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Highly selective solid-phase extraction and large volume injection for the robust gas chromatography–mass spectrometric analysis of TCA and TBA in wines

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Abstract

A reliable solid-phase extraction (SPE) method for the simultaneous determination of 2,4,6-trichloroanisole (TCA) and 2,4,6-tribromoanisole (TBA) in wines has been developed. In the proposed procedure 50 mL of wine are extracted in a 1 mL cartridge filled with 50 mg of LiChrolut EN resins. Most wine volatiles are washed up with 12.5 mL of a water:methanol solution (70%, v/v) containing 1% of $NaHCO_3$. Analytes are further eluted with 0.6 mL of dichloromethane. A 40 μL aliquot of this extract is directly injected into a PTV injector operated in the solvent split mode, and analysed by gas chromatography (GC)–ion trap mass spectrometry using the selected ion storage mode. The solid-phase extraction, including sample volume and rinsing and elution solvents, and the large volume GC injection have been carefully evaluated and optimized. The resulting method is precise (RSD (%) < 6% at 100 ng L^{-1}), sensitive (LOD were 0.2 and 0.4 ng/L for TCA and TBA, respectively), robust (the absolute recoveries of both analytes are higher than 80% and consistent wine to wine) and friendly to the GC–MS system (the extract is clean, simple and free from non-volatiles).

Keywords: 2,4,6-Trichloroanisole; 2,4,6-Tribromoanisole; Large volume injection; Solid-phase extraction; Wine; Cork taint

1. Introduction

One of the most significant organoleptic defects in wines is called cork taint which is related to a mouldy/musty off-flavour. Several substances have been suggested to be responsible for corkiness, such as geosmin, 2-methylisoborneol, guaiacol, 1-octen-3-one, 1-octen-3-ol, pyrazines, chloroanisoles and chlorophenols [1–3]. Among these, 2,4,6-trichloroanisole (TCA) has been blamed as the most contributory compound because of its frequent occurrence in tainted wines. According to the European QUERCUS project, at least 80% of spoiled wines analysed exhibited the presence of TCA [4]. This potent odorant can also produce sensorial alterations in other food products and its incidence in drinking water, coffee, raisins, chicken, etc. has been reported [5–7].

The sensory problems produced by TCA are closely associated with its low odour threshold. Some studies have been focussed on determining the detection threshold values for TCA in wines [8]. Although this value varies depending on the type of wine and the assessors, we can assume that TCA can be detected at concentrations lower than 10 ng/L. However, in some cases the quantities of the compound in tainted wines are not high enough to produce any alteration. This phenomenon was explained by the contribution of other substances (noticeable spoilage has been reported for 2,3,4,6-tetrachloroanisole (TeCA) at concentrations above 10 ng/L in still wines [9]) or through synergism [10].

Recently, 2,4,6-tribromoanisole (TBA) has been identified as a potent odour agent related to cork taint [9]. The findings have concluded that TBA can cause an intense musty odour in

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wines where the chloroanisole content is below its detection threshold. This compound is also perceived at low concentration level, especially in water samples, and can provide unpleasant aromas in many food products [11,12].

Gas chromatography (GC) is the most suitable technique for TCA and TBA analysis in wine. Detection is accomplished either with an electron-capture detector (ECD) [13–16] or mass spectrometry [10,17–26]. Due to the complexity of the matrix and the need of reaching the human sensory thresholds in wine, an effective pre-treatment of the sample is required. Regarding to TCA determination in wines, several extraction methods have been proposed: liquid–liquid extraction (LLE) [10,17–19], solid-phase extraction (SPE) [20], solid-phase microextraction (SPME) [13–16,21–23], stir bar sorptive extraction (SBSE) [24] and pervaporation (PV) [25,26]. SPME has become very popular in food analysis, especially for the extraction of volatiles and semivolatiles. This technique makes possible to save sample preparation time and to avoid the use of solvents. In addition, very good detection limits can be often achieved, as a consequence of the low background caused by the absence of solvent and of heavy contaminants. However, the influence of matrix composition, carry-over effects and sometimes poor reproducibility and linearity, are some of the most recurrent limitations in SPME [27]. SBSE follows the same principles as SPME, with the advantage of higher sensitivity because the film of sorbent which covers the magnetic stir bar is thicker, but the extracts obtained are more complex and dirty than those obtained by SPME. Both SPME and SBSE techniques have been employed for determining TCA at concentrations down to the ng/L level with SBSE showing limits of detection for TCA one order of magnitude lower, although the linearity was poorer [24].

