

The Analysis of EDTA in Dried Bloodstains by Electrospray LC-MS-MS and Ion Chromatography*

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Abstract

Analytical methods were developed to determine the presence of ethylenediaminetetraacetic acid (EDTA) in dried bloodstains to provide probative information when allegations of evidence tampering have been made in criminal cases. A simple screening method using ion chromatography to analyze stains was found to be quantitative to the 5 ppm level. The presence of EDTA was then confirmed using negative and positive ion mode liquid chromatography-tandem mass spectrometry (LC-MS-MS) methods. A blind trial of these methods on 42 samples correctly determined the bloodstains that did and did not contain the preservative EDTA. One interesting observation in these results was the adsorption and postanalysis release of EDTA in the chromatographic system. In order to avoid cross contamination of samples resulting from this phenomena, it was found to be necessary to use EDTA-free blood extracts as blanks in the LC-MS analysis of bloodstains.

Introduction

The collection of blood at crime scenes and for legal proceedings is a common practice used to inculcate or exculpate individuals associated with evidentiary blood at crime scenes. Allegations of "planting" blood evidence from collected reference specimens has occurred in some criminal investigations, and this issue may be resolved by the determination of exogenous components that would not ordinarily be present in authentic crime scene evidence. Ethylenediaminetetraacetic acid (EDTA, also known as edetic acid, C₁₀H₁₆N₂O₈, molecular weight, 292.24) (Figure 1), a chemical commonly added to collected blood specimens, can be used to implicate the origin of a dried bloodstain as coming from this type of preserved specimen tube. The purpose of the EDTA in the tube is to prevent coagulation and enzymatic degradation by chelating metals in the

blood that behave as catalysts and/or cofactors.

EDTA-preserved blood tubes use the salt forms of EDTA: the disodium, dipotassium, or tripotassium salt. The concentration of EDTA in its free acid form in a drawn blood tube is 1000-2000 mg/L (ppm), depending on the volume of blood and the capacity of the tube. The free acid and salt forms are all water soluble at this concentration. EDTA is stable on storage and on boiling in aqueous solutions, but it does decarboxylate when heated to temperatures of 150°C (1). It is an excellent complexing agent and forms water-soluble chelates with nearly all heavy metals. Therefore, aqueous extractions of dried bloodstains should readily isolate EDTA in solution.

EDTA is used as a chelating agent in a variety of materials, and several chromatographic methods have been developed for its determination. A number of the methods employ reversed-phase ion-pair liquid chromatography (LC) for analysis of EDTA in foodstuffs (2,3), water (4,5), radioactive waste (6), and pharmaceuticals (7). Gas chromatographic (GC) methods have also been developed for the detection of EDTA in foodstuffs (3). Ion chromatography (IC) is an additional logical approach for the analysis of EDTA (6). All of the previously mentioned LC methods use ultraviolet detectors and lack the specificity of liquid chromatography-mass spectrometry (LC-MS). An additional level of selectivity in the analysis of EDTA can be added by the use of LC-MS-MS. A simple extraction technique coupled with positive ion and negative ion LC-MS-MS methods was developed for the analysis of EDTA in preserved dried bloodstains. Pneumatically assisted electrospray (ES) was used to ionize the chromatographic effluent before mass spectral analysis of the charged species. A secondary method using ion chromatography was developed to provide a quantitative presumptive test for the presence of EDTA as well as corroborate the results of the LC-MS-MS analysis. In a blind trial conducted on 42 bloodstains using the same extraction protocol for IC and LC-MS-MS, problems occurred in the LC-MS-MS analysis. Subsequently, a refined extraction method was developed for LC-MS-MS analysis of dried blood.

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Experimental

Chemicals

American Chemical Society reagent-grade ammonium hydroxide, disodium EDTA, cupric sulfate, and high-performance liquid chromatography (HPLC)-grade acetonitrile were purchased from Sigma Chemical (St. Louis, MO). HPLC-grade methanol was obtained from EM Science (Gibbstown, NJ). Certified sulfuric acid (2.5M) and sterile Vacutainer blood tubes without additives (red top) and with EDTA (K₃) (lavender top) were obtained from Fisher Scientific (Fairlawn, NJ). Nanopure water from a Barnstead (Dubuque, IA) water purification system was used for samples and mobile phases. The deuterated standard of EDTA-d₁₂ (Figure 1) was purchased from Cambridge Isotope Laboratories (Andover, MA).

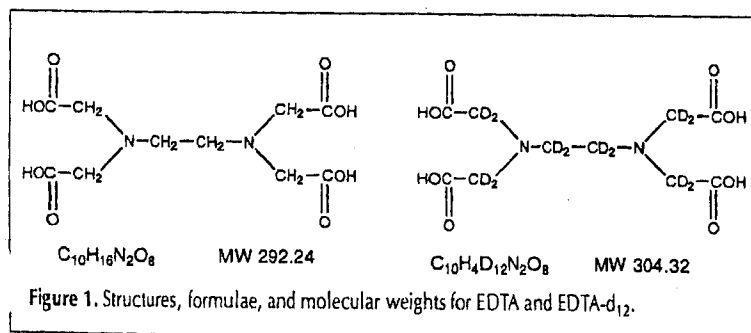
Instrumentation

Ion chromatographic analysis was carried out using a Waters 510 HPLC pump (Milford, MA) coupled to a Hamilton (Reno, NV) PRP X-100 ion chromatographic column. Sample detection was performed using a Spectroflow-773 tunable absorbance detector (Kratos Analytical Instruments, Westwood, NJ.) Instrument control and signal processing were performed using a Millennium 2010 chromatography manager (Waters, Milford, MA). Additional ion chromatographic analyses were performed using a Waters 600 analytical HPLC coupled to Waters 990 photodiode array detector.

