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Visualization and LC/MS Analysis of Colorless Pepper Sprays*†

ABSTRACT: Pepper sprays are used in a variety of circumstances, including criminal activity, self-defense, and law enforcement. As such, the presence or absence of pepper sprays on evidentiary materials is often important when determining the facts of an incident. When no visible stains are present on evidentiary materials, ascertaining the presence or absence of pepper spray can be a challenge to the forensic analyst. A method, based on a chemical derivatization of capsaicinoids using a diazonium salt, has been developed for the visualization of colorless, ultraviolet (UV) activated fluorescent dye-free pepper sprays on textiles. Identification of both the capsaicinoids and their derivatives is confirmed via extraction of the derivatized capsaicinoids followed by liquid chromatography/mass spectrometry (LC/MS) analysis. LC/MS analysis is conducted using a YMC Basic™ column and elution of the compounds using a gradient of 10 mM ammonium formate, pH 4.2 and methanol at 0.35 mL/min. Full-scan MS data are collected for the full 6.5 min LC analysis. Although this method is qualitative in nature, visual detection of as little as 50 µL of a 0.2% pepper spray (equivalent to ~0.1 mg) on a variety of garments is possible, and more than adequate signal-to-noise is obtained for reconstructed ion chromatograms on LC/MS analysis at these levels.

KEYWORDS: forensic science, liquid chromatography/mass spectrometry (LC/MS), oleoresin capsicum (OC), capsaicin, pepper spray, liquid chromatography (LC), mass spectrometry (MS), visualization

The term “capsaicinoid” refers to the family of molecular homologs and analogs purified from the oily resin oleoresin capsicum (OC) that is extracted from the *Capsicum* fruits having the structure shown in Fig. 1 (1,2). The most abundant members of this family are capsaicin and dihydrocapsaicin, which account for an estimated 80–95% of naturally occurring capsaicinoids (3,4). As such, subsequent references to capsaicinoids in this paper will refer to these two forms unless otherwise indicated. Interest in these molecules is generally due to the physiological reactions that they produce. In small amounts, they are responsible for the “heat” of some spicy foods. Capsaicinoids are also found in some homeopathic medical treatments, such as arthritis creams, which generally have concentrations ranging from 0.025 to 0.100%. At higher concentrations, they are the active ingredients in self-defense pepper spray products (3,5).

Pepper sprays manufactured using OC or of purified capsaicinoids extracted from the resin typically contain a variety of capsaicinoids, primarily capsaicin and dihydrocapsaicin as previously noted. Some pepper spray formulations are not made using this natural extract; instead, they are formulated using synthesized capsaicinoid molecules such as nonivamide (2,5,6). Nonivamide-based pepper sprays may also be formulated without the addition of

any kind of visible marker (e.g., CapTor®, Civil Defense Supply, Lincoln, England). While nonivamide-based pepper sprays were not evaluated here, they are expected to produce similar results when derivatized.

Pepper sprays are readily available to both law enforcement personnel and the general public for a variety of uses, including riot control and self-defense. In those instances, the presence or absence of pepper spray on an evidentiary garment may help determine the facts of the incident. Previous laboratory analysis of pepper sprays at the FBI Laboratory relied on visually inspecting the garments for either a colored dye as found, for example, in *CAP-STUN*® (Zarc International, Inc., Gaithersburg, MD), or examining the garments under a UV light source for pepper sprays that contained a UV-activated fluorescent marker. For example, Fox Labs Pepper Sprays (Fox Labs International, Clinton Township, MI) contain 2,5-(di-5-tert-butylbenzoyl) thiophenate [CAS# 7128-64-5], a general use fluorescent optical brightener, to permit UV visualization of the product. Once located, suspect stains were cut from the garment, extracted with methanol, and analyzed for capsaicin and dihydrocapsaicin via gas chromatography/mass spectrometry (GC/MS). Similar methods have been reported by other laboratories for the analysis of capsaicinoids from pepper sprays and other sources (2,5–9).

There are now a number of products on the market that do not contain a colored or UV-activated fluorescent dye, for example, MK4 First Defense® pepper spray (Defense Technology Corporation of America, Casper, WY). When no stains were visible on evidentiary garments submitted for pepper spray analysis, testing was limited to random sampling of areas of the garment for extraction and subsequent GC/MS analysis. Negative results from this type of blind sampling were always inconclusive. A presumptive method has been developed for the visualization of the capsaicinoids present in pepper spray, and a rapid liquid chromatography mass spectrometry (LC/MS) method for analytical confirmation.

