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Improved solid-phase extraction procedure for the isolation and in-sorbent pentafluorobenzyl alkylation of polyfunctional mercaptans Optimized procedure and analytical applications

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Abstract

A fast method for the determination of aroma-powerful polyfunctional thiols at ng L^{-1} level has been developed. Mercaptans are selectively retained in a 50 mg solid-phase extraction cartridge and derivatization takes place in the same cartridge at room temperature (25 °C) in 20 min by adding small amounts of pentafluorobenzyl bromide (PFBBr) and a strong alkali: 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU). The corresponding derivatives are further eluted and determined by gas chromatography–negative ion mass spectrometry (GC–NCI-MS). Isolation, derivatization, clean-up, elution and calibration conditions were examined. Carrying the reaction in the cartridge makes it possible to use water and non-polar reagents simultaneously, to avoid large volumes of toxic solvent, and to eliminate the excess of reagent. This was last accomplished by the reaction with mercaptoglycerol and further rinsing with a hydromethanolic solution. The method makes it possible to simultaneously determine 2-methyl-3-furanthiol (MF), 2-furfurylthiol (2-furanmethanethiol) (FFT), 4-mercapto-4-methyl-2-pentanone (MP) (as its methoxime), 3-mercaptohexylacetate (MHA) and 3-mercaptohexanol (MH). Absolute limits of detection were 0.2 (MF), 0.1 (FFT), 0.1 (MP), 0.3 (MHA) and 2 (MH) ng L^{-1}. Repeatability ($1\% < RSD < 20\%$) and linearity ($0.978 < R^2 < 0.999$) were satisfactory. Problems with matrix effects were solved by the use of deuterated analogues for MP, MHA and MH and by avoiding the oxidation of analytes and standards via addition of cysteine and EDTA. The different aspects of the method optimization are discussed.
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1. Introduction

Sulfur-containing molecules are an important class of compounds due to their biological significance and aromatic impact. Most volatile sulfur compounds have very powerful and penetrating odors that can be detected at very low concentrations. Leaving aside alkylmercaptans, with their characteristic putrefaction-related odors, there are several volatile or semi-volatile polyfunctional mercaptans which can be responsible for positive and pleasant odor and flavor nuances of different foods such as meat [1], coffee [2], grapefruit [3], passion fruit [4,5], green tea [6], onions [7], Iberian ham [8] or wine [9,10]. Some of these polyfunctional mercaptans have the lowest olfactory detection threshold known, which can be as low as 0.4 ng L^{-1} (in water solution). The presence of some of these compounds (4-mercapto-4-methyl-2-pentanone, 2-methyl-3-furanthiol, 3-mercaptohexanol, 3-mercaptohexylacetate and 2-furfurylthiol) in wine has been reported in certain wines over the years [9–13], but the role of these compounds in the sensory characteristics of wine is not yet well understood because of the difficulties involved in their analytical determination. These are related to the complexity of the sample matrix, the extremely low concentration levels that must be determined and the well-known instability and elusivity of these compounds [14].

In spite of their importance, there are just a few reports on methods for their analytical determination. The most commonly used strategies for the analysis of these compounds make use of *p*-hydroxymercurybenzoate, which has the property of forming water soluble reversible combinations with volatile thiols [9,15–17] or of the so-called covalent chromatography in which the Hg salt is fixed on a polycellulose matrix [18,19]. The use of this salt makes it possible to obtain very clean extracts and to

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get very high concentration factors. However, the poor signals shown by most of these compounds in the mass spectrometric detectors make necessary to handle large volumes of sample (200–500 mL), which in turn makes the procedures long, tedious and difficult. Because of these reasons, different strategies based on the formation of derivatives have been developed [14,20–23].

2,3,4,5,6-Pentafluorobenzyl bromide (PFBBr) has been the most recently proposed derivatization reagent of thiols [20,21]. This derivatization is a very good alternative since derivatives can be sensitively and selectively determined by negative ion mass spectrometry. Two methods based on this reagent have been previously reported; in the first one derivatives were formed directly in the solid-phase microextraction (SPME) fiber [21] but only two analytes could be determined and the dynamic range of the method was quite narrow. In the second one, the reaction took place in a benzene extract containing the analytes [20]. Although results were considerably better than those achieved with the SPME alternative, the method has shown some limitations in the long-term application, such as those derived from the excess of reagent and other wine extractables which could not be satisfactorily eliminated, the use of a carcinogenic solvent and the impossibility to get consistent results for 2-methyl-3-furanthiol.

In order to solve these limitations, the possibilities to carry out the derivatization reaction in a solid-phase extraction (SPE) sorbent have been explored. The potential advantages of such alternative include the possibility to introduce additional cleaning up steps, to carry out the reaction in a less matrix-dependent media, to reduce reagents and solvents and to semi-automate the process. Therefore, the main goals of this work are to optimize the derivatization of polyfunctional mercaptans in a SPE cartridge and to develop a fast, easy and reliable method to determine these compounds in wine.

2. Materials and methods

2.1. Reagents and standards

n-Hexane for organic trace analysis (UniSolv), dichloromethane (SupraSolv), methanol (SupraSolv) and ethanol, gradient grade for liquid chromatography (LiChrosolv) were from Merck (Darmstadt, Germany). Diethyl ether for instrumental analysis and mercaptoglycerol were from Fluka (Buchs, Switzerland). Anhydrous sodium sulfate was for analysis ACS-ISO quality from Panreac (Barcelona, Spain). Ethylenediaminetetraacetic acid disodium salt 2-hydrate (EDTA), L-cystein hydrochloride hydrate 99%, 1,4-dithioerythritol, octafluoronaphthalene 96% (OFN) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) were from Aldrich (Steinheim, Germany). *O*-Methylhydroxylamine hydrochloride purum >98% and PFBBr were from Fluka. 4-Mercapto-4-methyl-2-pentanone (MP) 1% PG and 3-mercaptohexylacetate (MHA) were from Oxford Chemicals (Hartlepool, UK). 2-Furfurylthiol (FFT), 3-mercaptohexanol (MH) and 1-hexanethiol were from Lancaster (Strasbourg, France). 2-Methyl-3-furanthiol (MF), 2-methyl-3-tetrahydrofuranthiol, 2,5-dimethylfuran-3-thiol and 2,4,6-trimethylbenzyl mercaptan were from Aldrich. 2-Phenylethanethiol and 4-methoxy-α-toluenethiol were from Fluka. [$^2H_2$]3-Mercaptohexanol (d-MH); [$^2H_5$]3-mercaptohexylacetate (d-MHA) and [$^2H_{10}$]4-methyl-4-mercaptopentan-2-one (d-MP) were kindly donated by Professor Baumes and synthesized in the Analyse des Volatils, Unité Mixte de Recherche, Sciences Pour l'Œnologie, INRA, Montpellier (France) according to the protocol described in [24].

