DM

# Exploring the Potential Sorbent Capacity of Iron Nanostructures to Isolate Urinary Endogenous Metabolites

MASTER DISSERTATION

**Mariana Pereira Santos**
MASTER IN NANOCHEMISTRY AND NANOMATERIALS

UNIVERSIDADE da MADEIRA
A Nossa Universidade
www.uma.pt

February | 2019

# Exploring the Potential Sorbent Capacity of Iron Nanostructures to Isolate Urinary Endogenous Metabolites

MASTER DISSERTATION

**Mariana Pereira Santos**
MASTER IN NANOCHEMISTRY AND NANOMATERIALS

SUPERVISOR
José de Sousa Câmara

CO-SUPERVISOR
João Manuel Cunha Rodrigues

# Exploring the potential sorbent capacity of iron nanostructures to isolate urinary endogenous metabolites

Dissertation submitted to the University of Madeira in fulfilment of the requirements for the degree of Master in Nanochemistry and Nanomaterials

By Mariana Pereira Santos

Work developed under the supervision of

Professor Dr. José de Sousa Câmara and

co-supervised by Professor Dr. João Manuel Cunha Rodrigues

Faculdade de Ciências Exatas e de Engenharia,

Centro de Química da Madeira,

Campus Universitário da Penteada, 9000-390 Funchal, Portugal;

February 2019

*“Start by doing what's necessary;*

*then do what's possible; and suddenly you*

*are doing the impossible.”*

**Francis of Assisi**

# 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.

Funchal, 28th February 2019

***Dedication***

*To my Parents and my Brothers for their unconditional love and support.*

# Conference Contributions

**November/December 2018 – Poster publication:**

Mariana P Santos, Catarina Silva, Ana Olival, João Rodrigues & José S Câmara, *Exploratory evaluation of the potential of magnetic NPs as powerful sorbents for extraction of cancer biomarkers*, presented at MAD-Nano 18 – 30$^{th}$ of November to 2$^{nd}$ of December 2018 – Madeira island, Portugal.

## Acknowledgements

To begin with, completing my Master degree without a doubt would not have been possible without others guidance and I'm so grateful to the people who helped me.

Firstly, I would like to express my sincere gratitude to my supervisor Prof. José S. Câmara for the continuous support of my Master study and related research, for his patience, motivation and immense knowledge. His passion for science and innovation provided the guidance that helped me in all the research and writing of this thesis. I could not have imagined having a better advisor and mentor for my Master study.

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

A special thanks must go to Catarina Silva and Ana Olival. Their rich experience and abundant knowledge in cancer detection on urine with nuclear magnetic resonance were extremely resourceful. From the nuclear magnetic resonance studies, I learnt the importance of patience and meticulousness in research.

I'm also deeply thankful to the laboratory technician Paula Andrade, for her support, her availability in providing the necessary tools for the elaboration of this work as well for her friendship.

To no forget, I want to thank CQM for providing me the tools and support in the development of this Master Project. This project was supported in part by FCT-Foundation for Science and Technology (projects PEst-OE/QUI/UI0674/2013, CQM, New-INDIGO/0003/2012 project and INN-INDIGO/0001/2015, Portuguese Government funds), and through Madeira 14-20 Program, project PROEQUIPRAM - Reforço do Investimento em Equipamentos e Infraestruturas Científicas na RAM (M1420-01-0145-FEDER-000008) and by ARDITI-Agência Regional para o Desenvolvimento da Investigação Tecnologia e Inovação, through the project M1420-01-0145-FEDER-000005 - Centro de Química da Madeira - CQM$^{+}$ (Madeira 14-20).

Last but not the least, I would like to thank my family: my parents and to my brothers and sister for supporting me spiritually throughout writing this thesis and my life in general. This accomplishment would not have been possible without them.
Thank you.

## Abstract

Cancer, one of the deadliest diseases of the world, is characterized by metabolic alterations that cause cells abnormal growth resulting in an uncontrollable proliferation. To reduce the mortality, increase the life quality and make the treatment more effective, early diagnosis is essential. Metabolomics is a promising area regarding cancer early diagnosis that detects a specific metabolite profile from biological samples using "case-control" studies. This profile consists of a panel of small molecules derived from a global or target analysis that is detected through high-resolution analytical methods like the proton nuclear magnetic resonance ($^1$H NMR). Urine is an appealing biofluid, obtained by a non-invasive way, rich in metabolites that reveals the recent homeostatic condition of an individual.

Magnetic nanoparticles (MNPs), namely the magnetite ($Fe_3O_4$) and maghemite ($Fe_2O_3$) have been widely used in oncology for tumour targeting and contrast agent for magnetic resonance image diagnosis. However, their uncoated sorption capacity towards cancer biomarkers remains unknown.

In this work, we aimed to evaluate the sorption capacity of uncoated magnetite and maghemite towards the extraction of different metabolites potential cancer biomarkers present on urine using the magnetic solid phase extraction followed by $^1$H NMR. To achieve this, the extraction methodology was optimised using spiked synthetic urine regarding the MNP type, amount, extraction time and temperature. The best optimization results were applied on urine samples of lymphoma and breast cancer patients and healthy volunteers to identify and quantify the potential biomarkers on a "case-control" study.

Regarding the results, the 20-30 nm magnetite showed best cost-effectiveness ratio being the optimal extraction conditions obtained by using: a ratio of 0.2 mg/ml to extract during 5 min at room temperature with the addition of 1 ml of ultrapure water as elution solvent. On "case-control" study, most of the potential biomarkers followed the same changes, regardless of the cancer type.


**Keywords:** Biomarkers; Cancer; Maghemite; Magnetite; NMR; Sorption capacity

## Resumo

O cancro, uma das doenças mais letais do mundo, é caracterizado por alterações metabólicas que causam o crescimento anormal das células levando à sua incontrolável proliferação. Para reduzir a mortalidade e aumentar a qualidade de vida, o diagnóstico precoce é essencial. A metabolómica, através do estabelecimento do perfil metabólico específico de amostras biológicas usando estudos de "caso-controlo" constitui-se como uma ferramenta promissora no diagnóstico precoce do cancro. Este perfil consiste num painel de pequenas moléculas derivadas de uma análise global ou alvo que é detetada através de métodos analíticos de alta resolução, como a ressonância magnética nuclear de protão ($^1$H RNM). A urina é um biofluído obtido de forma não-invasiva rico em metabolitos que expressam a condição homeostática de um indivíduo.

As nanopartículas magnéticas (MNPs), nomeadamente a magnetita ($Fe_3O_4$) e maghemita ($Fe_2O_3$) têm sido muito utilizadas na oncologia para o direcionamento tumoral e como agentes de contraste no diagnóstico de imagem por ressonância magnética. No entanto, a sua capacidade de sorção para metabolitos potenciais biomarcadores do cancro ainda permanece pouco explorada.

No presente trabalho, será estudada a capacidade de sorção da magnetita e maghemita não revestidas, na extração de potenciais biomarcadores do cancro presentes na urina, utilizando a extração magnética em fase sólida seguida de análise por $^1$H NMR. Para isso, a metodologia de extração foi otimizada, utilizando urina sintética fortificada, em relação ao tipo de MNP, à quantidade sorbente, ao tempo e temperatura de extração. Os melhores resultados da otimização foram aplicados nas amostras de urina de pacientes com cancro da mama e linfoma e voluntários saudáveis para identificar e quantificar os potenciais biomarcadores num estudo de "caso-controlo".

A magnetita 20-30 nm apresentou a melhor relação custo-eficácia, nas seguintes condições de extração: uma razão de 0,2 mg/ml para extrair durante 5 min à temperatura ambiente, adicionando 1 ml de água como solvente de eluição. No estudo do "caso-controlo", a maioria dos biomarcadores seguiu as mesmas mudanças, independentemente do tipo de cancro.


**Palavras-chave:** Biomarcadores; Maghemita; Magnetita; NMR; Capacidade de sorção

# Index

***Exploring the potential sorbent capacity of iron nanostructures to isolate urinary endogenous metabolites***

# List of figures

# List of tables

# List of acronyms, abbreviations and symbols

| | Definition |
|---|---|
| $^{1}$H NMR | Proton Nuclear Magnetic Resonance |
| $^{1}$H qNMR | Quantitative Proton Nuclear Magnetic Resonance |
| AA | Amino acid |
| Ala | Alanine |
| ALAC | Latic acid |
| AMYR | Myristic acid |
| Arg | Arginine |
| Asn | Asparagine |
| BC | Breast cancer |
| Cit | Citruline |
| CRCI | Cancer-related cognitive impairment |
| CT | Computed tomography |
| D2O | Deuterated water |
| DDS | Drug delivery systems |
| DoH | Declaration of Helsinki |
| dSPE | Dispersive solid-phase extraction |
| FA | Formic acid |
| FH | Fumarate hydratase |
| Gln | Glutamine |
| Gly | Glycine |
| His | Histidine |
| HMDB | Human Metabolome Data Base |
| HPLC | High-Performance Liquid Chromatography |
| IDH | Isocitrate dehydrogenase |
| IDO | Indoleamine 2,3- dioxygenase |
| ILE | Isoleucine |
| IONPs | Iron oxide nanoparticles |
| LEU | Leucine |
| LYM | Lymphoma |
| Lys | Lysine |
| MAT | Magnetoacoustic tomography |

| | |
|---|---|
| **MEPS** | Microextraction by packed sorbent |
| **Met** | Methionine |
| **MeOH** | Methanol |
| **MNPs** | Magnetic nanoparticles |
| **MRI** | Magnetic resonance imaging |
| **MS** | Mass spectrometer |
| **MSPE** | Magnetic solid-phase extraction |
| **NaOH** | Sodium hydroxide |
| **NMs** | Nanomaterials |
| **NMR** | Nuclear Magnetic Resonance |
| **NPs** | Nanoparticles |
| **Orn** | Ornithine |
| **PDH** | Pyruvate dehydrogenase |
| **PET** | Positron emission computed tomography |
| **Phe** | Phenylalanine |
| **ppm** | Parts per million |
| **Pro** | Proline |
| **PYR** | Pyruvate |
| **qNMR** | Quantitative Nuclear Magnetic Resonance |
| **SDH** | succinate dehydrogenase |
| **Ser** | Serine |
| **SPE** | Solid-phase extraction |
| **SPIONs** | Superparamagnetic iron oxide nanoparticles |
| **SU** | Synthetic urine |
| **SUCC** | Succinate |
| **TCA** | Citric acid cycle/ Krebs cycle |
| **Thr** | Threonine |
| **TMAO** | Trimethylamine N-oxide |
| **Trp** | Tryptophan |
| **TSP** | Trimethylsilylpropanoic acid |
| **Tyr** | Tyrosine |
| **US** | Ultrasound |
| **Val** | Valine |

# Chapter 1. Introduction

## Contents

# 1. Introduction

In this chapter, the main topics related with dissertation will be discussed. We will give a brief introduction of cancer disease and its impact, mentioning the current treatments and diagnosis as well the importance of early diagnosis. Right after we'll mention some of the metabolic mutations that cancer has, namely the Warburg effect and Krebs (TCA) cycle as well as amino acid metabolism and profile. To conclude the cancer introduction, we'll introduce metabolomics, explaining how it can help on the fight against cancer.

After the cancer introduction, we'll shortly introduce the biofluid urine, pinpointing its advantages and particularities regarding other non-invasive biofluids since is the biofluid chosen to develop the dissertation. Moreover, it is also mentioned some cancer diagnosis methodologies that are being developed using the metabolites found on this biofluid.

Then we introduce how nanotechnology is contributing to the fight against cancer, focusing on magnetic nanomaterials, namely the iron oxide nanoparticles, which were the nanoparticles studied on this dissertation.

The main techniques, magnetic solid-phase extraction (MSPE) and nuclear magnetic resonance (NMR), that were always used on this dissertation are also introduced through the explanation of their basic principles and why they were useful for the development of this dissertation.

To settle this chapter, the main objectives of this thesis will be presented.

## 1.1. Cancer overview

Cancer is a well-known disease to world population characterised by metabolic alterations that cause cells abnormal growth resulting in an uncontrollable proliferation leading to death [1,2]. In the United States and Europe, is the second leading cause of death only being surpassed by cardiovascular diseases [3,4]. At the United States in 2018 is estimated to appear 1.8 million new cases and 0.6 million deaths [3]. On Europe 1 in 4 deaths are caused by cancer [5]. During 2018 is estimated to kill 1.4 million [6], largely affecting the European economy and productivity with a loss of 75 billion [4]. At Portugal, it is predicted that during 2018 will appear 58 thousand cases of cancer, killing 29 thousand [7].

The most mortal cancer type in Europe and the United States is the lung cancer following by prostate on males and breast on females [1,3,6,8,9]. In Portugal, the most fatal cancer is prostate cancer on males and breast cancer on females succeeded by colorectum that affects both genders [7].

### 1.1.1. Current diagnosis

The current diagnosis methods, shown in Figure 1, holds several drawbacks. The imaging methods lack sensitivity and specificity in addition to the health risks for patients and high costs. Thermography beside of being more cost-effective holds a lower sensitivity and specificity than imaging methods. The methodologies that ensure the diagnosis are invasive methodologies (biopsy and cytology), yet they have a high cost and can lead to complications [9–15].

**Current cancer diagnostic methods:**

- Imaging methods:
  - Mammography;
  - Computed tomography (CT);
  - Magnetic resonance imaging (MRI);
  - Ultrasound (US);
  - Positron emission computed tomography (PET);
- Thermography;

*Figure 1 – Current cancer diagnosis methodologies* [9–15].

### 1.1.2. Current treatments

Currently, the cancer is treated in plenty of ways that are showed in Figure 2. However, most of them still have low selectivity and efficiency [16,17]. One of the main causes of death by cancer is the side effects of cancer treatments or relapse after the treatment [16–18]. Other death causes are the complications that cancer acquires on advanced stages such as cachexia [19,20], cancer-related cognitive impairment (CRCI) [21] or metastasis [12].

**Current cancer treatment procedures:**

- Chemotherapy – use of drugs to treat cancer;
- Surgery:
  - Curative (removes the tumour);
  - Palliative (side effect minimization);
  - Reconstructive (to restore a function that cancer damaged);
- Radiation – use of high-energy rays;
- Immunotherapy – use of antibodies to treat cancer;

*Figure 2 – Current cancer treatment procedures* [17,18,22,23].

### 1.1.3. Importance of early diagnosis

In order to improve the cancer treatment, decreasing its mortality and improve the life quality and expectancy, earlier diagnosis methodologies need to be developed [1,9,12,13,17,23–34]. The ideal diagnosis methodology must be non-invasive, inexpensive, fast and reliable to detect cancer on early stages and present a high degree of specificity/selectivity [12,13,28]. Figure 3 describes how early diagnosis contributes to better life quality and a mortality reduction [35].

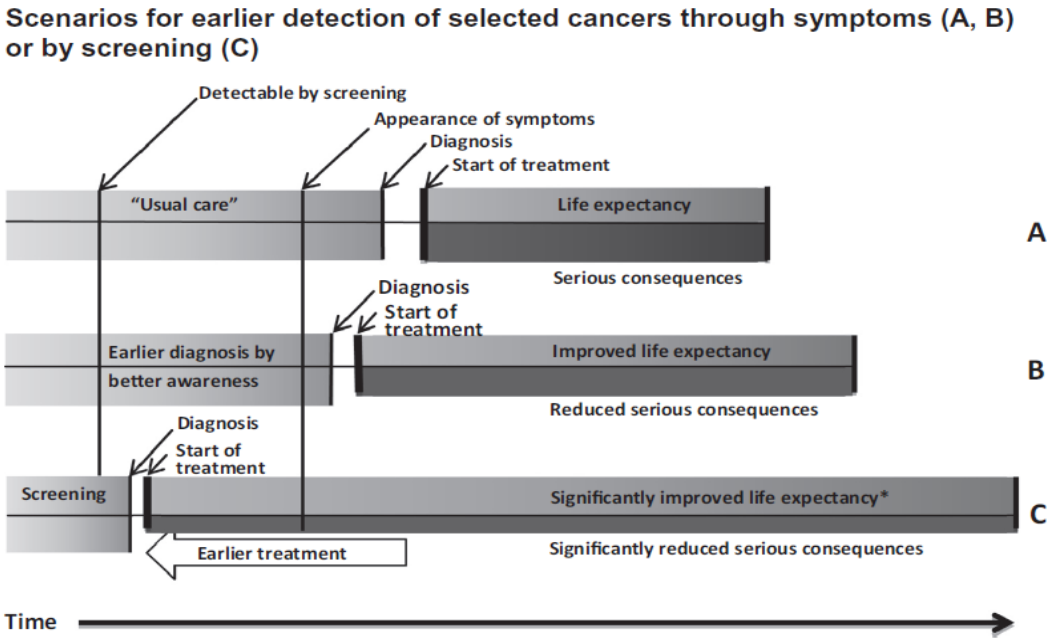



*Figure 3 – Scenarios of early cancer detection through symptoms (A and B) or by screening (C). (A) Time intervals between symptoms appearance, diagnosis, and the start of treatment of cancer can be weeks to months, as well depends upon access to specialized care. (B) The better awareness of cancer symptoms may increase life expectancy and reduce serious consequences of the disease. (C) Before symptoms appear, screening in people at-risk leads to even earlier diagnosis and treatment of cancer, increasing life expectancy and reducing the consequences of cancer* [35].

With the development of early diagnosis methodologies, it will be possible to create screening programmes towards risky populations, improving their life quality (Figure 3 C) [35]. Currently, several screening projects for early detection are being developed and applied taking, for example, the EUROCOURSE [35] and the EUROMED CANCER Network project [36].

## 1.2. Metabolic exchanges of cancer

In order to achieve the well-recognised uncontrollable and abnormal proliferation characteristic of cancer cells as well their great adaptability, they undergo to several mutations that will lead to metabolism changes (Figure 4) [1,2,9,37–44] that affect several molecular mechanisms, from protein expression to molecular signalling's, reflecting specific biochemical adaptations with the purpose of cancer cells acquire survival advantages [2,9,37–44].

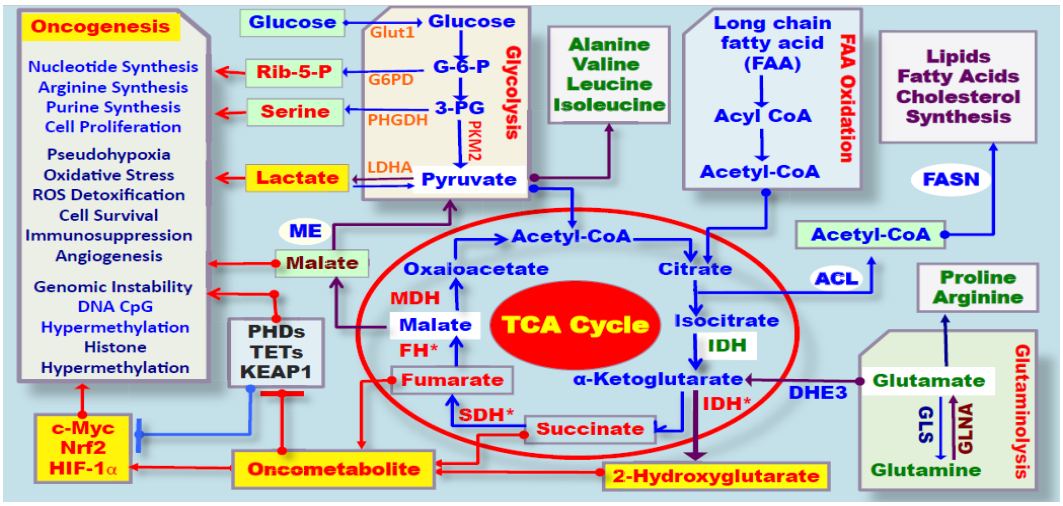



*Figure 4 – Overview of some cancer cells metabolism pathways. The metabolites in red colours represent possible cancer metabolites* [41].

### 1.2.1. Warburg effect and TCA cycle

One of the most well-known metabolism alterations that cancer does is the "Warburg effect", discovered at the 1920s [2,29–31,37,39–41,43–48]. This effect affects the glycolysis pathway by consuming a higher glucose amount where, regardless the oxygen presence, produces lactate instead of pyruvate (anaerobic glycolysis) leading to a growth advantage since it synthetizes energy and the metabolites (nucleotides, lipids

and amino acids) needed for the proliferation (Figure 5) [2,29,31,39–41,43–47]. Initially, it was believed that this effect happened due mitochondria mutations that prevented they realize the oxidative metabolism, however recent studies show that the cancer cells mitochondria is also functional despite being disconnected from glycolysis [2,31,40,41,44]. This disconnection happens due to inactivation of the pyruvate dehydrogenase complex enzyme (PDH) that is the starting point of the citric acid cycle (TCA cycle also called "Krebs cycle", where happens the oxidative metabolism and redox balance) [2,31].

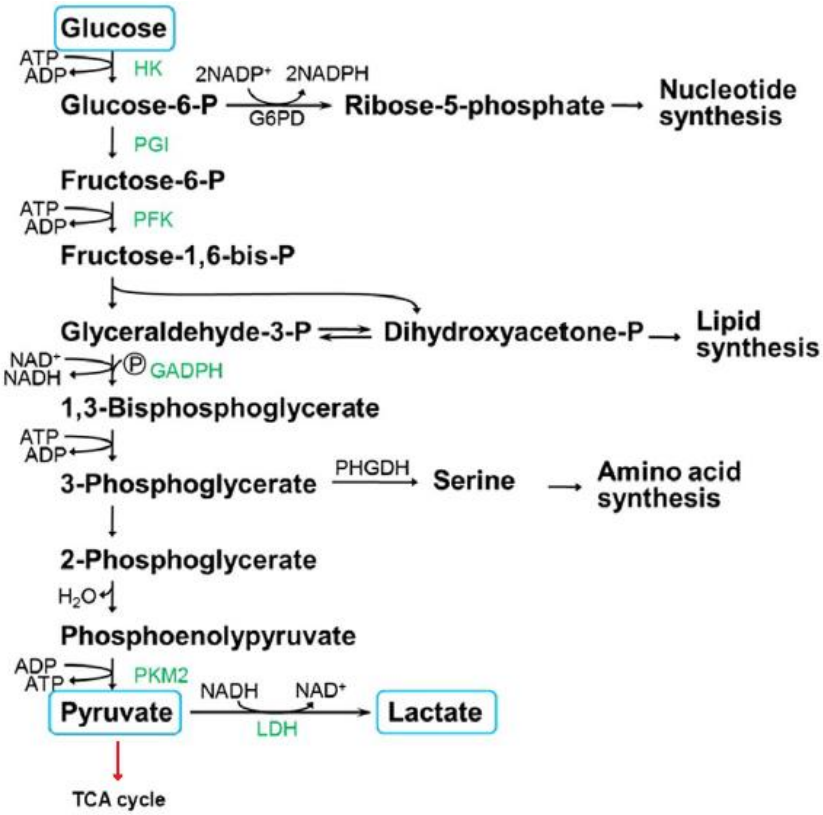



*Figure 5 – Glycolytic pathway that cancer cells use (Warburg effect) highlighting the metabolite synthesis used for proliferation. The red arrow is the pathway that healthy cells apply in the presence of oxygen to produce energy* [40].

These studies also found that the mitochondria is the major contributor of energy synthesis on cancer cells [31,39,41]. As an adaptation to PDH inactivation to realize the TCA metabolism, the mitochondria uses as an alternative carbon source the glutamine realizing the glutaminolysis (Figure 6) [2,31,45,47]. This metabolic alteration synthesises the aspartate and oxaloacetate as well as alters the production of TCA intermediates (nucleotides, AA and lipids precursors needed for proliferation) [29,31,43,47]. An example of a TCA intermediate that supports the proliferation is the

citrate that is used to produce acetyl-CoA and oxaloacetate as well is used on lipogenesis [47].

There're other mitochondria mutations that also are related to carcinogenesis, namely the mutations on the succinate dehydrogenase (SDH), isocitrate dehydrogenase (IDH) and fumarate hydratase (FH) [2,41,43,48]. On the SDH mutation case, it leads to a succinate accumulation that leads to an oncogenic signal transmission from mitochondria to the cytosol [41,43].

