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Quantitative Analysis of L-Abrine and Ricinine Spiked into Selected Food Matrices by Liquid Chromatography-Tandem Mass Spectrometry

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Abstract

Abrin and ricin are highly toxic and lethal proteins which have the potential to be used as bioterrorism agents.L-abrine and ricinine, molecular biomarkers for abrin and ricin, serve as useful biomarkers in assessing exposure and contamination.In this study, we developed a method for the quantitation of L-abrine and ricinine spiked into four food types: ground beef, chicken breast, hot dogs, and Eggbeaters®.This method involved sample homogenization, followed by polymeric reversed solid phase extraction, evaporation, and reconstitution.Determination and quantitation of L-abrine and ricinine was achieved using LC tandem mass spectrometry utilizing positive electrospray ionization. Quantitation was based upon fragmentation of m/z219→132 for L-abrine and m/z 165→138 for ricinine.The limit of detection achieved was 0.1 µg/L while the limit of quantitation was 0.50 µg/L for L-abrine and 0.30 µg/L for ricinine.A 7-point standard calibration curve showed good linearity for both analytes (r2 = 0.990) with a CV of <10% for replicates.Spiked fortified food matrices at 5 µg/L, 25 µg/L, and 50 µg/L yielded recoveries ranging from 93 – 119%. Validation results showed this method to be rapid, accurate, sensitive, and suitable for surveillance monitoring of food products suspected of abrin and ricin contamination.

Keywords: Abrin; ricin; L-abrine; Ricinine; Liquid chromatography; Mass spectrometry; Solid phase extraction;Quantitative analysis

Introduction

Ricin, an alkaloid of castor bean plants, is an extremely potent and deadly poison which has gained interest in the chemical emergency preparedness and response community due to its appearance in terrorism literature and its potential use as a chemical warfare agent [1]. The castor plant is widely available due to its many uses in the production of oils, lubricants, pharmaceuticals, cosmetics and engineering plastics [2].Harvested in many countries, including Asia, Africa and Europe, there is an overall production of 1 million metric tons of castor seeds which implies a total production of 10,000 metric tons of ricin each year [3]. While the most potent approaches of ricin poisoning are inhalation and injection, the most common approach has been found to be ingestion [4]. Currently there are no validated tests for the detection of ricin available to the clinical laboratories [5] and while the analysis of ricin, a large heterogeneous protein with glycosylation, has proven difficult, the identification of the ricinine has shown to be a complementary technique for the determination of castor bean extracts [1].

Abrin, another alkaloid chemical, can easily be isolated from the seed of the rosary pea plant [6]. Just like ricin and ricinine, crude abrin and abrine are easily obtained from their seeds using rather simple technology [7,8]. This simple isolation and the potential of its use to adulterate foods have made the detection of abrin and ricin in foods a top priority. However, abrin, like ricin, belongs to the large family of ribosome inactivating proteins containing two disulfide linked heterodimic chains [8], making it just as difficult as ricin to effectively isolate from contaminated matrices at low detection limits.Due to the high potential threat which abrin and ricin carry, both toxins are classified as category B Select Agents by the US Health and Human Services [8].

As it is mentioned, the primary analytes of interest to the chemical emergency preparedness community are that of abrin and ricin, however due to the difficulty of isolation and detection of these analytes from potentially tainted matrices, L-abrine and ricinine have proven to be useful markers in the detection of the primary analytes. In addition to the easier isolation of L-abrine and ricinine, they have also demonstrated longer stability in contaminated matrices thereby allowing for a larger window between the time of contamination and detection [9].

Materials and Methods

Chemicals and food products

L-abrine was purchased from Sigma Aldrich (St. Louis, MO) and ricinine was purchased from Accurate Chemical and Scientific Corporation (Westbury, NY). Isotopically-labeled L-abrineand ricinine were purchased from Cerilliant (Round Rock, TX) and used as internal standards for instrumental quantitation.Formic acid and HPLC grade solvents including acetonitrile and methanolwere purchased from Sigma Aldrich (St. Louis, MO), and used throughout the study.High purity water was supplied from within the laboratory using a Millipore Elix Water Purification System (Billerica, MA).Raw food products, including ground beef (15% fat), chicken breast, hot dog, and EggBeaters®, were purchased from a local grocery store.

