Solid Phase Extraction Based on Nanomaterials for Isolation of Urinary Volatile Metabolites

MASTER DISSERTATION

Zheng Qiao

MASTER IN NANOCHEMISTRY AND NANOMATERIALS

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Thesis submitted to the University of Madeira in order to obtain the degree of Master in Nanochemistry and Nanomaterials

by Zheng Qiao

Study performed under the supervision of Professor José S. Câmara

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“It was the best of times; it was the worst of times.”

---Charles John Huffam Dickens

Contents

Dedication 5

Declaration 6

Acknowledgments 7

ABSTRACT 9

List of Figures 13

List of Tables 15

List of Abbreviations 16

Thesis structure 19

CHAPTER I 20

1. Urinary Volatile organic metabolites (VOMs) as biomarkers of cancer diagnosis 21

1.1. Metabolic changes in cancers 22

1.2. Urinary VOMs as biomarkers for cancer diagnosis 25

1.3. Basic principles of SPE 26

1.4. Nanomaterials-based sample preparation in SPE 28

1.4.1. Metallic NPs 29

1.4.2. Metal organic framework materials 36

1.4.3. Carbonaceous NPs 37

1.4.4. Silica NPs 42

CHAPTER II 45

2.1. Introduction 46

2.2. Experimental 50

2.2.1. Chemicals and Reagents 50

2.2.2. Subjects and sample collection 51

2.2.3. Synthesis of Fe_3O_4@SiO_2-C_{18} NPs and Fe_3O_4@PPy NPs 51

2.2.3. Preparation of stock and working solution 55
2.2.4. NMs-based Sorbent Extraction Procedure 56
2.2.5. Chromatographic and Mass Spectrometric Conditions 57
2.2.6. Method validation 58
2.3. Results and Discussion 62
2.3.1 Optimization of NMs-based sorbent extraction approach 62
2.3.2 Method validation of urinary VOMs using NMs-based sorbent extraction approach 68
2.3.3 Quantification urinary cancer VOMs using NMs-based sorbent extraction with GC-qMS 71
2.4. Conclusions 75
CHAPTER III 77
References 80

Dedication

To my dearest parents,

for their incessant love and unwavering support to my lifelong pursuit of dreams and happiness.

To my beloved grandmother who is now home with the Lord.

Declaration

I hereby declare that this thesis is the result of my own work, is original and was written by me. I also declare that its reproduction and publication by Madeira University will not break any third party rights and that I have not previously (in its entirety or in part) submitted it elsewhere for obtaining any qualification or degree. Furthermore, I certify that all the sources of information used in the thesis were properly cited.

07/2014

Acknowledgments

Without doubt, completing my Master degree would not have been possible without the support of others, and I'm so grateful to the people who helped me. A general "thank you" would take a lot of pages, but a blanket statement is not enough to express my excited emotion. The following part, an attempt is to succinctly execute the former.

First and foremost, I would like to express heartfelt gratitude to my supervisor, Professor José S. Câmara, for his mentorship. His passion for science, decisive judgment as a leader, zeal for innovation, and commitment to education were sources of inspiration for me. As a novice in science, his tutelage promoted my metamorphosis to be a scholar with higher intellectual maturity to tackle problems through all my experiments.

I also need to thank Professor João Rodrigues for teaching me how to think and work like a researcher and for his useful lessons in my master career.

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ABSTRACT

Testing of urinary volatile organic metabolites (VOMs) is recognized as a useful medical approach since they are the end-products of metabolic processes and non-invasively sampling. Among the various techniques conventionally used for metabolic profiling, gas chromatography-quadrupole mass spectrometry (GC-qMS) is viewed as an effective approach for the testing of VOMs in urine due to its high sensitivity, peak resolution and reproducibility. Solid phase extraction (SPE), one of the sample preparation methods, has been proven to be a robust tool for the application in target VOMs concentration and separation from complex matrix.

The aims of this study are to explore new nanomaterials (NMs), such as inorganic and carbon-based nanoparticles magnetic NPs (MNPs), combined with GC-qMS in order to isolate and pre-concentrate the target urinary VOMs, reported as possible cancer biomarkers. Concerning to the nanosorbent, the best efficiency of extraction is achieved with nanohydroxyapatite (NHA) and magnetic Fe_3O_4@SiO_2-C_{18} NPs. Some experimental parameters such as, sorbent amount, adsorption and elution time, as nature of elution solvent, were investigated and compared. Under optimal conditions for nanohydroxyapatite (NHA) and magnetic Fe_3O_4@SiO_2-C_{18} NPs, the obtained results revealed a good linearity ($r^2 \geq 0.988$) within the linear dynamic range, for all urinary volatile metabolites under study. The projected strategy showed low limits of detection (LODs), ranging from 9.7 to 69.5 ng/L, and low limits of quantification (LOQs) from 32.4 to 231.6 ng/L. The methodology also afforded suitable results in terms of matrix effect (62.8–96.1%) and accuracy, higher than 70 % for the majority of the investigated VOMs. The precision, expressed as intra- and inter-day repeatability, was lower than 3 and 13 %, respectively. Together, the NHA and Fe_3O_4@SiO_2-C_{18} NPs-based sorbent extraction combined with GC-qMS in the current

study provides a new, reliable and high throughput strategy for the determination of VOMs in human urine.

Keywords: Nanohydroxyapatite; Magnetic nanoparticles; Volatile Organic Metabolites; Solid Phase Extraction; Gas Chromatography-Mass Spectrometry

Resumo

Os metabolitos orgânicos voláteis (VOMs) presentes na urina de pacientes oncológicos são de grande utilidade no diagnóstico da doença e na diferenciação entre diferentes patologias oncológicas, , uma vez que são produtos resultantes de processos metabólicos. Entre as diversas técnicas convencionalmente utilizadas para estabelecer o perfil metabólomico, a cromatografia em fase gasosa acoplada à espectrometria de massa (GC-qMS) é considerada uma abordagem eficiente para ensaios urinários de VOMs devido à sua elevada sensibilidade, resolução e reprodutibilidade. A extração em fase sólida (SPE), um dos métodos de preparação de amostras, demonstrou ser uma ferramenta robusta para a aplicação no isolamento e pré-concentração de VOMs em matrizes complexas.

Os objetivos deste estudo visam explorar novos nanomateriais (NMs), nomeadamente nanopartículas inorgânicas (NPs) e magnéticas à base de carbon (MNPs) , combinadas com GC-qMS com intuito de isolar e pré-concentrar VOMs presentes na urina de indivíduos oncológicos. Relativamente ao nanosorvente, foram estudados a nanohidroxiapatite (NHA) e MNPs Fe_3O_4@ SiO_2-C_{18}. Alguns parâmetros experimentais com influência na eficiência de extração, tais como a quantidade de sorvente, o tempo de adsorção, o tempo de eluição e o solvente de eluição, foram avaliados. Sob condições otimizadas, e para os 2 nanosorventes testados, os resultados obtidos demonstraram uma boa linearidade ($r^2 \geq 0,988$), na gama de concentrações usadas para todos os metabólitos voláteis urinários investigados. A estratégia proposta permitiu obter limites de deteção (LODs) entre 9,7 a 69,5 ng/L e limites de

quantificação (LOQs) entre 32,4 a 231,6 ng/L. O método proporcionou igualmente resultados satisfatórios em termos de efeito de matriz (62,8-96,1%) e recuperações (precisão) superiores a 70 % para a maioria dos VOMs estudados. A precisão intra- e inter-dia foi inferior a 3 e 13%, respetivamente. Verificou-se que os NMs usados como sorventes, NHA e Fe_3O_4@ SiO_2-C_{18}, combinado com GC-qMS forneceram uma estratégia vantajosa, confiável e de alta eficiência de extração na determinação de VOMs alvo na urina.

Palavras-chave: Nanohidroxiapatite; Nanopartículas magnéticas; Metabolitos orgânicos voláteis; Extração em fase sólida; Cromatografia em fase gasosa-espectrometria de massa

List of Figures

Fig. 1.1. Processes of Glycolysis. HK, hexokinase; PGI, phosphoglucose isomerase; PFK, phosphofructokinase; TPI, triosephosphate isomerase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; PGM, phosphoglycerate mutase; PK, pyruvate kinase; PDH: pyruvate dehydrogenase; LDH: lactate dehydrogenase.

Fig.1.2. Structures of biomarkers from the study of Silva *et al*.

Fig. 1.3. Typical procedures of SPE extraction.

Fig. 1.4. General procedure of Magnetic SPE.

Fig. 1.5. Schematic of sampling and desorption procedures on MOF-5 packed tube.

Fig. 1.6. (a) Models of GO and graphene sheets. The shadowed sections indicate the polar groups in the GO and graphene sheets. (b) Chemical routes to the synthesis of GO@silica and G@silica. NP-SPE, Normal-phase SPE; RP-SPE, Reversed-phase SPE; EDC, (*N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride; NHS, *N*-hydroxysuccinimide, DCC =*N*,*N*′-dicyclohexylcarbodiimide, DMF = dimethylformamide

Fig. 1.7. Main strategies of functionalization/modification of silica NPs.

Fig. 2.1 Chemical structures of the volatile metabolites used in the experiment

Fig. 2.2. Schematic illustration of the preparation of Fe_3O_4@SiO_2-C_{18} NPs..

Fig. 2.3. TEM microscopy of Fe_3O_4@SiO_2-C_{18} NPs.

Fig. 2.4. IR spectrum of Fe_3O_4@SiO_2-C_{18} NPs.

Fig. 2.5. TEM microscopy of Fe_3O_4@PPy NPs.

Fig. 2.6 IR spectrum of Fe_3O_4@PPy NPs.

Fig. 2.7. Scheme showing the route for NHA to the extraction of VOMs.

Fig. 2.8. Procedure for the extraction of VOMs by using Fe_3O_4@SiO_2-C_{18} NPs**.**

Fig. 2.9. Total ion chromatogram of fortified synthetic urine by NHA/GC-qMS under optimum conditions.

Fig. 2.10. Effect of sorbent amount of NHA on the adsorption efficiency of VOMs.

Fig. 2.11. Effect of sorbent amount of Fe_3O_4@SiO_2-C_{18} NPs on the adsorption efficiency of VOMs.

Fig. 2.12. Effect of adsorption time on the adsorption ability of VOMs in NHA.

Fig. 2.13. Effect of adsorption time on the adsorption ability of VOMs by Fe_3O_4@SiO_2-C_{18} NPs..

Fig. 2.14. Effect of elution time on the adsorption of VOMs by NHA.

Fig. 2.15. Effect of elution time on the adsorption of VOMs by Fe_3O_4@SiO_2-C_{18} NPs

Fig. 2.16. Effect of chemical nature of eluent systems on the adsorption of VOMs by NHA.

Fig. 2.17. Total ion chromatogram of cancer patient using NHA-based sorbent extraction with GC-qMS (peak identification: 1: hexanal; 2: undecane; 3: 4-heptanone; 4: heptanal; 5: decanal; 6: benzaldehyde; 7: 5-methyl-furfural; 8: phenol).

List of Tables

Table 1.1 Pre-concentration of metal ions by metal oxide NPs.

Table 1.2 Application of magnetic SPE for different analytes.

Table 2.1. Validation parameters for urinary cancer biomarkers using NHA-based sorbent extraction combined with GC-qMS.

Table 2.2. Validation parameters for urinary cancer biomarkers using Fe_3O_4@SiO_2-C_{18} NPs-based extraction with GC-qMS.

Table 2.3. Urinary cancer biomarker concentration (µg/L) in urine samples using NHA-based sorbent extraction combined with GC-qMS.

Table 2.4. Urinary cancer biomarker concentration (µg/L) in urine samples using Fe_3O_4@SiO_2-C_{18} NPs-based extraction with GC-qMS.

List of Abbreviations

Acetyl-CoA	Acetyl-coenzyme A
Akt	Serine/threonine kinase
BPA	Bisphenol A
BPF	Bisphenol F
BFDGE	Diglycidyl ether
CNTs	Carbon nanotubes
C_8	Carbon8
C_{18}	Carbon18
CPs	Chlorophenols
CT	Computed tomography
CTAB	Cetyltrimethyl ammonium bromide
DDTC	Diethyldithiocarbamate
FAAS	Flame atomic absorption spectrometry
HPLC	High-performance liquid chromatography
HPLC-DAD	High-performance liquid chromatography-Diode array detection
HPLC-FLD	High-performance liquid chromatography-Fluorescence detection
ICP-AES	Inductively coupled plasma-atomic emission spectrometry
ICP-MS	Inductively coupled plasma-mass spectrometry
LDH	Lactate dehydrogenase

LLE	Liquid-liquid extraction
LLME	Liquid-liquid microextraction
LODs	Limits of detection
LOQs	Limits of quantification
MALDI-TOF-MS	Matrix-assisted laser desorption ionization-time of flight-mass spectrometry
ME	Matrix effect
MOFs	Metal organic frameworks
MOF-5	$(Zn_4O(BDC)_3)$
MIL-101	Matériaux de l'Institut Lavoisier no. 101
MRI	Magnetic resonance imaging
MSPE	Magnetic solid phase extraction
MWCNTs	Multi-walled carbon nanotubes
NHA	Nanohydroxyapatite
NMs	Nanomaterials
N.R.	Not reported
OTMS	Octadecyltriethoxysilane
PAHs	Polycyclic aromatic hydrocarbons
PET	Positron emission tomography
PK	Pyruvate kinase
RSDs	Standard deviations
SPE	Solid phase extraction
SPME	Solid phase microextraction
SPNE	Solid phase nano-extraction

SWCNTs	Single-walled carbon nanotubes
TCA cycle	Citric acid cycle
TEM	Transmission electron microscopy
TEOS	Tetraethyl orthosilicate
TICs	Total ion chromatograms
Tp53	Tumor protein 53
VOMs	Volatile organic metabolites
γ-MPTMS	γ-mercaptopropyltrimethoxysilane

Thesis structure

Chapter I represents some topics which help to fully understand the purpose of this study. The details involved in this chapter are:

1. Why urinary volatile organic metabolites (VOMs) can be used as cancer biomarkers.

2. Why solid phase extraction (SPE) was selected in this study.

3. Recent advances of nanomaterials (NMs) as SPE sorbents.

Chapter II ascertains the application of Laponite, SiO_2, TiO_2, nanohydroxyapatite (NHA) and magnetic Fe_3O_4@SiO_2-C_{18} NPs as sorbents to extract target VOMs from urine samples. Some variables which influence the extraction efficiency, including sorbent amount, adsorption time, elution time, and types of elution solvent, were investigated. Through the results from urine sample test, it demonstrated that NHA and magnetic Fe_3O_4@SiO_2-C_{18} NPs are good candidates being sorbents for extracting VOMs. In addition, Fe_3O_4@PPy NPs were synthesized and characterized for the further investigation.

Chapter III deviating from the results from chapter II provides a detailed conclusion for the studies and a brief outlook for the further investigation.

CHAPTER I

INTRODUCTION

1. Urinary Volatile organic metabolites (VOMs) as biomarkers of cancer diagnosis

Cancer is a disease of DNA deregulation where endogenous (age, endogenous hormones, genetics and heredity, race) and exogenous (hazardous substances and chemicals, radiation, diet, food additives, lifestyle and behavior) factors are linked to its development.[1] Despite global efforts to limit the incidence of this disease, cancer has become the leading cause of death in the last five decades. Various forms of cancer, including lung-, prostate-, colon- and breast cancer, are now responsible for a quarter of all deaths among males and females through all the world.[2] The management of high-risk cancers requires diagnosis at an early stage. To date, current screening trials have primarily focused on imaging modalities, including computed tomography (CT),[3] magnetic resonance imaging (MRI),[3] positron emission tomography (PET),[4] endoscopy[5] and ultrasonography, [6] coupled with clinical analysis. In most cases, removal of cells or tissues is required resulting in unpleasant for patients, in addition, these methods are expensive and time-consuming.

Over the past few years, exploring non-imaging methods have been extensively investigated. Analysis of biomarker, a biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process, or of a condition or disease,[7] from metabolites present in biological samples allows an indication of abnormal biological processes, especially pathologic processes of cancer since it is well established that the transformation of normal cells to malignant cells is closely associated with important metabolic disturbances.[8] An emerging approach for diagnosing cancer relies on volatile organic metabolites (VOMs), viz. organic metabolites with relatively high vapor pressure can be detected in the headspace of

cancer cells, exhaled biological fluids (*e.g.*, blood or urine) or in the exhaled breath.[9] As mentioned above, the analysis on cancer cells and blood can be ascribed to invasive approach, whereas technologies related with exhaled breath are always expensive. Thus, analysis on urinary VOMs is viewed promising candidate for the detection and diagnosis of cancer. In this part, some molecular biology backgrounds about metabolic changes in cancer will be briefly addressed, followed by a simple summary on the recent advances in the discovery of urinary VOMs potential cancer biomarkers.

