Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography A, 1146 (2007) 242–250
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Quantitative determination of wine polyfunctional mercaptans at nanogram per liter level by gas chromatography–negative ion mass spectrometric analysis of their pentafluorobenzyl derivatives

Laura Mateo-Vivaracho, Juan Cacho, Vicente Ferreira *

Laboratory for Flavour Analysis and Enology, Department of Analytical Chemistry, Faculty of Sciences, University of Zaragoza, 50009 Zaragoza, Spain

Received 22 November 2006; received in revised form 1 February 2007; accepted 2 February 2007
Available online 8 February 2007

Abstract

A fast method for the determination of aroma-powerful polyfunctional thiols at nanogram per liter level has been developed and applied to wine. A small volume of wine (6 mL) was extracted with 1.5 mL of benzene containing four internal standards. Pentafluorobenzyl derivatives of mercaptans were formed in the extract by adding small amounts (100 mg L^{-1}) of pentafluorobenzyl bromide and a strong alkali: 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU). After washing with a water:methanol (5:1) solution 0.5 M in HCl, 20 μL of the extract was directly injected into a gas chromatograph. Derivatives were detected by negative ion mass spectrometry. The method makes it possible to simultaneously determine 2-furfurylthiol (2-furanmethanethiol) (FFT), 4-mercapto-4-methyl-2-pentanone (MP), 3-mercaptohexylacetate (MHA) and 3-mercaptohexanol (MH). Inconsistent results were obtained for 2-methyl-3-furanthiol (MF). Detection limits were 0.5 ng L^{-1} (FFT), 0.1 ng L^{-1} (MP), 0.6 ng L^{-1} (MHA) and 7 ng L^{-1} (MH), well below the corresponding odor detection thresholds. Method repeatability ($10\% < RSD < 17\%$) and linearity ($0.98 < R^2 < 0.999$) were satisfactory. The linear range was more than 2 orders of magnitude wide, covering the natural range of occurrence of these compounds in wine, and the slopes of the standard addition plots from different wines were very similar. The different aspects of the method optimization are discussed.

Keywords: Thiols; Mercaptans; Pentafluorobenzylbromide; Wine; Aroma; Flavour; GC–NCI–MS

1. Introduction

Mercaptans are compounds with strong odors, and quite often they are responsible for the sensory characteristics and quality of a product. Simple low-molecular-weight thiols, such as methanethiol or ethanethiol, have extremely unpleasant odors, which are most often linked to the existence of microbiological decomposition. On the other hand, polyfunctional mercaptans of higher molecular weight (4-mercapto-4-methyl-2-pentanone, 2-methyl-3-furanthiol, 3-mercaptohexanol, 3-mercaptohexylacetate, 2-fururylthiol) have, at low concentrations, powerful and penetrating aromas that are responsible for the particular sensory characteristics of products like mango [1], coffee [2], passion fruit [3,4], green tea [5], onions [6], cooked meat [7] and some wines [8–15]. The odor thresholds of these mercaptans are in the nanogram per liter level, which implies that these compounds can be detected, or can even be key aroma compounds, at extremely low concentrations.

The analytical determination of these compounds at these levels is particularly difficult, since different problems have to be overcome. A first problem is their poor "detectability". Most often, the mass spectra of these compounds lack characteristic ions of high *m/z*. In addition, their chromatographic properties are often also poor because of the adsorptive characteristics of the thiol function, which causes intense tailing peaks in some stationary phases or in chromatographic systems with residual active sites. A second problem is because of their instability. These compounds are elusive and can react with oxygen and other oxidants [16] and, in addition, form complexes and precipitates with many metal ions.

It is not surprising, therefore, that not many analytical methods have been described for the quantitative analysis of these compounds at trace level. The most commonly used strategies for the analysis of these compounds make use of the complexing

* Corresponding author. Tel.: +34 976762067; fax: +34 976761292.
E-mail address: vferre@unizar.es (V. Ferreira).

0021-9673/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2007.02.003

properties of the thiol group, particularly to certain forms of organic mercury. This idea was originally proposed by Full and Schreier, who used an agarose gel containing phenylmercuric ions [17], and has been recently specifically applied to the analysis of wine polyfunctional mercaptans [18]. A similar strategy, but using aqueous solutions of organomercury salts, has also been applied to the analysis of these compounds in wine. Darriet et al. [19] and Tominaga et al. [20,21], have introduced a selective extraction of thiols from an organic extract with an aqueous solution of *p*-hydroxymercurybenzoate. Different versions of this idea have been also proposed in the recent literature [22,23]. These strategies make it possible to obtain very good isolates containing almost exclusively the thiols present in the original sample, but do not solve the aforementioned problems of "detectability" and instability. As a consequence, a large sample has to be processed and the methods are very often long, expensive and complicated. Furthermore, as has been recently shown, the losses of mercaptans in some steps of the analytical procedures can be important function of the matrix composition, which seriously hampers the real quantitative ability of such methods. The analytical performance of the methods can be improved by using carefully optimized analytical procedures [23] or stable isotope dilution analysis [18]. Nevertheless, the methods are laborious and their setup complicated.

Formation of derivatives is an interesting alternative for improving "detectability" and stability, but again not many references for the derivatization of volatile thiols can be found in the scientific literature. Hoffman and Schieberle [16] proposed 4-vinylpyridine as derivatizating reagent, and recently, Ortín et al. [24,25] have developed a method using this reagent for the quantitative analysis of these compounds in wine. However, the gain in "detectability" is not very high, and the chromatographic properties of pyridine derivatives are quite complicated. More recently, 2,3,4,5,6-pentafluorobenzyl bromide (PFBBr) has been proposed as the derivatization reagent for the determination of polyfunctional mercaptans [26]. This reagent has a main advantage that its derivatives can be sensitively and selectively determined by electron-capture detection (ECD) or negative ion mass spectrometry. In that procedure, derivatives were formed directly in the solid-phase microextraction fiber, which made it possible to fully automate the method. Although that strategy is appealing, the limitations of the procedure (only two analytes could be determined, the linear ranges are quite limited and samples cannot be left in the autosampler tray) suggest that many aspects of the reaction are not well understood and should be further studied. The objectives of the work presented in this paper are to study different aspects of derivatization of mercaptans with PFBBr and develop procedure to quantify some polyfunctional mercaptans of wine which can be aroma active at the nanogram per liter level.

