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Mass Spectrometric Determination of the Predominant Adrenergic Protoalkaloids in Bitter Orange (*Citrus aurantium*)

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The predominant adrenergic protoalkaloid found in the peel and fruit of bitter orange, *Citrus aurantium*, is synephrine. Synephrine is reputed to have thermogenic properties and is used as a dietary supplement to enhance energy and promote weight loss. However, there exists some concern that the consumption of dietary supplements containing synephrine or similar protoalkaloids may contribute to adverse cardiovascular events. This study developed and validated a positive-ion mode liquid chromatography/tandem mass spectrometry (LC/MS/MS) method for the quantitative determination of the major (synephrine) and minor (tyramine, *N*-methyltyramine, octopamine, and hordenine) adrenergic protoalkaloids in a suite of National Institute of Standards and Technology (NIST) bitter orange Standard Reference Materials (SRMs): SRM 3258 Bitter Orange Fruit, SRM 3259 Bitter Orange Extract, and SRM 3260 Bitter Orange Solid Oral Dosage Form. The limit of quantitation (LOQ) for all protoalkaloids is approximately 1 pg on-column, except for octopamine (20 pg on-column). Additionally, the method has a linear dynamic range of ≥3 orders of magnitude for all of the protoalkaloids. Individual, as well as "total", protoalkaloid levels (milligrams per kilogram) in the NIST SRMs were determined and compared to the levels measured by an independent liquid chromatography/fluorescence detection (LC/FD) method. Satisfactory concordance between the LC/MS/MS and LC/FD protoalkaloid measurements was demonstrated. LC/MS/MS analysis of the protoalkaloids in the SRMs resulted in mean measurement imprecision levels of ≤10% coefficient of variation (% CV).

KEYWORDS: Bitter orange protoalkaloids; dietary supplements; hordenine; liquid chromatography; *N*-methyltyramine; octopamine; synephrine; tandem mass spectrometry; tyramine

INTRODUCTION

Biogenic protoalkaloids, such as synephrine, tyramine, *N*-methyltyramine, octopamine, and hordenine (**Figure 1**), are common constituents of *Citrus* plants (*1*). Synephrine is especially prevalent in the peel and fruit of bitter orange (*Citrus aurantium*). All of the listed protoalkaloids possess adrenergic (stimulant) activity; however, it is the adrenergic activity of synephrine and its purported ability to promote weight loss that has been the subject of much recent research (*2–5*). Synephrine is the predominant adrenergic protoalkaloid in bitter orange-containing dietary supplements, supplements that have been aggressively developed and marketed in the United States as weight-loss products. Bitter orange-containing dietary supplements have actually replaced Ephedra-containing dietary supplements, removed from the U.S. marketplace in 2004, as the most popular weight-loss products (*3*). The safety of bitter orange products has been a source of controversy because of the potential connection of synephrine to adverse cardiovascular events (*2, 3, 6–8*). Synephrine that is administered parenterally leads to a significant increase in blood pressure in humans; however, it is not clear what effect the ingestion of bitter orange products and products containing synephrine, as well as other adrenergic protoalkaloids, will have on cardiovascular health (*2*). Analytical methods are needed that can identify chemically or biologically active components in dietary supplements (*9, 10*). Specifically, analytical methods are needed that can detect and quantify the known adrenergic protoalkaloids in bitter orange products. It is important to assess both the identity and level of adrenergic protoalkaloids in bitter orange products to ensure accurate labeling and quality control. Additionally, accurate quantitative information regarding the protoalkaloids in bitter orange products will help to clarify the relationship between ingestion of bitter orange products and weight reduction.

Mass spectrometry-based methods have been used infrequently for the analysis of adrenergic protoalkaloids in bitter orange products/matrices, and most of the reported methods have

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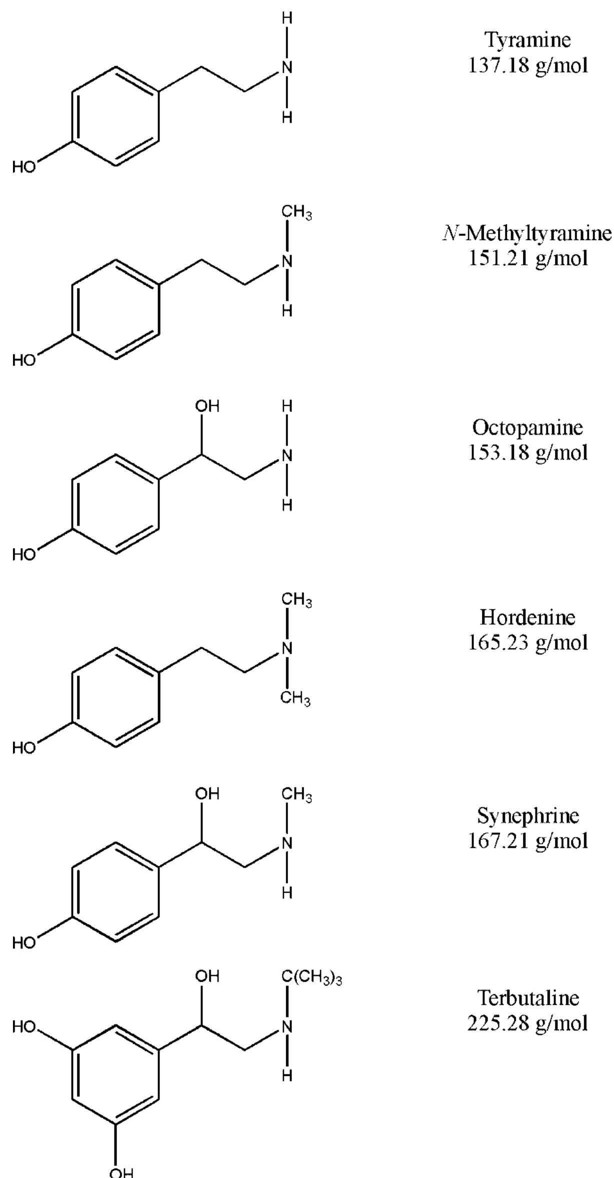