1.1. Metabolic changes in cancers

The Warburg effect explains one of the most important metabolic alterations in cancer cells related to the increase in aerobic glycolysis and the dependence to generate ATP by glycolysis. The glycolytic process converts one molecule of glucose into two molecules pyruvate and the free energy released is used to form two high-energy ATP. In normal conditions, pyruvate converts to acetyl-coenzyme A (acetyl-CoA), which provides the basis for the citric acid cycle (TCA cycle) and oxidative phosphorylation. Although cancer cells do not use glucose efficiently, they often convert pyruvate to lactate (**Figure 1.1**). Although the relationship between the increase in aerobic glycolysis and the development of cancer is still controversial, increased glycolysis has been consistently observed in many cancer cells of various tissues, indicating that this metabolic alteration is common in cancer. The Warburg effect can be viewed as a prominent biochemical symptom of cancer cells that reflects a fundamental change in their energy metabolic activity. As we will see in the next pages, several key factors, including pyruvate kinase, hypoxia, protein kinase B, tumor protein 53, lactate dehydrogenase, make contribution to the regulation of the energy metabolic activity.[10]

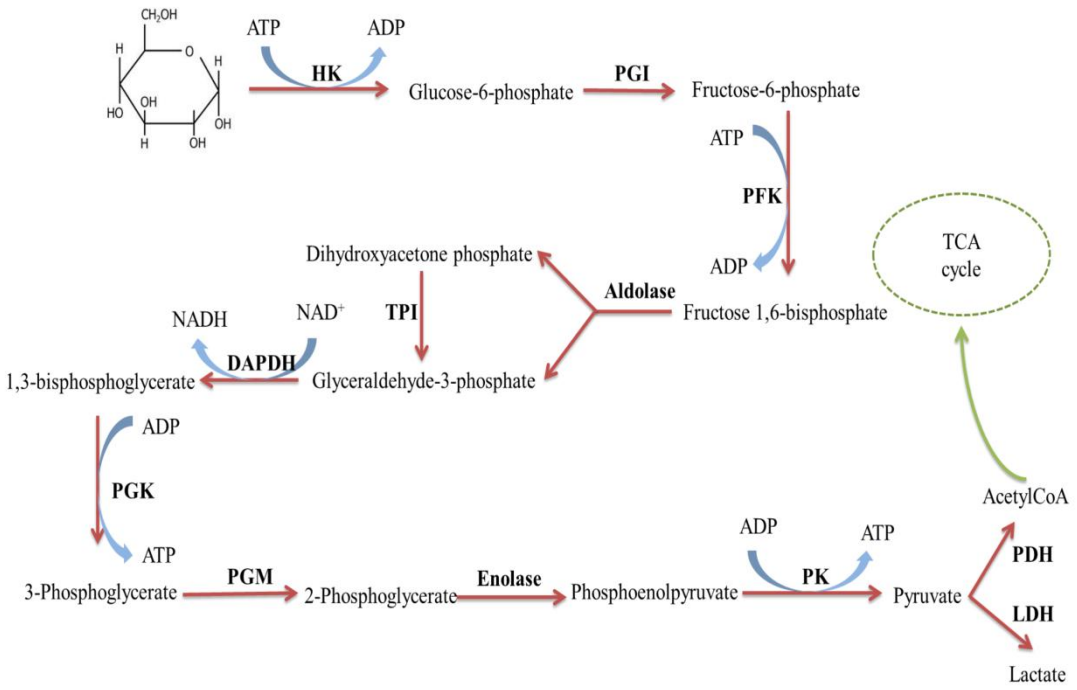

Fig. 1.1. Schematic processes of glycolysis. HK, hexokinase; PGI, phosphoglucose isomerase; PFK, phosphofructokinase; TPI, triosephosphate isomerase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; PGM, phosphoglycerate mutase; PK, pyruvate kinase; PDH: pyruvate dehydrogenase; LDH: lactate dehydrogenase. Adapted from Ref. [9].

Pyruvate kinase

Differing isozymes of pyruvate kinase (PK) essential in nutrient supply and general cell proliferation are expressed in different tissues. For example, PK type L (L-PK) is existed in the kidney, liver and intestine; PK type M1 is expressed in the organs which are strongly energy-dependent, such as brain and muscle; and PK type M2 (M2-PK) in differentiated tissue, including fat tissue, lung, pancreatic islets, retina, and in all normal cells which has a high rate of nucleic acid, as well as in most cancer cells.[11] Its tetrameric form of M2-PK is highly found in normal proliferating cells, but in cancer cells, M2-PK is predominantly in the dimeric form and therefore has been termed as cancer M2-PK. Such dimerization appears is induced by direct interaction between M2-PK and certain oncoproteins. The increases of cancer M2-PK in the plasma and in stool samples is a tool of patients for the early detection of colorectal cancer.[12]

Hypoxia

Cancer hypoxia is the situation where cancer cells are deprived of oxygen. For continuous growth and proliferation in challenging hypoxic situation, metabolism changes are found in cancer cells. Typically, inducible factor-1 (HIF-1) serve as a key regulatory factor which take responsibility for adaptive cellular responses, and then the expression of target genes related with angiogenesis, glycolysis, growth factor signaling, apoptosis, and metastasis are found. In addition, over-expression of HIF-1 takes responsibility of cervical, ovarian, endometrial stomach and breast cancers.[13]

Protein kinase B

The serine/threonine kinase (Akt) which plays a significant role in many cellular processes may contribute to tumorigenesis. Akt, for instance, is associated with the regulation of cell proliferation. Moreover, the regulation of glucose uptake in nanotransformed cells has relationship with Akt. Akt1 triggers the accumulation of NADH and lactate, and accelerates the consumption of glucose.[14] Thus, understanding Akt and its pathways is necessary for creating better therapies to cancer cells.

Tumor protein 53

Tumor protein 53 (Tp53), one of the earliest found anticancer gene, has generally been described as cancer suppressor gene. Tp53 is an important anticancer gene which can prevent cancer occurrence, on the other hand, it also has the ability to repair the defects of cell gene, which helps defected cancer cells undergoing chemotherapy become new ones. The loss of Tp53, moreover, results in increased glycolysis, impairing respiration and leaving glycolysis as the main source of ATP production.[15]

Lactate dehydrogenase

Lactate dehydrogenase (LDH) mediates the conversion of pyruvate to lactate in the

absence of oxygen. The over-expression of LDH indicates the metabolic alters compared to normal cells. Some investigations exhibited that the expression of LDH can be induced directly by oncogenes (c-Myc)[16] or indirectly through the activation of HIF-1.[17]

1.2. Urinary VOMs as biomarkers for cancer diagnosis

Sometimes, certain molecules originating from metabolic disturbances are observed in cancer cells relative to their counterparts, and their altered levels can be measured to establish a correlation with the diseased state. As mentioned above, the investigations on exploring urinary VOMs as biomarkers are viewed as a non-imaging, non-invasive, and non-expensive method, but relative few results have been reported.[18] Metabolites studies generally employ techniques such as nuclear magnetic resonance (NMR), high performance liquid chromatography-mass spectrometry (HPLC-MS) and gas chromatography-mass spectrometry (GC-qMS). Among all the techniques mentioned above, GC-qMS has been widely used in the identification and quantification of metabolites due to its high sensitivity, peak resolution and reproducibility.[19-21] Recently, the analytical platforms used for exploring urinary VOMs are mainly based on solid phase microextraction (SPME) and GC-qMS. For example, Silva *et al.*[18] studied the urinary metabolomics profile of 26 breast cancer patients and 21 healthy individuals with the purpose of studying VOMs as biomarkers in the early detection of cancer. 79 VOMs were detected, belonging to distinct chemical family, in control as well as breast cancer groups. The levels of some molecules, including 4-carene, 3-heptanone, 1,2,4-trimethylbenzene, 2-methoxythiophene, and phenol, increased and then were promoted as breast cancer biomarkers. In addition, the presence of dimethyl disulfide in lower amounts in cancer patients also was identified (see **Figure 1.2**). Thus, this investigation provided an evidence for the use of VOMs as valuable breast

cancer biomarkers.

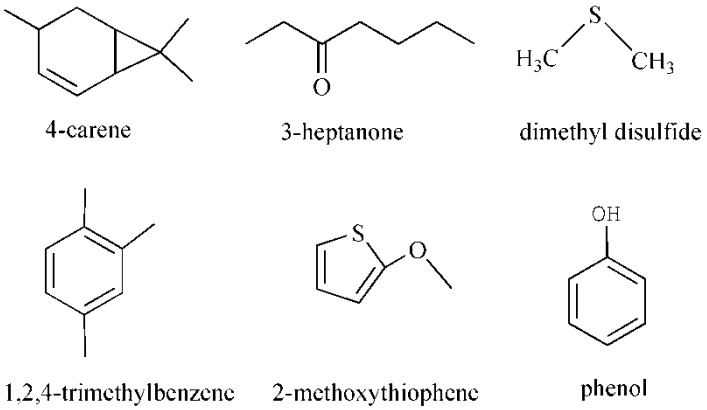

Fig.1.2. Structures of biomarkers from the work of Silva *et al*.[22]

In general, the main disadvantage of SPME is uncovered if the sensitivity is low with regard to the target concentrations of analyte. The occurrence of a huge number of interferents in the chromatogram which comes from endogenous trace substances in urine prevents the analysis of target VOMs at low concentrations (ppm to ppt).[23] Thus, in this study, solid phase extraction (SPE) coupled with GC-MS was selected as main technique. In the following part, the basic principles of SPE and NMs-based sample preparation are summarized and discussed in detail.

1.3. Basic principles of SPE

An analytical process, in general, can be divided into several steps: sample preparation (containing sampling, separation and pre-concentration), detection and data handling and treatment. Among all these procedures, Selective and sensitive sample preparation is a vital role. Making target analytes more suitable for separation or detection are the purposes of sample preparation. Various adsorption materials or solvents therefore have been used in different sample preparation methods, for example, liquid-liquid extraction (LLE),[24] liquid-liquid microextraction (LLME),[25] SPE,[26] and SPME.[27] Among these methods, SPE has received more attention in terms of simpler and faster operation, quicker phase separation, higher enrichment factors

with better recoveries, lower cost, and reduced consumption of organic solvents. The extraction can be carried out within a SPE cartridge/column (**Figure 1.3**),[28] or simply by dispersing sorbents in sample solution followed by collecting the analyte-adsorbed sorbents.[29] For the former method cumbersome packing of sorbent into the column and time-consuming loading of large-volume samples, however, are the disadvantages of this mode. But the latter method can avoid the problems mentioned above.

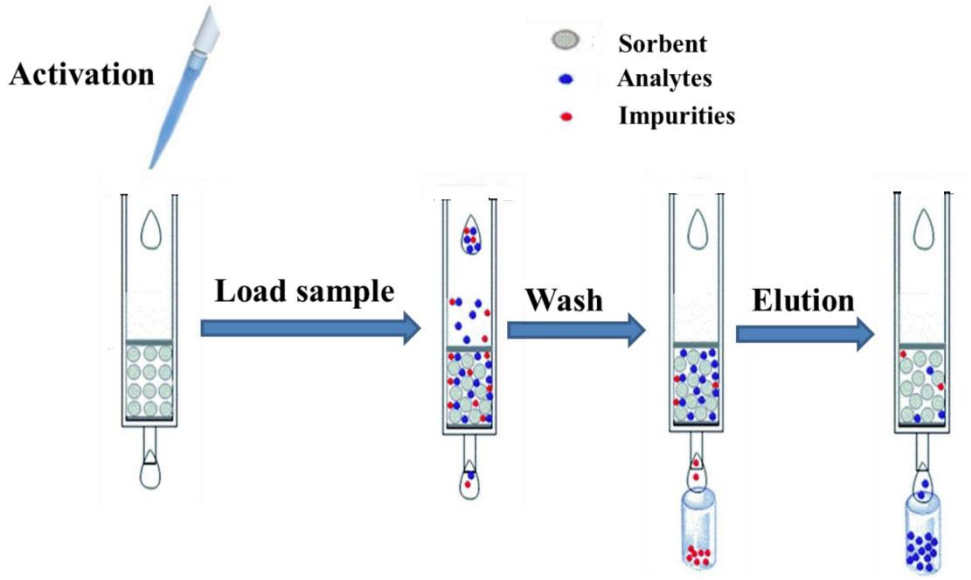

Fig. 1.3. Typical procedures of SPE extraction.

In SPE, the selection of appropriate sorbent, in large part, relies on the understanding of the mechanism of interaction between the sorbent and analytes. In general, the most common mechanisms in SPE depends on hydrophobic (non-polar interactions), hydrophilic (polar interactions) and cation-anion interactions.[30]

Hydrophobic interaction

In reversed phase, the non-polar functional groups of the sorbent operate according to the Van der Waals forces. In general, the analyte is typically mid- to non-polar. The non-polar solvent which have ability to disrupt the forces between the sorbent and analyte is usually selected as elution solvent.[31]

Hydrophilic interaction

Normal phase provide a polar sorbent and the extraction performance of an analyte under normal phase is mainly based on the interaction between polar functional groups of the analyte and polar groups of the sorbent. These processes generally involve hydrogen bonding and π-π interactions. Analyte adsorbed on the sorbent through these mechanisms is usually eluted by a solvent whose polarity is more than sample.[31]

Cation-anion interactions

The main mechanism of cation-anion interactions depends on the electrostatic attraction of the charged functional group of the analyte to the oppositely charged group of the sorbent. These interactions usually disrupted via modifying pH to neutralize analyte or sorbent, in addition, using more selective counter-ion to compete for ion-exchange binding sites.[32]

There are several factors which affect the final extraction results. The exploring suitable sorbent should firstly be taken into consideration since the SPE process could be achieved due to the affinity of analytes to sorbents.[30] In addition, the sorbent area is also a main factor responsible for the extraction performance because large surface area of sorbent means more activity of sorbent.[33] The meaning of pH is also very important. Unsuitable pH values affect the properties of sorbents, such as functional groups of sorbents may be cleaved.[30] Last, the selection of appropriate elution solvent plays vital role for the extraction results, for example, non-polar solvent is good candidate as elution solvent in reversed phase SPE.[34]

1.4. Nanomaterials-based sample preparation in SPE

When applying SPE method, the selection of the most appropriate sorbent is important to allow an efficient isolation and pre-concentration of the analytes and ensure the sensitivity, selectivity and precision of the results. Currently research in

SPE therefore is oriented on exploring novel sorbents with high capacity, good regenerability and surface area accessibility. NMs, materials, in principle, of which a single unit is sized between 1-100 nm, seem the ideal candidate to be SPE sorbent since they often exhibit, compared to their bulk counterparts, impressive changes, such as improved optical, electrical, thermal, magnetic, catalysis characteristics because of their ultra-small size effect.[35-39] Initially, target analytes and interferents were directly removed and concentrated by NMs in SPE. Different chemical groups can also be conjugated on the surface of NM in order to increase their affinity toward target compounds, which makes them suitable to extract target analytes in various samples, such as environmental and biological samples. In this part, some of the important NPs used as sorbents in SPE including metallic NPs, metal organic frameworks (MOFs), carbonaceous NMs and siliceous NPs are discussed due to their widely applications. It should be noted that this part is not a comprehensive survey, but rather discusses some advanced development on the application of NMs used in SPE.

