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A Simple Emulsification-Assisted Extraction Method for the GC–MS/SIM Analysis of Wine Markers of Aging and Oxidation: Application for Studying Micro-Oxygenation in Madeira Wine

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Abstract
Sotolon and the heterocyclic acetals of glycerol are known as potential aging and oxidation markers in fortified wines such as Madeira, Port, and Sherry. Thus, determining the evolution of these compounds under different oxidative aging conditions is important for fortified wine quality purposes. This study proposes a new methodology based on a miniaturized emulsification extraction followed by GC–MS/SIM, which was developed and optimized to follow the formation of sotolon and heterocyclic acetals in fortified wines that were submitted to traditional accelerated aging and micro-oxygenation. The optimization was achieved by means of a mixed-level factorial design, considering 3 factors: sample volume, extractant volume, and concentrated extract volume, by performing 19 experiments in duplicate. The extraction was optimized using 8 mL of wine sample, 5 mL of dichloromethane, concentrating the extract up to 10-fold. The method performance was evaluated for sotolon, using a matrix-matched calibration between 10 and 2000 μg/L. The selectivity was confirmed through the analysis of real samples. The methodology showed good linearity ($R^2 = 0.999$), high sensitivity (LOQ = 6.8 μg/L), recovery about 105%, and good precision (less than 8 and 9%, evaluated by the variation of intra- and inter-day measurements, respectively). This is the first methodology that revealed to be an excellent tool to simultaneously follow the formation of sotolon and heterocyclic acetals in Madeira wines, using an inexpensive, simple, efficient, and effective experimental layout. Indeed, it was shown that traditional accelerated aging and micro-oxygenation have impact on the formation of such molecules.

Keywords Alcoholic beverages · Sotolon · Heterocyclic acetals · Liquid–liquid extraction · Design of experiments

Introduction

Madeira wines are recognized as one of the world's great classic fortified wines and are characterized by a peculiar winemaking process. Their fermentation is stopped, by the addition of neutral spirit of vine origin up to 17–22% (*v*/*v*: mL ethanol/100 mL wine), according to the desired sweetness (dry to sweet wines are produced, with total sugars usually higher than 49.1 g/L) and desired marketing characteristics (Carvalho et al. 2015; Pereira et al. 2011). Traditionally, their aging process usually includes an artificial heating at about 45 °C for at least 3 months (*estufagem*), followed by aging in wood casks placed in sunshine-heated lodges (*canteiro*). More information about its processing can be found elsewhere (Pereira et al. 2016; Pereira et al. 2011). These strong oxidative conditions contribute to their specific organoleptic characteristics, quality, and longevity, as exceptional 100-year-old Madeira wines are easily found. Indeed, it is their longevity that has made them unique in the world of wine. Thus, the economic value of these wines is strongly associated with their age. Several aging-derived compounds have been pointed out as playing an important role on the aroma of these wines (Câmara et al. 2006; Campo et al. 2006; Pereira et al. 2014; Perestrelo et al. 2011); however, sotolon (3-hydroxy-4,5-dimethyl-2(5H)-furanone) is recognized as being the key

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odorant and has also been classified as a potential aging marker of these fortified wines (Câmara et al. 2006; Câmara et al. 2004). This chiral lactone is a powerful odorant, which can impart a nutty, caramel, curry, or rancid odor, depending on its concentration and enantiomeric distribution (Pons et al. 2008; Silva Ferreira et al. 2003). Despite being pointed out as a key odorant of other fortified wines (Collin et al. 2012; Martin et al. 1992; Silva Ferreira et al. 2003), the researchers' attention has also been directed to its off-flavor character, associated to the premature oxidative aging of young dry white wines, overlapping the expected fruity, flowery, and fresh character (Mayr et al. 2015; Pons et al. 2008; Pons et al. 2015).

Sotolon has been quantified above its odor threshold (10 μg/L in white wine, 19 μg/L in Port wine) in several wines, such as Botrytised (5–20 μg/L), Vin Doux Naturel (0.9–140 μg/L), Tokay (80–140 μg/L), French Vin Jaune (120–268 μg/L), Sherry (0–500 μg/L), Port (5–958 μg/L), and Madeira (0–2000 μg/L) (Gabrielli et al. 2015; Guichard et al. 1993; Lavigne et al. 2008; Pons et al. 2008). The determination of sotolon in wines has been done mainly using classical liquid–liquid extraction (LLE) and solid-phase extraction (SPE) followed by liquid or gas chromatography (Ferreira et al. 2003; Gabrielli et al. 2015; Gabrielli et al. 2014; Guichard et al. 1993). The main difficulty in quantifying this lactone lies in its low concentration found in wine and in its high boiling temperature (184 °C), affecting the sensitivity of analytical methods based on headspace sampling (DHS and SPME) (Gabrielli et al. 2015). Despite the efforts made, the formation pathways of sotolon in wine are not yet completely understood (Pons et al. 2010). However, in general, it is accepted that oxidation, sugar concentration, temperature, and storage time influence the formation of sotolon (Jacobson et al. 2013; Martins et al. 2013). Recently, Pereira et al. (2017) found that a high concentration of fructose (similar to what is found in sweet wines) in a fortified wine model solution (180 mL/L of ethanol, 6 g/L tartaric acid and pH 3.5) develops sotolon, at 70 °C for 1 month (overheating). Furthermore, a correlation has also been found between the production of sotolon and heterocyclic acetals (Castro et al. 2014), such as *cis*- and *trans*-5-hydroxy-2-methyl-1,3-dioxane (1,3-*cis/trans*-dioxane) and *cis*- and *trans*-4-hydroxymethyl-2-methyl-1,3-dioxolane (1,3-*cis/trans*-dioxolane), which are also strongly correlated with aging and oxidation in fortified wines (Câmara et al. 2003; Muller et al. 1978; Silva Ferreira et al. 2002). This kind of acetals is generated by the acid-catalyzed condensation reaction between glycerol and acetaldehyde, at wine pH, and have also been reported as one of the indicators of Port wine's aging, under oxidative conditions (Silva Ferreira et al. 2002). Additionally, they can also contribute to the aroma of very old fortified wines. There are some published experimental layouts focused on the analysis of these four heterocyclic acetals in fortified wines (Câmara et al. 2006; Perestrelo et al. 2011; Silva Ferreira et al. 2002) and more recently in table wines (Peterson et al. 2015). LLE and SPME combined with gas chromatography are often used.

