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Rapid simultaneous determination of amines and organic acids in citrus using high-performance liquid chromatography

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ABSTRACT

Rapid analytical method for the simultaneous separation and determination of amines and organic acids is a vital interest for quality control of citrus and their products. In the present study, a simultaneous high performance liquid chromatography (HPLC) method for the rapid separation of three amines and two organic acids was developed. Chromatographic separation of compounds was achieved using Xbridge C_{18} column at ambient temperature, with an isocratic mobile phase of 3 mM phosphoric acid at a flow rate of 1.0 mL min^{-1}. A photodiode array (PDA) detector was used to monitor the eluent at 223 nm and 254 nm with a total analysis time of 10 min. Extraction of amines and organic acids from citrus juice was optimized. The method was validated by tests of linearity, recovery, precision and ruggedness. The limit of detection (LOD) and limit of quantification (LOQ) for amines and ascorbic acid were determined to be 5 ng and 9.8 ng, respectively. All calibration curves showed good linearity ($R^2 \geq 0.9999$) within the test ranges. The recoveries of the amines and organic acids ranged between 84% and 117%. The identity of each peak was confirmed by mass spectral (MS) analysis. The developed method was successfully applied to analyze the content of amines and organic acids in six different species and two varieties of citrus. Results indicate that mandarin and Marrs sweet orange contain high level of amines, while pummelo and Rio Red grapefruit had high content of ascorbic acid (137–251 μg mL^{-1}) and citric acid (5–22 mg mL^{-1}). Synephrine was the major amine present in Clementine (114 μg mL^{-1}) and Marrs sweet orange (85 μg mL^{-1}). To the best of our knowledge, this is the first report on simultaneous separation and quantification of amines and organic acids in Marrs sweet orange, Meyer lemon, Nova tangerine, Clementine, Ugli tangelo and Wekiwa tangelo.

1. Introduction

Citrus fruits contain numerous naturally occurring compounds including flavonoids, limonoids, furocoumarins, vitamins, carotenoids, organic acids and amines. Synephrine, octopomine and tyramine are commonly occurring amines in citrus (Fig. 1). In recent years, an increased attention towards the importance of amines was observed due to their potential role in obesity prevention [1,2]. Synephrine is chemically similar to ephedrine based on which, several formulations and extracts with synephrine as the main ingredient are currently being promoted as weight reducing dietary supplements. Moreover, the ban of ephedrine containing substance by Food & Drug Administration [3] seems to have resulted in higher consumption of dietary supplements containing amines due to their relatively less health risks [4].

In addition to amines, citrus fruits contain a high content of organic acids such as ascorbic and citric acid (Fig. 1). Ascorbic acid is a potent antioxidant, anti-proliferative and anti-scurvy agent [5–7]. Moreover, citric acid imparts the tart flavor of citrus which makes it an important component of quality. Organic acids not only influence browning in citrus juices but also have a major role in quality control in the citrus processing industry [8,9]. Considering bioactivity of amines, several analytical methods were reported for determining their content in *Citrus aurantium* fruits [10–12] and dietary supplements [13–15]. However, very few reports on the levels of amines present in other citrus species have been reported. In a report evaluating the content of amines in orange juices, an ion pair agent was used for separation using a μBondapak C_{18} column [16]. The juice sample was initially purified on a C_{18} reverse-phase (Sep-Pak) cartridges, followed by the separations of synephrine and octopomine using an isocratic elution of 0.1 M acetate buffer and acetonitrile (91:9) with UV detection of 275 nm. Tyramine and other amines were analyzed separately using a gradient of 0.1 M acetate buffer and acetonitrile with a run time of 71 min. Use of dual methods for the separation of amines is time consuming

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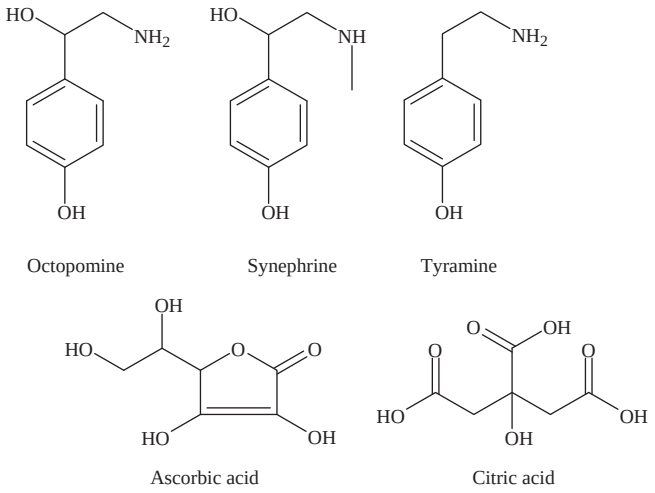

Fig. 1. Structures of amines and organic acids quantified in this study.

and may not be economically viable. A 'green' HPLC technique for the analysis of octopomine, synephrine and tyramine using ionic mobile phase was reported by Tang et al. [17]. While this method enabled the separation of amines, use of pyridinium and methylimidazolium salts in the mobile phase for routine purpose may not be safe due to their toxicity [18]. Moreover, due to the poor retention of amines on the column, buffers have been commonly used in mobile phase for the separation of amines [13,19,20]. Recently, Pellati and Benvenuti [21] analyzed aqueous extract of sour orange sample using a pentafluorophenylpropyl stationary phase without clear baseline separation between synephrine and tyramine.

