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Journal of Chromatography A, 1064 (2005) 187–191
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Determination of limonoate and nomilinoate A-ring lactones in citrus juices by liquid chromatography–electrospray ionization mass spectrometry

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Received 11 June 2004; received in revised form 30 November 2004; accepted 16 December 2004
Available online 6 January 2005

Abstract

The development of delayed bitterness in citrus products is a major problem to citrus producers and juice processors worldwide. A rapid and sensitive liquid chromatography–electrospray ionization mass spectrometry (LC–ESI-MS) method has been developed to quantify the recognized precursors of limonoid derived delayed bitterness, limonoate and nomilinoate A-ring lactones, in a wide variety of citrus juices. The limonoid A-ring lactones were isolated by solid-phase extraction from juice samples, analyzed by negative ion LC–ESI-MS and quantified utilizing the standard addition method.
Published by Elsevier B.V.

Keywords: Limonoids; Citrus; Solid-phase extraction; Juice analysis

1. Introduction

Excessive bitterness due to limonin and nomilin in a variety of citrus juices is a major problem in the citrus industry. Juices with higher concentrations than the human bitterness threshold of 6 ppm are considered of poor quality and of low market value for producers [1,2]. Prior to harvest or processing, limonin and nomilin exist in fruit tissues as their non-bitter precursors limonoate A-ring lactone (LARL) and nomilinoate A-ring lactone (NARL), respectively (Fig. 1) [3]. In citrus subjected to stress (juicing, squashing, bruising) or freezing, the limonoid A-ring lactones are gradually converted to the bitter aglycones limonin and nomilin by the activity of limonoid D-ring lactone hydrolase (E.C. 3.1.1.36) and the acidic pH of juice in a process known as "delayed bitterness" [4–6]. In general, LARL and NARL concentrations are greatest at the beginning of the season and decrease as the fruit matures. In 1989, Hasegawa et al. attributed the decrease in limonoid A-ring lactone concentrations to their conversion into limonoid glucosides and later identified UDP-limonoid glucose transferase (E.C. 2.4.1.210) as the enzyme responsible for the natural mitigation of delayed bitterness [7,8].

The economic significance associated with delayed bitterness has generated extensive research efforts directed to detecting and modulating bitter limonoid aglycones [6,9–13]. Methods for the quantification of limonoid aglycones and limonoid glucosides are well established and include a variety of methods from thin-layer chromatography (TLC) [6] to recently described LC–MS methods [14]. In contrast, analysis of limonoid A-ring lactones in citrus samples has been limited to indirect chemical and biochemical techniques [15–17], although the chromatographic separation of LARL from limonin [18] and MS detection of LARL [19] have been described. We now describe a rapid and reliable LC–electroscopy ionisation (ESI) MS method for the direct quantification of LARL and NARL in a wide variety of citrus juices.

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0021-9673/$ – see front matter Published by Elsevier B.V.
doi:10.1016/j.chroma.2004.12.046

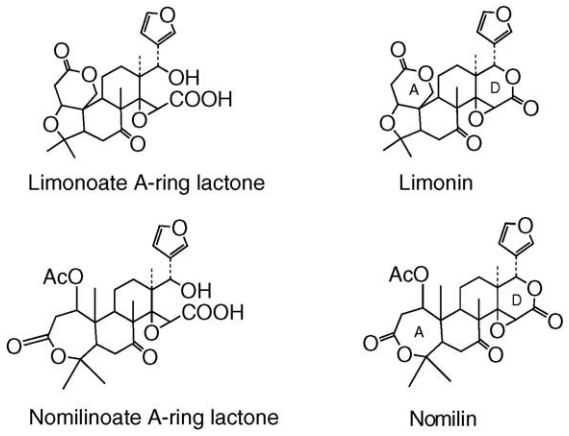

Fig. 1. Structure of limonoid A-ring lactones and aglycones of limonin and nomilin. The A- and D-rings of the aglycones are labeled.

2. Experimental

2.1. Chemicals

Water was distilled and deionized. Chromatographic solvents (Fisher, Pittsburgh, PA, USA) were HPLC grade. Carminic acid (96%) was purchased from Acros (Belgium). Limonin and nomilin were available from previous studies in our laboratory.

2.2. Preparation of solutions

Carminic acid (CA) was dissolved in water to a concentration of 100 ppm and stored protected from light in an unsilanized glass container at room temperature. Solid-phase extraction (SPE) elution solutions A and B were prepared by combining Tris–HCl (1 M, pH 8.0) with CH_3CN–water (30:70) and MeOH–CH_3CN–water (50:30:20), respectively, at a ratio 10 μL Tris to 1 mL of solution.

