Technical Note

Determination of Δ^9-THC in Whole Blood using Gas Chromatography–Mass Spectrometry

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Abstract

A simple and reliable liquid–liquid extraction method for the determination of Δ^9-tetrahydrocannabinol (THC) in whole blood utilizing gas chromatography–mass spectrometry in electron impact mode is described. The substance is derivatized with pentafluoropropionic anhydride in pentafluropropanol. The limit of detection is 0.5 ng/mL for a 1-mL specimen, with recovery greater than 70%. The intra-assay coefficient of variation (CV) is 3.1% to 5.2%, and the interassay CV is 6.4% to 9.5%, calculated at THC concentrations of 1, 5, and 25 ng/mL. The accuracy is between 95 and 97%. The optimization of extraction and derivatization conditions is detailed.

Introduction

In recent times, the analysis of the primary active component of cannabis, Δ^9-tetrahydrocannabinol (THC), has become more common in standard toxicological examinations, whereas previously 11-nor-Δ^9-carboxy-tetrahydrocannabinol (cTHC) had been the analyte of choice to indicate past use of cannabis (1).

The detection times for THC in blood (and plasma) are significantly shorter than for cTHC, due to the very rapid distribution of THC to tissues including sequestration in body fat (2). Concentrations of THC in plasma are known to decrease to under 1 ng/mL after 4–6 h of exposure to cannabis (3,4).

A number of methods have been published for analysis of THC using gas chromatography–mass spectrometry (GC–MS) in plasma and blood (2,4–11). Methods exist for fat (12) and hair (13,14). A number of these methods have utilized solid-phase extraction (SPE) (8,11,12); however, liquid–liquid extraction techniques (LLE) remain more popular.

Negative chemical ionization (NCI) was utilized by Foltz et al. (6) to detect sub-nanogram levels of THC. Tandem mass spectrometry (MS–MS) has also been used to detect extremely low THC at concentrations as low as 50 pg/mL (15), although these systems are also expensive.

Conventional electron impact MS (EI-MS) has been used to detect THC in the blood or plasma. These methods used 3–5 mL of matrix in order to achieve adequate sensitivity (2,5,7). Improvements in detection limits using EI-MS have been made using multiple extraction steps (11). Because it is more difficult to extract THC from blood, plasma has been the matrix of choice in most LLE methods. However, plasma samples are not always available, particularly in forensic cases. Additionally, the majority of assays published analyzed clinical samples, whereas postmortem blood presents additional difficulties such as variable quality and putrefaction that may affect reliable extraction and recovery (8,9,11).

Although most methods use silyl derivatives for the analysis of THC, the use of pentafluoropropanol (PFP) and pentafluoroproprionic anhydride (PFAA) has the advantage of not affecting column performance (11).

We report a validated, simple, and robust LLE method for extraction of THC from antemortem and postmortem blood, utilizing EI-MS of PFP derivatives.

Materials and Methods

Reagents

Methanol, toluene, ethyl acetate (Asia Pacific Specialty Chemicals Ltd., Clayton, Australia), and acetonitrile (Mallinckrodt, Rowville, Australia) were of analytical high-performance liquid chromatography (HPLC) grade. High purity n-hexane HiPerSolv™ (BDH Laboratory Supplies, Poole, England) was used. Anhydrous ethyl acetate was prepared by addition of a 1.5- to 2.5-mm molecular sieve (Ajax Chemicals, Auburn, Australia) to a sealed vessel containing ethyl acetate (99.5% purity) (Asia Pacific Specialty Chemicals Ltd.).

A 1M ammonium sulfate solution (Ajax Chemicals) was used to produce the buffer for blood extraction, adjusted to pH 4.5 by the addition of 0.1M sulfuric acid (Ajax Chemicals).

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For the derivatization process, 2,2,3,3,3-pentafluoro-1-propanol (PFP) (Sigma Aldrich Pty Ltd., Castle Hill, Australia), and pentafluoropropionic anhydride (PFAA) (Pierce Chemical Co., Rockford, IL) were used.

