Citrus Limonoids

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Citrus Limonoids

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Foreword

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Preface

Orange growers would love to be able to grow navel oranges as both a fresh produce crop and as a juice crop. Navel oranges are seedless, large, easy to peel, and can withstand mechanical harvesting and packaging—a favorite of fresh fruit consumers all over the world. Unfortunately, navel orange juice has a problem. Although it tastes just fine when it is freshly squeezed from the fruit, it will turn bitter in a few hours at ambient temperature, overnight when refrigerated. This "delayed bitterness" problem has been the target of a number of research efforts in the United States, Australia, Japan, Israel, Spain, Italy, China, Brazil, and other areas where citrus is grown as a commercial crop. We have learned a great deal about the limonoids, the chemical constituents responsible for delayed bitterness in citrus fruits, during the past 40 years. Much of the chemistry and biochemistry of limonoids has been elucidated, and a number of chromatographic techniques have been developed to lower the bitter limonoid levels in citrus juices.

However, as the research went forward on limonoids it was discovered that these compounds have interesting biological functionality in both the plants and the animals that consume them. Thus we stand at an interesting horizon, research is expanded to determine how these compounds function in the human diet. It may well prove that diets rich in citrus limonoids may prevent or deter the development of certain types of cancers.

This book is the first volume to bring together all the aspects of research on citrus limonoids—the chemistry, analysis, biochemistry, and biological activities. It will be of interest to the citrus industry, the food industry, the pharmaceutical industry, the health care industry, and to the general public. Much of the work presented in this book was initiated by the many collaborators working with Dr. Shin Hasegawa and his research group.

During the course of Dr. Hasegawa's biochemical research on limonoids in Pasadena, there were three major breakthroughs. First was the isolation of several species of bacteria that are capable of metabolizing limonoids. The biochemical conversion of citrus limonoids was first demonstrated in bacteria and five biodegradation pathways were established. These findings had significant contribution to elucidation of biosynthetic pathways of limonoids in *Citrus*. The second breakthrough was the discovery that the stems of lemon seedlings were an excellent tool for the preparation of ^{14}C-labeled limonoid substrates for radioactive tracer work, which were required for the study of the biosynthetic pathways of these compounds. The third breakthrough was the most important of all—the discovery that citrus plants have a natural debittering process that converts the limonoid aglycones to non-bitter limonoid glucosides during the final stages of fruit maturation.

For 25 years, Dr. Hasegawa was part of the staff at the U.S. Department of Agriculture (USDA), Agricultural Research Service, Fruit and Vegetable Laboratory in Pasadena, California, where much of his work on the biochemistry of limonoid accumulation was conducted.

The USDA Fruit and Vegetable Chemistry Laboratory: a Brief History

In 1910 the Citrus By-Products Laboratory was established by the Chemistry Bureau of the USDA on South Mission Road in Los Angeles at the request of the California Fruit Growers Exchange (now Sunkist Growers). The name was changed to the Los Angeles Fruit and Vegetable Chemistry Laboratory in 1921. The research group at this laboratory worked on projects aimed at evaluating and enhancing citrus products: juice, vinegar, butter, preserved peel products, and concentrates. The Citrus By-Products research group developed the Brix:acid maturity tests for citrus fruits that are still used today. Other early research projects included the use of ethylene gas as a greening agent; development of maturity standards for avocados, walnuts, and other fruits; dried fruit and vegetable products; determining the composition of lemon oil; and the development of the Davis colorimetric test for the measurement of flavonoid content in grapefruit juices.

In 1947, a fire badly damaged part of the Los Angeles Laboratory. A new facility was built on surplus government property in Pasadena. The new building was dedicated on April 14, 1949, as a field station of the Western Regional Research Laboratory in Albany, California. A number of significant projects have originated here including the invention of modern thin-layer chromatography, the characterization of citrus flavonoids, the development of dihydrochalcone sweeteners, the discovery of a new class of plant biochemical regulators, and the elucidation of the chemistry and biochemistry of citrus limonoid bitter principles.

The Fruit and Vegetable Chemistry Laboratory maintained a strong association with the citrus processing industry. Several industrial groups have financed the positions of scientists at the Laboratory, including the Desert Grapefruit Industry Board, Diamond Nut Growers Association, the California Lima Bean Association, the Date Administrative Committee, and the Citrus Advisory Board. By far the most enduring collaboration was with the Citrus Products Technical Committee, which first began supporting research as the Lemon Advisory Board (originally called the Lemon Products Technical Committee) in 1955.

In February of 1994, the President proposed the closure of the Pasadena laboratory, along with several other USDA research facilities. The Pasadena lab was officially closed in November of 1994, ending a long and successful research program.

We dedicate this book to the many scientists and staff who worked with us at the Fruit and Vegetable Chemistry Laboratory, especially those involved in the research efforts on natural products found in *Citrus*: Dr. Vincent Maier, Dr. Raymond Bennett, Dr. Robert Horowitz, Dr. David Dreyer, Dr. Henry Yokohama, Carl Vandercook, Dr. John Manthey, Dr. Brent Tisserat, Dr. Katherine Kanes, Dr. James Keithley, Dr. Jean Hsu, Zareb Herman, Chi Fong, Peter Ou, Jerry Klavons, Ed Orme, Dora Smolensky, Linda Brewster, Grace Choi, Sadie Vannier, and Karen Kwan.

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Chapter 1

Citrus Limonoid Research: An Overview

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The citrus limonoids are a rare natural compound group that has been thoroughly investigated on their biochemical nature, biological functions, food applications, importance in plant physiology, relationship in different plant species and cultivars, by-product recovery, and commercial applications. There is increased interest in limonoids as potential cancer preventing compounds in foods. This chapter summarizes the previous chemical and biochemical research on citrus limonoids and discusses future research projects which are examining the role these compounds play in both human and plant health.

Citrus fruits are one of the most popular foods in the world, with worldwide agricultural production over 100 million metric tons per year. While a significant quantity of citrus is consumed as fresh fruit, more and more of the crop is being consumed in the form of processed products such as juices, concentrates, flavored citrus beverages, and other food products. Besides being a favorite food, citrus fruits have been shown to possess many constituents which have important effects on the human health: vitamin C, carotenoids, folic acid, flavonoids, limonoids, potassium, high quality soluble fiber, and others. However, one of the long-standing problems in processed citrus products, especially orange and grapefruit juices, has been bitterness. The level of bitterness varies among the different cultivars. Bitter juices have a much lower market value and are sometimes treated with adsorption resins, blended with other nonbitter juices, or discarded. Bitterness in citrus juices is caused by two groups of chemicals: the flavanone neohesperidosides, such as naringin, found in species related to the pummelo, and the limonoids (1, 2).

Limonoids are highly oxygenated triterpenoids present in the Rutaceae and Meliaceae family plants. Limonin, the first characterized compound of this group of phytochemicals, has been known as a constituent of citrus since 1841 (3). It was isolated from navel orange juice in 1938 (4) and shown to be the bitter principle in navel orange juice in 1949 (5). The structure of limonin remained unknown for more than 120 years after its discovery. Its structure was finally determined by a

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combination of chemical methods and X-ray crystallography in the 1960s (6, 7). Limonin's chemical composition is $C_{26}H_{30}O_8$ with a molecular weight of 470.

Limonoids are important quality constituents of citrus fruits and have been shown to possess biological activities. One of the roles limonoid aglycones may play in the plants is as pest deterrents. They are abundant in young leaves and fruit when these tissues need to be protected from pathogen attack (8).

A comprehensive study of the taste thresholds of limonin was performed by Guadagni et al (9, 10). Carefully screened panels of judges who were consistently able to detect limonin at low levels were selected. About 30% of the panel members perceived bitterness in juices containing 2 ppm of limonin. 75% were able to detect bitterness at 6 ppm limonin, which is considered to be the taste threshold level. Limonin bitterness is especially acute in juice obtained from early season fruits, such as navel oranges. Juices from early to mid-season navel oranges can contain as much as 25 ppm of limonin (11). Grapefruit also has significant levels of limonin, an average of 15 ppm or more in the early season (11).

Research on the chemistry and biochemistry of citrus limonoids has made significant progress in recent years, providing new information about limonoids. Progress is being made on a genetic engineering solution to the problem of limonin bitterness in citrus juices. The limonoids are also proving to be important compounds in the human diet.

Analysis of Limonoids

Dreyer (12) made several initial significant contributions to the field of limonoid analysis, including a TLC analysis for limonoid detection and the use of NMR for determination of limonoid structure. These two methodologies were used by Hasegawa and Bennett to isolate and identify 30 additional limonoid aglycones and the 20 limonoid glucosides from citrus and its allied species.

The major analytical techniques for the detection and quantitative analysis of limonoids are HPLC introduced by Fisher (13), radioimmunoassay introduced by Mansell and Weiler (14), and recently HPLC-MS by Manners and Hasegawa (15). Most of these methods (except RIA) require sample preparation protocols using organic solvent extraction, partitioning and solid phase extraction. HPLC has become the most widely used method as it is accurate and reproducable. Both normal phase and reverse phase methods have been developed using both isocratic and gradient development protocols (16).

Delayed Bitterness in Citrus Juices

Navel oranges in general do not taste bitter if eaten fresh or if juice is squeezed from the fruit and consumed immediately. However, the juice becomes bitter within a few hours after juicing at room temperature or overnight if stored in a refrigerator. This gradual development of bitterness, or delayed bitterness, in navel orange juices is caused by the formation of limonin from a tasteless precursor. This delayed bitterness differentiates limonoid bitterness from flavanone neohesperidoside bitterness which occurs in citrus cultivars related to pummelo. Many other winter citrus may also yield

bitter juices due to limonin under certain horticultural conditions. Among 36 limonoid aglycones isolated from *Citrus* and its hybrids, only six are bitter (17). Limonin is the major limonoid found in most citrus fruit juices and is the major cause of delayed bitterness. Nomilin is also involved, but its role is very minor (18). Other bitter limonoids are not found in significant concentrations in the commercial citrus juices (11).

The mechanism of the delayed bitterness was not fully understood until 1968. The precursor theory was first put forth by Higby, after he first isolated limonin from Washington navel orange juice (4). Over the years evidence to support the precursor theory has accumulated. Maier and Beverly (1) finally identified limonoate A-ring lactone as the precursor of limonin in citrus fruit. A ring-closing reaction proceeds under acidic conditions below pH 6.5 and is accelerated by the enzyme, limonin D-ring lactone hydrolase (19). The delayed bitterness is an important economic problem in commercial citrus juice production. It lowers the quality and value of commercial juices and has significant negative economic impact to the citrus industry. Unusual weather and harvesting conditions which cause disruption of fruit tissues, such as freezing or mechanical harvester damage, can promote the acidic pH in the fruit and promote the conversion of limonoate A-ring lactone to limonin making bitter fruit.

Examination of the structures of both the bitter and nonbitter limonoids has revealed some of the structure requirements for the detection of bitterness in the mouth (17). Some other sweet and bitter compounds can alter the detection limits of limonoid bitterness (17). Sucrose is a weak suppresser of limonin bitterness. Other sweeteners such as neohesperidin dihydrochalcone, hesperidin dihydrochalcone, and aspartyphenylalanine methyl ester also act as suppressers. Citric acid has more pronounced suppressing effect on limonin bitter taste detection. Naringin, the flavonoid bitter principle in citrus, has been shown to be a depressor of limonin bitterness detection thresholds.

Biosynthesis of Limonoids

The limonoid biosynthetic pathways have been proposed based on radioactive tracer research by Hasegawa et al (20). Citrus seedlings have been used to prepare ^{14}C-labeled nomilin from labeled acetate and mevalonate (21). Nomilin is most likely the precursor of all other limonoids isolated from *Citrus* and its hybrids. Nomilin is biosynthesized in the phloem region of stem tissues via terpenoid biosynthetic pathways from acetate and mevalonate, via farnesyl pyrophosphate (22). It is then translocated from the stem to leaves, fruit tissues, peels and seeds, where it is further metabolized in each tissue to the other limonoids through at least four different pathways: the limonin pathway, the calamin pathway, the ichangensin pathway and the 7-acetate limonoid pathway (23, 24).

There are five groups of enzymes involved in the biosynthesis and biodegradation of limonoids in *Citrus* (24). One group is present only in the phloem region of citrus stem tissues dedicated to the production of nomilin. A second group which converts nomilin to the other limonoid aglycones occurs in all citrus tissues including leaves, stems, fruit juice sac segments, fruit peel, and seeds regardless of maturity. The activity of limonoid D-ring lactone hydrolase, which catalyzes the

lactonization of the D-ring, occurs in the seeds. Newly synthesized monolactones are converted to dilactones in the seeds by this enzyme during fruit growth. UDP-D-glucose:limonoid glucosyltransferase, which catalyzes the conversion of limonoid aglycones to their respective glucosides during maturation, occurs in fruit tissues and seeds. The activity of limonoid glucoside ß-glucosidase, which catalyzes the hydrolysis of limonoid glucosides to liberate limonoid aglycones and glucose during seed germination, occurs only in seeds.

Glucosidation of Limonoids: a Natural Debittering Process

Limonin bitterness is a problem in the early-season to mid-season winter fruit, but not in late season fruit. As the fruit ripens, the limonoate A-ring lactone concentration decreases (25). This natural debittering process was known for over a century, but the mechanism was not known until the discovery of limonoid glucosides in citrus tissues. In 1989, Hasegawa et al (26) discovered the presence of limonoid glucosides in citrus and determined that limonoid aglycones were converted to their respective glucosides in fruit tissues and seeds during the late stages of fruit growth and maturation (25). Since then, twenty limonoid glucosides have been isolated and identified from *Citrus* and its hybrids (24). Each of these compounds contains one D-glucose molecule attached to a corresponding aglycone via a ß-glucosidic linkage. The presence of the glucose group in this position prevents the formation of the closed D-ring, a key structure requirement for bitterness perception.

In both navel oranges and Valencia oranges grown in California, the glucosidation of aglycones begins in September and continues until fruit is harvested (25, 27). Juice from navel oranges harvested in early to mid-season has delayed bitterness, while the juice from Valencia orange does not. The difference lies in the short maturation period of the navel orange that can be as short as two months before the harvest season starts in November. During this short period, there is insufficient conversion of the aglycones to the glucosides. On the other hand, the Valencia orange has at least six months of maturation before its harvest season begins in March. During this extra 4 months of maturation, the limonoid aglycones are almost completely converted to the tasteless glucosides.

The enzyme responsible for this conversion has been identified as UDP-D-glucose: limonoid glucosyltransferase (28). Limonoid glucosides are found only in mature fruit tissues and seeds, not in immature fruit tissues and seeds, nor in leaves and stems. Sweet oranges in general have relatively high levels of limonoid glucosyltransferase activity. Consequently, they contain high concentrations of limonoid glucosides. In contrast, pummelo has very low activity levels of this enzyme, so juices from fruits harvested even at very late season have low levels of the limonoid glucosides and high levels of the bitter limonoid aglycones. The juices from mature pummelo fruits grown in California contained, on average, 18 ppm of limonin and 29 ppm of total limonoid glucosides (29). Pummelo hybrids such as grapefruit, Oroblanco, and sour orange also possess relatively low concentrations of limonoid glucosides, which indicates that the limonoid glucosyltransferase level of activity is very low in these cultivars as well (30).

Limonoid 17 ß-D-glucopyranoside ß-glucosidase catalyzes the hydrolysis of limonoid glucosides and liberates limonoids and glucose. This enzyme is involved in limonoid biodegradation and is present only in dormant mature seeds and germinating seeds (31). The limonoid glucosides stored in the fruit tissue are very stable. However, in seeds the glucosides are hydrolyzed during germination to liberate glucose and the limonoid aglycones. The limonoids may function as pesticides during the germination process, while the glucose would be used in the general metabolism. Crushing seeds during commercial juice processing may release this ß-glucosidase activity in the juice. This may increase the level of the bitter limonoid aglycones, such as limonin, by hydrolyzing the tasteless limonoid glucosides.

Commercial Juice Debittering Methods

A number of column and batch methods using adsorbent and ion exchange resins have been developed to remove the bitter principles from a variety of citrus juices (32, 33). Although these processes effectively remove the bitter constituents from excessively bitter juices, they are of limited use commercially. None of the debittering processes are truly specific for limonoids, and each method has its drawbacks. Of course, none of these debittering methods can be used on whole fruit. At present, the most widely used method of debittering is to blend bitter juice with nonbitter juice, diluting out the bitter taste.

A promising approach to this problem is to create new citrus varieties by genetic engineering. The procedures for the creation of transgenic citrus plants has already been firmly established. With the insertion of the proper genetic material, it may be possible to create transgenic citrus varieties which produce fruits free from limonin bitterness. Our extensive metabolic research has indicated that the insertion of one of three genes coding for specific limonoid metabolic enzymes may yield nonbitter fruit in transgenic plants. Two of these enzymes have been isolated, and several amino acid sequences of fragments of these enzymes have been determined. Based on this information, a gene for UDP-D-glucose: limonoid glucosyltransferase has been isolated from Satsuma mandarin (34). The insertion of this enzyme gene coding into citrus which is suffering from limonin bitterness, could enhance the natural limonin debittering activity and reduce aglycone concentrations, which would reduce the amount of delayed bitterness.

Biological Activities of Citrus Limonoids

Many furan-containing natural compounds, such as kahweol and cafesterol from green coffee beans, induce a detoxifying enzyme, glutathione S-transferase activity. Citrus limonoids, which contain a furan ring attached at the C17 position, have been shown to be inducers of this enzyme system in mice and rats (35). The ability of a substance to induce increased activity of glutathione S-transferase has been correlated with its inhibitory action against carcinogenesis. Citrus limonoids have been shown to inhibit the formation of chemically-induced neoplasia in forestomach, small intestine, colon, lung, skin and oral of laboratory animals (36, 37). They also inhibit the proliferation of human breast cancer cells in culture (38).

In general, commercial citrus juices contain limonoids below the bitterness taste threshold of 6 ppm. However, the juices contain very high concentrations of limonoid glucosides. Commercial orange juices contain a total limonoid glucoside concentration as high as 500 ppm with an average of 320 ppm (39). Fruit tissues and peels contain limonoid glucosides concentrations even higher than that of juices (27). Since a number of species of bacteria are present in the intestinal flora to hydrolyze limonoid glucosides and liberate limonoid aglycones, citrus juices and fruits are excellent sources of these compounds which seem to act as glutathione S-transferase inducers and inhibitors of chemically induced carcinogenesis. Limonoid glucosides themselves have been shown to have anticarcinogenic activity. Limonin glucoside is an inhibitor of the development of DMBA-induced tumor for oral carcinogenesis in hamster (36). A mixture of limonoid glucosides isolated from orange molasses are strong inhibitors for the proliferation of human breast cancer cells in culture (38).

Antifeedant activity of citrus limonoids against insects was first reported in 1982 (40). It has been firmly established that limonoid aglycones possess antifeedant activity against a number of insects including Colorado potato beetle, corn earworm, fall armyworm, spruce budworm and tobacco budworm (40, 41). A significant number of studies have been carried out on insecticidal activities of limonoids isolated from several plants from the Meliaceae family. Presently, the neem limonoid, azadirachtin, is commercially available for use as an insect repellant on food and ornamental crops. Research has only just begun on the insecticidal properties of limonoids from the plants in the Rutaceae family, including those from citrus. The activity of limonin is roughly 10 times less than that of azadirachtin (42). Limonin and ichangensin are, however, very potent antifeedant compounds against Colorado potato beetle (42). Limonoid glucosides have no antifeedant activity against insects (42).

The biosynthetic groups of limonoids in the citrus cultivars can be used in taxonomic studies of citrus and its near relatives. Limonoids can be classified into several biosynthetic groups, which can be used to tag specific species and cultivars in the Rutaceae family of plants. This analysis can be used to evaluate existing classification schemes or to modify those schemes. As citrus breeders are continually looking for new root stocks and new genetic traits, a limonoid analysis can provide important information in the nomination of new species to evaluate as candidates for breeding studies, especially with the advent of new non-sexual breeding techniques such as protoplast fusion. It is of interest to note that *Skimmiya japonica*, a Rutaceae species from the Toddalioideae subfamily, possesses three of the biosynthetic groups of limonoids: the limonin, the calamin and the ichangensin groups (43). This plant is the only one which has been found to contain more than two groups of citrus limonoids. Although this plant is classified in a subfamily which is fairly remote from *Citrus*, this species may have a close genetic similarity to *Citrus*.

The Future of Citrus Limonoid Research

Much of the basic information of the chemical nature and the biosynthetic steps involved in the formation of the citrus limonoids have been determined. We now stand at the beginning of a new age of nutritional research, the determination of the

roles that the chemical constituents of plant-derived foods play in the long term health and well being of the animals and humans that consume them. This work is just beginning and will be an exciting area of research. We feel the citrus limonoids will prove to play an important role in this arena. The emergence of new analytical and preparative techniques, such as benchtop LC-MS, have made the analysis of natural products such as the limonoids extremely accurate and efficient. These analytical techniques will carry natural product research into this next frontier and allow us to answer important questions about the healing and prevention of chronic diseases.

Limonoid glucosides are water soluble, tasteless, abundant in nature, and are safe natural compounds to consume. They may prove to be ideal food additives for cancer prevention and may be new profitable food additives produced from by-products in the citrus industry. In fact, a citrus juice producer and a health food company in Japan have already test marketed Satsuma mandarin juice products that have been fortified with additional limonoid glucosides— "LG 1000" and " LMG Plus," respectively.

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Chapter 2

Biochemistry of Limonoids in *Citrus*

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Significant progress has been made in understanding the biochemistry of limonoids in *Citrus*. Based on radioactive tracer work and chemical structures, biosynthetic pathways of each of 36 limonoids isolated from *Citrus* and its closely related genera have been elucidated. The glucosidation of limonoid aglycones to form 17β-D-glucopyranoside derivatives during maturation in fruit tissues and seeds has also been established. Also, sites of the limonoid biosynthesis and the process of limonoid accumulation have been established. Biochemical research is now focusing on bioregulation of biosynthesis and accumulation of limonoids for improved fruit quality.

Citrus limonoids are a group of chemically related, highly oxygenated, tetracyclic triterpenoids present in *Citrus* and its closely related genera. Citrus limonoids are responsible for the gradual development of bitterness in citrus juices after juicing, referred to as delayed bitterness (1, 2). They are biologically active, displaying anticarcinogenic activity in laboratory animals (3-6) and cultured human breast cancer cells (7), and antifeedant activity against insects (8,9).

The citrus limonoids occur as limonoid aglycones and limonoid glycosides (10, 11). Limonoid aglycones are glucosidated to limonoid glucosides during fruit maturation (12-14). This glucosidation process naturally debitters citrus bitter limonoid aglycones, and is catalyzed by the enzyme UDP-D-glucoside: limonoid glucosyltransferase (14). Limonoid glucosides such as limonin 17_-D-glucopyranoside **27** (Fig. 10), accumulate only in the fruit and seeds as major secondary metabolites (12-16).

Thirty-six limonoid aglycones and 17 limonoid glucosides have been isolated from *Citrus* and its closely related genera. The biosynthetic pathways of each of these 36 limonoid aglycones have been elucidated (17). Also, sites of the biosynthesis and the process of accumulation of both aglycones and glucosides have been established (17). Biochemical research on citrus limonoids is now focusing on the creation of transgenic citrus by genetic engineering techniques, which is free from limonoid bitterness and has improved nutritional value of the fruit (18). This review summarizes biosynthetic pathways and sites of biosynthesis of limonoids, accumulation of limonoids at various locations, and possible bioregulation of limonoids for improving fruit quality.

Delayed bitterness

Delayed bitterness in a variety of citrus juices is a major problem of the citrus industry and has a significant negative economic impact. Intact fruits do not normally contain bitter limonin **1**, but instead contain the nonbitter precursor of limonin, limonoate A-ring lactone **2** (19, 20). As shown in Fig. 1, this precursor is gradually converted to limonin after juice is extracted. This conversion proceeds under acidic conditions at pH below 6.5 and is accelerated by the action of the enzyme, limonoid D-ring lactone hydrolase. This enzyme has been isolated from *Citrus* and shown to be an extremely heat stable enzyme (21).

Biosynthesis

Thirty-six limonoid aglycones, which have been isolated from *Citrus* and its closely related genera, are classified into four groups: limonin, calamin, ichangensin and 7α-acetate limonoid (Table 1). Biosynthetic pathways of each group of these limonoids have been elucidated.

Substrates for Radioactive Tracer Work

Lemon seedlings are excellent tools for the biosynthetic preparation of radioactively labeled nomilin **3** which can be easily prepared using labeled acetate (22, 23) Up to 5% of labeled acetate can converted to nomilin. Other labeled limonoids can be prepared biochemically or chemically starting from ^{14}[C]-nomilin.

Limonin Group

The true *Citrus* species contain only the limonin group of limonoids. The biosynthetic pathways of each of 20 limonoid aglycones present in this group have been established utilizing radiolabeled substrates and chemical structure correlation (Fig. 2).

Acidic pH

Limonoid D-ring
lactone hydrolase

Limonoate A ring lactone **2**
(Nonbitter)
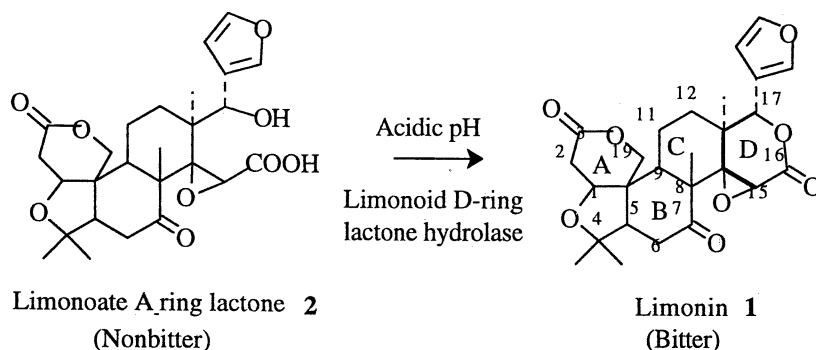
Limonin **1**
(Bitter)

Figure 1. Mechanism of delayed bitterness

Table 1. Biosynthetic classification of limonoids of *Citrus* and its closely related genera

Limonin Group
Limonin, nomilin, obacunone, deacetylnomilin, ichangin, deoxylimonin, citrusin, limonol, 7α-obacunol, deoxylimonol, nomilinic acid, deacetylnomilinic acid, obacunoic acid, deoxylimonic acid, isoobacunoic acid, epi-isoobacunoic acid, isolimonic acid, 19-hydroxydeacetylnomilinic acid, isoobacunoic acid diosphenol and 17-dehydrolimonoic acid A-ring lactone

Calamin Group
Calamin, retrocalamin, cyclocalamin, isocyclocalamin, methyl isoobacunoate diosphenol, 6-keto-7β-nomilol, 6-keto-7β-deacetylnomilol, methyl deacetylnomilinate, calaminic acid, retrocalaminic acid and cyclocalaminic acid

Ichangensin Group
Ichangensin

7α-Acetate Limonoid Group
Limonyl acetate, 7α-obacunyl acetate, 1-(10-19)abeo-obacun-9(11)-en-7α-yl acetate and 1-(10-19)abeo-7α-acetoxy-10β-hydroxyisoobacunoic acid 3,10-lactone
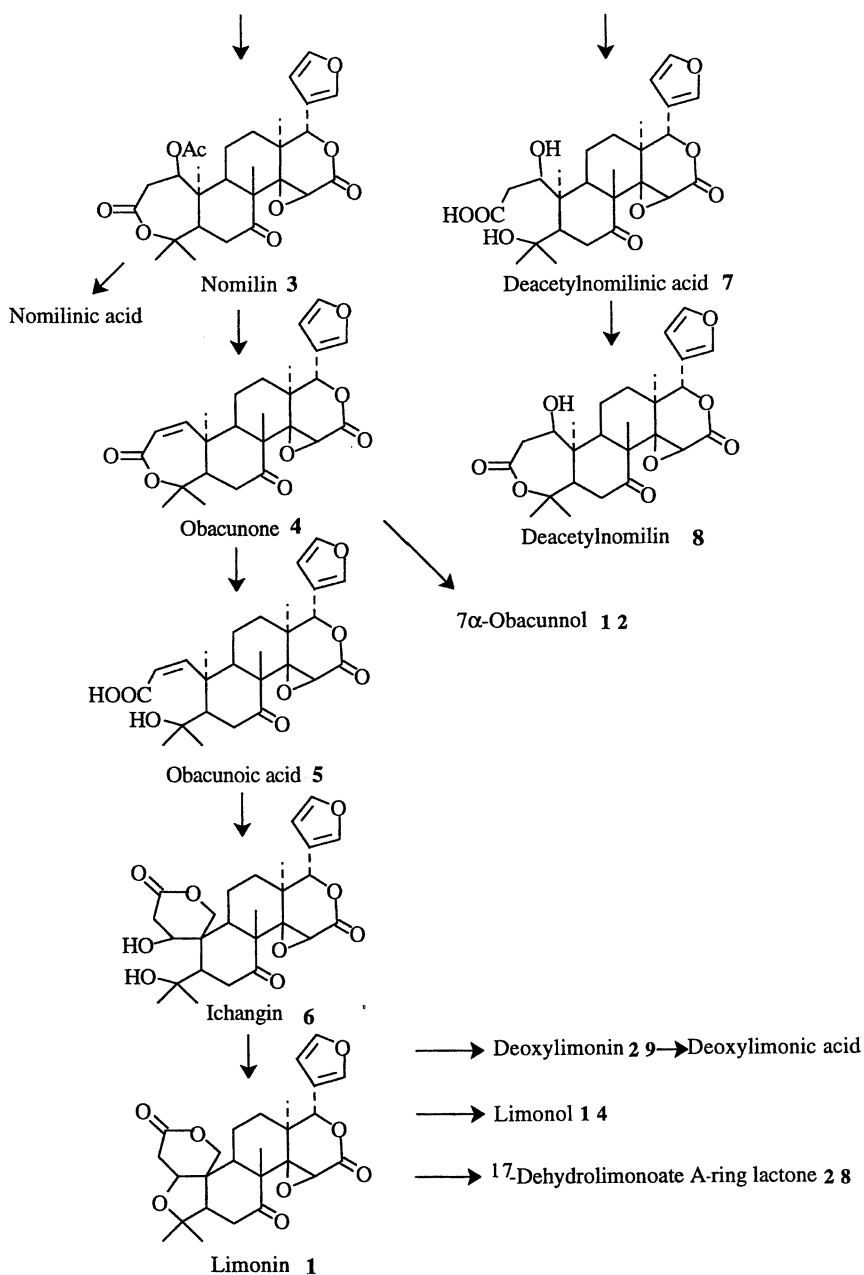
Figure 2. Biosynthetic pathways of the limonin group of limonoids

Limonin **1** , nomilin **3**, obacunone **4** and deacetylnomilin **8** are the major limonoids present in *Citrus*. Radioactive tracer work demonstrated the conversion of nomilin to obacunone (24) as well as the conversion of obacunone to obacunoic acid **5** (25). Trans-elimination of the acetyl group of nomilin gives obacunone, and the opening of the A-ring lactone of obacunone gives obacunoic acid. The enzymes involving in these two steps, nomilin transacetyleliminase and obacunone A-ring lactone hydrolase, have been isolated from bacterial cells (26), but they have not been isolated from *Citrus*. Radioactive tracer work also demonstrated the conversion of obacunoic acid to limonin (25). Ichangin is most likely the immediate precursor of limonin (27). Since labeled ichangin **6** has not been prepared, the step involving ichangin, however, remains hypothetical. Isoobacunoic acid **11** was once considered to be an intermediate between obacunoate and limonin (28). However, labeled isoobacunoic acid has never been shown to be converted to limonin.

Deacetylnomilinic acid **7** and nomilinic acid are the two major acidic limonoids widely distributed in *Citrus*. Deacetylnomilinic acid is most likely the initial precursor of all the limonoids present in *Citrus*. Tracer work demonstrated the conversion of deacetylnomilinic acid to nomilin in stem of *Citrus limon*.(29) (Fig. 2). Nomilin has been shown to be the major limonoid, which is synthesized in stems and translocated to other tissues (22, 23 30). Data accumulated suggest strongly that deacetylnomilinic acid is also most likely biosynthesized in stems and translocated to other tissues.

Deacetylnomilin is most likely biosynthesized from deacetylnomilinic acid. This compound is not directly involved in the biosynthesis of other major limonoids in *Citrus*. However, in *Citrus ichangensis* which has been classified with *Papedocitrus*, this compound is an important intermediate precursor of ichangensin **26** biosynthesis. Nomilinic acid is biosynthesized from nomilin (31).

Radioactive tracer work demonstrated that limonin is converted to deoxylimonin **29** (32) and 17-dehydrolimonoate A-ring lactone **28** (33). The latter compound has been shown to be the only limonoid which reacts negatively toward Ehrlich's reagent (34). Other minor limonoids such as limonol **14** and 7α-obacunol **12** are most likely biosynthesized from limonin and obacunone, respectively.

Sour orange (*Citrus aurantium*) seeds possess high concentrations of three acidic limonoids: isolimonic acid **10** (35) and deacetylnomilinic acid, and a unique acid 19-hydroxydeacetylnomilinic acid **9** (36). They also contain neutral limonoids such as limonin, deacetylnomilin and ichangin (35). All of the limonoids present in *C. aurantium* are derived biosynthetically from deacetylnomilinic acid (Fig. 3). Based upon structural rationale, the biosynthesis can be reasoned to proceed by the following steps. Hydroxylation of 19-methyl group of deacetylnomilinic acid gives 19-hydroxydeacetylnomilinic acid which forms ichangin by lactone ring closure. The 4-hydroxyl group of 19-hydroxydeacetylnomilinic acid cyclizes with the 19-hydroxyl to produce isolimonic acid. Limonin is formed from ichangin by cyclization.

Isoobacunoic acid **11**, which is present in grapefruit seeds, is derived from obacunoic acid or more directly by cyclization of deacetylnomilinic acid (28) (Fig. 4). The presence of isoobacunoic acid in seeds supports the hypothesis that this compound may be the immediate precursor of limonin. However,
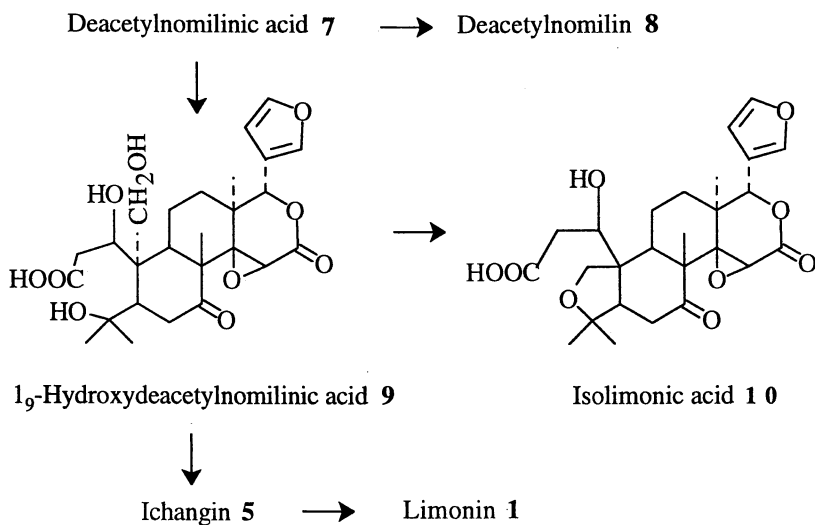
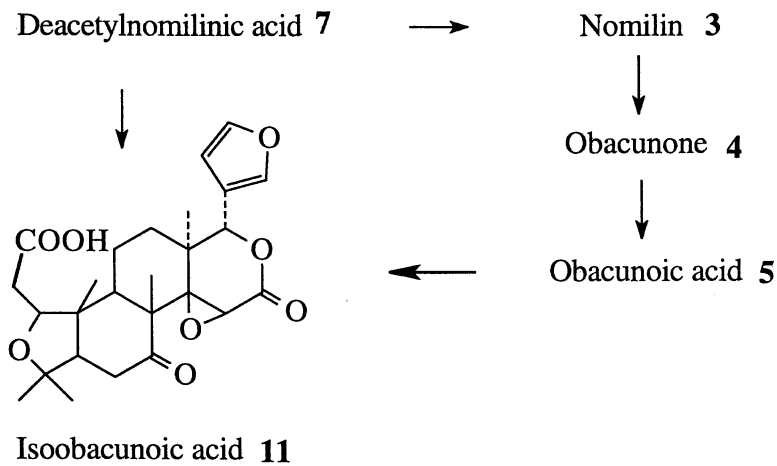Figure 3. Biosynthetic pathways of acidic limonoids in Citrus aurantium

Figure 4. Biosynthetic pathways of isoobacunoic acid

radioactive tracer has shown that [14][C]- isoobacunoic acid is not converted to limonin in *Citrus*. Epiisoobacunoic acid is found to be also a natural constituent of grapefruit seeds (28). This compound is the only known limonoid having the opposite configuration to limonin at C-1. and it is therefore unlikely to be involved in limonin biosynthesis.

7α-Acetate Limonoid Group

Poncirus and its hybrids contain the 7α-acetate limonoid group of limonoids although in very low concentrations (37). Limonyl acetate **15** and 7α-obacunyl acetate **13** can be speculated to be formed biosynthetically from limonol **14** and 7α-obacunol **12**, respectively (Fig. 5). *Poncirus* also contains two unique limonoids in which a methyl carbon has been inserted into a ring (37). The 7α-acetate limonoid 1-(10-19)abeo-obacun-9(11)-en-7α–yl-acetate **16** is structurally related to obacunone **4**. This compound is most likely the precursor of 1-(10-19)abeo-7α, 10β-dihydroxy isoobacunoic acid 3,10-lactone **17**. As shown in Fig. 6, opening the lactone ring of 1-(10-19)abeo-obacun-9(11)-en-7α-yl-acetate followed by addition of the 4-hydroxyl group to the 1,2 double bond, a shift of the 9,11 double bond to the 9,10-position, addition of the 3-carboxyl group at C-10 and finally lactonization would produce compound 1-(10-19)abeo-7α,10β-hydroxy isoobacunoic acid 3,10-lactone.

Calamin Group

The genus *Fortunella* possesses calamin **20** and its biosynthetically related limonoids (38,39). All of the *Fortunella* group of limonoids, except 6-keto-7β-nomilol **18** and 6-keto-7β-deacetylnomilol **19**, are methyl esters and all, except methyl deacetylnomilinate **24**, are oxygenated at C-6. The biosynthetic pathway of this group of limonoids has been established in calamondin, a hybrid of *Fortunella* and *Citrus*.

Radioactive tracer work demonstrated each of all of the biosynthetic steps which produce this group of limonoids starting from deacetylnomilinic acid (39-41) (Fig. 7). The limonoid 6-keto-7β-nomilol and 6-keto-7β-deacetylnomilol possess structural features of both the *Citrus* and the *Fortunella* limonoids, and represent a biosynthetic link between them.   In calamondin, labeled deacetylnomilinic acid was converted to nomilin **3** and 6-keto-7β-nomilol. Conversion of deacetylnomilinic acid to 6-keto-7β-nomilol involves the acetylation at C-1, closing of the A-ring and introduction of 6-keto-7 hydroxy moiety to the B-ring. Hydrolysis of the acetyl ester of 6-keto-7β-nomilol will give 6-keto-7β-deacetylnomilol.   Opening of the A-ring of 6-keto-7β-deacetylnomilol followed by methylation will give calamin **20**. Cyclization of calamin will give cyclocalamin **22**. Oxidation of the OH group at C-7 will cause enolization of the ketone at C-6 and formation of the double bond between C-5 and C-6.   This sequence will give methyl isoobacunoate diosphenol **23**. Calamin converts to retrocalamin **21** on silica gel (38). Retroaldol reactions are usually base-catalyzed. The ease with which this reaction occurs on the silica gel column under neutral conditions was surprising. This raised the possibility that
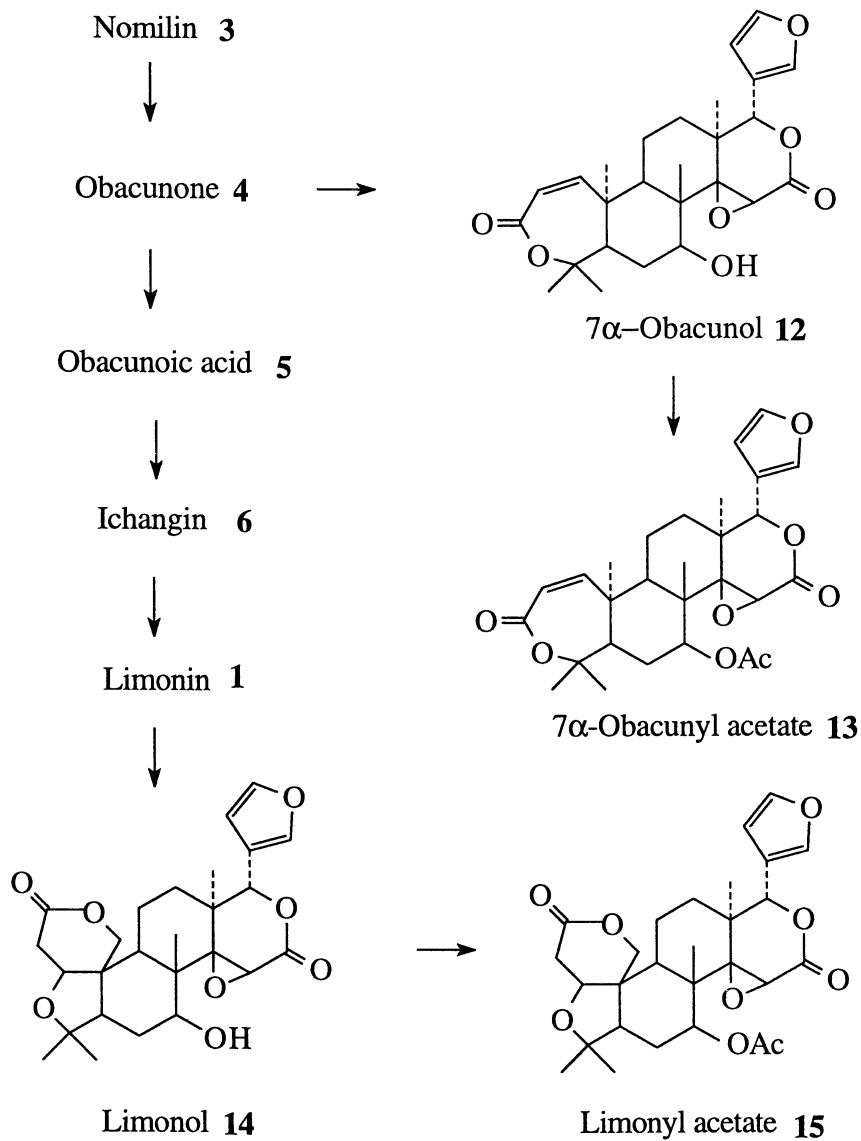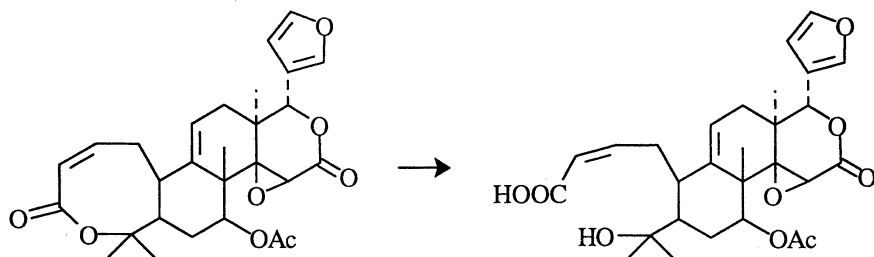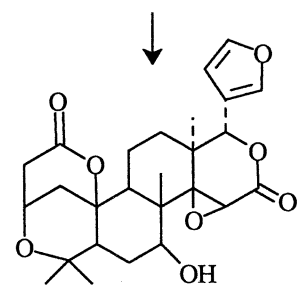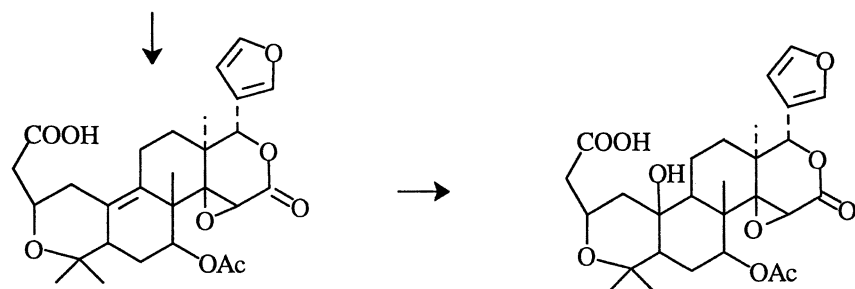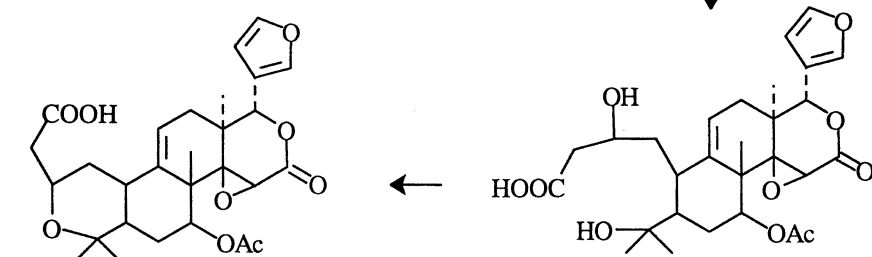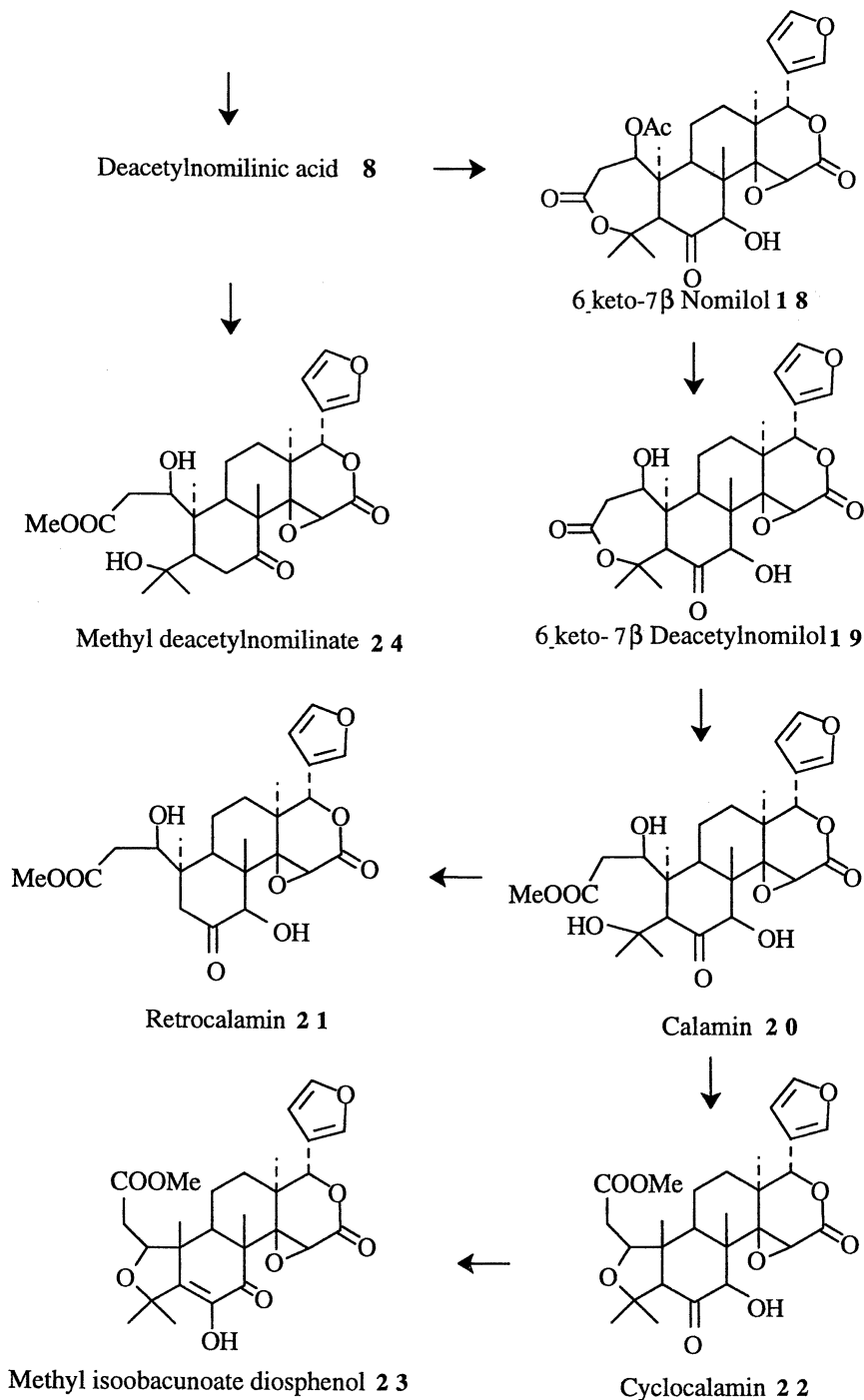
Figure 5. Biosynthetic pathways of the 7α-aceate limonoid group of limonoids

1-(10-19)abeo-obacun-9(11)-en-7a-yl-acetone **16**

1-(10-19)abeo-7α,10β-dihydroxy isoobacunoic acid 3,10-lactone **17**

Figure 6. Biosynthetic pathways of Poncirus limonoids

Deacetylnomilinic acid **8**

6 keto-7β Nomilol **1 8**

Methyl deacetylnomilinate **2 4**

6 keto- 7β Deacetylnomilol **1 9**

Retrocalamin **2 1**

Calamin **2 0**

Methyl isoobacunoate diosphenol **2 3**

Cyclocalamin **2 2**

Figure 7. Biosynthetic pathways of the calamin group of limonoids

retrocalamin may be an artifact produced from calamin during the isolation process. However, TLC analysis, which takes less than 20 min, showed that retrocalamin was present in the original extract prior to chromatography, and when a pure sample of calamin was subjected to the extraction conditions, it was not converted to retrocalamin. Tracer work also demonstrated this conversion in the fruit tissue of calamondin. The chemical conversions of calamin to cyclocalamin and cyclocalamin to methyl isoobacunoate diosphenol were also demonstrated (40).

Ichangensin

Citrus ichangensis has been classified with the *Citrus* subgenus, *Papedocitrus*. This species and its hybrids accumulate a unique limonoid, ichangensin **26** as the major limonoid (42-46) (Fig. 8). They also accumulate relatively high concentrations of deacetylnomilin **8** and deacetylnomilinic acid **7**. Unlike Citrus, they accumulate limonin **1** as a minor limonoid.   Radioactive tracer work has shown that labeled nomilin **3** is converted to deacetylnomilin and ichangensin in fruit tisssutes (43). The conversion of nomilin to deacetylnomilin is presumably catalyzed by nomilin deacetylase. This enzyme activity appears to be unique to this species and its hybrids, and has never been shown in any other *Citrus*   species or in bacteria.   Ichangensin is most likely formed by dehydrogenation of deacetylnomilinic acid, followed by decarboxylation.   In CHCl₃ solution ichangensin is present as two isomers: the keto **25** and ketal **26** forms (42). In fruit tissues, it is present only in the ketal form.

Limonoid Glucosides

Limonoids in citrus tissues and juices have been routinely analyzed by organic solvent extraction followed by TLC and HPLC methods. The aqueous portion from the solvent extraction was ignored. TLC analysis of an aqueous portion after solvent extraction showed that citrus fruit tissues contained high concentrations of water soluble, polar compounds giving an Erhlich-positive reaction on TLC, typical characteristic of limonoids (11).   Four of these compounds were then isolated from grapefruit seeds and characterized as the 17β-D-glucopyranosides of limonin, nomilin, deacetylnomilin and obacunone. Currently, 17 limonoid glucosides have been isolated from *Citrus* and its closely related genera (11, 47, 48) (Table 2). Each has one D-glucose molecule attached to the 17-position of limonoid molecule via a β-glucosidic linkage such as limonin 17β-D-glucopyranoside **27**.

Biosynthesis

Limonoid aglycones are converted to nonbitter 17β-D-glucopyranoside derivatives during maturation (12-14, 49, 50) . This process occurs only in fruit tissues and seeds. In navel oranges and Valencia oranges grown in California

Nomilin **3** $\longrightarrow$ Deacetylnomilin **8**

$\downarrow$

Deacetylnomilinic acid **7**

Ichangensin (ketal) **26**   Ichangensin (keto) **25**
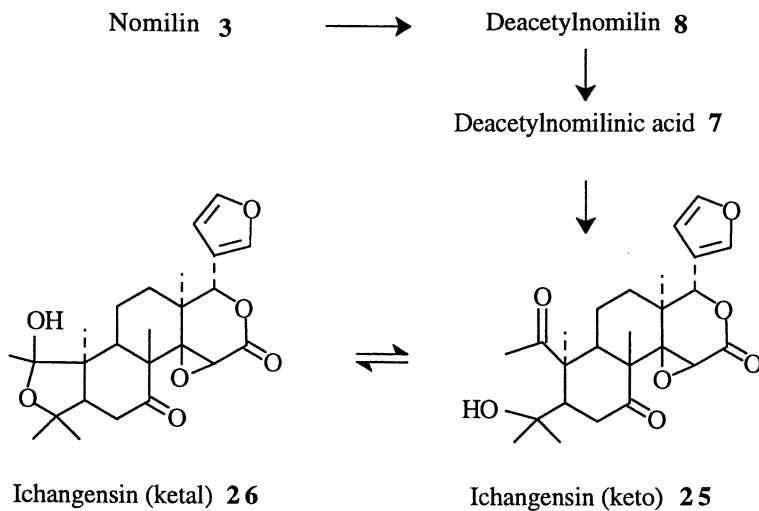
Figure 8. Biosynthetic pathways of ichanginsin

Table 2.   Limonoid glucosides occurring in *Citrus* and its closely related genera.

Monocarboxylic Acids

17β-D-Glucopyranosides of

1. Limonin	2. Nomilin
2. Deacetylnomilin	4. Obacunone
5. Ichangin	6. Ichangensin
7. Calamin	8. 6-keto-7β-deacetylnomilol
9. Methyl deacetylnomilinate	

Dicarboxylic acids

17β-D-Glucopyranosides of

10. Nomilinic acid	11. Deacetylnomilinic acid
12. Obacunoic acid	13. trans-Obacunoic acid
14. Iso-obacunoic acid	15. Epi-isoobacunoic acid
16. Isolimonic acid	17. 19-Hydrodeacetylnomilinc acid

(12, 13), the glucosidation of aglycones begins in September and continues until fruit is harvested (Fig. 9). Juice from navel oranges harvested in the early- to mid-season has delayed bitterness, while the juice from early- to mid-season Valencia orange does not. The difference lies in the short maturation period of the navel orange, which can be as short as two months before the harvest season starts in November. On the other hand, the Valencia orange has at least six months of maturation before the harvest season begins in March. During this extra four months of maturation, the aglycones are almost completely converted to the tasteless glucosides

The conversion of aglycones to glucosides is catalyzed by the enzyme UDP-D-glucose:limonoid glucosyltransferase (limonoid glucosyltransferase) (51) (Fig. 10). This enzyme activity was first recognized in navel orange (12) and shown to be present only in mature fruit tissues (50) and seeds (14). The activity is not present in immature fruit tissues and seeds. Nor has it been shown to occur in leaves, stems and roots. An evaluation of the substrate specificity using the isolated enzyme from navel oranges indicates that a single isoform appears to be responsible for glucosidation of all the limonoid aglycones to their respective glucosides (51).

Limonoid glucosides accumulate in fruit tissues and seeds in significant quantities (14, 15, 52-55). Sweet oranges in general have relatively high levels of limonoid glucosyltransferase activity. Consequently, they contain high concentrations of limonoid glucosides. In contrast, pummelo and its hybrids such as Oroblanco and Melogold contains low activity levels of this enzyme, so juices from fruits harvested at very late season still have a severe limonoid bitterness problem, and low concentrations of the limonoid glucosides (56, 57). Since limonoids possess chemopreventative activity against certain cancers (5, 6, 7), these research findings have made citrus and citrus products more attractive to the public.

Biodegradation

The limonoid glucosides accumulated in the fruit tissues are relatively stable. Radioactive tracer work showed that [14] [C]- nomilin glucosides are stable in albedo tissues of navel oranges, suggesting the absence of a limonoid glucoside β-glucosidase activity in the tissue (50). In seeds, however, the glucosides are rapidly hydrolyzed during germination. Both dormant and germinated lemon seeds hydrolyzed labeled nomilin glucoside to liberate glucose and nomilin (58). This is why young citrus seedlings do not contain limonoid glucosides. The hydrolysis is presumably catalyzed by a limonoid glucoside _-glucosidase, but it has not yet been isolated.

Commercial enzymes such as β-glucosidases from almonds and naringinase from mold do not hydrolyze the limonoid glucosides. However, a species of a bacterium isolated from soil by enrichment on limonoid glucoside as a single carbon source, exhibited limonoid glucoside _-glucosidase activity (11).

Most of citrus limonoid glucosides are stable during juice processing and normal storage periods. One known exception is nomilin glucoside which is converted to nomilinic acid glucoside at pH below 3 and to obacunone glucoside at pH above 8.
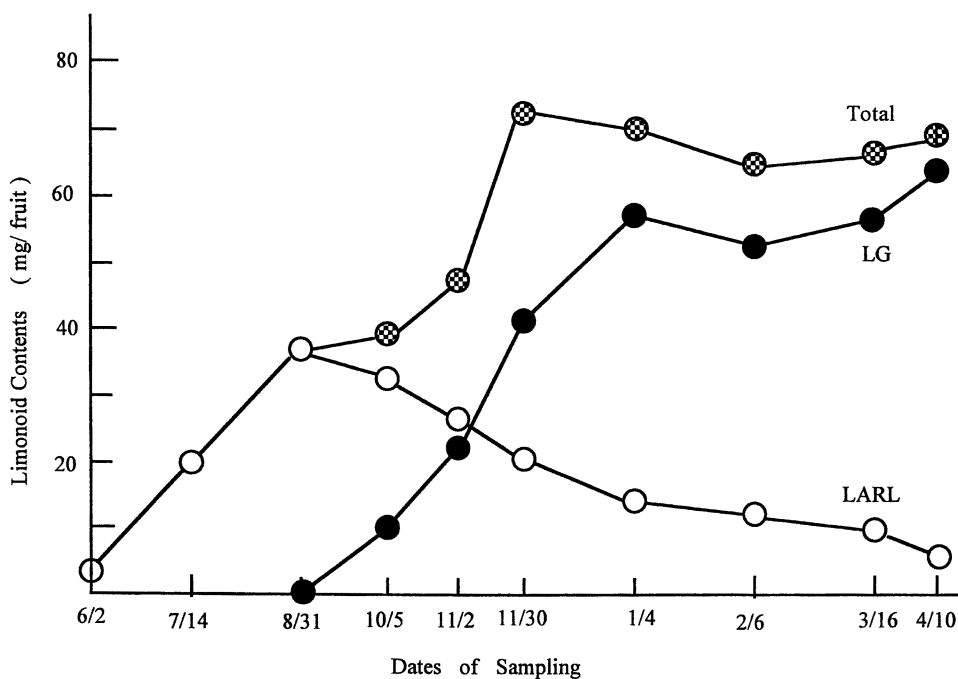
Figure 9. Changes in limonoate A-ring lactone 2 (LARL) and limonin 17β-D-glucopyra-noside 27 (LG) concentration in navel oranges during fruit growth and maturation. LG values are expressed as LARL by multiplying by 0.723. (Reproduced from reference 12. Copyright 1991, American Chemical Society.)
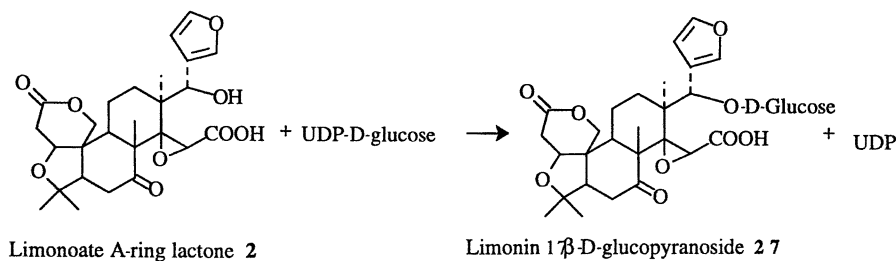

Limonoate A-ring lactone **2**   Limonin 17β-D-glucopyranoside **2 7**

Figure 10. Glucosidation of limonoid aglycone

Sites of Biosynthesis

Limonoids are present in *Citrus* in three chemical forms; limonoid monolactones (open D-ring aglycones such as limonoate A-ring lactone **2**), limonoid dilactones (D-ring closed aglycones such as limonin **1**) and limonoid glucosides such as limonin 17_-D-glucopyranoside **27**. Biosynthesis and accumulation of these limonoids and enzymes involved in the limonoid metabolism in various tissues are summarized in Fig 11.

