

Optimization of the HS-SPME-GC/MS technique for the analysis of volatile compounds in caprine Coalho cheese using response surface methodology

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Abstract

Caprine Coalho cheese presents great potential for a typical protected designation of origin, considering that this traditional Brazilian cheese presents a slightly salty and acid flavor, combined with a unique texture. This study optimized the HS-SPME-GC-MS methodology for volatile analysis of Coalho cheese, which can be used as a tool to help in the identification of the distinctive aroma profile of this cheese. The conditions of equilibrium time, extraction temperature and time were optimized using the statistical tool factorial experimental design 2³, and applying the desirability function. After the evaluation, it was concluded that the optimum extraction conditions comprised equilibrium and extraction time of 20 and 40 minutes, respectively; and ideal extraction temperature of 45 °C. The optimum extraction of volatile compounds in goat Coalho cheese captured 32 volatile compounds: 5 alcohols, 5 esters, 3 ketones, 6 acids, 3 aldehydes, 3 terpenes, and 7 hydrocarbons.

Keywords: dairy products; cheese; aromas; GC/MS.

Practical Application: Application of factorial design to optimize the extraction conditions of volatile compounds in caprine Coalho cheese.

1 Introduction

Caprine cheese is a product consumed worldwide, due to the growing demand for goat dairy products. Coalho cheese is a traditional, partially-cooked or cooked curd cheese which has been traditionally produced and consumed in Northeastern Brazil for over 150 years (Queiroga et al., 2013).

Caprine Coalho cheese is a very sought after product thanks to its slightly salty and acid flavour, alongside its “rubberized” texture. This unique texture is the result of its resistance to heat, which prevents it from melting when cooked. This allows the preparation of the popular “roasted Coalho cheese”, with its own distinctive volatile profile (Silva et al., 2012). In addition, caprine Coalho cheese is a product of great commercial value for a number of reasons: its simple low-cost production process, the high process yield, its low allergenic potential and high digestibility, as well as its excellent nutritional value (Oliveira et al., 2012; Santos et al., 2012).

On the other hand, caprine Coalho cheese presents great potential for a typical protected designation of origin (PDO), since the PDO label implies that the product has distinctive characteristics that are connected to traditional production methods. The PDO could be used as a tool of the to contribute to the economical sustainability of the Northeast region, especially the rural areas. It must be mentioned that even though it has been produced for over one century, Coalho cheese is still manufactured using non-standardized processes, which causes

variability mainly in sensory properties (Queiroga et al., 2013), and consequently in its aroma profile.

It is well known that the volatile profile is very important for the organoleptic quality of cheeses. It can be considered a finger print since the flavour of a cheese variety is the result of a specific balance between the volatile compounds produced during the manufacture process. According to Delgado et al. (2011), the basic flavor of any cheese is formed by the complex balance involving non-volatile and volatile substances from the microbiological and biochemical activities, which range from the raw material, the process and maturation. The volatile compounds are among the most important criteria of cheese quality for consumer acceptance. These compounds comprise the flavor characteristic of the same (González-Martín et al., 2014).

Gas chromatography coupled to mass spectrometry (GC-MS) has been extensively employed to identify and quantify aroma/flavor components in several types of foodstuffs, including cheeses. Traditional extraction methods for cheese volatiles, such as steam or vacuum distillation, are hardly used anymore (Moio & Addeo, 1998; Vandeweghe & Reineccius, 1990), mainly due to their poor recovery rate of highly volatile compounds and the disappearance of thermally sensitive compounds associated with the formation of artifacts (Concurso et al., 2008).

Headspace solid phase micro-extraction (HS-SPME) is a technique currently used for a great variety of cheeses, as it

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provides many advantages: it is an easy-to-use, relatively fast and sufficiently sensitive technique which does not require organic solvents. The results obtained with this technique are very promising and a considerable number of compounds have already been described in a great variety of cheeses (Pinho et al., 2003; Mallia et al., 2005; Concurso et al., 2008; Januszkiewicz et al., 2008); however, information regarding the volatile fraction and the use of SPME extraction of volatiles from caprine Coalho cheese (known to have a unique sensory profile) is not yet present in literature.

