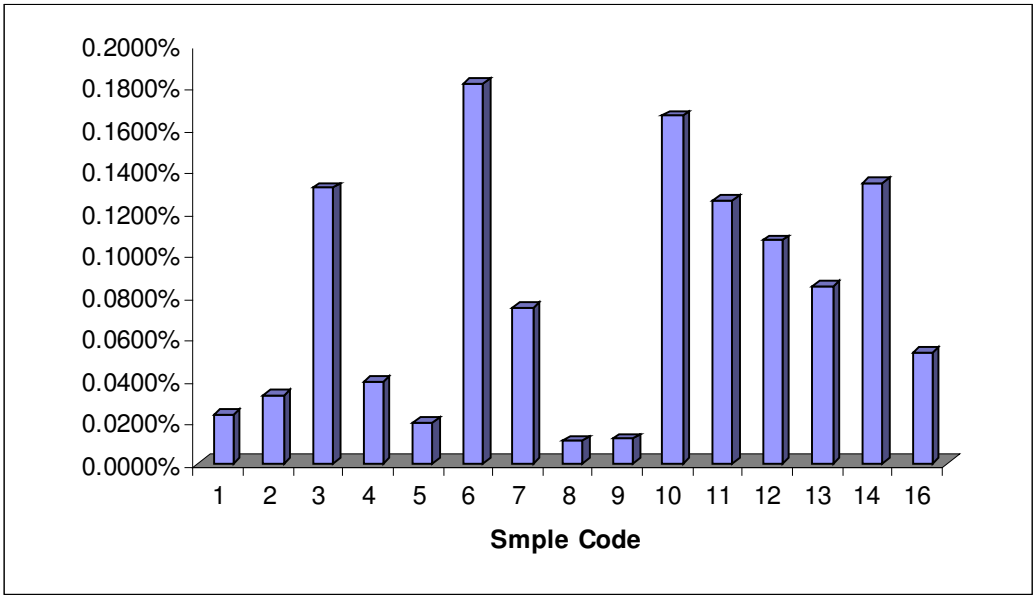

Figure 8.9. Comparison of total isoflavone contents of Edamame varieties from the 2003 growing season, New Jersey field data

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CHAPTER 9. NEW CATECHIN RESOURSE INVESTIGATION AND LC/MS (ION TRAP) DETERMINATION OF THE PREDOMINANT CATECHINS IN *ACACIA CATECHU*

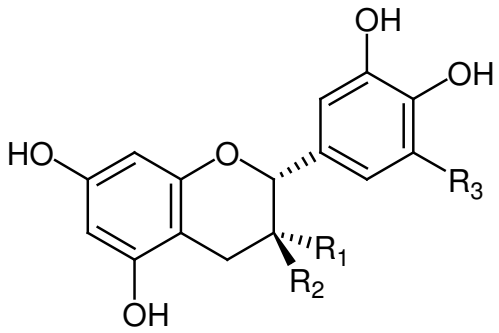
The following work was conducted as part of this dissertation and has been since published: Diandian Shen, Qingli Wu, Mingfu Wang, Yonghong Yang, Edmond J. Lavoie, James E. Simon. Determination of the predominant catechins in *Acacia catechu* by LC/ESI-MS. *Journal of Agricultural and Food Chemistry* **2006**, *54*, 3219-3224.

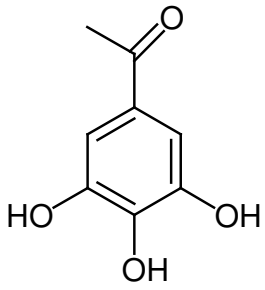
9.1. INTRODUCTION

Acacia catechu commonly known as catechu, Khail or Cutch tree is native to Southern Asia and widely distributed in India and Burma (Naik et al., 2003). Khail is a multipurpose leguminous tree providing wood, gum, tannin and dye, and also used for reforestation and land reclamation due to its wide adaptability and rapid growth rate (Kaur, Verma & Kant, 1998; Rout, Samantary & Das, 1995). The wood of *Acacia catechu* being hard is used in making furniture and agriculture implements (Kaur & Kant, 2000; Singhal & Joshi, 1984). Khair gum is a pale yellow mucilaginous gum exuded from the tree, yielding one of the best natural substitutes for gum Arabic (Rout, Samantary & Das, 1995). The plant extract has been used as an astringent, and for itching, indigestion and inflammations (Naik et al., 2003; Yadava & Sodhi, 2002). Heartwood extract of catechu is a traditional Chinese medicine called “Ercha” and is used in treatment of cough, dysentery, as well as topically for skin ulceration and lesions (Wang et al., 1999). Catechu is also known to be used as a component of the betel leaf chewed in India for the local

treatment of otitis and otorrhoea (Yang et al., 2004).

Previous studies have reported on the isolation and identification of several chemical constituents from the heartwood, bark, roots and leaves, the major components being catechins, caffeine, flavonol glycosides and other phenolic compounds (Sharma, Dayal & Ayyal, 1997; Sharma, Dayal & Ayyal, 1999; Deshpande & Patil, 1981; Azad, Ogiyama & Sassa, 2001; Yadav, 2001; Agarwal & Soni, 1988). Of the dietary flavonoids examined, catechins, naturally occurring flavan-3-ols, such as (+)-catechin, (-)-epicatechin, (-)-epicatechin-3-*O*-gallate and (-)-epigallocatechin-3-*O*-gallate (**Fig. 9.1**) exhibit strong antioxidant activities that are generally associated with free radical scavenging and metal chelation properties (Ricardo de Silva et al., 1991; Arpentine et al., 1992; Frankel, Waterhouse & Kinsella, 1993; Hu et al., 1995). These natural products have also been reported as anticarcinogenic (Liu & Castonguay, 1991; Blot, Chow & McLaughlin, 1996; Khokhar et al., 1997), antimutagenic (Liviero et al., 1994) and cardioprotective agents (Terencio, Sanz & Paya, 1991; Meunier et al., 1987). An inhibitory effect to mouse IV allergy was recently reported by Suzuki et al. from tea catechins by determining the mouse ear swelling ratios (Suzuki et al., 2000). Abe et al. found that green tea gallocatechines potently and selectively inhibited rat squalene epoxidase (SE), a rate-limiting enzyme of cholesterol biogenesis (Abe et al., 2000).





