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Quantitative analysis of 2-furfural and 5-methylfurfural in different Italian vinegars by headspace solid-phase microextraction coupled to gas chromatography–mass spectrometry using isotope dilution

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Abstract

A new method was developed for the determination of 2-furfural (2-F) and 5-methylfurfural (5-MF), two products of Maillard reaction in vinegar, with head-space solid-phase microextraction (HS-SPME) coupled to gas chromatography–mass spectrometry (GC–MS). A divinylbenzene (DVB)/carboxen (CAR)/polydimethylsiloxane (PDMS) fibre was used and SPME conditions were optimised, studying ionic strength effect, temperature effect and adsorption time. Both analytes were determined by calibration established on 2-furfural-d_4 (2-F-d_4). The method showed good linearity in the range studied (from 16 to 0.12 mg/l), with a regression coefficient r^2 of 0.9999. Inter-batch precision and accuracy were found between 14.9 and 6.0% and between −11.7 and 0.2%, respectively. Detection limit was 15 μg/l. The method is simple and accurate and it has been applied to a series of balsamic and non-balsamic vinegars.

Keywords: Vinegar; Isotopic dilution; Food analysis; Furfurals; 5-Methylfurfural

1. Introduction

Vinegar is a solution of acetic acid, obtained by a fermentation process from a variety of raw materials (especially white and red wines, apple juice, honey, malted barley, etc.). It is used as flavouring and preserving agent for a wide range of foods.

Italian traditional balsamic vinegar is typically produced in Reggio Emilia and Modena area (Italy) with a long and complex process, involving a time of at least 12 years. Unlike common vinegar, according to Italian laws, it must be produced from acetic acid fermentation of cooked grape must, then transferred into a cask where partial alcoholic fermentation occurs. The aging process takes place into little casks, made of different types of wood (oak, mulberry, chestnut or juniper). This process gives the characteristic flavour of this product, particularly

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appreciated to enrich the taste of a large variety of foods.

Furan derivatives are characteristic of the flavour (caramel like) of these vinegars. They are formed with Maillard reactions (or non-enzymatic browning) between reducing sugars and amino acid, that occur during the cooking of the grape must and during the aging period [1]. This reaction gives *N*-glycosilamines and *N*-fructosylamines in the first step, then their isomerisation leads to 1-amino-1-deoxy-2-ketoses or 1-amino-2-deoxy-2-aldoses (Amadori compounds) which are the precursors of these furan compounds.

Among these, we focused our attention on 2-furfural (2-F) from pentose sugars and 5-methylfurfural (5-MF) from hexose sugars. Their presence in balsamic vinegar is regular and some producers are interested in determination of the level of these molecules in the finished product in order to evaluate organoleptic properties and possible commercial frauds.

Different analytical methods were developed in the last years to determine the products from Maillard reaction in some foods, like milk, infant formulas [2], fruit juices [3], oils [4] and spirits [5]. They were initially analysed by spectrophotometric measurements [6,7], but these methods are time consuming and not specific. So other analytical methods for identification and quantification were developed, principally based on RP-HPLC [2,3] and GC–FID or gas chromatography–mass spectrometry (GC–MS) [4,8]. Among these, only the works reported by Lo Coco et al. [3] and by Goldberg et al. [5] described a quantitative method for the determination of 2-F with a considerable accuracy and sensibility.

Sample preparations were based on liquid–liquid extraction [8] or solid-phase extraction (SPE) [9], including steam-distillation extraction [4].

Solid-phase microextraction seems to be a valid alternative to traditional methods for the preparation of samples [10] because it is fast, inexpensive and solvent free. This technique has been successfully applied in combination with GC and HPLC to the determination of a wide variety of analytes from environmental [11,12], biological [13] and food [14–21] samples.

The literature reviewed for vinegars showed the development only of generic methods for screening analysis of flavours [22–24] with GC–MS but there was a lack of exclusive methods for the quantitation of specific Maillard products such as 2-F and 5-MF. Vinegar (especially balsamic vinegar) is a complex matrix, so an external calibration method in a synthetic matrix could mismatch with the real vinegar sample. For these reasons, standard additions method or isotopic dilution calibration [25–27], may be used for quantitative analysis by SPME.

Starting from these considerations, the aim of this work was to establish a new, specific method to evaluate the levels of 2-F and 5-MF in vinegars, (with particular attention to balsamic vinegars), by applying head-space SPME coupled to GC–MS. Isotope dilution calibration was performed, using 2-furfural-d_4 (2-F-d_4), so avoiding standard additions method.

