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Forensic Determination of Ricin and the Alkaloid Marker Ricinine From Castor Bean Extracts*

REFERENCE: Darby SM, Miller ML, Allen RO. Forensic determination of ricin and the alkaloid marker ricinine from castor bean extracts. J Forensic Sci 2001;46(5):1033–1042.

ABSTRACT: Liquid chromatography/mass spectrometry (LC/MS) and matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS methods were developed for the presumptive identification of ricin toxin and the alkaloid marker ricinine from crude plant materials. Ricin is an extremely potent poison, which is of forensic interest due to its appearance in terrorism literature and its potential for use as a homicide agent. Difficulties arise in attempting to analyze ricin because it is a large heterogeneous protein with glycosylation. The general protein identification scheme developed uses LC/MS or MALDI-TOF for size classification followed by the use of the same instrumentation for the analysis of the tryptic digest. Fragments of the digest can be searched in an online database for tentative identification of the unknown protein and then followed by comparison to authentic reference materials. LC fractionation or molecular weight cutoff filtration was used for preparation of the intact toxin before analysis. Extracts from two types of castor beans were prepared using a terrorist handbook procedure and determined to contain 1% ricin. Additionally, a forensic sample suspected to contain ricin was analyzed using the presented identification scheme (data not shown). The identification of the alkaloid ricinine by GC/MS and LC/MS was shown to be a complementary technique for the determination of castor bean extracts.

KEYWORDS: forensic science, ricin, toxin, ricinine, mass spectrometry

Ricin is a toxic plant lectin derived from the seeds of the castor bean plant, *Ricinus communis* L. (1–3). It is a protein with a molecular weight of over 60 000 Da and has substantial glycosylation (4–9). The structure and sequence of the protein is well established in the scientific literature (10–13). Ricin is deadly in submilligram amounts (100 μg) and has the threat to be a powerful biochemical warfare agent (2). Because there is no known antitoxin or vaccine for ricin, the potential threat of this compound is great.

Ricin came into the spotlight as a deadly agent in 1978 when Bulgarian diplomat Georgi Markov was injected with a metal sphere from the tip of an umbrella. He died three days later (14,15). This sphere contained a small hole in which 500 μg of ricin had been placed.

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The toxin is heterogeneous in terms of which sugars are attached in its glycosylation (8,9). Ricin contains three N-linked oligosaccharides, which vary among molecules of the protein. The presence of these sugars presents an analytical challenge for the identification of ricin due to the large number of variants that are possible.

The most common testing for ricin is performed by immunoassay (16–18), typically by enzyme-linked immunosorbent assay (ELISA). A particular advantage of ELISA is the visual or spectrophotometric indication of the result through the development of color by the enzyme bound to an antibody. Although the methods are sensitive, they could be subject to crossreaction and interferences. In addition, a more general screening method for an unknown sample is required beforehand so that the appropriate specific assay can be applied. An alternative is to develop a combined nonimmunoassay screen and chemical identification scheme for any unknown protein toxin. Mass spectrometry is a powerful tool in the scheme developed in this work, not only for a first screen of an unknown substance, but also for specific testing and confirmation of identity through comparison to authentic materials.

A strategy was developed to identify ricin in crude materials. Matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry and electrospray liquid chromatography/mass spectrometry (LC/MS) were used for both screening and identification of the protein material. Biochemical cleavage of the samples was necessary to facilitate the analysis. Digesting a protein into peptide fragments produces a unique mass spectral fingerprint pattern of the molecule (19,20). This pattern can be used to distinguish samples with similar molecular weights and presumptively identify an unknown through database searching. The results of the search can be followed by a comparison to an authentic standard. An identification scheme was developed to distinguish suspected plant toxins, but it can also be applied to other proteins that have the potential of being deadly.

Ricinine (Fig. 1) is a toxic alkaloid ($C_8H_8N_2O_2$, MW 164.1) that is derived from the leaves and seeds of the castor bean plant (21–26). The determination of ricinine in an unknown extract supplements the presumptive identification of ricin, since both compounds are derived from the same source. GC/MS and LC/MS analyses were developed for the analysis of ricinine in crude castor bean extracts.

Experimental

Electrospray LC/MS

A Hewlett-Packard 1100 HPLC system was used for all liquid chromatography (Palo Alto, CA). Data were acquired, and the system was controlled using ChemStation software (Hewlett-Packard). Online detection was performed with a diode array detector (DAD)

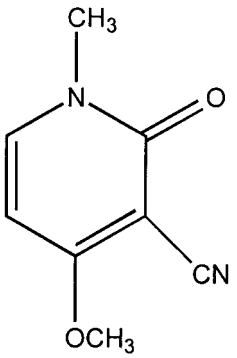

FIG. 1—*Structure of ricinine, MW = 164.1.*

equipped with a deuterium lamp for UV and a tungsten lamp for visible detection. Mass spectrometric detection bypassed the DAD with the column directly attached through polyetheretherketone (PEEK) tubing to the electrospray interface. Electrospray LC/MS with quadrupole ion trap detection was performed on a Finnigan LCQ (San Jose, CA) controlled by Navigator software (Finnigan MAT). Reversed-phase liquid chromatography was performed on a Jupiter C_{18} column, 2.1 × 150 mm, 300 Å, 5 μ (Phenomenex, Torrance, CA). A 2 mm SecurityGuard C_{18} guard cartridge (Phenomenex) was attached to the inlet of the column. HPLC-grade acetonitrile was obtained from Burdick and Jackson (Muskegon, MI), and ultrapure water was purified using an 18.5 MΩ system (Elga, Fisher Scientific, Pittsburgh, PA). Trifluoroacetic acid (TFA) (Sigma, St. Louis, MO) was used as a mobile phase ion-pairing additive. Linear gradients starting with 10 to 90% acetonitrile (intact proteins) and 10 to 60% acetonitrile (digestion fragments) were run at 300 μL/min.

Gas Chromatography/Mass Spectrometry

A Hewlett-Packard (Palo Alto, CA) 6890 GC and 5973 MSD were used for GC/MS analyses. The column was a HP 5-MS (5% phenyl, 95% methylsiloxane), 30 m × 250 μm I.D. × 0.25 μm film thickness (HP #190915-433). A 1 μL splitless injection was made with an injection port temperature of 250°C. Helium flow was 1.2 mL/min. Temperature programming was as follows: Initial T = 40°C, hold 2 min, ramp 15°C/min to 300°C, hold 5 min. Analytes were ionized in electron impact (EI) mode and the mass spectrometer scanned the range 50 to 500 μ at a rate of 2 scans/second.