G: Galloyl

Catechin (C): R_1 = H, R_2 = OH, R_3 = H
Epicatechin (EC): R_1 = OH, R_2 = H, R_3 = H
Gallocatechin (GC): R_1 = H, R_2 = OH, R_3 = OH
Epigallocatechin (EGC): R_1 = OH, R_2 = H, R_3 = OH
Catechin-3-O-gallate (CG): R_1 = H, R_2 = O**G**, R_3 = H
Epicatechin-3-O-gallate (ECG): R_1 = O**G**, R_2 = H, R_3 = H
Gallocatechin-3-O-gallate (GCG): R_1 = H, R_2 =O**G**, R_3 = OH
Epigallocatechin-3-O-gallate (EGCG): R_1 = O**G**, R_2 = H, R_3 = OH

Figure 9.1. Chemical structures of catechins

In *Acacia catechu*, catechins occur with many additional UV-absorbing phenolic compounds, making their chromatographic separation and detection difficult. The only reference available for the HPLC quantitative analysis of catechins from catechu was the LC/UV quantification of two catechins components (+)-catechin and (-)-epicatechin found in a traditional medicine extract of catechu (Wang et al., 1999). In this study, we procured botanically authenticated catechu heartwood and leaves plus a commercially available catechu product, catechu resin chunks, to quantitate the individual catechins. By

using LC/ESI-MS and LC/MS/MS, catechin and epicatechin in catechu heartwood as well as resin chunks, and catechin, epicatechin, epigallocatechin-3-*O*-gallate, and epicatechin-3-*O*-gallate in catechu leaves were selectively detected and quantified. In addition to these flavan-3-ols, other major components were identified or tentatively identified.

9.2. MATERIALS AND METHODS

9.2.1. Materials.

Standard compounds, (+)-catechin, (-)-epicatechin, (-)-epigallocatechin, (-)-epicatechin-3-*O*-gallate and (-)-epigallocatechin-3-*O*-gallate, and the internal standard glycitein were purchased from Sigma Chemical Co. (St. Louis, MO). The solvents methanol (MeOH) and acetonitrile (MeCN) used in this research were HPLC-grade and purchased from Fisher Scientific Co. (Fair Lawn, NJ). HPLC-grade water (18MΩ) was prepared using a Millipore Milli-Q purification system (Millipore Corp., Bedford, MA) and was used for preparing all solutions. Formic acid was purchased from Acros Organics (NJ). The catechu leaves were collected from Yunnan province, P. R. China, and authenticated by the botanical taxonomist, Dr. Yonghong Yang, Yunnan Agricultural University, China. The catechu heartwood sample was obtained as

dry wood powder from Shree Baidyanath Ayurved Bhawan Pvt. Ltd. (India). The catechu resin chunks (extract from catechu heartwood) were purchased as a commercial product of Chinese traditional medicine from 13 Moons store (Johnson City, NY). Both of these catechu heartwood sample and catechu resin chunks were authenticated microscopically by Dr. Qingli Wu in our research team, and the description and identification of catechu (Ercha) was further confirmed using Chinese Pharmacopoeia (1995 edition, part I). Product samples are also held in our botanical products library for future reference. A green tea sample was used as a comparative control source of catechu and was procured from P. R. Bigelow Inc. (Fairfield, CT).

9.2.2. Equipment.

HPLC separation was performed on a 250×4.6mm i.d., 5 μm, Polaris Amide C18 column (Varian Inc., Palo Alto, CA), 1100 Series LC/MSD system (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler, quaternary pump system, diode array, multiple-wavelength detector, thermostated column compartment, degasser, MSD trap with an electrospray ion source (ESI), and HP ChemStation software. Bruker Daltonics 4.1 and DataAnalysis 4.1 software were used for LC/ESI-MS and LC/MS/MS experiments.

9.2.3. Calibration Standards and Quality Control (QC) Samples.

The stock solution was prepared by dissolving the appropriate amounts of ~5.0 mg each standards, catechin, epicatechin, epicatechin-3-*O*-gallate, and epigallocatechin-3-*O*-gallate in 7 mL of diluent (0.1% formic acid water and MeOH, 3:7) with sonication for 15 min. The final volume of each solution was then diluted to 10 mL with the diluent at room temperature. Calibration standards were prepared by diluting the stock solution with the diluent and then spiking the same amount of internal standard glycitein. The calibration curves were established on 10 data points covering a concentration range for catechin, epicatechin, epicatechin-3-*O*-gallate, and epigallocatechin-3-*O*-gallate. QC samples were prepared by diluting the separated stock solutions with diluent and then spiking the same amount of known internal standards.

9.2.4. Sample Preparation.

All catechu and green tea samples were finely ground with a coffee grinder. About 100 mg powder was accurately weighed from each sample and placed into a 100 mL volumetric flask, and ~70 mL of diluent (0.1% formic acid water and MeOH, 3:7) was added. The sample was sonicated for 20 min (only for 70% methanol extraction) and allowed to cool down to room temperature, and then filled to the full volume with the diluent. The extract was transferred to a centrifuge tube and centrifuged at 12,000 rpm for 2 min to obtain a clear solution and filtered through a 0.45 μm filter. The injection

volume was 20 μL. The recovery test was performed by the addition of known quantities of standards corresponding approximately to 100% of the expected values in the original sample and then together extracting according to the same extraction method described above.

9.2.5. LC/MS Conditions for Identification.

The mobile phase for chromatographic separation consisted of solvent A (0.1% formic acid in water, v/v) and B (0.1% formic acid in acetonitrile, v/v) in gradient. The total running time was 50 min. The gradients were 0 min, 90% solvent A; 10 min, 80% solvent A; 30 min, 70% solvent A; 40 min, 80% solvent A. Prior to the next injection, the column was equilibrated for 10 min with 10% B. The flow rate was set to 1.0 mL/min with the column compartment maintaining at 25 °C, and the injection volume was 20 μL. The eluent was monitored by electrospray ion mass spectrometer (ESI-MS) under positive ion mode, and the sample was scanned from *m/z* 120-2200. ESI was conducted by using a needle voltage of 3.5 kV under an optimum collision energy level of 20%. The identity of flavonoids was based on their molecular ions in total ion chromatogram (TIC) and fragment ions in auto MS/MS spectra (Threshold, 30,000). Tandem MS/MS was conducted in the positive ion mode and under the collision energy level of 20%. Helium was used as nebulizer at 70 psi, and desolvation was achieved by using high-purity nitrogen (99.999%) heated to 350 °C at a flow rate of 12 L/min.