2. Experimental

2.1. Chemicals and materials

2-Furaldehyde and 5-methylfuraldehyde were purchased from Fluka (Buchs, Switzerland). Furfural-d_4 (99.5 at.% D) was supplied by CDN Isotopes (Point-Claire, Que., Canada). Methanol (HPLC grade) and NaCl (analytical grade) were obtained from Carlo Erba Reagenti (Milan, Italy) and Merck (Darmstadt, Germany), respectively.

2.2. Vinegar samples

Ten vinegar samples were used for our study: three white, three red, two balsamic vinegars, all available from local markets, and two balsamic vinegars obtained from Italian craftsmen.

2.3. Stock solutions

Stock solutions of 2-F, 2-F-d_4 and 5-MF were prepared in methanol at the final concentration of 1 mg/ml and stored a 4 °C in the darkness.

2.4. Equipment

2.4.1. SPME fibres

The fibre used in this study, coated with 2 cm–50/30 μm of divinylbenzene (DVB)/carboxen (CAR)/polydimethylsiloxane (PDMS), was purchased from Supelco (Bellefonte, PA, USA). The fibre was conditioned before its use by inserting it into the GC

injector overnight at 280 °C, according to supplier's instruction. The holder used was for manual injection and was also supplied by Supelco.

DVB/CAR/PDMS fibre has been chosen after previous trials at 20 °C and at extraction time of 30 min with four different types of fibres: a 100 μm polydimethylsiloxane (PDMS), a 75 μm carboxen CAR)/polydimethylsiloxane (PDMS), a 65 μm carboxen (CAR)/divinylbenzene (DVB) and a 65 μm carbowax (CW)/divinylbenzene (DVB) fibre, all purchased from Supelco. The first three fibres were not able to detect our analytes, also in accordance with Castro Mejías et al. [23], and the best results were obtained with DVB/CAR/PDMS fibre.

2.4.2. Instrumental conditions

HS-SPME analysis was performed with a Hewlett-Packard (Palo Alto, CA, USA) HP 6890N gas chromatograph coupled to a HP5973N mass-selective detector.

The separations were performed using a DB-WAX capillary column (J & W Scientific, Folsom, CA, USA), 60 m × 0.25 mm i.d., with a 0.25 μm coating. A split/splitless injector was used in the splitless injection mode; the injector temperature was set at 280 °C. Ultrahigh-purity helium was used as carrier gas at the flow rate of 1 ml/min, column head pressure was 22 psi (1 psi = 6894.74 Pa). The detector (transfer line) was set at 280 °C. The GC oven temperature was programmed as follows: from 110 °C increasing to 160 °C at a rate of 3°C/min. Run time was 17 min.

Spectra were acquired in electron impact (EI) mode at 70 eV, with electron multiplier voltage set at 1294 V. The analyses were performed without solvent delay, using selected ion monitoring (SIM) mode, monitoring *m/z* 96, 100, 110 (target ions) and *m/z* 67, 70 and 81 (qualifier ions) for 2-F, 2-F-d_4 and 5-MF, respectively.

2.5. Optimised headspace-SPME procedure

For sample preparation, 8 ml of vinegar sample were placed into a 16 ml glass-vial, adding NaCl in order to obtain a 40% (w/v) solution. Each sample was spiked with 20 μl of 2-F-d_4 stock solution. Then, the vial was capped with a PTFE-faced silicone septum and shaken to obtain a homogeneous mixture. The sample was maintained at 50 °C and the fibre was inserted through the vial septum and exposed to the sample headspace for 40 min to perform the extraction. Finally, the fibre was inserted in GC injector for desorption (10 min). This time was enough to ensure total desorption and no memory effect was observed when the same fibre was inserted for a second time.

2.6. Calibration

Calibration was established on furfural-d_4 in vinegar matrix, with eight calibration points over the expected concentration range of our compounds in vinegar samples. For this aim, calibration standards were prepared by adding an appropriate amount of stock solution of 2-F-d_4 to a vinegar matrix and then serially diluting with additional matrix in order to obtain concentrations ranging from 16 to 0.12 mg/l. Other dilutions were made to evaluate the LOD.

For each concentration level, five independent measurements were taken on different days in order to determine precision and accuracy. Each calibration curve was built plotting peak area versus concentration. Statistical analysis was developed with Excel software (Microsoft, Redmond, WA, USA). After recalculation of concentrations from regression curves, we evaluated precision as the relative standard deviation (R.S.D.%) of the recalculated concentrations and accuracy as [(mean calculate concentration − nominal concentration)/nominal concentration] × 100.