Similarly, several methods were described for the quantification of organic acids using different transduction systems such as spectrophotometer [22,23], colorimeter [24,25], and HPLC [26–29]. However, a method with the separation of both amines and organic acids would be of vital interest to monitor quality control in citrus processing industry and may also enhance the consumer preference for citrus consumption. To the best of our knowledge, currently an economical simultaneous technique for the extraction and separation of amines and organic acids is not available. This method will be less time consuming and economical. Since both amines and organic acids are polar compounds, we hypothesize that the use of a polar solvent would enable in simultaneous extraction. In continuation of our research on efficient HPLC method development for the quantification and identification of citrus bioactive compounds [30–32], a study was conducted to develop a rapid HPLC method for the extraction, separation and determination of amines and organic acids in citrus fruits.

2. Materials and methods

2.1. Reagents and standards

Octopomine, synephrine, tyramine, citric acid, and HPLC grade phosphoric acid were purchased from Sigma–Aldrich (St. Louis, MO). L-Ascorbic acid was purchased from Mallinckrodt (Paris, KY, USA). Nanopure water (NANOpure, Barnstead/Thermolyne, Dubuque, IA) was used for the sample preparation and HPLC analysis. The standard amines such as octopomine, synephrine, and tyramine were prepared in nanopure water to obtain 1 mg mL^{-1} stock solution. Citric acid was also dissolved in nanopure water. All the standards were sonicated for 30 s and serial dilutions were made with nanopure water. Ascorbic acid was prepared in 3% metaphosphoric acid.

2.2. Fruit samples

Six different species and two varieties of citrus fruits such as Marrs sweet orange (*Citrus sinensis* Tan.), Rio Red (*C. paradisi* Macf.), red fleshed pummelo (*C. grandis* Tan.), Meyer lemon (*C. limon* Tan.), Nova tangerine (*C. reticulata* Tan.), Ugli tangelo (*C. reticulata* × *C. paradisi*), and Wekiwa tangelo (*C. reticulata* × *C. paradisi*), were harvested in the month of November 2008 from Texas A&M-Kingsville Citrus Center (Weslaco, TX, USA). Clementine fruits (*C. clementina*) were harvested in the month of November 2008 from Placer County (CA, USA).

2.3. Instrumentation

The HPLC system consisted of a Waters 1525 HPLC series (Milford, MA, USA) connected to a Waters 2996 PDA detector and Waters 717 autosampler. The columns evaluated for the optimum separations of amines and organic acids were Xbridge C_{18} (3.5 μm, 4.6 mm × 150 mm i.d.) from Waters (Milford, MA, USA), Gemini C_{18} (5 μm, 250 mm × 4.6 mm i.d.) from Phenomenex (Torrance, CA, USA), and Luna C_{18} (5 μm, 250 mm × 4.6 mm i.d.) from Phenomenex (Torrance, CA, USA). An isocratic mobile phase of 3 mM phosphoric acid was used at a flow rate of 1.0 mL min^{-1}. The sample injection volume for the analysis of amines and organic acids was 10 μL. The amines and organic acids were detected at 223 nm and 254 nm, respectively. Chromatographic data was collected and processed using Empower2 software (Waters, Milford, MA, USA).

2.4. Sample preparation

Fruit samples of Clementine mandarins and Meyer lemons were peeled, blended for 3 min and homogenized for 30 s using a Polytron homogenizer (Brinkmann Instruments Inc., Westbury, NY, USA). Two solvents such as water and 3% meta phosphoric acid were used to optimize extraction efficiency of amines and organic acids. Ten milliliters of homogenized juice sample was diluted with 30 mL of water in a centrifuge tube and mixed for 15 min. Three milliliters of diluted sample was filtered under vacuum using a 0.45 μm membrane filter (Millipore Corp., Bedford, MA, USA). The residue was re-extracted with 1 mL of solvent and filtered. The procedure was repeated for another two times using 1 mL of solvent each time. Filtrate from all the extractions was pooled and 10 μL was injected to HPLC for analysis. Similarly, four extractions were performed using 3% meta phosphoric acid and analyzed by HPLC. The above extraction was conducted at 4 °C using an ice bath to prevent the degradation of ascorbic acid. The sample extracts were stored at −80 °C until analyzed.

2.5. Recovery and repeatability

To validate the sample preparation procedure, recovery studies were performed by adding known concentration of standard mixture of amines and organic acids to Meyer lemon and Clementine mandarin juice samples. These two species were selected based on high and low concentrations of the analyzed compounds present naturally. Meyer lemon juice (10 mL) was fortified by adding 0.40 mg of octopomine, 0.50 mg of synephrine, 0.2 mg of tyramine, 120 mg of citric acid and 0.25 mg of ascorbic acid. To evaluate the dependence of recovery on the concentration, Clementine mandarin juice was fortified with two different concentration levels of standard amines and organic acids. Ten milliliters of Clementine mandarin juice was fortified with 1.00 mg of octopomine, 1.00 mg of synephrine, 0.40 mg of tyramine, 0.50 mg of ascorbic acid and 260 mg of citric acid. Similarly, for the evaluation of different levels of concentration, 0.50 mg of octopomine, 0.50 mg of synephrine, 0.20 mg of tyramine, 0.25 mg of ascorbic acid and 130 mg of cit-

ric acid were added to 10 mL of Clementine mandarin juice. After the addition of standards to the respective juice samples, the volume was made up to 40 mL by adding 3% meta phosphoric acid and extracted using the resultant optimized extraction procedure and analyzed by HPLC. This analysis was evaluated on a different day using a different set of samples.

Repeatability was expressed as the relative standard deviation (%RSD) and was determined by repeating the extraction procedure and analysis five times.

