Detection of Fatty Acid Ethyl Esters in Skin Surface Lipids as Biomarkers of Ethanol Consumption in Alcoholics, Social Drinkers, Light Drinkers, and Teetotalers Using a Methodology Based on Microwave-Assisted Extraction Followed by Solid-Phase Microextraction and Gas Chromatography–Mass Spectrometry

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Abstract

Fatty acid ethyl esters (FAEE) are known to be a direct alcohol marker and are mainly investigated in hair samples for their ability to be incorporated into this matrix from sebum. The present study used an already developed methodology to provide and confirm information about the use of FAEEs in skin surface lipids as markers of alcohol consumption. The skin surface lipids were collected with Sebutapes® from the foreheads of teetotalers, light drinkers, social drinkers, and alcoholics. The samples were analyzed by direct solid-phase microextraction and gas chromatography–mass spectrometry for ethyl myristate, ethyl palmitate, ethyl oleate, and ethyl stearate. Relative FAEE/sebum allowed an evaluation of alcohol consumption. The ranges obtained for relative FAEEs in each category were as follows, teetotalers (0–13.85 pg/mg), light drinkers (11.10–26.80 pg/mg), social drinkers (20.55–86.55 pg/mg), and alcoholics (109.00–1243.40 pg/mg). A social drinker volunteer was monitored during a period of two months. The highest mFAEE/msebum were generally detected 7–9 days after the days of high alcohol consumption. From these results, a clear distinction of teetotalers, social drinkers, and alcoholics could be established with the methodology used.

Introduction

Alcohol consumption has health and social consequences via intoxication, alcohol addiction, and other biochemical effects of alcohol. There is increasing evidence that besides volume of alcohol, the pattern of the drinking is relevant for the effect on health. The diagnosis of chronic alcohol abuse is generally performed using some enzymatic and hematological parameters that lack sensitivity and specificity and are invasive to the patient. The most frequent methods used at present for diagnosis are based on indirect alcohol markers such as γ-glutamyltransferase (γ-GT), erythrocyte mean cell volume (MCV), or carbohydrate-deficient transferrin (1–3). All these methods have a common disadvantage; increased values can also originate from other pathological reasons. Diagnosis of alcoholism with these tests requires a complete clinical assessment. At present, recent ethanol consumption can be routinely detected with certainty only by direct measurement of ethanol concentration in blood or urine. Ethanol is eliminated rapidly from circulation, and the time span for this detection is in the range of hours. Various new markers have been proposed to expand the detection period, but their characteristics have not yet justified their use in routine medical practice. Direct alcohol biomarkers are valuable because these contain the carbon atoms of ethanol, so they can only be derived from alcohol. Some of the most important direct markers are ethyl glucuronide (4), phosphatidylethanol (5), and fatty acid ethyl esters (FAEEs) (6). Unfortunately, these compounds have a relatively short life span in blood. Therefore, in blood, they can be used only for detection of recent alcohol intake.

FAEEs are formed during non-oxidative metabolism of ethanol by the conjugation of ethanol to endogenous free fatty acids and fatty acyl-CoA. FAEE formation can be spontaneous but is most often catalyzed by microsomal acyl-CoA:ethanol O-acetyltransferase (AEAT) or cytosolic FAEE synthase that is
found throughout the body, which uses ethanol and free fatty acids as its substrates (7). FAEEs can be detected in hair, meconium, blood, and various organs (8–10). Several papers have shown that the concentration of FAEE in hair can be used for distinction between teetotalers and social drinkers on one hand and strong alcohol abusers on the other (11–13). The detection of FAEEs in hair presents some problems, as the susceptibility to contamination by the environment and the complexity of some extraction processes presented in the literature.

The incorporation of FAEEs into the hair was found to occur primarily from the lipid layer (sebum) on the hair surface, which is steadily regenerated by sebaceous glands (14). Consequently, FAEEs should also be detectable on the skin surface independent of the hair and should provide information about previous alcohol consumption.

