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Development and validation of method for heterocyclic compounds in wine: Optimization of HS-SPME conditions applying a response surface methodology

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ABSTRACT

Considering the importance of the heterocyclic compounds in terms of wine flavor, this study aims to propose a new rapid and solvent free method to quantify different classes of heterocyclic compounds, such as furans, thiophenes, thiazols and pyrazines, which are products of the Maillard reaction, in wines. The use of a central composite design and the response surface methodology to determine the best conditions allows the optimum combination of analytical variables (pH, NaCl and extraction time) to be identified. The validation was carried out using several types of wine as matrices. The method shows satisfactory repeatability (2.7% < RSD < 12%), reproducibility (2.8% < RSD < 12%), accuracy and specificity. The optimized method was applied to 29 French wines and significant concentrations of the different heterocyclic compounds were determined, mainly for red wines.

1. Introduction

Wine aroma originates from a large number of volatile compounds belonging to heterogenic chemical groups, which may be classified as primary (derived from grape), secondary (formed during the fermentation) and/or tertiary aromas (from chemical reactions or physical interactions with containers, such as barrels) [1]. However, the origins of many odorous molecules present in wines are still not well defined. Some wines have aromatic notes close to those of "coffee", "toasted", "roasted", which are associated with the Maillard reaction. The Maillard reaction is responsible for the characteristic flavor and color of many processed food products (baked, fried or roasted) [2]. Typically, a carbonyl compound derived from carbohydrate degradation is reacts with a single amino acid. This well-known reaction occurs between a reducing sugar and an amino acid, and the mechanism can be divided into the Amadori and Heyns rearrangement or into the Strecker degradation and melanoidin formation. The most odorous products of the Maillard reaction are heterocyclic compounds with 5 or 6 members, which contain nitrogen-, sulfur- and oxygen-, notably including the compounds belonging to 4 different classes: furans, thiazoles, thiophenes and pyrazines [3].

In wines, some of these compounds have been identified and quantified and in some cases their chemical generation pathways and origins have been studied. Pripis-Nicolau et al. [4] showed that some carbonyl compounds mixed with amino acids in wine-like solutions reacted, even if kept in a reducing medium and under mild conditions similar to those of in bottle wine aging (pH close to 3.5, temperature close to 20 °C, and aqueous medium). The authors observed that the most interesting molecules were produced in the presence of the sulfur amino acids, in particular cysteine, with the production of heterocycles such as pyrazines, alkylpyrazines, methylthiazoles, acetylthiazole, acetylthiazoline, acetylthiazolidine, trimethyloxazole, and dimethylethyloxazoles. These mixes generate notes described as 'popcorn', 'hazelnut', 'toasted' and 'roasted' which are known to contribute to the aged wine bouquet [5]. Later, Marchand, de Revel and Bertrand [6] also studied the products of the reaction between cysteine and dicarbonyl compounds, under similar conditions. The compounds formed in solution included five of the most abundant and odorous compounds, that is, thiazol, 2-acetylthiazole, trimethyloxazole, 2-furanmethanethiol and thiophene-2-thiol, which were identified and quantified in French wines from different origins.

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These are known products of the Maillard reaction that occurs in several agribusiness processes and leads to roasted food flavors. These molecules were described for the first time in the wines by these authors, and may play an important role in their flavor. Researchers studied the reaction between diacetyl and cysteine, and proposed new pathways for the generation of odorous heterocyclic compounds under wine-like physicochemical conditions [7,8].

Considering the importance of these compounds in terms of the flavor of wines, and that no analytical method to identify and quantify of 4 different families of heterocyclic compounds in single chromatographic run could be found in the literature, this study aimed to propose a new rapid and solvent-free quantification method to quantify different classes of heterocyclic compounds, such as furans, thiophenes, thiazoles and pyrazines, which are products of the Maillard reaction. However, there are several methods for their quantification described in the literature using gas chromatography (GC) and various detectors: GC/FPD, GC/NPD, GC/FID, and GC/MS [9,4], thus a method using GC coupled to mass spectrometry was developed and validated. In relation to the extraction method for the compounds, researchers have been using liquid–liquid extraction (LLE), solid-phase extraction (SPE) and solid-phase microextraction (SPME) [10–12]. In this study SPME was used because it offers many advantages over conventional sample preparation techniques. Simplicity, speed, solvent-free extraction and minimal sample manipulation are amongst the advantages offered by this technique [13]. As many factors can influence SPME extraction, a central composite design (CCD) using the response surface methodology (RSM) was applied to determine the best conditions. This methodology represents a combination of mathematical and statistical techniques aimed at optimizing the final response. The main advantage of this method is the reduced number of experiments required to provide sufficient information to obtain statistically acceptable results. The RSM and CCD provide a complete factorial investigation of the simultaneous, systematic and efficient variation of important components, identifying the possible interactions, main effects and optimal conditions of operation [14].

