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dissertation entitled

SEPARATION AND QUANTITATION OF LIMONOIDS AND FLAVONOIDS IN JUICE AND BY-PRODUCTS OF SWEET ORANGE (*Citrus sinensis*)

presented by

Korada Sunthanont Saipetch

has been accepted towards fulfillment
of the requirements for the

Ph.D. degree in Food Science

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Major Professor's Signature

Jan. 5. 2004

Date

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SEPARATION AND QUANTITATION OF LIMONOIDS AND FLAVONOIDS IN JUICE AND BY-PRODUCTS OF SWEET ORANGE (*Citrus sinensis*)

By

Korada Sunthanont Saipetch

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Food Science and Human Nutrition

2004

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ABSTRACT

SEPARATION AND QUANTITATION OF LIMONOIDS AND FLAVONOIDS IN JUICE AND BY-PRODUCTS OF SWEET ORANGE (*Citrus sinensis*)

By

Korada Sunthanont Saipetch

Two major classes of citrus phytochemicals, limonoids and flavonoids, have attracted considerable attention from science and industry because of their pharmacological properties. Large production of by-products accompanying orange juice production offers inexpensive starting materials for recovery of secondary metabolites with potential anticarcinogenic and cardioprotective activities.

The objectives of this study were 1) to determine limonoid and flavonoid content of orange juice and by-products resulting from commercial orange juice processing, and 2) to determine the influence of lime treatment on these phytochemicals.

Limonoid and flavonoid content was determined in various by-products (seed, peel, peel press cake, rag, and peel press liquid) and orange juice from commercially grown orange varieties (Hamlin, Parson Brown, and Valencia). Twenty one compounds in the categories limonoid aglycones, limonoid glucosides, flavanone glucosides, and polymethoxylated flavones were analyzed.

Seeds had the highest content of limonoids, while peel and peel press cake had the highest concentrations of flavonoids. Water removal by pressing extracted limonoid glucosides and polymethoxylated flavones from the peel into peel press liquid, but concentrated limonin in peel press cake. Average g/100g dry wt. of total contents in solid fractions from three varieties were 7.1 (flavanone glucosides), 4.1 (limonoid glucosides),

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1.2 (limonoid aglycones), and 0.26 (polymethoxylated flavones); and in liquid fractions were 0.150 (flavanone glucosides), 0.072 (limonoid glucosides), 0.009 (polymethoxylated flavones), and 0.002 (limonoid aglycones). Limonoid glucosides are rich in edible orange fraction and are extracted into the juice. The results show that rags containing seeds are good sources for limonoid aglycones and limonoid glucosides, while peels and peel press cake are good sources for flavanone glucosides and polymethoxylated flavones. Peel press liquid is a potential source for limonoid glucosides and polymethoxylated flavones after evaporation to the molasses end-product. Orange juice is a good source of limonoid glucosides.

In the lime study, limonoid and flavonoid content in waste products were measured before and after lime treatment. CaO was added to peel and rag, primary waste materials, so that the final concentration of CaO was 0.3% CaO (wet wt.). After 48 hours, the samples were pressed to yield press cakes and press liquids. Seeds were treated with 0.3% CaO (wet wt.) separately. These fractions were analyzed for the content of limonoid aglycones, limonoid glucosides, flavanone glucosides and polymethoxylated flavones.

With lime treatment, more limonoid aglycones (25%) and limonoid glucosides (12%) leached from press cake into press liquid (rag and peel). Overall, there was a trend for increased phytochemical content release from press cakes into press liquids due to lime treatment. In seed, lime treatment resulted in losses of limonoid glucosides, but had no effect on limonoid aglycones, flavanone glucosides or polymethoxylated flavones. Lime treatment resulted in increased phytochemical content in press liquids especially limonoids.

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ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Dr. Mark Uebersax for his continued support and guidance throughout my graduate studies at Michigan State University. His vision and insight were invaluable to my development as a well-rounded scientist.

I would like to especially thank Dr. Maurice Bennink for his guidance and unwavering confidence in me. It is safe to say that without his efforts, I would still be working in the lab. And in the years that I have spent at this great University, I am grateful that Dr. Bennink was not only my research advisor, but also my friend.

Special thanks to the members of my committee, Dr. Jerry Cash (Department of Food Science and Human Nutrition) and Dr. Ray Hammerschmidt (Department of Plant Pathology) for their valuable contribution throughout my graduate study.

I wish to express my appreciation to the Tropicana Products, Inc. (Bradenton, FL) for providing pilot plant space for me to prepare my samples and for their partial financial support for this research.

Thanks to scientists from the USDA [Dr. Gary D. Manners (Pasadena, CA), Dr. Mark A. Berhow (Peoria, IL), and Dr. John A. Manthey (Winter Haven, FL)] for their valuable limonoids and flavonoids standards.

I want to thank p. Mum, p. Yim and p. Kok for their warm support and yummy Thai foods. My big sisters were always there and willing to help. I would like to thank all my friends here at Michigan State University for their support and encouragement. All of whom made my being away from home a much better experience.

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encouragement, support
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always been true givers
everything you taught m
my little sister (Kook)
when I was down.

Finally, I would
for your endless love a
a big part of my succes

Many thanks to my Lab 110 gang. In the years spent pursuing this degree, I had the pleasure of spending it with four amazing friends: Abby, Kathy Lai Pui Kwan, Mar, and Mo. Actually, we spent so much time in the lab I think we should have paid rent. I will always remember even those occasional "little" moments that put our miserable lives as graduate students in perspective; that one day we too will live "normal" lives as well—the noodle soups at 2 am and watching taped Friends and the Bachelor with my gang. Your friendships, I will always cherish.

I want to thank my entire family. Especially to my mom-in-law (Mary Saipetch), my dad-in-law (Anan Saipetch), my brother-in-law (Poon) and n. Ohm for their love, encouragement, support, and belief in me. I am forever thankful to be a daughter of my mom (Suchada Sunthanont) and dad (Kampol Sunthanont). All my life, they have always been true givers. Thank you mom and dad for your unbelievable sacrifice and for everything you taught me. I would not have come to this far without you. Also thanks to my little sister (Kook) for always being there for me and occasionally helping me up when I was down.

Finally, I would like to thank Auan, my husband and my best friend. Thank you for your endless love and understanding. Your positive attitude and sense of humor were a big part of my success.

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APCI: atmospheric chem

BHT: butylated hydroxy

C: carbon-13 isotope

CE: capillary electropho

EI: electron impact ion

ESI: electrospray ioniza

-eV FAB: negative fast

+eV FAB: positive fast

DD: didymin

DNAG: deacetylnomil

DNM: deacetylnomilir

DNG: deacetylnomilir

ERT: eriocitrin

FAB: fast atom bomb

FTNMR: fourier trans

FCOC: Frozen concer

GC: gas chromatogra

H: deuterium

HD: hesperidin

HP: 3,4,5,6,7,8,3',4'

HPLC: high perform

LIST OF ABBREVIATIONS

APCI: atmospheric chemical ionization

BHT: butylated hydroxytoluene

^{13}C: carbon-13 isotope

CE: capillary electrophoresis

EI: electron impact inonization

ESI: electrospray ionization

-eV FAB: negative fast atom bombardment

+eV FAB: positive fast atom bombardment

DD: didymin

DNAG: deacetylnomilinic acid glucoside

DNM: deacetylnomilin

DNG: deacetylnomilin glucoside

ERT: eriocitrin

FAB: fast atom bombardment

FTNMR: fourier transform nuclear magnetic resonance

FCOJ: Frozen concentrate orange juice

GC: gas chromatography

^{1}H: deuterium

HD: hesperidin

HP: 3,4,5,6,7,8,3',4'-heptamethoxyflavone

HPLC: high performance liquid chromatography

EX: 3,5,6,7,3',4'-hexame

HP: 3,4,5,6,7,8,3',4'-hept

IR: infrared radiation

L: limonin

LC: Liquid chromatograp

LG: limonin glucoside

MS: mass spectrometry

MS/MS: tandem mass sp

NAG: nomilinic acid gl

NG: nomilin glucoside

NBT: nobiletin

NHD: neohesperidin

NFC: not from concent

NG: nomilin glucoside

NM: nomilin

NMR: nuclear magnet

NT: naringin

NT4'-G: naringin-4'

O: obacunone

OG: obacunone gluco

PDA: photodiode arra

PC: paper chromatog

S: sinensetin

HX: 3,5,6,7,3',4'-hexamethoxyflaovne

HP: 3,4,5,6,7,8,3',4'-heptamethoxyflavone

IR: infrared radiation

L: limonin

LC: Liquid chromatography

LG: limonin glucoside

MS: mass spectrometry

MS/MS: tandem mass spectrometry

NAG: nomilinic acid glucoside

NG: nomilin glucoside

NBT: nobiletin

NHD: neohesperidin

NFC: not from concentrate

NG: nomilin glucoside

NM: nomilin

NMR: nuclear magnetic resonance

NT: narirutin

NT-4'-G: narirutin-4'-glucoside

O: obacunone

OG: obacunone glucoside

PDA: photodiode array

PC: paper chromatography

ST: sinensetin

SME scutellarein tetra

TLC thin layer chroma

T tangeretin

UV Ultra violet

STME: scutellarein tetramethylether

TLC: thin layer chromatography

TT: tangeretin

UV: Ultra violet

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INTRODUCTION

Overwhelming evidence has indicated that a plant-based diet can reduce the risk of chronic disease, particularly cancer. In 1992, a review of 200 epidemiological studies (Block et al., 1992) showed that cancer risk in people consuming diets high in fruits and vegetables was only one-half that in those consuming fewer of these foods. It is apparent that there are components in a plant-based diet other than traditional nutrients that can reduce cancer risk. Steinmetz and Potter (1991a) identified more than a dozen classes of these biologically active plant chemicals, commonly termed "phytochemicals." The protective effects are commonly attributed to antioxidant activity, although recent work has shown additional role of these polyphenolic components of the higher plants (Hertog et al., 1993), which may act as antioxidants or agents of other complex mechanisms that contribute to their anticarcinogenic or cardioprotective actions. Although oranges, lemons, limes, and grapefruits are a principal source of such important nutrients such as vitamin C, folate, and dietary fibers, Elegbede et al. (1993) have suggested that another component is responsible for the anticancer activity. Citrus fruits are particularly high in classes of phytochemicals known as the limonoids (Hasegawa and Miyake, 1996) and flavonoids (Benavente-Garcia et al, 1997). Beside health-related properties, these compounds also have shown possibility to functionally serve as antioxidants, insecticidal agents and taxonomic tracers.

USDA National Agricultural Statistics Service has reported that the citrus production by eighteen major countries is approximately 73 million metric tons in 2002 (FAS/USD, 2003). Among the total citrus agronomic production classes, sweet orange

Citrus sinensis) accounts

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(*Citrus sinensis*) accounted for 68% of the total. Two major orange producing countries, Brazil and the United States, have contributed to 60% of the world production with 85% of the total as further processed products. Mediterranean countries have also started to produce and process oranges in significant amounts. These data show that there is also a large amount of processing by-product available. Approximately two million dry tons (dry basis) of these residues are generated annually in those two major citrus-processing countries (Grohmann et al., 1999). The major by-products include dried pulp, molasses, washed pulp solids, and essential oil. The peel residue is the primary fraction, accounting to almost 50% of the fresh fruit weight. This part of the fruit is the source of dried pulp, molasses, cold-press oils, d-limonene, pectin, potential seed derived products, and flavonoids (Braddock, 1995). Processing practices set minimum levels of by-product incorporation ("add back"), because of an impact on flavor, texture or appearance. Additionally, there are numerous regulatory standards to control for economic adulteration of high value juices. As a result, the bulk of these components with potential health benefits are processed into cattle feed for sale at 5-10% above the cost of processing (Widmer and Montanari, 1996). Therefore, these by-products would be an effective source of functional food additives or pharmaceutical products.

The goal of this project is to utilize sensitive analytical methods for identifying and quantifying the limonoids and flavonoids in edible and inedible fractions of oranges to enhance the potential utilization of phytochemical from citrus by-products obtained from commercial orange juice production.

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Citrus juice processing

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LITERATURE REVIEW

Citrus juice processing

Development of the frozen concentrated orange juice (FCOJ) industry is responsible for the most significant increase in citrus fruit consumption since World War II (Ting, 1980). This innovation solved many p roblems associated with citrus fresh fruits such as storage diseases, susceptibility to physiological disorders and requirements for rapid transportation. Citrus fruits and their products are an important source of vitamin C in the American diet, and are becoming increasingly more important to other developed and developing countries. The health-promoting aspects of citrus, together with its appealing color and delightful aroma and taste, make it the most popular of the processed fruit products. Improved technology of citrus production, processing, storage, and transportation have placed the product economically within reach of more consumers.

Citrus trees are cultivated in tropical and subtropical regions throughout the world. The trees can grow in a wide range of soil, yet growing conditions such as climate (temperature and rainfall), types of soil and cultural practices, have a large influence on the quality of fruit produced and juice extracted (Anonymous, 1998).

During 2001-2002, the world production of citrus fruit was approximately 73 million metric tons, of which 49% was marketed as fresh fruit and 42% was converted into processed products USDA/FAS (2003). In that same time period, the-top five citrus producing countries were Brazil, the United States, China, Mexico, and Spain, together producing approximately 74% of world production. Sweet orange (*Citrus sinensis*) has been the main citrus produced (68%), followed by

angerines (*Citrus re*

(*Citrus limon*) (6

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Cost efficiency

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tangerines (*Citrus reticulate*) (18%), grapefruit (*Citrus paradisii*) (5%), and lemon (*Citrus limon*) (6%). In the United States, of all sweet oranges produced, 15% were marketed as fresh produce. The remaining 85% accounted for processed products, primarily orange juice.

Cost efficiency and juice yield are very important to citrus juice processors. Extracting equipment has been developed to increase juice yield while maintaining juice quality. Process designs have been developed to effectively use energy. Further it is also very important to increase by-product applications to help maximize the profit and minimize the waste produced from this growing sector of the juice market (Anonymous, 1998).

One of the long-standing sensory problems in processed citrus products, especially orange and grapefruit juices, has been bitterness, generally associated with astringent "after taste". The level of bitterness varies among the different cultivars and cultural practices. Bitter juices have a much lower market value; therefore, debittering of citrus juice has been investigated extensively. Primary studies involved applications of adsorption and ion exchange techniques (McColloch, 1950, Chandler et al., 1968, Nisperos and Robertson, 1982, Couture and Rouseff, 1992). Other debittering methods include super critical carbon dioxide (Kimball, 1987), immobilized naringinase (Gray and Olson, 1981), and immobilized bacteria which metabolize limonoids (Hasegawa, 1987). Notwithstanding the fact that these techniques have been able to effectively remove bitter compounds from citrus, they are still not practical for commercial routine operation due to the additional steps of cleaning up and regeneration, column clogging, and associated

off-flavor problems

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Citrus by-products

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off-flavor problems. In addition to juice debittering treatments, preventions of bitterness formation were also studied at pre- and post-harvest levels. Treatment with auxin on fruit-bearing plants showed reduction of limonin precursor (limonoate-A-ring lactone) in Navel orange fruit by 10-23% (Hasegawa, 1988). Post-harvest treatments of citrus fruits with ethylene showed no effect on naringin content in Suisho-buntan (*Citrus grandis [L.] Osbeck*) fruits (Nishikawa et al., 2002), but substantial loss in limonoate-A-ring lactone in Navel orange, lemon, and grapefruit (Maier et al., 1973). It should be noted that a widely used debittering method has been to dilute the bitter juice with non-bitter juice (Hasegawa, 2000). The most promising solution to this problem is to create a new variety with high debittering activity and low aglycone concentration through genetic engineering (Hasegawa, 2000). Figure 1 shows a diagram highlighting of a typical commercial orange juice production and illustrating the primary products and by-products.

Citrus by-products

Accompanied by the increased production of orange juice, large amount of fruit residues, accounting for more than half of the fruit wet weight, are generated. Waste products from juice extractors consist of peel, internal membrane, rag (ruptured juice vesicle), and seed. Figure 2 presents a cross section of intact orange fruit showing common fruit parts and their terminology. The peel is the primary waste fraction by both weight and volume. Added value by-products such as pectin, essential oils, flavonoids, molasses, are extracted from portions of these wastes. The remainder has been used as cattle feed, both wet and dried forms (Maier, 1978) with the marketing price lower than its production cost.

Main products

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Clarif

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juice production

Storage o

Reprocessi

Figure 1: Orange j

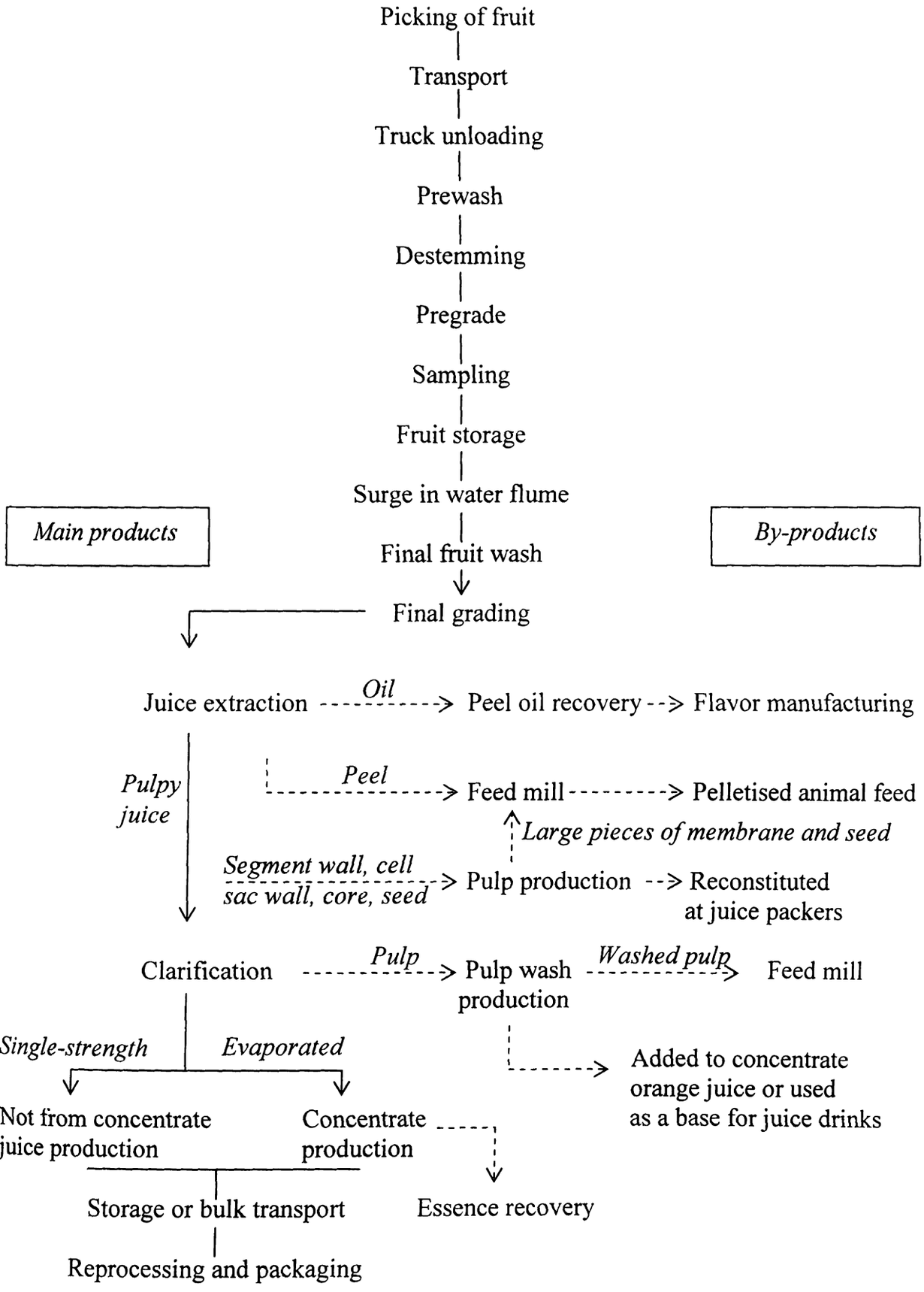

Figure 1: Orange juice production (after: Anonymous, 1998).

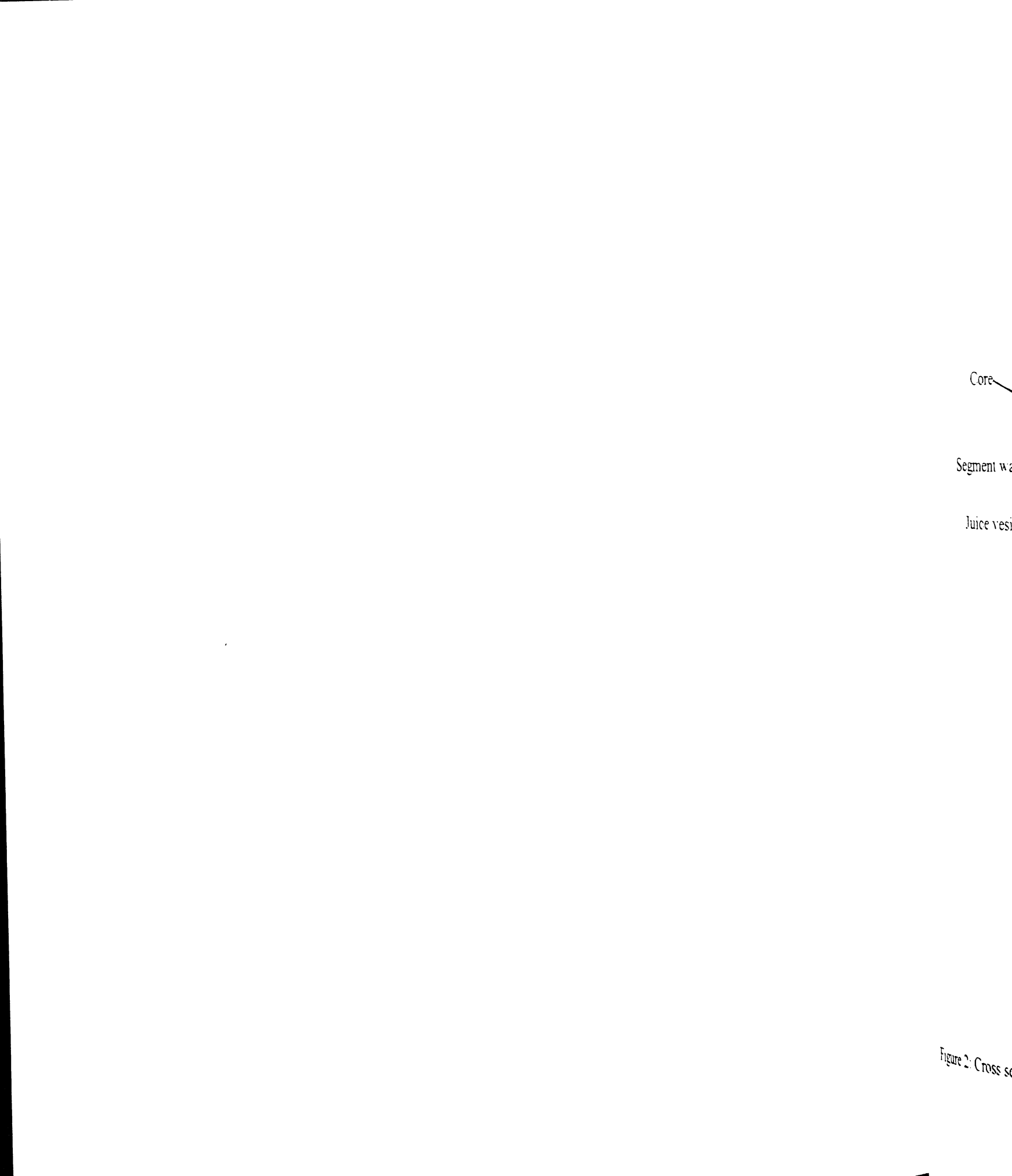

Figure 2: Cross se

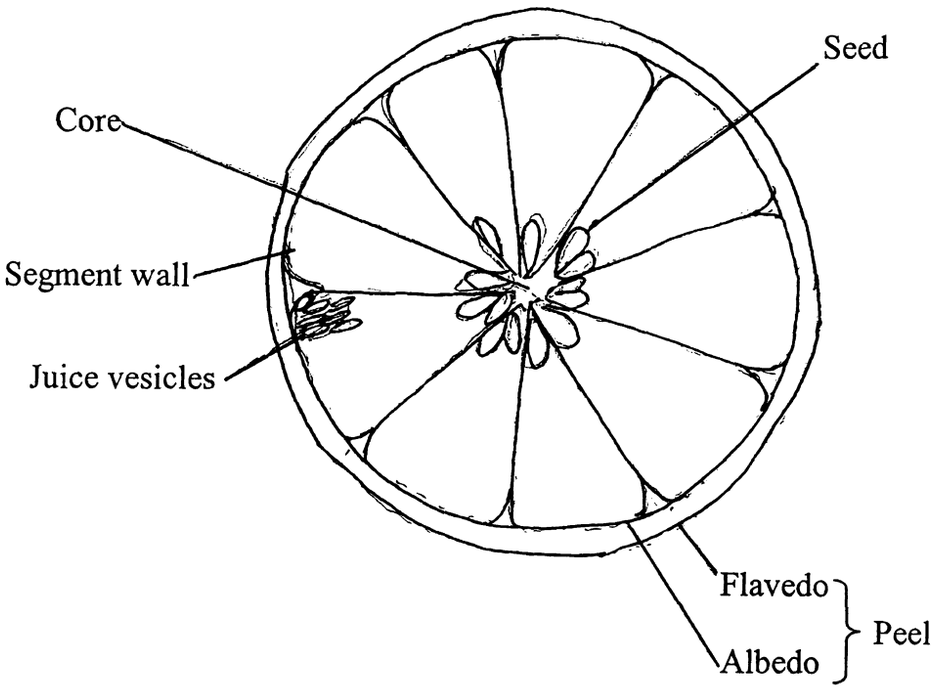

Figure 2: Cross section of intact orange fruit.

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Theoretical yields (wet weight basis) of products from Valencia oranges were: juice (55.74%), residue to feed mill (44.52% at 82% moisture), press cake (19.78% at 72% moisture), water evaporated in dryer (13.74%), finished dried pulp (6.02% at 8% moisture), press liquor (24.77% at 90% moisture), water evaporated in waste heat evaporator (19.11%), concentrated press liquor (5.15%), and limonene separated from evaporator condensate (0.28%) (Braddock et al., 1979).

Odio (1993) has described that the feed mill operation is very important to the efficiency of citrus processing plants, because it is the largest energy consuming point, it is the primary pollution control point (especially liquid wastes), and it produces the main by-products (cattle feed, molasses, d-limonene).

A detailed review of citrus feed mill operations was written by Braddock (1999). The operation begins with delivering the wet residue from the peel bin to the hammer mills to be chopped to optimum size (0.6-2 cm). Particles of too fine a mesh size can cause air pollution and yield lost, but too large pieces may not achieve dryness and can cause mold or so-called "spontaneous combustion". The chopped residues are blended with 0.2-0.5% lime (wet wt. basis) (primarily calcium oxide, CaO) to achieve a more rapid dehydration process. CaO is commonly used, because it readily hydrates with water in the residue, liberating heat and forming calcium hydroxide [$Ca(OH)_2$]. Further, lime neutralizes the peel acidity and de-esterifies the pectin. Traditionally, residence time from mixing and pressing ranges from 10-15 minutes.

Typically, during pressing processes, orange peel is fed on to the top opening of the extractor and the peel is pressed by the rotating screw, pushing toward the

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Limonoids in citrus

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exit die. Pulp is pushed out toward the side opening, while peel juice is collected from the bottom opening. The resulting pulp is termed "press cake" and the juice is termed "press liquid". Final pH of the press liquid is approximately 6.5-7.0 (Braddock, 1999). However, the pH of molasses (press liquid end product) was reported to be relatively more acidic (5-6) (Hendrickson and Kesterson, 1971).

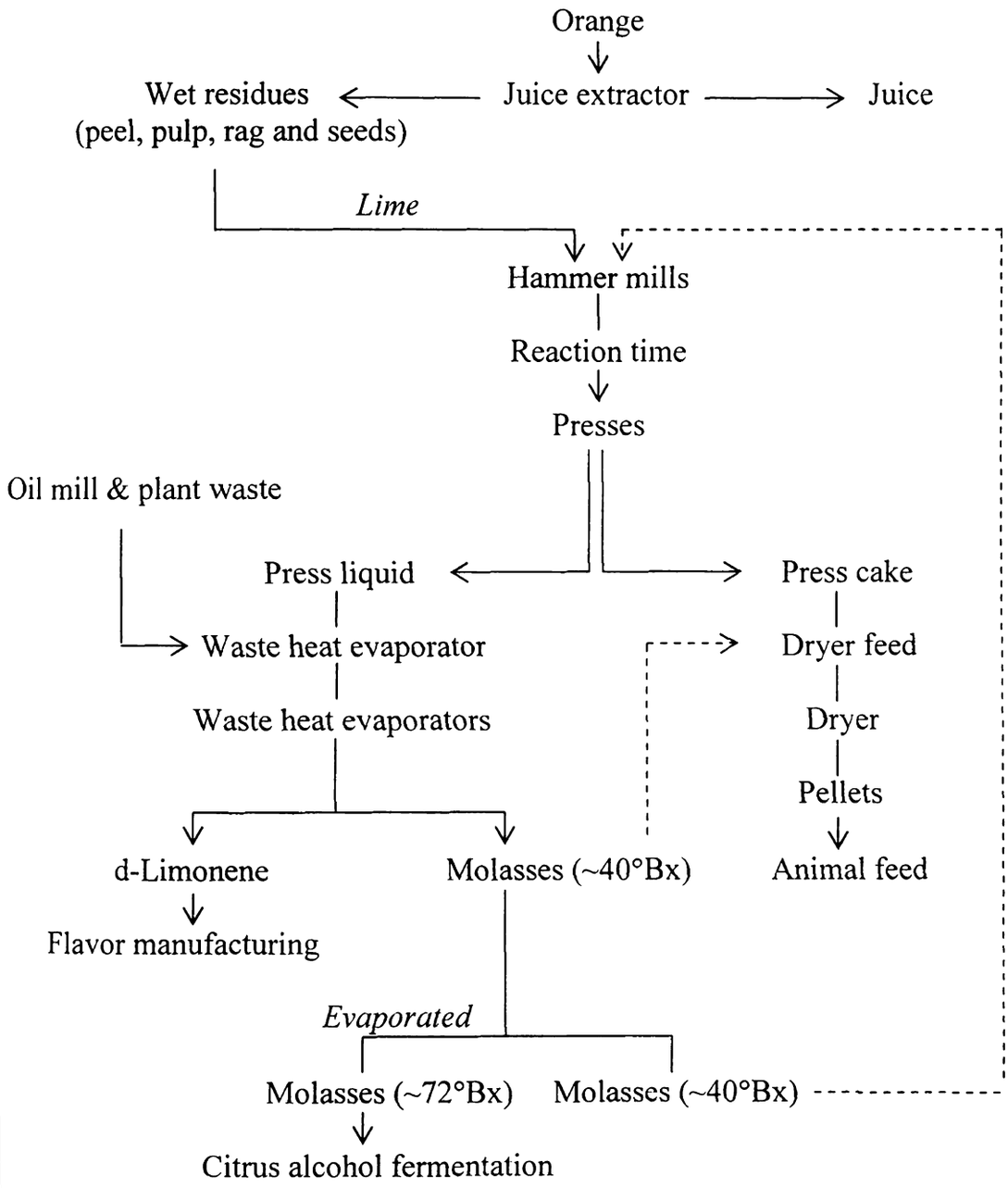
An innovative procedure enabling continuous lime addition and mixing produces more homogenous end products. Enhanced efficient lime reaction lowers press cake moisture, lowers power consumption, capital investment, maintenance, and requires less labor cost (Braddock, 1999). Figure 3 shows the typical flow diagram of citrus feed mill operations (Anonymous, 1998).

Limonoids in citrus products

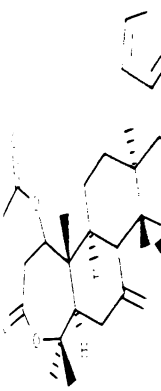
Limonoids are a group of highly oxygenated, tetracyclic triterpene derivatives, present in *Citrus* and its closely related genera that include fruits such as lemon, lime, orange, and grapefruit (Hasegawa et al., 2000). Limonin, the most predominant limonoids for all *Citrus*, was first discovered in 1841 (Bernay, 1841), and was reported to be a principle bitter compound in navel orange juice (Emerson, 1949). More than 120 years later, its chemical composition was elucidated to be $C_{26}H_{30}O_8$ with a molecular weight of 470 using chemical methods and X-ray crystallography (Arigoni et al., 1960 and Barton et al., 1961). Figure 4 and Figure 5 show chemical structure of major citrus limonoids and their glucosides.

According to Hasegawa (2000), limonoids are present in *Citrus* in three chemical forms: 1) limonoid monolactones (open D-ring aglycones such as limonoate A-ring lactone), 2) limonoid dilactones (D-ring closed aglycones such as limonin),

Figure 5: Feed mill



igure 3: Feed mill unit operations (after: Braddock, 1999).



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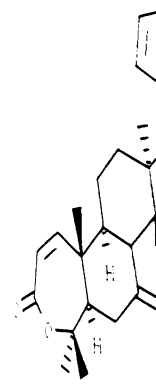

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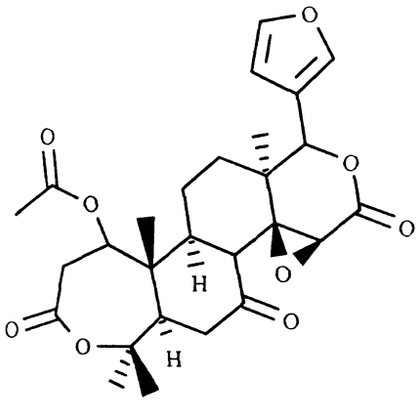

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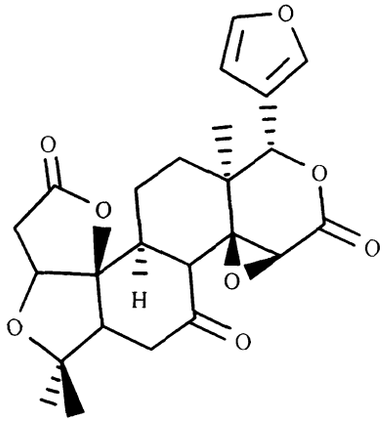
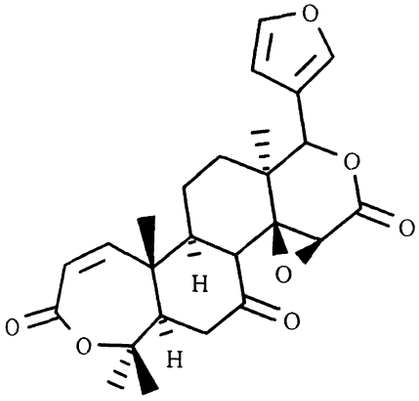


Limonin



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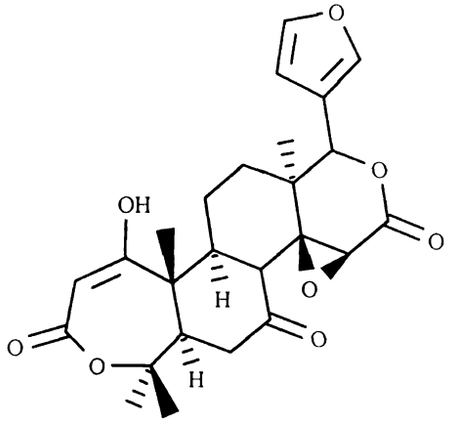

Deacetylnomilin

Figure 4: Chemical structures of the major citrus limonoid algycones.

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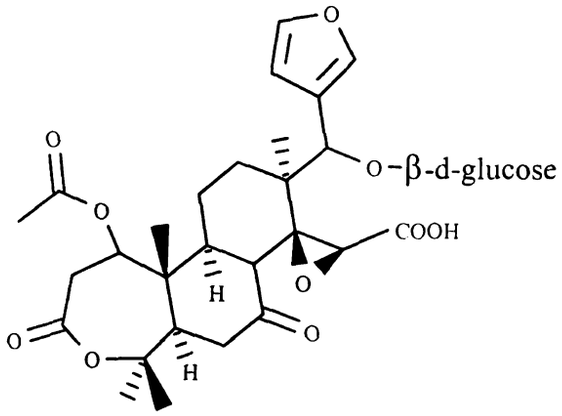
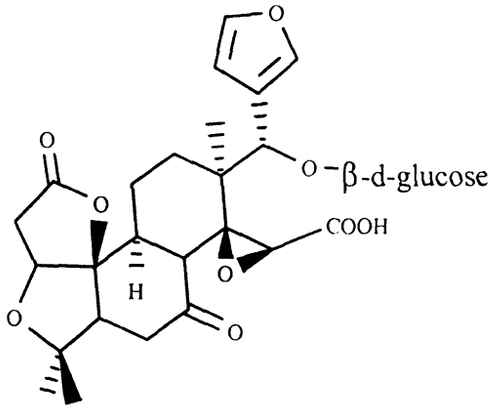
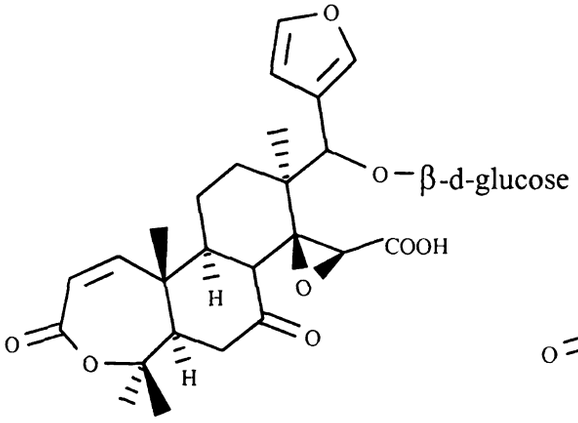


Nomilin glucoside



Limonin glucoside



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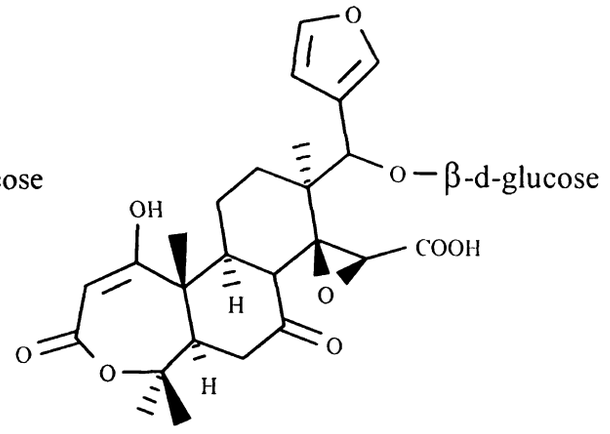

Deacetylnomilin glucoside

Figure 5: Chemical structures of the major citrus limonoid glucosides.

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Delayed bitterness

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and 3) limonoid glycosides such as limonin-17-β-D-glucopyranoside. These three forms are dependently synthesized and have different chemical characteristics such as solubility, pH stability, and taste perception.

An extensive review on biosynthesis and accumulation of citrus limonoids was written by Hasegawa (2000). There are three types of enzymes involved in the biosynthesis of limonoids: a) enzymes involved in the biosynthesis of limonoid monolactones from the precursor nomilinate A-ring lactone from stem, b) limonin D-ring lactone hydrolase, which lactonizes open D-ring of monolactones to form dilactones, c) UDP-D-glucose transferase which convert open D-ring to form limonoid glucosides during fruit maturation (Fong et al., 1993). The biosynthesis and accumulation of these limonoids and enzymes involved are summarized in Figure 6 (Hasegawa, 2000).

Delayed bitterness and glucosidation (natural debittering process)

Limonoids make up one of the bitter principles in citrus juice. Limonoid bitterness is termed "delayed bitterness", characterized by gradual development of bitterness in juice a few hours after its extraction. Fresh fruits do not posse this problem, because when intact, limonoids are present in monolactone forms, which are tasteless. Once fruit cells are ruptured, monolactones are exposed to an acidic environment, which causes D-ring to close, producing dilactone molecules. Some of the dilactone molecules are bitter; these include the major limonoids, limonin and nomilin. Limonin bitterness is a problem primarily in the early season to mid season winter fruit, but not is not present in late season fruit. As the fruit ripen, monolactone levels decrease (Hasegawa et al., 1991).

Figure 6: Biosynth

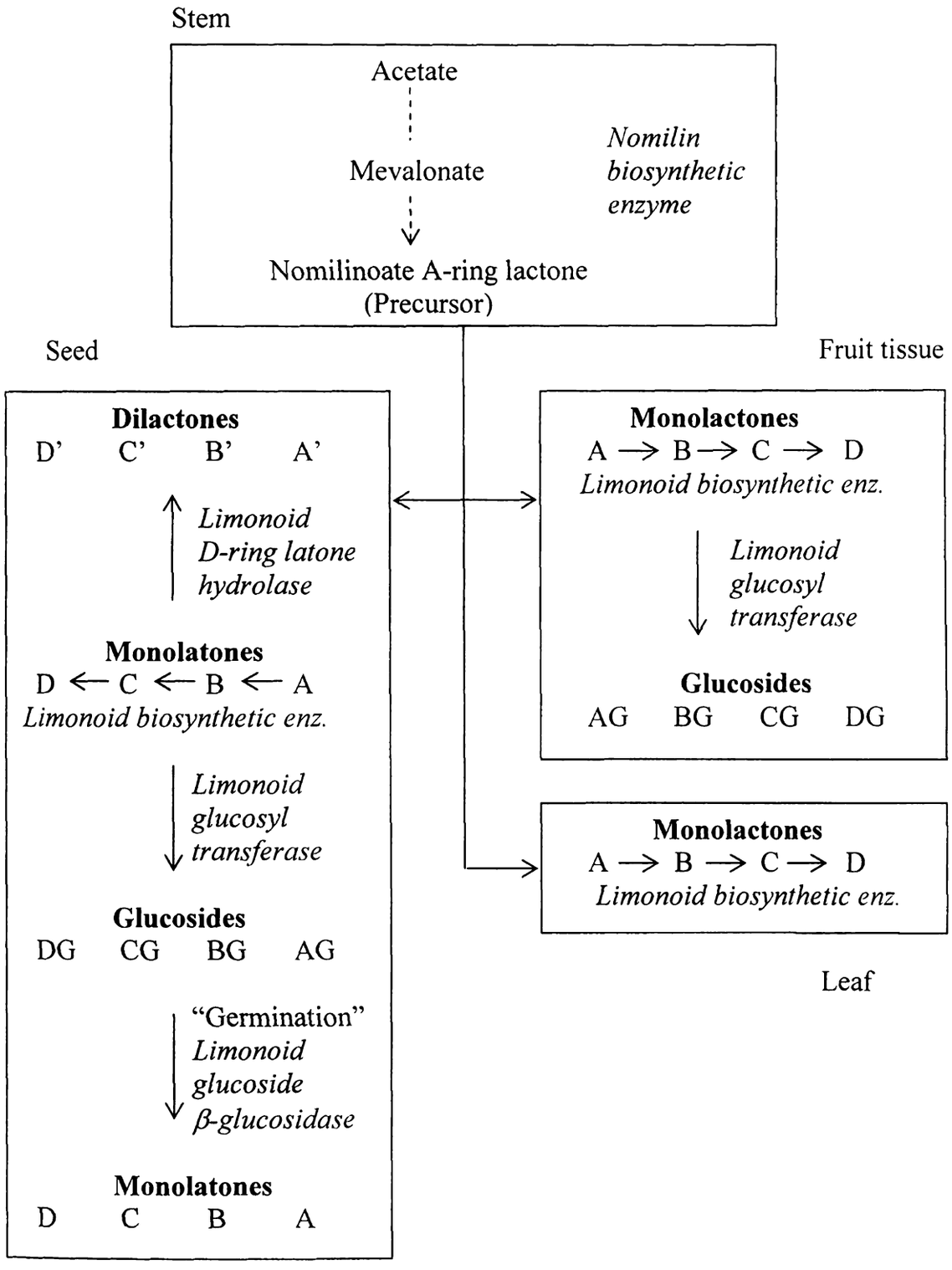

Figure 6: Biosynthesis and accumulation of limonoids in *Citrus* (after: Hasegawa, 2000).

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It has been described that monolactones are converted to their corresponding glucosides in fruit tissues and seeds during the late stage of fruit growth. During the maturation process, the glycosidation contributes to the reduction of bitterness in fruit tissue, since monolactones are no longer available. However, in seed, both glycosidation and lactonization occur simultaneously, therefore, dilactones produced from the lactonization process can still cause bitter taste in mature seed. Figure 7 shows glycosylation and lactonization processes in citrus fruit tissue and seed.

Twenty-one limonoid glucosides from *Citrus* and its hybrids have been isolated and characterized, in which one limonoid molecule is linked with one D-glucose molecule at the 17-position of the open limonoid D-ring by a β-glycosidic linkage (Ozaki et al., 1995). The closed D-ring structure is a key requirement for bitterness perception (Hasegawa et al., 2000). Molecules with no sugar moiety attached are termed "aglycones". Limonoid glycosides are almost tasteless and are water soluble, whereas some limonoid aglycones are extremely bitter and have very low solubility in water.

Biological activities of limonoids

Previous studies on limonoids focused on the removal of these bitter compounds through various techniques aimed at improving taste quality of citrus juices and thus enhance their commercial value. Recently, limonoids have been found to possess beneficial biological activities and engender positive health benefits in the diet.

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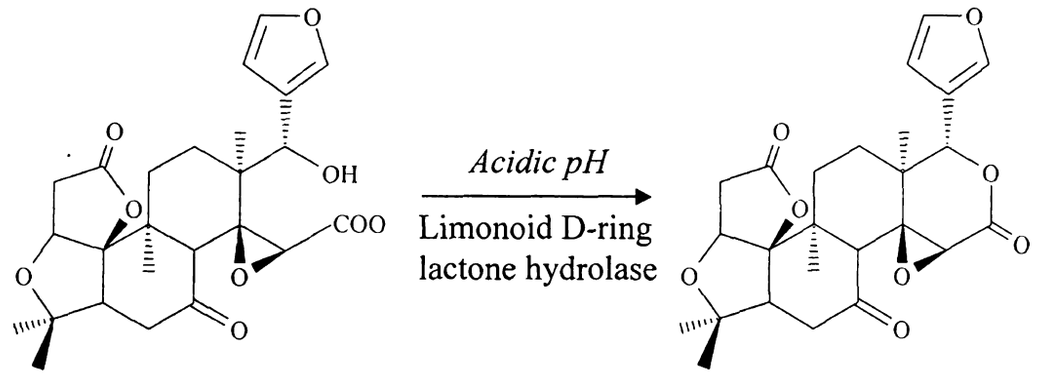
A. Lactonization



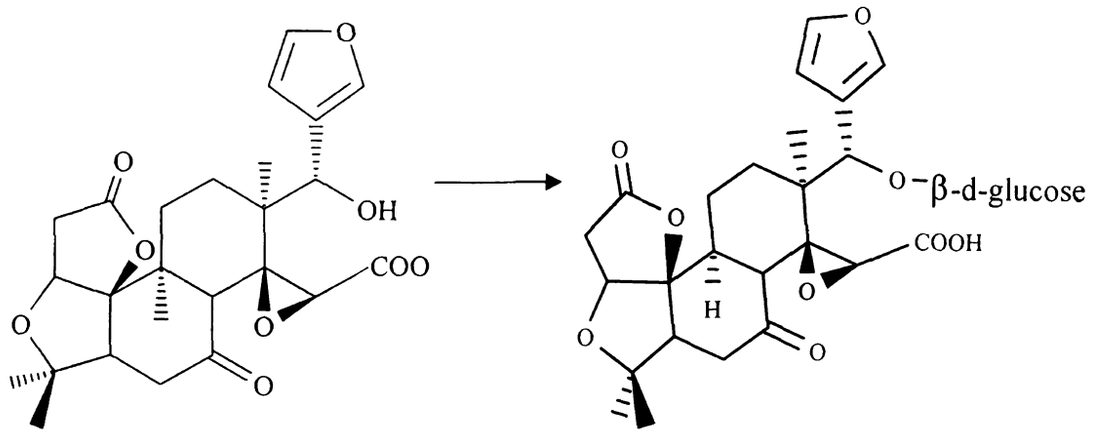
Limonoate A-ring lactone
(Nonbitter)

Limonin
(Bitter)

B. Glycosylation

+ *UDP-D-glucose*

+ *UDP*



Limonoate A-ring lactone
(Nonbitter)

Limonin glucoside
(Nonbitter)

Figure 7: Lactonization (A) and glycosylation (B) processes in citrus fruit (after: Hasegawa, 2000).

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Limonin and nomilin were first shown to have chemo-preventive activities such as inhibiting the development of neoplasia in the forestomach of mice (Lam and Hasegawa, 1989), inhibiting tumors in the buccal pouch of hamsters (Miller et al., 1989) and reduced skin cacinogenesis (Lam et al., 1994). Miller et al. (1994) have discussed the cancer chemo preventive activity of citrus limonoids in relation to chemical structure. The furan ring on a limonoid molecule is an important key for the induction of glutathione S-transferase (GST), an enzyme that catalyzes the conjugation of glutathione with electrophiles that include activated carcinogens, resulting in less reactivity, more water solubility, and facilitated excretion. Important structural features for GST induction are furan moiety, triterpene, and the A ring which is nonperpendicular to the plane of the molecule (Lam and Hasegawa, 1989). Numerous bacteria are present in the intestinal flora and available to hydrolyze limonoid glucosides and liberate limonoid aglycones, in addition, limonoid glycosides themselves have been shown to have direct anticancer activity (Hasegawa, 2000a). Limonoid glucoside has been found to inhibit the development of DMBA-induced tumor for oral carcinogenesis in hamster (Miller et al., 2000). Miller et al. (1992), in a study of the inhibition of hamster buccal pouch carcinogenesis, reported that the addition of glucose and the opening of the D-ring in limonin or nomilin do not modify the cancer chemopreventive activity of these two triterpenes. Limonoid glucosides, both individual and mixed, have been found to significantly inhibit human breast cancer cell proliferation (Tian et a., 2001). This finding is very important because the glucosides are more abundant in the fruits and are tasteless, whereas aglycones are concentrated in the seeds and some of

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them possess bitter off-flavor. Thus, the general consumption of the limonoid glucosides compared with the aglycones is relatively high.

Limonoids have also been shown to possess antifeedant activity against insects including Colorado potato beetle (Bentley et al., 1988), spruce budworm (Lepidoptera: Tortricidae) (Alford and Bentley, 1986), fall armyworm (Lepidoptera: Noctuidae) larvae (Mendel et al., 1993), and *Spodoptera frugiperda* larvae (Ruberto et al., 2002); as well as the common termite, *Reticulitermes speratus Kolbe* (Serit et al., 1991). These responses are very important because each is primary pests with high economic impact. Appropriate pest control in agricultural practice is very important indirectly to overall human health. This biological property of limonoids suggests consideration of the compounds as potential replacements for chemical insecticides. Mendel et al. (1993) investigated the limonoid structures and concluded that the furan system and epoxide group are necessary for feeding deterrence against fall armyworm (Lepidoptera: Noctuidae) larvae; and that nomilin was the most active among the limonoids tested. However, Murray et al. (1999) reported that limonoid glucosides have no antifeedant activities against insects. Thus, the assessment of insecticidal properties remains an active area of biological research.

Distribution information of neutral limonoids in the citrus seed obtained by HPLC analysis was found to be specific to species and cultivars. This implied the potential importance of the limonoid profiles of seeds as a chemotaxonomic tool in the development of new Citrus cultivars (Manners and Hasegawa, 1999).

Flavonoids in citrus f

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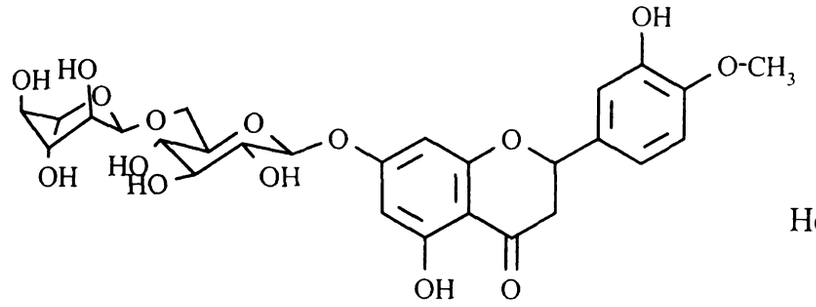
Flavonoids in citrus products

Flavonoids are one of the most widely distributed and diverse groups of polyphenolic compounds in the plant kingdom (Harborne et al., 1975). They are considered to be the most important natural plant pigments, particularly when considered with the carotenoids and the tetrapyrrole derivatives.

Flavonoids have a typical chemical structure consisting of two benzene rings enclosing a heterocyclic six-member ring containing an oxygen atom. They are distinguished by means of differences in the heterocyclic ring and added hydroxyl groups, which are free, methylated, or bound to sugars. Flavonoids found in citrus include flavanones, flavones, and flavonols, with much lower levels in the second and third groups (Ooghe et al., 1994 a). Citrus flavanones occur mostly as diglucosides, whereas methoxylated flavones occur as free aglycones. Figure 8 and Figure 9 show chemical structures of major citrus flavanone glucosides and Figure 10 shows chemical structures of major citrus polymethoxylated flavones, respectively.

Most citrus cultivars can be classified by the glycosylation patterns, which occur at the 7 position on the flavonoid skeleton, except for rutin which is glycosylated at the 3 position. The two main flavonoid glycosylation patterns that have been widely used are a) neohesperidosides (2-β-1-rhamnosyl-D-glucose), which are found only in species related to pummelo, and b) rutinosides (6-β-1-rhamnosyl-D-glucose), which are found in all species of citrus (USDA, 2002). The glycosylation contributes to increased polarity of the flavonoids, which is necessary for storage in plant cell vacuoles (Justesen et al., 1998). It is hypothesized that flavonoids in plants



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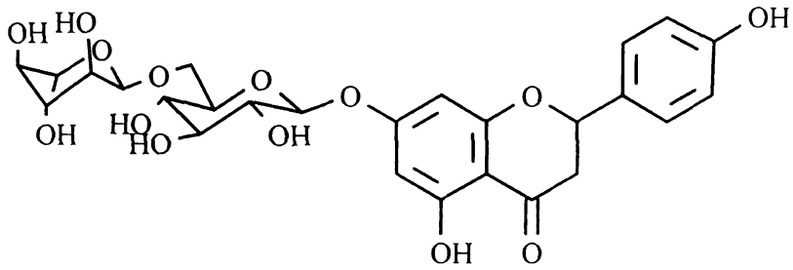

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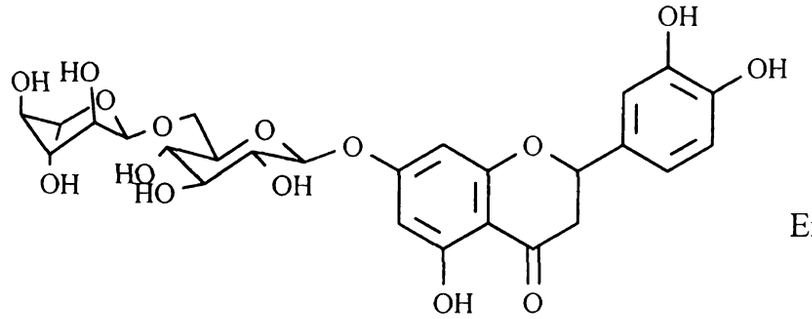
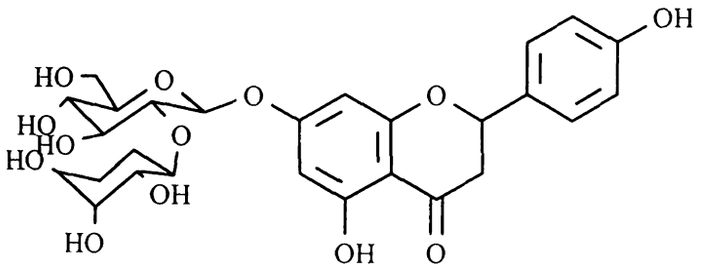


Eriocitrin

Figure 8: Chemical structure of the major citrus flavanone glucoside (rutinosides).



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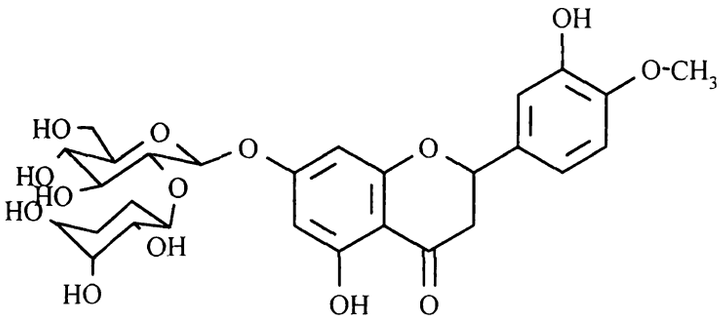

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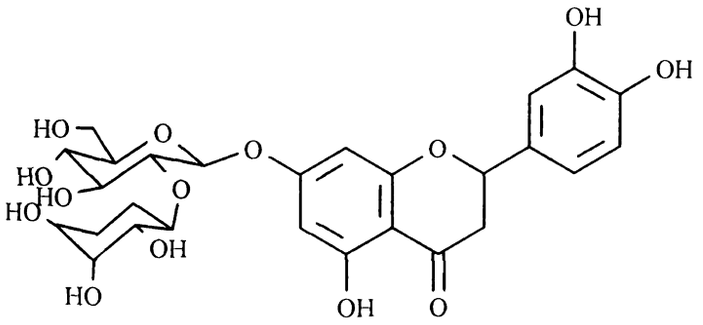
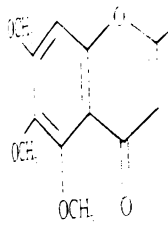


Neoeriocitrin

Figure 9: Chemical structure of the major citrus flavanone glucoside (neohesperidosides).



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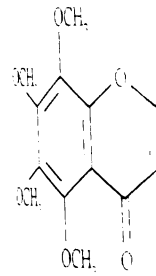

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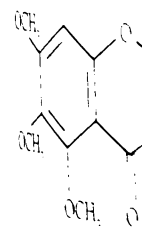
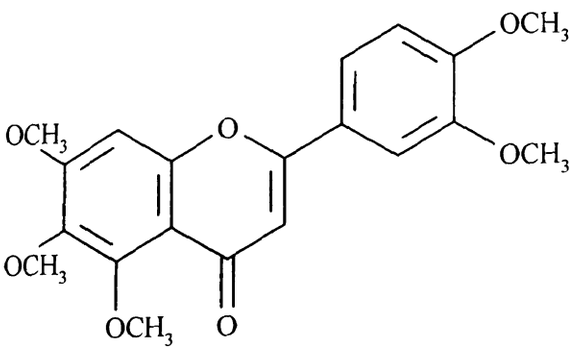


Tetra-O-methyl

Figure 10: Chem



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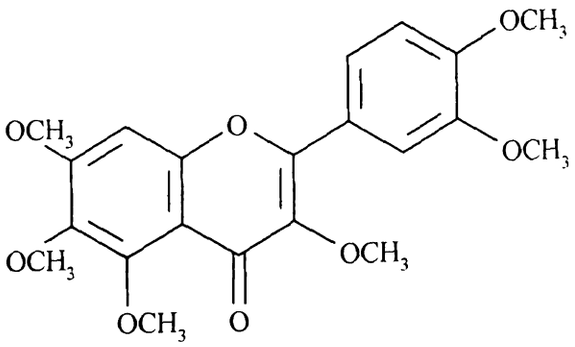
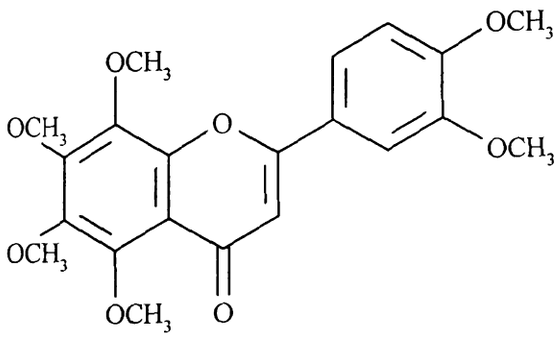


3,5,6,7,3',4'-Hexamethoxyflavone



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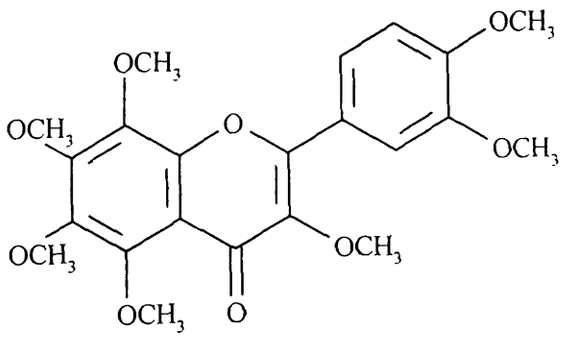
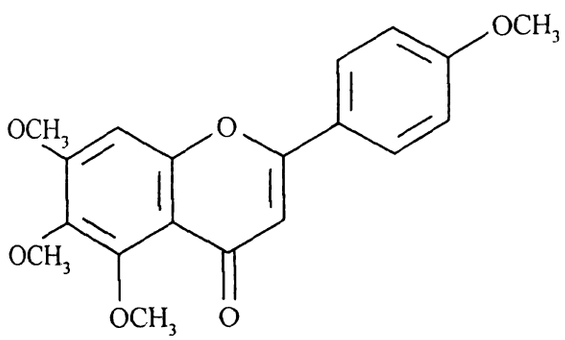


3,4,5,6,7,8,3',4'-Heptamethoxyflavone



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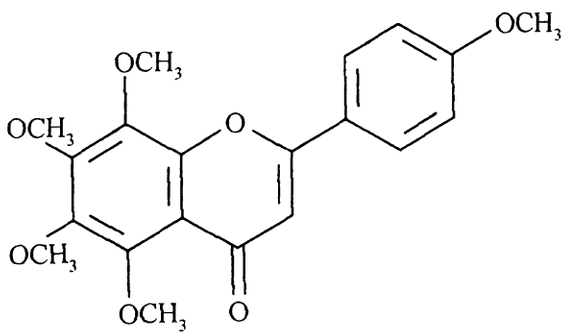

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Figure 10: Chemical structures of the major citrus polymethoxylated flavones

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Regarding flavonoid content during fruit growth, it is understood that flavonoid content in the whole fruit increases at the early stage of fruit development and then remains almost constant (Rouseff, 1980). Decreased flavonoid concentration as the fruit matures is due to the absolute content and its gradual dilution as a result of the increase in the size of the fruit (Kesterson an and Hendrickson, 1953). However, there is disagreement on juice flavonoid concentration and whether changes occur as the fruit matures (Rouseff, 1980). Rouseff and Dougherty (1979) observed a small but consistent decrease of naringin in grapefruit juice as the fruit matures under strictly controlled experimental conditions.

