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Solid phase microextraction, mass spectrometry and metabolomic approaches for detection of potential urinary cancer biomarkers—A powerful strategy for breast cancer diagnosis

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ABSTRACT

A sensitive assay to identify volatile organic metabolites (VOMs) as biomarkers that can accurately diagnose the onset of breast cancer using non-invasively collected clinical specimens is ideal for early detection. Therefore the aim of this study was to establish the urinary metabolomic profile of breast cancer patients and healthy individuals (control group) and to explore the VOMs as potential biomarkers in breast cancer diagnosis at early stage. Solid-phase microextraction (SPME) using CAR/PDMS sorbent combined with gas chromatography–mass spectrometry was applied to obtain metabolomic information patterns of 26 breast cancer patients and 21 healthy individuals (controls). A total of seventy-nine VOMs, belonging to distinct chemical classes, were detected and identified in control and breast cancer groups. Ketones and sulfur compounds were the chemical classes with highest contribution for both groups. Results showed that excretion values of 6 VOMs among the total of 79 detected were found to be statistically different ($p < 0.05$). A significant increase in the peak area of (−)-4-carene, 3-heptanone, 1,2,4-trimethylbenzene, 2-methoxythiophene and phenol, in VOMs of cancer patients relatively to controls was observed. Statiscally significant lower abundances of dimethyl disulfide were found in cancer patients. Bioanalytical data were submitted to multivariate statistics [principal component analysis (PCA)], in order to visualize clusters of cases and to detect the VOMs that are able to differentiate cancer patients from healthy individuals. Very good discrimination within breast cancer and control groups was achieved. Nevertheless, a deep study using a larger number of patients must be carried out to confirm the results.

1. Introduction

Breast cancer is a major health problem that affects quality of life in many developed countries of the world [1,2]. Despite the global efforts to reduce the occurrence of this disease, cancer has become the leading cause of death in the last 50 years being breast cancer the most common malignancy in women and the second most common cause of cancer-related mortality [1,3]. Numerous risk factors for breast cancer have been identified, such as hormone-related, and the only well-established diet-related risk factors: obesity and alcohol consumption [2,4,5]. Other factors include inheritance of high-penetrance susceptibility genes, increasing age, exposure to ionizing radiation, family history of breast cancer, higher socioeconomic status, and prior benign breast disease [6]. Taking into consideration genetic susceptibility, two major genes are associated to breast cancer: BRCA1 and BRCA2. Mutations in either of these genes increase a lifetime risk of breast cancer up to 60% and 85%. However, mutations in these genes account for only 2% to 3% of all breast cancers, and susceptibility alleles in other genes, such as TP53, PTEN, and STK11/LKB1, are even less common causes of breast cancer [7].

Environmental factors, including exposure to xenobiotic compounds, diet, electromagnetic fields, and lifestyle have been the subject of numerous scientific inquiries [6]. According to LaKind et al. [6] the timing of exposure to environmental risk factors is an important consideration when studying breast cancer etiology.

The lack of selective and specific serum tumor markers for breast cancer creates many problems for its molecular diagnosis in the early stages, evaluation of curative effect, prognosis, and the monitoring of recurrence, metastasis, and biotherapy [8]. The better clinical outcomes associated with early detection highlighted the need for highly sensitive and specific techniques [9].

Currently available diagnostic techniques for breast cancer detection include direct examination of the cytomorphology of exfoliated cells, and the molecular analysis of tumor biomarkers in nipple aspirate fluid (NAF) or in ductal lavage [10]. However, these methods are time-consuming, painful for patients and require skilled medical staff.

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Over the last few years, urine and breath analysis for the routine monitoring of metabolic disorders has attracted a considerable amount of scientific interest, due to painless, non-invasive sampling, and can be performed as often as needed [11]. Thousands of VOMs in trace amounts are present in human breath [12–16] and different studies have shown that the VOMs profile in patients with lung cancer can be discriminated from those of healthy subjects [12,17,18]. Urine has been the preferred biological fluid since compounds are concentrated by the kidney before excretion [19]. The relative enrichment of volatile components makes urine an attractive target for a volatile metabolomic profiling approach. Urinary metabolomic studies have been applied to breast, lung, prostate, colorectal, and liver cancer [20].

In recent years, there has been an enormous effort to develop specific and sensitive biomarkers for precise and accurate screening, diagnosis, prognosis and monitoring of high risk cancer to aid therapeutic decisions [21,22]. Different methods such as chemical interaction have been developed to analyze volatile metabolites and to compare them in healthy subjects and cancer patients, adsorptive binding, cold trapping and supercritical fluid extraction. The most successful in this field are SPME and the recently developed multi-bed sorption trap [23–25]. The SPME technique was developed by Pawliszyn in late 1989 as a new preconcentration technology, in which a fused coated silica fiber is used as the stationary phase [20,26]. This methodology presents several advantages when compared with conventional solvent extraction procedures. SPME is rapid, easy to use, solvent free, sensitive, and does not require any concentration step prior to analysis, preventing the production of artifacts [27].

In this study, urine samples, collected at the Hospital Dr. Nélio Mendonça (Haemato-Oncology Unit), from clinically diagnosed patients with breast cancer ($n=26$) were analyzed and compared to healthy normal controls ($n=21$), to provide comprehensive information on the VOMs which can be selected as potential cancer biomarkers. A comparative analysis of the urinary metabolic profiles between cancer patients and normal controls was carried out. Multivariate statistical methods were used to verify the metabolomic differences between healthy and patient cases and find related volatile metabolites that could be associated with a type of cancer (e.g., breast). This identification is indispensible for future work on the biochemical sources of these compounds and their metabolic pathways.