1.4.1. Metallic NPs

Metallic NPs involves a lot of pure inorganic metal, metal oxide NPs and organic/inorganic hybrid NPs, such as MOFs. Due to their chemical compositions, size, and surface structure characteristics, metallic NPs attracted attention in the application of sample preparation of SPE.[40]

i) **Metal NPs**

As one of the promising and reliable NMs, Au NPs has been used of the sample preparation of SPE due to their high surface-to-volume ratio, long-term stability, and compatibility with biomolecules.[41] In addition, some organic molecules containing thiol or amino groups can be easily conjugated onto the surface of Au NPs to form a

well-organized self-assembled monolayer.[42]

By making use of the affinity between polycyclic aromatic hydrocarbons (PAHs) and Au NPs, Wang *et al.*[43-45] developed solid phase nano-extraction (SPNE) technique to pre-concentrate PAHs the from water samples. The Au NPs in 20 nm diameter showed the best extraction efficiency. Through the entire extraction procedure, the consumption of organic solvents per sample is less than 100 μL. High-performance liquid chromatography (HPLC) and laser excited time-resolved Shpol'skii spectroscopy are the two ways for the analysis of the extraction. Similar method was employed to improve the analytical recovery and the detection limits of monohydroxy-PAHs (OH-PAHs) in urine sample.[46]

By simply mixing Au NP of 13 nm and Al_2O_3 particles of 50-100 μm, an Au NP-Al_2O_3 sorbent was introduced to extract mercury species from nature water.[47] Compared to the Al_2O_3 and Au NP sorbents, the Au NP-Al_2O_3 sorbent exhibited excellent extraction efficiency to mercury species and other tested metal ions. Inductively coupled plasma-mass spectrometry (ICP-MS) allowed the detection of mercury ions down to sub-ppq level in aqueous sample. In addition, sequential using of Al_2O_3 and Au NP-Al_2O_3 sorbents enjoy the selectively concentration of inorganic and organic mercury species. Karimipour *et al.*[48] developed a Au NPs-based sorbent conjugated activated carbon for pre-concentrating trace amounts of Co^{2+}, Cu^{2+}, Ni^{2+}, Fe^{2+}, Pb^{2+}, and Zn^{2+} in water samples. Under optimized conditions a pre-concentration factor of 200 was obtained for all the metal ions with detection limits of 1.4-2.6 ng/mL. Gunduz *et al.*[49] synthesized TiO_2@Au NPs functionalized with 11-mercaptoundecanoic acid which have ability to collect Cu^{2+} and Cd^{2+}. The extraction can be analyzed by the detection of flame atomic absorption spectrometry (FAAS). Due to the precipitate was slurried after discarding supernatant, it can be

directly aspirated into the flame for the determination of analytes without elution step, which is the most treasured advantages and the novelty of this established method [36].

Considering the similar properties of Ag with Au, organic compounds have been also purified with Ag NPs. In the presence of cetyltrimethylammonium bromide, a solution stable Ag NPs can be obtained by reducing $AgNO_3$ with $NaBH_4$ in an aqueous solution. Nonpolar organic compounds, for example, PAHs can be pre-concentrated on the surface of Ag NPs. The presence of Ag NPs, moreover, allows the enhancement of analyte luminescence, which has been demonstrated to be useful for the pre-concentration and direct analysis of trace amounts of PAHs in aqueous samples.[50] A method for separation and pre-concentration of Pb^{2+} and Cu^{2+} with cysteamine modified TiO_2@Ag NPs was reported by Baysal *et al.*[51] Similar to the TiO_2@Au NPs mentioned above, the ion-loaded slurry was separated and directly introduced into FAAS for detection.

ii) **Metal oxide NPs**

Metal oxide NPs including TiO_2, ZnO, ZrO_2, Al_2O_3, and CeO_2 have received much more attention because of their use in the application on extracting pollutants. These metal oxide NPs, in most cases, were selected as SPE sorbents for the enrichment of organic compounds and toxic heavy metal ions. Due to their fascinating properties: high chemical stabilities, high adsorption capacity and easy modification ability, these metal oxide NPs meet the requirement of SPE sorbents.[52]

The adsorption of toxic metal ions and rare earth elements on TiO_2 NPs has been reported recently. Baytak *et al.*,[53] for example, described a trace element pre-concentration procedure by using a minicolumn of yeast immobilized TiO_2 NPs for determination of multi-element (*e.g.*, Cr, Cu, Fe, Mn, Ni, and Zn) from water and the extractions were analyzed with inductively coupled plasma-atomic emission

spectrometry (ICP-AES). Some advantages of this established method, including long column reusability, high capacity of pre-concentration and lower memory effects, were observed. In addition, Al_2O_3 NPs have been also used as sorbent for analyzing metal ion in environmental waters.[54] Some examples are summarized in **Table 1.1**.

Table 1.1 Pre-concentration of metal ions by metal oxide NPs.

Samples	NPs	Detection	Analyte	LOD (ng/L)	Ref.
Environmental water	TiO_2	ICP-AES	Cu(II)	340	55
			Cr(III)	1140	
			Mn(II)	520	
			Ni(II)	1780	
Environmental water	Al_2O_3	ICP-MS	Mn(II)	6.7	54
			Ni(II)	38	
			Zn(II)	78	
			Pb(II)	27	
			Co(II)	8.2	
			Cd(II)	79	
Environmental water	ZrO_2	ICP-OES	Mn(II)	12	56
			Ni(II)	7	
Sediment	TiO_2	GFAAS	Se(IV)	160	57
			Se(VI)	140	

Rendering selectivity is important for the use of these NPs as sorbent in SPE. Along this line, physical or chemical modification of the sorbent surface with organic compounds is required. By coordination chemistry some molecules, such as (a) dithizone, (b) diethyldithiocarbamate (DDTC), (c) 1-(2-pyridylazo)-2-naphthol or (d) 8-hydroxyquinoline can be immobilized on TiO_2 NPs and these compounds have been used to pre-concentrate toxic metal ions.[57-59] Except TiO_2 NPs, Al_2O_3 NPs also can be modified by chelating molecules. The modification of Al_2O_3 NPs with dithizone has been reported for the pre-concentration of Pb(II) from drinking and nature water.[60] Another example comes from gallic acid. In this case, the gallic acid introduce selectivity to Al_2O_3 NPs for the pre-concentration of Fe(II) and Fe(III).[61]

iii) Magnetic SPE

It is well established that magnetic SPE is a rapid and easy sample preparation method. In this procedure, the magnetic sorbents are always directly dispersed in the sample solutions and the target analyte therefore is adsorbed on the surface of the sorbents which are separated from the aqueous solution by an external magnetic field. After, the target analytes are eluted for further determination. The general procedure is available in **Figure 1.4**.[62] Some problems with conventional SPE, such as cumbersome of the packing of columns and the time-consuming process of loading large-volume of samples, can be avoided. Typically, the magnetic sorbent was composed with a core material (*e.g.* Fe_3O_4) to collect by a magnetic field, and a supported NP to extract different analytes.[63] **Table 1.2** presented the application of magnetic SPE for different analytes in recent years.[64]

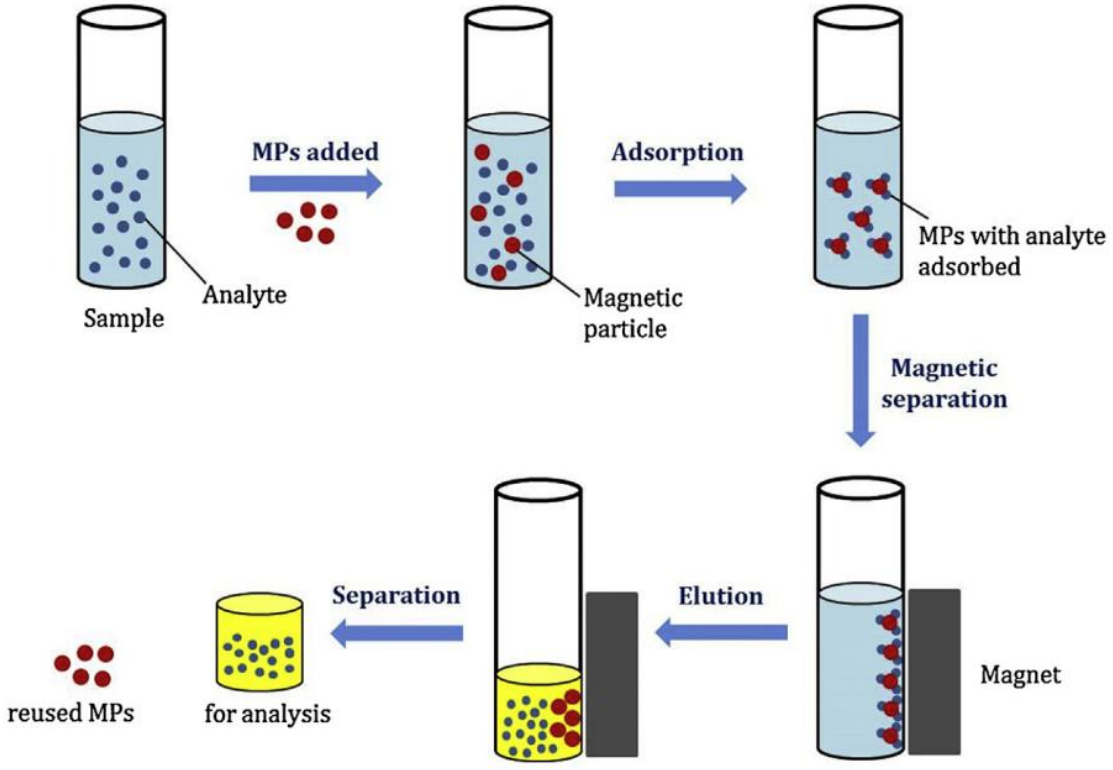

Fig. 1.4. General procedure of magnetic SPE.[55]

Table 1.2 Application of magnetic SPE for different analytes

Supported NPs	Functional monomer	Analytical method	Analyte	Limits of detection (ng/mL)	Limits of quantification (ng/mL)	Linear range (ng/mL)	Sample	Ref
Carbon	Fe_3O_4	GC-MS	PAHs	0.015-0.335	0.05-1.14	0.5-80	Water samples	65
MWCNTs	Fe_3O_4	GC-MS/MS	BPA, BPF and BFDGE	0.001-0.05	n.r.	0.01-50	Water samples	66
MWCNTs	Fe_3O_4	GC-MS/MS	PAEs	0.009-0.032	n.r.	0.05-10	Water samples	67
MWCNTs	Fe_3O_4	GC-MS	PAEs	0.0049-0.038	0.016-0.13	0.2-50	Bottled beverages, tap water and perfume samples	68
MWCNTs-OH	Fe_3O_4	HPLC-DAD	Aconitines	3.1-4.1	10.9-14.2	10.9-425.5	Serum samples	69
CNTs	Fe_3O_4@graphene	MALDI-TOF MS	Small molecule compounds	1.21-2.35	4.03-7.83	5-2000	Urine samples	70
Graphene	Fe_3O_4	HPLC	Carbamate pesticides	0.02-0.04	n.r.	0.1-50	Water samples	71
Graphene	Fe_3O_4	HPLC-UV	Neonicotinoid insecticides	0.01-0.006	n.r.	0.05-50	Water samples	72
Graphene	Fe_3O_4@SiO_2	HPLC-UV	Sulfonamide antibiotics	0.09-0.16	0.32-0.53	0.5-100	Water samples	73

Supported NPs	Functional monomer	Analytical method	Analyte	Limits of detection (ng/mL)	Limits of quantification (ng/mL)	Linear range (ng/mL)	Sample	Ref
C18	Fe_3O_4	GC-MS	PAHs	0.8-36	n.r.	10-800	Water samples	[74]
C18	Fe_3O_4@SiO_2	HPLC	Methylprednisolone	10	n.r.	100-4000	Rat plasma	[75]
Chitosan	Fe_3O_4	HPLC-DAD	Flavonoids	5.4-16.8	18-63	22.5-1575	Green tea beverage samples	[76]
Chitosan	Fe_3O_4@C_{18}	HPLC	PAEs	12.3-36.5	n.r.	0.1-10	Water samples	[77]
Polyaniline (PANI)	Fe_3O_4	GC-MS	Methylmercury	0.15	0.5	0.5-300	Water samples	[78]
Diphenyl	Fe_3O_4	GC-MS	PAHs	n.r.	0.00004-0.00039	0.0003-0.003	Urine samples	[79]
SiO_2-C18	Fe_3O_4@SiO_2	HPLC-FLD	PAHs	n.r.	n.r.	n.r.	Water samples	[80]
C18/NH_2	Fe_3O_4@SiO_2	HPLC-MS/MS	Anionic organic pollutants	n.r	n.r.	n.r.	Water samples	[81]
Schiff base L	Fe_3O_4@SiO_2	FAAS	Pb, Cd and Cu	0.14 0.19 0.12	n.r	0.32-320 0.63-300 0.28-400	Environmental and biological samples	[82]

1.4.2. Metal organic framework materials

MOFs may be defined as a class of organic-inorganic hybrid supermolecular materials that consist of ‘‘strong bonding” providing robustness, linking units that are available for modification by organic synthesis, and a geometrically well-defined structure.[83-85] The materials show different pore size and diverse topology by changing the structure; in addition, the modification of their internal surfaces is easy.

MOF-5 ($Zn_4O(BDC)_3$ (BDC^{2-}:1,4-benzenedicarboxylate) with 3D frameworks[86] was used as the sorbent for in-field sampling and pre-concentration atmospheric formaldehyde from air samples due to their large surface area and pore volume (**Figure 1.5**). By comparing those of commercial sorbents Tenax TA™ (organic polymer) and Carbograph 1TD™ (graphitized carbon black), it showed a good performance to analyze indoor and outdoor air. The good recovery of analysis, in general, after long-time transportation or storage is necessary especially when subjecting situations, such as a long distance between the sampling spot and laboratory and delay between sampling and analysis in the laboratory are inevitable. After sampling, MOFs showed a 90% recovery even 72 h storage at room temperature.[87]

Air-stable MIL-101, a chromium terephthalate-based mesoscopic MOF, even show stability in boiling water and changing conditions of solvent without causing structure change.[88] It was proved that MIL-101 can give much higher affinity and bigger adsorption capacity to volatile organic compounds (VOCs) than activated carbon. Another example about the adsorption of VOCs can be achieved by MIL-101 with various functional groups and polarities at atmospheric pressure, which showed higher affinity and bigger adsorption capacity to VOCs by comparing with activated carbon.[89-90]

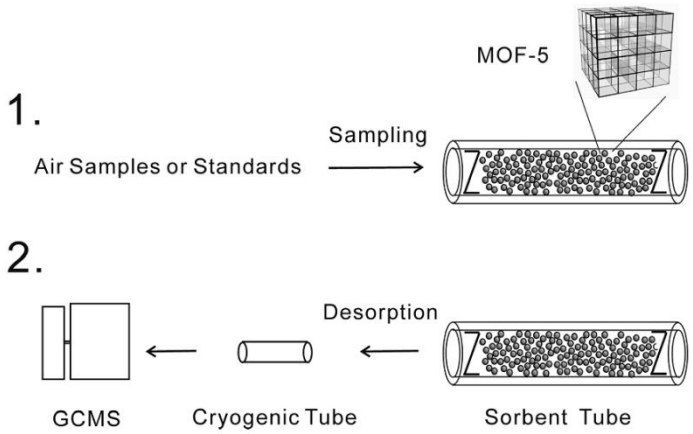

Fig. 1.5. Schematic of sampling and desorption procedures on MOF-5 packed tube.[75]

1.4.3. Carbonaceous NPs

Progress in materials science provides new tools in the analytical sample preparation. Recently, a large number of carbon allotropes have been reported in the literature.[91-94] Most of the carbonaceous NMs have been used as sorbents in different sample preparation techniques. Here, the interests mainly focus on the application of graphene and carbon nanotubes (CNTs) on SPE in recent three years.

***i)* Graphene**

Graphene, a monolayer of carbon atoms densely packed into a 2D honeycomb crystal lattice with a large specific surface area, show great adsorption ability.[95] The large delocalized π-electron system of graphene offer a strong affinity for π-π stacking interaction with carbon-based aromatic ring structures.[96] In addition, easily modification of graphene provide enhanced selectivity, the remarkable example is graphene oxide (GO).[97] Some literatures referred to graphene as good candidate sorbent in SPE have been reported.

As a pioneer work, Liu *et al.*[28] used graphene as sorbent for chlorophenols (CPs) extraction from water samples, using as eluent a solution of alkaline methanol. Compared to graphitic carbon, C_{18} silica and CNTs, higher recoveries of the analytes

were observed. Moreover, the graphene could be reused over 50 times without lacking of recoveries. In order to analyze lead in environmental water and vegetable samples Wang *et al.*[98] developed a SPE method based on graphene. Dithizone was used as chelating reagent before its determination by FAAS. The graphene is viewed as sorbents in SPE because of their outstanding advantages: fewer solvents and samples consumption, shorter pre-concentration time, and higher recoveries.