MALDI-TOF Mass Spectrometry

Desalted samples in either 100% water or an acetonitrile/water mix were analyzed by MALDI-TOF mass spectrometry using a PE Biosystems linear Voyager-DE instrument. A 100-position stainless steel sample plate was used for the deposition of sample and matrix. The matrix for the protein solutions was 10 mg/mL sinapinic acid (Sigma) in 50:50 acetonitrile:0.1% aqueous TFA. Peptide solutions as well as protein digests were analyzed with 10 mg/mL α-hydroxycinnamic acid in 50:50 acetonitrile:0.1% aqueous TFA as the matrix. One μL of sample was deposited onto the sample plate followed by 1 μL of the matrix solution; the spots were allowed to dry thoroughly. Samples were ablated with a pulsed nitrogen laser at 337 nm. The sample plate in the high-voltage ion source was held at 25 kV. The variable voltage grid was held at 92% of the grid voltage for delayed extraction. Ion extraction was delayed for 200 ns. Linear detection was performed using a multichannel plate detector at the end of the flight tube. Results were reported in GRAMS™ software (Galactic Industries, Salem, NH).

Samples and Cleanup

Standard solutions of ricin toxin were obtained from Sigma and were in a buffer solution of 0.01 M potassium phosphate pH = 7.2, 0.15 M sodium chloride, and 0.1% sodium azide. Ricinine was obtained from Latoxan (Valence, France). Desalting of proteins was achieved using 0.025 μm dialysis membrane filters (Millipore) in a 0.1% aqueous TFA solution for 1 hr. In addition to dialysis, Biomax molecular weight cutoff (MWCO) filters of 3 k to 30 k (Millipore) were used to clean up samples. For cleanup, 50 to 250 μL of sample were placed in the top of a MWCO filter mounted on an Eppendorf vial. The tube was centrifuged at 5000 rpm for 20 to 50 min until 10 to 25 μL of sample remained in the top of the filter. When necessary, an additional 100 μL of ultrapure water were placed in the sample reservoir with the concentrated protein and respun for further purification.

Digestion of Proteins

For protein digestions, 5 nmol of reduced or intact protein was mixed with 100 μL of 20 mM ammonium bicarbonate. A stock solution of trypsin which was treated to prevent self digestion (Promega, Madison, WI) was prepared by adding 40 μL of 50 mM acetic acid to 20 μg of trypsin. One μL of the stock enzyme solution was added to the protein solution, and the digest was run at 37°C for 12 to 16 h. Ten μL of a crude castor bean extract solution was also fractionated by liquid chromatography, and the ricin toxin peak was collected as it eluted from the LC column. The eluent was dried in a Speed Vac (Savant), reconstituted in 25 μL of water, and digested with trypsin. Digests were analyzed by both electrospray and MALDI-TOF MS. LC/MS was performed on a Jupiter C_{18} column with a 10 to 60% acetonitrile gradient with 0.1% TFA in water over 40 min. An injection volume of 5 μL was used.

Extraction of Toxin from Plant Material

Two samples of castor seeds (large- and small-grain) were extracted to analyze ricin from a crude extract of an authentic matrix. The extraction procedure was modified from a terrorist cookbook to simulate an actual case sample (27). Whole castor beans (seeds) were cracked with a pair of pliers to remove the outer hull. Due to the serious health risks involved with the handling of ricin, all steps were performed in an enclosed glove box (Scienceware, Bel-Art Products, Inc., Pequannock, NJ). Seven grams of hulled beans were ground up with 50 mL of acetone using a mortar and pestle until a milky slurry was obtained. The bean pulp slurry was placed in a sealed jar and allowed to sit for three days to remove the castor oil from the seeds. The mixture was poured through fluted filter paper, and the solids were dried thoroughly. The dried bean pulp was re-extracted overnight with 25 mL of acetone. After this solution was filtered and dried, the fine, white, solid material was placed into a sealed vial.

Quantitation of Ricin

A method for quantitation of ricin was developed using liquid chromatography with ultraviolet detection. Standard ricin solutions were prepared in concentrations from 25 to 500 μg/mL. Five μL injections were made onto a 2.1 × 150 mm Jupiter C_{18} column (Phe-

nomenex) equilibrated with 10% acetonitrile (0.1% TFA)/90% water (0.1% TFA) run with a 300 μL/min gradient to 90% acetonitrile (0.1% TFA) in 15 min. Detection was performed at 214 nm, and peak areas (retention time 11 min) were plotted against protein concentration. Sample carryover on the autosampler was an issue only for those samples that contained greater than 200 μg/mL of ricin. One blank injection was run between each standard from 25 to 200 μg/mL. For the higher concentration standards as well as the extract solution, two blank injections were made after each sample injection to prevent carryover problems.

Analysis of Ricinine

For GC/MS analysis, measures were taken to extract the alkaloid without the protein, which is not compatible with GC. A sample of the crude castor bean extract was mixed in chloroform, because proteins are not readily soluble in this solvent. A 9 mg sample of the crude material was extracted overnight with 0.5 mL of chloroform, dried with nitrogen to approximately 0.1 mL, and analyzed by GC/MS.

Results

LC/UV Quantitation of Ricin

Although an internal standard was not used for the quantitation of ricin, measuring the peak areas produced a linear calibration curve ($R^2 = 0.980$) over the range of 25 to 500 μg/mL. A crude castor bean extract was prepared in water to a concentration of 22 mg of powder/mL. Ricin in this sample was quantitated at 204 μg/mL, corresponding to 0.9% in the plant extract (Fig. 2).

