# Analysis of Limonoid Glucosides from *Citrus* by Electrospray Ionization Liquid Chromatography−Mass Spectrometry

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An electrospray ionization liquid chromatography−mass spectrometry (ESI-LC-MS) method for the detection and quantitation of limonoid glucosides has been developed. Negative ions $[M - H^+]^-$ characteristic of six limonoid glucosides can be detected and quantified from selected ion monitoring chromatograms using carminic acid as an internal standard. The described method has been applied to the analysis of limonoid glucoside content in various liquid and solid *Citrus* spp. samples as well as complex mixtures of partially purified limonoid glucosides. Rapid and sensitive qualitative screening of samples for limonoid glucosides can also be accomplished with slight modifications of the method.

**Keywords:** *Limonoid glucoside; limonin 17β-D-glucopyranoside; electrospray ionization; liquid chromatography−mass spectrometry; Citrus*

## INTRODUCTION

Limonoids are highly oxygenated triterpenes found exclusively in the Rutaceae and Meliaceae plant families. Limonoid aglycones, in particular limonin (**1**), have been known and studied since the 1940s in relation to the development of "delayed bitterness" in *Citrus* juices from edible species (*1*). However, the discovery and isolation of limonoid glucosides from *Citrus* did not occur until the late 1980s (*2*). The limonoid glucosides are found predominantly in fruit and seed tissues and are biosynthesized in *Citrus* during fruit maturation (*3*). This pathway involves the glucosylation of a limonoid glucoside precursor at C17 (Figure 1). The resulting limonoid glucoside is tasteless and water soluble (*1*); furthermore, the concentration of limonoid glucosides can exceed 300 ppm in citrus fruit and juices (*4*).

Recent research has established that *Citrus* limonoids (aglycones and glucosides) display significant inhibitory activity against cancerous tumors (*5*, *6*) and also induce glutathione-*S*-transferase activity (*7*) in animals. In vitro testing of these limonoids with human breast cancer cell lines has also shown high levels of inhibitory activity (*8*). These results have generated interest in *Citrus* limonoids as potential cancer chemopreventative agents in humans and have fostered the need to examine citrus sources for the nature and amounts of limonoids. Correspondingly, rapid and accurate analytical methods are necessary to quantify the occurrence of limonoids in citrus fruit, processed citrus products, and citrus-processing byproducts.

Current quantitative methodology for the analysis of limonoid glucosides includes a thin-layer chromatography (TLC) method utilizing Ehrlich's reagent as a specific detection reagent (*9*, *10*) and a reversed phase high-pressure liquid chromatography (HPLC) method that utilizes ultraviolet detection at 215 nm (*11*). Both of these analytical methods are restricted by selectivity and sensitivity in the quantification of limonoid glucosides from citrus sources. We have recently developed a qualitative electrospray liquid chromatography−mass spectrometry (ESI-LC-MS) method for the analysis of limonoid glucoside mixtures from citrus sources (*12*), and an ESI-LC-MS method has recently been reported for the screening of limonoid glucosides in a citrus species (*13*). We now report a rapid and sensitive ESI-LC-MS method for the quantitative analysis of six limonoid glucosides commonly present in citrus fruit samples and demonstrate the application of this method to wet and dry samples obtained from citrus sources.

## MATERIALS AND METHODS

**Materials and Chemicals.** HPLC grade acetonitrile, HPLC grade methanol, ammonium hydroxide, and formic acid were obtained from commercial sources. Carminic acid (96%) was purchased from Acros. Water was distilled and deionized prior to use. Limonoid glucoside standards were previously isolated in our laboratory (*2*, *14*).

**Instruments and Equipment.** An LC-MS system consisting of a Waters 2690 solvent/sample delivery system coupled to a Micromass LCZ mass spectrometer equipped with an electrospray ionization (ESI) probe was used. The tuning parameters for the ESI probe were obtained by infusing a 3 mg/L solution of limonin glucoside in the desired mobile phase into the MS and maximizing the signal at *m*/*z* 649.3. A Waters C18 XTerra guard column (2.1 × 20 mm, 3.5 μm particle size) was in-line between the Waters 2690 and the MS for quantitative analysis.