2. Material and methods

2.1. Chemicals and Standards

Standard compounds (numbers given in Table 1) were obtained from commercial sources as follows: numbers 1 to 13, 16 and18 to 24 (Sigma-Aldrich, Saint-Quentin-Fallavier, France); 15 (Alfa Aesar A Johnson Mattey Company, Bischheim, France); and 14, 17 and22 (Acros organics,Geel, Belgium). The internal standard (25) was supplied by CDN Isotopes (Quebec, Canada). All solvents were HPLC grade. Absolute ethanol and methanol (purity > 99%) were obtained from Merck (Darmstadt, Germany). Milli-Q water was obtained from a Milli-Q Plus water system (Millipore, Saint-Quentin-en-Yvelines, France). Sodium chloride (99%) was supplied by VWR-Prolabo (Fontenay-sous-bois, France).

2.2. Sample preparation and spiking

The optimization of the type of fiber, sample dilution and the optimization of extraction of heterocyclic compounds were carried out on red wines spiked with 100 μg L^{-1} of the standard solutions, prepared at 1000 mg L^{-1} in water/ethanol solution (50% v/v).

Liquid–liquid extractions (LLE) was carried out with dichloromethane, 50 mL samples of wine were extracted 3 times with 5 mL of solvent. The combined extracts were dried over anhydrous

Table 1
Flavor description, boiling point and ions monitored in SIM detection for each compound.

No	Compounds	Flavor description	BP (°C)[a]	Selected Ions[b]
N,S-heterocycle				
1	Thiazole	Popcorn, peanut	117–118 °C	**85**/58
2	4-Methylthiazole	Green, nutty	133–134 °C	**99**/71/72
3	2-Ethylthiazole	Green, nutty	148 °C	**113**/112/98
4	Benzothiazole	Rubber	231 °C	**135**/108
5	2-Acethylthiazole	Nutty, popcorn	89–91 °C/12 mmHg	**127**/99/112
6	2-Methylthiazole	Green vegetable	129 °C	**58**/99
N,O-heterocycle				
7	2,4,5-trimethyloxazole	Very ripe fruit, nutty	133–134 °C	**111**/96/82
O-heterocycle				
8	3-Acetyl-2,5-dimethylfuran	–	62 °C/0.25 mmHg	**123**/138/91
9	2,3-Dihydrobenzofuran	–	188–189 °C	**91**/121
10	2-Acetylfuran	Powerful, balsamic, burning, sweet	67 °C/10 mmHg	**110**/95
11	5-Methylfurfural	Sweet, caramel, nutty, spicy	187 °C	**110**/53/81
S-heterocycle				
12	3-Acetylthiophene	–	208–210 °C/748 mmHg	**111**/126
13	2-Acetylthiophene	Mustard-like, onion, malty, roasted	214 °C	**111**/126/83
14	2,3-Dimethylthiophene	–	142 °C	**97**/111/112
15	2,5-Dimethylthiophene	Green-like	134 °C/740 mmHg	**111**/95
N-heterocycle				
16	2-methylpyrazine	Nutty	135 °C/761 mmHg	**94**/67
17	Acetylpyrazine	Roasted, sweet	78–79 °C/8 mm Hg	**80**/122/43
18	2-Ethylpyrazine	Nutty, roasted	152–153 °C	**107**/108/80
19	2,6-Dimethylpyrazine	Nutty, sweet, roasted, chocolate	154 °C	**42**/108/67
20	2,3-Diethylpyrazine	Roasted, earthy	180–182 °C	**121**/136/80
21	2-Ethyl-3-methylpyrazine	Potato, burnt nutty, roasted, cereal	57 °C/10 mmHg	**121**/122/94
22	2-Acetyl-3-methylpyrazine	–	90 °C/20 mmHg	**93**/136/94
23	2,3,5-Trimethylpyrzine	Nutty, roasted peanut, cocoa, burnt	171 °C	**81**/122/42
24	2,3,5,6-Tretramethylpyrazine	Green, nutty, cocoa, musty, potato	190 °C	**54**/136
25	2-Methylpyrazine-d_6			**100**

[a] BP: Boiling point (not specified) was 760 mm Hg.
[b] Quantitative ions are marked in bold text and control ions are marked in regular character.

sodium sulfate, filtered and concentrated under a gentle nitrogen flow.

For the Stir Bar Sorptive Extraction (SBSE) 10 samples of red wine have been spiked with the heterocycles compounds at 10 levels (10, 20, 50, 80, 100, 150, 250, 500, 750 and 1000 μg L^{-1}). The spiked samples were submitted to extraction using a PDMS stir bar (Twister, 63 μL coating) (Gerstel, Mullheim an der Ruhr, Germany) during 1 h at 20 °C and desorption at 280 °C during 10 min.

