Universidade de Aveiro

Departamento de Química

2015/2016

Sónia Isabel Neto Pedro

Concentração e purificação de biomarcadores do cancro da próstata visando um diagnóstico precoce

Concentration and purification of prostate cancer biomarkers envisaging an early diagnosis

Universidade de Aveiro

Departamento de Química

2015/2016

Sónia Isabel Neto Pedro

Concentração e purificação de biomarcadores do cancro da próstata visando um diagnóstico precoce

Concentration and purification of prostate cancer biomarkers envisaging an early diagnosis

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre de Bioquímica, ramo de Bioquímica Clínica, realizada sob a orientação científica da Doutora Mara Guadalupe Freire Martins, Investigadora Coordenadora do Departamento de Química, CICECO, da Universidade de Aveiro, e co-orientação do Professor Doutor João Manuel da Costa Araújo e Pereira Coutinho, Professor Catedrático do Departamento de Química da Universidade de Aveiro.

“O valor de uma ideia está na utilização da mesma”

Thomas Edison

O júri

presidente

Prof. Dr. Pedro Miguel Dimas Neves Domingues
Professor Auxiliar com Agregação do Departamento de Química da Universidade de Aveiro

Drª. Mara Guadalupe Freire Martins
Investigadora Coordenadora do Departamento de Química, CICECO, da Universidade de Aveiro

Drª. Ana Margarida Nunes da Mata Pires de Azevedo
Investigadora Auxiliar no Departamento de Bioengenharia do Instituto Superior Técnico da Universidade de Lisboa

Agradecimentos

Gostaria de começar por agradecer aos meus orientadores, a Drª. Mara Freire, e o Prof. Dr. João Coutinho pelo notável acompanhamento, e pela aprendizagem que esta tese me proporcionou, agradeço igualmente disponibilidade e atenção que tiveram comigo ao longo do meu trabalho e principalmente o incentivo que me deram ao longo deste ano. Quero agradecer aos membros do Path, por toda ajuda e atenção.

Quero agradecer especialmente ao Matheus, por disponibilizar todo o seu tempo e paciência para me ajudar incondicionalmente, quero agradecer-lhe por ter acreditado em mim desde sempre e por me dar as palavras certas quando mais preciso, por me transmitir calma e pelo que me ensinou nesta tese e não só, por me motivar todos os dias a ser melhor, mas especialmente por ser aquilo preciso, amigo.

Quero agradecer com todo o carinho, à minha família de Aveiro, os meus amigos, que me apoiam em tudo e são pessoas extraordinárias, que me fazem sentir em casa todos os dias. Á Inês, por tudo, por ser como uma irmã, por fazer os meus dias, no laboratório e fora dele, os melhores, por estar sempre presente quando preciso. Quero agradecer aos quatro Joões da minha vida, por terem cada um na sua maneira um lugar especial no meu coração e pelo sorriso no rosto que me colocam todos os dias. Em especial, ao David João, que é das melhores pessoas que tenho, pelo apoio incondicional que me dá, pelo carinho, por me fazer acreditar no melhor e por tornar os dias sempre mais bonitos. E por último, à minha família de sangue, que eu tanto adoro. O meu maior agradecimento aos meus pais são eles que me dão força para tudo, agradeço por me terem apoiado sempre, por me fazerem querer ser melhor todos os dias, pelos conselhos e por me colocarem um sorriso no rosto em qualquer altura, agradeço especialmente pelo amor e carinho incondicional que me dão, que me faz tão feliz. Por serem o meu maior exemplo e por me fazerem acreditar que tudo é possível. Obrigada, por estarem presentes em cada segundo deste percurso. Um dia serei tão grande quanto vocês.

Palavras-chave

Cancro da próstata, marcadores tumorais, lactato desidrogenase, antigénio prostático específico, sistemas aquosos bifásicos, concentração, purificação, líquidos iónicos

Resumo

O cancro da próstata representa nos dias de hoje a terceira causa de morte mais comum entre os homens, sendo que, atualmente, não existe nenhum tratamento eficaz quando o tumor é diagnosticado já num estado avançado. Tendo em conta as elevadas taxas de mortalidade associadas ao cancro, a identificação e quantificação de biomarcadores tumorais em fluidos e tecidos humanos têm sido alvo de uma investigação intensa no sentido de efetuar diagnósticos mais fiáveis e evitar tratamentos invasivos excessivos. Alguns biomarcadores tumorais, como o antigénio prostático específico (PSA) amplamente utilizado para o diagnóstico do cancro da próstata, podem ser avaliados em conjunto com outros biomarcadores relevantes, surgindo neste sentido a identificação e quantificação da enzima lactato desidrogenase (LDH), que recentemente tem sido sugerida como uma excelente ferramenta de prognóstico e monitorização do tratamento do mesmo tipo de cancro. Tendo em conta que ambos os biomarcadores são proteínas e que os fluidos humanos são matrizes muito complexas, os métodos atuais de identificação e quantificação apresentam pouca seletividade e podem conduzir a falsos positivos ou negativos. Neste sentido, torna-se fundamental desenvolver alternativas eficientes para a identificação e quantificação de vários biomarcadores tumorais presentes na mesma amostra. Para este efeito, avaliaram-se sistemas aquosos bifásicos (SAB) constituídos por líquidos iónicos (LIs) como uma técnica alternativa de extração e purificação de PSA e LDH a partir de fluídos biológicos sintéticos e reais. Neste trabalho identificaram-se SAB promissores capazes de extrair e concentrar PSA a partir de amostras de urina, e SAB capazes de extrair e purificar LDH a partir de amostras de soro humano, num único passo, permitindo portanto a sua identificação e quantificação de ambos os biomarcadores em fluidos humanos por métodos analíticos mais expeditos, tal como cromatografia líquida de alta eficiência por exclusão molecular (SE-HPLC).

Keywords Prostate cancer, cancer biomarkers, lactate dehydrogenase, prostate specific antigen, aqueous biphasic systems, concentration, purification, ionic liquids

Abstract

Nowadays, prostate cancer is the third most common cause of death among men. Currently, there is no effective treatment when the tumor is diagnosed at an advanced stage. Given the high mortality rates associated with cancer, the identification and quantification of tumor biomarkers in human fluids and tissues have been the subject of intense research aiming more reliable diagnoses and to avoid excessive invasive treatments. In recent years, a special interest was also raised on the simultaneous analysis of several biomarkers for the same purpose, *i.e.*, in order to make a more accurate diagnosis. Therefore, certain tumor markers, such as prostate specific antigen (PSA) widely used for the diagnosis of prostate cancer, should be considered together with other relevant biomarkers, like lactate dehydrogenase (LDH), which has recently been indicated as an excellent prognostic and monitoring tool of treatment of the same type of cancer. Considering the current methods for the quantification and purification of these biomarkers, and since both are proteins and are present in complex biological media, they usually exhibit low selectivity and may lead to false positives or negatives. In this context, it is crucial to develop efficient and selective analytical methods for the identification and quantification of several tumor biomarkers present in the same biological sample. For this purpose, aqueous biphasic systems (ABS) composed of ionic liquids (ILs) were investigated as an alternative extraction and purification technique for these biomarkers from synthetic and real biological fluids. In this work, novel ABS capable of extracting and concentrating PSA from human urine, and SAB capable of extracting and purifying LDH from human serum, in a single-step, were identified, allowing thus the identification and quantification of both biomarkers in biological fluids using more expedite analytical equipment, such as size-exclusion high-performance liquid chromatography (SE-HPLC).

Index

1. General Introduction 1

1.1 Scope and objetives 3

1.2 Cancer update overview 4

1.2.1 Epidemiology 4

1.2.2 Cancer: molecular mechanisms 5

1.3 Prostate cancer 8

1.3.1 Diagnosis and molecular mechanisms 8

1.4 Prostate cancer biomarkers 9

1.5 Prostate-specific antigen (PSA) as a prostate cancer biomarker 12

1.6 Lactate dehydrogenase as a cancer biomarker 14

1.7 Complex matrix handling 16

1.7.1 Stability of LDH, and Total and Free PSA during Sample Storage 16

1.8 Identification and quantification of PSA and LDH by analytical methods 17

1.9 Aqueous biphasic systems as platforms of concentration and extraction of proteins 20

2. Extraction and concentration of PSA using IL-based ABS 23

2.1 Introduction 25

2.2 Experimental Section 28

2.2.1 Chemicals 28

2.2.2 Biological samples 28

2.2.3 Phase diagrams, tie-lines and concentration factors of PSA using synthetic samples 29

2.2.4 PSA extraction efficiency of IL-based ABS 30

2.2.5 SE-HPLC analysis of real samples 32

2.3 Results and discussion 33

2.3.1 Phase diagrams, tie-lines and concentration factors of PSA using synthetic samples 33

2.3.2 Extraction and concentration of PSA from human urine 37

2.4 Conclusions 39

3. Extraction and purification of LDH using IL-based ABS 41

3.1 Introduction 43

3.2 Experimental Section 46

3.2.1 Chemicals 46
3.2.2 Biological samples 46
3.2.3Phase diagrams, tie-lines and LDH extraction and purification with IL-based ABS 47
3.2.4 Extraction and purification of LDH from serum samples 49
3.2.5. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) 49
3.3 Results and discussion 51
3.3.1 Phase diagrams, tie-lines and extraction of LDH using IL-based ABS 51
3.3.2 Extraction and purification of LDH from human serum 57
3.4 Conclusions 61
4. Final remarks and future work 63
5. References 67
Appendix A 85
Appendix B 93
Appendix C 97
Appendix D 101

Index of figures

Figure 1.1- Statistical evaluation of cancer distribution in Portuguese men population in 2012 (Adapted from (10)). 5

Figure 1.2- Illustration of six cancer hallmarks originally proposed by Hanahan and Weinberg (Adapted from (15)).....6

Figure 1.3- Balance between growth factors and growth inhibitors; when growth factors are highly expressed, cancer occurs.....7

Figure 1.4- Gene fusion events are recurrent in prostate cancer. TMPRSS2-ERG fusion results in the upregulation of ERG transcription which then exerts its effects by binding target gene promoter regions, further resulting on their activation or inhibition and in th their activation or inhibition and in the generation of neoplastic phenotype (Adapted from (38))....11

Figure 1.5- Structure of prostate-specific antigen (PSA) forms (64). 13

Figure 1.6- Probability of PCa by total PSA levels in serum (Adapted from (71)). 14

Figure 1.7- Schematic representation of an ABS phase diagram: C = critical point; T = composition of the top phase; B = composition of the bottom phase; X, Y and Z = mixture compositions at the biphasic region; Fc: concentration factor on the top phase (Adapted from (130)). 21

Figure 2.1- Chemical structure of the studied IL: $[P_{4444}]Cl$. 28

Figure 2.2- Phase diagram for the system composed of $[P_{4444}]Cl$+ $C_6H_5K_3O_7$ + H_2O at 25ºC and atmospheric pressure (in wt%) (152) and respective TLs. The experimental binodal curve was adjusted using equation (1). 34

Figure 2.3- Different compositions along the same TL for the system $[P_{4444}]Cl$ + $C_6H_5K_3O_7$ + H_2O which allow to achieve different *CF* values 35

Figure 2.4- HPLC chromatogram of standard PSA aqueous solution (at 150 $g.mL^{-1}$), and top (IL-rich phase) and bottom (salt-rich phase) phases of the system formed by $[P_{4444}]Cl$ + $C_6H_5K_3O_7$ + aqueous solution of PSA at 150 $ng.mL^{-1}$ 36

Figure 2.5- ABS composed of $[P_{4444}]Cl$ + $C_6H_5K_3O_7$ + human urine (with added PSA at 150 ng/mL). 37

Figure 2.6 - HPLC profile of a standard PSA aqueous solution (at 150 $g.mL^{-1}$), a human urine sample, and top and bottom phases of the IL-based ABS after the extraction with PSA in aqueous solution and with human urine (PSA at 150 $ng.mL^{-1}$).....38

Figure 3.1- Chemical structure of the studied ILs: $[P_{4444}]Br$ and $[P_{4444}]Cl$.....46

Figure 3.2- Phase diagrams at 25 ◦C and atmospheric pressure (135), correlation of the binodal data using equation (1), and respective TLs for the systems composed of $C_6H_5K_3O_7/C_6H_8O_7$ + H_2O + $[P_{4444}]Br$ (●); $[P_{4444}]Cl$ (■).....52

Figure 3.3- Extraction efficiency (*EE%*) and precipitation efficiency (*PE%*) for LDH (■) and serum proteins (■) at 25ºC and pH 7.0 in the ABS composed of IL+ $C_6H_5K_3O_7/C_6H_8O_7$ + aqueous solution, using both ILs studied $[P_{4444}]Cl$ (A) and $[P_{4444}]Br$ (B). 53

Figure 3.4- Extraction efficiency of LDH (EE_{LDH}%) and precipitation efficiency of serum proteins ($PE_{SerumProteins}$%) for the ABS composed of $[P_{4444}]Br$+$C_6H_5K_3O_7/C_6H_8O_7$ from a model aqueous solution: LDH (■) and serum (■) for different points of mixture variating salt amount, at 25ºC and pH 7.0. 55

Figure 3.6- Pre-treatment of serum samples spiked with LDH using ABS composed of $[P_{4444}]Br$ + $C_6H_5K_3O_7/C_6H_8O_7$ (in the left) and of $[P_{4444}]Cl$ + $C_6H_5K_3O_7/C_6H_8O_7$ (in the right), evidencing the presence and absence of a third phase corresponding to precipitated protein. 58

Figure 3.7- SE-HPLC chromatogram of standard LDH and HSA aqueous solutions, serum sample (dilution 1:10 in PBS solution) without pre-treatment, and top and bottom phases of the ABS composed of $[P_{4444}]Br$ + $C_6H_5K_3O_7/C_6H_8O_7$ with serum samples. 59

Figure A.2.1- Phase diagram for the system composed by $[P_{4444}]Cl$(♦) +$C_6H_5K_3O_7/C_6H_8O_7$ + H_2O at 25◦C and pH= 7.0 (153).....91

Figure A.2.2- Phase diagrams for the systems composed by $C_6H_5K_3O_7/C_6H_8O_7$ + + H_2O+ $[P_{4444}]Br$ (●); + $[P_{4444}]Cl$ (■). at 25◦C and pH= 7.0 (135).....91

Figure C.1- Calibration curve for LDH in PBS aqueous solution in HPLC at 280 nm.....99

Figure D1.1- HPLC chromatogram of the top (–) and bottom phases (- -) of the ABS composed of $[P_{4444}]Br$ (A) or $[P_{4444}]Cl$ (B) + $C_6H_5K_3O_7/C_6H_8O_7$, at 25ºC and pH 7.0, corresponding to the extraction of LDH. 103

Figure D1.2- HPLC chromatogram of the top (–) and bottom phases (- -) of the ABS composed of $[P_{4444}]Br$ (A) or $[P_{4444}]Cl$ (B) + $C_6H_5K_3O_7/C_6H_8O_7$, at 25ºC and pH 7.0, corresponding to the extraction of serum.....103

Index of tables

Table 1.1- Characteristics that allow to differentiate benign a malignant neoplasms (Adapted from (20)). 7

Table 1.2- Comparison of literature methods for the purification and quantification of PSA in different matrices. 18

Table 1.3- Comparison of literature methods for the purification and quantification of LDH in different matrices. 19

Table 2.1- Weight fraction percentage (wt%) of the initial mixture and of the coexisting phases, and respective values of TLL. 33

Table 3.1- Weight fraction percentage (wt%) for the coexisting phases of ionic liquid (IL)+ potassium citrate (salt) + H_2O, and respective values of TLL. 51

Table A.1.1- Experimental weight fraction data for the binodal curve of the system composed of $[P_{4444}]Cl$ (1) + $C_6H_5K_3O_7$ (2) at (25±1) °C (153).……87

Table A.1.2- Experimental weight fraction data for the binodal curve of the systems composed of IL (1) + $C_6H_5K_3O_7$/$C_6H_8O_7$ (2) at (25±1) °C (136).……..89

Table A.3.1- Correlation parameters obtained by the regression of the experimental data through the application of equation (1) and respective standard deviations (σ) and correlation coefficient (R^2) (153)……92

Table A.3.2- Correlation parameters obtained by the regression of the experimental data thought the application of Eq. (1) and respective deviations (σ) and correlation coefficient (R^2) (136)……..92

Table B1.1- *CF* of the system composed of $[P_{4444}]Cl$ + $C_6H_5K_3O_7$ + H_2O at (25±1) °C ……...……...95

Acronyms

A2M - Macroglobulin

ABS - Aqueous biphasic systems

ACT - Antichymotrypsin

BPH - Benign prostate hyperplasia

CaLB - *Candida antarctica* Lipase B

CF - Concentration factor

CGH - Comparative genomic hybridization

CMIA - Chemiluminescent microparticle immunoassay

CRPC - Castration-resistant prostate cancer

CTC - Circulating tumor cells

DAD - Diode array detector

DTT- Dithiothreitol

DRE - Digital rectal examination

ECM - Extracellular matrix

EE2 - Ethinylestradiol

ELISA - Enzyme-linked immunosorbent assay

ERG - Erythroblast transformation- specific gene

FDA - Food and Drug Administration

FISH - Fluorescence in situ hybridization

fPSA - Free prostatic specific antigen

GlcNAc - *N*-acetylglucosamine

GSTP1 - Glutathione S-transferase P1

HIF-1 - Hypoxia inducible factor 1

hK2 - Hexokinase 2

HPLC - High performance liquid chromatography

HSA - Human serum albumin

IL - Ionic liquid

LDH - Lactate dehydrogenase

NADH - Nicotinamide adenine dinucleotide

PAP - Prostatic acid phosphatase

PCa - Prostate cancer

PCA3 - Prostate cancer antigen 3

PCR - Polymerase chain reaction

PEG - Polyethylene glycol

PI3K - Phosphoinositide 3-kinase

PIN- Prostatic intraepithelial neoplasia

PSA - Prostatic specific antigen

PTEN - Phosphatase and tensin homolog

QMS - Quantitative Microsphere System

TCA - Trichloroacetic acid

TMPRSS2 - Transmembrane protease, serine 2

tPSA - Total prostatic specific antigen

TRUS - Transrectal ultrasound

TL - Tie-line

TLL- *tie-line* length

VEGF - Vascular endothelial growth factor

$[C_4mim]Cl$ - 1-butyl-3-methylimidazolium chloride

$[C_2mim][EtOSO_3]$ - 1-ethyl-3-methylimidazolium ethylsulfate

$[C_2mim][N(CN)_2]$ - 1-ethyl-3-methylimidazolium dicyanamide

$[C_2mim][NO_3]$- 1-ethyl-3-methylimidazolium tetrafluoroborate

$[P_{4444}]Br$ - Tetrabutylphosphonium bromide

$[P_{4444}]Cl$ - Tetrabutylphosphonium chloride

$[P_{4441}][MeSO_4]$ - Tributyl(methyl)-phosphonium methylsulphate

$[P_{i(444)4}][Tos]$ - Tri(isobutyl)methylphosphonium tosylate

1. General Introduction

1.1 Scope and objetives

The accelerated pace of biomedical research faced in the past 50 years has resulted in the ability to alter cellular functions in predicable ways — providing the scientific and medical communities with innovative methods to detect and study diseases, as well as in the development of effective ways to treat serious medical conditions such as cancer (3).

Worldwide, prostate cancer (PCa) is one of the most common types of cancer being related with a high percentage of deaths among men (4). Early diagnosis is highly required to avoid these numbers. Biological molecules like biomarkers have many potential applications in oncology, including cancer screening, risk assessment, differential diagnosis, prognosis, and evaluation of response to treatment (5). Although the identification and quantification of cancer biomarkers can be helpful to improve the success rate of PCa treatment, many difficulties are associated to immuno(metric) assays kits due to their variation in specificity and binding affinity of the employed antibodies (6). Moreover, protein biomarkers secreted by tumors at very early stages of growth (when symptoms have not yet developed) appear at very low concentrations, a problem that needs to be bypassed by analytical methods (7).

Based on the aforementioned concerns, this work starts with an introduction referring to the prostate cancer epidemiology, followed by the understanding of the mechanisms that comprise the appearance and development of this disease. At the biomarker level, the key features of these biomolecules, and particularly for PSA and LDH, are highlighted to better understand their importance in prostate cancer. Finally, aqueous biphasic systems as alternative purification and concentration platforms are described.

The main goal of this MSc thesis consists on the development of efficient methods to pre-treat biological fluids so that the detection/quantification of prostate cancer biomarkers could be achieved by more expedite and less-expensive analytical equipment aiming at increasing the rate of early-stage diagnoses. For this purpose, aqueous biphasic systems (ABS) composed of ionic liquids and organic salts were studied, both in the extraction and concentration of the biomarker prostate specific antigen (PSA) from urine samples and in the extraction/purification of the biomarker lactate dehydrogenase (LDH) from human serum. As demonstrated in this work, the development of these pre-treatment strategies to purify and concentrate biomarkers from human fluids allowed the use of Size-Exclusion High Performance Liquid Chromatography (SE-HPLC) for their identification

and quantification, representing thus a remarkable alternative to the commonly and currently used immunoassays that display several drawbacks (mainly high cost due to the use of specific antibodies, and false negatives and positives results due to other proteins interferences).

1.2 Cancer update overview

1.2.1 Epidemiology

Nowadays, and worldwide, cancer is the leading cause of death, with approximately 14 million new cases each year (8). Just in 2012, 8.2 million deaths have been registered, being expected an increase up to 70% in the next two decades (9). The rate of death by cancer was found to be higher for men (205 *per* 100,000) than for women (165 *per* 100,000) (10). For both, the highest rate recorded worldwide was found in Denmark with 338 people *per* 100,000 being diagnosed with cancer in 2012. Comparing developed countries with developing countries, prostate, colorectal, female breast, and lung cancer rates are 2 to 5 times higher in the first type as a result of variations in an unequal set of risk factors and diagnostic practices. The opposite trend is true for cancers related to infections, such as stomach, liver, and cervical cancers (11).

In Portugal, the cancer death rate is 246.2 *per* 100,000 individuals (10), being the prostate cancer (PCa), large bowel, lung and bladder the types of cancer with higher incidence, prevalence and mortality among men population (Figure 1.1) (12). PCa is the third most common type of cancer in men, in all Europe, North America, and some parts of Africa. It has been estimated that 1.1 million men worldwide were diagnosed with PCa in 2012, accounting for 15% of the cancers diagnosed in men, with almost 70% of the cases (759,000) occurring in more developed regions (13).

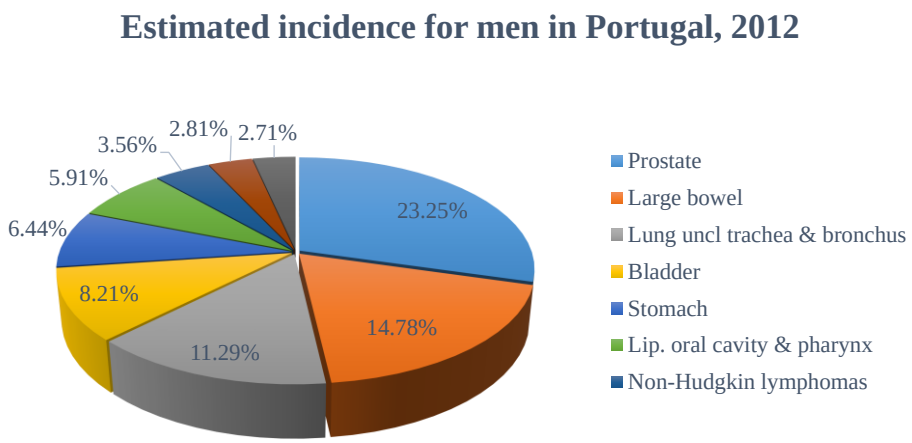

Figure 1.1- Statistical evaluation of cancer distribution in Portuguese men population in 2012 (Adapted from (10)).

1.2.2 Cancer: molecular mechanisms

Cancer is caused by DNA mutations that are acquired spontaneously or induced by environmental injuries (14). A low percentage of cancers are hereditary, remaining the majority of cancer cases a result of genetic mutations that arise from a variety of causes: toxins, UV radiation and other environmental agents and lifestyle factors, such as lack of physical activity, unhealthy diet or excessive alcohol consumption or smoking (8). During cancer progress, additional somatic mutations and genomic arrangements usually tend to accumulate (15), as well as epigenetic changes, such as increases in DNA methylation and alterations in histone modifications, as well as the variation of expression or function of key genes that are responsible for regulating growth, survival and senescence events (14).

