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Accurate quantitative determination of the total amounts of Strecker aldehydes contained in wine. Assessment of their presence in table wines

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ABSTRACT

Strecker aldehydes (SAs) are key determinants of wine shelf-life and can be present in unoxidized wines in odorless forms, such as hydroxyalkylsulfonates, imines or acetals. A robust and accurate method for the determination of total forms of SAs, based on the classical derivatization with *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine (PFBHA) and in the selective solid phase extraction of derivatives has been optimized and validated. Matrix effects have been solved by the use of adequate internal standards and by large-enough equilibration times under anoxic conditions. Method figures of merit are highly satisfactory in terms of detection limits (<0.1 μg/L), linearity ($R^2 > 0.997$), reproducibility (5–13%) and recoveries (RSDs, between 2 and 10%, for 3-methylbutanal, 14%). The analysis of total SAs in 108 Spanish wines revealed that between 52% and 70% of unoxidized red wines and likely a similar fraction of white wines, contain levels of SAs high enough to cause oxidative aromas if bound forms of SAs cleave.

1. Introduction

Strecker aldehydes (SAs), isobutyraldehyde, 2-methylbutanal, 3-methylbutanal, methional and phenylacetaldehyde, are five aroma powerful aldehydes derived from the amino acids, valine, isoleucine, leucine, methionine and phenylalanine, respectively. SAs are one of the most relevant groups of natural aroma compounds; not only they have relatively low odor thresholds and explicit odors, but also they can participate actively in odor × odor perceptual interactions, in particular methional and phenylacetaldehyde (Coetzee et al., 2015; Culleré, Cacho, & Ferreira, 2007; San-Juan, Ferreira, Cacho, & Escudero, 2011). They are relatively ubiquitous and play a notorious role on wine oxidation chemistry, since they have a direct implication in the oxidative aroma deterioration of wine (Bueno, Culleré, Cacho, & Ferreira, 2010; Culleré et al., 2007; Escudero, Hernandez-Orte, Cacho, & Ferreira, 2000; Ferreira, Hogg, & de Pinho, 2003). Methional is able to change the fruity perception of wines from fresh to overripen and, at higher levels, to raisin, while phenylacetaldehyde is able to suppress completely the fruity character of red wines, before delivering its typical honey notes (San-Juan et al., 2011). In white wines, methional has demonstrated a strong suppressive effect on grapefruit and guava descriptors (Coetzee et al., 2015).

These compounds can be naturally formed during fermentation, since they are normal intermediates in the Ehrlich amino acid catabolism pathway of yeast, and can be also formed by Strecker degradation of wine amino acids during wine oxidation (Bueno et al., 2018; Pripis-Nicolau, de Revel, Bertrand, & Maujean, 2000). Tracing their exact origin in wine is not easy because SAs form relatively strong complexes

Abbreviations: ANOVA, Analysis of Variance; ARW, Aged Red Wine; EI, Electron Impact; FID, Flame Ionization Detection; GC, Gas Chromatography; IS, Internal Standard; LD, Limit of Detection; LQ, Limit of Quantification; MS, Mass Spectrometry; OIV, International Organization of Vine and Wine; PFBHA, *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine; RA, Relative Area; RF, Response Factor; RSD, Relative Standard Deviation; RT, room temperature; SAs, Strecker Aldehydes; SIM, Single Ion Monitoring; SPE, Solid Phase Extraction; SPME, Solid Phase Microextraction; UPLC, Ultra High Performance Liquid Chromatography; YRW, Young Red Wine.

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with SO_2 (de Azevedo et al., 2007), so that they can be already present in unoxidized wine, forming non-volatile and odorless hydroxyalkylsulfonates (Bueno, Zapata, & Ferreira, 2014; Grant-Preece, Fang, Schmidtke, & Clark, 2013). Later, during wine storage, these associations are cleaved, as free SO_2 is depleted by oxidation or by its natural slow reaction to different wine components (Bueno, Carrascon, & Ferreira, 2016). In any case, if wine at bottling contained SAs as hydroxyalkylsulfonates, levels of free SAs will inevitably increase with aging time, impacting wine sensory characteristics. Alternatively, SAs can be also formed from wine amino acids if wine is exposed to O_2 (Bueno et al., 2018), as may occur if the closure of the bottle fails during transport, with the same sensory consequences. However, a correct diagnose of the ultimate cause of the origin of SAs is essential in order to look for a satisfactory solution and not to blame unfairly the closure, or the wine. Because of that, the reliable analysis of the real content of SAs in wine, regardless of the forms in which they can be present, is a most necessary analytical tool for the wine industry.

Apart from their strong interactions with SO_2, aldehydes can also form reversible associations with amines (imines) (Baert, De Clippeleer, De Cooman, & Aerts, 2015) or alcohols (acetals) (Ferreira, Barbe, & Bertrand, 2002), and also with nucleophilic positions of flavonoids (Es-Safi, Cheynier, & Moutounet, 2002). All these associations, in general weaker than those formed with SO_2, increase the number of potential chemical forms under which Strecker aldehydes are found. This makes that the analytical responses obtained by different analytical strategies can be very different, depending on the number and type of chemical forms of the aldehydes stimulated by the specific analytical stimuli. Analytical responses can be also poorly repeatable and show a strong dependence with time.

Because of the relatively polar and reactive character of aldehydes, whose chromatographic peaks easily tail on many gas chromatography (GC) phases, and because of their too-fragmented and poorly selective EI mass spectra, most analytical methods use different chemical derivatization reactions. The most common derivatization reagents for aldehyde analysis are 2,4-dinitrophenylhydrazine, 2-aminoethanethiol, 2,4,6-trichlorophenylhydrazine, pentafluorophenylhydrazine and *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine (PFBHA) (Osorio & Cardeal, 2013). Nevertheless, the latter is with difference the most employed for wine analysis (Culleré, Cacho, & Ferreira, 2004; Mayr et al., 2015; Moreira et al., 2019; Zapata, Mateo-Vivaracho, Cacho, & Ferreira, 2010; Zhang, Kontoudakis, Blackman, et al., 2019). Such derivatizations are often carried out in solid phase extraction (SPE) cartridges (Ferreira, Culleré, Loscos, & Cacho, 2006) or solid phase microextraction (SPME) fibers (Schmarr et al., 2008).

As the relevance of the bound forms of SAs has only recently been known, most methods developed in the past do not consider the existence of different chemical forms of these compounds. As a consequence, the analytical signal measured refers to the free fraction of SAs plus an indeterminate part of the bound fraction. There are, however, some recent reports which take into consideration the existence of different chemical species. One of them targets exclusively free forms by a careful analysis of the undistorted headspace fraction (Bueno et al., 2014), and provides an estimate of total forms by using some surrogates previously equilibrated. Other authors have suggested the use of *p*-benzoquinone to remove SO_2 before the analysis (Zhang, Kontoudakis, Blackman, et al., 2019), so that analyzed forms include native free forms plus those released from SO_2 adducts. However, *p*-benzoquinone could trigger Strecker degradation of amino acids (Rizzi, 2006), which would bias results. Most recently, the direct UPLC-MS analysis of aldehydes forming α-hydroxyalkylsulfonates, after the addition of extra amounts of SO_2 to ensure all aldehydes are under these forms has been also proposed (Zhang, Kontoudakis, & Clark, 2019). This promising method provided relatively good analytical characteristics, although method quantification limits for methional and phenylacetaldehyde were above 1.5 μg/L (above their odor thresholds), recoveries for methional were just acceptable and 2-methylbutanal was not quantified. This made us to explore a different way.

The main goal of the present paper is to develop a reliable and robust analytical procedure, based on the well-known derivatization of aldehydes with PFBHA, able to quantify total forms of Strecker aldehydes and to provide an assessment of the levels of SAs present in unoxidized Spanish commercial wines.