For the validation, the linearity and range were determined for white and red Bordeaux wine matrices. Repeatability assays were carried out with a red wine spiked at 10 μg L^{-1} with all of the compounds studied. Reproducibility assays were carried out on spiked (10 μg L^{-1}) red wine, carrying out the analysis at 2-day intervals for 21 days. The samples were individually frozen on the day of the first measurement. For the accuracy and specificity assays, the analytes were spiked (10 μg L^{-1}) in synthetic wine (hydroalcoholic solution (12% v/v), pH 3.5, tartaric acid 3 g L^{-1}), and white and red Bordeaux wines. Limits of detection (LOD, concentration for signal/noise = 3) and quantification (LOQ, concentration for signal/noise = 10) were calculated using the Chemstation data analysis software (Agilent Technologies, France). The identification of heterocyclic compounds in wines was performed by comparing retention times and mass spectra with those of pure standards and with the NIST mass spectra database. For the quantitative study 10 μL of internal standard solution, pyrazine-d_6 at 700 mg L^{-1} in ethanol solution (50% v/v), was added to 10 mL of the samples.

2.3. Solid-phase microextraction (SPME)—experimental design

The fibers used (Supelco, Bellefonte, PA, USA) were coated with various stationary phases and film thicknesses: divinylbenzene-carboxen-polydimethylsiloxane 50/30 μm (DVB/CAR/PDMS), polydimethylsiloxane-divinylbenzene 65 μm (PDMS/DVB), carboxen-polydimethylsiloxane 85 μm (CAR/PDMS), polydimethylsiloxane 7 μm and 100 μm (PDMS-7 and PDMS-100, respectively) and polyacrylate 85 μm (PA).They were conditioned before use by insertion into the GC injector as recommended by the manufacturer. To a 20 mL headspace vial was an added 10 mL sample of either wine, spiked wine or a blank hydroalcoholic solution of 12% (v/v). The solution was loaded onto an autosampler (see below). The basic program for the fiber selected consisted of swirling the vial at 250 rpm for 5 min at 40 °C, then inserting the fiber into the headspace for 40 min at 40 °C as the solution was swirled again, then transferring the fiber to the injector for desorption at 250 °C for 5 min.

The parameters tested in the experimental design to determine the best conditions for the extraction experiment were: pH, ionic strength and extraction time. To evaluate the effects and the interactions of these three variables, the response surface methodology was used together with a central composite design. The independent variables and their levels (−1, 0, 1) used for the experimental design were: pH (3.5, 5.5, 7.5), NaCl (0, 1.5, 3.5 g) and time extraction (20, 40, 60 min).

The design was constructed based on 3^3 factorial designs with 4 replications of the center point to estimate the experimental error, leading to 18 experiments, carried out in random order. All runs were carried out with 10 mL of spiked wine (100 μg L^{-1}), a Carboxen/PDMS fiber was used, which was selected in preliminary tests, and the extraction temperature was fixed at 40 °C to avoid the formation of new products of the Maillard reaction in the wine.

The response selected (y) was the geometric mean of all the areas of the individual peaks of all analytes, in order to obtain a unique set of optimum conditions for the extraction of all the target analytes. In order to estimate the response, an empirical model composed of a second-order polynomial was constructed (Eq. (1)):

$$y = \beta_0 + \sum_{i=1}^{k}\beta_i x_i + \sum_{i=1}^{k}\beta_{ii}x_i^2 + \sum_{i=1}^{k=1}\sum_{j>1}^{k}\beta_{ij}x_i x_j + \varepsilon \quad (1)$$

where y is the predicted response, β_0 the model constant, β_i the coefficients of the linear effects, β_{ii} the coefficients of the quadratic effects, β_{ij} the coefficients of the interaction between the factors, x_{ij} and x_j the independent coded variables, ε the error, k the number of variables considered, and i and j the coded factors of system.

The coefficients were calculated by regression analysis and their significance was verified using analysis of variance (ANOVA) with the Statistic (version 7.0) software program.

2.4. GC–MS analysis

All of the analysis was carried out using an HP 6890N (Agilent) gas chromatograph, coupled to a quadrupole mass spectrometer equipped with a Gerstel MPS2 autosampler. An HP5capillary column was used (30 m × 0.25 mm, 0.25 μm film thickness, SGE, Courtaboeuf, France) and the carrier gas was helium (N55), at a flow rate of 1.0 mL/min. The column oven temperature program was: initial temperature 40 °C for 4 min, then raised at 2 °C/min to 160 °C and held for 1 min, and finally ramped to 230 °C at a rate of 5 °C/min and held for 5 min. For the quantitative determination the selective-ion monitoring (SIM) mode was used. The interface was kept at 280 °C and the ionization mode was electron impact (70 eV). The analytes and internal standard (IS) were monitored according to the ions shown in Table 1. Prior to quantification in the SIM mode, the full scan mode (m/z 40–250) was used for the identification of all target compounds based on their mass spectra and GC retention times.

