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Analysis of Bitter Limonoids in Citrus Juices by Atmospheric Pressure Chemical Ionization and Electrospray Ionization Liquid Chromatography-Mass Spectrometry

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Improved analytical techniques for bitter limonoids in citrus and citrus juices can expedite the evaluation of freeze-induced citrus damage for citrus growers and juice quality for citrus juice producers. Microbore normal-phase and reverse-phase chromatography coupled to a mass spectrometer operating in a positive ion atmospheric pressure chemical ionization and electrospray ionization modes were found to be rapid, selective, and sensitive methods for the analysis of the bitter limonoids limonin and nomilin in citrus juices. Analysis was performed on a chloroform extract of citrus juice to which an internal standard was added. The methods are capable of detecting citrus limonoids in citrus juice in the 60−200 picogram range and quantifying citrus juice limonoids in concentrations as low as 120 picograms. An accurate "total limonoid bitterness" in citrus juice, as represented by the combined occurrence of limonin and nomilin, is easily determined by these methods.

KEYWORDS: Limonoid; citrus; liquid chromatography-mass spectrometry; analysis

INTRODUCTION

Delayed bitterness in citrus juices is primarily caused by the presence of bitter limonoids and continues to pose a major problem for citrus juice processors worldwide. The presence of bitter citrus triterpenoid limonoids in concentrations in excess of 6 ppm reduces the acceptability of citrus juices to consumers and forces citrus juice producers to lower the bitter limonoid content through juice-blending dilution or the removal of bitter limonoids.

Several analytical methods have been developed to quantify limonin (**1**) (**Figure 1**), the most abundant bitter limonoid occurring in citrus juices. The majority of these analytical methods utilize reverse-phase and normal-phase high performance liquid chromatography (HPLC) coupled to ultraviolet (UV) detection. Two immunoassay methods, one of which was developed commercially but is no longer marketed, quantify limonin in citrus juices (*1*, *2*). The early commercial immunoassay method offered speed and cost advantages over the HPLC techniques but lacked reproducibility (*3*). A more recent immunoassay method (*2*) offers greater specificity for limonin, but its complexity appears to restrict its application compared to simpler liquid chromatographic (LC) methods.

The HPLC reverse-phase methods that quantify limonin in citrus juices employ C-8, C18, and CN bonded silica stationary phases (*3*−*8*). The more recent LC methods employ solid-phase extraction (SPE) modules to obtain limonoid-enriched extracts from juices prior to reverse-phase HPLC analysis (*6*−*8*). C-18 SPE concentration of limonoids in citrus juice has been accomplished prior to chromatography (*7*) and during chromatography (*8*). One normal-phase method using CN bonded silica includes a chloroform liquid−liquid extraction of clarified citrus juice to isolate the bitter limonoids prior to chromatography (*9*). This normal-phase LC−UV analysis method reports success in quantifying nomilin (*2*) (**Figure 1**). All of the methods rely

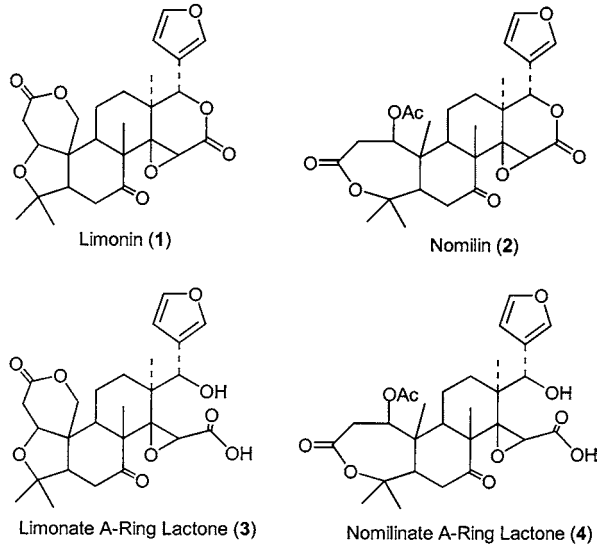

Figure 1. Prominent bitter limonoids present in citrus and their precursors.

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on ultraviolet (UV) detection at 210−215 nm and report high quantitative reproducibility in the detection of limonin with a detection limit near 2 ng (*6*).

