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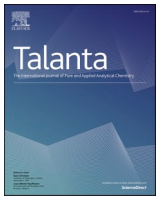

High-sensitivity quantification of trace carbonyl compounds in wine by a derivatization-free SPE-SBSE-TD-GC-GC-MS approach

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ABSTRACT

Trace carbonyl compounds play a critical role in wine aroma due to their extremely low sensory thresholds. The analytical challenge arises from the structural diversity of these compounds, which span a broad range of volatility and polarity, as well as from their occurrence at trace levels in a complex matrix. Current approaches frequently rely on derivatization to achieve adequate sensitivity and stability. In this work, a high-sensitivity, derivatization-free analytical strategy is proposed for the quantification of ketones, alkanals, alkenals, and alkadienals in wine, based on a dual preconcentration approach combining solid phase extraction (SPE) and stir bar sorptive extraction (SBSE), followed by thermal desorption and heart-cutting comprehensive gas chromatography coupled to mass spectrometry (TD-GC-(FID)-GC-MS).

Key parameters affecting extraction efficiency and chromatographic performance were systematically optimized, including SPE breakthrough volume, elution conditions, SBSE dilution factor, ionic strength, analyte load, and extraction time. The implementation of heart-cutting GC provided enhanced chromatographic resolution and selectivity for these carbonyls, while reducing interferences and instrumental noise, thereby improving overall sensitivity. The validated method demonstrated good linearity, repeatability (<10%), and reproducibility (<13%), with detection limits below reported sensory thresholds.

The method was successfully applied to 63 young red wine samples, enabling the quantification of trace carbonyls across a wide concentration range. By eliminating derivatization while achieving high sensitivity and robust performance in a complex matrix, the proposed methodology represents a significant analytical advancement for the determination of trace carbonyl compounds in wine.

1. Introduction

Wine possesses a highly complex aromatic profile shaped by a wide range of volatile compounds belonging to different chemical families [1,2]. Among these, trace carbonyl compounds, including aldehydes and ketones, play a crucial role in sensory perception due to their very low olfactory thresholds (≤μg/L) and their strong association with oxidative processes occurring during wine aging [3]. Furthermore, these compounds may impart undesirable aromas to wine, commonly described as "fatty' or "rancid,' which mask its positive organoleptic properties [4]. Alkenals mainly arise from the oxidation of unsaturated fatty acids [4], as well as ketones, alkanals, and alkadienals. Therefore, their accurate quantification is essential for understanding oxidation-related aroma evolution and for reliable aroma prediction.

In addition to oxidation-derived sensory deviations, green or vegetal aroma attributes constitute another relevant aspect of wine aroma quality. Six-carbon aldehydes, together with their corresponding alcohols and methoxypyrazines, are known contributors to vegetal, herbaceous, green, and unripe aromas in grapes, musts, and wines. Methoxypyrazines are particularly characteristic of certain grape varieties, such as Cabernet Sauvignon, Sauvignon Blanc, Merlot and Cabernet Franc [5–10]. In non-typically pyrazinic varieties, however, the presence of green-note aldehydes may lead to the expression of vegetal descriptors, which are often perceived as an aromatic defect. Moreover, some green odors traditionally attributed to six-carbon aldehydes may in fact originate from nine-carbon aldehydes. Compounds such as (*E*)-2-nonenal [11] and (*E,Z*)-2,6-nonadienal [12] are particularly noteworthy in this context. Furthermore, other carbonyl compounds have been specifically classified as potential contributors to green aromas in wine. As demonstrated by Arias-Pérez et al. [13], the

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"green vegetable" note in red wine can be induced by the concerted action of a complex pool of chemicals, including saturated and unsaturated aldehydes in the presence of acetaldehyde. However, since these compounds are often present at ubiquitous but very low concentrations, their individual monitoring remains a challenge. In this context, a high-sensitivity method for the quantification of trace alkenals and alkadienals would represent the missing piece to accurately assess their specific contribution to the green aromatic profile of wine.

The analysis of trace carbonyl compounds in wine poses considerable analytical challenges due to their diverse physicochemical properties and often poor chromatographic behavior, which can lead to peak tailing and low-specificity mass spectra. To overcome these limitations, most analytical strategies applied to wine analysis rely on derivatization through reaction with O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA). This approach has been integrated into the extraction step, enabling simultaneous derivatization and preconcentration. In solid-phase extraction (SPE), the derivatization reaction may occur directly on the sorbent prior to elution, allowing selective retention of the formed oximes [14,15]. Similarly, in solid-phase microextraction (SPME), the fiber can be pre-exposed to PFBHA vapors and subsequently placed in contact with the sample, where derivatization and extraction take place concurrently [15–18]. Despite their advantages, derivatization-based methods also present several drawbacks. Reagent-derived blanks may negatively affect sensitivity, particularly at trace concentration levels [15]. Furthermore, the formation of *(E)/(Z)* oxime isomer mixtures can complicate chromatographic separation and compromise selectivity, especially in complex matrices such as wine.

A promising preconcentration alternative is stir bar sorptive extraction (SBSE), which offers higher extraction efficiency compared to SPME due to its larger extractive phase volume [19]. Although various coatings are available for these stir bars, the most commonly used is polydimethylsiloxane (PDMS), making it a suitable technique for extracting low-polarity compounds [20]. This technique, commercially known as Twister, has demonstrated low detection limits and good reproducibility in aroma analysis across various food matrices [21–23], including wine [24,25].

Some studies have adopted a targeted strategy focused on a single compound in order to maximize analytical quality. For instance, Allamy et al. [26] successfully identified and quantified *(Z)*-1,5-octadien-3-one using SPME-GC-MS, achieving a limit of detection (LD) of 0.15 ng/L. Culleré et al. [27] developed a method for the determination of 1-octen-3-one based on PFBHA derivatization combined with SPE preconcentration and GC-MS/MS detection. The method provided a LD of 0.75 ng/L. In another example, Pons et al. [28] determined 3-methyl-2,4-nonadione using liquid-liquid extraction followed by GC-MS-CI, reaching a lowest quantifiable level of 2.9 ng/L. Overall, these methodologies demonstrate exceptional sensitivity toward their specific target analytes, allowing quantification at concentrations below their sensory thresholds in synthetic wine (1.2, 15 and 16 ng/L respectively). However, they are limited to a single compound and do not provide comprehensive coverage of the broader trace carbonyl profile present in wine.

Beyond single-compounds approaches, few derivatization-free multianalyte methodologies have been reported [29,30]. These two strategies (the former based on GC-QqQ-MS, the latter on LC-QqQ-MS), achieved very low LDs for several oxidation-related aldehydes in wine, well below the olfactory thresholds reported by Culleré et al. [4]. Although they expand analyte coverage compared to strictly single-target methods, their applicability remains confined to oxidation-related alkanals and alkenals, excluding other relevant carbonyl families such as ketones and alkadienals. Consequently, despite their high sensitivity, their analytical coverage remains relatively narrow.

