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Fatty Acid Ethyl Ester Synthesis in the Preparation of Scotch Whiskey

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GOSS, K. A., R. ALHARETHI AND M. LAPOSATA. *Fatty acid ethyl ester synthesis in the preparation of scotch whiskey.* ALCOHOL **17**(3) 241–245, 1999.—Fatty acid ethyl esters (FAEE), nonoxidative ethanol metabolites present in human organs commonly damaged by ethanol abuse, have been implicated as mediators of organ damage. FAEE are additives in various foods and beverages to provide flavor or fragrance, and therefore are common dietary lipid constituents. We hypothesized that FAEE could be generated during alcoholic beverage production because fatty acids are present within microorganisms and ethanol is generated during the fermentation process. In this report, we demonstrate that FAEE are present in commercially available scotch beverages, and that in the preparation of scotch, FAEE can be produced during the fermentation reaction as a result of FAEE synthase activity in the yeast. Following ingestion of scotch, preformed FAEE are delivered to GI tract. The consequences of ingestion of FAEE in scotch, if any, remain to be determined.

Ethanol Lipids Fatty acids Alcoholism Ethanol abuse Addiction

FATTY acid ethyl esters (FAEE) are esterification products of fatty acids and ethanol. A 1986 autopsy study (5) involving organs from subjects who were acutely intoxicated at the time of death showed that FAEE and the enzymes responsible for their synthesis are found selectively in organs injured by ethanol abuse, implicating FAEE as mediators of ethanol-induced organ damage. It has been demonstrated that FAEE packaged within a core of low-density lipoprotein particles (1,2) are toxic for intact hepatoblastoma cells in vitro (9) and for the pancreas in vivo in rats following intra-arterial infusion (11). These studies have raised concerns about the potential toxicity of orally ingested FAEE.

It has become popular to use FAEE, rather than triglycerides, as fatty acid supplements for patients. FAEE supplements can be used to deliver a concentrated form of one specific fatty acid, such as an n-3 fatty acid, as a treatment for certain inflammatory, thrombotic, or hyperlipidemic disorders (7). FAEE are also commonly added to foods and beverages as flavors or fragrances (3). We have recently shown that FAEE are rapidly hydrolyzed in the gastrointestinal tract at the level of the duodenum, but that most FAEE in the stomach remain intact (8). From this study, it was speculated that orally ingested FAEE may enter the circulation from absorption through the stomach wall and present a source of potential toxic FAEE into the circulation.

We hypothesized that FAEE could be generated during alcoholic beverage production because fatty acids are present within microorganisms and ethanol is generated during the fermentation process. In this report, we demonstrate that FAEE are present in commercially available scotch beverages, and that in the preparation of scotch, FAEE can be produced during the fermentation reaction as a result of FAEE synthase activity in the yeast used to prepare the scotch. With ingestion of scotch, preformed FAEE are delivered into GI tract. The toxicity of ingested FAEE in scotch, if any, remains to be determined.

METHOD

FAEE Isolation and Quantitation

An internal standard of 25 nmol of ethyl heptadecanoate (ethyl 17:0) (Nu-Check Prep, Elysian MN) was added to each