2. Material and methods

2.1. Reagents, standards and samples

2.1.1. Chemical standards and reagents

Ethanol, methanol, dichloromethane and hexane (GC quality) were supplied by Merck (Darmstadt, Germany), acetaldehyde ≥ 99.5% was from Sigma-Aldrich (Madrid, Spain), tartaric acid 99%, sodimun hydrogencarbonate and sodium metabisulfite 97% were from Panreac (Barcelona, Spain). Sodium hydroxide 99% was from Scharlau (Barcelona, Spain). Water with resistance of 18.2 MΩ·cm at 25 °C was purified in a Mili-Q system from Milipore (Bedford, Germany). The chemical standards and internal standards (IS) used for the analytical quantification were supplied by Merck, with the exception of deuterated compounds which were purchased from Eptes (Vevey, Switzertland). Chemical standards: 2-methylpropanal (isobutyraldehyde) ≥ 99%, 2-methylbutanal ≥ 95%, 3-methylbutanal ≥ 95%, phenylacetaldehyde ≥ 95% and 3-(methylthio)propionaldehyde (methional) ≥ 98%. Internal standards: 2-methylpentanal ≥ 98%, 3-methylpentanal ≥ 97%, phenyl-d_5-acetaldehyde ≥ 95%, 3-(methy-d_3-lthio)propionaldehyde (methional-d_3) ≥ 90%. O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA ≥ 98%) used as derivatization reagent for aldehydes was also supplied by Merck.

2.1.2. Working solutions

Analytes solution: an ethanol solution containing isobutyraldehyde, 2-methylbutanal, 3-methylbutanal and phenylacetaldehyde at a concentration of 20 mg/L, and methional at 10 mg/L.

Internal standards solution: an ethanol solution containing 2-methylpentanal, 3-methylpentanal, methional-d_3 and phenyl-d_5-acetaldehyde at a concentration of 10 mg/L.

A 10 g/L solution of PFBHA in ultrapure water was prepared daily.

Synthetic wine: 12% (v/v) in ethanol, 5 g/L tartaric acid and pH adjusted to 3.5 with sodium hydroxide 1 M.

2.1.3. Wine samples

For the method development and validation, seven red wines (5 young and 2 aged), 3 white and 3 rosé commercial wines were used. All of them were dry table wines with alcoholic degrees between 12.0 and 14.5% and pHs between 2.95 and 3.60. The method was further applied to the analysis of total Strecker aldehydes in 66 Spanish red wines and 42 Spanish white wines from 14 and 16 different production zones, respectively. All wines were commercially available and were made from 10 red and 13 white grape varieties. Vintages ranged from 2005 to 2020, pH ranged from 3.0 to 4.0, total SO_2 between 8 and 130 mg/L (8–85 mg/L for reds and 100–130 mg/L for whites) and ethanol concentration ranged from 11.5 to 14.5% (v/v). All the measurements were conducted in duplicate.

2.1.4. Total sulfur dioxide determination

Total sulfur dioxide was determined by using the aspiration-oxidation method recommended by the OIV (International Organization of Vine and Wine) (OIV, 2009). Briefly, 10 mL of sample was acidified with 5 mL of 25% H_3PO_4 and heated to 100 °C. Then, the acidified sample were bubbled with air for 15 min (with a flow of 600 ± 12 mL/min). The SO_2 released was collected in pear shaped flask containing 3 mL of neutralized hydrogen peroxide (3%) with two drops of mixed indicator (methyl red—methylene blue) in which sulfur dioxide was completely oxidized to sulfuric acid, turning the color of the

solution from green to purple. The sulfuric acid formed was titrated with standardized 0.01 M NaOH.

2.1.5. Total acetaldehyde determination

Total acetaldehyde was determined following the method previously described elsewhere (Bueno et al., 2018). The method is based on breaking the adducts directly in the injector port. One microliter of wine sample spiked with 2-butanol (100 mg/L) as internal standard was analyzed by gas chromatography with flame ionization detection (GC-FID).

2.2. Method optimization

2.2.1. Gas Chromatography-Mass Spectrometry conditions

The instrument used was a GC-2010 gas chromatograph coupled to a QP 2010 single quadrupole mass spectrometer from Shimazdu (Kyoto, Japan). The standard split/splitless injector was operated in splitless mode. The injection was kept at a temperature of 250 °C, and a pulse of pressure of 300 kPa was applied during 1.50 min splitless time (the column flow during splitless injection was 7.02 mL/min). The carrier gas was He at a constant linear velocity of 40.5 cm/s (≈1.26 mL/min flow rate) during the run. The column was a DB-WAX ETR 30 m × 0.25 mm i.d. × 0.5 μm film thickness, preceded by a silica precolumn from Supelco (Bellefonte, PA, USA) 3 m × 0.25 mm i.d. The chromatographic oven was held at 40 °C for 4 min, then raised to 250 °C at 10 °C/min, remaining at that temperature for 10 min. The electron impact (EI) ion source worked at 220 °C, while the interface was kept at 230 °C. The mass analyzer was operated in single ion monitoring (SIM) mode with the selected ions for each analyte shown in Table 1. The quantification was carried out by using a response factor calculated by the GC–MS analysis of spiked wines containing known amounts of the analytes. For 3-methylbutanal, whose two isomeric oximes appear separated in the chromatogram, the summed area of both peaks was considered in their quantification (Zapata et al., 2010).

Table 1
Masses of the ions selected for the determination of the compounds considered in the study and final concentrations added in samples.

Compounds	Oxime LRI	*m/z*	Added (μg/L)	IS
Analytes				
isobutyraldehyde	1492	195, 239, 250*	200	2-methylpentanal
2-methylbutanal	1573	195, 239*, 253	200	
3-methylbutanal	1596–1615	195, 239*, 266	200	3-methylpentanal
methional	2150	181, 252, 299*	100	methional-d_3
phenylacetaldehyde	2329	91, 181, 297*	200	phenyl-d_5-acetaldehyde
Internal standards				
2-methylpentanal	1643	195, 253*, 266	50	
3-methylpentanal	1698–1715	195, 253*, 266	50	
methional-d_3	2147	181, 252, 302*	50	
phenyl-d_5-acetaldehyde	2327	96, 181, 301*	50	

LRI: linear retention index, **m/z* of the ion used for quantification; IS: internal standard.

2.2.2. Derivatization reaction conditions

Two synthetic wines spiked with $5{\cdot}10^{-6}$ M of each analyte were prepared, one of them contained 0.001 M SO_2 (0.1 g/L) prepared using sodium metabisulfite and incubated at least 12 h at room temperature (Bueno et al., 2014). Each synthetic wine was split in 4 aliquots of 50 mL, to be analyzed at 2, 5, 12 and 24 h after their reaction at 35 °C with a high ($1.2 \cdot 10^{-3}$ M) or low ($8.4{\cdot}10^{-4}$ M) concentration of PFBHA. The experiment was carried out in duplicate. The oximes were then extracted by liquid–liquid extraction with two consecutive fractions of 1 mL of hexane. One microliter of each extract was injected in the chromatographic system.

2.2.3. Solid phase extraction conditions

SPE cartridges were prepared in 1 mL internal volume propylene tubes filled by 30 mg of LiChrolut EN® (styrene/divinylbenzene copolymer) enclosed by frits; all materials were supplied by Merck. A breakthrough volume study of the oximes of the aldehydes in the SPE bed was built by percolating a 50 mL volume of a synthetic wine after oximation with PFBHA through the SPE bed. The eluate was divided in consecutive 5-mL fractions, which were extracted with 0.5 mL of hexane and analyzed in the GC–MS system. In a second experiment, 100 mL of synthetic wine were spiked with the analytes and derivatized. Then, volumes of 4, 6, 8, 10, 12, 16 and 20 mL of the derivatized wine were loaded into SPE cartridges, dried, and eluted with 2 mL of hexane. The hexane fractions were analyzed by GC–MS.

