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Optimisation of a simple and reliable method based on headspace solid-phase microextraction for the determination of volatile phenols in beer

C. Pizarro*, N. Pérez-del-Notario, J.M. González-Sáiz

Department of Chemistry, University of La Rioja, C/Madre de Dios 51, 26006 Logroño, La Rioja, Spain

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ABSTRACT

A simple, accurate and sensitive method based on headspace solid-phase microextraction (HS-SPME) coupled to gas chromatography–tandem mass spectrometry (GC–MS/MS) was developed for the analysis of 4-ethylguaiacol, 4-ethylphenol, 4-vinylguaiacol and 4-vinylphenol in beer. The effect of the presence of CO_2 in the sample on the extraction of analytes was examined. The influence on extraction efficiency of different fibre coatings, of salt addition and stirring was also evaluated. Divinylbenzene/carboxen/polydimethylsiloxane was selected as extraction fibre and was used to evaluate the influence of exposure time, extraction temperature and sample volume/total volume ratio (V_s/V_t) by means of a central composite design (CCD). The optimal conditions identified were 80 °C for extraction temperature, 55 min for extraction time and 6 mL of beer (V_s/V_t 0.30). Under optimal conditions, the proposed method showed satisfactory linearity (correlation coefficients between 0.993 and 0.999), precision (between 6.3% and 9.7%) and detection limits (lower than those previously reported for volatile phenols in beers). The method was applied successfully to the analysis of beer samples. To our knowledge, this is the first time that a HS-SPME based method has been developed to determine simultaneously these four volatile phenols in beers.

1. Introduction

Beer is one of the most widely consumed alcoholic beverages in the word [1]. A crucial factor in consumer-acceptance of this product is its flavour.

Volatile phenols play an important role in the aromatic profile of beers. The two main volatile phenols conferring flavour in beer are 4-vinylguaiacol (4-VG) and 4-vinylphenol (4-VP). In addition, 4-ethylguaiacol (4-EG) and 4-ethylphenol (4-EP) can also appear in beers as a result of the reduction reaction of their corresponding vinylphenols [2]. The presence of these volatile phenols is appreciated in certain beers, whereas in others, when present at high concentrations, are considered as off-flavour that negatively affect their quality [2–4]. Hence, the establishment of the term phenolic off-flavour (POF) to describe beers with high levels of these compounds [5].

4-VG and 4-VP are the decarboxylation products of ferulic acid and p-coumaric acid, respectively [6,7]. These hydroxycinnamic acids are mainly associated with polysaccharides in the plant cell wall, more precisely of cereal grains employed in beer production [8]. During the beer production process, hydroxycinnamic acids can decarboxylate into their corresponding vinylphenols by two different ways: either by thermal impact (during high-temperature treatments such as wort boiling, whirlpool holding or pasteurisation) or by enzymatic decarboxylation (during fermentation). Usually, enzymatic decarboxylation is the predominant route for vinylphenol formation [9–11]. Hydroxycinnamic acids are flavour-inactive, having high threshold values in beer, around 600 mg/L [12]. Moreover, they are very appreciated for their antioxidant activity [13]. In contrast, volatile phenols are highly flavour-active compounds, the flavour threshold of 4-VG in beer is reported to be 0.3 mg/L, thereby producing, even at trace levels, a significant phenolic flavour in beer [12].

Due to the involvement of volatile phenols in beer flavour, simple and reliable methods that would enable routine analysis of these compounds are required.

The preferred analytical technique for the determination of volatile phenols is high performance liquid chromatography (HPLC) [2,3,10,11]. One of the main drawbacks for the determination of volatile phenols in beer by HPLC is the difficulty to reach low detection limits for the target analytes [2]. Moreover, this separation technique requires previous degasification and filtration steps, which are tedious, time consuming and can increase experimental error. Gas chromatography (GC) is preferred for volatile phenol determination in other alcoholic beverages such as wine or cider [14–16]. Analysis by GC is usually preceded by an extraction step.

* Corresponding author. Tel.: +34 941299626; fax: +34 941299621.
E-mail address: consuelo.pizarro@unirioja.es (C. Pizarro).

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Solid-phase microextraction (SPME) is a fast and sensitive technique for extraction of volatile compounds, which allows sample preparation-time to be reduced and does not require the use of solvents, thereby simplifying sample preparation. In addition, very good detection limits can often be achieved [17]. This technique has already been used satisfactorily in beer research to determine sulphur and selenium compounds [18,19], alcohols and esters [20], aldehydes [21] and the general profile of volatile compounds of beer [22–25]. However, to our knowledge, no literature can be found describing the optimisation of a SPME method for the analysis of volatile phenols in beer.

Beer is a very complex matrix and its analysis can present a particular problem due to the presence of CO_2 [26]. Foam formation can interfere in the determination of target compounds, negatively affecting method reproducibility. Several methods have been proposed to attempt to solve this problem, such as NaCl addition at low temperature [24], nitrogen bubbling [27], agitation [28] or ultrasonication [1,22]. The latter two are the most commonly employed decarbonation procedures. However, these decarbonation methods have several drawbacks, including that they are time consuming and that volatile compounds can be removed from the matrix, altering the actual composition of the sample, which can affect the determination of analytes. Therefore, the aim of this study was to develop a rapid, sensitive and selective method for the quantitative analysis of 4-EP, 4-EG, 4-VP and 4-VG, responsible for the presence of phenolic off-flavour (POF) in beers, using HS-SPME with GC analysis and detection by mass spectrometry. Several SPME parameters that influence the extraction process were optimised by means of experimental design methodology. To our knowledge, this is the first report of the development and validation of a headspace (HS) SPME procedure to determine simultaneously these four volatile phenols in beer.

2. Experimental

2.1. Chemicals

4-Ethylguaiacol, 4-ethylphenol, 4-vinylguaiacol and 4-vinylphenol were supplied by Aldrich Chemie (Steinheim, Germany). The purity of all standards was above 98%. Sodium hydroxide was obtained from Scharlau Chemie (Barcelona, Spain) and sodium chloride from Aldrich Chemie (Steinheim, Germany). Ethanol, methanol and L(+)-tartaric acid were purchased from Merck (Darmstadt, Germany) and ultrapure water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Standard solutions and samples

Individual stock standard solutions of each compound were prepared in methanol. Work solutions used for further studies were prepared by diluting different amounts of each stock standard solution. Standard and work solutions were stored in darkness at 4 °C.

