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Determination of capsaicin, dihydrocapsaicin, and nonivamide in self-defense weapons by liquid chromatography–mass spectrometry and liquid chromatography–tandem mass spectrometry

Christopher A. Reilly[a], Dennis J. Crouch[a,*], Garold S. Yost[a], Alim A. Fatah[b]

[a]*Center for Human Toxicology and Department of Pharmacology and Toxicology, University of Utah, Salt Lake City, UT 84112, USA*
[b]*Office of Law Enforcement Standards, National Institute of Standards and Technology, 100 Bureau Drive, Stop #8102, Gaithersburg, MD 20899-8102, USA*

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Abstract

Sensitive and selective liquid chromatography–mass spectrometry (LC–MS) and liquid chromatography–tandem mass spectrometry (LC–MS–MS) methods for the analysis of capsaicin, dihydrocapsaicin, and nonivamide in pepper spray products have been developed. Chromatographic separation of the capsaicinoid analogues was achieved using a reversed-phase HPLC column and a stepwise gradient of methanol and distilled water containing 0.1% (v/v) formic acid. Identification and quantification of the capsaicinoids was achieved by electrospray ionization single-stage mass spectrometry monitoring the protonated molecules of the internal standard (*m*/*z* 280), capsaicin (*m*/*z* 306), dihydrocapsaicin (*m*/*z* 308), and nonivamide (*m*/*z* 294) or by tandem mass spectrometry monitoring the appropriate precursor-to-product-ion transitions. The plot of concentration versus peak area ratio was linear over the range of 10–750 ng/ml using LC–MS and 10–500 ng/ml using LC–MS–MS. However, to accurately quantify the capsaicinoids in the pepper spray products calibration curves between 10 and 1000 ng were constructed and fit using a weighted quadratic equation. Using the quadratic curve, the accuracy of the assay ranged from 91 to 102% for all analytes. The intra-assay precision (RSD) for capsaicin was 2% at 25 ng/ml, 10% at 500 ng/ml, and 3% at 800 ng/ml. The inter-assay precision (RSD) for capsaicin was 6% at 25 ng/ml, 6% at 500 ng/ml, and 9% at 800 ng/ml. Similar values for inter- and intra-assay precision were experimentally obtained for both dihydrocapsaicin and nonivamide. The analysis of selected pepper spray products demonstrated that the capsaicinoid concentration in the products ranged from 0.7 to 40.5 μg/μl.

Keywords: Pepper spray; Capsaicin; Dihydrocapsaicin; Nonivamide; Octanoyl vanillamide

1. Introduction

Oleoresin capsicum-based self-defense products are used as less-than-lethal weapons by law enforcement agencies [1]. Also, it was estimated that nearly 15 million Americans carried these products for personal defense in 1996 [2]. In recent studies designed to assess the potency, safety and relative efficacy of various pepper spray products, it was demonstrated that pepper sprays have an approximate 10 to 26% failure rate [2–4]. Data obtained from field use of pepper spray products have demon-

*Corresponding author. Center for Human Toxicology, 20 S. 2030 E., Room 490, Salt Lake City, UT 84112, USA. Tel.: +1-801-581-5117; fax: +1-801-581-5034.

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strated similar rates of failure. It has also been reported that the efficacy of pepper spray products has varied unpredictably, particularly when the products were used to subdue individuals under the influence of illicit drugs or alcohol [2,3]. In addition to product failures, there have been several reports of respiratory complications, particularly in asthmatics, and even deaths following exposure to pepper sprays [2,5–7]. The cause of the product variability and adverse physiological responses has not been determined.