2. Experimental

2.1. Chemicals and reagents

Sodium chloride and 4-methyl-2-pentanol were purchased from Panreac (Barcelona, Spain) and Sigma (St. Louis, MO, USA), respectively. Helium of purity 5.0 (Air Liquid, Portugal) was utilized as the GC carrier gas. The Cimarec™ digital stirring hot plate was supplied by Thermo Scientific (Waltham, MA, USA). SPME holder for manual sampling SPME fibers [(polydimethylsiloxane (PDMS), polyacrylate (PA), divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS), carbowax/divinylbenzene (CW/DVB), carboxen/polydimethylsiloxane (CAR/PDMS) and polydimethylsiloxane/divinylbenzene (PDMS/DVB)], glass vials were purchased from Supelco (Bellefonte, PA, USA). The SPME fibers were preconditioned as recommended by the manufacturer at some degrees below each fibers maximum temperature before being used for the first time. Before the first daily analysis, the fibers were conditioned for 5 min at the operating temperature in the GC injector port, and the blank level checked. Prior to the first daily analysis, the fibers were conditioned for 5 min at the operating temperature in the GC injector port. The analyses were performed in triplicate.

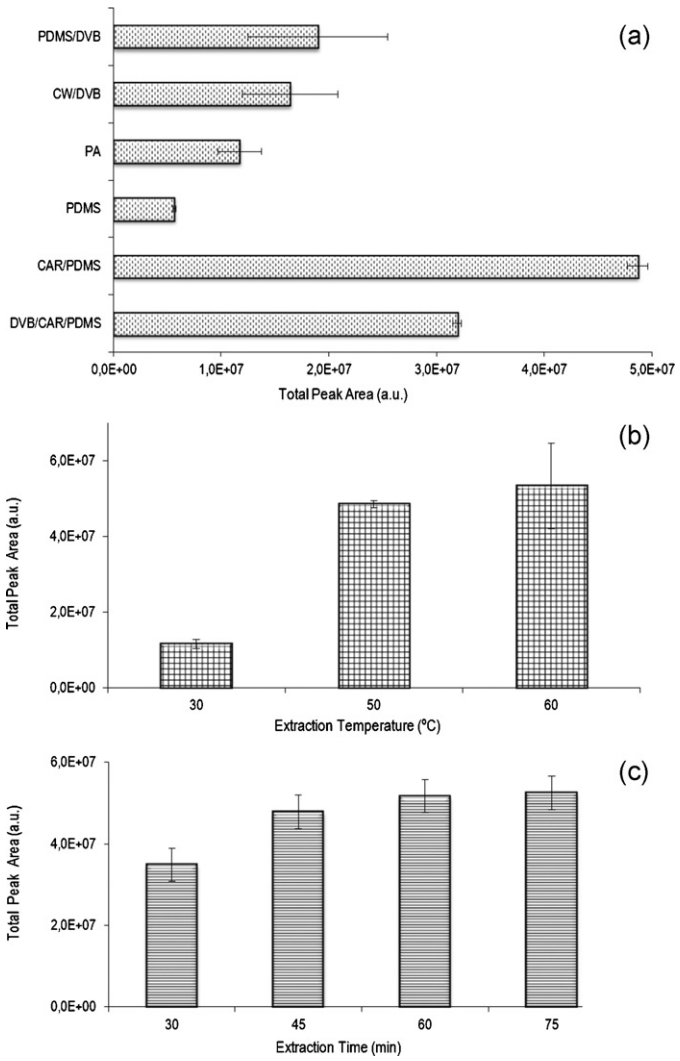

Fig. 1. Optimization of the SPME influencing-extraction parameters: (a) effect of fiber coatings (60 min of extraction time at 50 °C); (b) effect of extraction temperature (fiber: 75 μm CAR/PDMS; extraction time: 60 min); and (c) influence of the extraction time (fiber: 75 μm CAR/PDMS; extraction temperature: 50 °C), on SPME extraction efficiency of urinary volatile metabolites in a healthy individual. All assays were carried out with continuous stirring (800 rpm). Thermal desorption of metabolites were performed at 250 °C for 6 min.

2.2. Subjects and sample collection

The study included the following two groups: patients with breast cancer and healthy volunteers (controls). Normal controls ($n=21$, age = 44.2 ± 10.3 y (range 28–60 years), 18 male and 3 female) volunteered and were eligible to participate in study if they were 18 years of age or older and had no history of previously diagnosed cancer of any type. They were selected among the blood donors of the Hospital Dr. Nélio Mendonça (Funchal, Portugal). Breast cancer urine samples ($n=26$, age = 53.9 ± 9.6 y (range 31–74 years) were obtained from females with a diagnosis of invasive breast cancer were patients at Unit of Haematology–Oncology at the same institution.

Breast cancer patients underwent the diagnostic procedures such as breast physical examination, mammography and ultrasonography. Each individual (either patient or healthy volunteer) provided a sample of morning urine (after overnight fasting) in a 50 mL sterile glass container. The samples were frozen at −80 °C and stored until needed for experiments. Before the extraction procedure, the urine pH value was adjusted to 1–2. All

