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Evaluation of Extraction Techniques for the Forensic Analysis of Human Scalp Hair Using Gas Chromatography/Mass Spectrometry (GC/MS)*

ABSTRACT: Preliminary research using on-line supercritical fluid extraction/gas chromatography-mass spectrometry (SFE/GC-MS) has shown that the natural and artificial surface components of human scalp hair are reproducible and differentiable. Therefore, these components may be useful for individualization or determining demographic characteristics or both. However, it is not known how the efficiency and selectivity of on-line SFE/GC-MS compares to other extraction methods. In this study, ultrasound, Soxhlet, and pressurized-fluid extraction were used to extract 1 mg to 1.3 g portions of a composite hair sample taken from an Asian male between the ages of 10 and 18. Percent extractables ranged from 0.9% to 5.6%, depending on the solvent used, and tended to increase with solvent polarity. Chemical analysis using GC/MS showed that the extracts contained large proportions of free fatty acids, squalene, cholesterol, and various wax esters. Finally, comparisons to SFE/GC-MS showed that this method possesses adequate efficiency, no observable differences in selectivity, and greater potential for miniaturization.

KEYWORDS: forensic science, hair, lipids, sebaceous, gas chromatography-mass spectrometry

Current methods of forensic hair analysis rely upon comparisons of either hair morphology or DNA. Microscopic examination of hair can indicate species, race, body area (e.g., scalp or pubic), and method of removal (e.g., shed or plucked) (1,2). However, determining the age and/or gender of a subject based on hair morphology is not widely accepted (1,2). Most commonly, a comparison of the morphological characteristics of a known and unknown hair sample can be used to determine if they may have originated from the same source, although the microscopic characteristics of hair are not considered to be unique to individuals (1,2).

Genetic analysis, on the other hand, is not as informative about the characteristics of an unknown subject such as its species or race. However, DNA comparisons can form highly significant associations between known and unknown hair samples. In these techniques, nuclear DNA can be isolated from the cells found on the hair root/sheath, or mitochondrial DNA can be isolated from the cells in the hair shaft, followed by analysis, comparison, and assessment of any association (3,4). In some cases, it is also possible to identify gender based on the presence of Y chromosomes in root sheath cells (5,6).

Attempts to discriminate human hair through chemical analysis has included monitoring hair dye components (7,8), hair oxidation (9,10), hair proteins (11–14), and trace elements (15,16). However, these techniques are not commonly applied and they have met with limited success due to poor discrimination between individuals, the destructive nature of the tests, and the difficulty of detecting small changes in bulk hair composition.

Human Hair Lipids

The work presented here is predicated on the fact that all human hair is coated with a complex mixture of naturally occurring lipids and other anthropogenic materials such as shampoos and hair treatments. While there have been some reports analyzing various artificial residues (17–21), hair lipids have not been succesfully examined for their forensic utility. Luckily, there is considerable interest in the fields of dermatology, clinical chemistry, and cosmetic chemistry about the organic compounds found on human hair. Hence, much is known about the characteristics and trends observed in human hair lipids that may be useful for forensic purposes. Of course, this value will only be realized if an extraction and analysis methodology is developed that allows for the detection of such characteristics and there is clear understanding of any trends. While further research will be required to realize these goals, current knowledge about trends in human hair lipids is summarized below.

Human scalp hair is thought to acquire a coating of lipids largely through transfer from neighboring sebaceous glands (22,23). In addition, studies have shown significant qualitative and quantitative differences in the lipid content of hair based upon gender, race, and age. For example, while cholesterol is found at lower levels on hair than in skin, more cholesterol is present on children's hair than adult's (24). This observation has been confirmed by others, as well as observing lower levels of squalene in children's hair lipids (25). It is also known that the amount of surface lipids on hair increases during adolescence then noticeably decreases in older females (26). Furthermore, the total acid content was the highest in females, then mature males, then young males; the opposite trend

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was seen with paraffinic hydrocarbons (26). Finally, wax alcohols were lower in male children than in mature males (26).

Additional research has shown that African-American hair tends to have a greater total lipid content, a greater amount of saturated hydrocarbons, and a higher amount of mono- and triglycerides (26–28). In contrast, Caucasian hair had a higher ratio of squalene to nonsqualene hydrocarbons, and a higher amount of diglycerides and free fatty acids. Furthermore, yields of lipids tended to increase with age (from 8 to 18 years old) (26–28).

