CITRUS LIMONOIDS AND FLAVONOIDS: EXTRACTION, ANTIOXIDANT ACTIVITY AND EFFECTS ON HAMSTER PLASMA CHOLESTEROL DISTRIBUTION

A Dissertation

by

JUN YU

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2004

Major Subject: Horticulture

CITRUS LIMONOIDS AND FLAVONOIDS: EXTRACTION, ANTIOXIDANT ACTIVITY AND EFFECTS ON HAMSTER PLASMA CHOLESTEROL DISTRIBUTION

A Dissertation

by

JUN YU

Submitted to Texas A&M University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Approved as to style and content by:

Bhimanagouda S. Patil (Co-Chair of Committee)	Leonard M. Pike (Co-Chair of Committee)
Rosemary L. Walzem (Member)	Kevin Crosby (Member)
Edward G. Miller (Member)	Tim D. Davis (Head of Department)

August 2004

Major Subject: Horticulture

ABSTRACT

Citrus Limonoids and Flavonoids: Extraction, Antioxidant Activity and Effects on Hamster Plasma Cholesterol Distribution. (August 2004)

Jun Yu, B.S., Shangdong Institute of Building Materials;

M.S., Wuxi University of Light Industry

Co-Chairs of Advisory Committee: Dr. Bhimanagouda S. Patil
Dr. Leonard M. Pike

Four *in vitro* models were used to measure the antioxidant activity of 11 citrus phytochemicals. The citrus limonoids and bergapten showed very weak antioxidant activity. The flavonoids demonstrated mild, to moderate, to strong antioxidant activity. In addition to some other commonly accepted structural features our data indicated that the hydroxyl group in position 6 of ring A could also increase the antioxidant activity of flavonoids. Compared with the active flavonoids, limonoids are highly oxygenated triterpenoids, with fewer hydroxyl groups to stabilize unpaired electrons (or scavenge free radicals). Bergapten lacks a hydroxyl group. This is the first report on the antioxidant activity of limonoids and neoeriocitrin.

A feeding study using Syrian hamsters was followed to determine the effect of citrus limonoids and flavonoids on plasma cholesterol. Hamsters fed with limonin, limonin 17-β-D-glucopyranoside and grapefruit pulp significantly inhibited the increase of LDL/HDL-cholesterol (36.6%, 52.9% and 57% respectively) compared with the basal control (65.8%) and the pectin control (70%). Furthermore, hamsters fed with limonin had significantly larger LDL particle size (21.21 nm) compared with the control group

(19.96 nm). Further studies demonstrated that LDLs from hamsters fed with limonin and limonin 17-β-D-glucopyranoside were less susceptible to oxidation. These data suggest that limonin, limonin 17-β-D-glucopyranoside and grapefruit pulp have potential inhibitory effects against atherogenesis.

Supercritical CO_2 (SC-CO_2) was attempted to extract limonoids from grapefruit seeds and molasses. Limonin aglycone was successfully extracted with SC-CO_2 directly from grapefruit seeds with the yield of 6.3mg/g seeds at 48.3 MPa, 50˚C and 60 min with CO_2 top feeding; and the limonin glucoside was extracted using SC-CO_2 and ethanol as co-solvent from the defatted seeds with the yield of 0.73 mg/g seeds at 42 MPa, 52˚C, 45% ethanol (X_{Eth}=0.45) and 40 min with CO_2 top feeding; and limonin glucoside also was extracted using SC-CO_2 and ethanol with the yield of 0.61mg/g grapefruit molasses at 48.3 MPa, 50˚C and 10% ethanol (X_{Eth}=0.1), 40 min with CO_2 top feeding. CO_2 flow rate was around~5 l/min in experiments. The results demonstrated SC-CO_2 extraction of limonoids from citrus juice industry byproducts has practical significance for future commercial production.

DEDICATION

This dissertation is dedicated to my beloved father Bulou Yu for being the light and inspiration of my life, my mother Guangde Peng for her patient and spiritual instruction and my wife Shuyu Zhao for her silent support.

ACKNOWLEDGMENTS

I would like to express my deepest gratitude to my committee co-chairmen, Dr. Bhimanagouda S. Patil and Dr. Leonard M. Pike, for their advice, friendship and patience during completion of this dissertation. I would like also to express my sincere gratitude to committee members: Dr. Rosemary L. Walzem, Dr. Edward G. Miller and Dr. Kevin Crosby for their thoughtful comments and support throughout this study. I wish to express my special appreciation to Dr. Romeo Toledo and Dr. Rakesh Singh in the Department of Food Science and Technology, University of Georgia for their guidance and strong support to finish the supercritical extraction work, Dr. Yaowen Huang and Dr. Lin Ye for their kind HPLC technical support. All of the other technicians and graduate students in Dr. Toledo's lab also deserve my deep appreciation for their constant help and cooperation.

My immense gratitude also goes to all post docs, graduate students, technicians and staff member at the Citrus Center in Weslaco, Vegetable and Fruit Improvement Center in College Station and Dr. Walzem's lab. I would like to especially thank Dr. Qingguo Tian, Dr. Girijia Raman, Dr. Limin Wang, Dr. Kim Sungil, Jiaxing Li, Sheunyi Chen, Kendall Hood, Jairam Vandamala, and Shibu Poulose.

Finally, I wish to thank all my friends who have been always friendly and cooperative during my studies.

TABLE OF CONTENTS

Page

CHAPTER Page

LIST OF TABLES

LIST OF FIGURES

FIGURE Page

CHAPTER I

INTRODUCTION

Introduction to Supercritical Fluids

The observation of the occurrence of a supercritical phase was first reported by Baron Cagniard de la Tour (1822), where he noted visually that the gas-liquid boundary disappeared when the temperature of certain materials was increased by heating each of them in a closed container. Reports followed by Hannay and Hogarth (1879) who first demonstrated the solvating power of supercritical fluids for solids. They studied the solubilities of cobalt (II) chloride, iron (III) chloride, potassium bromide, and potassium iodide in supercritical ethanol (T_c=243˚C, P_c=63 atm) and found that the concentrations of the metal chlorides in supercritical ethanol were much higher than their vapor pressures alone would predict. They also found that increasing the pressure caused the solutes to dissolve and decreasing the pressure caused the dissolved materials to precipitate as “the snow”. Later, Buchner (1906) reported that the solubities of certain nonvolatile organic materials in CO_2 under supercritical conditions were orders of magnitude higher than expected based on vapor pressure considerations alone.

Extraction and separation of mixtures with supercritical fluids (SFs) was neglected during the first half of the twentieth century though the dissolving capacity of a supercritical fluid was known. The interest in SFs during this time focused on

---

This dissertation follows the style of Journal of the American Society for Horticultural Science.

process operations and not analytical chemistry. Zosel's patent (1964) promoted significant development of supercritical fluid extraction (SFE). This patent reported the decaffeination of green coffee with supercritical CO_2, where the beans were first soaked in water and then immersed in supercritical CO_2 (Zosel, 1971).

Since 1980, SFE has been rapidly developed for the extraction of hops (Laws et al., 1980), cholesterol from butter (Krukonis, 1988), perfumes and flavors from natural products (Coenan, 1983), residual solvents, monomers from polymers (Krukonis, 1985) and unsaturated fatty acids from fish oils (Krukonis, 1988), vegetable oils from plants and seeds and phytochemcials from vegetables, fruits and other plant sources (Rozzi et al., 2002; Yu and Qiu, 2000). Currently, the use of SFs in the following applications is under study: remediation of soil, demilitarization of propellants, chemical reaction and synthesis of polymers and organic chemicals, impregnation and in situ deposition of chemicals, removal of nicotine from tobacco, nucleation and particle size regulation and cleaning of electronic parts.

Supercritical fluid technology has been an interdisciplinary field utilized by chemical engineers, chemists, food scientists, materials scientists, agronomists, and scientists in biotechnology and environmental control. Presently, in addition to the application for the purpose of new product isolation and purification, considerable effort has been devoted to gaining a better fundamental understanding of molecular structure, phase behavior and co-solvent effects, solvation processes between solute and fluid phase and transport properties of supercritical fluids.

Properties of Supercritical Fluids

A phase diagram, as shown in Figure 1-1, can describe the physical stage of a substance of fixed composition. In this pressure-temperature diagram, there are three lines describing the sublimation, melting and boiling processes. These lines also define the regions corresponding to the gas, liquid and solid states. Points along the lines (between the phases) define the equilibrium between two of the phases. The vapor pressure (boiling) starts at the triple point and ends at the critical point (CP). The critical region has its origin at its critical point. So at this point, we can define SF as any substance that is above its critical temperature (T_c) and critical pressure (P_c). The critical temperature is therefore the highest temperature at which a gas can be converted to a liquid by an increase in pressure. The critical pressure is the highest pressure at which a liquid can be converted to a gas by an increase in the liquid temperature. Therefore, in the critical region, there is only one phase and it possesses some of the properties of both a gas and liquid such as higher diffusivities, lower viscosities, near zero surface tension and high solvating power.

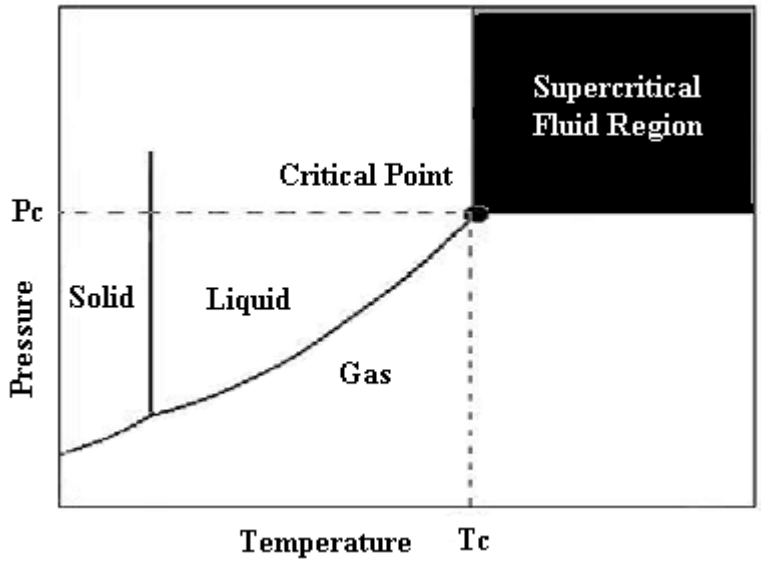


Figure 1-1. Phase (pressure-temperature) diagram for one single substance.

Subcritical fluid is found in the triangular region formed by the melting curve, the boiling curve and the line that define the critical pressure (Brogle, 1982). Among all the SFs, supercritical CO_2 has been the most thoroughly investigated and applied due to its lower critical temperature and pressure (31.1˚C and 7.38 MPa), lack of toxicity and combustibility, environmental compatibility and ready, commercial availability of highly pure material (Rozzi and Singh, 2002). Commonly used supercritical fluids with their critical point parameters are listed in Table 1-1.

The solvating power of SF is highly dependent on its temperature and pressure that determine the density of SF, i.e., the density of SF in the final form influences its solvating power. Additionally, solid sublimation also influences the solvating power of SF. Generally, the solvating power of a SF increases with density at a given temperature; however, the solvating power of a SF also increases with temperature at a given density. However, during operation, there are some other parameters influencing on the extraction efficiency such as extraction time, SF flow rate, co-solvent, SF feeding mode (the direction of SF applied to the sample to be extracted, for examples, top feeding and bottom feeding) and so on.

Table 1-1. Commonly used supercritical fluids with their critical point parameters.

Fluid	Critical Temperature (K)	Critical Pressure (bar)
Carbon dioxide	304.1	73.8
Ethane	305.4	48.8
Ethylene	282.4	50.4
Propane	369.8	42.5
Propylene	364.9	46.0
Trifluoromethane (Fluoroform)	299.3	48.6
Chlorotrifluoromethane	302.0	38.7
Trichlorofluoromethane	471.2	44.1
Ammonia	405.5	113.5
Water	647.3	221.2
Cyclohexane	553.5	40.7
n-Pentane	469.7	33.7
Toluene	591.8	41.0

Source: http://sunny.vemt.bme.hu/sfe/angol/supercritical.html

Supercritical Carbon Dioxide as a Solvent for Extraction

SC-CO_2 has been widely used in the food industry due to the advantages of this SF mentioned in last section. In an electronic search of literature indexed within the Food Science and Technology Abstracts between the years 1990 and 2003 using "supercritical fluids" and "carbon dioxide", returned a total number of 48 papers, of which about 52% focused on extraction, 31% on analysis and 17% on processing. Presently, SC-CO_2 has been used to extract a wide variety of analytes from botanical and other food samples. There were reports on SC-CO_2 extraction in food systems as (1) extraction of fats and oils from fish (Zosel, 1978), rice bran (Ramsay et al., 1991), egg yolk (Froning et al., 1990) and other food materials; (2) cholesterol extraction from beef (Chao et al., 1991), milk fat (Fujimoto et al., 1987), egg yolk (Froning et al., 1990); (3) fractionation of fats and oils from rice bran oil (Saito et al., 1991), canola oil (Temelli, 1992) and others; (4) refining of fats and oils with uses such as deodorization of soybean, paml kernel and peanut oils (Ziegler and Liaw, 1993; Zosel, 1979), deacidification of olive oil (Brunetti et al., 1989) and, extraction of tocopherols from soybean sludge (Lee et al., 1991); (5) flavour/aroma extraction from Hops (Gardner, 1993), onion (Moyler, 1993), lactones from milk fat (Rizvi et al. 1993); (6) miscellaneous extractions such as an essential oil from black pepper (Ferreira et al., 1999; Tipsrisukond et al., 1998), limonene and terpines from grapefruit (Poiana et al., 1998), carotenes from carrots (Chandra and Nair, 1997), polyphenolic compounds from grape seeds (Palma et al., 2000), and lycopene from tomatoes byproducts-skins and seeds (Rozzi et al., 2002).

Introduction to Citrus Limonoids

Limonoids are a group of highly oxygenated triterpenoids present mainly in the Rutaceae and Meliaceae families. Furthermore, citrus limonoids are a group of secondary metabolites that have not been found to have any direct function in plant growth and development, unlike primary metabolites such as amino acids and nucleotides, which have recognized roles in the processes of assimilation, respiration, transport and differentiation.

Naturally occurring limonoids as triterpenoids, share some characteristic structural features such as a furan ring attached to the D-ring at C-17, oxygen containing functional groups at C-3, C-4, C-16 and C-17, a 14,15-epoxide group (except in deoxylimonin), and a methyl or oxymethylene at C-19 (Maier et al., 1977). However, there is no general figure for the molecular weight of limonoids because of their diverse chemical structures. Limonin was the first isolated from navel orange juice in 1938 (Higby); and 20 years later, its structure was first determined (Arigoni et al., 1960). The chemical composition of limonin is $C_{26}H_{30}O_8$ with a molecular weight of 470.

Limonoids naturally occur in two chemical forms in citrus: limonoid aglycones and limonoid glucosides (Hasegawa et al., 1989; Maier et al., 1977). Limonoid aglycones are further classified into limonoid monolactones and limonoid dilactones. The former has an open D-ring such as limonoate A-ring lactone, and the later has a closed D-ring such as limonin. Presently, thirty-six limonoid aglycones and 17 limonoid glucosides have been isolated from citrus and its closely related genera.

The biosynthetic pathways of these limonoid aglycones have been established (Hasegawa and Miyake, 1996). Emerson (1948) first isolated nomilin using a radioactive tracer technique, and limonin was later demonstrated to be the predominant limonoid synthesized and accumulated in seedlings of lemon, Valencia orange, grapefruit and tangerine (Hasegawa et al., 1984).

The phloem region of stem was found to be the major site of nomilin biosynthesis. Epicotyl, hypocotyl and root tissues were also capable of biosynthesizing nomilin, but leaves, fruits and seeds did not show this capacity (Hasegawa et al., 1986a).

In the form of nomilinoate A-ring lactone, nomilin is biosynthesized via terpenoid biosynthetic pathways from acetate and mevalonate, via farnesyl pyrophosphate (Ou et al., 1988). This precursor is translocated from the stem to other sites such as leaves, fruit tissues, peels and seeds (Hasegawa et al., 1986b), where it is further metabolized in each tissue to the other limonoids.

Four different pathways are involved in limonoid biosynthesis: the limonin pathway, the calamin pathway, the ichangensin pathway and the 7-acetate limonoid pathway (Hasegawa and Miyake, 1996). True citrus species only contain the limonin group of limonoids. Limonin, nomilin, obacunone and deacetylnomilin are the major limonoids found in this group.

Limonoid aglycones are converted in fruit tissues and seeds to non-bitter 17-β-D-glucopyranoside derivatives during the late stages of fruit growth and maturation, (Fong et al., 1991; Fong et al., 1992; Fong et al., 1993; Hasegawa et al., 1991; Herman et al., 1991). The limonoid glucosides are stable in the fruit tissues

(Herman et al., 1991): however, it is different in both dormant and germinating seeds. In this tissue, a β-glucosidase activity specific for limonoid glucosides has been found (Ronneberg et al., 1995). This enzyme catalyzes the hydrolysis of limonoid glucosides during the seed germination process.

The biosynthesis and biodegradation of limonoids in Citrus are enzyme-regulated and occur in specific tissues (Hasegawa and Miyake, 1996). Several groups of enzymes are involved in these biological pathways. Enzymes related to the production of nomilin have been found only in the phloem region of citrus stem tissues, while enzymes responsible for the conversion of nomilin to the other limonoid aglycones occur in all citrus tissues regardless of maturity. The enzyme limonoid D-ring lactone hydrolase that catalyzes the lactonization of the D-ring (from monolactones to dilactones) is found in the seeds of citrus (Maier et al., 1969). UDP-D-glucose: limonoid glucosyl transferase, which converts limonoid aglycones to their respective glucosides during fruit growth and maturation (Fong et al., 1992; Hasegawa et al., 1991), occurs in fruit tissues and seeds.

Introduction to Flavonoids

Flavonoids are naturally polyphenolic phytochemicals which occur in edible fruits, vegetables, nuts, seeds, grains, tea, and wine. In plants, they have the role of protection against ultraviolet radiation, pathogens, and herbivores. They have long been known for their health benefits even before their isolation and structure elucidation. For example, a diet rich in flavonoids has been shown to have an inverse relationship with heart disease (Hertog et al., 1993 & 1995).

Structurally, flavonoids are usually characterized by a C_6-C_3-C_6 carbon skeleton. There is a basic structure that is common to all flavonoids which consists of a chromane skeleton and a phenyl substituent (Figure 1-2). Even with this common denominator there is a great diversity of flavonoids due to different hydroxylation and glycosylation positions. Presently more than 4000 flavonoids have been identified, which are separated further into many classes such as anthocyanins, flavans, flavanones, flavones, flavonols, isoflavonoids and others. Flavonoids generally occur as aglycones and glucosides (with sugar moieties). Most flavonoids exist as glucosides in plant sources, and many only differ by the linkage position of hydroxyls and the sugars. The biological activity of flavonoids is affected by these subtle structural differences (Rice-Evans et al., 1998). Specifically targeted in this dissertation are the citrus flavonoids, which have been studied for nearly two centuries, since the first description of hesperidin by Lebreton in1828 (http://www.ibiblio.org/herbmed/eclectic/kings/citrus-aura_cortex.html). Citrus flavonoids occur in high concentrations in the tissue and peel of citrus fruits and flavonoids intake can be substantial among citrus consumers.

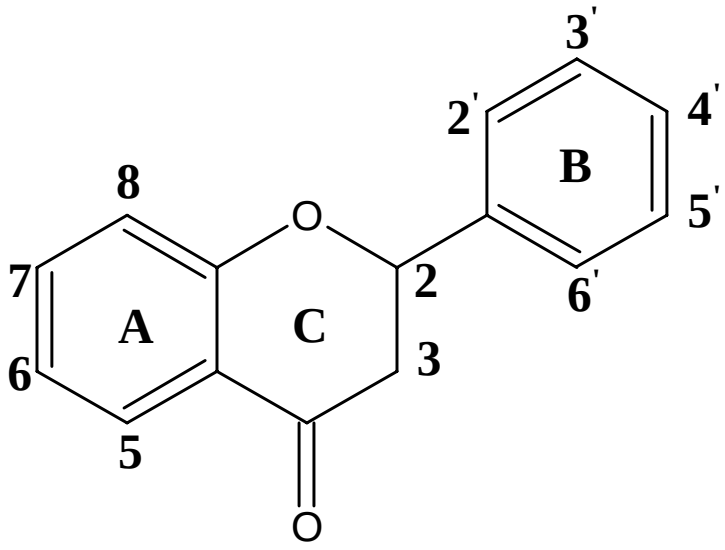


Figure 1-2. Basic ring structure of flavonoids with labeling.

Health Benefits of Citrus Limonoids and Flavonoids

Diets high in fruits and vegetables are associated with lower rates of a variety of diseases, particularly cardiovascular disease and certain types of cancer (Bors et al., 1990; Ness et al., 1997). Initially, it was assumed that this was due to the high fiber, low saturated fat, and cholesterol-free composition of citrus fruits (Kushi et al., 1995; Anderson et al., 1994). However, fruits and vegetables are also rich in vitamin E, C and a variety of bioactive compounds including limonoids and flavonoids (Cook and Samman, 1996; Hertog et al., 1992; Kurowska et al., 2000a), which are now hypothesized to be partly responsible for the prevention of coronary artery disease (Kinsella et al., 1993; Kurowska et al., 2000a). Citrus is one of the fruits that are consumed in large quantities in the United States. This factor increases the potential importance of the bioactive agents citrus contain.

Research with these compounds has shown that some limonoids could induce the detoxifying enzyme glutathione S-transferase in the liver of mice and rats (Lam et al., 1989). Citrus limonoids were also shown to inhibit the formation of chemically induced neoplasia in the oral cavity, forestomach, small intestine, colon, lung and skin of laboratory animals (Lam et al., 2000; Miller et al., 2000). Previous studies (Guthrie et al., 2001; Tian et al., 2001) have shown that limonoids can also inhibit the proliferation of breast cancer cells grown in culture. With the expanding research, more benefits of citrus limonoids to human health have been revealed. In one study with casein fed rabbits, it was found that the substitution of orange juice and grapefruit juice for drinking water significantly lowered cholesterol levels in the blood. Further work with HepG2 cells indicated that citrus limonoids (especially

limonin) were partly responsible for this effect on LDL cholesterol (Kurowska et al., 2000a). In humans, consumption of 750 ml of orange juice increased plasma HDL cholesterol concentrations (Kurowska et al., 2000b).

A number of *in vitro* studies suggest that flavonoids act as free-radical scavengers, modulate enzymatic activities, inhibit cellular proliferation, and possess antibiotic, anti-allergic, anti-diarrheal, anti-ulcer, and anti-inflammatory activities (Bravo, 1998). Flavonoids were also shown to scavenge peroxyl radicals, alkyl peroxyl radicals, superoxide hydroxyl radicals, and peroxynite in aqueous and organic environments (Chen et al., 1996; Duthie et al., 2000; Mira et al., 2002; Ng et al., 2000; Sanz et al., 1994). Furthermore, in the HepG2 cell culture experiments, flavonoids (especially naringenin) were also partly responsible for the lowering of LDL cholesterol (Kurowska et al., 2000a) and naringenin markedly decreased plasma cholesterol (Wilcox et al., 1998). Interestingly, several animal studies demonstrated both positive and negative effects in relation to cholesterol using grapefruit flavonones. Among the positive studies, naringenin and naringin significantly lowered the plasma total cholesterol and increased the HDL to total plasma cholesterol ratio in rats fed a high cholesterol diet (1%, w/w) (Lee et al., 2003; Seo et al., 2003). In another positive study, naringenin and naringin lowered the levels of plasma cholesterol (Choi et al., 2001) and hepatic cholesterol of male rats fed a high-cholesterol diet (Shin et al., 1999). In addition, several studies showed that naringenin and naringin had essentially no effect on plasma cholesterol levels. Lee and co-authors demonstrated that naringenin and naringin had no effects on plasma lipoprotein, total cholesterol, triglyceride, and high-density lipoprotein (HDL) in male

New Zealand White (NZW) rabbits (Lee et al., 2001). Furthermore, another report also indicated that naringenin could not attenuate aortic cholesterol accumulation in Watanabe heritable hyperlipidemic (WHHL) rabbits (Mortensen et al., 2001). However, very little information is available using limonoids or flavonoids in relation to distribution of plasma lipoprotein cholesterol in animals. In addition, very little is known about the antioxidant activity of citrus limonoids.