We have previously reported a normal-phase HPLC method for the quantification of a broad range of limonoids present in citrus seeds utilizing UV detection (*10*) and introduced liquid chromatography-mass spectrometry (LC-MS) for limonoid analysis using atmospheric pressure chemical ionization (APCI) and electron ionization mass spectral detection (*11*). We have also recently described an electrospray ionization liquid chromatography-mass spectrometry (ESI LC-MS) method for the quantification of limonoid glucosides in citrus juices and citrus juice byproducts (*12*). We now describe APCI LC-MS (normal-phase and reverse-phase) and ESI LC-MS (reverse-phase) methods for the quantification of limonoids in citrus juices.

MATERIALS AND METHODS

Solvents. Water was distilled and deionized. Solvents (Fisher) were HPLC grade. Tetrahydrofuran (THF) (Fisher) contained no stabilizer but was utilized with a 0.5% water content for chromatography and was therefore not considered to pose a problem of peroxide formation.

Juice Samples. Analysis samples of pink grapefruit and white grapefruit juice were generated from commercial frozen juices reconstituted according to package directions. A fresh fruit juice sample was obtained from Texas Rio Red grapefruit purchased from a local market. Juice was obtained from the fresh fruit by hand squeezing.

Thirty-two Washington navel oranges were obtained for a freeze-susceptibility study from the University of California at Davis Lindcove Research Center, Exeter, California. The oranges were randomly selected and divided into two groups (Group 1, eight oranges; Group 2, 24 oranges). The first group was immediately sliced and juiced by hand with the juice from all oranges combined. The remaining 24 oranges were placed in a freezer (−10 °C (14 °F), 9 h), removed, and thawed to room temperature. Eight of the thawed oranges were sliced and juiced by hand, and the juice was combined. Eight of the remaining 16 oranges were allowed to stand at room temperature for 24 h before slicing and juicing, and the last eight oranges were sliced and juiced after 48 h.

Standards. Limonoid standards were available in our laboratory. Podophyllotoxin was obtained from Aldrich Chemical Co. A stock solution of the bitter limonoids limonin (114.2 ng/μL) and nomilin (112.6 ng/μL) was prepared in THF. The stock solution was diluted with THF or MeOH to provide four standard solutions with limonoid concentrations ranging from ∼60 pg/μL to 6ng/μL. Podophyllotoxin (in THF or MeOH) was added as an internal standard to each standard solution to achieve a concentration of 167 pg/μL for the LC-MS analysis. All standard stock solutions were tightly sealed and stored under refrigeration between uses.

LC-MS System Parameters. *Mass Spectrometer Tuning.* All mass-spectrometer tuning was accomplished through optimization of a signal for a limonin protonated molecule at *m*/*z* 471.4 generated through the infusion of a solution of limonin into the mass spectrometer in the LC mobile phase (∼1 mg/L) at the analysis flow rate.

Normal Phase APCI LC-MS Analysis. Normal phase APCI LC-MS analysis of citrus juice limonoids was conducted on a Micromass LCZ single quadrapole mass spectrometer equipped with an APCI probe. The mass spectrometer was operated in the positive ion mode, with a probe temperature of 500 °C, cone voltage of 27 V, and corona voltage of 3.87 kV. Protonated molecules of the citrus limonoids were monitored by the mass spectrometer operating in the single ion monitoring (SIM) mode. In the analysis of the bitter limonoids, SIM was conducted at *m*/*z* 397.4 (podophyllotoxin), 471.4 (limonin), and 515.4 (nomilin).

The mass spectrometer was coupled to a Waters Alliance 2690 solvent/sample delivery system. HPLC was conducted on a 150 mm × 2 mm i.d deactivated Luna 3μ silica column (Phenomenex, Torrance, CA). The Luna column was deactivated by subjecting the column to 80% aq EtOH (1 h) followed by 90% aq EtOH (1 h) at flow rates that maintained a back pressure less than 3000 psi. The column was then flushed with 99.5% aq THF (30 min) and equilibrated with a mixture of cyclohexane and 99.5% THF (1:1, 0.4 mL/min, 3 h). The column was further equilibrated with the initiating gradient solvent mixture (cyclohexane/99.5% THF, 3:1, 1 h) before the first analytical HPLC run. The analytical chromatography was conducted as a linear gradient elution (flow rate = 0.4 mL/min, column temperature 22 °C): Time 0, cyclohexane/95.5% THF (3:1); time, 4 min, cyclohexane/99.5% THF (2:3); time, 6 min, cyclohexane/99.5% THF (2:3); time, 7 min, cyclohexane/99.5% THF (3:1). Total chromatographic run time was 11 min, to allow the column to reequilibrate between injections. Sample injection volume was 3 μL.

