

# focus on Chromatography

## Establishment of the Saliva Volatome Profile as an Exploratory and Non-invasive Strategy to Find Potential Breast Cancer Biomarkers

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Breast cancer (BC) is a major health problem worldwide. It is the most common malignancy in women and the second cause of cancer-related mortality. BC late diagnosis is a major cause of BC morbidity and improved diagnosis tools are needed. Here we present a sensitive assay aiming to identify volatile organic metabolites (VOMs) as biomarkers of BC development using saliva samples. Saliva is a promising source of VOMs that can be used as potential cancer biomarkers. The extraction of saliva VOMs was performed using dynamic headspace solid phase microextraction (dHS-SPME). Several parameters that influence the extraction were tested. These included the nature of the adsorptive phase, sampling temperature, extraction time, volume, pH, salt concentration and agitation.

### Introduction

Cancer is characterised by abnormal growth and development of normal cells beyond their natural boundaries. In the last 50 years, and despite the global efforts to decrease the incidence of this disease, cancer has become the leading cause of death. Particularly, breast cancer (BC) is the most common malignancy and the second most common cause of cancer-related mortality in women [1].

The diagnosis at an early stage allows the management of high-risk cancers and a much better prognosis, but to allow this it is necessary to find specific and sensitive biomarkers. In certain circumstances some molecules are differentially expressed in cancer cells relative to their normal counterparts, and their altered levels can be measured to establish a correlation with the diseased state [2,3]. A promising class of biomarkers for cancer early diagnosis are the volatile organic metabolites (VOMs) [4]. Saliva contains VOMs that can be used as potential cancer biomarkers and its sampling is easy and non-invasive. Moreover, when compared with blood, saliva contains less proteins, thus decreasing any potential risk of non-specific interference and hydrostatic interactions [5]. Therefore, the aim of this study was to establish the saliva volatome profile between BC patients and healthy individuals, and to explore VOMs as potential biomarkers in BC diagnosis at early stage. Dynamic headspace solid-phase microextraction (dHS-SPME) using Carboxen®/polydimethylsiloxane (CAR/PDMS) sorbent was selected as a powerful and highly sensitive strategy to isolate VOMs from saliva. Combined with GC-qMS, it was used to obtain metabolomic information patterns of BC patients and healthy individuals. Additionally, several parameters that influence the extraction such as the nature of the adsorptive phase, sampling temperature, extraction time, volume, pH, salt concentration and agitation were tested.

### Methods dHS-SPME Procedure

The saliva samples used in the optimisation of the dHS-SPME parameters were from normal subjects and kept at -80°C, being thawed at room temperature before use. For the selection of the best fibre, five SPME fibres (Supelco) with different stationary phases and film thicknesses were tested. These include polydimethylsiloxane (PDMS) (100 µm), polyacrylate (PA) (85 µm), divinylbenzene/Carboxen®/polydimethylsiloxane (DVB/CAR/PDMS) (50/30 µm), Carboxen®/polydimethylsiloxane (CAR/PDMS) (75 µm), and polydimethylsiloxane/divinylbenzene (PDMS/DVB) (65 µm). In the dHS-SPME optimisation, 2 ml saliva aliquots were placed in 4-ml headspace glass vials, adjusted to pH 1–2 with 0.25 ml of 5.0 M HCl, 0.2 g of NaCl added and stirred at 800 rpm. The sample vial was placed in a thermostat bath adjusted to 38.0 ± 0.1°C and the SPME fibre was introduced into the headspace for 45 min. After sampling, the SPME fibre was withdrawn into the needle, removed from the vial and inserted into the injector port (250°C) of the GC-qMS system for 6 min. The metabolites were thermally desorbed and transported directly to the analytical column. Each sample was analysed in duplicate.

A CAR/PDMS fibre was used to study the temperature and the time of extraction parameters. Extraction was performed at temperatures of 28, 38 and 48°C for 30, 45, and 60 min with a stirring rate of 800 rpm. The optimum conditions were determined by the totality of the peak areas obtained in each parameter, number of compounds extracted and reproducibility. The experimental design is represented in Figure 1.

### GC-qMS Analysis

The gas chromatograph (GC) system was composed of an Agilent Technologies 6890N Network (Palo Alto, CA, USA) equipped with a 30.0 m x 250 µm ID x 0.25 µm film thickness BP-20 (SGE, Dortmund, Germany) fused silica capillary column and interfaced with an Agilent 5975 quadrupole inert mass selective detector (MS). The oven temperature program was as follows: initial 60°C held for 5 min then ramped 2°C/min up to 150°C, held for 5 min, again ramped 15°C/min to 220°C, and held for 1 min. The total GC run time was 60 min. The column flow was maintained constant at 1 ml min<sup>-1</sup> using He (Helium N60, Air Liquide) as the carrier gas.

The injection port was operated in the splitless mode and held at 250°C. For the MS system, the operating temperatures of the transfer line, quadrupole and ionisation source were 270, 150 and 230°C, respectively. Electron impact mass spectra were recorded at a 70 eV ionisation voltage and the ionisation current was 10 µA. The acquisitions were performed in Scan mode (30–300 m/z), and the electron multiplier was set to the auto tune procedure. Metabolite identification was accomplished through manual interpretation of spectra and matching against the Agilent MS ChemStation Software (Palo Alto, CA, USA). This software was equipped with an NIST05 mass spectral library.

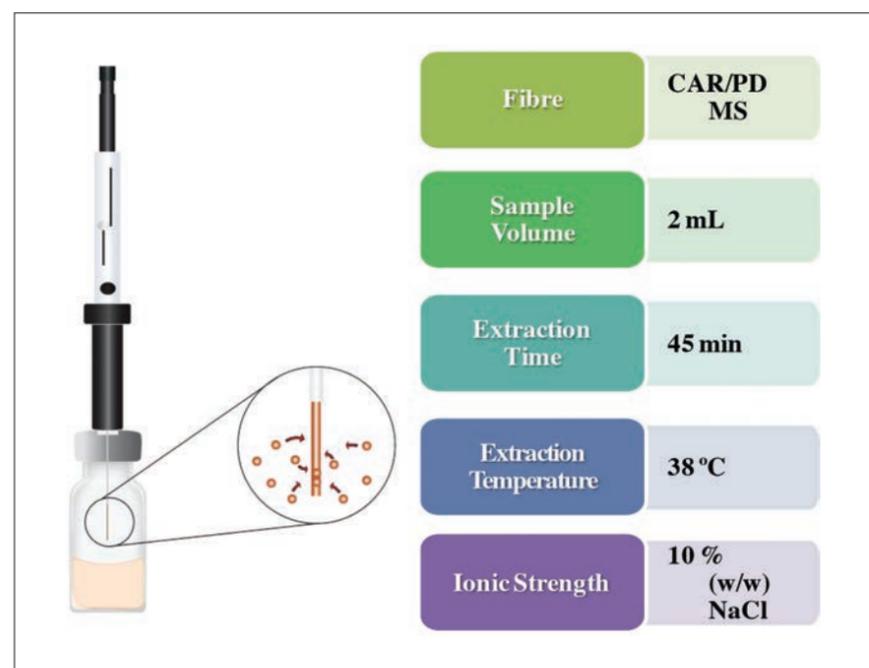


Figure 1. Experimental design

## Results and Discussion

This study involved the optimisation of the best experimental conditions to extract volatile metabolites from the saliva samples using HS-SPME. This was followed by a comparison of the metabolomic patterns from saliva of BC patients and healthy volunteers. This was achieved by the identification of the metabolites and through peak area differences.

### Optimisation of the dHS-SPME parameters

The optimisation of the parameters involved in HS-SPME was executed by selecting the conditions that achieved the maximum response in terms of metabolite peak area, number of metabolites detected and reproducibility.

### Fibre Selection

To select the most appropriate fibre to isolate volatile metabolites from saliva, five SPME fibres were tested. By comparing all of the tested fibres in terms of peak areas and the number of identified metabolites, the best efficiency was obtained using a CAR/PDMS coating as observed in Figure 2, whereas the lowest efficiency was obtained using the PDMS and PA fibres. Thus, the CAR/PDMS fibre was chosen as the SPME fibre for the following optimisation studies.

### Extraction Temperature and Time

Using an extraction time of 45 min with a 2 mL saliva sample, the effect of extraction temperature on SPME extraction efficiency was investigated at 28, 38 and 48°C. The best results were obtained at 38°C since the number of identified metabolites was higher.

To evaluate the influence of extraction time on the extraction capacity, different extraction times (30, 45 and 60 min) were tested using the previously selected fibre (CAR/PDMS) and 38°C. Of the three extraction times tested, the one with higher extraction efficiency was 45 min (Figure 2).

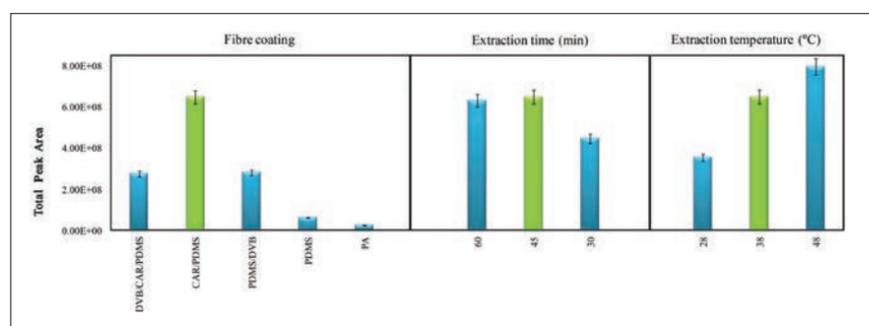


Figure 2. Influence of fibre coating, extraction time and temperature on the extraction efficiency of VOMs

### Ionic Strength and pH

Several sodium chloride concentrations were assayed (0, 5, 10, 15 and 20% (w/v)), the best results obtained were with a concentration of 10%. In order to study the influence of the sample pH on the efficiency of the HS-SPME extraction, samples were prepared at neutral, acidic (5M HCl) and basic (1M NaOH) conditions. As observed in Figure 3 acidic pH facilitates the extraction of volatile compounds.

### Volume Sample and Stirring

Three sample volumes (1, 2 and 4 mL) were analysed and it was found that the 2 mL samples allowed a more efficient extraction. Finally, two more assays were performed, extraction with and without agitation. It was verified that the agitation facilitated the extraction of the volatile compounds present in saliva (Figure 3). In summary, the best extraction conditions for the determination of the volatile profile of the saliva from BC patients and healthy volunteers were 2 mL saliva samples containing 10% of NaCl (w/v) and acidified to pH 1, extracted at a 38°C during 45 min, using a CAR/PDMS fibre and 800 rpm stirring.

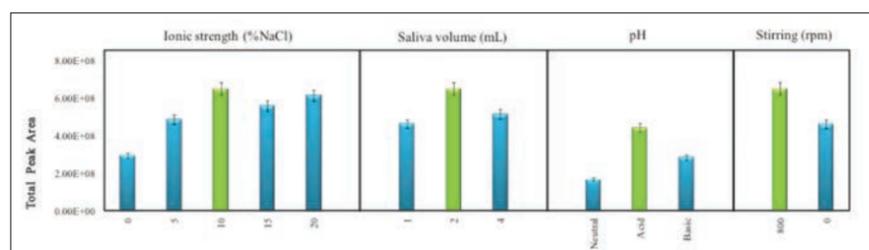


Figure 3. Effect of ionic strength, sample volume, pH and stirring on the extraction efficiency of VOMs

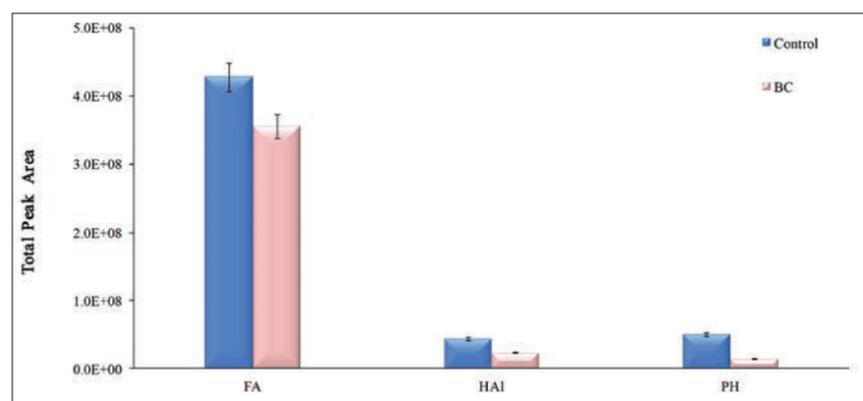
## Characterisation and Comparative Analysis of Salivary VOMs

Following the optimisation of the best extraction conditions, salivary volatile metabolites were analysed using dHS-SPME in combination with GC coupled to mass spectrometry with the aim to distinguish individuals with BC from healthy subjects. Different profiles for healthy subjects and BC patients were recognised, with significant differences in the fatty acids, higher alcohols and phenols (Figure 4).

The major metabolites identified in both groups were acetic acid, 2-ethyl-1-hexanol, propanoic acid, butanoic acid, methoxyphenyl-oxime and phenol (see Figure 5).

Overall, phenol and 2-ethyl-1-hexanol were the compounds that presented the greater difference between the healthy individuals and BC patients studied.

Figure 4. Distribution of major chemical families by healthy individuals (Control) and BC patients.



FA- Fatty Acids; HAI- Higher Alcohols; PH- Phenols

## Conclusions

Saliva presents significant advantages as a diagnostic tool when compared with blood or urine. Saliva sampling is easy, safe, and non-invasive and may be collected repetitively without discomfort to the patient. Here we optimised the characterisation of the volatile composition of saliva using dHS-SPME followed by GC-qMS analysis. Different experimental factors were evaluated and the most effective extraction was achieved using 2 ml of acidified saliva sample containing 10% NaCl (w/v), stirred and exposed to the CAR/PDMS fibre for 45 min, at 38°C. Preliminary results point to higher abundances of fatty acids, higher alcohols and phenols in saliva samples from healthy subjects than BC patients. Ongoing studies are focusing in individual volatiles that could be used as cancer biomarkers. This work reveals the potential of saliva as a promising biological fluid for cancer biomarkers research, particularly its suitability for the early disease diagnosis.

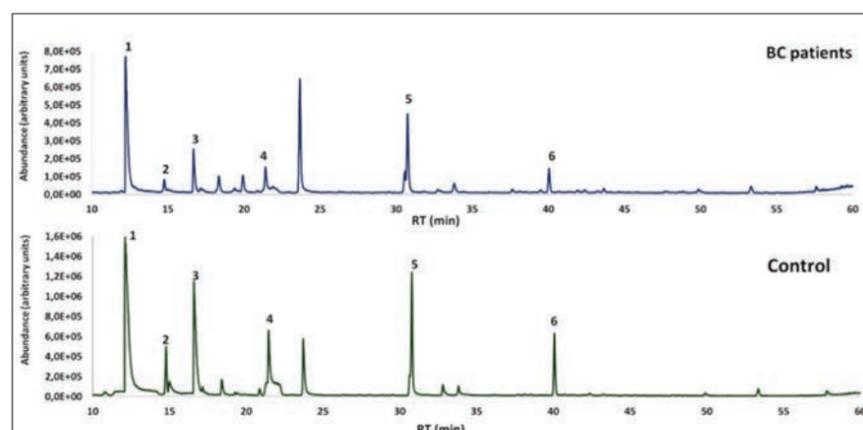


Figure 5. GC-qMS chromatograms from BC patients and healthy subjects (control) (major compounds: 1. Acetic acid; 2. 2-Ethyl-1-hexanol; 3. Propanoic acid; 4. Butanoic acid; 5. Methoxyphenyl-oxime; 6. Phenol).

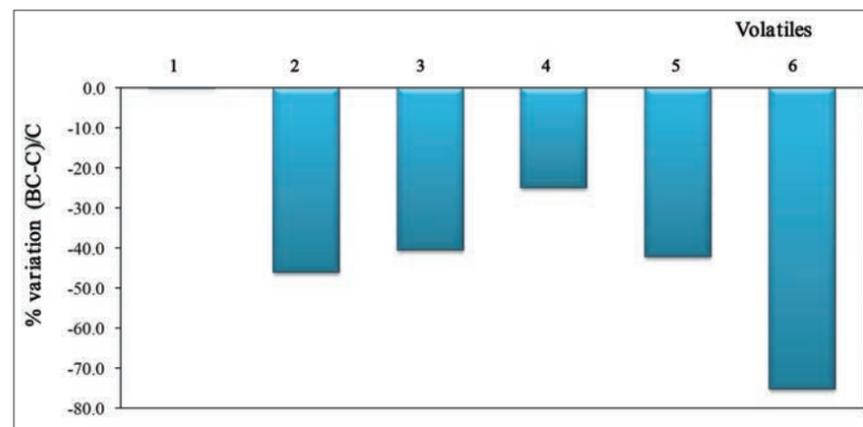


Figure 6. Variation of the main VOMs identified in healthy individuals and BC patients (major compounds: 1. Acetic acid; 2. 2-Ethyl-1-hexanol; 3. Propanoic acid; 4. Butanoic acid; 5. Methoxyphenyl-oxime; 6. Phenol).

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