The methodologies reported in literature to analyze these markers of aging and oxidation have come to depreciate the extraction step, since significant sample and extractant volumes and long-lasting extraction procedures are still used, while user- and environment-friendly extraction techniques have been emerging in the last decades. Current trends of modern analytical chemistry are toward the simplification and miniaturization of sample preparation procedures, minimizing the use of hazardous organic solvents and reagent consumption (Bosch Ojeda and Sánchez Rojas 2014). Table 1 presents the most relevant information about some procedures found in the literature for the analysis of sotolon and heterocyclic acetals.

This study sought to develop a new methodology for the simultaneous analysis of markers of aging and oxidation in Madeira wine. Furthermore, considering that sotolon and heterocyclic acetals are aging markers, and can elucidate if specific conditions are effective for accelerating wine aging, it was also our objective to study the feasibility of the use of micro-oxygenation for accelerating the aging of these wines, comparing it with the traditional thermal processing (*estufagem*). Thus, in this study, we propose a vortex-assisted emulsification extraction method, which was optimized to extract the target compounds from fortified wines, using an experimental design optimization procedure, namely full factorial design. The methodology was validated and successfully applied to quantify sotolon in these wines.

Experimental

Chemicals

All chemicals had a purity grade higher than 97%. Sotolon and sodium citrate tribasic dehydrate were purchased from SAFC (St. Louis, MO, USA), while 3-octanol (internal standard, IS) and sodium citrate dibasic sesquihydrate were obtained from Acros Organics (Geel, Belgium). Dichloromethane and ethyl acetate were obtained from Fisher Scientific (Loughborough, UK). Ultra-pure water (18 MΩ, type 1) was also used and was obtained from a Simplicity® UV ultra-pure water apparatus from Millipore (Milford, MA, USA). Absolute ethanol, sodium hydroxide, and anhydrous magnesium sulfate were obtained from Panreac (Barcelona, Spain) while *L*-(+)-tartaric acid was from Merck Co. (Darmstadt, Germany).

Standards and Samples

A sweet Tinta Negra wine (TN, a *Vitis vinifera* L. red grape variety), aged for 3 years (*estufagem* followed by *canteiro*),

Table 1 Extraction and validation parameters of some procedures found in the literature for the individual analysis of sotolon and heterocyclic acetals

Chemical compounds	Extraction technique	LOD	Recovery	Sample volume	Solvent volume	Salt addition	Chromatography technique	Ref
Sotolon	SPE	10 μg/L	60%	25 mL	30 mL of $(C_2H_5)_2O$	Dried with Na_2SO_4	HPLC–UV	Guichard et al. (1993)
	SPE	0.84 μg/L	73.8%	50 mL	6 mL of CH_2Cl_2	7.5 g of $(NH_4)_2SO_4$	GC–MS	Silva Ferreira et al. (2003); Martins et al. (2013)
	LLE - SPE	0.029 μg/L	> 89.5%	30 mL	3 × 40 mL of CH_2Cl_2	3 g of NaCl/dried with Na_2SO_4	HPLC–UV	Gabrielli et al. (2014)
	LLE	0.26/0.001 μg/L	75.5–87.7%	30 mL	2 × 20 mL of CH_2Cl_2	90 mg of NaCl/dried with Na_2SO_4	HPLC–UV/UPLC–MS	Gabrielli et al. (2015)
Heterocyclic acetals	LLE	–	–	50 mL	2 × 5 mL of CH_2Cl_2	Dried with Na_2SO_4	GC–MS	Silva Ferreira et al. (2002); Câmara et al. (2003)
	LLE	2.85–9.76 μg/L	27.1–77.1%	2 mL	2 × 1 mL of $CH_3COOC_2H_5$	200 mg of Na_2SO_4	GC–MS	Peterson et al. (2015)
	SPME	–	–	1 mL	–	0.5 g of NaCl	GC × GC–MS	Perestrelo et al. (2011)

LOD limit of detection, *SPE* solid-phase extraction, *LLE* liquid–liquid extraction, *SPME* solid-phase microextraction, $(C_2H_5)_2O$ diethyl ether, CH_2Cl_2 dichloromethane, $CH_3COOC_2H_5$ ethyl acetate, Na_2SO_4 sodium sulfate, $(NH_4)_2SO_4$ ammonium sulfate, *NaCl* sodium chloride, *HPLC* high-performance liquid chromatography, *UPLC* ultra-performance liquid chromatography, *GC* gas chromatography, *UV* ultraviolet detector, *MS* mass spectrometry

was selected to perform a factorial design, used for the optimization of the extraction procedure.

A standard stock solution of 200 mg/L was prepared by dissolving appropriate amounts of the sotolon standard in synthetic wine (containing 6 g/L of tartaric acid, 180 mL/L of ethanol ,and pH adjusted to 3.5, with a 1 M NaOH solution), which was then used to spike the fortified wines for the evaluation of method performance. The selected young fortified wine used to perform the matrix-matched calibration was a dry wine made from a white grape variety, Sercial (*Vitis vinifera* L.), which was previously checked to ensure the absence of quantifiable amounts of sotolon. For the evaluation of precision, two other sweet TN wines were analyzed, one 3 years old and another 5.

After undergoing a typical winemaking process, a sweet TN wine from the 2013 harvest was transferred to six 200 L vats. Since this was the first time that the impact of micro-oxygenation was studied in Madeira wines, three treatments were employed at different stages of wine aging. Figure 1 illustrates the sequence by which each wine was submitted to micro-oxygenation. The traditional accelerated aging was also studied (AAge), applying standard conditions, wherein the wine did not receive oxygen addition and was also used as control. Each experiment was conducted in duplicate. The first treatment (T1) was applied before e*stufagem* and lasted 78 days. During this period, a continuous flow of 66 mg O_2/L per month was applied to two vats (MOX1). Because there was no information regarding the best MOX conditions for Madeira wine, this flow rate was chosen since, at room temperature, it proved to be enough to elevate dissolved oxygen to saturation levels and consequently allowed the effect of oxygen addition to become more evident. Two more were reserved for another experiment (MOX2). When the T1 treatment was concluded, the second micro-oxygenation treatment (T2) was introduced and all wines were transferred to 200 L stainless steel tanks and heated at 45 °C for 4 months (*estufagem* period). At this stage, the two wine samples, previously reserved, underwent micro-oxygenation and the wine that had previously been oxygenated did not receive any more oxygen addition. During the T2 treatment, which lasted 60 days, a flow rate of 66 mg O_2/L per month was added to MOX2 wine. MOX2 wine underwent an additional period of micro-oxygenation for 65 days (T3 treatment), during which the oxygen flow rate was increased up to saturation: the flow rate was initially raised up to 660 mg O_2/L per month (increased by 10-fold) and was gradually increased up to a final flow rate of 1122 mg O_2/L per month. This wine was re-named MOX3. The continuous oxygen (Air Liquide, Portugal) flow rate was delivered by an Enartis Microox 2 (Enartis, Windsor, CA, USA) micro-oxygenation system, fitted with two dosing points connected to two stainless steel diffusers (Enartis, Windsor, CA, USA). The diffusers were suspended inside the tanks, near to the bottom but without

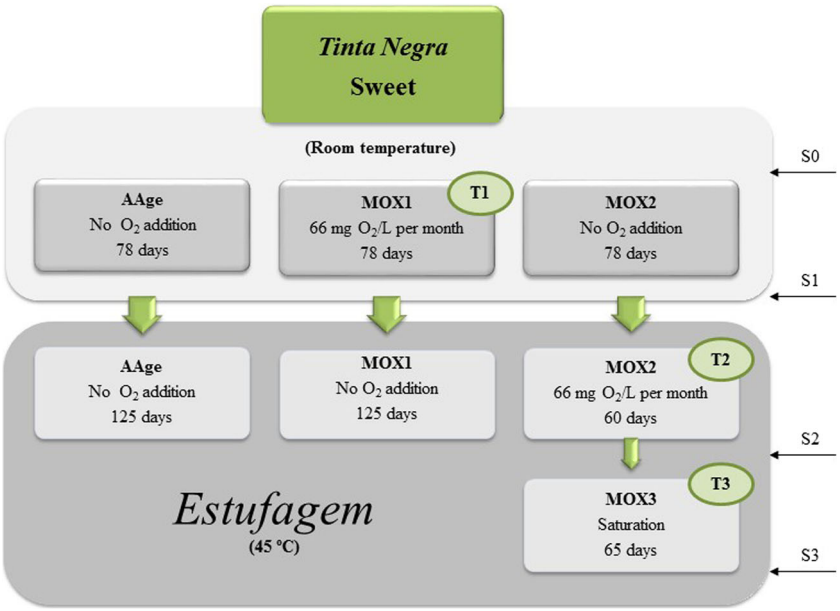

Fig. 1 Schematization of the micro-oxygenation treatments introduced before and during the *estufagem* process. T1 micro-oxygenation treatment 1; T2 micro-oxygenation treatment 2, and T3 micro-oxygenation treatment 3. S0 sampling before T1 treatment; S1 sampling before T2 treatment and beginning of *estufagem*; S2 sampling during *estufagem* and before T3 treatment; S3 sampling at the end of T3 treatment and *estufagem*

touching it. The resulting wines had ethanol contents between 17.7 and 18.5% and sugar content ranging from 90 to 97 g/L. Four sampling points were established: S0—before T1 treatment (at this stage only AAge was considered, taking one sample from each vat), S1—before T2 treatment and beginning of *estufagem*, S2—during *estufagem* and before T3 treatment, and S3—end of T3 treatment and *estufagem*.