Reverse-Phase APCI LC-MS Analysis. The reverse-phase APCI analysis was conducted on the same LC-MS system as the normal-phase analysis. The mass spectrometer was operated in the positive ion mode with a probe temperature of 600 °C, cone voltage of 29 V and corona voltage 3.9 kV. The mass spectrometer was operated in the SIM mode monitoring the same protonated molecules as the normal-phase method.

HPLC of the citrus juice limonoids utilized a 50 mm × 2 mm i.d. column packed with a 3 μ Hypersil BDS C-18 (Keystone, Bellefonte, PA) stationary phase. Chromatography of the limonoids was accomplished with a 4 mM formic acid/MeOH gradient, flow rate = 0.4 mL/min, and column temperature of 40 °C. The gradient conditions were time 0 min, formic acid/MeOH (55:45); time 3 min, formic acid/MeOH (30:70), time 4 min, formic acid/MeOH (55:45). Total chromatographic run time was 6 min to allow column reequilibration between runs. Sample injection volume was 3 μL.

Reverse-Phase ESI LC-MS Analysis. Reverse-phase ESI LC-MS analysis of citrus juice limonoids was conducted on a Thermo-Finnigan LCQ ion trap mass spectrometer equipped with an ESI probe. The mass spectrometer was operated in the positive ion mode, with a capillary temperature of 300 °C, capillary voltage of 16 V, and ion spray voltage = 5.2 kV. The mass spectrometer was operated in the SIM mode, detecting the protonated molecules at the same *m*/*z* values monitored in the reverse- and normal-phase APCI methods.

HPLC of the citrus juice limonoids utilized the same column as the APCI method. Chromatography of the limonoids was accomplished with the same 4 mM formic acid/MeOH gradient, flow rate = 0.4 mL/min, and column temperature of 40 °C used for the reverse-phase APCI LC-MS analysis. Sample injection volume was 3 μL.

Calibration Curves. Replicate (3×) chromatographic runs were conducted for each of the standard solutions (see above) using 3-μL injections. The peak areas of the mass spectral SIM detected limonoid ions were calibrated relative to the podophyllotoxin internal standard to generate calibration curves. The same standard solutions were stored to generate fresh calibration curves for each subsequent set of sample analyses.

Sample Preparation and Analysis. Juice from analyzed fruit was obtained by hand squeezing. Juice (2 mL) was transferred to a test tube and internal standard (40 μL, 167 ng/μL) was added. The juice was gently liquid/liquid extracted (30 s) with $CHCl_3$ (2 × 2.5 mL). The $CHCl_3$ extracts were combined, and an aliquot (100 μL) of the $CHCl_3$ soluble material was removed, evaporated to dryness, and redissolved in MeOH or THF (300 μL). Then 3 μL of the diluted extract was injected into the LC-MS.

Two blank LC-MS runs were performed to equilibrate the system prior to sample analysis. Fresh calibration curves for the limonoid standards were also generated before and after the sample analysis to minimize any variations in mass spectrometer response (*12*). APCI or ESI LC-MS quantified the samples through comparison of limonoid SIM peak areas to the standard calibration curves.

Analysis Validation. The LC-MS method was validated through standard spiking experiments. The recovery efficiency of $CHCl_3$ extraction of limonoids from citrus juice was determined through the LC-MS analysis of a $CHCl_3$ extract of an aqueous solution (5 mL) containing limonin (25 μg) and the internal standard podophyllotoxin (100 μL, 167 ng/μL). The limonin-containing aqueous solution (2 mL) was extracted with $CHCl_3$, according to the sample preparation protocol, and an aliquot of the $CHCl_3$ extract was dried and reconstituted with MeOH or THF and analyzed by LC-MS to determine the concentration of limonin. The efficiency of the $CHCl_3$ extraction and the accuracy

of the LC-MS analysis were further evaluated through spiking experiments using limonin in frozen orange juice. Podophyllotoxin standard (100 μL, 167 ng/μL) was added to the juice (5 mL), the juice sample (2 mL) was extracted with $CHCl_3$ and reconstituted in MeOH or THF, according to the sample preparation protocol, and the resulting sample was analyzed by LC-MS to establish a residual limonin content. Limonin (50 μL, 500 ng/μL) and podophyllotoxin (100 μL, 167 ng/μL) were added to a second juice sample (5 mL) that was subsequently extracted with $CHCl_3$, reconstituted, diluted, and analyzed by LC-MS as previously described. Each spiking experiment was conducted in triplicate, and the resulting samples were analyzed by the APCI and ESI LC-MS methods. Results of the aqueous limonin extract analysis and a comparison of the results of the two juice spiking analyses for limonin served to determine the efficiency of the limonin extraction and accuracy of the LC-MS analysis. A relative standard deviation of the replicates was determined from the comparative analytical results for limonin.