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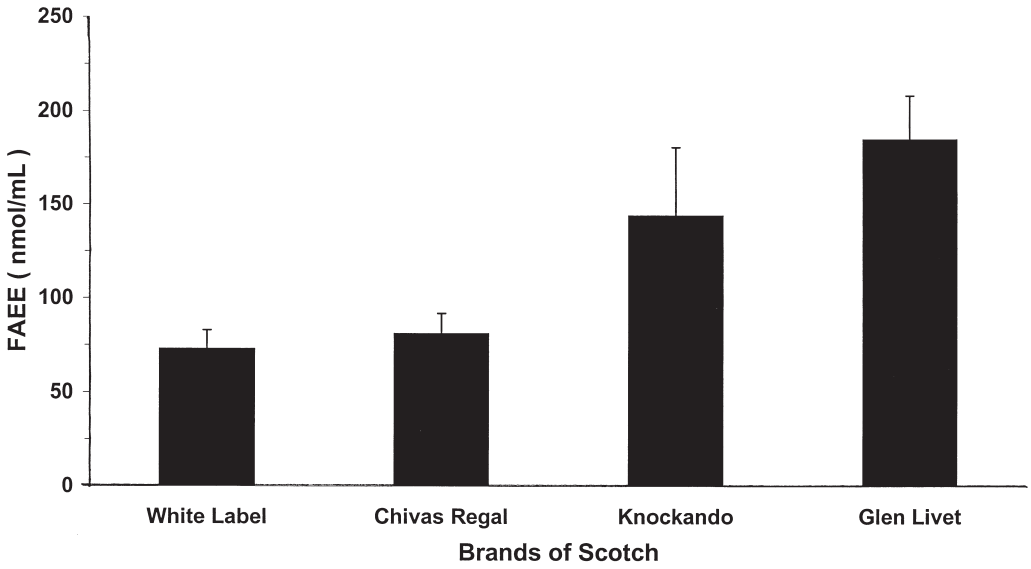

FIG. 1. Total FAEE in different brands of scotch. Error bars represent SEM from three assays.

sample. Lipids were extracted with a modified Folch method (10). The organic phase was concentrated by drying the sample completely under nitrogen gas. A 1–2-μl aliquot of the concentrated sample was injected into a Hewlett-Packard 5890 gas chromatograph with a WCOT Supelcowax capillary column (Supelco, Bellefonte, PA) coupled to a 5970 Mass Spectrometer (MS). The injector was maintained at 260 ℃, the MS detector was kept at 280 ℃, and the oven was heated from 150 ℃ to 250 ℃ increasing at a rate of 10 ℃/min, with maintenance at 250 ℃ for 6 min. Total ion chromatograms were generated using an ionization energy of 70 eV. Individual FAEE were identified by comparison to known FAEE standards (Nu-Check Prep).

Alcoholic Beverage Preparation

Alcoholic beverages were prepared in the laboratory using a 12.5% solution of dried malt extract (Munton and Fiston Co.) in water. This mixture, the "wort," was boiled for 1 h. Then 500 ml of this sterilized wort was filtered into jars and inoculated with 0.75 g of brewer's yeast (*S. cerevesiae*) (Munton and Fiston Co.), which had been reconstituted in 12.5 ml of water at 27°C for 30 min. The wort was allowed to ferment to completion at 27°C. Samples were collected at various time points and stored at −80°C until analysis. In a separate experiment, 100 ml of the fully fermented wort was distilled with a standard laboratory flask fitted with a water cooled con-