Another major analytical challenge arises from the complexity of the wine matrix that may cause co-elution and masking effects, thereby hindering accurate quantification of target analytes. To overcome these limitations, bidimensional chromatographic approaches (GC-GC) have gaining increasing attention in the analysis of trace compounds in wine [31–33]. GC-GC has been primarily applied to resolve co-elutions of specific compounds and has also been employed for large-volume injections [34]. Separation in a two-dimensional chromatographic system is accomplished by using two orthogonal columns, each with a different stationary phase, typically housed in separate ovens and connected in series. This configuration enables compounds that are insufficiently resolved in the first dimension to undergo additional separation in the second dimension while preserving their first-dimension retention characteristics. As a result, the technique provides a level of separation and sensitivity that cannot be attained with conventional one-dimensional gas chromatography [35].

In this context, the present study proposes the development of a unified analytical method for the simultaneous quantification of fifteen carbonyl compounds associated with fatty and green aromas in wine, encompassing alkenals, alkanals, ketones, and alkadienals. The method was designed, optimized, and validated to provide comprehensive coverage of these chemically diverse analytes within a single analytical procedure. To enhance analytical performance, the proposed approach combines SPE and SBSE as consecutive preconcentration steps, followed by thermal desorption and comprehensive two-dimensional gas chromatography coupled to mass spectrometry. This strategy aims to improve sensitivity and selectivity, enabling the reliable quantification of trace-level carbonyl compounds in complex wine matrices.

2. Material and methods

2.1. Solvents, standards and samples

2.1.1. Chemical standards and solvents

The solvents used in this study were hexane (Hx, organic trace analysis grade), and diethyl ether (Et, LiChrosolv HPLC grade), both purchased from Merck (Darmstadt, Germany); absolute ethanol (EtOH), supplied by VWR Chemicals® (Barcelona, Spain); dichloromethane (DCM) and methanol (MeOH), Distol Pesticide Residue Grade, purchased from Scharlab S.L. (Barcelona, Spain). Milli-Q water was obtained by purification of deionized water using a Millipore® purification system (Bedford, Germany).

The chemical standards used in this study were hexanal (98%), nonanal (95%), (*E*)-2-hexenal (98%), (*E*)-2-octenal (95%), (*E*)-2-nonenal (97%), (*E*)-2-decenal (95%), (*E*)-3-nonen-2-one (95%), 3-methyl-2,4-nonanedione (97%), (*E,E*)-2,4-heptadienal (88%), (*E,Z*)-2,6-nonadienal (95%), and (*E,E*)-2,4-nonadienal (85%), all purchased from Aldrich; and (*E,E*)-2,4-hexadienal (95%), (*E,E*)-2,4-octadienal (90%), (*E,E*)-2,4-decadienal (90%), and (*E,E*)-2,4-undecadienal (95%), obtained from SAFC (Darmstadt, Alemania). The internal standard used was 3-octanone (98%, Aldrich).

2.1.2. Working solutions and wine samples

Working solutions were prepared for method development and validation. SPE conditioning and washing solutions, as well as a synthetic wine matrix (12% v/v ethanol, 5 g/L tartaric acid, pH 3.5 adjusted with NaOH), were prepared following commonly used procedures for wine analysis.

Individual stock solutions of each target analyte were initially prepared in ethanol at approximately 1500 mg/L. These primary stock solutions were subsequently used to prepare intermediate mixtures and working standard solutions by appropriate dilution in the synthetic and real wine matrices.

An internal standard solution of 3-octanone was prepared by diluting 81 µL of a 1232 mg/L stock solution in ethanol to a final volume of 10 mL, yielding a 10 mg/L intermediate solution.

To evaluate the recovery, six Spanish red wines and mistelles from different vineyards across various regions (Cariñena, Somontano, Calatayud and Navarra) were analyzed. These wines, all produced in the 2024 vintage showed no noticeable off-flavors.

Additionally, the method was applied in 2025 to a larger set of 63 Grenache young red wines from grapes harvested in 2024 originating from different regions (Roussillon, Aragon, Navarre and La Rioja).

2.2. Method optimization

2.2.1. Solid phase extraction optimization

2.2.1.1. Breakthrough volume optimization. The Breakthrough Volume (BV) was defined as the maximum sample volume that can be loaded onto the SPE cartridge before analyte loss occurs, corresponding to less than 10% loss relative to reference [36,37]. BV was assessed by passing 50 mL of spiked wine (500 µg/L of each analyte) through a 70 mg Isolute ENV cartridge previously conditioned with 2 mL DCM, 2 mL MeOH, and 2 mL H_2O:EtOH (12%, v/v). This sorbent was selected based on its superior retention capacity for small volatile molecules in wine, as demonstrated by Culleré et al. [38], who identified high-surface-area styrene-divinylbenzene copolymers as the most efficient resins compared to other reversed-phase and mixed-mode sorbents. The percolate was collected in ten 5 mL fractions. In addition, 5 mL of the spiked wine was retained as reference. All fractions were analyzed by GC-MS (Shimadzu QP-2010, Shimadzu, Tokyo, Japan) after a liquid-liquid extraction with 1 mL DCM and analyzed sequentially.

2.2.1.2. Eluent type and volume optimization. For the selection of the optimal eluent type and volume, six 70 mg Isolute ENV cartridges were conditioned as described in the previous section and loaded with 15 mL of spiked wine (100 µg/L of each compound). After that, cartridges were washed with 1.5 mL of H_2O:MeOH (30%, v/v) containing 1% (w/v) $NaHCO_3$, then dried under vacuum. Elution was performed with 5 mL of different eluents (Et, Hx, Hx:EtOH 80:20, DCM:MeOH 95:5, EtOH, and MeOH), collecting eight 500 µL fractions per eluent. Fractions were analyzed by GC-MS.

2.2.1.3. GC-MS chromatographic conditions. Two microliters of sample were injected in splitless mode at 250 °C for 1.5 min. The chromatographic oven program was as follows: 40 °C (5 min), ramped at 2 °C/min to 85 °C, then at 4 °C/min to 145 °C, 8 °C/min to 200 °C, and finally at 20 °C/min to 230 °C (held for 20 min). Separation was performed on a ZB-WAX capillary column (30 m × 0.25 mm diameter × 0.5 µm film thickness; Agilent®), preceded by a 3 m × 0.25 mm deactivated silica pre-column. Helium was used as carrier gas at a constant linear velocity of 40.5 cm/s.

The analyses were carried out using a GC-MS system consisting of a gas chromatograph coupled to a quadrupole mass spectrometer with electron ionization (EI)ionization. The mass analyzer was operated in SCAN mode for compound identification and in Single Ion Monitoring (SIM) mode for quantification. Ion source and transfer line temperatures were 220 °C and 230 °C, respectively. SIM windows and target ions are detailed in Supplementary Material, Table A1.