However, aggregation of the directly used graphene maybe inconvenient no matter in handing and clean-up. To overcome this shortcomings, Zhang *et al.*[99] used sulfonated graphene sheets in μ-SPE method for pre-concentrating PAHs in water samples. The introduction of a small amount of *p*-phenyl-SO_3H groups eliminated the aggregation and water-resistance of the graphene. Under the optimized conditions, good extraction performance can be observed by using sulfonated graphene sorbents, which showed superiority to C_8 and C_{18} in differing sulfur content.

SPE sorbents related with graphene, such as graphene and GO bounded silica (G@silica and GO@silica) avoid not only aggregation but lose of sorbents from the SPE cartridge/column, especially under high pressure in online SPE systems. GO with much more polar moieties enjoys more hydrophilic character. Polar GO@silica therefore could be used for reversed-phase SPE of CPs while non-polar G@silica is more suitable for normal-phase SPE (**Figure 1.6a**). Liu *et al.*[31] developed new SPE sorbents by covalently binding GO sheets to silica and found that organic synthesis was more efficient than aqueous synthesis. These sorbents were used to extract various analytes from small molecules of pollutants to large biomolecules. After bounding on the surface of silica, the extraction efficiencies of graphene and GO was highly enhanced, it is interesting that graphene bounded silica is capable of extracting sticky proteins with large molecular weight and phosphorylated peptides, making

them particularly suitable for handling biological samples for MALDI-TOF-MS analysis (**Figure 1.6b**).

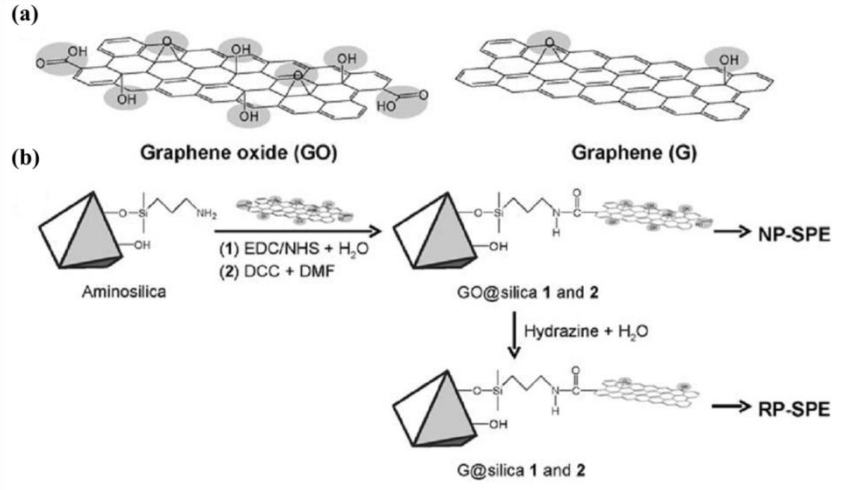

Fig. 1.6. (a) Models of GO and graphene sheets. The shadowed sections indicate the polar groups in the GO and graphene sheets. (b) Chemical routes to the synthesis of GO@silica and G@silica. NP-SPE, Normal-phase SPE; RP-SPE, Reversed-phase SPE; EDC, (*N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride; NHS, *N*-hydroxysuccinimide, DCC =*N*,*N*'-dicyclohexylcarbodiimide, DMF = dimethylformamide.[92]

It's hard to retrieve from a suspension even with high-speed centrifugation since graphene is an ultralight material. Endowing graphene with magnetic properties can avoid the use of cartridges. Luo *et al.*[73] prepared graphene-Fe_3O_4@SiO_2 magnetic composite for extraction of sulfonamide antibiotics from water samples. However, the composite formed by physical adsorption may not be stable enough to reuse. Zhao *et al.* [100] reported graphene-Fe_3O_4 composite by *in situ* co-precipitation of Fe^{2+} and Fe^{3+} in an alkaline solution in the presence of graphene. The results of the experiments showed that the sorbents could be reused over 12 times.

***ii)* Carbon nanotubes**

Without doubt, as the hottest carbon nanostructured materials, CNTs can be viewed as a graphene sheet derived from the scrolling up into a nanoscale-tube.[92, 101] In general, single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes

(MWCNTs) are two kinds of available CNTs. SWCNTs with diameters between 1 and 10 nm are cylinders made of a single layer of graphene sheets[102] while MWCNTs with larger size are concentric SWCNTs held together by *van der Waals* forces.[103]

Unique geometry structure, including high tensile strength, thermal conductivity and stability as well as high resilience, make concentrations to their good mechanical and thermal properties [104] CNTs are therefore have received more attention on the application in electronics, catalysis, biomedicine and analytical chemistry.[105-106] It has been well established that the modification of their side walls can clearly enlarge their potential. In general, it's possible for CNTs to be functionalized with non-covalent and covalent modification modes. Non-covalent modification as presented by π-π-stacking interaction and other physical interaction. Contrasting to non-covalent modification, fluorination, 1,3-dipolar addition, nucleophilic addition, free-radical addition, etc. can be modified by covalent modifications modes. Nowadays, CNTs used as sorbents in SPE mainly based on their ability to establish π-π interaction and *van der Waals* interactions with other molecules. Also, their large surface area, especially on the outside, and interstitial spaces within nanotubes bundles make them as promising sorbents in SPE. The use of CNTs as sorbents in SPE has been discussed in many excellent reviews.[103-104] Thus, in this part, interests mainly be focused on the typically innovations rather than providing a comprehensive collection of all the literatures.

Determination of inorganic[107-112] and organic compounds[113-115] by using CNTs as sorbents in SPE has been considerably reported recently. According to their use in SPE, 3 types of CNTs are available: as-grown, oxidized and functionalized CNTs. Lack of hydrophilicity and functional binding groups on the surface are responsible for the weak performances of the first type on metal ions. To increase the selectivity

for various target analytes, the oxidized and functionalized CNTs were used, in which the as-grown ones were oxidized (including hydroxyl, carboxyl, and carbonyl groups) and the oxidized ones were functionalized with various functional groups, respectively.

For extracting metal ions applications, Soylak and Unsal[116] took advantage of double-walled CNTs to extract Co(II), Cu(II), Ni(II), Pb(II), Fe(III) and Mn(II) from environmental samples. The obtained pre-concentration factor was 100, and when applying to determine these ions in authentic water samples and a reference material, satisfactory results were also obtained.

Oxidized CNTs (ox-CNT) exhibited a better extraction performance than as-grown ones. For example, oxidized SWCNTs introduced by concentrated HNO_3 were packed into a microcolumn as sorbents to pre-concentrate of trace Cu, Co and Pb in biological and water samples.[117] In addition, oxidized MWCNTs was used as SPE sorbents to pre-concentrate trace Rh ion in aqueous solution.[118] Initially, in the pH range of 3.2–4.7, Rh ions were mixed with 1-(2-pyridylazo)-2-naphthol (PAN) to form Rh-PAN complex, followed by adsorbing on the oxidized MWCNTs. The established method offered a higher enrichment factor and lower detection limit for rhodium ion compared with other sorbents. Moradi *et al.*[119] compared the extraction performance of Pb(II), Cd(II), and Cu(II) ions from aqueous solutions onto SWCNT and SWCNT-COOH, which showed SWCNT-COOH surfaces were more versatile than as-grown SWCNT ones. CNT, ox-CNT and l-alanine immobilized CNT (ana-CNT) were prepared by Savio *et al.*[120] to pre-concentrate Ni and Pb in water samples by using an on-line SPE technique. It demonstrated that the ox-CNT could be the best sorbent in terms of the highest capacity and linearity for both analytes when making a comparison with CNT and ana-CNT sorbents.

Pesticides in virgin olive oil samples were analyzed by using MWCNTs and carboxylated SWCNTs as sorbents.[121] It showed that carboxylated SWCNTs achieved low detection limits between 1.5 and 3.0 μg/L, and a fast single pre-concentration-elution step in less than 8 min. Li *et al.*[122] packed MWCNTs, SWCNTs, ox-MWCNTs and ox-SWCNTs in SPE cartridges to extract six polar OPPs from aqueous sample. The oxidation CNTs showed enhanced adsorption abilities to the analytes. By packing MWCNT into mini-column system, Vinas *et al.*[123] evaluated the speciation of four cobalamins representing the various forms of vitamin B12. The developed method could offer a pre-concentration factor of 33 to the analytes, which provided an excellent alternative for the analysis of cobalamins at trace-level.

1.4.4. Silica NPs

It's possible for SiO_2 NPs to introduce chemical modifications due to their high surface areas and intrinsic surface reactivity. **Figure 1.7** shows the general strategies. N-[3-(trimethoxysilyl)-propyl]ethylenediamine, for example, modified SiO_2 NPs have been reported for the pre-concentration of some toxic heavy metal ions such as Hg(II), Cu(II) and Zn(II).[124-126] 1-(2-pyridylazo)-2-naphthol modified SiO_2 NPs (SiO_2-PAN) as sorbents of SPE have been developed for the pre-concentration of trace amounts of Cd(II) in different water samples.[127] The adsorption capacity of SiO_2-PAN was found to be 60.57 μmol/g and the adsorption equilibrium was achieved within just 15 minutes.

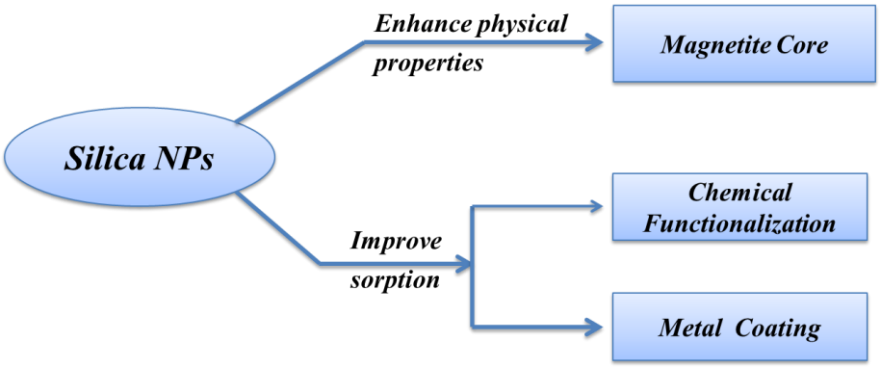

Fig. 1.7. Main strategies of functionalization/modification of silica NPs.

The application of thiol modified SiO_2 NPs for the pre-concentration of drugs and pesticides have also been investigated. Acetylsalicylic acid, *p*-dimethylaminobenzaldehyde and 5-sulfonylsalicylic acid can be conjugated to SiO_2 NPs.[128] In terms of metal coating, SiO_2@Au NPs have also been used for the pre-concentration of neutral steroids (testosterone, progesterone, and testosterone propionate) in urine samples. The pre-concentration of the analytes was more efficient than in a commercial C_{18}-bonded silica gel.[129]

Other applications come from the use of SiO_2 NP derivatives. For example, silica-coated magnetic NPs conjugated by γ-mercaptopropyltrimethoxysilane (γ-MPTMS) have been used for SPE sorbents of trace amounts of Cd, Cu, Hg, and Pb from biological and environmental samples.[130] Similar example related with extraction and pre-concentration of compounds from biological samples, rhein and emodin in urine and serum samples were analyzed with strategy that mixed hemimicelles of cetyltrimethyl ammonium bromide (CTAB) on silica-coated magnetic NPs.[131] With the use of the hemimicelles/NPs-based SPE, the detection limits reached for rhein and emodin were 0.2 and 0.5 ng/mL in urine samples and 7 and 10 ng/mL in serum samples, respectively, and satisfied recoveries were obtained.

In summary, NMs possessing unique physical and chemical properties make them

as promising sorbents in SPE. Although metallic NPs[43], MOFs,[87] carbonaceous NMs,[31] and silica NPs[127] have been widely used as sorbents in SPE, more and more NMs will be used for the sample preparation in SPE. Initially, NMs were used as sorbents in SPE.[43] And then, modification of NMs with various functional groups have ability to improve the extraction performance.[132] For the further investigation, novel NMs will be synthesized according to the demands of applications, for example, endowing selectivity of sorbents is a challenge for the environmental and biological applications. It should be noted that this part only offer a review for the NMs as sorbents in SPE, but NMs used on chemical sensor[133] and stationary phase[134] have become more attractive in analytical chemistry.

CHAPTER II

Experimental

2.1. Introduction

Early diagnosis is of great importance for reducing death rates since different forms of cancer (mainly lung-, prostate-, colon- and breast cancer), are now responsible for a quarter of all deaths among males and females throughout most of the world.[2] Many different methods are currently available for cancer identification, such as computed tomography (CT)[135], magnetic resonance imaging (MRI),[135] positron emission tomography (PET),[4] ultrasonography[6] and endoscopy,[5] which are collectively "physical visualization/detection" methods for the presence of cancer rather than focusing on the metabolites of the cancer. Metabolites which normally serve as potential biomarkers play a significant role in early detection and diagnosis of cancer because cancer is a disease which is known to alter cellular metabolism.[8] Recently, an emerging approach for diagnosing cancer relies on volatile organic metabolites (VOMs), viz. organic metabolites with relatively high volatility, which can be detected in exhaled biological fluids (*e.g.* blood, sputum or urine), and/or exhaled breath.[9, 136-139] Many groups have been reported some VOMs which can be divided into four compound families, for example, alkanes (*e.g.* undecane),[140] aldehydes (*e.g.* hexanal, heptanal, decanal, and benzaldehyde),[18, 140-142] ketones (*e.g.* 4-heptanone),[18] and aromatic compounds (*e.g.* 5-methyl-2-furfural and phenol).[18, 143] Thus, considerable efforts have been made towards the development of various methods for cancer VOMs detection.

Exhaled biological fluids test have been long recognized as a fast and non-invasive medical technique that make the link between specific VOMs associated with the detection of cancer and medical conditions possible. Normally, a majority of methods developed for VOMs monitoring in the exhaled biological fluids has been based on sorbent extraction.[144] In brief, the

extraction process could be achieved on account of the affinity of VOMs existing in exhaled biological fluids to solid sorbent. The sorbent is therefore the core of the extraction process that determines the efficiency and the selectivity of the extraction.

Among all kinds of sorbents, nanomaterials are viewed as good candidates for extracting VOMs in exhaled biological fluids of cancer due to their high surface area and reactivity,[145] presenting higher adsorption ability than their bulk counterparts.

Laponite ($[Na_{0.7}(Si_8Mg_{5.5}Li_{0.3})O_{20}(OH)_4]$), a relatively uniform disc-shaped synthetic clay having 25 nm in diameter and 1 nm thick,[146] is often used as sorbent due to they (1) are relatively monodisperse, (2) have a controlled chemical composition, and (3) have a large surface available for adsorption.[147] For example, the adsorption of cationic surfactant,[148] poly(ethylene oxide),[147] and polycyclic aromatic compounds[149] on laponite were investigated. However, for VOMs, no report is available so far.

Metal oxide NPs are a class of promising sorbent in SPE, for example, as an important industrial material, TiO_2 NPs have been widely used as sorbent to adsorb alcohols, DNA Oligonucleotides, arsenic.[150] In addition, SiO_2 NPs also have been selected as SPE sorbent due to their large specific surface area and high adsorption ability.[151] But the extraction performance of VOMs on these two NMs have not been reported.

Nanohydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, NHA) whose structure is hexagonal prism has non-toxicity, non-irritant property, non-solubility and also non-allergenic similar to the main inorganic components in human bones and teeth. They exhibits excellent biocompatibility and adsorption properties,[152] and

has been widely used as a sorbent for the adsorption and separation of biomolecules (*e.g.* bovine serum albumin),[153] and for removal of heavy metals (*e.g.* Pb^{2+}, Cd^{2+}) from contaminated solid and water.[154] In addition, the adsorption of succinylated lysozyme,[155] oxalic acid,[156-157] nitrobenzene,[158] and fluoride[159] could be achieved by employing NHA. However, to our knowledge, the extraction of VOMs in the exhaled biological fluids, particularly in urine, of cancer patients by NHA has not been reported.

Usually, intensive centrifugation at high speed is inevitable when applying NMs as sorbents in SPE, which may lead to co-precipitation of undesirable interferents, such as non-volatile metabolites (hormones, uric acid, urea, among other with high molecular weight), and even loss of some target analytes, which, in large part, restricts the application of these advanced materials, for example, undesirable interferents cause noise of the equipment. A simple, rapid, gentle and efficient method is therefore urgently needed. In past decades, separation technology based on magnetic NMs (MNMs) has become a powerful complement to sorbent-based extraction of urinary VOMs.