The LC-MS-MS work was performed on a Hewlett-Packard (Palo Alto, CA) HP 1090 ternary LC with autosampler connected to a Finnigan MAT (San José, CA) TSQ 700 triple-stage quadrupole MS using a Finnigan electrospray interface. Argon was used as a collision gas for MS-MS. The instrument was set up to scan the mass range in 0.5–2 s. A flow rate of 0.3 mL/min was used on a Hamilton (Reno, NV) PRP-1 polymeric column (2.1 × 150 mm).

Procedure

Test samples were made by drawing whole blood samples into unpreserved and EDTA-containing tubes. The bloodstains were prepared on the same day by applying between 2 and 50 µL of unpreserved or EDTA-containing blood onto sterile cotton linen. Additional samples were prepared using liquid whole blood from a laboratory volunteer.



¹The web address for the ChemPuter is <http://www.shef.ac.uk/~chem/chemputer/isotopes.html>.

For the initial study, 25 to 50% of the stained area (up to 1/2 cm²) was cut out of the cotton swatch. The cutting was placed in 50 or 100 µL of 0.025M copper (II) sulfate (enough to cover sample). The samples were soaked in solution for 3 or more hours before vortex mixing and centrifuging at 3000–9000 rpm for 10 min. After passing the sample through a 0.2-µm nylon syringe filter, injections of 25 µL were made for IC analysis. The IC mobile phase was 3mM sulfuric acid/methanol (95:5). The flow rate was 2 mL/min with a detector wavelength of 254 nm. This wavelength was subsequently changed following analysis by a UV photodiode array detector that indicated the peak absorbance maximum for the copper/EDTA complex occurs at 243 nm. Following IC, the residual copper extract samples were diluted with 25 µL of water and 1-µL injections were made for positive ion LC-MS-MS. This preparation method was used for all IC analyses and for the initial run by LC-MS-MS of the 42 blind trial samples. Subsequent LC-MS-MS analysis samples were prepared by the procedure given in the next paragraph.

A different extraction procedure was developed for LC-MS-MS analysis to eliminate copper (II) sulfate, which caused arcing problems in the electrospray interface, from the extract. This procedure was used to prepare samples for both negative and positive ion LC-MS-MS but not IC analysis. A portion (up to 1/2 cm²) of the bloodstain was extracted by inserting the sample into Millipore (Bedford, MA) Ultrafree-MC centrifugal filters made of a polysulfone membrane (type PTTK) with a nominal molecular weight cutoff of 30,000 Daltons. After the addition of 25 µL of water, the sample was allowed to sit at room temperature for 45 min. The filter tubes were centrifuged for approximately 10 min, and the filtrate was collected for analysis.

Positive ion LC-MS-MS data were collected by scanning for product ions of (M + H)⁺ at 293 u from 128–296 u at a collision offset of –20 V. The interface was set for a spray voltage of 4 kV, a sheath gas pressure of 90 psi, and an auxiliary gas flow of 5 units. The interface capillary was maintained at 200°C. A mobile phase of acetonitrile and water (5:95) with 0.06% ammonium hydroxide was used.

Negative ion LC-MS-MS data were acquired by scanning for the 300 u product ion from the iron adduct of EDTA at 344 u. A scan window of 298–302 u was employed. The collision offset for selected reaction monitoring of the 44 u mass loss was 20 V. The interface was set for a spray voltage of 4.5 kV, a sheath gas pressure of 50 psi, and an auxiliary gas flow of 5 units. The heated capillary was set for 200°C. The optimized-column mobile phase for negative ion was found to be acetonitrile and water (80:20) with 0.03% ammonium hydroxide.

The isotopic pattern calculation was performed on the ChemPuter from the Department of Chemistry at the University of Sheffield, Sheffield, England¹. The experimental isotope pattern was calculated by adding six scans together and the same number of background scans were subtracted to obtain the result.

