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New Applications of Mass Spectrometry for Drug and Lipid Analysis

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NEW APPLICATIONS OF MASS SPECTROMETRY FOR DRUG AND LIPID ANALYSIS

NEW APPLICATIONS OF MASS SPECTROMETRY FOR DRUG AND LIPID ANALYSIS

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Chemistry

By

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December 2012
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ABSTRACT

Mass spectrometry is an important tool used in many different disciplines and settings that include forensics, drug discovery, environmental analysis, and proteomics. Gas chromatography – mass spectrometry (GC-MS) and matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI – TOF MS) are two of the most important instruments used for analysis of compounds. Chapters 1 and 2 of this discussion use GC-MS for the investigation of synthetic cannabinoids in ‘K2’ incense products and the detection of metabolites in urine samples from individuals suspected of consuming these mixtures. Analytical standards were synthesized and used for identification and confirmation of structures. Detection of these compounds and their metabolites is important since ‘K2’ products are banned nationwide as a Schedule 1 substance. Chapter 3 uses LDI-TOF MS for the analysis of triacylglycerol (TAG) degradation products in fingerprint samples exposed to light/dark conditions on four different surfaces: stainless steel, glass, plastic, and iron. A standard of triolein was used for identification of products with the detection of C7:0 and C8:0 aldehyde and carboxylic acids through tandem mass spectrometry analysis. The age of a fingerprint was estimated through comparison of unsaturated and saturated TAGs. Analysis of TAGs in fingerprints is important as a possible dating technique which could provide investigative leads or a timeline of events in a criminal investigation. Chapter 4 uses MALDI-TOF MS for the development of a rapid separation technique to overcome suppression effects of TAGs by phosphatidylcholine (PC). A solid phase extraction (SPE) technique was developed to separate these classes of compounds using reference lipids and real samples (beef, egg yolk) as models. Because lipids play important roles in biological systems, a method to clearly detect and overcome suppression effects is important. Chapter 5 uses GC-MS for the analysis of fatty and resin acids in biomass fermentation process

waters. An extraction procedure was developed for detection and quantification of these compounds. Monitoring these acids in water samples is important because they can negatively impact environmental and industrial pathways.

This dissertation is approved for recommendation
to the Graduate Council.

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Chapter 4

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INTRODUCTION

Mass spectrometry is a technique used to detect and quantitate analytes in a sample. This method of analysis includes determining the elemental composition of a sample and elucidating the chemical structure of compounds. Because of the applicability to analyze a wide range of materials, mass spectrometry has been used in many different settings that include forensics, pharmaceuticals, proteomics, and environmental analysis.

Investigation of drugs of abuse and toxicological analysis of biological specimens are an important area in forensic science with more than 75 % of the evidence evaluated in crime laboratories being drug related [1]. Mass spectrometry provides the ability to identify and quantitate drugs and their metabolites in blood, urine, saliva, tissues, and hair. The two most common mass spectrometry techniques for these types of studies include gas chromatography – mass spectrometry (GC-MS) and liquid chromatography – mass spectrometry (LC-MS). These methods unequivocally identify compounds based on fragmentations in the mass spectra, can be used to test for many different analytes in a single injection, are highly sensitive, and have established protocols in the literature [1]. GC-MS, in particular, has long been used in the field of forensics. Under the Mandatory Guidelines for Federal Workplace Drug Testing Programs, it was the only permitted method for urine drug testing from 1988 to October 2010 [2]. GC-MS instruments are generally less expensive than LC-MS/MS systems, the chromatography is often sharper with improved resolution, and software libraries are more advanced allowing comparison to standards [3]. In contrast, LC-MS/MS is better adapted for a wide range of low to high polarity compounds that require sample preparation and derivatization for GC-MS analysis [4]. Regardless of the method of analysis, however, development of analogous technologies is

important for confirmation of results and to determine if one procedure is better suited for detection of a particular set of analytes.

Chapters 1 and 2 of this discussion use GC-MS for the analysis of synthetic cannabinoids and their urinary metabolites. Analysis of the methanolic extracts of these products with GC-MS is ideal because compounds are easily resolved with good sensitivity and selectivity [5].

Detection of urinary metabolites using GC-MS, however, has not been extensively studied allowing only tentative assignments of suspected analytes [6,7]. Development of a complementary method to existing LC-MS/MS techniques is thus of significant value for confirmation of results and as an alternative technology. The aim of this present work was to use GC-MS to analyze the contents of several 'K2' products and develop a method for the detection of metabolites in urine samples from individuals suspected of using these mixtures. Analytical standards were synthesized and used for identification and confirmation of structures.

Comparison to standards is important because of isomeric compounds with similar mass spectral and retention properties.

Analysis of lipids is important because of their widespread prevalence in biological systems, food products, and environmental pathways. These compounds have been studied using many different methods that comprise chromatography (thin layer chromatography (TLC)), spectroscopy (nuclear magnetic resonance (NMR)), and mass spectrometry techniques [8]. Due to its high sensitivity and speed of analysis, mass spectrometry is an ideal analytical method for lipid analysis [8]. In particular, matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a suitable method because sample preparation is quick since derivatization is not required, high performance liquid chromatography (HPLC) separation is not needed, spectra are easy to interpret and limited buffer or salt contamination is tolerated [9-11].

Chapter 3 of this report uses laser desorption/ionization time-of-flight mass spectrometry (LDI-TOF MS) for the analysis of triacylglycerol (TAG) degradation products in fingerprint samples. Monitoring these products is important for understanding fingerprint degradation as a potential dating method. TAGs have previously been analyzed in fingerprints using this technique for gender comparison studies and structure elucidation using tandem mass spectrometry [12]. Other fingerprint studies have included analysis of fatty acids, amino acids, and squalene [13-18]. Many of these methods require lengthy sample preparation, de-esterification of the TAGs, and derivatization of the fatty acids into volatile components. The LDI-TOF MS approach, however, is rapid since no solvent or matrix is required for analysis.

MALDI-TOF MS is used in chapter 4 of this discussion for the analysis of lipids in complex mixtures. Other methods for analyzing lipids have included LC-MS and coupling TLC with MALDI MS. These approaches are lengthy and the TLC method only reports phospholipids and no TAGs [8]. The reported MALDI MS technique uses solid phase extraction (SPE) cartridges to produce a few fractions from which lipids and classes of lipids can be characterized. The approach is rapid and inexpensive with direct analysis of the intact lipid.

Chapter 5 of this report uses GC-MS for the analysis of fatty and resin acids in biomass fermentation process waters. Previous methods for the detection of these compounds have included liquid-liquid extraction of samples followed by LC-MS or GC-MS analysis [19,20]. While LC-MS allows for direct injection of samples without the need for a derivatization step, poor separation is achieved between analytes [20]. In contrast, GC-MS analysis offers slightly better sensitivity, selectivity, linearity, and recoveries. A method of trimethylsilyl (TMS) derivatization followed by GC-MS was thus selected for analysis of water samples due to the improved analytical parameters.

Herein, new applications of mass spectrometry are described for drug and lipid analysis. A combination of GC-MS and MALDI-TOF MS is used for analysis of samples. In addition, analytical techniques of liquid-liquid extraction, SPE, and derivatization are described.

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Chapter 1

Analysis of synthetic cannabinoids in 'K2' herbal blends using gas chromatography – mass spectrometry

Abstract

Herbal incense products containing synthetic cannabinoids are a growing trend. JWH-018 (1-pentyl-3-(1-naphthoyl)indole) is one of numerous cannabinoids contained in these products marketed as 'K2' or 'Spice'. Monitoring these mixtures is a continuous challenge because of more than 100 different psychoactive compounds that could be present. An extraction procedure and gas chromatography – mass spectrometry (GC-MS) method were developed for isolation and detection of synthetic cannabinoids in various herbal blend mixtures and evidence submitted from a local drug court. An analytical standard of JWH-018 was synthesized in a two-step process to confirm proposed identifications. JWH-018 was detected in all of the samples with concentrations ranging from 2.7 – 14.1 mg/g. No other synthetic cannabinoids were detected in any of the samples.

1. Introduction

1.1 Cannabinoids

Cannabinoids are a group of compounds with diverse chemical structures but related pharmacological effects. Phytocannabinoids and synthetic cannabinoids represent the two most abundant groups [1]. Phytocannabinoids occur naturally in cannabis plants with the most common being Δ^9 -tetrahydrocannabinol (THC) found in marijuana. Since the isolation of THC in the 1960's, synthetic cannabinoids have been developed to determine structure-activity and receptor binding relationships [2-3]. Of particular importance is separating marijuana's undesirable psychoactive effects from its therapeutic properties: anti-emetic, anti-inflammatory, analgesic, and appetite stimulant effects. Comparisons between these two groups of cannabinoids are possible since both bind to the same cannabinoid receptors (CB₁ and CB₂). Correlations in pharmacological activity are thus frequently observed [3-5].

1.2 Synthetic cannabinoids

Two groups of synthetic cannabinoids that have been developed include the cyclohexanol (CP) and JWH series. The CP analogues were first developed by Pfizer in the 1970's and include CP-47,497 and CP-55,940 [1,3,6-7]. In the 1990's, John W. Huffman et al. created a large group of compounds that include naphthoylindoles, naphthylmethylindolels, naphthoylpyrroles, naphthylmethylindenes, and phenylacetylindoles. Examples of these cannabinoids include JWH-018 and JWH-073 from the naphthoylindole group and JWH-250 from the phenylacetylindole series [1].

Representative structures for some of these compounds are given in Fig. 1 [4]. The CP series has structural similarities to THC while JWH compounds have no resemblance. Numerous other analogues of these cannabinoids have also been synthesized especially with regard to the JWH series. This includes modification of the alkyl chain, indole ring, and naphthoyl moiety with various substituents. Differences in the binding affinity to the CB₁ and CB₂ receptors are also observed for these cannabinoids especially with respect to THC. JWH-018 has approximately a four-fold increased affinity to CB₁ and about a ten-fold affinity to CB₂ compared with THC [3-4,7]. The CB₁ receptor is located in the central nervous system and is responsible for psychoactive effects. The CB₂ receptor is primarily found in immune cells and, although its physiological role is not well understood, is involved in pain perception. An ideal synthetic target would thus have a low binding affinity at CB₁ and increased affinity at CB₂ [3,8].

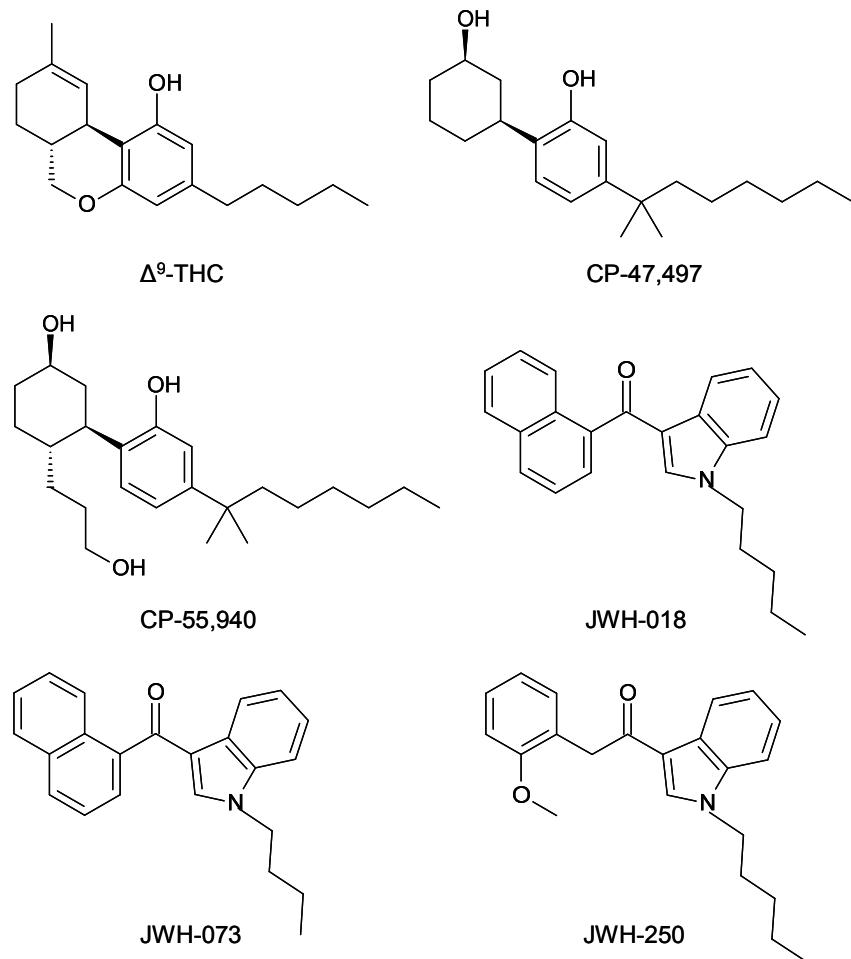


Fig. 1. Representative structures of cannabinoids found in marijuana or incense products.

1.3 Synthetic cannabinoids in ‘K2’ herbal products

Since 2004, herbal mixtures under the brand name ‘K2’ have been sold over the internet and in headshops. These products were first made available in several European countries and have now been distributed world-wide. While ‘K2’ is the generic name given to these mixtures, they are sold under a variety of names which include ‘Spice Diamond’, ‘Smoke’, ‘Spice’, ‘Space’, and ‘Chill-out’ [1,5,9]. These blends are a mixture of a green/brown plant material (0.5

– 3 g) that is packaged into a colorfully designed packet. Although they are advertised as herbal products and not for human consumption, many users' have smoked or taken them orally. These individuals have reported 'cannabis-like' effects similar to marijuana [4,9]. In 2008, Auwärter et al. conducted a self-experiment with an herbal product to gain insight into its pharmacological activity. One cigarette containing 0.3 g of 'Spice Diamond' was smoked. Approximately 10 min after use the first noticeable effects were observed in the form of reddened eyes, a significant increase in heart rate, dry mouth, and an alternation of mood and perception. These effects continued for about 6 hr with some minor symptoms reported the next day [10]. In addition to these effects, other users' reported profuse sweating, nausea, vomiting, headaches, tremors, insomnia, and depression [11].

After their emergence in 2004, the popularity of 'K2' products began to grow. The blends were easy to obtain with many vendors selling the products without an age restriction. The mixtures were also being reported as a 'legal' substitute for marijuana that could not be detected in routine urine tests [5-6]. By 2008, several laboratories began analyzing and identifying the additives in these products. The first detected compounds were two synthetic cannabinoids: the C8 homologue of CP-47,497 and JWH-018. While these substances were soon banned by several European countries, other cannabinoids began appearing in products. Among these were JWH-073, JWH-250, and JWH-019 (an alkyl homologue of JWH-018) [1,6]. Beginning in 2010, some states began issuing laws to make the sale of these products illegal. Some of these states included Kansas, Alabama, Missouri, Louisiana, and Arkansas. On March 1, 2011, the Drug Enforcement Administration (DEA) temporary classified five synthetic cannabinoids (JWH-018, JWH-073, JWH-200, CP 47,497, CP 47,497 C8 homologue) as schedule 1 substances. This ban was enacted for a year but was extended for an additional 6 months [4,6].

1.4 Analysis of ‘K2’ herbal blends

Synthetic cannabinoids in ‘K2’ products have been analyzed using both GC-MS and LC-MS techniques. These procedures involve extraction of the plant material in the ‘K2’ packet with methanol or ethanol, evaporation of the solution to dryness, and reconstitution of the residue [5,8-9,12-14]. GC-MS is an ideal method because these cannabinoids are easily resolved with good sensitivity and selectivity. Analysis of some samples is limited, however, because of the difficulty in obtaining reference standards. This permits only tentative assignments to be made with no quantitative measurements on the amount of substance in the packets [12,14]. The aim of this present work was to analyze the contents of several ‘K2’ products using GC-MS. JWH-018 was synthesized as a reference standard for comparison and was selected because of its prevalence in other samples as reported by several authors [8,12,14]. A ‘K2’ blend and the burnt material from a glass pipe used for smoking cannabis products were provided by a local drug court for testing.

2. Materials and methods

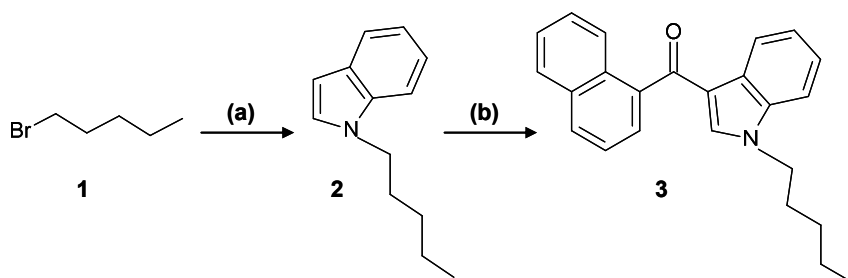
2.1. Reagents

The reagents and solvents were obtained from EMD chemicals (Gibbstown, NJ), Sigma Aldrich (St. Louis, MO), and TCI America (Portland, OR). NY). Three herbal blend packets (Astral Blast Fragrant Blend, Astral Blast Berry Blend, and Space Orbit) were purchased from a local headshop. Another packet (Astral Blast Berry Blend) and a glass pipe used to smoke cannabis were provided by an officer from the Arkansas Department of Community Corrections

(Fayetteville, AR). The 5-hydroxypentyl JWH-018 metabolite was synthesized as reported below.

2.2. Synthesis of JWH-018

JWH-018 was synthesized as indicated in Scheme 1: A nucleophilic substitution reaction was used to obtain product **2** by reaction of bromopentane (**1**) with indole [15]. Friedel crafts acylation of pentylindole (**2**) with 1-naphthoyl chloride and Me_2AlCl afforded product **3** in 73 % yield [3].



Scheme 1. Reagents and conditions for synthesis of JWH-018: (a) DMF, indole, KOH; (b) 1-naphthoyl chloride, CH_2Cl_2 , Me_2AlCl , 0°C .

2.3 Sample preparation

Methanol (45 mL) was added to 0.5 g of the herbal mixtures and vortexed for 2 min. After filtration to remove particulate matter (0.45 μm nylon membrane filter), the filtrate was collected. The extraction was repeated two additional times (2 x 45 mL) with collection of the filtrates. The extracts were then combined and evaporated to dryness. The residue was reconstituted in 4 mL methanol and was thereafter ready for analysis. For the smoking pipe, the burnt material was scraped from the glass surface of the pipe and extracted as described above.

2.4 Detection limits

Detection limits were calculated using the slope of a calibration curve (m) and the standard deviation of the response (σ) based on repeated blank measurements ($n=8$). The limit of detection (LOD) was calculated using criteria of $3 \sigma/m$ and the limit of quantification as $10 \sigma/m$ [16].

2.5 GC-MS analysis

GC-MS analysis was performed on a Varian 450-GC coupled to a 320 -MS triple quad mass spectrometer (Bruker Daltonics, Billerica, MA). Separation was achieved with a Phenomenex Zebron ZB-5HT Inferno column (30M x 0.25ID). The initial column temperature was set at 70 °C and was increased at a rate of 15 °C/min to 310 °C (held for 5 min). One μL injections were done with the injector at 310 °C in the split mode (10:1) with the transfer line temperature set at 320 °C. The mass spectrometer was operated in full scan mode with a scan range of m/z 50-500. To prevent any carry-over between different brands of incense, a methanol blank was injected between each sample.

3. Results and discussion

Synthesized JWH-018 was analyzed using $^1\text{H-NMR}$ and GC-MS for confirmation of structure. The $^1\text{H-NMR}$ for step a and b of the synthesized JWH-018 is given in Figs. 2 and 3. The spectra show characteristic signals including a methylene attached to a nitrogen atom at δ 4.09.

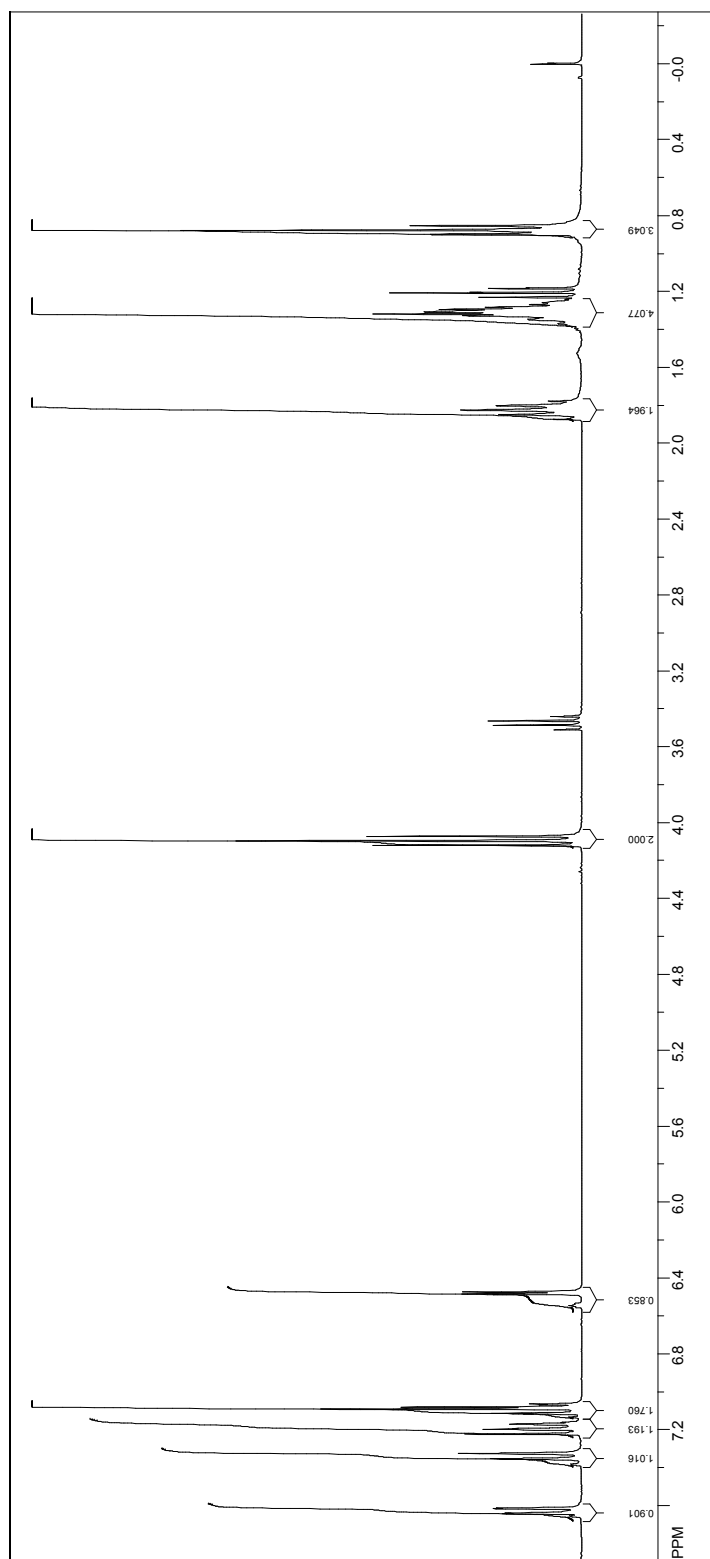


Fig. 2. ¹H-NMR of 1-pentylindole (step a) of the synthesis pathway.