3. Results and discussion

3.1. Method optimization

3.1.1. Extraction mode and sample dilution

The heterocyclic compounds that are extracted with this method are from different families of compounds with distinct chemical characteristics, mainly in terms of polarity. Thus, it was necessary to compare various SPME fibers to find a compromise that would provide the best option for the entire set of heterocyclic compounds studied. In addition to solid-phase microextraction (SPME), liquid–liquid extraction (LLE), with different proportions of sample:diclormethane (5:1, 5:3 and 5:5 v/v), and SBSE with a PDMS-coated stir bar were tested. LLE has been abandoned because of the time and solvent consumption and SBSE system was abandoned because of the low sorption rate of heterocycles on stir bar. The SBSE results showed that for levels lower to 100 μg L^{-1} the 4-methylthiazole, trimethyloxazole are not detected and the linearity was not good for the two other compounds (R^2 = 0.73 for 2-acetylthiazole and R^2 = 0.13 for ethylpyrazine). Considering the heterocycles levels in wines (most of time lower to 10 μg/L), the PDMS SBSE extraction technique is not efficient. When the compounds evaluated were not extracted by dichloromethane, it was not used to compare LLE with SPME fibers.

Fig. 1A shows that the Carboxen/PDMS fiber is the best option for all heterocyclic compounds and it presents greater specificity for thiophenes and thiazols. Yu et al. [15] used this same fiber for the analysis of sulfur heterocyclic compounds, produced through the Maillard reaction between cysteine and ascorbic acid. Pérez-Palacios et al. [16] also used the Carboxen/PDMS fiber for the extraction of furanic compounds from coated deep-fried products. Fan et al. [10] showed that Carboxen/PDMS fiber was the most sensitive type of fiber for pyrazines extraction of the Chinese liquor. However, there are not research that demonstrate

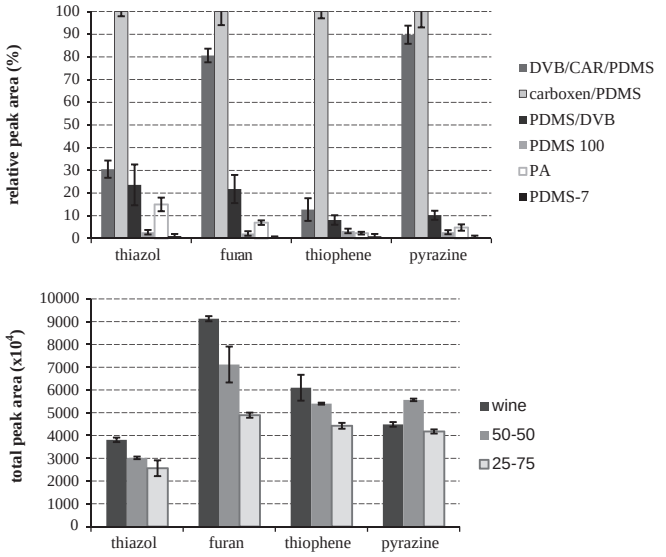

Fig. 1. Optimization on the extraction efficiency of heterocyclic compounds. Mean ± SD ($n=3$). (a) Relative peak area as a function of type of fiber for the extraction of heterocyclic compounds from wine, with the corresponding standard deviations. For each group, the ratio between the absolute peak area and the highest absolute area peak is presented along the *Y*-axis; (b) effect of the wine dilution using the headspace SPME method (50:50 and 25:75 water:wine).

its use for heterocyclic compounds extraction from different families (furan, thiophene, thiazol and pyrazine), determined in single chromatographic run.

The influence of ethanol on the adsorption of compounds by the SPME fiber is considered to result from the individual characteristics of each compound, such as the molecular weight, boiling point, molecular structure, solubility in the liquid matrix, and affinity for the fiber coating [17–19]. Because ethanol is one of the major constituents of wines, it was used to determine the extractability of the other compounds. Previous results in the literature describe the influence of the ethanol content on the efficiency of the SPME method [20,21]. A preliminary study was carried out on the possible effect of the ethanol content on the headspace SPME technique. For this, hydroalcoholic solutions (water:ethanol, pH 3.5, 3 g L^{-1} tartaric acid) with different ethanol contents (6, 12, 18 and 24% v/v), spiked with 50 μg L^{-1} of the standard compounds, were analyzed. A consistent decrease in the extraction yield (%) was observed with increasing ethanol content for all compounds available (data not show). Based on previous HS-SPME optimization studies it has been suggested that this reduced efficiency is due to ethanol directly competing with analytes for SPME binding sites [22,23].