In order to have capture of volatile compounds by the fibre sample analyzed is allocated in a tightly closed glass bottle and subjected to a given temperature until the formation of the headspace to occur during equilibration. After the fibre is exposed to the headspace for desorption to occur during a certain time of extraction (Delgado et al., 2011).

The extraction parameters equilibrium time, extraction time and extraction temperature should be tested so as to obtain the optimum conditions for that permits the capture of a greater amount of the compounds, since these factors are the most frequently reported in the literature as factors that influence the performance of the analysis (Ma et al., 2013).

Having said that, this study, through the use of response surface methodology (RSM), aimed to develop and optimize the Solid Phase Micro Extraction technique (SPME), so that it may be used to characterize the volatile fraction of Coalho cheese. The optimization of HS-SPME-GC/MS methodology could be used as a tool to help the identification of the distinctive aroma profile of Coalho cheese, and will be used in further studies on the aroma profile of raw and cooked caprine Coalho cheese.

2 Materials and methods

2.1 Standards

The homologous series of n-alkanes C8 - C25, used for calculating the linear retention index of volatiles, was supplied by Fluka (Sigma Aldrich Chemie GmbH, Steinheim, Germany).

2.2 Extraction of Coalho cheese volatile compounds by HS-SPME

Samples were prepared by weighing 20 g of grated Coalho cheese in 60 mL vials sealed with a polytetrafluoroethylene silicone septum (Supelco, Bellenfont, PA, USA). The system was submitted to an extraction temperature (T) of 20, 30, 45, 60, and 70 °C in a water bath and an equilibrium time (Eq) of 3, 10, 20, 30, and 37 minutes before fibre exposure. Prior to extractions, the fibre was conditioned to the gas chromatograph injector at the temperature of 270 °C for 60 minutes. Afterwards, the SPME fibre was exposed to the headspace, above the Coalho cheese sample for an extraction time (Ex), of 6, 20, 40, 60, and 74 minutes. Then the fibre was retracted into the needle and transferred to the gas chromatograph injector where analytes were desorbed. The values tested were all obtained from previous studies, in which they had yielded satisfactory results for SPME extraction in cheeses (Delgado et al., 2010; Hayaloglu et al., 2013; Kondyli et al., 2013).

Prior to the optimization of the SPME conditions for the analysis of volatile components, a screening was performed to select the appropriate fibre. Fibres were purchased from Supelco (Bellofonte, PA, USA). Four fibres were tested and compared: divinylbenzene/Carboxen/polydimethylsiloxane (DVB/CAR/PDMS) 50/30 µm; polydimethylsiloxane (PDMS) 100 µm; Carbowax/divinylbenzene (CW/DVB) 65 µm and polydimethylsiloxane/divinylbenzene (PDMS/DVB), 65 µm. Before extraction began, each extraction fibre was conditioned according to the manufacturer's prescription.

Of the four fibres evaluated, the divinilbenzeno/Carboxen/polidimetilsiloxano 50/30 µm (DVB/CAR/PDMS) fibre presented the best performance, with a higher number of volatiles in the cheese samples, and was therefore selected to be used in the HS-SPME volatiles analysis in Coalho cheese.

2.3 Optimal conditions for the extraction of Coalho cheese volatiles

To optimize extraction by HS-SPME, a central composite rotational design (CCRD) was used, in which three independent variables were analyzed: equilibrium time (Eq), extraction time (Ex) and extraction temperature (T): The design included seventeen trials which comprised eight factorial points, six axial points and three replicates of the central point for knowing the experimental error associated with measurements. The response surface model was elaborated using the following Equation 1:

$$y = \beta_0 + \beta_1 E_q + \beta_2 E_x + \beta_3 T + \beta_4 E_q^2 + \beta_5 E_x^2 + \beta_6 T^2 + \beta_7 E_q \cdot E_x + \beta_8 E_q \cdot T + \beta_9 E_x \cdot T \quad (1)$$

Where y is the response value predicted by the model; β_0 is the mean coefficient (or the constant) β_1 , β_2 and β_3 are the linear, β_4 , β_5 and β_6 the quadratic and β_7 , β_8 and β_9 the interaction coefficients. In this model Ex (extraction time), Eq (equilibrium time) and T (extraction temperature) are the independent variables. The dependent variable in this study is the number of peaks in the chromatograms.