The limit of detection (LOD) was defined as the amount of 2-F-d_4 which gives a signal three times higher than noise signal (S/N = 3), while the limit of quantification (LOQ) was defined as the lowest amounts of 2-F-d_4 which can be determined with a precision and accuracy ≤20% [28,29].

3. Results and discussion

3.1. GC–MS optimisation

In order to establish retention times and the characteristic spectra of our compounds, 10 μl of standard solutions containing 2-F, 2-F-d_4 and 5-MF, respectively, each at the concentration of 1 mg/l in methanol,

were injected into the GC–MS with the instrument in the full scan mode in the range of 27–400 amu, at a rate of 2.23 scan/s. The mass spectra of 2-F, 2-F-d_4 and 5-MF, showed the mass ion at *m*/*z* 96, 100, 110, respectively, as main peak and a less abundant peak at *m*/*z* 67, 70 and 81, respectively, derived from each molecular ion by the loss of CHO, (CDO for 2-F-d_4).

The selected ion monitoring (SIM) focused the mass selective detector on *m*/*z* 96, 100, 110 (target ions) and on *m*/*z* 67, 70 and 81 (qualifier ions) for 2-F, 2-F-d_4 and 5-MF, respectively. Retention times were 10.55 min for 2-F, 10.62 min for 2-F-d_4 and 13.37 min for 5-MF. Peaks detection was based on the retention times, the presence of the qualifier ion and the predetermined ratio between the target ion and the qualifier ion.

3.2. SPME parameters optimisation

SPME technique involves the optimisation of some experimental parameters (ionic strength, temperature and extraction time) that affect the extraction procedure and consequently its efficiency, reproducibility and sensitivity. This optimisation was achieved studying each factor separately and performing a series of experiments on vinegar matrix (8 ml) enriched with 20 μl of 2-F-d_4 stock solution in order to obtain 2.5 mg/l, in a 16 ml vial. Sample volume of 8 ml was chosen because it was the maximum volume to obtain the minimum headspace/sample volume ratio and, at the same time, allowing the fibre to be completely exposed into the headspace.

3.2.1. Salt effect

The addition of a salting-out agent can improve the efficiency of extraction, because the presence of dissociated ions decreases the solubility of analytes in matrix favouring their partition into the headspace.

The ionic strength effect was evaluated comparing the amounts of analytes extracted from a sample without salt and from a sample saturated with 40% (w/v) of NaCl at 20 °C and at extraction time of 30 min (Fig. 1). The saturation of the sample with NaCl increased peaks areas indicating an increase in the efficiency of extraction.

3.2.2. Effect of temperature

HS-SPME is controlled by analytes equilibrium between sample and headspace and between the headspace and the fibre. Extraction temperature affects these equilibria because its raising causes an increase in distribution rates but a decrease in distribution constant.

In order to establish the optimal conditions for the effectiveness of the extraction, we investigated the effect of temperature, varying it from 20 to 50 °C. The best results both for the amounts of analytes absorbed and the standard deviations were obtained at 50 °C (Fig. 2).

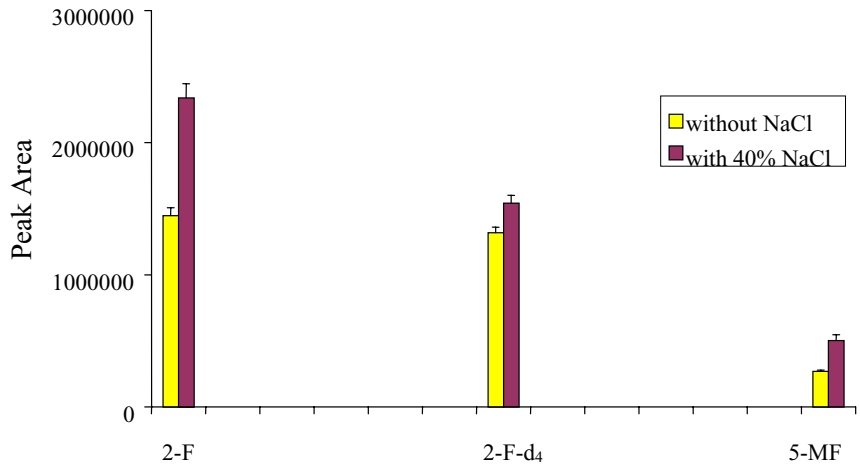

Fig. 1. Ionic strength effect on HS-SPME at 20 °C (mean ± S.D.), from 8 ml sample and 30 min extraction time ($n = 3$).

