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Optimization of Headspace Solid-Phase Microextraction and Gas Chromatography Coupled with Mass Spectrometry Technique for the Quantification of Volatile Compounds in a Fresh Cheese (Oaxaca)

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Abstract

The Head Space Solid Phase Microextraction Method (HS-SPME), coupled with Gas Chromatography and Mass Spectrometry (GC-MS) was optimized to quantify Volatile Organic Compounds (VOC's) in a fresh Oaxaca cheese during its shelf life of 22 days. Optimal extraction conditions were ascertained by means of a multiple linear regression analysis and the generation of a response surface. Conditions to reach the equilibrium of VOC's at the headspace were established to be 30 minutes of ultrasonic agitation at 50°C. The conditions for extraction were exposure of the fiber at room temperature for 30 minutes. Optimal extraction of volatile compounds released by the Oaxaca cheese allowed identification of 21 volatile compounds: 8 acids, 1 alcohol, 4 aldehydes, 4 ketones, 1 ester, and 3 lactones. Quantification of VOC's was carried out using the technique of standard addition. The limits of quantification ranged from 0.0033 to 0.0311 ng/μg. Ten compounds were identified with the heaviest concentration and are listed as follows, from heaviest to lightest: propionic acid, acetoin, acetic acid, diacetyl, capric acid, caprylic acid, δ-decalactone, δ-dodecalactone, γ-dodecalactone, butyraldehyde. During storage of the cheese, the most significant changes in the concentration of VOC's took place between day 1 and day 15. Based on the results obtained, it can be concluded that the optimization of the SPME makes it possible to isolate and quantify VOC's of fresh cheeses in an efficient and reproducible manner.

Keywords: SPME; Volatile organic compounds (VOC's); Fresh cheese; Oaxaca cheese

Introduction

The attributes of aroma in cheese represent decisive factors in determining its quality as well as consumer preference. The typical aroma of each cheese variety is the result of a complex balance among volatile and non-volatile chemical compounds produced by the components of the curd (fat, protein and remaining lactose). These compounds are generated during processing of the cheese, and their production continues during storage and ripening [1,2]. Secondary catabolic reactions that occur during storage and ripening are responsible for the unique aroma profile of a specific variety of cheese [3]. The type and concentration of aromatic components are used to typify cheeses and to set up parameters for good quality.

Volatile components of cheese are present in trace amounts. To analyze and quantify them, it is necessary to isolate and concentrate them. The Headspace Solid-Phase Microextraction (HS-SPME) method has been shown to successfully extract volatile compounds from various foods and beverages [4] to then be analyzed by Gas Chromatography (GC). These techniques are widely used since they allow isolation of volatile analytes of solid and liquid matrices easily and in short duration [5-7].

Ideally, extraction of volatile compounds requires optimization of the SPME process, carried out by standardizing the parameters involved and applying a statistical model that considers the interactions among them. In HS-SPME, the analyte must be in balance between the sample matrix and the gas phase to facilitate absorption or adsorption on the stationary phase of the fiber when exposed to the headspace. The time necessary to establish equilibrium in the headspace is influenced by the temperature at which extraction is performed, as well as the sort of fiber and its capacity to absorb analytes present in the matrix of samples [8]. Thus, the time of equilibrium, the time of extraction, and the temperature of extraction must all be standardized in order to obtain optimal conditions that allow for capture of a larger number of compounds [9-11].

The fraction of Volatile Organic Compounds (VOC's) in cheeses has been studied using extraction techniques, including static headspace, purge and trap, and solvent-assisted flavor evaporation extraction [12-15]. These techniques entail lengthy times or exhaustive pre-concentration stages, which often result in the appearance of artifacts, and complicate quantification of VOC's. Conversely, HS-SPME can reduce the time of analysis substantially as well as the number of steps of sample manipulation, all of which minimize the appearance of artifacts [16]. Thus, this technique is reliable, quick, and effective in enabling quantification of volatile compounds present in a complex matrix such as cheese.

VOC's have been thoroughly studied in ripened cheeses [17-19] in which aroma and flavor compounds indicate a period of time to establish the proper shelf life and to typify cheeses. This is particularly useful for cheeses with a designated origin. By contrast, few studies have focused on ascertaining VOC's in fresh cheeses.