Exposure to pepper sprays (or capsaicinoids) elicits intense physiological responses that include coughing and gagging, disorientation, erythema, lacrimation, temporary blindness, and intense pain [8]. The actions of pepper spray products are the result of the capsaicinoid analogues; capsaicin, dihydrocapsaicin, nordihydrocapsaicin, nonivamide, homocapsaicin, and homodihydrocapsaicin [9,10]. These compounds are found in oleoresin capsicum (OC). Oleoresin capsicum is the concentrated extract of ''hot peppers'' and is used to prepare pepper spray products [9]. Capsaicinoids interact with the vanilloid receptor and promote release of substance P, as well as several other cytokines and tachykinins [10–12]. Release of substance P from peripheral sensory neurons causes a sensation of intense burning and pain [10–12]. Capsaicin, dihydrocapsaicin, and nonivamide exhibit the greatest pungency of the capsaicinoid analogues and typically represent 85–95% of the total capsaicinoid content in hot peppers and pepper products [9,13,14].

Previous research has demonstrated that the concentration of the capsaicinoid analogues present in fresh peppers and OC varies in response to growth conditions, time of harvest, pepper type and geographical origin [13–15]. Because pepper spray products are prepared by dilution of food-grade OC pepper spray product manufacturers are not required to perform accurate analytical procedures to ensure consist product formulation. Currently, the accepted practice for establishing product potency (capsaicinoid content) is a taste test (The Scoville Organoleptic Test). This test is used to estimate total capsaicinoid concentration based on the minimum dilution required to ameliorate nociception [16]. Because the manufacture of pepper spray products involves diluting starting materials that may be qualitatively different and a subjective taste test is used for estimating the quantitative content, predictably, there will be variability in the concentrations of active ingredients in the pepper spray products. This variability may affect the performance and efficacy of the products as less-than-lethal deterrents.

The purpose of the research reported here was to develop sensitive and selective methods for the analysis of capsaicin, dihydrocapsaicin, and nonivamide in pepper spray products. Because nonivamide is often used as a synthetic substitute for OC in pepper spray products and because it may be improperly identified as capsaicin or nordihydrocapsaicin [17], it has been included in these analyses. We have applied these methods to the analysis of several OC-pepper spray products and have quantified capsaicin, dihydrocapsaicin, and nonivamide in these products.

2. Experimental

2.1. Reagents

Capsaicin, dihydrocapsaicin, and nonivamide (*n*-vanillylnonanamide) were purchased from Sigma (St. Louis, MO, USA). Octanoyl vanillamide (internal standard) was synthesized by condensation of octanoyl chloride and vanillamine, as previously described [18]. Octanoyl chloride and vanillamine hydrochloride were purchased from Aldrich (Milwaukee, WI, USA). Methanol (GC-Resolv) was purchased from Fisher Scientific (Fair Lawn, NJ, USA). *n*-Butyl chloride and 88% formic acid were purchased from J.T. Baker (Phillipsburg, NJ, USA). Distilled water (specific resistance=18.2 MΩ cm) was obtained using a Millipore Milli-Q Plus water purification system (Millipore, Burlington, MA, USA). The pepper sprays were purchased from independent distributors of the products.

2.2. Analytical standards

The analytical standards were prepared by weighing 1.0 mg of each capsaicinoid using a Cahn Model 4700 analytical balance (Cahn Instruments, Cerritos, CA, USA) and dissolving the compound in 10 ml of methanol. Prior to weighing each compound, the

balance was calibrated to ensure accuracy. Stock standards were prepared by serial dilution of a 0.1 mg/ml standard in methanol using volumetric pipettes and flasks. All standards were stored protected from light at −20°C for the duration of the study. Quality control samples were prepared from independent stock solutions at 25, 500 and 800 ng/ml in methanol.

2.3. LC–MS analysis

Analysis of capsaicin, dihydrocapsaicin, and nonivamide was performed using a Hewlett-Packard Series 1100 LC–mass selective detection system (Agilent Technologies, Palo Alto, CA, USA) [15]. Liquid chromatographic separation of the analytes and internal standard was achieved using a MetaSil Basic (100×3.0 mm, 3 μm particle size) C_2–C_8 reversed-phase high-performance liquid chromatography (HPLC) column (MetaChem Technologies, Torrance, CA, USA) and a stepwise gradient of methanol and distilled water containing 0.1% (v/v) formic acid. The column was equilibrated at a flow-rate of 0.25 ml/min with a mobile phase consisting of methanol–distilled water (57.5:42.5) containing 0.1% (v/v) formic acid at 40°C. The mobile phase was maintained at this composition for 7.9 min and then the methanol was increased to 65% over the duration of 0.1 min. At 10.2 min, the concentration of methanol was increased to 75%. After 16.0 min, the concentrations of methanol and water were returned to their initial concentrations. To ensure equilibration of the column, a 3-min delay time was programmed between samples. The autosampler injection volume was set at 5 μl.