9.2.6. LC/MS Conditions for Quantification.

HPLC conditions were the same as described. SIM mode (selected-ion monitoring) was applied and protonated molecular ions were isolated for analytes of catechin, epicatechin, epigallocatechin-3-*O*-gallate and epicatechin-3-*O*-gallate, respectively. The mass spectrometer was set into four time segments: 0-13min for catechin and epicatechin; 13-18.5min for epigallocatechin-3-*O*-gallate; 18.5-30min for epicatechin-3-*O*-gallate; and 30-50min for glycitein (IS). Scan range was set from *m/z* 200 to 500, and the isolation width was 1.0 *m/z*. Other MS parameters were the same as described for qualitative identification. An internal standard glycitein was added to the analytes before injection, and a plot of the peak area ratio (analyte/IS) versus analyte concentration for calibration standards resulted in equations of $y = 0.0279x + 0.0052$ ($r^2 = 0.999$) for catechin; $y = 0.0358x + 0.0049$ ($r^2 = 0.997$) for epicatechin; $y = 0.0073x - 0.0007$ ($r^2 = 0.998$) for epigallocatechin-3-*O*-gallate; and $y = 0.005x - 0.0014$ ($r^2 = 0.998$) for epicatechin-3-*O*-gallate. The concentrations of the QC and catechu samples were calculated from these linear equations. All analyses were carried out in triplicate.

9.3. RESULTS AND DISCUSSION

9.3.1. Characterization of the Major Components in Catechu by LC/MS and LC/MS/MS Method.

Positive LC/MS and the subsequent fragmentation of the predominant positive ions in auto MS/MS were used to obtain information about the molecular weights (Fig. 9.2), the molecular masses of conjugates and the masses of the sugar moieties bond to the aglycones. Whenever possible, the authentic standards and literature were applied to support the identification of peaks. The retro Diels-Alder (RDA) fragmentation ion can serve as a characteristic fingerprint for the presence of catechins in complex matrices (Wu, Wang & Simon, 2003). $[M+H\text{-galloyl}+H\text{-}H_2O]^+$ is also a general fragmentation pattern observed for all catechin gallates and gallocatechin gallates (Wu, Wang & Simon, 2003). Because epicatechin is an epimer of catechin, its mass spectrum is identical to that of catechin. The positive full scan LC/MS analysis produced peaks for catechin (peak 5) and epicatechin (peak 7) matching with the molecular ions at *m/z* at 291 under a 20% collision energy level (Fig. 9.3). In addition, in the second MS stage, the characteristic RDA fragmentations at *m/z* 139 further confirmed the structures (Fig. 9.4). The identities of epicatechin-3-*O*-gallate (peak 16) and epigallocatechin-3-*O*-gallate (peak 12) in catechu were confirmed on the basis of molecular ions at *m/z* 443 for epicatechin-3-*O*-gallate and 459 for epigallocatechin-3-*O*-gallate, and the fragmentation $[M+H\text{-galloyl}+H\text{-}H_2O]^+$ at *m/z* 273 for epicatechin-3-*O*-gallate and *m/z* 289 for

epigallocatechin-3-*O*-gallate.

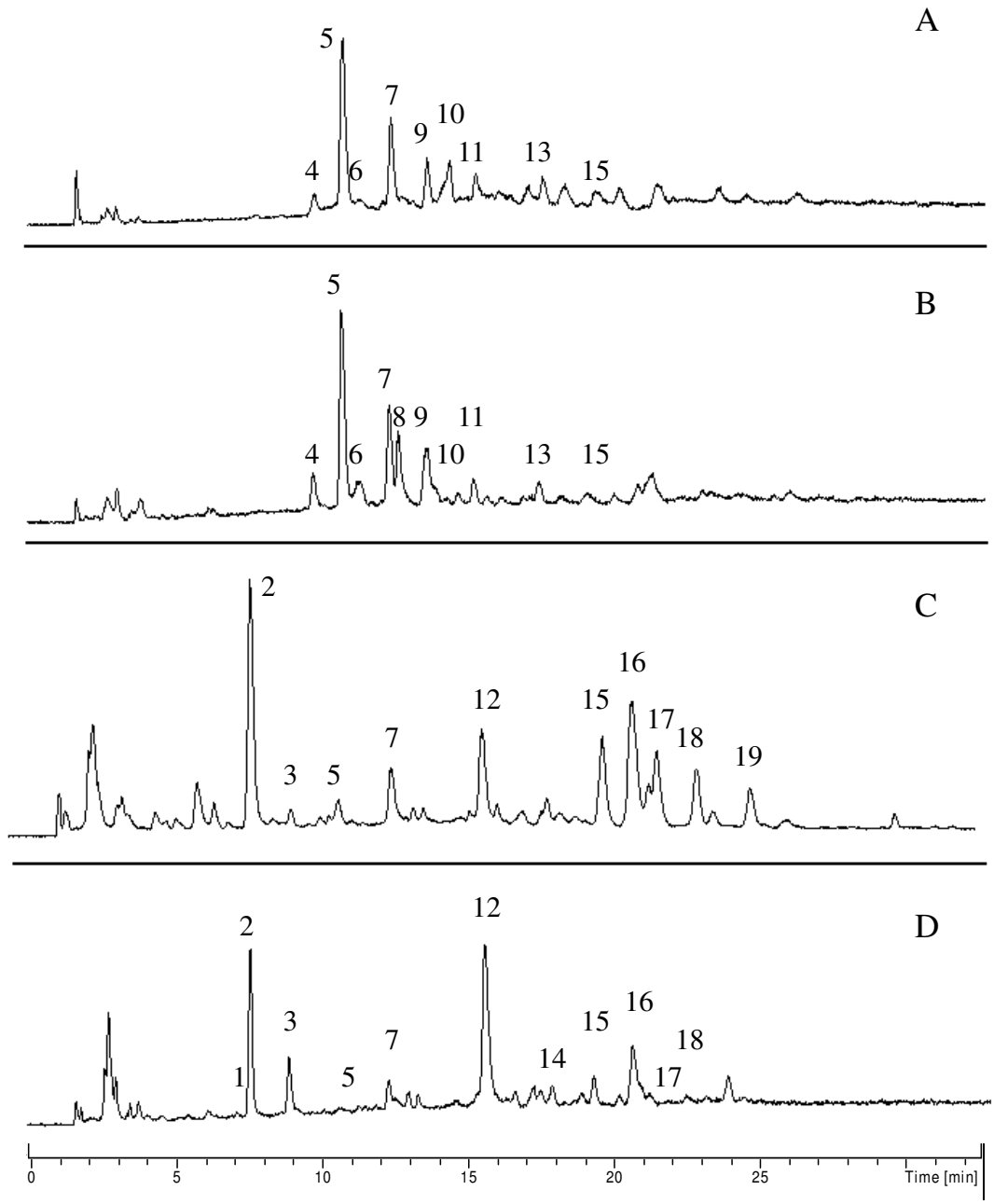


Figure 9.2. Total ion chromatogram (TIC) of catechu heartwood (**A**), catechu resin chunks (**B**), catechu leaves (**C**) and green tea leaves (**D**), scanned from *m/z* 120 to 2200. For identities, see Table 9.1.