Thus, to verify the influence of the ethanol in the wine with an alcohol level of approximately 12%, samples of white and red wine were diluted in water in two proportions, 50:50 and 25:75 water: sample, respectively, and compared with the undiluted wine samples (Fig. 1B). The maximum response for the more polar compounds (alkylpyrazines) was obtained when the solutions were diluted by a factor of 50% (v/v). However, for most compounds (furan, thiophene and thiazoles) the peak responses decreased with increasing dilution. Since in this method the aim is to determine 4 different classes of heterocyclic compounds in samples of white and red wine in a single chromatographic run, and based on the results obtained, samples were analyzed without dilution.

3.1.2. Response surface methodology

Once the SPME fiber had been chosen, and decided to analyze the wines without dilution, it was realized a central composite design (CCD) (3^3) with the objective of defining the best parameters for the extraction of compounds. The values for the total sum of the chromatographic area for all compounds analyzed (furan, thiophene, thiazole and pyrazine families) were considered as the dependent variables. The significant regression coefficients were negative, indicating that a response surface with a maximum point was obtained in the experimental design. A quadratic model was built through regression analysis described the mathematic relation between the independent and response variables (Eq. (2)).

$$\begin{aligned}\text{Total surface} = {} & 248117739 + 10238520^{*}\,\text{pH} - 8466366^{*}\,\text{pH}^2 \\ & + 31409833^{*}\,\text{NaCl} - 21449796^{*}\,\text{NaCl}^2 \\ & + 50089132^{*}\,\text{time} - 27032639^{*}\,\text{time}^2 \end{aligned} \quad (2)$$

The significance of the factors was confirmed by ANOVA, where it was possible to observe for all factors (pH, time (min) and NaCl (g)) that linear and quadratic effects were significant. The time of extraction was shown to have the weakest influence on the responses, and interactions between the variables were not observed (Table 2). These results showed that the model was significant ($F=42.7$ and $p<0.05$) and the lack-of-fit was not significant ($p>0.05$), indicating that the quadratic model was valid for this study. Moreover, the R^2_{pred} and R^2_{adj} values obtained (0.9382 and 0.9045, respectively) confirm this result.

For the graphical representation of the functions of this design, graphs are used which describe the individual and cumulative effects of the variables tested and their effect on the response. Fig. 2 shows the response surface graph in a three-dimensional plane for the regression model fitted to the data. The maximum response (surface area for all compounds) was obtained at pH 5.5 with 3 g of NaCl and applying an extraction time of 55 min, which represent the best conditions for the solid-phase microextraction.

It is important to assess the fitted model to ensure that it provides a sufficient approximation to the results obtained under the experimental conditions. The normality of the data was analyzed using a normal probability plot of the residuals and the difference between the observed values and those predicted from the regression. It was found that the experimental points were normally distributed around the curve, indicating that the normality assumption was satisfied. A determination coefficient (R^2) of 0.94 was obtained for this model, which indicates a good fit between the observed and the predicted response values. The plots of the residual *versus* the predicted values (plots not shown) showed that the residuals were scattered randomly around zero and did not have outliers, because all of the values are within the accepted range (−3 to +3) for the validation of the model [24]. Thus, the analysis of variance results were valid, since the model assumptions were satisfied.

Table 2
Analysis of variance for response surface quadratic model for the sum of the peak areas for the heterocyclic compounds in wine.

Source	Sum of squares	Degrees of freedom	Mean square	*F*-value	*p*-value
pH (L)	**2.0247E+15**	**1**	**2.0247E+15**	**5.26241**	**0.017095**
pH² (Q)	**3.7201E+15**	**1**	**3.7201E+15**	**5.80572**	**0.025412**
NaCl (L)	**1.1443E+16**	**1**	**1.1443E+16**	**22.68695**	**0.001421**
NaCl² (Q)	**3.8174E+15**	**1**	**3.8174E+15**	**10.59867**	**0.011604**
Time (L)	**3.9615E+16**	**1**	**3.9615E+16**	**76.26895**	**0.000023**
Time² (Q)	**1.6010E+16**	**1**	**1.6010E+16**	**29.89885**	**0.000596**
pH.NaCl	4.2823E+11	1	4.2823E+11	0.00076	0.978613
pH.time	1.3567E+12	1	1.3567E+12	0.00242	0.961945
NaCl.time	4.4744E+14	1	4.4744E+14	0.79919	0.397432
Lack of fit	4.4404E+15	5	8.8808E+14		0.452171
Pure Error	3.8520E+13	3	1.2840E+13		
Total	7.1714E+16	17			

Bold line shows significant difference ($p<0.05$).
L, linear effect.
Q, quadratic effect.

3.2. Analytical performance

3.2.1. Linearity and limits of detection and quantification

The linearity was evaluated, in a concentration range appropriate for wine contents according to Marchand et al. [6]. A Bordeaux white and red wine were spiked with the target compounds as listed in Table 1, using 7 levels of concentration prepared in duplicate. A correction was applied by subtracting the peak area ratios of the non-spiked wine sample (reference) from the spiked wine samples. The calibration curves were plotted as the relative peak areas (solute *versus* the corresponding internal standard) as a function of the concentration ratio (compound concentration *versus* internal standard concentration). The linearity for white and red wines was satisfactory, with coefficients of determination of greater than 0.99 in all cases.