Bond Elut-ENV resins, prepacked in a 50-mg cartridge (1 mL total volume) and semi-automated SPE Vac Elut 20 station were from Varian (Walnut Creek, CA, USA).

2.2. Proposed method

In a 20-mL screw capped vial, spike 10 mL of wine with 0.05 g of EDTA and 0.156 g of L-cystein clorhydrate and keep it shaking for 2 min. After this, spike the volume of wine with an ethanolic solution containing the internal standards so that the final concentration is 400 ng L^{-1} of 2-phenylethanethiol, 384 ng L^{-1} of d-MP, 800 ng L^{-1} of d-MHA, 54 μg L^{-1} of d-MH, stir the mixture for 15 s, and finally add 0.1 g of *O*-methylhydroxylamine. Purge the vial gently with pure nitrogen, seal it and incubate in a water bath at 55 °C for 45 min.

Six milliliters of this incubated sample is then loaded onto a 50-mg Bond Elut-ENV SPE cartridge (previously conditioned with 1 mL of dichloromethane, 1 mL of methanol and 1 mL of water). Some wine major volatiles are removed by rinsing with 4 mL of a 40% methanol–water solution 0.2 M in phosphate buffer at pH 7.7 and after this, with 1 mL of water. Mercaptans retained in the cartridge are directly derivatized by passing 1 mL of an aqueous solution of DBU (6.7%) and 50 μL of a 2000 mg L^{-1} solution of PFBBr in hexane, and letting the cartridge imbibed with the reagent for 20 min at room temperature (25 °C). Excess of reagent is removed by adding 100 μL of a 2000 mg L^{-1} solution of mercaptoglycerol in 6.7% DBU aqueous solution, and letting the cartridge again for 20 min at room temperature. The cartridge is then rinsed with 4 mL of a 40% methanol–water solution 0.2 M in H_3PO_4; and with 1 mL of water. Derivatized analytes are finally eluted with 600 μL of a solvent mixture (hexane 25% in diethylether) containing 375 ng L^{-1} of the chromatographic internal standard, OFN.

The eluate is finally washed with five-1 mL volumes of a brine (200 g L^{-1} NaCl water solution), transferred to a standard 2 mL autosampler vial and spiked with a small amount of anhydrous sodium sulfate. Twenty microliters of this sample is directly injected into the GC-negative chemical ionization (NCI) MS system.

2.2.1. GC–NCI-MS analysis

Apparatus: Shimadzu QP-2010 gas chromatograph with a quadrupole mass spectrometric detection system. Injector: Optic 3 from ATAS-GL (Veldhoven, The Netherlands); injection conditions: 20 μL of extract is injected in a packed liner for large volume injection. The initial temperature of the injector is 55 °C and after 17 s it is heated at 5 °C s^{-1} up to 250 °C, remaining at this temperature until the end of the analysis. The carrier gas is He, flowing through the column initially at

0.6 mL min^{-1}. Seventeen seconds after the injection the flow is increased to 1.8 mL min^{-1} for 3 min. After this period it is fixed at 1 mL min^{-1}. The split valve is opened at the first 17 s of analysis (split flow 100 mL min^{-1}), closed at the following 3 min, and opened again for the rest of the analysis (split flow 50 mL min^{-1})

The column is a Factor Four capillary column VF-5MS from Varian, 20 m × 0.15 mm ID, with 0.15 μm film thickness. The column initial temperature is 45 °C for 2 min, heated to 140 °C at 25 °C min^{-1}, then to 180 °C at 15 °C min^{-1}, then to 210 °C at 30 °C min^{-1} and finally to 300 °C at 250 °C min^{-1}; remaining at that temperature for 10 min. The ion source is operated in NCI mode using methane at 2 bar as reagent gas. The temperature of the ion source was 220 °C and the interface was kept at 290 °C. The analytes and internal standards ions are acquired in the single ion monitoring (SIM) mode from minute 5 to minute 12 at 0.20 s^{-1}/point: OFN is quantified with *m/z* 272; MF and FFT are quantified with *m/z* 274; MP is quantified with *m/z* 160; d-MP is quantified with *m/z* 170; MH is quantified with *m/z* 133; d-MH is quantified with *m/z* 135; MHA is quantified with *m/z* 175; MHA is quantified with *m/z* 180 and 2-phenylethanethiol is quantified with *m/z* 135.

2.3. Method development and validation

2.3.1. Method development

2.3.1.1. Solid-phase extraction. Different wine sample volumes (5–50 mL), washing up solutions (HCl 0.5 M or H_3PO_4 0.2 M at different pH values and H_3PO_4 0.2 M with 20, 30, 40 or 50% methanol and different pH values), washing up volumes (4, 6 or 8 mL), and elution solvent composition and volumes (benzene, hexane, diethyl ether, ethyl acetate and different combinations of them), were checked to ensure a 100% extraction of the analytes and a large removal of interfering compounds.

2.3.1.2. Derivatization. Different ways of applying the reagents (as vapors or dissolved in different amounts of different solvents), the mass of reagent in the cartridge (10–200 μg of PFBBr), the type and concentration of alkali, the presence of water, the reaction time and the elution solvent were considered and tested. The order in which PFBBr and DBU were applied was also studied by performing the analysis of standard samples or spiked wines with the DBU aqueous solution imbibed in the SPE cartridge before or after PFBBr addition (always after extraction). For the elimination of the excess of reagent, two different polar mercaptans (cysteine and mercaptoglycerol) were studied. The amount of mercaptan, the solvent in which it is delivered, the presence of alkali (DBU), the reaction time and the composition of the rinsing solvent were considered.