Electrospray LC/MS Analysis of Toxins

All components of the castor bean extract were resolved using reversed-phase liquid chromatography on the Jupiter C_{18} column. The column was equilibrated with 10% acetonitrile (ACN) containing 0.1% TFA, 90% water with 0.1% TFA. A gradient was run to 90% ACN in 15 min. Adding 0.1% TFA to the ACN in the mobile phase ionized the proteins more efficiently than ACN containing no ion-pairing reagent during the gradient run. Spectra were obtained for ricin and its separated chains. The intact protein produced spectral peaks that were very noisy, yet somewhat distinguishable (Table 1). Attempts at deconvolution of the spectrum estimated a molecular weight of 64 540 Da for ricin (Fig. 3), about 2000 Da larger than the expected molecular weight. The deconvoluted spectrum for the A chain produced a MW of 30 946 Da, consistent with the expected molecular weight. The B chain produced no distinct mass spectral peaks by LC/MS.

The heterogeneity of the sugar residues in ricin, in particular those of the B-chain, limits the use of electrospray LC/MS on the unmodified toxin and the B-chain. Options to solve this problem include: 1) development of an alternate mass analysis method, or 2) modification of ricin to yield a distinguishable electrospray spectrum.

TABLE 1—*Mass clusters observed for standard ricin toxin run by reversed-phase LC/MS.*

Mass Range (m/z)	Approximate Centroid (m/z)
1825–1860	1845
1775–1810	1790
1725–1750	1735
1675–1720	1690
1630–1660	1650
1610–1625	1615
1550–1590	1575
1510–1540	1525
1470–1490	1480

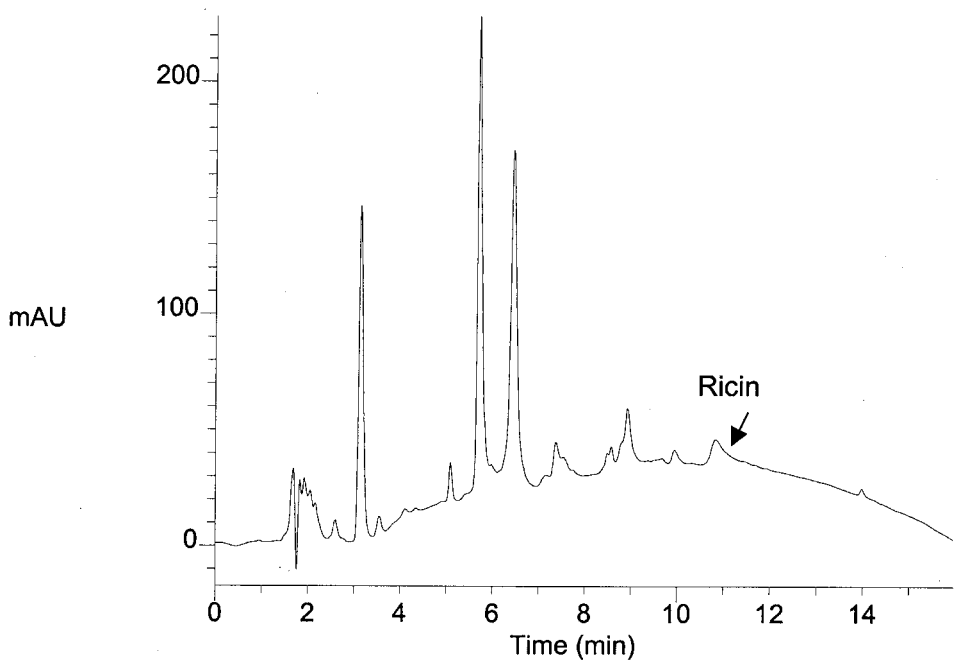

FIG. 2—*HPLC chromatogram using UV detection (214 nm) for castor bean extract. Ricin elutes at 11 min.*

