Journal of Chromatography A, 1230 (2012) 1–7

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Automated and quantitative headspace in-tube extraction for the accurate determination of highly volatile compounds from wines and beers

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ARTICLE INFO

Article history:
Received 5 September 2011
Received in revised form
24 November 2011
Accepted 13 January 2012
Available online 21 January 2012

Keywords:
Headspace
Microextraction
Dynamic extraction
Acetaldehyde
Diacetyl
Ethyl acetate

ABSTRACT

An automatic headspace in-tube extraction (ITEX) method for the accurate determination of acetaldehyde, ethyl acetate, diacetyl and other volatile compounds from wine and beer has been developed and validated. Method accuracy is based on the nearly quantitative transference of volatile compounds from the sample to the ITEX trap. For achieving that goal most methodological aspects and parameters have been carefully examined. The vial and sample sizes and the trapping materials were found to be critical due to the pernicious saturation effects of ethanol. Small 2 mL vials containing very small amounts of sample (20 μL of 1:10 diluted sample) and a trap filled with 22 mg of Bond Elut ENV resins could guarantee a complete trapping of sample vapors. The complete extraction requires 100× 0.5 mL pumping strokes at 60 °C and takes 24 min. Analytes are further desorbed at 240 °C into the GC injector under a 1:5 split ratio. The proportion of analytes finally transferred to the trap ranged from 85 to 99%. The validation of the method showed satisfactory figures of merit. Determination coefficients were better than 0.995 in all cases and good repeatability was also obtained (better than 7% in all cases). Reproducibility was better than 8.3% except for acetaldehyde (13.1%). Detection limits were below the odor detection thresholds of these target compounds in wine and beer and well below the normal ranges of occurrence. Recoveries were not significantly different to 100%, except in the case of acetaldehyde. In such a case it could be determined that the method is not able to break some of the adducts that this compound forms with sulfites. However, such problem was avoided after incubating the sample with glyoxal. The method can constitute a general and reliable alternative for the analysis of very volatile compounds in other difficult matrixes.

1. Introduction

Some of the most volatile compounds of beer and wine and many other fermented products, such as diacetyl (2,3-butanodione), acetaldehyde or ethyl acetate have remarkable sensory, toxicological and biochemical properties and are also relevant markers of microbiological state [1–3].

Toxicologically, diacetyl has been related to acute breath problems (bronchiolitis obliterans syndrome) in workers exposed to it [4,5] such as those working in popcorn [6], butter [7] and other dairy products. Remarkably, some authors have suggested that its rather pleasant aroma may cause a false feeling of safety [5]. Acetaldehyde is suspected to be carcinogenic and has been related to upper aerodigestive tract cancer [8,9]. There are some worrying studies trying to establish correlations between the risk of suffering such tumors with alcoholic beverage consumptions [10,11]. The risk would be linked not only to the natural content of acetaldehyde in the alcoholic beverage, but also to the fact that ethanol can be converted to acetaldehyde by salivary dehydrogenases. Such additional risk would be higher in those segments of people with a more active genotype [8]. Toxicological effects of ethyl acetate are less clear and contradictory. While a recent study warns that exposure to low levels of ethyl acetate and toluene of workers of the wood industry may be related to headaches, cough or nervousness [12], a previous study concluded that there were no adverse chemosensory effects at normal exposure levels [13].

From the sensory point of view the three of them have a relevant role on the aroma of fermented beverages. There is no doubt that diacetyl is a key aroma compound in all the fermented dairy products [14], but in wine and beer its role is more complicated. Diacetyl is most often considered just an off-flavor in beer production [15–17] while in the case of wine it is generally accepted that it has deep stylistic implications and that its role may range from positive to negative depending on the wine type and concentration [3,14,18]. Acetaldehyde on its part is quantitatively the most abundant carbonyl in beer and wine and traditionally it is considered to

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doi:10.1016/j.chroma.2012.01.037

be a major responsible of wine oxidation. However, its role on wine oxidative aroma is not really straightforward [19]. Similarly, ethyl acetate is usually considered just a problem [20], but it also could have a significant positive role on the perception of wine and beer fruity notes.

These compounds are also important in wine and beer technology, microbiology and biochemistry. Diacetyl may react with cysteine forming numerous odorants in Maillard or Maillard related processes [21]. Sulfite–acetaldehyde complexes seem to be the cause of sluggish malolactic fermentations [22], the rate of acetaldehyde production is closely related to the level of sulfur dioxide added to the wine [23] and that compound is known to have a key role on wine color stabilization. Ethyl acetate levels are related to the action of acetic acid bacteria.