As the naturally occurring lipids found on the surface of human hair are largely sebaceous in origin, hair can be used to indicate various diseases that affect lipid levels. These include cystic fibrosis, which causes decreases in octadecenoic, eicosenoic and other free fatty acids (29). Chronic alcohol abuse can also be indicated by the presence of fatty acid ethyl esters in hair lipids (30,31).

Forensic Analysis of Human Hair Lipids

An initial assessment of the ability of hair lipids to provide more individualistic data from a hair sample found that chromatographic resolution and sensitivity were not sufficient to distinguish individual hair strands (32). More recently, Benner, Goodpaster, and Degrasse have focused on extracting organic compounds from the surface of hair by coupling supercritical fluid extraction (SFE) to GC/MS (33,34). This research has shown that it is possible to obtain high-quality chromatograms from single human hairs that are reproducible within an individual and differentiable from other individuals. Differentiation was achieved based on the qualitative and quantitative differences between surface components found on various hair samples. In addition, the chemical profiles thus obtained provided information on the age of a subject based on the relative amounts of cholesterol and other lipids (33,34).

This report evaluates the use of liquid solvents for the extraction of hair organic components. These solvents are of interest as they may allow more efficient extractions, greater ease of use, and broader selectivity than supercritical CO_2 (33,34). Experiments were designed to find the most efficient (through measuring percent extractable mass) extraction method, as well as compare the efficiency and selectivity of these methods to SFE. The solvents tested included methanol, acetone, dichloromethane, and hexane, which were chosen because of their varying degrees of polarity. The extraction methods used were sonication, Soxhlet, pressurized fluid extraction (PFE), and solid-phase microextraction (SPME). Sonication was chosen because of its use in studies targeting drugs of abuse, while Soxhlet was chosen because of its use in exhaustively extracting many different sample types, including hair (35). PFE was chosen because of its speed and ease of use, while SPME was chosen because of its ability to interface easily with the GC/MS.

Each solvent was tested in combination with each method using portions from a composite hair sample that was taken from one individual between the ages of 10 and 18. In addition, the exhaustiveness of PFE was evaluated, and single portions of the composite hair sample were sequentially extracted with each solvent, in order of polarity. A sample size test was also conducted using PFE with methanol. Based on the results of these tests, the effectiveness of liquid solvents and their complementary extraction methods in combination with GC/MS analysis was determined.

Methods

Materials

HPLC-grade acetone, hexane, and methanol (J.T. Baker, Phillipsburg, NJ) and spectroscopic-grade dichloromethane (Honeywell Inc., Burdick & Jackson Division, Muskegon, MI) were used for the hair extractions.

Relatively large hair samples were provided by an Asian male at various intervals, spanning a period from age 10 to age 18, as a result of normal haircuts. The samples were wrapped in paper towels and stored in a large beaker for use in hair-related projects. Among other environmental variables, use of hair care products and diet were uncontrolled. As individual samples were not dated, all samples were combined in a large container and mixed to ensure large-scale homogeneity. While microheterogeneity may be a concern for such a broad composite sample, the constraints of federal ethics guidelines for human subject research make the above sample limitations largely unavoidable. Further discussion of these limitations and any observed effects on the data obtained are included below.

Ultrasonic Extraction

Four hair samples ranging from 556 mg to 1275 mg were ultrasonicated for 30 min in 50 mL of methanol, acetone, dichloromethane, and hexane, respectively. The extracts were then pipetted into TurboVap vessels for eventual solvent evaporation. The extracted hair samples were then washed with 10 mL of solvent five times to recover any remaining organic residue. The original sample jars remained loosely covered and the solvent was allowed to evaporate from the hair for 48 h before the jars were weighed.

Soxhlet Extraction

Four hair samples ranging from 485 mg to 850 mg were extracted individually by Soxhlet with 150 mL of each of the four solvents. Each Soxhlet apparatus was run continuously for 24 h with approximately three extractions performed per hour (i.e., approximately 72 extractions total). After 24 h, the extracts were transferred to TurboVap vessels, combined with rinses of the Soxhlet apparatus, and evaporated according to the procedures outlined below. The thimbles were covered and the solvent was given 48 h to evaporate before weighing.