**Qualitative MS Analysis.** The Waters 2690 was used to make direct injections (3−30 μL, depending on concentration) into a 0.3 mL/min flow of 0.5% $NH_4OH/CH_3CN$ (4:1) without a column. The mass spectrometer was scanned from 600 to 800 amu in the negative ion mode. For detection of small quantities of limonoid glucosides, selected ion monitoring (SIM) was used at selected masses.

**Quantitative LC-MS Analysis.** For quantitative results, standards or samples (20 μL) were injected onto the Xterra guard column and eluted isocratically with $CH_3CN$/4 mM HCOOH (15:85, 0.5 mL/min). Column outflow directed to the ESI probe on the mass spectrometer was monitored in SIM

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10.1021/jf001157d 
Published on Web 01/27/2001

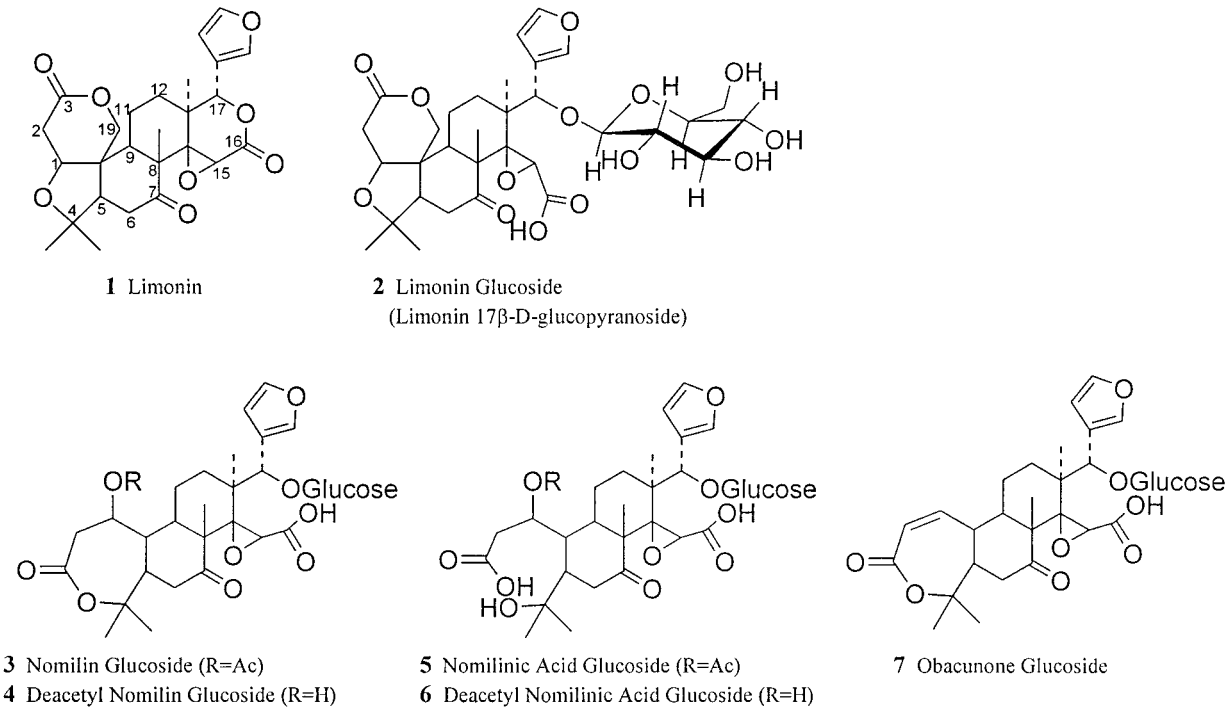


**Figure 1.** Structures of limonin and limonoid glucosides.

mode for seven negative ions (six glucosides and the internal standard carminic acid).