RESULTS AND DISCUSSION

Internal standard related calibration curves for limonin (**1**) and nomilin (**2**), based upon SIM data discrimination obtained by the APCI LC-MS, were linear over a range of 120pg−6 ng, and ESI LC-MS methods were linear over a range of 240pg−6 ng with correlation coefficients consistently greater than 0.98. Evaluation of the efficiency of the chloroform extraction recovery of limonin from a spiked aqueous solution showed recovery to be greater than 98%, and recovery of limonin from a spiked orange juice matrix as analyzed by all LC-MS methods was 96% or greater. Relative standard deviation for a replicate analysis of limonin in an aqueous solution was 5.9% for the normal-phase APCI LC-MS method and 7.9% and 8.2% for the reverse-phase APCI LC-MS method and ESI LC-MS method, respectively. The detection limit for limonin, as quantified in the mass spectrometer operating in the SIM mode for both normal and reverse-phase APCI LC-MS was 60 pg, and the limonin detection limit measured by ESI LC-MS was 180 pg.

The dynamic nature of the LC-MS system can often cause variation in ion detection even during the course of a single day. In the LC-MS analysis of limonoid glucosides obtained from liquid and solid samples (*12*), we found it necessary to have a reliable internal standard with physical and chromatographic properties similar to the analytes and to reestablish calibration curves often. The solubility and chromatographic character of the lignan, podophyllotoxin, are very similar to those of the limonoids, making it an ideal internal standard. The addition of the internal standard to citrus juice prior to chloroform extraction increases the accuracy of the analysis and reduces the impact of extraction errors.

Figure 2a shows a total ion current (TIC) chromatogram of the chloroform extract of navel orange juice plus the internal standard and the SIM-derived chromatograms of the same extract with detection confined to limonin, nomilin, and the internal standard (**Figure 2**, Parts b−d) obtained by normal-phase APCI LC-MS. **Figure 3a** displays a TIC chromatogram for the reverse-phase APCI LC-MS analysis of the chloroform extract of the navel orange juice. An ESI LC-MS TIC chromatogram is not shown, because it was very similar to the APCI LC-MS trace, reflecting the fact that chromatographic conditions for the two methods are identical. **Figure 3**, Parts b−d shows the SIM-derived chromatograms for the two bitter limonoids and the internal standard present in the extract as obtained by reverse-phase APCI or ESI LC-MS. The chromatographic results illustrated clearly establish the ability of all three LC-MS methods to selectively extract quantifiable data for the bitter limonoids, and the internal standard present in a chloroform extract of citrus juice.

Table 1. Concentration of Bitter Limonoids in Chloroform Extract of Citrus Juices Analyzed by Normal Phase APCI LC-MS

fruit juice	limonin (ppm)	nomilin (ppm)
fresh Washington navel	1.24	0.07
frozen Washington navel	1.78	0.08
frozen Washington navel (24 h)	7.22	0.12
frozen Washington navel (48 h)	8.21	0.15
fresh Texas Rio Red grapefruit	2.31	
reconstituted pink grapefruit	2.35	
reconstituted white grapefruit	11.75	1.22

The three LC-MS analytical methods were applied to a variety of citrus juice samples to determine the level of bitter limonoids. The comparative results of those analyses were found to be very similar. Analytical results for the normal-phase APCI LC-MS analysis are summarized in **Table 1**. These results confirm limonin as the primary bitter limonoid present in citrus juices. Concentrations ranging from 1.24 ppm in fresh navel oranges to 11.75 ppm in reconstituted frozen white grapefruit juice were observed.