As can be seen there are relevant reasons for the robust quantification of these compounds in fermented beverages. However, its accurate analysis is not straightforward due to a number of causes related mainly to their physicochemical similarity to ethanol which in fact is the most active solvent in a fermented beverage. Such similarity impairs not only the efficiency of liquid–liquid or liquid–solid extraction strategies [1,2,24–28] but also that of static or dynamic headspace methodologies [29–32]. In the former cases, it is difficult to extract quantitative amounts of the compounds and the most volatile compounds can be lost during solvent evaporation [29,33]. In the latter cases, the high levels of ethanol seriously limit the sensitivity and trappings efficiencies. An appealing strategy is the direct analysis by headspace solid phase microextraction (SPME), because of its simplicity, speed, possibility of automatization and user-friendliness. However, and as consequence of the high level of ethanol and other major volatiles, recoveries are usually very small and what is worse, they are strongly matrix dependent [25,29,34,35], so that accuracy only can be guaranteed by the use of very good internal standards. Deuterated standards have been used in wine [1], cheese [36], butter or air [37]. In some other cases, acetaldehyde and diacetyl were first derivatized to form their pentafluorobenzyl derivatives [38] to improve the mass spectrometric signal, but no recovery data were reported.

ITEX is a completely automatic solventless extraction technique for headspace sampling in which a headspace syringe with a needle body filled with a sorbent is used [39,40]. The analytes are extracted from the sample headspace by pumping it repeatedly through the sorbent. The needle body is surrounded by a heater used for the thermal desorption of analytes into the injection port of a GC system. A detailed description of the system has been reported by Jochmann et al. [39]. To the best of our knowledge, ITEX has been used previously only for the determination of volatile organic hydrocarbons from aqueous samples [39,40], for the analysis of hydroxyl methyl-derivatized and volatile organic compounds in blood and urine [41] and for the analysis of aliphatic hydrocarbons from petroleum source rock coupled with microwave-assisted nonionic surfactant extraction [42]. All these applications deal with problems classically solved by purge and trap or even by static headspace strategies, and demonstrate that ITEX can represent an economic alternative to classical purge and trap enrichments and that can be much more sensitive than static headspace techniques. In the present work, however, the analytical problem has not an obvious solution from static or dynamic headspace strategies, which have to face the aforementioned problems related to the presence of large amounts of ethanol. The aim of the present work is to study the ITEX extraction conditions that lead to the quantitative transference of the acetaldehyde, diacetyl and ethyl acetate contained in beer or wine to the ITEX trap, with the purpose of developing an automatic, accurate and fast analytical method for their quantitative determination.

Table 1
ITEX method parameters.

Parameters	
Incubation temperature (°C)	60
Incubation time (min)	5
Agitator speed (rpm)	250
Syringe temperature (°C)	40
Extraction volume (μL)	500
Extraction strokes	100
Extraction speed ($\mu L\,s^{-1}$)	160
Desorption temperature (°C)	240
TrapH FillSpeed ($\mu L\,s^{-1}$)	25
Desorption speed ($\mu L\,s^{-1}$)	50
Injection volume (μL)	500
Needle flush time (min)	20
Trap cleaning temperature (°C)	240

2. Experimental

2.1. Materials

Acetaldehyde 99%, ethyl acetate 99%, ethyl propanoate 99%, isoamyl alcohol 98%, isobutanol 99%, methyl 2-methylbutyrate 99%, 4-methyl-2-pentanol 99% were purchased from Sigma–Aldrich (Madrid, Spain). Diacetyl 99% and ethyl butyrate 98% were purchased from Fluka (Madrid, Spain). 2-Butanol 99% and ethanol LiChrosolv, were supplied by Merck (Darmstadt, Germany) and tartaric acid 99% by Panreac (Barcelona, Spain). Pure water was obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA).

Samples used in the study were one red and two white wines with alcoholic degrees comprised between 12% and 13% (v/v) and pHs ranging from 3.3 to 3.8; all of them were dry table wines with ages between 1 and 3 years old. One sample of beer was also used (4.5% (v/v) in alcohol). All samples were purchased in a local store.

2.2. Methods

2.2.1. Sample preparation

Two milliliters of wine or beer sample, 40 μL of internal standards solution (methyl 2-methylbutyrate 300 $mg\,L^{-1}$, 2-butanol 750 $mg\,L^{-1}$ and 4-methyl-2-pentanol 1250 $mg\,L^{-1}$) are pipetted into a 20 mL volumetric flask. This is brought to volume with a 5 $g\,L^{-1}$ tartaric acid solution. Twenty microliters of this sample are then transferred to a 2 mL vial and are encapsulated with a magnetic cap. The vial is then put in the sample tray of the Combi Pal automatic sampler which contains the ITEX accessory.