Neoplastic cells have genetic alterations that are heritable, passing through cell linage upon cell division. In fact, these cells gather conditions for evolution by Darwinian selection (16). Nordling and his co-workers (3), back in 1953, proposed that cancer was caused by a succession of mutations. After a quarter century of rapid advances on cancer research, it is now assumed that cancer is a disease involving dynamic changes in the genome (17). These knowledge allowed Hanahan and Weinberg (17), to suggest that the vast catalogue of cancer cell genotypes is a manifestation of essential alterations in cell physiology that collectively dictate malignant growth, known by the hallmarks of cancer (Figure 1.2).

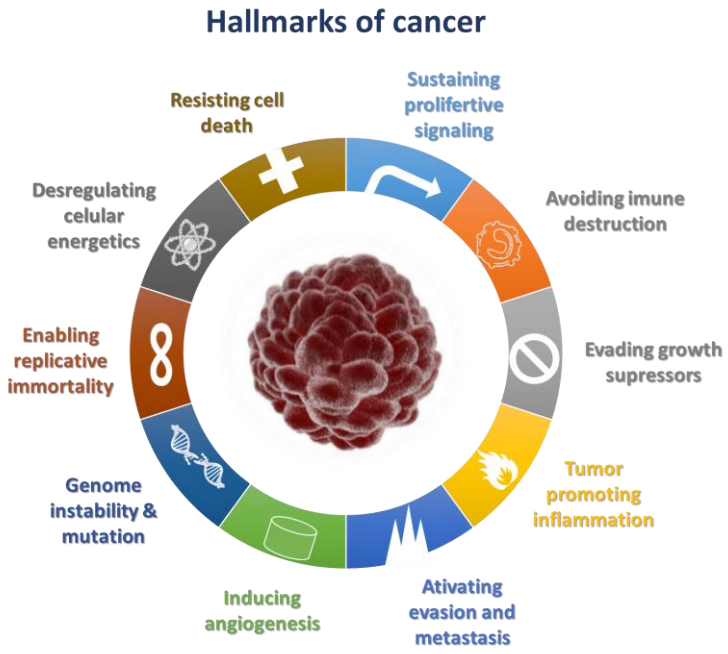

Figure 1.2- Illustration of six cancer hallmarks originally proposed by Hanahan and Weinberg (Adapted from (15)).

Like normal tissues, tumors require nutrients and oxygen to develop, as well as the ability to discharge metabolic wastes and carbon dioxide. Vascularization is essential for tumors to grow and is controlled by the balance between angiogenic and anti-angiogenic factors that are produced by tumor and stromal cells (14). If these cells have a deregulated control of cell proliferation they will proceed with adjustments of energy metabolism in order to complete cell growth and division (18). In normal tissues, cells control the production and release of growth promoting signals that instruct the cell to grow and divide; however, cancer cells use a number of strategies to drive their proliferation and become insensitive to normal growth regulators (14). In the cells cycle, tumors suppressors operate in order to limit the cell growth and proliferation. In general, when unbalanced expression occur it is verified an overproduction of growth factors along with cancer (Figure 1.3). A curious fact about cancer is that cancer cells develop alterations in their shape as well as in their attachment to other cells and to the extracellular matrix (ECM), reflecting thus local invasion, intravasion into blood and lymph vessels, transit through the vasculature, extravasation from the vessels and distant metastasis (18).

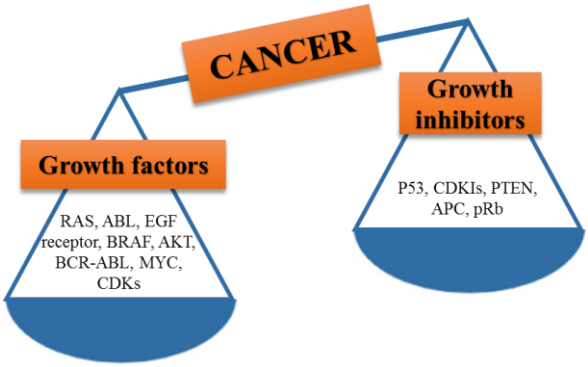

Figure 1.3 - Balance between growth factors and growth inhibitors; when growth factors are highly expressed, cancer occurs.

Only in rare situations, cells emerge and exhibit unlimited replicative potential, termed as immortalization (19). In the absence of p53 — the key mediator of the response to dysfunctional telomeres — cell death and cell cycle arrest responses are mitigated. Additionally, the accumulation of neoplastic cells may result not only in the activation of growth-promoting oncogenes or inactivation of growth-suppressing tumor suppressor genes but also in the mutation of genes that regulate apoptosis and avoid the immune system of evading eradication (14). Based on the knowledge of these factors, advances in cancer molecular biology have identified new ways to inhibit the growth of cancer, leading to new, more selective and less toxic forms of cancer chemotherapy (20).

To understand cancer it is necessary to know how to distinguish its forms, and thus, the division of neoplasms into benign and malignant categories is based on a judgment of tumors clinical behavior. Benign neoplasms are, in majority, encapsulated by a fibrous connective tissue capsule, while malignant ones are invariably non-encapsulated (21). In addition to the proliferative neoplastic cells that make up their parenchyma, both benign and malignant neoplasms have supportive stroma, made up of connective tissue and vascular supply. The characteristics of both types of cancer are summarized in Table 1.1.

Table 1.1 - Characteristics that allow to differentiate benign a malignant neoplasms (Adapted from (20)).

Characteristics	Benign	Malignant
Growth type	Expansive	Infiltrating
Growth speed	Slow	Fast
Stabilization	Frequent	Exceptional
Mitoses	Rare	Numerous
Evolution	Local	Local/General
Metastasizing	No	Yes
Spontaneous evolution	Usually favorable	Always fatal
Evolution after removal	No recurrences	Common recurrences

1.3 Prostate cancer

1.3.1 Diagnosis and molecular mechanisms

The majority of prostatic tumors, which have origin in prostatic epithelium, are within *adenocarcinomas*. The PCa progression is a process involving multiple molecular alterations, many of which can be reflected in changes of gene expression in the prostate carcinoma cells (22). Benign prostate hyperplasia (BPH), on the other hand, is the most common benign tumor found in men with age higher than 60 years-old (23). Men with early localized PCa often have no symptoms; yet, as cancer evolves in the gland, some urinary tract symptoms may develop, being commonly more associated with BPH (24).

Prostatic carcinomas are typically diagnosed by a histological evaluation of prostatic tissue taken by biopsy or by transrectal ultrasound (TRUS) guided biopsies (25). PCa is not only addressed by the histological evaluation, but it is also supported based on PSA values in serum and local findings based on digital rectal examination (DRE), as it will be discussed below. The risk of development of PCa increases with age, and depends on other factors, such as ethnics, diet, levels of testosterone and its main metabolite, 5-dihydrotestosterone, and family history (26,27).

Prostate is one of the accessory glands of reproduction, whereas its primary function is to secrete an alkaline fluid that is part of the ejaculate, which nourishes sperm and aids in its motility (28). The peripheral, central, transition, and fibromuscular are the four anatomic zones of prostate, with the peripheral zone comprising about 75% of the gland (29). Several studies already demonstrated that BPH usually develops in the transition zone, whereas 75% of PCa develops in the peripheral zone. The cytological characteristics of prostate adenocarcinomas include hyperchromatic, enlarged nuclei with prominent nucleoli and abundant cytoplasm (28, 29). The basal cell layer is absent in PCa, but present in normal glands and glands of BPH (30).

The progression of PCa can be described in terms of growth rate, hormone responsiveness, histology and metastatic ability (30). Some of the somatic alterations in PCa cells are genetic while others are epigenetic. However, both contribute to somatic genome alterations producing the cancer phenotype (31, 32). In fact, Singh *et al*. (33) reported, for the first time, the correlation between gene expression signatures and clinical outcomes with the intention to anticipate the clinical behavior of the disease. Years later,

True *et al.* (34), identified a 86-gene model capable of distinguishing low-grade from high-grade cancers, being the first research group publishing a study demonstrating that gene signatures could be used for pathological staging of PCa.

Prostatic carcinoma is characterized by the silencing of the pi-class glutathione S-transferase gene (GSTP1), which encodes a detoxifying enzyme (35). The silencing of GSTP1 results from aberrant methylation at the CpG island in the promoter-5' and may render prostatic cells sensitive to carcinogenesis driven by inflammation and dietary factors (36). This loss of function occurs in the vast majority of cases of high-grade prostatic intraepithelial neoplasia and prostate cancers (37). As happens with many cancers, evidences show that telomere shortening may be a nearly universal feature of early-stage PCa and may promote chromosomal instability leading to the disease progression (38). From genes located on chromosome 8p, it stands out the NKX3.1 gene, being its product a prostate restricted homeobox protein that is involved in the regulation of prostate development. This gene is expressed in normal prostate epithelium and is often decreased in prostatic intraepithelial neoplasia (PIN) lesions and in prostate tumor cells. Posterior studies on chromosome 8p, have identified two small common deleted regions at 8p23.1 and 8p21.3, which showed evidence linkage to hereditary prostate cancer (38). Moreover, alterations in the expression at the 8q24 locus have been observed, which includes the C-MYC proto-oncogene (39). This has been noted as one of the most common chromosomal abnormalities in PCa progression. A study carried out by Hawksworth *et al.* (39) revealed that higher levels of C-MYC mRNA are related to primary prostate tumors, and that the C-MYC locus is frequently amplified in advanced stages of PCa, but rarely in primary prostate tumors. In summary, the current knowledge on the PCa molecular mechanisms has contributed to the development and approval of various cancer biomarkers by the Food and Drug Administration (FDA) (40).

1.4 Prostate cancer biomarkers

In the last years, the interest in the use of biological markers to evaluate the risk of future diseases has significantly increased (41). According to the National Cancer Institute, a biomarker is "a biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process, or of a condition or disease" (42). These different types of biomolecules can be used do predict, detect, diagnose, distinguish benign from malignant tumors, or one type of malignancy from another, and also be used for prognostic

(43). They can be detected by screening technologies like micro-RNA expression profiling, gene expression profiling, genome hybridization, proteomic profiling, molecular imaging, assessing tumor specific antibodies and circulating tumor cells (44). Molecular alterations, such as mutations, can be uncovered by using DNA sequencing, mass spectrometry based genotyping or mutation specific polymerase chain reaction (PCR). Although biomarkers have numerous advantages, variability is a major concern (43). Variability applies regardless of whether the biomarker represents an exposure or effect modifier, a surrogate of the disease, or an indication of susceptibility. For that reason, there is no biological biomarker completely reliable, especially in early detection and diagnosis, and a well-established range is difficult to attain. However, a large number of biomarkers exists and that are currently used in clinical practice, being already approved by FDA (45).

The management of prostate cancer has undergone several dramatic changes as a result of the evolution of biomarkers used in screening, detection and prediction of the disease (31). Human prostatic acid phosphatase (PAP) (or serum acid phosphatase (AP) was reported as the first serum biomarker of prostate cancer (31). Although AP, with elevated levels in more than 70% of the patients, was linked with early-stages prostate cancer, both AP and prostate-specific AP (PAP, its subtype) were later found to be not enough sensitive for screening (46,47). Only many decades later, the PSA screening was proposed and latter approved by FDA as a more sensitive and specific PCa marker (45).

In 1999, Bussemakers *et al*. (48) were the first to publish their findings regarding a new prostate cancer–related gene, prostate cancer gene 3 (PCA3). Using the PCR method, authors demonstrated that this gene was overexpressed in prostate tumor tissues. In 2012, FDA also approved this biomarker for use in men in which PCa is suspected based on PSA levels and/or DRE and/or one or more negative biopsy results. In general, this assay comprises the determination of the levels of PCA3, PSA and RNA and is further based on the calculation of the ratio of PCA3 RNA molecules to PSA RNA molecules (PCA3 score) in post-digital rectal examination (DRE) urine specimens (49).

Nowadays, the research on cancer biomarkers and their use in diagnosis is focused on serum, urine, and tissue samples to improve accuracy and response to current clinical challenges in the decision of undergoing biopsy, invasive therapies and to alter therapeutic strategies (50). Many new PCa biomarkers have been recently suggested to be concomitantly used with markers that are already well-established and used, since the use of a group of biomarkers instead of one is a step forward to avoid overdiagnosis and

overtreatment (51). One of these examples is TMPRSS2-ERG (Figure 1.4); the fusion between the transmembrane protease serine 2 (TMPRSS2) gene and the v-ets erythroblastosis virus E26 oncogene homolog (avian) (ERG) gene is the predominant variant in approximately 40% to 80% of PCa cases (50). Quantitative levels of TMPRSS2-ERG in urine appear to be associated with clinically significant PCa. Moreover, the assessment of post-DRE urine TMPRSS2-ERG, in combination with urine PCA3, has shown to enhance the utility of serum PSA levels for predicting PCa risk and clinically relevant cancer on subsequent biopsy. On other hand, the dysregulation of PTEN, a tumour suppressor gene involved in cell cycle regulation, is consistently associated with poor prognosis in PCa (50).

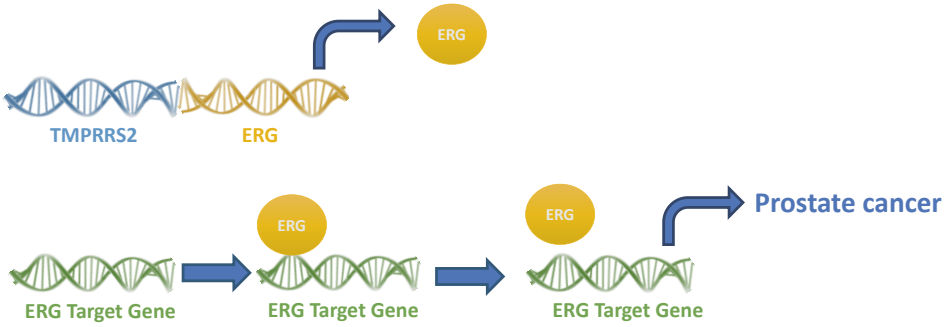

Figure 1.4 Gene fusion events are recurrent in prostate cancer. TMPRSS2-ERG fusion results in the upregulation of ERG transcription which then exerts its effects by binding target gene promoter regions, further resulting on their activation or inhibition and in the generation of neoplastic phenotype (Adapted from (40))

The most important source of error associated to biomarkers seems to be the failure on identifying factors that may alter their levels in body fluids or tissue (43). Up to now, no biomarker has been established as an ‘‘ideal’’ cancer screening tool that can meet the diagnostic and prognostic simultaneously (52). The validation of new cancer biomarkers for efficient cancer diagnosis and prognosis is quite necessary and demanding (53). Although, the PSA is currently the most consensual biomarker in the diagnosis of prostate cancer. It is expected that, in the next few years, more tests will become available to help clinicians in the prediction of aggressive PCa (49). In this sense, optimized quantification methods for cancer biomarkers are amply required and these should be based on more than one biomarker to improve accuracy in diagnosis.

1.5 Prostate-specific antigen (PSA) as a prostate cancer biomarker

PSA is a serine protease, with 33 kDa, and belongs to the human kallikrein gene family (54). The kallikrein family belongs to the largest cluster of peptidases in the human genome, being located at the chromosomal region 19q13.3-q13.4 (55). PSA is secreted by the prostatic epithelium and the epithelial lining of the periurethral glands. Its main function consists on the liquefaction of the seminal coagulum to allow the release of spermatozoa by the preferential hydrolysis of arginine- and lysine-containing substrates (56,57). Outside its physiological role, PSA may participate in the process of proliferation of normal and abnormal cells, and in particular, in the tumoral growth of prostate cells through the degradation of the insulin-like growth factor binding protein 3 and 4 (58). PSA expression is regulated by the androgen receptor, whose activity has a fundamental role in prostate tissue development and progression of prostate cancer (55).

PSA is a 237- residue glycoprotein with a single N-oligosaccharide chain linked to Asn61, close to the entrance of the catalytic cleft and close to the kallikrein loop, but far from the dimer interface (59). This *N*-linked sugar-moiety, of *N*-acetylglucosamine (GlcNAc), comprises about 8% of the total mass and is the major sugar-chain structure of PSA (60). PSA comprises 92% of peptides and 8% of carbohydrates, whereas the carbohydrate fraction is constituted by 4.84% of hexoses, 2.87% of hexosamines, and 0.25% of sialic acid (61). PSA also has in its composition His57, Asp102 and Ser195, sharing the catalytic triad characteristic of serine proteases; this catalytic site is conserved and located in a cleft between two β-barrels (59,62).

PSA is a glycoprotein that has several isoenzymes, two intact and nicked forms of PSA, in the isoelectric point (pI) range of 6.5–8 (63). This biomolecule is synthesized with a 17–amino acid leader sequence (preproPSA), that is cotranslationally cleaved to generate an inactive 244–amino acid precursor protein (proPSA; Figure 1.5) (64). Normal secretory epithelium, surrounded by basal cells and a basement membrane, secretes proPSA into the lumen where the pro-peptide is removed by hK2 to generate active PSA, by the cleavage of the N-terminal between the arginine at position 7 and isoleucine at position 8 of proPSA (65).

A fraction of the active PSA can diffuse into the circulation, where it is rapidly bounded by protease inhibitors (primarily alpha1-antichymotrypsin, ACT). Active PSA also undergoes proteolysis in the lumen to generate inactive PSA, which can enter the bloodstream and circulates in an unbounded state (free PSA). The majority (70% to 90%)

of PSA that enters the peripheral blood is intact and circulates as an 80- to 90-kDa complex with the protease inhibitor alpha1-antichymotrypsin (64). Minor amounts are complexed with other protease inhibitors, including alpha2-macroglobulin and alpha1-antitrypsin (64).

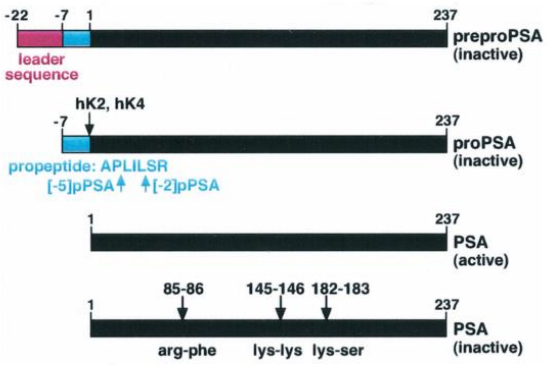

Figure 1.5 - Structure of prostate-specific antigen (PSA) forms (64).

The quantification of PSA in human fluids is a powerful tool for early PCa detection (67). The earliest report on the properties of antigens in the prostate is back to 1960 and was reported by the American urologist Rubin Flocks (68). Only in 1980, PSA was quantified in blood and studied for clinical uses as a marker of prostate cancer (32). However, only six years later, FDA approved PSA test for prostate cancer screening (54).

Despite nearly 20 years of advances in molecular biology, only total PSA (tPSA) and free PSA (fPSA) measurements have found a relatively broad and growing clinical role in the management of prostate cancer patients (69). An annual screening of PCa is extremely recommended for men with 50 or more years old, and in African American men with more than 40 years old (because ethnicity is a relevant factor) (70). Men with clinically localized prostate cancer are considered ‘‘very low risk’’ if they have PSA levels < 10 $ng.mL^{-1}$; men with ‘‘intermediate risk’’ have PSA values between 10 and 20 $ng.mL^{-1}$ in serum; and men with ‘‘high risk’’ of prostate cancer have PSA levels in serum > 20 $ng.mL^{-1}$ (71). More than in other cancer types, age is a significant factor that influences the PCa development and PSA baseline values. The serum PSA concentration seems to be directly correlated not only with the patient age but also with the prostatic volume, allowing the prediction of the disease stage and size of the tumor (72). PSA values > 4.0 $\mu g.L^{-1}$ in serum (and more recently > 2.5 $\mu g.L^{-1}$) has been defined in the literature as abnormal and it is frequently used as a cut-off. The diagnosis of PCa is usually performed based in abnormal DRE results or findings of high concentrations of PSA in serum (72). In

general, it is accepted that the levels of PSA can be indicative of the cancer risk, as summarized in Figure 1.6.

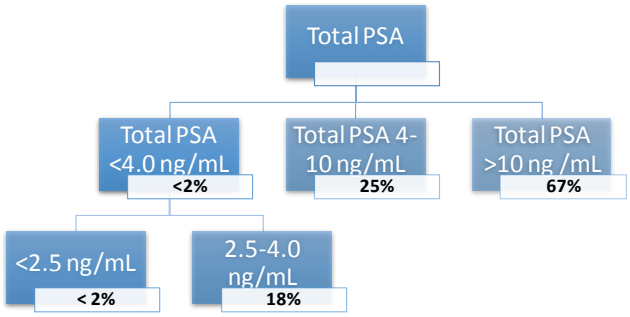

Figure 1.6 -Probability of PCa by total PSA levels in serum (Adapted from (71)).

After the detection of high levels of PSA in human serum, active surveillance is reacquired to avoid radical treatment (73). For that, biopsy or MRI analysis are then performed, as well as continued PSA quantification (73). However, PSA screening is still controversial because it leads to a significant number of false-positive results and to subsequent unnecessary biopsies and detection of clinically indolent tumors and subsequent overtreatment (67). Based on this information, a panel of diagnostic and prognostic biomarkers that could work in conjunction with PSA will be ideal (74). In this sense, new biomarkers, like lactate dehydrogenase, have been proposed in the past few years for PCa diagnosis and that can be included in the discussed biomarker panel (53).

1.6 Lactate dehydrogenase as a cancer biomarker

Lactate dehydrogenase (LDH), EC1.1.1.27, is a NAD^+-dependent enzyme of the oxidoreductases family, with a molecular weight between 115 kDa and 154 kDa (74). This enzyme catalyzes the last step of lactic fermentation, converting pyruvate to lactate (75). For this conversion, LDH presents an active center rich in hydrophilic and charged residues as glutamate, glutamine, aspartate, arginine and histidine (76).

LDH is usually present in the liver, cardiac muscle, kidneys, skeletal muscle and erythrocytes. It is composed of four polypeptide chains, each one encoded by a separated gene (M and H) (77), which associate into five different combinations of polypeptide subunits resulting in five molecular isoforms of LDH, and thus being found in different quantities depending on the originating tissue (78). However, some studies reported that the M form is usually present in anaerobic tissues, like skeletal muscle and liver (codified

by gene A), while the H form is typical of aerobic tissues, like cardiac muscle (codified by gene B), respectively located on chromosomes 11p15.4 and 12p12.2-p12.1.

It is well known that cancer and non-cancerous cells have different types of metabolism (79). In contrast to normal differentiated cells, which mainly perform mitochondrial oxidative phosphorylation to generate energy, most cancer cells resort to aerobic glycolysis, a phenomenon called "Warburg effect" (80). LDH levels and hypoxia are related by a tumor-driven angiogenesis pathway through the abnormal activation of the hypoxia inducible factor 1 (HIF-1) (76). Tumor hypoxia is enough to alter the gene expression of this factor and downregulate several genes, which encode for vascular endothelial growth factor, erythropoietin and many enzymes involved in glucose, iron, and nucleotide metabolism (81). A central player in the Warburg effect is lactate dehydrogenase-5 (LDH-5), which plays a crucial role in tumor maintenance, being thus considered a cancer biomarker (81).

According to the American Cancer Society, LDH is used as a cancer biomarker for germ cell tumors (82). Over recent years, the LDH use has undergone a high clinical importance being tested as a biomarker for myeloma, lung cancer, osteosarcoma and kidney cell carcinoma (83-86). The normal levels (healthy men) range between 100 and 300 $U.L^{-1}$, while levels higher than 300 $U.L^{-1}$ are usually associated with cancer (86). Although total LDH levels in blood may be a diagnostically meaningful parameter in cancer patients, the clinical significance of serum isoenzymatic patterns of LDH is still under discussion (81). On the other hand, Nishikawa *et al*. (87) proposed that the determination of LDH isoenzymes in the urine might be useful in diagnosing urinary tract malignancy, including early stage ones. Nevertheless, it is known that LDH-5 levels in urine or body fluids can be altered, especially elevated, when bacterial infections occur (81).

Studies on LDH isoenzymes have led to the conclusion that since certain diseases of the prostate gland have different isoenzyme distributions, LDH isoenzymes may distinguish PCa from BPH (88). LDH-5 levels increase in prostate cancer and LDH-1 decreases, while in BPH the contrary behavior is observed (88). Since LDH levels in presence of prostate cancer are rarely increased in early stages, LDH was tested for assessment of prognosis, therapeutic efficacy and evaluation of symptoms (89). In fact, some studies have reported that LDH is an excellent prognostic indicator for PCa patients with bone metastasis (90,91). Other studies referred that LDH-5 overexpression is

significantly linked to highly proliferating prostate carcinomas and can be used to monitor improvements in radiotherapy (92). It was also demonstrated that LDH levels and circulating tumor cells (CTC) count form a biomarker panel for castration-resistant prostate cancer (CRPC) (93). This biomarker panel proved to have high discriminatory power being thus an effective tool for discrimination between high-risk and low-risk patients with CRPC (94). More recent investigations stated that serum LDH high levels are associated with poor survival of patients with solid tumors, and could be used as a cost-effective prognostic biomarker in terms of prognostic of metastatic carcinomas, including prostate cancer (95). As a whole, the quantification of LDH seems to be advantageous when combined with PSA determination in biological samples and can improve the accuracy of PCa diagnosis.