Pressurized Fluid Extraction

Samples ranging from 203 mg to 305 mg were extracted in 1 mL cells with an automated pressurized fluid extractor. Extractions were carried out at 125°C and 13.79 MPa (2000 psi) with five extraction cycles; each cycle consisted of a 10 min static step followed by flushing of the extraction cell with solvent. The following PFE runs were executed: [1] Four separate hair samples were extracted individually with each of the four solvents; [2] a single hair sample was extracted with all four solvents sequentially and in order of polarity; [3] two hair samples were extracted twice each using either acetone or methanol to determine the exhaustiveness of a single extraction, and finally; and [4] using methanol, hair samples of different masses were extracted, from 1 mg to 408 mg. The remaining space in the extraction cells was filled with previously extracted granular anhydrous sodium sulfate (J.T. Baker, Phillipsburg, NJ).

Supercritical Fluid Extraction (SFE)

For the off-line procedure, a 1.62 mL SFE cell with a 30 cm $\times$ 50 μm inside diameter, deactivated restrictor was labeled and filled with one 269 mg sample of hair. The cell was filled with CO_2 at 40.53 MPa (5880 psi), heated to 125°C, extracted statically for 10 min, followed by a 15 min dynamic extraction (approximately 1.5

mL/min to 1.6 mL/min) into 50 mL of dichloromethane. The on-line technique used a 40 μL extraction cell filled with one 251 μg sample of hair connected to a 30 cm × 25 μm i.d. deactivated restrictor. The restrictor was inserted into the injector of the gas chromatograph and operated at a flow rate of 250 to 350 μL/min. All other SFE conditions were identical.

Solvent Evaporation and Residue Collection

The TurboVap vessels containing sample extracts were heated at 30 to 50°C (depending on solvent) under nitrogen then given 24 h to further evaporate at room temperature. All weighings were repeated five times. Measuring percent extractables by monitoring the loss of mass of the extracted hair samples showed inconsistent results, most likely caused by the slow evaporation of the solvent from the hair, even after 48 h. Therefore, monitoring the mass increase of the TurboVap vessels was used to determine the percent extraction values. After weighing the TurboVap vessels, sample residues were redissolved in the appropriate solvent by ultrasonicating for 10 min, then transferred to clean containers and stored at 0°C.

GC-MS Analysis

The extracts were characterized with a GC-MS system. The following conditions were used for analysis of the dichloromethane extracts:

Injection:	2.5 μL, cool on-column
Carrier gas:	helium, constant flow at 2 mL/min
Column:	60 m × 0.25 mm, 0.25 μm DB-XLB film (J&W Scientific, Folson, CA) 5 m deactivated retention gap
Oven program:	5 min hold at 50°C, 10°C/min until 340°C, 20 min hold at 340°C
MS temperature:	source @ 230°C, quadrupole @ 150°C
MS scan range (m/z):	40 to 550

For hexane, acetone, and methanol, the initial oven temperature was 60°C. For hexane, the solvent delay/temperature hold time was 6 min, while for acetone and methanol it was 7 min. The chromatograms were analyzed using data acquisition software and the NIST/EPA/NIH Mass Spectral Library Version 2.0. The identity of peaks was recorded only if a match score of 790 or greater was obtained. A Grob test mixture was used to monitor chromatographic performance and the accuracy of the MS system.

Solid Phase Microextraction

For SPME, hair samples were covered in methanol and the SPME fiber was exposed to the slurry with stirring. After extraction, the needle was inserted into the GC inlet and the fiber was desorbed for 5 min. The GC/MS run was conducted according to the same method as above. The SPME tests used a 65 μm PDMS-DVB fiber (Supelco Corp., Bellefonte, PA) for hair samples of 344, 173, and 86 mg. The 344 mg sample was extracted for 30 min, the 173 mg sample for 60 min, and the 86 mg sample for 120 min.

Results and Discussion

Percent Extractables

The measured percent extractables of the hair samples depended upon the method and solvent used. Overall, methanol recovered the largest amount of residue, followed by acetone, dichloromethane, and finally hexane. The percent extractables from sonication ranged from 0.9 to 1.8%, Soxhlet ranged from 1.1 to 4.1%, and pressurized fluid extraction ranged from 2.5 to 5.6% (see Table 1).