Recently, Pragst et al. (15) presented a study of the analysis of FAEE on skin surface lipids collected by a wipe-test and by a patch-test. In this analysis, samples were collected by a wipe-test from the foreheads of 13 teetotalers, 16 social drinkers, 10 cases of death with known recent alcohol misuse, and 5 cases of death without indications of alcohol misuse. The samples were analyzed by headspace solid-phase microextraction (HS-SPME) and gas chromatography–mass spectrometry (GC–MS) for ethyl myristate, ethyl palmitate, ethyl oleate, and ethyl steareate and by high-performance liquid chromatography (HPLC) with photodiode-array detection using squalene (SQ) as a natural reference substance to which the FAEEs concentrations were related. The ratio \(\text{mFAEE/mSQ}\) ranged between 0.16 and 1.12 ng/mg (mean 0.34 ng/mg) for the teetotalers and between 0.08 and 0.94 ng/mg (mean 0.37 ng/mg) for the social drinkers with no significant difference between the groups. For alcoholics, 2.4–24.2 ng/mg (mean 13.1 ng/mg) were found. In this study performed by Pragst and co-workers (15), the FAEEs in skin surface lipids were established as markers for medium-term retrospective detection of heavy drinking.

The present study seeks to provide and confirm information about the use of FAEEs in skin surface lipids as markers of alcohol consumption using an already developed and validated methodology by Díaz-Vázquez and co-workers (16). The skin surface lipids will be collected with Sebutapes. These collection devices are white, open-celled, microporous, hydrophobic films of polypropylene coated with an adhesive layer, with pores specifically designed to collect skin surface lipids (sebum) (17). Sebum is collected and accumulated passively in the Sebutapes pores and appears as translucent spots. The use of Sebutapes will allow the determination of the total of skin surface lipids collected per sample and the rate of total FAEEs per milligram of sample collected.

**Materials and Experimental Methods**

**Reagents and sebum model sample preparation**

The standards ethyl myristate, ethyl palmitate, ethyl oleate, and ethyl stearate and the solvents were obtained from Sigma–Aldrich (St. Louis, MO). All solvents, acetone, hexane, and dichloromethane, were HPLC grade.

Model matrixes of sebum with and without spikes of four FAEE standards were used for the optimization of some parameters of analysis (selection of the best SPME fiber and selection of the sebum collection device). Sebutapes were obtained from CuDerm (Dallas, TX). Synthetic sebum was obtained from Scientific Services (New York, NY). Its composition was as follows: 17% fatty acid, 44.7% triglycerides, 25.1% of wax monoesters, and 12.9% of squalene. A stock solution of the model matrix was prepared as follows: the synthetic sebum (0.016 mg/mL) was dissolved in dichloromethane and the standards of interest were spiked into the solution. An aliquot of 3 µL of the spiked sebum solution was taken, deposited on the Sebutapes, and the solvent allowed to evaporate. For the SPME optimization, an aliquot of model matrix spiked with the standards was diluted in nanopure water using a mixture of acetone/hexane (2:1) as a co-solvent (200 µL/4 mL nanopure water). The final concentration of the standards was 25 ng/mL. Nanopure water was obtained from a Barnstead purification system (mega-Pure™ System MP-3A with NANOpure ultrapure water system).

**Volunteers and sample collection**

Four categories of persons were defined to classify the samples under investigation: teetotalers (do not drink at all), light drinkers (drink very sporadically, 1 drink per month within the past 30 days), social drinkers (adults who have at least 1 drink of alcohol per week within the past 30 days), and alcoholics (adult men having more than two drinks daily and adult women having one drink daily). The definition of the categories were based on the definitions of the WHO organization and the CDC (18,19).

For each category, five or six volunteers were sampled during a period of seven days. One sample was taken from each volunteer daily. Consent forms and a questionnaire to monitor the age, sex, and alcohol consumption during the last seven days before sampling, the average consumption during the last two months, and the daily consumption during the sampling period were administrated to each participant. In addition, the use of cosmetic products on the face before the sampling period was recorded. The volunteers were required not to use creams, makeup, or other cosmetic products during the sampling period.