2.2.2. Stir bar sorptive extraction optimization

To optimize stir bar sorptive extraction (SBSE), the effects of extract dilution, extract volume, salt concentration, and extraction time were systematically evaluated [39]. Twister® stir bars (20 mm length, 1 mm film thickness) coated with 126 µL of polydimethylsiloxane (PDMS), purchased from Gerstel GmbH & Co. KG (Mülheim an der Ruhr, Germany), were used. Experiments were conducted in duplicate in 25 mL Erlenmeyer flasks. In each assay, varying extract volumes were diluted with ultrapure water to a fixed final volume, and different amounts of NaCl were added to adjust ionic strength [40]. Extraction was carried out by stirring at 1000 rpm for variable durations. Extracted analytes were analyzed by TD-GC-(FID)-GC-MS, and peak areas were compared across conditions. The tested parameters are summarized in Table 1.

2.2.3. TD-GC-(FID)-GC-MS chromatographic conditions

Volatile compounds were analyzed using a GC-GC system (Shimadzu, Tokyo, Japan) equipped with a TD-30R thermal desorption unit (Shimadzu), a Deans switch valve. The valve enabled selective transfer of analytes either to a flame ionization detector (FID) for first-dimension (^{1}D) detection or to the second-dimension (^{2}D) column coupled to a QP-2010 Plus quadrupole mass spectrometer. Twister® stir bars were thermally desorbed at 300 °C for 10 min (He 50 mL/min), cryofocused at −15 °C into the Tenax internal trap, and dry purged at 50 °C for 7.5 min. Compounds were injected to the first column via a heated line at 250 °C. Injection was performed in split mode (1:5) at constant pressure (220.6 kPa).

The GC-GC system included two orthogonal columns (polar and non-polar) housed in separate ovens. First oven program: 40 °C (5 min), ramped at 4 °C/min to 145 °C, 6 °C/min to 200 °C, and 8 °C/min to 240 °C (hold 20 min). The ^{1}D column was a polar ZB-FFAP (30 m × 0.25 mm × 0.25 µm; Phenomenex). The column outlet was connected to a FID (H_2 flow: 40 mL/min, air flow: 400 mL/min, and N_2 flow: 20 mL/min) at 270 °C.

The second oven program: 40 °C (25 min), ramped at 4 °C/min to 145 °C, 6 °C/min to 200 °C, and 8 °C/min to 300 °C (hold 5 min). The ^{2}D column was a non-polar ZB-5MSplus (30 m × 0.25 mm × 1 µm; Phenomenex). The second column was connected to a quadrupole mass spectrometer operating in EI ionization mode (ion source temperature: 200 °C, interface temperature: 240 °C), acquiring in SCAN (identification) or SIM (quantification) modes.

Eleven cut windows were applied via the Deans switch interface enabled selective transfer of analyte bands from the ^{1}D to the ^{2}D. Analytical details including retention times, cut windows, and SIM parameters are summarized in Supplementary Material, Tables A2 and A3.

2.3. Proposed method

Wine bottles were opened, and 15 mL aliquots were immediately transferred into 20 mL screw-capped vials equipped with a septum. Samples were spiked with 90 µL of the internal standard solution to achieve a final concentration of 60 µg/L. The spiked samples were loaded onto SPE cartridges previously conditioned with 2 mL of dichloromethane, 2 mL of methanol, and 2 mL of a 12% (v/v) aqueous ethanol solution. Matrix interferences were removed by washing with 1.5 mL of a 30% (v/v) methanol in water solution containing 1% (w/w) $NaHCO_3$. After drying the cartridges under vacuum, carbonyl compounds and internal standards were eluted with 1.2 mL of EtOH.

For the SBSE step, 400 µL of the ethanolic extract were mixed with 7.1 mL of Milli-Q water and 2 g of NaCl in 25 mL Erlenmeyer flasks. A PDMS-coated stir bar was used for extraction with stirring at 1000 rpm for 1 h. Analytes were then thermally desorbed and analyzed by TD-GC-(FID)-GC-MS.

Table 1
Summary of experimental factors and values used to optimize extraction.

Variable to optimize	Total volume (mL)	Extract volume (µL)	(%) NaCl	Extraction time (min)
Dilution factor	2.5; 5; 7.5; 10	200	20	60
Extract volume	7.5	100; 200; 400	20	60
Ionic strength	7.5	400	0; 13; 26	60
Extraction time	7.5	400	26	60; 120; 180

2.4. Method validation

2.4.1. Precision, linearity, detection limits and trueness

Repeatability and reproducibility were evaluated in synthetic and red wine spiked at 5 μg/L, by analyzing three Twister® stir bars from a single extract and three independent SPE extracts, respectively. Linearity in synthetic and real red wine was assessed using spiked samples at eight concentration levels (plus blank, two replicates) using weighted (1/x) calibration curves based on analyte/3-octanone response ratios. Detection or quantification limits (LD or LQ) were defined as signal-to-noise ratios of 3 and 10, respectively, using peak height and local baseline noise. Matrix effects were evaluated by comparing the slopes of calibration curves prepared in synthetic and real wines. Method trueness was evaluated through recovery experiments performed in six different spiked red wine samples.

Those figures of merit were also evaluated in mistelles samples.

2.5. Data treatment

Experimental data are presented as mean value ± standard deviation. Results were analyzed using analysis of variance (ANOVA), while mean values were compared using Tukey's test (XLSTAT for Excel, Version 19.03). A p-value ≤ 0.05 was considered statistically significant, and alphabetical letters were used in the figures to indicate significant differences between means.

2.6. Comparative experiments for SPE–SBSE preconcentration and heart-cutting GC configuration

To justify the technical requirements of the proposed methodology, three comparative studies were conducted. First, the need of the SPE clean-up was evaluated by comparing the proposed method against direct SBSE extraction of 6 mL of real wine spiked at 10 μg/L. Second, the sensitivity of the SBSE step was compared to direct thermal desorption of the SPE extract by loading 100 μL of the eluate onto Tenax® adsorbent tubes [41]. Finally, the necessity of the dual heart-cutting GC-GC configuration was assessed in real wine by simulating a monodimensional separation, maintaining the ^{1}D at a constant 240 °C to transfer the entire sample directly to the ^{2}D and MS detector.

2.7. Quantification of real red wine samples

A total of 63 young Grenache red wines from four different regions were analyzed. Quantification was performed using calibration curves prepared in a real young red wine matrix.

2.8. Odor detection thresholds

Odor detection thresholds were determined in synthetic wine following the ASTM E679-04 standard practice. A 3-alternative forced-choice method was employed using an ascending concentration series with a constant dilution factor. For each step, panelists were presented with three samples, only one of which was spiked, and were asked to identify the different sample. The individual odor detection thresholds were calculated as the geometric mean of the last concentration with an incorrect response and the first concentration with a correct one. The final group threshold for each analyte was determined as the geometric mean of the individual ones.

3. Results and discussion

The aim of this study was to develop a robust method for the determination of trace carbonyl compounds potentially involved in green aroma deviations in red wine. The method combined SPE and SBSE to enhance sensitivity, while GC-GC improved analytical selectivity and, in many cases, sensitivity through a significant reduction of background noise.