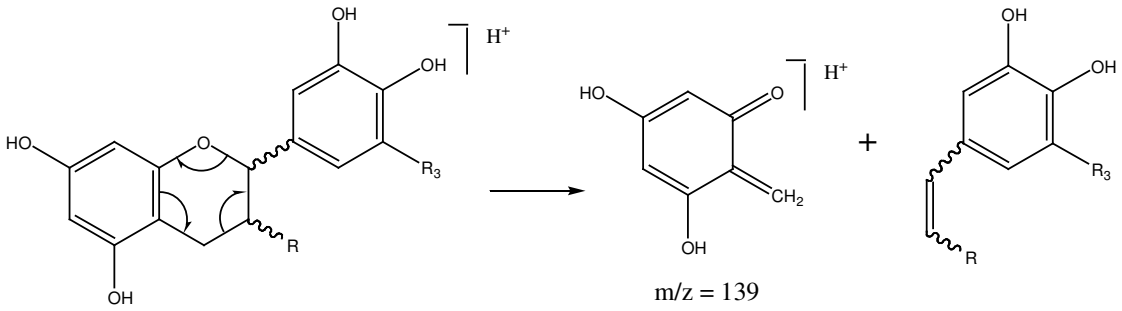


Figure 9.3. Proposed retro Diels-Alder fragmentation of catechins

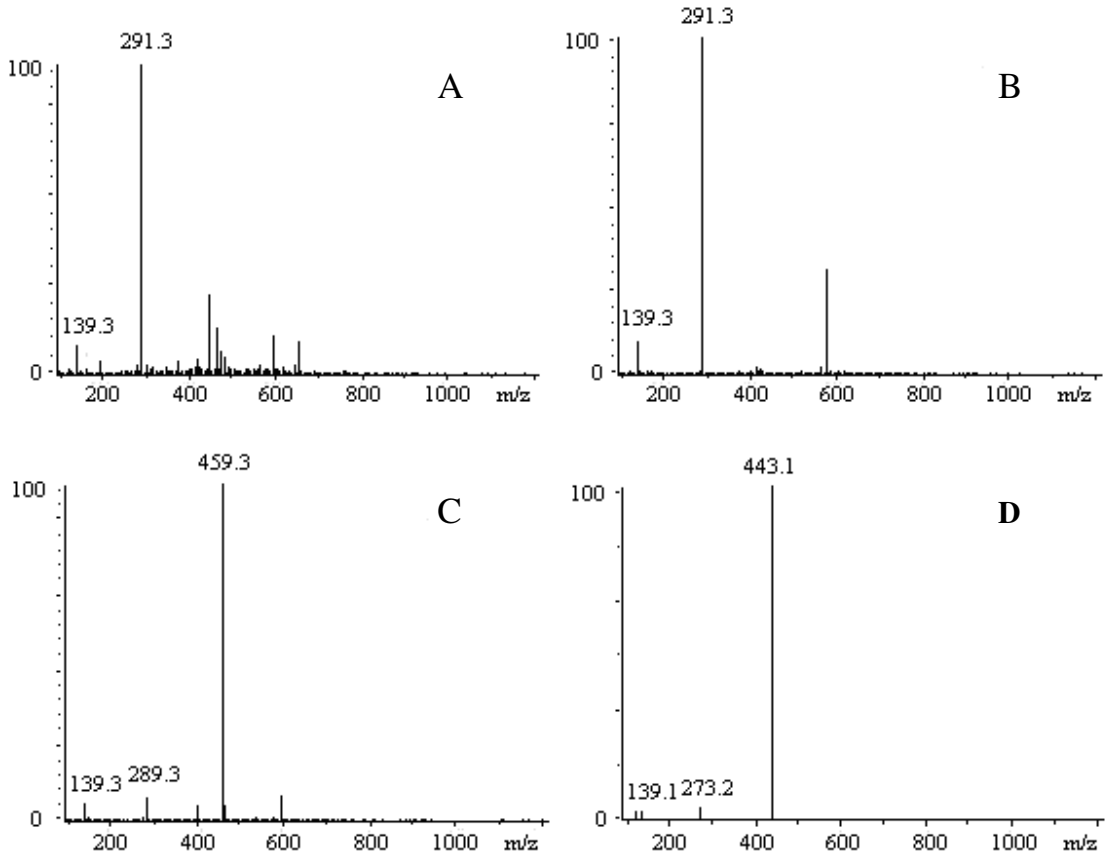


Figure 9.4. Representative positive ESI mass spectra obtained from the LC/MS total ion chromatograms of catechu leaf extract in Fig. 9.2. (A) C (peak 5 in Fig. 9.2). (B) EC (peak 7 in Fig. 9.2). (C) EGCG (peak 12 in Fig. 9.2). (D) ECG (peak 16 in Fig. 9.2).

Full-scan LC/MS produced two series of flavanol dimers with molecular ions at *m/z* 579 (peak 4 and 6) and *m/z* 581 (peak 9, 10, 11 and 13), respectively. The dimers with molecular ion peaks at *m/z* 579 appear to be procyanidin dimers on the basis of catechin-like RDA fragmentation $[M+H-152]^+$ at *m/z* 427 and a catechin-like monomer fragmentation at *m/z* 291 in MS/MS (**Table 9.1**) (Desphande & Patil, 1981; Maatta-Riihinen, Kamal-Eldin & Torronen, 2004, Nelson & Sharpless, 2003). The characterization of chalcan-flavan type dimers were based on the molecular ion peaks at *m/z* 581, the RDA fragmentation at *m/z* 429, the catechin-like monomer fragmentation at 291, and the previous structural elucidations (Bajadurai & Nayudamma, 1964; Santhanam, Ghosh & Nayudamma, 1965; Dalluge & Nelson, 2000).