Supercritical Fluids Extraction of Citrus Limonoids

As the investigation into the bioactive functions of limonoids, the demand for purified limonoids has significantly increased (Braddock and Cadwallader, 1992). Unfortunately, limonoids are still unavailable commercially. In the citrus industry, peels, seeds and molasses are the three major by-products that contain many bioactive compounds. These source materials have been neglected for years. It is economical and practical for the citrus industry to exploit high value-added products, especially bioactive compounds such as limonoids and flavonoids. Several extraction methods for limonoids were reported from citrus seeds using enzymes (Miyake et al., 1991), and from molasses using resin (Ozaki et al., 1995; Tian et al., 2003). In addition, limonoids were also extracted from citrus juice and molasses using supercritical CO_2 (Miyake et al., 2000). An extraction method for flavonoids was reported from citrus peel (Bocco et al., 1998; Coll et al., 1998). Recently a supercritical fluid extraction (SFE) of limonoids and flavonoids was also investigated using citrus peel, juices and molasses (Giannuzzo et al., 2003; Miyake et al., 2000); however, extraction parameters were not determined. Even though several extraction methods used

organic solvents and resins to isolate limonoids and flavonoids, limitations such as, solvent residue, environmental pollution, flammability and low efficiency continue to limit their usefulness. In view of increasing environmental concerns about the use of organic solvents in the extraction of natural products, there has been growing interest in alternative extraction techniques using supercritical fluids (Modey et al., 1996). Supercritical CO_2 has several advantages for extraction of plant bioactive compounds as mentioned above (Rozzi and Singh, 2002). Therefore, this technique has been widely used in the food processing industry.

Objectives of Study

The objectives of this study are as follows:

1. To extract limonoids and flavonoids from grapefruit seeds and molasses using supercritical carbon dioxide techniques and to obtain optimal extraction parameters.

2. To investigate the antioxidant activity of citrus limonoids and flavonoids *in vitro*.

3. To investigate the effects of citrus limonoids and flavonoids on plasma lipoprotein cholesterol *in vivo*.

CHAPTER II

SUPERCRITICAL FLUID EXTRACTION OF LIMONOIDS FROM GRAPEFRUIT SEEDS

Introduction

Citrus Limonoids are highly oxygenated triterpenoids abundantly present in Rutaceae (citrus fruits) and Meliaceae (neem) family plants. Presently thirty six-limonoid aglycones and seventeen limonoid glucosides have been isolated from Citrus and its hybrids (Hasegawa and Maier, 1996). It is well known that limonoid aglycones (mainly limonin and nomilin) impart bitterness to citrus products. For this reason, their presence in citrus juices remains problematic for the citrus juice industry. Studies have been conducted to remove bitter components from citrus juice (Hasegawa et al., 2000).

Despite increasing demand for limonoids with documented and potential health benefits, lack of well defined and economical extraction and purification methods have precluded commercial development of this market (Braddock and Cadwallader, 1992). For this reason improved methods of limonoid extraction and purification will be of interest to the citrus industry as it seeks to capture its share of the dietary phytochemical/human health benefits (Battinelli et al., 2003). In view of increasing environmental and consumer concerns about the use of organic solvents in the extraction of natural products, there has been a growing interest in alternative extraction techniques such as SC-CO_2 (Modey et al., 1996). In comparison to solvent extraction, SC-CO_2 extraction methods offer the advantages of non-toxicity, non-

flammability, no residual chemical, low-moderate operating temperatures and pressures, and even low-cost (Rozzi and Singh, 2002). Supercritical CO_2 extraction is now widely used in the food processing industry. Supercritical fluid extraction applications in the food industry were recently reviewed (Rozzi and Singh, 2002; Sihvonen et al., 1999).

The amount of limonoid aglycones and glucosides in grapefruit seeds can be up to ~1% (Braddock and Charles, 2001), making this material a good source for limonoids. Previous reports indicated feasibility of extracting limonoids from citrus juice and its by-products: molasses, peels and seeds (Braddock and Charles, 2001; Matthews et al., 1990; Miyake et al., 1991; Miyake et al., 1996; Miyake et al., 2000; Ozaki et al., 1995; Tian et al., 2001). Most of the previous studies focused on organic solvents, resins and enzymatic methods. Although SC-CO_2 extraction was used, the optimum operating parameters to maximize the extraction yield were not determined (Miyake et al., 1996; Miyake et al., 2000).

To the best of our knowledge, very little information is available on extraction of limonoids from grapefruit (*citrus paradisi*) seeds using supercritical fluid techniques. This paper focused on SC-CO_2 extraction of limonoids from grapefruit seeds and the determination of the optimum operating parameters of supercritical fluid extraction (SFE) of these bioactive compounds.

Materials and Methods

Materials. Grapefruit (*citrus paradisi*) seeds were obtained from fruits harvested at the Texas A&M University-Kingsville Citrus Center, Weslaco, TX. All solvents were HPLC grade (Fisher Scientific, Atlanta, GA). High purity CO_2 was used for supercritical fluid extraction (SFE) and SFE schematics are illustrated in Figure 2-1.

SFE extracts were analyzed by High Performance Liquid Chromatography (HPLC-UV2000, Thermo Hypersil-Keystone Company, USA) equipped with a reverse C18 Waters Spherisorb ODS column (250 mm x 4.6 mm) and Gas Chromatography (GC, HP6890 series, autosampler, hydrogen flame-ionization detector, Hewlett-Packard Co., Avondale, PA) equipped with a capillary column (DB-23, 30 m×250 μm×0.25 μm; J&W Scientific Co., Folsom, CA).

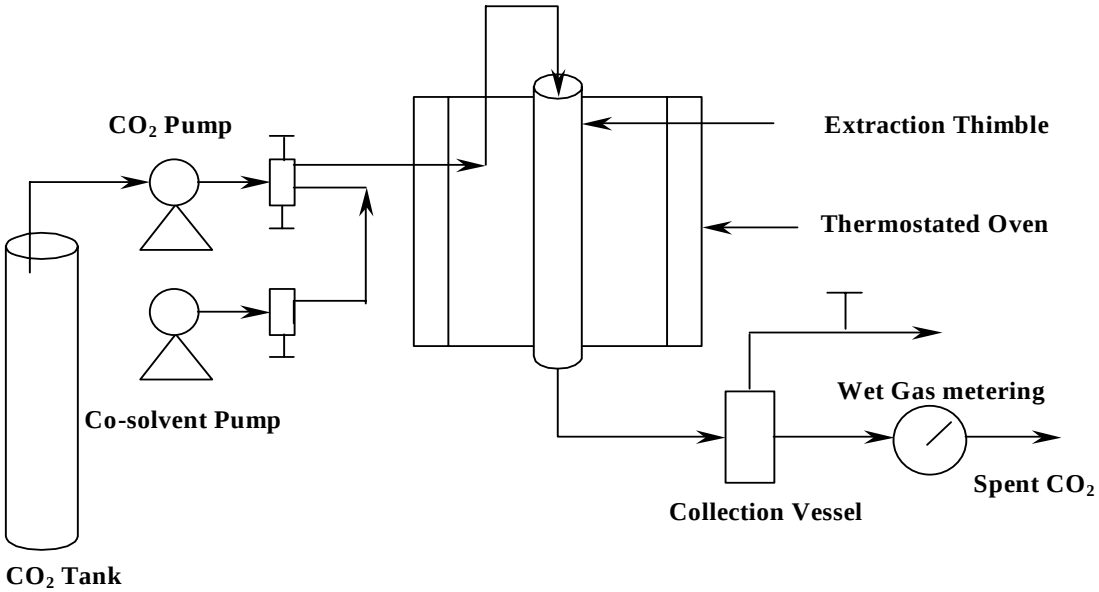


Figure 2-1. Schematics of the pilot scale supercritical fluid extraction unit used in this study.

Isolation of Limonoids. Limonin (Lim, >98% pure) and limonin 17-β-D-glucopyranoside (LG) (90% pure), from grapefruit seeds, were prepared and purified according to the procedures in our lab (Tian et al., 2001; Tian et al., 2003) as follows: 500 g of mature grapefruit seeds were collected and dried at 55˚C before being ground into powder with a Retch mill (Brinkmann, Westbury, NY). The powder was extracted (Soxhlet) overnight with hexane (solvent:seeds=3:1, extraction at 25˚C) to remove the oil. Solvent was changed to acetone and methanol sequentially to extract the treated seeds. The methanol fraction was evaporated to dryness in a rotary evaporator under vacuum (<60˚C), and the residue was partitioned with 1:1 methylene chloride-water using an ultrasonic sonicator. The methylene chloride fraction and the previous acetone fraction were combined and evaporated to dryness for purification of the limonoid aglycones. Limonin was purified by repeated crystallization in methylene chloride and isopropanol. Limonin glucoside in the aqueous fraction was isolated by successive column chromatography using XAD-2 and WA-30 resins, followed by medium pressure chromatography and preparative HPLC. The purity of the various fractions was determined using High Performance Liquid Chromatography (HPLC)-electrospray ionization mass spectrometry.

SFE of limonoids from grapefruit seeds was done in two different stages. First, limonoid aglycones were removed using SC-CO_2 followed by extraction of limonoid glucosides using SC-CO_2-ethanol co-solvents. Extraction of limonoids from grapefruit seeds was optimized by varying operating parameters according to the Box-Behnken design (3^3 factorial) (Box and Behnken, 1960).

Supercritical CO_2 Extraction. For the first extraction stage using SC-CO_2, 35 g grapefruit seeds (dried and flaked) were filled in the extraction thimble of a pilot scale supercritical fluid extraction (Figure 2-1). Glass wool was placed at both ends of the thimble to prevent plugging of the cap frits. Pressurized CO_2 was allowed to pass through the extraction thimble. Upon exiting the thimble the extract was passed through a micrometering valve that reduced the pressure to one atmospheric pressure (atm). The extract was then admitted into a collection vessel to trap the solute while CO_2 exitted the system through a gas meter at ambient pressure. Thus, the reported volumetric flow rate of CO_2 gas is measured at 1 atm and 25˚C.

The first stage SFE extraction conditions were pressures of 34.5 MPa (5000 psi), 41.4 MPa (6000 psi) and 48.3 MPa (7000 psi) at temperatures of 40, 50 and 60˚C for 20, 40 and 60 min with constant CO_2 (gas) flow rate of about 5 L/min. The extraction vessel was installed inside a temperature-controlled oven (Model 3119-005 Instron, Canton, MA), with a 5 m length coil of tubing inside the oven to preheat the CO_2 before contacting the sample. Sample temperature was monitored with a thermocouple inserted through a thermocouple well installed on the wall of the extraction vessel, and that extended into the center of the vessel. To determine the effect of the CO_2 feeding mode on extraction yield, SFE was carried out at 41.4 MPa, with approximately a 5 L/min CO_2 flow rate, at 50˚C sample temperature with CO_2 fed from bottom and top respectively.

The collection vessel consisted of a glass test tube (200 ml) contained within a pressurized and temperature-controlled cell maintained at 40˚C. The extract from the extraction thimble entered the collection vessel via a tube that extended into the

bottom of the vessel. The opening at the tip of the tube was welded shut and multiple 1 mm diameter holes were drilled on the side at 1 cm intervals up to 10 cm from the tip. This permitted the CO_2 extract to enter the collection vessel directed towards the wall and prevented lipids in the extract from blowing out of the collection vessel. Fifteen mL of methanol was added directly to the extract in the collection vessel, and aliquots from this solution were analyzed for limonoid aglycones. After each run, the residue from the first extraction stage was removed from the extraction vessel, combined, and subjected to re-extraction for limonoid glucosides and flavonoids.

The second stage extraction used SC-CO_2-ethanol co-solvents. CO_2 (5 L/min) at 1 atm and 25˚C and ethanol (liquid) (0.015 L/min=0.59 X_{Eth}) were used. Ethanol was pumped through a syringe pump (Model 260D, Isco Inc., Lincoln, NE). The volume of extract in the collection vessel was noted and an aliquot was taken for analysis of limonoid glucosides.

Limonoids Determination. Limonoids were analyzed using HPLC as a separation technique and UV absorption as a monitoring technique. Limonoid aglycones were eluted from a C18 Waters Spherisorb ODS column using an isocratic mobile phase, water: methanol (6:4) with a flow rate of 1 ml/min, with elution of all limonoids achieved after 70 min. Individual limonoids were analyzed in 20 µl of sample with elution of individual peaks being monitored at UV 210 nm. Limonoid glucosides were separated using the same C18 column with elution of individual compounds effected using a linear gradient with a flow rate of 1 ml/min which had a starting composition of 10% acetonitrile in 0.03 mM phosphoric acid and a final

composition of 24% acetonitrile in 0.03 mM phosphoric acid. Elution of limonoid glucosides within 20 µl aliquots were complete in 80 min, and elution time of individual eluates determined by monitoring UV 210 nm (Li, 2002)

Flavonoids Analysis. Citrus flavonoids were separated by HPLC using C18 Waters Spherisorb ODS column eluted with a mobile phase comprised of water: acetonitrile: acetic acid (81.5:18:0.5) flowing at a rate of 1 ml/min for 45 min. Injection volumes of 20 µl were used, with the elution of individual flavonoid compounds being monitored at UV 284 nm.

Fatty Acid Determination. Hexane extraction at the first stage of extraction netted a large quantity of neutral lipids in addition to limonoids. The fatty acid composition of this lipid was analyzed using gas chromatography. Lipid extracts were saponified with potassium hydroxide, and fatty acid methyl esters (FAME) were prepared by esterification using 3N methanolic HCl and overnight incubation in an oven operating at a temperature of 65˚C. And then FAME were analyzed using GC. The injector and flame-ionization detector temperatures were set at 250 and 270˚C respectively. Finally FAME were identified by comparison of their retention times with authentic standards [GLC-461]. This method was modified from Rozzi et al. (2002).

Statistical Analysis. The data collected were analyzed using the response surface analysis (SAS® for Windows® 9.0 version). The optimization data were collected according to Box-Bohnken Design (Box and Behnken, 1960) to optimize the parameters for both stages.

Results and Discussion

It was hypothesized that nonpolar or less polar molecules could be extracted from plants, vegetables and other food matrices using SC-CO_2, since SC-CO_2 is considered to be a nonpolar solvent and is compatible with nonpolar or slightly polar molecules. In order to extract polar molecules, polarity of the solvent system needs to be changed using the supercritical fluid modifier ethanol. Therefore, we chose SC-CO_2 directly to extract the non-polar limonoid aglycones from grapefruit seeds, while the more polar limonoid glucosides were extracted using the more polar combination of SC-CO_2-ethanol as co-solvents. The effect of SF feeding mode on extraction yield is shown in Table 2-1. The data show that the extraction yield of Lim was higher with bottom feeding of CO_2 compared to top feeding. However, top feeding of CO_2-ethanol resulted in a better extraction yield of LG than bottom feeding. This may be explained by the fact that CO_2 entering the lower part of a vertical extraction thimble had to overcome the weight of the seeds, prolonging the contact time of CO_2 and seeds, and increasing Lim solubilization in CO_2. On the other hand, top feeding of CO_2-ethanol guaranteed thorough permeation of ethanol through the seeds, which increased LG solubilization in CO_2-ethanol system.

Table 2-1. Effects of SFE feeding mode on extraction yield.

Mode	Extraction yield of Lim from grapefruit seeds (mg/g) [1]	Extraction yield of LG from spent grapefruit seeds (mg/g) [2]
Top feeding	4.3, 4.7 [3]	0.48, 0.54
Bottom feeding	5.2, 5.4	0.41, 0.45

[1] Extraction of Lim was under 41.4 MPa pressure, about 5 L/min CO_2 flow rate, 50˚C sample temperature with 40 min.

[2] Extraction of LG was under 41.4 MPa pressure, about 5 L/min CO_2 and ethanol (co-solvent) flow rate, 50˚C sample temperature, 20% ethanol with 40 min.

[3] Values were the results of the duplicate tests.

The effects of pressure, temperature and time on the yield from the first stage extraction are presented in Table 2-2. The extraction conditions that gave the maximum yield of Lim (6.3 mg/g dried seeds) consisted of 48.3 MPa, 50˚C and 60 min with CO_2 fed from the bottom of the extraction vessel at a flow rate of approximately 5 L/min. Limonin was the major component identified by HPLC analysis of extract fractions (Figure 2-2). For this reason Lim was selected as the target molecule for optimization of conditions for maximize the extraction yield. Response surface analysis (RSA) of the data in Table 2-2 demonstrated the total model regression R^2=0.99. It is hypothesized that the relationship between extraction yield and pressure, temperature and time was quadratic. The RSA graph showed that extraction yield first increased and then decreased when extraction pressure increased with system temperature (Figure 2-3). This could be due to the density change of SC-CO_2; generally a higher SC-CO_2 density dissolves more solute and has a higher saturation concentration.

Table 2-2. Supercritical CO_2 extraction of limonin (Lim) from grapefruit seeds: extraction conditions and yields (CO_2 flow rate was about 5 L/min).

Run	Pressure (MPa)	Temperature (°C)	Time (min)	Yield (mg/g sample)
1	34.5	40	40	2.8, 3.2[1]
2	48.3	40	40	4.1, 4.3
3	34.5	60	40	3.3, 3.7
4	48.3	60	40	4.5, 4.7
5	34.5	50	20	1.8, 2.2
6	48.3	50	20	3.5, 4.1
7	34.5	50	60	4.3, 4.9
8	48.3	50	60	6.1, 6.5
9	41.4	40	20	2.4, 2.6
10	41.4	60	20	3.6, 4.2
11	41.4	40	60	5.3, 5.7
12	41.4	60	60	4.7, 5.1
13	41.4	50	40	5.2, 5.4
14	41.4	50	40	5.1, 5.5
15	41.4	50	40	4.9, 5.3

[1]Values were the results of the duplicate tests.

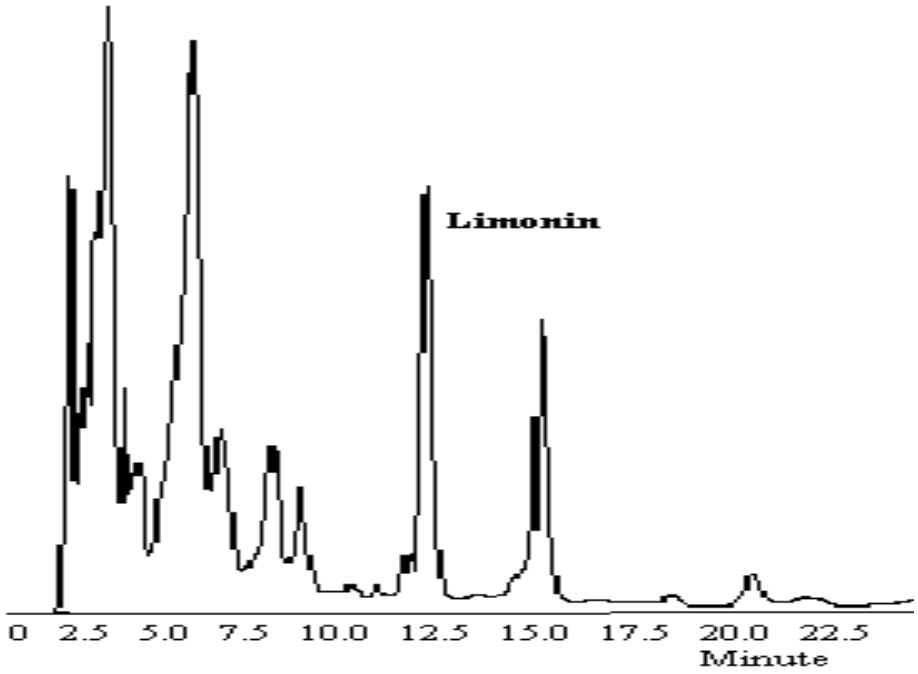


Figure 2-2. Typical HPLC chromatogram of the extract for limonoid aglycones analysis from grapefruit seeds using SC-CO_2.

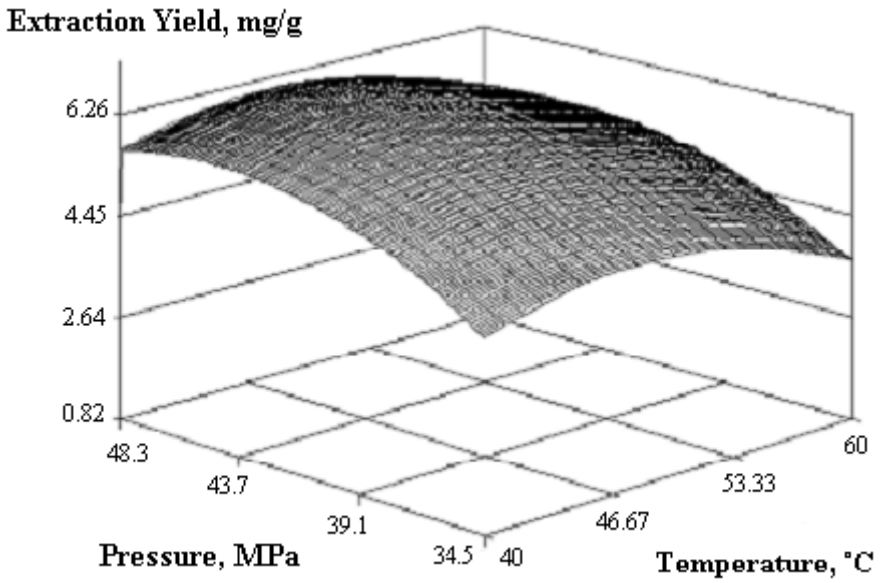


Figure 2-3. Response surface estimated for the extraction yield effects of pressure and temperature on limonin recovery from grapefruit seeds.

Extraction yield increased with time of extraction (RSA output graph not included). However, the amount of solute extracted ultimately leveled off as the extractable solutes in the sample are depleted. Nonetheless, the practical requirements such as extraction yield and operation cost, should be considered in deciding the extraction time needed.

Previous work from this laboratory (Shah et al., 2003) demonstrated that the time to exhaustive solute extraction was a function of CO_2 flow rate. High flow rates resulted in reduced solute yield/liter CO_2 and low flow rates required prolonged extraction time before maximal solute extraction was achieved. Therefore, in the present study we chose a CO_2 flow rate of approximately 5L/min. Finally, a maximum predicted yield of 6.8 mg/g dry and flaked seeds was determined by RSA under the following conditions, 46 MPa, 43˚C and 90 min. The optimum yield was close to that obtained in one of the treatments used in the experimental design. The treatment 48.3 MPa, 50˚C and 60 min with CO_2 bottom feeding, flow rate of approximately 5 L/min produced a yield of 6.3 mg Lim/g dry and flaked seeds, similar to the calculated RSA optimum.

However, because this extraction scheme required less extraction time, it would be more desirable economically.