2. Material and methods

2.1. Reagents and standards

n-Hexane for organic trace analysis (UniSolv) and ethanol, gradient grade for liquid chromatography (LiChrosolv), were from Merck (Darmstadt, Germany). Acetonitrile was supra-gradient HPLC grade from Scharlau (Barcelona, Spain); toluene of HPLC grade, ethyl acetate, methylisobutylketone (MIBK) and tetrahydrofuran (THF) for instrumental quality analysis were from Panreac (Barcelona, Spain). Diethylether for instrumental analysis was from Fluka (Buchs, Switzerland). Benzene of HPLC grade (+99.9%) was from Aldrich (Steinheim, Germany). Anhydrous sodium sulphate for ACS-ISO quality analysis was from Panreac. Tributyl, tripropyl and triethylamine and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) were from Aldrich. PFBBr was from Fluka. 3-Mercapto-3-methylbutyl formate, 3-mercapto-2-pentanone, 4-mercapto-4-methyl-2-pentanone (MP) 1% PG and 3-mercaptohexylacetate (MHA) were from Oxford Chemicals (Hartlepool, UK), 2-furfurylhiol (FFT), 3-mercaptohexanol (MH) and 1-hexanethiol were from Lancaster (Strasbourg, France), 2-methyl-3-furanthiol (MF) was from Aldrich, 2-phenylethanethiol and 4-methoxy-α-toluenethiol were from Fluka.

2.2. Proposed method

In a 13 mL screw-capped test tube, 6 mL of wine was extracted with 1.5 mL of benzene containing 200 ng L^{-1} of the internal standards and surrogates 3-mercapto-3-methylbutyl formate, 1-hexanethiol, 2-phenylethanethiol and 4-methoxy-α-toluenethiol. The air inside the tube was displaced by purging with N_2 during 2 min before closing the tube. Extraction was carried out in an automatic shaker for 15 min at a temperature below 10 °C; after this, the tubes were centrifuged (20 min, 6 °C and 2264 g) to let the phases separate. Nine hundred microliter of the supernatant organic phase was transferred to a clean, cold (4 °C) and dry 3 mL screw-capped Wheaton reaction vial. Then, 50 μL of the reagent solution (2000 mg L^{-1} PFBBr in benzene) and 50 μL of the alkali solution (20% DBU in benzene) were added. The mixture was left to react for 40 min at 4 °C. After this time, the vials were left to reach room temperature for 5 min, and the extract was washed with three 1-mL volumes of cleaning solution (0.5 M HCl, 20% of methanol). The organic phase was finally transferred to a standard 2 mL autosampler vial and spiked with a small amount of anhydrous sodium sulphate. Twenty microliter of this sample was directly injected into the GC–negative chemical ionization (NCI)–MS system.

2.3. 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 was injected into a packed liner for large volume injection. The initial temperature of the injector was 70 °C; and after 17 s, the injector was heated at 5 °C s^{-1} to 250 °C, remaining at this temperature until the end of the analysis. The carrier gas was He, flowing through the column initially at 0.6 mL min^{-1}. Seventeen seconds after the injection, the flow was increased to 1.8 mL min^{-1} for 3 min. After this period, the flow was fixed at 1 mL min^{-1}. The split valve was opened the

first 17 s of analysis (split flow 100 mL min^{-1}), closed the following 3 min and opened again for the rest of the analysis (split flow 50 mL min^{-1}).

The column was a Factor Four capillary column VF-5MS from Varian, 20 m × 0.15 mm I.D., with 0.15 μm film thickness. The initial temperature of the column was 70 °C for 3 min. The column was heated to 140 °C at 20 °C min^{-1}, to 180 °C at 15 °C min^{-1}, 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 was operated in NCI mode using methane at 2 bar as reagent gas. The temperature of the ion source was 200 °C, and the interface was kept at 320 °C. In the time segments given below, the following ions were acquired in single ion monitoring (SIM) mode for the different analytes: MF and FFT from 7.9 to 8.15 min, 113 and 274 at 0.25 s^{-1}; hexanethiol from 8.65 to 8.90, 115 and 278 at 0.25 s^{-1}; MP from 9.20 to 9.42 min, 131 and 194 at 0.2 s^{-1}; 3-mercapto-3-methylbutyl formate from 9.65 to 9.85; 147 at 0.2 s^{-1}; MH from 9.85 to 10.48 min, 133 at 0.35 s^{-1}; MHA and 2-phenylethanethiol from 10.30 to 10.65, 175, 194 and 135 at 0.2 s^{-1}; 4-methoxy-α-toluenethiol from 11.22 to 11.35 min, 314 at 0.30 s^{-1}.

Cleaning up of laboratory ware: The reaction and the extraction tubes were first thoroughly washed with water and detergent, and later with acetone (thrice). Tubes and vials were then filled with a small volume (1 mL) of benzene, closed with their corresponding caps and shaken for 5 min. The benzene was then discarded, and the material was finally washed with acetone.

2.4. Method validation

2.4.1. Internal standards, surrogates, linearity and matrix effects

In preliminary studies, the chromatographic and mass spectrometric properties of the PFBBr derivatives of 15 different mercaptans were determined. After discarding those giving tailing peaks or eluting too close to some of the analytes, five of them (3-mercapto-2-pentanone, hexanethiol, 3-mercapto-3-methylbutyl formate, 2-phenylethanethiol and 4-methoxy-α-toluenethiol) were selected and used as potential internal standards in different standard addition experiments. These experiments were carried out on six (using a preliminary extraction procedure consisting of two successive extractions of 9 mL of wine, pH 7, with benzene) or eight (following the proposed procedure) different wines. In the first experiment, the wines used were a red from Garnacha and Tempranillo, 13.5% ethanol; a rosé from Garnacha, 12.5% ethanol; and four whites (one from Macabeo, 12.5% ethanol; one from Chardonnay, 12.0% ethanol; one from Sauvignon blanc, 11.5% ethanol; and one from Verdejo, 11.5% ethanol). In the second experiment, two more wines were added: an aged red from Tempranillo, 12.5% ethanol and a white from Albariño, 11.0% ethanol. The wines spiked or not with variable amounts of analytes in the ranges shown in Table 4 were extracted with benzene containing a fixed amount of the potential internal standards (400 pg). The extracts were derivatized and analyzed following the proposed procedure. Five standard addition lines (one per potential internal standard) were built per analyte and wine. The standards for each analyte were finally selected as those providing the least dissimilar slopes between the eight (six in the case of the preliminary extraction procedure) different wines.

