J Forensic Sci, Sept. 2005, Vol. 50, No. 5
Paper ID JFS2005074
Available online at: www.astm.org

CASE REPORT

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The Determination of Nicotine and Sulfate in Supermarket Ground Beef Adulterated with Black Leaf 40*

ABSTRACT: In December 2002, approximately 250 lbs. of ground beef was adulterated with nicotine sulfate by a supermarket employee and subsequently sold to the public. Soon afterward, reports of illness associated with ground beef purchased at a single store were identified. Authorities suspected the ground beef was tainted with Black Leaf 40, a banned pesticide containing approximately 40% nicotine as nicotine sulfate. Ground beef submitted to our laboratory was analyzed in concert by high performance liquid chromatography-ultraviolet detection (HPLC-UV), gas chromatography-mass spectrometry (GC-MS), and high performance anion exchange chromatography with suppressed conductivity detection. GC-MS was used to identify the samples that contained nicotine. The nicotine was confirmed and quantitated by HPLC-UV. The sulfate was identified and quantitated by high performance anion exchange chromatography with suppressed conductivity detection. Our analysis revealed that the raw tainted beef contained about 350 mg/kg nicotine free base, a potentially lethal dose of nicotine per serving for an adult. Additionally, we found elevated sulfate levels in the samples that tested positive for nicotine, providing evidence that nicotine sulfate was the probable adulterant.

KEYWORDS: forensic science, food tampering, Black Leaf 40 insecticide, nicotine sulfate, ground beef, tissue analysis, high performance liquid chromatography-ultraviolet detection, gas chromatography mass spectrometry, high performance anion exchange chromatography

One of the largest incidents of domestic food tampering occurred in Byron Center, Michigan in late December, 2002, and early January, 2003. A cluster of food poisonings was identified and linked to raw ground beef sold from a single supermarket (1). In the course of the investigation, it was determined that the beef was intentionally adulterated with Black Leaf 40, a nicotine sulfate-based insecticide. At least 111 people from 36 families in the surrounding counties were sickened by the meat, experiencing intense burning in the mouth, nausea, vomiting, and dizziness (1,2). Four individuals sought medical attention: two from personal physicians, and two were evaluated and treated in hospital emergency departments. Although laboratory analysis determined that a quarter pound of the raw ground beef contained a potentially lethal dose of nicotine (2), no deaths were associated with this incident (3).

A supermarket employee ultimately plead guilty to dumping a portion of a bottle of Black Leaf 40 insecticide into approximately 250 lb. of raw ground beef as it was being mixed, ground, and wrapped into 1 to 3 lb. packages for sale (2). Black Leaf 40 is an aqueous-based insecticide containing nicotine sulfate with a nominal nicotine content of 40% as the free base. The product registration for Black Leaf 40, allowing its sale on the consumer market in the United States, was canceled in 1992 by the U.S. Environmental Protection Agency due to its toxicity (4).

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* A portion of this work (sulfate analysis) was presented in poster form at the 16th Annual International Ion Chromatography Symposium, San Diego, CA, 2003.

Received 18 Feb. 2005; and in revised form 2 May 2005; accepted 7 May 2005; published 3 Aug. 2005.

Prior to submission to our laboratory, samples of the adulterated ground beef were analyzed by two other laboratories (one private lab and one hospital-based lab). The labs found nicotine at or near a level of 300 mg/kg (0.3 mg/g) in the raw beef. Although nicotine was identified as the toxic agent in the beef, USDA investigators wanted an independent analysis from a government-based forensic laboratory to determine if the source of the nicotine could have been the Black Leaf 40 insecticide. The original container of Black Leaf 40 used in the criminal incident was not available for examination or analysis. The items of evidence received by our laboratory comprised three types related to the investigation: (1) ground beef items which were sought by investigators because they were known to have originated from the adulterated batch; (2) ground beef items which were returned by the consumers during the recall phase which may or may not have originated from the tampered batch; (3) control ground beef samples which were known to be unadulterated.

We report here on the concurrent determination of nicotine and sulfate in the ground beef samples. Special care was taken to adequately sample the ground beef evidence in order to assess the degree of homogeneity of the nicotine adulterant throughout the meat. Background levels of sulfate were determined in locally purchased, unadulterated ground beef samples to provide a baseline reference for comparison to the tainted samples.