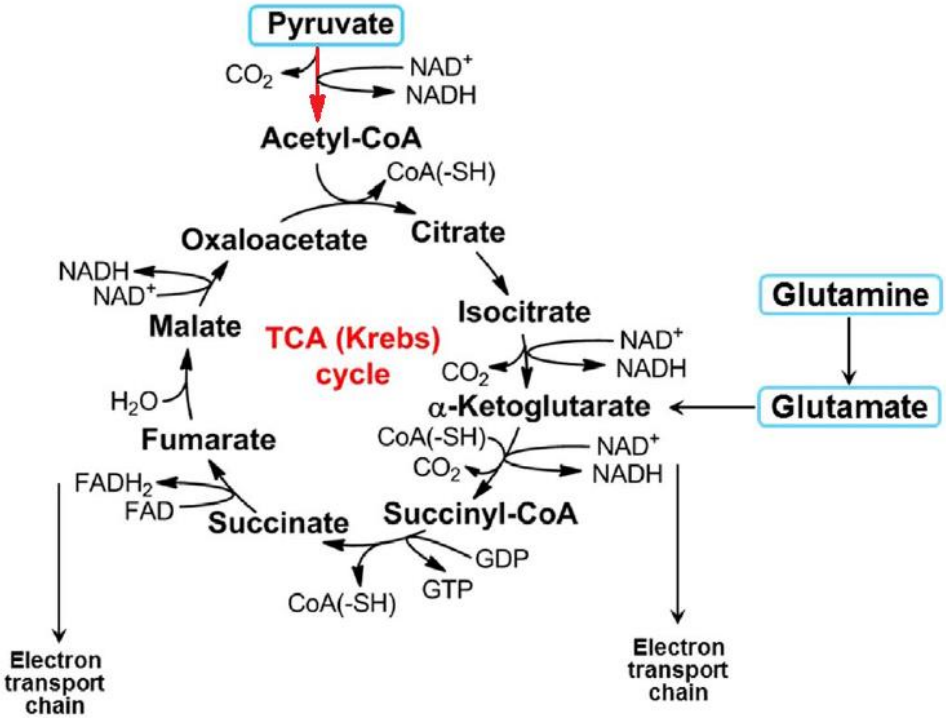



*Figure 6 – TCA cycle adapted to cancer cells. The red arrow represents the pathway that normal cells take but that on cancer cells is inactivated due to PDH inactivation* [40].

One of the uses of the lactate, aside from contributing to the acidic and hypoxic environment, is to produce citric acid. This acid is produced by the neighbour cells helping the tumour to maintain the microenvironment that supports the tumour progression. Another use is the pyruvate conversion made by the cancer cells that are more oxygenated (near the blood vessels). Pyruvate is an important metabolite that have several uses and may even reprogram the cancer cells metabolism since it is a common intermediate between TCA cycle and glycolysis. It can be used to produce

alanine by transamination [31]. Usually, the pyruvate is used on the TCA cycle to produce energy for cancer cells (Figure 7) [40,46,47].

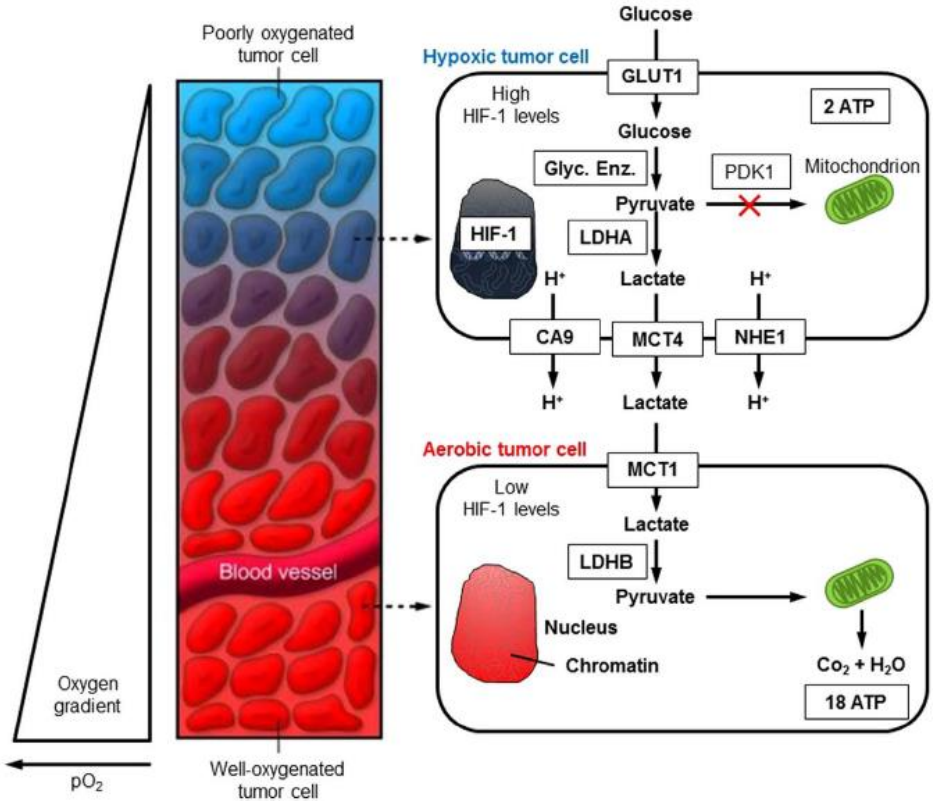



*Figure 7 – Representation of lactate use on cancer cells* [40].

### 1.2.2. Amino acid metabolism and profile

Amino acids (AA) are essential metabolites for several metabolisms, where they act as intermediates or as protein building blocks [28,37,49–51]. Since AA profile is widely dependant of metabolism conditions, metabolic changes caused by cancer affects the overall AA profile [37,43,46,49–51]. Since cancer needs AA for its proliferation, AA profile is a potential cancer marker, although some AA metabolic functions in cancer remain unknown [28,37,49–51].

An example of known AA alterations that is caused by carcinogenesis is the glutamine (Glu), that is abundant in blood and holds important functions on the organism such as nitrogen transport on plasma, AA level support in cells and proline production (which is needed to synthetize collagen) [31,40,46,51]. It can also be used to produce others AA, namely the alanine and arginine [40]. When the tumour is on hypoxic conditions (oxygen deprived), glutamine is used as a substrate for fatty acid synthesis [40,45]. On the tyrosine (Tyr), that is an aromatic AA originated from

phenylalanine hydroxylation, there're studies that report that disorders on tyrosine concentration might be related to carcinogenesis [52]. The tryptophan (Trp) presence is essential for immune T cells proliferation, therefore in order to cancer cells increase their survival they metabolize the Trp using the indoleamine 2,3- dioxygenase (IDO) thus inhibiting the immune cell proliferation [45]. Currently, there's some AA profile studies, showed in Table 1, that had been developed for cancer diagnosis.

*Table 1 – Alterations of amino acid profile in different types of cancer compared with controls. Keys: alanine (Ala), arginine (Arg), asparagine (Asn), citrulline (Cit), glutamine (Gln), glycine (Gly), histidine (His), isoleucine (Ile), ornithine (Orn), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tryptophan (Trp).*

| Cancer type | AA significantly decreased ($p < 0.05$) | AA significantly increased ($p < 0.05$) |
|---|---|---|
| **Lung** [53] | Asn, Cit, Gln, His, Trp | Ile, Orn, Phe, Pro |
| **Breast** [50] | Asp, Gln, Gly, His | Arg, Thr |
| **Breast** [49] | His, Pro, Thr | |
| **Pancreatic** [54] | Ala, His, Trp | Gly, Ile, Ser |

Is important to mention that there's still remains a lot unknown and much to learn regarding the cancer metabolomics [2,30,37,39,45,47,55]. Indeed there's several factors that affect the tumour metabolism, from the microenvironment to cell lineage and drug response [2,37,39,45,47,55]. An example of that is the reported cases where lung cancer cells *in vitro* prefer to use glutamine as TCA carbon source, whereas *in vivo* in mouse prefer to use the glucose [39]. Before the target, the cancer metabolism for diagnosis and therapy it is important to learn and identify the several compensatory pathways that the different types of cancer use to adapt and survive [2,39,40,45,47].

## 1.3. Metabolomics

Metabolomics is the research area where the small-molecule metabolites in the metabolome are detected, identified and quantified in different biological samples, namely biofluids (Figure 8). Since a metabolome is a set of metabolites (that can be endogenous or exogenous) that are derived from metabolism, it is possible to know the

overall health status of individuals. An alteration on the metabolite(s) concentration can indicate that there's a disease [28–31,49,55–65].

Metabolomics covers a wide range of metabolites (Figure 8), from metabolic intermediates to signalling molecules like hormones, for example. Due to that, it's difficult to estimate the number of metabolites that exist on human metabolome. Currently, on the biggest and most comprehensive metabolite database known as Human Metabolome Data Base (HMDB) there's more than 40,000 metabolites [60,61,65–71].

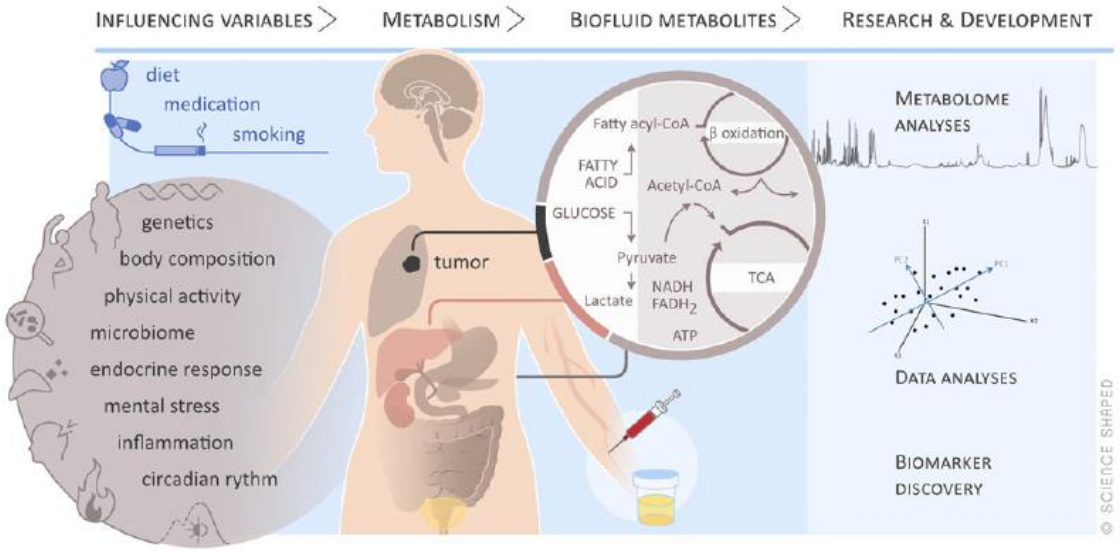



*Figure 8 – Example of how the metabolites found on different biofluids (e.g. blood and urine) can reflect the status of the entire individual organism, from health status to dietary habits [55].*

Regarding cancer, since is already known that cancer alters the metabolism in several pathways, metabolomics is a promising area that helps its early diagnosis [9,26]. Some of the known cancer changes are the alterations on AA and glucose uptakes needed for its proliferation [55]. With the detection of certain metabolites that will be known as biomarkers, it is possible to detect cancer, namely the type and stage that prevails [55]. Metabolomics also has the potential to monitor cancer treatment and its eventual recurrence [1,9,26,28–31,38,42,45,55,59,61,64,72].

According to the National Cancer Institute, a biomarker is "*a biological molecule found on body fluids or tissues that is a sign of normal or abnormal process or of a condition or disease*" [73]. In other words, a biomarker is a molecule that depending on

its presence, absence or concentration can indicate a disease as well it's stage and the therapeutic responses. In order to discover a biomarker, generally case-control studies are usually carried out for biomarker discovery [13,31,56,65,73].

Is important to mention that a biomarker doesn't have to be just a molecule. A biomarker also can be a set of molecules, where their concentrations will indicate the homeostatic condition of the individual, differentiating between the diseased and normal state. The ideal biomarker must fulfil some requirements namely the high specificity and sensitivity towards the disease (dropping this way the false positives and negatives cases), be economical and standardisable (providing good reproducibility) besides being ease of use (fast procedure) and provide clear results [13,28,29,73].

On metabolomics, there's two different approaches for metabolite detection: untargeted and targeted metabolomics (Figure 9).

**Metabolite approach detection:**

- ***Untargeted approach***
  - Applies minimal sample treatment to prevent loss of metabolites;
  - Measures as many metabolites as possible;
  - Doesn't have any knowledge of metabolites identity and nature;
  - Keeps many metabolites uncharacterized;
  - Is often related with hypothesis generating.
- ***Targeted approach***
  - Uses analytical standards to know the exact quantification of the metabolite;
  - Optimises the sample preparation methodology and analysis conditions to improve the detection and quantification;
  - Usually Is based on a specific hypothesis that follows a certain metabolic pathway(s).

*Figure 9 – Description of the different approaches used in metabolite detection* [60,61].

Regardless of the applied approach, the study of metabolomics follows the scheme represented in Figure 10 [60,61].

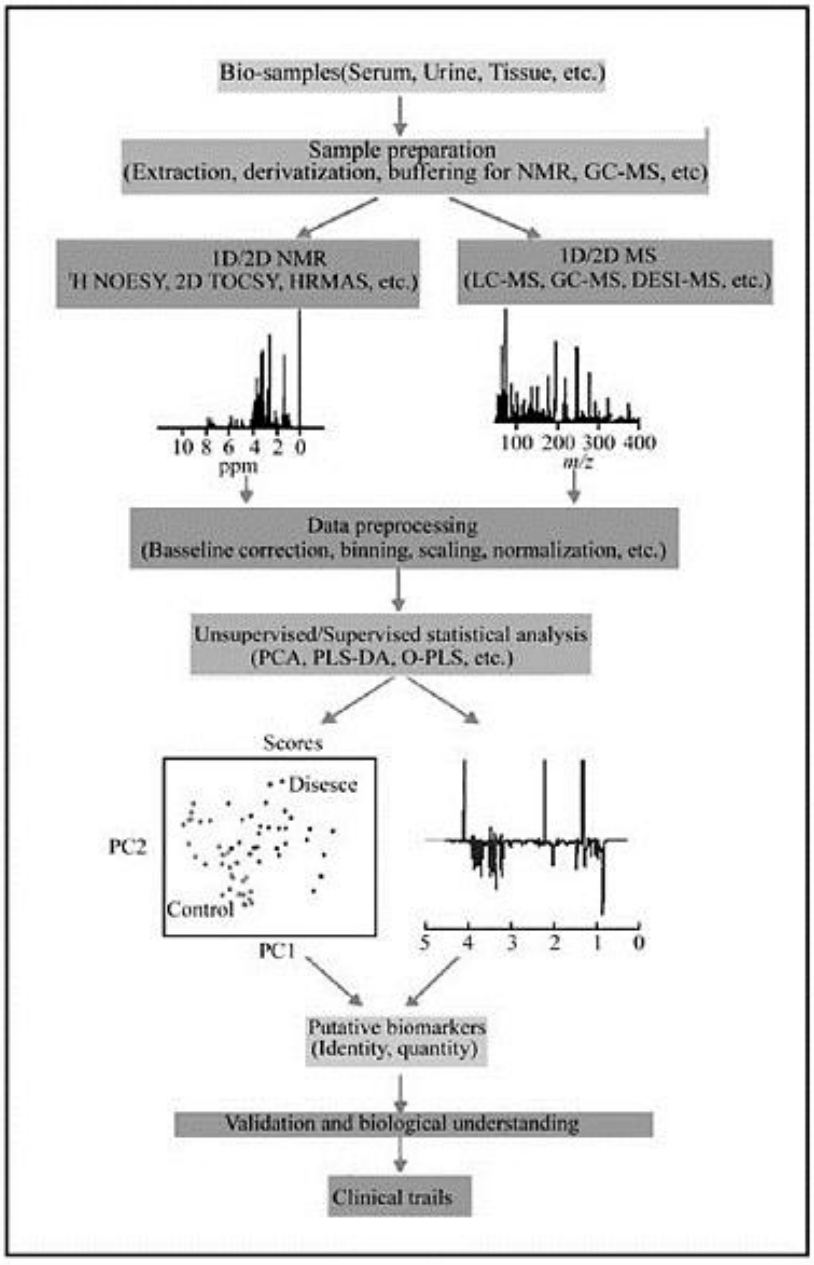



*Figure 10 – Flowchart of a metabolomic study* [30].

## 1.4. Urine as a source of biomarkers

On the development of the ideal cancer detection methodology, the use of non-invasive sampling procedures holds a high interest. This interest comes from the fact that these kinds of samples can be obtained on substantial amounts as well they can be

obtained as often as needed with better acceptance towards the patients since it is painless. Examples of non-invasive samples are the breath, saliva and urine [9,13].

Urine is an appealing biofluid due the high abundance and availability that can be easily collected and stored [15,27,29,31,38,61,62,64,65,73–75]. It has a high chemical diversity with a lower complexity (when compared with other biofluids) that facilitates the sample preparation for analysis and minimizes the interferences. Is primarily composed of urea, creatinine, small hydrophilic molecules like AA, soluble lipids, sugars, volatile compounds, organic acids and amines. It also has in lower amount hormones, proteins, metabolites, genetic material (DNA and RNA residues) and small inorganic salts that passed through the body reticuloendothelial filtration system [13,29,62,65,73,75–78].

This biofluid is rich in metabolites that derive from several body processes whereas kidneys help to concentrate on acquiring the same metabolite concentrations as plasma [1,38,52,64,65,73,74]. These metabolites reflect the recent (last 24 h) homeostatic condition of an individual, showing it's pathological or physiological state whereas can also show the individual gender, age, dietary habits, genotype, environmental exposures or drug intakes [27,29,38,52,57,62,64,65,73,76–79].

However, one of the most significant unresolved issues in the use of urinary metabolites for pathology diagnosis relies on the remarkable variance in urinary excretion volumes and subsequent variations in metabolite concentrations. This drawback can be countered with a standardization [62,73].

To develop a diagnosis strategy using urine, "case-control" studies are usually performed. On these studies, patients are compared with healthy controls where the differences detected can show a disease pattern which can be used for diagnosis [65]. Currently, several cancer diagnosis methodologies are being developed using "case-control" studies to detect urine metabolites from prostate [1,14,38,61,72,75,78,80–82], bladder [15,38,61,65,72,75,83–88], ovarian [61,77], lung [1,13,38,65,72,89], breast [1,38,61,72,90], kidney [38,65], colorectal [1,38,64,65,91], esophageal [65], liver [1,38,65] and colon [72] cancers.

## 1.5. Nanotechnology contributions towards cancer

An emerging and promising strategy in the metabolomics cancer field is the use of nanotechnology. Nanotechnology consists on the manipulation of the size or shape of a material at nanoscale level (size between 1-100 nm on at least one dimension) known as nanoparticles (NPs). This kind of materials have a high surface energy that leads to higher interactivity since there's more atoms available to interact. They have a wide range of applications in different areas of science and technology, where the fight against cancer is one of the most promising [16,92–102].

Regarding cancer, nanomedicine uses several NMs, such as quantum dots, liposomes, dendrimers, silica NPs, polymersomes, metallic NPs, carbon nanotubes and magnetic NPs (MNPs). These NMs usually are able to accumulate more on tumours than normal tissues. Currently, the main application of these NMs against cancer is the creation of drug delivery systems (DDS) (Figure 11 and Table 2) that transports the anticancer drug directly to the tumour, reducing, by this way, the side effects and improve the therapy efficiency.

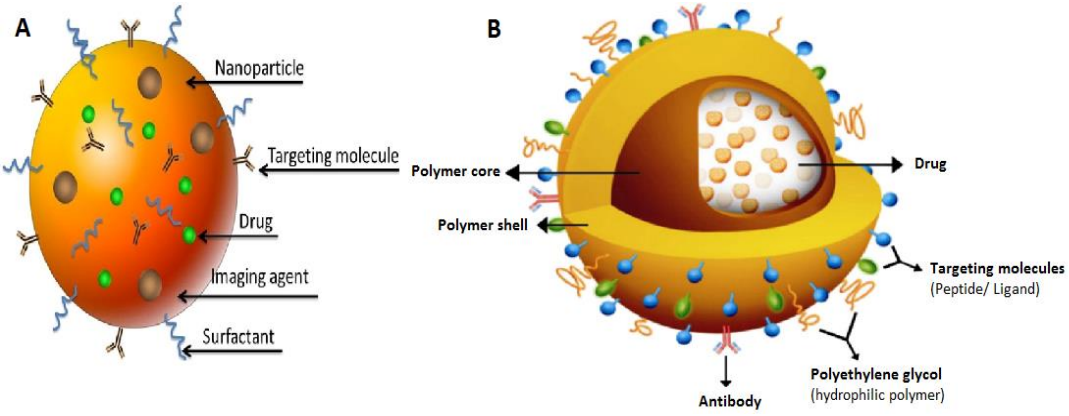



*Figure 11–Examples of drug delivery systems (DDS) made with: A – Nanoparticle; B – Polymeric nanohybrid* [16,17].

Moreover, these NMs can also act at cellular level when they are endocytosed by cells that leads to internalization of the anticancer drug. On Figure 12 is possible to observe some variations that DDS can have in order to fulfil their goal. Sometimes DDS can also diagnose the tumour directly, where these systems are known as theragnostics [16,17,92,93,95,96,99,103].

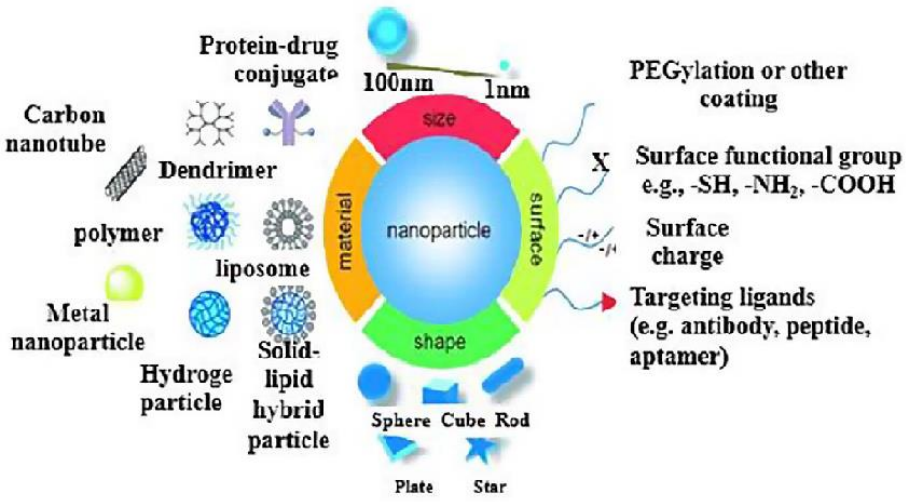
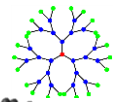
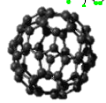
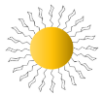
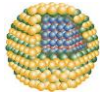
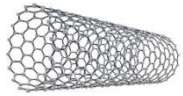
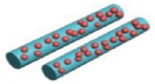
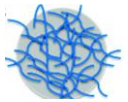
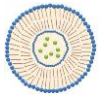



*Figure 12 – Possible variations of DDS* [96].

Another main use of NMs regarding cancer is the development of imaging agents that help to diagnose cancer with better accuracy. For this achievement, NMs are used as contrast agents that can be detected by CT, MRI, PET, US, magneto acoustic tomography (MAT) (an US variation) and radiodiagnosis. An example of NMs being used as contrast agents are the quantum dots that currently are used on MRI and PET [16,17,92,99,103].

*Table 2 – Classes of Drug delivery systems (DDS) and its composition* [16].

| Nanosystem | | Schematic representation | Size |
|---|---|---|---|
| **Dendrimers** | Poly(amidoamine) |  | 1-10 nm |
| **Fullerenes** | Carbon based nanocarriers |  | |
| **Inorganic nanoparticles** | Gold nanoparticles (AuNPs) |  | 2-100 nm |
| | Quantum dots |  | 1-20 nm |
| | Carbon nanotubes |  | Lengh of 40 nm |
| | Mesoporous sílica nanoparticles |  | 80-500 nm |
| **Polymer-based nanoparticles** | Polylactic acid (PLA) nanoparticles; poly(cyano)-acrylates, polyethyleneimine; polysaccharides including alginate, chitosan, gum arabic |  | |
| **Liposomes** | Phospholipids |  | 50-210 nm |

## 1.6. Magnetic nanoparticles

Magnetic nanoparticles (MNPs) are a class of NPs that can be manipulated under an external magnetic field. They are well known for their unique electrical, optical, chemical and magnetic properties that are advantageous to be used on a wide range of areas (like medicine, physics, biology and chemistry). These NPs are widely applied in many research fields where some of the main applications can go from sample preparation (Table 3 shows the improvement regarding conventional materials) to batteries and chemosensors [23,79,94,97,98,100,102,104–115].