All glass extraction tubes were silanized by immersion for 1 h in a 5% solution of Surfasil® (Pierce Chemical Co.) in toluene, followed by rinsing in methanol and oven drying prior to use.

Working standards

Stock solutions of THC (1 mg/mL) and the internal standard THC-d_3 (100 mg/mL) (Radian International, now Cerilliant, Round Rock, TX) were freshly diluted in methanol for each assay. Working standards were added to whole blood to produce a seven-point calibration curve with concentrations ranging from 1 to 100 ng/mL.

Quality controls

In order to ensure a measure of quality assurance, all assays performed contained in-house controls prepared by an independent analyst, with a new batch used every two months. A different THC stock solution lot was used by the independent analyst to prepare the controls. The designated "low" control contained a nominal 10-ng/mL level of THC, and the "high" control contained a nominal 50-ng/mL level. All controls were stored at –60°C.

GC–MS

GC–MS was utilized for all analyses of blood for THC. A model 6890 GC coupled with a 5973 mass selective detector was operated in electron impact mode (EI) and selected ion monitoring (SIM) (Agilent Technologies, Melbourne, Australia). A 30-m HP-5 MS fused-silica column coated with a 5% phenyl methyl silicone liquid phase with 0.25-mm i.d. and 0.25-mm film thickness (Agilent Technologies) was used. High-purity helium was used as the carrier gas.

Samples were injected (1 mL) using a model 7683 series autosampler and series injector (Agilent Technologies) operating in splitless mode, with temperature zones for the injector port set at 250°C, detector at 300°C, quadrupole at 150°C, and ion source at 250°C. The temperature program was set for an initial temperature of 70°C for 1 min, increasing at 20°C/min to 300°C, with the final temperature of 300°C held for 6 min. The total run time was 18.5 min.

In SIM mode, ions monitored for detection of THC-d_3 were *m/z* 463 and 420. Detection of THC was achieved by monitoring *m/z* 460, 417, and 445. The MS was activated at 9.5 min and turned off at 11.5 min. Dwell time was 20 ms/amu and solvent delay was set at 9.50 min.

Blood extraction procedure

Specimens were thawed at room temperature and placed on a Ratek rotator instrument (Ratek Technologies, Melbourne, Australia) for 15 min before use in order to mix the contents uniformly.

To each freshly silanized tube, 1 mL of blood (sample, control, or standard) was added after initial addition of 25 ng of THC-d_3 as the internal standard (IS). After allowing 5 min for equilibration of the sample, 1.0 mL of 1M ammonium sulfate buffer (pH 4.5) was added, followed by agitation on a vortex mixer (Ratek Technologies).

Samples were extracted with 7 mL n-hexane on a Ratek rotator for 60 min. After centrifugation at 3500 rpm for 10 min in a Sorvall centrifuge (Sorvall Instruments, Sydney, Australia), the tubes were placed in a Dynavac freeze bath (Dynavac, Wendouree, Australia) at –30°C in order to separate the supernatant from the frozen aqueous layer. The solvent layer was transferred to a fresh silanized extraction tube. Supernatants were then evaporated to dryness in a SpeedVac SVC200H sample concentrator (Savant Industries, Selby Australia Pty Ltd., Melbourne, Australia).

The dried extracts were derivatized by the addition of 25 mL PFP and 50 mL PFAA, capped, and placed in a heating block (Pierce) at 70°C for 25 min. Under a slow stream of nitrogen, the tubes were evaporated to dryness at room temperature. Residues were reconstituted with 100 mL anhydrous ethyl acetate and transferred to glass inserts inside autosampler vials for injection.

Using this method, up to 50 samples (including controls and standards) could be prepared daily for analysis.

Analyte identification

Relative retention times (RRT) to internal standard were calculated for THC, in addition to identification based on the relative abundance and ratio of the base and qualifier ions. For identification, retention times for sample peaks were required to be within 0.003 min of the corresponding retention time of the spiked standards in order to be deemed acceptable. In addition, the ratios for the two qualifier ions were required to be within 20% of the ratios calculated in the 25-ng/mL calibrator (used to establish ion ratios), in order for the identification of THC to be confirmed. The seven-point calibration curve was calculated by dividing the peak-area response of THC by the corresponding THC-d_3 peak-area response, and relating these back to the assigned values in the curve, at 1, 2, 5, 10, 25, 50, and 100 ng/mL.

