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Multiple headspace solid-phase microextraction of ethyl carbamate from different alcoholic beverages employing drying agent based matrix modification

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ABSTRACT

Multiple headspace solid-phase microextraction (MHS-SPME) combined with gas chromatography-nitrogen phosphorus detector is proposed to determine the toxic contaminant ethyl carbamate (EC) in various alcoholic beverages after matrix modification. The remarkable feature of this method is that matrix effect, which commonly appears in SPME-based analysis, is avoided by determining the total amount of the analyte in the sample. To increase the sensitivity of the method, a novel polyethylene glycol/hydroxy-terminated silicone oil fiber was developed by sol–gel technique and applied for the analysis. Owing to the high polarity and hydrophilia of EC, an important problem still remains because the adsorption by sample matrix causes low transport of EC to the headspace and thus invalidates MHS-SPME for quantification. Mixing with anhydrous sodium sulphate, the sensitivity of the method can be improved. A Taguchi's L_{16} (4^5) orthogonal array design was employed to evaluate potentially significant factors and screen the optimum conditions for MHS-SPME of EC. Under the optimized conditions, limit of detection of 0.034 mg L^{-1} was obtained. Relative standard deviation of replicate samples ($n = 6$) was 2.19%. The proposed method was linear in the range of 0.04–100 mg L^{-1}, and the coefficient of determination was 0.9997. The method was used to determine EC in various alcoholic beverages. The concentrations obtained were compared with those obtained by standard addition method and no statistically significant differences were observed.

1. Introduction

The presence of ethyl carbamate (EC) in alcoholic beverages and fermented foods is a problem that has caused public health concern in the past few years as EC was re-classified as a carcinogen (Group 2A) by the International Agency for Research on Cancer in 2007 [1]. Considering that alcoholic beverages represent the highest part of EC intakes, several countries have established limitations on their levels [2]. This has forced the scientific community to develop analytical procedures that can determine not only the presence of EC in different alcoholic beverage samples but also their concentrations with a good accuracy.

Chromatography has become an important tool in the quantitative analysis of EC in various matrices. An important problem in the method development by chromatographic techniques is the possible occurrence of matrix effects, especially for complex samples. In most cases, matrix effect is considered to be a suppression or enhancement of the analyte response due to the matrix constituents. In general, there are two forms of matrix effect by chromatographic techniques. The first one is caused by co-eluting compounds, which show similar chromatographic behavior (i.e. retention time). It can be controlled by the improvement of the chromatographic separation and specific detectors, for which multidimensional chromatography [3] and tandem mass spectrometric detector [4] emerge as the powerful analytical techniques. The other is caused by co-existing components in sample matrix, which affect the extraction of the analyte and lead to a poor recovery. This is often solved by improvements in sample pretreatment procedures. Liquid–liquid extraction (LLE) [5,6] and solid phase extraction (SPE) [4,7,8] are often used for the determination of EC in alcoholic beverages. Nevertheless, these techniques require extensive organic solvents and are time-consuming. Solid-phase microextraction (SPME), a versatile solvent-free extraction technique, represents a good alternative to the aforementioned techniques. It has been used to extract EC in beers [9], wines [3,9,10], stone-fruit spirits [11] and grape brandies [9].

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Unlike traditional sample preparation methods, such as LLE, SPE and Soxhlet extraction, SPME is a non-exhaustive extraction technique in which only a small portion of the target analyte is removed from the sample matrix. The different components and characteristics of the matrices cause considerable differences in the partition coefficients and release rates of the same analytes. It implies that a careful calibration is necessary to compensate for the matrix-effect error. Different measures were taken for headspace SPME (HS-SPME) of EC in alcoholic beverages. Zhang and Zhang [9] found that using an internal standard could eliminate the effect of ethanol. But the effects caused by the other compositions in different types of alcoholic beverages were not mentioned when the method was applied to real samples. Apart from adding a deuterated internal standard, Lachenmeier et al. [11] introduced a sample preparation by diluting the stone-fruit spirits samples to disrupt the ethanol micelles and to reduce the competitive influence. To a great extent, the internal standard method can compensate for the effect of complicated matrix, but cannot avoid it thoroughly, and systematic errors may occur in the quantification step. And also sometimes isotopically labelled standard substances may cause the matrix effect as well [12]. Besides, the external standard calibration with model wines [3] has also been proposed to remove the effects of complex samples. However, it cannot represent the real matrix absolutely.