For sample clean up, 10 mL volumes of real wine spiked with the analytes at levels indicated in Table 1 were oximated using the optimal derivatization conditions. The oximated volumes were further loaded in the SPE beds. After this, the SPE beds were washed up with methanol/water solutions at 40% or 60% (v/v) containing 1% (w/w) of $NaHCO_3$. The percolates were collected in 2-mL fractions, which were diluted with water 1:4 or 1:6, respectively, and were extracted with 1 mL of hexane. The hexane extracts were analyzed in the GC–MS system. In a second set of experiments, twelve SPE cartridges were loaded with 10 mL of a wine spiked with the analytes previously derivatized. The cartridges were then washed up with different volumes of the washing up solutions (between 0 and 15 mL), further dried and eluted with 2 mL of hexane. The hexane solutions were analyzed by GC–MS.

All conditions previously optimized were employed to determinate the optimal elution volume using a young red wine spiked with the analytes. The wine was derivatized and 10 mL were further percolated through the cartridge, which was washed up with 10 mL of the cleaning solution. The cartridge was then dried and then the oximes were eluted with 4 consecutive fractions of 0.5, 0.5, 0.2 and 0.3 mL of hexane which were further analyzed by GC–MS.

2.2.4. Preliminary assessment of matrix effects

A synthetic wine containing four concentration levels (0, 20, 100 and 150 μg/L of isobutyraldehyde, 2-methylbutanal, 3-methylbutanal and phenylacetaldehyde; 0, 10, 50, 75 μg/L of methional) of the analytes, plus three different real wines spiked at those same four concentration levels, were analyzed by the proposed procedure (except equilibration time). The slopes of the corresponding calibration graphs were compared via t tests.

After this experiment, the derivatization conditions were re-examined in real wine. For this, one wine spiked with the analytes and the internal standards was left to equilibrate for 24 h in anoxia. The wine was then derivatized at 35 °C at two different pHs (3.0 and 3.5), two different levels of PFBHA (10 and 20 g/L) and two different times (12 and 24 h). Derivatized aliquots were then extracted, washed and eluted and analyzed by GC–MS, as in previous experiments.

2.2.5. Internal standards equilibrium time

Two different commercial red wines were spiked with the analytes and with the internal standards solution at the levels indicated in Table 1. The wines were stored in anoxia at room temperature and

triplicate aliquots were analyzed following the proposed procedure at different times between 2 and 72 h. A second set of the same samples were stored in anoxia at 50 °C. In this case, triplicate aliquots were analyzed during 27 h.

2.3. Proposed method

Wine bottles are opened within the anoxic chamber and a 12 mL volume is poured into a 20 mL screw capped vial with septum, spiked with 60 μL of the internal standards solution, closed and incubated in a laboratory oven for 5 h at 50 °C. After this, the vial is cooled down to room temperature and spiked with 360 μL of 10 g/L PFBHA solution. The mixture is incubated 12 additional hours at 35 °C. Then, 10 mL of the derivatized sample are loaded onto the SPE cartridge, previously conditioned with 1 mL of dichloromethane, 1 mL of methanol and 1 mL of a 12% ethanol (v/v) aqueous solution. Polar compounds and underivatized reagent are removed by cleanup with 10 mL of a 60% methanol in water solution (v/v) containing 1% (w/w) $NaHCO_3$. The cartridge is dried under vacuum and oximes from the analytes and internal standards are finally eluted with 1.2 mL of hexane. Derivatives were analyzed by injecting 3 μL of the extract in the GC–MS system.

Quantification was carried out using response factors (RF, equation (1)) calculated in the analysis of a real wine and its pair spiked with known amounts of analytes. The response factors were calculated by equation (1), where C_{add} is the concentration added of each analyte, RA_{Add} is the relative area of the analyte in the spiked sample, RA_0 is the relative area of analyte in the original wine.

$$RF(\mu g/L) = \frac{\Delta C}{\Delta Signal} = \frac{C_{add}}{RA_{Add} - RA_0} \quad (1)$$

2.4. Method validation

2.4.1. Linearity, detection limits, precision and accuracy

Linearity was studied by standard addition to a commercial real wine using 6 levels of concentrations and three replicates at each level.

Limits of detection and quantification were defined as the amount of analyte that gives peaks three or ten times higher, respectively, than the standard deviation of the baseline signal measured in the proximities of the peak in real samples.

Repeatability was studied by evaluating the signal obtained in 16 determinations of a real red wine spiked with analytes at the levels indicated in Table 1 in groups of 4 replicates and on 4 different days. Reproducibility was calculated as the square root of the addition of the square of repeatability plus the square of the inter day repeatability.

The existence of matrix effects was assessed by a recovery study carried out on eight commercial wines (2 whites, 2 rosés, 2 young reds and 2 aged reds) spiked or not with known concentrations of all analytes (see Table 1). Each wine was analyzed by the proposed method at least 5 times along 1 month. In addition, one white wine was analyzed again 3 months later, and one red young wine was analyzed at least one time every month for 3 months. Wines were always kept protected from oxygen. pH, total SO_2 and total acetaldehyde were determined in these wine samples.

2.4.1.1. Sulfur dioxide influence. The specific influence of SO_2 was investigated in an independent experiment. Four commercial wines (young red, aged red, white and rosé) spiked with analytes as defined in Table 1 were studied at four different SO_2 concentrations (wine native SO_2 level and addition of 30, 60 and 90 mg/L of SO_2, respectively). Samples were held for 2 months in an anoxic atmosphere at 25 °C to ensure the complete equilibration between analytes an SO_2. Then, the samples were analyzed by the proposed method. All the experiment was carried out in duplicate.

2.5. Data treatment

Experimental data are shown as mean value ± standard deviation. Results were analyzed by analysis of variance (ANOVA), whereas mean values were compared by Tukey's test (SPSS Statistics v.15 IBM, Armonk, NY, USA). The value of $p \leq 0.05$ was considered statistically significant, and alphabetical letters were used to indicate the existence of significant differences between means in the figures.

3. Results and discussion

The main goal of this paper is to develop an accurate procedure for measuring the total contents of Strecker aldehydes of wines, regardless of the chemical form in which they could be. This was achieved by a careful re-examination and re-optimization of the classical derivatization procedure using PFBHA, seeking a higher yield of the reaction and an improved isolation of the oximes, and by using adequate internal standards well equilibrated in the wine matrix.

3.1. Reaction conditions

In previous works, the derivatization reaction was developed in the cartridge in which the analytes had been previously retained. This improved reaction yields, precision and reaction time with respect to the classical derivatization reaction carried out directly in the liquid phase (Culleré et al., 2004; Ferreira, Culleré, López, & Cacho, 2004). However, as hydroxyalkylsulfonates are poorly retained in the SPE bed, the in-cartridge derivatization strategy is suitable for the analysis of free forms of the aldehydes, but not for the analysis of total forms. Therefore, a direct derivatization in the liquid phase seems more advantageous, as the removal of free forms of the aldehydes by derivatization with the reagent should facilitate the cleavage of bound forms by displacement of the chemical equilibria. As sulfite-related equilibria are affected by diverse parameters, different conditions of time, pH and concentration of reagent were tried, at a fixed temperature of 35 °C. A brief summary of results obtained at wine pH is given in Fig. 1.

The figure shows the sum of the peak areas of all analytes, normalized to the maxima value measured in the experiment, which was obtained in the sample derivatized with 0.30 g/L of reagent for 24 h. As can be seen, the effects of SO_2 are only evident at short reaction times. As levels of reaction after 12 h do not significantly differ than those measured at 24 h, 12 h at 35 °C with 0.3 g/L of PFBHA were chosen as optimal derivatization conditions.

Regarding the different aldehydes, when no SO_2 is present, derivatization rates do not show differences between them. However, in samples with SO_2, isobutyraldehyde and 2-methylbutanal react faster than methional and phenylacetaldehyde, which only reach maxima signals after 12 h (Supp. Mat. Fig. S1). These results are consistent with the highest aldehyde-SO_2 equilibrium constants reported for the last two compounds (Bueno et al., 2014).