Table 9.1. Peak Assignment for the LC/MS and LC/MS/MS Analysis of Catechu and Green Tea

peak no.	t_R (min)	$[M+H]^+$ MS (*m/z*)	fragment ion MS/MS (*m/z*)	tentative identification
1	7.0	307	289, 139	(-)-gallocatechin
2	7.5	195	138	caffeine (std)[a]
3	8.8	307	289, 139	(-)-epigallocatechin (std)[a]
4, 6	9.7, 11.2	579	427, 409, 291	procyanidin dimers
5	10.6	291	273, 139	(+)-catechin (std)[a]
7	12.1	291	273, 139	(-)-epicatechin (std)[a]
8	12.5	329	279	unknown compound
9,10, 11,13	13.4,14.3, 15.1, 17.2	581	563, 429, 411, 291	chalcan-flavan dimers
12	15.4	459	289, 139	(-)-epigallocatechin-3-O-gallate(std)[a]
14	17.7	459	289, 139	(-)-gallocatechin-3-O-gallate
15	19.1	611	465, 303	quercetin hexose –deoxyhexoside
16	20.4	443	273, 139	(-)-epicatechin-3-O-gallate (std)[a]
17	20.9	465	303	quercetin hexoside
18	22.7	595	449, 287	kaempferol hexose –deoxyhexoside
19	24.3	449	287	kaempferol hexoside

[a]Compared with the standard.

The identification of flavonol glycosides was performed according to the MS spectra and the identities of flavonoids from catechu on previous studies (**Table 9.1**) (Sharma, Dayal & Ayyal, 1997; Sharma, Dayal & Ayyal, 1999; Deshpande & Patil, 1981). Peak 15 seems to be a quercetin hexose–deoxyhexoside based on positive molecular ion at *m/z* 611 in LC/MS, which fragmented to ions at *m/z* 465 (loss of a deoxyhexose) and 303 (loss of a hexose-deoxyhexose) in MS/MS. The molecular ion at *m/z* 465 and the fragmentation at *m/z* 303 for peak 17 indicated the presence quercetin hexoside. Peak 18 showed a positive molecular ion at *m/z* 595, which fragmented to ions at *m/z* 449 (loss of a deoxyhexose) and 287 (loss of a hexose-deoxyhexose), suggesting the presence of kaempferol hexose-deoxyhexoside. The identification of peak 19 as kaempferol hexoside was based on the molecular ion at *m/z* 449 and the fragmentation at *m/z* 287 (loss of hexose).

9.3.2. Quantification of the Catechins by LC/MS Method.

To generate more molecular ions and prevent further fragmentation, different collision energy levels were evaluated, and an optimal energy level of 20% was selected (Wu, Wang & Simon, 2003). Under SIM mode, protonated molecular ions $[M+H]^+$ were isolated for individual compound catechin and epicatechin at *m/z* 291, epigallocatechin-3-*O*-gallate at *m/z* 459, and epicatechin-3-*O*-gallate at *m/z* 443. Glycitein, a type of flavone, was used as internal standard in the analytes. The selective-ion chromatograms of catechins were successfully performed within 50 min

(**Fig. 9.5**). Lower limits of quantification for catechin, epicatechin, epicatechin-3-*O*-gallate and epigallocatechin-3-*O*-gallate were established by analyzing a set of working solutions across 10 different concentrations. The linearity of the calibration curves was found to be 0.32-41μg/mL for catechin, 0.34-43.8μg/mL for epicatechin, 2.55-81.6μg/mL for epicatechin-3-*O*-gallate, and 1.88-60μg/mL for epigallocatechin-3-*O*-gallate. On the basis of the calibration equations, the contents of catechin and epicatechin in catechu heartwood as well as resin chunks were quantitated, and the concentrations of catechin, epicatechin, epicatechin-3-*O*-gallate and epigallocatechin-3-*O*-gallate in catechu leaves were calculated (**Table 9.2**).

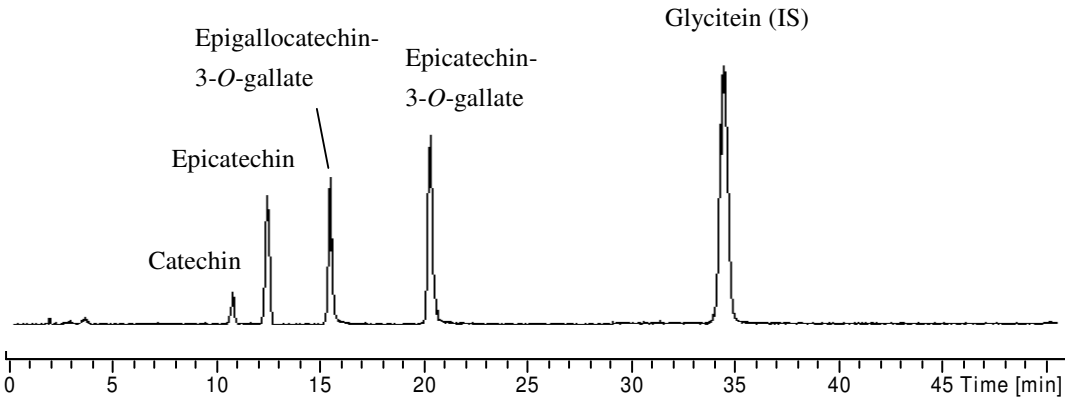


Figure 9.5. Representative selective-ion monitoring (SIM) chromatogram from catechu leaf extract set with four time segments: 0-13min at *m/z* 291 (catechin and epicatechin), 13-18.5min at *m/z* 459 (epigallocatechin-3-*O*-gallate), 18.5-30min at *m/z* 443 (epicatechin-3-*O*-gallate) and 30-50min at *m/z* 285 (glycitein).

Table 9.2. Quantification of the Predominant Catechins in Catechu and Catechu Products (mg/g)

	catechin[a]	epicatechin[a]	epigallocatechin-3-*O*-gallate[a]	epicatechin-3-*O*-gallate[a]
Catechu heartwood	20.00±0.32	4.63±0.12	ND[b]	ND[b]
Catechu resin chunks	74.76±1.07	14.14±0.42	ND[b]	ND[b]
Catechu leaves	1.07±0.05	4.09±0.22	9.10±0.47	20.45±0.58

[a]Mean value ± SD in triplicate. [b]Not detectable.