Figure 1. Chemical structures and relative masses for the five predominant bitter orange protoalkaloids (tyramine, *N*-methyltyramine, octopamine, hordenine, synephrine) and the internal standard (terbutaline).

focused only on the determination of synephrine (*11–15*). In an early paper, synephrine was positively identified in bitter orange extracts through the use of liquid chromatography/mass spectrometry (LC/MS), but could not be quantified because of poor chromatographic retention (*12*). Putzbach and co-workers (*15*) were able to resolve the problem of poor chromatographic retention and have developed a single-stage LC/MS procedure for the separation and determination of synephrine, tyramine, *N*-methyltyramine, and octopamine in bitter orange products. Recent studies based on liquid chromatography/tandem mass spectrometry (LC/MS/MS) methodology show good quantitative data for synephrine in bitter orange-containing dietary supplements and in bitter orange/sweet orange fruits (*11, 14*). The latest application of an MS-based method describes the quantitative determination of synephrine in dietary supplements through the use of gas chromatography coupled to MS (GC/MS) (*13*). The GC/MS method is sensitive and specific for synephrine; however, the required analyte derivatization step makes the analysis laborious.

We have undertaken the development and application of a positive-ion mode LC/MS/MS method for the simultaneous quantitative determination of synephrine and four minor adrenergic protoalkaloids (tyramine, *N*-methyltyramine, octopamine, and hordenine) in bitter orange products. Each of the protoalkaloids can theoretically exist as three different positional isomers (ortho, meta, para) and as two different optical isomers (dextro, levo). The present method has been optimized to measure the para positional isomers; optical isomerism of the detected protoalkaloids was not assessed. The method was developed and validated using a suite of new NIST bitter orange SRMs: SRM 3258 Bitter Orange Fruit, SRM 3259 Bitter Orange Extract, and SRM 3260 Bitter Orange Solid Oral Dosage Form. Value assignment of the SRMs is currently underway, and it is the intention that the LC/MS/MS method will assist in those value assignments.

MATERIALS AND METHODS

Tyramine (CAS Registry No. 51-67-2), *N*-methyltyramine (CAS Registry No. 370-98-9), octopamine (CAS Registry No. 770-05-8, hydrochloride salt), hordenine (CAS Registry No. 622-64-0, sulfate salt), and synephrine (CAS Registry No. 97-07-5, *d*/*l* racemic mixture) were obtained from ChromaDex (Santa Ana, CA). All protoalkaloid primary standards purchased were in the para (*p*) positional isomeric form. Terbutaline (CAS Registry No. 23031-32-5, hemisulfate salt), hydrochloric acid, and ammonium acetate were obtained from Sigma Chemical Co. (St. Louis, MO). HPLC-grade methanol was obtained from J. T. Baker (Phillipsburg, NJ). All other chemical reagents and solvents were ACS reagent grade unless stated otherwise. The identities of tyramine, *N*-methyltyramine, octopamine, hordenine, synephrine, and terbutaline were confirmed by direct-infusion MS and MS/MS analyses. SRM 3258, SRM 3259, and SRM 3260 were obtained from the Standard Reference Materials Group at NIST. SRM 3258 was prepared from unripe fruit that was processed to produce a dried, powdered material. SRM 3259 was prepared by extracting ripe fruit to yield a nominal synephrine level of 6% (mass fraction). SRM 3260 was prepared by grinding and blending a select number of commercially available bitter orange-containing dietary supplements, both tablets and capsules.

Reagent concentrations given in terms of percent are to be considered as mass fractions (grams per gram) in all listed procedures. Preparation of analyte stocks/standards, samples, and calibrants was performed gravimetrically in all procedures, except where noted otherwise. Additionally, all procedures were conducted under subdued lighting conditions.

Preparation of Protoalkaloid Stock and Calibration Solutions. One liter of terbutaline internal standard solution (0.1 ng/μL, solution A) was prepared in water. A set of five individual stock solutions (1000 ng/μL each) was prepared for each protoalkaloid primary standard (tyramine, *N*-methyltyramine, octopamine, hordenine, and synephrine) using solution A as diluent. Further dilutions of the protoalkaloid stock solutions were prepared using solution A as needed.

Five calibration solutions containing mixtures of the protoalkaloid primary standards were prepared by adding discrete masses of appropriately diluted protoalkaloid stock solutions to a constant mass of solution A. The calibrant concentration range (inclusive of all protoalkaloids) ranged from 2×10^{-4} to 10 ng/μL. The protoalkaloid stock solutions were stored at -80 °C, and the calibration solutions were stored at -20 °C until needed.