Beers were kept refrigerated (4 °C) until they were analysed. A synthetic beer solution was prepared by dissolving 11 g/L of L(+)-tartaric acid in a hydro-alcoholic solution (4%, v/v ethanol). The pH of the resulting solution was adjusted to 4.5 with NaOH. Both real and synthetic samples were spiked with different amounts of work solutions containing the target analytes. Optimisation experiments were performed with the spiked samples at 400 μg/L; while validation experiments (repeatability and reproducibility) were performed at 10 and 1500 μg/L levels.

2.3. HS-SPME procedure

One of the most important factors that control the efficiency of the extraction is the SPME fibre coating. There are several previously published methods to determine volatile phenols in other sample matrices, but these studies showed differing results as regards the efficiency of fibre coatings [15,16,29–32]. Taking into account that the presence of CO_2 in the beer samples could influence the extraction process, it was decided to carry out a detailed study of the efficiency of different fibres. In order to evaluate suitable extraction conditions for the determination of volatile phenols in beer, six different fibres were studied. The evaluated fibres were purchased from Supelco (Bellefonte, PA, USA) and were coated with different stationary phases: polydimethylsiloxane (PDMS, 100 μm), polydimethylsiloxane/divinylbenzene (PDMS/DVB, 65 μm), polyacrylate (PA, 85 μm), carboxen/polydimethylsiloxane (CAR/PDMS, 75 μm), divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS, 50/30 μm) and carbowax/divinylbenzene (CW/DVB, 70 μm). They were conditioned in accordance with the producer's specifications before use.

The main parameters that affect the SPME process were studied. SPME can be used in two principle modes: direct-extraction and headspace configurations. In the direct-extraction mode, the coated fibre is inserted directly into the sample and analytes are extracted directly from the sample matrix to the extraction phase. In the headspace mode the vapour above the matrix is sampled. This headspace mode protects the fibre coating from damage by interferences present in the sample matrix [17]. Taking into account matrix complexity, headspace extraction mode was preferred to direct extraction to prevent the direct contact of the fibre with the beer matrix and related matrix effects.

For each SPME analysis, beer aliquots (from 4 to 12 mL, depending on the experiment) were placed in a 20 mL headspace vial and then the vial was tightly sealed with a PTFE septum. Then, samples were incubated at corresponding temperature (from 40 to 90 °C) for 5 min before SPME extraction. After, the fibre was exposed to the headspace over the sample from 20 to 90 min, depending on the experiment. Once finished the extraction step, the fibre was retracted and the SPME device was removed from the vial. The SPME device was then inserted into the injection port of a GC/MS/MS system for thermal desorption at the maximum recommended operating temperature for each fibre. In order to check possible carryover of analytes from previous extractions, after the first desorption fibre was desorbed for a second time to check whether the process was complete. 5 min of desorption was revealed to be incomplete, whereas no compounds were present when the fibre was reinserted after 7 min desorption. Therefore, 7 min desorption was chosen for subsequent analyses. Blank runs were completed at least once daily before sampling to ensure no carryover of analytes from previous extractions and clean the fibre before the analysis in order to remove potential interferences.

2.4. Equipment and chromatographic conditions

The HS-SPME–GC/MS/MS analyses were performed with a Varian 3800 gas chromatograph (Walnut Creek, CA, USA) equipped with a Combipal Autosampler (CTC Analytics) and connected to an ion-trap mass spectrometer (Varian Saturn 2200). Compounds were separated using a CP-WAX 52-CB column (30 m × 0.25 mm I.D., 0.25 μm film thickness) from Varian. Helium, at a flow of 1 mL/min, was used as carrier gas. Oven temperature was programmed as follows: 35 °C for 2 min, heated at 20 °C/min to 170 °C and kept for 1 min and finally raised to 210 °C at 3 °C/min and held for 12 min. Injection was performed in splitless mode for 2 min and then split was set at 30 mL/min. An inlet of 0.75 mm I.D. was used and the injector temperature, after the optimisation stage, was fixed at 270 °C. The manifold, GC/MS interface and ion trap temperatures were set at 60, 280 and 200 °C, respectively. Mass spectra were obtained using electron impact ionisation (70 eV).

Table 1
Retention time and MS/MS detection parameters for volatile phenols using the proposed method.

Compound	Retention time (min)	Precursor ion (*m/z*)	Quantification ions (*m/z*)	CID parameters	
				Storage level (*m/z*)	Amplitude (V)
4-Ethylguaiacol	13.996	137	91	75	80
4-Ethylphenol	15.119	107	77	60	69
4-Vinylguaiacol	15.520	150	107	80	72
4-Vinylphenol	19.234	120	91	65	64

Precursor ions were isolated using a 3 amu isolation window and subjected to collision-induced dissociation (CID). For operating in MS–MS mode, the emission current was fixed at 80 μA and scan time at 0.46 s/scan. The other MS/MS parameters are summarised in Table 1.

2.5. Statistical analysis

The influence of each experimental factor was evaluated by means of central composite design (CCD). The construction and analyses of the experimental design for reaching the optimum conditions were carried out using the Nemrod-W statistical package [33]. The composite design consisted of several groups of experiments: 8 experiments of a full factorial design (2^3), 6 experiments of a "star" or axial design and 3 centre points (all experiments were performed in triplicate). The design was used to obtain the surface response fitting the data to a polynomial model, the evaluation of the effects of each factor and also the interaction effects between factors [34]. Besides, four test points were also included in the experimental design. These test points were used to verify the predictive capabilities of the model by comparing the experimental results obtained for these points with the predictions of the model.

The model function was

$$Y = b_0 + \sum_{i=1}^{n} b_i X_i + \sum_{i=1}^{n}\sum_{j=1}^{n} b_{ij} X_i X_j \quad (1)$$

where X_i were the studied factors, exposure time, extraction temperature and V_s/V_t; the response Y was the individual chromatographic peak area of each compound. The experimental domain was defined taking into account the results of the preliminary experiments.

3. Results and discussion

3.1. Preliminary experiments

Preliminary experiments were carried out to achieve good chromatographic separation of the volatile phenols, in which an adequate separation of the compounds was achieved in 35 min. The determination of these compounds was performed by GC–MS/MS. Optimised conditions for MS/MS detection are detailed in Section 2. These conditions were achieved using the automated method development (AMD) tool included in the software of the Saturn GC/MS Workstation.

