Extraction Parameters and Capillary Electrophoresis Analysis of Limonin Glucoside and Phlorin in Citrus Byproducts

Robert J. Braddock* and Charles R. Bryan

Citrus Research and Education Center, University of Florida, 700 Experiment Station Road, Lake Alfred, Florida 33850

Limonin glucoside (LG) and phlorin were extracted from citrus fruit tissues and assayed by capillary electrophoresis (CE). LG was determined in dried [1.20 ± 0.10 mg of dry weight (dw)] and wet peel residues (1.16 ± 0.04 mg of dw), orange juice finisher pulp (0.58 ± 0.03 mg of dw), dried grapefruit seeds (2.70 ± 0.15 mg of dw), and 50 °Brix molasses (2225 ± 68 mg/L). Phlorin was purified from orange peel residue and grapefruit albedo, and concentrations were determined in some citrus products. Phlorin and LG were extracted from residues with water/pectinase or with water solutions of methanol and ethanol. Efficient LG extraction from grapefruit seeds (2.40 ± 0.15 mg/g) was achieved with 50−65% methanol, solvent polarity $P' \approx 7-8$. Extracts were purified and concentrated by adsorptive resins and HPLC to obtain 95% pure compounds of LG and phlorin. CE analysis did not require extract purification beyond filtration. LG and phlorin migrated as anions in electropherograms containing peaks representing other citrus flavonoids and limonoid glucosides.

Keywords: *Limonoids; phlorin; citrus; capillary electrophoresis; orange juice*

INTRODUCTION

Reports of the supposed pharmacological activity of limonoid glucosides have resulted in a number of publications dealing with procedures for extraction of these compounds from citrus tissues and analysis by HPLC (*1*). The interest in phlorin stems from its potential as an adjunct to analytically detect adulteration of juice with water extracts of pulp and peel. Limonin glucosides and phlorin are found in the tissues of citrus fruit and water extracts of that tissue; thus, isolation, purification, and analysis may exploit their water solubility. The limonoid glucoside in highest concentration in most citrus fruit tissue is limonin 17-β-D-glucopyranoside (LG), with glucoside derivatives of nomilin and other limonoids present in lesser amounts (*2*, *3*). Although the limonin aglycone predominates in grapefruit seeds, nomilin glucoside (NG) is more abundant than LG in this tissue.

Published procedures to extract, purify, and recover LG have involved extraction of seeds with methanol using a Soxhlet method (*3*), orange juice, and fresh fruit tissue with 70% methanol (*4*) or buffer + methanol (*5*). LG in citrus molasses has been quantitated by using a procedure requiring resin adsorption and elution with methanol (*6*), and this method has been proposed as a potential commercial recovery procedure (*7*). Commercial recovery of LG from citrus fruit fractions may be difficult to economically implement, because whole oranges contain only ∼50 mg/fruit (*8*).

The phenolic glucoside and phlorin occurs naturally in most citrus fruit tissue, with highest concentrations in the albedo (*9*). Phlorin may be extracted from fresh tissue with water and is a component of the juice and juice-pulp-extractor residue streams in citrus processing plants. Phlorin, like LG, has questionable economic value to the citrus processing industry as a byproduct. It is chemically unstable during storage; however, there is some interest in using it for a juice adulteration marker. Specifically, HPLC analysis of phlorin has been studied in attempts to reliably detect addition of pulp-wash and/or aqueous peel extracts to pure orange juice (*9*−*11*).

Analytical methods for the determination of LG and phlorin in citrus fruit components and extracts have primarily involved HPLC. One study described an HPLC procedure separating limonin, nomilin, nomilinic acid, deacetylnomilinic acid, and obacunone glucoside (OG) (*12*). Limonoid aglycones and LG have also been determined in seeds from several citrus varieties by both TLC and HPLC (*3*). Combination of HPLC with mass spectral analysis was used to separate and identify LG in chloroform extracts of lemon peel (*13*). Capillary electrophoresis (CE) in conjunction with the detergent lauryl sulfate allowed separation and analyis of several limonoid glucosides from citrus seeds (*14*). CE has recently been used as an analytical tool to monitor flavonoids and phlorin present in citrus juices with the intention of estimating quality or adulteration (*15*). The present study defines solvent extraction parameters for recovering LG and phlorin from various citrus tissues and products, followed by quantitative CE analysis of these compounds.

MATERIALS AND METHODS

LG Extraction from Seeds. Grapefruit seeds were separated by hand from the juice extraction processing residue of seedy grapefruit (Duncan), washed with water, dried for 12 h at 60 °C in a vacuum oven to 0% moisture, comminuted dry in a blender, and stored at −6 °C for use as needed. LG was recovered as follows: dried seeds (25 g) were ground in a blender (Omni-Mixer, Sorvall, Newtown, CT) with dichloromethane (3 × 100 mL), followed by filtration to recover the cake, discarding the liquid containing lipids and limonoid

* Author to whom correspondence should be addressed [telephone (863) 956-1151; fax (863) 956-4631; e-mail rjbk@ufl.edu].