2.2.2. ITEX

Analyses were carried out with a commercial version of ITEX installed in a Combi Pal autosampler from CTC Analytics (Zwingen, Switzerland). A modified 2.5 mL headspace syringe was connected to the ITEX trap. The original sorbent material was replaced by 22 mg of Bond Elut ENV (Varian, Walnut Creek, USA). The ITEX extraction parameters are given in Table 1.

2.2.3. GC–MS conditions

The apparatus was a Shimadzu QP-2010 gas chromatograph with a quadrupole mass spectrometric detection system. The injector was a standard split/splitless injector set at 250 °C. The injection was carried out in split mode with a relation 1:5. The carrier gas was He at a constant linear velocity of 50 $cm\,s^{-1}$ (1.93 $mL\,min^{-1}$) during the run. The column was a DB-WAXetr capillary column from J&W, 30 m × 0.32 mm I.D., with 1.0 μm film thickness. The chromatographic oven was held at 35 °C for 5 min, then raised to 120 °C at 4 °C min^{-1}, then to 230 °C at 100 °C min^{-1} and finally the temperature was held at 230 °C for 3 min. Mass spectrometric detection was

in electron impact mode. The ion source temperature was 220 °C while the interface was kept at 240 °C. Detection was in scan mode from 1.2 min to 2.75 min for acetaldehyde and 4.0 to 5.7 min for ethyl acetate with m/z ranged from 35 to 120 in both cases. Finally between 6.9 and 29 min detection was in single ion monitoring (SIM) mode taking the ions with m/z 55, 57, 59, 60, 69, 70, 74, 86, 88 and 102 at 0.4 points s^{-1} for another analytes. The ions used in the analysis were 43 for acetaldehyde and ethyl acetate, 57 for ethyl propanoate, 86 for diacetyl, 88 for ethyl butyrate, 74 for isobutanol, 70 for isoamyl alcohol, 88 for methyl 2-methylbutyrate, 69 for 4-methyl-2-pentanol and 59 for 2-butanol. The other ions were used as qualifiers.

2.2.4. Method development and validation

A synthetic solution imitating wine (12%, v/v ethanol, 7 g L^{-1} glycerin, 5 g L^{-1} tartaric acid and pH adjusted to 3.5) and containing 33 mg L^{-1} acetaldehyde, 10.6 mg L^{-1} diacetyl, 12.1 mg L^{-1} ethyl acetate, 3.4 mg L^{-1} ethyl propanoate, 2.9 mg L^{-1} ethyl butyrate, 17.6 mg L^{-1} isobutanol and 18.7 mg L^{-1} isoamyl alcohol and 77.5 mg L^{-1} of acetic acid was used for method development. Different volumes of this solution (between 1 and 20 μL), diluted or not with water, brine or acidified water were tested in different vial volumes (5, 2 and 0.3 mL). Two different sorbent materials were tested: Bond Elut ENV from Varian and the standard Tenax TA originally contained in the trap. Different masses of Bond Elut ENV were also tried (between 2 and 23 mg). The trap temperature during the extraction process was set at 35 °C and during the desorption it was fixed at 240 °C for Bond Elut ENV and at 300 °C for Tenax TA. The vial extraction temperature was studied between 30 and 70 °C. In all extraction conditions a different number of pumping strokes was essayed and in most cases, the amount remaining in the trap was evaluated by performing successive extractions and by determining the rate of decrease of the area. In another set of experiments, the effect of the volume used in the extraction strokes was also evaluated together with the aspiration speed. Desorption process was optimized independently. Parameters considered were volume, speed, temperature and split ratio. Different chromatographic columns were also tried (DB-WAXetr 30 m × 0.20 mm and 0.25 μm; DB-WAXetr 30 m × 0.32 mm and 1.0 μm; DB-5 30 m × 0.20 mm and 0.25 μm), and the final phase was selected attending to polarity, phase ratio and phase thickness for achieving an adequate retention of the early eluting acetaldehyde and for limiting the effects of mass overloading caused by the high amounts of ethanol recovered.

Once the major parameters were optimized, an experiment for evaluating the existence of matrix effects was carried out. In that experiment, the samples were made by mixing a dearomatized non-volatile matrix from a wine or a beer (in both cases such matrixes were prepared by vacuum distillation followed by solvent extraction and further re-distillation), with the analytes, a certain level of ethanol (only for wine between 10 and 15%) and variable levels of major volatiles (acetaldehyde, ethyl acetate, isobutanol and isoamyl alcohol). The analysis of results led to the reoptimization of some parameters (particularly relevant were the number of extraction strokes, the dilution of the sample and the need for using internal standards for accounting for the interday variability of the GC–MS system).