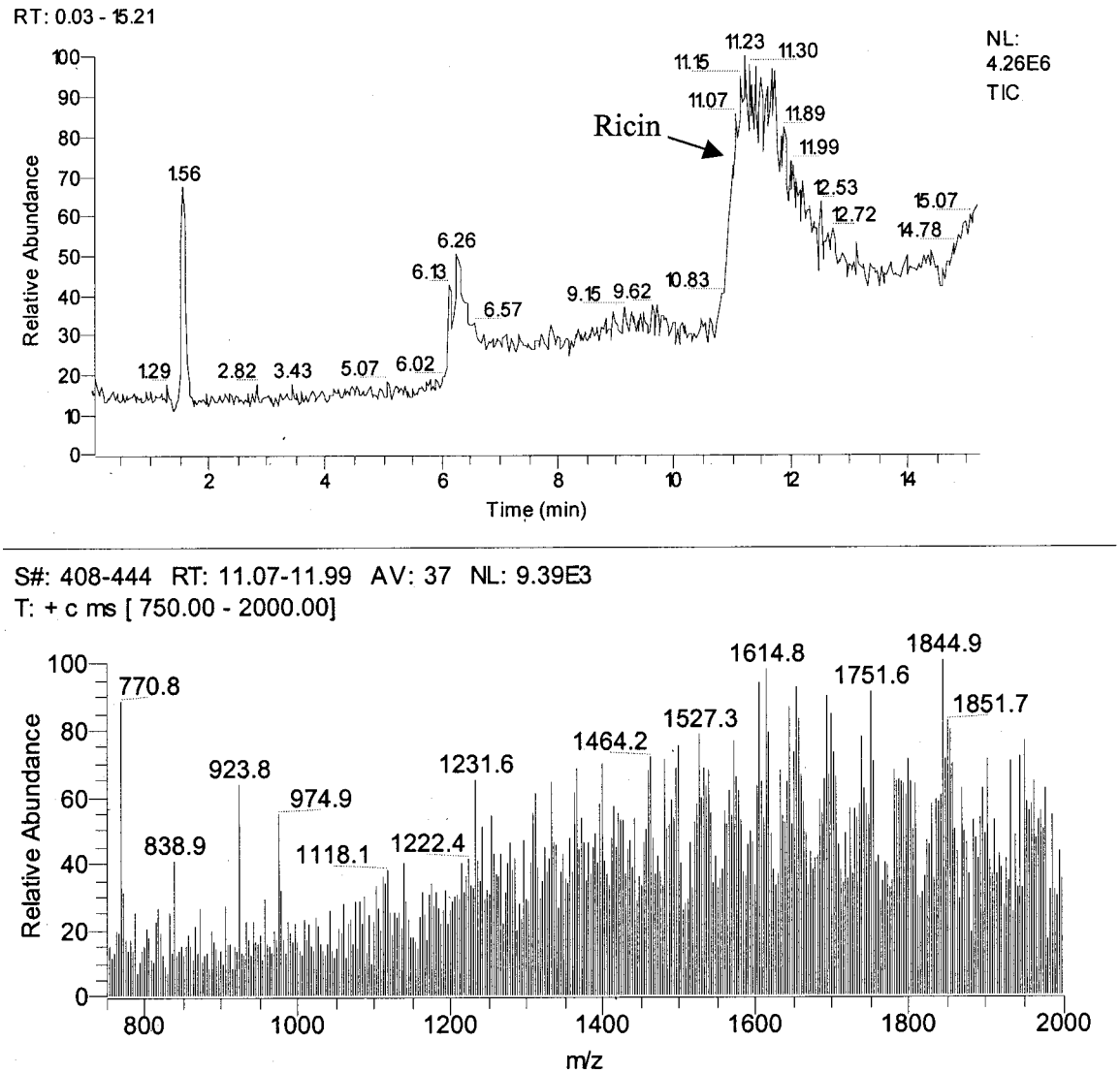

FIG. 3—*Detection of ricin toxin (11.3 min) separated by reversed-phase liquid chromatography. Jupiter C_{18} column, 10–90% ACN (0.1% TFA): 0.1% TFA in water, 0.3 mL/min, 15-min gradient to 90% ACN.*

MALDI-TOF MS Analysis of Ricin Standard

A standard ricin solution (Sigma) was analyzed by MALDI-TOF mass spectrometry to obtain a reference spectrum for the protein. The width of the molecular weight peak apex covered a mass range of approximately 300 μ, so an exact molecular weight was not determined. This variation is due to the large size, isotope effects, and heterogeneity of the protein. The ricin toxin produced a spectrum with a centroid at m/z 62 766, but the peak base width was broad (~4000 μ) and mass resolution was poor (Fig. 4), due in part to the isotopic profile and the mixture of glycosylated species. In contrast, the peaks produced from the separated A- and B-chains of ricin were narrower than the intact protein, less than 2000 μ in width. The peak widths were proportional to the molecular weights of the analytes. The A-chain produced a distinct spectrum with a doublet at m/z 30 855 and m/z 32 114. The difference of 1259 μ between the two peaks is most likely due to the glycosylated and deglycosylated moieties of the protein, because the A-chain of ricin only contains one sugar residue. The isolated B-chain of ricin, which has two sites of glycosylation, produced a molecular weight peak at m/z 31,768.

Sample Cleanup for MALDI-TOF

Initial MALDI-TOF MS analyses indicated that a great deal of interferences were present in the castor bean extracts. These components, which were smaller than 15 kDa but higher in concentration than ricin, completely inhibited the ionization of ricin and other compounds with higher molecular weights. A castor bean extract was spiked with a 100 μg/mL solution of ricin standard and analyzed by MALDI-TOFMS. No signal was detected for ricin, which confirmed ionization was suppressed for this protein. A method was developed for the cleanup of the extract solutions.

The high-resolving power of liquid chromatography was employed to isolate a ricin fraction from an aliquot of the crude extract solution. A 10 μL injection was made onto a 300 Å Jupiter C_{18} column and run with a gradient of 10 to 90% acetonitrile:0.1% TFA in 15 min. The ricin fraction was collected at a retention time of 10.4