10.1021/jf010737n CCC: $20.00
Published on Web 10/31/2001

aglycones. The cake was dried free of dichloromethane under a hood, and samples were blended three times each with either water or 65% methanol (100 mL) to extract LG and concentrated to dryness on a rotary vacuum evaporator at 40 °C; the residue was solubilized in water (150 mL) for further purification. The extraction efficiency of LG from defatted seeds was determined by extracting triplicate 1 g samples with 3 × 15 mL of water, or 20, 40, 65, or 95% ethanol, or 20, 40, 65, or 100% methanol. Recovery efficiencies of LG were determined by spiking samples (1 g) with 1 mg of purified LG and extracting with 3 × 15 mL of water or 65% methanol.

LG Extraction from Fresh or Dried Peel, Molasses, and Finisher Pulp. Commercially manufactured, dried (10% moisture) Valencia orange peel residue (dried citrus pulp) was comminuted in a Wiley mill to 1 mm particle size and finely ground to a powder. Triplicate dry samples (50 g) were blended with 500 mL of water or optionally with water solutions of ethanol and methanol at room temperature, filtered through cheesecloth, and repeated two times with 300 mL of solvent; extracts were combined and centrifuged (10000*g*, 10 min) and then filtered through Whatman No. 1 paper and 0.45 μm membrane filters (type HVLP, Millipore). The filtrates were analyzed by CE to determine LG concentrations. To determine recovery efficiencies, triplicate dried samples (2 g) were spiked with LG (1 mg) and extracted with 3 × 15 mL of solvent.

Fresh Valencia orange peel (flavedo + albedo) was the source for extraction of quantities of LG for purification and recovery studies. A mixture of peel (500 g) + water (1000 mL) was blended at high speed for 30 s in a stainless steel blender, poured into a 2 L beaker, and stirred for 30 min after the addition of 0.5 mL of a commercial pectolytic enzyme (Biopectinase 300L, 52,102 ADJU/mL, Quest International, Hoffman Estates, IL). The mixture was filtered through cheesecloth, the liquid recovered, and the retentate washed with water (800 mL) and stirred for another 30 min before the liquid was recovered. The combined liquids were centrifuged (10000*g*, 10 min) and then filtered (Whatman No. 1). The filtered liquid was divided into 250 mL portions and stored frozen until purification/CE analysis. For analytical screening of extractor peel residues for LG content, triplicate samples of wet residue (7 g) were extracted with either water or 25% ethanol (3 × 15 mL). This was replicated two times. Recovery efficiencies were determined by spiking samples with 1 mg of LG.

Commercial Valencia orange molasses (50 °Brix) was diluted with distilled water to 20 °Brix, the pH was adjusted to 3.5, and the mixture was refrigerated overnight, allowing precipitation of much of the hesperidin. The supernatant was centrifuged (10000*g*, 10 min) and filtered with Whatman No. 1 paper. Filtrates were diluted to 2 °Brix and centrifuged (14000*g*, 1 min), and then samples (5 mL) were filtered (0.45 μm) for CE analysis of the unpurified sample. Purification was performed by use of an adsorbent resin (XAD-16, Sigma, St. Louis, MO) similar to a procedure using C_{18} adsorbent (*6*). Elution of the resin with 25% ethanol resulted in a 2 °Brix molasses extract suitable for CE analysis. Each sample of molasses was analyzed three times.

Finisher pulp (7 g, 80% moisture) from juice extraction of pineapple and Valencia oranges was extracted similarly to the peel residue with 25% ethanol (3 × 20 mL) for the purpose of determining its LG and phlorin contents.

Purification of LG. Batch columns of an adsorbent resin (XAD-16) and a weak anionic exchanger (DEAE-cellulose, Sigma) were used for recovery and purification of LG. The adsorbent, XAD-16 (50 g wet weight, 90 mL bed volume) in a 350 mL sintered glass filter funnel, was initially conditioned with 3 × 100 mL of 95% ethanol and equilibrated with 4 × 100 mL of HPLC grade water. The DEAE-cellulose column (20 g wet weight, 40 mL bed volume) before sample application was generated to Cl^- form by washing with 200 mL of 0.5 N HCl, followed by rinsing with 200 mL of HPLC grade water (pH 6−7).

LG was purified by applying peel extracts (300 mL) to the XAD-16 column and eluting to the bed volume, discarding the eluate, adsorbing both LG and phlorin. First, the column was washed with water (700 mL), discarding 500 mL and then collecting the final 200 mL eluate for phlorin purification. Then the column was washed with 75% ethanol (450 mL), collecting the eluate containing LG, flavonoids, and other nonpolar impurities. The column was regenerated with water (600 mL) to remove the ethanol, another 300 mL extract applied, and the process repeated to obtain enough LG and phlorin for further purification. The combined ethanol extracts containing LG were evaporated to dryness (rotary vacuum evaporator at 40 °C) and dissolved in 100 mL of water.

Final purification consisted of applying the LG concentrate (pH 6−7, 100 mL) to the DEAE-cellulose column and washing with water (200 mL) to remove unbound impurities. The column was washed with 25 mM NaCl (pH 6−7, 160 mL), and LG was recovered by collecting the eluate. Regeneration for reuse was with 5 column volumes 0.5 N HCl, followed by water. The NaCl from the eluate, containing LG, was removed by application as described above to an XAD-16 column dedicated for that purpose. The 75% ethanol eluate contained the LG and was decolorized by stirring with 2% activated charcoal (Darco G-60, Fisher Scientific) and filtered. The filtrate was evaporated to dryness on a watch glass at room temperature for recovery of 85% LG crystals, determined by CE of standard solutions of pure LG.