2.3.2. Method validation

2.3.2.1. Internal standards, linearity and matrix effects. In preliminary studies, the chromatographic and mass spectrometric properties of the PFBBr derivatives of different mercaptans were determined. Because of the behavior of the analytes and the internal standards, deuterated analogues of the analytes were considered as the best internal standards for quantitative purposes. After discarding those giving tailing peaks or eluting too close to some of the analytes, four of them (d-MH, d-MHA, d-MP and 2-phenylethanethiol) were selected and used as potential internal standards in different standard addition experiments. These assays were carried out on white (from Verdejo and Sauvignon Blanc, D.O. Rueda 2006, 12.5% ethanol) and red wine (from Cabernet Sauvignon, Merlot and Cabernet Franc, France 2005, 13.5% ethanol). The wines spiked with variable amounts of analytes (MF: 0, 6.5, 13.3, 26.6, 53.2, 133 ng L^{-1}; FFT: 0, 6, 12.5, 25, 50, 125 ng L^{-1}, MP: 0, 5, 10, 20, 40, 100 ng L^{-1}, MH: 0, 115, 230, 460, 1150, 2500 ng L^{-1} and MHA: 0, 10, 30, 50, 100, 200 ng L^{-1}), and fixed amounts of internal standards (200 ng L^{-1} of 2-phenylethanethiol, 192 ng L^{-1} of d-MP, 400 ng L^{-1} of d-MHA and 27 μg L^{-1} of d-MH) were analyzed according to the proposed procedure. Per each analyte and wine, four standard addition lines (one per potential internal standard) were built. The standards for each analyte were finally selected as those providing the least dissimilar slopes between different wines.

2.3.2.2. Method repeatability and limits of detection. Four different wines (Sauvignon Blanc 2006 DO Rueda; Cabernet Franc, Cabernet Sauvignon and Merlot 2004, Bourdeaux; Sauterness 2002, Bordeaux; Merlot, Cabernet Franc, Cabernet Sauvignon and Petit Verdot 2003, Bourdeaux were spiked at two different levels (see Table 2), and five replicate analyses were carried out on each sample. The concentration of internal standards was 200 ng L^{-1} of 2-phenylethanethiol, 192 ng L^{-1} of d-MP, 400 ng L^{-1} of d-MHA and 27 μg L^{-1} of d-MH. Method limits of detection and quantification were determined by the analysis of real samples, spiked at low concentration levels of analytes, as the concentration of analyte in wine which would give a signal 3 or 10 times higher than the noise.

3. Results and discussion

3.1. Reaction optimization

As reported in a previous work [20] the formation of derivatives in a SPE cartridge filled with LiChrolut EN was a failure. This sorbent is a polystyrene-divinylbenzene copolymer with a microporous structure (<20 Å) and a high active surface (1200 m^2 g^{-1}). Previous studies have found that some compounds elute out of these resins giving tailing and distorted peaks [25] and, in particular, that the elution of some of the target mercaptans is problematic [15]. All these suggest that these resins exert strong secondary interactions with many polar compounds and particularly with the mercaptans. Some of those secondary interactions between the analytes and the sorbent may take place through the mercapto group, which could explain why the derivatives are not easily formed in these resins. The use of a sorbent with a non-microporous structure was then considered. Bond-Elut ENV resins are also a polystyrene-divinylbenzene (SDVB) copolymer, but they have a much higher pore size (450 Å), a much smaller surface area (500 m^2 g^{-1}), and in gen-

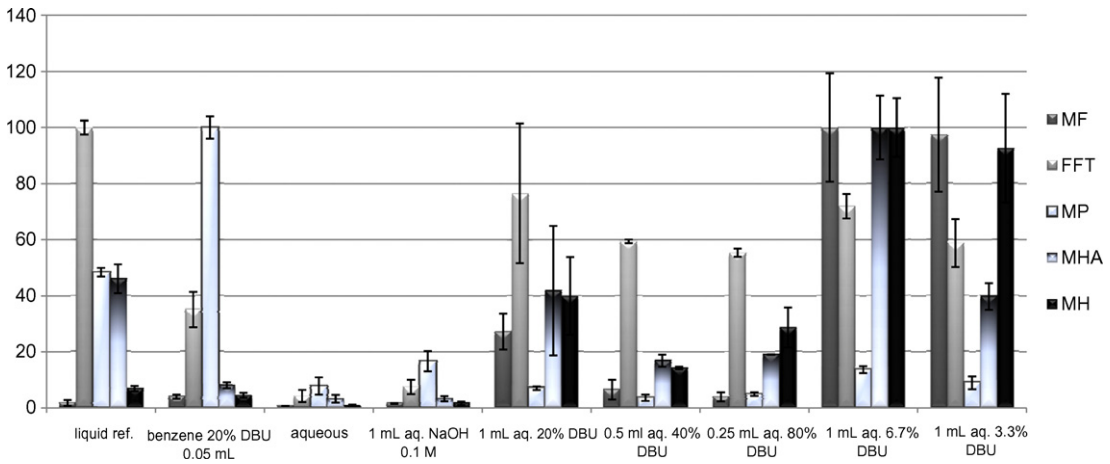

Fig. 1. Effect of the type and concentration of alkali and of the presence and volume of water on the relative yields obtained in the formation of PFB derivatives. In all cases 3 ng of the analytes were loaded in the cartridge (or in benzene in the liquid reference) and derivatives were formed by the addition of 50 μL of a 2000 mg L^{-1} solution of PFBBr in hexane.

eral, they have shown to exhibit a much better chromatographic behavior [25]. Preliminary experiments showed, however, that neither the conditions used to form the derivatives in the SPME fiber [21], nor those used to form them in a benzene extract [20], could be directly transferred to the solid sorbent. In the first case, in which the reagents (PFBBr and an alkali) are delivered in gas phase, the formation of derivatives was residual (less than 5% of those obtained in benzene). In the second case, the yields for mercaptohexylacetate were similar to those obtained in benzene, but those for the rest of mercaptans were just a 20%. These experiments also revealed that keeping a minimum speed in the elution of derivatives is critical and that the reaction rate is faster than that observed in benzene: Derivatives of MF, FFT, MH and MHA are formed in just 10 min, while that of MP requires 20 min.

3.1.1. Addition of alkali

After these preliminary trials, different aspects of the reaction were considered: (1) the type and concentration of alkali; (2) the presence of water; (3) the carrier in which the reagent is delivered; and (4) the concentration of reagent.