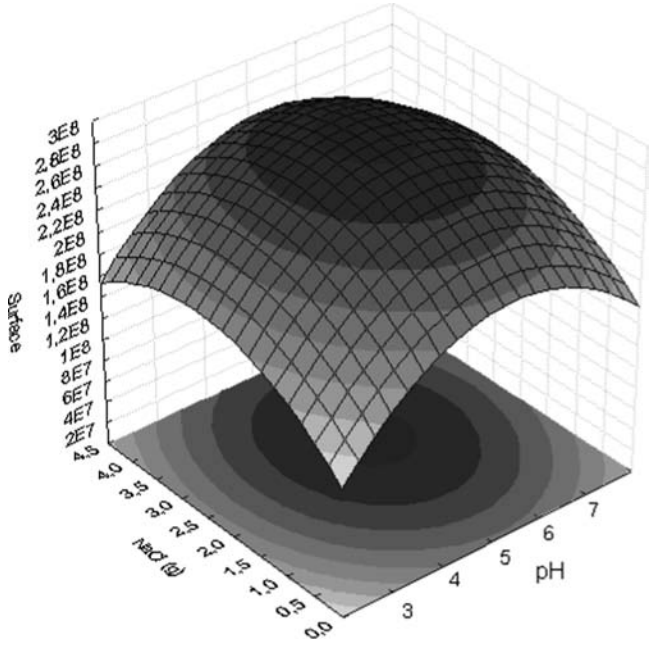

Fig. 2. Response surface graph for solid-phase microextraction of the heterocyclic compounds, using pH *versus* NaCl (g), at a fixed extraction time of 55 min.

3.2.2. Repeatability

To evaluate the repeatability of the determination, ten identical samples of spiked wine (white and red wine) (10 μg L^{-1}) were analyzed. The relative standard deviation of the area ratios was lower than 10% in all cases except for 2,6-dimethyl pyrazine (12.0%) for red wine; for most of the heterocyclic compounds the coefficient of variation was below 5%, which verifies the good precision of the method.

3.2.3. Reproducibility

The reproducibility of the method was tested using two determinations: (1) a spiked red wine (that used for the repeatability study) was measured 11 times at 2-day intervals over a 3 week period. The results are shown in Table 3 and the values are in most cases better than 10%, ranging from 5.7% to 10.9%. The reproducibility was also determined from the slopes of the curves, with 5 calibration curves being constructed on different days, and the RSD% was calculated based on the average slope value. The results were below 13%, varying from 2.8% to 12.7%.

3.2.4. Accuracy and specificity

The accuracy of the analytical method was evaluated by calculating the recoveries for the spiked samples. The analytes

Table 3
Linearity, detection and quantification limits, repeatability, reproducibility and accuracy of the method.