If an HPLC is available, an alternate preparative HPLC LG purification avoids the loss incurred during the above charcoal-decolorizing step. The procedure involved HPLC injection of 5 mL of sample (2.3 mg/mL LG) from the XAD-16 column. The HPLC (Perkin-Elmer series 4) conditions were as follows: inject sample, hold 1 min, ramp to 20% ethanol/80% water in 10 min with a linear gradient, Chromaflex column (Kontes, Vineland, NJ), 1 cm × 15 cm, packed with 40 μm C_{18}, 12 mL volume, maximum pressure = 0.7 MPa, 5 mL/min flow rate, manual injector with 5 mL loop, UV detection at 210 nm. A fraction collector sampled the LG peak. This procedure allowed accumulation of >95% pure LG.

Purification of Phlorin. Phlorin was recovered by extraction from grapefruit albedo, after peeling, to remove the colored flavedo. The flavedo contains oil and pigments, which make phlorin purification more difficult, especially from orange peel. Albedo (50 g) was extracted with water (250 mL) at high speed for 30 s, and 1 mL of pectolytic enzyme (Biopectinase 300 L) was added and stirred for 30 min, followed by heating to 90 °C to inactivate the enzyme. The liquid was centrifuged (10000*g*, 10 min) and then filtered (Whatman No. 1) to clarify, resulting in 185 mL of pH 3.42 extract. To recover and purify phlorin from the clarified extract, a procedure for the extraction of phlorin from navel orange peel, modified by use of an XAD-16 column, was used (*9*).

CE Analysis of LG and Phlorin. LG and phlorin contents of extracts and process steps were determined by CE analysis. Sample extracts (2−5 mL) were filtered (0.45 μm) and 1 mL microvials (15 × 45 mm) loaded into the autosampler of the CE instrument (P/ACE 2100 with System Gold Software, Beckman Instruments, Fullerton, CA). Run conditions were as follows: 75 mM borax, pH 9.4, 15 kV, 25 °C, 50 μm × 57 cm fused silica column, 214 nm detection, 5 s pressure injection. These conditions were suitable for quantitative analysis of either LG or phlorin. Purity and quantitation of LG and phlorin samples were determined by CE analyses and comparison to pure standard solutions in the range from 20 to 250 mg/L for LG and from 25 to 200 mg/L for phlorin. Although the minimum detection limits were low (LG = 2.0 mg/L; phlorin = 0.2 mg/L), sample extracts were much more concentrated, reflected by the range selected for the standard curves (Table 1). All quantities were determined at the proper dilution by comparison with a linear standard curve of peak area versus LG concentration; for example, before dilution, the range for seed extract was 0.2−2.0 mg/mL. Pure limonin, nomilin, and obacunone glucosides were donated (S. Hasegawa, USDA, Albany, CA), and phlorin was purified from fresh grapefruit albedo extract in the authors' laboratory and identified by comparison with literature UV spectra (*9*).

Phlorin purity and quantitation were by comparison with "citrate units" following a published procedure (*10*), where it was shown that at 214 nm, the absorbance of 10800 mg/L citric

Table 1. Statistical Data for Standard Curves of Limonin Glucoside (LG) and Phlorin Analysis by Capillary Electrophoresis

	CE peak area[a]	
LG (mg/L)		
2	0.025 ± 0.001	
22.9	0.214 ± 0.005	
45.8	0.442 ± 0.013	
91.6	0.929 ± 0.042	
229	2.329 ± 0.031	$r = 0.99$
phlorin (mg/L)		
1	0.034 ± 0.001	
2	0.085 ± 0.002	
10	0.338 ± 0.003	
25	0.873 ± 0.039	
50	1.709 ± 0.066	
100	3.352 ± 0.054	
200	6.681 ± 0.200	$r = 0.99$

[a] Area from absorbance at 214 nm, triplicate analyses.

acid solution = 300 mg/L phlorin. A standard curve between 2000 and 20000 mg/L citric acid (ACS anhydrous, Fisher Scientific) was generated for CE analysis of citrate/phlorin solutions to determine the concentration of phlorin extracts and the purity of dried phlorin solutions. Conditions of CE analysis for citrate quantitation were as follows: 20 mM borax, pH 9.1, −15 kV, 25 °C, 50 μm × 27 cm linear polyacrylamide column (Bio-Rad), 214 nm, 5 s pressure injection. Once phlorin purity was determined by citrate comparison based on the work of Johnson, further measurement of phlorin concentration and purity was by comparison with a standard solution of potassium sorbate (minimum = 99%, Sigma), where 100 mg/L sorbate = 112 mg/L phlorin. Use of sorbate as an internal standard was simpler, because lower concentrations were required and sorbate is not a component of citrus extracts. Phlorin standards ranged from 1 to 200 mg/L solutions (Table 1). CE analysis conditions for phlorin and sorbate were the same (75 mM borax, etc.) as above for LG and phlorin analysis.

RESULTS AND DISCUSSION

Use of CE for measurement of LG and phlorin in aqueous citrus fractions offers several potential advantages compared with HPLC. Some are inherent in the technique, for example, use of very small quantities of water or buffer, no use of organic solvents requiring disposal, simple sample preparation, low-cost silica columns, high resolution of sample peaks, and short retention times. Both LG and phlorin migrated as separate anions resolved in the electropherograms of a single injection. CE may also be used to monitor extraction and recovery efficiencies, determining yields of these compounds from various citrus substrates.