Moreover, the comparison of the signals obtained in the derivatization of a synthetic wine containing a large fraction of carbonyls forming adducts with SO_2 at wine pH (pH 3.5), with a second one at pH 2 revealed that acid pHs did not improve the reaction (Complete results in Supp. mat. Fig. S2).

3.2. SPE conditions

Derivatized aldehydes are further extracted by SPE using a cartridge filled with 30 mg of LiChrolut EN®. Breakthrough curves were built by the analysis of the oximes contained in consecutive fractions of the eluate. No oximes were detected in the first 25 mL, so that in can be concluded that breakthrough volumes for these compounds are above this value and that the oximes of the aldehydes are strongly retained in the sorbent. Furthermore, in a second experiment, seven cartridges were loaded with increasing volumes of derivatized wine, and oximes were

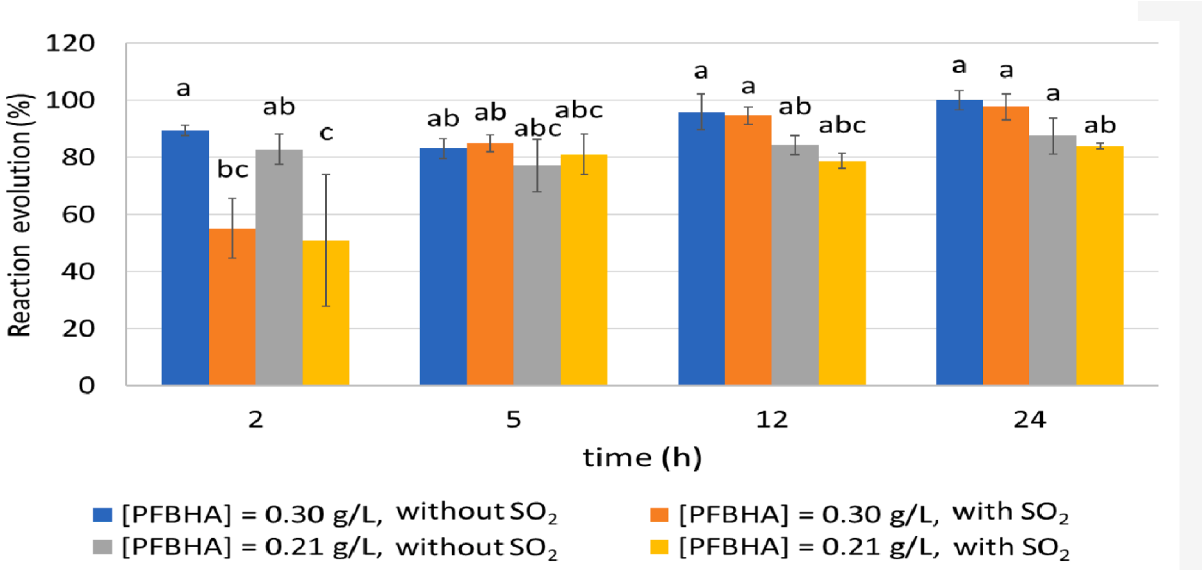

Fig. 1. Influence of the concentration of derivatizing reagent (PFBHA) and of reaction time on the average signals of Strecker aldehydes. Data are average signals for the five Strecker aldehydes normalized to the maxima level observed in the experiment. Different letters show significant differences ($p < 0.05$) among treatments (combination of the derivatization conditions and incubation time).

eluted and analyzed. The signals of the oximes increased linearly with the volume at last up the first 12 mL, demonstrating that the SPE bed was not saturated (Supp. Mat. Fig. S3). A 10 mL volume was consequently used as sample loading volume.

For removing polar volatiles of wine co-extracted with the oximes, a washing up with different volumes of aqueous-methanol solutions (40% and 60% in methanol) containing 1% of $NaHCO_3$ were studied. Results confirmed that oximes are so strongly retained in the sorbent that up to 12 mL of the cleaning solution containing 60% of methanol can be applied without any evident loss of analytes. The specific effect of the washing up is more clearly shown in Fig. 2.

The figure shows the measured areas of the oximes of the two most polar analytes (isobutyraldehyde and methional), normalized by the areas measured in the samples without any washing up, as a function of the volume of washing solution applied (complete results in Supp. Mat. Fig. S4). The figure clearly shows an improvement in the areas, particularly noticeable for isobutyraldehyde, with increasing volumes of the washing up solution. The improvement is due to the removal of interfering compounds affecting in different potential ways to the signals, particularly during sample vaporization and transference to the column and during ionization in the mass spectrometer. In the case of isobutyraldehyde, the improvement reaches a factor two, and is achieved with washing up volumes higher than 3 mL, and then remains stable. In the case of methional there is a continuous improvement, most notable with the more energic washing up (60% methanol), which stabilizes with washing up volumes of around 10 mL. The superimposed chromatograms make it possible to evaluate the amounts of interfering compounds removed by the washing up (Supp. Mat. Fig. S5). Nearly all polar compounds co-extracted with the oximes were removed, including higher alcohols, fatty acids and volatile phenols and also the oximes of acetaldehyde and unreacted PFBHA.

For elution, best results were obtained with hexane. Hexane is a weak solvent, so that hexane extracts are cleaner than those obtained with other more polar solvents such as dichloromethane (data not

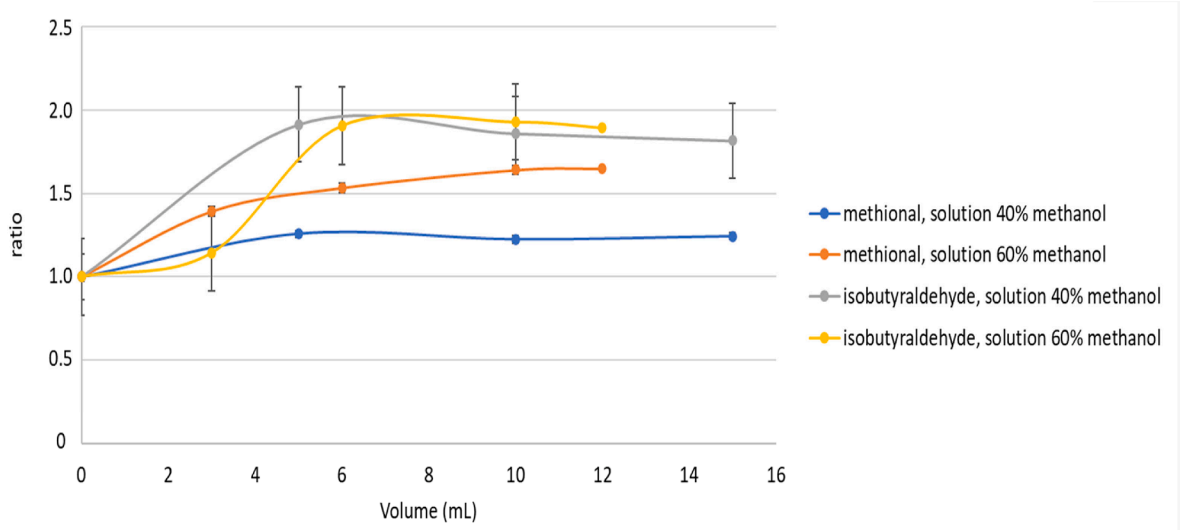

Fig. 2. Effects of different volumes of washing up solutions on the signals of isobutyraldehyde and methional finally eluted out of the cartridge. The superimposed chromatograms show the effects of the washing up on the chromatographic profile.

shown). Hexane has the additional advantage of not being chlorinated, so that it may be used even with electron capture detectors, and provides a reduced background in electron impact MS. Only using 0.5 mL of hexane, more than 90% of isobutyraldehyde, 2-methylbutanal and 3-methylbutanal were eluted, whereas it is needed 1 mL to achieve the same value for methional and phenylacetaldehyde. The oximes of the latter compounds were the last to elute, with a presence of 5–6% in the third fraction. Therefore, for quantitative elution, 1.2 mL of hexane had to be applied, (Supp Mat Fig. S6). The final chromatogram obtained with the optimized method can be seen in Fig. 3.