Based on these data, PFE was deemed the most efficient of the extraction methods tested. In addition, PFE used half the sample size and was executed four times faster than Soxhlet extraction. Hence, this method was further investigated for its suitability in exhaustively extracting small hair samples. First, the reproducibility of PFE was determined for methanol and acetone. In these tests, methanol recovered an average of 5.4% of the sample mass ($n = 6$ samples, standard deviation of 0.42%) while acetone recovered an average of 5.6% of the sample mass ($n = 4$ samples, standard deviation of 1.4%). These results differ from those of the other extraction methods in that the percent extractables of methanol and acetone are statistically indistinguishable.

Determinations of the exhaustiveness and scalability of PFE were also conducted. After completing one preliminary extraction, methanol recovered an additional 0.35 to 0.47% of the sample mass in a second extraction ($n = 2$ samples). Acetone recovered an additional 0.64 to 0.85% of the sample mass in a second extraction ($n = 2$ samples). In both cases, the mass of residue obtained after the second extraction was on the order of that obtained from a blank solvent sample (0.5 to 0.8 mg), therefore, the above extractions could be deemed exhaustive. Furthermore, no blank corrections have been made in any subsequent results.

Steadily reducing the sample size in the PFE method resulted in a minimum sample mass of 51 mg, which represents about 200 strands of hair each 3 cm in length. For sample masses less than 51 mg, the value of the residue was on the order of that obtained from a blank solvent sample. Overall, these results indicate that both methanol and acetone extractions are exhaustive. However, it is important to note that PFE cannot be scaled down to the small sample sizes that would be typical of a crime scene sample. In turn, this will limit its utility for analysis of evidential samples.

Table 1 also contains comparative data for an off-line supercritical fluid extraction (SFE) using carbon dioxide. These data demonstrate that the percent extractables for SFE with CO_2 (2.4%) was less than that obtained with polar organic solvents, but comparable to nonpolar organic solvents. This indicates that percent extractables tend to decrease with the polarity of the solvent used. The trend with polarity was confirmed when a sequential solvent series was used in PFE (see Fig. 1). The distribution of extractable material shows that the majority is polar in nature, consistent with previous research showing that many of the organic compounds on the surface of hair to have hydrophilic functional groups such as alcohols or carboxylic acids (28). In addition, a small fraction (i.e., approximately 10%) of the hair surface components is insoluble in polar solvents and consists of more paraffinic and/or lipophilic components (26,27).

It is possible that some of the polar extractable mass may consist of species such as salts and proteins, therefore, not all of the ex-

TABLE 1—*Comparison of extraction methodologies.*

Method	Percent Extractables (%)	Sample Amount (g)
Ultrasound	0.9–1.8	0.556–1.275
Soxhlet	1.1–4.1	0.485–0.850
Pressurized fluid extraction	2.5–5.6	0.203–0.305
Supercritical fluid extraction	2.4	0.269

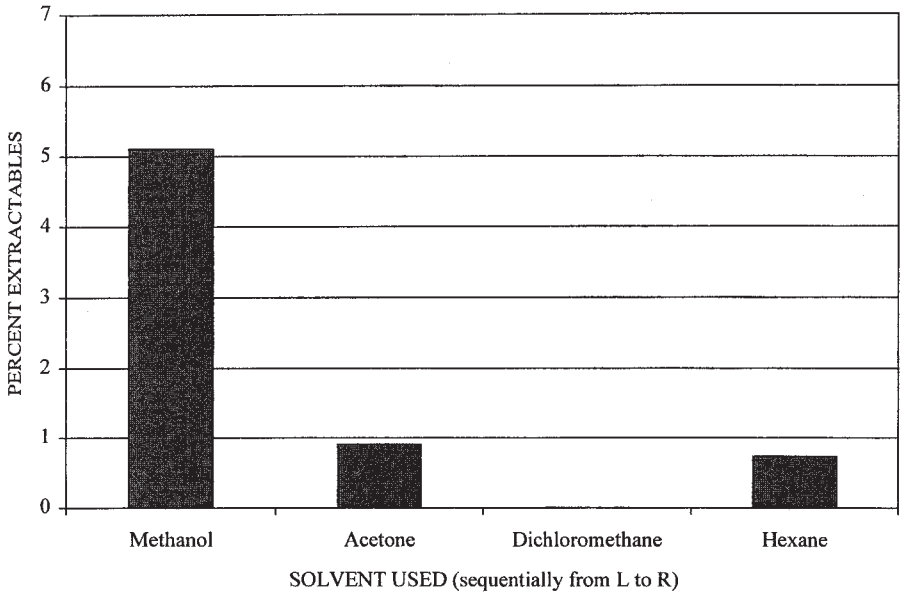

FIG. 1—*Sequential PFE using solvents in order of polarity (mean of two trials).*

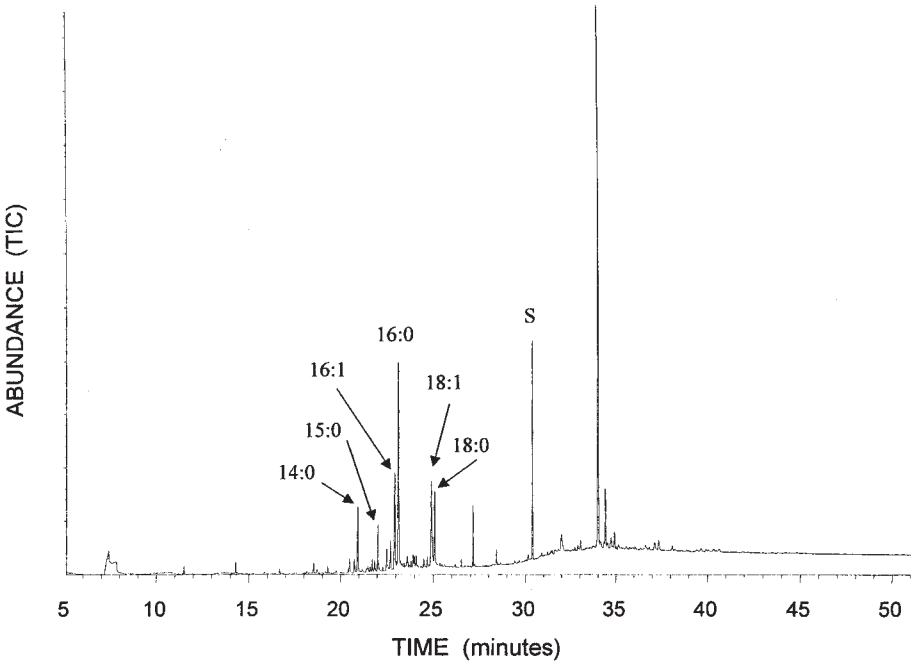

FIG. 2—*Methanol extract of a human hair sample using PFE (271 mg sample size).*

tractable mass would be sufficiently volatile for separation by gas chromatography. In fact, analysis of a methanol extract obtained from pressurized fluid extraction using liquid chromatography-mass spectrometry indicated that some protein-like components were present. However, these components were minor constituents of the total methanol extract. Furthermore, the relatively non-polar nature of the remaining extraction solvents (including carbon dioxide) supports the presumption that nearly all the extractable mass recovered using these solvents is amenable to gas chromatography.

Chromatographic Analysis

Generally, all the chromatograms from the liquid solvent extractions yielded similar results, so only the chromatograms from the pressurized fluid extracts are included here (see Figs. 2 through 5 for the chromatograms and Table 2 for peak identifications). All chromatograms have been scaled to the most intense peak (typically squalene). Overall, only naturally occurring lipids were detected on the hair samples. This indicates that any hair products

