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# Multi-target optimization of solid phase microextraction to analyse key flavour compounds in wort and beer

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## Abstract

Despite the literature comprises numerous studies dealing with the analysis of wort and beer flavour-related compounds by HS-SPME followed by GC-MS quantification, no generalized consensus exists regarding the optimal conditions for the extraction procedure. The complex chemistry nature of these matrices, the number of analytes, as well as the number and interactions among parameters affecting the extraction performance, requires the adoption of optimal experimental design protocols. This aspect is often overlooked and often not properly addressed in practice. Therefore, in the present work, the optimal conditions under which a range of wort and beer analytes can be extracted and quantified were analyzed. The optimal extraction conditions were presented at two levels of aggregation: global (untargeted) and key-flavour analysis. Experimental data was generated by Definitive-Screening-Design, followed by model development and optimization. Both approaches were compared and critically analyzed. For vicinal-diketones group, a complete validation study for the optimal conditions is presented.


**Keywords:** Optimization of analytical processes; Design of experiments; HS-SPME; Wort and beer; Flavour; Principal Component Analysis; Definitive Screening Designs;

## 1. Introduction

The global production of beer has been experimenting a steady and robust increasing trend in the last decade, establishing it in the top rank of the most consumed and popular alcoholic beverages. The increasing consumption and production volume allied to the market demand and consumer's preferences have also undergone changes which call for innovative technologies and a more comprehensive knowledge of the production process in breweries, in order to understand and better respond to the drivers of demand. In this regard, several studies have been focusing on the analysis of the volatile organic compounds (VOCs) formation during beer fermentation and storage conditions in order to eliminate or promote specific flavours in the final product.

The headspace solid phase microextraction (HS-SPME) is the most used extraction technique before gas chromatography coupled to mass spectrometry (GC-MS) quantification of beer volatile compounds (Andrés-Iglesias, Montero, Sancho, & Blanco, 2015; Braga, Zielinski, Silva, de Souza, Pietrowski, Couto, et al., 2013), both for its efficiency as well as operability. Table 1 shows a systematic overview of the parameters and the conditions usually adopted for the extraction of VOCs in beer and wort, as well as the optimization procedure adopted to set the optimal value of HS-SPME parameters. This literature review reveals that, despite being a widely used technique, it is still quite difficult to establish the optimal conditions for analysing beer VOCs. For example, regarding fiber coating, there are five references to different coatings as being optimal to quantify beer VOCs. The optimal extraction time and temperatures also present significant differences, varying from 30 to 60 min and from 30 °C to 60 °C, for the same fiber coating. Significant differences can also be found in the optimal values of the remaining extraction parameters, namely the degas and salt addition effect, the sample/vial volume ratio, the use of stirring and sample pre-incubation. This lack of agreement and consensus may be caused by the adoption of sub-optimal optimization practices, such as one-factor-at-a-time (OFAT) strategies (Bezerra, dos Santos, Santos, Novaes, Ferreira, & de Souza, 2016; Granato & Calado, 2013; Pereira, Reis, Leça, Rodrigues, & Marques, 2018). Besides completely overlooking factor interactions, OFAT

occasionally induce some parameters to be fixed and not considered in the optimization procedure.

Statistical design of experiment methodologies (DoE) overcome OFAT limitations, guaranteeing statistically meaningful results with reduced experimental effort (Ferreira, Silva Junior, Felix, da Silva, Santos, Santos Neto, et al., 2019). The most common used DoE methodologies in HS-SPME optimization procedures are the Full Factorial Design (FFD) and CCD (Central Composite Design). In general, such procedures are applied to two, up to four parameters, and to quantitative variables, such as time, temperature and sample/vial volume ratio. Fiber coating optimization was only considered by Leça *et al*. (2015) in the optimization of HS-SPME for quantifying just two beer off-flavour compounds. A new relevant addition to the DoE toolkit was recently introduced with the development of Definitive Screening Designs (DSD) (Bradley Jones & Nachtsheim, 2011). DSDs are a new class of three-level screening designs, with the capability for estimating quadratic effects of the model (if more than six factors are contemplated). In these designs, the main effects are completely decoupled from second-order interactions, making their estimation very efficient (as expected for a screening methodology). Furthermore, second-order interactions are also not aliased with each other, making the pattern of aliasing of these designs very interesting when compared to other alternatives. DSDs only require two more treatments than twice the number of factors, making them highly competitive from a cost-benefit perspective. The design matrix for DSDs is generated from numerical methods applied to solve D-optimal designs formulations or, more simply, using conference matrices (Xiao, Lin, & Fengshan, 2012). For more details, please refer to (Bradley Jones & Nachtsheim, 2011; B. Jones & Nachtsheim, 2013).

In the present work, we addressed the task of systematically finding the optimal conditions for quantifying eight groups of compounds (multi-target optimization). These groups include the major chemical families of VOCs that can be found in beer, namely the higher alcohols and esters, and less abundant molecules but presenting great importance in beer-flavour such as aldehydes, volatile fatty acids and vicinal diketones. The latter compounds, the vicinal diketones (diacetyl and 2,3-pentanedione) are also key markers for fermentation process monitoring in brewers. We also test the alternative untargeted methodology, where a compromise solution for all the

compounds (28 overall) is sought. Both approaches are compared and critically analysed. We also fully validate the quantification for the particular case of VDK, given their particular interest to beer producers and the higher quality of the models obtained.

This article is organized as follows. In the following section, the materials and experimental methods employed in this work are described in detail. Then, the methodological workflow for data generation and analysis is introduced. The results obtained are reported in the Section 3, including the optimal operation conditions for HS-SPME regarding untargeted (global) and multi-target analysis of key-flavour groups of compounds. Specific optimal conditions for one family (VDK) are then fully validated, and the method is applied to independent samples ranging from fermenting wort to different packed beer brands. The paper closes with a final section summarizing the main contributions and conclusions.