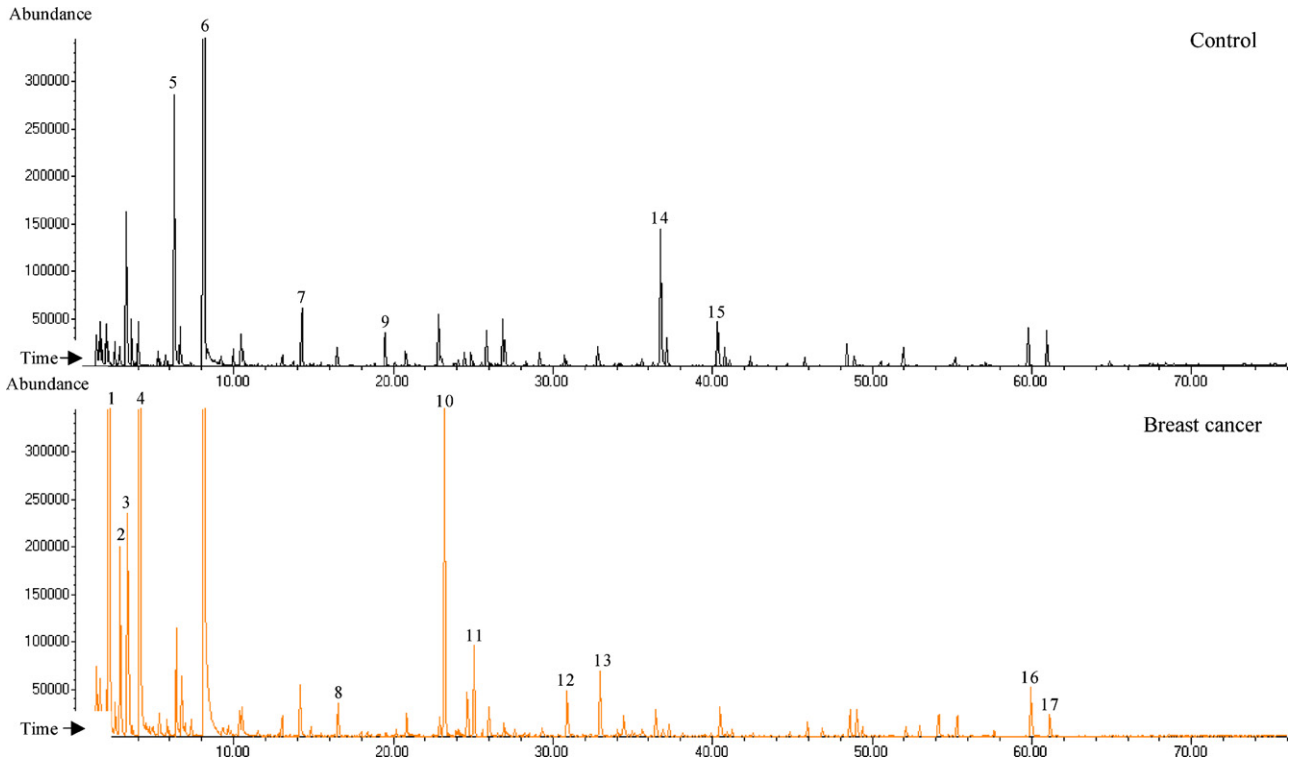

Fig. 2. Representative GC–qMS total ion chromatograms (TIC) (fingerprint signals) of urine volatile metabolites from a healthy person (a), and breast cancer patient (b). Extraction was performed using a CAR/PDMS fiber at 50 °C for 60 min. Peak assignment: (1) acetone; (2) 2-butanone; (3) ethyl alcohol; (4) 2-pentanone; (5) dimethyl disulfide; (6) 4-heptanone; (7) γ-terpinene; (8) 2-methoxy-tiophene; (9) dimethyl trisulfide; (10) linalool oxide; (11) 2,6-dimethyl-7-octen-2-ol; (12) isomenthol; (13) menthol; (14) D-carvone; (15) 3,4-benzaldeyde; (16) p-tert-butyl-phenol; (17) 2,4-bis(1,1-dimethylethyl)-phenol.

subjects signed an informed consent to participate in the study and the research was approved by the Ethics Committee of Funchal Hospital.

2.3. HS-SPME procedure

The nature of the adsorptive phase, the sampling temperature and the extraction time required to achieve equilibration between the analytes and the fiber were optimized by applying an univariate experimental design.

fiber selection was performed by testing and comparing the extraction efficiency of six SPME fibers (Supelco, Bellefonte, USA) to different stationary phases and various film thicknesses, including PDMS (100 μm), PA (85 μm), DVB/CAR/PDMS (50/30 μm), CAR/PDMS (75 μm), CW/DVB (70 μm) and PDMS/DVB (65 μm). A urine sample from a normal subject was used as the matrix for the optimization of the dHS-SPME parameters. Frozen urine samples were completely thawed at room temperature prior to use.

Briefly, 4 mL aliquots of urine sample adjusted to pH 1–2 with 500 μL of HCl were transferred to 8 mL sampling glass vial. After the addition of 0.8 g of NaCl and stirring (0.5 mm × 0.1 mm bar) at 800 rpm, the vial was capped with a PTFE septum and an aluminum cap (Chromacol, Hertfordshire, UK). The addition of salt increased the extraction efficiency for many metabolites, particularly the polar ones. The presence of salt can influence the adsorption in two ways: changing the properties of the phase bonding and decreasing the solubility of hydrophilic metabolites in the aqueous phase (salting-out effect). The salting-out effect is widely used to increase the sensitivity of an analytical methodology [28]. The vial was placed in a thermostat bath adjusted to 50.0 ± 0.1 °C and then the SPME fiber was inserted in the headspace for 60 min. After sampling, the SPME fiber was withdrawn into the needle, removed from the vial and inserted in the injector port (250 °C) of the GC–qMS system for 6 min where the analytes were thermally desorbed and transferred directly to the analytical column. Each sample was analyzed in triplicate. Blanks, corresponding to the analysis of the coating fiber not submitted to any extraction procedure, were run between sets of six analyses.

2.4. Gas chromatography–quadrupole mass spectrometry analysis (GC–qMS)

The SPME fiber with absorbed/adsorbed VOMs was inserted into the injection port of an Agilent Technologies 6890N Network gas chromatograph system (Palo Alto, CA, USA) where the metabolites were desorbed at 250 °C for 6 min. The gas chromatograph was equipped with a 30 m × 0.25 mm I.D. × 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. We employed the following chromatographic protocol for separation before MS analyses: 35 °C for 2 min, then programmed at 2.5 °C min^{-1} to 220 °C with a 5-min hold at this final temperature, for a total GC run time of 77 min. Column flow was constant at 1 mL min^{-1} using He (Helium N60, Air Liquid, Portugal) as carrier gas. The injection port was operated in the splitless mode and held at 250 °C.