MNMs have been widely applied in various research fields, including chemosensors,[160] catalysis,[161] drug delivery,[162] MRI,[163] and sample preparation.[63, 160, 164-165] Although a number of magnetic materials are available now (*e.g.*, iron, cobalt, nickel, magnetite, maghemite and alloys), Fe_3O_4 NPs are the most frequently used MNMs in sample preparation due to their easy preparation, surface modification and good recoverability.[166] Although bare Fe_3O_4 NPs can be directly used for isolation and pre-concentration of some target analytes, they are prone to the formation of large aggregates resulting in changes of their magnetic properties, in addition, their lack of selectivity makes them unsuitable for samples with complex matrices.[167] Thus, a

suitable coating and modification of the Fe_3O_4 NPs is needed to overcome the limitations mentioned above. Normally, coating with silica is considered to improve the stability and prevent oxidation of the Fe_3O_4 NPs. Furthermore, the modification of the silica-coated Fe_3O_4 NPs can be achieved by silanation using silane coupling agents (*e.g.*, C_{18}).[168] In this study, silica-coated Fe_3O_4 NPs functionalized by C_{18} (Fe_3O_4@SiO_2-C_{18}) NPs were successfully synthesized, though this sorbents have been widely used as sorbent for the adsorption and separation of methylprednisolone (MP),[75] lidocaine[169] and puerarin[170] from the complex matrix of rat plasma, and for removal of sudan dyes,[171] pesticide residues,[172] and polycyclic aromatic hydrocarbons (PAHs)[173] from water samples, in addition, the adsorption of ergosterol from cigarette could be achieved by employing Fe_3O_4@SiO_2-C_{18} NPs,[174] to our knowledge, the extraction of VOMs in the urine of cancer patients by Fe_3O_4@SiO_2-C_{18} NPs has not been reported.

Polypyrole (PPy), one of the most important conducting polymers, has been widely studied as SPE sorbents in many fields due to the formation of the π-π complex and/or hydrophobic interactions between PPy and analytes.[175-176] Considering the advantages of magnetic separation, Fe_3O_4@PPy NPs were synthesized and characterized by IR and TEM.

There are several experimental variables affecting the extraction performance, such as sorbent amount, adsorption time, elution time, and types of elution solvent. Thus, designing and optimization of the experimental variables should be taken into consideration. In this work, Laponite, TiO_2 NPs, SiO_2 NPs, NHA and Fe_3O_4@SiO_2-C_{18} NPs were used, for the first time, with the help of GC-qMS, to explore the optimum combination of experimental variables in order to investigate the

possibilities of these materials as an immobilization materials to extract VOMs from urine of cancer patients.

2.2. Experimental

2.2.1. Chemicals and Reagents

All the reagents used were of analytical regent grade and used without further purification. Nanohydroxyapatite was purchased from Aladdin Chemical Reagent Co, Ltd (Shanghai, China). Methanol (99.99%) or ethanol (99.99%) used as solvent in the whole experiment were purchased from Fisher Scientific (Loughborough, UK). Decanal (95%), hexanal (96%), heptanal (95%), undecane (99%), 5-methyl-2-furfural (98%), TiO_2 NPs, SiO_2 NPs, and laponite was obtained from Acros Organics (Geel, Belgium) while benzaldehyde (99%), phenol (99%) and 4-heptanone (96%, purity) were from Sigma-Aldrich (Madrid, Spain). The chemical structures of VOMs investigated in this study are shown in **Figure 2.1**.

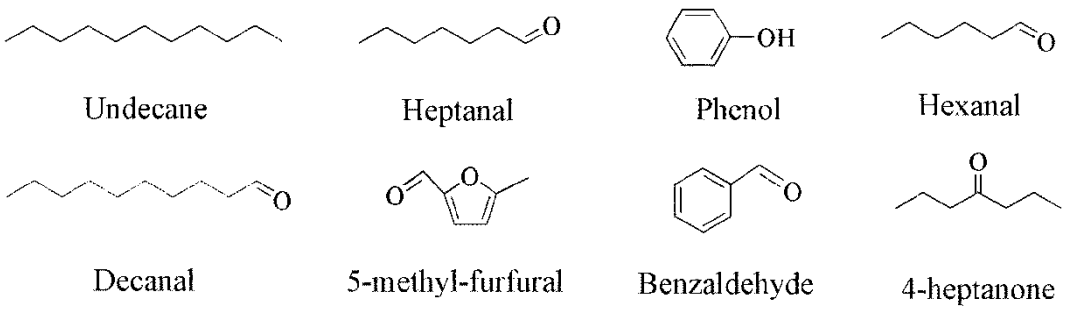

Fig. 2.1 Chemical structures of the volatile metabolites used in the experiment

$FeCl_3 \cdot 6H_2O$, $FeCl_2 \cdot 4H_2O$ and ammonia, used for the synthesis of Fe_3O_4@SiO_2-C_{18} NPs and Fe_3O_4@PPy NPs, were purchased from Sigma-Aldrich. Tetraethyl orthosilicate (TEOS) and ethanol were employed for covering the Fe_3O_4 NPs with a protective silica-based coating. Finally, n-octadecyltriethoxysilane (C_{18}) and toluene were used to introduce hydrophobic groups on the NP surface.[177] In the

case of Fe_3O_4@PPy NPs, PPy monomers were used to cover the surface of Fe_3O_4 NPs with the help of sodium dodecyl sulfate (SDS).[178]

2.2.2. Subjects and sample collection

Cancer patients (n=9, age=40.6 ± 7.4 years; non smokers) underwent different diagnostic procedures, such as breast physical examination, mammography and ultrasonography, MRI and chest X-ray and finally histologically diagnosed with primary cancer by the Haematology–Oncology Unit of the Dr. Nélio Mendonça Hospital. Each individual provided a sample of morning urine (after overnight fasting) in a 20 mL sterile PVC container. The samples were immediately frozen at −80°C and kept until being processed.

All cancer patients gave their written informed consent for inclusion in the study and the research was approved by the Ethics Committee of the Dr. Nélio Mendonça Hospital, being done in accordance with the Good Clinical Practice guidelines and with the ethical guidelines of the 2013 Declaration of Helsinki (DoH).[179] All data were analyzed anonymously throughout the study.

2.2.3. Synthesis of Fe_3O_4@SiO_2-C_{18} NPs and Fe_3O_4@PPy NPs

The procedures of the synthesis of Fe_3O_4@SiO_2-C_{18} NPs were prepared according to previously report by Alcudia-León *et al* (**Figure 2.2**).[180] The Fe_3O_4 NPs was obtained by co-precipitation method. Briefly, $FeCl_3 \cdot 6H_2O$ (0.09 mol, 24 g) and $FeCl_2 \cdot 4H_2O$ (0.05 mol, 9.8 g) were dissolved in 100 mL of water under gentle nitrogen atmosphere, vigorously stirred and maintained at 80 °C in a water bath for 30 min. Then, 50 mL of ammonia (25 wt.%) were added dropwise. The Fe_3O_4 NPs were separated with an external magnetic field, washed with water to remove the unreacted chemicals, and finally air dried.

To the surface coating, the Fe_3O_4@SiO_2 NPs were obtained by simply dispersing

Fe_3O_4 NPs in ethanol/water (50 mL/4 mL) solution in the presence of TEOS (2 mL) under a nitrogen atmosphere. The dispersion was stirred overnight and the Fe_3O_4@SiO_2 NPs were collected with an external magnetic field, thoroughly washed with water and dried. Finally, the surface modification of the Fe_3O_4@SiO_2 NPs was achieved by dispersing in 50 mL of anhydrous toluene containing 1% (v/v) of n-octadecyltriethoxysilane (C_{18}). The mixture was sonicated for 5 min and refluxed for 12 h. The obtained resultant Fe_3O_4@SiO_2-C_{18} NPs were washed several times with ethanol and air dried.

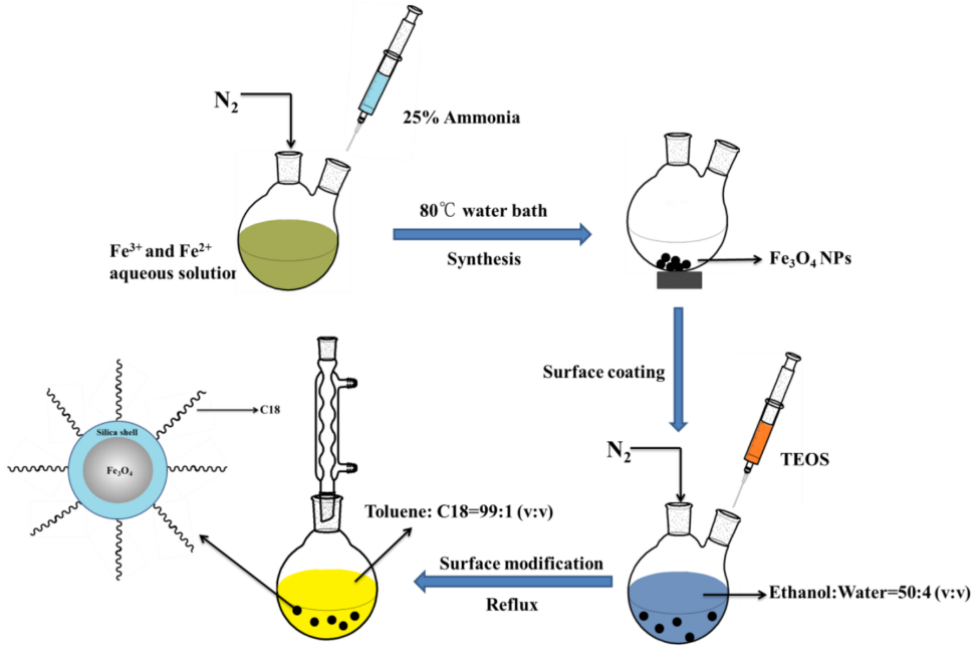

Fig. 2.2. Schematic illustration of the preparation of Fe_3O_4@SiO_2-C18 NPs.

In the case of the synthesis of Fe_3O_4@PPy NPs, 1.0 g (4.3×10^{-3} mol) of MNPs (Fe_3O_4 NPs), 9.1 g (0.03 mol) of $FeCl_3\cdot6H_2O$, and 100 mL of deionized water were added to a 250 mL flask. After sonification for 3 h, 20 mL of SDS solution (5.85 wt %) and 0.5 mL of PPy monomers were rapidly added, and the mixture was kept shaking for 12 h. Finally, the resultants were separated by external magnetic field and washed 3 times by deionized water.

2.2.4. Characterization of Fe_3O_4@SiO_2-C_{18} NPs and Fe_3O_4@PPy NPs

The synthesized Fe_3O_4@SiO_2-C18 NPs and Fe_3O_4@PPy NPs were characterized by FT-IR spectroscopy and Transmission Electron Microscopy (TEM). IR spectra were obtained on a Thermo Nicolet Magna-IR 750 fourier transform infrared spectrometer (Waltham, MA). The samples for IR measurement were prepared by mixing the sample powder with KBr and then pressing the mixture into transparent disks.

Specimens for TEM were prepared by releasing a drop of Fe_3O_4@SiO_2-C_{18} NPs and Fe_3O_4@PPy NPs in methanol onto 400-mesh copper grids coated by ultrathin carbon support films (Ted Pella; Redding, CA). Once the grids were dry, images were acquired using a Philips CM-12 microscope operating at an accelerating potential difference of 120 kV.

The Fe_3O_4@SiO_2-C_{18} NPs show a spherical morphology with an average diameter of 10 nm (**Figure 2.3**). The IR spectrum obtained under the attenuated total reflection sampling mode shows a characteristic band of Fe_3O_4 around 600 cm^{-1} ascribed to the Fe-O bonds and a strong absorbing region at 1200-1000 cm^{-1} which are corresponded to the Si-O-H and Si-O-Si bonds. Furthermore, the C-H stretching vibrating bands of octadecyl groups of the Fe_3O_4@SiO_2-C_{18} NPs can be observed at 2920 and 2850 cm^{-1} (**Figure 2.4**).

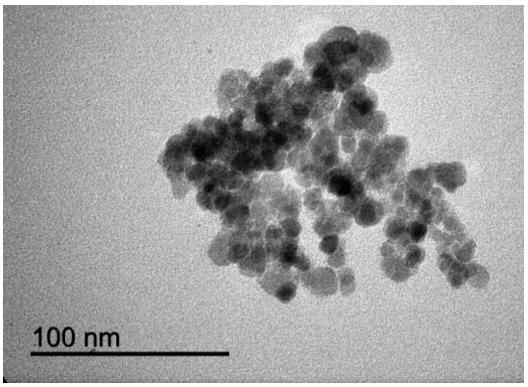

Fig. 2.3. TEM microscopy of Fe_3O_4@SiO_2-C_{18} NPs.

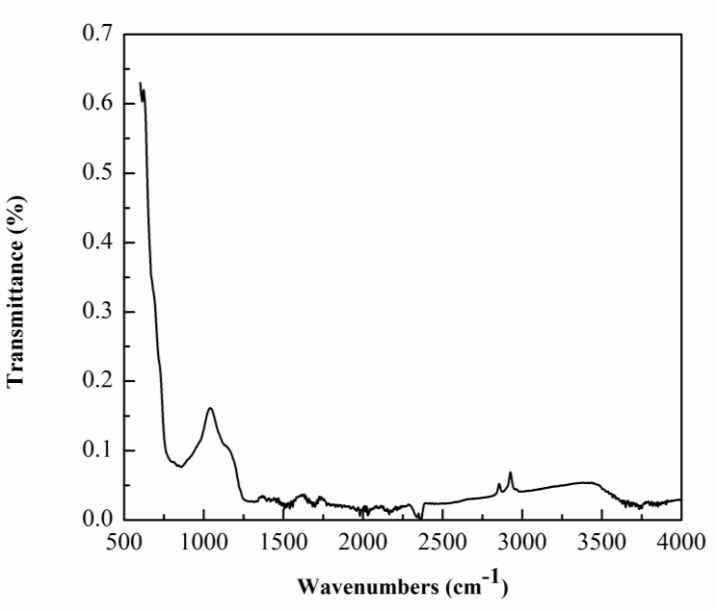

Fig. 2.4. IR spectrum of Fe_3O_4@SiO_2-C_{18} NPs.

For the Fe_3O_4@PPy NPs, from the TEM graphic, they showed spherical morphology with a mean size of about 15 nm (**Figure 2.5). Figure 2.6** shows the IR spectrum of Fe_3O_4@PPy NPs, the bands at 1455 and 1040 cm^{-1} can be assigned to the C-N ring stretching vibrations of the pyrrole ring. In addition, the peak at 1166 and 886 cm^{-1} are related to the C-H vibrations.

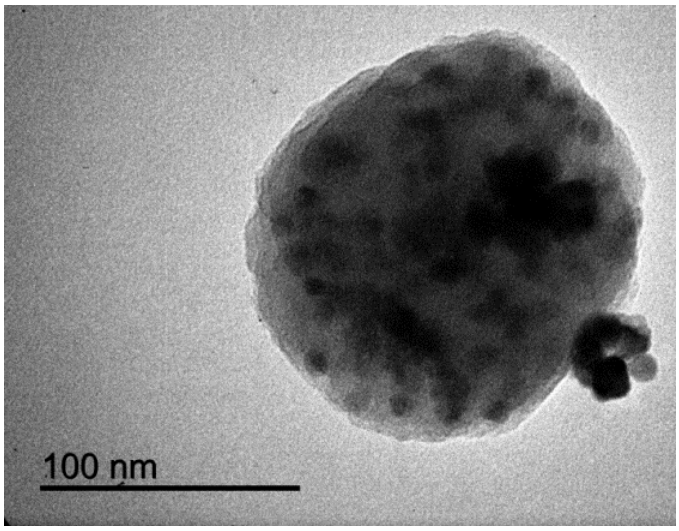

Fig. 2.5. TEM microscopy of Fe_3O_4@PPy NPs.

