FATTY ACID ETHYL ESTERS: TOXIC NON-OXIDATIVE METABOLITES OF ETHANOL AND MARKERS OF ETHANOL INTAKE

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1.   ABSTRACT

       This report describes the biochemistry of fatty acid ethyl esters (FAEE), non-oxidative metabolites of ethanol, and their clinical significance.   We review information regarding the enzymes responsible for FAEE synthesis and degradation, and the mechanisms involved with the intracellular and extracellular transport of FAEE and FAEE-mediated cytotoxicity.   Also a summary of reports on the emerging clinical significance and diagnostic utility of FAEE as short and long-term markers of alcohol consumption, and the methodological aspects of FAEE assessment is included.

2. INTRODUCTION

       Despite the fact that alcoholism is a major cause of disease, the mechanisms that underlie and influence the onset, progression and severity of alcohol-related disease are not well understood.   One hypothesis is that fatty acid ethyl esters (FAEE), esterification products of ethanol (alcohol) and fatty acids, are important mediators of ethanol-induced cytotoxicity, organ damage and disease. Substantial *in vitro* and *in vivo* evidence has emerged that FAEE contribute to ethanol related pathophysiology, and a number of different mechanisms for their toxic effects have been proposed.   In the last ten years there has been

impressive growth of our understanding of FAEE synthesis and degradation. Multiple enzymatic activities associated with FAEE synthesis and hydrolysis have been described. More recently, the potential diagnostic utility of FAEE as short- and long-term markers of ethanol intake have been investigated. A number of studies have shown that ethyl esters are readily detectable in serum and a wide variety of tissues after ethanol ingestion, and that they persist long after alcohol is no longer detectable. Thus FAEE, independent of their role in ethanol-induced organ damage, may play an important role in the diagnosis of alcohol associated disease because they serve as markers of ethanol intake. This review focuses on the major observations in the field, the development of methodologies related to FAEE identification and quantitation and the emerging clinical significance of FAEE.

3. ALCOHOLISM

Alcohol is the most widely used and frequently abused drug in the world. While the majority of alcohol consumers are able to benefit from the positive psychological and healthy effects of moderate alcohol consumption, a large number of individuals suffer from alcohol-related disease. Approximately 14 million Americans abuse alcohol or are alcohol dependent and more than 700,000 Americans receive treatment for alcoholism on any given day (1,2, 3). Alcohol abuse carries an estimated annual cost to society of more than $176 billion (4). In addition, alcohol consumption contributes to 105,000 deaths annually, making it the 3^d leading cause of preventable death in this country (5).

Alcoholism is defined by the consumption of alcohol at a level that interferes with physical or mental health; characterized by the development of alcohol dependence and of tolerance to increasing quantities of alcohol; and by the development of withdrawal symptoms upon cessation of alcohol consumption (6). Long-term pathological effects of alcohol ingestion depend on heredity, gender, concomitant disease, diet, alcohol consumption patterns, and whether the drinking is minimal, moderate, or heavy. According to the National Institute on Alcohol Abuse and Alcoholism, moderate drinking is defined as ≤2 drinks per day for men under the age of 65 and ≤1 drink/day for women and for men over the age of 65. A standard drink contains 12-14 grams of pure alcohol, which is equivalent to 12 ounces of beer, 4 ounces of wine, or 1.5 ounces of distilled spirits (7).

For the non-tolerant adult drinker, the characteristic clinical effects and behavioral changes following alcohol ingestion correlate with blood ethanol concentration. The effects can range from minor behavior changes, to coma, respiratory failure and death (8). Chronic alcohol abuse is associated with numerous pathophysiological effects, including pancreatitis, alcoholic hepatitis, cirrhosis, hyperlipidemia, hypertension, cardiomyopathy, arrhythmia, stroke, infection, fluid and electrolyte abnormalities, ascites, hematologic disorders and nutritional deficiencies. In addition, heavy drinking can have serious neuropathological effects (7,9,10,11).

Alcoholics may exhibit changes in personality and can experience altered mental functioning, including impaired perception, learning and memory. Two of the most severe and well-defined neurological effects of alcohol consumption are Korsakoff's Syndrome (KS) and Fetal Alcohol Syndrome (FAS). KS is a profound memory disorder, in which the individual loses short-term memory capabilities. FAS, which occurs in some pregnant women who consume alcohol, is the leading environmental cause of mental retardation in the western world and results in 4,000-12,000 babies born every year with physical and intellectual disabilities (11,12).

4. ALCOHOL PHARMACOKINETICS AND ALCOHOL METABOLISM

Alcohol is a small, uncharged molecule and is absorbed from the entire gastrointestinal tract (GI) through passive diffusion. Approximately 20% of an alcohol dose is absorbed directly from the stomach and the remaining 80% is absorbed rapidly and completely from the upper portions of the small intestine (primarily at the level of the duodenum). The rate of absorption is dependent on a number of factors including: volume ingested, type and concentration of the beverage ingested, the rate of alcohol ingestion, the presence and type of food in the stomach, gastric and hepatic metabolism, GI motility, the coexistence of GI disease, co-ingestion of drugs, and individual variation (8,13).

Once alcohol is absorbed, it distributes freely in body tissues and fluids in direct proportion to the blood alcohol concentration. Approximately 95% of the alcohol is metabolized before it is excreted and the remaining 5% is excreted unchanged, primarily by the kidneys into the urine and also through the lungs (8).

4.1. Gender differences in pharmacokinetics of alcohol

When men and women of the same weight consume the same amount of alcohol, women achieve higher blood alcohol levels. Women have a decreased volume of ethanol distribution compared to men because they have a lower proportion of total body water to fat. Women also have reduced gastric alcohol dehydrogenase enzyme activity and therefore a lower rate of "first-pass" metabolism (14,15). Interestingly, women can develop alcohol-induced liver disease after consuming lower amounts of alcohol over a shorter period of time than men. Women also have a higher mortality rate from cirrhosis than men (16,17).