Chemotaxonomic marking and authenticity

Flavonoids are present in Citrus fruits in two major classes: glycosylated flavanones and polymethoxylated flavones. They are found only in citrus and their fingerprint is specific of each species (Bocco et al., 1998). Flavanone glucosides have been used to categorize citrus and its hybrids (Tatum et al., 1974). The presence of various polymethoxylated flavones were also used to distinguish between nucellar and zygotic seedlings from leave extracts, taxonomic classification (Tatum et al., 1978), including differentiation between common species (Gaydou et al., 1987).

With respect to the citrus industry, there are two main reasons for the additions of non-*C. sinensis* juices to orange juice: 1) addition of cheap juice from grapefruit or sour orange to sweet orange juice to increase the financial profit

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(Rouseff et al., 1987) and 2) addition of juice from expensive hybrids such as tangerine to orange juice from early season oranges to improve the juice quality (Ooghe, and Detavernier, 1997).

According to Codex Alimentarius (1992), "orange juice and concentrated orange juice have to be obtained by a mechanical process from the endocarp of sound, ripe oranges (*Citrus sinensis*), preserved exclusively by physical means. The juice may contain up to 10% (m/m) of mandarin juice (*Citrus reticulata*)". The U. S. Food and Drug Administration (FDA) permit the addition of 10% (m/m) of mandarin (*C. reticulate*) or hybrids to pasteurized and canned orange juice. Frozen concentrated orange juice also may contain up to 5% (m/m) sour orange (*C. aurantium*) (Rouseff, 1988). However, most countries within the European Union do not allow any addition of non-*C. sinensis* juices (Ooghe and Detavernier, 1999).

Flavonoids are promising as means for determination of juice authenticity, because they are a) ubiquitous and present in measurable quantities, b) genetically specific, c) multiple and diverse, and d) mostly expensive to synthesize as a result of their structural complexity (Rouseff et al., 1987, Schnull, 1990, and Wade, 1992). Addition of small amount of *C. paradise*, *C. aurantium*, and/or *C. bergamia* juice may be detected by the presence of flavanone neohesperidosides, which are not present in sweet oranges (Ooghe et al., 1994a). However, for tangerines and its hybrids, which also do not contain these neohesperidosides, distribution patterns of polymethoxylated flavone offer more sensitive mean to detect their contamination (Ooghe et al., 1994b).

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Bitterness and precipitation problems

The flavonoids have significant influence on nearly every aspect of citrus fruit production and processing. Two main impacts are bitter taste of flavanone hesperidosides and low solubility in aqueous solutions of hesperidin (Horowitz, 1961).

Flavanone glucosides are present in citrus in two structural isomers: a) bitter flavanone neohesperidosides, and b) tasteless flavanone rutinosides. Major neohesperidosides are naringin and neohesperidin, which are commonly found in grapefruit and pummelo. Veldhuis et al., (1970) reported that some polymethoxylated flavones were bitter, but their concentration in orange juice was relatively low, therefore, they are considered not highly important contributors to the flavor of orange juice.

Differentiated from limonoid bitterness, flavanoid bitterness causes intact fruit to be bitter and also imparts immediate bitterness to the freshly prepared juice. Bitter flavonoids occur only in a few *Citrus* species (grapefruit, pummelo, sour orange, and Ponderosa lemon), but limonin occurs in all Citrus, even though it may not be present in sufficient amounts to cause highly bitter taste. Since flavonoids are not evenly distributed through out the fruit, extraction pressure and contact time between the juice and high flavonoid fractions (albedo, central core and segment membrane) play an important role on their final concentration in the juice (Rouseff, 1980).

Hesperidin, the most abundant flavonoid compound in lemons and sweet oranges, is the most insoluble of all citrus flavonoids (Rouseff, 1980). In intact

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fruits, hesperidin occurs as a soluble complex, which is destroyed during juice extraction, liberating free hesperidin (Horowitz and Gentili, 1977). Free hesperidin gradually precipitates as fine, white, needle-shaped crystals, which can only be dissolved by formamide, pyridine, or dilute alkali (Rouseff, 1980). Hesperidin crystals are found in frost damaged oranges (Hume, 1957) and concentrated orange juices during storage; and are found as a thin crust coating the evaporators used in production of frozen concentrate orange juice (USDA, 1962). Even though the presence of these hesperidin particles does not affect juice flavor, it results in visual appearance which is considered a major quality defect (Rouseff, 1980).

Biological activities of flavonoids

Although, high accumulation of unfavorable flavonoids results in lower-quality juice, it has been reported in many studies that these compounds possess beneficial biological activities, especially health-promoting functionalities.

Benavente-Garcia et al (1997) systematically described health-related properties of citrus flavonoids. These properties include a) antioxidant activities; b) cardiovascular properties; c) anti-inflammatory, d) antiallergic, and e) analgesic activities; and f) antimicrobial activities. Due to their antioxidant properties and their ability to absorb UV light, flavonoids may act in all stages of the carcinogenic process: damage to the DNA (initiation), tumor growth (promotion), and invasion (proliferation). Flavanone glycosides are not absorbed by humans or other mammals. Widmer and Montanari (1996) concluded that intestinal floras in the gut cleave off the disaccharides of hesperidin and naringin, producing hesperitin and naringinin that were absorbed. Numerous studies on different chemically induced

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cancers have reported that hesperidin was found to inhibit chemically induced colon cancer (Tanaka et al., 1997a and Miyagi et al., 2000) and esophageal cancer (Tanaka et al., 1997b). Flavonoids were also reported to have the protective effect against UV-induced DNA damage (Kooststra, 1994).

Polymethoxylated flavones (PMFs) have been found to possess pharmacodynamic properties (Ooghe et al., 1994). PMFs were found to be much more active than naringin, hesperidin, or their flavanone aglycones (Wall et al., 1988) and exhibit higher levels of biological activity than their hydroxylated counterparts (Attaway, 1994). Robbins (1974) found that isolated PMFs had greater anti-adhesive effects on red blood cells and platelets than did flavanone glycosides. Kandaswami et al (1991) examined quercetin, taxifolin, nobiletin and tangeretin on the *in vitro* growth of a human squamous cell, carcinoma cell line (HTB43). They found that nobiletin and tangeretin markedly inhibited cell growth and quercetin and taxifolin exhibited no significant inhibition. These differences in activity may be due to the relatively greater membrane uptake of the PMFs since methoxylation of the phenolic groups decreases hydrophilicity of the flavonoid. Several of PMFs have been demonstrated to possess antimicrobial, antiviral activities, anti-inflammatory properties and inhibit histamine release to reduce allergic reactions (Widmer et al., 1996).

In addition to the health potential roles of flavonoids mentioned above, some flavonoids may be suitable for industrial food ingredient sweetener application. Naringin and neohesperidin have been found to be able to convert into their corresponding dihydrochalcones which are 100 and 15,000 times sweeter than

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Sample preparation in citrus phytochemistry

Generally, prerequisite to qualitative and quantitative analyses of the natural phytochemicals is the extraction of these compounds from the plant tissue matrices. The critical goal is to obtain a sample extract uniformly concentrated in all components of interest and free from interfering components (Antolovich et al., 2000).

Recovery is complicated as fruit constitutes a natural matrix with a high enzyme activity, and therefore care must be taken to ensure correct extraction and the lack of chemical modification, which will result in artifacts (Macheix et al., 1990). The consistency of the relative compound profile between starting material and that of isolated extract provides a theoretical basis for analytical technique selection. Therefore, the conditions used should be as mild as possible to prevent oxidation, thermal degradation, and other chemical and biochemical changes in the samples (Antolovich et al., 2000).

Isolation of biological compounds is also complicated by their uneven distribution within various fruit tissue fractions. For instance, citrus peel contains high polymethoxylated flavones (Gaydou et al., 1987, Morin et al., 1991, and Dugo et al., 1996), where as citrus molasses (evaporated peel juice) contains high limonoid glycosides (Ozaki et al., 1995 and Hasegawa et al., 1996). Accumulation of soluble

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phenolics is greater in the outer tissues (epidermal and subepidermal layers) of the fruit than in the inner tissues (mesocarp and pulp) (Bengoechea et al., 1997). Hypothetical explanations at the subcellular level have been proposed that phenolic compounds may exist in the vacuole or within the cell wall (Yamaki, 1984); they may occur in soluble, suspended and/or colloidal forms and in covalent arrangement with cell wall components (Lichtenthaler and Schweiger, 1998). Therefore, the extractions of these phytochemicals may greatly influenced by these different subcellular-level accumulations (Antolovich et al., 2000).

With few exceptions (hesperidin and naringin), it is less complicated to work with liquid samples because the compounds present are most readily extractable. Mild heating may be needed to achieve complete dissolution. Extraction techniques include: a) liquid-liquid extraction, b) solid phase extraction and c) solvent extraction. In limited circumstances, no sample treatment is needed prior to analyses. In the case of solid samples, more extensive extraction is required to obtain complete recovery. These conditions range from a sequence of exhaustive extractions and preconcentration to supercritical fluid extractions and solid phase micro extractions (Antolovich et al., 2000).

It is essential to recognize that sample manipulation to increase selectivity of targeted compounds may result in a relative decrease of their sensitivity. The precise procedure selected depends on both natures of analytes (e. g. total limonoids, total flavonoids, glycosides, or aglycones) and sources of samples (e. g. peel, seeds, juice, or rag). The extraction procedure is simplified in analyses focusing only a

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Analyses of citrus limonoids and flavonoids

Citrus limonoids

Analyses of these particular compounds possess many technical problems including: a) their existence in the samples in minute quantities, b) the low ability to absorb UV light, c) the lack of commercial standards, and d) their presence in a complex matrix. Consequently, the isolation of limonoids becomes an important preliminary step essential to the quantitative analyses.

Limonoids occurs in both aglycone and glucoside forms. Limonoid glucosides are soluble in aqueous solution, due to an added glucose molecule, whereas their corresponding aglycones are much less nonsoluble. It is a common practice to specifically analyze each group independently. Solvent extraction of limonoid glucosides usually renders flavanone glucosides, likewise limonoid aglycones and polymethoxylated flavones are usually co-extracted. Thus, it is important to use analytical methods that effectively differentiate these compounds in the complex mixture.

Dreyer (1965) was the initial contributor who developed quantitative determination by thin layer chromatography (TLC), and structural elucidation by nuclear magnetic resonance to characterize the limonoid compounds in citrus. Since then, there have been 38 limonoid aglycones and 20 limonoid glucosides identified. The reddish-orange limonoid color developed with Ehrlich's reagent and by exposing it to hydrogen gas is specific enough to be readily differentiated.

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Subsequently developed TLC methods (Maier and Grant, 1970 and Tatum and Berry, 1973) are more specific, sensitive, and precise. TLC is suitable for routine quality control analyses because it is simple and rapid, however, there are analytical reproducibility problems associated with this technique. Also, there is no solvent system to separate limonoid glucosides; therefore TLC determines limonoid glucoside as a total value rather than as the individual species (Hasegawa and Berhow, 2000).

Recent quantitative techniques of limonoids include TLC (Ohta,1993), HPLC-UV (Fong et al., 1993, Ozaki et al., 1995, Hsu et al., 1998, McIntosh and Mansell, 1997, Hasegawa and Manners, 1999) radio immunoassay (RIA) (McIntosh, 2000), HPLC-MS (Schoch et al., 2001), and capillary electrophoresis (Moodley et al., 1995, Braddock and Bryan, 2001). Most of these methods, except for RIA, require sample preparation such as organic solvent extraction, partitioning, or solid phase extraction (Hasegawa et al., 2000).

HPLC is the most commonly used method, because it is accurate, reproducible, and highly accessible. The application of reverse phase HPLC is dominant in limonoid quantitative analyses not only for citrus limonoids but also for other compounds in plant extracts, as it provides higher separating efficiency for such complex mixtures through various mobile phase selections and it consumes less organic solvent compared to normal phase HPLC. Reverse phase HPLC has been developed for analytical determination of both limonoid aglycones and glucosides in citrus seeds, juice, peel, fruit tissue, and by-products. These techniques utilized C18 bonded silica columns with solvent mixtures of acetonitrile/water,

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acetonitrile/aqueous acid or mixtures of acetonitrile, methanol, tetrahydrofuran and water. Acetonitrile has been the solvent of choice because it has low UV absorption (190nm UV cutoff) and low viscosity (0.38 cP). Therefore, acetonitrile does not interfere with the limonoid detection (typically at 210 nm) and does not cause high back pressure problem to HPLC pump.

Rouseff and Fisher (1980) recently developed a normal phase HPLC system for analysis of limonoid aglycones. They used a combination of a CN bonded silica column with a ternary mobile phase (hexane/2-propanol/methanol). This HPLC system was able to effectively separate obacunone, nomilin, limonin, and deoxylimonin. Hasegawa and Manners (1999) used a spherical silica column with a binary mobile phase (cyclohexane/tetrahydrofuran) to resolve limonoid aglycones. The high selectivity achieved allows the option of commercial scale up for the separation of minor limonoid aglycones.

Determination of limonoids was achieved by UV detection at wavelengths lower than 220 nm. Identification of individual limonoids is based on a comparison of retention times to those of standards. Photodiode array detection offers a more advanced identification compared to UV-vis detection. This type of detector produces UV spectral data for each resolved compounds, thus aiding to screen flavonoid impurities that may be present and adjacent to limonoid compounds. Higher specification of limonoid analyses was achieved by application of LC-MS, where by the resolved compounds is immediately subjected to MS system. The identification of selected compounds is therefore dependent on retention time, structural and/or molecular weight data.

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The first comprehensive explanation of limonin structure determination using ^{1}H and ^{13}C NMR was achieved by Dreyer (1965). Since the evolution of NMR has enabled increased magnetic field strengths with corresponding large increase in resolution, a large number of limonoid aglycones and glucosides have been isolated and characterized. The development of Fourier transform NMR (FTNMR) techniques, which produce extensive intramolecular ^{1}H-^{1}H and ^{1}H-^{13}C correlations have led to more rapid structural assignments of the limonoids (Hasegawa et al., 2000b).

Limonoid structural characterization by mass spectrometry has been found in limited studies. Due to their nonvolatility, limonoids are not readily adaptable to be analyzed by basic EI-GC-MS instrumentation combinations necessary to acquire the fragmentation patterns. Derivatization of these high oxygenated molecules by addition of nonpolar moieties may produce large molecules that are still difficult to vaporize.

Two main mass spectrometric techniques are electrospray ionization mass spectrometry liquid chromatography (ESI-LC-MS) (Schoch et al., 2001, and Manners et al., 2003), and fast atom bombardment mass spectrometry (FABMS) (Sawabe et al., 1999). Among these techniques, LC-MS provides a high sensitivity as low as 42 picograms for analysis of citrus limonoids (Hasegawa et al., 2000). Hasegawa et al. (2000) described that EI-LC-MS was useful only for the analysis of limonoid aglycones, whereas ESI-LC-MS was found to be the most effective method for the analysis of limonoid glucosides. In addition, an established normal phase HPLC condition suitable for limonoid aglycone analyses introduced by Manner and

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Hasegawa (1999) was reported to be able to directly adapt to the atmospheric chemical ionization-mass spectrometry system (APCI-MS) (Hasegawa et al., 2000b). They concluded that the flow rates up to 2 ml/min were compatible with the APCI - LC-MS mode. Sawabe et al. (1999) successfully identified citrus limonoid glucosides using positive and negative FABMS. Both modes showed consistent results for establishing molecular weight of nomilinic acid 17-*O*-β-D-glucopyranoside, methyl nomilinate 17-*O*-β-D-glucopyranoside, and obacunone 17-*O*-β-D-glucopyraside.

In the field of limonoid structural analyses, mass spectrometry has been primarily used to obtain molecular weight information for their identification and confirmation. These results are supplemental to other techniques such as chromatographic retention, UV spectra, and NMR spectra.

Citrus flavonoids

Citrus flavonoid are more readily analyzed than limonoids, because the fact that their molecules contain potent chromophores, which highly absorb UV light. This UV absorption property allows higher specification for the flavonoid detection and higher flexibility for the mobile phase selections.

Analyses of citrus flavonoids generally deal with flavanone glucosides and polymethoxylated flavones as major compounds. Most flavanone glucosides are soluble compounds, except for hesperidin, which dissolve in formamide, pyridine, or dilute alkali (Rouseff, 1980), and narigin, which requires heating for complete dissolution in alcohol solutions. Polymethoxylated flavones are nonpolar compounds, which exist in much lower levels than the flavanoid glucosides.

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Earlier studies on flavonoid quantitative analyses involved spectrophotometric methods (Coustou and Babin, 1957), paper chromatography (Edward et al., 1957, Toshio and Shintaro, 1959) and thin layer chromatography (Tatum et al., 1957, Swift, 1967). Among those analyses, the most widely used technique was the "Davis test" introduced by Davis (1947). This method determines amount of naringin in grapefruit juice and total flavonoid in orange juice at 420 nm when the juice sample is incorporated with 4 N sodium hydroxide and 90% diethylene glycol. It has been known to be nonspecific but is simple, rapid and inexpensive (Ting and Rouseff, 1986).

Because of the number and diversity of flavonoids in citrus juice, the analytical techniques have been developed based on chromatography (Robards et al., 1997). Available methods for citrus flavonoid determinations include GC (Stremple, 1998), HPLC-UV/PDA (Ooghe et al., 1994a, Robards et al., 1997, Kawaii et al., 1999, Mouly et al., 1999), HPLC-Fluorescence (Robards et al., 1997), radioimmunoassay (Jourdan et al., 1982, and Barthe et al., 1988), capillary electrophoresis (Takei et al., 1998), GC-MS (Stremple, 1998), LC-MS (He et al., 1997, Robards et al., 1997, Ishii et al., 2000), and LC-PDA-MS (Baldi et al., 1995, Cuyckens and Claeys, 2002). The developed radioimmunoassay is very specific to 2-rhamnosyl-1-glucopyranose at the C-7 position but not with the isomeric 6-rhamnosyl-1-glucopyranose moiety. It appeared that this method was limited to rutinosides; however, it can be used to identify the stereochemistry of this disaccharide moiety at the C-7 position of flavonoids (Jourdan et al., 1982). Advanced techniques such as GC-MS, LC-MS, and LC-PDA-MS allow

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simultaneous quantitative determination and structural identification. The identifications are dependent on retention time, mass spectra, and UV spectra (when PDA is coupled). However, these sophisticated and expensive systems are still not very widely available and are not used for commercial juice quality control analyses.

The most commonly used method has been HPLC coupled with UV or PDA detector. The HPLC is a flexible tool for analysis of both polar and nonpolar nonvolatile compounds; therefore, it is readily adaptable to both flavanone glucosides and polymethoxylated flavones without the need for additional derivatization or heating. Photodiode Array Detector (PDA) has been used to obtain both chromatographic (absorbance as a function of time) data and spectral data (absorbance as a function of wavelength). This type of detector is a powerful tool compared to standard UV-vis detector, which produces chromatograms at only one or two wavelengths. Since UV spectra are relatively specific markers of flavonoids, HPLC coupled with PDA can provide effective systematic determination of flavonoids for both routine quality control analyses and method-development studies.

In recognition of the complexity of the flavonoids in most extracts, reverse phase HPLC with gradient elution has been the method of choice for separation of the flavonoids in citrus fruits. Under these conditions, the elution profile for flavonoids containing equivalent substitution patterns is flavanone glycosides, flavonol glycosides, flavone glycosides and subsequently the free aglycones in the same order. However, the separations between glycosides and aglycones are not

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adequate for the resolution of a complex mixture containing many compounds of each group. Therefore, it has been a common practice to separate the various classes of flavonoids in a preliminary extraction step (Robards et al., 1997).

Initially, flavonoid structures were established primarily on a combination of at least two of these techniques: a) physicochemical data (such as melting point or crystal characteristics) (Nishiura et al., 1971), b) chemical reactions (such as color development after addition of NH_3, NH_4Cl, $FeCl_3$) (Nishiura et al., 1971), c) IR spectra, d) UV spectra, e) TLC and f) PC. Development of NMR techniques has contributed an important improvement in structural elucidation of flavonoids, because they make possible the complete structural assignments including numbers of carbons, number of hydrogens, and their specific arrangement shown directly in NMR spectra. NMR can be used for elucidation and/or confirmation of chemical structures, and/or purity analysis of isolated compounds. A systematic review of the flavonoid structural identification using UV and NMR spectra was written by Mabry et al. (1970).

Mass spectrometric applications in citrus flavonoids are electron impact ionization gas chromatography mass spectrometry (EI-GC-MS) for analysis of polymethoxylated flavones (Rizzi and Boeing and Berahia et al., 1994, Stremple, 1998, Chen et al., 1998), liquid chromatography mass spectrometry (LC-MS) for analysis of flavanone glucosides (Baldi et al., 1995, He et al., 1997), or direct probe fast atom bombardment mass spectrometry (FABMS) for analysis of both major flavonoids. Fewer studies were found utilizing the FABMS, because prior purification of individual compounds has been a necessary step to obtain simpler

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mass spectral data required for molecular weight assignment. Tandem mass spectrometry instruments (MS/MS) offer higher specificity of the analysis with second order fragmentations than that obtained from singular MS. This system was demonstrated to produce consistent ion fragments that could be used as compound "finger prints". Trace amounts of naringin and its metabolites in human urine were analyzed by electrospray ionization mass spectrometry (ESI-MS), tandem mass spectrometry-mass spectrometry (MS/MS), and tandem mass spectrometry-mass spectrometry-mass spectrometry (MS/MS/MS) technique in an absorption study of flavonoids (Ishii et al., 2000). The absorptions of both pure naringin and hesperidin and those in grapefruit and orange juice were identified by positive chemical ionization-collisionally activated dissociation tandem mass spectrometry (PCI-CAD MS/MS). In addition to high compound specificity, these studies demonstrated that the mass spectrometric conditions used were very sensitive for the trace levels of targeted flavonoids.

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Part I: Screening for ma

1. Abstract

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Study I: Analytical methodology suitable for isolation and quantitation of limonoids and flavonoids in sweet orange

Part I: Screening for major limonoids and flavonoids

1. Abstract

Modified methanol extracts from orange fraction including: seeds, peels, peel press cake, rags, peel press liquid and orange juice were analyzed by HPLC using an extended gradient system, starting with 10% acetonitrile in 3 mM phosphoric acid and ending with 60% acetonitrile in 115 minutes. UV-visible absorbance was measured at 210 nm (limonoid detection), 280 nm (flavanone glucoside detection), and 340 nm (polymethoxylated flavone detection). Chromatographic separation (Rs = 0.9/N = 95,216) obtained allowed screening of major limonoids and flavonoids and estimation of their relative retentions.

Compounds within a detectable level were limonin, deacetylnomilin, nomilin, and obacunone (limonoid algycones); limonin glucoside, deacetylnomilinic acid glucoside, nomilinic acid glucoside, obacunone glucoside (limonoid glucosides); sinensitin, nobiletin, 3,4,5,6,7,8,3',4'-heptamethoxyflavone, scutellarein tetramethylether, and tangeretin (polymethoxylated flavones); eriocitrin, narirutin, hesperidin, and didymin (flavanone glucosides).

Results from this study suggested that to analyze various compounds possessing wide ranges of polarity, an improved HPLC system was required to separate compounds with similar chromatographic retentions.

2. Introduction

The presence of citrus limonoids and flavonoids has been known to be diverse among tissues, genetically specific among species, and quantitatively varied ranging from

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3. Materials and me

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ppm to percent units. Quantitative analyses on limonoids in sweet orange have been done primarily with the major compounds (limonin and nomilin). Compared to limonoids, flavonoids have been analyzed more extensively due primarily to the methodology and commercial standards available for their determination.

Our research has attempted to quantify the broad spectrum of limonoids and flavonoids that could be consistently detected in sweet orange. Therefore, it was important to conduct a screening study to select these compounds based on their relative amount present in diverse tissues of sweet orange and to estimate their relative chromatographic retention required for effective method optimization. Specific objectives are as follows:

1) To select limonoids and flavonoids present in sweet orange for the subsequent quantitative study,

2) To obtain relative retention of limonoids and flavonoids present in sweet orange, and

3) To select raw materials for isolation of unknown limonoids and flavonoids.

3. Materials and methods

3.1 Orange samples

Orange samples of Valencia variety including: 1) seeds, 2) peels, 3) peel press ke, 4) rags, 5) peel press liquid, and 6) orange juice were obtained from Tropicana ducts Company (Bradenton, FL). Descriptions of samples are as follows:

A single strength orange juice was pasteurized (95°C/2 sec), vacuum-sealed, and d at refrigerated temperature. Peel press liquid was prepared using a Vincent screw ® (Vincent Corporation, Tampa, FL). The pulp resulting from the pressing process

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is termed "press cake". The liquid squeezed from pulp is termed "press liquid" or "press liquor". Peel press liquid was pasteurized (95°C/2 sec), vacuum-sealed and stored at refrigerated temperature (2±1°C).

Rags (containing seeds), peels, and peel press cake were vacuum-sealed and frozen (-20°C). The samples were shipped in Styrofoam containers to the Department of Food Science and Human Nutrition, Michigan State University, E. Lansing, MI.

3.2 Sample preparation

Upon arrival, samples were immediately stored at –20°C for approximately one week before analyses. Juice samples were held at refrigerated temperature (2±1°C) until completely thawed. To ensure homogeneity, all containers of each sample were combined and mixed thoroughly, sub-sampled, and collected into 100 ml glass bottles, and then stored at –20°C until analyzed.

Samples of rags, peels, and peel press cake were freeze-dried, and then ground to pass 1 mm screen using a UDY-Mill (Chicago, IL). The ground samples were stored at –20°C until analyzed. Seeds were extracted twice with hexane (1:4, W/V) at room temperature to remove orange oil before being milled with a UDY-Mill and stored in glass vials as described above.

3.3 Studied compounds and standards

Studied compounds included limonoid glucosides, limonoid aglycones, flavanoid glucosides, and polymethoxylated flavones. Standards, kindly donated by scientists from USDA, Dr. Gary D. Manners (Pasadena, CA), Dr. Mark A. Berhow (Peoria, IL), and Dr. John A. Manthey (Winter Haven, FL), included deacetylnomilin (DNM), obacunone (O), limonin glucoside (LG), deacetylnomilinic acid glucoside (DNAG), nomilinic acid

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glucoside (NAG), obacunone glucoside (OG), obacunoic acid (OA), isoobacunoic acid (IOA), deoxylimonin (DL), 17-19-didehydrolimonoic acid (DDHLA), 19-dehydrolimonoic acid (DHLA), limolinic acid (LA), rutaevin (R), sinensetin (ST), nobiletin (NBT), 3,4,5,6,7,8,3',4'-heptamethoxyflavone (HP), and tangeretin (TT).

Limonin (L), nomilin (NM) hesperidin (HD), naringin (NG), neohesperidin (NHD), hesperitin (HT), diosgenin (DN), coumarin (CM), quercetin (QT) were purchased from Sigma Company (St. Louis, MO). Sinensetin (ST), scutellarein tetramethylether (STME), narirutin (NT), didymin (DD), and eriocitrin (ERT) were purchased from Extrasynthese, (Genay, France).

3.4 Extraction

Ground, freeze-dried orange parts (peel, peel press cake, and rag) (1 g) was extracted twice with 10 ml 70% methanol, and once with 10 ml 100% methanol; the supernatants were combined, and methanol was evaporated in the round bottom flasks at 40°C to 2-3 ml under vacuum. The evaporated extract was passed through C18 Sep-pak (1000 mg), which were preconditioned with 3 ml methanol and 10 ml water. The Sep-pak was washed with 10 ml water and the compounds were eluted with 10 ml methanol. Eluate was evaporated at 50°C under vacuum and reconstituted with 2 ml 10% acetronitrile in 3mM phosphoric acid (initial mobile phase). Additional c entrifugation (10,000X g/10 minutes) was done due to remaining residues, which was dissolved in 1ml acetronitrile. Therefore, there were two fractions analyzed a) fraction dissolved in 2 ml 10% acetronitrile in 3mM phosphoric acid, and b) fraction dissolved in 1 ml acetronitrile.

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Juice and peel press liquid were thawed at room temperature. The juice or peel press liquid (10 ml) was mixed with 23 ml methanol to obtain 70% methanol. The rest of extraction procedure was the same as solid fractions.

3.5 High performance liquid chromatography (HPLC) analysis

HPLC system consisted of two pumps model Waters 510 (controlled by Waters Pump Control Module), equipped with an injection system (Water 717 plus autosampler), and Water 996 Photodiode Array Detector (PDA). Absorption was measured from 200-400 nm and recorded at 210nm (limonoid detection), 280 nm (flavonoid glucoside detection) and 340 nm (polymethoxylated flavone detection). Millennium 32 software (Waters Company) was used for data acquisition and processing.

Mobile phase consisted of 10% acetonitrile in 3 mM phosphoric acid (solvent A) and acetonitrile (solvent B). Separation is achieved on C18 colunm (Alltima, Alltech: 5μ, 250mm x 4.6mm, 16 % carbon load, void time 2.02 minutes, packing lot number 2763) with gradient run starting with 0% B to 20% B in 20 minutes, and ending with 60% B at 115 minutes. Flow rate was 1 ml/minute. Injection volume was 10 μl.

Identification of limonoid aglycones (limonin, deacetylnomilin, nomilin, and obacunone), limonoid glucosides (limonin glucoside, deacetylnomilinic acid glucoside, nomilinic acid glucoside, and obacunone glucoside), flavanone glucosides (eriocitrin, narirutin, hesperidin, and didymin) and polymethoxylated flavones (sinensitin, nobiletin, heptamethoxyflavone, scutellarein tetramethylether, and tangeretin) were based on retention time, UV spectra and response factors of external standards.

4. Results and discussio

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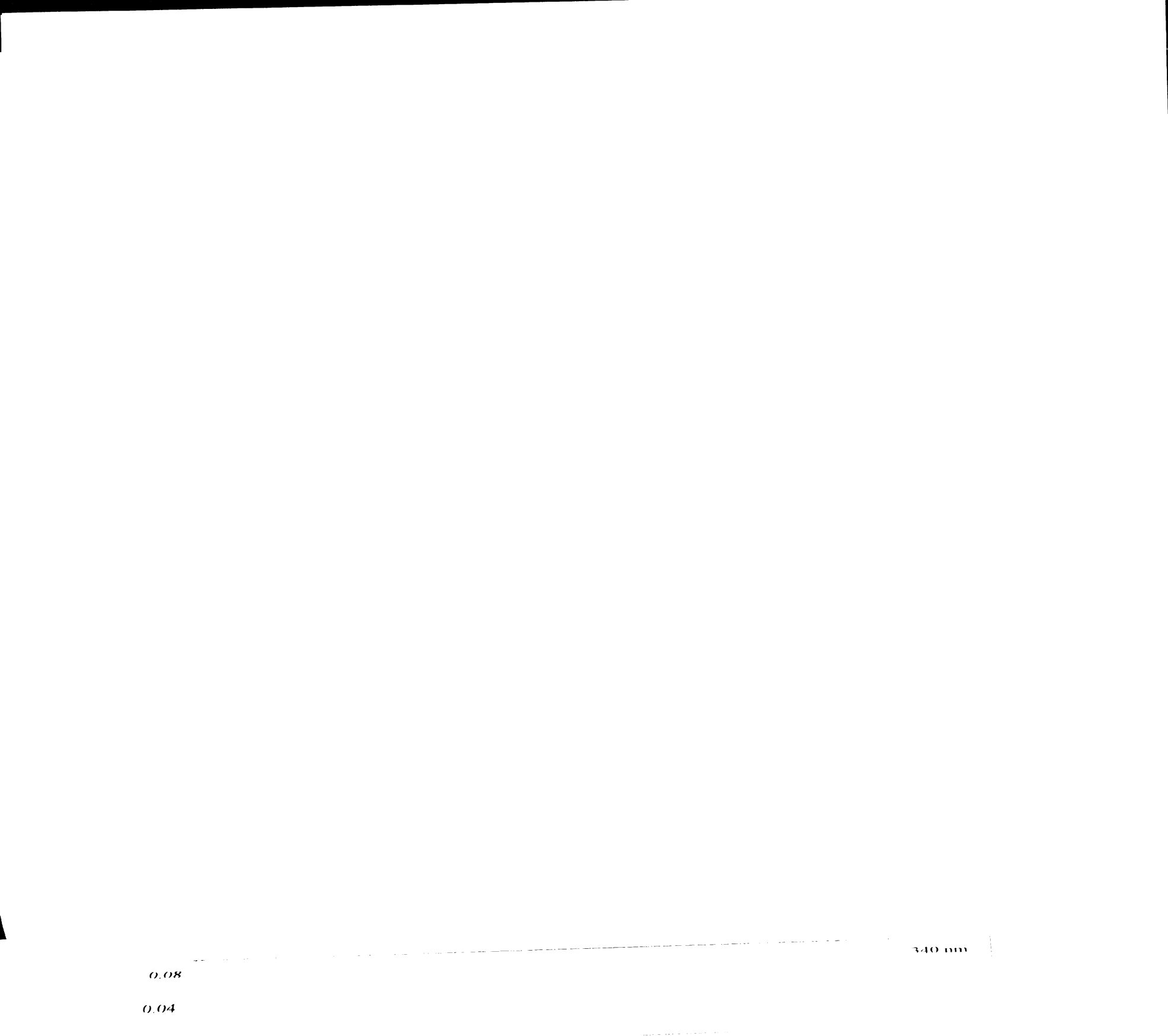
4. Results and discussion

Selections of the compounds (based on peak size) were determined from orange fractions containing highest content of limonoids and flavonoids. Peel had the highest flavonoids, while seed had the highest limonoids. Therefore, peel was used for the selection of flavonoids (flavanone glucosides and polymethoxylated flavones), whereas seed was used for the selection of limonoids.

Figures 11 to Figure 14 show chromatograms of polar compounds (reconstituted with 10% acetonitrile in 3mM phosphoric acid) and nonpolar compounds (reconstituted with 100% acetonitrile) from Valencia peel and seed extracts at 210, 280, and 340 nm, respectively. Chromatograms of other orange parts including: rag, press cake, peel juice, and juice are presented in Appendix I. Selected compounds are the labeled peaks. Retention times of the selected compounds were as follows: 40.7 (eriocitrin), 42.0 (limonin glucoside), 48.0 (deacetylnomilinic acid glucoside), 53.0 (narirutin), 57.8 (hesperidin), 64.6 (nomilinic acid glucoside), 67.6 (obacunone glucoside), 74.2 (didymin), 98.3 (deacetylnomilin), 100.6 (sinensitin), 102.6 (limonin), 106.6 (nobiletin), 109.5 (3,4,5,6,7,8,3',4'-heptamethoxyflavone/scutellarein tetramethylether), 109.7 (nomilin), 115.4 (tangeretin), and 117.2 (obacunone) minutes, respectively. There were coelution of limonin/unknown at 102.6 minutes; and 3,4,5,6,7,8,3',4'-heptamethoxyflavone/scutellareintetramethylether at 109.5 minutes.

The results showed that flavonoid glucosides and limonoid glucosides eluted in the similar region, and the same as limonoids and polymethoxylated flavones. Based on the chromatographic retention alone, flavonoid glucosides could be simultaneously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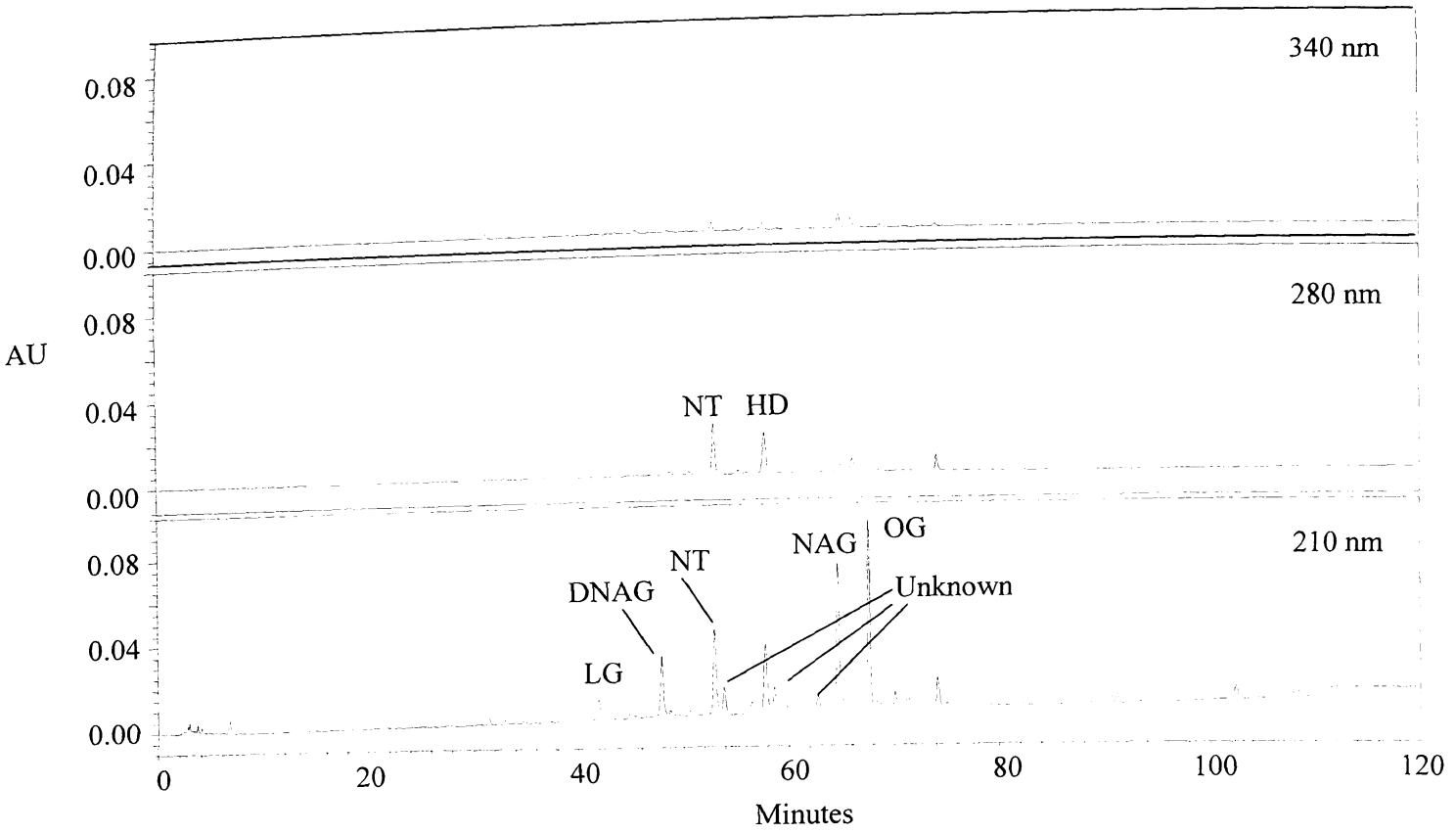

Figure 11: Polar compounds (reconstituted in 10% acetonitrile in 3 mM phosphoric acid) in seed extract at 340 nm (polymethoxylated flavones), 280 nm (flavanone glucosides), and 210 nm (limonoids). *NT = narirutin, HD = hesperidin, LG = limonin glucoside, DNAG = deacetylnomilinic acid glucoside, NAG = nomilinic acid glucoside, OG = obacunone glucoside.*


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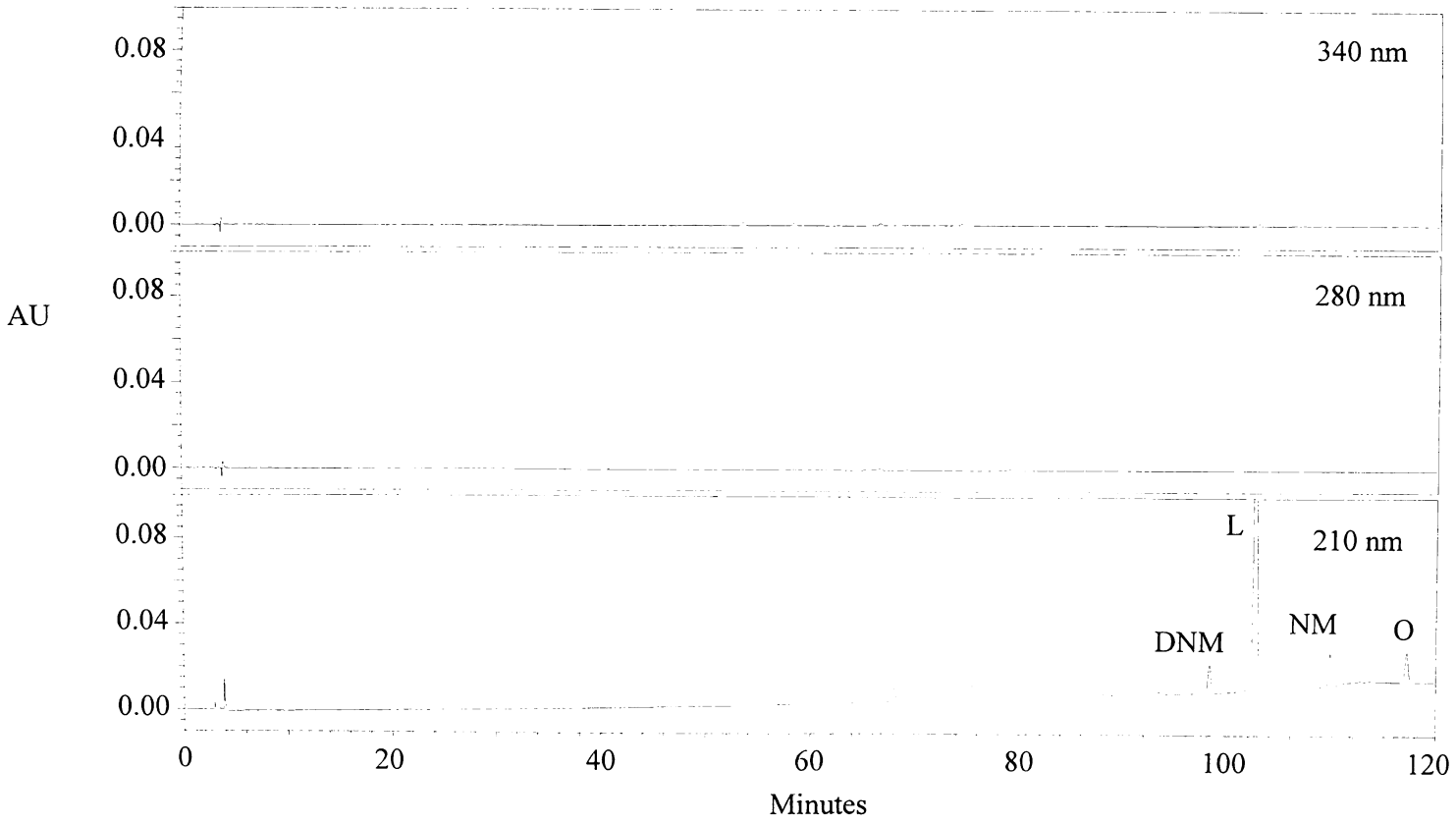
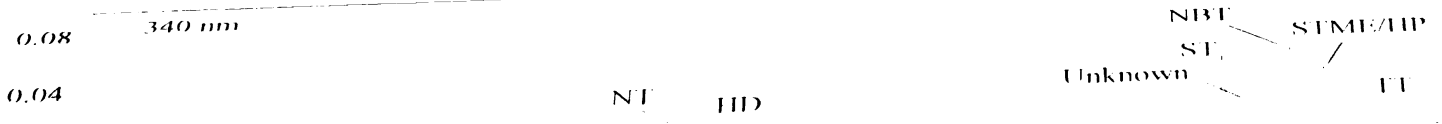


Figure 12: Nonpolar compounds (reconstituted in 100% acetonitrile) in seed extract at 340 nm (polymethoxylated flavones), 280 nm (flavanone glucosides), and 210 nm (limonoids). *L = limonin, NM = nomilin, DNM = deacetylnomilin, O = obacunone.*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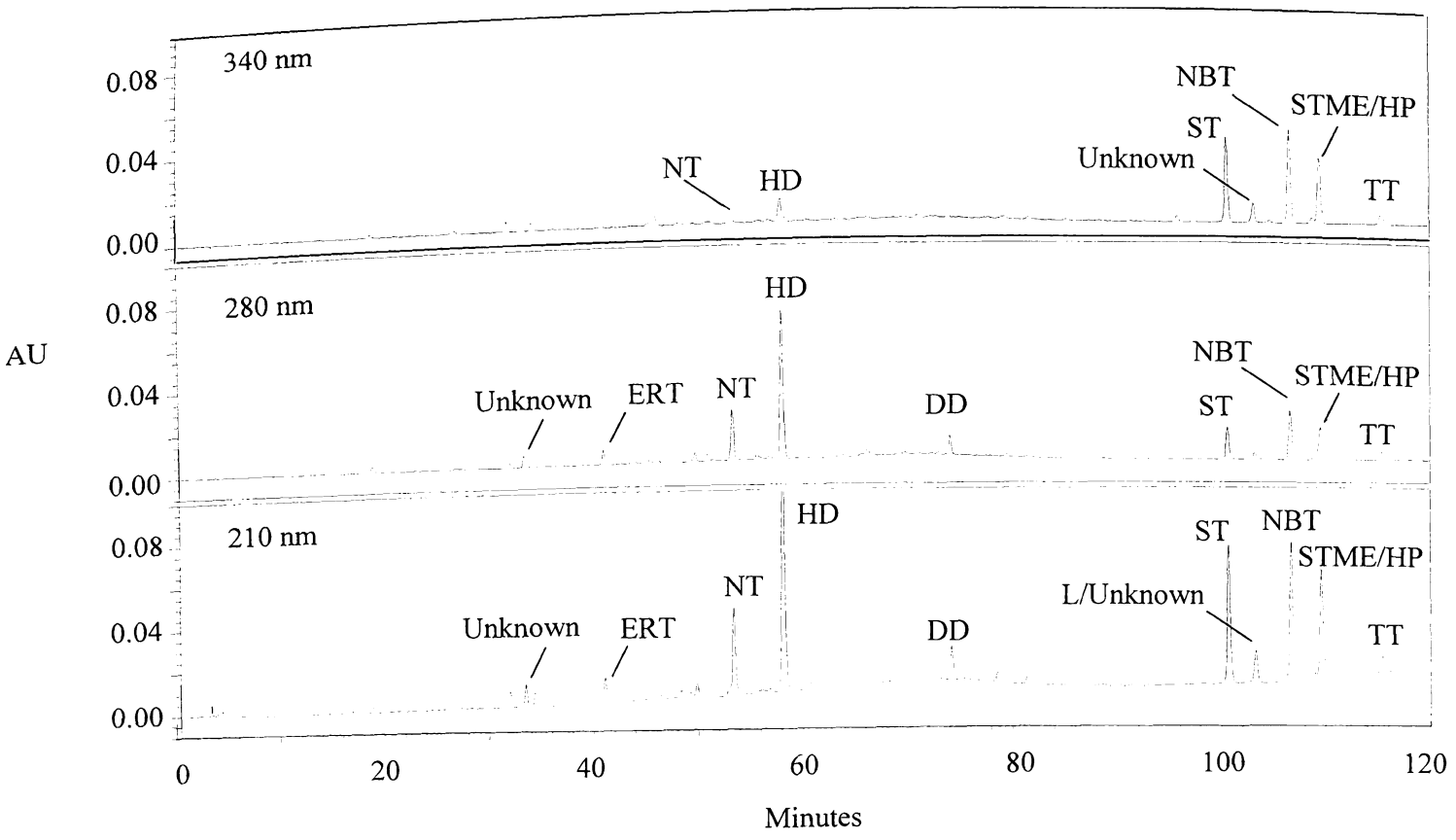
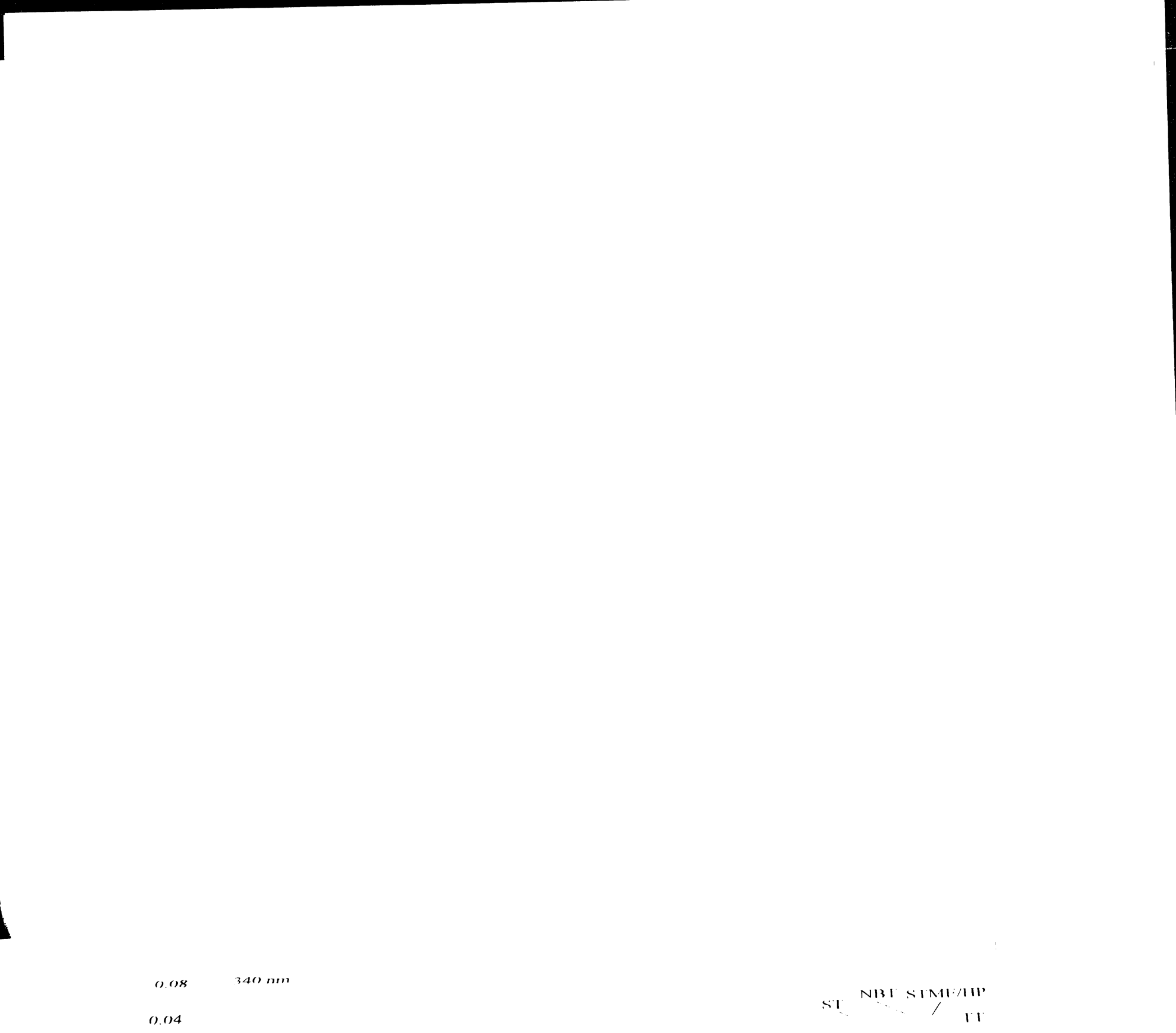


Figure 13: Polar compounds (reconstituted in 10% acetonitrile in 3mM phosphoric acid) in peel extract at 340 nm (polymethoxylated flavones), 280 nm (flavanone glucosides), and 210 nm (limonoids). *ERT = eriocitrin, NT = narirutin, HD = hesperidin, DD = didymin, L = limonin, ST = sinensitin, NBT = nobiletin HP = 3,4,5,6,7,8,3',4'- heptamethoxyflavone, STME = scutellarein tetramethylether, TT = tangeretin.*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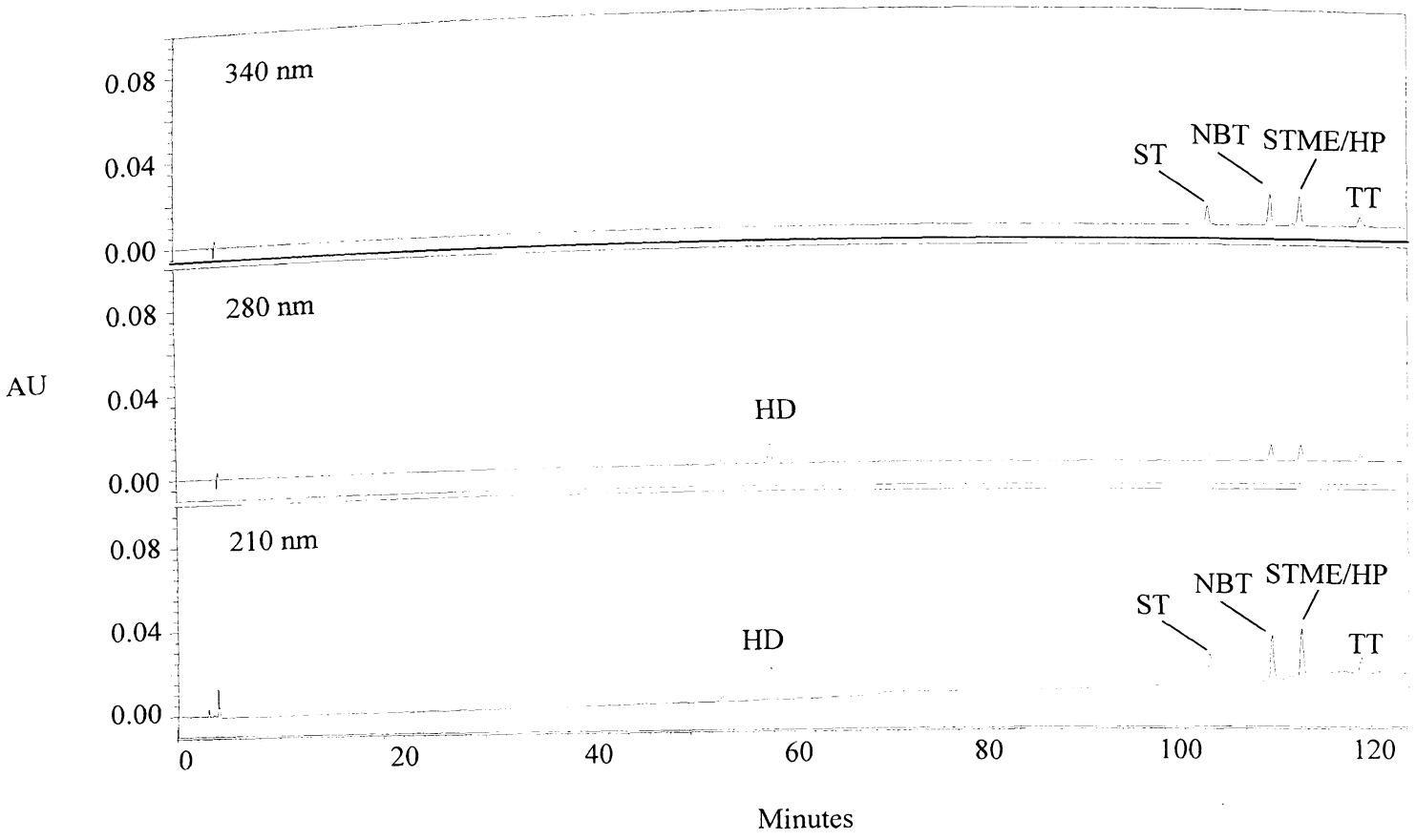

Figure 14: Nonpolar compounds (reconstituted in 100% acetonitrile) in peel extract at 340 nm (polymethoxylated flavones), 280 nm (flavanone glucosides), and 210 nm (limonoids). *HD = hesperidin, ST = sinensitin, NBT = nobiletin, HP = 3,4,5,6,7,8,3',4'-heptamethoxyflavone, STME = scutellarein tetramethylether, TT = tangeretin.*

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There were unknown peaks at 33.1, 53.8, 58.6, 62.7, and 103.2 minutes that existed in a comparable range with other identifiable compounds.

Based on chromatographic retention (Fong et al., 1993), and UV spectra of other available limonoid standards (Figure 15), unknown peaks at 58.6 and 62.7 min were identified as potentially deacetylnomilin glucoside and nomilin glucoside, respectively. Figure 16 shows UV spectra of unknown peaks at 58.6 and 62.7 minutes.

Based on chromatographic retention (Manthey and Grohmann, 1996, Robards et al., 1997 and Sendra et al., 1988), unknown at 33.1 and 103.2 minutes had the potential to be narirutin-4'-glucoside and 3,5,6,7,3',4'-hexamethoxyflavone, respectively.

UV spectrum of unknown at 103.2 minutes (Figure 17) was similar to that of 3,5,6,7,3',4'-hexamethoxyflavone presented in Figure 18 (Sendra et al., 1988), while UV spectrum of unknown at 33.1 minutes was similar to that of narirutin standard (Figure 19).

5. Conclusion

The HPLC gradient mobile phase used was suitable for estimation of relative retentions and selection of compounds (based on peak height), but not for quantitative analyses, since the achieved separation was relatively low. Based on the chromatographic retention alone, flavonoid glucosides could be simultaneously analyzed with limonoid glucosides; and limonoid aglycones could be simultaneously analyzed with polymethoxylated flavones.

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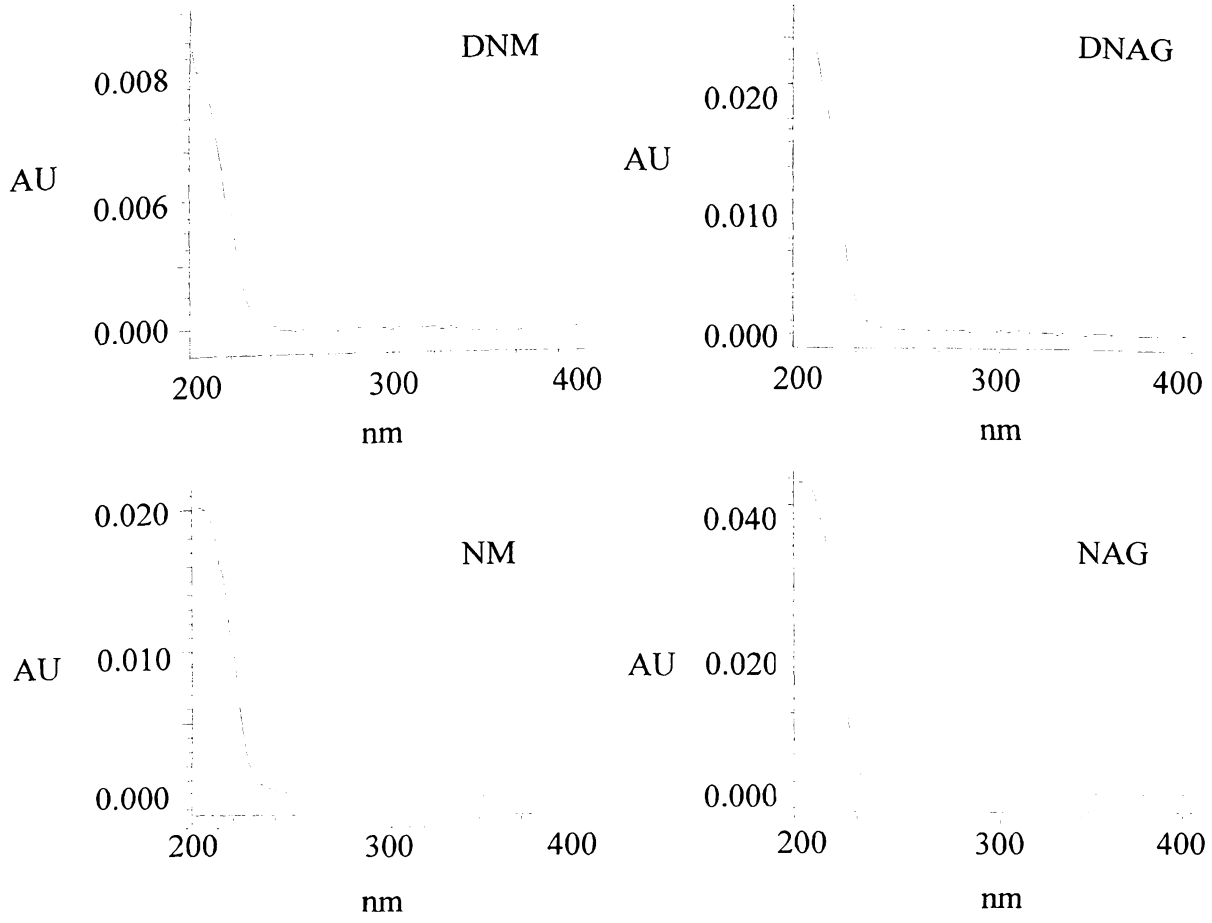

Figure 15: UV spectra of deacetylnomilin, nomilin, deacetylnomilinic acid, and nomilinic acid glucoside standards obtained from photo diode array detector.