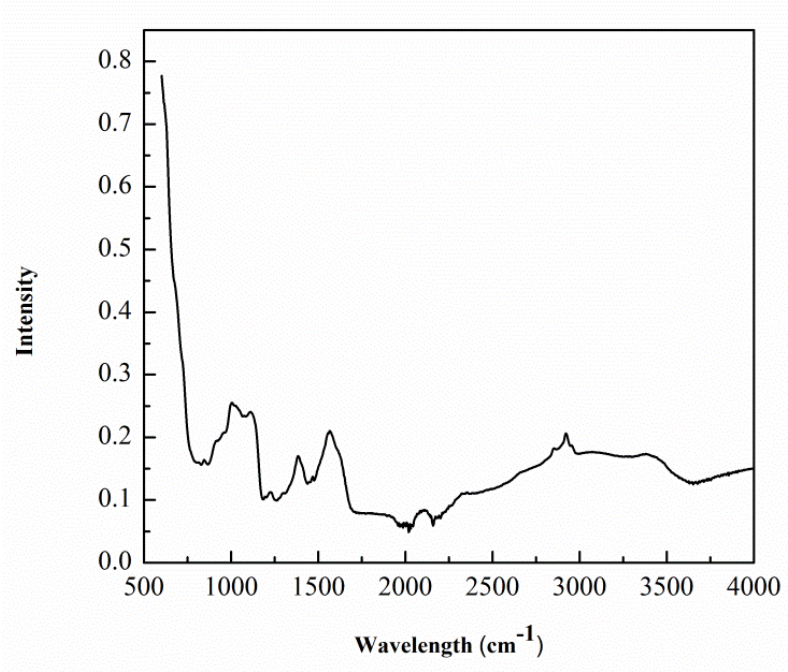

Fig. 2.6. IR spectrum of Fe_3O_4@PPy NPs.

2.2.3. Preparation of stock and working solution

Individual stock solution of target volatile metabolites were prepared in a hydroalcoholic solution (12% v/v) with a concentration of 500 µg/L, aliquoted in 8 mL vials and stored at −20°C in the dark until use. Working solutions containing the cancer biomarkers were prepared daily from the individual stock solutions by diluting them in the synthetic urine whose formula was described by Uppuluri *et al.*[181] The ranges of concentrations (see **Table 2.1 and Table 2.2, pages 79 and 80**) were selected according to the sensitivity of the GC-qMS towards each biomarker (as the physical-chemical characteristics of each compound affect its analytical signal, higher concentrations had to be used for some compounds in order to be possible their detection) and its concentration on biologic fluids. All samples were analyzed in triplicate.

2.2.4. NMs-based Sorbent Extraction Procedure

Batch techniques were employed for investigating the extraction process of VOMs in working standard solution. Normally, extraction can be carried out simply by dispersing NMs in working standard solution followed by collecting the analyte-adsorbed NMs by centrifugation. Adsorptions were carried out by taking different amounts of sorbent in 8 mL vials containing 500 µL working standard solution and 1 mL deionized water. The mixture solution was stirred with the help of magnetic stir at the constant temperature of 45 °C for 1 hour except where the effect of the contacting time was investigated. After each batch of the adsorption, the sorbent was separated from the solution and eluted by 1.5 mL elution solvent. The eluate was collected and evaporated to 200 µL under gentle stream of nitrogen before GC-qMS analysis (**Figure 2.7**). When using Fe_3O_4@SiO_2-C_{18} NPs as sorbents, ultrasounds (5 min) was selected for an efficiently dispersion and the sorbents were collected by magnetic separation (**Figure 2.8**).

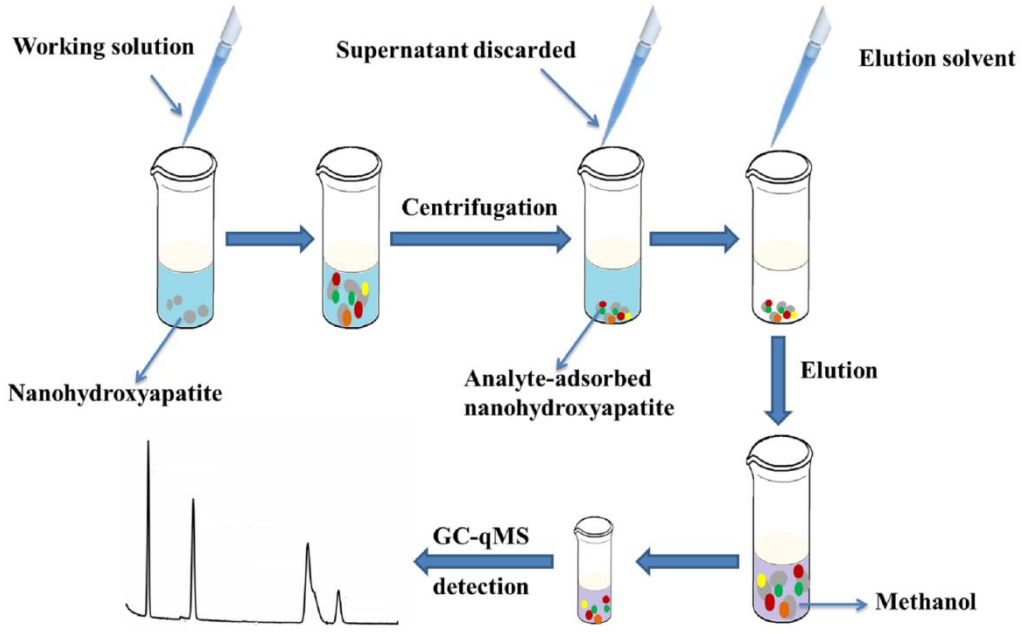

Fig. 2.7. Scheme showing the route to the extraction of VOMs.

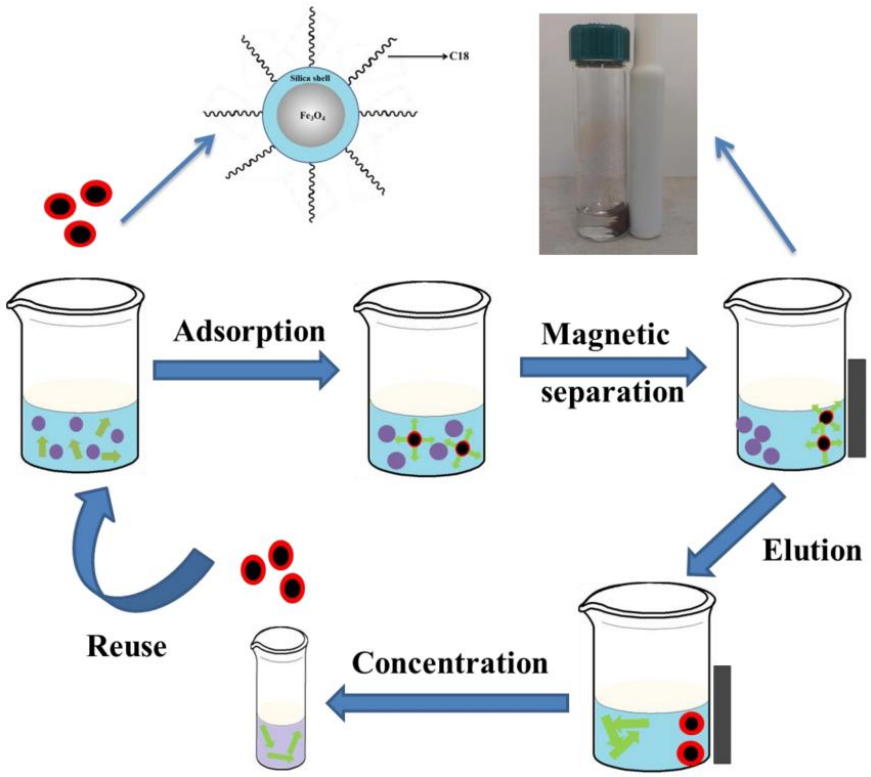

Fig. 2.8. Procedure for the extraction of VOMs by using Fe_3O_4@SiO_2-C_{18} NPs.

2.2.5. Chromatographic and Mass Spectrometric Conditions

The chromatographic separation was performed on an Agilent Technologies 6890N Network gas chromatography system (Palo Alto, CA, USA). The carrier gas was He (Helium N60, Air Liquid, Portugal) with flow rate of 1.3 mL/min. The gas chromatography was fitted with a 30 m × 0.32 mm I.D. × 0.25 μm film thickness, Carbowax-20 (SGE, Dortmund, Germany) fused silica capillary column and interfaced with an Agilent 5975 quadrupole inert mass selective detector. The oven temperature was: 60 °C for 4 min, followed by an increase of 1.20 °C/min to 100 °C, held for 2 min. 10.0 °C/min to 220 °C staying for 5-min at this temperature, for a total GC run time of 56.33 min. The splitless mode was used and the injector temperature was 250 °C.

For the 5975 MS system, the quadrupole temperature, transfer line temperature,

and MS source temperature were 150, 250, and 220 °C, respectively. Electron impact mass spectra were recorded at 70 eV ionization voltages and the ionization current was 10 μA. 2 min was used as solvent delay. Data acquisition was performed in Scan mode (30-300 *m*/*z*). The electron multiplier was set to the auto tune procedure. Identification of VOMs was accomplished through (*i*) manual interpretation of spectra and matching with the Agilent MS ChemStation Software, coupled with a NIST05 mass spectral library with a similarity threshold higher than 80 %, and (*ii*) comparison with commercially available standard samples when necessary.

2.2.6. Method validation

The NMs-based sorbent extraction approach to isolate urinary VOMs from cancer patients was validated for linearity, analytical limits including instrument LODs and method LOQs, intra- and inter-day precision, accuracy and matrix effect (ME).

Method linearity was evaluated by constructing a seven concentration levels calibration curve (peak area versus metabolite concentration), in triplicate, for each metabolite in synthetic urine, covering the concentration range of VOMs normally found in urine of cancer patients (**Table 2.1 and Table 2.2**). The sensitivity was assessed determining the LOD (the lowest metabolite concentration that produces a response detectable above the noise level of the system) and LOQ (the lowest level of metabolite that can be accurately and precisely measured) for each metabolite. LOD and LOQ were calculated with the data generated by linear regression results as follows: LOD=a+3$S_{a/b}$ and LOQ=a+10$S_{a/b}$, where "a" represents origin ordinate, "S_a" the origin ordinate variance and "b" the slope.[182]

The precision of the method (expressed as %RSD) was evaluated using synthetic urine spiked with a known concentration (10 μg/L) of each target VOMs. Seven replicates (n=7) was performed in the same day to obtain repeatability (intra-day

precision). The inter-day precision was based on the analysis of three successive replicates five different days (n=15).

The accuracy of the method was evaluated through a recovery study, using synthetic urine spiked with 10 μg/L of each VOM, and subjected to NHA-based sorbent extraction approach described in Section 2.4. The recovery values were calculated according to: % Accuracy = 100 × ($[\text{metabolite}]_{\text{after spiking}}$ - $[\text{metabolite}]_{\text{before spiking}}$)/[$\text{metabolite}]_{\text{added}}$); where $[\text{metabolite}]_{\text{after spiking}}$ is the metabolite concentration measured in spiked synthetic urine; $[\text{metabolite}]_{\text{before spiking}}$ is the metabolite concentration measured in the same unspiked urine, and $[\text{metabolite}]_{\text{added}}$ is the nominal volatile metabolite concentration added to synthetic urine. The targeted VOMs concentration in urine samples was previously determined using NHA-based sorbent extraction approach. The ME was estimated through the ratio between the slopes of the calibration curves obtained from synthetic urine-matched standard solution and those obtained by matrix matched calibration according to the following equation: $\%ME=b_{MMC}-b_{SUMSS}/b_{SUMSS}$; where: b_{MMC} is the slope of matrix-matched calibration, and b_{SUMSS} is the slope of synthetic urine-matched calibration standard solution. All the experiments were performed at least in triplicate.

Table 2.1. Validation parameters for urinary cancer biomarkers using NHA-based sorbent extraction with GC-qMS

RT (min)	Cancer biomarkers	Calibration equation	r^2 [a]	LOD (ng/L)[b]	LOQ (ng/L)[c]	% Recovery	Matrix effect (%)	Intra-day (*n* = 7)	Inter-day (*n* =15)
2.25	Hexanal	y = 5109.9x + 13750.2	0.993	35.7	119.0	85.9	94.6	2.2	12.9
2.52	Undecane	y = 6390.3x + 67654.2	0.994	43.5	144.9	84.4	93.5	1.0	10.1
3.41	4-Heptanone	y = 19002.0x - 3815.9	0.999	34.4	114.7	46.4	69.8	0.8	3.9
3.63	Heptanal	y = 6503.0x + 7566.0	0.997	69.5	231.6	70.9	72.9	1.6	5.4
14.92	Decanal	y = 6952.2x - 4142.1	0.997	9.76	32.5	55.7	92.7	1.8	8.2
16.12	Benzaldehyde	y = 15299.9x + 10787.0	0.995	41.9	139.6	40.4	75.1	2.1	7.9
21.35	5-Methyl-2-furfural	y = 6979.7x + 907.3	0.994	10.0	33.4	79.6	72.8	1.7	8.2
46.33	Phenol	y = 2301.9x + 4868.7	0.998	13.8	46.1	97.2	96.1	1.3	9.4

[a] Correlation coefficient, give an estimating how well the experimental points fit a straight line;

[b] Limit of detection;

[c] Limit of quantification. Values obtained from ordinary least-squares regression data.

Concentration range: 0.2 - 50.0µg/L

Table 2.2. Validation parameters for urinary cancer biomarkers using Fe_3O_4@SiO_2-C_{18} NPs-based sorbent extraction with GC-qMS

RT (min)	Cancer biomarkers	Calibration equation	r^2 [a]	LOD (ng/L)[b]	LOQ (ng/L)[c]	% Recovery	Matrix effect (%)	Intra-day (n = 5)	Inter-day (n =25)
9.62	Hexanal	Y = 41325x + 253000	0.988	39.2	130.6	82.6	91.5	2.8	10.3
10.04	Undecane	Y = 88098x + 204006	0.990	24.1	80.2	65.8	90.7	0.4	5.7
11.65	4-Heptanone	y = 92489x + 07173	0.991	19.1	63.6	58.5	62.8	0.7	8.6
14.59	Heptanal	Y = 42395x + 309518	0.992	50.8	169.2	72.1	71.5	1.2	5.8
39.44	Decanal	Y = 52543x + 299158	0.994	42.6	141.8	72.9	91.7	1.5	4.9
41.19	Benzaldehyde	Y = 40595x + 170828	0.995	9.7	32.4	99.1	89.5	0.8	2.4
43.75	5-Methyl-2-furfural	Y = 39381x+125457	0.991	57.3	190.9	42.6	78.6	1.3	6.4
52.21	Phenol	Y = 90506x + 120158	0.996	12.0	40.1	74.2	96.1	1.2	4.9

[a] Correlation coefficient, give an estimating how well the experimental points fit a straight line;

[b] Limit of detection;

[c] Limit of quantification. Values obtained from ordinary least-squares regression data.

Concentration range: 0.25 - 50.0µg/L

2.3. Results and Discussion

Laponite, SiO_2, and TiO_2 didn't give any peaks when applying these materials as sorbents in SPE. For the case of Laponite, the blockage of internal cavity caused by swell when meeting aqueous solutions probably make contribution to the poor extraction performance. For SiO_2 and TiO_2, lack of surface modification by functional groups, such as carbonaceous and thiol compounds, may be a main reason for the absence of peaks.

2.3.1 Optimization of NMs-based sorbent extraction approach

NHA possesses a rod-like morphology according to our previous report.[183] The target compounds in this experiment are widely detected under the optimum conditions by using GC-qMS analysis. The total ion chromatogram is shown in **Figure 2.9**. Compounds were identified from their molecular fragmentation and quantified from the peak area of their major fragment ions. All the chemical standards were separately identified.