*Table 3 – Improvements of MNPs on several analytical processes compared with conventional materials* [104] .

| Analytical step | Exploited property | Technique | Improvements |
|---|---|---|---|
| Sample Treatment | - Magnetic<br>- Thermal<br>- Chemical | - MNPs used in SPE and MSPE for the sorption of analytes or interferences.<br>- MNPs used as pseudo-stationary phase in LLE and MLLE for sorption of analytes or interferences. | - Packing into a cartridge is not required.<br>- Short time in the sample preparation by using a magnet.<br>- Good thermal stability and possibility of reusability for several times.<br>- High affinity, adsorption capacity and selectivity. |
| Sample separation | - Magnetic<br>- Thermal<br>- Chemical | - Introduction of MNPs in open-tubular CEC.<br>- Use of MNPs as pseudo-stationary phase in CEC.<br>- Introduction of MNPs in microfluidic channels. | - Easy packing with a magnet.<br>- Good stability.<br>- Increase in the retention of the analytes.<br>- Enhancement of the efficiency.<br>- Good separation ability. |
| Detection | - Electrical<br>- Chemical<br>- Magnetic<br>- Optical | - Modification of transducers by MNPs.<br>- Biomolecules-MNPs for biosensing and bioassays.<br>- FMNPs for diagnosis and therapy of a cancer cell. | - Improvement of the catalytic activity of analytes.<br>- Good electrical conductivity.<br>- Easy of synthesis and compatibility.<br>- Selective recognition of analytes.<br>- Good sensitivity. |

SPE = solid phase extraction; MSPE = micro-solid phase extraction; LLE = liquid-liquid extraction; CEC = capillary electrochromatography; FMNPs = fluorescent-magnetic nanoparticles.

On the field of nanomedicine, MNPs are highly researched due to their potential function at the molecular and cellular level of biological interactions, controlling their magnetism to achieve the desired uses. One of the most desired properties in this area is the superparamagnetism. Some of the research on this field is focused on the development of DDS, hyperthermia treatment or diagnostic methodologies, where MNPs are used as a contrast agent for MRI, as observed in Figure 13 [23,34,79,93,96–98,100,105,106,110,112,113,116–119].

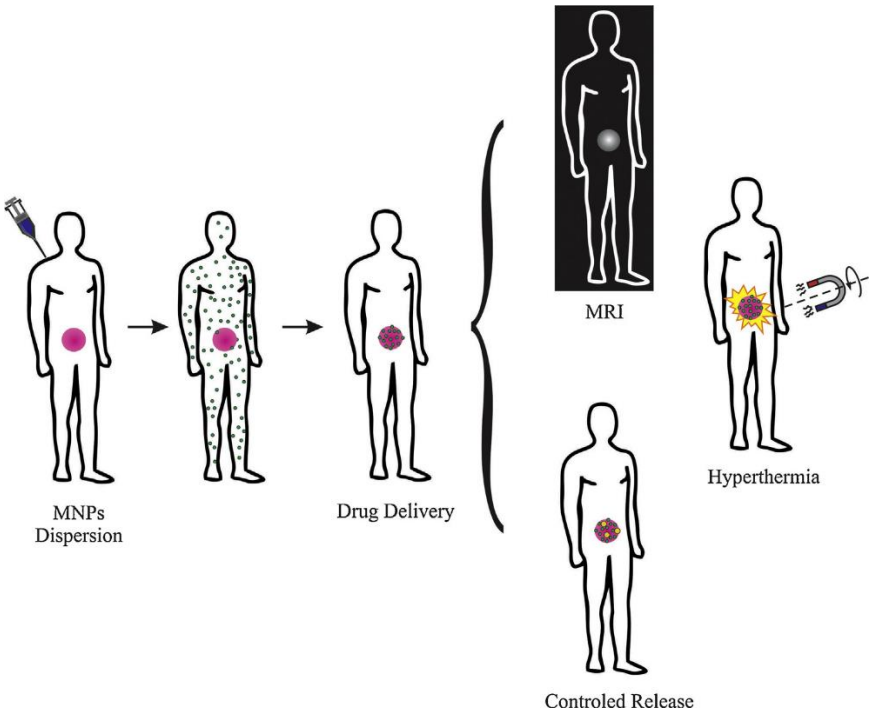



*Figure 13 – MNPs applications in cancer* [23].

MNPs properties and uses highly depend on the size, composition, surface chemistry and morphology. Regarding its magnetism, they are classified into five types (Figure 14): paramagnetic, diamagnetic, ferromagnetic, antiferromagnetic and ferrimagnetic. Paramagnetic materials have a weak magnetic moment and don't maintain it when the magnetic field is removed. The diamagnetic materials only show magnetic moments under strong magnetic fields. Ferromagnetic materials have a strong magnetic moment and can maintain a magnetic moment after the magnetic field is removed. Antiferromagnetic materials usually are made of two different elements (usually metal oxides) that have doesn't have a magnetic moment since the composed elements have magnetic moments on opposite directions, cancelling the magnetism. Ferrimagnetic materials are also composed of two elements that unlike antiferromagnetic, the magnetic momentums don't cancel since they have different magnitudes. When there's a magnetic field on antiferromagnetic and ferrimagnetic materials, they have the same behaviour as ferromagnetic. During superparamagnetism state, MNP behaves as a paramagnetic material with a giant spin [120].

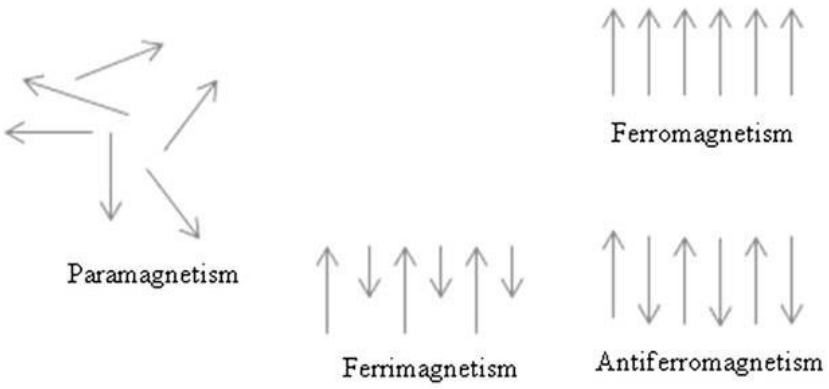



*Figure 14 – Types of magnetism* [121].

There are numerous MNPs that can be classified into three classes:

**Metals** – where only the metallic elements with magnetism are included, and they need an oxidation-protective layer. The only elements that fill this criterion are the ferromagnetic elements like cobalt, nickel and iron, where only iron isn't toxic and can have uses on medicine [115].

**Alloys** – Constituted with ferromagnetic alloys like FePt, CoPt or FeNi. This MNP class tends to agglomerate and have low magnetism, having no use on nanomedicine [115].

**Oxides** – On this class are included the metallic oxides, and mixed oxides with different crystal structures (e.g., ferrite oxide) are included. Their magnetism properties depend on the MNP composition. Some MNP from this class hold promising applications on nanomedicine regarding cancer like magnetic hyperthermia (that is a tumour localised treatment) and a contrast agent [115].

To characterise the MNPs, several techniques shown in Table 4 can be applied.

*Table 4 – Characterization techniques applied on magnetic nanoparticles showing their pros and cons* [104] .

| Technique | Working size range | Advantages | Disadvantages |
|---|---|---|---|
| Dynamic light scattering | 1 nm–5 µm | - Automated, short measuring time and less labour intensive.<br>- Extensive experience for routine work is not required.<br>- Non-destructive technique (the sample can be employed for other additional measurement).<br>- Extremely sensitive towards the presence of small aggregates. | - Time consuming in some case for MNPs analysis.<br>- Sensitive for mechanical disturbances.<br>- Lack of selectivity and relatively low signal strength. |
| Transmission electron microscopy<br>Scanning electron microscopy | 0.5 nm–1 µm | - It provides direct structural and size information of MNPs.<br>- It gives quantitative results on the size distribution of MNPs.<br>- Better resolution than SEM.<br>- Easy to use.<br>- MNPs can be seen as a 3D image.<br>- Images have a good depth of field.<br>- Other techniques can be coupled to SEM to complete the information about MNPs | - Suffer from sampling size involved.<br>- Expensive and time consuming.<br>- Images are in black and white.<br>- The resolution is not as good as the TEM. |
| Thermomagnetic measurement | 10 nm-50 nm | - It provides a very reliable magnetic size of the nanoparticles to be analysed. | - Indirect measurement.<br>- It relies on the underlying assumption of mathematical model used to calculate distribution.<br>- Small magnetic field applied for ZFC measurements.<br>- Neglects particle-particle- dipolar interactions which increase the apparent blocking temperature. |
| Dark-field microscopy | 5 nm-200 nm | - Direct visual inspection of optical signals emitted from MNPs. | - It needs an extra synthesis step by coating MNPs with a noble metal that exhibits SPR with a visible wavelength.<br>- Dependence on the ability to trace the particle optically.<br>- Size obtained are larger than those obtained by TEM or DLS. |
| Atomic force microscopy | 1 nm–1 µm | - It provides three-dimensional surface profile of MNPs.<br>- MNPs viewed by AFM not require special treatments.<br>- It does not need an expensive vacuum environment.<br>- It provides a higher resolution than SEM. | - It provides a single scan image.<br>- It requires several minutes for a typical scan.<br>- Low quality and possibility of image artefacts. |
| Acoustic spectrometry measurement | 1 nm–1 µm | -Good tool for studying MNPs with high polydispersity. | - Limited pathlength and high water absorption.<br>- Temperature dependence.<br>- Overlapping bands.<br>- Weak absorption at short wavelengths. |

### 1.6.1. Iron oxide NPs

Iron oxide nanoparticles (IONPs) are a type of MNPs acknowledged for their low-cost, controllable size, biocompatibility and nontoxicity, environmentally friendly nature and catalytic activity. The most common IONPs types are FeO, $Fe_2O_3$ (ferrite oxide) and $Fe_3O_4$ (magnetite). On the case of $Fe_2O_3$, there are four crystallographic phases denominated as hematite (α-$Fe_2O_3$), β-$Fe_2O_3$, maghemite (γ-$Fe_2O_3$) and ε-$Fe_2O_3$. Each crystallographic phase has different magnetic behaviour [115,122–126].

These MNPs have been widely used in several areas, depending on their size, shape, magnetism and surface properties. Some of their applications are: magnetic recording, humidity and gas sensors, microwave absorption, water treatment, catalysts, ferrofluids, inorganic pigments, pollutants extraction, magnetic seals and inks. On nanomedicine, they have been used as a cancer therapeutic agents, as contrast agent, magnetic hyperthermia and DDS development [34,95,109,121,123,125–128].

Among the different iron oxides types, only maghemite and magnetite have ferrimagnetism that is caused by their 3d spinel structure (on fcc cubic lattice subtype). These nanoparticles are one of the most promising materials regarding the cancer nanomedicine since they have colloidal stability, surface modification properties,

maximum surface area, low toxicity and biocompatibility. When they are smaller than 20 nm, they become superparamagnetic being known as superparamagnetic iron oxide nanoparticles (SPIONs). This property is highly desirable on nanomedicine since that prevents agglomeration inside blood vessels [9,34,56,69,79,93,95–98,102,113,115–122,124–133].

Nowadays on the cancer field, these NMs are used as MRI contrast agent (for cell and molecular imaging), magnetic hyperthermia (that can kill tumours) and DDS development, where usually they are functionalized or belong to the DDS core coated with another nanoparticle [23,134,135]. Sometimes, they can also be the shell of the DDS nanostructure [95,136,137]. Other applications on nanomedicine are tissue engineering/repair, cell labelling, detoxification, cell separation and isolation [9,23,92,95,115,121,122,126,127,129,130].

However, the sorption capacity of uncoated maghemite and magnetite remains vastly unknown, despite that there's a large research for nanosorbents to sample preparation [79,94,101,102,110–112,114,138,139]. SPIONs can be synthetized with several methodologies that are categorized as chemical, physical or biological methods. Figure 15 shows methodologies that each synthesis category has as well the frequency that they are applied. On the Figure 16 it is possible to observe a schematic representation of some chemical synthesis methodologies of SPIONs and Table 4 summarizes some synthesis techniques along with their benefits and disadvantages [119,125].

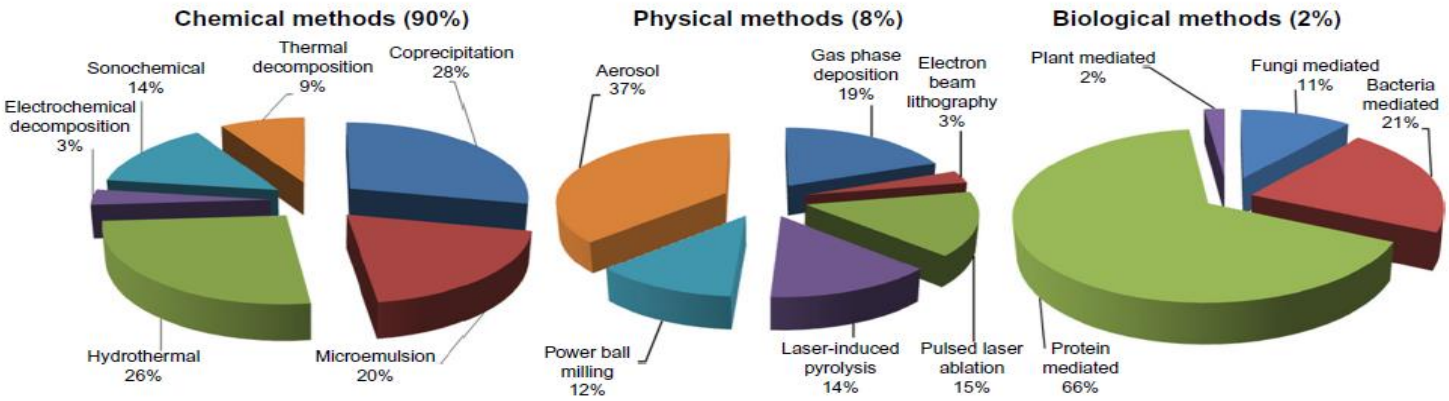



*Figure 15 – SPIONSs synthesis methodologies* [125].

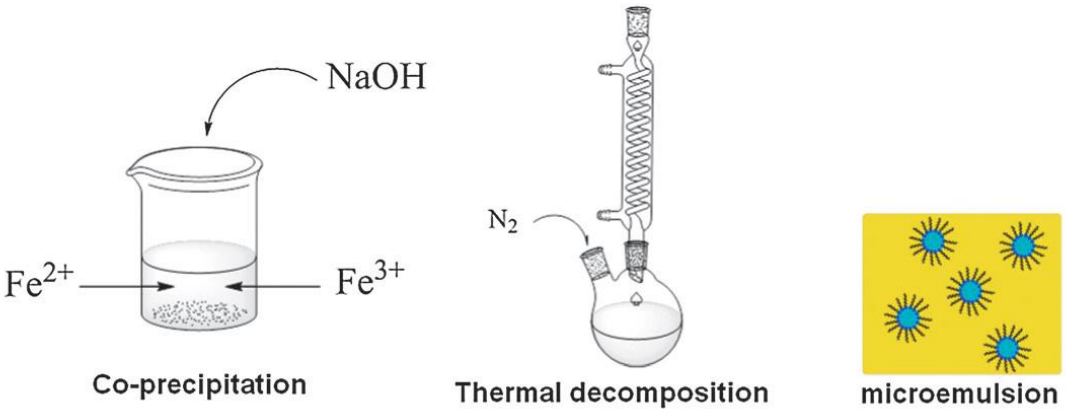



*Figure 16 - Schematic representation of some chemical synthesis methodologies of SPIONs* [132].

*Table 5 – Synthesis methodologies used to synthesize SPIONs*

| Synthesis methodology | Advantages | Disadvantages | References |
|---|---|---|---|
| **Co-precipitation** | - Rapid and simple methodology<br>- Easy to maintain the synthesis conditions (pH, temperature and ionic strength)<br>- Easy to scale up | - Large particle size variation<br>- Por crystallization<br>- Polydispersion | [23,93,100,104,108,110,111,114–117,119,121,123–130,132,133,135,137,140–143] |
| **Oxidation method (wet chemical)** | - Easy to obtain small and uniform NPs at the molecular level | - Synthesis takes a long time<br>- Expensive methodology | [116,119,125,127,143] |
| **Gas-phase deposition** | - Produces one-dimensional nanostructures with high purity<br>- Simple methodology | - Difficult to maintain the control of synthesis conditions and NP size<br>- Requires high temperatures and vacuum<br>- Produces low amount | [116,119,121,125,127] |
| **Hydrothermal** | - Good crystallization<br>- Easy product morphology control | - Requires high temperatures and pressure<br>- Produces a low amount when compared with co-precipitation | [23,93,104,110,114–116,119,121,125,127,128,143] |
| **Thermal decomposition** | - Synthetizes high-quality monodisperse np, with narrow size range and good crystallinity | - Demands high temperature<br>- Uses toxic and expensive iron precursors | [23,93,105,114,115,121,126,128,130,132,133,141–143] |
| **Water-in-oil microemulsion** | - Precise control of the size, shape and distribution | - Usage of large solvent amounts<br>- Produces a low amount when compared with co-precipitation<br>- Requires purification after synthesis | [23,93,114,115,121,123,125,128,130,132–134] |
| **Sol-gel** | - Easy to scale up<br>- Produces narrow size range, monodispersed np | - Requires heating for crystallization | [23,104,116,119,121,125,128,133,143] |
| **Flow injection** | - Synthetizes high-quality monodisperse NPs, with a narrow size range<br>- High reproducibility<br>- Precise control of the conditions | - Requires high temperatures and pressure with a segmented or continuous mix of reagents under a laminar flow regime in a capillary reactor | [114,116,119,121,125,133,143] |

## 1.7. Basic principles of MSPE

The need for preparation of biological samples is an essential step to lower the complexity of biological matrices as well it helps to pre-concentrate the desired compounds (Figure 17) [29,102,114]. To prepare the samples, there're several extraction methodologies such as solid-phase extraction (SPE), microextraction by packed sorbent (MEPS), dispersive solid-phase extraction (dSPE) or magnetic solid-phase extraction (MSPE), that can be applied, depending on the biological matrix and the target analytes. The ideal sample preparation technique should present a selective, fast, cheap and green extraction with a minimal sample loss and maximum recovery of the analyte [29,102].

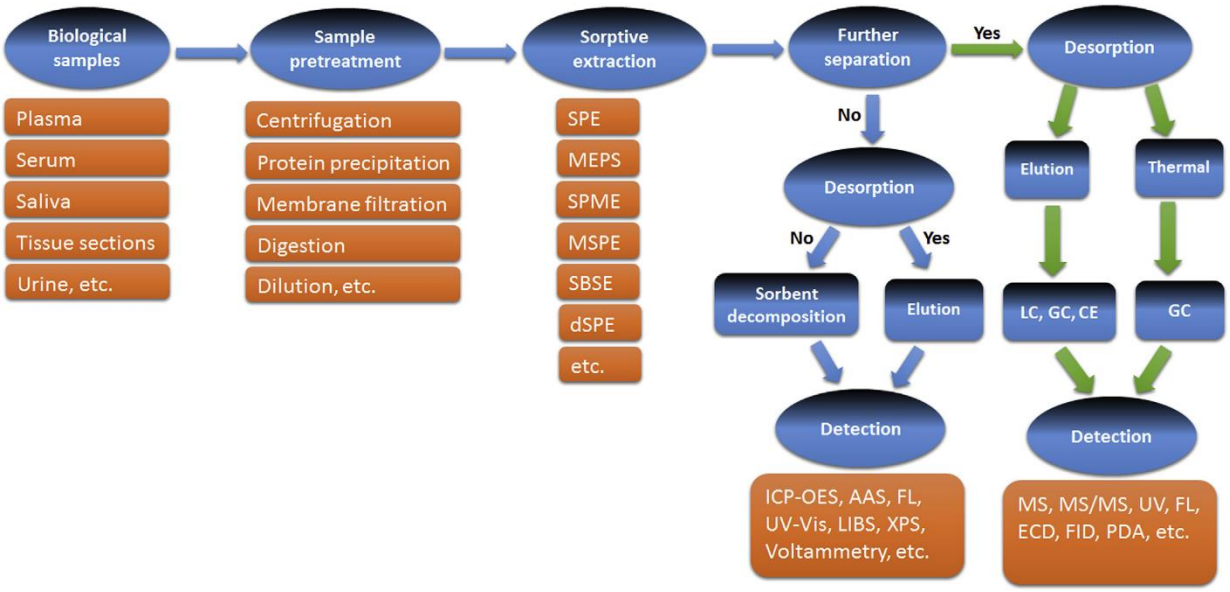



*Figure 17- Biological sample preparation for analysis* [102].

Magnetic solid-phase extraction (MSPE) is as extraction methodology acknowledged for its simplicity, speed and practicality [101,102,104,110,112,114,138]. This methodology uses MNP as sorbents, simplifying the extraction procedure since with the help of a magnet, they are easily separated, overcoming this way, the need for packing columns that SPE requires [101,102,104,110,112,114,139,144]. The MNPs that are used can be in core-shell, composite, in bare or modified with an organic or inorganic ligand [102,112,114,138].

The general procedure is shown on Figure 18, where MNPs are dispersed on the sample that contains analytes and interferences that will be sorbed with the help of incubation or auxiliary radiation (sonication, ultrasonication).

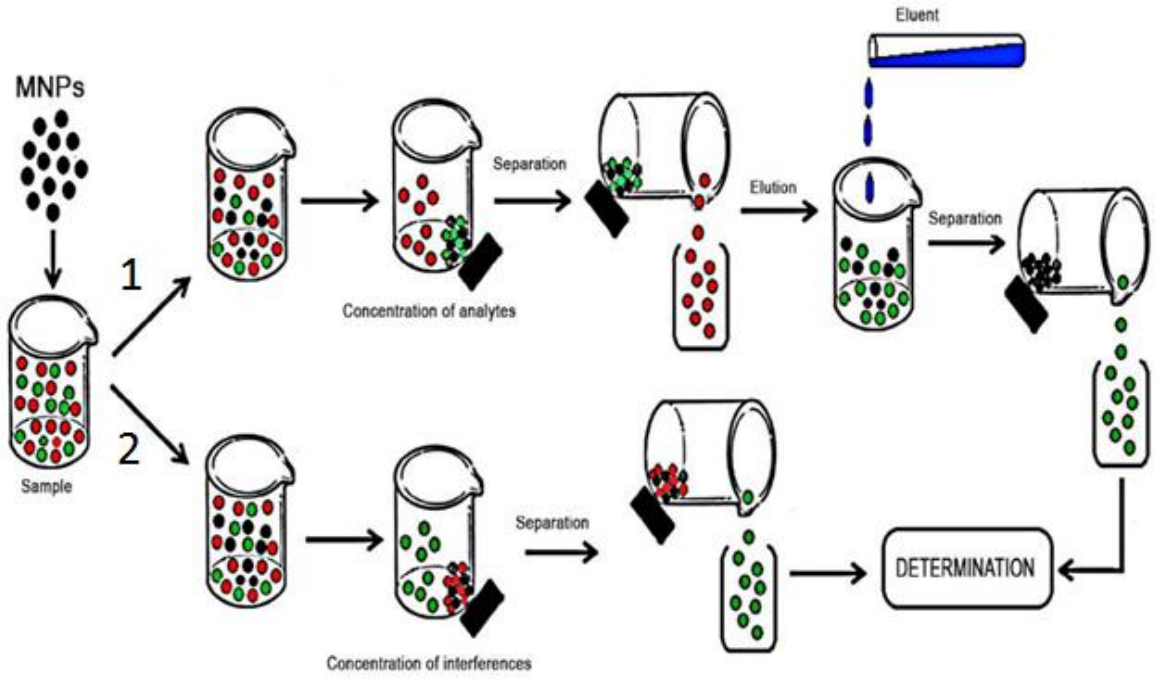



*Figure 18 - Magnetic solid phase extraction scheme* [104].

The MNPs will sorption the interferences or analytes. Depending on the extraction goal, there's a division of two procedures: [79,101,104,111,112,144]

1. If the goal is to pre-concentrate the analytes, MNPs will adsorb the analytes and retain them under a magnetic field while the sample is discarded. Then the analytes are eluted from the MNPs using an appropriate elution solvent, separating the MNPs from the solution with the help of a magnet to be analysed [79,104,111,112].
2. On the other hand, If the goal is to remove the interferences cleaning the sample, MNPs will retain them under a magnetic field while the sample is separated and analysed [104].