Preparation and Extraction of Samples. For each bitter orange SRM, six packages were selected for the quantitative determination of protoalkaloid levels. Two aliquots from each of the six packages were prepared and extracted. Development, optimization, and validation of the extraction procedures have been completely detailed in a separate publication (*16*); however, a brief description follows. For SRM 3258, a 50 mg sample was weighed into a 50 mL plastic centrifuge tube and spiked with 800 μL of terbutaline (1000 ng/μL, solution B). The sample was diluted with 40 mL of 1% aqueous HCl and vortex mixed, and the tube was sonicated at room temperature for 60 min. The sample was then centrifuged at 3000*g* for 10 min, and the supernatant was transferred into a clean 50 mL plastic centrifuge tube and stored on

ice. The remaining solid material was re-extracted via sonication (60 min, room temperature) using a fresh 40 mL portion of 1% aqueous HCl. Following centrifugation, 5 mL of the supernatant from the second extraction was combined with 5 mL of the supernatant from the first extraction. The combined extracts were vortex mixed, and a 5 mL aliquot was filtered through a 0.45 μm pore polytetrafluoroethylene (PTFE) membrane filter. The filtrate was diluted 1 to 100 (volume ratios, 1 mL of extract + 99 mL of water) with water and stored at 4 °C until analysis. For SRM 3259, the sample preparation was the same as for SRM 3258, except that the spike volume of solution B was 200 μL instead of 800 μL and the sample was extracted using a single 30 min sonication period instead of two 60 min sonication periods. The SRM 3259 filtrate was diluted 1 to 100 with water and stored at 4 °C until analysis. For SRM 3260, the sample preparation was the same as for SRM 3258, except that the spike volume of solution B was 80 μL instead of 800 μL and the sample was extracted using two 30- min sonication periods instead of two 60 min sonication periods. The SRM 3260 filtrate was diluted 1 to 10 with water and stored as before.

Analysis of Sample Extracts and Calibration Solutions. Sample extracts and calibration solutions were injected (2 μL) in duplicate onto the LC/MS/MS system. Analyte/internal standard peak area ratios (area/area) and mass ratios (milligrams/milligrams) were subjected to linear least-squares regression analysis to produce calibration curves and calibration equations from the calibration solutions. Protoalkaloid levels in the sample extracts were quantified on the basis of the relevant calibration equation and the protoalkaloid/terbutaline peak area response ratio detected in the sample extract.

Spiking Study for Ion Suppression. A stock solution containing all six protoalkaloids (1 ng/μL per protoalkaloid) was prepared in water. Samples of SRM 3258, SRM 3259, and SRM 3260 were prepared and diluted exactly as described previously. Two sets of spiked samples were prepared using the diluted extracts. Set 1 consisted of the diluted SRM extracts spiked with a known amount of protoalkaloid stock solution (200 μL of extract + 20 μL of protoalkaloid stock). Set 2 consisted of the diluted SRM extracts spiked with a known amount of water (200 μL of extract + 20 μL of water). A control sample was prepared by spiking water with a known amount of protoalkaloid stock solution (200 μL of water + 20 μL of protoalkaloid stock). All samples were analyzed by LC/MS/MS (five injections each), and the set 1 area responses were subtracted from the set 2 area responses to generate area responses for the protoalkaloids in the spike. The observed area responses for the protoalkaloids in the spike were compared to the area responses in the control sample for each of the diluted SRM extracts.

Determination of Method Linearity, Limit of Detection (LOD), and Limit of Quantification (LOQ). A stock solution (1 mL) containing 1.5 ng/μL of each protoalkaloid analyte standard (tyramine, *N*-methyltyramine, octopamine, hordenine, and synephrine) was prepared using solution A (terbutaline internal standard) as diluent. A set of 19 volumetric serial dilutions was prepared from the stock solution covering the range from 6×10^{-6} to 1.5 ng/μL analyte using solution A as diluent. Each standard was injected (2 μL) onto the LC/MS/MS system to estimate linear range, LOD, and LOQ of the method for each protoalkaloid analyte.

LC/MS/MS experiments were conducted on an Agilent 1100 series LC system coupled to an Applied Biosystems 4000 Q-Trap MS/MS system. The Q-Trap MS/MS system was operated as a triple-quadrupole MS/MS system in positive electrospray ionization mode. The LC system was outfitted with a binary pump, a variable-wavelength UV absorbance detector, a temperature-controlled (10 °C) autosampler, and an in-line mobile phase vacuum degasser. Samples were analyzed using a 150 × 4.6 mm, 5 μm Supelco Discovery HS-F5 (pentafluorylphenyl) analytical column (Supelco, Bellefonte, PA) with an attached 20 mm × 3 mm, 5 μm, HS-F5 guard column (Supelco) held at 35 ± 1 °C. The isocratic LC elution conditions were as follows (solvent percentages are volume fractions): mobile phase A, 10 mmol/L ammonium acetate in water; mobile phase B, 10 mmol/L ammonium acetate in methanol; A/B = 10:90; flow rate = 500 μL/min. All protoalkaloid analytes were detected and quantified on the basis of multiple-reaction monitoring (MRM) MS/MS in positive ion mode. The optimal MRM transition for each protoalkaloid and terbutaline was selected on the basis of collision-activated dissociation (CAD) MS/MS infusion studies. The analytes (10 ng/μL) were dissolved in 10:90 (volume fractions) water/methanol containing 10 mmol/L ammonium acetate and infused at a rate of 10 μL/min. For each analyte, protonated precursor molecules and compound-specific fragment ions were utilized to construct the MRM transitions. General MS/MS instrument parameters are described below, whereas analyte-specific MS/MS parameters are given in Supporting Information Table 1: dwell time = 150 ms/ion; source temperature = 450 °C; curtain gas flow = 40 kPa; gas 1 flow = 45 kPa; gas 2 flow = 50 kPa; CAD gas (nitrogen) = 8 kPa.