**Standard Curves.** Aqueous stock solutions (20 mg/L) were prepared by dissolving each limonoid glucoside in distilled, deionized water. Two "group" stock solutions were prepared by combining equal amounts of three different limonoid glucoside stock solutions [group 1, limonin glucoside (**2**), nomilinic acid glucoside (**5**), and nomilin glucoside (**3**); group 2, obacunone glucoside (**7**), deacetylnomilin glucoside (**4**), and deacetylnomilinic acid glucoside (**6**)]. The group solutions were used to decrease the number of runs required to generate the calibration curves. Concentrations of the individual limonoid glucosides in the group solutions were 6.67 mg/L. Standard curves were generated for each of the limonoid glucosides after the group solutions had been diluted and combined with 300 $\mu$L of MeOH and 200 $\mu$L of carminic acid solution (30 mg/L) to produce standard solutions (1.5 mL total volume, concentrations of individual limonoid glucosides were 0, 0.4, 1, 2, and 4 mg/L). One additional standard was made for limonin glucoside at a concentration of 8 mg/L. $[M - H^+]^-$ ions for the six limonoid glucoside ions (*m*/*z* 633.3, 649.3, 651.3, 669.3, 693.3, and 711.3) were monitored in SIM mode and the peak areas calibrated versus the internal standard. Standards were prepared and analyzed each day before and after each series of samples to minimize detector response variations. A series of five blanks was run to equilibrate the system before analysis of standards and samples.

**Analysis of Samples.** Solid samples (peel, seeds, etc.) were oven-dried and ground using a mill to pass a 2.0 mm mesh screen, and 100 mg portions were weighed into 10 × 50 mm cellulose extraction thimbles. Samples were extracted overnight in soxhlet extractors with MeOH (25 mL). The resulting solutions were diluted to 30 mL with MeOH, and an aliquot of the extract (300 $\mu$L) was added to water (700 $\mu$L) and carminic acid solution (30 mg/L, 200 $\mu$L) in an autosampler vial. The vials were capped with Teflon septa and analyzed by ESI-LC-MS as detailed above. Pulp samples were dried overnight in a vacuum oven at 60 °C and then treated as above. Wet samples (juice, molasses, wash water, etc.) were centrifuged in 1.5 mL microcentrifuge tubes (5 min at 16000*g*). The supernatant liquid was passed through a 0.45 $\mu$m filter into a clean tube. Concentrated viscous samples, such as 45 °Brix citrus molasses, were diluted 1:10 with water before centrifugation and filtration. Samples for injection were prepared by combining sample (75 $\mu$L), water (925 $\mu$L), MeOH (300 $\mu$L), and internal standard solution (200 $\mu$L) in an autosampler vial with a Teflon septum and analyzed as detailed above. Samples found to have concentrations higher than the highest standard were diluted 1:10 with water, combined with water, methanol, and carminic acid solution, and reanalyzed.

## RESULTS AND DISCUSSION

A typical mass spectrum from a qualitative analysis run is shown in Figure 2. The relative abundances of the characteristic ions roughly reflect the amounts of the limonoid glucosides found in the sample but are not consistent enough to use for quantitation. However, qualitative runs are generally faster and more sensitive than quantitative runs, and they have proven to be useful for screening samples for limonoid glucosides and for quick identification of individual limonoid glucosides. In contrast to the method reported by Tian and Ding (*13*), a column is not required, and sample preparation is simplified. The use of dilute $NH_4OH$ in the mobile phase aids the negative ionization of the acidic limonoid glucosides, resulting in detection limits as low as 40 pg in water for limonin glucoside using SIM. In complex samples such as dilute orange juice the detection limit increases to 200 pg.

A reconstructed total ion current chromatogram (RTICC) from the HPLC separation of a typical mixture of limonoid glucosides and individual SIM chromatograms are shown in Figure 3. The retention times of the individual limonoid glucosides are obtained from the SIM chromatograms (Figure 3b−h) and show that limonin glucoside (**2**) and carminic acid coelute and that there is considerable overlap between limonin glucoside and deacetylnomilinic acid glucoside (**6**) and between nomilinic acid glucoside (**5**) and obacunone glucoside (**7**). Peak areas for individual limonoid glucosides were measured from the SIM chromatograms (Figure 3b−h) and used for quantitation. The detection limit for the quantitative method was 2 ng, about an order of magnitude higher than that of the qualitative method.