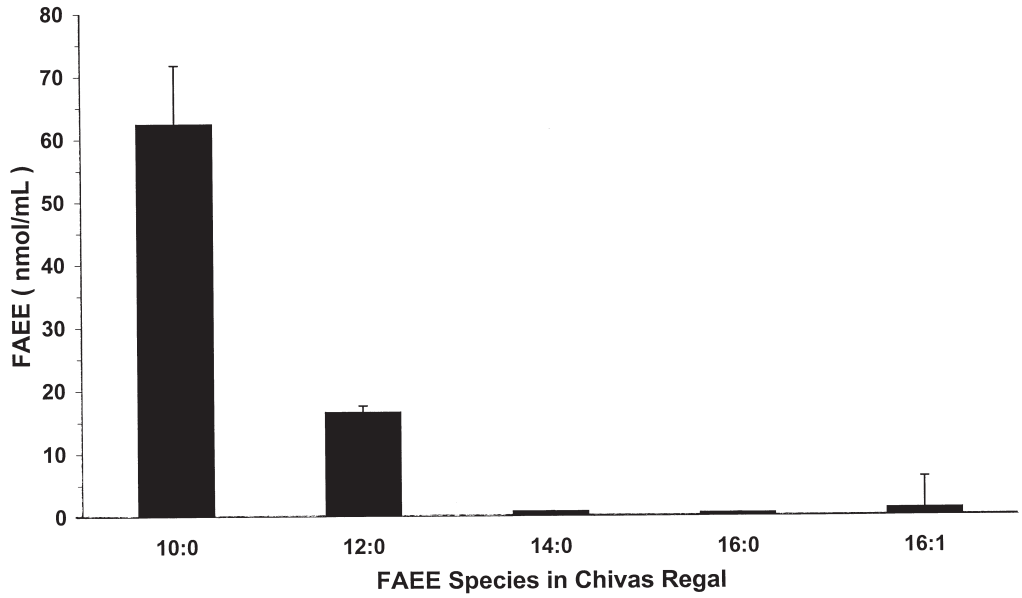

FIG. 2. FAEE species in Chivas Regal. Error bars represent SEM from three measurements.

denser to one half its volume. Three consecutive distillations were performed, and samples were collected from the condensate of each of these distillations.

Ethanol Quantitation

Alcoholic beverage samples (scotch, vermouth, white and red wine, beer, gin, vodka, and bourbon whiskey) were diluted 1:10 with a solution containing 1-propanol as an internal standard. Then 1 μl of this dilution was injected into a Hewlett-Packard 5890 gas chromatograph fitted with a packed column of 60/80 carbopack B/5% Carbowax 20m (Supelco, Bellefonte, PA) and a flame ionization detector. The injector and detector were maintained at 200°C. Ethanol quantitation was accomplished using peak area ratios to the internal standard (6).

FAEE Synthase Enzyme Assay

Briefly, the FAEE synthase assay was performed as described by Gorski et al. (4). [1-^{14}C]Oleate (0.1 μmol, 56 μCi/μmol) was dried to completion under nitrogen. Then 1.4 μmol (5.8 μl) of ethanol was added to each tube with 88.6 μl of water reconstituted yeast of different protein concentrations, bringing the total volume to 100 μl. After a 1-h incubation, a recovery marker of [9,10-^{3}H]ethyl oleate was added, and the sample was extracted using a modified Folch method (10). FAEE were isolated from the organic phase by thin-layer chromatography (TLC) using a petroleum ether/diethyl ether (75/5, v/v) solvent system and silica gel 60 plates (E. Merck, Darmstadt, Germany). The silica gel was scraped into scintillation vials and counted for the ^{14}C and ^{3}H using a Beckman LS5000 TD scintillation counter.