3.1. Solid phase extraction optimization

3.1.1. Breakthrough volume optimization

To assess the maximum sample volume that could be loaded onto the SPE cartridge without significant analyte loss, the BV was evaluated. BV was determined by analyzing target analytes in 5 mL effluent fractions. Hexane was initially tested for liquid-liquid extraction, but persistent emulsions prevented separation; DCM was therefore used. After extraction, fractions were analyzed by GC-MS. BV was defined as the maximum effluent volume containing <10% of the analyte amount present in the original sample. Assuming the maximum area corresponds to spiked wine not passed through the cartridge, percentage losses were calculated (Fig. 1; detailed in Supplementary Material, Table A4).

Under this criterion (<10% loss), the most problematic analytes were the lightest and most polar: (*E*)-hexenal, (*E,E*)-2,4-hexadienal and (*E,E*)-2,4-heptadienal. The hydrophobic resin retains more nonpolar compounds with higher Log P values [42], resulting in a BV that increases proportionally with Log P. Considering these results, a BV of 20 mL was selected, and a sample loading volume of 15 mL was established for SPE to allow a 5 mL washing margin, ensuring less than 10% loss of C6 unsaturated aldehydes.

3.1.2. Eluent type and volume optimization

Eluent selection for SPE took into account analyte properties, solid phase characteristics, and analytical compatibility, with polarity and elution strength as key factors to achieve complete analyte desorption. After loading 15 mL of spiked wine onto the SPE cartridge, retained compounds were eluted using 5 mL of selected solvents, and then collected in eight 500 μL fractions. The relative distribution of each analyte across the collected fractions was calculated with respect to the total eluted amount. Cumulative mean percentages were then plotted to assess elution efficiency (Fig. 2). The figure presents the cumulative elution profiles of four representative compounds, each corresponding to one of the chemical families studied (alkanals, alkenals, alkadienals, and ketones), thereby illustrating the efficiency of the different eluents in recovering target compounds.

The optimal eluent was defined as the one eluting ≥95% of analytes with the lowest possible volume. Several solvents reached the ≥95% threshold below 1 mL, while others, such as hexane, required larger volumes. Although DCM:MeOH 5% performed well as a control, it was excluded due to incompatibility with the Tenax sorbent in the thermal desorption trap.

More polar compounds (C6, log P < 2) were efficiently eluted with 0.5 mL of polar solvents (EtOH or MeOH), but not with hexane, whereas less polar compounds (C9, log P > 3) required larger volumes of the same polar solvents. Ultimately, ethanol was selected as the optimal eluent, achieving optimal recovery with only 1 mL.

3.2. Stir bar sorptive extraction optimization

Several parameters were systematically optimized to improve SBSE efficiency. All experiments were performed in duplicate under controlled extraction conditions. Representative results for four target compounds are shown in Fig. 3, while the results for the remaining compounds are provided in the Supplementary Material (Table A5).

The dilution factor significantly affected analyte response. As shown Fig. 3A, (*E,E*)-2,4-hexadienal was the only analyte that exhibited its maximum signal at 5 mL, whereas the remaining 14 compounds showed equal or significantly higher responses at 7.5 mL (Supplementary Material, Table A5). This increase at higher dilution is attributed to reduced competition from ethanol for the PDMS adsorption sites, favoring analyte adsorption into the Twister. Therefore, a final extract volume of 7.5 mL was selected as the best compromise for the overall method.

Regarding extract volume (Fig. 3B), increasing the amount of analyte

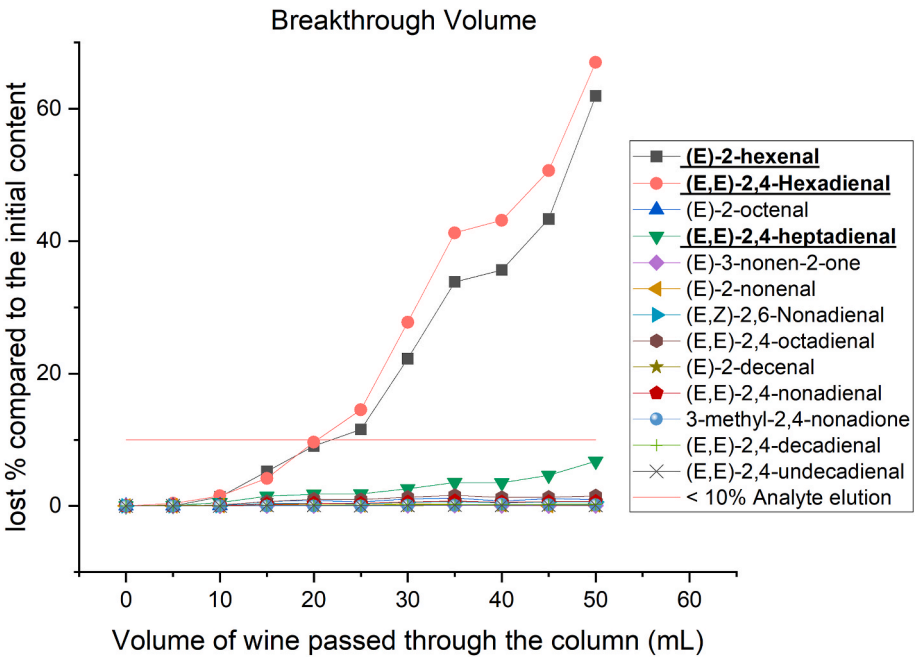

Fig. 1. Percentage of analyte loss versus loaded sample volume. The 10% loss threshold is shown as a horizontal red line; least-retained compounds are highlighted.