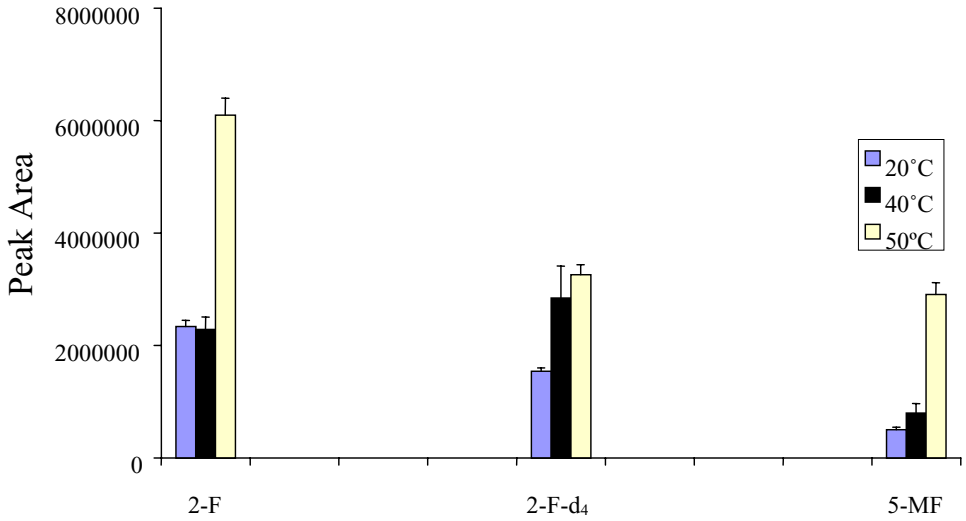

Fig. 2. Temperature effect on HS-SPME (mean ± S.D.), from 8 ml sample containing 40% of NaCl ($n = 3$).

3.2.3. *Effect of extraction time*

The extraction time affects the efficiency and the reproducibility of the extractions and an absorption-time profile was considered for 2-F, 5-MF and 2-F-d_4 at 20, 40 and 50 °C (Fig. 3). The time at which the peaks areas became constant was considered as the equilibration time. Both at 50 and 20 °C there were little variations in peaks areas after 40 min for 2-F, 5-MF and 2-F-d_4, while at 40 °C the equilibrium was not achieved even after 60 min.

Considering these trials, the best results were obtained at 50 °C, either for the amounts of analytes extracted, the reproducibility and time to achieve the equilibrium.

For these reasons, 50 °C and 40 min were selected as our operative conditions for the fibre extraction.

3.3. *Method calibration*

The method was linear in vinegar matrix over the range 0.12–16 mg/l and the r^2 value for 2-F-d_4 was 0.9999. As shown in Table 1, the accuracy and precision were ≤14.9 and ≤11.7%, respectively.

The limit of detection (LOD) was 15 μg/l.

In order to evaluate the applicability of 2-F-d_4 calibration also to the determination of 5-MF, standard addition method was applied, spiking a balsamic sample with standard solutions of 5-MF to obtain 4.8, 2.4, 1.2

Table 1
Precision and accuracy for 2-furfural-d_4

Nominal concentration (mg/l)[a]	Mean calculated concentration (mg/l)	Precision (R.S.D.%)	Accuracy (%)
16.00	16.03	7.9	0.2
8.00	7.88	11.6	−1.5
4.00	4.09	14.9	2.3
2.00	2.05	6.0	2.6
1.00	0.99	8.2	−0.8
0.50	0.51	8.1	1.7
0.25	0.25	9.3	−0.9
0.12	0.11	11.6	−11.7

[a] Nominal concentrations: expected concentrations of prepared standard solutions.