4.2. Rate of metabolism

Alcohol is metabolized by naive drinkers at an average rate of 3.3-3.9 mmol/L/hr (15-18 mg/dL/hr). There is wide inter-individual variability with rates ranging from 2.2 to 4.3 mmol/L/hr (10 - 25 mg/dL/hr) (13, 18). The rate of metabolism in the liver is independent of the concentration of alcohol in the blood (zero order kinetics) and is linear with time. A 70 kg person metabolizes 7-10 g alcohol/hour (15 ± 5 mg/dL/hour). It takes 1-2 hours to metabolize a 1-ounce glass of 80-proof whiskey (40% ethanol), 4-ounces of wine, or 12-ounces of beer (19).
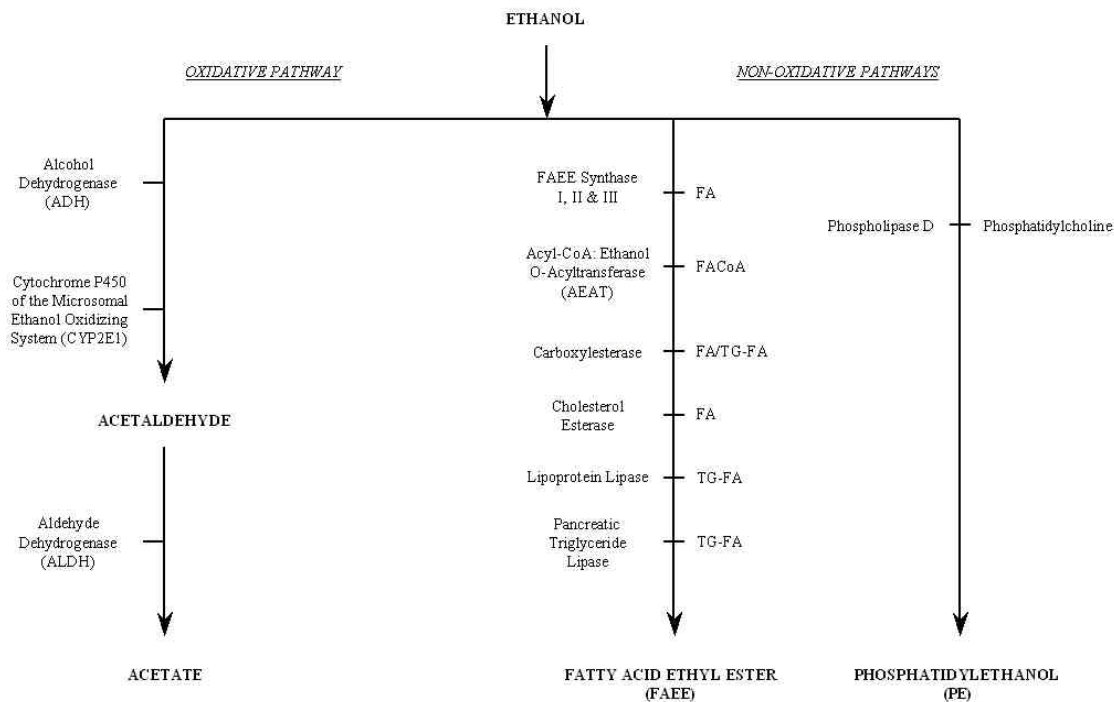
Figure 1. Oxidative and non-oxidative pathways of ethanol metabolism. Abbreviations – FA, fatty acid; FACoA, fatty acyl coenzyme A; TG, triglyceride.

4.3. Oxidative and non-oxidative alcohol metabolism

As shown in Figure 1 alcohol is metabolized by both oxidative and non-oxidative pathways. Alcohol is primarily metabolized in the liver via the two-step oxidative pathway. Alcohol dehydrogenase (ADH), the cytochrome P450 2E1 (CYP2E1) of the microsomal ethanol-oxidizing system (MEOS) and catalase can all convert alcohol to acetaldehyde. Acetaldehyde is a highly reactive compound which is converted by aldehyde dehydrogenase (ALDH) to acetate. CYP2E1 is inducible with chronic alcohol intake. It has a high K_m for ethanol (8-10 mmol/L compared to 0.2-2 mmol/L for hepatic ADH) (20). Catalase does not play a major role in human ethanol oxidation under physiological conditions (21,22).

Ethanol is metabolized non-oxidatively via at least two known pathways as depicted in Figure 1. One pathway leads to the formation of FAEE and the other to phosphatidylethanol. The first non-oxidative pathway, and the focus of this review, results in the esterification of ethanol with endogenous fatty acids or fatty acyl-CoA to form FAEE. The enzymology of FAEE synthesis is described in detail below. Two enzymes implicated as catalytic for FAEE synthesis are FAEE synthase and acyl-CoA: ethanol *O*-acyltransferase (AEAT). The other non-oxidative pathway for ethanol metabolism that leads to the synthesis of phosphatidylethanol requires the action of phospholipase D. Ethanol is inserted as a head group in place of choline on phosphatidylcholine to form phosphatidylethanol (23).

The oxidative and non-oxidative pathways are linked. Recently it was shown both *in vitro* and *in vivo* that

inhibition of ethanol oxidation by inhibitors of ADH, cytochrome P450 and catalase results in an increase in non-oxidative metabolism of ethanol and leads to an increased production of FAEE in the liver and pancreas (24).

Unlike FAEE, acetaldehyde is not in significant concentrations in the pancreas, heart or brain, and only negligible amounts are detected in the circulation following ethanol intake (25,26). This suggests that the pathological consequences of ethanol abuse in these organs are not due to oxidative metabolism of ethanol. Instead they may be attributed to either ethanol itself or to the non-oxidative products of ethanol metabolism. To explain some of the existing disparities, it has been hypothesized that FAEE are at least partly responsible for the observed organ damage in alcoholics (27,28,29).

5. FAEE SYNTHESIS AND DEGRADATION

Following ethanol intake, FAEE concentrations vary extensively in different organs, tissues and cells because of differences in fatty acid substrate availability, enzymatic capacity for synthesis and degradation of FAEE and the availability of carriers to transport FAEE. See Figure 2.

5.1. Enzymes associated with fatty acid ethyl ester synthesis

Since the 1965 report by Newsome and Rattray on the enzymatic esterification of ethanol with fatty acids by "porcine pancreatin", numerous FAEE synthases have been purified from several tissues. A number of enzymes which catalyze well known reactions have also been shown to promote FAEE synthesis. The relative physiologic
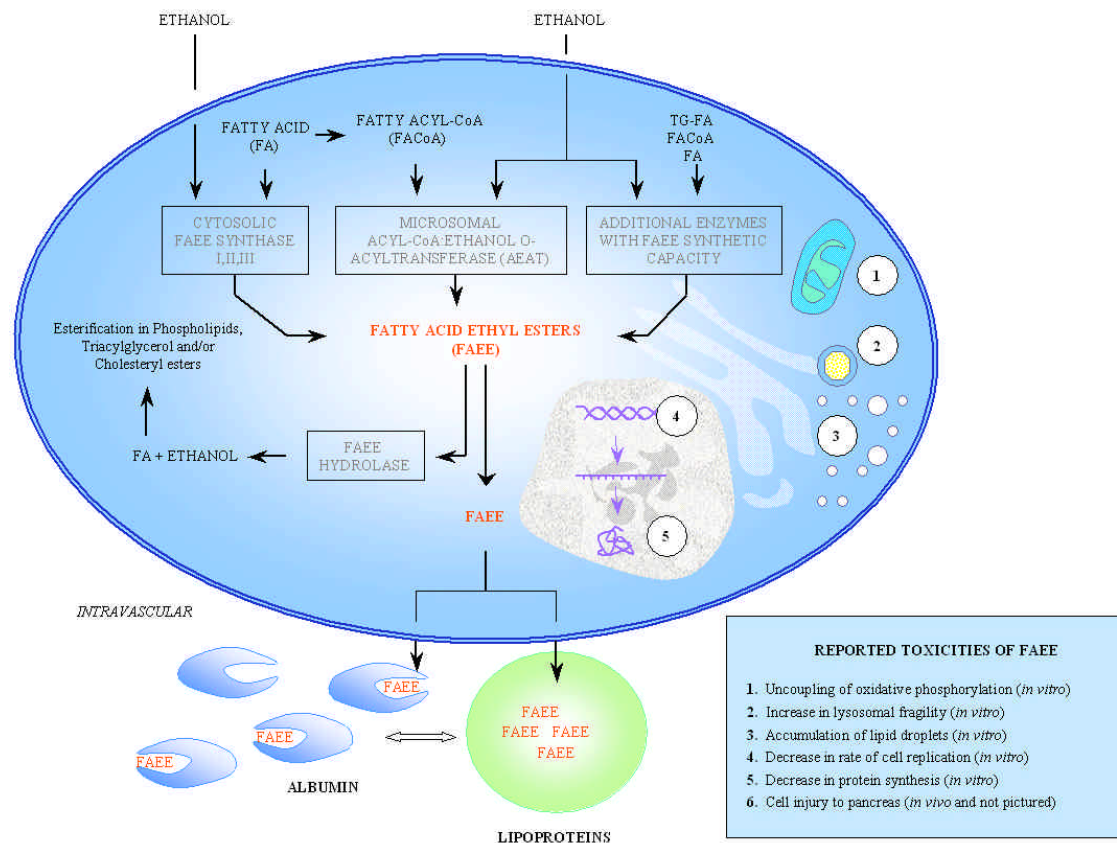
Figure 2. Current understanding of the synthesis, degradation and toxicities of fatty acid ethyl esters. Abbreviations: FA, fatty acid; FACoA, fatty acyl coenzyme A; FAEE, fatty acid ethyl esters; TG, triglyceride.

importance of these enzymes in FAEE synthesis *in vivo* is unknown (30,31,32,33,34).