Multiple HS-SPME (MHS-SPME) is proposed as a suitable alternative in order to avoid the matrix effect [13]. The quantitative approach of MHS-SPME is theoretically different from that of HS-SPME. This technique involves sampling repeatedly the same vial by HS-SPME. When a portion of the analytes in the headspace is removed in the first extraction, the equilibrium between the analytes in the sample and those in the headspace is disturbed. As the sample is intended to re-equilibrate, more analytes migrate from the sample into the headspace. The concentrations in the two phases will now be smaller than those during the first extraction, but the ratios of these concentrations in the two phases will be the same. The second extraction and analysis, thus, results in a smaller peak. By continuing this procedure it is possible to extract all the analytes from the sample. If carried out ad infinitum, all of the peak areas are summed up to get the total peak area, which corresponds to the total amount of the analyte in the sample. The use of MHS-SPME enables a complete recovery of the target compounds and therefore, the matrix effect is avoided by the exhaustive extraction. As the logarithms of the various area values from the consecutive analyses are plotted versus the number of extractions in a linear scale under certain circumstances, the total area value can be obtained by regression calculation from the areas obtained in only a few extraction steps [13]. In this way, the total area (A_T) can be calculated using the following mathematical Eq. (1) when the extraction is not exhaustive, or directly calculated as the sum of the areas of each individual extraction when it is exhaustive:

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

where A_1 is the peak area of the first extraction and β (constant) is calculated from the linear regression of the logarithms of the individual peak area:

$$\ln\ Ai = \ln\ A1 + (i-1)\ln\ \beta \quad (2)$$

where A_i is the relative peak area obtained in the ith extraction.

As described in the literatures, MHS-SPME has been applied to the determination of volatile compounds in different types of matrices including packaging materials [13], soils [14], tomato [15], oils [16] and wines [17–19]. To evaluate the applicability of the aforementioned MHS-SPME method, the results are usually compared with those obtained by standard addition method, and the concentrations gained by both methods for the analytes are statistically equivalent. However, MHS-SPME has certain drawbacks such as increased analysis time compared with one-step SPME. There is a way to reduce analysis time, to perform MHS-SPME under a non-equilibrium situation. The theoretical principals of MHS-SPME under both equilibrium [13] and non-equilibrium [20] conditions have been presented. In our study, a fast MHS-SPME method is developed under a non-equilibrium situation.

Although MHS-SPME would be a good approach in principle, the usefulness of this method is limited. It may be difficult to achieve the exponential decay in peak area for all analytes because some interferences exist. The decay is characteristic for each analyte and depends on sample matrix and extraction conditions [15]. For this method to be effective, analytes must be released easily from their matrix into the headspace. For volatiles, the main challenge is the possible trapping and adsorption of analytes on the micro-phases of matrix [21], while for semi-volatile analytes low volatility is also a major concern. EC is highly polar and hydrophilic, which is an important limiting factor in MHS-SPME. EC is easily soluble in water and alcohol. It is relatively involatile and stable in aqueous solutions. The most commonly used matrix-modification techniques in the case of liquid samples, such as NaCl addition [11,22–25], temperature [22–25] and pH [9,11,25] adjustment, were employed in our preliminary study. But satisfactory results were not achieved yet. It showed that the matrix retained EC strongly in aqueous system, which caused low migration of analyte to the headspace and deviations from linearity of $\ln A_i$ plots. Efforts should be made to reduce the interference of matrix components in the samples.

Additionally, a suitable SPME fiber is needed to provide an appropriate coating-sample distribution coefficient, since in MHS-SPME it is essential to extract a significant amount of analyte in relation to the total amount in order to observe an exponential decay of peak areas versus the number of extractions. The carbowax/divinylbenzene (CW/DVB) fiber was usually employed for the headspace extraction of EC in alcoholic beverages [9–11]. However, it is not commercially available because of the solvent instability, swelling and stripping of the coating [26]. Therefore, the development of effective extraction coatings is in urgent need nowadays.

The aim of this study was to develop a simple, sensitive and reliable method for the analysis of EC in different alcoholic beverages. MHS-SPME was employed to avoid matrix effect from different samples, and additionally, drying agent based matrix modification was introduced to reduce the interference of water and enhance EC amount in the headspace. This approach has not been used in SPME for this purpose. To increase the sensitivity of the method, a new SPME coating made from polyethylene glycol (PEG) and hydroxy-terminated silicone oil (OH-TSO) was developed with sol–gel technique and applied for the analysis of EC followed by gas chromatography-nitrogen phosphorus detector (GC-NPD).

2. Experimental

2.1. Chemicals and standard solutions

EC (>97%) was purchased from J&K Chemical Ltd. (New Jersey, USA). PEG-20M (average molar mass ranging from 14,000 to 16,000 g mol^{-1}), sodium hydroxide (NaOH), tartaric acid, sodium chloride (NaCl), anhydrous sodium sulphate (Na_2SO_4), ethanol and acetone were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), which were all analytical-reagent grade. OH-TSO, 3-(2-cyclooxypropoxyl) propyltrimethoxysilane (KH-560), tetraethoxysilane (TEOS), and poly (methylhydrosiloxane) (PMHS) were purchased from Wuhan University Silicone New Material Co., Ltd. (Wuhan, China). Trifluoroacetic acid (TFA) was purchased from Shanghai Chemical Factory, China. Ultrapure water

was obtained from a Milli-Q system from Millipore (Milford, MA, USA). A stock solution with 10 mg mL^{-1} of EC was prepared in acetone and stored at 4 °C. The standard solutions, used to prepare the calibration curve, were prepared by dilution of the stock solution with ultrapure water.