3.3. Evaluation and correction of matrix effects

Once the method was optimized, its ability to provide accurate signals of the analytes was initially checked. For this, several calibration plots were built and compared; one using synthetic wines and three more, obtained by standard addition experiments with three different real wines. Results of this experiment were highly frustrating, since the signals provided by the optimized method resulted to be strongly matrix dependent as can be seen in Supp. Mat. Table S1. Note that results were unsatisfactory even for those analytes for which an isotopomer was available as internal standard.

Attending to the quality of SPE step and to the cleanliness of the extracts obtained, it seems reasonable to assume that differences between matrixes were not introduced in the SPE or in the GC–MS analysis, but in the derivatization step. Therefore, results strongly suggest that the derivatization conditions optimized at the beginning of the work, were not good enough to derivatize completely all the aldehydes present in the samples. Different yields should be likely attributed to the different binding levels of the Strecker aldehydes in the different wines. Some fruitless efforts were further devoted to improve reaction yields with higher levels of derivatizing reagent, however, results were not satisfactory, since higher levels of PFBHA did improve the signals but did not make the relative signals to become more similar to those measured in the synthetic wine (Supp. Mat. Fig. S7).

Once it became clear that the reaction could not be easily improved, then the efforts concentrated on correcting matrix effects by ensuring that internal standards were well equilibrated within the wine matrix. Certainly, the lack of ability of the isotopomers to correct for a matrix effect, suggests that isotopomers and analytes are not equally distributed in the wine matrix between free and bound forms. It can be thought that native analytes are forming already strong associations with SO_2 and other wine components, and that the time given to equilibrate the internal standards is not enough to achieve a similar binding level.

This equilibration-time issue was examined by measuring the evolution of response factors analytes/internal standard as a function of the equilibration time for two different wines. Response factors were defined as the ratios between the signals per unit of concentration of spiked analyte to that of the corresponding internal standard. Equilibration time is the time elapsed at room temperature and complete anoxia between the addition of the internal standards and the derivatization reaction. Results for methional and 3-methylbutanal are given in Fig. 4a and 4b (detailed results in Supp. Mat. Table S2), respectively. In the case of methional a deuterated analogue was used as IS, while in the case of 3-methylbutanal, the IS was the homologous compound 3-methylpentanal.

Results demonstrate that equilibration time plays a key role both on the evolution with time of response factors and on their stability and reproducibility. In the case of methional, even if an isotopomer is used as internal standard, the evolution followed by response factors is clearly wine dependent, as seen in the figure. In addition, repeatability was very poor for equilibration times below 48 h, and response factors became stable and repeatable only after 48 h of equilibration. In the case of 3-methylbutanal the complete stabilization of response factors was achieved only after 72 h of equilibration.

In order to speed-up the process, the experiment was repeated at 50 °C. Results were compared with those obtained after 72 h of equilibration at room temperature and are summarized in Fig. 4c. As can be seen, 5 h of equilibration at 50 °C were enough to obtain response factors statistically equivalent to those observed after 72 h, so that 5 h of anoxic incubation at 50 °C was incorporated into the method procedure. The figure also shows that response factors of 3-methylbutanal and phenylacetaldehyde begin to change after incubation times higher than 8 h. The most serious divergence is observed for phenylacetaldehyde, whose response factor increases after this time. Since the incubation is carried out in complete anoxic conditions, such increase should be attributed to the formation of more phenylacetaldehyde by the Strecker degradation of wine phenylalanine induced by dicarbonyls already present in the wine, such as diacetyl, glyoxal or methylglyoxal. In the case of 3-methylbutanal, the decrease would be related to its potential degradation by a relatively selective reaction to other wine components, not affecting isobutyraldehyde, 2-methylbutanal or 3-methylpentanal.

3.4. Method validation

Method quality parameters were calculated after the optimum conditions were determined. Figures of merit for the method linearity, sensibility and precision are shown in Table 2. Linearity was obtained by the analysis of a commercial wine naturally containing low amounts of the analytes. For all analytes, linearity was satisfactory with determination coefficients better than 0.997. As can be seen, the linearity range covers the normal range of occurrence of these compounds in wine (Bueno et al., 2016; San Juan, Cacho, Ferreira, & Escudero, 2012). Method sensitivity was evaluated in terms of limits of detection (LD) and quantification (LQ). In all cases LQs were below 1 μg/L. Table 3 compares the figures of merit of this method with other methods for total SAs quantification that take into consideration the existence of different forms of these compounds (Bueno et al., 2014; Zhang, Kontoudakis, Blackman, et al., 2019; Zhang, Kontoudakis, & Clark, 2019). As can be seen in this table, the current method is the only one that reaches LQs for

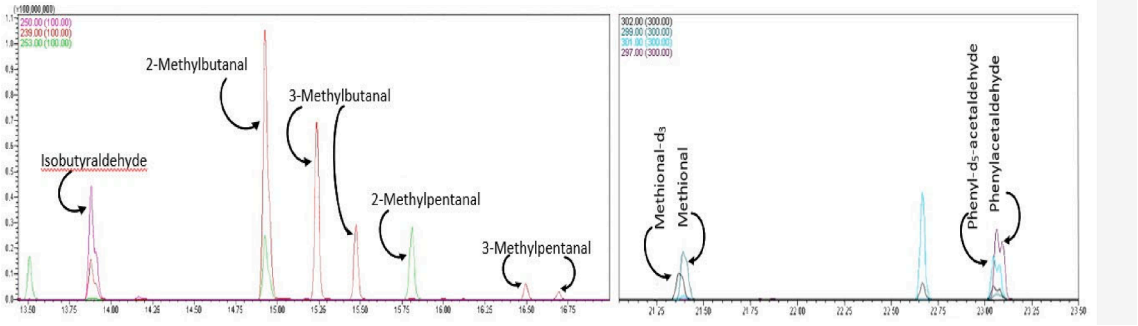

Fig. 3. MS ion chromatogram of target compounds obtained with the proposed procedure.