Development and Method Optimization

The biggest challenge was how to discriminate and determine the trace quantities of the target compounds from a complex matrix without using large volumes of toxic organic solvents and time-consuming procedures. The following extraction procedure was initially tested: 4 mL of ethyl acetate was added to 10 mL of wine spiked with the IS (500 mg/L of 3-octanol prepared in synthetic wine), which were placed into 50-mL PTFE centrifuge tubes, vortexed for 1 min and centrifuged for 4 min at 4400 rpm (Centrifuge Eppendorf 5702, NY, USA). Then, 3 mL of extract was concentrated, by evaporation up to 1 mL, under a gentle nitrogen stream. The addition of salts (1 g of sodium chloride and 4 g of anhydrous magnesium sulfate) and buffers (1 g of sodium citrate tribasic dehydrate and 500 mg of sodium citrate dibasic sesquihydrate) was also studied. The performance of dichloromethane was also tested to check its effectiveness to emulsify and extract the target compounds. Both solvents were chosen considering their effectiveness in extracting such compounds in wines (Ferreira et al. 2003; Gabrielli et al. 2014; Peterson et al. 2015). The extraction procedure was then optimized employing a design of experiments (DoE), namely a three-level full factorial design. Three experimental variables that could have some effect on the GC–MS–SIM response of sotolon and heterocyclic compounds were studied: the sample volume at three levels, 8, 10 and 15 mL; the extractant volume at three levels, 4, 5 and 8 mL; and the extract concentration at two levels, 3-fold (up to about 1 mL) or 10-fold (up to about 0.3 mL). The duplicates were randomly performed. The injections were also carried out in duplicate. A 3-year-old sweet Madeira wine was used for the method optimization. The data analysis was performed using the Matlab software, version R2016b, to estimate the effect of the factors on the sum of the sotolon and 1,3-*trans*-dioxolane peak areas, considering that both compounds showed lower responses.

Chromatographic Conditions

The GC–MS analysis was carried out in a TRACE GC Ultra gas chromatograph equipped with an ISQ single quadrupole (on electronic impact mode, at 70 eV) and a TriPlus autosampler (on liquid mode) from Thermo Scientific (Hudson, NH, USA). The column used was a TR-5MS 60 m × 0.25 mm with 0.25 μm film thickness from Thermo Scientific. Injections (1 μL) were performed in splitless mode using helium (Air Liquide, Algés, Portugal) as carrier gas, at a constant flow rate of 1 mL/min. The GC injector port was kept at 230 °C, while the transfer line and ion source were kept at 290 and 250 °C, respectively. The oven temperature was initially set to 40 °C for 2 min, raised 12 °C/min until 196 °C, raised again 30 °C/min until 260 °C, and held for 5 min, with a total run time of 22 min. Initially, some tests with the sotolon standard and old fortified wines were performed in TIC mode, in the range m/z 30–300, to ensure the retention time of the target compounds. The remaining chromatograms were then acquired in SIM mode recording five fragment ions for identification purposes, while, for quantification, a single fragment

Table 2 Retention time (t_R) and characteristic fragment ions of the target compounds. List of the recorded fragment ions and those used for quantification purposes

No.	t_R (min)	Compound	Characteristic fragment ions (m/z)	Recorded fragment ions (m/z)	Quantification fragment ions (m/z)
1	10.33	1,3-*cis*-dioxane	43, 44, 45, 57, 88, and 103	57, 87, 88, 103, and 117	103
2	10.60	1,3-*cis*-dioxolane	31, 43, 45, 57, 59, 87, and 103		
3	10.94	1,3-*trans*-dioxolane	31, 43, 45, 57, 59, 87, and 103		
4	11.61	1,3-*trans*-dioxane	43, 44, 45, 57, 88, and 103		
5	11.95	3-octanol (IS)	41, 55, 59, 83, and 101	59, 83, 101, 113, and 128	83
6	13.68	sotolon	43, 55, 83, 113, and 128		

ion was used, as described in Table 2. The semi-quantitative evaluation of heterocyclic acetals was expressed in terms of the IS concentration, since there were no standards commercially available for these compounds. The identification of the isomers was based on the retention order of each compound, using a chromatographic column of similar polarity, described by Silva Ferreira et al. (2002), who synthetized the heterocyclic acetals from glycerol and acetaldehyde and used them to identify each of the compounds.