2.4.2. Method repeatability and limits of detection

Two different wines (red from Garnacha and white from Verdejo) were spiked at two different levels as indicated in Table 5, and five replicate analyses were carried out on each sample. Method limits of detection and quantification were determined from the standard addition plots built previously as the concentration of analyte in wine which would give a signal 3 or 10 times higher than the noise, respectively.

2.4.3. Application

Thirteen different wines made with four different white varietals (three from Albariño, Macabeo and Verdejo, and four from Sauvignon blanc) were finally analyzed following the proposed procedure.

3. Results and discussion

This paper presents a new method for the determination of several aromatic mercaptans in wine. In the final method, mercaptans are first extracted from wine using benzene, are further derivatized with PFBBr and are finally analyzed by GC–NCI–MS. Although all the different steps of the method are extremely interdependent, for the sake of clarity, the different aspects of the method development will be treated separately.

3.1. Derivatization

The key feature of the method is the transformation of mercaptans into their corresponding pentafluorobenzyl derivatives by using PFBBr. The reaction is a nucleophilic substitution, NS_2 type, which proceeds via the corresponding thiolate. Similar reactions are frequently used in literature for the derivatization of fatty acids or phenols [27–29]. As these compounds are stable, in these cases, in most of the procedures, the reactions are carried out in aqueous media using quite energetic conditions (high concentration of alkali, high temperature). These conditions should not be used for the derivatization of thiols in wine, since the many fatty acids and phenols naturally occurring in wine would also react and the reactive mercaptans could undergo different oxidation or degradation processes. Consequently, different derivatization strategies were considered in the present study in order to carry out the reaction under more selective conditions, or in a phase in which the mercaptans could have been previously isolated from the interfering materials. The following list summarizes the different trials (detailed conditions are given in Table 1): (1) reaction in a two-phase liquid–liquid system [30], (2) reaction in a two-phase liquid–liquid system with a phase transfer catalyst [31], (3) reaction in a two-phase liquid–solid (polymeric sorbent) system [32], (4) reaction in a homogenous organic solvent system.

As none of the three first alternatives worked under the conditions assayed (see Table 1 for details), the following efforts concentrated on the development of the reaction in an organic

Table 1
Summary of the different studies carried out to determine the best reaction strategy for the formation of PFB derivatives of the selected polyfunctional mercaptans

Reaction medium	Reaction conditions
(1) Liquid–liquid	A 2 mL benzene solution containing 1 mg L^{-1} of analytes and 200 mg L^{-1} of PFBBr was energetically shaken with 1 mL of aqueous 0.1 M NaOH at room temperature. The reaction was controlled by taking small volumes (0.1 mL) of the organic phase at 0, 20, 40, 60, 80 and 100 min. These volumes were washed with 0.5 M HCl, diluted and analyzed by GC–ECD
(2) Liquid–liquid with phase transfer catalyst	As before, but the aqueous phase was 0.1 M in NaOH and 0.05 M in tetrabutylammonium chloride
(3) Polymeric sorbent–liquid system	1 μg of analytes was deposited in a 50 mg LiChrolut EN Solid Phase Extraction cartridge. This was then imbibed in 600 μL of a benzene solution containing 200 mg L^{-1} of PFBBr and 10% (w/v) of DBU. The cartridge was left in contact with the reactive solution for 20 min. After this time, excess DBU was removed by washing with HCl 0.1 M and derivatives were eluted with three consecutive 2 mL volumes of hexane and hexane/diethylether 1/1 and 1/4. These volumes were further diluted and analyzed by GC–ECD
(4) Homogeneous organic solvent	Hexane, hexane/diethylether 1/1, hexane/ethyl acetate 8/2, toluene, benzene, methyl-isobutyl ketone, tetrahydrofurane and acetonitrile were assessed. One milliliter volumes of the organic solvent containing 10 μg of each analyte were mixed in a clean and dry 3-mL screw-capped Wheaton vial with 20 μL of a 10,000 mg L^{-1} PFBBr solution and 10 μL of alkali (triethylamine, tributylamine or DBU). Reaction was left to occur for 40 min at room temperature. Immiscible solvents were directly washed with 1 mL of 0.5 M HCl. Miscible solvents were first diluted with hexane. The organic phases were dried, diluted and analyzed by GC–ECD

solvent. The synthesis of derivatives is usually carried out in a polar solvent such as acetonitrile [33] under relatively mild conditions; and, so far, this is the procedure used to synthesize reference standards for the derivatives. The procedure works relatively well as long as the analytes are at relatively high concentrations, independently of the solvent system used, but the yield of the reaction sharply decreases when the levels come down below 1 mg L^{-1}. At these low concentrations, the reaction kinetics follow more or less the model illustrated in Fig. 1. MF and FFT, which are the most reactive, reach their corresponding maxima in just 10 min, while the reaction rate for MP is the slowest. Once the maxima are reached, the concentrations of the derivatives begin to decrease, the decrement being clearer in the case of MF. This result suggests that there are several other side reactions apart from the formation of derivatives that become important at low concentrations. The kinetic trend shown in Fig. 1 was similar in all the solvents tested. However, the amount of derivative finally formed in the media was found to differ in the different solvents or solvent systems. As

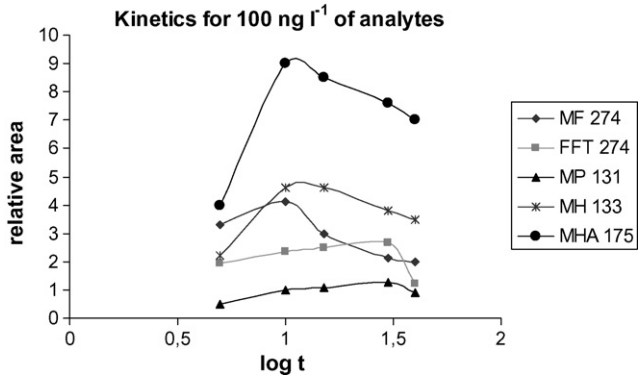

Fig. 1. Plot showing the typical kinetics found in the formation of pentafluorobenzyl derivatives of mercaptans when these are present at low concentration in any solvent (100 ng L^{-1}). In the example shown, the reaction medium was 1 mL of benzene containing 1% of tributylamine and 20 g L^{-1} of PFBBr.