Materials and Methods

Standards and Chemicals

Nicotine hydrogen tartrate (Catalog No. N-5260), nicotine sulfate aqueous solution (Catalog No. N-4001, nominally 48% nicotine sulfate, 37% nicotine free base), citric acid monohydrate (ACS reagent), 1-octanesulfonic acid sodium salt (ca. 98%), and

TABLE 1—*Summary of ground beef items of evidence with results.*

Item No.	Description	Average Nicotine* Content (mg/kg) HPLC-UV	Nicotine ID GC-MS	Average Sulfate† Content (mg/kg) IC
1	2 patties (SUSPECT)	ND	ND	28 (29,27)
2	large roll ground beef, unopened, dated for sale Mar 27 (CONTROL)	ND	ND	*NA*
3	1/2 opened package ground beef, dated for sale Jan 1 (SUSPECT)	390 (400,380)	DET	230 (240,220)
4	1/4 opened package ground beef, dated for sale label missing (SUSPECT)	320 (320,330,320)	DET	100 (98,110)
5	2 self-seal plastic bags ground beef (SUSPECT)	ND	ND	*NA*
6	13 individually wrapped patties (SUSPECT)	ND	*NA*	*NA*
7	1 self-seal plastic bag ground beef (SUSPECT)	300 (280,320)	DET	*NA*
8	unopened package ground beef, dated for sale Mar 27 (CONTROL)	ND	*NA*	32 (32,32)
9	3/4 opened package ground beef, dated for sale Jan 2 (SUSPECT)	360 (360,360)	DET	100 (99,104)
10	3/4 opened package ground beef, dated for sale Mar 27 (CONTROL)	ND	ND	32 (33,31)
11	1 plastic bag ground beef (SUSPECT)	ND	ND	26 (26,26)
12	cooked ground beef crumbles (SUSPECT)	200 (190,210)	DET	*NA*
14	1 self-seal plastic bag ground beef (SUSPECT)	370 (390,360)	DET	120 (120,110)
16	1 cooked meat loaf slice (SUSPECT)	160 (170,140,180)	DET	*NA*

Note: ND: not detected; DET: detected; *NA*: sample not analyzed by this technique.
*Nicotine content reported as free base. All results are the average of 2 or 3 trials, individual results reported in parantheses.
† Sulfate content results are the average of 2 trials, individual results reported in parantheses.

sodium carbonate (Catalog No. 22,353-0) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Concentrated hydrochloric acid (ACS reagent) and methylene chloride (Ultra Resi-Analyzed) were obtained from JT Baker (Phillipsburg, NJ). Hexane (Catalog No. H303), sodium hydroxide (Catalog No. S612), sodium hydroxide (50% w/w in water), and sodium sulfate (ACS reagent) were obtained from Fisher Scientific (Fairlawn, NJ). A certified mixed anion standard containing 20 ppm fluoride, 30 ppm chloride, 100 ppm nitrate, 150 ppm phosphate, and 150 ppm sulfate in water was obtained from Inorganic Ventures, Inc. (Lakewood, NJ). Reagent water (>18 MΩ*cm) was obtained using a MilliQ system (Millipore, Billerica, MA). *Note:* The nicotine sulfate aqueous solution was assayed by our laboratory at 39.7% w/v free base nicotine based on the nicotine hydrogen tartrate standard (used as received).

Sampling of Ground Beef Evidence

Sixteen items of evidence, detailed in Table 1, including 12 raw ground beef items, in varying forms, and 2 cooked ground beef items were submitted to our laboratory in March, 2003. The raw ground beef items included six packages of ground beef in the original supermarket labeling, ranging in weight from 0.7–14 pounds. Three of the supermarket labeled packages were provided as control samples, and consisted of ground beef dated for sale in a time frame (March 27, 2003) much later than the adulteration incident. All other items of evidence were considered suspect samples enumerated as: two supermarket labeled packages dated for sale in the time frame of the adulteration (January 1 or 2, 2003), one supermarket labeled package in which the dated for sale label was missing; four packages of raw ground beef in various other package types (e.g., self-seal plastic bags), ranging in weight from 0.3 to 3.5 pounds; and two items comprising groupings of individually wrapped raw hamburger patties. The two cooked ground beef items (Items 12 and 16) consisted of ca.1 pound of crumbled meat and a ca. 0.2 pound slice of meatloaf. The last two of the sixteen items comprised grease drippings and charcoal grill scrapings (Items 13 and 15), and were not analyzed. All items of evidence were received frozen and were thawed in a refrigerated environmental chamber (4°C) prior to sampling.

