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Analysis of Free Fatty Acids in Beer: Comparison of Solid-Phase Extraction, Solid-Phase Microextraction, and Stir Bar Sorptive Extraction

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Solid-phase extraction (SPE), solid-phase microextraction (SPME) using carbowax/divinylbenzen fiber, and stir bar sorptive extraction (SBSE) followed by solvent back extraction have been used for the extraction of free fatty acids (caproic, caprylic, pelargonic, capric, lauric, myristic, palmitic, stearic, oleic, linoleic, and linolenic acids) from beer. Subsequent gas chromatographic analyses with flame ionization detection were used for the determination of these compounds. Medium-chain fatty acids (caproic−lauric acid) were determined as free acids, and long-chain fatty acids (myristic−linolenic acids) were determined as methyl esters after methylation by BF_3−methanol 14%. Linearity, recovery, and repeatability of all methods have been determined and compared with the SPE method used as a reference (SPME method was used only for medium-chain fatty acid determination). All three procedures provide similar working parameters characterized by high repeatability (2.3−16.3%) and good linearity (correlation coefficient ranging from 0.9919 to 0.9999). Results of beer analyses obtained by using these three methods were highly correlated. Although all methods provide compatible alternatives, for medium-chain fatty acid analysis SPME may be a more appropriate technique due to its operational simplicity, repeatability, and low cost.

KEYWORDS: Solid-phase extraction (SPE); solid-phase microextraction (SPME); stir bar sorptive extraction (SBSE); solvent back extraction; free fatty acids; beer

INTRODUCTION

Beer consists of many volatile and nonvolatile compounds that affect beer flavor, a combination of odor and taste. Many of these aroma compounds are synthesized by yeasts during fermentation. Free fatty acids represent one group of these beer flavors that affect beer taste, the vitality of yeasts, and also the foam stability of beer (*1*−*3*).

Straight-chain acids such as hexanoic, octanoic, and decanoic acids are formed during the synthesis by yeasts of long-chain fatty acids during fermentation. Their formation is influenced by a number of factors, but yeast strain, original gravity, wort composition, and degree of aeration are the most important (*4*). These medium-chain fatty acids have rancid or goaty flavor characteristics. The aroma contributions for these acids are additive. Therefore, rancid and goaty off-flavors can occur even though none of the individual acids are present above threshold concentrations. Off-flavors due to these acids normally arise from excess formation during fermentation and not other causes such as infection or raw materials (*5*, *6*).

Long-chain fatty acids such as linoleic and linolenic acids are especially of great importance because their oxidative degradation, by means of either enzymatic or free radical reactions, may lead to the formation of a characteristic aging flavor (*7*, *8*).

Due to the involvement of fatty acids in beer flavor, quick and reliable methods for analyzing of these acids are desirable.

Although HPLC methods have been used for the analysis of fatty acids in beer (*9*), gas chromatographic methods are often applied. GC procedures for analyzing these acids in beer can involve different techniques for sample preparation. Methods used in the past have been based on steam distillation (*10*, *11*) and solvent extraction with small and large volumes of solvents such as chloroform, a mixture of chloroform and ethanol, dichloromethane, hexanol, and a mixture of ethyl acetate and *n*-pentane (*12*−*16*). The difficulty with techniques such as steam distillation is that the method is time-consuming and labor-intensive. Solvent extractions first of all suffer from problems of emulsion formation.

The above-mentioned problems could be successfully solved by solid-phase extraction (SPE). C18 bonded-phase columns are often used (*17*, *18*). These procedures are quite fast, minimizing volumes of organic solvents, and lead to good recovery and high reproducibility.

Solid-phase microextraction (SPME) is an alternative to these methods. This procedure is a solvent-free sample preparation technique for the extraction of volatile and semivolatile compounds. This method, developed by Arthur and Pawliszyn in

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1990 (*19*, *20*), is used in many applications for the determination of, for example, alcohols, esters (*21*), dimethyl sulfide (*22*), vicinal diketones (*23*), carbonyl compounds (*24*), and fatty acids (*25*) in beer.