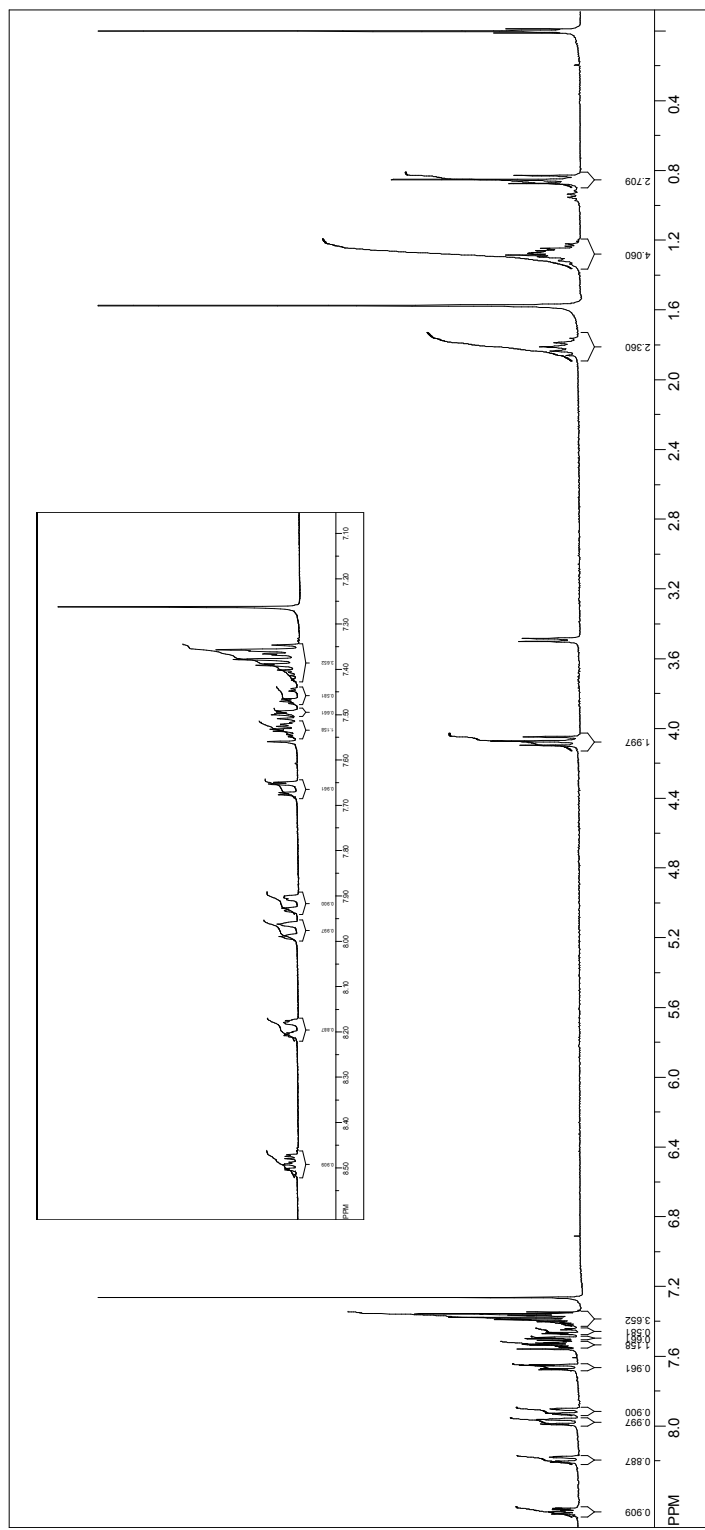


Fig. 3. ¹H-NMR of 1-pentyl-3-(-1-naphthoyl)indole (step b) of the synthesis pathway.

The mass spectrum of step a of the synthesized JWH-018 (Fig. 4a) indicates two ion peaks corresponding to the molecular ion at m/z 187 and loss of the butyl group at m/z 130. The GC chromatogram of step b (Fig. 5a) of the synthesis shows one major peak at 22.1 min. The mass spectrum (Fig. 5b) reveals several characteristic ion peaks corresponding to the molecular ion at m/z 341, a naphthalenyl fragment at m/z 127, a carbonylnaphthalenyl fragment at m/z 155, loss of the naphthalenyl moiety at m/z 214, and loss of the butyl group at m/z 284. Signal assignments for $^1\text{H-NMR}$ and GC-MS are both in agreement with those reported in the literature [8,12-13].

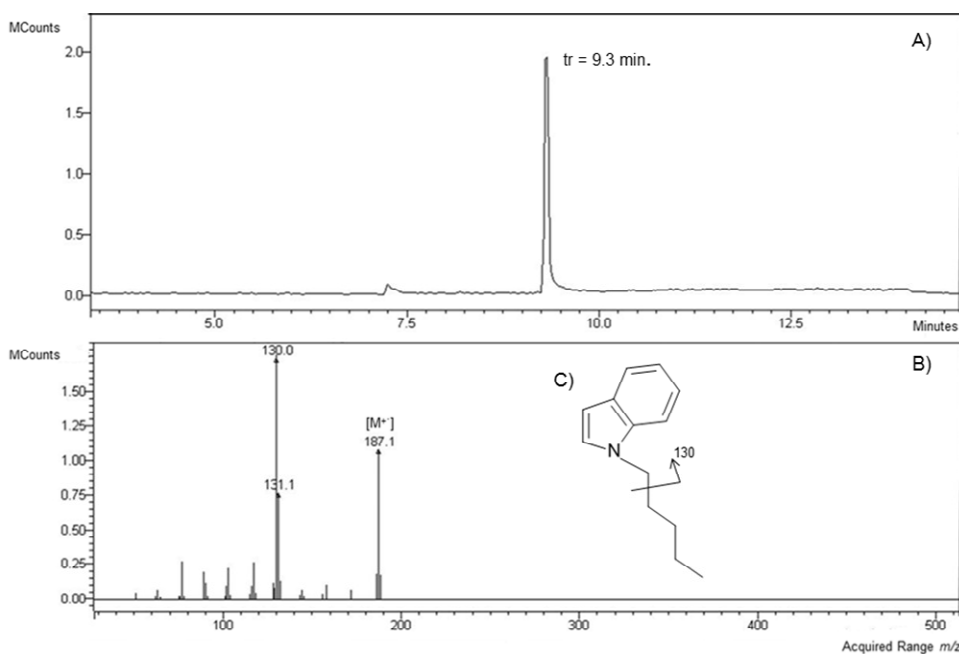


Fig. 4. Total ion chromatogram (a) and electron impact mass spectrum (b) of 1-pentylindole (step a of the synthesis pathway).

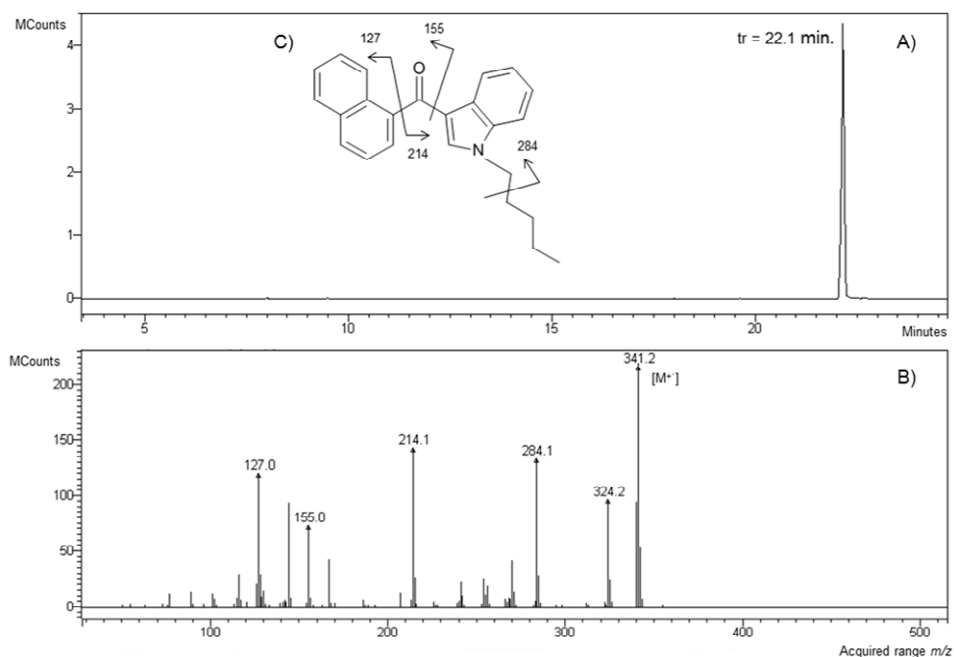


Fig. 5. Total ion chromatogram (a) and electron impact mass spectrum (b) of 1-pentyl-3-(1-naphthoyl)indole (step b of the synthesis pathway).

Using the synthesized JWH-018 as a reference standard, the material from three herbal blend packets was analyzed. These products appeared as a green-brown plant material with a fragrant berry aroma (Fig. 6). After extraction of these mixtures with methanol as described above, the extracts were analyzed with GC-MS. The GC-MS spectrum of one of these mixtures (Astral Blast Berry Blend) is shown in Fig. 7. Analysis of these products indicated a peak in the chromatogram with a retention time of approximately 22.1 min and ion signals with m/z values of 127, 155, 214, 284, and 341. Comparison of retentive and mass spectral fragmentation patterns with that of the synthesized standard (Fig. 5) conclusively indicates JWH-018 is present in all tested herbal blends. No other JWH compounds were identified in any of the mixtures as would be indicated by other peaks in the chromatogram with similar mass fragments. Hexane

was initially tested as a solvent but it did not adequately extract all of the cannabinoid from the sample matrix. This was demonstrated by extraction of an herbal blend mixture three times with hexane followed by one time with methanol. JWH-018 was detected in both of these solvent extracts with a higher concentration in methanol. Approximately 8 times more JWH-018 was present in the methanol extract through comparison of peak areas between samples. Analysis of samples with an extraction sequence of three times with methanol and one time with hexane indicated JWH-018 only in methanol and not in hexane. Extraction of the herbal blends three times with methanol was thus demonstrated to efficiently isolate all of the cannabinoid from the solid sample matrix. This solvent has also previously been used for the extraction of other herbal blends as reported in the literature [12,13].



Fig. 6. An illustration of the material and different ‘K2’ herbal products purchased from a local headshop and analyzed by GC-MS. Some packets indicated ‘not for human consumption’ on the back.

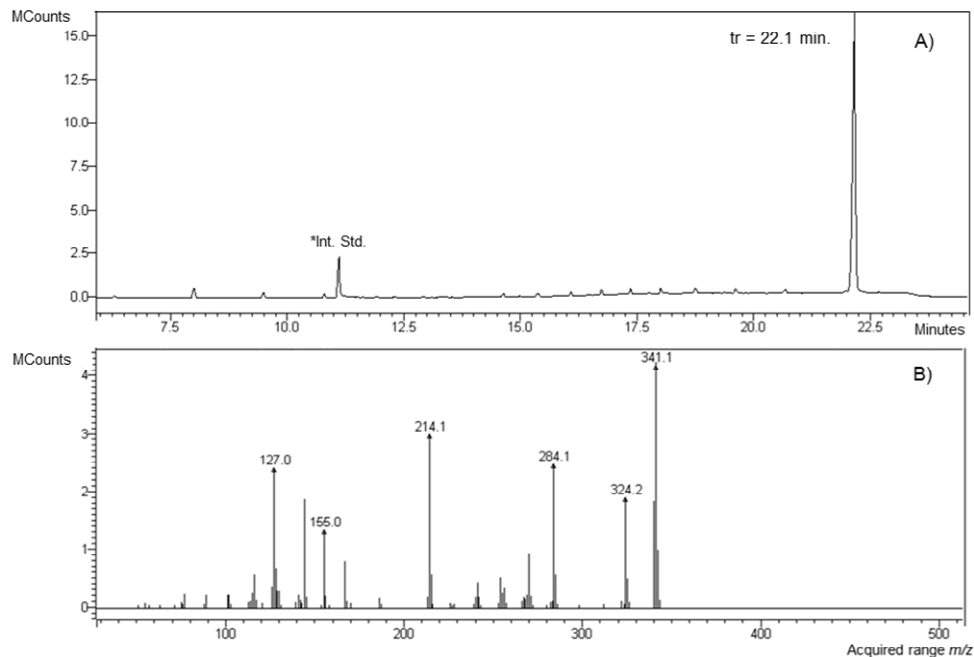


Fig. 7. Total ion chromatogram (a) and electron impact mass spectrum (b) of a methanolic extract of an Astral Blast Berry Blend packet containing JWH-018.

The concentration of JWH-018 in the herbal mixtures was calculated through addition of an internal standard (DPA, RF = 0.11) to the sample mixtures. An average of 10.1 ± 6.4 mg/g was detected in the three samples with the highest concentration in the Space Orbit sample at 14.1 mg/g followed by Astral Blast Berry Blend at 13.5 mg/g and Astral Blast Fragrant Blend at 2.7 mg/g. The addition of JWH-018 in ‘K2’ products has been reported to vary between different brands with reported concentrations ranging from 2-35 mg/g. In this report the most expensive blend (Space Orbit, \$18/g) had the highest concentration of JWH-018. No correlations, however, have been demonstrated between price and the composition of synthetic cannabinoid added to the mixture. Although both Berry Blend packets were the same brand of incense and purchased

for the same price (\$13/g), the concentration of JWH-018 differed by a large amount. Lindigkeit et al. have reported variations in the amount of synthetic cannabinoids between similar brands of incense. Some samples may contain a low dose but then a much higher amount the next time the product is used [1]. These irregularities make the herbal blends even more dangerous because individuals may experiment with different brands and amounts that they consume, resulting in an accidental overdose.

Detection limits and sample carry-over between different blends were examined through method analysis. The LOD was calculated as 0.26 ng/mL and the LOQ as 0.87 ng/mL. Detection of JWH-018 and other synthetic cannabinoids is typically not an issue, however, since they are present in high concentrations in herbal blends. Sample carry-over between different blends was calculated by assessment of a JWH-018 standard followed by injection of a methanol blank. A small peak, with the same retention time and mass fragmentation pattern of JWH-018, was detected in the blank. Comparison of peak areas indicated a carry-over of 0.34 % between samples. Injection of another methanol blank further reduced any leftover JWH-018 to less than 0.1 % amounts. Because of only a small carryover, only one methanol blank was injected between different samples.

After analysis of different 'K2' herbal blends, two samples submitted as evidence from a local drug court were examined. The material from an Astral Blast Berry Blend packet and the burnt residue from a pipe used to smoke cannabis were as extracted as described above. The methanolic extracts were then analyzed by GC-MS. JWH-018 was detected in both samples with a concentration of 11.5 mg/g in the Berry Blend packet. This same brand of incense contained 13.5 mg/g indicating variation in the amount of added synthetic cannabinoid as previously described. Detection of JWH-018 in the burnt material from the pipe indicates no degradation is

observed even after exposure to high temperatures. Although it cannot be explicitly determined if the pipe was used to smoke the herbal blend submitted as evidence, there are good indications that the two are linked together. This is based on the two items being discovered in close proximity to one another and both containing the same synthetic cannabinoid of JWH-018.

4. Conclusions

A procedure was developed for detection of synthetic cannabinoids in herbal blends using GC-MS. Methanol was determined to be an efficient solvent to isolate these compounds from the solid sample matrix. JWH-018, a common synthetic cannabinoid, was synthesized as an analytical standard for comparison to samples through retention properties and mass spectral fragmentation patterns. GC-MS analysis indicated four herbal blend samples and a pipe used for smoking cannabis all contained JWH-018. Quantitative analysis of the products indicated concentrations ranging from 2.7 -14.1 mg/g with variations between the same brands of incense observed. No other synthetic cannabinoids were detected in any of the samples. Although only one compound was detected, the method could be extended to include other cannabinoids or drugs of abuse provided proper analytical standards were used for comparison. In this case, adjustment of the GC temperature program would be required to compensate for similar retention properties between some compounds. This is especially important given the number of potential synthetic cannabinoids that could be added to the herbal blends.

5. Experimental

5.1 General

All molecular structures were confirmed using GC-MS and $^1\text{H-NMR}$. Conditions for GC-MS analysis are discussed above. $^1\text{H-NMR}$ spectra were recorded on a Bruker 300 MHz spectrometer using CDCl_3 (δ 7.26) as the solvent with TMS (δ 0.00) as the internal standard.

5.1.1. 1-pentylindole

To a stirred solution of 1-bromopentane (**1**) (4.5 g, 30 mmol) in 50 mL DMF was added indole (1.2 g, 10.2 mmol) and ground KOH powder (0.6 g, 10.7 mmol). The mixture was stirred overnight at room temperature. Water (100 mL) was added, and the product was extracted into ether (3 x 50 mL). The ether extracts were washed with water and dried with MgSO_4 . After concentrating the solution, product **2** (1.3 g, 68 %) was isolated through chromatography (petroleum ether followed by petroleum ether / ether, 10:1) as an oil [15]; $^1\text{H-NMR}$ δ 0.88 (t, 3H, CH_3), 1.26-1.38 (m, 4H), 1.82 (quintet, 2H), 4.09 (t, 2H, CH_2N), 6.49 (dd, 1H), 7.09 (m, 2H), 7.20 (dt, 1H), 7.32 (m, 1H), 7.62 (m, 1H). (An impurity of diethyl ether is also present; 1.21 (T, 3H), 3.48 (Q, 2H).

5.1.2. 1-pentyl-3-(1-naphthoyl)indole

To a stirred solution of **2** (0.09 g, 0.5 mmol) in 1.5 mL dry CH_2Cl_2 at 0°C under N_2 was added dropwise Me_2AlCl (1 M in hexanes, 0.75 mL, 0.75 mmol). After stirring the mixture for 30 min at 0°C , 1-naphthoyl chloride (0.12 g, 0.6 mmol) in 1.5 mL of CH_2Cl_2 was added. The reaction mixture was stirred at 0°C until the reaction was complete as indicated by TLC analysis

(approximately 1 hr). The mixture was poured into iced 1 M aqueous HCl and extracted with CH₂Cl₂ (3 x 50 mL). The extracts were washed with aqueous NaHCO₃ and then dried with MgSO₄. After evaporation of the solvent, chromatography (petroleum ether / ethyl acetate, 9:1) was used to obtain product **3** (0.12 g, 73 %) as an off-white solid [3]; ¹H-NMR δ 0.86 (t, 3H, CH₃), 1.21-1.34 (m, 4H), 1.82 (quintet, 2H), 4.09 (t, 2H, CH₂N), 7.34-7.39 (m, 4H), 7.46 (m, 1H), 7.49 (dd, 1H), 7.53 (td, 1H), 7.66 (dd, 1H), 7.90 (br d, 1H), 7.96 (br d, 1H), 8.20 (br d, 1H), 8.49 (m, 1H). (An impurity of water is also present; 1.56 (s, 2H).

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Chapter 2

Gas chromatography – mass spectrometry of JWH-018 metabolites in urine samples with direct comparison to analytical standards

Abstract

JWH-018 (1-pentyl-3-(1-naphthoyl)indole) is one of numerous potential aminoalkylindoles contained in products marketed as ‘K2’ or ‘Spice’. Investigation of the urinary metabolites from consumption of these compounds is important because they are banned in the United States and many European countries. An efficient extraction procedure and gas chromatography – mass spectrometry (GC-MS) method were developed for detection of ‘K2’ metabolites in urine from individuals suspected of using these products. Analytical standards were used to elucidate the structure-specific mass spectral fragmentations and retention properties to confirm proposed identifications and support quantitative studies. A procedure for the synthesis of one of these metabolites (5-hydroxypentyl JWH-018) was also developed. Results are comparable to existing LC-MS/MS methods, with the same primary metabolites detected. The specific metabolite hydrolysis products include 4-hydroxypentyl, 5-hydroxypentyl, and N-pentanoic acid derivatives.

1. Introduction

Since 2004, herbal mixtures under the brand names ‘Spice’, ‘K2’, and others have been sold via the internet and in ‘headshops’. Although these products are marketed as incense, the blends have been smoked in a manner similar to tobacco products giving users cannabis-like effects comparable to marijuana [1,2]. These psychoactive effects are a result of synthetic cannabinoids, including aminoalkylindole (AAI) and cyclohexylphenol (CP) compounds, added to the mixtures. One of the first and most commonly reported additives is JWH-018, an AAI with binding affinity to the CB₁ and CB₂ cannabinoid receptors [1-3].

Analysis of synthetic cannabinoids is relevant from both a clinical and a law enforcement perspective. Several studies have investigated their detection in seized material and in bodily fluids [1,3-11]. Reliable detection of AAI’s and the metabolites in a variety of substrates is critical because the numbers of severe episodes of intoxication are increasing at healthcare facilities. In addition, five synthetic cannabinoids (JWH-018, JWH-073, JWH-200, CP-47,497, and cannabicyclohexanol) have been classified as Schedule 1 substances by the U.S. Drug Enforcement Administration (DEA) [12]. These compounds, and other JWH analogues, are also banned in many European countries [13].

Several methods been reported for detection of JWH metabolites in urine samples using liquid chromatography and tandem mass spectrometry (LC-MS/MS) [6-9, 14]. The development of a second technique, using a different approach, would be useful for confirmation. We elected to develop a GC-MS method to complement the existing LC-MS approach because it has higher specificity in both the chromatographic and the mass spectral detection steps, and the instrument is usually less expensive. The GC chromatographic column provides an order of magnitude more theoretical plates for separation, and the mass spectral ionization step (electron impact (EI))

produces suppression-free spectra with higher structure specific information. The advantage of the LC-MS/MS technique is that it is uniquely amenable to direct analysis of aqueous solutions and is better suited for non-volatile compounds. With suitable extraction of the problematic components beforehand, GC-MS should provide a powerful and complementary approach for the characterization of these metabolites after separation from the urine matrix. This approach has not yet been extensively studied due to a lack of method validation studies (i.e. detection limits, recovery efficiency, and quantification) and the difficulty in obtaining proper analytical standards [10,11]. These limitations permit only tentative or semi-quantitative assignments of suspected urinary metabolites by GC-MS. Additionally, identification of some metabolites is difficult because of the presence of isomeric compounds with similar mass spectral fragmentation patterns and retention properties. This can lead to differences in the reported identity of metabolites from the same parent compounds. For example, the main urinary metabolites of JWH-018 reported by Sobolevsky et al. involve monohydroxylation on the indole ring [10] whereas Grigoryev et al. identify monohydroxylation on the pentyl chain [11]. The synthesis of the appropriate analytical standards is probably the best approach to resolve such differences. Even with reported standards, however, identification of the main metabolites and their relative abundance differ between studies. Chimalakonda et al. report detection of three metabolites that includes the following ranking by concentration: 4-hydroxypentyl > 5-hydroxypentyl > N-pentanoic acid derivatives [6]. Analyses of urine samples with a similar method by ElSohly et al. identify three main metabolites with the ranking as N-pentanoic acid > 5-hydroxypentyl > 6-hydroxyindole derivatives [14].

The aim of this present work was to develop a GC-MS method that is analogous and complementary to the existing LC-MS/MS method. A GC-MS method would be of significant

value for confirmation of LC-MS/MS results to determine the main urinary metabolites of JWH-018. This is especially important given the discrepancy between detected metabolites from different studies as described above. Development of a GC-MS method also provides an alternative technology that may be better suited to the existing equipment in a particular laboratory. Moreover, GC/MS instruments are generally less expensive than LC-MS/MS systems. GC-MS is a fundamental tool in forensic toxicology and was the only permitted method for urine drug testing from 1988 to October 2010 under the Mandatory Guidelines for Federal Workplace Drug Testing Programs. New guidelines now permit alternative technologies (LC-MS, GC-MS/MS, and LC-MS/MS) to be used as long as the methods are scientifically validated [15-17]. In this current report, three metabolite hydrolysis products (4-hydroxypentyl, 5-hydroxypentyl, and a carboxylated derivative of JWH-018) and the native compound of JWH-018 were used as reference standards for comparison to urine samples from suspected 'K2' users. These particular metabolites were chosen because of their abundance in urine samples from 'K2' users [6-9]. Method validation was completed by examining the efficiency of extracting the metabolites from urine samples along with calculation of detection limits.

2. Materials and methods

2.1. Reagents

The reagents and solvents were obtained from EMD chemicals (Gibbstown, NJ), Sigma Aldrich (St. Louis, MO), and TCI America (Portland, OR). N,O-Bis(trimethylsilyl) trifluoroacetamide + 10% trimethylchlorosilane (BSTFA + 10% TMCS) was purchased from Regis Technologies (Morton Grove, IL). Solid phase extraction (SPE) octadecyl (C18)

disposable extraction cartridges were manufactured by J.T. Baker (Phillipsburg, NJ). A certified negative control urine sample was obtained from Biochemical Diagnostics Inc. (Edgewood, NY). The 5-hydroxypentyl JWH-018 metabolite was synthesized as reported below because of availability at the time of analysis. As the study progressed, two additional standards (4-hydroxypentyl and N-pentanoic acid JWH-018 derivatives) were made commercially available by Cayman Chemical (Ann Arbor, MI). JWH-018 was synthesized as reported in the literature [18].