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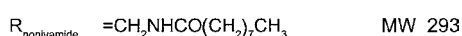
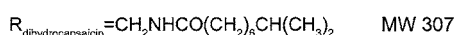
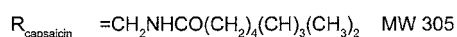
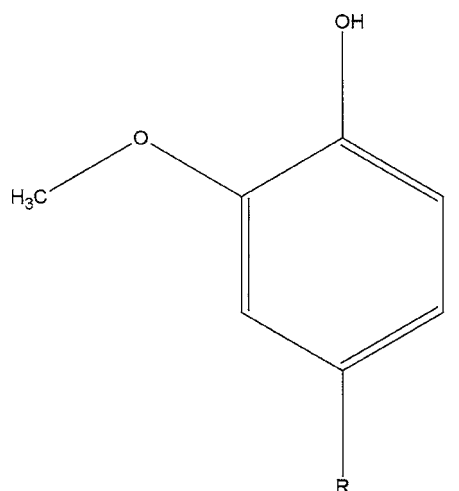


FIG. 1—Capsaicinoid structures and nominal molecular weights.

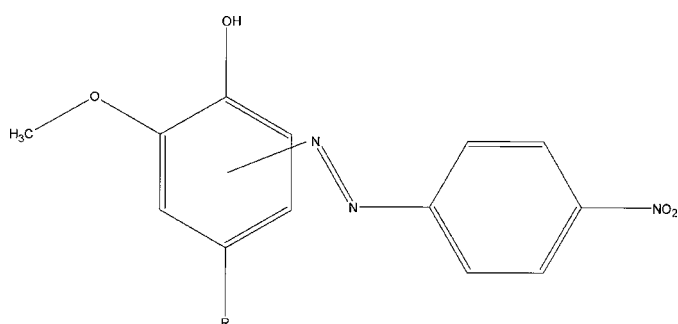


FIG. 2—Derivatization product of a capsaicinoid and 4-nitrobenzene diazonium tetrafluoroborate.

Experimental Methods

Visualization

Chemical derivatization of the capsaicinoids is used to create chromophores that are readily observed in the visible region of the electromagnetic spectrum. The questioned garments are first treated with 1 N NaOH, which is applied by misting a small amount of the base over the garment's surface. To date, this sparing application of base has not damaged any of the fabrics studied. A 0.2% aqueous solution of 4-nitrobenzene diazonium tetrafluoroborate (yellow color in solution) is then sprayed lightly over the entire garment. The electrophilic diazonium coupling reaction between a basic phenol and diazonium salt is normally *para* directed (10); however, in this case, the *ortho* coupled product is predicted. Additional work is ongoing to determine the final derivative structure; the present depiction of the derivative is shown in Fig. 2. This reaction occurs in the presence or absence of the sodium hydroxide solution; however, without the addition of base, the reaction produces a light orange stain that is not readily distinguishable from the yellowish stains produced by the diazonium solution.

The appearance of a violet stain provides the presumptive indication of the pepper spray location (Figs. 3a and 3b). This reaction is immediate and permits the visualization of the OC spray for extraction and subsequent LC/MS analysis.

Studies of the effects of washing and/or bleaching garments stained with pepper spray have demonstrated that very little pepper spray remains on fabrics after the bleaching process (6). To determine the effects of bleaching on the visualization reaction, multi-fiber fabric swatches (Testfabrics, Inc., West Pittston, PA) were each spiked in duplicate with 20 μL of MK4 First Defense[®] brand pepper spray and allowed to dry at ambient room conditions. The multi-fiber fabric (MFF) swatches used in this experiment were MFF#43 and MFF#49, composed of a Dacron 55 fill (fibers running in the vertical direction), and warp stripes (fibers woven horizontally into the fabric) of 13 different fibers. One swatch from each set was then placed in a clean beaker containing approximately 100 mL of 0.35% bleach (by volume) in water. The bleach concentration was estimated based on the manufacturer's instructions to dilute bleach for laundry at a ratio of 1 tablespoon to 1 gal (3.785 L); this produces a 0.39% bleach solution, approximately the value chosen for



FIG. 3—White, cotton T-shirt with dried colorless, UV dye-free pepper spray, before (left) and after (right) the derivatization process.

TABLE 1—Alternate sources of capsaicin.

Tabasco® hot sauce (McIlhenny Co., Avery Island, LA)
Tiger® hot sauce (Reily Foods Co., New Orleans, LA)
Texas Pete® hot sauce (T.W. Garner Food Co., Winston-Salem, NC)
Pace hot salsa (Pace Foods, Paris, TX)
Capzasin-P® analgesic cream (Chattem, Inc., Paris, TX)

this experiment. The solution was agitated for approximately 5 min, drained, and rinsed with approximately 2×100 mL deionized water. The test cloths were then removed from the flask and allowed to dry at ambient room conditions. After drying, the visualization reaction was performed on both the bleached and the control fabric swatches.