Mogelson purified cytosolic FAEE synthase from rabbit myocardium in 1984. The results of his study suggested that the active FAEE synthase enzyme has a MW of 50,000 Da, and that it is a soluble dimeric enzyme composed of two identical or nearly identical subunits (31). Two forms of FAEE synthase were identified in postmortem human brain homogenates in 1987. The investigators found FAEE synthase activity in 10 different anatomic locations of the brain, with gray-matter sites containing approximately twice the activity of white-matter sites (32). In 1989, three FAEE synthases were purified from human myocardium, and were named synthase I, II, and III, in accordance with their elution order from a DEAE-column. Synthase I is a dimer of identical subunits of 26,000 Da each. Synthase II is a monomer that dimerizes readily with a molecular weight of 65,000 - 67,000 Da. Synthase III, like synthase I, is a dimer of identical subunits of 26,000 Da each (33). Lange later cloned and sequenced the cDNA of human myocardial FAEE synthase III and reported that FAEE synthase III cDNA is highly homologous to the glutathione S-transferase pi-1 cDNA from Michigan Cancer Foundation (MCF) cells. He claimed that two of the three human myocardial FAEE synthases were glutathione S-transferases (35). This finding was contested by both

Sharma *et al.* and Board *et al.* Sharma's group showed that human pancreatic glutathione S-transferase isoenzymes belonging to alpha, mu, and pi classes do not express FAEE synthase activity (36). Board's group concluded from their investigation that the cDNA of human myocardial synthase III produced by Lange's group in 1991 might have resulted from a cloning artifact and that FAEE synthase and glutathione S-transferase are distinct proteins (37). In 1990 Riley *et al.* isolated a FAEE synthase from human pancreas (38). In 1996, a clarification in terminology for the numerous FAEE synthases was advanced by Treloar *et al.* Cytosolic FAEE synthase activity which uses ethanol and free fatty acids as substrates was designated FAEE synthase, and the microsomal FAEE synthase activity, which uses ethanol and fatty acyl-CoA as substrates, was termed acyl-CoA: ethanol *O*-acyltransferase (AEAT) (34).

Dan *et al.* assessed whether all fatty acids in HepG2 cells were equally available for FAEE synthesis and found that FAEE are generated from specific intracellular pools of fatty acids, and that not all fatty acids are utilized in FAEE synthesis equally. The fatty acids most recently incorporated into the cells were preferred as substrates for FAEE synthesis (39).

5.1.1. FAEE synthase
FAEE synthase catalyses the cytosolic esterification of ethanol to free fatty acids.

$$R-\overset{\overset{\textstyle O}{||}}{C}-O^{-} + CH_3CH_2OH \rightarrow RCOCH_2CH_3 + H_2O$$

Fatty acid + ethanol → FAEE + water
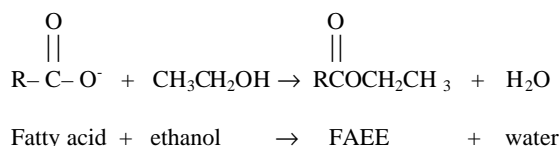
FAEE synthase is a family of enzymes present in almost all tissues with the highest level of activity reported in the pancreas (27,40). Pancreatic FAEE synthase is structurally and functionally different from hepatic FAEE synthase (40). FAEE synthase also has additional non-FAEE specific synthetic capabilities. FAEE synthase also catalyzes the production of cocaethylene, a cocaine metabolite generated in the presence of ethanol (41).

The presence of lipid binding proteins and membranes may effect FAEE production by delivering or sequestering the free fatty acid substrate and or the ethyl ester product in aqueous solution. It was reported that FAEE synthase activity was negligible in the presence of $\geq$ 8 mg/mL albumin presumably due to the binding of fatty acid substrate (40). (The normal concentration of albumin in plasma is on the order of 30-45 mg/mL). Similarly, bovine serum albumin (BSA) caused a large concentration-dependent decrease in human liver AEAT activity (42).

5.1.2. Acyl-CoA: ethanol *O*-acyltransferase (AEAT)

AEAT catalyzes the transesterification of ethanol and fatty acyl-CoA.

$$R-\overset{\overset{\textstyle O}{||}}{C}-O-ScoA + CH_3CH_2OH \rightarrow RCOCH_2CH_3 + CoASH$$

Fattyacyl-CoA + ethanol → FAEE + Coenzyme A
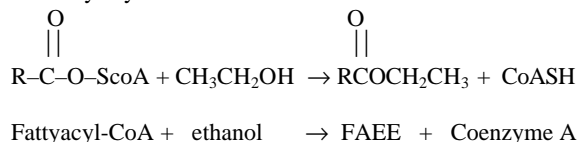
The reaction is thermodynamically favorable and involves the conversion of the activated thiol ester of the fatty acid to the oxygen ester. This reaction is similar to that which occurs in the biosynthesis of carboxylic ester bonds in triglycerides, phospholipids and cholesterol esters. Since fatty acids are rapidly esterified to fatty acyl-CoA when incorporated into cells, AEAT may be especially important in the formation of FAEE from these CoA esterified fatty acids (42).

Diczfalusy *et al.* found that AEAT activity was several-fold higher than FAEE synthase activity in a number of human organs and tissues, including heart, liver, duodenal mucosa, gastric ventricular mucosa, lung, adipose, and gall bladder. Interestingly, AEAT activity was more than 100 fold higher than FAEE synthase activity in the duodenal mucosa. Only in the pancreas were the synthetic activities of AEAT and FAEE synthase found to be comparable (43).

5.1.3. Non-FAEE specific enzymes with FAEE synthetic capacity

Enzymes other than FAEE synthase and AEAT have been reported to have FAEE synthetic capabilities. A number of enzymes, which hydrolyze fatty acids from triglycerides and phospholipids, have been shown to catalyze FAEE synthesis in the presence of circulating ethanol. The relative importance of enzymes with hydrolytic action, which makes the fatty acid substrate available for esterification with ethanol, versus esterifying enzymes which join the fatty acid or fatty acyl-CoA with ethanol, is not known. The hydrolytic enzymes known to catalyze FAEE formation include: lipoprotein lipase (LPL), cholesterol esterase, carboxylesterase (adipose and pancreatic) and carboxylester lipase (44,45,46,47,48). Tsujita and Okuda proposed that the lipase attacks the triacylglycerol forming an acyl-enzyme intermediate and that during deacylation, ethanol binds to the free fatty acid as an acceptor -forming an FAEE molecule (44). In support of this hypothesis Chang *et al.* showed that an isolated rat heart perfused with chylomicrons in the presence of ethanol synthesized FAEE as a result of LPL activity (49).