For the 5975 MS system, the operating temperatures of the transfer line, quadrupole and ionization source were 270, 150 and 230 °C, respectively; electron impact mass spectra were recorded at 70 eV ionization voltages and the ionization current was 10 μA. Data acquisition was performed in Scan mode (30–300 m/z). 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

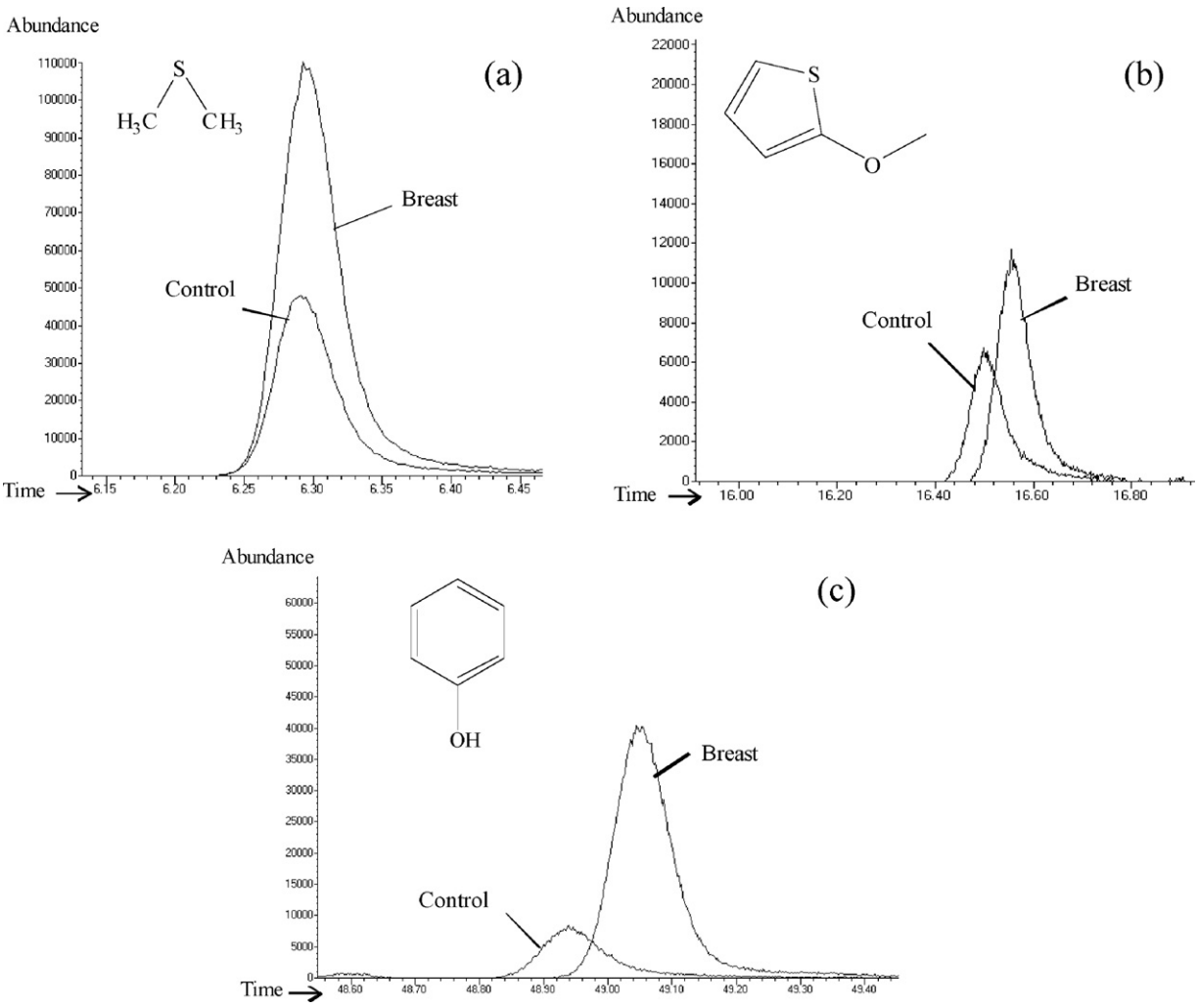

Fig. 3. Comparison between healthy and oncologic group of 3 illustrative metabolites selected from 79 compounds analyzed. Enlarged part of the chromatograms of Fig. 2 with peaks representing: (a) dimethyl disulfide (ion 94); (b) methoxythiophene (ion 114); and (c) phenol (ion 94).

Software, equipped with a NIST05 mass spectral library with a similarity threshold higher than 80% and comparison with commercially available standard samples when available. A series of C_8–C_{20} n-alkanes were analyzed using the same methodology (HS-SPME$_{CAR/PDMS}$/GC–qMS) in order to establish the retention indices (RI), and to confirm the identity of the metabolites by comparison with the literature.

2.5. Statistical analysis

Data statistical analysis was performed using the SPSS 17.0 package for Windows (SPSS Inc., Chicago, IL, USA). Significant differences among the groups were assessed with a one-way analysis of variance (ANOVA). The least square difference (LSD) test (p-value <0.05) was used to compare the means. Principal component analysis (PCA) was also applied to the analyzed groups to verify the distribution of the variables for the referred groups.

3. Results and discussion

An objective comparison among the metabolomic pattern found in urine from breast cancer patients and healthy volunteers has been established in terms of qualitative (identification by comparison of MS spectrum and *kóvats index*) and semi-quantitative (peak area ratio) differences, using HS-SPME$_{CAR/PDMS}$/GC–qMS methodology. Among other compound classes, aldehydes, ketones, terpenoids, volatile fatty acids, furan compounds, volatile phenols, benzene derivatives, sulfur-containing compounds, and naphthalene derivatives, were identified.

The optimization of the different parameters concerning in HS-SPME was performed by choosing the conditions that enabled the maximum response in terms of metabolite peak area, number of detected metabolites and reproducibility. Six SPME fibers were tested to select the most effective for isolation of volatile metabolites from urine. The results of the relative extraction efficiency for the tested fibers are shown in Fig. 1a.

By comparing all of the tested fibers in terms of chromatographic areas, the number of identified metabolites and relative standard deviation (R.S.D.) the best efficiency was obtained using a CAR/PDMS coating, whereas the lowest efficiency was obtained using the PDMS and PA fibers.

Temperature substantially affected the diffusion rates of VOMs. Raising the temperature progressively from 30 to 50 °C increased the number of extracted metabolites that were identified. Although there was a slight increase in the number of metabolites that were identified at 60 °C (2 more), the R.S.D. obtained therein was higher than those for the other investigated temperatures.

