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Comparative Analysis of Effect of Various Mobile Phase Solvent Systems in Forensic Examination of Pepper Spray Residues By HPTLC.

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ABSTRACT

The exploitation of plants, toxins and certain synthetic chemicals is incredible as the collective knowledge of chemical production has increased tremendously around the globe. Pepper spray is a lachrymatory agent, the active component of bell peppers, a plant product used in policing, riot control, crowd control, and self-defence by women. Incidences of substance-facilitated criminal activities like robbery, sexual assault & crime against women have been reported to be on rise in recent years. In this study we compared the efficiency of different extraction solvents for the extraction of the capsaicin from pepper spray residue found at the site of crime through Gas Chromatography-Mass Spectroscopy (GC-MS) and the efficiency of eight different mobile phases for High Performance Thin Layer Chromatography (HPTLC) analysis of capsaicin in pepper spray residue. Analysis revealed that the ethanolic and chloroform extracts contain capsaicin by conforming through GCMS. It was also found that solvent system Toulene:Ethylacetate (7:3) when used as mobile phase in HPTLC analysis distinctly separated the components present in pepper spray residue and the Retention factor (Rf) matched with the Rf of standard capsaicin which is 0.25. This finding is in agreement with what was indicated in literature.

Keywords: Defensive Devices, Pepper Spray, Capsaicin, High Performance Thin Layer Chromatography, Gas Chromatography-Mass Spectrometry, Mobile phase.

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INTRODUCTION

Incidences of substance-facilitated criminal activities such as robbery, sexual assault & crime against women have been reported to be on the rise in recent years. There has always been a dire need of handy small devices which can be used by women for self-defence. Aerosol defence spray are devices which can be used very easily and quickly by women in emergency situations. Exploiting the hotness of pepper, Kamran Loghman, invented pepper spray [1]. From then it is being used as a defensive device during attacks. Over decades' police are using it as an alternative to use force on criminals while drastically reducing the harm caused to them due to conventional weapons. It gradually went into the hands of criminals and burglars who now are using it very comfortably for criminal activities. The perpetrators employ highly unusual and very creative methods such as use of pepper spray or chilli powder for incapacitating the unsuspecting travellers/targets. Such irritants-facilitated robberies pose a threat for travel safety. Pepper spray consists of capsaicinoids like capsaicin, dihydrocapsaicin, nordihydrocapsaicin, nonivamide, homocapsaicin, homodihydrocapsaicin [2,3,4] which are extracted from a fruit called *capsicum annuum*, a non-climacteric bell pepper that belongs to the family solanaceae. Out of these, capsaicin and dihydrocapsaicin are responsible for about 90% of the total pungency in bell peppers [5,6]. Capsaicin a phenyl propane compound (trans -8-methyl-N-vanilyl-6-nonenamide) is a lipophilic alkaloid and crystalline having molecular formula $C_{18}H_{27}NO_3$ [7]. Dihydrocapsaicin (N-(4-Hydroxy-3-methoxybenzyl)-8-methylnonanamide) is a lipophilic, colourless and odourless compound having molecular formula $C_{18}H_{29}NO_3$ [8]. Capsaicin is 70 times hotter than piperidine (black pepper) and 1000 times hotter than zingerone (ginger). Capsaicin when comes into contact with human body, it binds to a transient receptor potential vanilloid 1 (TRPV1), which is mainly expressed in sensory neurons. This receptor is ligand operated, non-selective cationic channel present in the small fibres of nociceptive neurons [8]. After contact with the receptor TRPV 1, a series of reactions takes place which leads to increase in intracellular calcium levels that triggers release of neuropeptides like substance P and calcium gene related peptide (CGRP). Thus, the effects capsaicinoids cause on human body when bound to sensory neurons are coughing, disorientation, erythema, reddening of skin, lacrimation, temporary blindness, pain, inflammation and localised burning sensation [9,10]. This ability of capsaicinoids to produce adverse effects lead to the creation of pepper sprays that are widely used in self-defence. Pepper spray usually contains a 10% solution of oleoresin capsicum that is diluted in a suitable solvent like methylene chloride, methanol, trichloroethylene, isopropanol, freons, propylene glycol, ethanol or dimethyl ether and a gaseous propellant N_2 or CO_2 . These aerosol pepper sprays are the real attention these days as their use has ranged from criminal activities to as a defensive device. Reported cases and no regulations on pepper spray led to a greater attention by the public. A striking case series have been reported in media pertaining to the nefarious use of pepper sprays raising public awareness regarding the use of pepper sprays. So it is utmost important for forensic experts to be ready with tools and techniques for the detection, analysis and quantification of pepper spray. The conventional method of analysis of capsaicinoids were replaced by number of instrumental methods like High Performance Thin Layer Chromatography (HPTLC), Gas Chromatography-Mass Spectrometry (GC-MS) which are more reliable and accurate. Chromatographic methods particularly HPTLC and High-performance liquid chromatography (HPLC) are being used extensively for qualitative and quantitative analysis of capsaicin from fruit and also different commercial products [11,12,13].