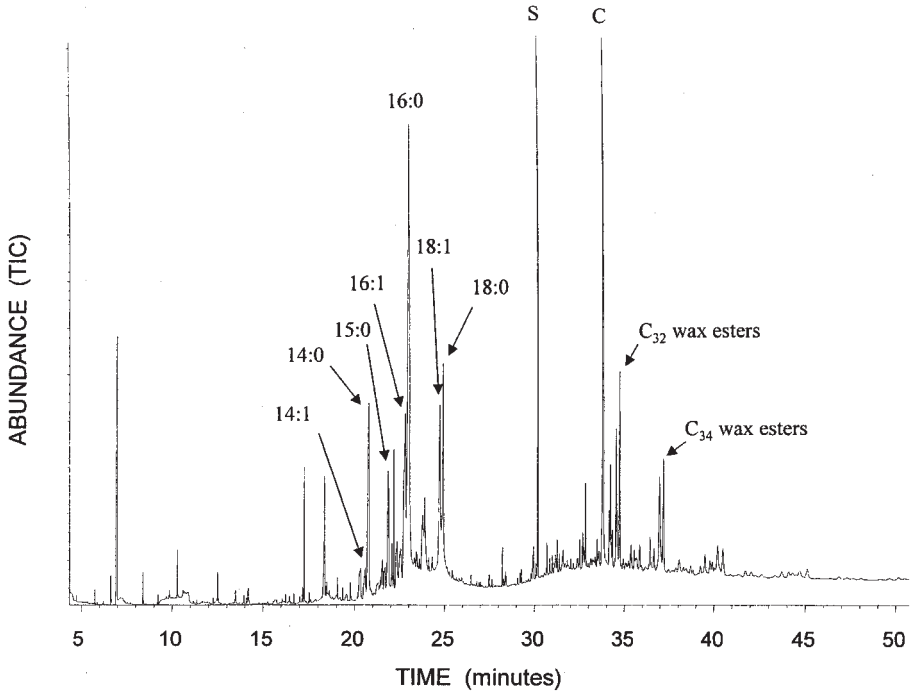

FIG. 3—*Acetone extract of a human hair sample using PFE (305 mg sample size).*

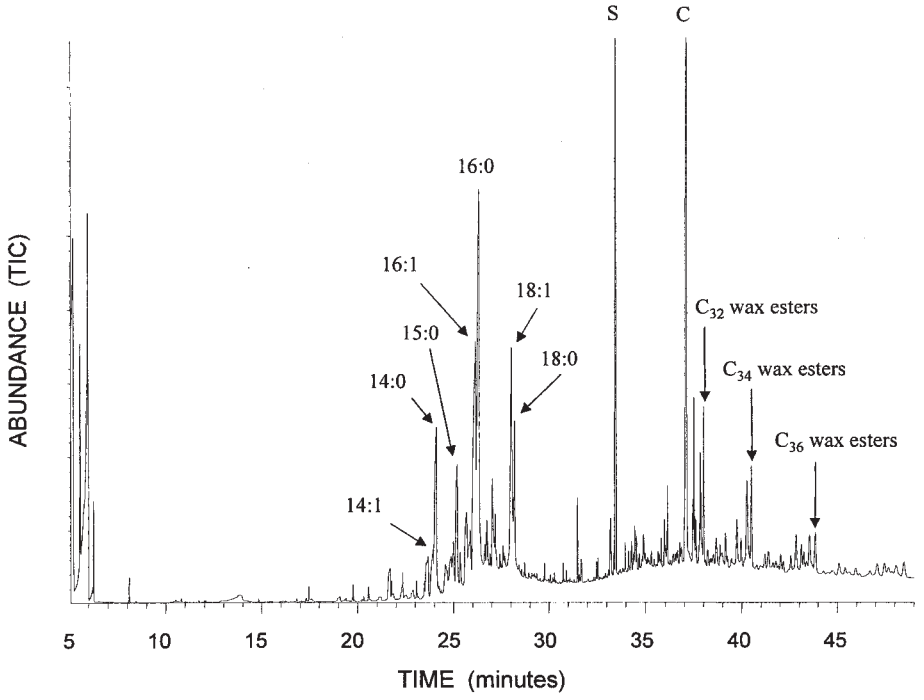

FIG. 4—*Dichloromethane extract of a human hair sample using PFE (263 mg sample size).*