is shown in Fig. 2, the worst results were found with non-polar solvents or with systems containing relatively high amounts of non-polar solvents. It should also be remarked that there is no best solvent, since different compounds follow different trends. MP and MHA (the least polar) seem to react better in benzene, while MF, FFT and MH (the most polar and/or reactive) prefer most polar solvents, such as MIBK or THF. Given the fact that benzene is a better extraction solvent than MIBK, since it extracts lesser amounts of polar compounds from wine, that THF is wine miscible, and that MP is one of the most difficult compounds to analyze because of its sensory significance at very low concentrations [9], benzene was finally selected as the reaction solvent.

The different parameters affecting the reaction, such as reagent concentration, type of alkali and concentration of alkali, were further investigated in order to find conditions leading to an optimal yield of derivatives. The results of the experiments were mostly frustrating, because none of the parameters seemed to be critical or appeared to exert any important effect on the other experimental parameters. For instance, four different alkalis were studied (triethylamine, tributylamine, tripropylamine and DBU); the minimum amounts (%) of alkali required to achieve maximum yields were the same in all the cases (1%), in all solvent systems and at nearly all the different concentrations of reagent studied. Moreover, the kinetics of the reactions were, in all cases, again similar to the one shown in Fig. 1. The single

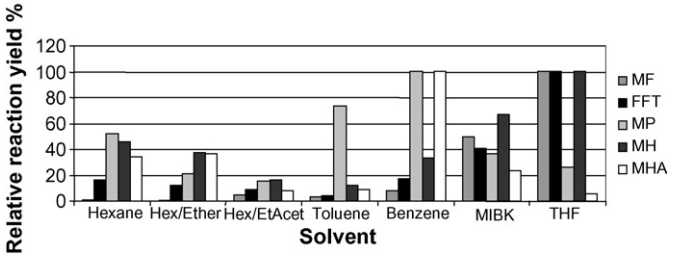

Fig. 2. Influence of the solvent on the relative yield of the derivatization (expressed as percentage of the maxima). Reaction systems were in all cases 1 mL of solvent containing 10 mg L^{-1} of analytes, 1% of tributylamine and 20 g L^{-1} of PFBBr.

Table 2
Summary of the experiments carried out to eliminate the excess of reagent from the reaction mixture

Procedure	Cleanup conditions	Results
Clean up on silica gel or a polymeric sorbent	The sorbents (500 mg activated silica gel or 50 mg LiChrolut EN) were packed in 3 or 1 mL reservoirs, respectively. Twenty microliter of the reaction mixture (containing 50% PFBBr) were deposited in the bed and were then chromatographied with 2 mL fractions of a hexane/diethylether elutropic series	Silica gel: 98% of the reagent can be eluted with hexane, but 50% of the MF-derivative coelutes with it. The derivative of FFT can be perfectly quantitatively recovered with 10% diethylether. The other derivatives require at least 75% diethylether LiChrolut EN: A 2% of diethylether is required to remove the reagent but 60 and 20% of MF and FFT are lost with it
Selective evaporation in a PTV injector	1–20 μL volumes of a benzene solution containing the PFB derivatives of the analytes at 500 $\mu g\,L^{-1}$ and 10 or 500 $mg\,L^{-1}$ of PFBBr were injected in a Programmed Temperature Injector packed with carbofrit™. Three isotherms: 50 °C (evaporation of benzene), 100–130 °C (evaporation of reagent) and 250 °C (evaporation and transfer of analytes) and different flow programs were assayed	It is possible to completely eliminate the reagent through the split valve (with a quantitative recovery of analytes) if its concentration is below 10 $mg\,L^{-1}$. If the concentration of reagent is higher, no more than 80% of it can be safely removed

parameter, on which the nature of the alkali was found to exert influence, was the minima concentration of reagent required to obtain maximum yields, as is shown in Fig. 3. For amine-type alkalis, such as triethyl- or tributylamines, the yield of the reaction drops at concentrations of PFBBr below 2 $g\,L^{-1}$. However, the yield for DBU was found to be constant at concentrations of PFBBr as low as 40 $mg\,L^{-1}$. This is a very important factor, since the presence of high amounts of reagent in the sample analyzed by GC may have adverse consequences on the chromatography, on the NCI–MS signal and on the stability of the chromatographic column. On the other hand, the elimination of the reagent is not straightforward, and none of the procedures studied (cleanup on silica gel or on polymeric sorbents, selective evaporation in the PTV injector—see Table 2 for details) worked properly. Fortunately, the amount of reagent finally required with DBU to get a satisfactory signal makes unnecessary the elimination of reagent. In regular routine work, the chromatographic system does not seem to be affected apart from the effects described for MH (see Section 3.3).

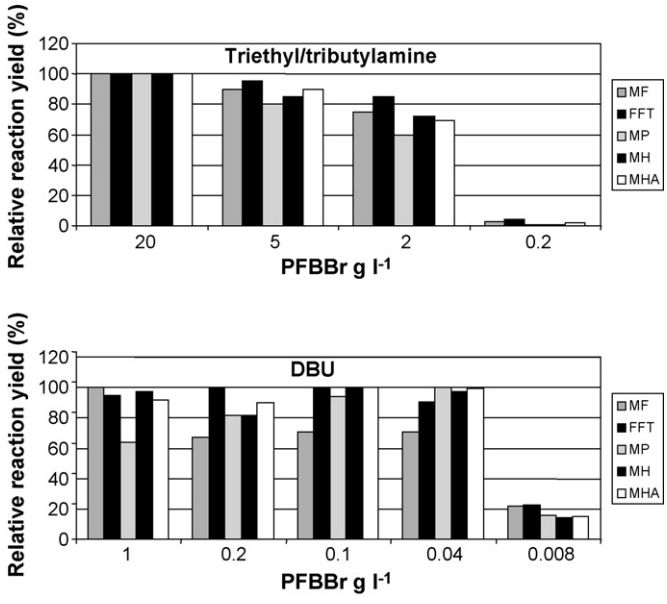

Fig. 3. Combined effect of the type of alkali (at 1% in the reaction mixture) and the concentration of reagent (PFBBr) on the amount of pentafluorobenzyl derivative formed (normalized to 100).