LG Extraction and Analysis. *Seeds.* Higher amounts of limonoids (aglycones + glucosides) in seeds (~1% dry weight) than in other fruit tissues might suggest they be considered as a commercial source for LG. The truth is that few seedy varieties of grapefruit (and oranges) are now processed for juice recovery, and it is not practical to remove seeds from juice extractor residue (*8*). However, recovering small quantities of LG from seeds for research studies is possible by adapting a Soxhlet extraction procedure (*3*) to include comminuting and rapid multiple extractions. The yield of LG was not affected by elimination of the hexane extraction step to remove neutral lipids, as dichloromethane removed both limonin and neutral lipids.

CE monitored the extraction efficiency of LG from dried grapefruit seeds with methanol and/or ethanol/water solutions. A typical CE electropherogram of a 65% methanol extract of defatted seeds illustrates the LG peak (1.81 mg/mL) from an extract (Figure 1). The scheme for dried seeds allowed comparison of LG extraction with water and solutions of methanol and ethanol, based on the solvent polarity, P' (*16*). Results monitored by CE indicated that extractions with water or alcohol solutions in the polarity range $P' \sim 6.5-10$ were the most selective for extracting LG. This range corresponds to 65−40% methanol ($P' = 7-8.2$), 40−25% ethanol ($P' = 6.5-8.5$), or water ($P' = 10$). The maximum amount (2.7 ± 0.15 mg of LG/g of dried defatted seeds) extracted from grapefruit seeds was obtained using 65% methanol (Figure 2). When seed meal samples were spiked (1 g) with pure LG (1 mg) and three consecutive extractions performed, the average LG recovery with 65% methanol was 99.7 ± 2.8% and with water as solvent was 81 ± 4.1%. The amount of LG was in the range previously reported for grapefruit seeds (*3*), using HPLC analysis involving enzyme hydrolysis and extraction steps not required during CE analysis. There is also wide variation in biosynthesis of these compounds with maturity for different fruit (*5*).

Dried Peel. Dried Valencia orange peel residue was used in a study designed to determine the best solvent for LG extraction from peel. Mimicking the seed study, a concentration range between 25% ethanol and 65% methanol and water extracted similar amounts of LG. Ethanol is less toxic than methanol, and lower concentrations (25%) were almost as effective as higher concentrations of methanol (65%) for extractions of peel.

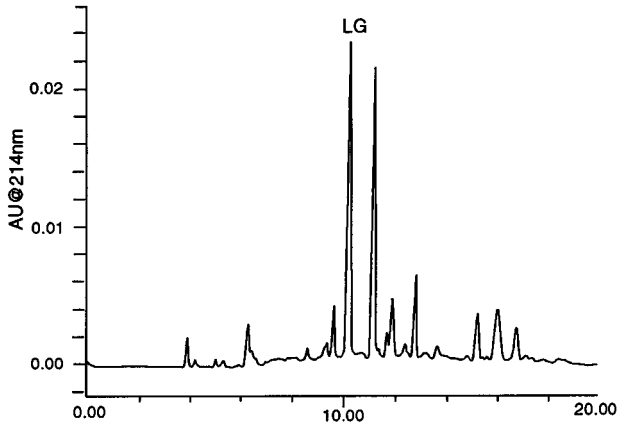

Figure 1. CE electropherogram indicating the LG peak from defatted grapefruit seed 65% methanol extracts evaporated to dryness and solubilized in water. Run conditions: 75 mM borax, pH 9.4, 15 kV, 25 °C, 50 μm × 57 cm fused silica column, 214 nm, 5 s pressure injection.

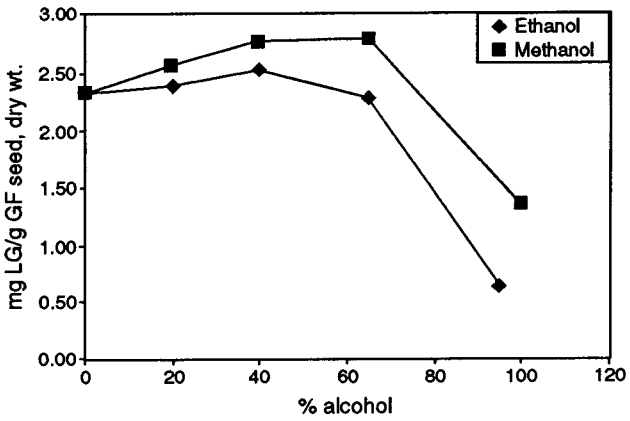

Figure 2. Recovery of LG from seeds with water solutions of ethanol or methanol.

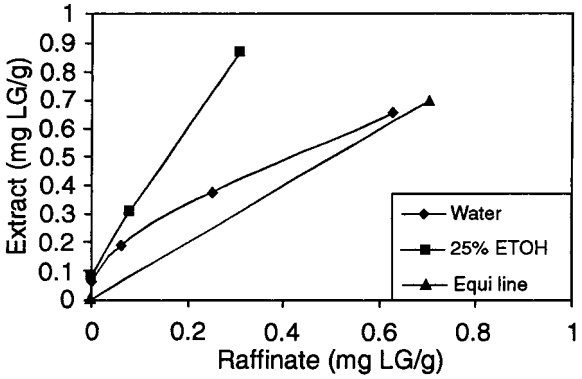

Figure 3. Extraction of LG from dried peel with 25% ethanol and water, illustrating LG in the feed and raffinate and the equilibrium line for extracting LG from the raffinate.