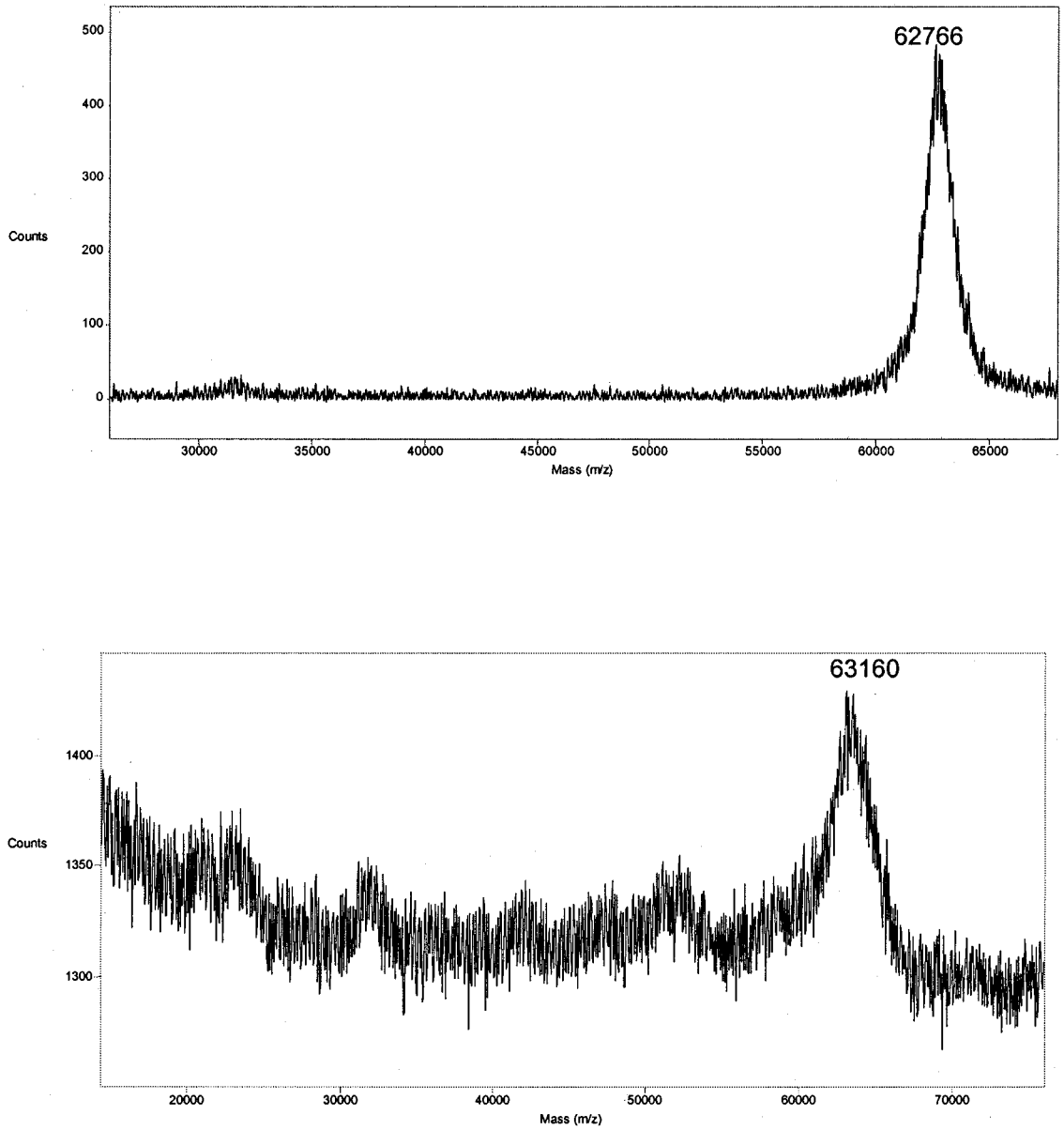

FIG. 4—*MALDI mass spectra of ricin standard (upper portion) and crude castor bean extract fractionated by LC, 10 to 11 min (lower portion).*

min to separate it from the other components in the extract. The collection time corresponds to the elution of a ricin standard. Fractions were concentrated in a Speed-Vac, and analyzed by MALDI-TOFMS (Fig. 4). The molecular ion in the isolated fraction sample was within 400 μ of the reference standard's mass (<1%).

Because the LC fractionation cleanup method was time-consuming, tedious, and subject to sample loss, an alternative method for the isolation of ricin was developed using molecular weight cutoff (MWCO) filtration. The MWCO utilizes a polysulfone membrane with a cutoff of 30 kDa. Sample is placed on the membrane and washed through, leaving molecules larger than the cutoff in the sample reservoir. This method is ideal for the castor bean extract purification. The main interferents with molecular weights of less than 15 kDa are below the cutoff and are separated from the analytes of interest. The higher molecular weight compounds remaining in the sample reservoir are easily removed and analyzed by MALDI-TOF MS. In addition, a larger sample volume can be purified, and a higher concentration factor can be obtained using MWCO filtration, which also requires less time to process and has higher sample throughput than LC fractionation.

Figure 5 illustrates the MALDI spectrum of the filtered crude castor bean extract for both small- and large-grain varieties. The higher molecular weight components were ionized more readily after the low-molecular weight interferents were removed. Although the MW limit of the filter was 30 kDa, a component at m/z 22 500 still remained in the filter. However, the components with molecular weights of less than 15 kDa were removed, which allowed the ricin to be ionized and detected using this method. In addition, the extract was concentrated four-fold from an original volume of 100 μL to a final volume of 25 μL. Ricin in the large grain sample produced a molecular ion at m/z 63 145 from the crude extract, which was consistent with the standard that was analyzed (<1%). The small grain extract produced a less distinct molecular peak at m/z 64 084, which is 2% higher than the mass of the standard.

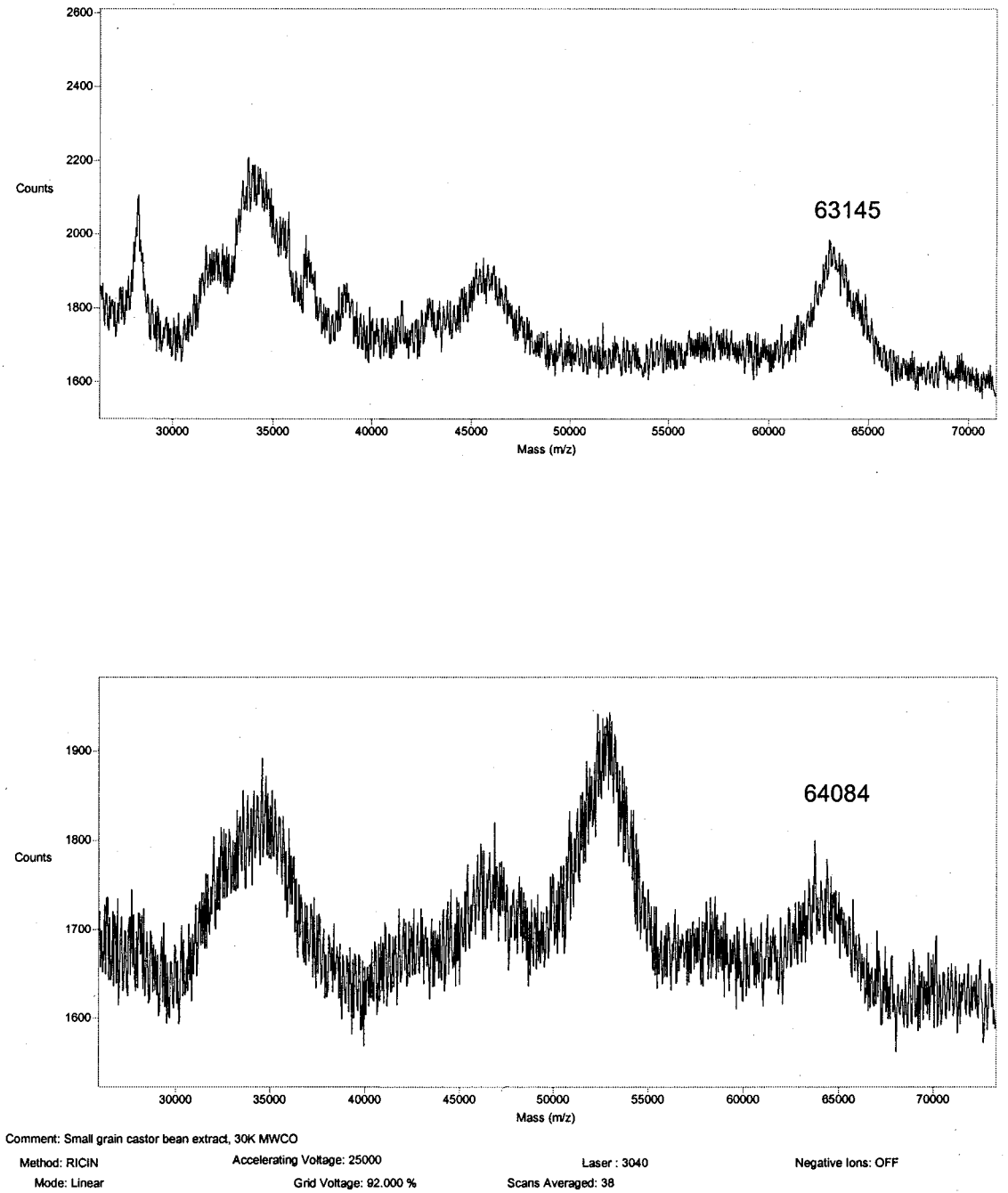

FIG. 5—*MALDI spectra of samples prepared using 30 KD_a MWCO filters. Large-grain castor seed extract (upper trace) and small-grain castor seed extract (lower trace).*