2.4 Statistical analysis

The response function (y) was used to perform regression analyses and analyses of variance (ANOVA) for the regression. The equation model was fitted to experimental data to yield the proposed model. Response surface graph and desirability parameters were generated for the response function (y), i.e. total number of peaks. The executed analyses, desirability and response surface were performed with STATISTICA 8.0 software (StatSoft Inc., 2007). After response surface analysis and graphing of the desirability for the maximum number of volatiles extracted, the proposed model was validated by performing new assays in triplicate. The results were compared with the estimated response (y) by Student's t-test ($p < 0.05$).

2.5 Gas chromatography-mass spectrometry conditions

For separation and identification of compounds extracted from Coalho cheese, a Varian 3800 gas chromatograph coupled to a Varian Saturn 2000R mass detector (Varian Inc., Palo Alto,

California) and VF-5 ms column (60 m × 0.25 mm × 0.25 μm) was used (Agilent, Santa Clara, California).

The temperature programming of the gas chromatograph oven started at 40 °C for 10 minutes, followed by a 5 °C/min ramp increase up to 240 °C, maintaining this temperature for 11 minutes, with a total running time of 61 minutes. This temperature programming was proposed after preliminary tests. The injector used was the split type with manual injection, maintained at a temperature of 250 °C. Helium was used as carrier gas at a constant flow rate of 1.0 mL/min.

The mass spectrometer was operated by electron impact at a source temperature of 200 °C and a ionization energy of 70 eV and a scan range from m/z 29 to m/z 400 at 3.33 scan/s. The identification of volatile compounds was based on the comparison of their mass spectra with those of compounds previously analyzed, with NIST/EPA/NIH Mass Spectral Database (Version 2008), or spectra published in journals. To confirm the identity of each component, the linear retention index (LRI) was calculated using the retention times of a homologous series of n-alkanes C8-C25 and also by comparing the LRI of authentic compounds analyzed under similar conditions (Atasoy et al., 2013; Hayaloglu et al., 2013; Madruga et al., 2013).

3 Results and discussion

3.1 Optimization of SPME parameters for volatiles in caprine Coalho cheese using response surface

The experimental results of the CCRD shown in Table 1 and in the Pareto chart (Figure 1) expose the importance of the variables analyzed and their interactions. The number of peaks is a very important variable for determining the optimization parameters of chromatographic analysis, the greater the amount of detected peaks, the greater the quantity of compounds trapped by the fiber and detected by gas chromatography.

The resulting R² in the model was 0.88421, and, according to the F test, this was a significant regression, bearing in mind that the $F_{\text{calculated}} > F_{\text{tabled}}$ and showed no lack of fit ($F_{\text{calculated}} < F_{\text{tabled}}$). Therefore, the Equation 2 for the dependent variable number of peaks was obtained.

$$y=26.47+1.69(\text{Ex})+2.20(\text{T})-1.29(\text{Ex}^2)-2.88(\text{T}^2)-2.13(\text{Eq}.\text{Ex}) \quad (2)$$

Where y is the response value predicted by the model. In this model, Ex (Extraction Time), Eq (Equilibrium Time) and T (Extraction Temperature) are the independent variables. The dependent variable in this study is the number of peaks in the chromatograms.

After analyzing the data, a response surface (Figure 2) was obtained with the purpose of finding the optimal region and thus verify which independent variable values produced the best response.

From the Pareto Chart (Figure 1), it can be observed that equilibrium time and the interactions between 1 and 3, and between 2 and 3 were not significant. Therefore, when creating the response surface, equilibrium time was fixed at the center point (20 minutes).

Based on the response surface (Figure 2), it can be observed that the region with the best response values is located between the marks of 40 minutes and 74 minutes of extraction time (Ex), and between the temperatures (T) of 38 °C and 70 °C. However, it is in the central region of the surface, where the central points of extraction time and temperature can be found, that the maximum response value can be seen.