3.2. Effect of carbonation

Several experiments were performed to determine if beer need to be decarbonated before volatile phenol analysis by HS-SPME–GC–MS/MS. Five replicates of a beer sample were decarbonated in an ultrasonic bath during 15 min [22] before HS-SPME extraction. Another five replicates of the same beer were extracted directly (without decarbonation) by HS-SPME under same extraction conditions. The results for both procedures were statistically equivalent (data not shown). Moreover, reproducibility of the method was not be affected by the presence of CO_2. It was concluded that carbonation does not significantly influence SPME sampling. Therefore, the proposed procedure does not include a degassing step, which is time consuming and can alter matrix composition.

3.3. Optimisation of microextraction conditions

The influence of several parameters on the efficiency of the microextraction step was evaluated using beer samples spiked with the target compounds (400 μg/L for each compound). Four factors were selected as potentially affecting the SPME efficiency: fibre coating, extraction temperature, extraction time and sample volume. Salt addition and sample stirring were also studied to explore their effect on the microextraction procedure [17].

3.3.1. Fibre selection

The effect of the fibre coating on extraction yield was evaluated using the spiked beer samples. Six types of commercial fibres coated with different phases (PDMS 100 μm, PDMS/DVB 65 μm, PA 85 μm, CAR/PDMS 75 μm, DVB/CAR/PDMS 50/30 μm and CW/DVB 70 μm) and five temperatures (30, 40, 60, 80 and 95 °C) were evaluated and their extraction efficiency for the target compounds was compared. It is noteworthy that a comparison among the fibre's performance is relative since it would have to be performed in the optimised conditions to each fibre [35]. At this evaluation stage, the extraction time was set at 60 min. Fig. 1 summarises the results of the fibre screening process. It was observed that, at low temperatures, the efficiency of the fibres was very low for all compounds. With higher temperatures the recovery of the compounds was higher, with the exception of PDMS, which showed low efficiency over all the range of temperatures studied. The CAR/PDMS fibre showed high efficiency at high temperatures, mainly for 4-EG and 4-EP, but this involved less reproducible results (the relative standard deviation between replicates was higher than that obtained with the other fibres). This behaviour of the CAR/PDMS fibre had already been observed in previous studies [16,36]. PDMS/DVB presented only intermediate efficiency for all compounds. CW/DVB and DVB/CAR/PDMS exhibited the highest efficiency in the extraction process for the majority of the volatile phenols. While DVB/CAR/PDMS fibre afforded better extraction results for guaiacol family compounds, CW/DVB fibre provided higher extraction of polar compounds. This was also observed for PA fibre, which exhibited high efficiency for more polar analytes such as 4-EP and 4-VP, presumably due to its higher surface polarity.

Since the main flavour-active volatile phenol in beer is 4-VG, and because the final objective of this study was the optimisation of HS-SPME applied to real beer samples, we focused mainly on the highest sensitivity for this compound. Taking into account this fact and the relative olfactory thresholds of the target compounds [2,11,29,37] DVB/CAR/PDMS fibre was selected as the compromise extraction fibre for volatile phenols. This fibre has been previously used for volatile compounds determination by SPME in beer and wort [25,35,38].

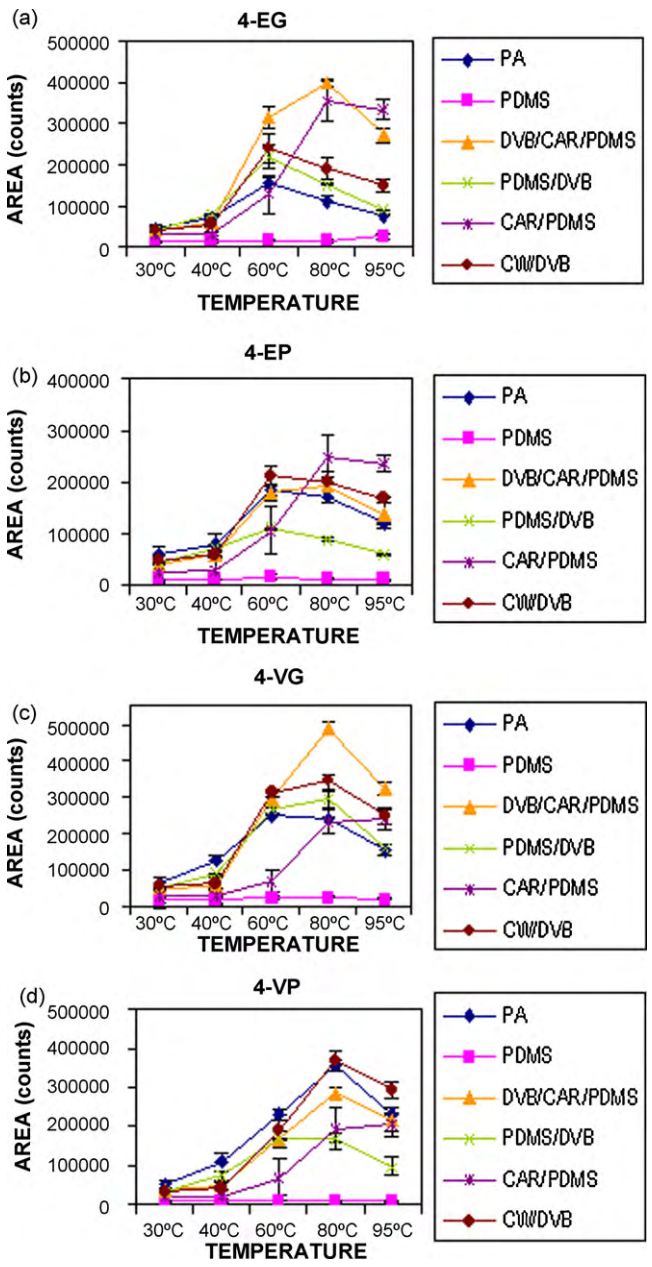

Fig. 1. Influence of fibre type and extraction temperature on the HS-SPME process: (a) 4-ethylguaiacol, (b) 4-ethylphenol, (c) 4-vinylguaiacol and (d) 4-vinylphenol.