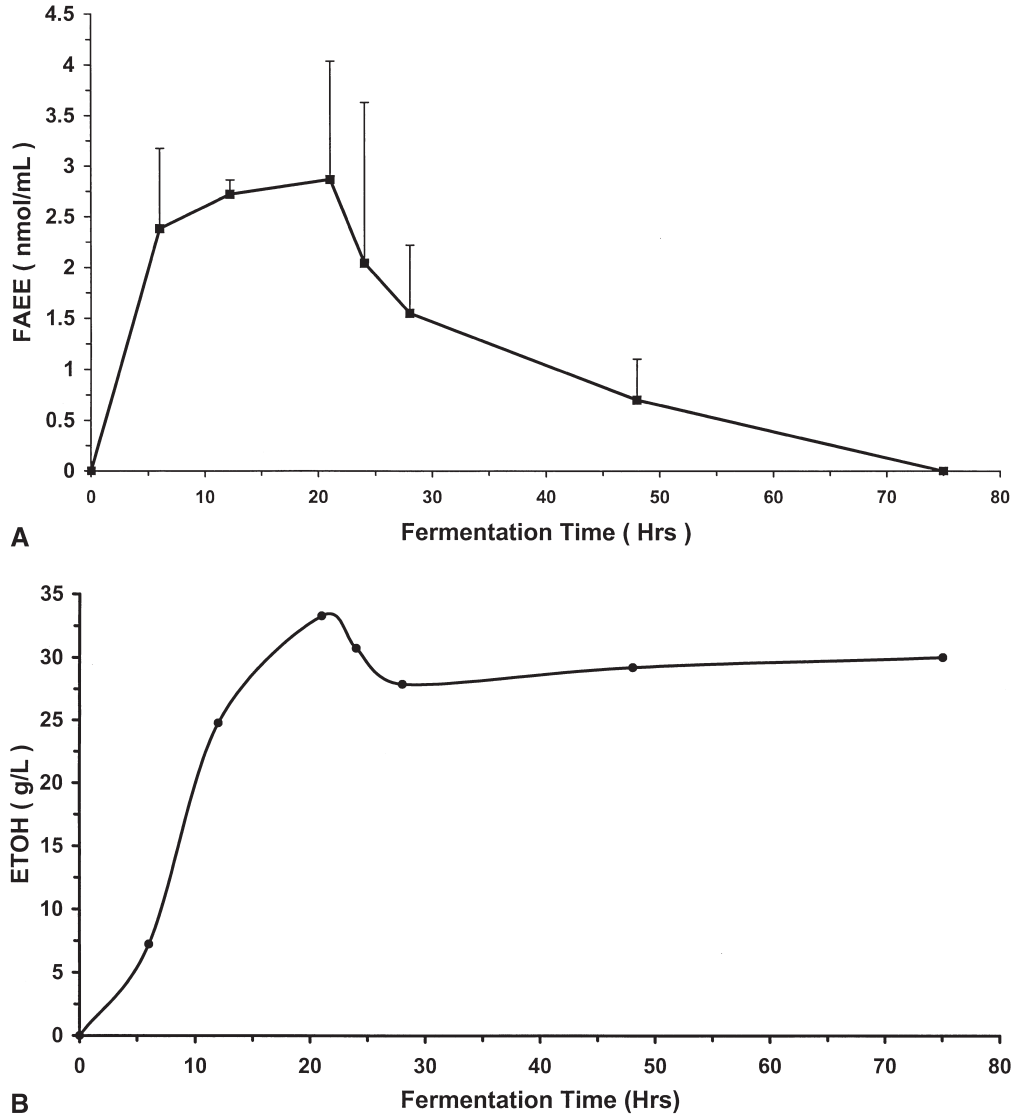

FIG. 3. (A) Time course for FAEE formation in the preparation of the Scotch Whiskey. Error bars represent triplicate SEM from three assays. (B) Time course for ethanol generation in the preparation of scotch whiskey.

RESULTS

Figure 1 shows the total FAEE present in four different brands of commercially available scotch whiskey. There was a significant range in concentrations among the four brands tested, with the largest amount present in Glen Livet scotch. Figure 2 shows the composition of the FAEE detected in our system using Chivas Regal scotch whiskey. The predominant FAEE species detected was ethyl 10:0 with substantial amounts of ethyl 12:0 and trace amounts of 14:0, 16:0, and 16:1. It is likely that the concentration of FAEE in these and other scotch beverages is somewhat higher, as ethyl ester species with less than 14 carbons are volatile and may have been lost in processing steps prior to GC-MS quantitation of FAEE. The identification of the peaks in the GC-MS chromatogram as FAEE was conclusively established by matching the electron impact spectra of the peaks in the chromatogram with the spectra of authentic ethyl esters within the mass spectrometry library. Figure 3 illustrates the time course for FAEE formation during the preparation of scotch whiskey in the laboratory. The FAEE concentration in the fermentation mixture (Fig. 3A) increased as the ethanol was generated (Fig. 3B) during the fermentation process. The decrease in ethyl ester concentration represents degradation of the FAEE, most likely enzymatically mediated, over time in the fermentation procedure. The data in Fig. 4 show that the yeast contain substantial quantities of an enzyme that esterifies free fatty acids and ethanol. There was a strong association between increasing concentrations of the yeast used in the preparation of scotch whiskey and FAEE synthesis in a standard FAEE synthase enzyme assay.