Stir bar sorptive extraction (SBSE), like the SPME method, is a solventless enrichment technique. SBSE is based on the sorption of analytes onto a thick film of polydimethylsiloxane (PDMS) coated on a stir bar (*26*). SBSE had been shown to have a much higher sensitivity than SPME due to the higher volume of PDMS phase, in which the amount of analyte extracted is proportional to the coating thickness, increasing the limit of detection during sampling (*27*).

After the enrichment step, the stir bar is usually transferred from the sample to a thermal desorption unit. The analytes are thermally desorbed and immediately delivered to a capillary column of a gas chromatograph. Sunstruck flavor (3-methyl-2-butene-1-thiol) and other sulfur compounds, esters, and stale-flavor carbonyl compounds were determined in beer by this method (*28*−*30*).

A small volume of organic solvent is another possibility for desorption of the stir bar (*31*). Utilization of solvent back extraction of the stir bar followed by gas chromatography leads to lower sensitivity than using the thermal desorption procedure, but no desorption unit is requested, and combination of SBSE and solvent back extraction can offer an effective and low-cost opportunity for the determination of compounds in no trace concentration levels. Thermal desorption also does not offer the opportunity of reanalysis, which is an important issue in many studies for validation purposes. Some beer flavors such as esters and medium-chain fatty acids have been determined in this way (*32*, *33*).

The aim of this work was to compare different extraction methods for the determination of free fatty acids in beer. The data obtained by headspace SPME technique and by SBSE with solvent back extraction were compared with SPE procedure used as a reference. The advantages and limitations of the compared methods are discussed.

EXPERIMENTAL PROCEDURES

Samples and Chemicals. Standards of analyzed compounds [hexanoic (caproic), heptanoic, octanoic (caprylic), nonanoic (pelargonic), decanoic (capric), dodecanoic (lauric), tridecanoic, tetradecanoic (myristic), pentanedecanoic, hexadecanoic (palmitic), heptadecanoic, oktadecanoic (stearic), *cis*-9-octadecenoic (oleic), *cis*-9,*cis*-12-octadecadienoic (linoleic), and *cis*,*cis*,*cis*-6,9,12-octadecatrienoic (linolenic) acids] and derivatization agent boron trifluoride (10% in methanol) were obtained from Sigma-Aldrich (St. Louis, MO) and were of > 99% purity. Analytical reagent grade ethanol was purchased from Lach-ner (Neratovice, Czech Republic); hexane, dichloromethane, chloroform, and methanol were from Merck (Darmstadt, Germany); purified water (Milli-RO 5plus) was from Millipore (Billerica, MA); and helium quality 5.0, hydrogen quality 5.0, and synthetic air were obtained from Messer (Prague, Czech Republic).

Beer samples were fresh commercial lagers of the pilsner type, produced and bottled in the Czech Republic.

SPE Analysis. As a reference method an SPE method described by Hage (*17*) was utilized with some changes. SPE columns Strata C18-E 200 mg/3 mL were purchased from Phenomenex (Torrance, CA). SPE columns were activated with 2.5 mL of methanol and washed by 5.0 mL of water. Then 20 mL of degassed beer sample with 1.0 mL of 1 M HCl and spiked with heptanoic and pentanedecanoic acids (each internal standard at a concentration of 1.3 mg/L) was applied. A volume of 5.0 mL of water was used for cleanup. The next step included drying under a mild nitrogen flush. Chloroform (2 × 0.5 mL) was used for fatty acid elution. For the determination of medium-chain fatty acids (C_6−C_{12}) 2 μL of this chloroform extract was injected to the GC column. For the determination of long-chain fatty acids (C_{14}−C_{18}) the chloroform extract was evaporated and methylated with 0.1 mL of BF_3−methanol 14% during 20 min at 95 °C. Reaction was stopped by the addition of 0.2 mL of water, and after shaking, finally fatty acid methyl esters were extracted with 0.2 mL of hexane in an ultrasonic bath for 2 min. By this procedure only free fatty acids were methylated, but not fatty acids bonded in triglycerides and phospholipids.