There are other readily available sources of capsaicin; it is found in foods (e.g., salsas or hot sauces) and can be found in some homeopathic medical treatments. To determine the effect that a secondary source of capsaicinoids would have on pepper spray analysis, clean white cotton T-shirts were treated with the substances described in Table 1, photographed, and then treated with 1 N NaOH and 0.2% nitrobenzene diazonium tetrafluoroborate.

Potential interferences were evaluated to determine (i) their effect on the visualization process, and (ii) whether false-positives would be generated by these substances during the visualization process. Substances tested included human body fluids (saliva, perspiration, urine, blood) and common garment stains such as cola, lemonade, coffee, and clay/dirt. Human saliva and perspiration were collected from six individual subjects, three male and three female. One lot each of human blood (preserved in EDTA) and human urine were tested. Potential interferences were applied in duplicate to sections of a clean white cotton T-shirt and allowed to dry at ambient room conditions. Next, 50–75 μ L of MK4 First Defense® colorless pepper spray was applied to one of the two sections treated with each substance and allowed to dry at ambient room conditions. Samples were then treated with 1 N NaOH followed by 0.2% nitrobenzene diazonium tetrafluoroborate and visually examined.

Extraction

Identification of the key capsaicinoids in pepper spray is confirmed by cutting small (approximately 10 mm \times 15 mm) sections from the stained areas and extracting in 250–500 μ L of 0.05% formic acid in 50/50 methanol/water. The extraction procedure is rapid and simple, consisting only of vortexing the sample in the extraction solvent for 30–60 sec. The pH of the extract is then verified to be between 4 and 5 for analysis on the LC/MS system. If the pH is above 5, additional clean extraction solvent may be added to the tube to lower the pH. This ensures that samples with basic pH are not introduced, since these samples can be damaging to the LC column (YMC Basic™ pH range 2–7). A portion of the liquid is removed from the extraction tube and used for LC/MS analysis. Note that both the extraction tube and the autosampler vial should be glass to avoid interactions between the derivatives and plastics.

Instrumentation

The analytical instrumentation used for LC/MS analysis consists of an HP1100 binary pump and autosampler fitted with a YMC Basic™ S-3, 2.0 \times 50 mm column (Waters Corporation, Milford, MA). Mobile phase A is 10 mM ammonium formate, pH 4.2 and

B is HPLC grade methanol. Mobile phase is pumped through the column at 0.35 mL/min along a gradient that pumps 65%B for 0.5 min, increases to 90%B at 1.5 min and returns to 65%B after 4.0 min. The LC run time is set to 6.5 min to allow the column to re-equilibrate to the initial chromatographic conditions. Injection volume is dependent on concentration and may vary from 2 to 20 μ L. The LC is interfaced to a Finnigan LCQ™ MS detector with an atmospheric pressure chemical ionization (APCI) source. APCI was selected because it provided greater response for the derivatives when compared to electrospray ionization. Analytical conditions are as follows: N₂ sheath gas (80 psi [552 kPa] at tank, 40 arbitrary units at LCQ™ software control), N₂ auxiliary gas (off), vaporizer temperature (450°C), capillary temperature (180°C), corona needle discharge current (5.5 μ A), scanning 50–500 m/z with no CID energy.

Results and Discussion

The present method was developed primarily for rapid qualitative assessments followed by definitive MS confirmation. Limitations of the method include difficulty observing the colored derivative stain generated by the visualization procedure on certain fabrics, including those that have been treated for outdoor use (e.g., water-repellent garments). Experiments were conducted to test the visibility of the derivative by spotting various fabrics with measured amounts of the actual pepper spray product. Visualization was successful with amounts as small as 50 μ L of MK4 (0.2% capsaicinoid content, \sim 0.1 mg) on all of the following fabrics: 100% cotton (white, yellow, blue denim), 100% acrylic (neon pink), 100% Dacron polyester (multihued and patterned), and a blend of 50/50 cotton/polyester (patterned white) (Fig. 4). The size of the spot associated with 50 μ L of MK4 First Defense® pepper spray varied due to the chemical characteristics of the fabric, but was generally approximately the size of a quarter.

Absolute limits of detection for the visualization reaction will depend on a variety of factors, especially those which influence the deposition of the pepper spray on the garment or the visibility of the derivative on the garment. Fabrics which are partially or fully composed of water-resistant fibers will absorb less of the pepper spray, reducing the amount available for derivatization. A more viscous formulation might not “spread” as readily along a given type of fabric, and a very thin fabric is likely to absorb less pepper spray than a heavier fabric. These factors are important to the visualization since a more concentrated area of pepper spray residue will generate a more intense stain on derivatization; faint color produced by less concentrated residues is less readily visible to the eye, especially on fabrics with similar colors, strong patterning or other potentially interfering stains.