Another parameter considered was the reaction temperature. Again, its effect was not significant, and similar signals were obtained in the range from 4 to 45 °C. As the extraction is carried out at low temperature (see next section), it was decided to use 4 °C as reaction temperature to ensure maximum repeatability.

3.2. Extraction

Different aspects of the extraction, such as wine pH, number of extractions and phase ratio were considered. Critical aspects of the study were the formation of emulsions (up to 0.7 mL of benzene per 10 mL of wine) and the likely oxidation of analytes during the extraction. Two different procedures were devised and tested. In the first procedure, greater emphasis was laid on classical criteria (enrichment efficiency and elimination of interferences); and, in the second procedure, the strategy laid the emphasis on fastness and simplicity.

Accordingly, optimal conditions in the first procedure were two successive extractions (with 1.5 and 1.0 mL of benzene) of 9 mL of wine adjusted at pH 7.0 previously. This procedure was not very fast but ensured a consistent recovery of organic phase, the chromatograms were very clean (wine fatty acids were not extracted), the concentration achieved during the extraction was satisfactory (about five times) and the extraction of analytes was nearly complete (the amount of thiols remaining in the wine after the two successive extractions was found to be less than 5% for MF, FFT, MP and AMH and around 15% for MH). The analytical signals obtained with this procedure were repeatable (RSD around 15%) and linear. However, they were found to greatly differ in different samples or even in batches as the standard addition experiments carried out on six different wines have shown (see Section 2.3). These results are shown in

Table 3
Linearity and matrix effects obtained by using the first extraction procedure (two successive extractions of wine at pH 7)

Analyte	Benzene			Wines		
	[a]Average slope	[a]RSD (%)	Average R^2	[b]Average slope	[b]RSD (%)	Average R^2
2-Furfurylthiol (FFT)	3535 ± 165	4.7	0.997	3714 ± 478	13	0.997
4-Mercapto-4-methyl-2-pentanone (MP)	231 ± 69	30	0.994	84.0 ± 39	47	0.936
3-Mercaptohexylacetate (MHA)	582 ± 17	2.9	0.994	485 ± 159	33	0.917
3-Mercaptohexanol (MH)	3130 ± 220	7.0	0.991	1957 ± 481	25	0.903

Data are absolute peak areas.
[a] Average and relative standard deviation of two slopes calculated in two independent experiments (six concentration levels in each case).
[b] Average and relative standard deviation of six slopes calculated in six standard addition experiments carried out on six different wines (five concentration levels in each case).

Table 3. As shown in the table, the slopes of the standard addition lines obtained with this procedure for FFT were relatively similar, which suggests that matrix effects were not significant for this compound. However, results in the case of MP were particularly worse. For this compound, even results in synthetic benzene solutions were not reproducible and the normalization of the areas by those of the internal standards did not bring about any improvement. Similar poor results were obtained with MF and MH. The dilution of the wine ameliorated, but not solved, these problems. Several observations suggested that the problems should be related to the redox state of mercaptans in the wine and to the likely oxidation of analytes during the extraction. The first is that the order in which internal standards and analytes are added to the wine has some influence, and that those added later appear to be more reproducible. The second is that the addition of relatively small amounts of SO_2 or mercaptoglicerine to the wine before the extraction has deep influence on the signal. It was not possible, in any case, to eliminate these effects, and it was decided to work out an extraction strategy that is different, faster and less exposed to oxygen.

This strategy consisted of a single extraction of 6 mL of untreated wine with 1.5 mL of benzene. The procedure was carried out at low temperature, under nitrogen and in darken tubes to avoid the effect of light and oxygen. In these conditions, results improved, and the slopes of the standard addition plots built in different wines were not very different, as indicated by the RSD values given in Table 4. It was not possible, however, to obtain consistent results for MF as will be discussed later.

3.3. Extract cleanup and chromatographic analysis

A relatively large volume (20 μL) of the derivatized extract should be injected to obtain the required sensitivity. This was carried out using the solvent split injection technique, as is detailed in Section 2. Although the technique worked well, three different problems were observed in the medium term. The first was that some of the injections, in an apparently random way, failed, causing dirty and distorted chromatograms. The second was found in some wine samples, in which the chromatographic peak of the 3-mercaptohexanol derivative appeared strongly distorted (see Fig. 4). The third was a progressive deterioration of the chromatographic efficiency, which was particularly evident, again, for 3-mercaptohexanol. The first problem was attributed to the random presence in some of the extracts of a small amount of emulsified water, and was solved just by adding dehydrating salt (see the proposed procedure). The second and third problems were attributed to the co-extraction of polar compounds which, by themselves or by interaction with the non-reacted reagent, would cause the appearance of strongly active (for hydrogen donors) sites in the pre-column or in the first centimeter of the column, either temporally (second problem) or permanently (third problem). These effects were minimized, although the third problem could not be completely neutralized, by washing the extract with a water:methanol (1:5) solution, as shown in Fig. 4. In spite of that, it was found that after a certain number of analyses of real samples, the peak for the 3-mercaptohexanol derivative appeared progressively broader and delayed, which eventually made its quantification impossible. The use of chro-

Table 4
Linearity and matrix effects obtained by using the second and definitive extraction procedure

Analyte	Benzene		Wines			
	[a]Slope	R^2	[b]Average slope	[b]RSD (%)	[c]Average R^2	Studied range (ng L^{-1})
2-Furfurylthiol (FFT)	0.1404	0.9951	$(0.1452 \pm 8.28) \times 10^{-3}$	5.2	0.9992	0.5–100
4-Mercapto-4-methyl-2-pentanone (MP)	0.0022	0.9931	$(0.0101 \pm 5.5) \times 10^{-4}$	5.5	0.9924	0.5–43
3-Mercaptohexylacetate (MHA)	0.0468	0.9899	$(0.0204 \pm 2.28) \times 10^{-3}$	12.3	0.9898	1–650
3-Mercaptohexanol (MH)	6.42	0.9902	7.69 ± 0.93	14.1	0.9865	10–1120

Data are peak areas normalized by that of the corresponding internal standard.
[a] Slope of the standard calibration graph built with seven concentration levels.
[b] Average and relative standard deviation of eight slopes calculated in eight standard addition experiments carried out on eight different wines (six concentration levels in each case).
[c] Average determination coefficients of the eight standard addition lines.