Similar strategies and approaches were used to optimize the second stage of extraction. In this series of experiments extractions were conducted in which the pressure was varied between 34.5-48.3 MPa, while the temperature varied from 40-60˚C and 10%-30% ethanol was added as the co-solvent in a Box-Behnken experimental design. Results of the experiment to examine the effect of treatment time of 10, 20, 40 and 60 min (data not included) on extraction yield showed no significant difference between 40 and 60 min under 41.4 MPa, 50˚C, 20% ethanol, CO_2 flow rate about 5 L/min with top feeding. Based on these results, 40 min was established as the extraction time for the second stage. Table 2-3 presents the experimental results and shows that the treatment 48.3 MPa, 50˚C and 30% ethanol with CO_2 top feeding, flow rate at approximately 5 L/min

Table 2-3. Supercritical CO_2 extraction of limonin glucoside (LG) from defatted grapefruit seeds: extraction conditions and yields, including CO_2 flow rate of ~5 L/min and extraction time at 40 min.

Run	Pressure (MPa)	Temperature (°C)	Ethanol (%)	Yield (mg/g sample)
1	34.5	40	20	0.31, 0.33 [1]
2	48.3	40	20	0.38, 0.42
3	34.5	60	20	0.36, 0.4
4	48.3	60	20	0.44, 0.48
5	34.5	50	10	0.17, 0.23
6	48.3	50	10	0.27, 0.33
7	34.5	50	30	0.56, 0.58
8	48.3	50	30	0.6, 0.64
9	41.4	40	10	0.18, 0.22
10	41.4	60	10	0.25, 0.31
11	41.4	40	30	0.53, 0.57
12	41.4	60	30	0.59, 0.63
13	41.4	50	20	0.47, 0.51
14	41.4	50	20	0.48, 0.53
15	41.4	50	20	0.52, 0.54

[1] Values were the results of the duplicate tests.

produced the maximum average extraction yield of 0.62 mg limonin glucoside/g defatted seeds. A response surface analysis of the data from the extraction yield study of LG from Table 2-3 demonstrated a high regression value R^2=0.9969 (Figure 2-4) supported our hypothesis that the relationship between extraction yield and pressure, temperature and molar concentration of ethanol was quadratic. Additionally, the results also indicated that the experimental design included what appear to be the optimal conditions to maximize yield. Figure 2-5 (response surface generated by RSA) shows that the two crucial factors in the second stage extraction are also pressure and temperature. However, when limonoid glucosides were the extraction targets, the co-solvent constituted an additional factor that influenced the extraction yield. Figure 2-6 shows the relationship between the RSA generated extraction yield and pressure and co-solvent percentage. The extraction yield increased with co-solvent percentage until the solubility of limonoid glucosides in the solvent system was achieved. The RSA predicted a maximum LG yield of 0.73 mg/g defatted seeds at 42 MPa, 52˚C and 45% ethanol with top feeding, CO_2 flow rate around 5 L/min and 40 min. A high percentage of ethanol (>30%) is crucial to obtain the higher extraction yield. The RSA optimum appears to be desirable in the extraction of LG compared to any of the individual treatments in the experimental design.

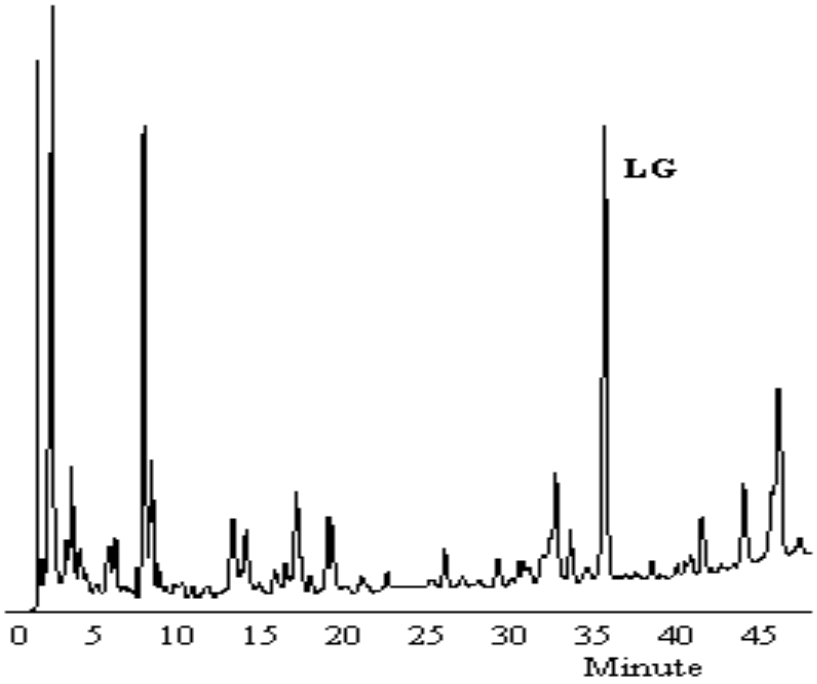


Figure 2-4. Typical HPLC chromatogram of the extract for limonoid glucosides analysis from defatted grapefruit seeds using SC-CO_2 and ethanol.

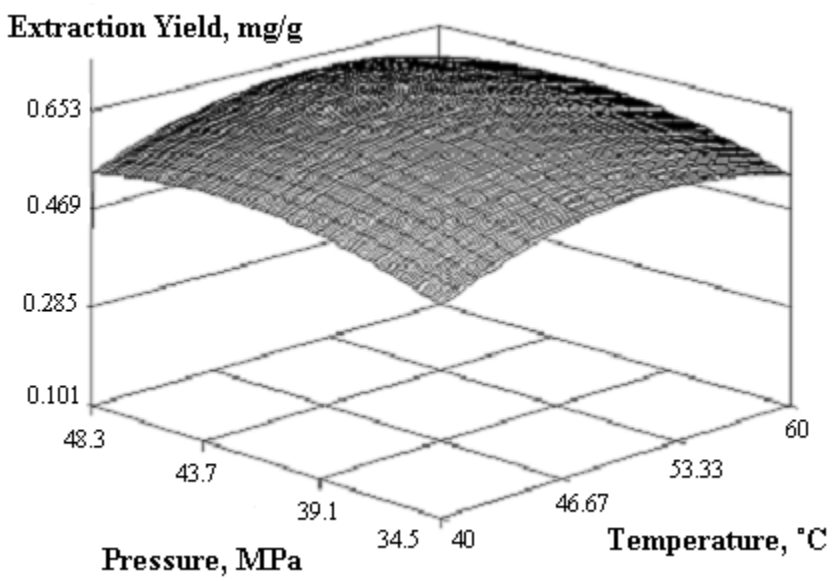


Figure 2-5. Response surface estimated for the extraction yield effects of pressure and temperature on limonin glucoside recovery from defatted grapefruit seeds.

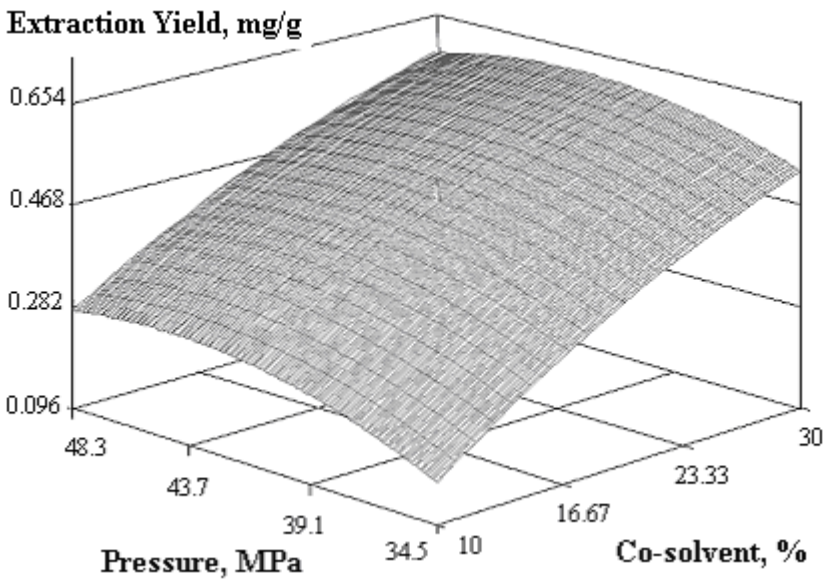


Figure 2-6. Response surface estimated for the extraction yield effects of pressure and co-solvent percentage on limonin glucoside recovery from defatted grapefruit seeds.

Limonoids and flavonoids would both be expected to be present in the co-solvent SC-CO_2-ethanol extract due to the similarity in compound polarity. After analyzing the extracts from the defatted seeds, we obtained the maximum extraction yield of 0.2 mg naringin/g defatted seeds under the conditions 41.4 MPa, 50˚C, 20% ethanol, 40 min and ~5 L CO_2/min. Figure 2-7 shows the typical HPLC chromatograph of flavonoids extracted, with naringin, the major component, varying in amount in the extract under different operating conditions.

The first stage of extraction extracted a large quantity of seed lipids in addition to Lim. The seed lipids are primarily non-polar or low-polarity triglycerides with high solubility in SC-CO_2. The extract obtained under the optimum conditions for the extraction of Lim (was solid) was analyzed by GC. The fatty acid composition in the grapefruit seed are C16:0 (Palmitic acid, 43.231%), C18:0 (Stearic acid, 8.734%), C18:1n9 (Oleic acid, 30.05%) and C18:2n6 (Linoleic acid, 13.71%). The fatty acid composition of the extract may vary under different extraction conditions. However, Rozzi et al. (2002) reported that no statistical difference was found in the fatty acid composition of supercritical fluid extracts under different extraction conditions; and also no difference of the fatty acid composition was observed between supercritical fluid extracts and liquid solvent extracts. Therefore, SC-CO_2 extraction is a good alternative to solid-liquid extraction of vegetable oils.

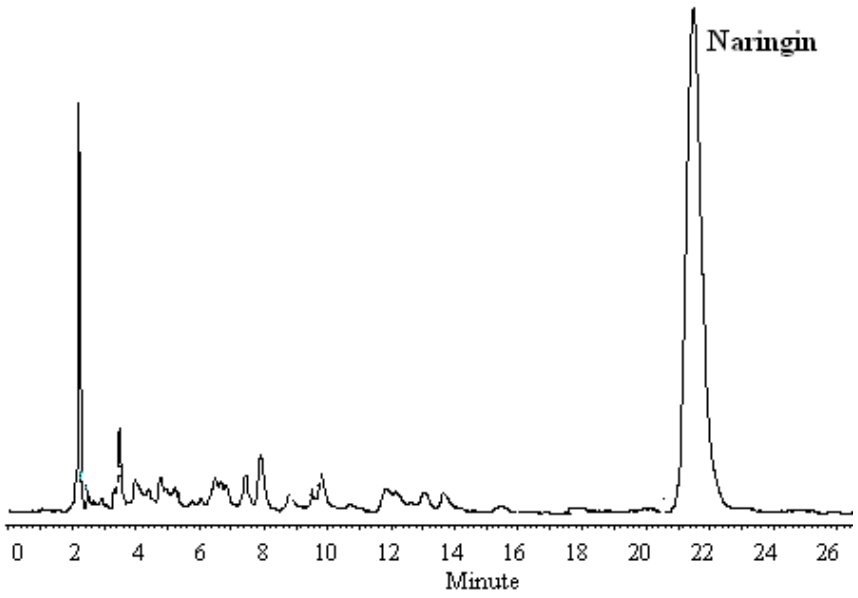


Figure 2-7. Typical HPLC chromatogram of the extract for flavonoids analysis from defatted grapefruit seeds using SC-CO_2 and ethanol.

Conclusion

The results indicate that limonoid aglycones, glucosides and flavonoids can be extracted with substantial success from grapefruit seeds with the SC-CO_2 technique. The extraction parameters optimized for aglycones via Response Surface Analysis (RSA) were 48.3 MPa, 50˚C and 60 min with CO_2 bottom feeding, flow rate at approximately 5 L/min with the extraction yield of 6.3 mg limonin/g dried grapefruit seeds. Optimum conditions for limonoid glucosides were 42 MPa, 52˚C, 45% ethanol in the SC-CO_2-ethanol solvent and 40 min with CO_2 top feeding, flow rate around 5 L/min resulting in a maximum predicted extraction yield of 0.73 mg limonin glucoside/g defatted seeds. According to R^2=0.99 and previous experience, this predicted value is reasonable and can be achieved. Additionally, we also obtained the maximum extraction yield for naringin of 0.2 mg/g defatted grapefruit seeds under the conditions 41.4 MPa, 50˚C, 20% ethanol, 40 min and ~5 L CO_2 /min. Besides these compounds, a large quantity of grapefruit seed oils were also obtained. The results demonstrated good potential for commercial production of limonoids and flavonoids from citrus seed by supercritical fluid extraction.

CHAPTER III

SUPERCRITICAL FLUID EXTRACTION OF LIMONOID GLUCOSIDES FROM GRAPEFRUIT MOLASSES

Introduction

Modern medicinal chemists have been hunting for complex molecules that have beneficial biological activity. The Kingdom *Planta* has been a promising source of structurally complex secondary metabolites. Limonoids are highly oxygenated triterpenoids primarily found in *Rutales*, especially in the families *Meliaceae* and *Rutaceae*. Chemically they belong to the family of terpenoids with isoprene units as the basic building block. It is well known that limonoids (mainly limonin and nomilin) impart bitterness to citrus juices and their presence in the juice has been a problem for the citrus juice industry for a long time. However, limonoid glucosides are water soluble, tasteless, abundant in citrus, and are safe natural compounds to consume (Hasegawa et al., 2000).

With the increasing interest in the bioactive functions of limonoids, the demand for these chemicals has significantly increased (Braddock and Cadwallader, 1992). Since the limonoid glucosides are not available commercially, extraction and purification of limonoids from juice processing plants byproducts could increase the potential value of the citrus crop. It was reported that molasses and peel contain 18% and 36% of total limonoid glucosides, respectively. Thus, citrus byproducts molasses and peel residues are excellent sources for limonoid glucosides. Furthermore,

molasses being liquid is the best potential industrial source for the extraction of limonoid glucosides (Miyake et al., 2000).

Compared with traditional extraction with liquid organic solvents, extraction with supercritical fluids such as CO_2 (SC-CO_2) has been of interest due to its environmental compatibility (Modey et al., 1996). Besides, SC-CO_2 has several other advantages such as non-toxicity, non-flammability, inability to leave residual chemical, low-moderate operating temperature and pressure (Rozzi and Singh, 2002). Therefore, it's been widely used in the food processing industry. Advantages of the supercritical fluid extraction technique and benefits in the food industry were recently reviewed (Rozzi and Singh, 2002; Sihvonen et al., 1999).

Recent studies have reported that limonoid glucosides could be extracted from citrus molasses using SC-CO_2 (Miyake et al., 1996; Miyake et al., 2000). Traditional methods to extract limonoid glucosides from citrus molasses for analytical purposes have also been explored (Braddock and Charles, 2001; Ozaki et al., 1995). However, very little information is available on the application of co-solvent, operating parameters and other factors influencing the extraction. Our research focused on SC-CO_2 and co-solvent extraction of limonoid glucosides from grapefruit molasses and attempted to optimize the operating parameters for SC-CO_2 and co-solvent extraction of these bioactive compounds.

Materials and Methods

Materials. Wet grapefruit molasses was obtained from fruits harvested at the Texas Citrus Exchange, Mission, TX. High purity CO_2 was used for supercritical fluids extraction (SFE). SFE schematics are illustrated in Figure 2-1. All solvents were HPLC grade (Fisher Scientific, Atlanta, GA). SFE extracts were analyzed by High Performance Liquid Chromatography (HPLC-UV2000, Thermo Hypersil-Keystone Company, USA) equipped with a reverse C18 Waters Spherisorb ODS column (250 mm x 4.6 mm).

Isolation of Limonoids. Limonin 17-β-D-glucopyranoside (LG) (90% pure), from grapefruit (*citrus paradisi*) seeds, was prepared and purified according to the procedures in our lab (Tian et al., 2003; Tian et al., 2001) at Texas A&M University-Kingsville Citrus Center, Weslaco, TX. Before extraction, seeds of mature grapefruits were collected and dried at 55˚C. 500 g of the dried seeds were ground into powder with a Retch mill (Brinkmann, Westbury, NY). The milled seeds were placed in a Soxhlet extractor and washed overnight with hexane (solvent:seeds=3:1, extraction at 25˚C) to remove the oil. Acetone and methanol were sequentially used to extract the treated seeds. The methanol fraction was evaporated to dryness with a rotary evaporator under vacuum (<60˚C), and the residue was partitioned with 1:1 methylene chloride-water using an ultrasonic sonicator. Limonoid glucosides in the aqueous fraction were isolated by successive column chromatography using XAD-2 and WA-30 resins, followed by medium pressure chromatography and preparative

HPLC. The purity of the various fractions was determined using high-performance liquid chromatography (HPLC)-electrospray ionization mass spectrometry.

SFE of limonoid glucosides were extracted from the wet grapefruit molasses by one-step extraction using SC-CO_2-ethanol co-solvents. Various operation parameters were investigated to extract limonoids. Box-Behnken design (3^3 factorial) was applied to seek optimal operation parameters.

Supercritical CO_2 Extraction. Wet grapefruit molasses (60 g) was loaded into the extraction thimble of a pilot scale supercritical fluid extraction apparatus (Figure 2-1). Glass wool was placed at both ends of the thimble to prevent plugging of the cap frits. The supercritical fluid was a mixture of CO_2 (~5 L/min at one atm and 25˚C) and ethanol (liquid) (0.015 L/min=0.59 X_{Eth}). Ethanol was pumped through a syringe pump (Model 260D, Isco Inc., Lincoln, NE). Pressurized CO_2 and ethanol were allowed to pass through the extraction thimble. Upon exitting the thimble the extract passed through a micrometering valve that reduced the pressure to atmospheric pressure, and finally the extract entered a collection vessel. CO_2 exitted the system to surrounding air through a gas meter at one atm. Thus, the reported volumetric flow rate is that of CO_2 gas at one atm and 25˚C.

The extraction conditions were optimized by operating the SFE unit at 34.5 MPa (5000 psi), 41.4 MPa (6000 psi) and 48.3 MPa (7000 psi) pressures, at the temperatures of 40, 50 and 60˚C with ethanol at the molar fraction of 10%, 20% and 30%, and CO_2 (gas) flow rate ~5 L/min. The extraction thimble was installed inside a temperature-controlled oven (Model 3119-005 Instron, Canton, MA), and a 5 m

length coil of tubing inside the oven preheated the CO_2 and ethanol before entering the thimble. Sample temperature was monitored with a thermocouple installed on the extraction thimble wall that extended into the center of the vessel. Thermocouple output was recorded by a digital data logger (Hydra 2635A, Fluke, Everett, WA). To determine the effect of operating time on the extraction yield, experiments were then carried out at 41.4 MPa pressure, 20% ethanol, ~5 L/min CO_2 flow rate, 50˚C sample temperature for 20, 40, 60 and 80 minutes respectively.

The collection vessel consisted of a glass test tube (200 ml) contained in a pressurized and temperature-controlled cell maintained at 40˚C. The extract from the thimble entered the collection vessel via a tube that extended into the bottom of the vessel. The opening at the tip of the tube was welded shut and multiple 1 mm diameter holes were drilled on the side at 1 cm intervals up to 10 cm from the tip. This permitted the entry of CO_2 and ethanol extract into the collection vessel to be directed towards the wall, and thereby prevent the extract from blowing out of the collection vessel. Aliquots from the extract were analyzed for limonoid glucosides. Residual molasses was finally discarded.

Limonoid Glucosides Determination. Limonoid glucosides were analyzed by HPLC. The column was eluted using a linear gradient flowing at a rate of 1 ml/min starting with 10% acetonitrile in 0.03 mM phosphoric acid and ending with 24% acetonitrile in 0.03 mM phosphoric acid for 80 min. Injection volumes of 20 µl were used in these analyses, with limonoids being detected at UV 210 nm.

Statistical Analysis. The data collected were analyzed using the response surface analysis procedures (SAS® for Windows® 9.0 version). The experiment data were collected according to Box-Bohnken Statistical Design (3^3 factorial) to optimize the parameters.

Results And Discussion

It was hypothesized that nonpolar or less polar molecules could be extracted from plants, vegetables and other food matrices using SC-CO_2, since SC-CO_2 is considered to be a nonpolar solvent and is compatible with nonpolar or slightly polar molecules. In order to extract polar molecules, the polarity of the SC-CO_2 solvent system needs to be increased. Since glucosidation of limonoid aglycones increases the polarity of limonoids, it is difficult to extract limonoid glucosides using neat SC-CO_2. However, it is possible to extract limonoid glucosides from citrus molasses using SC-CO_2-ethanol combinations as co-solvents. In this study, we used top feeding of the CO_2-ethanol co-solvents because bottom feeding allowed the molasses to blow into the tubing system and block the solvent flow. Top feeding guaranteed that all the co-solvents moved through the molasses with adequate contact time to maximize extraction efficiency.

Figure 3-1 demonstrates that extraction yield increased with increasing extraction time, with a plateau observed when the soluble components were exhausted. In this study, product yields from a 40 min extraction were not significantly different from those obtained with extraction times of 60 and 80 min, so 40 min was chosen as the treatment time for all subsequent extractions. Additionally,

CO_2 flow rate also influenced the extraction yield. Generally, higher flow rate led to wastage of CO_2 and lower flow rate led to inefficient extraction. According to the previous work (Shah et al., 2004), we chose the medium CO_2 flow rate ~5 L/min in this study.

The effects of the three key factors of pressure, temperature and co-solvent molar percentage were examined for the extraction of limonoid glucosides. The experimental limits for these studies were pressures within the range of 34.5-48.3 MPa, temperatures between 40-60˚C and co-solvent concentrations of 10-30% ethanol within a full 3^3 factorial design. The extraction yield was used as a dependent variable. Table 3-1 gives the experimental data and the 6^{th} treatment with 48.3 MPa, 50˚C and 10% ethanol producing the maximum average yield of 0.61 mg LG/g molasses. Since LG was one of the major components in the extract, we maximized the extraction yield of LG. Response surface analysis (RSA) of the data in Table 3-1 produced the prediction model between the extraction yield and the three key factors illustrated in Figures 3-2, 3-3 and 3-4. Figure 3-2 (output graph of RSA) shows that extraction yield of LG increased up to a maximum value and then dropped when the extraction pressure increased with the system temperature. This effect could be explained by the density change of the mixture of SC-CO_2 and ethanol. In general, higher supercritical fluid density allows greater solute dissolution and higher solute concentration at saturation.

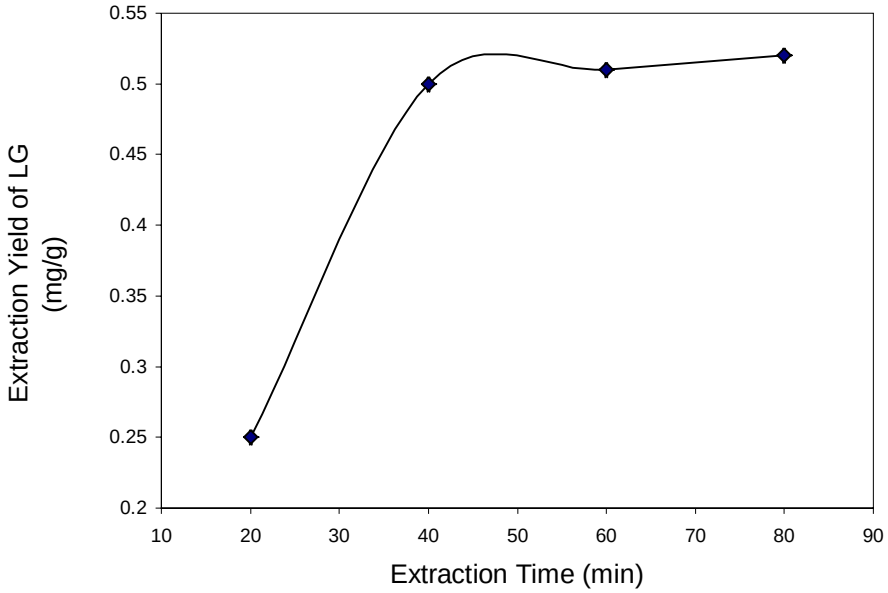


Figure 3-1: Effects of time on the extraction yield. The value of each point was the average of duplicate tests under the conditions: 41.4 MPa, 50°C, 20% ethanol with top feeding of CO_2 and ethanol at a flow rate of ~5 L/min.

Table 3-1. Supercritical CO_2 extraction of limonin glucoside (LG) from grapefruit molasses: extraction conditions and yields (CO_2 flow rate was ~5 L/min, extraction time 40 min).

Run	Pressure (MPa)	Temperature (°C)	Ethanol (%)	Yield (mg/g sample)
1	34.5	40	20	0.18, 0.22[1]
2	48.3	40	20	0.34, 0.38
3	34.5	60	20	0.47, 0.53
4	48.3	60	20	0.55, 0.57
5	34.5	50	10	0.5, 0.54
6	48.3	50	10	0.59, 0.63
7	34.5	50	30	0.4, 0.46
8	48.3	50	30	0.53, 0.57
9	41.4	40	10	0.41, 0.49
10	41.4	60	10	0.51, 0.55
11	41.4	40	30	0.45, 0.47
12	41.4	60	30	0.45, 0.51
13	41.4	50	20	0.50, 0.52
14	41.4	50	20	0.47, 0.51
15	41.4	50	20	0.48, 0.52

[1]Values are the duplicate extraction yields.

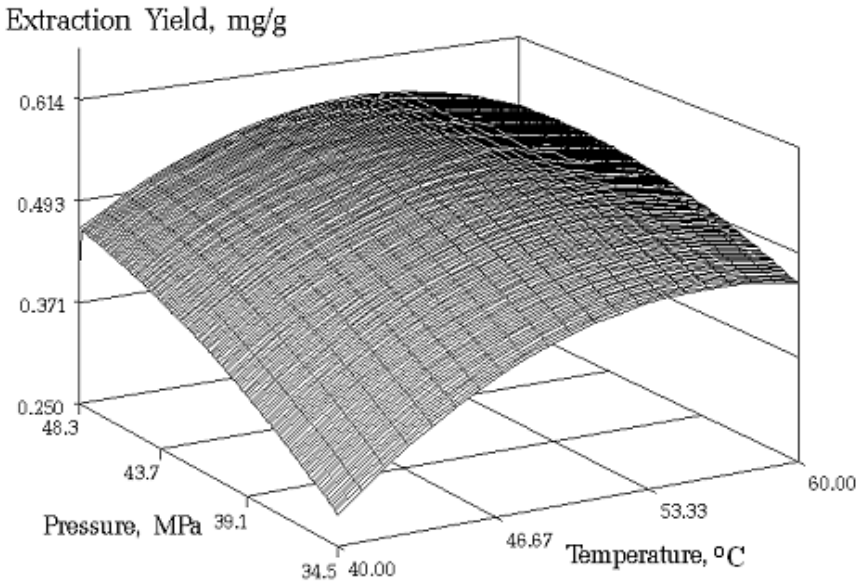


Figure 3-2: Response surface estimated for the extraction yield effects of pressure and temperature.

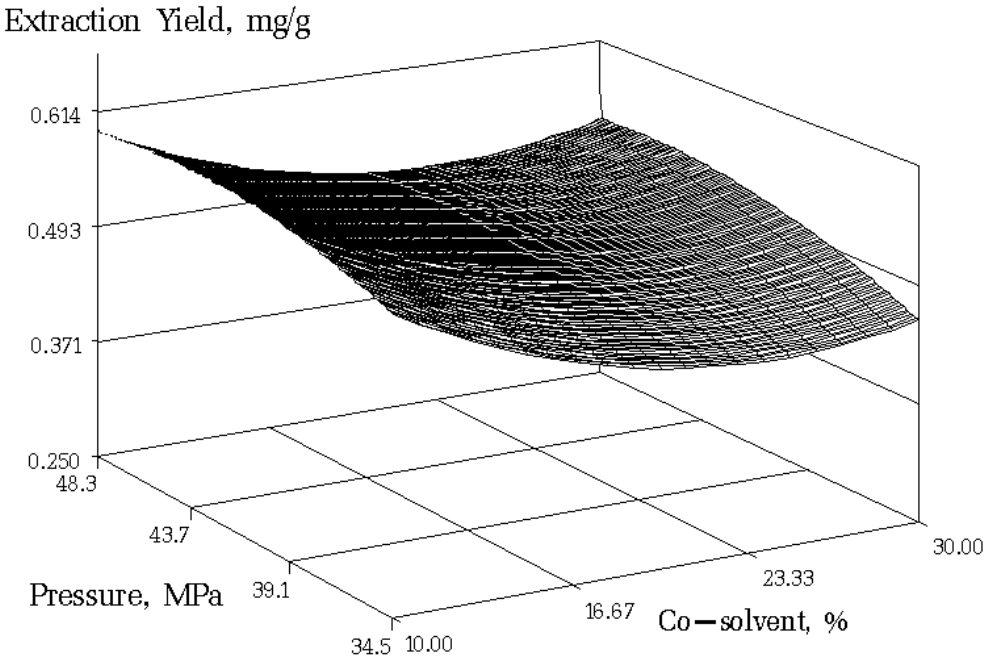


Figure 3-3: Response surface estimated for the extraction yield effects of pressure and co-solvent percentage.

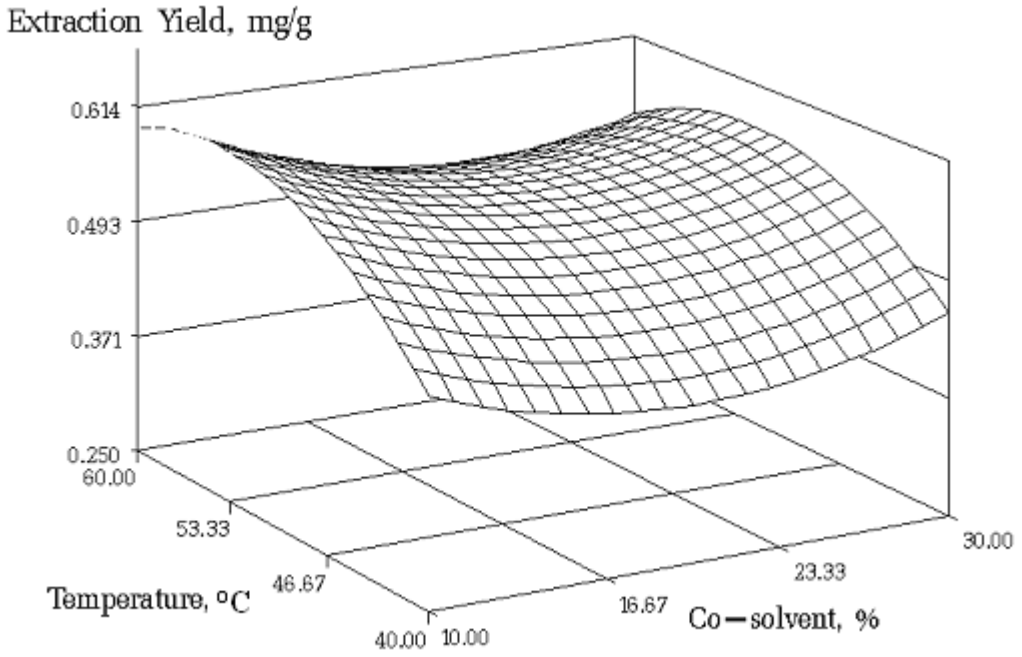


Figure 3-4: Response surface estimated for the extraction yield effects of temperature and co-solvent percentage.

Figure 3-3 demonstrates that the extraction yield of LG decreased as the molar percentage of the co-solvent ethanol increased from 10% to 30%, or when the extraction pressure increased from 34.5 to 48.3 MPa. This response may be specific to liquid extraction materials as results from extraction studies with solid materials showed that extraction yield increased directly with the percentage of co-solvent in the solvent system. The large amount of water present in liquid materials could possibly prevent the SC-CO_2 and 30% ethanol from extracting the target solutes because LG is hydrophilic, and water is immiscible with the SC-CO_2 and ethanol phase. Furthermore, because solvents with similar polarities are believed to be easily miscible, the polarity of the SC-CO_2 and 10% ethanol system was presumed to be similar to that of LG. Thus, increasing the percentage of ethanol co-solvent in the system increased the polarity of SC-CO_2 and therefore reduced its ability to dissolve the relatively less polar LG.

These results were further demonstrated in Figure 3-5A and 3-5B, where 10% ethanol showed higher extraction selectivity of LG with 15.154% in the extract; while 30% ethanol showed only 10.923%. Besides, the synergism of density and polarity of the SC-CO_2, the presence of ethanol also strongly affected extraction yields.

The extraction yield increased first and then decreased with the increase of both temperature and ethanol percentage. This behavior can be explained by the density change of the SC-CO_2 and ethanol system (Figure 3-4).

RSA produced the optimized conditions for the extraction of LG at 48.2 MPa, 53˚C and 22% ethanol with the predicted value of 0.54 mg/g that is a little lower than

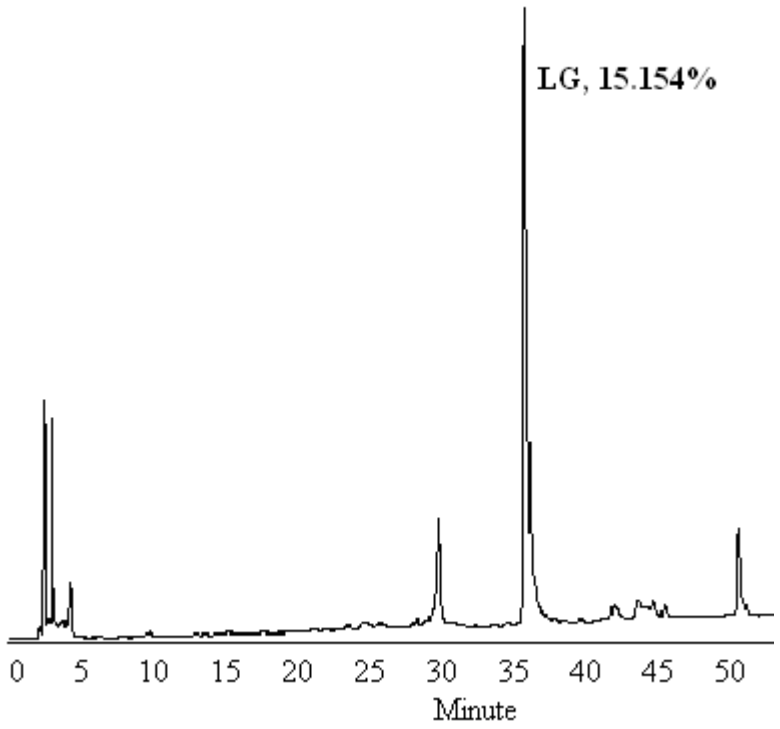


Figure 3-5A: Typical HPLC chromatogram of grapefruit molasses extracts obtained using supercritical CO_2 and 10% ethanol at a pressure of 48.3 MPa, and an extraction temperature of 50°C with a flow rate of ~5 L CO_2 /min. LG is limonin 17-β-D-glucopyranoside.

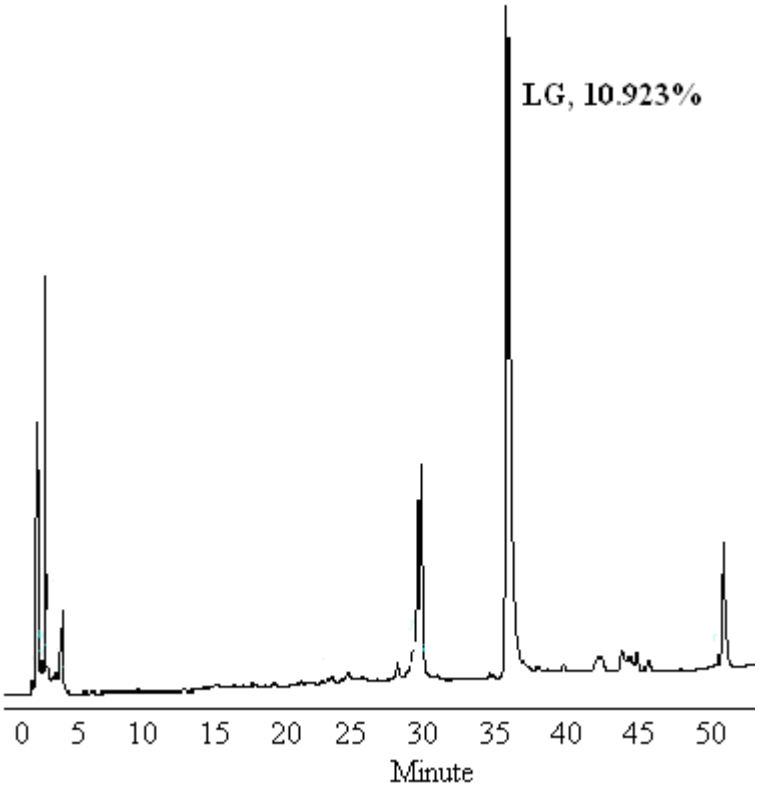


Figure 3-5B: Typical HPLC chromatogram of grapefruit molasses extracts obtained using supercritical CO_2 and 30% ethanol at a pressure of 48.3 MPa and an extraction temperature of 50°C with a flow rate of ~5 L CO_2 /min. LG is limonin 17-β-D-glucopyranoside.

the 6th treatment conditions. Therefore, the optimized conditions for the extraction of LG are 48.3 MPa, 50˚C, 10% ethanol, 40 min and ~5 L/min CO_2 flow rate.

Conclusion

The results indicate that limonoid glucosides can be extracted with substantial success from grapefruit molasses, a byproduct from the citrus juice industry, when supercritical CO_2 and ethanol act as co-solvents. Finally the extraction parameters were optimized via Response Surface Analysis (RSA) as the following: 48.3 MPa, 50˚C, 10% ethanol, 40 min and ~5 L/min CO_2 flow rate with an extraction yield of 0.61 mg limonin glucoside/g grapefruit molasses. The results suggest that supercritical fluid extraction might be used for the commercial production of limonoids.

CHAPTER IV

ANTIOXIDANT ACTIVITY OF CITRUS LIMONOIDS, FLAVONOIDS AND COUMARINS

Introduction

Diets high in fruits and vegetables are protective against a variety of diseases, particularly cardiovascular disease and some types of cancer (Ness and Powles, 1997). Reactive oxygen, e.g. superoxide and singlet oxygen radicals, and nitrogen, e.g. peroxynitrite and nitrogen dioxide radical species generated *in vivo* are known to alter cellular structure and function. These induced alterations are thought to cause chronic degenerative diseases including heart disease and cancer (Kinsella et al., 1993). Antioxidant phytochemicals are a class of nutrients thought to significantly contribute to protection against these diseases (Ross and Kasum, 2002). Citrus fruits are rich sources of vitamin C (ascorbic acid), an essential nutrient with well-described antioxidant properties. However, citrus also contain other phytochemicals with potential health-promoting properties (Guthrie et al., 2000; Lam et al., 1989; Lam et al., 2000; Miller et al., 2000; Tian et al., 2001). Citrus contains several flavonoids, coumarins and limonoids in significant concentrations. There is extensive literature describing the antioxidant activities of flavonoids from a variety of plant sources (Chen et al., 1996; Ng et al., 2000; Mira et al., 2002; Noroozi et al., 1998; Sanz et al., 1994). Indeed, flavonoids possess a wide range of activities *in vitro* (Bravo, 1998; German and Walzem, 2000). For example, this class of phytochemicals is known to act as free-radical scavengers, to modulate enzymatic activities, and inhibit cellular proliferation, as well as possessing antibiotic, anti-allergenic, anti-diarrheal, anti-ulcer, and anti-inflammatory activities (Duthie and

Crozier, 2000). Flavonoids were shown to be able to scavenge peroxyl radicals, alkyl peroxyl radicals, superoxide hydroxyl radicals, and peroxynitrite in aqueous and organic environments (Chen et al., 1996; Guthrie et al., 2000; Mira et al., 2002; Ng et al., 2000; Srimanth et al., 2002).

Coumarins, derived from a branch of the phenylalanine metabolism pathway that leads ultimately to furanocoumarin (psoralin) synthesis, are another class of phytochemicals found in citrus. Some coumarins have been shown to possess anticarcinogenic and antithrombotic activities (Fernandez-Puntero et al., 2001; Gunatilaka et al., 1994; Kostova et al. 2001). Limited data is available to demonstrate the antioxidant activities of coumarins (Fernandez-Puntero et al., 2001; Ng et al., 2000; Vladimirov et al., 1991).

Citrus limonoids are a group of highly oxygenated triterpenoids. Research with these compounds has shown that some limonoids could induce the detoxifying enzyme glutathione S-transferase in the liver of mice and rats (Lam et al., 1989).

Citrus limonoids were also shown to inhibit the formation of chemically induced neoplasia in the oral cavity, forestomach, small intestine, colon, lung and skin of laboratory animals (Lam et al., 2000; Miller et al., 2000).

Previous studies (Guthrie et al., 2001; Tian et al., 2001) have also shown that limonoids can also inhibit the proliferation of breast cancer cells grown in culture. However, the antioxidant activities of citrus limonoids have not yet been well documented.

The objective of this study was to determine the antioxidant activity of grapefruit (*citrus paradisi*) derived phytochemicals. The compounds selected included 2 limonoids; limonin (Lim) and limonin 17-β-D glucopyranoside (LG), 8 flavonoids; apigenin (Api), scutellarein (Scu), kaempferol (Kae), rutin trihydrate (Rut), neohesperidin (Neh), neoeriocitrin (Nee), naringenin (Ngn) and naringin (Ng), and the coumarin bergapten (Ber). The structures of these citrus phytochemicals are given in Figure 4-1.

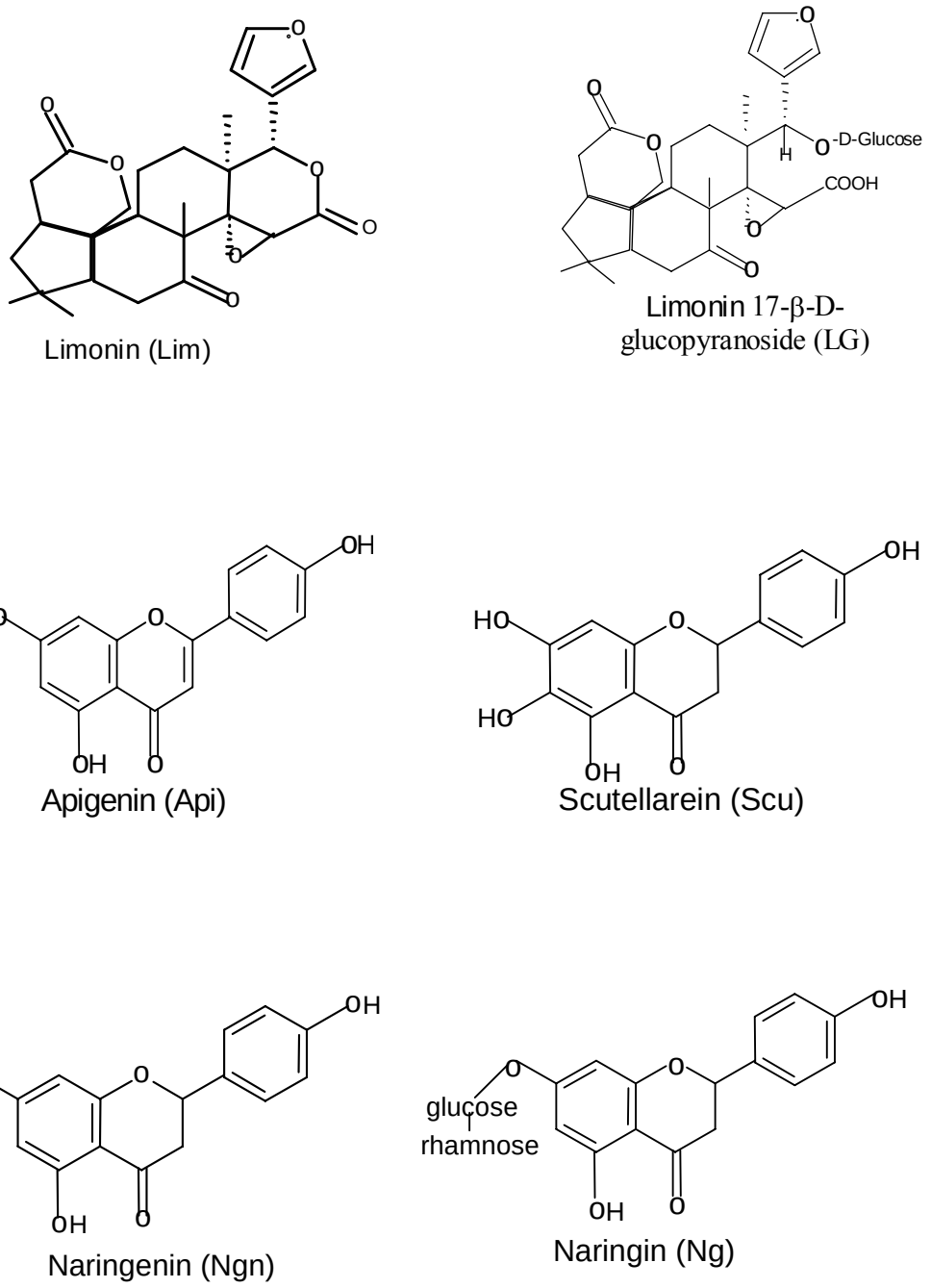
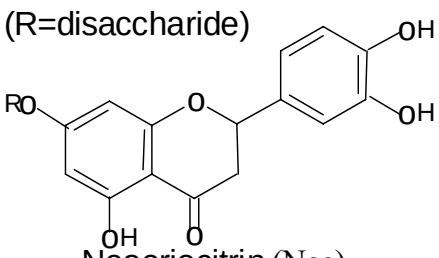
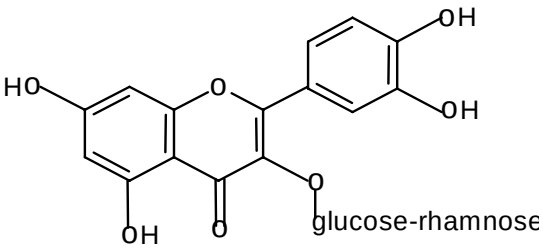


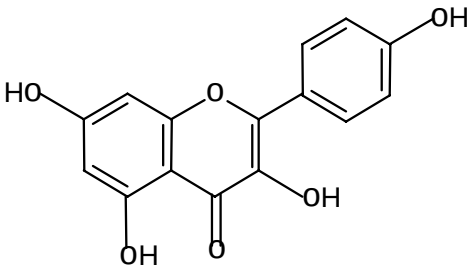
Figure 4-1. Structures of citrus compounds tested. Limonoids, limonin and limonin 17-β-D-gluocpyranoside; flavonoids, apigenin, scutellarein, kaempferol, rutin trihydrate, neohesperidin, neoeriocitrin, naringenin and naringin; and a coumarin, bergapten, tested for antioxidant activity.



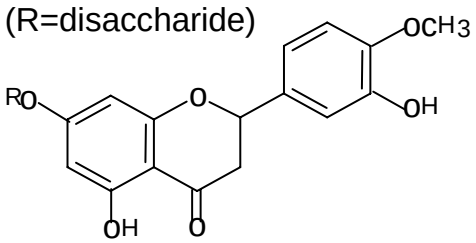
Neoeriocitrin (Nee)



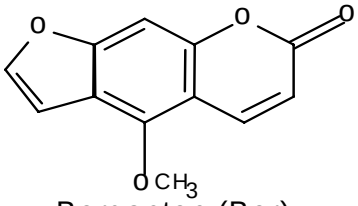
Rutin trihydrate (Rut)



Kaempferol (Kae)



Neohesperidin (Neh)



Bergapten (Ber)

Figure 4-1 Continued.