DISCUSSION

The results of these studies indicate that: 1) FAEE are present commercially available Scotch beverages, and 2) in the preparation of the scotch, FAEE can be produced as a result of an enzyme activity in yeast that promotes the esterification of ethanol and fatty acid. The data show that this esterification reaction occurs during the fermentation process, and that with increasing time of fermentation, the FAEE are degraded. The predominant FAEE species detected in these studies was a medium chain FAEE of 10 carbon and no double bonds. There were trace amounts of FAEE with 14 or more carbons. Essentially all of the FAEE generated during this process were saturated. It has been demonstrated that FAEE with at least 16 carbons are toxic to intact hepatoblastoma cells (9) and in vivo are toxic to the pancreas following intra-arterial infusion (11). It is not known if medium chain FAEE have the same toxicity as long chain FAEE.

FAEE are not present in most alcoholic beverages tested, possibly because they are synthesized in lower amounts or they are rapidly degraded. Scotch whiskey and vermouth were positive for FAEE in our studies, but wine (red and white), beer, clear liquors (gin and vodka), and bourbon whiskey were negative for FAEE.

It has been demonstrated that FAEE are rapidly hydrolyzed in the gastrointestinal tract at the level of duodenum (8). The majority of FAEE delivered into the gastrointestinal tract in rats remain intact in the stomach and it is possible, therefore, that FAEE can enter the circulation through the stomach wall. However, this same study demonstrated that FAEE in the circulation have a half-life of only 58 s. Thus, FAEE may appear in the circulation if there is absorption of FAEE through the stomach, possibly from ingested scotch, but they should not circulate for more than a few minutes (8). Although this study does not assess the toxicity of FAEE, it does clearly demonstrate that FAEE are ingested along with ethanol in scotch. These findings would support the performance of clinical studies to determine if FAEE appear in the circulation after ingestion of scotch. Because the FAEE present in the plasma are usually 16 carbons or greater, the presence of FAEE that have 10 carbons or less could be used as markers of FAEE originating in Scotch Whiskey.

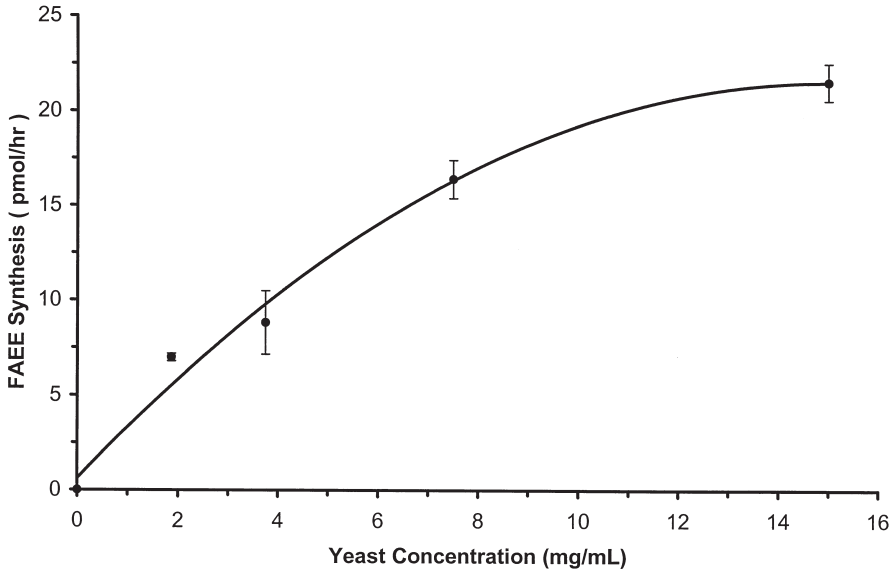

FIG. 4. The association between yeast concentration and FAEE production.

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