2.2. Synthesis of 5-hydroxypentyl JWH-018 metabolite

This JWH-018 metabolite was synthesized as indicated in Scheme 1: The hydroxyl group of 5-bromopentanol (**1**) was protected with TBDMSCl to give product **2** [19]. A nucleophilic substitution reaction was used to obtain product **3** by reaction of the protected bromopentane with indole [20]. Friedel-Crafts acylation of the protected pentylindole with 1-naphthoyl chloride and Me₂AlCl afforded product **4** [18]. Deprotection of product **4** with TBAF in THF produced product **5** in 71 % yield [19].

2.3. Urine samples of suspected ‘K2’ users

The urine samples analyzed in this study were collected from three individuals through a drug testing program operated by Employee Screening Management (Fayetteville, AR). No information regarding prior drug history or admittance to smoking any ‘K2’ products of the participants was provided. Samples were supplied with an assigned number with no personal

information exchanged. Samples were first analyzed by the Arkansas Department of Health, Public Health Laboratory using their established LC-MS/MS method [7].

2.4. Preparation of urine samples

A 1-mL urine sample containing an internal standard of bisphenol A (BPA, 900 ng/mL) was evaporated to dryness under N₂ at room temperature. Hydrolysis was completed by addition of trifluoroacetic acid (TFA) (0.6 % v/v, aq) to the dried residue. The sample was then heated for 40 min. at 100 °C. After cooling to room temperature, ammonium hydroxide (~ 4.5 mL, 28-30 %) was added to adjust the pH to ~9 as monitored with pH paper.

SPE cartridges (C18) were conditioned prior to analysis by rinsing the column with 3 mL methanol followed by 10 mL distilled water. The urine sample, prepared as described above, was then passed through the cartridge followed by rinsing with 10 mL distilled water. After discarding the above washing solutions, the analytes of interest were eluted with 4 mL methanol. The eluted methanol was evaporated to dryness at 60 °C (Centrivap Concentrator, Labconco, Kansas City, MO). The residue was then dissolved in 150 µL DMF and derivatized by addition of 150 µL BSTFA + 10 % TMCS. After heating for 25 min. at 70 °C, the samples were analyzed by GC-MS. To avoid contamination, a separate SPE cartridge was used for each sample.

2.5. Method validation

2.5.1. Specificity

Five urine samples, collected over five consecutive days, were obtained from a healthy individual and prepared as described above. Samples were analyzed using GC-MS to determine if any interfering peaks at the retention time of the internal or analytical standards were present.

2.5.2. SPE recovery

The efficiency of the SPE method was determined by evaluating a reference sample before and after extraction. A sample of synthesized metabolite (100 µg/mL), without any SPE preparation, was compared to a negative control urine sample spiked with the same concentration of metabolite. After extracting the urine sample according to the above procedure, both samples were derivatized and analyzed by GC-MS. The chromatographic areas of both samples were compared and % recovery was calculated as follows: (peak area of sample after extraction / peak area of sample before extraction) * 100.

2.5.3. Reproducibility

Reproducibility of the method was calculated by addition of synthesized metabolite (500 ng/mL) to three samples of negative control urine. Samples were then prepared and extracted using SPE as described above.

2.5.4. Detection limits

Detection limits were calculated using the slope of the calibration curve (m) and the standard deviation of the response (σ) based on repeated blank measurements ($n=8$). The limit of detection (LOD) was calculated using criteria of $3 \sigma/m$ and the limit of quantification (LOQ) as $10 \sigma/m$ [21].

2.6. GC-MS analysis of synthesized metabolite and urine samples

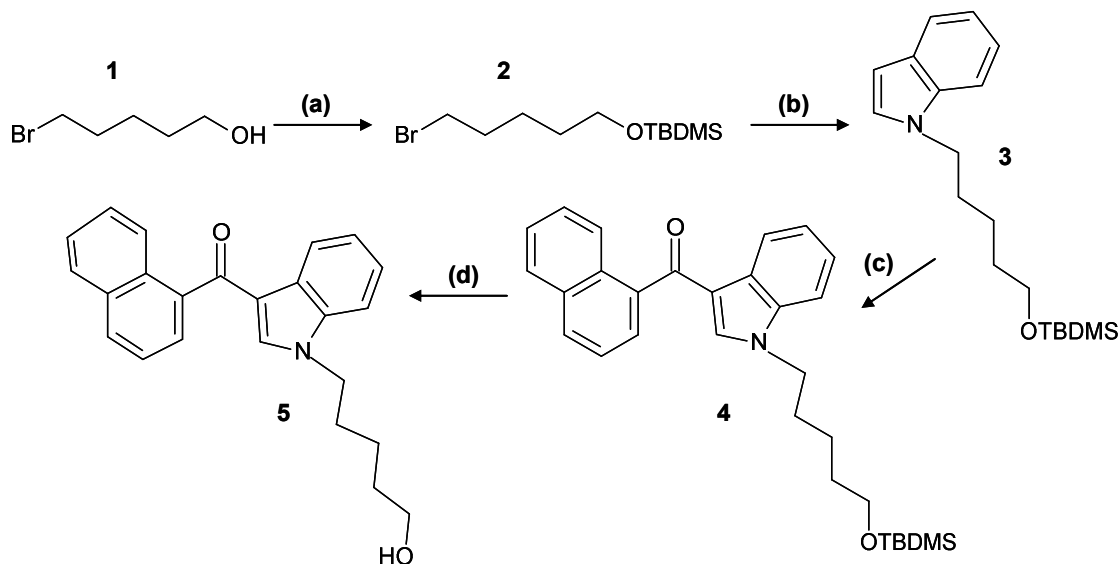
GC-MS analysis was performed on a Varian 450-GC coupled to a 320 -MS triple quad mass spectrometer (Bruker Daltonics, Billerica, MA). Separation was achieved with a Phenomenex Zebron ZB-5HT Inferno column (30M x 0.25ID). The initial column temperature was set at 180 °C and was increased at a rate of 20 °C/min to 320 °C (held for 12 min). One μ L injections were done with the injector at 310 °C in the split mode (10:1) with the transfer line temperature set at 320 °C. The mass spectrometer was operated under electron impact (EI) ionization in both full scan and SIM modes. Four ions were monitored for each analyte and are discussed below.

3. Results and discussion

The metabolism of JWH-018 appears to be consistent with cytochrome P450 oxidation followed by transformation to glucuronic acid conjugates [22]. Because of the variability of metabolic processes and differences in the time frame of urinary retention among individuals, the free metabolites in urine may include both the conjugates and their hydrolysis products. Deliberate hydrolysis of samples with an enzyme or acid then yields complete conversion of the free non-conjugated metabolite and also produces chemical structures more amenable to GC

separation. The addition of TFA and neutralization with ammonium hydroxide works effectively for this process.

Synthesis of the 5-hydroxypentyl JWH-018 metabolite is illustrated in Scheme 1. The route of synthesis is similar to JWH-018 but includes substitution of bromopentane with 5-hydroxybromopentane and protection and deprotection of the hydroxyl end group. Each product was confirmed through $^1\text{H-NMR}$ and GC-MS analysis with assignments in agreement with those reported in the literature [6-10, 23]. It is important to note that step d of the synthesis could be omitted as previous studies by the author's (not shown) have indicated that silylation of the hydroxyl group is necessary for better detection limits using GC-MS. The OTBDMS compound (product **4**) could then be used for analysis rather than derivatizing the final product.



Scheme 1. Reagents and conditions for synthesis of 5-hydroxypentyl JWH-018 metabolite: (a) TBDMSCl, imidazole, DMF, 0°C to room temperature; (b) DMF, indole, KOH; (c) 1-naphthoyl chloride, CH₂Cl₂, Me₂AlCl, 0°C; (d) THF, TBAF.

Method validation was completed using certified negative control urine samples spiked with the synthesized metabolite. Using the specified SPE procedure, a recovery of 91 % was achieved. It should be noted that this efficiency is based upon recovery of the free hydroxylated product and not the glucuronidated metabolite excreted from the body. Hydrolysis efficiency and recovery could be determined more precisely if glucuronidated JWH-018 compounds were more readily available. Efficiencies of >90 % for acid and enzyme hydrolysis of other glucuronidated compounds (i.e. morphine-6-glucuronide to morphine) have been reported using similar conditions [24]. Addition of TFA to the metabolite hydrolysis product, however, demonstrates

no decomposition or dehydration is observed. This is indicated by the high % recovery and observation of no additional products in the GC chromatogram. A reproducibility of 12 % (% CV, n = 3) was determined from recovery of synthesized metabolite in spiked samples. The LOD was calculated as 2.8 ng/mL and the LOQ as 9.24 ng/mL for the 5-hydroxypentyl metabolite.

A typical chromatogram containing native JWH-018 (N1), 4-hydroxypentyl (M1), 5-hydroxypentyl (M2), and N-pentanoic acid (M3) JWH-018 is shown in Fig. 1. Baseline resolution is achieved between compounds with elution of all analytes within 14 min. A mass spectrum for a TMS derivative of M2 is given in Fig. 2a with the structures and fragmentation for all metabolites in Fig. 2b. Ions at m/z 127 and 155 correspond to naphthalenyl and carbonylnaphthalenyl fragments. The ions at m/z 270 and 284 are produced from loss of the substituted butyl and pentyl side chains, respectively. These ions are characteristic for JWH-018 and the three metabolites analyzed in this study. Ions at m/z 127, 155, and 284 were then monitored for all urine samples of suspected 'K2' users in addition to the molecular ion for each analyte. The ion at m/z 341 was selected for N1, m/z 429 for M1 and M2, and the ion at m/z 443 for M3.

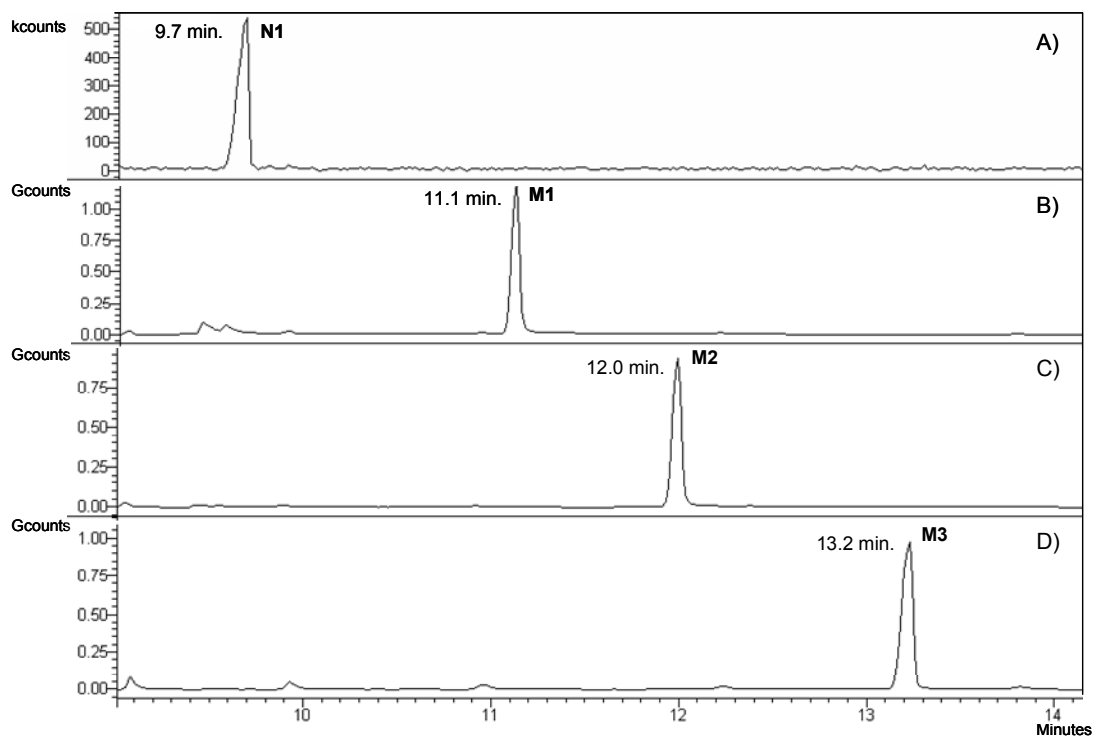


Fig. 1. Chromatograms produced from a) native JWH-018 (N1, 5 $\mu\text{g/mL}$), b) 5-hydroxypentyl (M1), c) 4-hydroxypentyl (M2), and d) N-pentanoic acid (M3) JWH-018 metabolites (3 $\mu\text{g/mL}$).

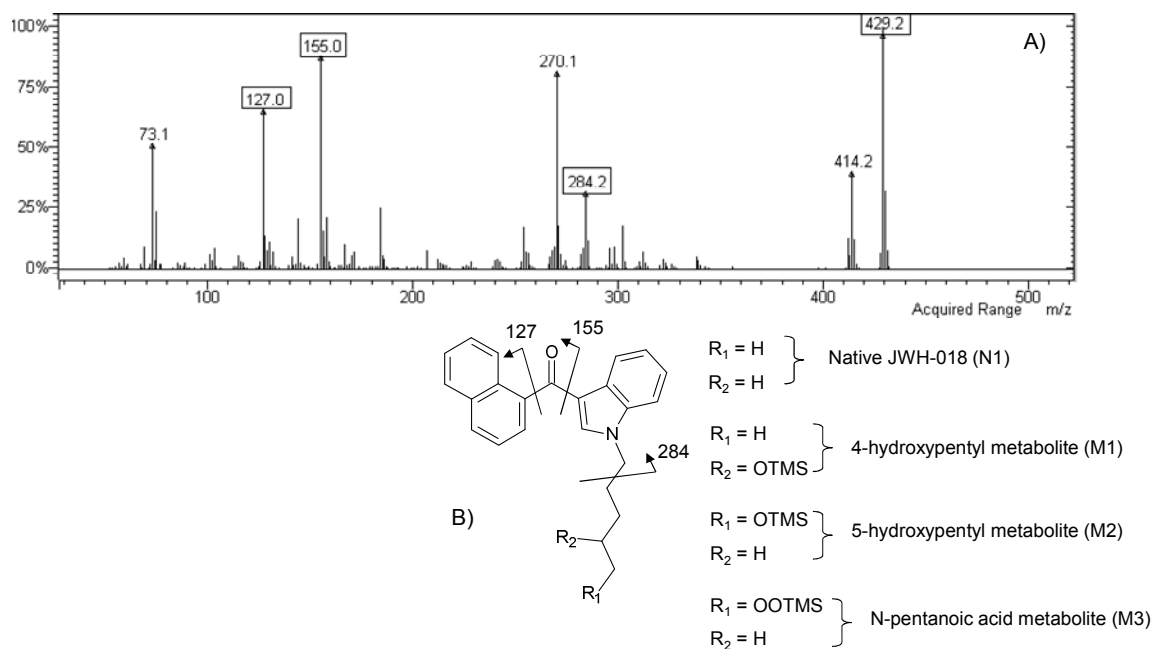


Fig. 2. a) Mass spectrum of 5-hydroxypentyl JWH-018 metabolite and b) the structure and resulting fragmentation for each of the analytical standards. Ions monitored during analysis are indicated by a rectangular box in the mass spectrum.

The chromatogram of a negative control urine sample is shown in Fig. 3a. Specificity of the method is demonstrated by the absence of interfering peaks at the retention times of the analytes of interest and the internal standard. A chromatogram of a urine sample (specimen 1) from a suspected 'K2' is presented in Fig. 3b. For clarification purposes, the chromatograms are expanded over a time range of 9-14 min. The internal standard, with a retention time of 5 min. is thus excluded. Retention times and mass spectra for this sample are consistent with those for the analytical standard (Fig. 1 and 2). Specimen 1 then contains M1, M2, and M3 but no N1. The

absence of N1 has been observed in other studies and is therefore not useful as an indicator of JWH-018 consumption [6-11]. This pattern is consistent with all of the samples except specimen 3 which contains only M1 and M2. Quantification of metabolites from specimen 1 and 3 indicates that M1 is excreted in the highest concentration followed by M2 then M3 (Table 1). This ranking is consistent with other reports that measured metabolites in samples using LC-MS/MS [6]. In specimen 2, however, M2 is excreted in a higher concentration followed by M1 then M3. Differences in the metabolites are consistent with individual differences in metabolism. No other metabolites for JWH analogues were detected in any of the samples. However, validation of the method was necessary to rule out the possibility that their absence in some experiments was due to experimental error.

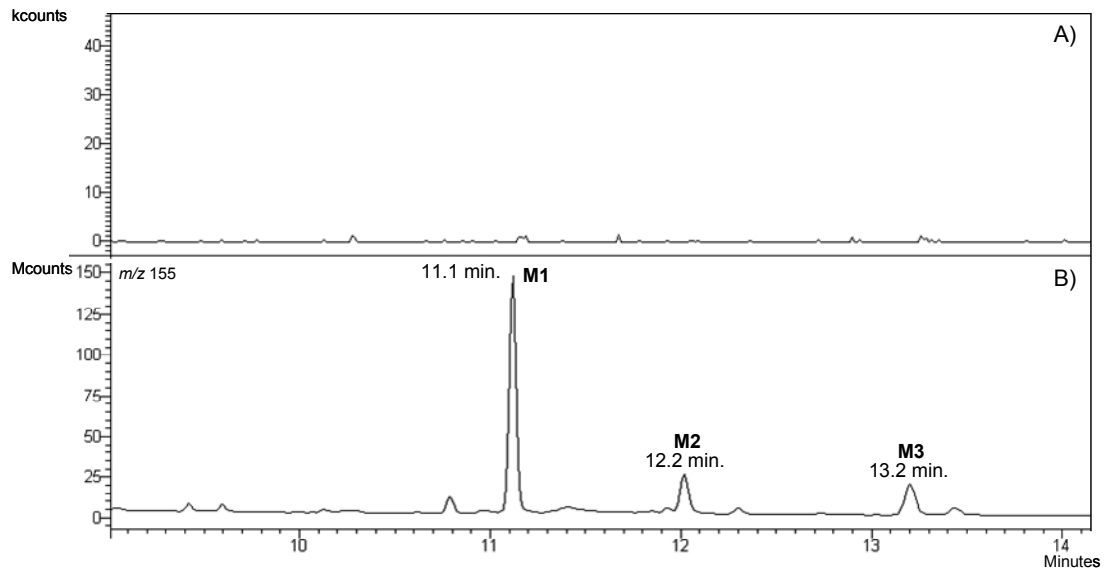


Fig. 3. Chromatograms resulting from a) a negative control urine sample and b) a sample taken from an individual suspected of consuming ‘K2’ products (specimen 1).

Table 1. Detection of JWH-018 metabolites in urine samples ^a

	M1	M2	M3
Specimen 1	485	126	25
Specimen 2	81	123	10
Specimen 3	40	16	---

^a Urinary concentration in ng/mL after acid hydrolysis and SPE; ---, not detected.

4. Conclusions

In this report, a procedure was established for detection of three JWH-018 urinary metabolites using GC-MS. Acid hydrolysis followed by SPE extraction was used for preparation of samples. Using this method, three metabolites were detected in urine samples from individuals suspected of using 'K2' products. These analytes were confirmed using analytical standards for comparison. A procedure for synthesis of one of the standards (M2) was also described. Identification of the sites of hydroxylation as occurring at positions 4 and 5 on the pentyl chain, with detection of a carboxylic acid derivative, are in agreement with those in urine samples analyzed by LC-MS/MS [6,7].

Previous studies using GC-MS were only able to determine the location of hydroxylation as somewhere on the alkyl chain or indole ring because of no direct comparison to standards. Analysis of samples using the described GC-MS method indicates it is a suitable technique for the detection of JWH-018 metabolites in urine and can be extended to other AAI if suitable standards are available. When compared to LC-MS/MS, similar results are achieved for sensitivity and reproducibility with identification of the same metabolites reported. For example, Moran et al. report detection limits of ~ 2 ng/mL with an analytical precision of ~10 % using enzyme hydrolysis [7]. In contrast, a detection limit of 2.8 ng/mL and a precision of 12 % were calculated using acid hydrolysis and SPE in this report. Analysis of urine samples suspected of containing JWH-018 metabolites could then be analyzed by either GC-MS or LC-MS/MS.

5. Experimental

5.1. General

All molecular structures were confirmed using GC-MS and ¹H-NMR. Conditions for GC-MS analysis are discussed above. ¹H-NMR spectra were recorded on a Bruker 300 MHz spectrometer using CDCl₃ as the solvent with TMS as the internal standard.

5.1.1. 5-bromopentoxy-tert-butyl-dimethyl-silane

To a solution of imidazole (1.2 g, 17.6 mmol) in 10 mL DMF was added 5-bromopentan-1-ol (**1**) (2.0 g, 12.0 mmol) and 1 M TBDMSCl in THF (15 mL, 15 mmol) at 0 °C under N₂. Stirring was continued overnight at room temperature. The mixture was diluted with Et₂O and washed with 1 N HCl, water, and brine. The filtrate was then dried with Na₂SO₄ and concentrated. Product **2** (2.4 g, 71 %) was afforded after purification by silica gel column chromatography (petroleum ether / Et₂O, 50:1) as a colorless oil [19].

5.1.2. tert-butyl-(5-indol-1-ylpentoxy)-dimethyl-silane

To a solution of **2** (8.4 g, 30 mmol) in 50 mL DMF was added indole (1.2 g, 10.2 mmol) and ground KOH powder (0.6 g, 10.7 mmol). The mixture was stirred overnight at room temperature. Water (100 mL) was added, and the product was extracted into ether (3 x 50 mL). The ether extracts were washed with water and dried with MgSO₄. After concentrating the solution, product **3** (2.0 g, 62 %) was isolated through chromatography (petroleum ether followed by petroleum ether / ether, 10:1) as an oil [20].

5.1.3. [1-[5-[tert-butyl(dimethyl)silyl]oxypentyl]indol-3-yl]-(1-naphthyl)methanone

To a stirred solution of **3** (0.16 g, 0.50 mmol) in 1.5 mL dry CH₂Cl₂ at 0 °C under N₂ was added dropwise Me₂AlCl (1 M in hexanes, 0.75ml, 0.75 mmol). After stirring the mixture for 30 min at 0 °C, 1-naphthoyl chloride (0.12 g, 0.63 mmol) in 1.5 mL of CH₂Cl₂ was added. The reaction mixture was stirred at 0 °C until the reaction was complete as indicated by TLC analysis (approximately 1 hr). The mixture was poured into iced 1 M aqueous HCl and extracted with CH₂Cl₂ (3 x 50 mL). The extracts were washed with aqueous NaHCO₃ and then dried with MgSO₄. After evaporation of the solvent, chromatography (petroleum ether / ethyl acetate, 9:1) was used to obtain product **4** (0.17 g, 72 %) as an off-white solid [18].

5.1.4. [1-(5-hydropentyl)indol-3-yl]-(1-naphthyl)methanone

To a solution of **4** (4.3 g, 9.1 mmol) in 10 mL of THF was added 1 M TBAF in THF (18.4 ml, 18.4 mmol). The reaction mixture was stirred for 2 hr and then quenched with MeOH. The mixture was washed with water and brine then dried with Na₂SO₄. After concentrating the solution, chromatography was used to give product **5** (2.3 g, 71 %) as an off-white solid [19].