Stability of derivatized samples

Triplicate sets of derivatized extracts were re-analyzed over a total period of seven days to determine their stability, when stored in a refrigerator at 4°C or at room temperature before injection.

Validation

Accuracy of the assay was determined by comparing the value of the intended spiked concentrations with the calculated results for the two in-house quality control specimens (n = 8 for each), which were prepared by an independent analyst in our laboratory. Intra-assay precision was calculated by analyzing up to 10 replicate THC specimens spiked at 1, 5, and 25 ng/mL and expressed as a coefficient of variation percentage (CV %). Reproducibility was assessed by the comparison of these same concentrations across separate assays (n = 5, 10, and 10, respectively), in addition to the two in-house controls analyzed over separate assays (n = 8 for each). A regression analysis of the points in the calibration curve required a correlation coefficient

of $r^2 = 0.99$ for the curve to be accepted. The THC recovery was calculated by dividing the peak-area responses of the 25-ng/mL and 100-ng/mL THC calibration standard with the peak-area responses of a corresponding 25 ng and 100 ng THC pure unextracted standard (derivatized without matrix), and reported as an average percentage. Recovery of THC-d_3 was similarly conducted, where peak-area responses were compared with the peak-area response of a 25 ng THC-d_3 pure unextracted standard.

Assay optimization

Eight buffers that were added to the blood samples prior to extraction were compared in conjunction with a selection of 10 extraction solvents that have appeared in the literature for extraction of THC.

A comparison was made between a single extraction for 60 min and a double extraction for 30 min each. When a double extraction was used, the two supernatants were combined before being reduced to dryness and subjected to further treatment. An initial experiment involved triplicate samples of THC-free blood spiked at 15 ng/mL THC extracted in one 6-mL volume of *n*-hexane for 60 min and with two 6-mL volumes of *n*-hexane for 30 min each. An additional experiment was performed where supernatants from the second extraction were not combined, but rather were analyzed separately. This was investigated using duplicate samples of six different concentrations of THC: 5, 10, 15, 25, 50, and 100 ng/mL. In addition, an assessment of the effect of extraction time on recovery was made. This compared extraction for 60 min to samples extracted with a single 30 min extraction time ($n = 16$).

An evaluation of the optimum length of time and temperature of derivatization was made by assessing assay performance at derivatization temperatures of 50°C, 60°C, 70°C, 80°C, and 90°C for periods of either 20 or 40 min. In further derivatization optimization, the effect of different ratios of PFP to PFAA was assessed. The volumes of PFP/PFAA (in microliters) were 5:50, 10:50, 25:50, 50:50, 50:25, and 50:10. Triplicates at each ratio were used to assess the magnitude of the peak response.

The use of standard pipettes with disposable plastic tips was compared to pipettes containing glass tips for the preparation and dispensing of standards, in order to examine whether surface type had an effect on the assay.

The effect of sonication of the blood sample prior to extraction as opposed to samples that had been thawed and gently mixed before use was examined. Two spiked samples and one case sample were sonicated by insertion of a probe into the specimen after thawing and held for approximately 30 s. These results were related to corresponding samples that were only gently mixed prior to use.