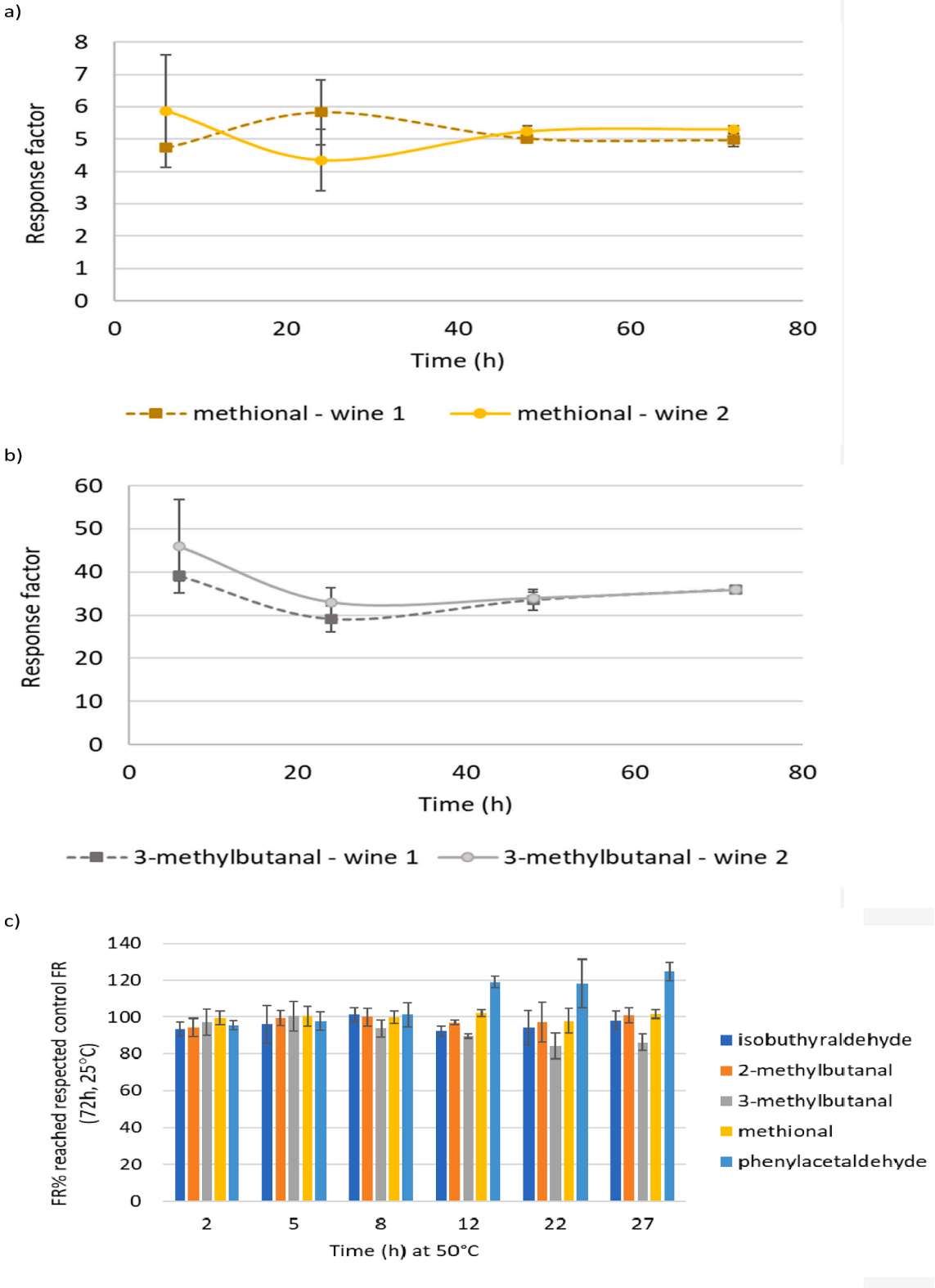

Fig. 4. Effects of the equilibration time in anoxia on the measured response factors: a) methional at 25 °C, b) 3-methylbutanal at 25 °C, c) general overview at 50 °C.

the five Strecker aldehydes below to their odor thresholds (Culleré et al., 2007; Escudero et al., 2000). This fact is relevant to be able to evaluate the importance of these aldehydes in the aroma of the wine. This great sensitivity also influences the lowest point of the linearity range.

Method precision was estimated by measuring the method repeatability and reproducibility (EURACHEM/CITAC, 2012). Repeatability was calculated as the within-batch variability, and reproducibility adds inter-day variability, as described in reference (Bueno et al., 2014). For this reason, when inter-day variability was not significant, both figures are the same. The worst results were obtained for

Table 2
Method quality parameters.

	Linearity[a]			LD[b] (μg/L)	LQ[c] (μg/L)	Repeatability RSD (%)[d]	Reproducibility RSD (%)[e]
	Slope	r^2	Range (μg/L)				
Isobutyraldehyde	9.49×10^{-3}	0.9971	0.2–279	4.15×10^{-3}	1.38×10^{-2}	12.8	12.8
2-Methylbutanal	1.92×10^{-2}	0.9992	0.2–214	2.73×10^{-2}	9.10×10^{-2}	8.78	8.84
3-Methylbutanal	5.36×10^{-2}	0.9987	0.2–221	2.57×10^{-2}	8.57×10^{-2}	6.33	6.33
Methional	3.77×10^{-3}	0.9981	1–119	5.16×10^{-2}	1.72×10^{-1}	1.83	5.30
Phenylacetaldehyde	7.56×10^{-3}	0.9988	1–232	6.39×10^{-2}	2.13×10^{-1}	4.35	6.58

r^2: determination coefficients.
[a] Six levels of analyte concentrations and three replicates in each level.
[b] Limit of detection calculated as the concentration giving a peak height three times the signal-to-noise ratio.
[c] Limit of quantification calculated as the concentration giving a peak height ten times the signal-to-noise ratio.
[d] Signal evaluation of 16 determinations in groups of 4 on 4 different days of a commercial young red wine.
[e] Squared root of the addition of the square of repeatability (as RSD) plus the square of interday repeatability (as RSD).

Table 3
Method quality parameters comparison.

Reference	Compound	LD (LD, μg/L)	LQ (LQ, μg/L)	Recovery %	Linearity range (μg/L)	Repeatability RSD (%)	Reproducibility RSD (%)
Bueno et al., 2014	isobutyraldehyde	1.10	3.67	89 ± 4	1.10–223	2.8	3.8
	2-methylbutanal	0.880	2.93	93 ± 2	1.01–261	2.5	3.4
	3-methylbutanal	0.520	1.73	92 ± 4	0.940–242	3.3	3.3
	methional	0.880	2.93	108 ± 10	0.880–93.0	10.7	10.7
	phenylacetaldehyde	0.670	2.23	85 ± 6	0.990–257	3.6	3.8
Zhang, Kontoudakis, Blackman, et al., 2019	isobutyraldehyde	0.262/0.262/ 0.262*	0.863/0863/ 0.863*	99/86#	1.05–20.93 and 20.93–209.3	4.5/4.0#	3.6/3.9#
	2-methylbutanal	–	–	–	–	–	–
	3-methylbutanal	0.685/2.73/ 4.11*	2.26/9.04/ 13.6*	103/ 102#	2.74–54.77 and 54.77–547.70	3.7/12.3#	2.8/3.2#
	methional	0.652/1.30/ 0.652*	2.15/4.30/ 2.15*	101/97#	2.61–52.13 and 52.13–521.3	6.8/10.9#	7.1/3.3#
	phenylacetaldehyde	0.085/2.56/ 1.28*	0.282/8.45/ 4.22*	97/99#	5.12–102.4 and 102.4–1024	6.5/8.5#	3.9/7.4#
Zhang, Kontoudakis, & Clark, 2019	isobutyraldehyde	0.10	0.34	111 ± 14	1.0–206.5	1.1/3.7#	1.9/1.1#
	2-methylbutanal	–	–	–	–	–	–
	3-methylbutanal	2.52	8.39	97 ± 9	2.5–251.7	2.1/2.3#	2.9/2.1#
	methional	0.49	1.62	113 ± 16	2.4–116.4	1.9/2.6#	1.2/1.9#
	phenylacetaldehyde	0.46	1.55	111 ± 2	6.4–222.9	2.3/2.6#	0.4/2.3#
This method	isobutyraldehyde	0.004	0.014	106 ± 10	0.2–279	12.8	12.8
	2-methylbutanal	0.027	0.091	105 ± 8	0.2–214	8.78	8.84
	3-methylbutanal	0.026	0.086	101 ± 14	0.2–221	6.33	6.33
	methional	0.052	0.172	99 ± 2	1–119	1.83	5.30
	phenylacetaldehyde	0.064	0.213	102 ± 10	1–232	4.35	6.58

*model wine/white wine/red wine; #white wine/red wine; Odor thresholds: isobutyraldehyde 6 μg/L, 2-methylbutanal 16 μg/L, 3-methylbutanal 4.6 μg/L, phenylacetaldehyde 1 μg/L (Culleré et al., 2007) and methional 0.5 μg/L (Escudero et al., 2000).

isobutyraldehyde, whose reproducibility is close to 13%, followed by 2-methylbutanal.

Finally, for the estimation of method accuracy, a recovery study with 8 different wines was carried out. The experiment included five complete and independent analytical determinations of each wine and of its corresponding spiked sample along 1 month. Samples were very different, covering different wine types (young red, aged red, rosé and white), pH ranges (3.0–3.6), and total acetaldehyde and SO_2 contents (8–44 mg/L and 21–129 mg/L, respectively). The average recoveries obtained in the study are reported in Table 4. As can be seen, recoveries were in all cases in the 80–120% range, except for 3-methylbutanal in wine ARW2. Furthermore, recoveries were in most cases in the range 85–115, with just 3-methylbutanal in three samples and isobutyraldehyde and phenylacetaldehyde in one sample each, out of this range. The slightly worse accuracy obtained for 3-methylbutanal was already evident in the specific pattern followed with equilibration time by the response factor of this compound, as it was seen in Fig. 4c. Average recoveries were not significantly different from 100%, as the *t* test shown in the table demonstrates. The standard deviation of the recoveries given in the table provides an estimation of the uncertainty

Table 4
Recoveries and average recoveries with their standard deviation obtained using the average response factor and statistical test for checking matrix effects.