5.1.4. FAEE synthesis in the blood

Given that LPL has FAEE synthetic capabilities and that LPL is localized on the lumenal side of the capillary endothelium, it has been suggested that LPL plays a significant role in the synthesis of circulating FAEE. Chang *et al.* suggested that FAEE found in the plasma after ethanol intake derives from LPL-mediated synthesis (44,49).

The direct production of FAEE by blood cells may contribute to circulating FAEE levels. Gorski *et al.* found that FAEE synthase activity in the blood is predominantly found within white blood cells; and that the highest synthase activity among white blood cells was found in the natural killer cells. This white blood cell synthase activity was also found to be inducible in naive drinkers. Gorski *et al.* also reported that reduced FAEE synthase activity was demonstrated in white blood cells of alcoholic individuals. Even though red blood cells produce less FAEE than white blood cells on a per cell basis, red blood cells may account for a significant portion of the total FAEE synthase activity in whole blood because they are more numerous than white blood cells (by 3 orders of magnitude) (50).

Aleryani *et al.* demonstrated that patients with liver or pancreatic disease release FAEE synthase into their plasma, presumably from injured or dead cells in the liver and pancreas. The circulating FAEE synthase activity that is independent of cells may also contribute to the formation of FAEE detected in the plasma after ethanol ingestion (51).

5.2. FAEE Hydrolysis

The hydrolysis of FAEE has been investigated in a number of studies. Hungund *et al*. reported that within 5 minutes of injecting rats with FAEE in an ethanol suspension unbound to lipid carriers, more than 95% of FAEE were removed from the circulation (52). Similarly, Saghir *et al*. demonstrated that FAEE are rapidly and extensively degraded by cellular elements in the blood, with a half-life for FAEE within a low density lipoprotein (LDL) particle of only 58 seconds. They also showed that FAEE administered directly into the rat stomach were rapidly degraded to free fatty acids and ethanol in the GI tract at the level of the duodenum, with limited hydrolysis

in the stomach (53). This study was particularly important because it explained the lack of toxicity of orally ingested FAEE used in fatty acid supplements.

In a subsequent study, Saghir *et al.* demonstrated that the cellular elements of the blood, at physiologic concentrations, are all capable of hydrolyzing FAEE and that cell free plasma contains minimal FAEE hydrolytic activity. On a per cell basis, the white blood cell fraction showed the highest capacity for FAEE degradation. In this study 71% of the FAEE within an LDL particle in human blood was hydrolyzed after 2 hours. The authors conclude that degradation of FAEE in the blood is rapid and extensive and that the hydrolysis is mediated by an activity present in cells (54). Consistent with these findings, Szczepiorkowski *et al.* showed that within 3 hours more than 90% of FAEE from LDL that was incorporated into intact human hepatoblastoma cells (Hep G2) were hydrolyzed to fatty acid and ethanol (55). Laposata *et al.* reported that the FAEE hydrolytic activity of cultured Fu5AH rat hepatoma cells is organelle/membrane-bound and enriched in the microsomal and mitochondrial-lysosomal fractions (56). This study supports previous studies by Lange and Sobel that investigated myocardial cell damage by FAEE. Lange and Sobel hypothesized that the myocardial cell damage was due to the local accumulation of free fatty acids following FAEE hydrolysis (57).

Diczfalusy *et al.* compared the FAEE hydrolytic capacity of different tissues. The highest FAEE hydrolytic activity was found in the liver and pancreas, with much lower activity in gastric ventricle and duodenal mucosa. The lowest activity was found in the heart and adipose tissue. They speculated that the accumulation of FAEE in adipose tissue might be a consequence of a very low turnover of fatty acids with a low FAEE hydrolytic activity (43).

6. INTRACELLULAR FAEE TRANSPORT

Kabakibi *et al.* developed a system in which HepG2 cells incubated with ethanol synthesize and release FAEE into the culture medium. They observed FAEE synthesis within 5 minutes of the addition of ethanol. FAEE synthesis reached a plateau after 2 hours of incubation. They showed that the intracellular transport of FAEE occurs by a mechanism independent of the vesicular transport pathway. They also reported that the cytosolic FAEE are associated with a 13,000 - 15,000 Da protein, which is possibly liver fatty acid binding protein (L-FABP). L-FABP was detected in the cytosol by Western blot analysis. The authors suggested that FAEE are rapidly transported to the plasma membrane, similar to the intracellular transport of free fatty acids, by a lipid carrier protein and are then delivered onto lipoprotein and albumin molecules outside the cell (58).

7. EXTRACELLULAR FAEE TRANSPORT

In the blood FAEE are transported in the core of lipoproteins and bound to albumin. Doyle et al. found that

albumin transports the majority of FAEE in plasma, because albumin has a much greater plasma concentration than lipoprotein particles (59). Later studies showed that the distribution of FAEE among their carriers in plasma/serum is dependent on FAEE concentration. As the FAEE concentration increases, the percentage of FAEE associated with albumin decreases and the percentage associated with lipoproteins increases. This is expected, given that lipoproteins can carry several thousand FAEE molecules and an albumin molecule can transport only a few FAEE molecules. Among the lipoproteins, low density lipoprotein (LDL) and very low density lipoprotein (VLDL) bind the largest amount of FAEE (60).

Using ^{13}C-NMR spectroscopy, Bird *et al.* analyzed ethyl oleate binding to small unilamellar phospholipid vesicles (SUV), human LDL, and albumin. The ethyl oleate was found in a nonaqueous environment, most likely the core of the lipoprotein, and was also found to be soluble up to 30 mole percent of the fatty acids within the phospholipids. The authors showed that within phospholipid bilayers, FAEE align parallel to the fatty acid moieties. They reported bi-directional movement between LDL and SUV (61). Similarly Doyle *et al.* reported that FAEE, like other lipids in the core of lipoproteins, can transfer between lipid carriers (59).

Albumin has a lower affinity for FAEE compared to free fatty acids. Free fatty acids displace FAEE from albumin. (Unpublished observation, Best C.A., Szczepiorkowski Z.M., Laposata M., May 2001). The albumin and lipoprotein FAEE pool contained a high percentage of saturated fatty acids as compared to serum free fatty acid or triglyceride pools (59).

Hasaba *et al.* showed that the addition of LDL, HDL and albumin to the culture medium of HepG2 cells stimulates the release of FAEE from cells. They reported that the extracellular addition of lipoprotein particles also stimulates the overall production of FAEE by HepG2 cells in a time and dose dependent manner. However, independent of the acceptor (lipoprotein or albumin) concentration and time of incubation, the cells retain a fixed amount of FAEE (62). Similarly, Kabakibi *et al.* found that increasing concentrations of HDL in the extracellular medium was associated with an increase in the amount of FAEE outside the cells (58).

8. STUDIES INDICATIVE OF FAEE-MEDIATED CYTOTOXICITY

In 1986, an autopsy study published in *Science* reported a correlation between organs associated with alcohol-related disease and the presence of FAEE. Examination of post-mortem tissues from individuals who had been intoxicated at the time of death revealed that FAEE were most abundant in the pancreas, liver, heart and brain. Similarly, FAEE synthetic activity was highest in these organs. In chronic alcoholics, significant quantities of FAEE were also found in the adipose tissue - a potential FAEE storage site. Little or no FAEE were detected in organs not typically damaged by alcohol. In addition, a

linear relationship was discovered between blood alcohol and pancreatic FAEE concentration (27). Absent from this report was a causal relationship between FAEE and cytotoxicity or organ damage.