To determine if the nicotine was distributed evenly throughout the ground beef, the initial sampling was made using duplicate 3 g portions for the raw ground beef and cooked ground beef items. Samplings were made from opposite ends or opposing positions, and the sampling positions were documented. For patty items, 3 g portions of two or three separate patties were sampled. For subsequent analyses, three to four additional samplings were made from selected items for nicotine spiking and limit of quantitation experiments, again documenting the sampling locations. For sulfate analysis, initial samplings were made using 20 g portions of six selected items. Additional samplings were made by taking 50–60 g portions of eight selected items, and subdividing these into two 25 g portions for analysis.

Extraction Procedure for HPLC-UV Determination of Nicotine Content

A 3 g portion of ground beef was accurately weighed into a polypropylene centrifuge tube. Then, 30.0 mL of 0.1 N HCl was added and the tube was capped and vortexed, causing the beef pieces to disperse. The tube was subsequently sonicated (15 min) and centrifuged (Fisher Marathon 21000 R, 10 min, 4500 rpm, 2270 RCF). For removal of fat, a 5.0 mL aliquot of the acid extract was transferred to a 20 mL glass scintillation vial, 5.0 mL of methylene

chloride was subsequently added, and the vial was capped and vortexed. After vortexing, the phases were allowed to separate, and ca. 1.0 mL of the aqueous acid layer was withdrawn using a polypropylene syringe and filtered (2 stage membrane: nylon/glass microfiber, 25 mm, 0.45 μm) for HPLC-UV analysis.

Additional Sample Preparation for GC-MS Identification of Nicotine

A second ca. 1.0 mL portion of the organically washed acid extract was taken and filtered (see previous section) into a 4 mL glass vial. The filtered extract was made basic with ca. 30 μL of 6.7 M NaOH, the vial capped and vortexed. The pH of the solution was checked with pH paper (pHydrion 1–12) and additional aliquots of base were added as necessary, to give a pH of ≥12. To back extract the free base nicotine, a 1.0 mL portion of hexane was subsequently added. After vortex mixing, the phases were allowed to separate. A portion of the hexane layer (top) was taken for GC-MS analysis.

Due to residual fat, approximately half of the samples congealed with the initial addition and vortexing with hexane. For all but two of these samples, an additional centrifugation step (10 min, 2270 RCF) allowed enough phase separation for a liquid portion of the hexane layer (ca. 100 μL) to be removed and analyzed. Another 1.0 mL of hexane was added to these latter two samples, but they remained semi-solid even after a second centrifugation and were not analyzed by GC-MS (Items 6 and 8 in Table 1, tested negative for nicotine by HPLC-UV). The authors believe these samples had a high fat content, and that additional organic washes (methylene chloride) in the initial sample preparation for HPLC-UV analysis may have alleviated this problem.

Extraction Procedure for Ion Chromatographic Determination of Sulfate Content

Twenty-five gram portions of ground beef were lyophilized (Lab Conco, Kansas City, Mo., Freeze Dry/Shell Freeze Freezone 6 operated in Automatic Mode) and ground with a mortar and pestle. One-gram portions of the resulting lyophilized beef were accurately weighed and mixed with about 10 g of a 1 mM sodium hydroxide solution followed by sonication (15 min) and centrifugation (10 min, 4500 rpm). To remove the fats/proteins, 3.0 mL of the resulting aqueous extract was added to 3.0 mL methylene chloride and vortex mixed. The mixture was then centrifuged to aid in the separation of the aqueous layer. A second organic wash was performed as above with 1.0 mL methylene chloride. The resulting aqueous extract was transferred to a 5.0 mL volumetric flask and diluted to the mark with high purity water. A portion of the resulting solution was filtered through a 0.45 μm nylon syringe filter (25 mm, Alltech, Deerfield, IL) for analysis by ion chromatography.

Equipment and Conditions for Nicotine Analyses

The high performance liquid chromatography ultraviolet detection (HPLC-UV) conditions for nicotine determination were adapted from a previous study (5). An Hewlett Packard Series 1100 Liquid Chromatograph with a diode array detector (HP G1315A) was used for all analyses. The analytical column was a C_{18} Synergi Max-RP (4 μm, 4.6 mm × 250 mm), with the column compartment thermostatted at 40°C. The mobile phase comprised 48:52 buffer: methanol at a flow rate of 0.8 mL/min. The buffer was 50 mM citric acid, 50 mM sodium octane sulfonate, adjusted to pH 3.0. The injection volume was 25 μL with a run time of 15 min. Detection was at 260 nm, with online UV spectra collected from 200 nm to 400 nm. Standard calibration curves were generated using 0.1 N HCl solutions of nicotine hydrogen tartrate in the range 4–140 μg/mL (values as free base nicotine).