Points 1 and 2 were considered simultaneously and a summary of the results obtained in the study can be seen in Fig. 1. As can be seen, the presence of the strong "organic" base (DBU) is essential and cannot be replaced by a strong inorganic base such as NaOH. A very interesting question is whether the formation of derivatives greatly improves when the DBU is delivered dissolved in water and that the volume of water also has a clear influence. The best results were obtained when this volume was 1 mL and when the concentration of DBU was 6.7%. These results suggest that the formation of a thin layer of water molecules near the surface of the sorbent favors the stabilization of the thiolate anion, and that such layer requires at least 1 mL of water to be formed (in a 50-mg cartridge). This volume roughly corresponds to 20 void volumes ($V_0 \approx 50$ μL in a 50 mg cartridge) or to 15 pore volumes (1.3 mL g^{-1}). The lesser amounts of derivates formed at higher concentrations of DBU suggest that derivatives may be degraded. In these final conditions, the formation of derivatives in the SPE cartridge was more efficient than it was in benzene, except in the case of MP. The improvement is particularly important in the cases of MF and MH.

3.1.2. Addition of the derivatization reagent

A parameter that has been demonstrated to have sometimes influence on the formation of derivatives on solid phases are the solvents in which the reagents are delivered [26,27]. The effect of such parameter is shown in Fig. 2. As can be seen, our results confirm the importance of this parameter and its effect on the selectivity. While the derivatives of MF, FFT and MP were very well formed and PFBBr was added dissolved in acetonitrile, those of MH and MHA were best formed when the solvent was hexane. In any case, and leaving aside again MP, the best results were obtained with hexane, which was selected for the subsequent studies.

The influence of the amount of derivatization agent can be seen in Fig. 3 and is relatively similar to the one observed in liquid media [20]. The formation of derivatives for MHA, MH and FFT increases with the level of PFBBr until a point in which it reaches a plateau at 100 μg, exactly the optimal level for the reaction in benzene. In the cases of MF and particularly in that of MP, the dependence is less clear. In these two cases, the results suggest that the excess of reagent provokes the degradation of the derivatives formed. In any case the reaction in the SPE cartridge does not seem to make it possible to reduce the level of reagent. The kinetics of the formation of derivatives were also similar and 20 min at room temperature with a small amount of PFBBr (100 μg) were chosen as optimal conditions.

3.1.3. Reagent elimination

The elimination of the excess of reagent is an important point, since the unreacted reagent can cause chromatographic problems, can shorten the working life of the chromatographic column and can also induce the degradation of the derivatives formed, as the results shown in Fig. 3 suggest. In fact, as it was

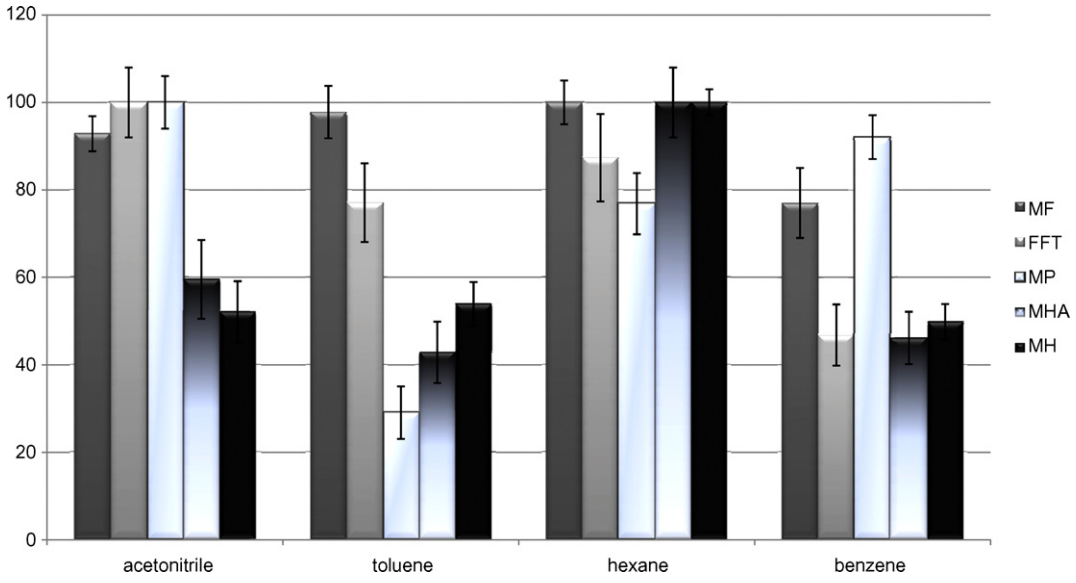

Fig. 2. Influence of the solvent used as carrier to deliver the reagent into the cartridge. In all cases 50 μL of a 2000 mg L^{-1} solution of PFBBr in a given solvent was added to the 50 mg cartridge containing 3 ng of each analyte.

aforementioned and in spite of the low amount of reagent used, this excess is most likely one of the reasons why the practical application of the liquid–liquid method in the long term is troublesome.

As chromatographically it was not possible to separate the reagent from the derivatives in the cartridge or in any other simple separation system, the strategy devised to eliminate the excess of reagent was to make it react with a mercaptan polar enough so that its derivative could be easily washed out of the cartridge after the reaction. L-Cystein and mercaptoglycerol were considered for this study, but only the latter was efficient at removing the excess of reagent and was taken further in consideration. A very interesting effect noted after the elimination of part of the reagent is that the signals of all the analytes improved but, such improvement is particularly large in the cases of MF and MP, the two compounds for which the excess of reagent seem to exert a most critical influence (see Fig. 3). In these two cases, the signals increase by factors as large as 7 or 3, respectively (data not shown). This result seems to confirm that the lack of consistence in the signals obtained for MF in the previously developed method can be attributed to the degradation of the derivative by the unreacted reagent. The study of the most adequate reaction conditions showed that the addition of 50 μL of an aqueous solution containing 100 μg of mercaptoglycerol and 6.7% DBU, eliminates nearly 99% of the excess of PFBBr after 20 min of reaction at room tempera-