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Chapter 3

Profiling of TAG degradation in fingerprint samples as a dating technique by laser desorption/ionization time-of-flight mass spectrometry

Abstract

Analysis of the chemical components of fingermarks is potentially important for investigating different techniques and reagents for visualization of prints and establishing a dating method for when the print was deposited. This latter application, in particular, would be useful in a forensic setting to help confirm or dispute a suspect's alibi. In the present work, triacylglycerols (TAGs) in fingermark samples were analyzed using laser desorption ionization (LDI) time-of-flight mass spectrometry (TOF MS). Only LDI appeared to be useful for this technique while conventional matrix-assisted laser desorption/ionization (MALDI)-TOF MS was not. Samples were analyzed under light and dark conditions over a time period of 112 hrs on four different surfaces: glass, plastic, iron, and stainless steel. Fingermarks from glass, plastic, and iron all showed similar rates of degradation while those from stainless steel were much more rapid. Additional products, resulting from TAG decomposition, were observed in the mass spectrum of these samples in the mass range of m/z 650-750. These same products were not observed in samples exposed to the dark. Analysis of an unsaturated TAG standard (triolein, C57:3) was exposed to the same conditions as the fingermarks to identify/confirm selected TAG degradation products. Formation of bound C7:0 and C8:0 aldehydes and carboxylic acids were observed and were consistent with those from fingermarks. The age of a fingermark was also estimated through analysis of a print with a known time of deposition. The print was determined to be between 16 and 112 hrs old through data comparison; the actual age of the print was 24 hrs.

1. Introduction

Fingermarks are the recovered traces of material transferred to other surfaces upon contact. The composition of this material is related to secretion of different glands in the body: eccrine, sebaceous, and apocrine glands. Sebaceous glands are appendages of the hair follicle that extend into the dermal layer of the skin. These glands secrete a clear lipid mixture, known as sebum, onto the skin surface [1,2]. The composition of sebum isolated from the gland consists mainly of TAGs, wax esters, squalene, and a small percentage of sterol esters. In addition to these lipids, sebum collected from the skin surface contains di- and monoacylglycerides, free fatty acids, and unesterified sterols. Differences in composition from the two areas are a reflection of lipolytic activity of the TAGs as they pass from the sebaceous gland through the duct. TAGs are broken down first into diacylglycerides (DAGs), then monoglycerides, and finally free glycerol with a molecule of free fatty acid being formed in each step. In addition to lipolytic activity in the sebaceous duct, the glycerides are broken down further on the surface of the skin by bacterial lipases [3].

The composition of skin surface lipids has been examined using various mass spectrometry (MS) techniques: gas chromatography (GC) – MS [4-9], liquid chromatography (LC) – MS [10], MALDI – mass spectrometry imaging (MALDI-MSI) [11], surface assisted (SA)LDI-TOF [12], direct analysis in real time (DART) [13], and desorption electrospray ionization (DESI) [13]. These studies have included analysis of fatty acids, amino acids, squalene, and external contaminants e.g. drugs, cosmetics, etc. In many of these investigations lengthy sample preparation, de-esterification of the TAGs, and derivatization of the fatty acids into volatile components was necessary.

Analysis of the chemical components contained in a fingermark is important in a forensic setting for determining investigative leads. Potential applications in this area include strategies for identification of prints through visualization reagents, detecting explosives or drugs of abuse, and a method for establishing the time a print was deposited. Verifying the age of a fingermark, in particular, would be useful to help confirm or dispute a suspects alibi. For example, an individual could assert that a fingerprint recovered from a crime scene was present for weeks before the crime was committed. Determining that the print was left hours before the offense could be critical in establishing a time-line of events and a subsequent conviction. At the present time, however, no established method is accepted for dating fingerprints or fingermarks [14].

Studies involving the age of a fingermark have included analysis of both visual and chemical changes over time. Differences in the adherence of fingerprints powders after development and variations in the appearance of sweat and other chemical traces have been previously investigated [14]. More recently changes in the chemical composition of fingermarks have been monitored using mass spectrometry. Wolstenholme et al. analyzed fatty acids in fingermarks using MALDI-MSI. In this study fatty acids, notably oleic acid, were investigated in fingermarks that had been aged for 7 days at 4°C, 37°C, and 60°C. As the temperature increased, lipid degradation was observed with little product remaining after exposure at 60°C [11]. Archer et al. examined fatty acids and squalene in fingermarks exposed to light and dark conditions over time using GC-MS. As the samples were aged, an initial increase in the amount of material was detected followed by a decrease to original levels or below. Levels of squalene were found to decrease more rapidly in the light with less depletion occurring in the dark. Levels of deposited material differed significantly between donors with large variations also observed in prints collected from the same donor [4]. These same authors have further investigated squalene and

squalene oxidation products in fingerprints using LC-MS. Compounds were analyzed over a 7 day time frame in prints exposed to light conditions. Squalene was rapidly depleted and after day 7 was no longer detected. The squalene oxidation products initially increased over 5 days and were no longer detectable after day 7 [10]. General results of these studies indicate that fatty acids and squalene are two compounds that are potential markers for determining the age of a fingerprint.

TAGs are potential components for accessing the time a print was deposited because of their abundance in fingerprints and because of oxidation effects. TAGs in fingerprint samples have been previously analyzed in fingerprint samples using LDI-TOF MS. In this study a method was developed for detection of TAGs using no solvent or matrix. The fatty acid profiles of some fingerprint TAGs were determined using tandem mass spectrometry (MS/MS) and the possibility of differences in the composition between male and female fingerprints was also investigated [15].

The mechanisms of oxidation for fatty acids in a TAG have been extensively studied. This route begins with an initiation step where a hydrogen atom in the fatty acid of a TAG is removed and a lipid alkyl radical is produced ($RH \rightarrow R\cdot + H\cdot$ where R is a lipid alkyl). Several factors affect this process with heat, metal catalysts, and light accelerating radical formation. The composition of the fatty acid is also important with oxidation of unsaturated compounds occurring more rapidly than saturated acids. This effect is a result of lower energies for removal of hydrogen atoms adjacent to double bonds. The lipid alkyl radical then reacts with atmospheric oxygen to form a lipid peroxy radical ($R\cdot + O_2 \rightarrow ROO\cdot$). This radical removes hydrogen from other lipid molecules and reacts with it to form hydroperoxides ($ROO\cdot + RH \rightarrow ROOH + R\cdot$). Homolytic cleavage between oxygen and the oxygen bond occur and the hydroperoxide

decomposes to form alkoxy and hydroxy radicals ($\text{ROOH} \rightarrow \text{RO}\cdot + \cdot\text{OH}$). Further decomposition of these radicals forms aldehydes, ketones, acids, esters, alcohols, and short chain hydrocarbons [16].

Monitoring decomposition products and the rate of their formation under different conditions is important for understanding fingerprint degradation as a potential dating technique. This report addresses the issue of age determination by observing degradation effects in TAGs from fingerprints under different experimental conditions. Our method is different from the alternative approaches using fatty acids, in that that we propose to detect changes from TAGs or TAG decomposition products. Variability between donors can potentially be reduced since TAGs are analyzed directly and require no solvent or derivatizing reagent.

2. Materials and methods

Herein, we demonstrate our approach for analyzing TAGs and their degradation products using a standard and fingerprints collected on various surfaces. The standard (triolein) was analyzed to identify specific products of degradation using MS/MS conditions. Using this approach, products observed in fingerprint samples were characterized. Fingerprint collection included obtaining samples on glass, plastic, iron, and stainless steel surfaces. A comparison of the relative intensities of TAGs, collected over several days, was used to compare the rate of degradation. The age of a known print was estimated using these rates.

2.1. Materials

Triolein (C54:3) was purchased from Sigma Aldrich (St. Louis, MO). All chemicals (hexane and 2,5-dihydroxybenzoic acid (DHB)) were analytical grade or better. Fingermarks were collected on four different materials from the laboratory: a plastic CD case (polystyrene), watch glass (borosilicate), a strip of iron (>99 % iron), and a piece of stainless steel (a MTP Multiprobe Adapter MALDI target disk).

2.2. Mass spectrometry analysis and MALDI targets

A Bruker Ultraflex II (Bruker Daltonic GmbH, Bremen, Germany) MALDI-TOF was operated in the positive-ion reflectron mode using an MTP Multiprobe Adapter MALDI target. Spectra were acquired in the m/z range of 400-1650 with adjustment of the sample position to produce intense ions for the TAGs. 1500 laser shots were collected for each spectrum. MS/MS spectra were obtained by operating the instrument in LIFT mode. It should also be noted that during analysis of fingermarks, samples were not mixed with a matrix but were instead analyzed by transfer of the fingermark onto the MALDI target. Triolein and matrix, (DHB), were mixed directly together (1 μ L each) in a ratio of 1:1, and 1 μ L of this mixture was applied to a MALDI target. A peptide standard was spotted on each target for calibration purposes.

Care was taken to ensure that the MALDI disks of the target were not contaminated with additional fingermarks during handling and analysis. Nitrile gloves were worn during sample collection, and the disks of the target were handled with tweezers. Before sample collection, the target disks were submersed in a mixture of deionized water and methanol and then a mixture of deionized water and acetone (approximately 50:50 v/v mixtures). In between each cleaning mixture, the target disks were wiped with a Kimwipe[®]. Using tweezers, the disks were replaced

onto the MALDI target for sample collection. Mass spectrometry analysis of the cleaned target confirmed that the cleaning procedure adequately removed all interfering fingerprint material from the target disks before their use.

2.3. Preparation of triolein samples

A sample of triolein was prepared by dissolving a small amount of the TAG in 2 mL hexane. Triolein was selected because it is an unsaturated TAG containing double bonds that are susceptible to oxidation. Using a stainless steel MALDI target disk, 500 μ L of the mixture was pipetted over each side of the disk and allowed to evaporate. This procedure was repeated using another target dish with the remaining TAG mixture. Disk 1 was then placed under a hood laboratory light while disk 2 was placed into a covered cardboard box. Samples were left under these conditions for 24 hours. After this time, one side of both disk 1 and 2 were “rinsed” with 1 mL hexane into separate centrifuge tubes. The “rinsing” procedure consisted of pipetting the hexane over the disk and collecting the run-off. Samples were stored in the freezer protected from light until analysis, and the MALDI disks, each still containing one side of deposited triolein, were returned to the light and dark conditions. After an additional 20 hours, the disks were removed and each side was again “rinsed” with 1 mL hexane and the run-off was collected. A triolein standard was also prepared in hexane as a reference without exposure to the light or dark conditions. The storage conditions of light and dark are the same as those for the fingerprint samples. The experimental layout for this procedure is shown in Fig. 1.

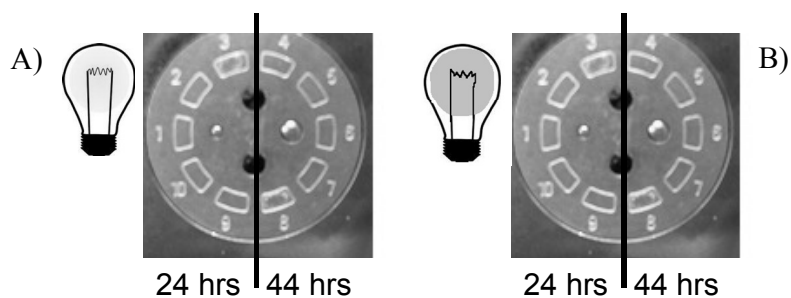


Fig. 1. Schematic illustrating the conditions for triolein degradation on the MALDI target under light (A) and dark (B) condition over 24 and 44 hrs.

2.4. Collection of fingerprint samples

Fingerprint samples were collected from individuals by having them undergo a typical “grooming procedure”. In this procedure the index finger was wiped once across the forehead, over the bridge of the nose from cheek to cheek, and across the chin area. This procedure was carried out to ensure that the finger was loaded with sebaceous secretions. This sample collection procedure was adapted from groups who have previously studied the composition of fingerprints [4-5, 11].

After undergoing the “grooming procedure”, individuals were instructed to press their index finger onto a glass surface. Volunteers were then asked to press the same finger onto the glass to overlap the previous print. This was done to ensure privacy since the fingerprint was now smeared. This process was repeated onto plastic, iron, and stainless steel surfaces with the “grooming procedure” followed in between each of the different conditions. Fingerprints were collected from five different individuals for each of the different samples surfaces. These samples were subsequently exposed to light conditions over time. To analyze lighting conditions,

additional samples were collected on each of the four surfaces and exposed to the dark over the same time period. The time of collection and analysis were the same for each of the different surfaces under light conditions. Samples exposed to the dark were collected the following week to ensure that an adequate amount of lipid material was transferred to the surfaces.

Immediately following sample collection, approximately one-fourth of the fingerprint material from each volunteer was directly transferred to the MALDI target using a Q-tip. This process consisted of simply rubbing the Q-tip onto the fingerprint and then wiping this material onto a well of the MALDI target. This transfer procedure was repeated for each fingerprint from the four different sample surfaces. Mass spectra were subsequently obtained for each sample. After each fingerprint was transferred, the four different sample surfaces were left for 16 hours at room temperature in the presence of light from a laboratory hood. After this time, another fourth of the fingerprint from each sample was transferred to the MALDI target with collection of mass spectra. This collection process was repeated for the glass, plastic, and iron surfaces an additional two times for a total of 35 and 86 hours exposed to light. The stainless steel surface was exposed to light for 14, 16, and 32 hours. The same sampling procedure described above was repeated for the surfaces held under dark conditions with collections occurring at the same periods of time. Additional fingerprints were collected and sampled at 112 hrs for all conditions described. The experimental layout for this procedure is shown in Fig. 2. A similar method has previously been shown to be effective for adequate transfer of fingerprints with low error < 6% [15].

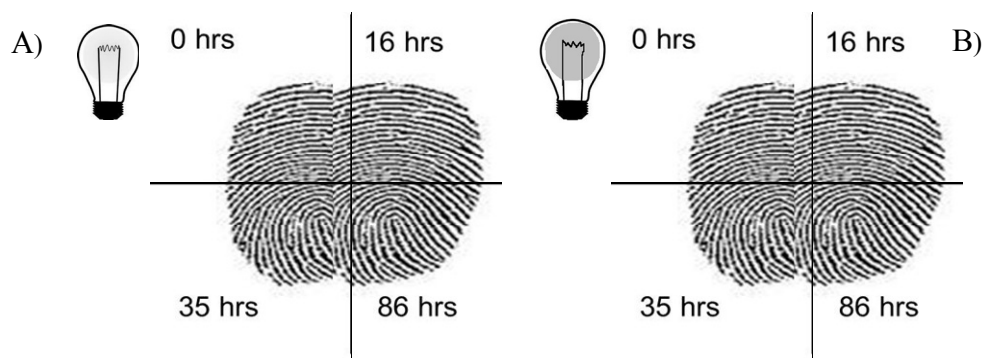


Fig. 2. A portion of the fingermark was collected from the different surfaces and transferred to the MALDI target under light (A) and dark (B) conditions.

3. Results and discussion

3.1. MS and MS/MS of triolein degradation

Fig. 3 shows MS spectra of the triolein sample immediately after preparation (Fig. 3A) and after 24 (Fig. 3B) and 44 hours (Fig. 3C) exposure to light. In the spectra, an intense peak is observed at m/z 907.7 for the sodiated adduct ion of triolein (C54:3). After 24 hours, additional peaks are observed in the m/z range of 650-850. These peaks are almost entirely absent in spectrum A and increase as the sample is exposed to light for a longer duration of time.

Comparison of relative intensities for the most abundant peak at m/z 797.5 compared to 907.7 is as follows: 5% for 0 hours, 28% for 22 hours, and 40% for 44 hours. Similar results also occur for the remaining peaks relative to m/z 907.7. The MS spectra of the triolein sample held under dark conditions also show some degradation products (spectra not shown).

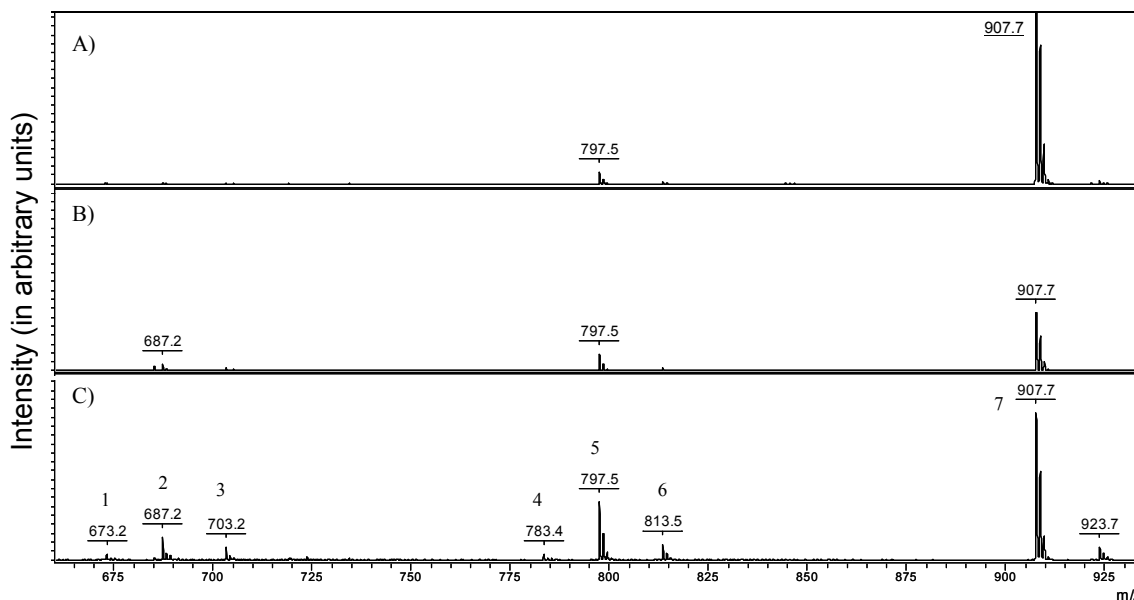


Fig. 3: Partial (m/z 675-925) MALDI-TOF mass spectra of a triolein sample held under light conditions for 0 (A), 24 (B), and 44 (C) hrs. Numbered peaks are sodiated ions and are assigned as follows: 1 – (C18:1, 1 heptanal group, 1 octanal); 2 – (C18:1, 2 octanal groups); 3 – (C18:1, 1 octanal, 1 C8:0 acid); 4 - (2 C18:1 groups, 1 heptanal); 5 – (2 C18:1 groups, 1 octanal); 6 – (2 C18:1 groups, 1 C8:0 acid); 7 – (3 C18:1 groups).

In order to characterize the structure of the peaks in the spectrum, MS/MS experiments were performed on ions at m/z 907.7, 797.5, and 687.2. The MS/MS spectrum of m/z 907.7 (C54:3) is shown in Fig. 4. The peak at m/z 602.8 corresponds to loss of a C18:1 fatty acid sodium salt residue (-RCOONa) with the corresponding C18:1 fatty acid residue at m/z 624.8 (-RCOOH). This is an expected result since triolein contains three C18:1 fatty acids and can therefore only lose a C18:1 group.

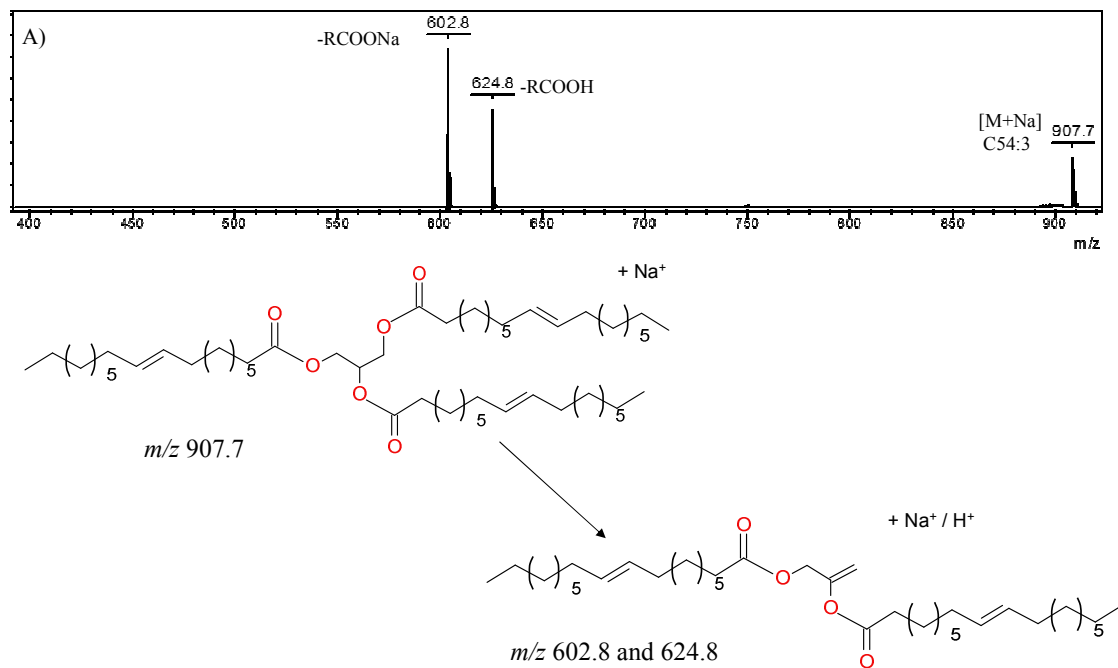


Fig. 4. MS/MS of a triolein sample monitored at m/z 907.7 and the resulting fragmentation pattern.

Prospective parent/fragment ion relationships can be considered for the remaining peaks (m/z 795.5 and 687.2) based on the mechanism of TAG degradation previously discussed. Among the two possible oxidation products of oleic acid (C18:1) are aldehydes including bound C7:0 aldehyde (heptanal) and C8:0 aldehyde (octanal). Fig. 5 gives the fragmentation of m/z 797.5. The peak at m/z 429.9 corresponds to loss of a C18:1 fatty acid sodium salt residue (-RCOONa) and the peak at m/z 514.9 gives the corresponding C18:1 fatty acid residue (-ROOH). This indicates the peak at m/z 797.5 (C48:2) is a sodiated ion containing two C18:1 fatty acids and octanal.

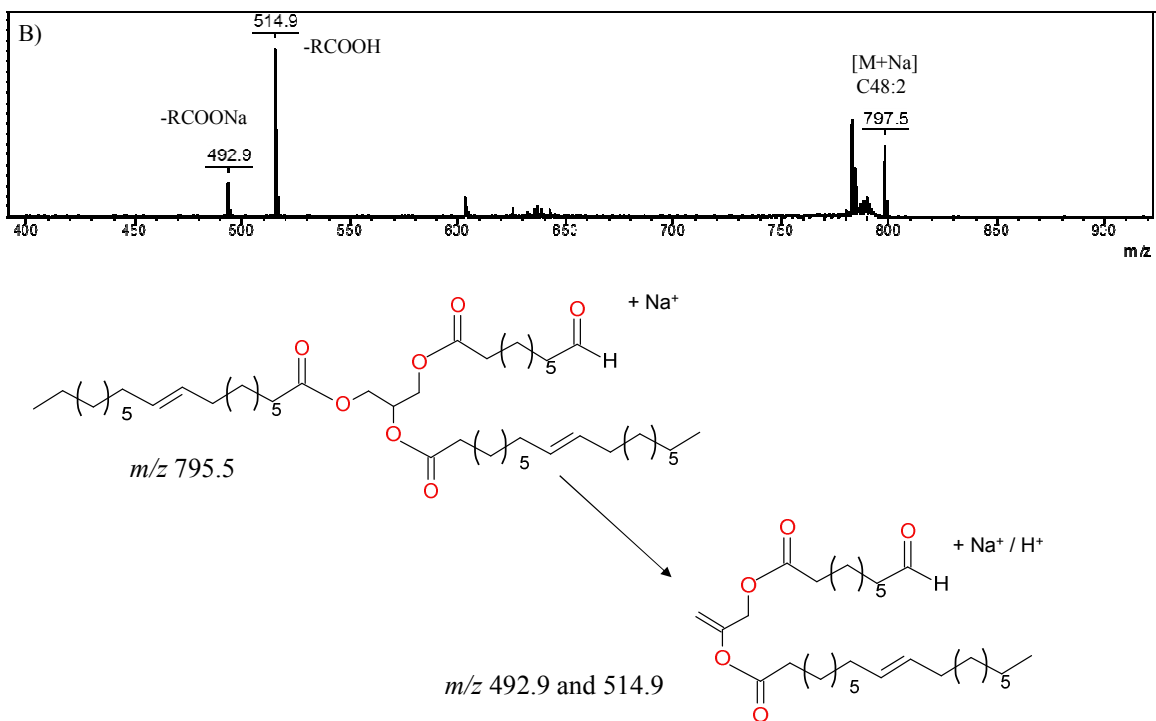


Fig. 5. MS/MS of a triolein sample monitored at m/z 797.5 and the resulting fragmentation pattern.