2.6. Precision and ruggedness

The precision of the HPLC system was determined by evaluating inter-day and intra-day injections of standard amines and organic acids. Six injections were performed for each day within three consecutive days. The %RSD of the retention times was evaluated for all the injections.

The ruggedness of the analytical method was evaluated by varying the HPLC systems and keeping all parameters such as column: Xbridge C_{18} column (3.5 μm, 4.6 mm × 150 mm i.d.), mobile phase (0.03 mM phosphoric acid), flow rate (1 mL min^{-1}) and detection constant. The two HPLC systems Waters 1525 HPLC series (Milford, MA, USA) and Agilent 1200 Series (Foster City, CA, USA) were evaluated for the separation of amines and organic acids using the developed method.

2.7. Quantification of amines and organic acids in citrus samples

Ten microliters of each sample was injected onto HPLC for the analysis of amines and organic acids. The elution and quantification of the target compounds was carried out using the optimized method. The concentration of the respective compound was calculated using the regression equation and dilution factor. The concentration of amines and ascorbic acid is represented as μg mL^{-1}, and citric acid is expressed as mg mL^{-1} of juice.

2.8. Mass spectral analysis

The individual peaks were collected from HPLC and subjected to mass spectral analysis. The analyses of octopomine, synephrine and citric acid were performed on MDS-Sciex QSTAR Pulsariquadrupole-time-of-flight (QqTOF) mass spectrometer (Toronto, Ontario, Canada). Analysis was performed under following conditions; collision gas: nitrogen, curtain gas: 20 psi, ion spray voltage: 4500 V, declustering potential: 10 V, focusing potential: 220 V, second declustering potential10 V, ion release delay: 11 μs, ion release width: 10 μs, resolution ion energy: 1 V, detector (MCP): 2150 V, and syringe pump flow: 7 μL min^{-1}. Mass spectral analysis of tyramine and ascorbic acid was performed on LCQ™ Deca (Thermoscientific) ion trap mass spectrometer. Ionization was done using the atmospheric chemical ionization (APCI) source. The source heater temperature was set at 450 °C, sheath gas flow was maintained at 80 units and auxiliary gas flow was set to 10 units, The discharge current: 4.5 μA, capillary temperature: 150 °C, capillary voltage: 46 V, and tube lens offset was 10 V. Amines and ascorbic acid were analyzed by positive mode and citric acid was analyzed in negative mode.

2.9. Statistical analysis

Data was analyzed using the General Linear Model (GLM) procedure with the Walter-Duncan K-ratio *t*-test (SAS, 2007). The analysis of variance differentiates the means by assigning different letters to the treatment means that are significantly different at the 95% level of probability ($P \leq 0.05$). The tests of linearity for the calibration equations and the *P*–*P* plots were determined using regression function in PASW Statistics 18, Version 18.0.0 (SPSS, Inc., Chicago, IL).

3. Results and discussion

3.1. Method development

Three C_{18} columns such as Xbridge, Gemini, and Luna in combination with organic solvents (acetonitrile and methanol) and modifiers (phosphoric acid, trichloroacetic acid, and acetic acid) were evaluated for the rapid separation of amines and organic acids in citrus juice. Due to the poor retention of the compounds on the column because of their high polarity, water seemed to be more ideal mobile phase. Use of acetonitrile, methanol and modifiers such as trichloroacetic acid and acetic acid did not yield optimum separations. In early trials, using water as a mobile phase resulted in peak tailing and poor separation of the compounds. The peak tailing may be due to the interaction between amines and the silanols on the surface of stationary phase [17]. Using (3 mM) phosphoric acid as a modifier coupled with a wide range of pH compatible Xbridge column reduced the peak tailing and a clear separation of amines and organic acids was observed (Fig. 2).

3.2. Sample extraction procedure

Homogenized Clementine mandarin and Meyer lemon juice were extracted with water and compared with 3% meta phosphoric acid extract to determine the optimum extraction procedure for simultaneous analysis of amines and organic acids. In Clementine mandarin juice, synephrine, tyramine, ascorbic acid and citric acid were detected. The extraction efficiency for octopomine was determined using Meyer lemon juice. Sample extraction with 3% meta phosphoric acid resulted significantly higher content of synephrine, tyramine, ascorbic acid and citric acid in comparison with water. No significant difference in the octopomine content was noticed between 3% meta phosphoric acid and water extraction. Therefore, 3% meta phosphoric acid seems to be an ideal solvent for the simultaneous extraction of both amines and organic acids (Fig. 3). Previous analytical methods suggest, water as an ideal solvent for the optimum extraction of amines [21,33]. For optimum simultaneous extraction of organic acids, water as a solvent is a limiting factor since ascorbic acid is highly unstable and requires acidic medium for stability [34]. Although both amines and organic acids are soluble in 3% meta phosphoric acid, the dense matrix of citrus juice limits optimum extraction in a single step. Results from monitoring successive extractions of the unfiltered residue of the juice suggest that re-extraction of the residue with 3 mL of 3% meta phosphoric acid was optimum for the complete extraction of amines and organic acids (Fig. S1).