Stems

Radioactive tracer work demonstrated the biosynthesis of nomilinate A-ring lactone (monolactone form of nomilin **3**) from acetate, mevalonate or farnesyl pyrophosphate in the phloem region of stem (23). No other tissues possess the group of enzymes involved in the biosynthesis of the limonoid from acetate. This precursor, nomilinoate A-ring lactone, is then translocated from stems to other tissues where other limonoids are biosynthesized from this compound independently (30). Deacetylnomilinic acid **7**, the precursor of nomilinoate A-ring lactone, is most likely also biosynthesized in the stem and migrated to other tissues. No attempt, however, has been made to show this translocation.

The biosynthesis of nomilinate A-ring lactone ceases in stems attached old leaves, but the biosynthesis continues in stems with fruit attached. The newly synthesized nomilin continues to migrate to fruit tissues and seeds, and is converted to other limonoids which will be glucosidated in maturing fruit tissues. This explains why the limonoid glucoside content in fruit continues to increase as long as the fruit is attached to the stem.

Leaves

Nomilinoate A-ring lactone from stems is converted to other limonoids in leaves. The biosynthesis of the various aglycones occurs actively when leaves are young and immature, but the biosynthesis ceases in older leaves. No glucosidation of aglycones occurs in leaves.

Fruit Tissues and Seeds

The monolactones are the only limonoids biosynthesized and accumulated in immature fruit tissues. Particularly, the concentrations of limonoid aglycones are very high in a small fruit. In the immature seeds, however, both the monolactones and the dilactones are biosynthesized and accumulated.

During fruit maturation, the glucosidation of the monolactones takes place in both the fruit tissues and seeds (12, 14). The dilactones accumulated in the seeds are not converted to glucosides and stored in the seed tissues. During
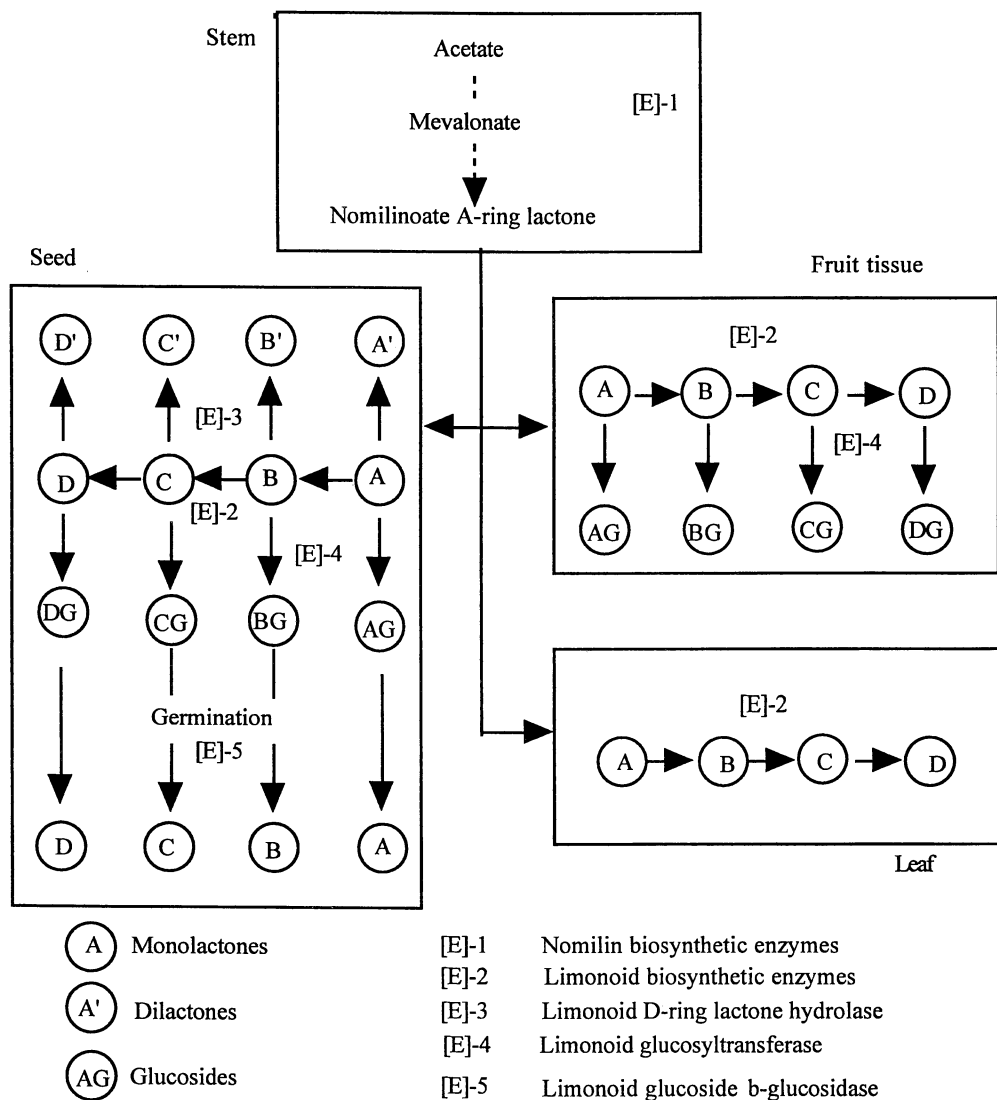
Figure 11. *Biosynthesis and accumulation of limonoids in Citrus*

maturation, the two processes, the biosynthesis of aglycones from nomilin and the glucosidation of the aglycones, are involved simultaneously in fruit tissues, while in the seeds, the three processes, the lactonization of monolactones, the aglycone biosynthesis and the glucosidation of aglycones, continues to take place concurrently. Each of these processes in the fruit tissues and seeds competes for the newly biosynthesized monolactones. The mature fruit tissues, therefore, accumulate glucosides only, while seeds accumulate both aglycones and glucosides.

There are also significant differences in the composition of limonoid glucosides and the ratio of limonoid aglycones to glucosides between the fruit tissues and seeds (15, 52, 53). Limonin glucoside is the predominant limonoid glucoside in the fruit tissue (52,53) , but its concentration in the seeds is very low (53). Nomilin glucoside is the major limonoid glucoside in the majority of seeds (53). The ratio of total aglycones to total glucosides in the mature fruit tissues is about 1 to 150 (52), whereas in seeds, the ratio is 2 to 1 (53). These data suggest strongly that there are separate biosynthetic pools in the seeds and fruit tissues.

The limonoid glucosides in the fruit tissues are stable (50). In seeds, however, there is _-glucosidase activity specific for limonoid glucosides in both dormant and germinating seeds (58). Detailed analysis of limonoid composition in seedlings shows that all of the limonoid glucosides in the seeds are hydrolyzed during the course of germination.

Bioregulation

Biosynthetic pathways and the accumulation of limonoids in *Citrus* may be altered to a certain degree by genetic engineering techniques or bioregulation using phytohormones.

UDP-D-Glucose: Limonoid Glucosyltransferase Gene

The conversion of limonoid aglycones to nonbitter glucosides occurs in fruit tissues during maturation. This process is catalyzed by the enzyme UDP-D-glucose: limonoid glucosyltransferase. Unfortunately, in certain citrus species there is not enough of this enzyme activity to reduce limonoate A-ring lactone below the bitterness threshold levels at time of harvest. The enhancement of this enzyme activity in citrus crops, which have bitterness problems, by genetic engineering techniques may result in lower levels of limonoate A-ring lactone at time of harvest, and simultaneously may increase the concentrations of limonoid glucosides in the fruit tissue.

This limonoid glucosyltransferase has been isolated from albedo tissues of two navel orange cultivars by a combination of $(NH_4)_2SO_4$ fractionation, affinity column and ion-exchange HPLC (51). Amino acid sequences of the N-terminal and three internal segments of the enzyme have been determined. The gene for this enzyme has been isolated and the insertion of the gene into the genome of citrus varieties which have a limonin bitterness problem is in progress (59).

Nomilin Deacetylase Gene

Citrus ichangensis possesses a unique enzyme, nomilin deacetylase, which catalyzes the conversion of nomilin **3** to deacetylnomilin **8** (43). This species and its hybrids accumulate deacetylnomilin, deacetylnomilinic acid **7** and ichangensin **26** in high concentrations. Limonin **1** is also accumulated although in a very low concentration . If this gene could be isolated, inserting it into citrus cultivars which have a bitterness problem may result in the accumulation of tasteless limonoids at the expense of limonin.

Limonoate Dehydrogenase Gene

Limonin is further metabolized to 17-dehydrolimonoate A-ring lactone **28** in *Citrus* although the conversion is a very minor occurrence (33). This enzyme catalyzes the dehydrogenation of the hydroxyl group at C-17 and produces nonbitter 17 dehydrolimonoids. The enzyme has not been isolated from *Citrus*, but it has been isolated from bacterial cells such as *Arthrobacter globiformis* (60) and *Pseudomonas* sp (61). The amino acid sequence of N-terminal of the enzyme isolated from *A. globiformis* is: Met-Pro-Phe-Asn-Arg-Leu-Glu-Asp-Glu-Val-Ala-Ile-Val-Val-Gly-Ala (62).

The disadvantage of using this gene would be that the reaction products, 17-dehydrolimonoids, are no longer capable of forming 17β-D-glucopyranoside derivatives. This may be harmful to the plants.

Inhibition of Limonoid Biosynthesis

Auxin phytohormones inhibit the biosynthesis of nomilin **3** from acetate (63). Radioactive tracer work using [14C]-labeled acetate showed the inhibition of up to 97% of nomilin **3** biosynthesis in the stem tissues of *Citrus limon*. All the auxins tested including indoleacetic acid, indolebutyric acid, naphthaleneacetic acid and 2,4,5-trichlorophenoxyacetic acid are potent inhibitors of nomilin biosynthesis. The inhibition was reversed with a cytokinin. Gibberellic acid had no effect on nomilin biosynthesis.

Biodegradation of Limonoids in Microorganisms

The biochemical conversion of citrus limonoids was first studied in microorganisms (34, 64). Using five species of bacteria isolated from soil by enrichment methods, limonin **1** was metabolized by at least four different pathways to produce nonbitter products: 17-dehydrolimonoate A-ring lactone **2 8** (60), deoxylimonin **29** (65), limonol **14** (66) and trans-19-hydroxyobacunoate **3 0** (67). Another bitter limonoid, nomilin **3**, was metabolized to nonbitter obacunone **4** (68). These findings have led to development of a bioreactor system

that uses immobilized bacterial cells for reduction of limonoid bitterness in the juice (69).

Summary

Significant progress has been made in understanding how, when and where limonoids are biosynthesized and accumulated in Citrus. During the course of biochemical research on limonoids, there were three major breakthroughs. First, we were able to isolate several species of bacteria which are capable of metabolizing limonoids. The biochemical conversion of citrus limonoids was first demonstrated in bacteria and five biodegradation pathways were established. These findings had significant contribution to elucidation of biosynthetic pathways of limonoids in *Citrus* Second, we found stems of lemon seedlings to be an excellent tool to prepare ^{14}C-labeled limonoid substrates for radioactive tracer work, which is the best way to study biosynthetic pathways of natural compounds. The third breakthrough was the most important of all, the discovery of limonoid glucosides, which are biosynthesized during fruit maturation and accumulate as major secondary metabolites. The discovery of nonbitter limonoid glucosides of bitter limonoid aglycones in mature fruit tissues has answered a long standing question of how a naturally occurring limonoid debittering proceeds. The presence of limonoid glucosides in citrus fruit in high concentration, which are beneficial to human health, has also made a significantly contribution to anticancer research.

Based on utilizing the accumulated biochemical knowledge, transgenic citrus trees that produce fruits free from limonoid bitterness, are being created by genetic engineering techniques. This technology should have commercial feasibility since this in situ debittering process during fruit development and maturation is all 'natural' and should be readily acceptable to both regulatory and consumer groups. This technology is applicable to the production of juices and non-juice forms of products as well as to fruit themselves.

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Chapter 3

Analysis of Limonoids by Thin-Layer Chromatography

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The single most useful method for detecting limonoids in plant extracts is thin layer chromatography (TLC). Developed plates sprayed with Ehrlich's reagent (dimethylaminobenzaldehyde solution) and exposed to hydrogen chloride gas reveal limonoids as reddish-orange spots. Concentrations as low as 0.5 _g of limonoid aglycones and 0.2 _g of limonoid glucosides can be detected, identified and quantified with TLC.

Limonoids can be best evaluated in plant extracts by TLC. The TLC method was first introduced for detecting limonoids in citrus tissues in 1965 by Dreyer (2). Since then, a combination of TLC and NMR spectroscopic analysis has resulted in isolation of over 50 limonoids from the Rutaceae family plants. The limonoid color, developed with Ehrlich's reagent and by exposing it to hydrogen gas, is characteristic enough that it can readily be differentiated. With the exception of the 17-dehydrolimonoids such as 17-dehydrolimoonoid A-ring lactone (4), all limonoid aglycones and glycosides isolated from *Citrus* and its related genera produce a reddish-orange color, suggesting that the D-ring of the limonoids is important for color formation with Ehrlich's reagent. In our experience, compounds detected by this method always have proven to be limonoids when they were isolated and characterized. Chandler (3) developed a different method, claiming to be more specific for limonoids. In his method, TLC plate was exposed to bromine vapor and sprayed with Tollen's reagent to visualize furanoids. However, no evidence was presented to indicate that this method is more specific for limonoids than Ehrlich's method.

The TLC method by Maier and Grant (10) is specific, sensitive and precise, and can quantify as little as 0.5 _g of limonin. Their method involves preliminary extraction, respotting of samples after the approximate limonoid content has been established, and comparison to a series of standards that are spotted onto the same plate. Data obtained by this method are comparable to those obtained by HPLC (8). The TLC method developed by Tatum and Berry (14) is simple and fast, and requires no sample preparation. The whole juice is spotted directly on a plate. Although not as reproducible, their method is suitable for routine quality control analyses. There is no TLC solvent system for separation of individual glucosides, but it can be used to estimate the total amount of limonoid glucosides (8, 12). Recently, the analysis of limonoids in fruit tissues and seeds has been reviewed (6, 9).

Citrus Limonoids

The solubility of limonoids is a critical factor for their extraction, isolation and analysis. There are two groups of limonoids in *Citrus*, limonoid aglycones and limonoid glycosides (Fig. 1). Thirty-six aglycones and 17 glucosides have been isolated from *Citrus* and its closely related genera (7). The aglycones occur in *Citrus* as neutral dilactones (both A- and D-rings closed), acidic monolactones (A-ring or D-ring closed) or dicarboxylic acids (both A- and D-rings open). The neutral dilactones are hydrophobic, while the acidic monolactones are soluble in water and some polar solvents. The dicarboxylic acid aglycones are mostly hydrophylic. The limonoid glucosides are all 17β-D-glucopyranoside esters of the acidic aglycones, and are soluble in water and alcohols, but insoluble in nonpolar solvents.

Limonoid Aglycone Analysis

Preparation of Samples

Juices

The juice sample is centrifuged to give a clear serum, 20 ml of which is loaded onto a C-18 Sep-Pak cartridge (Waters, Milford, MA). The cartridge is first washed with water followed by MeOH to elute the limonoids. Following evaporation, the residue is dissolved in CH_3CN for the analysis. Alternatively, a liquid/liquid extraction method can be employed. In this method, 100 ml of the clear juice serum is shaken twice with 50 ml of CH_2Cl_2 or EtOAc. The organic phase containing the aglycones is evaporated for analysis.

Seeds

The limonoid aglycones can be extracted from citrus seeds by buffer or solvent extraction. In the buffer extraction, seeds are ground with 10 V of 0.1 M Tris-buffer at pH 7.5. The homogenate is allowed to incubate overnight at the
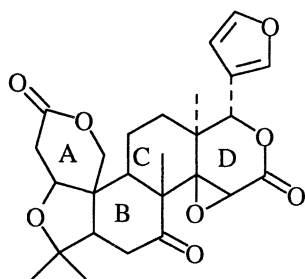
Limonin
Dilactone
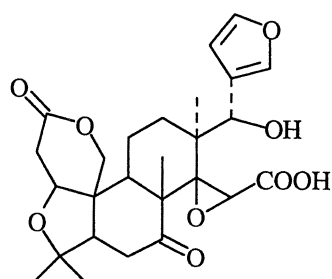
Limonoic acid A ring lactone
Monolactone (A-ring closed)
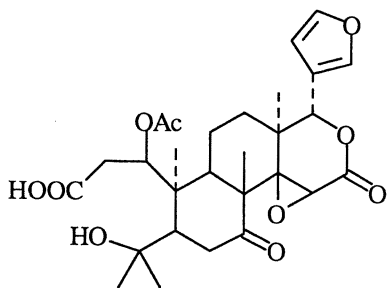
Nomilinic acid
Monolactone (D-ring closed)
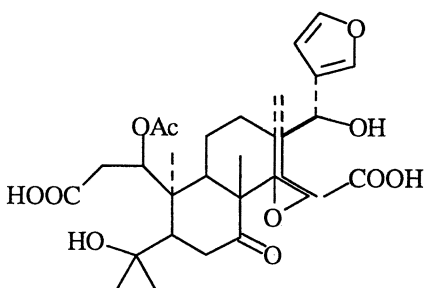
Nomilinoic acid
Dicarboxylic acid
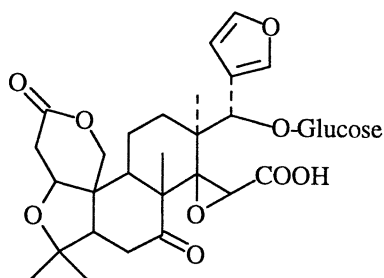
Limonin 17β-D-glucopyranoside

Figure 1. Chemical structures of limonoids

room temperature. During incubation, the dilactones are converted to monolactones by the action of limonoid D-ring lactone hydrolase which is present abundantly in the seed. The mixture is centrifuged and filtered through Celite to yield clear solution. The filtrate is then acidified to pH 3.0 with 1N HCl to close the D-ring, and extracted twice with CH_2Cl_2 or EtOAc. This method is very efficient and quantitative, and the resulting solution contains very little non-limonoid compounds (11).

In solvent extraction, seed meals are placed in a Soxhlet extractor and washed thoroughly with hexane to remove oily materials. Limonoids are extracted with acetone or other solvents such as EtOAc, and CH_2Cl_2. Acetone is the best solvent, but the solvent method is less efficient than the buffer method since some water-soluble limonoids (monolactones and diacidic limonoids) may not be fully extracted with solvents (11). The hexane also extracts some of the very non-polar limonoids such as obacunone (13) or methyl isoobacunoate diosphenol and methyl isoobacunoate present in the genus *Fortunella* and its hybrids (1). However, the solvent extraction method is recommended for large-scale extraction.

Acidic limonoids

Acidic aglycones (A-ring open), such as nomilinic acid and deacetylnomilinic acid, do not migrate in most of solvents developed for limonoid analysis. Therefore, diazomethane is used to methylate them before TLC analysis.

TLC Analysis

Solvent Systems

There are a number of solvent systems developed for analysis of the bitter limonoid, limonin (10,14). However, they are not suitable for separation analysis of the other limonoids. We have developed three solvent systems which can detect and separate practically all limonoids present in *Citrus* on silica plates; (1) EtOAc-cyclohexane (3 : 2); (2) EtOAc- CH_2Cl_2 (2 : 3) and (3) CH_2Cl_2-MeOH (3 : 97). The Rfs of limonoid aglycones and methyl esters of acidic limonoid aglycones are shown in Fig. 2, 3 and 4, respectively. Two solvent systems should be used to positively confirm the identity of a given limonoid aglycone.

Analysis

Limonoid aglycones can be detected, identified and semi-quantified using silica gel TLC in conjunction with standards. Samples prepared as described above are spotted onto a silica gel plate along with the appropriate standards and the plate is developed in one of the three solvents. The dried plate is then sprayed with Ehrlich's reagent (0.5% p-dimethylaminobenzaldehyde in EtOH) and exposed to HCl gas. HCl gas can be easily generated by mixing NH_4Cl with conc H_2SO_4. The limonoids produce very distinctive reddish-orange spots on the plate. As low as 0.5 _g can be detected. Estimates of the quantities of specific
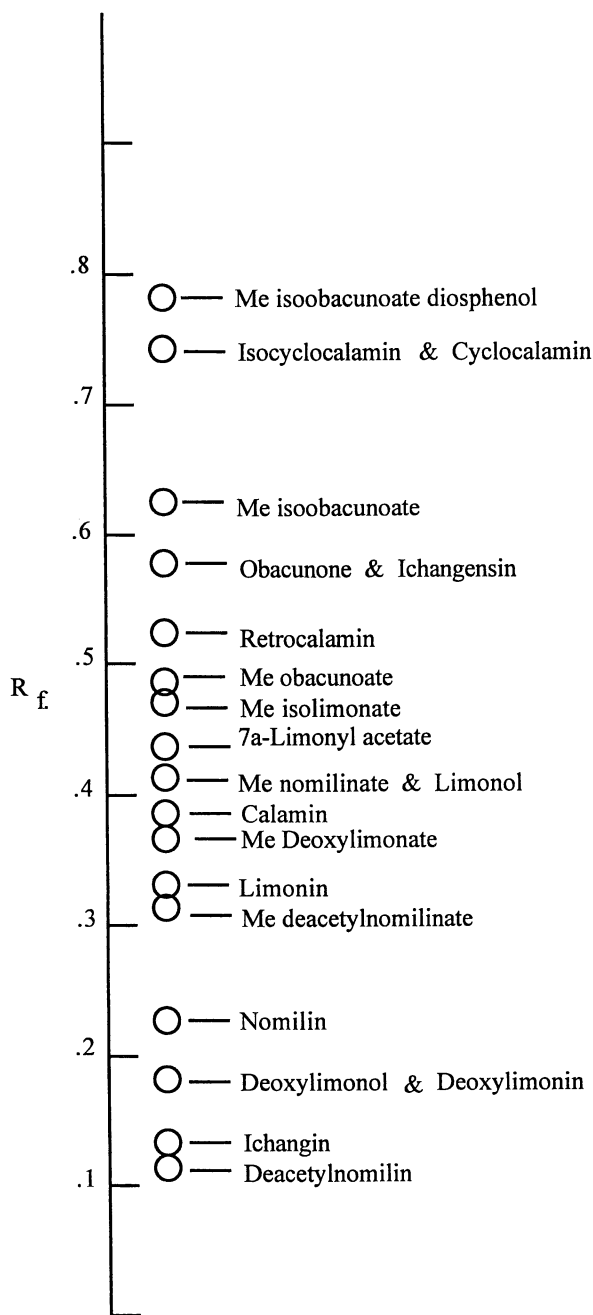
Figure 2. TLC R_fs of limonoid aglycones and methylated acidic limonoid aglycones on silica gel plates using EtOAc-cyclohexane (3 : 2)

R$_f$

.9 — ⭕ — Me isoobacunoate diosphenol

⭕— Cyclocalamin

⭕— Me isoobacunoate

.8 — ⭕ — Obacunone

.7 — ⭕ — Retrocalamin

⭕ — Me obacunoate & Me isolimonate

⭕— Ichangensin

.6 —

⭕= Nomilin
⭕= Limonin

.5 — ⭕ — Me nomilinate

⭕— Calamin
⭕= Deoxylimonin
⭕= Me deacetylnomilinate

.4 —

⭕ — Deacetylnomilin

.3 —

⭕ — Ichangin

.2 —

.1 —
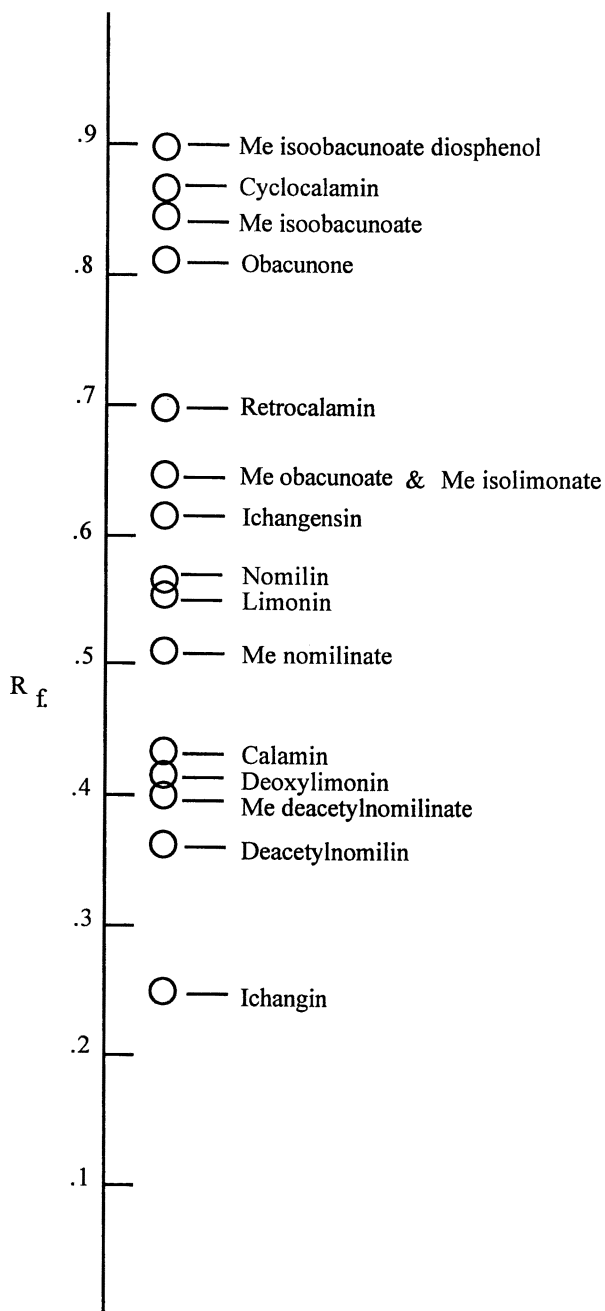
Figure 3. TLC R$_f$s of limonoid aglycones and methylated acidic limonoid aglycones on silica gel plates using EtOAc-CH2Cl2 (2 : 3)

R_f

.9

.8 — Me isoobacunoate diosphenol
— Cyclocalamin
— Me isoobacunoate

— Isocyclocalamin

— Obacunone

.7

.6 — Nomilin
— Retrocalamin
— Me isolimonate
— Limonin
— Ichangensin

.5 — Deoxylimonin

— Me obacunoate

.4 — Me deacetylnomilinate
— Me nomilinate
— Calamin
— Deacetylnomilin

.3

.2 — Ichangin

.1
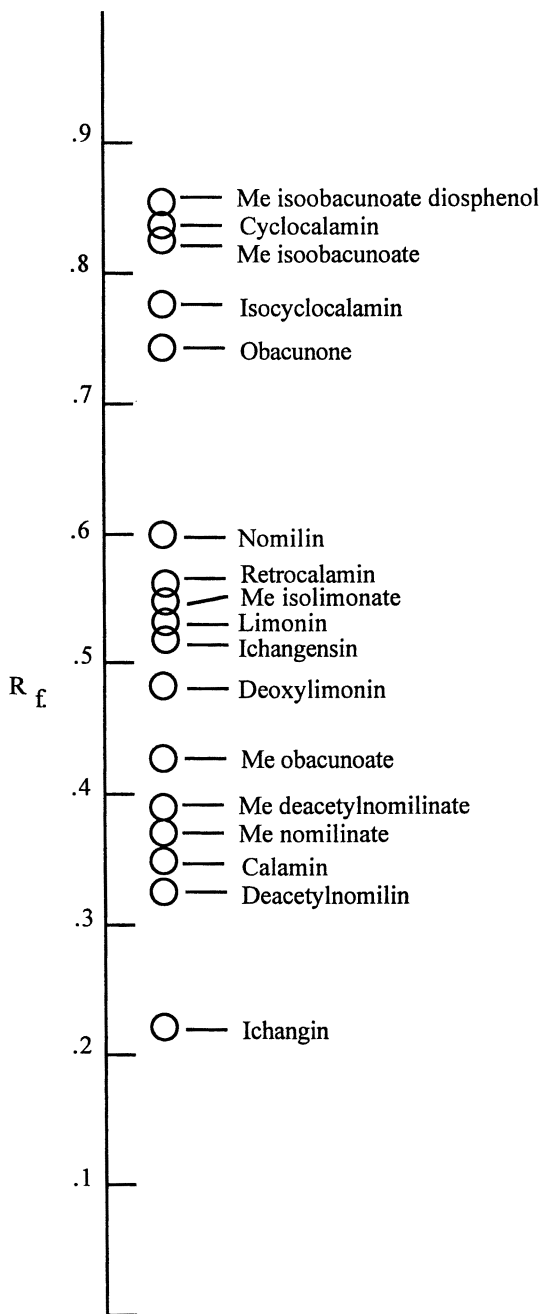
Figure 4. TLC R_fs of limonoid aglycones and methylated acidic limonoid aglycones on silica gel plates using CH2Cl2-MeOH (97 : 3)

limonoids can be accomplished by comparison of spot intensity to a series of standards, which are spotted onto the same plate. Positive identification of a limonoid requires chromatographic comparison in at least two different solvent systems.

Limonoid Glucosides

Limonoid glucosides are present in mature fruit tissues and seeds as major secondary metabolites. TLC is the easiest and most economical method for detecting limonoid glucosides in plant extracts. However, unlike aglycone analysis, there is no TLC solvent system for separation of glucosides. Therefore, TLC can not be used for identification of individual limonoid glucosides, but can be used to estimate the total amount of limonoid glucosides (8, 12). The solvent system we commonly use is EtOAC-methyl ethyl ketone-formic acid-H_2O (5: 3: 1: 1). For most citrus fruit tissues and seeds, three positive reddish-orange spots can be observed when the plate is developed in this solvent system after spraying the plate with Ehrlich's reagent and exposing to HCl gas. Limonoid glucosides react very positively toward Ehrlich's reagent and tend to produce more intensified, reddish spots than those of aglycones. Therefore, as little as 0.2 _g of limonoid glucosides can be detected.

For quantification of total limonoid glucosides, the intensity of three spots can be used by comparing spots produced by a series of known amounts of limonoid glucoside standards. The result of the total limonoid glucosides estimated by TLC tends to agree very well with that of HPLC analysis (8, 12).

Limonoid glucosides are easily extracted from juice. Ten to twenty ml of clear serum is loaded onto a C-18 Sep-Pak cartridge. After washed thoroughly with water, limonoid glucosides are eluted with MeOH. Following evaporation, the residue is dissolved in MeOH for analysis.

Since glucosides are soluble in water and alcohols, they can be extracted from seed meals with water or MeOH. Seeds are homogenized in 15-fold volume of H_2O. The mixture is centrifuged at 15,000 g for 20 min, and the supernatant is filtered. A portion of the filtrate is then loaded onto a Sep-Pak cartridge, and the cartridge is washed with several volume of H_2O. The limonoid glucosides are eluted with MeOH. We developed an alternative extraction method employing 70% MeOH to prevent the formation of pectin gels which occur often when H_2O is used for extraction (5).

Both limonoid aglycones and glucosides can be extracted from the same batch of seed meal (5). Following hexane wash for removal of oily materials, they can be extracted with MeOH. Following removal of MeOH by evaporation, the residue is shaken vigorously in CH_2Cl_2-$H2O$ (1 : 1). The H_2O fraction contains glucosides and the CH_2Cl_2 fraction contains aglycones. This procedure is recommended for large-scale extraction.

Conclusion

TLC is the most expeditious and economical method for detection and identification of limonoids in plant tissue extracts, and can also be used for identification and semi-quantification. The method can be scaled up for isolation and/or purification of small quantities of limonoids. Adaptation of TLC in limonoid research has made a significant contribution to discovery and isolation of new limonoid aglycones and glucosides from *Citrus* and its closely related genera.

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Chapter 4

LC-MS and NMR Techniques for the Analysis and Characterization of *Citrus* Limonoids

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This chapter describes liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) spectroscopy methods applied to the analysis and characterization of limonoids present in *Citrus* sp., citrus products and by-products of citrus processing. Electron ionization (EI) LC-MS and atmospheric pressure chemical ionization (APCI) LC-MS analysis of limonoid aglycones and electrospray (ESI) LC-MS analysis of limonoid glucosides are described. The NMR characteristics of limonoid aglycones and limonoid glucosides are discussed and the ^{13}C NMR chemical shifts of 22 limonoid aglycones and 17 limonoid glucosides tabulated for reference.

Liquid chromatography – mass spectrometry (LC-MS) and Fourier transform nuclear magnetic resonance spectroscopy (FT NMR) are two sophisticated methodologies available to natural products chemists for the isolation, characterization and analysis of complex natural products. Technological advances in the interfacing of mass spectrometers with liquid chromatography systems have produced a new breed of robust bench top instruments featuring simplified operation characteristics, high analytical sensitivity and broad applicability to natural products problems. Correspondingly, methods development in FT NMR coupled with NMR instruments of increasing field strength and microprobe technology now allow unequivocal structure determination on natural products in microgram amounts. This chapter will summarize the types of LC-MS instruments available and demonstrate the application of LC-MS to the analysis of limonoids in the fruit of *Citrus* spp. and from citrus processing products and by-products.. It will also review and summarize the NMR characteristics of limonoids obtained from *Citrus*.

LC-MS of Citrus Limonoids

Increasing evidence of the anticancer properties of limonoids (1-6) suggests they may play an important role in human health. The location of these compounds in *Citrus*, citrus products and by-products, their characterization and the investigation of their bioavailability and metabolism in humans requires methodologies capable of detecting and identifying limonoids at very low levels. LC-MS now has the capability to detect, identify and quantify limonoids from citrus sources and human biological fluids with high sensitivity.

Bench top, open access LC-MS instruments operating in electron ionization (EI), atmospheric chemical ionization (APCI) and electrospray (ESI) modes offer significantly increased sensitivity compared to ultraviolet (UV) detection and can provide specific molecular and chemical structural information about detected substances. These instruments are not reliant upon UV absorbing chromophores for analyte detection and response is thus independent of solvent ultraviolet absorptivity. In association with photodiode array detection, an LC-MS system can incorporate all of the desirable characteristics of UV and/or MS detection into a comprehensive LC-UV-MS system capable of high sensitivity, quantification and characterization of limonoids (LC-MS) and simultaneous semi-preparative or preparative methods development (LC-UV).

Mass spectrometers coupled to liquid chromatography systems offer some important advantages for the detection and characterization of organic natural products compared to UV detection, but cost three to five times more than UV diode array detectors. This chapter offers a brief description of the types of MS detectors available in bench top instruments and to the application of two of these detector types to the analysis and characterization of limonoids in citrus.

An extensive description of mass spectrometry is beyond the scope of this chapter. If the reader requires a more comprehensive information, several good texts describing mass spectrometry (eg. 7,8) and the coupling of mass spectrometry to liquid chromatography (eg.9-11) are available.

The analysis of ions formed from solvent/analyte mixtures in LC-MS instruments involves removal of the mobile phase followed by ion formation and mass analysis. Ion formation can be achieved by gas-phase analyte ionization (electron ionization (EI) or chemical ionization (CI)), by gas phase ion-molecule reactions of analyte molecules with mobile phase ions (atmospheric pressure chemical ionization (APCI)) or by ion evaporation of of small droplets (electrospray ionization (ESI)).

Two types of mass analyzers are presently utilized in bench top LC-MS instruments, transmission-quadrupole and quadrupole ion trap. The transmission quadrupole, the most common mass analyzer, relies upon a combination of radio frequency (RF) and direct current (DC) applied between opposing surfaces of four hyperbolically related parallel surfaces (metal rods) to act as a quadrupole mass filter. Ions extracted from the ion source are accelerated into the field of the quadrupole where they are subjected to varying DC voltages and RF amplitudes between the quadrupole surfaces. Ions produced by the source of an EI or CI mass spectrometer

proceed to the mass detector through the mass filter according to strength and amplitude of the DC and RF fields. The mass detector can be set to scan a specified mass range (total ion current (TIC)) or select an ion of a particular m/z (selected ion monitoring (SIM)) depending upon the DC and RF field settings. Differentiation and detection of ions produced by bench top EI LC-MS is accomplished with a single transmission quadrupole. When two or three transmission quadrupoles are in series (tandem quadrupole mass spectrometer), secondary mass analysis of fragments from a mass-selected precursor ion is possible and the techniques is known as mass spectrometry/mass spectrometry (MS/MS) analysis. Bench top instruments with single quadrupoles do not have MS/MS capability.

Quadrupole ion trap analyzers are the most recent additions to the field of commercial mass spectrometry. Unlike ion beam quadrupole mass analyzers, the ion trap quadrupole analyzer maintains all of the ions in separate orbitals within the boundaries of two hyperbolic surfaces. The orbiting ion mixture is destabilized by variations in the DC and RF field strengths to eject the ions sequentially by mass/charge ratio. The ejected ions proceed to the mass detector where they are recorded (TIC). Segment length adjustments allow monitoring at a particular m/z or pseudomolecular weight (SIM). These instruments are capable of conducting MS/MS experiments.

The analysis of individual components within a mixture of analytes utilizing LC-MS may or may not require prior chromatographic separation. In this chapter we will demonstrate the LC-MS quantitative analysis of *Citrus* limonoids with prior chromatographic separation and the qualitative analysis of these compounds without prior chromatography.

HPLC Analysis of Limonoids

High-performance liquid chromatography (HPLC) is a prominent analytical method for the analysis of limonoids in *Citrus*. The application of reverse phase HPLC methodology for the quantification of both limonoid aglycones and limonoid glucosides present in citrus seeds has recently been reviewed (12,13). Reverse phase HPLC has also been used extensively for the analysis of limonoid aglycones and limonoid glucosides in citrus juice, peel, and flesh and in citrus processing by-products (14-19). These procedures utilize C-18 bonded silica columns with solvent mixtures of acetonitrile/water, acetonitrile/aqueous acid or mixtures of acetonitrile, methanol, tetrahydrofuran and water as eluents. Most of the earlier methodology was intended for the quantification of the limonoid aglycone limonin (**1A**) as a measure of delayed bitterness in citrus juices. The separation and identification of secondary, non-bitter neutral limonoid aglycones has not generally been a consideration except when they interfere with the limonin analysis.

Until recently, the only normal phase separation and analysis of citrus limonoid aglycones had been reported by Rouseff and Fisher (20). They applied a CN bonded silica HPLC column with a ternary mobile phase of hexane, 2-propanol and methanol to resolve the limonoid aglycones obacunone (**5A**), nomilin (**10A**), limonin (**1A**) and deoxylimonin (**2**) more efficiently. We have recently reported a normal phase HPLC

method which utilizes a binary organic solvent system (cyclohexane/tetrahydrofuran) for the separation of neutral limonoid aglycones on a spherical silica column (21). This system achieves a highly selective separation of the limonoids and allows the option of scale up for the separation of minor limonoid aglycones in *Citrus* extracts.

All of the HPLC methods currently described for the analysis of *Citrus* limonoids rely upon ultraviolet (UV) detection to identify and quantify the limonoid aglycones or limonoid glucosides. The UV detection limit for limonoids separated by HPLC has been determined to be about 3.5 to 10 ng at 207 nm (20) and 30 ng at 215 nm (21). The identity of the individual limonoids is dependent upon a comparison of retention times with those of authenic standards.

LC-MS Quantitative Analysis of Chromatographically Separated Limonoids

Limonoid aglycones

The media and organic solvent system previously reported for the normal phase HPLC analysis of limonoid aglycones (21) were found to be directly adaptable to the analysis of these compounds by LC-MS. The LC-MS analysis was conducted in the APCI (positive ion mode) on a Micromass Platform LCZ LC-MS instrument. Since flow rates up to 2 ml/min are compatible with LC-MS in the APCI mode, the column (4.2mm x 250mm, 5μ spherical silica), solvent flow rates (1.5 ml/min) and gradient of the original LC-UV procedure were directly adaptable in the LC-MS determination. Pseudomolecular ions were observed for all standards with the exception of deoxylimonol (2) and 7-α-limonyl acetate (7C), which formed adducts with tetrahydrofuran. Substitution of a small bore column (2.0mm x 150mm, 3μ spherical silica) for the original column in the normal phase method allowed reduction in flow rates (0.4 ml/min) while a chromatographic separation of the limonoid aglycones similar to our LC-UV method was maintained (Fig 1). A detection limit of 0.6ng was determined for aglycone analysis in this LC-MS system in the TIC acquisition mode; a detection limit of 20pg was observed for the limonoid aglycone limonin (1A) in the SIM acquisition mode.

The small bore spherical silica column and normal phase chromatographic conditions used with the APCI LC-MS were directly applicable to the EI LC-MS analysis of the limonoid aglycones on a Waters Integrity particle beam LC-MS instrument. A detection limit of 5 ng was determined for the aglycones in the EI analysis method operating in the TIC data acquisition mode. The limonoid aglycones did not display molecular ions in the EI analysis, but they did display distinctive fragment ions of high abundance. Limonoid aglycones with intact B,C and D rings and with a carbonyl at C-7 display a prominent fragment ion reflective of the loss of m/z 123, the result of a two bond cleavage through ring D ((eg. limonin (1A), Fig 5). Ion detection in the SIM acquisition mode was about five times more sensitive (1 ng) than the TIC mode. SIM analysis was associated with primary EI fragmentation ions of the limonoid aglycones. A non-linear relationship between SIM ion intensities and limonoid concentrations prevented SIM acquisition as a quantifying method in EI LC-MS. The high solvent flow rates of the original, normal phase, limonoid aglycone analysis were also incompatible with the EI particle beam LC-MS instrument.

The limonoid aglycones were also efficiently separated for quantification by a small
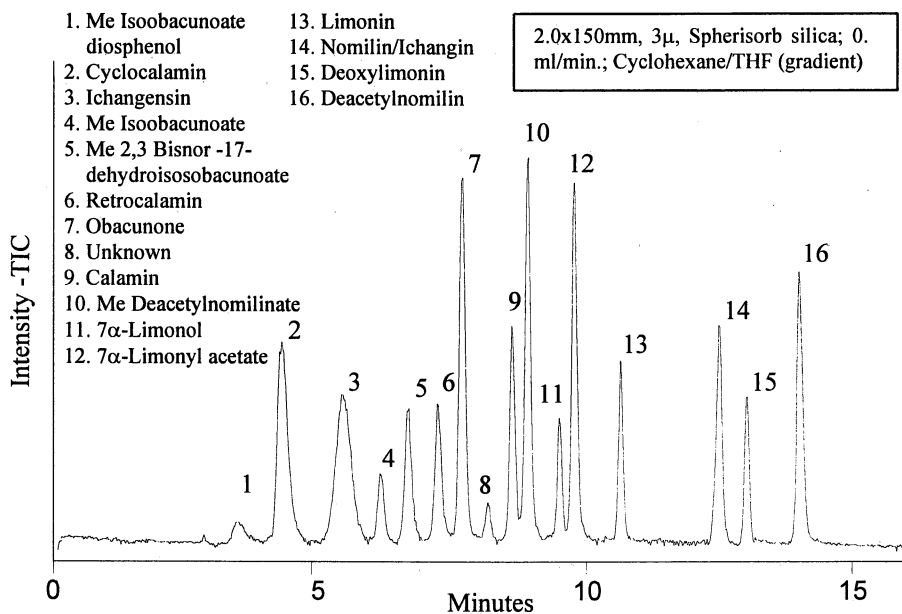
Figure 1. EI/APCI normal phase LC-MS of *Citrus* limonoid aglycone standards.

bore base deactivated C-18 spherical silica reverse phase column (2mm x 150mm, 3μ) eluted with a methanol/water gradient (0.4 ml/min). The chromatographic resolution of the limonoid aglycones was comparable to the normal phase system and sensitivity was similar. Linear calibration curves were established for limonoid aglycone standards in both the EI and APCI LC-MS instruments, utilizing TIC acquisition and the lignan podophyllotoxin as internal standard.

To demonstrate the application of the EI and APCI LC-MS methodology we analyzed the seeds of the *Citrus/Fortunella* hybrid *Calamondin reticulata* var. *austera* X *Fortunella* sp for limonoid aglycones. A limonoid aglycone extract of about 500 mg of dried seeds was prepared according to the method of Hasegawa et. al. (12). A 2μL injection of a 1 mL solution containing the isolated limonoid aglycones was introduced to the small bore spherical silica column operating in the EI LC-MS under normal phase conditions. EI LC-MS TIC traces of the chromatographically separated limonoid aglycones in the calamondin seed sample are shown in Figure 2. The seed extract was analyzed under the same conditions by the normal phase APCI LC-MS method. Quantification of individual limonoids (results not shown) were in close agreement between the two methods in the TIC mode.

Limonoid Glucosides

The LC-MS analysis of limonoid glucosides is currently restricted to ESI methodology. We have been unsuccessful in producing detectable ions for these
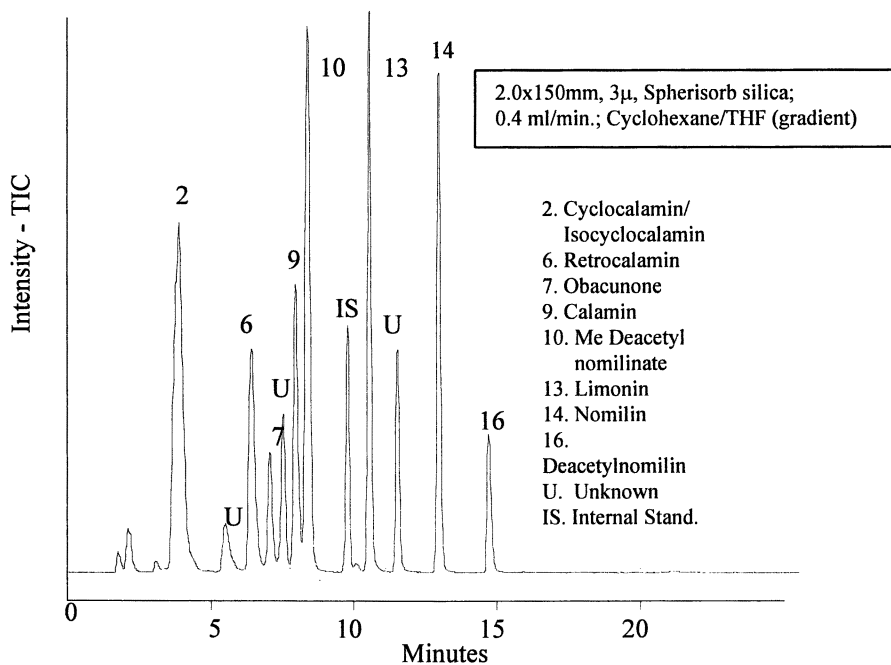
Figure 2. Normal phase APCI LC-MS of limonoid aglycones in calamondin seeds.

compounds in an EI particle beam instrument and we find extensive solvent adduct formation with gas phase ions generated in the APCI method. ESI LC-MS on the Micromass LCZ instrument produces consistent pseudomolecular ions (negative ion mode) for all of the limonoid glucoside standards separated on a base deactivated C-18 reverse phase column (2mm x 150mm), utilizing a 4 mmol formic acid/acetonitrile gradient (0.3mL/min). The C-18 reverse phase chromatographic separation and LC-MS analysis of limonoid glucoside standards is shown in Figure 3. Linear external standard calibration curves were generated for each of these standards in the TIC data acquisition mode and a detection limit of 400pg was determined for limonin glucoside (**1D**). Orange juice and orange molasses samples prepared according to the method of Ozaki et. al. (16) were analyzed for limonoid glucoside content utilizing ESI LC-MS in TIC mode (Figure 3) (analysis results not shown).

Direct Introduction LC-MS Qualitative Analysis of Limonoid Mixtures

      LC-MS chemical ionization mass spectrometric methods produce and detect distinct pseudomolecular ions for analyte constituents introduced in pure form or in mixtures. The ability to accurately quantify individual limonoids among a mixture of limonoids without prior chromatographic separation would markedly increase the

Limonoid Glucoside Standards

1. Limonin glucoside
2. Deacetylnomilin glucoside
3. Obacunoic acid glucoside
4. Epiisoobacunoic/
   isoobacunoic acid glucoside
5. Nomilin glucoside

6. Epiisoobacunoic/
   isoobacunoic acid glucoside
7. Nomilinic acid glucoside
8. Obacunoic acid glucoside

Citrus Molasses

Orange Juice

Hypersil BDS C-18, 2.0x 150mm, 3μl, 4mmol HOOC/Acetonitrile gradient.

Intensity - TIC

Minutes

Figure 3.   Reverse phase ESI LC-MS of limonoid glucoside standards and limonoid glucosides in orange molasses and orange juice extracts

efficiency of limonoid analysis from citrus extract mixtures.   In addition, the development and application of direct introduction APCI or ESI LC-MS methods offers the potential of much higher detection sensitivity of these compounds from citrus sources.

      Quantitative analysis of individual limonoid aglycones and limonoid glucosides in mixtures directly introduced to the MS has not yet been achieved. However, these *Citrus* components can be qualitatively analyzed in citrus extracts by EI/APCI and ESI methods respectively.   The limonoid character of aglycone mixtures, dissolved in methanol, can be qualitatively analyzed by direct introduction into the LC-MS operating in either the EI or the APCI mode. A snapshot of the EI TIC data (m/z range 250 to 500) of a direct introduction of a limonoid aglycone mixture displays an array of distinctive aglycone fragment ions. A similar snapshot of the APCI TIC data (M+1 range 300 to 600) for the direct introduction of an aglycone

mixture displays an array of M+1 ions for the aglycones (Fig. 4). Limonoid glucosides can also be swiftly analyzed by direct introduction LC-MS in methanol containing 0.5% ammonium hydroxide in the ESI mode. In this solvent combination, a detection limit of 42pg was established in the SIM acquisition mode. The limonoid glucoside analysis is also qualitative; however, the lower detection limit makes the analysis particularly sensitive in detecting minute amounts of limonoid glucosides. The ability to achieve this level of sensitivity is predicated upon consistency of the solvent/analyte matrix in the direct introduction method. Contaminates or competing ions can create matrix effects which may suppress or eliminate desired ions in the ESI LC-MS method.
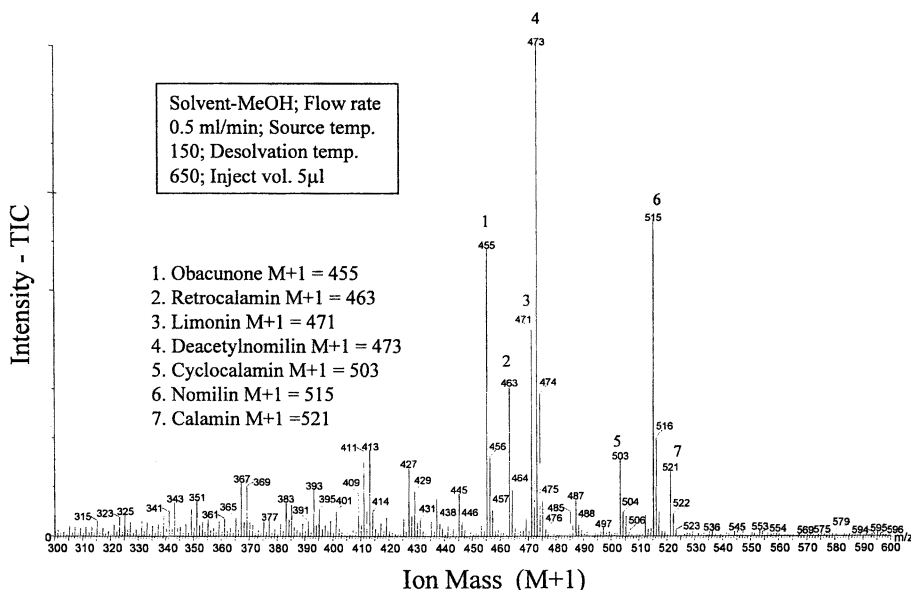
Figure 4. APCI LC-MS snapshot of direct introduction of limonoid aglycone mixture.

In each instance the ion arrays provide qualitative data for the relative amounts of the limonoid aglycones or glucosides present in the mixtures in less than a minute. The speed of this qualitative method is very useful for monitoring of preparative semi-preparative chromatographic separations of limonoids. Large numbers of individual fractions or the preparative chromatographic stream itself can be more swiftly and accurately monitored by direct introduction LC-MS than by thin layer chromatography (TLC) methods.

Direct introduction LC-MS can not distinguish analyte ions of the same molecular weight. Specific identification of individual limonoid glucosides of similar molecular weight will require chromatographic separation and comparison to standards.

LC-MS Instrument Summary

LC-MS provides highly sensitive means to analyze *Citrus*, citrus products and by-products and biological fluids for the presence of limonoid aglycones and limonoid glycosides present in levels as low as 42 picograms and structural and/or molecular weight data about the limonoids without solvent interference. The methods work well with small bore columns and can be coupled with an LC-UV system for methods development. Our examination of the EI and CI LC-MS instruments revealed that the EI particle beam instrument was useful only for the analysis of limonoid aglycones whereas ESI CI is found to be the most effective method for the analysis of limonoid glucosides. Glucoside analysis was efficiently achieved using the same solvent systems with base deactivated reverse phase C-18 currently used in analytical HPLC separations of these compounds from *Citrus* sources. APCI CI was effective for analysis of limonoid aglycones using the normal phase system previously developed for LC-UV limonoid analysis or by C-18 reverse phase HPLC. Preliminary data suggest that LC-MS may provide the ability to analyze limonoid content in *Citrus* source samples without chromatography.

NMR of Limonoids in Citrus

The characterization of limonin (**1A**) was the earliest application of NMR to the structural analysis of limonoids (22,23). Interpretation of this data was limited to the association of C-methyl and furan resonances to the limonin (**1A**) structure. In 1965, Dreyer (24) provided the first comprehensive description of NMR application to limonoid structure determination. In this description the 60 MHz ^{1}H NMR spectra of limonin (**1A**) and synthetic derivatives of limonin was utilized to establish general ^{1}H NMR characteristics for the limonoids.   Dreyer established the chemical shift relationships of the C-18 and C-24 methyl protons and the gem dimethyl protons at C-4 in the limonin type limonoids and the relationship of the C-19 methyl protons of the nomilin/obacunone limonoids to the limonin C-methyls. He further identified the C-1, C-15 and C17 protons of these compounds; however lack of resolution did not allow a detailed description of the NMR characteristics of the alkyl protons at C-1,2,5,6,9,11 and 12. The H-15 and H-17 proton resonances were examined in relationship to the functionality at C-7, the conformation of Rings A, A', B and C, and the configuration at C-17.

A large number of limonoid aglycones and glucosides have been isolated and characterized in the past 30 years (Fig 5,6). During that period, the evolution of NMR has seen significant increases in magnetic field strengths with correspondingly large increases in resolution. The development and application of Fourier transform NMR (FT NMR) methods which produce extensive intramolecular ^{1}H-^{1}H and ^{1}H-^{13}C correlations have led to more rapid chemical structure characterizations and stereochemical assignments of naturally occurring compounds. The application of these techniques to limonoids has confirmed the earlier observations for the C- methyl protons, the furan ring protons and the protons at C-1,2,15, and C-17 in limonoid aglycones (25-30) and limonoid glucosides (31,32). In addition, however, these

1A Limonin

2 Deoxylimonol

B

3B 7α-Limonol, R=α-OH
4B 7α-Limonyl acetate, R=α-OAc

C

5A Obacunone
6B 7α-Obacunol, R=α-OH
7B 7α-Obacunyl acetate, R=α-OAc

A/B

8A Methyl obacunoate

9A Methyl-*trans*-obacunoate

10A Nomilin, R=Ac
11A Deacetylnomilin, R=H

12A Ichangin

13C Retrocalamin

14A Ichangensin

15A Methyl isoobacunoate
15C Cyclocalamin
16A Methyl epiisoobacunoate

17A Methylnomilinate, R=Ac, R¹=H
18A Methyl deacetylnomilinate, R, R¹=H
20C Calamin, R, R¹=H

21A Methyl isolimonate

22A Methyl isoobacunoate diosphenol

Figure 5. Citrus limonoid aglycones

1D Limonin glucoside

D

E

5D Obacunone glucoside

8D Obacunoic acid glucoside

9D *trans*-Obacunoic acid glucoside

10D Nomilin glucoside, R=Ac
11D Deacetylnomilin glucoside, R=H
23E 6-Keto-7β-deacetylnomilol glucoside, R=H

12D Ichangin glucoside

14D Ichangensin glucoside

15   **16**

15D Isoobacunoic acid glucoside
16D Epiisoobacunoic acid glucoside

17D Nomilinic acid glucoside, R,R²=H, R¹=Ac
18D Deacetyl nomilinic acid glucoside, R,R¹,R²=H
18E Methyl deacetynomilinate glucoside, R=Me, R¹,R²=H
19D 19-Hydroxydeacetylnomilinic acid glucoside, R¹=H,R²=OH
20E Calamin glucoside, R=Me, R¹=H, R²=H

21B Isolimonic acid glucoside

Figure 6. Citrus limonoid glucosides

techniques have yielded extensive structural information through the correlation of interactions between protons (COSY), carbons and protons (HETCOR, HMQC, HMBC, NOSEY) and the differentiation of carbons and their multiplicity (DEPT). The DEPT and the COSY, NOSEY and HETCOR two dimensional NMR techniques have been routinely applied in the characterization of *Citrus* limonoids (29,30,33). A rigorous description of the application of these methods to structure determination of the limonoids is beyond the scope of this chapter. However, a general description of the application of NMR to the structural characterization of nomilin (**10A**) will serve both to demonstrate the NMR spectral character of these compounds and provide an example of how these new techniques are used to elucidate their structures.

The 400 mHz ^{1}H NMR of nomilin (**10A**) (Fig. 7) was chosen for a general discussion of limonoid NMR characteristics. In nomilin (**10A**), the chemical shifts of proton resonances with minimal multiplicity in the spectrum are consistent with the early NMR signal designations for limonoid made by Dreyer (24). The C-methyl resonances ($\delta 1.09$ (CH$_3$ (24)), $\delta 1.18$ (CH$_3$ (18)), $\delta 1.33$ (CH$_3$ (19)), $\delta 1.47$ (CH$_3$ (25α)), $\delta 1.55$ (CH$_3$ (25β))), the proton signal at C-15 ($\delta 3.80$), the proton signal at C-17 ($\delta 5.44$) and the furan proton resonances ($\delta 6.37$ (H-21), $\delta 7.39$ (H-22, H-23) are indicative of an intact limonoid structure. The acetate methyl resonance ($\delta 2.01$) is a unique feature of the nomilin NMR. Nomilin (**10A**), methyl nomilinate (**17A**), nomilinic acid (**17D**), 7α-obacunol acetate(**6B**) and 7α-limonyl acetate (**4B**) and corresponding glucosides are the only acetylated limonoids thus far described from *Citrus*.

The ^{1}H NMR of nomilin displays nine multiplet resonances centered at $\delta 1.53$, 1.62, 1.79, 2.48, 2.59, 2.78, 3.09, 3.20 and 5.01. These resonances are associated with the alkyl protons in rings A, B, and C and can be easily be assigned to H-12(a), H-11(a,b), H-12(b), H-9, H-6(a,b), H-5, H-2(a), H-2(b) and H-1 respectively by analysis of COSY, HETCOR and HMBC two dimensional experiments. Changes in the structure of ring A (eg. limonin (**1A**), calamin (**20C**)), or oxidation state at C-6 and C-7 significantly alter the chemical shifts of protons at C-1, C-2, C-3, C-5, C-6 and C-15 (eg. 6-keto-7β-nomilol, C-15 ($\delta 4.41$)). In the case of the limonin-type limonoids, only four C-methyls are observed. The C-19 methyl group becomes an ether methylene as a part of ring A and is shifted to lower field ($\delta 4.2$-4.5). Regardless of the structural changes, limonoid aglycones do not display more than 14 multiply coupled alkyl proton resonances and alkyl chain lengths are unlikely to be greater than three carbons. These characteristics indictate that two dimensional FT NMR techniques can fully elucidate these structures.

The chemical shift patterns of the resonances of minimal multiplicity in the ^{1}H NMR of limonoid glucosides closely follow the pattern of the aglycones (31). A comparison of the chemical shifts of the C-methyl protons, furan protons and the C-15 and C-17 protons of the corresponding glucosides display shifts to higher field (eg. C-15 ($\delta 3.02$ vs $\delta 3.44$); C-17 ($\delta 5.23$ vs. $\delta 5.44$). Changes in chemical shift of these protons is consistent with changes in ring D of the limonoid nucleus. In addition to the limonoid nucleus proton resonances, a resonance for the C-1 glucose anomeric proton of the glucose occurs at about $\delta 4.15$. The signals of the C-2 to C-6 carbinol protons of the glucosides ($\delta 3.0$ - $\delta 3.6$) can obscure some of the limonoid nucleus

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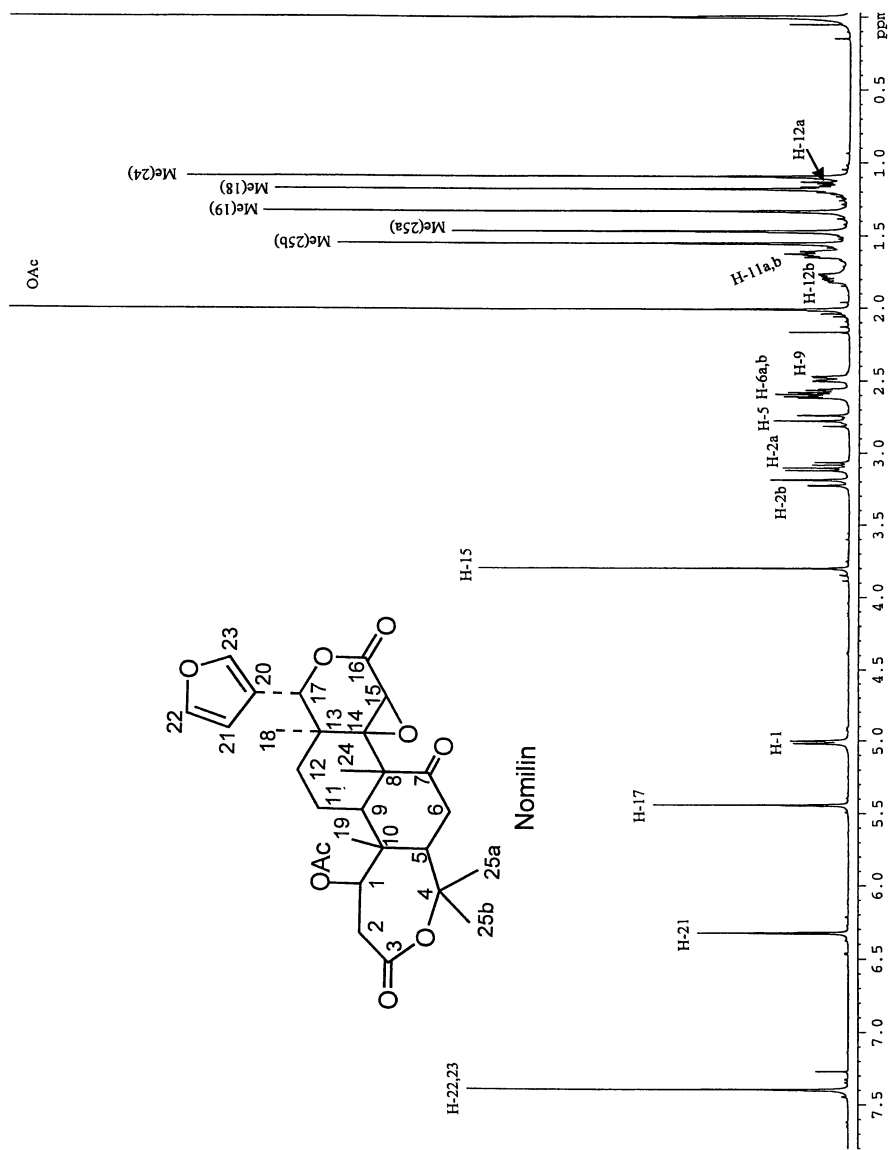
Figure 7. ^{1}H NMR (400 mHz) of nomilin

alkyl protons (generally H-2). However, the chemical shifts of these protons and their multiplicity can be determined by COSY and HETCOR NMR experiments.

Two dimensional FT NMR methods have been used successfully to assign the C-methyl resonances in the limonin-type glucosides and to establish the position of attachment of the glucose unit. ^{1}H-^{1}H COSY and NOSEY experiments showed strong correlation cross peaks between the protons on C-17 and on C-1 of the glucose moiety to establish the C-17 glucose substitution for the glucosides (29). C-methyl assignments were established by a combination of [1]H-^{13}C HETCOR and [1]H-^{1}H NOESY experiments (34). The C-18 methyl resonance was identified by its correlation with the protons at C-17 and C-1 of the glucose unit. The gem dimethyl groups at C-4 were differentiated by correlation with the protons at C-19 and C-15. The remaining methyl signal was assigned to the C-24 methyl and confirmed by [1]H-^{1}H NOESY correlation between the methyl and a proton at C-19.

The large number of quaternary carbons and carbons bonded to oxygen dominate the ^{13}C NMR spectra of limonoid aglycones and limonoid glucosides. Many of the limonoids have as many as nine quaternary carbons and the glucosides have up to 14 carbons bearing oxygen. The ^{13}C NMR chemical shifts of carbons present in limonoid aglycones and limonoid glucosides isolated from *Citrus* are presented in Tables 1 and 2. Molecular weights of each of the limonoids are included in the tables to aid in correlating LC-MS and NMR data for *Citrus* limonoids.

Although each of these groups of limonoids display unique ^{13}C NMR spectra, structural differences are associated more with changes in the A and B rings than in the C and D rings. The largest differences in chemical shift values for the ^{13}C resonances between limonoids in each group occurs among the C-1 to C-7 carbons. With the exception of deoxylimonol, the furan carbons, the C-8 through C-16 carbons and the C-18 and C-24 methyl groups display resonances in predictable chemical shift ranges for both the limonoid aglycone and limonoid glucoside nucleus. Resonances for the carbons of the furan ring and the glucoside are particularly consistent. As observed in the ^{1}H NMR spectra of these compounds, the difference between compounds with a C-19 methyl ($\sim\delta_c$ 10-15) and a C-19 methylene ($\sim\delta_c$ 60-68) are quickly identifiable. Retrocalamin (**13C**) and ichangin (**12A**) are the only limonoids whose basic structures have less than 25 carbons. Retrocalamin(**13C**) has no C-4 gem dimethyl group and ichangin (**12A**) is without a C-3 carbon.

LC-MS and NMR are powerful tools which can be applied to the isolation and characterization of *Citrus* limonoids. The LC-MS methodology provides a rapid and highly sensitive means to analyze for limonoid aglycones and glucosides in *Citrus* and citrus processing materials and offers chromatographic means to acquire purified limonoids. Modern NMR methods provide comprehensive means to characterize limonoids structurally and to elucidate their stereochemical properties. Further application of these two tools in *Citrus* research will broaden the chemical knowledge about these compounds, expedite the analysis and identification of new limonoids from *Citus* sources and aid in defining the nutritional role and metabolic fate of these compounds in humans.

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Table 1. ^{13}C NMR Chemical

C	$1A^b$	2^c	$3B^c$	$4B^c$	$5A^a$	$6B^a$	$7B^a$	$8A^a$	$9A^a$	$10A^a$
1	78.5	80.1	80.9	80.6	156.8	157.3	155.8	159.9	160.6	70.8
2	35.6	36.4	36.8	36.6	122.7	120.5	121.0	118.1	119.6	35.3
3	169.9	171.1	171.5	170.6	166.7	168.6	167.3	166.6	166.9	169.2
4	79.5	81.0	81.2	81.0	84.0	85.5	84.6	73.6	73.6	84.3
5	58.2	52.2	52.9	53.9	57.2	47.9	49.2	55.5	59.4	51.1
6	36.2	26.5	27.5	24.2	39.9	30.6	26.9	38.2	37.7	38.9
7	207.7	72.1	70.8	74.3	207.5	69.0	72.9	209.3	209.5	206.9
8	50.4	46.8	46.8	46.5	52.9	43.9	42.1	52.8	52.8	52.9
9	46.7	39.8	42.9	43.9	49.1	39.9	41.4	45.9	46.9	44.3
10	45.4	46.8	44.8	43.8	43.1	43.1	43.9	45.3	44.6	44.3
11	17.7	17.3	17.9	17.7	17.0	16.4	16.4	19.5	18.3	16.5
12	29.2	26.5	27.1	26.8	32.6	26.8	26.3	32.8	32.5	32.2
13	37.7	38.7	39.1	39.4	37.3	38.0	38.5	37.4	37.4	37.6
14	66.6	175.9	70.3	70.0	65.1	70.1	69.7	65.6	65.4	65.6
15	53.8	114.5	58.3	56.9	53.3	57.2	56.5	53.3	53.3	53.5
16	167.0	166.1	169.0	168.3	166.9	167.9	167.2	167.2	166.9	166.9
17	77.4	82.5	79.3	79.2	77.9	78.6	78.3	78.2	78.2	78.1
18	17.2	17.9	17.9	18.1	19.4	18.1	18.1	20.0	16.4	17.2
19	64.8	66.5	67.1	66.5	16.4	16.0	16.0	16.6	15.7	17.2
20	120.2	121.5	122.1	121.5	120.0	120.5	120.4	120.5	120.4	120.2
21	143.2	144.0	142.6	142.4	143.1	141.1	141.3	142.9	143.0	143.3
22	110.1	111.0	111.2	111.0	109.7	109.9	109.9	110.0	110.0	109.7
23	141.5	142.4	144.2	144.0	140.9	142.9	143.1	141.0	141.0	141.1
24	19.6	22.0	18.9	17.9	21.0	18.5	18.2	20.4	20.9	20.8
25a	29.8	30.7	30.7	30.7	32.0	32.0	32.1	32.5	31.0	33.5
25b	21.3	26.3	22.0	21.8	26.7	26.8	26.5	29.0	30.0	23.4
OAc(C=O)										169.2
OAc(Me)										20.8
MeO								51.7	51.5	
Mol. Wt.	470	456	472	514	454	456	498	486	486	514
Ref.	29	19	19	19	29	19	19	29	29	21

^{a}=CDCl$_3$, ^{b}=DMSO-d_6, ^{c}=CD$_3$CN

Shifts for Limonoid Aglycones in *Citrus*

11A[b]	12A[b]	13C[b]	14A[a]	15A[a]	15C[a]	16A[a]	17A[b]	18A[a]	20C[a]	21A[a]	22A[a]
68.5	70.3	71.4	105.9	82.1	85.8	83.6	76.2	74.6	73.0	74.9	82.8
39.0	39.0	37.0	24.8	36.0	37.6	36.7	38.5	37.0	36.9	39.5	35.9
170.6	171.3	172.6		171.2	171.7	170.9	171.1	174.1	172.3	173.4	171.4
83.7	71.7		79.8	78.1	81.5	80.4	73.0	74.4	71.8	83.7	80.3
49.3	49.6	43.9	54.2	59.7	68.8	55.6	51.6	51.3	63.8	48.4	144.6
39.2	38.2	209.5	36.8	36.0	209.3	36.4	35.0	39.5	210.6	37.2	138.0
208.4	209.3	80.5	208.8	207.9	82.6	208.0	209.9	210.8	80.5	211.8	196.1
52.0	52.1	46.7	50.2	50.2	48.4	51.1	51.9	52.4	48.2	52.4	47.8
43.6	48.0	37.5	41.2	45.3	46.2	43.1	43.4	44.4	41.6	35.8	45.2
44.3	45.4	43.7	49.3	45.3	46.7	46.9	45.7	45.6	51.0	48.7	46.9
16.8	17.7	17.0	16.7	17.3	20.4	17.9	18.4	18.8	17.7	18.6	19.4
31.2	29.2	28.9	27.8	28.0	32.1	30.1	31.2	31.8	27.5	28.0	31.1
36.9	37.7	38.3	39.6	38.6	38.1	38.1	36.8	37.4	38.2	39.2	37.9
65.8	66.0	69.8	69.1	66.4	66.4	66.5	65.7	66.1	71.6	69.0	66.0
52.9	53.8	53.1	55.7	54.7	51.4	54.0	52.8	53.3	54.7	55.3	52.7
167.0	167.0	167.6	167.9	167.0	167.1	167.0	166.8	167.5	167.6	167.4	166.9
77.6	77.4	77.8	78.1	77.9	78.1	77.7	77.8	78.4	77.7	78.1	77.8
16.2	15.8	18.7	19.2	18.4	20.7	19.4	16.0	17.0	17.7	16.8	18.8
16.1	64.8	13.8	14.8	11.8	14.5	17.7	16.0	16.6	13.1	68.7	15.3
120.3	120.2	120.6	120.7	120.0	120.2	120.1	120.3	120.5	120.3	120.3	120.2
143.1	143.2	141.5	143.0	142.6	141.1	142.9	143.2	143.1	141.5	143.0	141.1
110.1	110.1	110.3	110.0	109.5	109.9	109.6	110.2	109.9	110.1	109.7	108.8
141.3	141.5	143.3	141.2	140.8	143.2	140.9	141.4	141.1	143.2	141.1	143.2
20.1	20.6	19.4	19.2	19.5	21.1	20.6	20.2	20.7	19.0	19.5	20.4
32.9	32.7		31.9	29.2	30.3	31.6	32.4	34.0	32.7	28.9	26.2
23.3	26.1		23.5	22.9	23.4	23.5	28.7	27.7	27.5	24.2	24.6
							169.5				
							20.7				
		51.9		51.3	51.9	51.7	51.2	51.8	50.8	52.1	52.0
472	488	462	444	486	502	486	546	504	520	488	502
21	20	21	29	21	21	29	29	21	21	20	21

Table 2. ^{13}C NMR Chemical Shifts

C	1D[a]	5D	8D	9D	10D	11D	12D	14D
1	77.9	150.3	155.2	158.3	70.7	67.4	70.3	105.2
2	35.6	119.5	119.8	119.1	35.6	40.1	38.0	74.2
3	169.9	165.4	167.0	167.1	169.5	171.0	170.9	23.9
4	79.8	83.2	72.3	72.5	84.1	83.5	72.5	78.5
5	55.7	51.3	53.7	54.8	47.0	45.3	45.4	51.7
6	36.6	40.0	38.6	38.2	38.9	40.1	39.0	37.3
7	207.2	208.5	212.9	212.0	208.8	209.9	211.8	209.4
8	50.5	51.4	51.3	51.1	50.8	50.8	51.3	50.4
9	45.3	46.7	40.5	42.6	42.0	41.5	41.0	40.8
10	45.4	44.4	44.0	44.0	44.3	44.4	45.8	48.7
11	17.0	17.3	16.8	17.4	15.1	15.1	17.7	15.7
12	26.6	26.7	26.7	26.6	26.4	26.6	27.1	26.5
13	44.9	44.1	43.9	43.6	43.1	43.9	43.4	45.1
14	70.8	70.8	71.1	70.9	71.1	71.0	69.9	71.4
15	57.1	57.4	58.4	58.3	57.7	57.5	58.6	57.1
16	169.6	169.2	169.2	169.2	169.4	169.3	168.9	169.9
17	77.8	77.8	77.9	77.7	77.7	77.7	77.4	77.8
18	21.8	23.1	24.3	25.0	22.0	22.4	24.2	25.3
19	63.7	15.1	16.9	14.5	13.3	15.5	68.1	12.5
20	125.5	125.5	125.8	125.6	125.7	125.7	125.6	125.8
21	141.5	141.7	141.6	141.6	141.5	141.5	141.6	141.5
22	112.5	112.6	112.7	112.6	112.6	112.6	112.6	112.6
23	140.8	140.7	140.6	140.6	140.6	140.6	140.7	140.6
24	19.0	19.4	19.4	17.0	19.0	18.7	15.2	19.6
25a	30.4	29.9	30.2	30.1	32.3	32.1	33.7	32.1
26b	25.1	25.4	29.9	28.8	24.9	25.0	24.8	24.0
OAc(C=O)					169.2			
OAc(Me)					20.5			
Gluc C-1	104.4	104.4	104.4	104.4	104.4	104.5	104.3	104.4
Gluc C-2	74.1	74.1	74.2	74.2	74.2	74.2	74.2	74.2
Gluc C-3	76.9	77.0	77.0	77.0	77.0	77.0	77.4	77.1
Gluc C-4	70.9	70.6	70.7	70.7	70.6	70.8	70.7	70.7
Gluc C-5	76.2	76.2	76.2	76.2	76.1	76.1	76.2	76.2
Gluc C-6	61.6	61.6	61.7	61.7	61.7	61.7	61.7	61.7
Mol. Wt.	650	634	652	652	694	670	668	612
Ref.	25	25	26	26	25	25	27	23

[a] all samples run in DMSO-d_6 (90° C)

for Limonoid Glucosides in *Citrus*

15D	16D	17D	18D	18E	19D	20E	21D	23E
81.6	82.9	75.8	73.2	73.2	71.0	72.4	73.7	66.7
36.9	36.8	38.7	37.8	37.9	37.8	37.3	38.6	37.2
172.1	171.9	172.2	174.2	173.2	173.8	172.4	173.1	170.4
78.1	79.9	73.2	73.3	73.3	73.8	71.6	83.3	80.8
56.5	51.3	48.3	47.2	47.0	48.2	63.6	48.5	59.6
36.7	36.6	35.3	39.5	39.5	39.0	210.7	39.6	209.5
208.6	208.2	212.3	213.3	213.2	212.2	81.1	211.7	81.3
50.5	50.3	51.4	51.3	51.3	51.3	49.8	53.0	50.7
44.8	41.3	40.3	39.7	39.6	39.4	41.0	35.8	38.6
45.4	46.7	44.5	44.7	44.8	49.5	52.2	50.5	49.6
17.0	16.2	17.3	16.9	16.8	182.0	18.0	19.2	15.5
26.5	26.8	26.9	27.0	26.9	26.9	26.8	26.6	26.5
45.0	45.0	43.6	43.7	43.7	43.4	43.6	44.2	44.0
71.1	71.2	70.5	70.5	70.4	70.5	75.5	70.0	75.3
57.3	57.4	58.3	58.2	58.3	58.5	58.2	57.4	58.3
169.8	170.0	169.0	169.1	169.2	169.0	170.2	169.2	170.1
77.9	77.9	77.8	77.7	77.6	77.7	77.1	77.6	77.2
23.9	24.3	24.9	24.7	24.6	24.8	23.0	24.8	23.1
9.7	16.2	15.0	15.3	16.3	59.8	13.1	68.1	13.2
125.7	125.7	125.7	125.8	125.8	125.8	126.2	125.6	126.2
141.6	141.6	141.5	141.5	141.5	141.5	141.3	141.4	141.2
112.6	112.6	112.7	112.7	112.7	112.6	112.6	112.6	112.5
140.7	140.7	140.6	140.6	140.6	140.6	140.3	140.7	140.5
19.7	19.7	16.3	19.7	15.3	15.7	16.0	16.4	14.4
30.1	32.1	31.7	32.5	32.8	32.7	32.8	28.0	32.7
25.5	25.4	28.0	27.7	27.7	26.4	27.7	23.3	27.7
		169.2						
		20.6						
104.4	104.4	104.5	104.4	104.4	104.5	104.2	104.4	104.2
74.2	74.2	74.1	74.1	74.2	74.2	74.3	74.2	74.3
77.0	77.0	77.0	77.0	77.1	77.1	77.1	77.1	76.9
70.7	70.7	70.6	70.7	70.7	70.7	70.8	70.7	70.8
76.2	76.2	76.1	76.1	76.2	76.1	76.1	76.2	76.1
61.7	61.7	61.6	61.6	61.6	61.7	61.7	61.7	61.7
652	652	712	688	684	686	700	668	686
26	26	26	26	24	27	24	27	24

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Analysis of Limonin and Flavonoids in Citrus Juices and Byproduct Extracts by Direct Injection and In-Line Sample Clean-Up

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A cyano stationary phase was found to have better selectivity than a C-18 stationary phase for HPLC analysis of limonin in citrus juices. However, the cyano phase was found to be more susceptible to contamination by constituents contained in citrus juices under reversed phase conditions, requiring more attention to sample cleanup. An automated in-line sample preparation method using a switching valve was developed for analysis of limonin, flavanone glycosides and for screening methoxylated flavones, and coumarins in citrus juices. A rapid sample extraction technique utilizing sonication was also developed to allow screening and analysis of extracts from citrus seeds and peel byproducts for the compounds described above. Using the method, more than 1000 sample injections can be performed before replacement of the analytical column is required.

The development of reliable HPLC methods for quantifying limonin and naringin has facilitated measurement of bitterness in juice products in citrus quality control labs. Because limonin is present in citrus juices at low part per million levels and does not contain a good chromophore, detection is limited mostly to absorbance in the UV region below 220 nm. These factors require rigorous sample preparation to obtain an extract free of interference upon analysis. We reported on an earlier method utilizing a cyano (CN) column for reversed phase analysis of solid phase extracts (1). This method provides improved resolution and selectivity for limonin compared to previous methods (2,3,4). The earlier methods used solid phase extraction (SPE) for sample preparation and either octyl (C-8) or octadecyl (C-18) based stationary phases for analysis. We now describe a method that automates the SPE clean-up procedure for limonin and allows analysis of naringin and other flavanone glycosides in the SPE waste if desired.

Sample Preparation

Citrus Juices

It is necessary to heat juice to 90° C to dissolve any precipitated limonin or naringin and to convert A-ring lactone to limonin. Heating is most easily accomplished using a microwave oven. Citrus juice (5mL) was placed into a disposable screw cap centrifuge tube with the cap loosely sealed to allow expanding air to escape. The time required for heating samples to 90° C without allowing them to boil must be determined by trial and error for each microwave and was usually in the order of 3 to 6 seconds per sample. The microwave oven used should have a rotating carousel to ensure samples are heated evenly. The heated samples were removed from the oven and 40% aqueous acetonitrile (5 mL) added to each sample. Samples were filtered through a 25mm 0.45µ nylon filter with glass fiber prefilter (Whatman GD/X, Clifton, New Jersey; Titan #44525-NN, Scientific Resources Inc., Eatontown, NJ.) These filters eliminate the need to centrifuge samples prior to filtration.

Citrus By-Product Solids

The amount of sample extracted will depend on the expected concentration of limonin. Citrus peel and pulp material generally contain between 100 – 600 ppm limonin on a dry weight basis while dried seeds may contain as much as 8000 ppm limonin. For peel and pulp, 0.5-1.0g of material was used. For seed material approximately 0.1g was used. When the approximate concentration of limonin is not known, it may be necessary to extract several amounts to obtain an extract within the standard range.

An extraction solvent was prepared to contain 40% acetonitrile in 0.05% aqueous acetic acid using HPLC grade solvents and distilled water. The sample was weighed into a 25mL glass screw cap vial, extraction solvent (10 mL) introduced into the vial and sealed with a polypropylene lined cap. Samples were sonicated in a water bath for 60 minutes, removed from the bath, combined with distilled water (10 mL), mixed, and filtered through a 25 mm 0.45µ nylon filter with glass fiber prefilter.

Analytical

Equipment

The following equipment is necessary to perform the automated solid phase extraction and analysis with the configuration as shown in Fig. 1.
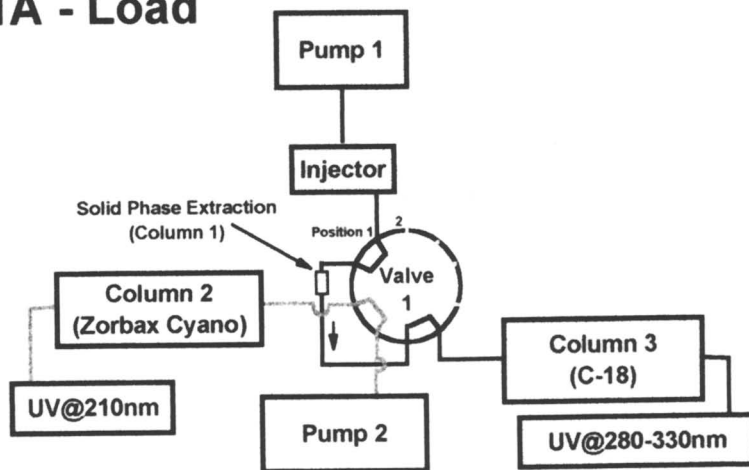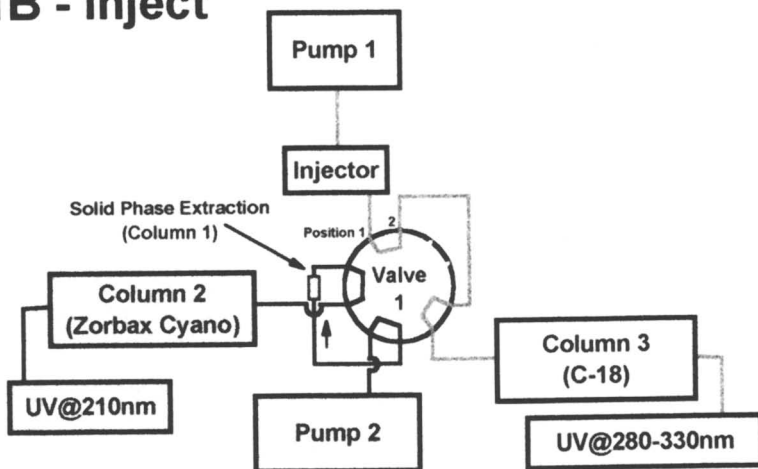
Figure 1. Instrument flow diagram of system for simultaneous analysis of limonin and naringin

Pump 1	- Liquid chromatography pump capable of pumping at 0.5 mL/min.
Pump 2	- Gradient pump capable of forming binary gradient and pumping at 1.2 mL/min.
Autosampler	- Automated sampling device capable of delivering 40μL injection of sample.
Valve 1	- 6, 8 or 10 port injection valve for automated solid phase extraction.
Detector 1	- UV detector capable of measuring absorbance at 280 – 330nm for naringin determination.
Detector 2	- UV detector capable of measuring absorbance at 205 – 215nm for limonin determination.
Integration	- Device capable of integrating 2 independent signals (detector 1 and detector 2).
Timer	- Device for control of valve timing if not built into another LC system component.
Column 1	- Upchurch 3.0 x 10mm C-18 (Oak Harbor, Washington) for solid phase extraction of limonin.
Column 2	- Zorbax 4.6 x 250mm Cyano (Hewlett Packard, Palo Alto, California) for limonin analysis.
Column 3	- Phenomenex 3.0 x 150mm C-18 Prodigy (Torrance, California) for naringin analysis.

Standard Preparation

Stock limonin standard: Prepare a 0.02% (w/v) solution of limonin in acetonitrile (HPLC grade). Accurately weigh 10mg limonin (LKT Labs, St. Paul, Minnesota) into a 50mL volumetric flask using an analytical balance. Make to volume and mix thoroughly. Standard is stable for 6 -12 months if properly sealed to avoid evaporation of acetonitrile.

Working standards: Accurately weigh 50 mg naringin (Indofine Chemical Co., Somerville, New Jersey) that has been dried in a vacuum oven into a 100 mL volumetric flask. Pipette 20mL limonin stock standard into the flask and mix until naringin is completely dissolved. Add 0.5% aqueous acetic acid to volume and mix thoroughly to give working standard with 40ppm limonin and 500ppm naringin. Standards containing 20 and 10ppm limonin respectively were obtained through dilution of first working standard 1:1 and 1:3 with 20% acetonitrile in aqueous 0.5% acetic acid.

Limonin and Naringin Analysis

At injection the system is configured as in Fig. 1A with valve 1 in the load position. A 10 port switching valve (Vici Valco, Houston, Texas) was used for valve 1. A 6 or 8 port switching valve may also be used with modifications (for a 6 port valve, port 2 would be connected directly to column 3 and the SPE extraction column would be connected to port 3). Solvent 1 flows from pump 1 to the sample injector,

through valve 1 into the limonin extraction column, back through valve 1 to the C-18 analytical column for naringin analysis, and finally to detector 1. Solvent 1 (water:acetonitrile (81:19), flowing at a rate of 0.5mL/min.) performs the in-line sample clean-up for limonin analysis and separates naringin from other flavanone glycosides. Injection volumes ranged from 40μL for citrus juices down to 10μL for peel and seed extracts containing large amounts of limonin and/or naringin. The event sequence that takes place on the instrument is outlined in Table 1. After 2.5 min. the clean-up sequence is complete and valve 1 is switched to the inject position (Fig. 1B). In this position limonin is back flushed from the SPE column onto the cyano analytical column where it is separated from coumarins, methoxylated flavones and other components by a gradient elution. At 18 minutes valve 1 is switched back to the load position to allow equilibration for SPE and analysis of the next sample.

If analysis of naringin is not required, column 3 and detector 1 may be eliminated and the line going to column 3 directed to waste. To reduce generation of waste solvent when naringin analysis is not being performed, pump 1 can be shut off when valve 1 is switched at 2.5 min. and then turned back on again at 18 min. to allow equilibration of the SPE column.

When first developed, separation of limonin from other components could be accomplished isocratically. However, due to changes in juice processing practices by the industry, it is now necessary to perform a gradient elution for limonin analysis to remove late eluting components that would interfere with subsequent injections. Solvent is pumped at a flow rate of 1.2 mL/min. from pump 2 and is programmed for the linear gradient profile outlined in Table II. While it may appear that sufficient time has not taken place for column equilibration between injections, the cyano column undergoes an equilibration time of 7 min. before limonin is transferred to the column for analysis due to the time required for injection and the SPE step.

Table I. Automated Extraction Timing Sequence for Valve 1

Time (min)	Valve	Event
0	Load	Inject Sample, start clean-up
2.5	Inject	Clean-up finished, load limonin onto cyano column
18	Load	Equilibrate column for next sample clean-up
20	Load	Column equilibrated, system ready for next sample

Table II. Gradient Profile Used for Limonin Analysis on the Cyano Column

Time (min)	Flow (mL/min)	% Water	% Acetonitrile
0	1.2	64	36
5	1.2	64	36
13	1.2	52	48
15	1.2	20	80
16	1.2	20	80
16.5	1.2	64	36
20	1.2	64	36

Linearity and Detection Limits

Using a 40µL injection volume, the 40ppm standard delivers 1.6µg limonin on column and a 4 point standard curve forced through the origin was found to be linear (R=0.9997) using UV absorbance at 210nm for detection. The detection limit using UV absorbance at 210 nm was found to be 0.15ppm using a 40µL injection or 6 ng limonin on column.

Measurement of naringin using a 40µL injection volume was found to be linear to 200ppm (8µg naringin on column) using 280nm UV absorbance for detection. Measurement to 500ppm in prepared samples required the use of a quadratic standard curve as the detector appeared to near the saturation point, making use of a 3 or 4 point standard curve necessary. Detector saturation may be avoided by setting the absorbance wavelength at 330 nm for naringin. This is well away from the UV absorption maximum of 282 nm for naringin and results in a five-fold decrease in sensitivity. At 330 nm the detection limit for naringin is 2ppm (80ng on column) and the standard response is linear to 1000ppm (40 µg on column) with a 40 µL injection. Grapefruit juice that has been produced by a "hard squeeze" or high extraction pressures will often exceed the linear range for naringin if UV detection at 280 - 290 nm is used. Extracts prepared from untreated commercial dried grapefruit peel contain approximately 4% naringin by weight. These must be diluted by a factor of 40 - 80 times the original sample weight to obtain an extract containing less than 1000ppm naringin. Another consideration is the limonin content. Dried peel waste material from seedless varieties generally will contain 300 - 600ppm limonin. Limonin content in the waste material from seedy citrus varieties will be higher as the seeds contain 5,000 – 10,000 ppm limonin by dry weight. Citrus waste materials containing a high proportion of seeds may need to be prepared with dilutions higher than required for naringin. Conversely, waste material from seedless varieties should be prepared at a dilution in the lower range required for naringin to maximize the limonin peak.

Recovery of Limonin and Naringin

The recovery efficiencies of limonin and naringin during the in-line SPE step were examined by comparing component retention of standards and a grapefruit juice on column 1 only. Naringin and other flavanone glycosides were traced going through column 1 used for the SPE cleanup step (Fig. 2). The switching time of valve 1 was based on the time necessary to elute naringin, hesperidin, and neohesperidin from the SPE column; while limonin, didymin and poncirin are retained. Limonin does not show up in the tracing as it is retained for better than 10 min using the specified SPE cleanup solvent. Naringin passes through the column in less than 2 min. There were no differences in peak area found for either naringin or limonin standards injected directly onto the analytical column when compared to use of valve switching at 2.5-3 min with SPE. Juice samples spiked with limonin were found to give 95 - 101% recoveries for added limonin. Careful monitoring of the timing is necessary to separate neohesperidin, didymin and poncirin in prepared samples. It is desirable to

Flavanone Retention on Precolumn Only
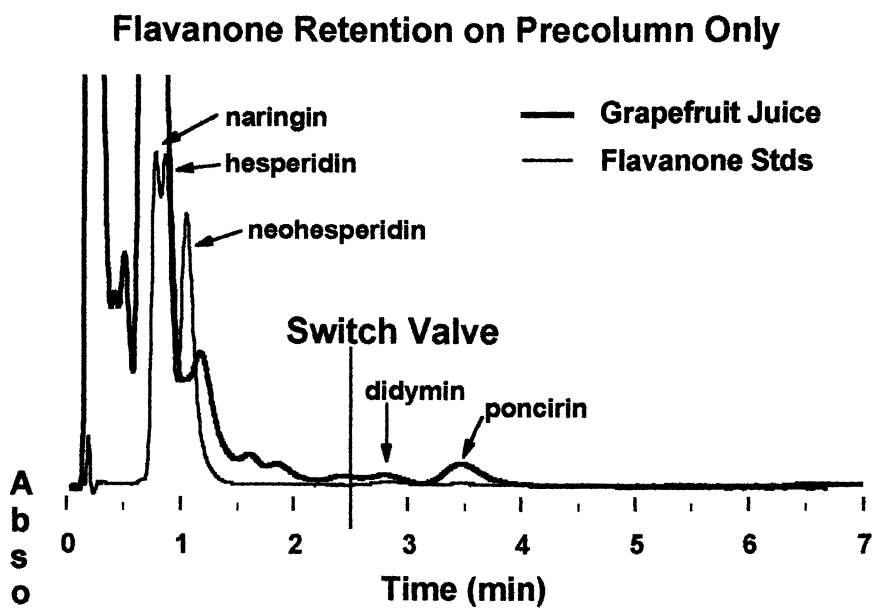
Figure 2. Separation of flavanone glycosides on C-18 precolumn. Limonin will elute after 10 minutes.

have didymin and poncirin still retained on the SPE column when switching takes place so they can be eluted quickly from the cyano column (Fig. 3 & 4). If they are allowed to pass onto the C-18 column along with naringin, they elute much later and may cause interference in subsequent analyses. The 3 x 10 mm C-18 column used for SPE from Upchuch Scientific (Oak Harbor, Washington) was found to be rugged and could be used for 100 or more injections before replacement was necessary.

Limonin and naringin were completely extracted from peel and seeds by following the recommended technique. Extractions using sonication were compared with exhaustive Soxhlet extraction of peel and seed material and yielded comparable results. Extraction by sonication had 2 advantages over those done by Soxhlet. Extractions were accomplished much faster and did not contain components that had a tendency to foul the cyano column. Analysis of orange and grapefruit peel extracts by Soxlet were found to give good quantitative results, but had a tendency to load the cyano column with components that were difficult to remove.

Filters with polyvinyldifluoride (PVDF) or teflon (PTFE) membranes should not be used to filter the sample extracts in 20% aqueous acetonitrile. The PVDF membranes were found to strongly absorb limonin from these sample extracts. While nylon filter membranes were found earlier to absorb naringin and other flavanone glycosides in aqueous samples, extracts in 20% acetonitrile did not show any loss of naringin or limonin from adsorption by nylon membranes. The described method does not quantitatively measure hesperidin in orange juice. Hesperidin is only sparingly soluble in aqueous solutions and most of the hesperidin present in orange juice occurs as precipitate in the juice cloud. Hesperidin solubility in aqueous solutions is not increased significantly on heating. Juice samples diluted 1:1 contain approximately 225ppm soluble hesperidin in the 20% acetonitrile preparation while orange juice may contain greater than 500ppm hesperidin. In order to obtain a quantitative measurement of hesperidin in orange juice, a higher dilution of the juice must be used.

Analytical Column Performance

Mobile Phase Solvents: Because limonin requires detection by UV at low absorbance wavelengths (200 – 220 nm), solvent requirements are stringent. With in-line automated SPE, use of high purity HPLC grade solvents is critical. This is especially true of water used to make up mobile phases. It should either be purchased through a reliable vendor, or if manufactured in the lab, passed through deionization cartridges, activated charcoal, and media specially made to remove trace organic materials. Trace impurities in the SPE mobile phase are concentrated on the SPE column during equilibration. Problems with interference in limonin analysis and screening of coumarins and polymethoxylated flavones can many times be traced back to the SPE mobile phase. Degassing mobile phase solvents with helium containing trace impurities can be another source of interference and degrade analytical performance.

A. Red Grapefruit Juice

B. White Grapefruit Juice
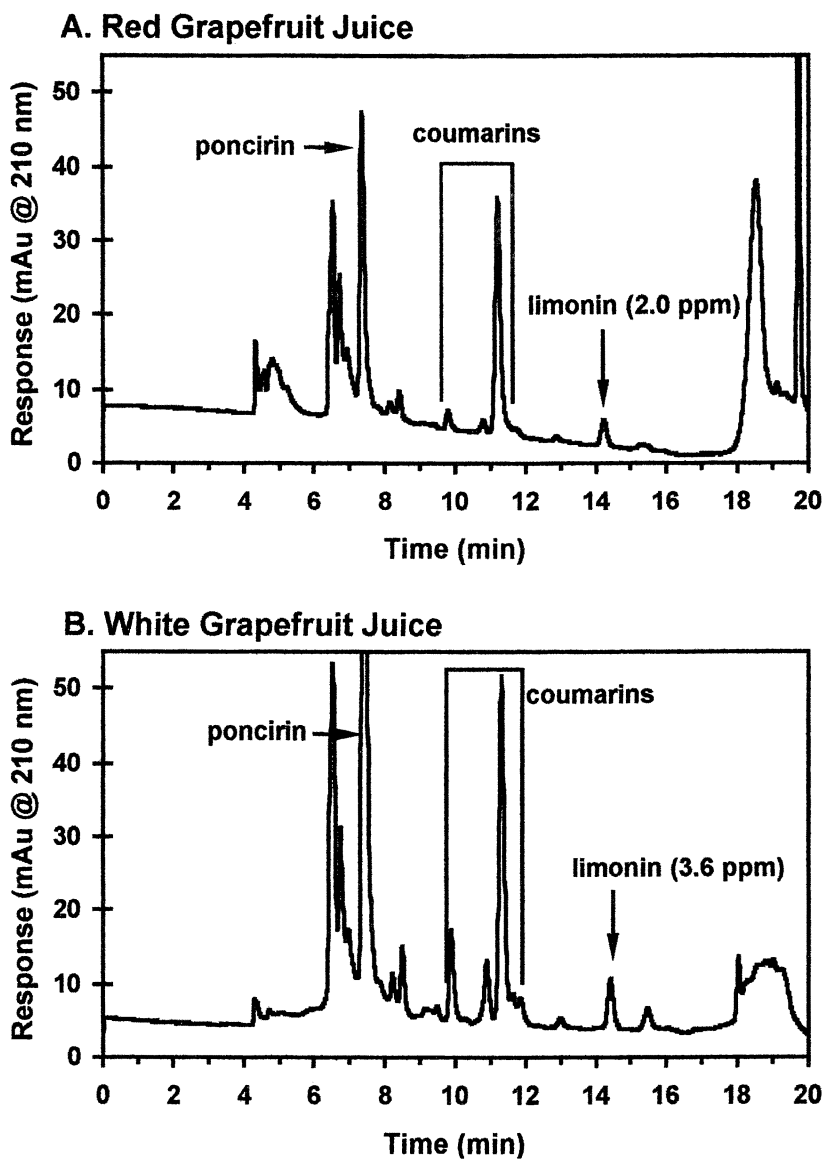
Figure 3. Separation of limonin and coumarins in red (A) and white (B) grapefruit juices on Zorbax Cyano column. Limonin amount indicated is amount measured in the extract.

A. Navel Orange Juice

B. Valencia Orange Peel (1+50)
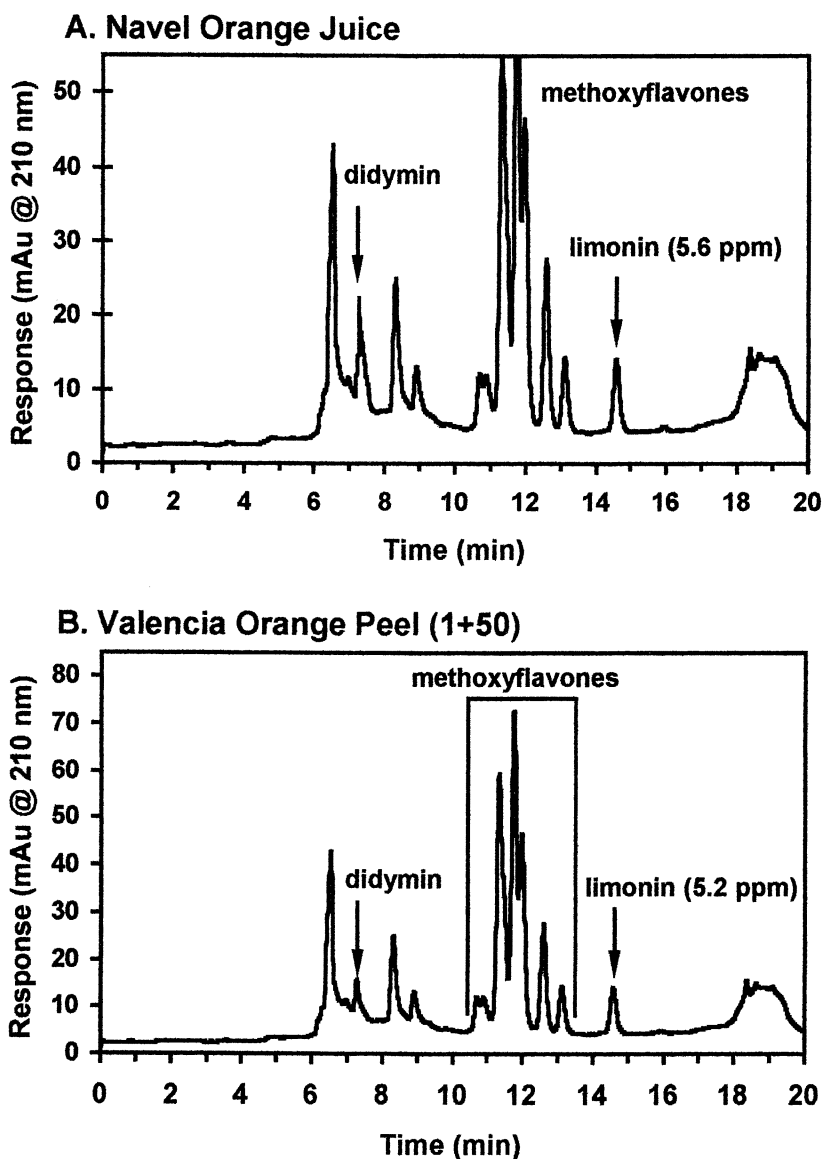
Figure 4. Separation of limonin on Zorbax Cyano column in Navel orange juice (A) and extract of valencia orange peel (B). Limonin amount indicated is amount measured in the extract.

Zorbax Cyano Column for Limonin: A number of cyano columns have been evaluated for performance, selectivity and ability to isolate limonin from other components in the SPE extract. Columns with a cyano phase other than Zorbax material or a Zorbax equivalent cyano phase manufactured by Whatman (Clifton, New Jersey) were not found to be suitable for limonin analysis. Separation of limonin from other citrus components in grapefruit (Fig. 3A &B) and orange juices (Fig 4A), and orange peel (Fig. 4B) are shown. Successful resolution of limonin from other components was monitored through the use of spectral scans of UV absorbance. Other cyano phase columns either produced tailing peaks or had an interfering peak that could not be resolved through manipulation of the gradient. Fortunately a number of companies are now licensed to carry Zorbax material and columns made with this material can be obtained through a number of chromatography equipment suppliers.

C-18 column for Naringin: Methods for analysis of naringin in citrus juices using a C-18 analytical column have been available for more than 20 years. Most C-18 stationary phases require addition of small amounts of acid to the mobile phase to suppress peak tailing and reduce peak width. This greatly improves the quality of separation that will be obtained on these columns for flavanone glycosides in citrus juice. In recent years a number of C-18 phases have been introduced based on column manufacture using materials of high purity and careful bonding of the C-18 phase. These materials have fewer problems with tailing and peak broadening during flavonoid analysis. Columns made with C-18 phase bonded to packings of Inertsil (SGE, Ringwood, Australia), Kromasil (Higgins Analytical, Mountain View, California) and Prodigy (Phenomenex, Torrance, California) have all been found to provide superior separation of flavanone glycosides in citrus juices without the need to add acid modifiers (Fig 5). The ability to perform the SPE extraction of limonin and analysis of naringin using water:acetonitrile without an acid modifier, provides a cleaner extract. This allows screening for polymethoxylated flavones and coumarins in citrus juices in addition to limonin analysis on the cyano column.

Applications for Analysis

The described method was developed primarily to measure bitterness in citrus juices. Limonin and naringin are the primary components causing bitterness in grapefruit juice, and limonin is the primary component causing bitterness in Navel orange juice. Poncirin is a minor flavanone glycoside in grapefruit that is also bitter and can be monitored. Because of the influence of grapefruit juice on absorption of calcium channel blocker drugs and the evidence associating coumarins contained in grapefruit juice as a likely cause, it may also be of interest to monitor coumarin content in grapefruit juice. The large peak at approximately 11.2min (Fig. 3) is 3,4-dihydroxybergamottin and the peak at 19.9 min is bergamottin (Fig. 3B). Unfortunately, there is no commercial source of 3,4-dihydroxybergamottin available, therefore quantitation of the coumarins is not practical on a routine basis. However, peak areas may be compared to assess relative amounts of these compounds between grapefruit juice varieties.

Flavonoids in Grapefruit Juice SPE Waste Stream

naringin
(655 ppm total in juice)

narirutin

hesperidin

neohesperidin

Time (min)

Response @ 330 nm
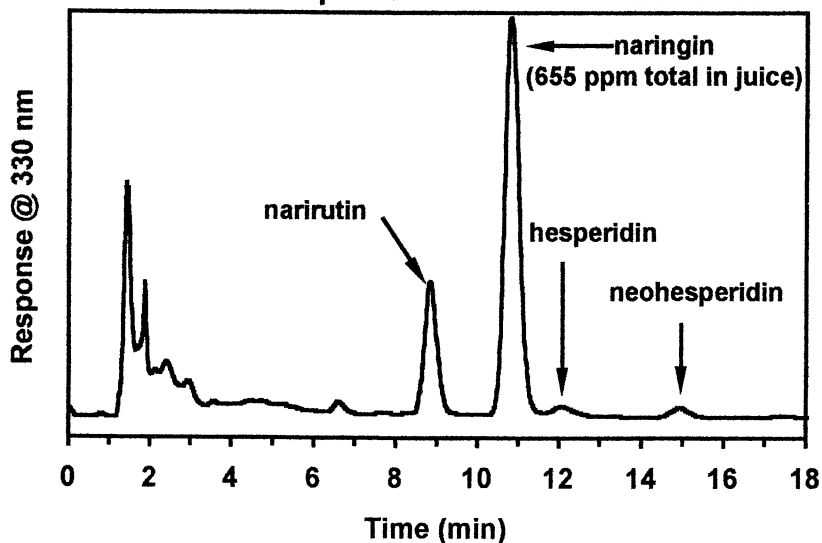
Figure 5. Separation of naringin and other flavanone glycosides in grapefruit juice from limonin SPE waste on Phenomenex C-18 column.

Hamlin Orange Seeds (1+400)

limonin (12.2 ppm)

Response (mAu @ 210 nm)

Time (min)
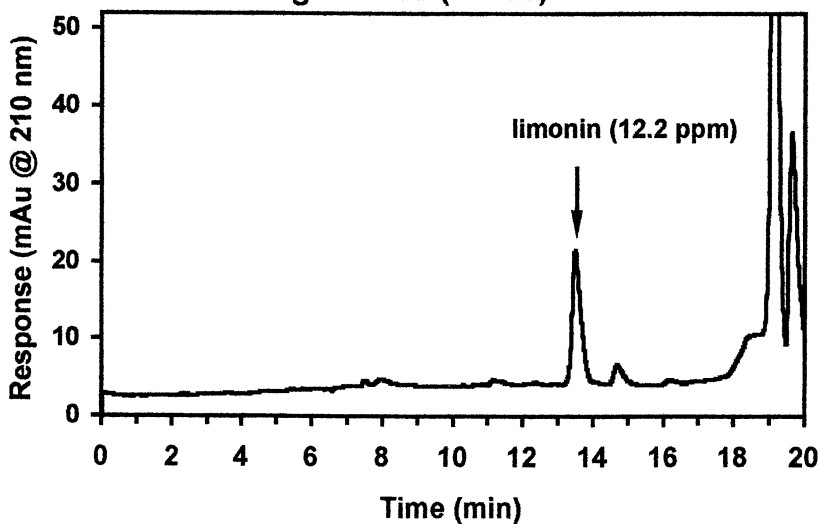
Figure 6. Separation of limonin in extract from Hamlin orange seeds on Zorbax Cyano column. Limonin amount indicated is amount measured in the extract.

The polymethoxylated flavones are another class of citrus compounds that one may wish to monitor because of their biological activity (5). They elute in the same region as coumarins but may be distinguished by UV absorption characteristics. Grapefruit does not contain appreciable amounts of polymethoxylated flavones and orange does not contain the coumarins present in grapefruit. Currently, work is in progress to add value to citrus waste streams. Peel byproducts (Fig. 4B) in the author's laboratory are monitored for reduced bitterness after treatment and the methoxylated flavones are also measured to evaluate content and feasibility for extraction. Citrus seeds contain large amounts of limonin and the method may be used to determine limonin content (Fig. 6) of various citrus seeds provided appropriate dilutions are made to give no more than 40ppm limonin in the extract.

References

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Chapter 6

Quantification of Limonin and Limonoate A-Ring Monolactone During Growth and Development of Citrus Fruit and Vegetative Tissues by Radioimmunoassay

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RIAs were developed using a polyclonal antibody raised against limonin-7-(O-carboxymethyl)oxime conjugated to BSA (*1, 2*). Using an iodinated tracer, sensitivity was 150 fmol limonin (less than 1 ppb) and only deoxylimonin showed significant cross-reactivity. The assay developed using ^{3}H-limonol as tracer had a detection limit of 0.22 ng (2.2 ppb). In both systems, there was no cross-reactivity with limonoate A-ring monolactone, therefore this compound could also be quantified. Since the RIAs required no prior purification of samples, up to 400 samples could be analyzed per day. Therefore, they were used to analyze natural variation of limonin levels in fruit and vegetative tissues, changes during growth and development, and variation in processed juice. RIA techniques can provide several advantages as compared to many other quantitative or qualitative methods. They are sensitive down to the fmol range, allow processing of hundreds of samples per day, and can be highly specific, depending on characteristics of antibodies used. The specificity of RIA frequently permits substances to be measured or detected in crude samples.

Limonoids are a group of triterpenoid compounds that are widely distributed in *Citrus* sp. and other members of the Rutaceae. The naturally occurring limonoids are diverse in their chemistry (Figure 1), yet characteristic structural features exist (*3*). These are: 1) a furan ring attached to the D-ring at C-17; 2) oxygen containing functional groups at C-3, C-4, C-7, C-16, and C-17; 3) a 14,15-epoxide group (except deoxylimonin); and 4) a methyl or oxymethylene at C-19.

One of the major areas of interest in the limonoids is the bitter properties of some of the naturally occurring forms. Limonin has long been known as a major bitter principle

in processed citrus (3), especially Navel oranges, Shamouti oranges, and grapefruit, and its presence greatly affects the consumer acceptance of these products. Limonin, nomilin, and ichangin are all bitter, while deacetylnomilin, obacunone, limonoate A-ring monolactone, and deoxylimonin (Figure 1) are regarded as non-bitter (4-7). As little as 0.5 ppm of limonin in juice can be detected by some people, however, below 6 ppm is normally considered to be non-bitter, 6-11 ppm is slightly bitter, and 18 ppm or more is very bitter (3). These values reflect a very narrow range of organoleptic tolerance when compared to the bitterness range of naringin, a flavonoid bitter principle in grapefruit. The range of organoleptic response to naringin is from 20-1500 ppm (8, 9).

The focus of the work described in this chapter was to learn as much as possible about the distribution and natural variation in limonin production by citrus fruit and vegetative tissues throughout growth and development. Additionally, studies were conducted to ascertain the natural variation in individual lots of freshly processed juice. Another goal was to determine the feasibility of rapid identification of low limonin producing plants that could be used in the development of new and improved strains of citrus.

Prior to 1980, only HPLC methods or semi-quantitative TLC methods were used for limonin detection (7, 10, 11). Neither of these methods were ideal for the planned studies. HPLC isrelatively sensitive, but fewer than 20 samples can be processed per day and each must be pre-purified. Our experimental designs required greater sensitivity and/or processing capacity than that afforded by either TLC or HPLC. Therefore, a radioimmunoassay for the detection of limonin in citrus, which is sensitive to the ppb range and allows the processing of over 400 unpurified samples per day, was developed by Mansell and Weiler (1, 2) in order to study the natural variation of limonin in citrus.

Immunoassay Technology: an Overview

Quantification of molecules by immunoassay exploits the specificity of the mammalian immune system towards perceived infection by foreign agents. Microbes and/or large macromolecules are recognized as "non-self" and antibodies are produced to neutralize the potential danger. Small compounds (usually <1000 Da) often are not immunogenic and must be conjugated to a larger compound, usually a protein carrier such as bovine serum albumin, in order to elicit an immune response from animals. These hapten-protein conjugates can result in the production of antibodies that are polyclonal in nature, but which contain populations of antibodies that are highly specific for certain regions or groups on the hapten (12, and ref. therein). Quantification in solutions of unknown concentration becomes possible via use of a specifically design tracer molecule in combination with the antibody and standard solutions of the molecule to be analyzed. Many immunoassays have been developed since the introduction of the method in 1959 by Yalow and Berson (13). Use of immunoassay technology for specific and sensitive quantification of small molecular weight plant compounds in impure plant extracts was first reported by Weiler and Zenk in 1976 (14). The field has grown since then to encompass dozens of compounds.

In general, a radioimmunoassay (RIA) involves the mixing of an extract of unknown concentration (diluting if necessary) with a defined amount of radiolabeled

tracer molecule and antibody. This mixture is allowed to incubate during which time the tracer and extract antigen compete for binding sites on the antibody. After incubation, the free and bound antigen are separated and the amount of radioactive tracer in either phase is determined. Quantification is done by comparing the results of the extracts of unknown concentration to results obtained using solutions of known antigen concentration (15).

The potential advantages for using immunoassay as a quantitative method include the fact that they can be extremely sensitive, measuring compounds present in the fmol range. In addition, processing of hundreds of samples per person per day is possible and rapid analysis (< 30 minutes) may be feasible. Immunoassays can be extremely specific, depending upon the characteristics of the antibodies produced and the tracer molecule used. This specificity allows for quantification from very crude extracts. If detection of a class of compounds rather than one specific compound is desired, an assay of more broad specificity may be able to be developed by changing the design of how the hapten-protein carrier conjugate is made and/or by altering the chemistry of the tracer molecule used in the assay.

Development of Immunoassays for Limonin

Immunoassays for limonin using both radiolabeled and non-radioactive tracers have been developed (U.S. pat. no. 4,305,923). Radioimmunoassays using both iodinated and tritiated tracers were developed for use in a research laboratory setting (1, 2). Since use of isotopes requires a more extensive lab set-up and is not allowed in food processing plants, immunoassays using enzyme-linked tracers were also developed (16, 17). Since most of the information on natural variation of limonin in Citrus tissues and juices presented by Mansell and collaborators was obtained using the RIAs, only those assays will be discussed in detail.

Synthesis of Tracer Molecules

One of the tracer molecules synthesized for RIA use was limonin-7-(O-carboxymethyl)oxime-tyrosine methyl ester (TME-limonin) labelled with ^{125}Iodine. TME-limonin was synthesized by reacting limonin-7-O-(carboxymethyl)oxime with triethylamine, isobutylchlorocarbonate, and tyrosine methylester as described in (2). Iodine-labelled TME-limonin was synthesized as in (2) and purified by TLC. The resulting product had a specific activity of 600-800 Ci/mmol.

Although the iodinated tracer was highly effective, its short half-life required that a new synthesis be performed every few months. Therefore, a tritiated tracer (half-life 12 years) was made by reducing the C-7 keto group with sodium-boro-tritide [NaB(^{3}H)$_4$] in the presence of pyridine and methanol (1) and subsequently purified by TLC and co-chromatographed with authentic limonol (specific activity = 20 Ci/mmol).
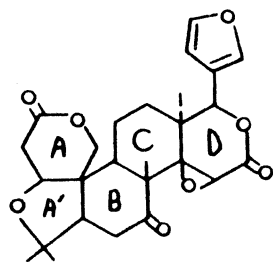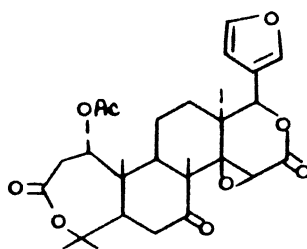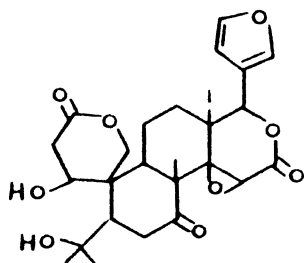
Figure 1. Structures of limonin, nomilin, ichangin, deacetylnomilin, obacunone, limonoate A-ring monolactone, deoxylimonin, nomilinic acid, isoobacunoic acid, and limonol.
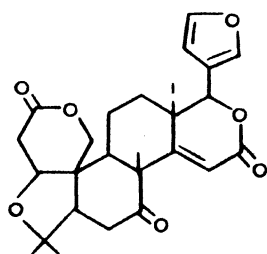
deoxylimonin
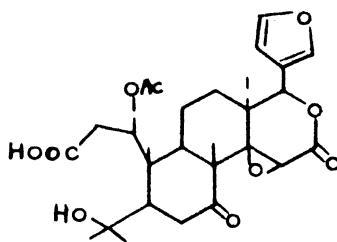
nomilinic acid
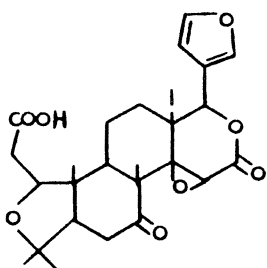
isoobacunoic acid
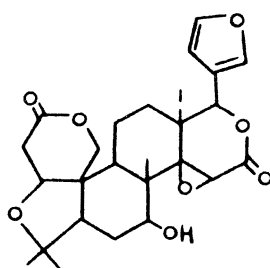
limonol

Figure 1.   Continued.

Synthesis of Limonin-BSA Conjugate

The keto group at carbon 7 of limonin was targeted for derivatization (Figure 1). Synthesis of limonin-7-(O-carboxy-methyl)oxime was achieved by refluxing limonin with aminooxyacetic acid. After purification, the oxime derivative was conjugated to bovine serum albumin (Figure 2) at a coupling ratio of limonin:protein of 16:1 (2).

Production of Antisera

Randomly bred rabbits were pre-immunized with weekly intradermal injections of 0.5 mg of the conjugate in an emulsion of Freund's complete adjuvant for four weeks. The rabbits received intramuscular boosters monthly and blood was collected at 3-4 week intervals from their ears. The blood was allowed to coagulate overnight at 4°C and the serum obtained after centrifugation (2). Subsequent batches of antisera were characterized and matched before being pooled.

Assay Characterization

Before an immunoassay can be utilized, it must be fully characterized so that the results will be meaningful. One of the first characters to determine is the potency (titre) of the antiserum. That is, at what dilution will the antiserum produce a linear binding kinetic with the tracer, thereby maximizing the accuracy of results? The goal is to determine the dilution at which the antiserum will bind 50% of the tracer. Since the dilution will vary depending upon the tracer concentration and specific activity, a new dilution factor must be determined each time a new batch of tracer is synthesized. The limonin antiserum had titres ranging from 400-30,000 assays per milliliter of serum (15).

In addition to antiserum titre, other RIA characters that must be determined include assay sensitivity, measuring range, unspecific binding of tracer, a measure of accuracy of the method (usually determined as percent recovery in "spiked" extracts), variation in replicate assays, and processing capacity (or "throughput") per worker and per day. The characteristics of the iodinated and tritiated tracer assays are presented in Table I (15). These assays are extremely sensitive, with detection limits in the pg (ppb) range, low coefficients of variation (2.5-3.0%), and high sample throughput (200-600 samples per worker per day).

Assay Specificity

One of the most critical characters to determine is the assay specificity and its potential for cross-reacting with structurally related compounds. In order to be thorough, testing must be done using both pure compounds as well as determining the cross-reactivity in crude tissue extracts. The first approach to characterize the cross reactivity of the limonin RIAs was to test different concentrations of pure compounds in order to
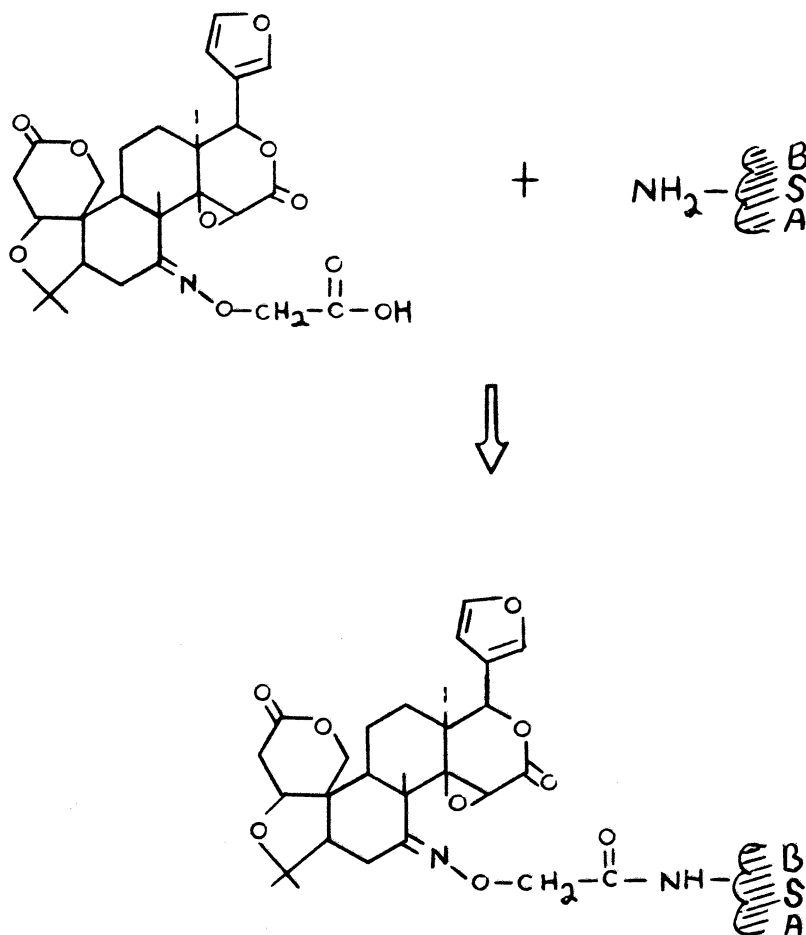
Figure 2. Schematic of synthesis of limonin-BSA conjugate.

Table I. Comparison of RIAs using Iodinated and Tritiated Tracers (*1*).

Assay Parameter	[125I]-limonin	[3H]-limonin
antiserum titre	27,000	1,350
measuring range (ng)	0.1-100	0.5-100
detection limit, pg (ppb)	71 (0.7)	220 (2.2)
unspecific binding	27%	1.1%
recovery of limonin	93%	97%
coefficient of variation (n=3, %B/Bo)	2.5 ± 1.8	3.0 ± 1.7
throughput	400-600	200-400

determine whether a compound does cross-react and, if so, to what extent. The latter was elucidated by determining the concentration of cross-reacting compound necessary to displace 50% of the tracer bound to the antiserum. Comparison of results obtained from these related compounds to those obtained from pure limonin permits one to calculate the percent cross-reactivity. The percent cross-reactivity is indicative of the amount of error in the apparent limonin concentration due to the presence of another compound recognized by the antibody *if this compound was present in a concentration equal to that of limonin.* After analysis of known compounds, the potential cross-reactivity of substances within the crude extracts was also tested. This was done in order to determine whether any unknown but interfering substance might be present in crude tissue extracts. Unpurified juice samples were also tested for the presence of unknown cross reacting substances.

The cross-reactivity of the limonin antiserum with several limonoids (Figure 1) is summarized in Table II. Only deoxylimonin cross-reacted strongly, most likely due to the fact that the area of difference in structure to limonin resides in the area used for hapten-protein conjugation. Thus, this region of the molecule that is distinct from limonin was likely "masked" to the antiserum. Although deoxylimonin occurs only in very low levels in citrus tissue (*4, 18*), any potential interference by this compound in the limonin RIAs was tested by chromatographing tissue extracts followed by determination of the presence of immunoreactive material on the TLC plate (*1*). Results obtained from a grapefruit seed extract (Figure 3) showed only one immunoreactive band which co-chromatographed with authentic limonin. Analyses of other tissues and juice gave similar results. Therefore, since deoxylimonin was not present or only present in levels below detection limits, the RIA could be used to determine the amount of limonin in crude extracts.

Use of Limonin RIA to Assay Limonoate A-Ring Monolactone

In the limonoid biosynthetic pathway, the natural non-bitter precursor is limonoate A-ring monolactone (Figure 1) (*7, 19*). This compound is very labile and can be converted to limonin enzymologically (*19*) or during the disruption of intact tissues. The A-ring
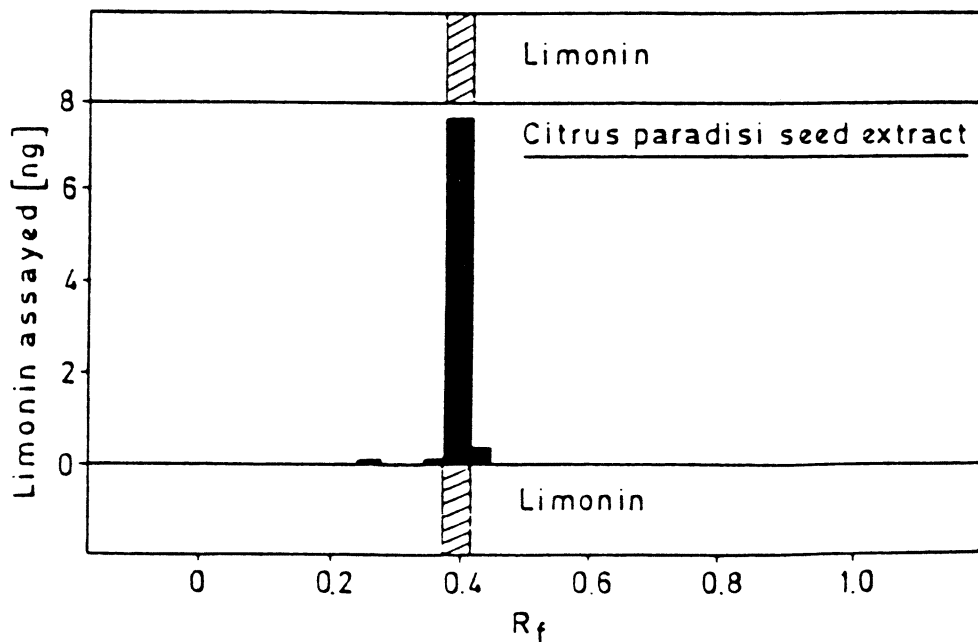
Figure 3. Distribution of immunoreactive material on a TLC of Citrus paradisi (white Marsh) seed extract after acidification. Reproduced with permission from reference (2).
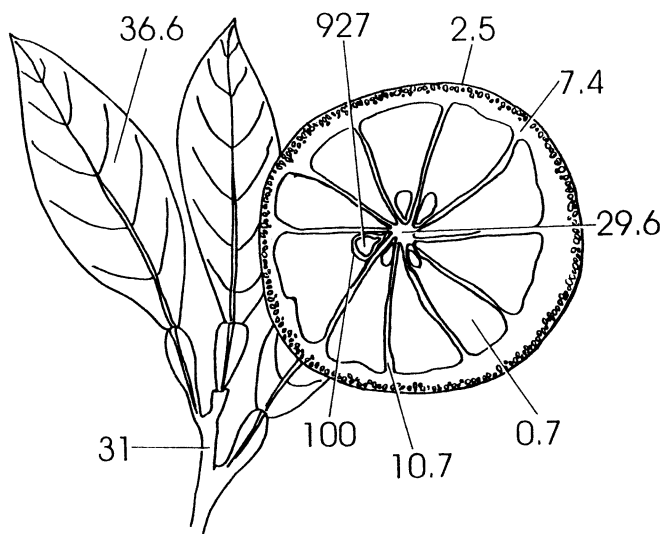
Figure 4. Distribution of limonin in a fruit and vegetative parts of White Marsh. Limonin concentration values are given as μg/100mg fr. wt. Reproduced with permission from reference (2).

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Table II. Cross-Reactivities of Limonin Antibody using Iodinated and Tritiated Tracers (1, 2, 20, 21).

Compound	nmol compound required to displace 50% [125I]-limonin	% Cross-reactivity	nmol compound required to displace 50% [3H]-limonin	% Cross-reactivity
limonin	4.2	100	6.3	100
deoxylimonin	15.5	27	6.3	100
deacetylnomilin	63.7	6.6	185	3.4
nomilin	447.5	0.9	1012	0.6
obacunone	1101	0.4	1454	0.4
nomilinic acid	>>2000	0	>>2000	0
isoobacunoic acid	>>2000	0	>>2000	0
limonoate A-ring monolactone				ca 0

monolactone conversion is affected by the acidity of the homogenate and will spontaneously be converted to limonin under acidic conditions. In fact, the actual identity of the endogenous form(s) was not absolutely certain. It was not clear whether the A-ring monolactone was the compound present in tissues and limonin formed only upon extraction, or whether some tissues contained only the A-ring monolactone and others contained only limonin. Another possibility was that the tissues contained some combination of the two compounds.

Since limonoate A-ring monolactone potentially could interfere with the assay, it was important to determine whether it cross-reacted with the antiserum. Due to the instability of limonoate A-ring monolactone, evaluation of whether or not this compound bound to the antiserum was done by carefully determining conditions of extraction from tissues that would prevent lactonization of the D-ring, yet preserve the integrity of endogenous limonin (20, 21). It was found that limonoate A-ring monolactone did not cross-react with the antiserum.

This finding proved to be very valuable as it permitted the quantification of both limonin and the A-ring monolactone with the same antibody. To utilize this property, extracts were first made in neutral conditions where the A-ring monolactone would not be converted to limonin, thus measuring endogenous limonin. An aliquot of the extract was then acidified to convert endogenous A-ring monolactone to limonin and assayed. The difference between the two analyses gave a measurement of the A-ring monolactone concentration in crude extracts.

Limonin Concentration in Different Varieties of Grapefruit Juice

One of the earliest studies conducted using the limonin RIA was designed to elucidate the variation of limonin in grapefruit juice and to determine whether or not there was any correlation between limonin content and other monitored taste factors such as acid and brix (*22, 23*). In one study (*23*), samples from more than 6500 truckloads of fruit were assayed for limonin (and naringin). This study alone represented approximately 6% of all the fruit harvested and processed into juice that season within the State of Florida. While the limonin content in juice varied between the different cultivars, neither acid nor brix correlated with limonin content and therefore could not be used as predictors of bitterness. The limonin content of the juice showed an overall decrease during the season as the fruit matured, although there was a spike in limonin content that correlated with a freeze in January of that season (*23*). In addition, a study to determine if there was any correlation between limonin and naringin content of grapefruit juice (*24*) showed that the concentration of one of these components could not be used to predict the other (r = 0.137).

Analysis of Natural Variation in Fruit and Vegetative Tissues

Determinations of cross-reactivity of the antiserum with pure samples of naturally occurring limonoids, coupled with evaluation of interfering compounds present in crude extracts via TLC separation and detection of immunoreactive bands (e.g, *2, 22*), showed that the RIAs were highly specific and able to accurately quantitate both limonin and limonoate A-ring monolactone in crude samples. The assays were highly sensitive and time-consuming prepurification of extracts was not necessary, so the use of these assays permitted the determination of natural variation in limonin production and accumulation in citrus.

Distribution in Fruit Tissues

During the initial analysis of limonin levels in fruit tissues (Figure 4), results showed that the seeds had the highest content. The second highest limonin content was in the pith followed by segment membranes, albedo, and flavedo. The lowest levels were found in the juice vesicles (*2*).

A more in-depth analysis of the distribution of limonin in fruit tissues was done by obtaining ten fruits from each of nine cultivars, carefully dissecting the fruit so that cross contamination of tissue parts was avoided, and randomly taking ten samples of each tissue from each fruit for analysis (*26*). This single study resulted in a total of 7200 individual samples that were analyzed for limonin content. After employing analysis of variance (ANOVA) and the Student Neuman Keuls test for location of statistically significant differences, individual cultivars were ranked according to the overall limonin content and the limonin content of each tissue (Table III). The varieties used most

Table III. Analysis of Limonin in Grapefruit Cultivars: ANOVA and SNK (26).

Tissue	Conclusions								
Flavedo	L	M	R	M	K	H	F	D	T
Albedo	M	L	W	K	R	H	F	T	D
S.M.	K	M	W	T	F	H	L	D	R
J.V.	L	M	K	R	F	H	W	D	T
O.S.C.	K	D	W	R	T	M	L	F	H
I.S.C.	K	D	W	F	H	M	R	T	L
Cotyledon	K	R	H	W	T	F	D	L	M
Pith	M	L	W	K	R	F	T	D	H
Overall	K	R	D	W	F	T	H	M	L

NOTE: Cultivars are ranked in order of descending limonin content. K=Davis Krome; D=Duncan; F=Foster Pink; L=Leonardy; M=Mott; T=Thompson Pink; R=Triumph; H=Wheeney; W=White Marsh; SM=segment membrane; JV=juice vesicle; OSC=outer seed coat; ISC=inner seed coat. Cultivars sharing underlining are not statistically different from each other.

commonly for juice (White Marsh, Duncan, and Thompson Pink) had the lowest juice vesicle limonin levels.

Statistical analyses were also performed in order to rank the limonin concentration in the different fruit tissues (Table IV). Results showed that the distribution of limonin in fruit tissues of the different cultivars followed similar trends. There was up to a 10-fold variation in limonin content *within the same tissue of the same fruit variety.* This variation in concentration could be due to random events or, if there is a pattern of accumulation within each tissue, it could be due to the location from which samples were taken.

In order to elucidate this point, we obtained Duncan, Marsh, and Thompson Pink grapefruit and performed a complete dissection of each fruit, mapping the axial and radial location of each sample (27). This resulted in 650-900 individual samples per grapefruit which were analyzed for both limonin and limonoate A-ring monolactone content (also naringin content). Overall, the average limonin content in fruit tissues showed the same relationship as in the earlier distribution studies. When considering

Figure 5. Limonin concentration (total limonin = limonin + limonoate A-ring monolactone) in juice vesicles, side segment membranes, and flavedo of the proximal, central, and distal Duncan fruit slices. Data are ppm limonin (23).
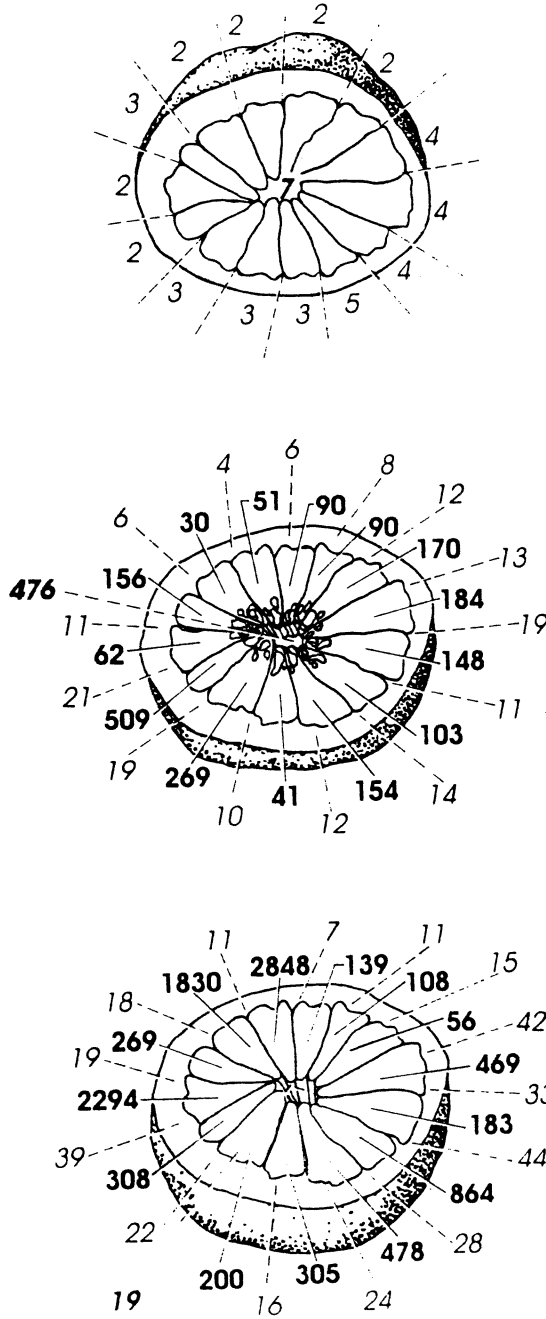
Figure 6. Limonin concentration (total limonin = limonin + limonoate A-ring monolactone) in albedo, pith, and outer (back) segment membranes of the proximal, central and distal Duncan fruit slices. Data are ppm limonin (23).

Table IV. Limonin in Grapefruit Cultivar Tissues: ANOVA and SNK (26).

Cultivar	Conclusions							
Davis Krome	C	I	O	P	M	A	F	J
Duncan	C	I	O	P	M	F	A	J
Foster Pink	C	I	O	P	M	F	A	J
Leonardy	C	I	O	P	F	M	A	J
Mott	C	I	P	O	M	F	A	J
Thompson Pink	C	I	O	P	M	A	F	J
Triumph	C	I	O	P	F	A	M	J
Wheeney	C	I	O	M	P	A	F	J
White Marsh	C	I	O	P	M	A	F	J
Overall	C	I	O	P	M	A	F	J

NOTE: Tissues are arranged in order of descending limonin content. F=flavedo; A=albedo; J=juice vesicles; M=segment membranes; P=central pith; O=outer seed coat; I=inner seed coat; and C=cotyledon. Tissues sharing underlining are not statistically different from each other.

the three-dimensional distribution, results from Duncan grapefruit (Figures 5, 6) clearly indicated the lack of a radial pattern of limonin accumulation and indicated the potential for axial distribution. Marsh and Thompson Pink fruit showed similar trends.

Statistical analyses (ANOVA) of these data supported the conclusion that there were statistically significant differences in the axial distribution of limonin in grapefruit tissues. In most tissues, higher concentrations of limonin were observed in the distal portions of the fruit. This distribution pattern was most dramatic in the less-seedy grapefruit varieties, White Marsh and Thompson pink. In contrast, the highest concentration of limonin in the pith tissue tended to be in the more central locations. It is not clear at this time if there is a relationship between the distribution of limonin in the pith and translocation into the seeds which are clustered in the central areas of the fruit. Comparison of overall content in each fruit tissue showed that the White Marsh and Thompson Pink grapefruit varieties had higher limonin concentrations in most all fruit tissues as compared to the Duncan (seedy) grapefruit. The exception to this trend is that Duncan grapefruit had a greater proportion of limonin in the seeds (27).

The percentage of limonin present in the non-bitter A-ring monolactone form in these fruit tissues is presented in Table V (27). Juice vesicles had the lowest limonoate A-ring monolactone content, probably due to the acidic properties of this tissue. Seeds tended to have the highest percentage of total limonin present in the A-ring monolactone form. While the precise percentages for the different tissues vary from each other, standard errors of samples within each tissue were very low. The low standard errors indicate that ratios of endogenous limonin and limonoate A-ring monolactone were nearly constant within each tissue and were independent of location within the fruit.

Table V. Average Percent of Total Limonin in Grapefruit Tissues That Was Limonoate A-ring Monolactone (27)

Tissue	Duncan	Marsh	Thompson
Flavedo	52.1 + 1.3 (140)	78.9 + 1.0 (108)	58.9 + 1.4 (108)
Albedo	51.6 + 0.9 (140)	61.4 + 1.1 (108)	60.4 + 0.9 (108)
Back membrane	53.5 + 1.0 (121)	62.9 + 1.0 (84)	60.9 + 0.9 (95)
Side membrane	52.3 + 0.9 (118)	58.2 + 1.2 (84)	56.1 + 1.0 (96)
Juice vesicles	6.9 + 1.4 (117)	15.0 + 2.2 (84)	14.6 + 1.9 (96)
Pith	61.6 + 4.3 (12)	57.6 + 3.0 (10)	63.0 + 1.8 (10)
Seeds	83.9 + 2.8 (51)	64.0 + 3.4 (26)	70.6 + 4.3 (10)

NOTE: Data are average percent + SE (n).

Seasonal Variation in Fruit

Monthly variation in the concentration of limonin (and naringin) in whole Duncan, Marsh, and Thompson Pink grapefruit was monitored over three growing seasons. Ten fruit per month were homogenized in buffer and extracted as previously described. Results using Duncan grapefruit are shown in Figure 7. Young fruit had the highest limonin concentration and the concentration decreased as the fruit matured and developed juice vesicles. It should be noted that the total amount of limonin per fruit continued to increase. Transient increases in limonin concentration corresponded with time of leaf flushing. During two of the seasons presented in Figure 7, the growing area experienced freezing conditions which resulted in a temporary decrease in the limonin content. These results further illustrate the dynamic nature of limonin (and naringin) metabolism in citrus fruit.

Distribution in Vegetative Tissues

Analysis of the distribution of limonin in vegetative tissues was performed by analyzing individual discs of leaves taken from a young branch as well as analyzing stem
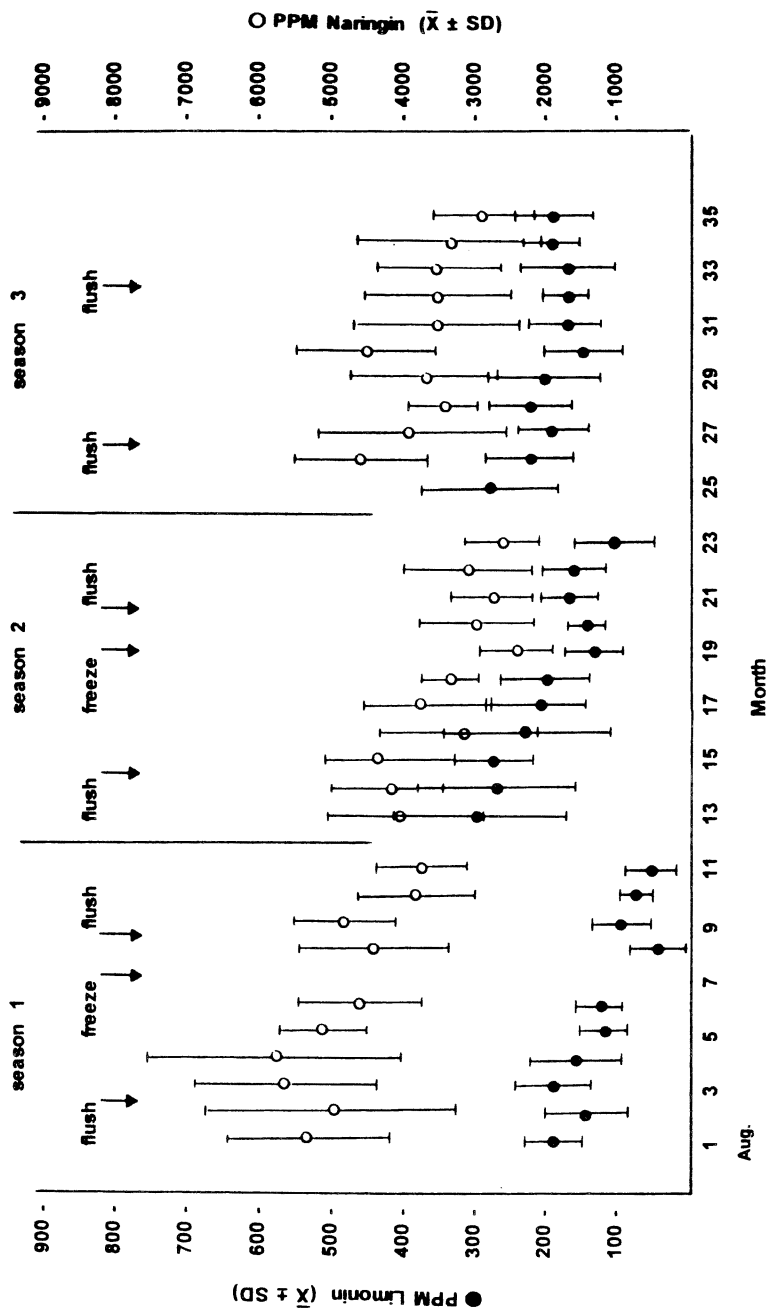
Figure 7. Limonin and naringin concentration in whole Duncan grapefruit over three growing seasons. (●) = ppm limonin per fruit and (○) = ppm naringin per fruit (data are average values ± SE)
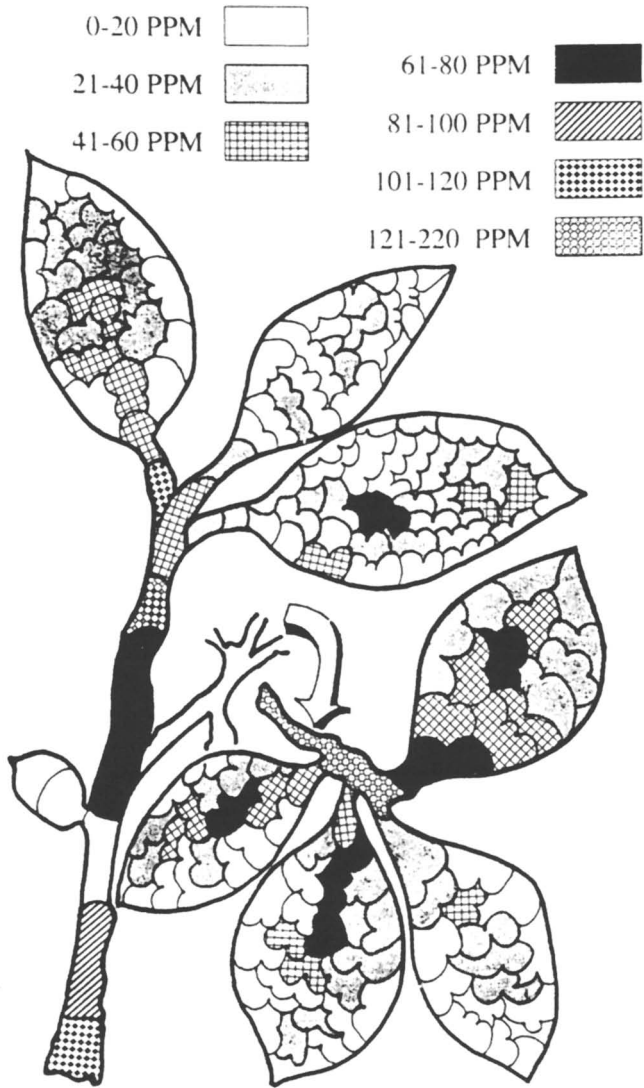
Figure 8. Concentration of limonin in grapefruit leaf disk samples (21).
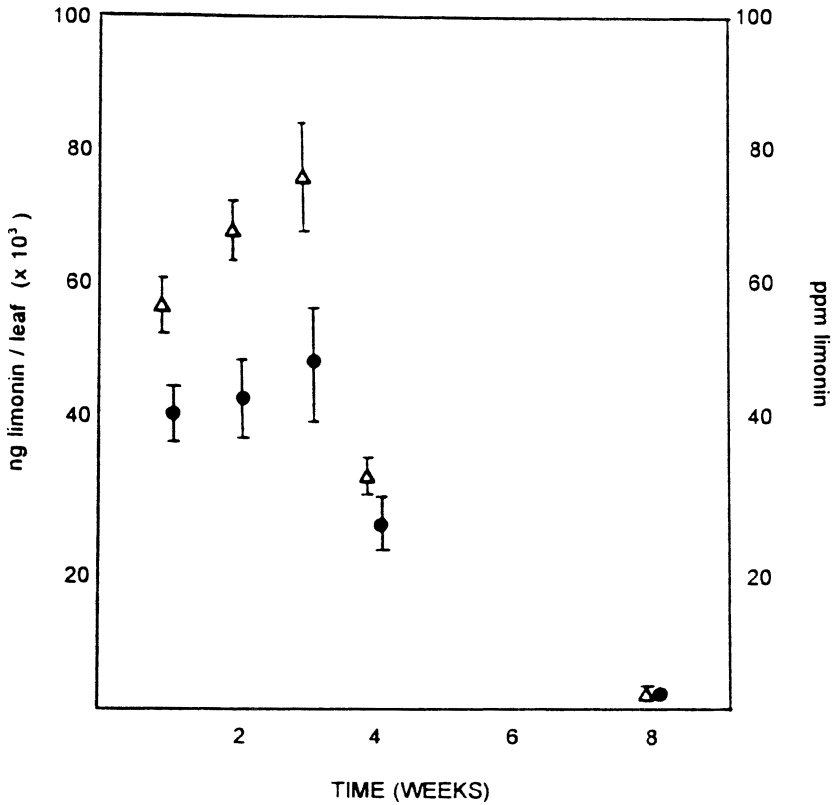
Figure 9. Limonin content of September flush leaves during development (21). (●) = nanograms of limonin per leaf and (△) = ppm limonin. (data are average values ± SE)

segments (*21*). Results from a flushing branch showed that limonin was more concentrated in areas adjacent to the conductive tissues (Figure 8). Since there was a pattern of limonin distribution within leaves, the disc-sampling technique was not further utilized. During subsequent determination of limonin content of leaves, each individual leaf was extracted in order to avoid errors due to location of subsampling. The results clearly demonstrated that flushing leaves have limonin concentrations 10-200 times greater than those found in mature leaves (*21*).

A study of the amount of limonin found in grapefruit leaves during growth and development showed that both the total amount of limonin per leaf and the concentration of limonin in the leaf increased during the first 3 weeks of growth after which it decreased as the leaves matured (Figure 9). The concentration of limonin in whole branches followed the same trend (*21*). It is not known whether the limonin found in mature leaves was residual limonin which was not translocated or whether the older leaves continued to synthesize low levels which may or may not continue to be transported. In addition, it was found that the relative amounts of limonin and its A-ring monolactone changed during leaf maturation, ranging from 50% monolactone in the youngest leaves increasing to 90% by the third week, and decreasing to 60% at maturity (*21*). The changes in limonin and limonoate A-ring monolactone observed during the growth and development of vegetative tissues emphasizes the dynamic nature of limonin metabolism in citrus.

Limonin Production in Callus Cultures and Regenerated Shoots

Plant tissue culture has emerged as a valuable tool for the propagation of plants. Woody plants have been notorious for their slow response to culture and regeneration, however recent advances in transformation technology coupled with micrografting have given some justification that pursuit of biotechnological manipulation of fruit quality may be successful. Monitoring of secondary metabolites such as those that contribute significantly to taste quality, for example, becomes paramount to evaluation of success of these endeavors. During the mid and late 1980's, the research program of Dr. Richard Mansell at the University of South Florida was heavily involved in citrus tissue culture, plant regeneration, and the evaluation of the production of bitter principles during these processes. Once again, the accuracy and sensitivity of RIA was utilized to monitor the limonin and limonoate A-ring monolactone (also the bitter flavonoid, naringin) content of citrus tissues during culture initiation, propagation, and shoot regeneration (*25, 28*).

Concentrations of limonin and limonoate A-ring monolactone were 100-10,000 fold lower in callus cultures as compared to the initial tissues from which the cultures were derived. In addition, most of the limonin present in the callus was in the form of the A-ring monolactone. After transplant of callus onto regeneration medium and incubation under a 16 hr light/8 hr dark regime at 25°C, buds began to form which further developed into regenerated shoots (Figure 10). The limonin levels in callus grown on regeneration medium were at least 70-fold higher than those in the original

Figure 10. Sketch of Duncan grapefruit callus on regeneration medium with buds and regenerated shoots.

callus, and formation of bud and shoots resulted in an additional 10-fold increase in limonin levels (Table VI). Results obtained with Thompson Pink grapefruit showed similar trends (25). Once again, limonoate A-ring monolactone accounted for the majority of the total limonin present in the buds and shoots (Table VI). Due to extremely low and uniform limonin levels in undifferentiated callus (0.1-1 ppm), determination of levels at this stage was not useful in evaluating possible transformants. It would be necessary to test regenerated shoots in order to ascertain whether metabolic potential has been altered.

Summary

Use of immunoassay as a method for the quantification of relatively small plant compounds has proven to be invaluable in studies designed toward understanding the natural dynamics of the production and accumulation these compounds in plants. The sensitivity and low cross-reactivity of the limonin RIAs developed by Mansell and Weiler enabled the in-depth study of natural variation in the production of limonin (and limonoate A-ring monolactone) in many different tissues as well as elucidating changes that occur during growth and development. The sensitivity of the assays, coupled with the ability to analyze crude extracts, should also be useful in evaluation of any future efforts toward crop improvement that would utilize biotechnological and tissue culture approaches.

Acknowledgement

Table VI. Limonin and Limonoate A-ring Monolactone Levels During Shoot Development in Duncan Grapefruit Callus on Regeneration Medium (25).

Sample	*n*	*Limonin*	*Limonoate A-ring Monolactone*	*Total Limonin*
Callus	10	0.8 ± 0.7 (0.1-2.0)	6.6 ± 6.7 (1.6-2.1)	7.4 ± 7.3 (1.8-23.0)
Buds	14	2.1 ± 1.4 (0.3-5.3)	38 ± 22 (4.1-69.0)	40 ± 23 (7.5-73.0)
Shoots	3	4.0 ± 3.1 (1.5-7.5)	73 ± 59 (32.0-141.0)	77 ± 58 (40.0-144.0)

NOTE: Data are average ppm $\pm$ std. dev. (range). Reprinted with permission from (25)

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Chapter 7

Extraction and Recovery of Limonoids with the Supercritical Carbon Dioxide Micro-Bubble Method

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A new technique using supercritical carbon dioxide (SC-CO$_2$) micro-bubble method to remove bitter limonoid aglycones from bitter juices has been developed. This procedure is also capable of isolating limonoid aglycones, purifying limonoid glucoside extracts obtained from citrus molasses and other sources, sterilizing the samples and inactivating enzymes in the samples. A pilot scale apparatus, which is capable of feeding a liquid sample and SC-CO$_2$ continuously, successfully purified a limonoid glucoside extract isolated from mandarin. The yeild of limonoid glucosides in the SC-CO2 step was 97%. The product was practically odorless, tasteless and in an aceptic state.

There are two groups of limonoids, the aglycones and their respective glycosides, present in *Citrus*. Excessive bitterness due to the limonoid aglycones, limonin and nomilin, lowers the quality and the value of citrus juices, which has a significant negative economic impact to the citrus industry. Limonoids have been also shown to possess anticancer activity in laboratory animals and cultured human beast cancer cells (1-6), and antifeedant activity against insects and termites (7-11). Due to the biological activities, the demand for limonoids has significantly increased in recent

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years (12). Limonoid glucosides, in particular, are ideal for use as functional food additives because of their non-bitter taste properties and water solubility. Citrus molasses is an excellent source of limonoid glucosides. (13).

Our research has focused on the recovery of limonoid glucosides from citrus juices and juice processing by-products, and on the removal of bitter limonoid aglycones from bitter citrus juices. To achieve the objective, we have developed the $SC\text{-}CO_2$ micro-bubble technique on a pilot scale. This new method is capable of treating liquid samples continuously with $SC\text{-}CO_2$.

Limonoid aglycones in citrus juices

Two limonoid aglycones, limonin and nomilin, which are responsible for delayed bitterness in citrus juices, were analyzed in several citrus juices (Table 1). Satsuma madarin and Valenicia do not have a bitterness problem although they are harvested at early- to mid-season. Most likely, they possess high activity of limonoid glucosyltransferase, which catalyzes the glucosidation of aglycones to form non-bitter limonoid glucosides during fruit maturation. Hassaku and Natsudaidai are typical late ripening citrus fruits in Japan. Juices extracted from Hassaku and Natsudadai harvested in Feb. had 25 ppm and 20 ppm of bitter aglycones, respectively, which are well above the bitterness threshold of 6 ppm (14-16). Navel orange and grapfruit aslo have a bitterness problem even when they were harvested rather in the late season.

The concentration of limonin and nomilin in Hassaku and Natsudaidai juices are so high that they have a severe delayed bitterness problem, similar to navel orange juices extracted from fruits harvested in early- to mid-season in the United States. It is difficult to consume 100% juice products from these varieties without dilution, blending with other juices or removing bitter limonoids from the juice. Therefore, Hassaku juices are routinely passed through limonoid adsoption resins in commercial operation to remove the bitter limonoids.

Table 1 Limonoid Aglycones in Citrus Juices

Fruits	Concentrations (ppm)*
Satsuma mandarin	4(processed in Nov.)
Hassaku	25(processed in Feb.)
Natsudaidai	20(processed in Feb.)
Navel orange	8(processed in Jan.)
Valencia orange	5(processed in Dec.)
Grapefruit	10(processed in Feb.)

* Total of limonin and nomilin

Limonoid glucosides in citrus juices

Limonoids are present mainly as glucoside derivatives in citrus juices (17). A ratio of limonoid aglycones to limonoid glucosides in commercial orange juices in the United States is about 1: 150. This ratio is determined by two factors: the time of fruit harvest and the activity of limonoid glucosyltransferase. Sweet oranges have generally relatively high activity of limonoid glucosyltansfease, but pummelo and its hybrids such as grapefruit, Natsudaidai, Oroblanco and Melogold have a low activity. Therefore, limonoid bitterness in navel oranges is a problem in the early-season to mid-season-harvested fruits (18-20), but not in late-season-harvested fruits. On the other hand, the bitterness problem in pummelo and its hybrids is very persistent and juices extracted from fruit harvested in very late season still have a severe bitterness problem.

The concentrations of limonoid glucosides in several citrus juices extracted from mature fruits are shown in Table 2. All of the juices contained high concnetraion of total limonoid glucosides. These values are much higher than those previously reported (21). Since limonoid glucosides display potential cancer chemopreventative activity, consumption of these juices could have a significant impact on human health and nutrition.

Table 2 Limonoid Glucosides in Citrus Juices

Fruits	Concentrations (ppm)*
Satsuma mandarin	215(Brix 9)
Hassaku	340(Brix 10)
Natsudaidai	360(Brix 10)
Navel orange	390(Brix 11)
Valencia orange	578(Brix 11)
Grapefruit	240(Brix 9)

* Total of limonoid glucosides

Limonoid glucosides in citrus juice processing by-products

The distribution of limonoid glucosides in various portions of Satsuma mandarin fruit juice processing products is shown in Fig. 1. The total concentration of limonoid glucosides in whole fruit was about 500 ppm. Only 24% of the original glucosides was retained in the concentrated juice. Dried peel residues contained limonoid glucosides highest, 36% of the total, and molasses contained 18%. Molasses and peel residues therefore, are excellent sources for limonoid glucosides. Citrus molasses is the best potential industrial source since it is already in a liquid state and an extraction step could be omitted to access the limonoid glucosides.