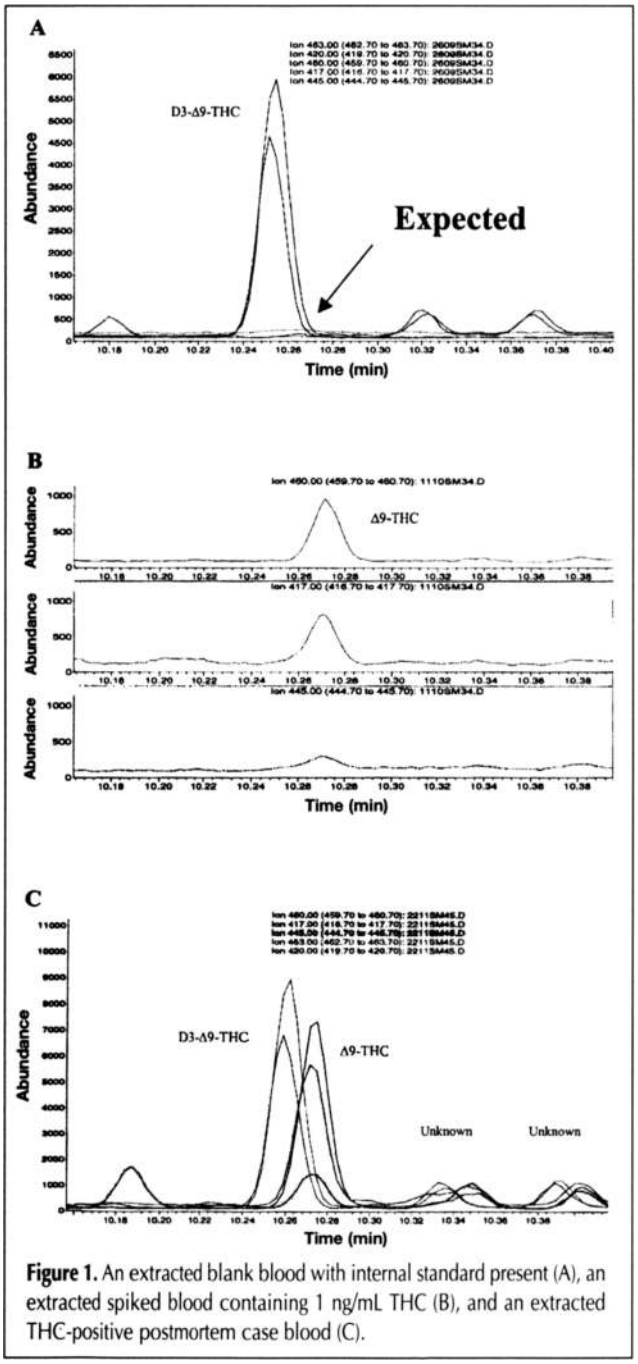

Figure 1. An extracted blank blood with internal standard present (A), an extracted spiked blood containing 1 ng/mL THC (B), and an extracted THC-positive postmortem case blood (C).

Results and Discussion

Using the method described, SIM analysis of both THC and THC-d_3 in postmortem blood using the nominated ions revealed chromatograms that were free of interference from putrefactive or other endogenous substances (Figure 1). Blank antemortem and postmortem blood show no peaks in the region eluted by THC. THC-d_3 and THC eluted at approximately 10.26 min and 10.28 min, respectively. For a 1-ng/mL THC spiked concentration in blood, a signal-to-noise ratio of at least 3:1 was achieved for each of the three ions.

The limit of detection (LOD), defined as being the minimum concentration equivalent to or greater than three times the background noise that could still be detected for each of the three ions, was estimated at 0.5 ng/mL for a 1-mL specimen. This value is better than many published methods using EI-MS and would be sufficiently sensitive to allow detection of recent cannabis use (16,17). The lower detection limit was possibly a result of a successful reduction in background ion response caused by the use of n-hexane. The limit of quantitation (LOQ),

defined as being the equivalent of the lowest calibration level that was able to be quantitated with a precision of at least ± 20%, was 1 ng/mL. As concentrations in routine analyses conducted in our laboratory have indicated higher THC concentrations than had initially been expected, a LOQ of 1 ng/mL was regarded as being sufficiently sensitive for use in routine work.

Accuracy for the 10 ng/mL (n = 8) and the 50 ng/mL (n = 8) in-house quality controls was between 95 and 97% of target amounts (Table I). Intra-assay precision conducted at 1-, 5-, and 25-ng/mL concentrations revealed a CV of 3.4% (n = 10), 5.2% (n = 10), and 3.1% (n = 6), respectively. Interassay reproducibility for 1, 5, and 25 ng/mL was calculated at 9.5% (n = 5), 7.4% (n = 10), and 6.4% (n = 10), respectively. The precision for the two in-house controls were similar to those of the standards, at 7.8% and 7.7% for the 10 and 50 ng/mL concentrations, respectively (n = 8 for each).