Procedure for the Method Performance Evaluation

The method was assessed in terms of linearity, sensitivity, precision, selectivity, and accuracy for sotolon. The method precision was also evaluated for the heterocyclic acetals.

The selectivity was appraised by the analysis of several fortified wines to ensure the absence of chromatographic interferences at the retention time of sotolon (SIM at m/z 83), which could compromise its quantification. A young fortified wine was then chosen to be used for the matrix-matched calibration.

To evaluate method linearity, a young fortified wine without quantifiable amounts of sotolon was spiked with known amounts of the standard at seven different concentration levels (10, 25, 50, 100, 200, 500, and 2000 μg/L). Each solution was prepared in duplicate and the extractions and injections as well (a total of eight injections for each concentration level). The matrix-matched calibration curve was obtained by an internal calibration, plotting the peak area ratio of each calibration solution (sotolon area/IS area) against the corresponding concentration, determining the method linearity (R^2).

Sensitivity was assessed by determining the LOD and the LOQ, which were calculated based on the linear regression as follows: $LOD = 3.3\ \sigma/b$ and $LOQ = 10\ \sigma/b$, in which σ is the standard deviation of the *y*-intercept and *b* is the slope.

Precision was evaluated based on inter- and intra-day repeatability, by the analysis of two fortified wines from different vintages (3- and 5-year-old sweet TN wines). Ten successive extractions were performed for each wine, to verify intra-day precision. In order to verify inter-day precision, the results of five extractions of each wine, in three different days, were analyzed. Both parameters were expressed as relative standard deviation (% RSD).

A recovery study evaluated accuracy, in which the 3-year-old sweet TN wine, also used in the precision study, was spiked with known amounts of sotolon standard at three different concentration levels (25, 200, and 2000 μg/L).

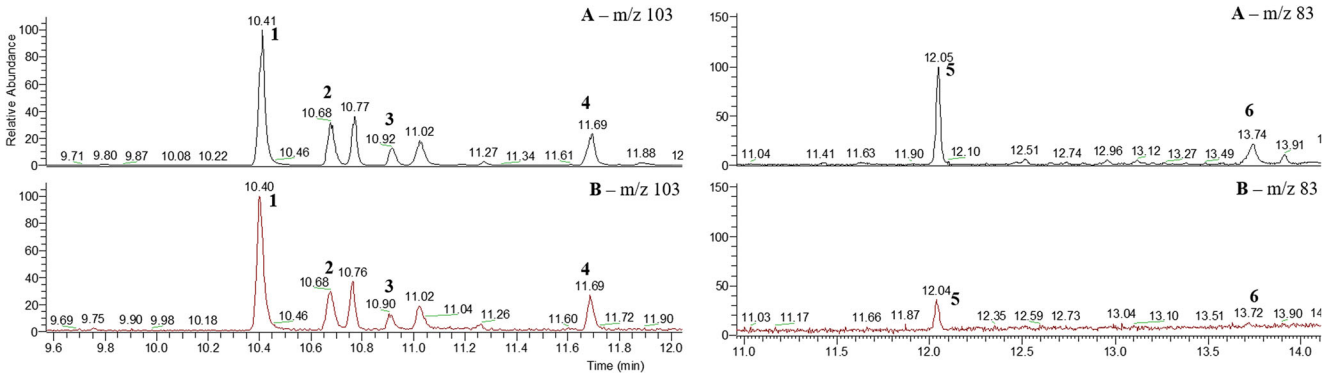

Fig. 2 Comparison of the GC–MS/SIM chromatograms of a wine extracted without (**a**) and with (**b**) the addition of salts and buffers (salts 1 g of sodium chloride and 4 g of anhydrous magnesium sulfate; buffers 1 g of sodium citrate tribasic dehydrate and 500 mg of sodium citrate dibasic sesquihydrate). Peak identification: 1 1,3-*cis*-dioxane; 2 1,3-*cis*-dioxolane; 3 1,3-*trans*-dioxolane; 4 1,3-*trans*-dioxane; 5 3-octanol (IS); 6 sotolon

Recovery was then calculated comparing the difference of the sotolon concentrations between the spiked and unspiked wines with the corresponding theoretical concentration of the spike, as follows:

$$\text{Recovery}\ (\%) = \frac{(\text{sotolon concentration of spiked wine} - \text{sotolon concentration of unspiked wine})}{\text{theoretical sotolon concentration of the spike}} \times 100$$

The target compounds were determined in the wines submitted to accelerated aging (22 wine samples) by applying the developed method, while also assessing its suitability.

Results and Discussion

Development and Method Optimization

After performing the initial procedure, as described in the "Development and Method Optimization" section, it was found that the salts and buffers reduce the extraction yield of sotolon; therefore, they were not included in the extraction procedure. Figure 2 illustrates this result. Dichloromethane was chosen over ethyl acetate to continue with the experimental layout, considering that it was found that it produces chromatograms with better peak shapes and less interferences at the retention time of sotolon and heterocyclic acetals. Furthermore, the diffusion of solvent by emulsification was better observed when dichloromethane was used. This phenomenon is known to significantly increase the interface area between the organic and aqueous phases, improving the analyte extraction from aqueous samples (Moradi et al. 2014).