Analysis of Digests

The digestion of proteins results in peptide fragment patterns that provide detailed composition information and de facto chemical fingerprints. The intact molecular weight is a preliminary identification step for the protein, but digesting ricin with trypsin and mapping the peptides by mass spectrometry provides more definitive identification of the toxin. The tryptic digest peaks of ricin produced in our analyses by both electrospray and MALDI-TOF mass spectrometry are outlined in Table 2.

The Sigma ricin toxin standard produced 11 mass peaks using electrospray versus 14 detected with MALDI. Of these peaks, only six were the same between the two techniques; selectivity was different between electrospray and MALDI-TOF MS. The peaks are named according to their origin on the A- or B-chain of the toxins. For example, A1 is the first peptide produced from the N-terminal end of the A-chain; B2 is the second fragment produced from the N-terminus of the B-chain, and so forth. The expected peptides and designations for the tryptic digest of ricin were calculated and are listed in Table 3.

The digested LC fraction corresponding to ricin that was collected from the crude castor bean extract produced ten fragments matching to known peptides for ricin when analyzed by MALDI-TOF MS. The peptides produced from the digest were entered into

TABLE 2—*Peaks produced by electrospray and MALDI-TOF MS for toxin digests.*

Protein	Peaks Produced, by Electrospray		Peaks Produced by MALDI	
Ricin toxin	A12	A14	A11	B18
	A8	A6	A1	B13
	A20	A11	A10	B20
	A23	B20	B1	A20
	A19	B13	B6	B10
	A13		B11	A9
			A19	A12
Ricin A-chain	A7	A20	A17	A10
	A14	A9	A5	A19
	A4-A5	A12	A7	A20
	A6	A23	A11	A9
			A6	A12
			A14	
Ricin B-chain	B13	B10	B2	B6
	B6	B20	B17	B18
			B16	B13
			B14	B20
Crude castor bean extract, LC fraction	Not detected		A7	B6
			A11	B19
			A6	B18
			A14	A23
			A10	A20

TABLE 3—*Predicted mass fragments and designations for ricin (RCA 60).*

Designation	Average Mass (m/z)	Designation	Average Mass (m/z)
A1	504.6	B1	1345.6
A2	2506.8	B2	444.6
A3	345.4	B3	876.0
A4	232.3	B4	347.4
A5	832.9	B5	1528.8
A6	1075.3	B6	1391.5
A7	897.1	B7	175.2
A8	3208.7	B8	618.7
A9	3309.5	B9	405.4
A10	1311.4	B10	2951.3
A11	1014.2	B11	1613.8
A12	3442.8	B12	6937.7
A13	1582.9	B13	2232.4
A14	1173.3	B14	1238.3
A15	276.3	B15	575.7
A16	288.4	B16	1176.3
A17	452.5	B17	611.8
A18	175.2	B18	1863.1
A19	1729.9	B19	717.8
A20	2260.5	B20	2278.7
A21	175.2		
A22	405.4		
A23	2213.7		
A24	934.1		

the Internet search program, MSFit, to match against the known proteins (http://prospector.ucsf.edu/ucsfhtml3.2/msfit.htm).

For the digested ricin standard, 14 out of the 25 masses that were entered into the program matched those for the known ricin precursor. The search for each of the digests selected ricin as the number one hit, as expected. In addition, the masses produced from the ricin collected from the crude castor bean extract also selected ricin toxin as the most likely identifiable protein from the database.

Determination of Ricinine

Electron impact GC/MS analysis was performed on the chloroform re-extract of the castor bean extract. An extracted ion trace of m/z = 164 corresponding to the molecular ion of ricinine indicated a match with a peak at 15 min. The mass spectrum from EI analysis matched the library reference (Wiley Mass Spectral Library) for ricinine with a match quality of 97 (Fig. 6). The identification was also verified through analysis of a ricinine standard (in a methanol/chloroform solution) which produced a mass spectral match.

In addition to GC/MS analysis, further experiments were conducted to determine if LC/MS^n could be used for identification of ricinine. Since ricinine is also slightly soluble in water, the aqueous sample of the castor bean extract that contained the protein fractions was analyzed by LC/MS. The same conditions that were used for the separation of ricin in the extract were applied, except the mass range was decreased to acquire lower masses. The ricinine eluted at 3 min with an $(M+H)^+$ at m/z 165.1 (Fig. 7). Utilizing the conditions optimized for the determination of ricin allowed both the toxin and the alkaloid marker to be analyzed simultaneously over the range of m/z 100 to 2000.

To produce more definitive information, tandem mass spectrometry (MS/MS) was performed on the ricinine precursor at m/z 165.1 as it eluted from the LC column. A relative collision energy of 12% resulted in product ions at m/z 138 (100%), 82 (8%), 110 (7%), and a precursor intensity of 1%. The ion at m/z 138 corresponds to $[(M+H)^+27]$ from loss of HCN on the phenyl ring. The details provided by MS/MS allows ricinine to be determined conclusively and complements the information obtained by single-stage MS.

The presence of ricinine in a sample is unique to the castor bean plant. Other similar toxic plants, such as the jequirity bean (*Abrus precatorious L.*), which contains the toxic protein abrin, do not contain ricinine in their seeds. The toxic plant lectins, ricin and abrin, exhibit similar characteristics although the proteins are not derived from the same source. The two proteins exhibit amino acid sequences which are 40% identical to one another and the undigested toxins produce almost identical MALDI mass spectra with molecular ions at m/z 62 766 and 62 589, respectively. Unique digest fragments in the abrin C sample observed by both LC/MS and MALDI were m/z 877.0 (A3), m/z 1682.8 (A10), m/z 538.6 (A11), m/z 1159.2 (A12), m/z 652.7 (A19), m/z 2747.1 (B14), and m/z 3840.2 (B16). The determination of both ricinine and ricin in an unknown sample links the questioned sample to the castor bean as the source of the material.