Gas chromatography mass spectrometry (GC-MS) analysis was used for identification and confirmation of nicotine. An Agilent 6890N Series Gas Chromatograph with 5973N Mass Selective Detector was used for all analyses. The GC column was a Restek Rtx-5MS (nominal length 35 m, ID 0.25 mm, film thickness 0.25 μm) with 5 m Integra Guard. Operating parameters consisted of constant pressure (12 psi) with a nominal carrier gas flow (helium) of 1.0 mL/min, and splitless injection (injection volume 1 μL, injector port temperature 250°C). The oven program was held at 75°C for 1.0 min, ramped at 10°C/min to 300°C; and then held at 300°C for 10 min (total runtime 33.5 min). Mass spectra were obtained in full scan mode (acquisition mass range 40–450 amu) with a 5 min filament delay. The transfer line temperature was 280°C, and the source and quadrupole temperatures were 230°C and 150°C, respectively. A nicotine standard was prepared by mixing 30 μL of a stock solution of nicotine hydrogen tartrate in methanol with base (30 μL 6.7 M NaOH) and hexane (940 μL, final nicotine free base concentration 40 μg/mL).

Equipment and Conditions for Sulfate Analyses

A Dionex DX-500 Chromatography system consisting of a GP-50 gradient pump, an LC-20 chromatography enclosure, an ED-40 conductivity detector equipped with a DS3 conductivity cell, ThermoSeparations AS3500 autosampler with Peaknet Version 5.21 software for instrument control, data acquisition, and calculation was used. A Dionex IonPac AS9-HC analytical column and guard were used with a 9 mM Na_2CO_3 mobile phase flowing at 1.3 mL/min and an injection volume of 25 μL. Suppressed conductivity detection was done with an ASRS-ULTRA I (current = 100 mA) in recycle mode. Standard calibration curves were obtained in the range of 3 μg/mL to 150 μg/mL using the mixed anion standard diluted in reagent water.

Nicotine Spike/Recovery and LOQ Experiments

For spike/recovery experiments, an aqueous spiking solution of nicotine sulfate (nominal concentration 4.0 mg/mL nicotine free base) was prepared by dilution of the concentrated nicotine sulfate solution (nominally 40% nicotine free base). Based on the assay results, two ground beef items (Items 4 and 16) which previously tested positive for nicotine were selected for additional assay work (one additional 3 g portion) and spike/recovery experiments (two additional 3 g portions). Aliquots of the nicotine sulfate spiking solution (4.0 mg/mL) were deposited onto the ground beef portions to provide an additional nicotine content (320 or 130 mg nicotine per kg ground beef) which was close to the assayed amounts. For LOQ (limit of quantitation) experiments, an item which previously tested negative for nicotine (Item 10) was spiked at ca. 20, 10, and 5 mg nicotine per kg ground beef (three separate 3 g ground beef portions), and an unspiked 3 g portion of ground beef was also analyzed. After spiking, all samples were thoroughly mixed with a glass rod to homogenize the nicotine and beef, and then prepared for HPLC-UV nicotine determination as previously described.

Sulfate Spike/Recovery

For sulfate spike/recovery experiments, an aqueous spiking solution of sodium sulfate (nominal concentration, 7020 μg/mL SO_4^{2-}) was prepared from solid sodium sulfate. To 25 g portions of ground

beef, 1.0 mL sodium sulfate spiking solution was added for a final concentration of approximately 280 mg/kg. The spiked portions were thoroughly mixed and allowed to stand for about 2.5 h. All spiked samples were then lyophilized and further prepared as detailed above for the unspiked portions.

Results and Discussion

Nicotine Detection and Determination

Nicotine is a naturally occurring alkaloid for which the free base form is miscible with water in all proportions, and is also soluble in both polar and nonpolar organic solvents (6). Salt forms of nicotine also have high water solubility. The major components of raw ground beef are water (in the range 50–75%), fat (in the range 5–30%), and protein (ca. 14–21%) (7). In general, cooking of ground beef reduces the water and/or fat content, causing a corresponding increase in the protein content (the protein content for cooked beef is in the range 25–30%). However, the retained fat content of cooked ground beef is still fairly high, in the range 5–20% (7).