Analyses of both medium- and long-chain fatty acids were carried out using a Chrompack CP 9001 gas chromatograph (Middelburg, The Netherlands) with a split/splitless injector and a flame ionization detector (FID) at the same chromatographic conditions. The gas chromatograph was equipped with autosampler Labio ASG 40 (Prague, Czech Republic). Compounds of interest were separated on a 30 m × 0.32 mm i.d. fused silica capillary column of Phenomenex ZB-Wax with 0.25 μm film thickness (Torrance, CA). The GC column was maintained at 120 °C for 2 min, ramped at a rate of 10 °C/min to 150 °C, then ramped at a rate of 30 °C/min to 200 °C, and held isothermally for 15 min. The splitless mode was used, and the split vent was opened after 0.25 min. Temperatures of the injector and the FID were 250 °C. The carrier gas was helium 5.0 quality with a column head pressure of 150 kPa at 80 °C.

SPME Analysis. The procedure developed by Horák et al. (*25*) was used with minor modifications. A manual SPME device and carbowax/divinylbenzene (CW/DVB) 65 μm fibers were obtained from Supelco Co. (Bellefonte, PA).

Before use, the fiber was preconditioned in the GC injection port at 250 °C for 0.5 h. Beer sample (4 mL) with addition of heptanoic acid as internal standard (final concentration = 1.3 mg/L) and 2 g of ammonium sulfate was placed in a 20 mL vial closed by a polytetrafluoroethylene (PTFE)/silicone septum purchased from Chromacol (Herts, U.K.). Before extraction, the vial was shaken manually for 10 s. Headspace SPME was carried out at 25 °C (ambient temperature). A sampling time of 30 min was used for the analysis. Each analysis was carried out three times.

After exposure, the fiber was thermally desorbed into a GC and left in the injection port at 250 °C for 5 min. The injector was equipped with a 0.75 mm i.d. inlet liner. The same chromatographic parameters were used as in the SPE method.

SBSE Analysis. The stir bar was supplied from Gerstel GmbH (Mülheim a/d Ruhr, Germany). A 10 mm long and 3.2 mm o.d. stir bar with a 0.5 mm thickness of polydimethylsiloxane (PDMS) coating was used.

The stir bar was conditioned in a glass tube at 300 °C for 60 min by passing helium through the tube (50 mL/min). Sample extraction was performed by placing 10 mL of sample spiked with internal standards (final concentration = 1.3 mg/L of each internal standard) in a 20 mL glass vial, adding a stir bar. The vial was crimped with a PTFE/silicone septum purchased from Chromacol. The extraction procedure was based on conditions described by Horák (*33*), stirring at 1000 rpm for 60 min at room temperature. After extraction, the stir bar was removed with forceps, rinsed briefly in distilled water, and dried with a lint-free tissue. For solvent back extraction the stir bar was placed into a 350 μL glass insert containing 200 μL of the solvent mixture dichloromethane/hexane, 50:50. This insert was immersed into a 2 mL vial closed by a PTFE/silicone septum and stirred at 1000 rpm for 40 min again.

For the determination of free medium-chain fatty acids (C_6−C_{12}) 2 μL of this extract was injected into the GC column and the same chromatographic parameters were applied as in the SPE method.

For the determination of long-chain fatty acids (C_{14}−C_{18}) this SBSE extract was evaporated and methylated with 0.1 mL of BF_3−methanol 14% during 20 min at 95 °C. Reaction was stopped by the addition of 0.1 mL of water, and after shaking in an ultrasonic bath, long-chain fatty acid methyl esters were extracted with 0.1 mL of hexane in an ultrasonic bath for 2 min. GC separation was the same as described above.