Solid-phase extraction is widely used in analytical laboratories for either sample extraction or sample clean up procedures. Many benefits of SPE have been commonly cited including its robustness, potential for automation, capacity for providing clean extracts and selective isolations and even a fractionation of the different sample components. For these reasons, SPE is a powerful pre-concentration technique that can be easily adapted for routine analysis. Good analytical methods based on selective SPE can be much more robust and selective than the SPME or SBSE alternatives and, in fact, many studies based on SPE procedures for monitoring different compounds in wine samples have been described [28–30].

An apparent drawback of SPE methods, when compared with their SPME or SBSE counterparts is the need of a powerful pre-concentration step. However, this is no longer a problem if a large volume injection technique (LVI) to introduce the sample in the GC is used [31]. This strategy makes it possible to simplify the sample preparation procedures, reduces analysis time and improves method detection limits. Among the different alternatives for LVI, a programmed temperature vaporizing injector (PTV) working in the solvent split mode, represents a convenient choice due to its low tendency to matrix effects and the, in general, long-term stability of analyte response [32]. A great number of research works have been published applying this technique for environmental analysis as well as for the determination of earthy-musty compounds in water samples [11].

Although SPE has been previously applied for TCA extraction from wine [20], the method developed does not take advantage of some of the previously cited characteristics: the extraction is not selective, the sorbents used are not the most adequate for wine extraction [29], and a small volume is injected. Therefore, the main goals of the present work are to develop and optimize a SPE–LVI GC–MS analytical method for the determination of TBA and TCA in wine samples at ng/l.

2. Materials and methods

2.1. Chemicals and reagents

Stock solutions of 2,4,6-trichloroanisole and 2,4,6-tribromoanisole (Sigma–Aldrich Química, Madrid) were prepared in ethanol (Merck, Darmstadt, Germany) at 1000 mg/L level and were stored in a refrigerated environment. As internal standard, deuterated 2,4,6-trichloroanisole ($^2H_5$-TCA) was employed, which was synthesised according to Pollnitz et al. [18].

Both dichloromethane and methanol were supplied by Merck (Darmstadt, Germany) and $NaHCO_3$ by Panreac (Barcelona, Spain). The high purity water was taken from a Milli-Q purification system (Millipore, Bedford, USA).

Polypropylene SPE reservoirs (1 mL total volume) equipped with polyethylene frits (20 μm porosity) in bulk were obtained from Supelco (Bellefonte, USA). The SPE tubes were packed with 50 mg of LiChrolut EN resins (40–120 μm) purchased from Merck.

2.2. Solid-phase extraction

Semiautomated solid-phase extraction procedure was performed with a VAC ELUT 20 station from Varian (Walnut Creek, USA). The SPE bed was formed by 50 mg of LiChrolut EN resins prepacked in a 1 mL reservoir. Resins were previously purified and conditioned by adding 1 mL of dichloromethane, 1 mL of methanol and finally, 1 mL of a 12% (v/v) ethanol/water solution. A known volume (50 mL) of the wine sample, to which previously the internal standard had been added, was loaded at a flow-rate of ∼2 mL/min. Afterwards, the sorbent was rinsed with 12.5 mL of a methanol/water mixture containing 1% $NaHCO_3$ to remove matrix interferences. The bed was then dried by letting air pass through (−0.6 bar, 10 min) and analytes were eluted with 0.6 mL of dichloromethane. A suitable volume of internal standard was added to the final extract prior to the gas chromatography analysis. To prevent the contamination of the sample, the cartridges were not reused.

For the purposes of method optimization and validation, a dry white wine, made from Xarel·lo and Macabeo varieties and purchased in a local store, was employed. The absence of TCA and TBA was checked before use.