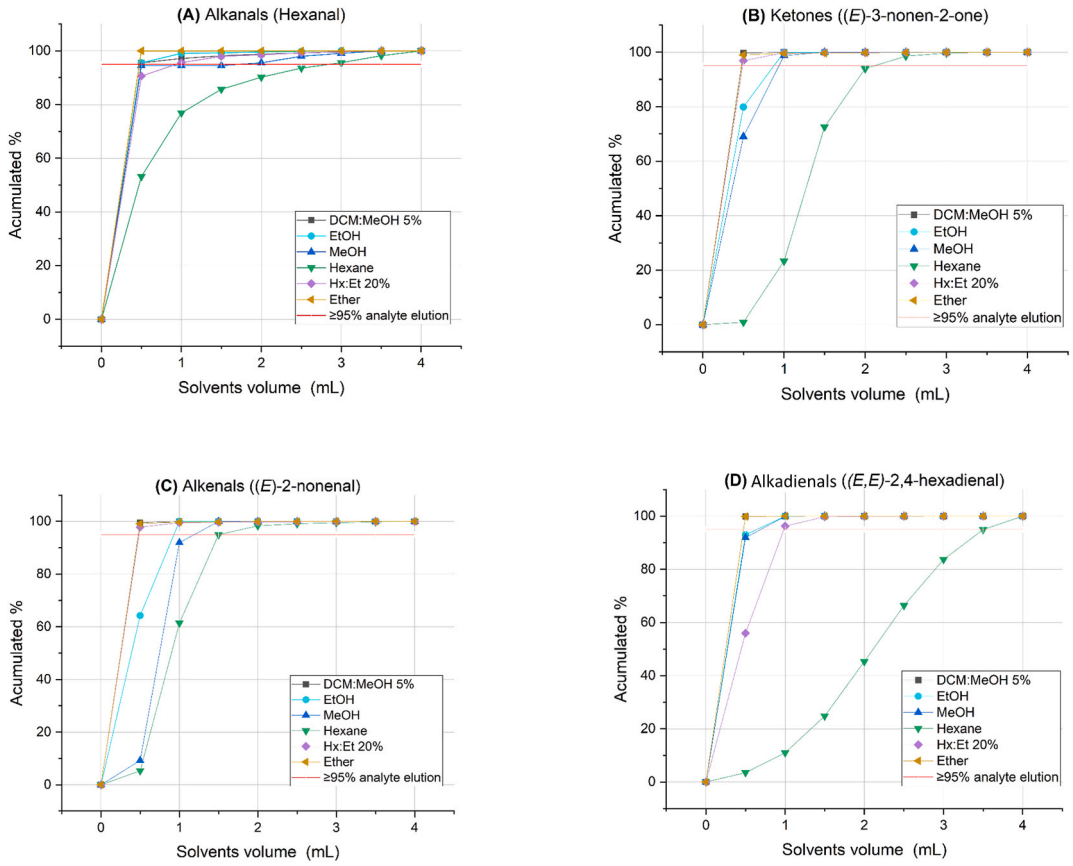

Fig. 2. Cumulative elution percentage of A) hexanal, B) (E)-3-nonen-2-one, C) (E)-2-nonenal, D) (*E,E*)-2,4-hexadienal as a function of eluent volume.

introduced into the system significantly improved signal intensity for all target compounds. A volume of 400 µL was established as the maximum practical amount that allowed duplicate SBSE extractions from a single 1 mL SPE eluate, and was thus adopted as the optimal volume.

Ionic strength was adjusted by NaCl addition to assess the salting-out effect (Fig. 3C). The addition of 2 g NaCl significantly improved recovery for seven analytes, indicating more efficient transfer of analytes to the PDMS phase. For the remaining compounds, no significant differences in signal intensity were observed regardless of the amount of NaCl added.

Extraction time (1-3 h) showed no statistically significant influence

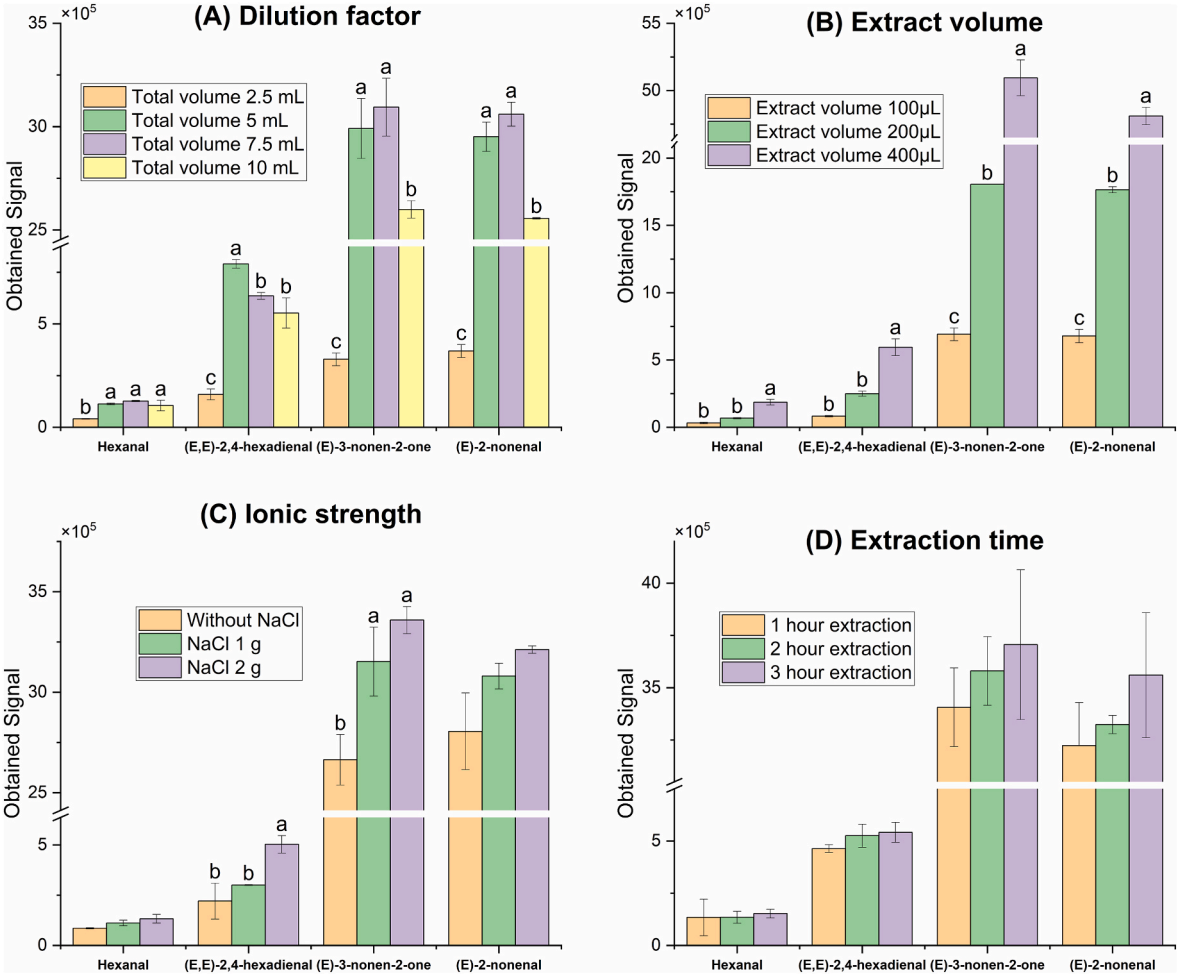

Fig. 3. Effect of A) dilution factor, B) extract volume, C) ionic strength and D) extraction time on four analyte signal responses (hexanal, (E)-3-nonen-2-one, (E)-2-nonenal, (*E,E*)-2,4-hexadienal) during SBSE optimization (n = 2). Letters indicate significant differences between treatments within the same compound according to one-way ANOVA.

on analyte response (Fig. 3D). Therefore, 1 h was selected as the optimal duration for improved efficiency. This choice is supported by previous studies [31,35]: Castejón-Musulén et al. applied 1 h extraction [35], and Wen, Y. et al. observed the highest signal at 1 h [31], indicating that shorter times do not improve recoveries of wine volatiles.

Consequently, the final optimized SBSE conditions were: 400 µL of SPE extract diluted to 7.5 mL with water, addition of 2 g NaCl, and a 1 h extraction time.

3.3. Method validation

After establishing the optimal extraction conditions, the method was validated for the quantification of 15 carbonyl compounds in synthetic and real red wine by evaluating its main analytical performance parameters. Figures of merit, including precision, linearity, sensitivity, matrix effects and recovery are shown in Tables 2 and 4.

Precision was satisfactory. Repeatability (RSD%) was below 10% for most analytes in real wine, with slightly higher values for (*E,E*)-2,4-heptadienal and (*E*)-2-nonenal. Reproducibility remained below 13% for all compounds in synthetic and real wine.

