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Multiple headspace solid-phase microextraction for quantifying volatile free fatty acids in cheeses

Arturo A. Rincón, Verónica Pino*, Juan H. Ayala, Ana M. Afonso

Departamento de Química Analítica, Nutrición y Bromatología, Universidad de La Laguna (ULL), La Laguna, Tenerife 38206, Spain

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ABSTRACT

Multiple headspace solid-phase microextraction (MHS-SPME) has been utilized for the quantitative determination of 9 volatile free fatty acids (FFAs) in cheeses, in combination with gas-chromatography and flame-ionization detection (GC-FID). Variables affecting HS-SPME and MHS-SPME were optimized to attain adequate sensitivity while allowing correct application of the MHS method. Thus, the MHS-SPME method was successfully performed when using 0.3 g of cheese and 1 mL of NaCl (sat. solution), which is subjected to four consecutive extractions using the carboxen–polydimethylsyloxane (CAR–PDMS) as the commercial SPME coating, 40 min of HS extraction time at 45 °C, and 6 min of desorption time in the GC injector at 290 °C. The MHS-SPME permitted the calculation of β values, which range from 0.72 ± 0.01–0.95 ± 0.02, depending on the cheese studied. Later, this β parameter is used to perform quantitation for the 9 volatile FFAs after just a single HS-SPME extraction, using an external solvent calibration curve. The validity of the utilization of an external solvent calibration was tested with aqueous standards of volatile FFAs, getting average recoveries higher than 81.2%. Quantitation by MHS-SPME was free of matrix interferences despite measuring a complex cheese sample. The optimized method was validated, presenting inter-day reproducibility values (as RSD in %) lower than 13%, and limits of detection down to 7 $\mu g\ kg^{-1}$. The method was also compared with a conventional extraction method such as solid-phase extraction for the studied cheeses elaborated with goat milk, generating comparable results. To our knowledge, this is the first time that MHS-SPME has been applied to volatiles in cheeses.

1. Introduction

Free fatty acids (FFAs) are compounds which highly contribute to the cheese aroma, also acting as precursors of other important aroma components such as methylketones, alcohols, aldehydes, and esters. The majority of volatile compounds including volatile FFAs in cheeses are formed during ripening [1,2]. FFAs are formed mainly as a result of the lipolytic enzyme action on glycerides. This becomes particularly important in raw milk cheeses, where the native lipase is not deactivated by pasteurization [2–4]. It has been shown that the flavor of these cheeses mainly comes from short and medium chain FFAs, and indeed they have been suggested as indices of ripening of cheeses [3,5].

Given the interest of studying the evolution of volatile FFAs during cheese ripening, it is important to take use of an adequate method to identify and quantify these volatiles in such a complex matrix. Volatile compounds in foods are generally analyzed by gas chromatography (GC) coupled to mass spectrometry (MS) with a prior step of extraction and/or preconcentration [6,7]. Among preconcentration methods, headspace-solid phase microextraction (HS-SPME) has proven to be quite successful regarding food aroma, specifically in the determination of compounds responsible of cheese flavor [4,8–13].

Nevertheless, it is also well known that the sensitivity and accuracy of the SPME-based methods are quite influenced by the "matrix effect", particularly with solid samples. In addition to this, SPME is a non-exhaustive extraction technique. To overcome these drawbacks regarding quantitation of volatiles using HS-SPME, it is possible to use internal standards [14], matrix-matched standards, and the standard addition method [15–22]. However, given the difficulty in mimicking solid samples, it is not practical the utilization of matrix-matched standards calibration. Standard addition and internal standard methods also present problems, as there are differences between the behavior of the native analytes and the spiked standards when dealing with complex solid samples.

As an improvement over HS-SPME, multiple HS-SPME (MHS-SPME) has been proposed to successfully perform quantitation of volatile analytes from solid samples [23,24]. This stepwise method implies the repeated use of HS-SPME in the same sample. Clearly, the total content of an analyte in the solid sample does not depend

* Corresponding author. Tel.: +34 922318990; fax: +34 922318003.
E-mail addresses: aarincon@ull.es (A.A. Rincón), veropino@ull.edu.es (V. Pino), jayala@ull.es (J.H. Ayala), aafonso@ull.es (A.M. Afonso).

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on sample composition if HS-SPME is carried out at infinitum with the same sample. In that utopic case the total peak area (A_T) of an analyte, obtained by summing all peak areas for each individual extraction, would directly reflect the total amount of the volatile analyte in the solid sample [24]. If the MHS-SPME method is successfully exhaustive after few extractions, A_T can be also obtained from the sum of the peak areas of such extractions, and in this case the scenario becomes practical. If the MHS-SPME is not exhaustive after few extractions, A_T can also be estimated, but in this case using the following equation:

$$A_T = \sum_{i=1}^{\infty} A_i = \frac{A_1}{1-\beta} \quad (1)$$

where A_1 is the peak area of the first extraction, and β is a constant value calculated from the slope of the linear regression of the logarithms of the individual peak areas, according to the following expression:

$$\ln A_i = \ln A_1 + (i-1) \times \ln \beta \quad (2)$$

where A_i is the relative peak area obtained in the ith extraction. In common practice, β can be calculated after few (3–5) consecutive HS-SPME extractions of the same sample.

Thus, it is possible to estimate A_T only measuring A_1 once β is experimentally known. The A_T value is then used to estimate the mass of analytes in the real solid sample employing a calibration curve obtained with standard solutions. This calibration can now be used despite the different behavior of samples and standard solutions [24].