Using this method, 25 random drivers positive for THC in 1999 revealed a THC concentration range of 2.1–252 ng/mL and a median concentration of 9.9 ng/mL. An assessment of 10 drivers (charged with driving under the influence of cannabis) revealed a THC concentration range of 5 and 20 ng/mL and a median concentration of 7.5 ng/mL.

The average recovery of THC was calculated at 73% (n = 13) in standards and 74% (n = 40) for the low and high in-house controls (Table II). The average internal standard recovery in standards and postmortem specimens was 82%.

The validation data were comparable to that observed in published methods in which validation data were supplied (4,6,8–11). Of these methods, only one utilized the same derivatizing agent as the described method (11). Recoveries were comparable or greater to other published methods in blood and plasma (4–11), although the methods used to calculate recovery have varied across publications, and thus direct comparisons are not always appropriate.

The ion ratios across the calibration curve were consistent, with a maximum of 20% relative variation allowed for m/z 417/445 and 445/460 ratios before the confirmation was rejected. An examination of 10 assays conducted using the same column revealed an average ion ratio (% ± S.E.M.) across the calibration curve of 80.2 ± 0.8% and 18.7 ± 0.4% for m/z 417/460 and m/z 445/460, respectively, indicating that the ratios were highly reproducible over time. There was no appreciable difference in ion ratios across the calibration range.

Derivatized extracts were found to be stable when re-analyzed after storage at both ambient temperature and 4°C for 1, 3, and 7 days, indicating that derivatized samples could be left for a number of days without significant deviation from the initial values.

Optimization of the described method provided significant improvements in assay performance. It was desirable to add a buffer prior to extraction because it assisted in precipitating proteins from the blood and consequently improved reproducibility (6). Of the buffers initially tested, sodium acetate at pH 4.5 extracted with n-hexane showed superior recovery and lower baseline noise compared to other solvents. An average recovery of 74% was recorded, compared with pH 9.2 tris buffer (67%), pH 9.5 sodium tetraborate (51%), pH 3.1 sodium acetate (34%), pH 5.0 sodium acetate (34%), and pH 7.4 PBS (16.5%) using n-hexane as solvent.

Once sodium acetate (pH 4.5) had been identified as the optimal buffer in the initial experiments, a further two buffers were evaluated. Zinc sulfate (1M) was found to cause the specimen to clump extensively, making a consistent extraction difficult, as reflected by an extremely poor average recovery of only 16%. Recoveries using ammonium sulfate (1M) were more comparable with sodium acetate and revealed slightly superior chromatography with fewer peaks in the proximity of THC. For this reason, it was chosen as the preferred buffer.

Initial experiments showed that the solvents n-hexane/ethyl acetate (5:1, 7:1, 8:1), heptane/ethyl acetate (4:1) and dichloromethane generally exhibited poorer recoveries with all buffer types. Neither iso-pentane nor heptane was comparable to n-hexane, with recoveries calculated at 29% and 53%, respectively. Cyclohexane also gave a lower average THC recovery than n-hexane (Table III).

Although n-hexane proved to give a reliable and efficient extraction from blood, polar solvents such as an n-hexane/ethyl acetate mixture are capable of extracting THC efficiently out of the matrix. However, such solvents have also been reported to increase extraction of unwanted components and endogenous materials in the blood, resulting in increased chromatographic interference (6) and thus a likely decrease in recovery.