After this, the method was evaluated for reproducibility by analyzing the same real sample 8 times along a 3-week period. Method linearity was evaluated at 6 different concentration levels ($n = 3$ for each level). Detection limits were estimated as the concentration of compounds that generated a signal of three times the signal-to-noise ratio (S/N = 3). Matrix effects and method accuracy were assessed by a recovery assay. Additionally, the real amount of analytes trapped in the trap was determined by performing successive extractions on the previously analyzed sample.

3. Results and discussion

3.1. Sorbent material and sample and vial volumes

One of the basic prerequisites of the present method is to be able to trap in the ITEX sorbent nearly quantitative amounts of the target volatile compounds present in the sample, so that potential matrix effects are minimized. For achieving this, it is necessary to avoid an excessive dilution of the sample vapors and also to guarantee that the trap does not become mass-saturated and that breakthrough volumes are not reached either. Avoiding dilution of sample vapors implies working in vials as small as possible. It should be taken into account that the headspace extraction process of the ITEX system involves in fact a dilution of the sample headspace. If the sample headspace would be, for instance, 20 mL and the pumping strokes were set at 1.0 mL, the sample headspace first is expanded to 21 mL (original headspace volume + extraction volume), then it can be assumed that the volatile material content in the 1 mL is fully trapped in the sorbent, so that the amount trapped in one sample stroke has been a mere 4.76% (4.76 = 1/21 × 100) of that content in the headspace. A relatively simple spreadsheet calculation shows that the number of 1.0 mL-pumping strokes required for transferring 95% of the headspace content would be in this case 62 strokes. On the other hand, a 2 mL headspace volume, would require just eight 1.0 mL-pumping strokes for trapping the 95%. Smaller headspace volumes would theoretically improve these numbers, but in the practical use, the vacuum caused by pumping out 1 or 2 mL of a 0.5 mL headspace is strong enough to cause that air comes in through the septum and further out and there is not real improvement. Therefore, standard 2 mL vials were used for the extraction of small volumes of sample (between 1 and 50 μL).

The mass saturation and breakthrough volumes for the different analytes in a given trap are functions of the extraction temperature, the trap material and dimensions, the number of extraction strokes and of course the volume of sample. Two different sorbent materials were considered, Tenax TA, which is the standard ITEX sorbent and Bond Elut ENV. This sorbent is seldom used for headspace trapping, but in a previous work it was found to have excellent trapping properties providing gas–solid distribution coefficients 2 orders of magnitude higher than those of Tenax TA [43]. The disadvantage of Bond Elut ENV is that its maximum working temperature is 240 °C, below the 300 °C maximum temperature that the standard ITEX system can reach, and below the maximum 340 °C maximum temperature of Tenax TA. Such disadvantage has a clear incidence on the elution profile, making the peak of the early eluting acetaldehyde to become clearly distorted.

In spite of that limitation, the trapping capacity of Bond Elut ENV was much better than that of Tenax TA and it was found to be critical for the quantitative trapping of the most volatile compounds (acetaldehyde, ethyl acetate and diacetyl). It should be noted that the high percentage of ethanol that the wine contents, together with the high volatility of those analytes and with the relatively high volumes of air "passing to and through" the small trap, makes that breakthrough volumes and/or mass saturation can be very easily achieved. In the case of Tenax TA, the breakthrough of the trap for acetaldehyde, ethyl acetate and diacetyl occurred after just 32-0.5 mL-pumping strokes. At that point the percentages of these compounds recovered in the trap were estimated to be less than 46, 72 and 50%, respectively. These figures are unsatisfactory, particularly taken into account that they were obtained with standard solutions. In the case of a trap containing 22 mg of Bond Elut ENV the breakthrough was not achieved in more than 150-0.5 mL strokes. Under those conditions satisfactory recoveries, even for the most volatile compounds can be achieved, as will be discussed in the next section.