After laundering, most of the bleached fabrics showed little to no indication of the presence of pepper spray. These results are similar to those found in previous studies (6). Faint stains were produced on a few of the bleached fabrics, indicating that the reaction proceeds, provided that a small amount of the capsaicinoids remains on the fabric. Results of the visualization reaction performed after bleaching fabrics treated with pepper spray are summarized in Table 2.

Additional observations about the nature of the interaction between capsaicinoids and fibers were noted during the MFF bleaching experiment. On some fibers (e.g., cotton), the stains had patterns with clear, well-defined edges, while other fibers (e.g., filament triacetate) did not appear to stain at all. Stain patterns are highly dependent on the chemical composition of the fabric fibers and

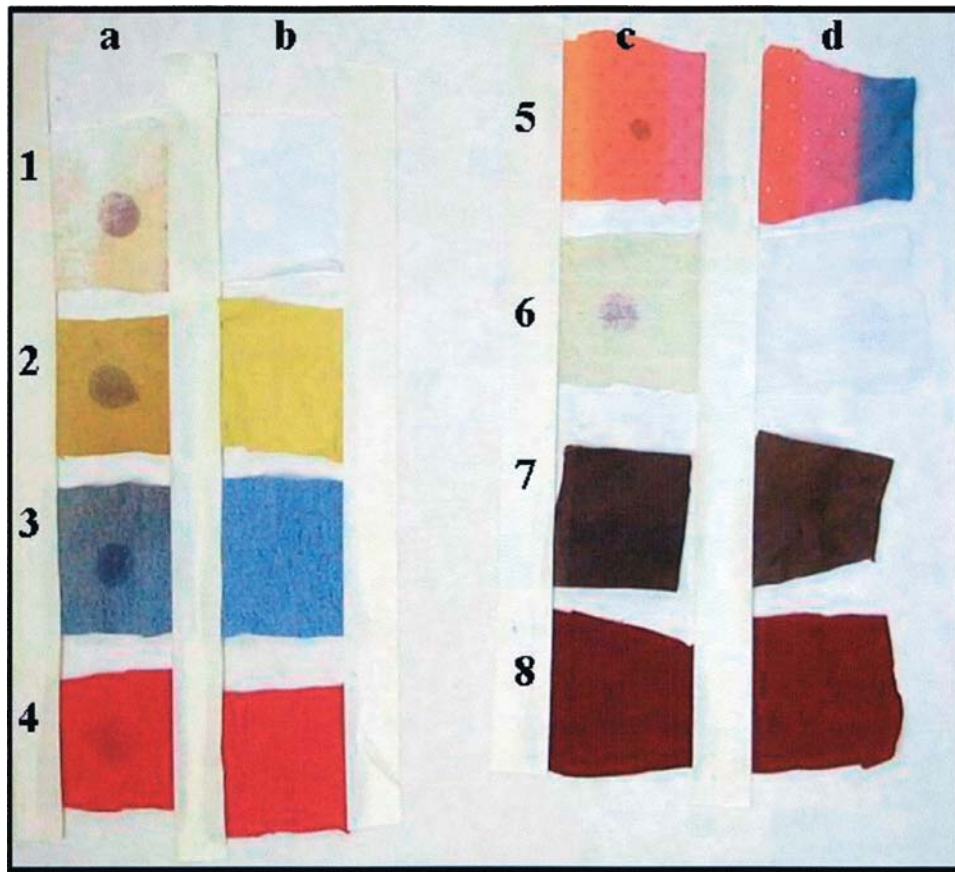


FIG. 4—Fabric swatches spiked with 50 μ L MK4 brand pepper spray. Columns a and c: swatch of each column after visualization reaction; Columns b and d: swatch of each column, no visualization reaction; Columns a and b, from top: white cotton, yellow cotton, blue cotton denim, pink acrylic; and Columns c and d, from top: multihued polyester, 50/50 cotton/polyester, brown leather, red nylon.

TABLE 2—Visualization results after bleaching.

MFF Warp Stripe Fiber	Color Indicator after Bleaching
SYNTHETIC FABRICS	
Spun diacetate	Faint
Filament triacetate	None
Nylon 6.6 polyamide	Faint
Dacron 54 polyester	Faint
Dacron 64 polyester	None
Creslan 61 acrylic	None
Orlon 75 polyacrylic	None
Spun viscose (rayon)	None
SEF modacrylic fire retardant fiber	None
Polypropylene	None
NATURAL FABRICS	
Bleached cotton	Faint
Spun silk	None
Worsted wool	None

provide strong support for performing a positive control test on the actual fabric in question prior to analysis via this method. Individual fabrics from evidentiary garments may be tested by spotting a small amount of a colorless OC spray or capsaicinoid standard on a small swatch cut from the garment and performing the visualization procedure. A small piece from an underturned hem is suggested for the control.