Preparation of standards

Assessment of standard purity: The sources of L-abrine and ricinine were analyzed on an Agilent Technologies 1100 HPLC coupled with an Applied Biosystems API 4000 quadrupole linear ion trap mass spectrometer (LC/MS/MS) controlled by Analyst software.

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Stock standard solutions of abrine and ricinine were prepared by dissolving 50 mg of each analyte into 50 mL of deionized water. Working standardsolutions were prepared by diluting 0.50 mL of the stock standard solution into 100 mL deionized water.Ten µL of this 5 µg/mL solution was injected into the HPLC and chromatographed using a mobile phase gradient program.The ion transitions for L-abrine were 219/132 (quantitation) and 219/187.9 (confirmation). The ion transitions for ricinine were 165/138 (quantitation) and 165/82 (confirmation).

Sample preparation and clean-up

Preparation of standards: A working standard solution of L-abrine and ricinine with a concentration of 5.0 µg/mL was used to prepare thirteen standards to construct a linear dynamic range (LDR), seven calibration standards, and six quality control (QC) samples. The LDR for L-abrine ranged from 0.10 µg/L to 800 µg/L and the seven calibration standards ranged from 0.50 µg/L to 200 µg/L. The LDR for ricinine ranged from 0.10 µg/L to 600 µg/L and the seven calibration standards ranged from 0.30 µg/L to 100 µg/L.

Preparation of samples: For solid food samples (i.e., ground beef, hot dogs, chicken) the matrix was homogenized while the liquid sample (i.e., egg beaters) was mixed and 5.0 g or 5.0 mL of each matrix sample was aliquoted into a 15 mL centrifuge tube.Each vial was spiked with the target analytes at the equivalent to its concentration level, vortexed and kept at 4°C overnight.

Liquid extraction: Samples were prepared by allowing the sample vials to sit at room temperature for 30 min and then adding10 mL water to each tube.The sampletubes were capped and vortexed for 30 seconds. The samples were then sonicated (Branson 3510, Bransonic, Dansbury, CT) for 30 min and centrifuged at 4000 x*g* for 30 min (Sorvall, Thermo Scientific, Waltham, MA).The supernatant was removed with a disposable glass pipette and passed through a 0.2 µm filter.A 1 mL aliquot was transferred to a test tube and spiked with 100 µL of L-abrine and ricinine internal standard mixture.

Solid phase extraction: Strata-X SPE columns (60 mg/3 mL; Phenomenex, Torrance, CA) were conditioned with 3 mL of methanol followed by 3 mL of water.The supernatant of the sample was loaded (1 mL) and allowed to drip through by gravity.The SPE columns were then washed with 5% methanol and the L-abrine and ricinine were eluted with 3 mL of acetonitrile.The eluent was then completely evaporated under a constant flow of nitrogen at 15 psi in a Zymark Turbo Vap evaporator (Caliper Life Sciences, Waltham, MA) at 65°C for 20 minutes or until completely dry.The extracts were reconstituted in 200 µL water, vortexed and transferred to an autosampler vial for analysis by LC/MS/MS.

The optimization of the clean-up protocol for SPE was evaluated extensively.The solid phase extraction cartridges were evaluated with the elution solvents of acetonitrile, ethyl acetate, acetone and methanol. Acetonitrile as the elution solvent resulted in the highest recoveries.