RESULTS AND DISCUSSION

Due to the thermal desorption of all sorptive compounds from the SPME fiber during the determination of free medium-chain fatty acids, the long-chain fatty acids cannot be determined without another extraction by SPME method. Therefore, the SPME procedure was compared with other techniques only for medium-chain fatty acid analysis.

Table 1. Comparison of Linearity, Recovery, and Repeatability of SPE, SPME, and SBSE Methods Used for Free Fatty Acid Determination in Beer

	SPE				SPME				SBSE			
compound	r^a	recovery (%)	RSD[b] (%)	repeatability RSD (%)	r	recovery (%)	RSD (%)	repeatability RSD (%)	r	recovery (%)	RSD (%)	repeatability RSD (%)
caproic acid	0.9996	107	4.4	4.9	0.9951	110	4.8	3.7	0.9935	57	5.8	6.7
caprylic acid	0.9999	107	4.7	4.3	0.9976	103	4.3	2.3	0.9919	84	5.2	6.2
pelargonic acid	0.9999	97	6.1	8.3	0.9983	105	7.1	8.1	0.9925	85	4.9	7.6
capric acid	0.9999	98	4.4	6.0	0.9983	98	4.5	4.8	0.9928	87	4.0	4.7
lauric acid	0.9998	103	4.1	5.8	0.9979	95	5.8	5.1	0.9995	89	3.2	4.1
myristic acid	0.9993	85	9.7	16					0.9998	94	8.5	15.2
palmitic acid	0.9978	125	14.9	12					0.9991	99	9.5	14.8
stearic acid	0.9971	108	11.2	14					0.9985	110	12.0	16.3
oleic acid	0.9986	105	11.2	11					0.9984	97	10.3	15.0
linoleic acid	0.9989	98	11.3	11					0.9981	102	9.8	13.8
linolenic acid	0.9988	110	11.4	12.5					0.9987	95	12.1	15.3

[a] r, regression coefficient of the standard curve. [b] RSD, relative standard deviation.

For method comparison the following parameters have been determined: linearity, recovery, and repeatability. Results of this comparison are shown in **Table 1**. For SPE, SPME, and SBSE procedures standard solutions prepared in a 5% v/v ethanol solution have been used.

For all methods, calibration curves have been measured throughout a range of the free fatty acids covering the concentration usually found in beers of pilsner type (concentration from 0.0015 to 8 mg/L for each compound). The correlation coefficients to straight line for all determined fatty acids were best for the SPE method (> 0.997). These coefficients for SPME procedure were > 0.995, and for SBSE for all but one compound were > 0.992.

This shows that all methods are characterized by high linearity in the examined concentration ranges.

The accuracy of the methods was investigated by conducting recovery tests. The tests were performed by measuring the natural concentrations of the fatty acids in five different beers. Then the same beers were spiked with a known amount of each acid (2.0 mg/L), and concentrations of fatty acids were determined again. The results showed that recoveries of the SPE and SPME methods for medium-chain fatty acids were similar in the ranges of 97−107 and 95−110%, respectively. For the SBSE method recoveries were poor and ranged from 57 to 89%. The lowest recoveries were found for medium-chain acids, especially for capric acid. For other higher fatty acids in homologous line the recoveries improved. The likely reason for these improving recoveries is related to the fact that the lower molecular weight acids are too polar and tend to stay behind the aqueous phase instead partitioning into the nonpolar polydimethylsiloxane phase on the stir bar. The recoveries of less polar long chain fatty acids were comparable as for SPE as SBSE and ranged from 85 to 125% and from 94 to 110%, respectively.

The repeatability of the methods was examined by repeating all procedures five times during the same day on the same beer sample. The results demonstrated that there are not significant differences among all methods. The procedures had good repeat ability for medium-chain fatty acids. Relative standard deviations (RSD) extended from 4.3 to 6.0% for SPE, from 2.3 to 5.1% for SPME, and from 4.1 to 6.7% for SBSE. Only for pelargonic acid did RSD show a worse value (about 8%), due to the very low concentration. The determination of long-chain fatty acids indicated a slightly inferior repeatability because after SPE and SBSE extractions another derivatization step was required. For SPE and SBSE methods RSD values ranged from 11 to 16% and from 13.8 to 16.3%, respectively.