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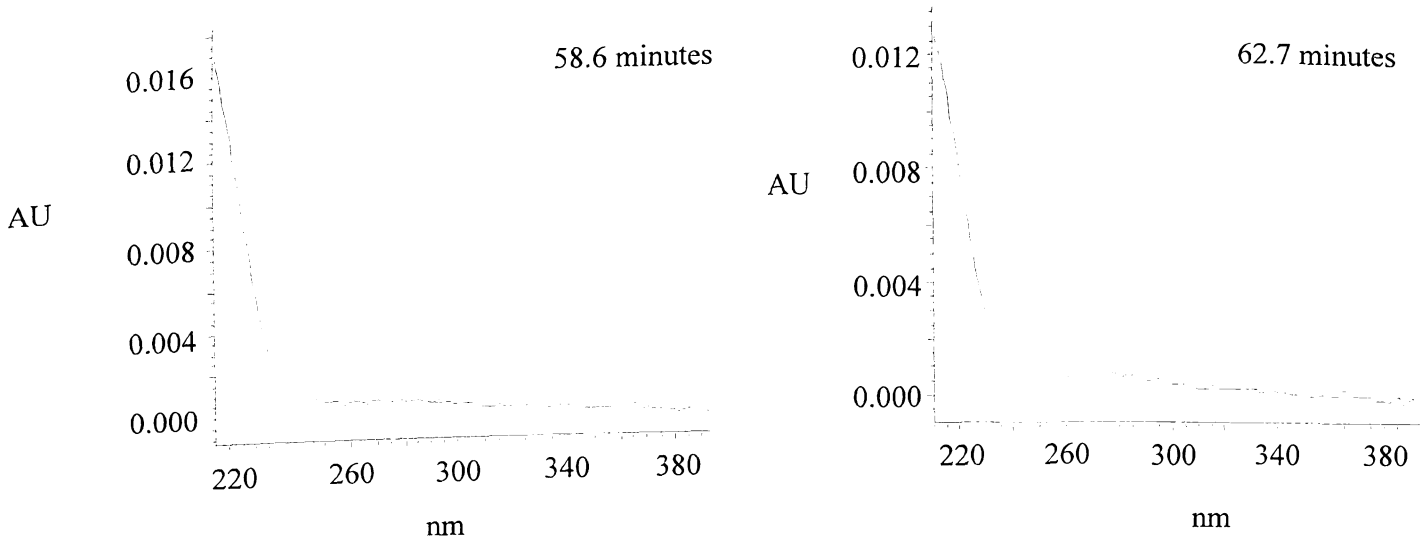

Figure 16: UV spectra of unknown peaks at 58.6 and 62.7 minutes obtained from photodiode array detector.

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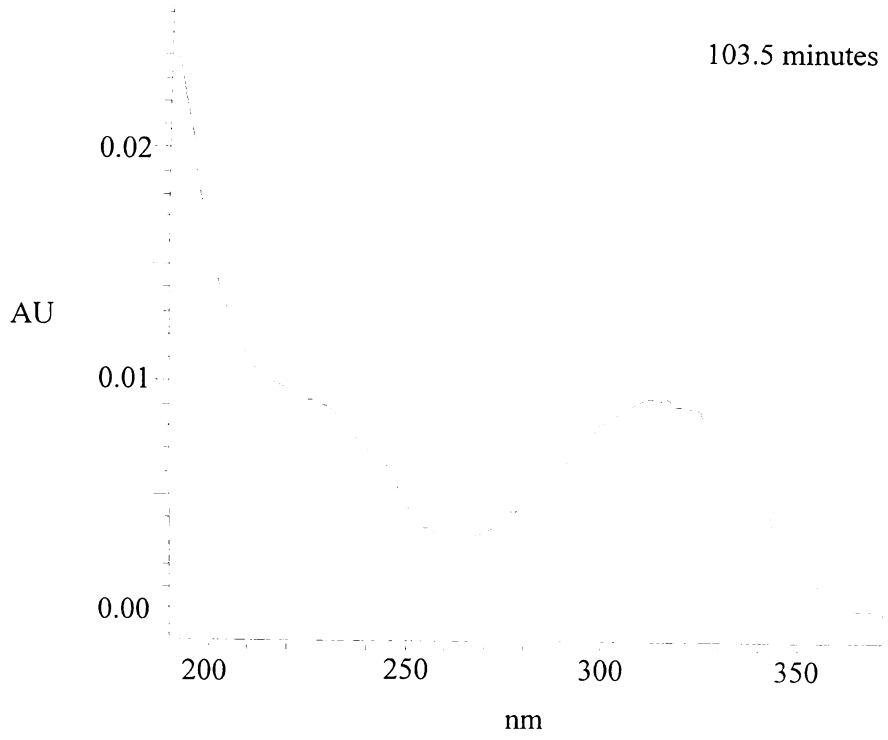

Figure 17: UV spectra of unknown peaks at 103.1 minutes obtained from photodiode array detector.

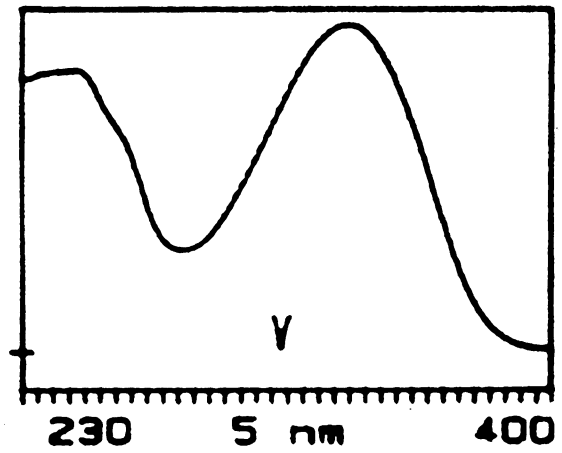

Figure 18: UV spectra of published 3,5,6,7,3',4'-hexamethoxyflavone (Sendra et al., 1988)

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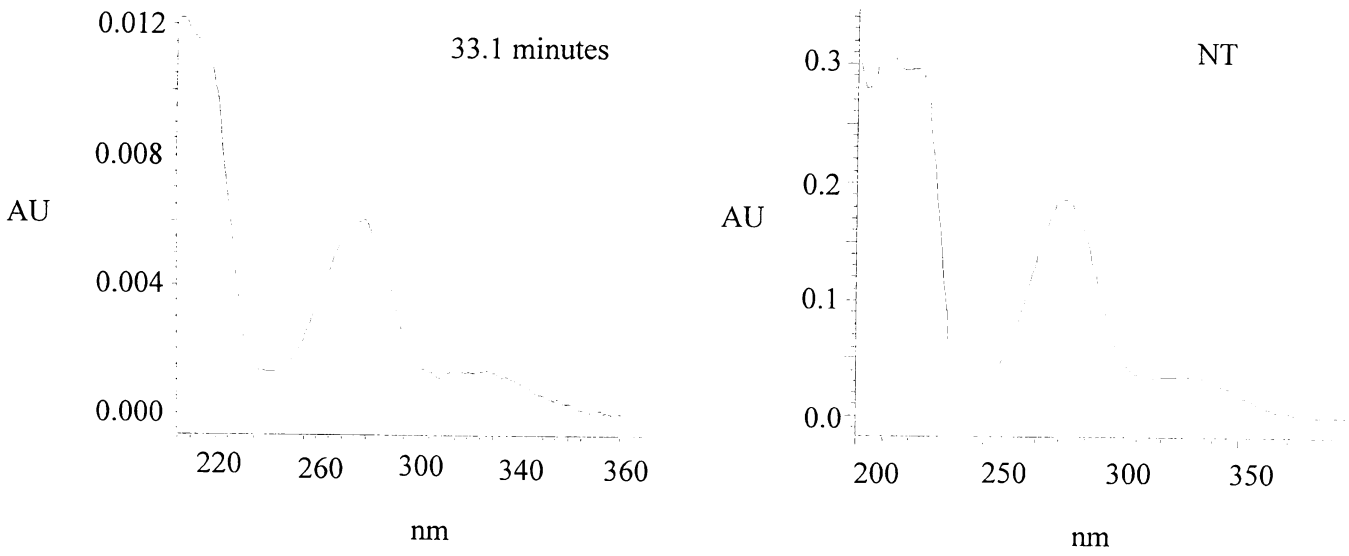

Figure 19: UV spectra of unknown peaks at 33.1minutes and narirutin standard obtained from photodiode array detector.

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

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Part II: Optimization of

1. Abstract

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2. Introduction

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Part II: Optimization of analytical methods

1. Abstract

Extraction and chromatographic conditions for limonoid aglycones/polymethoxylated flavones, limonoid glucosides, and flavanone glucosides were optimized using extracts of seeds, peels, and peel press liquid. Solvent extraction at high temperature (with additional steps for limonoid aglycones) was the primary technique. The obtained extraction techniques were rapid, inexpensive, and allowed acceptable recovery (greater than 90%). Reverse phase HPLC conditions were mainly gradient systems, except for analyses of seed limonoid aglycones which employed an isocratic system. Separations obtained were in the range of Rs = 0.6/N = 13,079 to Rs 1.1/N = 23,588 which was acceptable for these complex matrices.

2. Introduction

Even though quantitations of citrus limonoids and flavonoids in previous studies are relatively extensive, these two different citrus major phytochemicals have been analyzed separately. Distribution of limonoid aglycones and glucosides in Valencia oranges was reported by Fong et al. (1993). The analytical method used in this study is well established and have been used in numerous studies in their laboratory (Hasegawa et al., 1991 and Fong et al., 1992). However, in this study the fresh samples were analyzed and comparisons were based on the amount of individual limonoids per fruit, in which the water content of these tissues can be greatly varied.

Analyses of flavanone glucosides and polymethoxylated flavones have been done commonly in juice (Ooghe and Detavernier, 1999) and peel oil (Gaydou et al., 1987, Dugo et al., 1996, Chen et al., 1997, and Stremple, 1998). Extraction of

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compounds from these liquid samples is much simpler than from solid samples (such as peel, seed, and rag) because the compounds present are most readily extractable. Primary solvent used in flavonoid extraction were dimethylsulfoxide and dimethylformamide. They have high-UV cutoff, therefore producing large solvent front under wavelength used for detection of limonoids.

The objectives of this study were to optimized extractions and HPLC conditions suitable for types of samples and natures of compounds studied and thus incorporated the benefits and drawbacks of previous studies in the analytical methods. Specific objectives as follows:

1) To obtain a rapid extraction method with high selectivity and recovery,
2) To obtain a rapid chromatographic condition that allows separation resolution suitable for quantitative determination.

3. Materials and methods

3.1 Orange samples

Ground, freeze-dried orange peel and seed were selected for method optimization for solid fractions, while peel press liquid was selected for that for liquid fractions.

3.2 Extraction and analysis of limonoid aglycones and polymethoxylated flavones

3.2.1 Extraction

The extraction procedure of Fong et al. (1993) was modified from. Peel press liquids were thawed at room temperature and heated in a water bath (82°C for 30 min), then cooled to room temperature. Ten ml of peel press liquid was then mixed with 25 ml of 0.5 M Tris buffer (pH 8) for 15 minutes and then acidified to pH 2 with 1 N HCl.

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Ground, freeze-dried peel (1 g) was mixed with 25 ml of 0.5 M Tris buffer (pH 8) for 15 minutes and then acidified to pH 2 with 1 N HCl. Ground, freeze-dried seed (1 g) was mixed with 25 ml of 0.15 M Tris buffer (pH 8) overnight (20 hours), and then acidified to pH 2 with 1 N HCl. The acidified mixtures of peel, peel press cake, rag and seed were heated in a water bath (82°C for 30 min).

Ethyl acetate (25 ml) containing 200 ppm butylated hydroxytoluene (antioxidant) was added to all samples, shaken for 15 minutes, and the ethyl acetate layer was decanted. Ethyl acetate extraction was performed twice. The ethyl acetate layers were combined, evaporated to dryness, and reconstituted to 10 ml with methanol. Filtered extract (0.45μ nylon) was analyzed by HPLC.

Incorporation of heat (82°C for 30 min) to limonoid aglycone extraction was modified from method of McIntosh and Mansell (1997). To evaluate the recovery of extraction when heat was incorporated, a set of control was subjected to the same conditions but no heating applied. Figure 20 shows a flow diagram of limonoid aglycone and polymethoxylated flavone extraction.

3.2.2 Hydrolysis of limonoid glucosides

Ten ppm (mg/L) of limonin glucoside in 25 ml 0.15 M Tris buffer pH 8 was extracted by the method described above when heat (82°C for 30 min) was incorporated. Control was the buffer without limonin glucoside. Comparison of limonin content between spiked and control samples indicated whether there was occurrence of limonin glucoside hydrolysis.

Orange tissues

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1 g

Mix

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Figure 20: Flow

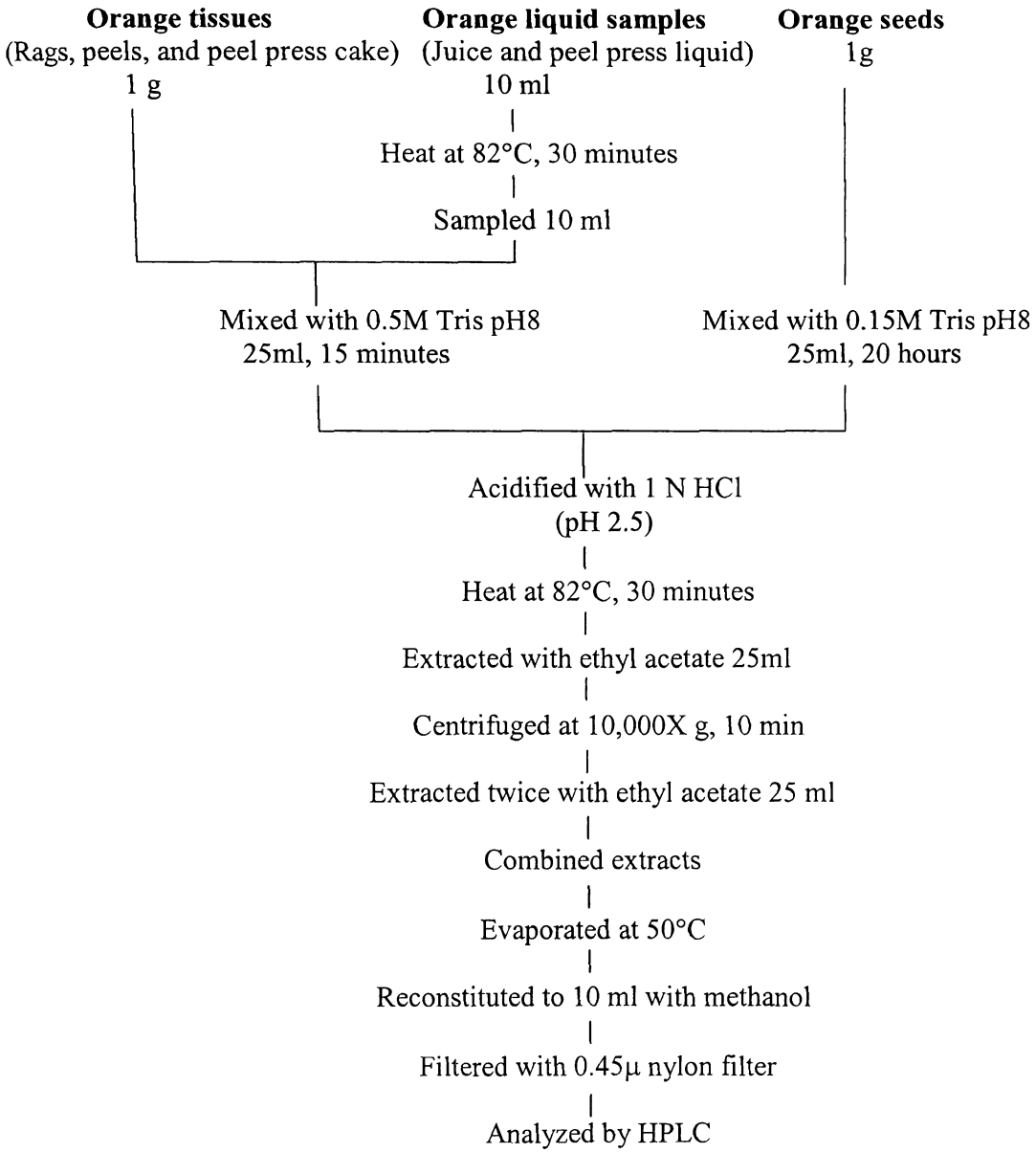

Figure 20: Flow diagram of limonoid algycone and polymethoxylatedflavone extraction.

3.2.3 Recovery

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3.2.4 HPLC

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3.2.3 Recovery

Recovery of limonoid aglycones and polymethoxylated flavones obtained from the extraction with heating (82°C for 30 min) was performed by spiking the samples with limonin (12.5 ppm) and scutellarein tetramethylether (2.5 ppm). The recovery was obtained by comparison of limonin and scutellarein tetramethylether content extracted from spiked and control samples.

3.2.4 HPLC

The mobile phases consisted of 3 mM phosphoric acid (solvent A) and acetonitrile (solvent B). Limonoid aglycones and polymethoxylated flavones were resolved with a gradient that started with 30% B, was 40% B in 20 minutes and ended with 50% B at 50 minutes. Flow rate was 1ml/min. Separation was achieved on a C18 column (Luna: C18, 5μ, 250 mm x 4.6 mm, 17.8 % carbon load, void volume 2.5 ml). Injection volume was 10 μl. Limonoid aglycones were detected at 210 nm, while polymethoxylated flavones were detected at 340 nm.

Since seeds are rich in limonoid aglycones and low in polymethoxylated flavones, limonoid aglycone analysis was carried out separately for seed extract. Separation of limonoid aglycones was achieved on C18 column (Alltima: C18, 5μ, 250 mm x 4.6 mm, 16 % carbon load, void time 2.02 minutes) and an isocratic mobile phase (acetonitrile/methanol/water, 10:41:49). Flow rate was 1ml/minute and injection volume was 10 μl.

Identification and quantitation of limonoid aglycones (limonin, deacetylnomilin, nomilin, and obacunone), were based on retention time, UV spectra and response factors of external standards. Identification of polymethoxylated flavones (sinensitin, nobiletin,

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3.3 Extraction

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3,4,5,6,7,8,3',4'-heptamethoxyflavone, scutellarein tetramethylether, and tangeretin) were based on retention time and UV spectra obtained with external standards. The quantitations of polymethoxylated flavones were based on the response factor determined for scutellarein tetramethylether.

3.2.5 Data analysis

Effect of heat treatment was determined by significant difference ($P \leq 0.05$) between limonin content extracted with and without heating using paired t test (Excel). Analyses were conducted in triplicate.

3.3 Extraction and analysis of limonoid glucosides

3.3.1 Extraction

Ground, freeze-dried seed (1 g) were mixed with 25 ml of 70% methanol for 15 minutes, and heated in a water bath (82°C for 5 min). The samples were centrifuged (10,000X g for 10 min), and the supernatants were decanted. The pellet was extracted again with 70% methanol. Combined supernatants were evaporated to approximately 2-3 ml at 40°C under vacuum, and reconstituted with 10 ml methanol. Filtered extracts (0.45μ nylon) were analyzed by HPLC.

Solvent extraction conditions using 70% methanol was studied at two pH levels (0.05 M Tris buffer pH 7.83 and purified water pH 4.4), three heating temperatures (room, 60°C, and 82°C), and two heating times (5 and 15 minutes). Figure 21 shows a flow diagram of limonoid glucoside extraction.

3.3.2 Recovery

Recovery of limonoid glucosides obtained from adjusted extraction was performed by spiking the sample with limonin glucoside (10 ppm), while the control was

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Figure 21: Flow

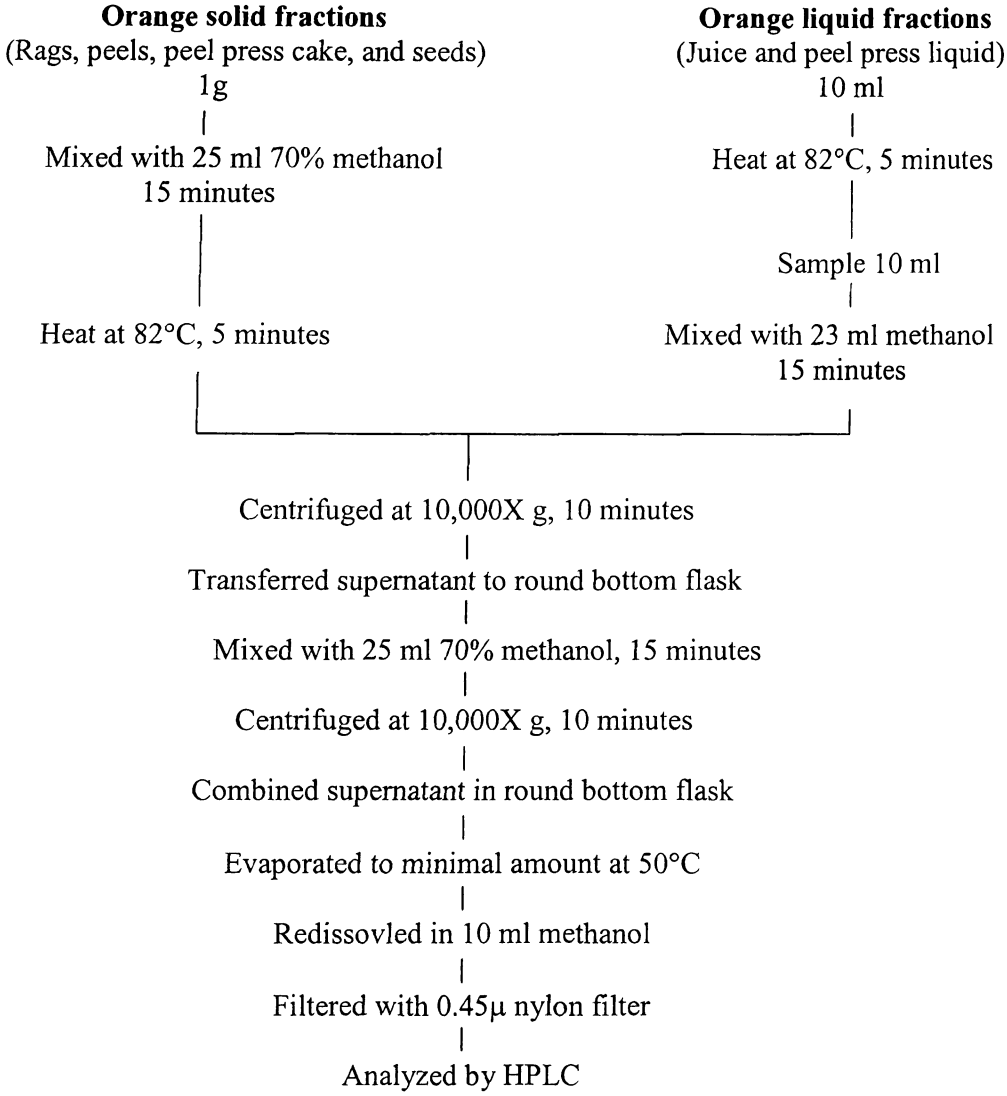

Figure 21: Flow diagram of limonoid glucoside extraction.

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added with the same amount of blank methanol. The recovery was obtained by comparison of limonin glucoside contents extracted from spiked and control samples.

3.3.3 High performance liquid chromatography (HPLC) analysis

The mobile phases consisted of 3 mM phosphoric acid (solvent A) and acetonitrile (solvent B). Limonoid glucosides were separated with linear gradient starting with 10% B and ending with 26% B in 70 minutes. Separation was performed on C18 column (Luna: C18, 5μ, 250 mm x 4.6 mm, 17.8 % carbon load, void volume 2.5 ml) with 1 ml/min flow rate and 10 μl injection volume. Limonoid glucosides were detected at 210 nm.

Identification and quantitation of limonoid glucosides (limonin glucoside, deacetylnomilinic acid glucoside, nomilinic acid glucoside, and obacunone glucoside) were based on retention time, UV spectra, and response factors obtained with external standards.

3.3.4 Data analysis

Different extraction conditions were compared for the highest recovery of limonoid glucosides using analysis of variance (ANOVA) with single factor (extraction condition) (Excel). Analyses were conducted in triplicate.

3.4 Extraction and analysis of flavanone glucosides

3.4.1 Extraction

Ground, freeze-dried peel (1 g) was mixed well with different modified solvents (25ml) [including 70%, 80%, 90% methanol in water; 70%, 80%, 90% methanol in 0.01 M sodium phosphate buffer (pH 7); dimethylformamide/methanol (1:1); and dimethylformamide/methanol (1:2)], heated (82°C for 5 min), and centrifuged at

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Figure 22: Flow

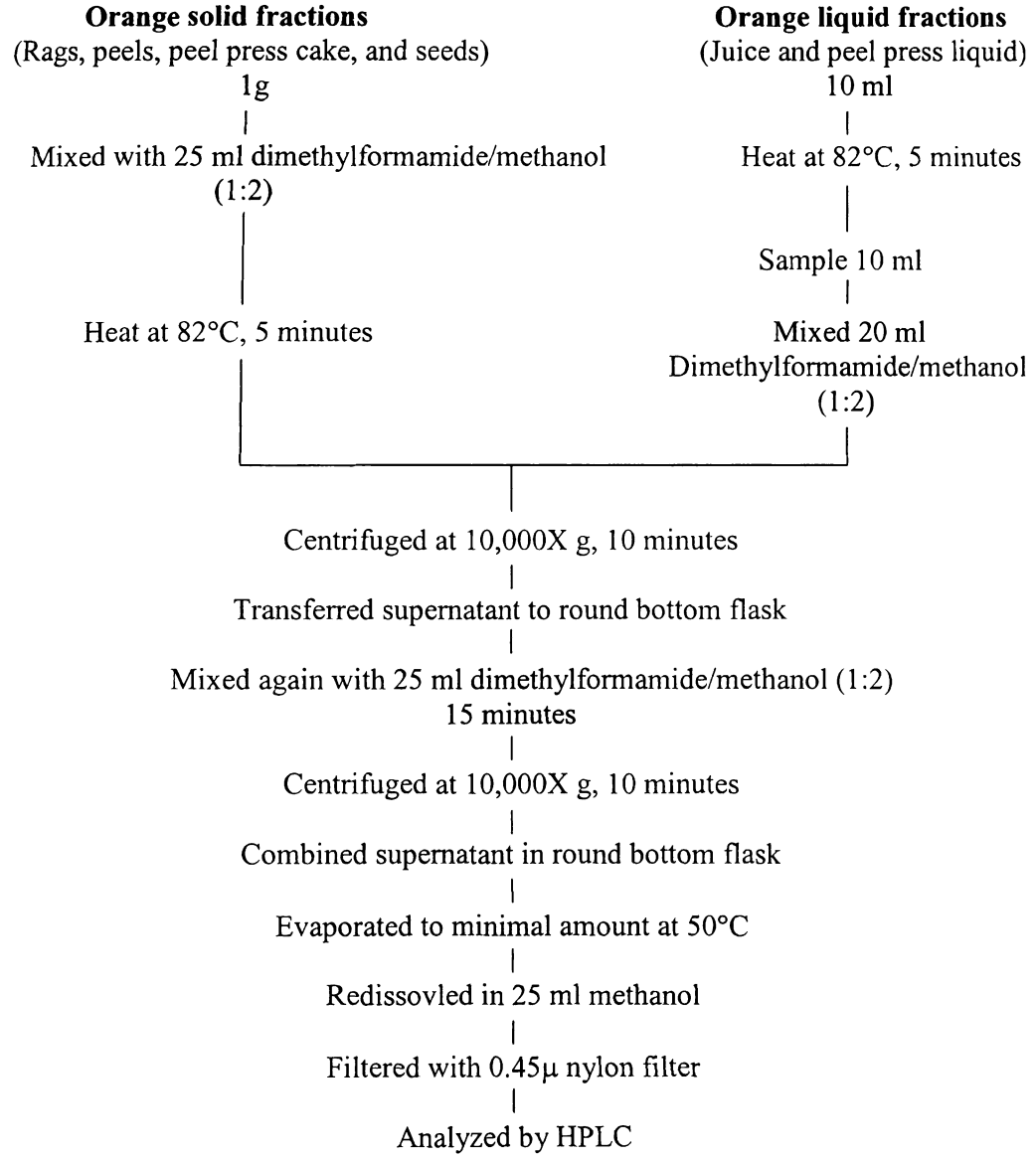

Figure 22: Flow diagram of flavanone glucoside extraction.

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10,000X g for 10 min. The pellet was extracted again and supernatants were combined, evaporated at 40°C under vacuum, and reconstituted with methanol to 10 ml (or 25 ml when dimethylformamide was used). Figure 22 shows flow diagram of flavanone glucoside extraction.

3.4.2 Recovery

Recovery of flavanone glucosides obtained from adjusted extraction was performed by spiking the sample with neohesperidin (10 ppm) and hesperidin (5 ppm), while the control was added with the same amount of blank methanol. The recovery was obtained by comparison of neohesperidin and hesperidin contents extracted from spiked and control samples. Neohesperidin was used because it is naturally absent from these sweet orange, whereas hesperidin was used because it was the most concentrated and insoluble flavanone glucoside in the sweet orange.

3.4.3 High performance liquid chromatography (HPLC) analysis

The HPLC analysis of flavanone glucoside was based on the method of Ooghe (1999). Flavanone glucosides were separated on C18 column (Alltima: C18, 5μ, 250 mm x 4.6 mm, 16 % carbon load, void time 2.02 minutes) with a mobile phase consisting of 0.01 M potassium phosphate monobasic (solvent A) and acetonitrile (solvent B). A linear gradient starting at 10%B and ending at 30% B in 60 minutes was used. Flow rate was 1 ml/min flow rate and injection volume was 10 μl. Flavanone glucosides were detected at 280 nm. Identification and quantitation of eriocitrin, narirutin, hesperidin, didymin were based on retention time, UV spectra, and response factors obtained with external standards.

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4. Results and

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3.4.4 Data analysis

Different extracting solvents were compared for the highest recovery of flavanone glucosides using analysis of variance (ANOVA) with single factor (extracting solvent) (Excel). Analyses were conducted in triplicate.

4. Results and discussion

Studied compounds were divided into three groups, based on their solubility and chromatographic retention: a) limonoid aglycones and polymethoxylated flavones; b) limonoid glucosides; and c) flavanone glucosides.

4.1 Extraction procedure

4.1.1 Limonoid aglycones and polymethoxylatedflavones

We adjusted Fong et al. (1993) method that was designed for limonoid aglycones. Adjusted method was subsequently verified for the recovery of polymethoxylated flavones. Limonoid aglycones and polymethoxylated flavones have common characteristics in that they both are nonpolar and neutral (carrying no charge).

Incorporation of heat (82°C for 30 min) in the extraction was evaluated. Heating was believed to improve dissolution of these nonpolar limonoids in freeze-dried samples, since the extraction method was originally used for fresh orange tissues. The results (Table 1) showed that there was a significant increase ($P \leq 0.05$) in limonin content due to heating.

The hydrolytic study was conducted to assure the absence of hydrolysis of β-glycosidic linkage on the limonoid glucoside molecules due to heat (82°C for 30 min). The hydrolysis would produce limonoid aglycones and result in the overestimation of limonoid aglycones. Result showed no peak of limonin in limonin glucoside extract

Table 1: Limonoid agl
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Sample	
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Seeds	
Peel press liquid	

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Table 2: Recovery o
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N=2

Table 3: Limonoid
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Extraction condi	
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Room temp. pH	
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60°C 5 min pH	
60°C 15 min w	
60°C 15 min pH	
82°C 5 min wa	
82°C 5 min pH	
82°C 15 min w	
82°C 15 min p	

LG = limonin gl
OG = obacunone
0.05 Tris buffer

Table 1: Limonoid aglycone content in seed, peel, and peel juice extracts with and without heating (82°C for 30 min).

Sample	Limonin (mg/Kg) ± %CV[1]	
	Without heating	With heating
Peels	180±2.9	342±11.5
Seeds	12301±1.2	12031±3.2
Peel press liquid	16±14.3	35±1.5

[1]N = 2, Heating resulted in a significant increase ($P \leq 0.05$) in limonin content (paired t test).

Table 2: Recovery of limonin (limonoid aglycone) and scutellarein tetramethylether (polymethoxylatedflavone) extracted under heating (82°C for 30 min).

Compound	Recovery (g/100g)[1]±%CV			
	Peel	Seed	Peel press liquid	Buffer
Limonin	91±7.9	94±5.7	95±1.3	-
Scutellarein tetramethylether	111±3.3	-	-	94±2.2

[1]N=2

Table 3: Limonoid glucoside content in sweet orange seeds extracted by different solvent extraction conditions.

Extraction conditions	mg/Kg ± %CV[1]			
	LG	Potential NMG	NAG	OG
Room temp./water[2]	13052±3.3	11999±0.3	7314±1.6	17157±4.3
Room temp./pH 7.8[3]	12295±5.4	974±33.0	7920±0.5	27730±2.1
60°C/5 min/water	13121±1.4	11839±0.3	7524±1.6	17282±3.8
60°C/5 min/pH 7.8	13355±2.1	454±17.9	8030±0.8	27390±1.6
60°C/15 min/water	13241±0.7	12046±0.3	7551±0.6	17286±0.5
60°C/15 min/pH 7.8	12858±2.3	756±28.3	8041±0.7	28618±0.8
82°C/5 min/water	13287±0.2	12169±0.2	7527±0.0	17308±0.2
82°C/5 min/pH 7.8	12654±6.4	565±8.7	7943±0.3	27950±2.9
82°C/15 min/water	13196±0.1	12141±0.2	7415±0.2	17618±0.1;
82°C/15 min/pH 7.8	12524±0.1	594±6.3	7821±0.8	28036±2.2

LG = limonin glucoside, NMG = nomilin glucoside, NAG = nomilinic acid glucoside, OG = obacunone glucoside, [1]N = 2, [2]70% methanol in water (pH 4.4), [3]70% methanol in 0.05 Tris buffer (pH 7.8)

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4.1.2 Limonoi

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which indicated that there was no hydrolysis of limonin glucoside under heating used. Recoveries of the adjusted extraction were approximately 93% for limonin and 102% for scutellarein tetramethylether (Table 2).

4.1.2 Limonoid glucosides

Unlike associated compounds, limonoid glucosides contain a carboxylated group. Initial attempts for their extraction were to use anion exchange to separate them from other neutral-polar compounds, primarily flavanone glucosides. However, anion exchange extraction used produced high variations and low recoveries of limonoid glucosides.

Extractions by 70% methanol at different pH, heating times, and heating temperatures were studied. At pH ~ 6.5 to 7, the carboxylate group on these molecules are completely ionized and more soluble, therefore higher recovery was expected. Results in Table 3 showed that at pH 7.5, there were a 90% decrease in potential nomilin glucoside and a 60% increase in obacunone glucoside compared to that at pH 4.4 at all heating levels and heating times. According to Hasegawa (2000), nomilin glucoside was converted to obacunone glucoside at pH $\geq$ 8 and nomilinic acid glucoside at pH $\leq$ 3. The increase in obacunone glucoside concentration could be contributed from the converted nomilin glucoside. As such, extraction at pH 7.5 was not analyzed.

Table 4 presents total limonoid glucoside content in sweet orange seeds extracted by 70% methanol at different heating temperature and heating time. There were no significant differences of limonoid glucoside content due to heating temperature and heating times. However, it was shown that when heating was applied extraction reproducibility was improved. Extraction by 70% methanol at 82°C for 5 minutes was

Table 4: Total limono
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Extraction
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N = 2. LSD $_{P\leq .05}$ =
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Table 5: Flavanone
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Extraction condi
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Table 4: Total limonoid glucoside content in sweet orange seeds extracted by 70% methanol at different conditions.

Extraction conditions	mg/Kg ± %CV[1]
Room temp.	87047[a]
60°C/5 min	87693[a]
60°C/15 min	88201[a]
82°C/5 min	88313[a]
82°C/15 min	88548[a]

[1]N = 2, $LSD_{(P\leq 0.05)}$ = 1300, Different superscripts indicate significant difference at P≤0.05 (ANOVA with single factor)

Table 5: Flavanone glucosides in sweet orange peel extracted by different solvent extractions.

Extraction conditions	mg/Kg±%CV[1]				
	potential NT-4'G	ERT	NT	HD	DD
90% methanol, water	519±10.8	354±3.2	1166±3.2	4194±6.2	332±3.3
80% methanol, water	522±3.9	379±6.3	1170±6.3	4157±24.0	317±14.1
70% methanol, water	522±0.7	383±5.1	1158±5.1	3586±7.8	293±10.4
90% methanol, pH 7[2]	502±6.9	361±18.3	1163±18.3	4670±65.6	360±57.9
80% methanol, pH 7[2]	538±0.5	371±0.0	1139±0.0	3592±7.5	295±2.0
70% methanol, pH 7[2]	527±0.2	370±3.9	1134±3.9	3614±14.5	310±14.7
DMF/methanol(1:1)	687±0.8	615±1.9	2057±1.9	27381±1.3	1371±1.3
DMF/ methanol (1:2)	613±0.4	586±0.2	1958±0.2	25915±0.5	1301±0.4

NT-4'-G = narirutin-4'-glucoside, ERT = eriocitrin, NT = narirutin, HD = hesperidin, DD = didymin, DMF = dimethylformamide, [1]N=2, [2]0.01sodiumphosphate (pH 7)

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selected, since it was a short heating extraction which resulted in low variation (Table 3). Under this extraction condition (70% methanol/ 82°C for 5 min), 90% (±5.9) recovery was obtained.

4.1.3 Flavanone glucosides

Flavanone glucosides are polar-neutral compounds. The difficulty of flavanone glucoside extraction was insolubility of hesperidin. Quantitative studies on flavanone glucosides (Kawaii et al., 1999; Ooghe and Detavernier, 1997; Manthey and Grohmann, 1996) have been primarily used dimethylsulfoxide (DMSO) and dimethylformamide (DMF) as extracting solvents to enhance hesperidin solubility.

The use of dimethylformamide was initially not preferable because of the toxicity and the high boiling point (153°C), which do not evaporate well and therefore resulting in lower detection sensitivity.

Table 5 shows flavanone glucosides in sweet orange peel extracted by different solvents. Table 6 shows total limonoid glucoside content in sweet orange seeds extracted by different solvent extractions. Significantly higher flavanone glucoside content ($P \leq 0.05$) was obtained when extracting solvent contained dimethylformamide. There was no significant difference ($P \geq 0.05$) between flavanone glucoside content extracted by dimethylformamide/methanol (1:1) and dimethylformamide/methanol (1:2). Therefore, dimethylformamide/methanol (1:2) was selected for extraction of flavanone glucosides in the subsequent studies, since less dimethylformamide was used. Table 7 shows recovery of neohesperidin and hesperidin in peel and peel press liquid extracted by dimethhylformamide/methanol (1:2). The recoveries were approximately 99% for hesperidin and 90% for neohesperidin.

Table 6: Total limonc
solvent extra

Extraction
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70% meth
90% meth
80% meth
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Dimethylforman
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N = 2. LSD $_{P\leq 0.05}$ =
difference at $P \leq 0.0$
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Table 7: Recovery
methanol

Compound
Neohesperidi
Hesperidin

N = 2

Table 6: Total limonoid glucoside content in sweet orange seeds extracted by different solvent extractions.

Extraction conditions	mg/Kg±%CV[1]
90% methanol, water	6565[a]
80% methanol, water	6545[a]
70% methanol, water	5942[a]
90% methanol, pH 7[2]	7057[a]
80% methanol, pH 7[2]	5936[a]
70% methanol, pH 7[2]	5956[a]
Dimethylformamide/methanol(1:1)	32112[b]
Dimethylformamide / methanol (1:2)	30376[b]

[1]N = 2, $LSD_{(P\leq 0.05)}$ = 3145, $LSD_{(P\leq 0.01)}$ = 4576, Different superscripts indicate significant difference at $P \leq 0.01$ (ANOVA with single factor), [2]0.01sodiumphosphate (pH 7), DMF = dimethylformamide

Table 7: Recovery of neohesperidin and hesperidin extracted by dimethylformamide /methanol (1:2)

Compound	Recovery (g/100g)±%CV[1]	
	Peel	Peel press liquid
Neohesperidin	94±4.7	104±1.4
Hesperidin	91±5.5	89±2.9

[1]N = 2

4.2 HPLC

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4.2 HPLC

Chromatographic conditions were separately adjusted for each compound group: a) limonoid aglycones and polymethoxylated flavones, b) limonoid glucosides, and c) flavanone glucosides.

Quantitation was based on "Peak height", instead of more common "peak area", because it accounts only peaks of interest when baseline resolution is not achieved. Standards for each group were analyzed before and after each series of samples to minimize detector response variations. A blank methanol was run to equilibrate the system before analyses.

It was observed that, for studied flavonoids and limonoids, improved separations were obtained using Luna column compared to Alltima column. Higher carbon load in Luna column (17.8%), compared that to Alltima column (16%), may contribute to this improved separation. Therefore, when analyzed complex mixtures (where the chromatograms include numerous peaks that were closely retained, Luna column was used.

4.2.1 Limonoid aglycones and polymethoxylatedflavones

Since Fong et al. (1993) condition was originally designed for separating limonoid aglycones in fruit tissues. To separate limonoid aglycones and polymethoxylated flavones, the mobile phase gradient was adjusted to be more extended (lower slope, %/min). Separation at 210 nm was Rs 0.94/ N 12,000.

Retention times were 27 (sinensitin), 27 (deacetylnomilin), 31 (unknown), 32 (limonin), 33 (nobiletin), 35 (3,4,5,6,7,8,3',4'-heptamethoxyflavone), 37.2 (scutellarein tetramethylether), 39 (nomilin), 47 (obacunone), and 42 (tangeretin) minutes. Figure 23


0.10
0.08
0.06
210 nm
Peel extract

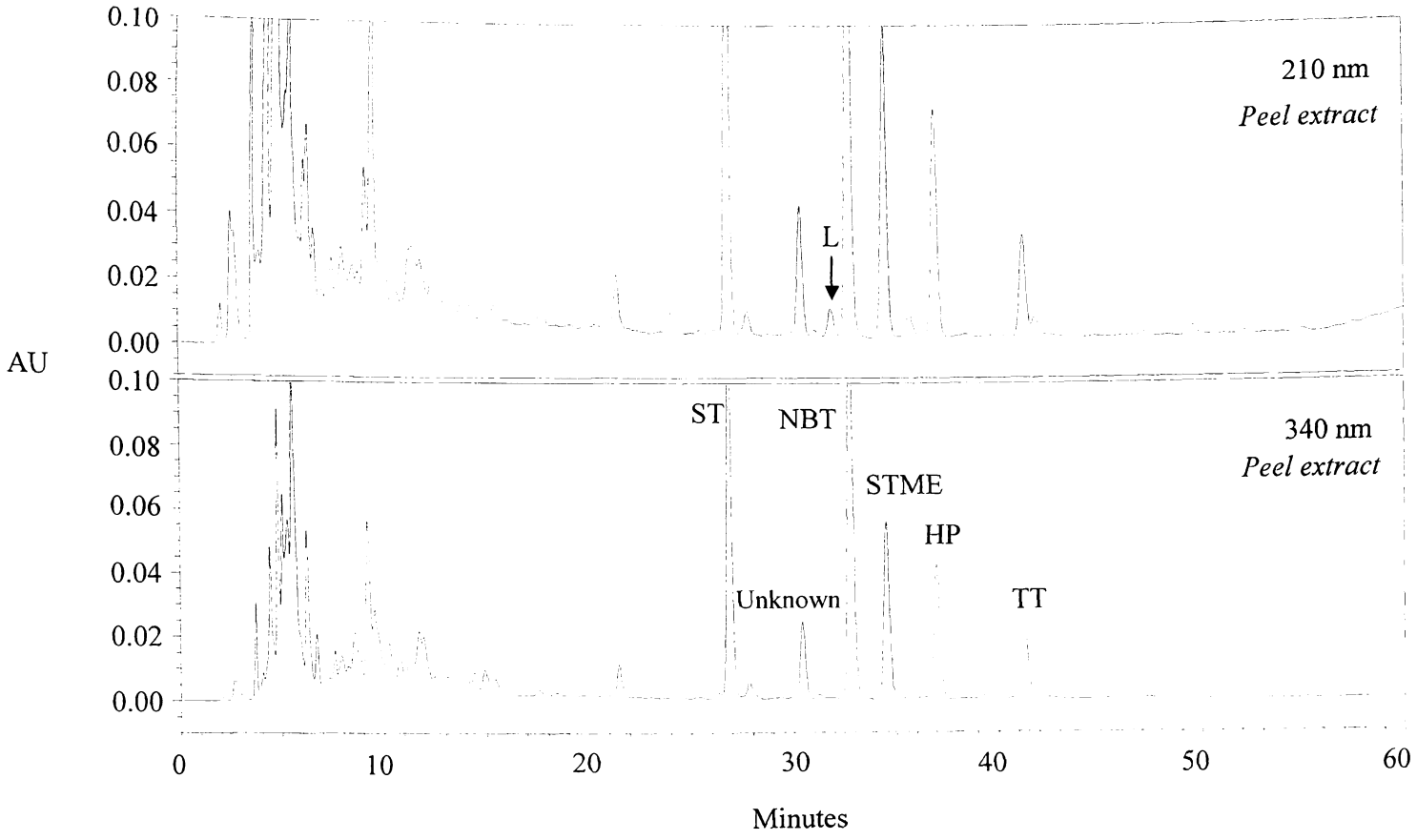

Figure 23: Limonoid aglycones (210 nm) and polymethoxylated flavones (340 nm) in peel extract (RT: 27.0 (ST), 27.4 (DNM), 30.6(unknown), 32.0 (L), 32.9(NBT), 34.7(SL), 37.2 (HP), 38.7 (NM), 47.0 (O), and 41.5 (TT) minutes [SP: C18, MP: 30% CH_3CN to 50% CH_3CN in 3mM H_3PO_4 in 50 min, 1 ml/min].
L = limonin, ST = sinensitin, NBT = nobiletin, HP = 3,4,5,6,7,8,3',4'-heptamethoxyflavone, STME = scutellarein tetramethylether, TT = tangeretin

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shows separation of limonoid aglycones and polymethoxylated flavones in orange peel extract under the gradient system at 210 nm (limonoid aglycone detection) and at 340 nm (polymethoxylated flavone detection). Figure 24 shows separation of polymethoxylated flavones in orange seed extract under the gradient system at 340 nm (polymethoxylated flavone detection).

For analyses of limonoid aglycone in orange seed, isocratic system was used. Separation at 210 nm was Rs 1.3/ N 1,131. Retention times were 18 (limonin), 21 (deacetylnomilin), 30 (nomilin), and 53 (obacunone). This system was not suitable for other samples which contained high flavonoid and low limonoid content, because limonin and deacetylnomilin were not separated from impurities, and obacunone coeluted with polymethoxylated flavones. Figure 25 shows separation of limonoid aglycones in orange seed extract under the isocratic system at 210 nm (limonoid aglycone detection).

4.2.2 Limonoid glucosides

Fong et al., (1993) was modified. Separation obtained was Rs 1.1/N 23,588. Retention time were 38 (limonin glucoside), 46 (deacetylnomilinic acid glucoside), 53 (unknown), 63 (unknown), 65 (nomilinic acid glucoside), and 68 (obacunone glucoside). Under the chromatographic system used, an addition of a glucose molecule on limonoid aglycones did not change the elution order of limonoids. Figure 26 shows separation of limonoid glucosides in orange seed and peel extracts at 210 nm (limonoid glucoside detection).

4.2.3 Flavanone glucosides

Figure 27 shows separation of flavanone glucosides in orange peel extract at 280 nm. Separation obtained was Rs 0.6/N 13,079. Retention times of interested flavanone


0.010
0.008
340 nm
Seed extract

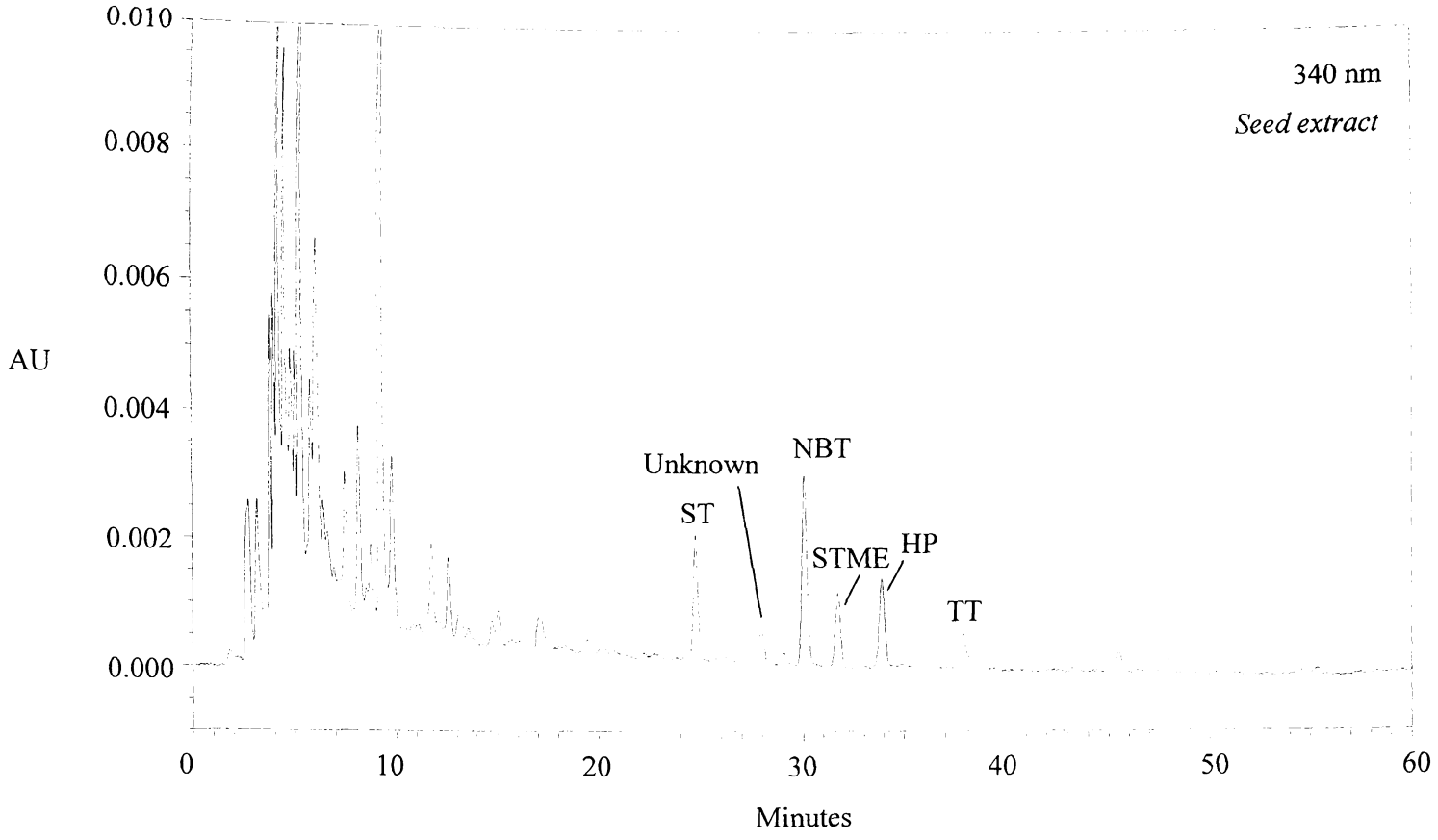

Figure 24: Polymethoxylated flavones at 340 nm in seed extract (RT: 27.0 (ST), 30.6(unknown), 32.9(NBT), 34.7 (STME) 37.2 (HP), and 41.5 (TT) min), [SP: C18, MP: 30% CH_3CN to 50% CH_3CN in 3mM H_3PO_4 in 50 min, 1 ml/min]. *ST = sinensitin, NBT = nobiletin, HP = 3,4,5,6,7,8,3',4'-heptamethoxyflavone, STME = scutellarein tetramethylether, TT = tangeretin.*

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210 nm

Seed extract

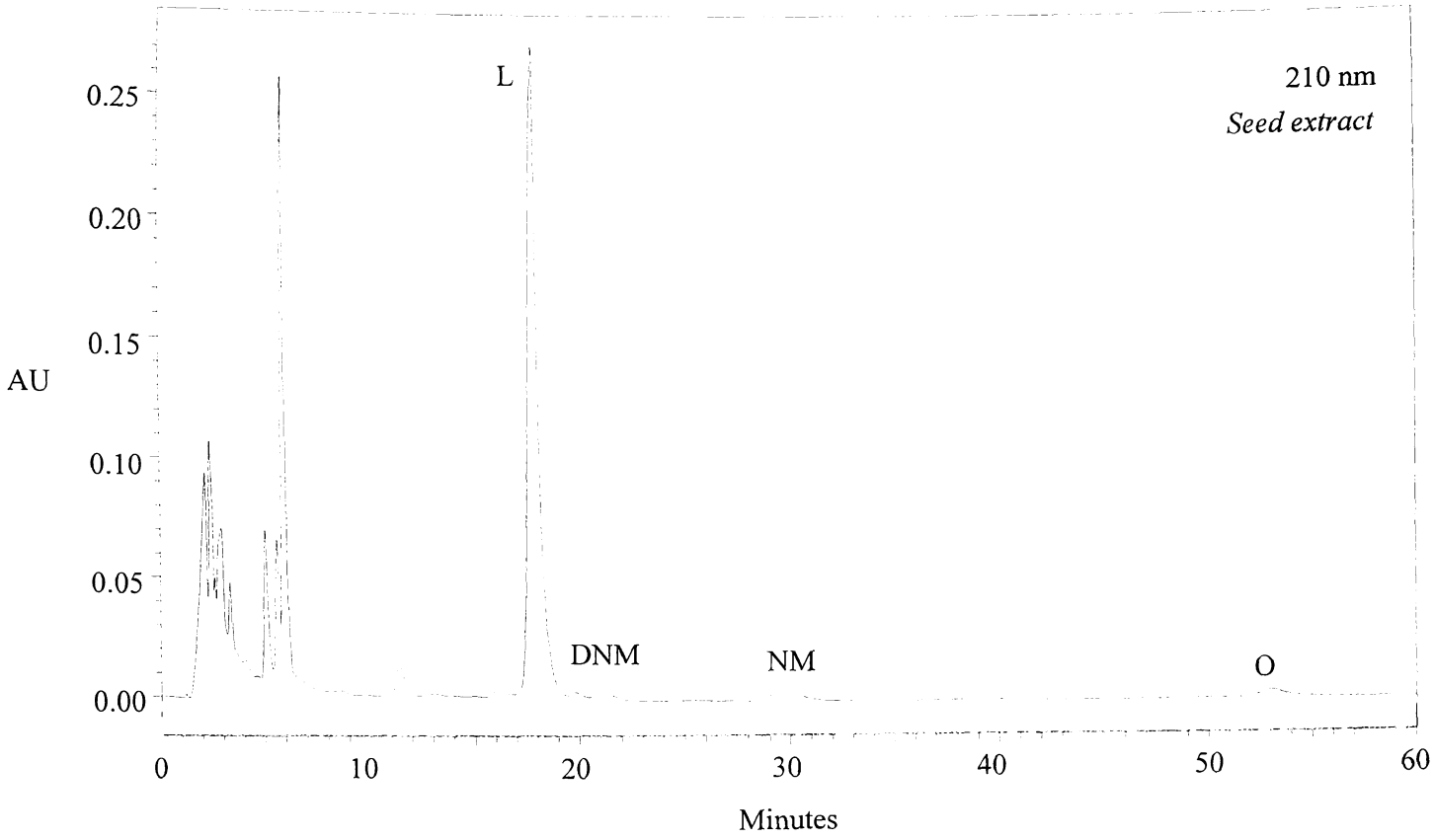

Figure 25: Limonoid aglycones at 210 nm in seed extract (RT: 17.7(L), 20.9(DNM), 29.7(NM), and 53.1(O) min) [SP: C18, MP: $CH_3CN/CH_3OH/H_2O$, 10:41:49, 1 ml/min]. *L = limonin, NM = nomilin, DNM = deacetylnomilin, O = obacunone*


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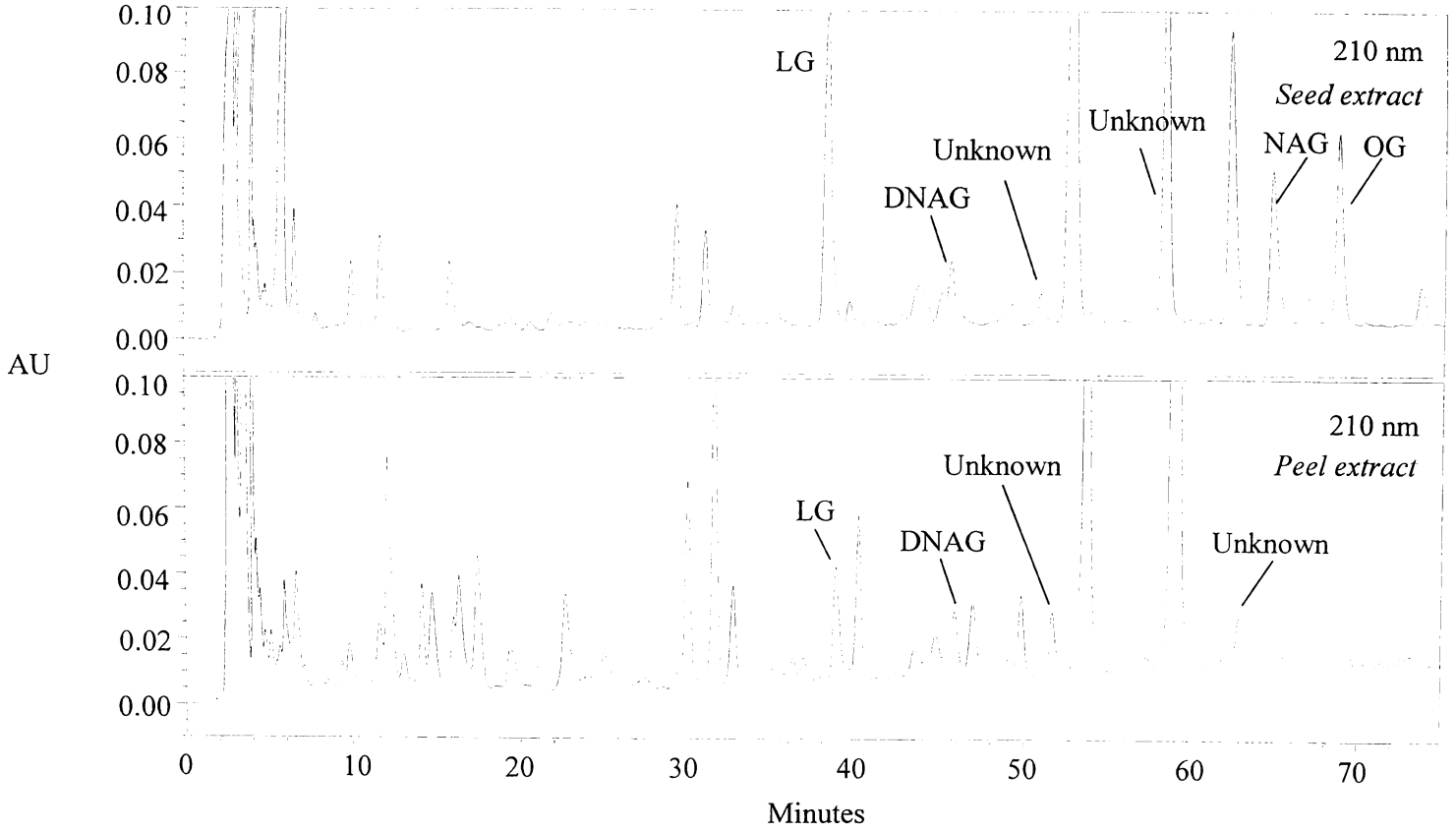
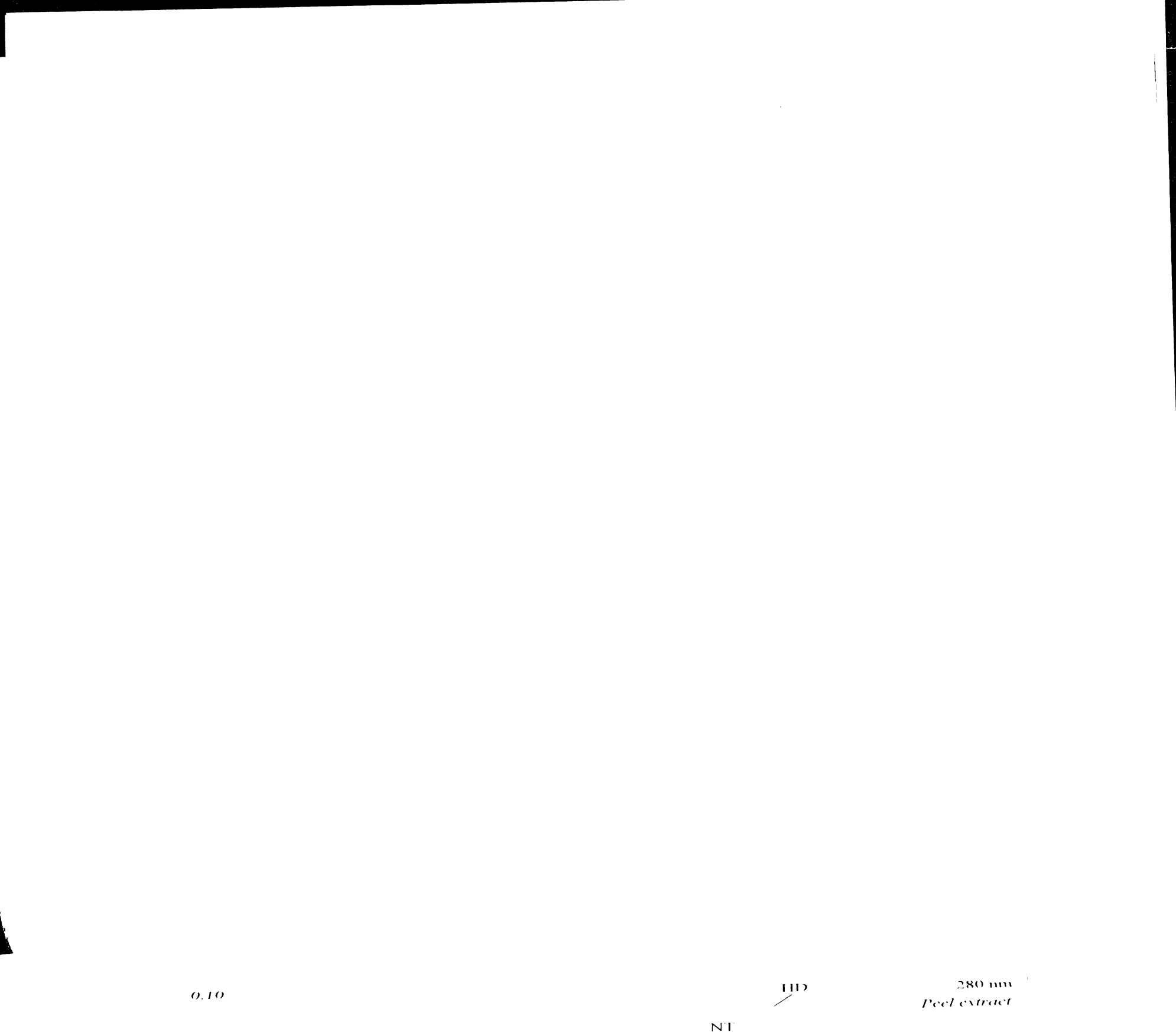


Figure 26: Limonoid glucosides in seed and peel extracts at 210 nm (RT: 37.98 (LG), 45.64 (DNAG), 52.7 (unknown), 62.57 (unknown), 65.3 (NAG), and 68.5 (OG) min), [SP: C18, MP: 10% CH_3CN to 26% CH_3CN in 3mM H_3PO_4 in 70 min, 1ml/min]. *LG = limonin glucoside, DNAG = deacetylnomilinic acid glucoside, NAG = nomilinic acid glucoside, OG = obacunone glucoside*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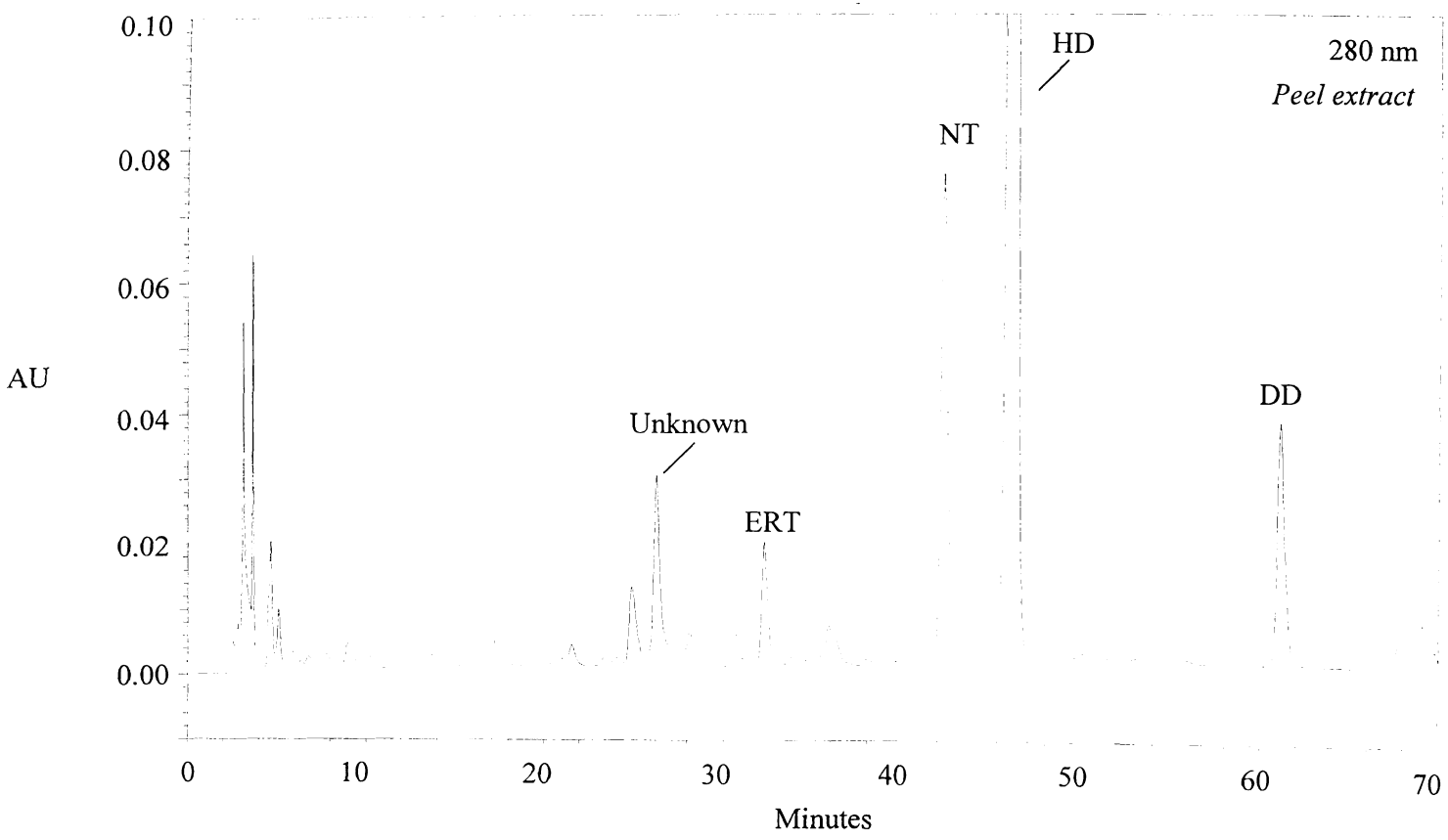

Figure 27: Flavanone glucosides at 280 nm in peel extract (RT: 26.2 (unknown), 34.1 (ERT), 42.1 (NT), 45.8(HD), 61.1(DD) minutes [SP: C18, MP: 10% CH_3CN to 30% CH_3CN in 0.01 M KH_2PO_4 in 60 min, 1ml/min]. *ERT = eriocitrin, NT = narirutin, HD = hesperidin, DD = didymin*

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5. Conclusio

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glucosides were 26 (potential narirutin-4'-glucoside), 34 (eriocitrin), 42 (narirutin), 46 (hesperidin), 61 (didymin) minutes.