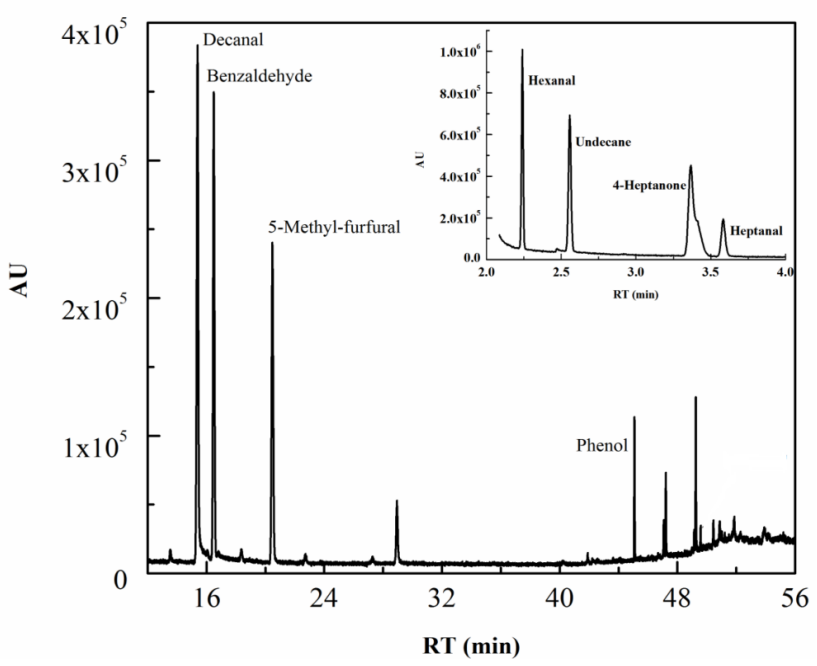

Fig. 2.9. Total ion chromatogram of fortified synthetic urine by NHA/GC-qMS under optimum conditions

Various batch experiments were conducted to evaluate the effect of several factors involved in the extraction performance. For the case of NHA-based sorbent extraction approach, the effect of the sorbent amount was investigated by varying the total amount of sorbent from 20 to 60 mg under an adsorption time of 30 min, elution time of 1 hour (methanol was used as elution solvent). Increase in sorbent amount increased the adsorption of VOMs (**Figure 2.10**), which could be attributed to an increase in available surface area and adsorption sites resulting from the increased concentration of nanohydroxyapatite. Thus, based on the present investigation, 60 mg of sorbent was selected as the optimum amount for further experiments.

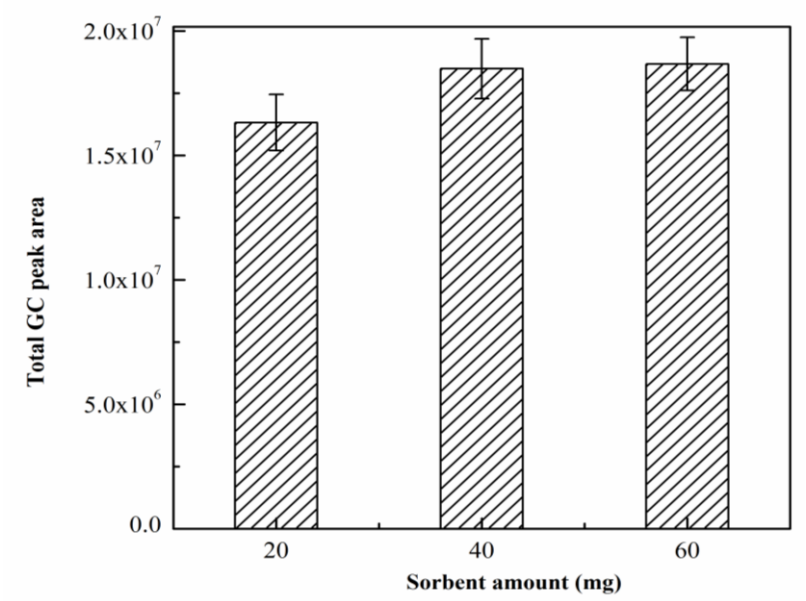

Fig. 2.10. Effect of sorbent amount of HNA on the adsorption efficiency of VOMs.

For the Fe_3O_4@SiO_2-C_{18} NPs-based sorbent extraction approach, the effect of the sorbent amount was studied by varying the total amount of sorbent from 20 to 60 mg under the adsorption time of 30 min, elution time of 30 min (methanol was used as elution solvent). Increase in sorbent amount increased the adsorption of VOMs (**Figure 2.11)** while the total GC peak area of 40 and 60 mg are almost same. Thus,

based on the present investigation, 40 mg of sorbent was selected as for further experiments as the optimum amount.

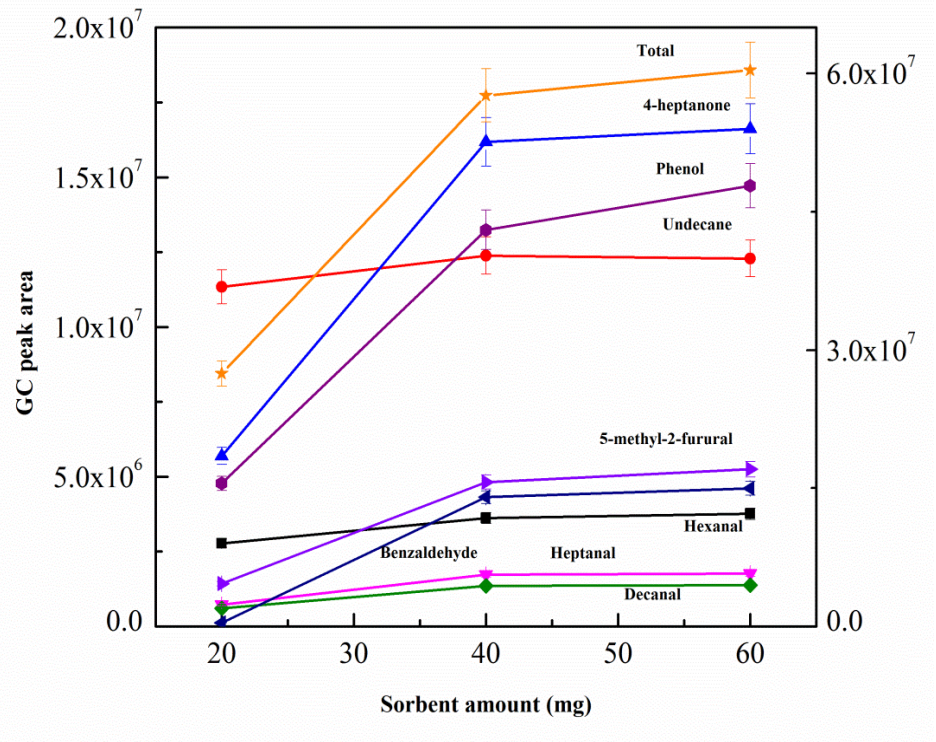

Fig. 2.11. Effect of sorbent amount Fe_3O_4@SiO_2-C_{18} NPs on the adsorption efficiency of VOMs.

The adsorption time is of vital importance for evaluating the extraction performance of sorbent. For the case of NHA-based sorbent extraction approach, the effect of the adsorption time of VOMs was investigated by varying the adsorption time from 1 to 3 hour while the sorbent amount was fixed at 60 mg and elution time was kept at 1 hour. The results indicated that the adsorption of VOMs onto nanohydroxyapatite was strongly time-dependent (**Figure 2.12**). The optimum peak area obtained when the adsorption time was 3 hour.

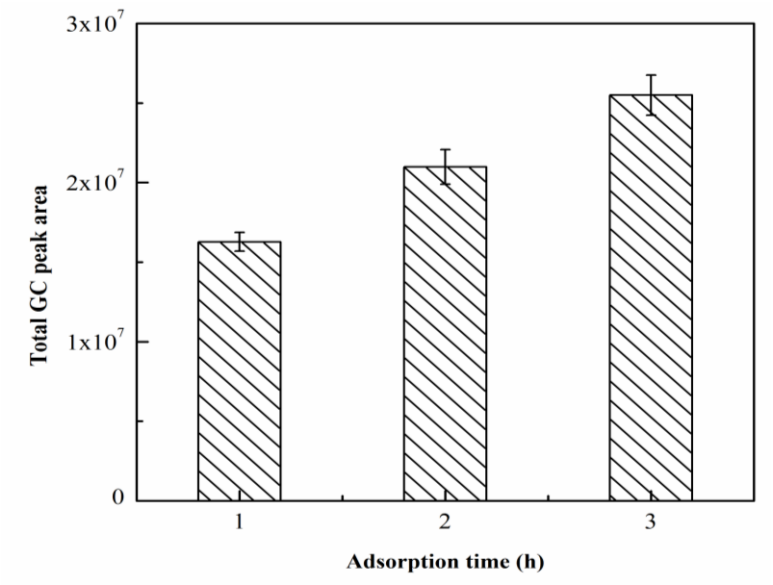

Fig. 2.12. Effect of adsorption time on the adsorption ability of VOMs in NHA

For the Fe_3O_4@SiO_2-C_{18} NPs-based sorbent extraction approach, this variable was studied in the interval from 10 to 60 min (10, 30 and 60 min) while the sorbent amount was fixed at 40 mg and elution time was kept at 30 min. The results indicated that the adsorption of VOMs onto Fe_3O_4@SiO_2-C_{18} NPs is time dependent (**Figure 2.13**). The optimum peak area obtained when the adsorption time is 60 min.

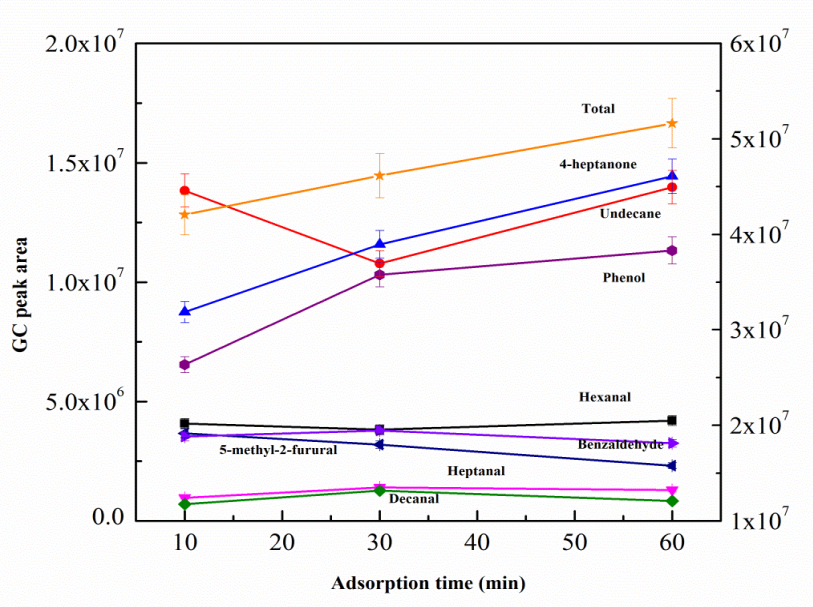

Fig. 2.13. Effect of adsorption time on the adsorption ability of VOMs by Fe_3O_4@SiO_2-C_{18} NPs.

Elution time is another significant variable which should be taken into consideration for the extraction process. In the experiment of NHA-based sorbent extraction approach, the elution time was investigated in the range of 1 to 3 hour at the sorbent amount of 60 mg, adsorption time of 3 hour. According to the results, the total peak area was almost same at different elution time (**Figure 2.14**), which revealed that the elution time is not time-dependent. Thus, elution time of 1 hour was viewed as suitable for the following experiments.

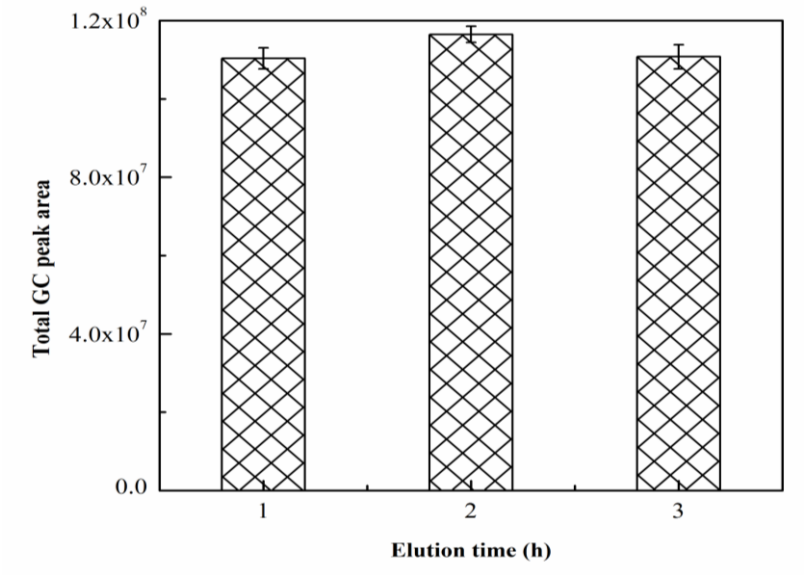

Fig. 2.14. Effect of elution time on the adsorption of VOMs by NHA.

For the Fe_3O_4@SiO_2-C_{18} NPs-based sorbent extraction approach, the elution time was investigated in the range of 10 to 60 min (10, 30 and 60 min) when the sorbent amount was 40 mg and adsorption time was 60 minutes. According to the results, the total peak area was almost same at different elution time for hexanal, benzaldehyde, heptanal, and decanal (**Figure 2.15**), which revealed that the elution time for these VOMs is not time-dependent. But for 4-heptanone, phenol, and undecane, the best extraction performance appeared when elution time was 30 minutes. Thus, elution time of 30 minutes was viewed as suitable for the following experiments.

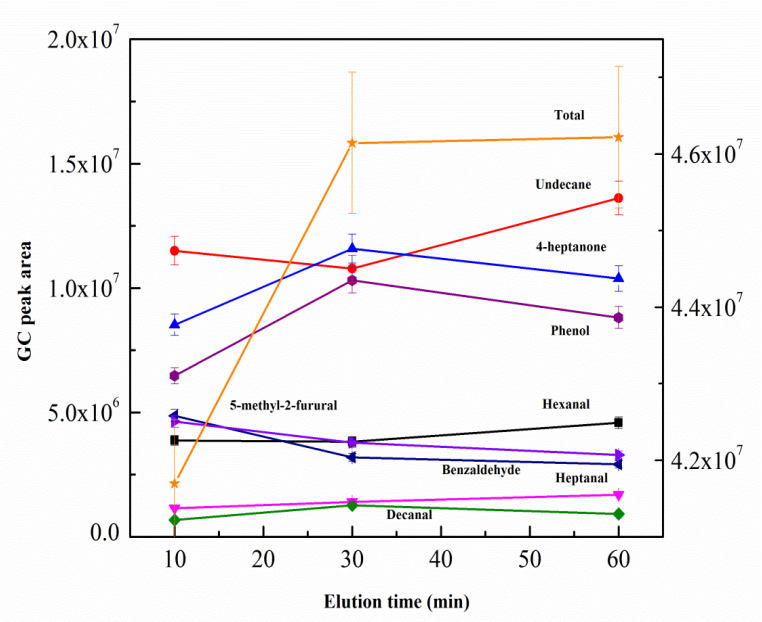

Fig. 2.15. Effect of elution time on the adsorption of VOMs by Fe_3O_4@SiO_2-C_{18} NPs.

Selection of the type of elution solvent also plays a vital role for the desorption of the analyte from nanohydroxyapatite sorbent. Four kinds of elution solvents including acetone, dichloromethane, methanol and acetonitrile were evaluated in both cases. Acetone is not appropriate because acetone appears in some biological fluids.[184] On the other hand, dichloromethane gives poor extraction efficiency. In the case of NHA-based sorbent extraction approach, the obtained results for the both candidate elution solvents are shown in **Figure 2.16**. For hexanal, undecane, and 4-heptanone, the extraction efficiencies using acetonitrile were higher than those using methanol, suggesting that methanol could be used as a good candidate for extraction. In the case of Fe_3O_4@SiO_2-C_{18} NPs-based sorbent extraction approach, after applying acetonitrile as elution solvent, it took more than 12 hours to separate sorbents by external magnetic field, which make them lose the advantage of magnetic separation without using intensive centrifugation. Thus, methanol was used as elution solvent for future experiments in both cases.

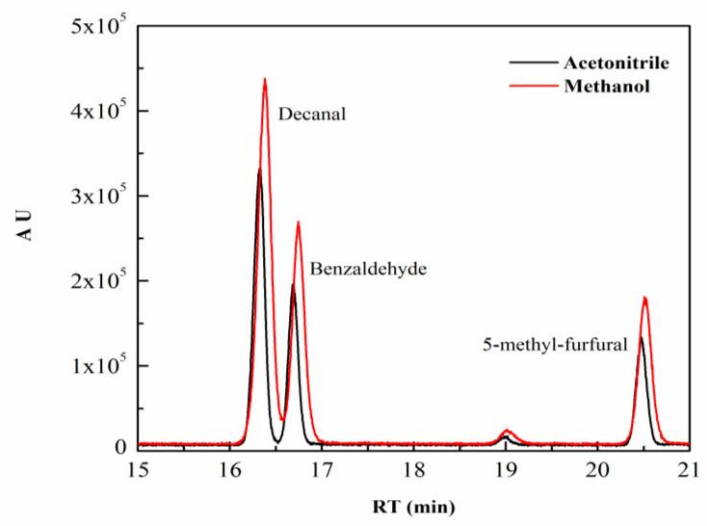

Fig. 2.16. Effect of chemical nature of eluent systems on the adsorption of VOMs by NHA.

