

# Rapid, accurate, and sensitive fatty acid ethyl ester determination by gas chromatography-mass spectrometry

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**Background:** Fatty acid ethyl esters (FAEEs) are useful markers of ongoing alcohol use and may be associated with alcohol-induced damage to the liver and pancreas. In this article, we describe a novel method for rapid determination of the three major FAEEs found in human plasma. **Methods:** Internal standard, ethyl heptadecanoate, was added to plasma samples, and FAEEs were isolated by acetone precipitation, hexane lipid extraction, and amino-propyl silica solid phase extraction. FAEEs were quantitated by gas chromatography-mass spectrometry (GC-MS) using a nonpolar dimethylpolysiloxane column. The accuracy, precision, specificity, and sensitivity of the assay were defined from plasma samples from recently drinking and abstinent persons, with and without the addition of FAEEs. **Results:** Individual FAEE peaks demonstrated excellent resolution. Instrument time was reduced by more than 60%. The lower limit of detection was 5 to 10 nM, and the lower limit of quantitation for each FAEE was 60 nM (for 22 samples with known concentration 60 nM,  $\times \pm$ SD:  $61 \pm 5.7$ ,  $57 \pm 5.7$ , and  $57 \pm 5.9$  nM, for ethyl palmitate, ethyl oleate, and ethyl stearate, respectively). Instrument precision (coefficient of variance, CV) for these three FAEEs was 0.3%, 0.4%, and 0.7%, respectively. Intra-assay precision (CV) for total FAEEs was less than 7%. FAEEs were absent in 49 samples from abstinent persons. FAEEs were detected in all 76 samples with associated positive blood alcohol levels. **Conclusions:** Our method of FAEE analysis is rapid and potentially useful in research and clinical studies. FAEE determination using this method is precise, accurate, sensitive, and specific and deserves broader application. (*J Lab Clin Med* 2006;147:133-138)

**Abbreviations:** BAL = blood alcohol level; CV = coefficient of variance; FAEE = fatty acid ethyl ester; GC-MS = gas chromatography-mass spectrometry; LLOD = lower limit of detection; LLOQ = lower limit of quantitation

**F**AEEs are nonoxidative metabolites comprised of an ethyl moiety esterified to any of several fatty acids in blood. These molecules can be detected at least 48 hours after ethanol consumption, have been shown to

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correlate with BAL<sup>1,2</sup> and may differentiate acute from chronic alcohol use.<sup>3</sup> Also, FAEEs in hair and skin lipids correlate with drinking history,<sup>4</sup> and FAEEs in meconium correlate with maternal alcohol exposure.<sup>5</sup>

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FAEEs may be toxic to various tissues. In rats, infusion of FAEEs causes edema, intracellular lipid accumulation, and organelle membrane distortion in the pancreas, and it is associated with increased serum amylase and activation of pancreatic zymogens.<sup>6,7</sup> Exposure of HepG2 cells to FAEEs impairs proliferation and protein synthesis, increases intracellular lipids, and distorts intracellular organelles and nuclear membranes.<sup>8</sup> FAEEs also uncouple mitochondrial oxidative phosphorylation<sup>9</sup> and have been implicated as mediators of toxicity in fetal alcohol syndrome.<sup>10</sup> For these reasons, FAEEs are candidates for monitoring alcohol use and alcohol-induced damage to tissues and organs.

Circulating levels of FAEEs in humans after exposure to alcohol vary from less than 50 nM to more than 3  $\mu$ M.<sup>11</sup> Determination of low FAEE concentrations requires sensitive and specific methods, such as GC-MS. Current GC-MS assays for FAEE use polar carbowax (Supelco, Bellefonte, Penn)<sup>12,13</sup> or ZB-WAX (Phenomenex, Torrance, Calif)<sup>14</sup> capillary columns and entail run times from 30 to 44 minutes.<sup>11,14</sup> In this article, we show that a less polar dimethylpolysiloxane capillary chromatography column offers excellent resolution with much shorter retention times. Last, we define the precision, accuracy, sensitivity, and specificity of this assay.