3.3. Method validation

3.3.1. Linearity, LOD and LOQ

Linear curves for all the standards (octopomine, synephrine, tyramine, and ascorbic acid) were prepared using six concentrations ranging from 9.8 ng to 312.5 ng and citric acid ranging 1.25 to 40 μg. with triplicate injections. The linear curves were obtained by plotting the standard concentration as a function of peak area obtained from HPLC analysis (Fig. S2). Good linear relationship and correlation coefficients were observed between the six different concentrations of amines as well as organic acids with their peak area responses. The correlation coefficient (R^2) of amines and organic acids were found to be >0.9999 (Table 1). The linearity for all the compounds was evaluated by residual graphs and normal probability. The residual plots corresponding to the respective

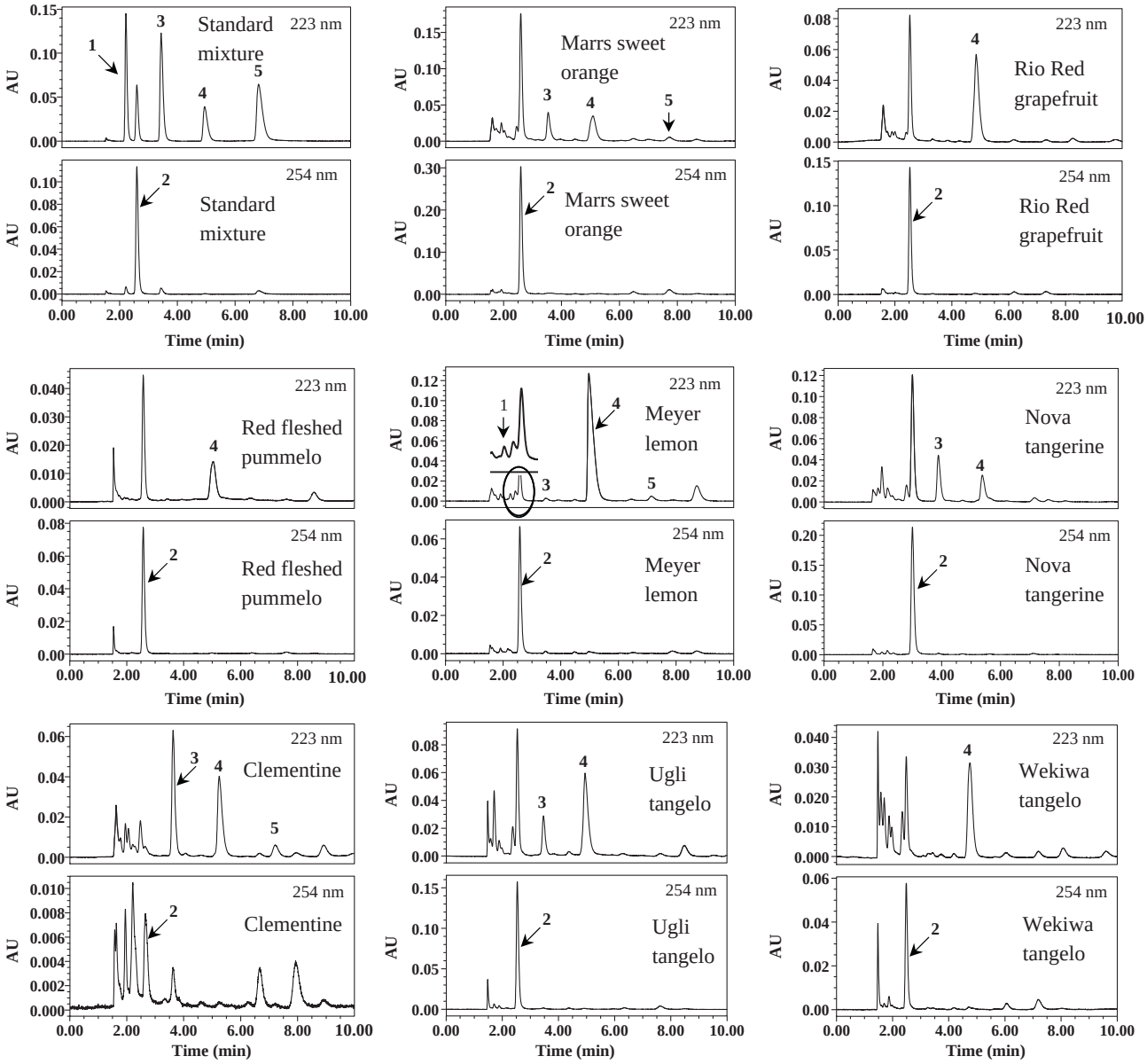

Fig. 2. HPLC chromatograms of standard mixture of amines and organic acids as well as different citrus species juice (peak identification: 1, (±)-octopomine; 2, ascorbic acid; 3, (±)-synephrine; 4, citric acid; 5, tyramine: detection, PDA at 223 and 254 nm).

compound linearity plot indicated random distribution of residuals (Fig. S3). Similarly, normal probability plots (*P*–*P* plots) were approximately linear for all the calibrations of the analyzed compounds (Fig. S4). The *t*-test ($P < 0.05$) also confirmed that there was no statistically significant difference in the predicted and observed values. The limit of detection or sensitivity was measured by injecting serial diluted standard solutions, considering the signal-to-noise ratio (3:1). The limit of quantification (LOQ) was determined as the lowest concentration which can be determined with an accuracy and precision of >95%. The LOD for the amines as well as ascorbic acid was determined as 5 ng while 63 μg was for citric acid. The LOQ for amines and ascorbic acid was determined to be 9.8 ng while 125 μg was for citric acid. The low LOD and LOQ values confirm that the method developed was sensitive to detect

Table 1
Linear ranges, coefficient of determination (R^2), limit of quantification (LOQ) and limit of detection (LOD) of the amines and organic acids.

Compounds	Regression equation	R^2	Linear range (ng)	LOQ (ng)	LOD (ng)
Octopomine	$y = 2683.6x - 7.7247$	0.9999	9.8–312.5	9.8	5
Synephrine	$y = 3271.2x + 14.736$	0.9999	9.8–312.5	9.8	5
Tyramine	$y = 3203.2x + 19.765$	0.9999	9.8–312.5	9.8	5
Ascorbic acid	$y = 2608.9x + 15.705$	0.9999	9.8–312.5	9.8	5
Citric acid[a]	$y = 26.421x + 3.8856$	1.0000	1.25–40.0[a]	1.25[a]	0.63[a]

x = concentration of the respective compounds.
y = peak area (AU)
[a] Citric acid concentration is expressed as μg.