## 1.8. Nuclear Magnetic Resonance Spectroscopy

NMR spectroscopy is a very versatile and powerful analytical technique that was first described in 1938 by Isidor Rabi and commercially available in 1952 by Varian. It's a well-known methodology used to identify molecules and discover their structure by measuring the nuclear magnetic interactions as well can be used to quantify the samples (qNMR) since each signal intensity is directly proportional to the number of atoms/nuclei present on that signal. This technique can be applied on small molecules as well as on large structures such as proteins. Currently, this technique is used on many fields such as medicine, physics, metabolomics, biochemistry, nanosciences and others (Figure 19) [25,42,58,60,145–147].

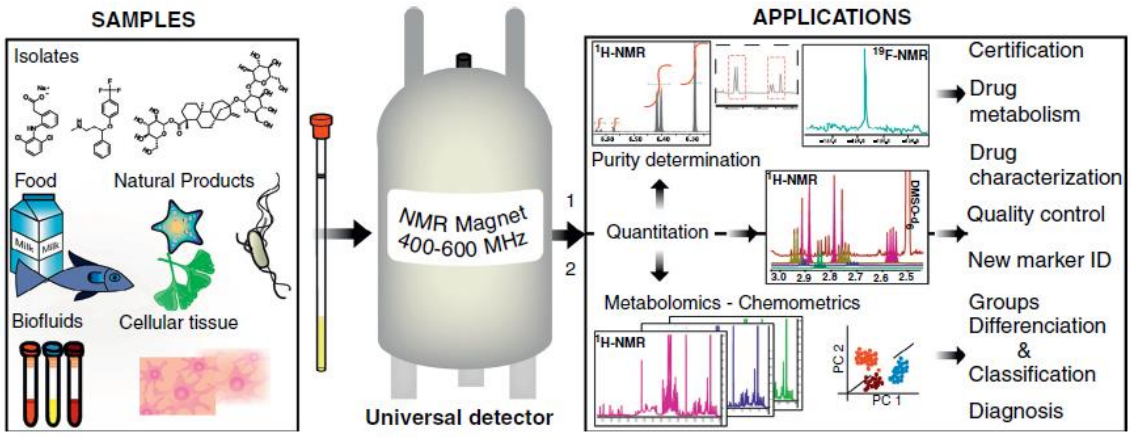



*Figure 19 – Examples of NMR applications* [25].

This technique relies on the nuclear spin of the atoms, which is a physical property where the atomic nuclei rotate on their own axis as a needle rotates towards magnetic fields. Usually, the nuclear spins are randomly oriented, depriving the macroscopic magnetization, but when are under a strong magnetic field, some of the nuclear spins become aligned parallelly to the applied field, generating a macroscopic ensemble magnetization (they become magnetized). In order to obtain the information of the sample, the sample is irradiated with radio waves pulses (also known as resonance frequencies) that will force the aligned atoms to leave their equilibrium position (parallel to the magnetic field) until the atomic nuclei become on a perpendicular plane. This influence creates a chemical shift (δ) that gets measured in parts per million (ppm). The δ values beside of depending on the external magnetic field, also depend of the atom microenvironment, generating this way a specific δ value that will indicate the atom

quantity and position as well their coupling, creating this way an NMR spectrum of the molecule(s) dissolved in a deuterated solvent [57,60,145,146].

The $^{1}$H (proton) NMR can provide important information on a sample (Figure 20). It is widely used for quantitative analysis ($^{1}$H qNMR) on metabolomics since it provides quantitative information between intra-molecular and inter-molecular resonances [55,57,147,148].

**Information avaliable on $^{1}$HNMR:**

- Chemical shifts;
- Signal multiplicites
- Homonuclear coupling constants
- Heteronuclear coupling constants
- First order or second-order of the signal (multiplicity)
- Half band-width of the signal
- Integral of the signal
- Stability of the signal

*Figure 20 – Summary of the information that $^{1}$HNMR can provide* [57].

On cancer metabolomics, the techniques that provide more data usually are the NMR and mass spectrometry (MS). On the Figure 21 it is possible to observe some of the advantages and drawbacks of each technique. The NMR also has the advantage of being a non-destructive and non-invasive methodology that can be used on characterization of complex biomolecules like proteins. To identify the metabolites, target analysis is widely used although that this methodology is slow and expensive [30,31,38,42,55,56,58,61–63,65,67–72,149,150].

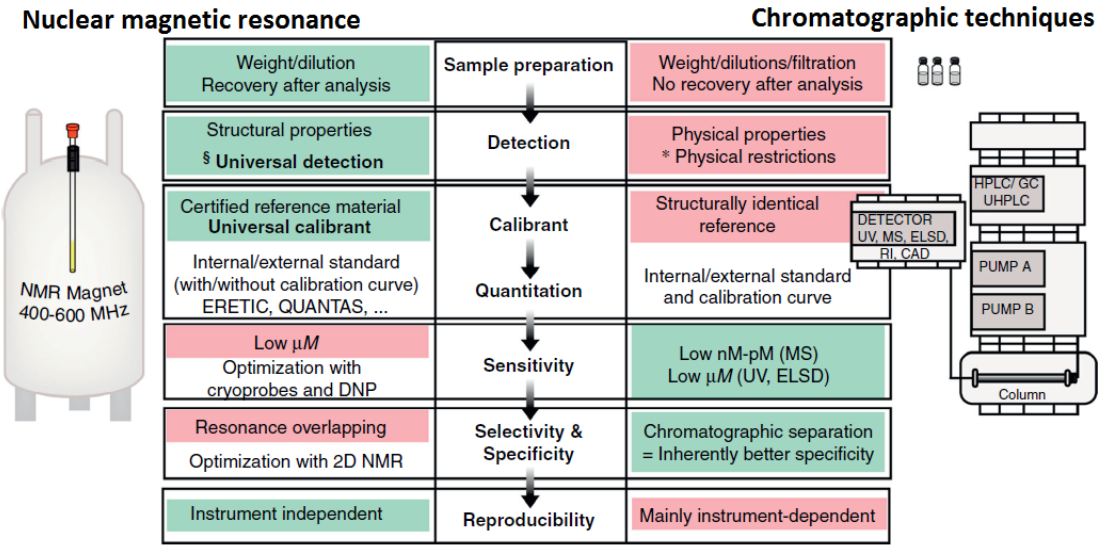



*Figure 21 – Comparison between NMR and chromatographic techniques. The properties highlighted in green are advantages and in red are drawbacks* [25,61].

The $^{1}$H qNMR is highly appealing on metabolomics since protons are present on all metabolites. It performs a fast analysis with high reproduction that identifies and quantifies many metabolites present on complex mixtures (e. g. body fluids such as urine) without sample preparation or with minimal preparation. Regarding human urine, NMR techniques can detect and quantify more than 200 compounds, being an attractive metabolic profiling technique for disease diagnosis and monitoring by applying/with the application of "case-control" studies. However, sometimes the metabolites can suffer a signal overlay on all their characteristic peaks, making them unable to be identified and quantified. In order to make a $^{1}$H qNMR for metabolomics, the use of internal standard (that resonances at 0 ppm) is essential since helps to calibrate all spectra's making easier the metabolites identification as well works as reference quantification peak, which is needed to quantify the metabolites. As reference substance for urine metabolomics, the Trimethylsilylpropanoic acid (TSP) is often used since is soluble on aqueous solutions in addiction that urine is a biofluid with low protein concentrations (TSP can bind with proteins, losing the reference function) [30,38,56–58,61–63,65,67–72,148–150].

## 1.9. Thesis objectives

The main objective of this Master thesis was to study the sorption capacity of the iron oxide NP, namely magnetite and maghemite, towards potential cancer biomarkers that can be found on urine. To reach that goal, a set of experiments were performed in particular:

- Preparation of a synthetic matrix (synthetic urine, spiked with some known potential cancer biomarkers) that will be used on the study of sorption capacity of iron oxide np. It will also be used on the optimization of biomarker extraction methodology;
- Creation of a metabolite library of potential cancer biomarkers used in this study. This library was used for metabolite identification using target analysis in order to know the biomarker characteristic peaks that could be used for identification and quantification. It was also used to know if the biomarkers suffered any overlay that prevent their identification;
- Optimization of the biomarker extraction methodology. On this objective, several parameters that could influence the extraction efficiency were tested:
    - Nanoparticle type and amount;
    - Elution solvent;
    - Ultrasound adsorption time and temperature;
- Application of the optimised conditions on a case-control study with real urine samples of control and cancer;

During the thesis research, a side objective was also performed in order to check the validity of the use of iron oxide np as cancer biomarkers sorbent:

- Compare the sorption capacity performance of iron oxide NP towards potential cancer biomarkers used on the study with the commonly used sorbents (LiChrolut and Amberlite XAD);

It is important to mention that the $^{1}H$ NMR analysis parameters were already previously optimized by our research group member Catarina Silva.

# Chapter 2. Materials and methods

## Contents

# 2. Materials and methods

In this chapter, we report how it was studied the iron oxide NPs sorption capacity towards cancer biomarkers. To make it more understandable, this section is divided into two main sections: the synthetic urine preparation and the extraction procedure with the iron oxide np. Furthermore, we describe the optimization methods in detail as such the characterization procedure. At the end of this section, it is presented with accuracy the application of the optimized methodology to real urine samples.

## 2.1. Materials and reagents

Alanine (Ala, 98 %), creatinine (98 %), L-isoleucine (Ile, 98 %), leucine (Leu, 98 %), L-lysine (Lys, 98 %), methionine (Met, 98 %), myristic acid (AMYR, 99.5 %), potassium chloride (99.5 %), Sodium Azide (99.5 %), Sodium Pyruvate (PYR, 99 %), Trimethylamine N-oxide (TMAO, 95 %), trimethylsilylpropanoic acid (TSP) (98 %), tryptophan (Trp, 98 %), tyrosine (Tyr, 98 %), urea (98 %) and valine (Val, 98 %) were provided by Sigma-Aldrich (Madrid, Spain). The calcium chloride (95 %), formic acid (FA, 98 %) lactic acid (ALAC, 85 %), potassium di-hydrogen phosphate (99 %), sodium carbonate (99.8 %), sodium chloride (99 %), sodium hydroxide (NaOH, 98 %) and sodium sulphate (99 %) was from Panreac AppliChem (Barcelona, Spain). The ammonium chloride (99.8 %), LiChrolut®, magnesium chloride (99 %) and trisodium citrate (CRI, 99 %) is produced by Merck. The ethyl lactate (ELAC, 95 %) and diethyl succinate (SUCC, 99 %) were provided from Acros Organics (Geel, Belgium). Methanol (MeOH, 99.9 %) and acetone (HPLC grade) were supplied by Fisher Scientific (Loughborough, UK). Amberlite® XAD® 2 was provided by Supelco (Bellefonte, PA, USA). The deuterated water ($D_2O$, 99.5 %) was provided by VWR (Radnor, PA, EUA). The ultra-pure (UP) water that was used on all experimental work was obtained through the Millipore Mili-Q direct 8 purification system with a resistivity higher than 18.2 MΩ.cm (at 25 ◦C).

The nanoparticles (Figure 22) were all purchased on US Research Nanomaterials, Inc (Houston, TX, USA) with analytical grade. This company provides some characterizations of the product, namely SEM images as shown on Figure 22.

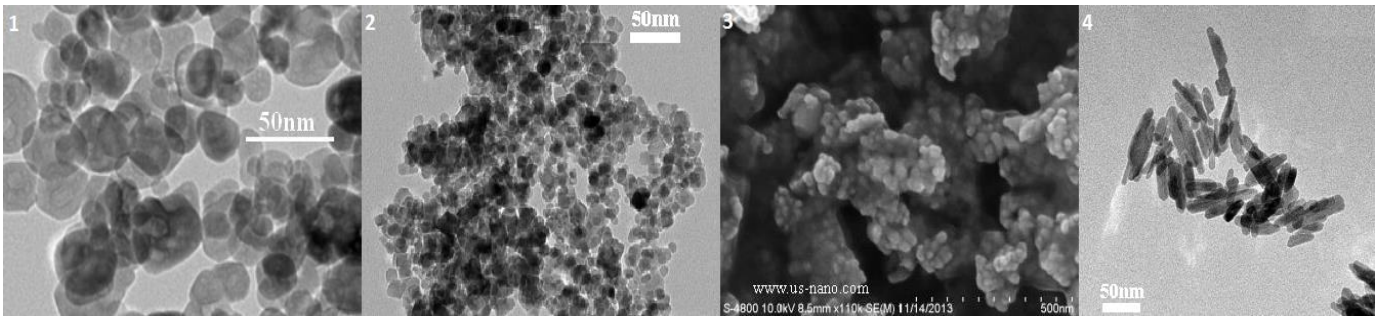



*Figure 22 – SEM images of the nanoparticles used on this study. Legend: 1- Maghemite (γ-$Fe_2O_3$) 20 nm; 2- Magnetite ($Fe_3O_4$) 15-20 nm; 3- Magnetite 20-30 nm; 4- FeOOH Nanorods 50x10 nm* [151–154].

To prepare the samples and standards, several materials and types of equipment, including an analytical balance Ohaus Pioneer and a microbalance Mettler Toledo AT20, were used. The pH was adjusted with Mettler Toledo™ EL20 Benchtop pH Meter for Teaching and Learning (Mettler Toledo AG, Switzerland). The samples were filtered on 0.2 µm membrane PTFE filters (Merck Millipore, Milford, MA, USA) and the centrifugation was carried out in the refrigerated centrifuge Sigma 3-30k equipped with a rotor 12154H. The different analysis were performed using an NMR Bruker Ultrashield 400 plus with the console Advance II+ 400 MHz.

For $^1$H NMR analysis, the TSP solution at 0.1% was prepared with a mix on $D_2O$ of 1.5 M monopotassium phosphate and 2 mM of sodium azide.

The real urine samples used on the final study were obtained from Urine bank (ACELab) frozen at −80 °C, collected previously at Hospital Nélio Mendonça within other studies. These samples were obtained from cancer patients (*n*=8, age=60.3 ± 10.2 years; Female non-smokers) that were diagnosed on 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, which were immediately frozen at −80 °C and kept until being processed.

All cancer patients gave their written informed consent for its volunteer participation in the study. 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) [155]. All data was analysed anonymously throughout the study.

## 2.2. Preparation of spiked synthetic urine

### 2.2.1. Synthetic urine preparation

The synthetic urine (SU) solution was prepared on UP water following the Wilsenach et al. [156] formula that is shown in Table 6. After preparing the SU solution, the pH was adjusted to 5.8 using FA at 10% or NaOH at 1M and filtered. The solution was divided on 5 ml aliquots in 8 ml vials that were stored on the fridge until being used.

*Table 6- Composition of synthetic urine* [156].

| Compound | Formula | Concentration | |
|---|---|---|---|
| | | g $L^{-1}$ | mM |
| *Calcium chloride-2-hydrate* | $CaCl_2 \cdot 2H_2O$ | 0.65 | 4.40 |
| *Magnesium chloride-6-hydrate* | $MgCl_2 \cdot 6H_2O$ | 0.65 | 3.20 |
| *Sodium chloride* | NaCl | 4.60 | 78.70 |
| *Sodium sulfate anhydrous* | $Na_2SO_4$ | 2.30 | 16.20 |
| *tri-Sodium citrate-2-hydrate* | $Na_3C_6H_5O_7 \cdot 2H_2O$ | 0.65 | 2.60 |
| *Sodium carbonate anhydrous* | $Na_2CO_3$ | 0.02 | 0.19 |
| *Potassium di-hydrogen phosphate* | $KH_2PO_4$ | 4.20 | 30.90 |
| *Potassium chloride* | KCl | 1.60 | 21.50 |
| *Ammonium chloride* | $NH_4Cl$ | 1.00 | 18.70 |
| *Urea* | $NH_2CONH_2$ | 25.0 | 417 |
| *Creatinine* | $C_4H_7N_3O$ | 1.10 | 9.7 |

## 2.3. Preparation of potential biomarker standard solutions

The potential biomarker standard solutions were prepared according to the proportions observed in Table 7. Each solution was dissolved on UP water apart from myristic acid that was dissolved on MeOH. On the cases where the standards were on the liquid state (marked with * on Table 7), the adjustment calculations were performed. All standard solutions were stored on the fridge protected with Parafilm® and only were used to spike SU once they reach room temperature and get vortex.

*Table 7 – Proportions used to prepare each biomarker standard solution.*

| Biomarker | Biomarker abbreviation | Volume (ml) | Mass (g) | Concentration (mg/ml) |
|---|---|---|---|---|
| *Alanine* | Ala | 20 | 0.2 | 10 |
| *Latic Acid* | ALAC | 18 | 1.9 | 100 |
| *Myristic Acid* | AMYR | 20 | 0.2 | 10 |
| *Ethyl Lactate* | ELAC | 18 | 2 | 100 |
| *Isoleucine* | Ile | 20 | 0.2 | 10 |
| *Leucine* | Leu | 20 | 0.2 | 10 |
| *Lysine* | Lys | 20 | 0.2 | 10 |
| *Methionine* | Met | 20 | 0.2 | 10 |
| *Pyruvate* | PYR | 20 | 0.2 | 10 |
| *Succinate* | SUCC | 20 | 0.1 | 100 |
| *Trimethylamine N-oxide* | TMAO | 20 | 0.2 | 10 |
| *Tryptophan* | Trp | 100 | 0.2 | 2 |
| *Tyrosine* | Tyr | 40 | 0.2 | 5 |
| *Valine* | Val | 20 | 0.2 | 10 |

### 2.3.1. Spiked of synthetic urine

SU was spiked with cancer potential biomarkers, each 5 ml aliquot of SU was added the equivalent of 1 mg of each biomarker, acquiring the final concentration of 0.2 mg/ml of each potential biomarker. The relation between the biomarker concentration and the quantity of standard solution used for spike of the SU is observed in Table 8.

*Table 8 - Relation between the potential biomarker concentration and the quantity of standard solution used for spike of SU*

| Concentration (mg/ml) | Quantity to add (µl) |
|---|---|
| **100** | 10 |
| **10** | 100 |
| **5** | 200 |
| **2** | 500 |

The biomarkers used to spike the SU were: Alanine (Ala), Ethyl lactate (ELAC), Isoleucine (Ile), Latic acid (ALAC), Leucine (Leu), Lysine (Lys), Myristic acid (AMYR), Methionine (Met), Tryptophan (Trp) and Valine (Val). This procedure always was done with the solutions at room temperature (23 ºC) after they being vortex. The spiked SU when wasn't applied extraction on the day was stored on the fridge.

All the potential biomarkers that were not used for the spike of urine, were used for target analysis for biomarker identification (Pyruvate (PYR), Succinate (SUCC), Trimethylamine N-oxide (TMAO) and Tyrosine (TYR)).

## 2.4. Extraction procedure

For the potential biomarker extraction, it was adapted the MSPE technique, where we disperse the MNPs on the sample, extract the potential biomarkers with the help of an ultrasound sonication and discard the sample solution while preserving the MNPs with the help of a magnet [79,104,111,138,139,144]. Then we add 1 ml of elution solvent on MNPs and we separate the MNPs by centrifuging for 15 min at 15,000 rpm (20627 rcf) and 10 °C and filtering through 0.22 µm PTFE syringe filters (BGB Analytik, VA, USA). On Figure 23 is observed the extraction scheme used in this study. Each extraction was carried out using spiked SU sample. Unspiked SU was used as a blank sample.

At the end of the extraction the nanoparticles were always dried on a lab oven for 2 days at 60 °C, then stored in vials separated by nanoparticle type for further studies.

To make the nanoparticles identification easier during all work, each type of nanoparticle was numbered: 1 - Maghemite ($Fe_2O_3$) 20 nm; 2 - Magnetite ($Fe_3O_4$) 15-20 nm; 3 - Magnetite 20-30 nm; 4 - FeOOH Nanorods 50x10 nm.

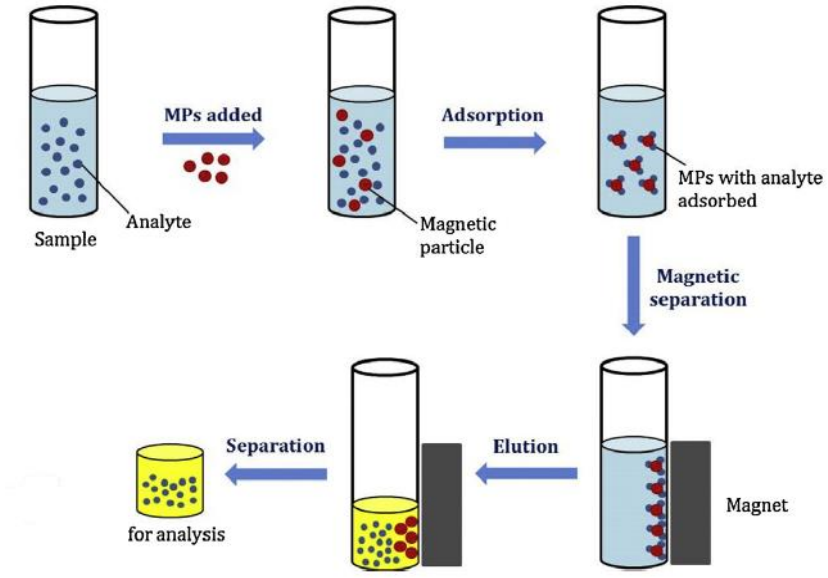



*Figure 23 – Magnetic solid phase extraction scheme. From* [112]

## 2.5. Optimization parameters

For the optimization of the experimental procedure, several parameters with influence on the extraction efficiency, namely extraction solvent, type of nanoparticles, nanoparticles amount, extraction time and temperature, were investigated.

The optimization followed a univariate design represented on Figure 24, where the optimal results of the previous parameter were always applied on the following parameter. Each extraction was performed using spiked SU sample. Unspiked SU was used as blank.

During the optimization, it was also tested the use of MNPs towards common sorbents such as LiChrolut® and Amberlite® XAD® 2. On this test, it was used 5 mg of each sorbent on SU and spiked SU extracting for 15 min on ultrasound. For elution, it was used 1 ml of UP water and the sorbents were separated with the help of centrifugation and filtration.

The conditions obtained at the end of optimization (a ratio of 0.2 mg/ml of nanoparticle 3 to extract during 5 min at room temperature with the addition of 1 ml of UP water as elution solvent) will be used to extract the real urine sample metabolites.

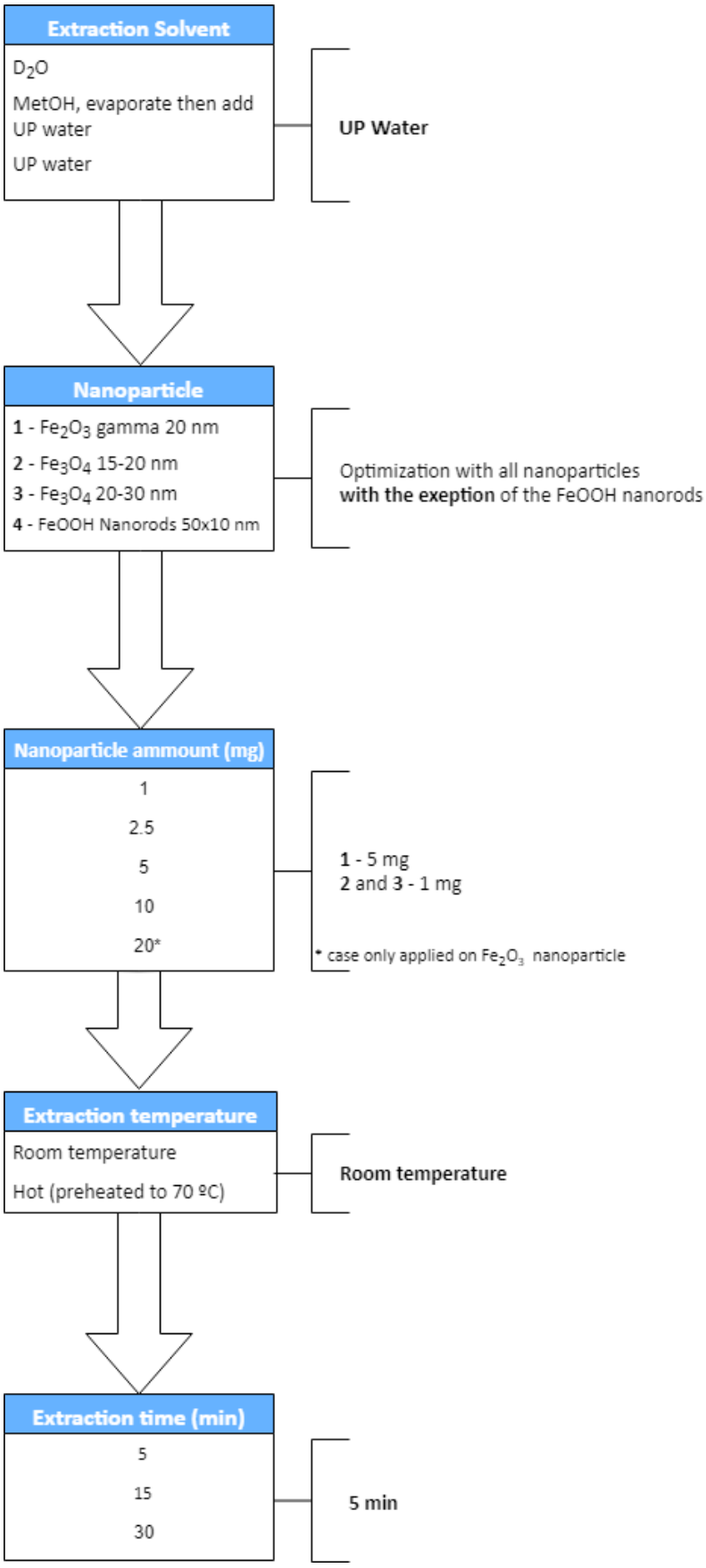



*Figure 24 – Flowchart of the optimization methodology applied, mentioning the parameters and the used conditions.*

## 2.6. $^{1}$H NMR Conditions

The $^{1}$H NMR conditions were 400 MHz for $^{1}$H nucleus observation, at 300 K probe temperature. On each sample, a standard 1D $^{1}$H NMR spectrum was acquired, using a water suppression pulse sequence with water irradiation during relaxation delay and mixing time ('noesypr1d' from Bruker library, SW 12.0153 Hz, TD 64 K data points, relaxation delay 5 s, mixing time 200 ms, 128 scans).