Then, a mixed-level factorial design was performed, with the objective of defining the suitable conditions for the extraction of the target compounds. Figure 3 depicts the results of the design of experiments (DoE), wherein the chosen factors were plotted against the response to visualize the variable combination that maximizes the GC–MS response of the two compounds that usually gave lower chromatographic peak areas, sotolon and 1,3-*trans*-dioxolane.

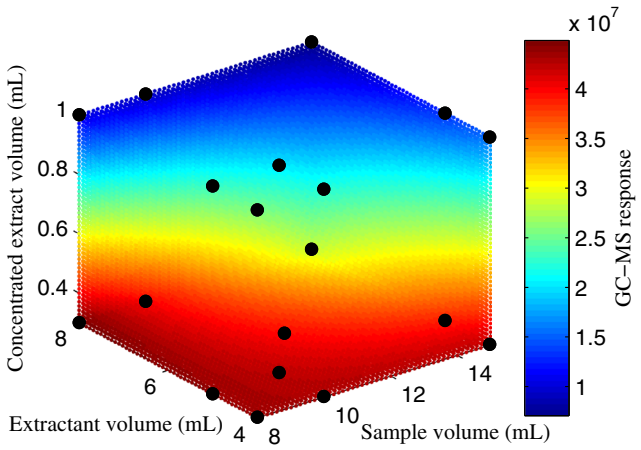

Fig. 3 Results of the design of experiment

Based on the results of the DoE, the final extraction procedure was as follows: 5 mL of dichloromethane was added to 8 mL of wine sample, spiked with 4 μL of IS (500 mg/L of 3-octanol prepared in synthetic wine). The mixture was vortexed for 1 min and then centrifuged for 10 min at 4400 rpm. After separation, 3 mL of the organic phase was transferred to graduated conic glass vials to evaporate the extract up to 0.3 mL, using a small nitrogen flow, concentrating it up to 10-fold. The gas chromatographic separation of all compounds was good and achieved in less than 22 min (Fig. 4). In general, the proposed extraction procedure uses smaller sample and solvent volumes than do most of the procedures described in Table 1, without the need for salt addition, while allowing the simultaneous analysis of sotolon and heterocyclic acetals in a single extraction step.

Method Performance Evaluation

The determination of sotolon and heterocyclic acetals in fortified wine samples can be obtained in approximately 20 min for sample preparation and less than 22 min for analysis run time. The emulsion formed between the organic and the aqueous phases increases the kinetics of compound dispersion. As far as we know, an emulsification-assisted extraction method has never been applied for the analytical determination of the target compounds. It is also the first time that the simultaneous analysis is reported. This section presents and discusses the results for the performance of the proposed methodology for the quantification of sotolon. The method performance results are summarized in Table 3 for the analysis of sotolon.

In regard to the analysis of sotolon, the performance of the methodology was fully evaluated. The selectivity of the proposed methodology was evaluated by the analysis of several fortified wines, from which a wine, absent of significant interferences at sotolon retention time, was chosen to be used to generate the matrix-matched calibration.

As can be observed, the methodology demonstrated good linearity in the range of 10–2000 μg/L, with a R^2 value higher than 0.999. The methodology also showed high sensitivity, with a LOD of 2.3 μg/L and a LOQ of 6.8 μg/L, lower than the sotolon odor threshold in a fortified wine (19 μg/L).

As previously mentioned, accuracy was verified by means of a recovery study. One of the wines used for the precision

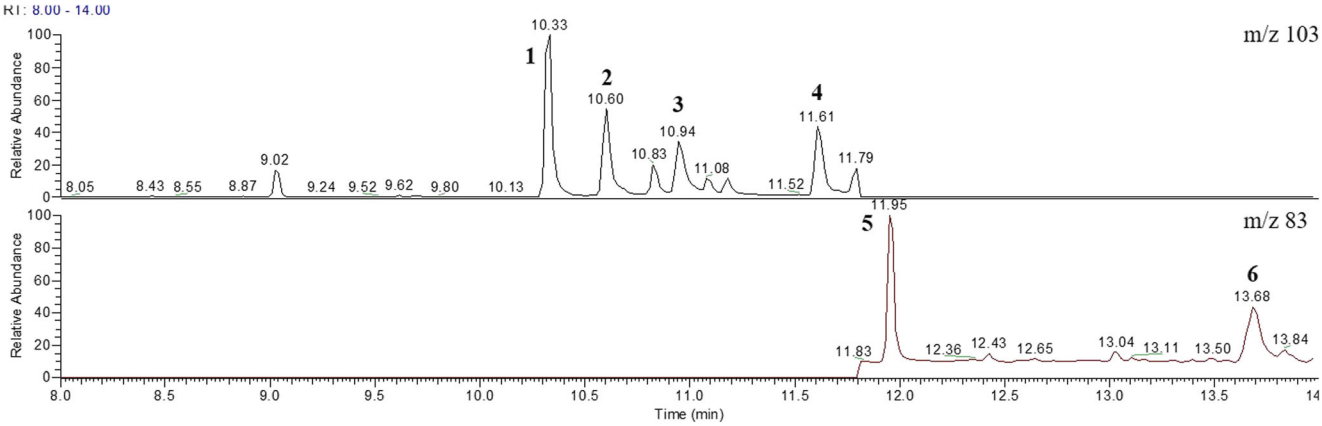

Fig. 4 Typical GC–MS/SIM chromatogram of the wine extract obtained by the optimized method. For peak identification, please see Fig. 2

assay was spiked with known amounts of the sotolon standard at three concentration levels, and the results showed that the average recovery was 105 ± 8%, which is well satisfactory. These results also reveal that the matrix complexity does not compromise the selectivity and sensitivity of the methodology.

