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ORIGINAL RESEARCH PAPER

INTERNAL OR EXTERNAL STANDARD TECHNIQUES FOR QUANTIFICATION OF FREE FATTY ACIDS (FFAs) IN RAW MILK AND KEFIR SAMPLES

Ahmet Dursun, Zehra Güler*

Hatay Mustafa Kemal University, Agriculture Faculty, Department of Food Engineering, Tayfur Sökmen Campus, 31034 Hatay, Turkey

*Corresponding author: zguler@mku.edu.tr

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Abstract: Free fatty acids (FFAs) can be analyzed by chromatographic methods as both qualitatively and quantitatively. The concentration of FFAs can be estimated by using internal or external standard techniques. The object of this study was to determine the recovery and repeatability of individual FFA (C_2-$C_{18:1}$) in milk and kefir, using internal or external standard (calibration curve). The FFAs adsorbed on aluminum oxide from samples are desorbed in isopropyl alcohol containing formic acid, which are analyzed using a gas chromatography-mass spectrometry (GC-MS) coupled with capillary column. The RSD of FFAs ranged from 2.77 % to 5.82 % for milk and from 1.02 % to 6.82 % for kefir. The recoveries of FFAs ranged from 82 % to 109.9 % and from 83.6 % to 109.3 %, respectively. The lowest recovery was obtained for hexadecanoic acid (82 %) in milk by the calculation relative to internal standard using relative correction factor. No significant differences in the concentrations of individual FFA were observed between internal and external standard techniques for milk but hexadecanoic and octadecenoic acids in kefir were higher at calculation by external standard compared to internal. In conclusion, the individual FFA showed a good repeatability in both kefir and milk. The high ethanol and acidity contents of kefir samples did not show a blocking effect on the alumina sorbent for isolation or releasing of FFAs.

Keywords: *correction factor, goat milk, kefir, recovery, repeatability*

INTRODUCTION

Kefir, a fermented milk product, has high ethanol and CO_2 contents, and acidic and yeasty flavor. While FFAs are considered as minor constituents of raw milk, they are an indicator of lipolysis in dairy products [1 – 3]. The various methods for extraction of FFAs from milk and milk products have been used for years since it is a critical process stage for FFA analysis. Salih *et al.* [4] used diethyl ether acidified with HCl for extraction of FFAs from milk, and reported high recoveries (99-105 %) for butanoic, decanoic, octadecanoic and *cis*-9-octadecenoic acids in a standard FFA mixture. Deeth *et al*. [5] proposed diethyl ether acidified with H_2SO_4 for the extraction of lipids and FFAs from dairy products such as butter, cheese and milk powder, where high recoveries (92-107 %) were obtained for FFAs (from C_4 to $C_{18:1}$). Some researchers [2, 6, 7] used ethanol with heptane/diethyl ether acidified with H_2SO_4 for FFAs extraction from dairy products, where an ion exchange aminopropyl column was used as adsorbent for the isolation of FFAs from lipids. The researchers have reported that use of aminopropyl column as an adsorbent for FFA isolation is suitable for the samples having a high lactic acid content (0.7-1.5 g ·100 g^{-1}) because of a good recovery ranging from 70 % to 101 % was obtained. However, the disadvantage of this method is a high detection limit for the individual FFA. Deeth *et al*. [5] utilized alumina deactivated with 4 % water as adsorbent. The high recoveries (>92 %) were obtained for FFAs in milk [5] and cheese [8], even when used a stainless steel column 10 % SP 216 PS (length 4 m, internal diameter 1/8 inch; Supelco Inc, Ballefonte, PE, USA) packed with Supelcopart 100/120 mesh for FFA analysis. This extraction method with slightly modifications has been used for milk [2, 9], yogurt [2, 10 – 12] and cheese [2, 13 – 15] for the last decade. However, the practicability of alumina adsorption technique in kefir is not known due to the high contents of ethanol as well as lactic acid. According to some researchers [2, 6, 16], the use of ethanol in the course of extraction can increase in lipid extraction efficiency and fatty acid yield. It is probably that ethanol, the less apolar than the other apolar solvents, can cause to extraction more polar lipids. In a previous study [17], the analysis of FFAs in Kefir has been based on the method described by de Jong and Badings [6]. In some studies, on Kefir [18 – 20], fatty acids were derivatized using two-step methylation procedure, and concentration was calculated in relative to internal standard, where did not made the method validation even for the probable losses during methylation process. However, in order to reduce possible analytical errors and also to improve the reliability and reproducibility of the analysis, the validation of a method is essential [21]. A comprehensive validation was undertaken by Mannitol *et al*. [2] who were investigated the performance of both derivatization method and the direct injection method of FFAs in many types of dairy products other than Kefir.

According to the most international guidelines, linearity, precision, limit of detection (LOD), limit of quantification (LOQ) and recovery are the parameters required for method validation [21 – 25]. Therefore, in this study a method for the quantification of FFAs (from C_2 to $C_{18:1}$) in dairy products with high ethanol and lactic acid such as kefir was described. The method encompasses a novel combination of procedures of extraction with acidified diethyl ether-hexane [4] and isolation with alumina oxide [5] and identification using a GC-MS coupled with a capillary column. This combination of techniques offers an improved method for the quantification of FFAs in kefir. The

assessments of precision (repeatability) and accuracy of method were carried out. For monitoring the effects of lactic acid and ethanol contents on repeatability and accuracy, besides milk, kefir was analyzed and the results were compared. In chromatographic analysis, the quantification of individual compound analyzed is calculated either relative to internal standard using correction factor or based on the calibration curve. In the present study, the both quantification methods were compared.