3.3.2. Sodium chloride addition

Having selected an extraction fibre for determination of volatile phenols, the effect of salt addition on target-compound analysis by HS-SPME was investigated.

Salt addition can improve the extraction efficiency since it modifies the solubility of the molecules into the sample matrix [17]. Analyte solubility usually decreases as ionic strength increases. A decrease in analyte solubility improves sensitivity by promoting analyte partitioning into the stationary phase, but the "salting-out" effect is compound-dependant. Moreover, NaCl has been used in beer analysis at low temperature to eliminate possible interferences due to the presence of CO_2 [24].

The influence of ionic strength of the matrix on the yield of the extraction was studied by adding different amounts of sodium chloride. Three different levels were evaluated in the analyses of beer: no sodium chloride addition; undersaturation (0.2 g/mL); and supersaturation (0.4 g/mL). Each experiment was performed in triplicate. Fig. 2 shows the influence of sodium chloride concentration on the efficiency of the SPME. Adding excess salt to the samples increased sensitivity for volatile phenols by shifting the equilibrium of volatile compounds to the headspace, with an acceptable reproducibility. Therefore, further extractions were performed with the addition of 0.4 g/mL of sodium chloride to the beer samples.

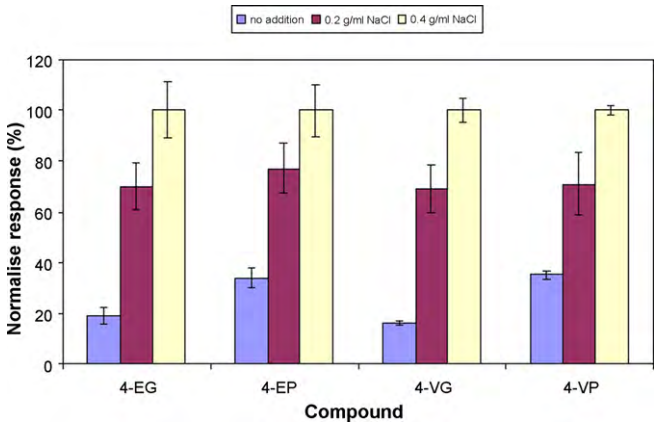

Fig. 2. Influence of NaCl concentration on the efficiency of SPME for 4-ethylguaiacol, 4-ethylphenol, 4-vinylguaiacol and 4-vinylphenol using a DVB/CAR/PDMS fibre ($n = 3$). Responses were normalized to the maximum signal achieved for each response.

3.3.3. Agitation of the sample

Mass transfer from a liquid sample to the headspace can also be accelerated by stirring of the sample and sample stirring may therefore improve the efficiency of the extraction process. For the present study, agitation of the sample was performed at 250 rpm using an autosampler equipped with a temperature-controlled vial agitator-tray. The recovery results obtained with agitation were compared with those obtained without sample agitation. Each experiment was carried out in triplicate. Fig. 3 shows the influence of sample stirring on the efficiency of the HS-SPME. For 4-EG and 4-EP no statistical difference in sensitivity was found between agitated and no-agitated samples. However, agitation of the sample was confirmed as a significant factor for the optimum performance of the SPME for 4-VG and 4-VP, speeding up mass transfer, increasing the response obtained for these compounds at similar extraction times. Therefore, in subsequent experiments the beer samples were continually agitated at 250 rpm during the extraction of the volatile phenols.

3.3.4. Effect of temperature

Temperature is one of the most important factors in a SPME method development, having a great influence on the amount of

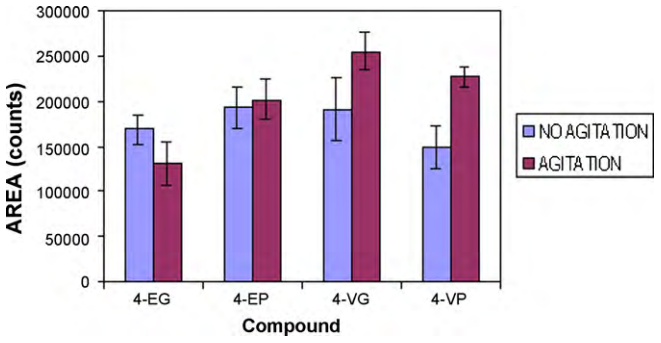

Fig. 3. Effect of stirring on the HS-SPME process for 4-ethylguaiacol, 4-ethylphenol, 4-vinylguaiacol and 4-vinylphenol using a DVB/CAR/PDMS fibre ($n = 3$).

Table 2
Experimental design matrix and response (mean values, $\times 10^{-1}$) obtained for volatile phenols ($n = 3$).

No. exp	Time (min)	Temperature (°C)	V_s/V_t	Results (area counts)			
				EG	EP	VG	VP
1	39	54	0.31	31643	19711	30956	20971
2	71	54	0.31	73669	33597	54040	26852
3	39	76	0.31	93117	38263	93517	49349
4	71	76	0.31	97717	40100	115967	69937
5	39	54	0.49	37329	21553	32113	19571
6	71	54	0.49	82804	36995	61366	25432
7	39	76	0.49	77859	35310	85333	47651
8	71	76	0.49	79996	38272	110347	67506
9	20	65	0.40	37486	21381	32804	20060
10	90	65	0.40	109900	45341	101567	67017
11	55	40	0.40	2864	12117	18259	11709
12	55	90	0.40	51663	28384	113967	79490
13	55	65	0.20	95625	37982	73408	41769
14	55	65	0.60	91518	41732	86393	46558
PC1	55	65	0.40	84844	36144	74563	41380
PC2	55	65	0.40	89567	39200	79372	42073
PC3	55	65	0.40	100905	40470	77737	43132
PT1	44	60	0.37	59760	30011	48029	28466
PT2	66	60	0.37	91691	35550	74799	41320
PT3	55	74	0.37	98137	40841	97269	54254
PT4	55	65	0.48	99729	42153	83534	45203

analyte extracted. Most SPME methods developed for beer analysis carry out the extraction at low temperatures, because high temperatures can cause the formation of artifacts due to the Maillard reaction, which can hinder the SPME [18]. Taking into account our experience in the determination of volatile phenols in other alcoholic beverages [14,16,39], for improved efficiency of the SPME for these compounds extraction at higher temperatures is required. However, high temperatures can cause the thermal descarboxylation of hydroxycinnamic acids forming volatile phenols [9]. Therefore, before multivariate optimisation of the HS-SPME, a temperature study was performed in order to assess an adequate working range of temperatures. For this purpose, the extraction profile depending on the temperature, for a commercial beer and for a synthetic beer solution, was studied at a fixed time (90 min). After the study, no differences were found in the temperature profile for volatile phenols between commercial beer and the synthetic beer solution. In this case, SPME efficiency in the beer matrix was not altered at higher temperatures. Moreover, formation of volatile phenols by thermal decarboxylation of hydroxycinnamic acids was not observed. It can be concluded from these observations that, for volatile phenols determination, it was possible to work in a broader range of temperatures than proposed previously.