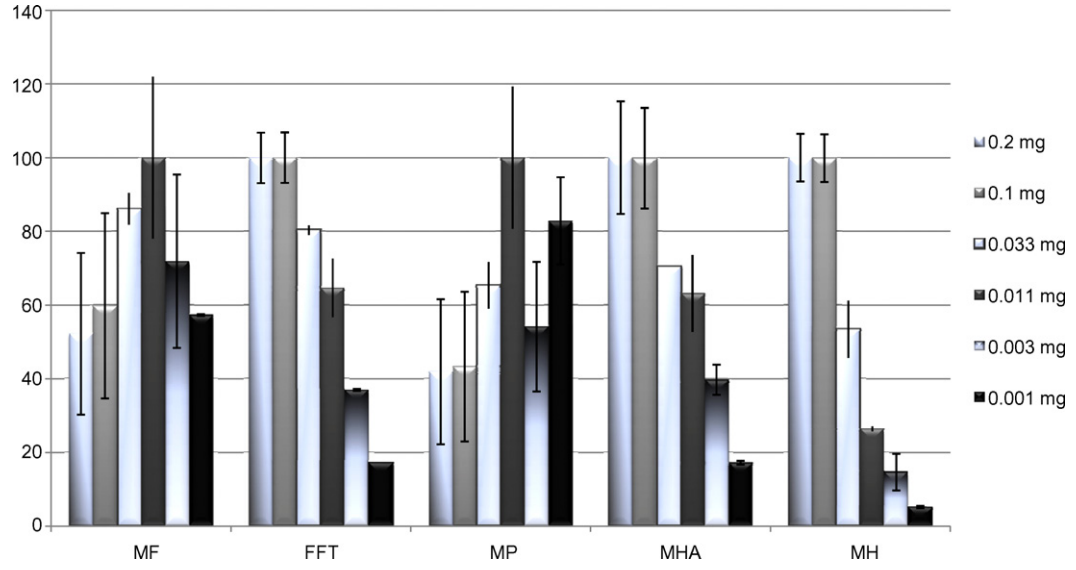

Fig. 3. Effect of the mass of reagent on the relative yields obtained in the in-cartridge solid-phase derivatization. In all cases the reagent was added dissolved in 50 μL of hexane to a 50 mg cartridge containing 3 ng of each analyte.

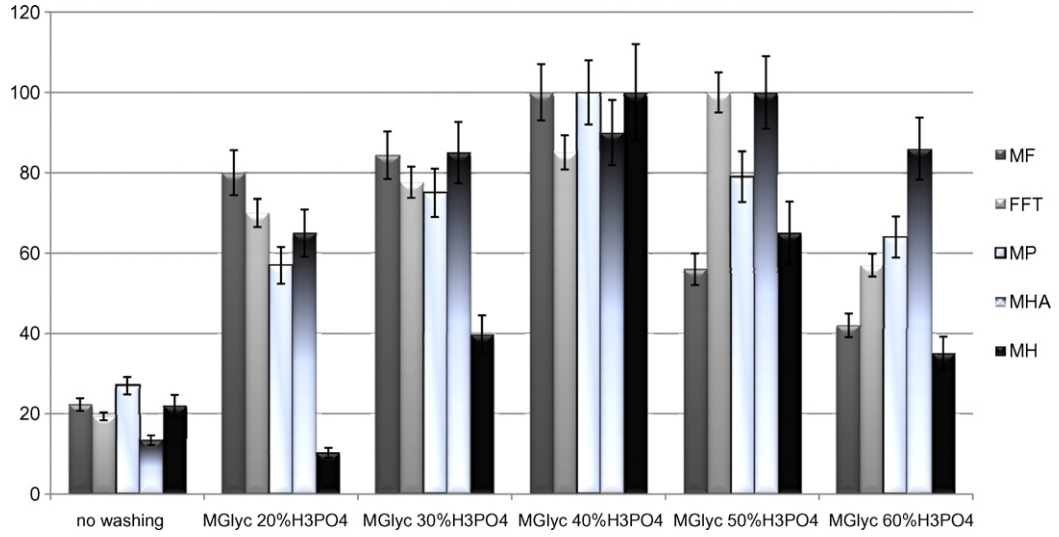

Fig. 4. Effect of rinsing the mercaptoglycerol derivative with 4 mL of different solutions on the signals obtained for the derivatives.

ture (data not shown). The derivative of mercaptoglycerol and the excess of DBU can now be easily removed by washing the cartridge with 4 mL of a rinsing solution 0.2 M in H_3PO_4 and 40% methanol, as shown in Fig. 4. A higher level of methanol in the rinsing solution brings about some losses in the derivatives, while the incomplete elimination causes important losses in the signals due to the broad elution peak of the derivatives of mercaptoglycerol.

3.2. Wine extraction

The retention properties of the analytes in a 50 mg BondElut-ENV bed were experimentally determined. The breakthrough volumes for the analytes contained in wine ranged between 12 (for MH) and 25 mL (for MHA) and, therefore, a loading volume of 6 mL was chosen in order to include a washing step in the protocol. The goals of such initial rinsing are to eliminate as many potential interferences as possible, such as fatty acids and phenols, and to ensure that the reaction takes place in a media as simple as possible. Best results were obtained with rinsing solutions buffered at pH 7.7 and containing 40% of methanol.

3.3. Oximation of MP

The yield obtained in the pentafluorobenzyl alkylation of MP has been systematically the lowest of all the mercaptans in all the studies carried out, the reaction rate has also been the smallest [20,21] and the general behavior of this compound the oddest, as it has been shown in Section 3.1. Such poorer ability to form the derivative may have two different causes. On the one hand, this compound is a tertiary mercaptan, which would mean that some side reactions related to the formation of a tertiary carbocation would be promoted. On the other hand, and as it is schematically shown in Fig. 5a, the proximity between the oxygen of the keto group and the hydrogen from the mercapto function may result in the formation of a strong intra-molecular hydrogen bond forming a stable cycle. The fact that in previous reports this compound has also shown the lowest affinity for

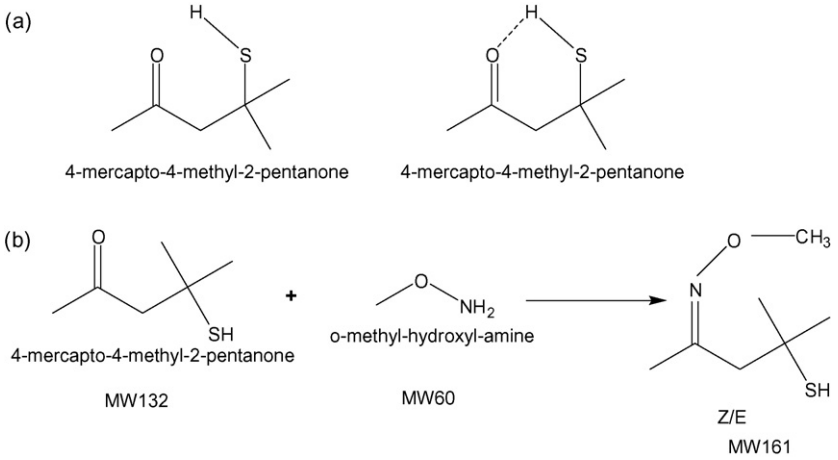

Fig. 5. Diagrams showing (a) the possibility of the existence of an internal hydrogen bond between the proton from the thiol and the oxygen from the keto group; (b) the oximation reaction to avoid such possibility.

binding *p*-hydroxymercurybenzoate [15] made us think that the formation of the cycle is the most relevant cause explaining the poor yields obtained in the derivatization. As a consequence, reactivity would improve if the keto function is protected by forming an oxime, which was easily achieved by adding *O*-methylhydroxylamine directly to the wine. The reaction that takes place can be seen in Fig. 5b. The methoximation is quantitative after 45 min at 55 °C and two isomeric methoximes (*cis* and *trans*) are formed with a molecular mass of 160 in a fixed ratio 1:4 (*syn*:*anti*), which provides a useful criterion to identify the compound. The methoximes react efficiently with PFBBr to form two pentafluorobenzyl derivatives whose NCI spectra show a most abundant ion at 160 *m/z*.