2.3. PTV GC–MS instrumentation

2.3.1. Gas chromatography–mass spectrometry conditions

Gas chromatographic analysis was performed with a CP-3800 chromatograph coupled to a Saturn 2200 ion trap mass-spectrometric detection system from Varian (Sunnyvale, CA, USA). A DB-WAXETR capillary column (J&W Scientific, Folsom, CA, USA) (30 m × 0.25 mm i.d., film thickness 0.5 μm) preceded by a 5 m × 0.25 mm uncoated (deactivated, intermediate polarity) precolumn from Supelco (Bellefonte, USA) was used. Helium was the carrier gas at flow rate of 1 mL/min. The oven temperature programme was 5 min at 60 °C, then increasing by 15 °C/min up to 220 °C and finally held at this temperature for 20 min. The MS-parameters were: both MS transfer line and chamber ionization temperature 200 °C, and trap emission current 80 μA. The global run time was recorded in full scan mode (45–360 *m*/*z* mass range), except in the segments where analyte isolation is accomplished. In these cases, in order to improve sensitivity and selectivity, the selected ion storage (SIS) mode was used. The mass range of these segments and the extracted ion chromatograms chosen for quantitative purposes are described in Table 1. The chromatographic data were analysed by Varian Saturn GC/MS Version 5.2 software.

2.3.2. Programmable injector conditions

The chromatograph was fitted with a 1093 septum-equipped programmable injector (SPI) from Varian. The insert, with internal diameter of 3.4 mm, was filled with ∼50 mg of silane treated glass wool (Supelco, Bellefonte, USA).

Large volume injections were performed at constant speed of 5 μL/s. The sample was introduced in a solvent split injection mode so as to increase the injection volume. During injection, the SPI was kept at 40 °C and a high split flow (100 mL/min) was applied with the aim of focusing the compounds on the insert packing. After most of the solvent had been removed, the split valve was closed. The injector was then heated to 300 °C at rate of 200 °C/min and the analytes were transferred from the insert to the column. As soon as the transference was completed, the split valve was opened (100 mL/min) and the injector temperature was kept constant at 300 °C. These conditions were maintained until the oven program was ended in order to prevent possible accumulation of interferences inside the injector. The overall evaluated parameters involved in large volume injection of TCA and TBA are summarized in Fig. 1 and subsequently examined in Section 3.

Table 1
MS segment description for the TCA and TBA analysis

Compound	Segment description		Quantitative fragments *m*/*z*
	Time (min)	Mass range (*m*/*z*)	
TCA	18æ21	190–220	195 + 210
$^{2}H_5$-TCA			217
TBA	28æ31	325–350	344

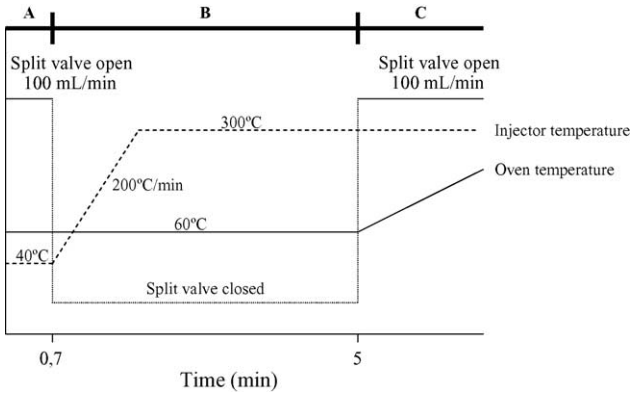

Fig. 1. Schematic overview of the large volume injection parameters (A). Sample introduction (B). Sample transfer (C) GC separation.

The influence of memory effect on the compound recoveries in successive injections was checked by injecting spiked real samples followed by blank samples. The glass wool plug replacement after ∼100 injections is advisable in order to avoid peak distortion and contamination by matrix components.

2.4. Method validation

2.4.1. Reproducibility

Method reproducibility was checked by the evaluation of intra-day (repeatability) and inter-day reproducibility with real wine samples spiked with known amounts of analytes. Intra-day reproducibility was determined by the replicated analysis of a wine spiked with 15 or 100 ng/L of analyte ($n = 3$ or 7, respectively). Inter-day reproducibility was determined by the analysis in three different days of four wine samples spiked with 5, 15 and 100 ng/L of analytes.