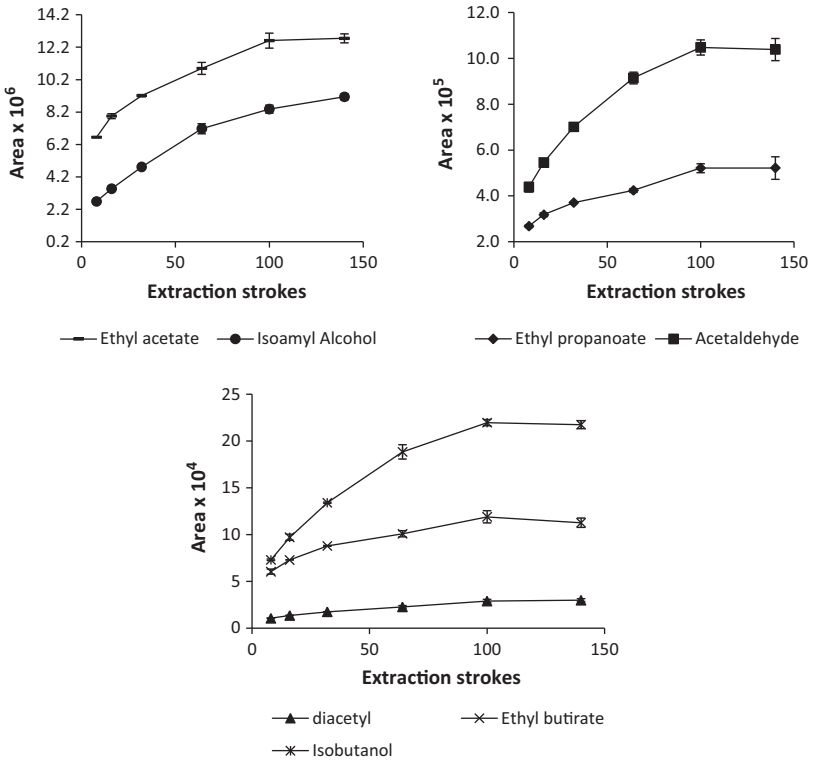

Fig. 1. Effect of the number of pumping strokes on the extraction of the recovered analytes ($n = 3$). Conditions were 0.5 mL extraction volume, plunger speed 100 μL s^{-1}, 10 μL diluted sample in 2 mL vial at 60 °C, desorption with 500 μL of He ejected at 50 μL s^{-1}.

As for the sample volume, this has to be quite small, since most ethanol will be co-extracted with our analytes, provoking the potential saturation of both the trap and the gas chromatographic column. Ten μL of diluted wine or beer (1–5 in an aqueous solution containing 5 g L^{-1} tartaric acid) was found to be a satisfactory sample volume at this point.

3.2. Other extraction variables

The relationship between the number of extraction cycles or pumping strokes and the signal from the analytes is given in Fig. 1. As shown in the figure, in nearly all cases a plateau is reached, with maxima levels of extraction at 100 pumping strokes. The relationship between area and pumping strokes can be better understood with the help of the scheme shown in Fig. 2. As can be seen, analytes are initially distributed between the sample and the gas phase within the vial. The pumping strokes have as effect the progressive transference of the analytes from the sample and headspace to the trap. In some cases, such transference may be complete at a number of pumping strokes below the critical number at which the breakthrough for that compound in the trap occurs (case 1 in the figure) and the mass of analyte retained in the trap can amount to 100%. In some other cases, however, the breakthrough of the trap takes place after a number of pumping strokes at which the transference has not been completed, so that increasing the pumping strokes does not further increase the mass of analyte retained in the trap, but provokes that a fraction of analyte is lost in the syringe barrel (case 2). This is the case of acetaldehyde, for instance, and the small drop in area observed at 150 strokes is due to the small amounts of analyte that have passed to the upper part in the syringe and are consequently lost. Compounds showing a clear plateau, such as ethyl acetate, behave as case 1. Only in the case of isoamyl compound a clear area increase between 100 and 150 extraction strokes is observed, because it is the least volatile and the compound which is more slowly released from the matrix. This compound in fact has not even reached the case 1 in the figure. In any case, 100 pumping strokes were taken as optimum. This corresponds to an extraction time of 24 min.

Extraction temperatures between 30 and 70 °C were essayed and the influence of this parameter, for the rest of parameters fixed, is given in Fig. 3. As shown in the figure, the best results are obtained at 60 °C in most cases. Lower temperatures lead in all cases to incomplete extractions (for this number of extraction cycles), since the compounds are more retained in the liquid phases. At higher temperatures, a slight reduction in the signal is observed in some cases, which however is not statistically significant because of the smaller precision observed at 70 °C. This higher imprecision (significant $p < 0.001$) must be attributed to the fact that breakthrough volumes are nearly reached, particularly in the cases of acetaldehyde, ethyl acetate and diacetyl, as a consequence of the higher proportion of ethanol present in the headspace at 70 °C. Once the breakthrough has occurred, an irreproducible amount of analyte is lost (w_1 in case 2 of Fig. 2). Only isoamyl alcohol was significantly more recovered at 70 °C, in accordance with its lower volatility. The addition of salt for improving the extraction was also evaluated, but there was no improvement.