These results indicated that all three methods provide similar working parameters.

Table 2. Concentration of Free Fatty Acids as Average of Three Replicates in Three Beer Samples Determined by Using SPE, SPME, and SBSE Methods and Their Differences after Analysis of Dispersion Test[a]

	sample A (mg/L)			sample B (mg/L)			sample C (mg/L)		
compound	SPE	SPME	SBSE	SPE	SPME	SBSE	SPE	SPME	SBSE
caproic acid	**3.00**	**2.90**	2.54	**2.48**	**2.39**	1.98	**2.85**	**2.78**	2.32
caprylic acid	**6.67**	**6.98**	**6.56**	**5.39**	**5.23**	**5.51**	**6.07**	**5.90**	**6.03**
pelargonic acid	0.04	0.03	0.03	0.03	0.02	0.03	0.02	0.02	0.01
capric acid	**2.00**	**1.90**	**2.17**	**1.17**	**1.00**	**1.02**	**1.65**	**1.49**	**1.61**
lauric acid	**0.41**	**0.37**	**0.45**	**0.59**	**0.57**	**0.69**	**0.72**	**0.75**	**0.64**
myristic acid	**0.08**		**0.10**	**0.06**		**0.06**	**0.05**		**0.04**
palmitic acid	**0.39**		**0.40**	**0.22**		**0.21**	**0.33**		**0.43**
stearic acid	**0.51**		**0.46**	**0.36**		**0.31**	**0.24**		**0.23**
oleic acid	0.05		0.03	0.02		0.02	0.01		0.01
linoleic acid	0.01		0.02	0.01		0.02	0.01		0.01
linolenic acid	0.01		0.01	0.01		0.01	0.01		0.01

[a] Bold characters indicate results without statistically significant differences.

All methods have been applied for the analysis of three different pilsner-type beer samples. Each analysis has been repeated three times. The concentrations as average of the triplets of the analyzed compounds are shown in **Table 2**. The internal standard method has been chosen for compound quantitation. As internal standards, heptanoic acid for the determination of medium-chain fatty acids (caproic−lauric acids) and pentanedecanoic acid for the determination of long-chain fatty acids (myristic−linolenic acids) were used. Three compounds were present in concentrations > 1 mg/L (caproic, caprylic, and capric acids), and four compounds were present in low concentration, < 0.05 mg/L (pelargonic, oleic, linoleic, and linolenic acids).

An analysis of variation (ANOVA) has been applied to compare statistical differences between results obtained by all three used procedures (**Table 2**). The results without statistically significant differences are marked by bold characters. Except for caproic acid determined by SBSE method, the determined concentrations of other analyzed fatty acids at a level of > 0.05 mg/L obtained by using of these methods are possible to regard as the same. Compounds at low concentration (< 0.05 mg/L) were not statistically tested.

The correlation of results of medium-chain fatty acids achieved by SPE and SPME methods and by SPE and SBSE procedures are presented in **Figures 1** and **2**. The SPME method gave a little better agreement with the SPE procedure in comparison with the SBSE method (correlation coefficients were 0.9983 and 0.9937, respectively).

Laboratories nowadays must give not only accurate and reproducible results but do so as rapidly as possible, so these

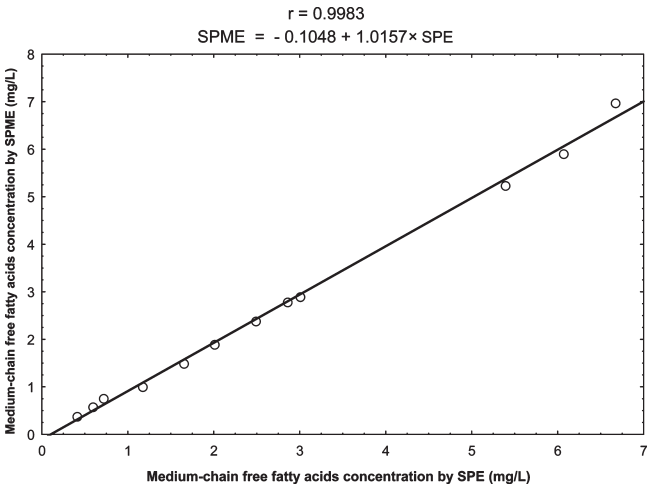

Figure 1. Linear regression for medium-chain free fatty acids in beer samples determined by SPE and SPME methods.