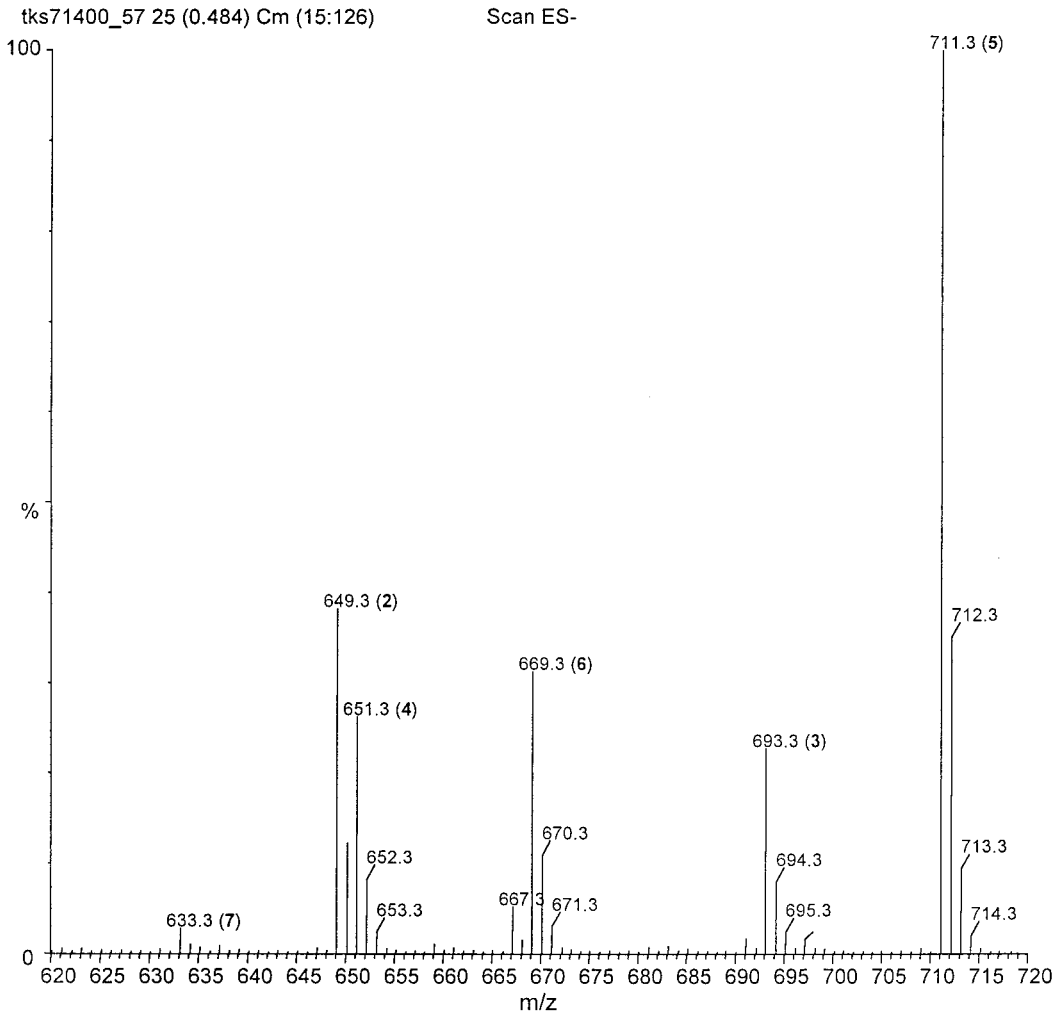


**Figure 2.** Mass spectral profile of a limonoid glucoside mixture: **2**, limonin glucoside; **3**, nomilin glucoside; **4**, deacetylnomilin glucoside; **5**, nomilinic acid glucoside; **6**, deacetylnomilinic acid glucoside; **7**, obacunone glucoside.

Individual calibration curves for the limonoid glucosides measured in the SIM mode are shown in Figure 4. All calibrations are linear, with $r^2$ values >0.99 in the range of the standards. In addition to calibration, the curves provide a means for troubleshooting, because nonlinear or badly correlated curves indicate a response problem with the mass detector.

The results of a replicate study involving three glucosides are shown in Table 1. Two wet samples and two dry samples were selected from industrial streams and analyzed six times over 2 days. Both wet and dry samples were evaluated to ensure that both types could be analyzed consistently in the same sample set using the same standards. The standard deviations are below 15% of the averages, down to the detection limit of 100 ppb (2 ng). Due to this relatively large variation, it is necessary to perform multiple runs of a sample to ensure accuracy. In a spike−recovery experiment, an industrial orange juice sample was spiked with 1300 ppb of limonin glucoside from a stock solution. The orange juice and the spiked sample were analyzed for limonin glucoside, and the percent recovery was calculated from the difference. The experiment was repeated on three consecutive days, and the average recovery was 92%.