Linearity was confirmed using spiked samples at eight concentration levels, obtaining coefficients of determination (R^2) higher than 0.96 for all compounds and higher than 0.98 for most. Weighted regression (1/x) improved accuracy at low concentrations, ensuring reliable calibration even for low-threshold compounds. For example, (*E,E*)-2,4-octadienal exhibited near-ideal linearity ($R^2 \approx 0.99$) in both matrices.

Sensitivity was high, with detection limits down to 1 µg/L in both, real wine and synthetic matrices. Quantification limits were generally below 80 ng/L in synthetic wine and ranged from 3 to 932 ng/L in real wine, allowing detection at or below sensory thresholds calculated using the forced-choice ascending concentration series method of limits, as described in ASTM standard E679-04. When compared to existing literature, our approach offers a significant advancement in analytical performance, achieving superior sensitivity and a broader analyte scope regarding green-note carbonyls in wine, without the need for sample derivatization, as detailed in Table 3.

Matrix effects, evaluated by slope comparison, were significant ($p < 0.05$) for 10 analytes, indicating signal variation due to matrix interferences. Therefore, quantification was performed using matrix-matched calibration curves. Method trueness was further evaluated through recovery experiments. Recovery, tested in six red wines, was within 80-120% for most compounds. Slightly higher recoveries (>120%) were observed for (*E*)-2-hexenal, (*E,E*)-2,4-hexadienal and (*E,E*)-2,4-heptadienal in some wines. Only one value, for hexanal, fell below 80%, likely due to sample-specific effects. Recovery results were consistent across samples.

Overall, the method proved to be sensitive, precise, and suitable for routine quantification of carbonyl compounds in complex wine matrices.

In the case of mistelles as a matrix, as detailed in Tables 5 and 6, precision results were comparable to those obtained for wine, with repeatability and reproducibility values (RSD%) ranging from 3 to 13%.

Table 2
Validation parameters (precision, linearity, sensitivity, and matrix effects) of the analytical method for carbonyl compound quantification in wine.

	Wine odor thresholds (µg/L)	Precision (RSD%) (n = 3)				Linearity (n = 2)				Sensitivity (µg/L) (n = 2)				Matrix effects
		Synthetic wine		Real wine		Synthetic wine		Real wine		Synthetic wine		Real red wine		
		Reprod.	Repeat.	Reprod.	Repeat.	Range (µg/L)	R^2	Range (µg/L)	R^2	LD	LQ	LD	LQ	Slope comparison (*p*-value)
Hexanal	53.05	8%	8%	10%	2%	70 - 0.280	0.997	70 - 0.096	0.991	0.007	0.023	0.009	0.029	<0.05
***(E)*-2-hexenal**	0.03	8%	11%	13%	8%	20 - 0.027	0.982	20 - 0.082	0.990	0.008	0.027	0.021	0.069	n.s.
***(E,E)*-2,4-hexadienal**	74.82	6%	12%	9%	5%	80 - 0.988	0.959	80 - 0.988	0.984	0.223	0.743	0.280	0.932	<0.001
***(E,E)*-2,4-heptadienal**	17.93	4%	7%	1%	13%	60 - 0.247	0.982	60 - 0.247	0.971	0.024	0.080	0.030	0.099	n.s.
***(E)*-2-octenal**	2.00	4%	9%	8%	5%	20 - 0.082	0.998	20 - 0.247	0.996	0.006	0.020	0.004	0.015	<0.001
Nonanal	8.50	9%	14%	1%	9%	30 - 0.123	0.993	30 - 0.370	0.969	0.002	0.007	0.003	0.009	n.s.
***(E,E)*-2,4-octadienal**	0.56	4%	2%	10%	0%	20 - 0.027	0.995	20 - 0.027	0.988	0.007	0.023	0.007	0.024	<0.01
***(E)*-3-nonen-2-one**	14.34	7%	1%	7%	2%	60 - 0.247	0.986	60 - 0.082	0.960	0.001	0.003	0.002	0.006	n.s.
***(E,Z)*-2,6-nonadienal**	0.02	7%	1%	10%	6%	10 - 0.041	0.993	10 - 0.123	0.985	0.012	0.040	0.018	0.058	<0.001
***(E)*-2-nonenal**	0.6*	8%	1%	7%	12%	20 - 0.082	0.982	20 - 0.246	0.991	0.022	0.073	0.073	0.242	<0.05
***(E,E)*-2,4-nonadienal**	0.07	6%	1%	10%	0%	10 - 0.041	0.994	10 - 0.014	0.983	0.011	0.038	0.001	0.003	n.s.
3-methyl-2,4-nonadione	0.02**	3%	2%	7%	2%	10 - 0.041	0.977	10 - 0.041	0.988	0.003	0.010	0.004	0.013	<0.001
***(E)*-2-decenal**	0.41	11%	1%	5%	7%	20 - 0.082	0.984	20 - 0.740	0.987	0.019	0.063	0.126	0.421	<0.001
***(E,E)*-2,4-decadienal**	0.03	9%	1%	1%	10%	10 - 0.041	0.985	10 - 0.041	0.989	0.011	0.036	0.006	0.018	<0.001
***(E,E)*-2,4-undecadienal**	0.16	13%	3%	4%	8%	20 - 0.082	0.990	20 - 0.027	0.982	0.010	0.033	0.002	0.008	<0.01

All odor detection thresholds presented in this study were determined in this work, except for *which was taken from Culleré et al. [4] and **which was obtained from Pons et al. [28].

Table 3
Comparison of the analytical performance of the developed method with previously reported methodologies for the determination of green-note carbonyls in wine. The assessment includes derivatization requirements, method scope (number of common analytes), and a sensitivity benchmark comparing the lowest LD reported in literature for a common analyte against the LD achieved in the present study.

Method	Derivatiz.	Number of common analytes	Sensitivity comparison between methods (µg/L)		Reference
			Lowest LD compound	This study LD (SPE-SBSE-TD-GC-(FID)-GC-MS)	
SPME-GC-MS (NCI)	Yes	3	0.004 *(E)*-2-hexenal	0.021	[15]
HS-SPME-GC-MS	Yes	6	0.031 *(E)*-2-hexenal	0.021	[43]
GC-MS	Yes	2	0.15 *(E)*-2-octenal	0.004	[14]
HS-SPME-GC-MS/MS	No	6	n.a.	-	[44]
HS-SPME-GC-ITMS	No	3	2.01 Hexanal	0.009	[45]
GC–QqQ–MS	No	5	0.009 *(E)*-2-octenal	0.004	[30]
LC–QqQ–MS	No	5	0.12 Nonanal	0.003	[29]

GC: Gas Chromatography; HS: Headspace; ITMS: Ion Trap Mass Spectrometry; LC: Liquid Chromatography; LD: Limit of Detection; MS: Mass Spectrometry; MS/MS: Tandem Mass Spectrometry; n.a.: not available; NCI: Negative Chemical Ionization; QqQ: Triple Quadrupole; SBSE: Stir Bar Sorptive Extraction; SPE: Solid-Phase Extraction; SPME: Solid-Phase Microextraction; TD: Thermal Desorption.