The precision was evaluated through intra- and inter-day repeatability. In this regard, the methodology proved to be suitably precise for the quantification of sotolon, with relative standard deviations (RSDs) inferior to 8 and 9%, respectively.

Since standards for the dioxane and dioxolane isomers were not commercially available, the methodology performance for these compounds was only evaluated in terms of precision. Intra- and inter-day precision for *cis*- and *trans*--dioxane and for *cis*-dioxolane had relative standard deviations lower than 13%. In the case of *trans*-dioxolane, these parameters were a little higher, reaching values of 17%. The results for the inter- and intra-day analysis are detailed in Table 4.

Table 3 Method performance parameters evaluated for the proposed methodology, within the validation range 10–2000 μg/L ($n = 4$ for each calibration point)

	Sotolon	
	Parameter	Result
Linearity	Linear regression	$y = 0.0028x - 0.011$
	R^2	0.999
Sensitivity	LOD	2.3 μg/L
	LOQ	6.8 μg/L
Accuracy	Recovery	
	25 μg/L	99.3%
	200 μg/L	114.9%
	2000 μg/L	102.1%
Precision	Intra-day precision	6.6–7.4%
	Inter-day precision	6.7–9.0%

R^2 correlation coefficient, *LOD* limit of detection, *LOQ* limit of quantification, *y* sotolon relative area, *x* concentration of sotolon in μg/L

Monitoring of Aging and Oxidation Markers in Madeira Wine

Finally, 22 Madeira wines were analyzed to study the effect of the traditional accelerated aging and of oxygen addition on the formation of sotolon and dioxane/dioxolane isomers in these fortified wines, evaluating the feasibility of micro-oxygenation for the accelerated aging. As far as the authors know, micro-oxygenation has never been applied to Madeira wines before and as such, it was important to understand how oxygen addition, by itself or combined with *estufagem*, would affect the target compounds.

The evolution of the concentrations of sotolon during the three micro-oxygenation treatments is presented in Fig. 5. The levels of sotolon remained almost unchanged during the 78 days of the T1 experiment. During the traditional accelerated aging (*estufagem*), the concentration of sotolon considerably increased to values well above its odor threshold in all wines (5- to 14-fold higher). These results were somehow expected, since the formation of sotolon is known to be favored by oxidative conditions such as temperature and oxygen exposure. The flow rate of 66 mg O_2/L per month, applied during the T1 treatment, did not have an immediate impact. In fact, it appears that the effect of the T1 treatment only became noticeable during *estufagem*, when the concentration of sotolon increased and reach values of 85.7 and 273.2 μg/L, at 2 and 4 months of heating, respectively. These concentrations are higher than those detected for the AAge wine (69.6

Table 4 Results of the intra- and inter-day precision assays for the heterocyclic acetals

	Intra-day precision	Inter-day precision
cis-Dioxane	4.9–8.9%	7.1–9.5%
trans-Dioxane	4.6–9.4%	10.6–12.8%
cis-Dioxolane	5.8–9.9%	8.9–8.9%
trans-Dioxolane	6.3–11.4%	16.6–16.8%