3.4. Matrix effects and other aspects of the final method

The application of the previously developed procedure to the analysis of these compounds in wine revealed a series of problems. The first one is about the existence of matrix effects, since different sensitivities were observed in different wines. The second problem was evident in some "difficult" wines in which the signals of some of the analytes appeared seriously distorted or even hidden by some analytical interferences, precluding the quantification of the analytes at low concentrations. In both cases, it was not possible to find a satisfactory solution by increasing the number of clean-up steps in the SPE cartridge and, therefore, two series of experiments were carried out. In the first one, different treatments were applied to the final extract, while in the second one the use of different internal standards was considered.

The most evident problem was found in the analysis of MP in some wines. The peaks of the two derivatives (one per oxime) appeared strongly distorted and overlapped, which was attributed to an incomplete elimination of the pentafluorobenzyl derivative of the mercaptoglycerol used to eliminate the excess of reagent. This problem could be solved by washing the extract with a brine (200 g L^{-1} NaCl in water), as shown in Fig. 6.

Six different mercaptans (1-hexanethiol, 2-phenylethanethiol, 4-methoxy-α-toluenethiol, 2-methyl-3-tetrahydrofuranthiol, 2,5-dimethylfuran-3-thiol and 2,4,6-trimethylbenzylmercaptane) were selected attending to the chromatographic properties of their derivatives and were checked as possible internal standards. Their ability to improve the method reproducibility and to compensate for the matrix effects was investigated by means of different standard addition experiments. However, each mercaptan seemed to follow a particular behavior that could not correct completely the different signal of the analytes in different wines. Because of this, a calibration with isotopic standards was assayed.

Surprisingly, the first results using d-MH, d-MHA and d-MP as internal standards were mostly frustrating because matrix effects persisted, as it is shown in Fig. 7a and b. The figures show the chromatograms obtained in the analysis of two wines originally containing low levels of MHA (in both cases

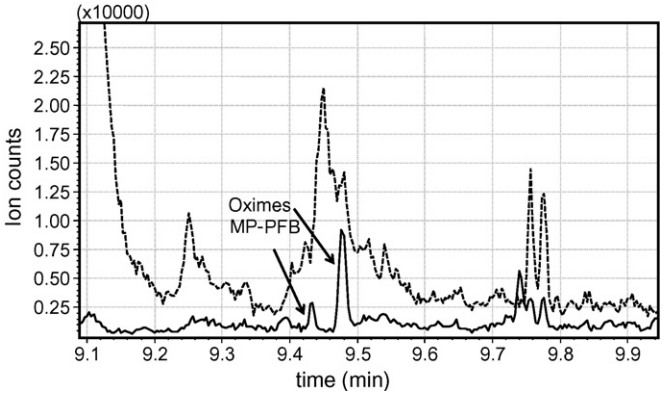

Fig. 6. The dotted chromatogram shows the distorted and overlapped MP-derivative peaks obtained in the analysis of a "difficult" wine spiked with 5 ng L^{-1} of this compound. The chromatogram below shows the effect of washing the extract with five 1 mL volumes of a brine (*m/z* 160 is shown).

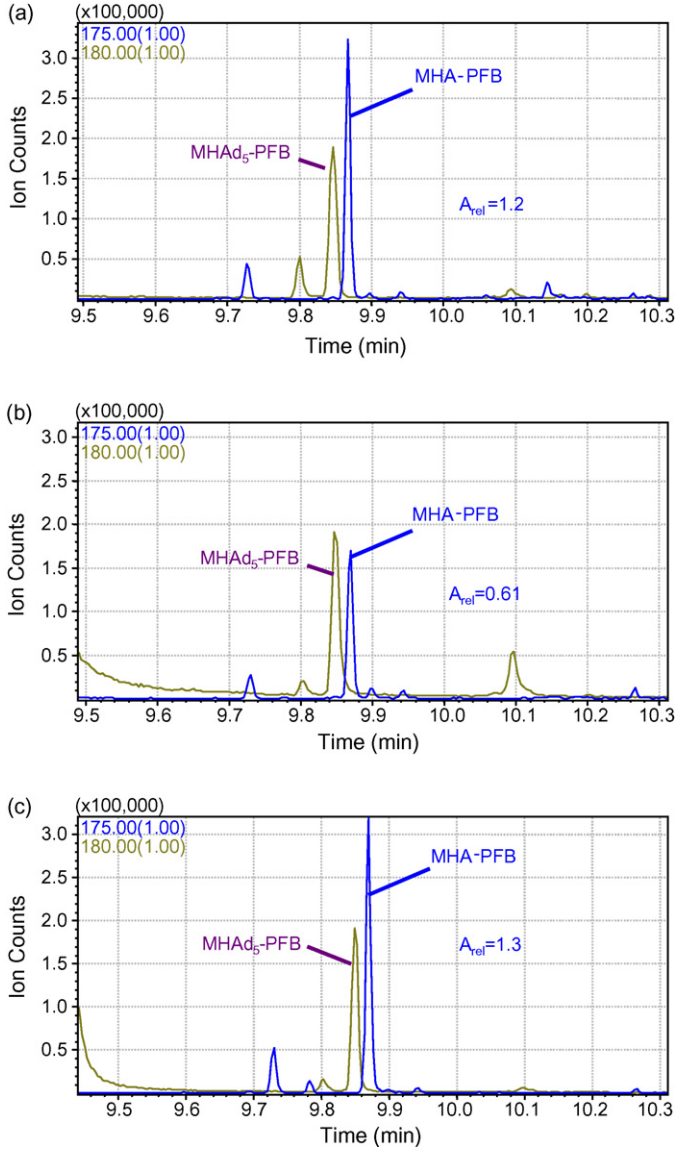

Fig. 7. Influence of the redox state of the wine sample on the signals obtained for the derivatives of MHA and of its deuterated analogue. Chromatogram (a) corresponds to the analysis of a red wine spiked with 400 ng L^{-1} of MHA and 500 ng L^{-1} of its isotopomer; (b) corresponds to an oxidized white wine, and (c) is this oxidized wine treated with 0.1 mg L^{-1} of 1,4-dithioerithreitol.