## METHODS AND MATERIALS

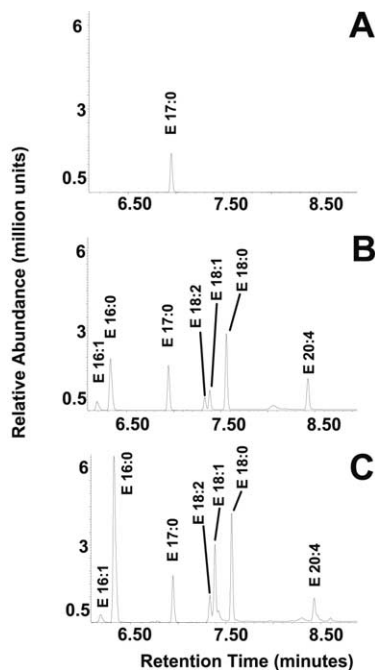
**Plasma samples.** To validate our method, we analyzed de-identified plasma samples. Seventy-six samples had an associated positive BAL, as determined by the University of Colorado Hospital clinical laboratory by a GC-flame ionization detector. Also, samples from 49 participants who reported no drinking in the previous 3 days (timeline follow-back instrument)<sup>15</sup> and had negative breath alcohol determination were analyzed for FAEE to determine specificity of the assay. Plasma samples from these abstinent persons were also spiked with low-concentration FAEEs to assess sensitivity and accuracy. This research was carried out according to the principles of the Declaration of Helsinki and was approved by the Colorado Multiple Institution Review Board. All participants gave informed consent.

**FAEE extraction and quantification.** A modified Folch<sup>16</sup> lipid extraction method revised by Bernhardt et al<sup>12</sup> and Zybko et al<sup>17</sup> was used for FAEE extraction from plasma. Internal standard, ethyl heptadecanoate (E 17:0, 125  $\mu$ L of a stock solution containing 4-nmol ethyl heptadecanoate/ $\mu$ L hexane) was added to 0.5-mL plasma samples to yield an internal standard concentration of 1000 nM. Four milliliters cold acetone was added, and samples were vortexed for 1 minute and centrifuged at 4000 rpm at 4°C in a Jouan CR422 centrifuge for 10 min. Lipids were extracted with 6 mL and then 2-mL hexane. Hexane extracts were combined, dried completely under nitrogen, and reconstituted in 300- $\mu$ L hexane. This was applied to 500-mg aminopropyl silicate solid-phase extraction columns (Bond Elut, Varian, Harbor City,

Calif) prepared with 4-mL methylene chloride followed by 4-mL hexane, using the method of Bernhardt et al,<sup>12</sup> as later modified by Soderberg et al.<sup>18</sup> FAEEs were eluted from the columns with 4-mL hexane and dried completely under nitrogen. The samples were reconstituted with 60- $\mu$ L hexane, 1  $\mu$ L was injected, and ions were analyzed. GC hardware used included an Agilent 6890 gas chromatograph (Agilent Technologies, Wilmington, Del), an Agilent 7683 automatic liquid sampler system, and a DB-1 (30 m, 0.25 mm internal diameter) dimethylpolysiloxane chromatography column (J & W Scientific, Folsom, Calif). Six ethyl acetate and six hexane rinses were performed automatically before and after each injection. GC oven temperature was held isothermally at 150°C for 30 s, increased from 150 to 250°C at 15°/min for 6.67 min, then held isothermally for 4 min and then cooled to 150°C over 1 min. The injection inlet and GC-MS interface were maintained at 260 and 280°C, respectively. Helium carrier gas flow rate was maintained at 1 mL/min throughout. FAEEs were quantified by mass spectrometry (Agilent 5973 mass spectrometer) using selective ion monitoring of base ions  $m/z$  88.1 and 101.1 for ethyl palmitate, ethyl heptadecanoate, ethyl oleate, and ethyl stearate. Calibration curves, retention times, and ion composition for the FAEEs were determined from commercial standards in blank plasma (Nu-Check Prep, Elysian, Minn). Standard purity of greater than 99% (thin layer chromatography and gas-liquid chromatography) was noted by the manufacturer.