In our laboratory's experience with extracting nicotine from a variety of polar and nonpolar food and plant matrices (8–10), we have obtained maximum recovery using acidic aqueous extracting solvents such as 0.1 N HCl. An obvious question at the outset of this work was whether the nicotine would have become bound up in the meat tissue. Given the high water content of ground beef, and the fact that meat products are weakly acidic, it was postulated that the nicotine would remain unbound and solubilized. However, any attempt to extract the nicotine with aqueous-based or organic solvents would result in interference from dispersed or solubilized fat. We elected to use an aqueous acidic extract to maximize the original recovery of nicotine from the sample matrix, while minimizing the amount of fat that was coextracted. Centrifugation of the acid extract prior to the methylene chloride wash facilitated separation of the liquid extract from the dispersed meat tissue. This was followed by a methylene chloride wash to further reduce the fat interference. Filtration of the acid extract after the methylene chloride wash appeared to reduce the residual dispersed fat content even further.

The washed and filtered aqueous acid extract was suitable for nicotine quantitation using HPLC-UV analysis. Figure 1 shows a comparison of typical chromatograms obtained for a nicotine-free ground beef sample (A) and a nicotine tainted ground beef sample (B). The nicotine-free sample did not have any significant interferences at the retention time of nicotine, facilitating nicotine detection and quantitation in the nicotine containing chromatograms. In order to obtain mass spectral confirmation (GC-MS), a portion of filtered extract was made basic and the nicotine was back extracted into hexane. At this stage, any residual fat would also be expected to back extract into the hexane. Resulting total ion chromatograms obtained for a negative (A) and a positive (B) ground beef sample are shown in Fig. 2. Positive identification of nicotine was based on a retention time and mass spectral match of the sample with a nicotine standard and additionally, a library mass spectral match (Wiley 7th edition). As previously discussed (see Materials and Methods section), residual fat precluded GC-MS analysis for 2 of the 14 items analyzed due to congealing of the fat in the hexane layer. As the particular items in question (Items 6 and 8, see Table 1) tested negative for nicotine by HPLC-UV, these items were not reanalyzed.

Nicotine was detected and quantitated in seven of the fourteen items tested, and was not detected in the remaining seven items

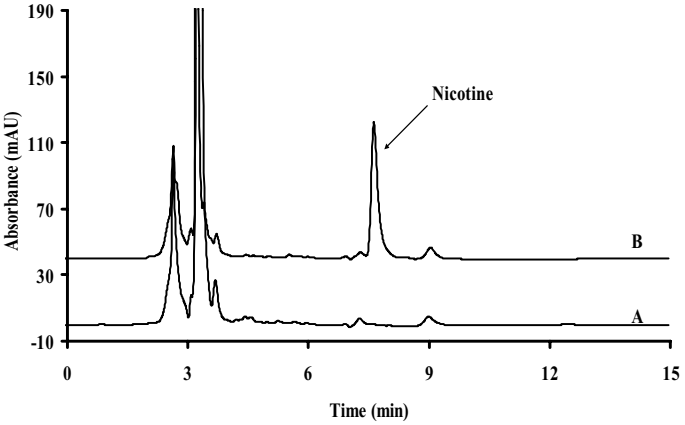

FIG. 1—*Comparison of HPLC-UV chromatograms obtained for a ground beef sample that was (A) negative and (B) positive for nicotine. Chromatographic conditions are as described in the experimental section.*

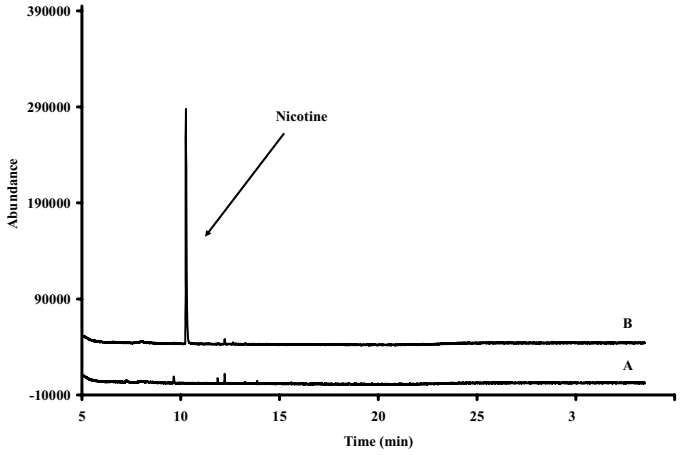

FIG. 2—*Comparison of GC-MS total ion chromatograms obtained for a ground beef sample that was (A) negative and (B) positive for nicotine. Chromatographic conditions are as specified in the experimental section.*

(Table 1, first two columns). Positive vs. negative results were consistent between HPLC-UV and GC-MS analyses with respect to the 12 items tested by both techniques. The nicotine content in the five raw ground beef items that tested positive ranged from 300 to 370 mg/kg. All of the supermarket-packaged raw ground beef items dated for sale on January 1 or 2 tested positive, while all three of the control samples (those which were dated for sale on March 27) tested negative. The nicotine content determined in the two cooked ground beef items was 200 and 160 mg/kg.