MATERIALS AND METHODS

Chemicals

The individual free fatty acid (from C_2 to $C_{18:1}$) except for C_{17} (≥98 %; Merck) and internal (C_{13}) standards were analytical grade (≥99 %) and purchased from Sigma GmbH (Steinheim, Germany). Aluminum oxide neutral, hydrogen chloride, diethyl ether, diisopropyl ether, anhydrous sodium sulfate and hexane were obtained from Merck (Darmstadt, Germany). Polypropylene column was purchased from Bio-Rad (Hercules, CA, USA).

Materials

Milk was obtained from Damascus (Shami) goats raised in Hatay province, Turkey. After milking, the milk was transferred immediately to laboratory at Food Engineering Department, Hatay Mustafa Kemal University using an ice box. Kefir was manufactured using conventional UHT (Ultra High Temperature) cow milk obtained from retail market. Kefir grains were obtained from Ankara University, Department of Dairy Science.

Production of Kefir

UHT milk (total solids 11.51 ± 0.03 %, protein 3.10 ± 0.16 %, fat 3.02 ± 0.15 %, carbohydrate 5.53 ± 0.02 %, ash 0.74 ± 0.01 % and *p*H 6.55 ± 0.03) was used in the manufacture of starter culture and natural kefir starter culture and kefir. Natural kefir starter culture was used for kefir production according to protocol described by Kök-Tasbaset *et al*. [26]. For the preparing of natural kefir starter culture, kefir grains were re-activated by culturing in UHT milk at a rate of 0.3 % over three growth cycles at 25°C for 22 h until *p*H 4.6. After each growth cycle, the grains were separated using sterile cheesecloth and re-inoculated into UHT milk at a rate of 0.3 %. After the third activation, the fermented milk (natural kefir starter culture) from which the grains had been separated, was inoculated into UHT milk at a rate of 3 % for the manufacture of Kefir. In a similar way to the production of natural kefir starter culture, the inoculated kefir milk was fermented at 25-26 ^{o}C for approximately 20 h up to *p*H 4.6. The kefir was stored at 5°C for overnight and then made the sampling. The kefir productions were made in six batches using two samples from each batch for analyses. The milk and kefir samples were stored at -18 ^{o}C until analysis.

*p*H measurement

For *p*H measurements, an Orion *p*H meter with a combined glass electrode and temperature probe (Thermo, Austin, TX, USA) was used. The *p*H meter was calibrated using standard buffer solutions at *p*H 4.0 and 7.0 (Merck, Darmstadt, Germany).

Ethanol analysis

The ethanol in UHT milk and kefir samples were analyzed according to the procedure reported by Dursun *et al*. [27]. Briefly, 10 g of milk or kefir was transferred into a 20 mL headspace vial containing 2.5 g NaCl (Agilent, Palo Alto, CA, USA). The vials sealed with a TFE-silicone septum (Agilent Palo Alto, CA, USA) were immediately frozen at –20 ºC until use. Prior to analysis, the frozen samples were thawed at 4 ºC overnight. A 75 µm fibre coated with Carboxen/Polidemethylsiloxane (CAR/PDMS, Supelco, Bellefonte PA, US) was used for the adsorption of ethanol from head space of milk or kefir samples. For this purpose, the sample vials were put in a water bath at 55 °C with continuous stirring. Vials containing milk or kefir samples were held at 55 °C for 30 min without fiber and for 20 min with fiber. Ethanol adsorbed on fibre was desorbed in an Agilent model 6890 gas chromatography and 5973 N mass spectrometry (MS) (Agilent, Palo Alto, CA, USA) equipped with a HP-INNOWAX capillary column (60 m × 0.25 mm id × 0.25 µm film thickness). The identity of ethanol was confirmed by the retention index calculated using the retention times of homologous series of n-alkanes C_5–C_{25}, and also by retention time (RT) and MS ion spectra of ethanol standard (Sigma-Aldrich, Milwaukee, WI, USA). Ethanol expressed as the percentage of integrated area.

Standard solution preparation

A stock standard mixture of pure free fatty acids containing 29.2 mg of C_2, 43.0 mg of C_4, 47.0 mg of C_6, 59.6 mg of C_8, 43.2 mg of C_{10}, 45.0 mg of C_{12}, 34.0 mg of C_{14}, 23.0 mg of C_{16}, 40.4 mg of C_{17}, 42.8 mg of C_{18}, 46.6 mg of $C_{18:1}$ and 44.0 mg of C_{13} (internal standard, IS) in diisopropyl ether by using 6 % formic acid was prepared in 100 mL calibrated volumetric flask. From this stock solution there were made serial dilutions within ranges indicated in Table 1. Six working solutions in triplicate were prepared for obtaining a wide concentration range.

Extraction and isolation of FFAs

The extraction and isolation of FFAs in milk and kefir were carried out according to the procedure reported by Güler *et al*. [9] as shown in Figure 1.