When an MS detector is used for quantifying samples, care must be taken to ensure that the detector response remains consistent. Small changes in pH, gas flow, solvent flow, or back pressure can result in significant response variations. The ESI source also must be cleaned regularly to maintain sensitivity. Unlike UV detection systems, the MS detector can display fairly large day-to-day variations in response. The use of an internal standard, in this case carminic acid, serves to minimize response variations. Carminic acid is a water-soluble anthraquinone used for food coloring and possesses a carboxylic acid group, a sugar subunit, and several phenolic hydroxyl groups. The acidity and solubility of carminic acid make it an ideal internal standard for limonoid glucosides because its behavior is quite similar to that of the limonoid glucosides under the ionization conditions.

The dynamic range of the method was limited to that of the calibration standards. It was found that at higher concentrations of limonoid glucosides, the MS detector did not exhibit a linear response, so samples were diluted to keep concentrations between the detection limit and the highest standard showing linear response. For limonin glucoside, this range was 100−8000 ppb, whereas for the other limonoid glucosides the range was 100−4500 ppb. Typically, two dilutions of each unknown (one as detailed above, plus a 1:10 dilution) were sufficient to bring the concentrations of the limonoid glucosides into the desired range. In some cases, an additional 1:100 dilution was necessary to bring highly concentrated samples into the calibration range.