Table 4
Recovery of the analytical method for carbonyl compound quantification in wine tested in six red wines.

	% Recovery					
	RW1	RW2	RW3	RW4	RW5	RW6
Hexanal	81%	89%	82%	91%	63%	86%
***(E)*-2-hexenal**	99%	131%	111%	132%	104%	103%
***(E,E)*-2,4-hexadienal**	104%	105%	106%	138%	118%	112%
***(E,E)*-2,4-heptadienal**	120%	127%	113%	120%	123%	113%
***(E)*-2-octenal**	103%	104%	86%	102%	100%	101%
Nonanal	95%	86%	113%	80%	97%	88%
***(E,E)*-2,4-octadienal**	109%	103%	97%	93%	89%	100%
***(E)*-3-nonen-2-one**	97%	90%	95%	91%	87%	93%
***(E,Z)*-2,6-nonadienal**	122%	117%	111%	111%	114%	116%
***(E)*-2-nonenal**	102%	96%	97%	94%	91%	99%
***(E,E)*-2,4-nonadienal**	82%	102%	110%	100%	105%	106%
3-methyl-2,4-nonadione	103%	94%	102%	93%	91%	103%
***(E)*-2-decenal**	108%	91%	108%	100%	83%	91%
***(E,E)*-2,4-decadienal**	82%	84%	78%	83%	83%	102%
***(E,E)*-2,4-undecadienal**	87%	115%	97%	90%	107%	102%

Linearity was maintained across slightly narrower working ranges than in wine, although R^2 values remained high, typically between 0.96 and 0.99. Method sensitivity in mistelles was generally lower than in wine. For example, the LD for hexanal increased from 9 ng/L in wine to 27 ng/L in mistelle; for (*E*)-2-nonenal, from 73 ng/L to 223 ng/L; and for (*E,E*)-2,4-undecadienal, from 2 ng/L to 22 ng/L, roughly one order of magnitude. As observed in wine, slope comparison between synthetic matrix and mistelles revealed significant differences, confirming the presence of matrix effects. Although recovery tests were performed, the results were not satisfactory, with several analytes showing recoveries outside the acceptable ±20% range. These results suggest strong and variable matrix effects among different mistelles, highlighting the need

Table 5
Validation parameters (precision, linearity, sensitivity, and matrix effects) of the analytical method for trace carbonyl compound quantification in mistelles.

	Precision (RSD%) (n = 3)		Linearity (n = 2)		Sensitivity (μg/L) (n = 2)		Matrix effects
	Reprod.	Repeat.	Range (μg/L)	R^2	LD	LQ	*p*-value
Hexanal	8%	10%	7.7 - 0.092	0.985	0.027	0.089	<0.05
***(E)*-2-hexenal**	7%	12%	2.2 - 0.082	0.969	0.022	0.073	<0.001
***(E,E)*-2,4-hexadienal**	5%	8%	2.9 - 0.32	0.988	0.081	0.271	<0.05
***(E,E)*-2,4-heptadienal**	9%	3%	2.2 - 0.082	0.998	0.020	0.066	n.s.
***(E)*-2-octenal**	9%	6%	2.2 - 0.082	0.996	0.024	0.08	<0.001
Nonanal	4%	13%	10 - 0.123	0.995	0.029	0.095	<0.001
***(E,E)*-2,4-octadienal**	12%	7%	6.6 - 0.082	0.972	0.011	0.037	<0.05
***(E)*-3-nonen-2-one**	13%	9%	6.6 - 0.082	0.999	0.010	0.032	n.s.
***(E,Z)*-2,6-nonadienal**	13%	9%	1.1 - 0.041	0.984	0.007	0.023	<0.001
***(E)*-2-nonenal**	13%	9%	6.6 - 0.74	0.986	0.222	0.743	<0.001
***(E,E)*-2,4-nonadienal**	13%	0%	1.1 - 0.041	0.993	0.010	0.036	n.s.
3-methyl-2,4-nonadione	10%	13%	1.1 - 0.041	0.995	0.007	0.025	<0.001
***(E)*-2-decenal**	12%	11%	2.2 - 0.082	0.985	0.019	0.064	<0.001
***(E,E)*-2,4-decadienal**	8%	8%	3.3 - 0.041	0.959	0.011	0.035	<0.001
***(E,E)*-2,4-undecadienal**	9%	9%	2.2 - 0.082	0.977	0.022	0.073	<0.01

Table 6
Recovery of the analytical method for trace carbonyl compound quantification in mistelles tested in six mistelles.

	% REC					
	M1	M2	M3	M4	M5	M6
Hexanal	116%	972%	117%	216%	152%	98%
***(E)*-2-hexenal**	157%	986%	265%	277%	246%	183%
***(E,E)*-2,4-hexadienal**	92%	198%	102%	141%	111%	103%
***(E,E)*-2,4-heptadienal**	62%	166%	65%	82%	67%	68%
***(E)*-2-octenal**	124%	388%	122%	43%	148%	136%
Nonanal	82%	181%	92%	106%	79%	44%
***(E,E)*-2,4-octadienal**	101%	294%	92%	150%	124%	117%
***(E)*-3-nonen-2-one**	131%	402%	144%	202%	161%	155%
***(E,Z)*-2,6-nonadienal**	194%	607%	227%	311%	255%	245%
***(E)*-2-nonenal**	50%	132%	66%	68%	59%	65%
***(E,E)*-2,4-nonadienal**	195%	576%	197%	317%	257%	232%
3-methyl-2,4-nonadione	85%	252%	88%	136%	124%	113%
***(E)*-2-decenal**	267%	585%	276%	442%	340%	245%
***(E,E)*-2,4-decadienal**	92%	250%	94%	154%	122%	109%
***(E,E)*-2,4-undecadienal**	258%	590%	224%	557%	420%	251%

to apply standard addition for accurate quantification in this matrix.

3.4. Contribution of sample preparation and chromatographic configuration to method sensitivity and selectivity

The analytical performance of the SPE-SBSE-TD-GC-GC-MS method was critically compared against simpler alternatives to highlight its advantages in terms of clean-up efficiency, sensitivity, and selectivity.

3.4.1. Effect of SPE–SBSE on clean-up and preconcentration efficiency

The integration of an SPE step prior to SBSE proved mandatory for wine analysis. Comparison of the ^{1}D-FID chromatograms, detailed in Supplementary Material (Fig. A1), revealed that direct SBSE introduces a high load of matrix interferences that compromise the chromatographic profile. The SPE stage selectively retains the target carbonyls while effectively removing these interferences. The combined SPE–SBSE approach resulted in analyte-dependent signal enhancements, with 10 out of 16 compounds exhibiting increases above 1.5-fold, and maximum signal gains of up to five-fold for selected analytes, as detailed in Supplementary Material (Table A6).