## 2. Materials and methods

In this section we present a detailed description of the materials employed in the study and the analytical procedures followed.

### 2.1 Materials, Reagents, Chemicals and Samples

The SPME fibers tested were purchased from Supelco (Bellefonte, PA, USA): a stableflex fiber core coated with 50/30 μm divinylbenzene-carboxen-polydimethylsiloxane (DVB/Car/PDMS), a 85 μm carboxen-polydimethylsiloxane (Car/PDMS) and divinylbenzene- polydimethylsiloxane (DVB/PDMS). Absolute ethanol (>99.8%) was purchased from Sigma–Aldrich (Steinheim, Germany). Ultra-pure water (conductivity of 18 MΩ) was obtained by the Simplicity®UV ultrapure water (type 1) apparatus from Millipore (USA). A calcium chloride ($CaCl_2.2H_2O$, ≥99.5%, Chem-Lab, Zedelgem, Belgium) aqueous solution (50 g/L) was also prepared for sample conservation purposes when yeast was present. One millilitre was added per 50 ml of sample collected during fermentation and then stored at -26 ºC until analysis. Sodium chloride (> 99.8%, Chem-Lab, Zedelgem, Belgium) was used in the HS-SPME extraction process. 1 g/L stock solution of 4-methyl-1-pentanol (97%, Sigma-Aldrich, Steinheim, Germany) was prepared by

dissolving this compound in synthetic beer and it was used as an internal standard (500 µg/L in the sample). The alkane solution (C7 to C30) was obtained from Supelco (Sigma Aldrich, St. Louis, MO, USA). Volatile organic compounds stock solutions were prepared in synthetic beer (SB) or in ethanol, whenever the solubility of the analytes was too low in aqueous solution. All the standards used have a purity grade of more than 97.0 %, except for trans-2-nonenal (95 %), adequate to GC-MS analyses. Trans-2-nonenal, ethyl isovalerate, 2,3-pentanedione, ethyl hexanoate, isoamyl acetate, isovaleric acid, ethyl caprylate, acetaldehyde and phenylethyl alcohol were purchased from Acros Organics (Geel, Belgium). Dimethyl sulphide, 2-methoxy-4-vinylphenol (vinyl guaiacol), phenylethyl acetate, isoamyl alcohol and isobutyraldehyde were purchased from Sigma-Aldrich (Steinheim, Germany). Diacetyl, ethyl butyrate, isobutyl acetate, octanoic acid, acetoin and isobutyl alcohol were purchased from TCI (Zwijndrecht, Belgium). Ethyl acetate was purchased from Fisher Scientific (Loughborough, United Kingdom).

The following matrixes were prepared to determine the presence of matrix effect: a) Lager beer from a local brewery with an ethanol content of 5.1%; b) a synthetic beer which was prepared by adding the adequate volume of absolute ethanol for a final concentration of 5.1% (v/v) in ultrapure water and by adjusting mixture pH to 4.0 with an aqueous solution of sodium hydroxide (98%, Panreac, Barcelone, Spain).

About 24 samples were considered in this study to evaluate the methodology applicability, including samples collected during the fermentation process and the final product. To be more precise, seven beer samples from different brands (labelled as samples 1 to 7) and several fermenting wort samples (samples 8 to 24), obtained from a local brewery were analysed.

**2.2 HS-SPME conditions**

Several qualitative and quantitative parameters were evaluated according to the literature review presented in Table 1. After this preliminary screening of factors, the following factors were selected to be contemplated in the analysis: the sample degasification influence (no degass and 15 minutes in ultrasonic bath), the sample/vial volume ratio (in particular, the sample volume in a 20 mL vial), the sample agitation (without and at 100 rpm), salt addition effect, the pre-

incubation and extraction time, as well as extraction temperature. The type of fiber was also considered, namely three different fiber coatings were studied (PDMS/DVB, CAR/PDMS and CAR/PDMS/DVB). A preliminary analysis using design of experiments revealed that, very clearly, CAR/PDMS was the best option among the different fiber coatings under analysis. Therefore, this factor was then kept fixed to CAR/PDMS during the study. In Table 2, the levels considered in the experimental design, for all parameters, are described. All SPME tests were carried out on a TriPlus autosampler, in SPME mode and were properly conditioned before using, according to the supplier indication.

Optimal extraction conditions were chosen after a careful statistical analysis of the experimental design results (Definitive Screening Design) from 18 randomly performed assays (see details in the next section). Once the optimized extraction conditions were found, in each vial, 5 µl of 4-methyl-1-pentanol (1 g/L) were added to the sample before properly sealing and homogenizing it in a vortex. Also, according to the ethanolic content of each sample, either the volume of ethanol was added or a dilution with water was performed to adjust ethanol concentration to 5.1 % and, consequently, uniformize the interference of this compound in the efficiency of the extraction.

### 2.3 HS-SPME optimization: multi-targeted and untargeted methodologies

The optimization of the operational conditions for simultaneously quantifying multiple components in a complex mixture, such as beer or wort, is an involved process. Most often, the research efforts are directed for quantifying a single component or family or components, for which the operation conditions are to be optimized. In this work we considered the case where multiple targets are considered, for which the optimal conditions, possibly different, need to be found. This is a gap found in the literature that we aim to mitigate in this work. Furthermore, we also considered the untargeted case where all compounds are to be considered simultaneously and a single set of conditions is to be proposed. This approach is often found in the literature when multiple compounds are present and will be further explored and critically analyzed in this work. The proposed systematic methodology consists of a logical sequence of steps. First, factors are selected (as described in section 2.2). Then a DSD design is set and executed in random order

(generating two plus twice as many experiments as factors). The experimental results are analyzed using multiple linear regression, after aggregating (summing) responses in families of compounds (multi-targeted approach) or into a single response, by computing the scores of the first principal component (PC1), in a Principal Component Analysis (PCA) (untargeted approach, also called, global approach). The obtained models are finally optimized using a desirability-based approach (in this case, the goal is to maximize the peak area of the chromatogram), leading to the optimal factor levels.