2.4.2. Linearity

GC–MS linearity was first determined by spiking real dichloromethane wine extracts (obtained following the proposed procedure) with known amounts of analytes in the range 0.1–50 ng/mL (which roughly corresponds to 1–500 ng/L in wine). The linearity of the complete method was finally evaluated by analysing real wines spiked with 0, 2, 5, 10, 20, 40 and 100 ng/L of analytes.

2.4.3. Recovery

Recovery was determined by comparing the absolute peak areas obtained in the analysis of wine samples spiked with 5, 15, 30 and 100 ng/L of analyte with those obtained in the analysis of dichloromethane solutions containing equivalent

amounts of analyte. This experiment was carried out in triplicate along 3 different days.

2.4.4. Method detection limits

Five different wines (two reds, a rosé and two white wines) were spiked with very low amounts of the analytes (1 or 2 ng/L) and analysed following the proposed procedure. The signal-to-noise ratio found in the analysis of these samples was used to calculate method detection limits as the concentration which should give a S/N ratio higher or equal to 3.

3. Results and discussion

3.1. SPE experiments

The main aim of the present work is to develop a robust and simple method for the routine analysis of TBA and TCA in wine samples at very low levels (ng/L). Particular attention has been paid to the dimensions of SPE system, so that small quantities of sample, sorbent and time are used. LiChrolut EN resins were used as sorbent because of its excellent capacity to retain volatile compounds from wine, and because of its extraordinary flexibility to provide clear cut fractions with the different wine components [28,30,33]. The mass of sorbent used was fixed as 50 mg, which are the smallest prepacked cartridges available. The breakthrough volume for both analytes in these cartridges was determined in preliminary experiences to be higher than 100 mL of wine, which can be considered enough to load safely a 50 mL volume of wine and to include a powerful washing up later. For the purpose of SPE method development wine samples spiked with 100 ng/L of the target compounds were utilized.

The washing up step was optimized by using different rinsing volumes and solvent mixtures containing water and methanol at different proportion and 1% $NaHCO_3$ to remove fatty acids [34]. The higher the methanol content, the cleaner

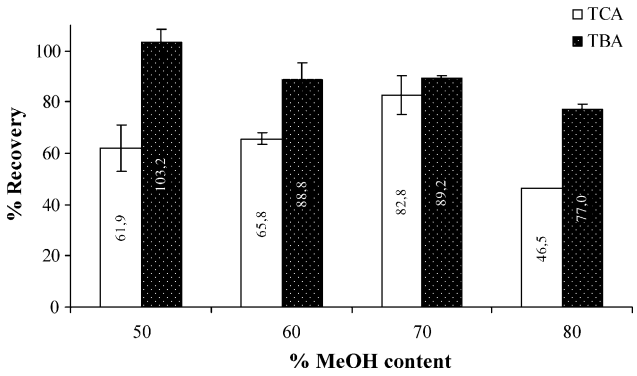

Fig. 2. Study of the effect of the methanol content of the washing solution on the recoveries achieved in the SPE isolation (TCA concentration 100 ng/L, $n = 2$).

the chromatogram, but when the rinsing solution contains 80% (v/v) of methanol, a decrease of the recoveries for the compounds of interest is obtained (Fig. 2). Therefore, 70% (v/v) of methanol represents the maximum strength of the washing up solvent. This solvent composition makes it possible to get an excellent selectivity using a minimum volume (12.5 mL). Solutions containing smaller proportion of methanol were also studied, but they require large volumes to achieve similar selectivity and recoveries are not better.

The effect of the washing up on the quality of the GC–MS signal is demonstrated in Fig. 3 by comparing the chromatographic profiles (reconstructed ion chromatograms) obtained by the injection of extract with (B) or without (A) the rinsing. Moreover, it must be recalled that the chromatogram shown in A comes from the injection of 1 μL of extract (coming approximately from 0.1 mL of wine), while the chromatogram shown in B comes from the injection of 40 μL of extract (equivalent to 4 mL of wine). As it can be seen, the washing up completely removes the large number of major compounds (concentrations between 1 and 200 mg/L) present in wine (fusel alcohols, ethyl esters, fatty acids) which make the GC–MS analysis of a trace component difficult or even