The mass spectrometer was equipped with an electrospray ionization source and was operated in the selected-ion monitoring (SIM) mode. The protonated molecules produced from capsaicin (m/z 306), dihydrocapsaicin (m/z 308), nonivamide (m/z 294), and octanoyl vanillamide (m/z 280) were monitored. The optimum conditions for the analysis of the analytes were as follows: fragmenter, 45 V, capillary voltage, 3500 V, gas temperature, 350°C, drying gas, N_2, gas flow-rate, 10 l/min, and nebulizer pressure, 25 p.s.i.g. (1 p.s.i.=6894.76 Pa).

Integration and quantification of the chromatographic peaks were performed using the HP Chemstation software package (revision A.06.03) (Agilent Technologies). Statistical analyses were performed using Microsoft Excel 2000 (Microsoft, Redmond, WA, USA).

2.4. LC–MS–MS analysis

Analysis of the capsaicinoids using LC–MS–MS was performed using a ThermoQuest TSQ tandem mass spectrometer (ThermoQuest, San Jose, CA, USA) combined with a Hewlett-Packard series 1100 HPLC system. The liquid chromatographic conditions used for separation of the analytes during LC–MS–MS were identical to those described above. The mass spectrometer was equipped with an electrospray ionization source and operated in the selected-reaction monitoring mode for precursor-to-product-ion transitions for capsaicin (m/z 306→137), dihydrocapsaicin (m/z 308→137), nonivamide (m/z 294→137), and octanoyl vanillamide (m/z 280→137). The collision gas was argon. The argon was set to a pressure of 3.75 mTorr and a collision energy of −15 V was used (1 Torr= 133.325 Pa).

Integration and quantification of the data were performed using the Xcalibur LCQuan software package (version 1.1) (ThermoQuest). Statistical analyses were performed using Microsoft Excel 2000.

2.5. Preparation of samples

The pepper spray products were equilibrated to −20°C and then carefully discharged into collection tubes cooled to −80°C. The tubes were immediately capped and the samples permitted to thaw on ice. The sample volume was recorded and the caps were removed to permit evaporation of the volatile carrier solvents. Solvent evaporation was performed under a fume hood for the duration of 1 h at room temperature. Following the evaporation of the solvents, the sample volume was reestablished by addition of the appropriate volume of methanol–*n*-butyl chloride (1:1). A 5-μl aliquot of the pepper spray product was diluted 50-fold in methanol–*n*-butyl chloride (1:1). A 10-μl aliquot of this solution was diluted to 1 ml using methanol containing 500 ng/ml octanoyl vanillamide. The samples were evaporated to dryness

under a stream of air at 40°C. The dried residues were reconstituted in 100 μl methanol–distilled water (60:40). The reconstituted samples were transferred to autosampler vials for analysis.

2.6. Accuracy and precision

The accuracy and precision of the assay were established by analyzing quality control samples at 25, 500 and 800 ng/ml ($n=5$) on 3 separate days. The accuracy was determined as the percentage of the target analyte concentration using the mean ($n=5$) assayed concentration in a single batch of samples. Intra-assay precision was expressed as percent relative standard deviation (RSD) and was calculated for each batch using the standard deviation of the assayed concentrations of each analyte at each concentration divided by the mean assayed concentration ($n=5$). Inter-assay precision (RSD) was determined by dividing the standard deviation of the assayed concentrations ($n=15$) for three separate replicate batches by the mean concentration ($n=15$).