Each NMR tube was filled with 540 µL of the sample and 60 µL of TSP (used as chemical shift reference) except for real urine samples, where the proportions were adapted towards sample quantity. To wash the NMR tubes, it was added and discarded distilled water once and ketone three times, letting the remaining ketone evaporate at room temperature for at least a day before reuse the tube.

To observe and calibrate the results obtained, the program topspin 4.0.4 was used.

### 2.6.1. Identification of biomarkers (target analysis)

In order to identify the cancer biomarkers on urine, a target analysis was made for each biomarker. On a 5 ml aliquot of SU, it was added the equivalent of 2 mg of a biomarker, acquiring the final concentration of 0.4 mg/ml. This solution was extracted for 15 min using 1 mg of the nanoparticle 3 and 1 ml of UP as an extraction solvent.

To identify the SU characteristic peaks, direct analysis of SU was performed without any extraction procedure.

### 2.6.2. Relative quantification of biomarkers

To quantify the metabolites, it was used the Chenomx NMR Suite software developed by Chenomx, Inc. (Edmonton, AB, Canada) that makes possible to quantify hundreds of metabolites that are present on the software database using as a base the internal standard peak. [55,57,62,63,68,69,71,147]

## 2.7. Application on real urine samples

Prior to the application of extraction methodology, the pH of all real urine samples was adjusted to 5.8, followed by a 15,000 rpm centrifugation for 10 minutes at 10 °C to

separate the precipitations. After centrifugation, the supernatants were extracted with the best result methodology (that is found at section 2.5), adapting the proportions of the methodology (NP quantity and elution volume) towards the sample volume available. As a consequence, the sample volumes also had to be adapted for $^{1}$H NMR analysis. A total of 12 urine samples, described in Table 9, were extracted on this work.

*Table 9 – Characterization of urine samples included on the study. All samples are from females*

| | Control | Breast | Lymphoma |
|---|---|---|---|
| **Number of samples (*n*)** | 4 | 4 | 4 |
| **Age (years) [range]** | 40 [26-46] | 53.5 [44-65] | 67 [58-72] |

# Chapter 3. Results and discussion

## Contents

# 3. Results and discussion

On this chapter, the main results and their interpretation will be presented. To start, the data obtained from the target analysis will be shown in order to know the characteristic peaks of each biomarker as well as the urine characteristic peaks and the sorbent peaks. Right after the sorbents, performance results will be displayed where the common sorbents and the nanosorbents will be compared based on biomarker quantification.

Then the results from the optimization methodology are presented. They follow the same order as the flowchart of Figure 24. Finally, the results obtained from the extraction of the real urine samples will be presented. The quantification of the control will be compared with urines from lymphomas and breast cancer in order to detect potential biomarkers.

## 3.1. Identification of biomarkers (target analysis)

The table 10 was made with the help of the target analysis and comparison of the data obtained with the Human Metabolome Database (HMDB) [60,65–71] biomarkers $^{1}H$ spectra. This table shows all the biomarkers characteristic peaks in ppm that will be used on the biomarker quantification on this study. Is important to mention that the lactic acid suffered overlay on all its peaks; meanwhile the myristic acid results didn't match with the HMDB results, leading to the exclusion of these biomarkers.

*Table 10 – Characteristic chemical shifts of potential target biomarkers. The identification peaks are highlighted. Keys: a - biomarker that cannot be identified due to overlay on all their identification peaks; b - characteristic peaks that doesn't match with HMDB characteristic peaks, making the biomarker not apt to be studied.*

| Biomarker | Biomarker initials | Chemical shift (ppm) |
|---|---|---|
| *Alanine* | Ala | **1.5**; 3.8 |
| *Latic Acid* | ALAC | 1.3; 4.1[a] |
| *Myristic Acid* | AMYR | 0.9; 1.6; 2.4; **3.1**; **3.5**[b] |
| *Ethyl Lactate* | ELAC | **1.3**; 4.1 |
| *Isoleucine* | Ile | **1.0**; 1.2; 1.9; 3.6 |
| *Leucine* | Leu | **0.9**; 1.7; 3.7 |
| *Lysine* | Lys | 1.5; 1.7; **1.9**; 3.0; 3.7 |
| *Methionine* | Met | **2.1;** 2.6; **3.9** |
| *Pyruvate* | PYR | **2.37** |
| *Succinate* | SUCC | **2.4** |
| *Trimethylamine N-oxide* | TMAO | **3.3** |
| *Tryptophan* | Trp | 3.3; 3.5; 4.1; 7.2; 7.3; **7.5; 7.7** |
| *Tyrosine* | Tyr | 3.0; 3.2; 4.0; **6.9**; 7.1 |
| *Valine* | Val | **1.04**; 2.3; 3.6 |

Regarding Chenomx database, all the biomarkers except PYR are present. This leads to a total of 11 biomarkers that will be quantified on urine samples result and a total of 8 biomarkers that will be quantified on optimization and sorbent performance.

Regarding urine, it was possible to identify the citrate (ppm 2.5 and 2.7), creatinine (ppm 3.1 and 4.2) and urea (ppm 5.8) that can be observed on Figure 25. Sometimes, in some published results, creatinine and citrate are used as cancer biomarkers. However, they won't be considered biomarkers on this study since they are one of the main urine constituents, making the standardization of their amounts difficult, making them being not suitable to be used as a biomarkers. [29,38,61,65]

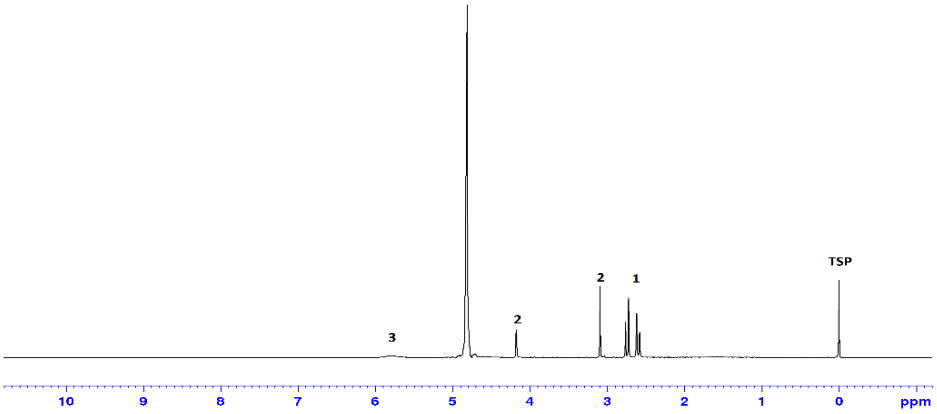



*Figure 25 – $^{1}H$ NMR spectra of synthetic urine in $D_2O$. Legend: 1 – Citrate; 2 – Creatinine; 3 – Urea.*

## 3.2. Sorbents performance

The sorbents performance towards the potential cancer biomarkers are displayed on Table 11 and Figure 26. Is possible to highlight that the nanoparticle 3 (Magnetite 20-30 nm) extracted the biomarkers with more efficiency, namely the Ile, Leu, Lys and Val.

This demonstrates that nanosorbents may be better than common sorbents. Aside from the simpler extraction methodology, the nanosorbents show a promising utility regarding the metabolite extraction that are potential cancer biomarkers.

*Table 11 – Sorbent efficiency towards the targeted potential biomarkers. Keys 1 - Maghemite (Fe2O3) 20 nm; 2 - Magnetite (Fe3O4) 15-20 nm; 3 - Magnetite 20-30 nm; 4 - FeOOH Nanorods 50x10 nm.*

| Biomarker | Concentration [mM] | | | | | | Best sorbent |
|---|---|---|---|---|---|---|---|
| | Maghemite 20 nm (1) | Magnetite 15-20 nm (2) | Magnetite 20-30 nm (3) | FeOOH Nanorods (4) | LiChrolut® | Amberlite® XAD® 2 | |
| ***Alanine*** | 3.56 | 2.79 | 3.84 | 3.08 | 4.08 | 4.26 | Amberlite® XAD® 2 |
| ***Ethyl Lactate*** | 3.43 | 2.42 | 4.16 | 4.44 | 3.85 | 3.92 | 4 |
| ***Isoleucine*** | 1.78 | 1.81 | 2.82 | 1.24 | 2.42 | 2.70 | 3 |
| ***Leucine*** | 2.30 | 2.07 | 3.64 | 1.94 | 2.75 | 2.78 | 3 |
| ***Lysine*** | 1.66 | 1.14 | 1.76 | 1.07 | 1.49 | 1.46 | 3 |
| ***Methionine*** | 1.39 | 2.09 | 1.72 | 0.92 | 1.35 | 1.42 | 2 |
| ***Tryptophan*** | 1.16 | 0.70 | 1.51 | 1.28 | 1.81 | 0.85 | LiChrolut® |
| ***Valine*** | 1.78 | 1.61 | 2.91 | 1.57 | 2.78 | 2.58 | 3 |

## 3.3. Extraction optimization

### 3.3.1. Extraction solvent

Observing the data present on Figure 27 is possible to see that only the UP water or deuterated water were suitable to elute the potential cancer biomarkers. Due to economic reasons, UP water was chosen as elution solvent for this study.

### 3.3.2. Nanosorbents

The results of the nanosorbents are shown on Figure 26 and Table 12 altogether with the common sorbents. It is possible to observe that the nanoparticle 3 stands out on the extraction of several biomarkers (Ile, Leu, Lys and Val).

The nanoparticle 2 was the nanosorbent that extracted more methionine, followed by nanoparticle 3. Meanwhile, the nanoparticle 1 doesn't stand out on any biomarker.

Aside from the nanorods (nanoparticle 4) being the nanoparticle that extracted more ethyl lactate, their extraction performance on other biomarkers is lower than the other nanosorbents. In addition, this NM is not magnetized, making the extractions made with this NM more difficult. Due to these reasons, this NM was discarded on this study.

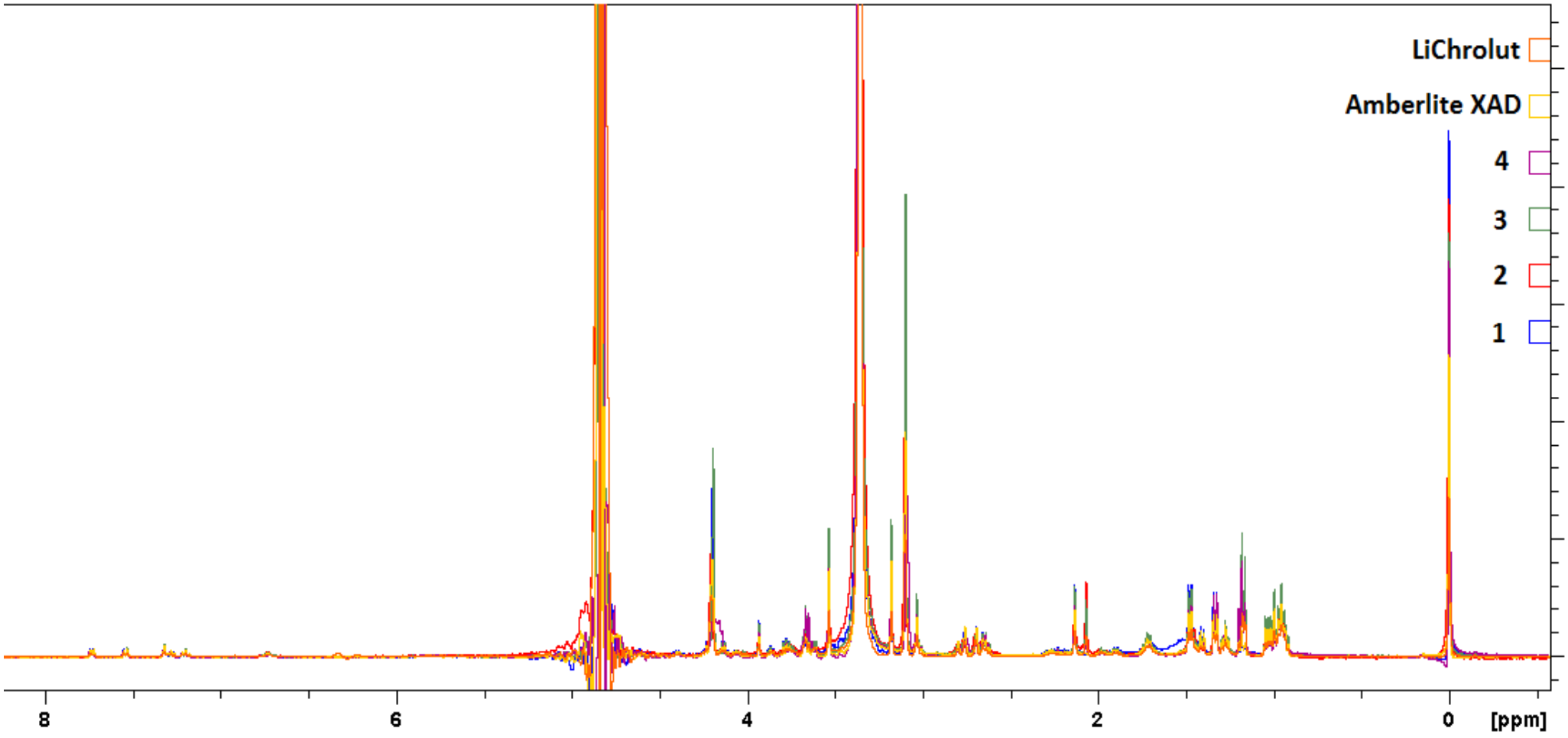



*Figure 26 -$^{1}$H NMR spectra of sorbent test in $D_2O$. Keys 1 - Maghemite ($Fe_2O_3$) 20 nm; 2 - Magnetite ($Fe_3O_4$) 15-20 nm; 3 - Magnetite 20-30 nm; 4 - FeOOH Nanorods 50x10 nm.*

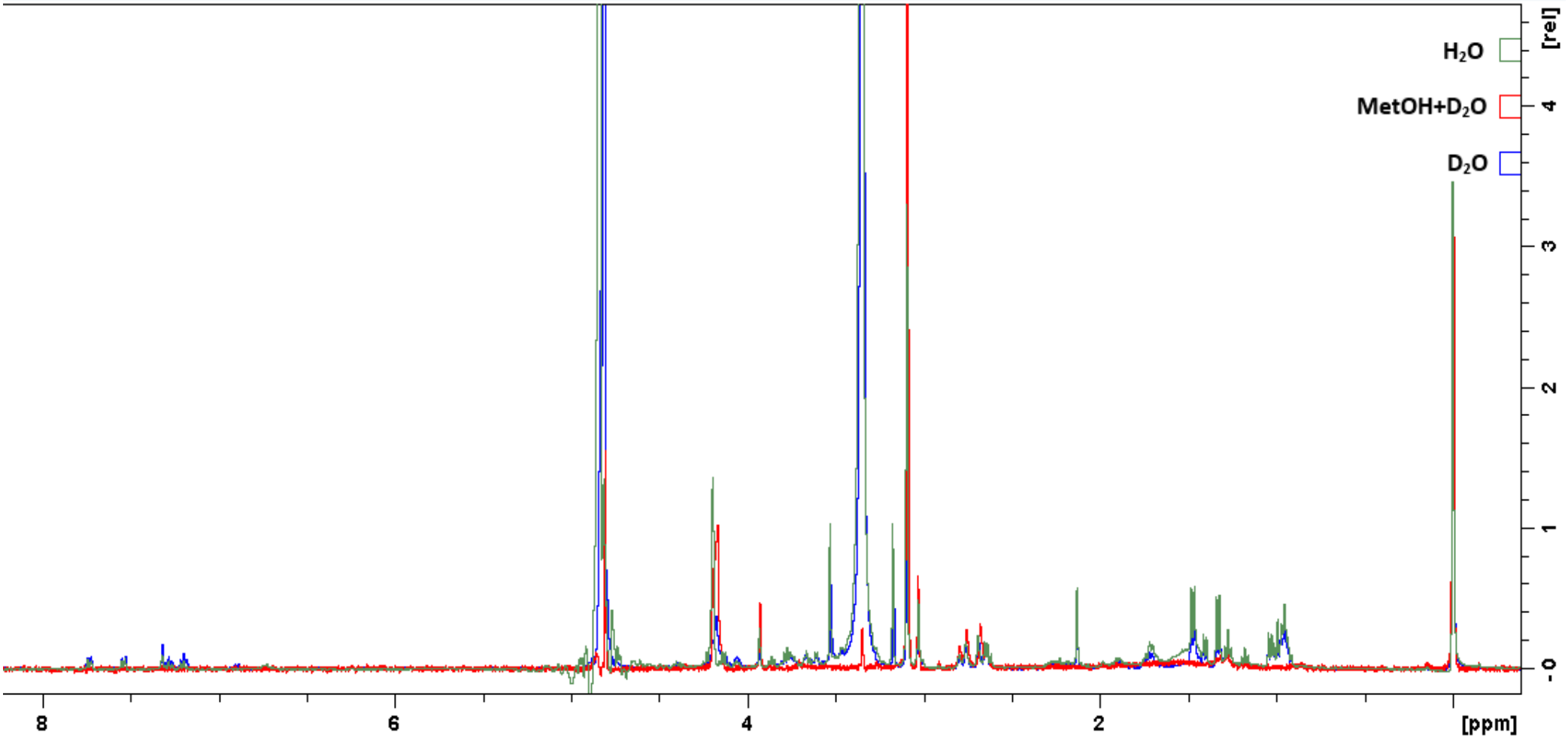



*Figure 27 - $^{1}H$ NMR spectra of solvent extraction optimization in $D_2O$*

### 3.3.3. Nanosorbents amount

The $^{1}H$ NMR spectra's that show the nanosorbents amount influence towards the potential cancer biomarkers are present on the Figures 28-30.

Observing the spectra of the nanoparticle 1 in Figure 28 is notable that as the nanosorbent amount increases, the biomarkers extraction efficiency increases. However, as present on the figures of annex B (page 106), the use of high amounts of nanosorbent leads to high variation degree in the biomarker extraction when doing replicas. Due to that, the ideal amount that balances the extraction performance and a low variation degree is the 5 mg.

On magnetite nanoparticles (nanoparticle 2 and 3), where the spectrums are found on Figures 29 and 30, is perceptible that the biomarker extraction efficiency doesn't change much regardless the amount of nanosorbent. For this reason, the ideal amount of magnetite nanoparticles is 1 mg.

### 3.3.4. Extraction temperature

Since the extraction at room temperature was already shown on previous results, namely on the Figures 26-30, only the extraction at hot temperatures is displayed on Figure 31. Observing the spectra is possible to see that the nanosorbents didn't manage to extract the biomarkers with the same efficiency as it extracts at room temperature, making the use of hot temperatures not suitable for extraction.

It was also observed that all the nanoparticles that underwent this extraction had partly lost their magnetic properties and agglomerated. An explanation for that is the rearrangement of the ordered magnetic moments, which decreases as the temperature increases. This makes the magnetic moments become disordered, losing their magnetization [120,121].

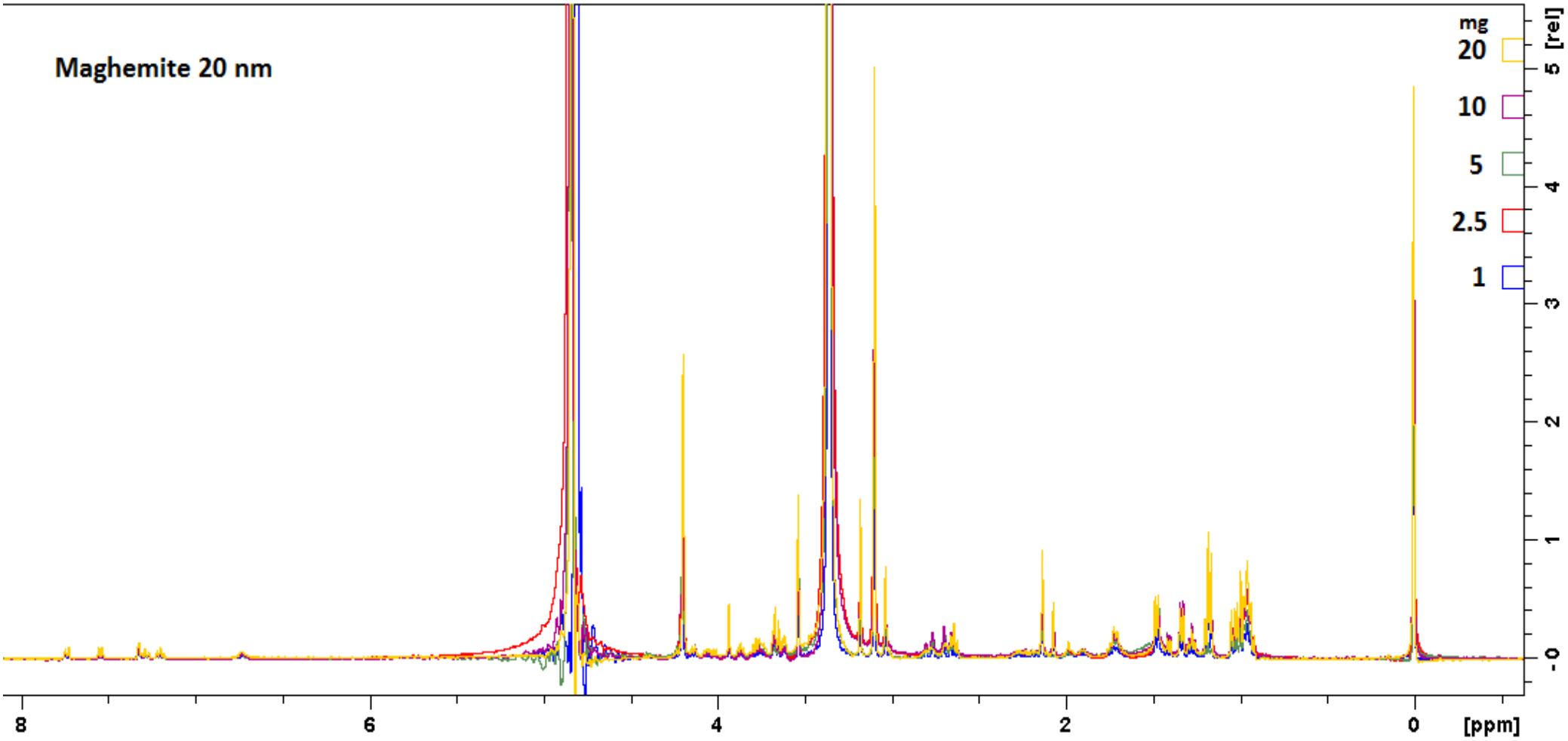



*Figure 28 - $^{1}H$ NMR spectra of nanoparticle 1 (maghemite 20 nm) extraction in $D_2O$ using different sorbent amounts (mg)*