Results and

A review of graphic approach to IC analysis of copper or iron from this analysis sample derivation. Recent applicable separation out using the

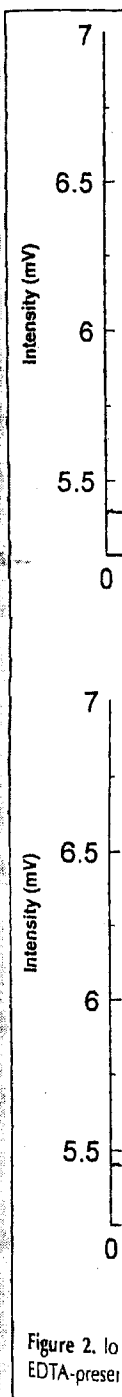


Figure 2. IC analysis of EDTA-preser

Results and Discussion

A review of the literature indicated that the best chromatographic approach for the determination of EDTA used HPLC or IC analysis of colored complexes formed between EDTA and copper or iron (2-6). Although procedures exist for performing this analysis by GC, these methods require time-consuming sample derivatization and are prone to matrix interferences (3). Recent applications published by Hamilton indicated acceptable separations of EDTA-copper complexes could be carried out using the ion exchange column PRP X-100 with sulfuric

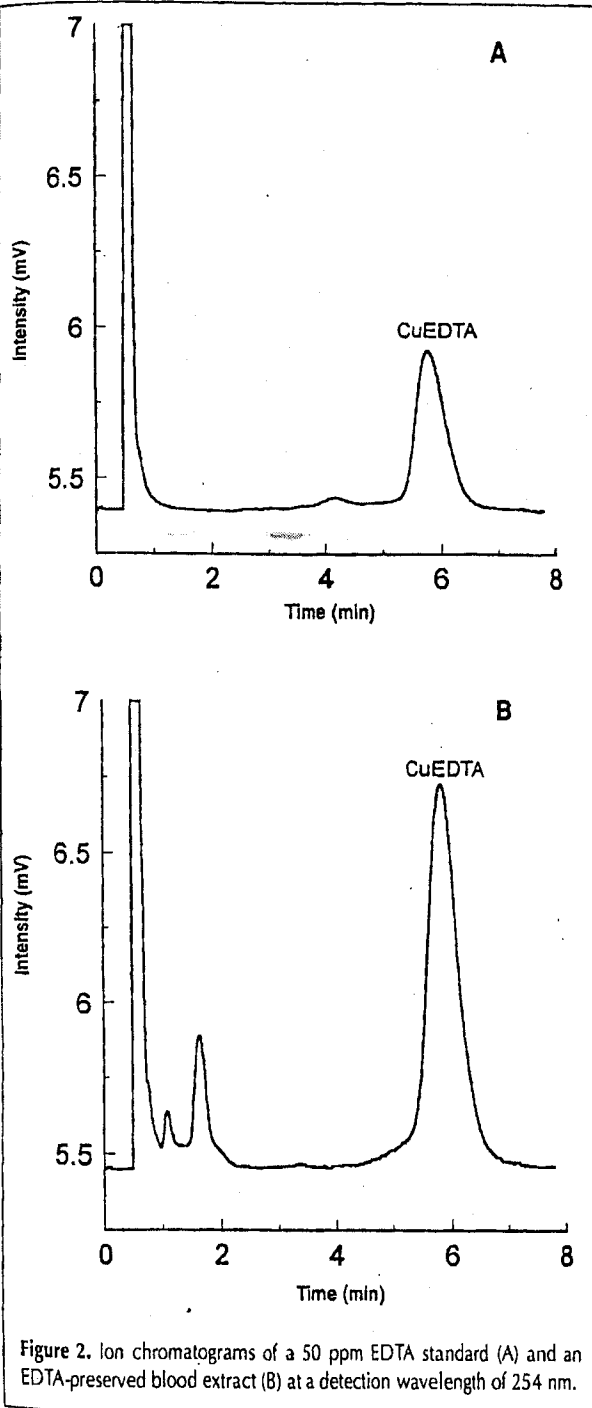


Figure 2. Ion chromatograms of a 50 ppm EDTA standard (A) and an EDTA-preserved blood extract (B) at a detection wavelength of 254 nm.

acid/methanol mobile phases (8). Initial IC testing with UV detection was carried out on standard samples of 50-100 ppm EDTA dissolved in 0.025M copper (II) sulfate. The results with this eluent system were encouraging; therefore, further tests were carried out on samples of liquid blood. IC samples were prepared from 200 μ L of whole blood (EDTA preserved) by first diluting it to 2 mL with water and then diluting the solution 1:1 with 0.05M copper (II) sulfate. This preparation was centrifuged for 7 min, which left a clear supernatant with a brown precipitate at the bottom. A large excess of copper (II) sulfate helped to ensure conversion of all free EDTA to the copper complex. The