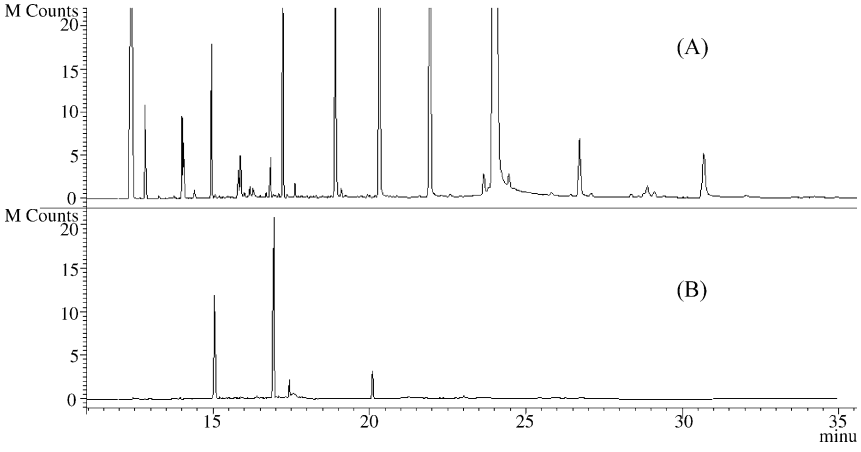

Fig. 3. Comparison between total ion chromatograms of wine extracts submitted or not to the clean up process. (A) Splitless injection of 1 μL of extract obtained by direct SPE, without applying washing step. (B) Chromatographic profile resulting of the injection (40 μL) of an extract obtained by the developed method. Peaks appearing in the profile do not correspond to analytes but to major wine volatile compounds.

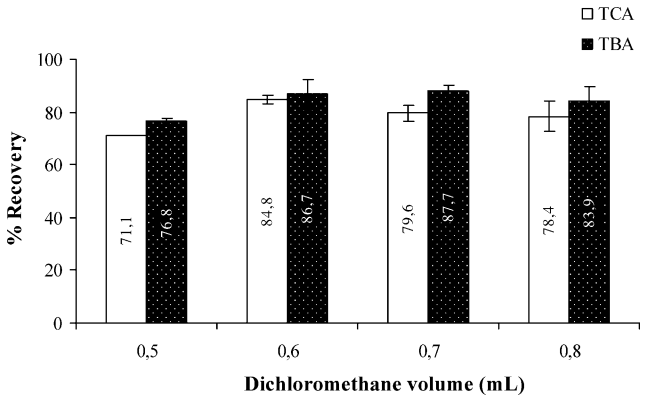

Fig. 4. Optimization of the solvent elution volume. Recovery results of the target compounds at different dichloromethane elution volumes (TCA and TBA at 100 ng/L; $n = 2$).

impossible, and in any case, disturb the long term performance of the GC–MS system. This selective isolation is a clear advantage of this method versus any other sample treatment alternative, including SPME or SBSE.

With the purpose of selecting the most suitable eluting solvent, wine extracts spiked with TCA and TBA were fractionated using the following series of solvents: pentane–pentane/dichloromethane (9:1)–dichloromethane. Results showed that TBA and TCA can be recovered in any of the solvents or solvent mixtures essayed. However, the elution volume increases with the nonpolar character of the solvent. From this point of view, the best results were achieved using dicloromethane as solvent, since both compounds can be totally eluted from the cartridge in a 0.6 mL volume, as shown in Fig. 4.

3.2. Large volume injection

There are several parameters with influence in the large volume injection. Some of them were fixed according to our experience or preliminary experiments. In particular, silanized glass wool was selected as insert packing due to its inertness to the studied compounds, an injection flow of 5 μL/s, a maximum injected volume of 40 μL (close to the maximum specifications of the injector), a split flow of 100 mL/min and initial temperature (40 °C) of the injector were fixed as a reasonable compromise.

The initial part of the optimization was carried out by the analysis of dichloromethane solutions or wine extracts obtained following the proposed procedure containing known amounts of TBA and TCA. The success of the transfer of the analytes from the injector to the GC–MS system was measured by comparing the areas obtained in the different large volume injections with that obtained in the injection of 1 μL of a 40× concentrated solution in classical splitless mode. All assays were performed in duplicate.