9.3.3. Validation of LC/MS Method with Selected-ion Chromatogram.

The precision (RSD) and accuracy (deviation from nominal concentration) were assessed by analyzing the quality control samples, catechin, epicatechin, epicatechin-3-*O*-gallate and epigallocatechin-3-*O*-gallate, by LLQ (lower limit of quantification), LQC (low quality control), MQC (medium quality control), and HQC (high quality control) (n = 6). Results showed an accuracy ranging from 1.06% to 11.76%, and the precision (RSD) varying between 1.60% and 9.36% for these four analytes (**Table 9.3**). The recovery test was validated by spiking the catechu leaf sample with known concentration of epicatechin and epicatechin-3-*O*-gallate, and the resulting combination was subjected to the entire extraction and analytical sequence. The recovery rates were calculated as 95.2% and 97.0% for epicatechin and epicatechin-3-*O*-gallate, respectively. These

validation studies showed the recommended method is reliable and sensitive for the quantification of catechins in *Acacia catechu*.

Table 9.3. Accuracy and Precision[a]

		analyte			
		C	EC	EGCG	ECG
LLQ (µg/ml)	nominal concn	0.32	0.34	1.88	2.55
	mean concn	0.30	0.37	1.90	2.25
	accuracy (%)	-6.25	8.82	1.06	-11.76
	precision (%)	9.26	9.36	9.32	8.41
LQC (µg/ml)	nominal concn	0.78	0.72	3.63	4.38
	mean concn	0.75	0.79	3.90	4.23
	accuracy (%)	-3.85	9.72	7.44	-3.36
	precision (%)	4.48	6.30	5.63	5.35
MQC (µg/ml)	nominal concn	4.67	4.33	14.5	17.5
	mean concn	4.49	4.58	13.87	16.68
	accuracy (%)	-3.85	5.77	-4.34	-4.68
	precision (%)	3.00	5.74	6.71	6.12
HQC (µg/ml)	nominal concn	28	26	58	70
	mean concn	27.56	24.47	55.12	67.95
	accuracy (%)	-1.57	-5.88	-4.97	-2.93
	precision (%)	1.68	4.98	1.60	5.13

[a]n = 6.

9.3.4. Distribution of Catechins and Other Flavonols in *Acacia catechu*.

Catechu was found to be a rich source for catechins which accumulate both in the heartwood and leaves. Catechin and epicatechin are the dominant secondary metabolites in catechu heartwood with 2.46% of the weight of dry wood and 8.89% of the resin chunks (heartwood extract), thus exhibiting both a simple and high concentration catechins profile. In contrast, catechin, epicatechin, epicatechin-3-*O*-gallate and epigallocatechin-3-*O*-gallate were the major flavan-3-ol components found in catechu leaves. Compared to catechu heartwood, gallated catechins are the major form according to catechins concentrations in leaves. Some low levels of flavanol dimers are present in catechu wood, such as procyanidin dimers and chalcan-flavan dimmers, whereas few dimers were found in leaves. We observed a similar chemical profile in catechu heartwood to that of catechu resin chunks, indicating that the qualitative composition can be characteristic of catechu products. In addition to catechins, the presence of several flavonol glycosides was also found in both catechu wood and leaves.

9.3.5. Comparison of Catechu Leaves and Green Tea Leaves.

Green tea is one of the plants which are well studied and recognized as an abundant source of catechins. Total catechins content in green tea ranges from 9 mg/g to 117 mg/g of dry weight of green tea leaves, varietal, environmental and processing dependent (Dalluge & Nelson, 2000). This is within the same range we now report for the catechins

in catechu leaves. The chemical profile of the catechins in catechu leaves and green tea were compared using the same method as described, indicating many similarities between both plant species relative to catechins accumulation. Seven catechins were found in green tea including epigallocatechin-3-*O*-gallate, epigallocatechin, epicatechin-3-*O*-gallate, epicatechin, catechin, gallocatechin and gallocatechin-3-*O*-gallate, of which the last three catechins were in trace amounts. In comparison, the catechu leaves contain five catechins, epicatechin-3-*O*-gallate, epigallocatechin-3-*O*-gallate, epicatechin, catechin and a trace amount of epigallocatechin (**Table 9.4**). Both species accumulated high concentration of gallated catechins. Epigallocatechin-3-*O*-gallate and epicatechin-3-*O*-gallate in green tea were quantified as 19.20±0.75 mg/g and 8.87±0.19 mg/g, respectively. Epigallocatechin-3-*O*-gallate is the highest catechins form in green tea, whereas epicatechin-3-*O*-gallate (20.45±0.58 mg/g) was the highest in catechu leaves (**Fig. 9.6**). In addition to the catechins, some flavonols, such as quercetin glycosides and kaempferol glycosides were also present in both green tea and catechu leaves. These findings show that catechu may have potential as an alternative source of natural catechins.

Table 9.4. The Presence of Catechins and other Flavonols in Catechu and Green Tea

compound	sample			
	catechu heartwood	catechu resin chunks	catechu leaves	green tea leaves
GC	-	-	-	T
EGC	-	-	T	+
C	+	+	+	T
EC	+	+	+	+
EGCG	-	-	+	+
GCG	-	-	-	T
ECG	-	-	+	+
Procyanidin dimers	+	+	-	-
Chalcan-flavan dimers	+	+	-	-
Quercetin glycosides	T	T	+	+
Kaempferol glycosides	-	-	+	T

(+) Present; (-) not detectable; T: trace amount.