It was determined that three consecutive extractions with either 25% ethanol or water extracted the maximum amount of LG (1.20 ± 0.10 mg/g of dried peel). The extractions performed were simple multistage contact where the feed (dried peel) was treated with consecutive solvent amounts in stages, where the peel from the first extraction (raffinate) is extracted with fresh solvent in a second extraction, etc. (*17*). Spiking with pure LG and extraction similar to that for seeds determined LG recovery with either solvent was approximately 96 ± 2.5%. Results of the experiments (Figure 3) indicate that 25% ethanol extracted more LG from the feed and raffinate in fewer stages than water. Both solvents were above the equilibrium line for extracting LG from the raffinate. The value (1.2 mg/g) seemed to be reasonable when compared to the amount of total limonoid glucosides estimated for dried peel residue (180 g/75 kg of dried peel) from Satsuma mandarin (*4*). In that study, it should also be mentioned that the amount in the dried peel was reduced by the amount in the press liquor stream.

Fresh Peel Residue. When fresh Valencia peel residue was used as the source of LG, extractions with 25% ethanol or water gave results (1.16 ± 0.04 mg/g, dried peel basis) similar to that from dried peel. If water was used as the extracting solvent with fresh peel residue, pectolytic enzyme treatment was necessary to reduce viscosity and foaming during subsequent blending, filtering, and centrifugation steps. However, water extraction facilitated CE analysis or application to adsorbent resins when recovery and purification were required, as transfer from methanol or ethanol solutions to water was eliminated. An electropherogram typical of a water or 25% ethanol crude extract, indicating LG and phlorin peaks, is presented (Figure 4). The LG peak does not resolve as well in the electropherograms of the crude peel extract as in the seed extract (Figure 1) because there are more interfering compounds in the peel.

A recent LC-MS quantitative study of the limonoid glucosides in some fruit components reported LG content in orange peel as 1.36% dry weight (*18*). This value is ~10 times greater than the value (1.16 ± 0.04 mg/g of dry weight) we reported above. Our analysis and other literature for mandarin peel residue [359 mg/L, wet weight (*4*)] and Valencia peel [200−300 mg/L, wet weight (*5*)] indicate the values of Schoch for dry samples must be in error. For conversion of milligrams per liter, wet weight, values to dry weight, citrus peel is ~75−80% moisture (*8*).

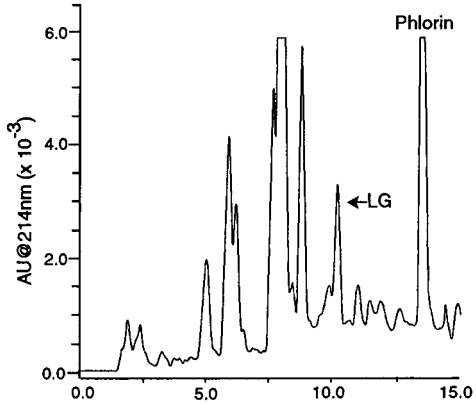

Figure 4. CE electropherogram typical of water or 25% ethanol peel residue crude extract, indicating the LG and phlorin peaks. Run conditions: 75 mM borax, pH 9.4, 15 kV, 25 °C, 50 μm × 57 cm fused silica column, 214 nm, 5 s pressure injection.

Molasses. Use of CE to analyze commercial 50 °Brix Valencia molasses (concentrated peel press liquor) required the sample to be diluted in the range of 1−2 °Brix, which was the normal concentration range after the purification procedure. Because of the high hesperidin concentration in citrus molasses, removal by precipitation before analysis or adsorption resulted in better resolution of the LG peak during CE analysis. Analysis of the 2 °Brix eluate allowed estimation of the LG concentration in the 50 °Brix molasses as ≈2225 ± 68 mg/L. This value is similar to that reported (*6*); however, it should be understood this commercial citrus byproduct has a variable composition resulting from many sources of citrus peel.

Finisher Pulp. The LG content of finisher pulp samples from pineapple orange juice recovery was 0.35 ± 0.01 mg/g of dry weight pulp, whereas that from Valencia orange was 0.58 ± 0.03 mg/g of dry weight pulp. Both samples were fresh from a juice-processing operation set up for similar finisher pressures. The results indicate that this fruit fraction contains much less LG than the peel or seeds, and there is variability between these two orange cultivars.

Purification and Analysis of LG. Purification of LG from peel extracts by adsorption and ion exchange resins and charcoal decolorizing by procedures modified from published studies (*4*, *7*) was found to be effective. The adsorption of LG by XAD-16 at pH ~4 allowed water washing to remove anionic constituents that bind to DEAE-cellulose. LG is anionic at pH 6−7 and binds to DEAE-cellulose; however, hesperidin and other flavonoid glycosides are not charged until pH >8−9. This allows water washing to remove these impurities, followed by elution of LG with 25 mM NaCl. The final purification step is accomplished by passing the eluate through an XAD-16 column for desalting. The capacity of the XAD-16 before breakthrough of LG was ~1.5 mg of LG/g of wet adsorbent. When the LG-containing XAD-16 effluent was further purified by DEAE-cellulose, the DEAE capacity before LG breakthrough was 6.5 mg of LG/g of wet DEAE-cellulose. CE analysis of the LG concentration of the crude extract before application to the XAD-16 determined the initial amount, where LG recovery efficiency from the final DEAE purification was 85 ± 5%. After these purification steps, losses were minimized by use of preparative HPLC for isolation of purest LG (95%) rather than use of activated charcoal for decolorizing (10−20% loss).