The first parameter studied was the transfer time. Results of this experiment are shown in Fig. 5. As can be seen, the transfer of TCA is complete at 2.5 min, while at least 3 min

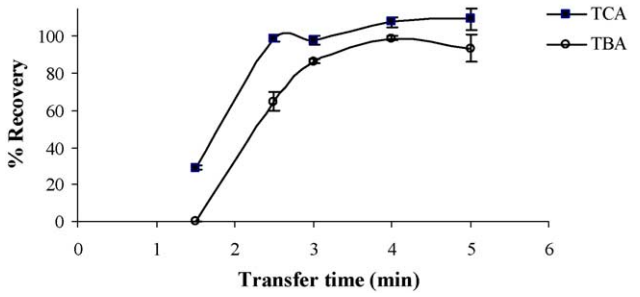

Fig. 5. Optimization of the analyte transfer time in the large volume injection. Recovery as a function of the transfer time ($n = 2$).

are required to get a good transfer of heavier TBA. It may be thought that these times are quite large, but the maximum heating capacity of our PTV is 200 °C/min. This means that the high temperature needs more than 1 min to be obtained, and probably more than 1.5 min to be really effective. For these reasons, 4 min were selected as a safe transfer time.

The second parameter considered was the solvent vaporization time or split time. An interval in the range from 0.5 to 3 min was tested as shown in Fig. 6. As can be seen, this parameter has hardly any influence on the transfer of TBA, a heavy compound well retained in the silanized wool kept at 40 °C. On the contrary, the transfer of TCA becomes incomplete at split times higher than 1 min. Although the best recovery was achieved at a split time of 0.5 min, the GC–MS profiles became much clearer if the split time is kept up to 0.7 min. Under these conditions, a reasonable GC–MS profile and high recoveries are obtained.

This result showed that an additional selectivity can be gained if volatile interfering compounds are flowed out the injector through the split valve during the splitless time. In order to maximize this effect, the injector was heated after the solvent evaporation time (0.7 min) to an intermediate temperature, and kept at such temperature up to 2 min, letting the split valve open along all this time. The results of the study can be seen in Fig. 7A and B. When standards in dichloromethane were injected (Fig. 7A) the recovery of analytes only decreased at temperatures higher than 100 °C for TCA. In contrast, when the same experiment was performed with spiked wine extracts, a diminution of recovery

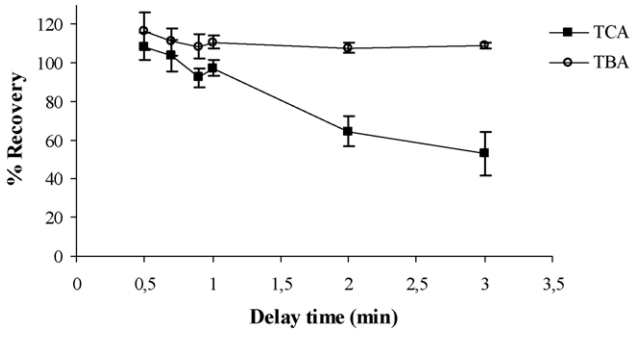

Fig. 6. Influence of split time on the recovery of TCA and TBA ($n = 2$).