The SBSE step (effectively sampling a 400 μL equivalent of the SPE extract) significantly outperformed direct thermal desorption of 100 μL of extract on Tenax. The dual preconcentration method (SPE-SBSE) achieved lower limits of detection, resulting in LOD reductions of up to 100 times relative to direct thermal desorption as detailed in Supplementary Material (Table A7).

3.4.2. Role of heart-cutting GC in improving chromatographic selectivity

The necessity of the bidimensional configuration was demonstrated through a simulated monodimensional analysis of real wine samples. In the monodimensional mode, some analytes including (*E,E*)-2,4-heptadienal, (*E*)-2-octenal, (*E,Z*)-2,6-nonadienal, (*E,E*)-2,4-nonadienal, 3-methyl-2,4-nonadione were masked by matrix co-elutions, as detailed in Supplementary Material (Fig. A2). Moreover, a progressively rising baseline in the monodimensional simulation obscured late-eluting compounds like (*E,E*)-2,4-decadienal and (*E,E*)-2,4-undecadienal, as detailed in Supplementary Material (Fig. A3).To evaluate selectivity, a systematic study of individual heart-cuts was performed as detailed in Supplementary Material (Table A8). This optimization allowed for the identification of inter-cut interferences, leading to a final multi-cut program that ensures a clean baseline and the high signal-to-noise ratios required for trace-level quantification. These results confirm that the dual heart-cutting GC-GC system is essential to overcome the complexity of the wine matrix.

3.5. Analysis of red wine samples

Target carbonyl compounds were quantified in 63 Grenache red wine samples using calibration curves constructed in a real wine matrix. On the one hand, several compounds were detected at concentrations below their olfactory thresholds, suggesting limited individual sensory relevance. On the other hand, notably, *(E,E)*-2,4-heptadienal and *(E,E)*-2,4-octadienal were frequently below the limits of detection. In addition, 3-methyl-2,4-nonadione was consistently below the limit of detection in all samples and was therefore not included in summary Table 7. Complete concentration data for all compounds in the 63 samples are provided in the Supplementary Material (Table A9).

The low occurrence of 3-methyl-2,4-nonanedione is consistent with previous findings [28], which demonstrated that this compound accumulates mainly during oxidative aging and reaches sensory relevance in oxidized or long-aged red wines. Its absence or very low concentration in the present study is therefore in agreement with the young age and limited oxidative evolution of the analyzed Grenache wines.

Odor activity values (OAVs) were calculated by dividing concentrations by odor thresholds. Compounds with OAVs $\geq$1 are considered individually odor-active, while those with 0.1 $\leq$ OAVs <1 may contribute via synergistic interactions. As shown in Table 7, most compounds exhibited OAVs <0.1 in the majority of samples. However, (*E*)-2-decenal, (*E*)-2-nonenal, *(E,Z)*-2,6-nonadienal, and (*E,E*)-2,4-undecadienal reached OAVs $\geq$1 in a significant number of cases, suggesting a relevant role in the wines' aroma, probably in green or fatty notes. Although (*E*)-2-hexenal and nonanal did not exceed OAVs = 1 in most samples, their frequent intermediate OAVs (0.1-1) point to possible synergistic effects. In particular, nonanal showed OAVs >1 in 6 samples

Table 7
Quantification results of 14 compounds in 63 real red wine samples: compound concentrations and sensory relevance based on OAVs. (Minimum, maximum, ratio$^{MAX/min}$ and median of concentrations and OAVs, as well as the distribution of samples according to OAV thresholds.)

	Concentration µg/L				OAV			Samples number		
	min	MAX	median	ratio$^{MAX/min}$	min	MAX	median	OAV<0.1	0.1≤OAV<1	OAV≥1
Hexanal	<LD	9.355	<LD	2185	<LD	0.176	<LD	58	5	0
***(E)*-2-hexenal**	<LD	9.865	<LD	951	<LD	328.825	<LD	0	35	28
***(E,E)*-2,4-hexadienal**	<LD	4.189	1.400	29	<LD	0.056	0.019	63	0	0
***(E,E)*-2,4-heptadienal**	<LD	0.600	<LD	40	<LD	0.033	<LD	63	0	0
***(E)*-2-octenal**	0.799	2.014	1.061	2	0.266	1.007	0.530	0	62	1
Nonanal	<LD	23.740	3.113	17678	<LD	2.793	0.366	18	39	6
***(E,E)*-2,4-octadienal**	<LD	0.058	<LD	15	<LD	0.103	<LD	61	2	0
***(E)*-3-nonen-2-one**	<LD	0.061	<LD	70	<LD	0.004	<LD	63	0	0
***(E,Z)*-2,6-nonadienal**	<LD	0.179	0.074	20	<LD	8.944	3.692	0	18	45
***(E)*-2-nonenal**	<LD	0.641	0.462	17	<LD	1.644	1.183	20	0	43
***(E,E)*-2,4-nonadienal**	<LD	0.086	0.014	166	<LD	1.235	0.201	6	54	3
***(E)*-2-decenal**	<LD	1.158	0.646	18	<LD	2.823	1.575	0	1	62
***(E,E)*-2,4-decadienal**	<LD	0.028	0.020	10	<LD	0.930	0.665	0	61	2
***(E,E)*-2,4-undecadienal**	<LD	0.532	0.527	448	<LD	3.326	3.292	21	0	42

Ratio$^{MAX/min}$ were calculated with values below the limit of detection assigned as LD/2.

and intermediate values in 39.

These results, shown in Table 7, support the idea that green or herbaceous aromas in wine may be attributed to the combined influence of several volatile compounds, even at sub-threshold levels, in line with the aroma vectorial perception model described in literature [46].

4. Conclusions

A sensitive and selective analytical method combining SPE, SBSE, and TD-GC-(FID)-GC-MS was developed and validated for the trace determination of 15 carbonyl compounds potentially involved in green aroma deviations in red wine. The combination of SPE–SBSE with two-dimensional GC provided enhanced selectivity and sensitivity, allowing the direct analysis of underivatized compounds and overcoming the limitations of previously reported methods. The method showed excellent analytical performance in terms of precision, linearity, recovery, and detection limits, and proved fully suitable for application to real wine matrices.

Application of the method to 63 red wines showed that most carbonyl compounds occurred at concentrations below their individual odor thresholds, with some compounds rarely detected, others present at intermediate levels, and only a few exceeding their sensory thresholds. These results indicate that the perception of greenness in red wine is unlikely to be driven by single compounds alone. Instead, they support the hypothesis of synergistic odor vectors, in which multiple sub-threshold carbonyls interact to generate a perceptible aroma. Moreover, the substantial variability in reported olfactory thresholds and the limited availability of descriptive sensory studies highlight the need for integrated approaches combining accurate quantification of these compounds with sensory analysis of real wine samples. The analytical method developed in this work provides a robust tool to support such studies. Furthermore, its application to a broad set of Garnacha wines offers insight into the natural occurrence ranges of these compounds in this variety.

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CRediT authorship contribution statement

María Buñuel-Escudero: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft. **Mónica Bueno:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing. **Vicente Ferreira:** Funding acquisition, Supervision, Writing – review & editing. **Ignacio Ontañón:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2026.129908.

Data availability

Data will be made available on request.

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