1.7 Complex matrix handling

1.7.1 Stability of LDH, and Total and Free PSA during Sample Storage

The problems encountered during analysis of complex biological samples are usually described as "matrix effects" (96). Complex samples, such as blood, display major barriers towards biomarkers discovery and their quantification. The main problem comprises the quantification of extremely low abundance (concentration) of the candidate markers in blood, which usually exist below the detection limits of conventional analytical techniques. In addition, the high abundance of resident proteins in blood, such as albumin which represent more than 50% of circulating plasma proteins, can mask the identification of rare biomarkers (97,98). The third challenge for biomarkers identification/quantification relays on the rapid degradation of the low abundance biomarkers - mainly because blood contains proteases which activity is enhanced when samples are collected (99). To handle these samples it is important to first remove the proteins content, which can be denatured using acids or heat, or ultra-filtration using cut-off membranes (100). Another possibility consists on the use of organic solvents such as trichloroacetic acid (TCA)/acetone mixtures (101).

Urine represents a way to perform non-invasive quantification of biomarkers for the early detection and prediction of prostate cancer prognosis (102). Urine contains a complex mixture of proteins, urea and high amounts of some ionic components (103). However, in urine samples, the variability of results can occur, especially in the presence of a large

plethora of components, such as organic compounds and electrolytes, and different pH values which can affect antibody binding and the assay performance (104). Recently, Struck-Lewicka *et al.* (105) demonstrated that urine metabolites are altered in prostate cancer, a result of the urea and tricarboxylic acid cycle, amino acid and purine metabolism, which play a crucial role in the pathogenesis of the disease. Therefore, the future perspectives in cancer biomarker detection fit within the development of an efficient detection platform with high sensitivity, versatility, high-throughput and capability to concentrate and isolate biomarkers.

In addition to matrix effects, the stability of cancer biomarkers, which are mainly proteins, plays a vital role. For instance, the LDH activity is dependent upon the storage temperature, storage medium and technique used for freezing (106). Some studies indicated a cold lability of the slower isoforms, *e.g.* LDH-4 and LDH-5, in both tissue homogenates and cord blood serum, with a greater loss of activity at -10°C than at 4°C (107). Shain *et al.* (108) evaluated the effect of storing human plasma and extracts of prostate at -90 °C on the activity of lactate dehydrogenase and isoenzyme distribution. Authors reported that these preparations can be thawed only once at 2 to 4 °C, and that they could be stored for as long as 165 days at -90 °C with no changes in isoenzyme distribution (108). Regarding PSA, parameters like pH and temperature may influence the stability of fPSA isoforms (109). Biological samples containing PSA should be stored at 4°C and analyzed within 8 h after collection or at -70°C and at pH of 5.5 for latter analysis. For tPSA, it was proved that it is stable at room temperature for 24 h, for almost a week when stored in a refrigerator at 4°C, and for 1 month at -20°C (109). However, this is not valid for fPSA since it is the most thermolabile isoform (109).

1.8 Identification and quantification of PSA and LDH by analytical methods

Since LDH and PSA biomarkers are primarily found in blood, and sometimes in urine at low abundance, features of analytical methods such as sensitivity, specificity and accuracy are critical to improve the identification and quantification of cancer biomarkers (33). In 1980, it was developed for first time, the enzyme-linked immunosorbent assay (ELISA) method for quantification of PSA (112). Since then, many other assays have been developed, not only for PSA, but also for the respective isoforms being currently commercially available (113,114). Although the ELISA method is extensively used in

routine clinical diagnostics, many challenges remain to improve, such as the increase of the method sensitivity, accuracy and reproducibility as well as its high cost (52). Usually, all commercial immunoassays, like the Acess Hybritech PSA assay, consist on the incubation of monoclonal anti-PSA antibodies for further binding to PSA in serum samples (110). PSA purification/fractionation can also be performed by liquid chromatography methods and by recently developed electrochemical biosensors (60). On the other hand, the LDH detection is usually performed with colorimetric and chromatographic methods, it can be also quantified by electrochemical methods (120-122). Tables 1.2 and 1.3 present a summary of the techniques and methods commonly applied in the identification and quantification of PSA and LDH.

Table 1.2 Comparison of literature methods for the purification and quantification of PSA in different matrices.

Purification/Concentration method	Isoform	Matrix	Sensitivity ($ng.mL^{-1}$)	Ref.
Immunoassay				
ELISA	PSA	Serum	0,1935	(115)
Sensitive rapid tandem bioluminescent enzyme immunoassay (BLIA)	PSA/ACT	Serum	0,3	(116)
Hybritech PSA assay	PSA/ACT	Serum	1800	(117)
Chromatography				
Liquid chromatography (LC) coupled with tandem mass spectrometry (MS-MS)	PSA	Serum	1	(118)
Biosensors				
Surface plasmon resonance (SPR)	PSA/ACT	Serum	18.1	(119)

Table 1.3 Comparison of literature methods for the purification and quantification of LDH in different matrices.

Purification/Concentration method	Isoform	Matrix	Sensitivity	Ref.
Chromatography				
Flow injection analysis with fluorimetric detector	LDH	Tissue	$0.1x10^{-3}$ $U.L^{-1}$	(120)
Reversed-Phase Liquid Chromatography Tandem Mass Spectrometry	L-LDH	Plasma	0.2 $\mu mol.L^{-1}$	(114)
Reversed-Phase Liquid Chromatography Tandem Mass Spectrometry	L-LDH	Urine	8.1 $nmol.mmol^{-1}$	(114)
Electrophoresis				
Isoelectric focusing	LDH-5	Tissue	10 $U.L^{-1}$	(121)
Biosensors				
Electrochemical biosensor with nanointerface	L-LDH	Tissue	4.73 $nmol.L^{-1}$	(122)

Even though the methods listed in Tables 1.2 and 1.3 are currently used, they display some disadvantages. Electrochemical/electrical detection strategies are currently available for both PSA and LDH, and in general they display a high sensitivity and simplicity combined with the use of miniaturized hardware (52). However, the application of impedance biosensors has been limited by their sensitivity to nonspecific adsorption, which often limits the applicability of these biosensors to complex matrices (52). Analytical techniques, like electrophoresis, are suitable to use since they simultaneously separate thousands of proteins and allow the comparative protein profiling between different crude biological samples (123). Nevertheless, they are incapable of detecting the majority of protein components found in low abundance in these samples due to their low detection limits. High-performance liquid chromatography (HPLC) presents high versatility, being able to detect biomarkers in the range from picograms to multigrams, combining speed, reproducibility, and sensitivity (34). However, despite these advantages, HPLC exhibits a limited effectiveness in bioanalytical applications, namely the lack the sensitivity required to quantify low levels of endogenous substances. Moreover, most of the times, HPLC requires complex and time-consuming samples purification and derivatization steps, which increases the cost and time effectiveness (124,125). For HPLC analysis it is required the use of special kits to eliminate highly abundant proteins and to allow the identification/quantification of low abundant biomarkers (123,126). An example of these

kits is the Cibacron blue F3GA based- and antibody-related methods. Evidences show that Cibacron blue F3GA based-resin method removes not only albumin from serum samples, but also every proteins having the same pI (123). Finally, colorimetric assays, as those used in LDH quantification, display low sensitivity and accuracy (124).

Based on the summarized information presented above, it is mandatory the development of alternative extraction/concentration techniques that can be coupled to analytical methods of higher accuracy and lower cost in order to reduce the costs associated with biomarkers quantification and sample purification, so that cancer diagnosis can be improved.

1.9 Aqueous biphasic systems as platforms of concentration and extraction of proteins

Aqueous biphasic systems (ABS) were originally discovered by the observation of the formation of two liquid phases by dissolving gelatin and agar in aqueous media (127). Only years later, Albertsson demonstrated that ABS can be formed with two polymers or a polymer and a salt in aqueous media, and that these systems can be used in the extraction and purification of proteins, viruses, cells, among others (128).

ABS are mainly composed of water and are thus more biocompatible and more environmentally benign than volatile organic solvents commonly used in the extraction of value-added compounds from aqueous media (129). ABS can be classified in polymer/polymer-, polymer/salt- and salt/salt-based systems, *i.e.*, two solutes structurally different, and both water-soluble, that separate into two coexisting phases above a given concentration: one of the aqueous phases will be enriched in one of the solutes while in the other phase there is prevalence for the second polymer or salt (130). The phase diagrams of ABS are usually determined in advance as they provide the necessary information about the minimum concentration of each component required for the formation of two phases, and the concentration of the components in the top and bottom phases, as well as the volume or weight ratio of the two phases (35). Figure 1.7 shows an example of a ternary phase diagram of an ABS, and where the binodal curve and three mixture compositions at the biphasic region (X, Y and Z) are presented. The mixtures X, Y and Z are along the same tie-line (TL) meaning that all initial mixtures present the same top phase composition (T_{IL}, T_{Salt}) and the same bottom phase composition (B_{IL}, B_{Salt}).

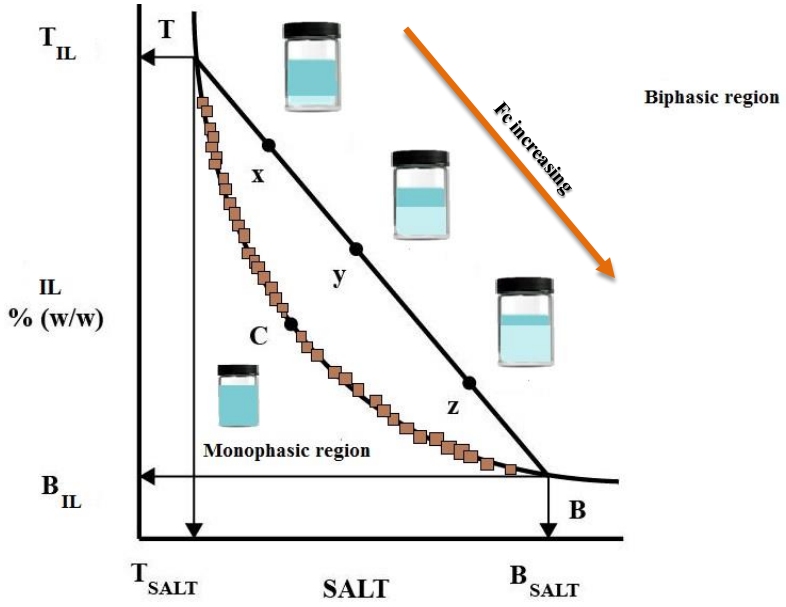

Figure 1.7- Schematic representation of an ABS phase diagram: C = critical point; T = composition of the top phase; B = composition of the bottom phase; X, Y and Z = mixture compositions at the biphasic region; Fc: concentration factor on the top phase (Adapted from (35)).

Since proteins are complex molecules which readily undergo denaturation and lose their biological activity when in contact with some polymers, salts or organic solvents, ABS are a biocompatible method of extraction (132). Nowadays, the ABS application in the separation of biomolecules has been amply reported in the literature, especially with ionic-liquid-(IL)-based ABS (131,132). In particular, IL-based ABS have been extensively studied as alternative methods for the extraction and purification of several proteins, such as bovine serum albumin, papain, lysozyme, trypsin, myoglobin, peroxidase, cytochrome c, γ-globulins, hemoglobin, and ovalbumin, without compromising the enzymatic activity or the protein stability (130, 131, 132).

Pei and co-workers, in 2009, demonstrated that it is possible to extract proteins, with 100% extraction efficiencies obtained in a single step, using ABS composed of ILs and salts (132). Authors also demonstrated by UV spectroscopy that chemical interactions between BSA and IL do not occur and there are no changes in the protein conformation (132). Although the extraction of proteins using IL-based ABS can serve as a guideline for the use of these systems in the extraction of enzymes, it must be recognized that the catalytic activity is a key parameter for the success of enzymes extraction (130). Therefore, it is mandatory to ensure that the interaction between the IL in the aqueous solution and the enzyme are balanced, meaning that they must have a strong ability of dissolving enzymes,

but not strong enough to break the structure and/or interact with the active site of the same (130). More recently, Du *et al.* (134) reported the use of ABS composed of [C_4mim]Cl and K_2HPO_4 for the extraction of proteins from biological samples, namely human urine, thereby increasing the possibility of ABS research for clinical use, particularly in clinical diagnosis. Passos *et al.* (135), later contributed to the advance in research of ABS, by concentrating endocrine disruptors from biological fluids up to 100 times in a single-step, using various ILs and K_3PO_4. More recently, ABS formed by phosphonium-based ILs have shown the ability to not only extract proteins of interest, but also to selectively denaturate proteins like albumin from complex matrices (136)
, encouraging thus the investigation of this type of systems for the extraction, purification and concentration of cancer biomarkers from biological fluids.

Considering the advantages of IL-based ABS stated before, this work aims the application of phosphonium-based ABS in the concentration and purification of prostate cancer biomarkers from synthetic samples and finally with human fluids, so that more expedite and less expensive equipment for their identification/quantification can be used. Both PSA and LDH cancers biomarkers are investigated in this work envisaging the development of more accurate diagnosis of PCa by the use of two biomarkers in two different types of fluids, instead of one biomarker and one type of human fluid as currently carried out.

2 . Extraction and concentration of PSA using IL-based ABS

2.1 Introduction

The discovery of room-temperature ionic liquids (ILs) was made by Paul Walden in 1914 (137). However, only in 2003, Rogers and co-workers have proposed ABS composed of ionic liquids (ILs) and inorganic salts as alternatives to systems mainly composed of polymers (138). When a salt is added to an aqueous solution of an IL, there is the formation of two phases, one enriched in the IL and the other mainly composed of water and salt, due to the preferential hydration of the salt ions which induce the ILs salting-out from aqueous media (130).

ILs are a class of compounds generally composed of large organic cations and an anion that can be organic or inorganic (139). Generally, these salts have a melting point below 100° C and exhibit reduced electrostatic forces, which difficult the formation of a regular crystalline structure. Due to the large number of potential cation-anion combinations, ILs are also known as "designer solvents". ILs can be classified into imidazolium-, pyridinium-, pyrrolidinium-, phosphonium-, sulfonium-based, among others (140,141). Despite the immense versatility inherent to the cation–anion permutations in ILs, most ABS have been attempted using imidazolium-based compounds; however, more recently, phosphonium-based ILs have emerged as potential alternatives in ABS formation and where studied in proteins extraction (130). The capacity of phosphonium-based ABS as prospective extraction media in biotechnological processes was also demonstrated by the high partition coefficients obtained with hydrophobic biomolecules (142).

The first reports of phosphonium-based ILs dates from the 70s, when Parshall (141) reported the synthesis of phosphonium-based ILs using stannate and germinate salts. Phosphonium-based ILs display some advantages, mainly higher thermal and electrochemical stabilities when compared with the largely investigated imidazolium-based counterparts (142). The lack of acidic protons turns them more stable, thus not reacting under reductive or alkaline conditions (143). Since these IL are less dense than water, they might be beneficial in several setups involving the separation of aqueous layers. Moreover, Louros *et al.* (144) reported that phosphonium-based ILs are more effective in the creation of ABS compared to the typical imidazolium-based class, meaning that lower amounts of ILs are required for their application in extraction, concentration and purification approaches.

Despite the evidences referred before, the use of IL-based ABS for the extraction, concentration and purification of cancer biomarkers from human fluids has not been

reported in the open literature. Even so, IL-based ABS seem to be a good alternative as a pre-treatment strategy of human fluids to allow the use of more accessible equipment for the quantification of cancer biomarkers in clinical laboratories. This requirement is mainly due to the high complexity of human fluids which inherently require their pre-treatment before the identification/quantification of target biomolecules, ie, to reduce interferences of other analytes. Protein purification methods are usually time consuming, expensive and employ volatile organic compounds hazardous to human health, environment and highly susceptible biomolecules such as proteins (145). In this sense, IL-based ABS also emerge as a more friendly alternative, since they are mainly composed of water and it is possible to maintain the pH of these systems by the addition of a proper biological buffer, allowing to conserve the stability of proteins.

The determination of PSA levels in human fluids has become a standard practice for prostate cancer recurrence monitoring. PSA is typically undetectable by most assays after surgery, and it is generally agreed that undetectable post-surgical PSA over time is indicative of a good prognosis (146,147). As long as PSA remains undetectable, the patient can be assured that there is no biochemical evidence of cancer recurrence. Indeed, the less sensitive the assay, the longer this assurance can be offered, while in fact, PSA could be present, yet below detectable levels (148). Nowadays, in clinical laboratories, total and free PSA are quantified by immunoassays, mainly by the chemiluminescent microparticle immunoassay (CMIA) (149). Although, immunoassays are susceptible to interferences like cross-reactivity with other drugs and metabolites, reaction with heterophilic antibodies, and influence of endogenous factors, like hematocrit or albumin, in the case of serum analysis (150). Interfering substances may lead therefore to false positive or false negative results.. In addition to CMIA, only one more method to quantify PSA is available, namely the Abbott Diagnostics and Quantitative Microsphere System (QMS™), having a sensitivity below 1μg.L^{-1}, and reported as to offer adequate accuracy and precision (151).

Since the methods described before require expensive equipment setup, the acquisition of antibodies and are usually time-consuming, alternative techniques are mandatory. IL-based ABS represent a potential alternative for the pre-treatment of samples before PSA analysis. ABS are easy to implement, of low cost and capable of simultaneous act as extraction, purification and concentration platforms. Indeed, recent reports have shown that IL-based ABS can be used to concentrate biomolecules of interest from biological fluids up to 100-times (135), and persistent pollutants from aqueous streams up

to 1000-fold (from $ng.L^{-1}$ to $\mu g.L^{-1}$) in a single-step (152). After the pre-treatment of human fluids with IL-based ABS, samples are envisaged to be simpler analyzed by more expedite equipment, such as SE-HPLC, avoiding thus the use of more expensive immunoassays.

In this section, IL-based ABS were used to simultaneous extract and concentrate PSA from synthetic and human urine samples in order to increase their values up to concentrations that can be quantified by SE-HPLC with an UV or DAD detector. Since human urine samples are not rich in proteins, in this section, the purification step was not attempted. Since the cut-off value of PSA in urine is 150 $ng.mL^{-1}$ (71) concentration factors of circa 250-fold must be studied in order to reach the limits of detection of more expedite equipment, like HPLC. As a first attempt, methods were optimized with synthetic samples and then applied to real human urine samples.

2.2 Experimental Section

2.2.1 Chemicals

The aqueous biphasic system studied in this section is composed of potassium citrate tribasic monohydrate, $C_6H_5K_3O_7 \cdot H_2O$, > 99 wt% pure, from Sigma-Aldrich and the ionic liquid tetrabutylphosphonium chloride ($[P_{4444}]Cl$), > 96 % pure, from Cytec Ind.. The chemical structure of the IL studied is represented in Figure 2.1. For HPLC analysis, sodium phosphate monobasic (NaH_2PO_4, purity: 99- 100.5%), sodium phosphate dibasic ($Na_2HPO_4 \cdot 7H_2O$, purity 98.2- 102.0 %) and sodium chloride (NaCl) were used to create the phosphate buffer solution at pH = 7. PSA dilutions were made by using phosphate buffered saline (PBS) aqueous solutions, 100 wt% pure from Sigma-Aldrich. Pure PSA, purity ≥ 95%, was obtained from Sigma-Aldrich Chemical Co..

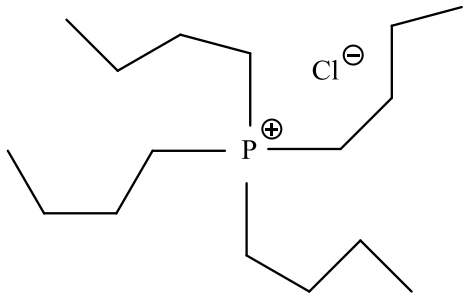

Figure 2.1 Chemical structure of the studied IL: $[P_{4444}]Cl$.

2.2.2 Biological samples

For the PSA quantification, 10 urine samples of random healthy patients were collected. The urine samples used were the first of the day. All samples collected belong to male individuals with ages between 20 and 30 years-old.

2.2.3 Phase diagrams, tie-lines and concentration factors of PSA using synthetic samples

The binodal curve of the studied ABS was taken from the literature (153), determined thought the cloud point titration method at 25 (±1) ºC and atmospheric pressure (Appendix A). The experimental procedure adopted by Passos *et al.* (153) consisted in the repetitive drop-wise addition of the aqueous salt solution ($C_6H_5K_3O_7$) into the aqueous solution of IL until the detection of a cloudy (biphasic) solution, followed by the dropwise addition of water until the observation of a limpid (monophasic region) (153). All the calculations considering the weight fraction of the phase-forming components were carried out discounting the complexed water in the commercial citrate-based salt. All additions were made under constant and controlled stirring and the ternary system compositions were determined by weight quantification of all components added within ± 10^{-4} g.

The respective *tie-lines* were then determined for this phase diagram, by the gravimetric method originally proposed by Merchuk *et al.* (154). For this purpose, a selected mixture at the biphasic region was chosen and prepared by weighting the appropriate amounts of IL+ salt + water and further stirred. The mixture was submitted to centrifugation for 10 min at 3500 rpm and at controlled temperature of 25 (±1) ºC and subsequently left in equilibrium at 25 (±1) ºC for 10 minutes to guarantee the equilibration of the coexisting phases. Lastly, the compositions of bottom and top phases, as well as the overall system composition were determined by the level-arm rule.

Experimental binodal curves were fitted by using equation. (1):

$$Y = A\exp[(BX^{0.5}) - (CX^3)] \quad (1)$$

where *Y* and *X* represent the IL and salt mass fraction percentages, respectively, and *A*, *B* and *C* are constants obtained by regression of the experimental binodal data.

Each individual tie-line was determined according the solution of the following system of four equations (2-5), and four unknown parameters (Y_T, Y_B, X_T and X_B):

$$Y_T = A\exp[(B \times X_T^{0.5}) - (C \times X_T^3)] \quad (2)$$

$$Y_B = A\exp[(B \times X_B^{0.5}) - (C \times X_B^3)] \quad (3)$$

$$Y_T = \frac{Y_M}{\alpha} - \frac{1-\alpha}{\alpha} \times Y_B \quad (4)$$

$$X_T = \frac{X_M}{\alpha} - \frac{1-\alpha}{\alpha} \times X_B \quad (5)$$

where in equations (2-5), *T*, *B* and *M* designate, respectively, the top phase, bottom phase and initial mixture; *X* represents the mass fraction of the inorganic salt, *Y* represents the weight fraction percentage of ILs and α is the ratio between the mass of the top phase and the total mass of the mixture experimentally determined. *A*, *B* and *C* are the constants obtained by the fitting of the experimental binodal curve by equation (1).

Each tie-line length (TLL) was determined through the application of the following equation:

$$TLL = \sqrt{(X_T - X_B)^2 - (Y_T - Y_B)^2} \quad (6)$$

In the studied system, the IL-rich phase corresponds to the top phase while the bottom phase is mainly composed of the organic salt and water.

2.2.4 PSA extraction efficiency of IL-based ABS

The ternary mixtures composition used in the partitioning experiments were chosen based on the phase diagram of the IL+ salt+ H_2O system used (153). The mixture point chosen in the biphasic region of the phase diagram was 30 wt% IL + 40 wt% salt + 30 wt% H_2O. All ABS were performed for a final weight of 50 g. Aqueous solutions of pure PSA, at concentrations of *circa* 150 ng.mL^{-1}, were used as the "water" added to each ABS. Each mixture was vigorously stirred, centrifuged for 10 min at 3500 rpm and at 25 (± 1) °C, to reach the complete PSA partitioning between the coexisting phases.

After a careful separation of the phases and their weight, the amount of PSA in each phase was quantified by SE-HPLC (Size Exclusion High-Performance Liquid Chromatography). Each phase was diluted at a 1:10 (v: v) ratio in a phosphate buffer solution before injection. A Chromaster HPLC (VWR, Hitachi) coupled to a DAD detector was used. SE-HPLC was performed with an analytical column (8 mm × 300 mm), Shodex Protein KW- 802.5. A 100 mM phosphate buffer in Milli-Q water (mobile phase) was run

isocrastically with a flow rate of 0.5 mL min $^{-1}$. The temperature of the column and auto-sampler was kept constant at 25 ºC. The injection volume was of 25 µL. The wavelength was set at 280 nm. The salt and IL interferences with the method of quantification were also verified and control samples were regularly analysed.