The MHS-SPME method has been successfully applied in the quantitation of a number of volatiles in a variety of solid matrices including soils [25], packaging materials [20,26,27], commercial rosemary extracts [28], cork stoppers [29], leaves [14], tomatoes [18], pickles [21], bread [30], sausages [31], mushrooms [32,33], and even a polar matrix such as nylon 6/6 [22]. However, to the best of our knowledge, the method has not been applied yet to the determination of volatiles in cheeses. In addition to this, this exhaustive MHS-SPME method has been hardly compared with other exhaustive extraction methods. Thus, Ezquerro and Tena [29] compared with Soxhlet for a group of four analytes present in cork stoppers, Groning and Hakkarainen [22] compared with microwave-assisted extraction for 2-cyclopentyl-cyclopentanone in nylon, and Oliveira et al. [20] compared with liquid-liquid microextraction for three radiolysis products in a packaging material. Here, we also intend to compare the performance of the MHS-SPME method in combination with GC and flame ionization detection (FID) *versus* a conventional and widely used exhaustive procedure for FFAs in cheeses such as solid-phase extraction (SPE) [34]. Specifically, the study will focus on a group of nine volatile FFAs, namely: acetic acid, propionic acid, *iso*-butyric acid, *n*-butyric acid, *iso*-valeric acid, *n*-valeric acid, *n*-hexanoic acid, *iso*-hexanoic acid, and *n*-heptanoic acid in chesses elaborated with raw milk. The MHS-SPME is comprehensively optimized and validated, and afterwards applied to the analysis of three different cheese samples obtained at different ripening times.

2. Experimental

2.1. Chemicals, reagents and materials

Volatile FFAs included in this study were acetic acid (99.8%), C_2; propionic acid (99.8%), C_3; *iso*-butyric acid (99.5%), *i*-C_4; *n*-butyric acid (99.8%), *n*-C_4; *iso*-valeric acid (99%), *i*-C_5; *n*-valeric acid (99.5%), *n*-C_5; *iso*-hexanoic acid (99.8%), *i*-C_6; *n*-hexanoic acid (99.8%), *n*-C_6; and *n*-heptanoic acid (99.0%), *n*-C_7; from Sigma-Aldrich (Steinheim, Germany). The volatile acid standard mix for FFAs, with an aqueous concentration of 10 mM in each FFA, was obtained from Supelco (Bellonte, PA, USA).

Cyclohexane (99.5%), chloroform (99.8%), heptane (99.0%), formic acid (98%), diethyl ether (99.7%), 2-propanol (99.5%), sulfuric acid (95%) and sodium chloride (99.5%) were also obtained from Sigma-Aldrich. Sodium sulfate (99%) was obtained from Scharlau (Barcelona, Spain).

Ultrapure water was obtained from the Milli-Q water purification system A10 Millipore (Watford, UK).

The external solvent calibration using GC-FID was carried out with all volatile FFAs individually dissolved in cyclohexane with concentrations ranging from 6–44 ng, being the range 20–70 ng for the calibration using acetic acid. Solutions were stored at 4 °C until analysis.

The volatile acid standard mix for FFAs was used as stock solution to prepare working aqueous standard solutions for HS-SPME and MHS-SPME, containing saturated NaCl under optimum conditions. Solutions were stored at 4 °C until analysis.

Amber glass headspace vials of 10 and 20 mL capped with PTFE/Butyl septa screw caps were supplied by CTC Analytics (Zwingen, Switzerland). Glass screw-capped tubes of 15 mL were supplied by SciLabware (Staffordshire, United Kingdom).

Two SPME fibers were used: carboxen–polydimethylsiloxane (CAR–PDMS, 75 μm of film thickness) and carboxen–divinylbenzene–polydimethylsiloxane (CAR–DVB–PDMS, 65 μm of film thickness), both obtained from Supelco.

The SPE cartridges used were aminopropyl-based, specifically Spe-ed NH_2 (500 mg, 3 mL) from Applied Separations (Allentown, PA, USA).

Several cheese samples were used in this study. One goat cheese sample (labeled as cheese 1) was acquired in a local supermarket. Other four cheese samples were kindly provided by the Animal Production and Forage Research Unit of the Canary Agronomic Research Institute (ICIA, Tenerife, Spain), and were elaborated according to the same pattern of traditional manufacturing of Canary cheeses. Thus, the amount of coagulant, raw goat milk, salt added, coagulation temperature, pressing, and storage were kept identical in all samples. These cheese samples were elaborated with different ripening times: one had 7 days of ripening (labeled as cheese 2), two had 45 days of ripening (labeled as cheese 3 and cheese 4) but coming with goats fed with different cattle foods, and another one had 90 days of ripening (labeled as cheese 5). All cheeses samples were stored at −18 °C until analysis.

The glassware used in this study was first washed with detergent and tap water, then with a mixture (1:1) of Derquim-Oxy supplied by Panreac (Barcelona, Spain) and sulfuric acid purchased from Sigma-Aldrich, and finally rinsed with ultrapure water. The non-graduated glassware and the vials were also dried in an oven at 550 °C during 2.5 h.

2.2. Equipment

It was utilized a gas chromatograph (GC) equipped with flame ionization detector (FID), Varian model CP-3800 Varian 450 (GC-FID) system, with a FFAP-CP capillary column (25 m × 0.32 mm I.D. × 0.3 μm) from Agilent Technologies (The Netherlands). The equipment also includes a Combi-Pal autosampler (CTC Analytics). The GC column was employed under the following temperature program: 40 °C for 2 min isothermal, then 25 °C min^{-1}–100 °C, then kept for 2 min, then subjected to 10 °C min^{-1}–240 °C, and then kept 8 min isothermal. The carrier gas was nitrogen, with a flow of 2 mL min^{-1}. The temperature of injector was maintained at 290 °C when using the CAR–PDMS and the DVB–CAR–PDMS fibers, altogether with 6 min for desorption time to avoid carry-over. The temperature of the injector was maintained at 280 °C when working in liquid

injection mode. For external solvent calibration, the injection volume was 2 μL. In all cases (solvent calibration or SPME) the splitless mode was utilized. The FID was kept at 280 °C, the make-up flow of nitrogen at 30 mL min^{-1}, the hydrogen flow at 30 mL min^{-1}, and the air flow at 300 mL min^{-1}. The workstation 6.9.3 Software (from Varian) was used for data acquisition.