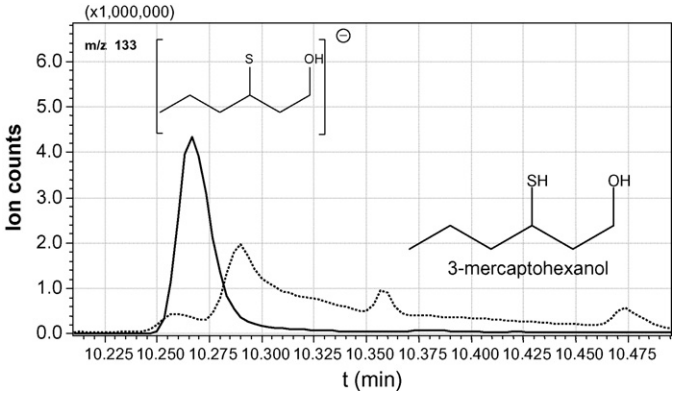

Fig. 4. Effect of washing a reacted wine benzene extract with a water:methanol mixture (4:1) on the chromatographic properties of the 3-mercapthexanol pentafluorobenzyl derivative (spiked at 500 ng L^{-1}). The dotted line corresponds to the chromatogram obtained in the analysis of the original extract.

matographic columns with a more polar phase (a CP-Wax was considered) did not solve this problem completely, and besides, in this more polar phase, the peak of the MP derivative was lost.

3.4. Method validation

3.4.1. Internal standards and surrogates

Five different mercaptans were selected attending to the chromatographic properties of their derivatives and were tested as possible internal standards. Their abilities to improve the method reproducibility and compensate for the possible matrix effects were investigated by means of different standard addition experiments. On the basis of the results of this study, 4-methoxy-α-toluenethiol was selected as internal standard for MF, FFT, MHA and MH, while 3-mercapto-3-methylbutyl formate was selected as internal standard for MP. Two other compounds (hexanethiol and 2-phenylethanethiol) were used as surrogates, and their area, normalized to that of 4-methoxy-α-toluenethiol, was used as quality control parameter.

3.4.2. Blanks

As the method is extremely sensitive and the quantitative determination of analytes at very low level is required, the study of blanks is compulsory. As expected, it was found that some pieces of the laboratory ware, particularly the caps of the extraction tubes or of the reaction vessels, were easily contaminated by the derivatives. This makes it necessary to follow a stringent cleaning procedure (see Section 2) and of course to keep an adequate separation between the reaction area and reaction material and the solutions in which standards are stored. Following these directions, the blanks did not represent a problem, except in the case of MF. For this compound and in some isolated batches of analyses, spurious peaks were found.

3.5. Reproducibility, limits of detection, linearity and matrix effects

Reproducibility was determined as repeatability at two different concentration levels and was found satisfactory, as can be seen in Table 5. Detection and quantification limits were determined by the analysis of different commercial wine 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 FFT, MP and MHA are below 1 ng L^{-1}, and quantification limits in these cases are below 2 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

Table 5
Method repeatability—expressed as RSD (%)—determined by the replicate analysis of commercial wines spiked at two different levels (five replications in both cases)

	Repeatability		LOD (ng L^{-1})	LOQ (ng L^{-1})
	Low level[a]	High level[b]		
2-Furfurylthiol (FFT)	12.2	10.1	≈0.5	≈1.4
4-Mercapto-4-methyl-2-pentanone (MP)	11.9	11.5	≈0.1	≈ 0.3
3-Mercaptohexylacetate (MHA)	17.0	15.6	≈0.6	≈1.9
3-Mercaptohexanol (MH)	15.2	10.6	≈7	≈20

Method detection and quantification limits determined by the analysis of real samples with low levels of analytes.
[a] Low level: FFT, 8 ng L^{-1}; MP, 1 ng L^{-1}; MHA, 10 ng L^{-1}; MH, 40 ng L^{-1}.
[b] High level: FFT, 40 ng L^{-1}; MP, 5 ng L^{-1}; MHA, 50 ng L^{-1}; MH, 800 ng L^{-1}.

Table 6
Average concentration levels of FFT, MP, MHA and MH in small sets of four different monovarietal Spanish wines

	FFT (ng L^{-1})		MP (ng L^{-1})		MHA (ng L^{-1})		MH (ng L^{-1})	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Albariño ($n=3$)	3.2[a]	3.4	10.9[a]	6.1	33[a]	21	134[a]	35
Macabeo ($n=3$)	<LQ[a]	–	2.1[a]	3.6	28[a]	13	114[a]	49
Verdejo ($n=3$)	9.1[b]	1.9	7.5[a]	5.9	216[b]	48	931[b]	136
Sauvignon blanc ($n=4$)	1.4[a]	0.43	1.8[a]	0.66	106[ab]	99	443[ab]	387

Different superscripts indicate a significant difference between the means.

is 60 ng L^{-1}, well above the method detection limits. Linearity was also satisfactory, with average determination coefficients better than 0.98 (see Table 4), and with linear ranges including the normal range of occurrence of these compounds in wine. As commented earlier, the existence of matrix effects was checked by carrying standard addition experiments in wines of very different types (reds, rosés and whites, as detailed in Section 2.3). The average slopes of the standard addition lines, together with the straight calibration lines obtained from the direct derivatization of benzene standard solutions, are shown in Table 4. As can be seen, except for FFT, there is a large disagreement between the slopes obtain in benzene or in wine. However, there is a quite good agreement in the slopes found in the analysis of very different wines. The relative standard deviations of the slopes were around 5% for FFT and MP, 12.3% for MHA and 14.1% for MH. These values are consistent with repeatability data, and suggest that matrix effects are not important for these compounds. The worst results were found, again, in the case of MF. In this case, the linearity obtained with synthetic solutions and with a limited number of wines was very good. However, these results were not consistent and different batches of analy-