The percentage extraction efficiency of PSA, EE_{PSA} %, is the percentage ratio between the amount of protein in the IL-rich aqueous phase to that in the total mixture, and is defined according to equation (7):

$$EE_{PSA}\% = \frac{[\mathrm{PSA}]_{IL} \times w_{IL}}{[\mathrm{PSA}]_{IL} \times w_{IL} + [\mathrm{PSA}]_{Salt} \times w_{salt}} \quad (7)$$

where [PSA] is the protein concentration, and w_{IL} and w_{salt} are the weight of the IL-rich and salt-rich aqueous phase, respectively.

To study the concentration factors achievable with the studied ABS, a long TL was chosen and several extractions were carried out at different initial compositions along the same TL, corresponding to different concentration factors, yet maintaining the composition of each phase. After the selection of the most appropriate TL, a weight balance approach was used to define the weight fraction of each phase-forming component, namely $[P_{4444}]Cl$ and $C_6H_5K_3O_7$, to be used in each extraction corresponding to a given concentration factor.

The percentages of ionic liquid and salt required in each of these extractions resulted in the desired concentration factor, defined by equation (8),

$$CF = \frac{w_{H2O}}{w_{IL}} \quad (8)$$

where w_{H2O} and w_{IL} correspond to the weight of the aqueous solution added containing PSA and the weight of the IL-rich phase (in which the protein is enriched) in the ABS.

ABS were prepared first using the theoretical weight percentages of salt, IL and solution containing PSA, provided by the lever-arm rule, to select a mixture that could allow a maximum concentration factor of 250-fold. The ternary mixtures were prepared, firstly as control without PSA, and further by adding an aqueous solution with a PSA concentration equal to the cut-off value found in urine for PCa, namely 150 ng.mL^{-1} (71). ABS were prepared with appropriate weight percentages aiming to achieve 5, 20, 50, 100, 150, 200 and 250-fold concentration factors. Each mixture was vigorously stirred,

centrifuged for 10 min and left to equilibrate for 10 min at 25 (± 1) ° C. The phases were carefully separated and weighted, and PSA was quantified by SE-HPLC in each phase. The predicted CF was then confirmed according to equation (8). It should be remarked that the lever-arm rule allows to determine the concentration factor achievable by the studied system, i.e., the change in the volume/weight of each phase along the same TL. Different initial compositions along the same TL have the same composition of each phase, compensated by a change in volume/weight of the phases.

2.2.5 SE-HPLC analysis of real samples

The adequacy of the developed methods to extract and concentrate PSA was then investigated with real human urine samples. Before the ABS preparation, a PSA solution of 1000ng.mL^{-1}, in PBS buffer, was used to dilute PSA in the urine sample, to achieve a final concentration of 150 ng.mL^{-1}. Thereafter, appropriate amounts of IL and salt were added to reach the initial mixture compositions selected that allow a *CF* of 250-fold. All ABS were performed for a final weight of 50 g. After obtaining the ABS, a careful separation of the phases was performed and their weight was determined. The amount of PSA in each phase was quantified by SE-HPLC, by the procedures described before.

2.3 Results and discussion

2.3.1 Phase diagrams, tie-lines and concentration factors of PSA using synthetic samples

The ternary phase diagram of the studied ABS was taken from the literature (153). The experimental weight fraction data and the referred phase diagram are presented in Appendix A. In the studied ABS, the top phase corresponds to the IL-rich phase, while the bottom phase is mainly composed of citrate-based salt and water. As evaluated in a previous work (1), for the cation core effect, ILs with the cation $[P_{4444}]^+$ show the highest ability for ABS formation when compared with imidazolium-, pyridinium-, and pyrrolidinium-based ILs (1). The same trend was evidenced by Sintra *et al.* (155) in their study with ammonium-, phosphonium-, cholinium-, imidazolinum- and pyridinium-based ILs and where the potassium citrate buffer ($C_6H_5K_3O_7/C_6H_8O_7$) at pH 7 was used instead of potassium citrate. $[P_{4444}]Cl$, being a quaternary phosphonium-based cation IL, presents a high ability to form ABS, since its composed of four butyl chains which are responsible for its higher hydrophobicity (1). On the other hand, being the citrate-based salt composed of a trivalent charged anion, it is a strong salting-out species according to the Hofmeister series (36), promoting therefore the preferential exclusion of the IL to the opposite phase (37).

For the studied systems the experimental binodal data were also fitted by the empirical relationship described by equation (1) by Passos *et al.* (153) . The regression parameters are presented in Appendix A. The experimental tie-lines, determined in this work, along with their respective length, are reported in Table 2.1.

Table 2.1 - Weight fraction percentage (wt%) of the initial mixture and of the coexisting phases, and respective values of TLL.

Weight fraction composition/ (wt%)							
IL + $C_6H_5K_3O_7$ + water							
IL	Y_T %	X_T %	Y_M%	X_M%	Y_B %	X_B %	TLL
$[P_{4444}]Cl$	69.01	3.46	30.16	30.44	0.61	50.94	93.35
	81.26	2.32	29.53	39.36	0.11	60.43	99.81

Both tie-lines obtained for this system, as well as the respective binodal curve, are depicted in Figure 2.2. The pH values of this system are in the alkaline region (pH 8–10) due to the use of the citrate-based salt. Even though, no denaturation or aggregation of PSA were observed during the SE-HPLC assays.

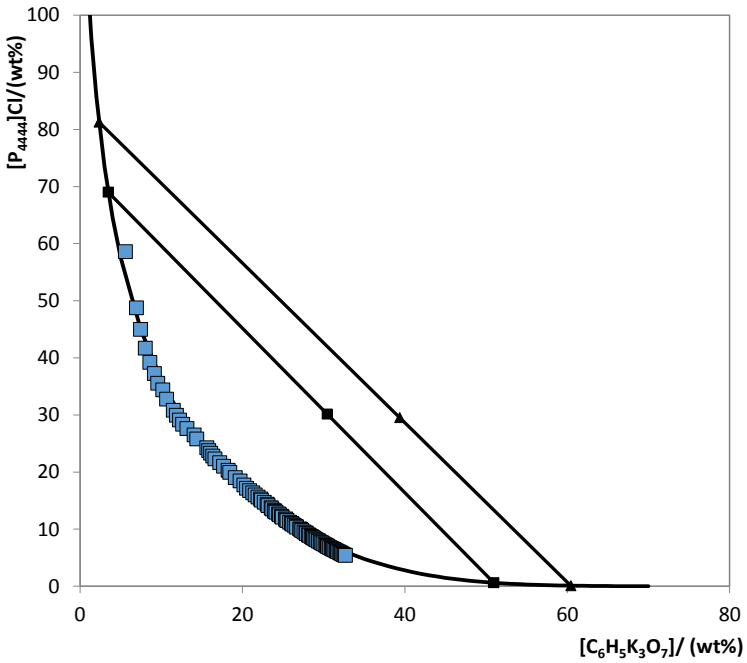

Figure 2.2 - Phase diagram for the system composed of $[P_{4444}]Cl + C_6H_5K_3O_7 + H_2O$ at 25ºC and atmospheric pressure (in wt%) (153) and respective TLs. The experimental binodal curve was adjusted using equation (1).

To verify the ability of the $[P_{4444}]Cl + C_6H_5K_3O_7 + H_2O$ system to reach several concentration factors, different initial mixture compositions along the same TL were tested as illustrated in Figure 2.3. After validation of the experimental *CF* achievable with pure water, the same ABS were tested with aqueous solution of PSA at 150 $ng.mL^{-1}$.

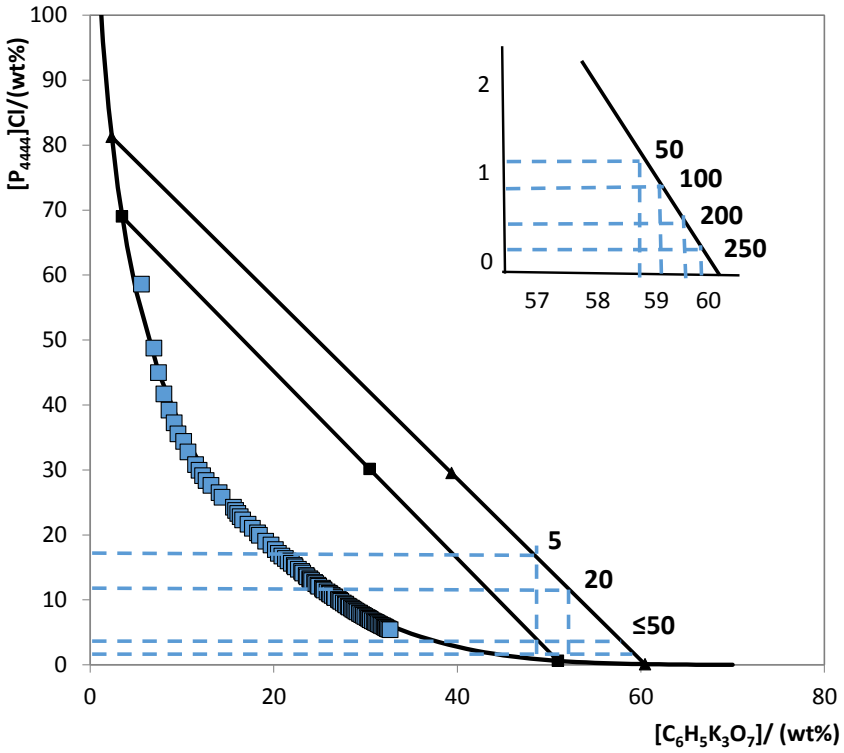

Figure 2.3 - Different compositions along the same TL for the system $[P_{4444}]Cl + C_6H_5K_3O_7 + H_2O$ which allow to achieve different *CF* values

CF were confirmed by weighting both the bottom and the top phase and by the application of equation (8). The capacity of the mixture to achieve the 250-fold *CF* was proven, allowing to reduce the volume of the IL-rich phase to a minimum capable of concentrate PSA 250 times and allowing, at the same time, its quantification by SE-HPLC. For the experiments with PSA, all *CF* tested were also achieved (Appendix B), demonstrating the ability of the system to extract and concentrate PSA in a single-step up to 250-fold.

The HPLC chromatograms of the IL- and salt-rich phase for a *CF* of 250 are shown in Figure 2.4. The PSA retention time is between 12 and 14 minutes, agreeing with the retention time of a standard PSA solution. These results show that all PSA is present at the IL-rich phase (no peak is detected at the salt-rich phase), meaning the studied system allows to completely extract and concentrate PSA in the IL-rich in a single-step. The *CF* of 250-fold allows to identify and quantify PSA by SE-HPLC with a DAD detector.

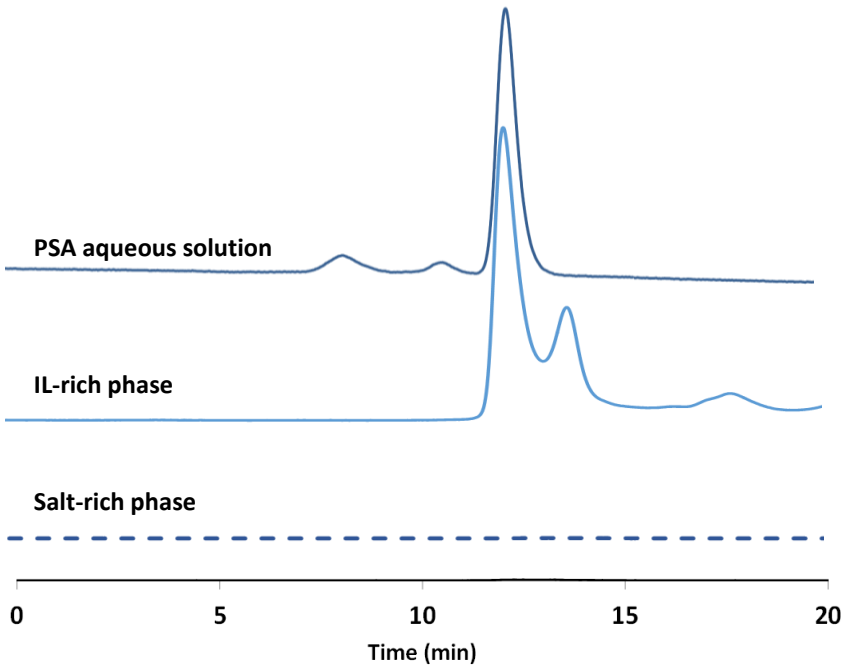

Figure 2.4- HPLC chromatogram of standard PSA aqueous solution (at 150 ng.mL^{-1}), and top (IL-rich phase) and bottom (salt-rich phase) phases of the system formed by $[P_{4444}]Cl$ + $C_6H_5K_3O_7$ + aqueous solution of PSA at 150 ng.mL^{-1} .

In summary, the obtained data show the possibility to extract and concentrate PSA up to 250-fold, in a single-step, using IL-based ABS. These findings highlight the possibility to apply these IL-based ABS to the early-stage diagnosis of prostate cancer. The studied ABS represents an additional advantage in PSA extraction and quantification, since it leads 100% of extraction efficiency with a reduced amount of IL used. Although the extraction of other proteins from human fluids with IL-based has been previously reported (134), extractions of 100% were not attained in single-step nor concentration factors up to 250-fold. The use of phosphonium-based ILs has proven to lead to promising results. According to these results, the extraction of PSA from real human fluids using the same and optimized system was further studied.

2.3.2 Extraction and concentration of PSA from human urine

The ABS composed of $[P_{4444}]Cl$ + $C_6H_5K_3O_7$ + water allows to concentrate an aqueous solution of PSA at 150 ng.mL^{-1} up to 250-fold, as demonstrated in the previous section. Therefore, the same mixture composition was used with human urine samples instead of using an aqueous solution synthetically prepared with PSA. Nevertheless, it should be remarked that urine samples of healthy patients were used and these samples were spiked with pure PSA at concentrations of 150 ng.mL^{-1} (Figure 2.5).

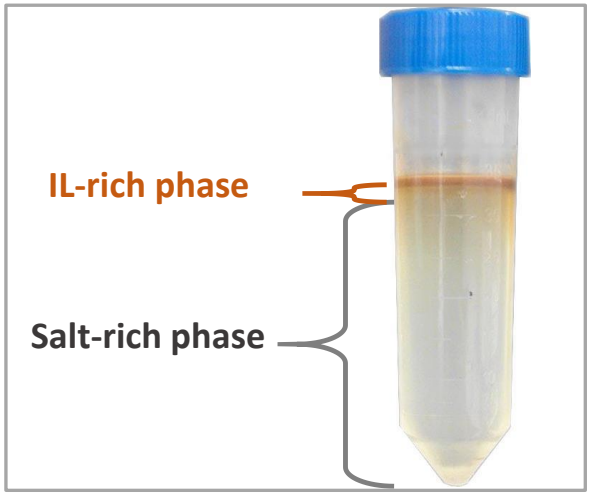

Figure 2.5- ABS composed of $[P_{4444}]Cl$ + $C_6H_5K_3O_7$ + human urine (with added PSA at 150 ng.mL^{-1}).

After the ABS equilibration, the top and bottom phases were separated and analysed by SE-HPLC, as well as a solution of pure PSA in PBS buffer and pure human urine samples. Results obtained are shown in Figure 2.6 and correspond to an aqueous solution containing standard PSA, to the urine sample with no pre-treatment, and to the top and bottom phases of the ABS for the synthetic system and for the extraction with real human urine samples.

The PSA peak in aqueous solution is evident between 12 and 15 min, being also seen in the top phase of the $[P_{4444}]Cl$-based ABS for both the synthetic and human urine samples. This peak is absent in the bottom phase of the same ABS, proving that all PSA migrates to the IL-rich phase, in single-step, even in the presence of a complex matrix such as urine. In addition, no metabolites or proteins are detected in the urine sample without pre-treatment. However, additional peaks are observed in the IL-rich phase after the extraction carried out from human urine. It is known that 2500 metabolites are present in

human urine (158), although at this stage it is not possible to identify them. Further studies by Mass Spectrometry are required to gather evidences on the major metabolites being simultaneously extracted to the IL-rich phase. Even so, the presence of these metabolites does not interfere with the identification and quantification of PSA in human urine samples after the pre-treatment strategy, meaning that IL-based ABS are remarkable systems to simultaneously extract and concentrate PSA from human fluids further allowing the quantification of PSA by SE-HPLC.

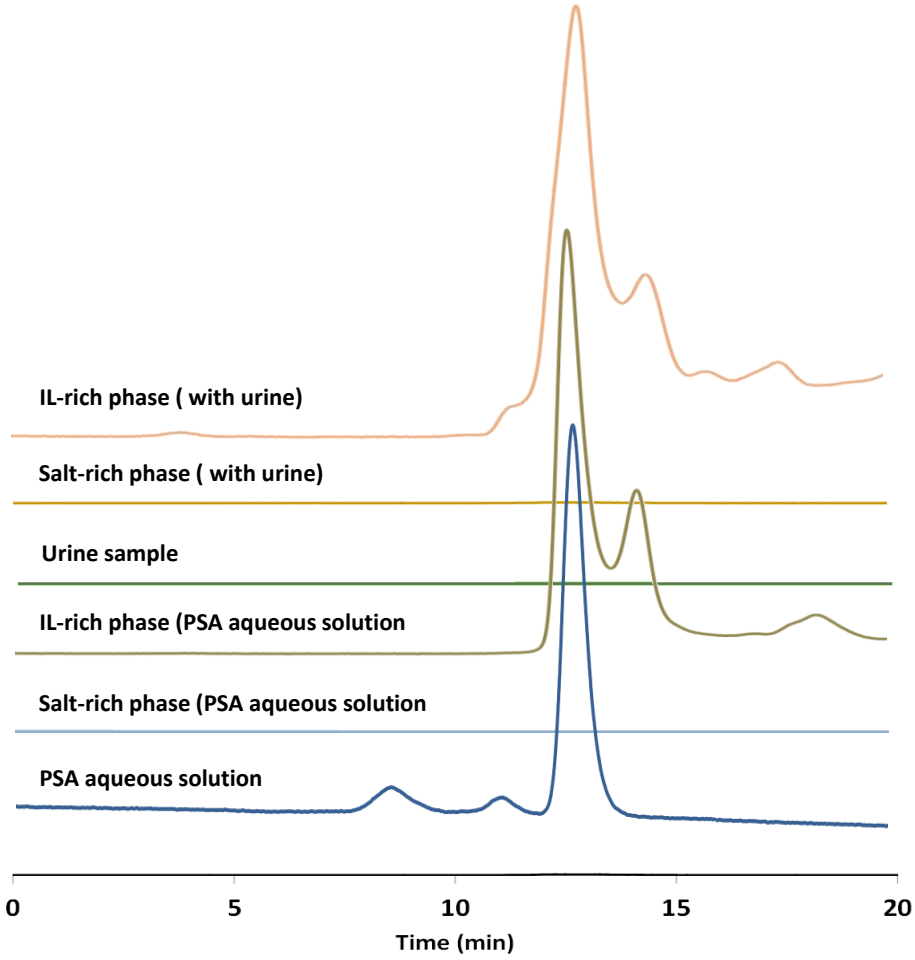

Figure 2.6 - HPLC profile of a standard PSA aqueous solution (at 150 ng.mL^{-1}), a human urine sample, and top and bottom phases of the IL-based ABS after the extraction with PSA in aqueous solution and with human urine (PSA at 150 ng.mL^{-1}).

2.4 Conclusions

The ability of IL-based ABS to completely extract and concentrate PSA up to 250-fold, in a single-step, from human urine samples, has been demonstrated. This achievement allows the identification and quantification of PSA by more expedite equipment, like HPLC with a DAD detector. Based on these results, human samples used for PCa early diagnosis can be pre-treated with IL-based ABS before proceeding for analytical quantification. Although other metabolites present in urine samples were also identified at the IL-rich phase, they do not interfere with the quantification of the target biomarker. Nevertheless, additional studies are required to identify the main metabolites present and being extracted to the IL-rich phase.

3 . Extraction and purification of LDH using IL-based ABS

3.1 Introduction

Serum is a source rich in several biochemical products that can act as biomarkers of diseases or of the physiological status of a patient (159). It is estimated that there are 10,000 different proteins in human serum, the majority of which are low-molecular-weight species (160). Albumin is the main protein found in mammals serum, being an anionic protein, with a large abundance of aspartate and glutamate residues; among all serum proteins, it has a molecular weight of 67 kDa and a longer-than-average half-life of approximately 20 days (161). Albumin is required to maintain the osmotic balance between intravascular and interstitial spaces; therefore, a deficiency in albumin ordinarily results in edema as water is redistributed to tissues. Albumin also functions as a transport protein for calcium, unconjugated bilirubin, thyroid hormones, and many drugs (161).

When particularly dealing with serum samples, the removal of interference proteins is an essential step in samples preparation for analysis (162). Protein precipitation followed by centrifugation is one of the most popular techniques for removing proteins (163). Proteins are usually less soluble when at their isoelectric point (pI); therefore, the selection of aqueous solutions of salt at pH values near the pI of the target protein is usually appropriate for their precipitation (163). However, developing a selective precipitation technique for a particular protein requires the identification of the appropriate precipitating agent and optimization of the precipitation procedure (164). Some reagents cause denaturation or adversely affect proteins bioactivity, and some form complexes that bind the protein tightly. Other methods lack the ability to selectively fractionate and enrich the desired protein (165). Moreover, the precipitation of proteins is a method not well-suited for high throughput analysis due to the multiple steps involved given the large number of proteins present in serum samples.

Proteins present in serum and plasma span a concentration range of 11 orders of magnitude, with the 20 most abundant proteins representing 97–99% of the total protein mass content (166). Hitherto, no single analytical method is capable of resolving all plasma or serum proteins, and no detection method can cover more than 4 or 5 orders of magnitude (167). Due to limitations in resolution, and because of the high abundance of a relatively small number of proteins, most analytical schemes for serum and plasma involve the depletion of high-abundance proteins to reduce both the complexity and dynamic range of the samples. On the other hand, for both laboratory and larger scales protein fractionation of biological samples, there is a crucial need to remove cellular proteins and

other components (168). It is especially important to remove proteases, as early as possible, to avoid the remaining proteins degradation.

Regarding the follow-up of patients with prostate cancer, lactate dehydrogenase (LDH) has been playing a pivotal role as a valuable biomarker (96). It is usually identified in serum samples (168). In serum, LDH is involved in the catalysis of the conversion of lactate into pyruvate. Due to its widespread localization in human tissues, the activity of this enzyme also considerably increases in blood after cell damage, which can be attributed to a large number of pathological conditions, including cardiac and liver injury, haematological disturbances, and cancer (169).

Several methods have been developed for the measurement of total LDH activity, categorized as either spectrophotometric or calorimetric (170). The bidirectional reaction is usually monitored spectrophotometrically by measuring either the increase in reduced NADH at 340 nm produced in the lactate-to-pyruvate reaction or by the decrease in NADH at 340 nm produced in the pyruvate-to-lactate reaction (168) Currently, LDH is quantified by kinetic assay systems by following the reversible reaction in both directions. The pyruvate kinase–lactate dehydrogenase coupled enzyme system has been successfully applied to the spectrophotometric determination of LDH activity (171). Although spectrophotometric assays are easy to perform and robust, they have some limitations. The reading must occur in the first seconds of reaction after adding serum samples, since most of the times all NADH is consumed even before the first reading (169). Furthermore, interferences display a significant role over the kinase activity when dealing with crude preparations or extracts (172).

Based on the exposed above, there is an essential need to develop alternative pre-treatment strategies to avoid interferences of main proteins present in plasma samples when the identification and quantification of biomarkers is required for diagnosis. Instead of quantifying the enzymatic activity of LDH, the quantification of the enzyme effective concentration could be seen as a more reliable approach.

In this section, the main goal consists on the development of pre-treatment methods of serum samples so that cancer biomarkers, such as LDH, could be extracted and major contaminant proteins removed at the same time. This strategy allows the reduction of interferences during the quantification of cancer biomarkers for diagnosis. To this end, IL-based ABS were investigated as a possible alternative to extract and purify LDH from serum samples. The major goal is to find an ABS able to completely extract LDH for the

IL-rich phase and that allows the simultaneous precipitation/removal of HSA. As a first attempt, synthetic samples where used and the best systems were finally applied to human serum samples.