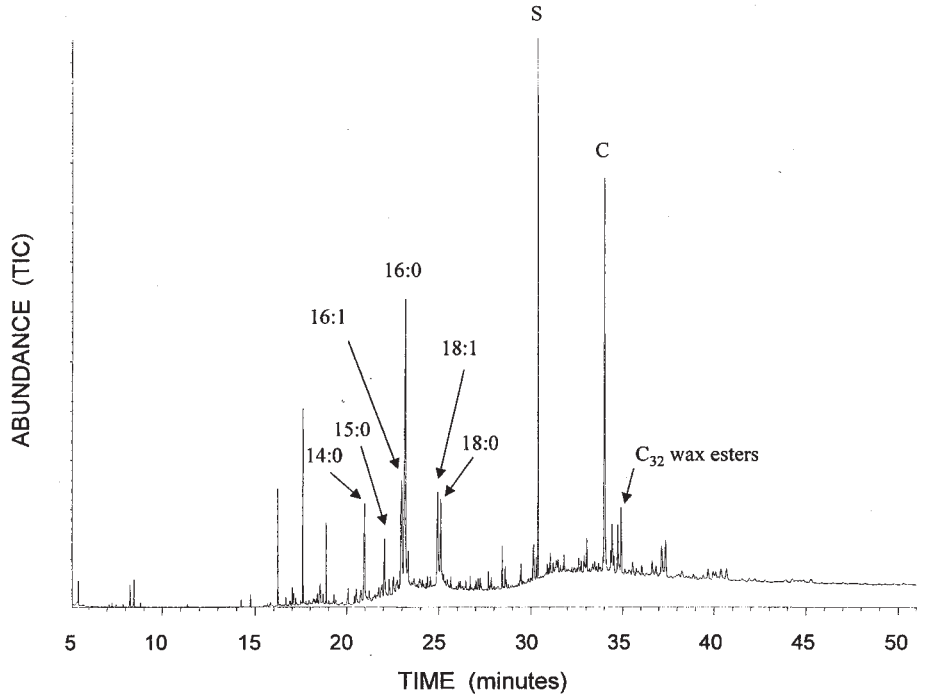

FIG. 5—*Hexane extract of a human hair sample using PFE (203 mg sample size).*

TABLE 2—*Lipids (abbreviations in brackets) as identified in human hair extracts.*

1.	Tetradecenoic acid	[14:1]
2.	Tetradecanoic acid	[14:0]
3.	Pentadecanoic acid	[15:0]
4.	Hexadecenoic acid	[16:1]
5.	Hexadecanoic acid	[16:0]
6.	Octadecenoic acid	[18:1]
7.	Octadecanoic acid	[18:0]
8.	Squalene	[S]
9.	Cholesterol	[C]
10.	C_{32} wax esters (e.g., hexadecanoic acid, hexadecylester)	
11.	C_{34} wax esters (e.g., hexadecanoic acid, octadecylester)	
12.	C_{36} wax esters (e.g., hexadecanoic acid, eicosylester)	

used by the subject did not deposit significant levels of residue, or that this particular hair sample was resistant to such deposition. Furthermore, chromatograms were reproducible under the same solvent and extraction conditions, indicating that temporal variation and/or microheterogenaity was minimal.

In general, as the solvent polarity decreased, the chromatograms yielded fewer fatty acids and sterols, and more waxes. Despite this difference, the presence of mainly even carbon numbers among the fatty acids for all solvents was consistent with the biological origin of the compounds. In addition, the presence of free fatty acids was consistent with the age of the subject from whom the hair samples were obtained (see above). The chromatograms of the second PFE runs for both methanol and acetone did not show identifiable peaks, confirming the exhaustiveness of the extraction. In addition, the chromatograms for samples as small as 51 mg did show identifiable peaks.

The chromatograms for SFE (see Figs. 6 and 7) showed that its response is similar to that of a non-polar solvent in PFE (i.e., higher relative yields of squalene and lower relative yields of cholesterol). Also, and of equal importance, the on-line SFE technique can successfully analyze sample sizes as small as 250 μg (see Fig. 7). This small sample size makes on-line SFE an attractive choice for trace evidence analysis of small hair samples.

Lastly, SPME was relatively inefficient at extracting lipids from the surface of the hair, resulting in large sample sizes and a limited number of compounds detected via GC-MS. In addition, desorbed siloxanes from the SPME fibers interfered with the detection and resolution of the targeted compounds.

Conclusions

The quantitative, qualitative, and practical results from this study show PFE with polar solvents such as methanol or acetone to be an efficient and exhaustive technique for extracting surface components from hair. However, chromatographic analysis indicates that intermediately-polar solvents such as dichloromethane were more successful at extracting a broad range of lipids from hair, including free fatty acids, sterols, and wax esters.

Despite the high extraction efficiency of PFE, it was not as sensitive as the on-line SFE technique demonstrated by Benner, Goodpaster, and Degrasse (33,34). The on-line approach has been able to analyze samples as small as 100 μg, representing a single strand of hair 1 cm in length. In addition, on-line SFE of hair also showed adequete selectivity for hair lipids. While PFE may have the potential for small-scale extractions (e.g., approximately 1 mg), SFE appears to be the most practical method to date because it can handle sample sizes of hair similar to those found at crime scenes.