The 12-port model Visiprep™ SPE vacuum manifold (with disposable liners) was obtained from Supelco. A vortex from Reax-Control Heidolph GMBH (Schwabach, Germany) was also employed.

2.3. HS-SPME and MHS-SPME procedures in combination with GC-FID

After adequate optimization of each of the variables implied, an amount of 0.3 g of cheese sample was thoroughly mixed with 1 mL of saturated NaCl and placed into a 20 mL glass vial, followed by stirring for few seconds using a vortex. The vial was then placed in the GC autosampler. In conventional HS-SPME only one extraction took place for each sample, and the remaining analysis conditions were identical to those of MHS-SPME. In MHS-SPME, four consecutive extractions were performed on the same sample at 45 °C for 40 min, using the CAR–PDMS fiber exposed in the headspace of the vial. After every extraction, fibers were subjected to GC desorption followed by FFAs determination.

2.4. SPE procedure in combination with GC-FID

Under optimum conditions, an amount of 1 g of cheese sample was placed in a screw-capped tube, and ground with 3 g of anhydrous Na_2SO_4 and 0.3 mL of H_2SO_4 (2.5 M). 3 mL of a mixture of diethyl ether:heptane (1:1, v/v) was added, and the tube was shaken for 3 min using a vortex. The supernatant was transferred to another screw-capped tube containing 1 g of anhydrous Na_2SO_4. This step was repeated three times. The combined supernatants were then loaded on the SPE cartridge, which was previously conditioned with 10 mL of heptane. 10 mL of a mixture of chloroform:2-propanol (2:1, v/v) was utilized to eliminate glycerides. FFAs were eluted with 5 mL of diethyl ether containing 2% (v/v) of formic acid. Finally, 3 mL of this eluate were diluted up to 5 mL with cyclohexane, and 2 μL of this solution were injected in the GC-FID. Each cheese sample was analyzed using SPE by triplicate.

3. Results and discussion

3.1. Optimization of the HS-SPME–GC-FID method

The optimum GC-FID chromatographic conditions for the selected group of volatiles FFAs are described in Section 2.2. Under these optimized conditions, the reproducibility as relative standard deviation (RSD in %) values of the retention times ranged between 0.05 and 0.24% (Table S1 of the Supplementary material).

It is necessary to adequately optimize the HS-SPME method prior to the MHS-SPME application for cheese samples. Thus, several variables affecting the microextraction process were first evaluated in a factor by factor approach: nature of the fiber coating, desorption conditions in the GC injector, extraction time, and extraction temperature.

Regarding the nature of the SPME fiber coating, the optimization study was accomplished with CAR–PDMS and DVB–CAR–PDMS for being the most used commercial SPME coatings for determining volatile FFAs in cheeses by HS-SPME–GC [4,8,13,35]. This screening study was performed using 4 g of cheese (commercial cheese 1, elaborated with goat raw milk), 45 min of extraction time and HS extraction at 50 °C, with a prior equilibration time of 10 min for the fiber in the HS of the vial containing the cheese sample (before heating). These values were fixed attending to common HS-SPME conditions for determining FFAs in cheeses [8,35]. Fig. 1 shows the obtained results under these experimental conditions. CAR–PDMS provided the best results in terms of extraction efficiency for the majority of volatile FFAs, except for *i*-C5 and *i*-C6, and so it was used thereafter.

Regarding desorption conditions in the GC injector, the injection mode (split/splitless), desorption time, and desorption temperature were tested. Optimum conditions to avoid carryover and ensure maximum signals were obtained using the splitless mode, 290 °C as injector temperature, and 6 min as desorption time for the CAR–PDMS fiber.

The influence of the extraction temperature and the extraction time were investigated using in this case 5 mL of working aqueous solutions of the FFA mix (1.6 mM) in the sample vial, to avoid any possible lack of reproducibility associated to the real cheese sample, and to select adequate conditions for all volatile FFAs independently on their content in the real cheese sample selected. In all cases, the equilibration time was kept to 10 min, to mimic that commonly used with cheese samples. The extraction time profiles for each FFA were studied from 20 to 70 min, fixing the extraction temperature at 50 °C. Results can be observed in Fig. 2(A). From these plots, it can be observed that 40 min is a sufficient time to ensure equilibration of volatile FFAs. Moreover, higher extraction times were not satisfactory for *n*-C_4, *i*-C_4, and C_3. The studies of the extraction temperature profiles were carried out keeping the extraction time fixed at 40 min, and working at three temperatures: 30, 45, and 65 °C. Higher temperatures were not tested because it is perfectly known this is against proper sorption on the SPME fiber. The lower temperature tested was 30 °C to avoid working at room temperature. Results can be observed in Fig. 2(B). The optimum extraction temperature was 45 °C. It must be comment that higher temperatures were not selected to avoid an important lack of sensitivity for C_3, *i*-C_6 and *n*-C_7, and thus intending their improvement.

The remaining variables that exert an effect in HS-SPME, such as cheese amount and salting-out effect, were specifically studied in MHS-SPME given its particularities.