Table 1 includes the results of bitter limonoid analysis in chloroform extracts of juice from fresh Washington navel oranges and laboratory frozen Washington navel oranges immediately after thawing and 24 and 48 h after thawing. This comparative analysis was conducted to quantify the recognized freeze-induced conversion of nonbitter limonate A-ring lactone (**3**) (**Figure 1**) present in citrus to bitter limonin (**1**). Under normal growing conditions, the limonate A-ring lactone is continuously converted to tasteless limonin glucoside as the fruit matures (*13*). Exposure of the fruit to mechanical damage and/or freeze-induced damage initiates enzyme catalyzed conversion of the limonate A-ring lactone to limonin (*14*). Chloroform extraction of citrus juices selectively isolates the water insoluble limonoids from the juice without removing water-soluble limonoid glucosides or limonate A-ring lactone. The extraction therefore selectivity allows determination of the degree of freeze-induced conversion of limonate A-ring lactone to limonin by the post-freeze analysis of limonin in a chloroform extract of the juice.

The analysis results (**Table 1**) confirms the freeze-induced formation of limonin with an observed increase in limonin of more than 30% in a frozen juice sample (1.78 ppm) immediately after thawing, compared to the fresh juice sample (1.24 ppm). Allowing the frozen oranges to stand for 24 and 48 h after thawing results in almost a seven-fold increase in limonin (8.21 ppm) after 48 h. The observed difference in limonin content of the juice of the fresh and frozen oranges demonstrates the susceptibility of navel oranges to freeze-induced bitterness, a major concern of California navel orange growers. The final concentration of limonin in the frozen oranges 24 and 48 h after thawing was well above the 6 ppm threshold of objectionable bitterness. A very small amount of nomilin (0.07 ppm) was observed in fresh navel orange juice. Slightly more nomilin (0.08 ppm) was present in the juice from the frozen orange immediately after thawing. The amount of nomilin remains low in the juice 24 h (0.12 ppm) and 48 h (0.15 ppm), even though the nomilin content is almost double the amount in the fresh juice. The appearance of nomilin (**2**) in the frozen juice may be attributed to enzymatic cyclization of nomilinate A-ring lactone (**4**), a compound recently detected in citrus by Japanese researchers (Dr. Yashuhiko Tomono, personal communication).

The analysis of the frozen orange juice illustrates the temperature dependence of the enzyme catalyzed formation of