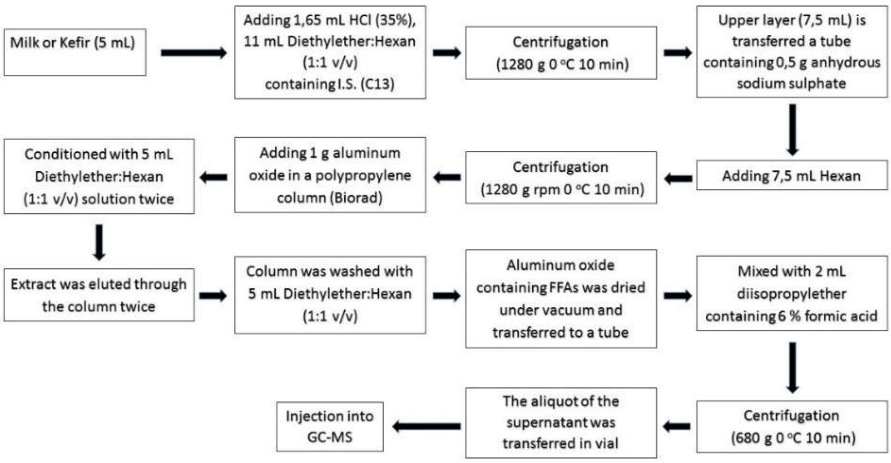

Figure 1. *The extraction and isolation of FFAs*

Table 1. *Regression equation of individual FFAs in milk and kefir samples (n=3)*

FFAs	Range [µg·g^{-1}] [Min-Max]	[1]Regression Equation [y=a+bx]	r	R^2	RSD of *f*	LOD	LOQ
C_2	4.5625-246	y= 0.1924x –0.0055	0.9994	0.9989	6.85	0.0495	0.1650
C_4	3.3594-215	y= 0.3768x –0.0034	0.9999	0.9998	2.11	0.0156	0.0521
C_6	3.6719-235	y= 0.5356x –0.0015	0.9999	0.9998	6.94	0.0049	0.0162
C_8	4.6563-298	y= 0.8083x +0.0035	0.9998	0.9997	4.41	0.0075	0.0250
C_{10}	3.3750-216	y= 0.8054x –0.0207	0.9999	0.9999	8.29	0.0445	0.1484
C_{12}	3.5156-225	y= 0.8609x –0.0279	0.9998	0.9997	5.71	0.0561	0.1871
C_{14}	5.3125-170	y= 0.7625x –0.0206	0.9999	0.9999	5.71	0.0468	0.1560
C_{16}	3.5781-115	y= 0.7022x –0.0187	0.9992	0.9985	6.92	0.0468	0.1538
C_{17}	3.1563-202	y= 0.7173x –0.0035	0.9997	0.9994	5.22	0.0085	0.0282
C_{18}	3.5781-214	y= 0.7140x –0.0184	0.9996	0.9992	8.79	0.0446	0.1488
$C_{18:1(cis\text{-}9)}$	3.6406-233	y= 0.4381x –0.0116	0.9996	0.9993	8.02	0.0459	0.1529

[1]Calibration curve for the ratio of individual FFA and internal standard (IS) peak areas as a function of the ratio of individual FFA and IS concentrations. y: FFA Area/IS Area; x: FFA Concentration (µg·g^{-1})/ Internal Standard Concentration (62.48 µg·g^{-1}); a: the independent term; b: the slope. r: Correlation coefficient. R^2: Determination coefficient. RSD of *f*: Relative standard deviation of response factor. LOD: Limit of detection. LOQ: Limit of quantification.

GC-MS analysis and conditions

Chromatographic separation was performed on an Agilent Model 6890 GC equipped with a 5973 N mass spectrometer (Agilent Technologies, USA). The injector was set at 250 ºC and a splitless injection mode was used. The separation of FFAs was carried out a DB-FFAP capillary column (30 m x 0.25 mm id; 0.25 µm film thickness; Agilent, USA). Helium was used as the carrier gas at a constant flow rate of 1.0 mL·min^{-1}. The oven temperature was initially held at 55 ºC for 2 min, then programmed from 55 ºC to 230 ºC at a ramp rate of 5 ºC·min^{-1} and held at 230 ºC for 20 min. The total run time was 57 min. The source temperature was fixed at 250°C and MS worked in electron impact mode (EI, 70 eV). In order to avoid the solvent peak, a solvent delay of 5 min was implemented. The mass spectra were acquired over the mass-to-charge (m/z) range

of 33-330. Two µL of extract was injected into GC-MS. Free fatty acids were identified by their retention times using authentic FFA standards (Aldrich Chemical Co., Steinheim, Germany) and the identification of FFAs were also confirmed by computer-matching of their mass spectral data against the Wiley7n.1 and Nist 02.L. GC-MS libraries (Agilent). The final FFA concentrations were expressed as $\mu g \cdot g^{-1}$ kefir or milk.

Statistical Analysis

For the statistical analysis, t-test was applied to compare differences between mean values ($P<0.05$) using SPSS statistical program (Version 22.00, SPSS, IBM, NY, USA). The calibration curve was drawn and the calculation of means, standard deviation (SD), relative standard deviation (RSD), limit of detection (LOD), limit of quantification (LOQ), response factor (*f*) and relative correction factor (RFC) and recovery were performed by using Microsoft Excel (Edition 2010; Microsoft, Redmond, WA, USA).

RESULTS AND DISCUSSION

The mean values of *p*H were 6.74 ± 0.13 and 4.22 ± 0.07 for analyzed milk and kefir samples, respectively. Ethanol (as % of all identified volatile compounds) was 2.0 ± 0.01 for milk and 23.5 ± 0.10 for kefir. These findings were consistent with the results obtained previously from goat milk [28] and kefir [1, 3].

Validation of the method

Peak identification of free fatty acids was carried out by comparison of retention times and ion spectra from authentic standards and also spectra from the Mass Spectral Database (Wiley7n.1/Nist02.L.). The quantification was performed based on the both external standard and internal standard methods to estimate the concentration of each individual FFA. The linearity of calibration curve of each individual free fatty acid was evaluated based on the relative standard deviation (RSD) of calibration response factors, correlation coefficient (r) and determination coefficient (R^2). The working solutions containing IS (C_{13}: 62.48 $\mu g \cdot g^{-1}$) were injected three times in GC-MS system coupled with capillary column to provide standard lines based on each fatty acid and internal standard peaks.

Linear regression curve for the individual acid covering a broad range of concentrations were calculated by using the ratio of individual fatty acid and internal standard peak areas (Standard area/IS area) as a function of the concentrations (Standard concentration/ IS concentration) ratio of individual fatty acid and internal standard used (Table 1). So the losses during injection and ionization were eliminated. Actually, calibration curve was drawn by taking into account GC-MS response factor with respect to internal standard. The data points from calibration curves were subjected to a least square regression analysis. The determination coefficient (R^2) of each individual free fatty acid was calculated. The coefficients of determination (R^2) obtained from all FFAs (except for C_{16}, 0.9985) were greater than 0.999. Correlation coefficient (r) for individual FFA was higher than 0.999, being a perfect correlation value. Additionally, the linearity of method was verified by calculating of response factor (*f*). From the

calibration curve with 6 points, response factor (*f*) for each individual free fatty acid were calculated by dividing the area under the peak at each point obtained in the chromatogram by the known corresponding concentration. The relative standard deviation (RSD) of calibration response factors of individual FFA was determined. The RSD values of *f* (%) ranged from 2.11 % to 8.79 % that considered as adequate to verify the linearity of the regression lines for analytical methods [23]. As shown in Table 1, RSD, r and R^2 values of free fatty acids varied from 2.11 % to 8.79 %, from 0.9985 to 0.9999 and from 0.9992 to 0.9999, respectively. The calibration curves showed an excellent linearity. Limits of detection (LOD) and quantification (LOQ) of free fatty acids were calculated by using following (Equations1 and 2), respectively.

$$\mathrm{LOD} = \left[\frac{3b}{a}\right] x \frac{1}{\sqrt{n}} \quad (1)$$

$$\mathrm{LOQ} = \left[\frac{10\mathrm{b}}{\mathrm{a}}\right] \mathrm{x} \frac{1}{\sqrt{\mathrm{n}}} \quad (2)$$

where a, b and n are the independent term, the slope and the number of replicates. As indicated in Table 1, LOD and LOQ ranged from 0.0049 to 0.0561 and from 0.0162 to 0.1871. The lowest and the highest LOD and LOQ values were obtained from C_6 and C_{12}, respectively.

Quantitative analysis

In chromatography, quantitative analysis is the determination concentration of a compound showing a response from a detector as a peak. One of the most common ways to quantify peak is the peak area. So, the concentration of a compound being analyzed is calculated based on peak area by using an internal standard or calibration curve (external). Internal standards are used to calculate relative response factor and to prepare the calibration curve, and to improve the precision of quantitative analysis. One of the easiest ways to eliminate the losses and variations during extraction/isolation, injection and ionization is to use the relative response or relative correction factors and an internal standard to calibrate the GC.
The internal standard selected should be similar to the analyte and have a similar retention time and derivatization. It must be stable and must not interfere with the sample components.

Determination of relative correction factor for individual FFA using IS (C_{13})

To relative correction factor (RCF) calculation, concentration of internal standard (C_{13}) as in course of calibration studies was 62.48 $\mu g \cdot g^{-1}$ FFA mixture solution with an internal standard containing low or high concentrations of FFAs showing in Table 2 were added into milk (n=6) or kefir (n=6) samples before the extraction process. In total, there were 12 spiked milk or kefir samples.
The free fatty acids from spiked kefir or milk samples were extracted/isolated and analyzed as in non-spiked kefir or milk. Each of FFA mixture solution containing internal standard, non-spiked milk or kefir and spiked milk or kefir was chromatographed three times with six repetitions. The area of individual FFA was

determined using (Spiked sample FFA area- Non-spiked sample FFA area). Then the concentration of individual fatty acid was calculated from (Equation 3).

$$\text{FFA Concentration} = \text{FFA Area} x \left[\frac{\text{IS Concentration}}{\text{IS Area}} \right] \quad (3)$$

The relative correction factor for individual FFA was obtained from the ratio of the real concentration of individual FFA to the concentration calculated versus IS. The mean correction factor for individual FFA in milk or kefir is shown in Table 2. The relative correction factors of FFAs, except for C_2, C_4, C_6 and *cis*-9-$C_{18:1}$, were almost equal to 1.0 in milk and kefir. The free fatty acids are far away from internal standard C_{13} did not show a good correction factor which was greater than 1.0. Thus, the concentration of individual FFA in a sample containing internal standard at the same concentration is calculated following (Equation 4).

$$\text{Concentration (A)} = \text{Peak Area A} \times \left[\frac{\text{Concentration IS}}{\text{Peak Area IS}} \right] x \text{RCF(A)} \quad (4)$$

Accuracy of method and recovery of free fatty acids

The milk or kefir samples as in course the determination of relative correction factor were spiked with known concentrations containing internal standard (C_{13}:62.48 µg·g^{-1}) at the minimum and maximum ranges indicating in Tables 3 and 4. The free fatty acids from spiked kefir or milk samples were extracted/isolated and analyzed as in non-spiked kefir or milk.