All flavanone glucosides found in detectable levels were tasteless rutinosides, which are found primarily in sweet oranges. Their relative retention is correlated to number of hydroxyl and methoxyl groups on the B ring. Compounds with more hydroxyl groups possess increased polarity (less retained in reverse phase) and those with more methoxyl groups are more nonpolar (more retained in reverse phase).

5. Conclusion

It was necessary to categorize limonoids and flavonoids studied into groups with similar chromatographic retentions: 1) polymethoxylated flavones and limonoid aglycones, 2) limonoid glucosides, and 3) flavanone glucosides.

Extraction of polymethoxylated flavones and limonoid aglycones was improved by heat (82°C for 30 min). The used of pH 7 resulted in structural instability of suspected nomilin glucoside. Reproducibility of limonoid glucoside extraction was improved by heating. Extraction of flavanone glucosides required dimethylformamide.

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Study II: Isolation

Part I: Isolation an nomilin glu

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2. Introduction

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Study II: Isolation and identification of selected limonoids and flavonoids

Part I: Isolation and identification of deacethylnomilin glucoside (DNG) and nomilin glucoside (NG)

1. Abstract

Two unknown peaks from seed extract having similar chromatographic retention to those of deacetylnomilin glucoside (DNG) and nomilin glucoside (NG) were identified for these two compounds. Ground seed from the Valencia variety was used as a raw material. Preliminary cleaning using liquid-liquid and anion exchange extraction resulted in a simpler limonoid glucoside mixture for the subsequent isolation by HPLC. Separation (Rs = 0.75/N = 5,575) was performed on analytical scale HPLC using a large sample load (40μl). Identification was confirmed based on their molecular weight information obtained from negative FABMS.

2. Introduction

Deacetylnomilin glucoside (DNG) and nomilin glucoside (NG) are among minor limonoid glucosides detected in sweet orange (*Citrus sinensis*). Quantitative analyses of limonoid glucosides were found in limited studies, due to the lack of the commercial standards. Fong et al. (1993) quantitatively analyzed both limonoid aglycones and their glucosides; the identification was based on the retention times of standard compounds purified in their laboratory. Identification of limonoid glucosides have been done primarily using nuclear magnetic resonance (NMR) established by Hasegawa (1989) and, in the less extent, electrospray ionization liquid chromatography mass spectrometry (ESI-LC-MS) (Schoch et al., 2001). Purified and concentrated (at least 5 mg/0.7ml) sample is required for NMR analyses, which provide detailed structural information for these complex structures. For ESI-LC-MS analyses, prior purification is not required and

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much less concentrated sample can be used (as small as picogram unit). This technique provides both chromatographic and molecular weight information. Fast atom bombardment mass spectrometry (FABMS), found in lesser extent (Sawabe et al., 1999) compared to those two techniques, since it requires preliminary purification step, it is less sensitive compared to ESI-LC-MS, and less structurally informative compared to NMR. However, operation of this instrument is very simple and relatively inexpensive. However, additional information such as UV spectra and chromatographic retention are required for the definitive conclusion.

The two unknowns present consistently in orange seed extracts had a similar chromatographic retention to those of deacetylnomilin glucoside (DNG) and nomilin glucoside (NG). We were interested to verify this assumption by using the appropriate techniques to identify these unknown peaks.

3. Materials and methods

3.1 Seed

Seed from Valencia variety was used. Freeze-dried seed was ground using coffee grinder and extracted with hexane extraction (1:4, W/V) twice at room temperature to remove orange oil. The ground seed was ground again to pass 1 mm screen using UDY-Mill and stored at –20°C until analyses.

3.2 Sample preparation and extraction

Seed powder was homogenized with 0.05 M Tris buffer pH 8 (1:10, W/V) with bio-homogenizer for 2-3 minutes. The mixture was acidified to pH ~ 2.5 with 1 N HCl. The mixture was extracted twice using ethyl acetate (containing 200 ppm butyrated hydroxytoluene). Nonpolar compounds partitioned into ethyl acetate fraction, which was

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separated by centrifuge at 5000X g for 10 min. Ethyl acetate fraction was a source for limonoid aglycones and buffer fraction was a source for limonoid glucosides. Figure 28 shows flow diagram of limonoid isolation from orange seed.

3.3 Preliminary purification for limonoid glucosides

To remove neutral impurities such as flavanone glucosides, sugars from the buffer fraction, the mixture was passed through anion exchange (75 ml). Anion exchange column was preconditioned with 50 ml 1 M acetic acid and 100 ml water. The buffer fraction was applied on to the top of the column, washed with 100 ml water, and limonoidglucosides were eluted with 50 ml 1 M sodium chloride.

To remove salts, each 10 ml of eluate was passed through the C18 Sep-Pak (1000 mg), which was preconditioned with 3 ml methanol and 10 ml water; the column was washed with 10 ml water and eluted with 6 ml. Methanol was evaporated; and the residue was dissolved in minimal amount of water, stored at -20°C until use.

3.4 Isolation of deacetylnomilin glucoside and nomilin glucoside by high performance liquid chromatography (HPLC)

Separations were done on C18 column (Luna column: C18, 5 μl, 250mm x 4.6 mm, 17.8% carbon load, Phenomenex). The linear gradient started at 15% and ended with 26% acetonitril in 3mM phosphoric acid in 60 minutes. Flow rate was at 1 ml/min. Injection volume was 40 μl. Limonoid glucosides were detected at 210 nm. The HPLC setup was mentioned in Study I/Part I (3.4).

Isolated fraction was evaporated at 40°C under vacuum and concentrated using C18 cartridge (500mg). Eluted methanol from C 18 cartridge was evaporated at 37°C under N_2 gas and stored at refrigerator until analyzed by mass spectrometer.

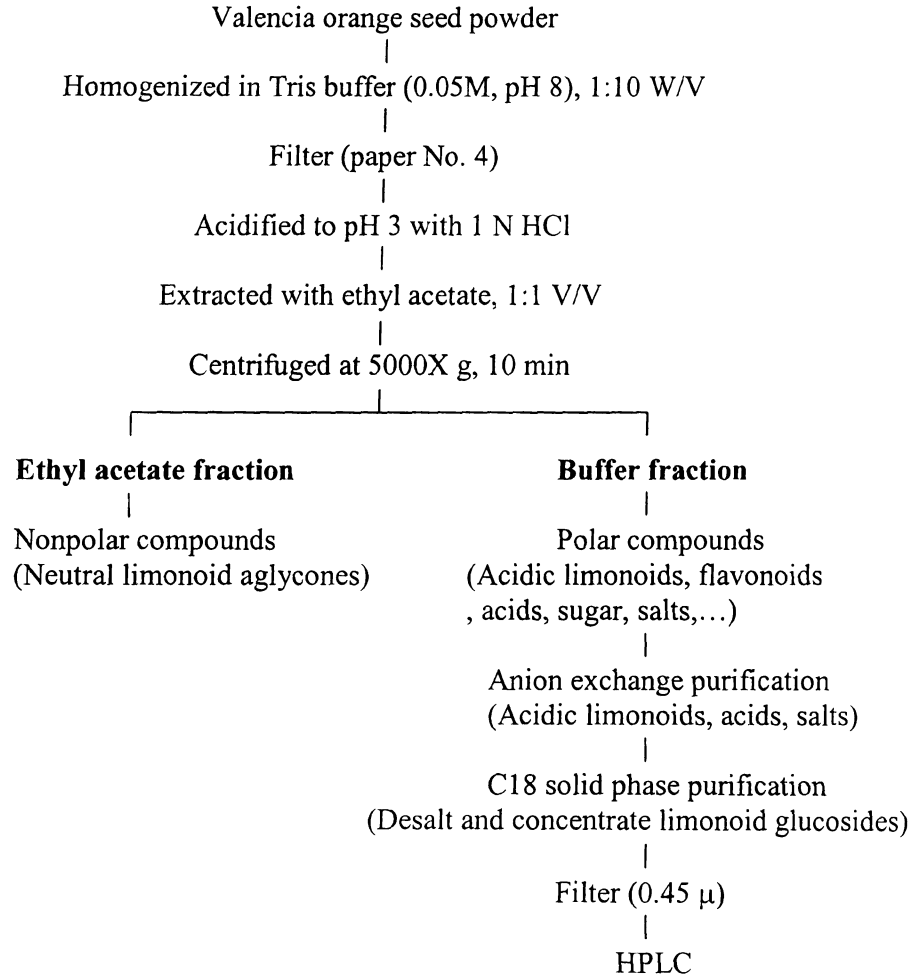

Figure 28: Flow diagram of limonoid isolation from orange seeds.

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3.5 Fast atom bombardment mass spectrometry

FAB mass spectra were obtained using a JEOL HX-110 double-focusing mass spectrometer (JOEL USA, Peabody, MA) operating in negative ion modes. Ions were produced by bombardment with a beam of Xe atoms (6 keV). Matrixes used were glycerol and m-nitrobenzyl alcohol (NBA). The accelerating voltage was 10 kV and the resolution was set at 3000. The instrument was scanned from m/z 0 to 1500, data were collected from m/z 50-1500. The sample was mixed with the matrix, which supported ionization on a probe tip and was then inserted into the instrument.

3.6 Standards

Detail on limonoid standards were mentioned in Study I/Part I (3.5).

4. Results and discussion

Seed was used for the purification of limonoid glucosides because it contains highest limonoid glucoside and low flavanone glucoside impurities. Preliminary purification of limonoid glucosides resulted in a simpler mixture for separation by HPLC. Different injection volumes were studied (10, 20, 40, and 80 µl) to discern maximum sample load. Separation of limonoid glucosides on C18 analytical column using 40 and 80 µl injection volumes (gradient system: 18% to 26 % acetonitrile in 3 mMH_3PO_4 in 40 mintues) are shown in Figure 29. Resolution (between the most difficult pair, unknown 2 and NAG) decreased with increasing injection volumes. The largest sample load that still maintained adequate resolution was 40 µl (Rs = 0.75/N = 5,575).

Retention times (RT) of limonin glucoside, deacetylnomilinic acid glucoside, nomilinic acid glucoside, and obacunone glucoside were 28, 33, 55, and 62 minutes, respectively. Based on published retention time (Fong et al., 1993), peak at 41 minutes


1.0
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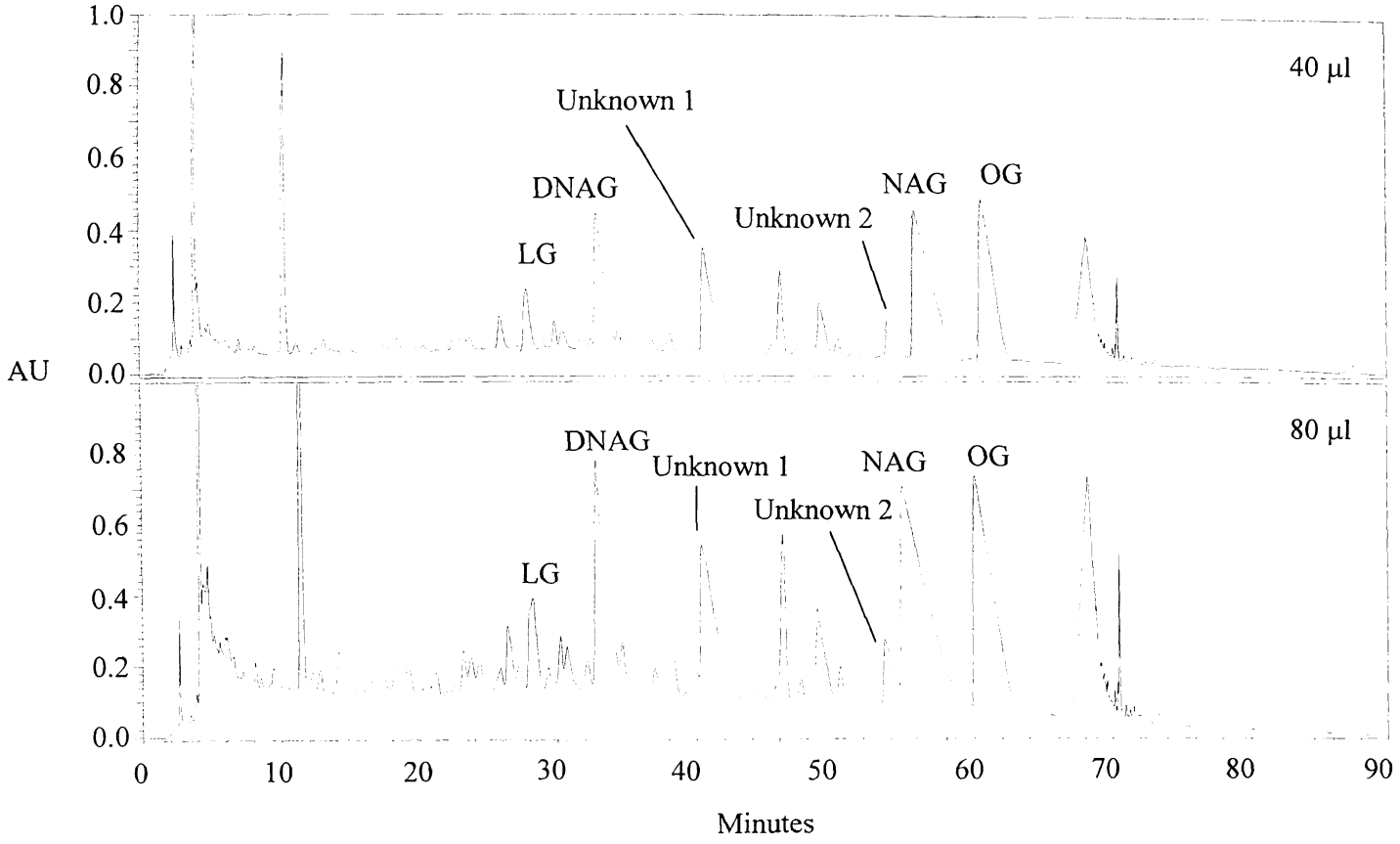

Figure 29: Separation of limonoid glucosides from polar fraction of seed extract by analytical HPLC (40 ml and 80 ml injection volumes), [SP: C18, MP: 18% to 26% CH_3CN 3 mMH_3PO_4 in 40 min, 1 ml/min]. *LG = limonin glucoside, DNAG = deacetylnomilinic acid glucoside, NAG = nomilinic acid glucoside, OG = obacunone glucoside.*

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(unknown 1) and 54 minutes (unknown 2) were potentially deacetylnomilin glucoside (DNG) and nomilin glucoside (NG). Figure 30 shows chromatograms of purified unknown 1 and unknown 2.

UV spectra of isolated unknown 1 (41 minutes) and 2 (54 minutes) (Figure 31) are similar to the typical UV spectra of limonoid compounds (deacetylnomilin, deacetylnomilinic acid glucoside, nomilin, and nomilinic acid glucosides) (Figure 15).

Confirmation of deacetylnomilin glucoside (DNG) and nomilin glucoside (NG) were based on molecular weight, determined by negative fast atom bombardment mass spectrometry (-eVFABMS) due to limited amount of purified compounds. Molecular weight of unknown 1 was found to be 652 (corresponding to deacetylnomilin glucoside); -eV FABMS showed a peak at m/z 651 $[M-H]^-$ in both glycerol and NBA matrices. The molecular weight of unknown 2 was found to be 694 (corresponding to nomilin glucoside); -eV FABMS showed a peak at m/z 693 $[M-H]^-$ in both glycerol and NBA matrices.

It should be noted that there was an occurrence of nomilinic acid glucoside in collected unknown 2 (nomilin glucoside) after 2 weeks storage at refrigerated temperature. This may be due to the conversion of nomilin glucoside to nomilinic acid glucoside at pH (~2.6) of mobile phase used. According to Hasegawa (2000), nomilin glucoside may be converted to nomilinic acid glucoside at pH ≤ 3.

5. Conclusion

Preliminary cleaning using liquid-liquid and anion exchange extraction resulted in a simpler limonoid glucoside mixture for the subsequent isolation by HPLC. Large sample load up to 40 μl can be used with optimized HPLC mobile phase to increase


0.008
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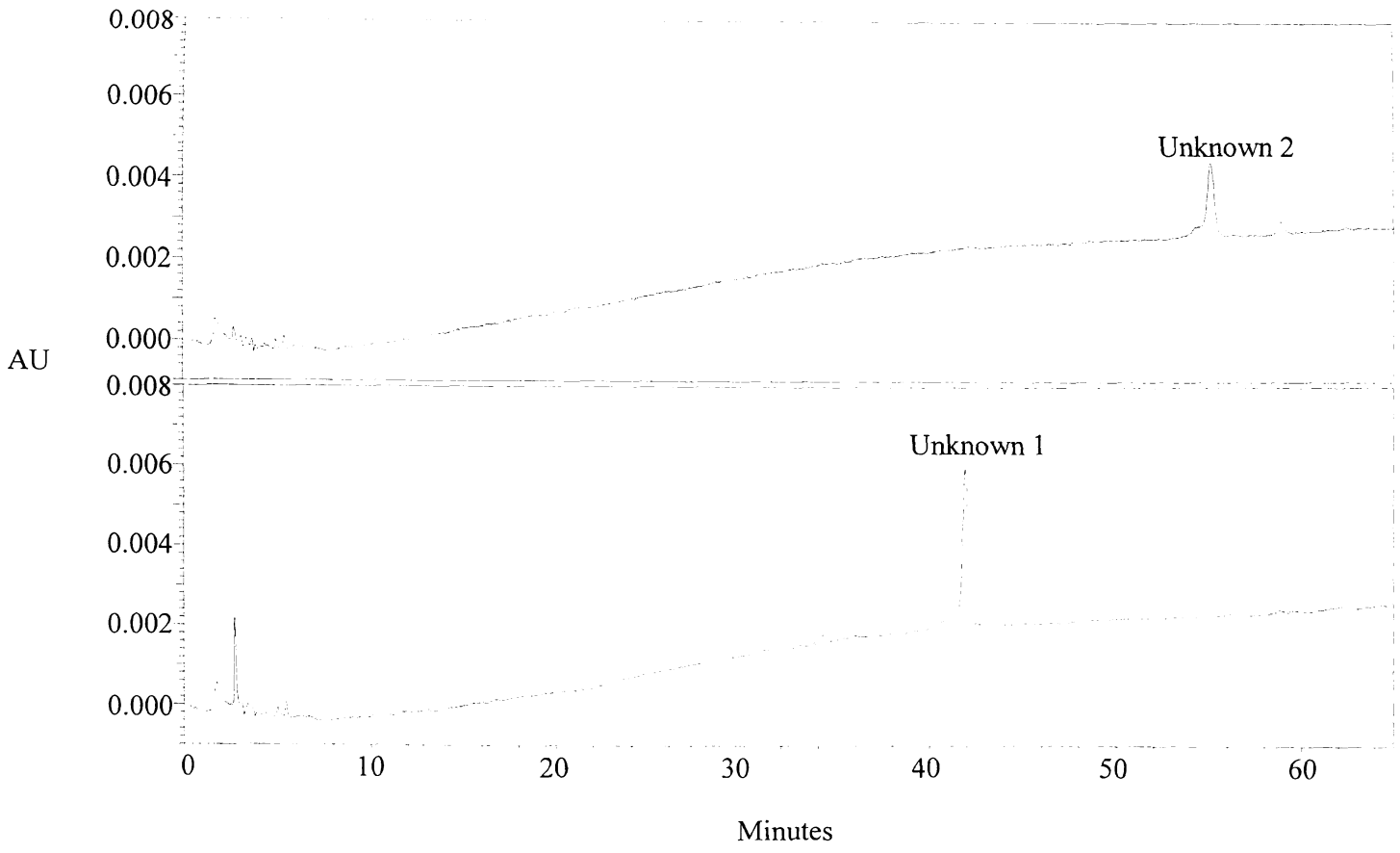

Figure 30: Purified unknown 1 (eluted at 41 minutes) and unknown 2 (eluted at 54 minutes) from seed extract at 210 nm [SP: C18, MP: 18% to 26% CH_3CN 3 mMH_3PO_4 in 40 min, 1 ml/min].

0.008

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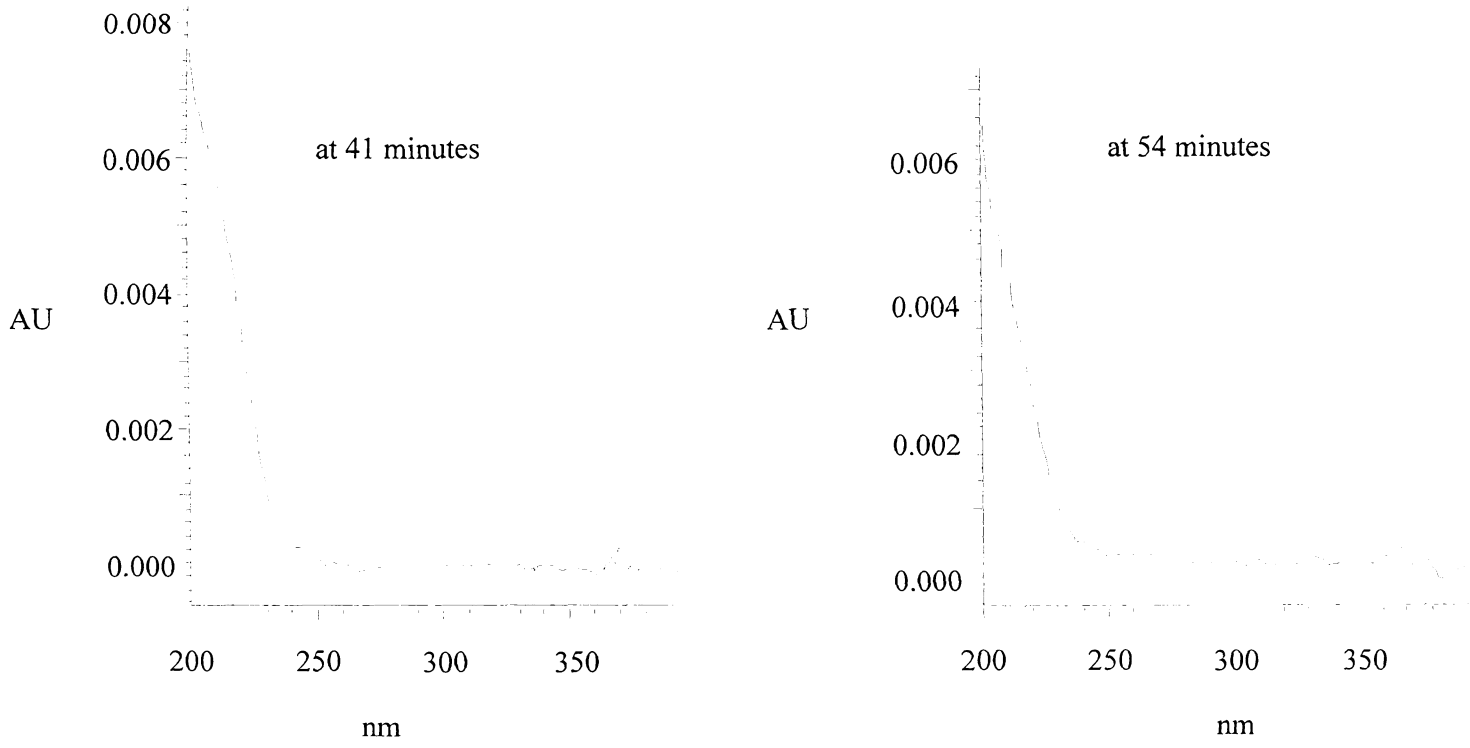

Figure 31: UV spectra of unknown 1 (41 minutes) and unknown 2 (54 minutes) obtained from photodiode array detector

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Part II: Isolation
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Part II: Isolation and identification of 3,5,6,7,3',4'-hexamethoxyflavone (HX) and narirutin-4'-glucoside (NT-4'-G)

1. Abstract

Two unknowns, having similar relative retention and UV spectra to those of 3,5,6,7,3',4'-hexamethoxyflavone and narirutin (subsequently demonstrate not to be narirutin) reported in previous study, were identified for these two compounds.

Orange peel from Valencia variety was used as a raw material. Preliminary purification included soxhlet extraction used to separate nonpolar and polar compounds; and column chromatography used to separate among the nonpolar/polar compounds. Final isolations were carried out using analytical HPLC. Sample loads were 100 µl for hexamethoxyflavone isolation and 20 µl for narirutin-4'-glucoside isolations. Both separation conditions optimized for HX and NT-4'-G produced resolved peak for HX (Rs = 1.4/N = 979) and well isolated single peak for NT-4'G, respectively. Identifications were based on molecular weight information using -eVFABMS and NMR spectral data.

2. Introduction

3,5,6,7,3',4'-Hexamethoxyflavone (HX) and narirutin-4'-glucoside (NT-4'-G) are minor flavonoids in sweet oranges. These two flavonoids have been reported in limited works (Gaydou et al., 1987, Manthey and Grohmann, 1996, Hsu et al., 1998) due to the unavailable commercial standards.

Identifications of flavonoids have been done through various spectrometric and chromatographic techniques. For routine analyses HPLC-PDA is primarily used (Ooghe et al., 1994a, Ooghe et al., 1994a, Ortuno et al., 1995, Bronner and Beecher, 1995, Ooghe and Detavernier, 1997, Kawaii et al., 1999). NMR is (Castillo et al., 1993, Ortuno et al., 1995, Miyake et al., 1997,Chen et al., 1997, Mitsuo et al., 1999) used when high

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informative details are needed and relatively concentrated sample is available (at least 5 mg/0.7 ml), but a series of purification steps prior to analysis is required for accurate interpretation. Highly conjugated structures of flavonoids which correspond to the distinctive UV spectrum is considered suitable identification tool when only small quantity of purified compound is available. An extensive review for systematic identification of flavonoids by application of UV and NMR techniques was written by Mabry et al. (1970). Other techniques include GC-MS (He et al., 1997, Stremple, 1998), LC-MS (Robards et al., 1997, Ishii et al., 2000, Dugo et al., 2000) and FABMS (Takashi et al., 1994, Miyake et al., 1997).

Two unknown peaks detected in comparable quantity with other identified flavonoids have demonstrated a potential to be hexamethoxyflavones and narirutin-4'-glucoside based on their chromatographic retention and UV spectra. We were interested to confirm this assumption by using additional techniques suitable to identify these compounds.

3. Materials and methods

3.1 Peel

Peels from Valencia variety was used. The peel was freeze-dried, and ground to pass 1 mm screen using UDY-Mill. The ground samples were stored at –20°C until analyses.

3.2 Sample preparation and extraction

Ground peel (35 grams) was refluxed with dimethyldichloromethane (1:50, W/V) for 24 hours, then evaporated to minimal under vacuum at 40°C. This dimethyldichloromethane fraction was a source of polymethoxylated flavones. Peel

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residue was further refluxed with methanol (1:50, W/V) for another 24 hours, then evaporated to minimal under vacuum at 40°C. This methanol fraction was a source of flavanone glucosides. Both fractions were stored at refrigerated temperature (2±1°C) until use.

3.3 Preliminary purification for polymethoxylated flavones by column chromatography

The residue from dimethyldichloromethane extract (1 g) was reconstituted with minimal hexane (20ml). To separate polymethoxylated flavones (nonpolar compounds), silica gel column chromatography was used. Silica powder from Sorbsil Chromatographic Media (Sorbsil C60 40/60H, synthetic amorphous silica, BET surface area 500-600 m^2/g, pore diameter 0.72-0.82 ml/g) was preconditioned in hexane overnight and slurry packed into glass columns (10 ml) .

Crude extract was applied to the top of the silica gel column followed by 50 ml of each solvent with increasing polarity: 100%hexane, hexane/toluene (8:2), hexane/toluene (6:4), hexane/toluene (5:5), hexane/toluene (4:6), hexane/toluene (3:7), hexane/toluene (2:8), hexane/toluene (1:9), 100% toluene, toluene/chloroform (8:2), toluene/chloroform (6:4), toluene/chloroform (4:6), toluene/chloroform (2:8), 100% chloroform, chloroform/ethylacetate (8:2), chloroform/ethylacetate (6:4), chloroform/ethylacetate (4:6), chloroform/ethylacetate (2:8), respectively. Nitrogen gas was introduced at the top of the column to speed up the flow of eluates through the column. These eighteen 50 ml fractions were collected and analyzed for the presence of polymethoxylated flavones. Figure 32 shows a flow diagram of flavonoid isolation from orange peel.

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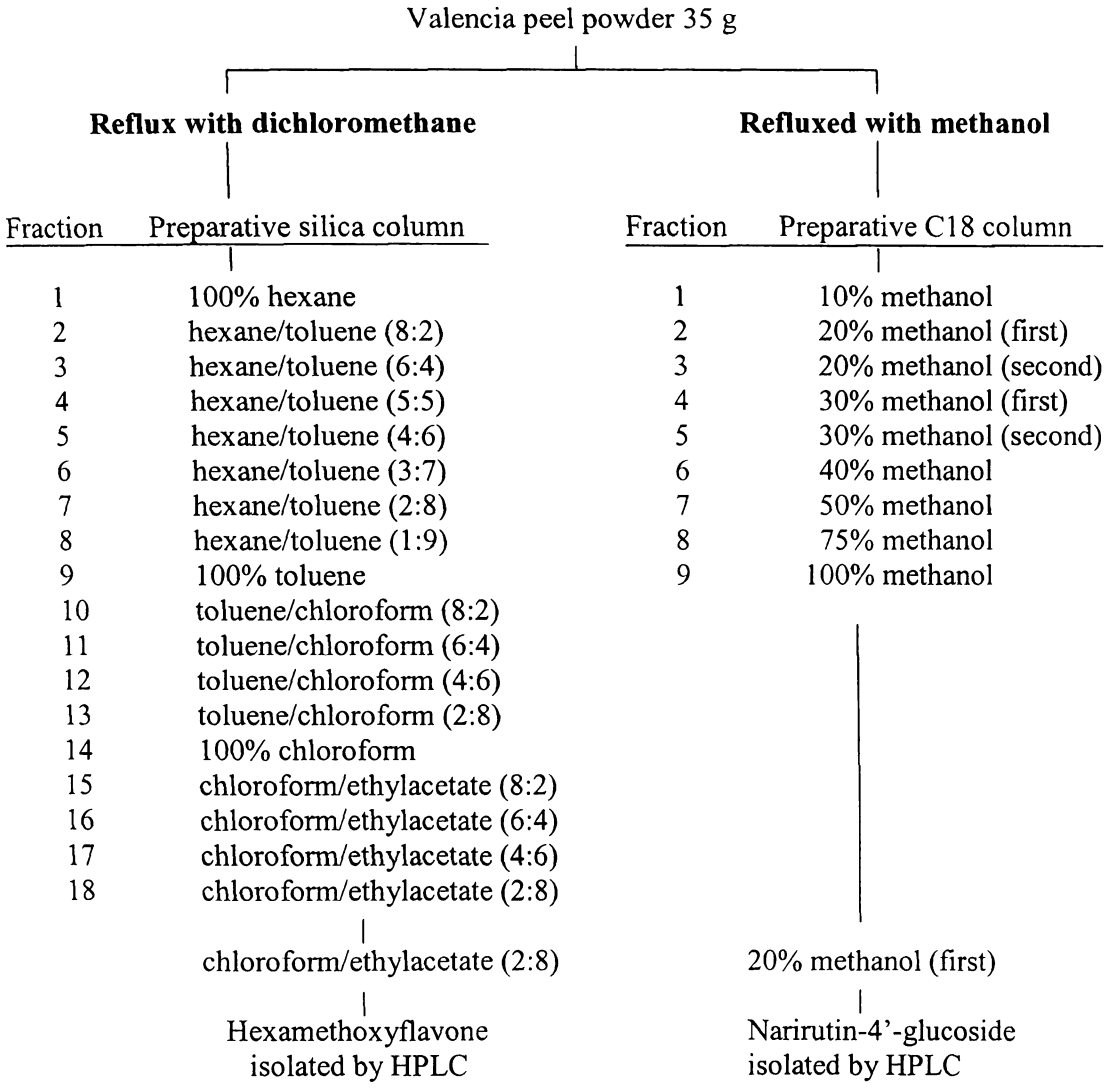

Figure 32: Flow diagram of flavonoid isolation from orange peels.

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3.4 Preliminary purification for narirutin-4'-glucoside by column chromatography

Residue from methanol fraction (1g) was reconstituted with minimal water (25 ml) and sonicated for 20 minutes. To separate flavanone glucoside (polar compound), separation was carried out on C18 column chromatography.

C18 (50 μm irregular-shaped silica, 60Å porosity, 6% carbon load, Alltech Company) particles (10 g) were soaked in purified water for 15 minutes and slurry packed on to 75-ml column (Alltech Company). The packed column was conditioned with 150 ml methanol and washed with 250 ml of purified water.

Crude extract was applied on the top of the column, washed with 250 ml water followed by 150 ml of each solvent with increasing methanol percentage: 10%, 20%, 20%, 30%, 30%, 40%, 50%, 75%, and 100%, respectively. Nine 150 ml fractions were collected and analyzed for the presence of narirutin-4'-glucoside (based on chromatographic retention and UV spectrum).

3.5 Purification of 3,5,6,7,3',4'-hexamethoxyflavone by high performance liquid chromatography (HPLC)

HPLC setting consisted of two-pump model Waters 515 (controlled by Waters 680 Automated Gradient Controller), connected with Waters 486 Tunable Absorbance Detector, and manual injection unit. Integration software was PEAKW32, version 2.08, SRI Inc.

Separation of polymethoxylated flavones was conducted on C18 column (Luna: C18, 5 μl, 250mm x 4.6 mm, 17.8% carbon load, Phenomenex) with isocratic mobile phase consisted of 1:1 [solvent A (water/acetonitrile/ propanol/acetic acid, 81:15:3:1):solvent B(water/acetonitrile/propanol/acetic acid, 40:56:3:1)] at 0.8 ml/min. Polymethoxylated flavones were detected at 340 nm. The collected eluate was

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3.6 Purification of narirutin-4'-glucoside by high performance liquid chromatography (HPLC)

HPLC setting was the same as that for purification of 3,5,6,7,3',4'-hexamethoxyflavone. Separation was achieved on C18 column (Luna: C18, 5 µl, 250mm x 4.6 mm, 17.8% carbon load, Phenomenex). Mobile phase used was gradient system starting with 15% acetonitrile and ending with 26% acetonitrile in 40 minutes at 1 ml/minute. An injection volume was 20 µl. The resolved peaks were detected at 280 nm. The HPLC setup was the same as that for 3,5,6,7,3',4'-hexamethoxyflavone purification mentioned above. The collected eluate was evaporated (50°C) to minimal amount and stored at refrigerated temperature until further analyses.

3.7 Fast atom bombardment mass spectrometry (MS)

200 µl of purified unknowns collected were evaporated to dryness at 37°C under N_2 gas. Methanol was added to the collected fraction to decrease its boiling point, and accelerating the evaporation. Unknown compounds were analyzed for their molecular weights using -eVFABMS. Conditions used were previously mentioned in identification of DNMG and NMG.

3.8 Nuclear magnetic resonance (NMR)

NMR analyses were conducted at Max T. Rogers NMR Facility, Michigan State University, E. Lansing. NMR spectra were acquired on a Varian VXR-500S spectrometer. The spectra were recorded in deuteriorated methanol (CD_3OD) at a temperature of 25°C. The 1H spectral width of 12ppm was acquired with a recycle time

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To ensure removal of solvent containing proton, samples were subjected to vacuum drying (room temperature/15 min). Residue was reconstituted with 0.7 ml deuteriorated methanol (CD_3OD) and transferred to NMR tube.

3.9 Standards

Detail on limonoid standards were mentioned in Study I/Part I (3.5).

4. Results and discussion

4.1 Hexamethoxyflavone (HX)

Separation of compounds in dimethyldichloromethane fraction using silica column chromatography resulted in isolated polymethoxylatedflavones in fraction 15 (ethylacetate/chloroform, 2:8).

Subsequent separation of fraction 15 using analytical HPLC with up to 100µl sample load was successful (Rs 1.4 /N = 979). Application of excess sample load, where common injection volumes for analytical HPLC is 10 or 20 µl, resulted in peak broadening. However, obtained separation allowed resolved peak of unknown 3 from sinensitin and nobiletin. Figure 33 shows separation of polymethoxylated flavones in fraction 15. Retention times (RT) were 21.4 (sinensitin), 26.6 (unknown 3), 30.9 (nobiletin), 34.8 (3,4,5,6,7,8,3',4'-heptamethoxyflavone), 38.1 (scutellarein tetramethylether), and 49.7 (tangeretin) minutes.

This separation condition demonstrated the advantages of analytical HPLC application for purification. The advantages of using analytical HPLC compared to preparative HPLC include 1) higher separation resolution (smaller packing materials, at


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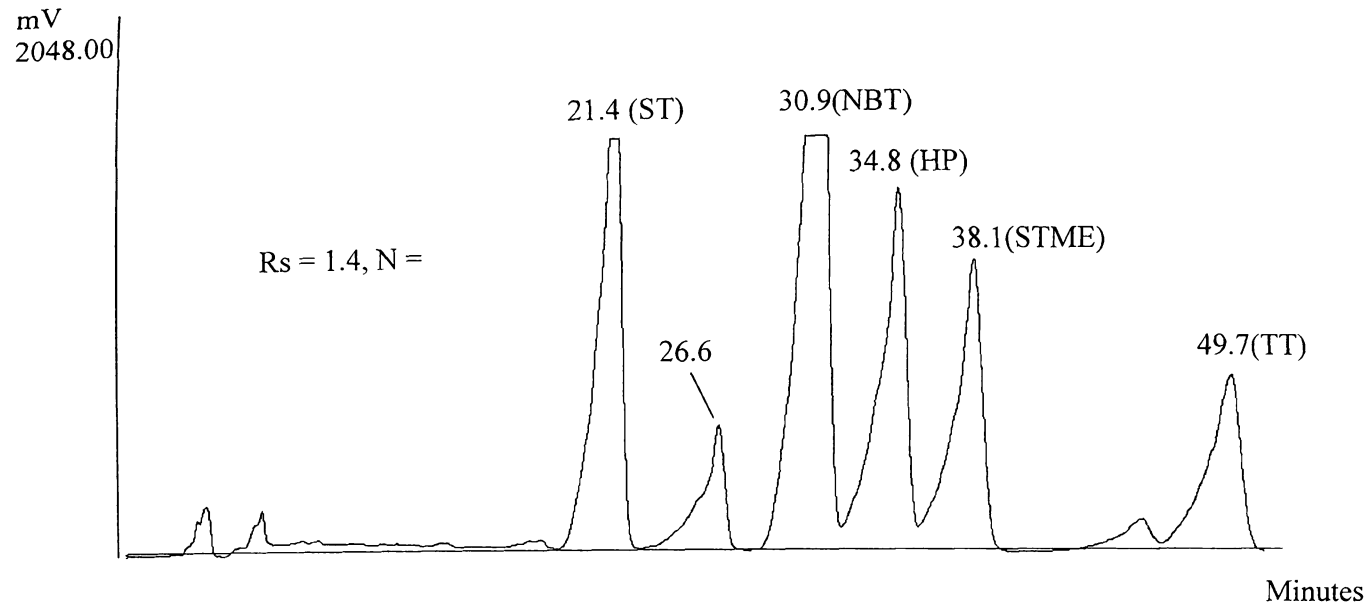

Figure 33: Separation of polymethoxylatedflavones (fraction 15) from nonpolar fraction of peel extract [SP: C18, 5 ml, MP: 1:1 ($H_2O/CH_3CN/C_3H_7OH/CH_3COOH$, 81:15:3:1) /($H_2O/CH_3CN$/ C_3H_7OH/CH_3COOH, 40:56:3:1)], 0.8 ml/min]. *ST = sinensitin, NBT = nobiletin, HP = 3,4,5,6,7,8,3',4'-heptamethoxyflavone, STME = scutellarein tetramethylether, TT = tangeretin.*

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least 2-time, greatly improves separation quality), 2) lower solvent consumption and organic waste generation (lower flow rate, at least 5 times, reduce the use of organic solvent, particularly important during method development), 3) shorter conditioning time (5-time column volume is ideally required for adequate conditioning), 4) minimized pump work.

Figure 34 shows polymethoxylated flavone standards and isolated unknown 3. It is shown that this separation allowed isolation of unknown 3 without other interfering polymethoxylated flavones. Both relative retention and UV spectrum of unknown 3 (Figure 35) were matched with those reported by Sendra et al.(1988) (Figure 18). Identical UV spectra taken from three different positions of the unknown-3 peak ensured that the isolated peak was relatively pure.

The molecular weight of unknown 3 was found to be 402 by FABMS (which corresponds to molecular weight of hexamethoxyflavone). Negative FABMS showed a peak at m/z 402 [M^-] and 494 [M^-+Gly] in glycerol matrix. There was no peak at m/z 402 in NBA matrix.

The obtained ^{1}H NMR (500MHz, CD_3OD) spectrum was δ 3.86, 3.88, 3.92, 3.94 (each 3H, s, OMe), 4.00 (6H, s, 2x OMe), 7.08 (1H, s, H-8), 7.12 (2H, d, J=9 Hz, H-5'), 7.75 (1H, d, J=2 Hz, H-2'), 7.79 (1H, dd, J=2 Hz,H-6'). The resulted NMR spectra was consistent to hexamethoxyflavone structure, based on Miyazawa et al.(1999) who identified three polymethoxylated flavones (tetra-O-methylscutellarein, sinensitin, and nobiletin) extracted from *C. aurantium* by EI-MS, ^{1}H- and ^{13}C-NMR.

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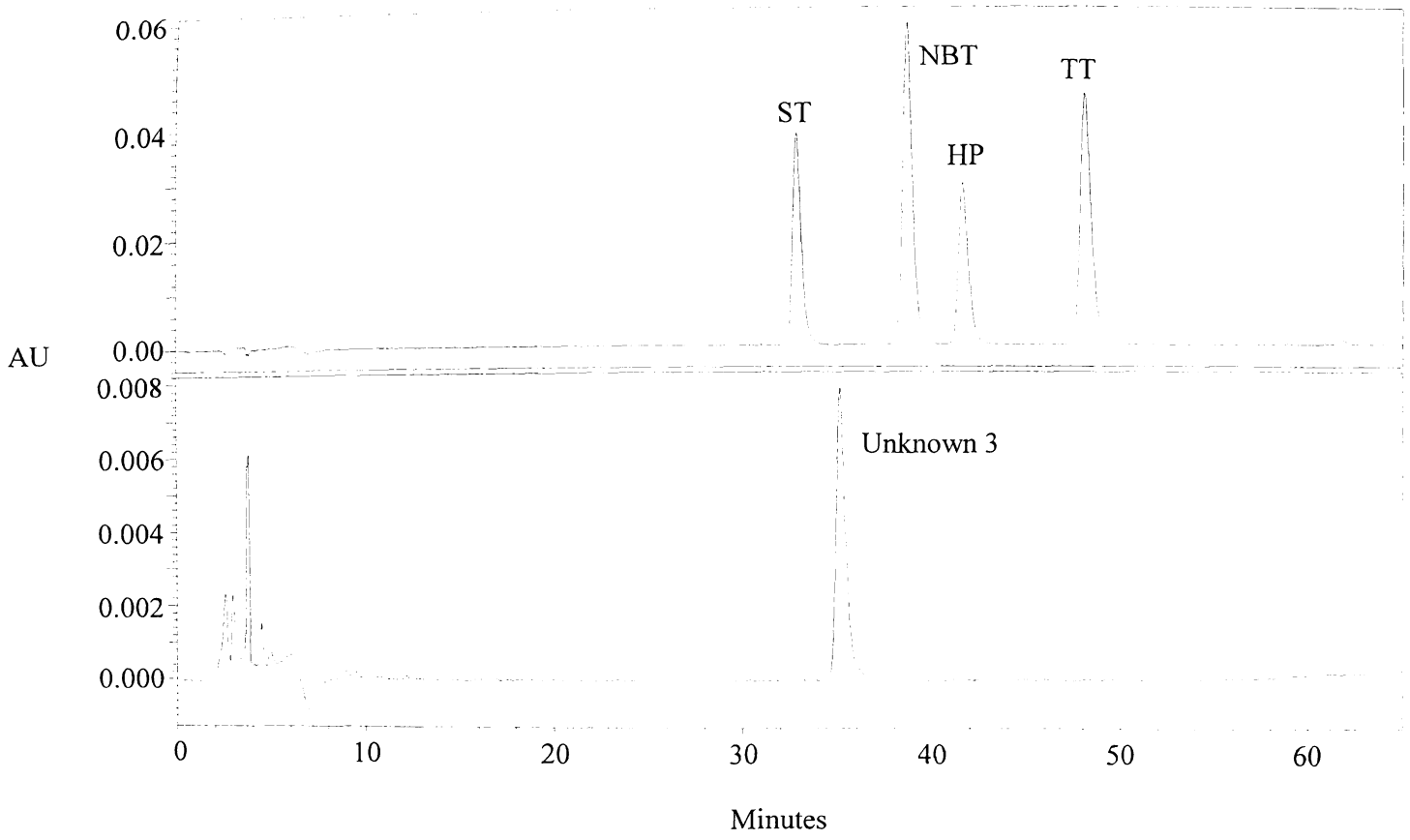

Figure 34: Separation of polymethoxylated flavone standards and purified unknown 3 at 340 nm.
ST = sinensitin, NBT = nobiletin HP = 3,4,5,6,7,8,3',4'-heptamethoxyflavone, TT = tangeretin.

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Figure 35: UV

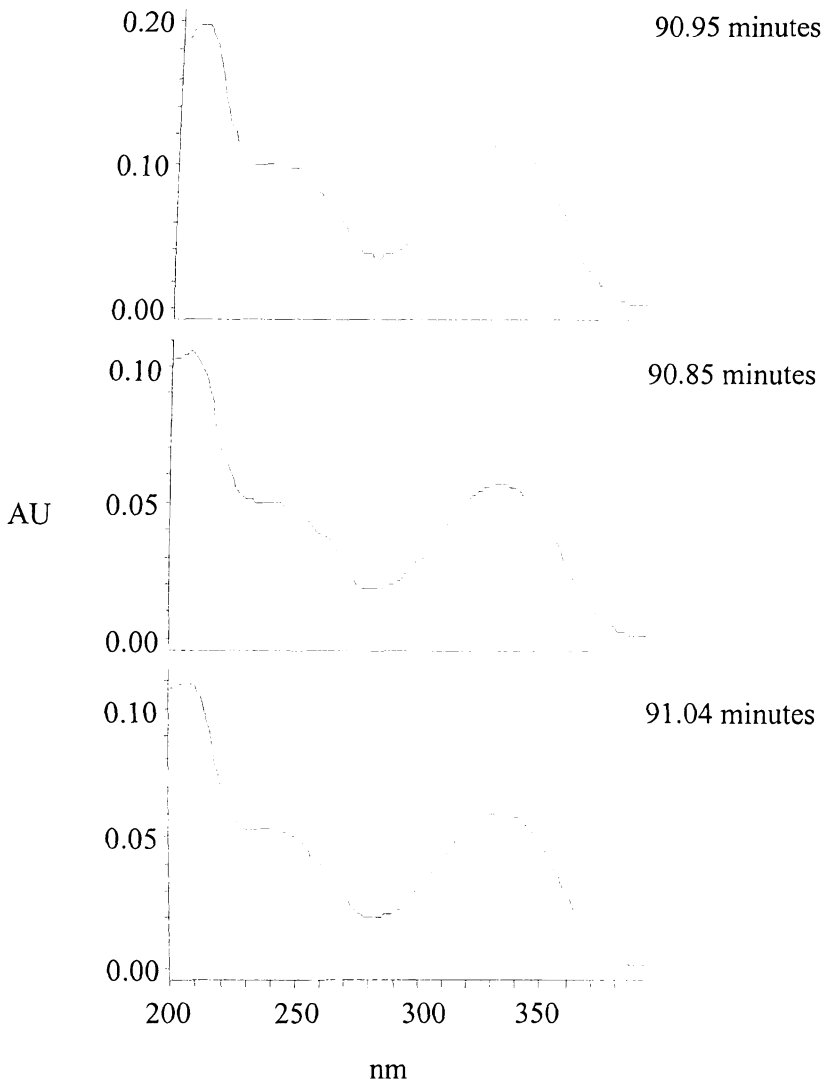

Figure 35: UV spectra of unknown 3 obtained from photodiode array detector.

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4.2 Narirutin-4'-glucoside (NT-4'-G)

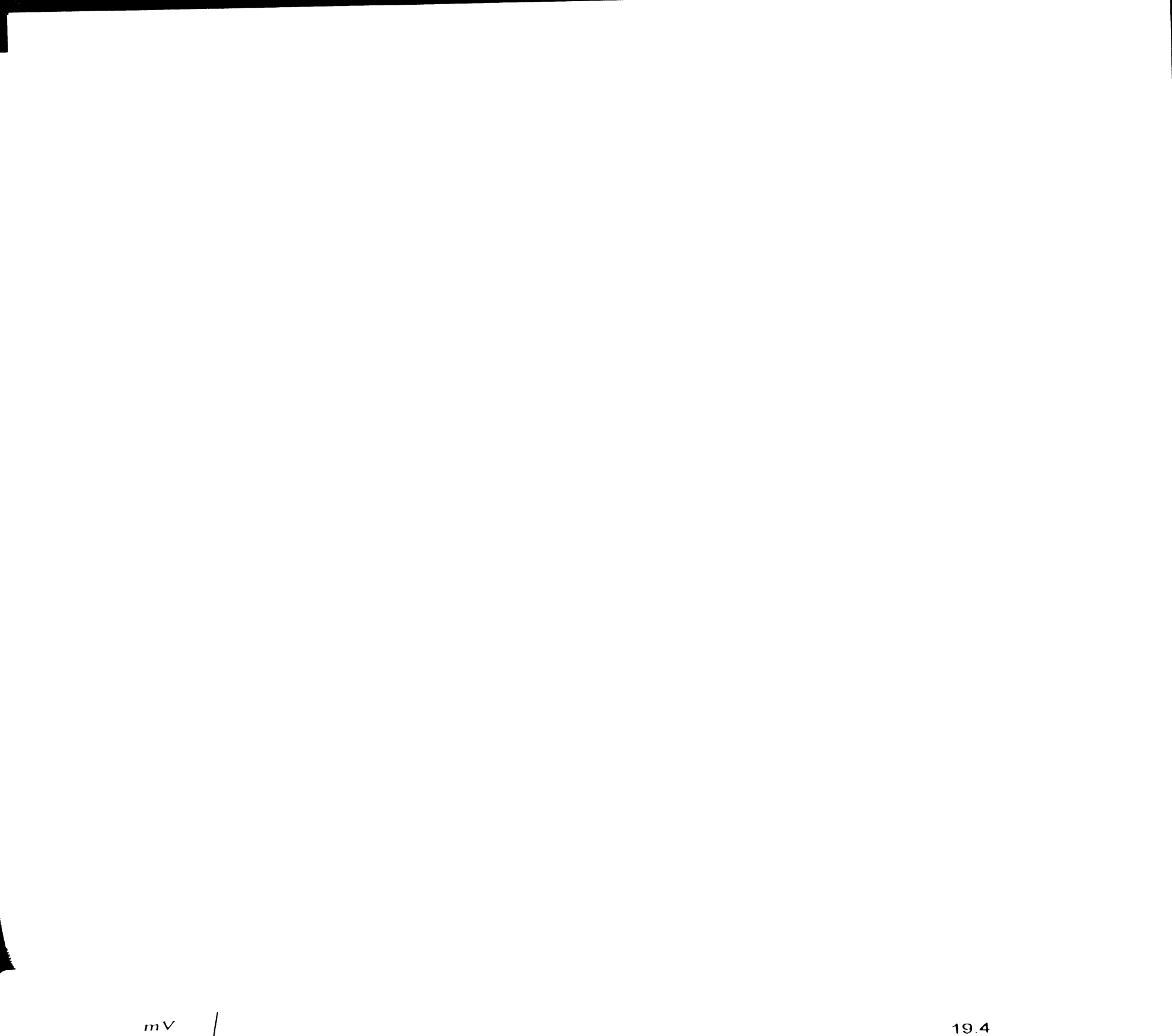
Preliminary isolation of methanol extract of Valencia peel by reverse phase column chromatography showed that fraction 2 (first 150 ml 20%methanol) contained an unknown 4 (potential narirutin-4'-glucoside).

The separation on reverse phase HPLC was achieved in 20 min. Injection volume of 20µl appeared to be the largest sample load to maintain adequate separation of unknown 4 in the fraction 2 (first 150 ml 20%methanol) (Figure 36). The increase of sample load may become less flexible when compounds to be separated are minimally retained, for example, separation of high polar compounds on highly nonpolar stationary phase such as C18 column.

Figure 37 shows chromatogram of purified unknown 4 (on C18 column using linear gradient starting from 15% to 26 % acetonitrile in 60 minutes). The HPLC condition used allowed isolation of unknown 4 with relatively high purification.

Figure 38 shows UV spectra of unknown 4 obtained from photo diode array detector. Identical UV spectra taken from three different positions of the unknown-4 peak indicate that the isolated peak was relatively pure.

The molecular weight of unknown 4 was found to be 743 (corresponding to narirutin-4'-glucoside molecular weight). -eVFABMS spectral data showed a peak at m/z 765 $[M-H+Na]^+$ and fragment ions at m/z 579 $[M-H\text{-glucose}]^+$, and m/z 625 $[M-H\text{-glucose}+2Na]^+$ in NBA matrice. The m/z 625 $[M-H\text{-glucose} + 2Na]^+$ was also found in glycerol. The presence of m/z 765 $[M-H+Na]^+$ and 787 $[M-H+2Na]^+$ was confirmed by Kumamoto et al. (1986) who purified and identified narirutin-4'-glucoside from Unshiu orange peels using -eVFAB and NMR.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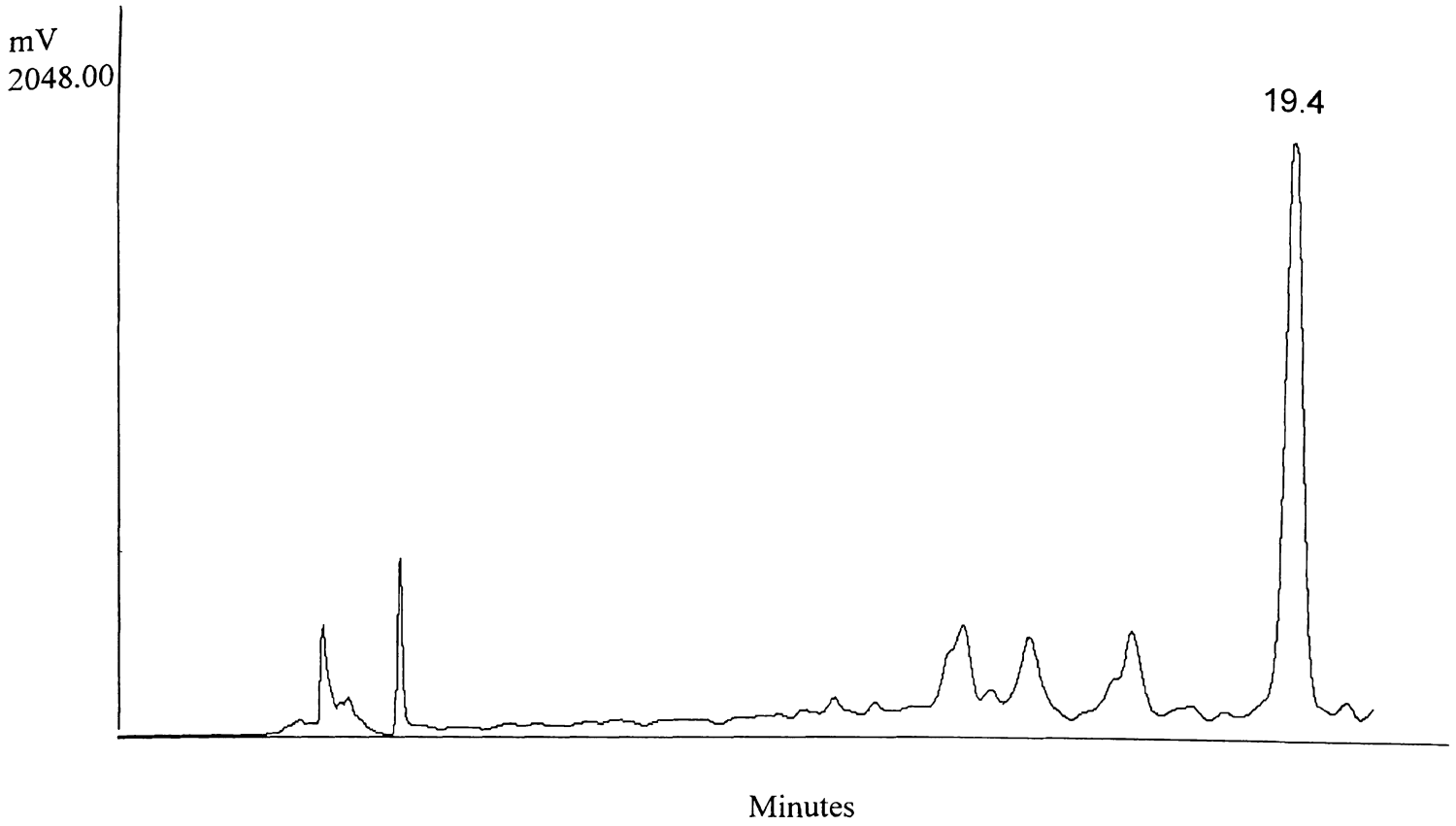

Figure 36: Separation of compounds (fraction 2) from polar fraction of peel extract at 280 nm (first 20% methanol) [SP: C18, MP: 15% to 26 % CH_3CN in 40 min, 1 ml/min].