2.3. Preparation of and quantification of LARL and NARL stock solutions

LARL and NARL stock solutions were prepared daily and generated enzymatically utilizing limonoid D-ring lactone hydrolase (LDLH) that was purified as previously described [4]. The reaction mixture consisted of purified LDLH (100 μL), Tris–HCl (120 μL, 1 M, pH 8.0), water (980 μL) and solid limonin or nomilin (2–3 mg). Following incubation at 30 °C (10 h), the reaction mixture was clarified using a centrifuge (14 000 × *g*, 5 min, 4 °C) and applied (1 mL) to a C-18e SPE column (500 mg, Phenomenex, Torrance, CA, USA) prewashed with MeOH (2 mL) and equilibrated in water (2 mL). The flow through and a water wash (2 mL) were discarded. A-ring lactones were eluted with solution A (1.5 mL). LARL and NARL concentrations were established by their acid catalyzed conversion to aglycones and quantification by fluorescence spectroscopy as previously described [20].

Standard solutions of limonin and nomilin (5–100 ppm) were prepared in 30% aqueous CH_3CN. Serial dilutions of LARL and NARL were prepared and an aliquot of each LARL and NARL sample (25 μL) and standard (25 μL) was transferred to a well in a Costar 3631 assay plate (Corning Inc., Corning, NY, USA) and combined with concentrated sulfuric acid (250 μL, Fisher). Standards and samples were plated in triplicate. After incubation (37 °C, 1 h), fluorescence was measured on a Molecular Devices Gemini-EM (Sunnyvale, CA, USA) plate reader. The plate was top read (λ_{ex} = 405 nm and λ_{em} = 460 nm) with a cut off of 455 nm used. Software supplied with the instrument was used for data analysis. Typical concentrations of LARL and NARL solutions generated ranged from 150 to 300 ppm. For the analysis presented here, LARL and NARL stock solutions were 308 and 174 ppm, respectively, and used without further adjustment. Solutions containing LARL, NARL or both to be used for the analysis of juice samples by the standard addition method were prepared from the LARL and NARL stock solutions by volume at LARL:NARL ratios of 1:0, 1:1 and 0:1. These three standard solutions are referred to collectively as the internal calibrators.

2.4. Juice samples

Fresh fruit juice samples were obtained from Valencia, Texas Valencia, Cara Cara navel oranges, Honey tangerines, and Rio Star grapefruits purchased from a local market in late February 2004. For each sample, juice from three fruits was combined after they had been sliced and juiced by hand. Hand juicing was accomplished using a glass juice reamer, taking care not to disrupt the albedo.

2.5. Sample preparation

Juice samples (10 mL) were clarified by centrifugation (16 000 × *g*, 5 min, 10 °C), the supernatant collected and filtered (0.45 μm, 25 mm GD/X, Whatman Clifton, NJ, USA). The filtered liquid was used to prepare three samples, one at each internal calibrator concentration, and consisted of juice (150 μL), CA internal standard solution (75 μL, 100 ppm), internal calibrator solution (15 μL) and water (1.26 mL). Samples were thoroughly mixed and loaded (1 mL) onto Strata-X solid-phase extraction (SPE) columns (30 mg, Phenomenex) that had been washed with MeOH (1 mL) and preconditioned with water (1 mL). Columns were washed sequentially with water (0.5 mL) and $CHCl_3$ (0.5 mL), and the limonoid A-ring lactones eluted with solution B (1 mL). Samples found to have concentrations greater than the highest internal calibrators were diluted 10:1 and reanalyzed as detailed above.

2.6. LC–MS

Mass spectrometer tuning was accomplished through optimization of a LARL signal at *m/z* 487.2 generated by introduction of a LARL solution into the mass spectrometer in the LC mobile phase at the flow rate used for analysis.

Reversed-phase LC–MS analysis of CA, LARL, and NARL was conducted on a Micromass LCZ single quadrapole mass spectrometer equipped with an ESI probe. The mass spectrometer was operated in the negative ion mode, with a capillary temperature of 500 °C, capillary voltage of 3.85 kV, and cone voltage of 24 V. For the analysis of the limonoid A-ring lactones, the mass spectrometer was operated in the single-ion monitoring (SIM) mode monitoring ions at *m/z* 490.9 (CA), 487.2 (LARL), and 531.2 (NARL).