Table 2. *Relative correction factor (RCF) of individual FFA in milk or kefir for extraction and isolation efficiencies, and GC-MS response*

FFAs	RC [µg·g^{-1}]	[1]Relative correction factors (RCFs)			
		Milk [Mean±SD]	RSD	Kefir [Mean±SD]	RSD
C_2	18.25-36.50	4.58±0.12	2.68	4.25±0.11	2.68
C_4	28.88-53.75	2.17±0.05	2.12	2.01±0.04	2.12
C_6	26.88-58.75	1.51±0.04	2.53	1.40±0.04	2.53
C_8	37.25-74.50	0.99±0.04	4.27	0.92±0.04	4.27
C_{10}	27.00-54.00	1.04±0.04	3.39	0.99±0.04	4.41
C_{12}	28.13-56.25	1.02±0.04	4.37	1.01±0.05	5.46
C_{14}	42.50-85.00	1.00±0.04	3.87	1.01±0.03	3.44
C_{16}	28.63-57.25	1.06±0.07	6.28	1.00±0.06	5.93
C_{17}	25.25-50.50	1.04±0.03	3.34	1.03±0.04	4.11
C_{18}	26.75-53.50	1.05±0.07	6.66	1.02±0.05	5.36
$C_{18:1}$ (*cis*-9)	58.25-116.5	1.71±0.11	6.46	1.59±0.10	6.46
Mean			4.18		4.25
SD			1.63		1.45

[1]Relative correction factor was calculated by the ratio of the real concentration (RC) of individual FFA to the theoretical concentration calculated versus internal standard (62.48 µg·g^{-1}).

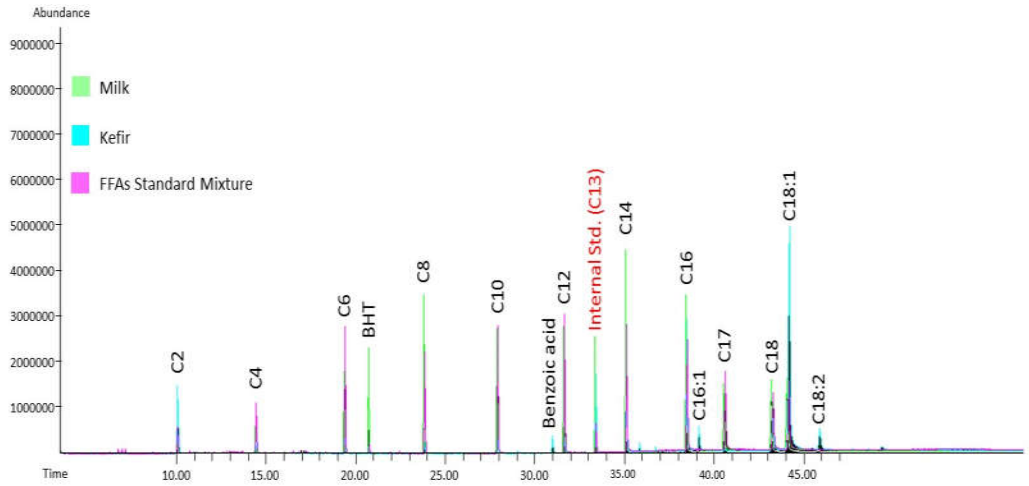

Figure 2. *A chromatogram from milk, kefir and FFAs standard solutions*

The concentration of each fatty acid was calculated not only using the calibration curve (external standard) but also by relative to the internal standard using a correction factor (Table 2). A chromatogram sample for the FFAs of milk, kefir and standard solvent used for calibration is shown in Figure 2.The recovery (%) was calculated by comparison of the pre- and post-spiked samples using following (Equation 5).

$$\text{Recovery (\%)} = \left[\frac{(C_1 - C_2)}{C_3}\right] \quad (5)$$

where C_1 represents the concentration obtained from kefir or milk samples fortified with standard solution of free fatty acids, C_2 concentration from non-spiked kefir or milk samples, and C_3 real concentration from standard solution of free fatty acids.

Table 3. *Recoveries of FFAs from milk (n=3)*

FFAs		External				Internal			
	RC [µg·g^{-1}]	TC [µg·g^{-1}]	Recovery^1 [%]	RSD [%]	Error [%]	TC [µg·g^{-1}]	Recovery^2 [%]	RSD [%]	Error [%]
C_2	18.25	16.7±1.24	91.3±6.81	7.46	8.71	16.6±1.23	90.8±6.76	7.44	9.17
	36.50	34.7±1.26	95.1±3.46	3.64	4.94	34.5±1.41	90±3.85	4.07	5.40
C_4	28.88	25.3±1.68	94.1±6.26	6.65	5.88	22.8±1.58	86.7±2.59	2.99	13.28
	53.75	58.2±1.16	108.3±2.16	2.00	-8.29	51.7±1.03	96.1±1.92	2.00	3.87
C_6	26.88	30.2±0.59	102.8±2.00	1.95	-2.82	26.7±0.29	90.9±0.98	1.08	9.13
	58.75	63.2±0.38	107.6±0.65	0.60	-7.60	55.0±0.33	93.6±0.56	0.60	6.39
C_8	37.25	40.7±1.06	109.2±2.84	2.60	-9.18	36.7±0.96	98.6±2.57	2.61	1.43
	74.50	75.1±4.74	100.7±6.36	6.31	-0.74	68.0±4.27	91.3±5.74	6.28	8.71
C_{10}	27.00	26.8±0.30	99.3±1.06	1.06	0.66	26.8±0.34	99.2±1.12	1.13	0.79
	54.00	50.0±0.62	92.6±1.15	1.25	7.36	49.6±0.25	92.6±1.56	1.69	7.37
C_{12}	28.13	29.2±1.27	103.9±4.50	4.33	-3.88	30.9±1.35	109.8±4.80	4.33	-9.89

	56.25	59.3±2.18	105.3±3.87	3.67	-5.33	60.2±2.32	107.2±4.13	3.67	-7.03
C_{14}	42.50	44.1±1.42	103.7±3.34	3.22	-3.68	41.7±1.34	98.0±3.16	3.22	1.96
	85.00	93.5±6.17	109.9±7.25	6.60	-9.94	88.4±5.83	104.0±6.86	6.60	-3.95
C_{16}	28.63	26.1±1.66	91.5±5.80	6.34	8.51	23.0±1.49	82.4±2.36	2.86	17.64
	57.25	58.6±3.57	102.4±3.39	3.31	-2.42	54.0±3.10	94.3±5.42	5.75	5.72
C_{17}	25.25	22.3±0.28	88.3±1.12	1.27	11.72	23.3±0.48	92.2±1.91	2.07	7.80
	50.50	48.1±1.85	95.3±3.66	3.85	4.74	50.2±2.09	99.5±4.14	4.16	0.52
C_{18}	26.75	27.4±2.76	102.5±9.62	9.37	-2.45	29.4±3.10	110±11.37	10.37	-9.71
	53.50	49.5±5.58	98.0±6.56	6.70	2.03	52.7±3.38	98.4±6.31	6.41	1.56
$C_{18:1\ (cis\text{-}9)}$	58.25	59.4±4.78	102.0±8.20	8.04	-2.02	49.3±4.15	87.5±2.29	2.62	12.55
	116.50	106±3.01	91.3±2.58	2.82	8.72	92.3±2.12	79.3±1.58	2.00	20.67
Mean	Low		99.0±6.63				95.2±9.14		
	High		100.6±6.61				96.0±8.21		

[1]Recoveries were calculated from concentration quantified by calibration curve.
[2]Recoveries were calculated from the concentration quantified by relative to internal standard using relative correction factor. TC: theoretical concentration, RC: real concentration ($\mu g \cdot g^{-1}$).

When calculated the concentrations according to external standard (calibration curve), recoveries in milk and kefir ranged from 88.3 % to 109.9 % and from 93 % to 108.1 %, respectively. When calculated the concentrations according to internal standard by using the relative correction factor, the recoveries ranged from 79.3 % to 109.9 % for milk and from 83.6 % to 108.9 % for kefir. In the both milk and kefir, a better recovery was obtained from the concentration calculated according to external standard compared to internal standard (Tables 3 and 4). Regardless of the calculation method and concentration spiked, the recoveries (from 92.4 % to 109.2 %) of FFAs in kefir were slightly better than those (from 90 % to 108 %) in milk. The present recovery values were better than those (from 70 % to 101 %) reported by de Jong *et al*. [7] for yogurt, in where amino propyl adsorbent was used for isolation of FFAs. All recovery values satisfy the performance criteria of acceptable limits of 70 – 110 % as recommended by European Commission [24].

Table 4. *Recoveries of FFAs from kefir (n=3)*

FFAs	RC [µg g^{-1}]	External				Internal			
		TC [µg·g^{-1}]	Recovery[1] [%]	RSD [%]	Error [%]	TC [µg·g^{-1}]	Recovery[2] [%]	RSD [%]	Error [%]
C_2	18.25	20.5±3.26	105.5±5.93	5.62	-5.50	20.4±3.23	105.0±6.34	6.04	-4.99
	36.50	36.8±3.29	100.9±9.00	8.93	-0.86	36.5±3.26	100.1±8.94	8.93	-0.10
C_4	28.88	29.1±2.00	104.4±4.75	4.55	-4.44	26.0±1.78	96.6±6.62	6.85	3.40
	53.75	54.4±3.90	101.2±7.25	7.17	-1.19	48.0±2.03	90.3±2.79	3.09	9.68
C_6	26.88	29.2±0.67	99.5±2.27	2.28	0.47	28.4±0.74	96.7±2.51	2.60	3.35
	58.75	58.0±1.69	98.8±2.87	2.91	1.24	56.9±1.42	96.9±2.41	2.49	3.08
C_8	37.25	39.1±1.35	103.5±1.97	1.90	-3.45	34.9±1.22	93.6±3.27	3.50	6.45
	74.50	69.7±4.06	95.3±3.39	3.56	4.69	64.2±3.66	88.0±1.93	2.19	12.03
C_{10}	27.00	26.6±1.87	98.4±6.94	7.06	1.63	26.9±1.87	99.6±6.93	6.96	0.44
	54.00	53.1±3.88	98.2±7.19	7.31	1.77	53.6±3.87	99.2±7.18	7.23	0.82

C_{12}	28.13	31.0±2.03	108.1±5.04	4.66	-8.12	30.1±2.17	107.4±4.01	3.60	-7.39
	56.25	59.7±0.72	106.1±1.28	1.21	-6.06	61.3±0.77	108.9±1.36	1.21	-8.90
C_{14}	42.50	44.5±1.96	104.6±4.62	4.41	-4.64	41.5±1.86	97.7±4.37	4.47	2.33
	85.00	92.4±3.44	108.7±4.05	3.72	-8.72	86.0±3.25	101.2±3.83	3.78	-1.20
C_{16}	28.63	27.3±0.61	95.4±2.13	2.23	4.60	23.9±0.53	83.6±1.85	2.21	16.39
	57.25	58.8±2.85	102.6±4.99	4.86	-2.63	50.4±2.49	88.1±4.34	4.93	11.89
C_{17}	25.25	25.3±0.04	100.1±0.15	0.15	-0.06	24.9±0.04	98.4±0.16	0.16	1.56
	50.50	47.0±4.15	93.0±8.21	8.83	7.02	46.0±4.46	91.0±8.84	9.71	8.97
C_{18}	26.75	27.5±0.88	102.8±3.28	3.19	-2.75	29.6±0.94	109.3±2.45	2.24	-9.33
	53.50	51.9±3.75	97.0±7.00	7.22	2.97	53.9±4.01	100.8±7.50	7.44	-0.76
$C_{18:1\ (cis\text{-}9)}$	58.25	56.8±4.28	97.4±7.35	7.54	2.56	51.5±3.72	88.4±6.38	7.22	11.63
	116.50	112±13.23	96.9±9.35	9.64	3.12	103±10.49	89.0±8.87	9.96	11.00
Mean	Low		101.8±3.90				98.2±8.26		
	High		99.8±4.72				96.0±7.73		

[1]Recoveries were calculated from concentration quantified by calibration curve.
[2]Recoveries were calculated from the concentration quantified by relative to internal standard using relative correction factor. TC: theoretical concentration, RC: real concentration ($\mu g \cdot g^{-1}$).

According to the student-t test, significant differences in recoveries of most FFAs such as C_2 (P<0.01), C_4 (P<0.001), C_{10} (P<0.05), C_{17} (P<0.05) and *cis*-9-$C_{18:1}$ (P<0.05) between kefir and milk were observed. It was probable that the high ethanol and acidity in kefir could be improved the extraction efficiency and FFA yields in course of extraction/isolation, as earlier reported [6, 16].

Repeatability of the Method for Milk and Kefir

FFAs in milk and kefir samples were analyzed by the above-mentioned procedure and their concentrations were calculated by the calibration curve or by relative to internal standard, taking into account the correction factors. As shown in Tables 5 and 6, the RSD for most FFAs was less than 5 %.The FFAs, quantified by relative to internal standard, displayed numerically a good average repeatability value in kefir.

Table 5. *FFAs found in goats'milk ($\mu g \cdot g^{-1}$)*

FFAs	Relative to IS		RCFs		Internal[1]		External[2]	
	Mean±SD	RSD	Mean±SD	RSD	Mean±SD	RSD	Mean±SD	RSD
C_2	2.41±0.12	4.96	4.58±0.12	2.68	11.71±0.40	3.45	11.47±0.49	4.24
C_4	6.02±0.25	4.10	2.17±0.05	2.12	13.03±0.53	4.10	13.32±0.52	3.93
C_6	7.06±0.34	4.84	1.51±0.04	2.53	10.66±0.52	4.84	10.72±0.51	4.76
C_8	12.27±0.48	3.93	0.99±0.04	4.27	12.15±0.48	3.93	11.88±0.48	4.02
C_{10}	18.94±1.03	5.43	1.04±0.04	3.39	19.69±1.07	5.43	20.42±1.02	5.00
C_{12}	7.66±0.45	5.82	1.02±0.04	4.37	7.82±0.45	5.82	9.15±0.41	4.53
C_{14}	20.38±0.84	4.11	1.00±0.04	3.87	20.38±0.84	4.11	23.07±0.88	3.81
C_{16}	84.38±3.71	4.39	1.06±0.07	6.28	89.44±3.93	4.39	97.79±4.22	4.32
C_{17}	1.21±0.03	2.77	1.04±0.03	3.34	1.25±0.03	2.77	1.65±0.04	2.26
C_{18}	59.70±4.54	7.61	1.05±0.07	6.66	67.45±3.23	4.79	68.51±3.60	5.25

$C_{18:1(cis\text{-}9)}$	26.01±2.09	7.63	1.71±0.11	6.46	47.80±1.73	3.62	53.94±1.79	3.32
Mean		5.05		4.18		4.29		4.13
SD		1.51		1.63		0.88		0.83

[1] The individual FFA was calculated by relative to internal standard (IS) and its corresponding correction factor (CF) was used. Internal standard was 62.48 μg·g^{-1} as in standard mixture. [2]FFAs were calculated using calibration curve.

No significant differences in the concentrations of each FFA between external and internal standard methods were observed for milk. However, there were significant ($P<0.05$) differences in concentrations of hexadecanoic and octadecenoic acids in kefir between the calculation methods (Table 6). Their concentrations were close to the maximum points of curves (Table 1). These upper limits can show a good repeatability but the determination of concentration using a calibration curve may cause possible deviations from the real value. Therefore, the unknowns should be measured only in the region of the curve. Regardless of the calculation methods, the best repeatability for FFAs as mean RSD value was observed in kefir compared with milk (Tables 5 and 6). The quantification of $C_{16:0}$, $C_{18:0}$ and *cis*-9-$C_{18:1}$ as previously reported by Mannion *et al*. [2] could be adversely influenced because of interaction with FFAP column phase, a phenomenon referred to as 'memory effect' that is more apparent for long-chain acids.

Table 6. *FFAs found in kefir (μg·g^{-1})*

FFAs	**Relative to IS**		**RCFs**		**Internal[1]**		**External[2]**	
	Mean±SD	**RSD**	**Mean±SD**	**RSD**	**Mean±SD**	**RSD**	**Mean±SD**	**RSD**
C_2	74.82±1.65	1.99	4.25±0.11	2.68	318.10±7.01	1.99	322.25±6.37	1.98
C_4	9.65±0.44	4.59	2.01±0.04	2.12	19.39±0.89	4.59	19.57±0.87	4.46
C_6	8.75±0.43	4.91	1.40±0.04	2.53	12.25±0.60	4.91	12.31±0.51	4.10
C_8	27.06±1.21	4.46	0.92±0.04	4.27	24.90±1.11	4.46	24.95±0.84	3.38
C_{10}	21.82±1.26	5.75	0.99±0.04	4.41	21.60±0.74	3.39	21.72±1.16	5.33
C_{12}	14.43±0.47	3.25	1.01±0.05	5.46	14.57±0.47	3.25	14.47±0.40	2.80
C_{14}	75.57±0.77	1.02	1.01±0.03	3.44	76.32±0.78	1.02	75.24±0.75	1.00
C_{16}	103.51±2.70	2.61	1.00±0.06	5.93	103.51±2.70^a	2.61	111.06±2.85^b	2.57
C_{17}	18.07±0.19	1.07	1.03±0.04	4.11	18.61±0.20	1.07	19.00±0.20	1.05
C_{18}	70.36±2.75	3.91	1.02±0.05	5.36	71.77±2.81	3.91	74.75±5.12	6.86
$C_{18:1(cis\text{-}9)}$	118.16±4.02	3.40	1.59±0.10	6.46	187.87±6.39^a	3.40	201.83±6.80^b	3.37
Mean		3.36		4.25		3.15		3.35
SD		1.56		1.45		1.34		1.78

[1]The individual FFA was calculated by relative to internal standard (IS) and its corresponding correction factor (CF) was used. Internal standard was 62.48 μg·g^{-1} as in standard mixture.
[2]FFAs were calculated using calibration curve. Different upper lower case superscript letters at the same row indicate the differences ($P<0.05$) between the quantification techniques.

The concentration of FFAs in milk and kefir could be determined using calibration curve according to the present method. However, the wider concentration ranges of hexadecanoic and octadecenoic acids and also acetic acid should be used for calibration curves. Care must be used at the upper limit of the curve to ensure that the data of unknowns are not collected outside of curve or close to the maximum point. Otherwise, the concentration of unknown is calculated by proportioning to the internal standard

using correction factor. Overall, in milk and kefir the method could be applied to the quantification of FFAs in chain lengths ranging from 2 to 18 carbon atoms. Since lactic acid in kefir was not detected in the present method, the removal of lactic acid from kefir or yogurt is not necessary during analysis of FFA.

CONCLUSIONS

This study is the first report on the method validation for analysis of FFAs in kefir. FFAs in kefir or milk were successfully analyzed by the proposed method using diethyl ether acidified with HCl (35 % v/v) for extraction and alumina oxide conditioned with diethyl ether/hexane for isolation, and a capillary DB-FFAP column (30 m x 0.25 mm id x 0.25 µm film thickness) for identification. The free fatty acids (C_2-$C_{18:1}$) with a recovery ranging from 92 % to 109 % and a repeatability lower than mostly 5 % could be analyzed in milk and kefir. Lactic acid in kefir did not cause any interference in the identification of FFAs. In contrast, low *p*H (4.22 ± 0.07) and high alcohol (23.5 %) in kefir provided a good reproducibility and recovery for FFAs in comparison to milk having high *p*H (6.74 ± 0.13) and low ethanol (2.0 %).The concentration of individual FFA in kefir or milk could be calculated by the calibration curve (external standard) or by internal standard method, taking into account correction factor for individual FFA. Care should be used at the upper limit of the calibration curve according to the milk product. It was concluded that the performance parameters showed total method adequacy for the detection and quantification of free fatty acids ranging from C_2 to C_{18} in milk and kefir. The application of the method was successfully applied to monitoring milk and milk products with different *p*H and ethanol contents.
This work confirms that the external standardization in a wide concentration range calibrated according to an internal standard may be a more effective technique than the internal standardization.

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