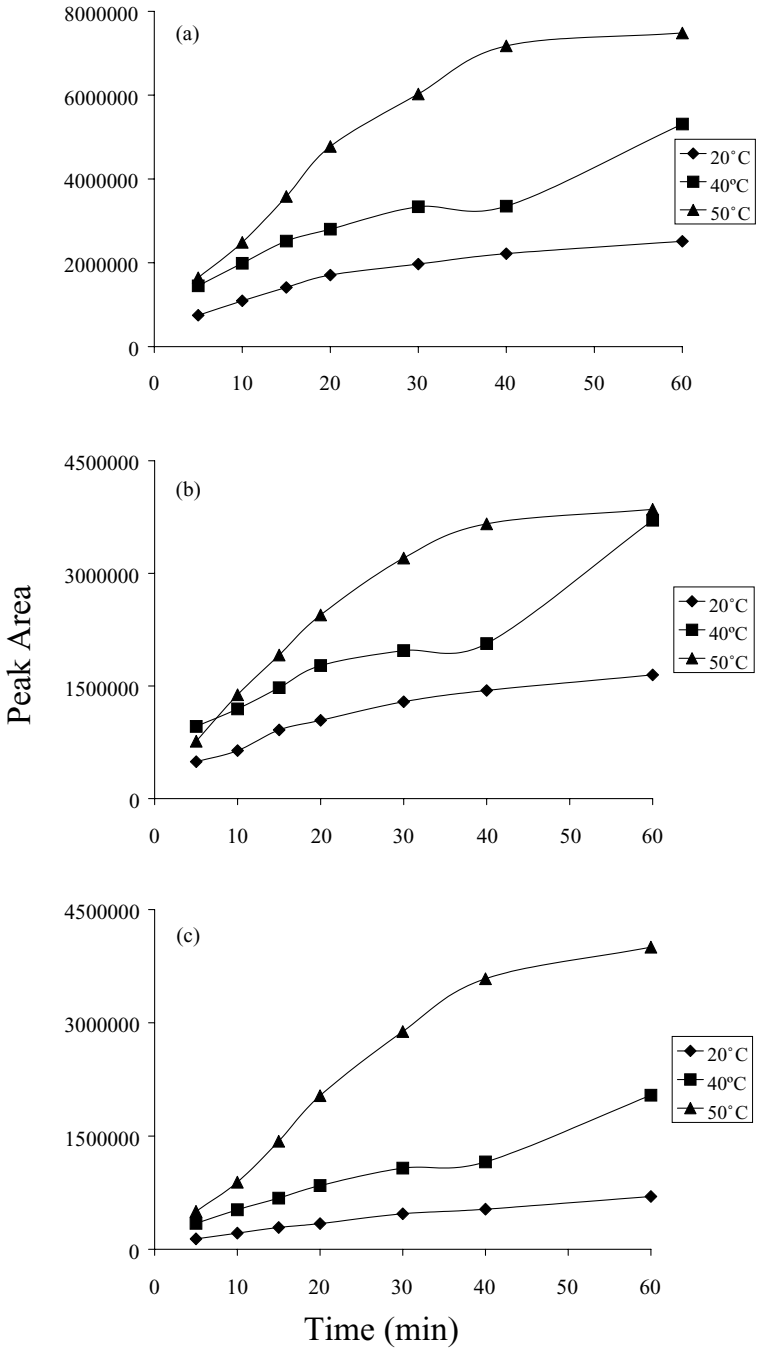

Fig. 3. Absorption-time profiles (mean ± S.D.) of 2-F (a), 2-F-d_4 (b) and 5-MF (c) at 20, 40 and 50 °C, from 8 ml sample solution containing 40% of NaCl ($n = 3$).

and 0 mg/l of added concentrations. The 5-MF concentrations determinated in the sample with standard addition method were compared with those determinated with 2-F-d_4 calibration, obtaining an accuracy of 1.6%. For this reason, 5-MF was determinated using the same calibration established on 2-F-d_4.

Recovery of the method was determined by adding to vinegar samples 2-F and 5-MF at known concentrations in the calibration range and extracting them as above described in the experimental section. The expected concentrations were compared to calculated concentrations and a recovery of 93% was obtained for both analytes.

3.4. Method application

The headspace SPME method developed for the determination of 2-F and 5-MF was applied to 10 vinegar samples. Two commercial balsamic vinegars (numbers 1 and 2) and two more aged balsamics (A, B), supplied from craftsmen producers, were analysed and the results were compared to six non-balsamic vinegars (three white and three red).

Typical SIM chromatograms of 2-F (peak 1), 2-F-d_4 (peak 2) and 5-MF (peak 3) obtained from a balsamic vinegar (a) and from a common vinegar (b) are shown in Fig. 4.