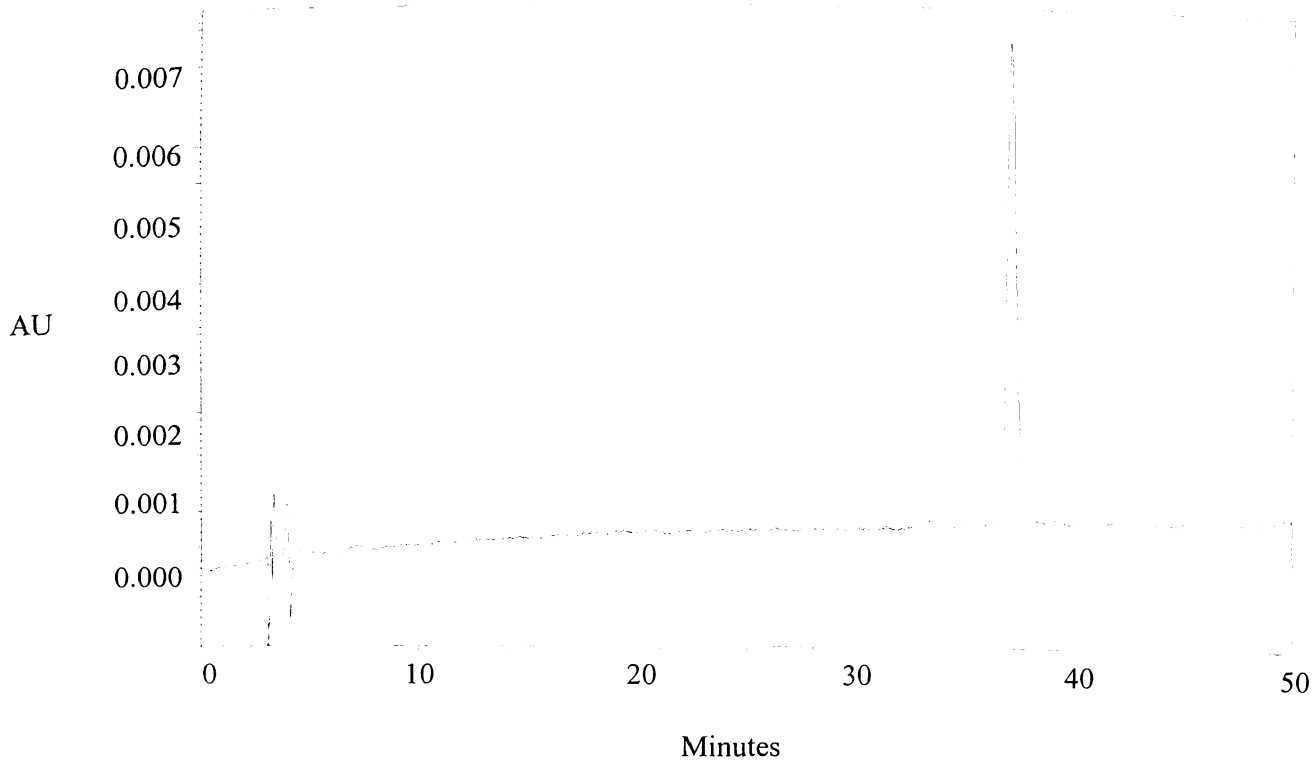

Figure 37: Purified unknown 4 from peel extract at 280 nm [SP: C18, MP: 15% to 26% CH_3CN in 60 minutes, 1 ml/min]

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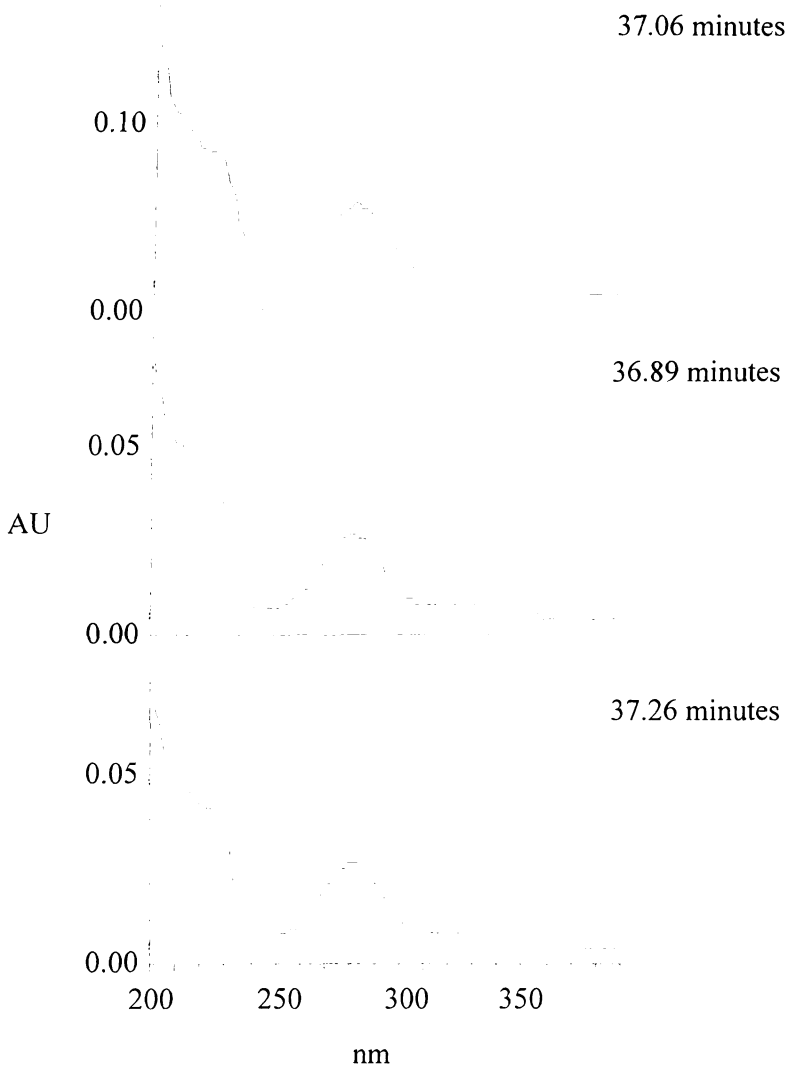

Figure 38: UV spectra of unknown 4 obtained from photodiode array detector.

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The result of ^{1}H-NMR (500MHz, CD_3OD) spectrum was δ 1.20 (3H, d, J=6 Hz, rhamnose-Me), 3.00 (1H, dd, J=3, 17 Hz, H-3), 4.65 [1H, d, J=1 Hz, α-rhamnose (H-1")], 5.50 (1H, dd, J=3, 12 Hz, H-2), 6.20 (2H, s, H-6, H-8), 7.04 (2H, d, J=8 Hz, H-2', H-6'). This NMR spectrum confirmed the presence of narirutin-4'-glucoside based on that reported by Kumamoto et al. (1986).

5. Conclusion

Preliminary purification including soxhlet extraction to separate nonpolar and polar compounds; and column chromatography to separate among the nonpolar/polar compounds resulted in a simpler polymethoxylated flavone and flavanone glucosides mixture for the subsequent HPLC isolation. Large sample load up to 100 ml can be use in 3,5,6,7,3',4'-hexamethoxyflavone isolation, but only up to 20 ml was allowed for narirutin-4'-glucoside isolation. Based on data from UV, -eVFABMS, and ^{1}H-NMR spectra, these two unknowns were 3,5,6,7,3',4'-hexamethoxyflavone and narirutin-4'-glucoside.

6. References:

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Study III: Distr
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Study III: Distributions of limonoids and flavonoids in edible and inedible fractions of sweet oranges (*Citrus sinensis*)

1. Abstract

Quantitative analyses of limonoids and flavonoids were determined on different fruit fractions including 1) seed, 2) peel, 3) peel press cake, 4) rag, 5) orange juice, and 5) peel press liquid from three commercial orange varieties (Hamlin, Parson Brown, and Valencia). Compounds analyzed were limonoid aglycones (limonin, nomilin, deacetylnomilin, and obacunone), limonoid glucosides (limonin glucoside, deacetylnomilinic acid glucoside, deacetylnomilin glucoside, nomilin glucoside, nomilinic acid glucoside, and obacunone glucoside), flavanone glucosides (narirutin-4'-glucoside, eriocitrin, narirutin, hesperidin, and didymin), polymethoxylated flavones (sinensitin, 3,5,6,7,3',4'-hexamethoxyflavone, nobiletin, scutellareintetramethylether, 3,4,5,6,7,8,3',4'-heptamethoxyflavone, and tangeretin).

Seeds had the highest concentrations of both limonoid aglycones and limonoid glucosides, and contained very low flavonoid levels. Peel and peel press cake had the highest concentration of polymethoxylated flavones and flavanone glucosides. Peel press liquid contained higher phytochemical content than juice with an exception of limonoid glucosides, suggesting that limonoid glucosides were highly extractable through commercial juice extraction. Water removal by pressing process in feed mill operation extracted limonoid glucosides and polymethoxylated flavones from the peel into peel press liquid, but concentrated limonoid aglycones in the peel press cake.

Flavanone glucosides were the predominant phytochemicals, followed by limonoid glucosides, limonoid aglycones, and polymethoxylated flavones. Valencia

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variety had the highest content of limonoids and polymethoxylated flavones, while Hamlin had the highest content of flavanone glucosides.

2. Introduction

There has been an increased interest in citrus secondary metabolites, since the discovery of pharmacological properties of certain compounds found exclusively in citrus products. The two major classes of secondary metabolites are limonoids and flavonoids. Among 36 citrus limonoids identified, 13 of them were detected in sweet oranges. Limonin and nomilin, previously described as the primary bitter compounds in orange juices exhibited anti-cancer properties (Hasegawa et al., 1994). It was reported that addition of glucose to limonoid glucosides does not modify the chemopreventive activity of limonoids (Miller et al., 1992). This is important because limonoid glucosides are more abundant and they are tasteless, thus there is higher consumption of these forms. Diets rich in citrus limonoids may prevent or deter the development of certain types of cancers (Lam and Hasegawa, 1989, Miller et al., 1992, Wattenberg and Coccia, 1991, Gould, 1993, Hasegawa et al., 1994, Lam et al., 1994, Miller et al., 1994, Miyagi et al., 2000).

Flavonoids are widely found in the plant kingdom, but there are some groups that are specific to *Citrus*, such as polymethoxylated flavones and several flavanone glucosides. Flavonoids have been reported to act as antioxidants, anti-inflamatory, antimicrobials, free radicals scavengers, antiallergic, and analgesic agents (Benavente-Garcia et al., 1997). Due to their antioxidant properties and their ability to absorb UV light, flavonoids may act in all stages of the carcinogenic process (Kandaswami et al., 1991). Epidemiological studies have suggested that flavonoid consumption is associated

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with a reduced risk of cancer (Kawaii et al., 1999) and heart disease (Benavente-Garcia et al., 1997).

During 2001-2002, the world production of citrus fruit was approximately 73 million metric tons, of which 49% was marketed as fresh fruit and 42% was converted into processed products (FAS/USDA, 2003). Thirty metric tons of processed oranges produce a large amount of residue. From the citrus industry standpoint, it is important to increase the utilization of by-products to help maximize profits and minimize wastes. Since limonoids and flavonoids have many potential beneficial properties, many fields such as nutritional science, phytochemistry, chemistry, food science, and the citrus industry need to know the distribution and concentration of these compounds in both edible parts and waste materials. The purpose of this study was to investigate the distribution and to determine the concentrations of these compounds in three commercial sweet orange varieties.

3. Materials and methods

3.1 Orange samples

Hamlin, Parson Brown, and Valencia varieties were studied. These three varieties, having different processing characteristics were selected to provide more complete results on phytochemical distribution in commercial sweet oranges. Table 72 (Appendix XI) presents processing qualities of orange varieties used in this research. Orange fractions including 1) seeds, 2) peels, 3) peel press cake, 4) rags, 5) peel press liquid, and 6) orange juice from these three varieties were obtained from Tropicana Products Company (Bradenton, FL).

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A single strength orange juice was pasteurized (95°C/2 sec), vacuum-sealed, and stored at refrigerated temperature. Peel press liquid was prepared using a Vincent screw press®, (Vincent Corporation, Tampa, FL). The pulp resulting from the pressing process is termed "press cake". The liquid squeezed from pulp is termed "press liquid" or "press liquor". Peel press liquid was pasteurized (95°C/2 sec), vacuum-sealed and stored at refrigerated temperature (2±1°C).

Rags (containing seeds), peels, and peel press cake were vacuum-sealed and frozen (-20°C). The samples were shipped in Styrofoam containers to the Department of Food Science and Human Nutrition, Michigan State University, E. Lansing, MI.

3.2 Sample preparation

Upon arrival, samples were immediately stored at –20°C for approximately one week before analyses. Juice samples were held at refrigerated temperature (2±1°C) until completely thawed. To ensure homogeneity, all containers of each sample were combined and mixed thoroughly, sub-sampled, and collected into 100 ml glass bottles, and then stored at –20°C until analyzed.

Samples of rags, peels, and peel press cake were freeze-dried, and then ground to pass 1 mm screen using a UDY-Mill, Chicago, IL. The ground samples were stored at –20°C until analyzed. Seeds were extracted twice with hexane (1:4, W/V) at room temperature to remove orange oil before being milled with a UDY-Mill and stored in glass vials as described above.

3.3 Studied compounds and standards

Studied compounds included limonoid glucosides, limonoid aglycones, flavanoid glucosides, and polymethoxylated flavones. Standards, kindly donated by scientists from

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USDA, Dr. Gary D. Manners (Pasadena, CA), Dr. Mark A. Berhow (Peoria, IL), and Dr. John A. Manthey (Winter Haven, FL), included deacetylnomilin (DNM), obacunone (O), limonin glucoside (LG), deacetylnomilinic acid glucoside (DNAG), nomilinic acid glucoside (NAG), obacunone glucoside (OG), obacunoic acid (OA), isoobacunoic acid (IOA), deoxylimonin (DL), 17-19-didehydrolimonoic acid (DDHLA), 19-dehydrolimonoic acid (DHLA), limolinic acid (LA), rutaevin (R), sinensetin (ST), nobiletin (NBT), 3,4,5,6,7,8,3',4'-heptamethoxyflavone (HP), and tangeretin (TT). Limonin (L), nomilin (NM) hesperidin (HD), naringin (NG), neohesperidin (NHD), hesperitin (HT), diosgenin (DN), coumarin (CM), quercetin (QT) were purchased from Sigma Company (St. Louis, MO). Sinensetin (ST), scutellarein tetramethylether (STME), narirutin (NT), didymin (DD), and eriocitrin (ERT) were purchased from Extrasynthese, (Genay, France).

3.4 Extraction and analysis of limonoid aglycones and polymethoxylated flavones

3.4.1 Extraction

The extraction procedure was modified from Fong et al. (1993). Juice and peel press liquid were thawed at room temperature and heated in a water bath (82°C for 30 min), then cooled to room temperature. The juice or peel press liquid (10 ml) was then mixed with 25 ml of 0.5 M Tris buffer (pH 8) for 15 minutes and then acidified to pH 2 with 1 N HCl.

Ground, freeze-dried orange parts (peel, peel press cake, and rag) (1 g) was mixed with 25 ml of 0.5 M Tris buffer (pH 8) for 15 minutes and then acidified to pH 2 with 1 N HCl. Ground, freeze-dried orange seed (1 g) was mixed with 25 ml of 0.15 M Tris buffer (pH 8) overnight (20 hours), and then acidified to pH 2 with 1 N HCl. The acidified

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mixtures of peel, peel press cake, rag and seed were heated in a water bath (82°C for 30 min).

Ethyl acetate (25 ml) containing 200 ppm butyrate hydroxytoluene (antioxidant) was added to all samples, shaken for 15 minutes, and the ethyl acetate layer was decanted. Ethyl acetate extraction was performed twice. The ethyl acetate layers were combined, evaporated to dryness, and reconstituted with 10 ml methanol. Filtered extract (0.45μ nylon) was analyzed by HPLC. A flow diagram of limonoid aglycone and polymethoxylated flavone extraction is presented in Figure 20 (Study I/part II).

3.4.2 High performance liquid chromatography (HPLC) analysis

The mobile phases consisted of 3 mM phosphoric acid (solvent A) and acetonitrile (solvent B). Limonoid aglycones and polymethoxylated flavones were resolved with a gradient that started with 30% B, was 40% B in 20 minutes and ended with 50% B at 50 minutes. Flow rate was 1ml/min. Separation was achieved on a C18 column (Luna: C18, 5μ, 250 mm x 4.6 mm, 17.8 % carbon load, void volume 2.5 ml). Injection volume was 10 μl. Limonoid aglycones were detected at 210 nm, while polymethoxylated flavones were detected at 340 nm.

Since seeds are rich in limonoid aglycones and low in polymethoxylated flavones, limonoid aglycones analysis was carried out separately for seed extract. Separation of limonoid aglycones was achieved on C18 column (Alltima: C18, 5μ, 250 mm x 4.6 mm, 16 % carbon load, void time 2.02 minutes) and an isocratic mobile phase (acetonitrile/methanol/water, 10:41:49). Flow rate was 1ml/minute and injection volume was 10 μl. The HPLC system was described in Study I/Part I (3.4).

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Identification and quantitation of limonoid aglycones (limonin, deacetylnomilin, nomilin, and obacunone), were based on retention time, UV spectra and response factors of external standards. Identification of polymethoxylated flavones (sinensitin, nobiletin, 3,4,5,6,7,8,3',4'-heptamethoxyflavone, scutellarein tetramethylether, and tangeretin) were based on retention time and UV spectra obtained with external standards. For 3,5,6,7,3',4'-hexamethoxyflaovne, identification was based on retention relative to other polymethoxylated flavones, which was verified by negative fast atom bombardment mass spectrometry (-eVFABMS) and nuclear magnetic resonance spectroscopy (NMR) in study II/part II. The quantitations of polymethoxylated flavones were based on the response factor determined for scutellarein tetramethylether.

3.5 Extraction and analysis of limonoid glucosides

3.5.1 Extraction

Juice and peel press liquid were thawed at room temperature, heated in water bath (82°C for 5 min), and cooled to room temperature. The juice and peel press liquid (10 ml) was mixed with 25 ml of 70% methanol for 15 minutes. Ground, freeze-dried orange parts (peel, peel press cake, rag, and seed) (1 g) were mixed with 25 ml of 70% methanol for 15 minutes, and heated in a water bath (82°C for 5 min).

The samples were centrifuged (10,000X g for 10 minutes), and the supernatants were decanted. The pellet was extracted again with 70% methanol. Combined supernatants were evaporated to approximately 2-3 ml at 40°C under vacuum, and reconstituted with 10 ml methanol. Filtered extracts (0.45μ nylon) were analyzed by HPLC. A flow diagram of limonoid glucoside extraction is presented in Figure 21 (Study I/part II)

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3.5.2 High performance liquid chromatography (HPLC) analysis

The mobile phases consisted of 3 mM phosphoric acid (solvent A) and acetonitrile (solvent B). Limonoid glucosides were separated with linear gradient starting with 10% B and ending with 26% B in 70 minutes. Separation was performed on C18 column (Luna: C18, 5μ, 250 mm x 4.6 mm, 17.8 % carbon load, void volume 2.5 ml) with 1 ml/min flow rate and 10 μl injection volume. Limonoid glucosides were detected at 210 nm. The HPLC system was described in Study I/Part I (3.4).

Identification and quantitation of limonoid glucosides (limonin glucoside, deacetylnomilinic acid glucoside, nomilinic acid glucoside, and obacunone glucoside) were based on retention time, UV spectra, and response factors obtained with external standards. For deacetylnomilin acid glycoside and nomilin acid glucoside, the identifications were based on retention relative to other limonoid glucosides which were previously verified by –eVFABMS in study II/part II. The quantitation of deacetylnomilin glucoside was based on the response factor determined for deacetylnomilinic acid glucoside, while that of nomilin glucoside was based on the response factor determined for nomilinic acid glucoside.

3.6 Extraction and analysis of flavanone glucosides

3.6.1 Extraction

Juice or peel press liquid was thawed at room temperature, heated in water bath (82°C for 5 min), and cooled to room temperature. The juice or peel press liquid (10 ml) was then mixed with 25 ml dimethylformamide/methanol (1:2) for 15 minutes. Ground freeze-dried orange parts (peel, peel press cake, rag, and seed) (1 g) was mixed with 25

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ml dimethylformamide/methanol (1:2) for 15 minutes, and then heated in a water bath (82°C for 5 min).

The samples were centrifuged (10,000X g for 10 min), and the supernatant was decanted. Extractions with dimethylformamide/methanol (1:2) were done twice. Combined supernatants were evaporated to approximately 15 ml at 50°C under vacuum, and reconstituted to 25 ml with methanol. Filtered extract (0.45μ nylon) was analyzed by HPLC. A flow diagram of flavonoid glucoside extraction is presented in Figure 22 (Study I/part II).

3.6.2 High performance liquid chromatography (HPLC) analysis

The HPLC analysis of flavanone glucoside was based on the method of Ooghe (1999). Flavanone glucosides were separated on C18 column (Alltima: C18, 5μ, 250 mm x 4.6 mm, 16 % carbon load, void time 2.02 minutes) with a mobile phase consisting of 0.01 M potassium phosphate monobasic (solvent A) and acetonitrile (solvent B). A linear gradient starting at 10%B and ending at 30% B in 60 minutes was used. Flow rate was 1 ml/min flow rate and injection volume was 10 μl. Flavanone glucosides were detected at 280 nm. The HPLC system was described in Study I/Part I (3.4).

Identification and quantitation of eriocitrin, narirutin, hesperidin, didymin were based on retention time, UV spectra, and response factors obtained with external standards. For narirutin-4'-glucoside, the identification was based on retention relative to other flavanone glucosides, which were previously confirmed by -eVFABMS and NMR in study II/part II. The quantitation of narirutin-4'-glucoside was based on the response factor determined for narirutin.

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3.7 Data analysis

The experimental design had two main effects (orange varieties and fruit parts), 22 observations (22 compounds), and 2 replications. Statistical analyses were conducted using 2-way analysis of variance (ANOVA) (Excel).

4. Results and discussion

Analysis of variance (ANOVA) of total phytochemical contents (limonoids and flavonoids) in Table 8 and Table 9 show significant differences among varieties, fractions and their interaction in both solid and liquid fractions ($P \leq 0.01$).

Total phytochemical content of sweet orange solid and liquid fractions were shown in Table 10 and Table 11 and Figure 39 and Figure 40. Seeds had the highest total phytochemical content; followed by peels, peel press cake, and rags. However, when considering total orange waste produced, seed contributes to phytochemical content at a much lower level than peel, since it accounts for only 0.5-1% of the fruit (wet wt.) (Braddock, 1999d), while peel accounts for almost 50% (wet wt.) (Braddock, 1995). For liquid samples, peel press liquid contained higher phytochemical content than orange juice.

Significant difference among varieties indicated that distribution patterns of these limonoids and flavonoids were specific even though they were in the same species (*Citrus sinensis*). In solid by-products, Valencia and Hamlin varieties contained significantly higher phytochemical content compared to Parson Brown variety, and in liquid products (juice and peel press liquid) Hamlin contained highest phytochemical content among three varieties.

Table 8: ANOVA of total phytochemical content (limonoids and flavonoids) in solid fractions of sweet oranges.

Source of Variation	*SS*	df	MS	F	P-value	F crit
		2	2078935	9	0.004**	4

Table 8: ANOVA of total phytochemical content (limonoids and flavonoids) in solid fractions of sweet oranges.

Source of Variation	SS	df	MS	F	P-value	F crit
Variety	4157870	2	2078935	9	0.004**	4
Fraction	1704686291	3	568228764	2544	4.4E-17**	3
Interaction	33396984	6	5566164	25	4.1E-06**	3
Error	2679666	12	223305			
Total	1744920812	23				

*significant difference at P≤0.05 **significant difference at P≤0.01

Table 9: ANOVA of total phytochemical content (limonoids and flavonoids) in liquid fractions of sweet oranges.

Source of Variation	SS	df	MS	F	P-value	F crit
Variety	17115	2	8557	128	1.2E-05**	5
Fraction	755101	1	755101	11352	4.6E-11**	6
Interaction	55558	2	27779	418	3.6E-07**	5
Error	399	6	66			
Total	828174	11				

*significant difference at P≤0.05 **significant difference at P≤0.01

Table 10: Total phytochemical content (limonoids and flavonoids) in solid fractions of sweet oranges.

Variety	Concentration (mg/Kg)[1]		Total phytochemicals for each variety
	Seeds	Rags	

Table 10: Total phytochemical content (limonoids and flavonoids) in solid fractions of sweet oranges.

Variety	Concentration (mg/Kg)[1]				
	Peels	Peel press cake	Seeds	Rags	Total phytochemicals for each variety
Hamlin	32460^{b}	30337^{c}	42040^{a}	22476^{d}	127315^{A}
Parson Brown	31319^{b}	30532^{c}	43832^{a}	18208^{d}	123892^{B}
Valencia	34048^{b}	29603^{c}	44901^{a}	18971^{d}	127524^{A}

[1]N=2, $LSD_{fraction\ (P\leq0.05)} = 594$, $LSD_{variety\ (P\leq0.05)} = 515$, different superscripts indicate significant difference at $P\leq0.05$

Table 11: Total phytochemical content (limonoids and flavonoids) in liquid fractions of sweet oranges.

Variety	Concentration (mg/Kg)[1]		
	Juice	Peel press liquid	Total phytochemicals for each variety
Hamlin	1004^{b}	1426^{a}	2431^{A}
Parson Brown	943^{b}	1333^{a}	2276^{B}
Valencia	786^{b}	1479^{a}	2266^{B}

[1]N=2, $LSD_{fraction\ (P\leq0.05)} = 12$, $LSD_{variety\ (P\leq0.05)} = 14$, different superscripts indicate significant difference at $P\leq0.05$

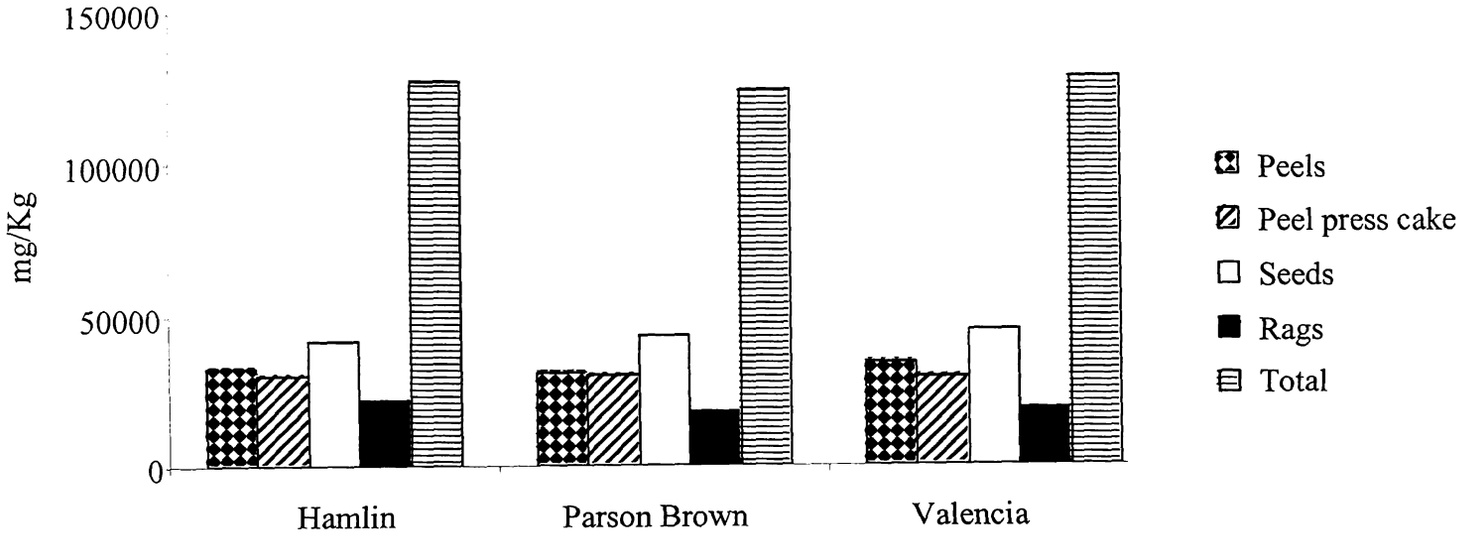

Figure 39: Phytochemical content (limonoids and flavonoids) in solid fractions of sweet oranges.

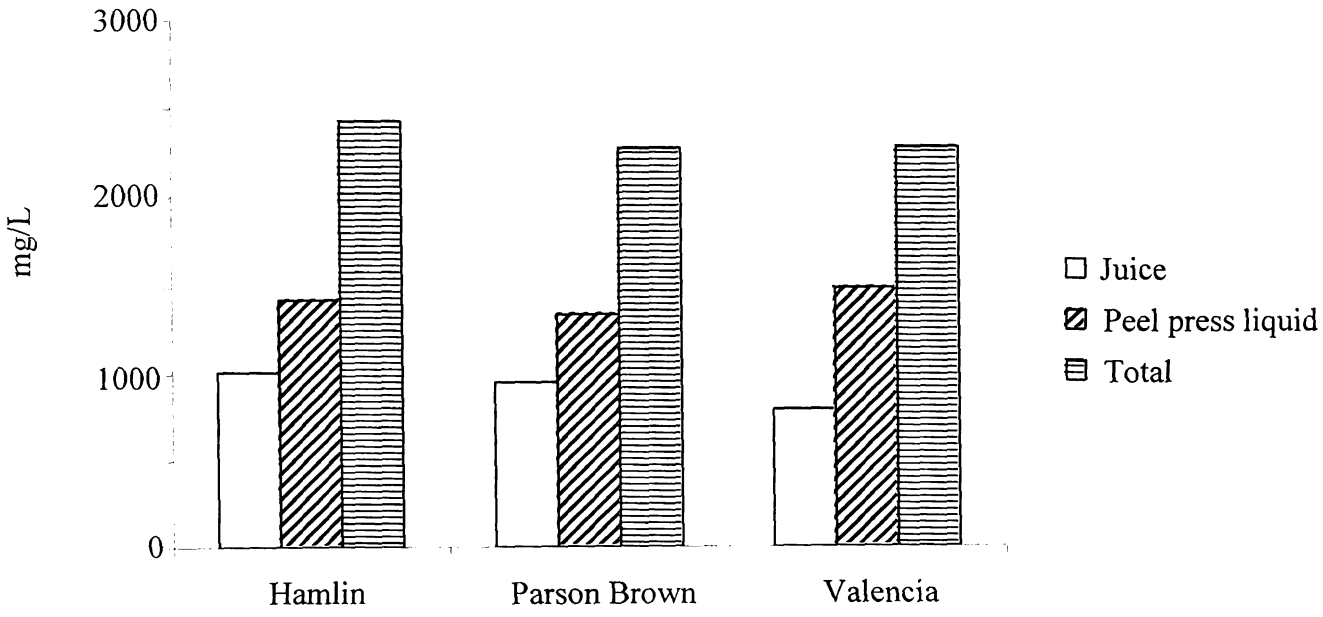

Figure 40: Phytochemical content (limonoids and flavonoids) in liquid fractions of sweet oranges.

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4.1 Limonoid aglycones

Limonoid aglycones occur in citrus seeds in two forms: dilactone (closed D-ring) and monolactones (open D-ring). The predominant form in mature seeds is dilactones, such as limonin, while in other orange fractions only monolactones, such as limonoate A-ring lactone (LARL), occur (Fong et al., 1993). The analytical method used in this study measured both forms.

ANOVA of total limonoid aglycone content in solid fractions (Table 12) detected significant differences among varieties, fractions and their interaction ($P \leq 0.01$). Table 13 and Figure 41 show total limonoid aglycone concentrations in solid fractions. Seeds had the greatest concentrations of limonoid aglycones. Limonoid algycone content in the seeds was at least 40-time higher than that of peel press cake (the second highest-limonoid aglycone fraction). There have been limited quantitative studies on limonoid aglycone found in juice and fruit tissues from sweet oranges. Most studies focus on limonin due to its bitterness problem.

Higher limonin concentrations were found in peel press cake compared to peel. The peel press cake is the pulp obtained after peel press liquid is pressed from the peel to reduce water in this waste residue. The extraction of soluble solids especially sugars (the primary constituents of peel, pulp, and rag dry solid reported by Braddock, 1999b) into the peel press liquid may concentrate limonin and explain why peel press cakes contain more limonin than peel.

Valencia contained the most total limonoid aglycones, followed by Parson Brown, and then Hamlin. Table 14 shows individual limonoid aglycone concentrations in solid fractions. Seeds were the only fraction containing measurable amounts of the

Table 12: ANOVA of total limonoid aglycone content in solid fractions of sweet oranges.

Source of Variation	SS	df	MS	F	P-value	F crit
				130	7.4E-09**	4

Table 12: ANOVA of total limonoid aglycone content in solid fractions of sweet oranges.

Source of Variation	SS	df	MS	F	P-value	F crit
Variety	6360162	2	3180081	130	7.4E-09**	4
Fraction	492493244	3	164164415	6708	1.3E-19**	3
Interaction	11578982	6	1929830	79	6.1E-09**	3
Error	293684	12	24473			
Total	510726073	23				

*significant difference at P≤0.05 **significant difference at P≤0.01

Table 13: Total limonoid aglycone concentrations in solid fractions of sweet oranges.

Variety	Sample	Concentration (mg/Kg)[1]	
		Total for each fraction	Total for each variety
Hamlin	Peels	87^{c}	9686^{C}
	Peel press cake	116^{c}	
	Seeds	9313^{a}	
	Rags	169^{c}	
Parson Brown	Peels	164^{c}	10464^{B}
	Peel press cake	440^{b}	
	Seeds	9724^{a}	
	Rags	135^{c}	
Valencia	Peels	342^{b}	14391^{A}
	Peel press cake	474^{b}	
	Seeds	13135^{a}	
	Rags	440^{b}	

[1]N=2, $LSD_{fraction\ (P\leq0.05)} = 197$, $LSD_{variety\ (P\leq0.05)} = 170$, Different superscripts indicate significant difference at P≤0.05

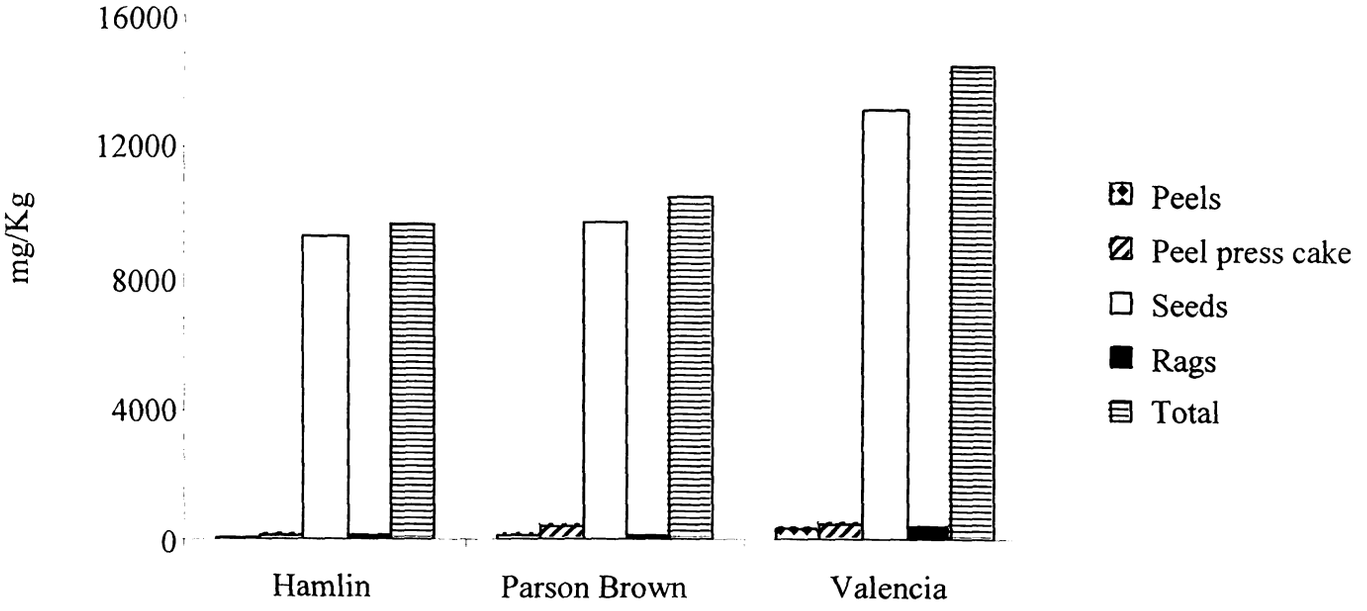

Figure 41: Limonoid aglycones in solid fractions of sweet oranges.

Table 14: Individual limonoid aglycone concentrations in solid fractions of sweet oranges.

Variety	Sample	Concentration (g/100g) ± %CV[1]	
		DNM	O

Table 14: Individual limonoid aglycone concentrations in solid fractions of sweet oranges.

Variety	Sample	Concentration (g/100g) ± %CV[1]			
		L	NM	DNM	O
Hamlin	Peels	0.01±9.9	[2]T	T	T
	Peel press cake	0.01±3.5	T	T	T
	Seeds	0.82±3.4	0.03±4.3	0.06±2.5	0.02±9.5
	Rags	0.02±1.0	T	T	T
Parson Brown	Peels	0.02±2.1	T	T	T
	Peel press cake	0.04±1.0	T	T	T
	Seeds	0.86±0.4	0.04±2.1	0.06±5.9	0.02±2.9
	Rags	0.01±12.2	T	T	T
Valencia	Peels	0.03±11.5	T	T	T
	Peel press cake	0.05±3.6	T	T	T
	Seeds	1.20±3.2	0.04±5.9	0.05±2.5	0.02±2.4
	Rags	0.04±4.3	T	T	T

L = limonin, NM = nomilin, DNM = deacetylnomilin, O = obacunone
[1]N = 2 [2]Trace

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minor limonoid aglycones (nomilin, deacetylnomilin, and obcunone). In seeds, limonin was the predominant limonoid aglycone, followed by deacetylnomilin, nomilin, and obacunone. According to Fong et al. (1993), nomilin, commonly known as a major limonoid aglycone, does not accumulate to a measurable concentration until late harvest season. Low nomilin levels in this study may indicate that the orange samples used were not in their late harvest season.

ANOVA of total limonoid aglycone content in liquid fractions (Table 15) showed significant differences among varieties, fractions and their interaction ($P \leq 0.01$). Table 16 and Figure 42 show total limonoid aglycone concentrations in liquid fractions. Peel press liquid contained higher limonoid aglycones than juice. Valencia contained highest limonoid aglycones, followed by Hamlin and Parson Brown.

Table 17 shows individual limonoid aglycone concentrations in liquid fractions. Limonin was the only limonoid aglycone detected in measurable quantity. Estimated levels of limonin in peel press liquid (10 to 35 ppm) are considered relatively low. Therefore, peel press liquid is not judged to be a good source for limonin. Limonin levels in juice were similar to those reported for Valencia orange juice (Widmer, 1993).

Estimated total limonin consumption for one serving (240 ml) of Valencia orange juice was 2 mg. Consumption of one Valencia orange fruit would be equivalent to 3.8 mg of limonin.

4.2 Limonoid glucosides

ANOVA of total limonoid glucoside contents in solid fractions (Table 18) detected significant differences among varieties, fractions and their interaction ($P \leq 0.01$). Table 19 and Figure 43 show total limonoid glucoside concentrations in solid fractions.

Table 15: ANOVA of limonoid aglycones in liquid fractions of sweet oranges.

	SS	df	MS	F	P value	F crit
					6.11E-09**	5

Table 15: ANOVA of limonoid aglycones in liquid fractions of sweet oranges.

Source of Variation	SS	df	MS	F	P-value	F crit
Variety	623	2	311	1641	6.1E-09**	5
Fraction	679	1	680	3581	1.5E-09**	6
Interaction	213	2	106	561	1.5E-07**	5
Error	1	6	0.2			
Total	1516	11				

*significant difference at P≤0.05 **significant difference at P≤0.01

Table 16: Total limonoid aglycone concentrations in liquid fractions of sweet oranges.

Variety	Sample	Concentration (mg/Kg)[1]	
		Total for each fraction	Total for each variety
Hamlin	Juice	1.5^{f}	13.7^{B}
	Peel press liquid	12.2^{b}	
Parson Brown	Juice	2.2^{e}	12.0^{C}
	Peel press cake	9.8^{c}	
Valencia	Juice	8.3^{d}	43.4^{A}
	Peel press cake	35.1^{a}	

[1]N=2, $LSD_{fraction\ (P\leq0.05)} = 0.6$, $LSD_{variety\ (P\leq0.05)} = 0.8$
Different superscripts indicate significant difference at P≤0.05

Figure 42: Limonoid aglycones in liquid fractions of sweet oranges.

Table 17: Individual limonoid aglycone concentrations in liquid fractions of sweet oranges.

Variety	*Sample*	Concentration (mg/Kg) ± %CV[1]					

Table 17: Individual limonoid aglycone concentrations in liquid fractions of sweet oranges.

Variety	Sample	Concentration (mg/Kg) ± %CV[1]			
		L	NM	DNM	O
Hamlin	Juice	1.5±10.7	[2]T	T	T
	Peel press liquid	12.2±3.2	T	T	T
Parson Brown	Juice	2.2±5.5	T	T	T
	Peel press liquid	9.8±1.6	T	T	T
Valencia	Juice	8.3±9.8	T	T	T
	Peel press liquid	35.1±1.5	T	T	T

L = limonin, NM = nomilin, DNM = deacetylnomilin, O = obacunone
[1]N = 2 [2]Trace

Table 18: ANOVA of limonoid glucosides in solid fractions of sweet oranges.

Source of Variation	SS	df	MS	F	P-value	F crit
					0.0000**	

Table 18: ANOVA of limonoid glucosides in solid fractions of sweet oranges.

Source of Variation	SS	df	MS	F	P-value	F crit
Variety	563005	2	281502	13	0.0009**	4
Fraction	3138366430	3	1.0E+09	49853	7.8E-25**	3
Interaction	17041362	6	2840227	135	2.6E-10**	3
Within	251808	12	20984			
Total	3156222606	23				

*significant difference at P≤0.05 **significant difference at P≤0.01

Table 19: Total limonoid glucoside concentrations in solid fractions of sweet oranges.

Variety	Sample	Concentration (mg/Kg)[1]	
		Total for each fraction	Total for each variety
Hamlin	Peels	4045^{f}	40725^{C}
	Peel press cake	2720^{h}	
	Seeds	29120^{b}	
	Rags	4838^{d}	
Parson Brown	Peels	2991^{g}	41212^{B}
	Peel press cake	1660^{i}	
	Seeds	32016^{a}	
	Rags	4545^{e}	
Valencia	Peels	5098^{c}	42198^{A}
	Peel press cake	2958^{g}	
	Seeds	29153^{b}	
	Rags	4989^{cd}	

[1]N=2, $LSD_{fraction\ (P\leq0.05)} = 182$, $LSD_{variety\ (P\leq0.05)} = 158$, Different superscripts indicate significant difference at P≤0.05

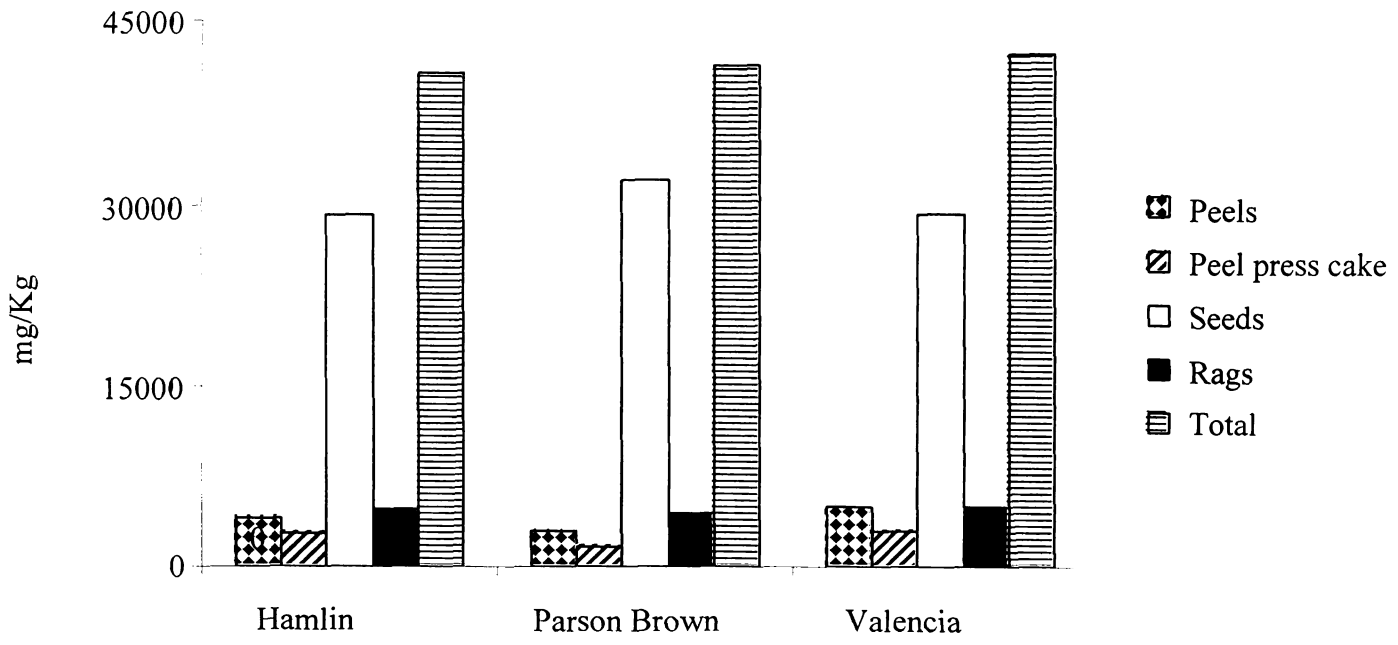

Figure 43: Limonoid glucosides in solid fractions of sweet oranges.

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Seeds had the highest content of limonoid glucosides. Rags contained more limonoid glucosides than peel. This may be partly due to crushing of seeds during juice extraction.

Limonoid glucosides in peel were significantly higher ($P \leq 0.01$) than those in peel press cake. The results suggest that water-soluble compounds like limonoid glucosides were extracted from the peel into peel press liquid during pressing process. Valencia contained highest limonoid glucoside contents, followed by Parson Brown and Hamlin.

Table 20 shows the individual limonoid glucoside concentrations in solid fractions. Nomilin glucoside was the predominant glucosides in seed, while limonin glucoside was the predominant in other orange fractions including juice and peel juice, confirming previous reports (Herman et al., 1990, Ozaki et al., 1991, Fong et al., 1993). Deacetylnomilinic acid and obacunoic acid were found in detectable levels only in the seeds.

ANOVA of total limonoid glucoside content in liquid fractions (Table 21) detected significant differences among varieties, fractions and their interaction ($P \leq 0.01$). Peel press liquid contained less limonoid glucosides than juice (Table 22 and Figure 44). However, these levels would be expected to increase many times in orange molasses (peel press liquid end product). Valencia contained highest total limonoid glucoside content, followed by Hamlin and Parson Brown.

Table 23 shows individual limonoid glucoside concentrations in liquid fractions. Predominant limonoid glucosides in liquid samples were limonin glucoside and nomilinic acid glucoside.

Table 20: Individual limonoid glucoside concentrations in solid fractions of sweet oranges.

Variety	Sample	Concentration (g/100g) ± %CV[1]					
		LG	DNAG	DNG	NG	NAG	OG
Hamlin	Peels	0.2±1.4	T[2]	T	0.1±2.0	0.05±4.2	T
	Peel press cake	0.2±1.3	T	T	0.1±1.4	0.05±6.5	T

Table 20: Individual limonoid glucoside concentrations in solid fractions of sweet oranges.

Variety	Sample	Concentration (g/100g) ± %CV[1]					
		LG	DNAG	DNG	NG	NAG	OG
Hamlin	Peels	0.2±1.4	[2]T	T	0.1±2.0	0.05±4.2	T
	Peel press cake	0.2±1.3	T	T	0.1±1.4	0.05±6.5	T
	Seeds	0.7±0.8	0.23±1.9	0.03±6.5	0.9±0.0	0.4±0.2	0.6±0.7
	Rags	0.2±12.3	T	0.02±3.7	0.1±3.8	0.1±2.5	T
Parson Brown	Peels	0.2±0.5	T	T	0.1±2.8	0.03±13.0	T
	Peel press cake	0.1±1.1	T	T	0.04±0.1	0.03±1.2	T
	Seeds	0.8±0.5	0.23±0.7	0.02±0.9	1.1±1.0	0.5±0.7	0.6±1.4
	Rags	0.2±0.6	T	0.01±1.9	0.2±0.9	0.1±0.2	T
Valencia	Peels	0.3±1.0	T	T	0.2±3.2	0.05±5.6	T
	Peel press cake	0.2±2.8	T	T	0.1±4.2	0.04±9.6	T
	Seeds	0.9±0.5	0.21±0.6	0.03±2.7	0.9±1.2	0.5±0.4	0.5±1.3
	Rags	0.2±1.3	T	0.01±0.1	0.1±3.2	0.1±1.4	T

LG = limonin glucoside, DNAG = deacetylnomilinic acid glucoside, DNG = deacetylnomilin glucoside, NG = nomilin glucoside, NAG = nomilinic acid glucoside, OG = obacunone glucoside, [1]N=2, [2]Trace

Table 21: ANOVA of limonoid glucosides in liquid fractions of sweet oranges.

Source of Variation	SS	df	MS	F	P-value	F crit
Variety	15042	2	7521	88	3.6E-05**	5
Fraction	43326	1	43326	508	5.0E-07**	6
Interaction	24469	2	12235	143	8.6E-06**	5

Table 21: ANOVA of limonoid glucosides in liquid fractions of sweet oranges.

Source of Variation	SS	df	MS	F	P-value	F crit
Variety	15042	2	7521	88	3.6E-05**	5
Fraction	43326	1	43326	508	5.0E-07**	6
Interaction	24469	2	12235	143	8.6E-06**	5
Error	512	6	85			
Total	83349	11				

*significant difference at $P \leq 0.05$ **significant difference at $P \leq 0.01$

Table 22: Total limonoid glucoside concentrations in liquid fractions of sweet oranges.

Variety	Sample	Concentration (mg/Kg)[1]	
		Total for each fraction	Total for each variety
Hamlin	Juice	424^{a}	746^{B}
	Peel press liquid	322^{d}	
Parson Brown	Juice	429^{a}	619^{C}
	Peel press liquid	190^{e}	
Valencia	Juice	403^{b}	786^{A}
	Peel press liquid	383^{c}	

[1]N=2, $LSD_{fraction\ (P \leq 0.05)} = 13$, $LSD_{variety\ (P \leq 0.05)} = 16$, Different superscripts indicate significant difference at $P \leq 0.05$

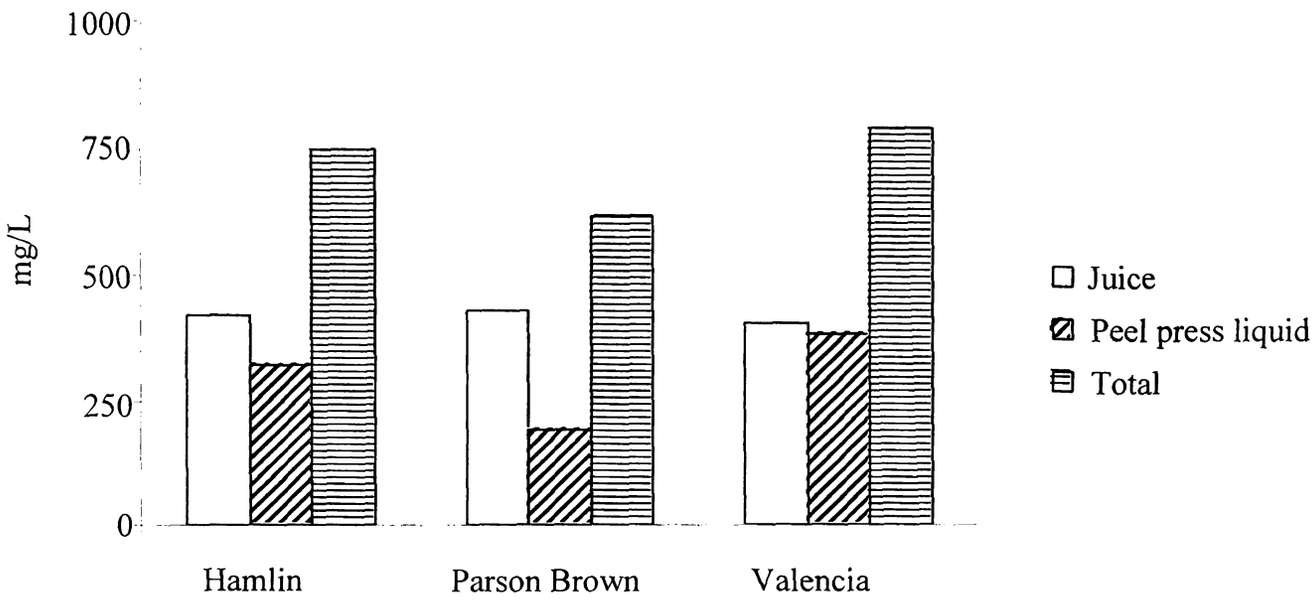

Figure 44: Limonoid glucosides in liquid fractions of sweet oranges.

Table 23: Individual limonoid glucoside concentrations in liquid fractions of sweet oranges.

Variety	Sample	Concentration (mg/L) ± %CV[1]					
		LG	DNAG	DNG	NG	NAG	OG
Hamlin	*Juice*	206±3.0	T[2]	111±1.68	39±2.1	166±2.1	T
	Peel press liquid	172±4.1	T	T	26±10.0	124±6.9	T

Table 23: Individual limonoid glucoside concentrations in liquid fractions of sweet oranges.

Variety	Sample	Concentration (mg/L) ± %CV[1]					
		LG	DNAG	DNG	NG	NAG	OG
Hamlin	Juice	206±3.0	T[2]	11±1.68	39±2.1	166±2.1	T
	Peel press liquid	172±4.1	T	T	26±10.0	124±6.9	T
Parson Brown	Peel	204±0.5	T	6±18.64	55±0.6	163±0.2	T
	Peel press cake	99±0.7	T	T	19±4.1	75±0.4	T
Valencia	Peel	237±1.4	T	7±33.21	20±7.0	138±0.3	T
	Peel press cake	212±0.0	T	T	31±5.4	139±0.0	T

LG = limonin glucoside, DNAG = deacetylnomilinic acid glucoside, DNG = deacetylnomilin glucoside, NG = nomilin glucoside, NAG = nomilinic acid glucoside, OG = obacunone glucoside, [1]N=2, [2]Trace

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Estimated total limonoid glucoside consumption for one serving (240 ml) of Valencia orange juice was 96 mg. Consumption of one Valencia orange fruit would be equivalent to 100 mg of limonoid glucoside.

4.3 Polymethoxylated flavones

ANOVA of total polymethoxylated flavones in solid fractions (Table 24) detected significant differences among varieties, fractions and their interaction ($P \leq 0.01$). Table 25 and Figure 45 show total polymethoxylated flavone concentrations in solid fractions. Polymethoxylated flavones are found mainly in the peel of citrus (Gaydou et al., 1987). The flavedo (external part of the citrus peel) is particularly rich in polymethoxylated flavones (Mouly et al., 1999). Peel and peel press cake contained significantly more polymethoxylated flavones than the edible parts of the fruit. Polymethoxylated flavone concentrations in Valencia peel as reported by Manthey and Grohmann (1996) were slightly lower than obtained in this study. Higher recovery obtained in this study may be attributed to utilization of heat (82°C for 30 min) during extraction. Valencia contained highest polymethoxylated flavone content, followed by Hamlin and Parson Brown.

ANOVA of total polymethoxylated flavone content in liquid fractions (Table 26) detected significant differences among varieties, fractions and their interactions ($P \leq 0.01$).

Table 27 and Figure 46 show total polymethoxylated flavone concentrations in liquid fractions. Peel press liquid had a higher concentration of polymethoxylated flavones than juice. The levels of polymethoxylated flavones in juices in this study were

Table 24: ANOVA of polymethoxylated flavones in solid fractions of sweet oranges.

Source of Variation	SS	df	MS	F	P-value	F crit
Variety	230184	2	115092	163	2.0E-09**	4
Fraction	9527229	3	3175743	4487	1.5E-18**	3
Interaction	390293	6	65049	92	2.5E-09**	3

Table 24: ANOVA of polymethoxylated flavones in solid fractions of sweet oranges.

Source of Variation	SS	df	MS	F	P-value	F crit
Variety	230184	2	115092	163	2.0E-09**	4
Fraction	9527229	3	3175743	4487	1.5E-18**	3
Interaction	390293	6	65049	92	2.5E-09**	3
Error	8494	12	708			
Total	10156200	23				

*significant difference at $P \leq 0.05$ **significant difference at $P \leq 0.01$

Table 25: Total polymethoxylated flavone concentrations in solid fractions of sweet oranges.

Variety	Sample	Concentration (mg/Kg)[1]	
		Total for each fraction	Total for each variety
Hamlin	Peels	1186^{c}	2292^{B}
	Peel press cake	1063^{e}	
	Seeds	26^{gh}	
	Rags	17^{h}	
Parson Brown	Peels	1322^{b}	2290^{C}
	Peel press cake	922^{f}	
	Seeds	21^{h}	
	Rags	25^{gh}	
Valencia	Peels	1900^{a}	3121^{A}
	Peel press cake	1126^{d}	
	Seeds	41^{gh}	
	Rags	55^{g}	

[1]N=2, $LSD_{fraction\ (P \leq 0.05)} = 33$, $LSD_{variety\ (P \leq 0.05)} = 29$, Different superscripts indicate significant difference at $P \leq 0.05$

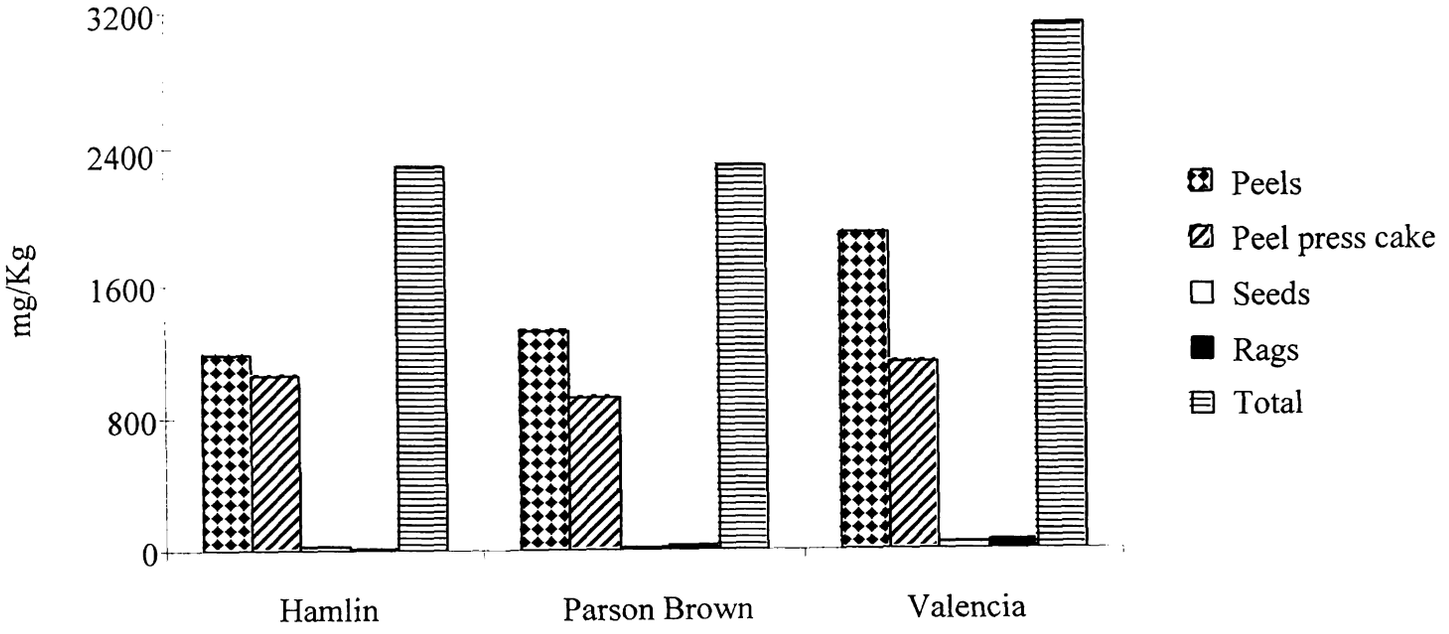

Figure 45: Polymethoxylated flavones in solid fractions of sweet oranges.

Table 26: ANOVA of polymethoxylated flavone concentrations in liquid fractions of sweet oranges.

Source of Variation	SS	df	MS	F	P-value	F crit
Variety	1634	2	817	1135	1.8E-08**	5
Fraction	11818	1	11818	16420	1.5E-11**	6
Interaction	3562	2	1781	2475	1.8E-09**	5

Table 26: ANOVA of polymethoxylated flavone concentrations in liquid fractions of sweet oranges.

Source of Variation	SS	df	MS	F	P-value	F crit
Variety	1634	2	817	1135	1.8E-08**	5
Fraction	11818	1	11818	16420	1.5E-11**	6
Interaction	3562	2	1781	2475	1.8E-09**	5
Error	4	6	0.7			
Total	17020	11				

*significant difference at P≤0.05 **significant difference at P≤0.01

Table 27: Total polymethoxylated flavone concentrations in liquid fractions of sweet oranges.

Variety	Sample	Concentration (mg/Kg)[1]	
		Total for each fraction	Total for each variety
Hamlin	Juice	19.9^{d}	57.6^{C}
	Peel press liquid	37.8^{c}	
Parson Brown	Juice	8.6^{e}	85.8^{B}
	Peel press liquid	77.2^{b}	
Valencia	Juice	6.6^{f}	114.8^{A}
	Peel press liquid	108.3^{a}	

[1]N=2, $LSD_{fraction\ (P\leq0.05)} = 1.2$, $LSD_{variety\ (P\leq0.05)} = 1.5$, Different superscripts indicate significant difference at P≤0.05

Figure 46: Polymethoxylated flavones in liquid fractions of sweet oranges.

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similar to those reported by Mouly et. al (1999). Among the three varieties studied, Valencia had the greatest polymethoxylated flavone content.