Each sample was collected from the center of the forehead of the volunteers. Before application, the foreheads were cleansed thoroughly with isopropyl alcohol swabs and allowed to dry for approximately 1 min. Sebutapes preweighed to five decimal places were applied to the forehead with tweezers. Sebum was collected for 45-min intervals in a room at 25°C. During the sampling period, additional Sebutapes were exposed to the same environment for use as blanks.

After the collection period, patches were removed, weighed, and stored in glass petri dishes, then sealed in plastic bags at −30°C until analysis. For each sample, the amount of skin surface lipids collected was determined. Although this method of collecting sebum is selective, other materials such as sweat and dead cells can also be present. The physical activity of the subject was limited to minimize sweat. When sebum weight was reported, it was not exclusively sebum.
Instrumentation

Microwave-assisted extractor. Microwave-assisted extraction was carried out using an Ethos Plus laboratory microwave extraction system (Milestone, Monroe, CT). The instrument is able to extract concurrently 10 samples under identical extraction conditions. This system is comprised of an Ethos laboratory microwave unit with a built-in magnetic stirrer and a fiber optic temperature sensor.

SPME. The SPME device and SPME fibers used were purchased from Supelco. Two different fiber types were purchased: polydimethylsiloxane (PDMS, 100 µm) and polydimethylsiloxane/divinylbenzene (PDMS/DVB, 65 µm). Fibers were conditioned in the injector of the GC for a period of time as indicated by the manufacturer before use. Initially new fibers were conditioned at 265°C for 2 h. A desorption period of 15 min at 265°C of sampled fibers was used to remove all analytes. The spiked model matrix was introduced into 5-mL vials. Sample vials were completely filled with solution in order to avoid headspace. Extraction time was 45 min, extraction temperature was 40°C, and the stirring rate was 1000 rpm. Samples were stirred on a digital stirring (± 20 rpm) hotplate. Others extraction times, temperatures, and stirring rates were performance by Díaz-Vázquez and co-workers (16).

GC–MS. All the extracts were analyzed with a Hewlett Packard 5890 series II-GC coupled with a 5971-MS system. The system was calibrated daily with perfluorotributylamine; system blanks and known standards were run to assure optimum performance and monitor sensitivity. External standards (n-tridecane and n-pentadecane) were used to normalize for changes in sensitivity in time. The analysis conditions were a J&W DB5 MS column (bonded and cross-linked 5%-phenylmethylpolysiloxane, 30-m column length, 0.32-mm column i.d., 0.25-mm film thickness). SPME injector inserts of 0.75-mm internal diameter for a Hewlett-Packard chromatograph (Supelco) were used. The GC temperature program was as follows: oven was initially 70°C for 5 min, 10°C/min until 140°C, 4 min at 140°C, 6°C/min until 200°C, 4 min at 200°C, 15°C/min until 280°C, and held at 280°C for 3 min. Samples were injected in the splitless mode with a solvent delay of 4 min. Both total ion detection and single ion monitoring were used. For the full scan acquisition mode, the MS was in the positive electron impact mode at 70 eV and the mass detection range was m/z 50–550. Carrier gas was helium at 0.9 mL/min. Standards were monitored with the full scan acquisition mode and peaks were confirmed with an IST/EPA/NIH Mass Spectral library. For sebum extract samples, the SIM mode with m/z 88 was selected because the retention time of the FAEEs standards had been confirmed by total ion detection.

Sample preparation and analysis

Each extraction was performed as reported by Díaz-Vázquez et al. (16). MAE proved to be an efficient and reproducible mode of extraction. The Sebutapes were transferred to the Ethos extraction vessels. A volume of 10 mL of a mixture of acetone/hexane (2:1) was added into the vessels and the microwave assisted extractions carried out using the following program, 5 min heating from 25°C to 60°C and then 5 min at 60°C. For each set of extractions a Sebutape without model matrix was extracted and used as a process blank. After extraction, the vessels were cooled down to room temperature before opening. The extract was transferred to tubes for pre-concentration. The sebutape and the extraction vessel were rinsed three times with 0.5 mL of the extraction solvent, and this was added to the preconcentration tube with the extract. The extracts were preconcentrated with an inert gas stream (N₂) to 1 mL and 1 µL of the solution was directly analyzed by GC–MS in the optimization of the MAE extraction parameters. For the SPME procedures, 0.20 mL of the extract was diluted in nanopure water to 4 mL.