Szczepiorkowski, Dickersin and Laposata showed that FAEE solubilized in LDL, a physiologic particle, exert toxic effects on HepG2 cells. HepG2 cells incubated with FAEE (600 μM ethyl oleate, or 800 μM ethyl arachidonate) showed a reduction in cell proliferation by 30% and 35%, measured by [methyl-^{3}H]thymidine incorporation. Similarly, protein synthesis, determined by [^{35}S] methionine incorporation into newly synthesized proteins, was decreased by 40% in HepG2 cells incubated with LDL containing ethyl oleate (400 μM). Electron microscopy revealed significant changes in cell morphology as well, including degeneration and accumulation of intracellular debris and distortion of the nuclear membrane. These changes were not evident in control samples. Upon incorporation into the cells the FAEE delivered in LDL were rapidly hydrolyzed, and the fatty acids were reesterified into phospholipids, triglycerides, and cholesterol esters, with a preference for triglycerides. This study provided evidence that FAEE may be a causative agent in ethanol-induced liver damage (55).

Werner *et al.* established a direct causal effect between FAEE and a pancreatitis-like injury *in vivo* in rats in 1997. In this study, rats received an intra-arterial infusion of FAEE (within LDL) in physiologic concentrations (10-30 μM). The FAEE were delivered as a bolus and then by continuous infusion for 1 hour through a cannula placed in the carotid artery and advanced through the aorta to the mesenteric artery. The rats were sacrificed 3-24 hours after the FAEE infusion and biochemical markers of organ damage were measured. Control rats received LDL reconstituted with cholesterol ester. The rats that received FAEE had significantly increased pancreatic edema (measured by the wet/dry ratio of the pancreas), had threefold higher levels of pancreatic trypsinogen-activating peptide (a biochemical marker for pancreatic cell damage), and increased vacuolization of acinar cells, relative to controls (63).

An *in vitro* study conducted by Haber *et al.* demonstrated that FAEE increased rat pancreatic lysosomal fragility and thus revealed a potential mechanism for alcohol-related pancreatic injury. Lysosomal destabilization may facilitate the release of lysosomal hydrolases into the cytosol and lead to the autodigestion of pancreatic cells and zymogens (64). In 1994 Ponappa *et al.* reported that isolated rat pancreatic acinar cells incubated with ethanol generated FAEE and concomitantly experienced a 20-30% decrease in protein synthesis (65).

Bora *et al.* investigated the link between FAEE and myocardial cellular and mitochondrial damage. They reported that myocardial cells showed gross deformation and enlargement after rat myocardium was injected with ethyl oleate (66). An earlier report demonstrated an FAEE-induced concentration-dependent reduction in the mitochondrial oxidative phosphorylation (57). The authors suggested that alcohol related cardiomyopathies are caused by the accumulation and persistence of FAEE in heart tissue (67).

Hungund et al. found FAEE in brain synaptosomal plasma membranes of mice treated with ethanol by inhalation. The authors showed that FAEE directly altered membrane anisotropy. They suggested that FAEE synthesis in neuronal membranes might be involved in the disordered membrane fluidity associated with chronic ethanol ingestion (68). Gubitosi-Klug and Gross demonstrated that the exposure of rat hippocampal cells, in culture, to physiologically relevant ethyl oleate and ethyl arachidonate concentrations results in alterations in voltage-dependent potassium current kinetics. Ethanol did not produce the same effect. This may be one of the pathological effects of FAEE in alcohol-associated impairment of the central nervous system activity (69).

FAEE have been detected in human placenta. Bearer *et al.* reported that human placentae have significant FAEE synthase activity. They showed that FAEE persist in placenta for up to 7 days following maternal ethanol intake (70). Consistent with this report, Hungund and Gokhale showed that FAEE accumulate in maternal and fetal organs following alcohol administration to pregnant rats (71). FAEE have also been detected in the meconium (the newborn's first stool) (72,73). The presence of ethyl linoleate and total FAEE levels in meconium correlate with maternal self-report of ethanol intake during pregnancy (72). However, it is still unknown whether FAEE play a contributory role in the embryopathy of fetal alcohol syndrome (FAS).

9. DIAGNOSTIC IMPLICATIONS AND CLINICAL UTILITY OF FAEE

Current biological markers for acute and chronic alcohol consumption, such as carbohydrate deficient transferrin (CDT), gamma-glutamyltransferase (GGT) and mean corpuscular volume (MCV), lack the sensitivity and specificity required for clinical use in screening for alcohol use and abuse. GGT and MCV values have unacceptably low levels of specificity, while CDT has limited utility, because it has inconsistent levels of sensitivity and is significantly less sensitive in women than in men (1,23,74,75). FAEE in the blood can serve as an excellent short-term confirmatory test for ethanol intake, because they appear rapidly after ethanol ingestion and remain detectable long after ethanol is no longer detectable (76).

9.1. FAEE: short-term and long-term markers of ethanol intake

Doyle *et al.* conducted a controlled clinical trial with seven healthy subjects (four men and three women). The healthy subjects ingested a weight-adjusted amount of ethanol as a mixture of vodka and fruit juice over a 90 minute period, at a fixed rate. The subjects all reached a peak blood alcohol level of more than 21.7 mmol/L (100 mg/dL) legal intoxication. Blood was collected through a peripheral vein catheter and FAEE levels were determined
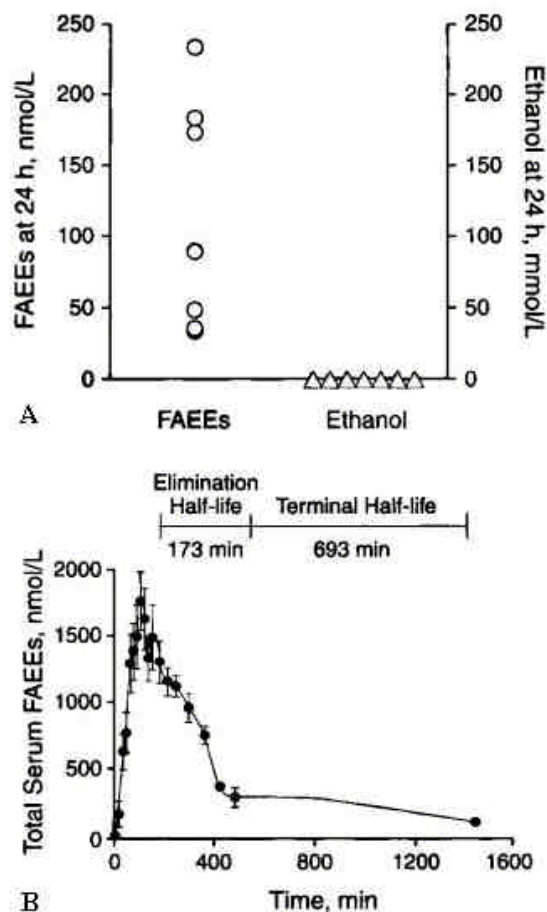
Figure 3. Panel A Serum fatty acid ethyl ester (FAEE) and ethanol levels 24 hours after the initiation of ethanol ingestion and 22.5 hours after cessation. **Panel B**. Composite time course for total serum fatty acid ethyl ester concentration over a 24-hour period. Each data point represents the mean FAEE values for seven subjects at the indicated time point; results are shown as ± SEM. Ethanol ingestion occurred during the first 1.5 hours of the time course. (Reproduced with permission, JAMA 276,1152-1156, 1996)

frequently over a 24 hour time period. See Figure 3. Blood alcohol concentrations and FAEE levels followed very similar decay curves, except FAEE were shown to have a very slow secondary elimination phase. FAEE persisted in the blood for at least 24 hours when alcohol was no longer detectable. Also in this report was a blinded comparison of 48 samples which were positive, negative, or equivocal for blood ethanol. All 20 samples positive for alcohol were FAEE positive; seven of seven equivocal for alcohol and classified as negative because the amount of ethanol was too low for accurate ethanol quantitation, were positive for FAEE; and 21 of 21 negative samples for alcohol were negative for FAEE (77).