3.2. Optimization of the MHS-SPME–GC-FID method

The MHS-SPME, as stepwise procedure, was carried out in this study subjecting the cheese sample to 4 consecutive extractions,

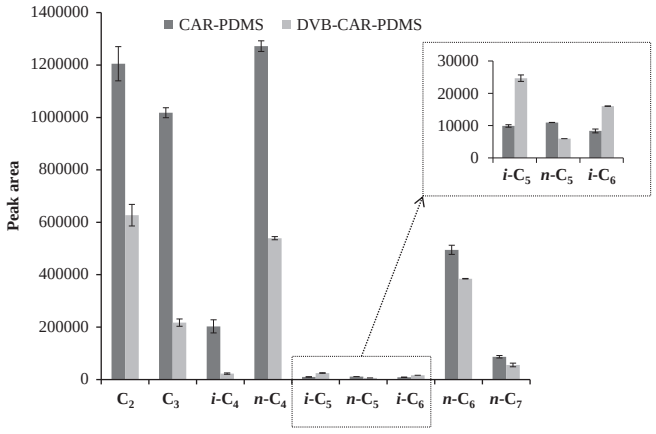

Fig. 1. Comparison of the extraction efficiency, expressed as chromatographic peak areas, for the studied commercial SPME fibers when determining volatile FFAs by HS-SPME–GC-FID. Extractions were performed by triplicate, under the following fixed parameters: 4 g of cheese (cheese 1), 10 min for the pre-equilibration time, 50 °C for the extraction temperature, 45 min for the extraction time, and the rest of conditions as described in the text.

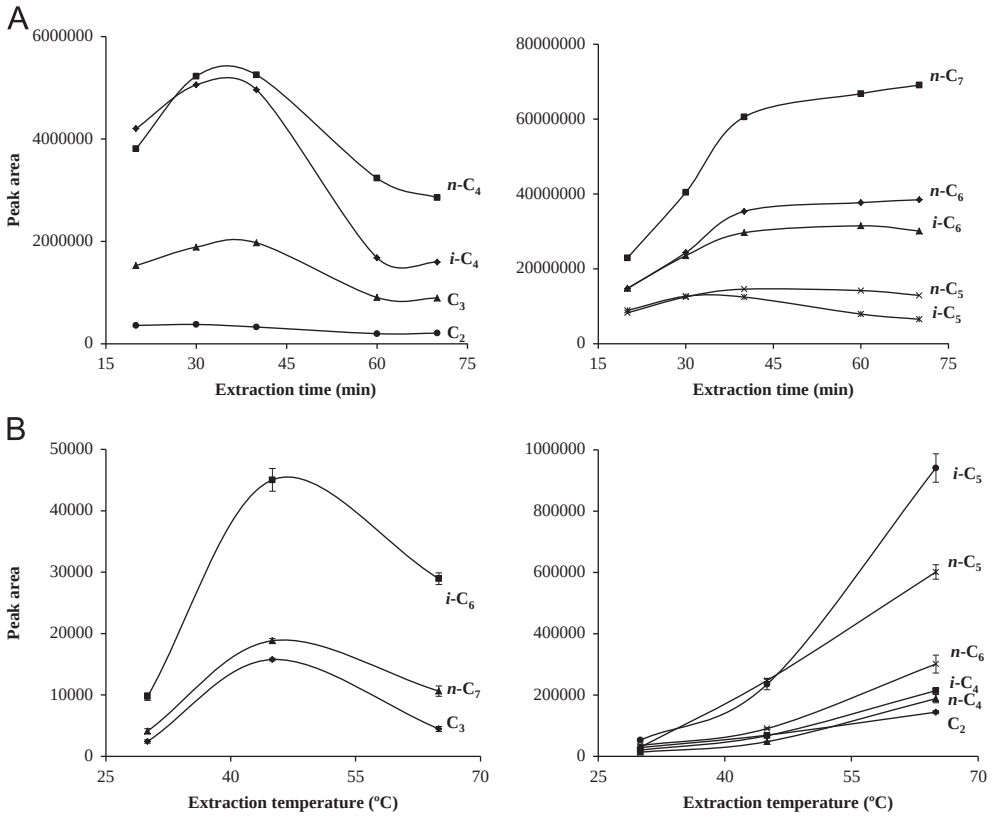

Fig. 2. Optimization of the HS-SPME–GC-FID method for the volatile FFAs studied, using the CAR–PDMS fiber, a pre-equilibration time of 10 min, a content of FFAs of 1.6 mM in the sample vial (5 mL of working aqueous standards), and rest of conditions as described in the text. (A) Influence of the extraction time at 50 °C, and (B) influence of the extraction temperature at 40 min (by triplicate).

using the conditions obtained for the HS-SPME optimization. The careful optimization of the MHS-SPME method was mainly centered in establishing the influence of the sample amount and of the salting-out effect.

3.2.1. *Influence of the cheese amount*

The amount of cheese sample placed in the vial to be subjected to MHS-SPME should ensure an exponential decay of the peak area for the volatile FFAs with the number of extractions. If the amount of cheese sample is too low, it is possible to observe sensitivity problems (low signals) and reproducibility problems (lack of homogeneity). If the amount of cheese sample is too large, the amount of volatile FFAs extracted by the SPME fiber is negligible compared to the total amount [24] (and so an exponential decay of the peak area would not be observed), being this accompanied by bad correlation coefficients of the logarithm of the peak-areas *versus* *i*-1 (Eq. (2)). It is pursued a difference of at least 5% between two consecutive extractions, because each single extraction must reduce significantly the amount of volatile FFAs in the cheese sample [18,24].

In order to determine the proper amount to perform MHS-SPME, the cheese sample amounts ranged from ~0.05 to ~0.5 g in the present study. Optimum conditions obtained in HS-SPME were used. Table 1 shows the obtained values for the correlation coefficients (r) of the plot corresponding to Eq. (2) when using different amounts of cheese (cheese 5). This study was performed by triplicate. Cheese 5 was selected for being the cheese obtained with the higher ripening time, and so with lower contents of volatile FFAs. If the method is valid for this cheese, it should perform even better with the remaining cheese samples studied. The optimal value was selected attending to the linearity observed,

Table 1
Influence of the cheese amount in the performance of the MHS-SPME method, evaluated by means of average β and r values.

Volatile FFA	Average[a] amount of cheese in the sample vial (g)							
	0.054 ± 0.004		0.13 ± 0.02		0.33 ± 0.02		0.52 ± 0.01	
	r	β	r	β	r	β	r	β
C_2	0.985	0.78	0.995	0.77	0.996	0.80	0.967	0.85
C_3	–[b]	–[b]	0.995	0.77	0.971	0.82	0.995	0.69
i-C_4	0.975	0.76	0.995	0.74	0.997	0.91	0.986	0.87
n-C_4	0.982	0.70	0.992	0.69	0.981	0.90	0.994	0.88
i-C_5	0.982	0.76	0.974	0.85	0.993	0.94	0.986	0.86
n-C_5	nd[c]	nd[c]	nd[c]	nd[c]	nd[c]	nd[c]	nd[c]	nd[c]
i-C_6	0.967	0.87	0.976	0.80	0.997	0.74	0.980	0.76
n-C_6	0.997	0.83	0.960	0.69	0.997	0.81	0.976	0.94
C_7	0.897	0.88	0.985	0.87	0.994	0.83	0.975	0.76

[a] $n=3$, $i=4$.
[b] It was not possible to obtain quantifiable areas after the third extraction.
[c] nd = non-detected.

the exponential decay of the areas, and then to the sensitivity achieved. The average amount of cheese of ~0.3 g was adequate to perform an efficient and reliable MHS-SPME, because it provided acceptable linearity with β values ranging from 0.74 to 0.94. It has already been pointed out that MHS-SPME can be considered feasible only for β values within the range 0.4–0.95 [24]. It must be commented here that the studied cheese did not present *n*-C_5 in quantities enough to be quantified by MHS-SPME within the amount of cheese studied. If an analyte had a β value below 0.4, it means it is exhaustively isolated in the first extraction (and so MHS is not required). If a β value is above 0.95, it means that the amount of analyte remains practically unaltered in the HS after

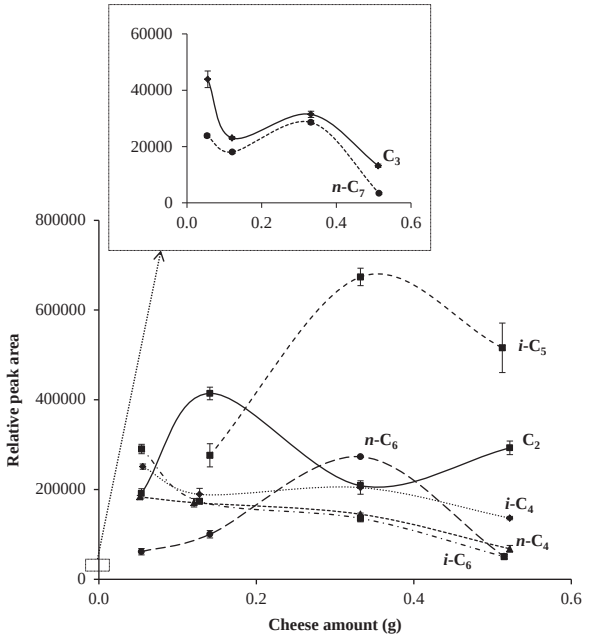

Fig. 3. Sensitivity of the MHS-SPME–GC-FID method working with different cheese amounts (cheese 5) in the sample vial. The relative peak area is obtained by dividing the first peak area (A_1) of MHS-SPME by the cheese amount exactly weighed in the sample vial. Experiments were performed by triplicate.

multiple extractions. Thus, cheese amount was fixed to a value of 0.3 g.

In order to evaluate properly the sensitivity when using different cheese amounts, it is important to carry out the comparison using the average relative peak area. The relative peak area is defined as the ratio of the first peak area (measured in the extraction 1 of MHS-SPME) *versus* the amount of cheese (cheese 5) specifically used. It should be kept in mind that it is hard to have exactly the same amount of cheese weighed in all sample vials. Thus, sensitivity is going to be affected by the accurate cheese amount utilized. The results associated to the utilization of the relative peak area are shown in Fig. 3. It can clearly be observed that ~0.3 g of cheese is an adequate amount to be employed, not only in terms of β and r values, but also in terms of sensitivity. In some cases, 0.05 was also adequate for several volatile FFAs (but not enough for *i*-C_5 or *n*-C_6) and it was accompanied by worse β and r values. In further studies, the relative peak-area was utilized to improve accuracy.

3.2.2. *Influence of the NaCl content*

It has been proposed the utilization of saturated solutions of salts with cheese samples not only as a way to improve the volatile FFAs content in the HS due to salting-out effect, but also to improve the homogenization of cheese samples [11,13,35]. In this sense, studies were carried out using three different volumes of a saturated solution of NaCl, which were added to an average

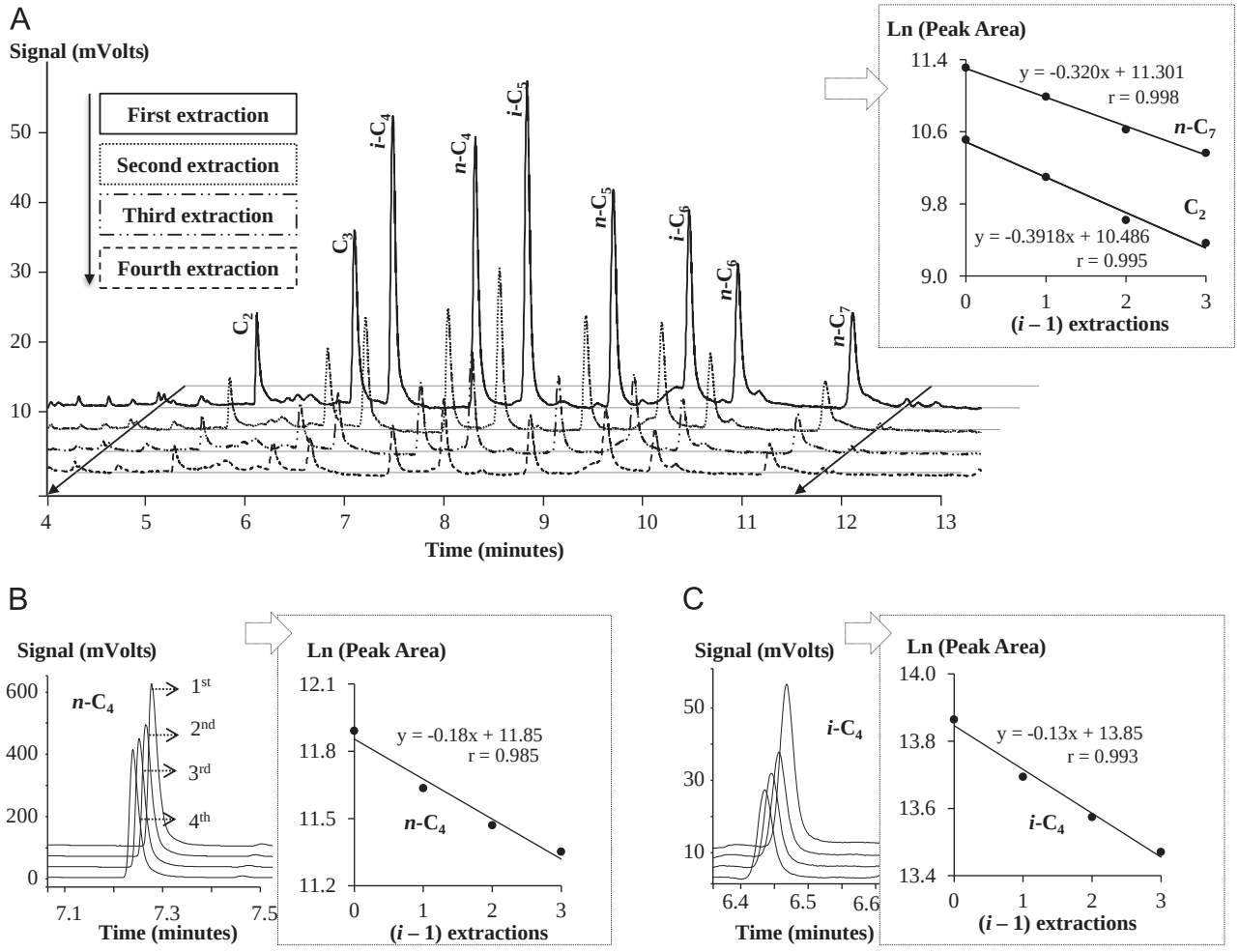

Fig. 4. (A) Chromatograms of four successive HS-SPME extractions using an aqueous standard solution of volatile FFAs (1.6 mM). Examples of decay in peak areas with cheese samples are included for two volatile FFAs in (B) cheese 2, and (C) cheese 3. The included sub-plots correspond to the application of Eq. (2). In all experiments, optimum MHS-SPME conditions were applied, including the adjustment of the ionic strength with NaCl.

amount of cheese (cheese 5) of ~0.3 g. The remaining conditions for the MHS-SPME were kept identical. Under these conditions, it was observed that the employment of 1 mL of NaCl (sat) to ~0.3 g of cheese was enough to ensure reproducibility and high r values (Table S2 of the Supplementary Material), and also better results when compared to those obtained in absence of NaCl. In this sense, the following conditions were used to perform MHS-SPME: ~0.3 g of cheese, 1 mL of saturated NaCl solution, 4 successive extractions using CAR–PDMS, a pre-equilibration time of 10 min, and 40 min of HS extraction time at 45 °C.

3.3. Quality analytical parameters of the optimized MHS-SPME–GC-FID method for quantifying volatile FFAs in cheeses

The MHS-SPME performance under optimal conditions can be observed in Fig. 4, in which the gradually decrease of peak-areas for the volatile FFAs between successive extractions is clear. Fig. 4(A) refers to the decrease in peak areas when measuring aqueous standards of the volatile FFA mix (5 mL, 1.6 mM), whereas Fig. 4(B) and (C) reflect such decrease for a volatile FFA in a specific cheese sample. Fig. 4 also includes typical plots related to Eq. (2), from which β is obtained.

Once β is obtained for a particular cheese sample, the total peak area (A_T) can be calculated using Eq. (1). The obtained values for β and A_T with three different cheese samples (2, 3 and 4) are shown in Table 2. It can be observed that β values ranged from 0.77 to 0.94 for cheese 2, from 0.72 to 0.95 for cheese 3, and from 0.90 to 0.94 for cheese 4. Regarding A_T values, they ranged from 1.00×10^5–8.24×10^7 for cheese 2, from 1.09×10^5 to 3.40×10^7 for cheese 3, and from 2.80×10^5 to 9.56×10^7 for cheese 4. It can be observed that the studied cheeses have a quite different contents on volatile FFAs.

Once A_T is known, it can be linked to a concentration using an external solvent calibration method. The quality analytical parameters of the external solvent calibration are included in Table S3 of the Supplementary material. These curves were obtained by injection of 2 μL of the standards prepared in cyclohexane, as described in the experimental section. With these calibrations, the calculated contents for volatile FFAs in these three cheeses can be observed in Table 3. It must be commented here that several observed A_T values (for some volatile FFAs) are above the studied linear range of the external solvent calibration curve. Nevertheless, quantification above de linear range of the external solvent calibration may be accepted because A_T is a theoretical value, not experimentally obtained by injection in the GC. This has been previously shown by Serrano et al., who concluded that the linear range might be extended to quantify high concentrations in samples without relevant errors [18].

In order to evaluate the feasibility of quantitation using external solvent calibration, β parameters corresponding to each volatile FFAs were also obtained extracting aqueous standards under optimum MHS-SPME conditions, followed by A_T determination (Eq. (1)) and an estimation of the concentration (by interpolation of the calculated A_T in the external solvent calibration to be tested). Recoveries can be obtained in this way by comparison between the estimated concentration values and those really used in the aqueous standard. The aqueous standards were prepared at two different concentration levels, all of them close to real values estimated in cheeses, each of them by triplicate. The MHS-SPME method for the aqueous standards was performed under optimized conditions (four consecutive extractions). The obtained

Table 2
Obtained values of β and A_T for three different cheese samples (2, 3 and 4), under the optimum MHS-SPME conditions.

Volatile FFA	Cheese 2		Cheese 3		Cheese 4	
	$\beta \pm SD$[a]	$(A_T \pm SD$[a]$)\times10^{-5}$	$\beta \pm SD$[a]	$(A_T \pm SD$[a]$)\times10^{-5}$	$\beta \pm SD$[a]	$(A_T \pm SD$[a]$)\times10^{-5}$
C_2	0.86 ± 0.03	643 ± 59	0.95 ± 0.02	340 ± 120	0.94 ± 0.01	238 ± 14
C_3	0.94 ± 0.01	68 ± 17	0.94 ± 0.01	56 ± 3	0.93 ± 0.01	154 ± 35
i-C_4	0.92 ± 0.01	13 ± 2	0.84 ± 0.01	8.88 ± 0.06	0.90 ± 0.01	9.9 ± 0.5
n-C_4	0.86 ± 0.01	318 ± 71	0.91 ± 0.01	220 ± 10	0.93 ± 0.01	270 ± 10
i-C_5	0.87 ± 0.02	1.08 ± 0.06	0.93 ± 0.01	134 ± 1	0.90 ± 0.01	8.3 ± 0.8
n-C_5	0.90 ± 0.02	1.0 ± 0.6	0.72 ± 0.01	1.09 ± 0.02	0.91 ± 0.01	9.8 ± 0.2
i-C_6	0.92 ± 0.01	824 ± 57	0.95 ± 0.02	61 ± 6	nq[b]	nq[b]
n-C_6	0.77 ± 0.03	200 ± 9	0.90 ± 0.01	187 ± 8	0.91 ± 0.01	956 ± 3
n-C_7	0.92 ± 0.01	121 ± 4	0.95 ± 0.01	319 ± 10	0.93 ± 0.02	2.8 ± 0.9

[a] Average of three independent experiments.
[b] Non-quantified.

Table 3
Contents of volatile FFAS in three different cheese samples, obtained by MHS-SPME–GC-FID and SPE–GC-FID.

Volatile FFA	Contents[a] (mg kg^{-1}) in cheese 2		Contents[a] (mg kg^{-1}) in cheese 3		Contents[a] (mg kg^{-1}) in cheese 4	
	SPE	MHS-SPME	SPE	MHS-SPME	SPE	MHS-SPME
C_2	297 ± 17	289 ± 39	243 ± 18	242 ± 9	128 ± 13	130 ± 12
C_3	12 ± 3	7 ± 2	11.8 ± 0.5	5.5 ± 0.5	18 ± 1	17 ± 4
i-C_4	nq[b]	0.60 ± 0.02	nq[b]	0.44 ± 0.01	nq[b]	0.49 ± 0.04
n-C_4	20 ± 1	17 ± 3	18 ± 2	14 ± 2	13 ± 1	15 ± 1
i-C_5	nq[b]	0.05 ± 0.02	9 ± 1	6 ± 1	nq[b]	0.36 ± 0.04
n-C_5	nq[b]	nq[b]	nq[b]	0.66 ± 0.01	nq[b]	0.55 ± 0.06
i-C_6	19.5 ± 0.4	18 ± 1	18 ± 1	16 ± 1	nq[b]	nq[b]
n-C_6	12 ± 1	11.7 ± 0.3	13 ± 2	12 ± 1	68 ± 5	58 ± 4
n-C_7	26 ± 3	8.9 ± 0.3	25 ± 1	24 ± 1	nq[b]	0.22 ± 0.09
Σ	387	353	356	319	226	222

[a] Average of three independent determinations for each sample in each method. Critical values ($n_1=3$, $n_2=3$, $\alpha_C=0.05$): $t_C=2.78$ and $F_C=39.0$.
[b] Non-quantified.

results are included in Table 4. It can be observed average recoveries of 81.2 and 82.1%, at each of the concentration levels used, with RSD values (in %) lower than 5.5%. Therefore, the validity of the external solvent calibration curve to estimate concentrations using A_T is demonstrated.