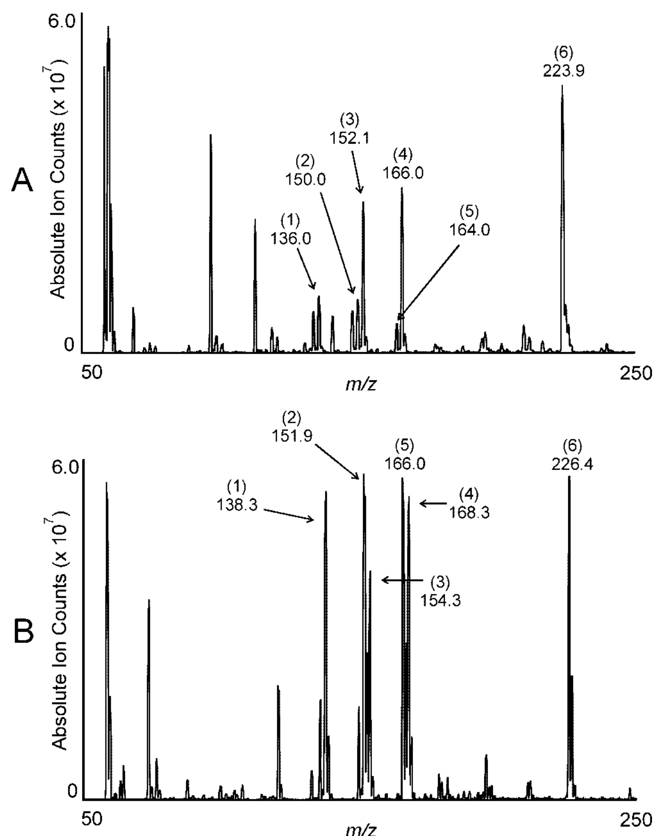

Figure 2. Relative negative and positive mode ionization efficiencies for a mixture containing the bitter orange protoalkaloids and terbutaline: (**A**) negative-ion mode full-scan profile; (**B**) positive-ion mode full-scan profile. Peak identities in both panels: (1) tyramine; (2) *N*-methyltyramine; (3) octopamine; (4) synephrine; (5) hordenine; (6) terbutaline.

Liquid chromatography combined with fluorescence detection (LC/FD) analyses was performed exactly as described previously (*16*). Briefly, protoalkaloids were separated using a 250 × 4.6 mm, 5 μm Ace 5 C_{18} Ultra Inert analytical column (Advanced Chromatography Technologies, Aberdeen, Scotland). Separations were performed using an isocratic LC mobile phase composition of 72% (volume fraction) of 10 mmol/L sodium dodecyl sulfate, pH 2.5, + 28% (volume fraction) acetonitrile at a flow rate of 1 mL/min. All analytes were detected on the basis of excitation at 273 nm and emission at 304 nm with a Jasco FP-1520 fluorescence detector.

RESULTS AND DISCUSSION

Full-Scan MS of Bitter Orange Protoalkaloids. Full-scan MS analysis on a standard mixture of bitter orange protoalkaloids and terbutaline utilizing negative-ion mode (**Figure 2A**) and positive-ion mode (**Figure 2B**) ESI produced distinctly different ion abundance profiles. The infusion solvent for both studies was 10:90 (volume fractions) water/methanol containing 10 mmol/L ammonium acetate, apparent pH 8. The detected ion counts (absolute scale) were significantly lower (≥10%) for the deprotonated protoalkaloids (**Figure 2A**) using negative-ion ESI compared to the protonated protoalkaloids using