The mass spectrometer was coupled to a Waters Alliance 2695 high-performance chromatography system using a Synergi (Phenomenex) Hydro-RP 80A column (50 mm × 2.00 mm I.D.; 4 μm). Chromatography of the analytes was accomplished with a 4 mM formic acid–MeOH (50:50) isocratic mobile phase at a flow rate of 0.4 mL/min, and column temperature of 36 °C. Total chromatographic run time was 2.3 min. Sample injection volume was 3 μL.

2.7. Determination of limits of detection and quantification

Instrumental limits of detection (LOD) and quantification (LOQ) were estimated in terms of baseline noise and instrument response. The LOD was defined as the lowest LARL and NARL concentrations that yielded a signal-to-noise ratio (S/N) of 3:1, whereas the LOQ was defined as the lowest concentration within the linear range of the MS response that exhibited a 10:1 S/N ratio. To determine the LOD and LOQ, serial dilutions of each standard containing CA (5 ppm) were injected (3 μL) under chromatographic conditions and data acquired in the negative SIM mode monitoring *m*/z 487.2, 531.2, and 490.9 for LARL, NARL, and CA, respectively.

2.8. Data analysis

Peak-area ratios of LARL/CA and NARL/CA were plotted as a function of the internal calibrator concentrations in ppm and a linear curve fit applied. Samples sets with an r^2 <0.95 were considered invalid and their analysis repeated. Concentrations of LARL or NARL were calculated by taking a ratio of the intercept over slope and multiplying it by the juice dilution factor.

3. Results and discussion

3.1. Preparation of LARL and NARL solutions

One reason for the general lack of methods for the direct quantification of LARL and NARL in citrus samples is the difficulty associated with generating and quantifying limonoid A-ring lactone standards. LARL and NARL solutions were generated enzymatically, purified by SPE, and their concentrations established by their acid catalyzed conversion to aglycones and quantification by fluorescence spectroscopy [20]. Previously described methods for the enzymatic generation of LARL and NARL were modified by omitting the methanol or acetonitrile co-solvent and directly adding solid limonin or nomilin as a suspension [4,21]. Capitalizing on the water solubility of limonoid A-ring lactones, solutions from 150 to 300 ppm were easily generated. In addition, the residual unreacted solid was recovered, washed with water and reused. Fluorescence detection was chosen because it was simple, rapid, and required no special solutions or extractions in contrast to colorimetric and HPLC methods [18,22].

3.2. LC–MS development

The dynamic character of the LC–MS system can cause variability in ion detection, and the use of internal standards to mitigate this variability has been reported for the LC–MS analysis of limonoid glucosides and aglycones [6,14]. CA, the same internal standard used for the limonoid glucoside analysis, was chosen for limonoid A-ring lactones because the chromatographic and MS ionization properties of limonoid A-ring lactones and limonoid glucosides are similar.

Because we had observed in situ conversion of limonin to LARL in the MS during ionization, chromatographic separation of LARL and NARL from their corresponding aglycones was necessary. In the course of examining a number of chromatographic conditions and stationary phases, we found that formic acid was essential for the separation of limonid A-ring lactones from their corresponding aglycones and for their ionization for MS detection. Methanol gave better peak shape than acetonitrile and ethanol. Increases in retention times had no effect on peak shape, but the Synergi stationary phase greatly reduced the tailing exhibited when using a C-18 stationary phases. In short, the best compromise between separation of NARL and LARL from their corresponding aglycones, run time and peak shape was achieved by use of a 4 mM formic acid–MeOH (50:50) isocratic mobile phase with a Synergi Hydro-RP 80A stationary phase. Under these conditions the order of elution was CA (0.48 min), LARL (0.64 min), NARL (0.91 min), limonin (1.60 min), and nomilin (2.5 min). Because analysis focused on CA, LARL and NARL, we determined that 2.3 min was the minimal run time. In contrast to the methodology of Merino et al. [18], no ion pair reagent was necessary and sample throughput was increased threefold by shortening chromatographic run times.