According to Pérez-Palacios et al. (2012), an increase in extraction temperature leads to the release of a greater quantity of volatile compounds into the headspace, since the rise in temperature is proportional to the increase in the analyte

Table 1. Central composite rotatable design (2³) with the independent variables, levels and experimental responses (Y = number of peaks) of the SPME extraction of volatiles in coalho cheese.

Assay	Independent Variables			Response function
	Equilibrium Time (minutes)*	Extraction Time (minutes)*	Extraction Temperature (°C)*	Number of Peaks
1	-(10)	-(20)	-(30)	16
2	+(30)	-(20)	-(30)	20
3	-(10)	+(60)	-(30)	24
4	+(30)	+(60)	-(30)	19
5	-(10)	-(20)	+(60)	23
6	+(30)	-(20)	+(60)	26
7	-(10)	+(60)	+(60)	30
8	+(30)	+(60)	+(60)	25
9	-α(3)	0(40)	0(45)	24
10	+α(37)	0(40)	0(45)	26
11	0(20)	-α(6)	0(45)	19
12	0(20)	+α(74)	0(45)	25
13	0(20)	0(40)	-α(20)	16
14	0(20)	0(40)	+α(70)	19
15	0(20)	0(40)	0(45)	29
16	0(20)	0(40)	0(45)	30
17	0(20)	0(40)	0(45)	28

*Coded independent variables and real.

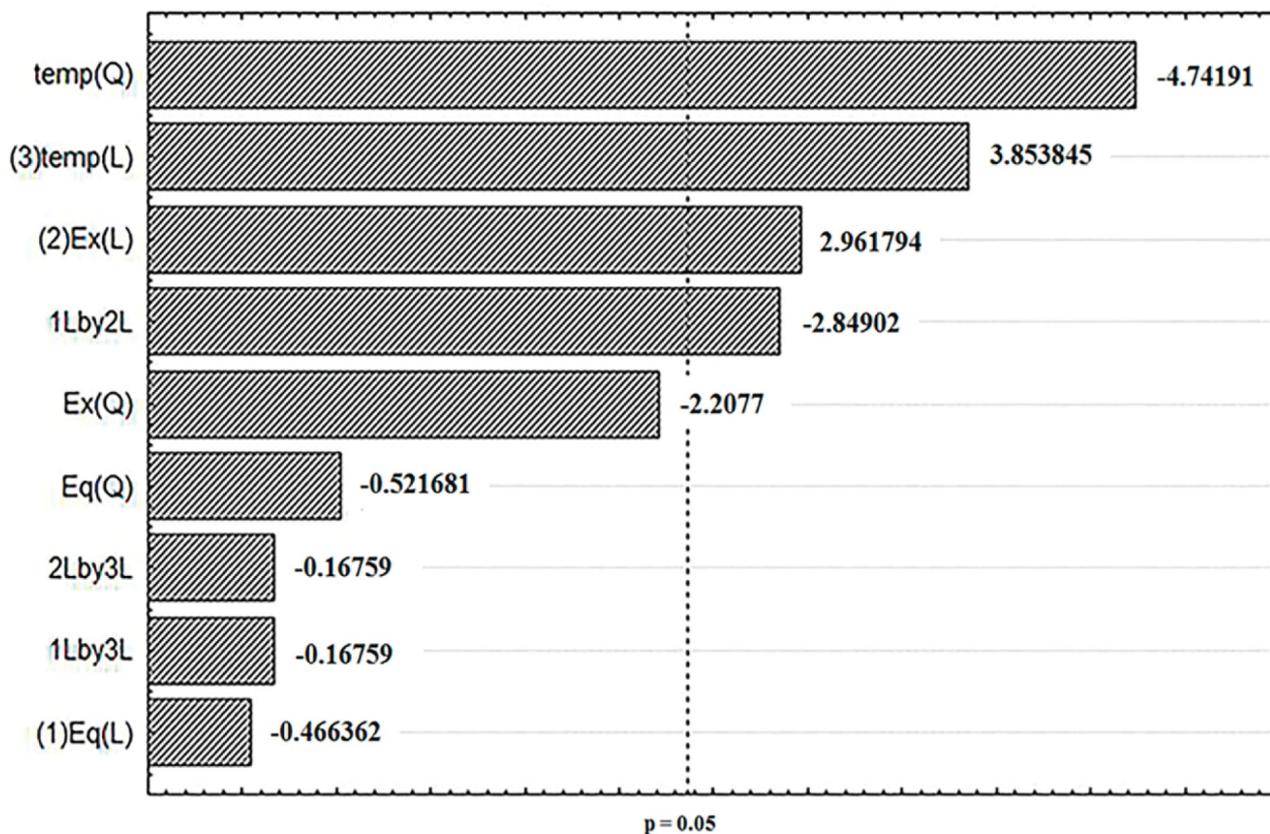


Figure 1. Pareto graphic of the dependent variable 'number of peaks'. *temp(Q): Extraction Temperature - Quadratic; temp(L): Extraction Temperature - Linear; Ex(L): Extraction Time - Linear; 1Lby2L: interaction between the parameters Equilibrium Time (Linear) and Extraction Time (Linear); Ex(Q): Extraction Time - Quadratic; Eq(Q): Equilibrium Time - Quadratic; 2Lby3L: interaction between the parameters Extraction Time (Linear) and Extraction Temperature (Linear); 1Lby3L: interaction between the parameters Equilibrium Time (Linear) and Extraction Temperature (Linear); Eq(L); Equilibrium Time - Linear.

diffusion coefficient. However, high temperatures can also lead to the formation of aromatic compounds derived from the Maillard reaction or from caramelization. It may also provoke the degradation of some of the compounds from the product.

Longer exposure of the fiber to the headspace enables greater capture of the compounds until polymer saturation. Therefore, it is usually necessary to have longer extraction times during analysis, before balance between the headspace and the fiber polymer is reached (Barros et al., 2012; Trujillo-Rodríguez et al., 2014).

3.2 Desirability parameter

Figure 3 shows that the optimal parameters of this study were located in the exact region of the center point. The desirability parameter indicates the optimal region, taking into account all the variables, at the same time. The optimization desirability value was 0.74793, which is considered a good, acceptable value, according to the values reported by Akhanazarova & Kafarov (1982).

Trujillo-Rodríguez et al. (2014), who studied the type of polymer used in extraction via SPME, reported that the

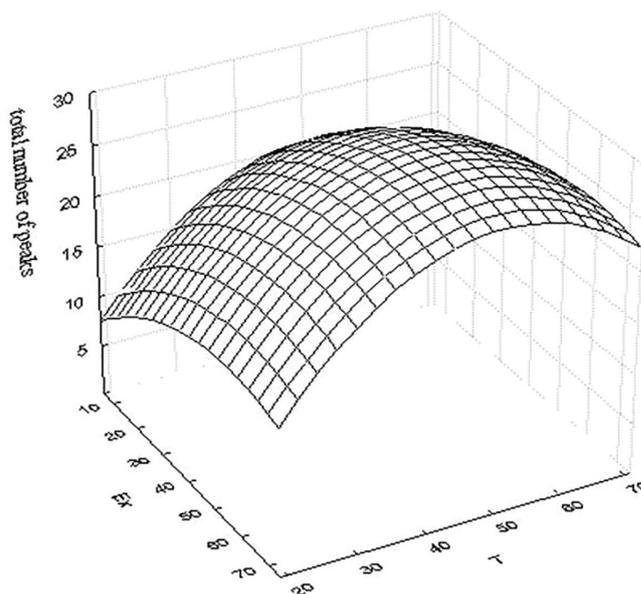


Figure 2. Response Surface for the dependent variable 'number of peaks'. Equilibrium time (Eq) was fixed at the center point. Ex (Extraction Time) and T (Extraction Temperature).