3.3.5. *Central composite design: evaluation of the influence of the exposure time, extraction temperature and V_s/V_t*

Taking into account the results of the preliminary experiments, an optimisation procedure was carried out to determine the influence of extraction time, extraction temperature and V_s/V_t ratio on the efficiency of the extraction of the target compounds. The influence of each experimental factor was evaluated by means of central composite design (CCD). DVB/CAR/PDMS fibre was selected and a narrower range of temperatures (40–90 °C) that provided the best extraction yields were selected. Extraction time was studied between 20 and 90 min and V_s (sample volume)/V_t (total volume) ratio was evaluated in the range 0.20–0.60. All experiments were performed randomly to minimise the effects of uncontrolled factors that may introduce bias into the measurements. The experimental conditions studied and the average value of the experimental responses obtained are shown in Table 2. The estimates of the coefficients for the models of each response were calculated by least squares linear regression and these models were analysed and validated by the analysis of variance (ANOVA) and the test points using Nemrod-W software [33]. It was demonstrated that the proposed mathematical models were significant for all compounds with 95% confidence, and correctly explain the behaviour of the compounds throughout the experimental domain. Therefore, the models were accepted and the results analysed in detail. Model coefficients for each response are shown in Table 3.

The reduced derivatives, 4-EG and 4-EP, showed a similar behaviour. For these compounds, time and temperature coefficients were statistically significant. Moreover, interaction coefficients time–temperature (b_{12}) and temperature–V_s/V_t (b_{23}) were also significant. Since interaction coefficients were significant, it was necessary to study the existing interactions with the help of response surface plots. By examining response surface for time–temperature interaction, it was possible to obtain very similar conclusions for both compounds (Fig. 4(a) and (b)). When V_s was fixed at 8 mL ($V_s/V_t = 0.4$) a maximum response zone could be identified (region in red). For both compounds, optimal responses were reached at temperatures between 60 and 75 °C, provided that time was over 55 min. Focusing on the interaction temperature–V_s/V_t, several conclusions can be drawn. When time was fixed at low value (around 30 min) responses were found far from their optimum values. By contrast, if time was set at 70 min, regardless of the temperature employed, optimal values for responses were achieved. This could probably reflect that equilibrium of extraction has been reached. As can be observed in Fig. 4(c), setting the time in a more appropriate value in terms of saving time (55 min), optimum values could be found for 4-EG between 65

Table 3
Estimates of model coefficients for the responses.

Coefficient	EG	EP	VG	VP
b_0	**90214.2**	**38078.5**	**76927.1**	**41020.5**
b_1	**14376.7**	**4858.3**	**14246.9**	**8803.6**
b_2	**13129.5**	**4331.0**	**24710.8**	**16371.6**
b_3	−1333.6	561.0	1424.5	263.3
b_{11}	**−3731.8**	**−1073.9**	**−2054.1**	269.6
b_{22}	**−13168.0**	**−3733.2**	**−2251.3**	686.0
b_{33}	409.9	282.7	587.2	413.8
b_{12}	**−10164.8**	**−3031.0**	−689.0	**3558.5**
b_{13}	74.4	360.1	1035.1	−114.7
b_{23}	**−6062.7**	**−1288.3**	−2832.0	−154.1

Bold numbers denote significant effects (5%).

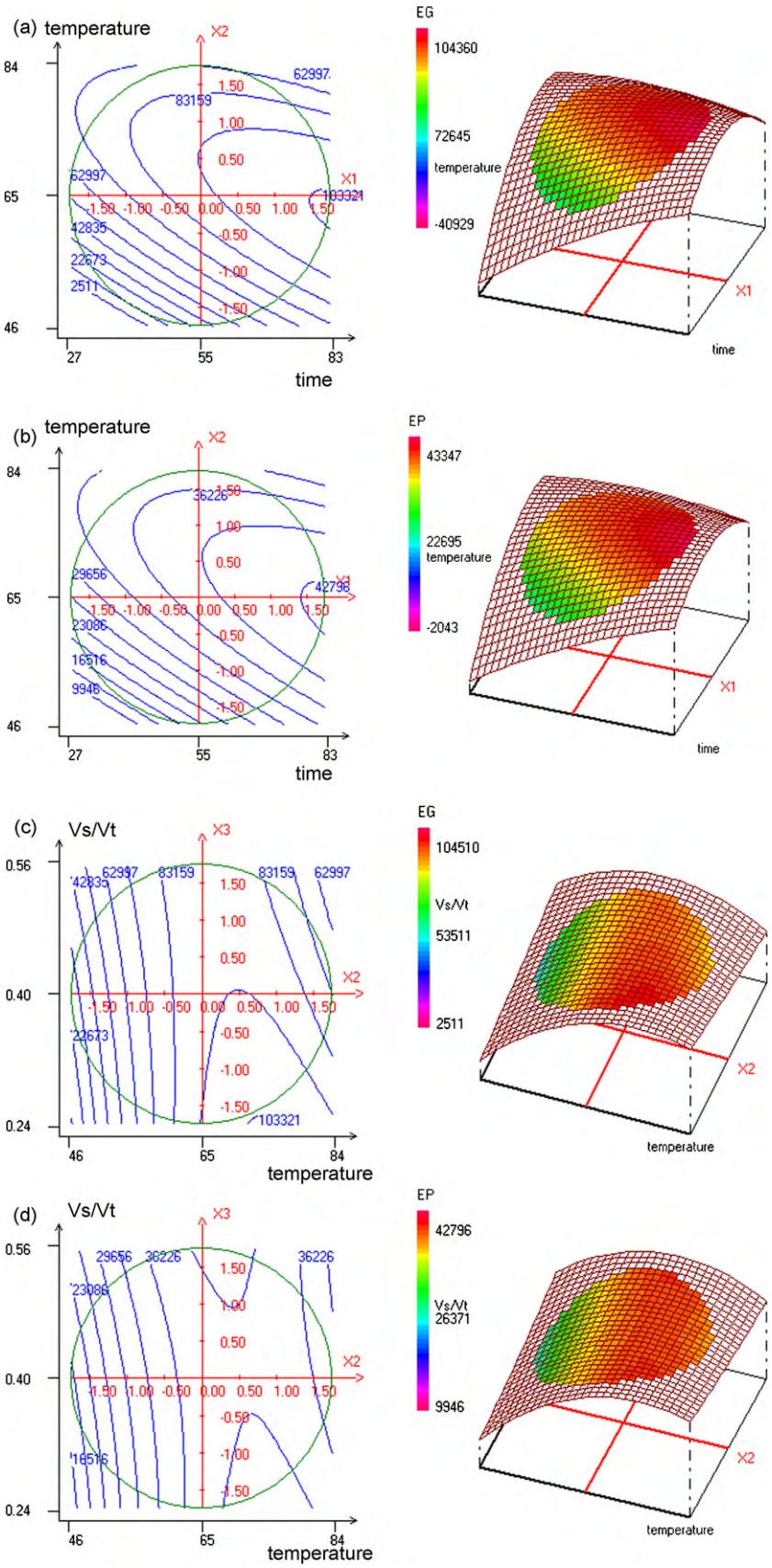

Fig. 4. Response surface plots for the chromatographic peak area as a function of extraction time and extraction temperature with a fixed V_s = 8 mL (V_s/V_t = 0.4) for 4-ethylguaiacol (a), 4-ethylphenol (b), 4-vinylguaiacol (e), 4-vinylphenol (f); and as a function of extraction temperature and V_s/V_t ratio with a fixed time (55 min) for 4-ethylguaiacol (c) and 4-ethylphenol (d).

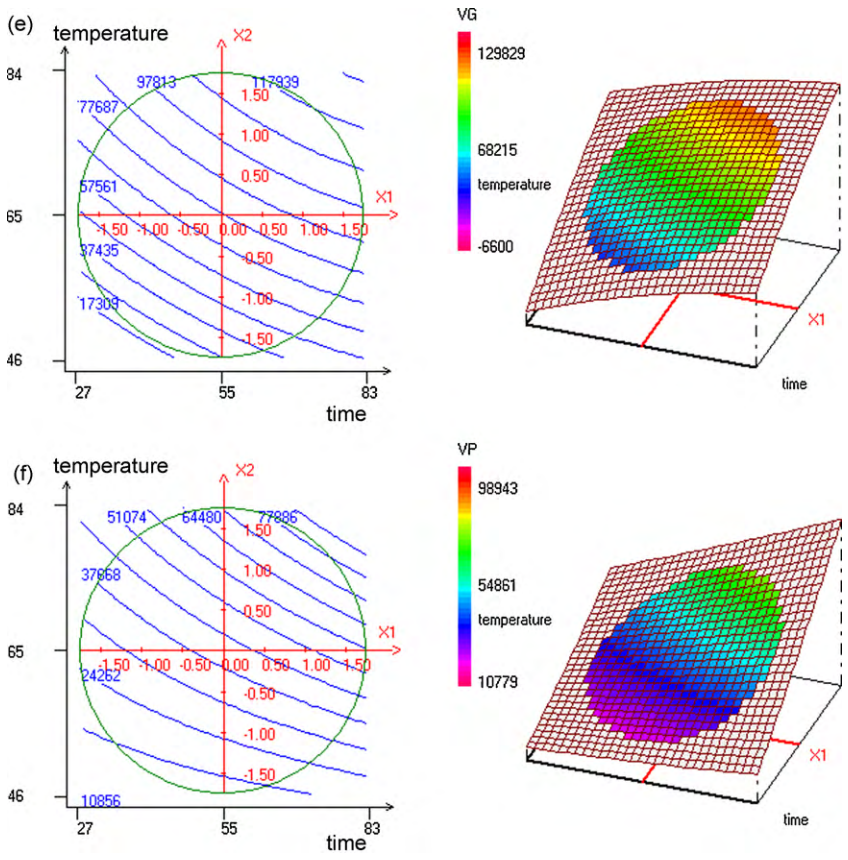

Fig. 4. (*Continued*).

and 85 °C, employing volumes under 8 mL. For 4-EP optimum values were reached between 60 and 80 °C, regardless of the volume employed (Fig. 4(d)). For 4-VG time and temperature coefficients were significant in their quadratic form. As can be seen in Fig. 4(e), extraction efficiency could be improved by employing longer times and higher temperatures: adequate responses were found for times over 50 min and temperatures over 70 °C. Finally, for 4-VP interaction coefficient time-temperature was significant. With times over 55 min and temperatures over 75 °C the responses achieved could be considered acceptable (Fig. 4(f)). Extraction temperature and time have a great influence on the amount of analyte extracted. The main objective was not to perform an exhaustive extraction but an extraction in adequate conditions that minimised extraction time while maximizing extraction efficiency, with special attention to 4-VG, which is the main flavour-active volatile phenol in beer. With these purposes in mind, an acceptable optimised compromise for the extraction of volatile phenols in beer corresponded to an extraction time of 55 min, an extraction temperature of 80 °C and the V_s/V_t ratio fixed at 0.30 (6 mL of beer).

3.4. Method performance

To our knowledge, this is the first time that a HS-SPME based method has been developed to determine simultaneously 4-EG, 4-EP, 4-VG and 4-VP in beers. In order to confirm that the method was suitable, it had to be evaluated. Linearity was evaluated using beer samples spiked with the target compounds at seven different concentration levels, from 2 at 4500 μg/L for 4-VG and from 2 to 2500 μg/L for the rest of the analytes. The beer was chosen because of their low native volatile phenol concentrations, without having noticeable off-flavours (as assessors indicated). The correlation coefficients varied between 0.993 and 0.999 over the linear range, so a directly proportional relationship between the extract amount of volatile phenols and initial sample concentration was demonstrated. Quantification and detection limits were calculated for the S/N ratio of 10 and 3, respectively (Table 4). The detection limits obtained for all compounds were lower than their olfactory thresholds. Moreover, detection limits were lower than those previously reported for volatile phenols in beers [2] and similar

Table 4
Linear range, correlation coefficients, limit of quantification (LOQ), limit of detection (LOD), repeatability, intermediate precision and recovery study of the proposed method ($n = 3$).