The MS/MS of the peak at m/z 687.2 is shown in Fig. 6. The peak at m/z 404.8 corresponds to loss of a C18:1 fatty acid residue (-ROOH). The peak at m/z 492.8 indicates loss of a sodiated octanal residue (-R₁OONa, R₁ is a C8:0 aldehyde) with the corresponding loss of a protonated residue at m/z 514.9 (-R₁OOH, R₁ is a C8:0 aldehyde). The peak at m/z 404.8 results from loss of a C18:1 residue (-R₂OOH, R₂ is a C18:1). This indicates the peak at m/z 687.2 is a sodiated ion containing one C18:1 fatty acid and two octanal groups (C39:1). Although MS/MS spectra were not collected of the additional peaks in Figure 5, inferences can be drawn based on the above results. For instance, the peak at m/z 813.5 most likely corresponds to a sodiated ion with two C18:1 residues and a bound C8:0 acid after oxidation of octanal. Likewise, the peak at

m/z 703.2 results from a sodiated ion containing one C18:1 residue, one octanal group, and one bound C9:0 acid. The peak at m/z 783.4 is likely a sodiated ion with two C18:1 residues and heptanal. Lastly, the peak at m/z 673.2 is as a sodiated ion containing one C18:1 residue, one heptanal, and one octanal. These fragmentation products of oleic acid are consistent with previous reports [16-17].

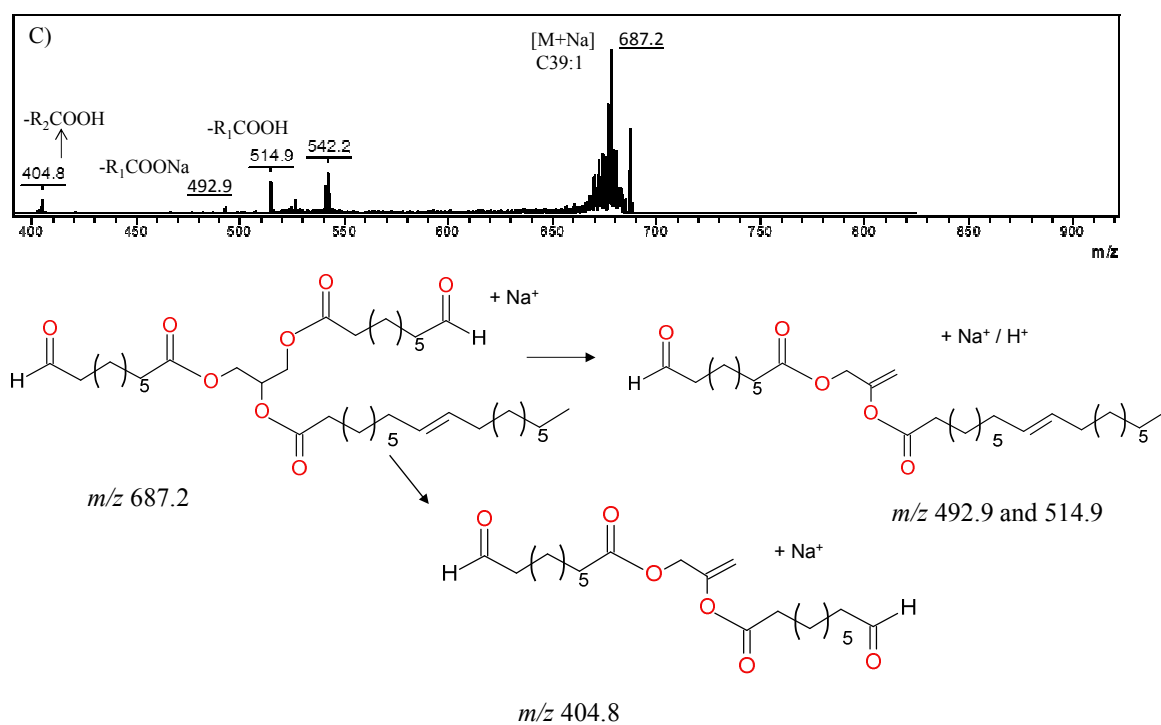


Fig. 6. MS/MS of a triolein sample monitored at m/z 687.2 and the resulting fragmentation pattern.

3.2. MS of fingerprint samples

Fig. 7 shows MS spectra of a fingerprint sample transferred from a glass surface immediately after collection (Fig. 7A) and after exposure to light conditions for 16 (Fig. 7B), 35 (Fig. 7C), and 86 (Fig. 7D) hrs. After 35 hrs in the light, the relative intensities of the TAGs begin to change with some TAGs undergoing a process of degradation. This change becomes more rapid after 35 hours and is clearly observed at 86 hours (Fig. 7D). Additional peaks are also observed in the mass spectrum of this sample particularly in the m/z range of 650-750. An enlarged view of the mass spectra centered at m/z 795-835 more visibly shows this change for the given time periods (Fig. 8). TAGs that decreased in intensity correspond to TAGs that were previously identified as having at least one unsaturated fatty acid through MS/MS experiments (e.g. m/z 799.5, 813.6, 825.5, and 827.6) (1). Other TAGs that were still present after 86 hrs (e.g. m/z 801.6, 815.6, and 829.6) were identified as containing all saturated fatty acids (1). This degradation process is an expected result since fatty acids that are unsaturated are oxidized more quickly than less unsaturated fatty acids (2). MS spectra of samples exposed to light for the plastic and iron surfaces showed a similar rate of degradation as for the glass. A decrease in intensity occurred after 35 hrs with a visible difference at 86 hrs (data not shown).

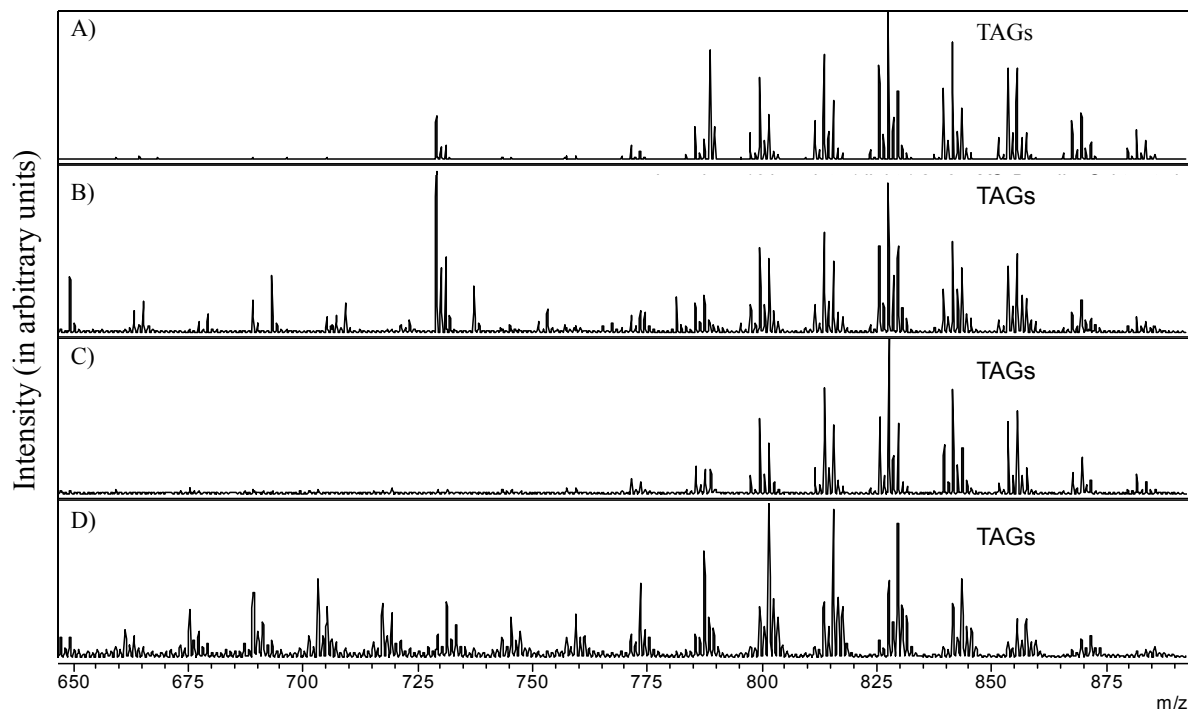


Fig. 7. LDI-TOF MS spectra of a fingermark sample on a glass surface held under light conditions following sample collection at 0 (A), 16 (B), 35 (C), and 86 (D) hrs.

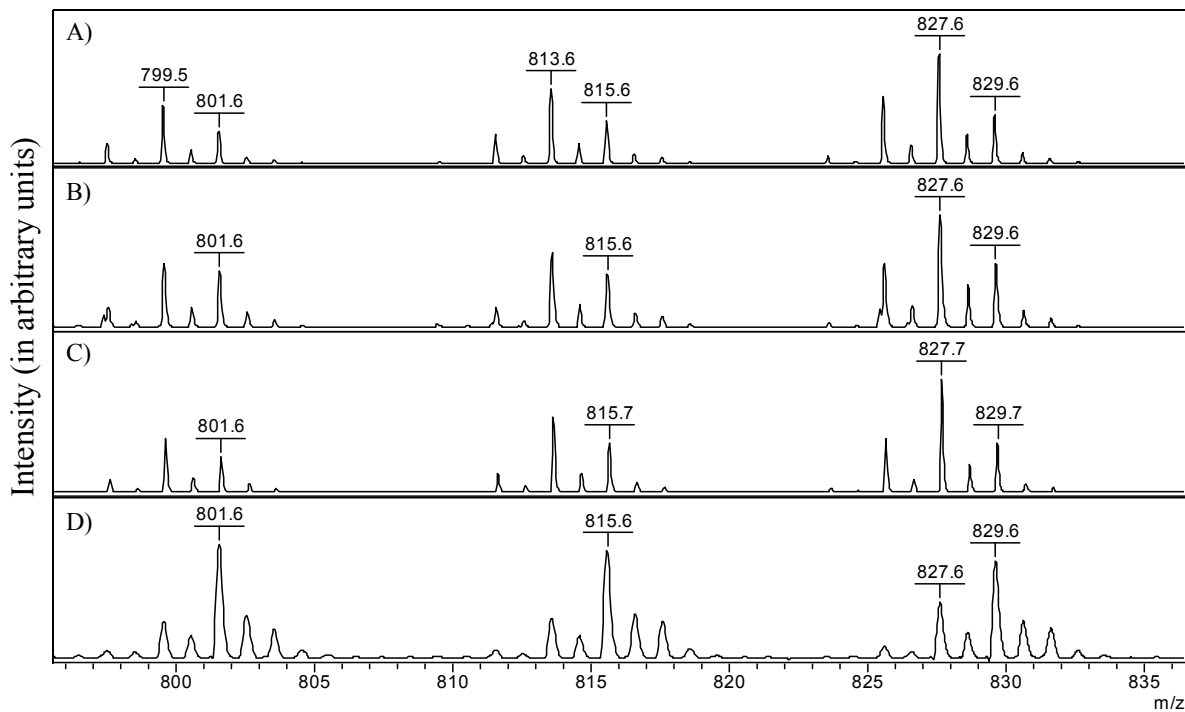


Fig. 8. Enlarged view of the mass spectrum of a fingerprint from a glass surface stored in the light after 0 (A), 16 (B), 35 (C), and 86 (D) hrs of time centered at m/z 795-835.

A degradation rate for each of the different surfaces was monitored by measuring the intensity of an unsaturated TAG at m/z 827.6 to a saturated TAG at m/z 829.6. These TAGs were selected because of their prevalence in fingerprint samples [15]. Fig. 9 shows the degradation for fingerprints on a glass surface over the 86 hr collection period. Data points are based on the average of five fingerprints collected at each time period. Error for each measurement ($\pm 30\%$) is calculated based on using a pooled standard deviation for all of the surfaces at each collection interval. As was observed in the mass spectra (Figs. 7 and 8), degradation begins around 35 hrs. At this time of collection, the relative intensity of m/z 827.6 to m/z 829.6 decreases to approximately 75%. Degradation rapidly continues after this time and by 86 hours the ratio of

unsaturated to saturated TAG is approximately 30 %. This rate of degradation closely resembles that for fingerprints transferred from plastic (Fig. 10) and iron (Fig. 11) surfaces. The rate of degradation at 35 hrs is approximately 60 % and at 86 hrs 27 % for these surfaces.

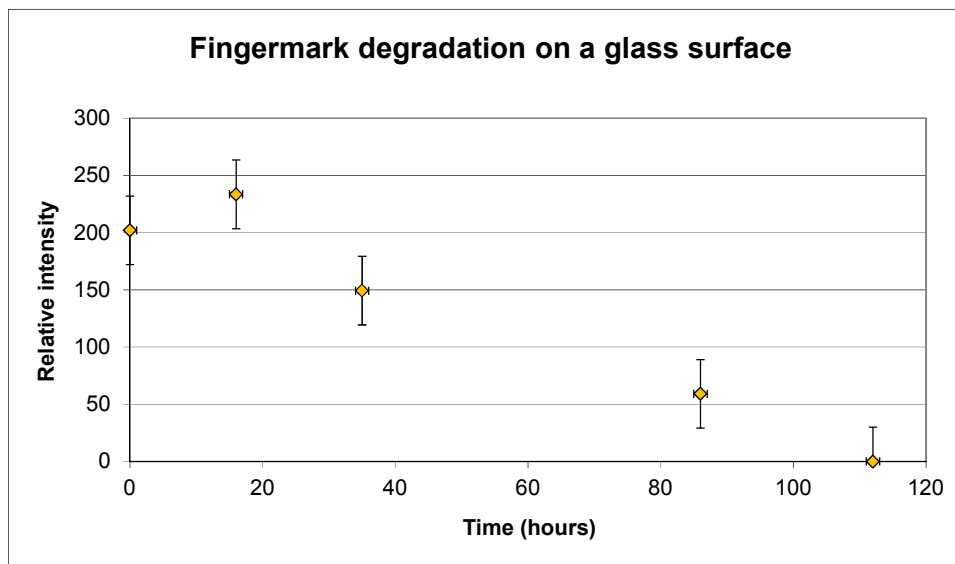


Fig. 9. Comparison of the relative intensities of TAGs from fingerprint samples at m/z 827.7 and m/z 829.7 over time on a glass surface held under light conditions.

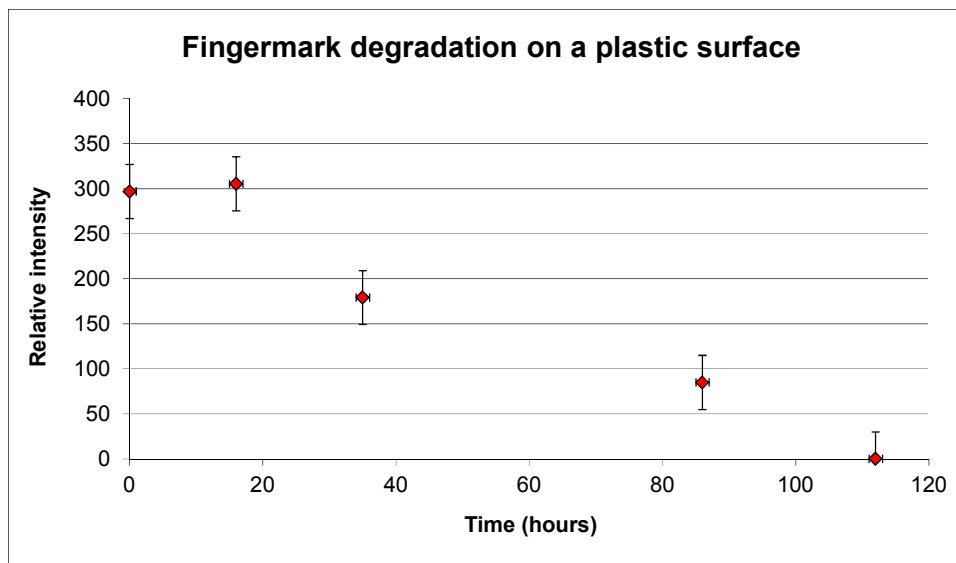


Fig. 10. Comparison of the relative intensities of TAGs from fingerprint samples at m/z 827.7 and m/z 829.7 over time on a plastic surface held under light conditions.

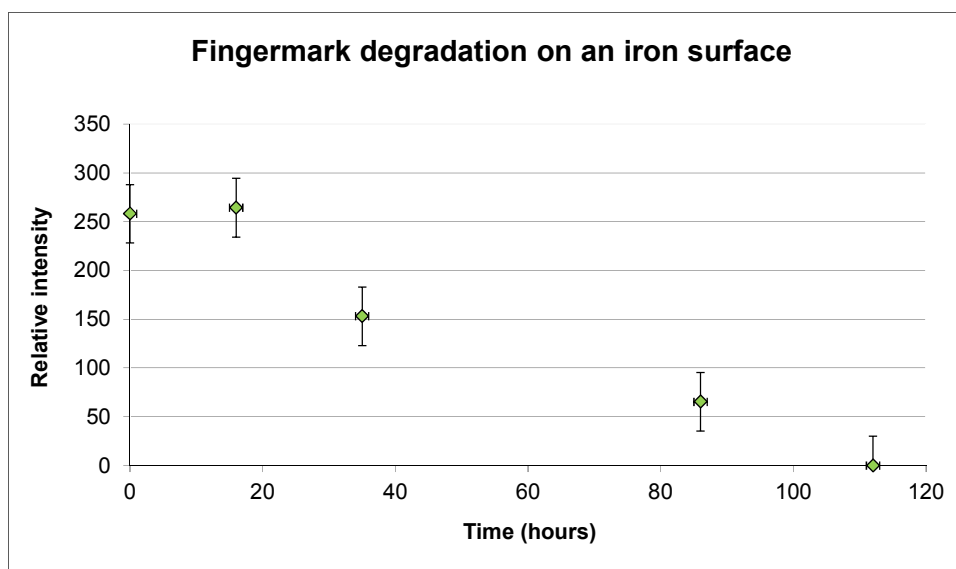


Fig. 11. Comparison of the relative intensities of TAGs from fingerprint samples at m/z 827.7 and m/z 829.7 over time on an iron surface held under light conditions.

Fig. 12 shows MS spectra of a fingerprint sample from a stainless steel surface immediately after collection (Fig. 12A) and after exposure to light conditions for 14 (Fig. 12B), 16 (Fig. 12C) and 32 (Fig. 12D) hrs. After 14 hrs in the light, the relative intensities of the TAGs begin to degrade very rapidly. An enlarged view of the mass spectra centered at m/z 795-835 clearly shows this rate of degradation (Fig. 13). After 32 hrs, all of the unsaturated TAGs (m/z 799.6, 813.6, 825.5, and 827.6) are almost entirely absent from the mass spectrum (Fig. 13D). Analysis of the degradation rate for fingerprints on a stainless steel surface is shown in Fig. 14. The relative intensity of unsaturated to saturated TAG is approximately 50 % at 14 hrs, 9 % after 16 hrs, and only 3% at 32 hrs.

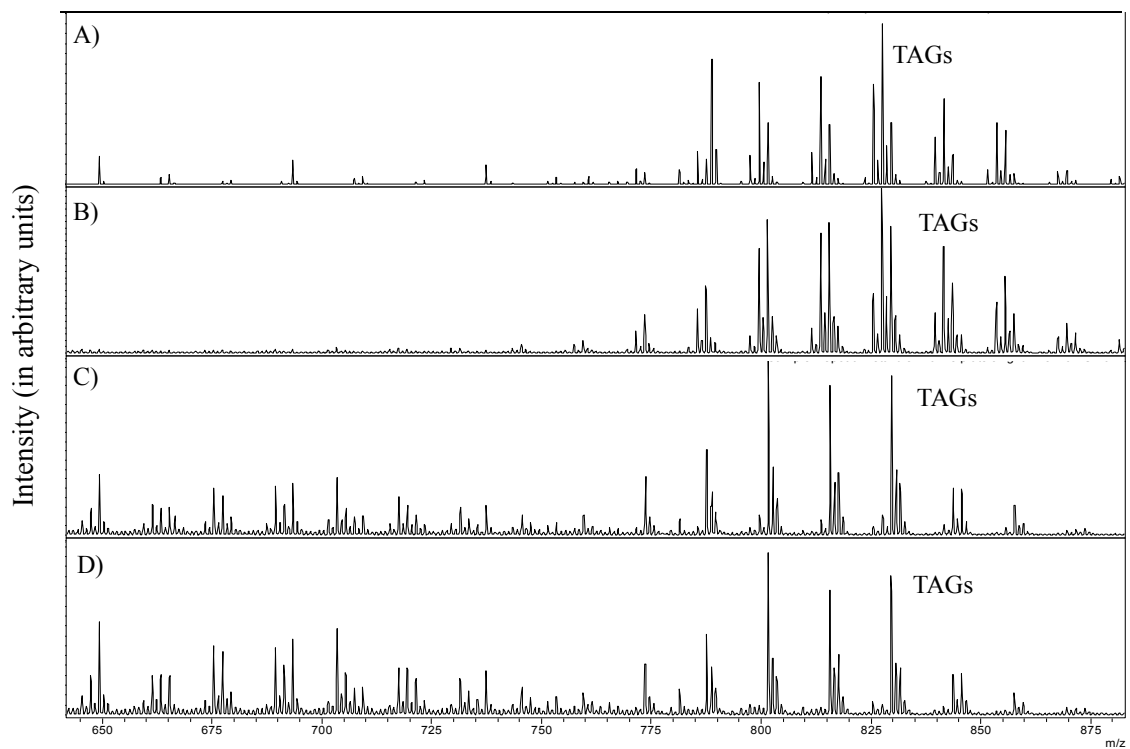


Fig. 12. LDI-TOF MS spectra of a fingermark sample on a stainless steel surface held under light conditions following sample collection at 0 (A), 14 (B), 16 (C), and 32 (D) hrs.

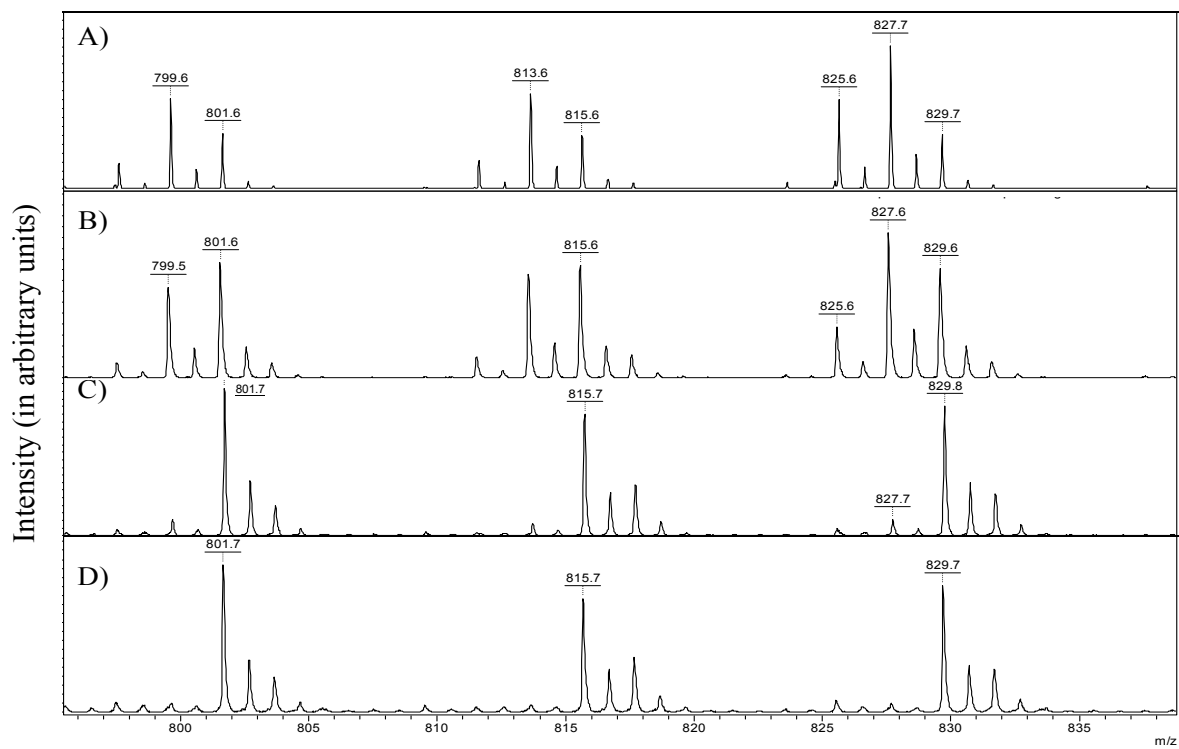


Fig. 13. Enlarged view of the mass spectrum of a fingerprint from a stainless steel surface stored in the light after 0 (A), 14 (B), 16 (C), and 32 (D) hrs of time centered at m/z 795-835.