	YRW1	YRW2	ARW 1	ARW 2	White 1	White 2	Rosé 1	Rosé 2	%R mean	s	t^a_{100}	*p*
Isobutyraldehyde	93	108	110	112	109	88	114	118	106	10	0,22	0.83
2-Methylbutanal	94	107	106	111	108	91	108	113	105	8	0,22	0.83
3-Methylbutanal	108	100	112	122	100	84	81	103	101	14	0,04	0.97
Methional	102	98	101	101	97	95	102	100	99	2	0,08	0.94
Phenylacetaldehyde	97	90	101	95	96	118	114	104	102	10	0,07	0.95

YRW: Young red wine; ARW: aged red wine; R% mean; average recovery; [a] t experimental value (95% significance) for the comparison of the average percentage for recovery versus 100%.

associated to the variability of the matrix. Worst results, as expected, are found for 3-methylbutanal, with s = 14, 14% in relative terms. In the rest of the cases, this figure ranges between 2 and 10%.

Although previous validation results were satisfactory, a specific study was carried out in order to demonstrate whether the proposed strategy can satisfactorily solve the specific matrix effects introduced by the SO_2 level of the wine. In this experiment, 4 wines were spiked with three different levels of SO_2, one fixed level of analytes, and were kept 2 months in complete anoxia in order to allow enough time for SO_2 binding. After this time, the wines were analyzed following the proposed procedure. Results of the experiment are summarized in Fig. 5, which shows the average concentrations of spiked analytes recovered in the four wines as a function of the levels of SO_2 added.

As can be seen, results in white and rosé wines (Fig. 5 a-b) were highly satisfactory, with no evidence of SO_2 influencing quantitative results obtained by the method. Results were also satisfactory for red wines, although in this case SO_2 levels exerted a slight but significant effect on the determined levels of aldehydes. In the aged red wine (Fig. 5c), determined levels of methional slightly increased with SO_2 level, contrary as expected, and levels of phenylacetaldehyde at low levels of SO_2 of addition were slightly but significantly higher. However, differences measured were of very low magnitude (<2%). In the case of the young wine (Fig. 5d), the effects of SO_2 were noticeable for isobutyraldehyde and 3-methylbutanal. In both cases, increased levels of SO_2 induced slight, but significant, decreases in the determined levels of analytes. However, the magnitude of the decreases was within the ranges of the repeatability (case of isobutyraldehyde) and matrix-effects (case of 3-methylbutanal), so that it can be concluded that the effects induced by SO_2 in this type of wines are not of relevance. It is apparent that a better internal standard for these two compounds would be convenient.

3.5. A study of total SAs in Spanish commercial wines

The method was finally applied to the analysis of SAs in 108 Spanish commercial wines (66 red and 42 white wines) produced from 23 varieties (10 for reds and 13 for whites) and several vintages. Results of the analysis are summarized in Fig. 6 (complete description in Supp. Mat. Table S3). Results reveal that all wines contained detectable amounts of total Strecker aldehydes. Furthermore, leaving aside 2-methylbutanal, levels of total SAs were most often above the corresponding odor thresholds of the free aldehydes. Phenylacetaldehyde is above threshold in all wines, methional in all but 4 whites and 1 red, 3-methylbutanal in all but 4 reds and 1 white, and isobutyraldehyde in all wines but in two whites. Nevertheless, the specific perceptual characteristics of these compounds makes that wines can naturally contain supra umbral levels of the free forms without displaying negative aroma characteristics. These become manifest at certain levels depending on the wine characteristics and, most likely, also on the particular relative profile of SAs. In the case of red wines, a recent sensory study demonstrated that aroma mixtures containing more than 14 μg/L of isobutyraldehyde, 12 μg/L of 2-methylbutanal, 8.5 μg/L of 3-methylbutanal, 4.0 μg/L of methional and 14 μg/L of phenylacetaldehyde were able to induce a clearly noticeable deterioration of red wine aroma models, regardless of the presence or absence of oaky aromas (Marrufo-Curtido et al., 2021). Strictly speaking, only 21% of the red wines analyzed have levels of the five SAs above those, because the levels of 2-methylbutanal contained in the mixture were too high. However, giving the aroma similarity of

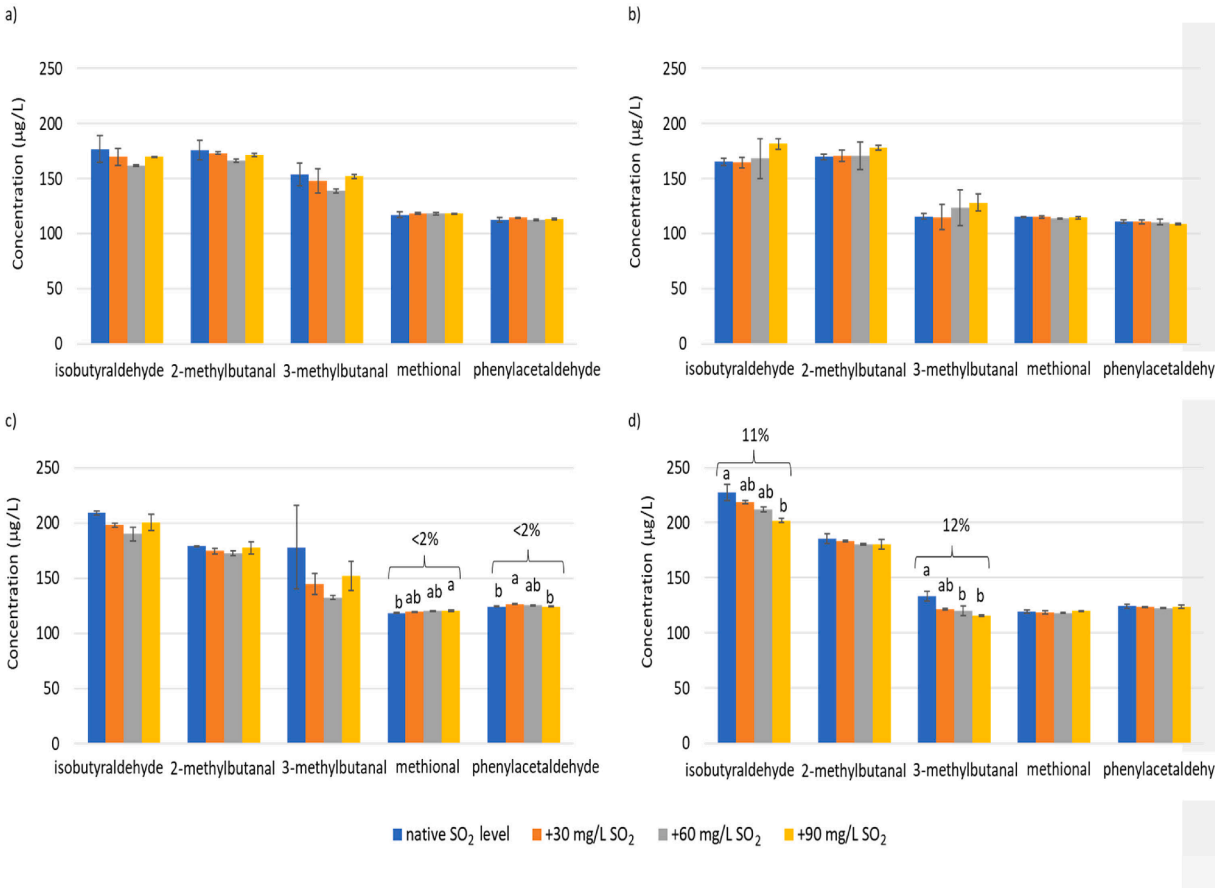

Fig. 5. Effects of SO_2 level on the determined concentrations of aldehyde using the optimized procedure. Four different wines: (a) rosé, b) white, c) aged red and d) young red) were spiked with analytes and with four different levels of SO_2 and were stored in anoxia for 2 months. After this period, they were analyzed. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