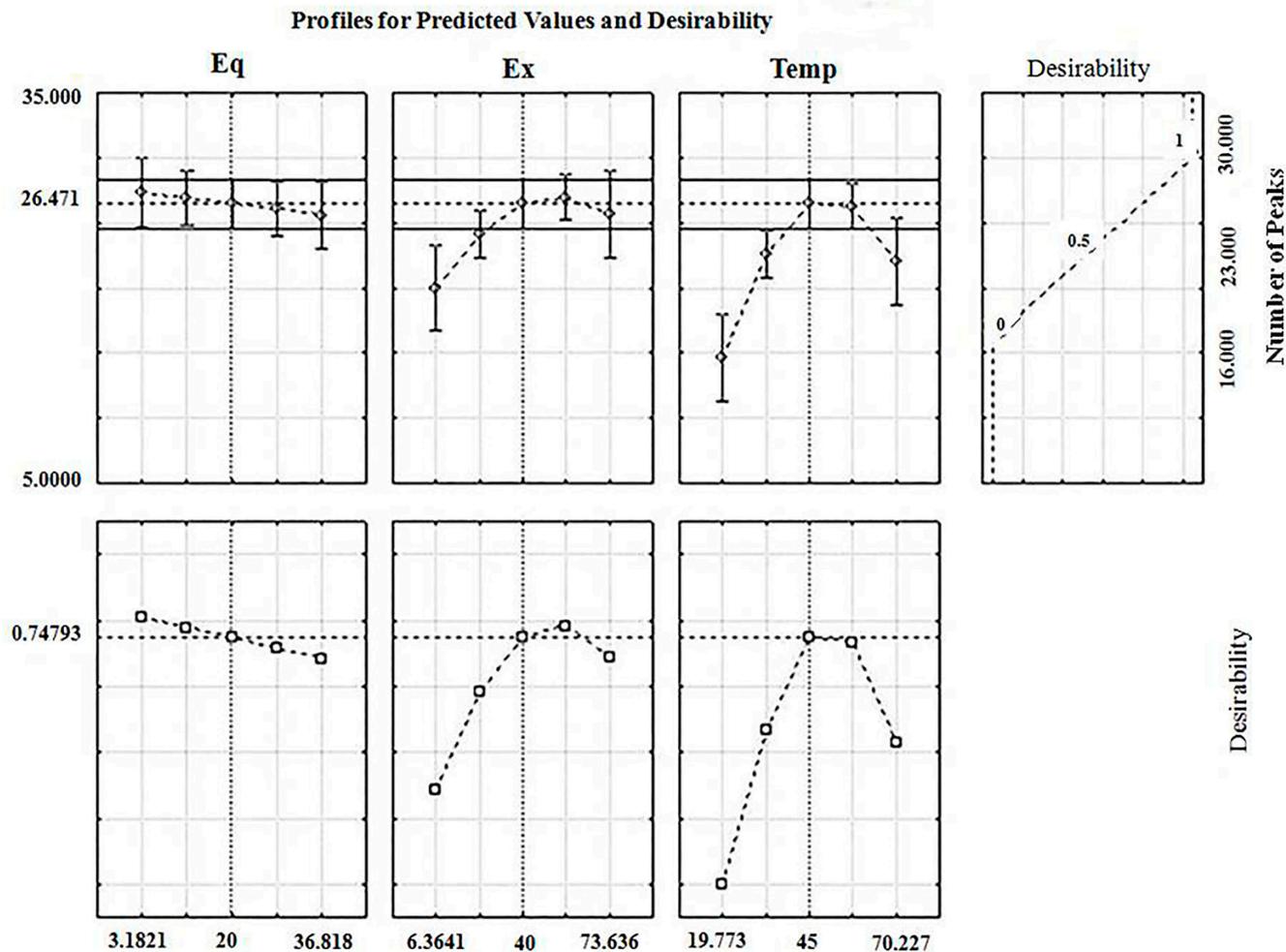


Figure 3. Profile of Predicted Values and Desirability for the number of peaks captured by HS-SPME-GC-MS in Coalho cheese. Eq (Equilibrium Time), Ex (Extraction Time) and Temp (Extraction Temperature).

optimal conditions for cheese extraction are obtained with a temperature of 45 °C and extraction time of 40 minutes. The desirability values these authors obtained are the same as the ones obtained in this study.

In their research on Gokceada cheese, a traditional type of goat cheese, Hayaloglu et al. (2013) reported that the ideal conditions for volatile compound extraction were obtained at 40 °C with extraction time of 30 minutes. These conditions differ from those found in this study. Bontinis et al. (2012) also reported a desirability value different from the one obtained in this study; they performed the extraction of volatiles from Xinotyri cheese, a traditional Greek goat cheese, at the temperature of 65 °C, with extraction time of 20 minutes.

