Rapid Determination of Hypoglycin A in Ackee by Liquid Chromatography/Tandem mass spectrometry

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Abstract

A simple liquid chromatography/tandem mass spectrometry (LC-MS/MS) analytical method was developed for the determination of toxic amino acid Hypoglycin A in ackee fruit. This free amino acid was retained on a mixed-mode column (Acclaim™ Trinity™ Q1 (3 µm, 100 x 3 mm) analytical column) without the need of pre-column derivatization. A 3-g test portion was shaken with ethanolic solution. After centrifugation, the sample extract was diluted and injected and analyzed within 15 min by LC-MS/MS. Two MS-MS transitions were monitored in the method for each target compound to achieve true positive identification. An isotopically-labeled internal standard of L-Leucine d3 was used to correct for matrix effect and/or instrument signal drift. The average recovery for all analytes at 17, 33, and 66 µg/g (n = 18) ranged from 70-120%, with a relative standard deviation of ≤ 20%.

Introduction

Hypoglycin A [(S)-2-amino-3-((S)-2-methylidenecyclopropyl)propanoic acid] (HG-A) is a plant toxin found in many plants including unripe ackee (*Blighia sapida*), litchi (*Litchi chinensis)* and other members of the Soapberry family (*Sapindaceae*) [1-2]. This compound may disrupt gluconeogenesis and β-oxidation of fatty acid [3]. It has been identified as the cause of an illness known as "Jamaican vomiting disease" [4]. This sickness results from the ingestion of immature ackee fruit. The patient will experience vomiting for 4 hours after ingestion followed by hypoglycemia [5]. It was found to be associated with an acute encephalitis syndrome in children in southern India during the litchi cultivation[6]. With the purpose to preventing HG-A consumption, the Food and Drug Administration (FDA) has defined concentration limits of HG-A in canned ackee not to exceed 100 µg/g of canned ackee [7]. There are no comparable restrictions based on toxicological limits for exporting or importing litchi or fruits of other *Sapindaceae* species. In 2017, the FDA issued an import alert for canned ackee imported to the US [8].

Like amino acid, HG-A is a compound that cannot be retained by conventional reversed-phase chromatography (Figure 1). The common methods used alcohol extraction [9-11] of fruit, and followed by derivatization with a reagent such as *o*-phthalaldehyde (OPA) [12], phenyl isothiocyanate (PITC) [9, 13-14], or dansyl chloride [15] followed by analysis on a reversed-phase column. It is known that most of the derivatization methods used for LC-MS have some drawbacks, such as derivative instability, lack of reproducibility, and they are time-consuming [16]. To eliminate the derivatization steps, ion-pairing reagents such as perfluoroheptanoic acid [17] and tridecafluoroheptanocid acid [18-20] were also used. The drawbacks include retention time instability and reduction of MS detector sensitivity due to ion suppression [21]. Hydrophilic interaction liquid chromatography (HILIC) was used to determine underivatized amino acid in plasma [22]. Rudolph et al. used HILIC technique to determine HG-A in serum[23]. A hybrid mixed-mode Acclaim P1 column was used to determine underivatized amino acid by the LC-MS/MS method [24]. The purpose of this study is to develop a simple LC-MS/MS method to determine HG-A in ackee, with a mixed-mode HPLC column without pre-column derivatization or using ion-pairing reagents.

Experimental

Chemicals and Reagents

1) Chemical standards of Hypoglycin A (85%) and L-Leucine d3 (99%) were obtained from Toronto Research Chemicals (Toronto, Ontario Canada).
2) Ethanol (95%), acetonitrile, water of HPLC grade, ammonium formate, and formic acid (98%) were purchased from Fluka (Buchs, Switzerland.).
3) A solution of 500 mM ammonium formate/formic acid (pH 2.9) was prepared as follows: 15.76 g of ammonium formate were dissolved in approximately 300 mL of HPLC water and adjusted with formic acid (approx. 28.3 mL) until the pH reached 2.9 (using pH meter), and the solution was diluted to 500 mL with water.
4) The HPLC mobile phase A was 1:9 water:acetonitrile + 5 mM ammonium formate pH 2.9. It was prepared by mixing 900 mL of acetonitrile with 90 mL of purified water and 10 mL of 500 mM ammonium formate pH 2.9.
5) The HPLC mobile phase B was 3:7 water:acetonitrile + 50 mM ammonium formate pH 2.9. It was prepared by mixing 700 mL of acetonitrile with 200 mL of purified water and 100 mL of 500 mM ammonium formate pH 2.9. The extraction solvent was 8:2 ethanol: water.

Standard Preparation

1) A stock solution of HG-A standard (1 mg/mL) was prepared in water (corrected for % purity). This solution was used to fortify a set of 3 g blank samples by pipetting 50, 100, and 200 μL to achieve the fortification levels of 17, 33, and 66 μg/g, respectively. A set of

HG-A standard solutions at 0.5, 1, 2, 5, 10, 20, and 40 μg/mL in acetonitrile was prepared from the HG-A stock solution (1 mg/mL).

2) An internal standard (IS) solution (L-Leucine d3 at 50 ng/mL) in acetonitrile was prepared from a stock solution of L-Leucine d3 in water at 1 mg/mL.
3) The calibration standard solutions used to construct a calibration curve were prepared by pipetting 5 μL of the HG-A standard solution (0.5 to 40 μg/mL) into a 900 μL plastic autosampler vial containing 245 μL of the IS solution and mixed well. The calibration represented concentrations of HG-A from 10 to 1000 ng/mL.

Sample Preparation and Extraction Procedure

Three different brands of canned ackee samples were obtained from a local market. The liquid portion was drained from the can, and the samples were minced with a food processor until they had a smoothie-like texture.

1) Weigh the representative portions in triplicate at 3 ± 0.1 g each into 50-mL polypropylene centrifuge tubes (Fisher Scientific, Pittsburgh, PA).
2) Fortify with 1 mg/mL of HG-A solution at the levels of 17, 33, and 66 μg/g, mixed briefly on a vortex mixer, and left to stand at room temperature for one hour.
3) Add the extracting solvent (20 mL) to each tube by using an automatic pipette.
4) The tubes were capped tightly and shaken for 20 min on a SPEX 2000 Geno grinder (SPEX Sample Prep LLC, Metuchen, NJ) at 2,000 stroke/min and then centrifuged at 4,130 rpm (3,000 x g) for 10 min using a Q-Sep 3000 centrifuge (Restek, Bellefonte, PA).
5) Pipette the sample extract (5 μL) into a 0.9 mL polypropylene autosampler vial containing 245 μL of the IS solution (50 ng/mL), cap the vial and vortex briefly.
6) Analyze the sample by LC-MS/MS along with the calibration standard solutions in 50 ng/mL of IS solution previously prepared. Three replicates of the same blank samples were analyzed in the same set to determine the concentration of HG-A incurred residue. This value was used to subtract from HG-A concentration found in the fortified sample to determine the % recovery.

Instrumentation

1) Liquid Chromatograph/Mass spectrometer- The instrument was equipped with two LC-20AD pumps, a Sil-20AC autosampler, and a CTO-20AC column oven (Shimadzu, Kyoto, Japan), coupled with a 6500 Q-TRAP mass spectrometer from AB SCIEX (Foster City, CA). The Analyst software (version 1.6) was used for instrument control and data acquisition. Nitrogen and air from TriGas Generator (Parker Hannifin Co., Haverhill, MA) were used for nebulizer and collision gas in LC-MS/MS. The MS determination was performed in positive mode with MS source condition as follows: curtain gas (CUR) of 40 psi, ion spray voltage (ISV) of 4500 volts, collisionally activated dissociation gas (CAD) is high, nebulizer

gas (GS1) of 50 psi, heater gas (GS2) of 55 psi, and source temperature (TEM) of 500 °C. Analyte-specific MS-MS conditions and LC retention times for the analytes are shown in Table 1.

2) HPLC column - An Acclaim™ Trinity™ Q1 (3 µm, 100 x 3 mm) analytical column from Thermo Scientific (Sunnyvale, CA) and a C18 SecurityGuard guard column (4 x 3 mm) from Phenomenex (Torrance, CA) were used for HPLC separation at 35 °C with a sample injection volume of 1 µL. The mobile phase was 100% A from 0 - 0.25 min at a flow rate of 0.5 mL/min then ramped down to 20% A from 0.25 – 5.5 min to elute the analytes. The column was flushed with 100% B for 5 min at a flow rate of 1 mL/min before equilibrated with 100% A for 4 min at the same flow rate for a total run time of approximately 15 min. A diverter valve connected between the HPLC column and the MS interface was used to direct the LC eluent to waste from 0 – 3 min and 5 – 15 min.

Results And Discussion

Chromatography and LC-MS/MS Optimization

Standard solution at 1 µg/mL in acetonitrile for HG-A and IS were infused into the mass spectrometer to obtain the optimum values for the MS parameters (Table 1). The Acclaim Q1 is a mixed-mode column possessing multiple retention mechanisms, including reversed-phase, anion-exchange, and cation exchange. It was previously used for glyphosate analysis in soybean and milk with an isocratic mobile phase of 50 mM ammonium formate pH 2.9 [25-26]. HG-A is an amino acid; therefore, it may behave similarly to glyphosate. To increase the retention time of glyphosate, a low ionic strength mobile phase (water) was used to retain glyphosate at the head of the column and elute non-ionic polar compounds [27]. The ionic strength of the mobile phase was then increased to elute glyphosate from the column. The ionic strength gradient was adopted and modified in this study. Water was replaced with 9:1 acetonitrile water containing 5 mM of ammonium formate to improve desolvation efficiency of the sample droplets and retain HG-A at the head of the column. This mobile phase enhanced the sample droplet desolvation by decreasing the surface tension and boiling point of the mobile phase [28]. Use of this mobile phase considerably improved the analyte response. The ammonium formate concentration was then increased to 50 mM to elute the analytes. Water was added to acetonitrile at the ratio of 3:7 to prevent ammonium formate from precipitating in 90% acetonitrile. A minimum of 10 column volumes of the initial mobile phase was used to equilibrate the column between injections to maintain retention time reproducibility.

The sample matrix may contain unknown compounds having the same target MRM transition and/or the same retention time as the analyte and cause the matrix suppression/enhancement. The sample extract was diluted 50 times with acetonitrile, and only 1 µL of the sample extract was injected to minimize matrix effect. Yet the repeat injection of the same sample (n = 10) gave peak area variation of HG-A of more than 30%. To minimize this unpredictable variation effect, L-Leucine d3 was used as an IS to compensate for matrix effect. This compound has a

retention time close to HG-A and is not present in the sample. This IS was inexpensive and significantly improved the precision of the method.

Method Performance

HG-A was extracted from the samples with 8:2 ethanol:water using the SPEX 2000 Geno grinder at 2,000 stroke/min for 20 min. This solvent was used in many studies with satisfactory results [9, 11, 15]. The Geno grinder provided vigorous shaking action for efficient extraction, and it can accommodate up to 15 50-mL tubes at a time. The mixed-mode Acclaim Q1 has been used to determine very polar pesticides that were not retained well on a reversed-phase column [25-27, 29-30]. It provided a unique, adjustable selectivity tool, using variation in pH, ionic strength or organic modifier to influence the separation selectivity of acids, bases, zwitterions and neutral molecules. It is versatile enough to retain a wide range of compounds. The stationary phase effectively retained HG-A on the column and yielded a narrow peak shape without the need for a derivatization step.

The method performance was evaluated by spiking HG-A in three blank ackee samples (in triplicate) at 17, 33, and 66 μg/g and analyzing on two different days. The average concentration of HG-A in each of the blank sample was calculated from three blank replicates. It was used to subtract the concentration of HG-A in the spiked samples. The final concentration was used to determine accuracy (recovery %) and precision (RSD %) of the method using the calibration curve in the solvent with IS at the concentration of 50 ng/mL. Table 2 shows the recovery data of HG-A in three different blanks performed on two different days. Method performance is summarized in Table 3. The calibration curve was a linear fit with 1/x weighing, and they all showed satisfactory linearity with a coefficient of determination (R^2) of more than 0.995. The chromatograms of HG-A standard at 200 ng/mL, blank ackee (no 2) and blank ackee spiked at 17 μg/g were shown in Figure 2. The LC-MS/MS gives a more accurate result than a traditional LC/UV method as it is free from background interference. The m/z ratio of confirmation ion (142.2/96) over the quantification ion (142.2/74) of the HG-A found in the sample matched with the m/z ratio found in the standard (within ± 20%)[31]. There was a small interference peak for the confirmation ion in the ackee blank just in front of the HG-A peak; however, it does not affect the accuracy of the ion ratio (by peak height). The method detection limit (MDL) was calculated according to the FDA guidelines with 7 replicates of the lowest calibration standard (10 ng/mL)[31]. The MDL was calculated by multiplying standard deviation of 7 replicates with a student t value at a confidence level of 99% with a degree of freedom of 6 (3.14). The MDL was calculated as 0.63 μg/g. The method quantification limit (MQL) was three times the MDL which was at 1.9 μg/g.

Conclusion

This study has demonstrated the versatility of the mixed-mode column for the determination of HG-A in ackee. Direct determination eliminates the need for lengthy derivatization or the use of the ion-paring reagent. The extraction procedure was simple, quick, and did not require matrix-matched calibration standards. The LC-MS/MS instrument used in this study was sensitive and selective. It provided accurate identification and quantification with minimum sample handling and cleanup.

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Table 1. Retention time and MRM conditions for LC-MS/MS analysis.

Analyte	Precursor Ion (m/z)	Product Ion (m/z)	DP	CE	EP	CXP	Retention Time (min)	dwell time
Hypoglycin.1	142.2	74	26	22	10	9	3.9	100
Hypoglycin.2	142.2	96	26	16	10	9	3.9	100
L-Leucine d3	132	86	26	14	10	9	3.8	100

Compound dependent parameters: DP = declustering potential, CE = collision energy, EP = entrance potential, CXP = collision cell exit potential

Table 2 Recovery data of three ackee blank samples spiked with HG-A and analyzed in two different days

spike level µg/g	matrix	Recovery (%) Day 1			Recovery (%) Day 2		
17	blank 1	105	115	112	107	108	106
	blank 2	102	100	98	112	86	96
	blank 3	84	84	102	100	96	86
33	blank 1	114	116	110	109	117	110
	blank 2	121	120	107	124	122	114
	blank 3	91	93	102	82	96	70
66	blank 1	113	111	107	118	113	111
	blank 2	113	112	120	105	108	106
	blank 3	107	99	89	92	105	109

HG-A found in blank 1, blank 2, and blank 3 were 10, 26, and 39 µg/g, respectively

Table 3 Method performance summary for all three different matrices at three levels.

Parameter	Fortification level (µg/g)		
	17	33	66
Intraday			
average recovery (n = 9)	100	108	108
std dev	10.7	11.0	9.1
RSD (%)	10.7	10.2	8.4
Interday			
average recovery (n = 18)	100	107	108
std dev	7.9	14.9	7.9
RSD (%)	8.0	14.0	7.4

Figure 1 Structure of Hypoglycin A

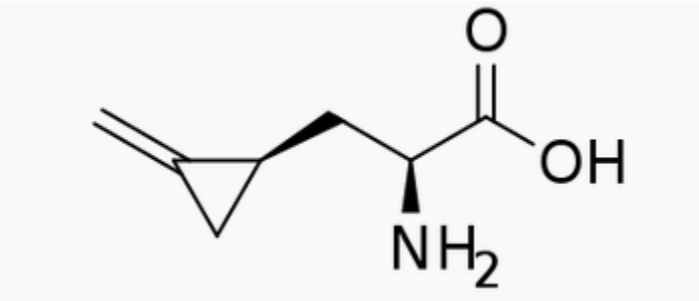
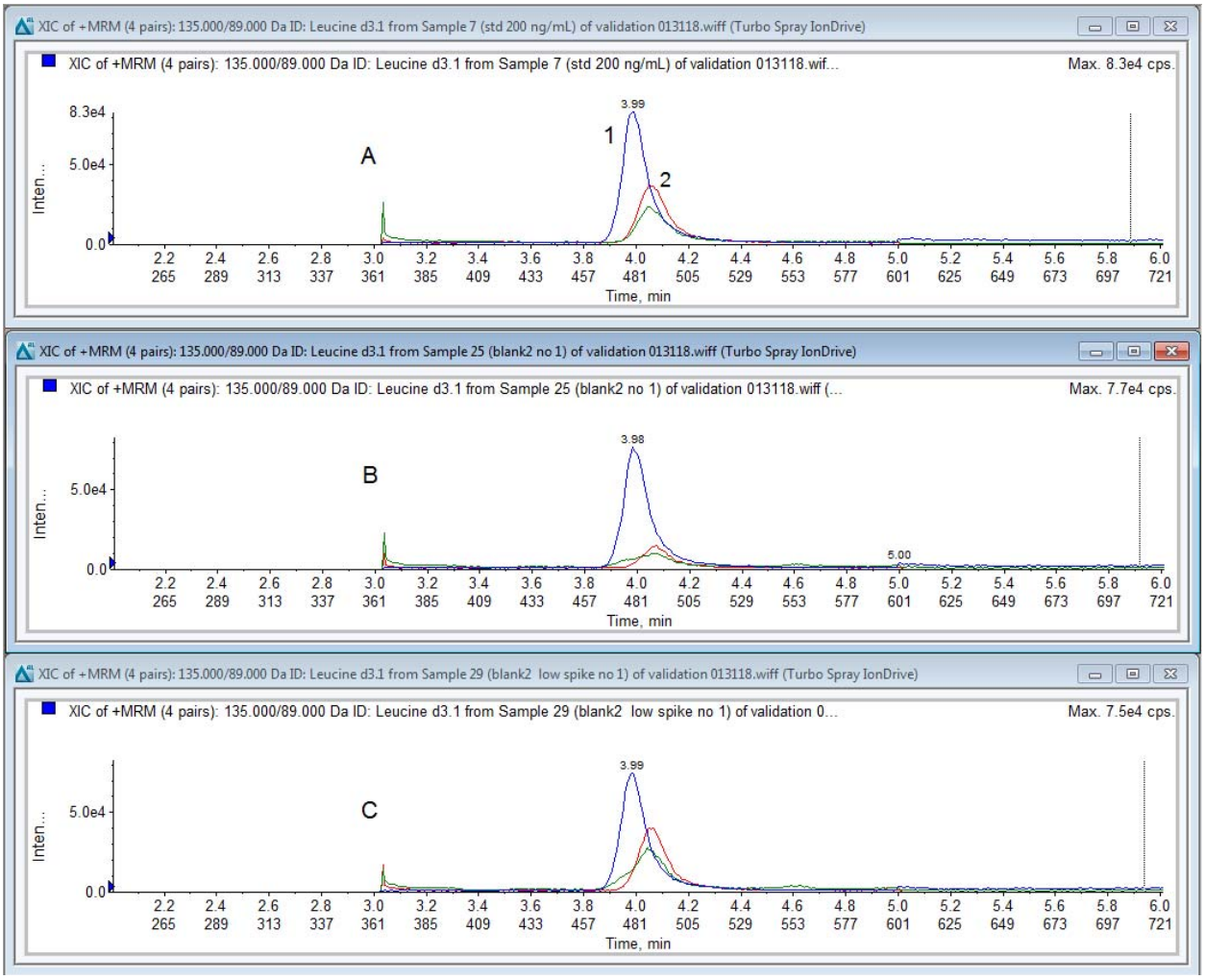


Figure 2 Extracted ion chromatograms of A) standard HG-A at 200 ng/mL + IS at 50 ng/mL, B) blank ackee no 2, and C) blank ackee no 2 spiked with HG-A at 50 ng/mL (17 µg/g).



Note: peak 1 (blue) is L-Leucine d3 (IS), peak 2 (red) is Hypoglycin A (HG-A) quantification ion 142.2/74, (green) confirmation ion 142.2/96.