Fig. 2a shows a total ion count (TIC) chromatogram obtained by reversed-phase LC–ESI-MS of a navel orange juice sample treated as described in the methods section. Fig. 2b–d show single-ion monitoring (SIM) mode chromatograms of the same sample derived from detection confined to CA,

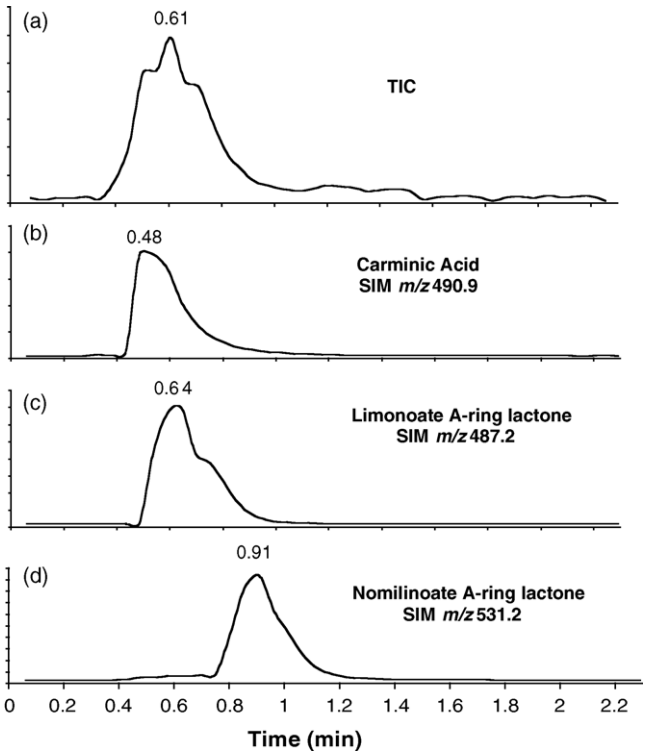

Fig. 2. Analysis of navel orange juice sample. Quantity of analyte listed in parentheses. (a) Total ion current LC–ESI-MS chromatogram; (b) single-ion monitoring of CA (17.9 ng); (c) single-ion monitoring of LARL (14.3 ng); (d) single-ion monitoring of NARL (3.0 ng).

LARL, and NARL. Fig. 2b–d are representative of the chromatograms obtained from the analyzed samples and similar results were obtained for standards. Although LARL (Fig. 2c) exhibited some shouldering, the peak shape was sufficient for consistent integration and quantification.

Instrumental detection (LOD) and quantification limits (LOQ) estimated in terms of signal-to-noise ratio were 40 and 75 pg, respectively. LC–MS response was linear over three orders of magnitude ($r^2 > 0.997$).

Evaluation of the efficiency of the SPE recovery of LARL (0.1–20.0 ppm), NARL (0.1–20.0 ppm) and CA (10.0 ppm) from a spiked aqueous solution showed recovery to be greater than 98%. Recoveries of LARL (0.1–20.0 ppm) and NARL (0.1–20.0 ppm) from spiked juice samples were linear over three orders of magnitude, but the percent recoveries were cultivar dependent and ranged from 63 to 87%. Rather than determining percent recovery for each cultivar, the analysis of samples was expedited by utilizing the standard addition method. To this end, three LARL and NARL concentrations were used to generate internal calibration curves. Calibrator solutions were added to samples prior to SPE to reduce the impact of extraction errors. For a typical three-point determination, r^2 values were greater than 0.98 and slopes for the standard addition method ranged from 0.62 to 0.94, and from 0.29 to 0.52 for LARL and NARL, respectively. The relative standard deviation for replicate analyses for this method was 7–10% and the LOD and LOQ were estimated to be 64 and 125 pg, respectively.

Table 1
LARL and NARL concentrations in citrus juices

Fruit juice	LARL (ppm)	NARL (ppm)
Valencia orange	2.4	2.0
Cara Cara navel orange	32.4	1.3
Texas Valencia orange	15.2	2.1
Honey tangerine	17.1	2.4
Rio Star grapefruit	17.8	1.5

Experimental details are described in Section 2.

The analytical method was applied to a variety of citrus juice samples to determine the level of LARL and NARL (Table 1). Concentrations of LARL ranged from 2.4 ppm in Valencia to 32.4 ppm in Cara Cara navel orange juices. Relative to LARL, a very small amount of NARL was observed in all analyzed fruits. If juice is left to stand, enzyme catalyzed conversion of non-bitter LARL and NARL to bitter limonin and nomilin is initiated. Our results show that four of the citrus fruits analyzed have enough LARL to generate limonin concentrations above the taste threshold level for bitterness (6 ppm). The juice of these fruits most likely will require post processing to lower the bitterness whereas Valencia juice is unlikely to require such additional processing.

4. Conclusion

A rapid and sensitive LC–ESI-MS method for the quantification of limonoate and nomilinoate A-ring lactones in a wide variety of citrus juices was described. This method provides a valuable tool for citrus growers and juice producers to evaluate the susceptibility of a fruit or juice to delayed bitterness.

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