3.3 Model validation

Applying the desirable value of each dependent variable to the equation of number of peaks (Equation 1), the result obtained was 26 peaks. With the optimal points reproduced in triplicate,

the mean of the experimental data was 30 peaks, and the standard deviation was 1. When submitted to ANOVA and to a t-test ($p < 0.05$), both predicted and experimental values showed no significant difference. This indicates that the proposed model was adjusted to the experimental data. Besides, the experimental values obtained were all within the 95% reliability limit.

3.4 Characterization of the volatile compounds found in caprine Coalho cheese

A total of 32 volatile compounds were detected after being extracted (through SPME) from caprine Coalho cheese: 5 alcohols, 5 esters, 3 ketone, 6 acids, 3 aldehydes, 3 terpenes and 7 hydrocarbons (Table 2). Among these compounds, the most abundant were decane and hexanoic acid. The latter is responsible for the characteristic smell of goat dairy products.

The alcohol compounds found in cheeses can be formed in a number of metabolic reactions: through fermentation, ketone reduction, aminoacid and fatty acid degradation, all performed by microbiological and biochemical activities (Kaminarides et al.,

2007; Bontinis et al., 2012). Most of these compounds are usually formed during storage, but are quickly dispersed due to their high volatility (Hayaloglu et al., 2013).

The most abundant alcohol compound found in the Coalho cheese was 2-Propanol, 1-methoxy. Delgado et al. (2010) also reported its presence in ovine cheese. The esters detected in the caprine Coalho cheese were ethyl acetate, ethyl butanoate, ethyl hexanoate, ethyl octanoate and ethyl decanoate. According to Bontinis et al. (2012), esters are originated in the esterification reaction between short chain fatty acids and ethanol. Among the esters found in the Coalho cheese ethyl butanoate, ethyl

hexanoate and ethyl octanoate were the most common. All three are originated from acids which possess the typical smell of caprine cheese. In their research, Delgado et al. (2011) also found high proportions of these three esters.

Only one ketone was found in the caprine Coalho cheese: 2-heptanone. This compound can be found in other types of cheese and has an herbaceous aroma. It is the product of β -oxidation of free fatty acids, which are released during lipolysis (Vandeweghe & Reineccius, 1990; Bontinis et al., 2012). Atasoy et al. (2013) who studied ovine, caprine and mixed Urfa cheese, reported the detection of this compound in both fresh and matured

Table 2. Volatile compounds in the headspace of in caprine Coalho cheese.