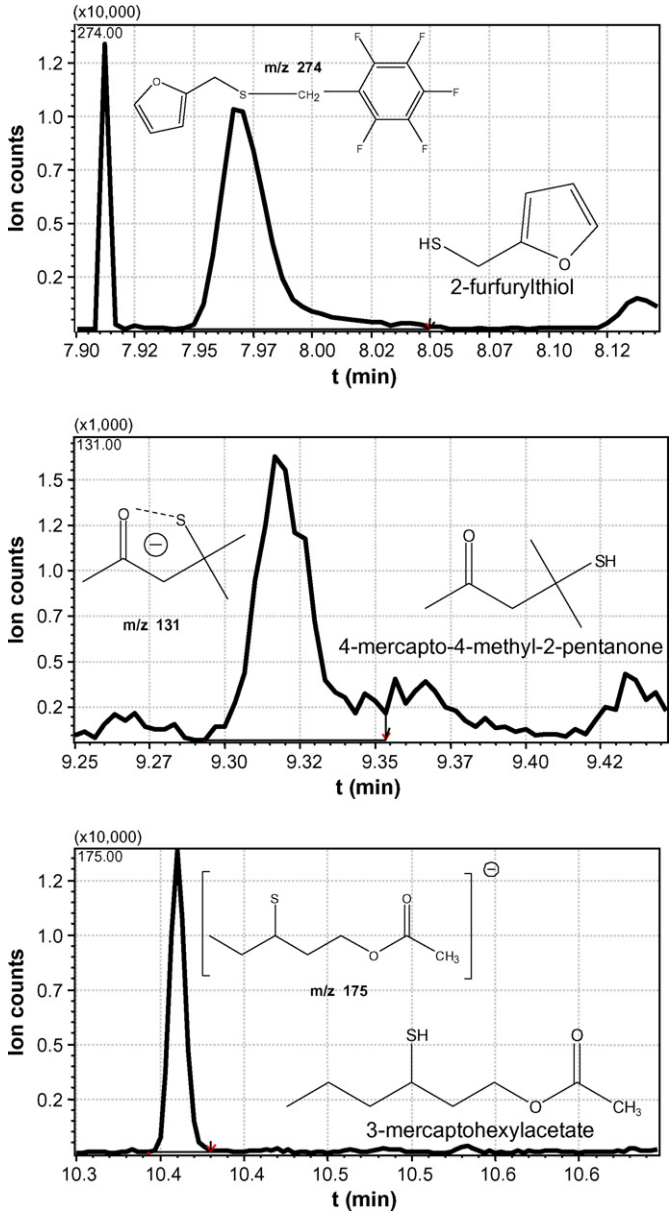

Fig. 5. Chromatograms obtained in the analysis, following the proposed procedure, of a wine containing 26 ng L^{-1} FFT, 4 ng L^{-1} MP and 66 ng L^{-1} MHA.

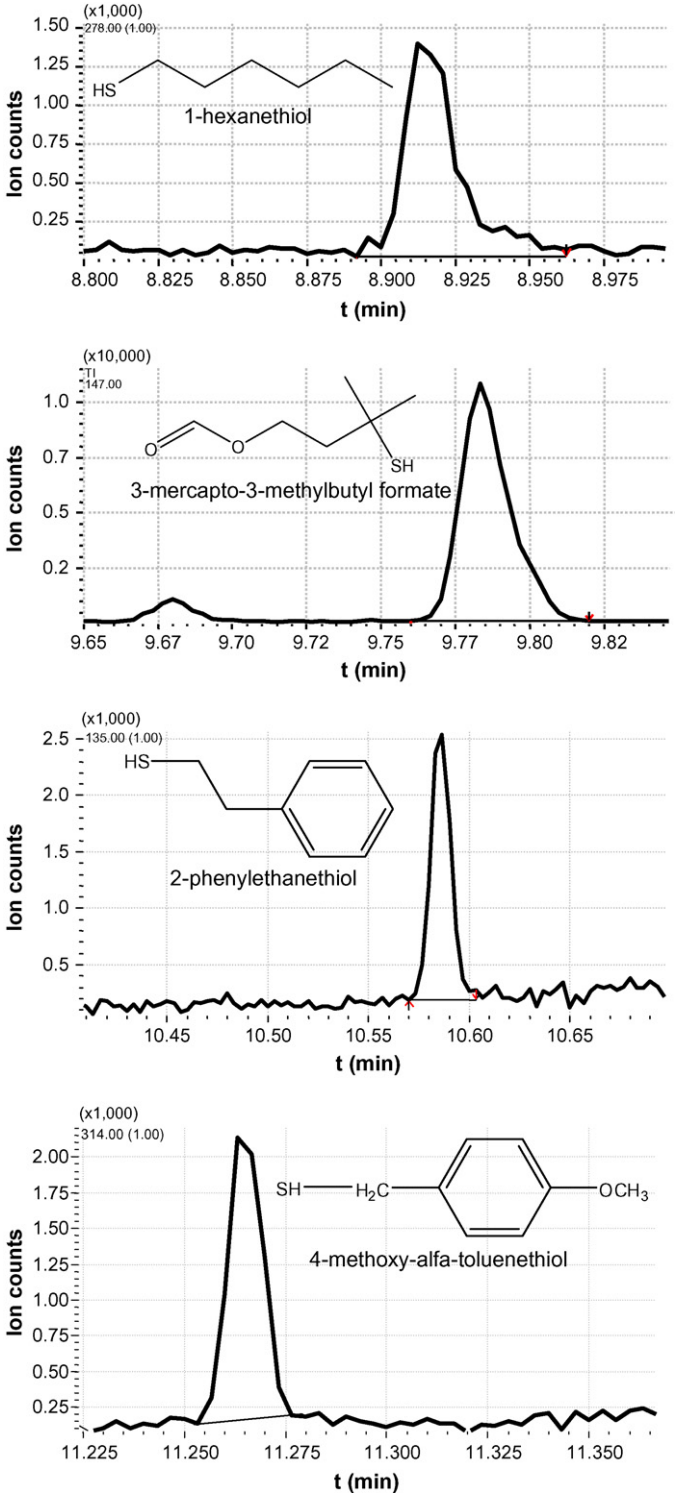

Fig. 6. Chromatograms corresponding to the four internal standards used in the study (200 ng L^{-1} each in the benzene extract).

ses eventually gave strange results. Although a systematic study was conducted to identify the source of this problem, no clear conclusions have been reached. Possible causes for this problem are contamination, existence of some unidentified interference or even the apparent instability of the pure standard.