2.3.1. Design of experiments

Being a data-driven methodology, it is very important to collect high quality data to support the subsequent steps of the workflow. In this sense, the Definitive Screening Design were applied using JMP-PRO ver. 12.1.0 (64-bit) (SAS Institute Inc.).

2.3.2 Multi-targeted and untargeted data analysis

In this work, two aggregation levels were considered. In multi-targeted analysis, the responses are the sums of compounds in each group (the most common chemical families of VOCs found in beer). In the untargeted or global methodology, the information of all the 28 compounds present are aggregated into a single response, by taking the linear combination of the compounds that most explains the original variability in all compounds. This is done through Principal Component Analysis (PCA). In the PCA nomenclature, the linear combinations are known as loading vectors or simply loadings (one such vector per principal component) and the result of the linear combinations applied to data are the scores (again, there is one score vector for each component). The importance of each component is analysed by the relative magnitude of the associated eigenvalue, which can also be used to compute the amount of variation of the original dataset it is able to explain (Granato, Santos, Escher, Ferreira, & Maggio, 2018). PCA summarizes the information of all analytes in uncorrelated Principal Components (scores) that explain most of the experimental variability – the global level of analysis.

2.3.3 Model building

In this stage, a model is derived that relates the experimental factors with the responses. As data was generated using DoE, polynomial models are to be estimated, consisting of combinations of main effects, second-order interactions and eventually quadratic factors. These models are estimated by Ordinary Least Squares (OLS), assisted with variable selection methods, such as the Forward Stepwise Variable Selection methodology (Draper & Smith, 1998) that sequentially selects the effects to include in the model that improve, in a statistically significant manner, its explanatory capability. At each round, irrelevant variables can also be discarded, if their effect ceases to be statistically relevant. The algorithm stops when no variables are added or removed from the model.

2.3.4 Optimization

Finally, the optimization of the estimated model is made by maximizing the selected desirability function (Ballus, Meinhart, de Souza Campos, Bruns, & Godoy, 2014; Candioti, DeZan, Cámara, & Goicoechea, 2014). This leads to the optimal settings for the experimental factors (in our case, the seven HS-SPME factors presented in Table 3; the blanks cells in this table indicate that the corresponding factors (indicated in the rows) do not have a statistically tangible effect on the quantification of the specified compounds (indicated in the columns) within the experimental domain explored).

## 2.4 Chromatographic conditions

All analyses to identify and quantify VOCs were carried out using a GC-MS system, the TRACE GC Ultra gas chromatograph coupled with an ISQ single quadrupole from Thermo Scientific (Hudson, NH, USA). We have employed a capillary column, TRB-WAX 60 m × 0.25 mm ID DF = 0.25 (Teknokroma, Spain). The carrier gas was helium at a constant flow rate of 1 mL/min. The injector port was kept at 260 °C, in splitless mode, while the transfer line and the ion source were maintained at 260 and 240 °C, respectively. The oven temperature program started at 40 °C, hold for 2 min, then increased up to 250 °C at 4 °C/min and it was finally kept at 250 °C for 5 min. The total GC run time rounded about 60 min. The first chromatograms were obtained in full scan mode (total ion count) in order to obtain the retention time ($t_R$s) of each target compound. After

the confirmation of the $t_R$s, the analyses were always performed with the characteristic and major ions of each analyte and characteristic ions were used for quantification purposes (presented in Results and Discussion section, Table 4).

The mass spectrometer was operated in electron impact (EI) mode at 70 eV. The selective ion monitoring (SIM) operating mode was used with the characteristic ions for each target compound. Each calibration point was extracted in triplicate within the validation range of each analyte.

### 2.5 Validation of the analytical methodology

The optimal experimental condition for implementing HS-SPME-GC-MS for the quantifying family of VDKs was fully validated in terms of selectivity, linearity, sensitivity, matrix effect, precision and accuracy, for compounds found in beer samples and for which chemical standards are available. Each VOC compound area ratio (VOC area/IS area), from all increasing standard solutions, was plotted against the corresponding concentration in order to obtain the respective calibration curves. Selectivity was confirmed by the absence of chromatographic interferences at the $t_R$s of the VOCs in a local lager beer, used for the matrix-matched calibration and several other commercial beers and fermentation samples.

The linearity ($R^2$), limit of detection (LOD) and limit of quantification (LOQ) were computed based on a linear regression approach, following the IUPAC recommendations. The values obtained for these quantities were: LOD = 3.3 $\sigma/b$ and LOQ = 10 $\sigma/b$, where $\sigma$ stands for the standard deviation of the regression and $b$ is the slope. A matrix effect (ME) study was also carried out in order to verify the influence of beer matrix in the extraction of target compounds, when compared to SB. The ME was calculated according to Leça *et al* (2015).

Intra-day precision was determined by the quantification of 10 successive replicates of SB spiked with three different standard solutions (low, intermediate and high concentrations of each analyte) and inter-day precision was assessed by performing the same analysis in 3 different days over a week. The values were expressed in terms of relative standard deviation. A recovery study was carried out (at the above-mentioned three levels of concentrations) to determine the method's accuracy, by computing the percentage of variation between the expected theoretical

concentrations, in each level, and the mean values obtained for the concentrations, by applying the proposed methodology.

Carry-over was also tested by running a blank sample after extracting the highest concentrated working standard solution of each analyte, which allowed us to adjust the fiber conditioning temperature and time.