Chromatography is a technique used to separate different components of a substance based on their affinity towards the solvent. They have a stationary phase usually thin layer of silica gel coated on an aluminium plate and a mobile phase. The mobile phase flows through the stationary phase and carries the components of the substance with it. HPTLC is a rapid inexpensive and accurate method of detection and evaluation of different compositions that are present in herbals, drugs etc [14]. It does not require sample clean up and one can use crude extracts directly, dissolving in a suitable solvent. Mobile phase in HPTLC analysis shows a significant effect on the separation of different compounds present in a sample. The scientific results of exhibits examined depends upon the successful extraction and separation of the capsaicinoids from the exhibits which can be further detected using sophisticated techniques like HPTLC and GC-MS. The most critical parameter is choosing the right mobile phase for separation. Thus, in this work we used Eight different mobile phases for HPTLC analysis of capsaicin which is the active constituent of the pepper spray, to test their efficiency in separating different compounds present in it. We also evaluated the efficiency of different extraction solvents in extracting capsaicin from an aqueous solution of sample.

MATERIALS AND METHODS

Chemicals and Reagents

The solvents Methanol, Hexane, Diethyl ether, Chloroform, Acetic acid, Benzene, Toluene, Acetone, Ethyl acetate, Acetonitrile, Petroleum ether and also Anhydrous sodium sulphate were purchased from Merck India and all solvents used were of HPLC grade. Capsaicin standard was purchased from M/S Sigma Aldrich. Three pepper spray canisters were purchased from local vendors which were from the same manufacturer but different batches.

Sample Preparation

Stains were made on 5 unstained cotton swatches outdoors with the nozzle of the canister nearly touching the swatch. The duration of discharge is approximately three seconds. After application the stains were allowed to dry at room temperature for five hours. These cotton swatches were then dipped in 10 ml of distilled water in extraction bowls and left overnight.

Sample Extraction procedure

Ethanol, Methanol, Chloroform, Diethyl ether, Acetonitrile were used as extraction solvents to determine the effective extraction solvent for the extraction of capsaicinoids from cotton swatches. Extraction bowls having stained cotton swatches with distilled water were taken and added with 10 ml of each solvent respectively. Using a glass rod these swatches were beaten up to allow the chemical to get dissolved in the solvents. These solutions were taken in volumetric flasks and vigorously shaken, the organic solvent layers were separated and passed through anhydrous sodium sulphate to remove any moisture present and left at room temperature to concentrate them. The left 2-3 ml of solutions were packed in vials for GC-MS analysis.

GC-MS Analysis of Prepared Extracts

An Agilent 7890 B GC System – with Agilent 5977A MSD gas chromatograph was used with nonpolar capillary column Agilent HP5-MS Ultra Inert (5% - phenyl methyl siloxane, 30.0 cm × 0.25mm × 0.25 μm) to analyse all the samples. The setting of the instrument was: Oven Temperature initially programmed at 100 °C for 1.5 minutes, and then programmed up to 300 °C at a rate of 7 °C/min. The injector temperature was 280 °C, Injection mode: split-less, Injected volume: 1μL, Carrier Gas – Helium (99.99%), Flow Rate – 1ml/min, Total run time for a cycle was 40 min. Operating parameters for mass detector -Ionization potential of 70 eV, Interface temperature - 200°C; Detector Temperature - 300°C. The acquisition of spectra was between 50-800 m/z. The instrument accesses the international library software of National Institute of Standards and Technology (NIST).

Gas chromatograms obtained for the extracts gave numerous peaks, without distinct separation that could not be interpreted without coupling to mass spectrometry. The easiest way to analyse is by full scan method or TIC (Total Ion Count) because it is possible that in the chromatogram the peaks with low resolution could not separate properly. Assigning chromatographic peaks was accomplished by comparing the mass spectra obtained with reference compounds of known structure stored in the spectra library (NIST) and by comparison to those reported in the literature. The analysis allowed the identification of large capsaicin and Dihydrocapsaicin with a high probability.