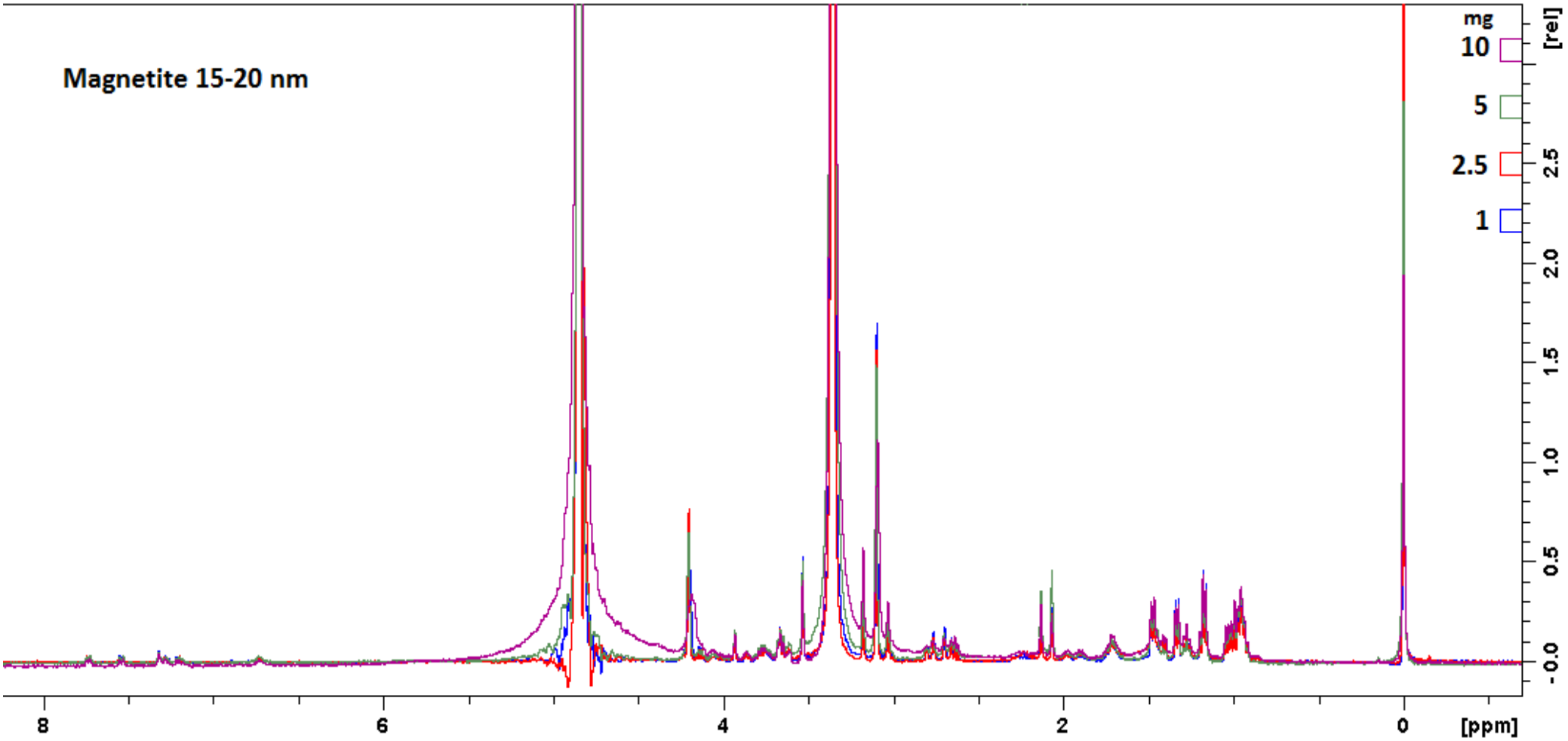



*Figure 29 - $^{1}H$ NMR spectra of nanoparticle 2 (magnetite 15-20 nm) extraction in $D_2O$ using different sorbent amounts (mg)*

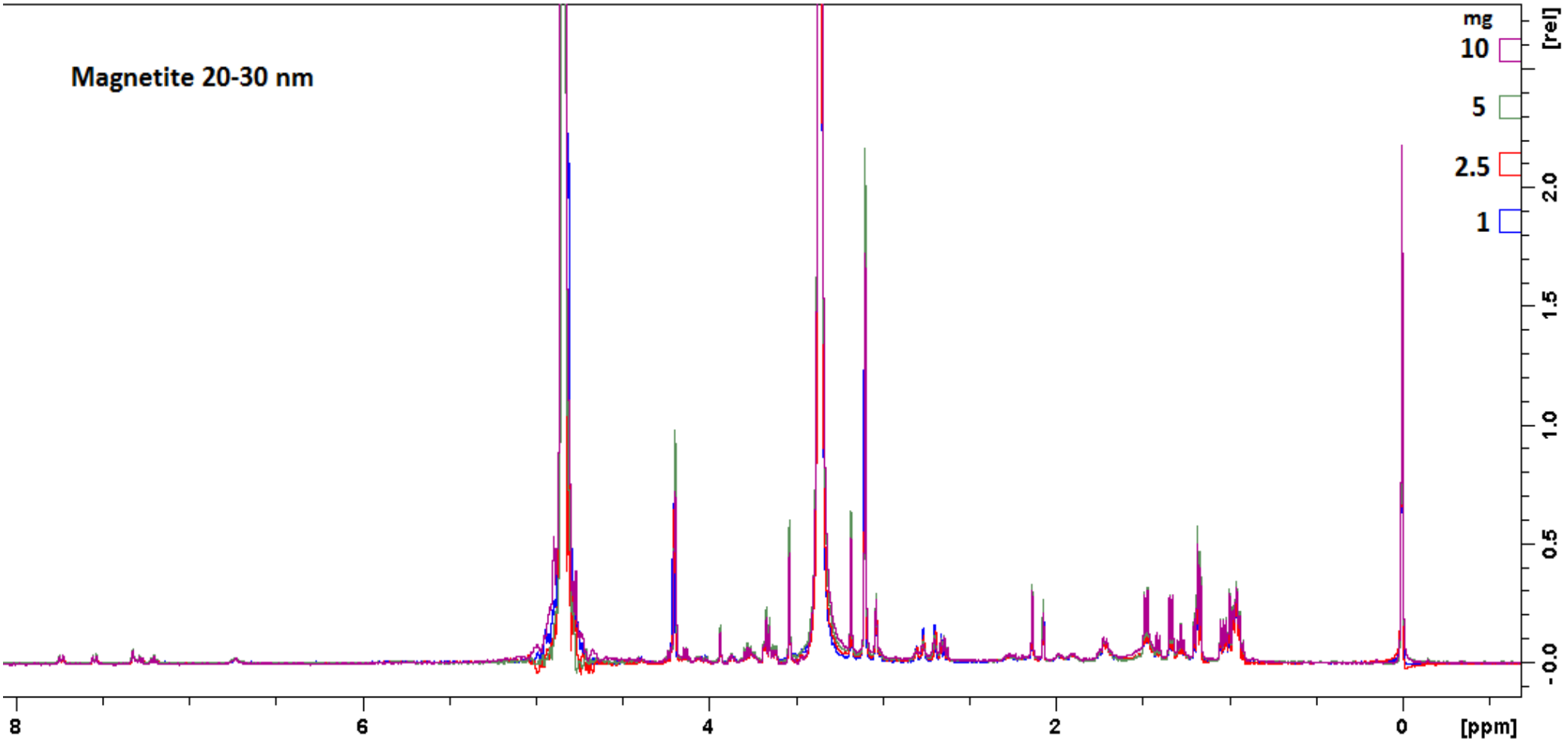



*Figure 30 - $^{1}H$ NMR spectra of nanoparticle 3 (magnetite 20-30 nm) extraction in $D_2O$ using different sorbent amounts (mg)*

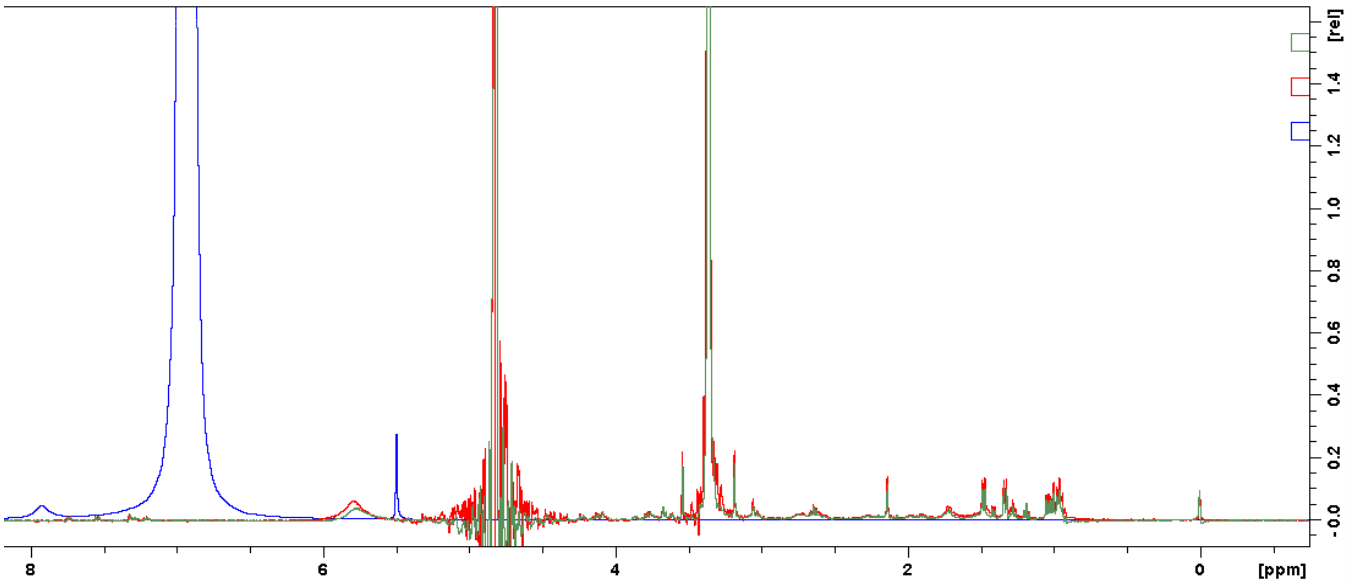



*Figure 31 - $^{1}H$ NMR spectra of extraction in $D_2O$ carried out at 70 ºC*

### 3.3.5. Extraction time

As observed on the Figures 32-34, is it notable that the lower extraction time leads to a better extraction performance on any nanosorbent, with the best extraction time being 5 minutes. Knowing that, Figure 35 was made as a comparison of the nanosorbents under their optimum extraction conditions.

Observing the data, the nanoparticle 1 and 3 are the nanosorbents that have better extraction efficiency, being that the extraction performance between these nanoparticles is very similar. Considering the cost-effectiveness ratio, which is an important parameter for scale up and routine analysis, the best nanosorbent to use is the nanoparticle 3 since on its optimum conditions uses 5x less amount than nanoparticle 1 and/ as well is the cheapest nanosorbent.

The best extraction conditions that were used on urine extraction are: a ratio of 0.2 mg/ml to extract during 5 min at room temperature with the addition of 1 ml of UP water as elution solvent.

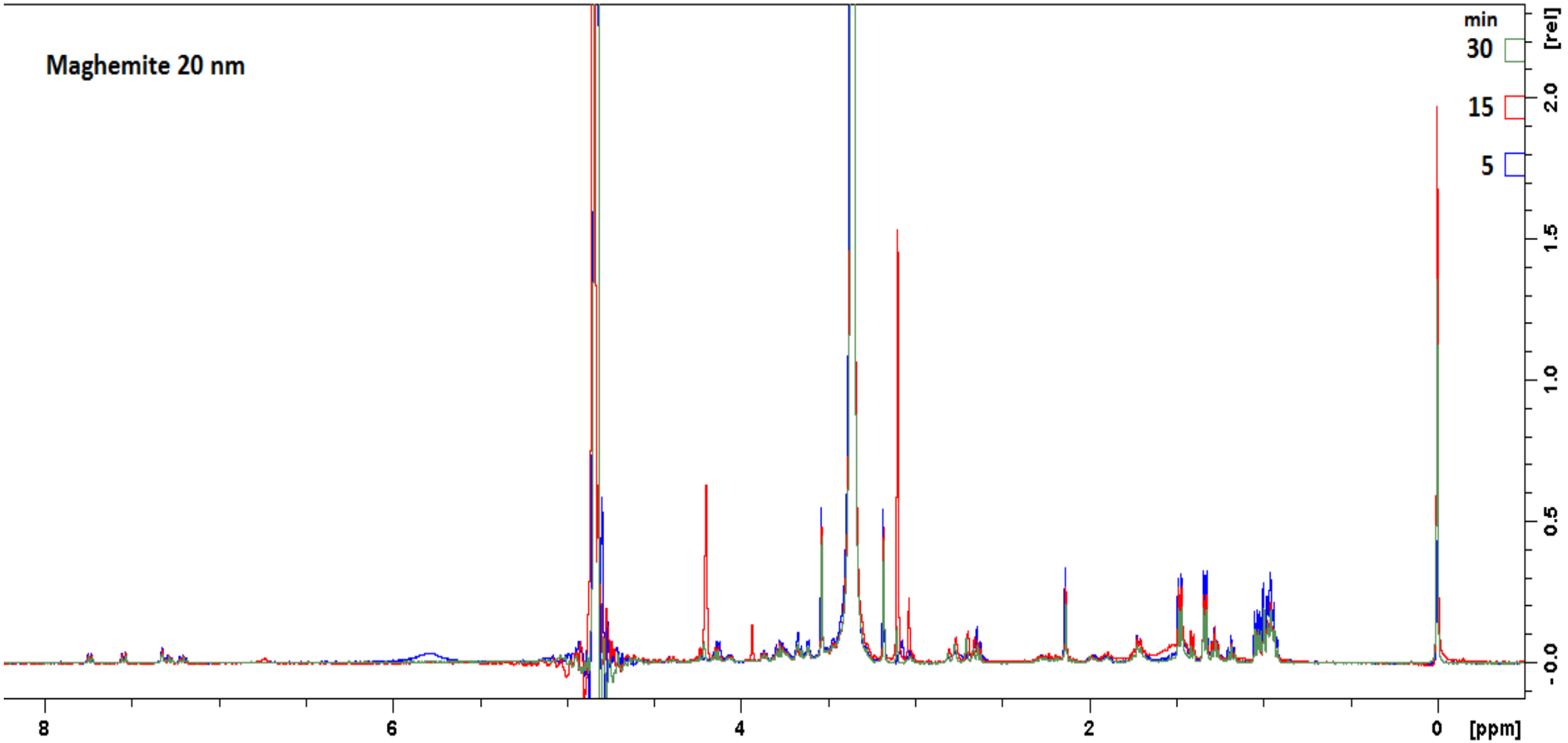



*Figure 32 - $^{1}H$ NMR spectra of nanoparticle 1 (maghemite 20 nm) extraction in $D_2O$ under different extraction time (min)*

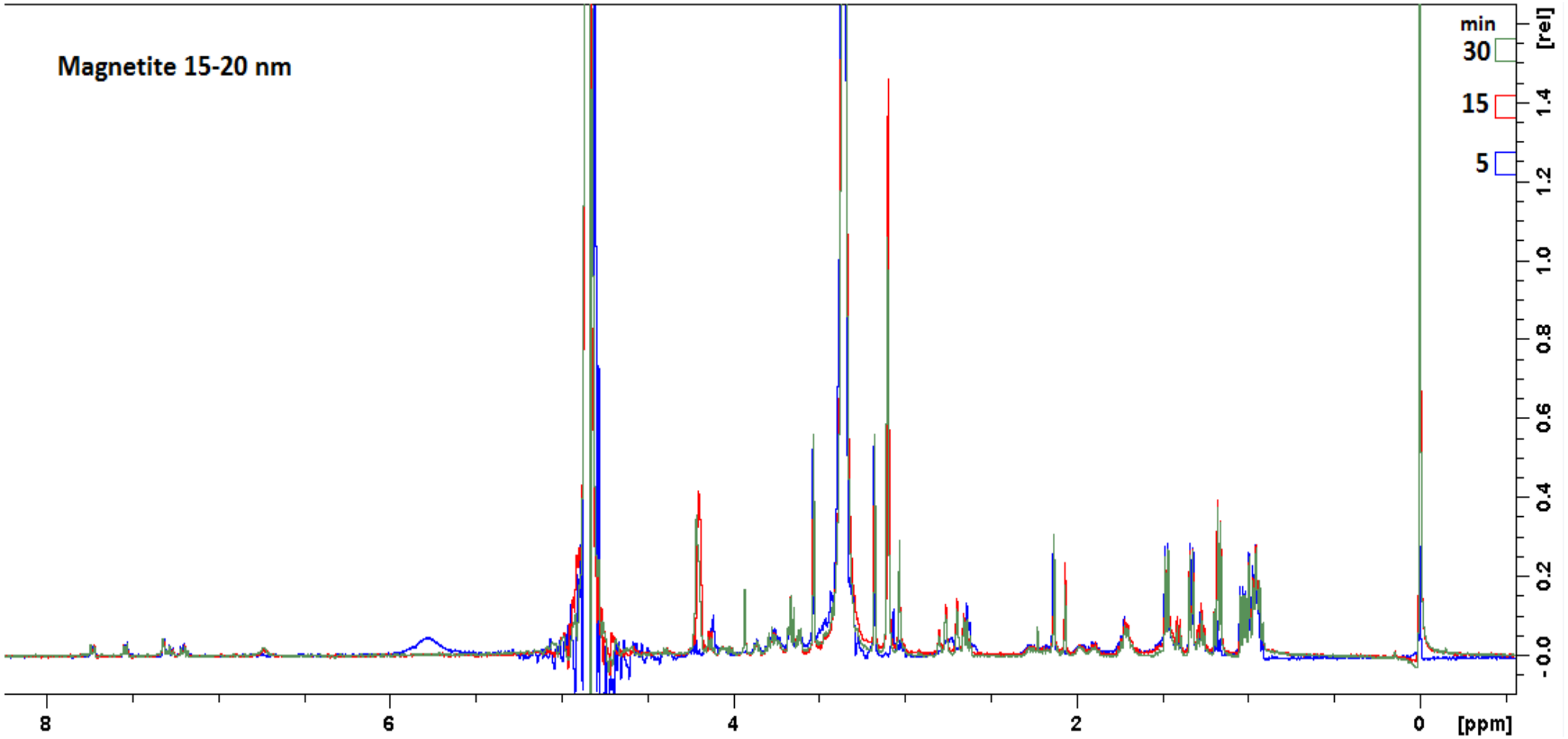



*Figure 33 - $^{1}H$ NMR spectra of nanoparticle 2 (magnetite 15-20 nm) extraction in $D_2O$ under different extraction time (min)*

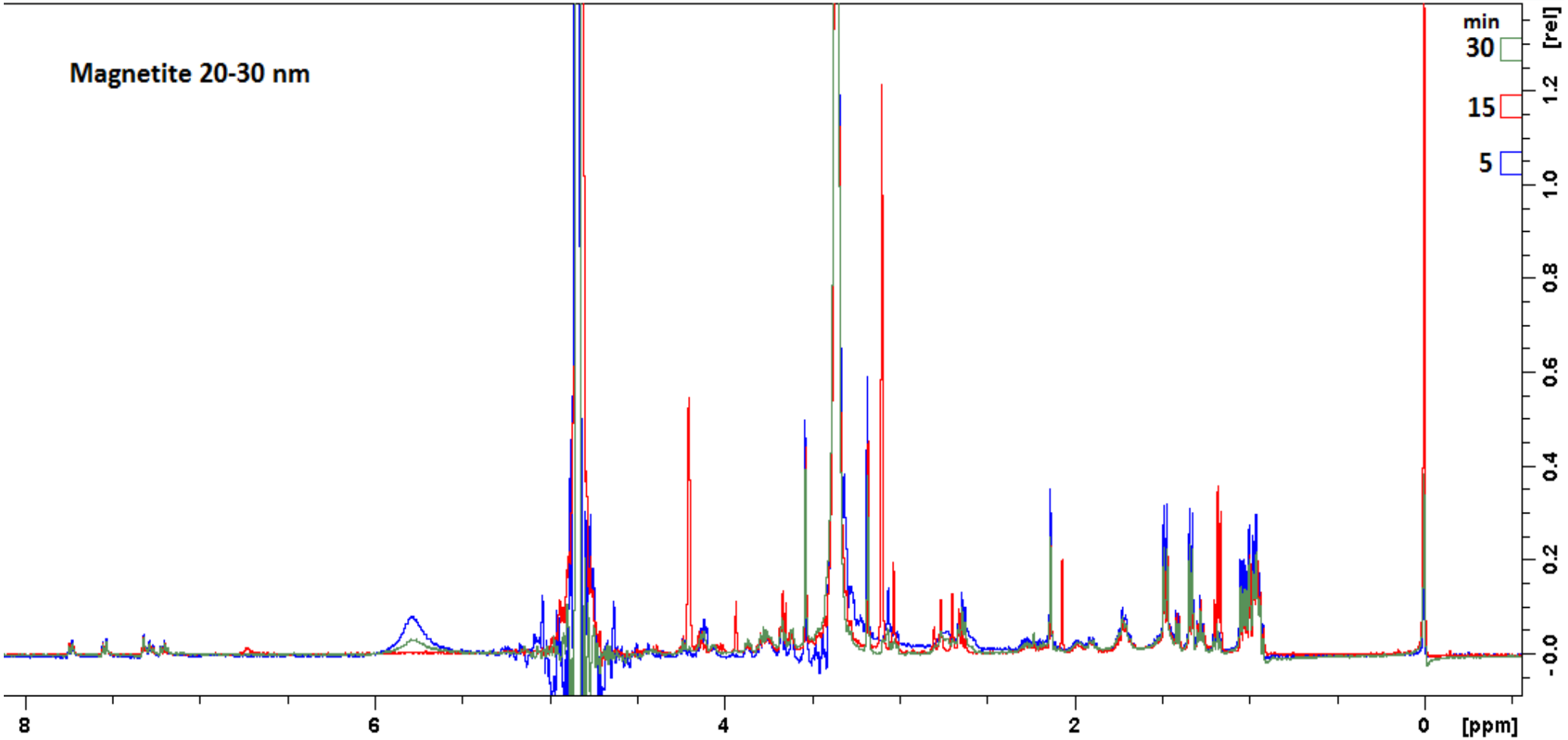



*Figure 34 - $^{1}H$ NMR spectra of nanoparticle 3 (magnetite 20-30 nm) extraction in $D_2O$ under different extraction time (min)*

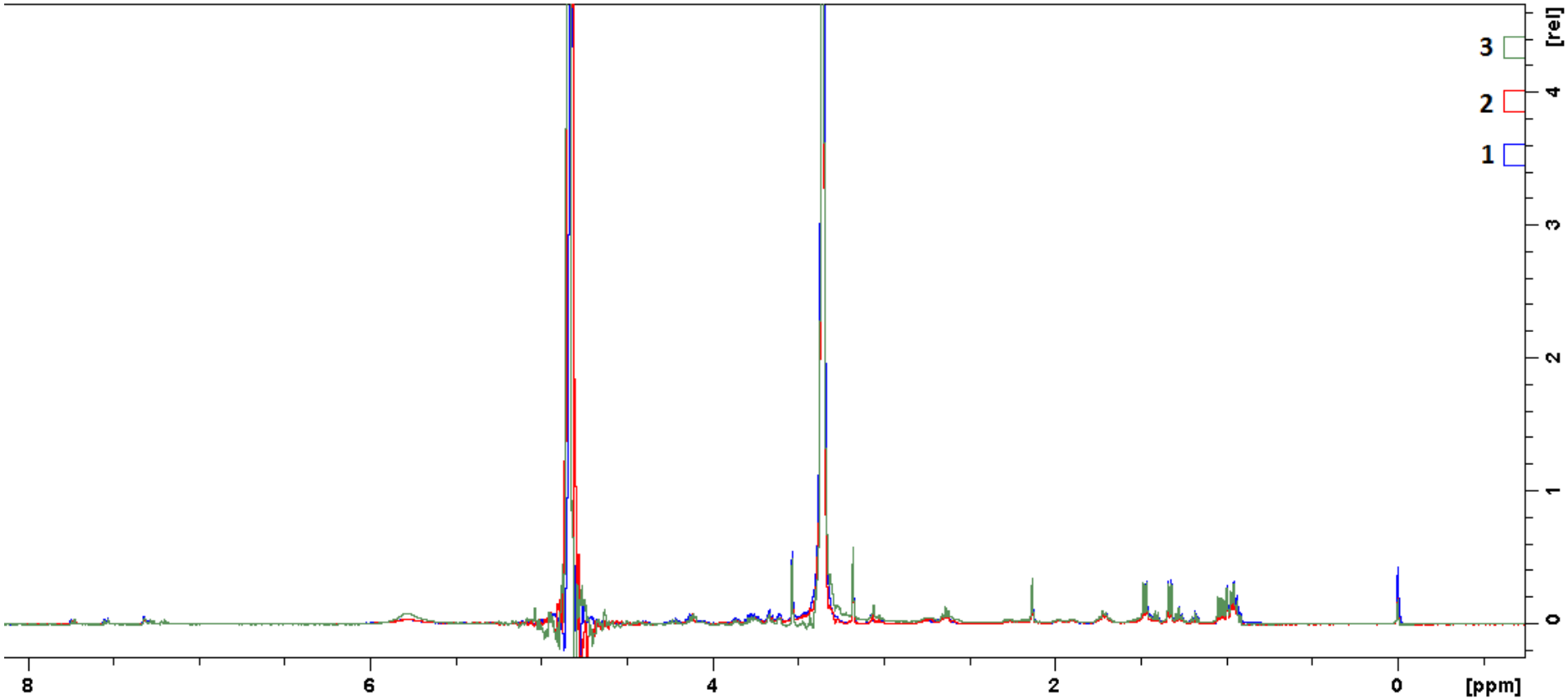



*Figure 35 -$^{1}$H NMR spectra of best optimization conditions in $D_2O$. Keys 1 - Maghemite ($Fe_2O_3$) 20 nm; 2 - Magnetite ($Fe_3O_4$) 15-20 nm; 3 - Magnetite 20-30 nm; 4 - FeOOH Nanorods 50x10 nm.*

## 3.4. Application on real urine samples

On Table 12, we observe a comparison of the biomarker's quantification of real urine samples. On breast cancer (BC), it is notable that the majority of the potential cancer biomarkers have a lower amount on breast cancer, regardless if they are AA (Ala, Leu, Lys, Met, Tyr and Val) or belong the other pathways (ELAC), which is expected since cancer cells consume the metabolites in order to proliferate.