Instrumental conditions

LC/MS/MS: The Applied Biosystems API 4000 quadrupole linear ion trap mass spectrometer was optimized for L-abrine and ricinine ionization and analysis from food samples.The instrument was first calibrated using positive and negative polypropylene glycol (PPG) solutions per manufacturer's specifications.After tuning the instrument with a 100 µg/L solution of L-abrine and ricinine in water + 0.1% formic acid, optimized settings were as follows: positive ion mode ESI with ion spray voltage at 5100 V, collision gas at high, curtain gas at 20 L/hr, ion source gas 1 at 65 L/hr, ion source gas 2 at 10 L/hr and the interface temperature at 550 °C.The collision energy setting was 25 eV for Ricinine 165→138, 41 eV for Ricinine 165→82, 25 eV for labeled Ricinine 171→85, 29 eV for L-abrine 219→132, 17 eV for L-abrine 219→187.9 and 17 eV for labeled L-abrine 223→187.9.The declustering potential for all ricinine transitions was 51 V, 31 V for L-abrine 219→132 and 46 V for L-abrine 219→187.9 and 223→187.9.The collision cell exit potential for all ricinine transitions was 8 V, 16 V for L-abrine 219→132 and 12 V for L-abrine 219→187.9 and 223→187.9.Dwell time was set a 100 m/sec for all transitions.

An Agilent 1100 HPLC system consisting of a quaternary pump, in line mobile phase degasser, temperature controlled autosampler (maintained at 20°C), and column heating compartments was utilized for chromatography.Mobile phase A consisted of 10% methanol in water + 5mM formic acid and mobile phase B consisted of acetonitrile + 5mM formic acid.Twenty µL were injected onto a 2.0 mm x 100 mm i.d., 2.5 µm, Polar Reverse Phase Phenyl analytical column (Phenomenex, Torrance, CA).The column was maintained at40°C ± 5°C throughout the chromatographic run with a 3 min equilibration time between samples.The gradient mobile phase conditions were as follows: 93% A at 0 min (hold for 0.5 min) to 50% A within 2 min (hold for 1 min), return to 93% A in 0.01 min (hold for 3 min) for a total run time of 6 min with a 0.300 mL/min flow rate maintained throughout.L-abrine eluted at 3.00 min and ricinine eluted at 4.2 min.

Quantitation was calculated using a PC equipped with Analyst software by linear regression with no weighting from 0.5 µg/L to 200µg/L for L-abrine and from 0.30 µg/L to 100 µg/L forricinine with n ≥ 6 measurements per standard.A standard curve was prepared for each individual sequence run per matrix and six quality control samples were included at the beginning and end of each run.A linear dynamic range consisting of 13 standards and a blank was also included in each run.L-abrine and ricinine was analyzed using multiple reaction monitoring (MRM) and the following transitions were monitored: L-abrine, m/z 219 → 132 (quantitation ion), m/z 219 → 187.9 (confirmation ion) and 223 → 187.9 (internal standard); ricinine, m/z 165 → 138 (quantitation ion), 165 → 82 (confirmation ion) and 171 → 85 (internal standard).

Method verification

Three sets of food samples were used in the method verification procedures.Aliquots (1 g) of each matrix were spiked at three levels to obtain 5.0 µg/L, 25.0 µg/L and 50.0 µg/L concentrations (n = 6 per spike level per food sample) and were also left unfortified (n = 6) to include control samples.The food samples were extracted with water as described above in *Liquid Extraction* and using the SPE procedure described above with analysis by LC/MS/MS.

Statistical analysis

All statistical analyses were completed using SPSS Statistics software from IBM.

Results and Discussion

Method characteristics

Figure 1are example chromatograms generated from the different food spike-recovery experiments demonstrating sufficient separation/detection of native and labeled L-abrine and ricinine by LC/MS/MS in 6 minutes at a sample concentration of 40 µg/L.It also shows the three ion ratios measured for each analyte, including the quantitation, confirmation and internal standard ion ratios.Figures 2 and 3 are example chromatograms of the quantitation and confirmation ion

ratios of L-abrine and ricinine respectively. Figures 4 and 5 exhibit extracted ion chromatograms of the L-abrine and ricinine and the resulting MRM.Tables 1 and 2 summarize native and labeled L-abrine and ricinine LC/MS/MS instrumental parameters and SRM configurations.L-abrine and ricinine analyzed by LC/MS/MS was quantified using a linear calibration curve with no weighting (Figures 6 and 7).The calibration curves had a minimum r^2 value of 0.9900 within the calibration range of 0.50 – 200 µg/L for L-abrine and 0.30 – 100 µg/L for ricinine for all extracted matrices demonstrating excellent method linearity.Instrumental detection limits were assigned to the molecular ions when their lowest abundance confirmation ion signal-to-noise (S/N) ≥ 3 as determined by the S/N script of the AB Sciex Analyst data analysis software, version 1.6 (Framingham, MA).Samples used in the determination of instrumental detection limits were standard solutions analyzed from several batches over several days.The instrumental detection limit was 0.10 µg/L for both analytes, L-abrine and ricinine. The analytes were considered quantitative when they calibrated with $r^2 \geq 0.9900$, their lowest abundance confirmation ion had S/N > 3 and had reproducible and accurate quantitation (± 20% of their true value) as assessed by quality control verification standards. Figures 8 and 9 summarizes L-abrine and ricinine spike recoveries obtained for liquid and SPE extraction methods from chicken, ground beef (15% fat), hot dog and EggBeaters®.The recoveries of the analytes were tested against a plethora of different solvent combinations to determine the most effective combination in eluting the analytes from the SPE columns.Combinations tested included ethyl acetate, methanol, acetonitrile, water, and hexanes with the most effective combination being methanol, water and acetonitrile. These extraction methods used in tandem yielded decent recoveriesranging from 80-120% over all matrices analyzed.The goal of this study was to develop and validate a method for the analysis of L-abrine and ricinine in a variety of food matrices.The data presented strongly indicate that a liquid extraction coupled with a dispersive SPE sample cleanup and LC/MS/MS is a fast, selective, efficient and precise method for the determination of L-abrine and ricinine in food matrices.This method demonstrates good potential for use in monitoring exposure of abrin, L-abrine, ricin and ricinine in food.

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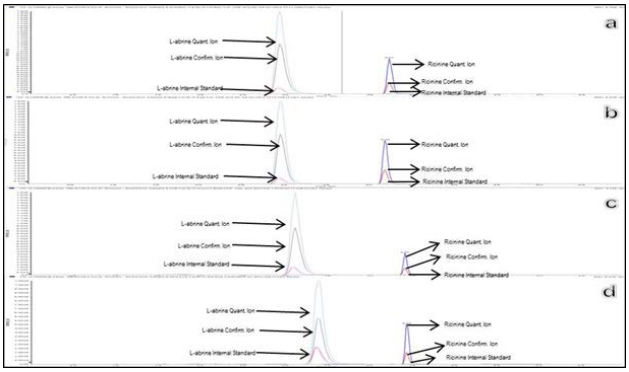

Figure 1: Total Ion Chromatograms of L-abrine (Rt = 2.97 min) , ricinine (Rt = 4.22 min) and L-abrine and ricinine labeled internal standards (Rt = 2.97 min and 4.22 min respectively) extracted from (a) raw chicken, (b) Eggbeaters®, (c) raw ground beef, and (d) hot dog. The different colors in the chromatograph are indicative of the various ion ratios (i.e. quantitation ion ratio, confirmation ion ratio, and internal standard ion ratio).

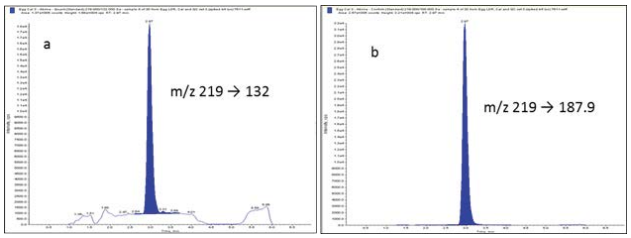

Figure 2: Typical chromatogram of L-abrine quantitation (a) and confirmation (b) ions.

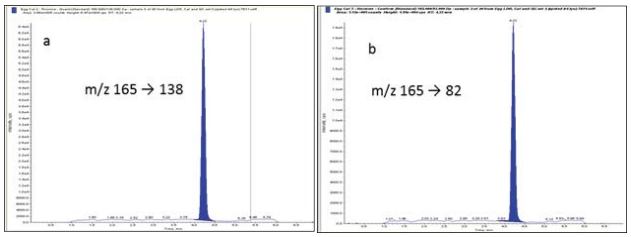

Figure 3: Typical chromatogram of ricinine quantitation (a) and confirmation (b) ions.