## 3. Results and discussion

In this section, the results obtained during the implementation of the workflow described in Section 2.3 are presented. The responses for the multi-targeted analysis consist of the sums of peak areas for all the compounds in each one of the eight groups. At the global (untargeted) level, the compounds are analysed simultaneously, as a whole, considering their mutual correlations. The analysis at the global level was conducted using PCA. The first component (PC1) explains over 40% of original variability whereas the first three components explain altogether 80%. The experimental results are scattered in the two-dimensional PC1-PC2 plane, not showing any obvious outlier or abnormal experiment (data not shown). The same was observed for the distribution of residuals around the PCA subspace (using the squared distance to the PCA subspace – not shown) which also did not reveal any outlying observations.

The loadings for the first three components are presented in Figure 1. These are the coefficients of the linear combinations for each PC and codify mutual correlation affinities. The first PC, representing the main overall correlation trend, has only positive coefficients, meaning that it is a sort of weighted mean of all analyte responses. The higher the coefficients, the stronger the correlations between the analyte responses. Esters, such as isobutyl acetate, isoamyl acetate, ethyl butyrate, ethyl hexanoate and ethyl caprylate (central part of the plot) are less represented in PC1 and more in PC2, which seem to represent a contrast between the quantification of these analytes and all the other components. 1-butanol shows a rather peculiar behaviour, widely different from any other analyte (it has a low magnitude loading coefficient in the first PC, meaning that the

variation of this analyte is not strongly correlated with the dominant sources of structured variability present in data). In summary, PC1 has a limited ability to represent all the compounds present, and any solution based on it may be limited in terms of the performance achieved for the less well-represented compounds. By seeking the best compromise between compounds with different patterns of interaction with the HS-SPME factors, PC1 also becomes a mixture of effects, with a rather erratic dependency upon experimental factors, potentially leading to poorer models.

### 3.1 Model building and optimization

For the multi-targeted analysis, a regression model was developed for explaining each one of the eight groups of compounds such as: VDKs, acetaldehyde, acetoin and 2.3-butanodiol, majority and minority esters, higher alcohols and fatty acids. In Table 3, the model figures of merit and the optimal HS-SPME settings for each case are presented. Also presented are the results for the global (untargeted) approach (last column). The best model corresponds to the estimation of the VDKs (more specifically, the sum of diacetyl and 2,3-pentanedione), based on the seven experimental factors and two-way interactions. The resulting model is significant (p-value = 0.0028) with a good quality of fit ($R^2$ = 0.99; Predicted $R^2$ = 0.92; see Figure 2). The models targeting other components present less explanation power but are still significant (significance level of 0.01), as well as the factors identified, which enable the determination of the most suitable operation conditions.

Regarding the global level (last column of Table 3), the quality of the model is, as expected from the discussion above, quite limited (low $R^2$). The global response is PC1, which is a result of the combination of the behaviour of all analytes with possibly conflicting dependencies upon the experimental factors. Therefore, it represents a sort of compromise solution that does not optimally fulfil the conditions to quantify any analyte, with the exception perhaps of those that most structure the variation originated in the DOE.

In summary, Table 3 presents our proposal for setting the extraction conditions under multi-targeted and untargeted conditions, which are valid for the extraction of volatile organic compounds from beer using a Car-PDMS fiber. It was found that carbonation does not significantly influence SPME sampling, in accordance with previous results reported by Pizarro, Pérez-del-Notario (Pizarro et al., 2010). Concerning the sample volume, the results suggest that 10 mL in a vial with 20 mL of capacity is the best option, since the extraction was not favoured when smaller sample amounts were used. Regarding the extraction time and temperature, the results indicate that these factors have a significant impact for almost every chemical families that were analysed, and their settings were established according to VDKs best results. The agitation and incubation time factors were found to be relevant only for VDKs and acetoin, and therefore their settings were established based on the results of these compounds.

### 3.2 Validation and independent testing

In this section, we complete the cycle of developing an analytical procedure, by fully validating a pre-selected target. The proposed multi-targeted approach leads to optimal conditions for different families of compounds. Among these, VDKs are a family of analytes with considerable interest for beer producers. Therefore, we selected this target to illustrate the usefulness of the proposed methodology. In this case, we take the optimal conditions in Table 3 and conduct a complete validation study and compute the figures of merit for the optimal conditions. The analytical methodology validation was carried out for analytes which chemical standards are available. Additionally, three other compounds were also considered in this phase (dimethyl sulfide, isobutyraldehyde, trans-2-nonanal) since these are common off-flavours in beer and chemical standards are available.

#### 3.2.1 Assessment of matrix effects

Following the extraction conditions described above, injections of standard solutions prepared in beer and in synthetic beer were analysed and calibration curves for all the analytes in study were determined in both cases. From these results, the matrix effect was studied by comparing the

deviations between the slopes of the calibration curves resultant from beer and synthetic beer. It was observed that this parameter was lower than 15% for most of the target compounds, confirming that matrix effect does not occurs (Table 4). For that reason, SB was used for the quantification of VOCs and for the validation of this methodology

3.2.2 Figures of merit of the methodology proposed

The selectivity of the methodology was confirmed by the absence of interferents at the retention times associated to each VOC in the calibration solutions, as well as the absence of any coeluted compounds with target compounds when beer was analysed using the same methodology. The parameters of each analyte linear regression curve, as well as validation results can be found in Table 4. Chromatograms resultant from a standard solution and from a lager beer sample injection are shown in Supplementary material section. All the regression curves were found to exhibit good scores in terms of linearity, $R^2$ (higher than 0.999 for all compounds analysed). LOD and LOQ values (evaluated from the standard deviation of the regression line) were appropriate for the linear ranges established for the target compounds, which most of the times were found both in wort fermenting samples and in several commercial beer from different brands and types (Table 4). The developed quantification method shows a good linearity in the concentration range of interest and recovery mean values ranging from 84.76 (diacetyl) and 114.86% (ethyl decanoate), confirming the method accuracy. The methodology precision was evaluated in terms of intra-day and inter-day precision, where variations were expressed as curve residual standard deviation values, which were generally lower than 10%.

3.2.3 Application of methodology to wort and beer samples

The optimized HS-SPME methodology was successfully applied to the quantification of VOCs in 7 beers and in 17 fermentation samples (Supplementary material section). The focus of this work relies on the possibility of applying a single analytical method to quantify the VOCs that are typically resultant from alcoholic fermentation of beer wort and other significant aromas such as the off-flavours dimethyl sulphide, isobutyraldehyde, trans-2-nonenal and vinyl guaiacol.

Sporadically, some analytes are present bellow their LOQ in specific samples but, once these are fermentation compounds, it was not expected to detect them in early fermentation samples. This was observed in some higher alcohols and esters, namely phenylethyl alcohol, ethyl acetate and ethyl butyrate. Furthermore, analytes such as the vicinal diketones (diacetyl and 2,3-pentanedione), which monitoring is crucial during the fermentation process, were quantifiable in all the samples that were analysed. These results reinforce the adequate applicability and performance of the developed methodology, particularly in terms of the wide linear range in which samples collected during the entire brewing process can fit.

## 4. Conclusions

The present study reports a multi-targeted and global methodologies for establishing the optimal extraction conditions regarding HS-SPME for quantifying a wide variety of VOCs in beer, including wort to beer fermentation samples, as well finished beers.

The proposed methodology consists of a sequence of steps, starting with the identification of experimental factors, application of a suitable design of experiments methodology (Definitive Screening Design) to plan the experimental trials, followed by their randomized execution, multi-targeted and global analysis of results (i.e., at different levels of aggregation of analyte information), and finally optimization leading to the optimal operation settings. The optimal settings can be flexibly used in the future, depending on the specific purpose of the analysis.

In addition, for illustration purposes, one of the conditions was fully validated and tested.

The optimal HS-SPME settings proposed in this study can now be adopted for performing key-flavour quantification studies in wort and beer. Moreover, the procedure can be replicated in the future for different target analyte or families of analytes (or even for the global approach), to establish the optimal conditions for operating the analytical techniques.

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**Figure Captions**

**Figure 1**. Principal Component Analysis: loading vectors for PC1, PC2 and PC3.

**Figure 2**: Predicted versus Observed plot for the model developed to predict the VDKs, together with several figures of merit, including the $R^2$, Predicted $R^2$, RMSE and p-value for the ANOVA F-test.

**CRediT author statement**

Ana C. Vieira – Investigation; Writing - Original Draft; Visualization

Ana C. Pereira – Conceptualization, Formal analysis, Resources; Writing - Original Draft; Writing - Review & Editing; Project administration; Funding acquisition.

José C. Marques – Resources, Writing - Review & Editing; Supervision; Project administration; Funding acquisition

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**Highlights:**

- Adoption of an optimal experimental design protocol to HS-SPME procedure
- Multi-targeted and global methodologies for establishing the optimal conditions
- Good results in terms of figures of merit of analytical methodology proposed
- Volatile organic compounds quantification in fermenting wort and beer samples

**Table 1**: Review of methodologies applied to beer VOC extraction by HS-SPME followed by GC analysis. The parameters that were optimized are highlighted in bold.

| **Main analytes** | **Optimization** | **Degas** | **Sample/vial volume** | **Salt addition** | **Fiber** | **Stirring** | **Extraction temperature** | **Incubation time** | **Extraction time** | **Derivatization** | **Reference** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| beer volatiles | - | yes | 5 mL | 1.35g NaCl | PDMS/DVB | 1200 rpm | 50 °C | 5 min | 30 min | - | (G. da Silva, Augusto, & Poppi, 2008) |
| beer volatiles | - | - | 10/20 mL | - | - | - | 85 °C | - | 30 min | - | (Huimin, Hongjun, Xiuhua, & Bing, 2012) |
| beer volatiles | - | - | 15/20 mL | 5g NaCl | PDMS | - | 50 °C | 10 min | 30 min | - | (Kleinová, Geršl, & Mareček, 2015) |
| beer esters | - | - | 10 mL | - | PA | - | 37 °C | 30 min | 60 min | - | (Techakriengkrai, Paterson, Taidi, & Piggott, 2006) |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| beer volatiles | - | - | 5/20 mL | - | PDMS/DVB | - | 50 °C | 26 min | 2 min | yes | (Rossi, Sileoni, Perretti, & Marconi, 2014) |
| beer volatiles | - | yes | 10/20 mL | - | PDMS/DVB | 500 rpm | 40 °C | - | 10 min | - | (Jiao, Ding, Shi, Chai, Cong, & Zhu, 2011) |
| beer volatiles | - | - | 5/20 mL | 1.75g NaCl | DVB/CAR/PDMS | - | 45 °C | 20 min | 40 min | - | (Riu-Aumatell, Miró, Serra-Cayuela, Buxaderas, & López-Tamames, 2014) |
| alcohols and esters | - | yes | 10 mL | - | PA | - | 60 °C | - | 50 min | - | (Jeleń, Wlazły, Wąsowicz, & Kamiński, 1998) |
| aldehydes | - | - | 10/20 mL | - | PDMS | - | 20 °C | - | 20 min | yes | (Vesely, Lusk, Basarova, Seabrooks, & Ryder, 2003) |
| beer volatiles | - | yes | 5g/20 mL | 2g NaCl | DVB/CAR/PDMS | - | 20 °C | 5 min | 10 min | - | (Giannetti, Boccacci Mariani, Torrelli, & Marini, 2019) |
| hop volatiles | - | - | 8/20 mL | 2.4g NaCl | DVB/CAR/PDMS | yes (incubation only) | 40 °C | 5 min | 30 min | - | (Richter, Eyres, Silcock, & Bremer, 2017) |
| beer volatiles | - | yes | 10/20 mL | 2g NaCl | Car-PDMS | - | 60 °C | - | 20 min | - | (Stefanuto, Perrault, Dubois, L'Homme, Allen, Loughnane, et al., 2017) |
| beer volatiles | - | yes | 10/20 mL | 3g NaCl | DVB/CAR/PDMS | 500 rpm | 60 °C | 10 min | 30 min | - | (Alvim, Gomes, Garcia, Vieira, & Machado, 2017) |
| beer volatiles | OFAT | - | 10/20 mL | **3.5g NaCl** | **DVB/CAR/PDMS** | 500/250 rpm | **40 °C** | 10 min | **30 min** | - | (Saison, De Schutter, Delvaux, & Delvaux, 2008) |
| beer volatiles | OFAT | - | 10/40 mL | 3g NaCl | DVB/CAR/PDMS | - | **60 °C** | 60 min (70 °C) | **30 min** | - | (G. C. da Silva, da Silva, da Silva, Godoy, Nogueira, Quiterio, et al., 2015) |
| beer volatiles | OFAT | - | 30/60 mL | 9g NaCl | **DVB/CAR/PDMS** | - | **30 °C** | - | **60 min** | - | (Rodrigues, Caldeira, & Câmara, 2008) |
| beer volatiles | OFAT | yes | **2 mL beer + 2 ml water /10 mL** | **1.7g NaCl** | **DVB/CAR/PDMS** | 500 rpm | **30 °C** | **5 min** | **5 min** | - | (Cajka, Riddellova, Tomaniova, & Hajslova, 2010) |
| beer volatiles | OFAT | yes | **5g/15 mL** | 2g NaCl | **Car/PDMS** | yes | 20 °C | **30 min** | **30 min** | - | (Pinho, Ferreira, & Santos, 2006) |
| wort volatiles | OFAT | - | 10/20 mL | **3.5g NaCl** | **DVB/CAR/PDMS** | 250 rpm | **45 °C** | 10 min | **30 min** | - | (De Schutter, Saison, Delvaux, Derdelinckx, Rock, Neven, et al., 2008) |
| beer volatiles | OFAT | - | 5/15 mL | 2g NaCl | **PDMS** | 870 rpm | 24 °C | - | **45 min** | - | (Charry-Parra, Dejesus-Echevarria, & Perez, 2011) |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| beer volatiles | OFAT | - | **10/20 mL** | **3g NaCl** | PA | - | **40 °C** | - | **30 min** | - | (Li, Liu, Kun-Farkas, & Kiss, 2015) |
| beer volatiles | OFAT | yes | 10/20 mL | 2g NaCl | PDMS/DVB | 400 rpm | 40 °C | 10 min | 30 min | - | (Martins, Brandão, Almeida, & Rocha, 2015) |
| beer volatiles | OFAT | **yes** | **15/30 mL** | **2g NaCl** | **DVB/CAR/PDMS** | - | **40 °C** | 10 min | **40 min** | - | (Biazon, Brambilla, Rigacci, Pizzolato, & dos Santos, 2009) |
| volatile phenols | OFAT | - | **6 mL/20 ml** | **2.4g NaCl** | **DVB/CAR/PDMS** | **250 rpm** | **80 °C** | 5 min | **55 min** | - | (Pizarro, Pérez-del-Notario, & González-Sáiz, 2010) |
| sulphur compounds | OFAT | - | **10/15 mL** | - | **Car/PDMS** | - | **45 °C** | - | **32 min** | - | (Hill & Smith, 2000) |
| trans-2-nonenal | OFAT | - | 5/20 mL | **1.5g NaCl** | **PDMS/DVB** | yes | **60 °C** | - | **20 min** | - | (Svoboda, Mikulíková, Běláková, Benešová, Marova, & Nesvadba, 2011) |
| trans-2-nonenal | OFAT | yes | 10/23 mL | - | Car/PDMS | yes | 50 °C | 15 min | **90 min** | - | (Scherer, Wagner, Kowalski, & Godoy, 2010) |
| phthalates | OFAT | - | 4/15 mL | - | PDMS/DVB | 250 rpm | **95 °C** | | **100 min** | - | (Carnol, Schummer, & Moris, 2017) |
| beer esters | OFAT | - | 10/20 mL | **5g NaCl** | DVB/CAR/PDMS | 800 rpm | **40 °C** | - | **60 min** | - | (Horak, Culik, Kellner, Jurková, Čejka, Hašková, et al., 2010) |
| beer trihalomethanes | OFAT | yes | **20/40 mL** | **4g NaCl** | Car/PDMS | **1000 rpm** | **30 °C** | 8 min | **15 min** | - | (Santos & Carasek, 2013) |
| carbonyl compounds | CCD | - | 5/20 mL | - | PDMS/DVB | 250 rpm | **45 °C** | **7 min** | **20 min** | **yes** | (Moreira, Meireles, Brandao, & de Pinho, 2013) |
| vicinal diketones | O-DOE | - | **5/20 mL** | - | **Car/PDMS** | **yes** | **30 °C** | **5 min** | **25 min** | - | (Leça, Pereira, Vieira, Reis, & Marques, 2015) |
| beer volatiles | FFD | - | 8/10 mL | 3g NaCl | **IL-1 butenyl** | 900 rpm | **35 °C** | **10 min** | **15 min** | - | (González-Álvarez, Blanco-Gomis, Arias-Abrodo, Pello-Palma, Ríos-Lombardía, Busto, et al., 2013) |
| beer volatiles | CCD | - | **6/20 mL** | 1.8g NaCl | DVB/Car/PDMS | - | **44.8 °C** | 10 min | **46.8 min** | - | (Rodriguez-Bencomo, Muñoz-González, Martín-Álvarez, Lázaro, Mancebo, Castañé, et al., 2012) |
| beer volatil | CCD | ye | 10/20 | **2.6g** | Car-PDMS | - | **50 °C** | - | **45** | - | (Nešpor, Karabín, Hanko, & Dostálek, |

es s mL **NaCl** **min** 2018)

**Table 2**. Experimental domain explored to optimize the HS-SPME extraction performance.

| Factor | Qualitative/Quantitative | Levels |
|---|---|---|
| Type of Fiber | Qualitative | **L1** - PDMS/DVB; **L2** -CAR/PDMS; **L3** - CAR/PDMS/DVB |
| Degass | Qualitative | **L1** - No; **L2** - Yes (15min) |
| Sample volume | Quantitative | **L1** - 5 mL; **L2** - 10 mL |
| Adition of salt | Qualitative | **L1** - No, **L2** - Yes |
| Agitation | Qualitative | **L1** - No; **L2** - Yes |
| Pre-incubation time (min) | Quantitative | **L1** - 0; **L2** - 5; **L3** - 10 |
| Extraction time (min) | Quantitative | **L1** - 20; **L2** - 30; **L3** - 40 |
| Extraction temperature (°C) | Quantitative | **L1** - 40; **L2** - 50; **L3** - 70 |

**Table 3**. Optimal levels for the experimental factors optimizing HS-SPME performance.

| | VDKs | Acetaldehyde | Acetoin | 2-3 Butanediol (R,S) | Majority Esters | Minority Esters | Alcohols | Fatty Acids |
|---|---|---|---|---|---|---|---|---|
| **l** | p-value=0.0028 $R^2$=0.99 | p-value =0.0158 $R^2$=0.59 | p-value =0.0069 $R^2$=0.80 | p-value =0.0211 $R^2$=0.56 | p-value =0.0044 $R^2$=0.68 | p-value =0.001 $R^2$=0.60 | p-value =0.0116 $R^2$=0.61 | p-value =0,001 $R^2$=0,70 |
| | No | | No | | No | No | No | No |
| | 10 mL | | 10 ml | | | | | |
| | Yes (3.3g NaCl) | Yes | | Yes | Yes | No | Yes | No |
| | Car-PDMS | Car-PDMS | Car-PDMS | Car-PDMS | Car-PDMS | Car-PDMS | Car-PDMS | Car-PDMS |
| | No | | Yes | Yes | No | No | | Yes |
| | 0 min | | | | 10 min | | | |
| | 20 min | 40 min | 40 min | | | 40 min | | 20 min |
| **)** | 40 °C | 70 °C | 40 °C | 70 °C | 40 °C | 70 °C | 70 °C | 70 °C |

**Table 4.** Figures of merit for the HS-SPME/GC-MS methodology to quantify VOC in samples ranging from wort to beer (* indicates compounds not considered in DSD analysis).

| $t_R$ (minutes) | Analyte | Kovats index | Identification/ quantification mode (m/z) | Concentration range in literature | Matrix effect (%) | Linear range | $R^2$ | LOD | LOQ |
|---|---|---|---|---|---|---|---|---|---|
| 4.66 | **Acetaldehyde (mg/L)** | 676 | 29, 42, **43**, 44, 45 | 1.7-40 (Brányik, Vicente, Dostálek, & Teixeira, 2008; Charry-Parra, Dejesus-Echevarria, & Perez, 2011; Kunze & Manger, 2010; Preedy, 2009) | 27.21 | 1.26-150.89 | 0.999997 | 0.35 | 1.18 |
| 5.04 | **Dimethyl sulfide (µg/L)*** | 740 | 47, 61, **62** | 2-215 (Preedy, 2009; Scarlata & Ebeler, 1999; Svoboda, Mikulíková, Běláková, & BeneŠOvÁ, 2017) | 11.02 | 1.00-112.31 | 0.999999 | 0.20 | 0.68 |
| 5.63 | **Isobutyraldehyde (µg/L)*** | 810 | 41, 43, **72** | 3.8-146.8 (Malfliet, Van Opstaele, de Clippeleer, Syryn, Goiris, de Cooman, et al., 2008; Moreira, Meireles, Brandao, & de Pinho, 2013; Saison, De Schutter, Delvaux, & Delvaux, 2008) | 4.46 | 1.00-100.02 | 0.999997 | 0.23 | 0.77 |
| 6.71 | **Ethyl acetate (mg/L)** | 887 | **43**, 61, 70, 88 | 0.29-60.9 (Jeleń, Wlazły, Wąsowicz, & Kamiński, 1998; Kleinová, Geršl, & Mareček, 2015; Kunze & Manger, 2010) | 15.94 | 4.99-62.46 | 0.999921 | 729.24 | 2.43 |
| 8.67 | **Diacetyl (µg/L)** | 976 | 42, 43, **86** | 8-1180 (Brányik, Vicente, Dostálek, & Teixeira, 2008; Briggs, Hough, Stevens, & Young, 1999; Kunze & Manger, 2010) | 2.34 | 10.00-999.78 | 0.999997 | 2.72 | 9.06 |
| 9.58 | **Isobutyl acetate (µg/L)** | 1014 | **43**, 56, 73 | 30-10120 (Jeleń, Wlazły, Wąsowicz, & Kamiński, 1998; Kunze & Manger, 2010; Nykänen & Suomalainen, 1983) | 13.10 | 100.05-2501.15 | 0.999899 | 33.54 | 111.79 |
| 10.24 | **Ethyl butyrate (µg/L)** | 1044 | 60, **71**, 88 | 40-300 (Boulton & Quain, 2001; Kunze & Manger, 2010; Phiarais, Mauch, Schehl, Zarnkow, Gastl, Herrmann, et al., 2010) | 15.73 | 20.30-406.00 | 0.999824 | 7.04 | 23.46 |
| 10.94 | **2,3-pentanedione (µg/L)** | 1074 | 43, 45, 57, **100** | 10-900 (Briggs, Hough, Stevens, & Young, 1999; Krogerus & Gibson, 2013; Leça, Pereira, Vieira, Reis, & Marques, 2015) | 17.43 | 25.05-500.56 | 0.999901 | 6.89 | 22.95 |
| 11.15 | **Ethyl isovalerate (µg/L)** | 1083 | 57, 85, **88** | Not available; it was established by determining its concentration in wort, fermenting wort and beer samples by external addition method | 0.76 | 1.00-25.06 | 0.999989 | 0.11 | 0.36 |
| 12.12 | **Isobutyl alcohol (mg/L)** | 1123 | 41, 42, **43**, 55, 74 | 0.3-59.92 (Charry-Parra, Dejesus-Echevarria, & Perez, 2011; Cheong, Wackerbauer, & Kang, 2007; Jeleń, Wlazły, Wąsowicz, & Kamiński, 1998; Kunze & Manger, 2010) | 3.46 | 1.00-49.86 | 0.999990 | 0.23 | 0.76 |
| 12.96 | **Isoamyl acetate (mg/L)** | 1157 | 61, **70**, 87 | 0.08-6.6 (Charry-Parra, Dejesus-Echevarria, & Perez, 2011; Kunze & Manger, 2010; Saison, De Schutter, Delvaux, & Delvaux, 2008) | 0.77 | 0.20-5.00 | 0.999967 | 0.04 | 0.13 |
| 15.86 | **Isoamyl alcohol (mg/L)** | 1249 | 42, **55**, 70 | 2.5-70 (Charry-Parra, Dejesus-Echevarria, & Perez, 2011; Kunze & Manger, 2010; Preedy, 2009) | 3.52 | 1.25-149.99 | 0.999997 | 0.37 | 1.24 |

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| 16.67 | **Ethyl hexanoate (µg/L)** | 1270 | 60, **88**, 99 | 50-1500 (Jeleń, Wlazły, Wąsowicz, & Kamiński, 1998; Kunze & Manger, 2010; Preedy, 2009; Saerens, Delvaux, Verstrepen, & Thevelein, 2010) | 7.88 | 20.03-500.83 | 0.999961 | 4.38 | 14.58 |
| 18.69 | **Acetoin (mg/L)** | 1323 | 43, **45**, 88 | 1-86 (Baxter, Hughes, & .Hornsey, 2001; Kunze & Manger, 2010) | 32.35 | 1.01-75.60 | 0.999991 | 0.33 | 1.09 |
| 23.44 | **Ethyl caprylate (µg/L)** | 1429 | **88**, 101, 127 | 40-4000 (Preedy, 2009; Saerens, Delvaux, Verstrepen, & Thevelein, 2010) | 13.60 | 199.99-4999.68 | 0.999994 | 18.72 | 62.41 |
| 26.73 | **Trans-2-nonenal (µg/L)** | 1477 | **55**, 70, 83 | 0.28-20.28 (Moreira, Meireles, Brandao, & de Pinho, 2013; Svoboda, Mikulíková, Běláková, BeneŠOvÁ, Marova, & Nesvadba, 2010) | 20.75 | 0.50-62.60 | 0.999999 | 0.11 | 0.37 |
| 29.74 | **Ethyl decanoate (µg/L)** | 1552 | **88**, 101, 155 | 20-2550 (Hiralal, Pillay, & Olaniran, 2013; Horak, et al., 2010) | 2.12 | 99.99-749.94 | 0.999646 | 20.42 | 68.05 |
| 31.38 | **Isovaleric acid (µg/L)** | 1612 | 41, **60**, 87 | 100-3400 (Cheong, Wackerbauer, & Kang, 2007; Jackson & Linskens, 2002; Kunze & Manger, 2010) | 24.20 | 199.98-2499.74 | 0.999893 | 32.90 | 109.68 |
| 35.00 | **Phenylethyl acetate (µg/L)** | 1731 | 43, 91, **104** | 100-6790 (Hiralal, Olaniran, & Pillay, 2014; Saerens, Delvaux, Verstrepen, & Thevelein, 2010) | 43.54 | 96.18-4809.12 | 0.999993 | 18.57 | 61.91 |
| 37.60 | **Phenylethyl alcohol (mg/L)** | 1828 | **91**, 92, 122 | 2.76-138.11 (Kunze & Manger, 2010; Preedy, 2009; Riu-Aumatell, Miró, Serra-Cayuela, Buxaderas, & López-Tamames, 2014) | 16.21 | 1.25-149.94 | 0.999993 | 0.56 | 1.87 |
| 41.55 | **Octanoic acid (µg/L)** | 1983 | **60**, 73, 101 | 2000-12000 (Cheong, Wackerbauer, & Kang, 2007; Kunze & Manger, 2010) | 9.22 | 350.35-10010.00 | 0.999979 | 65.59 | 218.65 |
| 44.56 | **Vinyl guaiacol (µg/L)** | 2109 | 107, 135, **150** | 7-1112 (Pizarro, Pérez-del-Notario, & González-Sáiz, 2010; Vanbeneden, Delvaux, & Delvaux, 2006; Vanbeneden, Saison, Delvaux, & Delvaux, 2008) | 16.44 | 11.12-1112.00 | 0.999999 | 1.97 | 6.57 |