2.7. Stability

The effect of various storage conditions on sample stability was determined for each analyte using quality control samples. Quality control samples ($n=5$) at 25, 500 and 800 ng/ml were stored at either room temperature for 24 h, subjected to a single freeze–thaw cycle, or stored overnight at room temperature in autosampler vials (methanol–distilled water, 60:40). The samples were then analyzed as described. Stability was assessed by comparing the mean assayed concentration ($n=5$) for the stability standards to the mean concentration of untreated quality control standards.

3. Results

3.1. LC–MS

The analysis of capsaicin, dihydrocapsaicin, and nonivamide by LC–MS was sensitive to less than 10 ng/ml. A typical selected ion monitoring profile obtained from the analysis of a 200 ng/ml calibrator by LC–MS is shown in Fig. 1. As shown in the figure, all analytes were separated either chromatographically or by m/z and each produced the expected protonated molecule.

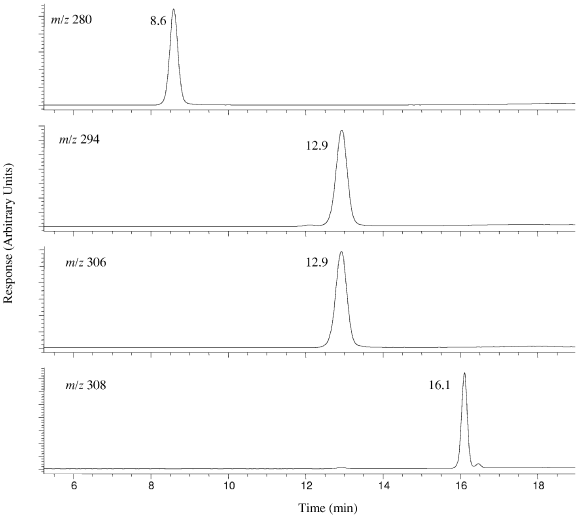

Fig. 1. Representative selected ion monitoring profile obtained from analysis of a 200 ng calibrator containing octanoyl vanillamide (500 ng), capsaicin, dihydrocapsaicin, and nonivamide by LC–MS. The identities of the peaks are octanoyl vanillamide (m/z 280), nonivamide (m/z 294), capsaicin (m/z 306), and dihydrocapsaicin (m/z 308).

The plot of peak area ratio versus calibrator concentration was linear over the range of 10–750 ng/ml. However, to account for the anticipated concentrations of the capsaicinoids in the pepper spray products, calibration curves between 10 and 1000 ng/ml were used and were fit with a non-linear quadratic equation with weighting of $1/y^2$. This procedure was used for quantification since it produced a more accurate regression fit over a larger concentration range ($r^2 \geq 0.999$). Calculation of the calibrator concentrations from the curves yielded values that were typically ≤5% of the fortified concentration. An administrative lower limit of quantitation (LLOQ) was set at 10 ng/ml, however, calibrators at 1/2 LLOQ were routinely included in the assay and the calculated concentrations were typically within 10% of their fortified concentration. The inter- and intra-assay precisions were ≤10% RSD and the accuracy ($n=5$) was ≥88% for all analytes at all quality control concentrations. The data for inter- and intra-assay precision and accuracy are summarized in Table 1.

Table 1
Accuracy and intra- and inter-assay precision (RSD) for quality control standards containing capsaicin, dihydrocapsaicin, and nonivamide

Target concentration (ng/ml)	Capsaicin		Nonivamide		Dihydrocapsaicin	
	Accuracy (% target)	RSD (%)	Accuracy (% target)	RSD (%)	Accuracy (% target)	RSD (%)
Intra-assay ($n=5$)						
25	92	2	92	2	91	5
500	101	10	99	10	102	10
800	100	3	98	2	101	3
Inter-assay ($n=15$)						
25	96	6	94	7	97	10
500	101	6	98	6	102	7
800	100	9	97	9	99	8

The effects of various storage conditions on the stability of the analytes are summarized in Table 2. The experimentally determined concentrations for the stability samples ($n=5$) that were stored at either room temperature, in autosampler vials (in methanol–distilled water, 60:40, at room temperature), or subjected to freeze–thaw conditions were essentially not affected. The calculated concentrations of the analytes in these samples were within 9% of the values obtained for untreated quality control standards (Table 2).