HPTLC Analysis of Prepared Extracts

Based on the GC-MS analysis we found Chloroform to be the most effective extraction solvent to extract pepper spray residue and thus used it as a solvent for HPTLC analysis. The sampling and analysis of TLC plates were done using CAMAG Automatic TLC Sampler 4 (Camag, Muttens, Switzerland). Six narrow bands (6mm) having four microliter of the samples, two tracks of each sample and standard capsaicin (1 mg/ml in methanol) were sprayed on pre-coated 10x10 Silica gel 60 F254 TLC plates. Methanol was used as a solvent and nitrogen gas was provided as pressure for spraying the sample homogeneously across the application area. After application, the plates were dried at room temperature for 5 minutes. Now, these plates were developed in a CAMAG HPTLC 10x10 twin trough chamber saturated with mobile phase.

The different mobile phases used were given in Table 1. The samples were allowed to move, till the solvent front reaches 80mm on the plate and then were removed for drying.

Table 1: Different solvents used to make mobile phases for HPTLC analysis

Solvent System	Solvents Used	Ration taken	Reference
A	Petroleum ether: Chloroform: Acetonitrile	40: 45: 15	15
B	Benzene: Methanol	80: 05	16
C	Hexane: Diethyl ether	80: 30	17
D	Chloroform: Methanol: Acetic acid	9.5: 0.5: 0.1	18
E	Toluene: Ethyl Acetate	7: 3	19
F	Chloroform: Methanol: Acetic acid: Hexane	2.85: 0.15: 0.15: 1	20
G	Toluene: Chloroform: Acetone	5.5: 2.6: 1.9	21
H	Methanol: Water	9.5: 0.5	22

RESULTS AND DISCUSSION

Solvent Extraction

The samples and Capsaicin standard were analysed by GC-MS. In this study presence of capsaicin and dihydrocapsaicin were confirmed present in ethanolic extract and capsaicin in chloroform extract by way of comparison of Retention time (Fig. 1 & 2) and matching with MS fragmentation. The peaks detected were identified as capsaicin and dihydrocapsaicin on their respective retention times and GC-MS spectrum, the principle peak at m/z were given (Fig. 1 & 2). The capsaicinoids obtained from extracts of both ethanol and chloroform were given in Table 2.

Table 2- Capsaicinoids obtained in Ethanolic and Chloroform extracts of pepper spray residue.

Solvent used	Compound name	Retention time (min)	Molecular weight (kg/mol)	m /z ratio	Probability
Ethanol	Capsaicin	26.545	305.2	137,152,151,122,305,195,55,41	91.1%
	Dihydrocapsaicin	26.799	307.2	137,151,138,195,152,307,122,43,153,41	75%
Chloroform	Capsaicin	26.661	305.2	137,207,138,55,152,69,57,97,41,83	65%

Fig. 1 - Chromatogram, Mass spectrum and structure of capsaicinoids of pepper spray residue ethanolic extracts identified by GC-MS.

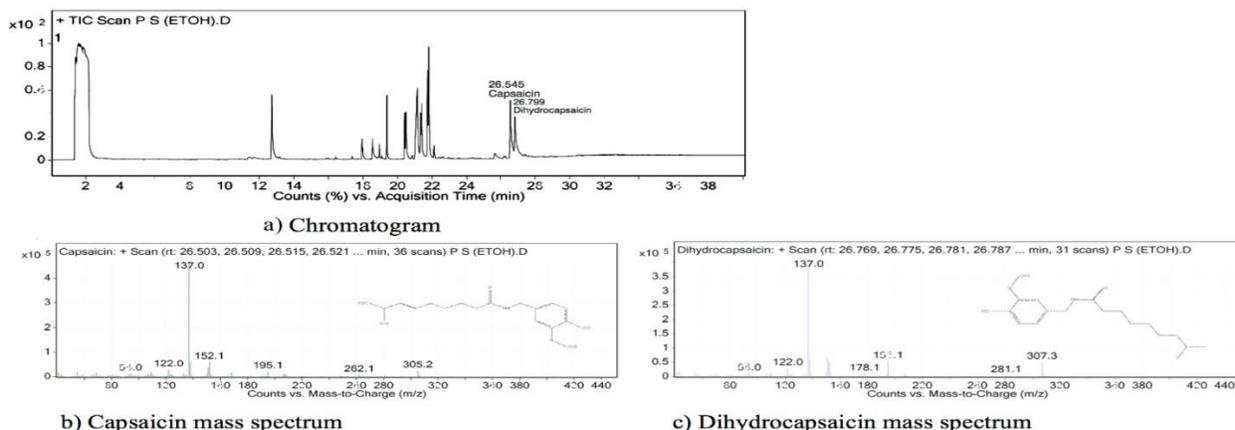
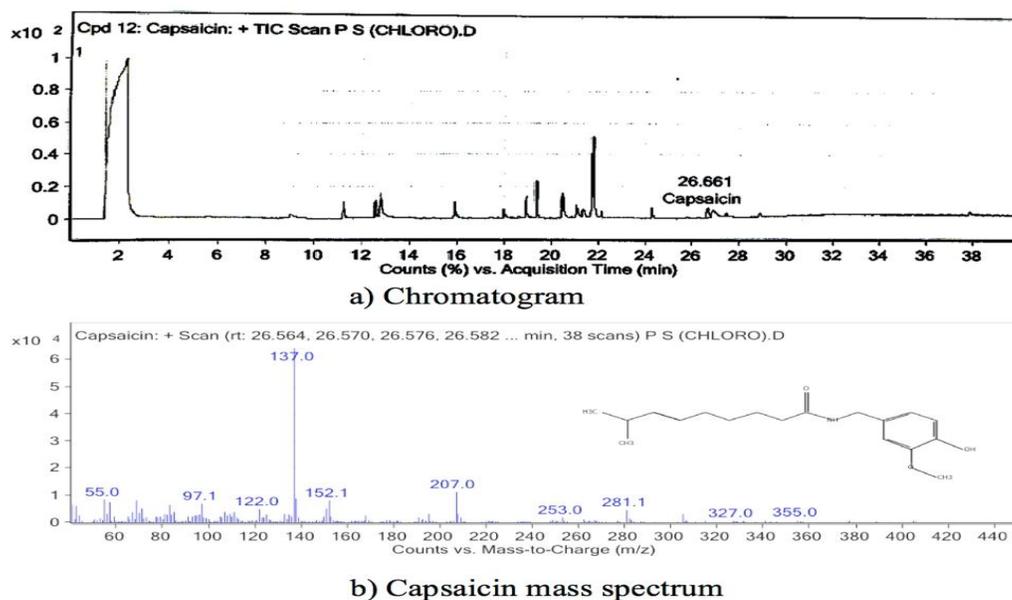


Fig. 2 - Chromatogram, Mass spectrum and structure of capsaicinoids identified by GC-MS in the chloroform extracts of pepper spray residue.



HPTLC Analysis

The compositions of mobile phase were obtained by exhaustive literature search and optimised to establish a precise and suitable method for HPTLC analysis of pepper spray. The plates developed through different mobile phases were visualised under UV 254 nm, UV 366 nm and white light with 85-point exposure using CAMAG TLC visualizer 4. It was observed that the R_f values of capsaicin were different for plates developed in different mobile phases and corresponded to the literature. The TLC plates were scanned using deuterium light (200-400nm) and the R_f of each spot was calculated. The output spectra of samples under different mobile phases were obtained, recorded and analysed. The developed plates and UV absorption spectra for various mobile phases were given (Fig. 3 & 4). Through literature search it was found that capsaicinoids absorb maximum at a light of wavelength 281 nm and 227 nm [22]. So, we obtained the R_f of those bands which had λ_{max} at 281 nm or 227 nm which corresponds to capsaicin. The different R_f 's obtained for different mobile phases were given in Table 3. TLC analysis for mobile phase solvent system F was done and the R_f was found to be 0.83. Out of eight mobile phases used, 95%

methanol was not successful in separating the compounds. Capsaicin, the active ingredient of pepper spray residue was best separated using the mobile phase E. It was found that the R_f of band corresponding to capsaicin standard was the same as the one found in pepper spray residue analysed using mobile phase system E (Fig. 3)

Table 3: R_f values of capsaicin identified in pepper spray residue developed using various mobile phases through HPTLC.

Solvent System	Solvents Used	Retention factor
A	Petroleum ether: Chloroform: Acetonitrile	0.53
B	Benzene: Methanol	0.38
C	Hexane: Diethyl ether	0.38
D	Chloroform: Methanol: Acetic acid	0.32
E	Toluene: Ethyl Acetate	0.49
F	Chloroform: Methanol: Acetic acid: Hexane	0.83
G	Toluene: Chloroform: Acetone	0.61
E	Methanol: Water	-

Fig. 3 - TLC plates developed using different mobile phases.