Materials and Methods

Materials. Limonin (Lim, >98% pure) and limonin 17-β-D-glucopyranoside (LG) (90% pure) were purified according to procedures in our lab (Tian et al., 2001; Tian et al., 2003) from grapefruit (*citrus paradisi*) seeds at the Texas A&M University-Kingsville Citrus Center, Weslaco, TX. Other citrus compounds: rutin trihydrate (Rut), apigenin (Api), scutellarein (Scu), kaempferol (Kae), neohesperidin (Neh), neoeriocitrin (Nee), naringenin (Ngn) and naringin (Ng) and bergapten (Ber) were purchased (Indofine Chemical Company, Somerville, NJ). Other chemicals and reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Spectra measurements were obtained using a DU 640 UV-Vis spectrophotometer (Beckman Coulter, USA).

Isolation of Limonoids. Seeds of mature grapefruits were collected and dried at 55˚C. 500 g of the dried seeds were ground into powder with a Retch mill (Brinkmann, Westbury, NY). The milled seeds were placed in a Soxhlet extractor and washed overnight with hexane (solvent:seeds=3:1, extraction at 25˚C) to remove the oil. Acetone and methanol were sequentially used to extract the defatted seed residue. The methanol fraction was evaporated to dryness with a rotary evaporator under vacuum (<60˚C), and the residue was partitioned with 1:1 methylene chloride-water using an ultrasonic sonicator. The methylene chloride fraction and the previous acetone fraction were combined and evaporated to dryness for purification of the limonoid aglycones. Limonin was purified by repeated crystallization in methylene chloride and isopropanol. Limonin glucoside in the aqueous fraction was isolated by successive column chromatography using XAD-2 and WA-30 resins, followed by medium pressure chromatography and

preparative HPLC. The purity of the various compounds was determined using high-performance liquid chromatography (HPLC)-electrospray ionization mass spectrometry.

β-Carotene-Linoleic Acid Model System. This experiment was carried out by the method of Emmons et al., (1999) and Chen and Ho (1995). Emulsion was prepared by dissolving β-carotene (5 mg) in 50 ml of chloroform, and 3 ml was added to the mixture of linoleic acid (40 mg) and Tween 40 (400 mg). Chloroform was removed under a stream of nitrogen gas and oxygenated water (100 ml) was added to the emulsion and vigorous mixing with a vortex-type mixer. Aliquots (3 ml) of the β-carotene-linoleic acid emulsion were mixed with 40 µl of sample solution (10 µM) and incubated in a water bath at 50°C. Oxidation of the emulsion was monitored with a spectrophotometer by measuring absorbance at 470 nm over a 60-minute period. The negative control contained 40 µl of ethanol in place of the compounds. The antioxidant activity is expressed as percent inhibition relative to the negative control after 60 minutes incubation using the following equation:

$$AA=100\ (DR_C- DR_S)/\ DR_C,$$

where AA is the antioxidant activity, DR_C is the degradation rate of the control [=(ln(a/b))/60], DR_S is the degradation rate in the presence of the sample [=(ln(a/b))/60], a is the initial absorbance at 0 time, and b is the absorbance at 60 minutes. All the citrus compounds were evaluated at the final concentration of 10 µM and Rut served as the positive control.

DPPH Radical Scavenging Activity. Samples were tested individually at a final concentration of 10 μM by addition to an ethanolic solution of 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical (100 μM). The mixtures were vigorously mixed and left to stand in the dark for 30 minutes. The absorbance of the resulting solution was measured using a spectrophotometer at 517 nm against blank samples without DPPH, the negative control (Blois, 1978; Chen and Ho, 1995; Yen and Duh, 1994) and Rut served as the positive control.

Superoxide Radical Scavenging Activity. The superoxide radical scavenging effect of the citrus compounds was determined by monitoring the reduction of nitro blue tetrazolium (NBT) (Nishikimi et al., 1972). The reaction mixture contained the test compound (10μM) in combination with PMS (5-Methylphenazinium methosulfate) (20 μM), NADH [Adenosine 5'-(trihydrogen diphosphate)] (156 μM), and NBT (50 μM) in phosphate buffer (0.1 M, pH7.4) in a final volume of 2.5 ml (Nishikimi et al., 1972). The samples were incubated at ambient temperature for 5 minutes and reaction product formation measured at 560 nm using a spectrophotometer. The blank sample (the negative control) was run without NBT and Rut served as the positive control.

Hamster Low Density Lipoprotein (LDL) Oxidation by Conjugated Diene Formation. Male Syrian Golden Hamsters (SASCO-strain, Charles River Laboratories, Wilmington, MA 01887) fed with a casein-based diet (Xu et al., 1998) for 7 wks were used as plasma donors. Plasma was harvested from cardiac blood, drawn into tubes containing sufficient EDTA to inhibit blood clotting. Following centrifugation at 2500 g

for 20 min at 4°C, prepared plasma was stored at 4°C prior to LDL isolation and preparation for the conjugated diene formation assay (Walzem et al., 1995). Conjugated diene formation was used as an endpoint, following initiation of oxidation by the addition of $CuSO_4$ (10 μM) to the mixture of individual citrus compounds (10 μM) and LDL (20 μg protein/ml) in 0.1 M phosphate buffered saline (PBS) (pH=7.4, in 0.15 M NaCl). Copper-catalyzed oxidation of LDL was monitored by continuous measurement of absorbance at 234 nm with a spectrophotometer at 37°C. Lag time was calculated as the intersection of baseline slope with that of the slope during propagation of conjugated diene formation. The blank sample (the negative control) was run without compounds and Rut served as the positive control.

Statistical Analysis. All tests were run in triplicate, and values were expressed as means ± SEM. Differences among treatments were evaluated by one-way ANOVA (SAS® for Windows® 9.0 version) with post hoc means ranking test using Duncan. Mean values differing by $P \leq 0.05$ were considered significant.

Results

The antioxidant activity of 2 citrus limonoids, 8 flavonoids and the courmarin bergapten as measured by the bleaching of β-carotene are presented in Figure 4-2. At the final concentration of 10 μM, Lim, LG and Ber inhibited <7% of the carotene bleaching observed in control incubations. Flavonoids exhibited a much greater antioxidant activity, with Scu, Kae and Rut inhibiting carotene bleaching by 51.3%, 47.0%, and 44.4% respectively.

The DPPH free radical scavenging potentials of the 11 citrus chemicals at the concentration of 10 μM were given in Figure 4-3. Flavonoids showed much stronger DPPH radical scavenging activities than limonoids. Rut showed the highest activity (32.18%), followed by Scu (18.32%), Nee (17.18%) and Kae (12.79%). With Lim and LG, the free radical scavenging activities were 0.5% and 0.25% respectively. Naringin, Ngn and Ber demonstrated negative activity; however, Ng, Ngn and Ber showed 16.5%, 17.3% and 12.6% scavenging activity respectively at the concentration of 20 μM.

Figure 4-4 illustrates the ability of the 11 citrus compounds to scavenge superoxide. Citrus phytochemicals at the final concentration of 10 μM variably diminished the *in vitro* superoxide production. The limonoids and bergapten inhibited the production of superoxide by 2.5-10% compared to control incubations. In contrast, flavonoids inhibited superoxide formation by 38-60%. Among the flavonoids, Rut, Scu, Nee, and Neh inhibited superoxide formation by 64.08%, 52.06%, 48.3%, and 37.7% respectively.

The ability of the 11 citrus compounds to prevent copper-initiated accumulation of conjugated diene fatty acid oxidation products in hamster LDL is given in Table 4-1. The average initiation time for LDL incubated in the absence of citrus phytochemicals was 120 minutes. As shown in Table 4-1, 10 μM LG did not inhibit LDL oxidation; Lim and Ber, however, did offer some protection against LDL oxidation, increasing lag time to 345 min (3-fold) and 160 min (33% increase), respectively. Compared to the limonoids, flavonoids showed much stronger protection against LDL oxidation. This was especially true for Rut and Nee, where lag time increased 23-fold to 2800 min, while Scu and Kae increased lag time to 18-fold (2140 min) and 15.7 fold (1879 min), respectively.

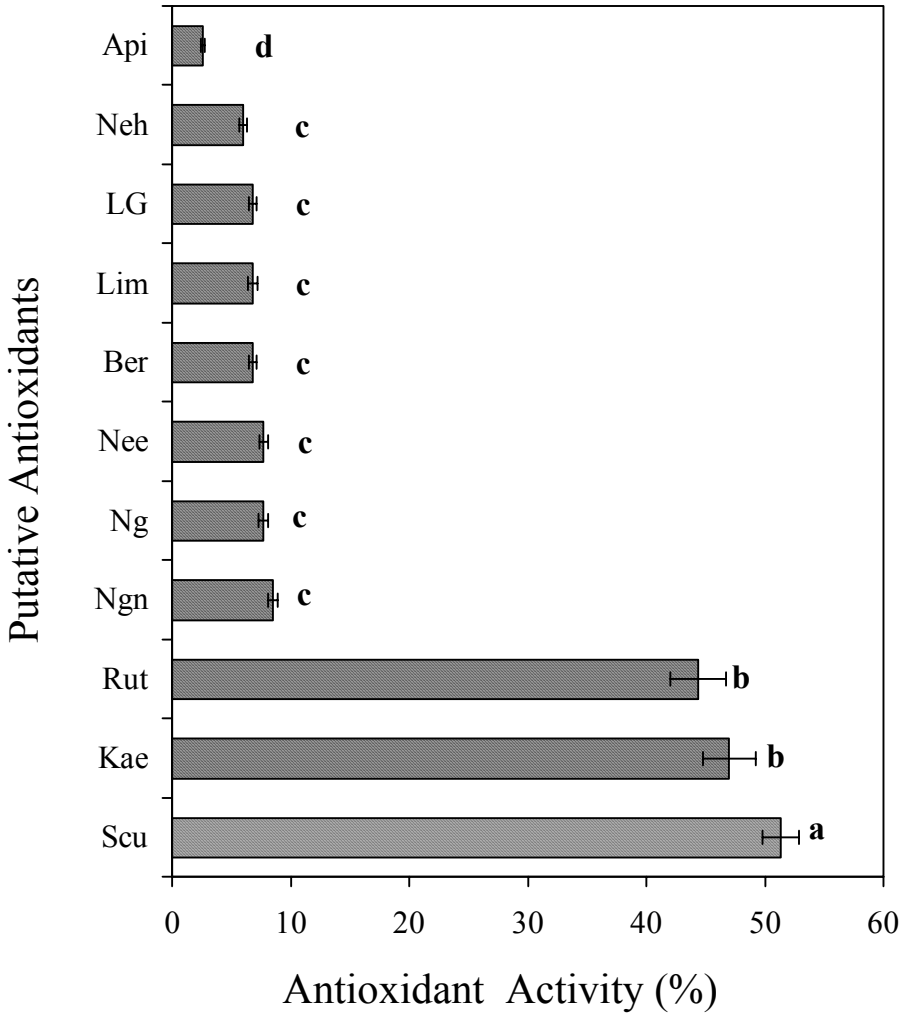


Figure 4-2. Antioxidant activity of limonoids, flavonoids and a coumarin in a β-carotene-linoleic acid bleaching assay system. All the compounds tested were at the final concentration of 10 μM. Values are means ± SEM of triplicate determinations of the auto-oxidation of the linoleic acid/β-carotene emulsion expressed as percentage inhibition of auto-oxidation observed in the absence of test compounds. Values with different letters are significantly different at $P \leq 0.05$.

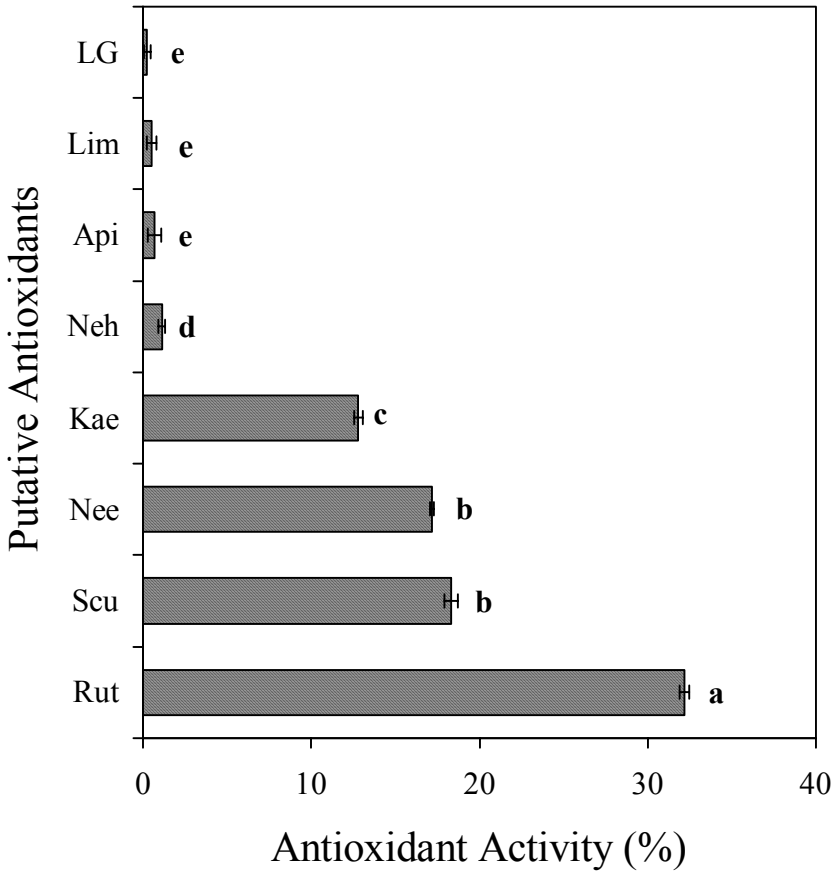


Figure 4-3. Ability of citrus limonoids, flavonoids and one coumarin to scavenge DPPH radicals. All the compounds tested were at the final concentration of 10 μM. The values represent the percentage of DPPH reduction observed in negative control incubations lacking citrus compounds; and Rut served as the positive control. Values are means ± SEM (n=3). Values with different letters are significantly different at $P \leq 0.05$. Bergapten, Ng and Ngn demonstrated negative activity.

Table 4-1. Effect of citrus limonoids and flavonoids on initiation time for copper-mediated conjugated diene formation in hamster LDL.

Compound [1]	Initiation Time (Min) [2]
Rut	2800±20.3 a [3]
Nee	2800±22.4 a
Scu	2140±15.6 b
Kae	1879±14.8 c
Neh	400±5.1 d
Lim	345±4.8 e
Api	340±5.1 e
Ber	160±4.3 f
Ngn	150±4.2 f
Ng	150±5.1 f
LG	120±3.5 g
Control	120±3.2 g

[1]The concentration of compounds was 10 μM.
[2]Each value is the mean ± SEM of triplicate measurements.
[3]Values with different letters are significantly different at $P \leq 0.05$.

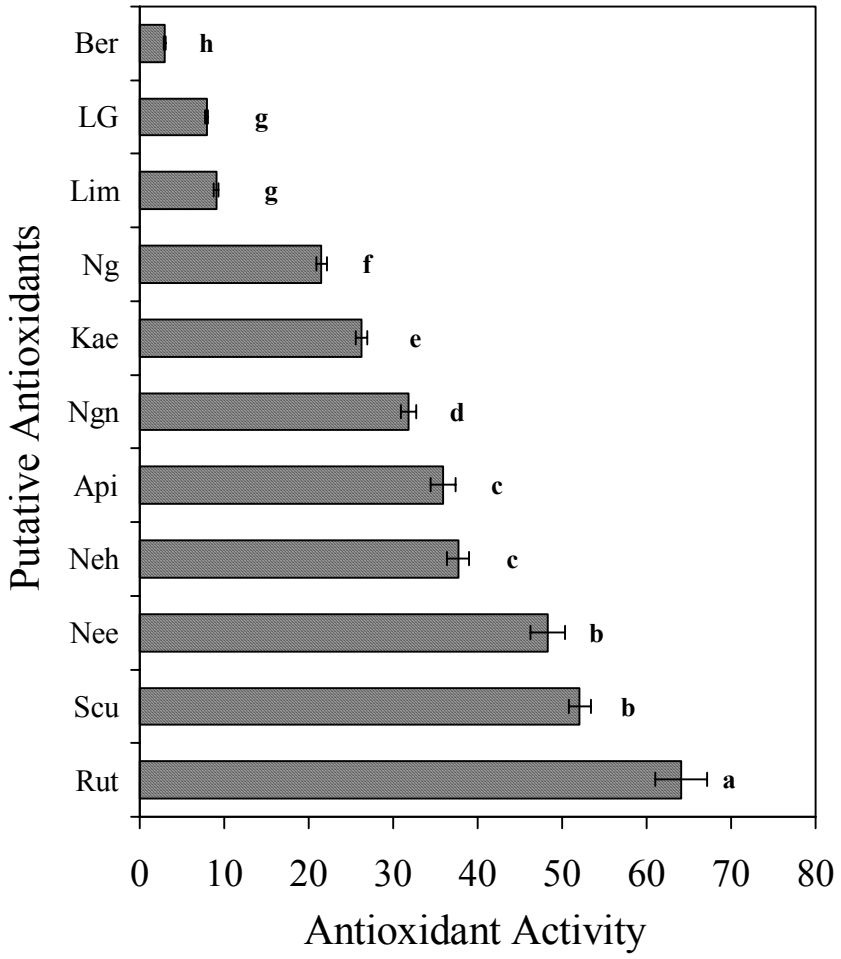


Figure 4-4. Ability of limonoids, flavonoids and a coumarin to inhibit, NBT (nitro blue tetrazolium) reduction. All the compounds tested were at the final concentration of 10 μM. The values represent the percent inhibition of NBT reduction observed in control incubations lacking citrus compounds. Values are means of triplicate determinations ± SEM. Values with different letters are significantly different at $P \leq 0.05$.

Discussion

The bleaching of β-carotene is a free radical mediated phenomenon resulting from the hydroperoxides formed from linoleic acid. The linoleic acid free radical formed by the abstraction of a hydrogen atom from one of its diallylic methylene groups attacks the highly unsaturated β-carotene molecules that will then lose their double bonds by oxidation (Singh et al., 2002; Unten et al., 2000). The presence of antioxidants can hinder the extent of β-carotene bleaching by neutralizing the linoleic acid free radical and other free radicals formed in this system.

In the DPPH assay, antioxidants react with DPPH, a stable free radical, and convert it to α, α-diphenyl-β-picry hydrazine. The degree of discoloration indicates the scavenging potentials of the test compounds. It is believed that the DPPH radical scavenging activity of antioxidants is directly correlated to their hydrogen-donating ability (Blois, 1978; Chen and Ho, 1995; Nakanishi et al., 2002; Yen and Duh, 1994).

In the superoxide assay, the superoxide generated by PMS and NADH easily decomposes into stronger oxidative species, such as singlet oxygen, hydroxyl radical and hydrogen peroxide, each of which can directly initiate lipid oxidation (Trisciuoglio et al., 2002). Antioxidants in this assay work as scavengers on superoxide and its decomposition products.

In the LDL assay, lipid oxidation generally involves three phases: initiation, propagation and decomposition. Chain breaking antioxidants are thought to be particularly beneficial as they terminate the free radical reaction through the donation of hydrogen atoms or electrons to prevent further peroxide formation. In some settings antioxidants can also chelate metal ions such as Cu^{2+} and prevent the initiation of lipid

oxidation (Steinberg et al., 1989). Oxidative modification of LDL is thought to play an important role in the pathogenesis of atherosclerosis and coronary heart diseases (Duh, 1998). Dietary antioxidants that protect LDL from oxidation may therefore reduce atherogenesis and coronary heart diseases (German and Walzem, 2000).

The present data demonstrated that each antioxidant differed in its anti-oxidative capacity toward different sources of free radicals and other oxidants. The antioxidant activities of the 11 citrus phytochemicals were tested with four different model systems. The β-carotene-linoleic acid bleaching method relies on oxygen mediated linoleic acid free radical formation; the DPPH method is based on DPPH free radical mediated oxidation; while the superoxide method relies on oxidation induced by superoxide and its decomposition products. The LDL method assesses lipid oxidation initiated by copper mediated Fenton chemistry. Material testing in multiple *in vitro* assay systems is considered advantageous to assess the antioxidant properties of food components as different reaction mechanisms can be evaluated.

A comparison of the activity of the 11 chemicals in the four assays provides some insight on potential mechanisms of action. For example, Nee demonstrated strong antioxidant activity in the hamster LDL system and weak activity in the β-carotene-linoleic acid system. It is possible that Nee not only scavenged the radicals (offered hydrogen or electrons) but also chelated the Cu^{2+} in the LDL assay inhibiting inhibited Fenton chemistry-mediated oxidation. However, in the β-carotene-linoleic acid method, it appears that Nee only released limited hydrogen or electrons to the system and demonstrated the weak antioxidant activity.

The data presented in this experiment indicated that the marked antioxidant activity of some flavonoids seemed to be due to the fact that the active chemicals are polyphenol compounds containing a chromanol ring system with the capacity to stabilize unpaired electrons and thereby scavenge free radicals.

Pietta (2000) and Bors et al. (1990) suggested that the radical-scavenging activity of flavonoids depended on the structure and substituents of the heterocyclic rings and the B ring. The major determinants for radical-scavenging capability were found to be (a) the presence of a catechol group in ring B, which has better electron-donating properties and is a radical target, and (b) a 2, 3-double bond conjugated with the 4-oxo group, which is responsible for electron delocalization. The presence of a 3-hydroxy group in the heterocyclic ring also increases the radical-scavenging activity, while additional hydroxyl or methoxyl groups at positions 3, 5, and 7 of rings A and C seem to be less important (Bors et al., 1990; Pietta, 2000). In addition to the location and total number of hydroxyl groups, the solubility of the phenolics in the test medium may significantly affect their ability to act as antioxidants (Chen et al., 1996). For example, antioxidant activity of flavonoids in lard appears to be related to the number of *o*-dihydroxy grouping in the A and B rings (Scott, 1997) whereas a lack of conjugation between the B and C rings is a major influence in aqueous media (Rice-Evans et al., 1996).

The results from the present studies indicated that the hydroxyl group in position 6 of ring A (as in scutellarein) could increase the antioxidant activity of flavonoids. These structural features contribute to increase the antioxidant capability of parent flavonoids. Thus, flavonols and flavones containing a catechol group in ring B are highly active, with flavonols more potent than the corresponding flavones because of the presence of the 3-

hydroxyl group. Glycosylation of this group, as in Rut, reduces the radical-scavenging capacity. Methylation of the catechol moiety hydroxyl and the presence of only one hydroxyl in ring B diminish the activity as in Neh, Api, Kae and Scu. Flavanones, such as in Ngn and Ng, due to the lack of conjugation provided by the 2, 3-double bond with the 4-oxo-group, are weak antioxidants.

Limonoids are highly oxygenated triterpenoids with fewer hydroxyl groups than flavonoids. Both of these structural features probably contribute to the weak antioxidant activity seen with these compounds. However, the poor aqueous solubility of Lim may have limited its antioxidation capacity in the present study. Our data demonstrated that Lim had relatively stronger antioxidant activity than LG, especially in the LDL oxidation assay system. Glycosylation is known to limit the antioxidant activity of the flavonoids kaempferol (Plumb et al., 1999) and quercetin (Noroozi et al., 1998) *in vitro*.