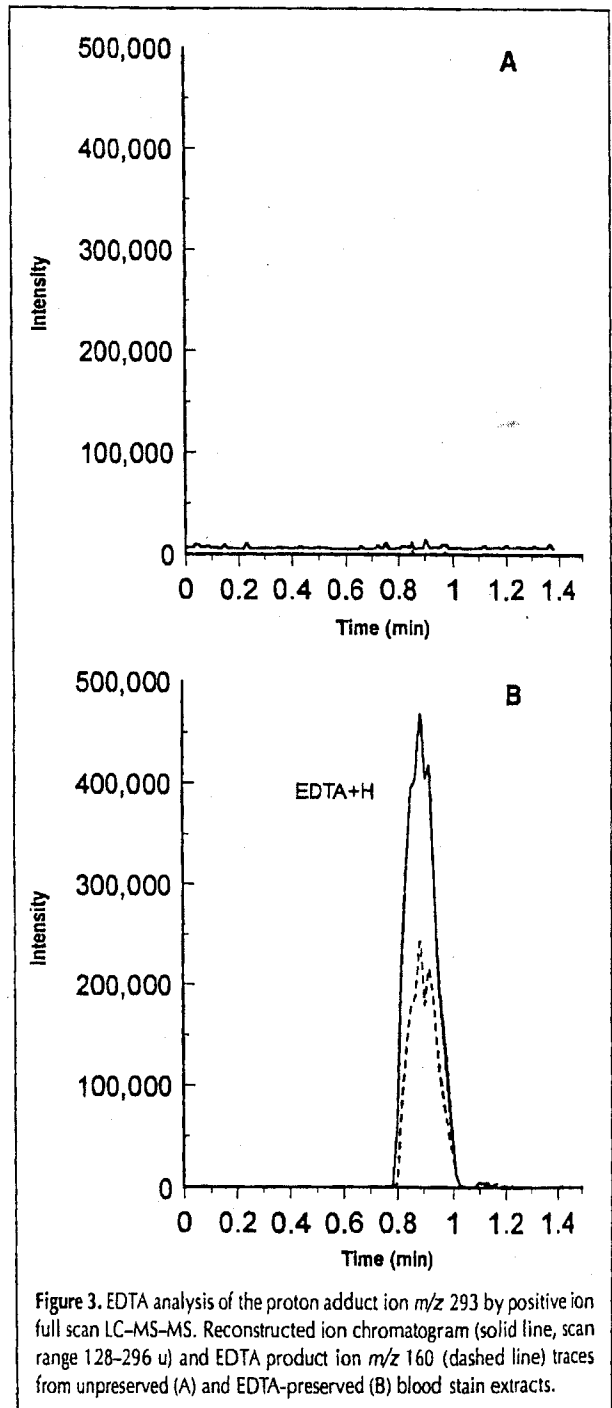


Figure 3. EDTA analysis of the proton adduct ion m/z 293 by positive ion full scan LC-MS-MS. Reconstructed ion chromatogram (solid line, scan range 128-296 u) and EDTA product ion m/z 160 (dashed line) traces from unpreserved (A) and EDTA-preserved (B) blood stain extracts.

result of this analysis is shown in Figure 2. Similar analyses using FeCl_3 and $\text{Fe}_2(\text{SO}_4)_3$ to complex with EDTA did not produce precipitates and showed large interfering peaks. As a result, all further tests were performed using Cu(II) sulfate at a concentration of 0.025M in each sample. In a set of serial dilutions in water, the analysis was shown to have a linear range from zero to greater than 500 ppm EDTA with a minimum detectable quantity of 5 ppm EDTA for the injected sample.

A Hamilton PRP-1 column was used to separate EDTA for MS detection in order to reduce interference from other compounds in the blood. Other blood components were retarded on the column and minimized peak overlap with EDTA, which has a short retention time. IC mobile and stationary phases were not used for the MS procedure because of the strong buffer ion concentrations required for ion exchange that led to electrical arcs in the electrospray interface. An additional reason

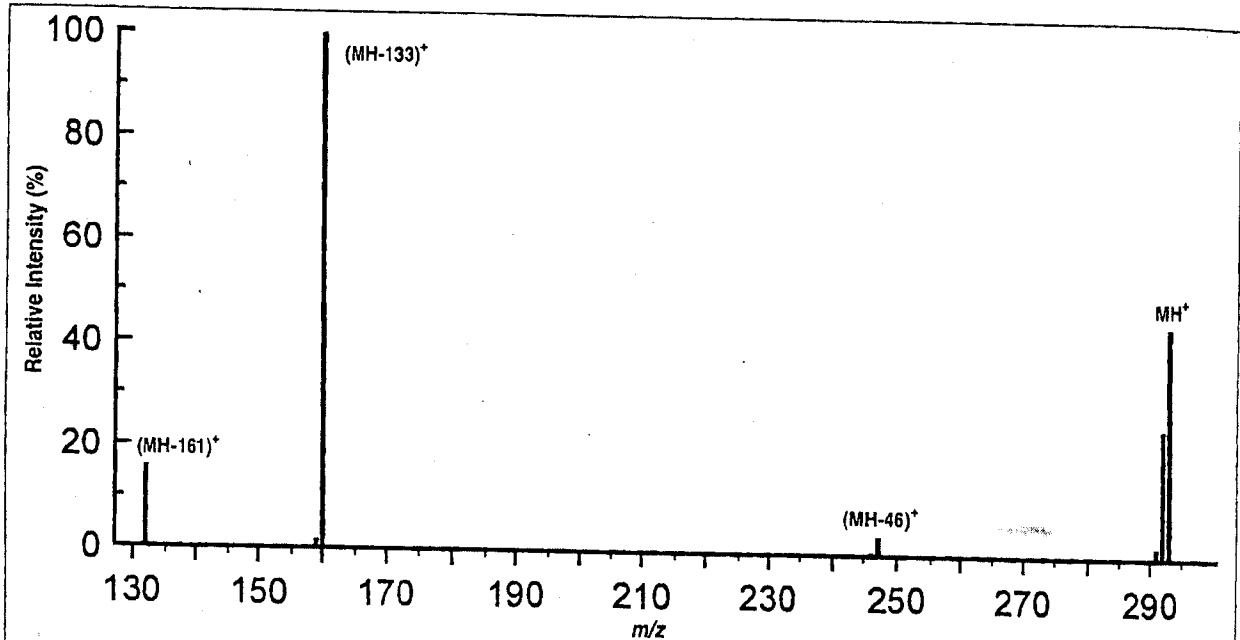


Figure 4. Positive ion LC-MS-MS product spectrum from collision-induced dissociation of EDTA ion $(M+H)^+$ at m/z 293.