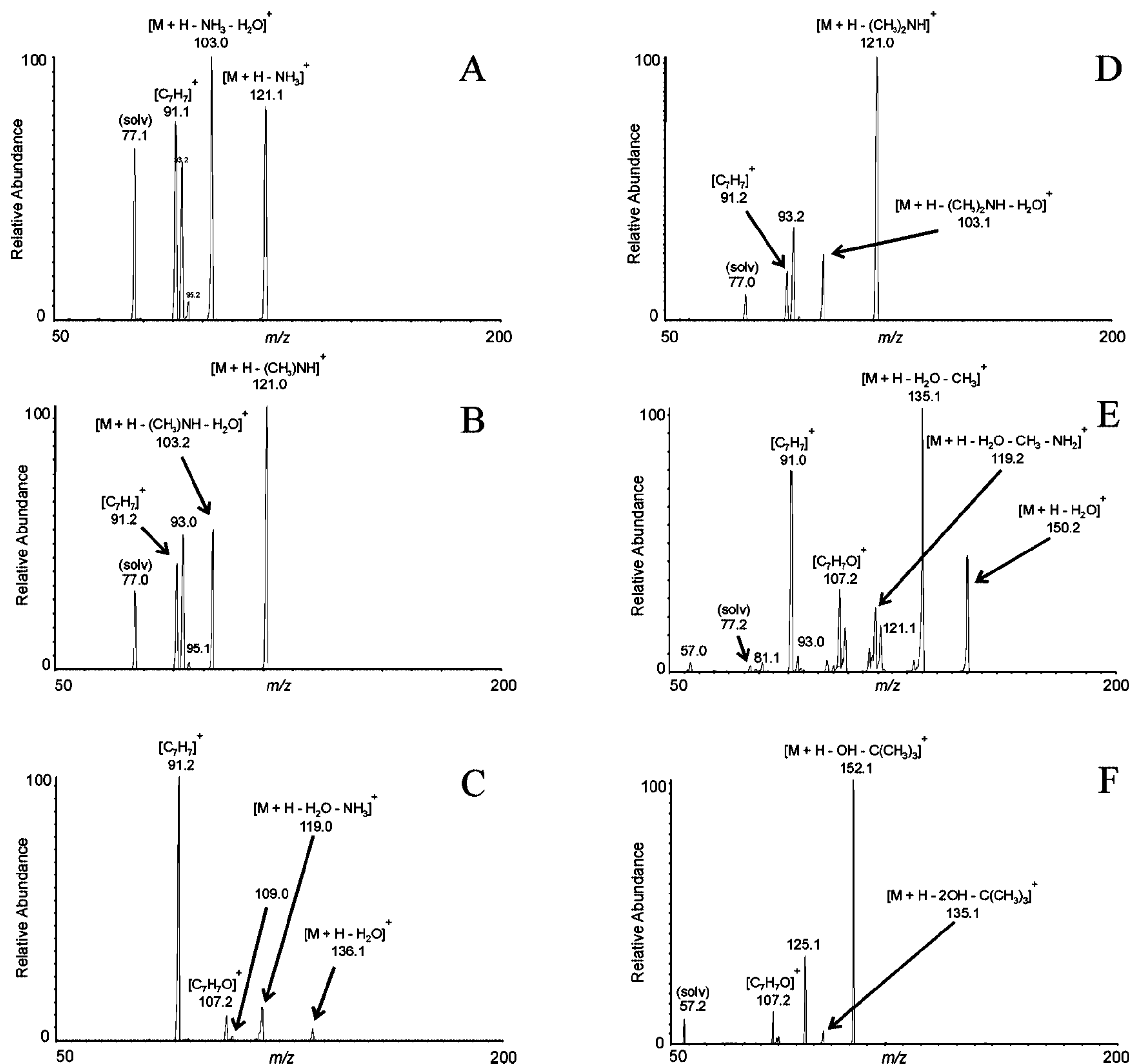

Figure 3. CAD MS/MS profiles for the bitter orange protoalkaloid and terbutaline protonated precursor molecules: (**A**) tyramine, precursor molecule, *m/z* 138; (**B**) *N*-methyltyramine, precursor molecule, *m/z*152; (**C**) octopamine, precursor molecule, *m/z*154; (**D**) hordenine, precursor molecule, *m/z*166; (**E**) synephrine, precursor molecule, *m/z* 168; (**F**) terbutaline, precursor molecule, *m/z* 226. Solv = ion present in solvent.

positive-ion ESI (**Figure 2B**). This phenomenon was also observed when individual solutions of the protoalkaloids were analyzed under negative- and positive-ion mode ESI. On the basis of the molecular structures of the protoalkaloids (**Figure 1**), these results might suggest that protonation of the alkyl nitrogen proceeds more readily than deprotonation of phenolic or alkyl hydroxyl groups under the experimental conditions. Nevertheless, it is interesting to note that synephrine and octopamine both have greater ion abundances in negative-ion mode (**Figure 2A**), compared to the other protoalkaloids in negative-ion mode, and this observation may be due to deprotonation of the alkyl hydroxyl group. Because of the higher ion abundances observed in positive-ion mode, all additional MS experiments were conducted using this mode.

CAD MS/MS Profiles of Bitter Orange Protoalkaloids. To establish the optimal MRM transitions for quantitation of the bitter orange protoalkaloids in sample extracts, CAD MS/MS profiles of the individual protoalkaloids and terbutaline were collected (**Figure 3**). The analytes (10 ng/μL) were dissolved in 10:90 (volume fractions) water/methanol containing 10 mmol/L ammonium acetate and infused at a rate of 10 μL/min. Profiles based on CAD MS/MS of the protonated analytes illustrate that one fragment ion, the tropylium ion ($[C_7H_7]^+$, *m/z* 91), is common to, but not diagnostic of, the protoalkaloids (**Figure 3A–E**). The tropylium ion forms due to an α-cleavage relative to the aromatic ring and is highly stable and characteristic of aromatic hydrocarbons with side chains (*14, 17*). In the present work, formation of the tropylium ion is generally more pronounced on the protoalkaloids with an α-hydroxyl group on the side chain. The neutral loss of an H_2O molecule, either alone or in conjunction with other molecules, is also common to the protoalkaloids (**Figure 3A–E**). The loss of H_2O is a characteristic fragmentation pathway for analytes containing a benzylic hydroxyl group (*18*). Other fragment ions that have been tentatively identified in this work and in other publications (*14, 19, 20*) are labeled in **Figure 3**.