Secondary sources of capsaicinoids (e.g., foods, homeopathic medical treatments) were differentiable from colorless pepper spray

stains. Prior to derivatization, salsa and hot sauce were clearly visible as orange-to-brown stains; however, when a colorless pepper spray (e.g., MK4) was applied to the shirts, there was a clear difference in the stain patterning after derivatization (Fig. 5). This stain pattern difference (before and after derivatization) should permit distinction between stains caused by pepper spray or those caused by a secondary capsaicinoid source (e.g., salsa and hot sauce).

The analgesic cream Capzasin-P® was applied to a clean white cotton T-shirt to simulate transfer from a topical application of the cream; after drying, no stain was visible on the sample. On derivatization, a faint violet stain developed, indicating the presence of capsaicinoids. While the violet color indicator is a presumptive identification of pepper spray, there is a clear difference between the intensity of the stain created by the analgesic cream and the pepper spray. The concentration of capsaicinoids in the analgesic creams range from 0.025 to 0.100%, an order of magnitude lower than typical pepper spray capsaicinoid content. (Pepper spray brands used in this study had capsaicinoid contents of 0.2% and OC concentrations as high as 10%, as specified by the manufacturers.) Stain patterns may also provide additional evidence to identify the source of the capsaicinoids.

Potential interferences were evaluated to determine (i) their effect on the visualization process, and (ii) whether false-positives would be generated by these substances during the visualization process. Results of these experiments are presented in Table 3. As expected, the presence of blood can mask the color indicator for the presence of pepper spray in the same way that dark-colored fabrics

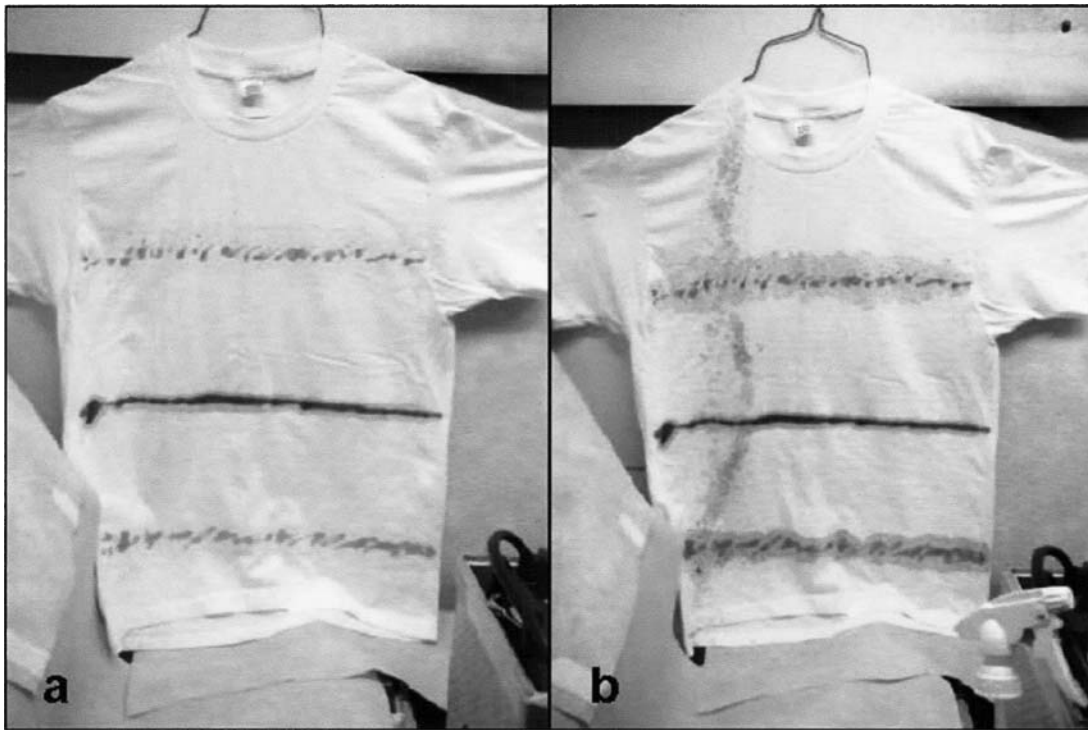


FIG. 5—White cotton T-shirt stained with (from top) Tiger® hot sauce, Tabasco® hot sauce, and Texas Pete® hot sauce applied in horizontal lines. Colorless, UV dye-free MK4 First Defense® Pepper Spray was applied in a vertical stripe on the left side of the shirt: (a) prior to visualization, (b) after visualization.

can disguise the presence of the violet indicator. There was no interference beyond potential color masking, and the derivatization procedure was not affected for pepper spray surrounding the actual bloodstain.