9.2. Gender differences in serum FAEE levels

Recently, Soderberg *et al.* investigated FAEE serum levels in men versus women (78). The peak level of FAEE was twofold higher in men than women, and the authors suggested that the observed difference may be due to differences in the activity of enzymes responsible for FAEE synthesis or enzymes catalyzing FAEE hydrolysis in men and women (78).

9.3. Postmortem FAEE detection and assessment of premortem ethanol intake

Alcohol related deaths may occur up to six times as often as reported on death certificates because it is often difficult or impossible to document premortem ethanol intake (79). Blood is not always attainable for postmortem assessment of ethanol intake before death. The peripheral blood may be coagulated by the time the autopsy is performed. Another potential problem in forensic cases is that the blood alcohol level may be artifactually elevated when extensive decomposition or putrefication has occurred, because ethanol is generated by bacteria. Urine and/or vitreous humor ethanol measurements can be used. However both are also associated with a number of confounding variables (80).

It has been suggested that FAEE analysis may be useful for forensic applications because adipose tissue samples are readily obtainable and FAEE have a long half-life in adipose tissue (approximately 16.5 hours) (81). Refaai *et al.* assessed the diagnostic value of FAEE concentrations and speciation in solid organs and tissues as markers of premortem ethanol intake (82). These investigators evaluated 31 cases from the Massachusetts Medical Examiner's Office and the Department of Pathology at the Massachusetts General Hospital. Postmortem intervals ranged from 5-29 hours (mean of 16 hours). In all cases blood ethanol concentrations were determined at autopsy. The presence of FAEE in liver and adipose correlated in all cases but one with the presence of ethanol in the blood. They concluded that the mass of FAEE in liver and adipose tissue can serve as postmortem markers of premortem ethanol intake when blood samples cannot be obtained (82).

9.4. FAEE speciation: Potential use in differentiating between chronic and social drinking patterns

Compared to binge drinkers, chronic alcoholics have higher serum concentrations of ethyl oleate (83). This is consistent with the observation that oleic acid is the predominant fatty acid found in the plasma of chronic alcoholics (84). Ethyl oleate analysis may help in the distinction between binge drinkers and alcoholics. The ability to differentiate between binge drinkers and alcoholics is important because the clinical management of these two groups of patients is significantly different. A chronic alcoholic who presents with an elevated blood ethanol concentration would be monitored for withdrawal associated complications and would be advised to enter an alcohol detoxification program. A binge drinker with an elevated blood ethanol concentration may require more immediate and critical treatment measures, such as dialysis. Binge drinkers have greater mortality and morbidity from very high alcohol concentrations, and alcoholics have a higher withdrawal associated mortality risk. Thus, FAEE measurement may be clinically useful in the emergency

room setting by providing a tool to choose appropriate case management protocols (83).

9.5. FAEE: A biomarker for prenatal ethanol exposure

Drinking during pregnancy can result in a wide spectrum of effects termed fetal alcohol spectrum disorders (FASD). The term describes the full range of birth defects attributable to in utero alcohol damage, from subtle effects on a number of behavioral, educational and psychological tests to severe defects including fetal alcohol syndrome (FAS) and alcohol-related birth defects (ARBD) (85). FAEE have been detected in meconium suggesting that in utero exposure to alcohol can be assessed. Clinically this is important because identifying alcohol-exposed neonates is difficult, and children born with FASD are in need of interventions and supportive care that can reduce their cognitive and behavior deficits. Early identification of infants at risk for FASD can help minimize secondary disabilities- including mental health problems, school problems, legal difficulties and problems with drugs and alcohol, associated with alcohol-related birth effects (86).

9.5. FAEE analysis in treatment programs

Blood and hair monitoring of FAEE levels can be useful in assessing compliance with alcohol abstinence programs and in determining eligibility for transplant organs. Alcoholism treatment programs require abstinence. Also, transplant recipients in queue for organ donations, particularly the liver, are required to abstain from drinking alcohol in order to receive an organ donation. However, the methods to assess compliance are problematic because individuals may abstain long enough before presenting to be tested and have a blood ethanol of zero. FAEE analysis is potentially useful in these settings because FAEE persist in circulation long after alcohol is metabolized and is no longer detectable (77).

FAEE hair analysis, like the hair analyses used in the detection of illegal and medical drug abuse cases, may be useful in the retrospective detection of alcohol consumption (87). Hair testing is non-invasive, and potentially provides a long-term record of substance intake (provided that the hair is long). Pragst *et al.* developed a method for the analysis of hair FAEE that is based on the extraction of the hair sample by a dimethylsulphoxide (DMSO) / n-hexane mixture, separation and evaporation of the n-hexane phase and application of headspace solid phase microextraction in combination with gas chromatography-mass spectrometry (SPME-GC/MS) (88). The investigators reported finding FAEE in hair samples of alcoholics. One limitation to FAEE hair testing is that low FAEE concentrations were also detected in hair taken from teetotalers, possibly as a result of sample contamination by ethanol containing hair care products (89).

10. FAEE METHODOLOGY

Laposata *et al.* developed a method for FAEE purification, detection and quantitation. Briefly a quantitative internal standard of ethyl heptadecanoate (E17:0) is added to each sample as a recovery marker and the lipids are extracted with acetone/hexane (2/8, v/v). The

FAEE are then isolated by solid phase extraction using Bond Elut-LRC aminopropyl columns. Finally, the FAEE are separated, identified and quantitated by gas chromatography-mass spectrometry (GC-MS) GC separation of various FAEE is based upon the chain length as well as the degree of unsaturation (77,90,91,92).

10.1. FAEE Isolation and quantitation

Extraction of lipids is initiated by the addition of 2 mL of acetone to 0.5 mL of sample, followed by the addition of 50 µL (1 nmol) of E17:0. After vortex mixing for 1 minute, the samples are centrifuged at 650 x g for 5 minutes at $4°C$. The acetone layer is transferred to a fresh 15 ml conical glass tube, and 6 ml of hexane is then added to the tube, which is vortexed again for 1 minute and centrifuged at 100 x g for 5 minutes at $4°C$. The supernatant is aspirated and saved in a separate tube. The remaining lower phase is washed with 2 mL of hexane, mixed for 1 minute, and centrifuged at 100 x g for 5 minutes at $4°C$. The supernatant is removed and pooled with the saved supernatant. The hexane extract is evaporated to dryness under nitrogen, resuspended in 200 µL of hexane and then applied to a conditioned aminopropyl - silica column (Bond-Elut LCR, Varian Diagnostics) (77,91,92).

10.2. Solid phase extraction (SPE)

The SPE procedure for FAEE purification employed is a method modified from that described by Kalunzny *et al.* (90). The aminopropyl - silica columns are placed on a Vac-Elut vacuum apparatus set at 10 kPa. The Bond-Elut column is first conditioned with 4 mL of dichloromethane followed by 4 mL of hexane. Immediately after the solvent reservoir is empty, 200µL of the sample is applied to the column followed by 4 mL of hexane and an additional 4 mL of dichloromethane. The hexane and dichloromethane fractions are then combined, evaporated under nitrogen, and resuspended in a small amount of hexane for GC-MS analysis (91).