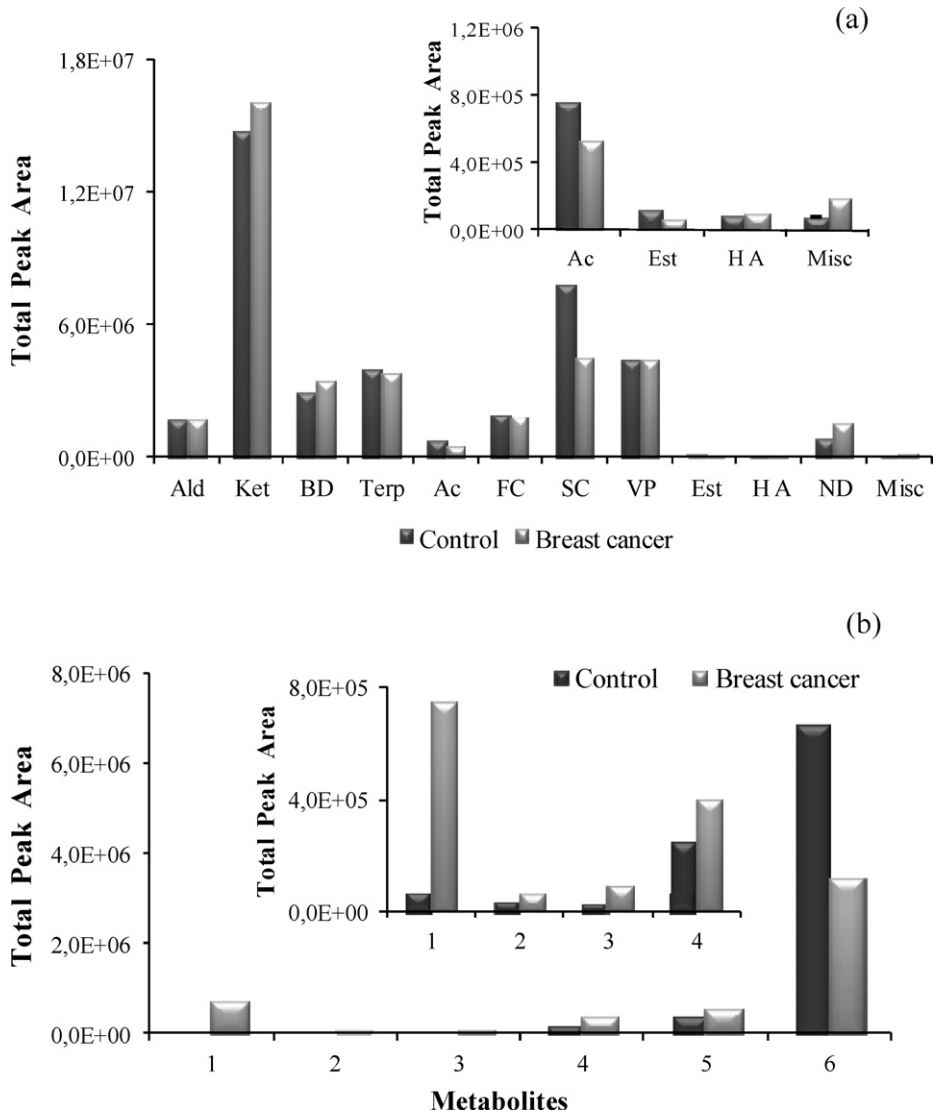

Fig. 4. Average levels of metabolites excreted in urine samples from normal subjects ($n = 21$) and breast cancer patients ($n = 26$) (a) Chemical families identified in control and breast cancer groups; (Ald – aldeydes, Ket – ketones, BD – benzene derivates, Terp – terpenoids, Ac – acids, FC – furanic compounds, SC – sulfur compounds, VP – volatile phenols, Est – esters, HA – higher alcohols, ND – naphthalene derivates, Misc – miscellaneous). (b) Average areas for statistically significant metabolites identified in breast cancer patients. Numbered bars correspond to: (1) 3-heptanone; (2) 1,2,4-trimethylbenzene; (3) (−)-4-carene; (4) 2-methoxythiophene; (5) phenol; (6) dimethyl disulfide.

As outlined in Fig. 1b, the temperature was fixed at 50 °C for the extraction of urinary volatile metabolites from healthy volunteers and cancer patients.

The sorption time profiles for volatile metabolites indicated that a sampling time of greater than 45 min was necessary to reach equilibrium. Fig. 1c demonstrates that the equilibrium between the samples and fiber was established in 60 min. With additional extraction time, there was no obvious increase in the peak area. On the basis of the results, 60 min was chosen as the optimal extraction time for further analysis.

3.1. Characterization and comparative analysis of urinary volatile metabolites

From the typical GC–qMS total ion chromatograms (TICs) (Fig. 2) a large diverse set of metabolites could be distinguished in the urine from a healthy person (control group) and from breast cancer patient.

Different urinary GC–qMS profiles for healthy subjects and breast cancer patients could be recognized. Seventy-nine volatile metabolites, found in urinary composition of both the breast cancer and the healthy subjects, included a variety of chemical structures which are potentially involved in several biological functions, for example in pheromonal communication (2-heptanone) [29]. Some metabolites that have previously been reported in human urine (dimethyl disulfide, methanethiol, and 2-methylbutanoic acid) were also identified [29–33]. The enlarged peaks in the TIC of some of the significant metabolites are represented in Fig. 3 and these facilitate the differentiation of metabolomic profiles.

The peak areas range (minimum, maximum and median values) of the urinary volatile metabolites found in cancer patients and healthy subjects are summarized in Supplementary Table 1. Identification was performed by using NIST05 library through comparison of the fragmentation patterns of the compounds with standard mass chromatogram and verified by reference compounds when available.