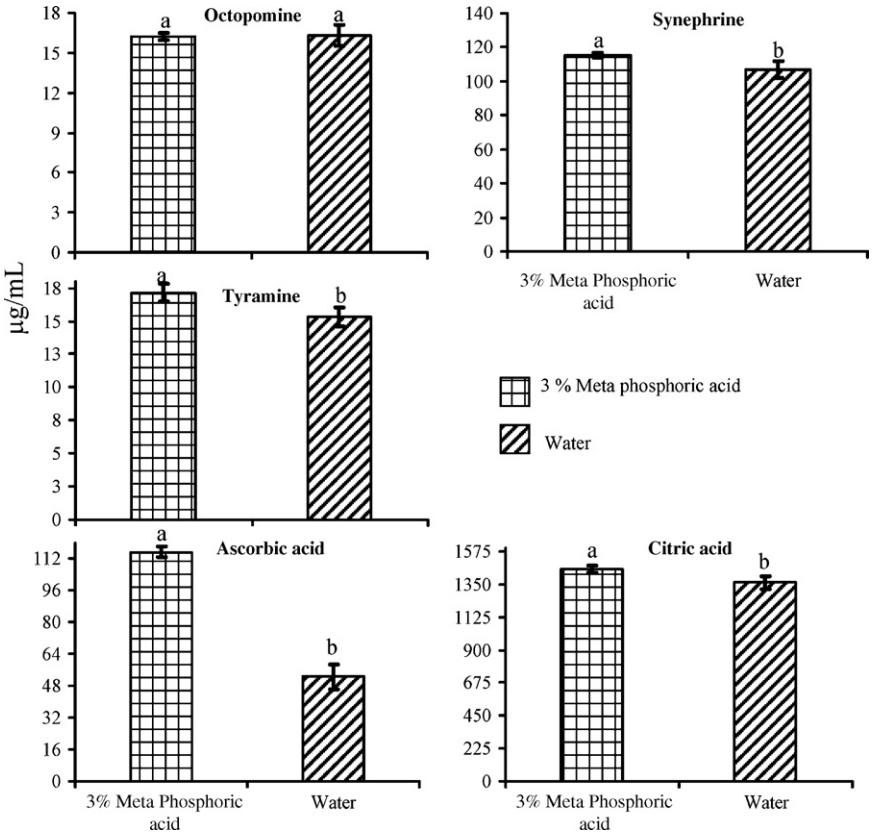

Fig. 3. Extraction of amines and organic acids by two solvents. Octopomine and ascorbic acid were quantified in Meyer lemon juice. Synephrine, tyramine and citric acid were quantified in Clementine mandarin juice. Different letters indicate significant differences at $P < 0.05$ and similar letters indicate no significant differences $P < 0.05$.

and quantify samples containing low concentrations of amines and organic acids. Peaks from the sample were identified by comparing the UV spectra and retention time (t_R) with those obtained from the individual standard samples. The results were confirmed by spiking the sample with standards for the detection of peak enhancement.

3.3.2. Recovery and repeatability

To evaluate the recovery test, known concentration of standard solutions was added to the Clementine mandarin and Meyer lemon juice. This fortified sample mixture was extracted and analyzed by the optimized HPLC method. Results obtained from the recovery analysis are summarized in Table 2. The mean recovery of the analytes was compared with the actual quantity of the analytes present in the sample. The recovery percentage for the analytes ranged between 84.01 and 117.28%, indicating the reliability and accuracy of the developed method. The %RSD for the recovery of all the amines ranged between 0.50 and 4.25. Among the analyzed organic acids, citric acid had a low %RSD of 0.16 in the recovery analysis for Meyer lemon juice, whereas %RSD of ascorbic acid ranged in between 10.56 and 1.05 for recovery analysis in Meyer lemon and

Table 2
Recovery studies of amines and organic acids from citrus juices.[a]

Variety compound	Actual amount present in the juice sample (mg 10 mL^{-1})	Amount of standard added to the sample (mg 10 mL^{-1})	Amount expected (mg 10 mL^{-1})	Amount determined (mg 10 mL^{-1})	Recovery (%)	RSD[b] (%)
Meyer lemon						
Octopomine	0.16 ± 0.01	0.40	0.56	0.55	98.71	3.03
Synephrine	0.01 ± 0.00	0.50	0.51	0.46	90.89	3.30
Tyramine	0.10 ± 0.00	0.20	0.30	0.31	102.24	1.90
Ascorbic acid	0.88 ± 0.01	0.25	1.13	0.95	84.01	10.56
Citric acid	598.99 ± 5.38	120.00	718.99	0.70	97.57	0.16
Clementine						
Octopomine	ND	1.00	1.00	0.86	86.24	1.01
Synephrine	1.24 ± 0.04	1.00	2.24	1.99	88.63	0.50
Tyramine	0.15 ± 0.01	0.40	0.59	0.55	93.21	4.25
Ascorbic acid	0.19 ± 0.01	0.50	0.70	0.59	90.45	4.46
Citric acid	142.58 ± 3.38	260.00	402.58	472.15	117.28	1.73
Octopomine	ND	0.50	0.50	0.48	96.82	0.89
Synephrine	1.24 ± 0.04	0.50	1.74	1.66	95.41	1.45
Tyramine	0.15 ± 0.01	0.20	0.39	0.57	93.33	3.72
Ascorbic acid	0.19 ± 0.01	0.25	0.40	0.37	92.71	1.05
Citric acid	142.58 ± 3.38	130.00	267.58	311.57	114.30	0.41

ND, not detected.