Table 28 and Table 29 show individual polymethoxylated flavone concentrations in solid and liquid fractions. Primary polymethoxylated flavones in these sweet oranges were nobiletin and sinensitin, which accounted for approximately 36 and 27 % of total polymethoxylated flavones in both solid and liquid fractions.

Ooghe (1999) described criteria for authentic orange juice. Seven compounds are consistently present in sweet orange juice - sinensitin, 3, 5, 6, 7, 3', 4'-hexamethoxyflavone, nobiletin, 3,4,5,6,7,8,3',4'-heptamethoxyflavone, scutellarein tetramethylether, tangeretin, and one unidentified minor compound. The results obtained in this study are consistent with this criterion for all orange fractions.

Estimated total polymethoxylated flavone consumption for one serving (240 ml) of Valencia orange juice was 1.7 mg. Consumption of one Valencia orange fruit would be equivalent to 2.1 mg of total polymethoxylated flavones.

4.4 Flavanone glucosides

ANOVA of total flavanone glucoside contents in solid fractions (Table 30) detected significant differences among varieties, fractions and their interactions ($P \leq 0.01$).

Table 31 and Figure 47 show total flavanone glucoside concentrations in solid fractions. Peel and peel press cake contained the highest levels of flavanone glucosides, while seed contained the lowest levels. The results reported here are consistent with the report by Manthey and Grohmann (1996) for peel and are higher than those reported by Kawaii et al. (1999) who quantitatively determined flavonoids in edible fruit parts

Table 28: Individual polymethoxylated flavone concentrations in solid fractions of sweet oranges.

Variety	Sample	Concentration (mg/Kg) ± %CV[1]					
		ST	HX	NBT	HP	STME	TT
Hamlin	Peels	309±2.9	52±0.3	477±3.1	145±2.9	144±3.2	57±3.0
	Pulp press cake	279±5.7	43±2.1	432±5.4	140±4.7	115±4.9	55±5.4
	Pulp					4.8±9.0	1.9±32.3

Table 28: Individual polymethoxylated flavone concentrations in solid fractions of sweet oranges.

Variety	Sample	Concentration (mg/Kg) ± %CV[1]					
		ST	HX	NBT	HP	STME	TT
Hamlin	Peels	309±2.9	52±0.3	477±3.1	145±2.9	144±3.2	57±3.0
	Peel press cake	279±5.7	43±2.1	432±5.4	140±4.7	115±4.9	55±5.4
	Seeds	4.8±10.3	1.2±18.2	9.8±8.2	3.6±11.9	4.8±9.0	1.9±32.3
	Rags	3.3±2.3	0.9±7.1	6.6±3.6	1.9±0.4	3.1±3.7	1.1±8.9
Parson Brown	Peels	386±4.	86±4.9	457±4.4	164±4.8	167±4.6	62±4.8
	Peel press cake	261±1.8	61±6.5	322±4.1	128±1.7	103±7.5	46±0.8
	Seeds	4.7±17.1	1.6±26.5	6.9±43.5	2.9±67.0	3.1±42.1	1.3±89.3
	Rags	5.3±3.1	1.4±7.9	8.3±5.5	4.1±0.3	3.9±2.7	1.7±4.3
Valencia	Peels	586±0.5	116±0.2	626±0.6	270±0.6	204±0.2	98±0.0
	Peel press cake	332±2.0	64±2.0	376±1.8	176±1.7	115±1.6	64±1.2
	Seeds	9.3±6.3	2.9±0.9	14±9.3	5.6±7.0	6.4±11.9	2.4±15.5
	Rags	12±26.3	3.5±19.0	18±16.5	8.7±7.1	8.5±8.1	4.3±2.8

ST = sinensitin, HX = 3,5,6,7,3',4'-hexamethoxyflavone, NBT = nobiletin, HP = 3,4,5,6,7,8,3',4'-heptamethoxyflavone, STME = scutellarein tetramethylether, TT = tangeretin, [1]N=2

Table 29: Individual polymethoxylated flavone concentrations in liquid fractions of sweet oranges.

Variety	*Sample*	Concentration (mg/Kg) ± %CV[1]					
		ST	HX	NBT	HP	STME	TT
Hamlin	*Juice*	5.8±4.6	1.0±4.7	8.3±4.1	1.9±3.7	2.3±3.7	0.5±7.5
		10.6±4.7	1.7±4.1	15.4±4.4	4.0±3.9	4.5±4.4	1.6±2.4

Table 29: Individual polymethoxylated flavone concentrations in liquid fractions of sweet oranges.

Variety	Sample	Concentration (mg/Kg) ± %CV[1]					
		ST	HX	NBT	HP	STME	TT
Hamlin	Juice	5.8±4.6	1.0±4.7	8.3±4.1	1.9±3.7	2.3±3.7	0.5±7.5
	Peel press liquid	10.6±4.7	1.7±4.1	15.4±4.4	4.0±3.9	4.5±4.4	1.6±2.4
Parson Brown	Juice	2.1±0.4	0.6±0.3	3.0±0.1	1.1±0.5	1.4±0.4	0.5±3.7
	Peel press liquid	23.2±2.1	5.3±0.9	26.0±1.2	8.8±0.6	10.1±0.3	3.7±6.2
Valencia	Juice	1.5±5.8	0.4±4.0	2.2±3.1	0.8±2.3	1.1±2.6	0.4±4.5
	Peel press liquid	34.5±0.8	6.8±0.2	36.4±0.8	12.9±0.8	12.4±0.6	5.2±0.0

ST = sinensitin, HX = 3,5,6,7,3',4'-hexamethoxyflavone, NBT = nobiletin, HP = 3,4,5,6,7,8,3',4'-heptamethoxyflavone, STME = scutellarein tetramethylether, TT = tangeretin, [1]N=2

Table 30: ANOVA of flavanone glucosides in solid fractions of sweet oranges.

Source of Variation	SS	df	MS	F	P-value	F crit
Variety	12106128	2	6053064	32	1.6E-05**	4
Fraction	2346946647	3	782315549	4134	2.4E-18**	3
Interaction	16289708	6	2907951	15	5.3E-05**	3

Table 30: ANOVA of flavanone glucosides in solid fractions of sweet oranges.

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Variety	12106128	2	6053064	32	1.6E-05**	4
Fraction	2346946647	3	782315549	4134	2.4E-18**	3
Interaction	17447704	6	2907951	15	5.3E-05**	3
Error	2270569	12	189214			
Total	2378771048	23				

*significant difference at P≤0.05 **significant difference at P≤0.01

Table 31: Total flavanone glucoside concentrations in solid fractions of sweet oranges.

Variety	Sample	Concentration (mg/Kg)[1]	
		Total for each fraction	Total for each variety
Hamlin	Peels	27141^{a}	74613^{A}
	Peel press cake	26437^{b}	
	Seeds	3581^{f}	
	Rags	17453^{d}	
Parson Brown	Peels	26843^{b}	69927^{B}
	Peel press cake	27510^{a}	
	Seeds	2071^{g}	
	Rags	13503^{e}	
Valencia	Peels	26709^{b}	67814^{C}
	Peel press cake	25045^{c}	
	Seeds	2573^{g}	
	Rags	13488^{e}	

[1]N=2, $LSD_{fraction\ (P\leq0.05)} = 547$, $LSD_{variety\ (P\leq0.05)} = 474$, Different superscripts indicate significant difference at P≤0.05

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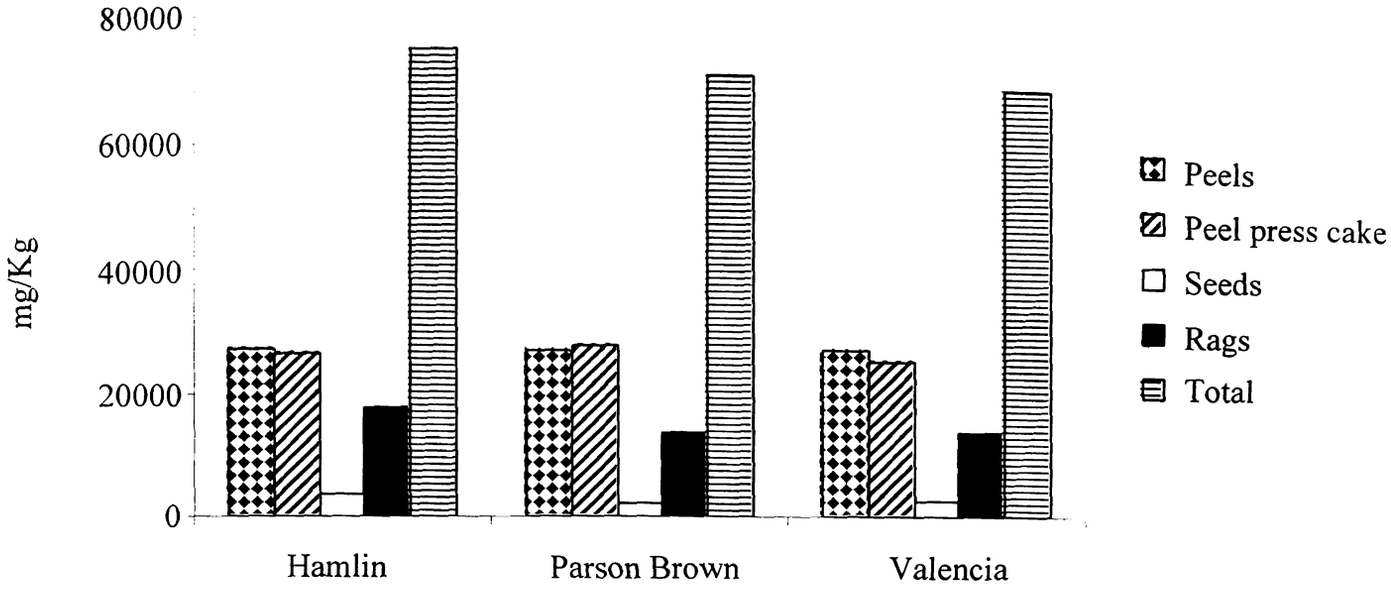

Figure 47: Flavanone glucosides in solid fractions of sweet oranges.

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without using heat in their extraction procedure. Among three varieties, Hamlin had the greatest flavanone glucoside content in solid fractions.

ANOVA for total flavanone glucoside contents in liquid fractions (Table 32) detected significant differences among varieties, fractions and their interaction ($P \leq 0.01$). Table 33 and Figure 48 show total flavanone glucoside concentrations in liquid fractions. Peel press liquid had higher flavanone glucoside content than juice by at least two times. Similar to the solid fractions, Hamlin had the highest flavanone glucoside content compared among three varieties.

Table 34 and Table 35 show individual flavanone glucoside concentrations in solid and liquid fractions, respectively. Hesperidin and narirutin were the predominant compounds in the sweet oranges studied with hesperidin having the greatest amounts.

Seed was the only fraction that had a higher concentration of narirutin than hesperidin. Flavanone glucosides found in these three cultivars were all rutinosides, the nonbitter forms. Rutinosides are found in all *Citrus*, while the bitter neohesperidosides are found in species related to pummelo (Ooghe, 1999).

Estimated total flavanone glucoside consumption for one serving (240 ml) of Valencia orange juice was 88 mg. Consumption of one Valencia orange fruit would be equivalent to 236 mg of total flavanone glucosides.

5. Conclusion

Different orange fractions exhibited varying concentrations of limonoids and flavonoids. Seeds had the greatest concentration of limonoids (aglycones and glucosides), while peels and peel press cake had the highest concentrations of polymethoxylated flavones and flavanone glucosides. However, it may not be effective

Table 32: ANOVA of flavanone glucosides in liquid fractions of sweet oranges.

Source of Variation	SS	df	MS	F	P-value	F crit
Variety	48122	2	24061	243	1.8E-06**	5
Fraction	887998	1	887998	8960	9.4E-11**	6
		2	2060	21	0.002**	5

Table 32: ANOVA of flavanone glucosides in liquid fractions of sweet oranges.

Source of Variation	SS	df	MS	F	P-value	F crit
Variety	48122	2	24061	243	1.8E-06**	5
Fraction	887998	1	887998	8960	9.4E-11**	6
Interaction	4120	2	2060	21	0.002**	5
Error	595	6	99			
Total	940835	11				

*significant difference at P≤0.05 **significant difference at P≤0.01

Table 33: Total flavanone glucoside concentrations in liquid fractions of sweet oranges.

Variety	Sample	Concentration (mg/Kg)[1]	
		Total for each fraction	Total for each variety
Hamlin	Juice	559^{c}	1613^{A}
	Peel press liquid	1054^{a}	
Parson Brown	Juice	503^{d}	1559^{B}
	Peel press liquid	1056^{a}	
Valencia	Juice	369^{e}	1322^{C}
	Peel press liquid	953^{b}	

[1]N=2, $LSD_{fraction\ (P\leq0.05)}$ = 14, $LSD_{variety\ (P\leq0.05)}$ = 17, Different superscripts indicate significant difference at P≤0.05

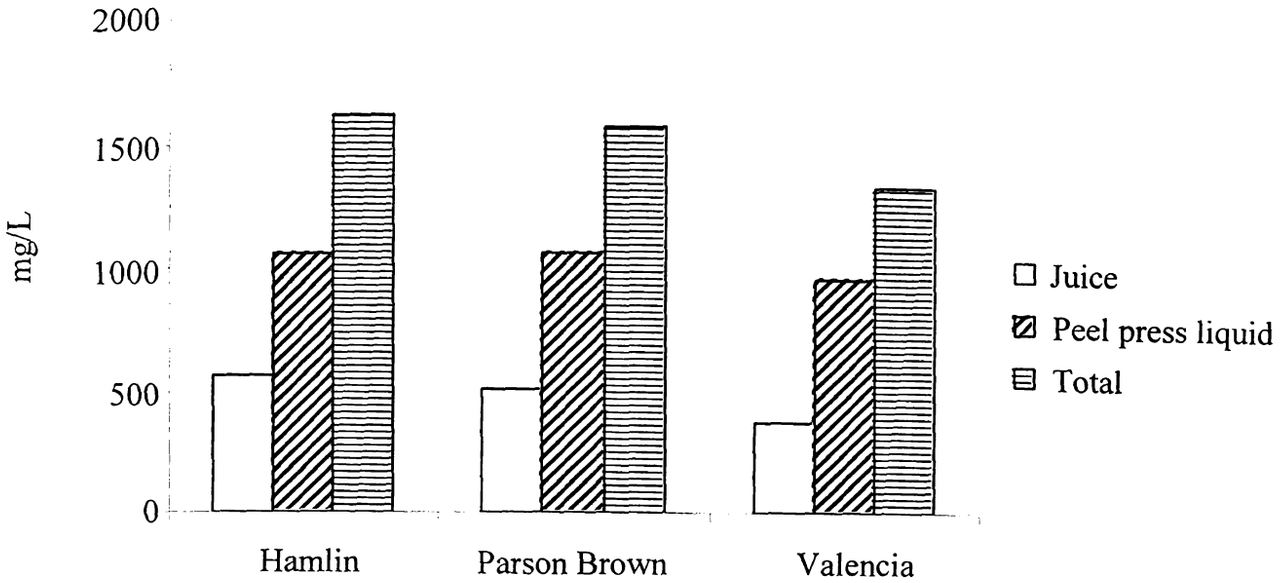

Figure 48: Flavanone glucosides in liquid fractions of sweet oranges.

Table 34: Individual flavanone glucoside concentrations in solid fractions of sweet oranges.

Variety	Sample	Concentration (g/100g)±%CV[1]				
		NT-4'-G	ERT	NT	HD	DD
Hamlin	Peels	0.11±0.6	0.11±0.2	0.20±0.5	2.15±0.2	0.14±0.0
		0.07±2.3	0.07±3.2	0.17±0.1	2.20±0.4	0.13±0.4

Table 34: Individual flavanone glucoside concentrations in solid fractions of sweet oranges.

Variety	Sample	Concentration (g/100g)±%CV[1]				
		NT-4'-G	ERT	NT	HD	DD
Hamlin	Peels	0.11±0.6	0.11±0.2	0.20±0.5	2.15±0.2	0.14±0.0
	Peel press cake	0.07±2.2	0.07±3.2	0.17±0.1	2.20±0.4	0.13±0.4
	Seeds	0.04±0.9	0.01±1.1	0.17±1.0	0.12±1.2	0.03±1.1
	Rags	0.09±1.8	0.04±0.1	0.28±1.1	1.23±1.3	0.12±1.0
Parson Brown	Peels	0.09±0.4	0.09±0.2	0.19±0.2	2.20±0.5	0.12±0.4
	Peel press cake	0.06±3.6	0.06±1.1	0.15±1.8	2.37±2.4	0.11±4.6
	Seeds	0.02±0.5	0.01±1.3	0.09±0.2	0.07±0.3	0.01±0.1
	Rags	0.05±0.1	0.02±0.9	0.19±0.2	0.99±0.4	0.09±0.0
Valencia	Peels	0.09±0.8	0.06±0.2	0.22±0.0	2.19±0.2	0.11±0.2
	Peel press cake	0.05±1.7	0.04±0.1	0.16±0.2	2.15±0.1	0.10±0.2
	Seeds	0.02±0.0	0.01±0.7	0.11±0.1	0.10±0.2	0.02±0.1
	Rags	0.06±1.3	0.02±0.8	0.21±0.5	0.98±0.2	0.08±0.1

NT-4'-G = narirutin-4'-glucoside, ERT = eriocitrin, NT = narirutin, HD = hesperidin, DD = didymin, [1]N=2

Table 35: Individual flavanone glucoside concentrations in liquid samples of sweet oranges.

Variety	Sample	Concentration (mg/L) ± %CV[1]				
		NT-4'-G	ERT	NT	HD	DD
	Juice	33±3.6	12±3.0	77±3.2	410±3.5	27±2.8
				125±0.5	737±1.0	55±0.2

Table 35: Individual flavanone glucoside concentrations in liquid samples of sweet oranges.

Variety	Sample	Concentration (mg/L) ± %CV[1]				
		NT-4'-G	ERT	NT	HD	DD
Hamlin	Juice	33±3.6	12±3.0	77±3.2	410±3.5	27±2.8
	Peel press liquid	79±0.2	59±0.5	125±0.5	737±1.0	55±0.2
Parson Brown	Juice	23±5.2	9±8.1	57±0.2	391±0.6	22±0.5
	Peel press liquid	67±3.5	50±1.2	116±2.6	776±0.7	47±1.3
Valencia	Juice	15±1.5	5±6.7	39±0.2	294±0.2	15±1.8
	Peel press liquid	62±0.4	33±0.2	137±0.5	675±0.2	46±0.8

NT-4'-G = narirutin-4'-glucoside, ERT = eriocitrin, NT = narirutin, HD = hesperidin, DD = didymin, [1]N=2, [2]Trace

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to isolate seeds from the waste stream, since seeds account for a small part of the total waste. In addition, the use of seed to isolate limonoids would require additional steps such as isolation and grinding.

Peel press liquid contained higher phytochemical content than juice with the exception of limonoid glucosides, suggesting that limonoid glucosides were highly extractable through commercial juice extraction. Pressing process in feed mill operation extracted limonoid glucosides and polymethoxylated flavones from the peel into peel press liquid, but concentrating limonoid algycones in peel press cake.

High content of limonoid glucosides in juice indicated their intake through orange juice consumption would be high.

Valencia had the greatest concentrations of limonoids (aglycones and glucosides) and polymethoxylated flavones, and Hamlin had the greatest concentration of flavanone glucosides.

Flavanone glucosides were found as the predominant group in both solid and liquid samples, accounting for approximately 60% of total phytochemicals studied, followed by limonoid glucosides, limonoid aglycones, and polymethoxylated flavones.

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Study IV: Effect of lime treatment on of limonoid and flavonoid content in by-products from orange juice process

1. Abstract

Waste streams from orange juice manufacturing provide inexpensive raw materials to produce value-added by-products for health, pharmaceutical, and a variety of other industries. Limonoid and flavonoid in waste products were measured before and after lime treatment. Peel and rag, primary waste materials, were treated with 0.3% CaO (wet wt.), pressed to yield press cakes and press liquid. These fractions were analyzed for the content of limonoid aglycones, limonoid glucosides, flavanone glucoside and polymethoxylated flavones.

With lime treatment, more limonoid aglycones (25%) and limonoid glucosides (12%) leached from press cake into press liquid (both in rag and peel). There was a trend showing increased phytochemical content were released from press cakes into press liquids due to lime treatment. In seed, lime treatment (0.3% CaO wet wt.) resulted in loss of limonoid glucosides, but had no effect on limonoid aglycone, flavanone glucoside and polymethoxylated flavone content. The results suggested that lime treatment resulted in increased phytochemical content in press liquid especially limonoids.

2. Introduction

During 2001-2002, the world production of citrus fruit was approximately 73 million metric tons, of which 49% was marketed as fresh fruit and 42% was converted into processed products (FAS/USDA, 2003). With such significant amounts of fruit being processed, large quantities of waste materials are produced. Waste products include peel, rag, core, seed, and pulp. These residues, accounting for 50% of the fruit weight (Anonymous, 1998); have been used or converted into a variety of end products

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(Braddock, 1999). Most dried pulp (final form) of the remained waste materials is used for animal feeds. These dried end products have lighter weight and longer shelf life than the raw material and thus enable stable shipment and storage prior to use.

Direct lime treatment has been used widely in fruit and vegetable processing to facilitate the dehydration of pulp, clarification of juice, and for other pectinacious materials (Braddock, 1999). During feed mill operations, where orange waste materials are processed, lime is used to aid the dewatering process of waste materials. Lime treated waste materials are subsequently pressed to remove water (approximately 10% moisture reduction) and then dried to about 10% final moisture content. Lime treatment is a necessary processing aid to reduce energy consumption and to increase drying rate.

Waste materials from orange juice processing are rich sources of limonoids (Ozaki et al., 1995, Hasegawa et al., 1996, Braddock, 1999, Braddock and Bryan, 2001) and flavonoids (Manthey and Grohmann, 1996, Braddock, 1999). These principal secondary metabolites, specifically found in *Citrus* species, have been demonstrated to have pharmaceutical and industrial applications. Claimed beneficial properties for flavonoids include antioxidant, anticancer, anti-inflamatory, antimicrobial, free radical scavengers, anti-allergic, and analgesic properties (Benavente-Garcia et al., 1997), as well as sweetening agents (Horowitz, 1986, Bar et al., 1990, and Borrego et al., 1991). Limonoids have been shown to have chemopreventive activities (Lam and Hasegawa, 1989, Miller et al., 1989, Lam et al., 1994, Miller et al., 2000, Tian et a., 2001), and antifeedant activities (Alford and Bentley, 1986, Bentley et al., 1988, Serit et al., 1991, Mendel et al., 1993, Ruberto et al., 2002).

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Citrus flavonoids are more soluble (Di Mauro et al., 2000) and limonoid aglycones are more stable (Miyake et al., 1993) under alkali conditions. Since commercial citrus waste streams are generally lime-treated, the objective of this study was to investigate influences of lime treatment on limonoid and flavonoid content in major waste materials (peel, rag, and seed).

3. Materials and methods

3.1 Waste samples

Waste materials from three orange varieties (Hamlin, Parson Brown, and Valencia) were obtained from the Tropicana Products Company (Bradenton, FL). Peel and rag with seed were vacuum-sealed and shipped frozen to Michigan State University. These waste samples were stored at -20°C until sample preparation.

3.2 Sample preparation

3.2.1 Lime treatment

Samples were thawed at room temperature. Seeds were manually separated from rag. Half-cut peels were sliced into approximately 12 mm-wide sections with a mechanical slicer. Each sample was mixed thoroughly with 0.3% CaO (wet wt), which prepared as slurry by addition of water (10 ml). For example, one kg of peel was added with 3 g of CaO which was initially mixed with 10 ml of water. The control samples were mixed with the same amount of water as used in the CaO slurry. Both lime-treated and control samples were incubated for two days.

3.2.2 Pressing

To simulate the industrial pressing process that partially extracts liquid from the residues, each sample ± lime treatment was processed with a domestic juice extractor

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(Juicerator). Liquid was recovered by centrifugal force which pressed the residue against a screen to expel the fluid juice. The liquid is termed "pressed liquid", while the remaining pulp is termed "pressed cake". The press liquid was stored at -20°C until analyzed. The press cake materials were freeze-dried, ground to pass through a 1mm screen using a Wiley Mill, and stored in a desiccated chamber at -20°C until analyzed.

3.3 Studied compounds and standards

Studied compounds included limonoid glucosides, limonoid aglycones, flavanoid glucosides, and polymethoxylated flavones. Standards, kindly donated by scientists from USDA, Dr. Gary D. Manners (Pasadena, CA), Dr. Mark A. Berhow (Peoria, IL), and Dr. John A. Manthey (Winter Haven, FL), included deacetylnomilin (DNM), obacunone (O), limonin glucoside (LG), deacetylnomilinic acid glucoside (DNAG), nomilinic acid glucoside (NAG), obacunone glucoside (OG), obacunoic acid (OA), isoobacunoic acid (IOA), deoxylimonin (DL), 17-19-didehydrolimonoic acid (DDHLA), 19-dehydrolimonoic acid (DHLA), limolinic acid (LA), rutaevin (R), sinensetin (ST), nobiletin (NBT), 3,4,5,6,7,8,3',4'-heptamethoxyflavone (HP), and tangeretin (TT).

Limonin (L), nomilin (NM) hesperidin (HD), naringin (NG), neohesperidin (NHD), hesperitin (HT), diosgenin (DN), coumarin (CM), quercetin (QT) were purchased from Sigma Company (St. Louis, MO). Sinensetin (ST), scutellarein tetramethylether (STME), narirutin (NT), didymin (DD), and eriocitrin (ERT) were purchased from Extrasynthese, (Genay, France).

3.4 Moisture content analyses

The AOAC (1990) method for moisture in animal feed (7.003) was followed. Samples (2 g) were dried (50°C) under vacuum condition for 12 hours.

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3.5 Extraction and analysis of limonoid aglycones and polymethoxylated flavones

3.5.1 Extraction

The extraction procedure of Fong et al. (1993) was modified from. Press liquids were thawed at room temperature and heated in a water bath (82°C for 30 min), then cooled to room temperature. Ten ml of press liquid was then mixed with 25 ml of 0.5 M Tris buffer (pH 8) for 15 minutes and then acidified to pH 2 with 1 N HCl.

Ground, freeze-dried press cake (peel or rag) (1 g) was mixed with 25 ml of 0.5 M Tris buffer (pH 8) for 15 minutes and then acidified to pH 2 with 1 N HCl. Ground, freeze-dried seed (1 g) was mixed with 25 ml of 0.15 M Tris buffer (pH 8) overnight (20 hours), and then acidified to pH 2 with 1 N HCl. The acidified mixtures of peel, peel press cake, rag and seed were heated in a water bath (82°C for 30 min).

Ethyl acetate (25 ml) containing 200 ppm butylated hydroxytoluene (antioxidant) was added to all samples, shaken for 15 minutes, and the ethyl acetate layer was decanted. Ethyl acetate extraction was performed twice. The ethyl acetate layers were combined, evaporated to dryness, and reconstituted to 10 ml with methanol. Filtered extract (0.45μ nylon) was analyzed by HPLC. A flow diagram of limonoid aglycone and polymethoxylated flavone extraction is presented in Figure 20 (Study I/part II).

3.5.2 High performance liquid chromatography (HPLC) analysis

The mobile phases consisted of 3 mM phosphoric acid (solvent A) and acetonitrile (solvent B). Limonoid aglycones and polymethoxylated flavones were resolved with a gradient that started with 30% B, was 40% B in 20 minutes and ended with 50% B at 50 minutes. Flow rate was 1ml/min. Separation was achieved on a C18 column (Luna: C18, 5μ, 250 mm x 4.6 mm, 17.8 % carbon load, void volume 2.5 ml).

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Injection volume was 10 µl. Limonoid aglycones were detected at 210 nm, while polymethoxylated flavones were detected at 340 nm.

Since seeds are rich in limonoid aglycones and low in polymethoxylated flavones, limonoid aglycone analysis was carried out separately for seed extract. Separation of limonoid aglycones was achieved on C18 column (Alltima: C18, 5µ, 250 mm x 4.6 mm, 16 % carbon load, void time 2.02 minutes) and an isocratic mobile phase (acetonitrile/methanol/water, 10:41:49). Flow rate was 1ml/minute and injection volume was 10 µl. The HPLC system was described in Study I/Part I (3.4).

Identification and quantitation of limonoid aglycones (limonin, deacetylnomilin, nomilin, and obacunone), were based on retention time, UV spectra and response factors of external standards. Identification of polymethoxylated flavones (sinensitin, nobiletin, 3,4,5,6,7,8,3',4'-heptamethoxyflavone, scutellarein tetramethylether, and tangeretin) were based on retention time and UV spectra obtained with external standards. For 3,5,6,7,3',4'-hexamethoxyflaovne, identification was based on retention relative to other polymethoxylated flavones and was verified by negative fast atom bombardment mass spectrometry (-eVFABMS) and nuclear magnetic resonance spectroscopy (NMR) in study II/part II. The quantitations of polymethoxylated flavones were based on the response factor determined for scutellarein tetramethylether.

3.6 Extraction and analysis of limonoid glucosides

3.6.1 Extraction

Press liquids were thawed at room temperature, heated in a water bath (82°C for 5 min), and cooled to room temperature. Ten ml of press liquid was mixed with 25 ml of 70% methanol for 15 minutes.

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Ground, freeze-dried solid fractions (peel press cake, rag press cake, and seed) (1 g) were mixed with 25 ml of 70% methanol for 15 minutes, and heated in a water bath (82°C for 5 min).

The samples were centrifuged (10,000X g for 10 minutes), and the supernatants were decanted. The pellet was extracted again with 70% methanol. Combined supernatants were evaporated to approximately 2-3 ml at 40°C under vacuum, and reconstituted to 10 ml with methanol. Filtered extracts (0.45μ nylon) were analyzed by HPLC. A flow diagram of limonoid glucoside extraction is presented in Figure 21 (Study I/part II).

3.6.2 High performance liquid chromatography (HPLC) analysis

The mobile phases consisted of 3 mM phosphoric acid (solvent A) and acetonitrile (solvent B). Limonoid glucosides were separated with linear gradient starting with 10% B and ending with 26% B in 70 minutes. Separation was performed on C18 column (Luna: C18, 5μ, 250 mm x 4.6 mm, 17.8 % carbon load, void volume 2.5 ml) with 1 ml/min flow rate and 10 μl injection volume. Limonoid glucosides were detected at 210 nm. The HPLC system was described in Study I/Part I (3.4).

Identification and quantitation of limonoid glucosides (limonin glucoside, deacetylnomilinic acid glucoside, nomilinic acid glucoside, and obacunone glucoside) were based on retention time, UV spectra, and response factors obtained with external standards. For deacetylnomilin acid glycoside and nomilin acid glucoside, the identifications were based on retention relative to other limonoid glucosides and subsequently verified by –eVFABMS in study II/part II. The quantitation of deacetylnomilin glucoside was based on the response factor determined for

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deacetylnomilinic acid glucoside, while that of nomilin glucoside was based on the response factor determined for nomilinic acid glucoside.

3.7 Extraction and analysis of flavanone glucosides

3.7.1 Extraction

Press liquids were thawed at room temperature, heated in water bath (82°C for 5 min), and cooled to room temperature. Ten ml of press liquid was then mixed with 25 ml dimethylformamide/methanol (1:2) for 15 minutes.

Ground, freeze-dried solid parts (peel press cake, rag press cake, and seed) (1 g) was mixed with 25 ml dimethylformamide/methanol (1:2) for 15 minutes, and then heated in a water bath (82°C for 5 min).

The samples were centrifuged (10,000X g for 10 min) and the supernatant was decanted. Extractions with dimethylformamide/methanol (1:2) were done twice. Combined supernatants were evaporated to approximately 15 ml at 50°C under vacuum, and reconstituted to 25 ml with methanol. Filtered extract (0.45μ nylon) was analyzed by HPLC. A flow diagram of flavonoid glucoside extraction is presented in Figure 22 (Study I/part II).

3.7.2 High performance liquid chromatography (HPLC) analysis

The HPLC analysis of flavanone glucoside was based on the method of Ooghe (1999). Flavanone glucosides were separated on C18 column (Alltima: C18, 5μ, 250 mm x 4.6 mm, 16 % carbon load, void time 2.02 minutes) with a mobile phase consisting of 0.01 M potassium phosphate monobasic (solvent A) and acetonitrile (solvent B). A linear gradient starting at 10%B and ending at 30% B in 60 minutes was used. Flow rate

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was 1 ml/min and injection volume was 10 µl. Flavanone glucosides were detected at 280 nm. The HPLC system was described in Study I/Part I (3.4).

Identification and quantitation of eriocitrin, narirutin, hesperidin, didymin were based on retention time, UV spectra, and response factors obtained with external standards. For narirutin-4'-glucoside, the identification was based on retention relative to other flavanone glucosides and subsequently confirmed by -eVFABMS and NMR in study II/part II. The quantitation of narirutin-4'-glucoside was based on the response factor determined for narirutin.

3.8 Data analysis

The paired-t test was to determine the differences significant difference in limonoid and flavonoid content between control and lime-treated samples. Since there were a limited number of samples, potential variety differences and the potential interaction between treatment and variety were not tested. Analyses were conducted in duplicate. Quantitative comparisons of the limonoid and flavonoid content between press cake and press liquid are based on the dried weight of raw materials.

4. Results and discussion

Calcium oxide (CaO, lime) is commonly used to treat citrus waste materials, as it readily hydrates with water in the residues, and forms calcium hydroxide [$Ca(OH)_2$]. The recommended concentration of lime ranges between 0.2-0.5% (wet wt. basis). Lime treatment reduces waste acidity and de-esterifies pectin in the waste materials (Braddock, 1999).

The pKa of pectin is between 3.55 and 4.10, depending on the degree of esterification (Plaschina et al., 1978). Neutralization prevents protonation of carboxylate

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groups on the pectin molecule and prevents formation of hydrogen bonding, a condition that is favorable for hydration. De-esterification of pectin under basic condition produces pectic acids which react with the calcium ions (Ca^{2+}) in lime to form calcium pectate salt, liberating water and methanol during subsequent pressing (Braddock, 1999).

Moisture contents of peel and rag raw materials were approximately 66% and 79%, respectively (Table 36). These initial moisture values are relatively low, especially for peels, compared to industrial data (80-82%) (Anonymous, 1998 and Braddock, 1999). The raw materials were frozen and stored before analyzed; therefore some reduction in water holding capacity and/or direct moisture loss may have occurred. The moisture contents estimated in press cake with and without lime treatment were approximately 64% in peel and 75% in rag. Moisture reduction by pressing (2 % in peel and 7% in rag) in our study is relatively low compared to industrial data (10%) (Anonymous, 1998 and Braddock, 1999). Lime treatment had no significant effect ($P \geq 0.05$) on moisture content of peel press cake but resulted in significantly decreased moisture content ($P \leq 0.05$) in rag press cake (Table 37). The small loss in water content due to lime treatment may be because peels were drier than commercial peels. Further, the pH values of lime-treated press liquids (Table 38), ranged from 5.07 to 5.71. The relatively acidic press liquids suggest that less than optimal cross-linking between calcium ions and pectin molecules were achieved and that minimal de-esterification occurred.

Table 39 and Table 40 show limonoid aglycone concentrations in press cakes and press liquids of peel and rag with and without lime treatment. Limonin was the only compound detected in measurable quantity in both peel and rag samples. There was a trend ($P \leq 0.05$) for lime treatment to decrease limonin content in press cakes

Table 36: Moisture content of raw materials prior to pressing process.

Sample	Variety	Moisture content (%) ± Std[1]
Peels	Parson Brown	65.6±0.2
	Valencia	65.4±0.2

Table 36: Moisture content of raw materials prior to pressing process.

Sample	Variety	Moisture content (%) ± Std[1]
Peels	Parson Brown	65.6±0.2
	Valencia	65.4±0.2
Rags	Hamlin	82.5±0.2
	Parson Brown	79.1±0.4
	Valencia	77.0±0.8

[1]N = 6

Table 37: Moisture content of press cakes recovered from pressing process (with and without lime treatment).

Sample	Variety	Moisture content (%) ± Std[1]	
		Control	Lime
Peel press cake	Hamlin	N/A[2]	N/A
	Parson Brown	63.6±0.0	63.2±0.3
	Valencia	63.9±0.2	63.8±0.2
Rag press cake	Hamlin	75.2±0.9	74.8±0.5
	Parson Brown	77.3±1.0	75.9±1.0
	Valencia	74.9±2.0	73.6±0.6

[1]N = 6; Lime treatment had no significant effect ($P \geq 0.05$) on moisture content of peel press cake, but resulted in a significant decrease ($P \leq 0.05$) of moisture content in rag press cake., [2]Not available

Table 38: pH and Brix values of press liquids (with and without lime treatment).

Sample	Variety	pH value		Brix value	
		Control N/A	Lime N/A	Control N/A	Lime N/A

Table 38: pH and Brix values of press liquids (with and without lime treatment).

Sample	Variety	pH value		Brix value	
		Control	Lime	Control	Lime
Peel press liquid	Hamlin	N/A[1]	N/A	N/A	N/A
	Parson Brown	3.8	5.4	14.6	14.2
	Valencia	3.9	5.7	14.2	14.0
Rag press liquid	Hamlin	3.7	5.1	10.4	10.0
	Parson Brown	4.0	5.6	11.0	9.8
	Valencia	4.3	5.1	11.2	11.0

[1]Not available

Table 39: Limonoid aglycone content in peel press cakes and press liquids (with and without lime treatment).

Sample	Variety	Treatment	mg/Kg peel (dried wt)			
			LM	DNM	NM	O
		Control	N/A[2]	N/A	N/A	N/A
						N/A

[1] %CV

Table 39: Limonoid aglycone content in peel press cakes and press liquids (with and without lime treatment).

Sample	Variety	Treatment	mg/Kg peel (dried wt) ± %CV[1]			
			LM	DNM	NM	O
Peel press cake	Hamlin	Control	N/A[2]	N/A	N/A	N/A
		Lime	N/A	N/A	N/A	N/A
	Parson Brown	Control	61±6.7	T[3]	T	T
		Lime	51±7.4	T	T	T
	Valencia	Control	124±3.0	T	T	T
		Lime	108±1.0	T	T	T
Peel press liquid	Hamlin	Control	N/A	N/A	N/A	N/A
		Lime	N/A	N/A	N/A	N/A
	Parson Brown	Control	7.0±2.2	T	T	T
		Lime	9.6±3.3	T	T	T
	Valencia	Control	15±0.8	T	T	T
		Lime	17±8.7	T	T	T

L = limonin, NM = nomilin, DNM = deacetylnomilin, O = obacunone, [1]N=2, Lime treatment resulted in significant decrease ($P \geq 0.05$) in limonin content in peel press cake and significant increase limonin content in press liquid ($P \geq 0.05$)., [2]Not available, [3]Trace

Table 40: Limonoid aglycone content in rag press cakes and press liquids (with and without lime treatment).

Sample	*Variety*	*Treatment*	mg/Kg rag (dried wt) ± %CV[1]			
			LM	DNM	NM	O
			41±6.0	T[2]	T	T

Table 40: Limonoid aglycone content in rag press cakes and press liquids (with and without lime treatment).

Sample	Variety	Treatment	mg/Kg rag (dried wt) ± %CV[1]			
			LM	DNM	NM	O
Rag press cake	Hamlin	Control	41±6.0	T[2]	T	T
		Lime	36±4.9	T	T	T
	Parson Brown	Control	208±4.3	T	T	T
		Lime	177±2.2	T	T	T
	Valencia	Control	179±2.3	T	T	T
		Lime	155±3.9	T	T	T
Rag press liquid	Hamlin	Control	6.1±1.3	T	T	T
		Lime	7.4±3.4	T	T	T
	Parson Brown	Control	13±3.8	T	T	T
		Lime	17±0.3	T	T	T
	Valencia	Control	13±8.0	T	T	T
		Lime	16±0.5	T	T	T

L = limonin, NM = nomilin, DNM = deacetylnomilin, O = obacunone, [1]N=2, Lime treatment resulted in significant decrease in limonin content ($P \geq 0.05$) in rag press cake and significant increase in limonin content ($P \leq 0.05$) in rag press liquid., [2]Trace

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(approximately 14% in both peel and rag) and to increase limonin content in press liquids (approximately 24% in peel and 22% in rag).

Table 38 presents the Brix (°Bx) values of press liquids from peel and rag. °Bx measurement is a rapid method commonly used to measure orange juice and molasses concentration (soluble solid content). °Bx values of press liquid were ~11 in rag and ~14 in peel. In feed mill operation, press liquids are evaporated to 72°Bx to produce molasses (the press liquid end products). Microbial spoilage is prevented by the low water activity in the molasses. The 72°Bx molasses is 5-6 times more concentrated than press liquids.

Since levels of limonin in press cakes were approximately 7 times higher than that in press liquids, press liquid may not be a direct source for limonin. However, the limonin concentration would be expected to be increased significantly in 72°Bx.

Comparison of the total limonin content from press cake and press liquid showed that there was significant lower ($P \leq 0.5$) total limonin concentration in lime-treated samples compared to controls (Table 41). Loss of total limonin was approximately 10% in peel and approximately 11% in rag. Thus, it can be concluded that lime treatment results in the degradation of limonin.

Table 42 and table 43 show limonoid glucoside concentrations in press cakes and press liquids of peel and rag with and without lime treatment. Limonin glucoside, nomilin glucoside, and nomilinic acid glucoside were detected in measurable quantity, with limonin glucoside being the primary compound.

Lime treatment resulted in a significant decrease ($P \leq 0.05$) in limonoid glucoside content in press cake and a significant increase ($P \leq 0.05$) in press liquid from both rag and peel samples. These results indicate that limonoid glucosides were released into

Table 41: Total limonoid aglycone content[1] in peels and rags (with and without lime treatment).

Sample	*Variety*	*Treatment*	mg/Kg peel or rag (dried wt)[1]			
			LM	DNM	NM	O
			N/A[2]	N/A	N/A	N/A

Table 41: Total limonoid aglycone content[1] in peels and rags (with and without lime treatment).

Sample	Variety	Treatment	mg/Kg peel or rag (dried wt)[1]			
			LM	DNM	NM	O
Peels	Hamlin	Control	N/A[2]	N/A	N/A	N/A
		Lime	N/A	N/A	N/A	N/A
	Parson Brown	Control	68	T[3]	T	T
		Lime	60	T	T	T
	Valencia	Control	138	T	T	T
		Lime	125	T	T	T
Rags	Hamlin	Control	221	T	T	T
		Lime	194	T	T	T
	Parson Brown	Control	192	T	T	T
		Lime	170	T	T	T
	Valencia	Control	47	T	T	T
		Lime	43	T	T	T

L = limonin, NM = nomilin, DNM = deacetylnomilin, O = obacunone, [1]N = 2, Lime treatment resulted in significant decrease ($P \leq 0.05$) in total limonin content in both peel and rag residues., [2]Not available, [3]Trace

Table 42: Limonoid glucoside content in peel press cakes and press liquids (with and without lime treatment).

Sample	Variety	Treatment	mg/Kg peel (dried wt) ± %CV[1]				
			LG	DNAG	NG	NAG	OG
			N/A[2]	N/A	N/A	N/A	N/A

Table 42: Limonoid glucoside content in peel press cakes and press liquids (with and without lime treatment).

Sample	Variety	Treatment	mg/Kg peel (dried wt) ± %CV[1]				
			LG	DNAG	NG	NAG	OG
Peel press cake	Hamlin	Control	N/A[2]	N/A	N/A	N/A	N/A
		Lime	N/A	N/A	N/A	N/A	N/A
	Parson Brown	Control	300±6.4	T[3]	114±3.5	131±1.8	T
		Lime	234±1.3	T	94±7.0	130±2.7	T
	Valencia	Control	568±0.0	T	190±1.4	179±19.7	T
		Lime	512±1.7	T	142±3.9	165±3.0	T
Peel press liquid	Hamlin	Control	N/A	N/A	N/A	N/A	N/A
		Lime	N/A	N/A	N/A	N/A	N/A
	Parson Brown	Control	218±1.7	T	69±0.8	87±0.7	T
		Lime	224±2.9	T	78±7.5	116±1.6	T
	Valencia	Control	314±3.5	T	114±1.3	133±2.8	T
		Lime	347±0.7	T	107±4.0	141±0.2	T

LG = limonin glucoside, DNAG = deacetylnomilinic acid glucoside, DNG = deacetylnomilin glucoside, NG = nomilin glucoside, NAG = nomilinic acid glucoside, OG = obacunone glucoside, [1]N=2, Lime treatment resulted in significant decrease ($P \leq 0.05$) in limonoid glucoside content in peel press cake, but significant increase ($P \leq 0.05$)in peel press liquid., [2]Not available, [3]Trace

Table 43: Limonoid glucoside content in rag press cakes and press liquids (with and without lime treatment).

Sample	*Variety*	*Treatment*	mg/Kg rag (dried wt) ± %CV[1]				
			LG	DNAG	NG	NAG	OG
				[2]	6312.8	2191.6	T

Table 43: Limonoid glucoside content in rag press cakes and press liquids (with and without lime treatment).

Sample	Variety	Treatment	mg/Kg rag (dried wt) ± %CV[1]				
			LG	DNAG	NG	NAG	OG
Rag press cake	Hamlin	Control	265±1.3	T[2]	63±2.8	219±1.6	T
		Lime	244±2.2	T	55±1.2	204±0.5	T
	Parson Brown	Control	390±0.3	T	187±0.1	301±2.6	T
		Lime	293±2.9	T	132±3.5	226±1.1	T
	Valencia	Control	442±1.4	T	98±2.2	362±0.8	T
		Lime	372±3.6	T	77±5.8	293±2.7	T
Rag press liquid	Hamlin	Control	183±1.7	T	35±2.6	147±1.3	T
		Lime	211±1.1	T	39±1.6	168±2.6	T
	Parson Brown	Control	123±2.8	T	53±2.1	100±1.3	T
		Lime	143±1.5	T	58±1.3	111±1.2	T
	Valencia	Control	155±0.6	T	28±2.8	123±0.0	T
		Lime	174±2.2	T	34±0.2	137±0.2	T

LG = limonin glucoside, DNAG = deacetylnomilinic acid glucoside, DNG = deacetylnomilin glucoside, NG = nomilin glucoside, NAG = nomilinic acid glucoside, OG = obacunone glucoside, [1]N=2, Lime treatment resulted in significant decrease ($P \leq 0.05$) in limonoid glucoside content in rag press cake, but significant increase ($P \leq 0.05$) in rag press liquid[3]., [2]Trace

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press liquid due to lime-treatment. Limonoid glucosides possess one or two carboxylate groups depending on the particular limonoid with approximate $pKa_1 = 2.7$ and $pKa_2 = 4.7$ [based on those reported for limonoic acid (USDA, 2003)]. Lime-treated samples had a pH 5.07-5.71, so that all of first carboxyl group and a significant fraction of the second group are ionic that results in increased compound solubility in aqueous solutions.

Comparison of the total limonoid glucoside content from press cake and press liquid showed that there was significant loss ($P \leq 0.5$) of total limonoid glucosides in lime-treated samples compared to controls (Table 44). Loss of total limonoid glucosides was approximately 5.2 % in peel and 8.6 % in rag. Thus, it can be concluded that lime treatment results in a small degradation of limonoid glucoside compounds.

Levels of limonoid glucosides in press liquids may be increased up to 5 times in 72°Bx molasses. Hasegawa et al. (1996) suggested that press liquids could be a good source for limonoid glucosides. Even though limonoid glucosides are reported to be stable through juice processing conditions (Hasegawa, 2000), evaluation of limonoid glucoside losses due to heat evaporation during molasses production has not been conducted.

Table 45 and Table 46 show polymethoxylated flavone concentrations in press cakes and press liquids of peel and rag with and without lime treatment. Polymethoxylated flavones detected were sinensitin, 3,5,6,7,3',4'-hexamethoxyflavones, nobiletin, 3,4,5,6,7,8,3',4'-heptamethoxyflavone, scutellareintetramethylether, and tangeretin. Sinensitin and nobiletin were the principal compounds.

Lime treatment effect on polymethoxylated flavone content was limited. A decrease in polymethoxylated flavone content (approximately 2.6%) in peel press cake

Table 44: Total limonoid glucoside content in peels and rags (with and without lime treatment).

Sample	Variety	Treatment	mg/Kg peel or rag (dried wt)[1]				
			LG	DNAG	NG	NAG	OG
			N/A[2]	N/A	N/A	N/A	N/A

Table 44: Total limonoid glucoside content in peels and rags (with and without lime treatment).

Sample	Variety	Treatment	mg/Kg peel or rag (dried wt)[1]				
			LG	DNAG	NG	NAG	OG
Peels	Hamlin	Control	N/A[2]	N/A	N/A	N/A	N/A
		Lime	N/A	N/A	N/A	N/A	N/A
	Parson Brown	Control	519	T[3]	183	218	T
		Lime	458	T	172	245	T
	Valencia	Control	882	T	303	312	T
		Lime	860	T	249	306	T
Rags	Hamlin	Control	513	T	241	401	T
		Lime	437	T	190	337	T
	Parson Brown	Control	598	T	127	486	T
		Lime	546	T	111	431	T
	Valencia	Control	449	T	98	367	T
		Lime	454	T	94	372	T

LG = limonin glucoside, DNAG = deacetylnomilinic acid glucoside, DNG = deacetylnomilin glucoside, NG = nomilin glucoside, NAG = nomilinic acid glucoside, OG = obacunone glucoside, [1]N = 2, Lime treatment resulted in significant reduction ($P \leq 0.05$) in total limonoid glucoside content in both peel and rag residues., [2]Not available, [3]Trace

Table 45: Polymethoxylatedflavone content in peel press cakes and press liquids (with and without lime treatment).

Sample	Variety	Treatment	mg/Kg peel (dried wt) + %CV[1]					
			ST	HX	NBT	HP	STME	TT
					N/A	N/A	N/A	N/A

Table 45: Polymethoxylatedflavone content in peel press cakes and press liquids (with and without lime treatment).

Sample	Variety	Treatment	mg/Kg peel (dried wt) ± %CV[1]					
			ST	HX	NBT	HP	STME	TT
Peel press cake	Hamlin	Control	N/A[2]	N/A	N/A	N/A	N/A	N/A
		Lime	N/A	N/A	N/A	N/A	N/A	N/A
	Parson Brown	Control	106±1.1	26±1.3	128±0.7	51±0.5	50±0.6	20±0.9
		Lime	101±0.1	24±0.4	124±0.1	53±0.1	49±0.2	21±0.7
	Valencia	Control	164±4.2	32±4.6	192±4.4	88±4.7	65±4.5	35±5.0
		Lime	154±0.3	30±0.1	193±0.8	84±1.0	65±0.4	35±0.6
Peel press liquid	Hamlin	Control	N/A	N/A	N/A	N/A	N/A	N/A
		Lime	N/A	N/A	N/A	N/A	N/A	N/A
	Parson Brown	Control	38±1.4	8±1.2	34±1.8	9±1.3	10±1.3	2.2±0.7
		Lime	39±0.1	8±1.4	34±0.1	10±0.2	10±0.2	2.2±1.1
	Valencia	Control	58±1.1	10±1.3	50±1.0	16±0.8	12±0.9	3.7±0.3
		Lime	55±7.1	9±7.2	49±7.1	15±7.1	12±7.5	3.4±7.5

ST = sinensitin, HX = 3,5,6,7,3',4'-hexamethoxyflavone, NBT = nobiletin, HP = 3,4,5,6,7,8,3',4'-heptamethoxyflavone, STME = scutellarein tetramethylether, TT = tangeretin, [1]N=2, Lime treatment resulted in significant decrease ($P \leq 0.05$) in polymethoxylated flavone content in peel press cake, but no significant difference ($P \geq 0.05$) on polymethoxylated flavones in peel press liquid., [2]Not available

Table 46: Polymethoxylatedflavone content in rag press cakes and press liquids (with and without lime treatment).

Sample	*Variety*	*Treatment*	mg/Kg rag (dried wt) + %CV[1]					
			ST	HX	NBT	HP	STME	TT
			0.04±4.0	0.27±4.9	1.91±1.0	0.83±1.0	1.07±1.4	0.51±0.3

Table 46: Polymethoxylatedflavone content in rag press cakes and press liquids (with and without lime treatment).

Sample	Variety	Treatment	mg/Kg rag (dried wt) ± %CV[1]					
			ST	HX	NBT	HP	STME	TT
Rag press cake	Hamlin	Control	0.94±4.0	0.27±4.9	1.91±1.0	0.83±1.0	1.07±1.4	0.51±0.3
		Lime	0.81±1.8	0.24±13.8	1.70±2.7	0.67±7.1	0.94±0.5	0.40±3.3
	Parson Brown	Control	2.21±6.3	0.67±6.1	3.43±6.4	1.95±8.7	1.67±5.8	0.86±18.0
		Lime	2.06±0.9	0.64±7.7	3.33±4.5	1.79±6.6	1.64±2.8	0.82±11.1
	Valencia	Control	3.77±4.7	1.14±0.2	6.53±5.2	4.25±3.9	3.28±3.9	1.94±7.2
		Lime	3.88±5.5	1.25±4.8	6.64±4.5	4.32±5.3	3.30±3.5	2.27±18.5
Rag press liquid	Hamlin	Control	0.13±9.0	0.04±15.3	0.26±7.3	0.07±8.6	0.13±13.7	0.03±0.5
		Lime	0.15±2.9	0.04±1.6	0.27±3.1	0.07±9.3	0.13±7.8	0.04±3.4
	Parson Brown	Control	0.16±15.4	0.05±16.1	0.21±16.9	0.06±27.4	0.11±16.2	0.03±35.8
		Lime	0.19±2.2	0.06±0.2	0.25±3.5	0.07±16.6	0.13±5.7	0.04±31.8
	Valencia	Control	0.22±5.0	0.07±5.2	0.32±4.6	0.09±0.7	0.15±5.3	0.03±1.5
		Lime	0.26±1.1	0.08±8.5	0.35±1.1	0.10±0.3	0.17±3.8	0.04±2.2

ST = sinensitin, HX = 3,5,6,7,3',4'-hexamethoxyflavone, NBT = nobiletin, HP = 3,4,5,6,7,8,3',4'-heptamethoxyflavone, STME = scutellarein tetramethylether, TT = tangeretin, [1]N=2, Lime treatment had no effects ($P \geq 0.05$) on polymethoxylated flavone content in rag press cake, but resulted in significant increase in rag press liquid.

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and a small increase in polymethoxylated flavone content (approximately 13%) in rag press liquid were statistically detected ($P \leq 0.05$). Similar to limonoid aglycones, these polymethoxylated flavones have a tendency to remain in press cake since they are not water soluble. The polymethoxylated flavones were minimally leached into press liquid during pressing process. The concentration of polymethoxylatedflavones would be expected to be much greater in molasses. There was a small degradation ($P \leq 0.5$) of total polymethoxylated flavones due to lime treatment (Table 47). Loss of total polymethoxylated flavones was approximately 2.4 % in peel and 3.7 % in rag.

Table 48 and Table 49 show flavanone glucoside concentrations in press cakes and press liquids of peel and rag with and without lime treatment. Flavanone glucosides detected in rag and peel samples included narirutin-4'-glucoside, eriocitrin, narirutin, hesperidin, and didymin, with hesperidin being the major compound.

The effect of lime treatment on flavanone glucoside content was minimal. A significant increase ($P \leq 0.05$) in flavanone glucoside content due to lime treatment was observed only in rag press liquid (approximately 19%).

Peel is a better source for flavanone glucosides compared to rag and seed, and press cake contained higher flavanone glucoside content than press liquid. It was observed that hesperidin was approximately 48 times higher in peel press cake than in peel press liquid. Its concentration may be greatly increased in molasses. However hesperidin has a limited solubility and with concentration of the press liquid to produce molasses, hesperidin would become saturated and the hesperidin would be expected to precipitate.

Table 47: Total polymethoxylated flavone content in peels and rags (with and without lime treatment).

Sample	*Variety*	*Treatment*	mg/Kg peel or rag (dried wt)[1]					
			ST	HX	NBT	HP	STME	TT
			N/A[2]	N/A	N/A	N/A	N/A	N/A

Table 47: Total polymethoxylated flavone content in peels and rags (with and without lime treatment).

Sample	Variety	Treatment	mg/Kg peel or rag (dried wt)[1]					
			ST	HX	NBT	HP	STME	TT
Peels	Hamlin	Control	N/A[2]	N/A	N/A	N/A	N/A	N/A
		Lime	N/A	N/A	N/A	N/A	N/A	N/A
	Parson Brown	Control	144	33.8	162	61	60	22
		Lime	140	32.0	159	62	59	24
	Valencia	Control	222	42.3	243	105	77	39
		Lime	208	39.01	242	100	77	38
Rags	Hamlin	Control	2.4	0.7	3.6	2.0	1.8	0.9
		Lime	2.2	0.7	3.6	1.9	1.8	0.9
	Parson Brown	Control	4.0	1.2	6.8	4.3	3.4	2.0
		Lime	4.1	1.3	7.0	4.4	3.4	2.3
	Valencia	Control	1.1	0.3	2.2	0.9	1.2	0.5
		Lime	1.0	0.3	2.0	0.7	1.1	0.4

ST = sinensitin, HX = 3,5,6,7,3',4'-hexamethoxyflavone, NBT = nobiletin, HP = 3,4,5,6,7,8,3',4'-heptamethoxyflavone, STME = scutellarein tetramethylether, TT = tangeretin, [1]Lime treatment resulted in significant reduction ($P \leq 0.05$) in total polymethoxylated flavone content in peel and rag residues., [2]Not available

Table 48: Flavanone glucoside content in peel press cakes and press liquids (with and without lime treatment).

Sample	Variety	Treatment	mg/Kg peel (dried wt) ± %CV[1]				
			NT4G	ERT	NT	HD	DD
		Control	N/A[2]	N/A	N/A	N/A	N/A

Table 48: Flavanone glucoside content in peel press cakes and press liquids (with and without lime treatment).

Sample	Variety	Treatment	mg/Kg peel (dried wt) ± %CV[1]				
			NT4G	ERT	NT	HD	DD
Peel press cake	Hamlin	Control	N/A[2]	N/A	N/A	N/A	N/A
		Lime	N/A	N/A	N/A	N/A	N/A
	Parson Brown	Control	110±3.9	131±3.0	293±1.5	3751±2.3	203±2.5
		Lime	98±0.9	122±1.0	291±0.4	3821±0.0	200±0.2
	Valencia	Control	99±2.2	91±0.6	335±0.1	3647±0.2	195±1.3
		Lime	87±0.1	105±0.4	305±0.3	4017±0.2	192±0.4
Peel press liquid	Hamlin	Control	N/A	N/A	N/A	N/A	N/A
		Lime	N/A	N/A	N/A	N/A	N/A
	Parson Brown	Control	22±6.7	17±8.5	44±8.1	84±10.7	7.7±12.2
		Lime	24±3.5	21±0.4	50±0.5	91±0.5	8.3±0.0
	Valencia	Control	25±1.9	13±0.3	59±0.8	63±0.5	11.0±0.6
		Lime	24±2.8	19±0.8	56±3.1	81±2.9	8.6±1.5

NT-4'-G = narirutin-4'-glucoside, ERT = eriocitrin, NT = narirutin, HD = hesperidin, DD = didymin, [1]N=2, Lime treatment had no effect ($P \geq 0.05$) on limonin content in both peel press cake and press liquid., [2]Not available

Table 49: Flavanone glucoside content in rag press cakes and press liquids (with and without lime treatment).

Sample	Variety	Treatment	mg/Kg rag (dried wt) ± %CV[1]				
			NT4G	ERT	NT	HD	DD
				49±2.4	303±0.5	1935±0.3	166±0.2

Table 49: Flavanone glucoside content in rag press cakes and press liquids (with and without lime treatment).

Sample	Variety	Treatment	mg/Kg rag (dried wt) ± %CV[1]				
			NT4G	ERT	NT	HD	DD
Rag press cake	Hamlin	Control	79±0.7	40±2.1	303±0.5	1935±0.3	166±0.2
		Lime	65±2.2	39±0.2	277±0.2	1800±0.4	154±1.3
	Parson Brown	Control	57±1.1	35±1.4	317±0.0	1819±0.5	183±0.6
		Lime	46±0.1	36±1.1	288±0.7	1804±0.6	176±0.2
	Valencia	Control	70±1.9	31±0.5	304±0.9	1908±1.2	166±0.0
		Lime	59±2.5	33±0.0	296±0.9	1953±0.9	171±0.6
Peel press liquid	Hamlin	Control	22±3.4	9.5±0.7	67±0.5	43±0.2	8.2±0.2
		Lime	26±4.0	13±2.1	83±0.0	60±0.8	10±0.4
	Parson Brown	Control	5.9±1.7	4.5±1.0	38±2.7	32±2.9	5.7±2.4
		Lime	6.8±0.1	7.1±2.3	47±0.8	33±0.5	6.6±0.8
	Valencia	Control	12±1.4	4.0±1.3	34±0.5	21±0.8	4.4±0.4
		Lime	11±0.7	5.7±0.5	40±0.1	21±1.0	4.8±0.3

NT-4'-G = narirutin-4'-glucoside, ERT = eriocitrin, NT = narirutin, HD = hesperidin, DD = didymin, [1]N=2, Lime treatment effects on flavanone glucoside content in rag press cake were not significant ($P \geq 0.05$), however the treatment resulted in significant increase ($P \leq 0.05$) in flavanone glucoside content in rag press liquid.

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Table 50 presents the total flavanone glucoside content from press cake and press liquid in peel and rag residues with and without lime treatment. There were no significant effects of lime treatment ($P \leq 0.5$) on the total flavonoid glucosides in either peel or rag residues. Degradation of flavanone glucosides due to lime treatment is not significant.

Table 51 Table 54 show distributions of limonoids and flavonoids in orange seeds with and without lime treatment. Since seed is a part of waste residue and may not be homogeneously distributed within the rag fraction, seeds were studied independently. Influence of lime treatment was studied without pressing process, as it already had relatively low moisture content. Lime treatment resulted in a significant decrease ($P \leq 0.05$) in limonoid glucoside content in seed, while no effects ($P \geq 0.05$) were observed on the concentrations of other compounds (limonoid aglycones, polymethoxylated flavones, and flavanone glucosides).

It should be noted that seeds are a particularly rich source of limonoid aglycones and glucosides. The data presented in Table 51 and Table 52 are expressed as g/100g while the data in most tables are expressed as mg/Kg. However, when considering total orange waste produced, seeds account for only 0.5-1% of the fruit (wet wt.) (Braddock, 1999), while peels account for almost 50% (wet wt.) (Braddock, 1995).