The metabolomic origin and physiological function of most VOMs are still not known. Their origins lie in a variety of endogenous biochemical pathways and exogenous sources (environmental, unhealthy lifestyle habits, biological agents), however,

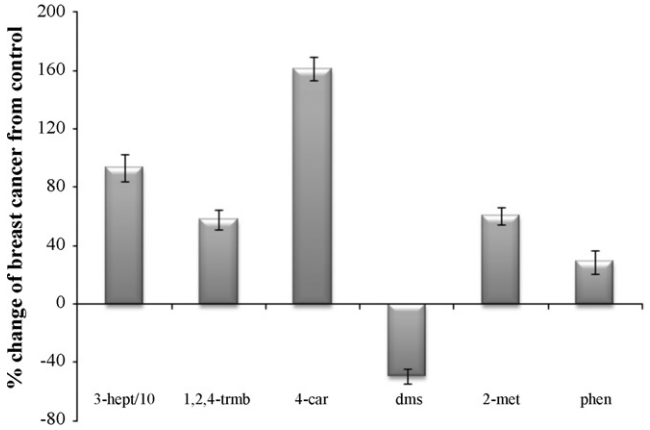

Fig. 5. Percentage of change of breast cancer patients from normal subjects. Bars correspond to: (3-hept): 3-heptanone; (1,2,4-trm): 1,2,4-trimethylbenzene; (4-car): (−)-4 carene; (dms): dimethyl disulfide; (2-met): 2-methoxithiophene; and (phen): phenol.

the chemical pathways of generation have not yet been explained. Some of the endogenous markers were derived from the mevalonic acid pathway of cholesterol synthesis (e.g. unsaturated hydrocarbons like isoprene), from glucose metabolism (e.g. acetone) and from oxygen free radical-mediated lipid peroxidation of fatty acids (e.g. aldehydes, and linear and branched saturated hydrocarbons). The source of the VOMs that were identified to be derivatives of naphthalene is yet unknown; they might derive from degradation products of steroids. Further research would be required to determine which of these metabolites are of tumor origin and which originate from normal metabolic processes and are either down- or up-regulated by the tumors.

Variation in the peak areas of identified metabolites clearly showed differences in the relative amounts of various metabolites (Supplementary Table 1, Fig. 4a and b). The identified metabolites belong to distinct chemical families, namely aldehydes, ketones, terpenoids, acids, alcohols, benzene derivatives, furan and sulfur-containing compounds, phenols, esters, naphthalene derivatives and miscellaneous (Fig. 4a). Like others, we noted a variation in the range of compounds extracted from urine of examined subjects.

We observed relatively consistent changes for many metabolites and for both groups with the most common pattern being a decreased production (down-regulation) in the cancer groups and either an increased production (up-regulation) or negligible change in the control group. For example, volatile sulfur-metabolites like dimethyl disulfide, are generated in humans by incomplete metabolism of methionine in the transamination pathway, which is down-regulated dramatically as a consequence of neoplastic cells presence (Supplementary Table 1). The same conclusion was reported by Catarina et al. [31] for the urinary metabolomic pattern of 33 cancer patients from leukemia, colorectal cancer and Hodgkin lymphoma. Thus, an overall down-regulation of this metabolite may be a common feature of tumor growth.

For the control group, 18 male and 13 female urine samples were analyzed. The largest chemical classes identified with highest contribution for the urinary metabolomics profile were ketones and sulfur compounds (Fig. 4a). The major metabolites of these chemical families are 3-heptanone and methanethiol, respectively. The origin of 4-heptanone is still unknown but is derived probably from an exogenous source [17]. It has been reported that 4-heptanone is produced from the in vivo metabolism of plasticizers in man [34]. Hexanal, which has also been identified in the samples, arises as a breakdown product of lipid peroxidation that is formed as a result of oxygen free radical (OFR) activity. The specificity of this marker for different cancers is under investigation [35]. Esters and higher alcohols were the chemical families with lowest contribution for the volatile metabolomic profile in breast cancer and healthy subjects.

Ketones, sulfur compounds and volatile phenols, were the chemical groups with highest contribution for the metabolomics volatile profile of the breast cancer group (Fig. 4a). Sulfur-containing compounds like ethyl mercaptane, dimethylsulfide, or dimethyldisulfide are responsible for the characteristic odor in the breath of cirrhotic patients [34]. Sulfur-containing compounds are generated in humans by incomplete metabolism of methionine in the transamination pathway [17,29]. The experimental data indicated that breast cancer patients had higher levels of 3-heptanone, 2,2,4-trimethylbenzene, (−)-4-carene, 2-methoxythiophene and phenol than control group (Fig. 4b). There was an increase of profile for all cancer patients when compared to controls except for dimethyl disulfide, an abundant compound present in control group individuals (Fig. 5).

The fragment-ion m/z values of the identified urinary metabolites with the highest abundance within each fragmentation

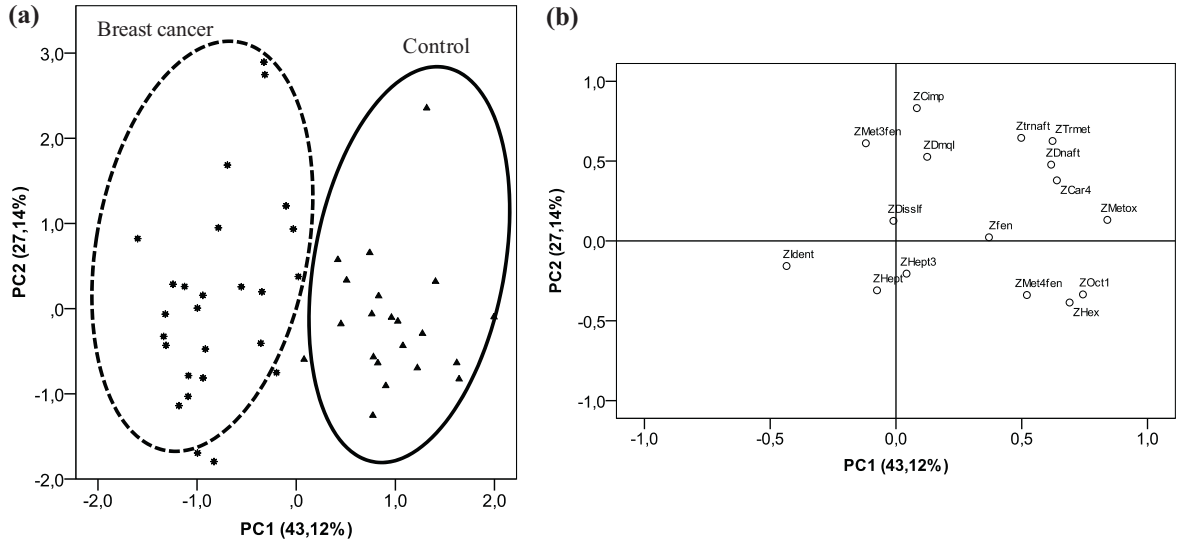

Fig. 6. Separation of cancer patients and healthy individuals based on PCA scores scatter plot. (a) Loadings of variables on the PC1–PC2 plane (70.28% of total variance; (b) factor scores for PCA analysis showing the similarity groups for healthy persons (control group), and cancer patients as described by variables represented in Fig. 4a.

