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Determination of Biogenic Amines in Fruit, Vegetables, and Chocolate Using Ion Chromatography with Suppressed Conductivity and Integrated Pulsed Amperometric Detections

INTRODUCTION

Biogenic amines play critical roles in human and animal physiological functions, and are related to food spoilage and safety.¹ Consumption of low concentrations of biogenic amines in the average diet is not dangerous, but consumption of high concentrations can result in hypotension (histamine, putrescine, cadaverine), hypertension (tyramine), migraines (tyramine, phenylethylamine), nausea, rash, dizziness, increased cardiac output, and increased respiration.^{2,3} Biogenic amines are known to occur in a wide variety of foods, such as fish, meat, dairy, fruits, vegetables, and chocolate.⁴ The determination of biogenic amines in food products is critical to assess potential health risks before consumption.

Determinations of biogenic amines are often accomplished by reversed-phase HPLC followed by UV or fluorescence detection. Because most biogenic amines lack a suitable chromophoric or fluorophoric group, however, either pre- or postcolumn chemical derivatization is required for detection. The most common derivatizing agents are dansyl chloride,^{3,5-7} benzoyl chloride,⁸⁻¹¹ and o-phthalaldehyde (OPA).^{5,12} These derivatization

procedures are time-consuming, laborious, can produce potential by-product interferences, and sometimes under- or over-estimate the amount of amines.^{6,13}

Application Note 183 (AN 183) describes the use of the IonPac[®] CS18, a weak acid cation-exchange column, with suppressed conductivity, integrated pulsed amperometric detection (IPAD), and UV for the detection of underivatized biogenic amines in meat and cheese. The CS18 allows separation of biogenic amines without the use of highly-concentrated acidic eluents or organic solvents while still providing resolution of closely eluting peaks such as putrescine and cadaverine. The milder separation conditions allow the use of suppressed conductivity to detect many underivatized biogenic amines. IPAD can detect these same amines, as well as those that lack a charge after suppression, and has recently been used to detect biogenic amines in chocolate.¹⁴ UV can confirm the presence and concentrations of weakly-retained aromatic biogenic amines that coelute with other amines and amino acids. In this update, the procedures described in AN 183 are used to determine biogenic amine levels in kiwi fruit, spinach, and chocolate.

EQUIPMENT

Dionex ICS-3000 system consisting of:
DP Dual Pump with in-line degas option
DC Detector/Chromatography module (dual temperature zones) with conductivity and electrochemical cells
Electrochemical cell consisting of a pH/Ag/AgCl reference electrode and a conventional Au electrode (PN 063722)
EG Eluent Generator module
EluGen[®] EGC II MSA cartridge (P/N 058902)
AD25 UV-Vis Absorbance Detector with 10-mm cell
Mixing tee, 3-way, 1.5 mm i.d. (P/N 024314)
Knitted reaction coil, 125 μ L (P/N 053640)
Two 4-L plastic bottle assemblies for external water mode of operation
Chromleon[®] 6.7 Chromatography Management Software
Blender (household or industrial strength type)
Centrifuge (Beckman Coulter, Brea, CA)
Vortex mixer (Fisher Scientific)

REAGENTS AND STANDARDS

Reagents

Deionized water, type I reagent grade, 18 M \cdot cm resistivity or better
Sodium hydroxide, 50% (w/w) (Fisher Scientific, SS254-1)
Methanesulfonic acid, 99% (Dionex Corporation, P/N 033478)

Standards

Dopamine hydrochloride (Sigma Chemical Co., H8502)
Serotonin hydrochloride, \geq 98% (Sigma Chemical Co., H9523)
Tyramine, 99% (Aldrich Chemical Co., T90344)
Putrescine dihydrochloride, \geq 98% (Sigma Chemical Co., P7505)
Cadaverine dihydrochloride, $>$ 98% (Sigma Chemical Co., C8561)
Histamine, \sim 97% (Sigma Chemical Co., H7125)
Agmatine sulfate, 97% (Aldrich Chemical Co., 101443)
 β -phenylethylamine, 99% (Aldrich Chemical Co., 128945)
Spermidine trihydrochloride, $>$ 98% (Calbiochem, 56766)
Spermine tetrahydrochloride, \geq 99% (Calbiochem, 5677)

CONDITIONS

Columns: IonPac CS18 Analytical, 2 x 250 mm (P/N 062878)
IonPac CG18 Guard, 2 x 50 mm (P/N 062880)
Eluent:* 3 mM MSA from 0–6 min, 3–10 mM from 6–10 min, 10–15 mM from 10–22 min, 15 mM from 22–28 min, 15–30 mM from 28–35 min, 30–45 mM from 35.1–45 min
Eluent Source: EG Eluent Generation module
Flow Rate: 0.30 mL/min
Temperature: 40 $^{\circ}$ C (lower compartment)
30 $^{\circ}$ C (upper compartment)
Injection Volume: 5 μ L
Detection:** Suppressed conductivity, CSRS[®] ULTRA II (2 mm), AutoSuppression[®] external water mode, power setting–40 mA and/or UV-Vis detection set at 276 nm
Background
Conductance: 0.4–0.5 μ S
Conductance
Noise: 0.2–0.3 nS
System
Backpressure: \sim 2500 psi

Postcolumn Addition:

Detection: Integrated pulsed amperometry, conventional Au electrode
Postcolumn
Reagent Flow: 100 mM NaOH at 0.24 mL/min
IPAD
Background: 40–50 nC
IPAD Noise: 60–70 pC (without suppressor installed)
 \sim 210 pC (with suppressor installed)

*The column was equilibrated at 3 mM MSA for 5 min prior to each injection.

**This application note discusses three separate detection configurations: IPAD, suppressed conductivity-IPAD, and UV-IPAD.

Waveform

Time (s)	Potential (V vs. pH)	Gain Region	Ramp	Integration
0.000	+0.13	Off	On	Off
0.040	+0.13	Off	On	Off
0.050	+0.33	Off	On	Off
0.210	+0.33	On	On	On
0.220	+0.55	On	On	On
0.460	+0.55	On	On	On
0.470	+0.33	On	On	On
0.536	+0.33	Off	On	Off
0.546	-1.67	Off	On	Off
0.576	-1.67	Off	On	Off
0.586	+0.93	Off	On	Off
0.626	+0.93	Off	On	Off
0.636	+0.13	Off	On	Off

SYSTEM PREPARATION AND SETUP

Installation of the IPAD, suppressed conductivity, and UV detectors is described in detail in AN 183. When working with MSA and NaOH, be sure to wear gloves to prevent exposure. Follow all precautions to prevent backflow of NaOH, as this can result in permanent damage to the column.

PREPARATION OF SOLUTIONS AND REAGENTS

Eluent Solution

Generate methanesulfonic acid (MSA) online by pumping high quality deionized water (18 M -cm resistivity or better) through the EGC II MSA cartridge. Chromeleon software will track the amount of MSA used and calculate the remaining lifetime.

Alternately, prepare 10 mM MSA by adding 0.961 g of concentrated MSA to a 1-L volumetric flask containing approximately 500 mL of deionized water. Bring to volume and mix thoroughly. Prepare 100 mM MSA by adding 9.61 g of concentrated MSA to a 1-L volumetric flask containing approximately 500 mL of deionized water. Bring to volume and mix thoroughly. Degas the eluents and store in plastic labware. The 3 mM MSA eluent is produced by proportioning between 10 mM MSA and deionized water. The gradient is proportioned between the 100 mM MSA solution and deionized water.

Postcolumn Base Addition Solution for IPAD

100 mM Sodium Hydroxide

Prepare 100 mM sodium hydroxide (NaOH) solution by adding 8 g of 50% w/w NaOH to approximately 800 mL of degassed deionized water in a 1-L volumetric flask and bring to volume. NaOH pellets, which are coated with a thin layer of sodium carbonate, must not be used to prepare this solution. The 100 mM NaOH solution should be stored under helium in a pressurized container at all times.

Acid Extraction Solutions

100 mM Methanesulfonic Acid

Add 4.81 g of MSA to a 500-mL volumetric flask containing approximately 300 mL of deionized water. Bring to volume and mix thoroughly. Store solution in plastic labware.

5% and 1.5% Trichloroacetic Acid

Prepare 5% trichloroacetic acid (TCA) by adding 25 g of TCA to a 500-mL volumetric flask containing about 300 mL of deionized water. Bring to volume and mix thoroughly. Store the solution in plastic labware.

Prepare 1.5% TCA by adding 30 mL of the 5% TCA solution to a 100-mL volumetric flask containing approximately 50 mL deionized water. Bring to volume and mix thoroughly. Store solution in plastic labware.

STANDARD PREPARATION

Prepare biogenic amine stock standard solutions at 1000 mg/L each by dissolving 123.8 mg of dopamine hydrochloride, 100 mg of tyramine, 182.7 mg of putrescine dihydrochloride, 171.4 mg of cadaverine dihydrochloride, 96 mg of histamine, 120.7 mg of serotonin hydrochloride, 172.7 mg of agmatine sulfate, 100 mg of phenylethylamine, 175.3 mg of spermidine trihydrochloride, and 172.1 mg of spermine tetrahydrochloride in separate 100-mL volumetric flasks. Bring each to volume with deionized water. Store stock solutions at 4 °C and protected from light. Prepare working standard solutions for generating the calibration curve with an appropriate dilution of the stock solutions in 3 mM MSA. These solutions should be prepared fresh weekly and stored at 4 °C when not in use.

SAMPLE PREPARATION

Spinach

Spinach extracts were prepared by adding 5 g of ground sample to a 50-mL centrifuge tube, followed by 20 mL of 100 mM MSA. The mixture was homogenized on a vortex mixer for 1 min and centrifuged at 6000 rpm for 20 min at 4 °C. The supernatant was decanted and filtered with a 0.2- μ m filter into a 50-mL volumetric flask. An additional 20 mL aliquot of MSA was added to the tube and the extraction procedure was repeated. The supernatant was again filtered into the flask, and the flask was brought to volume with deionized water. The extract was further diluted 1:1 with deionized water before analysis.

Kiwi Fruit and Chocolate (70% Cocoa)

The kiwi fruit and 70% cocoa chocolate extracts were prepared by adding 5 g of ground sample to separate 50-mL centrifuge tubes followed by 10 mL of 100 mM MSA. The samples were extracted as described for the spinach and the undiluted extracts were analyzed.

Dark and Milk Chocolate

The chocolate extracts were prepared by adding 2 g of ground sample to separate 15-mL centrifuge tubes followed by 4 mL of 100 mM MSA. These mixtures were homogenized with a vortex mixer for 1 min and centrifuged at 6000 rpm for 30 min at 4 °C. The supernatants were removed and filtered with a 0.2- μ m filter into separate flasks and diluted 1:1 with deionized water.

RESULTS AND DISCUSSION

Separation and Detection of Biogenic Amines

Figure 1 shows the separation of biogenic amines with suppressed conductivity, integrated pulsed amperometric, and UV detections (not connected in series). Dopamine, tyramine, and serotonin cannot be detected by suppressed conductivity because they lack a positive charge after suppression. Therefore, IPAD was required to detect all 10 biogenic amines. Tyramine was also monitored by UV detection to confirm its presence in samples that had previously been identified as containing tyramine by IPAD.

Electrolytically-generated MSA eluent was used to simplify the method and streamline the process of developing an optimum gradient for the separation of

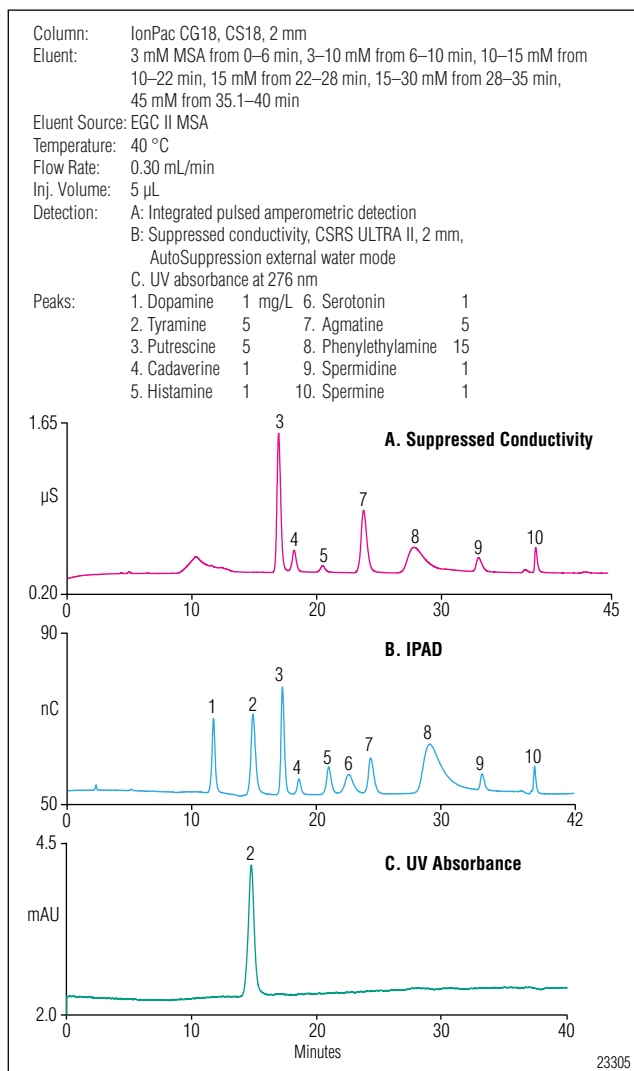


Figure 1. Separation of biogenic amines and detection by A) suppressed conductivity, B) IPAD, and C) tyramine by UV detection.

the target biogenic amines. An electrolytically generated eluent has not been used with IPAD in previous studies due to the production of oxygen by the generation of the MSA eluent. Dissolved oxygen in the eluent stream can result in significant changes in the background signal and therefore should be removed. Oxygen was removed by passing the eluent stream through the eluent channel and external water through the Regen channel of the EG degas device. This appeared to remove the oxygen created by the EG since no erratic changes in the background were observed. The EG simplified the method development by only requiring the addition of DI water, thus avoiding potential errors and inconsistencies that can occur when manually preparing eluents off-line.

Table 1. Linearity and Limits of Detection of Biogenic Amines

Analyte	IPAD Only			Suppressed Conductivity Detection			IPAD (post-suppression)			UV		
	Range (mg/L)	Linearity (r ²)	LOD (µg/L)	Range (mg/L)	Linearity (r ²)	LOD (µg/L)	Range (mg/L)	Linearity (r ²)	LOD (µg/L)	Range (mg/L)	Linearity (r ²)	LOD (µg/L)
Dopamine	0.1-5	0.9999	20	0.1-5	—	—	—	—	—	—	—	—
Tyramine	0.2-10	0.9999	80	0.2-10	—	—	—	—	—	0.2-10	0.9997	110
Putrescine	0.2-10	0.9979	50	0.2-10	0.9986	3.5	0.2-10	0.9974	97	—	—	—
Cadaverine	0.1-5	0.9999	70	0.1-5	0.9997	5.3	0.25-5	0.9997	160	—	—	—
Histamine	0.1-5	0.9999	40	0.1-5	0.9998	18	0.1-5	0.9998	88	—	—	—
Serotonin	0.1-5	0.9998	70	—	—	—	—	—	—	—	—	—
Agmatine	0.2-10	0.9998	170	0.2-10	0.9999	9.0	0.5-10	0.9999	290	—	—	—
Phenylethylamine	1-20	0.9999	400	1-20	0.9999	81	5--20	0.9999	1090	—	—	—
Spermidine	0.1-5	0.9999	80	0.1-5	0.9993	4.0	0.25-5	0.9996	140	—	—	—
Spermine	0.1-5	0.9996	50	0.1-5	0.9990	9.0	0.1-5	0.9998	90	—	—	—

Table 2. Biogenic Amine Concentrations in Food Products Determined by IPAD^a

Sample	Tyramine		Putrescine		Cadaverine		Histamine		Serotonin		Agmatine		Spermidine		Spermine	
	Amount Found (mg/kg)	Recov. (%)	Amount Found (mg/kg)	Recov. (%)	Amount Found (mg/kg)	Recov. (%)	Amount Found (mg/kg)	Recov. (%)	Amount Found (mg/kg)	Recov. (%)	Amount Found (mg/kg)	Recov. (%)	Amount Found (mg/kg)	Recov. (%)	Amount Found (mg/kg)	Recov. (%)
Chocolate (70% Cocoa)	<DL ^b	—	6.9±0.1	91.1	<DL	—	3.3±0.1	87.6	7.3±0.03	91.0	<DL	—	9.8±0.3	102.5	9.8±0.2	95.8
Dark Chocolate	<DL	—	<DL	—	<DL	—	<DL	—	<DL	—	<DL	—	0.4±0.0	103.2	0.5±0.1	99.4
Milk Chocolate	<DL	—	<DL	—	<DL	—	<DL	—	<DL	—	<DL	—	<DL	—	<DL	—
Spinach Leaves	<DL	—	7.8±0.1	107.6	<DL	—	61.0±1.5	93.8	<DL	—	<DL	—	48.5±1.8	102.0	6.6±0.3	98.2
Kiwi Fruit	<DL	—	3.1±0.1	96.1	<DL	—	1.9±0.1	91.3	9.2±0.3	95.4	<DL	—	7.5±0.1	105.5	1.9±0.1	98.0

^aTyramine determined by either UV or IPAD as noted.

^b<DL = less than the detection limit.

System Performance

The linear ranges for suppressed conductivity, IPAD, and UV detection were evaluated by tabulating peak area versus concentration. Calibration curves were prepared for each biogenic amine in 3 mM MSA using five increasing concentrations. The calibration data and LODs for the three detection configurations are summarized in Table 1. For more details, and for reproducibility and precision measurements, see AN 183.

Determination of Biogenic Amines in Food Products with IPAD

Biogenic amine concentrations for the foods analyzed in this update are listed in Table 2. The total biogenic amine concentrations for chocolate containing 70% cocoa, dark chocolate, and milk chocolate were 37.1, 0.9, and 0 mg/kg, respectively. This suggests that most of the biogenic amines detected were derived from the cocoa present in the chocolate. The 70% cocoa sample contained putrescine, histamine, serotonin, spermidine, and spermine at concentrations of 6.9, 3.3, 7.3, 9.8, and 9.8 mg/kg, respectively (Figure 2). These values are in agreement with previously published analyses of amines

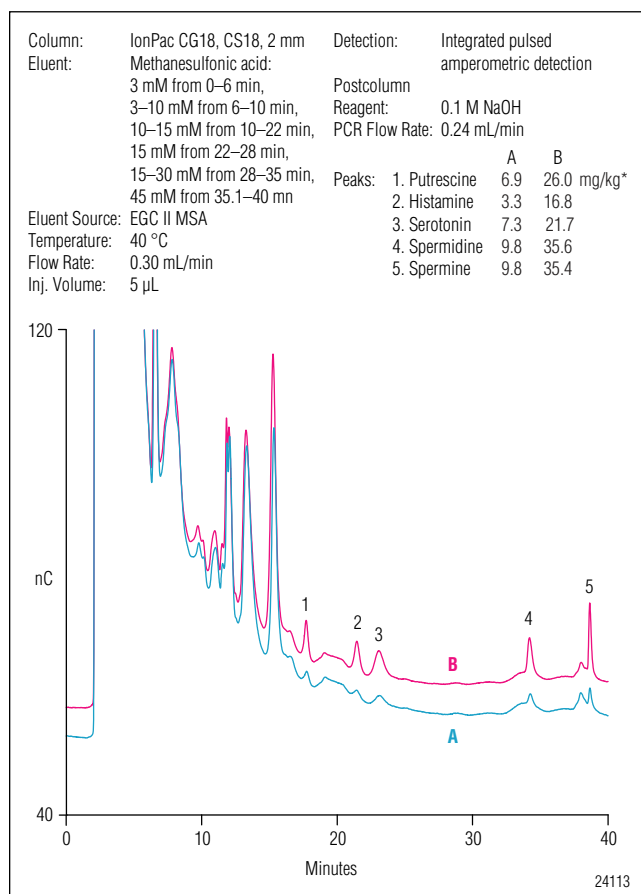


Figure 2. Detection of biogenic amines in chocolate containing 70% cocoa by IPAD. (A) Unspiked sample. (B) Spiked sample. *Concentrations calculated based on a dilution factor of 1:22.

in chocolate with the exception of serotonin. Previous findings have reported serotonin in chocolate in the range 16–61 mg/kg.^{14,15} Percent recoveries for biogenic amines spiked into the 70% cocoa sample ranged from 87.6 to 102.5% (Table 2).

Biogenic amines are also widespread in plant material that is commonly used for food, such as fruits and vegetables.² Limited information is available on the amine content in fruits. In this study, putrescine, histamine, serotonin, spermidine, and spermine were detected in kiwi fruit. Serotonin was detected at a concentration of 9.2 mg/kg, about 25% more than in the 70% cocoa sample. Putrescine, spermidine, and spermine are common polyamines that may serve specific protective roles in plants adapted to extreme environments.¹³ Previous studies have indicated a range of 1–4 mg/kg serotonin in passion fruit.¹⁶

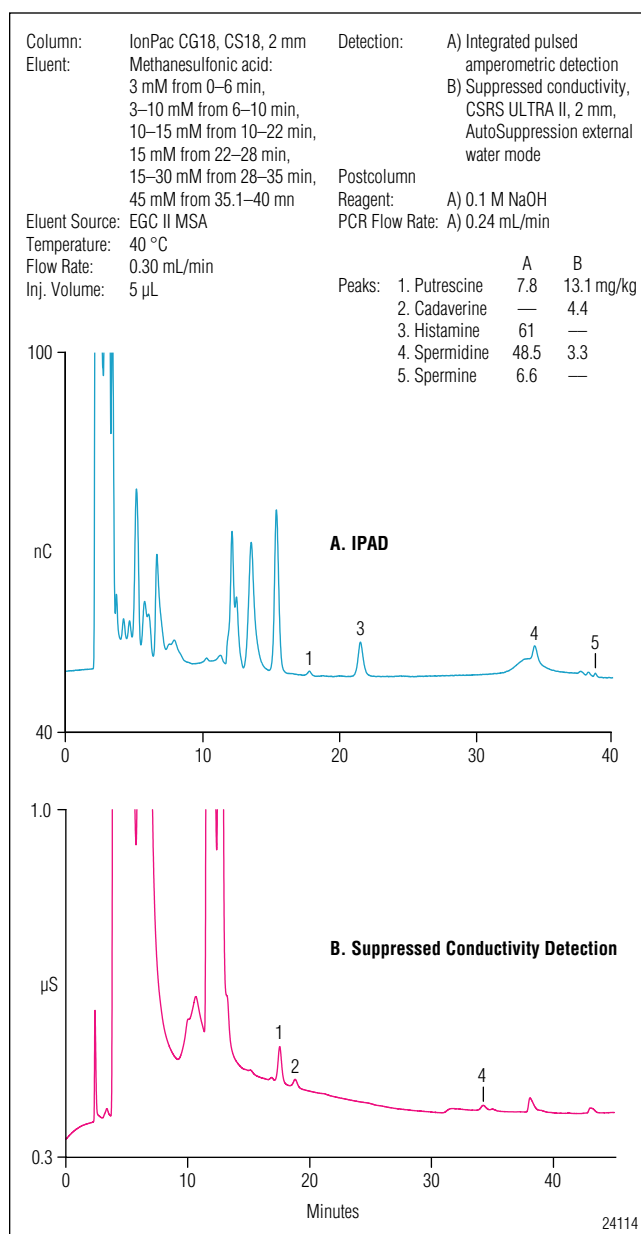


Figure 3: Separation of biogenic amines in spinach and detection by (A) IPAD (fresh sample) and (B) suppressed conductivity (after 3 weeks storage at 4 °C).