Compound	Linear range (μg/L)	Correlation coefficient (r^2)	LOQ S/N = 10 (μg/L)	LOD S/N = 3 (μg/L)	Repeatability (RSD%) $n = 5$		Reproducibility (RSD%) $n = 5$		Average recoveries ±RSD (%)	
					Low level[a]	High level[b]	Low level[a]	High level[b]	Low level[a]	High level[b]
4-Ethylguaiacol	2.42–2309	0.999	0.02	0.01	5.75	4.79	8.52	9.69	96.5 ± 5.1	95.7 ± 3.5
4-Ethylphenol	2.54–2704	0.998	0.06	0.02	3.84	5.24	6.31	6.90	98.7 ± 3.8	95.5 ± 4.3
4-Vinylguaiacol	2.62–4516	0.996	0.04	0.01	8.93	6.31	9.13	7.73	95.1 ± 6.2	97.1 ± 3.8
4-Vinylphenol	2.28–2226	0.993	0.03	0.01	4.17	6.52	7.71	8.97	97.6 ± 5.8	96.8 ± 7.1

[a] Spiked concentration 10 μg/L.
[b] Spiked concentration 1500 μg/L.

Table 5
Previously reported detection and quantification limits, linear ranges and correlation coefficients for volatile phenols in beer and other alcoholic beverages. .

Compound	Method performance				
	HPLC (beer) (Ref. [2])	MHS-SPME GC/MS (wine) (Ref. [14])	HS-SPME GC/FID (wine) (Ref. [15])	HS-SPME GC/MS (cider) (Ref. [16])	HS-SPME GC/FID (wine) (Ref. [30])
4-Ethylguaiacol	126[a]	0.18[a], 0.06[b]	80[a], 18[b]	0.03[a], 0.01[b]	5[a], 1[b]
	Up to 20000[c]	2.74–706[c]	15–3011[c]	2.71–2263[c]	40–400[c]
	0.9979[d]	0.997[d]	0.9991[d]	0.994[d]	0.980[d]
4-Ethylphenol	50[a]	0.20[a], 0.06[b]	81[a], 19[b]	0.08[a], 0.02[b]	5[a], 2[b]
	Up to 20000[c]	2.76–1714[c]	17–3041[c]	2.72–1829[c]	200–1800[c]
	0.9999[d]	0.995[d]	0.9989[d]	0.994[d]	0.989[d]
4-Vinylguaiacol	56[a]	0.66[a], 0.20[b]	68[a], 15[b]	0.26[a], 0.08[b]	
	Up to 20000[c]	3.60–762[c]	50–3144[c]	2.90–1474[c]	
	0.9996[d]	0.994[d]	0.9995	0.993[d]	
4-Vinylphenol	18[a]	0.40[a], 0.12[b]	15[a], 5[b]	0.09[a], 0.03[b]	
	Up to 20000[c]	2.80–760[c]	48–3853[c]	2.67–1801[c]	
	0.9984[d]	0.999[d]	0.9990[d]	0.998[d]	

[a] Quantification limit (μg/L).
[b] Detection limit (μg/L).
[c] Linear range (μg/L).
[d] Correlation coefficient.

Table 6
Results of an analysis of commercial beer samples by the HS-SPME–GC/MS/MS proposed method ($n = 3$).

Compound	Concentration ± SD (μg/L)				
	Beer A	Beer B	Beer C	Beer D	Beer E
4-Ethylguaiacol	n.d.	814 ± 13	n.d.	421 ± 9	n.d.
4-Ethylphenol	n.d.	335 ± 6	n.d.	65 ± 5	n.d.
4-Vinylguaiacol	1114 ± 15	565 ± 8	52 ± 4	424 ± 8	82 ± 5
4-Vinylphenol	351 ± 8	71 ± 5	7 ± 2	61 ± 7	n.d.

or even lower than those previously reported for other alcoholic beverages [14–16,30] (Table 5). The precision of the method was evaluated studying repeatability and reproducibility for all compounds at two different concentration levels, as shown in Table 4. For repeatability, 5 extractions were performed on the same day at the optimum conditions obtained for each factor; the relative standard deviations (RSDs) ranged from 3.8% to 8.9%. Reproducibility was studied by calculating the peak areas obtained over 5 days in optimum conditions; day-to-day precision was between 6.3% and 9.7%. Recoveries higher than 95% were obtained for all compounds.

3.5. Application of the method to real samples

The established HS-SPME method, previously optimised and validated, was applied for the content-analysis of volatile phenols in different beers. In order to avoid possible matrix effects, the quantitative analysis was performed by the standard addition method. Each determination was performed in triplicate. The levels of the analysed compounds in the beer samples are shown in Table 6. As shown in Table 6, all beers presented contamination with 4-VG, although its levels were over its odour threshold only for beers A, B and D. Beers B and D presented a marked phenolic off-flavour, containing significant amounts of volatile phenols, as sensorial analyses previously highlighted. The levels of the target compounds found in beers C and E were also coherent with the sensory trial, since no noticeable volatile phenols content was detected; 4-VG was found in both beers above its olfactory threshold, as occurred for 4-VP in beer C.

4. Conclusions

In this paper, the influence of different parameters on the HS-SPME process has been assessed to optimise a procedure for the analysis of 4-EG, 4-EP, 4-VG and 4-VP in beers. In preliminary experiments, the effect of beer carbonation was assessed, leading to the conclusion that carbonation does not influence SPME sampling; adding excess salt to the samples increased volatile phenols sensitivity. Therefore, extractions were performed by adding 0.4 g/mL of sodium chloride. Moreover, the positive effect of stirring beer samples was shown. According to the evaluation of coating affinities for the analytes when extractions were performed at different temperatures, DVB/CAR/PDMS was selected as the most suitable fibre. In these preliminary studies, the range of temperatures was successfully reduced for its further optimisation by CCD design together with the extraction time and V_s/V_t ratio. After the optimisation step, an optimal compromise situation was found at 80 °C for extraction temperature, 55 min for extraction time and 6 mL of beer (V_s/V_t 0.30). Owing to the good reproducibility (in terms of RSD, between 6.3% and 9.7%), satisfactory linearity (correlation coefficients between 0.993 and 0.999), and detection limits (lower than those previously reported for volatile phenols in beers), the methodology developed in this study could be used in the future to detect possible contaminations in beers. In addition, with this method it is also possible to compare volatile phenol content from different types of beers or to monitor the evolution of a particular beer during aging. To our knowledge, this is the first report of the development and validation of a HS-SPME procedure to determine simultaneously the four compounds considered in this study (not only 4-VG), which are implicated in the phenolic off-flavour in beer.