A number of methods have previously used multiple solvent extractions of the single sample (4,5,18). In a specific experiment conducted to assess the benefit of multiple extractions, the average THC recovery using a single-step extraction with n-hexane was similar (94%, n = 3) to the recovery when two successive extractions using n-hexane were combined (87%, n = 3). These results formed no part of the method validation conducted, and thus are not included in Table I. When supernatants from the second extraction were analyzed separately, the increase in recovery was only 5.2% (n = 12). A single-step extraction was therefore seen as preferable because it was more time ef-

Table I. Assay Accuracy and Reproducibility

	Analyte	N	Spiked concentration (ng/mL)	Detected concentration (ng/mL) ± SEM	Accuracy (%)	CV (%)
Intra-assay (spiked samples)	Δ^9-THC	10	1.0	0.97 ± 0.01	N/A	3.4
		10	5.0	5.08 ± 0.08	N/A	5.2
		6	25.0	25.98 ± 0.29	N/A	3.1
Interassay (spiked samples)	Δ^9-THC	5	1.0	0.97 ± 0.04	N/A	9.5
		10	5.0	5.09 ± 0.12	N/A	7.4
		10	25.0	25.00 ± 0.51	N/A	6.4
Inter-assay (in-house controls)	Δ^9-THC	8	10.0	9.67 ± 0.16	97	7.8
		8	50.0	47.43 ± 0.80	95	7.7

ficient and allowed for more than 50 specimens to be processed daily.

In contrast, a comparison of samples extracted for 30 min with those extracted for 60 min revealed substantial increases from 56% (n = 16) to 73% (n = 13) for THC and 61% (n = 16) to 83% (n = 19) for THC-d_3. Because of the substantially increased recovery of THC (17%), the longer extraction time was retained in the final method; however, shorter times could be used if sensitivity was not an issue.

Acylation of THC with the alcohol-anhydride reagent mixture of PFP and PFAA was the chosen method of derivatization of the phenolic hydroxy group. This derivatization technique has been used in published methods for detection of cannabinoids in blood (11), hair (14,19), and urine (20,21). Derivatizing at temperatures between 50°C and 90°C made only a small difference to the recovery of the analytes; however, a 20-min derivatization time at 70°C to 90°C (n = 3 at each temperature) revealed slightly lower baseline noise than at 50°C or 60°C. This could be possibly due to higher temperatures increasing the completeness of derivatization. Leaving the tubes to derivatize for 40 min (at 50°C to 90°C) rather than for 20 min did not have any significant effect on the recovery of THC and the THC-d_3.

In an attempt to increase the signal-to-noise response in the chromatography, the use of any plastic in the listed method was replaced with glass equivalents. Specifically, the disposable polypropylene tips used to pipette drug standards, derivatizing agents, reconstitution solvent and the final transfer of reconstituted samples to autosampler vial inserts were substituted with fixed glass pipettes. Although it has been reported that plastics can prompt the loss of cannabinoids in pipetting and in transferring THC-containing liquids (22,23), a comparison of pipettes using plastic tips and pipettes using glass tips indicated that there were slightly lower recoveries when dispensing with glass. Average THC recovery (± S.E.M.) over a range of concentrations with glass pipettes was 84.7 ± 7.2% of the responses using pipettes with plastic tips (n = 8). Because the techniques utilizing plastic proved more time efficient and less subject to cross-contamination, they were retained in the final method.

Sonication of blood was conducted to assess whether assay recovery might be increased because of a decrease in the size of solid particles; however, this was found to have no effect on recovery compared to samples that were gently mixed. Notably, the quality of chromatography was reduced after sonication because of increased baseline noise.

Two unidentified peaks were observed that eluted shortly after both the target THC and THC-d_3 peaks. These gave similar mass spectral characteristics (Figure 1), with the first of the peaks eluting approximately 0.06 min and the second at approximately 0.11 min after the target analyte. Furthermore, statistical analysis of ion ratios in both peaks for the m/z 417 (n = 18) and m/z 445 (n = 18) qualifiers relative to m/z 460 revealed that they were consistent throughout all the volume permutations, with S.E.M. values ranging between 0.10% and 5.4%. Although consistent, the ratios in the two additional peaks themselves were markedly different from each other.