A false-positive is generated if the violet color indicator develops on derivatization in the absence of capsaicinoids. Because the diazonium coupling reaction used in the visualization procedure is nonspecific, it is possible that other common garment stains may yield a false-positive during the presumptive analysis. Positive confirmation of capsaicinoid presence is performed via the LC/MS analysis previously described. Color development on derivatization did occur on several samples (Table 3): human saliva, perspiration, and urine. A faint reddish color was developed on derivatization of the single lot of human urine tested. On derivatization, three of the six human perspiration samples produced faint orange-to-red

“speckles.” Four of the six human saliva samples produced similar coloration patterns. The color indicator and pattern developed during the visualization differed markedly from the characteristic strong violet color produced by the capsaicinoids; LC/MS analysis can be used to confirm or reject questionable presumptive identifications.

Confirmatory Analysis via LC/MS

A chromatogram produced from a 3 μL injection of an MK4 First Defense® colorless pepper spray extract is shown in Fig. 6. It is worth noting that this method produces sufficiently resolved chromatographic peaks for identification within a total analytical run time of 6.5 min. The total ion chromatogram (TIC) is

TABLE 3—Potential interferences.

Potential Interference	Amount Applied to Fabric	Interference?	False Positive?
Human blood (EDTA preserved)	250 μL	Heavy stains could partially mask color indicator	No
Human urine	250 μL	No	} some faint rose-to-red coloration observed; no violet stains generated by derivatization
Human saliva	~1 mL	No	
Human perspiration	100–1000 μL	No	
Cola	250 μL	No	No
Lemonade	250 μL	No	No
Coffee	250 μL	No	No
Ethanol	50 μL	No	No
Isopropanol	50 μL	No	No
Water	50 μL	No	No
CS tear gas	50 μL	No	No
Soil (high clay content)	~50–100 mg	No	No