3.2 Experimental Section

3.2.1 Chemicals

In this section, ABS formed by the potassium citrate buffer at pH = 7, composed of potassium citrate $C_6H_5K_3O_7 \cdot H_2O$> 99 wt% pure, from Sigma-Aldrich, and citric acid $C_6H_8O_7$, 100 wt% pure, from Misher scientific, in a ratio 25:1, were investigated. Two ILs were studied to form ABS with the citrate buffer, namely tetrabutylphosphonium bromide ($[P_{4444}]Br$), > 96 % pure, and tetrabutylphosphonium chloride ($[P_{4444}]Cl$), > 96 % pure, both from Cytec Ind.. The chemical structures of the ILs studied are represented in Figure 3.1. For HPLC analysis, sodium phosphate monobasic (NaH_2PO_4, purity: 99- 100.5%), sodium phosphate dibasic ($Na_2HPO_4 \cdot 7H_2O$, purity 98.2- 102.0 %) and sodium chloride (NaCl, purity 99.9%) were used to prepare a phosphate buffer solution at pH=7. For LDH analysis, an aqueous solution of Lactate Dehydrogenase from bovine heart (1000 $U.L^{-1}$), from Sigma-Aldrich, was employed. LDH dilutions were prepared with aqueous solutions of phosphate buffered saline (PBS), 100 wt% pure, from Sigma-Aldrich.

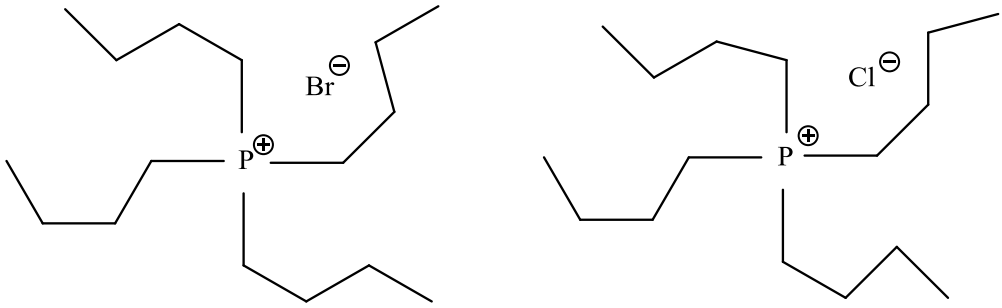

Figure 3.1- Chemical structure of the studied ILs: $[P_{4444}]Br$ and $[P_{4444}]Cl$.

3.2.2 Biological samples

Sterile-filtered serum human samples from male individuals, AB type of plasma, from Sigma-Aldrich were used.

3.2.3 Phase diagrams, tie-lines and LDH extraction and purification with IL-based ABS

The binodal curves required to infer on the mixture compositions needed to form two-phase systems were taken from the literature (136), determined thought the cloud point titration method at 25 (±1) ºC and atmospheric pressure (Appendix A). The experimental procedure adopted was described before in section 2.2.3.

Tie-lines were determined by the gravimetric method and using the lever-arm rule originally proposed by Merchuk *et al* (154). For this purpose, a selected mixture at the biphasic region was chosen and prepared by weighting the appropriate amounts of IL+ salt + water, followed by stirring. Similar experimental conditions were adopted to the ones described in section 2.2.3. The experimental binodal curves were fitted by equation 1, the TLs determined by equations (2-5), and the TLL determined through the application of equation (6), previously described in section 2.2.3.

For the partitioning experiments, it was chosen a specific ternary mixture composition based on the phase diagrams used in this section, namely 30 wt% IL+ 30 wt% salt+ 40 wt% of LDH aqueous solution/serum for both IL-based systems, all systems were performed for a final weight of 1 g. The LDH aqueous solution used was prepared by dilution of the same in PBS aqueous solution to achieve a final concentration of 100 U.L-1, used to prepare the synthetic samples. For systems with human serum, serum sample was used by addition to the system in its pure state. Each mixture was vigorously stirred, centrifuged for 10 min at 3500 rpm and at 25 (± 1) ° C, to reach the complete partitioning of the enzyme between the coexisting phases.

After a careful separation of the phases, the amount of LDH in each phase was quantified by SE-HPLC according the procedure described in section 2.2.4. The calibration curve for LDH is represented in Appendix C. The limit of detection of LDH was found to be 25 $U.L^{-1}$. The salt and IL interferences with the method of quantification were also verified and control samples were regularly analysed.

The percentage extraction efficiency of the studied systems for LDH, EE_{LDH}%, is the percentage ratio between the amount of protein in the IL-rich aqueous phase to that in the total mixture, and is defined according to equation 9:

$$EE_{LDH}\% = \frac{[LDH]_{Il} \times w_{IL}}{[LDH]_{Il} \times w_{IL} + [LDH]_{Salt} \times w_{Salt}} \times 100 \quad (9)$$

where [LDH] is the LDH concentration, and w_{IL} *and* w_{salt} are the total weight of the protein in the IL-rich and in the salt-rich aqueous phase, respectively.

The percentage extraction efficiency of serum proteins, $EE_{\text{SerumProteins}}\%$, is the percentage ratio between the amount of protein in the IL-rich aqueous phase to that in the total mixture, and is defined according to equation 10:

$$EE_{SerumProteins}\ \% = \frac{[SerumProteins]_{Il} \times w_{IL}}{[SerumProteins]_{Il} \times w_{IL} + [SerumProteins]_{Salt} \times w_{Salt}} \times 100 \quad (10)$$

where [SerumProteins] is the serum proteins concentration, and w_{IL} *and* w_{salt} are the total weight of the protein in the IL-rich and in the salt-rich aqueous phase, respectively.

The percentage of precipitation efficiency of LDH, $PE_{\text{LDH}}\%$, was evaluated basing in the amount of LDH detected in IL-rich phase and salt-rich phases, since no LDH was quantified in both phases, all LDH was admitted to be present in the third phase, being result of the percentage ratio between the amount of protein in the third phase and the amount present in the total mixture.

$$PE_{LDH}\% = \frac{[LDH]_{3rdPhase}}{[LDH]_{total}} \times 100 \quad (11)$$

where [LDH] is the concentration of LDH.

The percentage of precipitation efficiency of serum proteins, $PE_{\text{SerumProteins}}\%$, was evaluated basing in the amount of serum proteins detected in IL-rich phase and salt-rich phases, since no serum proteins were quantified in both phases, all serum proteins were admitted to be present in the third phase, being result of the percentage ratio between the amount of protein in the third phase and the amount present in the total mixture.

$$PE_{SerumProteins}\ \% = \frac{[SerumProteins]_{3rdPhase}}{[SerumProteins]_{total}} \times 100 \quad (12)$$

where [SerumProtein] is the concentration of serum proteins.

3.2.4 Extraction and purification of LDH from serum samples

After extractions with both IL-based ABS in the point of mixture refered in previous section was possible to select the best IL-based ABS to use in real sample treatment. From the [P_{4444}]Br-based phase diagram and respective tie-lines determined in the previous section, it was possible to select several mixture points in the biphasic region, by varying the IL and salt percentages. Several initial mixture compositions formed by 30 wt% IL+ 15, 20 and 30 wt% salt + respective wt% of LDH solution and serum were tested, and for the variation of IL, the points of mixture 20, 30 and 45 wt% IL+ 30 wt% salt + respective wt% of LDH solution and serum were evaluated. The LDH aqueous solution used was prepared by dilution of the same in PBS aqueous solution to achieve a final concentration of 100 $U.L^{-1}$, used to prepare the synthetic samples. For systems with human serum, serum sample was used by addition to the system in its pure state. After optimization, the best point of mixture was selected for the extraction of LDH and precipitation of serum protein from serum samples. The extraction and purification of LDH in real samples was performed by the dilution of 1:10 of initial LDH solution directly in the serum sample to reach a concentration of 100 $U.L^{-1}$.

Each mixture prepared was vigorously stirred, centrifuged for 10 min at 3500 rpm and at 25 (± 1) ° C. After the separation of the phases, the amount of LDH in each phase was quantified by SE-HPLC by the procedure described in section 2.2.4. The presence of serum contaminants proteins was also confirmed. The salt and IL interferences with the analytical method were also verified and control samples were regularly analysed.

3.2.5. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Human serum proteins present in third phase of the IL-based ABS were analysed by SDS-PAGE. The serum proteins were diluted 2:1 (v:v) in PBS buffer before its aplication in SDS-PAGE method thanks to the high protein content of the sample. Samples of the aqueous phases of each ABS containing ovalbumin were diluted at 2:1 (v:v) in a dissociation buffer consisting of 2.5 mL of 0.5 M Tris-HCl pH 6.8, 4.0 mL of 10 % (w/v) SDS solution, 2.0 mL of glycerol, 2.0 mg of bromophenol blue and 310 mg of dithiothreitol (DTT). This overall solution was heated at 95ºC for 5 min to denature the proteins by reducing disulfide linkages, and thus overcoming some forms of the tertiary

protein folding and breaking up the quaternary protein structure. Electrophoresis was run on polyacrylamide gels (stacking: 4 % and resolving: 20 %) with a running buffer constituted by 250 mM Tris-HCl, 1.92 M glycine, and 1 % SDS. The proteins were stained with Coomassie Brilliant Blue G- 250 0.1 % (w/v), methanol 50 % (v/v), acetic acid 7 % (v/v) and water 42.9 % (v/v). All gels were placed in an orbital shaker at a moderate speed during 2-3 h at room temperature. The gels were further distained in a solution containing acetic acid at 7 % (v/v), methanol at 20 % (v/v) and water at 73 % (v/v) in an orbital shaker at a moderate speed during 3-4 h at room temperature. SDS-PAGE Molecular Weight Standards, namely marker molecular weight full-range from VWR, were used as protein standards.

3.3 Results and discussion

3.3.1 Phase diagrams, tie-lines and extraction of LDH using IL-based ABS

The phase diagrams for the systems consisting of IL + H_2O + $C_6H_5K_3O_7/C_6H_8O_7$ (pH = 7.0) were determined at 25 ◦C and atmospheric pressure (136) and are depicted in in Appendix A. All the calculations considering the weight fraction of the phase-forming components were carried out discounting the complexed water in the commercial citrate-based salt. The phase diagrams reported before (136) for the systems constituted by phosphonium-based ILs allow the evaluation of both ILs ability to form ABS, following the order $[P_{4444}]Br$ > $[P_{4444}]Cl$. This is in close agreement with the trend previously described in the literature for ABS composed of phosphonium-based ILs +K_3PO_4 + H_2O (144). The Cl^- anion presents a higher aptitude to be hydrated or to form hydration complexes, and thus more salt is required to induce the salting-out when compared with bromide-based ILs (168).

The experimental data corresponding to the binodal curves were fitted using equation (1) and this correlation is shown Appendix A. The regression parameters estimated by least-squares regression, standard deviations (σ) and correlation coefficients (R^2) are displayed in Appendix A. The experimental TLs in each ABS, along with their respective length, and at the compositions for which the extraction studies were conducted are reported in Table 3.1. These allow to identify the content of each component in the coexisting phases for each initial mixture composition. The tie-lines obtained for both systems are depicted in Figure. 3.2.

Table 3.1 - Weight fraction percentage (wt%) for the coexisting phases of ionic liquid (IL)+ potassium citrate (salt) + H_2O, and respective values of TLL.

Weight fraction composition/ (wt%)							
IL + $C_6H_5K_3O_7/C_6H_8O_7$ + H_2O							
IL	Y_T %	X_T %	Y_M%	X_M%	Y_B %	X_B %	TLL
$[P_{4444}]Cl$	80.98	0.83	28.82	29.10	0.56	44.43	91.48
	75.06	1.20	27.08	27.09	1.08	41.11	84.05
$[P_{4444}]Br$	58.75	0.05	29.17	29.38	0.02	58.30	82.72
	52.02	0.21	19.99	20.30	1.74	31.75	59.35

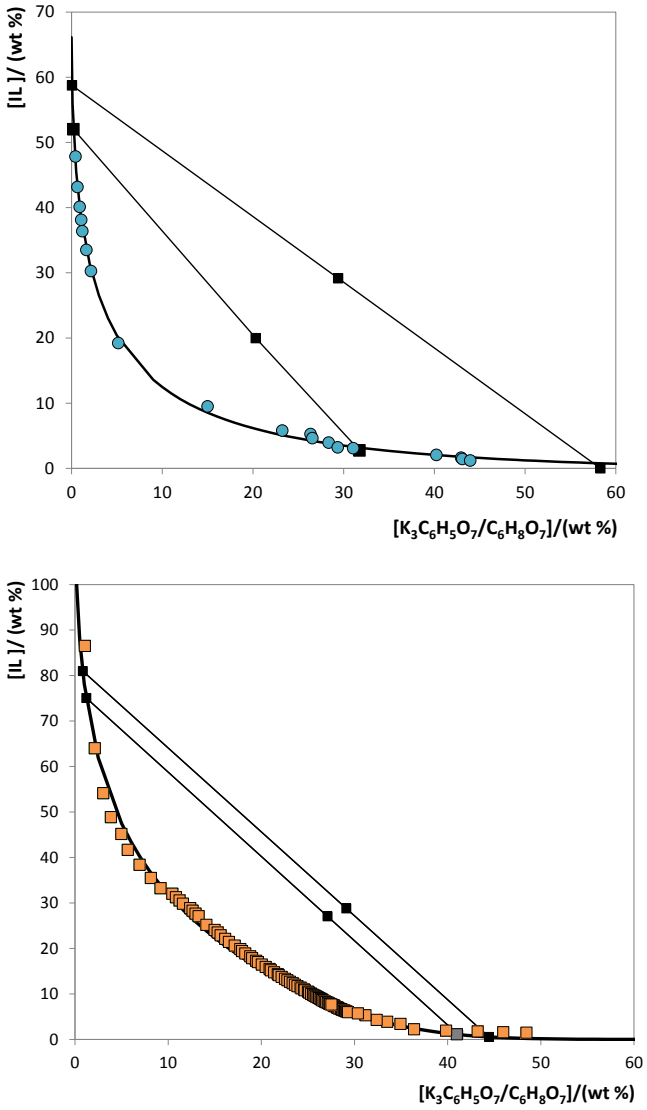

Figure 3.2- Phase diagrams at 25 ◦C and atmospheric pressure (2), correlation of the binodal data using equation (1), and respective TLs for the systems composed of C_6H_5 $K_3O_7/C_6H_8O_7$ + H_2O + $[P_{4444}]Br$ (●); $[P_{4444}]Cl$ (■).

The extraction efficiencies of LDH and serum proteins with the studied IL-based ABS were initially addressed for a mixture composition of 30 wt% of C_6H_5 $K_3O_7/C_6H_8O_7$, 30 wt% IL and 40 wt% of an aqueous solution containing either LDH at 100 $U.L^{-1}$ or human serum, and calculated according equations (9) and (10) and efficiency of extraction accordinh equations (11) and (12). Both aqueous solutions were tested using both ILs: $[P_{4444}]Cl$ and $[P_{4444}]Br$. The results obtained are depicted in Figure 3.3.

In this mixture composition, it is possible to verify that both types of IL-based ABS lead to the complete extraction of LDH to the IL-rich phase, with extraction efficiencies of

100% attained in a single-step and with no precipitation of this protein in both systems studied.

However, in the serum protein extraction, it was possible to verify that although the system $C_6H_5K_3O_7/C_6H_8O_7$ + $[P_{4444}]Cl$ is able to extract completely not only LDH but also serum proteins with extraction efficiencies of 100% attained in a single-step, the $[P_{4444}]Br$-based ABS, does not extract serum proteins for IL-rich phase, contrarily it leads to the complete precipitation of major serum proteins (also macroscopically visible, precipitates at the interface).

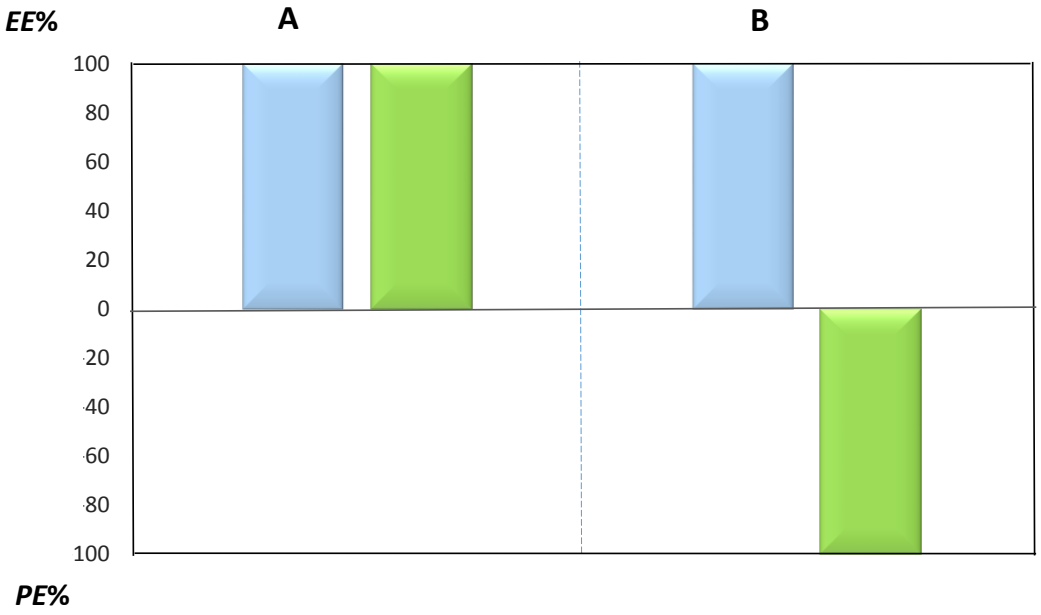

Figure 3.3- Extraction efficiency (*EE%*) and precipitation efficiency (*PE%*) for LDH (■) and serum proteins (■) at 25ºC and pH 7.0 in the ABS composed of IL+ $C_6H_5K_3O_7/C_6H_8O_7$ + aqueous solution, using both ILs studied $[P_{4444}]Cl$ (A) and $[P_{4444}]Br$ (B).

Concordant results were obtained by HPLC analysis of LDH aqueous solution and serum sample after the pre-treatment with IL-based ABS (Appendix D). The extraction of LDH from a standard aqueous solution displays a peak between 15 and 17 minutes in the HPLC chromatogram, corresponding to LDH, and is seen in both top phases of the $[P_{4444}]Cl$-based and $[P_{4444}]Br$-based ABS. The extraction efficiency of 100% of both systems is further confirmed by the absence of the same peak in the chromatograms corresponding to the bottom phases. For extractions carried out from serum samples, in $[P_{4444}]Cl$-based ABS an evident peak between the 17 and 18 minutes is verified. The bottom phase of this ABS presents no visible peaks, confirming that the extraction occurs with 100% efficiency. In serum extraction with $[P_{4444}]Br$-based ABS, no peaks are

observed in the HPLC chromatogram, confirming that serum proteins are precipitated with 100% efficiency.

This behaviour of precipitation was recently reported by Pereira *et al.* (136), who demonstrated that [P_{4444}]Br-based ABS induces the complete precipitation of bovine serum albumin (BSA). In this work (136), a large variety of ABS were able to completely extract BSA for the IL-rich phase – with extraction efficiencies of 100%, with the exception of [P_{4444}]Br. This IL, as the most hydrophobic IL investigated, led to the complete precipitation and/or denaturation of BSA. The data presented in this previous work (136) and in the present work indicate that changes in the protein structure may result from specific interactions occurring between the protein and this particular IL. Hydrophobic interactions responsible for proteins precipitation have been also reported with polymers, organic solvents and salts (174,175). Protein stability and enzyme activity can be strongly influenced by the type of salts present in the aqueous solution (176). For instance, Dryer *et al.* (177) reported that a dehydrogenase is more stable in the presence of ILs with more hydrophilic or salting-out-inducing anions, like Cl^-. Br^- has a weaker ability to hydrogen-bond with water and to form hydration complexes, since it is a weaker salting-out anion than Cl^- according to the Hofmeister series (176), a feature that seems to be responsible for promoting the protein denaturation. Although [P_{4444}]Br leads to the complete precipitation of serum proteins in a single-step, at the same time, it allows the complete extraction of LDH to the IL-rich phase and is capable to maintain the stability of the target biomarker. It is expected that different ions interact in different ways with different proteins, resulting in the precipitation of some proteins and enhancing the stability of others. Still, the understanding of the molecular-level mechanisms responsible for this phenomenon requires more detailed investigation.

Given the ability of [P_{4444}]Br-based ABS to extract with 100% efficiency LDH while precipitating completely the serum proteins, this system was selected for further studies in the biomarkers quantification in human serum, since it's the most advantageous in serum sample treatment while maintaining the extraction efficiency.

In order to optimize this IL-based ABS for serum sample treatment, reducing the amount of salt and IL required to form ABS is needed, while keeping their ability to completely extract LDH and to completely precipitate serum proteins, in a single-step. In this sense, a series of ABS containing variable concentrations of [P_{4444}]Br (15-45 wt%) and salt (10–30 wt%) were then evaluated. The effect of the salt concentration through the

extraction efficiency of LDH and serum protein precipitation is shown in Figure 3.4. All compositions studied allow the complete extraction of LDH and complete precipitation of serum proteins, without observing the precipitation and/or denaturation of the target biomarker.

In the extraction/precipitation of serum proteins from serum samples, the mixture composed of 30 wt% of IL and 10 wt% of salt presented a relatively small top-phase compared to the bottom phase, which did not allow to separate the interface compose of precipitated serum proteins; however, through the quantification of bottom and top phases for same mixture composition by HPLC, it was possible to infer that there was no serum proteins present, meaning that it was completely precipitated. However, this system is not suitable for further studies due its relatively small top-phase, which makes difficult LDH quantification.

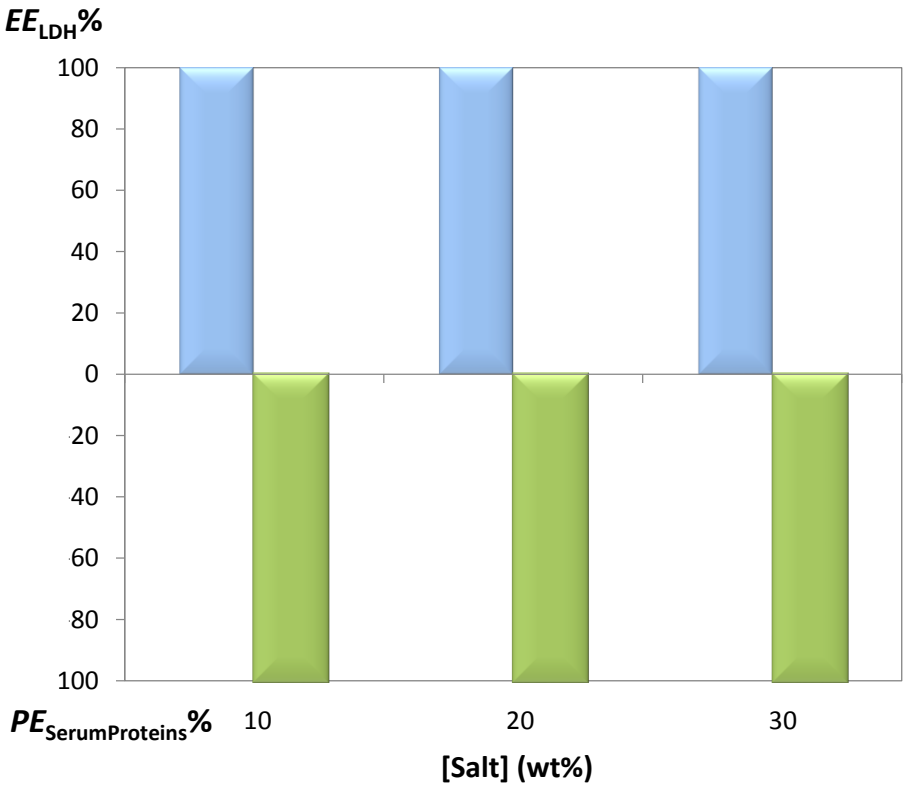

Figure 3.4- Extraction efficiency of LDH (EE_{LDH}%) and precipitation efficiency of serum proteins ($PE_{SerumProteins}$%) for the ABS composed of $[P_{4444}]Br+C_6H_5K_3O_7/C_6H_8O_7$ from a model aqueous solution: LDH (■) and serum (■) for different points of mixture variating salt amount, at 25ºC and pH 7.0.

Once the variation of the salt amount has been reported to drive the migration of proteins to the top phase due to a decrease in the difference of the electrostatic potential between the coexisting phases (178). Basing in this observations, and once low salt concentrations have less interference in proteins stability (179), the salt percentage chosen for further extractions with real samples, was 20% of $C_6H_5K_3O_7/C_6H_8O_7$. The effect of the IL concentration through the extraction and precipitation efficiency of the LDH and serum proteins for the studied systems was also investigated. The results obtained are presented in Figure 3.5.