10.3. FAEE identification and quantification by gas chromatography-mass spectrometry (GC-MS)

GC-MS analysis is performed on a Hewlett-Packard 5890 gas chromatograph coupled to a Hewlett-Packard 5971 mass spectrometer equipped with a Supelcowax 10 capillary column. The oven temperature is maintained at $150°C$ for 2 minutes, ramped at $10°C$/min to $160°C$, ramped again at $2°C$/min to $180°C$ and held for 7 minutes, and then is finally ramped at $15°C$/min to $230°C$, where it is held for 21 minutes. The injector and mass spectrometer are maintained at $260°C$ and $280°C$, respectively. Carrier gas flow rate is maintained at a constant 0.8 mL/min throughout. Total ion chromatograms are generated using an ionization energy of 70 eV. Single ion monitoring is performed, quantifying appropriate base ions for individual FAEE species (i.e., ions 67, 88, and 101 for ethyl palmitate (E16:0), ethyl heptadecanoate (E17:0), ethyl stearate (E18:0), ethyl oleate (E18:1) and ethyl linoleate (E18:2); and ions 79 and 91 for ethyl arachidonate (E20:4), ethyl eicosapentaenoate (E20:5) and ethyl docosahexaenoate (E22:6)). See Figure 4. FAEE quantification is determined
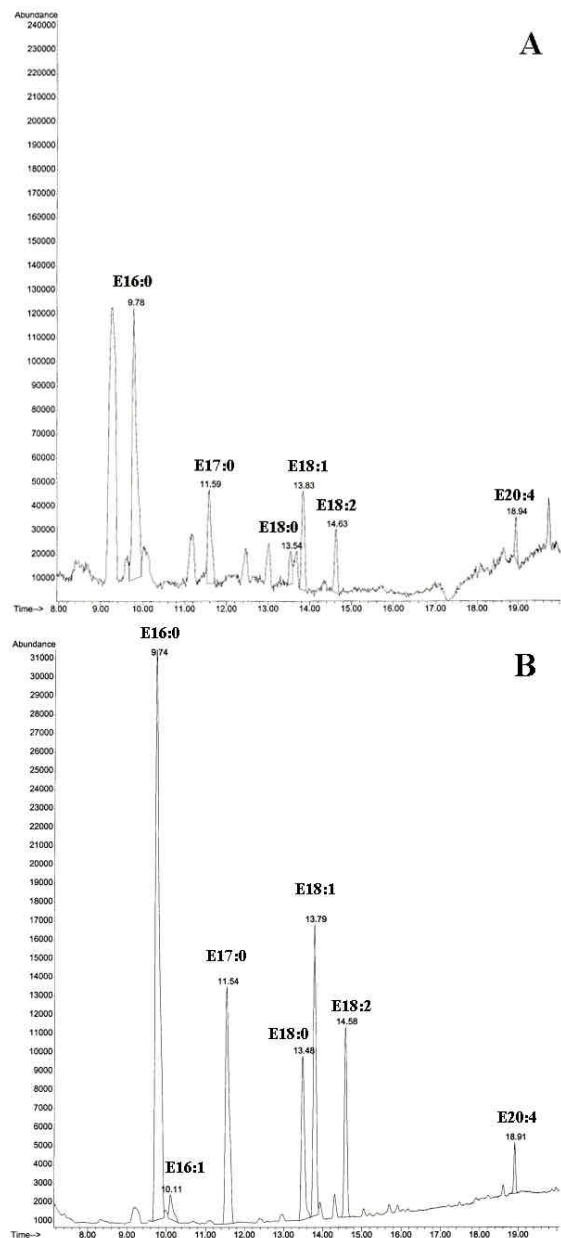
Figure 4. Detection and quantitation of fatty acid ethyl esters in serum by single ion monitoring. Gas chromatographic analysis of the ester fraction of lipids extracted from a representative ethanol positive serum sample. **Panel A** shows a scan of all ions (50-500 AMU) and **Panel B** shows a scan monitoring single ions of the identical sample. Base ions 67, 88 and 101 are selected for ethyl palmitate (E16:0), ethyl palmitoleate (E16:1), ethyl heptadecanoate (E17:0-the internal standard), ethyl stearate (E18:0), ethyl oleate (E18:1), ethyl linoleate (18:2); and ions 79 and 91 are selected for ethyl arachidonate (E20:4). E16:0, E16:1, E17:0, E18:0, E18:1, E18:2 and E20:4 peaks are shown. Detailed operating conditions of gas chromatographic analysis are described in the FAEE methodology section.

by interpolation of the slope generated from individually prepared standard curves comparing areas of varying concentration of E16:0-E22:6 to fixed concentrations of internal standard (E17:0). Mass relationships are obtained for each FAEE species using its individual standard curve. Total FAEE mass is determined by addition of the masses of the individual FAEE (E16:0-E22:6) (92, 93).

10.3.1 Identification of FAEE by single ion monitoring (SIM)

Figure 4A shows a total ion scan generated by gas chromatography-mass spectrometry (GC-MS) of lipids extracted and purified from an ethanol positive serum sample. The lipids were extracted with acetone and hexane after the addition of E17:0 - an internal standard. The extracts were evaporated under nitrogen, purified by solid phase extraction, and analyzed by GC-MS. Shown are relative peak intensities versus the mass/charge ratio. Total ion monitoring permits the identification of FAEE peaks by matching the electron impact spectra of the samples compounds with those of standards in the mass spectra library (92,93). Figure 4B shows a scan monitoring single ions of the identical sample (ions 67, 88, and 101 for E16:0, E17:0, E18:0, E18:1 and E18:2; and ions 79 and 91 for E20:4, E20:5 and E22:6). The prominent FAEE are comparable in amount to their corresponding fatty acids in serum, with relatively large amounts of ethyl palmitate, ethyl oleate and ethyl stearate. Single ion monitoring (SIM) allows greater resolution and sensitivity in the detection of FAEE by reducing baseline noise. SIM is especially advantageous when working with small sample amounts. Identification of FAEE is dependent on retention time, and upon the proportions/ distribution of the selected ions.

The electron impact spectra for the two most abundant FAEE in serum, ethyl palmitate and ethyl oleate, are shown in Figure 5, panels A and B, respectively. The major ions from each of the FAEE peaks (88 and 101) are characteristic of ethyl esters and is the basis for selecting these ions for SIM. The molecular ions from each of these peaks in the tracing reflect the exact molecular weight of the FAEE $--M^+ = 284$ for E16:0 and $M^+ = 310$ for E18:1.

The fatty acid <u>ethyl</u> ester mass spectrum resembles the fatty acid <u>methyl</u> ester (FAME) spectrum, except that the most abundant ion is at m/z = 88 for FAEE instead of 74 for FAME. Figure 5 panels C and D show the mass spectrum for methyl palmitate (M16:0) and methyl oleate (M18:1). The representative mass spectra show the characteristic fragmentation pattern of M-31 and the McLafferty ion, M/Z 74 for FAME (95).