The limits of detection of the four esters (3–10 pg per sample) and of quantification (10–30 pg per sample) were estimated from 3 and 10 times the baseline noise, respectively. Calibration curves were generated with concentrations equivalent to 10 to 1000 pg/sample with correlation factors which ranged from 0.9878 to 0.9958. The reproducibility of the measurement was determined by the six-time analysis of six sebum model matrix samples spiked with the FAEE standards extract on the same day. The relative standard deviation of the sum of

Figure 1. Relative FAEE concentration in the skin surface lipids collected daily from the forehead of a social drinker by the Sebutape test and alcohol consumption during a period of 52 days.
the fatty acid ethyl esters levels (m$_{\text{FAEE}}$) was 3.0% at a concentration of 100 pg/sample.

Results and Discussion

Selection of the SPME fiber

In order to optimize the detection limits and specificity of the applied method, prior to the analysis of the volunteer's skin surface lipids samples, an experiment was conducted in order to select the best SPME fiber for our purposes. Two fibers, the PDMS (polydimethylsiloxane) phase and the PDMS/DVB (polydimethylsiloxane/divinylbenzene), were tested using a sebum model matrix spiked with the four FAEEs under study. Higher signals were obtained for most of the FAEEs with the red fiber (PDMS). Based on these results the PDMS fiber was selected for the study.

Sampling period and alcohol consumption correlation

In order to confirm the relation between the alcohol consumption and time of release of FAEEs reported previously by Pragst and co-workers (15) and to demonstrate the effectiveness of our approach, a social drinker volunteer was monitored over a period of two months. No cosmetics or ethanol-containing products were used during the sampling period. A sebum sample was collected daily, and alcohol consumption was recorded. After 25 days of sampling, the volunteer was strictly abstinent for 18 days (until day 43). The results of this study are shown in Figure 1. During the period of abstinence, the m$_{\text{FAEE}}$/m$_{\text{sebum}}$ of the volunteer was in the range of teetotalers and light drinkers (see Figure 1). From the graph presented in Figure 1, one can notice that the highest m$_{\text{FAEE}}$/m$_{\text{sebum}}$ tended to be detected 7–9 days after the days in which high alcohol consumption were reported. This time delay is in agreement with the transition period of sebum formation/secretion reported in the literature (20,21). It can be concluded from this study that, for persons with alcohol consumption, high m$_{\text{FAEE}}$/m$_{\text{sebum}}$ was detected approximately 1–2 weeks after the alcohol was ingested. The results obtained were consistent with those previously observed.

The highest m$_{\text{FAEE}}$/m$_{\text{sebum}}$ was detected on day 50, a day preceded by 8 days of considerable alcohol consumption. This fact demonstrated the magnification effect and accumulation effect of FAEEs that is caused by the periodic consumption of alcohol. This result shows the applicability that the monitoring of FAEEs during a longer period could have in order to...
establish alcohol consumption patterns of the individual. Based on this preliminary study, the volunteers were monitored for a period of seven days, and the alcohol consumption history during the last seven days before sampling, the average consumption during the last months, and the daily consumption during the sampling period were recorded.

**FAEE detection with the sebutape test**

Sebum samples were collected and analyzed as previously described. It was shown by these blanks that Sebutapes exposed to the sampling environment did not lead to the detection of FAEEs. Figure 2 presents a typical SIM chromatogram obtained for one of the volunteers. This is the chromatogram of the sample for day 50, which correlates with the consumption of 130g of alcohol on day 43.

The ratio of \( \text{m}_{\text{FAEE}} / \text{m}_{\text{skin surface lipids}} \) was used as a relative FAEE concentration for interpretation and comparison with alcohol intake. The skin surface lipid amounts were in the range of 0.5–2.5 mg. In the analysis the ratio of FAEE/mg sebum was reported in order to normalize for variations between samples. The proposed method for monitoring FAEE in skin surface lipids by SPME–MAE–GC–MS analysis proved to be sufficiently sensitive for the identification in all samples including those of the teetotalers. Although an internal standard was not used, a relative comparison can be made because the conditions were the same in the samples analyzed.