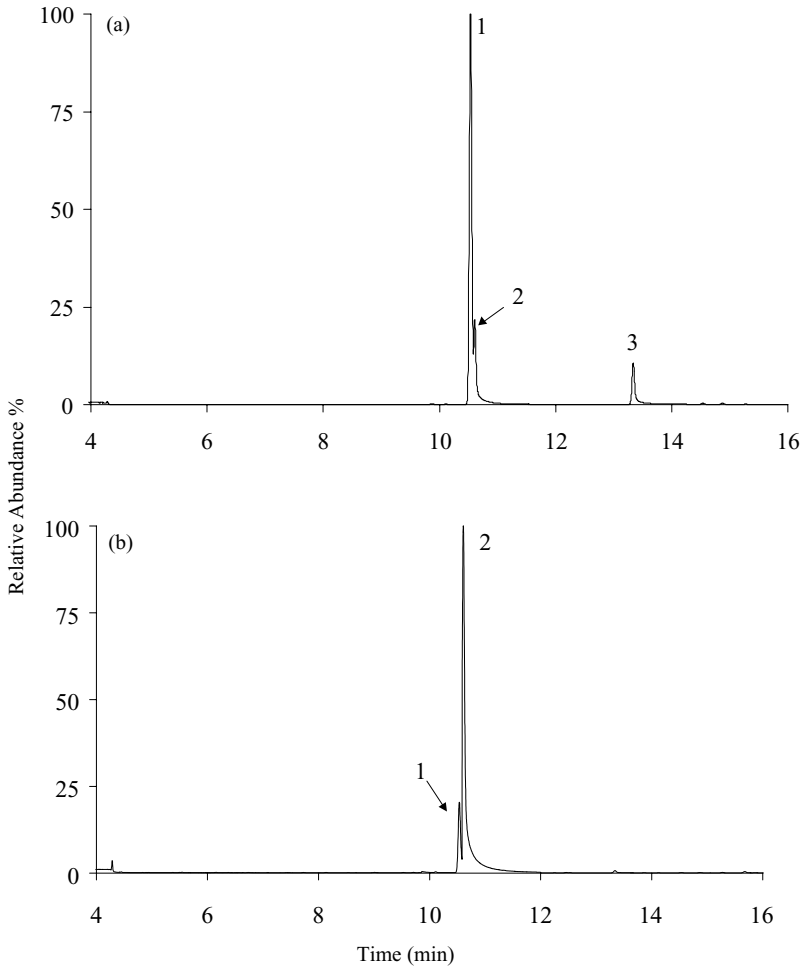

Fig. 4. SIM chromatograms relative to the GC–MS analysis of 2-F (peak 1), 2-F-d_4 (peak 2) and 5-MF (peak 3) in a balsamic vinegar sample (a) and a common vinegar sample (b).

Table 2
Concentrations and standard deviations of 2-F and 5-MF in vinegar samples ($n = 3$)

Vinegar sample	Concentrated ± S.D. (mg/l)	
	2-Furfural	5-Methylfurfural
White number 1	0.31 ± 0.03	<LOQ
White number 2	1.35 ± 0.02	<LOQ
White number 3	0.55 ± 0.05	<LOQ
Red number 1	0.34 ± 0.03	<LOQ
Red number 2	0.89 ± 0.02	<LOQ
Red number 3	0.57 ± 0.07	<LOQ
Balsamic number 1	2.63 ± 0.18	1.88 ± 0.18
Balsamic number 2	6.63 ± 0.10	2.30 ± 0.23
Balsamic A	14.19 ± 0.12	2.42 ± 0.24
Balsamic B	8.00 ± 0.49	1.08 ± 0.11

The results (Table 2) showed that 2-F was present in all the samples analysed, ranging from 14.2 to 2.6 mg/l in balsamic samples and from 1.4 to 0.3 mg/l in non-balsamic samples.

5-MF was present only in balsamic samples, ranging from 2.4 to 1.1 mg/l, while in common vinegars the 5-MF content was under the limit of quantification ($\leq$30 μg/l). This gap in concentration of our analytes between balsamic and non-balsamic vinegars can be explained by the process of must cooking typical of balsamics. As showed in Table 2 for sample A and B, the aging period also affects the increase of concentration.

4. Conclusions

HS-SPME combined to GC–MS was successfully applied to the determination of 2-furfural and 5-methylfurfural in vinegar samples. This methodology was very simple, fast and showed good repeatability, linearity and sensibility. These results for accuracy, precision and sensibility could be compared only to those obtained by Lo Coco et al. [3] for HPLC quantification of 2-F, though this method was applied to fruit juices and not to vinegar.

Salt effect, temperature and time of absorption were separately investigated in order to achieve optimisation of SPME parameters. Both the analytes can be quantified with GC–MS using isotope dilution calibration with 2-F-d_4. As we expected, the study of real vinegar samples revealed higher concentration levels of both analytes in balsamic vinegars than in non-balsamic vinegars.

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