Table 1
Method linearity

Analyte	Wines			
	Average slope[a]	RSD (%)	Average[a] R^2	Calibrated range (ng L^{-1})
2-Methyl-3-furanthiol (MF)	$0.0125 \pm 2.81 \times 10^{-3}$	22	0.9787	0–133
2-Furfurylthiol (FFT)	$0.00277 \pm 4.59 \times 10^{-5}$	1.6	0.9938	0–125
4-Mercapto-4-methyl-2-pentanone (MP)	$0.0109 \pm 7.07 \times 10^{-5}$	0.65	0.9986	0–100
3-Mercaptohexylacetate (MHA)	$0.00195 \pm 4.52 \times 10^{-5}$	2.3	0.9981	0–200
3-Mercaptohexanol (MH)	$2.24 \times 10^{-5} \pm 4.86 \times 10^{-6}$	22	0.9719	0–2500

Data are peak areas normalized by that of the corresponding internal standard.
[a] Average of two slopes calculated in two independent experiments in two different wines ($n = 6$).

below 40 ng L^{-1}) and spiked with 500 ng L^{-1} of this compound and with 400 ng L^{-1} of the deuterated standard. The first wine (shown in Fig. 7a) was a normal red wine, while the second one (Fig. 7b) was an oxidized white wine. In such a wine, it was clear that the mercaptans spiked to the wine were oxidized and that the oxidation rate was faster for the analytes than for their deuterated analogues (the spiking was carried out with ethanolic solutions just immediately before the oximation of MP with *O*-methylhydroxyamine). Obviously, it makes no sense to think that deuterated analogues are more stable, but it can be thought that the oxidation rate is related to the redox state and possible presence of free radicals in the spiking solutions, which is definitely something difficult to assess and control in very diluted solutions. However it should be taken into account that the analyte spiking solution was a mixture of the mercaptans, one of which, MF, is particularly reactive, and may promote the oxidation of the rest. Although such oxidation was not evident by GC–MS analysis of the stock solutions, a small amount of the disulfide of MF was easily detected. Whatever the case, the signals were recovered after adding 1,4-dithioerythritol to the wine just before the wine was spiked with the standards and further analyzed (Fig. 7c). Nevertheless, this solution was not satisfactory because a progressive deterioration of the chromatography occurred, due to the dirtiness caused by the continuous introduction of 1,4-dithioerythritol into the chromatographic column. The problem was satisfactorily solved including the addition of EDTA and L-cystein to the wine instead.

3.5. Method validation

Linearity was satisfactory for three of the analytes (FFT, MP and MHA), with average determination coefficients better than 0.993 (see Table 1), and with linear ranges including the normal range of occurrence of these compounds in wine. In the two other cases (MF and MH), however, linearity was relatively poor. The existence of matrix effects was checked by carrying standard addition experiments in different wines. The average slopes of the standard addition lines in two different wines (red and white, described in Section 2.3.2.1) are shown in Table 1. These slopes came from peak areas normalized by those of the corresponding internal standard: d-MHA for MF, FFT and MHA, d-MP for MP and d-MH for MH. There is a good agreement in the slopes found in the analysis of very different wines in the cases of FFT, MP and MHA, while the agreement for the cases of MF and MH is, again, less satisfactory. The relative standard deviations of the slopes were around 1.6% for FFT, MP and MHA and between 20 and 23% for MF and MH. In both cases part of the apparently poor results are attributed to cross contamination and blank contamination, a problem which could not be completely resolved.

Reproducibility was determined as repeatability at two different concentration levels for four different wines (described in Section 2.3.2.2) and can be seen in Table 2. At low concentration levels, RSDs are below 11% for FFT, MP and MHA, while at high concentration levels, this value is lower than 7% in all cases except for MH. This poor result can be attributed

Table 2
Method repeatability, detection and quantification limits and levels of analytes in the blanks

Analyte	Repeatability, RSD (%)		LOD (ng L^{-1})	LOQ (ng L^{-1})	Concentration of analytes found in blanks (ng L^{-1})	
	Low level[a]	High level[b]			After[c]	Long after[d]
2-Methyl-3-furanthiol (MF)	14.5	1.8	≈0.2	≈0.5	1.5–4	<0.8
2-Furfurylthiol (FFT)	8.5	4.9	≈0.1	≈0.3	1–5	<1
4-Mercapto-4-methyl-2-pentanone (MP)	11.0	6.1	≈0.1	≈0.3	0.5–1	n.d.
3-Mercaptohexylacetate (MHA)	5.1	3.6	≈0.3	≈0.8	0.8–3	<0.5
3-Mercaptohexanol (MH)	19.2	15.6	≈2	≈6	5–19	<3

Repeatability is expressed as RSD and was determined by the analysis of four real wines spiked at two different levels ($n = 5$ in both cases). Method detection and quantification limits were determined by the analysis of real samples spiked with low levels of analytes.
[a] Low level: MF 5 ng L^{-1}, FFT 2 ng L^{-1}; MP 1 ng L^{-1}; MHA 5 ng L^{-1}; MH 40 ng L^{-1}.
[b] High level: MF 80 ng L^{-1}, FFT 75 ng L^{-1}; MP 60 ng L^{-1}; MHA 90 ng L^{-1}; MH 800 ng L^{-1}.
[c] After a standard addition experiment.
[d] Two weeks after such experiment.

to the poor peak shape for this compound, as is shown in the chromatogram in Fig. 8d.