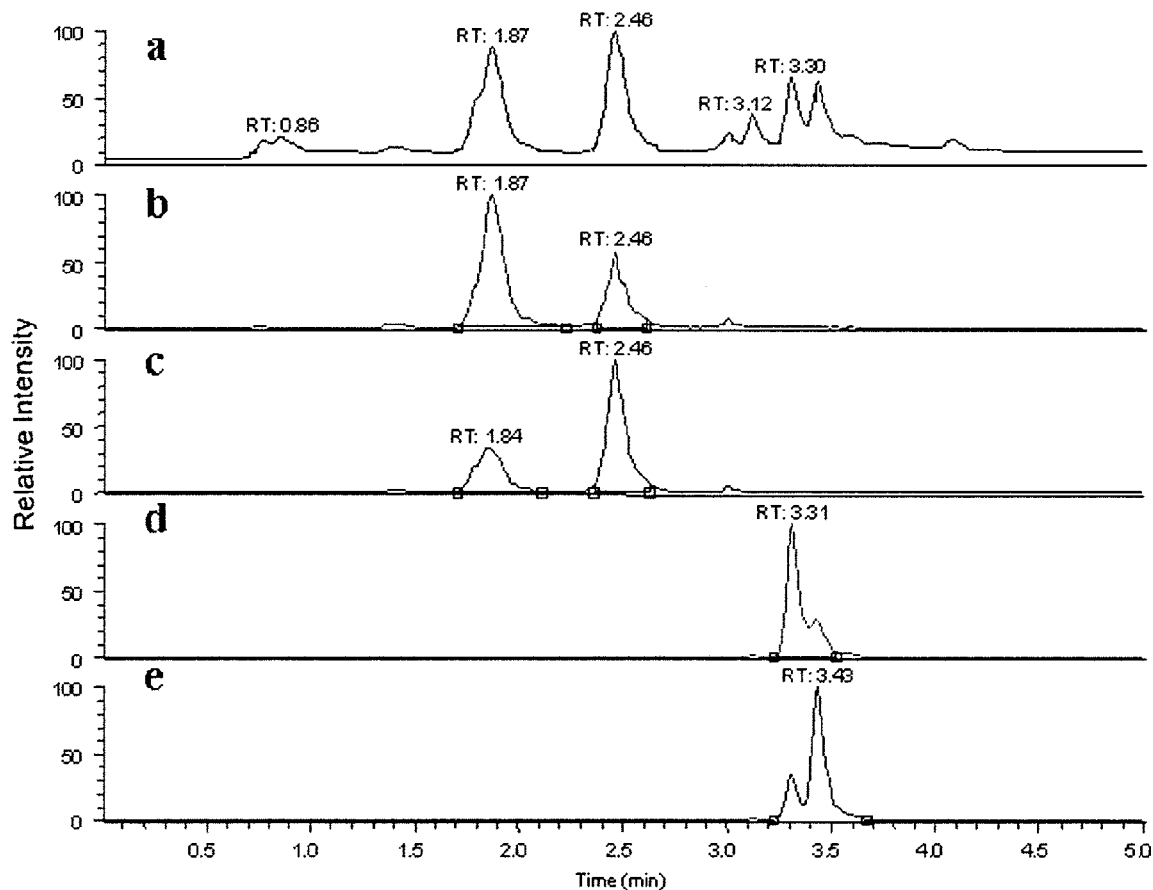


FIG. 6—Chromatogram generated from pepper spray derivatized with nitrobenzene diazonium tetrafluoroborate (a) Total Ion Current (TIC), (b–e) extracted ions of interest: (b) capsaicin (137+170+306), (c) dihydrocapsaicin (137+172+308), (d) capsaicin derivative (286+455), (e) dihydrocapsaicin derivative (286+457).

shown in the top trace. Reconstructed ion chromatograms for capsaicin (m/z 137 + 170 + 306), dihydrocapsaicin (m/z 137 + 172 + 308), capsaicin derivative (m/z 286 + 455) and dihydrocapsaicin derivative (m/z 286 + 457) are shown in Figs. 6b–6e, respectively.

As shown in Fig. 6, the signal-to-noise of the peaks in the extracted ion chromatograms is more than adequate. Given the levels of capsaicinoids found in pepper sprays, determining the absolute limit of detection for the LC/MS portion of the assay was not considered necessary. Previous work (2,5–8) has shown successful LC/MS analysis of capsaicinoids at levels several orders of magnitude less than those found in extracts prepared via this method. During this study, all samples that produced a violet color on derivatization generated clearly visible capsaicin peaks and spectra on LC/MS analysis. The protonated molecules for the capsaicinoids are consistent with previously published data (2,5,6,8) and logical fragment ions are obtained for the capsaicinoid derivatives (Fig. 7). The $[M + H]^+$ ion is observed for each compound of interest, and work is ongoing to confirm the proposed fragments via MS/MS and accurate mass analysis.

The chromatographic and mass spectral identification of capsaicin and dihydrocapsaicin are described in detail here. Pepper sprays may also contain other capsaicinoid molecules, whether from an extract of the resin or produced synthetically (e.g., nonivamide). Because the diazonium reaction occurs at the vanillyl moiety of the capsaicinoid molecules, derivatization is expected to occur regardless of which capsaicin analogues are present. The MK4 First

Defense[®] pepper spray examined in this study contained at least five different capsaicinoids. Chromatographic peaks and mass spectra consistent with derivatives for each of these molecules were found (unpublished data). While nonivamide was not identified in the MK4 First Defense[®] samples analyzed during this study, it is expected to behave in an analogous manner. Experiments are planned to confirm the derivatization results for capsaicinoids present in this formulation and in other formulations, for example, nonivamide based pepper sprays such as CapTor[®].

The derivatization reaction should be performed with the minimum amount of solutions needed to lightly cover the garment surface. Oversaturation can lead to gross discoloration of the fabric and may make the interpretation of color development more difficult. Limiting the amount of base and diazonium salt solution that are applied also reduces potential salt build-up in the heated capillary of the mass spectrometer. Additionally, it is preferable to show the presence of both derivatized and unreacted capsaicinoids during LC/MS analysis. Atomizers or bottles with fine mist spray dispensers are the preferred means of applying the solutions used in the visualization reaction. Analysts are strongly urged to perform this reaction on test samples to gain familiarity and proficiency in the method prior to performing evidence examinations.