Other parameters which were found to exert just a minor influence on the recovery, but which had a relevant effect on analysis time were the pumping speed and the pumping volume. The effect of both parameters is seen in Fig. 4. In the case of the pumping speed, it can be observed that passing from 50 to 160 μL s^{-1} implies a slight reduction on the extraction efficiency. In the worst case (ethyl butyrate) the recovery is just 11% smaller, but the gain in the extraction speed is a factor 3.2. As recovery can be further improved by increasing the number of pumping strokes, a pumping speed 160 μL s^{-1} was selected. More or less the same result is observed for the extraction volume. There is an increase, particularly notable in the cases of isobutanol and isoamyl alcohol (up to 20%) but just

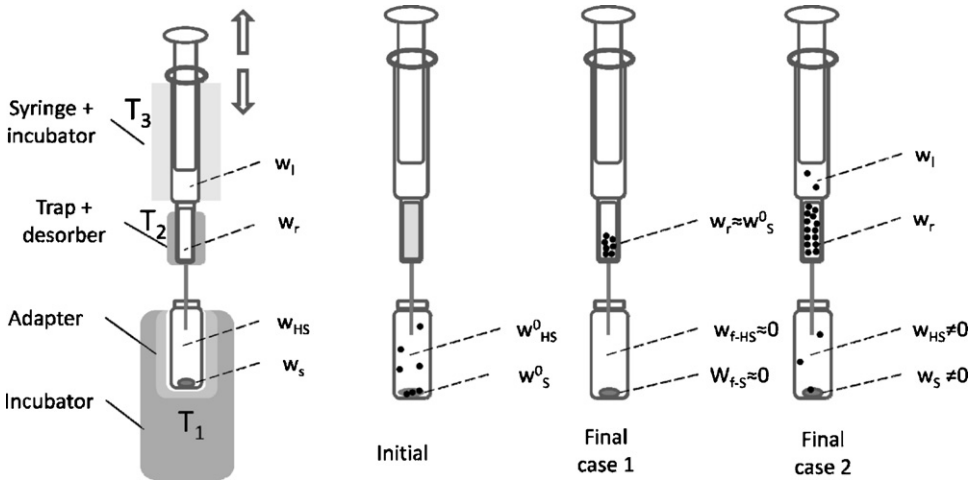

Fig. 2. Schematic representation of the ITEX process. T_i, temperatures in different zones of ITEX device; *w* represents the mass of an analyte in: the sample (s), the headspace (HS), the trap (r) and in the syringe (l) respectively. Superscript 0 and subscript f indicate initial or final condition. In the final case is possible to get two scenarios, in case 1 the extraction is completed before reaching the breakthrough of the trap, and case 2 the breakthrough situation is reached before the extraction is completed.

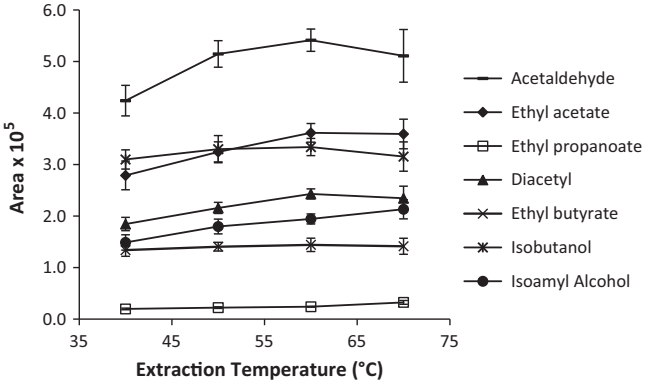

Fig. 3. Effect of temperature on the extraction of volatile compounds from wine ($n = 3$).

marginal in the rest of cases, when passing from 500 to 2000 μL, that implies increasing the extraction time by a factor near 4. In addition, it should be noted that isobutanol and isoamyl alcohol are not critical analytes in the present method, and that a higher recovery of these compounds will be paralleled by higher recoveries in ethanol, which is not convenient for the method robustness. Consequently, 500 μL were retained as optimal extraction volume.

Desorption temperature is limited by the maximum stability of the Bond Elut ENV polymer of 240 °C. The parameter with a higher influence on this process is the speed of the plunger during the desorption process. On the one hand, such speed cannot be very high because that would mean overloading the GC injector. On the other hand, a too slow speed would mean an unnecessary band broadening. For the slow split ratio, set at 1:5 for sensitivity, 50 μL s^{-1} was found to be a satisfactory value. Optimum desorption volume was found to be 500 μL.

3.3. Preliminary assessment of matrix effects

With the previous parameters fixed, an incomplete factorial experiment involving different ethanol levels (6, 10, 12 and 15% v/v), two levels of major volatile compounds and different matrixes (synthetic wine, red wine matrix, synthetic beer and beer matrix) was carried out. The Analysis of Variance of the data set, summarized in Table 2, revealed that the most influential factor was the level of ethanol, which exerted a significant effect on the extraction efficiency of all compounds except isoamyl alcohol. In all cases what was observed was a neat decrease on the recoveries at highest levels of ethanol (15%, v/v), which could be attributed to a decrease of the breakthrough volumes and trapping efficiencies due to the presence of ethanol in the trap. In addition, a significant effect of the matrix was also noted in the extraction of acetaldehyde and diacetyl (see Table 2), which were slightly better extracted from real wine matrixes than from synthetic ones. In order to minimize these effects, the dilution of the sample was then increased from 1:5 to 1:10 so that the volume of sample in the vial was increased to 20 μL. The purpose of this dilution is to reduce the influence of the matrix and to increase the volume of water which will retain a higher amount of ethanol decreasing the risk of saturation of the trap. After this correction, the effects of both the ethanol content and of the matrix on the extraction efficiencies were eliminated. In addition, three internal standards (ethyl 2-methylbutyrate, 2-butanol and