There are some reports on the antioxidant activities of Api, Kae, Rut, Neh, Ngn, and Ng. Like the present study these earlier reports also found different activities in different antioxidant assays. Kaempferol has been reported to have stronger antioxidation capacity than Api, and Api was stronger than Rut in the comparison of the total antioxidant activities of flavonoids in human lymphocytes using the comet assay (Noroozi et al., 1998). In a human LDL oxidation assay, Kae showed weaker antioxidant activity than Rut (Brown et al., 1998). In addition, the stoichiometry of the reaction of the flavonoids with the galvinoxyl free radical using electron spin resonance (ESR) spectroscopy showed the antioxidant potential as Rut>Kae>Api; however, the kinetic measurements demonstrated the antioxidant potential as Kae>Rut >Api, where Api showed little reactivity in both measurements, compared with Rut (McPhail et al., 2003).

In addition, Rut has often served as a positive standard for DPPH and other radical scavenging assays (Badami et al., 2003; Kim et al., 2002; Mensor et al., 2001), where it demonstrated good antioxidation capacity. Rutin in the present LDL oxidation assay increased the lag phase about 23-fold and Kae increased it about 18-fold compared to the control, supporting the literature that Rut is more effective than Kae against lipid peroxidation. In the thiobarbituric acid assay using rat brain and kidney homogenates, Ng and Ber have also been reported to inhibit lipid peroxidation slightly at 10.09% and 22.15% respectively (Ng et al., 2000). Naringin and Ngn were reported consistently as having little antioxdant effect in lipid peroxidation systems (Cholbi et al., 1991; Mora et al., 1999); however, Ng and Ngn were reported to perform inconsistently in the DPPH assay (Bao et al., 2004; Chang et al., 2002). It has also been reported that Scu is more potent than kaempferol-3-O-gal in inhibiting lipid peroxidation dependent on Fe^{3+}-ADP/NADPH (Sanz et al., 1994). Neohesperidin has been reported as an alkylperoxyl radical-scavenger (Sawa et al., 1999). In the present study, Ng and Ngn both demonstrated little antioxidant activity in the lipid peroxidation system with inhibition of oxidation less than 25% and negative activity in the DPPH assay at the concentration of 10 μM. Bergapten demonstrated mild antioxidant activity ranging from 0-30% compared to the controls in the different assays. However, Ng, Ngn and Ber all showed less than 20% antioxidant activity in the DPPH assay at the concentration of 20 μM. With Api there was considerable variation in antioxidant activity ranging from 0 to 3-fold when compared to the negative controls. The present data with these flavonoids are again consistent with previous publications.

Conclusion

To the best of our knowledge this is the first study on the antioxidant activity of citrus limonoids. In all of the *in vitro* assays, Lim and LG were very weak antioxidants. However, as has been observed for flavonoids, the limonin aglycone possessed a relatively stronger antioxidant capacity than the limonin glucoside, especially in metal-initiated lipid oxidation. This is the first report on the antioxidant activity of neoeriocitrin (Nee) within metal-initiated lipid oxidation, superoxide radical and DPPH radical scavenging assays. This citrus compound proved an effective antioxidant in each of these assays, ranking 2nd, 3rd and 3rd strongest, respectively of the 11 compounds tested in each assay system. The data further indicated that the hydroxyl group in position 6 of ring A (as in scutellarein versus naringenin) could increase the antioxidant activity of flavonoids. By comparison, the citrus flavonoids demonstrated mild to strong antioxidant activity. The coumarin bergapten provided little to no protection against DPPH or metal-initiated lipid radical mediated oxidation, and inhibited less than 10% of the oxidation in the carotene-bleaching and NBT-superoxide assays.

CHAPTER V

CITRUS LIMONOIDS AND FLAVONOIDS INFLUENCE ON HAMSTER PLASMA LIPOPROTEIN CHOLESTEROL

Introduction

Diets high in fruits and vegetables are protective against a variety of diseases, including cardiovascular disease (Bors et al., 1990; Ness and Powles, 1997). The protective properties of fruits and vegetables are attributed, at least in part, to the high fiber and low saturated fat content (Anderson et al., 1994; Kushi et al., 1995). In addition, fruits and vegetables are also rich in vitamin E, C and a variety of phytochemicals including limonoids and flavonoids (Cook and Samman, 1996; Hertog et al., 1992; Kurowska et al., 2000). Some of these chemicals have also been hypothesized to be partially responsible for the inhibitory effects of fruits and vegetables on the development of atherosclerosis and coronary artery disease (Kinsella et al., 1993; Kurowska et al., 2000). In a recent study with casein fed rabbits, it was found that the substitution of orange juice and grapefruit juice for drinking water significantly lowered cholesterol levels in blood. Further work with HepG2 cells indicated that citrus limonoids (especially limonin) and flavonoids (especially naringenin) were responsible for the reduction in LDL cholesterol (Kurowska et al., 2000a). In humans, consumption of orange juice was found to increased plasma HDL cholesterol concentrations (Kurowska et al., 2000b). Interestingly, several animal studies demonstrated both positive and negative effects in relation to cholesterol using grapefruit flavonones. Among the positive studies,

naringenin and naringin significantly lowered the plasma total cholesterol, the levels of hepatic cholesterol and increased the HDL to total plasma cholesterol ratio in rats fed a high cholesterol diet (1%, w/w) (Choi et al., 1991; Lee et al., 2003; Seo et al., 2003; Shin et al., 1999). Several other studies using naringenin and naringin showed negative effects on plasma cholesterol levels. Lee and co-authors demonstrated that naringenin and naringin had no effects on plasma lipoprotein, total cholesterol, triglyceride, and high-density lipoprotein (HDL) in male New Zealand White (NZW) rabbits (Lee et al., 2001). Furthermore, one study also indicated that naringenin could not attenuate aortic cholesterol accumulation in WHHL rabbits (Mortensen et al., 2001).

To the best of our knowledge, very little information is available using limonin alone or in combination with other limonoids or with flavonoids to reduce or change the distribution of plasma lipoprotein cholesterol in animals.

The present study focuses on the effects of citrus limonoids, flavonoids and grapefruit pulp on hamster plasma cholesterol distribution, LDL particle size and the susceptibility of LDL particles to oxidation.

Materials and Methods

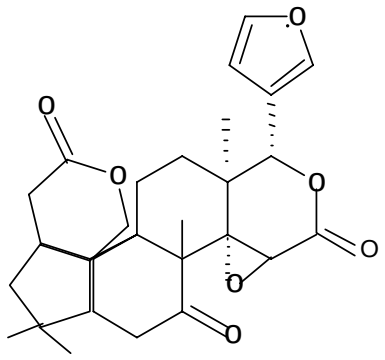
Sources of Citrus Compounds. Limonin (Lim, >98% pure) and limonin 17-β-D- glucopyranoside (LG) (90% pure), were purified according to previously established procedures with some modifications (Tian et al., 2001; Tian et al., 2003) from grapefruit seeds at the Texas A&M University-Kingsville Citrus Center, Weslaco, TX. Before extraction, seeds of mature grapefruits were collected and dried

at 55˚C. 500 g of the dried seeds were ground into powder with a Retch mill (Brinkmann, Westbury, NY). The milled seeds were placed in a Soxhlet extractor and washed overnight with hexane (solvent:seeds=3:1, extraction at 25˚C) to remove the oil. Acetone and methanol were sequentially used to extract the treated seeds. The acetone extract contained about half of the limonoid aglycones, whereas the methanol extract contained all the limonoid glucosides and the remaining aglycones. The methanol fraction was evaporated to dryness with a rotary evaporator under vacuum (<60˚C), and the residue was partitioned with 1:1 methylene chloride-water using an ultrasonic sonicator. The methylene chloride fraction and the previous acetone fraction were combined and evaporated to dryness for purification of the limonoid aglycones. Lim was purified by repeated crystallization in methylene chloride and isopropanol. LG in the aqueous fraction was isolated by successive column chromatography using XAD-2 and WA-30 resins, followed by medium pressure chromatography and preparative HPLC.

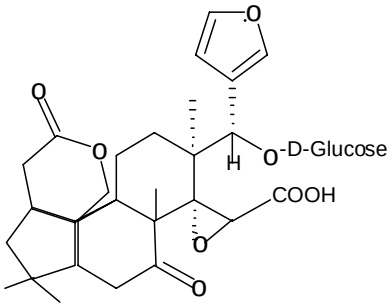
Naringenin (Ngn, 95% pure) and naringin (Ng, 95% pure) were also isolated and purified using flash chromatography. A known quantity of grapefruit molasses and an equal quantity of methanol (1:1) were added and the mixture was stirred for 60 min. The slurry was allowed to stand for the solids to settle and filtered under vacuum. The solids were washed with fresh methanol and dried under suction. The filtrate was concentrated by distillation to remove methanol. The residual liquid obtained was loaded on a DOWEX-50 resin column. The DOWEX-50 column was connected to a SP-70 collection column of similar size. The columns were flushed with deionized water**,** until most of the sugars were removed. The SP-70 column was

flushed with 100% methanol to elute the adsorbed compounds, which were concentrated under vacuum at 40-45^{o}C. The residual liquid was weighed and cooled under stirring to effect the crystallization of naringin. The slurry containing naringin was filtered under vacuum. The filtrate obtained was weighed and lyophilized to obtain the mixture of flavonoid glucosides. Analysis of compound purity was assessed throughout the extraction procedure using high-performance liquid chromatography (HPLC).

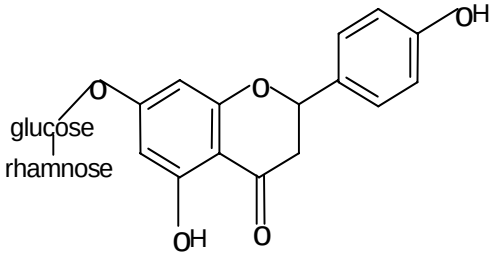
Instrumentation. A Thermo Finnigan (San Jose, CA) HPLC with a photodiode array detector set at 210 nm and 285 nm was used for monitoring the fractions. The freeze-dried fractions after chromatography were also analyzed for purity. UV spectra were taken in the region of 200-400 nm. Chromatographic conditions were as follows: column Novopak C-18 column, 2.1 x 150 mm, 5 μm (Waters, Milford, MA, USA); eluant, (A) 10% acetonitrile (0.03 M phosphoric acid) (B) 24% acetonitrile (0.03M phosphoric acid). The gradient elution had the following profile 0-60 min 100% (A)-100% (B). The flow rate was 1.0 ml/min; temperature was 25^{o} C. The purity of the various fractions was determined using HPLC-electrospray ionization mass spectrometry. Structures of these tested compounds were shown in Figure 5-1.



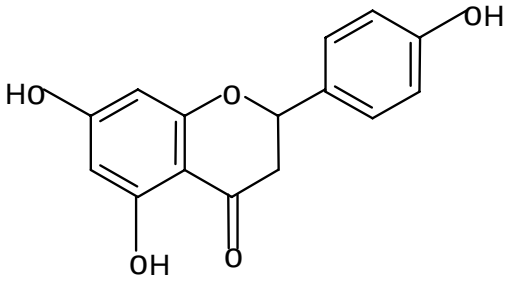
Limonin Aglycone (Lim)



limonin 17-β-D-glucopyranoside (LG)



Naringin (Ng)



Naringenin (Ngn)

Figure 5-1. Structures of citrus chemicals tested in this experiment.

Animals. Twenty-one male Syrian golden hamsters (SASCO, Inc., Madison, WI), 7-9 wks old with a weight of 70-80 g, were randomly housed in wire-top cages, three per cage, in an environmentally controlled room maintained at 20-22°C and 60% relative humidity. A constant 12-h alternating light-dark cycle was maintained throughout the experimental period. The animals were given free access to diets and water. Feed consumption was measured daily and the animals were weighed weekly. Hamsters were fed a commercial rodent chow for 1-week; followed with a hypercholesterolemic diet (basal diet) for 2-weeks. Later animals were randomly assigned to 7 groups of 3 each, with equivalent mean body weight and LDL/HDL cholesterol per group. Each group of animals was then fed for 4 weeks with basal diet or basal diet supplemented with citrus compounds. After 7 weeks of feeding, the animals were terminated for blood collection with cardiac puncture. The protocol for use of animals was approved by the Animal Care and Use Committee of the Texas A&M University, College Station (protocol number 2001-310).

Diets. The diets were formulated by adding 152 g of a blended lipid (90 g butterfat, 35 g vitamin E-stripped corn oil, 25 g fish oil and 2 g cholesterol) plus a basal dose of 3 IU vitamin E to 848g of a powdered fat-and vitamin E-free semi-purified diet base (Dyets, Bethlehem, PA). The citrus compounds added per kg of diet to basal diet, were 200 mg Lim, LG, Ng and Ngn respectively. Grapefruit pulp (10.49 g, freeze-dried, an amount quantified equivalent to 200 mg naringin) was added per kg of diet to basal diet.

The series of treatment diets consisted of Lim, LG, Ng, Ngn and grapefruit pulp diets, and the control diets containing only lipids and basal diet; control 1(C1). As grapefruit also containing pectin, the second control (C2) was used to monitor this factor. Cholesterol, vitamin E, citrus compounds and grapefruit pulp were dissolved or mixed in the lipid blend prior to mixing with the powdered base. Other dietary ingredients were purchased from Dyets (Table 5-1).

Table 5-1. Composition of the experimental diets (g/kg) used for hamster feeding.

Component	C 1*	Lim	LG	Ng	Ngn	Pulp	C 2
Corn Starch	496.93	496.93	496.93	496.93	496.93	496.93	496.93
Vitamin Free Casein	200.13	200.13	200.13	200.13	200.13	200.13	200.13
Cellulose	100.06	99.86	99.86	99.86	99.86	89.57	98.89
Mineral Mix	34.94	34.94	34.94	34.94	34.94	34.94	34.94
Vitamin Mix	10.00	10.00	10.00	10.00	10.00	10.00	10.00
DL-Methionine	2.97	2.97	2.97	2.97	2.97	2.97	2.97
Choline Bitartrate	2.97	2.97	2.97	2.97	2.97	2.97	2.97
Citrus Pectin	0	0	0	0	0	0	1.17
Butterfat	90	90	90	90	90	90	90.00
V_E-stripped Corn Oil	35	35	35	35	35	35	35.00
Fish Oil	25	25	25	25	25	25	25.00
Cholesterol	2	2	2	2	2	2	2.00
Vitamin E	3 IU	3 IU	3 IU	3 IU	3 IU	3 IU	3 IU
Limonin	0	0.2	0	0	0	0	0
Limonin Glucoside	0	0	0.2	0	0	0	0
Naringin	0	0	0	0.2	0	0	0
Naringenin	0	0	0	0	0.2	0	0
Grapefruit Pulp	0	0	0	0	0	10.49**	0

* The abbreviations of C1, Lim, LG, Ng, Ngn, Pulp and C2 represent control 1, limonin, limonin 17-β-D-glucopyranoside, naringin, naringenin, grapefruit pulp and control 2 treatment diets respectively.

** This amount of grapefruit pulp (10.49 g) is equivalent to 0.2 g naringin.

Plasma Collection and Lipoprotein and Total Triglycerides Analysis. After 3 weeks of feeding, the feed was removed for 15-h prior to anesthetizing the animals with O_2 and isofluorane. Blood was collected at the sinus vein using a 100 μl capillary tube. At the end of the 7-week feeding period, the feed was removed again for 15-h prior to anesthetizing the animals. Blood was drawn by open chest cardiac puncture into EDTA-treated syringes. Plasma was collected from the whole blood following centrifugation at 2500 rpm for 20 min at 4°C. Size exclusion chromatography (SEC) was used to analyze total cholesterol distribution of lipoproteins using a Superose^{TM}6 column (Pharmacia Biotech, NJ, USA) and 0.15 M NaCl and 0.02 % NaN_3 as the elution buffer. The chromophore developed by a cholesterol enzyme assay was monitored at 505 nm as described by German et al. (1996). The loading volume of samples was 20 μl. Total cholesterol and total triglycerids were measured by enzymatic methods using commercial kits (Sigma, St, Louis, MO).

Low Density Lipoprotein (LDL) Particle Diameter Analysis. Low density lipoprotein (LDL) was separated from 0.5 ml plasma at 148,600 g for 20 hrs at 14°C in a Beckman (CA, USA) rotor (type, 50.4) as described by Walzem et al. (1994) using density gradient centrifugation by adding NaBr to a basal density (NaCl, density, 1.052 g/ml) solution containing 0.01%EDTA and 50 kU/l streptomycin and penicillin. Separated LDL was stored at 4°C. The particle diameter distributions of plasma LDL were measured by dynamic light scattering using a Microtrack Series 250 Ultrafine Particle Analyzer (Clearwater, FL) with background solution density of

1.052 g/ml (at 20°C) as described by Walzem et al. (1995). Individual LDL diameter distributions were converted to population percentiles prior to statistical analysis in order to normalize raw data. Particle diameters observed at the 50^{th} percentile (medians) were used for statistical comparisons.

Low Density Lipoprotein (LDL) Oxidation Analysis by Conjugated Diene Formation. After LDL particle size analysis, all LDL samples of each group of animals were pooled. The pooled LDL was dialyzed in the dark for 30 hrs at 4°C against four changes of 0.1 M phosphate-buffered saline, pH 7.4 in 0.15 M NaCl (PBS). Conjugated diene formation was used as an endpoint, following initiation of oxidation by the addition of $CuSO_4$ (40 μM) to the solution of LDL (20 μg protein/ml) in 0.1 M PBS. Copper-catalyzed oxidation of LDL was monitored by continuous measurement of absorbance at 234 nm with a DU 640 UV-Vis spectrophotometer (Beckman Coulter, USA) thermostated at 37 °C (Walzem et al., 1995). Lag time was calculated as the intersection of baseline slope with that of the slope during propagation of conjugated diene formation.

Statistical Analysis. Data were analyzed by ANOVA (SAS® for Windows® 9.0 version). Mean values were expressed as Mean ± SEM and values differing by $P \leq 0.05$ were considered significant.

Results

Food Intake and Growth. There were no differences in food intake for the animals fed the seven control and treatment diets (Table 5-2). The weight gain profiles for the hamsters in the seven groups were similar (no significant differences, Figure 5-2).

Lipoprotein Cholesterol Distribution and Total Triglycerides. Plasma total cholesterol (TC) and total triglycerides (TG) are shown in Tables 5-3 and 5-4 respectively. Plasma TC was increased for all seven groups of animals in terms of initial TC and final TC. There were no significant differences between the groups. Final plasma TG concentrations were not different for the different dietary groups. Figure 5-3 shows the LDL-cholesterol to HDL-cholesterol (LDL/HDL-C) distribution change before and after treatment with the citrus phytochemicals. Figure 5-4 demonstrates the typical HPLC profile of hamster lipoprotein distribution after the treatment with hypercholesterolemic diet. The LDL/HDL-C distribution for each dietary group was increased. The C1 dietary group was increased 65.8% and the C2 dietary group increased about 70%. The differences between the control groups were not significant. Percentage increases for animals fed the Lim, LG and grapefruit pulp diets were 36.6%, 52.9%, and 57%, respectively. The data for the Lim and LG groups were significantly different from the C1 dietary group ($P \leq 0.05$). The pulp dietary group was significantly different from the C2 dietary group ($P \leq 0.05$). LDL/HDL-C values for the Ngn and Ng dietary groups were increased by 70.4% and 76.1%,

Table 5-2. Diets consumption of seven groups of hamsters during the treatment of phytochemical diets.

Hamster Groups	C 1	Lim	LG	Ng	Ngn	Pulp	C 2
Daily Consumption (g/hamster)	10.45±0.46	10.28±0.39	10.57±0.5	10.39±0.43	10.42±0.41	10.39±0.4	10.51±0.49

Note: Each group had 15 hamsters and diet consumption was monitored daily. The data were expressed as Mean ± SEM. No statistical differences between the different diet groups were found, $P \leq 0.05$. The abbreviations of C 1, Lim, LG, Ng, Ngn, Pulp and C 2 represent control 1, limonin, limonin 17-β-D-glucopyranoside, naringin, naringenin, grapefruit pulp and control 2 diet groups respectively.

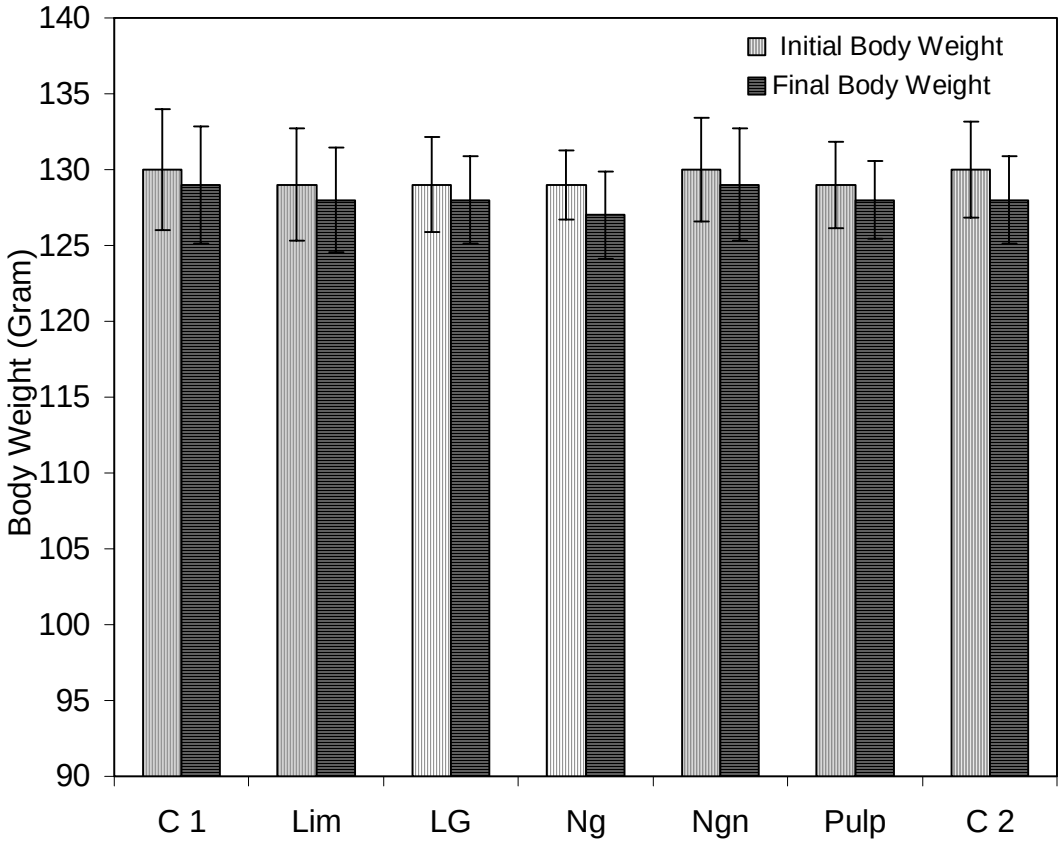


Figure 5-2. The body weight change of seven diet groups of hamsters before and after citrus compounds treatment. Each group had 15 hamsters and the body weight of each hamster was monitored twice a week. The data were expressed as Mean ± SEM. No statistical differences between the different diet groups were found, $P \leq 0.05$. The abbreviations of C1, Lim, LG, Ng, Ngn, Pulp and C2 represent control 1, limonin, limonin 17-β-D-glucopyranoside, naringin, naringenin, grapefruit pulp and control 2 diet groups respectively.

Table 5-3. Effect of citrus functional compounds on hamster plasma total cholesterol (TC).

TC	C 1	Lim	LG	Ng	Ngn	Pulp	C 2
Initial TC (μg/ml)	187±54	173±24	179±27	187±27	204±34	185±28	187±24
Final TC (μg/ml)	855±102	976±130	879±95	873±132	818±107	959±127	882±134

Note: Each group had 15 hamsters and each measurement was duplicated. The data were expressed as Mean ± SEM. Mean values differing by $P \leq 0.05$ were considered significant. No statistical differences between the different diet groups were found. The abbreviations of C1, Lim, LG, Ng, Ngn, Pulp and C2 represent control 1, limonin, limonin 17-β-D-glucopyranoside, naringin, naringenin, grapefruit pulp and control 2 diet groups respectively.

Table 5-4. Effect of citrus chemicals on hamster plasma total triglycerides (TG).

TG	C 1	Lim	LG	Ng	Ngn	Pulp	C 2
Final TG (μg/ml)	972±247	934±224	851±189	870±234	751±216	1052±255	940±225

Note: each group had 15 hamsters, and each measurement was duplicated. The data were expressed as Mean ± SEM. One-way ANOVA was applied. Mean values differing by $P \leq 0.05$ were considered significant. No statistical differences between the different diet groups were found. The abbreviations of C1, Lim, LG, Ng, Ngn, Pulp and C2 represent control 1, limonin, limonin 17-β-D-glucopyranoside, naringin, naringenin, grapefruit pulp and control 2 diet groups respectively.

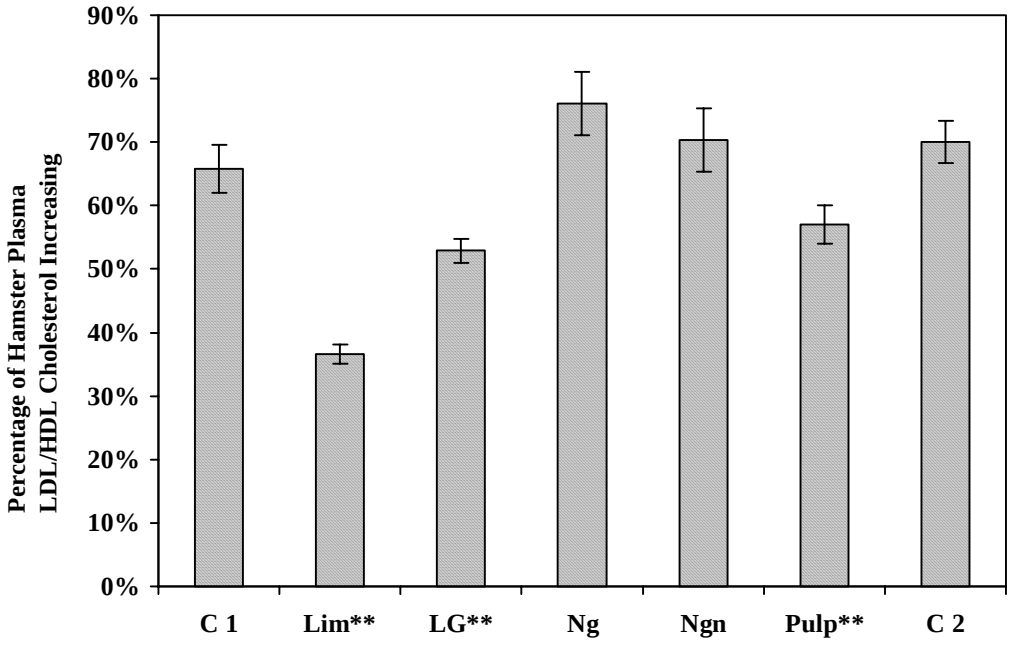


Figure 5-3. The relationship between the various diets and hamster plasma LDL/HDL-cholesterol after 7 weeks feeding. Each group had 15 hamsters. Data were expressed as Mean ± SEM (** means were statistically different from controls, $P \leq 0.05$). The abbreviations of C1, Lim, LG, Ng, Ngn, Pulp and C2 represent control 1, limonin, limonin 17-β-D-glucopyranoside, naringin, naringenin, grapefruit pulp and control 2 diet groups respectively.

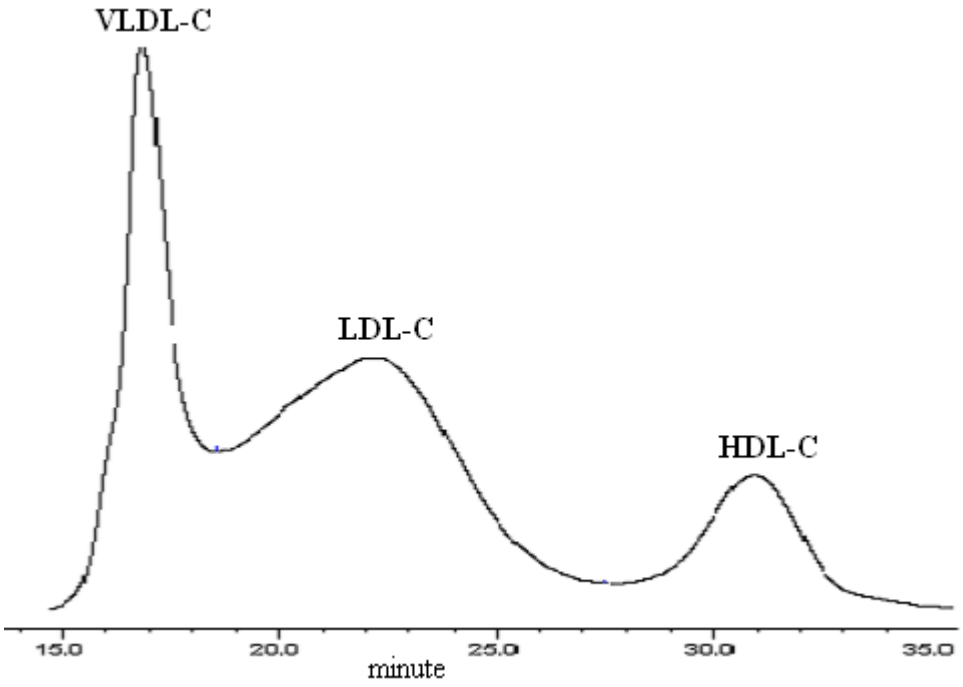


Figure 5-4. Typical HPLC chromatogram profile of hamster plasma lipoprotein cholesterol distribution after the treatment with hypercholesterolemic diet.

respectively, and these two dietary groups did not differ significantly from the C1 dietary group ($P \leq 0.05$).

Low Density Lipoprotein (LDL) Particle Diameter Analysis. Figure 5-5 gives the LDL particle diamters for all of the dietary groups. Particle diamters was highest in the animals fed the Lim diet (21.21±0.35 nm). This value was significantly different ($P \leq 0.05$) when compared to the C1 dietary group (19.96±0.29 nm). There were no significant differences for animals fed the LG diet (20.28±0.43 nm), Ng diet (19.91±0.29 nm), Ngn diet (20.09±0.59 nm), pulp diet (19.98±0.18 nm) and C 2 diet (19.54±0.41 nm).

Low Density Lipoprotein (LDL) Oxidation by Conjugated Diene Formation. Lag time of LDL oxidation by conjugated diene formation from all dietary groups is given in Figure 5-6. Lag times of animals fed the Lim diet (68.75±7.03 min) and LG diet (48.75±2.14 min) were significantly longer ($P \leq 0.05$) than the C1 group of animals (37.5±4.25 min). The lag times for the other dietary groups were not significantly different: Ng diet (41.25±5.35 min), Ngn diet (48.75±4.29 min), Pulp diet (42.5±4.29 min) and C2 diet (36.75±4.14 min).

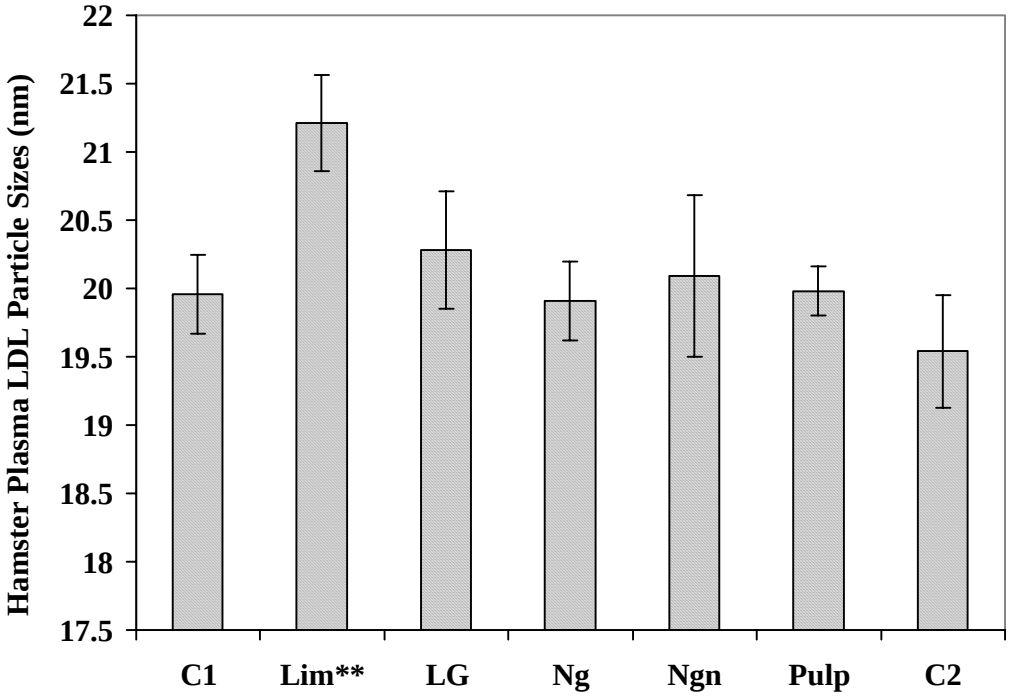


Figure 5-5. Plasma LDL diameter as determined by dynamic laser light scattering for hamsters fed diets containing various citrus components. Values are the medians of population distributions. Each group had 15 hamsters. Data were expressed as Mean ± SEM (** means were statistically different from controls, $P \leq 0.05$). The abbreviations of C1, Lim, LG, Ng, Ngn, Pulp and C2 represent control 1, limonin, limonin 17-β-D-glucopyranoside, naringin, naringenin, grapefruit pulp and control 2 diet groups respectively.

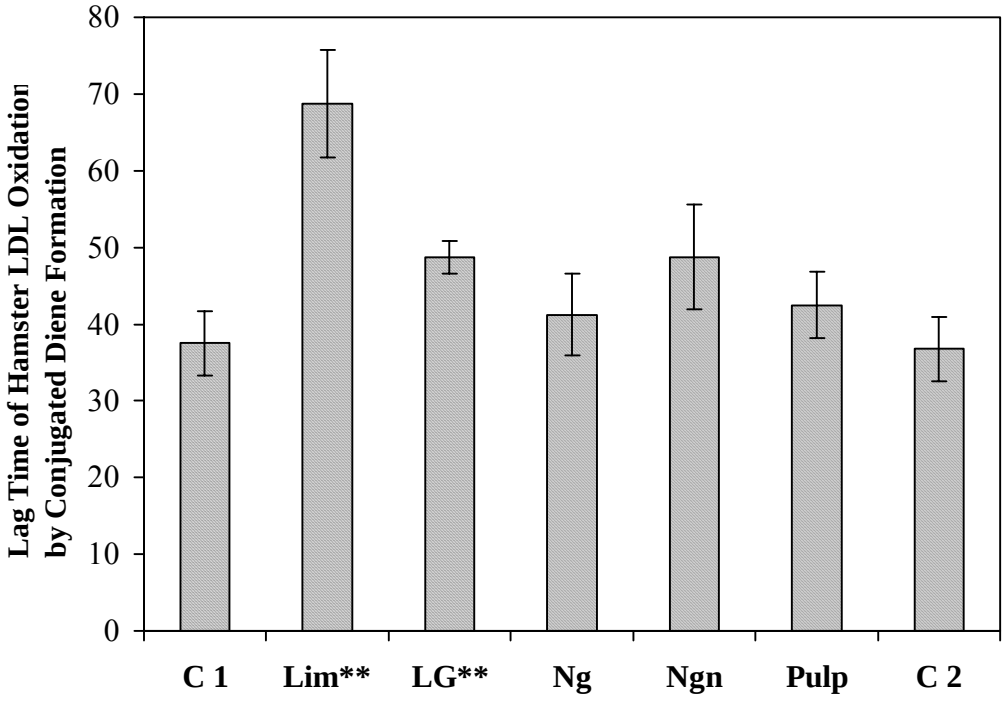


Figure 5-6. The effect of citrus component addition to a basal hypercholesterolemic on the lag time of hamster plasma LDL to Cu^{2+} initiated oxidation as monitored by conjugated diene formation. LDL isolated from each of the 15 hamsters in each group was pooled prior to triplicate assay. Values are means ± SEM (** means were statistically different from controls, $P \leq 0.05$). The abbreviations of C1, Lim, LG, Ng, Ngn, Pulp and C2 represent control 1, limonin, limonin 17-β-D-glucopyranoside, naringin, naringenin, grapefruit pulp and control 2 diet groups respectively.

Discussion

Our present study demonstrated that animals fed the Lim, LG, and grapefruit pulp diets had significantly lower LDL/HDL-C ratios when compared to the corresponding animals fed the control diets. The greatest effect was seen with the Lim diet followed by the LG diet and the grapefruit pulp diet. The Ng and Ngn diets were ineffective. The data with the flavonoid diets and the pectin control diet (Robert, 1997) suggest that the activity of the grapefruit pulp diet may be due to the limonoids, which are present in this diet at low levels. It is also possible that the limonoids and flavonoids were acting synergistically. The fact that limonin worked better than limonin 17-β-D-glucopyranoside agrees with what has been reported with other phytochemicals, the aglycone and the corresponding glucoside (Noroozi et al., 1998; Plumb et al., 1999). One possible explanation for this set of results is uptake with the aglycone being easier to absorb (Plumb et al., 1999). Further work is needed on the bioavailability of citrus limonoids.

The results of LDL particle diameter analyses demonstrated that LDL particle diameters from Lim fed animals were the largest and significantly different from the control animals. No other statistical differences were found. Larger LDL particles are less susceptible to oxidation than smaller ones (Tribble et al., 1992). Any delay in the oxidation of LDL particles could, in turn, inhibit the development of atherosclerosis (Jialal and Devarj, 1994). Furthermore the LDL oxidation experiments showed that LDLs from animals fed the Lim and LG diets had significantly longer lag times, while the lag times for the LDLs from animals fed the Ng, Ngn and Pulp diets showed no significant differences when compared to the controls. The data with the

Lim diet may be due to the larger LDL particle diameter. Larger LDL particles are associated with longer oxidation lag time because of increased stability.

None of the experimental diets reduced either plasma total cholesterol or triglycerides. The data with Ng and Ngn agree with the results of two earlier investigations (Lee et al., 2001; Shin et al., 1999); however, in other studies (Choi et al., 1991; Seo et al., 2003) the flavonoids reduced plasma total cholesterol and/or triglycerides. Two factors may have contributed to the two sets of results. One is the concentration of the phytochemicals in the diets. Based on an earlier study on arterial plaque formation (Xu et al., 1998), we formulated diets containing 200 mg of the test chemical per kg of food. The doses used in the earlier studies (Choi et al., 1991; Seo et al., 2003) were considerably greater. The second factor that may have affected the results was the animal models used in the experiments. We used hamsters instead of rats (Choi et al., 1991; Seo et al., 2003; Shin et al., 1999) or rabbits (Lee et al., 2001). The hamster is an excellent choice as a model since it is possible, through dietary modification, to change hamster LDL/HDL-cholesterol ratios to mimic changes seen in humans (German et al., 1996; Surette et al., 1992).

Conclusion

In general, the present paper demonstrates that limonin and limonin 17-β-D-gluocpyronoside could lower hamster plasma LDL/HDL-C and prolong the lag time of LDL oxidation. Limonin was also found to be able to increase plasma LDL particle diameter. Besides, grapefruit pulp also could lower hamster plasma LDL/HDL-C. From an earlier study (Kurowska et al., 2000), one possible explanation for these results is the apo B lowering potential of limonoids. Further research will need to be done to test this possibility in the hamster model.

CHAPTER VI

OVERALL SUMMARY AND CONCLUSIONS

Supercritical CO_2 (SC-CO_2) has several advantages for extraction of plant bioactive compounds including lack of toxicity, flammability, chemical residual, while requiring only low to moderate operating temperatures and pressures. Therefore, I used this technique to extract limonoids and flavonoids from grapefruit (*citrus paradisi*) seeds and molasses. Experiments using factorial and Box-Behnken designs (3^3 factorials) were used to optimize extraction conditions for citrus limonoids and flavonoids. These optimized methods were then used to successfully isolate several citrus phytochemicals. The isolated citrus limonoids and flavonoids were used to investigate both antioxidant activities *in vitro* and effects on cholesterol and lipoprotein biology in a hypercholesterolemic hamster model *in vivo*. These studies support the following results and conclusions.

Supercritical CO_2 Extraction of Citrus Limonoids from Grapefruit Seeds

Limonoid aglycones, glucosides and flavonoids can be extracted with substantial success from grapefruit seeds with SC-CO_2 technique. The extraction parameters and other results were obtained as follows:

(1) Limonoid aglycones-48.3 MPa, 50˚C and 60 min with CO_2 bottom feeding, flow rate at approximately 5 L/min with the extraction yield of 6.3 mg limonin/g dried grapefruit seeds;

(2) Limonoid glucosides-42 MPa, 52˚C, 45% ethanol and 40 min with CO_2 top feeding, flow rate around 5 L/min resulting in a predicted extraction yield of 0.73 mg limonin glucoside/g seeds.

(3) The maximum extraction yield of naringin 0.2 mg /g defatted grapefruit seeds was obtained using 41.4 MPa, 50˚C, 20% ethanol, 40 min and ~5 L CO_2 /min.

(4) Large quantities of grapefruit seed oils were also obtained with the predominant fatty acids C16:0 (Palmitic acid, 43.231%) and C18:1n9 (Oleic acid, 30.05%).

Supercritical CO_2 Extraction of Limonoid Glucosides from Grapefruit Molasses

Limonoid glucosides can also be extracted with substantial success from grapefruit molasses with supercritical CO_2 and ethanol as co-solvent. The extraction parameters were optimized via Response Surface Analysis (RSA) and are as the follows: 48.3 MPa, 50˚C, 10% ethanol, 40 min and ~5 L/min CO_2 flow rate with an extraction yield of 0.61 mg limonin glucoside/g grapefruit molasses. The results demonstrated practical significance for further application of supercritical fluid extraction of limonoids in commercial production.

Antioxidant Activities of Citrus Limonoids, Flavonoids and Coumarins

To the best of my knowledge this is the first study on the antioxidant activity of citrus limonoids.

(1) In all of the *in vitro* assays, Lim and LG were very weak antioxidants. However, as has been observed for flavonoids, the limonin aglycone possessed a

relatively stronger antioxidant capacity than the limonin glucoside, especially in metal-initiated lipid oxidation.

(2) The present report is the first known to the author on the antioxidant activity of neoeriocitrin (Nee) using metal-initiated lipid oxidation, superoxide radical and DPPH radical scavenging assays. This citrus compound proved an effective antioxidant in each of these assays, ranking 2nd, 3rd and 3rd strongest, respectively of the 11 compounds tested in each assay system.

(3) By comparison, the citrus flavonoids demonstrated mild to strong antioxidant activity.

(4) The hydroxyl group in position 6 of ring A (as in scutellarein versus naringenin) appears to increase the antioxidant activity of flavonoids.

(5) The coumarin, bergapten, provided little to no protection against DPPH or metal-initiated lipid radical mediated oxidation, and inhibited less than 10% of the oxidation in the carotene-bleaching and NBT-superoxide assays.

Influences of Citrus Limonoids and Flavonoids on Hamster Plasma Lipoprotein Cholesterol

The following conclusions were obtained:

(1) Limonin and limonin 17-β-D-gluocpyranoside lowered hamster plasma LDL/HDL-C and prolong the lag time of LDL oxidation.

(2) Limonin was also found to be able to increase plasma LDL particle diameter.

(3) Grapefruit pulp also lowered hamster plasma LDL/HDL-C.

(4) One possible explanation for these results is the apo B lowering potential of limonoids. Further research will be needed to test this possibility in the hamster model.

LITERATURE CITED

Anderson, J.W., B.M. Smith, and N.J. Gustafson. 1994. Health benefits and practical aspects of high-fiber diets. Am. J. Clin. Nutr. 59:1242-1247.

Arigoni, D., D.H.R. Barton, E.J. Corey, O. Jeger, L. Caglioti, S. Dev, P.G. Ferrini, E.R. Glazier, A. Melera, S.K. Pardhan, K. Schaffner, S. Sternhell, J. F. Templeton, and S. Tobinaga. 1960. The constitution of limonin. Experientia 15:41-84.

Badami, S., M.K. Gupta, and B. Suresh. 2003. Antioxidant activity of the ethanolic extract of Striga orobanchioides. J. Ethnopharmacol. 85:227-230.

Bao, H., H.F. Ren, H. Endo, Y. Takagi, and T. Hayashi. 2004. Effects of heating and the addition of seasonings on the anti-mutagenic and anti-oxidative activities of polyphenols. Food Chem. 86:517-524.

Battinelli, L., F. Mengoni, M. Lichtner, G. Mazzanti, A. Saija, C.M. Mastnonni, and V. Vullo. 2003. Effect of limonin and nomilin on HIV-1 replication on infected human mononuclear cells. Planta Med. 69:910-913.

Blois, M.S. 1958. Antioxidant determinations by the use of a stable free radical. Nature 181:1199-1201.

Bors, W., W. Heller, C. Michel, and M. Saran. 1990. Flavonoids as antioxidants: Determination of radical-scavenging efficiencies. Meth. Enzymol. 186:343-355.

Box, G.E.P. and D.W. Behnken. 1960. Some new three level designs for the study of quantitative variables. Technometrics 2:455.

Braddock, R.J. and K.R. Cadwallader. 1992. Citrus by-products manufacture for food use. Food Technol. 46:105-110.

Braddock, R.J. and R.B. Charles. 2001. Extraction parameters and capillary electrophoresis analysis of limonin glucoside and phlorin in citrus byproducts. J. Agric. Food Chem. 49:5982-5988.

Bravo, L.B. 1998. Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. Nutr. Rev. 56:317-333.

Brogle, H. 1982. CO_2 in solvent extraction. Chem. and Ind. 19:385-390.

Brown, J.E, H. Khodr, R.C. Hider, and C.A. Rice-Evans. 1998. Structural dependence of flavonoid interactions with Cu^{2+} ions: implications for their antioxidant properties. Biochem. J. 330:1173-1178.

Brunetti, L., E. Daghetta, I. Fedeli, I. Kikic, and L. Zandrighi. 1989. Deacidification of olive oils by supercritical carbon dioxide. J. Amer. Oil Chem. Soc. 66:209-217.

Buchner, E.H. 1906. Die beschrankte mischbarkeit von flussigkeiten das system diphenyamin und kohlensaure Z. Phys. Chem. 56:257-260.

Cagniard de la Tour, C., 1822. Exposé de quelques résultats obtenus par l'action combinée de la chaleur et de la compression sur certains liquides, tels que l'eau, l'alcool, l'éther sulfurique et l'essence de pétrole rectifiée. Ann. Chim. Phys. 21:127-178.

Chandra, A. and M.G. Nair. 1997. Supercritical fluid carbon dioxide extraction of alpha-and beta-carotene from carrot (*Daucus carota* L.). Phytochemical Anal. 8:244-246.

Chang, W.C., S.C. Kim, S.S. Hwang, B.K. Choi, H.J. Ahn, M.Y. Lee, S.H. Park, and S.K. Kim. 2002. Antioxidant activity and free radical scavenging capacity between Korean medicinal plants and flavonoids by assay-guided comparison. Plant Sci. 163:1161-1168.

Chao, R.R., S.J. Mulvaney, M.E. Bailey, and L.N. Fernando. 1991. Supercritical carbon dioxide conditions affecting extraction of lipid and cholesterol from ground beef. J. Food Sci. 56:183-187.

Chen, C.W. and C.T. Ho. 1995. Antioxidant properties of polyphenols extracted from green and black teas. J. Food Lipids 2:35-46.