5. Conclusion

Analyses of limonoids and flavonoids in press cake and press liquid (peel and rag) demonstrated that lime treatment did not appreciably alter their content in citrus waste. However, when the extract from both rag and peel were analyzed, there were significant losses of limonoid aglycones (~11% in peel and rag), limonoid glucosides (~5% in peel

Table 50: Total flavanone glucoside content in peels and rags (with and without lime treatment).

Sample	Variety	Treatment	mg/Kg peel or rag (dried wt)[1]				
			NT4G	ERT	NT	HD	DD
			N/A	N/A	N/A	N/A	N/A

Table 50: Total flavanone glucoside content in peels and rags (with and without lime treatment).

Sample	Variety	Treatment	mg/Kg peel or rag (dried wt)[1]				
			NT4G	ERT	NT	HD	DD
Peels	Hamlin	Control	N/A[2]	N/A	N/A	N/A	N/A
		Lime	N/A	N/A	N/A	N/A	N/A
	Parson Brown	Control	133	45	337	3835	211
		Lime	123	34	340	3912	208
	Valencia	Control	124	32	394	3710	206
		Lime	111	29	362	4098	201
Rags	Hamlin	Control	63	52	354	1851	189
		Lime	53	43	336	1837	183
	Parson Brown	Control	82	42	339	1929	170
		Lime	70	38	336	1974	176
	Valencia	Control	101	58	369	1979	174
		Lime	91	54	360	1860	164

NT-4'-G = narirutin-4'-glucoside, ERT = eriocitrin, NT = narirutin, HD = hesperidin, DD = didymin, [1]Lime treatment had no effect ($P \geq 0.05$) on total flavanone glucoside content in peel and rag residues., [2]Not available

Table 51: Limonoid aglycone content in seeds (with and without lime treatment).

Variety	Treatment	g/100g + %CV[1]			
		L	DNM	NM	O
			0.19±0.1	0.09±4.4	0.09±3.5

Table 51: Limonoid aglycone content in seeds (with and without lime treatment).

Variety	Treatment	g/100g ± %CV[1]			
		L	DNM	NM	O
Hamlin	Control	1.58±6.0	0.19±0.1	0.09±4.4	0.09±3.5
	Limed	1.50±2.9	0.16±2.0	0.06±1.5	0.05±4.7
Parson Brown	Control	1.64±3.4	0.20±5.1	0.10±6.0	0.08±0.8
	Limed	1.62±1.6	0.20±1.5	0.10±1.2	0.11±4.5
Valencia	Control	1.83±1.2	0.15±0.3	0.08±1.5	0.07±6.8
	Limed	1.78±0.6	0.19±5.5	0.11±4.7	0.11±1.4

L = limonin, NM = nomilin, DNM = deacetylnomilin, O = obacunone, [1]N=2, Lime treatment effect on limonoid aglycone content in seed was not significant ($P \geq 0.05$).

Table 52: Limonoid glucoside content in seeds (with and without lime treatment).

Variety	Treatment	g/100g ± %CV[1]					
		LG	DNAG	DNG	NG	NAG	OG
Hamlin	Control	0.56±0.3	0.26±1.5	T[2]	0.46±2.3	0.48±2.0	0.49±3.4
	Limed	0.49±1.2	0.22±0.9	T	0.40±0.1	0.38±1.5	0.48±0.5
Parson Brown	Control	0.64±0.3	0.23±3.5	T	0.73±1.3	0.49±4.1	0.60±4.1
	Limed	0.59±0.6	0.21±0.4	T	0.71±0.0	0.45±2.0	0.55±0.6
Valencia	Control	0.69±1.8	0.23±0.4	T	0.53±0.9	0.49±2.1	0.56±2.4
	Limed	0.70±0.8	0.19±0.0	T	0.63±1.0	0.44±2.0	0.53±0.5

LG = limonin glucoside, DNAG = deacetylnomilinic acid glucoside, DNG = deacetylnomilin glucoside, NG = nomilin glucoside, NAG = nomilinic acid glucoside, OG = obacunone glucoside, [1]N=2, Lime treatment resulted in decreased limonoid glucoside content in seed ($P \leq 0.05$)., [2]Trace

Table 53: Polymethoxylatedflavone content in seeds (with and without lime treatment).

Variety	Treatment	mg/Kg	%CV				
		ST	HX	NBT	HP	STME	TT
					0.5	8.2	0.6	8.5	

Table 53: Polymethoxylatedflavone content in seeds (with and without lime treatment).

Variety	Treatment	mg/Kg± %CV[1]					
		ST	HX	NBT	HP	STME	TT
Hamlin	Control	1.1±5.3	0.6±17.7	1.5±20.3	0.5±8.2	0.6±8.5	T[2]
	Lime	0.9±4.3	0.4±11.8	1.1±1.0	0.4±9.1	0.4±11.3	T
Parson Brown	Control	7.7±1.9	1.8±11.4	8.9±12.3	4.0±0.26	4.3±4.9	1.3±6.5
	Lime	7.1±1.5	2.1±2.5	9.8±1.8	3.8±1.8	4.2±0.2	1.6±0.5
Valencia	Control	4.2±3.9	1.3±16.9	5.2±0.7	2.1±5.7	2.3±6.0	0.5±5.9
	Lime	4.5±6.5	1.5±14.2	5.8±4.1	2.1±4.4	2.6±3.3	0.5±13.8

ST = sinensitin, HX = 3,5,6,7,3',4'-hexamethoxyflavone, NBT = nobiletin, HP = 3,4,5,6,7,8,3',4'-heptamethoxyflavone, STME = scutellarein tetramethylether, TT = tangeretin, [1]N=2, Lime treatment effect on polymethoxylated flavone content in seed was not significant ($P \geq 0.05$)., [2]Trace

Table 54: Flavanone glucoside content in seeds (with and without lime treatment).

Variety	Treatment	mg/Kg± %CV[1]				
		NT4G	ERT	NT	HD	DD
Hamlin	Control	46±0.2	28±0.8	493±0.5	288±4.1	71±0.6
	Lime	26±4.8	22±1.9	367±1.7	212±1.1	56±1.9
Parson Brown	Control	28±1.9	18±1.8	259±0.1	237±3.3	48±6.0
	Lime	24±3.1	19±0.5	241±1.7	185±1.4	45±3.1
Valencia	Control	31±2.5	15±6.3	276±2.1	251±0.2	46±2.1
	Lime	34±0.4	19±6.6	321±1.9	266±2.1	55±2.6

NT-4'-G = narirutin-4'-glucoside, ERT = eriocitrin, NT = narirutin, HD = hesperidin, DD = didymin, [1]N=2, Lime treatment effect on flavanone glucoside content in seed was not significant ($P \geq 0.05$).

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and ~13% in rag), and polymethoxylated flavones (~9% in peel and ~3% in rag) due to lime treatment.

Lime treatment had the greatest effect on limonoid glucosides. It was demonstrated that lime treatment resulted in a significant migration of limonoid glucosides from solid wastes in to press liquids.

Lime treatment resulted in loss of limonoid glucosides in seeds, but lime treatment did not change limonoid aglycone, polymethoxylated flavone, flavonoid glucoside of seeds.

The extent of compound leaching from pressed solids to pressed liquids during pressing process depends on the nature of the compounds (such as solubility) and physical characteristics of raw materials (such as surface area).

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RESEARCH CONCLUSION

Study I: Analytical methodology suitable for isolation and quantitation of limonoids and flavonoids in sweet orange

- Limonoid aglycones and polymethoxylated flavones had similar chromatographic retention.
- Limonoid glucosides and flavanone glucosides had similar chromatographic retention.
- Screenings of compounds from different orange fractions found four unknowns with potential to be deacetyl nomilin glucoside, nomilin glucoside, narirutin-4'-glucoside, and 3,5,6,7,3',4'-hexamethoxyflavone.
- Extraction of polymethoxylated flavones and limonoid aglycones was improved by heating (82°C for 30 min).
- 70% methanol in water was suitable for limonoid glucoside extraction.
- Extraction of flavanone glucosides required dimethylformamide.

Study II: Isolation and identification of selected limonoids and flavonoids

- Preliminary extractions and purifications of orange seeds and peels provided a simpler and more concentrated extract for purification of unknowns by analytical-scale HPLC.
- Based on UV, MS, and NMR spectra, four unknowns were identified to be deacetyl nomilin glucoside, nomilin glucoside, narirutin-4'-glucoside, and 3,5,6,7,3',4'-hexamethoxyflavone

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Study III: Distributions of limonoids and flavonoids in edible and inedible fractions of sweet oranges (*Citrus sinensis*)

- Flavanone glucoses were the predominant phytochemicals, followed by limonoid glucosides, limonoid aglycones, and polymethoxylated flavones.
- Valencia had highest phytochemical content, except for flavanone glucoside content which was highest in Hamlin.
- The results show that rags containing seeds are a good source for limonoid aglycones and limonoid glucosides, while peel and peel press cake are good sources for flavanone glucosides and polymethoxylated flavones.
- Peel press liquid is a potential source for limonoid glucosides and polymethoxylated flavones after evaporation to the molasses end-product.
- Orange juice is a good source of limonoid glucosides.

Study IV: Effect of lime treatment on of limonoid and flavonoid content in by-products from orange juice process

- With lime treatment, more limonoid aglycones (25%) and limonoid glucosides (12%) leached from press cake into press liquid (both in rag and peel).
- There was a trend showing increased phytochemical content were released from press cakes into press liquids due to lime treatment.
- In seed, lime treatment (0.3% CaO wet wt.) resulted in loss of limonoid glucosides, but had no effect on limonoid aglycone, flavanone glucoside and polymethoxylated flavone content.
- The results suggested that lime treatment resulted in increased phytochemical content in press liquid especially limonoids.

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RECOMMENDATIONS FOR FUTURE RESEARCH

- In this research the lime treatment study was conducted using three sweet orange varieties to represent the whole orange population and lime treatment. Effects on phytochemical content were analyzed using paired t-test to compare between lime-treated samples and controls, since limited samples were available. It would be more statistically meaningful to employ either 1) randomize complete block design [(3 blocks (3 varieties) and 2 factor (lime treatment and orange fractions] to remove the effects of variety in order to make the effects of lime and orange fraction more apparent, or 2) factorial design (3 varieties X 2 lime treatments X 2 waste fractions) to obtained the influence of variety, lime treatment, waste fraction, and their interaction.
- Addition of lime to citrus waste is limited, because the lime-treated waste is primarily used for the animal feed. However to improve recovery of limonoids and flavonoids in press liquid, higher lime concentrations, more effective mixing between lime and waste materials (continuous mixing and application of smaller size of waste material) and different types of lime could be studied.
- Orange-byproducts are rich sources of flavonoids and limonoids but isolation of these compounds are very expensive. It may be warranted to conduct the absorption study (*in vitro* or *in vivo*) using orange by-products directly such as freeze-dried peel powder, freeze-dried rag-with-seed powder, or spray-dried orange molasses. Direct application of these wastes would be greatly profitable to citrus industry.

APPENDICES

APPENDIX I

Screening of limonoids and flavonoids in different orange fractions.

340 nm

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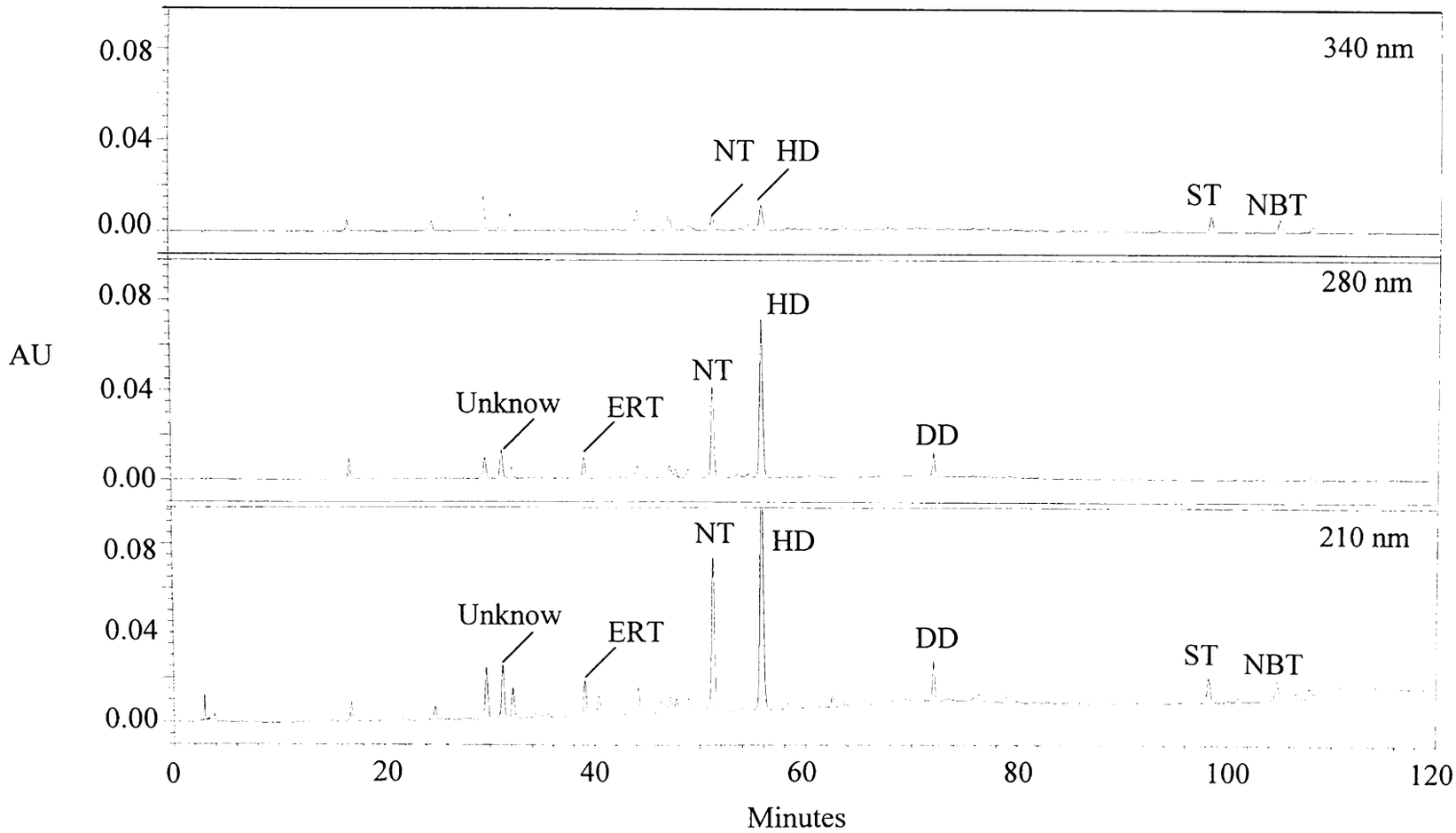
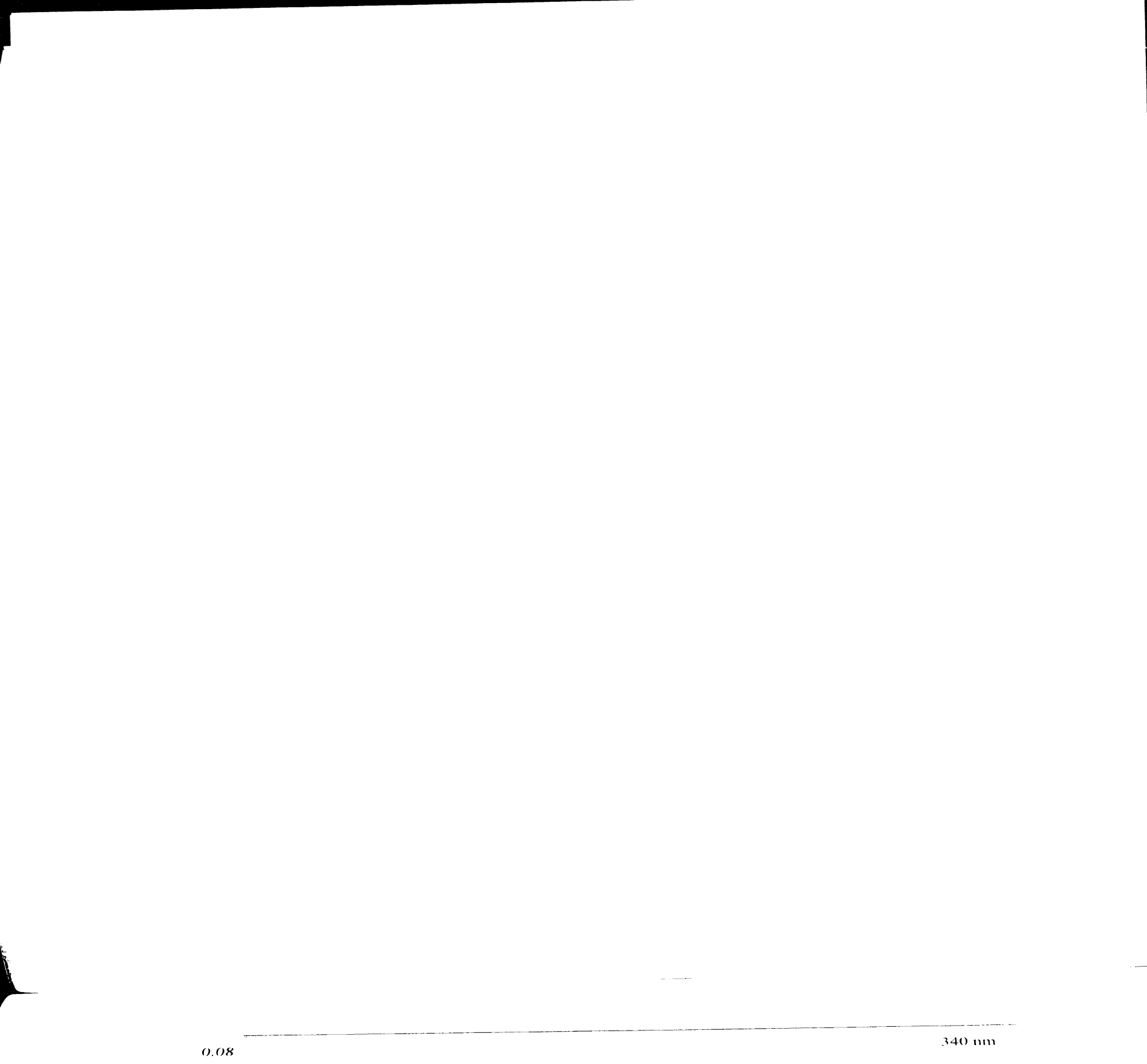


Figure 49: Polar compounds (reconstituted in 10% acetonitrile in 3mM phosphoric acid) in peel press cake extract at 340 nm (polymethoxylated flavones), 280 nm (flavanone glucosides), and 210 nm (limonoids). *ERT = eriocitrin, NT = narirutin, HD = hesperidin, DD = didymin, ST = sinensitin, NBT = nobiletin.*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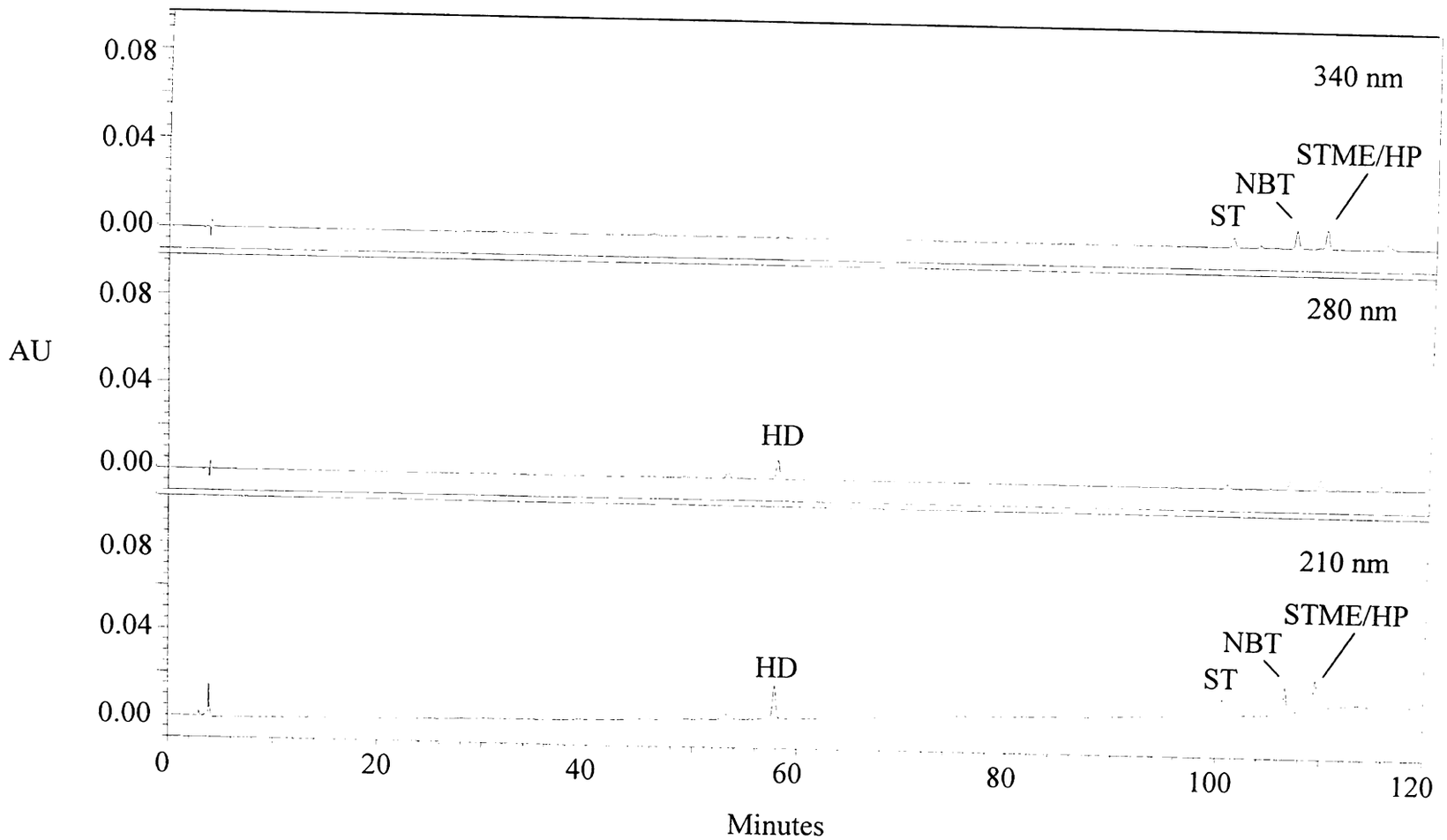

Figure 50: Nonpolar compounds (reconstituted in 100% acetonitrile) in peel press cake extract at 340 nm (polymethoxylated flavones), 280 nm (flavanone glucosides) and 210 nm (limonoids). *HD = hesperidin, ST = sinensitin, NBT = nobiletin HP = 3,4,5,6,7,8,3',4'- heptamethoxyflavone, STME = scutellarein tetramethylether.*

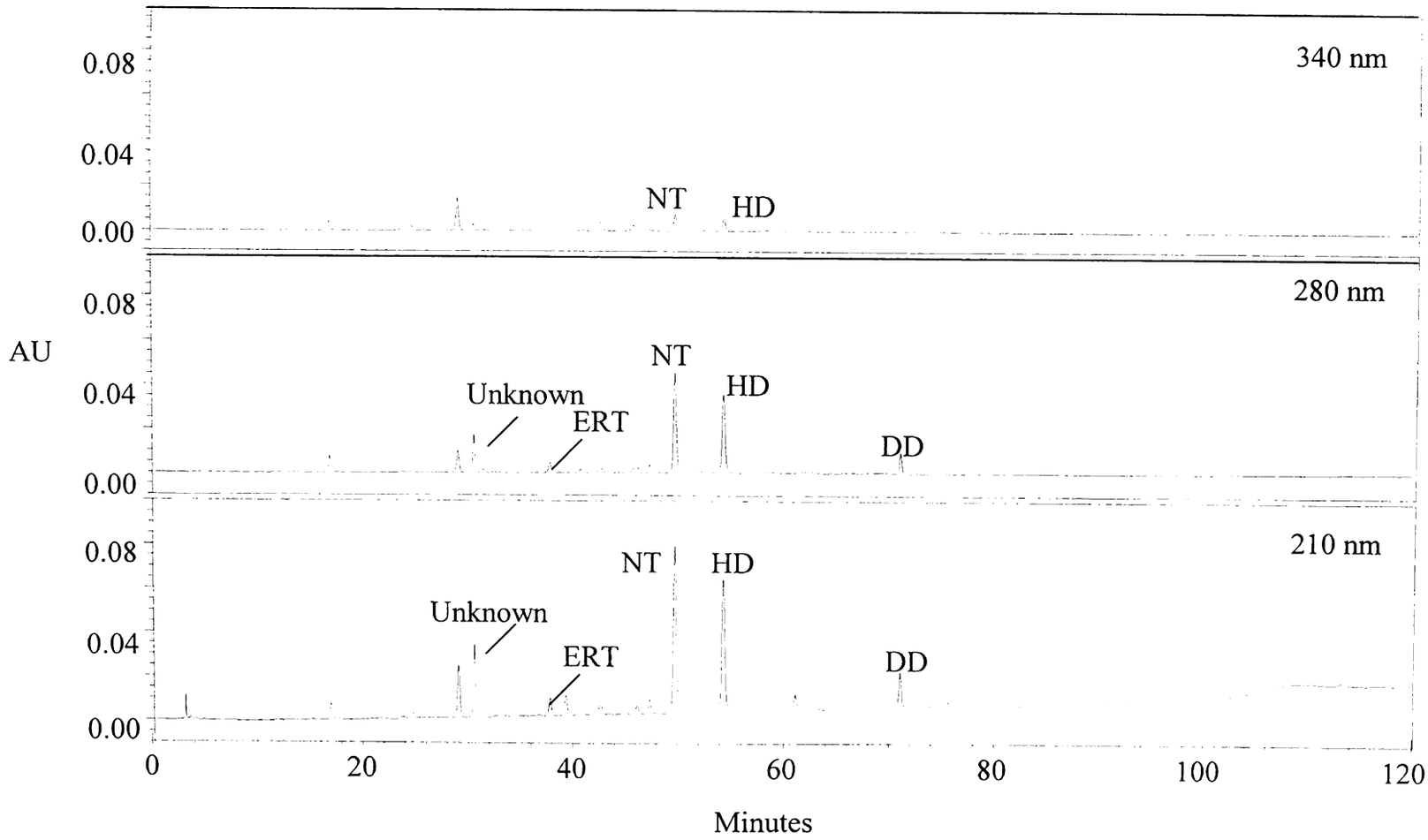

Figure 51: Polar compounds (reconstituted in 10% acetonitrile in 3mM phosphoric acid) in rag extract at 340 nm (polymethoxylated flavones), 280 nm (flavanone glucosides), and 210 nm (limonoids). *ERT = eriocitrin, NT = narirutin, HD = hesperidin, DD = didymin.*.

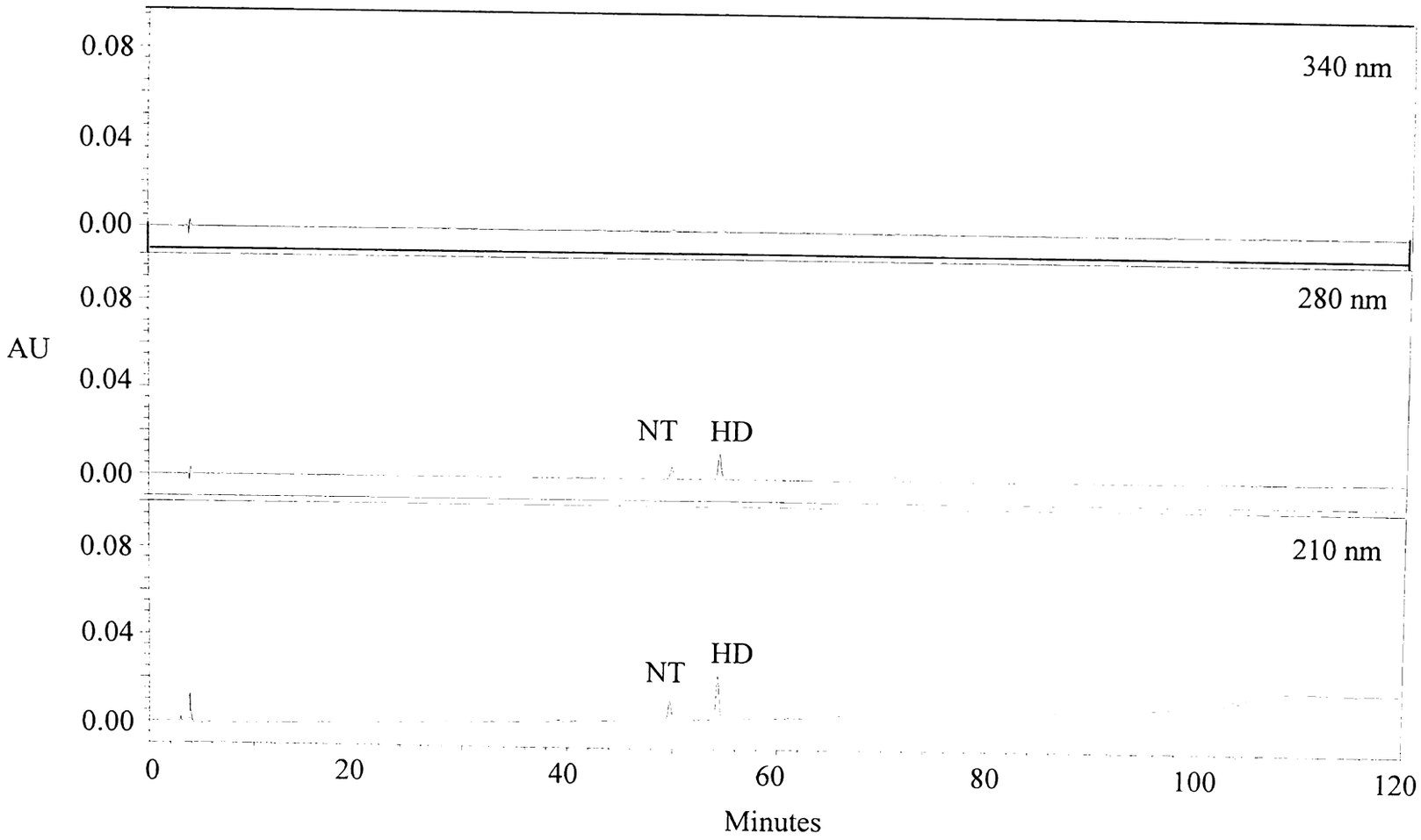

Figure 52: Nonpolar compounds (reconstituted in 100% acetonitrile) in rag extract at 340 nm (polymethoxylated flavones), 280 nm (flavanone glucosides), and 210 nm (limonoids). *NT = narirutin, HD = hesperidin.*

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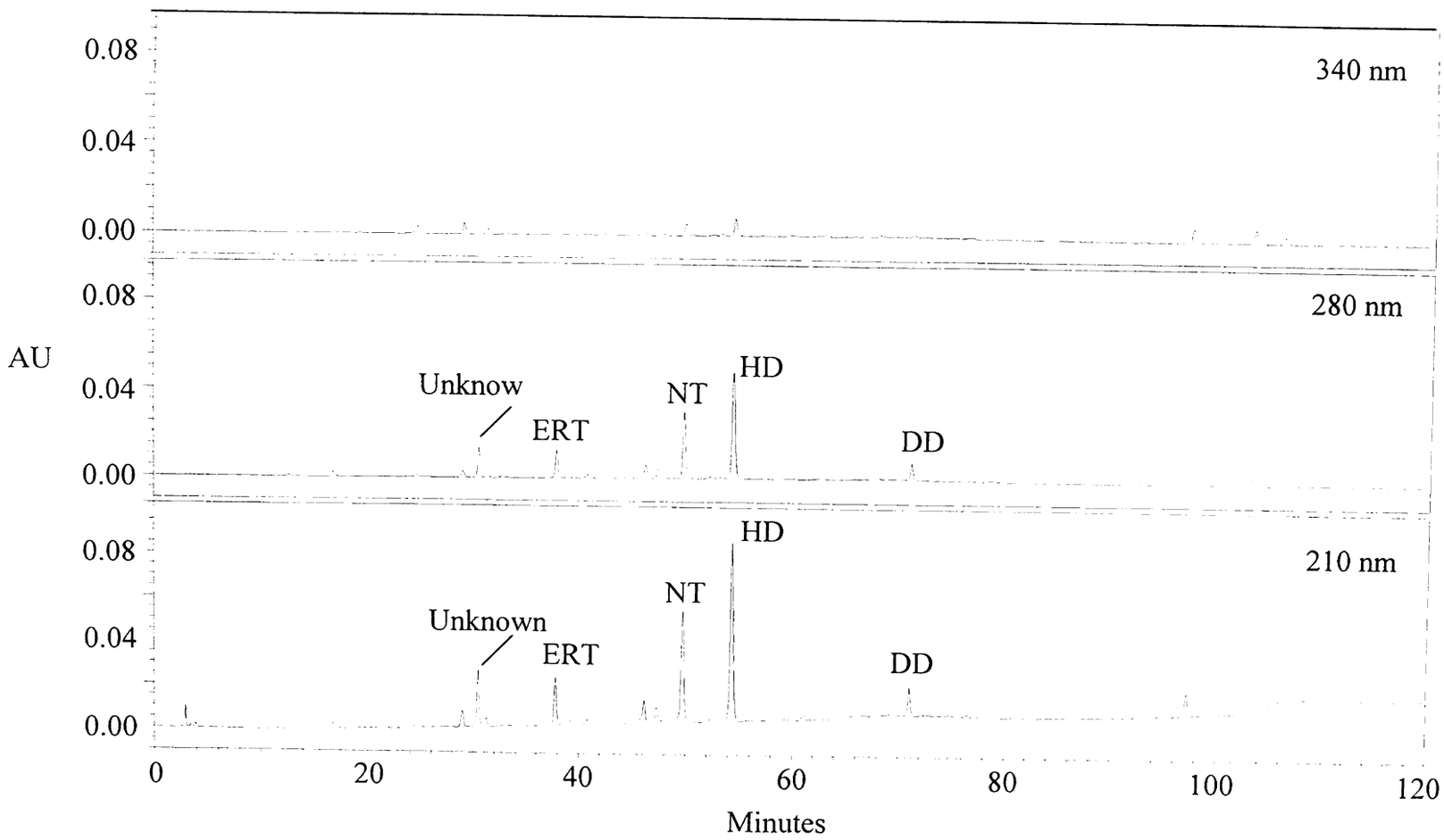
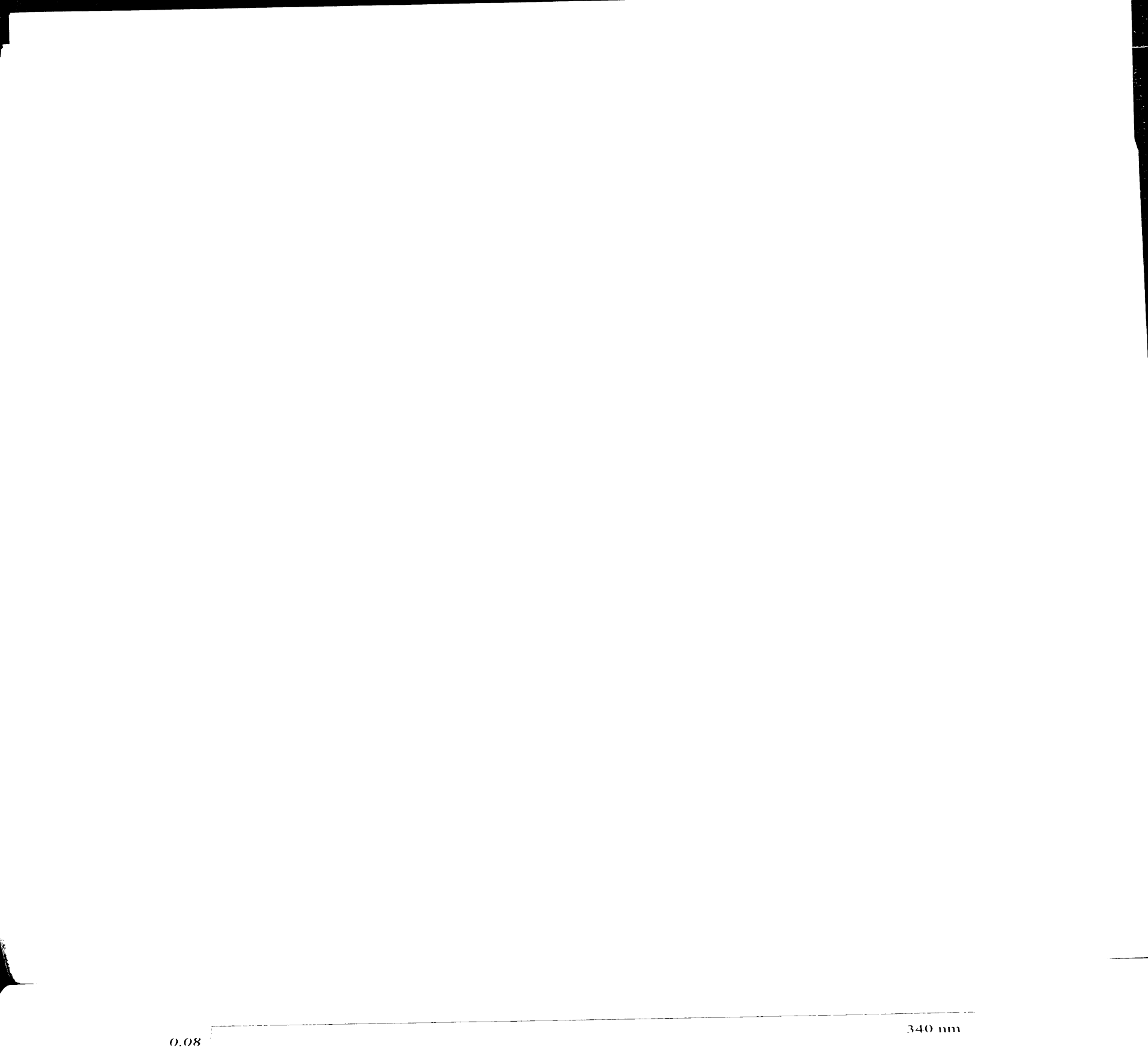


Figure 53: Polar compounds (reconstituted in 10% acetonitrile in 3mM phosphoric acid) in peel press liquid extract at 340 nm (polymethoxylated flavones), 280 nm (flavanone glucosides), and 210 nm (limonoids). *ERT = eriocitrin, NT = narirutin, HD = hesperidin, DD = didymin.*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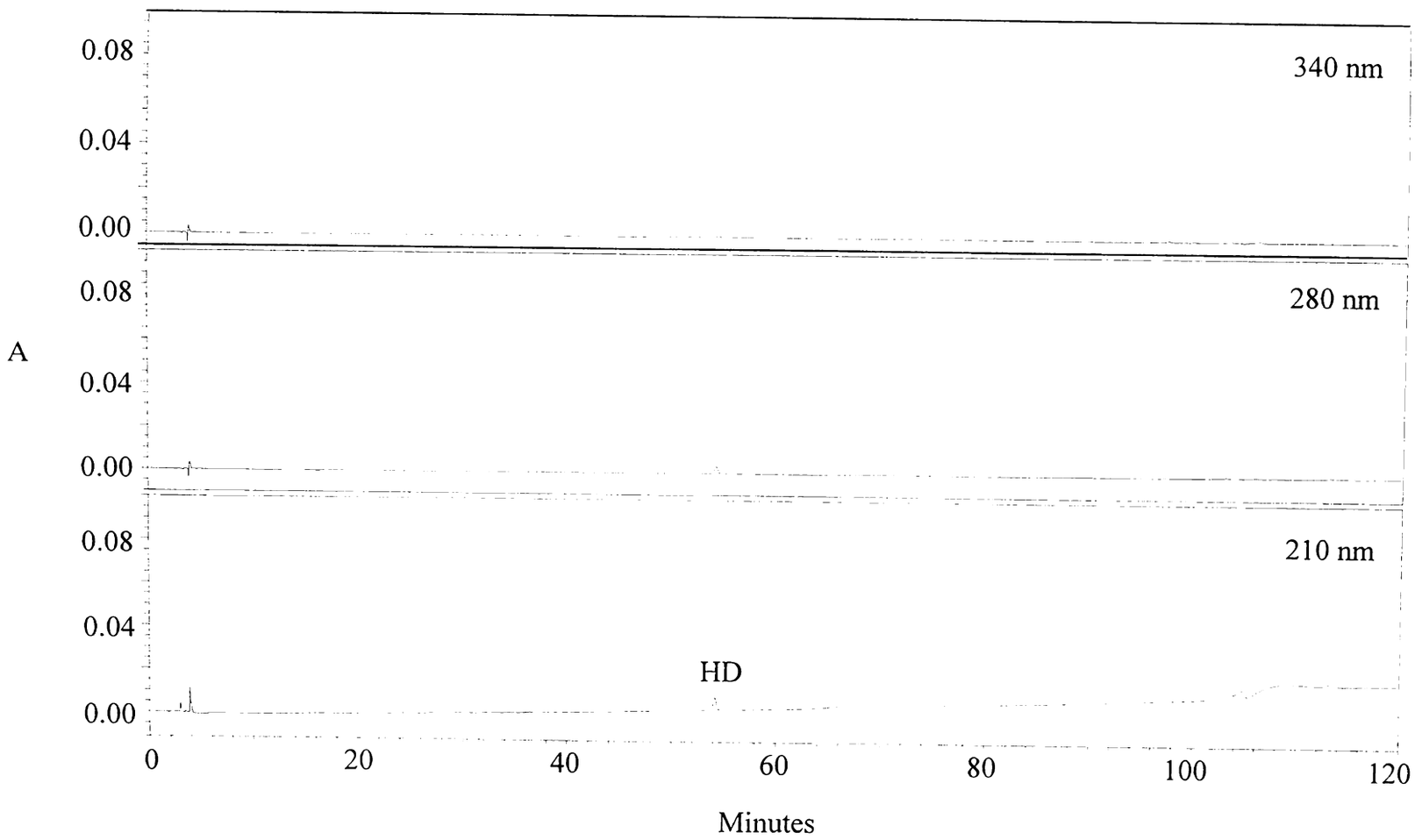
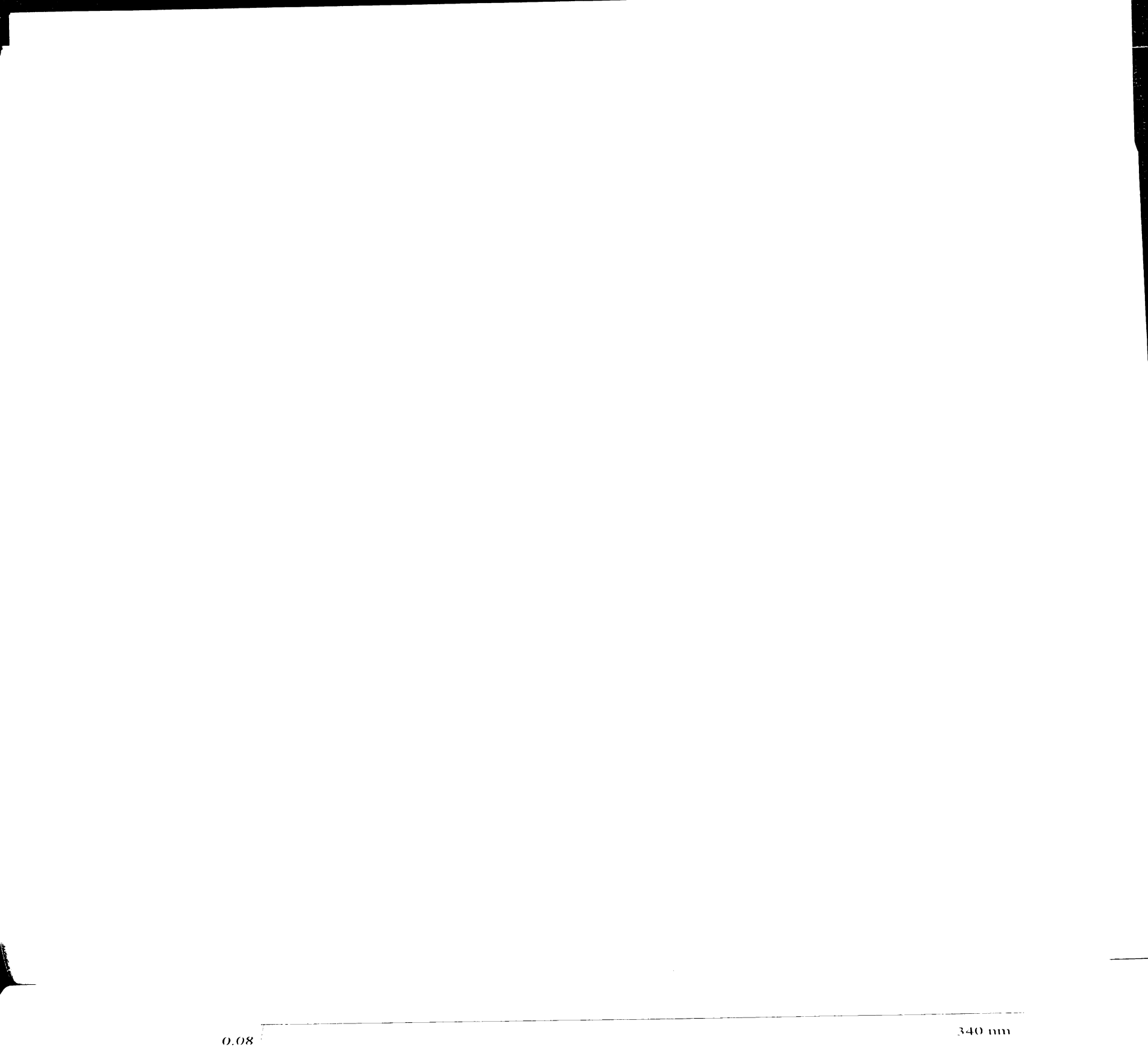


Figure 54: Nonpolar compounds (reconstituted in 100% acetonitrile) in peel press liquid extract at 340 nm (polymethoxylated flavones), 280 nm (flavanone glucosides), and 210 nm (limonoids). *HD = hesperidin.*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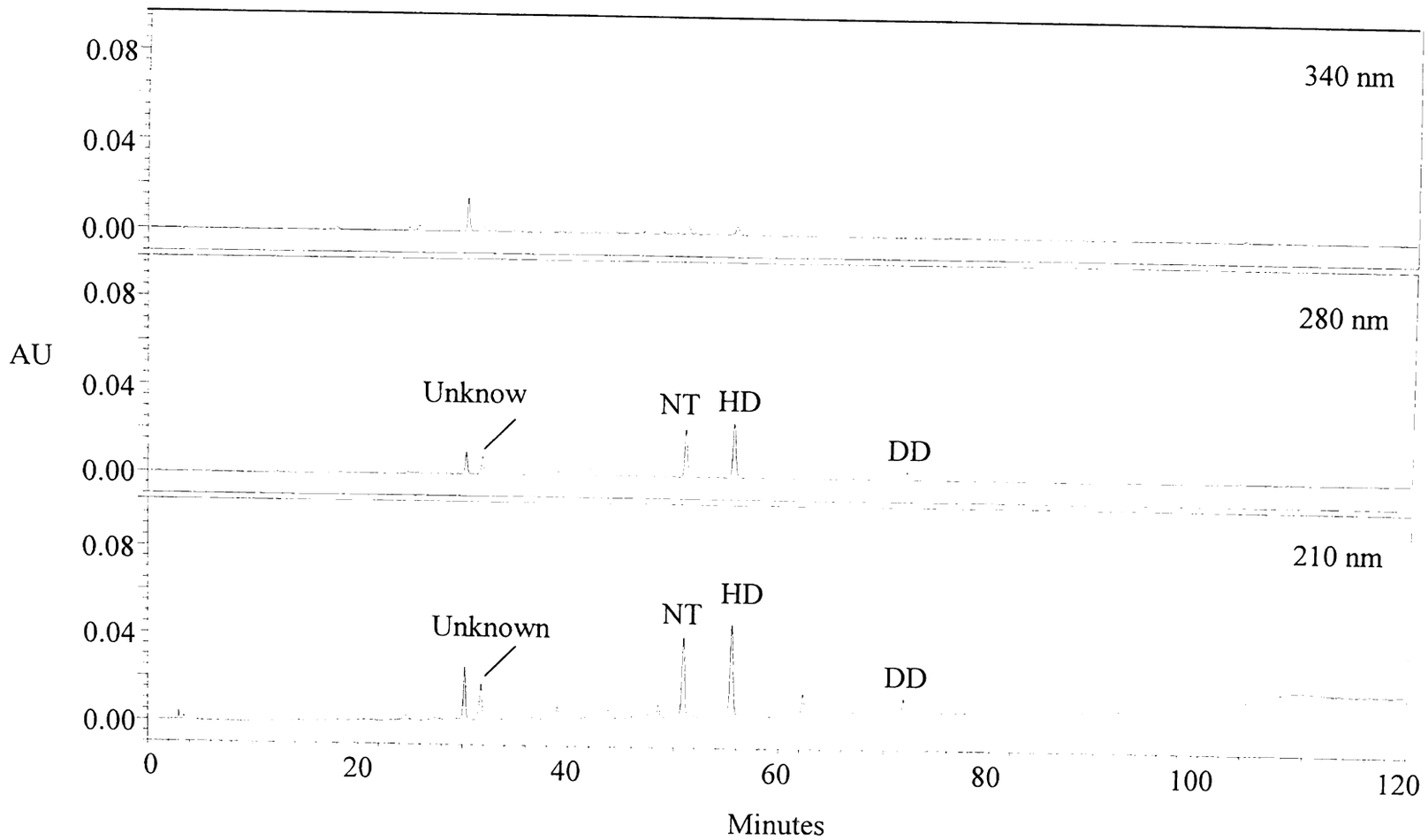

Figure 55: Polar compounds (reconstituted in 10% acetonitrile in 3mM phosphoric acid) in orange juice extract at 340 nm (polymethoxylated flavones), 280 nm (flavanone glucosides), and 210 nm (limonoids).). *NT = narirutin, HD = hesperidin, DD = didymin.*

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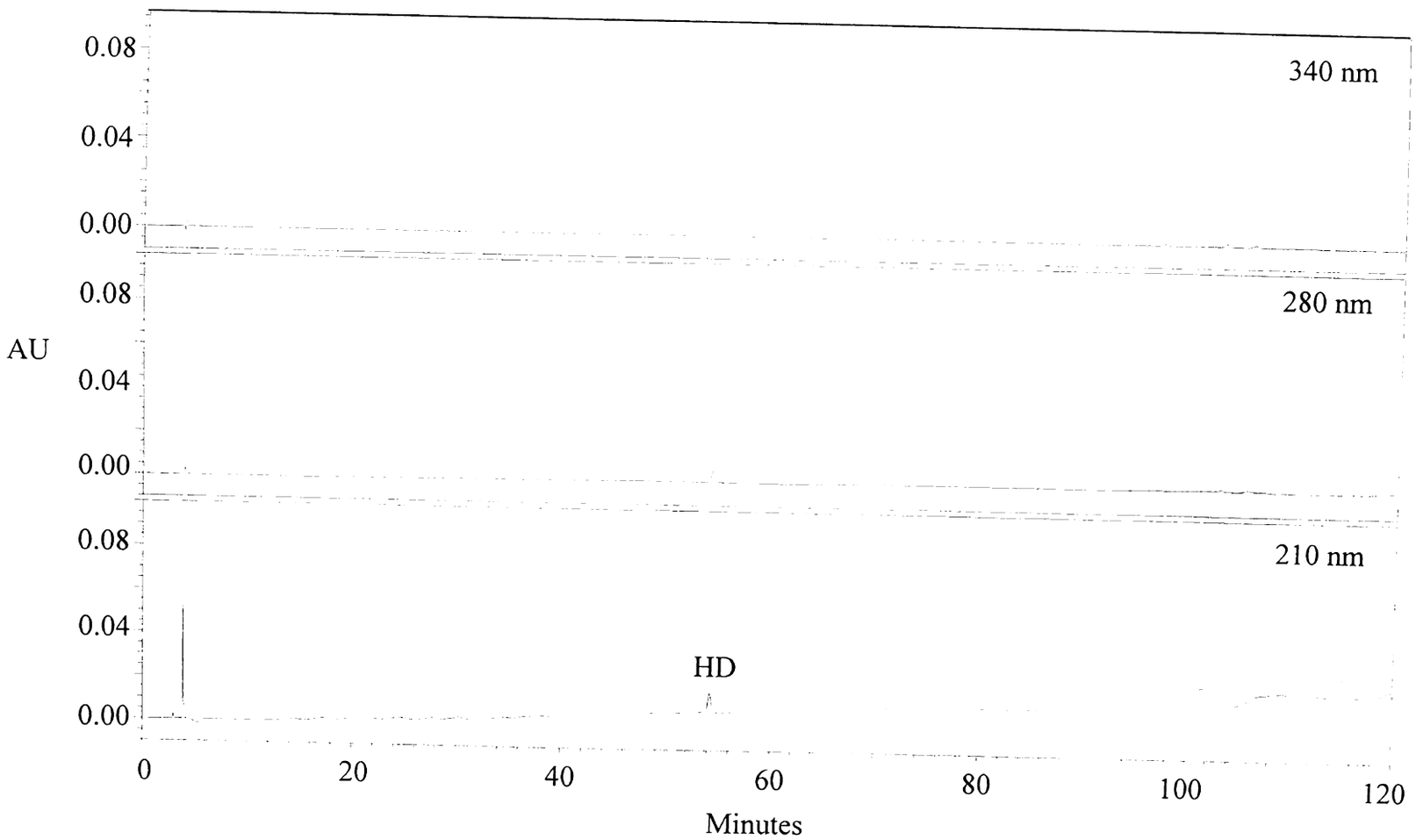

Figure 56: Nonpolar compounds (reconstituted in 100% acetonitrile) in orange juice extract at 340 nm (polymethoxylated flavones), 280 nm (flavanone glucosides), and 210 nm (limonoids). *HD = hesperidin.*

APPENDIX II

Purification of limonoid aglycones by
preparative high performance liquid chromatography.

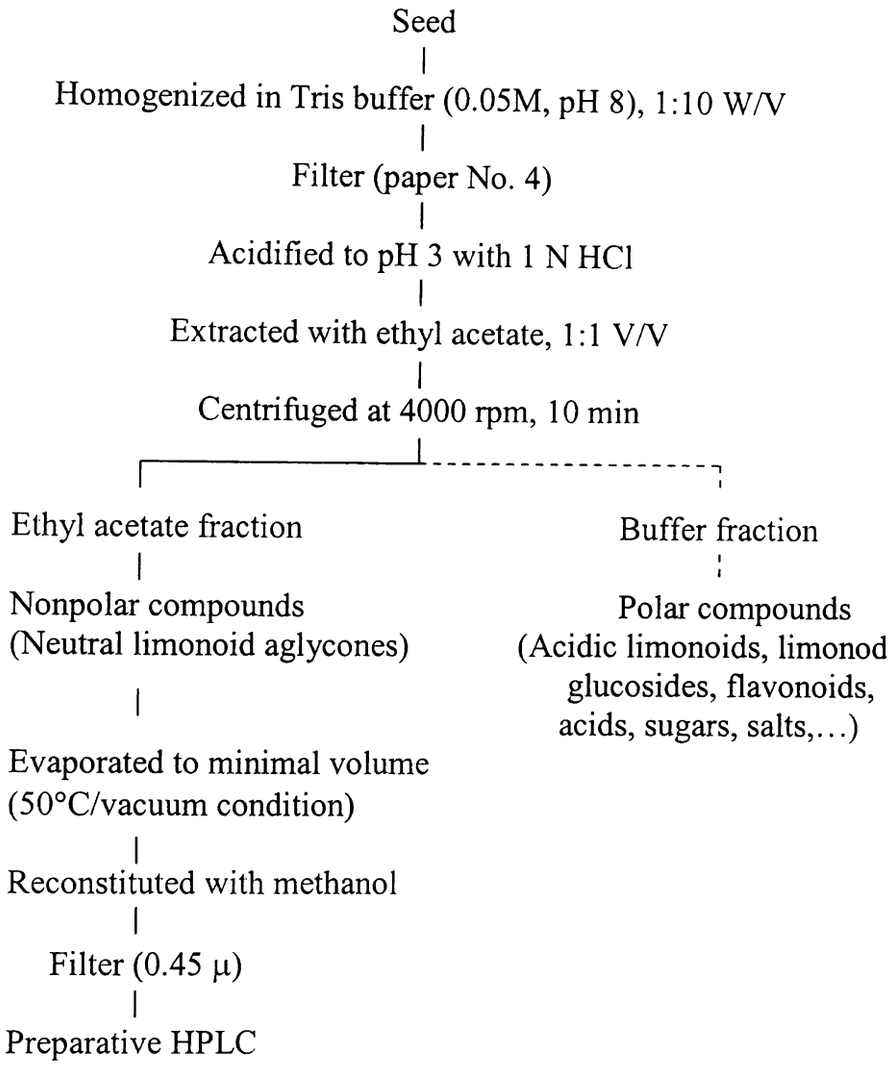

Figure 57: Flow diagram for the isolation of limonoid aglycones from orange seeds.

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HPLC condition

Column: Econosphere (C18, 10μ, 250mmx22mm, Alltech)

Mobile phase: acetonitrile/methanol/water (10:41:49), Fong et al. (1993)

Flow rate: 10ml/min

Injection volume: 100 μl

Detection: 210 nm

Identification: based on retention time of standards

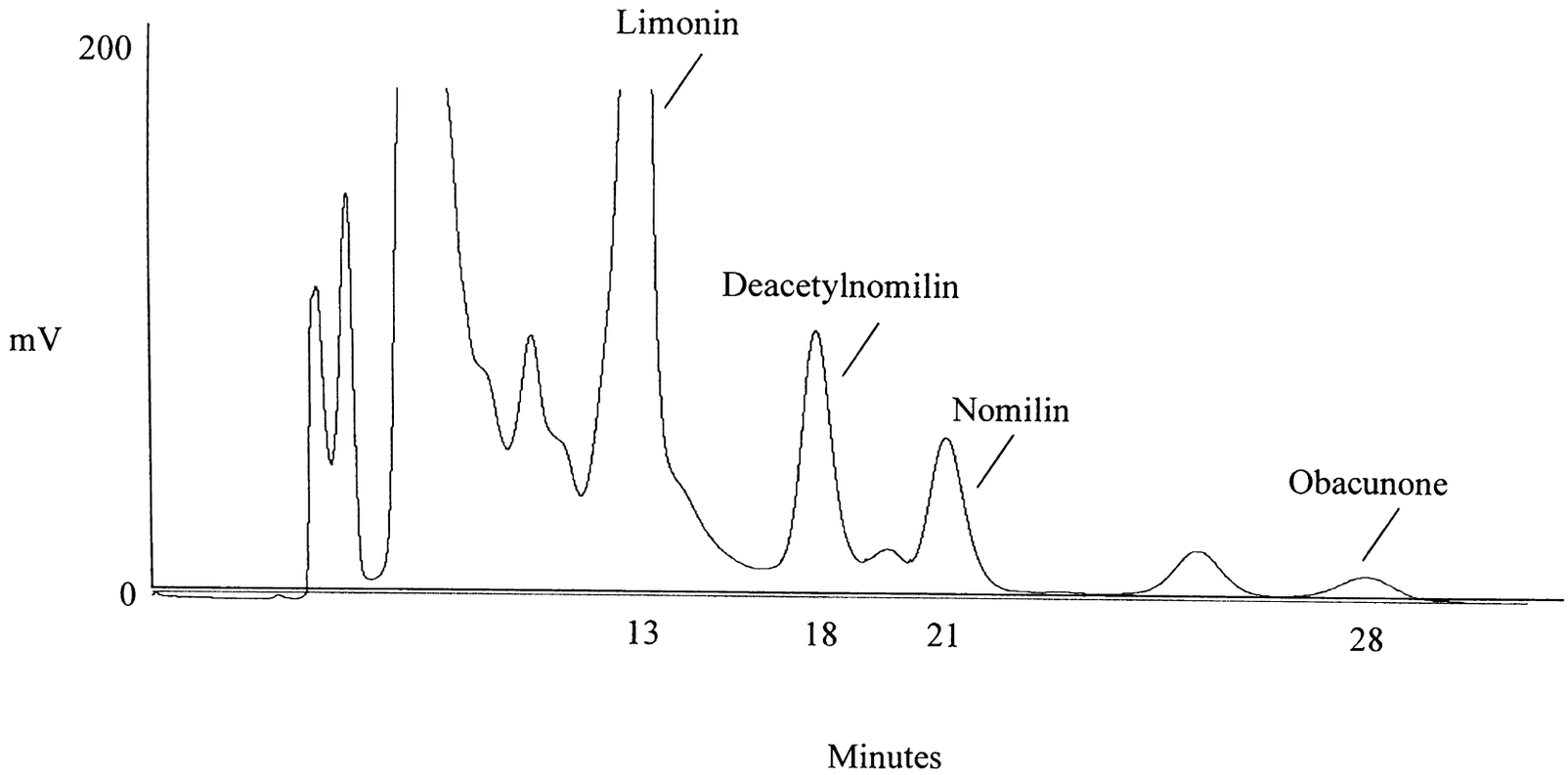


Figure 58: Separation of limonoid aglycones (isolated from Valencia orange seed) on preparative HPLC by isocratic system consisted of acetonitrile-methanol-water (10:41:49) at 10 ml/min.

APPENDIX III

Purification of limonoid glucosides by
preparative high performance liquid chromatography.

Extraction of limonoid glucosides

Flow diagram for the limonoid glucoside isolation from orange seeds is present in Figure 28 (Study II/part I).