Absolute detection and quantification limits were determined by the analysis of real samples containing low amounts of the analytes and correspond to the level of compound in wine required to give signals 3 or 10 times higher than the noise, respectively. Detection limits for MF, FFT, MP and MHA are below 0.3 ng L^{-1}, and absolute quantification limits are in these

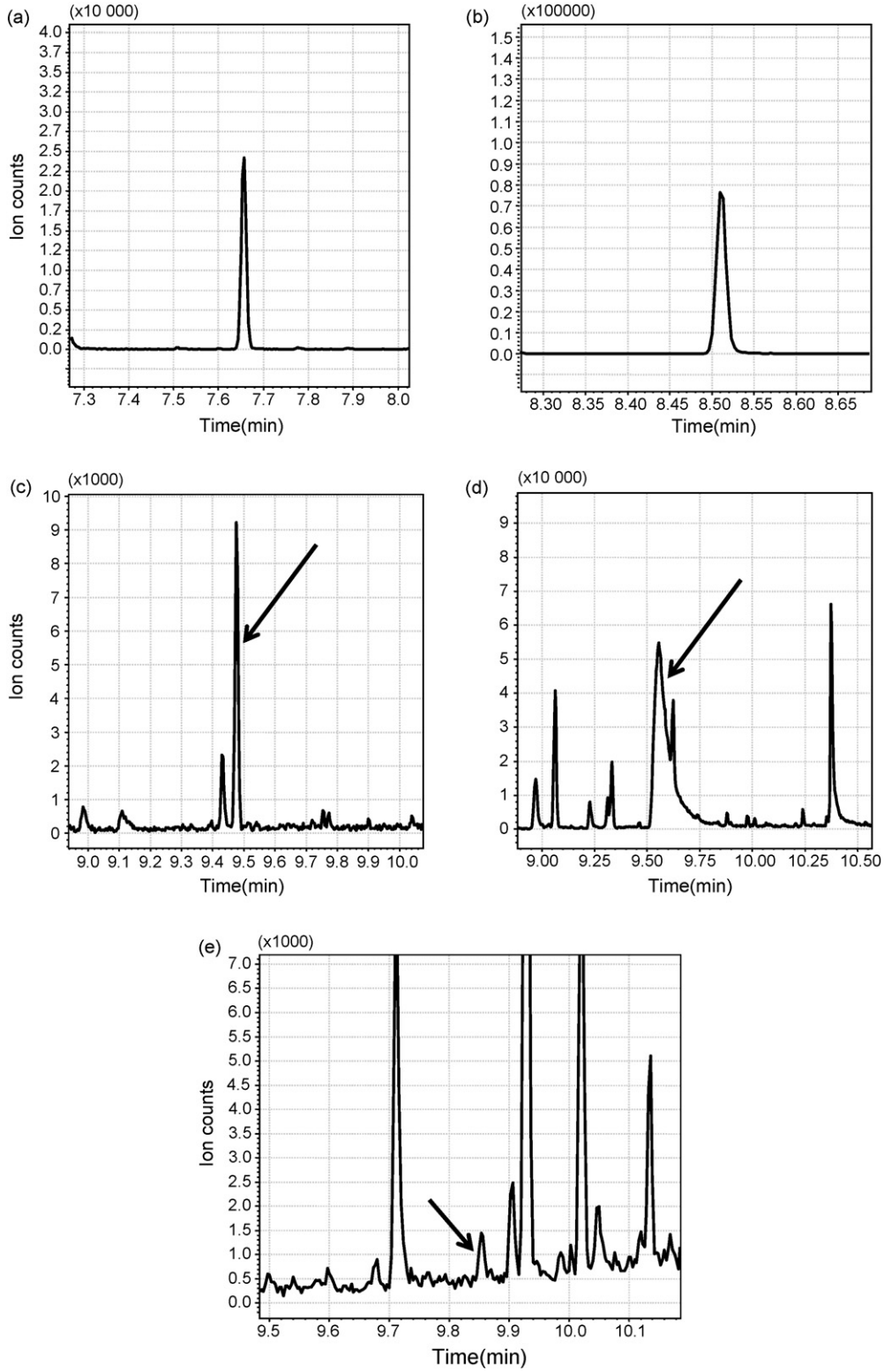

Fig. 8. Typical chromatograms obtained in the analysis of a red wine containing (a) 15 ng L^{-1} of MF, *m/z* 274; (b) 10 ng L^{-1} of FFT, *m/z* 274; (c) 10 ng L^{-1} of MP (spiked), *m/z* 160; (d) 40 ng L^{-1} of MH, *m/z* 133 and (e) 4 ng L^{-1} of MHA, *m/z* 175.

Table 3
Recoveries obtained in a spiking experiment carried out on three different wines spiked at different levels

Level (ng L^{-1})	MF			FFT			MP			MHA			MH		
	Co	10	50	Co	10	50	Co	10	50	Co	10	100	Co	30	300
Sauternes	5.6	111	105	8.2	97	89	3	94	97	37.2	90	104	2040	–	89
Mouton C	9.2	118	91	<LOQ	97	98	<0.5	107	110	<LOQ	112	99	257	123	94
Sauvignon	4.3	47	68	2.3	117	110	1	98	108	45.7	74	92	624	78	97

Co refers to the concentration of analyte found in the wine (ng L^{-1}).

cases below 1 ng L^{-1}, which can be considered satisfactory. The detection limit for MH is slightly higher, but the aroma of this compound is less powerful and its detection threshold is 60 ng L^{-1} [28], which is well above the method detection limit. However, most often the real detection limits are determined by the levels of the analytes found in the blanks, levels that can be particularly high after the manipulation of the analytes in the laboratory. For instance, after the standard addition experiments, the levels in the blanks were those shown in Table 2, and it took 2 weeks to have blanks with levels approaching the detection limits.

Finally, the accuracy of the proposed method was tested by the analysis of spiked and unspiked samples. The results of the study are shown in Table 3. As can be seen, in most cases recoveries are satisfactory, given the difficulties of the analysis. It can be observed, however, that the recoveries for MF in the Sauvignon wine are quite low, which suggests that matrix effects are not fully solved in this case and that for an accurate quantification, an specific isotopomer for this compound should be used.

4. Conclusions

The present method solves some of the limitations of previous procedures for the analysis of polyfunctional mercaptans at ultratrace level. The derivatization reaction, now carried out in the SPE cartridge, has been particularly improved. It has been shown that the presence of small amounts of water and the nature of the solvent in which the reagent is delivered exert a strong influence on the yields. The sorbent as support for the reaction makes it possible to eliminate the excess of reagent, which has been shown to exert a pernicious influence on the signals of the compounds. The SPE format makes it possible to handle up to 12 samples simultaneously. The method has been applied to the analysis of five important mercaptans present in wine at very low concentrations. Problems related to the existence of matrix effects, most likely due to changes in the redox state of the mercaptans spiked to the wine, have been solved by the use of deuterated internal standards, although more research should be carried out to understand these processes and control the stability of the stock solutions and the spiking process.

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