The recent technique of LC-MS may allow qualitative identification of complex mixtures of limonoid glucosides in a sample extract (*18*). However, monitoring LG extractions and resin purification by CE provides certain data not easily available by other analytical procedures. From the CE migration of various pH eluates from the DEAE column, it was determined that LG was anionic down to pH 5.0, whereas hesperidin (the major constituent of the extract) was not charged until pH ~9.0. Thus, the DEAE resin favors selective retention of LG during application of the XAD-16 water eluates. The XAD-16 resin allows separation of sugars, salts, and other hydrophilic impurities from LG, whereas DEAE-cellulose removes cations and hesperidin (flavonoids) and the charcoal removes other impurities, including the phlorin (see Figure 4). CE electropherograms indicated the presence of contaminants in LG extracts before and after charcoal purification, where charcoal removed most contaminants, except OG (Figure 5a,b). The purest LG (95%) was obtained after preparative HPLC (Figure 5c).

Although the method presented here demonstrates CE separation of LG and OG, at pH 9.4 nomilin glucoside degrades to OG (*19*); thus, electropherograms may not show a nomilin glucoside peak. It is possible to separate these glucosides by CE at lower pH through the use of the detergent SDS (*14*). However, use of SDS will not allow simultaneous separation of both LG and phlorin and has lower sensitivity for LG. By using the CE method described here, the minimum detection limit (10× baseline noise) for LG was 2.00 ± 0.08 mg/L with a migration time coefficient of variation (CV) = 1.32%. Standard curve ranges for CE LG analyses were from 2 to 229 mg/L (Table 1).

Purification and Analysis of Phlorin. An efficient method of isolation and purification of phlorin from Valencia peel extracts involved collecting the final 200 mL of water fraction from the initial XAD-16 cleanup during the LG isolation process. Evaporation of this fraction and final drying on a watch glass produced 90% purity phlorin crystals. This process is easily monitored by CE from the initial water waste fraction to the final determination of >90% purity. Additional purification by charcoal or preparative HPLC resulted in >95% purity and a UV spectrum similar to that of phlorin (*9*).

An interesting use of DEAE-cellulose allowed purification of phlorin in the waste eluate washed with water from the XAD-16 column cleanup. This procedure required generating the DEAE column in OH^- form with 0.1 N NaOH and equilibrating at pH 10 by adding 5 column volumes of water adjusted to pH 10 with NaOH. The XAD eluate (300 mL adjusted to pH 10) was applied to the DEAE column and washed with 300 mL of water (discard), monitoring the pH. Additional water (200 mL) eluted the phlorin. The pK_a of phlorin was estimated by CE to be ≈8.0. Once the pH reached this point during washing, phlorin was released from the DEAE-cellulose.

To quantify the amount of phlorin in extracts and in several citrus fractions and samples, the recovered phlorin from Valencia peel or grapefruit albedo extracts was dried and used as a standard. The minimum detection limit for phlorin was 0.25 ± 0.01 mg/L with a migration time CV = 1.04%, whereas standard curve concentrations ranged from 1 to 200 mg/L (Table 1). Phlorin is very hygroscopic and difficult to weigh accurately; thus, the calibration procedure (Materials and Methods) using sorbate solutions was used to determine the phlorin content of several byproducts. Phlorin produced via this method could be dried for use as a standard and maintained in a vacuum desiccator as a white crystalline material for 2−3 months before gradual degradation through oxidation and browning. The above purities in raw and purified albedo extracts (Figure 6a,b), as well as the phlorin concentration, was determined in dried Valencia peel residue (1.30 ± 0.06 mg/g of dry weight), fresh grapefruit albedo (9.70 ± 0.65 mg/g of dry weight), fresh orange albedo (10.78 ± 0.14 mg/g of dry weight), fresh peel residue (13.6 ± 0.08 mg/g of dry weight), fresh midseason orange juice finisher pulp (0.23 ± 0.01 mg/g of dry weight), fresh Valencia orange juice (8 mg/L), fresh Murcott tangerine juice (33 mg/L), commercial not from concentrate orange juice (30 mg/L), commercial orange juice from concentrate (67 mg/