MRM transitions for quantitation are usually selected on the basis of unique precursor ions and appropriately intense and stable product (fragment) ions. In most cases, it is preferable to avoid the selection of product ions that form due to nonspecific loss of H_2O molecules. However, when one is working with low relative mass analytes (<250 g/mol), the selection of product ions that include the loss of water is sometimes unavoidable because low relative mass analytes have few fragmentation pathways. For tyramine (**Figure 3A**), the most intense product ions result from the combined neutral loss of H_2O and NH_3 from protonated tyramine molecules; the tyramine MRM transition was selected on the basis of these product ions.

For *N*-methyltyramine (**Figure 3B**), the most intense product ions result from the loss of CH_3NH from protonated *N*-methyltyramine molecules; however, the product ions resulting from the loss of CH_3NH were demonstrated to be inappropriate for the *N*-methyltyramine MRM transition when tested by LC/MS/MS. As a replacement, the stable tropylium ion was selected as the product ion for the *N*-methyltyramine MRM transition. Octopamine (**Figure 3C**) formed few stable product ions under the tested experimental conditions. However, the profile showed the presence of high-intensity tropylium ions; therefore, the tropylium ion was selected as the product ion for the octopamine MRM transition. For hordenine (**Figure 3D**), the most intense product ions result from the loss of $(CH_3)_2NH$ from protonated hordenine molecules; however, as previously observed for *N*-methyltyramine during LC/MS/MS testing, the product ions resulting from the loss of $(CH_3)_2NH$ were demonstrated, via LC/MS/MS analysis, to be inappropriate for quantitation. Once again, the tropylium ion was selected as the substitute product ion for the hordenine MRM transition. Synephine (**Figure 3E**) generated the largest number (six) of identifiable product ions, with the most intense product ions resulting from the combined loss of H_2O and CH_3 from protonated synephrine molecules; the synephrine MRM transition was selected on the basis of these product ions. The terbutaline MRM transition was constructed from the protonated analyte and an unidentified product ion at *m/z* 125 (**Figure 3F**). The intense product ions (*m/z* 152) produced by the combined loss of OH and $C(CH_3)_3$ could not be used for the terbutaline MRM transition because of the poor peak shapes observed during LC/MS/MS testing. A summary of the selected protoalkaloid and terbutaline MRM transitions utilized for the LC/MS/MS method is given in Supporting Information Table 1.

Development of the MRM LC/MS/MS Method. Previously, it has been shown that strongly basic analytes, similar in nature to the bitter orange protoalkaloids, can be effectively retained on fluorinated chromatographic stationary phases under high organic mobile phase conditions (*21–23*). On the basis of this previous research, an LC method was developed that demonstrated good retention of the protoalkaloids on a pentafluorophenyl LC column under high organic, isocratic mobile phase conditions. MRM mode LC/MS/MS analysis of protoalkaloid standards resulted in narrow, symmetrical peaks for tyramine, octopamine, and synephrine and broad, split peaks for *N*-methyltyramine, hordenine, and terbutaline. Initial MRM transitions were constructed using the most intense protoalkaloid product ions produced during the CAD MS/MS infusion studies. After testing of various chromatographic parameters (LC mobile phase composition, LC column temperature, organic acid modifier, etc.), it was determined that the source of the split peaks was the selected MRM transition for *N*-methyltyramine, hordenine, and terbutaline. The product ion at *m/z* 121 dominates the CAD MS/MS spectra for both *N*-methyltyramine (**Figure 3B**) and hordenine (**Figure 3D**) and is the obvious choice for building each MRM transition. However, when the tropylium ion at *m/z* 91 was substituted for the product ion at *m/z* 121, the broad, split peaks observed for the *N*-methyltyramine and hordenine MRM transitions convert to narrow, single peaks. The reason for this peak shape conversion is not clear, but could possibly be due to instability of the *m/z* 121 product ion or the presence of an unknown isobaric ion at *m/z* 121. Similarly, when the intense product ion (*m/z* 152, **Figure 3E**) utilized for the preliminary terbutaline MRM transition was changed to a less intense product ion (*m/z* 125), the MRM transition converted from a split peak to a single peak. The final MRMs for the protoalkaloids and terbutaline were compound-specific, and cross-channel analyte interference was not evident within the linear dynamic range of the method (see Method Performance and Measurement Characteristics).

Representative chromatograms from the extraction and MRM mode LC/MS/MS analysis of bitter orange protoalkaloids in SRM 3258, SRM 3259, and SRM 3260 are shown in Supporting Information Figure 1, panels A, B, and C, respectively. All five analytes and terbutaline were easily detected in the SRM extracts within an analysis time of 10 min. A spiking study using the diluted extracts was conducted to detect ion suppression of the protoalkaloids due to SRM matrix components. All spiked protoalkaloid responses in the diluted SRMs were equivalent (within 1 standard deviation) to the expected responses in the control sample. No evidence of significant (>10%) ion suppression effects was detected for the five analytes and terbutaline in the diluted SRM extracts. All SRM extracts, when analyzed without dilution, produced broad, skewed protoalkaloid peaks, symptomatic of ion suppression and/or column overloading. Additionally, significant protoalkaloid carry-over from injection to injection was observed when undiluted extracts were used. By appropriately diluting the samples and incorporating a syringe needle-wash with 10% aqueous ascorbic acid solution before each sample injection, symmetrical protoalkaloid peaks were produced and protoalkaloid carry-over was completely eliminated (Supporting Information Figure 1).