Table 1
Identification mode, fragment-ion *m*/*z* with the highest abundance match percentage to the NIST05 library and the frequency of occurrence of the identified metabolites in breast cancer patients and normal controls.

Metabolites	ID[b]	*m*/*z*	Match percent (%)	Frequency of occurrence (%)	
				Breast cancer	Control
Methanethiol[a]	MS	47	90	100.0[c]	100.0
Furan[a]	St. MS	68	91	100.0	100.0
Acetone[a]	St. MS	43	90	100.0	100.0
2-Methylfuran[a]	MS	82	88	100.0	100.0
Ethyl acetate	St. MS	43	85	77.8	71.4
2-Butanone[a]	St. MS	43	85	100.0	100.0
2-Methyl-butanal	MS	57	81	63.0	71.4
3-Methyl-butanal	St. MS	44	81	40.7	28.6
2.5-Dimethyl-furan[a]	MS	96	85	100.0	100.0
2-Pentanone[a]	St. MS	43	86	100.0	100.0
Methyl isobutyl ketone	St. MS	43	93	88.9	100.0
Toluene[a]	St. MS	91	86	100.0	100.0
1-(2-Furanyl)ethanone[a]	St. MS	95	80	100.0	100.0
Dimethyl disulfide[a]	St. MS	94	97	100.0	100.0
3-Hexanone	St. MS	43	84	96.3	100.0
Hexanal[a]	St. MS	44	90	100.0	100.0
Geraniol oxide	MS	139	90	88.9	90.5
4-Heptanone[a]	St. MS	71	91	100.0	100.0
3-Heptanone	St. MS	57	95	81.5	95.2
α-Terpinene	St. MS	121	87	81.5	52.4
1,4-Cineol	St. MS	111	84	74.1	61.9
Limonene	St. MS	68	84	85.2	28.6
2-Heptanone	St. MS	43	83	96.3	100.0
Heptanal	St. MS	44	80	22.2	61.9
γ-Terpinene	MS	93	89	70.4	57.1
m-Cymene	St. MS	119	97	96.3	100.0
3,8-p-Menthadiene	MS	79	83	88.9	0.0
2,2,6-Trimethyl-cyclohexanone	MS	82	88	74.1	76.2
2-Methoxytiophene[a]	MS	114	89	100.0	100.0
1,2,4-Trimethylbenzene	MS	105	90	92.6	95.2
Dimethyl trisulfide[a]	St. MS	126	91	100.0	100.0
2-Methyl-5-(methylthio)furan	MS	128	91	96.3	100.0
Nonanal	St. MS	57	80	96.3	100.0
1,2,3,4-Tetrahydro-1,5,7-trimethyl naphthalene	MS	159	90	81.5	81.0
p-Cymene[a]	MS	132	97	100.0	100.0
Linalyl oxide	MS	59	82	92.6	90.5
Dihydrolinalool	MS	73	84	14.8	76.2
Acetic acid[a]	St. MS	43	90	100.0	100.0
Furfural	St. MS	96	91	85.2	100.0
2,6-Dimethyl-7-octen-2-ol	St. MS	59	90	92.6	100.0
1,2,3,4-Tetramethyl-benzene	MS	119	80	51.9	66.7
Decanal	St. MS	57	86	85.2	100.0
Bornylene	MS	93	86	44.4	14.3
Vitispirane I[a]	MS	192	82	100.0	100.0
Vitispirane II[a]	MS	192	85	100.0	100.0
1,2,3,4-Tetrahydro-1,1,6-trimethyl naphthalene[a]	MS	159	82	100.0	100.0
1-Octanol[a]	St. MS	56	90	100.0	100.0
Menthol	MS	71	84	66.7	71.4
2-Furanmethanol	St. MS	98	80	59.3	85.7
2-Methyl butanoic acid	St. MS	74	83	81.5	81.0
Anisole	MS	134	85	74.1	14.3
(+)-4-Carene	MS	93	80	44.4	71.4
2-Methyl-3-phenyl-2-propenal	St. MS	145	80	88.9	100.0
4-(1-Methylethyl)-1-cyclohexene-4-carboxaldehyde	MS	109	85	33.3	19.0
3-Carvomenthenone	MS	82	80	37.0	33.3
D-Carvone	MS	82	80	66.7	52.4
1,2-Dihydro-1,1,6-trimethyl-naphtalene[a]	St. MS	157	97	100.0	100.0
1-(4-Methylphenyl)ethanone	MS	119	94	14.8	14.3
4-(1-Methylethyl)-benzaldehyde	MS	133	84	58.3	9.5
3,4-Dimethyl-benzaldehyde[a]	MS	133	95	100.0	100.0
β-Damascenone[a]	St. MS	69	97	100.0	100.0
p-Cymen-8-ol	MS	43	90	74.1	100.0
2-Methoxy-phenol	St. MS	109	80	66.7	66.7
Butyl butanoate	MS	71	80	25.9	4.8
2,2,4-Trimethyl-1,3-pentadienol diisobutyrate	MS	71	85	14.8	0.0
2,7-Dimethyl-quinoline	St. MS	157	85	77.8	85.7
Hexanoic acid	St. MS	60	89	48.1	90.5
2,6-Dimethyl-naphtalene	MS	156	98	92.6	76.2
1-Ethyl-3,5-diisopropyl-benzene[a]	MS	175	88	100.0	0.0
Phenol[a]	St. MS	94	91	100.0	100.0
Octanoic acid	St. MS	60	92	55.6	95.2
4-Methyl-phenol	St. MS	107	90	96.3	95.2
1,4,5-Trimethyl-naphtalene	MS	155	86	96.3	57.1