The nicotine content was lower on average in the cooked ground beef items (two items, average 180 mg/kg) vs. the raw ground beef items (five items, average 350 mg/kg). It is possible that the cooking process may have volatilized some of the nicotine. While the boiling point of pure nicotine is 247°C, nicotine is volatile with steam (6), which would be released during the cooking process. For the meat loaf item (Item 16), the nicotine may also have been diluted by the addition of the other meat loaf components.

Results for duplicate or triplicate nicotine determinations were reproducible, agreeing within 0.6–15% on a relative basis (based on calculating [difference between duplicate trials/average] $*$ 100% for duplicate determinations, and relative standard deviation, RSD, for triplicate determinations). The reproducible results for the nicotine content determinations for the individual items, and the relatively

TABLE 2—*Spike/recovery and LOQ results for HPLC-UV determination of nicotine.*

Item No.	Description	Previously Determined Average Nicotine Content (mg/kg)	Additional Nicotine Content Due to Spiking (mg/kg)	Nicotine Recovery (% of spiked amount recovered
4	1/4 opened package ground beef, dated for sale label missing (SUSPECT)	320	320	105 (107, 104)
16	1 cooked meat loaf slice (SUSPECT)	160	130	120 (118, 121)
10	3/4 opened package ground beef, dated for sale Mar 27 (CONTROL)	ND	5	82
			10	109
			20	91

* Average of duplicate trials for Items 4 and 16 with individual results noted in parantheses; single trials for Item 10.

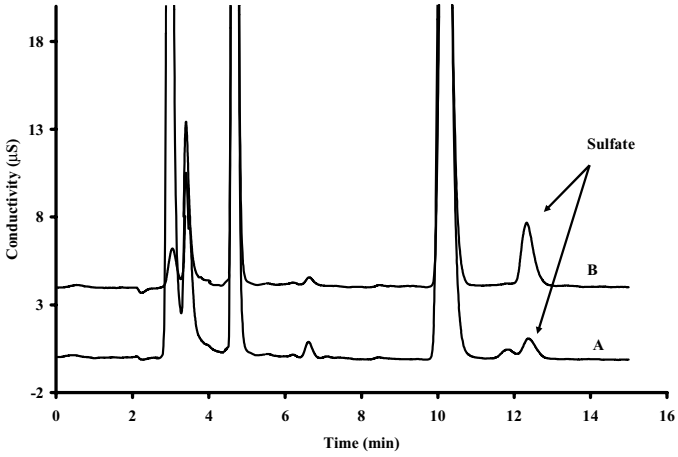

FIG. 3—*Comparison of HPAEC-suppressed conductivity detection chromatograms obtained for a ground beef sample that was (A) negative and (B) positive for nicotine. Chromatographic conditions are as detailed in the experimental section.*

narrow range of nicotine contents found across the various raw ground beef items (from 300–390 mg/kg), indicated that the poison was fairly evenly distributed throughout the beef. Recovery of nicotine in spike/recovery experiments was quantitative for both raw and cooked ground beef items (see Table 2, Items 4 and 16). The limit of quantitation (LOQ) was estimated at 5 mg/kg (0.005 mg/g) based on spiking lower amounts of nicotine into one of the unadulterated, raw ground beef control items (Table 2, Item 10). The average recovery for all nicotine spiking and LOQ experiments was 101%.

The "probable lethal dose" of nicotine by ingestion is 0.5–1.0 mg/kg body weight (11), and the lethal dose for adults is typically cited in the range of 30–60 mg nicotine (1,12,13). Using the average nicotine contents determined for the raw or cooked beef items, the amount of nicotine in a quarter pound (0.113 kg) portion of raw beef was 40 mg, or in cooked beef was 20 mg. These results indicate that the potential for consuming a lethal dose of nicotine existed in this case.

Sulfate Determination

Free sulfate anion is naturally present in biological tissues. Ion chromatography has been used to determine sulfate in a variety of biological matrices (14–15). Rozman et al determined concentrations of sulfate in rat organ tissues (kidney, intestine, lung, liver) to be 50–70 mg/kg (15). Studies have revealed that skeletal muscle tissue contains less sulfate than other tissues such as organs, and cartilage (14).

Initially, the question to be answered centered on the baseline expected concentration of sulfate in ground beef. Consequently, would the addition of nicotine sulfate to ground beef increase the concentration of sulfate enough to indicate the presence of a nicotine sulfate (Black Leaf 40) adulterant? An early attempt in our laboratory at the quantitation of sulfate in the ground beef submitted for testing was unsuccessful because the sulfate concentration detected for duplicate analyses was not reproducible (data not shown). In this initial work, the sulfate was extracted from 3 g portions of the suspect ground beef using a water shake method followed by solid phase extraction cleanup. The suspected problem was lack of homogeneity in the beef portions tested and an insufficient extraction procedure.

The extraction method was revised by lyophilizing and grinding the ground beef in 25 g portions prior to further sample treatment. In addition, a basic extraction with sonication was used in place of the shake extraction with water (14). These changes resulted in more reproducible data for duplicate analyses. Using the modified conditions, testing was first performed on locally purchased ground beef. Analysis was performed in duplicate on four separate portions of freeze dried ground beef. Sulfate concentrations in these ground beef portions were observed to be 25–39 mg/kg for 8 determinations.

Sulfate analysis was then performed on 8 of the 16 items submitted to our laboratory for analysis using the optimized extraction conditions. Four of the samples previously tested positive for nicotine and four of the samples previously tested negative for nicotine as described above (see Table 1). Figure 3 shows chromatograms for ground beef samples that tested negative (A) and positive (B) for nicotine. Sulfate is easily identified in both chromatograms. The increase in sulfate peak area was the only significant difference noted for chromatograms of the nicotine-tainted ground beef versus nicotine-free ground beef. The relative reproducibility range was 0.3–12% ([difference between duplicate trials/average]∗100%). The results of analyses for all samples are shown in Table 3. The beef was also spiked with 280 mg/kg sulfate (as Na_2SO_4) and percent recovery ranged from 78–100%, with similar ranges observed for both the unadulterated and tainted beef samples.

As shown in Table 3, samples that were positive for nicotine also showed elevated levels of sulfate (90–320 mg/kg). The samples that were negative for nicotine had sulfate concentrations similar to that found for the locally purchased ground beef (26–32 mg/kg). The association between the elevated levels of sulfate, and the presence of nicotine in the adulterated items, is readily apparent when the results are graphed (see Fig. 4). However, we also evaluated the results obtained for nicotine and sulfate in the adulterated ground beef to determine if a stoichiometric relationship existed between the nicotine and the elevated sulfate levels. Mole-based calculations of the nicotine and sulfate results for the tainted beef items gave a range of 2.0–2.4 mmol/kg for nicotine, and 0.73–2.1 mmol/kg for sulfate (after correction of the sulfate values for background levels). The molar ratio of nicotine: sulfate varied in the range from 3:1 to 1:1, with an average of 2.3:1. The significance, or lack thereof, of these mole-based calculations, required consideration of whether a prior stoichiometric relationship existed between nicotine and sulfate in the Black Leaf 40 insecticide.

TABLE 3—*Spike/recovery results for sulfate determination by ion chromatography.**

Item No.	Description	Sulfate Content (mg/kg)†	Sulfate Recovery‡ (% of spiked amount recovered
1	2 patties (SUSPECT)	28	87
8	unopened package ground beef, dated for sale Mar 27 (CONTROL)	32	95
10	3/4 opened package ground beef, dated for sale Mar 27 (CONTROL)	32	85
11	1 plastic bag ground beef (SUSPECT)	26	78
3	**1/2 opened package ground beef, dated for sale Jan 1 (SUSPECT)**	**230**	**86**
4	**1/4 opened package ground beef, dated for sale label missing (SUSPECT)**	**100**	**100**
9	**3/4 opened package ground beef, dated for sale Jan 2 (SUSPECT)**	**100**	**92**
14	**1 self-seal plastic bag ground beef (SUSPECT)**	**120**	**78**

* All items spiked at 280 mg/kg sulfate.
† Results are the average of 2 trials.
‡ Results are reported for one trial only.
Items in bold tested positive for nicotine.