Method Performance and Measurement Characteristics. The LC/MS/MS method demonstrated picogram level LODs (≤6 pg on-column) and LOQs (≤20 pg on-column) for the bitter orange protoalkaloids (see Supporting Information Table 2). Additionally, the method exhibited a linear dynamic range that extended over 3 orders of magnitude (∼6–3000 pg on-column) for each analyte (see Supporting Information Table 2). To evaluate the performance of the method for the quantitative determination of bitter orange protoalkaloids, the method was applied to the analysis of the five protoalkaloids in SRM 3258, SRM 3259, and SRM 3260. For confirmatory purposes, protoalkaloid levels in each of the NIST SRMs were also determined using a recently developed method based on LC/FD (*16*). The LC/MS/MS versus LC/FD individual protoalkaloid levels (milligrams per kilogram), as well as total protoalkaloids levels (milligrams per kilogram), in each of the NIST SRMs are compared in **Table 1** for SRM 3258, SRM 3259, and SRM 3260, respectively. The imprecision (coefficient of variation, % CV) and combined (u_c) and expanded (U) uncertainties of the measurements are also given in **Table 1**. Both type A factors and type B factors were evaluated for the assignment of u_c and U. However, it was determined that the type B contributions for each SRM were much smaller than the measurement uncertainty; hence, the reported values for u_c and U do not include type B factors. It should be noted that the LC/FD method is incapable of detecting or quantifying hordenine; therefore, no levels are reported for hordenine in **Table 1**.

For SRM 3258, the *N*-methyltyramine, synephrine, and total protoalkaloid levels were in excellent agreement between the LC/MS/MS and LC/FD methods (the percent difference between the two methods, inclusive of U, was ≤2%). Additionally, the LC/MS/MS method detected and measured a small level (<0.2% mass fraction) of hordenine in SRM 3258. On the other hand, there was a ∼3-fold difference (48 versus 18 mg/kg) for the mean tyramine levels between the two methods. Finally, the LC/FD method returned octopamine levels that were approximately 20% higher than the levels determined by the LC/MS/MS method. Concerning the discordance between the mean

Table 1. Quantification of Bitter Orange Protoalkaloids in SRM 3258 (Bitter Orange Fruit), SRM 3259 (Bitter Orange Extract), and SRM 3260 (Bitter Orange Solid Oral Dosage Form)[a]

	LC/MS/MS[b] (mg/kg)	CV (%)	u_c[c] (mg/kg)	U[d] (mg/kg)	LC/FD[e] (mg/kg)	CV (%)	u_c (mg/kg)	U (mg/kg)
SRM 3258								
tyramine	47.7[h]	11	2.2	4.4	17.9	7	0.5	1.0
N-methyltyramine	178.7	8	5.8	11.7	176.4	1	0.7	1.3
octopamine	120.9	8	4.1	8.2	145.0	<1	0.2	0.5
hordenine	12.2	13	0.7	1.3	ND[f]			
synephrine	8848.8	2	55.0	110.1	8842.8	1	22.0	44.0
total protoalkaloids[g]	9208.4	2	59.0	118.0	9182.1	<1	22.6	45.3
SRM 3259								
tyramine	757.5	3	7.7	15.5	757.9	1	2.7	5.4
N-methyltyramine	4352.0	2	31.4	62.8	5236.9	1	12.7	25.5
octopamine	840.3	3	9.8	19.6	933.1	1	2.0	4.0
hordenine	19.1	6	0.5	1.0	ND			
synephrine	71537.9	1	303.3	606.6	70348.6	1	136.7	273.3
total protoalkaloids	77506.8	1	312.4	624.8	77276.4	1	148.3	296.5
SRM 3260								
tyramine	172.8	2	1.3	2.7	169.6	1	0.7	1.3
N-methyltyramine	555.6	1	3.1	6.3	759.5	<1	1.2	2.4
octopamine	148.0	4	2.2	4.5	187.9	1	1.0	2.1
hordenine	5.9	4	0.1	0.2	ND			
synephrine	18132.4	1	56.8	113.6	17930.7	<1	26.6	53.2
total protoalkaloids	19014.8	1	56.5	113.0	19047.7	<1	27.7	55.4

[a] Levels shown are the mean from six samples (two preparations of each sample). [b] Levels based on the current method. [c] Combined uncertainty. [d] Expanded uncertainty based on a coverage factor of $k = 2$. [e] Levels based on analyses using liquid chromatography coupled with fluorescence detection. See ref *16*. [f] ND, not detected. [g] Total protoalkaloids = tyramine + *N*-methyltyramine + octopamine + hordenine + synephrine for LC/MS/MS and tyramine + *N*-methyltyramine + octopamine + synephrine for LC/FD. [h] The reported tyramine level is based on a tyramine signal quantified at the method LOQ (LC/MS/MS LOQ = 1 pg of tyramine on-column).