3.2. LC–MS–MS

We also validated the analytical method using tandem mass spectrometry to increase our confidence in the identification and quantification of the capsaicinoids in unknown samples. In addition, the quantification of the capsaicinoids using LC–MS–MS was more sensitive and exhibited greater accuracy at lower analyte concentrations. A mass spectrum of the product ions generated by collision-induced dissociation of capsaicin (m/z 306) is shown in Fig. 2. A typical selected-reaction monitoring profile from the analysis of the capsaicinoids by LC–MS–MS is shown in Fig. 3. The calibration curves for the analysis of the capsaicinoids by LC–MS–MS were linear over the range of 10–500 ng/ml. However, to extend the upper limit of quantitation to 1000 ng/ml the data were fit using a quadratic equation weighted $1/y^2$. Calibration curves generated in this manner exhibited a correlation coefficient (r^2) that was

Table 2
Stability of capsaicin, dihydrocapsaicin, and nonivamide following various storage conditions of quality control standards

Treatment and target concentration (ng/ml)	Capsaicin, % control	Nonivamide, % control	Dihydrocapsaicin, % control
24 h room temperature			
25	98	97	94
500	92	92	93
800	97	95	96
24 h autosampler			
25	108	109	101
500	100	103	102
800	104	104	101
Freeze–thaw			
25	100	100	104
500	109	108	107
800	102	102	102

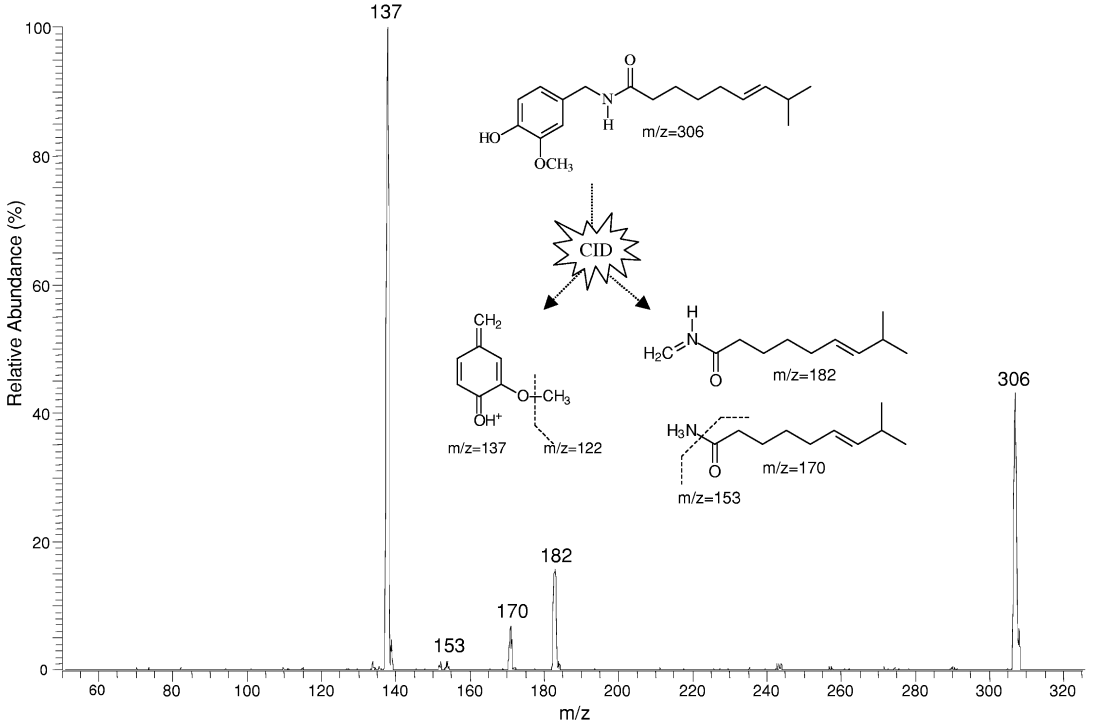

Fig. 2. Mass spectrum of the product ions produced from collision-induced dissociation of capsaicin. The data were generated by introducing a 10 ng/ml solution of capsaicin at a rate of 10 μl/min into the LC flow consisting of methanol–distilled water (57.5:42.5) containing 0.1% formic acid using a syringe pump. The LC flow was directed into the mass spectrometer at a flow-rate of 0.25 ml/min and data for 25 scans collected. The inset represents the proposed fragmentation pathway for the precursor ion of capsaicin (m/z 306).