The presence of these peaks was found to be linked with com-

Table II. Assay Recoveries for all Blood Samples

Analyte	Source	N	Spiked concentration (ng/mL)	Recovery (%)
Δ^9-THC	Calibration curve	13	100.0	73.0
	In-house controls	20	10.0	71.0
	In-house controls	20	50.0	76.0
Δ^9-THC-d_3	Calibration curve	5	25.0	83.0
	Actual cases	10	25.0	82.0
	In-house controls	10	25.0	93.0

Table III. Recovery of Δ^9-THC in all Buffer and Solvent Types

	Recovery of THC (%)							
Buffer/Solvent	Sodium acetate pH 3.1	Sodium acetate pH 4.5	Zinc sulfate pH 4.5	Ammonium sulfate pH 4.5	Sodium acetate pH 5.0	PBS pH 7.4	Tris buffer pH 9.2	Sodium tetraborate pH 5.0
n-Hexane/EA (5:1)	35	65	**21**	n/a	27	**23**	45	16
n-Hexane/EA (7:1)	n/a	64	15	n/a	31	14	43	19
n-Hexane/EA (8:1)	n/a	60	15	n/a	31	15	50	25
n-Hexane/EA (9:1)	33	64	13	n/a	27	13	44	27
n-Hexane	34	**74**	16	**72**	34	17	**67**	**51**
iso-Pentane	25	29	n/a	n/a	33	n/a	n/a	n/a
Heptane	**57**	53	n/a	n/a	**46**	n/a	n/a	n/a
Heptane/EA (4:1)	n/a	42	20	n/a	40	20	31	34
Dicloromethane	n/a	34	12	n/a	12	12	33	33

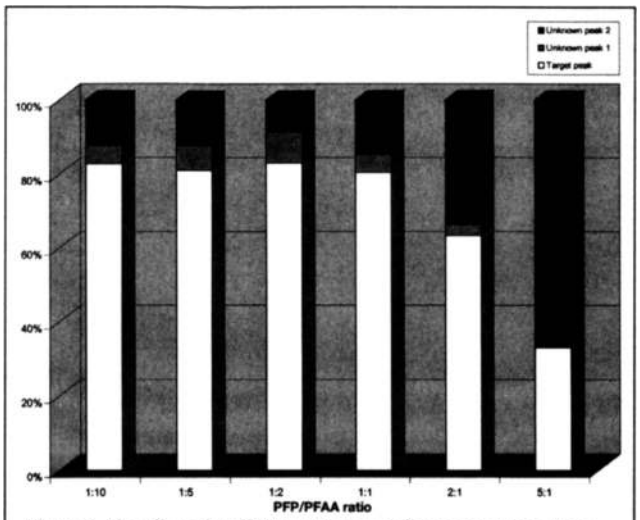

Figure 2. The effect of PFP/PFAA ratios on peak response proportions.

pleteness of the derivatization procedure, where the relative area of these peaks for both the analyte and the IS were found to change with the ratio of PFP to PFAA used in the derivatization. A PFP/PFAA volume ratio of 5:1 revealed a second peak double the size of the first peaks, compared with only 10% at a 1:2 ratio. There was little difference in the peak responses between the 1:10, 1:5 or 1:2 ratios. The first unknown peak remained very small throughout most of the variations and did not appear at all at a 5:1 ratio of PFP/PFAA (Figure 2).

Thus, high proportions of PFP to PFAA showed a notable drop in the response for THC, in addition to a large response increase in the second unknown peak. Conversely, at higher proportions of PFAA to PFP, chromatography was far superior for THC, as was the response for the target peaks. The fact that a statistical analysis of ion ratios in the two additional peaks indicated consistency for the *m/z* 417/460 and *m/z* 445/460 ratios suggests that the peaks could be caused by an isomer or analogue of THC. However, the exact explanation behind this phenomenon is unknown. It is of interest that similar peaks were present in the chromatograms presented by Felgate et al. (11), where the same derivatizing agents were used.

Because concentrations of THC in the blood can rapidly decrease after smoking to the low nanogram-per-milliliter levels (24), a sensitive and reliable MS assay was developed that was suitable for detection and quantitation of THC in whole blood from clinical and postmortem samples. Using 1 mL of blood, the LOQ was 1 ng/mL and the LOD was 0.5 ng/mL.

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