Conclusions

Colorless pepper sprays may be visualized via derivatization of capsaicinoid molecules under basic conditions with a diazonium salt

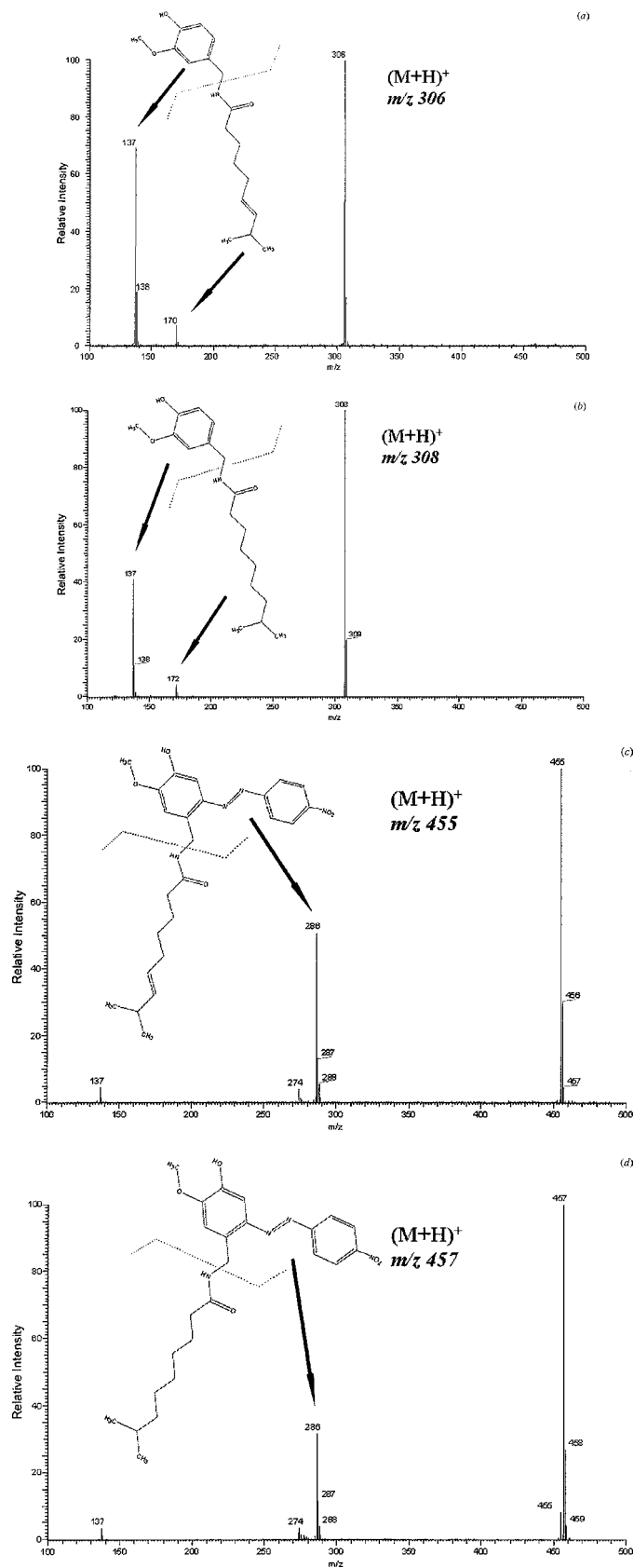


FIG. 7—MS spectra (a) capsaicin, (b) dihydrocapsaicin, (c) capsaicin derivative, (d) dihydrocapsaicin derivative. The $[M+H]^+$ ion is present for each compound of interest. Work is ongoing to confirm the spectra and proposed fragment ions of the derivatives via MS/MS and accurate mass determination.

solution to produce a violet chromophore. Interferences from common garment stains are negligible, except in cases where the stain is overly dark (e.g., blood) and overwhelms the violet color indicator that is developed at the location of the pepper spray stain. None of the interference tests yielded false-positives. If false-positives are obtained via the visualization procedure, they will be eliminated by the confirmatory LC/MS analysis.

The garment fabric plays as great a role in the visualization procedure as any potential interference. Some fabrics are designed to repel liquids; these fabrics are typically found in outerwear, e.g., nylon or Gortex®. Because liquid is repelled from the garment, little to no capsaicin may be available within the fibers for visualization and analysis, even if the garment has been heavily treated with pepper spray. Other fibers, such as the cotton fibers of a T-shirt or pair of blue jeans, will readily absorb the capsaicinoids. Previous studies (6) have shown that these capsaicinoids are present even after laundering, provided that no bleach was used.

Amounts of 50 μL ($\sim 100 \mu\text{g}$) are readily visible on fabrics that absorb the pepper spray and as little as 20 μL ($\sim 40 \mu\text{g}$) can be visualized on light-colored garments.

The derivatization of the capsaicinoids to produce a chromophore is a rapid, sensitive, and reliable method to identify sampling locations on evidentiary garments for subsequent LC/MS confirmatory analysis. Because the derivatization takes place almost immediately, this method can provide a presumptive identification of pepper sprays in less than an hour. Extraction and LC/MS analysis for confirmation take only slightly longer, providing a rapid and sensitive method for identifying colorless, UV-activated dye-free pepper sprays on evidentiary materials.

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