One kind of fresh cheese where VOC's are generated during storage is the Oaxaca cheese [20]. It is an especially demanded style of Mexican cheese of the pasta filata group [21]. Owing to its cultural importance, there is interest in quantifying the volatile compounds responsible for its flavor and aroma during its shelf life, since in most fresh cheeses the aroma profile changes rapidly due to their high moisture content. For this reason, the objective of this study was to optimize the SPME method to analyze a fresh cheese, using Oaxaca cheese as a prime example.

The results of this study are significant not only because the technique is viable and replicable and it is applicable to quantify volatile compounds in similar fresh cheeses, but also since fresh cheeses are difficult to study because their compounds change rapidly.

Materials and Methods

Production of Oaxaca cheese

Fresh cow's milk was obtained and used from the dairy plant in the Department of Veterinary Medicine and Zootechnics of the Universidad Autónoma del Estado de México (UAEMex). Three kinds of cheese were manufactured in the facilities of the pilot food factory in the department of chemistry, in 10-kilogram batches, following a standardized process [22]. Type I (MC), used pasteurized milk and mesophilic lactic cultures (*Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *cremoris*) (R-704, Chr. Hansen, Horsholm, Denmark). Type II (TC), used pasteurized milk and thermophilic lactic cultures (*Streptococcus salivarius* ssp. *termophilus*) (ST1.14, Chr. Hansen, Horsholm, Denmark). Type III (RM) used naturally acidified raw milk.

For cheeses manufactured with pasteurized milk, the milk was pasteurized at 72°C for 15 s, cooled to 34°C and inoculated with a 1% (v/v) of lactic cultures. Food calcium chloride (0.02%) (Hansen, Denmark) was added, and the inoculated milk was ripened for 60 min at 34°C. Diluted pure chymosin (0.22 mL/ kg milk, dissolved 1:20 in distilled water (Hansen, Denmark) was added to the ripened milk. Following a 40 min set, the gel was cut with stainless steel wire knives into 1 cm lengths. The curd was gently stirred for approximately 250 min for cheese made with thermophilic culture, and 310 min for mesophilic culture. When pH in the whey reached 5.4 (± 0.03), the whey was drained. When pH was approximately 5.2 (in whey draining of the curd), the curd was transferred to a plastic tub, and was kneaded twice by hand in a volume (20% of the initial milk volume) of warm (80°C and 77°C) water. Each kneading was performed for 3 min. The hot curd was stretched into a long strand 2.5-3.0 cm wide and 0.7-0.8 cm thick, which was then allowed to cool for 20 min on a dry, stainless steel surface. The cold strand was weighed and salted by dry salting (1.8% salt, w/w). The salted curds were rolled up into approximately 500 g balls and allowed to drain for 2 hours at room temperature. The cheese balls were packed in polyethylene bags and placed in cold storage at 4°C. The same process conditions were used for the cheese made with raw milk, except that the milk was not pasteurized and the time of stirring was approximately 140 min.

Standards used in the study

21 aromatic compounds were used for a standard addition method to identify and calculate the concentration of VOC's supplied by Ventós (Barcelona, Spain), with Chromatography purities between 78% and 99%, dissolved in ethanol. Analytes were selected according to previously published studies [1,23,24].

Selecting and conditioning the fiber

Three kinds of fibers with different stationary stages were tried: (1) divinylbenzene / Carboxen / polydimethylsiloxane (DVB/CAR/ PDMS) 50/30 μm; (2) polydimethylsiloxane (PDMS) 100 μm; and (3) polydimethylsiloxane/divinylbenzene (PDMS/DVB) 65 μm (Supelco, Bellefonte, PA, USA). The fibers were conditioned as specified by the manufacturer. The best extraction performance was obtained with DVB/CAR/PDMS. Before extraction, the fiber was conditioned in the GC injector at 220°C for 60 minutes.

Optimized extraction using HS-SPME

A factorial design 2^{4-1} was used, in which four factors at two levels were analyzed: equilibrium temperature (30°C and 50°C), equilibrium time (10 min and 30 min), extraction time (20 min and 40 min), and desorption time (1 min and 5 min). The experiment was carried out in triplicate. These parameters exhibited the most influence in optimizing the technique variations [9,25,26]. The response variable was indicated by the area of the peaks of six analytes representative of components in the cheese (acetaldehyde, diacetyl, acetic acid, acetoin, valeric acid, and caprylic acid).

To prepare the sample, 20 g of grated cheese (homogenized by quartering) were placed in 75 mL vials sealed with a polytetrafluoroethylene silicone septum (Supelco, Bellefonte, PA, USA). Each vial underwent the corresponding conditions expressed in the experimental design. Extraction was performed using the fiber DVB/ CAR/PDM and by exposing it to the headspace of the vial. Then, the fiber was transferred to the GC injector to desorb the extracted analytes.

Results of optimizing the extraction by HS-SPME were analyzed by means of a multiple linear regression model and response surface using Stat graphics Centurion XVI (Statpoint Technologies Inc., Virginia, USA).

Chromatographic methods

Volatile compounds were analyzed by gas chromatography (Perkin Elmer, model Autosystem XL) with a split-off injector and FID. Identity of the compounds was also verified with a GC (Varian, CP-3900, 101909) coupled with a mass selective detector (Varian, Saturn 2100T). In both cases, a capillary column Stabiliwax-MS, RESTEK brand, 30 m × 0.25 mm ID and 0.25 μm df was used, with helium as a carrier gas with a flow of 1 mL/min. The injector was kept at 230°C, using manual injections. Samples were desorbed in the injection port of the GC for 5 min. Program conditions were maintained as follows: 35°C kept for 8 min, and a temperature increase with two ramps: the first from 15°C/min. up to 130°C, kept for 10 min, and the second from 7°C/min. up to 230°C and kept for 15 min, with a total functioning time of 52.5 min. Temperature of the transference line of the GC/MS was 230°C. The mass spectrometer operated at an energy of electronic impact of 10 mV and a data gathering frequency of 1 scan per second over a mass interval of 10-400 m/z.

Identifying volatile organic compounds (VOC's)

The following compounds were used for the standard additions: acetaldehyde, diacetyl, 2-3 pentanedione, methylamyl ketone, acetic acid, acetoin, propionic acid, furfural, decanal, butyraldehyde, butyric acid, isovaleric acid, valeric acid, capric acid, ethyl laurate, caprylic acid, δ-decalactone, sulfurol, γ-dodecalactone, δ-dodecalactone. 0.1 μL of each of the analytes was injected separately with the injector in split-off mode to obtain retention time, as well as to check their chromatographic purity.

To identify volatile compounds in the cheese using the GC, retention times of reference compounds were compared with retention times of a cheese sample with standard additions of the analytes-except for furfural, which was added to a separate cheese sample, as it was noticed that it reacted with the other analytes.

Using GC-MS, identification of volatile compounds was undertaken by comparing the mass spectra with the standard spectra of previously identified volatile compounds, as well as with the mass spectra data base contained in the NIST library of the equipment. All samples were analyzed twice using the same fiber.

Quantifying volatile organic compounds in Oaxaca cheese

Quantification of VOC's was carried out based on the technique of standard addition. A standard solution was prepared by mixing 1 μL of each of the 21 reference compounds. Increasing volumes of standard solution (0.2, 0.4, 0.6, 0.8, and 1.0 μL) were added to 20 g of cheese (made with raw milk) contained in a 75 mL vial, which was placed in a water bath with ultrasonic agitation at 50°C for 30 min. Extraction of VOC's was made by exposing the selected fiber to the headspace of the vial for 30 min at room temperature. Desorption of VOC's was carried out by placing the fiber in the injection port of the GC for 5 min. A calibration curve of each of the 21 volatile compounds identified in raw milk cheese was created by using known concentrations of standard additions. Using linear regression, each peak area in the chromatogram was used to determine the unknown concentrations of the VOC's in the

Compound	RT (min)	Mp (°C)	Bp (°C)	MW (g/mol)	δ(g/cm³)	Polarity	Solubility in water	Vpress (hPa at 20°C)
Acetaldehyde	5.52	-123.0	20.4	44.1	0.78	P	U	990
Diacetyl	6.85	-3.0	88.0	86.0	0.99	P	LSW	65
2-3 Pentanedione	9.85	-52.0	111.0	100.1	0.96	P	LSW	28.5
Methylamyl ketone	12.71	-35.5	151.0	114.2	0.82	P	LSW	4.5
Acetic acid	14.49	17.0	118.1	60.0	1.05	P	U	15
Acetoin	16.53	15.0	143.0	88.0	1.01	P	U	ND
Propionic acid	17.20	-21.0	141.0	74.1	0.99	P	U	3.9
Furfural	17.72	-36.0	162.0	96.1	1.16	P	LSW	2.3
Decanal	18.49	-5.0	209.0	156.2	0.83	AP	IN	0.2
Butyraldehyde	19.30	-99.0	74.8	72.1	0.80	P	LSW	122
Butyric acid	20.89	-5.0	163.0	88.1	0.95	P	U	1.0
Isovaleric acid	22.25	-26.0	176.0	102.1	0.92	P	LSW	0.5
Valeric acid	28.67	-34.0	185.0	102.0	0.93	P	LSW	0.2
Capric acid	28.88	-4.0	205.0	116.2	0.92	P	LSW	0.3
Ethyl laurate	33.84	-10.0	272.0	228.4	0.86	AP	IN	ND
Caprylic acid	36.87	17.0	238.0	144.2	0.91	AP	IN	0.1
Lactic acid	37.71	16.8	125.0	90.1	1.21	P	U	0.0041
δ-decalactone	38.56	-25.0	284.0	170.3	0.97	AP	IN	ND
Sulfurol	39.77	ND	280.0	143.2	1.19	P	U	ND
γ-dodecalactone	40.69	7.0	291.0	198.3	0.93	AP	IN	ND
δ-dodecalactone	41.34	-7.0	285.0	198.0	0.94	AP	IN	ND

RT: Retention Time; Mp: Melting point; Bp: Boiling point; MW: Molecular weight; δ: Density; Vpress: Vapor pressure. P: Polar; AP: apolar; U: Unlimited; LSW: Lightly Soluble in Water; IN: Insoluble; ND: Non-determined.

Table 1: Physiochemical properties of VOC identified in Oaxaca cheese.

Compound	RT (min)	Equation	r²	VC%	DL ng/μg	QL ng/μg	Recovery percentage
Acetaldehyde	5.52	y = 3746772.02x + 103620.78	0.987	2.7	0.00293	0.00887	90.87
Diacetyl	6.85	y = 709059x + 5646.6	0.945	15.2	0.00737	0.02234	78.06
2-3 Pentanedione	9.85	y = 3577799.04x - 1499.9	0.955	22.6	0.00835	0.02529	78.24
Methylamyl ketone	12.71	y = 4714614.17x + 5056.69	0.974	15.1	0.00709	0.02148	82.27
Acetic acid	14.49	y = 159671x + 849.14	0.982	8.9	0.00554	0.01680	92.88
Acetoin	16.53	y = 163518x + 2671	0.973	7.1	0.00570	0.01728	88.44
Propionic acid	17.20	y = 107829x + 14763	0.977	1.7	0.00670	0.02029	95.68
Furfural	17.72	y = 4528428.9x + 68216	0.983	8.0	0.00782	0.02369	76.34
Decanal	18.49	y = 1906911.89x + 13981	0.964	10.1	0.00888	0.02690	80.03
Butyraldehyde	19.30	y = 723336x + 53.96	0.962	22.9	0.00403	0.01220	59.79
Butyric acid	20.89	y = 412505x + 27452	0.970	4.3	0.00919	0.02785	64.84
Isovaleric acid	22.25	y = 1255034.82x + 51734	0.951	7.1	0.01027	0.03111	89.36
Valeric acid	28.67	y = 1028655.31x + 51205	0.979	4.0	0.00675	0.02045	78.33
Capric acid	28.88	y = 3284654.32x - 39023	0.983	9.5	0.00960	0.02911	89.84
Ethyl laurate	33.84	y = 5407740.25x + 26006	0.971	5.8	0.00111	0.00337	80.64
Caprylic acid	36.87	y = 1764166.67x + 17995	0.973	10.9	0.00812	0.02460	79.72
Lactic acid	37.71	y = 775833.6x + 12405	0.974	6.0	0.00413	0.01253	66.04
δ-decalactone	38.56	y = 1000821x + 8093	0.973	8.3	0.00510	0.01545	85.25
Sulfurol	39.77	y = 4565606.08x - 14564.63	0.963	15.5	0.00574	0.01740	93.44
γ-dodecalactone	40.69	y = 559289x + 21414	0.987	2.6	0.00630	0.01909	74.65
δ-dodecalactone	41.34	y = 649639x + 18079	0.983	5.1	0.00641	0.01944	82.59

RT: Retention Time; r²: Correlation Factor; VC: Variation Coefficient; DL: Detection Limit; QL: Quantification Limit. y: Ratio of Compound area /area of the added Standard; x: Ratio of Compound Concentration /Concertation of added Standard.

Table 2: Validation parameters including regression equations in order to quantify aromatic compounds in cheese determined by SPME-GC.

samples. The data obtained was used to ascertain the concentration of VOC's in cheeses produced with mesophilic and thermophilic cultures. While the cheeses were stored, quantification was carried out on days 1,8,15 and 22.

To explain the behavior of the parameters for the validation of the quantification method, the data was analyzed including the coefficient of variation, recovery percentage, and detection and quantification limits. The physiochemical characteristics of molecules identified in the cheeses was considered in Table 1.

Statistical analysis

Parameters with the most influence on extraction efficiency were found out by means of multiple regression analysis considering a correlation factor (R^2) of over 0.8. The significant effect was verified through an ANOVA (α=0.05). Optimal values for these parameters were calculated by generating a response surface.

To determine significant differences among the concentrations of VOC's during storage, an ANOVA and a multiple comparison analysis by LSD were applied. In all cases, Stat graphics Centurion XVI (Statpoint Technologies Inc., Virginia, USA) was used.

Compound	RM				MC				TC			
	Day 1	Day 8	Day 15	Day 22	Day 1	Day 8	Day 15	Day 22	Day 1	Day 8	Day 15	Day 22
Acetaldehyde	BLQ	BLQ	0.0183b	0.0187b	BLQ	BLQ	0.0075b	0.0138b	BLQ	BLQ	0.0031b	BLQ
Diacetyl	BLQ	BLQ	0.0520b	0.0497b	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	0.0014	0.0033
2-3 Pentanedione	0.0007a	0.0010b	0.0012b	0.0014bc	0.0007	0.0010	0.0012	0.0015	0.0012ab	0.0026ab	0.0016b	0.0009a
Methylamyl ketone	BLQ	BLQ	0.0013a	0.0230b	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
Acetic acid	0.0026a	0.0286a	0.0683b	0.0054a	0.0195	0.0126	0.0183	0.0192	0.0140	0.0119	0.0136	0.0157
Acetoin	BLQ	0.0262a	0.3868b	0.2581c	0.0373a	0.1424ab	0.5444b	0.2120ab	0.0389	0.0257	0.3638	0.1099
Propionic acid	BLQ	0.7484c	0.5354ac	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
Furfural	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
Decanal	BLQ	0.0085b	0.0005a	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
Butyraldehyde	0.0008	0.0004	0.0010	0.0018	0.0017	0.0010b	0.0011	0.0006	0.0020	0.0022	0.0015	0.0005
Butyric acid	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
Isovaleric acid	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
Valeric acid	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
Capric acid	0.0145	0.0150	0.0160	0.0135	0.0128ab	0.0127a	0.0128ab	0.0131b	0.0130	0.0135	0.0130	0.0145
Ethyl laurate	0.0020a	0.0185b	0.0025a	0.0020a	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
Caprylic acid	0.0175	0.0120	0.0005	0.0075	0.0047a	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
Lactic acid	0.0095a	0.0165ab	0.0370b	0.0245ab	0.0241a	BLQ	BLQ	BLQ	0.0285	0.0130	0.0010	0.0095
δ-decalactone	0.0010a	0.0310ab	0.0840b	0.0095ab	0.0273	0.0050	0.0192	0.0071	0.0330	0.0225	0.0265	0.0090
Sulfurol	0.0065a	0.0100a	0.0295b	0.0065a	0.0098	0.0088	0.0049	0.0045	0.0045a	0.0080bc	0.0085c	0.0050ab
γ-dodecalactone	0.0100a	0.0200a	0.3215b	BLQ	BLQ	BLQ	0.0328	BLQ	BLQ	BLQ	0.0230c	BLQ
δ-dodecalactone	0.0320a	0.0350a	0.0850b	BLQ	BLQ	BLQ	0.0331b	BLQ	BLQ	BLQ	0.0210b	0.0005ab