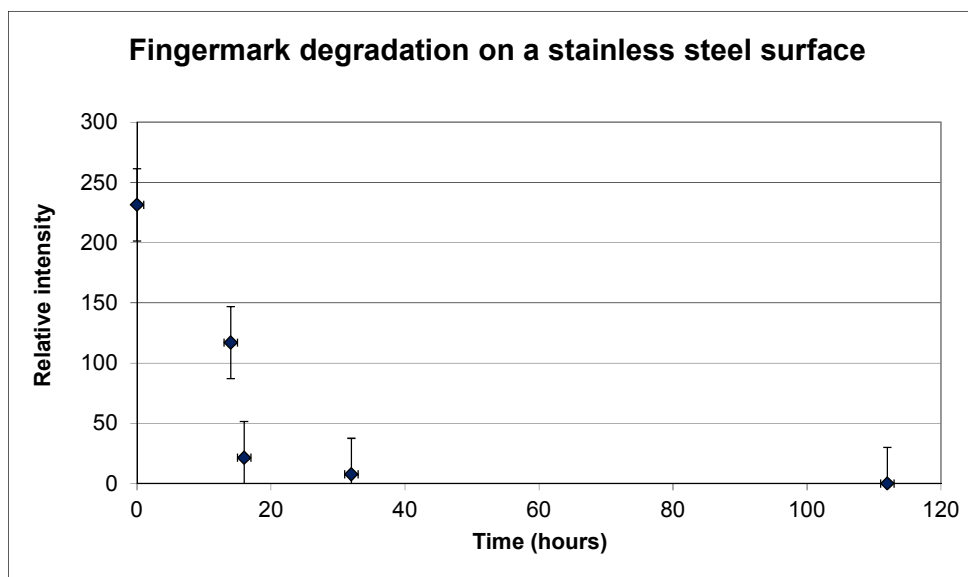


Fig. 14. Comparison of the relative intensities of TAGs from fingermark samples at m/z 827.7 and m/z 829.7 over time on a stainless steel surface held under light conditions.

Fig. 15 shows a comparison of the four different surfaces that were sampled for fingermark degradation. Initial rates were greater than 100 % for samples because relative intensities were calculated based on the ratio of the more abundant TAG at m/z 827.6 to the TAG at m/z 829.6. Rates begin to decrease over time because the TAG at m/z 827.6 begins to disappear while that for m/z 829.6 remains. Glass, plastic, and iron surfaces all show similar rates of degradation. It is unclear at this time why an increase in relative intensity is observed for all surfaces, except the stainless steel, at 16 hrs. After 86 hrs exposure to the light, approximately 30 % of unsaturated to saturated TAG is observed. Fingermarks sampled from a stainless steel surface, however, show a much more rapid rate of degradation. After just 32 hrs of exposure to light conditions, only a small fraction of unsaturated TAG remains. Degradation rates are based

on 32 hrs for stainless steel and 86 hrs for the other surfaces because little to no unsaturated TAG remains at this time. Analysis of samples exposed to light or dark conditions for longer periods of time (112 hrs) reveal that TAGs, unsaturated or saturated, no longer remain. This may be due to another degradation or polymerization mechanism and should be further investigated.

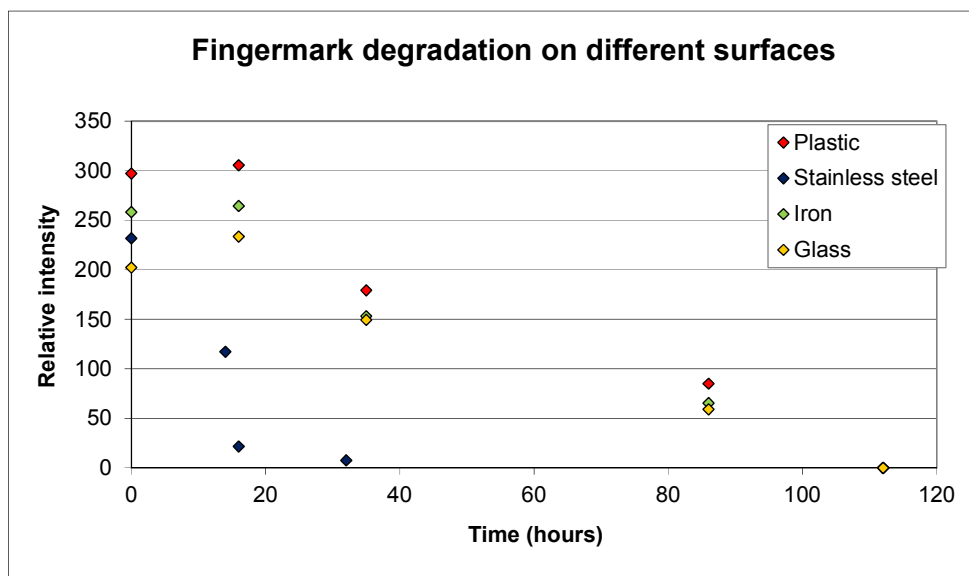


Fig. 15. Comparison of the relative intensities of TAGs from fingerprint samples at m/z 827.6 and m/z 829.6 over time on a various surfaces held under light conditions.

Fig. 16 shows MS spectra of a fingerprint sample (centered between m/z 795-835) transferred from a glass surface immediately after collection (Fig. 7A) and after exposure to dark conditions for 16 (Fig. 7B), 35 (Fig. 7C), and 86 (Fig. 7D) hrs. Samples exposed to these

conditions showed little signs of degradation for the four different surfaces. This lack of decomposition indicates the acceleration of oxidation for samples held under light conditions.

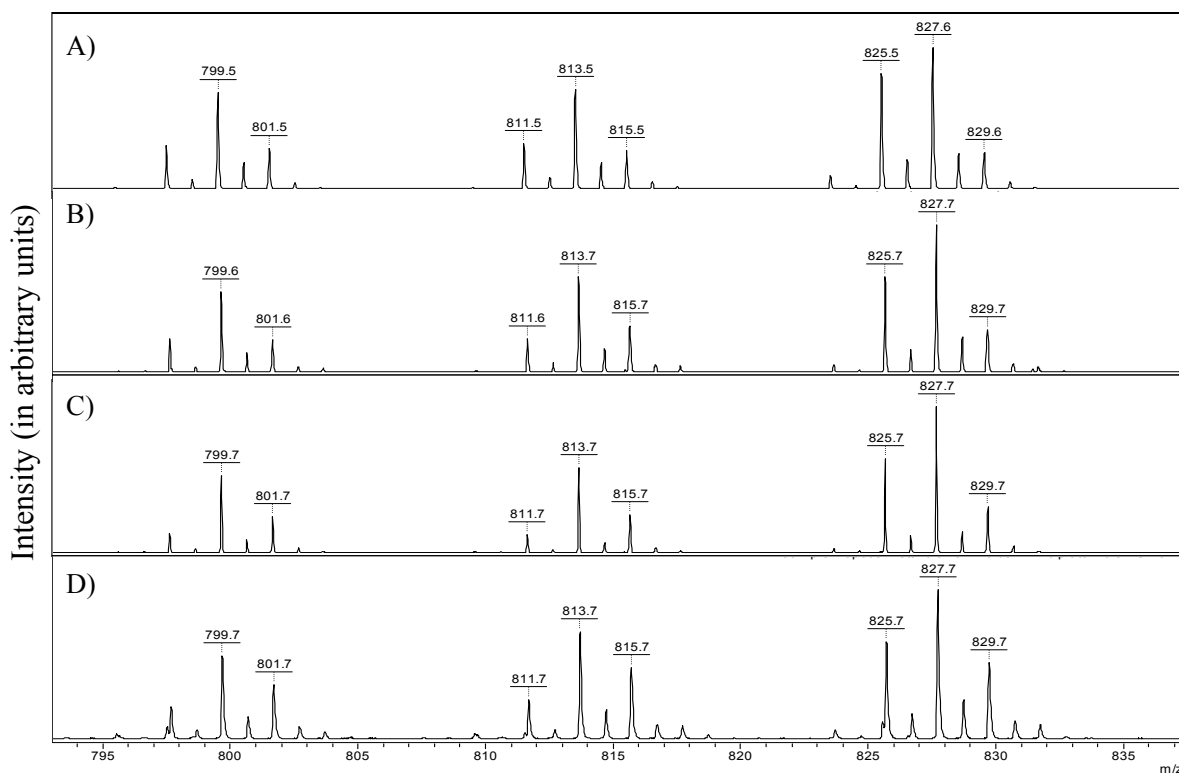


Fig. 16. Enlarged view of the mass spectrum of a fingerprint from a glass surface stored in the dark after 0 (A), 16 (B), 35 (C), and 86 (D) hrs of time centered at m/z 795-835.

3.3. Comparison of standard and fingerprint degradation

After studying the degradation products of triolein, a similar mechanism is observed for fingerprints. These products are observed in samples at m/z 650-750 after 16 hours for the stainless steel surface and 86 hours for plastic, glass, and iron surfaces. Fig. 17 shows a MS spectrum of a fingerprint after 16 hrs for the stainless steel surface indicating these products. Using the composition of TAGs containing unsaturated fatty acids, some assignments can be

made. The TAG at m/z 799.6 was previously determined to contain fatty acids C14:0, C16:0, C16:1 [15]. Because C16:1 is unsaturated, it will undergo oxidation and subsequent degradation as did the C18:1 in triolein. Products of this degradation likely contain heptanal at m/z 675.5 and octanal at m/z 689.5 as is observed in the MS spectrum. Overlap in the products can occur from other TAGs degrading in a similar manner. For example, the TAG at m/z 813.6 either has the composition C14:0, C16:0, C17:1 or C15:0, C16:0, C16:1 [15]. Decomposition of fatty acid C17:1 would also lead to the peaks at m/z 675.5 and 689.5 described above. Decomposition of C18:1 instead leads to 689.5 (heptanal) and 703.5 (octanal). Observation of these products, with disappearance of some TAGs, thus indicates that unsaturated fatty acids undergo a process of degradation to form bound C7:0 and C8:0 aldehydes and carboxylic acids.

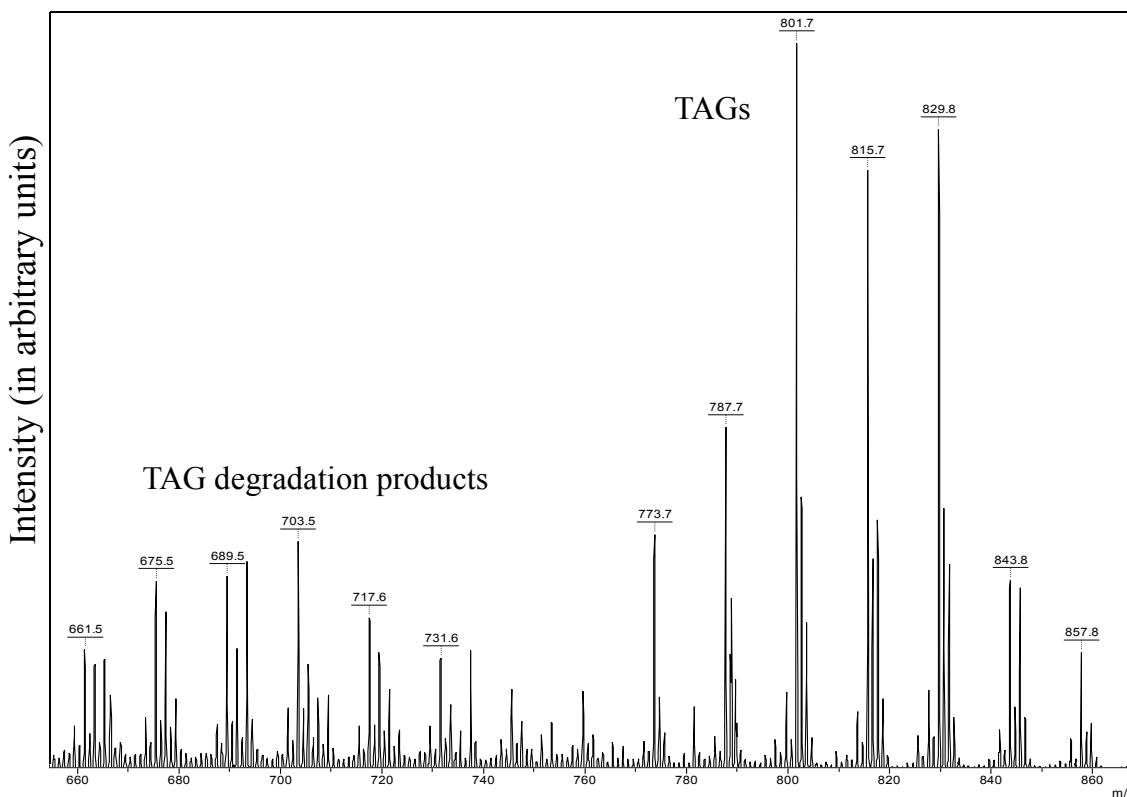


Fig. 17. MS spectrum of a fingerprint after exposure for 16 hrs in the light on a stainless steel surface; TAG degradation products are observed at m/z 650-750.

3.4. Analysis of a fingerprint's age with a known sample

The possibility of dating a fingerprint was accessed using a print in which the age was known. The following scenario is a potential application for ageing this print in a forensic setting. A victim is discovered murdered in her home. Several pieces of evidence are gathered including an object thought to be involved with the crime. Several prints are identified including one collected from this object. Conditions at the scene are noted as follows: lights in the house are on and the temperature of the room is approximately 25° C. The medical examiner estimates the time of death between 20-30 hrs prior to discovery of the body.

The print from the object is analyzed using LDI-TOF MS. The intensity of the TAGs at m/z 827 and 829 are compared with a ratio of 19 % calculated. Comparison of this value to Fig. 14, due to the stainless steel surface of the object and light conditions, indicates the print is between 16 and 112 hrs old.

The actual age of the print in this study is 24 hrs. Comparison to the corresponding data, based on light and surface conditions, indicate the age of a print can be estimated within a certain interval of time. If the ratio had been >87 %, it could be concluded the print was less than 14 hrs old. Analysis of the print's age is especially important in this situation since the time of death (20-30 hrs) is estimated as occurring around the time the print was deposited. This could lead to possible investigative leads including a timeline of events. Similar relationships can be made for the other samples surfaces.

4. Conclusions

Exposure of fingerprint samples to different surfaces resulted in mass spectra with TAGs that began to degrade over time and at different rates. These TAGs were determined to be unsaturated with oxidation occurring after exposure to light conditions. Degradation products from fingerprint samples were compared to a standard of triolein. MS/MS analysis of the standard revealed that degradation occurs to form C7:0 and C8:0 aldehyde and carboxylic acid products. A similar mechanism of degradation was observed for fingerprint samples. The ratio of an unsaturated (m/z 827.6) and saturated (m/z 829.6) TAG were compared for four different surfaces over 112 hrs. Glass, plastic, and iron surfaces all showed similar rates of TAG degradation while those for the stainless steel surface showed a much more rapid change. At 112 hrs TAGs in fingerprints could no longer be detected. Fingerprints exposed to dark conditions showed little difference between 0 and 112 hrs indicating light is necessary for degradation. The age of a known print was determined through data comparison based on surface and light conditions. The print was estimated to be between 16 and 112 hours old with the actual age being 24 hrs. This could be important for determining investigative leads and a timeline of events in a criminal case. Future studies include analysis of prints visualized with various fingerprint powders. Preserving the ridge detail of a print is a critical aspect for comparison to a suspect or a fingerprint database. It is thus important for a fingerprint to be photographed before the print is disturbed. While this method destroys a small part of the print, most of the ridge detail is preserved. The feasibility of determining the age of a fingerprint, however, is still demonstrated.

5. References

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Chapter 4

A rapid separation technique for overcoming suppression of triacylglycerols by phosphatidylcholine using MALDI-TOF MS

This research was originally published in the Journal of Lipid Research.

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Abstract

Phospholipids and triacylglycerols (TAGs) are important classes of lipids in biological systems. Rapid methods have been developed for their characterization in crude samples including matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). For mixtures, MALDI often selectively shows only some components. For example, phosphatidylcholine (PC) suppresses detection of other lipids. Most rapid MS methods detect either TAGs or phospholipids but not both. Herein, we demonstrate a simple approach to rapidly screen mixtures containing multiple lipid classes. To validate this approach, reference lipids [PC, tripalmitin (PPP), phosphatidyl-ethanolamine (PE)] and real samples (beef, egg yolk) were used. In a binary mixture with a strong suppressor (PC), PPP was greatly suppressed. After a simple separation, suppression was virtually eliminated. A mixture of nominally non-suppressing lipids (PE and PPP) was not adversely affected by separation. Ground beef and egg yolk were used to demonstrate detection of known lipid compositions where other methods have missed one or more lipids or lipid-classes. Separation was performed using solid phase extraction (SPE) with a PrepSep florasil column. A ten minute separation allows rapid screening for lipids and changes in lipids. It is sufficient to clearly detect all lipids and overcome suppression effects in complex lipid mixtures.

1. Introduction

Lipids play important roles in biological systems. Analysis allows probing of both their biological roles and as use in foodstuffs or other products. Examples of applications where it is important to detect multiple lipid classes (i.e. phospholipids and TAGs) include foods analysis [1-3], cell biology [4], health effects [5], taxonomy [6] and other fields [7-11]. However, because of different chemistries, it is often difficult to characterize one class of lipids in the presence of another, especially using rapid methods. Elegant methods have been developed for quantitative analysis in complex mixtures but they are typically difficult to implement and time consuming [8, 12]. There has been much recent interest in rapid MALDI MS analysis of lipids due to its speed of analysis and high sensitivity [5,13]. Sample preparation is quick because derivatization is not required (i.e. no silylation), high performance liquid chromatography (HPLC) separation is not needed, limited buffer or salt contamination is tolerated [9, 11, 13], and spectra are easy to interpret [13].

MALDI MS has functioned as both a qualitative and semi-quantitative approach to measure and track effected biology related phenomena. For example, Lay *et al.* demonstrated a rapid method for the analysis of edible oils by MALDI-MS that allowed determination of the relative abundances of TAGs with sufficient accuracy to correctly identify blind coded samples of various oils [15]. Gidden *et al.* have also reported using MALDI-MS to rapidly differentiate *Escherichia coli* and *Bacillus subtilis* based on the phospholipid profile and monitor changes in lipid content during the growth phases of the bacteria [16]. However, this rapid MALDI-MS approach cannot currently be applied to experiments requiring the detection of multiple lipid classes in an unresolved mixture. Attempts to use direct MALDI-MS on such complex mixtures invariable results in entire classes of lipids being missed because of suppression effects.

Suppression in MALDI-MS is well known and has been demonstrated amongst lipid classes as well as within members of a single class. For example, phospholipids containing quaternary amines can suppress detection of other phospholipids in the sample [7,11,13,17-20]. The classical solution to this problem is chromatographic resolution of mixture components using HPLC. Fuchs *et al.* have reported alternative methods to HPLC by coupling thin layer chromatography (TLC) with MALDI-MS. TLC/MALDI-MS worked well for phospholipids, but TAGs that were expected in the samples were not reported [18]. Likewise MALDI-MS of brain lipid extracts, using a silica gel cation exchanger for separation of components, have also been reported for the detection of phospholipids, but the detection of TAGs was again not reported [19].

Because some biological phenomena cause significant changes in the ratios of specific lipids within a class [14-16], a simple and rapid MALDI-based measurement technique is needed to monitor such changes. A rather lengthy LC-MS approach has been used for the separation of lipids, including phospholipids and TAGs [12]. While this approach is appropriate for quantitative analysis, it is not suitable for MALDI-MS or rapid screening. It is also not needed for measurements of large changes in relative lipid composition. The approach we report is the application of simple SPE cartridges (rather than TLC or ion exchange) to produce a few fractions from which lipids and classes of lipids can be characterized by rapid and direct MALDI-MS analysis. SPE approaches have been used for separation of different lipid classes in foods [21-22], oils [23], and biological tissues [23-25], most often after fatty acid methyl esterification to facilitate GC separation. In this case, we report MALDI-MS analysis of the intact lipids. Using a simple SPE separation, we report detection of both TAGs and phospholipids in a mixture.

2. Materials and methods

Herein, we demonstrate our approach using specific lipid standards and complex mixtures (beef and egg yolk) similar to those used in prior studies. A positive control for suppression included a mixture of PC (strong suppressor) and PPP (reference TAG). A negative control involved a mixture of PE (not a strong suppressor) and PPP. Comparison of the positive control spectra before and after SPE separation illustrates the extent of suppression whereas spectra from the negative control tests for potential losses during separation or analysis.

2.1. Materials

Tripalmitin (PPP), L- α -phosphatidylcholine, distearoyl (PC), and L- α -phosphatidylethanolamine, dioleoyl (PE) were purchased from Sigma Aldrich. PrepSep florisil extraction columns (14 mL volume capacity, 1 g pre-packed florisil) were purchased from Fisher Scientific. All chemicals (hexane, 2-propanol, 2,5-dihydroxybenzoic acid (DHB), and methanol) were analytical grade or better. Ground beef (80/20 – 80% lean, 20% fat) and hen eggs were purchased locally and used as obtained.

2.2. Lipid controls

Lipid standards were measured to the nearest 0.001 mg. Each lipid (1.500 mg) was placed in separate centrifuge tubes with 5 mL hexane/2-propanol (1:1 v/v) and vortexed several minutes to dissolve the solids. Mass spectra were then collected of the individual lipids. Afterwards, the PC solution was evaporated to approximately 1 mL under N₂ and added to the PPP sample. A mass spectrum was subsequently collected of this lipid mixture. (Volume adjustment was used to ensure the sample volumes were approximately the same as when the

lipids were analyzed individually). PC was selected as the positive control phospholipid in this experiment because of its prevalence in samples [11,18] and because of its reported suppression effects on other phospholipids [7,11,13,17-20]. A sample of PE and PPP was also analyzed. In this case suppression was not expected. This pair (negative control) of compounds was used to test for loss of signal from sample manipulation or during separation.

2.3. Beef and egg yolk lipids

The beef sample (0.25 g) was vortexed with 5 mL hexane/2-propanol (1:1 v/v) for 10 min. Insoluble tissue was removed and a mass spectrum was obtained from this crude beef lipid extract. The egg white and yolk were separated from a single hen egg. The yolk (0.05 g) was vortexed with 5 mL hexane/2-propanol (1:1 v/v) for 10 min. The extract was separated from the insoluble solid matter using a pipet. A mass spectrum was obtained of this crude egg yolk lipid extract.