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References

[1] C.W. Ye, J. Gao, C. Yang, X.J. Liu, X.J. Li, S.Y. Pan, Anal. Chim. Acta 641 (2009) 64.
[2] N. Vanbeneden, F. Delvaux, F.R. Delvaux, J. Chromatogr. A 1136 (2006) 237.
[3] S. Coghe, K. Benoot, F. Delvaux, B. Vanderhaegen, F.R. Delvaux, J. Agric. Food Chem. 52 (2004) 602.
[4] N. Vanbeneden, F. Gils, F. Delvaux, F.R. Delvaux, J. Agric. Food Chem. 55 (2007) 11002.
[5] P.A. Thurston, R. Tubb, J. Inst. Brew. 87 (1981) 177.
[6] T. Heresztyn, Arch. Microbiol. 146 (1986) 96.
[7] P. Chatonnet, D. Dubourdieu, J.N. Boidron, M. Pons, J. Sci. Food Agric. 60 (1992) 165.
[8] M.M. Smith, R.D. Harley, Carbohydr. Res. 118 (1983) 65.

[9] N. Vanbeneden, T. Van Roey, F. Willems, F. Delvaux, F.R. Delvaux, Food Chem. 111 (2008) 83.
[10] N. Vanbeneden, D. Saison, F. Delvaux, F.R. Delvaux, J. Agric. Food Chem. 56 (2008) 11983.
[11] N. Vanbeneden, F. Gils, F. Delvaux, F.R. Delvaux, Food Chem. 107 (2008) 221.
[12] M.C. Meilgaard, MBAA Tech. Q 12 (1975) 151.
[13] H.M. Pascoe, J.M. Ames, S. Chandra, J. Am. Soc. Brew. Chem. 61 (2003) 203.
[14] C. Pizarro, N. Pérez-del-Notario, J.M. González Sáiz, J. Chromatogr. A 1143 (2007) 176.
[15] R. Castro-Mejias, R. Natera-Marín, M. garcía-Moreno, C. García-Barroso, J. Chromatogr. A 995 (2003) 11.
[16] C. Pizarro, N. Pérez-del-notario, J.M. González Sáiz, J. Sep. Sci. 32 (2009) 176.
[17] J. Pawliszyn, in: Solid-Phase Microextraction-Theory and Practise, Wiley-VCH, New York, 1997.
[18] P.G. Hill, R.M. Smith, J. Chromatogr. A 872 (2000) 203.
[19] N. Campillo, R. Peñalver, I. López-García, M. Hernández-Córdoba, J. Chromatogr. A 1216 (2009) 6735.
[20] H.H. Jelen, K. Wlazły, E. Wasowicz, E. Kaminski, J. Agric. Food Chem. 46 (1998) 1469.
[21] P. Vesely, L. Lusk, G. Basarova, J. Seabrooks, D. Ryder, J. Agric. Food Chem. 51 (2003) 6941.
[22] G.A. Silva, F. Augusto, R.J. Poppi, Food Chem. 111 (2008) 1057.
[23] M. Liu, Z. Zeng, B. Xiong, J. Chromatogr. A 1065 (2005) 287.
[24] O. Pinho, I. Ferreira, L. Santos, J. Chromatogr. A 1121 (2006) 145.
[25] D. Saison, D.P. De Schutter, F. Delvaux, F.R. Delvaux, J. Chromatogr. A 1190 (2008) 342.
[26] J.A. Ragazzo-Sanchez, P. Chalier, D. Chevalier, M. Calderon-Santoyo, C. Ghommidh, Sens. Actuators B 140 (2009) 29.
[27] T.C. Pearce, J.W. Gardner, S. Friel, P.N. Bartllet, N. Blair, Analyst 118 (1993) 371.
[28] D. Méndez-Pérez, G. González-Alatorre, E. Botelló-Álvarez, E. Escamilla-Silva, J.F.J. Alvarado, Food Chem. 107 (2008) 1348.
[29] S. Vichi, A. Romero, J. Tous, E.L. Tamames, S. Buxaderas, J. Chromatogr. A 1211 (2008) 1.
[30] N. Martorell, M.P. Martí, M. Mestres, O. Busto, J. Guasch, J. Chromatogr. A 975 (2002) 349.
[31] M.C. Monje, C. Privat, V. Gastine, F. Nepveu, Anal. Chim. Acta 458 (2002) 111.
[32] A. Romano, M.C. Perelló, A. Lonvaud-Funel, G. Sicard, G. de Revel, Food Chem. 114 (2009) 15.
[33] D. Mathieu, J. Nony, R. Phan-Tan-Luu, NEMROD-W, version 2001, LPRAI, Marseille, 2001.
[34] G.A. Lewis, D. Mathieu, R. Phan-Tan-Luu, Pharmaceutical Experimental Design, Marcel Dekkler, New York, 1999.
[35] C.L. Biazon, R. Brambilla, A. Rigacci, T.M. Pizzolato, J.H.Z. Dos Santos, Anal. Bioanal. Chem. 394 (2009) 549.
[36] F. Vilaplana, M. Martínez-Sanz, A. Ribes-Greus, S. Karlsson, J. Chromatogr. A 1217 (2010) 359.
[37] S. Boutou, P. Chatonnet, J. Chromatogr. A 1141 (2007) 1.
[38] D.P. De Schutter, D. Saison, F. Delvaux, G. Derdelinckx, J.M. Rock, H. Neven, F.R. Delvaux, J. Chromatogr. A 1179 (2008) 75.
[39] C. Pizarro, N. Pérez-del-notario, J.M. González Sáiz, J. Chromatogr. A 1166 (2007) 1.