Data are the mean value of each sample analyzed in duplicate. [a,b,c]Different letters in the same row indelicate significant differences between the values of the mean (P<0.05); values with no letter indicate there are no significant differences. Arbitrary units (area of the peak) were used to assess the amounts of each compound. BLQ=Below limit quantification. RM=Cheese produced with raw milk; MC=Cheese produced with a mesophilic culture; TC=Cheese produced with a thermophilic culture

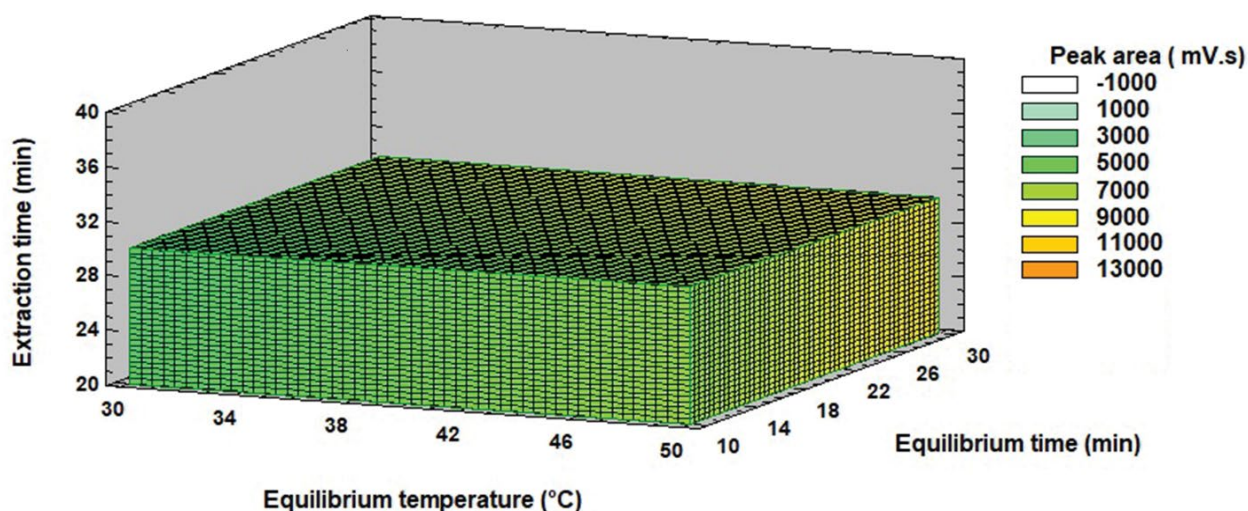**Table 3:** Changes in the concentration of VOC in Oaxaca cheeses during storage (ng/μg).

Figure 1: Response surface estimated to maximize the response in the validation of the HS-SPME-GC-MS technique considering the three factors that had a significant effect.