In the spinach leaves, histamine and spermidine were the primary biogenic amines detected at concentrations of 61 and 48.5 mg/kg, respectively (Figure 3). The highest concentration of spermidine in any of the unstored food products analyzed (including those tested in AN 183) was found in spinach. High spermidine levels have been reported in other green vegetables.⁵

Table 3. Biogenic Amine Concentrations in Stored Food Products Determined by Suppressed Conductivity and IPAD

Suppressed Conductivity Detection												
Sample	Putrescine		Cadaverine		Histamine		Agmatine		Spermidine		Spermine	
	Amount Found (mg/kg)	Recov. (%)	Amount Found (mg/kg)	Recov. (%)	Amount Found (mg/kg)	Recov. (%)	Amount Found (mg/kg)	Recov. (%)	Amount Found (mg/kg)	Recov. (%)	Amount Found (mg/kg)	Recov. (%)
Spinach Leaves ^a	13.1±0.1	105.8	4.4±0.0	90.5	<DL ^b	—	<DL	—	3.3±0.1	88.5	<DL	—
Kiwi Fruit ^c	1.7±0.0	98.5	<DL	—	<DL	—	<DL	—	9.5±0.0	93.6	1.7±0.1	95.3
IPAD (post-suppression)												
Sample	Putrescine		Cadaverine		Histamine		Agmatine		Spermidine		Spermine	
	Amount Found (mg/kg)	Recov. (%)	Amount Found (mg/kg)	Recov. (%)	Amount Found (mg/kg)	Recov. (%)	Amount Found (mg/kg)	Recov. (%)	Amount Found (mg/kg)	Recov. (%)	Amount Found (mg/kg)	Recov. (%)
Spinach Leaves ^a	12.7±0.3	100.8	4.9±0.1	95.0	<DL	—	<DL	—	<DL	—	<DL	—
Kiwi Fruit ^c	<DL	—	<DL	—	<DL	—	<DL	—	8.0±0.8	102.7	1.5±0.0	95.3

^aStored at 4 °C for 3 weeks.

^b<DL = less than the detection limit.

^cStored at 4 °C for 2 weeks.

Changes in Biogenic Amine Concentrations in Food Products during Storage at 4 °C Detected Using Suppressed Conductivity-IPAD

The kiwi fruit and spinach samples were reanalyzed after storage (Table 3). Significant changes in the biogenic amine content of spinach leaves were observed after three weeks refrigeration at 4°C. The spermidine concentration decreased from 48.5 to 3.3 mg/kg and histamine and spermine were completely degraded (Figure 3). The complete degradation of 61 mg/kg histamine presented the most interesting result. To confirm the accuracy of these results, the sample was reanalyzed using only IPAD, which confirmed the absence of histamine. Earlier studies have shown a change in the concentration of putrescine, spermidine, and spermine after three weeks refrigeration of some vegetable products.¹⁷ Although Leuschner et. al. have demonstrated that some microbial species degrade histamine,¹² no data could be found on the correlation of histamine degradation in vegetables. Storage of the kiwi fruit for two weeks at 4 °C resulted in an approximately 82% decrease in putrescine, 25% increase in spermidine, and no change in the spermine concentration. Histamine was completely degraded in the kiwi fruit after storage.

CONCLUSION

The IonPac CS18, a polymeric weak acid cation-exchange column, was used to separate biogenic amines in a variety of food samples, with detection by IPAD, suppressed conductivity, and UV. The described method uses a simple electrolytically generated MSA eluent without requiring the use of solvents or aggressive eluent systems that have been reported previously. In addition, the method results in good precision and recovery over a wide range of sample matrices and avoids the need for complex and long derivatization procedures. The use of three different detection configurations provides additional information and confirms the identification of tyramine to increase confidence in the analytical results. Suppressed conductivity had exceptionally low LODs for the main biogenic amines of interest without chromatographic interferences from common cations and amines present in many of the food samples. In addition to the amines detected by conductivity, IPAD allows the detection of dopamine, serotonin, and tyramine, which can be confirmed with a UV detector.

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