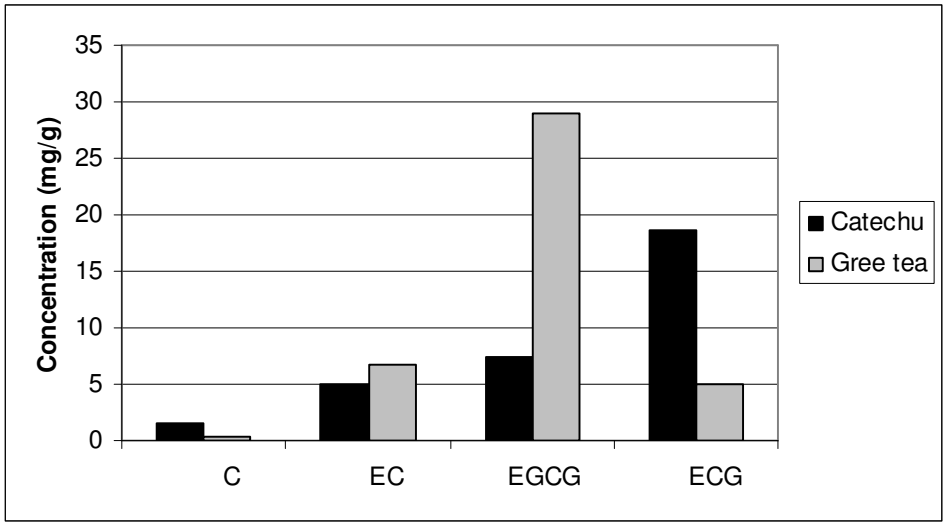


Figure 9.6. Comparison of levels of catechins in catechu and those in green tea.

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CHAPTER 10. CONCLUSIONS

Oregano is a well recognized and highly valued food seasoning and spice in western and Middle Eastern cuisine. Oregano is a rich source of aromatic volatile compounds extracted as essential oils which enter commerce for a variety of applications such as aromas, flavorings and more recently for their purported biological activities. This research focused on the non-volatile polyphenol bioactive components which may contribute to the health and functional attributes of the plant. In part, this research which first began in 2001, was based upon the hypothesis that bioactive compounds from oregano and other members of the Lamiacae family were not exclusively due to the better known and recognized volatile oils, but also due to the presence of bioactive polyphenols. Secondly, the spent biomass following essential oil distillation of oregano has long been viewed as waste products used as soil amendments, animal feed or in some regions even dried and later used as fuel to fire and charge the next distillation. We hypothesized that this spent material could be a source of nonwater soluble polyphenols, while the water remaining in the alembic could be a serve of water soluble polyphenols. Meanwhile, the development of anti-inflammatory natural dietary supplements from food sources was gaining increased interest, as safety is always a prime consideration, drugs intended for chronic disease treatment through oral delivery require greater and longer-term testing. This increased interest stimulated the search for natural plant-based anti-inflammatory "phytopharmaceuticals" to be used in the form of dietary supplements. Consumers perceive that such products will be lower cost and safe. Those in the private sector also see opportunity in such products as they would be easier to introduce into this market

than the traditional pharmaceutical marketplace.

We successfully identified the anti-inflammatory constituents in oregano as rosmarinic acid, oleanolic acid and ursolic acid by bioactivity-guided isolation, LC/MS and NMR techniques. The presence of rosmarinic acid is well recognized in oregano. Our research confirmed its presence and shows the importance of this compound as an anti-inflammatory agent in oregano. Our studies were the first to identify oleanolic acid and ursolic acid in oregano, though both acids have been reported in other plant species. The three organic acids identified in oregano were tested on the LPS-induced nitrite production assay and the Western Blotting of LPS-induced iNOS and COX-2 protein levels in murine cells. Each of these constituents exhibited stronger or comparable anti-inflammatory activities compared to the control indomethacin, a recognized anti-inflammatory agent. Subsequently, we found the combination of the three compounds, and even just two of these constituents in a particular ratio appeared syngergistic resulting in a higher level of potency on nitrite production assay alone after adjusting for concentration. The inhibition of these compounds through other inflammatory cell signaling pathways such as the 5-lipoxygenase inhibitory activities of rosmarinic acid and oleanolic acid was observed on our lipoxygenase screening assay as well.

We next reviewed the pharmacokinetic features, bioavailability and safety issues for these

bioactive ingredients. Given these positive pharmacokinetic and safety evidence reported in the literature for each of the three phenolic acids, we concluded it would be suitable to develop a dietary supplements product as a potential complimentary and alternative phytomedicine from this plant. We then conceived a process of extracting the bioactive water soluble compounds while still recovering the essential oil using steam or hydro-distillation material of oregano.

HPLC quantitation of organic acids is usually difficult due to the retention time shift, peak broadening, and inability to achieve adequate resolution on the chromatogram. The challenge of co-quantitation of the anti-inflammatory constituents in oregano varieties resides on the chemical similarity of oleanolic acid and ursolic acid, and the drastic difference of polarity between rosmarinic acid and the two triterpenoid acids. No HPLC method has yet been reported to co-quantitate these three organic acids. We successfully achieved a LC/MS quantitation of rosmarinic acid with oleanolic acid and ursolic acid by employing a tandem column system of two different stationary phases: first a Synergi Polar-RP column and downstream a Microsorb ODS column. The detection of rosmarinic acid was optimal under negative ion mode of SIM, while oleanolic acid and ursolic acid were sensitive to positive ion mode. The simultaneous quantitation was obtained by setting two time segments in one run to facilitate the ESI polarity switch. For the investigated analytes rosmarinic acid, oleanolic acid and ursolic acid, good linearities (r^2 > 0.999) were obtained for each calibration curve. Validation for this method showed a

precision (relative standard deviation) ranging from 4.84% to 6.41%, and the recoveries varied from 92.2% to 100.8% for the three analytes.

A quantitative survey of these anti-inflammatory constituents in different oregano varieties was performed, showing oregano as a significantly rich source of rosmarinic acid, oleanolic acid and ursolic acid. Rosmarinic acid was the predominant compound in the varieties of *O. vulgare* ssp. *hirtum* and *O. vulgare*, ranging from 13.73 mg/g to 63.69 mg/g on leaf dry weight. The average levels of oleanolic acid and ursolic acid in these two species were 1.96 mg/g and 6.72 mg/g, as calculated from 22 different sources and varieties. The varieties of *O. syriacum,* known in the Middle East as 'Zatar' showed a distinctly high content level of triterpenoid acids, with oleanolic acid averaging 9.40 mg/g in seven different sources and ursolic acid averaging 24.07 mg/g. Furthermore, we investigated the anti-inflammatory contents in different breeding lines from a Greek oregano variety GO1 (*O. vulgare* spp. *hirtum*). These breeding lines all accumulated high concentration of rosmarinic acid, with RSD = 28%. The RSDs for oleanolic acid and ursolic acid were both around 20%. We also found that although oleanolic acid and ursolic acid content levels may vary among different lines, the ratios of oleanolic acid to ursolic acid appeared to be a constant with little variation. These findings led us to conclude that there is major genetic variation in oregano relative to the accumulation of these compounds. We also identified a stable rich source of oregano that exhibited potent anti-inflammatory activity and from which an improved variety rich in anti-inflammatory

contents could be developed.