Regarding reproducibility, the study was carried out with a real cheese sample (cheese 5) by duplicate each day, and during three different non-consecutive days. The obtained inter-day precision values (as RSD in %) can be observed in Table 5, and varied between 6.6 and 13%.

Limits of detection (LODs) and limits of quantitation (LOQs) were also estimated for the optimized MHS-SPME method. They were estimated using a signal-to-noise ratio (S/N) of 3 and 10, respectively, from the chromatograms corresponding to the first extraction of real cheese samples, but measuring the noise in a time interval adjacent to the peak. The calculated LOD values range from 0.007 mg kg^{-1} for i-C_5 to 0.043 mg kg^{-1} for n-C_7, as it can be observed in Table 5. LOQ values down to 0.009 mg kg^{-1} for i-C_5 have been obtained. Literature limits of detection for volatile FFAs using HS-SPME have been reported to be of 0.545 mg kg^{-1} for n-C_7 using the CAR–PDMS fiber [35], and ~0.21 mg kg^{-1} for n-C_4 and ~0.12 mg kg^{-1} for n-C_6 using the PA fiber [9]. Clearly, the MHS-SPME method provides better sensitivity. There is no current literature method for volatile FFAs and MHS-SPME to compare with.

3.4. Comparison with other exhaustive extraction method

Cheese samples were also analyzed by an exhaustive extraction procedure for FFAs, such as SPE in combination with GC-FID [34]. Table 3 includes the obtained results from SPE as well as those obtained by the optimized MHS-SPME method. In general, it can be observed good agreement between both techniques for the three cheeses studied. A F-test was first utilized to verify the homogeneity of the variances for both methods. Given the fact that the variances are homogeneous if $F_{exp} < F_c$, and being in this case $n_1=3$, $n_2=3$, $\alpha_C=0.05$ and so $F_c=39.0$, it is possible to conclude that the variances are homogeneous. Afterwards, a t-test was employed to compare among the contents obtained by SPE and MHS-SPME. They are comparable if $t_{exp} < t_c$, and having in this case 4 degrees of freedom (n_1+n_2-2) while being $\alpha_C=0.05$ and so $t_c=2.78$, it is possible to conclude that both methods gave the same values. Exceptions were only observed for n-C_7 in cheese 2, and C_3 in cheese 3. Given the statistically concordance among the techniques, it must be highlighted the simplicity of the MHS-SPME approach, accompanied by an important decrease in the sample preparation time and organic solvents consumption if compared to SPE.

Average contents for volatile FFAs using the MHS-SPME method are 353 mg kg^{-1} for cheese 2, 319 mg kg^{-1} for cheese 3, and 222 mg kg^{-1} for cheese 4. It can be observed a decrease in the average content while increasing the ripening time of the cheeses studied.

Table 4
Estimation of performance of the optimized MHS-SPME method using aqueous FFAs standards.

Volatile FFAs	$\beta \pm$ SD[a]	ng Added[b]	R (%) $\pm$ SD[a]	ng Added[b]	R (%) $\pm$ SD[a]
C_2	0.94 ± 0.02	613	77 ± 9	1225	65 ± 8
C_3	0.92 ± 0.04	675	80 ± 1	1350	71 ± 3
i-C_4	0.92 ± 0.02	915	89 ± 12	1829	75 ± 12
n-C_4	0.91 ± 0.05	978	71 ± 9	1956	67 ± 12
i-C_5	0.91 ± 0.03	1064	62 ± 2	2128	98 ± 15
n-C_5	0.91 ± 0.03	1057	91 ± 11	2113	78 ± 5
i-C_6	0.92 ± 0.02	1201	98 ± 6	2402	93 ± 14
n-C_6	0.89 ± 0.04	1201	90 ± 7	2402	96 ± 14
n-C_7	0.81 ± 0.02	1353	71 ± 4	2706	96 ± 1
Average R (%)			81.2		82.1

[a] $n=3$, each measured by MHS-SPME using 4 consecutive extractions under optimized conditions.
[b] Amount of FFAs in the aqueous standard (prepared with NaCl sat.).

Table 5
Reproducibility and sensitivity achieved with the optimized MHS-SPME method.

Volatile FFA	LOD (mg kg^{-1})	LOQ (mg kg^{-1})	RSD[a] in % (average amount in cheese 5, in mg kg^{-1})
C_2	0.018	0.109	8.0 (50.2)
C_3	0.018	0.038	7.4 (0.72)
i-C_4	0.014	0.023	9.6 (0.42)
n-C_4	0.018	0.023	6.6 (22.0)
i-C_5	0.007	0.009	8.6 (0.10)
n-C_5	0.011	0.149	9.2 (0.29)
i-C_6	0.031	0.058	11 (0.03)
n-C_6	0.013	0.018	7.8 (34.0)
n-C_7	0.043	0.086	13 (3.23)

[a] $n=5$.

4. Conclusions

MHS-SPME has been used for the first time in the quantitation of volatile FFAs from complex solid samples such as cheeses. The overall method is characterized for performing quantitation of volatiles while minimizing matrix effects, and for including important advantages in sample preparation such as automation, low requirements of sample amount (~0.3 g), and elimination of the organic solvent consumption and tedious sample treatments during the extraction step.

The optimized MHS-SPME–GC-FID method has been adequately validated, presenting limits of detection down to μg kg^{-1} values, and acceptable inter-day precision values (RSD values lower than 13%) despite the fact of using low amount of cheese samples, making it feasible for real cheese samples analysis. Furthermore, the MHS-SPME method showed comparable quantitative results with that of a conventional and established extraction method such as SPE.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.05.032.

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