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Headspace - SPME as a screening system for yeast metabolic compounds - A case study

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Abstract

Aim: This study aimed to identify and quantify the fusel alcohols secreted by the opportunistic yeasts *Candida albicans* and *Candida dubliniensis* to further get insights into the amino acid catabolism of those two organisms.

Methods and Results: Fusel alcohols were screened in cell - free supernatants of yeasts cultivated in RPMI medium. For simultaneous identification and quantification of these alcohols, a Headspace - Solid Phase Micro Extraction (HS - SPME) coupled with gas chromatography - mass spectrometry (GC - MS) methodology, previously developed to analyse the volatile composition of fruits, was followed (Coelho et al. 2006). Results obtained show that both *C. albicans* and *C. dubliniensis* produced isoamyl alcohol and 2-phenylethanol, end – products of the catabolism of the amino acids leucine and phenylalanine, respectively.

Significance and Impact of the study: The HS – SPME used herein allows the simultaneous identification and quantification of compounds, requires small sample volume, has high sensitivity and reproducibility, is simple and rapid and is solvent free. This revealed to be a powerful tool for the screening system of yeast metabolic compounds.

1 Introduction

The recognition that the catabolism of amino acids to their respective fusel alcohols plays an important role in (off-) flavour and aroma of yeast fermented products motivated the scientific interest in this field. Whereas in most eukaryotes the carbon skeletons derived from amino acids enter the tricarboxylic acid cycle, in the yeast *Saccharomyces cerevisiae* the degradation of the aromatic amino acids (tryptophan, phenylalanine and tyrosine) and the branched-chain amino acids (valine, leucine and isoleucine) occur through the Ehrlich pathway (recently reviewed by Hazelwood et al. 2008). This pathway comprises an initial step of transamination of the amino acid to the corresponding α-keto acid, followed by its decarboxylation to the respective aldehyde and the reduction of the aldehyde to form the corresponding fusel alcohol. Research developed so far aimed to identify (1) the end - products of amino acids catabolism, (2) the enzyme(s) involved in the metabolic process and (3) the bioactivities of fusel alcohols (Dickinson 1996;Dickinson et al. 1997;Dickinson et al. 2003).

Despite the efforts done to identify metabolites in yeast/ fungal extracts, there is a scarce knowledge concerning the metabolism of amino acids in *Candida albicans* and *Candida dubliniensis*, two closely related opportunistic fungal pathogens. A number of techniques have been employed for fungal metabolites profiling including paper chromatography, thin layer chromatography, capillary electrophoresis and the mainly applied high performance

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liquid chromatography (HPLC) and gas chromatography - mass spectrometry (GC – MS) (Frisvad et al. 2008). Although chromatographic methods are very sensitive and selective their major disadvantage is the need of sample preparation. Traditional methods used for sample concentration and purification are time consuming, expensive and require a large volume of hazardous organic solvents (Lanças 2003). However, in the beginning of the last decade solid phase microextraction (SPME) technology was introduced to overcome some of these difficulties. SPME is a microextraction technique based in a equilibrium partition of the compounds between the sample matrix and the extracting phase, a polymer-coated fused fiber and can be directly coupled with HPLC or GC - MS (Arthur and Pawliszyn 1990). This technique was successfully applied for polar and non - polar compounds in gas, liquid and solid samples from environmental or biological sources (Ulrich 2000).

The objective of this work was to identify and quantify the fusel alcohols: isoamyl alcohol and 2-phenylethanol, secreted by *C. albicans* and *C. dubliniensis*, using headspace - SPME coupled with GC – MS, to further get insights into the amino acid catabolism of those two microorganisms.

2 Material and Methods

Chemicals. Standard alcohols were obtained from Sigma. Isoamyl alcohol stock solution was prepared in water whereas the 2-pheneylethanol was prepared in methanol.

Organisms. Two *Candida* species were used in this study: *C. albicans* CECT 1472 (Colección Española de Cultivos Tipo, Spain) and *C. dubliniensis* CBS 7987 (Centraalbureau voor Schimmelcultures, Germany).

Media and culture conditions. Prior to each experiment, both *Candida* species were maintained at 37°C in Sabouraud dextrose agar. After, cells were grown in Sabouraud dextrose broth at 37°C for 24 h on a mechanical shaker at 130 rpm. Subsequently, cells were washed twice with ultrapure water and counted in a hematocytometer. Standardized cell suspensions were prepared at a cell density of 1×10^6 cells/ml in 1×RPMI 1640 medium (Sigma) buffered with morpholinepropanesulfonic acid (MOPS– Sigma) (final concentration 0.165 M for pH 7.0). For culture supernatants preparation, 120 ml of standardized cell suspensions were inoculated in 300 ml flasks and incubated at 37°C, 130 rpm. After 24 h cell – free supernatant fractions were filter sterilized (0.22 µm) and stored at 4°C.