Discussion

Two samples of castor beans, large- and small-grain, were extracted with acetone to remove the oil from the seeds and obtain ricin in its natural matrix. A quantitation method for ricin was developed using reversed-phase liquid chromatography with ultraviolet detection at 214 nm. Running a gradient elution from 10 to 90% acetonitrile with 0.1% TFA over 15 min on a 300 Å C_{18} column provided the best separation and elution of ricin from matrix interferents. The crude castor bean extracts were each determined to contain approximately 1% ricin by weight.

Electrospray LC/MS produced mass clusters rather than distinct centroid peaks for the unmodified glycoproteins. Random, noisy

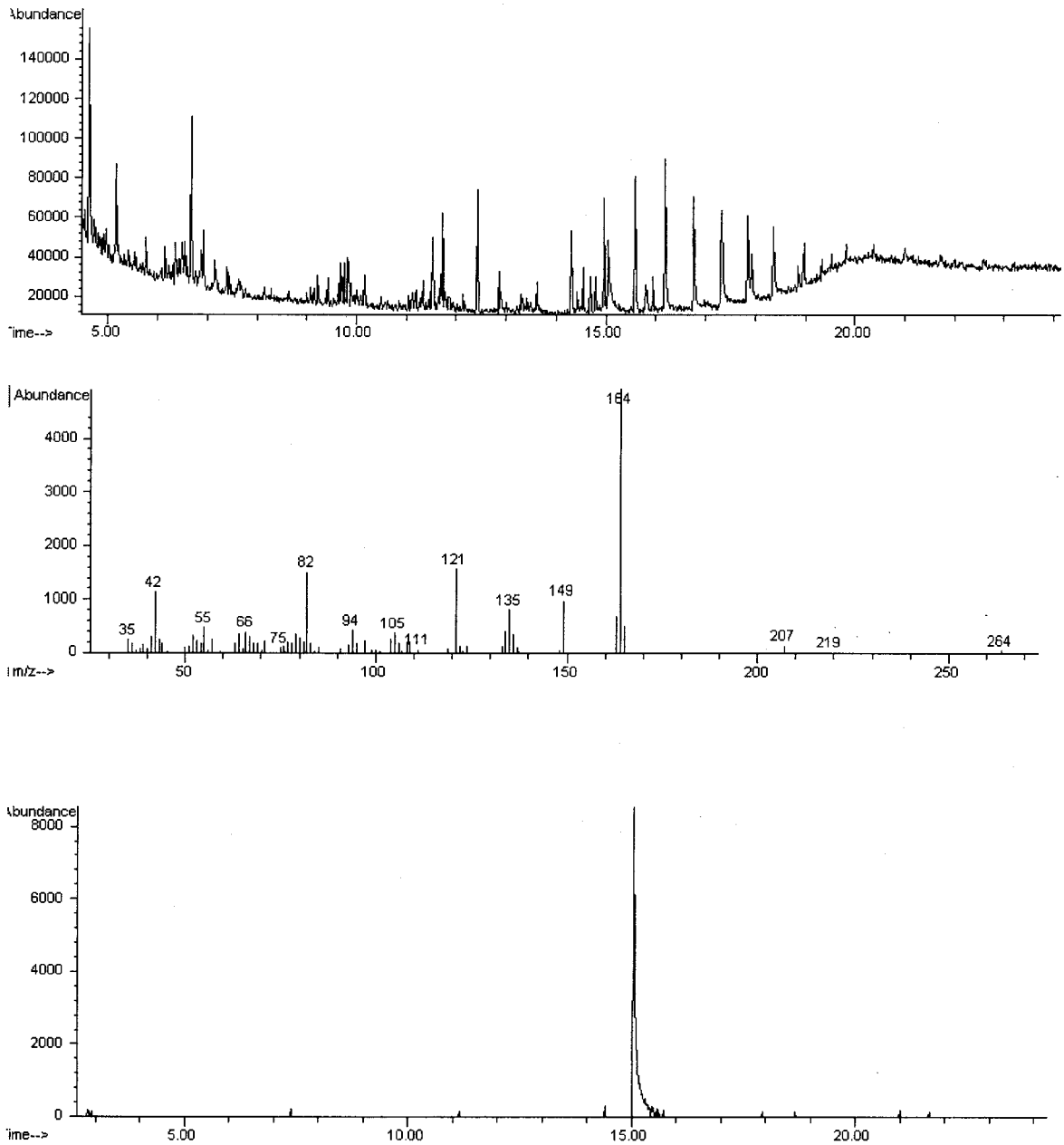

FIG. 6—*GC/MS analysis of a castor bean extract that was re-extracted overmight with chloroform. Upper panel: total ion current. Middle panel: Spectrum of ricinine in El mode. Lower panel: extracted ion current for m/z = 164 shows ricinine with an elution time of 15 min.*

mass spectral peaks were produced due to the presence of three heterogeneous sugar residues. In addition, these sugar residues were apparently not completely accessible, which inhibited enzymatic cleavage of the oligosaccharides. MALDI-TOF mass spectrometry was a more powerful tool for the determination of these difficult analytes, because it provided an intact $(M+H)^+$ determination rather than poorly defined multiply charged ions. The major problem associated with MALDI-TOF MS was the presence of low-molecular weight interferents that inhibited ionization of the larger proteins, including ricin. Two methods for cleanup of the extracts were developed. The first method is the fractionation of ricin using liquid chromatography. Using this method allowed a purified sample of ricin to be collected as it eluted from the column. The intact sample was analyzed by MALDI-TOF MS, then digested with trypsin and analyzed by MALDI. The peptides produced by tryptic digestion were correlated with known masses in a protein database. Ricin was presumptively identified in the crude extract from the database by correlating the molecular weight of the intact protein and matching of 23% of the expected tryptic fragments.

The second cleanup method for the castor bean extracts was molecular weight cutoff filtration. The low-molecular weight interferents were separated from the larger proteins by filtration through a 30 kDa membrane. Ricin from the crude sample after filtration was ionized more efficiently by MALDI and produced a spectrum that was consistent with that of the standard. MWCO filtration was a quicker method of purification than fractionation by LC. In addition, using MWCO filtration offered a ten-fold concentration of the sample, which provided a stronger mass spectrometric signal.

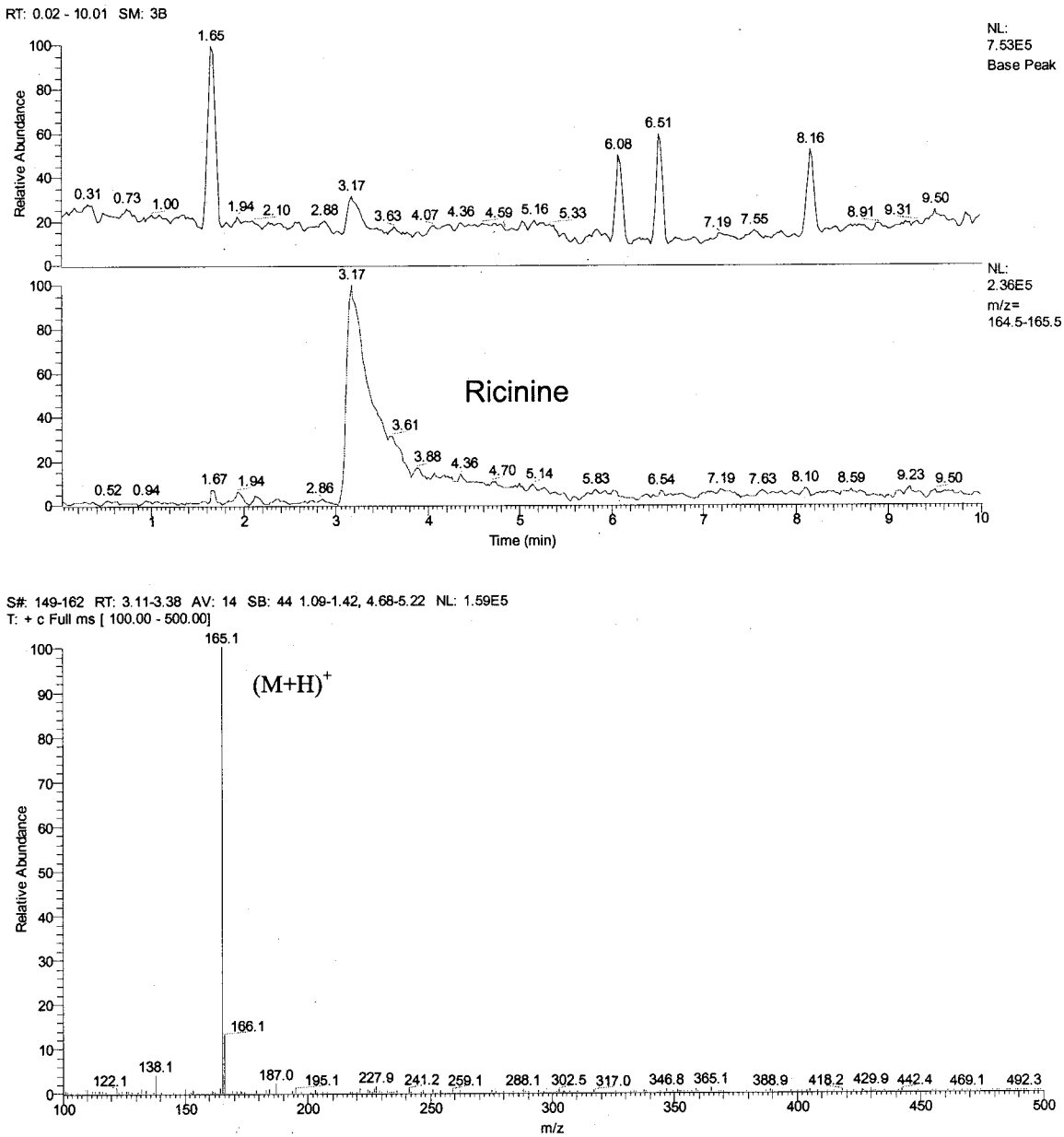

FIG. 7—*LC/MS analysis of a castor bean extract with a scan range of m/z 100 to 500. Ricinine elutes at 3.1 min with a spectrum at m/z = 165.1.*

A unique alkaloid, ricinine, was determined from the crude castor bean sample. GC/MS analysis of a chloroform solution of the extracted material produced a distinct EI spectrum for ricinine. A spectral standard was matched to ricinine in the extract. In addition, the alkaloid was concurrently analyzed by LC/MS under the same chromatographic conditions that were used for separation of ricin in the extract. Tandem mass spectrometry of the precursor ion at m/z 165 generated a major product ion of m/z 138. The determination of ricinine is complementary to the identification of ricin in a suspected castor bean sample, as the presence of both are unique to a common source.

Conclusion

The methods that were developed for the analysis of plant lectins are fundamental steps in the systematic approach for determination of biological toxins in complex matrices. Methods for general characterization of protein material could be accomplished using Fourier Transform Infrared spectroscopy (FTIR), and size determined by size exclusion chromatography (SEC) and MALDI-TOF MS as the first steps taken in the approach. Samples were purified by LC fractionation and molecular weight cutoff filtration before analysis by electrospray LC/MS and MALDI-TOF MS. Digestion of the proteins into characteristic fragments provided detailed information to support the unambiguous identification of the samples through protein database searches. Although ricin and abrin have almost identical intact molecular weights as determined by MALDI-TOF, the two toxins were distinguished due to differences in their amino acid sequences.

Specific enzymatic cleavage of each protein with trypsin produces unique fragments that can be correlated for the presumptive

identification of the intact toxins. Additionally, the analysis of unknowns with standard reference materials is a valuable correlation that supports this identification.

The methods developed in this work for the analysis of biochemical toxins provide specific analyses that are rapid and robust. Tedious steps such as gel purification, amino acid sequencing, and immunoassay are all avoidable with the development of a simple extraction and mass spectrometric analysis. Therefore, sample preparation time is greatly reduced. In addition, proteins that are still in their complex matrices can also be analyzed. The approach developed in this work has potential applications to a wide range of unknown toxic substances. The methods presented were applied to a forensic case sample that was originally processed using typical immunological methods. Data among each technique were consistent with one another and supported the identification of ricin.

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