2.2. Samples

Eight wines (dry red) were purchased from four well-known wineries in China (expressed as A, B, C and D): Wine A, Wine B, Wine C (C1, C2, C3), and Wine D (D1, D2, D3). The wines labelled with different numbers derived from different Vitis vinifera L. varieties. The ethanol contents of these wines under study ranged from 11.5% to 12% (v/v). Healthcare wine E (35%, v/v, ethanol) was produced from many kinds of precious medicinal materials, based on the traditional Chinese medicine theory. Chinese spirits A (45%, v/v, ethanol; strong aromatic) and Chinese spirits B (52%, v/v, ethanol; mild aromatic) were brewed by distilling fermented grain, such as sorghum, wheat and corn. Samples of Healthcare wine and Chinese spirits were prepared by diluting them with ultrapure water to obtain a final ethanol content of 12% (v/v). The synthetic wine was prepared containing 6 g L^{-1} tartaric acid, 12% of ethanol in ultrapure water and adjusting the pH to 3.5 with 1 M NaOH. The optimization of SPME conditions was performed in synthetic wine. All samples were stored at 4 °C in the dark place in sealed glass vials completely filled (without headspace) to avoid analyte losses.

2.3. Instruments and chromatographic conditions

The experiments were performed using an SP-6890A capillary GC system (Shandong Lunan Ruihong Chemical Engineering Instrument Co., Ltd., Tengzhou, China) equipped with a capillary split/splitless injector system and two detectors, a flame ionization detector (FID) and a NPD. Since the NPD offered better sensitivity and selectivity for EC than FID, it was used in this study. Compounds were separated using an AE-FFAP capillary column (30 m × 0.32 mm × 0.33 μm, Lanzhou ATECH technologies Co., Ltd., Lanzhou, China). Online data collection and processing were done on Chromatopac model N2000 (Hangzhou Mingtong Technology Co., Ltd., Hangzhou, China). The column temperature program was 80 °C held for 1 min, heated to 100 °C at 10 °C min^{-1}, then to 120 °C at 5 °C min^{-1}, and finally raised at 10 °C min^{-1} to 190 °C, held for 4 min. The temperatures were 200 °C for the injector and 250 °C for NPD. Nitrogen was used as carrier gas at a linear velocity of 12–15 cm s^{-1} in the splitless mode for all the analyses.

2.4. SPME fibers

The PEG/OH-TSO sol solution was prepared by mixing 100 mg of PEG, 90 mg of OH-TSO, 100 μL of TEOS, 50 μL of KH-560, 10 mg of PMHS, 300 μL of acetone and 60 μL of TFA containing 5% water in a polypropylene tube. The pre-treating and coating of fused-silica fibers and other operations were the same as Li et al. [27]. The fiber was then placed in a desiccator at room temperature for 12 h and then conditioned at 220 °C under nitrogen for 2 h in the GC injection port. The thickness of the PEG/OH-TSO fiber was measured using a microscopy and found to be 40 μm. The morphology of the fiber surface was evaluated by a field emission scanning electron microscope (FE-SEM, Hitachi S-4800) at an acceleration voltage of 5.0 kV. The commercially available fibers were purchased from Supelco (Bellefonte, PA, USA). The fiber coatings were 60 μm polyethylene glycol (PEG), 50/30 μm divinylbenzene/carboxen/polydimethylsiolxane (DVB/CAR/PDMS), 65 μm PDMS/DVB, and 85 μm polyacrylate (PA). Prior to use, all the fibers were conditioned following the manufacturer's recommendations.

Table 1
Designation of factors and levels in experimental design by Taguchi's orthogonal array.

Levels	Factors		
	A: Temperature (°C)	B: Extraction time (min)	C: Na_2SO_4 (mg μL^{-1})
1	20	10	0
2	35	25	0.4
3	50	40	0.8
4	65	55	4.0

2.5. MHS-SPME optimization

2.5.1. Design of the orthogonal test

A Taguchi's L_{16} (4^5) orthogonal array design was employed to evaluate potentially significant factors and screen the optimum conditions for HS-SPME of EC. The extractions were done in the 10-mL glass vials using the PEG/OH-TSO fiber. 100 μL of synthetic wine spiked at a level of 100 mg L^{-1} EC served as the sample in these experiments. Table 1 illustrates the assignment of the factors and levels of the orthogonal test. 16 experimental trials were conducted and randomly carried out trying to eliminate effect of extraneous or nuisance variables. The peak areas were considered as the experimental response. After each extraction, the extracted compounds were desorbed at 200 °C in the injection port for 4 min. Throughout the study, no analyte residues were found to be left on the fiber after each desorption. All the determinations were performed in triplicate except extra explanations. The average values and the standard deviations were reported.