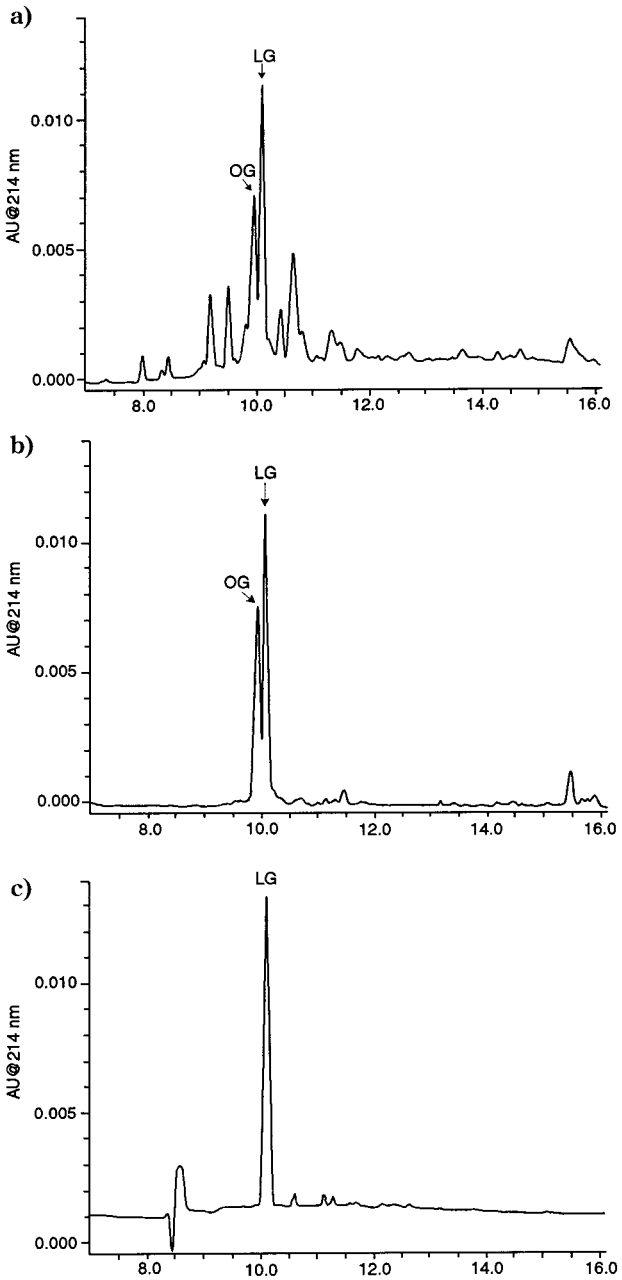

Figure 5. CE electropherograms resolving peaks of obacunone (OG) and LG in extracts of peel residue before (a) and after (b) charcoal purification and after preparative HPLC (c). Run conditions: 75 mM borax, pH 9.4, 15 kV, 25 °C, 50 μm × 57 cm fused silica column, 214 nm, 5 s pressure injection.

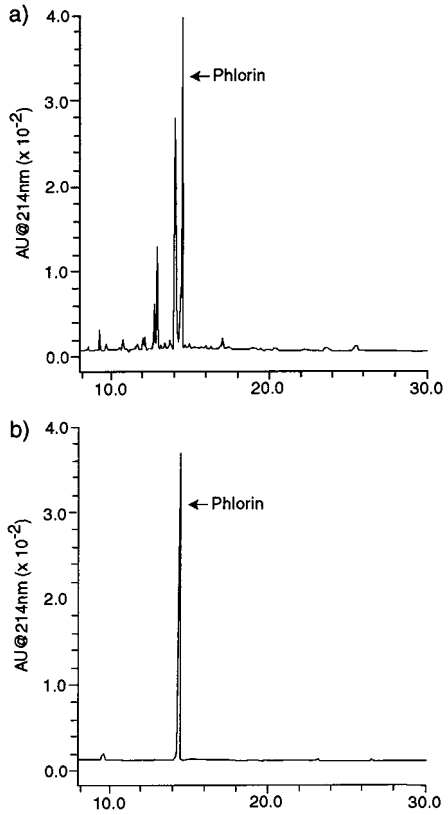

Figure 6. CE electropherograms resolving the phlorin peak from raw unpurified (a) and purified (b) water/pectinase extracts of grapefruit albedo. Run conditions: 75 mM borax, pH 9.4, 15 kV, 25 °C, 50 μm × 57 cm fused silica column, 214 nm detection, 5 s pressure injection.

L), and 50 °Brix orange molasses (6136 ± 140 mg/L). Although the source of phlorin standards was unexplained, one study reported 10 °Brix commercial molasses has 600−900 mg/L phlorin (*20*).

Quantitative profiles show that the amount of phlorin present in citrus products may be determined by CE. These data might be interpreted that low phlorin content in dried peel residue implies destruction during thermal dehydration, because fresh peel has high amounts, juice finisher pulp has very low quantities, and the amount in juices varies considerably. The amount in fresh peel (13.6 mg/g of dry weight) is near the amount of hesperidin in orange or naringin in grapefruit peel residue (*8*). If there were uses for phlorin from citrus, the concentration might allow economical recovery from the residue.

Use of phlorin has been proposed as an index (using HPLC) of orange juice adulteration with water extracts of pulp or peel (*9*, *10*). The juice byproduct, pulpwash, is obtained by water-washing finisher pulp, which has a low phlorin concentration. The implication of this is that, should commercial pulpwash samples have high phlorin contents, they might contain peel or core extracts. Many citrus processors no longer produce pulpwash concentrate as a stand-alone product; most pulpwash goes in-line into the juice stream for frozen concentrate manufacture. A detailed description of the pulp and core washing processes and product properties has been published (*8*). On the basis of the wide variability of processing parameters, extractor variables, juice composition and product variability, and cultural, biological, and geographical differences of fruit, it would be very difficult to prove juice adulteration with extracts of fruit components based only on phlorin analysis.