tyramine levels, the LC/MS/MS analytical signal for tyramine in SRM 3258 extracts corresponded to the analytical signal for tyramine at the method LOQ (see Supporting Information Table 2, LOQ = 1 pg of tyramine on-column). Higher noise levels and signal variability at the LOQ could have contributed to inaccuracy of the tyramine measurement; however, independent determinations of tyramine based on LC/UV and single-stage LC/MS procedures did not support this possibility. LC/UV measurements conducted by an outside collaborating laboratory and LC/MS measurements conducted at NIST using an alternative NIST method resulted in mean SRM 3258 tyramine levels of 35 and 26 mg/kg, respectively. The wide range of possible values for tyramine (18–48 mg/kg), inclusive of all measurement methods, suggests that the determination of low levels of tyramine is problematic and warrants further investigation. The imprecision (CV) of the LC/MS/MS determinations ranged from 8 to 13%, whereas the LC/FD determinations had imprecisions that ranged from <1 to 7%, inclusive of all protoalkaloids.

For SRM 3259, the tyramine, synephrine, and total protoalkaloid levels were in excellent agreement between the LC/MS/MS and LC/FD methods (the percent difference between the two methods, inclusive of U, was ≤2%). Additionally, the LC/MS/MS method detected and measured a small level (≤0.05% mass fraction) of hordenine in SRM 3259. The level of synephrine (>70000 mg/kg) in SRM 3259 was the highest of the three SRMs; however, the level was consistent with the manufacturer's stated synephrine level (6% mass fraction or 60000 mg/kg). On the other hand, the LC/FD method returned *N*-methyltyramine and octopamine levels that were approximately 20 and 11% higher, respectively, than the levels determined by the LC/MS/MS method. The imprecision (CV) of the LC/MS/MS determinations ranged from 1 to 6%, whereas the LC/FD determinations had imprecisions that were all about 1%, inclusive of all protoalkaloids.

For SRM 3260, the tyramine, synephrine, and total protoalkaloid levels were in excellent agreement between the LC/MS/MS and LC/FD methods (the percent difference between the two methods, inclusive of U, was ≤2%). Additionally, the LC/MS/MS method detected and measured a small level (≤0.05% mass fraction) of hordenine in SRM 3260. On the other hand, the LC/FD method returned *N*-methyltyramine and octopamine levels that were approximately 37 and 27% higher, respectively, than the levels determined by the LC/MS/MS method. The imprecision (CV) of the LC/MS/MS determinations ranged from 1 to 4%, whereas the LC/FD determinations had imprecisions that ranged from <1 to 1%, inclusive of all protoalkaloids.

In summary, a positive-ion mode LC/MS/MS method for the quantitative determination of the five known adrenergic protoalkaloids (tyramine, *N*-methyltyramine, octopamine, hordenine, and synephrine) in bitter orange products has been developed. The optimized method is capable of measuring picogram levels of each protoalkaloid in different types of bitter orange matrices. The method has been tested on processed fruits (NIST SRM 3258), fruit extracts (NIST SRM 3259), and a combination of dietary supplements (NIST SRM 3260). The method exhibited satisfactory mean measurement imprecision values of ≤10% CV for the different matrices, with most measurements exhibiting imprecision values of ≤5% CV. In comparison, the independent LC/FD method exhibited excellent mean measurement imprecision values of ≤2% CV for the different matrices, with most measurements exhibiting imprecision values of ∼1% CV. The LC/MS/MS method performed extremely well when compared against the LC/FD method for the determination of tyramine, synephrine, and total protoalkaloid levels (milligrams per kilogram) in all SRM extracts, excluding the tyramine levels in SRM 3258. The LC/FD method measured higher octopamine levels (≥11% higher) than the LC/MS/MS method for all three SRMs. Additionally, the LC/FD method measured higher *N*-methyltyramine levels (≥20% higher) than the LC/MS/MS method for SRM 3259 and SRM 3260. The statistically significant differences between the mean LC/MS/MS and LC/FD levels (the mean protoalkaloid levels do not overlap within the assigned U values) for octopamine and *N*-methyltyramine could be due to the heterogeneity of the reference materials themselves. The apparent level of octopamine in each of the SRMs is around 1% (mass fraction), and it is

conceivable, because of the inherent complexity of the *C. aurantium* matrix, that low levels of coeluting fluorescent components could have increased the relative response of octopamine in the SRM extracts, thus contributing to the higher LC/FD determinations. The measurement of higher *N*-methyltyramine levels via the LC/FD method could be the result of a similar phenomenon. Nevertheless, the reported LC/MS/MS method provides a second, independent method for the quantitative determination of the major (synephrine) and minor (tyramine, *N*-methyltyramine, octopamine, hordenine) adrenergic protoalkaloids in bitter orange-containing dietary supplements, and the measured protoalkaloid levels for the LC/MS/MS and LC/FD methods demonstrate satisfactory agreement.

Supporting Information Available: Table 1 provides analyte-specific MS/MS instrument parameters for the LC/MS/MS method. Table 2 provides information regarding the analytical performance characteristics of the LC/MS/MS method. Figure 1 shows representative MRM chromatograms resulting from the analysis of SRM 3258, SRM 3259, and SRM 3260. This material is available free of charge via the Internet at http://pubs.acs.org.

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