Number	Compounds	C ₁ ^a	CV ^b	C ₂ ^c	CV	C ₃ ^d	CV	LRI ^e	ID ^f
Acohol									
1	Ethanol	4.42	73	3.73	20	1.13	43	< 600	A
3	2-Butanol	ND ^g	-	21.16	1	ND	-	< 600	A
8	3-methyl-1-Butanol	0.35	3	3.67	12	1.07	10	760	A/B
11	1-Pentanol	ND	-	0.16	104	0.55	87	< 600	A
26	2-methyl-1-Decanol	1.78	18	ND	-	ND	-	1160	A/B
Esters									
4	Ethyl Acetate	0.87	63	1.77	99	2.01	70	< 600	A
12	Ethyl Butanoato	3.38	14	8.99	1	4.82	54	808	A/B
19	Ethyl Hexanoato	4.48	13	6.32	1	10.24	8	1007	A/B
28	Ethyl Octanoato	5.50	6	2.11	14	4.86	34	1198	A/B
30	Ethyl Decanoato	3.10	15	ND	-	ND	-	1404	A/B
Ketones									
2	2-pentanone	3.64	18	3.4	18	1.70	53	< 600	A
7	3-hydroxy-2-Butanone	3.91	99	8.73	14	ND	-	722	A/B
15	2-Heptanone	2.32	7	4.2	12	1.53	11	899	A/B
Acids									
5	Acetic acid	ND	-	0.89	5	15.20	3	< 600	A
13	Butanoic acid	2.96	10	4.02	32	3.10	20	823	A/B
14	Pentanoic acid	ND	-	3.09	6	0.73	23	880	A/B
20	Hexanoic acid	16.00	20	7.60	71	13.76	60	1016	A/B
27	Octanoic acid	1.47	50	0.02	2	4.17	34	1198	A/B
29	Decanoic acid	0.27	3	ND	-	ND	-	1401	A/B
Aldehydes									
6	3-methylbutanal	0.61	54	0.95	38	0.26	7	< 600	A
17	Benzaldehyde	5.86	117	ND	-	ND	-	986	A/B
21	2-octenal	ND	-	ND	-	1.85	45	1033	A/B
Terpenes									
16	α -Pinene	2.21	3	5.51	19	5.11	3	945	A/B
31	Copaene	0.83	141	1.32	14	2.56	65	1406	A/B
32	Caryophyllene	2.95	141	6.64	11	7.50	8	1460	A/B
Hydrocarbons									
9	3-ethyl-2-methyl Pentane	0.29	73	0.12	39	0.91	67	765	A/B
10	Tolueno	0.18	21	0.66	33	0.59	30	789	A/B
18	Decane	14.14	8	1.38	10	5.60	1	1003	A/B
22	3-methyldecane	1.15	5	ND	-	1.78	7	1059	A/B
23	4-methyl-1-Decene	2.10	2	ND	-	ND	-	1069	A/B
24	Undecane	9.16	0	3.56	9	8.97	11	1104	A/B
25	4-methydecane	5.02	23	ND	-	ND	-	1118	A/B

^a Artisanal caprine cheese. ^b % CV, percentage of coefficient of variation. ^c caprine cheese - 1. ^d caprine cheese - 2. ^e Linear retention indices. ^f A, mass spectrum and LRI agree with those of an authentic compound ran on VF-5ms column. B, mass spectrum agrees with reference spectrum in the NIST/EPA/NIH mass spectral database and LRI agree with those in the literature (Adams, 2007; Madruga et al., 2013; Mottram, 2014). ^g ND, not detected.

samples. Hayaloglu et al. (2013) also found 2-heptanone in caprine Gokceada cheese.

Acids are compounds that can be formed through lipolysis, proteolysis and lactose fermentation. It is the presence of acids such as hexanoic acid, octanoic acid and decanoic acid that gives goat dairy products their distinctive smell. These three acids are also known as caproic acid, caprylic acid and capric acid respectively (Bontinis et al., 2012; Hayaloglu et al., 2013). The acids found in caprine Coalho cheese were butanoic acid, hexanoic acid, octanoic acid and decanoic acid; hexanoic acid was the most abundant of these four. Butanoic acid is known in literature for its rancid, unpleasant smell, and its presence in cheese samples is therefore undesirable (Delgado et al., 2011). The aldehydes found in the Coalho cheese were 3-methylbutanal, heptanal and benzaldehyde, the last being the most abundant. Benzaldehyde has a typical almond-like aroma and was also detected in cheeses by various authors in their research (Delgado et al., 2010; Wolf et al., 2010). Aldehydes are known as transitory volatile compounds, because they are quickly converted into acids or alcohols. Some are formed through the Strecker reaction, such as 2-methylbutanal (Atasoy et al., 2013; Kondyli et al., 2013).

Three types of terpene were detected in the Coalho cheese: α -pinene, copaene and caryophyllene. According to Wolf et al. (2010), terpenes are compounds produced by plants and therefore, their presence in milk or in dairy products is due to whatever vegetables the animal has eaten. This way, the presence of these compounds may help in determining the geographical origin of the cheese. The other aromatic compounds found in the caprine Coalho cheese were 10 hydrocarbons, of which decane was the most common.

4 Conclusions

The optimal conditions for SPME extraction of volatile compounds from caprine Coalho cheese were obtained with equilibrium time of 20 minutes, extraction time of 40 minutes, and extraction temperature of 45 °C. Optimization was confirmed by the desirability parameter, which attained a value considered good and acceptable by literature. Therefore, the model obtained was validated. In the optimal conditions, 32 volatile compounds from caprine Coalho cheese were detected.

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