Orange juice and orange juice processing byproducts

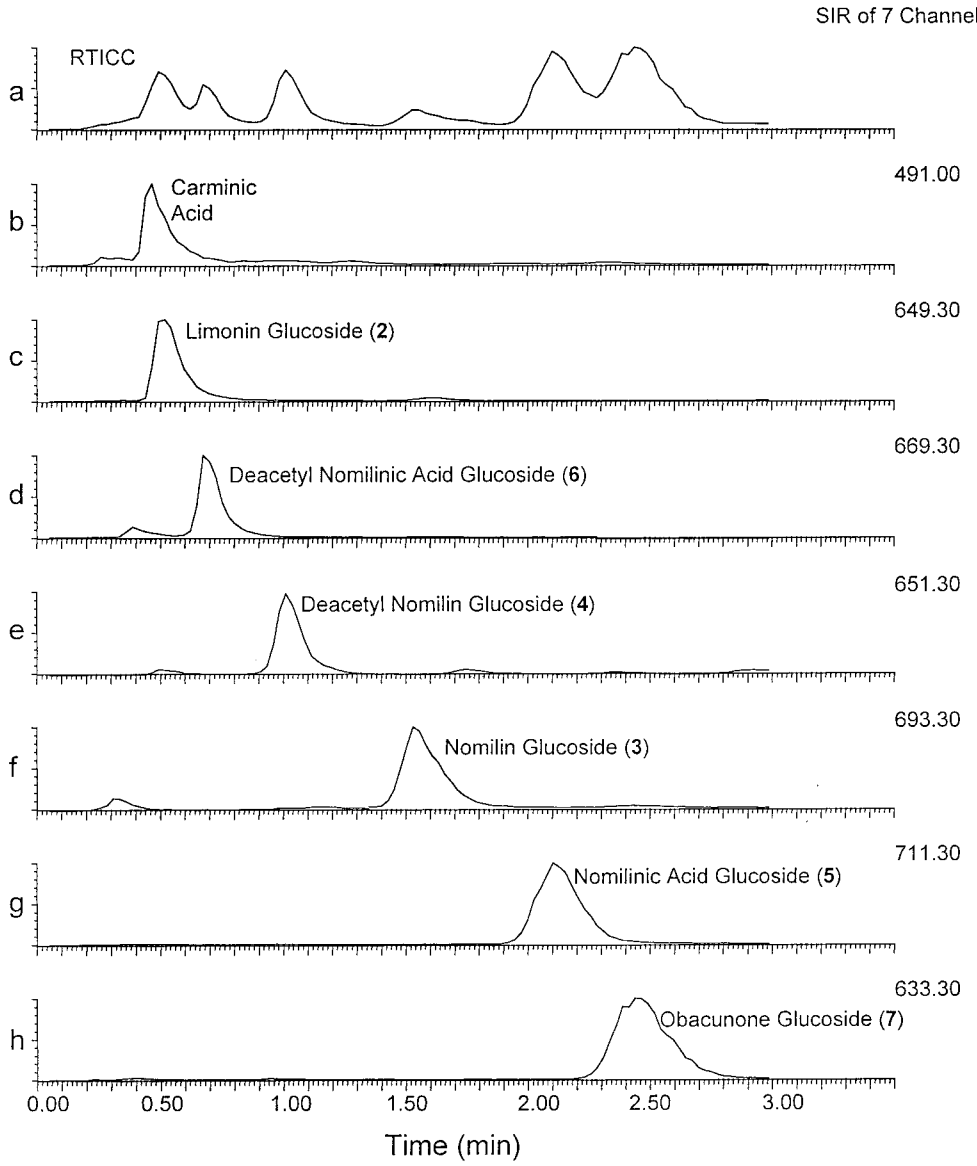


**Figure 3.** (a) RTICC of a sample containing limonoid glucosides; (b−h) SIM chromatograms of carminic acid and individual limonoid glucosides.

were selected to demonstrate the application of the LC-MS quantitative method. A summary of the results obtained from these samples is shown in Table 2. The analyses revealed that, as expected, limonin glucoside (**2**) was the predominant glucoside in nearly all of the orange-derived samples. The other five glucosides were present in smaller, sometimes trace, amounts. Although not all 17 known limonoid glucosides can be measured with this method, those commonly found in significant amounts in orange fruit are quantitated. Other limonoid glucosides were often detected in qualitative runs but generally occurred in trace amounts only. The most abundant of these minor components were obacunoic acid glucoside and its isomers, which are isomeric with deacetylnomilin glucoside but readily separated chromatographically.

As Table 2 shows, limonoid glucosides are found in significant amounts in both liquid and solid process streams. Citrus molasses contain large amounts of limonoid glucosides, but these originate from the peel and other solid byproducts of juice production. Of the solid samples, the highest concentrations were found in the seeds, but because seeds make up a small percentage of the weight of the fruit, larger amounts of limonoid glucosides are present in the other solid fractions. This analytical method enables mass balances to be performed on fruit and process streams to determine the location of the glucosides and also allows liquid and solid samples to be compared directly. As shown in Table 2C, partially purified mixtures can also be analyzed and the percentages of each limonoid glucoside calculated. The values obtained for juice samples in Table 2A generally agree with analyses of other juice samples performed using TLC, HPLC, and gravimetric methods (*4*, *10*). Only the six most predominant glucosides were measured, but as other limonoid glucoside standards are isolated in the future they can be easily added to the analysis method. The LC-MS method was successfully applied to other citrus juices (data not shown) to establish the general applicability of the method to *Citrus*.

Capitalizing on LC-MS as an analytical tool for separation and quantitation, our method was developed as a rapid, sensitive alternative to the previous, lengthy HPLC methods. In contrast to HPLC-UV methods for analysis of limonoid glucosides, LC-MS offers lower