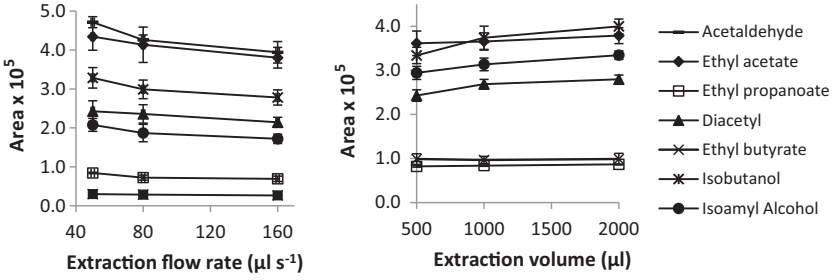

Fig. 4. Influence of extraction flow rate and extraction volume on the extraction of volatile compounds (50 strokes, 60 °C) ($n = 3$).

Table 2
Summary of the results of the ANOVA study carried out to check the existence of different matrix effects (the matrix itself, the level of ethanol and the levels of major volatiles) on the signals per unit of concentration obtained with the initial procedure (10 μL of sample diluted 1:5).

Compound	Matrix[a]		% Alcohol[b]		Volatile level[c]	
	F	*p*(*F*)	*F*	*p*(*F*)	*F*	*p*(*F*)
Acetaldehyde	7.292	**0.002**	40.248	**0.000**	1.962	0.171
Ethyl acetate	0.383	0.685	17.126	**0.000**	0.03	0.863
Ethyl propanoate	2.275	0.071	10.405	**0.000**	0.158	0.694
Diacetyl	5.766	**0.007**	35.883	**0.000**	0.382	0.541
Ethyl butyrate	0.698	0.505	4.055	**0.027**	4.008	0.054
Isobutanol	2.071	0.143	6.109	**0.006**	0.018	0.894
Isoamyl alcohol	0.009	0.991	0.919	0.409	0.387	0.538

Values in bold indicate a significant effect of parameter in the extraction efficiency.
[a] Four matrixes: water/ethanol 6%, v/v, beer, synthetic wine and red wine.
[b] Four ethanol levels: 6, 10, 12 and 15%, v/v.
[c] Two levels of major volatiles. Low level (10, 9, 5.2, 2.2 and 5.5 mg L^{-1}) and high (19.7, 51.7, 19.7 and 153.5 mg L^{-1}) for acetaldehyde, ethyl acetate, isobutanol and isoamyl alcohol, respectively. Concentrations of ethyl propanoate, diacetyl and ethyl butyrate were 2.13, 2.01 and 1.98 mg L^{-1} in all cases, respectively.

Table 3
Linearity, detection limits, reproducibility and extraction yield of the ITEX method.

Compound	Linearity ($n = 6$)			Detection limit (mg L^{-1})	Reproducibility (%RSD, $n = 8$)	Extraction yield (%)
	Slope	R^2	Range (mg L^{-1})			
Acetaldehyde[a]	0.0587	0.9949	3.9–49.4	0.03	13.1	85
Ethyl acetate[a]	0.0310	0.9996	10.3–129.2	0.07	8.1	95
Ethyl propanoate[a]	0.2821	0.9998	0.4–5.3	0.005	8.3	99
Diacetyl[a]	0.0118	0.9992	0.4–5.0	0.025	7.1	89
Ethyl butyrate[a]	0.0821	0.9997	0.4–4.9	0.01	4.7	97
Isobutanol[b]	0.0069	0.9997	3.9–49.5	0.13	4.4	92
Isoamyl alcohol[b]	0.0355	0.9991	30.7–383.7	0.01	2.2	90

[a] Area values were corrected by internal standard: methyl 2-methylbutyrate.
[b] Area values were corrected by internal standard: 4-methyl-2-pentanol.

4-methyl-2-pentanol) were also added in order to improve method repeatability and for assessing inter-batch deviations.