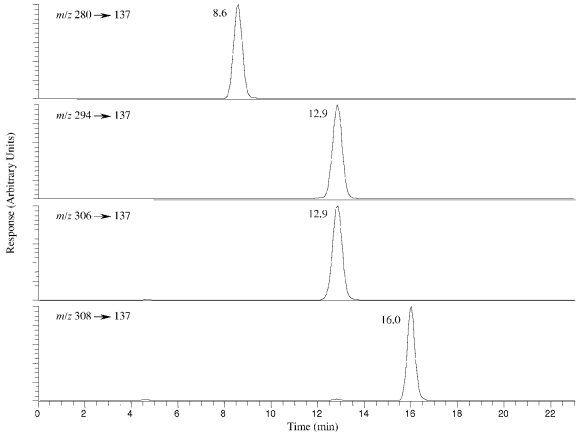

Fig. 3. Representative selected-reaction monitoring profile obtained from analysis of a 200 ng calibrator containing capsaicin, dihydrocapsaicin, and nonivamide by LC–MS–MS. The identities of the peaks are octanoyl vanillamide (m/z 280→137), nonivamide (m/z 294→137), capsaicin (m/z 306→137), and dihydrocapsaicin (m/z 308→137).

typically ≥0.997. The accuracy of the LC–MS–MS assay was ≥90% (n=5) and the intra-assay precision ≤8% RSD for all analytes at the three quality control concentrations. Data that compare the assayed concentrations of the quality control standards using both LC–MS and LC–MS–MS are presented in Table 3. In general, values obtained using LC–MS–MS were within 10% of the values observed when LC–MS was used.

3.3. Analysis of pepper spray products

The data for the analysis of several pepper spray products are presented in Table 4. The analysis of selected samples of pepper spray products by LC–MS demonstrated variability in the concentrations of the capsaicinoids. The total concentration of capsaicin, dihydrocapsaicin, and nonivamide in the samples ranged from 0.7 to 40.5 μg/μl. The relative proportions of each analyte (45–55% capsaicin, 40–

Table 3
Comparison of calculated concentrations of capsaicin, nonivamide, and dihydrocapsaicin in quality control standards using LC–MS and LC–MS–MS

Target concentration (ng/ml)	Capsaicin, % LC–MS value	Nonivamide, % LC–MS value	Dihydrocapsaicin, % LC–MS value
25	104	107	102
500	95	95	91
800	93	94	90

50% dihydrocapsaicin, and ~2% nonivamide) were consistent with previous data for fresh peppers and OC and did not exhibit significant variability in the products tested.

4. Discussion

The development of sensitive and selective methods to accurately quantify the concentrations of the capsaicinoids in pepper spray products is necessary to ensure consistent product composition. Knowledge of the concentrations of each analogue, as well as the total capsaicinoid concentration, is needed to predict the potency and efficacy.

We have developed sensitive and selective methods for the detection and accurate quantification of capsaicin, dihydrocapsaicin, and nonivamide in pepper spray products using LC–MS and LC–MS–MS. Using LC–MS, the assayed concentrations for the fortified quality control standards were ≥88% of the target concentrations for all analytes (Table 1). The method produced nearly identical values for the quality control standards on separate days, exhibiting an inter-assay precision of less than 10% RSD (Table 1). In addition, the samples were not affected by various storage and handling conditions (Table 2). Similar data were obtained when using either LC–MS or LC–MS–MS to identify and quantify the amount of analytes present in the samples (Figs. 1–3 and Table 3).