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                    ┌──────────────────┐
                    │   Citrus Fruit   │  (100%)
                    └──────────────────┘
                <In-line Juice Extractor>
                            │
                            ▼
          ┌─────────────────┴─────────────────┐
          ▼                                     ▼
  ┌──────────────────┐              ┌──────────────────┐
  │ Finishing Process│              │  Liming Process  │
  └──────────────────┘              └──────────────────┘
          │                                     │
          ▼                                     ▼
  ┌──────────────────┐              ┌──────────────────┐
  │  Centrifugation  │              │ Pressing Process │────────┐
  └──────────────────┘              └──────────────────┘        │
          │                                     │               ▼
          ▼                                     ▼        ┌──────────────┐
  ┌──────────────────────┐        ┌──────────────────┐   │  Evaporation │
  │ Single Strength Juice│        │  Drying Process  │   └──────────────┘
  └──────────────────────┘        └──────────────────┘          │
          │                                  │                   │
          ▼                                  │                   │
  ┌──────────────────┐                       │                   │
  │   Evaporation    │                       │                   │
  └──────────────────┘                       │                   │
 (24%)    │               (36%)              │    (18%)          │
          ▼                                  ▼                    ▼
  ┌──────────────────┐        ┌──────────────────────┐  ┌──────────────────┐
  │ Concentrated Juice│       │  Dried Peel Residue  │  │  Citrus Molasses │
  └──────────────────┘        └──────────────────────┘  └──────────────────┘
```
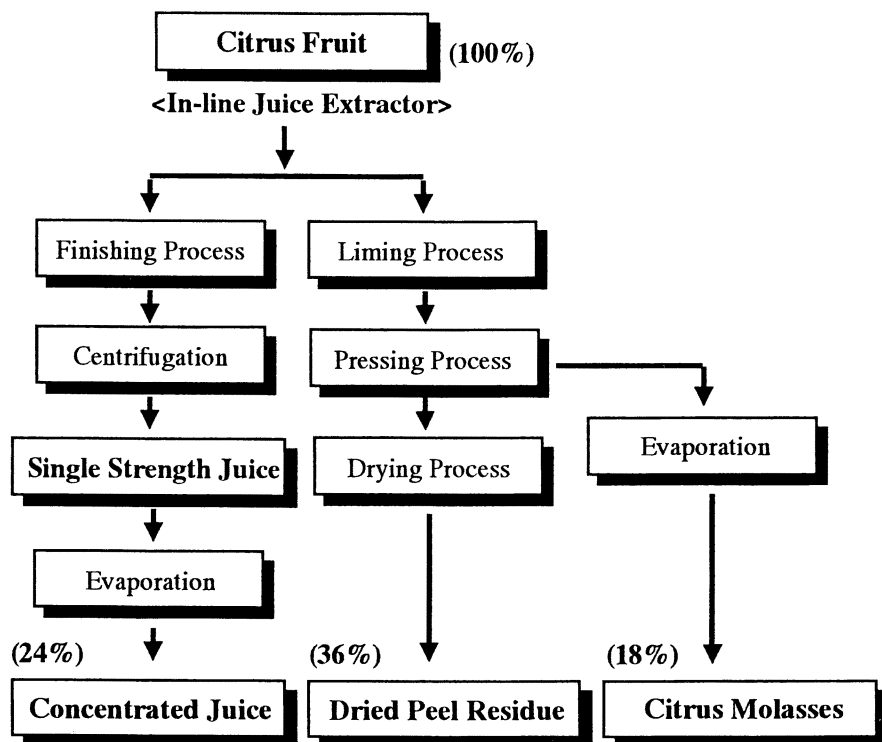
*Figure 1.   Distribution of Limonoid Glucosides in Satsuma Mandarin Juice
           Processing*
           Parentheses show the distribution of limonoid glucosides

Limonoid glucosides in citrus molasses

The concentrations of limonoid glucosides in citrus molasses from various origins, including Valencia orange, Hamlin orange, Pineapple orange and Satsuma mandarin (13,22), are shown in Table 3. All of the samples contained high concentrations of limonoid glucosides, averaging 1940 ppm of total limonoid glucosides at Brix 40.

Citrus molasses are currently utilized as cattle feeds or for alcohol fermentation. However, because of their biological activity, the demand for limonoid glucosides has increased significantly in recent years. Routine procedures for isolation of limonoid glucosides from citrus molasses have been developed on a commercial basis (23), and several industries are interested in mass production of these compounds. Molasses now represents a substantial value added by-product for the citrus industry.

Table 3 Limonoid Glucosides in Orange and Mandarin Molasses

Molasses	Concentrations (ppm / Brix 40)				
	LG	NG	NAG	OG	Total
Hamlin & Pineapple (12/92)	964	477	82	67	1590
Hamlin & Pineapple (1/93)	800	415	176	116	1507
Hamlin & Pineapple (2/93)	1084	836	338	133	2391
Valencia (4/93)	1284	465	149	83	1981
Valencia (5/93)	1000	469	113	56	1638
Valencia (6/93)	1214	514	186	86	2000
Satsuma mandarin (1/91)	1133	533	444	268	2378
Hassaku (3/92)	932	495	504	113	2044

LG:Limonin Glucoside, NG:Nomilin Glucoside, NAG:Nomilinic Acid Glucoside, OG:Obacunone Glucoside

Table 4 Limonoid Glucosides in Commercially Debittered Hassaku Juices

Samples	Concentrations (ppm)				
	LG	NG	NAG	OG	Total
Treated Mean	54.6	7.4	19.4	-	81.4
S. D.	9.2	2.5	4.3	-	13.8
Untreated Mean	146.2	80.0	109.3	4.7	340.2
S. D.	22.3	16.8	21.9	2.4	29.9

LG:Limonin Glucoside, NG:Nomilin Glucoside, NAG:Nomilinic Acid Glucoside, OG:Obacunone
Twenty treated samples and 10 untreated samples were analyzed.

Removal of limonoid glucosides in commercial debittering process

Hassaku juice is commonly passed through an absorbent, HP-20 to remove bitter limonoids from juices in commercial operation in Japan. This treatment is, however, not specific for bitter limonoids and removes also other nutrients, flavonoid compounds, as well (24). The concentrations of limonoid glucosides in untreated and resin treated Hassaku juices are shown in Table 4. Untreated juices contained an average of 340 ppm of total limonoid glucosides which was reduced to 80 ppm after adsorption resin treatment. This significant reduction in limonoid glucoside levels during the adsorption resin operation has led us to develop a new method for limonoid debittering of citrus juices.

The supercritical carbon dioxide (SC-CO$_2$) micro-bubble method

The supercritical fluid process can be summarized as follows; a liquid or solid mixture is brought into contact with a dense gas under supercritical conditions, and consequently compounds in the liquid or in the solid mixture are selectively solubilized in the supercritical fluid. The basic principles have been reviewed (25-28).

Supercritical fluids generally have a density similar to liquids and a viscosity similar to gases. Therefore, they have a solvent power similar to that of liquids and have mass transfer characteristics like those of gases. Therefore, extraction efficiency is very high. Carbon dioxide has several advantages such as its non-toxicity, non-flammability, no residual chemical problem, low-moderate operating temperature and pressure, and low-cost. It is of course possible to recycle the carbon dioxide.

A new technique using supercritical carbon dioxide (SC-CO$_2$) micro-bubble has been developed for the extraction of limonoid aglycones and the purification of limonoid glucoside extracts (Fig. 2). Supercritical carbon dioxide (SC-CO$_2$) is supplied into sample solution through a micro pore filter. As a result, SC-CO$_2$ forms microbubbles and the technique is designated a micro-bubble SC-CO$_2$ method. Microbubbling of CO$_2$ increases CO$_2$ concentration in sample solution dramatically. Thus, the extraction efficiency can be greatly improved (29-30). We have developed pilot scale equipment capable of feeding a liquid sample and SC-CO$_2$ continuously. We used this technique on debittering of citrus juices and purification of the limonoid glucoside extracts obtained from citrus juices and mandarin molasses. The sample solution moves from the bottom to the top together with the microbubbles of SC-CO$_2$ in the extractor. In other words, they contact with each other while they move in the same direction (parallel-flow contact). By this treatment, CO$_2$ concentration in the sample solution is significantly increased. Thus, the extracting efficiency and deodorizing efficiency are greatly improved. Furthermore, the microbubbles of SC-CO$_2$ can penetrate into the cells of microorganisms, so that the microorganiams and the enzymes are effectively inactivated (31-36).

SC-CO$_2$, which contain limonoid agylcones or odor compounds, is taken out from the outlet in the upper port of the reactor. On the other hand, the sample solution containing limonoid glucosides is taken out from the another outlet when it comes near the surface level in the extractor.
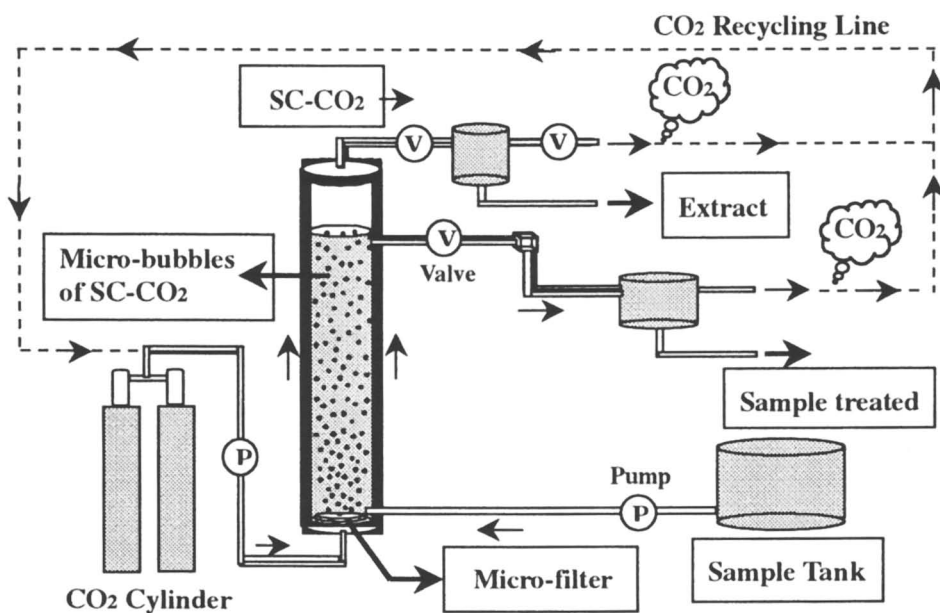
Figure 2.   Supercritical Carbon Dioxide Micro-bubble continuous Flow System
SC-CO2 : Supercritical Carbon Dioxide

Application of SC-CO$_2$ micro-bubble method

The SC-CO$_2$ micro-bubble treatment can be used for limonoid aglycone extraction and limonoid glucoside purification (Fig 3). When the bitter fresh juices are treated with the SC-CO$_2$ micro-bubble method, limonoid aglycones are effectively extracted and simultaneously microorganisms are sterilized and enzymes are inactivated. So, it is possible to produce sterilized non-bitter Hassaku fresh juices.

The limonoid glucoside solution, which is obtained from citrus molasses through the absorption resin, is potential source for industrial recovery of limonoid glucosides, though it contains contaminants. Flavor compounds are major impurities in the purification process. The micro-bubble SC-CO$_2$ treatment can effectively remove the contaminants from the extract solution. And the extract is in an aseptic state. Therefore, the treated preparation is ideal for use as food additives.

Recovery data of Limonoid Glucosides from Satsuma Mandarin Molasses

The limonoid glucoside recovery data from 1000 Kg of Satsuma mandarin molasses using a combination of centrifugation, adsorption resin chromatography and SC-CO$_2$ purification treatment are shown in Table 5. The procedures resulted in the yield of 95 Kg of limonoid glucoside extract containing 1530 g of limonoid glucosides with an overall 65% recovery. In this experiment, a major loss occurs at centrifugation and resin adsorption stages. Losses of the SC-CO$_2$ stages were minimal (i.e. 3%). The yield at the resin adsorption stage can be increased by manipulating resin volume and elution volume.

Table 5 Recovery Data of Limonoid Glucosides from Satsuma Mandarin Molasses

Treatment process of molasses	Limonoid glucosides in each process (g)
1. Before Treatment	2350 (100%)
2. Centrifugation (3000g)	2020 (86%)
3. Resin Adsorption (2000L/H)	- -
4. Elution(EtOH)	1630 (69%)
5. Evaporation	1580 (67%)
6. SC-CO2 Treatment	1530 (65%)

Data was obtained from 1000kg of Satsuma mandarin molasses.
Parentheses shows the yield of limonoid glucosides.

Citrus Fruit

<Extraction of Limonoid Aglycones> <Purification of Limonoid Glucosides>

Fresh Juice

SC-CO2 MB Treatment*
  Extraction of Limonoid Aglycones
  Sterilization of Microorganisms
  Inactivation of Enzymes

Peel Residue

Molasses

Resin Adsorption Treatment

Extract Solution

SC-CO2 MB Treatment*
  Removal of Contaminants
  Deodorization of Flavor Compounds
  Sterilization of Microorganisms

Fresh Juice Product

Limonoid Glucoside Extract

Figure 3. Application of Supercritical Carbon Dioxide Micro-bubble Method
     * Supercritical Carbon Dioxide Micro-bubble Treatment

Summary

Research on biological activities of citrus limonoids has made significant progress in recent years and the demand for citrus limonoids has increased considerably. A new technique using supercritical carbon dioxide ($SC\text{-}CO_2$) micro-bubble method has been developed for the extraction of limonoid aglycones from bitter citrus juices and the purification of limonoid glucosides isolated from juice processing by-products. Our pilot scale equipment, which is capable of feeding a liquid sample and $SC\text{-}CO_2$ continuously, removes bitter limonoids from citrus juices, purifies limonoid glucoside isolates, sterilizes the products and also inactivates enzymes present in samples. Limonoid glucosides isolated from mandarin molasses were odorless and practically tasteless. The recovery in the $SC\text{-}CO_2$ process was 97%, however, the overall yield was 65%. Plans to develop an industrial scale system and wider application of the method are underway.

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Chapter 8

Limonoid Glucosides of Satsuma Mandarin (*Citrus unshiu* Marcov.) and Its Processing Products

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The cultivation of Satsuma mandarin (*Citrus unshiu* Marcov.) was originated in Japan and is one of the most important parts of Japanese agriculture. We determined changes in the amounts of limonoid aglycones and glucosides in the Satsuma mandarin fruit tissues during fruit growth and maturation as well as the contents of limonoid glucosides in commercial juices and juice processing by-products. Mature Satsuma mandarin fruit contains relatively low concentrations of bitter limonoid aglycones and high concentrations of non-bitter 17β-D-glucoside derivatives, such as limonin 17β-D-glucopyranoside and nomilin 17β-D-glucopyranoside. The concentration of limonoid glucosides in the fruit tissues is about 150 times higher than that of aglycones. Therefore, with the exception of early ripening cultivars, limonoid bitterness is very infrequently in Satsuma mandarin juices and its other processed products. The Satsuma mandarin juice processing by-products are also rich sources of limonoid glucosides. Molasses, for example, contains over 2000 ppm of total limonoid glucosides.

The first description of *Citrus* in Japan was documented in the *Kojiki* and *Nihon shoki*, the oldest Japanese publications. According to these records, they were first introduced into Japan in the fourth century as a medicine for perennial youth. There is a lot of folklore related to the medicinal property of *Citrus*, and many plants

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belonging to the *Rutaceae* family including *Citrus* are used as traditional medicine in eastern Asian countries(*1*). Modern chemistry revealed that many kinds of chemical components of the citrus fruit can contribute to the human health. Some compounds present in the citrus fruit have been found to possess useful biological activities. They include vitamins, minerals, fibers, organic acids, essential oils and some other secondary metabolites (*2, 3*). Intake of citrus fruit appears to be much more important than it had been traditionally believed.

With the exception of world citrus production, the citrus processing industry has continued to grow. The recycling of citrus processing waste has become increasingly important with the growth of the citrus processing industry. However, only dried pulp, molasses, washed pulp solid and essential oil are commercially manufactured from the waste. There are many useful compounds contained in the waste which are not currently reclaimed. Limonoid glucosides are one of the most important of these compounds, because of their important biological activities including potential cancer chemopreventative activity (*4*). Limonoid glucosides are tasteless and water soluble, unlike some of the limonoid aglycones which are extremely bitter and water insoluble. This characteristic makes the glucosides ideal for use as functional food additives. Our research on limonoid glucosides of Satsuma mandarin, is directed toward the development of an industrial scale recover system of these compounds from citrus processing waste.

Cultivation of Satsuma mandarin (*Citrus unshiu* Marcov.) originated in Japan and the variety seems to have been derived from a citrus variety imported from China about 400 years ago. The name of "Satsuma" comes from the ancient name of the Kagoshima prefecture where the variety was established. Today, Satsuma mandarin is commercially grown in South Korea, China, the Mediterranean coastal countries, Spain and United States. It is the most popular fresh citrus fruit and processed juice product consumed in Japan. In the mid-1970s, the annual production of Satsuma mandarin reached 3.6 million tons and it was over 85 % of the entire Japanese citrus production. Due to the promotion of the citrus juice industry by the Japanese government in the early '70s, the juice production also reached to a peak in 1975. Approximately 1 million ton of Satsuma mandarin was processed in this year. After the peak, growers reduced Satsuma mandarin cultivation and production has decreased to around 1 million tons in recent years. However, it is still one of the most important parts of the Japanese agriculture.

For Satsuma mandarin, the limonoid bitterness problem occurs only infrequently in juices extracted from fruits of early ripening cultivar (*5, 6*) and very early ripening cultivar (*7, 8*). It had been believed that mature Satsuma mandarin fruit contains very little limonoid. Fong *et al.* (*9*) previously reported that commercial juices of Valencia orange, grapefruit and lemon contained high concentrations of limonoid glucosides. For instance, the total concentrations of limonoid glucosides in commercial orange juices were averaged at 320 ppm, whereas those of limonoid aglycones are generally under 6 ppm. It is expected that Satsuma mandarin contains high concentrations of limonoid glucosides as well as Valencia orange.

Limonoid glucosides of frozen concentrated juices of Satsuma mandarin.

We first analyzed Satsuma mandarin frozen concentrated juices for their limonoid glucoside content during the 1990 processing season and found that they contained very high concentrations of 17β-D-glucopyranosides of limonin (LG), nomilin (NG), nomilinic acid (NAG) and obacunone (OG) (*10*) (Table I).

Table I. Limonoid Glucosides of Satsuma Mandarin Concentrated Juices

Samplin date	Limonoid Glucosides Concentrations (ppm)				
	LG	NG	NAG	OG	Total
Oct. 20, 1990	124	47	41	0.7	213
Oct. 30, 1990	133	56	47	0.7	237
Nov. 10, 1990	140	63	43	0.5	247
Nov. 20, 1990	139	59	44	0.6	242
Nov. 30, 1990	146	69	50	0.8	266
Dec. 10, 1990	141	63	48	0.6	253
Dec. 20. 1990	134	44	39	0.3	217
Jan. 11, 1991	113	30	34	0.1	177
Jan. 19, 1991	112	39	38	0.2	189
Feb. 1, 1991	123	48	45	0.2	216
Feb. 15, 1991	114	36	32	0.1	182
Mean	129	50	42	0.4	223
S. D. 12	12	5	0.2	28	

Values show at the converted rate of 9.0° Brix. 17β -D-glucopyranosides of limonin (LG), nomilin (NG), nomilinic acid (NAG) and obacunone (OG).

Analysis of 11 samples showed that the juices contained an average of 223 ppm of total limonoid glucosides ranging from 182 to 266 ppm. This average concentration was lower than that of commercial orange juices sold in United State, but higher than that of grapefruit and lemon juices (*9*).

LG was the predominant limonoid glucoside in all juice samples, averaging 129 ppm or 58% of the total. This agrees with other citrus juices (*9, 11*). LG contents in orange, grapefruit and lemon juices are reported to be 56, 63 and 66% of the total, respectively (*9*). LG concentration was followed by NG, NAG and OG in order of decreasing concentration. Glucosides of deacetylnomilin and deacetylnomilinic acid were also present in the juices, but they were not quantitatively analyzed. Their concentrations appeared to be higher than that of OG.

The conversion of limonoid aglycones to their glucosides occurs in fruit tissues during late stage of fruit growth and maturation (*12*). This conversion has been demonstrated in navel oranges and Valencia oranges. The high concentration of

limonoid glucosides and the low concentration of limonoid aglycones (less than 3 ppm) clearly show that the glucosidation of the aglycones is also occurring in Satsuma mandarin.

The Satsuma mandarin juices contained fairly consistent concentrations of limonoid glucosides throughout the 1990 processing season. The analytical values of limonoid glucosides in juices were fairly consistent throughout the 1990 processing season. This disagrees with reports that limonin glucoside contents of Valencia orange (12) and navel orange (13) increase as the fruit maturation progresses. The reason may be that the processing plant received fruit which had already reached progressed levels of maturity.

Changes in limonoid glucosides and limonoate A-ring lactone (LARL) of Satsuma mandarin during fruit development.

A typical change in the weight during the course of Satsuma mandarin fruit development is shown in Figure 1. There are at least two sharp increases in fruit weight during the fruit development. The first increase occurred between September and October, due mainly to an increase in the flesh weight. The second one occurred between November and December where a sharp increase in both of the flesh and peel weight was observed. It has been suggested that the maturation of the flesh tissue occurs prior to that of peel tissue (14). Generally, the late ripening type of Satsuma mandarin is fully matured and ready to be harvested in late November.

Changes in LG contents of flesh and peel during the fruit development is shown in Figure 2. LG was first detected at the beginning of August in the flesh and in the beginning of September in the peel. The LG content of flesh increased sharply from the middle of September and reached a maximal level by the middle of October, and kept this level until the harvest season. Interestingly, LG content of peel sharply increased in November where the sharp increase in peel weight was observed. This synchronized increase between tissue weights and LG content suggests that the biosynthesis of LG is strongly related to tissue maturity. In a mature fruit, the content of LG was 17 mg in flesh and 11 mg in peel. These values are similar to those of Valencia orange grown in the United States (13).

Changes in the LARL content behaved a similarly to the LG content both in the flesh and the peel until they reach their maximal levels. The content of LARL was much lower than that of LG throughout the development of the fruit, approximately one eighth in the flesh and one fifth in the peel until they reached their maximal levels. Whereas the LG content remained at the maximal level, the LARL content sharply decreased after it reached its maximal level. It was previously reported that Valencia orange and navel orange accumulate LARL in flesh tissues until the glucosidation began (12, 13). However, Satsuma mandarin does not accumulate LARL. This may indicate that Satsuma mandarin does not synthesize LARL until the late season, and newly biosynthesized LARL in the late season is immediately converted to LG.
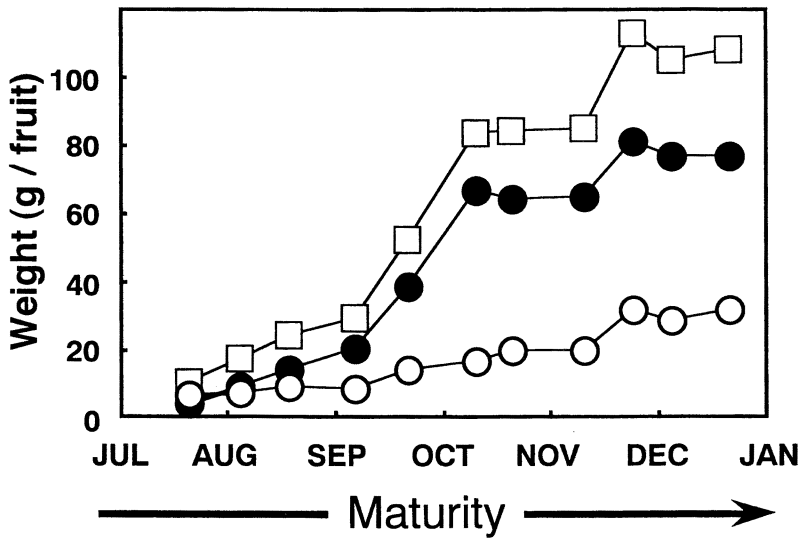
Figure 1. Increase in Fruit Weigh during Satsuma Mandarin Fruit Development.

Open squares represents changes in the weight of whole fruit. Open and solid circles indicates those of peel and flesh respectively.
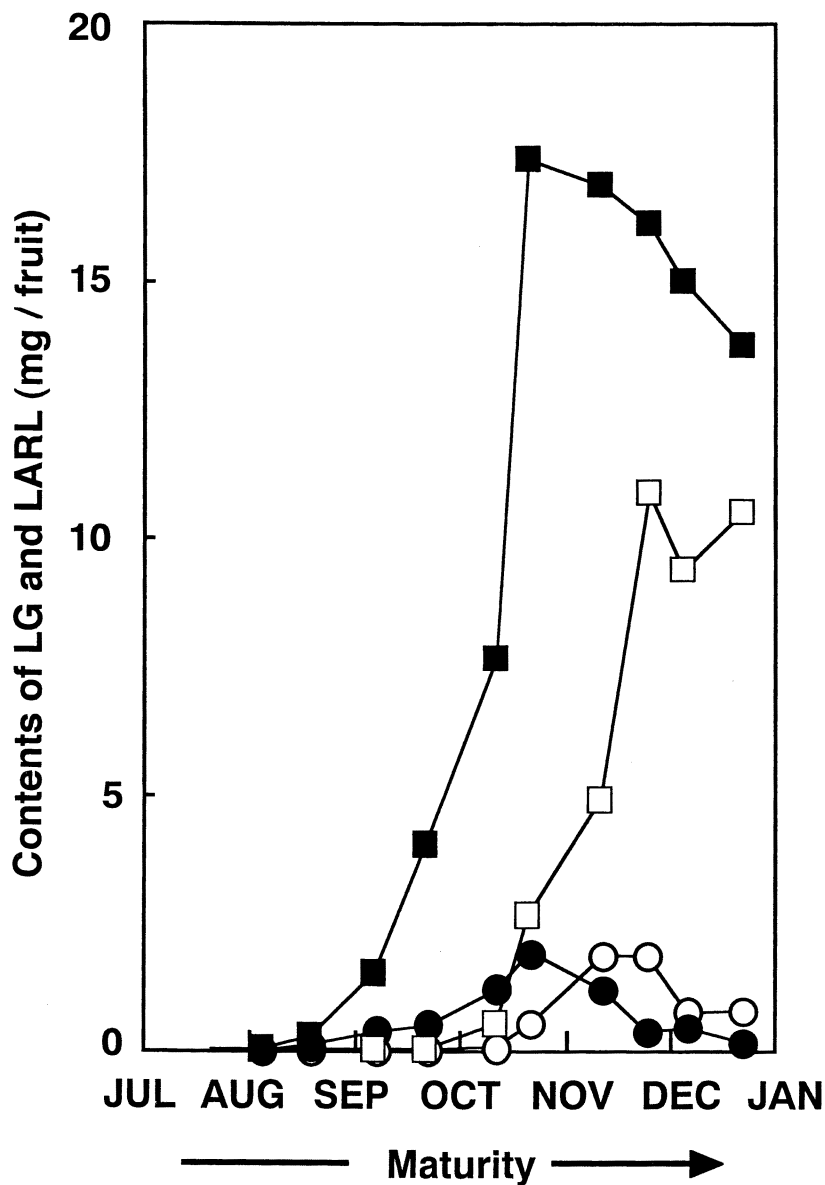
Figure 2. Changes in Content of Limonin 17β-D-glucopyranoside (LG) and Limonoate A-ring Lactone (LARL) during Satsuma Mandarin Fruit Development.

Squares indicate changes in LG contents and circles indicate changes in LARL. Solid symbols represent flesh and open symbols represent peel.

Limonoid glucosides of Satsuma mandarin fruit.

Satsuma mandarin fruit contains not only LG, but also the glucosides of nomilin, nomilinic acid and obacunone. The limonoid glucoside composition of mature Satsuma mandarin fruit is shown in Table II. The composition was similar to that of frozen concentrated juices. However, the concentrations of total limonoid glucosides, 460 ppm in flesh and 500 ppm in peel, are about twice those obtained from the concentrated juice analyses. This difference between raw fruit and processed products indicates that large amounts of limonoid glucosides are still retained in the processing waste or lost during the processing.

Table II Composition of Limonoid Glucosides in Mature Satsuma Mandarin Fruit.

Fruit tissues	Concentrations of Limonoid Glucosides (ppm)				
	LG	NG	NAG	OG	Total
Flesh	186	144	126	6	462
Peel	360	131	trace	11	502

17β -D-glucopyranosides of limonin (LG), nomilin (NG), nomilinic acid (NAG) and obacunone(OG).

Distribution of limonoid glucosides in processed juice products and by-products.

The juice processing by-products of Satsuma mandarin were analyzed to evaluate their suitability as sources of limonoid glucosides (15). Table III shows concentrations of limonoid glucosides in the products of juice processing. Analyses were conducted weekly throughout the processing season and the averaged values are indicated. For dried peel residue and molasses, the values are estimated by TLC, because of poor resolution in the HPLC analysis.

The total limonoid glucoside concentration of single strength juice was 215 ppm. This was consistent with the previous results. The concentrated juice contained over 1400 ppm of glucosides while the pulp contained less than 300 ppm. The limonoid content of whole fruits sampled from the juice plant agreed with that of fruit collected from the orchard.

The residue products contained high concentrations of limonoid glucosides. The dried peel residue contained over 3500 ppm while the molasses contained close to 2500 ppm.

Table III Concentrations of Limonoid Glucosides in Satsuma Mandarin Juice Processing By-products and Products.

Samples	Concentrations of Limonoid Glucosides (ppm)				
	LG	NG	NAG	OG	Total
Whole fruit	237	159	88	7.2	491
Juice products:					
Single strength juice	99	41	72	2.3	213
Finisher pulp	112	62	28	1.8	204
Centrifugal pulp	173	57	61	2.6	294
Residue products:					
Intact peel residue	359	235	168	7.0	769
Pressed peel residue	488	281	153	58.3	980
Dried peel residue	-	-	-	-	3561 *
Press liquor	255	120	100	60.3	535
Molasses	-	-	-	-	2486 *

*Values show estimates by TLC. 17β -D-glucopyranosides of limonin (LG), nomilin (NG), nomilinic acid (NAG) and obacunone(OG).

The composition of the whole fruit and the press liquor were quite similar except for the concentration of obacunone glucoside. The concentration of this glucoside in the press liquor was 8 fold higher than that found in the fruit itself. This may be due to the conversion of nomilin glucoside into obacunone glucoside during the liming process. Nomilin glucoside has been shown to be the most unstable limonoid glucoside and this type of conversion has been demonstrated previously (16).

A typical juicing procedure of Satsuma mandarin fruit and its material balance during the processing is summarized in Figure 3 (17). When 1,000 kg of Satsuma mandarin is processed, 83 kg of concentrated juice, 47 kg of finisher pulp, and 3 kg of centrifugal pulp is obtained. The peel residue from the juice extraction goes through a liming, pressing, and drying process producing 75 kg of dried peel residue and 36 kg of citrus molasses.

Based upon the concentration data obtained above, the yields of limonoid glucosides from 1,000 kg of Satsuma mandarin fruit have been estimated (Table IV). There is about 500 g of total limonoid glucosides present in 1000 kg of Satsuma mandarin fruits. Only about 120 g is retained in the concentrated juice which is about 24% of the original. Dried peel residue contains 180 g, which is 36% of the total, and citrus molasses contains about 90 g, 18 % of the total. These two by-products are good candidates for recovering limonoid glucosides. However, citrus molasses is a
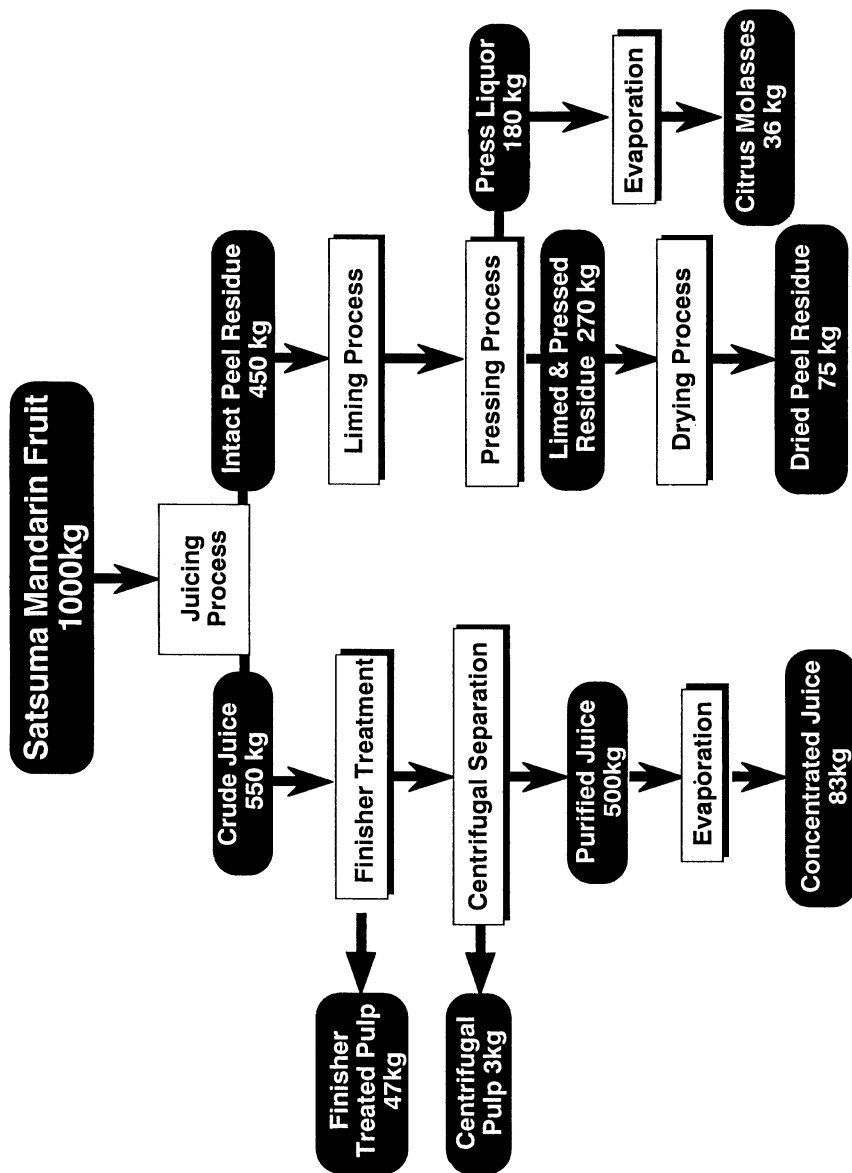
Figure 3. Schematic Diagram of Satsuma Mandarin Juice Processing and Model Balance Sheet.

Table IV Estimated Limonoid Glucosides Yields* of Processing By-products.

By-products	Estimated amount of total limonoid glucosides(g)	Ratio(%)
Fruit	500	-
Finisher pulp	10	2
Centrifugal pulp	1	0.5
Concentrated juice	120	24
Citrus molasses	90	18
Dried peel residue	180	36
Lost in the process	-	19.5

* from 1000kg of Satsuma mandarin fruit.

better source for recovery, since it is already in a liquid form and an extraction step could be omitted.

Limonoid glucosides contents of citrus molasses from various origins, including Valencia orange, Hamlin, and pineapple were found to be similar to those from Satsuma mandarin and the total concentrations averaged 2200 ppm at 37 °Brix (*18*). The development of a limonoid glucoside recovery system using citrus molasses should be a major contribution to the citrus industry, since citrus molasses is a major processing component from most of the processors.

Citrus seeds, Another candidate of recovery source of limonoids glucosides.

Although Satsuma mandarin is not seedy, citrus seeds are well known as a rich source of limonoid aglycones. They are also expected to be that of limonoid glucosides. Limonoid glucosides and aglycones in seeds of nine edible citrus fruit including, Fukuhara orange, Hyuuganatsu, Sanbokan, Shimamikan, Grapefruit, lemon, Valencia orange and tangerine, were measured to evaluate their suitability as sources of limonoid glucoside recovery (*19*).

All the citrus seeds analyzed contained 17 β -D-glucopyranosides of the major limonoids found in common citrus species (Table V). They included glucosides of limonin, nomilin, obacunone, deacetylnomilin, nomilinic acid and deacetylnomilinic acid. The total concentration of limonoid glucosides in the seeds ranged from 0.31 to 0.87 % and averaged 0.61 % of the dry weight (Table V and Table VI). This concentration is approximately 20-fold higher than in commercial citrus juice (*9, 11*). Among the seeds analyzed, Valencia orange contained the highest amount of total glucosides, followed by Fukuhara, grapefruit, Shimamikan and lemon in this order. Hyuhganatsu was the lowest.

Unlike fruit tissue in which limonin glucoside is the predominant glucoside (*11*), the concentration of limonin glucoside in the seeds was relatively low. In Hyuhganatsu seeds, the concentration of limonin glucoside was not measurable under

the condition used, and grapefruit and lemon seeds contained a moderate amount of limonin glucoside.

Nomilin glucoside was the major glucoside in the majority of the seeds (Table V). Particularly, in Fukuhara and Valencia orange, nomilin glucoside made up 42 % and 51 % of the total limonoid glucosides, respectively. However, the glucosides of obacunone and deacetylnomilinic acid were the major glucosides in Shimamikan and tangerine, respectively. It was generally believed that the limonoids in the seed are translocated from the fruit tissues to be stored. However, this finding supports the theory that the limonoids are biosynthesized in the seed independently and are not translocated from the fruit tissues.

Citrus seeds were confirmed to be an excellent source for both of limonoid aglycones and glucosides. However, because the concentrations of glucosides in citrus seeds were generally less than half of those of aglycones, citrus seeds appears to be more suitable to be used as a source of limonoid aglycones.

Table V Limonoid Glucosides in the Seeds of Some Commercial Citrus Varieties

Seeds	Limonoid Glucosides (mg g of dried seed)						
	LG	DAG	DG	NG	NAG	OG	Total
Fukuhara orange	0.51	0.28	1.32	3.22	0.98	1.09	7.40
Grapefruit	1.48	0.75	0.68	2.01	0.89	0.86	6.67
Hyuuganatsu	trace	0.42	0.37	1.10	0.76	0.65	3.31
Lemon	1.44	0.14	0.55	1.53	1.39	1.49	6.54
Sanbokan	0.51	0.37	0.89	1.13	0.55	0.90	4.36
Shimamikan	0.37	0.48	0.69	1.89	1.29	2.35	7.08
Tangerine	0.90	1.69	0.93	0.42	0.96	0.45	5.36
Valencia orange	0.59	0.13	1.69	4.48	0.98	1.06	8.94

17β-D-glucopyranosides of limonin (LG), deacetylnomilinic acid (DAG), deacetylnomilin (DG), nomilin (NG), nomilinic acid (NAG) and obacunone (OG).

Conclusion

It was confirmed that limonoid glucosides are one of major secondary metabolites in mature fruit tissues of Satsuma mandarin, like other edible citrus fruit previously reported (*12, 13*). Satsuma mandarin fruit contained high concentrations of limonoid glucosides and only a trace amount of aglycones. The total concentrations of limonoid glucosides in the frozen concentrated juices averaged 223 ppm, which is about 100 folds higher than that of aglycones and about same as that of L-ascorbic acid (vitamin -C) which is well known as one of the most important nutrients of Satsuma mandarin. Satsuma mandarin also contains , β-cryptxanthine,

Table VI Total Limonoid Aglycones and Glucosides in the Seeds of Some Commercial Citrus Varieties

Seeds	Glucosides(%)	Aglycones(%)	Total (%)	Aglycones/ Glucosides
Fukuhara orange	0.77	1.59	2.36	2.1
Grapefruit	0.70	2.39	3.09	3.4
Hyuuganatsu	0.31	0.90	1.22	2.9
Lemon	0.64	1.26	1.90	2.0
Sanbokan	0.42	0.68	1.10	1.6
Shimamikan	0.68	1.23	1.91	1.8
Tangerine	0.53	1.23	1.76	2.3
Valencia orange	0.87	1.48	2.35	1.7
Average	0.61	1.35	1.96	2.2

Values are indicated on a dry weight basis.

the predominant carotenoid responsible for the bright reddish color of the fruit. This carotenoid is reported to possess five times higher cancer preventive activity than that of β-carotene (20). The high yield contents of limonoid glucosides and β-cryptxanthine suggest that Satsuma mandarin should be considered as an excellent source of these important biologically active compounds.

It was previously reported that Valencia orange and navel orange accumulate LARL in flesh tissues until glucosidation began (12, 13). However, Satsuma mandarin does not synthesize LARL until biosynthesis of limonoid glucosides begins in the late season. Newly biosynthesized LARL in the late season is immediately converted to LG and LARL is not accumulated in the fruit tissue of Satsuma mandarin. This could be a main reason why delayed bitterness problem occurs infrequently in Satsuma mandarin juices.

About 20 % of the total limonoid glucosides present in Satsuma mandarin whole fruit is lost during juice processing and one fourth is retained in the final product, concentrated juice. The others are in by-products, especially in citrus molasses and dried peel residue. Limonoid glucosides in citrus molasses can be recovered by the combination of industrial absorbent and anion exchange resin. The development of the limonoid glucoside recovery system using citrus molasses should be a major contribution to the citrus industry.

Citrus seeds were found to contain not only aglycones but also glucosides of limonoids at high concentrations, and these compounds comprise nearly 1 % of the fresh weight of grapefruit seed. For individual limonoid glucosides, unlike fruit tissue where limonin glucoside predominates (9, 11), the concentration of limonin glucoside is relatively low in the majority of the seeds. This finding supports the theory that the limonoids are biosynthesized in the seed independently and are not translocated from the fruit tissues.

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Commercial Debittering Processes to Upgrade Quality of Citrus Juice Products

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Bitterness in citrus juice is primarily due to the presence of limonin in navel orange, and both limonin and naringin in grapefruit. Commercial systems using styrene/divinylbenzene resins to remove or decrease the levels of these bitter substances are used worldwide to upgrade juice quality. Byproducts from juice extraction (pulpwash, core wash and peel wash) which contain even higher levels of bitter substances, can be upgraded by this technique also, and sold as beverage bases. There are at least 34 commercial debittering units in operation worldwide, and most use a two-step process involving ultrafiltration to clarify the juice and a neutral resin bed to remove bitter and off-flavor components from the clarified juice, followed by recombination of the pulp and juice prior to concentration in an evaporator.

Bitterness in citrus has been a long-standing problem that affects consumer acceptance, especially in juice products (1). While some bitterness is expected in grapefruit juice, excessive bitterness is undesirable in this product and any bitterness in most citrus juices lowers the quality. Bitterness in grapefruit is caused by two types of bitter compounds, limonoids and flavonoids. The major limonoid is limonin, which is found in high levels in navel orange and grapefruit juices and is also present in many other citrus as well. It is formed from a nonbitter precursor present in the juice sacs,

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limonoic acid A-ring lactone (Figure 1), once the juice has been expressed from the fruit and allowed to stand or is heated during processing (2). This is the reason that navel orange fruit is not bitter when eaten, but the juice from the fruit is bitter.

Grapefruit on the other hand is bitter when eaten as the fresh fruit because it contains the bitter flavonoid naringin (Figure 1) in addition to the nonbitter precursor of limonin. Grapefruit juice becomes more bitter when standing or heated during processing as limonin bitterness forms in addition to the naringin bitterness already present.

Both naringin and the precursor of limonin are present in citrus at much higher levels in immature fruit and their amounts gradually decrease as the fruit becomes more mature. They are also present at higher levels in the peel, membranes and seeds than in the juice itself (3,4). In juice processing, the earlier the fruit is harvested and the harder the fruit is squeezed during juice extraction, the higher the levels of bitter components are in the product.

There have been many attempts through the years to eliminate or control levels of bitterness in citrus juice products. Some of these, such as use of enzymes, are not legal in Florida while others are not economically feasible. In the future, genetic engineering offers hope to solve this problem but at present use of legally approved processing aids is the only effective means available to control bitterness in citrus products. The historical development and current use of such processing aids are the subjects of this chapter.

Historical Development

Early attempts to reduce bitterness in citrus involved a wide variety of methodologies. Enzyme preparations, some now available commercially, were found to convert naringin to nonbitter products at room temperature, and at the pH level found in grapefruit juice (5,6). Hasegawa and co-workers reported an enzyme system capable of converting limonin to nonbitter substances (7), and developed an immobilized enzyme column for using this system to debitter navel orange juice (8).

Manipulation of limonoid synthesis in the fruit is another approach to decreasing bitterness in citrus juice. Ethylene treatment of fruit accelerated limonoid metabolism in navel oranges, grapefruit and lemons, resulting in lowered bitterness in juice expressed from treated fruit (9). Treatment of developing fruit with growth regulators such as triethylamine derivatives, indoleacetic acid and other auxins, or abscisic acid resulted in marked inhibition (up to 95%) of limonoid synthesis (10,11).

Experimental methods for removing bitterness from the juice include supercritical carbon dioxide extraction of juice, which reduced limonin content from 17.5 ppm to 7 ppm (12), and addition of cyclodextrin to encapsulate bitter components and thus mask the bitter taste (13). Column technologies for juice debittering include column packings of activated carbon, nylon polymers (14), Florisil (15), and cyclodextrin polymers (16). None of these methods has been adopted commercially.

The first commercial debittering unit for citrus juice was installed in a plant in
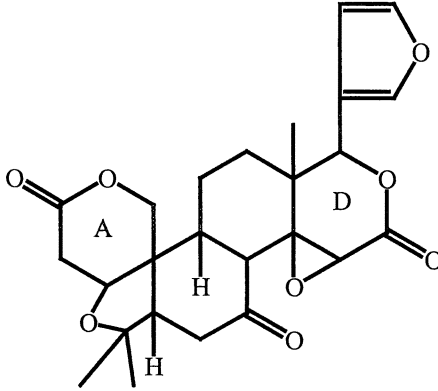
**LIMONIN
(bitter)**
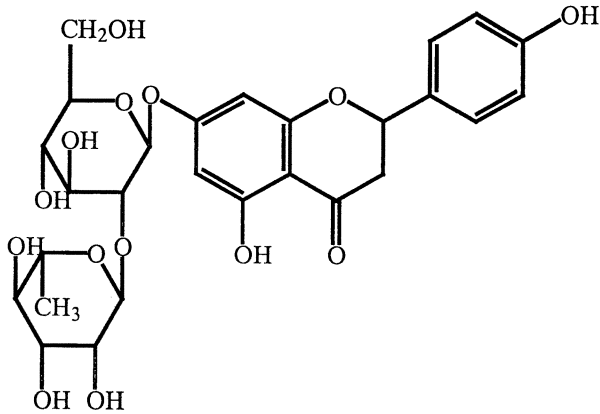
**NARINGIN
(bitter)**

Figure 1. Limonoids and flavonoids in citrus
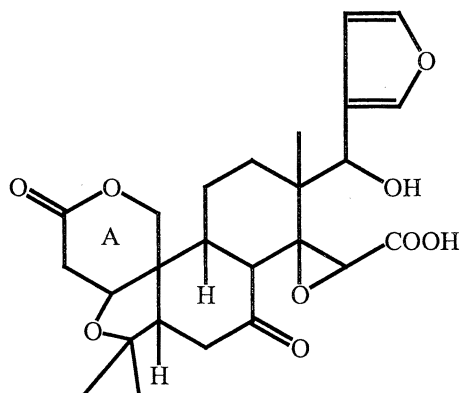
LIMONOIC ACID A-RING LACTONE
(tasteless)
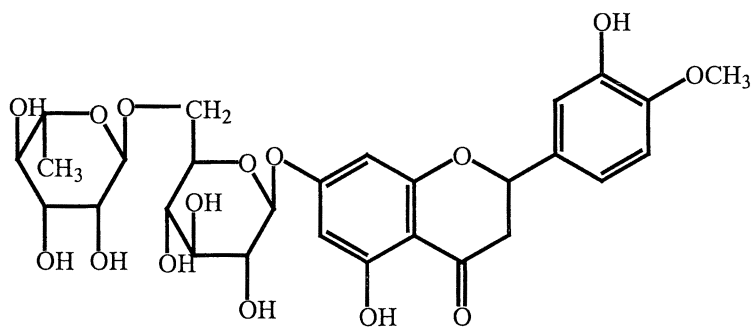
HESPERIDIN
(tasteless)

Figure 1.   Continued.

Australia in the late 1970s, based on the research of Johnson and Chandler (17,18). The debittering unit consisted of 12 long cylindrical columns packed with cellulose acetate polymer which was one of the few polymers approved for food use at that time (19). The juice was passed through the columns in series to remove the bitter components, and columns were regenerated by washing with water. There was a continuing problem of column plugging because of the pulp present in the juice, and the process was soon abandoned. In the early 1980's, these authors determined the debittering and deacidification capacity of a large number of other resins as well (20,21). Some of these were of the styrene-divinylbenzene type now used commercially. During that same time period, Maeda and co-workers (22) studied 33 types of neutral and ion exchange resins for their capacities to deacidify and debitter juice from the Hassaku mandarin hybrid fruit grown in Japan. They found styrene-divinylbenzene and acrylonitrile-divinylbenzene to be the most effective resins for decreasing naringin (60%) and limonin (75-90% reduction).

In 1988 the first commercial debittering unit used in the United States was installed in California using a styrene-divinylbenzene polymer that was approved for food use at that time (23). That unit, which is still in use in 1999, uses two stage centrifugation of the juice to remove most of the pulp which would plug the column, followed by passage of the clarified juice through one of two identical columns packed with a hydrophilic styrene-divinylbenzene adsorbent. After four hours the juice stream is diverted to the second column and the first column is regenerated by washing with one percent caustic solution. The clarified debittered juice is recombined with the pulp removed by centrifugation to provide the debittered juice for concentration. The level of peel oil should be reduced to 0.01-0.02 percent oil to prevent absorption of oil by the resin, which shortens its capacity (24).

Shortly after the first unit was installed, a second unit based on a slightly different principle was installed in another plant in California. This system utilized ultrafiltration for clarification of the juice. The clarified juice was passed downflow for 20 hours through three identical columns packed with a neutral styrene-divinylbenzene resin with a regeneration step which lasted four hours. Up to 200 gallons of juice per minute could be processed in that system (25).

Another commercial debittering system in limited use in Australia is based on a system developed for removal of color and acid from grape and other deciduous fruit juices which are then used to prepare juice drinks. This system uses two or three identical columns and a large hopper located above them. Centrifuged juice is debittered by passing it through one of the resin columns until the resin needs to be regenerated. Flow is diverted to a second column while the first is regenerated by pumping it as a slurry up into the hopper prior to washing with dilute caustic solution. Use of the funnel-shaped hopper is more efficient at removing pulp particles than leaving the resin in the column. That column can be packed with a different resin for a different use such as deacidification, if desired, and the regenerated resin can be stored for later use (25). Alimentech, Ltd., which first applied this system to citrus juice debittering, was recently acquired by Bucher-Guyer, Ltd.

A resin in cage system developed in Australia has been used to debitter orange juice and to debitter and deacidify grapefruit juice (26). The juice does not require

pulp removal because the resin is placed inside a stainless-steel cage with small mesh and the resin cage is agitated slowly inside a column as pulpy juice flows through. A neutral styrene-divinylbenzene polymer is used for debittering, and a weak base resin is used for reduction of acidity (26). The cage containing the resin beads is removed from the column for regeneration. This type of system has been used in Israel to debitter peel wash.

The styrene-divinylbenzene resins used in these processes fall under the category of processing aids as defined by 21CFR 177.2710 (27). Neutral resins such as XAD-4, XAD-16, Dyolite S861 and S866 and others are suitable as long as they satisfy the U.S. Food and Drug Administration requirements for food contact use (21,28).

Current Methodology

Most debittering units in use currently by citrus processors are based on a method described in detail by Wethern (14) as illustrated by the simple schematic system in Figure 2 (29). The oil level of the feed juice is reduced using a commercial deoiler. Then the juice containing 25 ppm of limonin, and naringin in grapefruit (see below), flows into the ultrafiltration system where the pulp and some of the juice are separated. Hollow fiber "shell and tube" ultrafiltration (UF) cartridges employing cross-flow filtration are generally used. Several UF cartridges in parallel form an UF module and a multistaged system of up to form modules affords a more energy-efficient system than single-stage filtration (14).

The clarified juice, still containing 25 ppm of limonin, flows into the debittering column packed with styrene-divinylbenzene polymer. The stainless steel column packed with resin beads is used in a down-flow mode with a distribution head that permits maximum filling of the column with resin. The column flow rate and temperature (about 50°C) of the feed juice are optimized according to the conditions of the resin (14). The debittered juice exiting the column is recombined with the pulp and the small amount of residual juice accompanying the pulp. Since that juice still contains 25 ppm of limonin, the final mixture contains limonin at a low level (5 ppm) which is below the reported taste threshold of 6.5 ppm (30). When necessary, the UF system can be operated at very high levels of pulp concentration in order to adjust the level of bitter components in the blended product to an acceptable level (31). Two identical columns are installed for continuous operation and one column is regenerated while the other column is in use for debittering. One percent caustic solution is normally used for regenerating the column, usually in an up-flow mode. The caustic solution can be recovered using a ceramic recovery unit to reduce the volume of caustic in waste disposal.

The naringin content in grapefruit juice can be lowered by 40% using the debittering process to decrease the bitterness in early season juice, which can exceed the maximum legal limit of 600 ppm in early-season fruit, as measured by the Davis test (32). The Davis test value is ~2.1 times the true naringin value, since it measures both naringin and its nonbitter isomer, the limit of naringin itself is about 285 ppm

(33). A level of 200-350 ppm of naringin in grapefruit can be maintained year-round using debittering techniques (29). The flavor threshold of naringin is estimated to be 20 times higher than limonin in water (30). By extrapolation to grapefruit juice, this range of 200-350 ppm provides moderate bitterness, which is a desirable characteristic in grapefruit juice (34).

A recent improvement in column design permits more uniform introduction of the juice stream onto the top of the resin bed. The column can be completely filled with resin, thus eliminating a large void area above the resin bed required in previous columns to aid in backflushing of the resin bed during regeneration. The styrene-divinylbenzene resin columns are generally used during a six-to-twelve hour cycle before regeneration. Ultrafiltration is the method of choice for clarification of juice because it eliminates all particulate matter and bacteria that might contaminate the resin column. This increases column life and minimizes bacterial growth in the column even though the caustic wash during regeneration helps to sterilize the system.

It is possible to recover limonin and naringin and other flavonoids as byproducts from column regeneration. Naringin is a precursor for dihydrochalcone sweeteners (35), which are used commercially in Europe; other flavonoids and limonoids have potential in the production of hypernutritious foods (36). Glucosides of limonin and other limonoids found in citrus juices have shown biological activity including anticarcinogenic properties, as described in several other chapters in this volume. They are present in greater quantities in most citrus juices than are the limonoid aglycones, and can potentially be removed from the debittering column during regeneration. Miyake et al. (37) discuss a method for recovery of limonoid glucosides from citrus juices based on supercritical CO_2 extraction.

Upgrading Byproducts

There are several byproducts recovered during citrus juice processing that can be used in preparation of citrus flavored beverages (Table 1). They are often bitter and astringent, but their quality can be upgraded using the debittering process (29,31). The most widely recovered byproduct is pulp wash, also called water-extracted soluble orange solids or WESOS (37). When orange juice is extracted from the fruit, the pulp content is too high and the juice and excess pulp are separated in a finisher. The separated pulp has a spongy character and thus retains some of the juice. Most of this juice can be recovered by washing the pulp in several stages with water, separating the pulp and water-extracted juice each time in a finisher. The water extracted soluble solids can legally be added back to the same juice from which the pulp was obtained if the juice is to be concentrated for making frozen concentrated orange juice. If not, the pulp wash can be concentrated separately and sold as a beverage base for use in citrus flavored drinks where it is used to provide natural orange cloud as well as natural sugars and acids.

Other products for use as beverage base ingredients include core wash and peel wash (31,38). During extraction of juice in an FMC extractor, a hollow perforated

stainless-steel tube cores the fruit prior to mashing the fruit to remove the juice. The pressed core plug can be isolated as a fraction separate from the peel and processed in the same way that pulp is processed to recover pulp wash. The core wash cannot be added legally to the juice directly, but must be concentrated and sold as a beverage base. It has a higher amount of limonin present than does pulp wash. Peel wash is another byproduct recovered by grinding the peel into small pieces, extracting it with water and separating the washed peel in a finisher. As with core wash, peel wash must be sold as a beverage base only. It contains such a high level of limonin that it must be debittered to be a usable product. It is also sold as a concentrated product. Table 1 lists the juice and those byproducts from juice extraction, the typical limonin content of each and the percentage of the total sugars present in the whole fruit which is contained in each of these fractions.

Table I. Upgrading citrus juice byproducts

	Limonin (ppm)	Sugars(%)[a]
Juice	2-30	50-60
Pulp wash	10-50	4-6
Core wash	40-100	12-15
Peel wash	100-250	20-25

a. Percent of total sugars present in whole fruit

Other Uses

A debittering column provides additional benefits for upgrading citrus products. The column removes other substances that adversely affect juice quality (29). One important use is the reduction of the level of hesperidin in clarified lemon juice (Figure 1). Clarified lemon juice can become hazy in appearance if the level of hesperidin is too high, resulting in an inferior quality product. The hesperidin is partly removed by the resin column as shown in Figure 3. Both hesperidin and naringin are only partly removed by the resin column during a 20 hour cycle, with slightly more hesperidin being removed. Limonin was almost totally removed during the 20 hour cycle, as shown in this figure.

When citrus fruit suffer freeze damage they must be removed from the tree and processed into juice immediately. The fruit often is of poor quality with high levels of hesperidin as well as off flavor components (39) such as coumarins, which can contribute astringent and other harsh flavor notes to the juice (40). The juice can be put through a debittering column to remove the off flavor constituents and lower the

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Typical Debittering Process
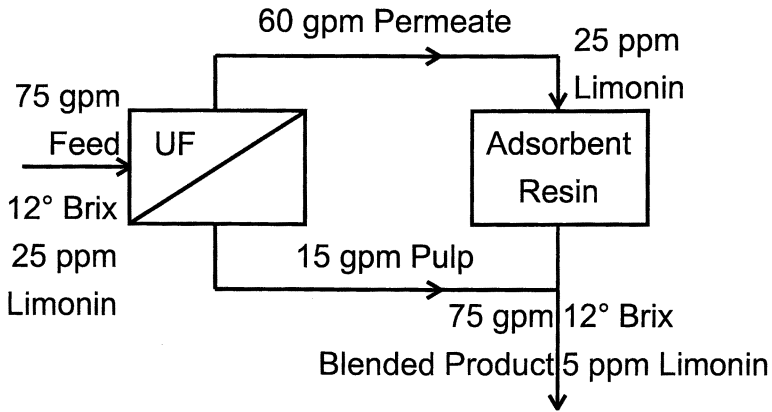
Figure 2. Schematic of typical commercial citrus juice debittering unit.
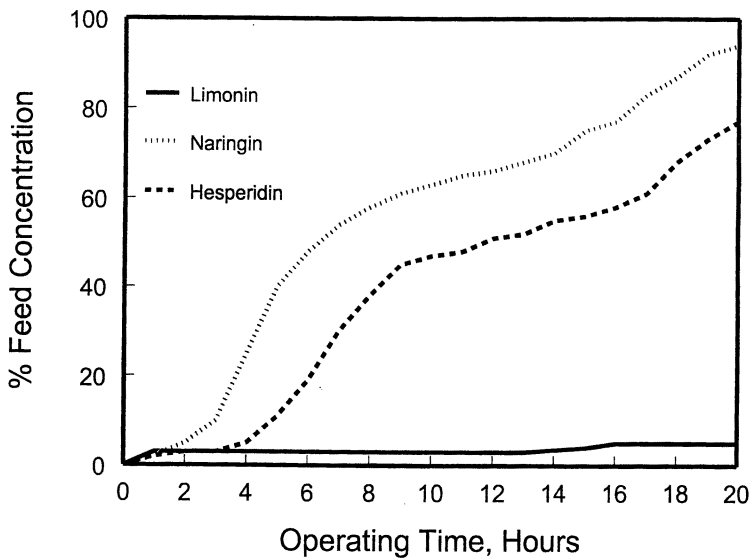
Figure 3. Percent removal of bitter components and hesperidin from juice during the debittering process.

hesperidin content to prevent formation of hesperidin crystals which will lower quality. By this treatment, juice that would only be suitable as a low-cost beverage base can be upgraded to juice suitable for production of concentrated orange juice for manufacture (29). The harsh and astringent flavor of barely mature orange and grapefruit is also reduced by treatment with the resin column. Reduction of bitter and astringent components often makes the juice taste sweeter even though the sweetness and acidity levels are unchanged (16,41).

Cost of Debittering

Estimated cost of operating a combined technology debittering system has been reported. Factors included were deoiling and pasteurizing, electricity, cleaning chemicals, UF membrane replacement, water labor and loss of yield, to afford an estimate of $0.03 or less per single strength gallon of juice (14). Another cost estimate reported a range of $25-30 per ton of concentrated juice product. This estimate included replacement membranes and resin, cleaning chemicals, utility costs and manpower based on a 5,000 liter per hour per unit operating for 100 days per year (31). These authors also estimated debittering/upgrading costs to be 4-5% of concentrated juice valued at $600-800 per ton. The market value for juice would be increased by 12-15% and up to 25% for by products.

Worldwide Units in Use

At present, there are about 34 debittering units in place worldwide for upgrading of citrus juices and juice byproducts. Table 2 lists the units in place worldwide, and their uses in various products. There may be a few additional units in use not accounted for in this table. Almost all of these listed are based on the principal of ultrafiltration to clarify and sterilize the juice followed by debittering with a neutral styrene/ divinylbenzene resin column and recombination of the pulp and clarified juice, unless a clarified juice is the desired product. At present, the European Union has not approved debittering for use with citrus juices sold within the European community, and so the units in place in Italy and Spain await approval.

It is clear that the debittering process is well-established for upgrading citrus products worldwide, and that new uses are discovered as quality problems arise. The expected approval of this process by the European Union in the near future and the increasing demand for low priced juice drinks in developing countries will undoubtedly expand its use worldwide.

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Table II. Units installed worldwide

Country	no.	uses
Argentina	3	lemon juice
Brazil	7	orange juice pulp wash, core wash
China	1	orange byproducts
Cyprus	1	grapefruit, orange
Greece	2	orange juice
Israel	4	orange juice, pulp wash, peel wash
Italy	4	orange juice, pulp wash, lemon juice
Mexico	3	lime juice, orange pulp wash
Pakistan	1	orange juice, pulp wash
Spain	2	orange juice
U.S.	5	orange juice, grapefruit juice, pulp wash, core wash, lime juice
Others	1-3	-

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Chapter 10

Citrus Limonoids: Increasing Importance as Anticancer Agents

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Limonoids have repeatedly been shown to possess anticancer activity in animals. As an outgrowth of this research, scientists are now working on the development of new foods containing elevated concentrations of limonoids. To recapture the additional funds needed to produce these products claims will have to be made on potential health benefits. This paper will review our research on the antineoplastic activity of 12 citrus limonoids. The data from these experiments have shown that some of the structural features associated with the limonoid nucleus are critical to cancer chemopreventive activity. The data from our recent studies on the effect of limonoid glucosides in mammalian systems will also be presented. Research with animals and human cells grown in culture suggest that these citrus chemicals are safe at elevated concentrations.

Research in several different laboratories has shown that the addition of green coffee beans to the diets of experimental animals can inhibit the development of carcinogen-induced tumors (*1-5*). The data from these experiments have shown that green coffee beans contain multiple cancer chemopreventive agents. Some of the active agents are present in the green coffee bean oil and others are present in the defatted portion of the bean. Further studies have shown that roasting does not destroy but may actually enhance antineoplastic activity (*6*).

Two of the cancer chemopreventive agents in the oil fraction have been isolated (*7,8*). The two chemicals, kahweol and cafestol, are diterpenes, which are structurally similar. The only difference is an extra double bond, which is found in the more active agent, kahweol. The anticancer activity of these coffee chemicals was demonstrated in animal models for mammary and oral carcinogenesis (*2,9*). Other studies suggested that both of these chemicals are type A blocking agents

(*5,7,10*), chemicals that can increase the activity of detoxifying enzymes (*1*). One of these enzymes, glutathione S-transferase, protects the organism from the action of chemical carcinogens. Further research with kahweol (*10*) indicated that certain portions of this chemical's structure are critical to its activity as a type A blocking agent. One of these structural features is a furan ring, the second is the extra double bond. The work with kahweol led to the idea that other terpenes containing a furan ring might also be cancer chemopreventive agents.

Over 50 limonoids present in the Rutaceae and Meliaceae families have been isolated and identified (*11*). Each of these chemicals is a highly oxidized triterpene containing a furan ring. Additional double bonds are found throughout the multi-ring structures. The structural similarities between limonoids and kahweol led to research on the cancer chemopreventive activity of these citrus chemicals.

Cancer Chemopreventive Activity

The hamster cheek pouch model for oral carcinogenesis (*12)* that was utilized in our earlier research on coffee beans and coffee chemicals (*3-6,9*) was used to test twelve citrus limonoids for cancer chemopreventive activity. In each of these experiments, the solution of the limonoid, like the solution of the carcinogen, 7,12-dimethylbenz[a]anthracene (DMBA), was painted on the surface of the buccal pouches. Depending on the molecular weight of the limonoid, the concentration of the solutions ranged from 2.5-3.5% (w/w). The solvent for the nonpolar limonoids was dimethyl sulfoxide. The solvent for the polar limonoids was water.

The same procedure was used in each experiment. The left buccal pouches of the hamsters in the experimental groups were painted five times per week. Two or three times per week the pouches were painted with the 0.5% solution of DMBA dissolved in heavy mineral oil. On alternate days, the solutions of the limonoids were applied to the pouches. The pouches of the animals in the control group were treated with the solvent used to dissolve the limonoid being tested for antineoplastic activity. In each experiment, there were a total of 71 treatments, 35 with the solution of DMBA and 36 with the solution of the citrus limonoid or the solvent used to dissolve the limonoid (control group).

Two to three days after the last treatment, the hamsters were sacrificed. The pouches were excised and the tumors when present were counted and measured (length, width, and height). The sum of the three measurements divided by six was used to calculate an average radius for each tumor. Since the tumors tend to be spherical in shape, this figure was then used to calculate an approximate volume for the tumor. The formula for the volume of a sphere, $4/3\pi r^3$, was used for this calculation. The sum of the volumes of all the tumors in one pouch was defined to be the animal's total tumor burden (*9,13*). After the gross tumor data had been collected, the tissues were fixed in 10% formalin, embedded in paraffin, and processed by routine histological techniques. The data from the macroscopic and microscopic observations were used to assess differences in tumor incidence, number, size, burden, and type. Chi-square analysis and the Student's t-test were

used to compare the results of the control groups and the groups treated with limonoids.

Tumor Data

The data from the experiments (5,14-18) showed that four of the citrus limonoids, limonin, limonin 17-β-D-glucopyranoside, limonin carboxymethoxime, and deoxylimonin, significantly inhibited the development of the DMBA-induced oral carcinomas. With each of these chemicals, there was a significant reduction in tumor burden. The overall inhibition ranged from 50-60%. The effect on tumor number was considerably less ranging from a 15-30% inhibition. The reason for this difference could be seen during the experiments. In the tissues treated with these chemicals, there was a delay in tumor onset of one to two weeks. The active limonoids seemed to be extending the promotion phase of carcinogenesis. The structures of limonin, limonin 17-β-D-glucopyranoside, limonin carboxymethoxime, and deoxylimonin are given in Figure 1.

Based on the following observations three of the limonoids, nomilin, nomilin 17-β-D-glucopyranoside, and obacunone (Figure 2) were classified as having partial activity. One, the three chemicals are structurally similar. Two, data from another laboratory demonstrated that nomilin and possibly obacunone are active in other tumor models (19-22). Nomilin exhibited antineoplastic activity in a model for benz[a]pyrene-induced forestomach tumors in ICR/Ha mice, benz[a]pyrene-induced lung tumors in A/J mice, and a two-stage mouse model for DMBA-induced skin tumors (19-21). In the model for skin tumors, the results with nomilin and limonin showed that nomilin was more effective during initiation and limonin was more active during the promotion phase of carcinogenesis. In addition, it was found that both nomilin and obacunone could induce increased levels of glutathione S-transferase activity in a variety of different tissues in ICR/Ha mice (22). Three, the data with these limonoids in the hamster cheek pouch model were similar. With each of these chemicals, there was a 20-30% reduction in tumor number. No effect on tumor volume was seen. For this reason, the overall reduction in tumor burden was similar to the reduction in tumor number. As compared to the control groups there was no apparent delay in the appearance of the tumors in the groups treated with nomilin, nomilin 17-β-D-glucopyranoside, and obacunone. These results suggest that the treatment with these chemicals affected primarily the initiation phase of carcinogenesis.

The data for the five remaining limonoids demonstrated that these chemicals were inactive. In each case, a comparison to the control group showed that the treatment with these limonoids had no effect on tumor incidence, number, burden, or type. The chemicals lacking antineoplastic activity included limonol, deoxylimonic acid, ichangensin, 17,19-didehydrolimonoic acid, and nomilinic acid 17-β-D-glucopyranoside. The structures of the inactive limonoids are given in Figure 3.

LIMONIN

LIMONIN 17-β-D-GLUCOPYRANOSIDE

R=CH$_2$OH

DEOXYLIMONIN

LIMONIN CARBOXYMETHOXIME

NOCH$_2$COOH
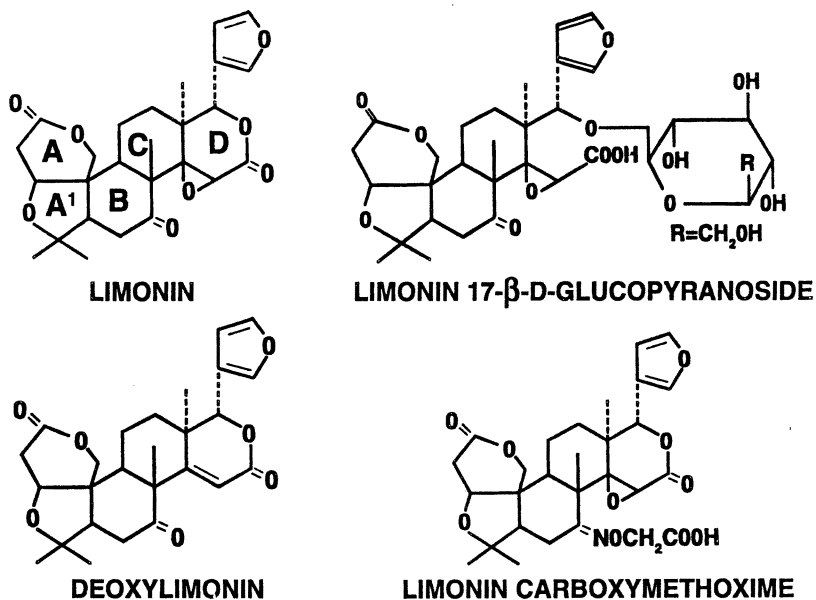
Figure 1. Limonoids with significant antineoplastic activity.

NOMILIN

OBACUNONE

NOMILIN 17-β-D-GLUCOPYRANOSIDE

R=CH$_2$OH
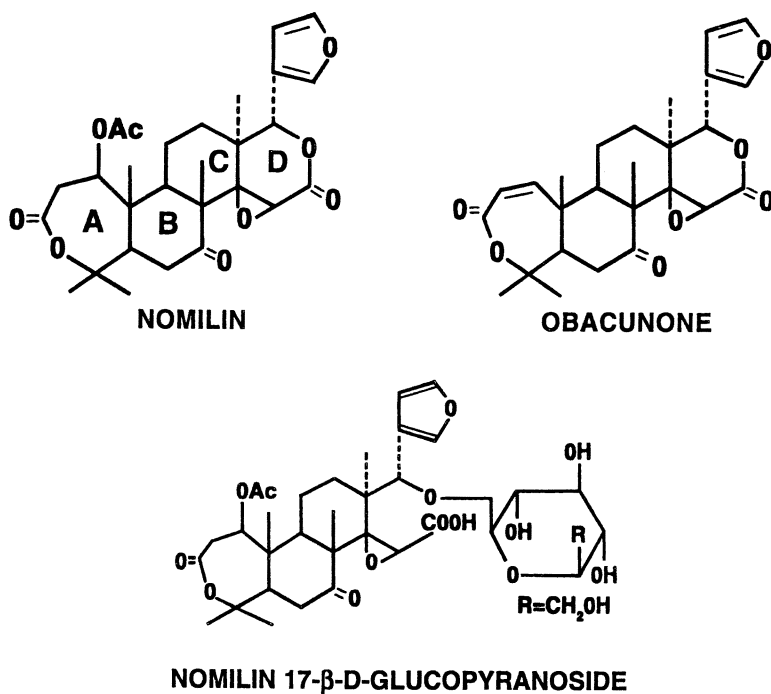
Figure 2. Limonoids with partial activity.
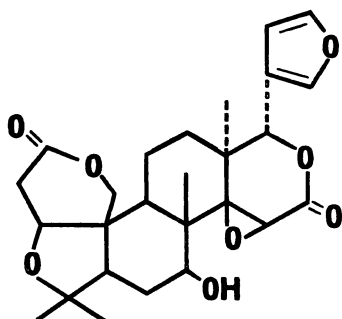
LIMONOL
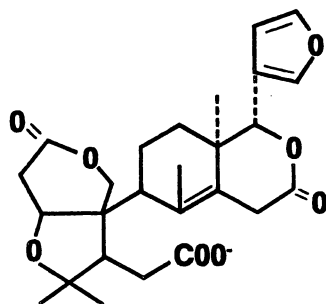
DEOXYLIMONIC ACID

ICHANGENSIN

17, 19 DIDEHYDROLIMONOIC ACID

$R=CH_2OH$
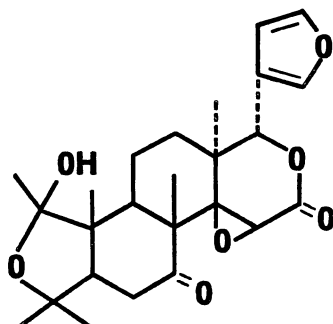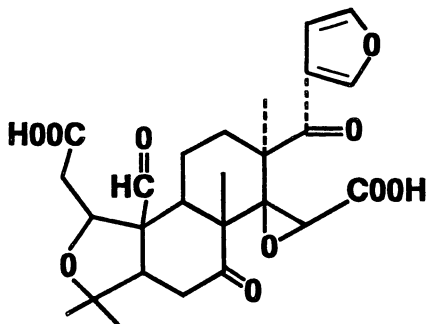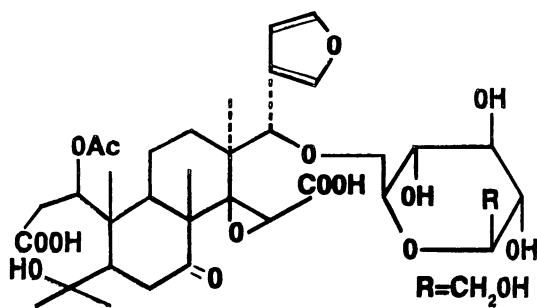
NOMILINIC ACID 17-β-D-GLUCOPYRANOSIDE

Figure 3. Inactive limonoids.

Structure-Activity Relationships

Besides helping to identify new citrus limonoids with cancer chemopreventive activity, the data from our earlier experiments suggested that some of the structural features found in citrus limonoids were critical to antineoplastic activity. In this section, we will focus on the limonoid nucleus. In limonin (Figure 1), the limonoid nucleus is a collection of five rings labeled A, A', B, C, and D. In nomilin (Figure 2), the limonoid nucleus contains four rings. Instead of separate A and A' rings, nomilin has a large A ring and the B, C, and D rings. Two of the limonoids, nomilin and obacunone, classified as having partial activity contain intact these four rings. The primary differences between obacunone and nomilin are some minor modifications to the large A ring. The third chemical classified as having partial activity, nomilin 17-β-D-glucopyranoside, contains the A, B, and C rings; however, the D ring is opened and a molecule of glucose has been added at C-17. The data suggest that major modifications can be made to the D ring without any apparent effect on anticancer activity. Looking at the inactive limonoids (Figure 3), it can be seen that nomilinic acid 17-β-D-glucopyranoside is structurally similar to nomilin 17-β-D-glucopyranoside. The primary difference is that the A ring is opened in nomilinic acid 17-β-D-glucopyranoside. This suggests that an intact A ring is required for the activity seen with these chemicals in the hamster cheek pouch model.

Similar comparisons can be made for the five rings in the limonoid nucleus in limonin. The data with limonin, limonin 17-β-D-glucopyranoside, and deoxy-limonin (Figure 1) suggest once again that modifications can be made to the D ring without any apparent loss of activity. The results with 17,19-didehydrolimonoic acid and possibly ichangensin (Figure 3) suggest that an intact A ring is required for antineoplastic activity. The results with deoxylimonic acid (Figure 3) are complicated by the fact that modifications are found in the B and C rings. Further research is needed to determine whether the opening of the B ring or the addition of the double bond to the C ring contributed to the loss of biological activity.

The results with limonin, limonin carboxymethoxime, and limonol are extremely interesting since these data not only suggest that the B ring is linked to cancer chemopreventive activity but pinpoints a double bond at carbon number 7 as being critical to antineoplastic activity. With limonol (Figure 3), a minor modification changes this double bond into a single bond and leads to a complete loss of activity. In contrast, a major modification is found in limonin carboxymethoxime (Figure 1) but the double bond is retained and biological activity is not affected. The explanation for these two sets of results is not known; however, some evidence suggests that if the double bond at carbon number 7 is changed into a single bond, the spatial configuration of the B ring to the rest of the rings in the limonoid nucleus is altered. Such a change might account for the loss of antineoplastic activity.

Potential Significance for Humans

Evidence from a large number of epidemiological studies (*23*) has shown that the consumption of citrus is protective against a wide range of cancers in humans. Significant reductions in risk were found for cancers of the oral cavity, larynx, esophagus, stomach, pancreas, lung, colon, and rectum. Initially it was assumed that vitamin C was the active agent in citrus. Vitamin C is a free radical scavenger and plays an active role in sparing or reconstituting the active forms of vitamin E and other antioxidants. In two of the studies (*24,25*), the investigators tested this assumption. In these studies, additional evidence was provided on the relative importance of citrus fruit consumption to similar levels of vitamin C intake from other sources. In both cases, the results were the same. Citrus fruit consumption was more closely linked to risk reduction. The authors concluded that citrus fruits contain not one but two or more anticancer agents.

Out of all of the limonoids tested for cancer chemopreventive activity most of the interest has centered on the limonoid glucosides. There are a number of reasons for this interest. Unlike some of the aglycones, including limonin and nomilin which are bitter (*26-30*), the limonoid glucosides are tasteless (*11,31*). Most of the aglycones, specifically the neutral limonoids, are insoluble in water, while the limonoid glucosides are water-soluble (*11,31,32*). Another plus is human consumption. Commercial citrus juices contain very high concentrations of mixed limonoid glucosides (*33,34*). The average concentrations in orange, grapefruit, and lemon juices are 320, 190, and 82 ppm, respectively. The major glucoside derivative is limonin 17-β-D-glucopyranoside with average concentrations of 180 ppm in orange juice, 120 ppm in grapefruit juice, and 54 ppm in lemon juice. Using the figures for orange juice, it has been calculated that one glass of orange juice contains similar amounts of vitamin C and mixed limonoid glucosides. By comparison, the concentration of the aglycones in orange juice is very low, 2-3 ppm. Finally, research on by-products from juice processing plants has shown that the concentration of mixed limonoid glucosides is extremely high in both the seeds and citrus molasses (*35-37*). On a dry weight basis, the average concentration of mixed limonoid glucosides in seeds from lemons, grapefruits, tangerines, and oranges was 0.61 %. In the same study (*35*), it was found that the average concentration of the aglycones in the seeds was 1.35%. Concentrations of mixed limonoid glucosides exceeding 3,000 ppm have been found in orange molasses.

When one combines the positive features of the limonoid glucosides with the earlier data on cancer chemopreventive activity (*5,14-22*) and the recent data indicating that citrus juices and citrus limonoids (aglycones and glucosides) can inhibit the growth of cancer cells (*38,39*), it is not surprising investigators are starting to think about functional foods. Using the techniques developed for the isolation of mixed limonoid glucosides (*35-37*), scientists in Japan have already isolated large quantities of these chemicals and used them to produce a test product (*40*). Specifically the mixed limonoid glucosides were added to one of the company's products, a mandarin orange juice. Through this procedure, they were able to artificially increase the concentration of mixed limonoid glucosides by approximately 5-fold, 220 ppm to 1,000 ppm. The test product was called *LG 1000*.

Results from consumer focus groups indicated that this change in product composition had no affect on color, texture, or taste. A second possible product is an outgrowth of the earlier work on limonoid bitterness (26-30). This work on "delayed bitterness" led to a complete description of the movement and metabolism of these chemicals in citrus plants. This basic understanding of the biochemistry of these chemicals in citrus (41-44) has now led to new research on the development of transgenic varieties of citrus that are free of limonoid bitterness containing elevated concentrations of mixed limonoid glucosides (45-47).

Limonoid Glucosides: Systemic Effects

The successful marketing of any functional food depends to a large extent on health claims. These claims are needed to offset the increased costs associated with the production of the new food. Before any claims can be made clinical trials are needed to establish whether or not the chemicals are effective in humans. Clinical trials have not yet been initiated with the limonoid glucosides. Another problem for the development of foods enriched in limonoid glucosides is basic information on the pharmacokinetics of these chemicals in mammalian systems. Research is needed to determine how effectively these chemicals are absorbed in the gastrointestinal tract. Once absorbed, how are the chemicals distributed throughout the body? How rapidly are the chemicals metabolized? Do any of the metabolites have anticancer activity?

To start to gather evidence on the uptake, distribution, turnover, and metabolism of limonoid glucosides in mammalian systems, we have initiated a series of experiments. The first experiment was designed to see what effect, if any, a long-term exposure to limonoid glucosides might have on the general health of the animals (48). In this study, 84 female Syrian golden hamsters (Lak:LVG strain) were separated into one of three equal groups. The animals in the control group were fed the semi-purified AIN76A diet (ICN Biochemicals, Aurora, OH). The animals in the two experimental groups were fed the same diet supplemented with either 0.05% or 0.5% of a limonoid glucoside mixture extracted from citrus molasses. The limonoid glucoside mixture contained limonin 17-β-D-glucopyranoside (35.9%), nomilinic acid 17-β-D-glucopyranoside (32.0%), nomilin 17-β-D-glucopyranoside (12.7%), deacetylnomilinic acid 17-β-D-glucopyranoside (8.7%), deacetylnomilin 17-β-D-glucopyranoside (6.6%), and obacunone 17-β-D-glucopyranoside (4.1%). The animals remained on their respective diets throughout the course of the experiment.

Sixty of the 84 animals (20/group) were used in an experiment on oral carcinogenesis. These animals remained on their respective diets for 105 days and were sacrificed. Blood samples were taken from the remaining animals (8/group) on day 45. Once a week the hamsters in the three groups were weighed. Twenty-four hour food intake data were collected on six separate occasions.

The data showed that there were no significant differences in the weight-gain profiles for the three groups. Similarly only minor differences in food intake were found. Combining the two sets of data it was calculated that an adult weighing 60

kg would have to consume 1.6 g of mixed limonoid glucosides per day to equal the exposure for the hamsters on the diet containing 0.05% of the limonoid glucoside mixture. This is equivalent to approximately 20 glasses of orange juice per day. The blood samples were analyzed at an outside laboratory (Professional Animal Laboratories, Irvine, CA). The complete blood counts for the three groups were normal. The mean values for an abbreviated series of blood chemistries (SGOT, LDH, total protein, glucose, creatinine, uric acid, and calcium) were normal. Taken together these data suggest that the long-term exposure to the limonoid glucoside mixture had no apparent effect on the general health of the animals.

Limonoid Glucosides: Human Cells

One way to test the possible effects of chemicals on humans is with cells grown in culture. We are currently in the process of completing a series of experiments with three human cell lines, a gingival fibroblast cell line, the Chang liver cell line, and a mammary gland cell line. Each of the cell lines was obtained from the American Type Culture Collection in Manassas, VA. These cell lines were chosen because they are normal cells and they were derived from tissues in which limonoids have already demonstrated anticancer activity.

The procedure with each of the cell lines was the same. The cells were plated as monolayer cultures in complete media in 60 mm petri dishes. The cultures were grown in 5% CO_2/95% air at 37°C with media changes every 2-3 days. At mid log phase, 12 dishes were selected at random and separated into 4 equal groups. The media over the cells were replaced at this time. Complete media were added to the three dishes in group 1, the control group. The media added to the cells in groups 2, 3, and 4 contained 20 (group 2), 200 (group 3), or 2,000 µg/ml of limonin 17-β-D-glucopyranoside (group 4). At the same time, 12 control dishes were prepared. These dishes, which did not contain cells, were separated into four equal groups. Complete media were placed in the three dishes labeled group 1a. The media added to the dishes labeled groups 2a, 3a, and 4a contained 20 (group 2a), 200 (group 3a), and 2,000 µg/ml of limonin 17-β-D-glucopyranoside (group 4a).

After 48 hrs., the media were removed. The cells (groups 1-4) still attached to the petri dishes were washed three times with phosphate buffered saline (5 ml per wash). A 0.25% solution of trypsin (3 ml/dish) was then used to release the cells from the dishes. The cell suspension was centrifuged. The pelleted cells were resuspended in 0.5 ml of 0.4 N perchloric acid. Following a second centrifugation, the resulting pellets were analyzed for DNA content (49).

All of the solutions including the media (groups 1-4 and 1a-4a), the three washes with phosphate buffered saline, the trypsin solutions, and the 0.5 ml solutions of 0.4 N perchloric acid were analyzed for limonin 17-β-D-glucopyranoside content using the basic HPLC techniques developed in Dr. Hasegawa's laboratory (34-37). Ultra spin cellulose centrifuge filters (Alltech Associates, Deerfield, IL) were used to remove high molecular weight contaminants (MW > 10 kD) from the samples. Following the one-hour centrifugation, 25 µl samples were loaded onto a C-18 reversed-phase Spherisorb ODS-2, 5 µm (250 x 4.6 mm) column

(Alltech Associates, Deerfield, IL). The column was eluted with a linear gradient starting with 15% CH₃CN in 3 mM H₃PO₄ and ending with 40% CH₃CN in 3 mM H₃PO₄ over 60 min. The flow rate was 1 ml/min. The limonin 17-β-D-glucopyranoside was detected by UV absorption at 210 nm and quantified by both peak height and peak area as determined by standard runs. The retention time for limonin 17-β-D-glucopyranoside was 25.2 min.

The basic data from the first two cell lines, the gingival fibroblast cell line and the Chang liver cell line have been collected and analyzed. The data for these two cell lines were similar. The samples from the mammary gland cell line have not yet been collected. From the initial data we make the following tentative conclusions.

- The limonin 17-β-D-glucopyranoside was stable in the complete growth media (groups 2a-4a) and the media over the cells (groups 2-4). Very little, if any, breakdown was detected.
- The three washes with phosphate buffered saline completely removed all of the residual limonin 17-β-D-glucopyranoside (groups 2-4).
- Limonin 17-β-D-glucopyranoside was not detected in the trypsin solutions collected from above the pelleted cells.
- Small amounts of limonin 17-β-D-glucopyranoside appeared to have been taken up by the cells.
- Microscopic observations before the cells were harvested and the data from the DNA determinations showed that the two-day exposure to limonin 17-β-D-glucopyranoside had no effect on the growth or general morphology of the cells

Additional experiments will be needed to determine more precisely how much of the limonin 17-β-D-glucopyranoside was taken up by the cells. In addition, the samples will also need to be analyzed for metabolites of limonin 17-β-D-glucopyranoside. One possible metabolite is the aglycone, limonin. Nevertheless, the initial results from these two experiments, one with mixed limonin glucosides and the other with limonin 17-β-D-glucopyranoside, suggest that these citrus limonoids are not toxic in mammalian systems.

Conclusions

The fact that the technology is now in place to create functional foods, designer foods, and nutraceuticals containing elevated concentrations of citrus limonoids has led to a considerable amount of commercial interest in these chemicals. This interest is heightened by the fact that these chemicals have consistently shown cancer chemopreventive activity in a variety of different tumor models. Unfortunately, what is lacking is detailed information on how these active agents are handled in mammalian systems. The preliminary studies described in this paper provide some basic information on stability and safety; however, the results on uptake by cultured cells indicate that the limonoid glucosides might not be efficiently absorbed in the intestinal tract. Comparative studies on the absorption

rates of the limonoid glucosides and the aglycones are needed. Further research is also needed on the distribution and metabolism of these chemicals in the body. This research will help to pinpoint the citrus limonoid or limonoids that need to be tested clinically.

As indicated earlier, the problem with "delayed bitterness" led after a considerable amount of time and effort to a complete description of the movement and metabolism of citrus limonoids in citrus plants. A similar effort is now needed for mammalian systems.

Acknowledgments

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Citrus Limonoids Obacunone and Limonin Inhibit the Development of a Precursor Lesion, Aberrant Crypt Foci, for Colon Cancer in Rats

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The typical bitter limonoids obacunone and limonin occurred widely in citrus have been reported to shorten the sleeping time in mice and limonin has been shown to inhibit forestomach and buccal pouch carcinogenesis in rodents. Their ability to prevent azoxymethane (AOM)-induced aberrant crypt foci (ACF) and to induce detoxification (phase II) enzymes, such as glutathione S-transferase (GST) and quinone reductase (QR) was investigated using male F344 rats. Obacunone or limonin was administered continuously in the diet starting one week prior to the first of two weekly 20 mg/kg s.c. injections of AOM and until the rats were killed four weeks later ("initiation" feeding). In addition, rats were fed the diet containing each chemical for four weeks, starting two weeks after the second AOM exposure ("postinitiation" feeding). At a concentration of 0.02% or 0.05% of obacunone and limonin diets caused significant reduction (55-65% by "initiation" feeding and 28-42% by "postinitiation" feeding) in the yield of ACF. The ability of both compounds when administered during "initiation" or "postinitiation" stage to reduce the proliferating cell nuclear antigen (PCNA)-labeling index in the crypts and to increase GST and QR activities in the liver and colonic mucosa correlated well with the prevention of ACF. These results indicate that the citrus limonoids obacunone and limonin are possible chemopreventive agents against colon carcinogenesis.

Colon cancer is the third most malignant neoplasm in the world (*1*) and the second leading cause of cancer deaths in the USA. In Japan, colon cancer incidence has been increasing, being the third leading cause of cancer deaths. It is well known that dietary factors can modulate the development of certain types of human cancer, including colon cancer (*2*). Also, epidemiological data suggest that ingestion of some constituents from vegetables and fruits may contribute to reduction of cancer incidence in humans (*3*). Since an inverse relationship between the intake of fruits/vegetables and human colon cancer has been suggested, primary prevention, including chemoprevention utilizing the active compounds in edible plants is important for reducing this malignancy.

Among possible chemopreventers for cancer development, certain inducers of phase II detoxifying enzymes including glutathione *S*-transferase (GST) and quinone reductase (QR) are considered to be promising chemopreventive agents against cancer (*4*). Our search for effective cancer chemopreventive compounds in edible plants revealed that several compounds able to induce phase II drug-metabolizing enzymes exert inhibitory effects on chemically induced colon carcinogenesis (*5-7*).

Limonoids are a group of triterpene derivatives present in the Rutaceae and Meliaceae families. Limonoids including obacunone (Figure 1) and limonin (Figure 1) are also found in citrus seeds (*8*), commercial citrus juices (*9*), and *Phllodendron amurense* (Kihada) (*10*). For example, commercial orange juices contain an average of 320 ppm of total limonoid glucosides: the major glucoside in citrus juices is limonin 17-O-β-D-glucopyranoside, being over 50% of the total limonoid glucosides in the juices, followed by other limonoid glucosides including obacunone (*11*). Limonoids including obacunone and limonin are considered to be responsible for delayed bitterness in citrus juices and processed products. Among 38 limonoid aglycones, 23 neutral and 15 acidic, reported to occur in citrus and its hybrids (*11*), obacunone and limonin could enhance glutathione *S*-transferase (GST) activity in various organs of mice (*12*), including liver (*12-13*). Limonin was reported to enhance small intestinal GST activity (*14*). Obacunone and limonin have been shown to shorten the sleeping time induced by α-chloralose and urethane (*15*) or anesthetics (*16*). Limited data are available on the cancer preventive properties of limonin in rodent models. Limonin inhibits 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced hamster buccal pouch carcinogenesis (*17*). Also, limonin 17-β-D-glucopyranoside could inhibit the development of DMBA-induced buccal pouch tumors in hamsters (*18*). To our knowledge, there are no studies indicating that the dietary administration of limonin and obacunone has been tested in a colon cancer model or in any other carcinogenesis model other than forestomach, buccal pouch, lung, and skin (*19*).

Early preneoplastic lesions, aberrant crypt foci (ACF), for colon carcinoma have consistently been observed in the colon of rats exposed to colon carcinogens (*20*) and are present in the colonic mucosa of patients with colon cancer (*21*). Thus, ACF are putative precursor lesions from which adenoma and adenocarcinoma may develop (*22*). There is evidence that several inhibitors in laboratory animals suggesting that ACF induction can be used to evaluate novel agents for their potential chemopreventive properties against colon carcinoma (*23*).
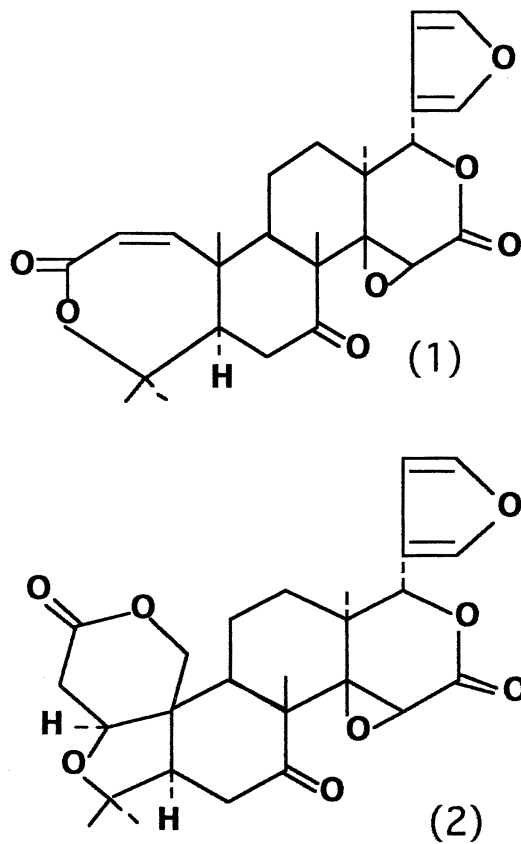
Figure 1. *Chemical structures of obacunone (1) and limonim (2).*

The present study was designed to evaluate the inhibitory activities of obacunone and limonin on azoxymethan (AOM)-induced ACF formation in the rat colon. The major goal of this study was to determine whether these natural agents are conceivably effective chemopreventive agents in pre-clinical efficacy studies and, eventually in human clinical trials.

Materials and Methods

Animals, Diets, Carcinogen and Test Compounds. AOM was obtained from Sigma Chemicals (St. Louis, MO). Obacunone (99.9% pure) and limonin (99.9% pure) were isolated from the barks of *Phllodendron amurense* (Kihada). Male F344 rats aged 4 weeks were purchased from Japan SLC Inc. (Hamamatsu, Japan). The rats were held in quarantine for 1 week and had access to powdered basal diet, CE-2 (CLEA Japan, Inc. Tokyo, Japan). They were randomly distributed by wt into various dietary groups and were transferred to an animal holding room where they were housed in plastic cages, three rats/cage, under controlled conditions of a 12-h light/12-h dark cycle, 50% relative humidity and 21°C room temperature. Experimental diets were prepared by mixing obacunone (0.02% and 0.05% w/w) or limonin (0.02% and 0.05% w/w) with a basal diet, CE-2 and the test compounds in diets were quite stable.

Experimental Procedure. At 5 weeks of age, groups of rats were fed the basal diet, CE-2 or experimental diets containing obacunone (0.02% or 0.05%) and limonin (0.02% or 0.05%), as shown in Figure 2. At 6 weeks of age, all animals except those given experimental diets alone and the untreated rats received AOM s.c. once weekly for 2 weeks at a dose of 20 mg/kg body wt per week. Animals intended for vehicle treatment were given an equal volume of normal saline. The animals given AOM and fed the experimental diet for 4 weeks after the start were sacrificed at week 4. The rats given AOM and basal diet were fed the experimental diets for 4 weeks, starting week 4 and sacrificed at week 8 when they were 13 weeks of age. The other groups consisted of rats given the experimental diets alone or untreated rats. At each sacrifice point (week 4 and 8), animals were killed by CO_2 euthanasia and their colons were removed, flushed with normal saline, opened from cecum to anus, and fixed flat between two pieces of filter paper in 10% buffered formalin. After staining with 0.2% methylene blue for 30 sec, ACF were observed through a light microscope, counted and recorded (6).

PCNA-labeling Index. Formalin-fixed colonic tissue in the distal 2 cm from anus was cut out from each colon and embedded in paraffin. Serial cross-sections of 3 mm each were cut parallel to the mucosal surface and mounted onto gelatin-coated glass slides. The paraffin was removed with xylene and the tissue sections placed in 2N HCl at 37°C for 30 min. Endogenous peroxidase was quenched by placing the tissue sections in 3% H_2O_2 (Sigma Chemical Co.) for 30 min. Anti-PCNA
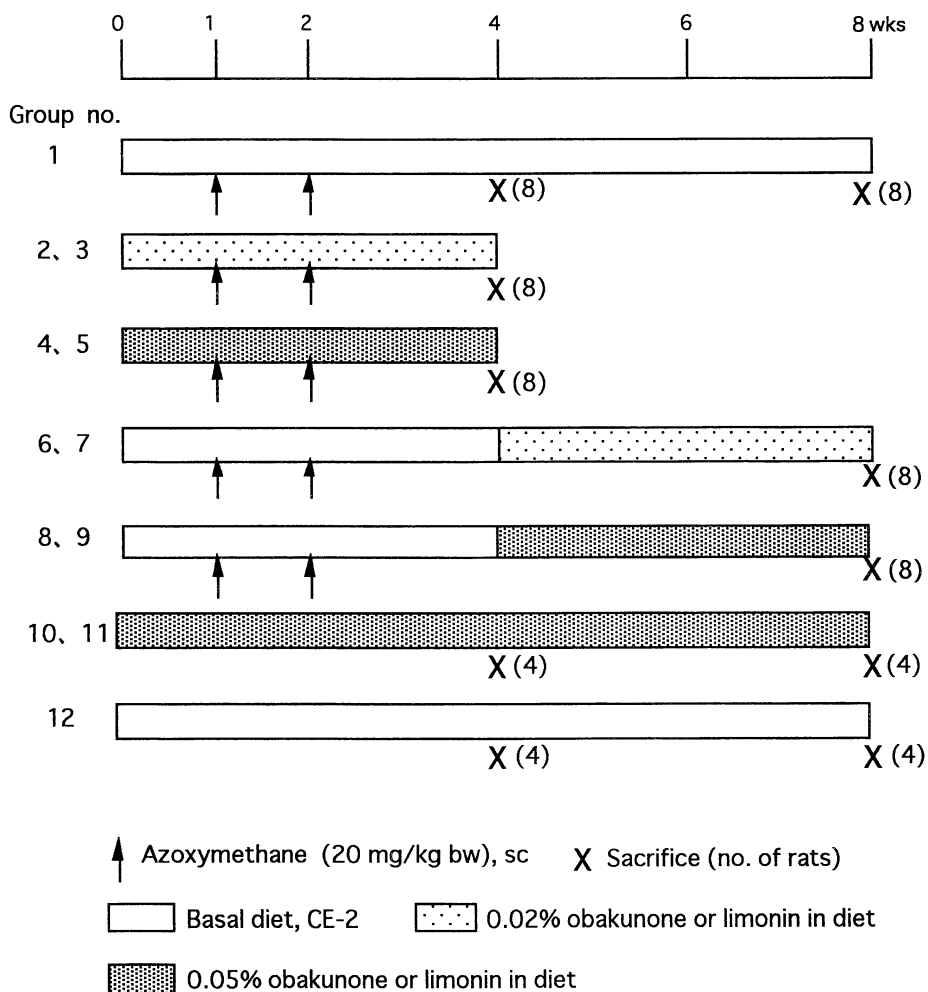
Figure 2. *Experimental protocol.*

antibody (Dako Co., Kyoto, Japan) was used with the avidin-biotin complex method. The immunohistochemical staining was performed according to the method described in our previous study (24). The number of PCNA-labeled and unlabeled cryptal cells in the cross-sections of the crypts in an ACF and of "normal-appearing" crypts were recorded from the mouth to the base of crypts. The total number of cells in each crypt was calculated by adding the number of labeled and unlabeled cells. The PCNA-labeling index was determined by dividing the number of PCNA-positive cells by the total number of cells in a crypt×100.

GST and QR Activities in Liver and Colon. At sacrifice, livers and colonic mucosa removed at each sacrifice time point were used for measuring GST and QR activities according to a standard procedure (6).

Statistics. All results were expressed as the means ± SD and were analyzed by Student's *t*-test or Welch's *t*-test. Differences were considered statistically significant at p<0.05.

Results

General Observation. At week 4 and week 8, the body weights of rats given AOM and the experimental diets except those fed 0.02% limonin containing diet at week 8 were slightly lower than rats treated with AOM alone, possibly due to lowered food intake (data not shown). However, administration of the experimental diets alone did not produce any gross changes in the liver, kidney, intestine and lungs.

Aberrant crypts. Rats receiving the control or experimental diets alone showed no evidence of ACF formation in the colonic mucosa. AOM alone treatment induced, on the average, 143 ACF/colon and 26 foci containing multiple (four or more) aberrant crypts/focus at week 4 (Tables I and II), and 149 AFC/colon and 53 foci containing multiple (four or more) aberrant crypts/focus at week 8 (Tables III and IV). As expected, ACF were predominantly observed in the distal colon. Efficacy endpoints used in the current study were inhibition of total occurrences of ACF as well as reduction of number of multicrypts (four or more) of aberrant crypts. Administration of obacunone together with AOM significantly inhibited total occurrence of ACF/colon (65%, p<0.001, Table I) and of multicrypt clusters containing four or more crypts/focus (65-73%, p<0.05, Table II). Limonin administration during AOM exposure also significantly suppressed the total number of ACF/colon (55-56%, p<0.001, Table I) and the number of multicrypts (four or more) of aberrant crypts (71-77%, p<0.02 to 0.001, Table II). Similarly, dietary obacunone treatment after AOM exposure significantly inhibited total number of ACF/colon (39-42%, p<0.005 to 0.002, Table III) and of multicrypt clusters containing four or more crypts/focus (61-68%, p<0.001, Table IV). Also, limonin administration after AOM treatment significantly reduced the total number of

Table I. Effecs of Dietary Obacunone and Limonin during AOM Exposure on ACF Formation at Week 4

Group no.	Treatment (no. of rats examined)	Total no. of ACF/colon (incidence)	No. of ACF/cm^2	Total no. of aberrant crypts/colon	No. of aberrant crypts/focus
1	AOM alone (8)	143 ± 14[a] (8/8)	9.36 ± 1.64	335 ± 22	2.36 ± 0.17
2	AOM + 0.02% obacunone (8)	50 ± 8 [b] (8/8)	4.58 ± 0.63 [b]	107 ± 17 [b]	2.16 ± 0.05 c
3	AOM + 0.05% obacunone (8)	50 ± 11 [b] (8/8)	3.35 ± 1.43 [b]	109 ± 22 [b]	2.17 ± 0.09 [c]
4	AOM + 0.02% limonin (8)	65 ± 14 [b] (8/8)	6.44 ± 1.36 [d]	128 ± 27 [b]	2.01 ± 0.06 [b]
5	AOM + 0.05% limonin (8)	63 ± 6 [b] (8/8)	5.08 ± 0.47 [b]	123 ± 16 [b]	1.97 ± 0.18 [b]
10	0.05% obacunone (4)	0	0	0	0
11	0.05% limonin (4)	0	0	0	0
12	No treatment (4)	0	0	0	0

[a] Mean ± SD.

[b,c] Significantly different from group 1 by Student's t-test or Welch's t-test ([b]$p<0.001$, [c]$p<0.02$, and [d]$p<0.002$).

Table II. Effect of Dietary Obacunone and Limonin on Size of ACF Induced by AOM at Week 4

Group no.	Treatment (no. of rats examined)	% of ACF containing:			
		1 crypt	2 crypts	3 crypts	4 or more crypts
1	AOM alone	45.4 ± 4.9 [a]	20.9 ± 3.2	14.0 ± 1.9	18.4 ± 3.0
2	AOM + 0.02% obacunone	41.9 ± 3.2	26.5 ± 2.3 [b]	16.9 ± 1.5 [c]	14.7 ± 2.8 [d]
3	AOM + 0.05% obacunone	45.7 ± 6.0	21.8 ± 8.7	14.6 ± 3.1	17.9 ± 2.8
4	AOM + 0.02% limonin	49.9 ± 3.5 [d]	23.5 ± 7.0	15.8 ± 2.3	11.5 ± 4.8 [b]
5	AOM + 0.05% limonin	47.9 ± 6.1	25.1 ± 6.0	16.9 ± 2.4 [e]	10.1 ± 4.1 [f]

[a] Mean ± SD.

[b-f] Significantly different from group 1 by Student's t-test ([b]$p<0.002$, [c]$p<0.005$, [d]$p<0.05$, [e]$p<0.02$, and [f]$p<0.001$).

Table III. Effects of Dietary Obacunone and Limonin after AOM exposure on the Development of ACF at Week 8

Group no.	Treatment (no. of rats examined)	Total no. of ACF/colon (incidence)	No. of ACF/cm²	Total no. of aberrant crypts/colon	No. of aberrant crypts/focus
1	AOM alone (8)	149 ± 36 a (8/8)	11.76 ± 2.75	436 ± 70	3.27 ± 0.13
6	AOM → 0.02% obacunone (8)	91 ± 10 b (8/8)	8.75 ± 1.05 c	219 ± 38 d	2.41 ± 0.20 d
7	AOM → 0.05% obacunone (8)	87 ± 13 e (8/8)	7.23 ± 1.04 e	220 ± 35 d	2.52 ± 0.04 d
8	AOM → 0.02% limonin (8)	107 ± 33 f (8/8)	8.49 ± 2.15 c	216 ± 71 d	2.30 ± 0.11 d
9	AOM → 0.05% limonin (8)	102 ± 27 c (8/8)	7.82 ± 1.29 b	209 ± 55 d	2.25 ± 0.23 d
10	0.05% obacunone (4)	0	0	0	0
11	0.05% limonin (4)	0	0	0	0
12	No treatment (4)	0	0	0	0

a Mean ± SD.

b-f Significantly different from group 1 by Student's t-test or Welch's t-test (b$p<0.005$, c$p<0.02$, d$p<0.001$, e$p<0.002$, and f$p<0.05$).

Table IV. Effect of Obacunone and Limonin on Size of ACF Induced by AOM at Week 8

Group no.	Treatment (no. of rats examined)	% of ACF containing:			
		1 crypt	2 crypts	3 crypts	4 or more crypts
1	AOM alone	20.8 ± 1.4 [a]	23.0 ± 2.7	20.4 ± 2.1	35.8 ± 3.2
6	AOM → 0.02% obacunone	29.7 ± 4.5 [b]	29.5 ± 3.4 [b]	22.4 ± 5.3	18.4 ± 5.4 [b]
7	AOM → 0.05% obacunone	25.5 ± 4.5 [c]	29.2 ± 5.1 [d]	21.4 ± 2.9	23.8 ± 4.2 [b]
8	AOM → 0.02% limonin	29.6 ± 5.5 [e]	30.9 ± 3.1 [b]	20.7 ± 2.9	18.8 ± 4.8 [b]
9	AOM → 0.05% limonin	30.8 ± 7.1 [e]	36.6 ± 1.8 [b]	18.6 ± 3.4	13.9 ± 6.5 [b]

[a] Mean ± SD.

[b-e] Significantly different from group 1 by Student's t-test or Welch's t-test ([b] $p < 0.001$, [c] $p < 0.05$, [d] $p < 0.01$, and [e] $p < 0.005$).

ACF/colon (28-32%, p<0.05 to 0.02, Table III) and the number of multicrypts (four or more) of aberrant crypts (62-74%, p<0.001, Table IV).

PCNA-labeling Index in ACF and "Normal-Appearing" Colonic Crypts. The ACF induced by AOM had 1.25- to 1.97-fold PCNA-labeled nuclei when compared with "normal-appearing" crypts (Figure 3). The PCNA-labeling indices in ACF at week 4 and 8 were significantly decreased by feeding of test compounds, obacunone and limonin (p<0.05 to 0.001, Figure 3). Test compounds did not affect the PCNA-labeling index in normal crypts of rats. Also, test chemicals reduced the size of the proliferative compartment in "normal-appearing" crypts (data not shown).

GST and QR Activities in Liver and Colonic Mucosa. Obacunone feeding (0.02% and 0.05%) together with AOM exposure significantly elevated liver GST and QR activities elevated as compared with AOM alone group at week 4 (p<0.005 to 0.001, Figure 4). Similarly limonin feeding (0.02% and 0.05%) significantly increased liver QR activity elevated when compared with AOM alone group (p<0.001, Figure 4). At week 8, dietary obacunone after AOM administration significantly increased liver GST and QR activities elevated as compared with AOM alone group (p<0.05 to 0.001, Figure 5). Dietary feeding of limonin at a dose of 0.05% significantly elevated liver GST and QR activities elevated as compared with AOM alone group (p<0.01 to 0.001, Figure 5). GST and QR activities in the colonic mucosa of rats given test compounds together with AOM exposure (week 4, Figure 6) or after AOM treatment (week 8, Figure 7) were significantly greater than those of rats given AOM alone (p<0.05 to 0.001).

Discussion

The present study was undertaken to evaluate the chemopreventive ability of dietary obacunone and limonin against ACF formation in rat colon. Since ACF are putative preneoplastic lesions for colon carcinoma and multiplicity of four or more aberrant crypts/focus has been fairly consistent predictor of colon tumor outcome (22,25), we used this criterion to evaluate these compounds for their potential chemopreventive properties. Dietary obacunone and limonin, either during or after the carcinogen exposure, clearly inhibit experimentally induced-ACF in rats. This is the first study to demonstrate that dietary administration of obacunone suppress a model of chemically-induced carcinogenesis. Topically applied limonin or limonin 17-β-D-glucopyranoside has previously been shown to inhibit DMBA-induced hamster buccal pouch tumorigenesis (17,18). In these studies, a bitter citrus limonoid nomilin and its glucosides nomilin 17-β-D-glucopyranoside and nomilinic acid 17-β-D-glucopyranoside were ineffective (17,18). In B[a]P-induced mouse forestomach tumorigenesis, gastric intubation of nomilin, which is the more active liver GST inducer, could inhibit the development of forestomach tumors, but limonin, which is a weak liver GST inducer, did not (14). Limonin and nomilin are effective in
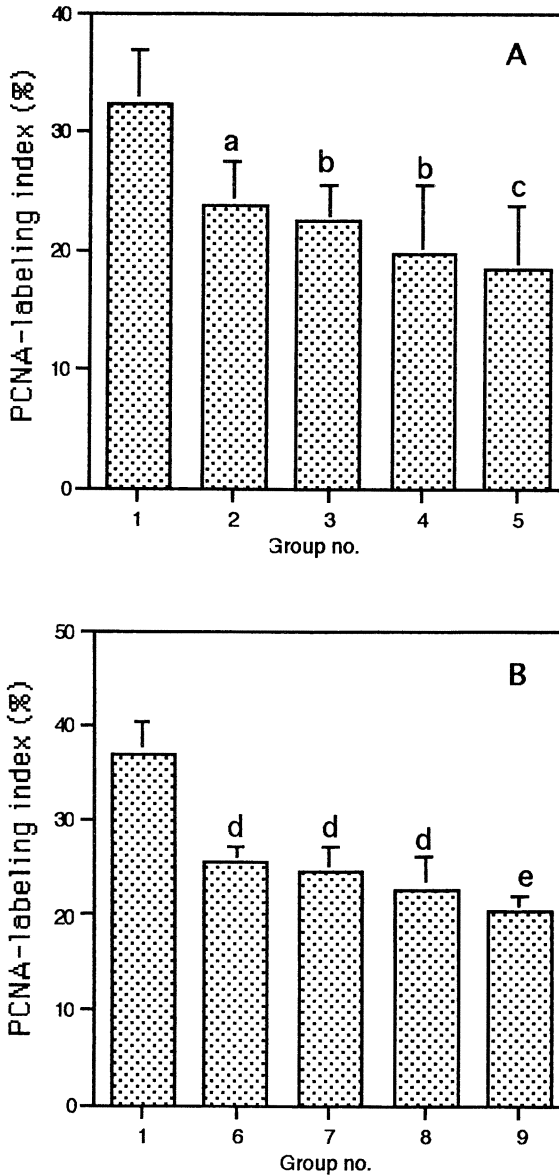
Figure 3. PCNA-labeling index in ACF at week 4 (A) and week 8 (B). Group 1, AOM alone; Group 2, AOM + 0.02% obacunone; Group 3, AOM + 0.05% obacunone; Group 4, AOM + 0.02% limonin; Group 5, AOM + 0.05% limonin; Group 6, AOM → 0.02% obacunone; Group 7, AOM → 0.05% obacunone; Group 8, AOM → 0.02% Limonin; and Group 9, AOM → 0.05% limonin, a, $p<0.05$; b, $p<0.02$; c, $p<0.01$; d, $p<0.005$ and e, $p<0.001$.
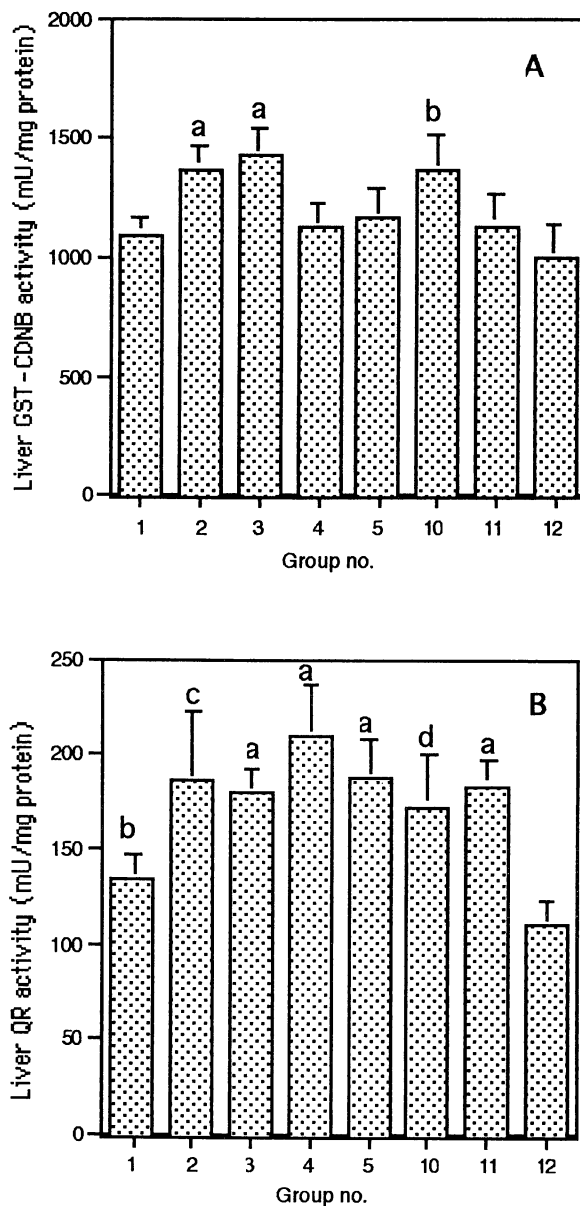
Figure 4. *Liver GST (A) and QR (B) activities at week 4, Group 1, AOM alone; Group 2, AOM + 0.02% obacunone; Group 3, AOM + 0.05% obacunone; Group 4, AOM + 0.02% limonin; Group 5, AOM + 0.05% limonin; Group 10, 0.05% obacunone; Group 11, 0.05% limonin; and Group 12, untreated, a, p<0.001; b, p<0.02; c, p<0.005; and d, p<0.01.*
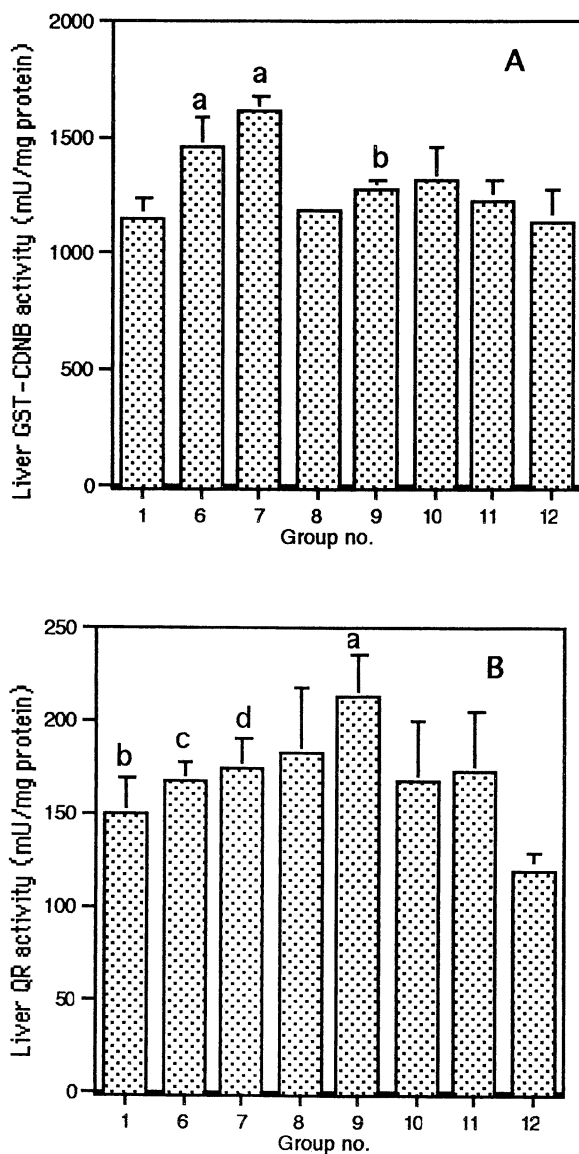
Figure 5. *Liver GST (A) and QR (B) activities at week 8. Group 1, AOM alone; Group 6, AOM → 0.02% obacunone; Group 7, AOM → 0.05% obacunone; Group 8, AOM → 0.02% limonin; Group 9, AOM → 0.05% limonin; Group 10, 0.05% obacunone; Group 11, 0.05% limonin; and Group 12, untreated, a, p<0.001; b, p<0.01; c, p<0.05; and d, p<0.02.*
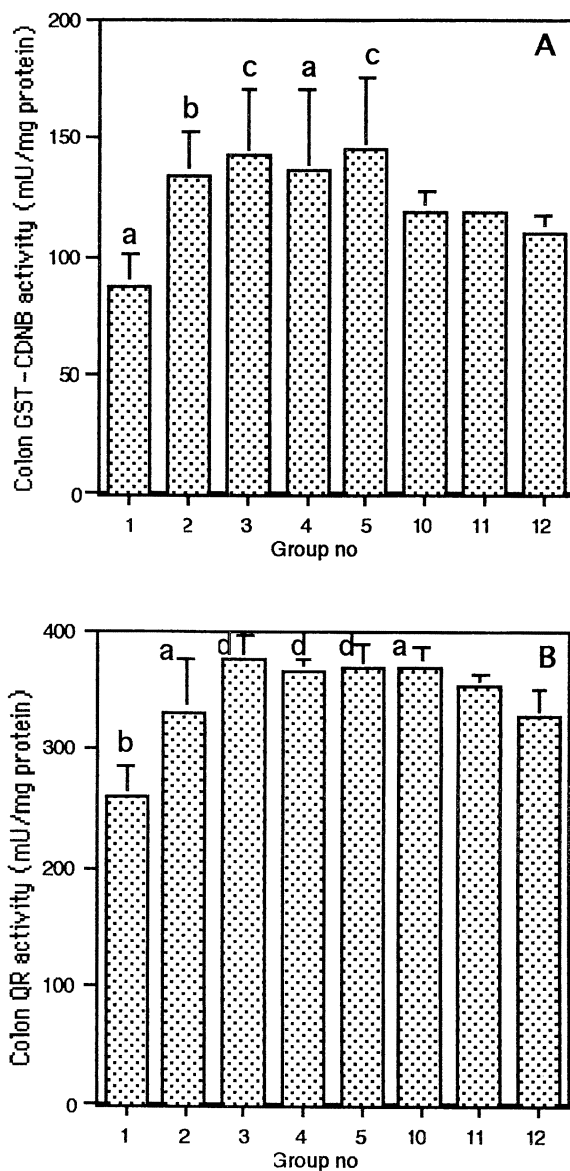
Figure 6. *Colonic mucosal GST (A) and QR (B) activities at week 4. Group 1, AOM alone; Group 2, AOM + 0.02% obacunone; Group 3, AOM + 0.05% obacunone; Group 4, AOM + 0.02% limonin; Group 5, AOM + 0.05% limonin; Group 10, 0.05% obacunone; Group 11, 0.05% limonin; and Group 12, untreated, a, p<0.05; b, p<0.01; c, p<0.02; and d, p<0.001.*
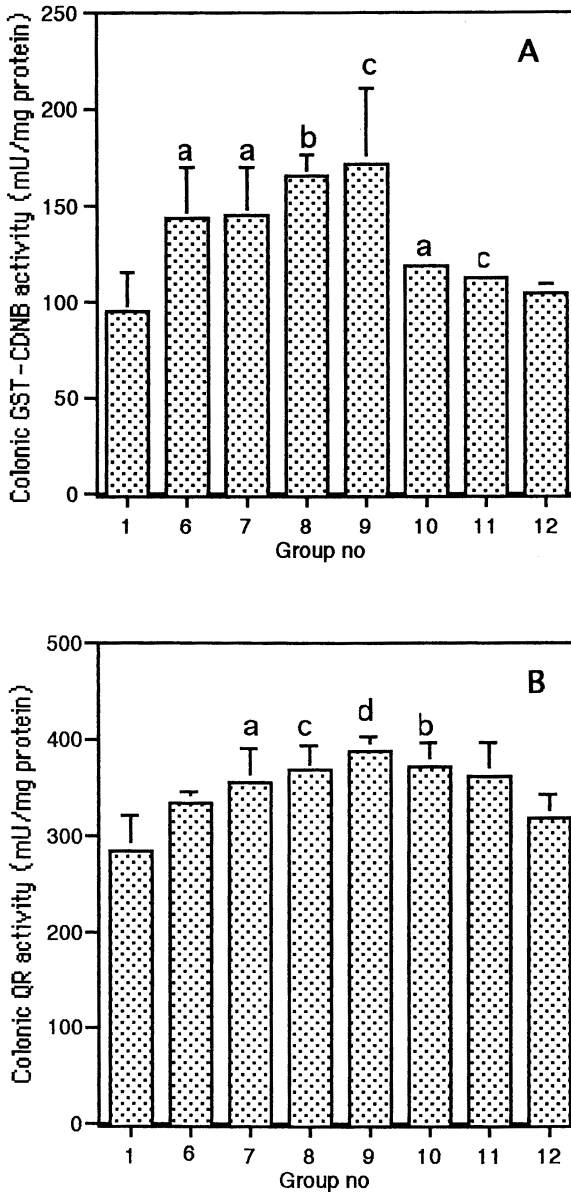
Figure 7. *Colonic mucosal GST (A) and QR (B) activities at week 8. Group 1, AOM alone; Group 6, AOM → 0.02% obacunone; Group 7, AOM → 0.05% obacunone; Group 8, AOM → 0.02% limonin; Group 9, AOM → 0.05% limonin; Group 10, 0.05% obacunone; Group 11, 0.05% limonin; and Group 12, untreated. a, p<0.001; b, p<0.01; c, p<0.02; and d, p<0.05.*

inhibiting chemical carcinogenesis in other organs, such as skin and lung (*19*). One of the advantages of obacunone and limonin is that, unlike synthetic chemopreventive agents, they are naturally occurring compounds that are produced endogenously in edible plants and thus present in human foods (*11*).

The exact mechanism(s) involved in the suppressing effects of AOM-induced colon tumorigenesis by dietary obacunone and limonin is not fully known. In the present study, dietary feeding of both compounds elevated liver GST and QR activities, although the capability was greater in obacunone than in limonin. In other studies, limonin was shown to elevated GST activities in small intestine (*12, 14*). Also, obacunone was reported to induce GST activity in liver, small intestine, forestomach, lung, and colon (*12*). It is possible that dietary obacunone and limonin induce an increase in the activity of detoxifying enzymes GST and QR in liver and/or colonic mucosa. In addition, the modulatory effects of both compounds on liver cytochrome P450 2E1 (*26*) and/or methylazoxymethanol dehydrogenases (*27*), which are pertinent to the AOM metabolism, may also contribute to the inhibition of ACF. In recent study using a long-term animal model of colon carcinogenesis, dietary obacunone inhibited the occurrence of colonic adenocarcinoma by 65-82% and feeding of limonin by 82-92%. Thus, these compounds might appear to be blocking agents (*28*).

Conclusion

This study demonstrates that dietary administration of obacunone and limonin inhibit the formation of preneoplastic lesions in the colon, with induction of liver GST and QR. The results in a recent study using a long-term animal model of colon carcinogenesis have confirmed the findings in the present experiment. Further experiments, including pre-clinical efficacy and mechanistic studies are warranted to fully evaluate these natural compounds for their cancer preventive properties and to understand their mode of action.

Acknowledgments

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Chapter 12

Inhibition of Human Breast Cancer Cells by Citrus Limonoids

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Citrus limonoids are a class of chemically related compounds present in lemon, lime, orange and grapefruit. We have shown that nomilin and a limonoid glucoside mixture are potent inhibitors of prolifertation of estrogen receptor-negative (ER-) and –positive (ER+) human breast cancer cells in culture. In the present experiments, we tested deacetylnomilin, deoxylimonin, ichangin, isoobacunoic acid, limonol, limonin carboxymethoxime, methyl deoxylimonate, methylnomilinate methyl deacetylnomilinate, methyl isolimonate, nomilin glucoside, nomilinic acid glucoside, 7a-obacunol, obacunone and obacunone glucoside for their ability to inhibit the proliferation of MDA-MB-435 ER- and MCF-7 ER+ human breast cancer cells. In ER- cells, limonin methoxime and deacetylnomilin were the most effective inhibitors having IC50s of 0.02 and 0.07 ug/mL respectively. In ER+ cells, deacetylnomilin, obacunone and methyl nomilinate were the most effective inhibitors of proliferation having IC50s of 0.005, 0.009 and 0.01 ug/mL respectively. Maximum tolerated dose studies were conducted in nude mice for limonin and the glucoside mixture. We found that this dose was 2% of the diet for limonin and 4% of the diet for the glucoside mixture. These results suggest that citrus limonoids have important anti-cancer activity.

Breast cancer is the second most common cause of cancer related deaths in North American women (1). Modern preventative and treatment methods are limited and the investigation of natural, low toxicity dietary components for their anti-cancer properties is of great interest. There is general

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agreement that plant-based diets rich in whole grains legumes, fruits and vegetables, reduce the risk of various types of cancer, including breast cancer, and a variety of compounds produced by plants have been investigated for their anti-cancer activity (2-7). Our results have shown that citrus flavonoids inhibit the proliferation of ER- and ER+ human breast cancer cells *in vitro* (8). In addition to the *in vitro* studies, we have previously reported that giving orange juice or naringin (the glycoside form of the flavonoid, naringenin, in grapefruit) to rats delayed the development of mammary tumors induced by 7,12-dimethylbenz(a)anthracene (DMBA) (8). In a more recent experiment, MDA-MB-435 ER- human breast cancer cells were injected into the mammary fat pads of nude mice. Giving the animals orange juice or grapefruit juice instead of water was found to reduce the incidence of tumors at the site of injection by more than 50% and to inhibit markedly metastases to the lymph nodes and lungs (9). The constituent flavonoids from orange or grapefruit juice appeared to be less effective inhibitors of cancer development and metastases in this experiment. Both orange and grapefruit juice contain other bioactive components (Table 1).

**Table 1. Average Amounts of Bioactive Compounds
in Orange Juice and Grapefruit Juice (mg/L).**

Component	Orange Juice	Grapefruit Juice
Hesperidin	205	85
Naringin	18	246
Methoxylated flavones	6	<5
Limonene	330	330
Limonoid glucosides	366	198
Limonin glucoside	209	137
Limonin	3	10
Total carotenoids	18	59
Hydroxycinnamic acids	80	89
Ascorbic Acid (Vit. C)	284	250

These include the limonoids, which are one of the two bitter principles found in citrus fruits, including oranges, grapefruits, lemons and limes (10-12). They are also present as glucose derivatives in mature fruit tissues and seeds, and are one of the major secondary metabolites present in citrus. Citrus limonoids were observed to inhibit the proliferation of human breast cancer cells more effectively than the flavonoids (9) and may be largely responsible for the anti-cancer effects of the juices.

Our interest in limonoids began with the observation that orange and grapefruit juice inhibited the growth and metastases of human breast cancer

cells injected into the mammary fat pad of nude mice and that this inhibition was not completely due to their constituent flavonoids (9). Limonoids have been shown to have anti-cancer activity (13-16). Nomilin reduced the incidence of and number of chemically-induced forestomach tumors in mice when given by gavage (14). Addition of nomilin and limonin to the diet inhibited lung tumor formation in mice and topical application of the limonoids was found to inhibit both the initiation and promotion phases of carcinogenesis in the skin of mice (15).

In the present experiments, we investigated a number of naturally-occurring and synthetic limonoids for their ability to inhibit the proliferation of MDA-MB-435 ER- and MCF-7 ER+ human breast cancer cells in culture. We have also conducted studies in animals to determine the maximum tolerated dose for limonin and the limonoid glucoside mixture.

Citrus Limonoids against Human Breast Cancer Cells in Culture

Cell Culture

MDA-MB-435 estrogen receptor-negative human breast cancer cells were maintained at 37°C in minimum essential medium (alpha modification) containing 3.7 g of sodium bicarbonate per litre, supplemented with 10% v/v fetal calf serum and 1% v/v fungizone (antibiotic/antimycotic, 10 000 units/mL penicillin G sodium, 10 000 µg/mL streptomycin sulphate and 25 µg/mL amphotericin B in 0.85% saline), in a humidified atmosphere of 5% carbon dioxide. Stock cultures were seeded at a density of 2×10^5 cells and allowed to multiply for 48-72 hours.

MCF-7 estrogen receptor-positive human breast cancer cells were maintained in minimum essential medium (alpha modification) containing 3.7 g of sodium bicarbonate supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 10 µg/mL insulin and 1% v/v fungizone (antibiotic/antimycotic, 10 000 units/mL penicillin G sodium, 10 000 µg/mL streptomycin sulphate and 25 µg/mL amphotericin B in 0.85% saline). Cells were grown to confluence at 37°C in a humidified atmosphere containing 5% carbon dioxide and were passaged weekly using 0.25% trypsin.

Experiments on cell proliferation

The effects of each of the limonoids on the proliferation of MDA-MB-435 ER- and MCF-7 ER+ human breast cancer cells were examined by determining the incorporation of [³H] thymidine into growing cells. MDA-MB-435 cells were plated at a density of 2×10^4 cells/well in 96-well, flat-bottomed tissue culture plates in a total volume of 200 µL of medium and incubated at 37°C, with or without the test compounds. The plates were incubated for 48 hours at 37°C and [³H] thymidine was then added to determine the number of dividing cells at each concentration. The cells were reincubated for 4 hours, after which the medium and excess radiolabel were removed. The cells were trypsinized and harvested onto a glass fiber filter paper, and the radioactivity was counted. The percentage of dividing cells

was determined by comparing the number of disintegrations per minute of the treated cells (average of 3 wells/concentration) with that obtained for the control cells. The concentrations at which 50% growth inhibition occured was determined (IC_{50}) for each compound (17).

We initially tested the effects of limonin, nomilin, limonin glucoside, and a glucoside mixture (limonin-30%, nomilinic acid-33.8%, nomilin-12.7%, obacunone-8.2%, deacetylnomilin-6.6%, deacetylnomilinic acid-8.7%) from orange molasses on the proliferation of MDA-MB-435 ER- and MCF-7 ER+ cells. The IC_{50} values for each limonoid are given in Tables 2 and 3.

Table 2: Effect of Limonoids on the Proliferation and Viability of MDA-MB-435 Estrogen Receptor-Negative Human Breast Cancer Cells in Culture.

Limonoid	IC_{50} (µg/mL)	LC_{50} (µg/mL)
Limonin glucoside	75	500
Limonin	12	80
Nomilin	0.4	3
Glucoside mixture	0.08	6

Table 3: Effect of Limonoids on the Proliferation and Viability of MCF-7 Estrogen Receptor-Positive Human Breast Cancer Cells in Culture.

Limonoid	IC_{50} (µg/mL)	LC_{50} (µg/mL)
Limonin glucoside	35	125
Limonin	2	63
Nomilin	0.05	2
Glucoside mixture	0.05	4

In ER- cells, the most potent inhibitor was the glucoside mixture having an IC_{50} of 0.08 ug/mL followed by nomilin and limonin. Limonin glucoside was the least effective having an IC_{50} of 75 ug/mL. The limonoids inhibited ER+ cells more effectively (Table 3). Both nomilin and the glucoside mixture were the most effective having an IC_{50} of 0.05ug/mL (Table 3). We have

also tested the effects of a number of naturally occurring and synthetic limonoids for their effect on cell proliferation. In ER- cells, limonin methoxime and deacetylnomilin were the most effective inhibitors having IC_{50}s of 0.02 and 0.07 ug/mL respectively (Table 4, Figure 1). In ER+ cells, deacetylnomilin, obacunone and methyl nomilinate were the most effective inhibitors of proliferation having IC_{50}s of 0.005, 0.009 and 0.01 µg/mL respectively (Table 5, Figure 2). Tamoxifen, a drug widely used in the treatment of hormone responsive breast cancers, acts mainly by competing with estrogen for its receptor. The IC50s for tamoxifen were 90 µg/mL in ER- cells and 0.04 µg/mL in ER+ cells (Tables 4 and 5). Our data indicate that citrus limonoids are potent inhibitors of both cell types and may act via an estrogen receptor-independent pathway. Nomilin and the glucoside mixture are comparable in their inhibition of MCF-7 cells to tamoxifen. However, deacetylnomilin, obacunone and methyl nomilinate are more effective in inhibiting ER+ cells than tamoxifen (Table 4).

Table 4: Effect of Limonoids on the Proliferation of MDA-MB-435 Estrogen Receptor-Negative Human Breast Cancer Cells in Culture.

Limonoid	IC_{50} (µg/mL)
Limonin methoxime	0.02
Deacetylnomilin	0.07
Deoxylimonin	0.78
Nomilin glucoside	0.78
Isoobacunoic acid	1.95
7-a obacunol	3.13
Ichangin	3.13
Limonol	3.13
Obacunone	3.13
Nomilinic acid glucoside	3.13
Obacunone glucoside	6.25
Limonin carboxymethoxime	10.3
Methyl deacetylnomilinate	12.5
Methyl deoxylimonate	25.0
Methyl nomilinate	25.0
Methyl isolimonate	25.0
Tamoxifen	90.0
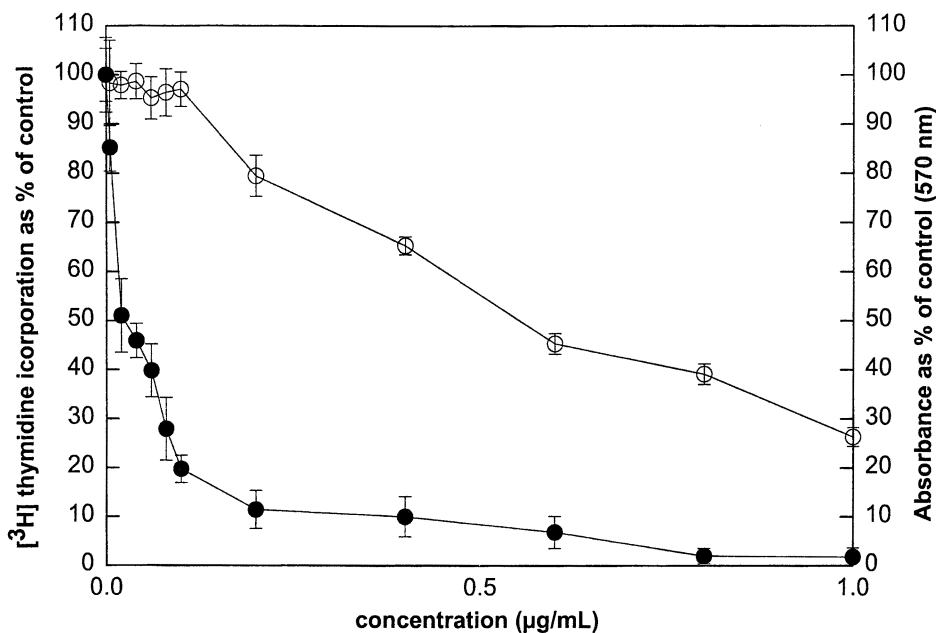
Figure 1. Effect of limonin methoxime on the proliferation (•) and survival (0) of MDA-MB-435 estrogen receptor-negative human breast cancer cells in culture as determined by the incorporation of [³H] thymidine and MTT respectively. Results are the averages of 3 separate experiments ±SEM.

TABLE 5: Effect of Limonoids on the Proliferation of MCF-7 Estrogen Receptor-Positive Human Breast Cancer Cells in Culture.

Limonoid	IC_{50} (µg/mL)
Deacetylnomilin	0.005
Obacunone	0.009
Methyl nomilinate	0.01
7-a obacunol	0.15
Nomilin glucoside	0.78
Isoobacunoic acid	0.78
Nomilinic acid glucoside	1.95
Obacunone glucoside	1.95
Limonin carboxymethoxime	2.50
Deoxylimonin	2.50
Methyl deacetylnomilinate	2.95
Ichangin	3.13
Limonin methoxime	3.13
Limonol	6.25
Methyl deoxylimonate	12.5
Methyl isolimonate	12.5
Tamoxifen	0.04

Experiments on Cell Growth

The effect of limonoids on the growth of both types of cells was also studied. MDA-MB-435 and MCF-7 cells were plated at 1 x 10^4 cells/dish in 60 mm dishes, with or without the test compounds at their IC_{50} concentration (determined in the proliferation assays) in a total volume of 7 mL. The cells were removed by trypsinization at the specified times and counted using a hemocytometer (17).

The ability of limonoids to suppress MDA-MB-435 and MCF-7 cell growth was evident when the cells were grown in the presence of the limonoids at their IC50 values for 10 days, as illustrated for nomilin in Figure 3. The effects were clearly apparent after 2 days of treatment, but cell growth was impeded over the entire period of 10 days.

Animal Studies

Maximum Tolerated Dose

Groups of nude mice NCR nu/nu were maintained in a pathogen free animal facility. After a period for acclimitization, they were placed on test diets containing limonin or a glucoside mixture at the initial level of 0.5% by
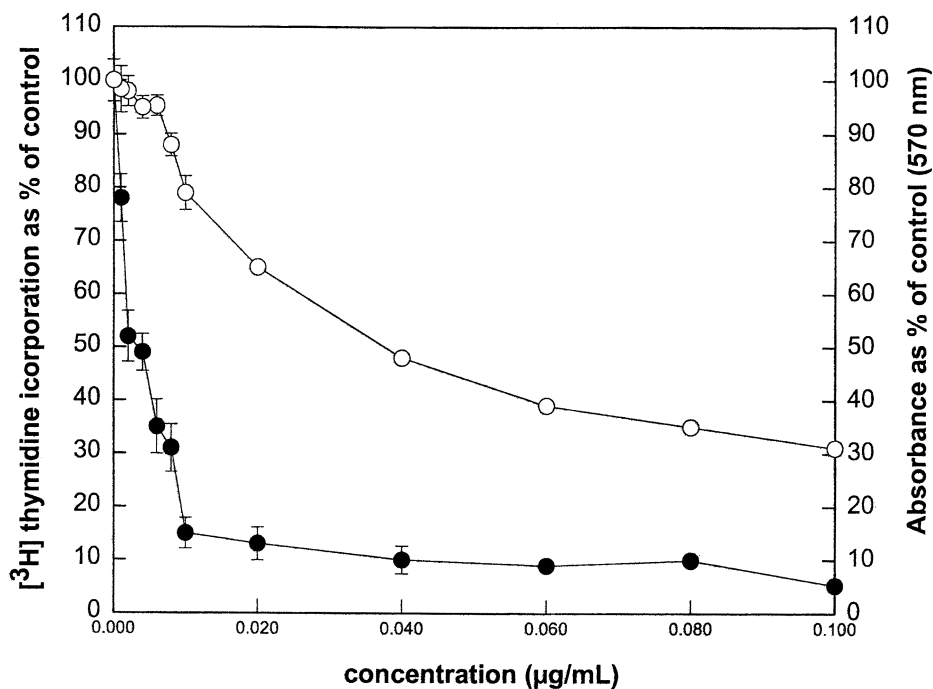
Figure 2. Effect of deacetylnomilin on the proliferation (●) and survival (0) of MCF-7 estrogen receptor-positive human breast cancer cells in culture as determined by the incorporation of [^{3}H] thymidine and MTT respectively. Results are the averages of 3 separate experiments ±SEM.
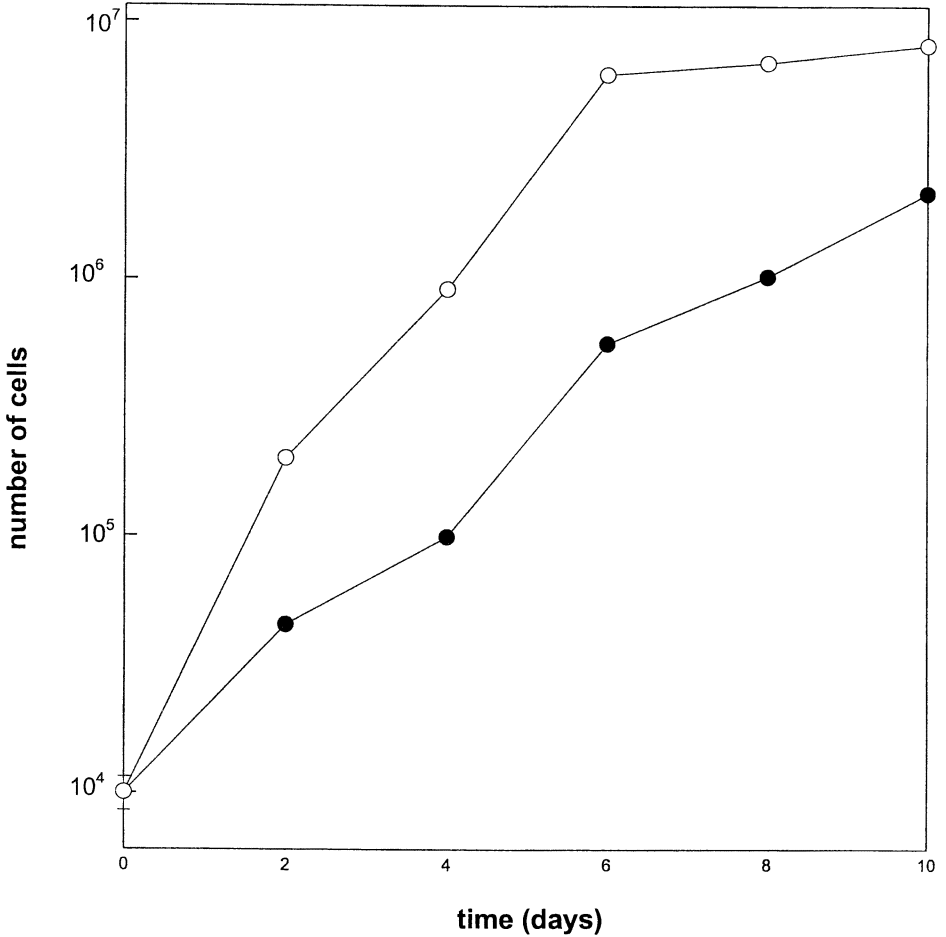
Figure 3. Growth of MDA-MB-435 estrogen receptor-negative human breast cancer cells in presence (•) and absence (0) of nomilin at the concentration that inhibited cell proliferation by 50%. Results are the averages of 3 separate experiments ±SEM.

weight. The animals were monitered daily for changes in weight, activity, mobility, and overall health. They were observed for 2 weeks and if no signs of toxicity were observed the level of limonoids in the diet was doubled for other groups of animals. This doubling was repeated until signs of toxicity appeared. The dose ranged from 0.5% to 8% for limonin or the glucoside mixture by weight. At autopsy, liver samples were examined histologically for signs of toxicity.

No significant weight loss was observed for the glucoside mixture at the 8% level. However, histological examination of the liver samples showed signs of early degeneration at 8% and 4% levels. The groups receiving limonin exhibited weight loss and signs of inactivity at the 8% level. Also, liver toxicity was observed at 4% levels for limonin. Based on these findings, we concluded that the maximum tolerated dose is 2% by weight for limonin and 4% by weight for the glucoside mixture. It is also important to note that the duration for these studies was short due to availability of limonoids.

Summary and Conclusions

Our results have shown that orange juice and grapefruit juice inhibit the proliferation and metastases of MDA-MB-435 ER- human breast cancer cells injected into nude mice and that this inhibition is only partially due to their constituent flavonoids. Limonoids are also present in the juices. We have recently reported limonoids to be more potent inhibitors of these cells than the flavonoids *in vitro*. We have also shown that citrus limonoids are effective at inhibiting MCF-7 ER+ human breast cancer cells in culture with nomilin and the glucoside mixture having the lowest IC50s. In testing other naturally-occurring and synthetic limonoids, we found that limonin methoxime and deacetylnomilin were the most effective inhibitors of proliferation in ER- cells and deacetylnomilin, obacunone and methyl nomilinate were the most effective inhibitors of proliferation in ER+ cells. In addition, when citrus limonoids were compared to tamoxifen, nomilin and the glucoside mixture inhibited ER+ cells to the same extent, whereas deacetylnomilin, obacunone and methyl nomilinate were much more effective. *In vivo*, we determined the maximum tolerated dose for limonin (2%) and the glucoside mixture (4%).

The inhibition of both ER- and ER+ cells by these limonoids has important clinical implications, since most breast cancers are heterogeneous and consist of both cell types. Tamoxifen is effective in arresting the growth of ER+ cells and thus is widely used in the treatment of hormone-responsive tumors, but is relatively ineffective in treating ER- tumors. Therefore, an alternative treatment regimen, using a growth inhibitor of ER-, coupled with an anti-hormonal drug would effectively target both types of cells. As normal constituents of food, the limonoids are relatively non-toxic and may significantly reduce the risk of cancer if ingested on a regular basis. They are also relatively inexpensive compared to other drugs used in chemotherapy. The limonoids could thus prove to be an important new means of treatment and/or prevention of breast cancer.

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Chapter 13

Regulation of Apo B Production in HepG2 Cells by Citrus Limonoids

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In rabbits, dietary orange juice or grapefruit juice reduced the hypercholesterol-emia associated with elevation of LDL (low-density lipoprotein) cholesterol, induced by feeding casein. To determine whether this effect could be partly due to constituent limonoids, the ability of these compounds to lower overall production of apo B (the structural protein of LDL) was tested. Using cultured human liver cells HepG2, fourteen naturally occurring limonoid aglycones and glucosides, a limonoid glucoside mixture isolated from orange molasses, and five synthetic limonoids were evaluated. Limonin and methyl deoxylimonate very effectively reduced the production of medium apo B by HepG2 cells. Nomilin, deoxylimonin and methyl isolimonate reduced moderately, and other limonoids had practically no effect on the production of medium apo B. The apo B-lowering potential of limonin was 30% greater than the apo B-lowering potential of two citrus flavonoids, hesperetin and naringenin. The effects of limonin, hesperetin and naringenin were dose-dependent but limonin, unlike the citrus flavonoids, did not cause decreases in the cellular synthesis of cholesteryl esters. The results suggest that limonin, along with citrus flavonoids, could contribute to cholesterol-lowering action of citrus juices. Limonin might be liberated from its glucoside derivative, limonin 17-D-glucopyranoside, which is present in high concentrations in citrus juices, by the action of bacteria in digestive tract.

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Elevated levels of blood cholesterol are known to be one of the major risk factors associated with coronary heart disease, the leading cause of death in North America. Dietary intervention has been proven to play an important role in prevention and treatment of hypercholesterolemia. Current dietary recommendations focus on reduced intake of saturated fat and cholesterol but numerous studies also demonstrated a role of other common macro-and micronutrients, such as carbohydrates, protein and vitamins, in modulation of cholesterolemic responses *(1, 2)*. However, during recent years, a growing interest has also been shown in investigating possible cardioprotective effects of plant-derived food products and their minor nutritive and non-nutritive constituents *(3, 4)*.

Epidemiological studies indicate that the consumption of fruit and vegetables is associated with reduced risk of cardiovascular disease *(5)*. The beneficial effects have been postulated to be due to minor components, especially flavonoids. These compounds have been suggested to exert their cardioprotective action mainly as antioxidants and as inhibitors of platelet aggregation *(3)*. Some dietary flavonoids were also reported to lower the risk of heart disease by reducing hypercholesterolemia associated with elevation of atherogenic lipoproteins, VLDL (very low-density lipoprotein) and LDL (low- density lipoprotein). Flavonoids from soybean, consisting mainly of the isoflavone, genistein, have been shown to decrease blood levels of VLDL + LDL cholesterol in Rhesus monkeys (6) and VLDL but not LDL cholesterol in rabbits (7). Other reports demonstrated that in rats, total cholesterol levels were reduced by feeding plant extracts rich in flavonoids *(8, 9, 10, 11)*. The two principal citrus flavonoids, hesperetin from oranges and naringenin from grapefruit, are structurally similar to genistein, which suggests that they could act as cholesterol-lowering agents. In support, it has been demonstrated that in rats, blood cholesterol levels were reduced by feeding diets supplemented with: i) a flavonoid extract from Prunus davidiana containing hesperetin 5-O-glucoside (9), ii) a hesperetin glucoside, hesperidin, abundant in orange juice *(10)*, iii) a mixture of citrus flavonoids containing largely hesperidin and naringin, a naringenin glucoside abundant in grapefruit juice *(11)*.

Citrus limonoids are present in immature fruit tissues as limonoid aglycones and in mature fruit tissues mainly as glucoside derivatives (limonoid glucosides). The conversion of aglycones to their respective glucoside derivatives occurs during late stages of fruit growth and maturation. Limonoid glucosides are major secondary metabolites in the mature fruit tissues and seeds. Commercial orange juices in the United States, for example, contain an average of 320 ppm of mixed limonoid glucosides *(12)*. By-products such as molasses and seeds produced by juice processing plants are excellent sources for limonoids.

Citrus limonoids possess several biological activities such as anticancer activity in mice, hamsters and cultured human breast cancer cells *(13, 14)* and antifeedant activity against insects *(15)*. We examined here the cholesterol-lowering properties of both limonoid aglycones and glucosides using cultured human liver cells HepG2.

Cholesterol-lowering Activity of Dietary Citrus Juices

The effect of orange and grapefruit juices on cholesterol metabolism was investigated in a rabbit model of experimental hypercholesterolemia *(16)*. In this study, animals were fed a semipurified, low-fat, cholesterol-free, casein-based diet which produces hypercholesterolemia with a raised LDL fraction, similar to that observed in humans *(17)*. The control group received water to drink and the experimental groups were given either orange juice or grapefruit juice (reconstituted concentrates made up to two times the regular strength). To ensure that the juice consumption did not affect the intake of other food components, diets given to the experimental groups were modified to compensate for additional sugar present in the juices and for any changes in the food intake observed during the study *(16)*.

The results (Figure 1) showed that after 3 weeks, the animals given citrus juices had significantly lower LDL cholesterol levels than those given water (43% and 32% reduction for orange juice and grapefruit juice, respectively). This was associated with significant decreases in liver cholesterol esters (42% decrease in each juice group) but not with increases in fecal excretion of cholesterol and bile acids *(16)*. The above data suggest that the reduction of LDL cholesterol was unlikely to be due to citrus pectins acting as cholesterol sequestrants in the intestine. Instead, the results indicated that changes in LDL cholesterol and in liver cholesterol esters might be induced by other juice components. Previous experiments demonstrated that the cholesterol-lowering responses produced by citrus juices were not likely due to their constituent sugars *(18)* or due to vitamin C, which is not a required nutrient in the rabbit *(19)*. It was therefore possible that the effects were related to minor juice components, such as limonoids and flavonoids, affecting metabolism of LDL in the liver.

Cholesterol-lowering Effect of Citrus Limonoids

Fourteen naturally occurring citrus limonoid aglycones and glucosides, a limonoid glucoside mixture isolated from orange molasses, and five other synthetic limonoids were examined for the cholesterol-lowering potential in HepG2 cells. HepG2 cells are neoplastic human liver cells that can secrete as well as catabolize lipoproteins similar to LDL *(20)*. Confluent HepG2 cells were preincubated for 24 h in a lipoprotein-free medium in which the fetal bovine serum was replaced by albumin to inhibit cell proliferation and to stimulate synthesis of cholesterol-containing lipoproteins. Cells were subsequently incubated for another 24 h in the same medium in the presence or absence of limonoids at the highest concentrations sustaining 100% cell viability, as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay *(21)*. All limonoids were added to the cell culture medium after solubilization in dimethyl sulfoxide (DMSO). At the end of the incubation, culture media were collected and the concentration of the LDL structural protein, apo B, was measured by Elisa *(22)*. Amount of lipoprotein-associated apo B in the media (net apo B secretion) was determined as described previously (22), calculated per mg cell protein and expressed as percent of control.
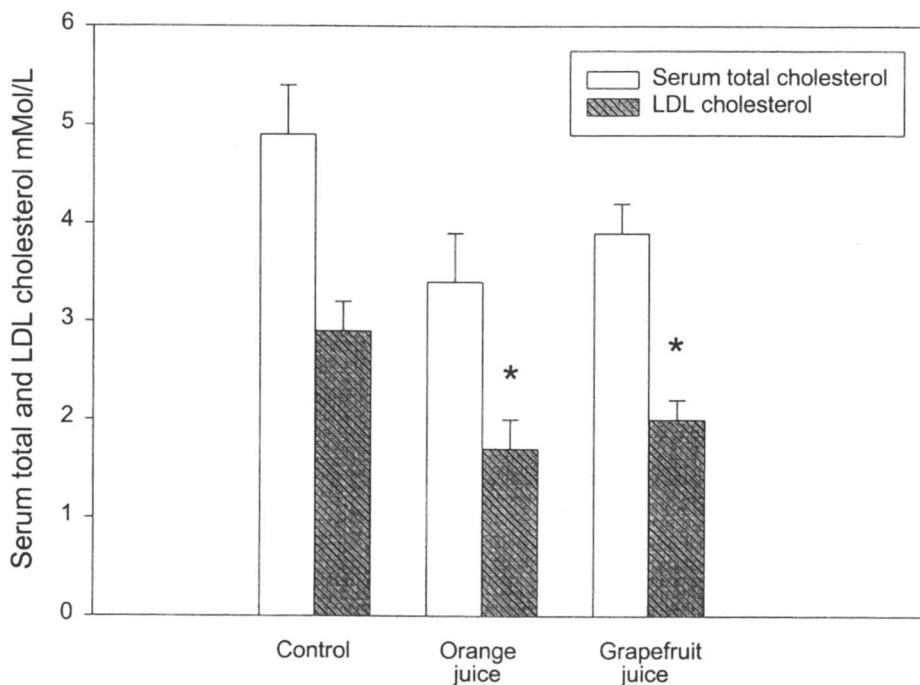
*Figure 1. Effect of dietary citrus juices on serum total and LDL cholesterol levels in rabbits with experimental hypercholesterolemia. Values are means ±SEM. Twelve animals per group. *-Significantly different from control, p < 0.05 (Data from reference 16.).*

The results (Table I) showed that among natural limonoids, limonin, nomilin and deoxylimonin induced either a substantial or moderate reduction of medium apo B. Limonin was the most active, reducing medium apo B by 74% when added to cells at a concentration of 50 μg/mL. At the same concentration, nomilin and deoxylimonin reduced medium apo B levels by 40% and 26%, respectively. Among the five synthetic limonoids, two methylated derivatives, methyl deoxylimonate and methyl isolimonate, were also active, inducing 70% and 34% apo B reduction, respectively. Other natural and synthetic limonoids and limonoid glucosides had little or no effect on medium apo B (Table I). The lack of activity of several free limonoids could be related to their structure whereas limonoid glucosides were most likely not available to cells due to the presence of sugar residues.

Limonin and nomilin are two major limonoid aglycones present in citrus seeds *(12)*. They are, however, present in citrus juices at relatively low concentrations (a few ppm). Instead, limonin and nomilin are present in citrus juices in high concentrations as glucoside derivatives, limonin 17β-D-glucopyranoside and nomilin 17β-glucopyranoside *(12)*. Evidence now suggests that several species of bacteria in the human intestinal tract can hydrolize the limonoid glucosides liberating the aglycones *(23)*. This increases the likelihood that limonin and nomilin might be absorbed into the blood in relatively high concentrations.

The apo B-lowering limonoid methyl esters, methyl deoxylimonate and methyl isolimonate, are synthetic. However, most of limonoids found in the genus *Fortunella* are methyl esters. Thus, *Fortunella* limonoids, such as calamin, cyclocalamin, retrocalamin, methyl isoobacunoate diosphenol and methyl deacetylnomilinate might be effective in lowering apo B *(23)*. Another possible food source for potentially active cholesterol-lowering limonoids could be kumquats (*Fortunella*) and their juices *(24)*.

Mechanism of action of limonin

The mechanism by which limonin exerts its apo B-lowering effect in cells was investigated further and the responses were compared to those obtained previously for hesperetin and naringenin *(22)*. For evaluation of the apo B-lowering potential, confluent HepG2 cells were incubated with or without increasing, non-cytotoxic concentrations of limonin. As shown in Figure 2, limonin, like hesperetin and naringenin, reduced the apo B content in medium of HepG2 cells in a dose-dependent manner. However, the apo B-lowering effect of limonin was greater than that of the citrus flavonoids. The IC_{50} concentration was 2.0-2.4 times lower for limonin than for both citrus flavonoids (20.5 μg/mL vs. 43.0 μg/mL and 48.5 μg/mL for limonin, hesperetin and naringenin, respectively).

To determine whether exposure to limonin altered intracellular lipid metabolism, confluent HepG2 cells were incubated for 24 h with or without the highest non-cytotoxic concentration of limonin. During the last 5 h of the incubation, ^{14}C-acetate
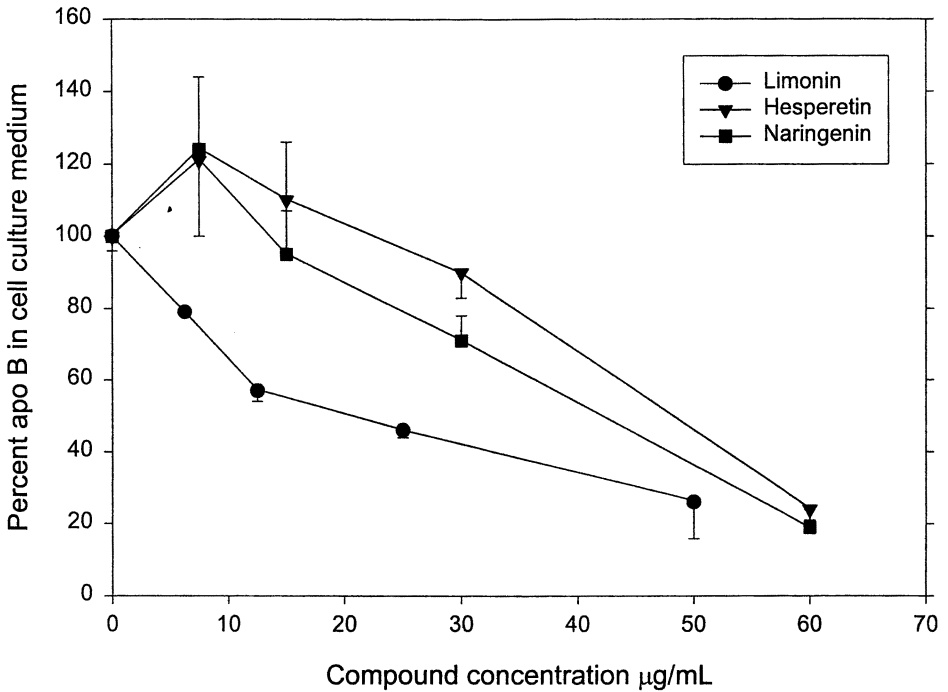
Figure 2. Changes in medium apo B in response to increasing doses of limonin and two citrus flavonoids, hesperetin and naringenin. Confluent HepG2 cells were incubated for 24 h in the presence or absence of either 0-50 μg/mL of limonin or 0-60 μg/mL of the citrus flavonoids. Apo B in the culture media was quantified by Elisa and expressed per mg cell protein before conversion to percent control. Values are means ±SEM, n = 3. Data for citrus flavonoids are from reference 13.

Table I. Effects of Citrus Limonoids on Net Apo B Secretion by HepG2 Cells

Compound	Origin[1]	Percent medium apo B reduction at the highest non-toxic concentration[2]
Limonin	N	74 ± 19[3]
Nomilin	N	40 ± 6
Obacunone	N	0
Ichangin	N	0
Deoxylimonin	N	26 ± 1
Limonol	N	0
7α-obacunol	N	0
Methyl deacetylnomilinate	N	0
Deacetylnomilin	N	0
Iso-obacunoic acid	N	0
Limonin glucoside	N	0
Nomilin glucoside	N	0
Mixture of limonoid glucosides[4]	N	12 ± 13
Obacunone glucoside	N	0
Nomilinic acid glucoside	N	0
Methyl deoxylimonate	S	70 ± 7
Methyl isolimonate	S	34 ± 2
Methyl nomilinate	S	0
Limonin methoxine	S	0
Limonin carboxymethoxine	S	0

[1]N – natural compound, S – synthetic compound
[2]The highest non-toxic concentration is 25 µg/mL for limonol and iso-obacunoic acid, 50 µg/mL for the remaining compounds and mixed glucosides.
[3]Values are means ± SD, n = 4.
[4]The mixture consists of limonin glucoside (30.0%), nomilinic acid glucoside (33.8%), nomilin glucoside (12.7%), obacunone glucoside (8.2%), deacetylnomilin glucoside (6.6%) and deacetylnomilinic acid glucoside (8.7%).

(0.5 μCi/mL) was added to the medium. The extraction and separation of cellular lipids was done as previously described *(22)*.

The results (Figure 3) showed that at the highest non-toxic concentration (50 μg/mL), limonin had no effect on [14]C-acetate incorporation into cellular cholesterol, cholesteryl esters and triacylglycerols. However, both flavonoids added to cells at the highest non-toxic levels (60 μg/mL) induced a significant, approximately 50% decrease in the incorporation of [14]C-acetate into cellular cholesteryl esters without causing significant changes in label incorporation into free cholesterol and triacylglycerols *(22)*. The results suggest that the mechanism by which limonin lowers medium apo B is different than that proposed for citrus flavonoids. According to our previous studies, hesperetin and naringenin could reduce the net apo B secretion by inhibiting the synthesis of cellular lipids, especially cholesteryl esters. These flavonoids could therefore act by interfering with the availability of neutral lipids that are required for the assembly of apo B-containing lipoproteins *(22)*. In contrast, the apo B lowering action of limonin might be mediated via increases in the intracellular degradation of apo B prior to lipoprotein assembly and secretion or via up-regulation of LDL receptors responsible for catabolism of apo B-containing lipoproteins.

Our results in vitro suggest that the cholesterol-lowering responses induced in rabbits by dietary citrus juices could be related to their high content of limonoids as well as hesperetin and naringenin. Hesperetin and naringenin but not limonin could also be responsible for decreases in liver cholesteryl esters produced in animals given the juices. Further studies are now needed to better understand the mechanism of action of limonin in HepG2. Experiments are also planned to determine whether limonin and other limonoids, alone or in combination with flavonoids, can lower LDL cholesterol in animals.

Conclusions

In the present study, we showed for the first time that limonin and some other natural and synthetic limonoids have cholesterol-lowering properties in HepG2 cells, and that in this system, limonin acts via a different mechanism than citrus flavonoids. Dietary citrus juices, which contain significant quantities of limonoids and flavonoids, have the ability to counteract the elevation of LDL cholesterol induced in rabbits by feeding a hypercholesterolemic, casein-based diet. The effect could be partly due to limonoids and flavonoids in the juices.

Limonin and other limonoids are found in citrus juices mainly as glucoside derivatives and the cholesterol-lowering activity in vitro has been observed only for aglycone forms of the selected compounds. It will be important to determine whether limonoid glucosides can be absorbed as aglycones after liberation of sugars by the intestinal flora. Studies on bioavailability of citrus limonoids and mechanism of absorption are important future research objectives.
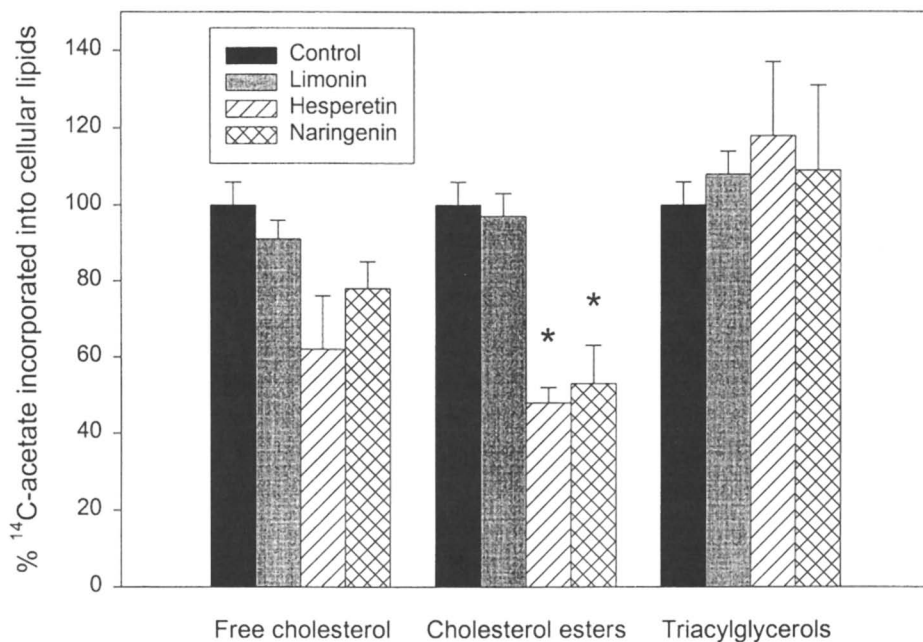
Figure 3.   Effect of limonin vs. citrus flavonoids on rates of incorporation of radiolabeled acetate into intracellular lipids. Confluent cells were incubated for 24 h in presence vs. absence of limonin (50 µg/mL), hesperetin (60 µg/mL) or naringenin (60 µg/mL). ^{14}C-acetate (0.5 µCi/mL) was added for the last 5 h of the incubation. Cellular lipids were then extracted, separated by thin-layer chromatography and counts in each lipid fraction were measured. Values are means ±SEM, n = 3. * - Significantly different from control, p < 0.05. Data for citrus flavonoids are from reference 13.

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Chapter 14

Limonin and Nomilin Inhibitory Effects on Chemical-Induced Tumorigenesis

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Citrus Limonoids are furan containing triterpenes present in common edible citrus fruits such as lemon, lime, orange and grapefruit. Limonin and nomilin, two of the most abundant limonoids, have been found to inhibit chemical-induced carcinogenesis. Both compounds are inducers of glutathione S-transferase, a major detoxifying enzyme system. The increased enzyme activity was correlated with the ability of these compounds to inhibit carcinogenesis. Nomilin was found to reduce the incidence and number of tumors per mouse of forestomach tumors induced by benzo[a]pyrene (BP). Topical application of the limonoids was found to inhibit both the initiation and the promotion phases of carcinogenesis in the skin of SENCAR mice. Nomilin appeared to be more effective at the initiation stage while limonin was more potent as an inhibitor at the promotion phase of carcinogenesis. Administration of nomilin and limonin to the diet or by gavage inhibited BP-induced and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced lung tumor formations, respectively, in A/J mice. These findings suggest citrus limonoids are potential cancer chemopreventive agents.

Introduction

Citrus limonoids are furan containing triterpenes. The most abundant limonoids in Rutaceous plants that include the commonly edible fruits lemon, lime, orange, and grapefruit, are limonin and nomilin (1). These two compounds are present in citrus fruits mostly as their glucosides (2). While the aglycones are intensely bitter and are one of the bitter principles that contribute to the unpleasant taste of the fruits and juices, the glucosides, on the other hand, are not organoleptic. Despite their unpleasant taste which is a major concern in the citrus industry (3), limonin and nomilin are potentially beneficial substances to human health. They have been

investigated as cancer chemopreventive agents in mice (4-6), hamsters (7-10), and cell culture systems (11). The results so far appear to be encouraging.

Structural Features Of Citrus Limonoids

One of the characteristic features of citrus limonoids is the presence of a furan ring (Fig. 1) (12). The D-ring lactone of a highly oxygenated triterpene is attached to the
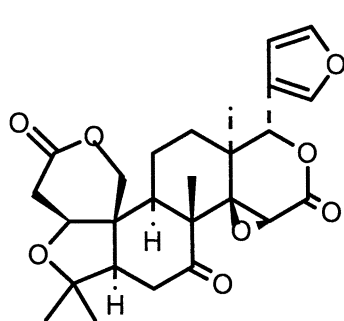
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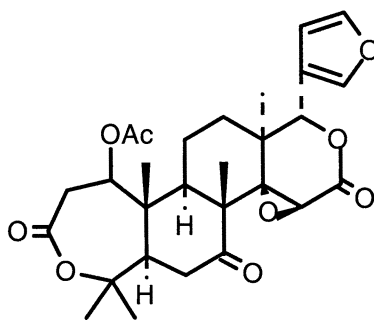
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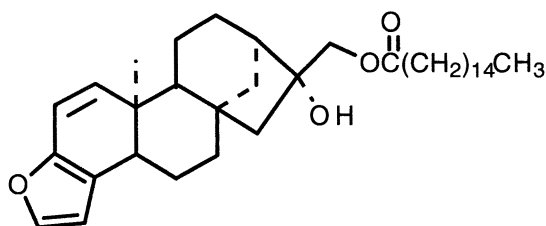
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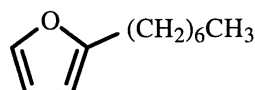
2-Heptylfuran

Figure 1. Furan containing natural products that show cancer chemopreventive potential.

3-position of the furan moiety. The furan function plays an important role that defines the biological activity of citrus limonoids. Mono-substituted furan compounds such as 2-n-heptylfuran are known to inhibit chemically induced carcinogenesis (13). Furan containing natural products such as kahweol and cafestol from coffee beans inhibit carcinogenesis in the hamster cheek pouch (10) and the rat mammary glands (14). The inhibitory activity of these furan compounds is attributed, in part, to their ability to induce the detoxifying enzyme system, glutathione S-

transferase (GST) (4-6, 13, 15, 16). Conversion of the furan function to dihydro- and tetrahydro-furan by stepwise hydrogenation eliminates the inducing properties of cafestol acetate (16). The loss of GST inducing property suggests the diminishing activity of cancer chemoprevention as well.

Mutagenicity Of Citrus Limonoids

The highly oxygenated triterpene moiety of limonoids possesses an epoxide on the D-ring and cyclic esters on both the A and D rings. The presence of the epoxide and the cyclic ester functions often raise concerns that they may bind to macromolecules to induce mutation, thereby, causing potential carcinogenic or teratogenic effects. To alleviate such concerns, we have used the bacterial mutagenicity testings, the Ames test and forward mutation method, to determine the mutagenicity of citrus limonoids. The Ames test was performed according to the methods of Maron and Ames (17). Three tester strains, TA98, TA100, and TA1535 were used in the presence of S9. The forward mutation assay was performed according to the method of Thilly and coworkers (18,19) using the *S. typhimurium* strain TM677 strain. Neither limonin nor nomilin show mutagenic activity at concentrations of 5 µg per plate (Table I).

Glutathione S-Transferase Induction

The Phase II enzyme system, GST, is a major detoxifying enzyme that catalyzes the conjugation of the endogenous tripeptide, glutathione, with reactive electrophiles (20, 21). Since many activated carcinogens are electrophiles, the induction of GST is considered an enhancement of carcinogen detoxification by an increase of carcinogen glutathione conjugate formation and their subsequent excretion (21). Many furan-containing natural products including the citrus limonoids have been shown to induce GST to relatively high levels (13, 16, 22).

Limonin and nomilin have been found to induce increased GST activity in the liver and small intestinal mucosa of mice given at 5 mg and 10 mg per animal every other day for a total of 3 doses (4,22). The level of GST was determined using the universal substrate chlorodinitrobenzene (CDNB) (21,22).

The results showed that limonin, the compound with the orthogonal A ring to the plane of the molecule was inactive in the liver. Nomilin, on the other hand, was quite active even at a low dose of 5 mg per animal (Table II). The relative inducing activity in the small bowel mucosa was higher than that in the liver for both limonin and nomilin. No appreciable elevation of GST activity was detected in the forestomach.

The GST enzyme system consists of three main classes of isoenzymes. They are the α, µ, and π class. Using a chromatofocusing fast protein liquid chromatographic (FPLC) technique some of the isoenzymes in the α and m classes can be separated as distinct peaks (5).

Limonin given in the diet at 0.25 and 0.50 % altered the FPLC profile in such a way that the relative intensity of the α and µ isoenzymes differed from that of the normal control level. No dose response factor can be observed, however, suggesting that the change is saturated at a very low dose level.

Table I. Limonoids Mutagenicity Testing using the Ames Test[a]
and Forward Mutation Method[b].

Chemicals[c]	TA98[d]	TA 100[d,e]	TA1535[d,f]	TM677[b,f,g]
S9 control	18	99	12	94
Limonin	15	97	15	83
Nomilin	13	85	13	89

a. The Ames bacterial testing was performed according to the methods of
    Maron and Ames (9).
b. Forward mutation assay (Thilly and coworkers (10,11)).
c. 5 µg each of limonin or nomilin per plate.
d. The values are number of revertants per plate.
e. Average of 3 experiments.
f. Average of 2 experiments.
g. The mutagenicity is expressed as mutant fractions, i.e. the average number
    of 8-azaguanine-resistant (mutant) clones from three plates divided by the
    average number of 8-azaguanine-sensitive clones from three plates.

Table II. Limonin and Nomilin Induction of Glutathione S-transferase Activity in the Liver and Small Bowel Mucosa of Female ICR/Ha Mice.

Compounds	Dose[a]	GST Activity[b]	
		Liver	*Small Bowel Mucosa*
Control	- -	1.15±0.19	0.33±0.04
Limonin	5 mg	1.29±0.35	0.45±0.08[e]
Limonin	10 mg	1.24±0.23	0.45±0.09[e]
Nomilin	5 mg	2.86±0.60[d]	1.00±0.03[c]
Nomilin	10 mg	3.96±0.81[d]	1.39±0.15[c]

a Limonin and nomilin were given as fine suspension in 0.3 mL cottonseed oil every other day for a total of 3 doses. Control was given cottonseed oil only.
b The GST activity (μmol/min/mg protein) was determined according to the method of Habig et al. using CNDB as the substrate (21). Mean ± S.D. (n = 3).
c $P < 0.001$.
d $P < 0.005$.
e $P < 0.05$.

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Nomilin, on the other hand, induced the μ class of isoenzyme, in particular 3-3 and 3-4 at concentration of 0.27 % addition to the diet. The relative intensity of the isoenzymes indicate the induction of the μ class of isoenzyme are closely associated with the inhibition of carcinogenesis.

Inhibition Of Benzo[a]pyrene (BP)-Induced Forestomach Tumors

The high GST inducing activity of the limonoids suggests that they are potential chemopreventive agents. An experiment designed to test their effectiveness as tumor inhibitors was carried out with female ICR mice (4). The limonoids were given by gavage three times a week at 5 and 10 mg/animal for 4 weeks. Two additional administrations before and one after the carcinogen were given. The carcinogen, BP, was given at 1 mg/animal twice a week on the days when the limonoids were not given. Eighteen weeks after the first dose of BP, the animals were sacrificed. The forestomach was excised and the tumors were counted. The tumors were confirmed histopathologically.

The results showed a significant decrease of the tumor incidence as a result of nomilin treatment (Table III). At 10 mg dose, nomilin was found to reduce the number of tumors/animal by greater than 50%. Limonin, under similar conditions, was somewhat less effective.

The results indicate the inhibitory effects of limonin and nomilin follow the same trend as their ability to induce GST activity. Nomilin being a more potent inducer of GST is more active as an inhibitor of carcinogenesis than the less effective limonin. The inability of limonoids to elevate GST activity in the forestomach does not appear to be an important factor in the inhibition of carcinogenesis in this target tissue. A significant enhancement of GST activity in the major detoxifying center, namely the liver, appears to be positively correlated with the inhibitory potential of limonoids not only in the portal of entry but also at a remote site such as the lung.

Inhibition Of DMBA-Induced Hamster Cheek Pouch Tumors

Under other experimental conditions, it has been determined that limonin was a more potent inhibitor of DMBA-induced carcinogenesis in the hamster's cheek pouch (7). The hamster study suggested that limonoids may be inhibiting at the promotional stage of carcinogenesis as well. An experiment using the two-stage skin tumor model initiated with DMBA and promoted with TPA (23) confirmed that nomilin was more effective as an inhibitor at the initiation stage of carcinogenesis and limonin was more effective at the promotional stage.

Inhibition Of Two-Stage Skin Carcinogenesis

To test the hypothesis that limonoids may be inhibiting at the promotional stages of carcinogenesis, the two-stage skin tumor model initiated with DMBA and promoted with TPA (20) was used. Both limonin and nomilin were tested (5).

**Table III. Limonin and Nomilin Inhibition of BP-induced
Forestomach Tumorigenesis in ICR/Ha Mice[a]**

compounds	Dose[b]	No. of mice[c]	% of Mice with tumors[d]	No. of tumors per mouse[e]
CONTROL	- -	18	100	3.6±0.8
Limonin	5 mg	16	88	3.1±0.8
	10 mg	19	84	2.5±0.6
Nomilin	5 mg	18	83	2.3±0.4
	10 mg	19	72[e]	1.7±0.4[f]

a BP (1 mg/0.2 mL of corn oil) was administered by gavage on Tuesdays
   and Thursdays for 4 weeks.
b Limonin and nomilin, in 0.3 mL corn oil in the form of fine suspension
   were administered by gavage on Mondays, Wednesdays and Fridays for
   4 weeks.
c Indicates effective number of mice at the termination of the experiment.
d All tumors >0.5 mm were included, Mean+ S.E.
e χ^2 analysis, P<0.025
f Students "t" test compared to control, P<0.05

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In this experiment, a total of twelve groups were used. The first 3 groups were the solvent and limonoids only controls. High doses (1 mg) of limonin and nomilin were applied on the back of the animals throughout the experimental period to check whether these compounds alone cause any unusual responses from the animal. No adverse effects were found. The remaining nine experimental groups are listed in Table IV. Group 1 was the positive control where 100 nmol of DMBA was applied and ten days later promoted with 5 mg of TPA twice a week for 12 weeks.

Groups 2 to 5 were designed to test the inhibition of initiation. In these groups, the limonoids were applied before and on the same day of DMBA treatment. No inhibitors were given to animals at the promotion stage. Groups 6 to 9 were designed to test the inhibition of promotion. They were not treated with limonoids during the DMBA application. The inhibitors were given, instead, one hour before each TPA treatment for the entire promotional period.

The tumors on the dorsal skin of the animals were recorded each week. Group 1 was found to have the highest number of tumors/animal from the beginning to the end of the experiment. At sacrifice, the average number of tumors/mouse was 20.6. Limonin given topically at 1 mg per application was the high dose group (LIMH) that showed a 15 % inhibition of tumor/mouse. The low dose group at 0.25 mg/animal had similar inhibition as that of the high dose group.

Nomilin at 1 mg/dose, on the other hand, showed 22.8 % inhibition of skin tumor formation than that of the LIMH group. At 0.25 mg/dose, there was no significant change from that of the control.

The inhibition by limonoids at the promotion phase of carcinogenesis indicated that limonin at 1 mg/dose applied one hour before TPA treatment inhibited the number of tumors/mouse throughout the course of the experiment. At sacrifice, the inhibition was greater than 43 %. With 1/4 of the dose, inhibition was found to be 32 %. Nomilin, the compound that showed higher inhibitory activity at the initiation stage and in other tumor model experiments was found to be less effective than limonin in reducing tumors at the promotional phase. No significant difference was found in the low and high dose groups.

These data showed that nomilin was more effective as an inhibitor at the initiation stage of carcinogenesis and limonin was more effective at the promotional stage.

Inhibition Of Benzo[a]pyrene-Induced Lung Tumors

In addition to inhibition of forestomach and skin tumors, citrus limonoids are also effective in preventing lung tumorigenesis induced by BP (6) and the tobacco specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (24).

Dietary limonoids inhibition of BP-induced lung tumorigenesis was carried out in A/J mice (6). Limonin and nomilin, at various concentrations in the diet (limonin, 0.125, 0.25, 0.5 %; nomilin, 0.068, 0.135, 0.27 %), were fed to A/J mice before and during the BP administration (Table V). The carcinogen was given by gavage at 1 mg/animal twice a week for 4 weeks. Three days after the last dose of BP the animals were returned to normal lab chow. The experiment was terminated 18 weeks after the first dose of BP. The weight gain of the animals at the end of the experiment was similar for all experimental groups. Under these experimental conditions, 100% of

Table IV. Limonin and Nomilin Reduction of Two-stage Carcinogenesis.

Group	Inhibitor-Initiator[a]	Inhibitor-Promotor[a]	Effective No. of mice	% of Mice with tumors	No. of tumors per mouse	% inhibition
1	None - DMBA	None - TPA	20	95	20.6±2.4	-
2	LIMH - DMBA	None - TPA	20	100	17.4±2.3	15.0
3	LIML - DMBA	None - TPA	19	100	17.4±1.7	15.5
4	NOMH - DMBA	None - TPA	19	100	15.9±2.2	22.8
5	NOML - DMBA	None - TPA	20	100	18.8±2.0	8.7
6	None - DMBA	LIMH - TPA	20	100	11.6±2.0[b]	43.7
7	None - DMBA	LIML - TPA	20	95	14.0±2.2	32.0
8	None - DMBA	NOMH - TPA	20	95	15.2±2.1	26.2
9	None - DMBA	NOML - TPA	20	95	14.4±2.0	30.1

a. All chemicals were dissolved in 200 µl acetone and applied topically. The inhibitors, limonin and nomilin, were given in high (LIMH, NOMH, 1 mg each) and low (LIML, NOML, 0.25 mg each) doses. The doses for DMBA and TPA were 100 nmol and 5 µg, respectively.

b. Values are mean±S.E., P<0.01.

Table V.   Dietary Limonin and Nomilin Inhibiton of BP-induced Lung Tumorigenesis.

Chemicals[a] (%)	Effective No. of Mice	% of Mice with tumors	No. of tumors per Mouse	% Inhibition
BP	17	100.00	11.9	
BP-Limonin (0.125)	19	100.00	10.8	9.2
BP-Limonin (0.25)	16	100.00	8.1	31.9
BP-Limonin (0.5)	18	100.00	6.1	48.7
BP-Nomilin (0.068)	18	100.00	14.9	
BP-Nomilin (0.135)	16	100.00	5.7	52.1
BP-Nomilin (0.27)	12	83.30	2.9	75.6

a. Citrus limonoids were added to the diet at the percentage indicated.   The
   experimental diets were given one week before, during and 3 days after the
   carcinogen administration. BP, at 1 mg/0.2 ml corn oil, was given by gavage
   twice a week for 4 weeks.

the animals developed pulmonary adenoma with the exception of the 0.27% nomilin group that had 83% incidence. Yet, the 17 % reduction in tumor incidence in this group was not statistically different from the control group. The number of tumors per animal, however, was significantly reduced for both the limonin and nomilin groups. The reduction was dose dependent. At 0.5%, the limonin group had 6.1 tumors /animal compared to 11.9 in the control group, a 48.7 % reduction. At 0.25%, 8.1 tumors/animal (32 % reduction) and at 0.125% only 9.2 % reduction was obtained.

The dosages used for nomilin were half those of limonin because it was anticipated to be more active judging from the previous forestomach tumor experiment (4). At 0.27%, 2.9 tumors/animals were found in this group when compared with 11.9 in the control group, which represented an inhibition of 75%. In the 0.135% group, 5.7 tumors/animal were found (a 52 % reduction) and no protection was obtained at the lowest dose of 0.068%. At 0.27%, it was estimated that each animal consumed approximately 10 mg/day of nomilin. At this level, a reduction of forestomach tumor was also found which is similar to that observed in a previous forestomach experiment using ICR mice (Table III). Similar to the inhibition of forestomach tumors, the protection of lung carcinogenesis by limonoids was positively correlated with the induction of GST activity and reduction of BP-DNA adducts formation in the lung (6).

Inhibition Of NNK-Methylation Of Liver And Lung DNA.

The carcinogen, NNK used in the lung tumorigenesis model, has been determined to undergo metabolic activation to a reactive intermediate which methylates the guanine base of DNA. A reduction of O6-methylguanine has been correlated with the inhibition of carcinogenesis in the lung of A/J mice by benzylisothiocyanate and other chemopreventive agents (25). The inhibition of NNK-induced DNA methylation in mice by citrus limonoids has been determined (2). Table VI shows the inhibition of DNA methylation in the lung of A/J mice. At 2 and 1 mmol/kg b.w. per dose nomilin reduced the O6-MeG level to 30 and 17 % that of the control. Limonin, at similar doses, reduced O6-MeG to 22 and 31 %, respectively. Under similar conditions, the level of 7-MeG was also reduced.

Inhibition Of NNK-Induced Tumorigenesis

NNK is the most potent carcinogenic nitrosamine so far found in tobacco and tobacco smoke.(26) The organ-specific effect of NNK in the induction of lung tumors in all animal species tested regardless of the route of administration strongly supports its possible role in the development of lung cancer among smokers.(27-30) In the U.S. alone, there are about 53 million cigarette smokers exposed to NNK daily, plus many more who are affected by second-hand smoke. Therefore, it is of great importance to discover compounds which can effectively alleviate the carcinogenic action of NNK. The discovery that citrus limonoids inhibit NNK-induced DNA methylation in the mouse lung and liver suggest the anticarcinogenic potential of these compounds against NNK-induced lung tumor formation.

Table VI. Inhibition of DNA Methylation in the Lung of A/J Mice.

Group	O^6-MeG[a]	Test/control	% Inhibition
NNK	0.073	1.00	–
2.0 Nomilin[c]	0.051	0.70	30
1.0 Nomilin	0.061	0.83	17
2.0 Limonin	0.057[b]	0.78	22
1.0 Limonin	0.051[b]	0.70	30

a. pmoles of O^6-MeG/nmoles of Guanine.
b. Significantly differnet from NNK group by Student's 2 tailed "t" test, P≤0.05.
c. Designates 2.0 or 1.0 mmol of limonoid/kg body weight.

In a protection experiment (24), the limonoids were suspended in cottonseed oil and administered by gavage. Limonin and nomilin at 1 mmol/kg body weight and 0.5 mmol/kg b.w. each was administered by gavage to A/J mice once every two days for a total of 3 doses. Since a combination of limonin and nomilin has been found to exert synergistic effect on the inhibition of proliferation of breast cancer cells in culture, this study also included two groups treated with combinations of 0.5 mmol/kg b.w. and 0.25 mmol/kg b.w. of each of the two limonoids. Two hours following the last dose of inhibitors, a single dose of NNK at 2 mg/0.1 ml of saline solution was given to animals in all groups except those in vehicle and inhibitors-only controls. The experiment was terminated 16 weeks after the administration of the carcinogen.

The average body weight gain of the control and experimental groups over the length of the experiment was similar. The deviation from the absolute control was within 10% through out the experimental period and at termination of the experiment indicating a lack of toxicity from the limonoids treatment.

Table VII shows the inhibition of high and low dose of individual limonoids and in 50/50 combination of the two compounds. The average number of lung adenomas in the carcinogen control group was 12.8±1.2. A small but significant reduction of 23 % tumor multiplicity in the high dose limonin group was observed. A similar reduction of 21 % in the low dose limonin group suggest that the effect of limonin on lung tumor inhibition is moderate.

The inhibition of lung tumorigenesis by low dose of nomilin was greater than 36 %. This statistically significant figure, unfortunately, was over shadowed by the much less percentage of protection (16.4%) with the high dose of nomilin. This reverse dose dependent phenomenon was also found with the high (27.3%) and low (14.1%) dose combination treatment of limonin and nomilin. We attribute this to the exceptional difficulty in solubilizing the limonoids in for the administration. The higher the dose the more difficult it was to deliver the proper dosage.

On the other hand, the detoxification of NNK may not be affected by the induction of GST activity; but rather, is the result of P450 metabolism followed by glucuronidation (32,33). This would explain the borderline inhibition by limonin and nomilin although the latter is capable of inducing much higher GST enzyme activity.

Summary

Limonin and nomilin are two most prevalent limonoids from citrus. They have been determined to induce the activity of the detoxifying enzyme, GST. The enzyme inducing property of the limonoids parallel the activity of the inhibition of tumor formation when the compounds were given to the animals in the diet or by gavage. More than four different tumor models which include the forestomach, oral cavity, lung and skin, have been used to assess the anticarcinogenic effects of limonoids. In general, nomilin is a more potent inhibitor than limonin except in the case where promotion is involved. Then, limonin is a potent inhibitor in the promotion phase of carcinogenesis. More recent work with human breast cancer cell lines further indicate the potential usefulness of citrus limonoids in the prevention of human cancer. Limonoids exist mainly as glycoside at very high concentration in juices (2, 31). The human consumption of these compounds is regular and quite substantial.

Table VII. Limonin and Nomilin Inhibiton of NNK-induced Lung Tumorigenesis.

Chemicals[a]	Effective No. of Mice	No. of tumors per Mouse	% Inhibition
NNK	26	12.8±1.2	-
NNK-Limonin (1.0)	18	9.9±1.2[b]	22.7
NNK-Limonin (0.5)	20	10.1±1.3	21.1
NNK-Nomilin (1.0)	20	10.7±1.3	16.4
NNK-Nomilin (0.5)	20	8.1±0.9[b]	36.7
NNK-Lim(0.5)Nom(0.5)	20	11.0±1.2	14.1
NNK-Lim(0.25)Nom(0.25)	20	9.3±0.9[b]	27.3

a. Citrus limonoids, suspended in 0.3 ml corn oil, were administered by gavage at the dosage (mmol/kg) indicated in parenthesis every two days for a total of 3 doses. NNK was given two hours following the last dose of limonoids at 2 mg/0.1 ml saline.
b. Significantly differed from the control (Student's "t" test), P≤0.5

The cumulative effects of citrus limonoids on cancer chemoprevention remained to be determined.

Acknowledgment

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Chapter 15

Prospects for Citrus Limonoids in Insect Pest Management

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Limonin and other citrus limonoids act as insect repellents, feeding deterrents, growth disrupters, and reproduction inhibitors against several pest species across a wide range of agricultural crops. We have investigated how these allelochemic properties can be deployed as ecologically sound methods of insect pest management and reduce dependence on conventional, synthetic insecticides in potato production. In a multi-stage process of study and product development, we demonstrated that field populations of Colorado potato beetles, *Leptinotarsa decemlineata* (Say), could be effectively managed using larvicidal applications of limonoids. Adult repellent and oviposition deterrent effects of limonoids can prevent these pests from colonizing and reproducing in potato fields and thereby reduce or eliminate the need for further pest control efforts. Limonoids also appear to act as synergists to enhance the effectiveness of other biological and conventional insecticides. Research and development approaches and limitations of these materials in agricultural pest management are described.

The theoretical basis for using natural plant compounds like the citrus limonoids for the management of insect pests is within the concept of coevolution advanced by Ehrlich & Raven in 1964 (*1*). The coevolutionary process of plants and insects has resulted in a number of plant defenses, including an estimated hundreds of thousands of plant-produced chemicals (*2*). Plant-feeding insects select and recognize their food based on plant chemical characteristics acting as attractants and phagostimulants that promote host finding and feeding. Other phytochemicals may also inhibit feeding, disrupt growth and development, and modify behavior by acting as insect deterrents, repellents or toxins (*3, 4, 5*). The identification and development of these biologically active allelochemicals into effective, insect pest management components have been the goals of natural product chemists, entomologists and agriculturists for decades, but commercial successes have been relatively rare.

Synthetic chemical insecticides, with their greater than fifty years of commercial dominance, have set the standards for assessment of all materials for controlling

insect pests. In the initial screening for biological activity, biases and limits can be set with the establishment of the single variable of measurement, which is usually mortality. It is this narrow perspective that is partly responsible for much of the failure, perceived and real, of natural product use in agriculture today. Natural product effects can be toxicological, physiological and behavioral, and they do not necessarily kill the insect rapidly or at all. Therefore, different criteria should be used to evaluate them. Beyond the identification and biological screening of a natural product, there is a diverse and staged process of bioassays and product development necessary to bring these materials to the market as reliable insect pest management tools (*3, 4, 5, 6*).

Researchers take different perspectives when evaluating and developing natural products. The approach we took in the early years of our research was the multiple modes of action view, i.e., the single compound exerts action against different insect species and life stages by a variety of biochemical and behavioral mechanisms. The complexity of the interactions between insects and plants makes it is necessary to investigate a wide range of behavioral and physiological responses of each insect species to these materials if we are to successfully use them. And as insect management tools, it will be the composite of these multiple effects that characterize the biological activity of the single compound and ultimately determine the best deployment strategy in the field.

An equally important, alternative view in evaluation and development is that these materials, by their very origin, are not single-variable defenses, but rather components of a complex plant defense system, and their use as insect management tools should be in combination with other strategies. Adams & Bernays (*7*) found that combinations of feeding deterrents were additive in their effects on the feeding behavior of *Locusta migratoria*. And Jermy et al. (*8*) found that several chemical classes of compounds were active in three plant species from the sagebrush community against Colorado potato beetle, *Leptinotarsa decemlineata*. These studies demonstrated that many plant species produce mixtures of defensive materials, which present the insect herbivore with a wide range of obstacles (*3*).

In consideration of using mixtures of plant chemicals for insect control, Gershenzon (*9*) outlined the potential advantages of combinations over single chemicals. These are that mixtures are likely more stable than that of single compounds, may act as synergists to provide greater toxicity or deterrence at lower concentrations, could slow the development of resistance to plant defenses by insects, and may be effective against a wider range of insects. Developing strategies for using mixtures of natural products in insect pest management would require a very different, and certainly a more complex integration of biological and chemical factors than for single compounds.

The phytochemical class of terpenoids has been demonstrated to function both as allelopathic and anti-herbivore agents (*10*), and one triterpenoid that has received a great deal of study and experienced some commercial success is azadirachtin. This is found in plants such as the Indian neem tree (*Azadirachta indica*), and it is the primary allelochemical constituent of several neem products. Commercial uses of the neem products are as soil amendments, fertilizer adjuvants, soap and toothpaste ingredients, human and animal pharmaceuticals, and pesticides. As an insecticide,

neem products are active against over 100 species of insects as antifeedants, growth disrupters, and repellents, and they have been used in several formulations and in combinations with many other insect and disease control measures. Structural modifications of azadirachtin itself have increased its stability and greatly expanded its use potential. After twenty years of development, neem continues to attract attention of agriculturists, environmentalists and the pesticide industry (*11, 12*).

The citrus limonoids, also triterpenoids, have been known to be active against insects since the early work of Klocke and Kubo (*13*), who determined that limonin, obacunone and nomilin disrupted development of larvae of the corn earworm (*Helicoverpa zea*) and fall armyworm (*Spodoptera frugiperda*). Since their work, citrus limonoids acting as behavior-altering and toxic allelochemicals have been shown to be active against many agricultural insect pests (*13, 14, 15, 16*). And the limonoids were recently found to increase mortality and reduce adult emergence of the mosquito *Culex quinquefasciatus* (*17*). With an estimated annual availability of 300 metric tons in the U. S. (*13*), citrus limonoids from citrus seeds have the potential as a readily available source of natural products for many insect pest management programs around the world.

We will outline the more than 10 years of study and product development undertaken at the University of Maine for citrus limonoids in the management of the Colorado potato beetle, a major and widely distributed pest of potatoes and a persistent challenge to all attempts of management. I will review the process of discovery and development of biological applications of these materials, making particular reference to the bioassay procedures used. Attempts to apply the findings from laboratory, greenhouse, and small-plot field studies into the management of the Colorado potato beetle by integrating citrus limonoids with other components of insect pest management systems will be described.

Feeding Reduction Bioassays

Based on the work of Kubo and Klocke (*13*), we first evaluated limonin as an antifeedant against several insect species. In that early period, we were surveying tropical and medicinal plants, their extracts, and the pure chemical constituents for insect feeding reduction activity. With Colorado potato beetle larvae, we found that application of pure limonin to potato leaf disks resulted in almost 90% feeding depression with dosages ≥ 30 μg /cm^2, but only in the no-choice bioassay (*16*). In a no-choice bioassay arena, the insect is exposed to either treated or untreated foliage alone, but in the choice situation, the insect is exposed to an equal amount of treated and untreated foliage. In this first study, a maximum of only 64% feeding reduction was elicited by limonin in choice tests at dosages up to 100 μg/cm^2, demonstrating the no-choice test was more sensitive than the choice assay procedure. In a later study, limonin, obacunone and nomilin, were compared in Colorado potato beetle larval feeding assays. Results from choice and no-choice assays showed no significant differences in feeding reduction among the three limonoids, but as in the earlier work, greater activity was found with the no-choice bioassay (*18*).

With other insect species, however, the choice arena was the most sensitive means of detecting limonoid activity as an antifeedant. Mexican bean beetles (*Epilachna varivestis*) and fall armyworm larvae showed higher feeding reduction activity in choice arenas (*19, 20*). Although choice tests are the most common, Bernays (*21*) warns that choice tests are not relevant except with a mobile insect. For Colorado potato beetles in the larval stage, this criticism is appropriate, but with the mobile adult stage, choice studies can be of value. In fact, epilimonol, a limonin derivative, reduced adult beetle feeding greater in the choice assay conditions than in the no-choice bioassays (*22*).

Growth and Development Bioassays

Following the feeding studies, we determined the impact of long-term exposures of the limonoids on insect growth and development. Initially, limonin and five derivatives were fed to Colorado potato beetle larvae for three days in the laboratory. Limonin, epilimonol, and limonin diosphenol significantly reduced feeding, delayed larval development, and reduced adult emergence following the three-day exposure of 30 μg/cm^2 of leaf foliage for each chemical; growth rate was not reduced, and mortality in soil as pre-pupae and pupae was not increased (*23*). These activities were seen in a series of later laboratory, field cage, and field plot studies using a crude citrus limonoid extract containing about 80% limonin. The extract consistently delayed Colorado potato beetle larval development, increased mortality and decreased insect seasonal densities in direct response to application dosages (*24, 25*).

After examining our data from the first study, we proposed that the level of beetle feeding reduction was directly related to the amount of limonin consumed (*16*). While the level of antifeedant activity of limonin was not considered particularly high when compared with other phytochemicals such as azadirachtin, we observed that insects in the no-choice arenas had greatly reduced movement and many regurgitated a few hours following consumption of limonin-treated foliage. In the no-choice bioassays, the data indicated that the insects fed until an equal amount of the limonin was consumed at each biologically active dosage. But in the choice arenas where the insects were likely to feed on both untreated and treated foliage, less limonin was consumed and overall feeding activity was greater.

To test whether feeding reduction was caused by stimulation of mouthpart chemoreceptors, limonin, epilimonol, and limonin diosphenol were applied onto whole mouthparts of fourth instars, and feeding consumption was compared to normal insects. Also, in a series of laboratory bioassays, starvation levels were tested to simulate different levels of antifeedant effects of the active limonoids (*23*) using a method described by Blau et al. (*26*). Both of these studies indicated that the primary mode of action of both epilimonol and limonin diosphenol was at the host acceptance level via the chemosensory system, and the observed growth reduction was caused mainly by feeding deterrence. The same conclusions could not be made for limonin. Therefore, we concluded that the primary mode of action of limonin against Colorado potato beetle larvae was post-ingestive.

Adult Reproduction and Behavioral Activity Studies

Adult responses to limonoids were initially studied when epilimonol was tested in short and long term bioassays against Colorado potato beetle adults for feeding reduction and oviposition suppression (22). Feeding was reduced by 65% at a dosage of 30 μg/cm^2 in the short-term no-choice assays. However, overall feeding was even more greatly reduced in the choice tests, but there was no evidence of preference for untreated foliage. In the long-term exposure tests, 10 μg/cm^2 of epilimonol evoked significant mortality and completely suppressed oviposition for up to 25 days. Even with females that had begun to lay eggs, oviposition was terminated after four days of epilimonol exposure. Importantly, epilimonol's effectiveness as an antifeedant was maintained for 9 days and as an oviposition suppressant for 25 days, indicating no habituation by the adults.

In later laboratory and greenhouse experiments with a crude citrus limonoid extract (approximately 78% limonin and 18% nomilin), adult oviposition suppression was comparable to that found with epilimonol in the earlier study (25). In the laboratory assays, the oviposition rate decreased with increasing dosage and exposure time. After three days of feeding on limonoid-treated foliage, oviposition was temporarily suppressed, but resumed after a period of feeding on untreated foliage. Oviposition suppression time also increased with increasing dosage. In the greenhouse tests, significantly fewer adult beetles were observed on limonoid-treated plants compared with control plants held in the same cages. The mean number of egg masses deposited on all plants significantly declined with increasing limonoid dosage, indicating the presence of a limonoid-treated plant appeared to interfere with oviposition on both the control and treated plants in the cages.

Colorado potato beetle adults often colonize potato fields from the field margin at the beginning of the growing season. The ability of crude limonoids to act as a repellent, thus disrupting beetle colonization behavior, was first suggested in greenhouse studies. We observed that adults exhibit a fast, erratic walking gait upon initial exposure to limonoid-treated plants but adopt a quiescent posture, often off the plant, after longer exposure (25). In another study, we found that season-long densities of adults were significantly lower in small field plots treated with 3.6 kg/ha of crude grapefruit extract than in untreated, control plots (27).

Field Application Patterns

Considering the apparent ability of adult beetles to react to the presence or consumption of limonoids and the beetles' behavior of colonizing field edges, one approach was to use a perimeter treatment of the limonoids to repel beetles and thereby prevent the colonization of the potato field by adults. In Maryland, rows treated with the insecticide imidacloprid (Admire®) and planting NewLeaf® Bt potatoes around the perimeter of a potato field provided effective control of first generation, colonizing beetles and reduced insecticide treatment by 70% (28, G. Dively, pers. communication).

Investigating similar strategies (Figure 1), we applied citrus limonoid extract to the center of field plots, leaving the perimeter rows untreated (Figure 1b), and we found 55% more adults and 82% more eggs in the untreated areas than the limonoid-treated rows. We also treated the perimeter rows, leaving the plot center untreated (Figure 1c), and we found 74% fewer adults and 81% fewer eggs in the center of these plots than in control, untreated plots. Two-row wide strips sprayed with limonoids (Figure 1d) had 38% fewer beetles and 50% fewer eggs than untreated strips. The treatment of the entire plot with the citrus limonoids (Figure 1a) resulted in a 60% reduction in adult density and 50% fewer eggs than the untreated, whole plots.
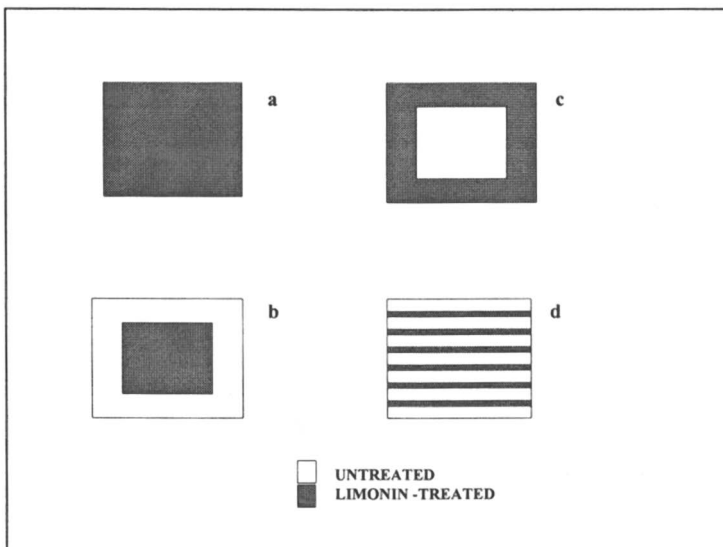
Figure 1. Application patterns of citrus limonoids in potato fields.

Field Trials

With the findings of multiple effects under laboratory, greenhouse, and small field-plot conditions, application of citrus limonoids under commercial field conditions became the focus of our efforts. Initially, we proposed a season-long management program (Figure 2) using the citrus limonoids applied at times during the growing season when their biological effects could be maximized (*24, 25*). The effects of the crude citrus limonoid extract on natural populations in the field indicated significant adult beetle density decrease in response to increasing application dosage and frequency (*25*). Adult beetle densities were significantly lower in the treated plots compared with the control plots beginning five days after application, lasting until day eight with the low rate of 3.6 kg/ha and until day 11 with

the high rate of 10.8 kg/ha. The effect of treatment on egg mass density was also apparent beginning on day five after application, when both low and high limonoid treatments were significantly lower than control. On days 8 and 11, only the high rate of limonoid treatment was significantly lower than control. This study indicated that oviposition in the limonoid-treated plots was the result of decreased adult densities rather than declines in the oviposition rate or egg mass size.
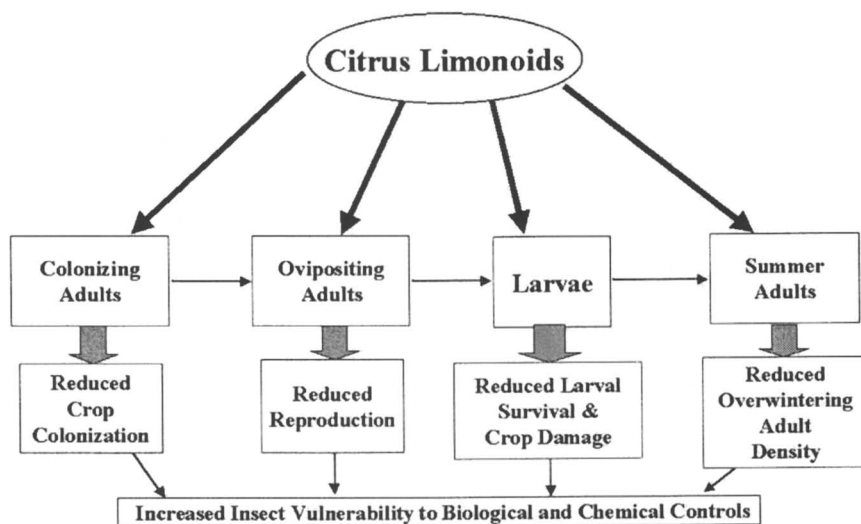
Figure 2. Season-long strategy of citrus limonoid use for insect pest management and the effects on the different life stages of Colorado potato beetles.

Among estimated beetle larval seasonal densities, which reflect the sum of effects occurring over the entire sampling period, significant linear trends of decreasing larval density with increasing limonoid application dosage and increasing application frequency were found (24). Plant defoliation was significantly reduced in response to limonoid dosage and application frequency. Three applications of 3.6 and 10.8 kg/ha of the crude extract, separated by three days each, maintained defoliation ratings significantly lower than controls for 36 days. The extract did not show any phytotoxicity when applied to potato foliage at levels as high as 100 μg/cm^2, and we saw no habituation to the crude material by adults or larvae.

Synergism Studies

Inhibition of feeding represents a strong physiological stress on feeding stages of phytophagous insects. Thus, allelochemicals may increase the susceptibility of the

pests to pathogens, predators, and insecticides (*3*). In earlier studies, plant extracts as antifeedants increased the effect of *Bacillus thuringiensis* on the larvae of *Spodoptera littoralis* (*29*). And in experiments with *Tribolium castaneum*, neem oil synergized several insecticides (*30*). In the second study, some insecticidal activities were antagonized, however, indicating that there is no general trend in the combined action of antifeedants and insecticides.

We first undertook a study to determine the compatibility and possible synergism of citrus limonoids with *B. thuringiensis* endotoxin, a biorational insecticide currently used in Colorado potato beetle management programs. In the laboratory, prior exposure to the limonoids did not alter larval mortality, but larval development was significantly delayed (*27*). In field tests, rates of adult beetle colonization and oviposition were significantly lower in plots treated with the endotoxin following treatment with limonoids. Throughout the experiment, densities of early instars in the limonoid and limonoid + endotoxin treatments remained significantly lower than the endotoxin alone and control treatments, but this was probably due to the lower egg densities in limonoid treatments. Conclusions from these experiments indicate that limonoids applied before the endotoxin do not interfere with the endotoxin's insecticidal activity. Rather, disruptive effects of these materials on reproduction are synergistic (*27*).

We expanded our limonoid – insecticide synergism studies to include the synthetic insecticides azinphosmethyl, endosulfan, esfenvalerate, and oxamyl, and in laboratory experiments we found that prior exposure to the citrus extract increased the susceptibility of Colorado potato beetle larvae to each insecticide (*5*). These results led us to explore the optimal field strategy for combining the use of natural products with insecticides, which requires precise application of lower dosages of both the limonoids and the insecticides at the most critical periods of insect susceptibility. These efforts are underway presently.

Structure - Activity Relationships, Structural Modifications, Model Synthesis, and Limonoid Field Stability