2.4. SPE separation

Lipid classes (standards or extracts) were separated from each other using disposable PrepSep florisil extraction columns. To prevent overloading the column, no more than 50 mg of lipids by weight (given 1 g florisil packing) was added to any given cartridge [26]. Columns were pre-conditioned by washing with 5 mL hexane/2-propanol (1:1 v/v) with no collection of the filtrate. The lipid samples were added to the column, and the filtrate (which should contain TAGs) was collected by vacuum filtration into an Erlenmeyer flask. The column was then washed with 2.5 mL aliquots of hexane to remove any traces of undesirable compounds. This wash filtrate was collected and discarded. Lastly, the phospholipids were eluted with 5 mL 2-

propanol (70%) and collected. Phospholipid and TAG fractions were analyzed by MALDI MS as described below.

2.5. Mass spectrometry analysis

A 1M DHB matrix solution was prepared by dissolving 2,5-DHB in 90% methanol. Samples and matrix (1 μ L each) were mixed directly together in a ratio of 1:1 and 1 μ L of this mixture was applied to a stainless steel MTP Multiprobe Adapter MALDI target. A Bruker Ultraflex II (Bruker Daltonic GmbH, Bremen, Germany) MALDI TOF was operated in the positive-ion reflectron mode. Spectra were acquired from m/z 400-1650 with adjustment of the sample position and laser power to produce intense ions. The laser power was then kept constant, and 1500 laser shots were obtained for each sample. Mass spectra were plotted to the same vertical scale.

3. Results

3.1. Lipid standards

Intense ions are observed for PC as protonated [PC+H] and sodiated [PC+Na] adducts at m/z values of 790.6 and 812.6 (data not shown). Smaller peaks are also observed for PC as the protonated [2(PC)+H] and sodiated [2(PC)+Na] dimers. Gas-phase dimers are often observed using soft ionization MS and have been reported for MALDI MS analysis of lipids [27]. Typically, they arise from electrostatic attraction resulting in the sharing of a single ionizing cation between two analytes. PPP ions are observed (not shown) as sodiated adducts [PPP+Na] at m/z 829.7. TAGs, in general, are exclusively observed as salt-cationized ions rather than

protonated molecules by MALDI MS because the protonated molecule is unstable and decomposes by rapid unimolecular decay [28].

Using approximately 300 ng of each lipid, mass spectra were readily obtained. The ratio of the measured absolute peak intensities (PC:PPP) for the sodiated adduct ions was approximately the same for optimized spectra obtained using the same number of laser shots and identical conditions. However, when the PC and PPP were mixed in approximately equal proportions, a ratio of approximately 5:1 was observed for these two ions (not shown). This represents a significant suppression of the TAG in the mixture. Based on a report by Lou et al, suppression of PPP (or any TAG) by PC is expected because of a more favorable competition for charge by its quaternary ammonium group [20]. A simple SPE separation was used to recover the lipids for re-analysis, in two fractions, presumably without analyte-induced suppression. After rapid SPE separation of PC and PPP, the MALDI TOF mass spectra again gave an approximately 1:1 ratio for the intensities of the sodium-adduct ions in the two spectra. Suppression effects were also seen using smaller amounts of PC (1:4 ratio) and likewise signal intensity were also recovered after SPE separation. In summary, the two analytes were readily detected with the expected relative abundances before and after separation but not in the mixture. For other pairs of lipids in which PC was present, the same sort of suppression would be expected [7, 11, 13, 17-20].

Some lipid mixtures do not show significant suppression in MALDI-MS spectra. The mass spectrum of a mixture of PE and PPP, for example, had the expected peak ratios of each lipid class. While the SPE extraction would not be needed for this specific mixture, the extra step did not result in significant sample loss. Spectra of individual components were similar both

before and after separation and the mixture spectrum was well represented by simple addition of the two individual component spectra.

3.2. Beef lipids

Ground beef has previously been shown to contain both phospholipids and TAGs, with the latter comprising 75-90% of the total lipid content [29]. Of the different phospholipid classes, PC is the most abundant, comprising approximately 50% of the total phospholipids [30]. A crude lipids fraction from “80:20” ground beef was analyzed by MALDI-TOF MS. The mass spectrum in Fig. 1a shows the lipids extracted from the beef sample before SPE separation. As expected because of the abundant PC, phospholipids are detected in the m/z range of 740-810 whereas TAGs, which should be detected between m/z 800-1000, are almost entirely absent even though known to be present in much greater abundance. The only TAGs that were detected give small peaks at m/z 855.8 and 881.8. Interpretation of this mass spectrum, without knowledge of the effects of ion suppression, might lead to the conclusion that TAGs are only present in ground beef lipids in low abundance. Using this protocol it might be possible to monitor changes in the phospholipid ratios but not changes in TAGs. For some applications (and for lipidomic studies) measurement of the relative changes of TAG as well as phospholipids is important [14-16].

Mass spectra of the two SPE separated fractions are shown in Figs. 1b and 1c. In Fig. 1b, a complex set of TAGs are clearly detected. After this simple separation, changes in the TAGs composition, if any, could be easily determined from peak ratios in this spectrum. Assignment of peaks in the spectrum is in good agreement with the known fatty acids in ground beef [31]. The carbon and double bond numbers from the TAG spectrum (1b) range from C44:1-54:2, corresponding to fatty acids with carbon chains of C14:0-C18:1, which are approximately 90%

of fatty acids in ground beef [31]. PE and PC, which account for approximately 80% of the phospholipids in ground beef [30], are also observed in great abundance in the phospholipid spectrum (1c) for the same fatty acids. The most abundant TAGs and phospholipids are denoted in the spectra for the separated fractions (Fig. 1b and 1c).

Fig. 1d is a calculated summation of the peak intensities in Figs. 1b and 1c. Considering that the TAGs are expected to be present in significantly greater abundance than the phospholipids in this sample, the expected intensities for TAG ions in Figure 1d should probably be larger. In other words, the relative abundances of the TAGs remain less than expected based on reported values, even after separation. Most likely this can be explained by different ionization efficiencies in the matrix. In the absence of suppression, lower ionization efficiencies are nevertheless expected for less polar compounds compared to more polar ones or preformed ions.

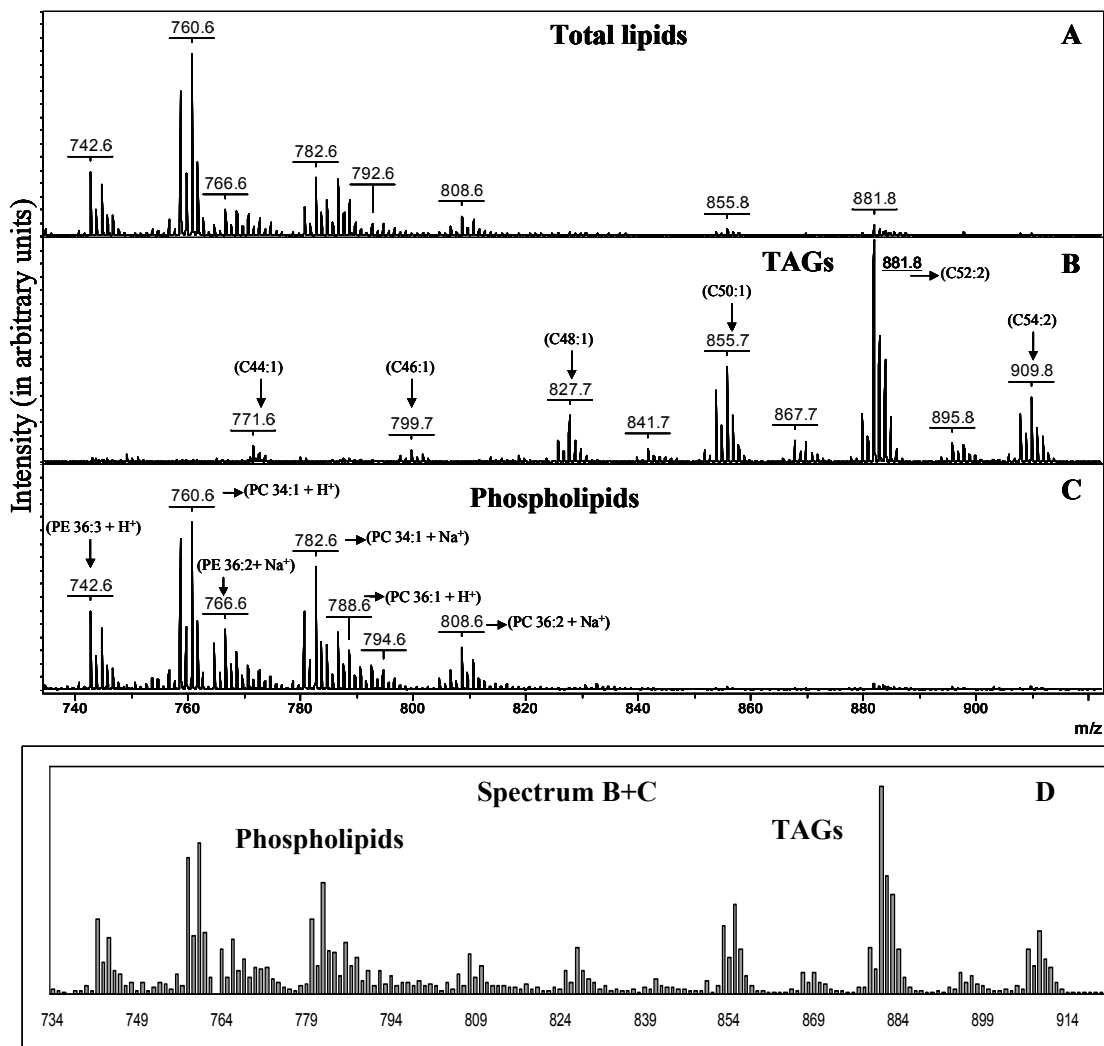


Fig. 1. Partial (m/z 740-920) MALDI-TOF mass spectra of a ground beef lipids mixture (A), the TAGs (B) and phospholipids (C) fraction collected from the SPE cartridge, and a simulated spectrum (D) from the summation of the spectra (B & C) from the two lipid fractions showing the expected peak intensities for total lipids in the absence of suppression effects. Numbers in parenthesis indicate the number of carbon atoms and double bonds, respectively, in the fatty acid side chains.

Generally, TAGs and phospholipids are observed in different m/z regions of the mass spectrum. However, there is some overlap which can make interpretation of spectra more difficult, especially for ions of different abundances and because of isotopic peaks. For example, if a sample contained the TAG 14:0/16:0/18:0 + Na⁺ (m/z 829.7) and PC 18:1/20:4 + Na⁺ (m/z 830.6) overlap would exist between the carbon isotope peak of the TAG and the monoisotopic peak from PC. In the beef lipid extract, two known TAGs (m/z 771.6 and m/z 799.7) (29) are observed overlapping the mass region normally associated with phospholipids. Even if there were no suppression effects, the overlap of members of these two classes could make it difficult to detect both. Simple SPE separation clearly resolves this issue. Moreover, knowledge of the lipid class often allows unambiguous assignment of the lipids based on molecular weight and known fatty acid composition. Thus, in addition to the reduction of suppression, separation minimizes problems that may arise from overlap in the mass regions for members of the two lipid classes. By minimizing this overlap, our approach greatly simplifies interpretation of the spectra.

3.3. Egg yolk lipids

TLC/MALDI-MS of egg yolk lipids was recently reported to demonstrate minimization of suppression and a separation method well suited for MALDI MS analysis (18). Egg yolks contain approximately 30% lipids by weight [32] with phospholipids (31%) and neutral lipids (65%) being the most abundant species. Neutral lipids in this case include TAGs, diacylglycerols, monoacylglycerols, fatty acids, carotenoids, and sterols [33]. Of the different phospholipid classes, PC, which frequently causes problems with suppression, is the most abundant comprising approximately 26% of the total phospholipids [32]. In the TLC/MALDI-

MS report, reduction of suppression by phospholipids was achieved but the abundant TAGs were not reported [18]. Herein, we demonstrate the ability to detect both phospholipids and TAGs using a SPE approach rather than TLC.

Fig. 2a shows the mass spectrum of the lipids extracted from the egg yolk before application of SPE separation. Phospholipids are detected in the m/z range of 758-810 whereas TAGs, which should be detected between m/z 840-920, are almost entirely absent even though present in a greater abundance. The only TAGs that were detected give small peaks at m/z 879.8 and 881.8. As with the ground beef lipids, interpretation of this mass spectrum might lead to the conclusion that TAGs were either present in low abundance or absent.

Mass spectra of the two separated fractions from a 15 mg-equivalent sample are shown in Figs. 2b and 2c. In Fig. 2b, the TAGs are clearly detected and two additional groups of TAGs (C50:3-50:1 and C54:5-54:2) that were not previously detected in Fig. 2a are observed. The fatty acids in the separated TAG fraction range from C50:3-54:2, corresponding to fatty acids with carbon chains of C16:0-C18:2, which represent approximately 95% of fatty acids in egg yolk [31]. The most abundant TAGs are labeled in the spectrum from this fraction.

Fig. 2c shows the fraction containing the phospholipids. Little difference is observed in this spectrum compared to the same m/z range in the total egg yolk sample (Fig. 2a). Biologically important changes in the phospholipid composition could probably be detected in the crude mixture but separated fractions would be needed to detect any changes in the TAG composition. The most abundant phospholipids are labeled in the spectrum for the separated fraction (Fig. 2c).

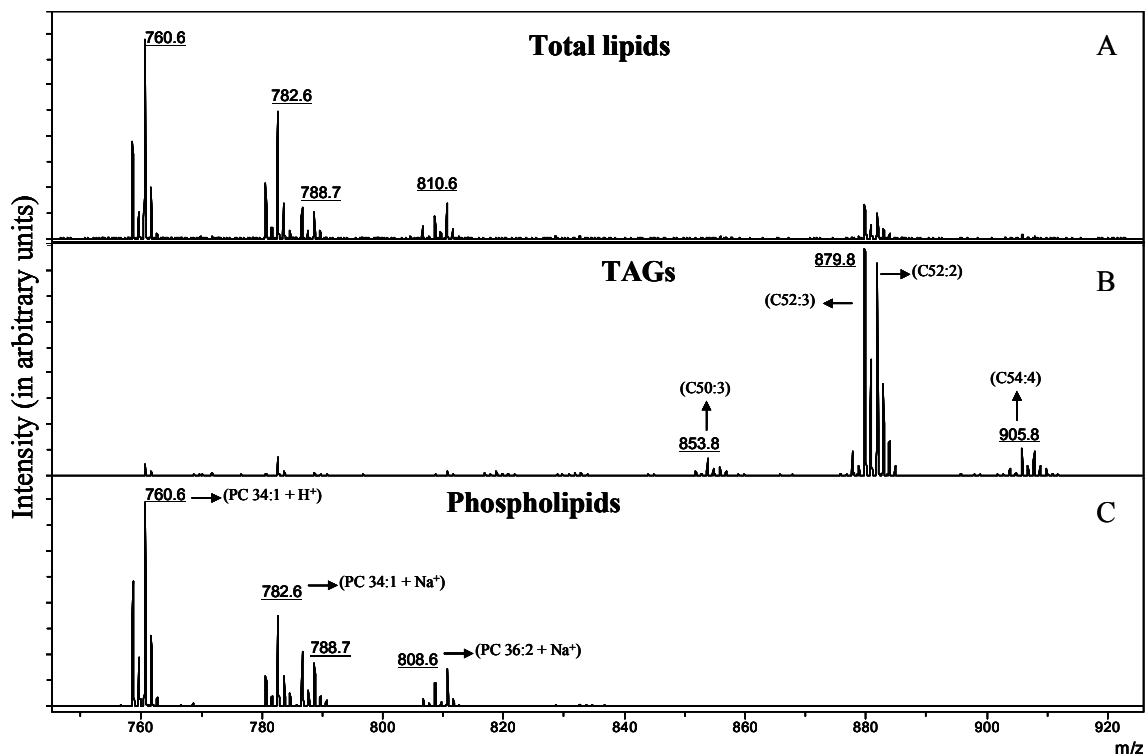


Fig. 2. Partial (m/z 740-920) MALDI-TOF mass spectra of an egg yolk lipids mixture (A), the TAGs (B) and phospholipids (C) fraction collected from the SPE cartridge. Numbers in parenthesis indicate the number of carbon atoms and double bonds, respectively, in the fatty acid side chains.

Fuchs *et al.* reported analysis of egg yolk lipids by MS analysis of the extracts before and after TLC separation using MALDI and MALDI imaging respectively [18]. Our observed phospholipids (Fig. 2a & 2c) are in good agreement with those observed in their direct MALDI analysis of a total lipids fraction. It should be noted that in this previous study by Fuchs, no TAGs were reported in the samples (by MALDI or TLC imaging) despite their careful analysis of PC suppression effects on phospholipids from a total lipids extract. Their analysis using direct

MALDI and MALDI TLC imaging only detected phospholipids and completely missed the TAGs which were more abundant in the sample. It is likely that the TAGs were completely suppressed by direct MALDI (without separation) and ran off of the TLC plate when analyzed by TLC/MALDI imaging. The advantage observed using TLC/MALDI was that some additional phospholipids were detected when the phospholipids themselves were separated by TLC. This highlights a limitation of the simple two step SPE technique. It readily detects abundant members of each fraction, but does not have sufficient chromatographic resolution to prevent all suppression within a SPE fraction. This can, perhaps, be resolved using different solvents and multiple elution steps if more detailed information about either phospholipids or other lipid classes is needed. Clearly TLC and SPE separations are complementary. The SPE approach provides rapid separation into classes, both of which can be screened by MALDI-MS, whereas the TLC approach provides more resolution but may miss components (classes) with very different polarity. Future analysis will focus on development of more complex SPE techniques of this sort.

4. Discussion

Analysis of a sample containing both phospholipid and TAG components by rapid MALDI-TOF MS resulted in mass spectra dominated by the phospholipids (in particular PC). For a lipid extract of beef and egg yolk, most TAGs, while present in greater abundance than the phospholipids, were not detected from an unresolved lipid/sample mixture. While relative changes in phospholipid composition might be detectable by rapid MALDI on the mixture, variations in the TAGs could not. With simple SPE separation, mixture resolution was sufficient to allow detection of both TAGs and phospholipids from a complex mixture known to contain ion-suppressing components, such as PC. While the SPE approach does not have the resolving power of the comparable rapid TLC approach, it provides broader coverage for mixtures containing analytes with disparate polarities. With either approach spectra are easier to interpret because mass overlap of lipids/classes is minimized by the separation. Separation using the disposable, pre-packed columns is fast and easy. This step required acquisition of two MALDI spectra rather than one, but the separation itself took only about ten minutes.

Future studies will focus on additional lipid class separations in different samples, suppression by other phospholipids, a solvent system or additional SPE cartridge to separate the phospholipid classes, and a ZipTip® (Millipore) procedure that allows fractionation of the lipids during MALDI plate spotting rather than by using an SPE cartridge.

5. References

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Chapter 5

Examination of fatty and resin acids in biomass fermentation process waters using gas chromatography – mass spectrometry

Abstract

Fatty and resin acids are potential compounds present in process waters from biomass fermentation and paper mill facilities. An extraction procedure and gas chromatography – mass spectrometry (GC-MS) method were developed for detection and quantification of these compounds in water samples. Using this procedure, six different fatty and resin acids were detected with extraction recoveries of 97 % (% CV, n=8). Fatty acids were detected in all samples while resin acids were only present in those from the paper mill wastewater. Total concentration of fatty acids ranged from 131-2714 $\mu\text{g/L}$ with palmitic and stearic acid being detected in all of the samples. Resin acids were present at a range of 235-1308 $\mu\text{g/L}$ with dehydroabietic (DHA) being the most abundant. Monitoring these concentrations is important since accumulation in water systems can negatively impact environmental and industrial systems. Effective treatment technologies are an important component so processed waters can be safely released into surrounding areas or be recycled for use in other manufacturing pathways.

1. Introduction

As the population of the world continues to rise, alternative sources of energy are needed to meet increases in the cost and demand for fossil fuels. Production of fuels using biomass materials is considered valuable since the source is renewable and greenhouse gas emissions are reduced. Production of biofuels, such as ethanol, first begins with a source. Biomass is biological material that includes products from paper and chemical mills, animal waste, and agricultural crops such as corn, wheat, and sugarcane. These raw materials are converted into glucose which is fermented and distilled to produce ethanol. The complexity of this process depends on the source of the feedstock with cellulosic materials (from wood and paper mills) being the most difficult. These resources contain complex carbohydrate polymers (cellulose and hemicellulose) and lignin that must be pre-treated with enzymes to allow access to simpler sugars that can then be fermented [1-2]. The biofuels produced in this pathway are used in many different areas including industry and transportation (Fig. 1) [3].

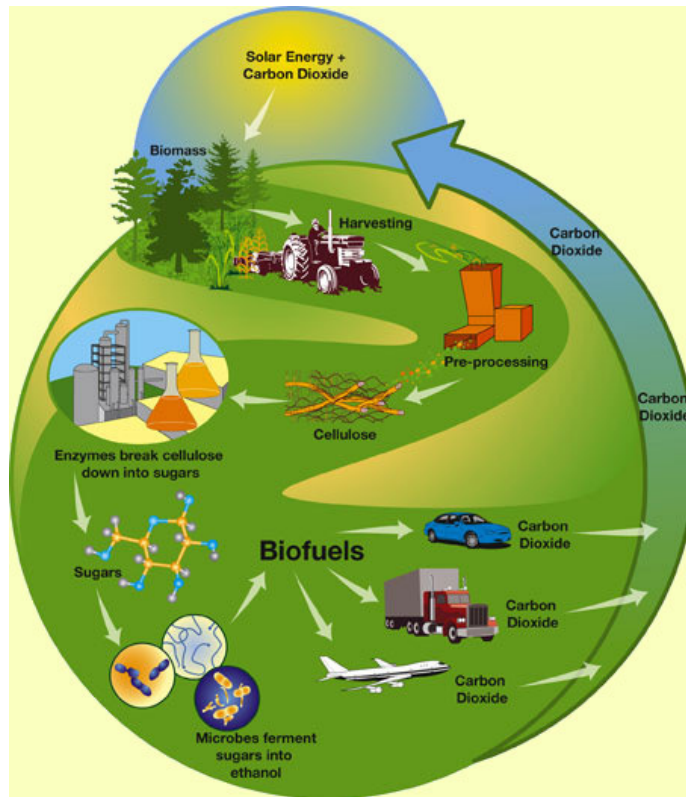


Fig. 1. Pathway for production of biofuels from cellulosic feedstock [3].

During the production of ethanol from biomass products, residual liquid is generated from the distilleries. This distillery spent wash can be toxic depending on the type of feedstock and the technology used in the manufacturing process [4]. For example, facilities that use softwood as in input stock have wastewater that contains fatty and resin acids. These compounds, which can be released during the production process, are naturally present in coniferous trees such as pines, spruces, and firs. These wood extractives are also present in effluents and closed water circuits from paper mill facilities. During the manufacture of pulp and paper, these compounds are released into the surrounding water systems. They are very resistant to chemical degradation and survive the pulping and bleaching process [5-8].

Fatty acids are long chain carboxylic acid, which can be saturated (C16:0 – palmitic and C18:0 – stearic acids) or unsaturated (C18:1 – oleic and C18:2 – linoleic acids). Resin acids are tricyclic diterpenoids with a carboxylic acid substituent. These compounds can be divided into two groups based on their structure: aromatic and non-aromatic resin acids. Aromatic resin acids include DHA, 12- and 14-chlorodehydroabietic, and 12,14-dichlorodehydroabietic acid. Non-aromatic resin acids, all with a molecular formula of $C_{19}H_{29}COOH$, can be designated into two categories. Some acids have an isopropyl substituent on the C-13 with two conjugated double bonds present in the ring system (palustric, levopimaric, abietic, and neoabietic acids). The other group of non-aromatic resin acids has a vinyl and methyl group in the C-13 position with two non-conjugated double bonds present (pimaric, sandaracopimaric, and isopimaric acids) [5-8]. Representative structures of these compounds are given in Fig. 2.