Unlike previous analytical techniques for the analysis of capsaicin and its analogues, these methods permitted the unique identification of nonivamide in the presence of capsaicin. Previous work using GC–MS or HPLC to quantify the capsaicinoids in pepper products has been limited by the inability to chromatographically separate or to differentiate nonivamide by mass [19–22]. As a result, nonivamide may have been misidentified as either nordihydrocapsaicin (due to the same mass in GC–MS) or capsaicin (due to similar chromatographic properties in HPLC) [19–22]. The methods described here permitted the differentiation of nonivamide and capsaicin by mass-to-charge ratio (Figs. 1 and 3).

Comparison of the values obtained for the analysis of quality control standards using either LC–MS or LC–MS–MS (Table 4) indicated that either method was suitable for the analysis of the capsaicinoids in pepper spray products. However, analysis of an independent and dilute (fourfold) set of quality

Table 4
Concentrations and relative percentages of capsaicin, nonivamide, and dihydrocapsaicin in selected pepper spray products, as determined by LC–MS

Pepper spray sample	Total (mg/ml)	Capsaicin (%)	Nonivamide (%)	Dihydrocapsaicin (%)
Spray 1a	40.5	49.4	2.4	48.2
Spray 1b	35.9	49.4	2.3	48.3
Spray 2a	5.1	48.3	2.3	49.4
Spray 2b	4.8	48.9	2.5	48.6
Spray 3	0.7	55.8	2.1	42.1

The numbers 1–3 represent pepper spray products from three different manufacturers. The letters a or b signify products from the same manufacturer but different product lots.

control samples and calibrators using LC–MS–MS exhibited an increased linear dynamic range for the assay as well as increased accuracy and decreased intra-assay variability (data not shown). Because of this, LC–MS–MS may be useful in identifying the capsaicinoids in dilute or very small samples that may be encountered in forensic analysis. In general, however, either LC–MS or LC–MS–MS can be used to quantify the capsaicinoid analogues in pepper spray products.

Based on our results, pepper spray products exhibit variability in their capsaicinoid content (Table 4). The average percentages for capsaicin, dihydrocapsaicin, and nonivamide were 51 ± 4.0, 47 ± 4.0 and $2.2\pm0.2\%$, respectively. Although the relative concentrations of these capsaicinoid analogues in the individual products were essentially the same, the total capsaicinoid concentration varied significantly. The concentrations of capsaicin, dihydrocapsaicin, and nonivamide ranged from 0.4–20, 0.3–19.6 and 0.015–0.97 $\mu g/\mu l$, respectively. As predicted, we observed differences in the capsaicinoid concentrations in different pepper spray products. These data indicate that a lack of accurate analytical methods for quality control in the manufacture of pepper spray products yields products that have variable capsaicinoid content. As a result, individual pepper spray weapons may exhibit variability in their efficacy and potential to cause adverse physiological responses.

5. Conclusions

These methods for the analysis of capsaicin, dihydrocapsaicin, and nonivamide permit the identification and quantification of the three principal active components in pepper spray products, as well as the qualitative identification of nordihydrocapsaicin, homocapsaicin, and homodihydrocapsaicin. The dynamic range for the assays was 10–1000 ng/ml. The methods utilize a simple dilution technique for sample preparation and analysis. The LLOQ of 10 ng/ml and upper limit of quantification of 1000 ng/ml were sufficient for the simultaneous quantification of these analytes in the pepper spray products that were evaluated. Extending the dynamic range of the assay to higher concentrations of capsaicin and dihydrocapsaicin would be desirable to account for products containing substantially different concentrations of the capsaicinoids, but due to the limitations in the dynamic range of the instrumentation this was not feasible.

As predicted, we demonstrated variability in the capsaicinoid concentration in the different pepper spray products. Based on these data, we propose that variability in the capsaicinoid concentrations in different pepper spray products may contribute to the unpredictability in product efficacy and failure to deter attack. In addition, the demonstration of variability in the concentration of capsaicinoids in different products may provide insight into the possible causes of the unpredictable efficacy and potential to elicit adverse physiological responses that frequently occur from exposure to pepper spray products.

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