Future work may include the use of derivitization agents in on-line SFE, which has not yet been successful due to persistent background and memory effects. Such agents do, however, provide in-

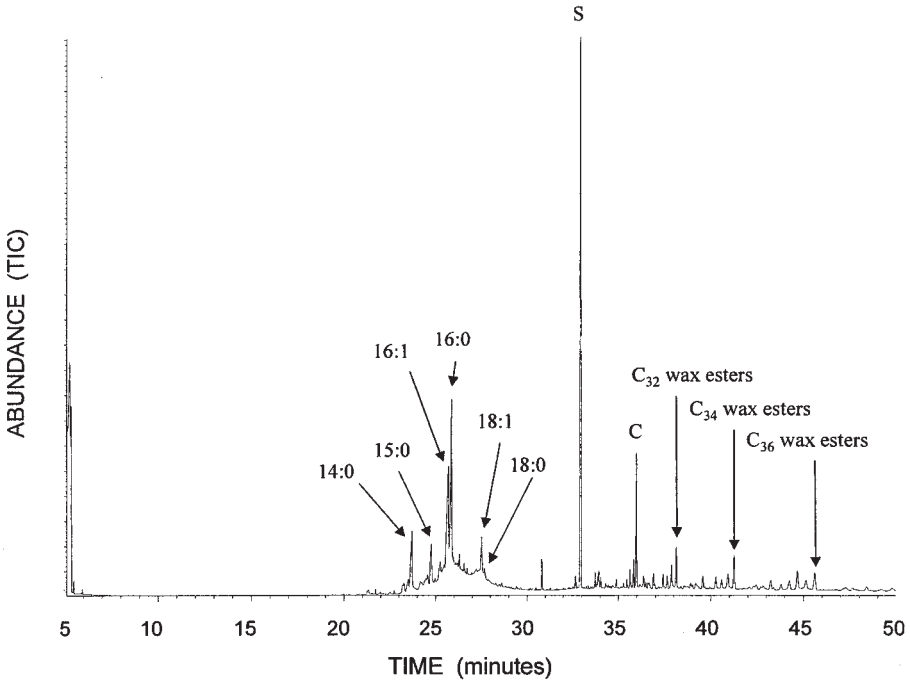

FIG. 6—*Carbon dioxide extract of a human hair sample using off-line SFE (269 mg sample size).*

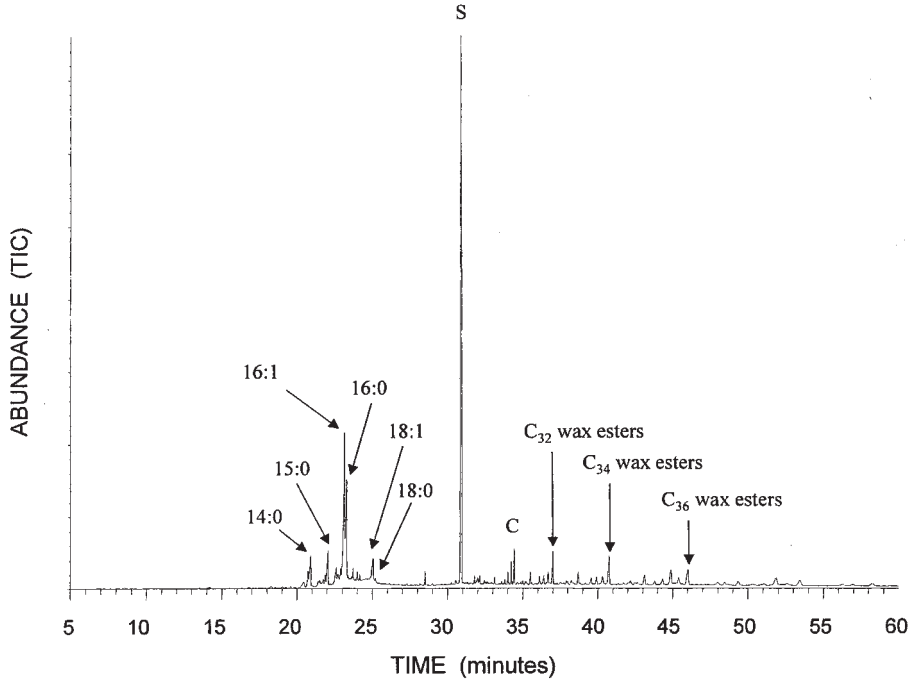

FIG. 7—*Carbon dioxide extract of a human hair sample using on-line SFE (251 μg).*

creased sensitivity and chromatographic resolution in off-line SFE (36). Direct insertion may also be useful, whereby a sample is heated and any desorbed compounds analyzed by GC/MS. However, this method may lack the ability to analyze the larger molecular weight species found on hair, whose elution temperatures are routinely above 260°C.

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