[a] Results are mean ± standard deviation values of three replications of each sample.

[b] RSD (%) = relative standard deviation; (standard deviation/mean) × 100.

Table 3
Intra-day and inter-day variation for retention time of amines and organic acid.

Compound	Intra-day precision[a]						Inter-day precision[b]	
	Day 1		Day 2		Day 3			
	t_R (min)	RSD (%)[c]	t_R (min)	RSD (%)[c]	t_R (min)	RSD (%)[c]	t_R (min)	RSD (%)[c]
Octopomine	2.16	0.9	2.21	0.9	2.18	0.5	2.19	1.2
Ascorbic acid	2.50	1.1	2.59	1.1	2.62	0.7	2.56	3.5
Synephrine	3.32	0.9	3.44	0.9	3.39	0.7	3.38	1.8
Citric acid	4.75	1.0	4.96	1.0	4.84	0.6	4.85	2.2
Tyramine	6.52	1.0	6.83	1.0	6.73	0.7	6.69	2.3

[a] Results are mean values of four separate injections of standard sample within each day.
[b] Results are mean values of injections of standard sample in three consecutive days.
[c] RSD (%) = relative standard deviation; (standard deviation/mean) × 100.

Clementine, respectively. The low %RSD obtained in the recovery of standard amines and organic acids evaluated in two different concentrations added to the Clementine mandarin juice further validate the sample extraction procedure.

Repeatability of the extraction procedure was determined by repeating the extraction procedure five times using the same Clementine sample and analyzed by HPLC. The RSD (%) values for synephrine, tyramine, ascorbic acid and citric acid were determined to be 1.22, 3.73, 1.74 and 9.94, respectively. The high RSD (9.94%) for citric acid could be due to its presence in high concentration in samples. Further diluting the sample was not ideal since octopomine and tyramine could not be detected due to low concentrations. The low RSD (%) of values for other compounds demonstrated good repeatability. Thus, the method could be used for the quantification of both high concentrations of amines and organic acids.

3.3.3. Precision and ruggedness

The precision of the HPLC system was determined by evaluating inter-day and intra-day injections of standard solution consisting of octopomine, synephrine, tyramine, citric acid and ascorbic acid (Table 3). The RSD of the retention times for intra-day ranged in between 0.5% and 1% for all the compounds and the inter-day variation ranged in between 1.2% and 3.5%.

The ruggedness of the present analytical method was evaluated by varying the HPLC systems without changing sample extraction procedure [35]. No change in the resolution of the peaks was observed for the same column. The RSD (%) values ranged between 0.19 and 1.14% in both the HPLC systems (Table 4). The results from the tests of precision and ruggedness demonstrate that the method is precise and rugged and could be used for analysis of commercial samples.

3.4. Analysis of citrus fruits samples

The developed optimized method was used for the quantification of amines and organic acids in six different species and two varieties of citrus. All the samples were extracted and analyzed in triplicate. The HPLC chromatograms of the analyzed citrus species are presented in Fig. 2. Table 5 demonstrates the variation of amines and organic acids among citrus species. Octopomine was detected only in the Meyer lemon (16.29 $\mu g\,mL^{-1}$). Synephrine was the predominant amine in most of the analyzed citrus species, but was not detected in grapefruit, pummelo and Wekiwa tangelo. Clementine mandarin had the highest content (114 $\mu g\,mL^{-1}$) of synephrine while Meyer lemon had the lowest content (2.75 $\mu g\,mL^{-1}$). In both grapefruit and red fleshed pummelo, amines were not detected. However, it was interesting to note the presence of synephrine in Ugli tangelo variety (46.88 $\mu g\,mL^{-1}$) and its absence in Wekiwa tangelo. Tangelos are a hybrid between tangerine (*C. nobilis var.* deliciosa) and grapefruit (*C. paradisi*). The absence of synephrine in Wekiwa tangelo may be due to hereditary characteristic of the parent crosses. Wekiwa tangelo is a cross between grapefruit and Sampson tangelo [36], and based on our analysis amines were not detected in grapefruits. Tyramine was detected in Clementine (17.0 $\mu g\,mL^{-1}$), Marrs sweet orange (4.82 $\mu g\,mL^{-1}$) and Meyer lemon (9.22 $\mu g\,mL^{-1}$). Among organic acids, citric acid was the predominant of the two with the high concentration determined in Meyer lemon (52.94 $mg\,mL^{-1}$), which is characteristic of acidic fruits. Pummelo had low citric acid content (5.44 $mg\,mL^{-1}$) which seems to be the less tart variety among the analyzed citrus species. Marrs sweet orange had high ascor-

Table 4
Retention (t_R) times and RSD (%) of amines and organic acids for ruggedness.[a]

Compound	Waters 1525 Xbridge		Agilent 1200 Xbridge	
	t_R (min)	RSD (%)[b]	t_R (min)	RSD (%)[b]
Octopomine	2.16	0.21	1.95	0.21
Ascorbic acid	2.45	0.19	2.17	0.28
Synephrine	3.32	0.21	2.84	0.48
Citric acid	4.74	0.40	3.83	0.91
Tyramine	6.52	0.55	5.38	1.14

[a] Results are mean values of five separate injections of sample for each individual HPLC system.
[b] RSD (%) = relative standard deviation; (standard deviation/mean) × 100.