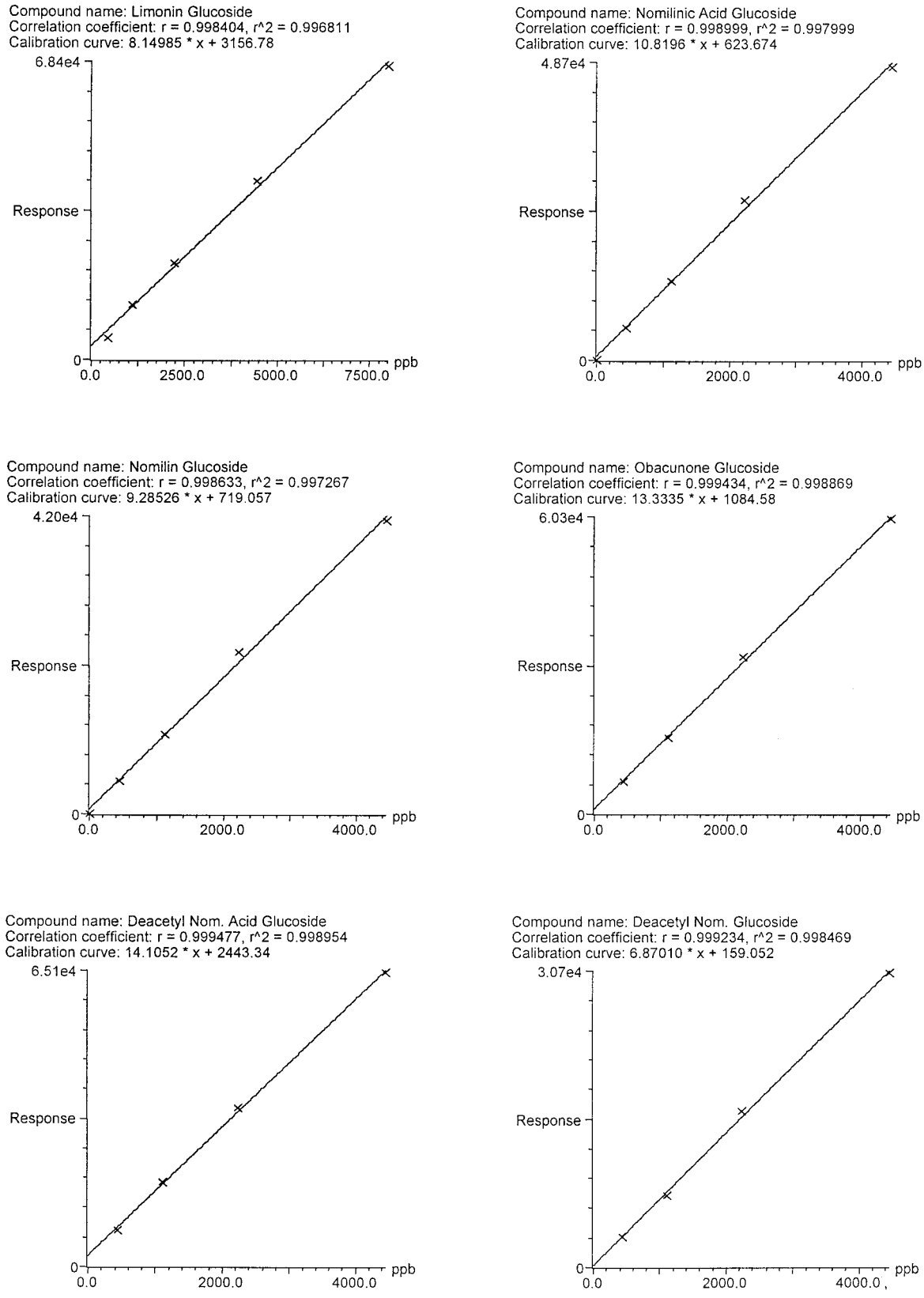


**Figure 4.** Individual calibration curves for limonoid glucoside quantitation.

detection limits, simpler and more definitive peak identification, and shorter analysis times. The sensitivities of the methods described here (2 ng for quantitative, 200 pg for qualitative) compare favorably with the limits of quantitative TLC (0.2 $\mu$g) (*9*) or quantitative HPLC-UV (6 ng) (*15*). Previously reported LC-MS methods for limonoid glucosides have employed qualitative detection only (*12, 13*).

Compound identification is simplified by the use of LC-MS. In qualitative mode, a profile of the limonoid glucosides present in a sample can be obtained by looking for the characteristic $[M - H^+]^-$ ions. Employing

**Table 1. Replicate Study**[a]

| | limonoid glucoside,[b] ppb | | | |
|---|---|---|---|---|
| sample | limonin | nomilinic acid | nomilin | total |
| 831 (wet) | 2330 ± 120 | 610 ± 50 | 1870 ± 140 | 4800 ± 170 |
| 844 (wet) | 3720 ± 130 | 980 ± 50 | 2040 ± 150 | 6740 ± 290 |
| 869 (dry) | 3900 ± 240 | 570 ± 80 | 520 ± 40 | 4990 ± 310 |
| 870 (dry) | 4170 ± 140 | 330 ± 20 | 250 ± 30 | 4740 ± 120 |

[a] $N = 6$. [b] Values are mean ± SD.