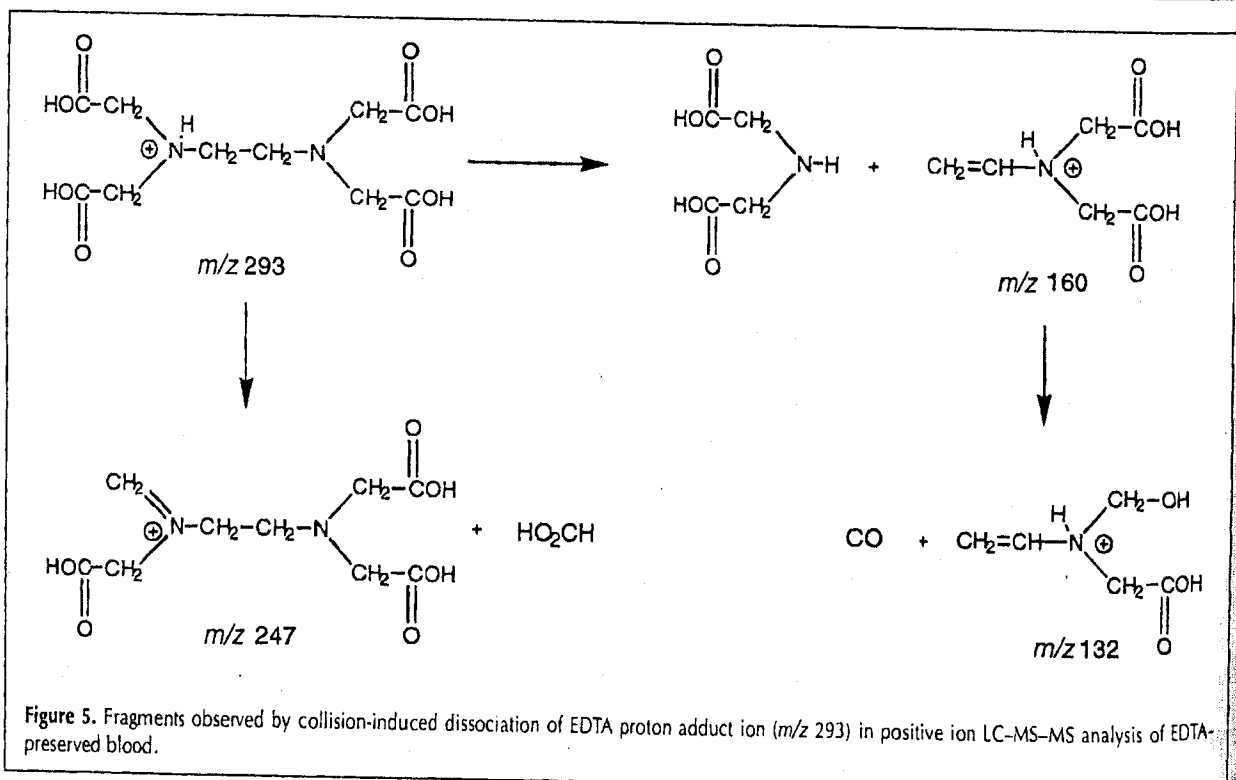


Figure 5. Fragments observed by collision-induced dissociation of EDTA proton adduct ion (m/z 293) in positive ion LC-MS-MS analysis of EDTA-preserved blood.

for keeping the ionic strength of the mobile phase minimized is higher ion concentrations may suppress analyte ionization (9).

Standard sample solutions (10–100 ppm) of disodium EDTA in water yielded abundant adduct ions by electrospray sample loop injection (flow injection analysis, FIA) or LC–MS in the positive ion mode. However, analyses of disodium EDTA with or without chromatography gave variable spectra as a result of the formation of numerous metal complexes with EDTA. The EDTA adduct ion ($M + H$)⁺ at m/z 293 was observed even though the mobile phase was alkaline. Other charged species observed in standard sample solutions and their proposed identities were as follows: m/z 315, ($M + Na$)⁺; m/z 317, ($M - 2H + Al^{III}$)⁺; m/z 337, ($M - H + 2Na$)⁺; m/z 346, ($M - 2H + Fe^{III}$)⁺; m/z 368, ($M - 3H + Fe^{III} + Na$)⁺; and m/z 390, ($M - 4H + Fe^{III} + 2Na$)⁺. The electrospray interface is constructed of stainless steel and aluminum and accounts for the presence of Fe^{III} and Al^{III} complexes in analyses of disodium EDTA. The electrolytic nature of electrospray has been shown to form iron complexes from the stainless steel spray needle (10).