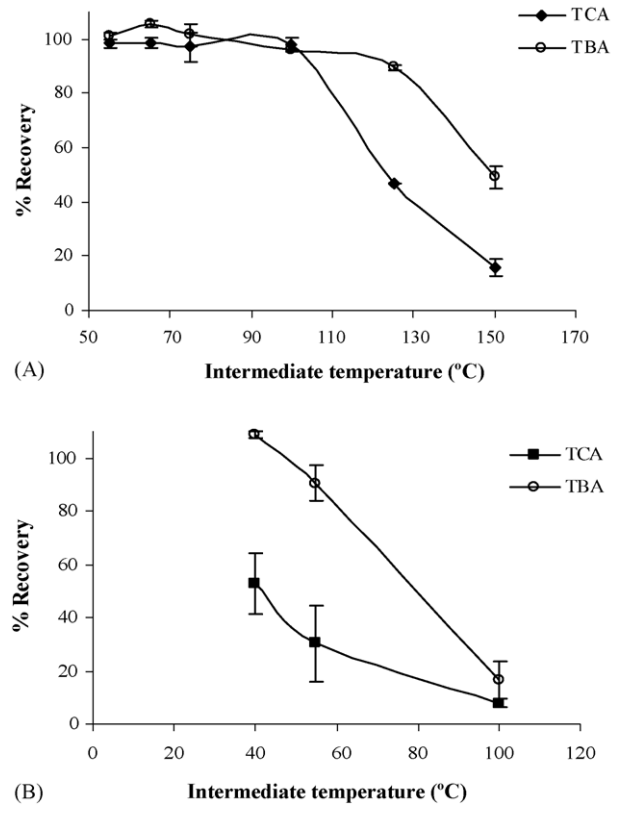

Fig. 7. Effect of keeping the injector at an intermediate temperature during the split time on analyte recovery ($n = 2$). (A) Injection of standards prepared in dichloromethane. (B) Injection of a real wine extract spiked with the analytes.

was observed (Fig. 7B), whichever temperature was assayed. This unexpected result just demonstrates that the evaporation of an extract and of a synthetic solution in the glass insert is very different. It can be suggested, that in a clean solution, analytes are well retained in some active point remaining

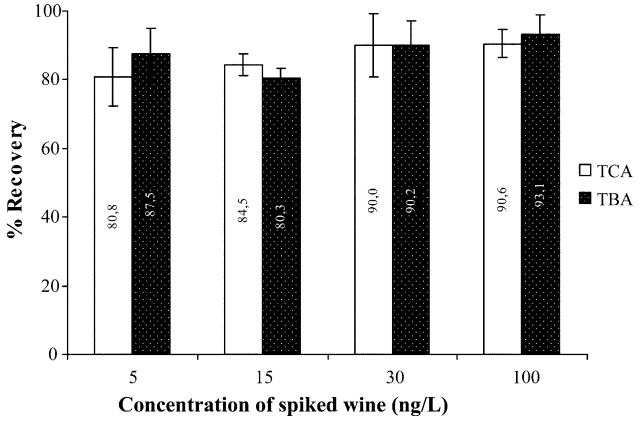

Fig. 8. Mean recoveries (calculated by comparing absolute chromatographic areas) and their relative standard deviation ($n = 3$) obtained in the analysis following the proposed procedure of three different wines spiked at different concentration levels of TBA and TCA.

in the glass wool packed in the insert, but when a sample is injected, other components compete for active sites and make the vaporization of analytes to take place before.

Under these finally optimized conditions, very good recoveries were obtained in the injection, with relative standard deviation for absolute areas below 10.1 and 7.5% for TCA and TBA, respectively.

3.3. Method validation

Method reproducibility data, referred to both intra- and inter-day precision, can be found in Table 2. As shown in the table, the average RSD (%) is below 6% and holds approximately along the whole dynamic range of the method, being a little bit higher at lower concentrations. Data in the table also indicate that inter-day variability is not much higher than intra-day variability, which suggests that set-up parameters

Table 2
Method precision data

Concentration (ng/L)	TCA		TBA	
	Intra-day RSD (%)	Inter-day RSD (%)	Intra-day RSD (%)	Inter-day RSD (%)
5		8.2 ($n = 3$)		7.9 ($n = 3$)
15	3.14 ($n = 3$)	5.14 ($n = 3$)	2.98 ($n = 3$)	6.84 ($n = 3$)
100	4.15 ($n = 7$)	5.82 ($n = 3$)	5.90 ($n = 7$)	3.05 ($n = 3$)

Table 3
Method linearity

	Compound	Linear range	a (S_a)	b (S_b)	r^2
GC–MS	TCA	0.09–47.7[a]	0.10 (0.04)	2.64 (0.02)	0.993
	TBA	0.17–42.2[a]	0.03 (0.02)	0.870 (0.009)	0.993
Complete method	TCA	4.65–92.92[b]	0.09 (0.03)	0.231 (0.005)	0.995
	TBA	4.60–92.04[b]	0.03 (0.01)	0.076 (0.002)	0.994

Linear ranges, regression coefficients and determination coefficients for the GC–MS analysis and for the whole procedure. a = Intercept; S_a = standard deviation of a; b = slope; S_b = standard deviation of b.

[a] Concentration in ng/mL referred to concentration in the extract.

[b] Concentration in ng/L referred to concentration in the wine.