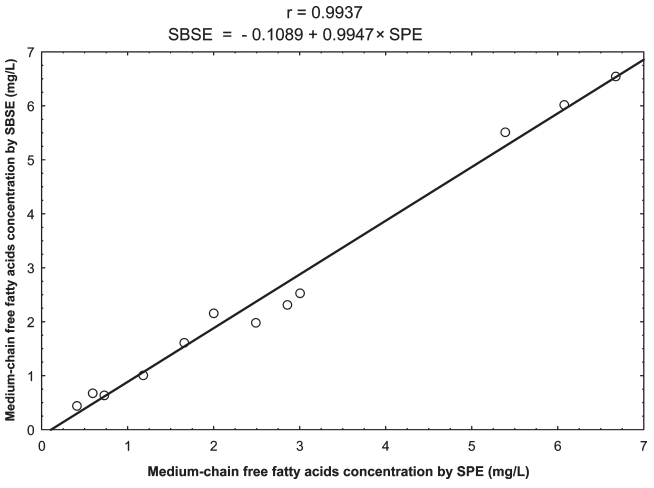

Figure 2. Linear regression for medium-chain free fatty acids in beer samples determined by SPE and SBSE methods.

Table 3. Comparison of Time Consumption for the Extraction of Medium-Chain Fatty Acids from Beer by SPE, SPME, and SBSE Methods

	time (min)		
operation	SPE	SPME	SBSE
condition	2	5	60
sampling	25[a]	30	60
reexraction			40
clean-up	2		
drying	5		
elution	2		
total time (min)	36[a]	35	160

[a] Time can be slightly changed depending upon beer sample.

sample preparation techniques were also compared from a time consumption point of view. For medium-chain fatty acid analysis results per sample are given in **Table 3**. The SPME method is the fastest, but it allows only the determination of medium-chain fatty acids. The derivatization process, which is necessary for long-chain acid determination, is the same for SPE as for SBSE, so there are no differences in time consumption.

Table 4 shows the consumption of solvents, which as is well-known is dropped from the SPE technique to become the solventless SPME procedure.

Table 4. Comparison of Solvent Consumption for the Extraction of Medium-Chain Fatty Acids from Beer by SPE, SPME, and SBSE Procedures

	volume (mL)		
solvent	SPE	SPME	SBSE
methanol	2.5		
water	2 × 5.0		
chloroform	2 × 0.5		
hexane			0.1
dichloromethane			0.1
total volume (mL)	3.5[a]	0	0.2

[a] Water consumption is not included.

In conclusion, the SPME method may be a more appropriate technique for routine quality control of medium-chain fatty acids in beer due to its operation efficiency (simplicity, repeatability, solventless, low time consumption, and low cost). Results obtained by this and the SPE method are also highly correlated. On the other hand, the fiber is fragile and its use requires careful manipulation. The great disadvantage of this procedure in comparison with the SPE and SBSE methods is the impossibility of determining also long-chain fatty acids after the same common extraction step. The stir bar used in the SBSE method is much more robust than the SPME fiber. SBSE is also simple and needs only 100 μL of solvent consumption, but it takes a lot of time. However, the method can be performed in parallel at room temperature. The working parameters of all introduced methods were similar. The obtained concentrations from all determined compounds corresponded except caproic acid determined by SBSE. Therefore, all techniques provide compatible alternatives, especially for medium-chain fatty acid analysis.

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