**Table 2. Concentrations of Limonoid Glucosides in Orange Samples**

A. Wet Samples

| | limonoid glucoside,[a] ppm (mg/L) | | | | | | |
|---|---|---|---|---|---|---|---|
| sample | **2** | **3** | **4** | **5** | **6** | **7** | total |
| PR molasses | 4600 | 270 | 830 | 910 | 220 | 1130 | 7960 |
| PR juice 1 | 720 | 50 | 10 | 80 | 0 | 10 | 870 |
| PR juice 2 | 710 | 70 | 20 | 90 | 0 | 10 | 900 |
| NT juice 1 | 380 | 30 | 10 | 50 | 0 | 0 | 470 |
| NT juice 2 | 410 | 40 | 20 | 60 | 0 | 0 | 530 |

B. Dry Samples

| | limonoid glucoside, dry wt % | | | | | | |
|---|---|---|---|---|---|---|---|
| sample[b] | **2** | **3** | **4** | **5** | **6** | **7** | total |
| PR peel | 1.36 | 0.00 | 0.12 | 0.09 | 0.00 | 0.01 | 1.57 |
| PR frits | 0.74 | 0.00 | 0.07 | 0.03 | 0.00 | 0.00 | 0.84 |
| PR core | 1.36 | 0.00 | 0.03 | 0.13 | 0.00 | 0.01 | 1.53 |
| PR flavedo | 2.66 | 0.00 | 0.19 | 0.03 | 0.00 | 0.02 | 2.90 |
| PR albedo | 0.96 | 0.00 | 0.05 | 0.04 | 0.00 | 0.01 | 1.07 |
| NT peel | 1.10 | 0.00 | 0.09 | 0.12 | 0.00 | 0.01 | 1.32 |
| NT frits | 1.02 | 0.00 | 0.08 | 0.06 | 0.00 | 0.00 | 1.16 |
| NT core | 2.12 | 0.01 | 0.07 | 0.18 | 0.00 | 0.02 | 2.40 |
| NT flavedo | 2.95 | 0.00 | 0.23 | 0.02 | 0.00 | 0.00 | 3.20 |
| NT albedo | 1.07 | 0.00 | 0.09 | 0.04 | 0.00 | 0.00 | 1.20 |
| NT seeds | 4.17 | 0.01 | 0.55 | 0.49 | 0.09 | 0.34 | 5.65 |

C. Limonoid Glucoside Mixtures[c]

| | limonoid glucoside, dry wt % | | | | | | |
|---|---|---|---|---|---|---|---|
| sample | **2** | **3** | **4** | **5** | **6** | **7** | total |
| MK1 LG mix | 7.0 | 0.0 | 8.3 | 4.1 | 17.5 | 0.0 | 36.9 |
| MK2 LG mix | 30.2 | 0.9 | 6.7 | 5.2 | 3.4 | 0.0 | 46.4 |
| MK3 LG mix | 32.7 | 2.4 | 7.3 | 14.5 | 7.0 | 0.1 | 63.9 |
| SH1 LG mix | 13.3 | 3.7 | 6.6 | 17.8 | 5.2 | 0.0 | 46.6 |
| T1 LG mix | 28.3 | 1.0 | 5.9 | 4.8 | 0.3 | 0.0 | 40.3 |

[a] **2**, limonin glucoside; **3**, nomilin glucoside; **4**, deacetylnomilin glucoside; **5**, nomilinic acid glucoside; **6**, deacetylnomilinic acid glucoside; **7**, obacunone glucoside. [b] PR, Pera Rio; NT, Natal. [c] Proprietary samples.

an acidic aqueous mobile phase with a short column allows partial separation of the individual ion peaks. In the RTICC the peaks are not fully separated, but quantitation can be obtained from the individual SIM traces. In the above method, individual run times are 4 min, and because the solvent flow is isocratic, no postrun re-equilibration of the column is necessary. In comparison, HPLC-UV methods for limonoid glucoside analysis typically use a solvent gradient and run times in excess of 40 min (*11*).

The analysis method detailed here is a quick and sensitive way to quantify limonoid glucosides in a variety of samples. In addition to orange samples, other fruit samples can easily be analyzed and the method extended to measure limonoid glucosides other than the common ones listed here. This method should prove to be valuable both for the isolation of new limonoid glucosides and for the detection and identification of limonoid glucoside metabolites in human and animal systems.

ACKNOWLEDGMENT

We thank Cargill, Inc., for providing samples and raw materials and Virginia Galan and Marlene Hidalgo for their assistance.

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Received for review September 19, 2000. Revised manuscript received December 11, 2000. Accepted December 18, 2000.

JF001157D