Compounds	Range (μg L^{-1})	R^2	LOD[a] (μg L^{-1})	LOQ[a] (μg L^{-1})	Repeatability (RSD%)[b]		Reproducibility (RSD %)		Recovery (%)		
					WW[c]	RW[d]	Wine	Slope[e]	SW[f]	WW[c]	RW[d]
Thiazole	99.5–0.1	0.997	0.263	0.534	3.2	4.6	10.9	2.8	93.1	82.7	92.8
4-Methyl thiazole	110.0–0.01	0.998	0.387	1.09	4.9	3.5	9.8	9.5	91.8	85.7	98.7
2-Ethyl thiazole	90.8–0.01	0.997	0.096	0.211	6.5	3.7	7.9	5.5	99.2	107.5	121.9
Benzothiazol	112.0–0.01	0.995	0.056	0.157	5.3	5.3	8.9	11.4	90.3	88.4	72.5
2-Acetyl thiazole	96.9–0.01	0.994	0.469	1.406	7.9	5.8	9.9	9.4	87.3	79.7	72.9
2-Methyl thiazole	76.1–.009	0.998	0.277	0.523	4.5	9.9	6.1	8.7	100.3	104.9	109.1
2,4,5-Trimethyloxazole	88.0–0.05	0.999	0.786	2.056	5.5	5.6	6.5	9.1	97.4	83.2	99.7
5-Methyl furfural	84.1–0.05	0.998	0.081	0.208	5.7	3.5	7.0	10.1	93.4	89.4	77.5
3-Acetyl-2,5-dimethylfuran	109.2–0.01	0.998	0.054	0.356	4.7	4.8	8.8	10.4	86.2	85.3	75.9
2,3-Dihydrobenzofurane	62.1–0.01	0.996	0.043	0.113	4.8	3.7	7.5	8.6	91.9	92.1	86.7
2-Acetylfuran	121.0–0.01	0.995	0.776	2.167	5.4	8.1	9.8	9.4	88.1	76.8	81.3
3-Acetyl thiophene	94.6–0.01	0.998	0.423	1.983	5.7	3.7	9.9	12.4	95.4	97.8	81.5
2-Acetyl thiophene	84.7–0.05	0.995	0.295	0.982	6.3	3.8	7.5	12.3	91.6	83.3	80.1
2,3-Dimethyl thiophene	97.6–0.05	0.997	0.102	0.345	8.3	4.8	8.2	3.9	98.7	104.8	115.2
2,5-Dimethyl thiophene	89.0–0.01	0.998	0.132	0.402	7.2	5.2	8.9	5.5	99.2	98.6	101.5
2-Methyl pyrazine	83.8–0.01	0.996	0.367	1.222	5.6	5.0	5.7	2.8	96.5	101.6	109.4
2-Acetylpyrazine	88.7–0.05	0.994	0.673	1.352	8.8	8.1	7.9	11.9	80.3	82.9	90.3
2-Ethylpyrazine	90.2–0.05	0.994	0.523	1.097	5.4	8.6	7.1	5.3	98.2	94.6	98.7
2,6-Dimethyl pyrazine	78.4–0.04	0.996	0.154	1.719	5.8	12.0	10.5	12.5	93.7	118.5	90.5
2,3-Diethyl pyrazine	73.9–0.009	0.996	0.189	0.629	6.7	4.7	6.0	7.7	80.2	79.5	91.9
2-Ethyl-3-methyl pyrazine	76.5–0.05	0.996	0.229	0.765	5.7	6.3	9.6	11.2	94.8	90.1	87.9
2-Acetyl-3-methyl pyrazine	76.4–0.05	0.997	0.251	0.819	6.9	8.9	9.1	10.4	88.5	85.3	80.0
2,3,5-Trimethyl pyrazine	76.4–0.009	0.996	0.281	0.732	4.6	2.7	7.9	10.2	94.9	108.1	92.8
2,3,5,6-Tetramethyl pyrazine	48.5–0.1	0.998	0.304	0.854	6.1	6.13	9.5	8.9	100.4	116.0	98.2

[a] Limits of detection and quantification, respectively.
[b] Relative standard deviation.
[c] White wine.
[d] Red wine.
[e] Angular coefficient of calibration curve.
[f] Synthetic wine (hydroalcoholic solution (12% v/v), pH 3.5, tartaric acid 3 g/L).

were spiked (10 μg L^{-1}) in synthetic wine, white and red Bordeaux wines. The recoveries for almost all samples were around 100% indicates good accuracy and specificity of the method.

These results for the validation of the method show good agreement with data from other researchers who also developed and validated methods for the determination of heterocyclic compounds in different food and beverages matrices. Keim et al. [9] developed and validated a method for the determination of 5 nitrogenous heterocyclic compounds in samples of red and white wines. Marchand et al. [6] validated a method to analyze 7 heterocyclic compounds of different chemical families present in red and white wines. The results for both of these validation methods are in good agreement with those observed in this study, although they used liquid–liquid extraction to extract the compounds. The SPME, provides several benefits compared to conventional extraction methods such as LLE, including elimination of the use of (often toxic) solvents, higher sensitivity and easy automation. Research carried out to develop and validate a method for the quantification of heterocyclic compounds produce from the Maillard reaction in some food products, such as potato chips [25], fish fried [16] and palm sugar [26], provided data consistent with those obtained in our study in terms of analytical performance. It should be emphasized, that in these studies cited previously only some heterocyclic compounds were optimized applying the proposed method. Also, no analytical method to identify and quantify of 4 different families of heterocyclic compounds in single chromatographic run could be found in the literature.

3.3. Wine analysis

The optimized and validated method described above was applied to 29 French wine samples of different origins, types and vintages (Table 4). The quantification of heterocyclic compounds was based on the calibration curves obtained in the linearity experiments for each heterocycle. Mostly, the wines analyzed presented significant concentrations of the different heterocyclic compounds, mainly for red wines. Note that the threshold of these compounds in wine is still unknown, except for the thiazole (38 μg L^{-1}), 4-methylthiazole (55 μg L^{-1}), acetylthiazole (3 μg L^{-1}) trimethyloxazole (17 μg L^{-1}), and 2-thiophenethiol (0.8 μg L^{-1}) [6]. Although some compounds were identified in the wines at relatively low concentrations and lower than the sensory threshold, researchers have demonstrated a possible synergy between the molecules present in concentrations lower than their sensory threshold when they are chemically similar [27].