Results and Discussion

Optimal conditions in the HS-SPME to extract VOC's

In this study, the area of peaks generated by each VOC's in the chromatograms was considered a response variable. Based on the proposed experimental design, it was found that the only variables with a significant impact on the response variable ($\alpha=0.05$) were the time and temperature used in the ultrasonic bath. The region with the best response values was at 50°C and 30 min equilibrium time (Figure 1). In a similar manner, forty minutes of exposure of the fiber to the headspace in the vial was needed to maximize capture of the raw milk cheese VOC's. 5 min of exposure of the fiber in the GC injector to attain the maximum desorption of volatiles. It was observed that 5 min of desorption and 50°C at the ultrasonic agitation bath improved peak signals in the chromatograph, possibly because the liberation of VOC's in the fiber matrix were optimized. To the extent that increase in temperature increases liberation of more volatile compounds in the headspace, as the diffusion coefficient of the analyte increases [27]. Furthermore, all these factors allowed certain volatiles to be concentrated selectively in the fibers with simultaneous displacement of others [16,28], which may exclude some analytes of low molecular weight [29]. In samples treated at temperatures over 50°C, the response signal in the GC decreased, possibly because the melted fat concentrated on the surface of the cheese and made liberation of VOC's difficult. A longer exposure of the fiber to the headspace generated a stronger response signal. These results agree with other studies that suggest that in order to reach equilibrium between the headspace and the polymer of the fiber, more than 30 minutes [30,31] and temperatures in the range between 40°C and 60°C is needed [17,32].

Volatile organic compounds identified in Oaxaca cheese

A total of 21 volatile compounds were detected by means of the HS-SPME-GC-MS method: 8 acids, 1 alcohol, 4 aldehydes, 4 ketones, 1 ester and 3 lactones (Table 2). The compounds identified arise from the degradation of the curd components by the enzymes present (microbial, remaining chymosin and alkaline protease) to low-weight molecules that could be identified by means of the employed technique [33,34]. During processing and storage, these compounds appear and disperse rapidly, owing to their high volatility [25]. Esters originate in the esterification reaction between short chain fatty acids and ethanol [34]. Ketones are produced through β-oxidation of free fatty acids generated over lipolysis [34,35], while acids are compounds that can be produced by lipolysis, proteolysis, and lactose fermentation. Aldehydes are known as transient volatile compounds, as they quickly turn into acids or alcohols; some are produced by means of the Strecker reaction such as butyraldehyde [26,36]. Terpenes are compounds produced by plants and their presence in milk or dairy products and is due to the type of feed given to the cows [37]. Identifying the terpenes present may be useful to find out the geographic origin of the cheese.

Quantified volatile organic compounds (VOC's)

The regression equations and the validation parameters of the technique of adding a standard are shown in Table 2. The quantification of aroma compounds in the cheeses were obtained by extrapolating from the calibration curves of each compound. The curves of the standards of the 21 compounds quantified showed R^2 between 0.945 and 0.987. Based on these values, the SPME method presents a convenient linearity by which to estimate the concentration of VOC's in fresh cheeses.

The detection limit indicates the minimal concentration of an analyte that can be detected, but not necessarily quantified, under the study conditions. The quantification limit represents the minimal concentration of an analyte that can be ascertained with acceptable accuracy and precision. Results (Table 2) show that both the Detection Limit (DL) and Quantification Limit (QL) are at levels of ng/μg. The DL presented an interval from 0.00111 to 0.01027 ng/μg, whereas the QL presented an interval from 0.00337 to 0.03111 ng/μg.

The Coefficient of Variation (CV) of the compounds analyzed is in the interval from 1.7% to 22.9%, with a mean of 9.2 and a median of 8.0. The recovery percentage is between 59.79% and 95.68%. This variability may be due the equilibrium that is established between the distribution coefficients of the three main phases comprised in the system analysis (sample, headspace, and fiber). Such equilibriums are affected by four phenomena: (1) physiochemical characteristics of the cheese (ionic strength, pH, salt concentration, content of dissolved organic matter), (2) physiochemical nature of the analyte (molecular weight, polarity, vapor pressure), (3) selectivity of the fiber coating, and (4) conditions of analysis (time and temperature of extraction and desorption). Thus, propionic, acetic, capric, and valeric acids, as well as acetaldehyde and acetoin, represent compounds with the highest recovery percentages. The behavior of these molecules may be present because they have lower melting and boiling points, higher vapor pressure, and higher volatility (Table 1). All of these elements promote wider diffusion among three phases (cheese, headspace, and fiber) as their extraction is optimized.