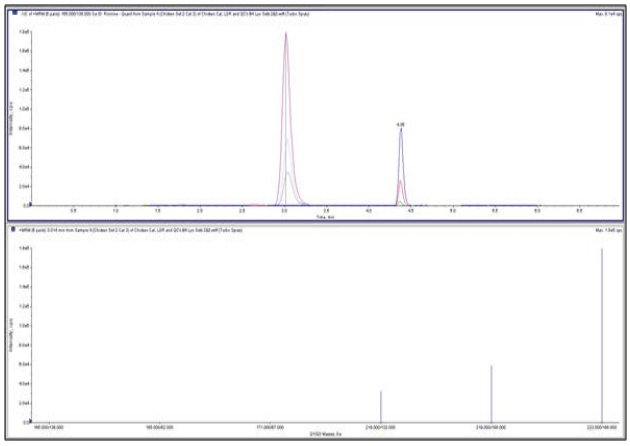

Figure 4: Typical extracted ion chromatogram and MRM transitions for L-abrine.

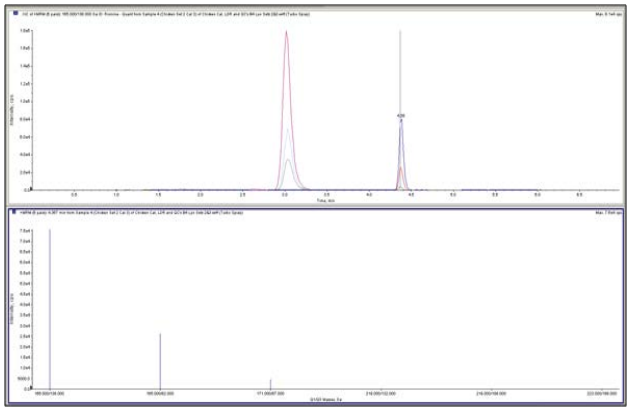

Figure 5: Typical extracted ion chromatogram and MRM transitions for ricinine.

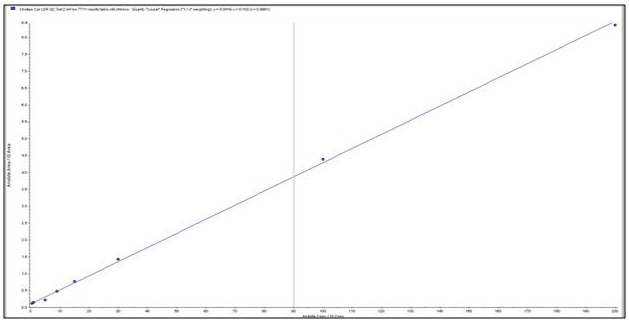

Figure 6: Typical calibration curve for L-abrine. Linear regression equation is equivalent to y=0.0149x + 0.102; r^2=0.9981

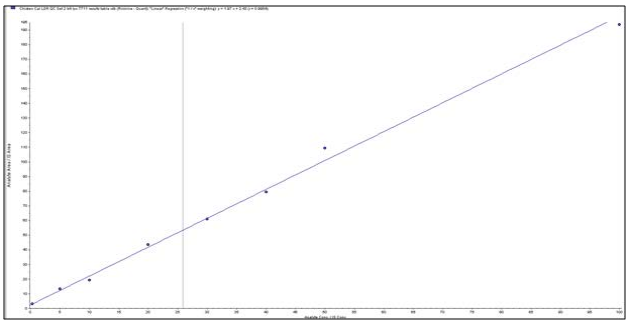

Figure 7: Typical calibration curve for ricinine. Linear regression equation is equivalent to y=1.97x + 2.43; r^2=0.9984