Biomass dry weight measurements. At the end of incubation, 1 ml of the planktonic culture was filtered through pre-weighted filters (0.45 µm) and washed three times with ultrapure sterilized water. Filters were dried at 60°C until constant weight and cell dry weight (CDW) was determined. This was repeated at least four times.

Supernatant alcohols analyses. Supernatant fusel alcohols were extracted using HS - SPME and analysed by GC - MS using a method adapted from Coelho et al. (2006). Supernatant fractions (20 ml) were introduced into a 60 ml bottle, together with 4 g of NaCl and a 20 × 5 mm stirring bar. After sealing the vial with a teflon septum and an aluminium cap, it was placed in a water bath at 40±0.1 ºC for a period of 15 min (200 rpm) to transfer the compounds from the sample to the headspace. The headspace volatiles were extracted along 45 min with a SPME carbowax-divinylbenzene fused silica fibre (CW-DVB, 65 µm- Lot P334957) (Supelco). The GC - MS parameters were established according to Coelho *et al*. (2006). *Candida* fusel alcohols were identified by comparison of their mass spectra and retention times with those of data system library (Wiley 275) and of reference substances. A control analysis was done with growth medium. Compounds quantification was performed by preparation of RPMI solutions containing pure standards and using the same conditions applied to samples. For each compound appropriate concentration ranges were chosen in order to include sample concentrations. All measurements were made with, at least, two

replicates. Blanks, corresponding to the analysis of the coating fibre not subjected to any extraction procedure, were run between sets of three analyses. Alcohols analyses were carried out in duplicate for each sample with results agreeing within ≤ 15 %.

3. Results and Discussion

RPMI 1640 medium was chosen as growth medium. This medium contains all 20 amino acids and nitrate but not ammonium ions. This combination may force *Candida* cells to get its nitrogen from amino acids and in the process secrete fusel alcohols such as isoamyl alcohol (from leucine) and 2-phenylethanol (from phenylalanine). Establishment of the HS - SPME methodology for the identification and quantification of these compounds in *C. albicans* and *C. dubliniensis* cell – free supernatants will be presented and discussed below.

Establishment of the HS - SPME GC - MS methodology

Fiber selection. The fiber polarity, porosity and surface area are critical parameters in SPME, thus several types of coating fibers with different film thicknesses are commercially available (1998b). In this study, a 65 µm CW - DVB fused silica fibre was used. The CW - DVB coating fibre is recommended for small and polar molecules (molecular weight between 40 and 275). The CW - DVB coating fibre was selected because it is a mixed coating that contains a liquid polymer and solid particles. This type of coating combines the absorption properties of the liquid polymer with the adsorption properties of porous particles, which are composed of macro (>500 Å), meso (20-500 Å) and microporous (2-20 Å). The mutually synergetic effect of adsorption and absorption to the stationary phase promotes a high retention capacity and, consequently, a higher sensitivity than fibres based on absorption only (Kataoka et al. 2000).

Extraction mode. Compounds extraction can be achieved exposing the fiber in the headspace (vapour phase) surrounding the sample, headspace (HS) - SPME, or introducing it directly in the sample matrix, direct immersion (DI) - SPME (Zhang and Pawliszyn 1993). In HS - SPME mode equilibrium is attained more rapidly and the prevention of the fiber direct contact with the sample minimizes background noise and can extend the fiber life time. Additionally, HS - SPME is more sensitive for the analysis of volatile compounds of complex matrices (Kataoka et al. 2000). In this work the HS - SPME mode was used. In a three system analysis (sample – headspace – CW - DVB coating fiber), the transference occurs from the liquid matrix to the headspace, and from that to the coating fiber, which promote the sorption of the compounds in the coating fiber.