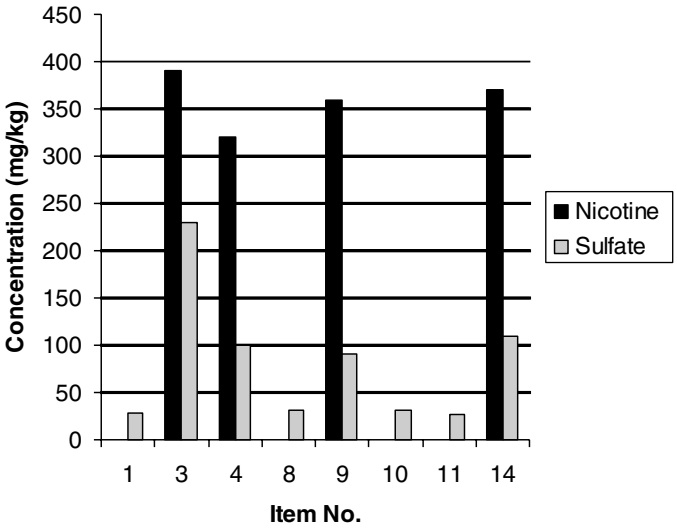

FIG. 4—*Comparison of nicotine and sulfate levels for eight submitted items of evidence in which nicotine was detected (Items 3, 4, 9, 14) or not detected (Items 1, 8, 10, 11). See also Table 1.*

Complete neutralization of nicotine free base with sulfuric acid will produce nicotine sulfate salt with a 2:1 molar ratio of nicotine to sulfate, owing to the presence of two titratable protons for nicotine: pyrrolidine ring pK_a 7.84 and pyridine ring pK_a 3.04 (6). In order to avoid either incomplete neutralization or the addition of excess acid, acid must be added to a specific endpoint which corresponds to a precise final solution pH. As a good estimate, the solution pH must be adjusted 2 full pH units below the relevant pKa. In this case, complete conversion to the 2:1 salt would require a solution pH of approximately 1.0 (i.e., 2 units below the pyridine ring pK_a of 3.04).

As previously mentioned, nicotine-based pecticides such as Black Leaf 40 are no longer available for purchase on the US market, and the original container of Black Leaf 40 used in this incident was not recovered. Although we could not obtain a sample of Black Leaf 40 for examination, we did have available the standard grade concentrated, aqueous nicotine sulfate (Sigma Chemical), which is labeled with nominal contents of 48% w/v nicotine sulfate, and 37 % w/v free base nicotine. We experimentally determined the nicotine content, sulfate content, and made pH measurements for the standard grade nicotine sulfate solution. The nicotine content was 39.7% w/v ($n = 2$), corresponding to a molarity of 2.4 M. The sulfate content was 19.0% w/w ($n = 4$), corresponding to a molarity of 2.0 M. The pH for a 10 fold dilution of the solution ($n = 2$) was 3.7. Although the nominal values listed on the label correspond to a 2:1 mole ratio between nicotine and sulfate, the measured values showed a nicotine:sulfate mole ratio of 1.2: 1.0., and the pH indicated incomplete conversion to the 2:1 salt. The presence of excess sulfate with incomplete conversion to the 2:1 salt could result from different scenarios, including overtitration with sulfuric acid and back adjustment of the pH with another agent, or the sulfate originating from multiple sources (e.g., sulfuric acid and a sulfate salt). However, clearly there is no stoichiometric relationship between nicotine and sulfate in the standard grade nicotine sulfate solution. Rather, the solution represents the equivalent of an imprecise mixture of 1:1 and 2:1 nicotine:sulfate mole ratio salts.

Based on the results for the standard grade nicotine sulfate material, the mole ratios for nicotine and sulfate in the tainted beef samples would not be expected to show a precise stoichiometric ratio. The observed mole ratios averaged 2.3: 1 nicotine:sulfate. However, the adulteration of the beef with a nicotine sulfate based pesticide should result in measurably elevated sulfate levels for the tainted samples, as was observed (Fig. 4). Thus, the nicotine and sulfate results are consistent with the scenario of a nicotine sulfate based formulation being used to adulterate the ground beef in question.

Conclusions

In this work, ground beef suspected to be adulterated with Black Leaf 40, a nicotine sulfate containing insecticide, was submitted for analysis. Analyses performed by HPLC-UV, GC-MS, and ion chromatography agreed well, showing that the samples that tested positive for nicotine also had significantly elevated sulfate levels. These results are consistent with the scenario that a nicotine sulfate-based formulation, such as Black Leaf 40, was the source of the contamination in the ground beef. Our data for nicotine confirm the findings obtained by other laboratories that previously analyzed the suspect ground beef and indicate that the insecticide was well distributed throughout the meat. Although there were no deaths associated with the tainted ground beef, the concentration of nicotine found was such that the possibility for a fatal poisoning existed in this case.

Acknowledgment

The authors would like to thank Dr. Douglas T. Heitkemper for helpful discussions and suggestions.

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