**FAEE identification.** Calibration curves were constructed in the following fashion. FAEE standards in hexane were added to 0.5-mL blank plasma to yield final concentrations of 20, 30, 40, 100, 1000, 3000, 4000, 5000, 6000, and 7000 nM. Calibration samples were extracted and analyzed as noted above to assess for instrument linearity. The amount ratios (analyte:internal standard) were plotted on the abscissa; abundance ratios (analyte:internal standard) were plotted on the ordinate. These calibration curves were updated with each batch of unknowns by reanalyzing a low and high point on the curve. Peaks were identified by two criteria: retention time and base ion ratio. For classification as an FAEE peak, suspected peak retention times fell within 0.02 min of the respective FAEE standards. The base ion ratio (eg,  $m/z$  101.1:  $m/z$  88.1 for ethyl palmitate) for any suspected FAEE peak fell within a 6% difference of the expected fragment ion ratio of standards in blank plasma to support classification as FAEE. To identify abundances, we also used the “*Q*” value descriptor within the Chemstation software, a relative measure of confidence in analyte identity that assesses retention time, and ion composition via an established algorithm. The “*Q*” value is expressed as a percentage, with a perfect match of retention time and fragment ion ratios expressed as 100%.

**Sensitivity, specificity, and accuracy.** We spiked plasma samples from the abstinent participants with 5 ( $n = 6$ ), 10 ( $n = 6$ ), 30 ( $n = 6$ ), 40 ( $n = 6$ ), and 60 nM concentrations (volumetric) ( $n = 22$ ) of the three predominant FAEEs to determine the LLOQ, LLOD, and within-run accuracy. Specificity determination was carried out on non-spiked samples from 49 abstinent persons, separate from the sensitivity analysis. We defined the LLOQ as having at least five times the response compared with



**Fig 1.** Selected ion chromatograms from plasma samples; ions 79.1, 88.1, 91.1, 101.1, and 117.1 were monitored. (A) Ethyl heptadecanoate (E 17:0, 1000 nM) used as an internal standard. (B) 1000 nM each of ethyl palmitoleate (E 16:1), ethyl palmitate (E 16:0), E 17:0, ethyl linoleate (E 18:2), ethyl oleate (E 18:1), ethyl stearate (E 18:0), and ethyl arachidonate (E 20:4) in blank plasma. (C) Plasma sample with an associated BAL of 570 mg/dL.

baseline noise and an accuracy of at least 90%. The LLOD was defined as abundance peaks at the appropriate retention times with at least three times the response of baseline.

**Precision.** Instrument precision was determined by analyzing 10 serial injections from each of three extracted samples with associated BALs of 25, 50, and 522 mg/dL, respectively. Within-run precision was determined using two sets of plasma samples: 10 from a sample with associated BAL of 20 mg/dL and the 22 samples used in the accuracy assessment.

**Postpreparative stability.** Ten samples with associated BAL ranging from 20 to 440 mg/dL were analyzed at baseline and after 24 hours of storage at room temperature in punctured autosampler vials, thus, exposed to room air. Means were compared using a Student *t*-test for paired data.

## RESULTS

**FAEE identification, specificity, sensitivity, and accuracy.** Selected ion chromatography with this method produced excellent resolution of several FAEEs, and in particular the three of primary interest, ethyl palmitate, ethyl oleate, and ethyl stearate, (Fig 1) the predominant FAEEs in human plasma after alcohol ingestion. Ethyl palmitoleate, ethyl linoleate, and ethyl arachidonate were identified by retention time and ion ratios but not quantitated. All 76 BAL (+) samples had measurable FAEE levels. None of the non-spiked 49 samples from

abstinent persons had two or more FAEE species present in concentrations greater than 17 nM. Seventeen of these 49 samples had FAEE concentrations of 17 nM or less for one or two FAEEs. No FAEE were detectable in 32 of the samples from abstinent participants. Ethyl palmitate, ethyl oleate, and ethyl stearate at 60-nM concentrations were accurately quantified in blank plasma samples (taken from the abstinent persons). Observed means ( $\pm$ SD) were  $61 \pm 5.7$  nM,  $57 \pm 5.7$  nM, and  $57 \pm 5.9$  nM, yielding accuracies of  $93 \pm 6\%$ ,  $92 \pm 7\%$ , and  $91 \pm 5\%$ , respectively, and establishing the LLOQ. The LLOD was in the 5–10 nM range for the three predominant FAEEs. Our identification criteria yielded “*Q*” values greater than 94% for expected FAEE abundance peaks. Instrument assay time (injection to injection) was 11.17 min. Linear regression of peak area to known mass of individual FAEEs yielded calibration curves with Pearson’s correlation coefficients (*r*) = 0.998, 0.998, and 0.989, for ethyl palmitate, ethyl oleate, and ethyl stearate, respectively (Table I).