The biomarkers that don't follow that pattern are the TMAO and Trp, that have higher amounts on BC meanwhile SUCC and Ile amount remains unchanged. On the case of Ile, is important to note that this metabolite usually was below the limit of detection or wasn't present on urine, being unfit for use as a biomarker. Regarding Ala metabolite, the behaviour didn't follow what has already been reported by Simińska et al. [28].

Unlike BC, most biomarkers have higher amounts on lymphoma (LYM) than control (Met, TMAO, Trp, Tyr and Val). The biomarkers that don't follow that pattern are the Ala, ELAC and Leu, that on lymphoma have lower amounts while Ile, Lys and SUCC remain unchanged.

Is remarkable that among the different types of cancer studied, the biomarker majority (Ala, ELAC, Ile, Leu, SUCC, TMAO and Trp) follows the same behaviour. However, the biomarkers Met, Tyr and Val had a higher amount on LYM than BC meanwhile the Lys remain unchanged on LYM while on BC had a lower amount.

It is important to note that few urine samples were used, leading to a high standard deviation in all cases. To obtain more sturdy results that will lead to better observations, it is necessary to extract more urine samples.

*Table 12 - Biomarker quantification of real urine samples*

| Biomarker | | Concentration in urine samples [mM] | | | | | | | | | Status | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Control (n=4) | | | Breast cancer (BC) (n=4) | | | Lymphoma (LYM) (n=4) | | | | |
| | | Average | SD | Range | Average | SD | Range | Average | SD | Range | Control x BC | Control x LYM |
| ***Alanine*** | ***Ala*** | 0.54 | 0.32 | 0.17-0.87 | 0.32 | 0.12 | 0.16-0.45 | 0.32 | 0.13 | 0.20-0.47 | ↓ | ↓ |
| ***Ethyl Lactate*** | ***ELAC*** | 0.58 | 0.14 | 0.44-0.71 | 0.36 | 0.09 | 0.30-0.49 | 0.47 | 0.13 | 0.34-0.65 | ↓ | ↓ |
| ***Isoleucine*** | ***Ile*** | 0.01 | 0.01 | 0-0.02 | 0.01 | 0.01 | 0-0.02 | 0.01 | 0.01 | 0-0.02 | – | – |
| ***Leucine*** | ***Leu*** | 0.05 | 0.06 | 0-0.13 | 0.03 | 0.03 | 0.01-0.07 | 0.03 | 0.02 | 0.01-0.05 | ↓ | ↓ |
| ***Lysine*** | ***Lys*** | 1.22 | 0.47 | 0.58-1.69 | 1.08 | 0.63 | 0.48-1.94 | 1.22 | 0.54 | 0.71-1.99 | ↓ | – |
| ***Methionine*** | ***Met*** | 0.57 | 0.62 | 0.13-1.49 | 0.23 | 0.09 | 0.15-0.36 | 3.86 | 4 | 0.21-7.75 | ↓ | ↑ |
| ***Succinate*** | ***SUCC*** | 0.09 | 0.03 | 0.07-0.13 | 0.09 | 0.05 | 0.04-0.14 | 0.09 | 0.05 | 0.04-0.14 | – | – |
| ***Trimethylamine N-oxide*** | ***TMAO*** | 0.21 | 0.12 | 0.05-0.33 | 0.55 | 0.55 | 0.17-1.37 | 0.31 | 0.25 | 0.10-0.66 | ↑ | ↑ |
| ***Tryptophan*** | ***Trp*** | 6.21 | 3.93 | 2.94-11.8 | 8.14 | 3 | 4.34-11.03 | 10.12 | 10.36 | 4.55-25.66 | ↑ | ↑ |
| ***Tyrosine*** | ***Tyr*** | 0.27 | 0.15 | 0.07-0.43 | 0.1 | 0.02 | 0.07-0.12 | 0.37 | 0.24 | 0.12-0.58 | ↓ | ↑ |
| ***Valine*** | ***Val*** | 0.06 | 0.05 | 0-0.11 | 0.02 | 0.01 | 0.01-0.03 | 0.07 | 0.04 | 0.02-0.10 | ↓ | ↑ |

# Chapter 4. Conclusions and future work

# 4. Conclusions and future work

In this thesis, it was explored a novel use of uncoated maghemite and magnetite as nanosorbents to explore its potential to isolate urinary metabolites potential cancer biomarkers present in urine samples from breast and lymphoma cancer patients and control group. These nanosorbents, namely the 20-30 nm magnetite, showed better results when compared with common sorbents, showing a promising utility regarding the metabolite extraction of potential cancer biomarkers.

Regarding the optimization with the optimum conditions, the nanosorbent that showed the best cost-effectiveness ratio was the 20-30 nm magnetite. The ideal conditions for the extraction with this nanosorbent were: a ratio of 0.2 mg/ml to extract during 5 min at room temperature with the addition of 1 ml of UP water as elution solvent.

On case-control study, most of the biomarkers followed the same changes, regardless of the cancer type. However, it is important to note that few urine samples were used, leading to high standard deviations in all cases. In the future, it will be necessary to apply the extraction methodology optimized in this thesis on a statistically significant quantity of urine samples (control and cancer) in order to obtain more robust and confident results. There will also be the need of applying a multiple extraction test to see the retention capacity of magnetite. It would be very interesting to test the extraction of different types and stages of cancer in order to study possible significative alterations. In addition, a washing methodology for magnetite recover would be developed in order to evaluate its potential reutilization.

# References

## References:


1. Silva CL, Passos M, Câmara JS. Investigation of urinary volatile organic metabolites as potential cancer biomarkers by solid-phase microextraction in combination with gas chromatography-mass spectrometry. Br J Cancer. 2011;105:1894–904.
2. Anderson NM, Mucka P, Kern JG, Feng H. The emerging role and targetability of the TCA cycle in cancer metabolism. Protein Cell. 2018;9:216–37.
3. Siegel RL, Miller KD, Jemal A. Cancer Statistics , 2018. CA Cancer J Clin. 2018;68(1):7–30.
4. Hanly P, Soerjomataram I, Sharp L. Measuring the societal burden of cancer : The cost of lost productivity due to premature cancer-related mortality in Europe. Int J Cancer. 2015;136:136–45.
5. Union E. 1 in 4 deaths caused by cancer in the EU28. Eurostat. 2014;(November):9–12.
6. Malvezzi M, Carioli G, Bertuccio P, Boffetta P, Levi F, Vecchia C La, et al. European cancer mortality predictions for the year 2018 with focus on colorectal cancer. Ann Oncol. 2018;29:1016–22.
7. World Health Organization. Portugal Cancer statistics. Int Agency Res Cancer. 2018;270:1–2.
8. Cronin KA, Lake AJ, Scott S, Sherman RL, Noone A, Howlader N, et al. Annual Report to the Nation on the Status of Cancer , Part I : National Cancer Statistics. Cancer. 2018;124:2785–800.
9. Qiao Z, Perestrelo R, Reyes-Gallardo EM, Lucena R, Cárdenas S, Rodrigues J, et al. Octadecyl functionalized core-shell magnetic silica nanoparticle as a powerful nanocomposite sorbent to extract urinary volatile organic metabolites. J Chromatogr A. 2015;1393:18–25.
10. Levy A, Dayan A, Ben-David M, Gannot I. A new thermography-based approach to early detection of cancer utilizing magnetic nanoparticles theory simulation and in vitro validation. Nanomedicine Nanotechnology, Biol Med. 2010;6:786–96.
11. Fu L, Ke H-T. Nanomaterials incorporated ultrasound contrast agents for cancer theranostics. Cancer Biol Med. 2016;13:313–24.
12. Zhou J, Huang Z, Kumar U, Chen DDY. Review of recent developments in determining volatile organic compounds in exhaled breath as biomarkers for lung cancer diagnosis. Anal Chim Acta. 2017;996:1–9.
13. Ramos ÁG, Antón AP, Sánchez N, Luis J, Pavón P, Cordero BM. Urinary volatile fingerprint based on mass spectrometry for the discrimination of patients with lung cancer and controls. Talanta. 2017;174:158–64.
14. Guo J, Yang J, Zhang X, Feng X, Zhang H, Chen L, et al. A Panel of Biomarkers for Diagnosis of Prostate Cancer Using Urine Samples. Anticancer Res. 2018;38:1471–77.
15. Lei T, Zhao X, Jin S, Meng Q, Zhou H, Zhang M. Discovery of potential bladder cancer biomarkers by comparative urine proteomics and analysis. Clin Genitourin Cancer. 2013;11:56–62.
16. Coelho SC, Pereira MC, Juzeniene A, Juzenas P, Coelho MAN. Supramolecular nanoscale assemblies for cancer diagnosis and therapy. J Control Release. 2015;213:152–67.
17. Sheikhpour M, Golbabaie A, Kasaeian A. Carbon nanotubes: A review of novel

strategies for cancer diagnosis and treatment. Mater Sci Eng C. 2017;76:1289–304.
18. Zhang H, Chen J. Current status and future directions of cancer immunotherapy. J Cancer. 2018;9(10):1773–81.
19. Loumaye A, Thissen J. Biomarkers of cancer cachexia. Clin Biochem. 2017;50(18):1281–8.
20. Sandri M. Protein breakdown in cancer cachexia. Semin Cell Dev Biol. 2016;54:11–9.
21. Mayo SJ, Kuruvilla J, Laister RC, Ayala AP, Alm M, Byker W, et al. Blood-based biomarkers of cancer- related cognitive impairment in non- central nervous system cancer : protocol for a scoping review. BMJ Open. 2018;1–5.
22. Kosheleva OK, Lai TC, Chen NG, Hsiao M, Chen CH. Selective killing of cancer cells by nanoparticle-assisted ultrasound. J Nanobiotechnology. 2016;14(1):1–12.
23. Lima-tenório MK, Gómez EA, Ahmad NM, Fessi H, Elaissari A. Magnetic nanoparticles : In vivo cancer diagnosis and therapy. Int J Pharm. 2015;493(1–2):313–27.
24. Olesen F, Rose P, Nafees S, Rijswijk E Van, Hiom S, Muth C, et al. The Aarhus statement: improving design and reporting of studies on early cancer diagnosis Clinical Studies. Br J Cancer. 2012;106(7):1262–7.
25. Simmler C, Napolitano JG, McAlpine JB, Chen SN, Pauli GF. Universal quantitative NMR analysis of complex natural samples. Curr Opin Biotechnol. 2014;25:51–9.
26. Qiao Z, Perestrelo R, Shi X, Rodrigues J, Câmara JS. An exploratory study to evaluate the potential of nanohydroxyapatite as a powerful sorbent for efficient extraction of volatile organic metabolites, potential biomarkers of cancer. Anal Chim Acta. 2014;6:6051–7.
27. Feng S, Zheng Z, Xu Y, Lin J, Chen G, Weng C, et al. A noninvasive cancer detection strategy based on gold nanoparticle surface- enhanced Raman spectroscopy of urinary modfied nucleosides isolated by affinity chromatography. Biosens Bioelectron. 2017;91:616–22.
28. Siminska E, Marcin K. Amino acid profiling as a method of discovering biomarkers for early diagnosis of cancer. Amino Acids. 2016;48:1339–45.
29. Silva C, Cavaco C, Perestrelo R, Pereira J, Câmara JS. Microextraction by Packed Sorbent (MEPS) and Solid-Phase Microextraction (SPME) as Sample Preparation Procedures for the Metabolomic Profiling of Urine. Metabolites. 2014;4:71–97.
30. Hai-wei GU, Yun-peng QI, Ning XU, Jian-hua D, Yan-bo AN, Huan-wen C. Nuclear Magnetic Resonance Spectroscopy and Mass Spectrometry-Based Metabolomics for Cancer Diagnosis. Chinese J Anal Chem. 2012;40:1933–7.
31. Lubes G, Goodarzi M. GC–MS based metabolomics used for the identification of cancer volatile organic compounds as biomarkers. J Pharm Biomed Anal. 2018;147:313–22.
32. Neal RD, Tharmanathan P, France B, Din NU, Cotton S, Fallon-Ferguson J, et al. Is increased time to diagnosis and treatment in symptomatic cancer associated with poorer outcomes? Systematic review. Br J Cancer. 2015;112:S92–107.
33. Dutheil F, Rouanet L, Mulliez A, Naughton G, Fontana L, Druet-cabanac M, et al. Urine cytology screening of French workers exposed to occupational urinary tract carcinogens : a prospective cohort study over a 20-year period. BMJ Open. 2017;7:1–10.

34. Mariappan L, Shao Q, Jiang C, Yu K, Ashkenazi S, Bischof JC, et al. Magneto acoustic tomography with short pulsed magnetic field for in-vivo imaging of magnetic iron oxide nanoparticles. Nanomedicine Nanotechnology, Biol Med. 2016;12:689–99.
35. Anttila A, Lo S, Ponti A, Suonio E, Villain P, Willem J, et al. Towards better implementation of cancer screening in Europe through improved monitoring and evaluation and greater engagement of cancer registries. Eur J Cancer. 2015;51:241–51.
36. Giordano L, Bisanti L, Salamina G, Park RA. The EUROMED CANCER network : state-of-art of cancer screening programmes in non-EU Mediterranean countries. Eur J Public Health. 2015;26:83–9.
37. Manig F, Kuhne K, Neubeck C Von, Schwarzenbolz U, Yu Z, Kessler BM, et al. The why and how of amino acid analytics in cancer diagnostics and therapy. J Biotechnol. 2017;242:30–54.
38. Carrola J, Rocha M, Barros S, Gil AM, Goodfellow BJ, Carreira IM, et al. Metabolic Signatures of Lung Cancer in Biofluids : NMR-Based Metabonomics of Urine research articles. J Proteome Res. 2011;10:221–30.
39. Heiden MG Vander, Deberardinis RJ. Review Understanding the Intersections between Metabolism and Cancer Biology. Cell. 2017;168:657–69.
40. Kalyanaraman B. Teaching the basics of cancer metabolism : Developing antitumor strategies by exploiting the differences between normal and cancer cell metabolism. Redox Biol. 2017;12:833–42.
41. Liao Y. Cancer metabolism as we know it today A prologue to a special issue of Cancer Metabolism. Genes Dis. 2017;4:4–6.
42. Kaushik AK, Deberardinis RJ. Applications of metabolomics to study cancer metabolism ☆. Biochim Biophys Acta - Rev Cancer. 2018;1870:2–14.
43. Sreedhar A, Zhao Y. Dysregulated metabolic enzymes and metabolic reprogramming in cancer cells ( Review ). Biomed REPORTS. 2018;8:3–10.
44. Jiang B. Aerobic glycolysis and high level of lactate in cancer metabolism and microenvironment. Genes Dis. 2017;4:25–7.
45. Lukey MJ, Katt WP, Cerione RA. Targeting amino acid metabolism for cancer therapy. Drug Discov Today. 2017;22:796–804.
46. Kato Y, Maeda T, Suzuki A, Bada Y. Cancer metabolism: New insights into classic characteristics. Jpn Dent Sci Rev. 2018;54:8–21.
47. Danhier P, Ba P, Payen VL, Grasso D, Ippolito L, Sonveaux P, et al. Cancer metabolism in space and time : Beyond the Warburg effect ☆. Biochim Biophys Acta. 2017;1858:556–72.
48. Frezza C, Gottlieb E. Seminars in Cancer Biology Mitochondria in cancer : Not just innocent bystanders. Semin Cancer Biol. 2009;19:4–11.
49. Cheng F, Wang Z, Huang Y, Duan Y, Wang X. Investigation of salivary free amino acid profile for early diagnosis of breast cancer with ultra performance liquid chromatography-mass spectrometry. Clin Chim Acta. 2015;447:23–31.
50. Gu Y, Chen T, Fu S, Sun X, Wang L, Wang J, et al. Perioperative dynamics and significance of amino acid profiles in patients with cancer. J Transl Med. 2015;13:35.
51. Song Y, Xu C, Kuroki H, Liao Y, Tsunoda M. Recent trends in analytical methods for the determination of amino acids in biological samples. J Pharm Biomed Anal.

2018;147:35–49.
52. Yongli Y. Study on the new Analytical Method of Tyrosine and its Metabolites in Urine from Breast Cancer patients. Nanchang University; 2017.
53. Kim HJ, Jang SH, Ryu J-S, Lee JE, Kim YC, Lee MK, et al. The performance of a novel amino acid multivariate index for detecting lung cancer: A case control study in Korea. Lung Cancer. 2015;90:522–7.
54. Fukutake N, Ueno M, Hiraoka N, Shimada K. A Novel Multivariate Index for Pancreatic Cancer Detection Based On the Plasma Free Amino Acid Profile. PLoS ONE. 2015;1–15.
55. Madssen TS, Euceda LR, Giskeødegård GF, Moestue SA, Tessem MB. NMR - based metabolomics of biofluids in cancer. NMR Biomed. 2018; p. e3927.
56. López-lópez Á, López-gonzálvez Á, Clive-baker T, Barbas C. A review of validated biomarkers obtained through metabolomics. Expert Rev Mol Diagn. 2018;18:557–75.
57. Dona AC, Kyriakides M, Scott F, Shephard EA, Varshavi D, Veselkov K, et al. A guide to the identification of metabolites in NMR-based metabonomics/metabolomics experiments. Comput Struct Biotechnol J. 2016;14:135–53.
58. Yang Q, Qiu H, Guo W, Wang D, Zhou X, Xue D, et al. Quantitative $^{1}$H-NMR method for the determination of tadalafil in bulk drugs and its tablets. Molecules. 2015;20:12114–24.
59. Zhang J, Bowers J, Liu L, Wei S, Gowda GAN, Hammoud Z, et al. Esophageal Cancer Metabolite Biomarkers Detected by LC-MS and NMR Methods. PLoS One. 2012;7: e30181.
60. Agin A, Heintz D, Ruhland E, Barca JMC De, Zumsteg J, Moal V, et al. Metabolomics – an overview . From basic principles to potential biomarkers ( part 1 ). Med Nucl. 2016;40:4–10.
61. Bujak R, Struck-lewicka W, Markuszewski MJ, Kaliszan R. Metabolomics for laboratory diagnostics. J Pharm Biomed Anal. 2015;113:108–20.
62. Emwas A, Roy R, Mckay RT, Ryan D, Brennan L, Tenori L, et al. Recommendations and Standardization of Biomarker Quanti fi cation Using NMR-Based Metabolomics with Particular Focus on Urinary Analysis. J Proteome Res. 2016;15:360–73.
63. Smolinska A, Blanchet L, Buydens LMC, Wijmenga SS. NMR and pattern recognition methods in metabolomics : From data acquisition to biomarker discovery : A review. Anal Chim Acta. 2012;750:82–97.
64. Wang Z, Lin Y, Liang J, Huang Y, Ma C, Liu X, et al. NMR-based metabolomic techniques identify potential urinary biomarkers for early colorectal cancer detection. Oncotarget. 2017;8:105819–31.
65. Emwas A, Luchinat C, Turano P, Tenori L, Roy R, Salek RM, et al. Standardizing the experimental conditions for using urine in NMR-based metabolomic studies with a particular focus on diagnostic studies : a review. Metabolomics. 2015;11:872–94.
66. Human Metabolome Database (HMDB).
67. Eghbalnia HR, Romero PR, Westler WM, Baskaran K, Ulrich EL, Markley JL. Increasing rigor in NMR-based metabolomics through validated and open source tools. Curr Opin Biotechnol. 2017;43:56–61.
68. M. Cassiède, P. Mercier PS et al. Comparison of computational approaches for

identification and quantification of urinary metabolites in 1H NMR spectra. Anal Methods. 2018;10:2129–37.
69. Ranjan R, Sinha N. Nuclear magnetic resonance ( NMR ) - based metabolomics for cancer research. 2018;19:1–21.
70. Edison AS, Markley JL, Bru R, Eghbalnia HR, Powers R, Raftery D, et al. The future of NMR-based metabolomics. Curr Opin Biotechnol. 2017;43:34–40.
71. Kostidis S, Addie RD, Morreau H, Mayboroda OA, Giera M. Quantitative NMR analysis of intra- and extracellular metabolism of mammalian cells : A tutorial. Anal Chim Acta. 2017;980:1–24.
72. Zhou J, Wang Y, Zhang X. Metabonomics studies on serum and urine of patients with breast cancer using 1 H-NMR spectroscopy. Oncotarget. 2017;1–9.
73. Resina MR. IDENTIFICATION OF NEW URINE BIOMARKERS FOR THE DETECTION OF PROSTATE CANCER. Universitat Autònoma de Barcelona (UaB); 2011.
74. Silva CL, Passos M, Câmara JS. Solid phase microextraction, mass spectrometry and metabolomic approaches for detection of potential urinary cancer biomarkers - A powerful strategy for breast cancer diagnosis. Talanta. 2012;89:360–8.
75. Wu D, Ni J, Beretov J, Cozzi P, Willcox M, Wasinger V, et al. Urinary biomarkers in prostate cancer detection and monitoring progression. Crit Rev Oncol Hematol. 2017;118:15–26.
76. Capelli L, Taverna G, Bellini A, Eusebio L, Buffi N, Lazzeri M, et al. Application and Uses of Electronic Noses for Clinical Diagnosis on Urine Samples : A Review. Sensors. 2016;16:1–24.
77. Zvarik M, Martinicky D, Hunakova L, Lajdova I, Sikurova L. Fluorescence characteristics of human urine from normal individuals and ovarian cancer patients. Neoplasma. 2013;60:1779–87.
78. Rigau M, Olivan M, Garcia M, Sequeiros T, Montes M, Colás E, et al. The present and future of prostate cancer urine biomarkers. Int J Mol Sci. 2013;14:12620–49.
79. Bianchi F, Chiesi V, Casoli F, Luches P, Nasi L, Careri M, et al. Magnetic solid-phase extraction based on diphenyl functionalization of Fe3O4 magnetic nanoparticles for the determination of polycyclic aromatic hydrocarbons in urine samples. J Chromatogr A. 2012;1231:8–15.
80. Wang L, Skotland T, Berge V, Sandvig K, Llorente A. Exosomal proteins as prostate cancer biomarkers in urine: From mass spectrometry discovery to immunoassay-based validation. Eur J Pharm Sci. 2017;98:80–5.
81. Maraldo D, Garcia FU, Mutharasan R. Method for quantification of a prostate cancer biomarker in urine without sample preparation. Anal Chem. 2007;79(20):7683–90.
82. Skotland T, Ekroos K, Kauhanen D, Simolin H, Seierstad T, Berge V, et al. Molecular lipid species in urinary exosomes as potential prostate cancer biomarkers. Eur J Cancer. 2017;70:122–32.
83. Xylinas E, Kluth LA, Rieken M, Karakiewicz PI, Lotan Y, Shariat SF. Urine markers for detection and surveillance of bladder cancer. Urol Oncol Semin Orig Investig. 2014;32:222–9.
84. Tilki D, Burger M, Dalbagni G, Grossman HB, Hakenberg OW, Palou J, et al. Urine markers for detection and surveillance of non-muscle-invasive bladder cancer. Eur Urol. 2011;60:484–92.