The structure of limonin is complex, containing a number of polar functional groups that are potential sites for interaction with receptors. We prepared ten structural analogs as synthetic modifications of limonin, and each was evaluated for antifeedant activity against Colorado potato beetle larvae in no-choice bioassays. We determined that the furan and epoxide groups on the C and D rings, respectively, were primary structures responsible for the antifeedant activity (*31*). Mendel et al. (*20*) showed that limonin, obacunone, and nomilin, which differ only in the structure of the A-ring, are similar in their effectiveness as larval feeding inhibitors, and, therefore, the A-ring structural characteristics are probably not critical to the types of biological activity measured in these tests. The structure - activity relationships established in these studies provided a basis for the design of a model antifeedant. We prepared two synthetic compounds based on the C and D rings of limonin and the two essential furan and epoxide groups, and both were shown to have antifeedant activity comparable to that of limonin in no-choice feeding bioassays (*32*).

Limonin in its double sodium salt form was evaluated for antifeedant activity, and the ineffectiveness of topical application of the limonin salt compared with limonin demonstrated that the integrity of the D-ring is essential for the antifeedant activity of limonin (*33*). Feeding and growth reduction with the uptake of limonin salt indicated the limonin salt was absorbed by potato leaves through petioles, translocated to other leaf tissues, and converted to limonin at low pH within potato leaves. The conversion resulted in high levels of both feeding and growth disruption. The limonin salt was not effective by topical application, as it did not cause significant feeding or growth reduction. There was no determination of the fate of the limonin salt in other potato tissues, such as the tuber itself, however.

In the Murray et al. study (*27*), limonoid antifeedant activity rapidly decreased in the field, as evidenced by increased consumption in bioassays of field-collected foliage. Nearly 50% loss in antifeedant activity occurred after 3 days, and little or no activity was observed by 8 days after treatment applications. In a later Murray et al. study (*24*), field stability assays indicated that limonoid activity declined to near zero within six to nine days after the last application. Azadirachtin has been photostabilized with the addition of UV light absorbers (*34*), and structural modifications of azadirachtin have dramatically increased this compound's half-life (J. Immaraju, pers. communication). Utility of the citrus limonoids will be greatly expanded with improvements in their environmental stability.

Multiple Strategy Model

While evaluation of the effects of the single compound provides a simple, direct measure of natural product performance for chemists, biochemists, toxicologists and physiologists, these chemicals most likely exist naturally in the plant as components of complex mixtures. Each chemical interacts with other substances and may have additive, antagonistic, or synergistic effects on the insect. The approach of incorporating an allelochemical into a multi-component system of integrated pest management may be a more fruitful approach for citrus limonoids.

Of the many substances screened for antifeedant activity, citrus limonoids are only moderate in the level of Colorado potato beetle feeding reduction caused. It is, however, the diversity of biological activities that distinguishes the citrus limonoids from the other natural products we have investigated. Citrus limonoids have several modes of action and effects on insect populations: they reduce feeding and fitness in larvae and adults, act as repellents and reduce reproduction in adults, and importantly, do not inhibit or reduce activity of other control measures. We, therefore, propose a multiple strategy model of citrus limonoid use in IPM, an approach to natural product use that integrates multiple, alternative strategies.

For example, citrus limonoids may be useful as an adult repellent and inhibitor of egg-laying, to reduce field colonization early in the season. Limonoids might further be incorporated into pest management programs by applying to plants as a foliar spray, either alone or in combination or rotation with other biological or conventional insecticides. Alternatively, with genetic engineering technology or development of a water-soluble active formulation, limonoids might be expressed by the plant or taken

up as a systemic material from in-furrow applications. Studies demonstrating the effectiveness of a strategy using a repellent-deterrent application followed by larvicidal-antifeedant applications of limonoids alone or in combination with other agents have recently been completed (Murray, et al., unpublished data).

Thus, citrus limonoids have tremendous potential as alternatives to conventional insecticides in pest management programs. The effectiveness of these unique plant compounds has been demonstrated in numerous studies. The primary impediment to acceptance and utilization of citrus limonoids for pest management is the lack of a commercially available, formulated product. Although several inquiries have been made in this arena, no producers have yet stepped forward to formulate and distribute an affordable agricultural product. However, changes in the availability of a number of conventional insecticides that are anticipated following adoption of tougher environmental and human health standards for all pesticides may lead to increased demand for alternative pest management products. Our knowledge of the effects of citrus limonoids on insects such as the Colorado potato beetle will be essential to the development and adoption of the citrus limonoid-based pest management programs.

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Chapter 16

Limonoids and the Chemotaxonomy of Citrus and the Rutaceae Family

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Introduction

The purpose of most taxonomic studies is to sort living things into a system of classification for easy reference. These systems are hierarchical with several superimposed categories beginning at the species level. Species are defined as a population of individuals who share a large number of genetic factors and can normally mate and produce progeny, which are also similar genetically. The higher categories are somewhat arbitrary classifications based on decreasing "commonalties," genus, family, order, superorder, class, etc. The older systematic classifications, from which most modern systems are derived, were primarily based on gross morphological features. Modern phytochemical analysis has only begun in earnest 50 years ago or so, and is currently being applied to taxonomic analysis as "chemotaxonomy."

The Order Rutales, as defined by Meeuse (1), is currently considered to be composed of the following families: Rutaceae, Simaroubaceae, Cneoraceae, Meliaceae, and Burseraceae. The classification of the Rutaceae dates back to the 1862 work of Bentham and Hooker (2) and has been modified by a number of researchers over the years (1, 3). The Rutaceae contains species mostly found in the tropics or subtropics, and is composed of the subfamilies Rutoideae, Dictyolomatoideae, Spatheliodeae, Flindersioideae, Toddaliodeae, Rhabdodendroideae, and Aurantioideae, as described by Engler in 1931 (4).

The commercially important genus *Citrus* is a member of the Aurantioideae subfamily, which is composed of species that are trees or shrubs with persistent leaves (in all but three cases). Most of the genera are believed to have originated in the

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monsoon belt of Southern and Eastern Asia, and has spread out through the islands of the Western & Southern Pacific. *Citrus* and its closely related genera *Poncirus, Fortunella, Microcitrus, Eremocitrus,* and *Clymenia,* have had a long history of discussion as to their proper classification. Basically there are two systems of Citrus taxonomy, developed in the early 1930s and 40s, used today: that of Walter T. Swingle (3), and of Tyozabura Tanaka (5-7). These systems have been subsequently discussed and modified by several other authors (8-10).

Plants synthesize a wide variety of chemicals, numbering well into the hundreds of thousands, perhaps even millions. Secondary metabolites have been defined historically as naturally-occurring substances that do not seem to be vital to the immediate survival of the organism that produces them; i.e. they are not an essential part of the process of building and maintaining living cells. The emerging picture from recent research, however, is that these secondary metabolites play pivotal roles in the eco-chemical functionality of the plant, as determinants in the way a particular species deals with stress in its environment. Many plants commit large amounts of physical resources to the synthesis and accumulation of secondary compounds.

Secondary compound production in plants may be triggered or altered in response to stresses, such as changes in light or temperature, competition, herbivore pressure, and pathogenic attack. They also may be critical to a plant's ability to survive and reproduce. Plants have incorporated many secondary metabolites into specialized physiological functions such as reproduction and intracellular signaling.

Comparative plant phytochemistry has become a valuable tool in plant classification (11), and is being used by botanists, chemists, and physiologists to improve on and develop a better understanding of the relationships in the classical plant classification schemes. Of special interest to taxonomists is that these secondary metabolites are often functionally unique at the species level, and often different compounds are responsible for identical functions in different species. Secondary metabolites have been generally grouped according to gross chemical structure and/or biosynthetic origin. These groups include essential oils, terpenoids, alkaloids, phenylpropanoids, amines, and amides among others. Individual compounds from these classes have many different functions in the plants, and many different effects on the microbes and herbivores that feed on them.

Much of this "emerging picture" for secondary metabolite biosynthesis and function has come about from studies initiated by the development and use of some key techniques in the years that followed the Second World War (12): the widespread availability of radioactive isotopes of carbon, hydrogen, sulfur and phosphorus for research during the 1950s; the development of new chromatographical techniques for the isolation and quantification of plant natural products during the 1950s and 60s, especially thin-layer chromatography, gas chromatography, and later, high pressure liquid column chromatography; the development of protocols for the isolation of specific enzymes involved in secondary metabolite biosynthesis from plant tissues during the 1960s; and the development of plant tissue and cell culture techniques during the 1970s. With the advent of these tools, and the recent interest in the field of plant natural products, research in this field has exploded in recent years.

Much of the chemotaxonomic work in the Rutaceae family has centered on two groups of secondary metabolites: the phenylpropanoid compounds, which includes the phenolic acids, flavonoids, lignans, etc., and the terpenoids, which includes the

mono-, di-, and tri-terpenoids. This chapter will principally discuss chemotaxonomic work over the past 20 years as it is related the taxonomic classification of the Rutaceae, especially in delineating the relation ship of a number of *Citrus* species and varieties. It will focus on the research concerning the limonoids, a class of phytochemicals that are only found in members of the Rutaceae and Meliaceae.

Another area of intensive chemotaxonomic research in recent years has centered on flavonoids in the Aurantiodaceae. While not as informative as the limonoid research, never the less flavonoid research has also contributed to the systematic evaluation of *Citrus* and its relatives (13-16). Other chemical families that have been evaluated in a number of Rutales species include the alkaloids, coumarins, acetophenones, chromones, and the quassinoids (11, 17).

Several chemotaxonomic reviews of the Rutaceae have been published, including the work of Dryer (18-20), Da Silva (21, 22), and Waterman (23, 24). A significant book on the chemotaxonomy of the Rutales was published in 1983 (25). There has been some debate over whether or not chemotaxonomic results should result in a reordering of the classic taxonomy schemes. It is our observation that while a few species may be misplaced, the limonoid analysis in general seems to reinforce much of the classical taxonomy.

The other plant family that has significant levels of limonoids is the Meliaceae, although they are biosynthetically distinct from most of the limonoids found in the Rutaceae. The limonoids of the Meliaceae are discussed extensively in several reviews (26-28), as this family contains among others, the neem plant *(Azadirachta indica)*. The limonoids of the neem have been commercially exploited for their insect repellent activity.

Additional taxonomic information is being provided by molecular biology and genetic mapping. These techniques provide unique genetic fingerprints that are being evaluated by a number of molecular biology techniques, including RFLP analysis. As more genes are mapped, cloned and sequenced, this very specific information will be used in modern systematic analysis of all plants, including those in the Rutaceae.

The limonoids are highly oxygenated triterpenoids present in the Rutaceae and Meliaceae family plants. The Rutaceae plants produce a homogenous group of closely related, largely A- and D- ring secolimonoids. The basic biosynthethic patterns and distributions are not as variable as found in the Meliaceae, and are dominated by the widespread appearance of limonin. Limonoids possess a striking and diverse set of biological activities (28). Species of the genus *Citrus* accumulate limonoids, some of which are the major bitter principles in a variety of citrus juices. These compounds have been found to possess anticarcinogenic activity in laboratory animals and cultured human breast cancer cells, and act as insect antifeedant agents, as discussed in other chapters of this book.

Over 50 limonoids have been isolated from the Rutaceae. Thirty-six have been isolated from *Citrus* and its closely related genera as discussed in the previous chapter on biosynthesis. The botanical distribution of the various plants among the major subfamilies of the Rutaceae correlates remarkably well with their limonoid content. In general, the subfamilies can be ranked in order according to their increasing ability to effect the C-19 oxidation of limonoids, e.g. Toddalioideae, Aurantioideae, and Rutoideae. Additional information on the botanical distribution of limonoids provides a sound basis for their use as chemotaxonomic indicators in this family. A great deal

of work on the chemotaxonomy of the Rutaceae was actively carried out in the 1970s and 1980s by several groups around the world (19, 22, 24, 25).

Limonoid research conducted at the USDA Fruit and Vegetable Chemistry Laboratory in Pasadena (circa 1965-1994) centered on the analysis of limonoids in the genus *Citrus* and its closely related genera (29, 30). Research in this lab and others have elucidated the structures of several of these limonoids. Much of this recent work has not been summarized and will be discussed in more detail here, including the first publication of a number of recent limonoid analyses performed on several additional Aurantioideae species by the authors, which will be cited as: Hasegawa, unpublished.

Based on radiolabel studies as discussed previously in the chapter in this book on biosynthesis, five major limonoid biosynthetic pathways have been determined in the Aurantioideae, although there are a few additional pathways found in the other major subfamilies—the Toddalioideae and Rutoideae.

The principle pathway in the true citrus species produces limonin (see representative structures in Figures 1 and 2). Limonin and its related compounds are widely distributed, present not only in the Aurantioideae, but in the other major subfamilies of the Rutaceae as well. Nearly all the members of the Tribe Citrae analyzed contain limonoids of the limonoid group, except *Atalantia* and *Severinia*. Most of the genera of subtribes Balsamocitrus and Triphasiinae examined contain only the limonin group of limonoids. Except for a few of the *Clausena* species, the tribe Clauseneae seems to produce little or no limonoids.

A second biosynthetic group was found in the genus *Fortunella* and its hybrids by Hasegawa et al. (31). In addition to accumulating the limonin group of limonoids, this species also accumulates the calamin group.

A third biosynthetic group is found in the species *Poncirus* and its hybrids. In addition to accumulating the limonin group, these species accumulate the 7α-acetate group of limonoids, but in very low concentrations.

A fourth biosynthetic pathway results in the unique limonoid ichangensin, found only in the species *Citrus ichangensis* and its hybrids (32).

The fifth pathway results in the atalantin group of limonoids, found in *Atalantia* species and *Severinia buxafolia*.

1.1 Aurantioideae: Tribe Citrae, Subtribe Citrinae

The thirteen genera of the subtribe Citrinae have been classified into three groups: the primitive-citrus fruit trees, the near-citrus fruit trees, and the true-citrus fruit trees.

1.1.1 True-Citrus Fruit Trees

The true-citrus fruit trees includes six genera: *Fortunella, Eremocitrus, Poncirus, Clymenia, Microcitrus,* and *Citrus*. The six genera of this group are all closely related and all, except the little studied genus *Clymenia,* have been successfully crossbreed with each other. The taxonomic relationships delinated from limonoid analysis for *Citrus* species and its closely related species is illustrated in Figure 3 (based on Scora (10).
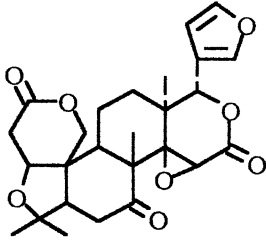
Limonin (general *Citrus*)
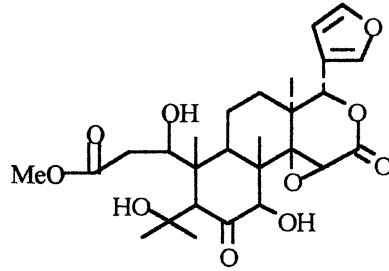
Calamin *(Fortunella)*
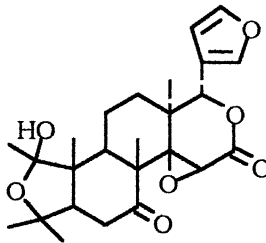
Ichangensin *(C. ichangensis)*
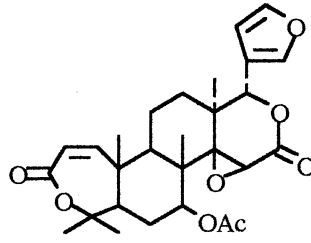
7α-Obacunyl acetate *(Poncirus)*
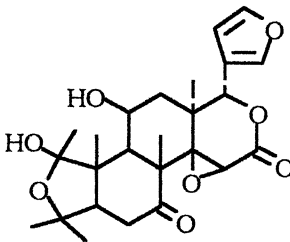
Clausenolide *(Clausenia)*
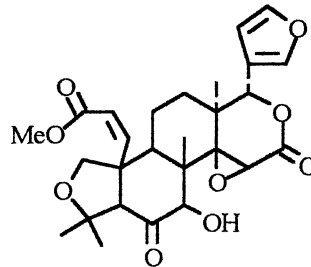
Atalantin *(Atalantia)*

Figure 1. Examples of limonoid structures from different biosynthetic classes.
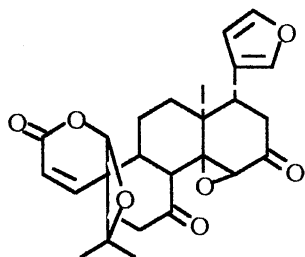
Acidissimin
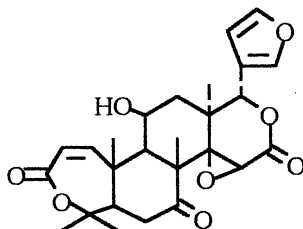
Zapoterin
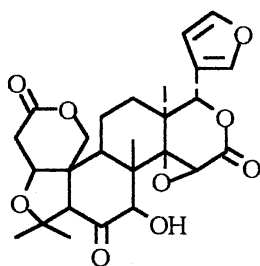
Rutaevin
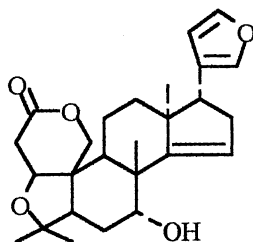
Tecleanin
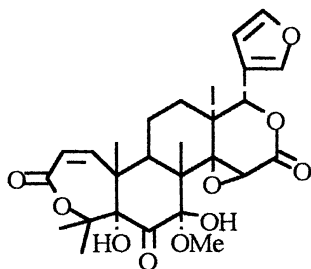
Harrisonin
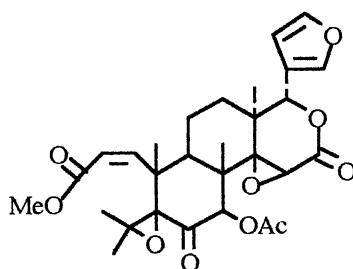
Spathelin

Figure 2. Examples of limonoid structures from different biosynthetic classes.
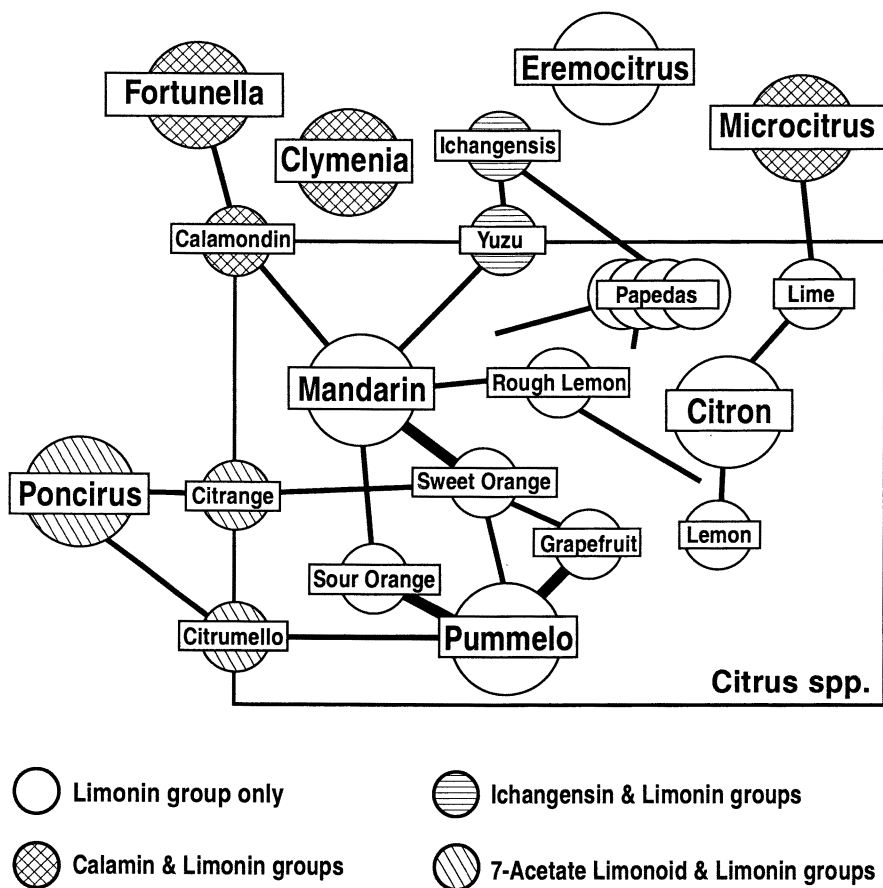
Figure 3. Chemotaxonomic relationships among the True-Citrus Tree species and hybrids. All species within the box are in the Citrus genus.

Fortunella

Species in the genus *Fortunella* and its hybrids contain the calamin group of limonoids (33-35). This includes calamin (Figure 1), and associated compounds retrocalamin, cyclocalamin, methyl deacetylnomilinate, methyl isoobacunoate diosphenol, 6-keto-7α-deacetylnomilol and 6-keto-7α-nomilol. These compounds were first isolated from seeds of calamondin *(Citrus reticulata* var. austera x *Fortunella sp.).* This species contains high concentrations of both the limonin and calamin groups of limonoids and has been used for establishing the biosynthetic pathways of the calamin group. Limonoids in the genus *Fortunella* have been intensively investigated, including *F. crassifolia, F. hindsii, F. margarita, F. japonica* and *F. polyandra.* These species contain all the calamin group of limonoids. In addition, trace amounts of limonin was found in these species, indicating that the genus also possesses the limonin biosynthetic pathway. *Fortunella obovata,* Chanshou kumquat, is a dwarf species commonly grown as a potted ornamental plant in China. This species is described as a chance hybrid between two species of *Fortunella,* but the limonoid analysis data suggest that instead it is a hybrid of *Fortunella* and one of the citrus species because it contains large amounts of limonin, nomilin and deactylnomilin in addition to calamin. Biogeneric hybrids of *Fortunella* species with *Citrus* species both contain the calamin and limonin limonoids. Eustis limequat *(Fortunella japonica* x *Citrus aurantifolia* 'Mexican") contains limonin, nomilin, obacunone, calamin, retrocalamin, cyclocalamin, 6-keto-7α-deacetylnomilol and methyl-isoobacunone. Sinton citrangequat *(Fortunella sp.* x *(Citrus sinensis* x *Poncirus trifoliata))* contains calamin, retrocalamin, cyclocalamin, methyl-isoobacunone, limonin, onomioin, and obacunone. It is of interest that *Microcitrus australasica, Microcitrus virgata* and *Clymenia polyandra* contain the limonin group and trace amounts of the calamin group such as calamin, retrocalamin, cycloclain, methyl-iso and 6-keto-7α-deacetylnomilol.

Eremocitrus

Eremocitrus glauca, the Australian desert lime, is used as rootstock for *Citrus aurantium* and other species of citrus. This species contains low concentrations of the citrus limonoids limonin and deacetynomilin (19).

Poncirus

Although the genus *Poncirus* is closely related to other genera of true citrus fruit trees which are found in only tropical or subtropical regions, this genus is native far into the temperate zone, into northeastern Asia. This genus and its hybrids provide valuable rootstock varieties for commercial citrus. Limonoid concentrations in seeds of *Poncirus* species are very low and the composition is different from that of the *Citrus* species. This genus and its hybrid, *(Poncirus trifoliata* x *(Citrus sinensis* x *Citrus paradisi)),* contain the 7α-acetate limonoid group's limonoids, such as 7α-obacunyl acetate, limonyl acetate, 1-(10-19)-abeo-obacun-9(11)-en-7α-yl acetate and 1-(10-19)abeo-7α-acetoxy-10β-hydroxyisoobacunoic acid 3,10-lactone (29, 36). The

occurrence of these limonoids distinguishes *Poncirus* cultivars from true *Citrus* species and other Rutaceae plants.

Clymenia

Clymenia is a relatively recently-described genus, of which only one species is known, *Clymenia polyandra*. Tanaka (7) considered it to be a hybrid of *Citrus macroptera* and *Citrus medica*. Later, Swingle (3) was convinced that this is a new genus, possibly having descended from a remote ancestral species to the genus *Monanthocitrus*, since the very close resemblance of leaves and petioles of this species to those from the genus *Monanthocitrus*, which is classified in the subtribe Triphasiinae. Analysis of limonoids here, however, suggest that *Clymenia polyandra* may instead be a hybrid of a *Citrus* and a *Fortunella* species, since it contains both the limonin and calamin groups of limonoids, including limonin, nomilin, obacunone, deacetylnomilin, calamin, retrocalamin, cyclocalamin, Me-isoobacunoate diosphenol and Me- deacetylnomilinate. (Hasegawa, unpublished).

Microcitrus

It is speculated that the six species of the genus *Microcitrus* are the result of millions of years of slow evolution from a primitive type in isolated regions of Australia and New Guinea. Like *Clymenia polyandra, Microcitrus australasica* possesses both the limonin and calamin groups of limonoids including limonin, nomilin, obacunone, calamin, retrocalamin, 6keto-7α-deacetylnomilol and Me-deacetylnomilinate (Hasegawa, unpublished).

Citrus: True-Citrus species

Limonoids present in the genus *Citrus* have been intensively investigated in terms of flavor, compositions, biochemistry, biological functions, by-product utilization and process chemistry. *Citrus* species and cultivars analyzed for limonoids include those of commercial importance. These include sweet orange *(C. sinensis)*, grapefruit *(C. paradisi)*, lemon *(C. limon)*, lime *(C. aurantifolia)*, sour or bitter orange *(C. aurantium)*, pummelo *(C. grandis)*, mandarins, (such as *C. reticulata, C. unshiu, C. tachibana,* and *C. deliciosa)*, and citrons *(C. medica)*, and several other hybrid species such as the Santa Barbara Rangpur lime, the Philippine red lime, and the rough lemon *(C. jambhiri)*.

Nineteen limonoids, all related biosynthetically to limonin, have been isolated from the genus *Citrus*, particularly from seeds of grapefruit, which are rich in limonoids (29, 30, 37-38). Among them, limonin, nomilin, obacunone and deacetylnomilin are the major limonoids present in *Citrus* species. Six limonoids have been shown to be bitter, but only limonin and nomilin are involved in the delayed bitterness problem of citrus juices. Limonin is the most predominant and widely distributed limonoid, and is the major cause of bitterness in a variety of citrus juices. The limonoids of the following genera have been examined and shown to contain only the limonin group of limonoids.

Citrus: Papeda-Citrus Species

The Citrus subgenus Papeda has been classified by Tanaka (7) into two sections: the section Papeda and the section Papedocitrus. The species in the Papedocitrus section are intermediate in character between the subgenus Citrus and subgenus Papeda species, with flowers much like those in the subgenus Citrus, but leaves like those of the subgenus Papeda. Papeda cultivars such as *Citrus latipes, Citrus hystrix, Citrus webberii,* and *Citrus macropylla* contain the limonin group including limonin, nomilin, obacunone, deacetylnomilin and ichangin (Hasegawa, unpublished).

A Papedocitrus species, *Citrus ichangensis* the Ichang papeda, contains an unique limonoid, ichangensin, in addition to the limonin group (32, 37). Ichangensin is biosynthesized from nomilin via deacetylnomilin and deacetylnomilinic acid. *Citrus ichangensis* contains relatively high concentrations of these two intermediate precursors in addition to ichangensin. Ichangensin has been found only in *Citrus ichangensis* and its hybrids such as Yuzu (*Citrus junos*): (*Citrus reticulata* var. *austera* ? x *C. ichangensis*), Sudachi (*Citrus sudachi*): (*C. ichangensis* x *C. reticulata* ?) and Kabosu (*Citrus sphaerocrpa*) (38-41). These species contain also relatively high concentrations of deacetylnomilin and deacetylnomilinic acid. *Citrus ichangensis* may be a unique citrus species that is unlike the other papedas.

Citrus: hybrids

Many hybrids between Citrus species and species of other genera have been created. Citrumelo *(Poncirus trifoliata* x *Citrus paradisi)* is an artificial hybrid between pummelo and the trifoliate orange. It contains limonin, deacetylnomilin and ichangin. It is of interest that nomilin and obacunone were not detected in the seeds. This limonoid compositional pattern was also observed in seeds of Rubidoux and Pomeroy trifoliate varieties, which are both hybrids of *Poncirus* and *Citrus* species. In addition, Citrumelo seeds contains very low concentrations of a single limonoid glucoside. Since the true *Poncirus* species does not contain any limonoid glucosides, the presence of limonin glucoside in citrumelo must be inherited from pummelo which does contain low levels of limonoid glucosides. This same limonoid compositional pattern was also observed in seeds of Rubidoux trifoliate and Pomeroy trifoliate, both hybrids of *Poncirus* and *Citrus* species, they contain limonin, deacetylnomilin and a trace amount of ichangin. No nomilin and obacunone, however, were detected. The total limonoid content in the seeds of these trifoliate oranges was approximately 20-fold less than that found in grapefruit seeds.

Fortunella obovata is described as a chance hybrid between two species of *Fortunella* (3), but the limonoid analysis suggests that this is a hybrid of *Fortunella* and *Citrus* species because it contains a large amount of both the limonin group (limonin, nomilin and deactylnomilin) and the calamin group (calamin, cyclocalamin, methyl deacetylnomilin and retrocalamin) of limonoids. Biogeneric hybrids of *Fortunella* with *Citrus* species contain both the calamin and the limonin groups of limonoids (Hasegawa, mostly unpublished data). Eustis limequat *(Fortunella japonica* x *Citrus aurantifolia*) contains limonin, nomilin, obacunone, calamin,

retrocalamin, cyclocalamin, 6-keto-7α-deacetylnomilol and methyl-isoobacunoate diosphenol. Citrangequat [*Fortunella* sp. x (*Citrus sinensis* x *Poncirus trifoliata*)] contain calamin, retrocalamin, cyclocalamin, methyl-isoobacunoate diosphenol, limonin, nomilin and obacunone.

Faustrimedins *(Microcitrus australasica* x *(Fortunella* sp. x *Citrus reticulata* 'Calamondin')) is a trigenic hybrid of *Citrus, Fortunella* and *Microcitrus*. Analysis of limonoids showed, however, the presence of only the limonin group including deacetylnomilin, nomilin, obacunone and limonin in order of decreasing concentration (Hasegawa, unpublished). The fortunella-specific limonoid metabolism genes may be recessive or masked by the dominant citrus limonin group metabolic genes. However, unlike many species of the genus *Citrus*, limonin is not the most abundant limonoid in this hybrid.

Citrus halimii contains the typical *Fortunella* type of limonoids, including calamin, retrocalamin, cyclocalamin, methyl isoobacunoate diosphenol, 6-keto-7α-deacetylnomilol, methyl-deacetylnominilate and a trace amount of limonin (Hasegawa, unpublished), suggesting that this species belongs to the genus *Fortunella* rather than the genus *Citrus*.

It is of interest to note that Borneo Rangpur lime may also be a fortunella hybrid as it contains calamin glucoside, in addition to the above limonoids (Hasegawa, unpublished).

1.1.2 The Near-Citrus Trees

The near-citrus fruit trees comprises two genera: *Citropsis* and *Atalantia*. The genus *Citropsis* is closely related to *Citrus*, as all its species have leaves or leaflets very similar to those of *Citrus* species in shape, venation and texture. The limonoid composition certainly supports this classification. *Citropsis gilletiana* contains the limonin group including limonin, nomilin, obacunone, deacetylnomilin, nomilinate, isolimonate, deoxylimonate, deacetylnomilinate and isoobacunoate (Hasegawa, unpublished). The seeds contain limonin glucoside. Limonoids present in *Atalantia* are, however, completely different. The genus *Atalantia* contains only the atalantin group of limonoids; there are no citrus limonoids in this genus. The atalantin group includes atalantin, atalantolide, dehydroatalantin, and cycloepiatalantin (20, 42). *Atalantia ceylanica* contains atalantin, dehydroatalantin and cycloepiatalantin, and *Atalantia citroides* contains cycloatalantin, dehydrocycloatalanin and isocycloatalantin (Hasegawa, unpublished).

1.1.3 Primitive-Citrus Trees

The primitive-citrus fruit trees comprises five genera: *Severinia, Pleiospermium, Burkillanthus, Limnocitrus* and *Hesperethusa*. This group appears to be a mixture of limonoid types. *Severinia buxifolia* contains only isocycloatalantin which is also present in *Atalantia* species (Hasegawa, unpublished). Like in *Atalantia*, none of the other groups of limonoids are present, hence *Severinia* is more like *Atalantia* in limonoid composition. It should be noted that Dreyer (43) could not find any limonoids in *Severinia buxifolia*. *Hesperethusa crenulata*, however, contains only the

limonin group including limonin, nomilin, obacunone, deactylnomilin, and ichangin. It does not contains any of the atalantin group limonoids (Hasegawa, unpublished). *Pleiospermium alatum* contains the calamin group including 7α-limonyl acetate and 1-(10-19)abeo-7α-acetoxy-10ß-hydroxyiso-obacunoic acid-3,10-lactone (44).

1.2 Aurantioideae: Tribe Citrae, Subtribe Balsamocitrinae

This subtribe is comprised of seven genera. Six species, each from different genera, have been analyzed for limonoids. All of the genera analyzed here, with the exception of the genus *Balsamocitrus*, contain the limonin group such as limonin, nomilin, obacunone, and deacetylnomilin, although concentrations are quite low (Hasegawa, unpublished). *Swinglea glutinosa*, possesses limonin, nomilin and deacetylnomilin. *Aeglopsis chevalieri* contains only limonin, as no other limonoid aglycones and glucosides were detected. *Aeglopsis chevalieri* which is found in Uganda and the Sudan, possesses limonin, nomilin and obacunone. *Aegle marmelos* seeds is rather unique as there was no limonin detected; they contain only obacunone. On the other hand, *Afraegle paniculata* which is also found in Africa, possesses limonin, nomilin, obacunone, deacetylnomilin, nomilinic acid and deacetylnomilinic acid. *Balsamocitru. dawei* did not contain any limonoids. *Limonia acidissima* (previously named *Feronia limonia*) contains limonin (Hasegawa-unpublished), acidissimin (Figure 1), a unique limonoid to this species in the root bark, and obacunone in the stem bark (44, 45).

1.3 Aurantioideae: Tribe Citrae, Subtribe Triphasiinae

Limonin has been found in *Pamburus missionis* (Hasegawa, unpublished, though Dreyer (19) did not detect any limonoids in the fruit of this species), in *Luvunga eleutherundra*, (19, 46), and in *Triphasia trifoliata* (Hasegawa, unpublished). Unfortunately, very little other analytical work has been done with species from this subtribe.

1.4 Aurantioideae: Tribe Clauseneae

The tribe Clauseneae is comprised of three subtribes: Micromelinae, Clauseninae, and Merrilliinae. The subtribes Micromelinae and Merrilliinae each have only one genera, *Micromellum* and *Merrillia*, respectively. These two species have not been examined for limonoid content. The subtribe Clauseninae comprises three genera: *Glycosmis*, *Clausena*, and *Murraya*. One species from each of these genera—*Glycosmis pentaphylla*, *Clausena lansium*, and *Murraya koenigii* have been analyzed for limonoid composition. None of these species contained limonoids, as limonoids were not detected when seeds were extracted by the buffer method which has been shown to be the most effective method for extraction of limonoids from seeds of citrus species (Hasegawa, unpublished data). Dreyer (19) also found limonoids to be generally absent from this subtribe. However, five limonoids were found in the bark and roots of *Clausena anisata*— limonin, zapoterin (shown in Figure 2), clausenolide (shown in Figure 1), clausenolide-1-ethyl ether and clausenarin. (20, 24, 47). It may be that while the seeds and fruit of these species do not accumulate limonoids, but the woody tissues do to some extent.

2. Toddaliodeae, Tribe Toddalieae

The rutaevin group (shown in Figure 2) contains various combinations of C-6- and C-7 -oxygenated substituents. This group of limonoids has been found in a number of the Toddaliodeae along with the limonin group.

2.1 Tribe Toddalieae: Subtribe Phellodendron

Phellodendron amurence contains the limonin group including limonin, obacunone and nomilin (20, 48-51). *Fagaropsis glabra* contains limonin, limonin diosphenol and rutaevin (52, 53).

2.2 Tribe Toddalieae: Subtribe Pteleinae

Several species of the genus *Helietta* contain limonin (54).

2.3 Tribe Toddalieae: Subtribe Toddaliinae

Species of the genus *Sargentia* contain rutaevin and limonin diosphenol (55). *Casimiroa edulis* and *Casimiroa tetrameria* contain zapoterin, obacunone, 7α-obacunol, deacetyl nomilin, nomilin, and limonin (18, 56). *Vespris bilocularis,* and *Vespris louisii* contain limonin and methyl epiisoobacuonate (55, 56-58).

Skimmia japonica is very unique. It contains limonoids from three true-citrus tree limonoid groups: limonin, nomilin, deacetylnomilin, obacunone, deacetylnomilinic acid, nomilinic acid, isoobacunoic acid, isoobacunoic acid diosphenol from the limonin group; calamin, retrocalamin, cyclocalamin, methyl isoobacunoate diosphenol from the calamin group; and ichangensis from the ichangensin group (59). It is of interest to note that this is the only species which possesses more than two groups of limonoids.

2.4 Tribe Toddalieae: Subfamily Amyridinae

Teclea grandifolia contains deacetylazadirone, 7-deacetylproceranone, and axadirone (60). *Teclea ouabanguiense* contains tecleanin (shown in Figure 2), 7-deacetylproceranone, 7-deacetylazadirone, 7-deacetoxy-7-oxoazadirone, and ouabanginone (61). *Teclea verdoornina* also contains these limonoids (58).

3.1 Rutoideae, Tribe Xanthoxylene

3.1.1 Tribe Xanthoxylene: Subtribe Evodiinae

The*Tetradium* genus contains a large group of related species found in Asia that has been reclassified recently. These species were once labeled in the genus *Euodia* (also spelled *Evodia)* which has about 120 species. *Tetradium glabrifolium* (formally *Euodia meliaefolia, Euodia glauca)* contains limonin, rutaevin, limonin diosphenol, glaucin A, glaucin B, and isolimonexic acid (62, 63). *Tetradium trichotomum* contains limonin and limonexic acid (64). *Euodia rutacarpa, Euodia daniella, Euodia hupenhensis,* and *Euodia fraxinifolia* all contain limonin, limonin diosphenol, and

rutaevin (17-19). *Tetradium rutacarpa* also contains limonin glucosides (67). It is of interest that only the Asian species of the *Tetradium/Euodia* group contain limonoids, these chemicals have not been found in the African members of this genus (23).

3.1.2 Tribe Xanthoxylene: Subtribe Choisyinae

Choisya arizonica, Choisya mollis, and *Choisya ternata* species native to Arizona, contain limonin and rutaevin (19).

3.1.3 Tribe Rutaea: Subtribe Dictamninae

Dictamnus albus and *Dictamnus angustifolius* contain obacunone, obacunoic acid, limonin, rutaevin, 7α-nomilyl acetate, evodol, and fraxinellone (20, 66). *Dictamnus dasycarpus* also contains limonoids (67).

3.2 Rutoideae: Tribe Diosmeae: Subtribe Calodendrinae

Calodendron capensis contains limonin, limonin disophenol and rutaevin (18).

3.3 Rutoideae: Tribe Cusparieae: Subtribe Pilocarpine

Esenbeckia hartmanii and *Esenbeckia feberifuga* has limonin, rutaevin, and limonin diosphenol (19, 68).

4. Spatheliodeae: Subfamily Spathelioideae

Spathelia sorbifolia contains the unique limonoid spathelin (shown in Figure 2) (69).

5. Dictyolomatoideae: Subfamily Dictyolomatoideae

Dictyoloma vandellianum contains deacetylspathelin and two gamma-hydroxybutenolide derivatives in the fruit and stems (70).

6. Flindersioideae: Subfamily Flindersioideae

Some *Flindersia* species contain a tetracyclic triterpene, flindercarpin 2, which appears to be an intermediate in limonoid biosynthesis (71).

7. An odd ball from the Family Simaroubaceae

Harrisonia abyssinica, which has been classified in the Simaroubaceae, has been shown to contain the limonoids limonin, harrissonin (shown in Figure 2), and 12ß-acetoxyharrisonin (72, 73). There is a close parallel in the chemistry of this plant and the genus *Spathelia*. This species may be very closely related to the Rutaceae family.

Conclusions

The limited distribution of the limonoids in plant species, the relative ease of their chemical analysis and identification, and the variation in structural complexity make this class of compounds excellent chemotaxonomic markers. Because the limonoids are related to one another biosynthetically, this allows for correlation with the various levels of taxa to a degree not possible with many other phytochemicals. Rutaceae species in general produce limonoids related biosynthetically to limonin. Modification of the C-methyl groups through oxidation is an unequivocal structural feature of the limonoids. The Rutaceae species are very limited in this aspect, with such oxidation occurring only at the C-19 methyl group. This oxidation occurs at a progressively higher degree in the Cneoraceae species and in the Meliaceae species. It seems to be a good rule of thumb to say that if one species of a genus produces limonoids, all the species in that genus will produce limonoids. The qualitative limonoid pattern for a particular individual plant can be used to elucidate its parentage and its position in the taxa. This methodology has worked very well for the examination of various citrus hybrids. It can be used to earmark species, even those that would not have been considered from a classical taxonomic viewpoint such as *Skimmia japonica* and *Harrisonia abyssinica.* which are potentially genetically compatible for the new molecular biology-based breeding programs.

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Chapter 17

Genetic Evaluation and Modification of the Accumulation of Limonoids in Citrus

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Abstract. UDP-D-glucose:limonoid glucosyltransferase (LGTase) is a key enzyme in the regulation of limonoid glucoside accumulation in citrus fruit. We have isolated a cDNA clone for LGTase which will be used to create transgenic citrus plants. We have isolated a full length cDNA clone (CitLGT) from a cDNA library prepared from the albedo of mature satsuma mandarin (Citrus unshiu Marc.). The isolated CitLGT has 1732 bp and codes for 511 amino acid residues which corresponds to the molecular mass of 57.5 kD of the purified LGTase protein. The gene expression was investigated by northern analysis on the transcriptional level. The accumulation of mRNA for CitLGT increases in albedo as fruit matures. Transgenic plants containing additional copies of the LGTase gene will be important for the examination of the accumulation of limonoids in citrus. The prospect of creating citrus species that have reduced limonoid bitterness by genetic transformation is discussed.

The creation of commercial citrus fruit that has reduced limonoid bitterness is an important objective of citrus breeding. Limonoid bitterness in citrus juices, or in citrus fruit damaged by frost in the early stage of maturation, is a serious problem in the citrus industry. Currently, the bitter limonin component in citrus juices can be removed by adsorption methods during processing. However, a natural debittering process in maturing fruit has been identified. Limonoid aglycones are converted to glucosides in fruit and seed during the latter part of fruit maturation (1). The reaction is catalyzed by the enzyme UDP-D-glucose:limonoid glucosyltransferase (2). Compared to the constitutive activities of the biosynthetic pathways from nomilin to limonin, LGTase activity increases in fruit pulp and albedo as maturation progresses

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(2). This explains why limonin bitterness occurs in juice extracted from some early maturing (mid-season) fruit, such as navel orange (2). However, juices extracted from early maturing satsuma mandarins rarely have as much of a bitterness problem as navel orange juice (3). The regulatory mechanisms involved in the natural debittering process in different Citrus species has not yet been fully characterized.

Limonin glucoside is the major limonoid glucoside accumulated during fruit development (4) and consistently accounts for 70% of the total limonoid glucosides observed (5,6). LGTase is a key factor regulating limonin glucoside accumulation in fruit. The observed biological activities of limonin glucoside, especially anti-cancer activity in laboratory animals (7,8) and in cultured human breast cancer cells model studies (9), indicate the importance of these compounds for human health. We have isolated and characterized citrus LGTase gene in an effort to analyze the molecular mechanism of limonoid glucoside accumulation in citrus. This gene will be used to create transgenic citrus plants that may have lower levels of bitter limonoids through the enhancement of the natural debittering process (10).

LTGase gene isolation from citrus fruit

The isolation of total mRNA from fruit requires a special procedure of extraction and purification due to the high polysaccharide content in fruit tissue. Using the phenol-chloroform extraction method, we have successfully isolated high quality mRNA from fruit pulp or rind of citrus. We have used this mRNA to construct cDNA libraries for several Citrus species (11). In research on the characterization of citrus genes, several full length cDNA clones have been obtained, sequenced, and expressed in citrus cell culture including metallothionein-like gene (12), sucrose phosphate-synthase gene(13), carotenoid-associated protein gene (14) and chalcone synthase gene (15). Random sequencing analyses have also been performed on the cDNA libraries derived from mature fruit albedo and pulp (12, 16) and from immature fruit segments (17) to catalogue the expressed gene repertories. More than 50% of the clones obtained contain complete coding sequences with their translation initiation codons so that these libraries can be used to isolate full length cDNA clones. The gene sequences in these cDNA libraries from mature fruit albedo cover a wide range of expressed genes involved in secondary metabolism, such as the isoprenoid biosynthetic pathway, isoprenoid cyclization, and plant steroid synthesis (16). The isolated mRNA from citrus fruits can be used to investigate the transcription expression levels of specific genes during the course of fruit maturation by the northern blot analysis (12-15).

It is important to characterize the genetic   isoforms of any given gene product which may have different gene structures and expression patterns. In the case of the enzyme sucrose phosphate synthase (18), there are 3 genetic isoforms which differ in sequence structures, expression stages and genome localization. In the case of the enzyme chalcone synthase, there are two types of gene isoforms, which are regulated differently during the course of embryogenesis in citrus suspension culture (15). Therefore, the copy number and transcription patterns of any specific gene should be confirmed before further detailed analysis of the genetic expression or before the

manipulation of plant genetic sequences by gene engineering to attempt to alter physiological events in the plant cell.

Strategy for LTGase gene isolation and expression analysis

1. Isolation of LGTase cDNA clone from citrus

LGTase has been isolated from the albedo tissue of Newhall navel orange *(Citrus sinensis* Osb.) (19). The amino acid sequence of the N-terminal region of the purified enzyme was determined by an automated Edman's method from digested peptide segments separated by SDS-PAGE and blotted on PVDF membrane (20). Several internal peptide sequences were determined after digestion with *S. aureus* V8 proteinase (21). A pair of degenerate primers, based on the N-terminal and internal amino acid sequences, were used to generate a PCR-amplified DNA fragment from both citrus genomic DNA or first strand cDNA (22). A cDNA library derived from albedo of mature satsuma mandarin fruit *(Citrus unshiu* Marc.) (16) was used to isolate full length a cDNA clone (CitLGT) which encodes the full length of the LGTase gene (23). The CitLTG clone is composed of 1732 bp which would produce a protein with a deduced molecular mass of 57.5 kD, corresponding to that of the purified LGTase enzyme (19).

The CitLGTase clone has been confirmed as a clone for the citrus LGTase enzyme—it contains the motif sequences which are common to the N terminal region amino acid sequence and to UDP-binding domain amino acid sequence of other glucosyltransferase genes (23). Furthermore, the fusion protein produced by the expression of CitLGT possesses the enzymatic activity to catalyze the transfer of glucose from UDP-glucose to limonin.

2. The genomic structure of the LGTase gene

Southern blot analysis for citrus genomic DNA suggested that LGTase gene is in single locus in citrus genome. PCR fragments covering full coding region of LGTase genomic DNA has shown similar sequences in many other Aurantioideae species (23). This suggests that there are no functionally significant substitutions in the amino acid sequences of LGTase genes among *Citrus* and its allied species, although there are a variety of limonoid glucosides produced. Independent from the production of different limonoid group in *Citrus junos, Fortunella crassifolia* or the non-production of limonoids in *Glycosmis pentaphylla,* the genomic sequence of the LGTase gene may be conserved throughout most of the species of the Aurantioideae subfamily. The results supported the prediction that LGTase most likely accepts all the citrus limonoid aglycones as substrates and converts them to their respective glucosides (19).

It is possible that the bitter limonin aglycones remaining in sweet orange or grapefruit are not the result of low activity of LGTase in these species. The analysis of the regulation system of LGTase activity will provide new avenues for the analysis of limonoid accumulation, and subsequent conversion to the glucoside forms.

Furthermore, genetic analysis on aglycone/glucoside ratio in the segregating generations of the cross hybridizations between sweet orange and mandarin can be used to investigate the genetic factors regulating bitter limonoid aglycone accumulation.

3. Expression analysis of limonoid GTase gene

The accumulation of LG in fruits is initiated during the transition from fruit cell expansion to final maturation. The LTGase enzymatic activity has only been detected in mature fruit and seed, and not in leaves or immature fruit. As suggested by Fong et al. (2), the glucosylation of limonoids occurs in the albedo, juice sacs, and seeds as maturation proceeds. The expression of the LGTase gene at the transcriptional level has been investigated by northern blot analysis for mRNAs probed with CitLGT, from Washington navel fruit and satsuma mandarin fruit at different stages of development (23). The limonin glucoside content in satsuma mandarin increases at an earlier stage than that of navel orange (3, 24). This suggests that the timing of the onset of LTGase gene transcription is different among the various *Citrus* species, and influences the activity of LGTase during fruit development. How the transcription of LGTase gene is differentially regulated among the species is not clear. Furthermore, the transcriptional activity was detected in leaf and stem tissues where no measurable levels of limonin glucoside have been detected. Future research on this type of genetic analysis and analysis of regulatory factors for LGTase gene will provide us with important evidence concerning the molecular aspects of LG accumulation and breeding.

Prospects for the creation of transgenic citrus free from limonin bitterness

1. Tissue culture and the genetic transformation system in citrus

The genetic transformation using the isolated LGTase gene will be the most effective method to confirm the action of the gene in citrus plants. Enhancement of the LGTase activity by genetic engineering to provide additional copies of the gene is expected to result in reduced aglycone concentrations (10). An effective plant tissue culture system and an effective gene delivery system has been established in citrus which is required for successful genetic transfer. As embryogenesis can be induced from ovular callus of citrus by changing sugar composition (25, 26), the callus culture system has become a reliable tool for the somatic cell manipulation. Using this system, considerable work has been performed resulting in the genetic transformation of several citrus species. Suspension cell cultures from embryogeneic callus have been transformed using the *Agrobacterium*-mediated method (27) and plantlets have been obtained. The success of the *Agrobacterium*-mediated gene delivery method is largely dependant on the host-range of the bacterial strain for citrus species. Both sweet orange and grapefruit are good candidates for the method using strain LBA4404 (27). *Agrobacterium* strain A281 is more virulent to citrus (28), and the

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EHA105 strain (29) (with disarmed plasmid from A281) was also used for citrus transformation of lime *(C. aurantifolia)* (30). These methods were used to transform the mandarin species.

The direct gene transfer to protoplast from suspension cells in citrus have also been reported using the polyethyleneglycol method (31, 32) or the electroporation method (33). The particle bombardment method has been used as gene delivery system in tangelo (34).

The range of candidate species has become wider using the direct gene transfer methods, but the efficiency of obtaining successfully transformed plants through somatic embryogenesis has been rather low with these methods. To improve this low plant regeneration efficiency, the regeneration system of adventitious buds from in vitro-cultured stem segments used in Carrizo citrange (35) may be used. A similar method in which epicotyl segments were inoculated with *Agrobacterium* resulted in much higher number of transformed plantlets (36).

These gene delivery systems have been successfully applied to citrus to produce transformed plants carrying expressed foreign genes such as the CTV coat protein in sour orange (37) and human epidermal growth factor (hEGF) in trifoliate orange (38). Because these foreign genes are functional in the transformants, similar procedures of genetic transformation can be applied using the isolated LGTase gene to create transformants in citrus varieties with enhanced LTGase activity.

2. Assay of LG production in transfomed citrus plants and tissues

Because the LGTase gene is a single copy in the citrus genome, it is expected that the control of transformant gene expression will be simpler than those of transformed genes with a number of isoforms or complex gene family structures. The *in vivo* assay system for the activity of the introduced LGTase gene must be established, because citrus transformants will take many years to mature before producing fruit that can be examined for altered limonin glucoside accumulation.

The cell suspension culture method previously used for the evaluation of the LGTase gene in citrus should also be appropriate for the assay of transformant gene activity. Undifferentiated citrus callus cells usually do not contain significant levels of major secondary metabolites (39, 40). The flavonoid and limonoid content rapidly decreases during callus induction and subculture from intact fruit tissue (41), possibly because of a lack of key biosynthetic steps, such as chalcone synthase in flavonoid production (15). Limonin content decreases during callus production from original fruit tissue, indicating that no biosynthesis takes place in the callus without a phloem tissue system (42). Some metabolic steps are maintained in the active state—the cell suspension system has been used for the bioconversion of exogenously added flavanones to their respective glucosides (43). Sweet orange suspension callus cells convert exogenously added nomilin to obacunone and limonin (unpublished observations). In theory, transformed Washington navel orange callus cells containing the constitutively expressed LGTase gene should be able to convert exogenously added limonin to limonin glucoside.

Biosynthesis of limonin and nomilin from   mevalonate and acetate has been confirmed only in phloem tissue where glucosylation activity has not been detected. It

will be interesting to examine regenerated shoots and plantlets, transformed with LGTase gene in both *Agrobacterium*-inoculated epicotyles and suspension cells for altered accumulation of limonoid glucosides.

It may take several years to assess the effects of introduced gene on LG accumulation in fruit due to the long juvenile period of plants regenerated from embryonic culture or by adventitious bud formation. However, an effort to by-pass the juvenile period of these transformation procedures has been reported (43). Stem segments obtained from green-house grown adult trees were transformed by *Agrobacterium* and the adventitious shoots subsequently regenerated were promoted by *in vitro* shoot tip grafting. The regenerated shoots were then top-grafted on mature tree branches. The procedure promoted the flowering and fruiting of transformed shoots within 14 months after inoculation. This technique may be effective for the rapid evaluation of LGTase gene transformants for altered limonoid glucoside accumulation in fruit.

3. Future steps in the genetic modification of LG accumulation

There have been numerous experiments reported in which citrus species have been transformed with foreign genes which have subsequently expressed their proteins. However, as yet there have been no transgenic experiments reported using genetic material isolated from citrus. Transgenic plants with the citrus LGTase gene will provide pioneering methodology for the examination of the regulation of gene expression in citrus. This will provide us with new information about the interaction between the original genetic material and introduced gene. Furthermore, these experiments will show us how limonoid accumulation might be modified through alteration of the isoprenoid pathway, which may in turn have an effect on the other plant triterpenoid and steroid metabolism. Limonoids are synthesized from mevalonate via the isoprenoid pathway (45). There is no information about the competitive relationship of biosynthesis between limonoids and other plant isoprenoids, such as the steroids, which are both produced from geranyl pyrophosphate via squalene. In recent analysis of mutant panels of Arabidopsis, key steps of plant steroid biosynthesis has been identified in relation to the dwarfing phenotypes. Because of the important role of plant steroids on the growth, the over-expression of LGTase gene in sweet orange may influence the balance of those pathways in the transformed citrus plants. There is a report that maize lines transformed to enhance carotenoid synthesis were weak growing plants due to low GA biosynthesis (46). The metabolites may have been funneled from GA biosynthesis to the carotenoid biosynthesis. To avoid such side effects in LGTase transformants, understanding the proper regulation of transformed gene will be important.

The promoter region of the gene largely influences the specific timing and transcription expression levels of the gene product. However, fruit-specific promoters have not been isolated. The native LGTase gene appears to be expressed specifically in maturing fruit. This specific regulation of the LGTase gene expression will be important for the investigation of the role of limonin glucoside accumulation in citrus fruit. In many cases of plant genetic transformation, the inserted genes have been driven by the CaMV 35S protein gene promoter and are expressed constitutively in all the plant tissues. The use of CaMV35S promoter with the LGTase gene would allow it to be transcribed constitutively in all tissues and stages in the transformed citrus

plants. In such cases, newly generated nomilin on stem phloem may be converted directly to nomilin glucoside or other limonoid glucosides. The production of limonoid glucosides in stem or leaf has not been known so that the translocation of the glucoside could be analyzed. Equally important will be the characterization of the natural LGTase promoter. Recently, a metallothione-like protein gene of citrus was shown to be expressed strongly in fruit during development and maturation as compared to the other tissues (12). The use of a transformant assay using a mature fruit-specific promoter will help to unravel the molecular aspects of limonoid accumulation in citrus, and possibly more about the control of secondary metabolism in general.

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