Chen, Z.Y., P.T. Chan, K.Y. Ho, K.P. Fung, and J. Wang. 1996. Antioxidant activity of natural flavonoids is governed by number and location of their aromatic hydroxyl groups. Chem. Phys. Lipids 79:157-163.

Choi, J.S., T. Yokozawa, and H. Oura. 1991 Antihyperlipidemic effect of flavonoids from prunus-davidiana. J. Nat. Prod. 54:218-224.

Cholbi, M.R., M. Paya, and M.J. Alcaraz. 1991. Inhibitory effects of phenolic compounds on CCl_4-induced microsomal lipid peroxidation. Experientia 47:195-199.

Coenan, H., R. Hagen, and M. Knuth. 1983. Method for obtaining aromatics and dyestuffs from bell peppers. U.S. Patent 4,400,398.

Cook N.C. and S. Samman. 1996. Flavonoids-chemistry, metabolism, cardioprotective effects, and dietary sources. J. Nutr. Biochem. 7:66-76.

Duthie, G. and A. Crozier. 2000. Plant-derived phenolic antioxidants. Curr. Opin. Lipidol. 11:43-47.

Emerson, O.H. 1948. The bitter principles of citrus fruit. I. Isolation of nomilin, a new bitter principle from the seeds of orange and lemons. J. Amer. Chem. Soc. 70:545-549.

Emmons, C.L., D.M. Peterson, and G.L. Paul. 1999. Antioxidant capacity of oat (*Avena sativa* L. extracts) 2. *In vitro* antioxidant activity and contents of phenolic and tocol antioxidants. J. Agric. Food Chem. 47:4894-4898.

Fernandez-Puntero, B., I. Barroso, I. Iglesias, J. Benedi, and A. Villar. 2001. Antioxidant activity of Fraxetin: *in vivo* and *ex vivo* parameters in normal situation versus induced stress. Biol. Pharm. Bull. 24:777-784.

Ferreira, S.R.S., Z.L. Nikolov, L.K. Doraiswamy, M.A.A. Meireles, and A.J. Petenate. 1999. Supercritical fluid extraction of black pepper (*Piper Nigrum* L.) essential oil. J. Supercritical Fluids 14:235-245.

Fong, C.H., S. Hasegawa, C.W. Coggins Jr., D.R. Atkin, and M. Miyake. 1992. Contents of limonin 17-β-D-glucopyranoside in fruit tissue of Valencia orange during fruit growth and maturation. J. Agr. Food Chem. 40:1178-1181.

Fong, C.H., S. Hasegawa, Z. Herman, and P. Ou. 1991. Biosynthesis of limonoid glucosides in lemon (Citrus limon). J. Sci. Food Agric. 54:393-395.

Fong, C.H., S. Hasegawa, M. Miyake, Y. Ozaki, C.W. Coggins Jr., and D.R. Atkin. 1993. Limonoids and their glucosides in Valencia orange seeds during fruit growth and development. J. Agr. Food Chem. 41:112-115.

Froning, G.W., R.L. Wehling, S.L. Cuppett, M.M. Pierce, L. Nicmann, and D.K. Seikman. 1990. Extraction of cholesterol and other lipids from dried egg yolk using supercritical carbon dioxide. J. Food Sci. 55:95-98.

Fujimoto, K., A. Shisikura, K. Arai, N. Kaneda, and S. Saito. 1987. A process for cholesterol-reduced fat production. Japanese Patent No. 62-134042.

Gardner, D.S. 1993. Commercial scale extraction of alpha-acids and hop oils with compressed carbon dioxide, p. 84-100. In: M.B. King and T.R. Bott (eds.). Extraction of Natural Products Using Near-Critical Solvents, Blackie, Glasgow.

German, J.B. and R.L. Walzem. 2000. The health benefits of wine. Annu. Rev. Nutr. 20:561-593.

Gunatilaka, A.A., D.G. Kingston, E.M. Wijeratne, B.M. Bandara, G.A. Hofmann, and R.K. Johnson. 1994. Biological activity of some coumarins from Sri Lankan Rutaceae. J. Nat. Prod. 57:518-520.

Guthrie, N., K. Morley, S. Hasegawa, G.D. Manners, and T. Vandenberg. 2000. Inhibition of human breast cancer cells by citrus limonoids, p. 164-174. In: M.A. Berhow, S. Hasegawa and G.D. Manners (eds.). Citrus Limonoids-Functional Chemicals in Agriculture and Foods. Amer. Chem. Soc., Washington, DC.

Hannay, J.B. and J. Hogarth. 1879. On the solubility of solids in gases. Proc. Roy. Soc. (London) 29:324-328.

Hasegawa, S., R.D. Bennett, Z. Herman, C.H. Fong, and P. Ou. 1989. Limonoid glucusides in citrus. Phytochemistry 28:1717-1724.

Hasegawa, S., R.D. Bennett, and V.P. Maier. 1984. Biosynthesis of limonoids in Citrus seedlings. Phytochemistry 23:1601-1603.

Hasegawa, S., M.A. Berhow, and G.D. Manner. 2000. Citrus limonoids research: an overview, p.1-8. In: M.A. Berhow, S. Hasegawa and G.D. Manner (eds.), Citrus Limonoids-Functional Chemicals in Agriculture and Foods. Amer. Chem. Soc., Washington, DC.

Hasegawa, S., Z. Herman, E.D. Orme, and P. Ou. 1986b. Biosynthesis of limonoids in citrus: sites and translocation. Phytochemistry 25:2783-2785.

Hasegawa, S., V.P. Maier, Z. Herman, and P. Ou. 1986a. Phytohormone bioregulation of nomilin biosynthesis in Citrus limon seedlings. Phytochemistry 25:1323-1325.

Hasegawa, S. and M. Miyake. 1996. Biochemistry and biological functions of citrus limonoids. Food Rev. Int. 12:413-436.

Hasegawa, S., P. Ou, C.H. Fong, Z. Herman, C.W. Coggins Jr., and D.R. Atkin. 1991. Changes in the limonoate A-ring lactone and limonin 17-β-D-glucopyranoside content of navel oranges during fruit growth and maturation. J. Agric. Food Chem. 39:262-265.

Herman, Z., C.H. Fong, and S. Hasegawa. 1991. Biosynthesis of limonoid glucosides in navel orange. Phytochemistry 30:1487-1488.

Hertog, M.G., H.B. Bueno-de-Mesquita, A.M. Fehily, P.M. Sweetnam, P.C. Elwood, and D. Kromhout. 1996. Vegetables, fruit, and cancer prevention: a review. J. Am. Diet Assoc. 5:1027-1039.

Hertog, M.G.L., E.J.M. Feskens, P.C.H. Hollman, M.B. Katan, and D. Kromhout. 1993. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen elderly study. Lancet 342:1007-1011.

Hertog M.G., P.C. Hollman, and M.B. Katan. 1992. Content of potentially anticarcinogenic flavonoids of 28 vegetables and 9 fruits commonly consumed in the Netherlands. J. Agric. Food Chem. 40:2379-2383.

Hertog, M.G., D. Kromhout, C. Aravanis, H. Blackburn, R. Buzina, F. Fidanza, S. Giampaoli, A. Jansen, A. Menotti, S. Nedeljkovic et al. 1995. Flavonoid intake and long-term risk of coronary heart disease and cancer in the seven countries study. Arch. Intern. Med.155:381-386.

Higby, R.H. 1938. The bitter constituents of navel and Valencia oranges. J. Amer. Chem. Soc. 60:3013-3018.

Jialal, I. and S. Devarj. 1996. The role of oxidized low density lipoprotein in atherogenesis. J. Nutr.126:1053S-1057S.

Kim, D.O., K.W. Lee, H.J. Lee, and C.Y. Lee. 2002. Vitamin C equivalent antioxidant capacity (VCEAC) of phenolic phytochemicals. J. Agric. Food Chem.50:3713-3717.

Kinsella, J.E., E. Frankel, B. German, and J. Kanner. 1993. Possible mechanisms for the protective role of antioxidants in wine and plant foods. Food Technol. 47:85-89.

Krukonis, V.J. 1985. Processing of polymers with supercritical fluids. Polym. News. 11:7.

Krukonis, V.J. 1988. Processing with supercritical fluids: overview and applications, p. 30. In: B.A. Chapentier and M.R. Sevenants (eds.). Supercritical Fluid Extraction and Chromatography, Techniques and Applications, Amer. Chem. Soc., Washington, DC.

Kurowska, E.M., C. Banh, S. Hasegawa, and G.D. Manners. 2000b. Regulation of apo B production in HepG2 cells by citrus limonoids, p. 175-184. In: M.A. Berhow, S. Hasegawa and G.D. Manners (eds.). Citrus Limonoids-Functional Chemicals in Agriculture and Foods. Amer. Chem. Soc., Washington, DC.

Kurowska, E.M., N.M. Borradaile, J.D. Spence, and K.K. Carroll. 2000a. Hypocholesterolemic effects of dietary citrus juices in rabbits. Nutr. Res. 20:121-129.

Kurowska, E.M. and J.A. Manthey. 2004. Hypolipidemic effects and absorption of citrus polymethoxylated flavones in hamster with diet-induced hypocholesterolemica. J. Agric. Food Chem. 52:2879-2886.

Kushi, L.H., E.B. Lenart, and W.C. Willett. 1995. Health implications of Mediterranean diets in light of contemporary knowledge. 1. Plant foods and dairy products. Am. J. Clin. Nutr. 61:1407-1415.

Lam, L.K.T. and S. Hasegawa. 1989. Inhibition of benzo[a]pyrene-induced forestomach neoplasia in mice by citrus limonoids. Nutr. Cancer 12:43-47.

Lam, L.K.T., S. Hasegawa, C. Bergstrom, S.H. Lam, and P. Kenney. 2000. Limonin and nomilin inhibitory effects on chemical-induced tumorigenesis, p. 185-200. In: M.A. Berhow, S. Hasegawa and G.D. Manners (eds.). Citrus Limonoids-Functional Chemicals in Agriculture and Foods. Amer. Chem. Soc., Washington, DC.

Lam, L.K.T., Y. Li, and S. Hasegawa. 1989. Effects of citrus limonoids on glutathione S-transferase activity in mice. J. Agr. Food Chem. 37:878-880.

Laws, D.R., N.S. Bth, and A.G. Wheldon. 1980. Hop extraction with carbon dioxide. U.S. Patent 4,218,491.

Lee, C.H., T.S. Jeong, Y.K. Choi, B.H. Hyun, G.T. Oh, E.H. Kim, J.R. Kim, J.I. Han, and S.H. Bok. 2001. Anti-atherogenic effect of citrus flavonoids, naringin and naringenin, associated with hepatic ACAT and aortic VCAM-1 and MCP-1 in high cholesterol-fed rabbits. Biochem. Biophys. Res. Commun. 284:681-688.

Lee, H., B.H. Chung, and Y.H. Park. 1991. Extraction of tocopherols from soybean sludge by supercritical carbon dioxide. J. Amer. Oil Chem. Soc. 68:571-573.

Lee, M.K., S.S. Moon, S.E. Lee, S.H. Bok, T.S. Jeong, Y.B. Park, and M.S. Choi. 2003. Naringenin 7-O-acetyl ether as inhibitor of HMG-CoA reductase and modulator of plasma and hepatic lipids in high cholesterol-fed rats. Bioorgan. Med. Chem. 11:393-398.

Li, J.X. 2002. Citrus Limonoids: Seasonal Changes and Their Potential in Glutathione S-Transferase Induction. MS Thesis, Texas A&M University, College Station.

Maier, V.P., R.D. Bennett, and S. Hasegawa. 1977. Limonin and other limonoids, p. 355-396. In: S. Nagy, P.E. Shaw and M.K. Veldhuis (eds.). Citrus Science and Technology. AVI Publishing, Westport, CT.

Maier, V.P., S. Hasegawa, and E. Hera. 1969. Limonin D-ring-lactone hydrolase: a new enzyme from citrus seeds. Phytochemistry 8:405-407.

Matthews, R.F., R.L. Rouseff, M. Manlan, and S.I. Norman. 1990. Removal of limonin and naringin from citrus juice by styrene-divinylbenzene resins. Food Technol. 44:130-132.

McPhail, D.B., R.C. Hartley, P.T. Gardner, and G.G. Duthie. 2003. Kinetic and stoichiometric assessment of the antioxidant activity of flavonoids by electron spin resonance spectroscopy. J. Agric. Food Chem. 51:1684-90.

Mensor, L.L., F.S. Menezes, G.G. Leitao, A.S. Reis, T.C. dos Santos, C.S. Coube, and S.G. Leitao. 2001. Screening of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. Phytother. Res. 15:127-130.

Miller, E.G., S.E. Taylor, C.W. Berry, J.A. Zimmerman, and S. Hasegawa. 2000. Citrus limonoids: Increasing importance as anticancer agents, p. 132-144. In: M.A. Berhow, S. Hasegawa and G.D. Manners (eds.). Citrus Limonoids-Functional Chemicals in Agriculture and Foods. Amer. Chem. Soc., Washington, DC.

Mira, L., M.T. Fernandez, M. Santos, R. Rocha, M.H. Florencio, and K.R. Jennings. 2002. Interactions of flavonoids with iron and copper ions: a mechanism for their antioxidant activity. Free Radical Res. 36:1199-1208.

Miyake, M., S. Ayano, Y. Ozaki, H. Maeda, Y. Ifuku, and S. Hasegawa.1991. Extraction of neutral limonoids from citrus seeds. Nippon Nogeik. Kaishi. 6:987-992.

Miyake, M., N. Inaba, S. Ayano, K. Maruyama, and S. Hasegawa. 1996. Method for manufacturing limonoid glucosides. U.S. Patent 5,734,046.

Miyake, M., M. Shimoda, Y. Osajima, N. Inaba, S. Ayano, Y. Ozaki, S. Hasegawa. 2000. Extraction and recovery of limonoids with the supercritical carbon dioxide micro-bubble method, p.96-106. In: M.A. Berhow, S. Hasegawa and G.D. Manner, (eds.), Citrus Limonoids: Functional Chemicals in Agriculture and Foods, Amer. Chem. Soc., Washington, DC.

Modey, W.K., D.A. Mullholland, and M.M. Raynor. 1996. Analytical supercritical fluid extraction of natural products, Phytochem. Analysis 7:1-15.

Mora, A., M. Paya, J.L. Rios, and M.J. Alcaraz, 1999. Structure-activity relationships of polymethoxyflaones and other flavonoids as inhibitorof non-enzymatic lipid peroxidation. Biochem. Pharmaco. 40:793-797.

Mortensen, A., V. Breinholt, T. Dalsgaard, H. Frandsen, S.T. Lauridsen, J. Laigaard, B. Ottesen, and J.J. Larsen. 2001. 17 beta-estradiol but not the phytoestrogen naringenin attenuates aortic cholesterol accumulation in WHHL rabbits. J. Lipid Res. 42:834-843.

Moyler, D.A.1993. Extraction of flavours and fragrances with compressed carbon dioxide, p.140-143. In M.B. King and T.R. Bott, (eds.). Extraction of Natural Products Using Near-Critical Solvents, Blackie, Glasgow.

Ness A.R., and J.W. Powles. 1997. Fruits and vegetables, and cardiovascular disease: a review. Int. J. Epidemiol. 26:1-13.

Ng, T.B., F. Liu, and Z.T. Wang. 2000. Antioxidative activity of natural products from plants. Life Sci. 66:709-723.

Nishikimi, M., N.A. Rao, and K. Yagi. 1972. The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. Biochem. Biophys. Res. Commun. 46:849-854.

Noroozi, M., W.J. Angerson, and M.E. Lean. 1998. Effects of flavonoids and vitamin C on oxidative DNA damage to human lymphocytes. Am. J. Clin. Nutr. 67:1210-1218.

Ou, P., S. Hasegawa, Z. Herman, and C.H. Fong. 1988. Limonoid biosynthesis in the stem of Citrus limon. Phytochemistry 27:115-118.

Ozaki, Y., S. Ayano, N. Inaba, M. Miyake, M. Berhow, and S. Hasegawa. 1995. Limonoid glucosides in fruit, juice and processing by-products of satsuma mandarine (citrus-unshiu marcov), J. Food Sci. 60:186-189&194.

Palma, M., L.T. Taylor, B.W. Zoecklein, and L.S. Douglas. 2000. Supercritical fluid extraction of grape glycosides. J Agric. Food Chem. 48:775-779.

Pietta, P.G. 2000. Flavonoids as antioxidants. J. Nat. Prod. 63:1035-1042.

Plumb, G.W., K.R. Price, and G. Williamson. 1999. Antioxidant properties of flavonol glycosides from tea. Redox Report 4:13-16.

Poiana, M., V. Sicari, and B. Mincione. 1998. Supercritical carbon dioxide extraction of grapefruit flavedo. Flavour-and-Fragrance-Journal 13:125-130.

Ramsay, M.E., J.T. Hsu, R.A. Novak, and W.J. Reightler. 1991. Processing rice bran by supercritical fluid extraction. Food Technol. 45:98-104.

Rice-Evans, C. A., N. J. Miller, and G. Paganga. 1996. Structure-antioxidant activity relationships of flavonoids and phenolic-acids. Free Radical Biol. Med. 20:933-956.

Rice-Evans, C.A. and L. Packer. 1998. Flavonoids in Health and Disease, Marcel Dekker, New York.

Rizvi, S.S., Z.-R. Yu, A. Bhaskar, and L. Rosenberry. 1993. Phase equilibria and distribution coefficients of d-lactones in supercritical carbon dioxide. J. Food Sci. 58:996-1000.

Robert A.B. 1997. Reassessment of some fruit and vegetable pectin levels. J. Food Sci. 62:225-229.

Ronneberg, T.A., S. Hasegawa, C. Suhayda, and Y. Ozaki. 1995. Limonoid glucoside β-glucosidase activity in lemon seeds. Phytochemistry 39:1305-1307.

Ross, J.A. and C.M. Kasum. 2002 Dietary flavonoids: bioavailability, metabolic effects, and safety. Annu. Rev. Nutr. 22:19-34.

Rozzi, N.L. and R.K. Singh. 2002. Supercritical fluids and the food industry. Comprehensive Rev. Food Sci. Food Safety 1:33-44.

Rozzi, N.L., R.K. Singh, R.A. Vierling, and B.A. Watkins. 2002. Supercritical fluid extraction of lycopene from tomato processing byproducts. J. Agric. Food Chem. 50:2638-2643.

Saito, N., Y. Ikushima, K. Hatakeda, S. Ito, and T. Goto. 1991. Fractional extraction of rice bran oil and their ester with supercritical carbon dioxide. J. Agric. Chem. Soc. Japan 65:153-161.

Sanz, M.J., M.L. Ferrandiz, M. Cejudo, M.C. Terencio, B. Gil, G. Bustos, A. Ubeda, R. Gunasegaran, and M.J. Alcaraz. 1994. Influence of a series of natural flavonoids on free radical generating systems and oxidative stress. Xenobiotica 24:689-99.

Sawa, T., M. Nakao, T. Akaike, K. Ono, and H. Maeda. 1999. Alkylperoxyl radical-scavenging activity of various flavonoids and other phenolic compounds: Implications for the anti-tumor-promoter effect of vegetables. J. Agric. Food Chem. 47:397-402.

Scott, G. 1997. Antioxidants in Science, Technology, Medicine and Nutrition. Albion Publishing, Chichester, U.K., p. 208-215.

Seo, H.J., K.S. Jeong, M.K. Lee, Y.B. Park, U.J. Jung, H.J. Kim, and M.S. Choi. 2003. Role of naringin supplement in regulation of lipid and ethanol metabolism in rats. Life Sci. 73:933-946.

Shah, A., C.C. Akoh, R.T. Toledo, and M. Corredig. 2004. Isolation of a phospholipid fraction from inedible egg. J. Supercrit. Fluids. In press.

Shin, Y.W., S.H. Jeong, T.S., K.H. Bae, N.H. Jeoung, M.S. Choi, S.H. Lee, and Y.B. Park. 1999. Hypocholesterolemic effect of naringin associated with hepatic cholesterol regulating enzyme changes in rats. Int. J. Vitam. Nutr. Res. 69:341-347.

Sihvonen, M., E. Jarvenpaa, V. Hietaniemi, and R. Huopalahti. 1999. Advances in supercritical carbon dioxide technologies. Trends Food Sci. Technol. 10:217-222.

Temelli, F. 1992. Extraction of triglycerides and phospholipids from canola with supercritical carbon dioxide and ethanol. J. Food Sci. 57:440-442.

Tian, Q.G., D. Barbacci, S. Schwartz, and B.S. Patil. 2003. Electron ionization of mass spectrometry. Rapid Commun. Mass Sp.17:2517-2522.

Tian, Q., E.G. Miller, H. Ahmad, L. Tang, and B.S. Patil. 2001. Differential inhibition of human cancer cell proliferation by citrus limonoids. Nutr. Cancer 40:180-184.

Tipsrisukond, N., L.N. Fernando, and A.D. Clarke. 1998. Antioxidant effects of essential oil and oleoresin of black pepper from supercritical carbon dioxide extractions in ground pork. J. Agric. Food Chem. 46:4329-4333.

Tribble, D.L., L.G. Holl, P.D. Wood, and R.M. Krauss. 1992. Variations in oxidative susceptibility among six low density lipoprotein subfractions of differing density and particle size. Atherosclerosis 93:189-99.

Vladimirov, I.A., E.A. Parfenov, O.M. Epanchintseva, and L.D. Smirnov. 1991. The antiradical activity of coumarin reductones. Biull. Exsp. Biol. Med. 112:472-475.

Walzem, R.L., P.A. Davis, and R.J. Hansen. 1994. Overfeeding increases very low density lipoprotein diameter and causes the appearance of a unique lipoprotein particle in association with failed yolk deposition. J. Lipid Res. 35:1354-1366.

Walzem, R.L., S. Watkins, E.N. Frankel, R.J. Hansen, and J.B. German. 1995. Older lipoproteins are more susceptible to oxidation: A linking mechanism for the lipid and oxidation theories of atherosclerotic cardiovascular disease. Proc. Nati. Acad. Sci. USA 92:7460-7466.

Xu, R., W.H. Yokoyama, D. Irving, D. Rein, R.L. Walzem, and J.B. German. 1998. Effect of dietary catechin and vitamin E on aortic lipid accumulation in hypercholesterolemic hamsters. Atherosclerosis 137:29-36.

Yen, G.C. and P.D. Duh. 1994. Scavenging effect of methanolic extracts of peanut hulls on free-radical and active-oxygen species. J. Agric. Food Chem. 42:629-632.

Yu, J. and A.Y. Qiu. 2000. Extraction of soybean germ oil with supercritical-CO_2 technique. J. China Oils 25:19-21.

Ziegler, G.R. and Y.-J. Liaw. 1993. Deodorization and deacidification of edible oils with dense carbon dioxide. J. Amer. Oil Chem. Soc. 70:947-953.

Zosel, K. 1964. Method for separation of mixtures. German Patent 1,493,190.

Zosel, K. 1971. The process for the decaffeination of green coffee beans. French Patent 2,079,261.

Zosel, K. 1978. Separation with supercritical gases: practical applications. Agnew. Chem. Int. Ed. Engl. 17:702-705.

Zosel, K. 1979. Process for deodorizing fats and oils. US Patent 4,156, 688.

VITA

Name	Jun Yu
Permanent Address	Chuanpeng Cun, Haihe, Sheyang, Yancheng City, Jiangsu Province, 224365, P. R. China
Birth Date/Location	March, 1973/Yancheng
Parents	Bulou Yu and Guangde Peng
Family	Wife, Shuyu Zhao Son, Dan
Education	
M.S.	Department of Food Science and Technology, Wuxi University of Light Industry, Wuxi, China
B.S.	Department of Chemistry, Shangdong Institute of Building Materials, Ji'nan, China
Professional Experience	
May 2001 to present	Graduate Research Assistant, Texas A&M University