2.5.2. Volume of sample

MHS-SPME was performed with 100, 20 and 10 μL of spiked Wine A or water in 10 mL sealed vials containing two levels of Na_2SO_4 addition (4.0 mg μL^{-1} and 0.8 mg μL^{-1}). Three consecutive extractions were performed at 35 °C with a PEG/OH-TSO fiber immersed in the headspace for 10 min. Linearity obtained with different volume of sample was evaluated by the correlation coefficient of the linear plot $\ln A_i$ versus $(i-1)$. Sensitivity was compared by using the normalized peak area for the first extraction. For each modified sample, a normalized area value of 100 was assigned to the extraction providing the highest peak area. The normalized area values for the rest were calculated as the percentage of the highest one: dividing the corresponding area by the highest one and multiplying by 100.

2.6. Quantification by MHS-SPME

20 μL of sample was placed into a 10-mL glass vial containing 80 mg of Na_2SO_4. Then, three consecutive extractions were performed at 35 °C for 10 min with a PEG/OH-TSO fiber and the total area was calculated using Eqs. (1) and (2). For calibration in the MHS-SPME study, 20 μL of EC aqueous solutions were used. The MHS-SPME procedure employed to analyze calibration standards was the same as the one described above. The concentrations of EC in the samples were calculated with the values of A_T by using the calibration curve obtained in standard solutions.

2.7. Quantification by standard addition

The standard addition method was accomplished by spiking the samples with three levels of known quantities of EC. 20 μL of the spiked samples were analyzed by HS-SPME in triplicate under extraction conditions optimised previously for the multiple headspace mode. A plot of the responses versus the concentrations

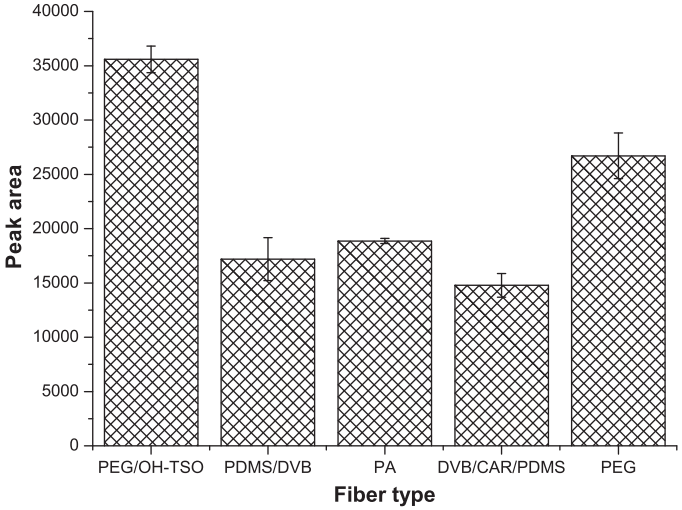

Fig. 1. Comparison of peak areas of EC with PEG/OH-TSO and commercial fibers. HS-SPME condition: sample, 20 μL Wine A; spiking level, 50 mg L^{-1}; extraction temperature, 35 °C; extraction time, 10 min.

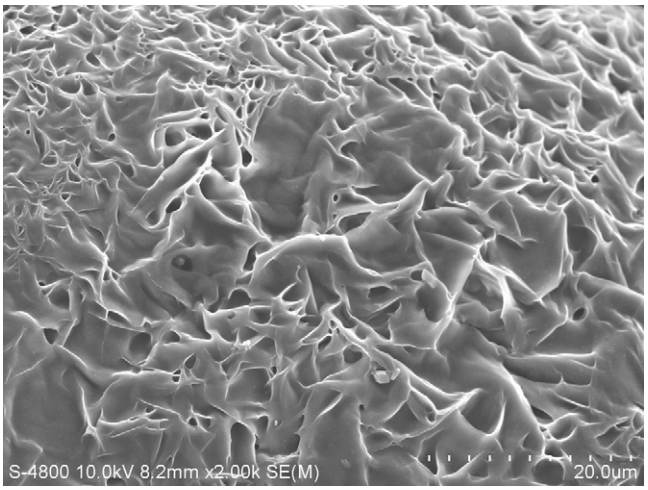

Fig. 2. Scanning electron micrograph (2000× magnification) of the sol–gel PEG/OH-TSO fiber.

of analyte addition was then developed, and the *x*-intercept in the plot represented the unknown concentration of the sample.

2.8. Statistics

Analysis of variance (ANOVA), homogeneity of variances and *t*-tests were performed to evaluate significant differences by using SAS system Version 8.0 (SAS Institute Inc, Cary, NC). The difference was considered to be statistically significant with values of $P < 0.05$.

3. Results and discussion

3.1. Comparison of PEG/OH-TSO fiber with commercial SPME fibers

As EC is a relatively polar and hydrophilic compound with an octanol–water partition coefficient of −0.15 [11], polar and bi-polar SPME fiber coatings have been evaluated in the previous literatures. A comparative study using five commercial fibers (CW/DVB, PDMS, PDMS/DVB, PA and CAR/PDMS) for the headspace extraction of EC from the synthetic wine showed that CW/DVB fiber was the best among these coatings [10]. Similar results were obtained in stone-fruit spirits [11]. However, the CW/DVB fiber is discontinued by Supelco. In this study, the extraction efficiency of the newly made PEG/OH-TSO fiber is compared with those of some commercial counterparts in Fig. 1.

The data in Fig. 1 shows that the PDMS-containing fibers were not suitable for extraction of EC, and both of the PEG-containing fibers had good extraction capacity. Among these fibers, the highest response was obtained with the sol–gel PEG/OH-TSO fiber. It might be attributed to the moderate degrees of hydroxyl groups in the coating, which can enhance the polarity of the fiber, and thus enhance the selectivity for polar compounds [28]. Fig. 2 presents the scanning electron micrograph of PEG/OH-TSO fiber surface. The sol–gel coating possesses a porous and folded structure, which significantly increases the available surface area on the fiber. Consequently, with such a coating structure, even an apparently thin coating is able to provide enhanced stationary-phase loadings and, therefore, high fiber sample capacity.

Table 2 shows the solvent stability of the coating. The fiber was used for direct SPME of EC in water and ethyl acetate, respectively. After six extraction and desorption cycles, the experimental response fluctuated about 3% in the terms of relative standard deviation (RSD). In fact, similar to the other sol–gel fibers [25,27], this new fiber can be dipped in ethyl acetate or water for 1 h without loss of extraction efficiency. It should be mentioned that commercial SPME fibers are not normally recommended to be exposed to organic solvent media. The average lifetime of the fiber was above 200 uses for HS-SPME.

3.2. Effect of water and salts

EC is soluble in water, and the addition of salt can increase the ionic strength of the aqueous solution and decrease the solubility of EC in theory. Fig. 3 illustrates the influence of water and salts on HS-SPME of EC. It shows that the response of the analyte decreased with increasing the volume of water, and increased significantly with the increase of NaCl and Na_2SO_4. The larger the amount of salt was added, the higher the extraction efficiency was obtained. Moreover, the responses were almost doubled by addition of Na_2SO_4 compared with those by the same amount of NaCl. However, too much salt could lead to a bad repeatability (with a RSD of 27.7% when 1000 mg of Na_2SO_4 was added).

The phenomenon can be explained by the shackles by water, which restrain the volatilization of EC through hydrogen-bond interactions. The addition of salts could change the activity coefficient of the analyte in the aqueous phase and, in this way, improve the extraction efficiency. The addition of Na_2SO_4 has double influence. It not only increases the ionic strength of water, but also desiccates the sample by addition of ample amount. Bearing these

Table 2
Influence of solvent on the extraction ability of PEG/OH-TSO fiber.

Extraction number	Peak area[a]	
	In water	In ethyl acetate
1	21504	29026
2	20576	30032
3	22416	30415
4	20952	28291
5	21089	29529
6	20520	28208
Average	21176	29250
RSD	3.33%	3.10%

[a] Direct-SPME condition: sample volume, 6 mL; spiking level, 100 mg L^{-1}; extraction temperature, 35 °C; extraction time, 10 min.

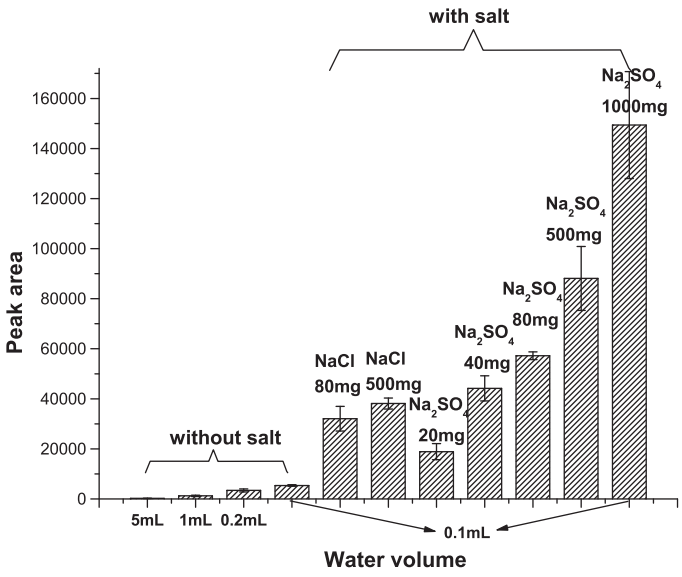

Fig. 3. Preliminary study of the influence of water and salts on HS-SPME of EC. HS-SPME condition: spiking level: 10 μg; extraction temperature, 35 °C; extraction time, 10 min.

results in mind, we decided to include sample volume and amount of Na_2SO_4 in the subsequent study.