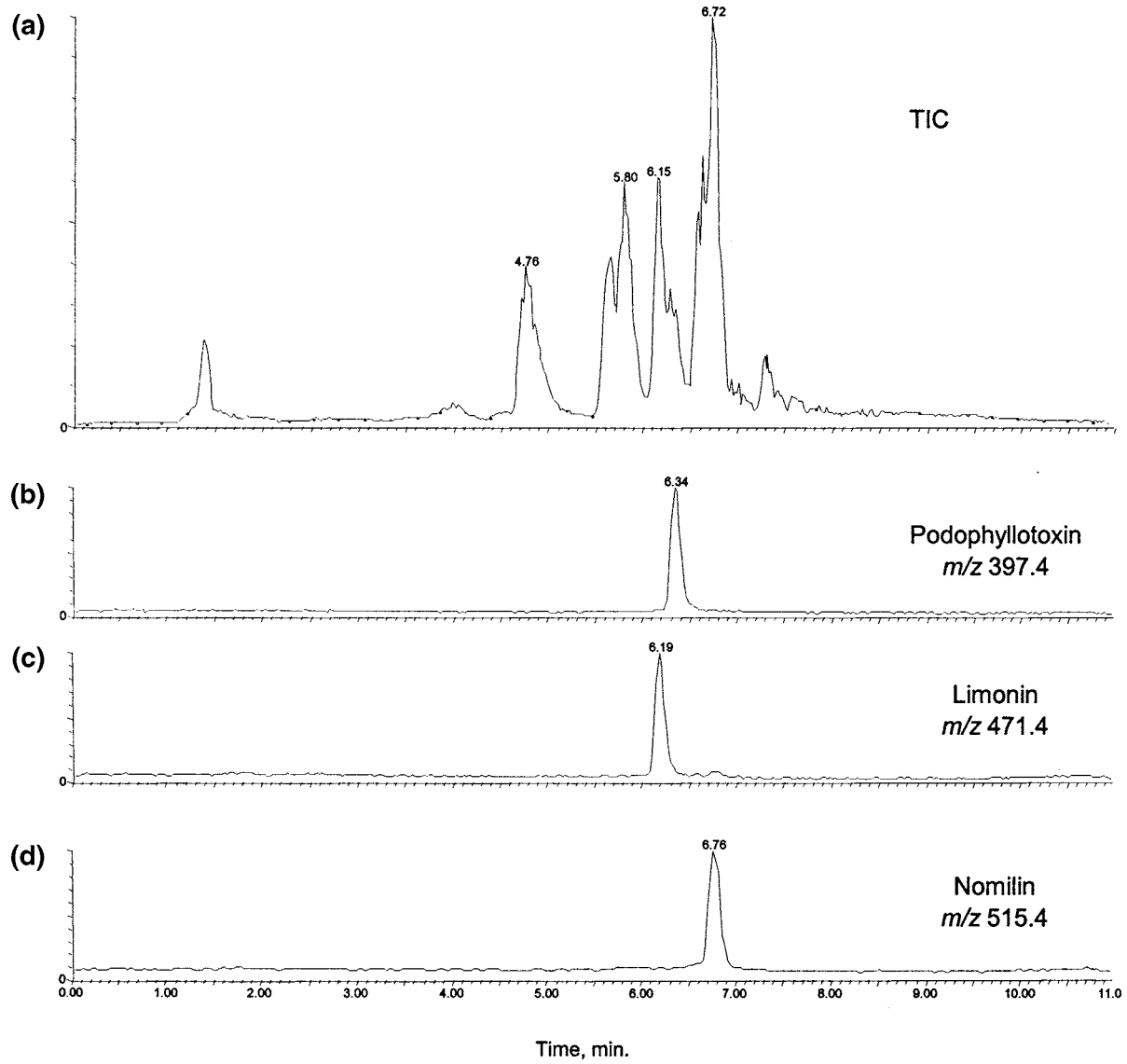

Figure 2. Total ion current normal-phase APCI LC-MS chromatogram for chloroform extract of navel orange juice and single ion monitor protonated molecule ions for limonin, nomilin, and podophyllotoxin.

limonin from limonate A-ring lactone. The deactivation of the enzyme responsible for the D ring lactonization of limonate A-ring lactone to limonin in the juice from navel oranges exposed to freezing conditions could allow salvaging of juice from freeze impacted oranges with a bitterness level suitable for use in blended orange juices. There is currently no means to salvage these oranges following a freeze event.

Analysis results for the other juices showed that two of the three grapefruit juices have levels of limonin slightly higher than the navel orange, with Texas Rio having 2.31 ppm and reconstituted Pink having 2.35 ppm. Analysis of the reconstituted white grapefruit juice showed a very high level of limonin (11.75 ppm) and the highest amount of nomilin of all the juices analyzed. The high values for limonin and nomilin indicate that the juice would be extremely bitter; this was confirmed by an empirical taste test.

The LC-MS methods for the analysis of limonoid described here have distinct advantages over normal-phase or reverse-phase LC−UV methods, most notably, efficiency and superior analytical selectivity and sensitivity. An accurate analysis of bitter limonoids is easily conducted in 6−11 min, because of the discriminative capability of SIM data processing to detect the limonoids without interference from coeluting compounds present in the same extract. The ability to selectively identify specific protonated molecular ions allows the accurate determination of a "total limonoid bitterness", representing the combined concentrations of limonin and nomilin in the citrus juice, such as reconstituted white grapefruit juice (**Table 1**). Coupling of the SIM data discrimination with high resolution chromatography should provide a rapid and reliable means to quantify a broad range of limonoids in citrus juices, seeds, and plant tissues. The LC-MS methods are significantly more sensitive for the detection of limonoids than all other current methods of limonoid analysis. With detection limits near 60 pg, the APCI methods are over 30 times more sensitive than UV detection methods, with a detection limit of ~2 ng. The ESI method is more than 10 times more sensitive than the LC-UV methods. Little difference was noted in the accuracy of the reverse-phase versus the normal-phase methods. There is no major advantage for the normal-phase method or the reverse-phase method in the accuracy of bitter limonoids in citrus juices. The normal phase does, however, require the use of organic solvents with more stringent safety requirements related to their use and handling.