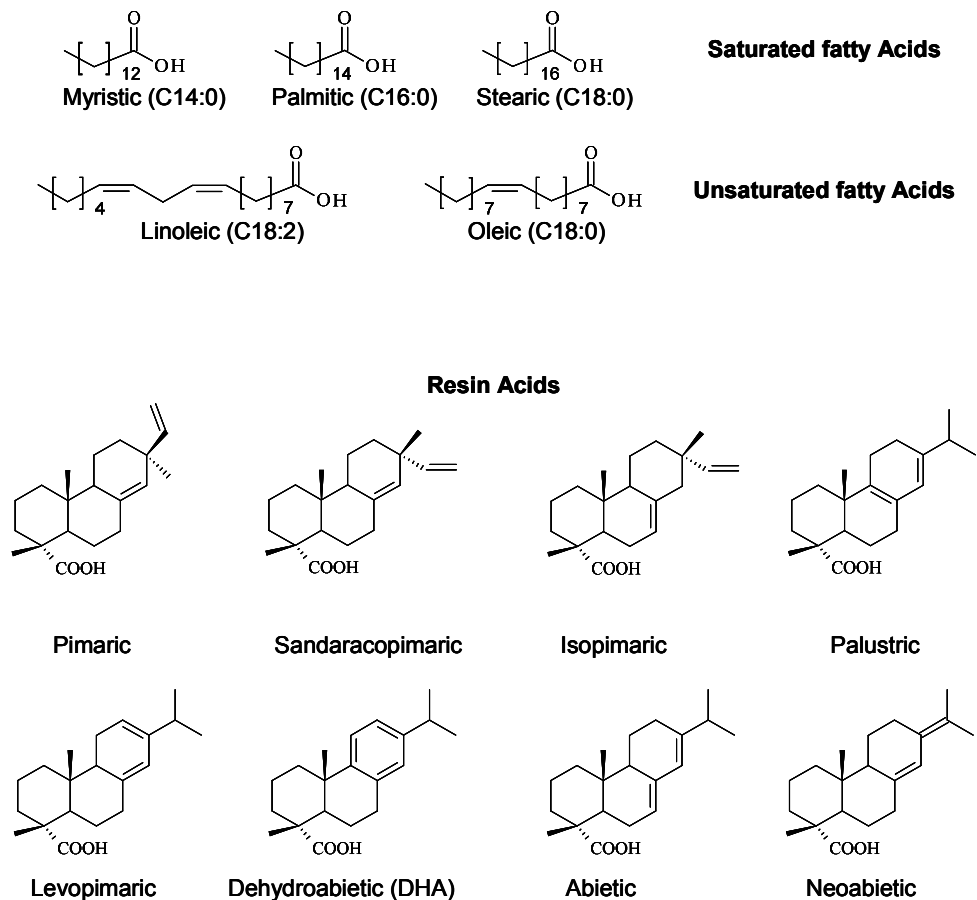


Fig. 2. Structures of fatty and resin acids present in distillery and paper mill waste water.

Fatty and resin acid detection and quantification are important from both a manufacturing and environmental vantage point. For every liter of ethanol produced, 10 to 30 liters of waste water is produced [9]. It is essential to treat this waste so that the large volumes of water can be reused in the biofuels process. In the paper industry these compounds are mainly responsible for pitch deposition during the production process. High levels of the materials can negatively affect the composition of the paper and thus the overall product quality. Environmentally these compounds have been identified as major contributors to the toxicity of effluents to fish [6].

Different technologies using anaerobic and aerobic methods are used for treatment of wastewater to overcome these challenges. Anaerobic digestion is most commonly used as the first step in management. In most cases, however, a combination of anaerobic and aerobic systems is used. This arrangement helps to maximize the advantages of each technique and overcome the disadvantages of using either independently [4, 10-12]. Depending on the concentration of compounds, a pre-treatment step may also be necessary before these methods can be used. Some materials, including fatty acids and glycerides, produce adverse environments that are toxic for anaerobic microorganisms. Knowledge of these substances can be used to evaluate the potential effects and lead to effective measures for their control [13].

In the literature, methods used for the detection of fatty and resin acids include liquid-liquid extraction using methyl tert-butyl ether (MTBE) [5-6, 14-15], derivatization to methyl [15-16], trimethylsilyl [5-6], or pentafluorobenzyl [8,14] esters, and analysis with GC-MS [5-6]. Derivatization is necessary to convert the polar O-H groups into thermally stable, nonpolar groups. Among derivatization techniques, silylation is the most widely used technique. Addition of the trimethylsilyl (TMS) group in this technique is the most popular and versatile silyl group agent [17]. The preferred reagent for TMS addition for acids, alcohols, amines, and phenols is a combination of BSTFA (N,O-bis(trimethylsilyl) trifluoroacetamide) and TCMS (trimethylchlorosilane). BSTFA replaces active hydrogens in the sample with a $-\text{Si}(\text{CH}_3)_3$ (TMS) group. TMCS acts as a catalyst by increasing the reactivity of BSTFA. In the reaction, nucleophilic attack upon the silicon atom of the silyl donor produces a bimolecular transition state. The leaving group of the silyl compound is lost from the transition state during the reaction [18]. Liquid chromatography/mass spectrometry (LC-MS) has also been used for the analysis of fatty and resin acids. Although LC-MS offers an advantage of direct injection without the need

for a derivatization step, the method provides less information about the exact composition of the water sample due to poor separation [5]. In contrast, analysis with GC-MS offers slightly better sensitivity, selectivity, linearity, and recoveries.

A method of TMS derivatization followed by GC-MS was chosen for analysis in this report because of the improved analytical parameters. Twenty-three water samples from a paper mill and biomass fermentation facility were submitted for testing. Detection of fatty and resin acids, along with calculation of corresponding concentrations for each compound, was performed. Preparation and analysis of sample was based on a similar method by Rigol et al [6]. This method has previously been used for the detection of fatty and resin acids in water samples from a paper mill facility [19].

2. Materials and methods

2.1. Reagents

HPLC grade MTBE and margaric acid at a purity of 98% were purchased from Sigma Aldrich (St. Louis, MO, USA). BSTFA + 10% TCMS was purchased from Regis (Morton Grove, IL, USA).

Twenty-three water samples from a paper mill and biomass fermentation facility were submitted for analysis. Because samples were submitted, no information regarding the exact location of the collection sites was provided. Samples were provided in 10 mL quantities in plastic centrifuge tubes and were stored in the dark until analysis. Samples varied in color from yellow to a dark brown with some samples containing suspended particles and sediment.

2.2. Sample preparation

Because of the presence of suspended particles and sediment, water samples were filtered through Whatman nylon membrane filters (0.45 μm). As samples were filtered, a small volume of deionized water was used for washing. The measured pH of the samples varied from 6.5-10; a pH range suitable for efficient extraction of fatty and resin acids [5,14]. Samples were thereafter ready for liquid-liquid extraction and derivatization followed by GC-MS analysis.

2.3. Liquid – liquid extraction and derivatization

For GC-MS analysis, 4 mL water samples were added to a separatory funnel. To each water sample, a 4 mL volume of MTBE containing margaric acid (C17:0) was added. Margaric acid was used as an internal standard because it is unusual to find in water samples, and it does not coelute with other samples [5]. The two layers were gently mixed and the clear MTBE layer was collected. Two additional extracts of 4 mL of MTBE (without the added margaric acid) were added to the water sample and the process was repeated. The combined MTBE extracts were evaporated under a gentle stream of nitrogen to dryness. For derivatization, 200 μL of BSTFA + 10% TCMS was added to the residue. The solution was pipetted into a GC insert and placed in a GC vial. The solution was heated to 70° for 25 min. and was subsequently ready for analysis. The overall scheme for this procedure is shown in Fig. 3.

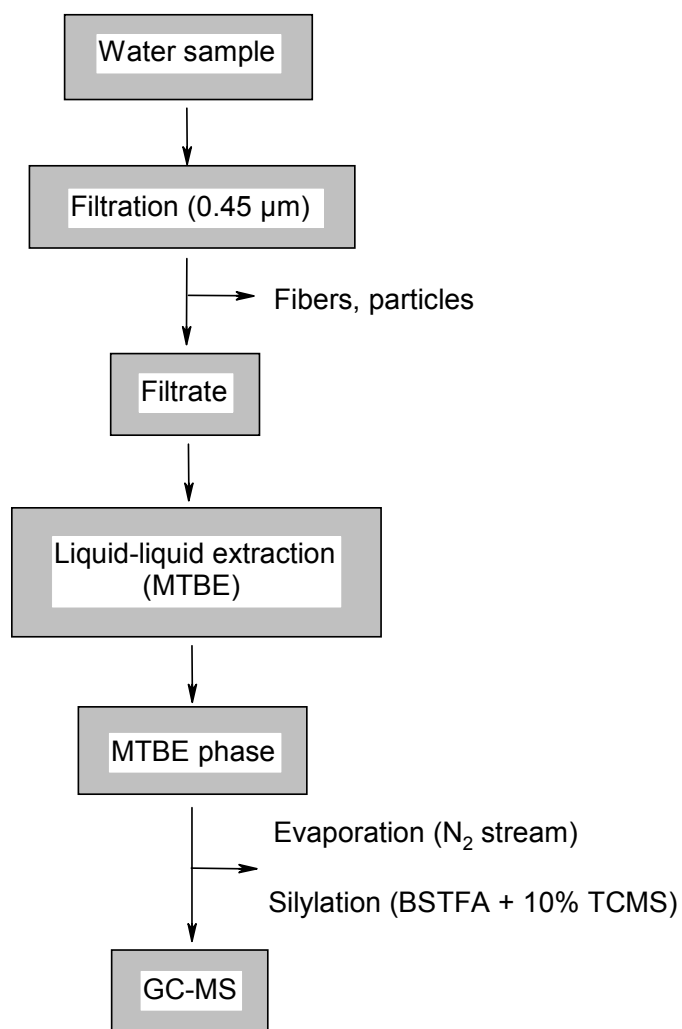


Fig. 3. Diagram of the procedure used for extraction of the fatty and resin acids from wastewater.

2.4. Extraction recovery

Extraction recovery was determined through analysis of a water sample fortified with the internal standard. A sample of margaric acid (25 μg/L) was compared to a blank water sample spiked with the same concentration of fatty acid. After extracting the water sample according to the above procedure, both samples were derivatized and analyzed by GC-MS. The

chromatographic areas of both samples were compared and % recovery was calculated as follows: (area of sample after extraction / area of sample before extraction) * 100. This procedure was repeated an additional seven times to allow for precision measurements (n = 8).

2.5. GC-MS analysis of water samples

A GC-MS instrument (Varian 450-GC) coupled to a 320 -MS triple quad mass spectrometer (Bruker Daltonics, Billerica, MA) was used for analysis. Separation was achieved with a Phenomenex Zebron ZB-5HT Inferno column (30M x 0.25ID). The initial column temperature was set at 120°C for 2 min. and was increased at a rate of 8°C/min to 275°C (held for 7 min). An autosampler was used for sample injection (1 µL) at 250°C under the split mode (10:1). Full scan data were obtained by scanning from m/z 45 to 500 at a rate of 1.5 scans/s.

Quantitative analysis was performed by addition of an internal standard (margaric acid, 25µg/mL in MTBE) to each water sample. An area correction factor was applied to correct for differences in the areas of the internal standard between samples. Sample concentrations for fatty and resin acids were reported in µg/L.

3. Results and discussion

Fatty and resin acids were analyzed in water samples from a biomass fermentation and paper mill facility using GC-MS. The analyzed samples can be divided into three groups based on the source of biomass and the type of technology used for treatment. Samples 1-18 are from a fermentation facility that utilized sugarcane as its source and samples 19-23 are from a paper mill with softwood as the input. Samples 1-18 can be further divided into two groups with

samples 1-15 being treated through an anaerobic process and 16-18 through sequential anaerobic and aerobic processes.

Identification of compounds was done in the full scan mode by analysis of relative retention times and monitoring ions in the mass spectrum (Table 1). For the fatty acids, ions related to TMS derivatives ($[\text{TMS}]^+$ (m/z 73), $[\text{COOTMS}]^+$ (m/z 117), $[\text{CH}_2\text{-CH}_2\text{-COOTMS}]^+$ (m/z 145) were observed at the highest intensity. The m/z values corresponding to these fragments were the same for each of the different compounds so they were not particularly useful for discrimination. The most selective peaks for identification were from loss of a methyl group ($[\text{M-CH}_3]^+$) and the molecular ion ($[\text{M}]^+$). With respect to the resin acids, ions were observed for the $[\text{TMS}]^+$, $[\text{M}]^+$, and $[\text{M-CH}_3]^+$ groups. Other ions were also present for elimination of the carboxyl moiety as CO_2 or HCO_2 with the TMS group. The relative intensity and m/z values for these ions were all used for identification.

Table 1. Fragmentation patterns of the fatty and resin acids detected in water samples; m/z values with relative abundances (%) in parenthesis.

Compound	Retention time (min.)	Mw	[M] ⁺	[M-CH ₃] ⁺	[CH ₂ -CH ₂ -COOTMS] ⁺	[COOTMS] ⁺	[TMS] ⁺
Fatty acids							
Lauric [1]	10.6	200	272 (4)	257 (51)	145 (22)	117 (93)	73 (100)
Myristic [2]	13.2	228	300 (2)	285 (40)	145 (20)	117 (100)	73 (92)
Pentadecylic [3]	14.4	242	314 (3)	299 (44)	145 (28)	117 (100)	73 (67)
Palmitic [4]	15.6	256	328 (5)	313 (51)	145 (30)	117 (100)	73 (90)
Margaric (1S) [5]	16.8	270	342 (7)	327 (66)	145 (30)	117 (100)	73 (77)
Linoleic [6]	17.5	280	352 (2)	337 (19)	--	117 (31)	73 (100)
Stearic [7]	17.8	284	356 (5)	341 (49)	145 (31)	117 (100)	73 (95)
Resin acids							
					[M-TMS-CO ₂] ⁺ a	[M-TMS-CO ₂ -CH ₃] ⁺	
Pimaric [8]	18.7	302	374 (7)	359 (9)	257 (18)	241 (11)	73 (100)
Sandaracopimaric [9]	18.9	302	374 (9)	359 (8)	257 (7)	241 (21)	73 (100)
Isopimaric [10]	19.0	302	374 (5)	359 (14)	256 (50)	241 (79)	73 (100)
Palustric [11]	19.2	302	374 (18)	359 (20)	257 (10)	241 (64)	73 (100)
DHA [12]	19.6	300	372 (5)	357 (5)	255 (6)	239 (100)	73 (27)
Abietic [13]	19.9	302	374 (10)	359 (10)	256 (100)	241 (48)	73 (54)

^a Loss of CO₂ or HCO₂.

A typical chromatogram of a water sample is shown in Fig. 4. This sample contained many of the compounds from Table 1. Fatty acids of lauric, myristic, pentadecyclic, palmitic, linoleic, and stearic acid were detected in the range of 15-18 min. Resin acids of pimaric, sandaracopimaric, isopimaric, palustric, levopimaric, DHA, and abietic were observed between 18-20 min. All compounds were thus eluted within 20 min with good separation between most compounds. Extraction recoveries were determined through analysis of fortified samples of blank water with margaric acid. Recoveries of 97 % were calculated with a precision (% CV n = 8) of 10 %. These same fatty and resin acids have been observed by Latorre et al. in distillery wastewater [5].

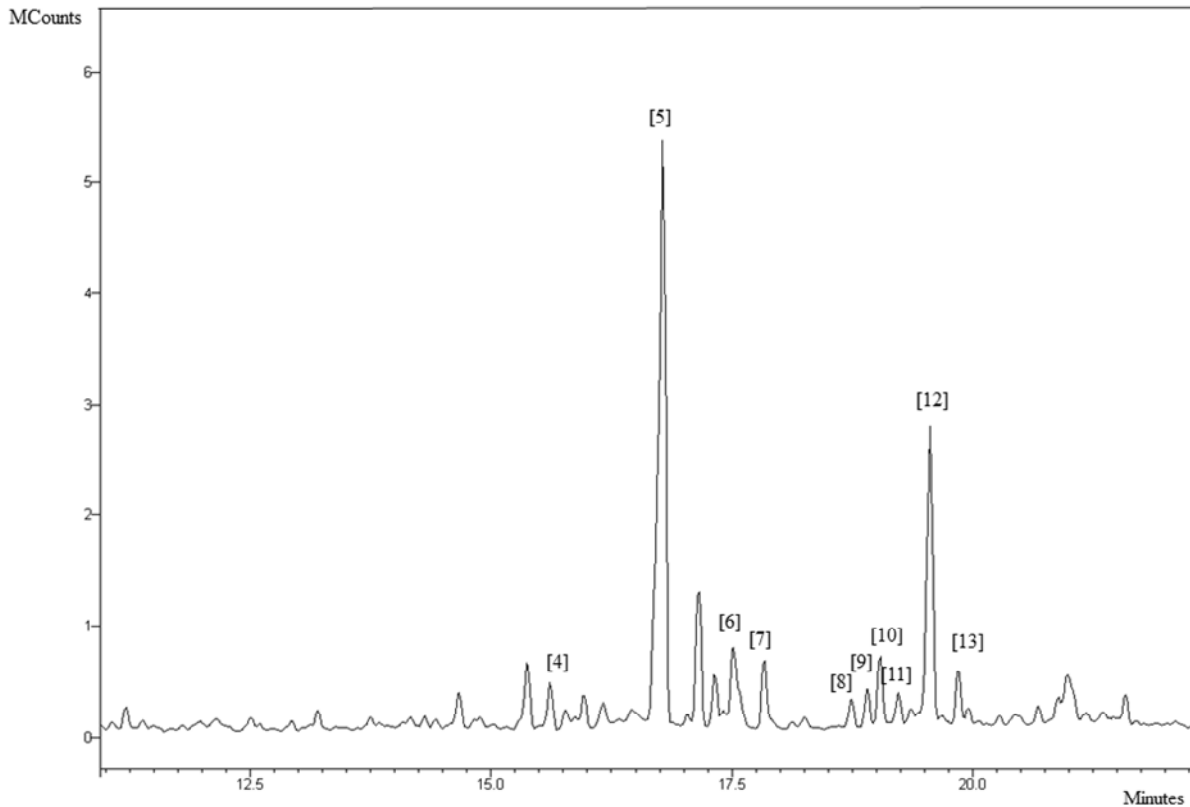


Fig. 4. Total ion chromatogram of a water sample (sample 19) from a paper mill facility. Fatty acids: palmitic [4], margaric (IS) [5], linoleic [6], stearic [7]; Resin acids: pimaric [8], sandaracopimaric [9], isopimaric 10], palustric [11], dehydroabietic [12], abietic [13].

The concentration of fatty and resin acids in water samples are given in Tables 2 and 3. Fatty acids ranged from 8-1410 $\mu\text{g/L}$ with an average of 245 $\mu\text{g/L}$. Palmitic and stearic acid were detected in all of the samples with the latter having the highest average concentration. A lower concentration of fatty acids was detected in samples treated with an anaerobic/aerobic process rather than just an anaerobic process only. Other reports have demonstrated that anaerobic pretreatment significantly improves performance of the aerobic process for purification of

complicated wastewater. Resin acids were detected between 66-799 $\mu\text{g/L}$ with an average of 236 $\mu\text{g/L}$. DHA was calculated as the most abundant resin acid followed by isomeric and abietic acid. This agrees with previous works stating that resin acid with conjugated double bonds can easily undergo isomerization forming thermodynamically more stable isomers with DHA and abietic being the favored final products. The absence of other resin acids in samples thus indicates this rearrangement process. Resin acids were only detected in samples from the paper mill facility since softwood, rather than sugarcane, was used as the feedstock. Resin acids account for 0.2 – 0.8 % of the total weight of the wood from coniferous trees and are released into the water during the production process [7]. Total concentrations of fatty acids were detected as levels ranging from 131-2714 $\mu\text{g/L}$ and resin acids at 235-1308 $\mu\text{g/L}$. These concentrations levels are significant because the 96-h LC_{50} values for salmon or rainbow trout range from 2000 to 8000 $\mu\text{g/L}$ for fatty acids and from 400 to 1700 $\mu\text{g/L}$ for the common resin acids [14].

Table 2. Concentration of fatty acids ($\mu\text{g/L}$) in water samples from a biomass fermentation and paper mill facility.

Sample	Lauric	Myristic	Pentadecylic	Palmitic	Linoleic	Stearic	Sample Color
1				287		404	Yellow
2				70		102	Yellow
3				65		96	Yellow
4		126		468		601	Yellow
5		53		216		329	Light amber
6		42		194		316	Amber
7		22		117		170	Amber
8	50	36	14	145		191	Amber
9		135	50	469		767	Amber
10		300	105	892		1040	Amber
11		267	107	930		1410	Amber
12	93			93		149	Dark amber
13		30		121		161	Dark amber
14		14		62		84	Dark amber
15		152		602		799	Brown (Dirty)
16		15		66		75	Light yellow
17		22		104	8	137	Light yellow
18				58		73	Yellow
19				106	125	154	Cloudy yellow
20				57		62	Cloudy yellow
21				304		64	Light brown
22		167	54	561		818	Cloudy brown
23		131	63	517		732	Cloudy brown

Table 3. Concentration of resin acids ($\mu\text{g/L}$) in water samples from a paper mill facility.

Sample	Pimaric	Sandaracopimaric	Isopimaric	Palustric	DHA	Abietic
19	66	85	160	70	799	128
20					235	
21					268	
22					315	

4. Conclusions

In this chapter, a method was developed for the determination and quantification of fatty and resin acids in biomass fermentation and paper mill process waters using GC-MS. Extraction followed by derivatization was used for preparation of samples. Identification of compounds was made through analysis of relative retention times and mass spectral fragmentation patterns. Using this method, six fatty and resin acids were detected in samples. Fatty acids were detected in all of the samples with the most abundant being stearic and palmitic acid. Resin acids were detected in only the paper mill samples due to softwood being used as the source rather than sugarcane. Total concentrations of fatty acids ranged from 131-2714 $\mu\text{g/L}$ and resin acids at 235-1308 $\mu\text{g/L}$. Monitoring the levels of these compounds is important due to paper manufacturing and environmental concerns.

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CONCLUSIONS

Procedures were developed for analysis of drugs and lipids using a combination of GC-MS and MALDI-TOF MS. In chapter 1, a method was developed for detection of synthetic cannabinoids in herbal incense products using GC-MS. JWH-018, a common synthetic cannabinoid, was synthesized as an analytical standard for comparison to samples. GC-MS analysis indicated four herbal blend mixtures and a pipe used for smoking cannabis all contained JWH-018. JWH-018 was detected at concentrations ranging from 2.7-14.1 mg/g. In chapter 2, a GC-MS procedure was established for detection of three JWH-018 metabolites in urine from individuals suspected of consuming herbal blends. Analytes were confirmed using synthesized and commercially available analytical standards. The specific metabolite hydrolysis products were confirmed as the 4-hydroxypentyl, 5-hydroxypentyl, and N-pentanoic acid derivatives. These analytes are in agreement with a LC-MS/MS procedure reported in the literature [1]. In chapter 3, TAGs in fingerprint samples were analyzed using LDI-TOF MS under light/dark conditions on four different surfaces. Fingerprints exposed to the light showed similar rates of degradation for glass, plastic, and iron surfaces while those from stainless steel were much more rapid. Samples exposed to the dark showed little change over the 112 hr sampling period. The age of a known print was estimated to be between 16 and 112 hrs old through data comparison with the actual age being 24 hrs. In chapter 4, complex mixtures were analyzed using MALDI-TOF MS. Reference lipids and real samples (beef and egg yolk) were used for analysis. Mass spectra were dominated by phospholipids (particularly phosphatidylcholine) while TAGs were not detected. With a simple SPE procedure, fractions were collected for each lipid class. With this technique, resolution was sufficient to allow detection of both TAGs and phospholipids. In chapter 5, an extraction procedure and GC-MS method were developed for detection and

quantification of fatty and resin acids in process waters from biomass fermentation and paper mill facilities. Using this method, six fatty and resin acids were detected in samples. Total concentrations of fatty acids ranged from 131-2714 $\mu\text{g/L}$ and resin acids at 235-1308 $\mu\text{g/L}$. Mass spectrometry is an ideal technique for these types of studies because of the structural information gained and the high sensitivity.

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