HPLC condition

Column: Econosphere (C18, 10μ, 250mmx22mm, Alltech)

Mobile phase: 17.5 % acetonitrile in 3 mM phosphoric acid, 10 ml/min

Injection volume: 100 μl

Detection: 210 nm

Identification: based on retention time of standards

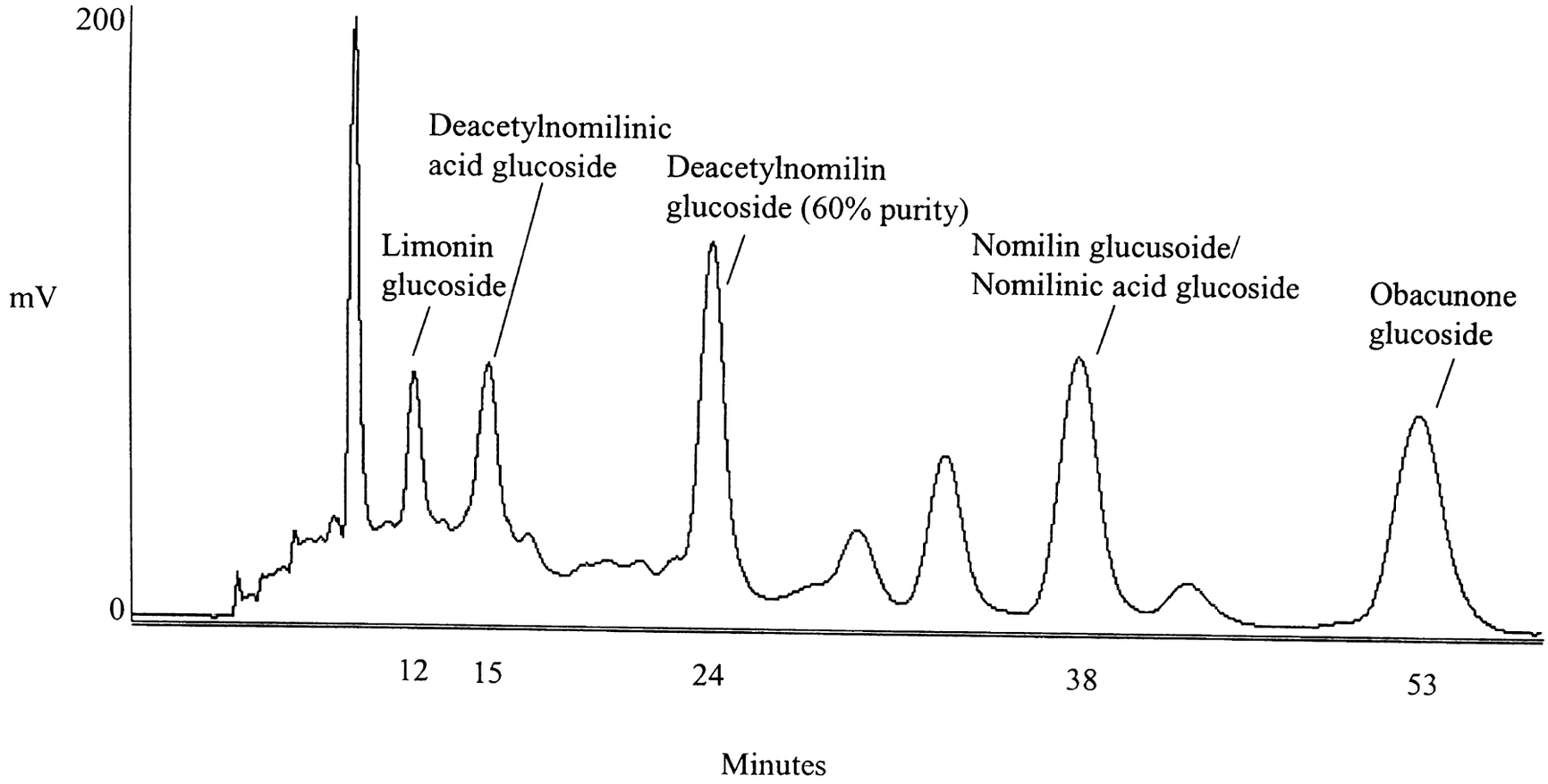


Figure 59: Separation of limonoid glucosides (isolated from Valencia orange seed) on preparative HPLC by 17.5 % acetonitrile in 3 mM phosphoric acid at 10 ml/min.

APPENDIX IV

Purification of polymethoxylated flavones by preparative high performance liquid chromatography.

Extraction of polymethoxylated flavones

Flow diagram for the polymethoxylated flavone isolation from orange peels is present in Figure 32 (Study II/part II).

HPLC Conditions

Column: Econosphere (C18, 10μ, 250mmx22mm, Alltech)

Mobile phase: 65% B

Where, A = water/acetonitrile/ propanol/acetic acid (81:15:3:1)

B = water/acetonitrile/propanol/acetic acid (40:56:3:1)

Injection volume: 100 μl

Flow rate: 8 ml/min

Detection: 340 nm

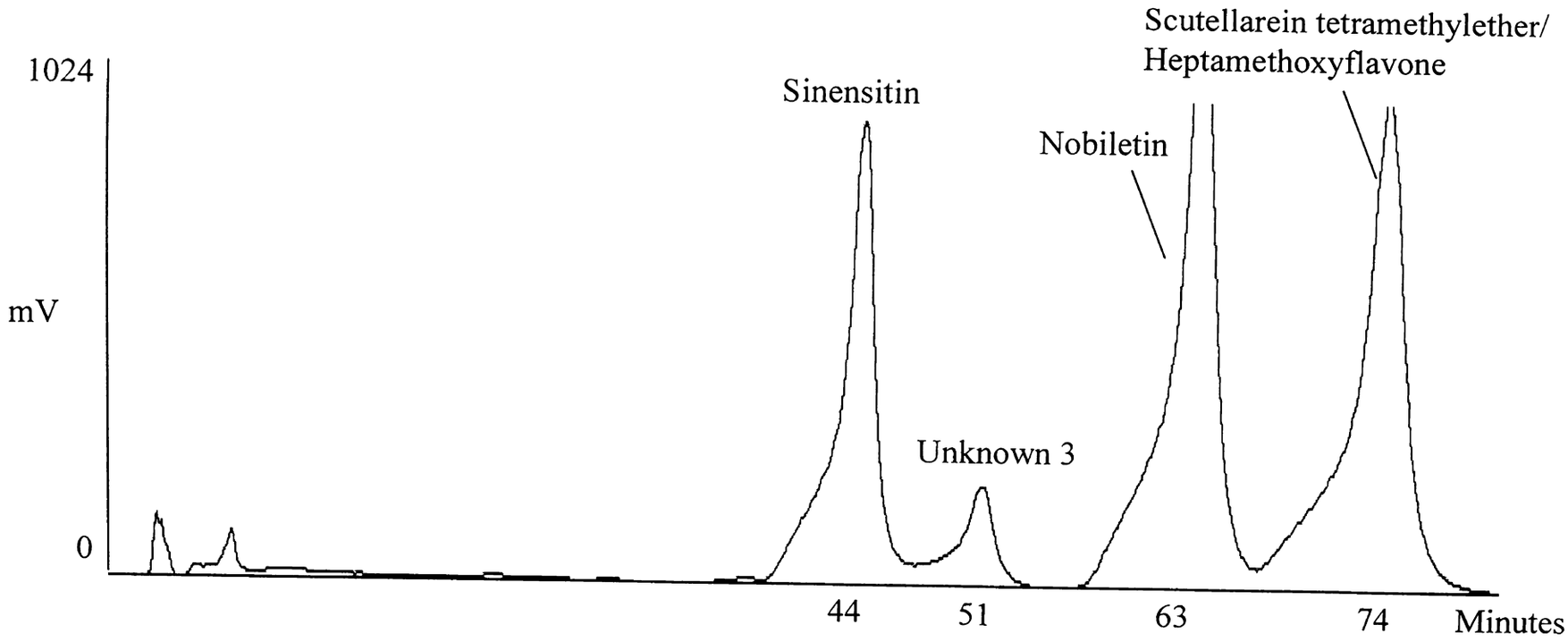
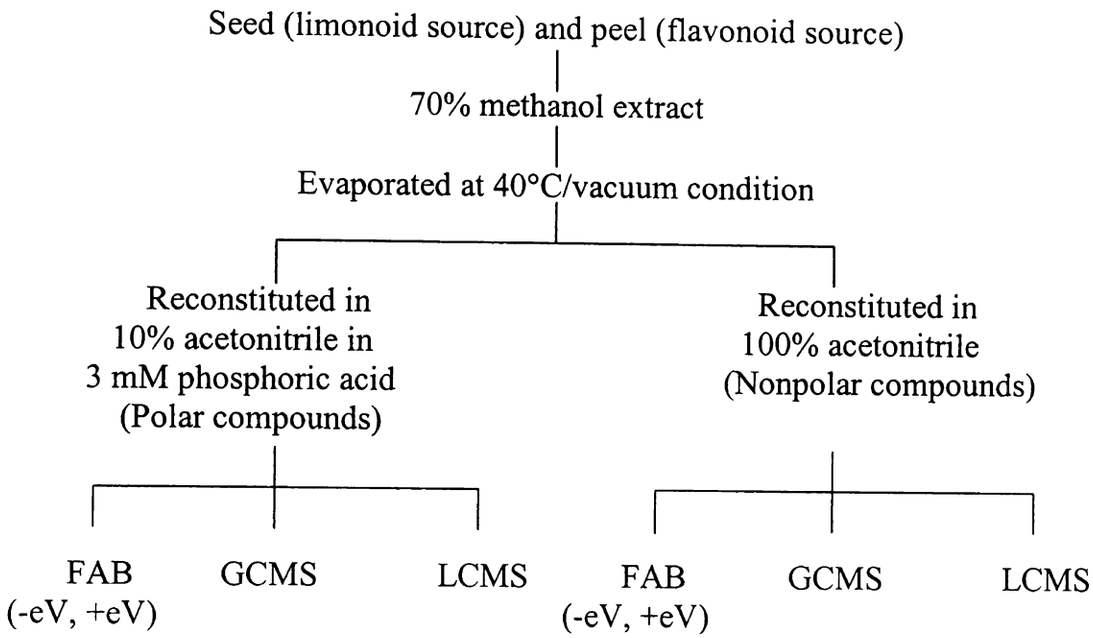


Figure 60: Separation of polymethoxylated flavones (isolated from Valencia orange peel) on preparative HPLC by 65 % B [A=H_2O-CH_3CN-C_3H_7OH-CH_3COOH (81:15:3:1) and B =H_2O-CH_3CN-C_3H_7OH-CH_3COOH(40:56:3:1)] at 8 ml/min

APPENDIX V

Preliminary trials of mass spectrometric techniques on flavonoids and limoniods extracted from sweet orange (*C. sinensis*).

Sample preparations



Direct probe fast atom bombardment mass spectrometry (FABMS)

The mass spectra were obtained using a JEOL HX-110 double-focusing mass spectrometer (JOEL USA, Peabody, MA) operating in the both positive and negative ion modes. Ions were produced by bombardment with a beam of Xe atoms (6 keV). Matrixes used were glycerol and m-nitrobenzyl alcohol (NBA). The accelerating voltage was 10 kV and the resolution was set at 3000. The instrument was scanned from m/z 0 to 1500, data were collected from m/z 50-1500. The sample was mixed with the matrix, which supported ionization on a probe tip and was then inserted into the instrument.

Electron impact ionization gas chromatography mass spectrometry (EI-GC-MS)

Gas chromatography column were a) 3/18: 30m DB1 0.32mm ID, 0.25um film b) 4/10: 30m DB5 0.32mm ID, 0.25um film. Mobile phase was helium gas. Program started with 50°C for 10 minutes, then 320°C for 3 minutes for 3/18 column; and started

with 50°C for 10 minutes, then 320°C for 30 minutes for 4/10 column. Flow rate was set at 1 ml/minutes. Injection volume was 5 μl.

The mass spectrometry condition was consisted of JEOL-AX-505H double focusing mass spectrometer coupled to a Hewlett-Packard 5890J gas chromatograph via a heated interface approximately 280°C, ion source temperature 220°C, electron energy 70eV, and m/z range 45-750.

Electrospray liquid chromatography mass spectrometry (ESI-LC-MS)

Liquid chromatography column used was self-packed Vydac, C18 reverse-phase capillary column with 75 μ ID and approximately 5-8 cm in length. Mobile phases were 0.1% formic acid in 2% acetonitrile (solvent A) and 0.1% formic acid in 95% acetonitrile (solvent B). Program started from 20% to 60%B in 30 minutes, then 95%B in 5 minutes, then down to 2%B and equilibrating for 20 minutes at 200 ηl/min. Injection volume was 0.5 μl for peel and 1 μl for seed extracts.

The mass spectrometry program used was a 60 minute run with triple play to acquire a full scan, a zoom scan (to determine the charge state), and MS/MS spectrum (to look at fragmentation at 35% normalized collision energy). The settings for the MS (tune file) were: spray voltage 2.4, and capillary temperature of 93°C.

Table 55: Positive ions produced from Valencia seed extract by positive FABMS/glycerol matrix

Compounds	MW	m/z						
		$[M+H]^+$	$[M+Na]^+$	$[M+K]^+$	Dimer $[2(M-H)+H]^+$	$[[M-H]+Gly]^-$	$[[M-H]+2Gly]^-$	$[[M-H]+3Gly]^-$
NG	694	-	-	-	-	787	-	970
DNG	652	-	676	691	-	-	837	-
NT-4'-G	742	-	765	-	-	835	927	-
HX	402	403	425	441	-	-	587	-
LG	650	-	673	-	-	-	835	-
DNAG	670	671	693	-	-	-	855	-
NAG	712	-	735	751	1423	-	897	-
OG	634	-	657	673	-	-	-	-
L	470	471	-	-	939	-	-	-
NM	514	515	-	-	-	-	-	-
DNM	472	473	-	-	-	-	657	-
O	454	555	477	-	-	-	-	-
HD	610	-	-	-	-	-	-	-
NT	580	-	603	619	-	673	765	857
ERT	596	-	619	-	-	-	-	873
DD	594	595	617	-	-	-	-	-
NT	402	403	425	441	-	-	587	-
ST	372	-	395	411	-	-	557	-
HP	432	433	455	471	-	-	-	-
STME	342	-	-	381	-	-	527	619
TT	372	-	395	411	-	-	557	-

Table 56: Positive ions produced from Valencia seed extract by positive FABMS/*m*-nitrobenzyl alcohol matrix.

Compounds	MW	m/z						
		$[M+H]^+$	$[M+Na]^+$	$[M+K]^+$	Dimer $[2(M-H)+H]^+$	$[MH+NBA]^+$	$[MH+2NBA]^+$	$[MH+3NBA]^+$
NG	694	695	-	-	-	-	-	-
DNG	652	652	-	-	-	-	-	-
NT-4'-G	742	743	-	-	-	-	-	-
HX	402	-	425	441	-	556	-	-
LG	650	-	673	-	-	-	-	-
DNAG	670	671	-	709	-	-	-	-
NAG	712	713	735	751	1423	-	-	-
OG	634	735	-	673	-	-	-	-
L	470	471	-	-	-	-	-	-
NM	514	515	-	-	-	668	-	-
DNM	472	473	-	-	-	626	779	-
O	454	455	-	-	-	-	-	-
HD	610	611	-	-	-	-	-	-
NT	580	581	-	-	1159	-	-	-
ERT	596	597	-	-	-	750	-	-
DD	594	595	-	-	-	-	-	-
NT	402	-	425	441	-	556	-	-
ST	372	-	395	-	743	-	679	-
HP	432	-	455	471	-	-	793	-
STME	342	-	-	-	-	-	-	-
TT	372	-	395	-	743	-	679	-

Table 57: Negative ions produced from Valencia seed extract by negative FABMS/glycerol matrix.

Compounds	MW	m/z				
		$[M-H]^-$	Dimer $[2(M-H)-H]^-$	$[[M-H]+Gly]^-$	$[[M-H]+2Gly]^-$	$[[M-H]+3Gly]^-$
NG	694	693	-	-	-	-
DNG	652	651	-	743	-	-
NT-4'-G	742	741	-	833	-	-
HX	402	401	-	-	-	-
LG	650	649	-	-	833	-
DNAG	670	669	-	-	-	-
NAG	712	711	-	803	-	-
OG	634	633	-	725	817	-
L	470	469	-	561	653	-
NM	514	513	-	605	-	-
DNM	472	471	-	563	-	-
O	454	453	-	-	-	-
HD	610	609	-	-	-	-
NT	580	579	-	671	-	-
ERT	596	-	-	-	-	-
DD	594	593	-	-	-	-
NT	402	401	-	-	-	-
ST	372	-	-	-	-	-
HP	432	-	-	-	-	-
STME	342	-	-	-	-	-
TT	372	-	-	-	-	-

Table 58: Negative ions produced from Valencia seed extract by negative FABMS/*m*-nitrobenzyl alcohol matrix.

Compounds	MW	m/z				
		$[M-H]^-$	Dimer $[2(M-H)-H]^-$	$[[M-H]+NBA]^-$	$[[M-H]+2NBA]^-$	$[[M-H]+3NBA]^-$
NG	694	693	-	-	-	-
DNG	652	651	-	-	-	-
NT-4'-G	742	-	-	-	-	-
HX	402	401	-	-	-	-
LG	650	649	-	-	-	-
DNAG	670	669	-	-	-	-
NAG	712	711	1421	-	-	-
OG	634	633	-	786	-	-
L	470	469	-	622	-	-
NM	514	513	-	666	-	-
DNM	472	471	-	-	-	-
O	454	453	-	-	-	-
HD	610	609	-	-	-	-
NT	580	579	-	-	-	-
ERT	596	595	-	-	-	-
DD	594	593	-	-	-	-
NT	402	401	-	-	-	-
ST	372	371	-	-	-	-
HP	432	-	-	-	-	-
STME	342	341	-	494	647	-
TT	372	371	-	-	-	-

Table 59: Positive ions produced from Valencia peel extract by positive FABMS/glycerol matrix.

Compounds	MW	m/z						
		$[M+H]^+$	$[M+Na]^+$	$[M+K]^+$	Dimer $[2(M-H)+H]^+$	$[MH+Gly]^+$	$[MH+2Gly]^+$	$[MH+3Gly]^+$
NG	694	695	-	-	-	-	-	-
DNG	652	-	-	-	-	745	-	-
NT-4'-G	742	743	-	-	-	835	-	-
HX	402	403	425	-	-	-	-	-
LG	650	-	-	-	-	743	835	-
DNAG	670	-	-	-	1339	-	855	-
NAG	712	713	735	-	-	805	897	-
OG	634	-	-	-	-	727	-	-
L	470	471	-	-	-	-	-	-
NM	514	515	-	553	-	-	-	-
DNM	472	473	-	-	-	-	-	-
O	454	455	477	-	-	-	-	730
HD	610	611	-	-	-	-	795	887
NT	580	581	-	-	-	-	-	857
ERT	596	-	-	-	-	-	-	-
DD	594	595	-	-	-	-	-	-
NT	402	403	425	-	-	-	587	-
ST	372	373	-	-	743	465	557	-
HP	432	433	455	471	-	525	-	-
STME	342	343	-	-	-	435	527	-
TT	372	373	-	-	743	465	557	-

Table 60: Positive ions produced from Valencia peel extract by positive FABMS/*m*-nitrobenzyl alcohol matrix.

Compounds	MW	m/z, relative intensity (%)						
		$[M+H]^+$	$[M+Na]^+$	$[M+K]^+$	Dimer $[2(M-H)+H]^+$	$[MH+NBA]^+$	$[MH+2NBA]^+$	$[MH+3NBA]^+$
NG	694	-	-	-	-	-	-	-
DNG	652	-	-	-	-	-	-	-
NT-4'-G	742	-	-	781	-	-	-	-
HX	402	403	425	-	-	-	-	-
LG	650	651	-	-	-	-	-	
DNAG	670	-	-	-	-	-	-	
NAG	712	713	-	-	-	-	-	
OG	634	-	-	-	-	-	-	1094
L	470	-	-	-	-	-	-	-
NM	514	-	-	-	-	-	-	974
DNM	472	-	-	-	-	626	779	932
O	454	-	-	-	-	-	-	-
HD	610	611	-	-	-	-	-	1070
NT	580	581	603	-	-	-	887	-
ERT	596	597	-	-	-	750	-	1056
DD	594	595	-	-	-	-	-	1054
NT	402	403	425	-	-	-	-	-
ST	372	373	395	-	-	-	-	832
HP	432	-	-	-	-	-	-	-
STME	342	343	-	-	-	496	-	-
TT	372	373	395	-	-	-	-	832

Table 61: Negative ions produced from Valencia peel extract by negative FABMS/glycerol matrix.

Compounds	MW	m/z				
		$[M-H]^-$	Dimer $[2(M-H)-H]^-$	$[[M-H]+Gly]^-$	$[[M-H]+2Gly]^-$	$[[M-H]+3Gly]^-$
NG	694	693	-	-	-	-
DNG	652	651	-	743	-	-
NT-4'-G	742	741	-	833	-	-
HX	402	401	801	-	-	-
LG	650	649	-	-	-	-
DNAG	670	669	-	-	-	-
NAG	712	711	-	803	895	-
OG	634	633	-	561	-	-
L	470	-	-	-	653	-
NM	514	-	-	-	-	-
DNM	472	-	-	563	-	-
O	454	-	-	545	-	-
HD	610	609	-	-	-	-
NT	580	579	-	-	-	-
ERT	596	595	-	-	-	-
DD	594	593	1185	-	-	-
NT	402	401	801	-	-	-
ST	372	371	-	463	-	-
HP	432	431	-	-	-	-
STME	342	341	681	433	-	-
TT	372	371	-	463	-	-

Table 62: Negative ions produced from Valencia peel extract by negative FABMS/*m*-nitrobenzyl alcohol matrix.

Compounds	MW	m/z				
		$[M-H]^-$	Dimer $[2(M-H)-H]^-$	$[[M-H]+NBA]^-$	$[[M-H]+2NBA]^-$	$[[M-H]+3NBA]^-$
NG	694	693	-	-	-	-
DNG	652	651	-	-	-	-
NT-4'-G	742	-	-	894	-	-
HX	402	-	801	555	-	-
LG	650	649	-	-	-	-
DNAG	670	669	-	-	-	-
NAG	712	711	-	-	-	-
OG	634	-	-	-	-	-
L	470	-	-	-	-	-
NM	514	-	-	-	-	-
DNM	472	471	-	-	-	-
O	454	-	-	-	-	-
HD	610	609	-	-	-	-
NT	580	579	-	-	-	-
ERT	596	595	-	748	-	-
DD	594	593	-	746	-	-
NT	402		-	-	-	-
ST	372		-	-	-	-
HP	432		-	-	-	-
STME	342		-	-	-	-
TT	372		-	-	-	-

Table 63: Electron impact ionization gas chromatography-mass spectrometry data of crude seed extract (5µl).

Compounds	Retention (min)	MS data (m/z, relative intensities)				
		MW	$M^{+\cdot}$	$[M-CH_3]^+$	$[M-CH_2O]^+$	$[A-CH_3]^+$
Tangeretin	23.00	372	372, 94	357, 100	341, 8	-
3,3',4',5,6,7-hexamethoxyflavone	23.00	402	-	387	-	-
3,3',4',5,6,7,8-heptamethoxyflavone	25.41	432	-	417	-	-
Sinensitin	25.41	372	-	357	-	-
Nobiletin	25.41	402	-	387	-	-

Table 64: Electron impact ionization gas chromatography-mass spectrometry data of crude peel extract (5µl).

Compounds	Retention (min)	MS data (m/z, relative intensities)				
		MW	$M^{+\cdot}$	$[M-CH_3]^+$	$[M-CH_2O]^+$	$[A-CH_3]^+$
Tetra-O-methylscutellarein	23.04	342	342, 5	327, 100	311,3	-
Tangeretin	23.04	372	372, 29	357, 100	341,13	-
3,3',4',5,6,7-hexamethoxyflavone	24.06	402	402, *	387, 100	371, 14	-
3,3',4',5,6,7,8-heptamethoxyflavone	24.06	432	432, 43	417, 100	402, *	-
Sinensitin	24.15	372	372,7	357, 100	341, 22	-
Nobiletin	24.15	402	402, 31	387, 100	371, 8	225, 5

Table 65: Electrospray ionization liquid chromatography-mass spectrometry data for crude presscake extract (0.5 µl).

RT* (min)	Compounds	MW	$[M+H]^+$	Dimer $[2(M-H)+H]^+$
1-18	Hesperidin	610	611	-
	Narirutin	580	581	-
	Didymin	594	595	-
	Deacetylnomilinglucoside	652	653	-
	Nomilin	514	515	-
	Hesperitin	302	303	-
	Eriocitrin	596	597	-
18-24	Narirutin-4'-glucoside	473	474	-
24-25.5	Nomilinglucoside	694	695	-
25.5-27.5	Nomilinicacid glucoside	712	713	1423
27.5-30	Limoninglucoside	650	651	-
	Limonin	470	471	-
30-31.5	Sinensitin	372	373	-
	3,3',4',5,6,7-Hexamethoxyflavones	402	403	-
31.5-33	Nobiletin	402	403	-
33-35	3,3',4',5,6,7,8-Heptamethoxyflavone	432	433	-
	Tetramethyl-O-scutellarin	342	343	-
	Obacunone	454	455	-
35-40	Tangeretin	372	373	-

*Retention time

Table 66: Electrospray ionization liquid chromatography-mass spectrometry data for crude seed extract (1 μl).

RT* (min)	Compounds	MW	$[M+H]^+$	Dimer $[2(M-H)+H]^+$
N/A	Limonin	470	471	-
N/A	Nomilin	514	515	1029
N/A	Obacunone	454	455	-
N/A	Deacetylnomilin	472	473	-
N/A	Hesperidin	610	611	-
N/A	Didymin	594	595	-
N/A	Narirutin	580	581	-
N/A	Eriocitrin	596	597	-
N/A	Limoninglucoside	650	651	-
N/A	Deacetylnomilinglucoside	652	653	-
N/A	Nomilinicacid glucoside	712	713	1423
N/A	Nomilinglucoside	694	695	-
N/A	Deacetylnomilinic acid glucoside	670	671	-
N/A	Obacunoneglucoside	634	636	-
N/A	Methylnomilinicacid	727	728	-
N/A	Nobiletin	402	403	-
N/A	Hexamethoxyflavone	402	403	-
N/A	Tangeretin	372	373	-
N/A	Sinensitin	372	372	-
N/A	Tetramethyl-O-scutellarin	342	343	-
N/A	Heptamethoxyflavone	432	433	-

*No separation obtained

APPENDIX VI

Limonoid and flavonoid content in rag and orange juice prepared domestically.

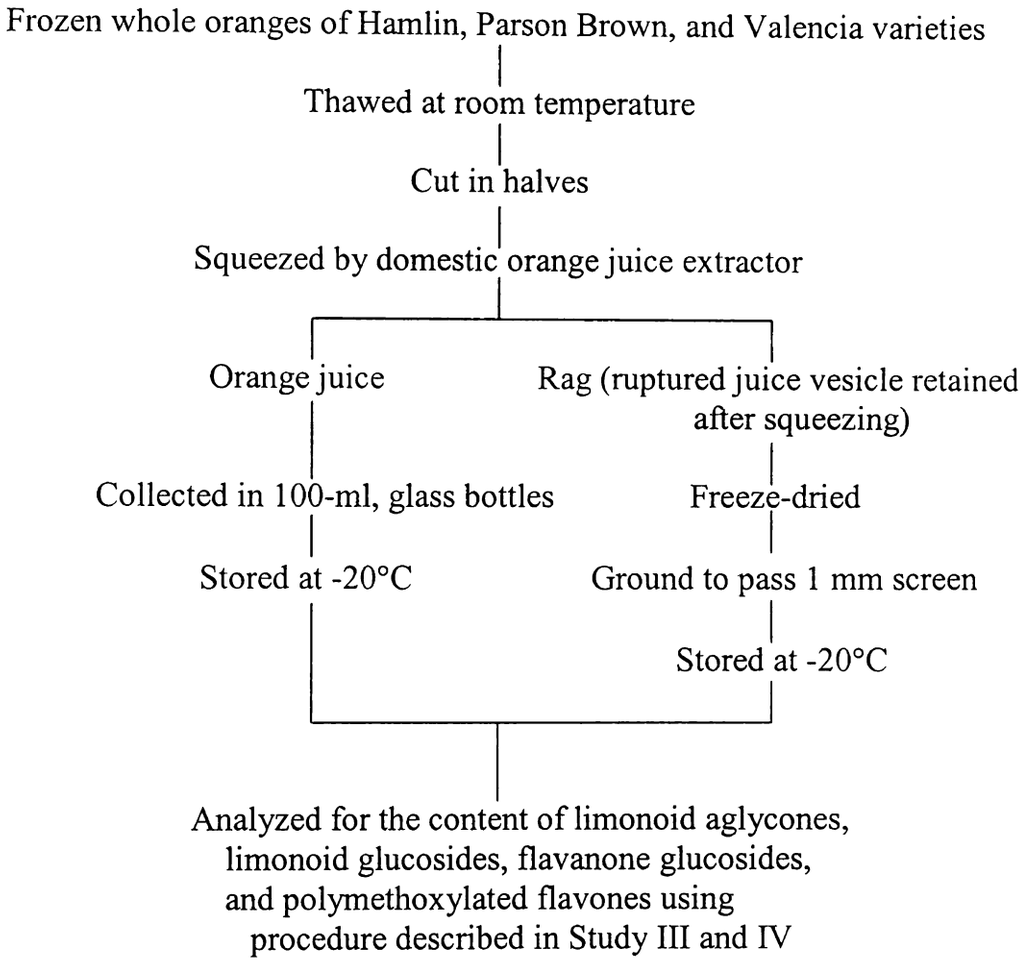


Figure 61: Flow diagram for the sample preparations and analyses of domestically prepared orange juice and rags.

Table 67: Individual limonoid aglycone concentrations in domestically prepared juice and rags of sweet oranges.

Variety	Sample	ppm (mg/Kg or mg/L) ± %CV[1]			
		L	NM	DNM	O
Hamlin	Rags	70±4.5	T[2]	T	T
	Juice	4.9±5.1	T	T	T
Parson Brown	Rags	108±5.1	T	T	T
	Juice	4.5±0.4	T	T	T
Valencia	Rags	177±8.8	T	T	T
	Juice	9.0±4.4	T	T	T

L = limonin, NM = nomilin, DNM = deacetylnomilin, O = obacunone, [1]N = 2, [2]Trace

Table 68: Individual limonoid glucoside concentrations in domestically prepared juice and rags of sweet oranges.

Variety	Sample	ppm (mg/Kg or mg/L) ± %CV[1]					
		LG	DNAG	DNG	NG	NAG	OG
Hamlin	Rags	2153±2.9	T[2]	105±5.6	978±0.6	744±3.8	T
	Juice	367±2.2	T	11±0.1	87±2.8	217±2.6	T
Parson Brown	Rags	1792±1.6	T	75±7.0	1024±2.0	708±0.8	T
	Juice	264±3.5	T	8.8±4.2	88±0.7	193±2.4	T
Valencia	Rags	2141±1.1	T	60±±6.2	847±4.0	554±0.0	T
	Juice	326±3.7	T	9.0±12.3	37±1.4	178±0.9	T

LG = limonin glucoside, DNAG = deacetylnomilinic acid glucoside, DNG = deacetylnomilin glucoside, NG = nomilin glucoside, NAG = nomilinic acid glucoside, OG = obacunone glucoside, [1]N=2, [2]Trace

Table 69: Individual polymethoxylated flavone concentrations in domestically prepared juice and rags of sweet oranges.

Variety	Sample	ppm (mg/Kg or mg/L) ± %CV[1]					
		ST	HX	NBT	HP	STME	TT
Hamlin	Rags	28±1.2	5.0±5.1	43±0.8	12±0.5	13±1.0	3.7±4.0
	Juice	0.6±14.2	0.1±14.6	1.0±9.0	0.3±14.0	0.5±6.9	0.2±12.2
Parson Brown	Rags	19.6±0.8	4.5±2.4	21.3±0.8	6.1±1.9	7.4±2.6	1.7±4.4
	Juice	0.7±4.4	0.2±1.6	0.7±4.0	0.2±1.8	0.3±1.7	0.07±4.2
Valencia	Rags	30±2.8	6.9±4.2	32±3.3	11±5.2	11±4.5	3.5±9.1
	Juice	1.9±5.8	0.4±8.3	2.0±10.9	0.6±19.6	0.6±14.5	0.2±37.7

ST = sinensitin, HX = 3,5,6,7,3',4'-hexamethoxyflavone, NBT = nobiletin, HP = 3,4,5,6,7,8,3',4'-heptamethoxyflavone, STME = scutellarein tetramethylether, TT = tangeretin, [1]N=2

Table 70: Individual flavanone glucoside concentrations in domestically prepared juice and rags of sweet oranges.

Variety	Sample	ppm (mg/Kg or mg/L) ± %CV[1]				
		NT-4'-G	ERT	NT	HD	DD
Hamlin	Rags	722±0.4	261±1.3	1608±1.0	9615±1.5	832±0.5
	Juice	81±1.5	37±0.8	149±0.1	482±0.0	37±0.8
Parson Brown	Rags	629±1.4	260±1.3	1635±0.0	9502±1.3	779±0.7
	Juice	26±2.2	10±5.6	68±3.4	403±4.0	20±4.0
Valencia	Rags	473±1.2	171±2.8	1485±1.0	8791±0.1	632±0.3
	Juice	21±6.1	8.0±0.4	54±0.3	372±1.6	16±1.5

NT-4'-G = narirutin-4'-glucoside, ERT = eriocitrin, NT = narirutin, HD = hesperidin, DD = didymin, [1]N=2

APPENDIX VII

Chromatographic retention and UV spectra of minor citrus limonoids compared to limonin and nomilin (common limonoids).

HPLC condition

Column: C18 (Alltima, Alltech: 5μ, 250mmx4.6mm, 16 % carbon load)

Mobile phase:

Flow (ml/min)	Minute	% Acetonitrile
1	0	30
1	40	50
1	50	50

Injection volume: 10 μl

Detection: 210 nm

Identification: based on retention time of standards

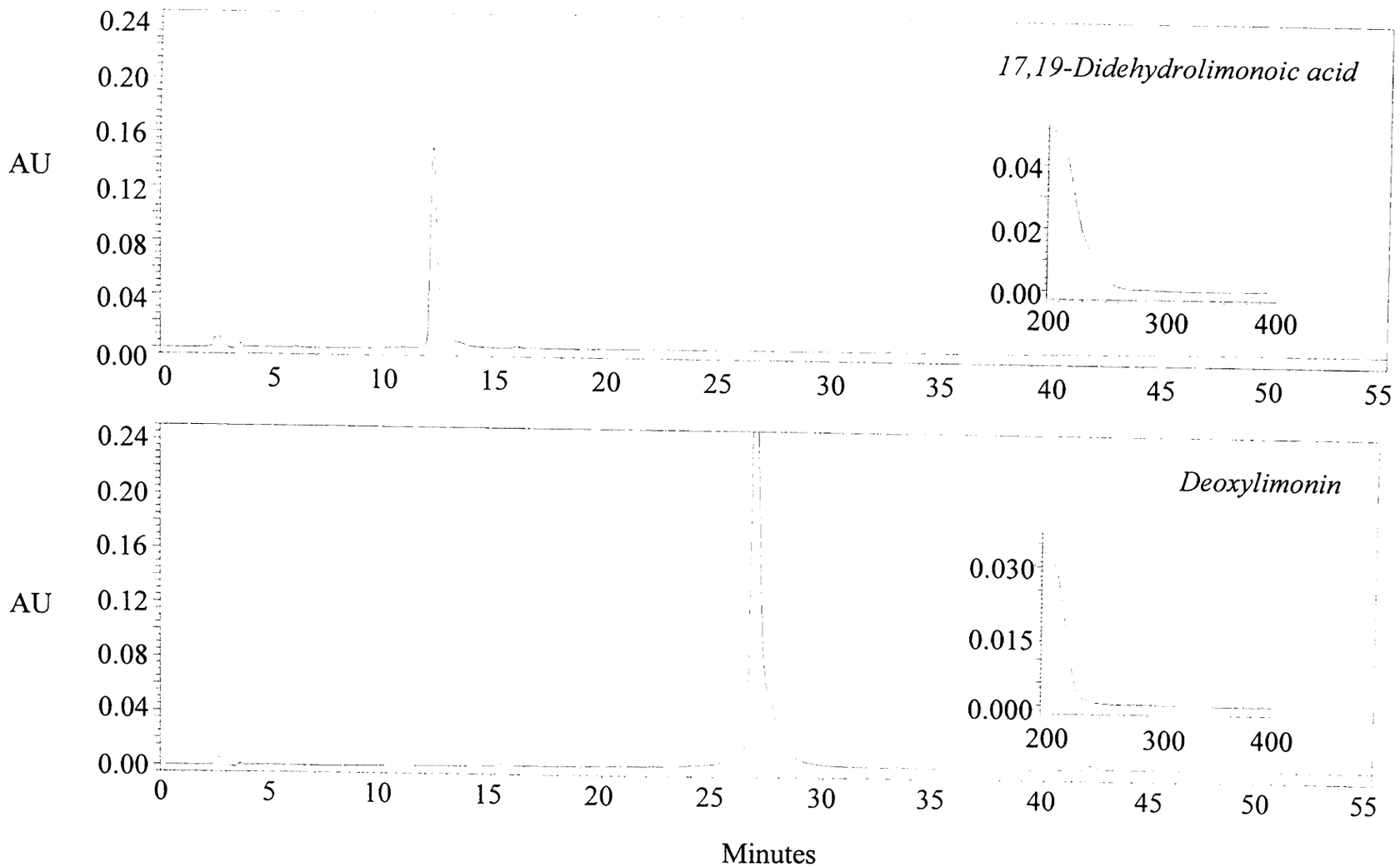


Figure 62: Chromatograms and UV spectra of 17, 19- didehydrolimonoic acid (12.23 min) and deoxylimonin (26.82 min).

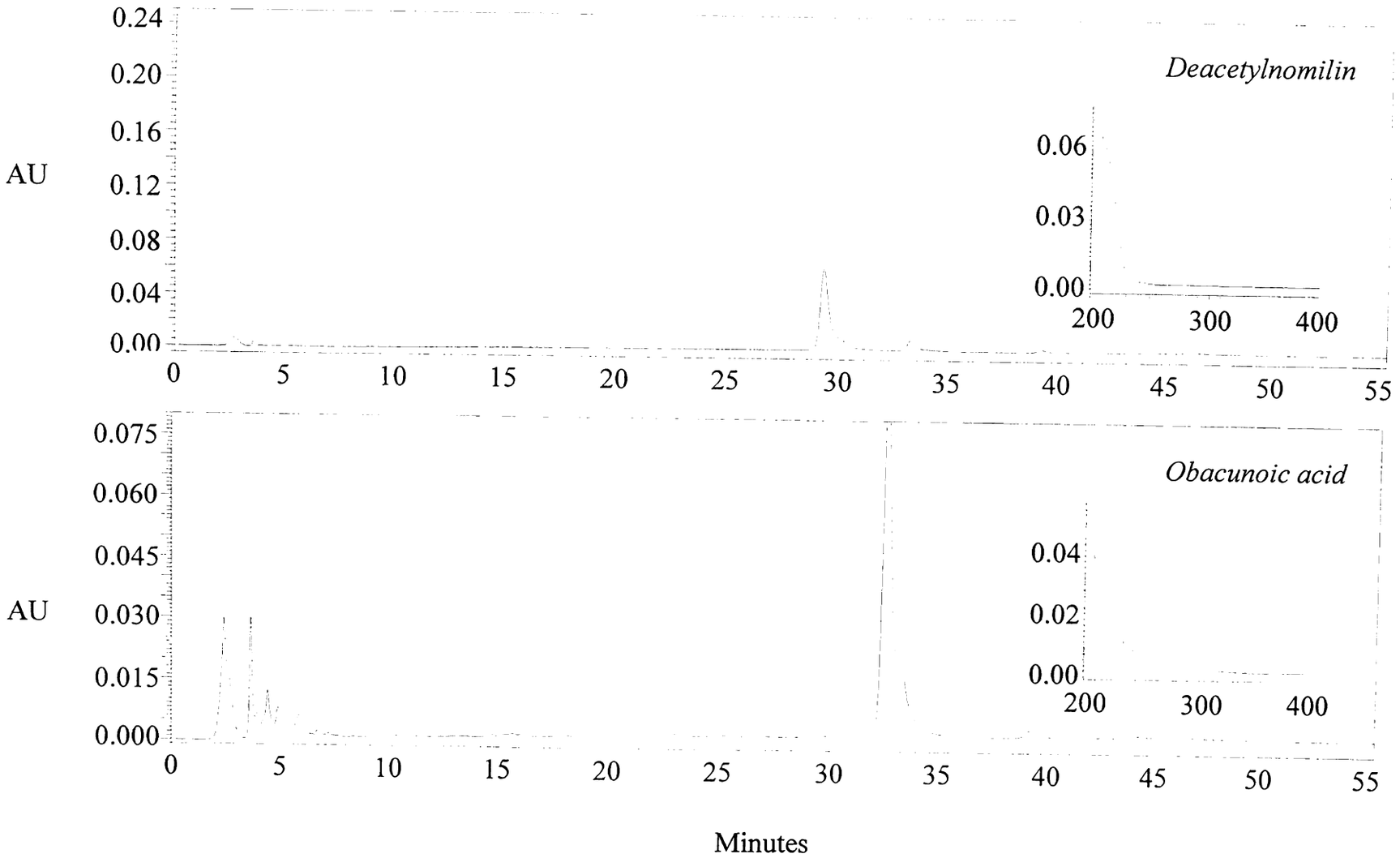


Figure 63: Chromatograms and UV spectra of deacetylnomilin (29.21 min) and obacunoic acid (32.42 min).

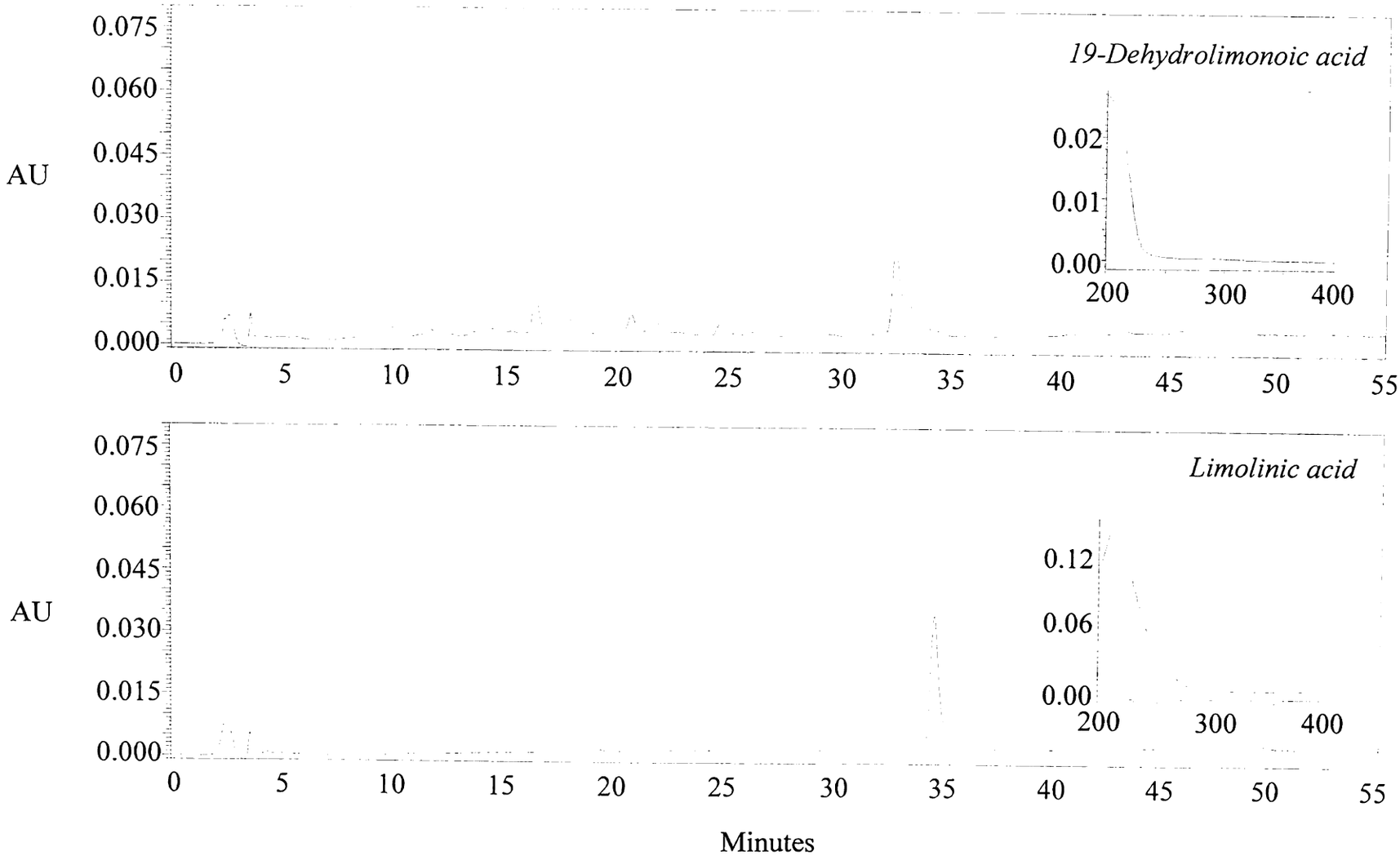


Figure 64: Chromatograms and UV spectra of 19-dehydrolimonoic acid (32.63 min) and limolinic acid (34.63 min).

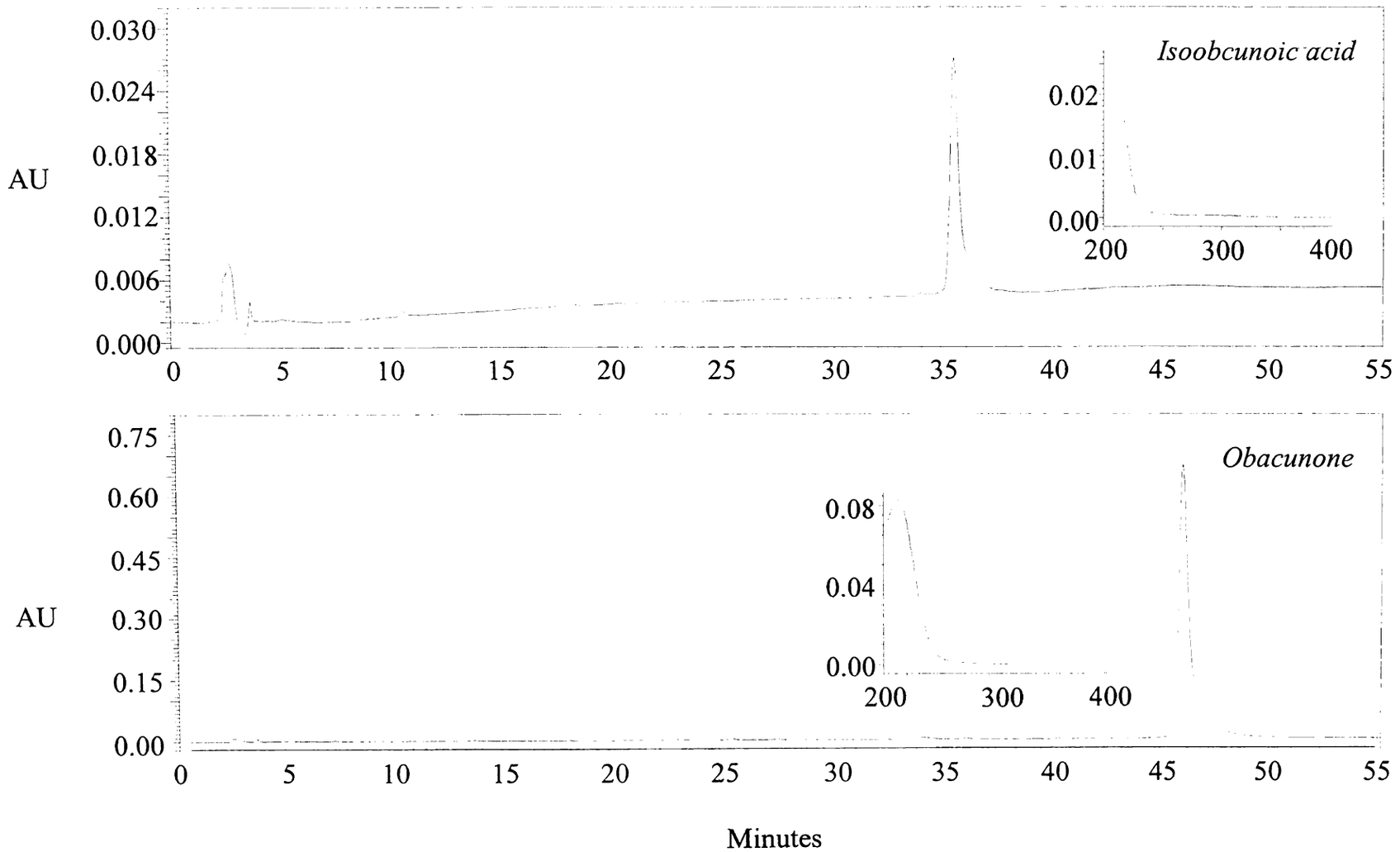


Figure 65: Chromatograms and UV spectra of isoobcunoic acid (35.45 min) and obacunone (45.89 min).

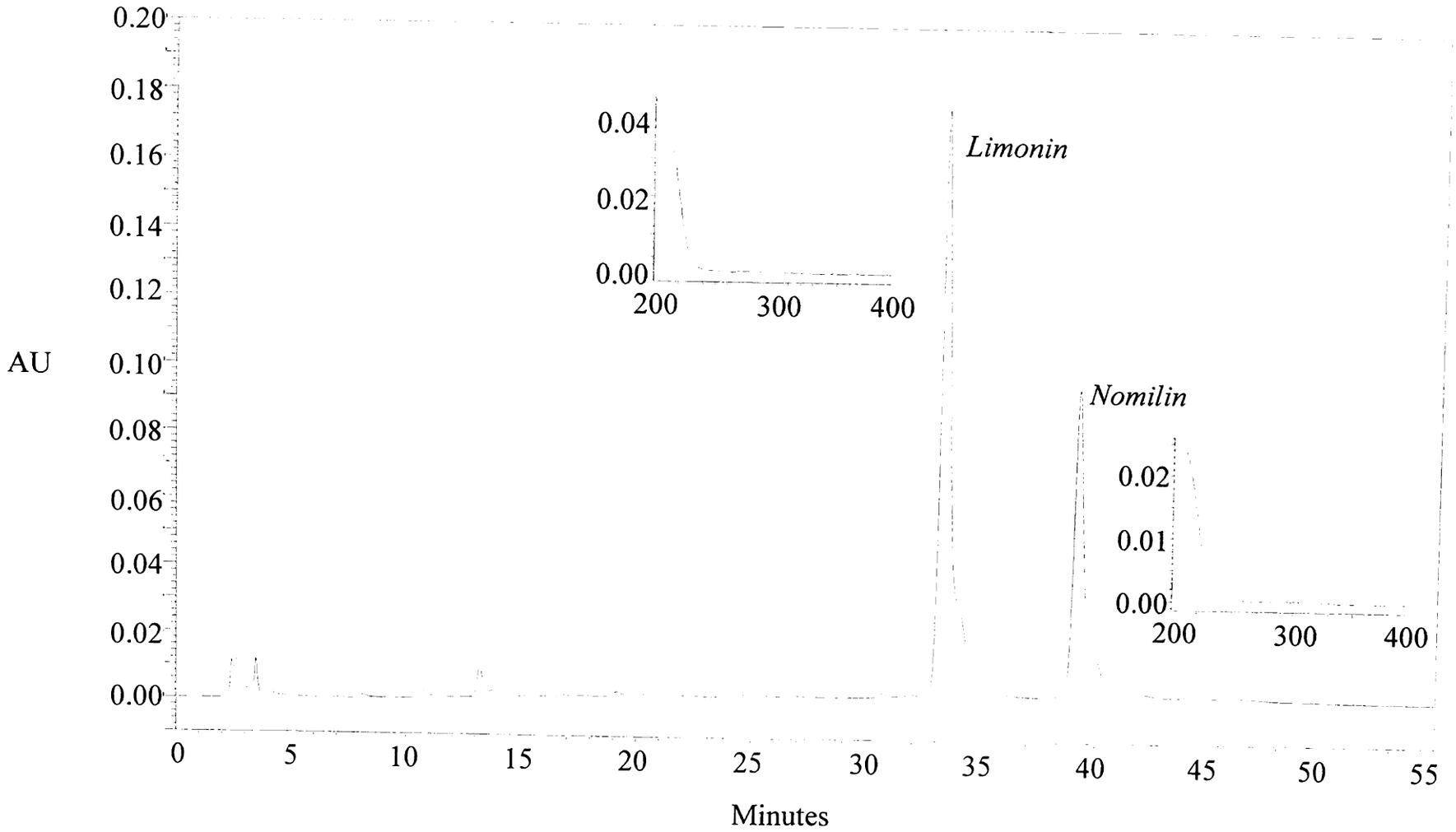


Figure 66: Chromatograms and UV spectra of limonin (33.25 min) and nomilin (39.10 min).

APPENDIX VIII

Oil content in studied sweet orange seeds.

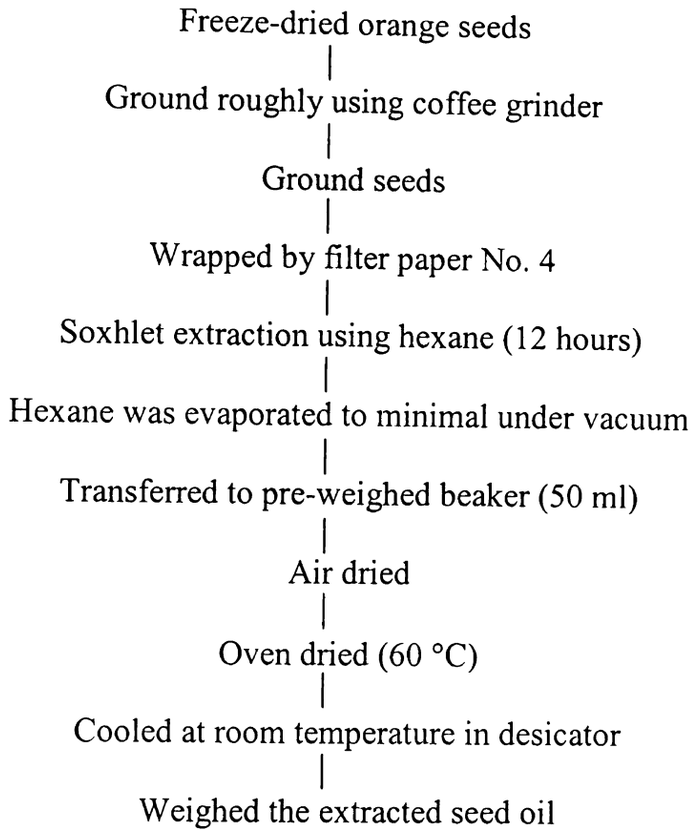


Figure 67: Flow diagram of orange seed oil extraction for estimation of oil content.

Table 71: Oil content in studied sweet orange seeds.

Variety	% Seed oil ± Std[1]
Hamlin	32.14 ± 2.42
Parson Brown	35.23 ± 0.36
Valencia	31.64 ± 0.32

[1]N = 3

APPENDIX VIIII

Processing qualities for juice production of studied orange varieties.

Table 72: Processing qualities of studied orange varieties.

Characteristics	Hamlin	Valencia	Parson Brown
Harvest season	Oct-Jan Early mature.	Feb-Jun Latest maturing of all oranges.	Oct-Jan
Quality of orange	Larger and less seedy than Parson Brown, dual purpose variety, high juice content, thin rind, more cold tolerant fruit tree	Medium to large, well color and thin rind, high juice content, 2-4 seeds per fruit, excellent shipping and storing quality	Seedy (15 seeds per fruit), small, smooth thin and well color rind, high juice content
Quality of processed orange juice	Light color, Sweet but light flavor	Good color, good flavor	Not good color, Poor quality
Processing application	Blending variety for both NFCOJ and FCOJ	Main variety for both NFCOJ[1] and FCOJ	For FCOJ[2] only

[1]Not from concentrate orange juice
[2]Frozen concentrate orange juice

APPENDIX X

Calculations.

- Plate No.

$$N = 16[(t/W)^2]$$

t...Retention time
W...Bandwidth

- Resolution (Rs)

$$Rs = \frac{2(t2-t1)}{W1+W2}$$

t_1 and t_2...Retention times of the first and second adjacent bands
W_1 and W_2...Baseline bandwidths

- Concentration of a compound (%) in an extract analyzed by HPLC:

$$\text{Compound} = \frac{(Rs)(CF)(Vtotal)}{(Vinj)(\text{Amount of sample})} \times 100$$

Rs.....Detector response value for the test sample
Vtotal...Total volume of solution (ml)
Vinj.....Volume of unknown injected solution (ml)
CF.....Calibration factor from the slope of standard calibration curve (g/AU or HU)

- Standard deviation (S)

$$S = \sqrt{\Sigma (u-\bar{u})^2/(n-1)}$$

n...number of observations
u...observation
$\bar{u}$...mean of observation

- Coefficient of variation (%CV)

$$CV = \frac{\text{Standard deviation}}{\text{Mean}} \times 100$$

- %Moisture content

$$\% = \frac{1- \text{Wt. of sample after drying}}{\text{Wt. of sample before drying}} \times 100$$

APPENDIX XI

Summary of HPLC instrumentation diagnostics used during this research.

Table 73: Summarized HPLC trouble shootings.

Problem	Cause	Solution
No peaks or very small peaks	Detector off	Check detector
	Broken connection between detector	Check connection
	No flow	Check flow
	No sample/wrong sample	Check sample Be sure it is not degraded
	Wrong setting on recorder or detector	Check the setting.
Inconsistent retention time	Leak	Check fittings, pump, and seals for leaks
	Change in mobile phase composition	Check. Make new
	Air bubble in the pump	Check flow Prime pump Check and change seals Be sure the mobile phase is degassed
	Temperature fluctuations	Stabilize column temp Use column oven
	Sample overloading	Dilute sample
	Sample dissolved in a solvent that is incompatible with the mobile phase	Dissolve sample in the mobile phase whenever possible. Adjust
Change of separation or loss of resolution	Leak	Contamination of the mobile phase. Prepare new one
	Obstructed guard or column	If guard column is obstructed, change the filter or repack it. If the analytical column is obstructed reverse it and flushes disconnected from the detector or replace the column.

Table 73: Summarized HPLC trouble shootings.

Problem	Cause	Solution
Peak splitting	Contamination of column or guard	Remove guard column. If the problem is solved replace it. If not go next.
	Plugged column	If guard column is obstructed, change the filter or repack it. If the analytical column is obstructed reverse it and flushes disconnected from the detector or replace the column.
	Plugged inlet frit	Replace. If the problem persists discard column
Peak tailing	Active sites within the column	Test with standard test mixture. If ok, add competing base or acid modifier.
	Wrong pH	Correct.
	Wrong column	Change.
	Void volume at inlet	May need repacking.
	Wrong sample solvent	Dissolve sample in mobile phase
Peak fronting	Column overload	Dilute the sample.
	Wrong pH	Correct
	Sample solvent incompatible with mobile phase	Dissolve sample in mobile phase
	Void volume at inlet	May need repacking
	Wrong sample solvent	Dissolve sample in mobile phase
Rounded peaks	Detector outside linear dynamic range	Reduce sample
	Gain too low	Adjust
	Column overloaded	Dilute the sample.
	Time constants (detector, recorder) too high	Reduce
	Wrong sample solvent	Dissolve sample in mobile Phase.

Table 73: Summarized HPLC trouble shootings.

Problem	Cause	Solution
Base line drift	Fluctuation of column temp.	Stabilize. Use column oven.
	Contamination of mobile phase	Use HPLC grade solvent. Degas.
	Air bubble in the detector cell	Use HPLC grade solvent. Degas.
	Air bubble in the detector	Clean cell. If necessary use a pressure restrictor at outlet.
	Plugged detector outlet line	Replace
	Default mixing	Check mixer unit. Check flow rate and composition
	Plugged detector outlet line	Replace.
	Strongly retained materials	Flush column with strong solvent.
	Un-optimized detection	Optimized detector.
Base line noise	Air bubbles	Flush system, prime pumps, degas mobile phase.
	Pump pulse	Use a pulse damper.
	Incomplete mixing	Promote complete mixing
	Electronic	Check electronic equipment in the same line.
	Leak	Check fitting, pump, and seals for leaks.
Broad peaks	Altered mobile phase	Make new.
	Low flow rate	Increase.
	Leak	Check fittings, pump, and seals for leaks.
	Incorrect detector settings	Check and correct
	Column overload	Dilute sample.
	Void volume. Tubing too long or to wide	Use 0.010" tubing. Shorten path.
	Low buffer concentration	Increase
	Column or guard column contamination	Replace.
	Void volume at inlet	Repack

Table 73: Summarized HPLC trouble shootings.

Problem	Cause	Solution
Change in peak height	Sample deterioration	Use fresh sample
	Leak	Check fittings, pump, and seals for leaks
	Non-reproducible sample volume	Ensure loop is completely filled
	Low detector response	Check detector settings and operating conditions
Negative peaks	Recorder connections	Check polarity
	Refractive index of mobile phase higher than that of solute	Change mobile phase
	Vacancy peaks	Originate from great difference in composition between sample solvent and mobile phase. Dissolve sample in mobile phase
	Mobile phase more absorptive than sample components	Use mobile phase that is transparent at the wavelength used
Ghost peaks	Contamination of injector or column	Always flush injector after each injection.
	Retained compound from previous injection	Flush column with strong solvent after operation to remove late eluting compounds
No flow	Pump off	Start pump
	Flow interrupted	Check reservoirs for position of the inlet tubing
		Check loop for air bubble
		Check degassing of mobile phase
		Check compatibility of the mobile phase components
	Leak	Check fittings, pump, and seals for leaks
	Air bubble in the system	Disconnect column and prime pump
		Flush system with 100% methanol or isopropanol

Table 73: Summarized HPLC trouble shootings.

Problem	Cause	Solution
Low pressure	Leak	Check fittings, pump, and seals for leaks
	Flow interrupted	Check reservoirs for position of the inlet tubing Check loop for air bubble Check degassing of mobile phase Check compatibility of the mobile phase components
	Air bubble in pump	Disconnect column and prime pump Flush system with 100% methanol or isopropanol
	Worn pump seals	Replace seals Check pistons and replace if necessary
High pressure	Pump, injector, tubing	Disconnect column, run pump at 25 ml/min: Is the pressure minimal? Go to next step If pressure still high check systematically from detector to pump for obstruction.
	Obstructed column or guard column	If guard column is obstructed, change the filter or repack it. If the analytical column is obstructed reverse it and flush disconnected from the detector or replace the column.

After: http://www.dq.fct.unl.pt/qof/hplcts1.html