Table 1 (*Continued*)

Metabolites	ID[b]	*m/z*	Match percent (%)	Frequency of occurrence (%)	
				Breast cancer	Control
2-Methoxy-4-vinylphenol	St. MS	135	80	44.4	52.4
Decanoic acid	St. MS	60	80	59.3	100.0
p-Tert-butyl-phenol[a]	MS	135	96	100.0	100.0
2,4-Bis(1,1-dimethylethyl)-phenol[a]	MS	191	98	100.0	100.0
Benzenecarboxilic acid	St. MS	105	90	25.9	85.7
Indole	MS	117	88	92.6	100.0

[a] Metabolites identified in all 47 studied subject.
[b] Metabolite identification using standard compound (st) or mass spectra of the NIST library search (MS).
[c] Means that the metabolite was identified in all subjects of the corresponding group.

Table 2
Potential volatile marker metabolites found in the urinary volatile composition of the two groups by total significance of one-way ANOVA and LSD post hoc test for multiple comparisons.

Metabolites	Mean[a] values of peak areas ($n=3$)			One way significance		LSD (multiple comparison test)
	Breast (C)	%Change[b]	Control (E)	F	p^*	
3-Heptanone	746,735	934.2	72,205	16.490	<0.001	$C-E$ ($p<0.001$)
1,2,4-Trimethylbenzene	65,728	57.9	41,623	3.124	0.032	$C-E$ ($p=0.038$)
(+)-4-Carene	94,104	161.0	36,060	3.808	0.017	$C-E$ ($p=0.002$)
Dimethyl disulfide	3,480,273	−49.5	6,892,194	4.667	0.005	$C-E$ ($p=0.002$)
2-Methoxytiophene	397,321	60.6	247,376	5.211	0.003	$C-E$ ($p=0.001$)
Phenol	557,866	29.1	432,130	3.376	0.023	$C-E$ ($p=0.002$)

[a] Average value from 3 replicates; RSD lower than 20%.
[b] Percentage change of cancer from normal, calculated from the arithmetic mean values of each group. Positive and negative percentages indicate higher levels of metabolites in cancer patients and healthy subjects, respectively.
[*] Statistical p-value calculated using the LSD test (significance at $p<0.05$).

pattern, the matching percentage of the NIST library and their frequency of occurrence in cancer patients and normal controls were listed in Table 1.

One-way ANOVA, ($p<0.05$) was carried out for both groups using SPSS version 17.0. The excretion values of all 79 volatile metabolites were compared between breast cancer group and the control group. The difference in levels of six from 79 metabolites identified including, 3-heptanone, 1,2,4-trimethylbenzene, (−)-4-carene, dimethyl disulfide, 2-methoxythiophene and phenol, were statistically significant ($p<0.05$) in cancer patients and control groups while others did not show any significant differences (Table 2)

The high inter-individual variability in urinary profiles and their large complexity make any attempt of visual comparison of these spectra an unsuccessful task. Applying, multivariate analysis allows to find consistent variation patterns within the dataset. In order to study the principal sources of variation among results, detect sample clustering and possible outliers, and to establish if the cancer patients could be distinguished from healthy individuals, exploratory principal component analysis was applied to the GC–qMS peak area obtained for the urinary volatile metabolites of both groups. PCA is an unsupervized projection method used to visualize the dataset and display the similarity and difference. After preliminary statistical analysis, PCA of the data showed that variables described in Table 2 were enough to describe subsets with similar characteristics, related to the health condition of the subjects.

Fig. 6a shows the scatter plots on the plane defined by first (PC1) and second (PC2) principal components and the factor scores for PCA analysis showing the similarity groups for healthy persons (control group), and cancer patients as described by variables represented in Fig. 4a.

Although this set of variables explains only 70.28% of the variability along the two first PCs, it is enough to divide the set of cases in two subsets according to health conditions of the subjects, breast cancer versus healthy individuals. These results indicate a great potential for early diagnosis of the studied cancer types using non-invasive urinary metabolomic analysis (Fig. 6b). The scores scatter plot shows that PC1, accounting for 43.12% of the total variability, split the samples into two different groups. The group located in PC1 positive comprises the samples corresponding to the healthy individuals (control). The group located in PC1 negative and PC2 (27.14% of the total variability) corresponds to the breast cancer patients. These results showed that the set of cases can be divided into two groups according to the clinical condition of the subjects. The variables phenol, 3-heptanone, and 1,2,4-trimethylbenzene seems to play an important role in healthy individuals group (control). Possible biomarkers of breast cancer patients may be selected from heptanal, dimethyl disulfide and 2-methyl-3-phenyl-2-propenal, respectively.

4. Conclusions

The present study showed that SPME coupled to GC–qMS is a simple, rapid, sensitive and solvent-free method useful for the establishment of urinary volatile metabolomic pattern characteristic for breast cancer patients and normal subjects (cancer-free). A CAR/PDMS fiber was found to be more sensitive for volatile metabolites than other coating phases.

Seventy-nine VOMs belonging to several derivatives were detected and identified in both control and breast cancer groups namely aldehydes, ketones, terpenoids, acids, alcohols, benzene derivates, furan compounds, sulfur compounds, phenols, esters and naphthalene derivates.

Different VOMs profiles for healthy subjects and breast cancer patients could be recognized by multivariate analysis, and possible biomarkers could be established. Between the breast cancer group ($n=26$) and the control group ($n=21$), excretion values of 6 among the total of 79 detected compounds were found to be different with statistical difference ($p<0.05$, LSD test). The

identification of volatile biomarkers in urine for disease diagnosis is an area of great promise however it is based on limited prior human research. The data in this paper are consistent with the hypothesis that diagnostically useful volatile compounds are produced in patients with cancer and secreted into the urine, thus providing support for this diagnostic approach in the context of different types of cancer. The ability to easily collect and store urine samples will be a major advantage of this approach.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2011.12.041.

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