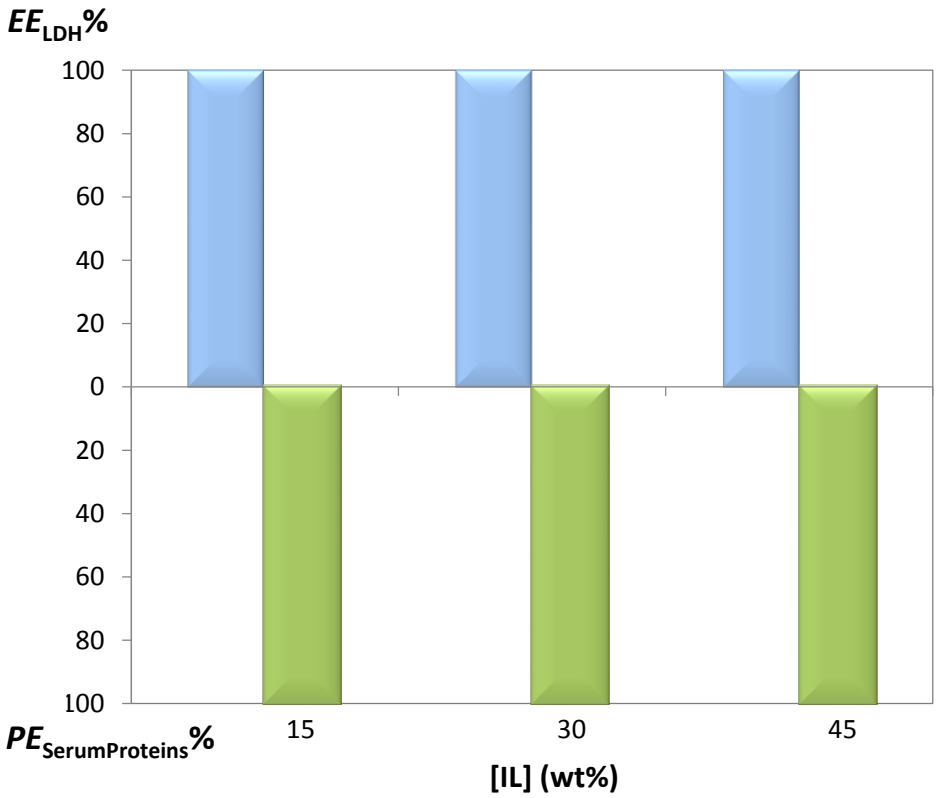

Figure 3.5- Extraction efficiency of LDH (EE_{LDH}%) and precipitation efficiency of serum proteins ($PE_{SerumProteins}$%) for the ABS composed of $[P_{4444}]Br$ and $C_6H_5K_3O_7/C_6H_8O_7$ from a model aqueous solution: LDH (■) and serum (■) for different points of mixture variating IL amount, at 25ºC and pH 7.0.

The initial mixture formed by 30 wt% of $C_6H_5K_3O_7/C_6H_8O_7$, 15 wt% of IL and using directly a serum sample, presented relatively low volume top-phase, not allowing the possibility to separate serum proteins in the interface from top-phase. However, during the quantification by HPLC, no serum proteins were detected in both phases, meaning that serum proteins have undergone a precipitation effect.

It should be remarked that in this mixture points there are some losses of protein. The mass balance for LDH extractions reveal losses between -5.75-2.02% for the mixture compositions referred before. Although the mixture formed by 30 wt% of IL+ 30 wt% salt is the one which leads to a lower loss of protein while maintaining a good separation of the coexisting phases, the mixture corresponding to 45 wt% of IL + 20 wt% of salt has additional advantages, namely a larger volume of the top-phase (IL-rich where LDH is enriched) and a well-defined third phase corresponding to precipitation of serum proteins, displaying thus additional benefits when carrying out the quantification of both proteins.

In summary, the system $[P_{4444}]Br + C_6H_5K_3O_7/C_6H_8O_7$ (pH=7) seems promising to the extraction of LDH to the IL-rich phase and simultaneous precipitation of serum proteins (eliminating thus the major contaminant protein) from serum samples. Even though, further studies are required to evaluate if there are other serum proteins, present in lower content, which are concomitantly precipitated in the process. The obtained data represent a step forward towards the quantification of serum biomarkers, like LDH, since the sample purification can be achieved in a single-step. However, the applicability of these systems in real serum samples is required to guarantee their widespread use in early-stage PCa diagnosis.

3.3.2 Extraction and purification of LDH from human serum

Since the initial mixture formed by 45 wt% $[P_{4444}]Br$ + 20 wt% $C_6H_5K_3O_7/C_6H_8O_7$ allows the best extraction *versus* precipitation and a better phases' separation for further analysis, according to the investigations carried out in the previous section, it was then applied to carry out the simultaneous extraction of LDH and precipitation of serum protein from serum samples. To this end, LDH was added to serum samples to reach a concentration of 100 $U.L^{-1}$. After the ABS preparation with real serum samples, both phases were separated and analysed by SE-HPLC.

Figure 3.6 represents the macroscopic appearance of an extraction carried out with the optimized ABS from a human serum sample, with both ILs. It is visually evident the precipitation of serum proteins as a third phase with $[P_{4444}]Br$-based ABS, a phenomenon not visible with $[P_{4444}]Cl$-based ABS.

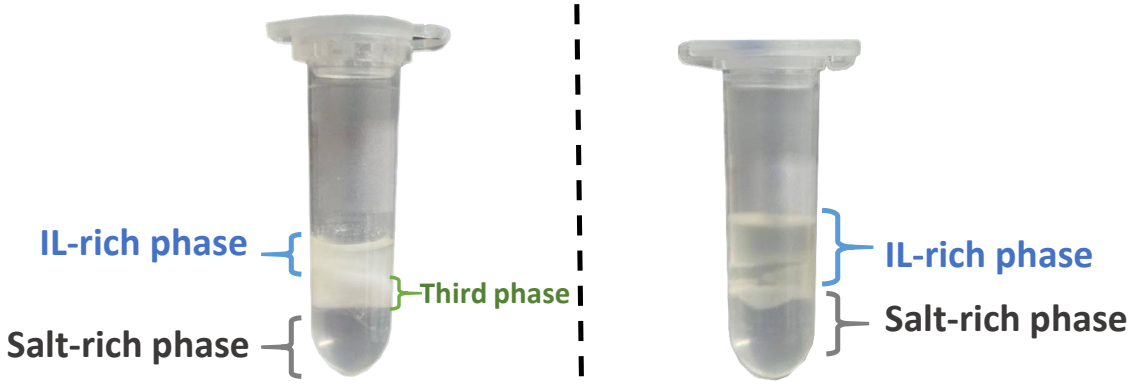

Figure 3.5 – Pre-treatment of serum samples spiked with LDH using ABS composed of $[P_{4444}]Br$ + $C_6H_5K_3O_7/C_6H_8O_7$ (in the left) and of $[P_{4444}]Cl$ + $C_6H_5K_3O_7/C_6H_8O_7$ (in the right), evidencing the presence and absence of a third phase corresponding to precipitated protein.

The SE-HPLC chromatograms used for the LDH and serum proteins quantification in each phase confirm the presence of LDH, only at the IL-rich phase, as well as the absence of LDH aggregates or the protein fragmentation – Figure 3.6. The SE-HPLC chromatograms for standard solutions of both proteins as well as for a serum sample (with no pre-treatment) are also shown in Figure 3.6. From the protein profile in serum samples it is evident that the major contaminant protein is HSA. LDH is not detectable since the commercial serum samples used correspond to healthy volunteers. For this reason it should be remarked that before the ABS pre-treatment step, serum samples were spiked with LDH to reach a final concentration of 100 $U.L^{-1}$. Less intense peaks are also visible in the HPLC profile of the human serum samples; however, further studies, are required to identify which proteins are present.

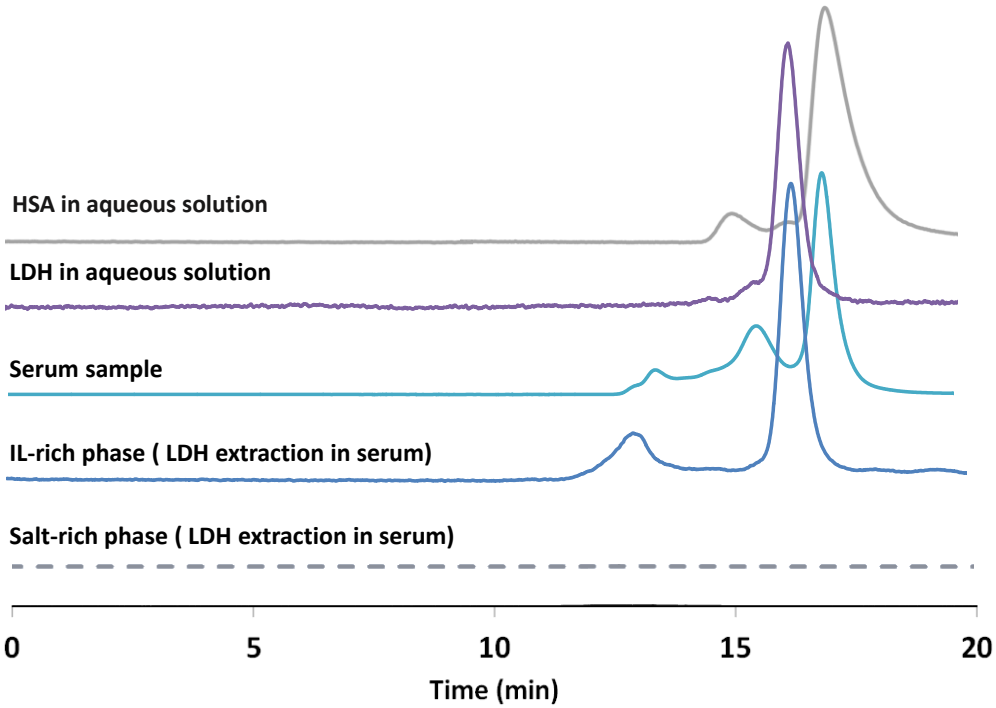

Figure 3.6- SE-HPLC chromatogram of standard LDH and HSA aqueous solutions, serum sample (dilution 1:10 in PBS solution) without pre-treatment, and top and bottom phases of the ABS composed of $[P_{4444}]Br + C_6H_5K_3O_7/C_6H_8O_7$ with serum samples.

The peak of LDH in aqueous solution at concentration of 100 $U.L^{-1}$ has a retention time of 16.48 minutes in the HPLC chromatogram. This peak is also evident in the HPLC profile of the IL-rich phase and is absent in the salt-rich phase. In summary, and from the results obtained, it is clear that IL-based ABS allow the complete extraction of LDH to the IL-rich phase in a single-step, and at the same time allow the removal of at least two proteins from serum samples which act as major contaminants (retention times of 15.88 and 17.26 min), acting simultaneously as extraction and purification platforms. Since human serum presents at least 375 distinct proteins, and albumin is in major content in serum samples, is possible to infer that the major contaminant with the highest peak, must correspond to this specific protein (180). Basing in HSA retention time, of 17.26 minutes, is possible to conclude that this protein has the same retention time that one of the major contaminants found in serum sample chromatogram. Therefore, the IL-based ABS allows the complete removal of the HSA from the IL-rich and salt-rich phase. However, other proteins of high concentration can be precipitated as well, having similar retention times.

To evaluate which proteins are precipitating in third phase, SDS-PAGE was performed, and the respective results are depicted in Fig. 3.7.

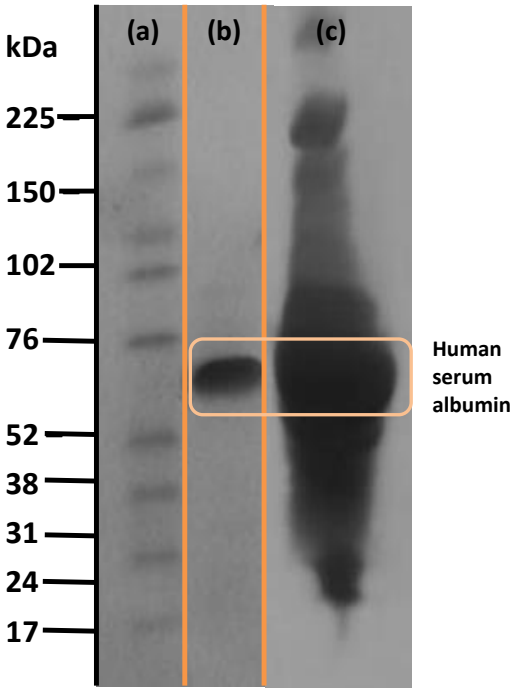

Figure 3.7-SDS-PAGE analysis of serum proteins in third phase of IL-based ABS. (a) Molecular weight marker; (b) HSA sample, 1g.L^{-1}; (c) human serum proteins precipitated in third phase of the IL-based ABS (diluted 2:1 in PBS buffer).

According to literature, SDS-PAGE presents approximately 3700 distinct protein spots for human serum proteins, basing in the observation of Figure 3.7, not only HSA is precipitated in the third phase, but proteins of high concentration in serum like, IgG, antitrypsin, transferrin, haptoglobin, fibrinogen, alpha2-macroglobulin, may be precipitated as well (180,181). Which is in accordance with the results obtained, once these proteins appear in SDS-PAGE gel spots from 150kDa to 17 kDa.

The referred observations allows to conclude that the IL-based ABS studied represents an effective pre-treatment method for complex samples such human serum, being able to remove the high protein content that interferes in cancer biomarkers quantification, like LDH.

3.4 Conclusions

Taking into account the previous results, it is possible to conclude that IL-based ABS have a remarkable potential to be applied as pre-treatment strategies of human fluids in cancer diagnosis. Optimized ABS allow the simultaneous extraction of the target biomarker (LDH) and the precipitation of the most abundant protein in human plasma (HSA). This pre-treatment approach allows the analysis of serum samples by HPLC, and making use of its effective concentration instead of enzymatic activity. This fact is particularly relevant since effects of solvents on enzymatic activity and respective interferences are completely eliminated. At this stage, the validation of the proposed pre-treatment method needs to be more deeply evaluated by their application with a wide number of samples, and the correct identification of all precipitated proteins and those at the IL-rich phase need to be pursued.

4. Final remarks and future work

IL-based ABS were here proposed as pre-treatment methods of human fluids for the extraction, concentration and purification of cancer biomarkers. IL-based ABS allow the complete extraction and concentration of PSA up to 250-fold, a single-step, to the IL-rich phase. This remarkable achievement allows the identification and quantification of PSA in human urine samples at cut-off values indicative of prostate cancer. Nevertheless, it is still needed to characterize and identify the remaining urine metabolites also present in the IL-rich phase. IL-based ABS also allowed the complete extraction and purification of LDH from human serum, a single-step. In this particular case, optimized IL-based ABS allow the complete extraction of the target biomarker and the simultaneous and complete precipitation of the major contaminant protein present in serum, which leads to a purified sample for further analysis. However, and as stated before with the PSA samples, also in this type of studies it is still required to identify the remaining contaminant proteins which are precipitating along with HSA.

The results presented in this work reinforce the opportunity of using IL-based ABS as pre-treatment strategies of human fluids for a more reliable and accurate PCa diagnosis. The developed pre-treatment methods allow the identification and quantification of PSA and LDH in human fluids by more expedite equipment, such as HPLC. The future routine use of HPLC for biomarkers quantification, instead of the currently applied immunoassays, will provide more reliable result while avoiding the high current number of false positive and negative results. Nevertheless, much more work is required on this direction to implement the combined pre-treatment-quantification approach in clinical laboratories. A widespread and deep validation of the technology still needs to be conducted by applying them in the treatment and analysis of a significant number of human fluids from PCa patients. It should be highlighted that samples from a group of healthy individuals and prostate cancer volunteers are being currently collected, and that we continue to work on this subject in the near future. In a first approach, ABS capable of extracting LDH and precipitating human albumin (as the major contaminant) from serum in a single step will be investigated. As a second approach, ABS for the extraction and concentration of PSA from urine samples will be studied. Both biomarkers will be identified and quantified by HPLC-DAD, and both approaches will be applied to real samples collected from 50 control and 50 prostate cancer volunteers (40-85 years-old men) from Centro Hospitalar Cova da Beira, Covilhã, Portugal. The samples already started to be collected and are being

preserved at -80ºC. The samples collection was approved by the University of Beira Interior, Faculty of Health Sciences Ethics Committee (CE-FCS-2015-021).

5. REFERENCES

1. Exploring cancer proliferative signaling pathways. (2015) Scientific T. Accessed on September 29, 2015; Available from: http://www.thermofisher.com/content/dam/LifeTech/global/promotions/global/images/aai-2015/aai pdfs/CancerProliferativePathwaysHandbook-r2.pdf.
2. World Health Organization. (2012) Prostate Cancer Estimated Incidence, Mortality and Prevalence Worldwide in 2012. Accessed on September 29, 2015; Available from: http://globocan.iarc.fr/pages/fact_sheets_cancer.aspx.
3. Henry, N. L., Hayes, D. F. (2012) Cancer biomarkers. Molecular Oncology; 6(2):140–6.
4. Thomas, C. M. G, Schmitt, M., Sweep, F. (2006) Analytical Aspects of Biomarker Immunoassays in Cancer Research; 17–31.
5. Longo, G. (2014) Detected twice for good measure A DNA that conducts. Nature Publishing Group; 9(12):959–60.
6. World Health Organization (2015) Cancer. Accessed on October 3, 2015; Available from: www.who.int/mediacentre/factsheets/fs297/en/.
7. World Cancer Research Fund International (2012) Worldwide data. Accessed on October 3, 2015; Available from: www.wcrf.org/int/cancer-facts-figures/worldwide-data.
8. World Cancer Research Fund International (2012) Data for cancer frequency by cancer. Accessed on October 3, 2015; Available from: www.wcrf.org/int/cancer-facts-figures/data-cancer-frequency-country.
9. Jemal, A., Bray, F., Ferlay, J. (2011) Global Cancer Statistics: 2011. CA: A Cancer Journal for Clinicians; 61:1:69–90.
10. International Agency for Research on Cancer. World Health Organization (2012) Acessed on October 4, 2015; Available from: http://eco.iarc.fr/eucan/Country.aspx?ISOCountryCd=620.
11. Ferlay, J., Shin, H., Bray, F., Forman, D., Mathers, C., Parkin, D. M. (2010) Estimates of worldwide burden of cancer in 2008: Globalcan 2008. International Journal of Cancer; 127, 2893–2917.
12. Kumar, V., Abbas, A., Aster J. (2013) Robbins Basic Pathology. Elsevier Saunders; 9th edition.
13. Yates L. R., Campbell P. J. (2013) Evolution of the cancer genome. Nature Reviews Genetics; 13 (11):795–806.
14. Pepper, J. W., Findlay, C. S., Kassen, R., Spencer S. L., Maley C. C. (2009) Cancer research meets evolutionary biology. Evolutionary Applications; 2(1):62–70

15. Hanahan, D., Weinberg, R. A. (2011) Hallmarks of cancer: the next generation. Cell. Elsevier Inc.; 144(5):646–74.
16. Hanahan, D., Weinberg, R. A., Francisco, S. (2000) The Hallmarks of Cancer. Cell. Elsevier Inc.; 100:57–70.
17. Lane, D. P. (1992) Cancer. p53, guardian of the genome. Nature. 358:15–16.
18. Wu, H., Chang, D., Huang, C. (2006) Targeted-therapy for cancer. Journal of cancer molecules; 2(2):57–66.
19. Sirica, A. (1989) The pathobiology of neoplasia. Plenum Press; 1:25–38.
20. Baba, A. I., Câtoi, C. Comparative Oncology. Academic Press. 2007.
21. Luo, J., Duggan, D.J., Chen, Y., Sauvageot, J., Ewing, C.M., Bittner, M.L., Trent, J. M., Isaacs, W. B., (2001) Human prostate cancer and benign prostatic hyperplasia: Molecular dissection by gene expression profiling. Cancer Research; 61(12): 4683–4688.
22. Ziada, A., Rosenblum, M., Crawford, E. D. (1999) Benign prostatic hyperplasia: an overview. Urology; 53(3):1–6.
23. Spickett, I., Robertson, J. (2010) Prostate cancer: the ongoing challenge. Men's Health; 20.
24. Dunn, M. W. and Kazer, M.W. (2011) Prostate cancer review. Semin Oncology Nurs; 27(4):241–50.
25. Damber, J., Aus, G. (2008) Prostate cancer. The Lancet; 371:1710-1721.
26. Bostwick, D. G., Burke H. B., Djakiew, D., Euling, S., Ho S., Landolph, J., Morrison, H., Sonawane, B., Shifflett, T., Waters, D. J., Timms, B. (2004) Human prostate cancer risk factors. Cancer, 2371–490.
27. Loeb, S., Schaeffer, E. M. (2009) Risk Factors, Prevention and Early Detection of Prostate Cancer. Prim Care; 36:603–621.
28. Turner, B. Drudge-Coates L. (2010) Prostate cancer: risk factors, diagnosis and management. Cancer nursing practice; 9(10):29–35.
29. Bhavsar, A., Verma, S. (2014) Anatomic Imaging of the Prostate. BioMed Research International; 2014.
30. Srigley, J. R. (2004) Benign mimickers of prostatic adenocarcinoma. Modern pathology; 17:328–48.
31. Coffey, D. S., James P., Karr R. G., Molecular and Cellular Biology of Prostate Cancer. Springer Sci. 2012;
32. Sadikovic, B, Squire, J. A, Zielenska M. (2008) Cause and Consequences of Genetic and Epigenetic Alterations in Human Cancer. Current Genomics; 9:394–408.

33. Singh, D., Febbo, P. G., Ross, K., Jackson. D. G., Manola, J., Ladd, C., Tamayo, P., Renshaw, A. A., D'Amico, A. V., Richie, J. P., Lander, E. S., Loda, M., Kantoff, P. W., Golub, T. R., Sellers, W. R. (2002) Gene expression correlates of clinical prostate cancer behavior. Cancer Cell; 1(2):203–9.
34. True, L., Coleman, I., Hawley, S., Huang, C., Gifford, D., Coleman, R., Beer, T. M., Gelmann, E., Datta, M., Mostaghel, E., Knudsen, B., Lange, P., Vessella, R., Lin, D., Hood, L., Nelson, P. S. (2006) A molecular correlate to the Gleason grading system for prostate adenocarcinoma. Proceedings of the National academy of Sciences; 103(29):10991–6.
35. Meiers, I., Shanks, J. H., Bostwick D. G. (2007) Glutathione S-transferase pi (GSTP1) hypermethylation in prostate cancer: review 2007. Pathology; 39:299–304.
36. Bastian, P. J., Palapattu, G. S., Lin, X., Yegnasubramanian, S., Mangold, L. A., Trock, B., Eisenberger, M. A., Partin, A. W., Nelson, W. G. (2005) Preoperative serum DNA GSTP1 CpG island hypermethylation and risk of early prostate-specific antigen recurrence following radical prostatectomy. Clinical cancer research; 11:4037–4043.
37. Cookson, M. S., Reuter, V. E., Linked, I., Fair, W. R. (1997) Glutathione Stransferase Pi (GST-pi) class expression by immunohistochemistry in benign and malignant prostate tissue. Journal of Urology; 157: 673–6.
38. Chang, B. L., Liu, W., Sun, J., Dimitrov, L., Li, T., Turner, A. R., Zheng, S. L., Isaacs, W. B., Xu, J. (2007) Integration of Somatic deletion analysis of prostate cancers and germline linkage analysis of prostate cancer families reveals two small consensus regions for prostate cancer genes at 8p. Cancer Research; 67(9):4098–103.
39. Hawksworth, D., Ravindranath, L., Chen, Y., Furusato, B., Sesterhenn, I. A., McLeod, D. G., Srivastava, S., Petrovics, G. (2010) Overexpression of C-MYC oncogene in prostate cancer predicts biochemical recurrence. Prostate cancer prostatic Dis Nat; 13(4):311–5.
40. Katsogiannou, M., Ziouziou, H., Karaki, S., Andrieu, C., Henry de Villeneuve, M., Rocchi, P. (2015) The hallmarks of castration-resistant prostate cancers. Cancer Treatment Reviews: 41(7):588–97.
41. Ward, J. B., Henderson, R. E. (1996) Identification of needs in biomarker research. Environ Health Perspect; 104:895–900.
42. National Cancer Institute at the National Institutes of Health (2012) Biomarkers. Accessed on November 1, 2015; Available from: http://www.cancer.gov/publications/dictionaries/cancer-terms?CdrID=45618.