Negative ion FIA–MS (sample loop injection) or LC–MS generated several ions with samples of disodium EDTA including ($M - H$)⁻ at m/z 291 and the doubly charged ion ($M - 2H$)⁻² at m/z 145. Adduct ions were also observed in negative ion mode with the same metals (Fe^{III} and Al^{III}) as positive ion mode. Complexed species indicated from FIA–MS or LC–MS and their proposed identities were: m/z 313, ($M - 2H + Na$)⁻; m/z 315, ($M - 4H + Al^{III}$)⁻; m/z 335, ($M - 3H + 2Na$)⁻; and m/z 344, ($M - 4H + Fe^{III}$)⁻. The negative ion spectra were also poorly reproducible in the relative intensity of the various ions for standard runs of disodium EDTA.

Positive ion LC–MS was also examined using a series of mixtures of acetonitrile and 0.06% ammonium hydroxide. The mobile phase (5:95) was selected as it gave the best response and consistency for EDTA analysis. This mobile phase gave ($M + H$)⁺ ions at m/z 293 for the disodium EDTA standard (10 to 100 ppm) and was the base peak for EDTA in preserved blood samples. No prominent fragment ions were observed in the spectrum of EDTA, and thus it was necessary to analyze samples by LC–MS–MS to obtain structurally significant ions for identification (Figure 3). In the blood samples, no interferences were found in the reconstructed ion (RIC) trace for the MS–MS of ion 293. MS–MS of the 293 ion generated three product ions with a base peak at 160 u and two smaller ions at

masses 132 and 247 u (Figure 4). The three-product ions (132, 160, 247) and their associated losses are consistent with the known EDTA- d_{12} spectrum which has product ions at m/z 140, 168, and 259, respectively (Figure 5). The neutral losses correspond to carbon monoxide, a di-carboxylic acid secondary amine, and formic acid.

A blind trial of the analysis procedures was performed independently by IC and by positive ion LC–MS–MS. Forty-two dried bloodstain extracts prepared for IC analysis were analyzed in the blind study to determine if EDTA preserved blood could be distinguished from unpreserved blood spots. Although the samples were diluted with 25 μ L of water before LC–MS–MS, some electrical arcs still occurred in the electrospray interface because of the copper (II) sulfate in the samples. The volume of the original bloodstain samples ranged from 2 to 50 μ L. Although all stains containing EDTA ($n = 21$) were correctly identified using the IC technique, LC–MS–MS correctly determined 20 of the 21 positive samples. Both techniques correctly identified all of the negative samples ($n = 21$). A stain sample that gave a positive result in the IC test had indications of EDTA by the LC–MS–MS procedure but was considered too weak to be called positive on a single result. This was an extract of half of the smallest EDTA blood spot (2 μ L) in the 42 samples (i.e., approximately 1 μ L). At the time of the testing all other positives gave significant area counts (several hundred thousand) for the 160 u product ion, and both 132 and 247 u ions were present. The false-negative result had an area count of 80,000 for ion 160, and both other product ions were present. A conservative decision was made to interpret the sample as negative until further testing could be completed.

A revised extraction method was developed for LC–MS–MS after the initial testing to eliminate the cupric sulfate-induced arcing problems in the interface and to obtain more concentrated sample extracts. A simple procedure was devised to extract the stains in centrifugal filters after a 45-min soaking in water. A molecular weight cutoff of 30,000 Da was chosen to remove particulate matter, blood cells, and large proteins from the filtrate while still maintaining an adequate flow through the filter disc. A retest by positive ion mode LC–MS–MS of several dried stains (preserved and unpreserved blood spots) produced positive results for all of the EDTA containing blood with area counts of several hundred thousand for the 160 ion and negative results for all unpreserved spots. The sample

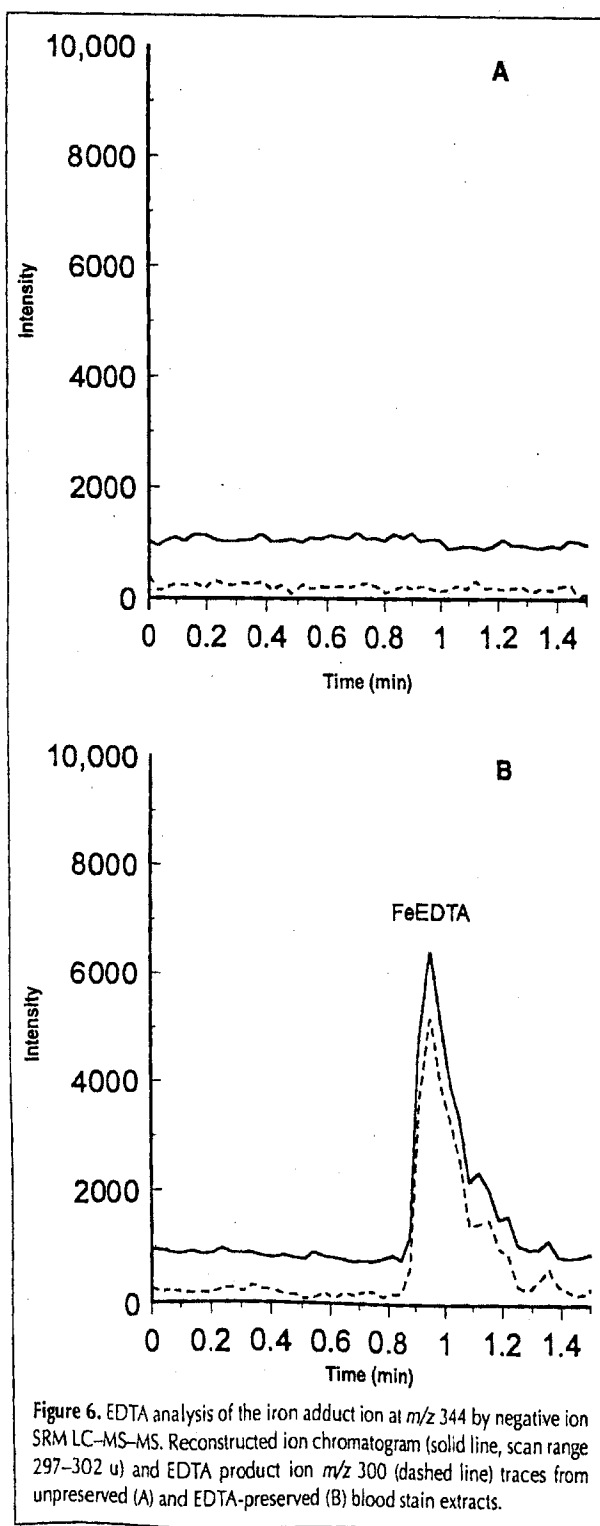
that previously had been deemed a negative (2- μ L EDTA blood spot) gave an area count of 1,000,000 for ion 160 by the revised method. Two of the positive samples were extracted a second time, and it was found that, on average, 91% of the EDTA response (peak area for 160 product ion) was in the first extract.

In the negative ion mode, the ferric ion complex with EDTA at m/z 344, ($M - 4H + Fe^{III}$)⁻, consistently appeared in analyses of the disodium EDTA standard and was the base peak for EDTA in preserved blood samples. The identity of the 344 ion was verified by comparing the calculated isotopic formula for ($C_{10}H_{12}N_2O_8Fe$) with experimental data (Table I). No prominent structural

Table I. Calculated and Experimental Intensity (%) of the Molecular Ion Cluster ($C_{10}H_{12}N_2O_8Fe$) for the Iron Complex with EDTA ($M - 4H + Fe^{III}$)⁻ Observed by Negative Ion LC–MS–MS for an EDTA Standard

m/z	Predicted intensity	Experimental intensity
342	6.3	6.4
343	0.8	2.1
344	100	100
345	14.7	14.7
346	2.9	2.1
347	0.3	0.6
348	0	0.2

peaks were observed in the LC-MS spectrum of this complex. MS-MS of the 344 ion gave a strong signal for the product ion at m/z 300 ($M - CO_2$). A second LC-MS-MS method, in negative ion mode, was developed to screen for the presence of EDTA in bloodstains by observing the mass-to-charge ratio transition from 344 to 300 in the selected reaction monitoring mode (SRM). This method of MS-MS operation is selective



and sensitive because the instrument scans over a limited mass range while measuring a specific transition resulting from collision-induced dissociation. The MS-MS ion trace of m/z 300 and the RIC show no extraneous signals other than the major peaks for the EDTA derived species from blood extracts (Figure 6). Some known ($n = 4$) and unknown ($n = 2$) blood samples were successfully tested as a trial of the negative ion method.

A comparison of negative ion and positive ion LC-MS-MS of a blood stain revealed an 80-fold difference in intensity of EDTA signal for the 160 ion (positive) over the 300 ion (negative). Because of the use of MS-MS in both techniques, responses from other components in the blood were not observed, and, therefore, both analyses were deemed selective. The positive ion mode is a better means of confirmation because there are three structurally significant product ions compared with only one for the negative ion mode.

The IC, negative ion and positive ion LC-MS-MS methods were used on investigative case samples ($n = 9$) to determine if evidentiary crime scene blood might have been tampered evidence. A phenolphthalein-presumptive test for blood (11) was positive on all tested stains ($n = 4$). In addition, all extracts of the stains appeared red in color as supportive evidence for the presence of blood. All three techniques were negative for EDTA in the bloodstains. However, in the positive ion SRM (m/z transition 293 to 160) LC-MS-MS analysis of one of the stains from a sock, a small peak appeared with the correct retention time for EDTA. IC and negative ion results did not indicate EDTA in the sample and positive ion full scan LC-MS-MS could not confirm all three EDTA product ions from the parent ion at 293 u. In known EDTA samples, the three techniques had consistently agreed, and intensities were strong enough for the known samples to confirm the three product ions by MS-MS of the parent ion at 293 u. A potential criticism of the evaluation of the results is the inability to determine the exact quantity of blood in a sample. In these studies, it was found that sample sizes as small as 1 μ L of blood generated more than adequate signal for EDTA. A sample of this size would leave a dried blood spot of 0.1 cm^2 on a swatch of cotton linen.