Except for the data reported above, CE analysis was not used to compare or quantify the amounts of LG and phlorin in the different component parts or byproducts of a wide spectrum of various citrus fruits. It was our intention to offer a CE analytical procedure useful for the study of these compounds. We conclude that CE is a very good analytical technique for this purpose, allowing high-resolution separations of both compounds with minimum sample preparation. CE analysis may be performed directly using methanol or water extracts and concentration by evaporation, if necessary to increase detection sensitivity. This study also provided additional information regarding extraction, isolation, and purification of these compounds. There has been some speculation that LG may have economic value. Familiarity with processes for extraction and recovery of compounds from natural substrates (e.g., citrus residues) should make one aware that the concentrations of limonoid glucosides reported here are too low for economic commercial recovery as byproducts.

LITERATURE CITED

(1) Berhow, M. A.; Hasegawa, S.; Manners, G. D. *Citrus Limonoids: Functional Chemicals in Agriculture and Food*; ACS Symposium Series 758; American Chemical Society: Washington, DC, 2000.

(2) Hasegawa, S.; Bennett, R. D.; Herman, Z.; Fong, C. H.; Ou, P. Limonoid glucosides in citrus. *Phytochemistry* **1989**, *28*, 1717−1720.

(3) Ozaki, Y.; Fong, C. H.; Herman, Z.; Maeda, H.; Miyake, M.; Ifuku, Y.; Hasegawa, S. Limonoid glucosides in citrus seeds. *Agric. Biol. Chem*. **1991** *55* (1), 137−141.

(4) Ozaki, Y.; Ayano, S.; Inaba, N.; Miyake, M.; Berhow, M. A.; Hasegawa, S. Limonoid glucosides in fruit, juice and processing by-products of Satsuma mandarin (*Citrus unshiu* Marcov.). *J. Food Sci*. **1995**, *60* (1), 186−189, 194.

(5) Fong, C. H.; Hasegawa, S.; Coggins, C. W., Jr.; Atkin, D. R.; Miyake, M. Contents of limonoids and limonin 17-β-D-glucopyranoside in fruit tissue of Valencia orange during fruit growth and maturation. *J. Agric. Food Chem*. **1992**, *40*, 1178−1181.

(6) Hasegawa, S.; Fong, C. H.; Miyake, M.; Keithly, J. H. Limonoid glucosides in orange molasses. *J. Food Sci*. **1996**, *61* (3), 560−561.

(7) Ifuku, Y.; Maeda, H.; Miyake, M.; Inaba, N.; Ayano, S.; Ozaki, Y.; Maruyama, K.; Hasegawa, S. Method for manufacturing limonoid glucosides. U.S. Patent 5734046, 1998.

(8) Braddock, R. J. *Handbook of Citrus By-Products and Processing Technology*; Wiley: New York, 1999.

(9) Louche, L. M.-M.; Gaydou, E. M.; Lesage, J.-C. Determination of phlorin as peel marker in orange (*Citrus sinensis*) fruits and juices. *J. Agric. Food Chem*. **1998**, *46*, 4193−4197.

(10) Johnson, R. L.; Htoon, A. K.; Shaw, K. J. Detection of orange peel extract in orange juice. *Food Aust*. **1995**, *47* (9), 426−432.

(11) Hammond, D. A.; Lea, A. G. H.; Rousseau, L. Detection of the addition of pulpwash to orange juice using two new HPLC procedures. *Ber. Int. Fruchtsaft-Union, Wiss.-Tech. Komm*. **1996**, *24*, 233−237.

(12) Herman, Z.; Fong, C. H.; Ou, P.; Hasegawa, S. Limonoid glucosides in orange juices by HPLC. *J. Agric. Food Chem*. **1990**, *38*, 1860−1861.

(13) Baldi, A.; Rosen, R. T.; Fukuda, E. K.; Ho, C. T. Identification of nonvolatile components in lemon peel by high-performance liquid chromatography with confirmation by mass spectrometry and diode-array detection. *J. Chromatogr. A* **1995**, *718*, 89−97.

(14) Moodley, V. E.; Mulholland, D. A.; Raynor, M. W. Micellar electrokinetic capillary chromatography of limonoid glucosides from citrus seeds. *J. Chromatogr. A* **1995**, *718*, 187−193.
(15) Cancalon, P. F. Analytical monitoring of citrus juices by using capillary electrophoresis. *J. AOAC Int.* **1999**, *82* (1), 95−106.
(16) Snyder, L. R. Classification of the solvent properties of common liquids. *J. Chromatogr. Sci.* **1978**, *16* (June), 223−234.
(17) Perry, R. H.; Chilton, C. H. *Chemical Engineers' Handbook*, 5th ed.; McGraw-Hill: New York, 1973.
(18) Schoch, T. K.; Manners, G. D.; Hasegawa, S. Analysis of limonoid glucosides from citrus by electrospray ionization liquid chromatography−mass spectrometry. *J. Agric. Food Chem.* **2001**, *49*, 1102−1108.
(19) Hasegawa, S.; Fong, C. H.; Herman, Z.; Miyake, M. Glucosides of Limonoids. Chapter 8. In *Flavor Precursors: Thermal and Enzymatic Conversions*; ACS Symposium Series 49; American Chemical Society: Washington, DC, 1992.
(20) Manthey, J. A.; Grohman, K. Phenols in citrus peel byproducts. Concentrations of hydroxycinnamates and polymethoxylated flavones in citrus peel molasses. *J. Agric. Food Chem.* **2001**, *49*, 3268−3273.

Received for review June 5, 2001. Revised manuscript received September 22, 2001. Accepted September 22, 2001. This research was supported by the FL Agricultural Experiment Station and approved as J.S. No. R-08185.

JF010737N