43. Mayeux, R. (2004) Biomarkers: potential uses and limitations. NeuroRx;1(2):182–8.
44. Sawyers, C. L. (2008) The cancer biomarker problem. Nature; 452(7187):548–52.
45. Füzéry, A. K., Levin, J., Chan, M. M., Chan, D. W. (2013) Translation of proteomic biomarkers into FDA approved cancer diagnostics: issues and challenges. Clinical Proteomics; 10(1):13.
46. Madu, C. O., Lu, Y. (2010) Novel diagnostic biomarkers for prostate cancer. Journal of Cancer. (1):150–77.
47. Bishop, M. C., Hardy, J. G., Taylor, M. C., Lemberger, M. L., Wastleand, R. J. (1985) Bone Imaging and Serum Phosphatases in Prostatic Carcinoma. British Journal of Urology; 317–24.
48. Bussemakers M. J. G., van Bokhoven, A., Verhaegh, G. W., Smit F. P., Karthaus, H. F. M., Schalken, J. A., Debruyne, F. M. J., Ru, N., Isaacs, W. B. (1999) DD3: a new prostate-specific gene, highly overexpressed in prostate cancer. Cancer Research; 59 (23):5975–9.
49. Sartori, D A., Chan, D. W. (2014) Biomarkers in prostate cancer: what's new? Current Opinion Oncology;26(3):259–64.
50. Crawford, E. D., Ventii, K., Shore, N. D. (2014) New Biomarkers in Prostate Cancer. Oncology.
51. Hakama, M., Auvinen, A., Day, N., E, Miller, A. B. (2007) Sensitivity in cancer screening. Journal of Medical Screening; 14(4):174–7.
52. Wu, L., Qu, X. (2015) Cancer biomarker detection: recent achievements and challenges. Chemistry Society Review; 44(10):2963–97.
53. Alvarez, M. A. (2014) Prostate cancer biomarkers. Journal of clinical ultrasound; 9(1):95–8.
54. Hernández, J., Thompson. I. M. (2004) Prostate-specific antigen: A review of the validation of the most commonly used cancer biomarker. Cancer.; 101(5):894–904.
55. Thorek, D. L. J., Evans M. J., Carlsson, S. V., Ulmert, D., Lilja, H. (2013) Prostate-specific kallikrein-related peptidases and their relation to prostate cancer biology and detection. Established relevance and emerging roles. Journal of Thrombosis and Haemostasis; 110(3):484–92.
56. Lilja, H. (1985) A kallikrein-like serine protease in prostatic fluid cleaves the predominant seminal vesicle protein. Journal of Clinical Investigation.; 76(5):1899–903.

57. Lilja, H,. Oldbring, J., Rannevik, G., Laurell, C. B. (1987) Seminal vesicle-secreted proteins and their reactions during gelation and liquefaction of human semen. Journal of Clinical Investigation; 80(2):281–5.
58. Cohen, P., Graves, H. C., Peehl, D. M., Kamarei, M., Giudice, L. Rosenfeld, R. G. (1992) Prostate-specific antigen (PSA) is an insulin-like growth factor binding protein-3 protease found in seminal plasma. Journal of Clinical Endocrinology & Metabolism; 75:1046–53.
59. Ménez, R., Michel, S., Muller, B. H., Bossus, M., Ducancel, F., Jolivet-Reynaud, C., Stura, E. A. (2008) Crystal structure of a ternary complex between human prostate-specific antigen, its substrate acyl intermediate and an activating antibody. Journal of Molecular Biology; 376(4):1021–33.
60. Sumi, S., Arai, K., Yoshida, K. (2001) Separation methods applicable to prostate cancer diagnosis and monitoring therapy. Journal of Chromatography B; 764:445–55.
61. Armbruster, D. A. (1993) Prostate-specific antigen: Biochemistry, analytical methods, and clinical application. Clinical Chemistry; 39(2):181–95.
62. Parracino, A., Neves-Petersen, M. T., di Gennaro, A. K., Pettersson, K., Lövgren, T., Petersen, S. B. (2010) Arraying prostate specific antigen PSA and Fab anti-PSA using light-assisted molecular immobilization technology. Protein Science; 19(9):1751–9.
63. Kumar, V., Hassan, M. I., Singh, A. K., Dey, S., Singh, T. P., Yadav, S. (2009) Strategy for sensitive and specific detection of molecular forms of PSA based on 2DE and kinetic analysis: a step towards diagnosis of prostate cancer. Clinica Chimica Acta 403(1-2):17–22
64. Balk, S. P. (2003) Biology of Prostate-Specific Antigen. Journal Clinical Oncology: 383–91.
65. Lilja, H. (2003) Biology of prostate-specific antigen. Urology; 62 (5):27–33.
66. Lilja, H., Christensson, A., Dahlén, U., Matikainen, M. T., Nilsson, O., Pettersson, K., Lövgren T. (1991) Prostate-specific antigen in serum occurs predominantly in complex with α1-antichymotrypsin. Clinical Chemistry; 37(9):1618–25.
67. Schlomm, T. (2015) The Era of Prostate-specific Antigen-based Personalized Prostate Cancer Screening Has Only Just Begun. European Association of Urology; 68(2):214–5.
68. Rao, A. R, Motiwala, H. G., Karim, O. M (2008) The discovery of prostate-specific antigen. BJU International; 101(1):5–10.
69. Shariat, S. F., Canto, E.I., Kattan, M. W., Slawin, K. M. (2004) Beyond prostate-specific antigen: new serologic biomarkers for improved diagnosis and management of prostate cancer. Reviews Urology; 6(2):58–72.

70. Luboldt, H., Schindler, J.F., Rübben, H. (2007) Age-Specific Reference Ranges for Prostate-Specific Antigen as a Marker for Prostate Cancer. European Association of Urology. 2007 Feb;5(1):38–48.
71. Oesterling, J. E., Jacobsen, S. J., Chute, C. G., Guess, H A., Girman, C. J., Panser, L. A. Lieber M. M. (1993) Serum prostate-specific antigen in a community-based population of healthy men. Establishment of age-specific reference ranges. JAMA; 270:860–4.
72. Linton, K. D., Catto, J. W. F. (2013) Prostate cancer. Renal and urological surgery; 31 (10):516–22.
73. Velonas, V., Woo, H., Remedios, C., Assinder. S. (2013) Current Status of Biomarkers for Prostate Cancer. International Journal of Molecular Sciences.;14 (6):11034–60.
74. Jaenicke, R., Knof, S., (1968) Molecular Weight and Quaternary Structure of Lactic Dehydrogenase. 3. Comparative Determination by Sedimentation Analysis, Light Scattering and Osmosis. European Journal of Biochemistry; 4(2):157–63.
75. Scartozzi, M., Faloppi, L., Bianconi, M., Giampieri, R., Maccaroni, E., Bittoni, A., Prete, M., Loretelli, C., Belvederesi, L., Baroni, G., Cascinu, S. (2012) The role of LDH serum levels in predicting global outcome in HCC patients undergoing TACE: Implications for clinical management. PLoS One.; 7(3):1–8.
76. Adams, M. J., Buehner, M., Chandrasekhar, K., Ford, G. C., Hackert, M. L., Liljas, A. Structure-function relationships in lactate dehydrogenase. Proceedings of national Academy of Sciences;70 (7):1968–72.
77. Markert, C. L. (1963) Lactate Dehydrogenase Isozymes: Dissociation and Recombination of Subunits. Science; 140(3573):1329–30.
78. Phan, L. M., Yeung, S. J, Lee, M. (2014) Cancer metabolic reprogramming : importance, main features, and potentials for precise targeted anti-cancer therapies Cancer metabolism : major remodeling of cellular energy production and metabolic. Cancer Biology & Medicine; (11):1–19.
79. Vander Heiden, M. G., Cantley, L. C., Thompson, C. B (2009) Understanding the Warburg Effect: The Metabolic Requirements of Cell Proliferation. Science; 324(5930):1029–33.
80. Maxwell, P. H., Dachs, G. U., Gleadle, J. M., Nicholls, L. G., Harris, A. L., Stratford, I. J., Stratford I. J., Hankinson O., Pugh C. W., Ratcliffe P. J. Hypoxia-inducible factor-1 modulates gene expression in solid tumors and influences both angiogenesis and tumor growth. Proceedings of national Academy of Sciences; 94(15):8104–9.

81. Augoff, K., Hryniewicz-jankowska, A., Tabola, R. (2015) Lactate dehydrogenase 5: An old friend and a new hope in the war on cancer. Cancer Letters; 358(1):1–7.

82 National Cancer Institute at the National Institutes of Health. Tumorous markers (2015) Acessed on November 8, 2015. Available from http://www.cancer.gov/about-cancer/diagnosis-staging/diagnosis/tumor-markers-fact-sheet.

83. Ziaian, B., Saberi, A., Ghayyoumi, M. A., Safaei, A., Ghaderi, A., Mojtahedi, Z. (2014) Association of high LDH and low glucose levels in pleural space with HER2 expression in non-small cell lung cancer. Asian Pacific Journal of Cancer Prevention: 1617–20.

84. Chen, J., Sun, M-X., Hua, Y-Q., Cai, Z-D. (2014) Prognostic significance of serum lactate dehydrogenase level in osteosarcoma: a meta-analysis. Journal of Cancer Research and Clinical Oncology; 0–5.

85. Girgis, H., Masui, O., White, N. M., Scorilas, A., Rotondo, F., Seivwright, A, Gabril, M., Filter, E. R., Girgis A. H., Bjarnason, G. A., Jewett, M. A., Evans, A., Al-Haddad, S., Siu, K. M., Yousef, G. M. (2014) Lactate dehydrogenase A is a potential prognostic marker in clear cell renal cell carcinoma. Molecular Cancer; 13(1):101.

86. Hermes, A., Gatzemeier, U., Waschki, B., Reck, M. (2010) Lactate dehydrogenase as prognostic factor in limited and extensive disease stage small cell lung cancer - A retrospective single institution analysis. Respiratory Medicine;104 (12):1937–42.

87. Nishikawa, A., Tanaka, T., Takeuchi, T., Fujihiro, S., Mori, H. (1991) The diagnostic significance of lactate dehydrogenase isoenzymes in urinary cytology. British Journal of Cancer; 63(5):819–21.

88. Flocks, R. H., Schmidt, J. D. (1961) Lactate Dehydrogenase Isoenzyme Patterns of Prostatic Cancer and Hyperplasia. Journal Surgical Oncoly; 161–7.

89. Prout, G. R., Macalalag, E. V., Denis, L. J. Preston, L. W. (1965) Alterations in serum lactate dehydrogenase and its fourth and fifth isozymes in patients with prostatic carcinoma. Journal of Urology; 94:451–61.

90. Naruse, K., Yamada, Y., Aoki, S., Taki, T., Nakamura, K., Tomiume, M., Zennami, K., Katsuda, R., Sai, S., Nishio, Y., Inoue, Y., Noguchi, H., Honda, N. (2007) Lactate Dehydrogenase is a Prognostic indicator for Prostate Cancer patients with Bone Metastasis. Hinyokika Kiyo.; 53(5):287-92.

91. Brown, J. E., Cook, R. J., Lipton, A., Colema, R. E. (2012) Serum Lactate Dehydrogenase Is Prognostic for Survival in Patients with Bone Metastases from Breast Cancer : A

Retrospective Analysis in Bisphosphonate-Treated Patients. American Association for Cancer Research; (8):1–8.

92. Koukourakis, M. I., Giatromanolaki, A., Panteliadou, M., Pouliliou, S. E., Chondrou PS, Mavropoulou, S., Sivridis, E. (2014) Lactate dehydrogenase 5 isoenzyme overexpression defines resistance of prostate cancer to radiotherapy. British Journal of Cancer; 110 (9):2217–23.
93. Scher, H. I., Heller, G., Molina, A., Attard, G., Danila, D. C., Jia, X., Peng, W., Sandhu, S. Riisnaes, R., McCormack, R., Burzykowski, T., Kheon, T., Fleisher, M., Buyse, M., Bono J. S. (2015) Circulating Tumor Cell Biomarker Panel As an Individual-Level Surrogate for Survival in Metastatic Castration-Resistant Prostate Cancer. Journal of Clinical Oncology; 33(12):1348–55.
94. Sidaway, P. (2015) Prostate cancer: New biomarker panel prognosticates patient survival. Nature Reviews Clinical Oncology; 12(6):310–310.
95. Petrelli, F., Cabiddu, M., Coinu, A., Borgonovo, K., Ghilardi, M., Lonati, V., Sandro, B. (2015) Prognostic role of lactate dehydrogenase in solid tumors: A systematic review and meta-analysis of 76 studies. Acta of Oncology; 54(7):961–70
96. Bylda, C., Thiele, R., Kobold, U., Volmer, D. A. (2014) Recent advances in sample preparation techniques to overcome difficulties encountered during quantitative analysis of small molecules from biofluids using LC-MS/MS. Analyst;139(10):2265.
97. Lopez, M. F. (2005) High-Resolution Serum Proteomic Profiling of Alzheimer Disease Samples Reveals Disease-Specific, Carrier-Protein-Bound Mass Signatures. Clinical Chemistry; 51(10):1946–54.
98. Bock, J. (1949) Serum Protein Concentration and Relative Albumin Percentage in Normal Individuals. Acta physiological; 18:231-237;
99. Ayache, S., Panelli, M., Marincola, F. M., Stroncek, D. F. (2006) Effects of storage time and exogenous protease inhibitors on plasma protein levels. American Journal of Clinical Pathology;126:174–84.
100. Bylda, C., Thiele, R., Kobold, U., Volmer, D. A. (2013) Simultaneous quantification of acetaminophen and structurally related compounds in human serum and plasma. Drug Test and Analysis
101. Wu, X., Xiong, E., Wang, W., Scali, M., Cresti, M. (2014) Universal sample preparation method integrating trichloroacetic acid/acetone precipitation with phenol extraction for crop proteomic analysis. Nature Protocols; 9(2):362–74.

102. Truong, M., Yang, B., Jarrard, D. F. (2013) Toward the detection of prostate cancer in urine: a critical analysis. Journal of Urology; 189(2):422–9.
103. Yaroshenko, I., Kirsanov, D., Kartsova, L., Sidorova, A., Borisova, I., Legin, A. (2015) Determination of urine ionic composition with potentiometric multisensor system. Talanta; 131:556–61.
104. Taylor, T. P., Janech, M. G., Slate, E. H., Lewis, E. C., Arthur, J. M., Oates, J. C. (2012) Overcoming the Effects of Matrix Interference in the Measurement of Urine Protein Analytes. Biomark Insights; 7: 1-8.
105. Struck-Lewicka, W., Kordalewska, M., Bujak, R., Mpanga, A. Y., Markuszewski, M. Jacyna, J., Matuszewski, M., Kaliszan, R., Markuszewski, M. J. (2015) Urine metabolic fingerprinting using LC–MS and GC–MS reveals metabolite changes in prostate cancer: A pilot study. Journal of Pharmaceutical and Biomedical Analysis; 111:351–61.
106. Jacobs, E., Hissin, P. J., Propper, W., Mayer, L., Sarkozi, L. (1986) Stability of lactate dehydrogenase at different storage temperatures. Clinical Biochemistry; 19(3):183–8.
107. Kreutzer, H. H., Fennis, W. H. S. (1964) Lactic dehydrogenase after storage in blood serum tempertaures. Clinical Chimica Acta; 9:64–8.
108. Shain, S. A., Boesel, R. W., Klipper, R. W., Lancaster, C. M. (1983) Creatine kinase and lactate dehydrogenase: stability of isoenzymes and their activity in stored human plasma and prostatic tissue extracts and effect of sample dilution. Clinical Chemistry; 29(5):832–5.
109. Arcangeli, C. G., Smith, D. S., Ratliff, T. L., Catalona, W. J. (1997) Stability of serum total and free prostate specific antigen under veryng storage intervals and temperatures. Journal of Urology; 158:2182–7.
110. Arneth, B. M. (2009) Clinical Significance of Measuring Prostate-Specific Antigen. Lab Med; 40(8):487–91.
111. Kuriyama, M., Wang, M. C., Papsidero, L. D., Killian, C.S., Shimano, T., Valenzuela, L., Nishiura, T., Murphy, G.p., Chu, T. M. (1980) Quantitation of Prostate-specific Antigen in Serum by a Sensitive Enzyme Immunoassay. Cancer Research; 40:4568–662.
112. Acevedo, B., Perera, Y., Ruiz, M., Rojas, G., Benítez, J., Ayala, M., Gavilondo, J. (2002) Development and validation of a quantitative ELISA for the measurement of PSA concentration. Clinical Chimica Acta; 317(1-2):55–63.
113. Ahmed H. A., Azzazy. H. M. (2013) Power-free chip enzyme immunoassay for detection of prostate specific antigen (PSA) in serum. Biosensors and Bioelectronics; 49:478–84.

114 Scheijen, J. L. J. M., Hanssen, N. M .J., van de Waarenburg, M. P. H., Jonkers, D. M A. E., Stehouwer, C. D. A., Schalkwijk, C. G. (2012) L (+) and D(-) lactate are increased in plasma and urine samples of type 2 diabetes as measured by a simultaneous quantification of L(+) and D(-) lactate by reversed-phase liquid chromatography tandem mass spectrometry. Experimental Diabetes Research: 234812.

115. Cheow, L.F. Ko, S. H. Kim, S. J. Kang, K. H. Han., J. (2011) Increasing the Sensitivity of ELISA using Multiplexed Electrokinetic Concentrator. Analytical Chemistry.; 82(8):3383–8.

116. Poon, C-Y., Chan, H-M., Li, H-W. (2014) Direct detection of prostate specific antigen by darkfield microscopy using single immunotargeting silver nanoparticle. Sensors Actuators B Chemical: 737–44.

117. Stenman, U. H., Leinonen, J., Alfthan, H., Rannikko, S., Tuhkanen, K., Alfthan, O. (1991) A complex between prostate-specific antigen and alpha 1-antichymotrypsin is the major form of prostate-specific antigen in serum of patients with prostatic cancer: assay of the complex improves clinical sensitivity for cancer. Cancer Research; 51(1):222–6.

118. Simon, R., Passeron, S., Lemoine, J., Salvador, A. (2014) Hydrophilic interaction liquid chromatography as second dimension in multidimensional chromatography with an anionic trapping strategy: application to prostate-specific antigen quantification. Journal of Chromatography; 1354:75–84.

119. Cao, C., Kim, J.P., Kim, B. W, Chae, H., Yoon, H. C., Yang, S. S. Sim, S. J. (2006) A strategy for sensitivity and specificity enhancements in prostate specific antigen-α1-antichymotrypsin detection based on surface plasmon resonance. Biosensors Bioelectronics: 2106–13.

120. Nandakumar, M. P., Nandakumar, R., Mattiasson, B. (2000) Fluorimetric quantification of intracellular lactate dehydrogenase during fermentation using flow injection analysis. Biotechnology Letters; 22(18):1453–7.

121. Pettit, S. M., Nealon, D. A.., Henderson, A. R. (1981) Purification of lactate dehydrogenase isoenzyme-5 from human liver. Clinical Chemistry; 27(1):88–93.

122. Nesakumar, N., Thandavan, K., Sethuraman, S., Krishnan, U. M., Rayappan, J. B. B. (2014) An electrochemical biosensor with nanointerface for lactate detection based on lactate dehydrogenase immobilized on zinc oxide nanorods. Journal of Colloid Interface Science; 414:90–6

123. Sahab, Z. J., Semaan, S. M., Sang, Q. A. (2007) Methodology and applications of disease biomarker identification in human serum. Biomarker Insights; 2:21–43.
124. Sapan, C. V., Lundblad, R. L., Price, N. C. (1999) Colorimetric protein assay techniques. Biotechnology and applied Biochemistry; 29:99–108.
125. McMaster, M. C. (2006) HPLC: A pratical user's guide. Analytical Chemistry;Second Ed.:3–13.
126. Bai, Y. J., Gao, X. Y., Lu, J. Q., Zhang, H. G. (2010) A LC-MS-based method for quantification of biomarkers from serum of allergic rats. Molecules; 15:3356–65
127. Freire, M. G., Pereira, J. F. B., Francisco, M., Rodríguez, H., Rebelo, L. P., Rogers, R. D., Coutinho, J. A. (2012) Insight into the interactions that control the phase behaviour of new aqueous biphasic systems composed of polyethylene glycol polymers and ionic liquids. Chemistry; 18(6):1831–9.
128. Albertson, P-A. (1958) Particle fractionation in liquis two-phase systems;The composition of some phase systems and the behaviour of some model particles in them aplication to the isolation of cell walls from microorganisms. Biochimica et Biophysica Acta; 27(958):378–95.
129. Willauer, H. D. , Huddleston, J. G., Rogers, R. D. (2002) Solute Partitioning in Aqueous Biphasic Systems Composed of Polyethylene Glycol and Salt: The Partitioning of Small Neutral Organic Species. Ind Eng Chem Res. American Chemical Society: 1892–904.
130. Freire, M. G., Cláudio, A. F. M., Araújo, J. M. M., Coutinho, J. A. Marrucho, I. M., Lopes, J. N. C., Rebelo, L. P. (2012) Aqueous biphasic systems: a boost brought about by using ionic liquids. Chemistry Society Reviews; 41(14):4966–95.
131. Raja, S., Murty, V. R., Thivaharan, V., Rajasekar, V., Ramesh, V (2012). Aqueous Two Phase Systems for the Recovery of Biomolecules – A Review. Science Technology: 1(1):7–16.
132. Pei, Y., Wang, J., Wu, K., Xuan, X., Lu, X. (2009) Ionic liquid-based aqueous two-phase extraction of selected proteins. Separation and Purification Technology; 64(3):288–95.
133. Bai, Z., Chao, Y., Zhang, M., Han, C., Zhu, W., Chang, Y., Li, H. Sun, Y. (2013) Partitioning Behavior of Papain in Ionic Liquids-Based Aqueous Two-Phase Systems. Journal of Chemistry; 2013:1–6.
134. Du, Z., Yu, Y-L., Wang, J-H. (2007) Extraction of proteins from biological fluids by use of an ionic liquid/aqueous two-phase system. Chemistry; 13(7):2130–7.

135. Passos, H., Sousa, A. C. A., Pastorinho, M. R., Nogueira, J. A., Rebelo, P. N., Coutinho, J. A. P., Freire M. G. (2012) Analytical Methods Ionic-liquid-based aqueous biphasic systems for improved detection of bisphenol A in human fluids †‡. Analytical methods; 4:2664–7.

136. Pereira, M. M., Pedro, S. N., Quental, M. V., Lima, A. S., Coutinho, J. A. P., Freire, M. G. (2015) Enhanced extraction of bovine serum albumin with aqueous biphasic systems of phosphonium- and ammonium-based ionic liquids. Journal of Biotechnology; 206:17–25.

137. Walden, P., (1914) Molecular weights and electrical conductivity of several fused salts. Bulletin of the Russian Academy of Sciences; 405–22.

138. Gutowski, K.E., Broker, G. A., Willauer, H. D., Huddleston J. G., Swatloski, R. P., Holbrey, J. D., Rogers, R. D. (2003) Controlling the aqueous miscibility of ionic liquids: aqueous biphasic systems of water-miscible ionic liquids and water-structuring salts for recycle, metathesis, and separations. Journal of the American Chemistry Society; 125(22):6632–3.

139. Earle, M. J., Esperança, J. M. S. S., Gile, M. A., Lopes, J. N. C., Rebelo, L. P. N., Magee, J. W., Seddon, K. R., Windegren, J. A. (2005) The distillation and volatility of ionic liquids. Nature; 439:831–4.

140. Parshall, W. G. (1977) Inorganic Syntheses; 17, 110.

141. Manzer, L. E., Parshall, G. W., (1976) Lewis acid adducts of trans-hydrocyanobis(triethylphosphine)platinum. Inorganic Chemistry; 15(12):3114–6.

142. Fraser, K. J., MacFarlane, D. R. (2009) Phosphonium-Based Ionic Liquids: An Overview. Australian Jouranl of Chemistry; 62(4):309.

143. Ermolaev, V., Miluykov, V., Rizvanov, I., Krivolapov, D., Zvereva, E., Katsyuba, S., Sinyashin, O., Schmutzler, R. (2010*)* Phosphonium ionic liquids based on bulky phosphines: synthesis, structure and properties. Dalton Transactions; 39(23):5564–71.

144. Louros, C. L. S., Cláudio, A. F. M., Neves, C. M. S. S., Freire, M. G., Marrucho, I. M., Pauly, J., Coutinho, J. A. P. (2010) Extraction of biomolecules using phosphonium-based ionic liquids + K(3)PO(4) aqueous biphasic systems. International Journal of Molecular Sciences; 11(4):1777–91.