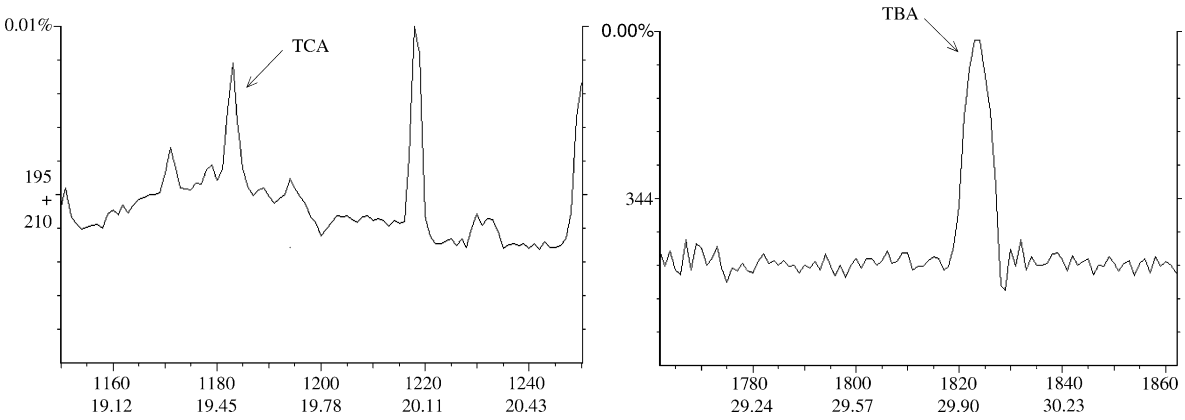

Fig. 9. GC–MS chromatogram obtained in the analysis, following the proposed procedure, of a wine (50 mL) spiked at 3 ng/L of TCA and 7 ng/L of TBA.

and particular batch conditions do not exert a strong influence on the results. This is a good indicator of method robustness.

The linearity of the developed method was determined in two independent studies. In the first one, real wine extracts, obtained following the proposed procedure, were spiked with known amounts of analytes to check the linearity of the GC–MS system. These results, expressed as concentration of analyte in the extract can be found in Table 3. In the second study, the linearity of the whole method was determined by the analysis of wine samples spiked with known amounts of the analytes. Results are also given in Table 3. Data in the table indicate that method linearity is satisfactory, with determination coefficients greater than 0.99 in all cases. Linearity extends well above the natural range of existence of the analytes.

Another quality parameter studied was the total recovery of the sample preparation scheme. For this purpose, different wines were spiked at different concentration levels (5, 15, 30 and 100 ng/L) and analyzed following the procedure. The absolute areas obtained in these analysis, were compared to those obtained in the analysis of dicloromethane solutions containing equivalent amounts of the compounds. The results of this experiment are shown in Fig. 8. As it can be seen, recoveries are in all cases very high and seem to be independent of the concentration level, and of the particular composition of the wine sample.

Detection limits of the method were calculated by analyzing five different wines spiked with very low levels of TCA and TBA (1 and 2 ng/L) and were found to be 0.2 and 0.4 ng/l for TCA and TBA, respectively. These values are similar or even lower than those found in the literature [9] and in any case, are well below the odour detection threshold of these components, which makes it possible to use the method for routine control of these compounds. In addition, in all the cases and even at such low concentrations, the SIS spectra obtained were very clear and showed the characteristic isotopic pattern of ions containing 3 halogens, which facilitated the confirmation of the identity of the analytes. The unspiked wines did not show the existence of any interference.

Fig. 9 illustrates a gas chromatogram from a wine spiked with 3 ng/L of TCA and 7 ng/L of TBA. Analyte peaks were unequivocally identified by retention time and by the mass spectra recorded in the SIS mode.

4. Conclusions

In this study, a fast, robust and selective SPE method coupled to large volume injection has been developed for the determination of TCA and TBA in wine samples. Extraction, clean-up and elution studies were conducted with LiChrolut EN (50 mg) resins. The selective SPE method developed makes it possible to get an extract virtually free from major wine compounds and containing quantitative recoveries of TCA and TBA.

The large volume injection of this extract has been also optimized. Under the best conditions 40 μL of real wine extracts can be injected with very good precision for absolute areas. The method is reproducible, sensitive and robust and represents an effective alternative for routine determination of the selected analytes in wine.

Acknowledgements

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