The discovery of the compounds in oregano that were responsible largely for the plants anti-inflammatory action, and the research that showed this species to be a rich source of rosmarinic acid, oleanolic acid and ursolic acid inspired us to look for additional sources of these natural anti-inflammatory agents within the same family of Lamiaceae. Following a detail review of the other plant candidates, we selected mint (*Mentha* spp.), which we showed also contain these three anti-inflammatory organic acids identified by LC/MS.

Mint is a significant agricultural crop grown primarily for its essential oils produced from the leaves, and the non-volatile components are generally viewed as waste products. Thirty-five *Mentha* spp. and varieties, including all the commercial species used in the production of essential oil distillation as well as many specialty varieties from around the world were collected, vegetatively propagated, and both grown in our greenhouses for authentication and field transplanted into the Rutgers Agricultural Experiment Station research farms. We developed and validated an LC/MS (MRM mode) method to co-quantitate these three organic acids. An analytical survey with our mint germplasm was then performed to quantitate these three organic acids in the different mint varieties. Results showed several mint varieties were rich sources of these anti-inflammatory acids, including spearmint (*Mentha spicata*), Persian mint (*M.* × *piperita*), chewing gum mint

(*M.* × *piperita*), orange mint (*M.* × *piperita*), apple mint (*M.* × *villosa*), Egyptian mint (*M.* × *villosa*), fuzzy spearmint (*M. spicata*), grapefruit mint (*M.* × *piperita*), green curly mint (*M.* × *piperita*), sweet mint (*M. aquatica* × *M. suaveolens*), regular mint (*M. spicata*) and Scotch spearmint (*M.* × *gracilis*). Additionally, we identified seven major phenolic components (flavonoids) from the non-volatile components of these mint varieties by using LC/UV/MS and NMR techniques. The LC/UV method was also validated and shown to be accurate, precise and sensitive for the quantitation of the seven predominant flavonoids (eriocitrin, luteolin 7-*O*-rutinoside, luteolin 7-*O*-glucoside, narirutin, isorhoifolin, hesperidin and diosmin).

To facilitate the frequent quality control requirement and material pre-evaluation, a NIR technique was developed for the fast quantitation of anti-inflammatory constituents in oregano and mint. Based on the quantification data obtained from LC/MS analysis, we collaborated with scientists in Analytical Spectral Devices, Inc. (Boulder, CO) and successfully developed a NIR method for the determination of rosmarinic acid, oleanolic acid and ursolic acid in both oregano and mint. Full Cross validation and True Test Set validations were used to evaluate the calibration models. Samples were randomly assigned to either calibration or validation sets for creation of the true test set, 50 samples were used for the calibration set and 16 for the validation set. Savitzky-Golay 1^{ST} derivative with 11 point second order smoothing was used as a pretreatment in PLS1 models. Results showed that NIR was feasible to quantitate the anti-inflammatory acids

in oregano and mint. Full Cross validation and True Test Set results confirmed the predictive ability for combing these three sample types into a single calibration, with r = 0.89 for rosmarinic acid, 0.94 for oleanolic acid and 0.95 for ursolic acid (Full cross); and r = 0.94 for rosmarinic acid, 0.87 for oleanolic acid and 0.90 for ursolic acid (True test set).

In addition to the dietary supplement development, we also developed HPLC methods for other bioactive phenolic compounds. The applied study, an analytical survey of isoflavones in Tofu-type soybeans cultivated in North America, was part of a larger program entitled “Human Food Soybean Systems” which seeks to provide information for farmers who are changing from conventional animal feed agronomic production to food grade production for Tofu soybean market. Tofu-type soybeans may vary from conventional soy varieties in terms of seed size, protein content and plant growth. At present, the concentration of nutritional components such as protein and oil content in soybeans are major considerations for growers in switching from traditional soybean varieties used for animal feed to human food. This study was conducted to begin to analyze the isoflavone content so that this factor may also be considered a component of quality in soybeans. On the basis of HPLC coupled with ESI/MS detection, total 20 isoflavones, including some trace amount constituents which were not detectable in whole soybean seed extract, were detected and identified by dissecting the whole seeds to seed coats, cotyledons and hypocotyls. Differences in isoflavone profiles by seed part

were also compared by qualitative analysis with LC/MS. A simple and low-cost method using HCl hydrolysis during sample extraction and LC/UV as a detection was developed to quantitate total isoflavones in soybeans, which was validated by recovery test and applied to further determine the total isoflavone contents of different Tofu-type soy varieties grown in several field locations over two growing seasons, as well as some conventional soy varieties as a comparison. The information obtained will be valuable for the evaluation of the potential of dietary soybean, distribution of isoflavones in soy seeds, as well as the effect of variety, location, and sowing date on the isoflavone contents. One of our goals was to first show the importance of including nutritional/nutraceutical applications screening in any varietal development and new crop introduction as well as to provide a platform to learn new analytical techniques.

In the investigation of catechins from traditional green tea, we sought to identify new plant sources of the same catechins from other botanicals. After searching, we found a leguminous tree, catechu (*Acacia catechu*), native to Southern Asia and widely distributed in India and Burma, was a rich source of catechins. High-performance liquid chromatography coupled with electrospray ionization mass spectrometry (LC/ESI-MS) method under selected-ion monitoring mode (SIM) was used to develop a method to quantitate the predominant catechins, (+)-catechin (C), (-)-epicatechin (EC), (-)-epicatechin-3-O-gallate (ECG) and (-)-epigallocatechin-3-O-gallate (EGCG) in this medicinal plant catechu (*Acacia catechu*). Other major secondary products including

caffeine, flavanol dimers and flavonol glycosides were also identified by their molecular ion peaks and fragmentation peaks using LC/MS and LC/MS/MS. For the investigated ion concentration ranges of C, EC, ECG, and EGCG, good linearities ($r^2 > 0.99$) were obtained for each calibration curve. Validation for this method showed an accuracy ranging from 1.06% to 11.76%, and the precision (RSD) varying between 1.60% and 9.36% for these four analytes. This is the first study to describe the quantitative determination of all predominant catechins in catechu heartwood and leaves and shows this plant to be a rich source of the 'green tea catechins' but which are found in different ratios than in green tea.

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PUBLICATIONS

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