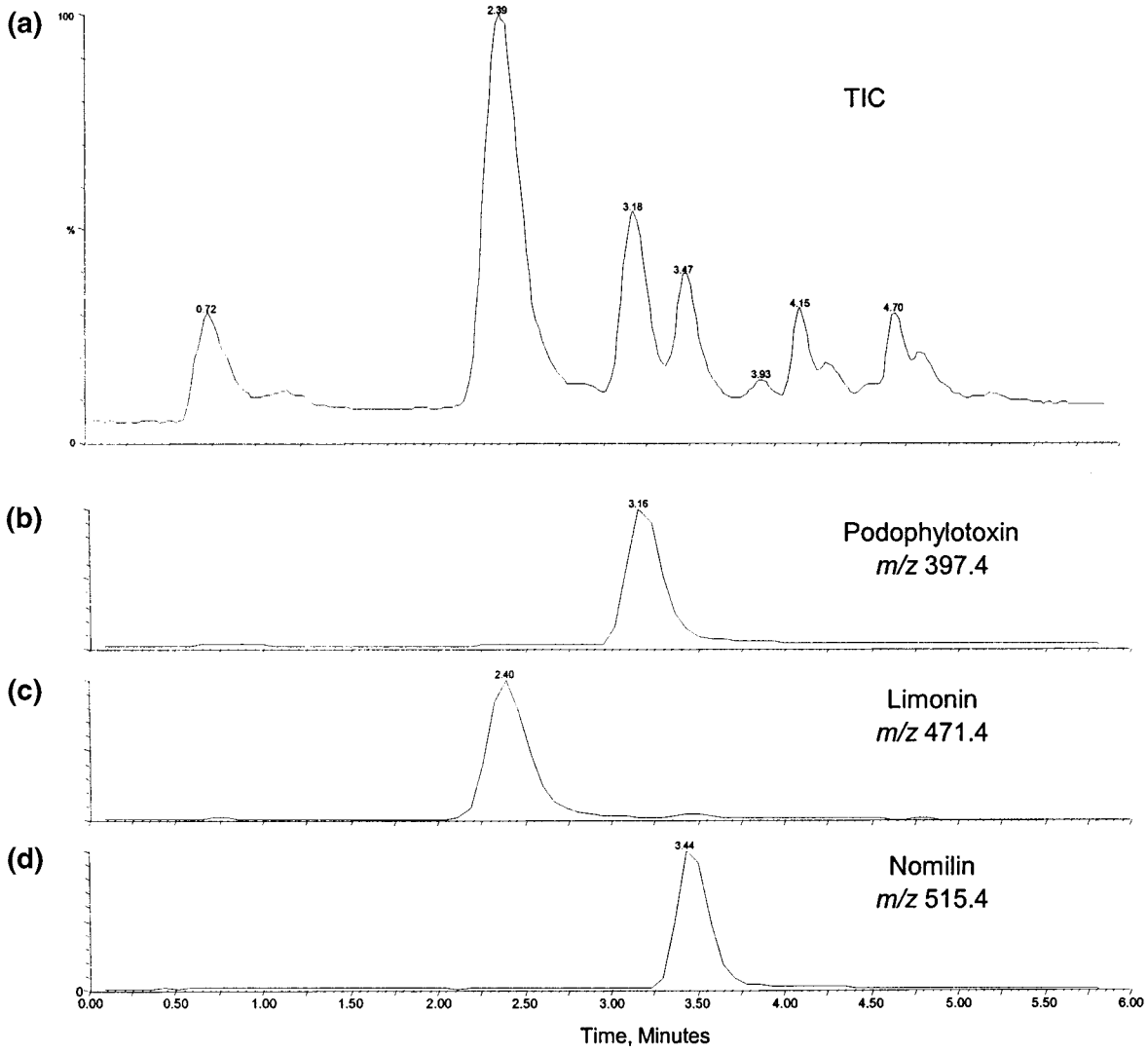

Figure 3. Total ion current reverse-phase APCI LC-MS chromatogram for a chloroform extract of navel orange juice and single ion monitor protonated molecule ions for limonin, nomilin, and podophyllotoxin.

Current LC-UV-based analytical methods, when applied to routine analysis of materials with large amount of limonoids, remain an economical method to quantify limonin in citrus juice. Nevertheless, the LC-MS methods described here offers higher selectivity, significantly higher sensitivity, and the ability to directly analyze all of the limonoid aglycones in chloroform extracts of citrus juices. The LC-UV analysis methods have not been capable of accomplishing this degree of limonoid analysis from citrus juices. The sensitivity and selectivity of the limonoid LC-MS methods is consistent with requirements to quantify very low levels of limonoids among a broad spectrum of other substances in complex chemical or biological matrixes and should be useful for analyzing limonoid metabolites in human and animal biological fluids as part of a study of the bioavailability of in citrus juice limonoids.

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