In Figure 3, the average \( \text{m}_{\text{FAEE}} / \text{m}_{\text{sebum}} \) per subject are presented. The ranges observed for each category were as follow, teetotalers (0–13.85 pg/mg), light drinkers (11.10–26.80 pg/mg), social drinkers (20.55–86.55 pg/mg), and for alcoholics (109.00–1243.40 pg/mg). From these results a clear distinction between teetotalers, social drinkers, and alcoholics can be established using the proposed methodology. There is not a clear difference between teetotalers and light drinkers in the total FAEE/mg sebum.

Small concentrations of FAEEs were also found in skin surface lipid samples of teetotalers in a previous investigation (15). The amounts of FAEEs in teetotalers may be produced by external ethanol accumulation from cosmetic residues on the stratum corneum, medicines ingested or the diet of the subjects. Furthermore, the formation of traces of ethanol from activated acetate in the sebum glands cannot be excluded since the sebocytes are able to transform it to the corresponding fatty alcohols in the wax ester formation (22). The overlap of FAEEs/mg of sebum of teetotalers and light drinkers categories may also be because the alcohol consumption of some individuals in the light drinker’s category was too low to produce enough FAEE to obtain a significant difference.

In addition to determining the total FAEEs/mg for each category, the presence and amount of each of the fatty acids esters were compared. The average amounts of each FAEE detected per person and category are shown in Figure 4. From this graph, it can be seen that only two FAEEs were detected in teetotalers and light drinkers, ethyl myristate, and ethyl palmitate, under the analytical conditions of the method. In social drinkers and alcoholics, the four FAEEs under study were detected. From these results, it can be concluded that the heavier FAEEs can be used as biomarkers in persons with a high consumption of alcohol. When the total amount of FAEEs is segmented in the contribution of each FAEE, a characteristic profile is obtained for each category under study. This profile can be used as a pattern to obtain information about the drinking behavior of the tested person.

For the social drinkers and alcoholics, the amount of FAEE/mg sebum detected and the reported amount of alcohol were related. Because it is known that the sebum has a transition time of about one week between sebum production and appearance on the skin surface lipid, the FAEE detected by each subject was related to the alcohol consumption reported seven days prior to the sampling. This fact confirmed that FAEEs in skin surface lipids can be used for medium-term retrospective determination of alcohol consumption.

**Conclusions**

The proposed method for monitoring FAEE in skin surface lipids by SPME–MAE–GC–MS analysis proved to be sufficiently sensitive for the identification of FAEEs in all samples including those of the teetotalers. The sampling of sebum from the forehead using the sebutape as the collection device appeared to be an appropriate sampling method. It allowed the quantization of the collected skin surface lipids and the determination of sebum excretion rates for each subject under analysis. In addition it allowed the acquisition of fresh skin surface lipids samples and in consequence cleaner samples. The FAEEs were normalized using the total amount of skin surface lipids collected in each sample. Relative
amounts of FAEE/skin surface lipids allowed an evaluation with respect to alcohol consumption. The ranges observed for the relative FAEEs in each category were as follows: 0–13.85 pg/mg for teetotalers, 11.10–26.80 pg/mg for light drinkers, 20.55–86.55 pg/mg for social drinkers, and 109.00–1243.40 pg/mg for alcoholics. From these results, a clear distinction between teetotalers, social drinkers, and alcoholics could be established using the proposed methodology. When the total amount of FAEEs is segmented into the contribution of each FAEE, a characteristic profile is obtained for each category under study. This profile can be used as a pattern to obtain information about the drinking behavior of the person tested.

The most relevant characteristic of the profiles is the presence of the four FAEEs in the social drinkers and alcoholics and the presence of only two fatty acids, ethyl myristate and ethyl palmitate, in the other categories. The profile of social drinkers and alcoholics under study differs in terms of the amount of FAEE. The results of this study confirm sebum as a suitable matrix for the detection of alcohol consumption and abuse by FAEE analysis. The detection window in sebum of at least one week is also confirmed through our results.

**References**