**Precision.** Within-run precision (CV) for each FAEE was as follows: ethyl palmitate, 4.7% and 9.5%; ethyl oleate, 12% and 10%; ethyl stearate, 12% and 10%; and total FAEE, 6.1% and 6.9%, *n* = 10 and 22, respectively. Precision of the instrument (CV) for each of three BALs (25, 50, and 522 mg/dL) was as follows: ethyl palmitate, 0.7%, 0.5%, and 0.4%; ethyl oleate, 1.5%, 0.8%, and 0.6%; and ethyl stearate, E 18:0, 1.2%, 1.0%, and 0.2%.

**Postpreparative stability.** Mean changes in ethyl palmitate, ethyl oleate, and ethyl stearate levels after 24 hours of exposure to room air and room temperature were 2.7%, 3.4%, and 2.3%, respectively. None were statistically significant differences (*P* = 0.28–0.93).

## DISCUSSION

**Advantages of the new method.** Published methods for FAEE analysis in plasma entail 30–40 min of instrument time for analysis of each sample. A more rapid increase in oven temperature yielded run times one third that of other methods, without sacrificing peak resolution.

The option to use a nonpolar chromatography column may increase the feasibility of FAEE analysis. Dimethylpolysiloxane columns, noted for high-temperature limits and utility in a wide range of applications, are considered an “industry standard.”<sup>19</sup>

We monitored base ions *m/z* 88.1 and 101.1 because these were the most abundant in the analytes and present in negligible amounts in non-ethanol exposed plasma. Monitoring only two ions allows for optimization of dwell time to increase resolution, and calculation of a distinct ion ratio for each FAEE. At very low

**Table 1.** Characteristics of GC-MS analysis calibration curves for FAEE standards in non-ethanol exposed plasma

FAEE species	Retention time (min)	<i>m/z</i> monitored	Adj. <i>r</i>	Slope (SEE)	<i>y</i> -intercept (SEE)	Highest calibration (nmol/l)	Root mean square error (SEE of abundance ratio)
Ethyl palmitate	6.34	88.1, 101.1	0.998	0.775 (0.017)	0.078 (0.062)	7000	0.128
Ethyl heptadecanoate <sup>a</sup>	6.95	88.1, 101.1	—	—	—	—	—
Ethyl oleate	7.39	88.1, 101.1	0.998	0.132 (0.003)	0.010 (0.010)	7000	0.021
Ethyl stearate	7.57	88.1, 101.1	0.989	0.811 (0.040)	0.243 (0.149)	7000	0.305

<sup>a</sup>Ethyl heptadecanoate used as internal standard. SEE: standard error of estimate; Adj. *r*: *r*-value corrected for degrees of freedom; *m/z*: mass to charge ratio.

FAEE concentrations, detection of base ions was necessary, as molecular ions were usually not measurable.

**Sensitivity.** Although we could easily identify seven FAEEs, we opted to define the characteristics of our assay for ethyl palmitate, ethyl oleate, and ethyl stearate. These are the predominant species in human plasma after alcohol ingestion,<sup>20</sup> and they likely hold the most promise for use as indicators of alcohol use in that matrix. Our lower limit of quantification, 60 nM, was similar to that reported by a group using headspace solid-phase microextraction GC-MS for skin lipid analysis (3 ng in 500- $\mu$ L solvent, or 20 nM).<sup>4</sup> Laposata's group uses a GC-MS assay that can quantitate at least eight FAEEs with a LLOQ of approximately 6–18 nM, but it requires more than 44 min per injection.<sup>1</sup> The rapid run time of our assay may well be worth the slight decrease in sensitivity in some clinical applications. Our LLOQ is roughly one order of magnitude lower than that reported using a GC-flame ionization detector instrument in the analysis of meconium.<sup>14</sup> Our LLOQ is below a threshold concentration (32 ng/g, or approximately 108 nM) proposed for identification of ethyl oleate in meconium by GC-tandem MS,<sup>21</sup> given the assumption that the mass of plasma and meconium are similar.

Our calibration curves for ethyl palmitate, ethyl oleate, and ethyl stearate become less linear above calibration concentrations of 5000 nM. This can be shown by excluding the two highest calibration concentrations, which yields improvements in the *r* values (0.998, 0.997, and 0.994 for ethyl palmitate, ethyl oleate, and ethyl stearate, respectively). Standard error of the estimate for slope also improved markedly (0.013, 0.003, and 0.027, respectively). Thus, quantitation with this instrument is probably optimal below concentrations of 5000 nM using the reported assay. We have not found reports of *r* values or slopes for FAEE calibration curves in the literature.