All of the molecules investigated in this study are known to be products of the Maillard reaction [28]. They all present odorous notes close to those which develop in roasted food. The presence of some compounds, such as thiazoles, pyrazines, trimethyloxazole and 2-acetylthiazole, has already been reported by other researchers [4,6,8], produced through reactions between amino acids and carbonyl compounds under wine aging conditions. The values found in this study were lower than those reported by other researchers [6], who identified compounds such as thiazole, 2-acetylthiazole and trimethyloxazole in French wines, in concentrations higher than those obtained in our study. The same authors reported the presence of considerable amounts of 2-acetylthyazole for wines from Pomerol and Saint-Emilion, which contained >3 μg L^{-1} on average, which is the odor threshold value in water. White Burgundy wines, Champagne and Alsace contained on average between 1.4 and 1.8 μg L^{-1} of these compounds. On the other hand, red wines from Médoc, Burgundy and Provence wines and fortified wines contained <1 μg L^{-1} of 2-acetylthiazole. Researches also identified nitrogenous compounds in different wines, with levels close to those identified in our study [9]. These authors observed that the concentrations varied as follows: trimethyloxazol 0.2–0.7 μg L^{-1}, 4 methylthiazol 0.2–0.9 μg L^{-1} and 2-acetylthiazol 0.2–0.4 μg L^{-1}. Considering all of the wines studied, these authors affirmed that the fortified wines, such as Port, Madeira,

Table 4
Range of heterocyclic compounds (μg L^{-1}) in wine samples.

Compounds	Bordeaux (red wine)[a]	Bourgogne (red wine)[b]	Alsace (white wine)[c]	Bordeaux (white wine)[d]
Thiazole	0–8.1	0–2.9	0–0	0–1.5
4-Methyl thiazole	0–10.2	0–1.1	0–0	0–1.1
2-Ethyl thiazole	0–0.22	0–0.75	0–0	0–0
Benzothiazol	0.2–1.5	1–1.6	0.9–3.5	0–1.99
2-Acetyl thiazole	0–1.8	0–1.5	0–1.9	0–1.4
2-Methyl thiazole	0–0.4*	0–0.5	0–0.4*	0–0.4*
2,4,5-Trimethyloxazole	0–0.4*	0–0.89*	0–0	0–0
5-Methyl furfural	1.5*–17	4–27	5–9	2–250
3-Acetyl-2,5-dimethylfuran	0–0.9	0–0.9	0–0.8	0.8–0.9
2,3-Dihydrobenzofurane	0–0.7	0.6–0.67	0–0.6	0–0.6
2-Acetylfuran	6–50.2	9.9–24.5	1*–6.2	2.2–21
3-Acetyl thiophene	0–2.5	0–2.1	0–2	0–2
2-Acetyl thiophene	0–2.2	0–1.75	0–2.8	0–0
2,3-Dimethyl thiophene	0–0.3	0–0	0–0	0–0
2,5-Dimethyl thiophene	0–0.7	0–0.6	0–0.5	0–0
2-Methyl pyrazine	0–1.2	0–0.9*	0–0.4*	0–0
2-Acetylpyrazine	0–5	0–3.7	0–0	0–0
2-Ethylpyrazine	0–0.6*	0–1.18	0–0	0–0
2,6-Dimethyl pyrazine	0–4	0–3.0	0–0	0–3.5
2,3-Diethyl pyrazine	0–0.8	0–0.7	0–0.6	0–0.6
2-Ethyl-3-methyl pyrazine	0–0.8	0–0.7	0–0	0–0
2-Acetyl-3-methyl pyrazine	0–1.15	0–0.98	0–0	0–0
2,3,5-Trimethyl pyrazine	0–0	0–0.94	0–0.8	0–0.8
2,3,5,6-Tetramethyl pyrazine	0–1.1	0–0	0–0	0–0

[a] $n=10$.
[b] $n=6$.
[c] $n=6$.
[d] $n=7$.
* LOD < value < LOQ; 0: value < LOD.

and Rivesaltes, in general, had the highest concentrations of nitrogenous heterocyclic compounds. The most abundant heterocyclic compounds in these types of wines have been found to be 2,4,5-trimethyloxazole, 2,4-dimethylthiazole and 2-acetylthiazole. Mo et al. [12] identified different classes of heterocyclic compounds that contribute to the aroma of Chinese rice wine, including benzothiazol, trimethylpyrazine, tetramethylpyrazine and 2.3-dimethylpyrazine, which were also identified in our study in different French wine samples.

4. Conclusions

This study shows the development of an HS-SPME method and its validation by GC–MS. The method allows the simultaneous determination of 24 heterocyclic compounds in the wine in the single chromatographic run. The use of a central composite design and the response surface methodology allowed the optimum combination of the analytical variables (pH, NaCl and extraction time) to be determined. The reliability is reported using validation criteria considering the linearity, repeatability, reproducibility and accuracy.

This method constitutes a fundamental tool for gathering useful information concerning the identification of the main heterocyclic compounds in wines and their potential influence on the wine odor.

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