Extraction conditions and sample modification. In general, sample volumes used in SPME extraction range from 4 to 120 ml (Krutz et al. 2003). In contrast, traditional techniques such as the described by Hornby et al. (2001) for the purification of farnesol from *C. albicans* cell – free supernatant require larger sample volume, specifically five litres. In SPME the amount of compound extracted and/or the extraction time can be increased by manipulation of some parameters. First, stirring liquid samples facilitates the transfer of the compounds to the coating fiber. Second, as a typical guideline, sample modification by the addition of a soluble salt such as NaCl or Na_2SO_4 decreases compound solubility in the matrix, favouring its extraction onto the fiber due to the salting – out effect. Third, for HS - SPME the compound concentration in the vapour phase is dependent on the extraction temperature, and samples are usually heated (Kataoka et al. 2000). Thus, over this study, all samples were extracted using a magnetic stirring for agitation that was maintained at 200 rpm, after the addition of 4 g of NaCl of 20 ml of sample and in a water bath operating at 40±0.1 ºC. In order to achieve accurate and precise analyses, sampling parameters such as sampling time, vial size, magnetic bar size and sample volume were kept constant (see Material and Methods section) intra and inter assays. The extraction procedure was completed within one hour/ sample, demonstrating that SPME is a rapid technique.

Fusel alcohols discrimination. Prior to sample analysis, the methodological approach described was tested using a standard mixture containing isoamyl alcohol and 2-phenylethanol (Figure 1). Under the GC - MS conditions used (Coelho et al. 2006), the signal (total ion current) obtained exhibited resolved peaks at 9.5 min for isoamyl alcohol (Figure 1, I) and at 55.7 min for 2-phenylethanol (Figure 1, II), demonstrating that this is a rapid screening procedure for the simultaneous extraction and identification of these compounds.

.External standardization. The quantitation approach when using SPME is dependent on the sample matrix, its complexity and extraction method being used. In the case of a liquid and simple sample an external standard calibration is recommended (1998a). Standard mixtures were prepared in a clean matrix sample (RPMI medium) and analysed under the same conditions as those of samples. This allows the GC peak area data to be used as an indirect approach to estimate the relative content of each compound. Standard curves were generated for GC - MS peak areas versus concentration of each compound (R^2>0.98). Estimated limit of quantification (LOQ) and limit of detection (LOD) were: 0.08 mg/l and 0.02 mg/l for isoamyl alcohol and 0.04 mg/l and 0.01 mg/l for 2-phenylethanol. Thus, using quantitative HS – SPME GC – MS a linear response was obtained for a wide range of concentrations and the LOD levels were found suitable for the analysis (see below).

HS - SPME GC - MS *Candida* sample analyses

Qualitative analysis. The growth medium was analysed for possible interference and was shown to have no interfering compounds into or near the retention times of those compounds (Figure 2, I). Figure 2 (II) shows a typical chromatogram obtained for *C*. *albicans* and *C. dubliniensis*. Comparison of GC retention times and mass spectra of pure standard compounds (such as those presented in Figure 1) allowed the identification of isoamyl alcohol and 2-phenylethanol in these two samples, showing that these two fusel alcohols are produced and secreted into the extracellular medium by *C. albicans* and *C. dubliniensis*.

Quantitative analysis. The detector response from the sample was compared to the response from the target compounds in the calibration curve. Values were standardized against cell dry weight. In *C. albicans* cell - free supernatants isoamyl alcohol was found at 5.65 mg/ g of CDW and 2-phenylethanol at 0.90 mg/ g of CDW. For *C. dubliniensis*, fusel alcohols concentrations (mg/ g of CDW) were: 6.06 for isoamyl alcohol and 1.88 for 2-phenylethanol. This evidences that quantitative HS – SPME GC – MS using matrix-matched calibration curves allowed determination of both fusel alcohols in *Candida* spp samples.

Conclusion

The HS - SPME is a microextraction technology that combines sample preparation and pre-concentration into one single step. The HS – SPME strengths include: (i) the simultaneous identification and quantification of compounds, (ii) small sample volume requirements, (iii) high sensitivity and reproducibility, (iv) simplicity, (v) speed, (vi) no solvent needs. Moreover, it is compatible with compound separation and detection by several systems and it can be automated. This sampling technology revealed to be a powerful tool for the screening system of yeast metabolic compounds.