2.3.2 Method validation of urinary VOMs using NMs-based sorbent extraction approach

The method performance parameters were calculated for urinary cancer biomarkers using concentrations usually found in human urine. To demonstrate the feasibility of the present approach for determination of urinary levels of VOMs from cancer patients and to test its practicability, the method was fully validated considering the linearity, LODs, LOQs, accuracy, intra- and inter-day precision and matrix effect. The analytical figures of merit of the NHA-based sorbent extraction approach and Fe_3O_4@SiO_2-C_{18} NPs-based sorbent extraction approach are summarized in **Table 2.1 (see page 79)** and **Table 2.2 (see page 80),** respectively.

Linearity of the method was established on spiked synthetic urine samples prepared and analyzed using the NHA-based sorbent extraction approach and Fe_3O_4@SiO_2-C_{18} NPs-based sorbent extraction approach in the range of 0.2 to 50 µg/L and 0.25 to 50µg/L for each cancer VOM, respectively (seven calibrators evenly distributed, three replicates). The GC-qMS system gave linear response over the studied range of concentrations for both cases and the least-squares linear regression

analysis of the data provided excellent correlation coefficient value ($r^2 \geq 0.993$) and ($r^2 \geq 0.988$) for the case of NHA-based sorbent extraction approach and Fe_3O_4@SiO_2-C_{18} NPs-based sorbent extraction approach, respectively. The calibration was performed using synthetic urine-matched calibration standards prepared as described in Section 2.2.3. The quantification of the VOMs was performed using the means of equation of calibration curve, obtained from ordinary least-squares regression data (see **Table 2.1 and Table 2.2, pages 79 and 80, respectively**). The LODs of the volatile metabolites, calculated as $a+3S_{a/b}$, were ranged from 9.8 ng/L for decanal to 69.5 ng/L for heptanal of NHA-based sorbent extraction approach and from 9.7 ng/L for benzaldehyde to 57.3 ng/L for 5-methyl-2-furfural of Fe_3O_4@SiO_2-C_{18} NPs-based sorbent extraction approach. The achieved LODs showed that the both proposed analytical approaches show a suitable sensitivity according to the VOMs levels commonly found in the urine of cancer patients. The LOQs varied from 32.5 ng/L for decanal to 231.6 ng/L for heptanal of NHA-based sorbent extraction approach and from 32.4 ng/L for benzaldehyde to 190.9 ng/L for 5-methyl-2-furfural of Fe_3O_4@SiO_2-C_{18} NPs-based sorbent extraction approach. To determine the recovery (as accuracy) of the analytical methodology synthetic urine was spiked with a known amount (10 μg/L) of each volatile metabolite. The obtained results are summarized on **Table 2.1** and **Table 2.2** for NHA-based sorbent extraction approach and Fe_3O_4@SiO_2-C_{18} NPs-based sorbent extraction approach, respectively. In all cases spiked and non-spiked aliquots were processed in triplicate. The obtained recoveries ranged from 40.4 % for benzaldehyde to 97.2 % for phenol of NHA-based sorbent extraction approach and from 42.6 % for 5-methyl-2-furfural to 99.1 % for benzaldehyde of Fe_3O_4@SiO_2-C_{18} NPs-based sorbent extraction approach.

The precision of the method was measured through intra-day and inter-day precision, expressed by the relative standard deviation (%RSD) and calculated using the measurement of peak area of each cancer biomarker in synthetic urine. Intra-day precision was evaluated by analyzing in the same day 3 replicates of synthetic urine spiked with VOMs at 10 μg/L. The intra-day precision was assessed analyzing, in triplicate, during 3 consecutive days, synthetic urine spiked at the same level. Good precision was achieved with RSD values lower than 3 % for intra-day precision, and 13 % for inter-day precision in the case of NHA-based sorbent extraction approach (**Table 2.1**). In the case of Fe_3O_4@SiO_2-C_{18} NPs-based sorbent extraction approach, RSD values lower than 3 % for intra-day precision, and 13 % for inter-day precision were obtained.

In order to evaluate the ME on the analytical response of VOMs, the slopes of calibration graph obtained with matrix-matched standards were compared with those obtained with solvent-based standards calculating the matrix-to-solvent slope ratio for each volatile metabolite. The obtained results (**Table 2.1 and Table 2.2**) showed that the matrix do not influence the extraction efficiency for hexanal, undecane, decanal and phenol for both cases. In opposite, for 4-heptanone, benzaldehyde, heptanal and 5-methyl-2-furfural extraction, a significant ME was observed.

In summary, compared with the Fe_3O_4@SiO_2-C_{18} NPs-based sorbent extraction approach coupled with GC-qMS system, combination NHA-based sorbent extraction with GC-qMS system showed to be an improved strategy, with excellent recoveries, sensitivity and repeatability, which make it possible to use as a suitable approach to quantify the target urinary cancer VOMs.

2.3.3 Quantification urinary cancer VOMs using NMs-based sorbent extraction with GC-qMS

Under the optimized conditions, the established method was applied to quantify the urinary biomarker cancer. Urine samples were collected from 9 cancer patients for NHA/GC-qMS, including 5 breast cancer and 4 lung cancer patients. Six urine samples from breast cancer patients were used for Fe_3O_4@SiO_2-C_{18} NPs/GC-qMS. A typical GC-qMS total ion chromatograms (TICs) from a cancer patient spiked urine of NHA/GC-qMS is depicted in **Figure 2.17**. All samples were analyzed in triplicate and the results are illustrated in **Table 2.3 and Table 2.4 for** NHA/GC-qMS and Fe_3O_4@SiO_2-C_{18} NPs/GC-qMS, respectively.

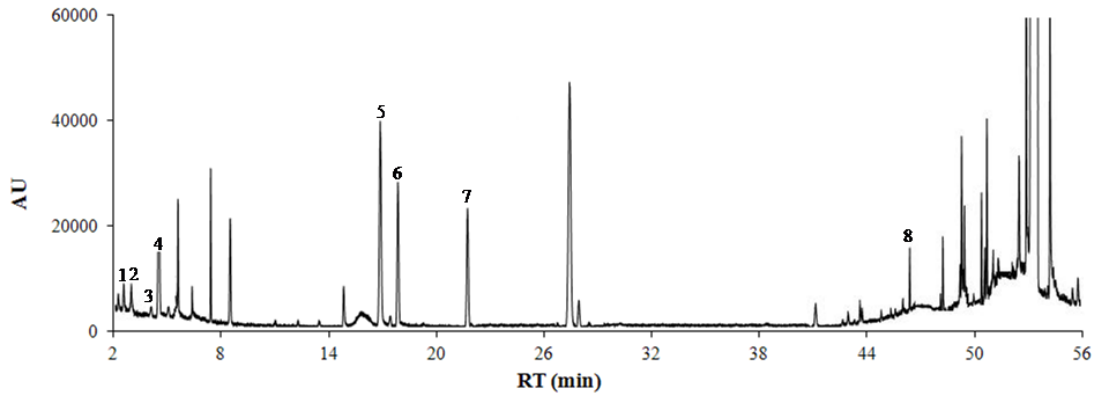

Fig. 2.17. Total ion chromatogram of cancer patient using nanohydroxyapatite-based sorbent extraction with GC-qMS (peak identification: 1: hexanal; 2: undecane; 3: 4-heptanone; 4: heptanal; 5: decanal; 6: benzaldehyde; 7: 5-methyl-furfural; 8: phenol).

These selected cancer biomarkers are representative of each chemical family since the metabolomic origin and physiological function of most VOMs are still unknown. Their origins lie in a variety of endogenous biochemical pathways and exogenous sources (environmental, unhealthy lifestyle habits, biological agents); however, 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).

As observed in **Table 2.3 and Table 2.4**, the urinary cancer biomarkers were detected and quantified in all samples analyzed in both cases, with exception of undecane, as its concentration in some urine samples was lower than its LOQ. The results demonstrate that some biomarkers showed remarkable differences between cancer patients, so they can be used as useful cancer biomarkers. On average, for the case of NHA/GC-qMS, a higher concentration was determined for phenol (14.9 μg/L), followed by benzaldehyde (13.1 μg/L), decanal (10.7 μg/L), hexanal (10.5 μg/L), heptanal (9.7 μg/L), undecane (8.8 μg/L), 5-methyl-2-furfural (7.5 μg/L) and 4-heptanone (4.6 μg/L). For the case of Fe_3O_4@SiO_2-C_{18} NPs/GC-qMS, concentration was determined in the consequence: benzaldehyde (43.5 μg/L), phenol (27.4 μg/L), hexanal (8.5 μg/L), heptanal (4.8 μg/L), decanal (4.2 μg/L), 4-heptanone (4.2 μg/L) 5-methyl-2-furfural (4.1 μg/L), and undecane (0.9 μg/L). In general, it was possible to observe that the biomarkers resulting from oxygen free radical-mediated lipid peroxidation of fatty acids showed higher concentration in cancer patients. Lipid peroxidation generates a large number of metabolites, including VOMs, from oxidation reactions catalyzed by enzyme systems associated with cytochrome P450.[185] For example, hexanal and heptanal are end metabolites of the lipid peroxidation processes of fatty acid ω3 and ω6.[186]

Table 2.3. Urinary cancer biomarkers concentration (μg/L) in urine samples using NHA-based sorbent extraction with GC-qMS

RT	Cancer	Concentration (μg/L) (%RSD)								
(min)	Biomarkers	C1	C2	C3	C4	C5	C6	C7	C8	C9
2.25	Hexanal	19.15 (3)	21.47 (5)	5.83 (5)	10.17 (9)	7.28 (7)	6.32 (5)	17.39 (8)	3.26 (2)	3.28 (3)
2.52	Undecane	4.22 (4)	4.96 (4)	14.44 (3)	13.79 (6)	< LOQ	< LOQ	6.50 (7)	< LOQ	< LOQ
3.41	4-Heptanone	4.97 (2)	5.80 (8)	3.90 (2)	5.79 (3)	4.09 (6)	5.90 (4)	3.83 (3)	1.74 (1)	5.23 (4)
3.63	Heptanal	8.62 (7)	8.87 (6)	6.47 (9)	18.08 (2)	6.03 (5)	6.83 (3)	28.12 (2)	0.92 (2)	2.99 (1)
14.92	Decanal	12.41 (4)	13.93 (3)	9.62 (4)	21.53 (3)	7.38 (4)	5.35 (2)	21.90 (8)	1.54 (3)	2.35 (2)
16.12	Benzaldehyde	9.12 (5)	10.10 (2)	15.29 (4)	30.43 (4)	7.99 (3)	13.03 (7)	20.61 (6)	3.10 (3)	8.18 (4)
21.35	5-Methyl-2-furfural	3.98 (2)	3.93 (1)	6.37 (1)	14.32 (6)	3.37 (2)	6.49 (4)	24.41 (3)	1.55 (4)	2.94 (1)
46.33	Phenol	12.15 (4)	11.82 (3)	15.96 (2)	30.55 (1)	13.49 (6)	14.29 (5)	23.71 (8)	4.72 (7)	7.06 (2)

< LOQ – lower of limit of quantitation

Table 2.4. Urinary cancer biomarkers concentration (µg/L) in urine samples using Fe_3O_4@SiO_2-C_{18} NPs-based sorbent extraction with GC-qMS

RT (min)	VOMs	m/z	Concentration (µg/L)					
			C1	C2	C3	C4	C5	C6
10.14	Hexanal	56	7.70	10.33	5.18	10.31	9.35	8.10
10.25	Undecane	57	0.57	1.65	0.16	1.04	1.03	0.78
11.8	4-Heptanone	71	3.47	2.92	4.16	6.09	3.95	4.55
14.73	Heptanal	44	9.01	<LOQ	<LOQ	1.64	2.83	5.64
39.37	Decanal	44	2.29	3.64	6.51	<LOQ	3.26	5.47
41.08	Benzaldehyde	106	24.77	49.04	25.08	59.95	56.64	45.67
43.67	5-Methyl-2-furfural	110	<LOQ	0.80	2.21	2.86	10.40	<LOQ
52.11	Phenol	94	14.06	18.55	17.38	47.08	39.59	27.87

< LOQ – lower of limit of quantitation

2.4. Conclusions

In this study, some sorbents in SPE such as laponite, TiO_2 NPs, and SiO_2 NPs were investigated. But for these sorbents, they showed poor extraction performances due to either the blockage of internal cavity when meeting aqueous solution for laponite or lack of surface modification by functional groups.

The present investigation shows that optimal experimental conditions for extracting VOMs potential biomarkers of cancer. NHA possessing high surface area and strong adsorption ability was for the first time proven to be a highly efficient sorbent for the enrichment of VOMs. The figures of merit of the NHA/GC-qMS analytical strategy showed satisfactory results in terms of selectivity, linearity ($r^2 \geq 0.993$), instrumental LODs (from 9.76 to 69.5 ng/L), LOQs (from 32.5 to 231.6 ng/L), accuracy (40.4 – 97.2%) and intra- and inter-day precision, lower than 3% and 13 %, respectively. The data obtained suggest that ME is observed for some metabolites which indicated the possibility of using matrix-matched standards as quantification technique. The established analytical approach has been successfully applied to the measurement of urinary VOMs from cancer patients.

$Fe_3O_4@SiO_2$-C_{18} NPs were successfully synthesized by co-precipitation and surface modification methods and first time used as sorbent in SPE to extract VOMs from urine samples. Due to the magnetic core, the NPs could be easily isolated from the matrix by means of external magnetic field, which avoid intensive centrifugation. The established method showed results on selectivity, linearity ($r^2 \geq 0.988$), instrumental LODs (from 9.7 to 57.3 ng/L), LOQs (from 32.4 to 190.9 ng/L), accuracy (42.6 – 99.1%) and intra- and inter-day precision, lower than 3% and 11 %, respectively. Compared to the NHA/GC-qMS analytical method, this method offer superior extraction performances obtaining LOD values at low concentration.

Fe_3O_4@PPy NPs were synthesized by coating PPy monomers onto the surface of magnetic core with the help of SDS. This sorbent will be tested to extract VOMs in the further investigation.

CHAPTER III

Conclusions

SPE, one of the effective methods for the sample preparation, has been widely used for the isolation and pre-concentration of target analytes due to their unique advantages, including lower cost (lower solvent and reagent consumption), greater recoveries (minimal sample transfer), and faster protocol (fewer steps).[187-189] In SPE process, sorbents play important role for the determination of extraction efficiency. Among all the materials, NMs, including inorganic NPs, carbonaceous NPs, and magnetic NPs, are viewed as promising sorbents in SPE for the isolation and pre-concentration of urinary VOMs.

The study presented in this thesis is largely on the effort to develop novel NMs as sorbents in SPE to extract urinary VOMs from cancer patients. Its foundation is mainly based on the successful exploration of urinary cancer biomarkers by Silva *et al*.[18] In this study, commercially available NHA was for the first selected as sorbent to extract VOMs from urine sample. With the help of GC-qMS, NHA showed good extraction efficiency. During the extraction process, centrifugation is an inevitable step, which may lead to copreciptation of unwanted interferents and/or loss of some target VOMs. Thus, developing a simple, rapid, gentle and efficient method is needed. Magnetic Fe_3O_4@SiO_2-C_{18} NPs were synthesized and used as sorbents to isolate and pre-concentrate urinary VOMs. Magnetic NPs can be separated quickly from large-volume samples by external magnetic field instead of centrifugation. Compared to the NHA, magnetic Fe_3O_4@SiO_2-C_{18} NPs exhibited lower extraction efficiency, which probably ascribe to the less adequate hydrophobic groups on the surface of the magnetic NPs.

To sum up, some pioneer works using NMs as sorbents to extract urinary VOMs have been done, for the further investigation, some new sorbents are tested, for example, polypyrole (PPy), one of the most important conducting polymers, has been

widely studied as SPE sorbents in many fields due to the formation of the π-π complex and/or hydrophobic interactions between PPy and analytes.[175-176] Considering the advantages of magnetic separation, Fe_3O_4@PPy NPs were synthesized and characterization. In addition, carbonaceous NPs including graphene and nanocarbon tubes, even magnetic carbonaceous NPs, are also promising for the application of isolating and pre-concentrating urinary VOMs due to their high surface-to-volume ratio, easy surface modification, and reactivates.

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