**Specificity.** We qualified FAEE concentrations less than the LLOQ as trace. Because trace abundances of individual FAEE were detected in some abstinent per-

sons, concomitant presence of at least two FAEE species in concentrations at least above the LLOQ was established as a criterion for defining positive FAEE levels with this assay. By this definition, our assay produced no false positives. Similarly, most studies report that FAEEs are not measurable in participants not recently exposed to alcohol.<sup>22</sup> However, one group using headspace GC-MS reports very low levels of FAEE in hair samples of teetotalers.<sup>23</sup> We believe that the trace FAEEs found in our non-ethanol-exposed samples are related to carryover of FAEEs on the automatic injector system and in the GC-MS inlet. A series of experiments using sequential manual and automatic injections of FAEEs and hexane, with six manual or automated needle rinses of hexane, ethyl acetate, or methyl tert-butyl ether followed by six rinses with hexane point to the automatic injector needle and the inlet as sources of contamination from previous analyses. For example, trace amounts of ethyl palmitate were detected in two manual hexane injections after manual injection of hexane containing ethyl palmitate (2 ng/ $\mu$ L). The manual injection needle had been rinsed six times each in methyl tert-butyl ether, ethyl acetate, and hexane between injections. Also, nonreported or forgotten alcohol consumption in the study participants, even in the form of mouthwash, could theoretically result in low-level FAEE detection. Hair cosmetics containing ethanol may also be contributory.<sup>24</sup> It is also possible that differences in fatty acid substrate between persons could lead to differences in FAEE levels and potentially explain FAEE persistence for more than 3 days in some persons.<sup>20</sup> In any case, these trace abundances in blank plasma are significantly less than our LLOQ and generally lack the presence of more than one FAEE species. We feel that these trace FAEE levels are a result of the sensitivity of the instrument and that, if defined properly, do not detract from the value of the assay.

**Assay validity and precision.** Our data reflecting within-assay precision compare favorably with a previous re-

port, supporting the consistency of the extraction method, and the validity of our hardware changes. Salem et al reported a within assay CV of 17% for measurement of seven FAEE species.<sup>12</sup> The within-assay CV for the three FAEEs that we analyzed was 6.1% and 6.9%. This difference in precision is due in part to the fact that we analyzed three FAEEs vs. seven, and potentially to the newer model GC-MS we used. Potential improvements in chromatography due to a more uniform sample injection with an automatic injection system in our assay, versus manual injection (personal communication, Laposata laboratory) must also be considered. We feel that use of the dimethylpolysiloxane chromatography column in conjunction with the GC-MS instrument used offers excellent precision for FAEE analysis, as well as a desirable resolution at similar levels of performance as previous assays using polar columns.

FAEEs are stable for at least 24 hours in automated injection vials exposed to room air and room temperature. This reassures that postpreparative samples could be re-analyzed if an instrument or software dysfunction occurred during automated analysis. This also suggests that between-run (day-to-day) precision is high for our assay.

**Importance of defining FAEE positivity.** Bisaga et al<sup>25</sup> report no difference between FAEE levels after overnight abstinence and 13 hours after ingestion of three to six drinks in heavy drinkers. Their interpretation is that a limited FAEE detection period will minimize the clinical utility of FAEE as a diagnostic tool. However, the key point may be that FAEEs were detectable at baseline and 13 hours after drinking and that both of these FAEE levels would likely be different than those of a teetotaler group. It is likely that these heavy drinkers had detectable FAEEs after 12 hours of abstinence, at levels similar to those 13 hours after the drinking experiment. It is possible that FAEEs may diffuse out of body storage compartments (likely adipose) for at least 44 hours in heavy drinkers,<sup>26</sup> which supports the use of current FAEE assays in a qualitative (positive or negative) versus quantitative manner in clinical alcohol detection settings. Inter-individual variation in FAEE metabolism<sup>11</sup> also supports this notion. These points make it imperative that the factors that aid in characterizing very low FAEE levels, such as accuracy, LLOQ, thresholds for ion ratios, and retention times be well described.

Herein, we describe a precise, accurate, timesaving, sensitive, and specific assay for the measurement of FAEEs. This method may be readily applied to studies of FAEEs in alcohol-exposed populations.

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