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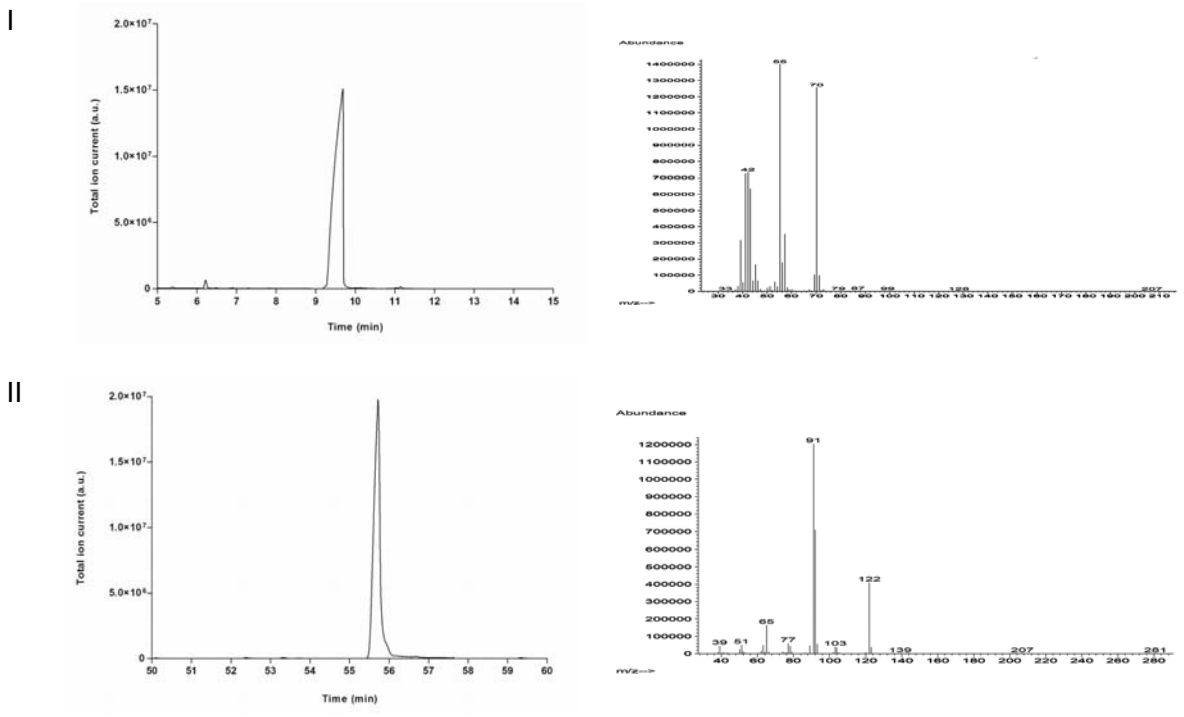

Figure 1: Discrimination of fusel alcohols using HS - SPME GC - MS. Typical chromatograms (total ion current) (A) and mass spectrum (B) of isoamyl alcohol (I) and 2-phenylethanol (II) obtained for a standard solution.

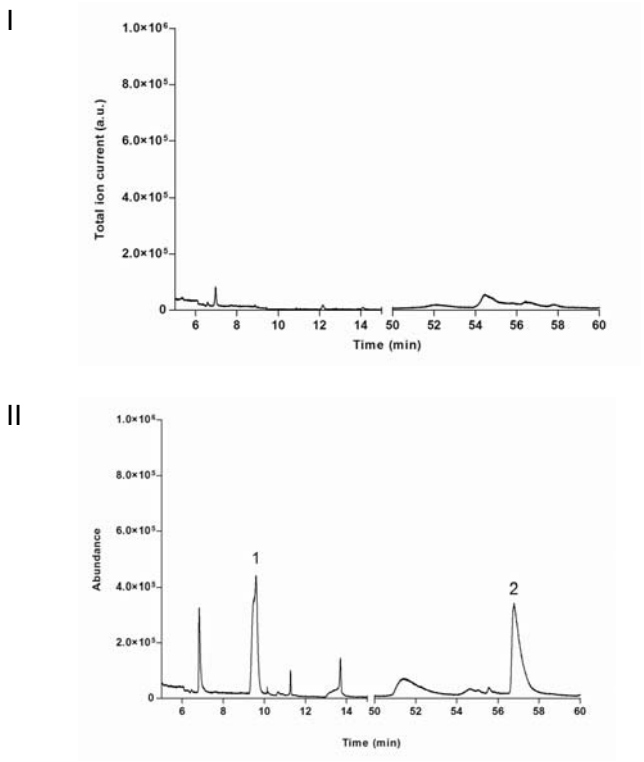

Figure 2: Typical HS - SPME-GC – MS chromatograms (total ion current) for the determination of the fusel alcohols 1, isoamyl alcohol and 2, 2-phenylethanol in *C. albicans* and *C. dubliniensis* samples inoculated in RPMI medium showing no interfering peaks at fusel alcohols retention times (I) and a cell - free supernatant sample (II).