Furthermore, the utilized fiber (DVB/CAR/PDMS) exhibits an affinity with volatile and semi-volatile organic compounds, both polar and a polar from 3 to 20 carbon atoms with a molecular weight of 40-275 g/mol. These compounds can be retained in macro-, meso-, and micro-pores of the solid part of the fiber. The selected fiber had the best performance in the extraction of volatile compounds responsible for the aroma of Oaxaca cheese, since its coating shows an affinity with all the identified compounds. It can be suggested that the possibility of the analyte to be trapped in the fiber depends on its concentration at the headspace and that the concentration would depend on diffusion attained under the extractive conditions (time, temperature, agitation).

The linearity of the estimated curves to calculate concentration of analytes presented R^2 values from 0.957 to 0.987. These correlations are adequate when taking into consideration that variation in the sensitivity of SPME is high.

Compounds with the heaviest concentration (Table 3) in RM cheese, in decreasing order, were presented as follows: propionic acid >δ-dodecalactone >δ-decalactone >acetic acid >acetoin >γ-dodecalactone >ethyl laurate >lactic acid >capric acid >caprylic acid >sulfurol, decanal >2-3 pentanedione >butyraldehyde. In MC cheese, they were: acetoin >capric acid >acetic acid >sulfurol >δ-decalactone >2-3 pentanedione >butyraldehyde; while in TC cheese, they were: acetoin >δ-decalactone >lactic acid >acetic acid >capric acid >sulfurol >butyraldehyde >2-3 pentanedione. Quantification of VOC's in cheeses during their time of storage verified that the parameters established in the technique validation are indeed viable. The results are reproducible and there is homogeneity in the data.

In fresh cheeses, aroma is an attribute that changes rapidly, with a characterization that depends on the type, quantity, and concentration of the present volatile and semi-volatile compounds. These compounds result from the degradation of cheese components by the microbial enzymes present. In this study, we found that the type and amount of VOC's in storage cheeses are maintained, but the concentration changes (Table 3). The highest concentration and variation of VOC's was observed in the RM cheese. The MC and TC cheeses had a similar

behavior, although the concentration was higher in the TC cheese. The concentration of acetaldehyde, diacetyl, acetoin, acetic acid, and capric acid increased in all three kinds of cheese, while propionic acid, butyraldehyde, lactic, butyric, isovaleric and valeric acid decreased at the end of storage.

The ANOVA results and multiple comparisons showed changes in the concentration of VOC's during storage. In the RM cheese, there were significant changes in 17 VOC's, whereas in 4 compounds, no significant differences were noticed (valeric, capric, caprylic acids, and butyraldehyde). In the MC cheese, 9 compounds presented significant differences: 2-3 pentanedione, sulfurol, γ-dodecalactone, methylamyl ketone, acetic acid, propionic acid, butyraldehyde, valeric acid, and γ-decalactone. In the TC Cheese, significant changes occurred in 6 compounds: acetaldehyde, butyric acid, 2-3 pentanedione, sulfurol, γ-dodecalactone, and δ-dodecalactone. The greatest RM cheese changes could have occurred because different types of microbial flora, as well as different enzymes, were present degrading the cheese components, which led to a faster transformation in molecules when compared to the MC and TC cheeses. The three kinds of cheese showed the most significant changes between day 1 and day 15 of storage.

Conclusions

Results suggest that the HS-SPME-GC method can be used to identify and quantify the profile of volatile and semi-volatile compounds responsible for the aroma of fresh cheeses. Validation was attained by the parameters that intervene in the method whose sensitivity can limit detection and quantification on the order of ng/μg. The experiment showed a good linear correlation in the intervals of concentrations of all compounds used, demonstrating their applicability in analyzing other fresh cheeses.

In the Oaxaca cheese, the number of volatile compounds remained practically constant during storage. Nevertheless, increases and decreases in the concentration of some volatile compounds affected the aroma profile. These factors can contribute to the understanding of unpleasant tastes in Oaxaca cheese-bitter, musty, or rancid-which are sufficient to justify a shelf life of 22 days for this particular cheese.

Acknowledgements

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