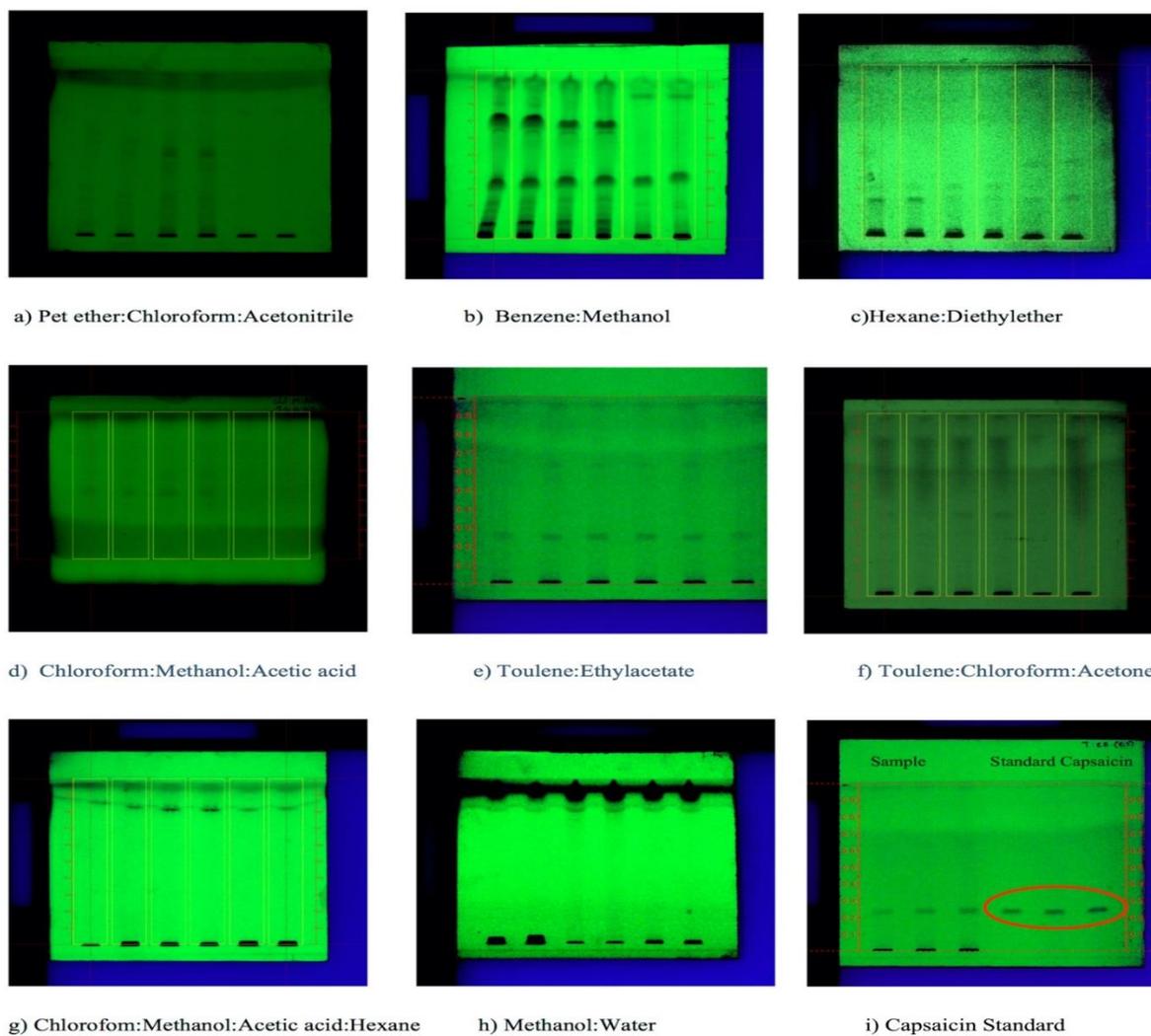
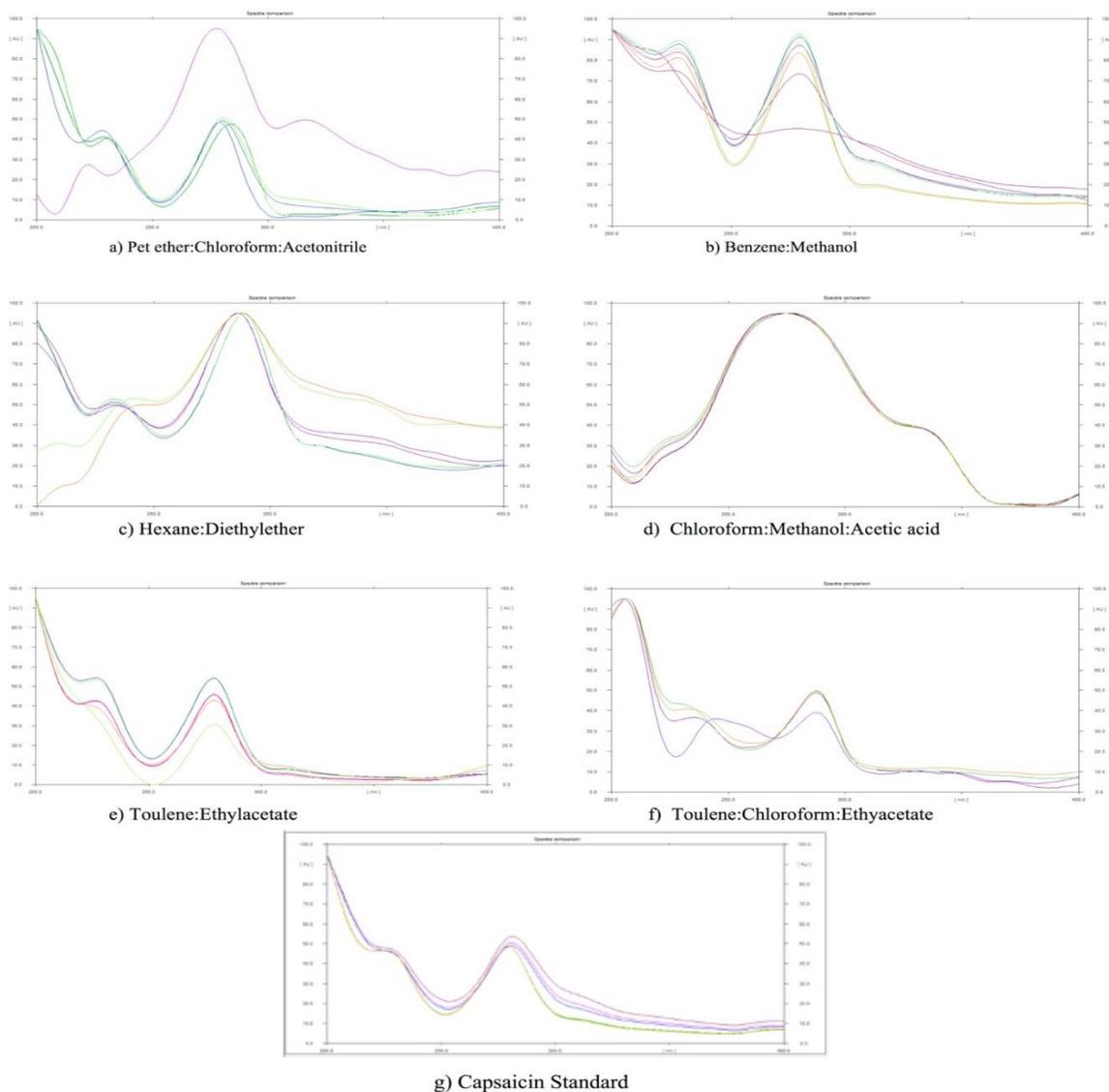


Fig. 4 - UV absorption spectrums for chloroform extracts of pepper spray residue developed using various mobile phases showing the absorption peaks of capsaicin.



CONCLUSION

HPTLC analysis of capsaicinoids was found to be an easy and effective process for its detection and quantification. The only prerequisite for this technique is to select the most effective mobile phase for the best separation and detection of the compounds. The main goal of this study is to identify the suitable mobile phase for HPTLC analysis of Pepper spray residue. Our studies showed that chloroform and ethanol are the best solvents to extract capsaicinoids from pepper spray residue found at the site of crime. On performing HPTLC analysis of pepper spray residue using 8 mobile phases, we found that the mobile phase Toluene: Ethyl Acetate (7:3) is very effective in separating capsaicinoids in the residue from the complex matrix whereas the mobile phase Methanol: Water is not successful in separation. The other systems studied suffered from tailing or incomplete separation. The R_f of capsaicin in the plate developed using mobile phase Toluene: Ethyl Acetate was found to be 0.25. Thus, the aim of the study to find the best extraction solvent to extract capsaicin and the best mobile phase for HPTLC analysis of pepper spray residue has been fulfilled.

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