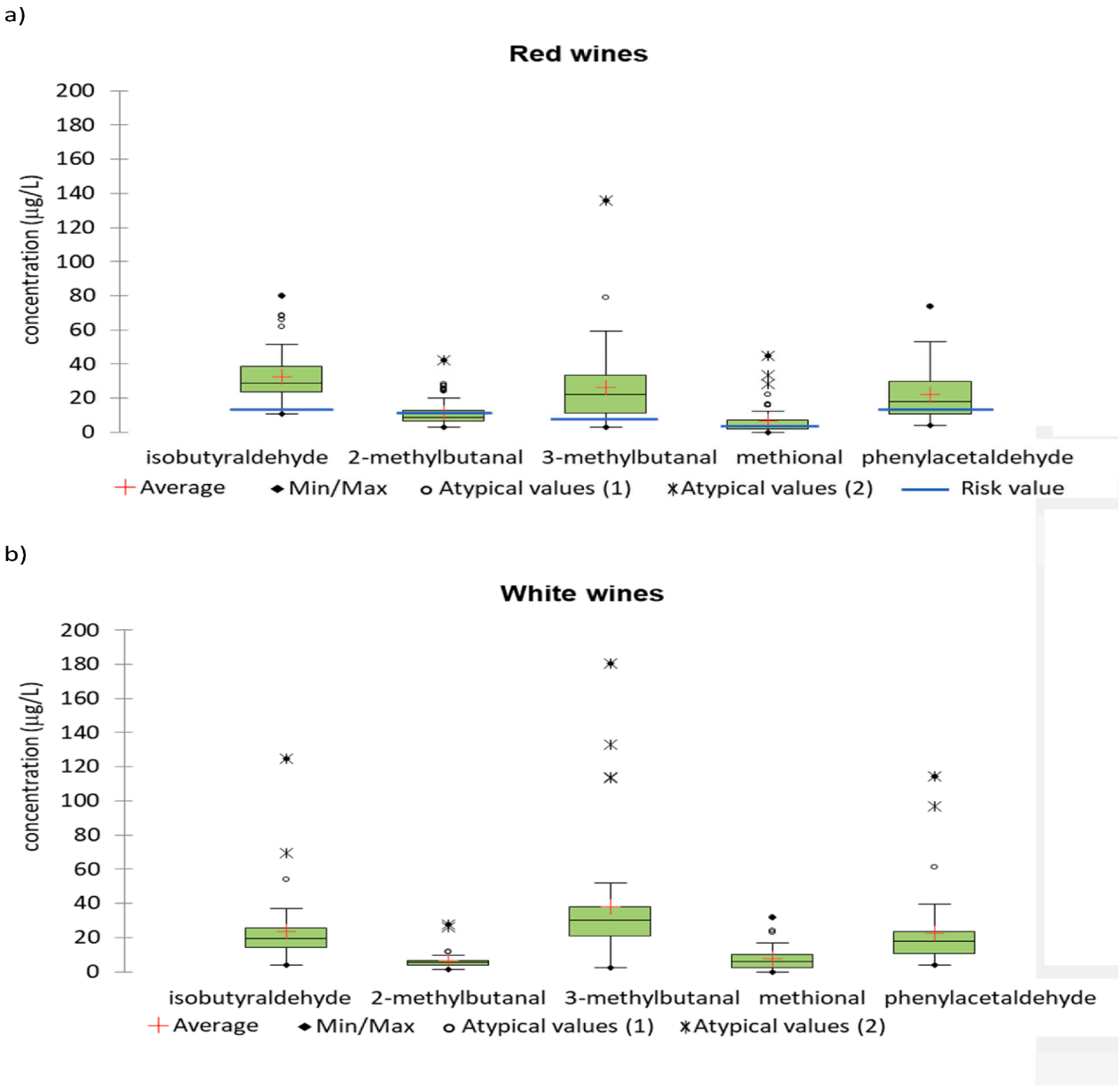

Fig. 6. Boxplots showing the range of levels of total SAs present in Spanish non-oxidized wines. a) Red wines; b) white wines. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

malty/yeasty between isovaleraldehyde, 2-methylbutanal and 3-methylbutanal (Moore, Forrester, & Pelosi, 1976), these aroma compounds can be gathered into the same wine aroma vector (Ferreira, De-la-Fuente-Blanco, & Sáenz-Navajas, 2021). It can be suggested that a very conservative estimate is that at least 48% of red wines contain levels of total SAs potentially risky. This percentage corresponds to the fraction of red wines with their three aroma vectors (isoaldehydes, methional, phenylacetaldehyde) above their risk levels. In addition, as some wines have very high levels of methional and phenylacetaldehyde, the fraction of red wines containing risky levels of total SAs is surely above 65%. There are no equivalent sensory studies in white wines, but results suggest that the fraction of wines containing worrying levels of total SAs could be equivalent to that of red wines. Another aspect that should be also mentioned, is that levels of total SAs in some unoxidized wines are in the range or well above of free levels of SAs measured in oxidized wines (Culleré et al., 2007). This suggests that oxidative aroma can readily be developed without the necessary concourse of the Strecker degradation of amino acids during wine oxidation. This means that the natural decay of free SO_2 with time, due to its different spontaneous reactions to wine constituents in anoxic conditions (Ontañón et al., 2020), can cause the apparition of oxidized notes by the simple cleavage of sulfite-bound forms of SAs.

Finally, results reported in Fig. 6 are of the same order of magnitude as those previously reported in literature (Bueno et al., 2016; Bueno et al., 2018; Bueno et al., 2014; Mayr et al., 2015; San Juan et al., 2012; Zhang, Kontoudakis, Blackman, et al., 2019; Zhang, Kontoudakis, & Clark, 2019), although some remarks can be made. On the one hand, mean values reported for 3-methylbutanal, methional and phenylacetaldehyde in Fig. 6 are lower than those reported in reference (Bueno et al., 2016). This was expected, since the method employed in this study (Bueno et al., 2014) used surrogate standards to estimate total levels but were left to equilibrate just for 14 h. As seen in Fig. 4, real equilibration times are higher, which indicate that such method should provide overestimated values. On the other hand, results for 3-methylbutanal in Fig. 6 are also much smaller than those reported by Zhang et al. using the UPLC-MS analysis of the sulfonates of SAs (Zhang, Kontoudakis, & Clark,

2019). This discrepancy should be attributed to the fact that reported values by Zhang are out of the reported linear range, and also to the fact that the IS for such compound was deuterated benzaldehyde. The interactions of benzaldehyde with SO_2 have been reported to be 30 times smaller than those of 3-methylbutanal (Bueno et al., 2014). Therefore, if a similar chemical behavior is assumed for deuterated benzaldehyde, its interactions with SO_2 should also be much weaker than those of the compound it supposedly controls.

4. Conclusions

The proposed procedure, using a variation of the classical PFBHA derivatization method, provides an accurate, selective, sensitive and reliable determination of the total levels of Strecker aldehydes contained in wine. The keys to avoid the strong matrix effects exerted by SO_2 and other wine components on the yield of the derivatization reaction is the use of adequate internal standards and large-enough equilibration times under strict anoxia. The application of the method to the analysis of total SAs in 108 Spanish wines, has revealed that between 48% and 65% of unoxidized red wines and likely a similar fraction of white wines, contain levels of SAs high enough to cause oxidative aromas if bound forms of SAs cleave. This will occur with the natural decay of free SO_2 by spontaneous reaction to wine components, without the necessary concourse of external O_2.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All relevant data are published as supplementary material.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2022.112125.

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