145. Kozlowska, K., Polkowska, Z., Przyjazny, A., Namiesnik, J. (2003) Analytical procedures used in examining human urine samples. Polish Journal of Environmental Studies; 12(5):503–21.

146. Diamandis, E. P. (1998) Prostate specific antigen- its usefulness in clinical medicine. Trends in Endocrinology & Metabolism; 9:310–6.

147. Sia, M., Pickles, T. Morton, G., Souhami, L., Lukka, H., Warde, P. (2008) Salvage radiotherapy following biochemical relap after radical prostatectomy proceedings of the Genito-Urinary Radiation Oncologists of Canada consensus meeting. Cancer Urological Association Journal; 2:500–7.

148. Wilson, D. H., Hanlon, D. W., Provuncher, G. K., Chang, L., Song, L., Patel, P.P., Ferrell, E.P., Lepor, H., Partin, A.W., Chan, D. W. Sokoll, L. J., Cheli, C. D., Thiel R. P, . Fournier, D. R., Duffy, D. C. (2011) Fifth-Generation Digital Immunoassay for Prostate-Specific Antigen Using Single Molecule Arrays. Clinical Chemistry; 57:1712–21.

149. Zhang, Q. Y., Chen, H., Lin, Z., Lin, J. M. (2012) Comparison of chemiluminescence enzyme immunoassay based on magnetic microparticles with traditional colorimetric ELISA for the detection of serum alpha-fetoprotein. Journal of Pharmaceutical Analysis; 2(2):130–5.

150. Tate, J., Ward, G. (2004) Interferences in immunoassay; 25:105–20.

151. Shipkova, M., Vogeser, M., Ramos, P. A., Verstraete, A. G., Orth, M., Schneider, C., Wallemacq, P. (2014) Multi-center analytical evaluation of a novel automated tacrolimus immunoassay. Clinical Biochemistry; 47(12):1069–77.

152. Dinis, T. B. V., Passos, H., Lima, D. L. D., Esteves, V. I., Coutinho, J. A. P., Freire, M. G. (2015) One-step extraction and concentration of estrogens for an adequate monitoring of wastewater using ionic-liquid-based aqueous biphasic systems. Green Chemistry.;17(4):2570–9.

153. Passos, H., Ferreira, A. R., Cláudio, A. F. M., Coutinho, J. A. P., Freire, M. G. (2012) Characterization of aqueous biphasic systems composed of ionic liquids and a citrate-based biodegradable salt. Biochemical Engineering Journal; 67:68–76.

154. Merchuk, J. C., Andrews, B. A., Asenjo, J. A. (1998) Aqueous two-phase systems for protein separation. Studies on phase inversion. Journal of Chromatography B: Biomedical Sciences and Applications; 711(1-2):285–93.

155. Sintra, T. E., Cruz, R., Ventura, S. P. M., Coutinho, J. A. P. (2014) Phase diagrams of ionic liquids-based aqueous biphasic systems as a platform for extraction processes. Journal of Chemical Thermodynamics; 77:206-213.

156. Zhang, Y., Cremer, P. S. (2006) Interactions between macromolecules and ions: the Hofmeister series. Current Opinion in Chemical Biology; 10(6):658–63.

157. Kumar, A., Venkatesu, P. (2014) Does the stability of proteins in ionic liquids obey the Hofmeister series? International Journal of Biological Macromolecules; 63:244–53.

158. Bouatra, S., Aziat, F., Mandal, R., Guo, A. C., Wilson, M. R., Knox, C., Bjorndahl, T. C., Ramanarayan, K., Saleem, F., Serihun, P. L., Liu, P., Dame, Z. T., Poelzer, J., Huynh, j., Yallou, F. S., Psychogios, N., Dong, E., Bogumil, R., Roehring, C., Wishart, D. S. (2013) The Human Urine Metabolome. PloS One; 8(9).

159. Steel, L. F., Trotter, M. G., Nakajima, P. B., Mattu, T. S., Gonye, G., Block, T. (2013) Efficient and specific removal of albumin from human serum samples. Molecular & Cellular Proteomics; 2(4):262–70.

160. Merrell, K, Southwick, K., Graves, S. W., Esplin, M. S., Lewis, N. E., Thulin, C. D. (2004) Analysis of low-abundance, low-molecular-weight serum proteins using mass spectrometry. Journal of Biomolecular Techniques.; 15(4):238–48.

161. Bertholf, R. L. (2014) Proteins and Albumin. (2014) Laboratory Medicine.; 45(1):25–45.

162. Chang, M., (2011) Automated Sample Preparation by Protein Precipitation for High Throughput Bioanalysis. Agilent Technologies.

163. Roe S. (2001) Protein Purification Techniques. 2nd Ed. Oxford Univ Press; 4(8):238.

164. Coligan, J. E., Dunn, B. M., Ploegh, H. L., Speicher, D. W., Wingfield P. T. (1996) Current protocols in protein science. Proteins Structure, Function and Genetics; 24(3):409–409.

165. Burgess, R. R. (2009) Protein Precipitation Techniques. Chapter 20 1st Ed. Methods in Enzymology. 331-342.

166. Anderson, N. L. (2002) The human plasma proteome: history, character, and diagnostic prospects. Molecular & Cellular Proteomics; 1(11):845–67.

167. Paulus, A., Freeby, S., Academia, K., Thulasiraman, V., Wehr, T., Liu, N., Roth, S., Smith, K. (2009) Accessing Low-Abundance Proteins in Serum and Plasma With a Novel, Simple Enrichment and Depletion Method; Bio-Rad 6.

168. Venderlinde, R. E. (1985) Measurement of Total Lactate Dehydrogenase Activity. Academy of Clinical and Labotarory Science; 15.

169. Buonocore, R., Avanzini, P., Aloe, R., Lippi, G. (2015).Analytical imprecision of lactate dehydrogenase in primary serum tubes. Annals of Clinical Biochemistry; 0(0):1–4.

170. Burd, J. F., Usategui-Gomez, M. (1973) A colorimetric assay for serum lactate dehydrogenase. Clinical Chemistry Acta.; 46(3):223–7.

171. Wiener Lab. (2013) Optimized UV method (SFBC) for the determination of lactate dehydrogenase (LDH) in serum or plasma. Journal of Chemical Information and Modeling.; 53(9):1689–99.

172. Bisswanger, H. (2014) Enzyme assays. Perspectives in Science; 1(1-6):41–55.

173. Cláudio, A. F. M., Marques, C. F.C., Boal-Palheiros, I., Freire, M. G., Coutinho. J. A. P. (2014) Development of back-extraction and recyclability routes for ionic-liquid-based aqueous two-phase systems. Green Chemistry; 16(1):259.

174. Arakawa, T., Kita, Y., Timasheff, S. N. (2007) Protein precipitation and denaturation by dimethyl sulfoxide. Biophysical Chemistry; 131(1-3):62–70.

175. Kumar, V., Sharma, V. K., Kalonia, D. S. (2009) Effect of polyols on polyethylene glycol (PEG)-induced precipitation of proteins: Impact on solubility, stability and conformation. International Journal of Pharmaceutics; 366(1-2):38–43.

176. Zhang, Y., Furyk, S., Sagle, L. B., Cho, Y., Bergbreiter, D. E., Cremer, P. S. (2008) Effects of Hofmeister Anions on the LCST of PNIPAM as a Function of Molecular Weight. Journal of Physics; 111(25):8916–24.

177. Dreyer, S. E. (2008) Aqueous two-phase extraction of proteins and enzymes using tetraalkylammonium-based ionic liquids. Rostock.

178. Nascimento, K. S., Rosa, P. A. J., Nascimento, K. S., Cavada, B. S., Azevedo. A. M., Aires-Barros, M. R. (2010) Partitioning and recovery of Canavalia brasiliensis lectin by aqueous two-phase systems using design of experiments methodology. Separation and Purification Technology; 75(1):48–54.

179. Date, M. S., Dominy, B. N. (2013) Modeling the Influence of Salt on the Hydrophobic Effect and Protein Fold Stability. Communications in Computational Physics; 13(1):90–106.

180. Pieper, R., Gatlin, C. L., Makusky, A. J., Russo, P. S., Schatz, C. R., Miller, S. S., Su, Q., McGrath, A. M., Estock, M. A., Parmar, P. P., Zhao, M., Huang, S. T., Zhou, J., Wang, F., Esquer-Blasco, R., Anderson, N. L., Taylor, J., Steiner, S., (2003) The human serum proteome: Display of nearly 3700 chromatographically separated protein spots on two-dimensional electrophoresis gels and identification of 325 distinct proteins. Proteomics; 3(7):1345–64.

181. Schneider, S., Pechtl, I. (2016) Affinity Removal of the 14 Most Abundant Proteins in Human Plasma Using the Agilent 1260 Infinity Bio-inert Quaternary LC System. Proteomics & Protein Sciences. Agilent Tecn.

Appendix A

Experimental binodal data

A.1. Experimental Binodal Data for the system composed by IL+ salt+ H_2O.

Table A.1.1. Experimental weight fraction data for the binodal curve of the system composed of $[P_{4444}]Cl$ (1) + $C_6H_5K_3O_7$ (2) at (25±1) °C (153).

$[P_{4444}]Cl$	
100 w_1	100 w_2
58.615	5.560
48.752	6.944
44.978	7.422
41.69	8.008
39.23	8.576
37.264	9.152
35.572	9.565
34.403	10.185
32.774	10.653
30.815	11.472
29.945	11.865
29.106	12.239
28.391	12.628
27.604	13.153
26.488	14.022
25.787	14.359
24.266	15.595
23.756	15.842
23.301	16.071
22.823	16.325
22.317	16.602
21.646	17.189
21.036	17.666
20.328	18.224
19.959	18.434

19.044	19.115
18.453	19.702
17.747	20.163
17.163	20.506
16.763	20.881
16.383	21.245
16.018	21.566
15.632	21.918
15.301	22.229
15.083	22.318
14.701	22.663
14.377	23.025
14.133	23.141
13.756	23.523
13.55	23.612
13.266	23.904

Table A.1.2. Experimental weight fraction data for the binodal curve of the systems composed of IL (1) + $C_6H_5K_3O_7$/$C_6H_8O_7$ (2) at (25±1) °C (136).

[P_{4444}]Br		**[P_{4444}]Cl**	
100 w_1	100 w_2	100 w_1	100 w_2
47.839	0.437	56.699	1.319
43.160	0.659	47.438	2.980
40.112	0.898	41.147	4.204
38.124	1.066	38.327	5.482
36.379	1.189	35.791	6.057
33.514	1.641	31.067	7.538
30.257	2.153	29.428	8.173
19.226	5.164	28.183	9.020
9.502	15.005	26.944	9.657
5.789	23.234	25.574	9.946
5.251	26.355	23.989	11.278
4.639	26.556	23.048	11.725
3.943	28.360	22.412	12.032
3.209	29.345	21.228	12.931
3.088	31.056	20.521	13.370
2.053	40.229	19.996	13.912
1.629	42.954	19.329	14.166
1.431	43.091	18.534	14.899
1.191	43.959	18.024	15.111
		17.212	15.937
		16.718	16.236
		16.092	16.718
		15.644	17.001
		15.208	17.350
		14.663	17.862
		14.484	17.635
		13.788	18.604
		13.221	18.808
		12.766	19.299
		12.286	19.792

12.257	19.183
11.473	19.096
10.896	19.616
10.488	19.697
9.979	20.579
9.709	20.918
9.220	21.536
8.809	21.902
8.451	22.391
8.001	23.208

A.2. Phase diagrams for the systems composed of IL+ salt+ H_2O.

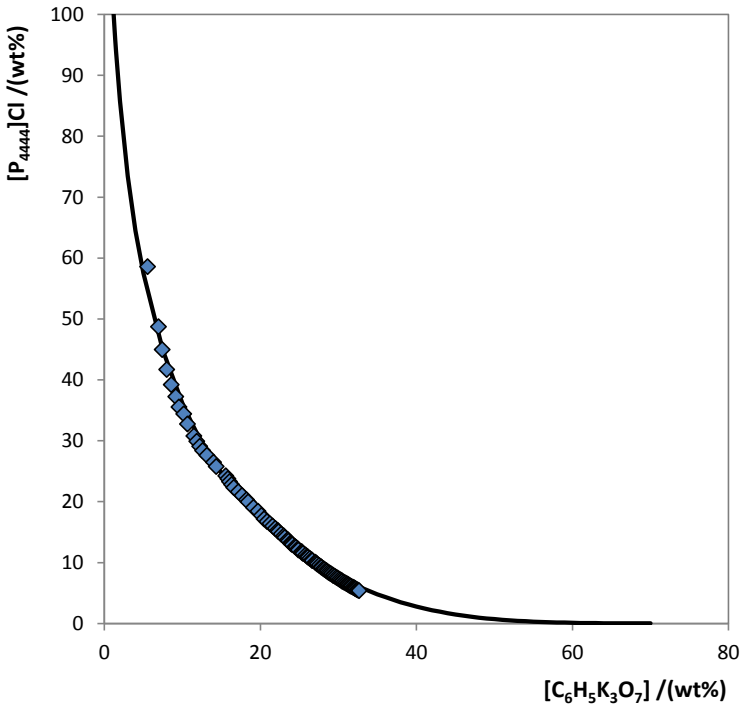

Figure A.2.1- Phase diagram for the system composed by $[P_{4444}]Cl$(♦) +$C_6H_5K_3O_7/C_6H_8O_7$ + H_2O at 25◦C and pH= 7.0 (153).

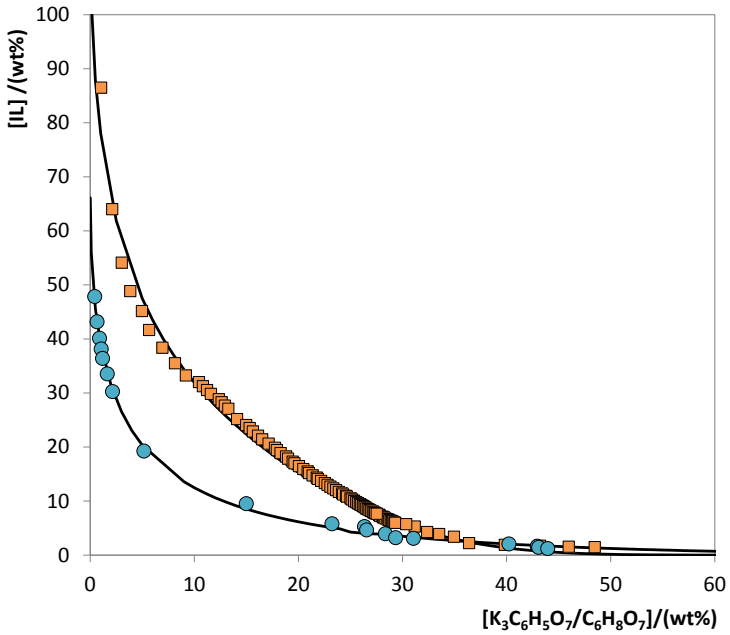

Figure A.2.2- Phase diagrams for the systems composed by $C_6H_5K_3O_7/C_6H_8O_7$ + H_2O + IL at 25◦C and pH= 7.0: $[P_{4444}]Br$ (●); + $[P_{4444}]Cl$ (■) (136).

A.3. Correlation parameters for the systems composed of IL+ salt+ H_2O.

Table A.3.1- Correlation parameters obtained by the regression of the experimental data through the application of equation (1) and respective standard deviations (σ) and correlation coefficient (R^2) (153).

IL	$A \pm \sigma$	$B \pm \sigma$	$10^5 (C \pm \sigma)$	R^2
[P_{4444}]Cl	170.0 ± 5.7	-0.484 ± 0.011	1.64 ± 0.14	0.994

Table A.3.2- Correlation parameters obtained by the regression of the experimental data thought the application of Eq. (1) and respective deviations (σ) and correlation coefficient (R^2) (136).

IL	**A ± σ**	**B ± σ**	10^5 **(C ± σ)**	R^2
[P_{4444}]Cl	116.40 ± 2.65	−0.399 ± 0.009	3.05 ± 0.20	0.985
[P_{4444}]Br	66.13 ± 0.98	−0.527 ± 0.013	0.21 ± 0.24	0.998

Appendix B

Experimental data for CF

B.1. Experimental data for the concentration factors in ABS composed of IL + salt + H_2O.

Table B1.1. *CF* of the system composed of $[P_{4444}]Cl$ + $C_6H_5K_3O_7$ + H_2O at (25±1) ˚C.

	$[P_{4444}]Cl$						
Theoretical CF	5	20	50	100	150	200	250
w IL/ g	0.1415	0.2038	0.8574	0.4589	0.3246	0.2572	0.2218
w Salt/ g	0.5191	2.882	25.64	29.92	30.02	30.07	30.34
w H2O/g	0.3570	1.919	19.51	19.63	19.66	19.67	19.83
w top/ g	0.0732	0.0834	0.3495	0.2032	0.1258	0.1007	0.0792
w bottom/ g	0.9188	4.694	44.83	48.85	49.13	50.29	49.93
Real CF	4.880	23.01	55.82	96.60	156.3	195.3	250.4

Appendix C

SE-HPLC calibration curves

C.1 SE-HPLC calibration curve for LDH.

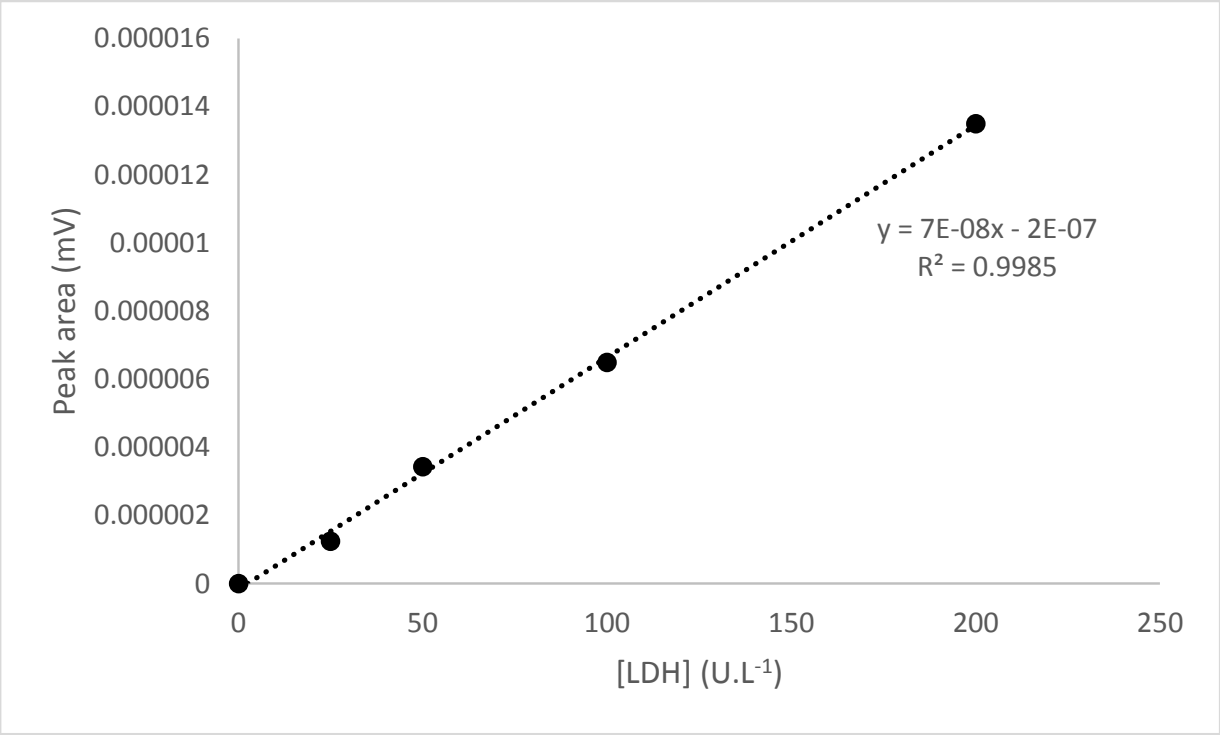

Figure C.1. Calibration curve for LDH in PBS aqueous solution by SE-HPLC at 280 nm.

Appendix D

SE-HPLC profiles

D.1 SE-HPLC chromatograms for extractions of LDH from synthetic samples and serum.

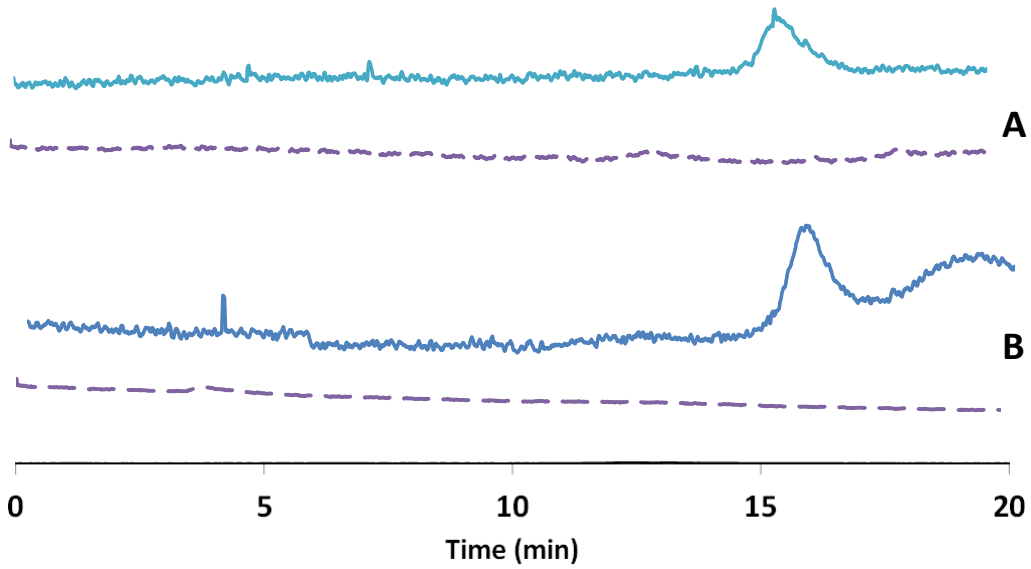

Figure D1.1- HPLC chromatogram of the top (–) and bottom phases (- -) of the ABS composed of $[P_{4444}]Br$ (A) or $[P_{4444}]Cl$ (B) + $C_6H_5K_3O_7/C_6H_8O_7$, at 25ºC and pH 7.0, corresponding to the extraction of LDH.

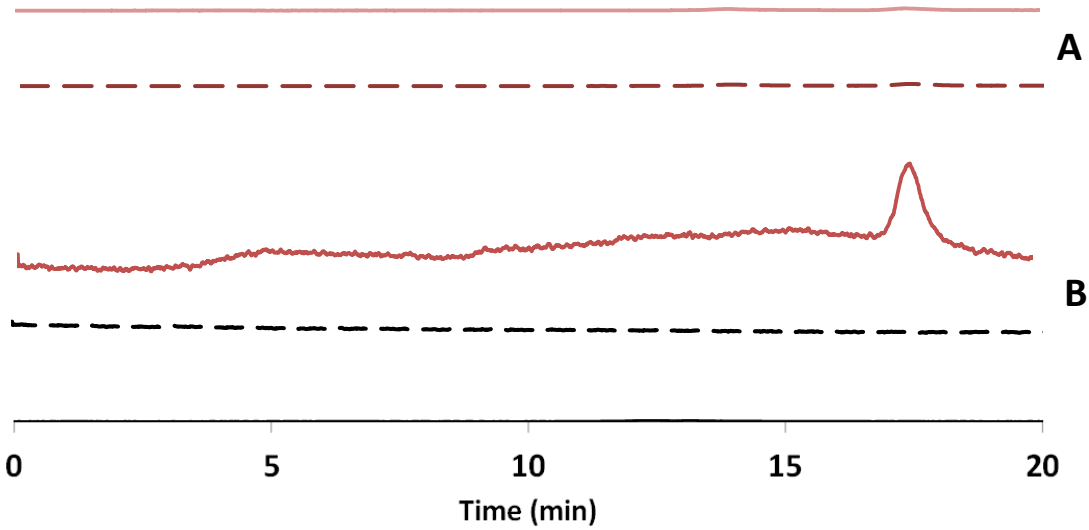

Figure D1.2- HPLC chromatogram of the top (–) and bottom phases (- -) of the ABS composed of $[P_{4444}]Br$ (A) or $[P_{4444}]Cl$ (B) + $C_6H_5K_3O_7/C_6H_8O_7$, at 25ºC and pH 7.0, corresponding to the extraction of serum.