Table 5
Content of amines and organic acids in eight citrus juice samples.

Species (common name)	Octopomine ($\mu g\,mL^{-1}$)[a]	Synephrine ($\mu g\,mL^{-1}$)[a]	Tyramine ($\mu g\,mL^{-1}$)[a]	Ascorbic acid ($\mu g\,mL^{-1}$)[a]	Citric acid ($mg\,mL^{-1}$)[a]
C. sinensis Tan. (Marrs sweet orange)	ND	85.17 ± 2.69	4.82 ± 2.87	565.21 ± 6.90	15.28 ± 0.34
C. paradisi Macf. (Rio Red grapefruit)	ND	ND	ND	250.82 ± 6.27	21.89 ± 1.89
C. grandis Tan. (Red fleshed pummelo)	ND	ND	ND	137.16 ± 1.90	5.44 ± 0.14
C. limon Tan. (Meyer lemon)	16.29 ± 0.26	2.75 ± 0.60	9.22 ± 0.44	115.23 ± 2.81	52.94 ± 1.11
C. reticulata Tan. (Nova tangerine)	ND	78.28 ± 6.36	ND	363.60 ± 4.23	7.31 ± 0.43
C. clementina Tan. (Clementine)	ND	114.61 ± 2.89	17.00 ± 0.72	16.08 ± 3.06	14.42 ± 0.47
C. reticulata × *C. paradisi* (Ugli tangelo)	ND	46.88 ± 5.78	ND	262.32 ± 0.26	19.92 ± 1.12
C. reticulata × *C. paradisi* (Wekiwa tangelo)	ND	ND	ND	95.97 ± 1.98	11.66 ± 0.17

ND, not detected.
[a] Data presented is mean ± standard deviation values of three replications of each sample.

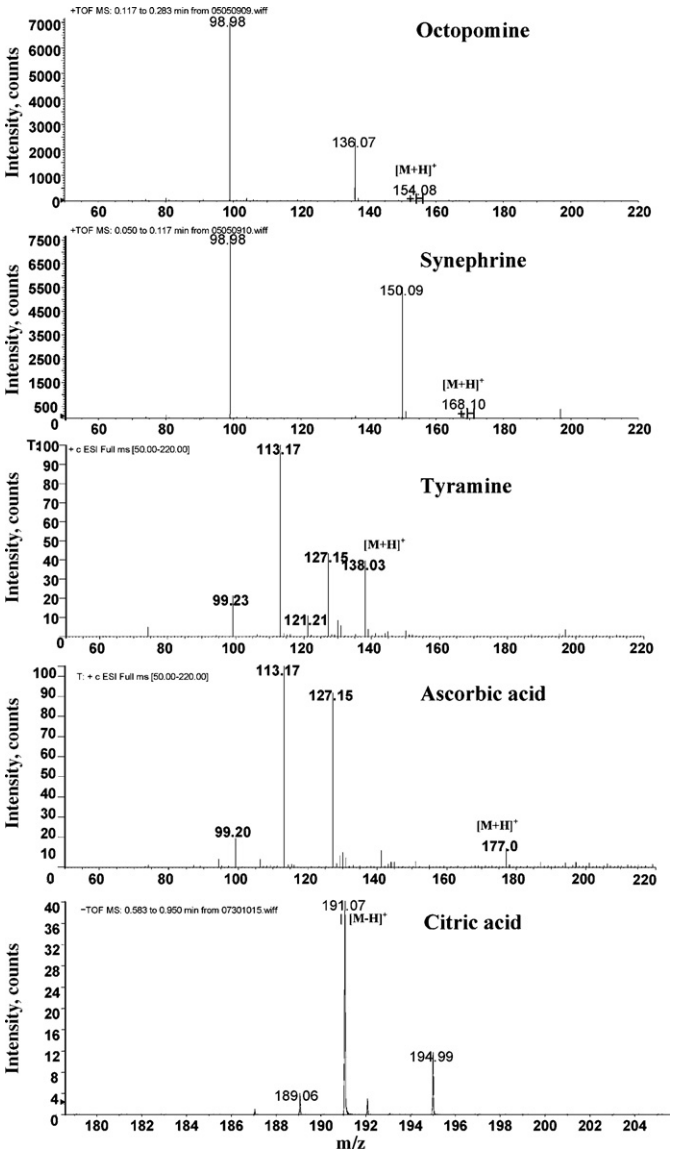

Fig. 4. Mass spectra of amines and organic acids. Each compound was collected from HPLC and analyzed by mass spectra.

bic acid content (565.21 μg mL^{-1}) followed by Nova tangerine (363.60 μg mL^{-1}).

3.5. Mass spectral analysis

The identity of pure peaks collected from HPLC peaks was confirmed by the mass spectral analyses (Fig. 4). The mass spectrum of octopomine shows a molecular ion $[M+H]^+$ at m/z 154.08, an intense adduct ion $[M+H-H_2O]^+$ at m/z 136.07. Synephrine generated molecular ion $[M+H]^+$ at m/z 168.10 and prominent product ions as a result of loss of H_2O, $[M+H-H_2O]^+$. Tyramine generated molecular ion $[M+H]^+$ at m/z 138.03 and an intense adduct by the loss of NH_3, $[M+H-NH_3]^+$ at m/z 121.21 from protonated tyramine molecule. The mass spectra of ascorbic acid and citric acid show a molecular ion $[M+H]^+$ at m/z 177.0, and $[M-H]^+$ at m/z 191.07, respectively.