10.4. Recent technique modifications

The effects of type of collection tube, storage time, and storage temperature on FAEE concentrations in blood have been investigated. Samples should not be collected in ethylenediaminetetra-acetic acid (EDTA)-containing vacuum tubes, as EDTA lowers the FAEE concentration recovered. It is necessary to freeze plasma or serum at -4ºC or -80ºC if more than 4 hours is required before FAEE analysis (96). It is also necessary to remove
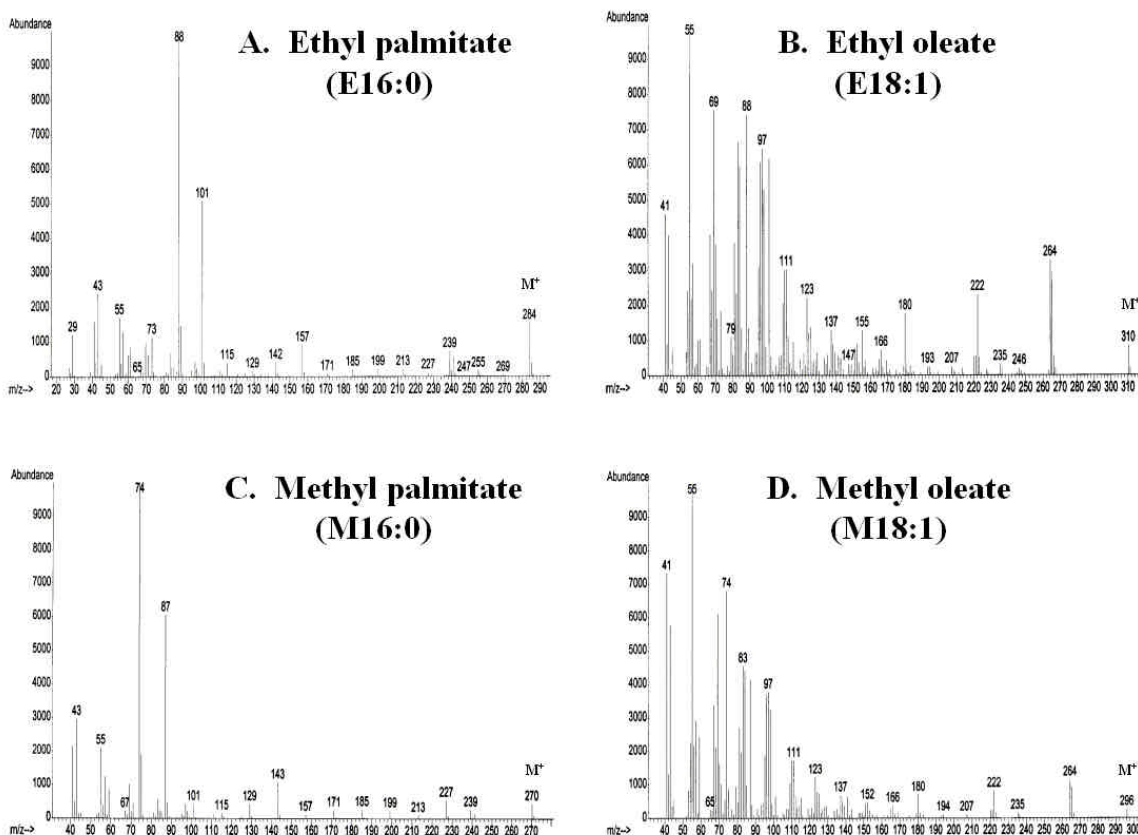
Figure 5. The electron impact spectra generated by GC-MS for selected fatty acid ethyl esters and the corresponding methyl esters. **Panel A**, ethyl palmitate (E16:0); **Panel B**, ethyl oleate (E18:1); **Panel C**, methyl palmitate (M16:0); and **Panel D**, methyl oleate (M18:1).

the plasma or serum from cells within 4 hours from collection. If plasma or serum is left on cells at room temperature, FAEE are artifactually generated (78). The sensitivity of the FAEE quantitation assay has been improved by reducing the sample volume size from 1.0 mL to 0.5 mL and increasing the relative amount of solvent used in the first extraction step (92).

11. NATURAL OCCURRENCE OF FAEE

Natural FAEE production in microorganisms and insects has been documented. Ethyl esters have been extracted from *Rhizopus arrhizus*, and found in the scents/sprays of bees and beetles where they likely function as pheromones (97,98,99,100). Different FAEE have been identified as components of the volatile territory-marking scents produced by *Bombus terrestris* and *B. lucorum* (99). The predominant FAEE of the "assembling scent" of the beetle *Trogoderma garnarium* acts as a repellent for *Tribolium castaneum* (100).

12. FAEE IN FOODS, BEVERAGES AND COSMETICS

FAEE are added to various commercial foods, beverages, hair care products, and lotions because they provide flavor, fragrance and foam-like qualities. Also

FAEE can be generated during alcoholic beverage production as a result of the FAEE synthase activity in yeast and because fatty acids are present within microorganisms and ethanol is generated during the fermentation process. Goss *et al.* showed that FAEE are present in commercially available scotch whiskies and are produced during the preparation of scotch (101). There was a wide range in total FAEE concentration among different brands of scotch. The largest amount was detected in Glen Livet scotch, and the lowest level of FAEE was detected in White Label scotch. The investigators also noted that there was a strong association between yeast concentration and FAEE synthesis. Increasing concentrations of yeast was associated with increasing levels of FAEE synthase activity (101). FAEE have also been detected in wine, but have not been detected in beer, gin, vodka or bourbon whiskey (101,102).

12.1. FAEE utilization in fatty acid supplements

The widespread health benefits of n-3 fatty acids in fish oil have been described, particularly in the prevention and treatment of cardiovascular disease, in inflammatory diseases such as arthritis, and in a number of psychiatric disorders including depression. FAEE capsules were introduced because oral preparations of FAEE permits

the use of a higher concentration of n-3 fatty acids relative to other fatty acids when compared to triglyceride supplements (103). Also in contrast to free fatty acids, there have been no reports of acute toxic effects associated with oral FAEE intake, presumably because the FAEE are extensively degraded in the gastrointestinal tract and the liberated fatty acids are then esterified in phospholipids, triglycerides and cholesterol esters as supported by the studies by Saghir *et al.* (53).

13. PERSPECTIVES: EMERGING BIOLOGICAL AND CLINICAL SIGNIFICANCE OF FAEE

The first compelling connection between FAEE and alcohol abuse was noted in the autopsy study published in Science in 1986 (27). The organs commonly damaged by ethanol abuse were found to have the highest levels of FAEE synthase activity and the highest concentrations of FAEE among many different tissues tested. This report sparked numerous FAEE cytotoxicity studies. FAEE were shown to uncouple oxidation in purified mitochondria (67), to increase the fragility of isolated pancreatic lysosomes (64) and to decrease the rate of liver cell replication and protein synthesis (55). See Figure 2. Physiologically relevant FAEE concentrations were also shown to effect human neuronal potassium channel activation (69). More recently, an *in vivo* study showed that FAEE produce a pancreatitis-like injury in rats and the study provided direct evidence that FAEE produce organ-specific toxicity (63). Increasing evidence continues to emerge that FAEE contribute to ethanol-induced organ damage and a mechanistic explanation for FAEE induced cell injury is on the horizon.

The significance of FAEE as markers of ethanol intake has only recently been recognized. A clinical trial in 1996 demonstrated that serum FAEE concentrations closely parallel blood ethanol concentrations, and that FAEE persist for at least 24 hours after ethanol intake (long after alcohol is no longer detectable) (77). See Figure 3. Since then a number of clinical settings have been identified that could benefit from monitoring ethanol use by FAEE assessment. They include measuring meconium FAEE levels to assess prenatal alcohol exposure (73) and postmortem hair and adipose FAEE quantitation to assess premortem ethanol intake (82). The years ahead could bring a robust clinical assay for detecting ethanol intake in a number of different settings.

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