3.3. Optimization of MHS-SPME variables

3.3.1. Extraction temperature, extraction time and Na_2SO_4 addition

The extraction conditions for MHS-SPME were optimized by a Taguchi's orthogonal array, and ANOVA was performed to identify the influence of individual factor to variance of outcomes. From ANOVA results in Table 3, it was observed that the effects of extraction temperature and Na_2SO_4 addition were statistically significant at $P \leq 0.05$, while extraction time did not have a significant effect ($P > 0.05$). The percentage contribution (PC%) of a factor was calculated by the "individual factor" sum of squares over a "total" sum of squares. It indicates the influence degree of each factor on the result. As shown in Table 3, the most important factor contributing to the extraction efficiency was Na_2SO_4 addition (52.8%), followed by extraction temperature (28.6%) and extraction time (9.2%). The average of responses for each factor at a level was also calculated to probe into the effect of each factor and screen the optimum level. From Table 3, the most efficient value of extraction temperature was 35 °C, extraction time was 55 min, and Na_2SO_4 addition was 4.0 mg μL^{-1}. It is worth noting that the amount of analyte extracted increased significantly with the increase of Na_2SO_4. The results showed that mixing of a sample with Na_2SO_4 (i.e. simultaneous increasing the ionic strength and sopping up the water) improved the sensitivity of the method, which was consistent with those obtained in the preliminary experiment. When 65 °C was tested as extraction temperature, an important decrease was observed in the response of the analyte, possibly due to the negative temperature effect on the coating-headspace partition coefficient of analyte. Similar results were also observed in previous HS-SPME studies [10]. Extraction time did not have a significant effect within the studied range. For time-economic reason, it was decided to work under non-equilibrium conditions and use an extraction time of 10 min.

Table 4
Optimization of sample volume and Na_2SO_4 addition in MHS-SPME.

Matrix[a]	Na_2SO_4 (mg μL^{-1})	Fitting parameters[b]	Sample volume (μL)		
			100	20	10
Water	4.0	r	NL[c]	0.9966	–[d]
		A_1	88.4	100	
	0.8	r	NL	NL	
		A_1	60.3	80.7	
Real wine	4.0	r	NL	0.9967	0.9961
		A_1	68.6	100.0	78.2
	0.8	r	NL	0.9945	0.9962
		A_1	73.8	93.4	87.7

[a] Spiked level: 100 μg mL^{-1}.
[b] r: correlation coefficient of the linear plot ln Ai versus $(i-1)$; A_1: mean of normalised area (%) for the first extraction.
[c] NL: not linear ($r < 0.95$).
[d] No available data.

3.3.2. Volume of sample

The volume of sample placed in the vial must be appropriate to observe an exponential decay of the peak areas with the number of extractions. If the mass is too low, the sensitivity of the method can be decreased. If the mass is too large, a poor correlation coefficient (r) is obtained for the linearity of ln A_i plot, and even the exponential decay is not observed for the analyte. Since a large amount of water reduced the volatilization of EC as described in Section 3.2, volumes of sample less than 100 μL were tested to reduce the total amount of water in the vials. Additionally, the amount of other matrix components which may spoil the linearity was also reduced. Table 4 shows the correlation coefficients and the normalized signals for different volumes of water and wine samples. Based on the results, 20 μL of sample with 4.0 mg μL^{-1} Na_2SO_4 was selected since it provided the highest peak area and the best correlation coefficient.

3.4. Eliminating matrix effect by MHS-SPME

The composition of alcoholic beverages is very complex and different, which may affect the extraction of EC. To check the matrix effect in detail, several samples (water, nine wines and two Chinese spirits) were spiked at three concentration levels within the linear range and the slopes of the linear calibration equations were

Table 3
ANOVA table for identifying the optimum levels and significant factors on variance of NPD response.

Factor	Peak area[a]				Analysis of variance[b]				
	Level 1	Level 2	Level 3	Level 4	DF	SS	F	P	PC (%)
A	14554	26688	25135	12624	3	6.20×10^8	6.09	0.030*	28.6
B	20573	15451	17964	25013	3	2.00×10^8	1.97	0.220	9.2
C	7442	16628	25833	29098	3	1.14×10^9	11.23	0.007**	52.8
Error					6	2.03×10^8			9.4
Total					15	2.17×10^9			100

[a] The average peak area of a factor at a level.
[b] DF = degrees of freedom; SS = sum of squares; PC (%) = percentage contribution.
* Significant at $P \leq 0.05$.
** Significant at $P \leq 0.01$.