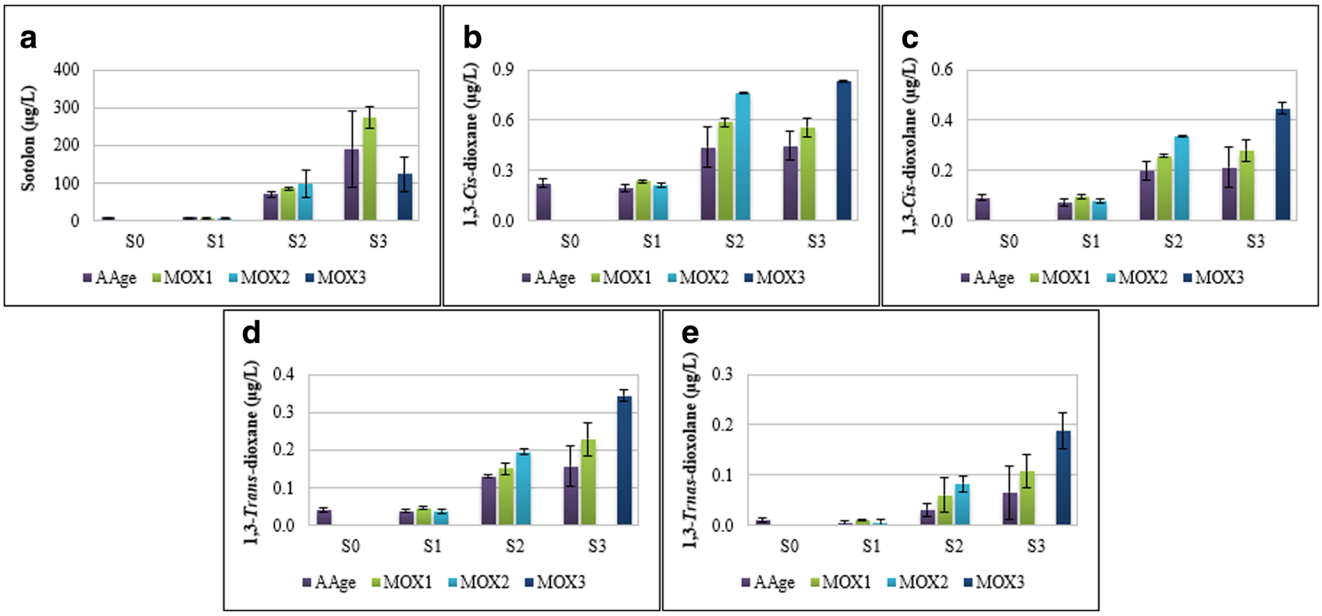

Fig. 5 Evolution of sotolon (**a**) and wine heterocyclic acetals (**b** 1,3-*cis*-dioxane; **c** 1,3-*trans*-dioxane; **d** 1,3-*cis*-dioxolane; **e** 1,3-*trans*-dioxolane) during the three micro-oxygenation treatments applied to a Tinta Negra Madeira wine. At each stage, $n = 8$

and 191.2 µg/L) at the same periods. When a similar flow rate was applied during the first 60 days of *estufagem* (T2 treatment), the combination of oxygen exposure and higher temperature (45 °C) had a positive effect on the formation of sotolon, increasing its concentration from 8.0 to 97.9 µg/L. During the T3 treatment, the wine saturation was reached and the levels of sotolon continued to increase; however, its evolution was much slower and the concentration of sotolon measured in MOX3 at S3 was only 123.0 µg/L, much lower than those found for the AAge and MOX1 wines at the same stage. The sotolon increase varied between 15- and 34-fold.

The impact of thermal processing and micro-oxygenation on the evolution of the heterocyclic acetals was also evaluated (Fig. 5). Results show that *cis*-dioxane was the most predominant isomer, at all measuring stages. Moreover, the concentrations of the *cis*- isomers were always superior to the concentrations of the *trans*- isomers. These results are somewhat similar to those described in previous studies for Madeira wines made from the white grape varieties (Câmara et al. 2003). In regard to the effect caused by temperature elevation and oxygen addition on these compounds, the first observation that is worth mentioning is that all isomers presented a very similar behavior. Once again, the T1 treatment had a very subtle impact; however, during *estufagem*, the MOX1 wine developed higher concentrations of all isomers when compared to the AAge wine. Thus, *estufagem* has a considerable impact on the formation of these molecules in Madeira wines, greatly increasing their amount, between 3- and 37-fold. It was also found that the exposure to oxygen influences the formation rate of these heterocyclic compounds. Their formation was favored by the combination of temperature elevation (45 °C) and oxygen exposure, since MOX2 and MOX3 wines presented the highest levels at S2 and S3 stages, respectively. The fact that the formation of 1,3-*cis*- and 1,3-*trans*-dioxane and 1,3-*cis*- and 1,3-*trans*-dioxolane has revealed a positive dependence upon temperature and oxygen is in agreement with the findings reported in literature (Castro et al. 2014; Silva Ferreira et al. 2002), which determines that extreme oxidative conditions favor the production of these compounds.

Conclusion

The optimized methodology showed to be effective for the quantification of sotolon in fortified wines, allowing the simultaneous analysis of heterocyclic compounds, in a simple and fast way. It was shown that the methodology can be successfully applied to monitor the aging process of fortified wines. Its application can be further extended to the analysis of other kind of wines, since its sensitivity is quite low. The methodology also provided good linearity, precision, and accuracy, comparable with methodologies previously proposed. The main advantages of the proposed methodology are its speed and simplicity, without using many chemicals to extract the target molecules or complex experimental layouts, minimizing the analysis time and costs.

The study confirms that the *estufagem* process promotes the formation of sotolon and heterocyclic acetals. The combination of temperature elevation and oxygen exposure favor their formation in higher extension. Thus, the results suggest that micro-oxygenation allied with thermal processing can be

a feasible procedure for the Madeira wine's accelerated aging. Despite needing further studies, namely to evaluate the impact on the sensorial profile and to optimize the potential introduction of micro-oxygenation to the traditional aging process, sotolon levels were enhanced up to 14-fold higher than its odor threshold, which can be seen as a positive effect.

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Compliance with Ethical Standards

Conflict of Interest Ana I. Freitas declares she has no conflict of interest. Vanda Pereira declares she has no conflict of interest. João M. Leça declares he has no conflict of interest. Ana C. Pereira declares she has no conflict of interest. Francisco Albuquerque is an employee of the Madeira Wine Company, S.A. José C. Marques declares he has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent Not applicable.

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