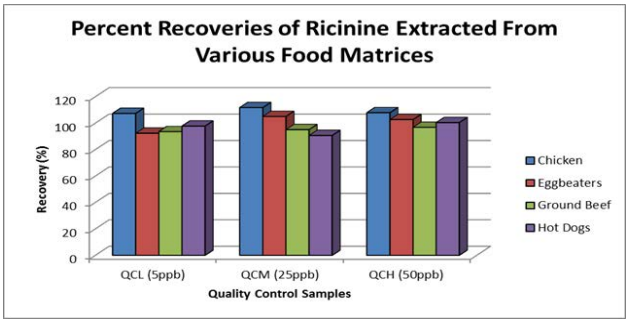

Figure 8: Average recoveries of ricinine from various food matrices. All matrices attained recoveries between 80-120%.

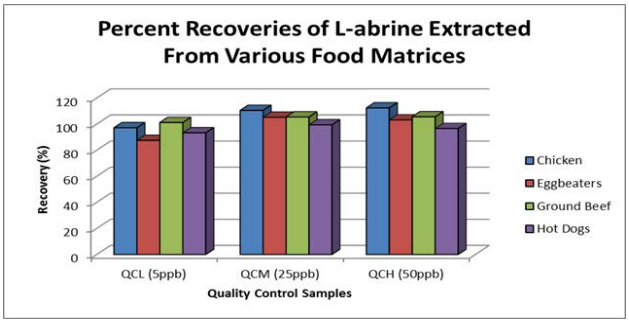

Figure 9: Average recoveries of L-abrine from various food matrices. All matrices attained recoveries between 80-120%.

Parameter	Setting			
LC Method Pump 1	Gradient			
	Reservoir A = 10% MeOH in water, 5 mM formic acid			
	Reservoir B = ACN, 5 mM formic acid			
	Time (min)	%A	%B	Flow Rate (µL/min)
	0.0	93	7	300
	0.5	93	7	300
	2.0	50	50	300
	3.0	50	50	300
	3.01	93	7	300
	6.0	93	7	300
Column Oven	Temperature ranges from 40 °C			
Column Type	Polar RP Phenyl column, 2x100 mm, 2.5µm particle size			
Injection Volume	20 µL			
Needle Rinse Time	5 - 10 seconds, using flushport			
Rinse Solvent	Mobile Phase A			
Injection Mode	Standard			
Autosampler Tray Temp.	20 °C			
Typical Retention Time	L-Abrine = 2.1 min			
	Ricinine = 3.5 min			
HPLC Degasser	Powered for continuous use (there are no settings)			
MS Scan Mode	MS/MS selected reaction monitoring (SRM)			
Ionization Type	Turbo-ionspray (similar to ESI)			
Ion Polarity Mode	Positive ion			
Scan Type	100 msec per channel			
Collision Gas (CAD)	High or "12"			
Curtain Gas (CUR)	20			
Ion Source Gas 1 (GS1)	65			
Ion Source Gas 2 (GS2)	10			
Ion Spray Voltage (IS)	5100			
Temperature (TEM)	550 (interface heater on)			
Declustering Potential (DP)	see Table 5			
Entrance Potential (EP)	10			
Collision Energy (CE)	see Table 5			

Table 1: LC/MS/MS Parameters and Settings.

Analyte	Retention Time (min)	Parent Ion (amu)	Center Ion (amu)	Collision Energy (eV)	Declustering Potential (DP)	Collision Cell Exit Potential (CXP)
Native Ricinine	4.38	165	138	25	51	8
Native Ricinine	4.38	165	82	41	51	8
Labeled Ricinine	4.38	171	85	25	51	8
Native L-abrine	3.01	219	132	29	31	16
Native L-abrine	3.01	219	187.9	17	46	12
Labeled L-abrine	3.01	223	187.9	17	46	12

Table 2: Tandem Mass Spectrometer SRM Configuration.

Disclaimer

The findings and conclusions published in this paper are those of the authors and do not represent the views of the Maryland Department of Health and Mental Hygiene.The use of trade names is for identification only and does not constitute endorsement by the Laboratories Administration, Maryland Department of Health and Mental Hygiene, or the USDA-FSIS.

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