A study was conducted to determine if the small unconfirmed signal observed in the case sample could result from a carry-over in the system. An EDTA- d_{12} standard was used for this work to prevent interference from endogenous levels of EDTA. After several injections of the EDTA- d_{12} standard (500 ppm), blank samples of different substances were run. No signal by LC-MS-MS was observed after a single injection of blanks such as water, mobile phase, or salt solutions. However, injection of EDTA free blood extracts, following a blank injection, gave a response for the EDTA- d_{12} compound that decreased with each repetition (Figure 7). The low signal in the case mentioned above was likely a result of an interference resulting from the previously injected standard. It is theorized that, when a blank blood matrix is injected onto the system, residual EDTA adsorbed onto sites in the column and tubing is released by competing metal ions in the blood extract. Therefore, it is recommended that the only suitable blank after injections of EDTA is EDTA-free blood extract. Multiple injections of EDTA-free blood extract should be made until no observable

EDTA peak is detected before any case samples are analyzed by this method.

EDTA is an additive in a variety of foods, such as pickles, canned mushrooms, and salad dressings, and potentially could occur in human blood at low levels. However, in a study of radio-labeled EDTA, ingested samples were found to be eliminated mainly by excretion with minimal gastrointestinal tract

absorption (12). Thus, it is unlikely that measurable quantities would be found in the blood by the employed techniques. Attempts to measure EDTA in unpreserved blood by the use of samples up to 1 mL in volume were negative. A search of the literature did not find any measurements of EDTA in blood from dietary consumption levels.

Another concern in the analysis of bloodstains is the stability of EDTA in the dried spots after extended periods of storage. Samples of EDTA preserved bloodstains ($n = 2$) were analyzed after 2 years of storage at room temperature. LC-MS-MS analysis of the additive-free samples were negative and the preserved samples were positive for EDTA.

Conclusion

Methods described herein demonstrate the ability to determine if tampering of bloodstains may have occurred using EDTA-preserved blood. The coupling of a quantitative technique with the specificity of the LC-MS-MS procedures is a powerful analytical protocol. Specific procedures were developed to identify and control the effect of matrix interference on the samples. Matrix interference can be minimized by injecting EDTA-free blood extracts before the analysis of any samples. The accuracy of the determination was supported through the use of simulated investigative case samples. Experiments on aged, dried blood indicate it is possible to determine EDTA in stains after at least 2 years of storage.

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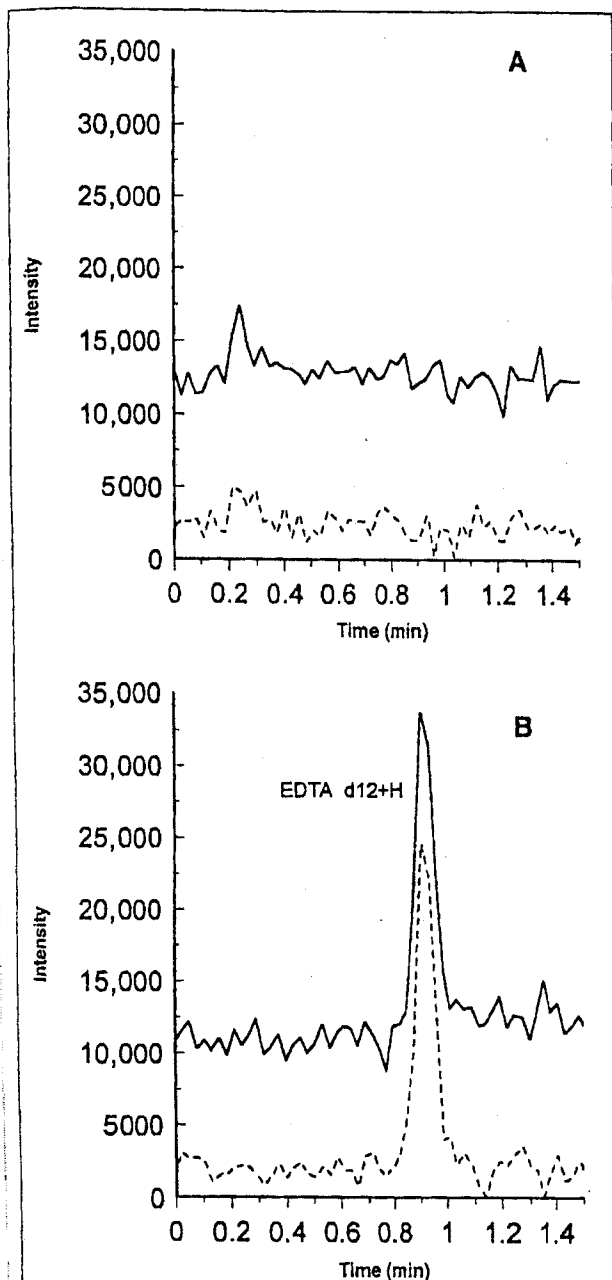


Figure 7. Sample carry over experiment to demonstrate the matrix effect of blood extracts. The top chart (A) is an injection of mobile phase after five injections of 500 ppm EDTA- d_{12} standard. The chart on the bottom (B) shows the results of injecting an extract from a non-EDTA-preserved blood tube after the blank analysis in (A). EDTA- d_{12} analysis of the proton adduct ion m/z 305 by positive ion full scan LC-MS-MS. Reconstructed ion chromatogram (solid line) and EDTA- d_{12} product ion m/z 168 (dashed line) traces.

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