3.4. Method validation

Results of the different validation assays are summarized in Table 3. The repeatability of the method was better than 7% for all the studied compounds even using absolute areas. However, the reproducibility of those absolute areas was unsatisfactory (10–20%) which was attributed to the GC–MS system. However, internal standards adequately correct these figures, that in all cases ($n = 8$) are better than 13.1%. Worst results were obtained for acetaldehyde, which can be attributed to its deficient peak shape. Linearity was determined by analyzing six different concentration levels spiked on a previously dearomatized red wine matrix. Linear ranges covered the natural range of occurrence of these compounds and linearity was satisfactory, with determination coefficients better than 0.999 in all cases except acetaldehyde, for which this coefficient was 0.995. Detection limits are in all cases several orders of magnitude below the normal ranges of these compounds in wine. Finally, the extraction yield was estimated to be in all cases between 85 and 99%, which can be considered highly satisfactory given the high volatility of these compounds.

Matrix effects were assessed via the analysis of reconstituted wine and beer samples containing different known levels of analytes and different alcoholic degrees, and also via a recovery experiment carried out on real wines and beers. The signal increments obtained in these cases were compared with the signals obtained in the analysis of a reference synthetic solution containing known amounts of analytes in a wine-like media (12% ethanol, 5 g L^{-1} tartaric acid, 7 g L^{-1} glycerin and pH = 3.5). Results are given in Table 4. Recoveries in nearly all cases are high and average recoveries do not significantly differ from 100 in all cases, except acetaldehyde. Exceptional low recovery values for this compound were obtained in two white wine samples. Excluding these values, the average recovery for this compound was 94.5 with RSD of 9.3%. It was hypothesized that those low recoveries were caused by the presence of a complex with sulfites. For breaking such complex, glyoxal was added to the wine and it was experimentally determined that the complex was completely broken after incubating the sample at 50 °C for eight hours. Recoveries after this were 105 and 102 for the samples VB1 and VB2, respectively. Complex of sulfite with

Table 4
Study of matrix effect through standard recovery test.

Compound	VR 150_10	VR 80_14	CR 80_6	CR 100_8	C 50_4.5	VR 100_50	VB1 50_12	VT 50_13	VB2 50_12	Recovery $n = 9$	RSD (%)
Acetaldehyde	105.0	108.8	88.9	91.1	89.2	92.4	31.6	90.2	26.3	80.4	37
Ethyl acetate	119.5	105.9	103.2	106.3	100.9	107.0	102.1	102.1	114.1	106.8	6
Ethyl propanoate	105.3	97.0	95.1	97.3	94.2	111.3	108.3	106.7	110.8	102.9	7
Diacetyl	104.9	109.2	95.3	101.6	89.7	105.5	89.9	108.6	91.1	99.5	8
Ethyl butyrate	99.7	95.3	90.6	93.4	97.5	104.7	113.9	108.7	104.0	100.8	8
Isobutanol	94.4	87.9	89.4	98.9	87.7	92.5	97.1	112.4	86.3	94.1	9
Isoamyl alcohol	108.5	102.0	102.3	106.1	97.8	96.2	101.1	109.4	97.6	102.3	5

Samples are labeled as VR, reconstructed wine; CR, reconstructed beer; VT, red wine; VB, white wine; and C, beer. The two numbers below the sample code refer to the level of volatiles and the alcoholic content of the sample, respectively. The 100% of the concentration of volatiles corresponds to 19.75 g L^{-1} acetaldehyde, 51.68 g L^{-1} ethyl acetate, 2.13 g L^{-1} ethyl propanoate, 2.01 g L^{-1} diacetyl, 1.94 g L^{-1} ethyl butyrate, 19.80 g L^{-1} isobutanol and 153.5 g L^{-1} isoamyl alcohol. Real wines or beers were spiked with known quantities of analytes.

diacetyl was apparently already broken by the sample dilution and the 60 °C extraction temperature. It was then concluded that the current procedure gives an estimation of the free acetaldehyde in wine sample and a total estimation of diacetyl levels, and that if the total acetaldehyde levels are required, the sample must be first pretreated with glyoxal.

4. Conclusions

The proposed method makes it possible an accurate and automated determination of some of the most volatile compounds produced in alcoholic fermentation using the in tube sorbent extraction system. It has been shown that the standard Tenax TA sorbent becomes very easily saturated by the ethanol vapors not making possible a quantitative recovery of the volatiles present even in 2 μL of sample. Bond Elut ENV resins were found to be efficient enough for such recovery, even though the different methodological aspects, particularly sample dilution and sample and vial volumes have to be thoroughly controlled. It can be hypothesized that the method can be applied to the analysis of acetaldehyde, diacetyl, ethyl acetate and other highly volatile compounds in many other samples.

Acknowledgments

This work has been funded by the Spanish Government, project AGL2010-22355. J.Z. has received a grant from the Universidad de Zaragoza-Banco Santander Central Hispano.

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