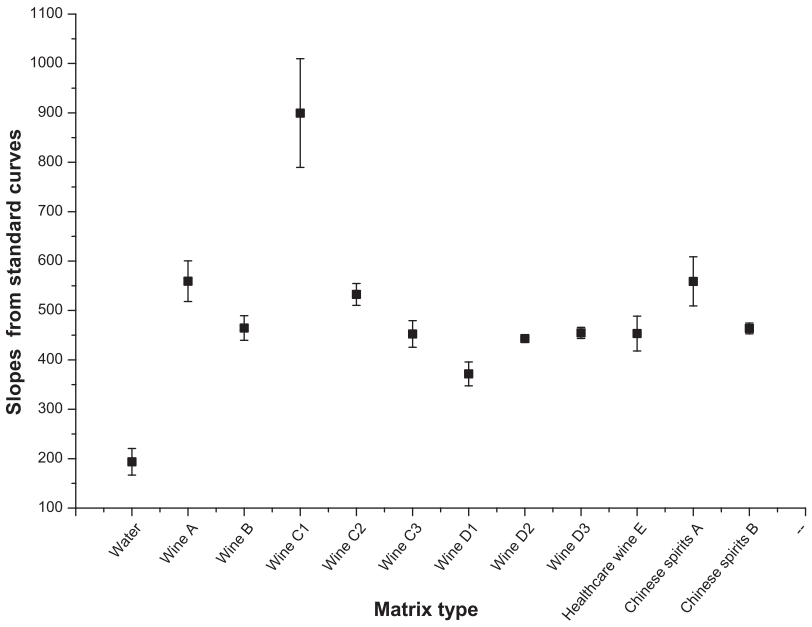

Fig. 4. Slopes and corresponding standard deviations from standard curves obtained in different samples.

Table 5
Statistical comparison of the total peak areas from different matrices by MHS-SPME.

Matrix	Total peak area	Homogeneity of variances	Analysis of variance
Spiking level: 100 μg mL^{-1}			
Water	299028 ± 38559	F = 0.99	F = 0.46
Wine A	279065 ± 30824	P = 0.41	P = 0.64
Wine C1	273924 ± 45911		
Spiking level: 5 μg mL^{-1}			
Water	18075 ± 616	P = 0.31	F = 0.37
Chinese spirits A	19637 ± 3205	F = 1.40	P = 0.78
Chinese spirits B	17821 ± 3812		
Healthcare wine E	17214 ± 3059		

obtained. As shown in Fig. 4, the slopes gained in different matrices were significantly different (data of ANOVA not shown), revealing the existence of systematic error in quantification by one-step SPME if one of these matrices was employed for establishing the linear calibration equation.

Table 5 shows the total peak areas of EC obtained by MHS-SPME from different matrices with two spiking levels. Statistical tests were applied to check the difference among these total peak area values. The first test was the Levene's test of homogeneity of variances. It showed that the variances were homogeneous for the two levels. The second test was the ANOVA for homogeneous samples to determine whether the total peak areas were statistically equivalent. No significant differences in these values were observed among different matrices at each spiking level. The use of MHS-SPME avoided the matrix effect by determining the total amount of the analyte in the sample. Therefore, the aqueous standard solution was selected in the method validation and calibration step.

Table 6
EC contents in alcoholic beverages using both MHS-SPME and standard addition (SA) methods.

Sample	Ethanol content (v/v, %)	Concentration ± SD (μg L^{-1})[a]		Significance of difference (P)	
		MHS-SPME	SA	Levene's test	t-Test
Wine A	11.5%	50.7 ± 1.1	58.9 ± 7.0	0.12	0.11
Wine B	12%	98.6 ± 0.8	79.1 ± 11.6	0.12	0.05
Wine C1	12%	66.7 ± 2.3	62.8 ± 5.9	0.17	0.4
Wine C2	12%	70.8 ± 2.2	71.8 ± 7.5	0.18	0.76
Wine C3	12%	105.2 ± 1.4	103.0 ± 28.1	0.11	0.74
Wine D1	12%	106.6 ± 2.1	113.0 ± 9.4	0.13	0.22
Wine D2	12%	102.1 ± 1.5	101.0 ± 21.7	0.12	0.43
Wine D3	12%	52.6 ± 3.6	53.9 ± 4.0	0.78	0.34
Healthcare wine E	35%	38.3 ± 2.6	33.0 ± 6.2	0.18	0.24
Chinese spirits A	45%	n.d.[b]	n.d.	–[c]	–
Chinese spirits B	52%	36.5 ± 0.2	38.1 ± 3.4	0.12	0.48

[a] Mean of three replicates ± standard deviation.
[b] Not detected.
[c] No available data.