4. Conclusion

For the first time, a rapid simultaneous separation as well as determination of amines and organic acids in citrus juice was achieved. The developed HPLC method demonstrates that, 3% meta phosphoric acid can be used for the simultaneous extraction of organic acids and amines. The method is precise and rugged combined with high recovery and repeatability. The simultaneous extraction and analysis of samples provides an economical method for analysis of large number of samples in short duration of time. Thus, this method has potential of being applied as an analytical technique for quality control in citrus fruits processing industries.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2010.10.063.

References

[1] E. Fontana, N. Morin, D. Prévot, C. Carpéné, Comp. Biochem. Physiol. C: Pharmacol. Toxicol. Endocrinol. 125 (2000) 33–44.
[2] C. Carpéné, J. Galitzky, E. Fontana, C. Atgié, M. Lafontan, M. Berlan, Naunyn Schmiedeberg's Arch. Pharmacol. 359 (1999) 310–321.
[3] C. Rados, FDA Consum. 38 (2004) 6–8.
[4] A. Fugh-Berman, A. Myers, Exp. Biol. Med. 229 (2004) 698–704.
[5] M.W. Roomi, V. Ivanov, T. Kalinovsky, A. Niedzwiecki, M. Rath, Oncol. Rep. 13 (2005) 421–425.
[6] M. Levine, C. Conry-Cantilena, Y. Wang, R.W. Welch, P.W. Washko, R. Dhariwal, J.B. Park, A. Lazarev, J.F. Graumlich, J. King, L.R. Cantilena, Proc. Natl. Acad. Sci. U.S.A. 93 (1996) 3704–3709.
[7] B. Frei, Am. J. Clin. Nutr. 54 (1991) 1113Sb–1118.
[8] B. Kacem, J.A. Cornell, M.R. Marshall, R.B. Shireman, R.F. Matthews, J. Food Sci. 52 (1987) 1668–1672.
[9] K.M. Clegg, J. Sci. Food Agric. 17 (1966) 546–549.
[10] B. Avula, V. Joshi, A. Weerasooriya, I. Khan, Chromatographia 62 (2005) 379–383.
[11] F. Pellati, S. Benvenuti, M. Melegari, J. Pharm. Biomed. Anal. 37 (2005) 839–849.
[12] F. Pellati, S. Benvenuti, M. Melegari, F. Firenzuoli, J. Pharm. Biomed. Anal. 29 (2002) 1113–1119.
[13] M.C. Roman, J.M. Betz, J. Hildreth, J. AOAC Int. 90 (2007) 68–81.
[14] J. Santana, K.E. Sharpless, B.C. Nelson, Food Chem. 109 (2008) 675–682.
[15] K. Putzbach, C.A. Rimmer, K.E. Sharpless, L.C. Sander, J. Chromatogr. A 1156 (2007) 304–311.
[16] S.M. Vieira, K.H. Theodoro, M.B.A. Glória, Food Chem. 100 (2007) 895–903.
[17] F. Tang, L. Tao, X. Luo, L. Ding, M. Guo, L. Nie, S. Yao, J. Chromatogr. A 1125 (2006) 182–188.
[18] P. Stepnowski, A.C. Skladanowski, A. Ludwiczak, E. Laczynska, Human Exp. Toxicol. 23 (2004) 513–517.
[19] M. Ganzera, C. Lanser, H. Stuppner, Talanta 66 (2005) 889–894.
[20] R.A. Niemann, M.L. Gay, J. Agric. Food Chem. 51 (2003) 5630–5638.
[21] F. Pellati, S. Benvenuti, J. Chromatogr. A 1165 (2007) 58–66.
[22] K. Srividya, N. Balasubramanian, Analyst 121 (1996) 1653–1655.
[23] Y.B. Enaam, M.E. Kamla, F.A. Hassan, A.S. Gamal, Analyst 116 (1991) 861–865.
[24] I.V. Terekhova, N.A. Obukhova, Mendeleev Commun. 15 (2005) 38–40.
[25] M.L. Antonelli, G. D'Ascenzo, A. Laganà, P. Pusceddu, Talanta 58 (2002) 961–967.
[26] C. Zhanguo, L. Jiuru, Chromatogr. Sci. 40 (2002) 35–39.
[27] M.A. Kall, C. Andersen, J. Chromatogr. B: Biomed. Sci. Appl. 730 (1999) 101–111.
[28] M.L. Vázquez-Odériz, M.E. Vázquez-Blanco, J. López-Hernández, J. Simal-Lozano, M.A. Romero-Rodríguez, J. AOAC Int. 77 (1994) 1056–1059.
[29] M.A.R. Romero, O.M.L. Vazquez, H.J. Lopez, L.J. Simal, J. Chromatogr. Sci. 30 (1992) 433–437.
[30] J.L. Perez, G.K. Jayaprakasha, K.S. Yoo, B.S. Patil, J. Chromatogr. A 1190 (2008) 394–397.
[31] A. Vikram, G.K. Jayaprakasha, B.S. Patil, Anal. Chim. Acta 590 (2007) 180–186.
[32] Q. Tian, E.G. Miller, G.K. Jayaprakasha, B.S. Patil, J. Chromatogr. B 846 (2007) 385–390.
[33] K. Hashimoto, T. Yasuda, K. Ohsawa, J. Chromatogr. A 623 (1992) 386–389.
[34] Y.C. Lee, J.R. Kirk, C.L. Bedford, D.R. Heldman, J. Food Sci. 42 (1977) 640–643.
[35] The United States Pharmacopeia, National Formulary 18, 23rd edition, United States Pharmacopeial Convention, Rockville, USA, 1995.
[36] W. Reuther, H.J. Webber, L.D. Batchelor, The Citrus Industry, Univ. Calif. Press, Berkeley, 1967.