3.6. Application

The method has been applied to the determination of these compounds in different Spanish wines made with different grape varietals. Results of the study are summarized in Table 6, and the corresponding chromatograms for analytes and internal standards are shown in Figs. 5 and 6, respectively. The signal obtained for MH is given in Fig. 4. The table shows that FFT and MP are present at extremely low level in those wines. Surprisingly, the levels of MP in the Spanish Sauvignon blanc wines were the lowest, which contrasts with the reported importance of this compound in the French wines [12] made with this variety. The levels of MHA and MH are relatively high in wines from Sauvignon blanc and Verdejo, which would support the characteristic tropical fruit aroma nuances of these wines [34].

4. Conclusions

The present method resolves some of the limitations of previous procedures for the analysis of polyfunctional mercaptans at ultratrace level. Although the method is not fully automated, it is relatively fast and simple, requires a small volume of sample and the number of compounds that can be determined simultaneously is higher than that of a previous report. Leaving aside 2-methyl-3-furanthiol, for which inconsistent results were found, the linear range is satisfactory, and the sensitivity is very good. The proposed procedure makes use of the formation of derivatives in a benzene extract, and although the procedure has been validated for wine, it should be expected that it be useful for the analysis of these compounds in different matrixes.

Acknowledgements

This work has been funded by the Spanish MEC, project AGL2004-06060/ALI. L.M. has received a grant from the Regional Government of Aragón.

References

[1] A.S. Buettner, P. Schieberle, J. Agric. Food Chem. 47 (1999) 5189.
[2] P. Semmelroch, W. Grosch, Food Sci. Technol. Lebens. Wissen. Technol. 28 (1995) 310.
[3] K.H. Engel, R. Tressl, J. Agric. Food Chem. 39 (1991) 2249.
[4] P. Werkhoff, M. Güntert, G. Krammer, H. Sommer, J. Kaulen, J. Agric. Food Chem. 46 (1998) 1076.
[5] K. Kumazawa, K. Kubota, H. Masuda, J. Agric. Food Chem. 53 (2005) 5390.
[6] M. Granvogl, M. Christlbauer, P. Schieberle, J. Agric. Food Chem. 52 (2004) 2797.
[7] R. Kerscher, W. Grosch, J. Agric. Food Chem. 46 (1998) 1954.
[8] P. Darriet, T. Tominaga, E. Demole, D. Dubourdieu, C. R. Acad. Sci. III 316 (1993) 1332.
[9] A. Escudero, B. Gogorza, M.A. Melus, N. Ortin, J. Cacho, V. Ferreira, J. Agric. Food Chem. 52 (2004) 3516.
[10] V. Ferreira, N. Ortin, A. Escudero, R. Lopez, J. Cacho, J. Agric. Food Chem. 50 (2002) 4048.
[11] H. Guth, J. Agric. Food Chem. 45 (1997) 3027.
[12] T. Tominaga, R. Baltenweck-Guyot, C.P. Des Gachons, D. Dubourdieu, Am. J. Enol. Vitic. 51 (2000) 178.
[13] T. Tominaga, L. Blanchard, P. Darriet, D. Dubourdieu, J. Agric. Food Chem. 48 (2000) 1799.
[14] T. Tominaga, G. Guimbertau, D. Dubourdieu, J. Agric. Food Chem. 51 (2003) 1016.
[15] T. Tominaga, G. Guimbertau, D. Dubourdieu, J. Agric. Food Chem. 51 (2003) 1373.
[16] T. Hofmann, P. Schieberle, W. Grosch, J. Agric. Food Chem. 44 (1996) 251.
[17] G. Full, P. Schreier, Lebensmittelchemie 48 (1994) 1.
[18] R. Schneider, Y. Kotseridis, J.L. Ray, C. Augier, R. Baumes, J. Agric. Food Chem. 51 (2003) 3243.
[19] P. Darriet, T. Tominaga, V. Lavigne, J.-N. Boidron, D. Dubourdieu, Flavour Frag. J. 10 (1995) 385.
[20] T. Tominaga, C.P. des Gachons, D. Dubourdieu, J. Agric. Food Chem. 46 (1998) 5215.
[21] T. Tominaga, A. Furrer, R. Henry, D. Dubourdieu, Flavour Frag. J. 13 (1998) 159.
[22] T. Tominaga, D. Dubourdieu, J. Agric. Food Chem. 54 (2006) 29.
[23] V. Ferreira, N. Ortin, J.F. Cacho, J. Chromatogr. A 1143 (2007) 190.
[24] N. Ortín, V. Ferreira, J. Cacho, in: Presented at the 11th Meeting on Instrumental Analysis (JAI), Barcelona, 15–17 November 2005, p. 203 (abstracts).
[25] N. Ortín, Ph.D. Thesis, University of Zaragoza, Zaragoza, 2006.
[26] L. Mateo-Vivaracho, V. Ferreira, J. Cacho, J. Chromatogr. A 1121 (2006) 1.
[27] M.H. Jia, W.W. Wu, R.A. Yost, P.A. Chadik, P.W. Stacpoole, G.N. Henderson, Anal. Chem. 75 (2003) 4065.
[28] Z. Kuklenyik, J. Ekong, C.D. Cutchins, L.L. Needham, A.M. Calafat, Anal. Chem. 75 (2003) 6820.
[29] O. Lerch, P. Zinn, J. Chromatogr. A 991 (2003) 77.
[30] S. Nakamura, M. Takino, S. Daishima, Analyst 126 (2001) 835.
[31] D. Landini, F. Rolla, Synthesis 8 (1974) 565.
[32] M.T. Cummins, R.J. Wells, J. Chromatogr. B 694 (1997) 11.
[33] P.J. Baugh, Gas Chromatography: A Practical Approach, IRL Press at Oxford University Press, Oxford, New York, 1993, p. 133.
[34] E. Campo, V. Ferreira, A. Escudero, J. Cacho, J. Agric. Food Chem. 53 (2005) 5682.