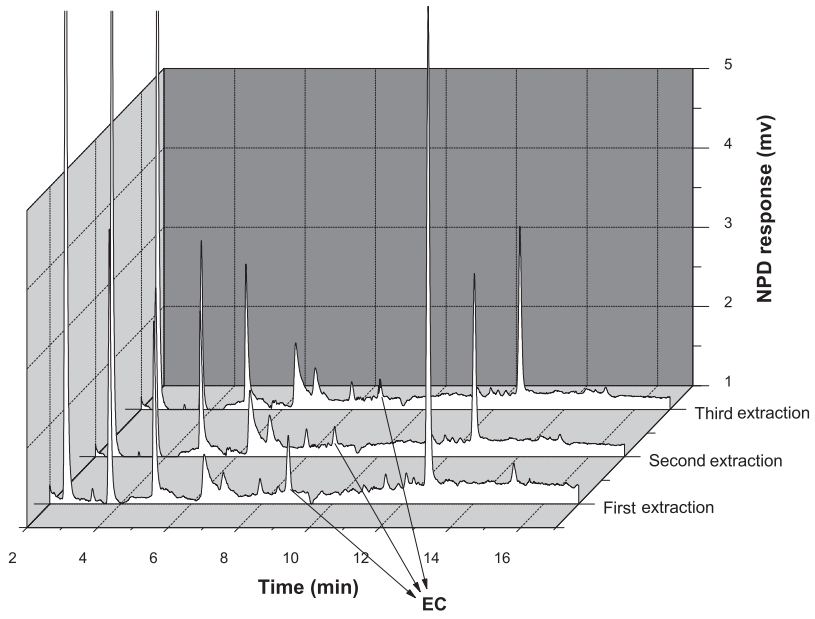

Fig. 5. HS-SPME–GC-NPD chromatograms of three consecutive extractions of EC from Wine D1.

3.5. Features of the method

The linearity of the total peak area versus the concentration of EC was studied for aqueous standard solutions using a PEG/OH-TSO fiber. And it was 0.04–100 mg L^{-1}, including four orders of magnitude. The calibration equation was $y = 537801x - 18930$. And the coefficient of determination was 0.9997, showing a high degree of correlation between the concentration and peak area. The precision experiments at the spiking level of 10 mg L^{-1} gave a RSD of 2.19% ($n = 6$). The limit of detection (LOD) was calculated for a signal-to-noise ratio (S/N) of 3 from the first extraction of the most diluted standard solution, and it was 0.034 mg L^{-1}. It is similar to that obtained by Lachenmeier et al. [11] using HS-SPME/GC-tandem mass spectrometry in stone-fruit spirits. By contrast, the present method requires no dedicated and expensive instrumentation, thus minimizing the costs of analysis per sample. For regulatory purposes, mass spectrometric detector as well as using isotope-labelled internal standards would be necessary. However, purely for survey purposes, the current approach will be a good alternative.

3.6. Analysis of real samples

A variety of alcoholic beverages were analyzed by MHS-SPME/GC-NPD. The total peak areas were interpolated in the calibration equation obtained using aqueous standard solutions. The results were summarized in Table 6. EC was found in all kinds of alcoholic beverages except Chinese spirits A. In all wines under study, EC ranged from 38.3 (Healthcare wine E) to 106.6 μg L^{-1} (Wine D1), exceeding the international limit established by Canada (30 μg L^{-1}) and USA (15 μg L^{-1}). EC levels in the Chinese spirits varied considerably. A single distillate spirits sample contained 36.5 μg L^{-1}, but another Chinese spirits, with higher ethanol content, did not contain detectable EC.

To further evaluate the accuracy of the MHS-SPME method, concentrations obtained were statistically compared with those obtained for the same samples by the standard addition method. From Table 6, the concentrations gained using both methods for all alcoholic beverages were statistically similar at 95% confidence level. But the calibration of the standard addition method is more laborious than that of MHS-SPME, for which calibration is required for each sample analyzed. Moreover, quantitative analysis of low-content analytes in solids is still a difficult task even though the standard addition method is used. This is mainly due to the difficulties in preparing spiked samples for calibration as it is often impossible to properly mix solid matrices with analytes or internal standards [14]. Fig. 5 shows the chromatograms of successive extractions of EC in Wine D1.

4. Conclusions

Generally speaking, matrix interference is one of the most important problems for trace analysis in complex samples. The use of MHS-SPME enables a complete recovery of the target compound and therefore the matrix effect, present when a SPME-based method is used for quantitative analysis, is avoided. In the case of EC, serious effect of water in the alcoholic beverages restrained its volatilization to the headspace. The employment of a sol–gel-coated PEG/OH-TSO fiber and the addition of Na_2SO_4 validated the MHS-SPME for quantification. The results indicate that MHS-SPME has a great potential for EC quantification directly from complex samples due to its simplicity, sensitivity, reliability, ease of operation and environmental protection, especially for the analysis of a large number of samples in different matrices.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.06.011.

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