85. Smalley DM, Sheman NE, Nelson K, Theodorescu D. Isolation and identification of potential urinary microparticle biomarkers of bladder cancer. J Proteome Res. 2008;7:2088–96.
86. Duriez E, Masselon CD, Mesmin C, Court M, Demeure K, Allory Y, et al. Large-Scale SRM Screen of Urothelial Bladder Cancer Candidate Biomarkers in Urine. J Proteome Res. 2017;16:1617–31.
87. Yosef HK, Krauß SD, Lechtonen T, Jütte H, Tannapfel A, Käfferlein HU, et al. Noninvasive Diagnosis of High-Grade Urothelial Carcinoma in Urine by Raman Spectral Imaging. Anal Chem. 2017;89:6893–9.
88. Chou R, Gore JL, Buckley D, Fu R, Gustafson K, Griffin JC, et al. Urinary biomarkers for diagnosis of bladder cancer: A systematic review and meta-analysis. Ann Intern Med. 2015;163:922–31.
89. Zhang C, Leng W, Sun C, Lu T, Chen Z, Men X, et al. Urine Proteome Profiling Predicts Lung Cancer from Control Cases and Other Tumors. EBioMedicine. 2018;30:120–8.
90. Beretov J, Wasinger VC, Millar EKA, Schwartz P, Graham PH, Li Y. Proteomic analysis of urine to identify breast cancer biomarker candidates using a label-free LC-MS/MS approach. PLoS One. 2015;10:1–21.
91. Qiu Y, Cai G, Su M, Chen T, Liu Y, Xu Y, et al. Urinary metabonomic study on colorectal cancer. J Proteome Res. 2010;9:1627–34.
92. Dai Z. Nanomedicine has opened new avenues for cancer diagnosis and therapy. Cancer Biol Med. 2016;13:297–8.
93. Kandasamy G, Maity D. Recent advances in superparamagnetic iron oxide nanoparticles (SPIONs) for in vitro and in vivo cancer nanotheranostics. Int J Pharm. 2015;496:191–218.
94. Pyrzynska K. Use of nanomaterials in sample preparation. Trends Anal Chem. 2013;43:100–8.
95. Brigger I, Dubernet C, Couvreur P. Nanoparticles in cancer therapy and diagnosis☆. Adv Drug Deliv Rev. 2012;64:24–36.
96. Aftab S, Shah A, Nadhman A, Kurbanoglu S, Ays S. Nanomedicine : An effective tool in cancer therapy. 2018;540:132–49.
97. Gawali SL, Dutta B, Barick KC, Hassan PA. Surface Engineering of Iron Oxide Nanoparticles for Cancer Therapy. Biomed Res J. 2017;4:49–66.
98. Cole AJ, Yang VC, David AE. Cancer theranostics : the rise of targeted magnetic nanoparticles. Trends Biotechnol. 2011;29:323–32.
99. Bayford R, Rademacher T, Roitt I, Wang SX. Emerging applications of nanotechnology for diagnosis and therapy of disease : a review Emerging applications of nanotechnology for diagnosis and therapy of disease : a review. Physiol Meas. 2017;38.
100. Dinali R, Ebrahiminezhad A, Manley-harris M, Ghasemi Y, Berenjian A. Iron oxide nanoparticles in modern microbiology and biotechnology. Crit Rev Microbiol. 2017;43:493–507.
101. Tian J, Xu J, Zhu F, Lu T, Su C, Ouyang G. Application of nanomaterials in sample preparation. J Chromatogr A. 2013;1300:2–16.
102. Ahmadi M, Elmongy H, Madrakian T. Nanomaterials as sorbents for sample preparation in bioanalysis : A review. Anal Chim Acta. 2017;958:1–21.
103. Hartshorn CM, Bradbury MS, Lanza GM, Nel AE, Rao J, Wang AZ, et al.

Nanotechnology Strategies To Advance Outcomes in Clinical Cancer Care. ACS Nano. 2018;12:24–43.
104. Ríos Á, Zougagh M. Recent advances in magnetic nanomaterials for improving analytical processes. TrAC - Trends Anal Chem. 2016;84:72–83.
105. Yousaf MZ, Yang-Long H, Song G. Magnetic nanoparticle-based cancer nanodiagnostics. Chinese Phys B. 2013;22: 0275506.
106. Shubayev VI, Pisanic TR, Jin S. Magnetic nanoparticles for theragnostics ☆. Adv Drug Deliv Rev. 2009;61:467–77.
107. Castillo-García M., Aguilar-Caballos M., Gómez-Hens A. Nanomaterials as tools in chromatographic methods. Trends Anal Chem. 2016;82:385–93.
108. Meng X, He Z, Zhao J, Lin Y, Liu X. Oleic Acid Surface Modification in the Preparation of Magnetic Nanoparticles by a Chemically Induced Transition. IEEE Trans Magn. 2018;54: A2041.
109. Lin J-H, Wu Z-H, Tseng W-L. Extraction of environmental pollutants using magnetic nanomaterials. Anal Methods. 2010;2:1829–2056.
110. Xie L, Jiang R, Zhu F, Liu H. Application of functionalized magnetic nanoparticles in sample preparation. Anal Bioanal Chem. 2014;406:377–99.
111. Bagheri H, Afkhami A, Saber-tehrani M, Khoshsafar H. Preparation and characterization of magnetic nanocomposite of Schiff base / silica / magnetite as a preconcentration phase for the trace determination of heavy metal ions in water , food and biological samples using atomic absorption spectrometry. Talanta. 2012;97:87–95.
112. Giakisikli G, Anthemidis AN. Magnetic materials as sorbents for metal / metalloid preconcentration and / or separation . A review. Anal Chim Acta. 2013;789:1–16.
113. Coral DF. Una guía para el estudio de nanopartículas magnéticas de óxidos de hierro con aplicaciones biomédicas . Parte I. Ing y Cienc. 2017;13:229–49.
114. He J, Huang M, Wang D, Zhang Z, Li G. Magnetic separation techniques in sample preparation for biological analysis : A review. J Pharm Biomed Anal. 2014;101:84–101.
115. Biehl P, Lühe M Von Der, Dutz S, Schacher FH. Synthesis, Characterization, and Applications of Magnetic Nanoparticles Featuring Polyzwitterionic Coatings. Polymers (Basel). 2018;10:91.
116. Hajba L, Guttman A. The use of magnetic nanoparticles in cancer theranostics: Toward handheld diagnostic devices. Biotechnol Adv. 2016;34:354–61.
117. Xu C, Sun S. New forms of superparamagnetic nanoparticles for biomedical applications ☆. Adv Drug Deliv Rev. 2013;65:732–43.
118. Wu M, Huang S. Magnetic nanoparticles in cancer diagnosis , drug delivery and treatment ( Review ). Mol Clin Oncol. 2017;7:738–46.
119. Xu J, Sun J, Wang Y, Sheng J, Wang F, Sun M. Application of Iron Magnetic Nanoparticles in Protein Immobilization. Molecules. 2014;19:11465–86.
120. Issa B, Obaidat IM, Albiss BA, Haik Y. Magnetic Nanoparticles : Surface Effects and Properties Related to Biomedicine Applications. Int J Mol Sci. 2013;14:21266–305.
121. Teja AS, Koh P. Synthesis , properties , and applications of magnetic iron oxide nanoparticles. Prog Cryst Growth Charact Mater. 2009;55:22–45.
122. Lassoued A, Saber M, Dkhil B, Ammar S. Synthesis , structural , morphological , optical and magnetic characterization of iron oxide ( α -Fe 2 O 3 ) nanoparticles

by precipitation method : Effect of varying the nature of precursor. Phys E Low-dimensional Syst Nanostructures. 2018;97:328–34.
123. Ruusunen J, Ihalainen M, Koponen T, Torvela T. Controlled oxidation of iron nanoparticles in chemical vapour synthesis. J Nanoparticle Res. 2014;16:2270.
124. Ansari OM, Ahmad MF, Shadab GGHA, Siddique HR. Superparamagnetic iron oxide nanoparticles based cancer theranostics : A double edge sword to fight against cancer. J Drug Deliv Sci Technol. 2018;45:177–83.
125. Ali A, Zafar H, Zia M, Phull AR, Ali JS, Hussain A. Synthesis, characterization, applications, and challenges of iron oxide nanoparticles. Nanotechnol Sci Appl. 2016;9:49–67.
126. Chen Z. Synthesis of monodisperse organic- and water-soluble iron oxide nanoparticles using Fe ( OH ) 3 as precursor. J EXP NANOSCI. 2014;9:406–14.
127. Meng Q, Liu J, Zhang C, Wang Z, Liu J, Wang Z. Chemical Synthesis and Characterization of Magnetic Nanoparticles. In: International Conference on Manipulation, Manufacturing and Measurement on the Nanoscale (3M-NANO). Changchun; 2015. p. 12–5.
128. Belaïd S, Stanicki D, Elst L Vander, Muller RN, Laurent S. Influence of experimental parameters on iron oxide nanoparticle properties synthesized by thermal decomposition : size and nuclear magnetic resonance studies. Nanotechnology. 2018;29:16.
129. Khmara I, Kubovcikova M, Koneracka M, Kalska-szostko B, Zavisova V. Preparation and Characterization of Magnetic Nanoparticles. ACTA Phys Pol A. 2018;133:704–7.
130. Budime P, Poklar N. Multifunctional superparamagnetic iron oxide nanoparticles : Promising tools in cancer theranostics. Cancer Lett. 2013;336:8–17.
131. Bakhtiary Z, Saei AA, Hajipour MJ, Raoufi M, Vermesh O, Mahmoudi M. Targeted superparamagnetic iron oxide nanoparticles for early detection of cancer : Possibilities and challenges. Nanomedicine Nanotechnology, Biol Med. 2016;12:287–307.
132. Gallo J, Long J, Aboagye EO, Gallo J. Magnetic nanoparticles as contrast agents in the diagnosis and treatment of cancer. Chem Soc Rev. 2013;42:7816–33.
133. Tombácz E, Turcu R, Socoliuc V, Vékás L. Magnetic iron oxide nanoparticles : Recent trends in design and synthesis of magnetoresponsive nanosystems. Biochem Biophys Res Commun. 2015;468:442–53.
134. Maleki H, Simchi A, Imani M, Costa BFO. Size-controlled synthesis of superparamagnetic iron oxide nanoparticles and their surface coating by gold for biomedical applications. J Magn Magn Mater. 2012;324:3997–4005.
135. Ruggiero MR, Geninatti S, Sieni E, Sgarbossa P. Iron oxide / PLGA nanoparticles for magnetically controlled drug release. Int J Appl Electromagn Mech. 2017;53:53–61.
136. Xing L, Brink GH, Chen B, Schmidt FP, Haberfehlner G, Hofer F, et al. Synthesis and morphology of iron – iron oxide core – shell nanoparticles produced by high pressure gas condensation. Nanotechnology. 2016;27:215703.
137. Li L, Mak KY, Leung CW, Leung CH, Ruotolo A, Chan KY, et al. Synthesis and Morphology Control of Gold/Iron Oxide Magnetic Nanocomposites via a Simple Aqueous Method. IEEE Trans Magn. 2014;50:2300505.
138. Yang F, Long Y, Shen R, Chen C, Pan D, Zhang Q, et al. Ultrasonication extraction

coupled with magnetic solid-phase clean-up for the determination of polycyclic aromatic hydrocarbons in soils by high-performance liquid chromatography. J Sep Sci. 2011;34:716–23.
139. Liu J, Yuan B, Feng Y. Determination of hexanal and heptanal in human urine using magnetic solid phase extraction coupled with in - situ derivatization by high performance liquid chromatography. Talanta. 2015;136:54–9.
140. Jadhav SA, Patil S V. Facile synthesis of magnetic iron oxide nanoparticles and their characterization. Front Mater Sci. 2014;8:193–8.
141. Lodhia J, Mandarano G, Ferris NJ, Eu P, Cowell SF. Development and use of iron oxide nanoparticles (Part 1): Synthesis of iron oxide nanoparticles for MRI. Biomed Imaging Interv. 2010;6:e2.
142. Coral DF. Una guía para el estudio de nanopartículas magnéticas de óxidos de hierro con aplicaciones biomédicas . Parte II. Ing y Cienc. 2017;13(26):207–32.
143. Chaki SH, Malek TJ, Chaudhary MD, Tailor JP, Deshpande MP. Magnetite Fe3O4 nanoparticles synthesis by wet chemical reduction and their characterization. Adv Nat Sci Nanosci Nanotechnol. 2015;6:035009
144. Gao Q, Luo D, Bai M, Chen Z, Feng Y. Rapid Determination of Estrogens in Milk Samples Based on Magnetite Nanoparticles / Polypyrrole Magnetic Solid-Phase Extraction Coupled with Liquid Chromatography À Tandem Mass Spectrometry. J Agric Food Chem. 2011;59:8543–9.
145. Bruker. What is NMR. 2013;
146. Bruker. What is NMR? Nuclear Magnetic Resonance. Bruker. 2015;8.
147. Bharti SK, Roy R. Quantitative 1H NMR spectroscopy. TrAC - Trends Anal Chem. 2012;35:5–26.
148. Sigma-Aldrich. QUANTITATIVE NMR - Technical Details and TraceCERT® Certified Reference Materials.
149. Hamid A, Edoardo E, Xin S, Ryan G, Vitor TM, Martins AP, et al. Recommended strategies for spectral processing and post-processing of 1D 1 H-NMR data of biofluids with a particular focus on urine. Metabolomics. 2018;14:1–23.
150. Sokolenko S, Mckay R, Chang D, George B, Aucoin MG. Understanding the variability of compound quantification from targeted profiling metabolomics of 1D- 1 H-NMR spectra in synthetic mixtures and urine with additional insights on choice of pulse sequences and robotic sampling. Metabolomics. 2013;9:887–903.
151. US Research Nanomaterials I. Iron Oxide Nanopowder / Nanoparticles (Fe2O3, gamma, high purity, 99.5+%, 20 nm)
152. US Research Nanomaterials I. Iron Oxide Nanopowder / Nanoparticles (Fe3O4, high purity, 99.5+%, 15-20 nm)
153. US Research Nanomaterials I. Iron Oxide Fe3O4 Nanopowder / Nanoparticles (Fe3O4, 98+%, 20-30 nm)
154. US Research Nanomaterials I. Fe(III) Oxide Hydroxide Nanorods / Yellow Iron Oxide Nanorods / FeOOH Nanorods (Fe2O3H2O, alpha, 98%, 50nmx10nm).
155. World Medical Association. World Medical Association Declaration of Helsinki. JAMA. 2013 Nov 27;310:2191–4.
156. Wilsenach JA, Schuurbiers CAH, van Loosdrecht MCM. Phosphate and potassium recovery from source separated urine through struvite precipitation. Water Res. 2007;41:458–66.

# Annexes

## A. Poster presented at MAD-Nano 18


Figure 1A – Poster presented at MAD-Nano 18 – 30th of November to 2nd of December 2018 – Madeira island, Portugal. *Mariana P Santos, Catarina Silva, Ana Olival, João Rodrigues & José S Câmara*, Exploratory evaluation of the potential of magnetic NPs as powerful sorbents for extraction of cancer biomarker

## B. Supplementary data regarding the nanoparticle 1 (maghemite 20 nm) amount

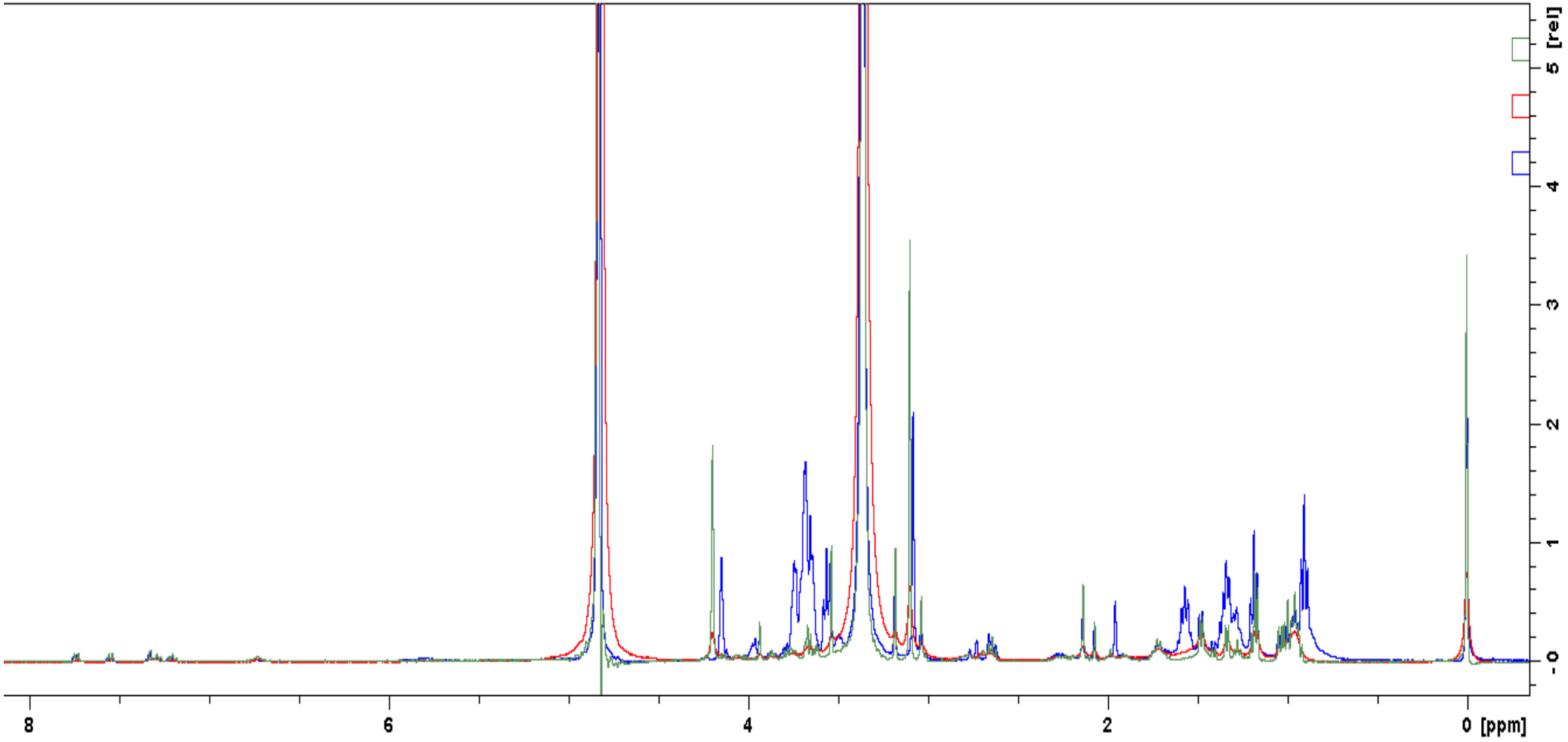



*Figure 2A - $^{1}H$ NMR spectra of potential cancer biomarkers after extraction (n=3) from SU with 20 mg of maghemite 20 nm (nanoparticle 1) in $D_2O$.*

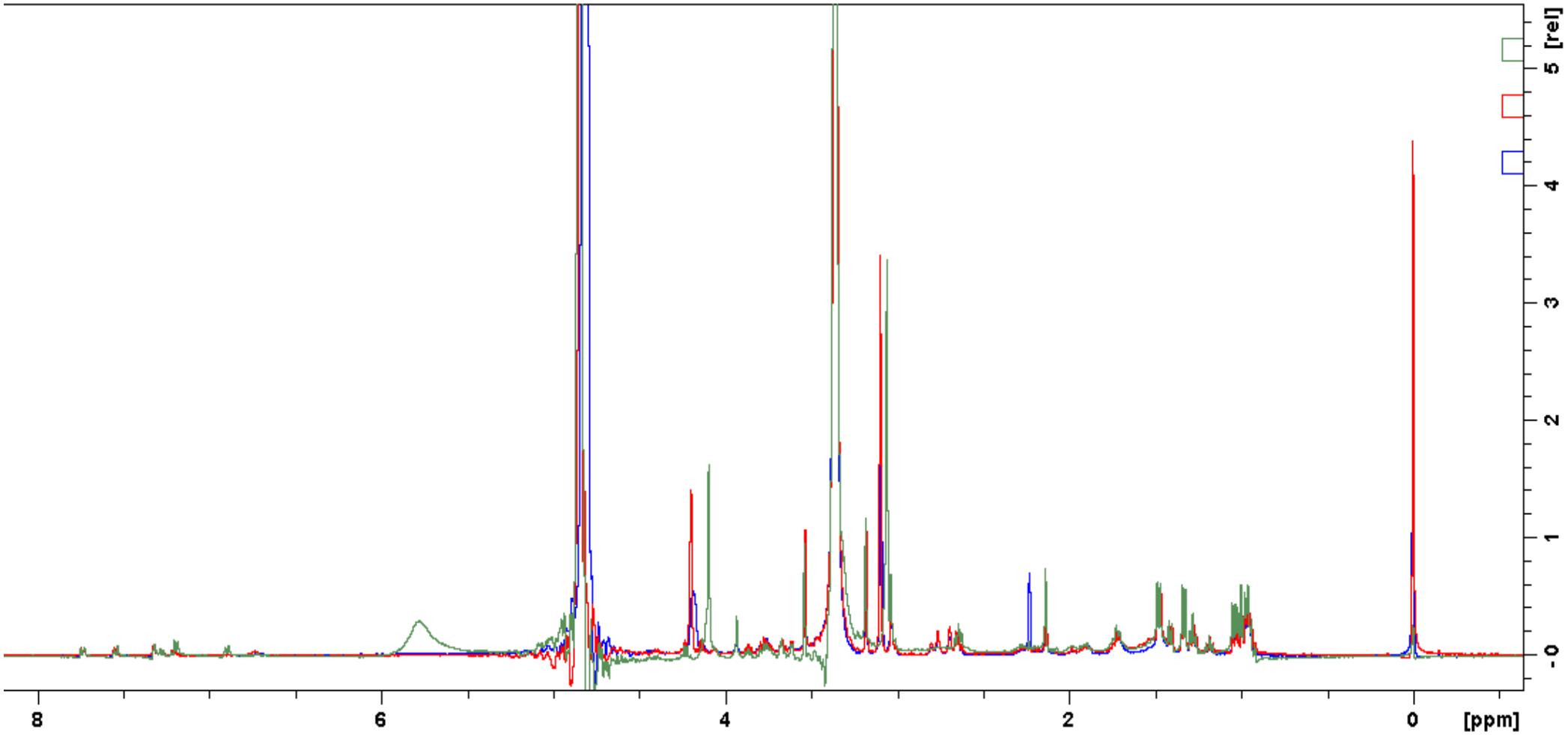



*Figure 3A - $^{1}H$ NMR spectra of potential cancer biomarkers after extraction (n=3) from SU with 5 mg of maghemite 20 nm (nanoparticle 1) in $D_2O$.*

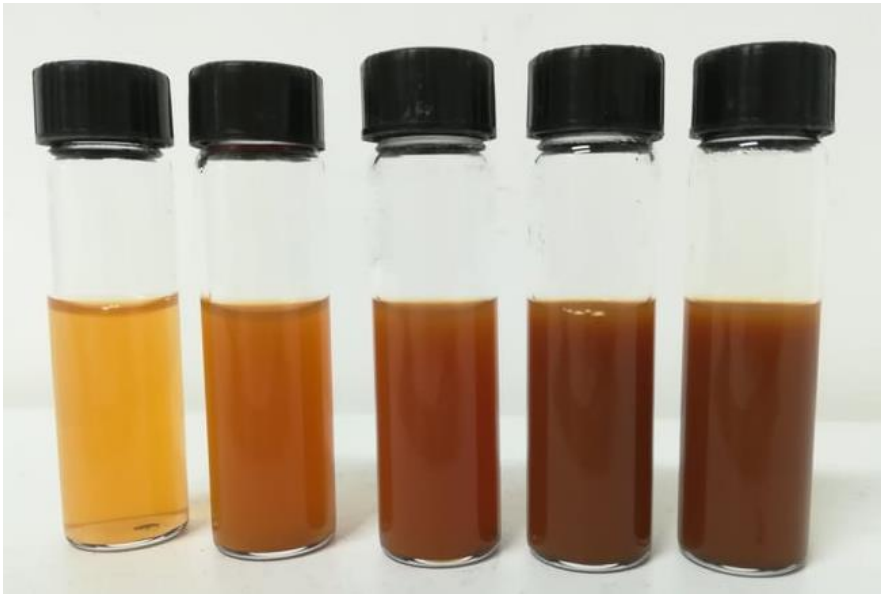


*Figure 4A – Relation of the maghemite different amount (1 mg, 2.5 mg, 5 mg, 10 mg and 20 mg) on different samples.*




UID/QUI/00674/2013



CQM +M1420-01-0145-FEDER-000005



Co-financed by: