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Porphyrins analysis in biological fluids: development and application of different methodologies

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“Nothing in life is to be feared, it is only to be understood.

Now is the time to understand more, so that we may fear less.”

Marie Curie

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Resumo

A exposição a metais pesados, tais como o chumbo, arsénio e manganês, constitui uma potencial ameaça para a saúde humana. A persistência destes metais no meio ambiente, juntamente com o seu uso intensivo pelas sociedades modernas, tem ao longo dos anos vindo a aumentar significativamente. Esta exposição pode ocorrer através do ar, alimentos ou água contaminada, e varia com o tipo de metal e com o nível de exposição a que estamos sujeitos. A toxicidade dos metais pesados reside essencialmente na formação de espécies reactivas de oxigénio, que desempenham um papel determinante para o desenvolvimento de efeitos adversos, tais como efeitos neurotóxicos e cancerígenos. O desenvolvimento de biomarcadores, que possibilitem a detecção de exposições continuadas, e/ou elevadas a metais pesados, e que possam assinalar atempadamente o aparecimento de efeitos adversos na fase inicial, enquanto o efeito tóxico for ainda reversível, é essencial para a saúde humana. Alguns metais interferem em pontos-chave da via metabólica da síntese do heme, alterando a excreção de porfirinas e de ácido delta-aminolevulínico (ALA) na urina; e promovem a oxidação das porfirinas reduzidas (porfirinogénios), que irão acumular-se em tecidos alvo, causando efeitos tóxicos no organismo. Concentrações urinárias de ALA superiores a 15 mg/L urina, podem provocar danos cerebrais em trabalhadores expostos, alucinações e convulsões. Neste sentido, o perfil de porfirinas e a concentração de ALA na urina são considerados biomarcadores de exposição e efeito para metais pesados.

As porfirinas são compostos macrocíclicos, que desempenham um papel preponderante no metabolismo dos organismos vivos, participando em processos como a fotossíntese e transporte de oxigénio. No organismo, uroporfirina (uro), heptaporfirina (hepta), hexaporfirina (hexa), pentaporfirina (penta) e coproporfirina (copro) são produzidas em excesso, e por isso excretadas normalmente pela urina e fezes, em determinadas concentrações. No entanto, a excreção de porfirinas urinárias pode ser anormalmente elevada devido a vários factores: doenças como as porfirias, ingestão de determinados fármacos, e exposição ambiental a químicos, em particular metais pesados. Considerando as características estruturais e químicas das porfirinas é possível desenvolver métodos que permitam a sua extracção dos fluidos biológicos, e a sua posterior identificação e quantificação, possibilitando obter o perfil de porfirinas de cada indivíduo. A detecção de porfirinas através de métodos espectrofotométricos é eficaz e frequentemente utilizada, uma vez que estas apresentam um espectro característico com bandas na região do visível (400 a 750 nm), correspondentes à banda de Soret e bandas Q. Posteriormente, através de cromatografia líquida de alta eficiência (HPLC), foram desenvolvidos métodos altamente sensíveis que permitem a separação e identificação das várias porfirinas e a determinação da sua concentração em amostras biológicas. Novos e mais eficientes métodos que permitam uma melhor extracção e quantificação das porfirinas em diferentes fluidos biológicos, são necessários. O desenvolvimento de condições e meios de conservação eficazes, sem exporem as porfirinas a ambientes que interfiram com a sua fluorescência e absorvência, são igualmente necessários. Estes métodos serão uteis para melhorar o diagnóstico diferencial das porfirias, e aumentar a sensibilidade e especificidade das porfirinas como biomarcadores.

Este trabalho teve como objectivos determinar as melhores condições de preservação de porfirinas em amostras de urina e avaliar vários métodos de extracção e quantificação de porfirinas, tendo em vista a obtenção de resultados mais rigorosos. Também, através da análise do perfil de porfirinas e do nível de ALA na urina de cada indivíduo, propomos biomarcadores de exposição e efeito, em subpopulações expostas em diferentes contextos de exposição a metais.

Amostras de urina de voluntários saudáveis, foram recolhidas e guardadas nas mesmas condições de temperatura (4º, -20º e -80 ºC), armazenamento (em alíquotas ou pool) e sem ou com conservantes (Na_2CO_3 ou HCl) ao longo de 90 dias. O perfil urinário de porfirinas determinado por HPLC, de cada amostra armazenada na respectiva condição, foi obtido no dia de recolha (dia 0), ao fim de uma semana (dia 7), ao fim de um mês (dia 30) e três meses depois (dia 90). Ao comparar as concentrações de porfirinas nas mesmas amostras em diferentes condições de conservação, é possível identificar quando houve perdas/ganhos de concentração de uro, hepta, hexa, penta e copro, em relação ao dia 0. As condições que não revelem diferenças significativas ($p>0.05$) são consideradas condições óptimas de conservação. Copro conseguiu manter-se estável a -20ºC e hepta a -80 ºC durante 90 dias, sem a adição de conservantes às amostras de urina. Na presença de Na_2CO_3, a copro permaneceu estável a -20 ºC e -80ºC e a hexa a -80 ºC, ao longo de 90 dias. Quando se utilizou HCl, a fracção uro manteve-se estável a -80ºC durante 90 dias. As amostras conservadas com Na_2CO_3 e HCl apresentaram concentrações mais estáveis ao longo do tempo do que amostras sem conservante. As amostras armazenadas em alíquotas apresentaram menos diferenças significativas de concentração em comparação com as denominadas de *pools* de urina.

O método de Soulsby (1974) permite quantificar a concentração total de copro em urina, enquanto que o método de Elder (2001) permite quantificar a concentração de porfirinas totais, ambos através de espectrofotometria UV-Vis. A principal diferença entre os métodos, é que o método Soulsby utiliza éter e vários passos de extracção, enquanto o método Elder apenas acidifica a urina com HCl, não havendo qualquer extração. De forma a estudar cada método, utilizámos padrões de copro I e copro III e uro I para obter os respectivos espectros em UV-Vis, quando aplicados em matriz de água e urina. Também analisámos uma mistura equimolar constituída pelos 3 padrões. Analisámos 55 amostras de urina de indivíduos saudáveis, pelos métodos de Soulsby, Elder e HPLC como forma de comparar os diferentes métodos e inferir sobre a qualidade de resultados. O método de Soulsby falhou na extracção e quantificação de uro I, mas foi bem-sucedido para a copro I e III. O método de Elder permitiu quantificar os padrões de copro e uro, individualmente e sob a forma de mistura de padrões. Os métodos de Soulsby e HPLC apresentaram uma relação linear, ainda que fraca (R^2=0,268), para a determinação de copro total na urina. Quando comparados, os métodos de Elder e HPLC para a análise de porfirinas totais, estes também apresentaram uma fraca relação linear entre si (R^2= 0,086).

Na última parte do trabalho, foram voluntariamente cedidas amostras de urina de 63 indivíduos de uma amostra de população portuguesa, divididos em vários grupos de acordo com o nível e forma de exposição a metais pesados: pessoas que vivem numa cidade de uma ilha portuguesa (Grupo I), pessoas que vivem numa grande área urbana (Grupo L), mineiros (Grupo M), pessoas que vivem numa área rural (Grupo A), técnicos hospitalares de Raios-X (Grupo R), pessoas que vivem numa área urbana não-industrializada (Grupo S), pessoas que vivem numa área urbana industrializada (Grupo V) e fumadores (Grupo F). Determinámos o perfil urinário de porfirinas por HPLC e os níveis de ALA na urina por um método colorimétrico. Estes valores foram combinados usando análise discriminante para criar um modelo capaz de identificar se um individuo tem um contexto de exposição igual a uma área/grupo específico. Estes biomarcadores foram posteriormente integrados em procedimentos preditivos recorrendo a ferramentas estatísticas. Aplicaram-se 3 procedimentos preditivos: o primeiro combinando todos os grupos com excepção do grupo I; o segundo, combinando apenas os grupos expostos ambientalmente (Grupo L, A, S e V), e o terceiro considerando apenas os grupos expostos activamente (Grupo M, R e S). Pela combinação dos biomarcadores determinados, o primeiro procedimento, apenas conseguiu classificar correctamente 76,8% dos individuos. No segundo

procedimento, já foi possível classificar correctamente 96,8% dos indivíduos expostos ambientalmente nos diferentes contextos, em áreas urbanas industrializadas e não-industrializadas, numa área rural e numa grande área urbana, de Portugal. No terceiro procedimento, a discriminação de indivíduos expostos activamente a metais pesados de forma ocupacional (mineiros ou técnicos de Raios-X) ou estilo de vida (fumadores), foi alcançada com uma classificação de 100%.

Do nosso estudo podemos concluir que para garantir a conservação de todas as porfirinas, especialmente as copro e uro, é necessário adicionar um conservante. Enquanto o Na_2CO_3, aumenta o pH do meio e conserva os analitos sob a forma de porfirinogénios, o HCl, acidifica a urina e promove a conversão de porfirinogénios a porfirinas. Sugerimos ainda que as amostras sejam armazenadas em alíquotas para garantir uma maior estabilidade das porfirinas urinárias. Este trabalho veio confirmar que a uro não é solúvel em éter, e por isso os métodos espectrofotométricos que o utilizem na extração não o conseguem quantificar. No entanto, estes métodos já são eficazes para análises de copro. As porfirinas totais conseguem ser detectadas em urina acidificada com HCl por espectrofotometria UV-Vis, e estimadas quantitativamente. O método de preferência deve ser HPLC, uma vez que permite a separação de cada porfirina e a sua quantificação individual, mas os métodos espectrofotométricos podem ser utilizados para uma análise rápida e preliminar. A combinação dos perfis de porfirinas com os níveis de ALA na urina pode ser utilizada como biomarcador de exposição e efeito de metais pesados, na amostra de população estudada, uma vez que os resultados dos procedimentos preditivos foram satisfatórios. A aplicação destes biomarcadores pode ser uma ferramenta útil para prever o tipo de exposição e a magnitude dos efeitos tóxicos provocados pelos metais em populações expostas, sendo por isso necessário continuar a investir no estudo das porfirinas e nas suas aplicações em Toxicologia Preditiva.

Palavras-chave:

Porfirinas; Conservação de amostras de urina; Análise de porfirinas na urina; Metais pesados; Biomarcadores.

Abstract

Throughout our lives we are simultaneously exposed to single or multiple sources of metal mixtures in environmental or occupational contexts. Lead (Pb), Arsenic (As) and Manganese (Mn) are classified as human carcinogens and may also cause neurotoxic effects. These metals induce porphyrins and delta-aminolevulinic acid (ALA) accumulation, that results from the interference at specific points of the heme synthetic pathway. For this, porphyrins and ALA can be used as biomarkers of exposure and effect, contributing to prevent the risk of neurotoxicity in human populations. The aim of this work was to determine the best preservation conditions of urinary porphyrins and the most accurate method of extraction and quantification of specific and total porphyrins. We also intended to provide biomarkers for heavy metals, through the characterization of urinary porphyrins profiles and urinary levels of ALA, in a Portuguese sub-population sample. We experienced various storage conditions (aliquots or pools subjected to freeze-thaw cycles), different temperatures (4ºC, -20 ºC and -80 ºC), and two different preservatives, over time. The addition of a preservative, like HCl or Na_2CO_3, to urine aliquots preserved at -80ºC succeeded to stabilize uroporphyrin (uro) and coproporphyrin (copro) for at least 90 days. Through the analysis of copro (I and III) and uro (I) standards we conclude that spectrophotometric methods, that use ether to extract the porphyrins from urine, are not effective for uro determination, while copro, uro and total porphyrin can be detected in acidified urine by spectrophotometry, without extraction. However, HPLC should be the method of choice, once the various porphyrins are able to be separated and individually quantified through this method. We combined the urinary porphyrin profiles and ALA levels of 63 subjects, using discriminant analysis, to create a model aiming to identify whether an individual is in an exposure context according with a specific area/group. It was possible to correctly classify 96,8% of individuals environmentally exposed to metals in an urban, rural and industrial context, and 100% of the individuals actively exposed to metals (workers or smokers). The investigation of porphyrins and their precursors seems to provide tools for the development of Occupational Toxicology, highlighting predictive biomarkers of effect, and consequently the improvement of public health.

Keywords:

Porphyrins; Urine sample storage; Porphyrins analysis in urine; Heavy Metals; Biomarkers.

Initial Statement

Some results of this thesis were presented in a communication in poster, in an international congress:

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List of Abbreviations

(ε) Molar Extinction Coefficient

AIP Acute Intermittent Porphyria

ALA Delta-Aminolevulinic acid

ALAD Delta-Aminolevulinic Acid Dehydratase

ALAS Delta-Aminolevulinic Acid Synthase

As (III^{-}) Arsenite

AS (V) Arsenate

As Arsenic

ASTDR Agency for Toxic Substances and Disease Registry

Ba Barium

BEI Biological Exposure Index

Cd Cadmium

Co Cobalt

Copro Coproporphyrin

Cr Chromium

Creat Creatinine

Cu Copper

DL Detection Limit

DNA deoxyribonucleic acid

EPP Erythropoietic Protoporphyria

FAB-MS Fast Atom Bombardment Mass Spectrometry

GFAAS Graphite Furnace Atomic Absorption Spectrophotometry

HBV Hepatitis B Virus

HCl Hydrochloric Acid

HCP Hereditary Coproporphyria

HCV Hepatitis C Virus

Hepta Heptaporphyrin

Hexa Hexaporphyrin

Hg Mercury

HIV Human Immunodeficiency Virus

HPLC High Performance Liquid Chromatography

HPLC/ESI MS/MS HPLC/Electrospray Ionization Tandem Mass Spectrometry

LCMS Liquid Chromatography-Mass Spectrometry

LC-MS/MS Liquid Chromatography Tandem Mass Spectrometry

MALDI-TOF Matrix-Assisted Laser Desorption Ionization/Time-of-Flight Mass Spectrometry

MMT Methylcyclopentadienyl Manganese Tricarbonyl

Mn Manganese

Mo Molybdenum

Ni Nickel

OEL-TWA Occupational Exposure Limit-Time Weighted Average

Pb Lead

PBG Porphobilinogen

PBG-D Porphobilinogen Deaminase

PEL Permissible Exposure Limit

Penta Pentaporphyrin

QL Quantification Limit

RFLPs Restriction Fragment Length Polymorphisms

ROS Reactive Oxygen Species

SD Standard Deviation

U Urinary

Uro III-S Uroporphyrinogen III Synthase

Uro Uroporphyrin

Uro-D Uroporphyrinogen Decarboxylase

UV-Vis Ultraviolet-Visible

V Vanadium

VDR Vitamin D Receptor

VP Variegate Porphyria

WHO World Health Organization

Zn Zinc

List of Chemical Formulas

(H_2O_2) Hydrogen peroxide

(Na_2CO_3) Sodium Carbonate

(O_2^-•) Superoxide ion

(OH•) Hydroxyl Radical

Chapter I

General Introduction

1.1 Porphyrins

The porphyrins are a class of naturally occurring macrocyclic compounds, which play a very important role in the metabolism of living organisms. The porphyrin molecule contains four pyrrole rings linked via methine bridges (Fig. 1.1) (Biesaga et al., 2000). The size of the porphyrin-macrocycle is perfectly suited to bind almost all metal ions and indeed many metals can be inserted in the center of the macrocycle, forming metallo-porphyrins that play key roles in several biochemical processes, like photosynthesis, oxygen transport and in various redox reactions (Biesaga et al., 2000; Giovannetti, 2012).

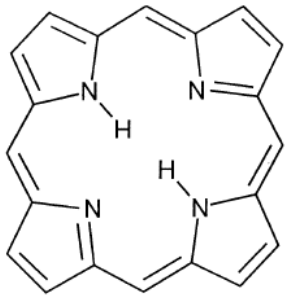

Fig. 1.1 Porphyrin structure (Biesaga et al., 2000).

From the perspective in coordination chemistry, the porphyrin ligand has turned out to be very versatile, and almost all metals have been combined with porphyrins. Such complexes have been used in a variety of applications as models for biological electron transport, oxygen transport, metalloenzymes and in cancer medicine and photodynamic therapy (Biesaga et al., 2000; Goldoni, 2002; Wiglusz et al., 2004). In recent years, (metallo)porphyrins and porphyrin-metal interfaces have become a major interest for applications in opto-electronics, data storage and solar cells (Goldoni, 2002).

Porphyrins, in the reduced form, porphyrinogens, are formed in mammalian tissues as intermediates in the biosynthesis of heme. In most tissues, porphyrinogens with 8-, 7-, 6-, 5-, and 4-carboxylated side chains are produced more than required for heme biosynthesis and are excreted as porphyrins in the urine (Woods, 1996)**.** The pattern of porphyrin excretion in normal individuals is represented in Table 1.1.

Table 1.1 Normal concentrations of porphyrins in urine for human subjects (Abe and Konaka, 1989).

Porphyrin	Sex	nmol/L	nmol/g creatinine
Uroporphyrin	M	5.5-50.8	6.0-17.3
	F	5.1-39.0	5.3-18.6
Heptaporphyrin	M	2.7-16.8	2.3-6.1
	F	2.4-13.6	2.7-6.2
Hexaporphyrin	M	<1.0	<1
	F	<1.0	<1
Pentaporphyrin	M	1.0-8.0	1.0-3.3
	F	1.3-5.3	1.0-4.2
Coproporphyrin I	M	22.2-167.1	27.2-78.3
	F	23.5-117.9	24.2-73.2
Coproporphyrin III	M	63.0-358.6	46.3-207.0
	F	44.3-247.2	40.2-137.8

In relation to the electronic absorption of porphyrins, they display extreme intense bands, the so-called Soret or B-bands, in the 380–500 nm range with molar extinction coefficients of $10^5\,M^{-1}$ cm-[1]. Moreover, at longer wavelengths, in the 500–750-nm range, their spectra contain a set of weaker, but still considerably intense Q bands with molar extinction coefficients of $10^4 M^{-1}cm^{-1}$(Fig. 1.2) (Biesaga et al., 2000; Giovannetti, 2012; Polo et al., 1988).

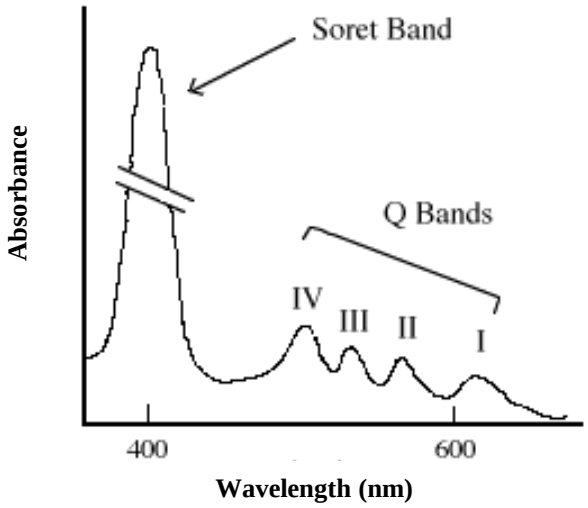

Fig. 1.2 Ultraviolet-Visible (UV-Vis) spectrum characteristic of porphyrins, with representation of the Soret and Q bands, between 350-750 nm (Goldoni, 2002).

Electronic absorption and spectra emission of porphyrins are sensitive to processes such as metalation, protonation (pH), substitution, or dimerization, which make porphyrins useful sensors of their environmental surroundings (Wiglusz et al., 2004). The change in spectra upon addition of acid or basic substances can generally be attributed to the attachment or the loss of protons (Giovannetti, 2012). For the main peak of the excitation spectra, fluorescence depends on pH and is minimal near

pH 5 and near pH 7-7.5 for coproporphyrin (copro) and uroporphyrin (uro), respectively (Polo et al., 1988).

Porphyrinogens are the actual products of porphyrin metabolism in vivo, and fully half of the porphyrins in urine may be present in this form. These colorless nonfluorescent compounds require oxidation to porphyrins before spectrophotometric or fluorometric analysis (Westerlund et al., 1988).

1.2 Porphyrias

Eight enzymes bring about heme synthesis from glycine and succinyl CoA. The biosynthetic pathway begins in the mitochondria and after three cytoplasmic stages the final step of heme formation takes place again in the mitochondria (Fig. 1.3). The first enzyme, delta-aminolevulinic acid synthase (ALAS), is the rate limiting enzyme in the production of heme and is controlled via negative feedback regulation by the intracellular heme concentration (Fateen et al., 2011).

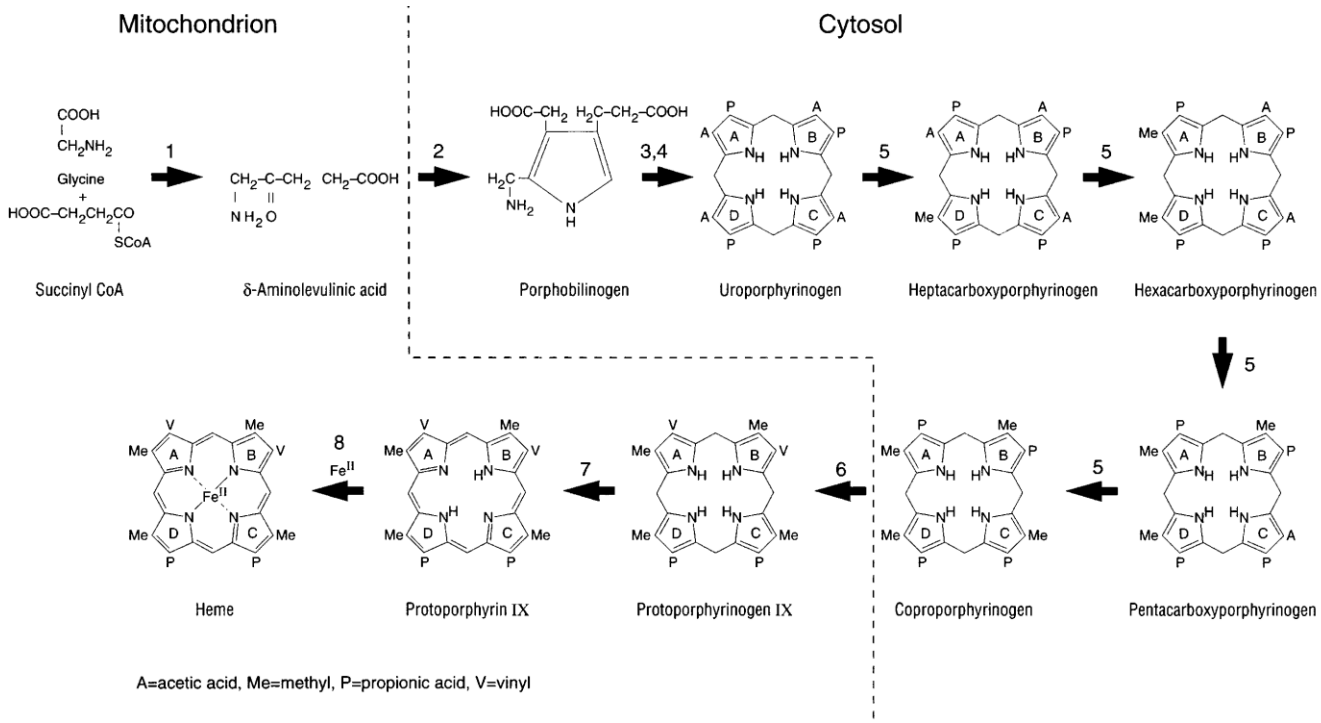

Fig.1.3 Heme biosynthetic pathway. Steps are catalyzed by (1) aminolevulinic acid synthetase (ALAS), (2) ALA dehydratase (ALAD), (3) uroporphyrinogen I synthetase, (4) uroporphyrinogen III cosynthetase, (5) uroporphyrinogen decarboxylase, (6) corporpophyrinogen oxidase, (7) protoporphyrinogen oxidase, and (8) ferrochelatase. Adapted from Woods et al, 1996.

Enzyme defects in the heme biosynthetic pathway cause genetic diseases called porphyrias which are characterized by the excessive production, accumulation and excretion of porphyrins and/or porphyrin precursors (Danton and Lim, 2006). Porphyrias are often classified as hepatic or erythropoietic, according to the organ where heme precursors accumulate. Specific patterns of accumulation of the precursors delta-aminolevulinic acid (ALA), porphobilinogen and porphyrins are associated with characteristic clinical features: acute neurovisceral attacks, skin lesions or both (Table 1.2) (Deacon and Elder, 2001; Fateen et al., 2011).

Table 1.2 Clinical classification of porphyrias: mode of genetic transmission, enzyme deficiency, tissues involved, clinical manifestations and patterns of overproduction of porphyrins and heme precursors in urine. Adapted from (Deacon and Elder, 2001; Hindmarsh, 2003; Woods, 1996).

Porphyria	Mode of genetic transmission	Enzyme deficiency	Tissues	Clinical Manifestation	Porphyrins and Heme precursors in urine
Acute Porphyrias					
Acute Intermittent Porphyria (AIP)	Auto Dom	PBG deaminase	Liver	Viscero-Neurological	ALA, Copro
ALA dehydratase Porphyria	Auto Recess	ALA dehydratase	Liver	Viscero-Neurological	ALA, Copro
Variegata Porphyria	Auto Dom	Protoporphyrinogen oxidade	Liver	Viscero-Neurological, Photosensitivity	ALA, PBG, Copro
Hereditary Coproporphyria	Auto Dom	Coproporphyrinogen oxidase	Liver	Viscero-Neurological, Photosensitivity	ALA, PBG, Copro
Non-Acute Porphyrias					
Congenital Erythorpoietic	Auto Recess	Uroporphyrinogen-III synthase	Erythroid cells	Photosensitivity	Copro
Porphyria Cutanea tarda	Sporadic Auto Dom	Uroporphyrinogen descarboxylase	Liver	Photosensitivity	Uro and Hepta
Erythropoietic protoporphyria	Auto Dom	Ferrochelatase	Erythroid cells and liver	Photosensitivity	Copro
Hepatoerythropoietic porphyria (HEP)	Auto Recess	Uroporphyrinogen descarboxylase	Erythroid cells and liver	Photosensitivity, Viscero-Neurological	Uro and Hepta

Auto Dom= Autosomal Dominant; Auto Recess= Autosomal Recessive

Treatment of porphyrias is based on symptomatic relief, together with carbohydrate (glucose) loading and in more severe attacks, heme therapy, which repress ALAS. However, glucose treatment is not as efficient as exogenous heme administration to achieve clinical remission in serious acute intermittent porphyria attacks (Sardh et al., 2009).

Although the porphyrias are uncommon disorders of heme biosynthesis, the wide range and variability of their clinical features leads to their inclusion in the differential diagnosis of many diseases. The porphyrias constitute a group of diseases for which the clinician requires the assistance of the laboratory to make a diagnosis. Therefore, every acute hospital laboratory needs to have simple, reliable methods for their exclusion or for the identification of those few patients who need more specialised further investigation (Deacon and Elder, 2001).

Clinical and biochemical diagnosis of the porphyrias is a difficult task and is currently based upon review of the clinical features and measurement of the various porphyrin metabolites in body fluids. Different techniques are available for the diagnosis of the porphyrias and the identification of

the eventual defect(s), ranging from simple biochemical measurement of ALA and porphobilinogen (PBG) through enzymatic assays to the investigation of genetic abnormalities (Deacon, 1988; Hindmarsh, 2003). A proposed algorithm for the biochemical investigation of acute porphyrias is proposed by Hindmarsh (2003).

Since the porphyrin excretion pattern is characteristic for each of the hereditary porphyrias, High Performance Liquid Chromatography (HPLC) porphyrin profiles have been used for their differential diagnosis (Deacon, 1988).

Except for the use of erythrocyte porphobilinogen deaminase activity to confirm the diagnosis of acute intermittent porphyria (AIP), enzyme assays have little to offer in the routine investigation of these diseases. Enzyme assays are often difficult to perform, and the relevant tissues are difficult to obtain. Genetic analysis provides a more accurate diagnosis and other benefits in AIP, variegate porphyria (VP), hereditary coproporphyria (HCP), and erythropoietic protoporphyria (EPP) (Hindmarsh et al., 1999a).

1.3 Changes in urinary porphyrins excretion

Usually changes in urinary porphyrins excretion are a sign of porphyria or intoxication with heavy metals or halogenated aromatic hydrocarbons (Table 1.3).They can also occur in individuals infected by some viruses such as Hepatitis B Virus (HBV), Hepatitis C Virus (HCV) and Human Immunodeficiency Virus (HIV) or exposed to illicit or therapeutic drugs like alcohol, to prolonged therapeutic use of estrogens, to iron overload, and as adverse effects of drugs like anticonvulsants or anaesthetics (Alves et al., 2009; Doss et al., 2000; Ford et al., 1981).

Drugs and chemicals, heavy metals and alcohol are known to be significant and frequent factors in the induction and maintenance of subclinical and clinical disturbances of porphyrin metabolism, including hepatic porphyrias. Especially acute hepatic porphyrias are triggered by drugs and/or alcohol and have therefore been designated as “pharmacogenetic diseases”(Moore, 1998).

Chronic alcoholics have an increased urinary excretion of copro, mainly isomer 3, but a normal urinary excretion of uro, ALA, and PBG. Ethanol administration to normal subjects results in increased activity of leucocyte ALAS and erythrocyte porphobilinogen deaminase, the two rate-controlling enzymes of the heme pathway. Ferrochelatase, the enzyme that inserts iron into protoporphyrin to form heme, shows very marked depression, existing also a prolonged depression in uroporphyrinogen decarboxylase too (Alves et al., 2009; Moore, 1998).

Table 1.3 Various causes and conditions related to changes in urinary porphyrin excretion (Doss et al., 2000).

(1) Intoxications: alcoholism (acute and/or chronic); foreign and environmental chemicals, such as hexachlorobenzene, polyhalogenated biphenyls, dioxins, vinyl chloride, carbon tetrachloride, benzene, chloroform; heavy metals such as lead, arsenic, mercury, etc.; drugs
(2) Liver diseases: e.g. cirrhosis, hepatitis, fatty liver, cholestasis, alcoholic liver syndromes, drug injury, cholangitis, haemochromatosis
(3) Haematological diseases: e.g. haemolytic, drug-induced, sideroachrestic, sideroblastic, aplastic anaemias; ineffective erythropoiesis (intramedullary haemolysis); pernicious anaemia; thalassaemia; leukaemia, erythroblastosis
(4) Infectious diseases: acute poliomyelitis
(5) Diabetes mellitus
(6) Disturbance of iron metabolism: e.g. haemosiderosis, idiopathic haemochromatosis
(7) Hereditary hyperbilirubinemias: e.g. Dubin–Johnson syndrome, Rotor's syndrome
(8) Malignancies: e.g. hepatocellular tumours, hepatic metastases, pancreatic carcinoma, lymphomatosis and other neoplastic processes
(9) Myocardial infarction
(10) Adverse effects of drugs: analgesics, sedatives, hypnotics, antibiotics, sex hormones, i.e. estrogens and oral contraceptives, and narcosis
(11) Pregnancy
(12) Fasting

1.4 Heavy Metals

Heavy metals are naturally found on earth's crust and have been used in many different applications over decades (Yousaf et al., 2016). Generic sources of heavy metals include mining and industrial production such as foundries, smelters, oil refineries, petrochemical plants and chemical industry, untreated sewage sludge, disperse sources such as metal piping, traffic and combustion by-production from coal burning power plants. Atmospheric emissions are probably the most harmful to the environment, and consequently, to human health due to either the great quantity involved, or their widespread dispersion which may originate many different exposure pathways (Dinis and Fiúza, 2011). Heavy metals exposure result from many unusual activities and sources and can come in contact with humans by a wide range of processes and pathways.

1.4.1Environmental Exposure to heavy metals

Heavy metals such as lead (Pb), mercury (Hg), cadmium (Cd) and arsenic (As) constitute a significant potential threat to human health. The environmental persistence of metals in concert with their intensive use by modern society have, over the years, created metal accumulation in the biosphere. The exposure may be through air, food or drinking water and depends on the metal type, its elemental/inorganic/organic form and exposure level (Kakkar and Jaffery, 2005; Kozlowska et al., 2003; Scherer, 2005; Yousaf et al., 2016).

Most of heavy metals bind to the sulfhydryl groups of biological molecules thus inhibiting enzyme activity, disrupting cellular transport and causing changes in protein functions. The toxicity of heavy metals includes the blocking of functional groups of important molecules, e.g. enzymes, polynucleotides, transport systems for essential nutrients and ions, and substitution of essential ions,

from cellular sites. While assessing the risk due to heavy metal exposure, factors such as age, sex, nutrition, socio-economic status, exposure conditions, duration of exposure, genetic variability and susceptibility have to be considered for a realistic approach (Calderón et al., 2003; Kakkar and Jaffery, 2005).

Assessing the risk of pollutant exposure in human populations involves the measurement of specific chemical residues in soil/water/air or in tissues of habituating populations. The use of biological markers or biomarkers may provide important information concerning human´s exposure (Kakkar and Jaffery, 2005).

1.4.1.1 Heavy Metals in urban environments

In an urban environment, trace metals can be emitted from numerous anthropogenic sources. Activities with a noticeable impact on the urban environment typically include traffic-related activities, industry-specific activities, the disposal of municipal waste (incineration or landfill), and the corrosion of construction/building materials. Those deposited on land in an urban setting can be readily relocated and dispersed by wind, rain, and surface runoff (Wong et al., 2006).

The prolonged presence of these contaminants in urban environment, particularly in urban soils, and their proximity to the human population, can significantly amplify the exposure of the urban population to metals (Zhang et al., 1999). The direct health impact of trace metal contamination of the urban environment are usually difficult to assess due to the complexity of the medical factors involved (Vlahov and Galea, 2002).

Compelled by the growing environmental and health awareness of the general public, assessment of an array of trace metals in soils, sediments, water, air, as well as food are demanded by regulatory guidelines. Those routinely regulated trace metals include As, Barium (Ba), Cd, Cobalt (Co), Chromium (Cr), Copper (Cu), Hg, Pb, Molybdenum (Mo), Nickel (Ni), Vanadium (V), and Zinc (Zn) (Wong et al., 2006).

1.4.2 Occupational exposure to heavy metals

Major sources of occupational exposure to heavy metals are smelters, mining activities and hazardous waste sites. Pyrometallurgical nonferrous metal production is the major global source of airborne As, Cd, Cu, Zn, and Pb. The metallurgical are also primary sources of Cd, Ni, and Pb for aquatic ecosystems, whereas for soil the most important sources of metals worldwide are mine tailings, smelter wastes, and atmospheric fallout. As a result, metal accumulation in air, water, soil, and food (fish, grains, etc.), may be higher than background levels in areas located in the vicinity of smelters and mines (Calderón et al., 2003).

Around smelters and mining areas, metals are commonly present as mixtures. Mixtures can influence expected adverse health effects because their components can individually have affinity for the same organs or, together, overwhelm a particular mechanism the body uses to defend itself against toxic substances. Thus, sometimes, metal mixtures can interact in the body in such a way that the combined toxicity is more serious than the individual toxicity of each metal alone. This way, low doses that might not individually cause adverse health effects, together may become a public health issue (Calderón et al., 2003).

Potentially high Pb levels may occur in the following industries: Pb smelting and refining industries, battery manufacturing plants, steel welding or cutting operations, construction, rubber products and plastics industries, printing industries, firing ranges, radiator repair shops, and other industries requiring flame soldering of Pb solder. In these work areas, the major routes of Pb exposure are inhalation and ingestion of Pb bearing dusts and fumes. A permissible exposure limit (PEL) for Pb of 50 μg/m3 in the workplace was established (Agency for Toxic Substances and Disease Registry, 2005).

Occupational exposure to As has been reported in several industries, particularly in nonferrous smelting, electronics, wood preservatives, As production, glass manufacturing, and application of arsenical pesticides. Those at primary risk include workers in numerous industries, including manufacture and use of insecticides, weed killers, wood preservatives, glass production, metal refining and in some medicines. (Kakkar and Jaffery, 2005; Rodríguez et al., 2003).

Occupational exposure to Manganese (Mn) occurs in various industrial processes such as welding, ferroalloy steel production and dry cell battery manufacturing. Mn is also found at high concentrations in several other industrial settings, such as the production of Mn-containing organic pesticides, fireworks, ceramics, glass, leather, textiles, paint, and cosmetics (Andrade et al., 2015; Bader et al., 1999). A significant number of studies have shown that welders are exposed to high concentrations of Mn-containing welding fumes which leads to increased prevalence of neurobehavioral symptoms among them. Several neurobehavioral symptoms have also been reported among the ferroalloy smelting workers exposed to Mn (Hassani et al., 2016)**.**

The current Occupational Exposure Limit-Time Weighted Average for Mn (OEL-TWA; 0.2 mg/m3) seems not to provide sufficient protection against the neuropsychological effects. So, an adjustment is needed to modify the current OEL-TWA of Mn for a better protection of exposed workers (Hassani et al., 2016).

1.4.2.1 Mine Workers

Mining plays an important role in both local and national economies, however, in some locations the activity is still performed in an uncontrolled way, giving rise to soil erosion and serious environmental contamination (Coelho et al., 2012).

Activities associated with mining are also hazardous activities, in both the environmental and public health contexts. Mining activities may adversely affect the health of miners and the communities living near mine sites, and these effects may persist even after the mine is closed (Coelho et al., 2012).

The mine workers are exposed to a large amount of dust with various potentially toxic substances for eight hours/day and six days/week over a lifetime of work (Coelho et al., 2012). The common toxicants present in the mining environment are Pb, Hg, Cd, Mn, As, aluminium and fluoride. Inhalation and absorption through the skin are common routes of exposure, and the conditions of work often intensify exposure due to increased respiration rates and repetitive exposure to toxicants. Low-dose chronic exposure of toxic substances results in the accumulation of toxicants in the body. Hence, there is a need to monitor the mining environment as well as the miners due to the exposure to these toxicants (Dhatrak and Nandi, 2009).

1.4.3 Heavy Metals in Cigarette Smoke

From the point of conception and throughout life, humans experience a broad range of physical, chemical and biological exposures. The health effects of such exposures will depend not only on dose but also on their interaction with each chemical and with the characteristics of the individual, such as age, sex and lifestyle (Berglund et al., 2011; Ellis et al., 2012).

The use of cigarettes constitutes one of the major causes of morbidity and mortality in the world. Tobacco smoke has several toxic properties, including genotoxic, and carcinogenic properties. Containing 4000 identified chemical compounds, cigarette smoke is very harmful for human health. Among these toxic materials there are heavy metals, particularly Cd and Pb. Several heavy metals found in tobacco smoke such as Cd, Cr, Pb, and Ni also accumulate in tissues and fluids through smoking (Ashraf, 2012; Kossowska et al., 2010; Lazarević et al., 2012).

Heavy metal content in tobacco depends on soil properties, atmospheric conditions, and requirements for tobacco farming (use of pesticide and fertilizer). Tobacco plants absorb Pb and As from soil and concentrate these metals in leaves. For this reason, there are large variations in the content of metals in tobacco between countries (Lazarević et al., 2012).The mean content of Pb in cigarettes is 1.26 μg/g and As is 0.11 μg/g (Ashraf, 2012; Lazarević et al., 2012; Pappas, 2011).

Tobacco is an important source of Pb in passive smokers (children and adolescents). Blood Pb levels were 14% and 24% higher in children who lived with 1 or with 2 or more smokers than in children living with non-smokers (Lazarević et al., 2012; Pappas, 2011).

As is methylated and eliminated from the body through the urine. Cigarette smoking lowers methylation capacity of As and its elimination from the body. Cigarette smoking can also act synergistically with As exposure to cause DNA damage in lungs. Mortality risk from heart disease, skin lesion, bladder cancer, and lung cancer associated with exposure to As is higher among smokers (Lazarević et al., 2012).

Mn (II) complexes have been studied in tobacco. U.S. Environmental Protection Agency Reports stated that compounds of Mn were suspected of inducing or exacerbating asthma. Mn(II) has been shown to cause pulmonary inflammation in rats, though not as strongly as Cu or Ni (Pappas, 2011).

Smokers had lower copro and uro + copro porphyrin levels, however there are few published data relating changes in urinary porphyrin excretion to the smoking habit. Further studies are needed to properly address these findings (Alves et al., 2009).

Biomonitoring of the exposure to complex mixtures such tobacco smoke is a challenge since these exposures have many constituents and people is exposed to more than one type of mixtures. Also, a panel of biomarkers of effect should cover the major known adverse effects of smoking (e.g., oxidative stress, inflammatory processes, lipid peroxidation, lipometabolic disorders and mutagenic effects) (Scherer, 2005).

1.4.4 Sources and toxicity of Pb, As and Mn

1.4.4.1 Pb

Pb is one of the most abundant toxic metals in the Earth's crust. It has been used since prehistorical times and has become widely distributed and mobilized in the environment. Exposure to Pb in the environmental and occupational settings continues to be a serious public health problem (Kakkar and Jaffery, 2005). At high exposure levels, Pb produces alterations in cognitive development in children; and kidney damage, anaemia and hypertension mainly in adults. About 50% of Pb is absorbed through the inhalation of dusts, 10–15% orally absorbed, out of which 90% accumulated in the bones (Ahamed and Siddiqui, 2007; Kakkar and Jaffery, 2005).

Acute toxicity of Pb is rare but may occur with adulterated food or drink. Wrist drop may occur as late sign of intoxication in exposed workers. Other effects include reduced life span of erythrocytes, impairment of proximal tubular function, reduced sperm count and mobility. In babies from exposed pregnant women a reduced birth weight can be observed (Kakkar and Jaffery, 2005).

Oxidative stress appears to be a possible mode of action of Pb toxicity. Oxidative stress occurs when generation of free radicals (i.e. substances with one or more unpaired electrons) exceed the capacity of antioxidant defence mechanisms (i.e. pathways that provide protection against harmful effect of free radicals) (Ahamed and Siddiqui, 2007).

The participation of free radicals in Pb toxicity may occur at different levels: (i) inhibition of delta-aminolevulinic acid dehydratase (ALAD) that leads to the accumulation of its substrate ALA, which can be quickly oxidized to generate free radicals as superoxide anion (O_2^{-}•), hydroxyl radical (OH•), and hydrogen peroxide (H_2O_2), and (ii) stimulation of ferrous ion initiating membrane lipid peroxidation (Ahamed and Siddiqui, 2007).

1.4.4.2 As

As is a common environmental contaminant widely distributed around the world. Elevated As levels in the environment are attributable to both natural and anthropogenic sources, including geothermal discharges, industrial products and wastes, agricultural pesticides, wood preservatives and mine drainage.

Human exposure to this metalloid comes from well water and contaminated soil, from occupational exposure, and from fish and other marine organisms rich in methylated As species. Researchers observed that concentrations more than 300 μg/L were associated with arsenical lesions (Rodríguez et al., 2003).

Human oral exposure to As has been mainly related to bladder, kidney, liver and skin cancer, while exposure through inhalation has been linked to lung cancer. As exposure is also associated with peripheral neuropathy and peripheral vascular disorders such as black foot disease, a disorder which results in gangrene of the lower extremities. Once absorbed, As is stored in the liver, kidney, heart and lung, while lower amounts are present in muscle and neural tissue. Two to four weeks after As ingestion, it is incorporated into the nails, hair, and skin by binding to keratin sulfhydryl group (Ng et al., 2005; Rodríguez et al., 2003).

Acute exposure to As in humans has been shown to result in memory problems, difficulty concentrating, mental confusion and anxiety. In children chronically exposed to As, urine levels of this metalloid were inversely correlated with verbal Intelligence Quotient (IQ) scores, including verbal comprehension and long-term memory (De Vizcaya-Ruiz et al., 2009; Rodríguez et al., 2003).

Reactive oxygen species generation (ROS) associated with As exposure is known to play a fundamental role in the induction of its adverse health effects and diseases (De Vizcaya-Ruiz et al., 2009). Better and more epidemiological studies are required for the identification of biomarkers for As exposure and health effects (Kakkar and Jaffery, 2005).

1.4.4.3 Mn

Mn is an essential element for the proper functioning of plants, animals, and humans. A deficiency of Mn can lead to serious health effects, such as the impairment of neurological functions, seizures, osteoporosis, and mental retardation. However, in the presence of high Mn concentrations, it is a neurotoxin and can cause irreversible neurological disease, for example, in cases of industrial exposure. Prolonged and subclinical Mn exposure is also suspected to cause cancer, neurologic and psychiatric disorders, including Parkinson's- and schizophrenia-like symptoms (Andrade et al., 2015). Methylcyclopentadienyl manganese tricarbonyl (MMT), an organometal, was introduced to unleaded gasoline as an antiknock agent. Levels of Mn in the urban environment were therefore anticipated to rise (Kakkar and Jaffery, 2005).

Epidemiological data suggest that high Mn concentrations in drinking water may be associated with neurological impairment. Nevertheless, neurotoxic effects induced by Mn in humans emerge mostly subsequent to inhalation exposure where the metal can enter the brain through the olfactory pathways, providing a direct path into brain tissue (Bader et al., 1999). High inhaled Mn levels may lead to manganism, a neurodegenerative disorder that resembles Parkinson disease in both symptomatology and some of the underlying cellular mechanisms. In the early stages of intoxication, the symptoms may be reversible (Andrade et al., 2015; Bader et al., 1999).

1.5 Biomarkers

A biomarker is defined as a xenobiotically induced alteration in cellular or biochemical components or processes, structures or functions, which is measurable in a biological system or sample (Gil and Pla, 2001; Marchiset-Ferlay et al., 2012).

Validation criteria for a biomarker include intrinsic qualities such as specificity, sensitivity, knowledge of background level in the population, existence of dose–response relationships, degree of inter- and intra-individual variability and knowledge of the kinetics, confounding and modifying factors. In addition, properties of the sampling and analytical procedures are of relevance, including constraints and non-invasiveness of sampling, stability of sample as well as simplicity, high sensitivity, specificity and quickness of the analytical method (Gil and Pla, 2001; Scherer, 2005).

Non-invasive methods are preferred over invasive methods because of greater acceptability. They are expired air, saliva, semen, urine, sputum, hair, nails, faeces, and breast milk. Invasive methods may yield samples from blood, lung tissue, bone marrow, amniotic fluid, liver tissue, bone, follicular fluid or adipose tissue (Kakkar and Jaffery, 2005).

Biomarkers of exposure allow measurement of the internal dose by chemical analysis of the toxic compound or metabolite in body fluids or excreta such as blood, urine and exhaled air. Internal dose may also mean the amount of a chemical stored in one or several body compartments or in the whole body. This usually applies to cumulative toxic chemicals (Gil and Pla, 2001). The measurement of the 'external dose' is for example Pb air concentration at the workplace or in the residential, rural or urban areas, multiplied by the time spent at these places. The 'biologically effective dose' (or 'target dose') is the amount of agent reaching the target organ and target cells (Scherer, 2005).

Biomarkers of susceptibility serve as indicators of a sensitivity of individuals to the effect of a xenobiotic or to the effects of a group of compounds. They can be genetic markers that include alterations in chromosomal structure, such as restriction fragment length polymorphisms (RFLPs), polymorphism of enzyme activities, and others (Scherer, 2005).

Response or effect biomarkers are indicative of biochemical changes within an organism due to exposure to the xenobiotic. The ideal biomarker should be detected early and be able to show adverse effects before they are irreversible. Those are the most studied biomarkers and they include modifications in some parameters of blood composition, alterations of specific enzyme activities, the appearance of DNA adducts, localized mRNA and protein increased and the appearance of specific antibodies against a xenobiotic or a particular cellular fraction (Gil and Pla, 2001).

As conclusion, we may say that the markers of biological toxicity represent an important tool in toxicology for three main reasons: they allow estimation of the biological effect on the target tissue, they are markers of subclinical alterations and sensible indicators of pathology being useful in diagnostic and preventive strategies, and they consider inter- and intra-individual variability in xenobiotics response (Gil and Pla, 2001).

1.5.1 Biomarkers of Pb, As and Mn

1.5.1.1 Pb

The ideal biomarker of Pb exposure would be a measurement of total Pb body burden. Biomarkers of exposure in practical use today are measurements of total Pb levels in tissues or body fluids, such as blood, bone, urine, or hair (Agency for Toxic Substances and Disease Registry, 2005).

Measurement of Pb in blood is the most widely used biomarker of Pb exposure. The biological exposure index (BEI) for Pb in blood of exposed workers is 30 μg/dL (Abadin et al., 2007).

The development of non-invasive techniques for measuring Pb concentrations in bone has enabled the exploration of bone Pb as a biomarker of Pb exposure in children and in adults. Pb in bone is considered a biomarker of cumulative exposure to Pb because Pb accumulates in bone over the lifetime and most of the Pb body burden resides in bone (Abadin et al., 2007).

Pb inhibits several enzymes in heme biosynthetic pathway, including ALAD, coproporphyrinogen oxidase and ferrochelatase. Because Pb effectively inhibits ALAD activity, resulting in accumulation of ALA in blood and urine, urinary ALA has also been used as a biomarker of Pb exposure or a marker of early biologic Pb effect. ALA can have neurotoxic activity and may contribute to Pb-induced neurotoxicity. Blood Pb level indicates recent exposure, whereas bone Pb

level, which represents 90–95% of Pb burden in adults and 80–95% in children, may be a better biomarker (Kakkar and Jaffery, 2005).

1.5.1.2 As

The most commonly employed biomarkers for identification and quantification of As exposure are total As in hair or nails, blood, and total or specific metabolites of As in urine. Urinary As concentration has been used as a key biomarker of exposure. As levels in both hair and nails are elevated within one to a few weeks after acute poisoning, and return to background levels within a few months. Blood As is typically used as an indicator of As exposure. Since As is rapidly metabolized and excreted in the urine, the sum of As metabolites are used as biomarkers of recent As exposure (Kakkar and Jaffery, 2005; Marchiset-Ferlay et al., 2012).

The reference value for inorganic arsenicals is currently 2.1 µg As/kg per day, and the drinking water guideline is 10 µg As/l (Rodríguez et al., 2003).

1.5.1.3 Mn

Mn levels in biological samples, such as blood and urine, have been investigated as biomarker of exposure, but their suitability is highly controversial. While they may indicate average levels of exposure on a group basis, they are not suitable for individual assessment. Another limiting factor is the rapid rate of Mn clearance from the body because excess of Mn in blood is rapidly removed by the liver and excreted into the bile. Thus, Mn levels in blood have been used as an indicator of recent exposure, generally less than 1 month. Since the urinary excretion of Mn is approximately 3 % of total excretion, urinary Mn has limited clinical validity (Andrade et al., 2015; Hassani et al., 2016).

The number of studies on biomarkers of Mn exposure has been increased during recent two decades, but suitable biomarkers are not well reached (Hassani et al., 2016).

1.5.2 Porphyrins as biomarkers of metal exposure and effect

Porphyrins can be utilized to afford a measure of xenobiotic exposure and potential toxicity in humans for chemicals that: i. inhibit specific heme biosynthetic pathway in target tissues, ii. induce metal facilitated oxidation, iii. reduce porphyrins that accumulate in tissue cells because of impaired porphyrin metabolism. Changes in porphyrin excretion patterns are largely metal specific, correlate with metal concentrations in tissue cells, and occur before the onset of target tissue injury. Thus, investigators have suggested that urinary porphyrin profile measurements can be utilized as biomarkers of metal exposure and toxicity in human subjects (Geier et al., 2011; Woods, 1996). As it was mentioned before, of particular importance with respect to the utility of porphyrins as biomarkers of metal effects in target tissues is the property of some specific metals, not only to impair porphyrin(ogen) metabolism, but also to facilitate the oxidation of reduced porphyrins wich subquently accumulate in tissue cells. Evidence indicates that the prooxidant action of metals wich promotes porphyrinogen oxidation, may also underlie the oxidation of other cellular constituents, such as lipids and proteins (Bradley and Richard, 2013).

Reduced porphyrins (porphyrinogens) are readly oxidized to the corresponding porphyrins by free radicals, such as reactive oxygen species. It has been postulated that chemicals which promote the

formation of endogenous reactive oxidants just as H_2O_2 promote porphyria via direct porphyrinogen oxidation. Numerous studies have shown that porphyrinogenic metals can, in vivo, initiate oxidative events by uncoupling mithocondrial and mircrossomal electron transport processes, by increasing H_2O_2 production and other endogenous reactive oxidants, or by redox cycling (Bradley and Richard, 2013). These events lead to free radical formation with consequent porphyrinogen oxidation and potential damage to cell constituents.

In heme biosynthetic pathway the most vulnerable steps to heavy metal inhibition are those in which uroporphyrin decarboxylase and coproporphyrinogen oxidase are involved. The result of these inhibitions is the specific elevation of copro and pentaporphyrin (penta) in the urine (Geier and Geier, 2007).

Urinary porphyrins may be a measure of the presence of Hg in the body (or Hg body-burden) owing to the level of disruption of the heme synthesis pathway. The presence of Hg inhibits specific enzymes that are necessary for the heme synthesis pathway to progress. This inhibition or interference results in a ‘‘backlog’’ and an increase urinary excretion of specific porphyrins. The level of the excess of these metabolites measured in the urine correlates with the level of disruption of this pathway and indicates the extent of Hg tissue burden. Specifically, Hg body-burden has been demonstrated to be associated with elevations in urinary copro, penta, and by the expression of an atypical porphyrin – precoproporphyrin (also known as keto-isocoproporphyrin) not found in the urine of unexposed populations (Geier et al., 2011; Miller and Woods, 1993; Woods, 1996).

In human studies, a comparable change in the urinary porphyrin profile was observed among subjects with occupational exposure to Hg at levels above 20 μg/L (Woods, 1996). Porphyrin profile measurements may be useful in assessing Hg body burden as well as the biological effects of Hg over an extended period after cessation of actual exposures (Pingree et al., 2001; Woods, 1996).

The enzymes of the heme biosynthetic pathway are highly susceptible to alterations induced by As (Marchiset-Ferlay et al., 2012). An expressive increase of total urinary uro, which was dependent of significant increases of copro excretion, has been observed in subjects who drank high contaminated As-water (300 or 400 μg As/L) comparatively to low As-exposed subjects (14 μgAs/L). The increased urinary excretion of total porphyrins was mainly due to copro III and uro III; the copro/uro and coproIII/coproI ratios were also significantly higher. A direct relationship between time-weighted As exposure and alterations in urinary porphyrin excretion ratios was observed (Marchiset-Ferlay et al., 2012; Ng et al., 2005).

As caused increased activity in porphobilinogen deaminase (PBG-D) and uroporphyrinogen decarboxylase (Uro-D) in peripheral blood erythrocytes. An increased activity in PBG-D and uroporphyrinogen III synthase (Uro III-S) in the liver, and a significant decrease in renal Uro-D and hepatic and renal coproporphyrinogen oxidase (Copro-O) activities were found in arsenate (As (V)) treated mice. These inhibitions were more pronounced in animals exposed to arsenite (As (III)) (Ng et al., 2005).

Pb exposure produced a differential decrease of protoporphyrin, copro and uro excretion. These Pb effects can be mainly explained by inhibition of the enzyme ALAD, resulting in a decreased monopyrrole supply for porphyrin biosynthesis, and probably by inhibition of Uro-D enzyme (Quintanilla-Vega et al., 1995).

1.5.3 ALA as a biomarker of metal exposure and effect

ALA is synthesized from glycine and succinyl CoA by the action of ALAS. Porphobilinogen is biosynthesized from ALA by the action of ALAD (Makino et al., 2000)**.** Pb exposure promotes the activity of ALAS and inhibits the activity of ALAD. The effects of ALAS induction and ALAD inhibition result in an increased blood ALA level, and subsequently an increased ALA urinary excretion (Ahamed and Siddiqui, 2007; Gil and Pla, 2001; Quintanilla-Vega et al., 1995).

It has been recognized that the urinary ALA level markedly increases with blood Pb level above 40 µg/dl. No relationship between urinary ALA and blood Pb has been obtained for blood Pb levels less than 20 µg/dl, which is considered to be a threshold for inducing adverse health effects (Makino et al., 2000)**.** The limits for urinary ALA are set at 5 to 15 mg/l for workers in general (Higashikawa et al., 2000)**.**

Studies are being carried out to see whether certain genes can make an individual more vulnerable to Pb exposure. The genes being studied that could affect Pb toxicity are ALAD and Vitamin D receptor (VDR) genes (Quintanilla-Vega et al., 1995).The effects of ALAD polymorphism on Pb toxicity are divers and complex. This could lead to differences in susceptibility to Pb among human population (Calderón et al., 2003). Individuals carrying one or two copies of ALAD2 allele presented higher blood Pb levels than individuals with only ALAD1 form of gene, but still individuals with ALAD1 homozygotes may experience more severe Pb effects in brain, bone, and hemopoiesis. The VDR exists in several polymorphic forms in humans and may affect Pb accumulation in bones. At least three genotypes of VDR gene have been identified (Kakkar and Jaffery, 2005). Inhibition of ferrochelatase and coproporphyrinogen oxidase by Pb have also been reported. The inhibition of these enzymes results in protoporphyrin accumulation in erythrocytes and increased urinary excretion of ALA and copro (Quintanilla-Vega et al., 1995)**.**

Increased ALAS levels in blood and changes in ALAD activity were observed upon exposure to both As (III) and (V) being considered that ALAD activity in blood could be used to estimate its enzymatic activity in the brain (Abadin et al., 2007).

Few information is available on Mn's propensity to affect heme biosynthetic pathway. One study suggested that Mn can interfere with heme biosynthesis by inhibiting ALAS activity. In addition, Mn has been shown to inhibit liver and erythrocytes ALAD and competitively inhibit ferrochelatase (Andrade et al., 2014, 2015; Hassani et al., 2016).

1.6 Urine as a biological sample for determination of biomarkers

Urine is a fluid produced in the kidneys in the amount of about 1500 mL a day. In addition to water (95%), it contains harmful and unnecessary products of metabolism (mainly urea). Urine is a biological material whose analysis can be a source of valuable information about the functioning of the human body and about the consequences of environmental pollutants on it (Kozlowska et al., 2003).

Thus far, blood has not been used as extensively as urine in the investigations of biological markers in this sample. This results from the fact that the collection of blood samples is an invasive process and the volume of sample collected does not exceed 20 mL. In some countries it is difficult to obtain

permission to collect blood samples for research analysis. Due to its properties and the method of sample collection, urine is used most often for the assessment of environmental and occupational exposure (Kozlowska et al., 2003)**.**

Timed urine sampling, (i.e., 24 h urine collection, which is the gold standard for measuring biomarkers in urine), is generally not feasible in studies involving large numbers of individuals and hence spot urines samples (single voided urine) are more commonly collected. The main drawback with spot urine samples is the variation in dilution due to differences in the state of hydration, related to fluid intake, physical activity and temperature. To compare concentrations of urinary biomarkers, both in individuals or in groups of people over time, it is essential to consider the variations in dilution (Alessio et al., 1985; Barr et al., 2005; Marchiset-Ferlay et al., 2012).

It is therefore common practice to correct the analytical values determined from spot urine samples according to reference parameters (specific gravity, osmolarity or creatinine). These reference parameters are measured on the same urine samples used for the analysis of the biological indicator (Alessio et al., 1985; Barr et al., 2005).

The most widely used method is creatinine (creat) adjustment that involves dividing the analyte concentration (micrograms analyte per liter urine) by the creat concentration (grams creat per liter urine). Analyte result is then reported as weight of analyte per gram of creat (micrograms analyte/creat). The World Health Organization (WHO) recommends that if a sample is too dilute (creat concentration < 30 mg/dL) or too concentrated (creat concentration> 300 mg/dL), another urine void should be collected and analysed for creat and the target chemical. (Barr et al., 2005)

For an individual, the creat-adjusted concentration of an analyte should be compared with a "reference" range derived from persons in a similar demographic group (e.g., children with children, adults with adults) (Barr et al., 2005).

Most often, the method used for the determination of metals in urine is Atomic Absorption Spectroscopy (AAS). The determination of metals in a sample requires suitable pre-treatment. Among the most commonly used methods of preparation of urine samples for metal determination, are: wet digestion, microwave-assisted digestion, ultrasound-assisted digestion in oxidizing mixtures, as well as the simplest method, which is sample dilution (Kozlowska et al., 2003).

1.7 Methods for porphyrin determination

Methods for porphyrin analysis are based on the fluorometric or spectrophotometric properties of porphyrin molecules. As it was mentioned before, when dissolved in acid, porphyrins display two strong fluorescent emission bands when exposed to light close to 400 nm, one at 600 to 610 nm and another at 640 to 660 nm. All porphyrins also display an absorption spectrum consisting of a major band near 400 nm, the Soret band, and four smaller bands of decreasing intensity between 500 and 630 nm (Biesaga et al., 2000).

Spectrophotometric and spectrofluorometric methods using a variety of extraction, separation and reaction techniques have been developed to measure the concentrations of the various porphyrins and their precursors in urine, faeces, whole blood and plasma (Sies et al., 2015). Methods used for quantifying total urine porphyrin fall into two equally represented categories, fluorometric and

spectrophotometric. Assay conditions for the fluorometric methods are divided in two subgroups: those that use HPLC with fluorometric measurement to report total urine porphyrins and constituents, and those that use a spectrophotometric method. Those using spectrophotometry employed a variety of extinction coefficients and a variety of concentrations of hydrochloric acid (HCl), from 10% w/v, 2.8 M through to concentrated HCl (Aarsand et al., 2011).

In the beginning, (decade of 1950) screening tests were based on visual inspection for red fluorescence of porphyrins under an ultraviolet lamp. Years later, simple alternative screening procedures involving spectrophotometric scanning were adopted, overcoming many of the problems encountered with the traditional methods (Deacon, 1988). The difference in the solubility and isoelectric points of the porphyrins makes it possible to perform separations by preferential extraction into organic solvents from aqueous buffered solutions. Organic solvents serve the dual function of isolating the porphyrins from urine, as well as separating them from each other (Henry and Fernandez, 1966). For several years, quantitative determinations of urine porphyrins rely on either column or thin-layer chromatography or multiple extractions of urine with organic and aqueous solvents (Ford et al., 1981). Both methods require considerable time, and the solvent-extraction procedures are quite tedious. No procedure fully and effectively separates the compounds being analysed, when limited to adapted methods of the clinical laboratory routine. Moreover, minor products of porphyrin metabolism not usually measured by these methods, may have clinical significance (Schreiber et al., 1983).

Advances in the technology of HPLC makes this technique advantageous for porphyrin assay (Schreiber et al., 1983). In HPLC methods, porphyrins are separated into uro, copro, and heptaporphyrin (hepta), hexaporphyrin (hexa), and penta, without exposing the porphyrins to an adverse environment. Sample preparation is simple, involving no extraction or derivatization of the porphyrins (Ford et al., 1981). The analysis is fast and does not require a lot of techician time once specimens are prepared. Calculations involve measuring peak heights and use of a simple formula (Schreiber et al., 1983).

The analysis of porphyrins demands the use of a fluorescence detector, since fluorescence is more sensitive and more specific than absorbance. With a visible spectrophotometric detector, the quantification is imprecise and numerous interfering substances in urine make interpretation of complete chromatograms difficult, although its sensitivity is adequate to detect porphyrins (Schreiber et al., 1983). The HPLC system separates the porphyrins from other natural components of human urine that would interfere with fluorometric quantitation (Ford et al., 1981). Most laboratories analyse porphyrin components using reverse phase, gradient elution HPLC with fluorescent detection excitation wavelengths ranging between 402 and 410 nm, and emission between 615 and 630 nm (Sies et al., 2015). Precision of the HPLC test is acceptable, especially when urine porphyrin concentrations are increased. Lack of proper calibration of standards is possibly the prime cause of variability in urine porphyrin assays (Zuijderhoudt et al., 2000).

Several techniques have been developed for determining urinary porphyrins by thin-layer chromatography, ion-pair HPLC, capillary electrophoresis with fluorescence, matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF), and fast atom bombardment mass spectrometry (FAB-MS). More recently, liquid chromatography-mass spectrometry (LCMS), and tandem mass spectrometry (LC-MS/MS) have been used to determine porphyrins in biological matrices (blood, urine and feces) because of their high sensitivity and specificity (Hur et al., 2014).

HPLC with fluorometric detection allows the determination of plasma concentrations of uro I and III, hepta III, hexa III, penta III, and copro I and III (Hindmarsh et al., 1999a).

HPLC/electrospray ionization tandem mass spectrometry (HPLC/ESI MS/MS) provides additional resolution and specificity for the unequivocal identification of porphyrins, thus significantly improving the diagnostic application of porphyrin profile analysis. The ability to resolve the type I and type III porphyrin isomers coupled with the characteristic tandem mass-spectrometry fragmentation patterns allows the porphyrin profiles in blood, urine and faeces to be accurately analysed with confidence (Danton and Lim, 2006).

Second-derivative spectroscopy has been used to measure urinary total porphyrins and the copro and uro fractions (Buttery et al., 1995; Van De Giessen and Van Wijk, 1990). Second-derivative spectroscopy is a simple, rapid, and inexpensive procedure. The results are accurate and precise and compare well with those determined by HPLC (Buttery et al., 1995).

A rapid and accurate spectrofluorometric method for screening and quantification of urinary porphyrins was developed by Westerlund et al. Total porphyrin content is determined at the isosbestic point for uro and copro, and the mole fractions of uro and copro are estimated from the wavelength of the maximum signal (Westerlund et al., 1988).

Magnetic circular dichroism is a suitable technique for semi quantitative determination of total porphyrins in urine and faeces, being fast, reliable, and involving little sample preparation. The sensitivity of the assay must distinguish normal from above-normal concentrations of total porphyrins in urine and faeces (Ivanetich et al., 1984).

Chapter 2

Aim

Metals, particularly heavy metals such as Pb, Mn and As, constitute a significant potential threat to human health (Yousaf et al., 2016). Metal environmental persistence along with their intensive use by modern society has, over the years, altered the distribution of metals in the biosphere. Human exposure to metals may be through air, food or drinking water and varies with the type of metal, its elemental/inorganic/organic form and exposure level (Kozlowska et al., 2003).

Assessing the risk of heavy metal exposure in human populations, involves the measurement of the specific metal in soil/water/air or in fluids of exposed populations, which apart from being time consuming is often not a good indicator of bioavailability (Ng et al., 2005). The use of biological markers or biomarkers may provide important information concerning human exposure to metals or other chemicals. Once validated through laboratory studies, biomarkers can provide direct measurement of exposure levels and/or actual effects of chemicals upon living organisms. The use of a careful selected combination of biomarkers may lead to a better risk assessment (Kakkar and Jaffery, 2005).

The investigation on human biological samples presents a great analytical challenge. In order to determine metal concentrations in such samples, there must have time-consuming sample preparation procedures (Kozlowska et al., 2003). The analytical method to be developed for biomarker´s determination should be specific, sensitive, simple, fast, robust and little expensive. These requirements are sometimes difficult to complete (Scherer, 2005).

Usually changes in urinary porphyrins excretion are a sign of xenobiotic exposure, that caused an alteration on heme biosynthesis. Urinary porphyrins profiles´ measurements may serve as a biomarker of metal exposure and effect in human subjects (Woods, 1996). The urinary levels of ALA, a precursor of porphyrins synthesis in heme biosynthetic pathway, have also been accepted in occupational health as a marker of Pb biochemical effect (Higashikawa et al., 2000).

Spectrophotometric and spectrofluorometric methods using a variety of extraction, separation and reaction techniques have been developed to measure the concentrations of the various porphyrins and their precursors in urine, faeces, whole blood and plasma (Sies et al., 2015). These laboratory tests´ results can, in general, be compromised by a variety of factors, including specimen integrity (reflecting conditions of specimen collection, processing, transport and storage), analytical quality control practices, and limitations of the analytical methods (Woolf et al., 2017). In order to use the full potential of these markers, it is important to establish the best sample urine storage conditions and standard methods for the analysis of urinary porphyrins (Zuijderhoudt et al., 2000).

It is needed to analyse porphyrins and ALA levels in urine samples from healthy and environmentally/occupationally exposed individuals, and to establish reference values related to gender, age and personal habits, allowing the use of porphyrins and ALA as biomarkers of exposure to porphyrinogenic agents (Zuijderhoudt et al., 1995).

The aim of this work was to determine the best preservation conditions of urine samples and the most appropriate method of extraction and quantification of porphyrins, in order to provide the most accurate analysis. We also intend to recommend biomarkers of exposure and effect to heavy metals, through the characterization of urinary porphyrins profiles and urinary levels of ALA, in a sample of Portuguese sub-populations.

There is the expectation that this work may contribute for a better identification and protection of metal exposed individuals in order to avoid their adverse health effects, bringing benefits for the public health. It is important to continue the investigation about the potential of porphyrins as biomarkers, once they can provide vital information on health effects associated with exposure to several environmental pollutants, as well as occupational or life habits.

2.1 Objectives

The main objective of this study is to explore, compare and apply different methodologies for the analysis of porphyrins in urine biological samples, and to infer about their use as biomarkers of exposure/effect to heavy metals.

The specific objectives are:

1. To study different storage conditions of urine samples over time to obtain reliable urinary porphyrin profiles.

2. To explore, evaluate and compare porphyrin quantification methodologies and, this way, obtain accurate results in urinary porphyrins concentrations.

3. To determine urinary porphyrins profiles and levels of ALA in urine of samples of individuals from a sample of Portuguese sub-populations, differently exposed to heavy metals.

4. To assess the use of urinary porphyrin profiles and ALA urinary levels as biomarkers of exposure/effect, and their ability to predict and classify the context of exposure of subjects to heavy metals.

Chapter 1 gives a general introduction that includes a state of art of porphyrins; a summary of the genetic diseases porphyrias, environmental metals sources, metal toxicity and the use of porphyrins and their precursors as biomarkers, was developed. Analytical methodologies for the determination of porphyrins were also reviewed. We also extended the advantages and main particularities on the use of biomarkers in urine.

Chapter 2 covers the general and specific aims of the work.

Chapter 3, develops and explores different storage conditions of urine samples (temperature, preservatives, containers) over time, to obtain the most accurate analysis of the urinary porphyrins profile.

Chapter 4, focus on methodologies for the analysis of porphyrins. We have studied methods for total porphyrin quantification in urine, methods that only measure the concentration of urinary copro, and methods that separate all the porphyrins in urine

In chapter 5 we used urinary porphyrins profiles and ALA levels in urine as biomarkers of exposure/effect to study and distinguish Portuguese sub-populations exposed, in different contexts, to heavy metals. We obtained prediction models that discriminate and classify these subpopulations.

Chapter 6 comprises the final remarks of this thesis and outlines the future works´ perspectives.

Chapter 3

Study of urinary porphyrins preservation in different conditions over time

3.1 Background

The collection of urine samples aiming the analysis of changes in urinary porphyrins excretion, is a non-invasive method with great acceptability, because a correct evaluation and interpretation of the results may provide a porphyrin diagnostic or inform about exposure to xenobiotics.

However, urine is a very complex matrix, with many interfering substances, and porphyrins are very unstable molecules (Kozlowska et al., 2003). It is known that all the samples must be protected from light until the time of analysis, because urinary porphyrin concentrations can decrease by up to 50% if kept in the presence of light for 24 hours (Deacon and Elder, 2001; Woolf et al., 2017). Adequate methods of storage and preservation of urinary porphyrins are essential for an accurate diagnosis.

Due to the risk of contamination, preservation of the sample should be kept as simple as possible and storage time in a refrigerator should be minimized. Freezing is a logical choice to delay the decomposition of biological matrices, for that low density polyethylene containers are suitable for long-term storage of samples at - 20°C or below (Christensen, 1995).

An important factor that should be considered is the time of storage, as porphyrins are photo-degradable, serious errors may be introduced during the interval between the collection of the samples and the determination of porphyrins concentration in urine (Christensen, 1995). Specimen collection laboratories often have difficulty in the selection of the amount of preservatives to add to random urine samples (Hindmarsh et al., 1999a).

Previous work has already been done to provide reference methods for storage of urinary porphyrins. Deacon and Elder (2001) drew various conclusions: i- porphyrins are best analysed in a fresh, random sample (10–20 ml) collected without preservative; ii- twenty-four hour collections offer little advantage, delay diagnosis, and increase the risk of losses during the collection period; and iii- porphyrins are stable in urine in the dark at 4 ºC for up to 48 hours and for at least a month at −20 ºC.

Literature also summarized other results: freeze-thaw cycles of urine samples can also result in a marked decrease in porphobilinogen concentration and a corresponding increase in total urinary porphyrins; all samples should be protected from light by wrapping labelled containers in black plastic or tinfoil immediately after collection and transported to the receiving laboratory as soon as possible; urine and blood samples should be stored at 4º C also light protected (Woolf et al., 2017).

Since there is not a universal method of urine samples' storage and conditions may vary from laboratory to laboratory, we will study different storage conditions over time to obtain reliable urinary porphyrin profiles of subjects. We will study variables like sample storage time, temperature of storage and addition of preservatives.

3.2 Material and Methods

3.2.1 Chemicals

Chemicals were obtained from the following sources: 2-Propanol for HPLC (C_3H_8O; 99.5%), methanol HPLC grade (CH_4O; ≥99.9%), phosphoric acid (H_3PO_4), sodium phosphate dibasic (Na_2HPO_4) and Sodium carbonate anhydrous (Na_2CO_3) from Sigma–Aldrich; Hydrochloric acid p.a. (HCl; 37%) from Panreac; Pure standards of uro-, hepta-, hexa-, penta- coproporphyrins (10 nM) where obtained from Porphyrin products, Frontier Scientific.

3.2.2 Experimental Design

Samples (urine) of three healthy volunteers (women) were obtained from the laboratory of Clinical Analysis of the Faculdade de Farmácia, Universidade de Lisboa. All the samples were collected in 25-ml aseptic containers and stored in the dark at 4º C for few hours. Then the samples were kept in different conditions of temperature (4º, -20º, -80º C), type of storage (aliquots or pool), preservation (without preservative, with Na_2CO_3 and with HCl) and time, according to the experimental design described in Fig.3.1. According to the mentioned work plan, the first analysis (urinary porphyrin profile) was proceeded 2 hours after the samples collection (t=0), the next analysis took place 1 week, then 1 month and the last was performed three months after the collection (t =7, t= 30 and t=90 days); in short, each of the different conditions was analysed on days 0, 7, 30 and 90.

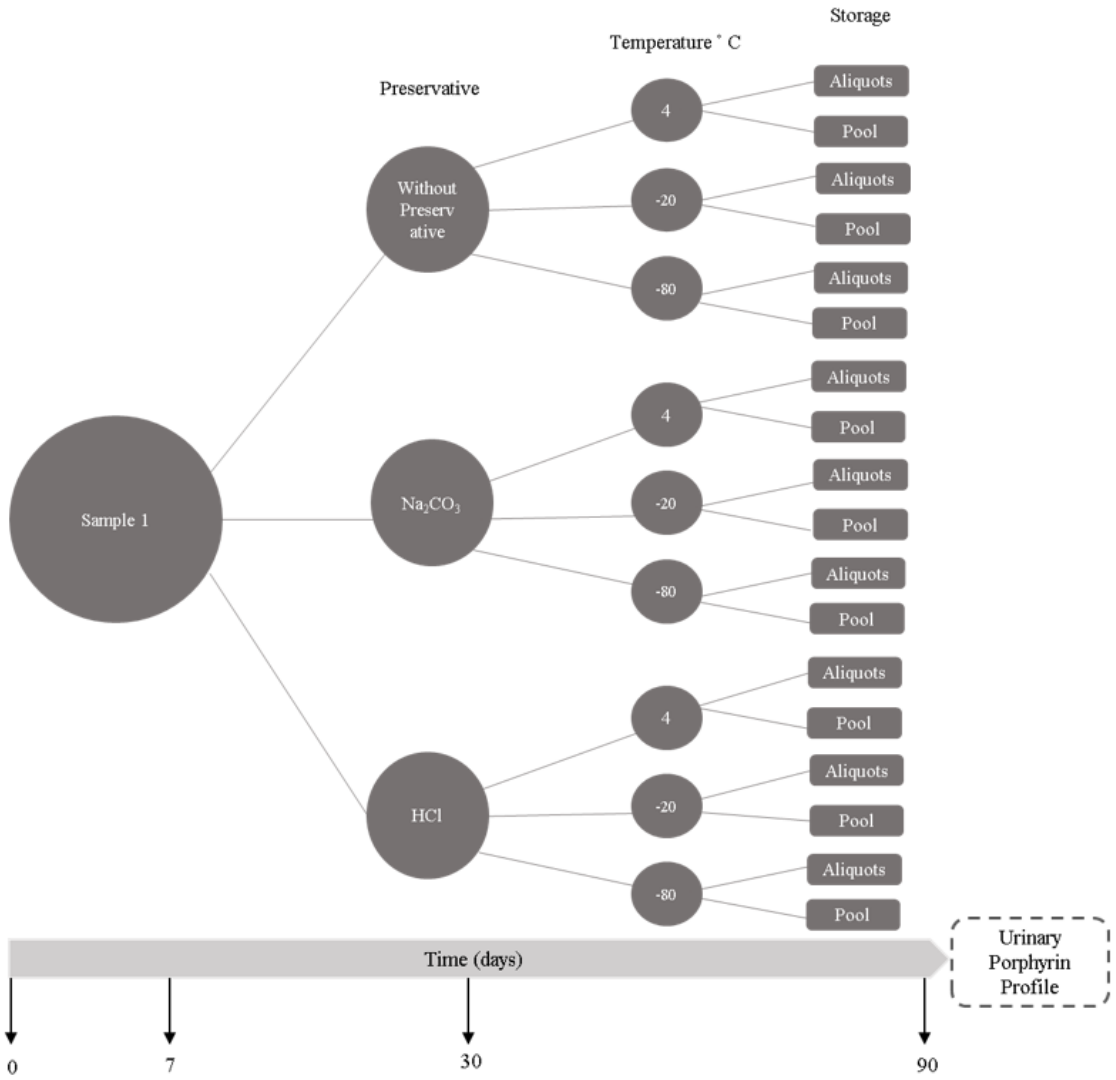

Fig. 3.1. Experimental design of the study of porphyrins preservation in urine samples. Each pool and aliquots were analysed on days 0, 7, 30 and 90.

3.2.3 Preservation of urine samples

For the analysis of aliquots, 1 mL of urine was stored in Eppendorf tubes with: no preservative, 0,5 mL of HCl (6 M) (Ford et al., 1981) or 0,5 ml of sodium carbonate (Na_2CO_3) (2 M) (Zuijderhoudt et al., 1995).

For the analysis of pools, urine was stored in plastic tubes of 5 ml with no preservative or with the proportional volume of HCl or Na_2CO_3 as preservatives, as mentioned before. To proceed the analysis, 750 µL of each sample were transferred to Eppendorf tubes and the remaining volume was stored in the conditions referred to above. The urine was subjected to freeze-thaw cycles between analyses.

The addition of Na_2CO_3 as preservative at the begining of the preservation process, is used to achieve a solution with pH above 7, obtaining an alkaline medium, where the porphyrinogen form prevail. On the contrary, when we add HCl, the medium is acidified, which promotes the oxidation of porphyrinogens to porphyrins. Preserving the same urine samples with Na_2CO_3 or HCl, we can

compare the differences of porphyrin concentrations when preserved in the form of porphyrinogens or porphyrins.

3.2 4 Urinary Porphyrin Profile

For the analysis of urinary porphyrin profile (levels of uro, hepta, hexa, penta and copro), 20 µL of HCL 10 M was added to 750 µL of each sample (urine), mixed in the vortex and kept in the dark for 30' (Woods et al., 1991).

Given that porphyrins are photosensitive compounds, during the handling of all the samples protection from light was assured. Chromatographic porphyrin analysis was performed by HPLC in a Hewlett Packard Agilent 1100 HPLC system equipped with a quaternary pump, solvent degasser, fluorescence detector and sampler (50 µL). Separation was accomplished on a LiChrospher 100 Merck RP18 column (125mm x 4mm, 5µm), fitted with an identically packed column (4 x 2 mm) maintained at room temperature. The mobile phase consisted of methanol 100% (solvent A) and sodium phosphate monobasic (50 mM), pH 3.5 (solvent B). A gradient was used starting at the mobile phase A: B (30:70%) until 3 min, changed linearly to A: B (80:20%) until 10 min, maintaining this proportion for 3 more minutes and after, the column was equilibrated with 30% of solvent A for 5 min. The flow rate was 1.0 mL/min. The fluorescence detector was set at an excitation wavelength of 395nm and an emission wavelength of 620nm. Data acquisition, processing and instrument control was performed by a Chemstation Software.

A dried porphyrin standard was recovered with HCl (3M) and after the appropriate dilutions of this stock solution, a calibration curve was obtained with the standards 0.015, 0.02, 0.04, 0.07 and 0.2 µmol/L and the established R^2 was 0,999. The determined quantification limits were: 0.11 µmol/L for uro, 0.12 µmol/L for hepta, 0.09 µmol/L for hexa, 0.09 µmol/L for penta and 0.08 µmol/L for copro. All the results were expressed as µmol of porphyrin per g of urinary creat.

Urinary levels of creat were determined by a colorimetric method with a Randox (CR510) commercial kit.

3.2.5 Statistical Analysis

Statistical analysis was performed with the SPSS 24.0 statistical package for Windows (SPSS, Inc., Chicago, IL, USA). Data are expressed as means ± standard deviations (SD).

After verification of data adequacy for parametric methods, normal distribution by Kolmogorov-Smirnov test and homogeneity of variance with Levene's test, all groups were compared by T-student tests. The significance of the results was considered when p- values were less than 0.05.

3.3 Results

3.3.1. Study of porphyrins stability in urinary samples

The following figures represent the variation of porphyrins concentration over time in the conditions described in the experimental design (Fig.3.1). Mean and SD of triplicate assays are presented in Tables A1.1 to A1.6, in Appendix 1.

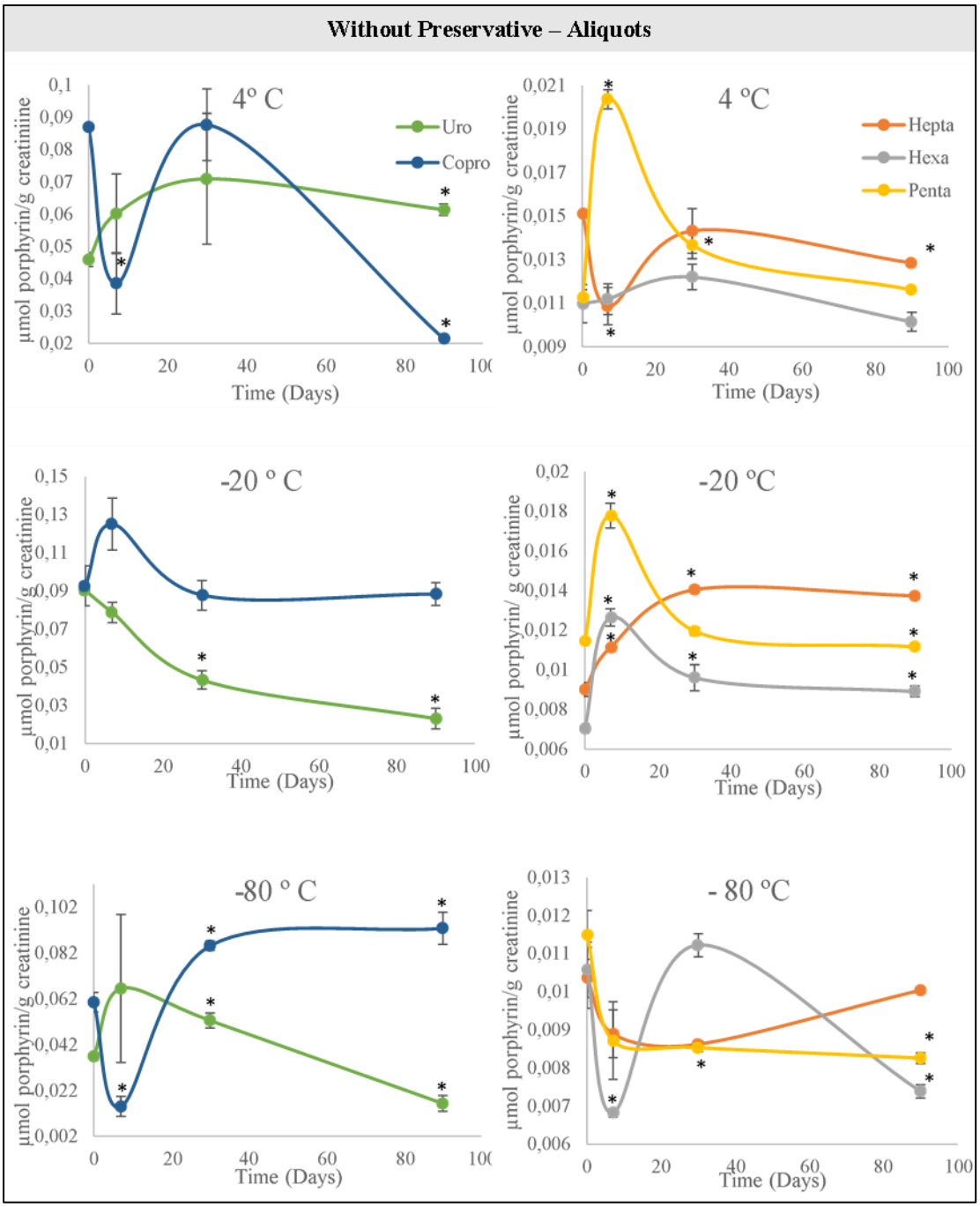

Fig. 3.2 Stability of Uro-, Hepta-, Hexa-, Penta- and Coproporphyrin over time (on days 0, 7, 30 and 90) in urine aliquots preserved at different temperatures (4º, -20º and -80º C), without addition of preservative. Data represent the mean ± SD. All the porphyrins concentrations correspondent to times t=7, t=30 and t=90 were compared, by T-student Test, to the initial concentration (t=0). When p<0,05 the difference is significative and signalized with *.

The stability of porphyrins in urine aliquots preserved at different temperatures, without any additional preservative, was represented in Fig.3.2. The copro fraction was stable at -20 ºC, without significative differences in concentration along time. The hepta fraction was stable at -80 ºC until at least 90 days. Uro only can be saved for 30 days at 4ºC.

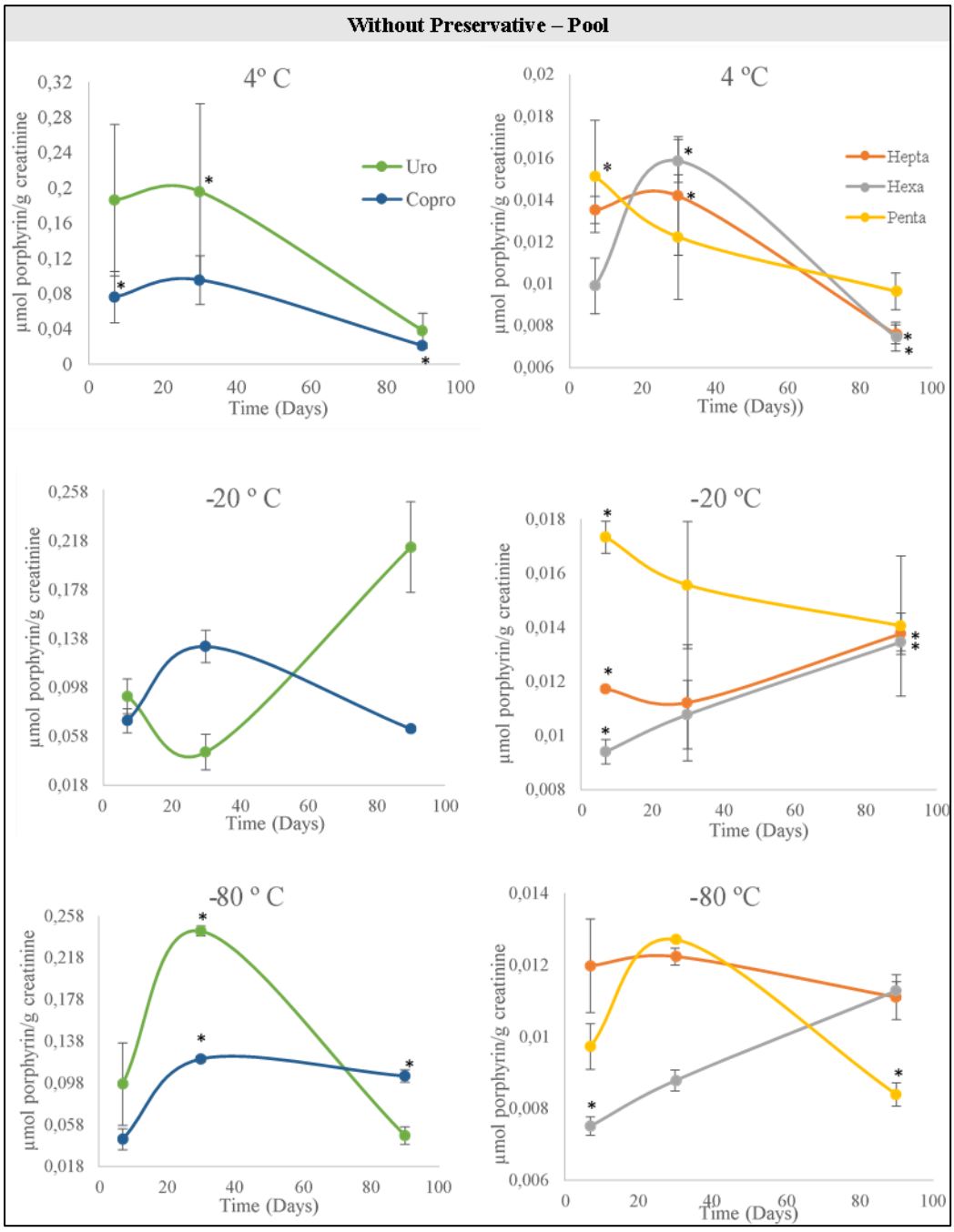

Fig. 3.3 Stability of Uro-, Hepta-, Hexa-, Penta- and Coproporphyrin over time (on days 0, 7, 30 and 90) in a pool of urine preserved at different temperatures (4º, -20º and -80º C), without addition of preservative. Data represent the mean ± SD. All the porphyrins concentrations correspondent to times t=7, t=30 and t=90 were compared, by T-student Test, to the initial concentration, correspondent to t=0. When p<0,05 the difference is significative and signalized with *.

When urine samples were storage in a pool at -20ºC without preservative, there were no significant differences between the initial and final concentrations of uro and copro (Fig.3.3), so we can preserve copro and uro for at least 90 days in these conditions. At -80 ºC, the hepta fraction did not present significative oscillation of the concentration over time, which indicates that it is possible to maintain hepta for at least 90 days. Penta could be preserved for 30 days in -80ºC, without danger of concentration's loss.

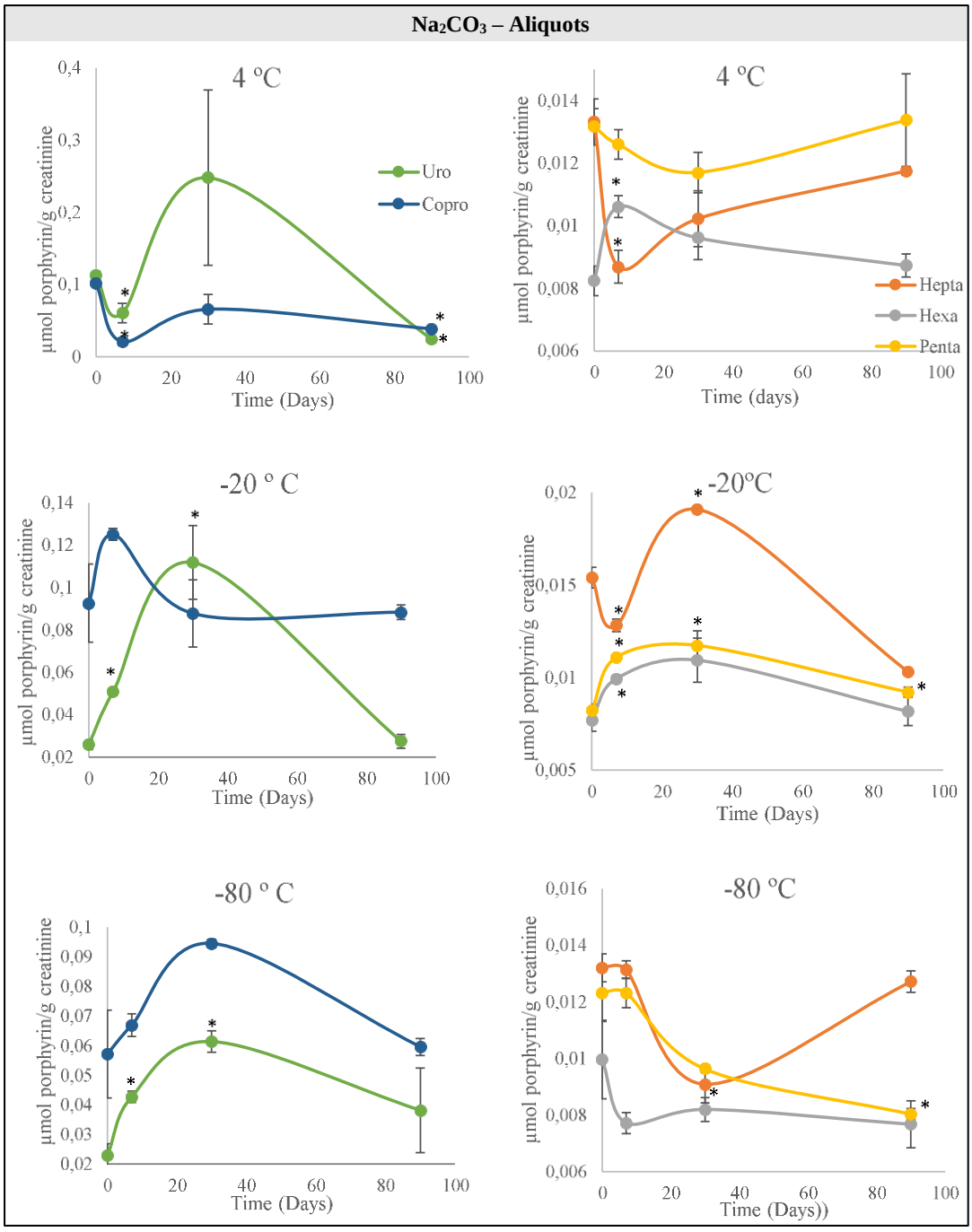

Fig. 3.4 Stability of Uro-, Hepta-, Hexa-, Penta- and Coproporphyrin over time (on days 0, 7, 30 and 90) in urine aliquots preserved with Na_2CO_3 at different temperatures (4º, -20º and -80º C). Data represent the mean ± SD. All the porphyrins concentrations correspondent to times t=7, t=30 and t=90 were compared, by T-student Test, to the initial concentration, correspondent to t=0. When p<0,05 the difference is significative and signalized with *.

The stability of porphyrins preserved with Na_2CO_3, which promote increase of pH, is presented in Figure 3.4. Copro was stable at -20 ºC and -80 ºC, the penta fraction was stable at 4ºC and hexa was stable at -80ºC, over 90 days.

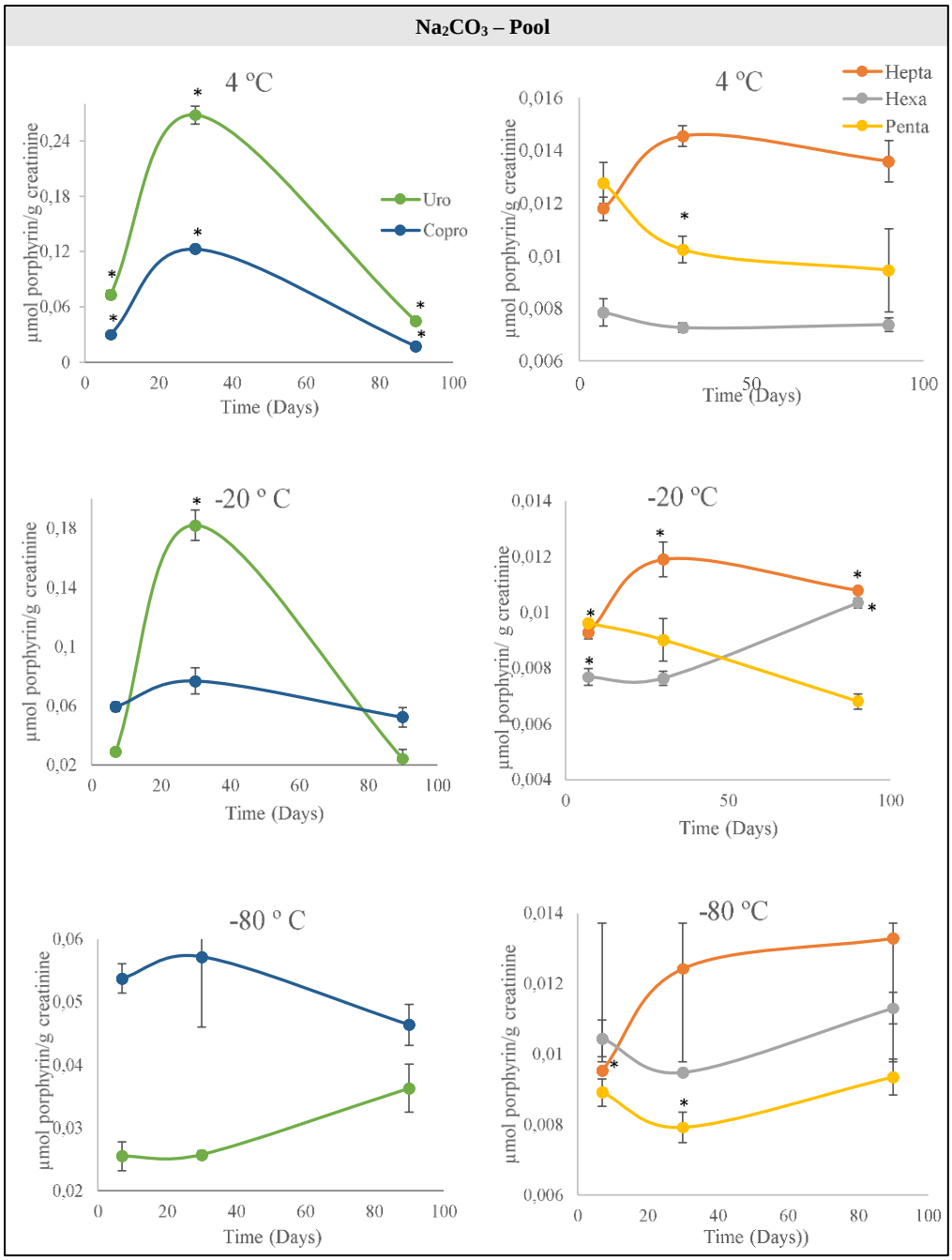

Fig. 3.5 Stability of Uro-, Hepta-, Hexa-, Penta- and Coproporphyrin over time (on days 0, 7, 30 and 90) in a pool of urine preserved with Na_2CO_3 at different temperatures (4º, -20º and -80º C). Data represent the mean ± SD. All the porphyrins concentrations correspondent to times t=7, t=30 and t=90 were compared, by T-student Test, to the initial concentration, correspondent to t=0. When $p<0,05$ the difference is significative and signalized with *.

In a pool of urine with Na_2CO_3 as preservative, uro, copro and hexa concentrations were stable at -80 ºC (Fig.3.5). At 4 ° C, hepta and hexa showed no significant differences in concentration over all the time. Copro was stable at -20 ° C and -80 ° C, and the same has occurred in urine aliquots with Na_2CO_3. These results indicated that we can preserve for at least 90 days copro at -20ºC and -80 ºC, in urine aliquots or urine pool.

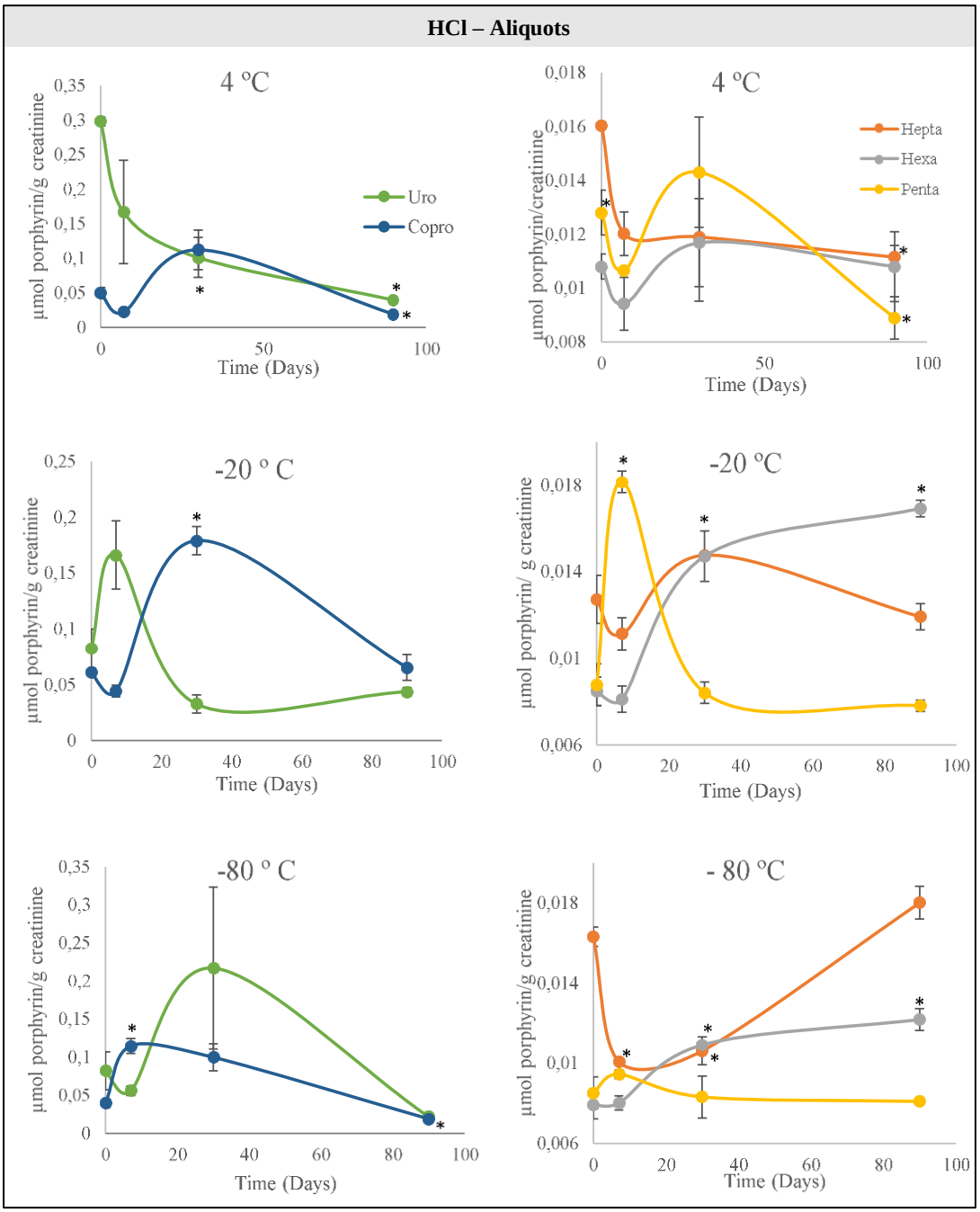

Fig. 3.6 Stability of Uro-, Hepta-, Hexa-, Penta- and Coproporphyrin over time (on days 0, 7, 30 and 90) in urine aliquots preserved with HCl at different temperatures (4º, -20º and -80º C). Data represent the mean ± SD. All the porphyrins concentrations correspondent to times t=7, t=30 and t=90 were compared, by T-student Test, to the initial concentration, correspondent to t=0. When p<0,05 the difference is significative and signalized with *.

The stability of porphyrin concentration in acidic media with HCL, in urine aliquots is shown in Figure 3.6. The uro fraction showed stability at -20 ° C and -80 ° C, while the copro fraction showed significant concentration oscillations at 4º, -20 º and -80ºC after days 0 and 7. This behavior was inverse to the one observed in alkaline environment (Fig.3.4). We can conserve for at least 90 days in HCl, uro at -20ºC and -80ºC, hexa at 4ºC and penta at -80 ºC.

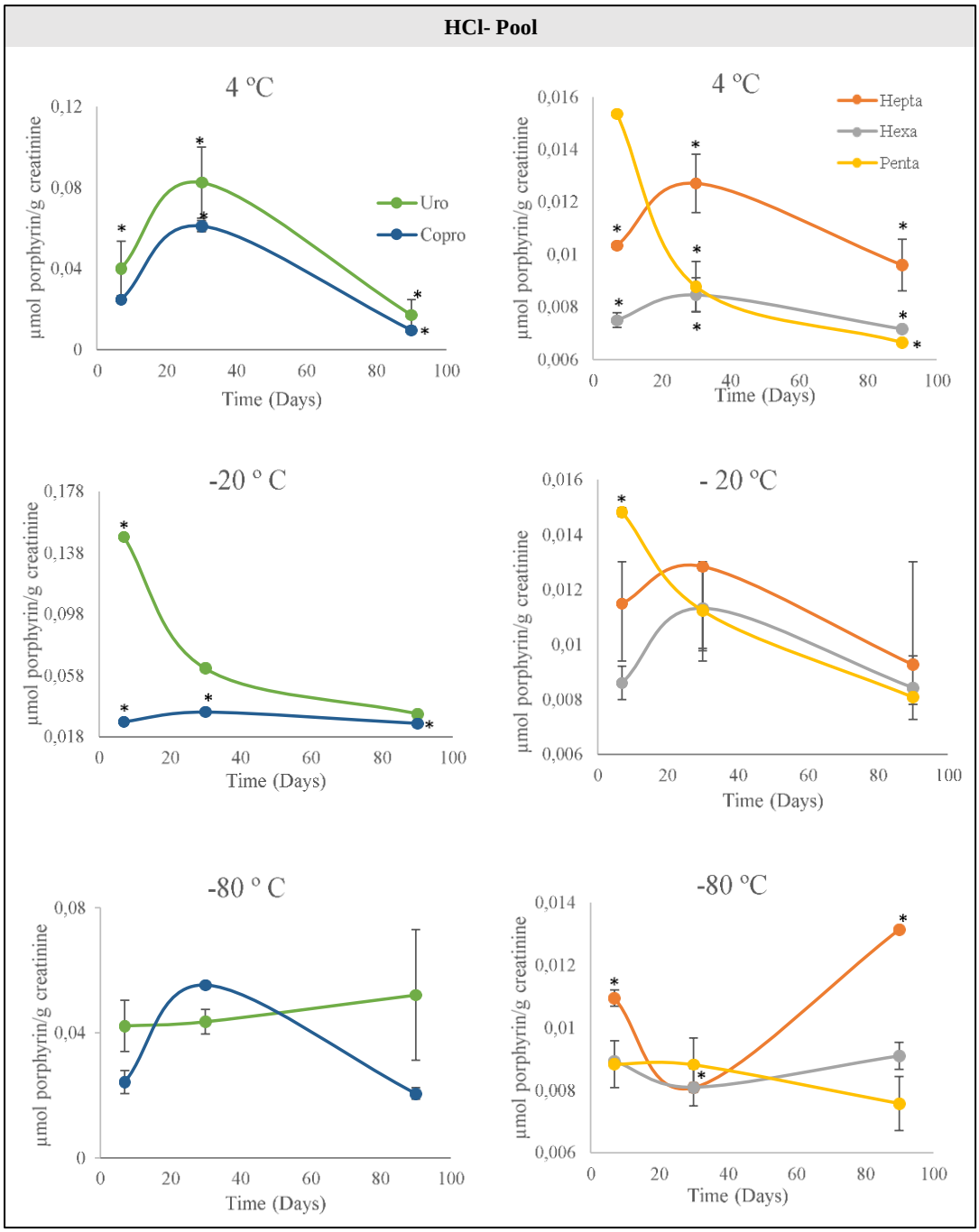

Fig. 3.7 Stability of Uro-, Hepta-, Hexa-, Penta- and Coproporphyrin over time (on days 0, 7, 30 and 90) in a pool of urine preserved with HCl at different temperatures (4º, -20º and -80º C). Data represent the mean ± SD. All the porphyrins concentrations correspondent to times t=7, t=30 and t=90 were compared, by T-student Test, to the initial concentration, correspondent to t=0. When p<0,05 the difference is significative and marked with *.

Concentration of uro and copro was stable at -80 ° C for 90 days in a urine pool with HCL as preservative (Figure 3.7). Uro fraction lost its stability at -20 ° C, compared with urine aliquots (Figure 3.6). Hepta and hexa showed stablity at -20 ° C for 90 days. In these conditions, we can preserve uro and copro at -80 ºC, and hepta and hexa at -20 ºC, for 90 days.

3.3.2 Percent differences in uro- and coproporphyrin concentrations over time

The following sub-chapters present the differences, in percentage, of the uro and copro concentrations over time in the conditions described on experimental design (Fig.3.1).

The mean concentrations and the concentrations correspondent to the time t=0 are showed in Tables A1.1 to A1.6, in Appendix 1.

Since the predominant porphyrin in urine of healthy individuals is copro, followed by uro at minor concentrations, we decided only to present results for copro and uro.

In urine aliquots, at all the temperatures, there wasn't differences of uro and copro 'concentration higher than 5%, which indicates that uro and copro can be conserved for at least 7 days (Table 3.1). Uro can be saved for 90 days at 4ºC and Copro for 90 days at -20 ºC, without significant differences of concentration, in absence of preservatives.

Table 3.1 Differences in percentage (%) of the concentrations of uro- and coproporphyrin over time (on days 7, 30 and 90) compared with the initial concentration (t=0), in urine aliquots and in urine pool, without preservative. The differences of percentage above 5% are marked in bold.

		4 ºC		**-20 ºC**		**-80 ºC**	
	Time (Days)	**Uro**	**Copro**	**Uro**	**Copro**	**Uro**	**Copro**
Aliquots	**7**	1,42	-4,84	-1,17	3,24	2,96	-4,53
	30	2,50	0,08	-4,70	-0,48	1,56	**7,02**
	90	1,55	**-6,54**	**-6,73**	-0,42	-2,06	0,76
Pool	**7**	4,55	-4,27	0,05	-2,17	**6,01**	-4,88
	30	3,44	3,66	-4,52	3,91	**20,67**	**7,71**
	90	-0,81	**-6,61**	**12,26**	-2,84	1,07	-1,64

Both in urine aliquots and urine pool, the same conditions of temperature presented differences of uro and copro' concentration superior to 5% (Table 3.2). In the presence of Na_2CO_3, Uro and Copro could be preserved for 90 days at -80 ºC, and copro can also be preserved for 90 days at -20 ºC, without differences of concentration higher than 5%.

Table 3.2 Differences in percentage (%) of the concentrations of uro- and coproporphyrin over time (on days 7,30 and 90) compared with the initial concentration (t=0), in urine aliquots and in urine pool, with Na_2CO_3. The differences of percentage above 5% are marked in bold.

		4 ºC		-20 ºC		-80 ºC	
	Time (Days)	**Uro**	**Copro**	**Uro**	**Copro**	**Uro**	**Copro**
Aliquots	**7**	-5,23	**-8,09**	2,49	1,71	1,99	0,98
	30	**13,53**	-3,58	**8,59**	5,32	3,85	3,74
	90	**-8,85**	**-6,31**	0,16	1,27	1,53	0,24
Pool	**7**	-3,93	**-7,11**	0,32	-0,11	0,26	-0,34
	30	**15,53**	2,14	**15,63**	1,64	0,29	0,01
	90	**-6,84**	**-8,41**	-0,16	-0,82	1,34	-1,08

In samples with HCl as preservative, the uro fraction revealed an increase of concentration on day 30 (19,43%) in urine aliquots, compared with t=0. Copro suffered an increase of 11,86% on day 30 (-20ºC, aliquots) (Table 3.3).

Table 3.3 Differences in percentage (%) of the concentrations of uro- and coproporphyrin over time (on days 7,30 and 90) compared with the initial concentration (t=0), in urine aliquots and in urine pool, with HCl. The differences of percentage above 5% are marked in bold.

		4 ºC		-20 ºC		-80 ºC	
	Time (Days)	**Uro**	**Copro**	**Uro**	**Copro**	**Uro**	**Copro**
Aliquots	**7**	**-13,07**	-2,75	**8,37**	-1,67	-2,63	**7,51**
	30	-1,15	1,10	0,70	**11,86**	**19,43**	4,27
	90	**-7,28**	**-8,21**	1,81	0,52	-0,09	-3,83
Pool	**7**	**-7,26**	**-7,66**	**12,27**	-3,26	1,93	-3,29
	30	-3,02	-4,03	3,69	-2,62	2,06	-0,19
	90	**-9,56**	**-9,17**	0,73	-3,37	2,92	-3,65

3.3.3 Differences of percentages in uro- and coproporphyrin concentrations over time comparing the added preservatives

The addition of both preservatives caused variations of uro and copro' concentrations above 5 %. When Na_2CO_3 was added to urine aliquots, the initial concentration of copro and uro increased 6,7% and 1,4% respectively, compared with samples without preservative, at 4ºC (Table 3.4).

However, on the 7th day, the addition of Na_2CO_3 did not make any difference on uro concentration, compared with the same samples without preservative. Uro initial concentration, increased 25,2% in urine aliquots after the addition of HCl, but concentration of uro at -80ºC, in urine pool, decreased 20%, on the 30th day.

Table 3.4 Differences in percentage (%) of the concentrations of uro- and coproporphyrin over time (on days 7, 30 and 90 days), preserved in Na_2CO_3 or HCl, compared with the concentration of urine aliquots and pool without preservative added. The differences of percentage above 5% are marked in bold.

			4 ºC		-20 ºC		-80 ºC	
		Time (Days)	**Uro**	**Copro**	**Uro**	**Copro**	**Uro**	**Copro**
Aliquots	**Na_2CO_3**	**0**	**6,70**	1,40	**-6,40**	-3,20	-1,40	-0,30
		7	0,00	-1,80	-2,80	-4,70	-2,40	5,20
		30	**17,70**	-2,20	**6,90**	2,60	0,80	1,00
		90	-3,70	1,70	0,40	-1,50	2,20	-3,30
	HCL	**0**	**25,20**	-3,70	-0,80	-3,20	4,50	-2,00
		7	**10,70**	-1,70	**8,70**	**-8,10**	-1,10	**10,00**
		30	3,00	2,40	-1,00	**9,10**	**16,40**	1,50
		90	-2,10	-0,20	2,10	-2,20	0,60	**-7,40**
Pool	**Na_2CO_3**	**7**	-1,80	-1,40	**-6,20**	-1,20	**-7,20**	1,00
		30	**18,80**	-0,10	**13,70**	-5,50	**-21,80**	**-6,40**
		90	0,60	-0,40	**-18,90**	-1,20	-1,20	-5,90
	HCl	**7**	-5,10	-1,90	5,80	-4,30	-5,50	-2
		30	0,20	**-6,30**	1,80	**-9,80**	**-20**	**-6,6**
		90	-2,10	-1,10	**-18,00**	-3,70	0,4	-8,4

3.4 Discussion

3.4.1. Study of porphyrins stability in urinary samples

Urine porphyrin measurement is important for the diagnosis of porphyrias or for the use of porphyrins as biomarkers of exposure to xenobiotics like heavy metals. It is central for the pharmaceutical and medical community as well as many research laboratories, to obtain accurate results when analysing urinary porphyrins; nevertheless, urine is a very complex matrix, with many interfering substances, and porphyrins are very unstable molecules. This way, we attempted to investigate the best

storage conditions (time, preservatives, and temperature) of porphyrins in urine samples capable of providing analysis with reliable results.

We tested various storage conditions (aliquots or pools subjected to freeze-thaw cycles at four different times), temperature (4ºC, -20 ºC and -80 ºC), and preservative (without preservative, with HCl 6M and with Na_2CO_3 2 M) for 90 days.

Without any additional preservative (Fig. 3.2 and 3.3), copro was stable at -20ºC both in aliquots and pools, and hepta was stable at -80ºC at both conditions too. This means that copro can be preserved at -20 ºC and hepta at -80 ºC for 90 days, without addition of preservative, at urine pool or aliquots. In this case, the storage method (aliquots or pool) wasn't significant for the stability of these porphyrins. Although in uro the storage method is important, because uro was stable at -20ºC under the pool condition but not at aliquots condition.

Samples were acidified with HCl, to neutralize negative charges on the carboxylic acid side chains of the porphyrins (Sullivan et al., 2015) and enhance the spontaneous oxidation of porphyrinogens to porphyrins (Perkins and Johnson, 1989). Na_2CO_3 has been added to samples to keep the medium near neutral or slightly alkaline, making it impossible the transformation of porphyrinogen into porphyrins (Schreiber et al., 1983).

In samples preserved with Na_2CO_3 (Fig. 3.4 and 3.5), copro was stable at -20 ºC and -80 ºC, however uro was only stable at -80ºC when samples where storage as a pool. Copro was already stable at -20 ºC without additional preservative. The hexa fraction presented stability at -80 ºC. In summary, when in the presence of Na_2CO_3, we can preserve copro at -20 ºC and -80 ºC and hexa at -80 ºC, until 90 days, independent of storage form (pool or aliquots).

HCl as preservative, succeeded to keep uro stable at -20 and -80 ºC, in urine aliquots, and copro and uro stable at -80 ºC, in urine pool, for 90 days (Fig. 3.6 and 3.7). By this way, the preservation of uro at -80 ºC is guaranteed for 90 days in urine aliquots and pool, when HCl is the preservative selected.

We suggest that it is necessary to add a preservative, like HCl or Na_2CO_3, for the preservation of porphyrins because the porphyrin's concentrations presented less significant differences with the supplement of preservatives than without preservative. We also recommended to store the urine samples in the form of aliquots, once we obtained less variations of porphyrins' concentrations in samples as aliquots than as pools.

The conclusions obtained in this chapter, are very important for research labs for example, because they sometimes need to preserve urine samples for 90 days or more and obtain reliable results in their studies. Our results suggested, there is no need to recall new samples of human or animal subjects at least for 90 days.

Previous works have shown that porphyrins in urine, faeces and whole blood are stable for up to 4 days if stored protected from light at 4ºC and up to 2 days if stored at room temperature (Woolf et al., 2017). We go further and admit that at least the urinary uro, copro, hexa and hepta fractions can be preserved up to 90 days at the specific conditions described before. Further studies are needed to

substantiate these findings, since porphyrins are quite unstable molecules, varying with pH, ionic strength, matrix and solvents (Polo et al., 1988).

3.4.2 Percent differences in uro- and coproporphyrin concentrations over time

In normal subjects, uro and copro predominate and they are the porphyrins with more importance for diagnostic purposes, so their stability in urine samples is very important to obtain valid results (Hindmarsh et al., 1999a).

When comparing the mean differences of uro and copro' concentrations, in the absence of preservative, we can observe that in the first seven days the concentrations of uro and copro decreased, but without differences above 5 % (Table 3.1). This decrement can be explained by degradation of porphyrins by light.

With Na_2CO_3 as preservative of urine samples, copro and uro had fewer differences of concentration in urine aliquots than in urine pool (Table 3.2). Uro was most stable at 4 ºC and copro at -80 ºC. The differences of uro and copro concentration were very oscillating over time in basic environment. This can be explained by the fact that a large fraction of porphyrins in urine are associated with precipitates present in alkaline samples, and if the samples are not thoroughly mixed, these precipitates can interfere with the results of the analysis (Woolf et al., 2017).

In acid medium, uro was most stable at urine pool while copro was most stable at urine aliquots (Table 3.3). In the first 30 days, porphyrin concentration increased by mean 3%, because of oxidation of porphyrinogens to porphyrins enhanced by acidification of urine (Perkins and Johnson, 1989).

Other works suggested that freeze-thawing of urine samples, particularly repeated cycles, can result in a marked decrease in porphobilinogen concentration and a corresponding increase in total urinary porphyrin (Woolf et al., 2017). In our work, we obtained similar results, once the uro and copro concentration increased 2% in the total of urine samples freeze-thaw cycles between analyses.

3.4.3 Differences of percentages in uro- and coproporphyrin concentrations over time comparing the added preservatives

Several urine preservatives have been validated based on their physicochemical properties for use in the determination of biochemical analytes. To inhibit degradation and preserve the stability of urine chemical substances, laboratories refrigerate urine during the sample collection period and freeze the sample until the time for the necessary analyses, and use preservatives (Feres et al., 2011). In the measurement of urinary parameters, different urinary preservatives, like HCl and Na_2CO_3, are used for different purposes for example, to increase solubility, prevention of bacterial growth, stability of metabolites, etc (Yilmaz et al., 2008).

In this subchapter, we tried to answer the following question: "Is it necessary to add preservative to urine samples for a better preservation of urinary copro and uro?". To answer to this question, we obtained the differences of uro and copro concentrations with and without preservatives, that are summarized in Tables 3.4.

The addition of Na_2CO_3 to urine aliquots and urine pool decreased the initial concentration of uro and copro compared with urine samples without preservative (Table 3.4). This decrement can be observed in the following 30 days. This event can be explained by the decreased of porphyrins' fluorescence in basic/neutral environments. Fluorescence for uro, for example, is minimal near pH 7-7,5 (Polo et al., 1988). Westerlund et al. (1988) have suggested that losses of porphyrin may result from matrix effects and (or) incomplete oxidation of porphyrinogens to porphyrins in the urine samples before analysis. These explanations can be applied to our results too.

When HCl is added to urine samples in aliquots, the initial concentration of uro and copro increased compared to the initial concentration without preservative added (Table 3.4). During the first 30 days, uro and copro were also higher than concentrations in samples without preservative. The fluorescence of porphyrins increases with decreasing pH, and in acid environment, porphyrinogens are easier oxidized to porphyrins. These facts can explain the increase of porphyrin' concentration. However, in urine pools, when HCl was added, the concentration of porphyrins decreased at 7, 30 and 90 days. This loss of porphyrins from solutions with acidic pH appeared to be due to a combination of micro precipitation and aggregation effects (Perkins and Johnson, 1989).

With our results we can answer our initial question for this subchapter. We conclude that additional preservative is useful for the stability of uro and copro in urine samples. By adding Na_2CO_3, the pH of the urine becomes basic, and porphyrinogen forms predominate. The conservation of analytes in the form of porphyrinogens, might provide more resistance against photo-degradation. The addition of HCl 6M promotes acidification of urine, which intensifies absorbance, facilitates conversion of porphyrinogens to porphyrins, and dissociates Zn–porphyrin chelates (Deacon and Elder, 2001). Moreover precipitates, such as calcium salts, tend to adsorb porphyrins, especially copro, but HCl dissolved this precipitated material (Ár et al., 2010).

Chapter 4

Insights into methodologies of porphyrin determination

4.1 Background

Changes in urinary porphyrin excretion may be the result of hereditary diseases (porphyrias), and/or from environmental or occupational exposure to xenobiotics (Alves et al., 2009).

Although the porphyrias are uncommon disorders of heme biosynthesis, the wide range and variability of their clinical features leads to inconclusions in their diagnosis and differentiation; therefore, every clinical laboratory needs to have simple and reliable methods for their correct diagnosis (Deacon and Elder, 2001; Sies et al., 2015). Also having the ability to evaluate xenobiotics´ exposure that affect heme metabolism, it is important for the pharmaceutical and chemical industries as well as many research laboratories (Alves et al., 2009).

In normal subjects, uro and copro predominate. Only small amounts of hepta, hexa, and penta are present, with predominance of III-isomer (Hindmarsh et al., 1999b). However, since the porphyrin excretion pattern is characteristic for each of the hereditary porphyrias, and changes in porphyrin excretion patterns can be xenobiotic specific, it is important to have methods that allow the differentiation and quantification of porphyrins.

Methods for porphyrin analysis are based on the fluorometric and spectrophotometric properties of porphyrin molecules (Sullivan et al., 2015). Spectrophotometric and spectrofluorometric methods utilising a variety of extraction, separation and reaction techniques have been developed to measure the concentrations of the various porphyrins and their precursors in urine, faeces, whole blood and plasma. Many methods have been developed 'in house' and conditions may vary from laboratory to laboratory (Sies et al., 2015).

There are spectrophotometric methods that have been improved to achieve rapid and accurate methods for quantitative determination of porphyrins in urine (Zuijderhoudt et al., 1995). However, conventional Ultraviolet-Visible (UV-Vis) detection for porphyrin-related compounds is complicated due to their varying and overlapping absorbance maxima and extinction coefficients. At the same time, the presence of an array of background metabolites, which also absorb in the UV-Vis range, is especially problematic in urine samples, complicating analyte identification and reliable quantification (Ford et al., 1981).

The difference in the solubility and isoelectric points of the porphyrins makes it possible to perform separations by preferential extraction with organic solvents from aqueous buffered solutions. Organic solvents serve the dual function of isolating the porphyrins from urine, as well as separating them from each other (Henry and Fernandez, 1966), but HPLC is the longstanding technique of choice for separating porphyrins. It remains a staple technique in many toxicology, pharmacology, and research laboratories because it is flexible and ubiquitous. However, conventional HPLC can suffer from high levels of run to run variability, necessitating the use of internal or external standards for compound identification and a significant investment of time/funds for optimizing the choice of the column, solvent, and run conditions (Sullivan et al., 2015; Zuijderhoudt et al., 2000).

Many of the analytical methods used are, in principle, similar, but substantially quite varied and adapted to each individual laboratory. There are currently no international methodological guidelines for porphyrins' determination. Standardisation of assay conditions, especially for total

porphyrins, should lead to improved consistency and reliability of results among laboratories, enable harmonised reference intervals, and enhanced diagnostic accuracy (Sies et al., 2015).

In this chapter, we provided a brief study about the behaviour of copro isomers I and III and uro I in the UV-Vis spectrum. We evaluated the capacity of two different spectrophotometric methods for the detection and quantification of total porphyrins in urine, one using extraction of porphyrins in organic solvents and the other using acidification of urine. We also compared fluorometric and spectrophotometric methods for quantification of copro and total porphyrins.

4.2 Material and Methods

4.2.1 Chemicals

Chemicals were obtained from the following sources: Diethyl ether ($C_4H_{10}O$) and ethanol (C_2H_6O) from Sigma–Aldrich; Acetic acid ($C_2H_4O_2$) and hydrochloric acid p.a. (HCl; 37%) from Panreac; Iodine resublimed from BDH; Pure standards of uro I, copro I and copro III were obtained from Porphyrin products, Frontier Scientific.

4.2.2 Simplified method by Soulsby and Smith for the quantitative determination of urinary coproporphyrin

50 µL of acetic acid and 1250 µL of diethyl ether were added to 500 µL of each urine sample, shacked (15'') and centrifuged (2500 rpm, 2'). After centrifugation and phase separation, 1250µL of a hydrochloric solution of iodine (1% in HCl 5%) was added to the organic phase and after new agitation, centrifugation and phase separation, the final aqueous phase was placed in a water bath (37 ˚C, 5'). A spectrum between 350 nm and 450 nm was recorded and absorbances were measured at 380 nm and 430 nm, corresponding to impurities' maximum absorbances, and at 401 nm, corresponding to the Soret band, in a UV-500 Spectronic Unicam spectrophotometer with Vision 32 Software.

Total copro were expressed in µg/g creat and were calculated according to the expression: Copro (µg/L) = [2 x A_{401} – (A_{430} + A_{380})] x 2,093 x 1,064 x 1000, and corrected for creat (Soulsby and Smith, 1974).

4.2.3. Semiquantitative determination of total porphyrins by Deacon and Elder method

Urine was mixed to resuspend any sediment material and an aliquot (4 mL) was blended with 1 mL of concentrated HCl. Undissolved material was then removed by centrifugation before transferring the clear supernatant to a 1 cm cuvette and recording the absorption spectrum between 350 nm and 450 nm against air in the reference cuvette. If a peak was present in the 400 nm region, the total porphyrin concentration was: A x 2500 nmol/litre, where A was the peak absorbance above a baseline drawn between two suitable points (Fig. 4.1).

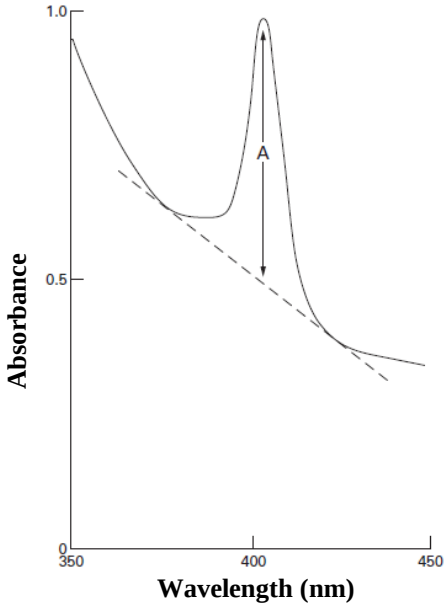

Fig. 4.1 Absorption spectrum of acidified urine showing the procedure for the measurement of corrected absorbance (A) of the porphyrin peak (Deacon and Elder, 2001).

The factor 2500 is derived from the volume of urine, the volume of acid, and a millimolar extinction coefficient of 500, which is approximately that of a 7/1 (mol/mol) mixture of copro and uro in 2.3M HCl. The porphyrin composition of this mixture resembles that of normal urine (Deacon and Elder, 2001)**.**

Total porphyrins were expressed in µmol/g creat,

4.2.4 Total porphyrins in urine by HPLC

Total porphyrins in urine by HPLC were calculated using the sum of the uro, hepta, hexa, penta and copro concentrations, obtained through the urinary porphyrin profile (Woods et al., 1991). This method was previously described in chapter 3.

Total porphyrins were expressed in µmol/g creat.

4.2.5 Statistical Analysis

Statistical analysis was performed with the SPSS 24.0 statistical package for Windows (SPSS, Inc., Chicago, IL, USA).

After verification of data adequacy for parametric methods, normal distribution by Kolmogorov-Smirnov test, and homogeneity of variance by Levene's test, correlations between the porphyrins determination methods were performed by the Pearson's Correlation tests. The correlations were considered significant when $p<0.05$.

4.3 Results

4.3.1 Study of copro- and uroporphyrin standards by UV-Vis spectrometry

Since the aim of this sub-chapter was to study and characterize the copro and uro isomers in UV-Vis, we started to see the linearity of the standards in aqueous solution, by linear regression analysis, in the Soret peak, and the respective spectrum with (Soulsby method) and without extraction.

4.3.1.1 Coproporphyrin I

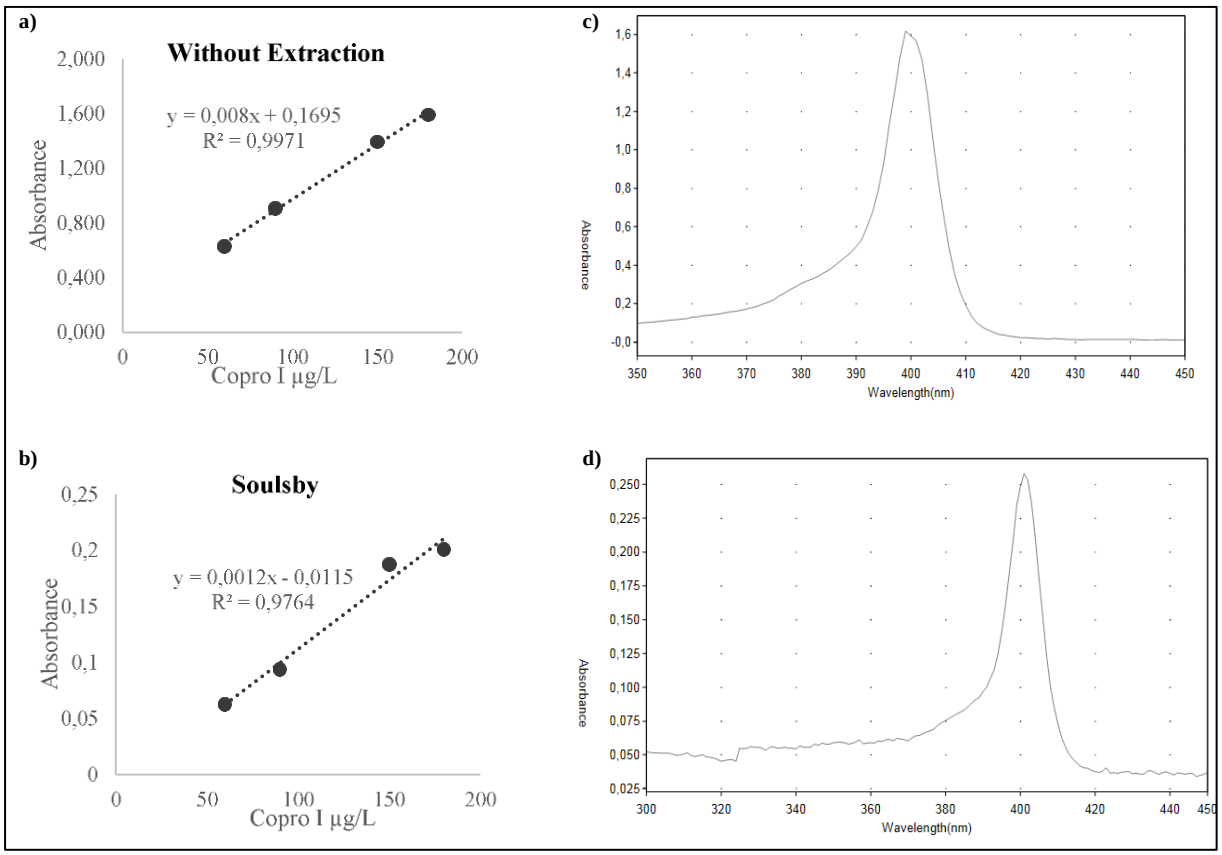

Fig. 4.2 Linearity of coproporphyrin I standards (60, 90, 150 and 180 µg/L) in a water matrix at 400 nm: a) without extraction method; b) with Soulsby' extraction method; UV-Vis spectrum of coproporphyrin I (180 µg/L) in a water matrix: c) without extraction method; d) with Soulsby' extraction method.

The UV-Vis spectrum of copro I shows a Soret' peak at 400 nm in both cases: without an extraction method and with the Soulsby' method (Fig.4.1). The linearity of standards was better achieved without extraction (R=0,997). The recovery rate for copro I by the Soulsby method was 12%.

4.3.1.2 Coproporphyrin III

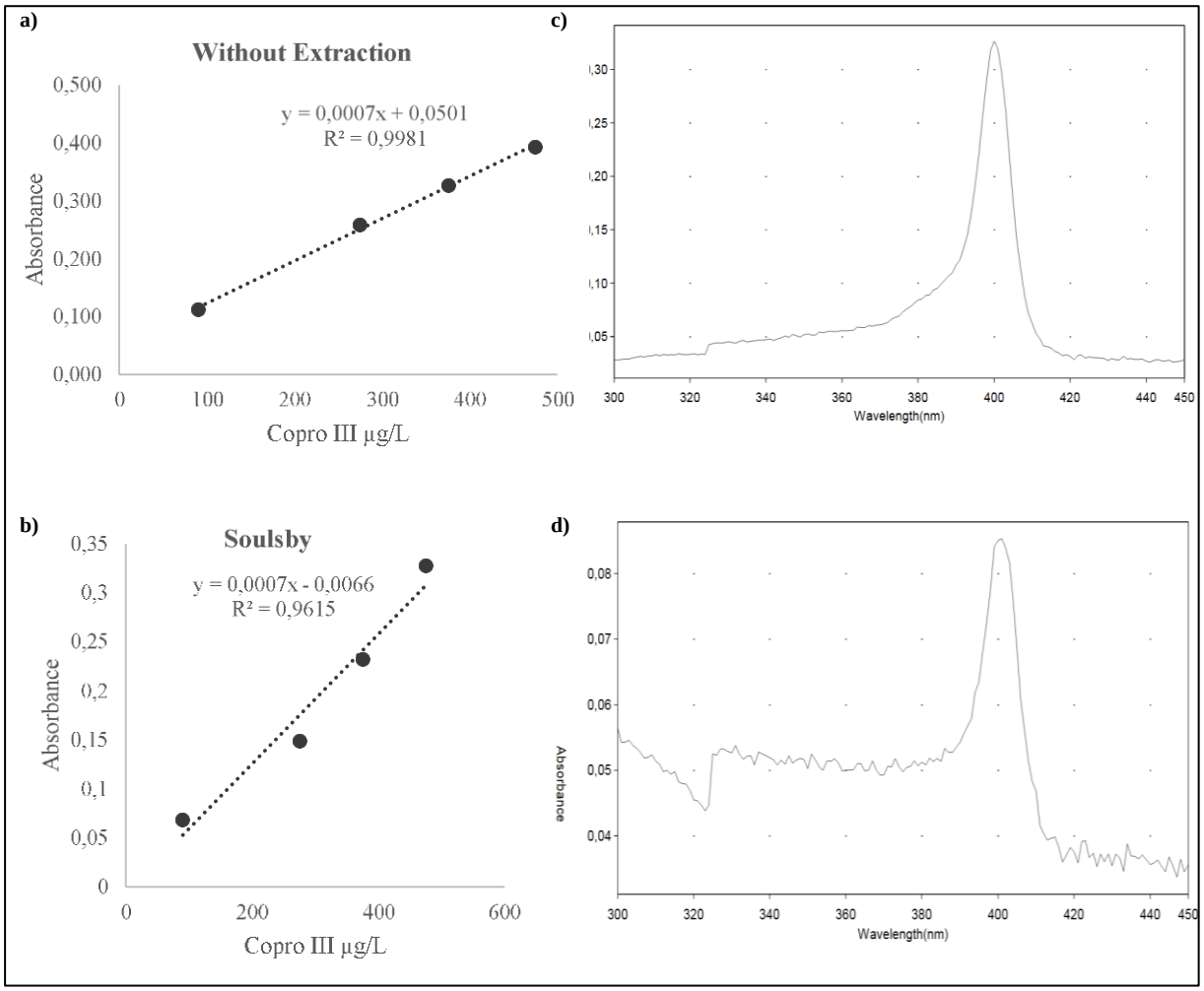

Fig. 4.3 Linearity of coproporphyrin III standards (90, 275, 375 and 475 µg/L) in a water matrix at 401 nm a) without extraction method b) with Soulsby' extraction method; c) UV-Vis spectrum of coproporphyrin III (375 µg/L) in a water matrix without extraction method d) UV-Vis spectrum of coproporphyrin III (475 µg/L) in a water matrix with Soulsby' extraction method.

Copro III standards showed a Soret peak at 400 nm in both cases, without extraction and with Soulsby extraction. The linearity is better achieved without extraction method (R^2=0,998) (Fig. 4.3). The recovery rate was 68%, when copro III was extracted by the Soulsby method.

4.3.1.3 Uroporphyrin I

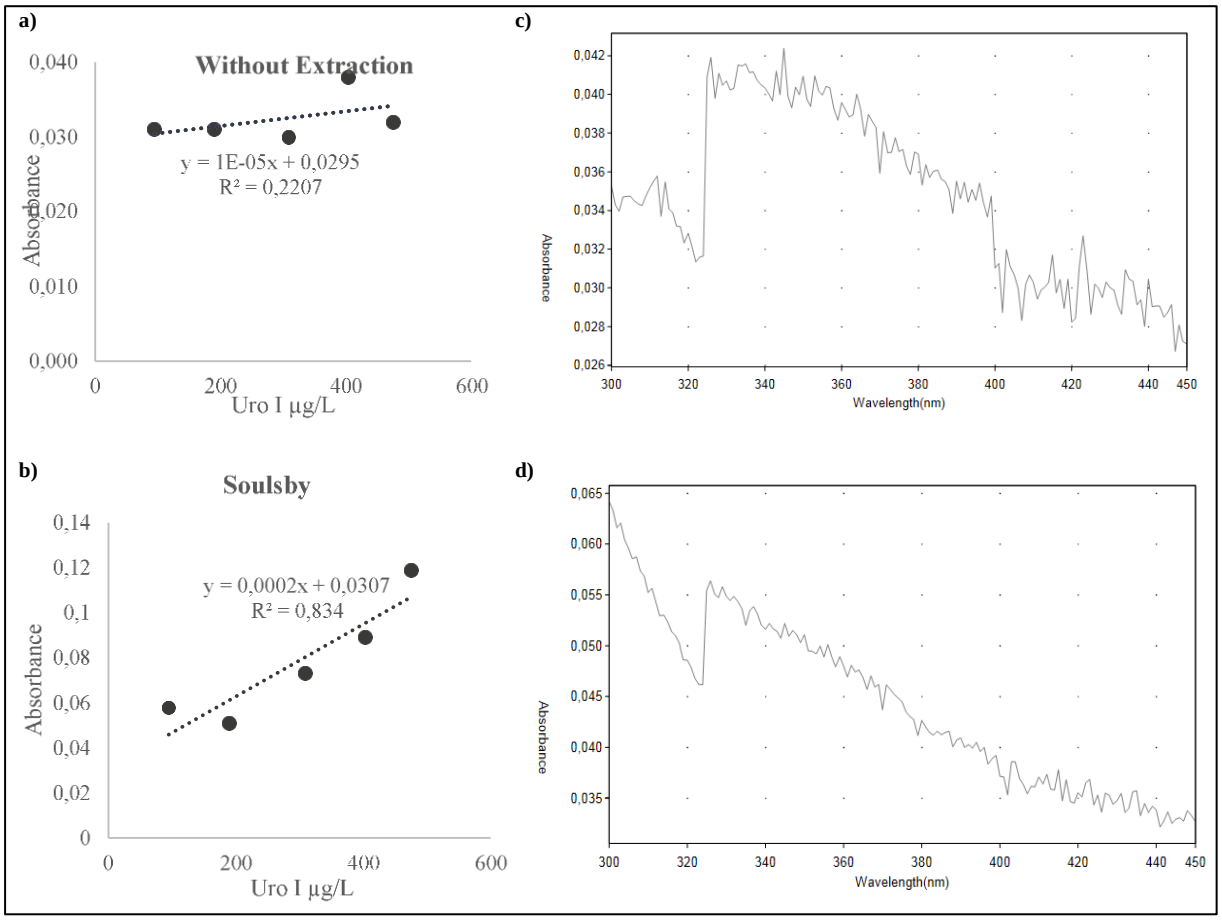

Fig. 4.4 Linearity of uroporphyrin I standards (180, 150, 90 and 60 µg/L) in a water matrix at 401 nm: a) without extraction method; b) with Soulsby' extraction method; UV-Vis spectrum of uroporphyrin I (180 µg/L) in a water matrix: c) without extraction method; d) with Soulsby' extraction method.

We were unable to obtain a defined spectrum for uro I. In this way, we cannot define the wavelength of the Soret peak. We didn't obtain a good linearity for the uro I standards, when we assumed 401 nm as the Soret Peak (Fig. 4.4).

4.3.2 Determination of the molar extinction coefficient under experimental conditions

The porphyrins in acid solutions obey Beer's law up to an absorbance of about 1 (Rimington, 1960). In this way, we could determine the molar extinction coefficient (ε), under our experimental conditions, by the following formula:

$$A = c \times \varepsilon \times l \Leftrightarrow \varepsilon = \frac{c \times l}{A} \quad (l = 1\ cm) \Leftrightarrow \varepsilon = \frac{c}{A}$$

ε is express in $M^{-1}\,cm^{-1}$.

We used several concentrations of standards (Fig. 4.2 a) and Fig.4.3 a)) in a water matrix, without any extraction method, and the respective absorbance values at the Soret peak, to calculate ε.

The Soret maximum bands of copro I and III, 401 and 400 nm respectively, were coincident with the values from literature. The ε values of our experimental conditions were close to the values mentioned in the literature for copro I and III (Table 4.1).

We can not determine the Soret maximum band and the ε value of uro I due to the reasons referred to above.

Tabel 4.1. Comparison of Soret maximum band and molar extinction coefficients (ε) of Coproporphyrin I and III and Uroporphyrin I, under our experimental conditions vs values reported in the literature.

	Soret Max. Experimental (nm)	**Soret Max. Literature (nm)**	**ε Experimental (M^{-1} cm^{-1})**	**ε Literature (M^{-1} cm^{-1})**
Copro I	401	400-401 (With et al., 1977)	$5,8x10^5$	$4,78x10^5$ (Rimington, 1960)
Copro III	400	399,5 (Rimington, 1960)	$6,4x10^5$	$4,89x10^5$ (Rimington, 1960)
Uro I	nd	406 (Henry and Fernandez, 1966; Rimington, 1960)	nd	$4,7x10^5$ (Zuijderhoudt et al., 2000)

nd=not determined

4.3.3 Comparison between the two different spectrophotometric methods for determination of porphyrins in urine

Since the Soulsby method is often used for the quantification of total porphyrins (Leahy and Brien, 1982), although it was described for the quantification of copro, we tried to clarify this issue. For that, we analysed standards of copro I, III and uro I through the Soulsby method, and compared that results with the results obtained by the Elder method, which quantifies total porphyrins by UV-Vis spectrometry (Deacon and Elder, 2001).

4.3.3.1 Coproporphyrin I

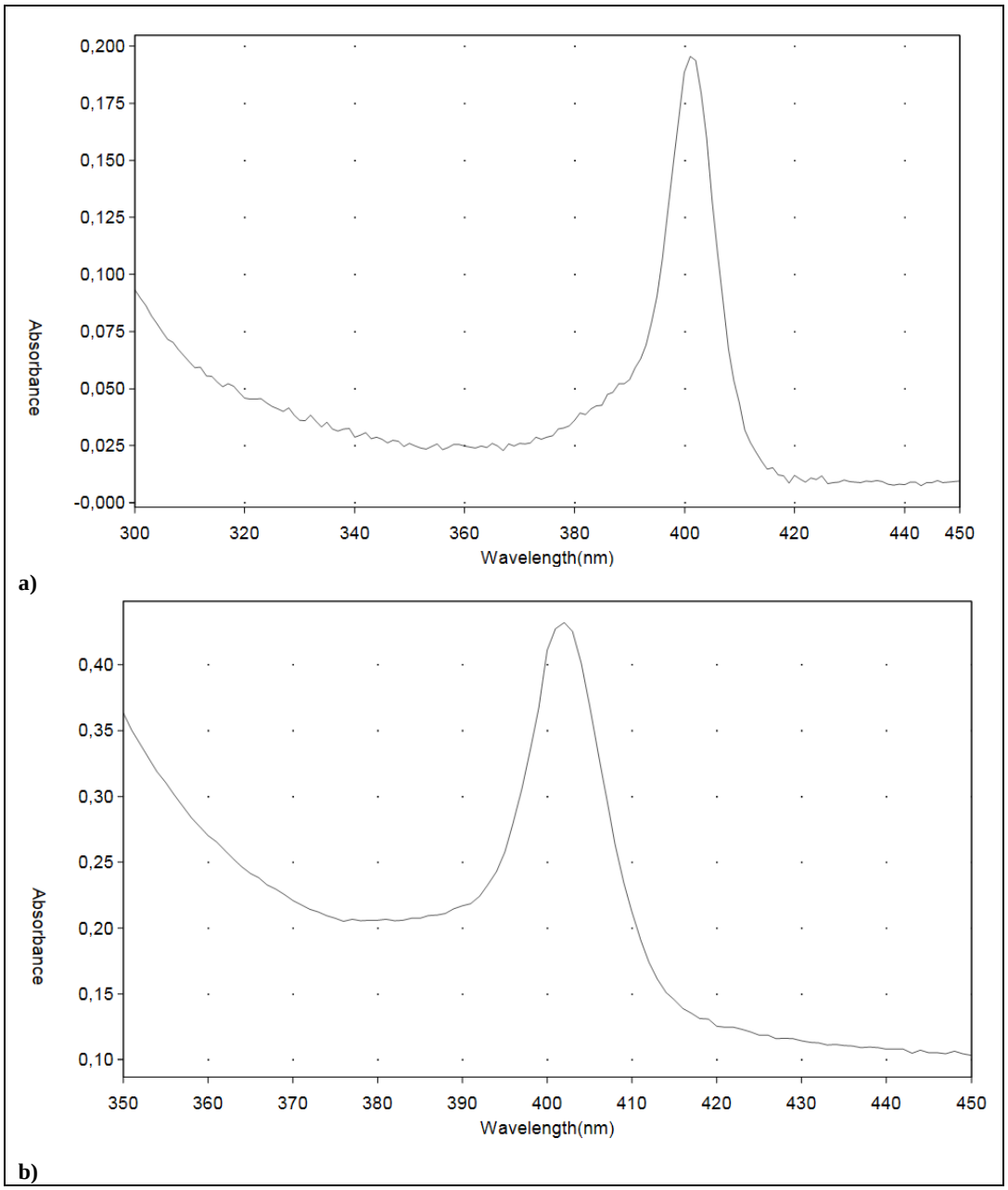

Fig. 4.5 UV-Vis spectra of coproporphyrin I (6 mg/L) in a urine matrix: a) with Soulsby' extraction method; b) with Elder's method.

The Copro I Soret peak (401 nm) was visible in the spectrum correspondent to the Soulsby extraction method and in the spectrum of Elder method (Fig. 4.5). Both methods can determine Copro I in urine samples.

4.3.3.2 Coproporphyrin III

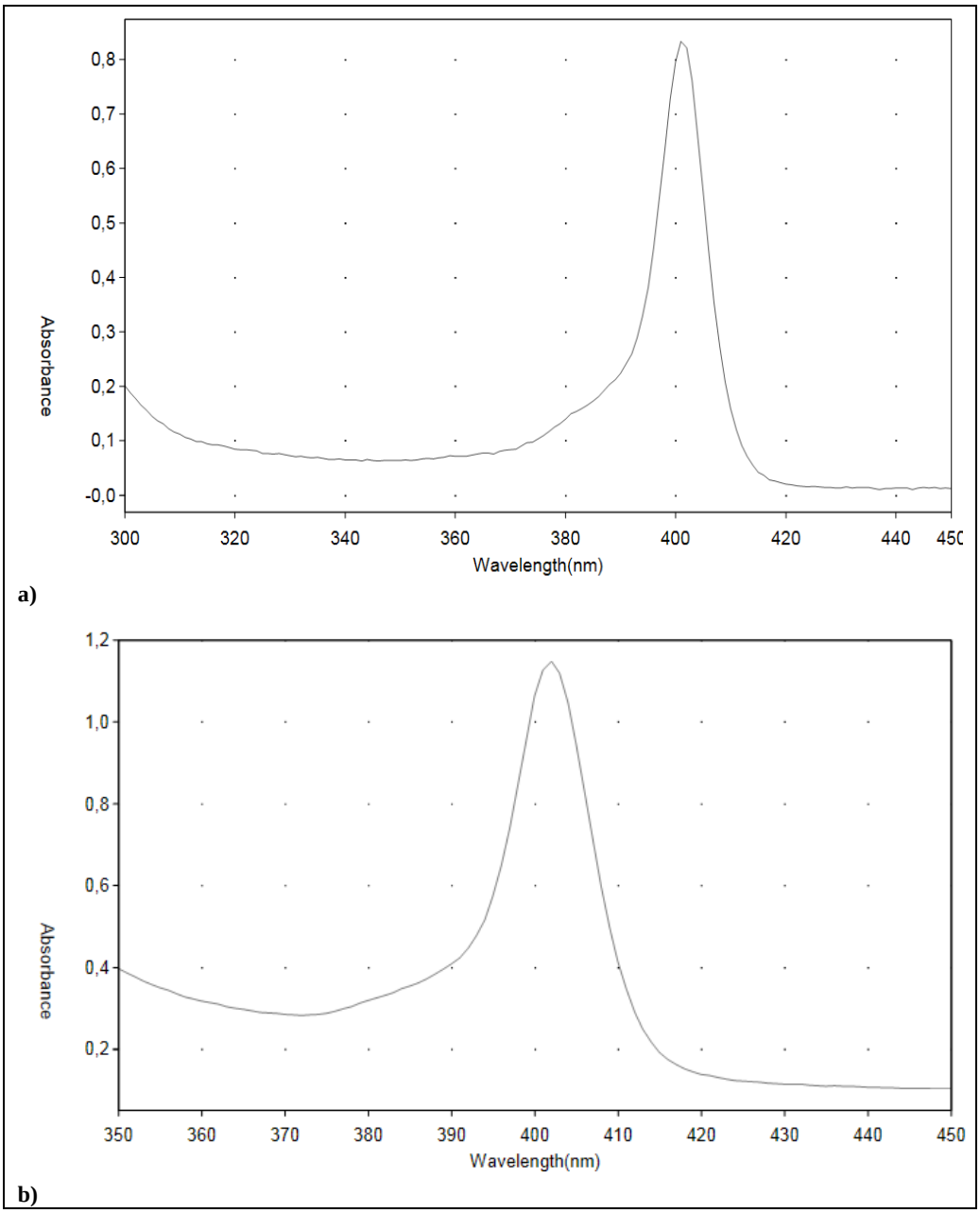

Fig. 4.6 UV-Vis spectra of coproporphyrin III (9.5 mg/L) in a urine matrix: a) with Soulsby' extraction method; b) with Elder's method.

Both methods, Soulsby and Elder, determined the Copro III standard added to urine; the Soret peak of copro III (400 nm) was visible in both spectra (Fig. 4.6).

4.3.3.3 Uroporphyrin I

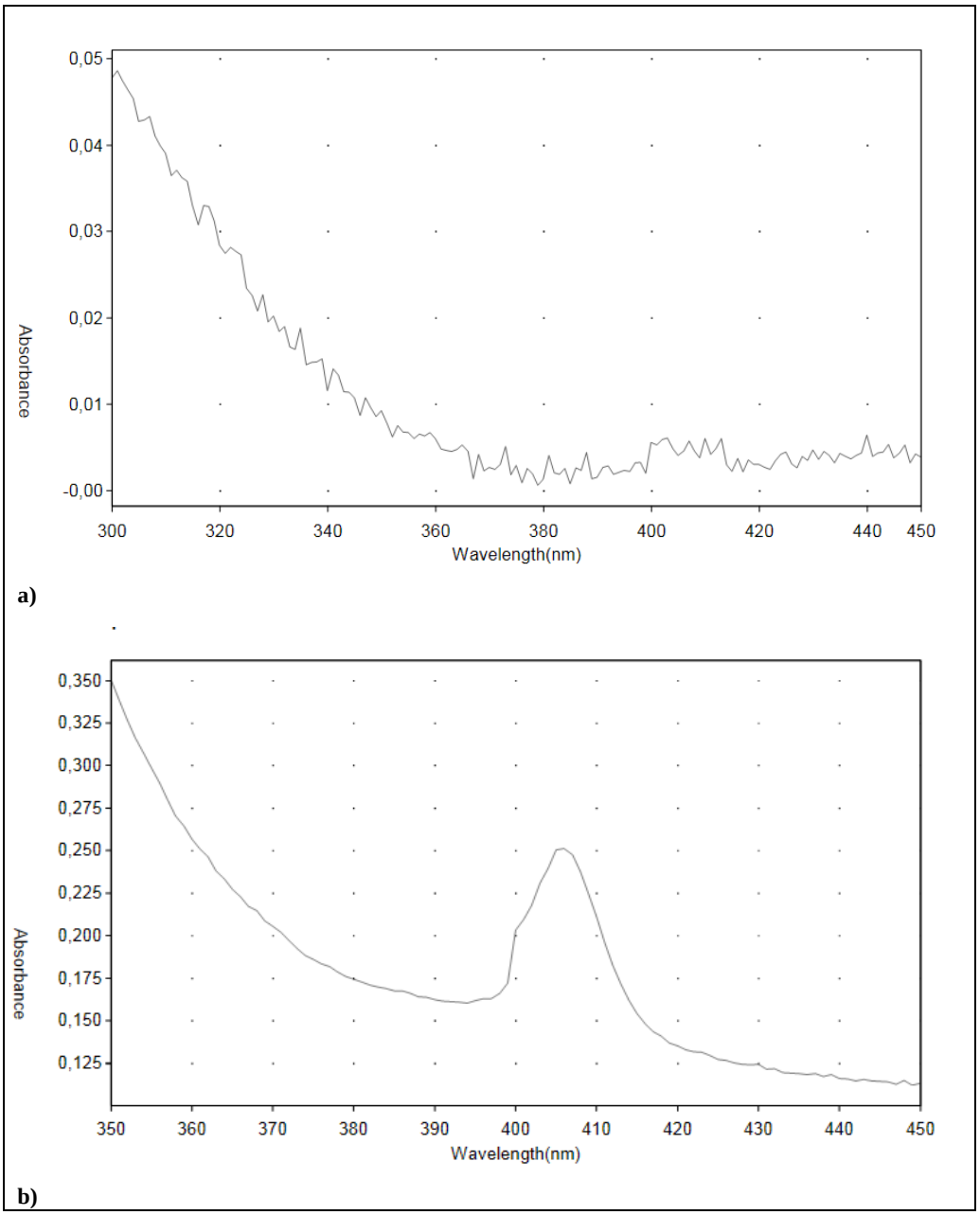

Fig.4.7 UV-Vis spectra of uroporphyrin I (9.5 mg/L) in a urine matrix: a) with Soulsby' extraction method; b) with Elder's method.

The Uro I peak was not visible in the spectrum correspondent to the Soulsby method. However, on the spectrum of the Elder method the Uro I peak was visible. This way, we can assume that the Soulsby method can not extract or determine Uro I.

4.3.3.4 Standards Mixture in water matrix

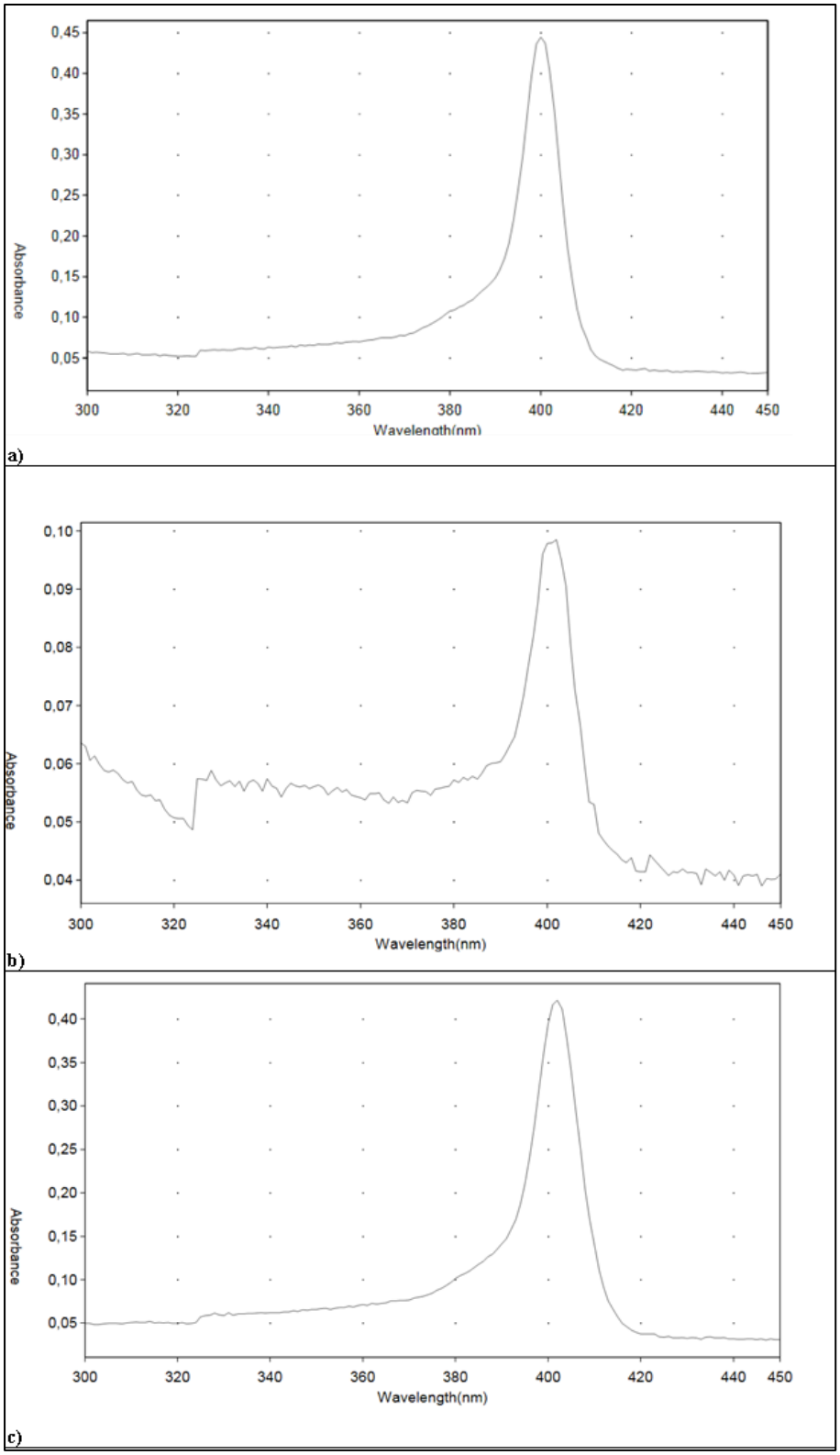

Fig.4.8 UV-Vis spectra of a standards equimolar mixture (230 µg porphyrins/L) in a water matrix a) without an extraction method; b) with Soulsby' extraction method; c) with Elder's method.

We added an equimolar mixture of standards of copro I, copro III and uro I, with a total concentration of 230 µg porphyrins/L, to a water matrix. The peak of the mixture, without extraction, had an absorbance value of 0,437. The mixture peak decreased to an absorbance of 0,095 when we used the Soulsby method. However, the peak presented an absorbance of 0,425 when we used the Elder method (Fig. 4.8).

4.3.3.5 Standards Mixture in urine matrix

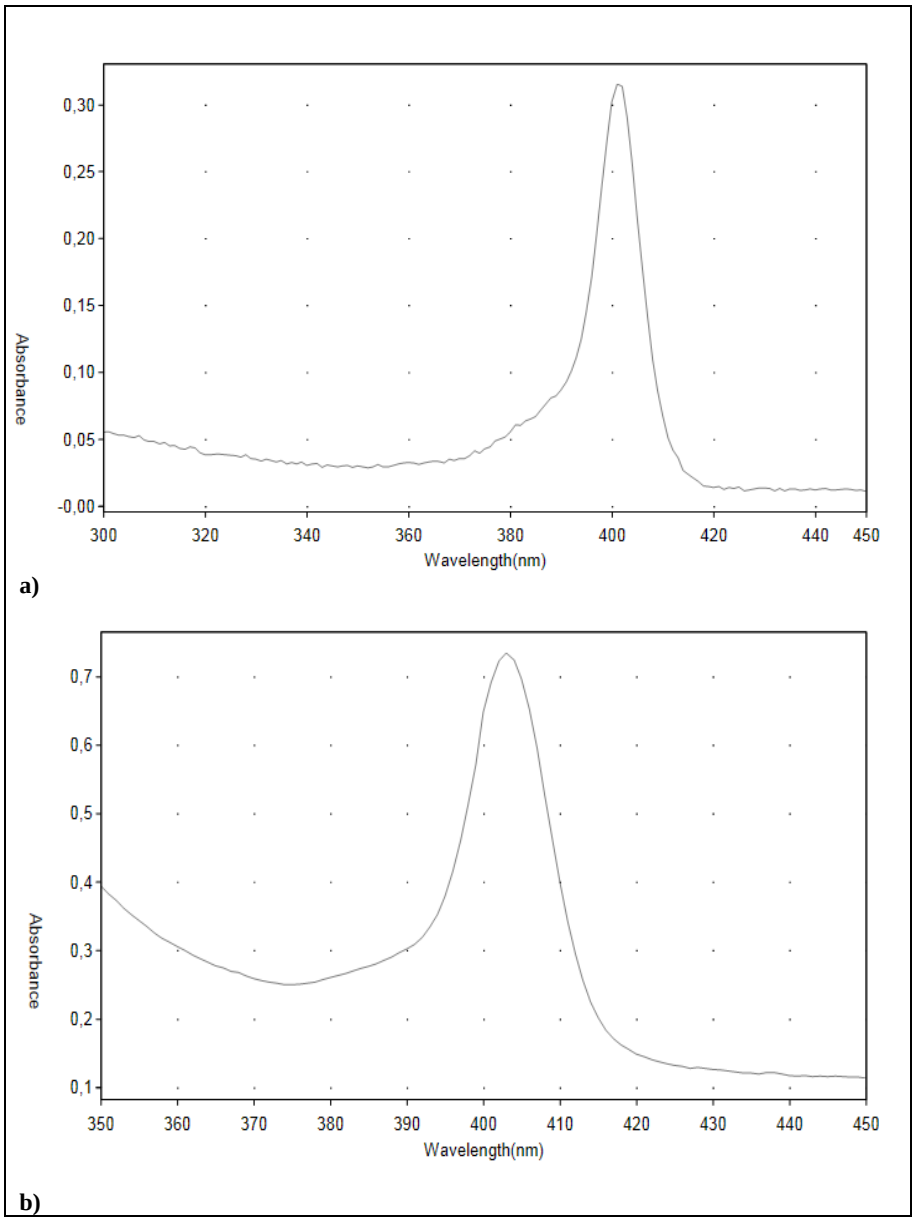

Fig.4.9 UV-Vis spectra of a standards equimolar mixture (230 µg porphyrins/L) in a urine matrix a) with Soulsby' extraction method b) with Elder's method.

We added a standard equimolar mixture (230 µg porphyrins/L) to a urine matrix. When we used the Soulsby method, the mixture peak had an absorbance value of 0,307. However, when we used the Elder method, the peak had an absorbance of 0,740 (Fig. 4.9). The same urine was used in both methods.

4.3.4 Comparison between Soulsby and HPLC methods for coproporphyrin quantification

We compared two methods for copro quantification: a spectrophotometric method by Soulsby and a HPLC method by Woods (1991). The concentrations of each sample analysed by the two methods are shown in Table A2.1, in Appendix 2.

We compared the Quantification and Detection Limits obtained for the Soulsby method and by the HPLC method (Table 4.2). The HPLC allows the detection and quantification of lower amounts of copro than Soulsby.

Tabel 4.2 Quantification Limit (QL) and Detection Limit (DL) obtained for coproporphyrins standards in a water matrix, analysed by the spectrophotometric method (Soulsby) and the HPLC method (Woods).

Method	QL (µmol/L)	DL (µmol/L)
Soulsby	0,143	0,05
HPLC	0,085	0,03

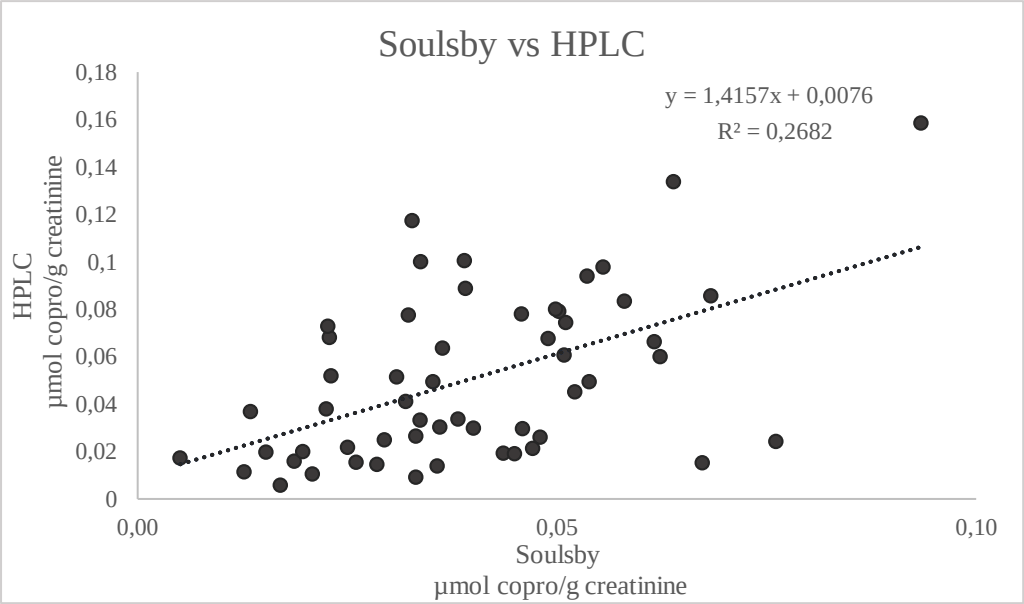

Fig. 4.10 Comparison of urine coproporphyrin concentrations (N=63) determined by the Soulsby 'method (*x*) and by HPLC (*y*).

Figure 4.10 illustrates the relation between the Soulsby method and the HPLC method through the analysis of 63 samples. A significant ($p<0.05$) Pearson correlation ($r= 0,491$) was determined between the two methods, with a significant ($p<0.05$) linear coefficient of determination ($R^2=0.268$) showing a weak linear relationship between the two variables. When we compare the two methods we

also observe that the mean of copro concentration is higher by HPLC (0,05 µmol/g creat) than by Soulsby (0,04 µmol/g creat) method.

The mean values and correlation test's results are presented in Appendix 2, Table A2.2.

4.3.5 Comparison between Elder and HPLC methods for total porphyrin quantification

We compared two different methods for total porphyrin quantification: a spectrophotometric method (Elder) and the HPLC method (Woods). The concentrations of each sample analysed by the two methods are presented in Table A2.2, in Appendix 2.

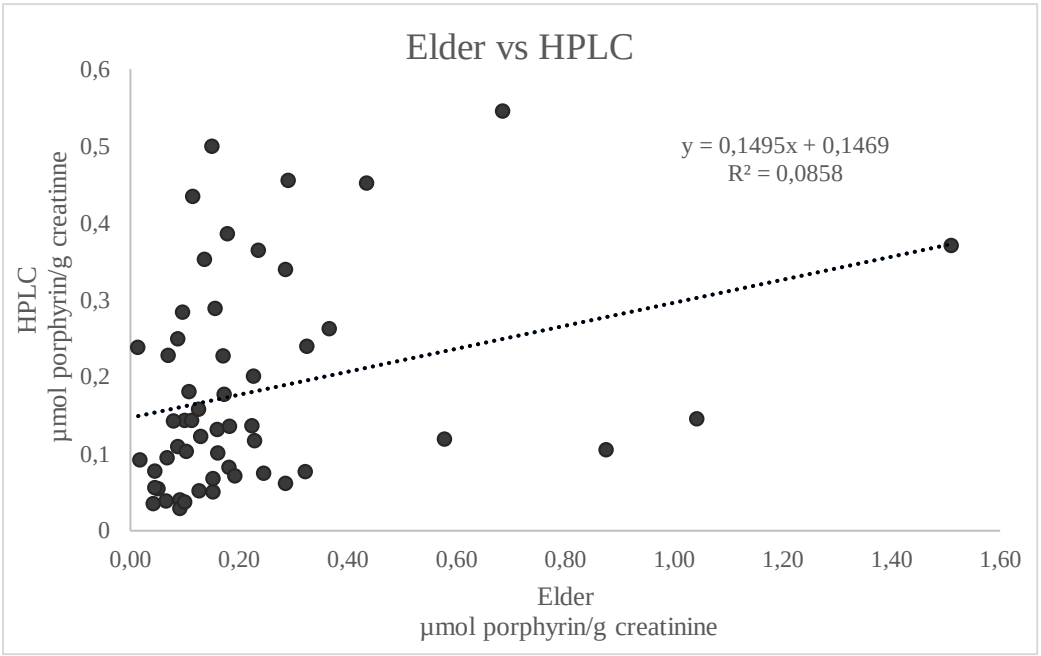

Fig. 4.11 Comparison of total porphyrin concentration (N=55) determined by the Elder method (*x*) and by HPLC (*y*).

Figure 4.11 illustrates the relation between the Elder method and the HPLC method in 55 samples. A significant ($p<0.05$) Pearson correlation ($r= 0,293$) was determined between the two methods, with a significant ($p<0.05$) linear coefficient of determination ($R^2=0,086$) showing a very weak linear relationship between the two variables. The mean of total porphyrin concentration is higher by the Elder (0,228 µmol/g creat) method than by the HPLC (0,181 µmol/g creat) method.

The mean values and correlation test's results are presented in Appendix 2, Table A2.3.

4.4 Discussion

4.4.1 Study of copro- and uroporphyrin standards by UV-Vis spectrometry

Standards of copro I (180 µg/L) in aqueous solution showed a Soret peak at 400 nm, with absorbances values of 1.6 and 0.250, without and with an extraction method, respectively (Fig.4.2). The main difference when using an extraction method, is the addition of diethyl ether to the sample preparation, commonly used to extract porphyrins from biological samples. In fact, we saw a remarkable intensity decrease of copro I with the Soulsby method, where we used diethyl ether and extraction steps, compared with the same concentration of copro I in aqueous solution without extraction. When we applied the extraction method of Soulsby, we observed an average loss of 78% of copro I. The ether used in the Soulsby method together with losses from extraction steps, may be the reasons for the decline of copro I intensity. These results agree with studies led by Polo et al. (1988).

The copro III standards also showed a Soret peak at 400 nm, without and with an extraction method (Fig.4.3). For copro III, the recovery rate was 68%, higher than the result obtained for copro I. The reduction of 32% of copro I absorbance corroborates the belief that the diethyl ether diminishes the fluorescence of porphyrins (Polo et al., 1988). However, this result can also be explained by loss of copro I in the extraction steps and weak efficiency of the extraction, once the correct separation of phases can be difficult to achieve by the operator.

We support the conclusion that to increase sensitivity of the Soulsby method and optimize the measurement conditions, once an extraction into organic solvent is made, a back extraction into aqueous medium- background correction- may be necessary (Polo et al., 1988). A more intense extraction, concerning time, should be made.

Copro can be measured more accurately than uro (Zuijderhoudt et al., 2000).The analysis of uro I standards in aqueous solution, with and without extraction method, revealed many problems. Through the spectra obtained, it was impossible to define the Soret peak and the respective absorbances. We believe that the problems we faced for uro I determination were related to the solubility of uro in aqueous solutions and its behaviour in organic solvent.

Aqueous solubility of the porphyrins, uro including, are determined by the acid/base characteristics of three reactive centers of the porphyrin molecule: the pyrrole ring, the carboxylic acid side chains, and the pyrrolenine nitrogens. Of the three reactive centers, the carboxyl groups and pyrrolenine nitrogens appear to be decisive in determining the solubility of porphyrins in aqueous solution. Ionized porphyrins are soluble in aqueous solution and uncharged porphyrins are soluble in organic solvent. Solubility in organic solvents would be optimal between pH 2 and 5, and maximum aqueous solubility is expected when the porphyrins are in the dicationic form (pH<2) or carboxylate form (pH>8) (Perkins and Johnson, 1989). A possible explanation for the inability to detect the Soret peak of uro I standard in an aqueous matrix (Fig.4.4 c), would be the pH value of the solution that should be between 2 and 8, where the porphyrins are not water soluble. Critical alterations in sample pH or matrix as a result of dilution may have affected the uro solubility (Polo et al., 1988). We should have used a pH <1, to guarantee the solubility of uro I in aqueous matrix (Perkins and Johnson, 1989).

When we applied the Soulsby' method to uro I in aqueous matrix, we added diethyl ether, like was described by the method. However, uroporprhyrins are not ether-soluble (Rimington and

Sveinsson, 1950). This information explains why we couldn't extract and obtain a defined spectrum of uro I, when we used the Soulsby method (Fig.4.4). Rimington (1950) also suggested that the uro is much more difficult to separate into a state of purity. We used pure uro I standards, so the separation of uro from the solvent used in the Soulsby method may not have been efficient (Rimington and Sveinsson, 1950).

4.4.2 Determination of the molar extinction coefficient under experimental conditions

The porphyrin spectra are markedly sensitive to conditions like matrix, pH, ionic strength and type of solvent (Polo et al., 1988); that is why it is necessary to know the molecular extinction coefficient (ε) and the maximum Soret band of porphyrins standards, under the conditions applied for the porphyrins measurement. The Soret peaks and the calculated ε values for copro I and III obtained in this work, are presented in Table 4.1 together with the values from the literature. We can also say that our values agree with the literature's values.

All the standards used were dissolved in HCl 1M. However, the intensity of porphyrin fluorescence also depends on ionic strength, reaching sharp maxima at 0.1 mol/L (for uro) and 1 mol/L (for copro) (Polo et al., 1988).

4.4.3 Comparison between the two different spectrophotometric methods for determination of porphyrins in urine

When comparing Soulsby method with Elder method, the main difference between these two methods concerns the sample preparation. Soulsby´s uses diethyl ether to extract porphyrins from urine and with separation of phases, while Elder´s method only consists in acidifying the urine samples with HCl, without extraction steps.

Both methods succeed to determine standards of copro I and III in urine matrix, showing defined Soret peaks (Fig. 4.5 and 4.6). However, the intensity of the peak in the Soulsby' method was half of the intensity in the Elder method: copro I and copro III lost 30% of its intensity. This can be explained by the decrease of porphyrins' intensity in organic solvent like ether, and losses from extraction steps (Perkins and Johnson, 1989).

In relation to uro I, Soulsby and Elder´s methods had different behaviours (Fig.4.7). The Soulsby method could not extract uro I standard from urine, because the respective spectrum did not present a peak at 400 nm region. However, in the Elder method we saw a defined peak at 406 nm, the value mentioned in literature for the uro' absorbance peak (Table 4.1). Because of this, we can conclude that the Elder method enables the determination of total porphyrins (uro + copro), while Soulsby's method can only be used for copro determination.

4.4.3.1 Standards Mixture in water matrix

We also tested a standard mixture of copro I, III and uro I, at equimolar concentrations, using Soulsby and Elder´s methods (Fig.4.8). The peak absorbance was maximum (0.45) when the mixture was not subjected to the extraction method and minimum for the Soulsby method (0.10). Elder method had a peak with an absorbance of 0.40. These differences can be explained for the extraction used in the Soulsby method or the inability of Soulsby method to extract and quantify uro I. Once Elder´s

obtained a very close value compared with standards without extraction, we concluded that Elder can quantify both copro and uro. The difference of 0,05 may result from matrix effects. Incomplete oxidation of porphyrinogens to porphyrins in the urine samples before analysis does not account for the effect illustrated, because we used porphyrin standards that contained no porphyrinogens.

4.4.3.2 Standards Mixture in urine matrix

When we tested the standards mixture in urine matrix, we also obtained a peak with less intensity in the Soulsby method compared with the Elder method (Fig.4.9). The absorbance's difference was about 60%. We believe that this difference is explained by the loss of porphyrins in the extraction but also to the absence of the uro I fraction in the Soret peak. We conclude that the Soulsby method does not extract nor determines uro I, thus determining only copro. In the meantime, the Elder method can be used for the determination of total porphyrins, provided a correction is made for background absorbance (Deacon and Elder, 2001).

4.4.4 Comparison between Soulsby and HPLC methods for coproporphyrin quantification

HPLC is the longstanding technique of choice for separating porphyrin precursors. It remains an essential technique in many toxicology, pharmacology and research laboratories, because it is flexible and ubiquitous. However, conventional HPLC can suffer from high levels of run to run variability, necessitating the use of internal or external standards for compound identification and a significant investment of time/funds for optimizing the choice of the column, solvent, and run conditions (Sullivan et al., 2015; Westerlund et al., 1988).

UV-Vis detection for porphyrins is complex due to variable absorbance peak and extinction coefficients. At the same time, the presence of an array of background metabolites, which also absorb in the UV-Vis range being impossible to separate from the analytes, is especially problematic in urine samples, complicating analyte identification and reliable quantification (Sullivan et al., 2015). The differentiation between the uro and the copro by spectrophotometric methods becomes impossible especially when intermediate forms of porphyrin are present in urine samples (Van De Giessen and Van Wijk, 1990).

It is of particular importance for toxicology, pharmacology, and research laboratories to have highly flexible methods for the quantification of porphyrins. These should ideally use widely available instruments and provide reasonably rapid analyses (Sullivan et al., 2015).

We compared two methods for copro determination: Soulsby method (spectrophotometric) and Woods method (HPLC). The average value for copro concentration was higher by HPLC than by the Soulsby method, when 63 urine samples were analysed. Our explanation for this, is the loss of copro because of extraction steps used in the Soulsby method. Spectrophotometry failed to detect lower concentration of porphyrins in urine which were detected by HPLC, because HPLC detection and quantification limits are lower for HPLC than Soulsby (Table 4.2).

Generally, copro concentration should preferably be determined using the HPLC method. This is because the various porphyrins can, then, be separated and individually quantified. However, HPLC method is expensive, so a high quality screening method is also important (Zuijderhoudt et al., 1995). We examined such a method (Soulsby) and compared it with an HPLC method. The copro

concentration measured using the Soulsby test correlated slightly with results obtained using the HPLC method (Fig. 4.10). Because there is not a strong linear relationship between the two methods, we did not attempt to standardize the values determined by the Soulsby method to the values determined by the HPLC method.

4.4.5 Comparison between Elder and HPLC methods for total porphyrin quantification

For total porphyrins quantification, we compared a spectrophotometric (Elder) with an HPLC (Woods) method. The total average concentration of porphyrins is higher by the Elder method than by HPLC method. This phenomenon suggests an underestimation of urine porphyrins when using the HPLC method; however, we believe that contamination of samples with strongly absorbing urine components, other than porphyrins, and urine turbidity may lead to a super estimation of porphyrin concentration by spectrophotometry. Also, the absorbance of total porphyrins may be higher than the sum of each porphyrin alone.

In the Elder method, total porphyrins concentration is estimated by measuring all porphyrins together at one wave-length. With HPLC the porphyrins are separated and measured individually. The sum of these uro, hepta, hexa, penta and copro values constitutes the total urine porphyrins concentration. The two methods showed a very weak linear relationship (Fig. 4.11). That way, it is not recommended to standardize the values found with the Elder method to the values found with the HPLC method.

The Elder method is reproducible but only semiquantitative because inaccuracies are produced by the correction for background absorbance, particularly at low porphyrins concentration. The detection limit depends on the amount of the background absorbance but concentrations of approximately 50 nmol/litre should be detected (Deacon and Elder, 2001).

We conclude that HPLC should be the method of choice for a correct quantification of total porphyrins in urine. However, the Elder method can be used to perform rapid, less expensive and preliminary analysis of urine samples, particularly in case of expected high total porphyrins concentrations. Increased concentrations require additional HPLC analyses to identify individual porphyrins and to distinguish increased porphyrin excretions from porphyrias (Zuijderhoudt et al., 2000).

Chapter 5

Multi-biomarker approach to assess exposure to heavy metals in a sample of Portuguese population

5.1 Background

The environment continuously contains a variety of heavy metals from natural and anthropogenic sources; the release of these metals from anthropogenic sources continues to increase due to a rapid industrialization and urbanization in many regions of the world (Kozlowska et al., 2003). These metals do not exist individually but essentially as joint mixtures, and during the day we are simultaneously exposed to single or multiple sources of these mixtures in several environmental, occupational and food contexts (Scherer, 2005).

Pb, As and Mn have a high degree of toxicities and are ranked among the priority metals that are of public health importance. They are classified as human carcinogens and may also induce neurotoxic effects (Andrade et al., 2014). It is important to know if simultaneous exposure to these metals produce additive or synergistic interactions or even new effects that are not seen in exposures of single components (Wang and Fowler, 2008). Also important is to select biomarkers that permit preventing and predicting negative health effects of these mixtures in exposed populations (Andrade et al., 2015).

Several metals induce porphyrins accumulation, that results from the interference with specific points of the heme biosynthetic pathway and when in excess, heme precursors are neurotoxic (Andrade et al., 2014). Pb exposure promotes an increase of urinary and blood ALA, which can lead to neurotoxic effects (Quintanilla-Vega et al., 1995)**.** For this, porphyrins and ALA can be used as biomarkers of exposure and effect, contributing to prevent the risk of neurotoxicity in human populations.

Exposed populations should be studied using a combination of biomarkers, to efficiently detect and diagnose early metal poisoning (Kakkar and Jaffery, 2005). The integration of different biomarkers promises to contribute to an improved diagnostic performance over single markers that may be lacking in sensitivity and/or specificity (Wang and Fowler, 2008). Nevertheless, analysing several biomarkers can cost a lot of time and money, due to the need of eventually collecting more than one biological sample (e.g. blood, urine or hair) and the use of more than one analytical technique. The determination of a porphyrin urinary profile simply requires the collection of one sample type, the urine (a non-invasive method and that does not need a technician for the collection) and one single HPLC analysis. The same urine can be used to determine ALA levels, by a simple spectrophotometric method. In this view, we propose to use porphyrins along with ALA determinations as a multi-biomarker approach.

5.2 Material and Methods

5.2.1 Chemicals

Chemicals were obtained from the following sources: Ethyl acetate ($C_4H_8O_2$), ethyl acetoacetate p.a. ($C_6H_{10}O_3$) , phosphoric acid (H_3PO_4) and Pb for AAS standard solution from Sigma–Aldrich; perchloric acid ($HClO_4$), ALA standard, sodium acetate ($C_2H_3NaO_2$), p-dimethylaminobenzaldehyde ($C_9H_{11}NO$), magnesium matrix modifier [$Mg(NO_3)_2 \cdot 6H_2O$] for Graphite Furnace Atomic Absorption Spectrophotometry (GFAAS), nitric acid 65% suprapure (HNO_3) and HCl for ultra-trace analysis from Merck; Acetic acid ($C_2H_4O_2$) and hydrochloric acid p.a. (HCl; 37%) from Panreac; Iodine resublimed from BDH;

5.2.2. Biological Samples

Biological samples (urine) from eight different groups were obtained from specific sources, according to the information described in Table 5.1. The groups were constituted by Portuguese individuals, living in different areas (urban, rural, industrial) and with different exposure levels to heavy metals (mine workers, healthcare technicians, smokers).

Table 5.1 Description and origin of the groups whose biological samples were analysed in this chapter.

Group	N	Origin	Description
I	7	Clinical Laboratory of Analysis, Açores (Portugal)	People living in an urban area of a Portuguese island
L	8	Clinical Laboratory of Analysis of the Faculdade de Farmácia, Universidade de Lisboa	People living in a big urban area
M	12	Occupational Health Department from a mining company (Portugal)	Workers from a mining company
A	5	Clinical Laboratory of Analysis, Alentejo (Portugal)	People living in a rural area
R	5	Volunteers from a Lisbon Hospital	x-rays workers
S	7	Clinical Laboratory of Analysis, (Portugal)	People living in a non-industrialized urban area
V	11	Clinical Laboratory of Analysis, (Portugal)	People living in an industrialized urban area
F	7	Workers from Faculdade de Farmácia,UL	Smokers

All the samples (urine) were stored at –80°C until the analysis. The samples were collected in 25-mL aseptic containers.

5.2.3 Urinary Porphyrin Profile

The porphyrin profiles were determined as previously described in Chapter 3.

5.2.4 Analysis of Pb in urine

Only x-rays workers' group was analysed for urinary Pb concentrations, because we had additional information about a possible exposure to Pb by this group.

Sample preparation

The determination of trace amounts of metals by AAS requires quite often oxidative decomposition of organic matter-mineralization- through the digestion of biological samples. The process is accompanied by the conversion of metallic xenobiotics to their ionic form (Kozlowska et al., 2003). Therefore, urine (2.5 mL) was digested with 1.25 mL of an acidic mixture of 1:1 (v/v) 65% HNO_3: HCl at 100°C for 30 minutes, in a water bath; the digested solutions were transferred to volumetric flasks (25 mL) and the volume completed with deionized water and kept at 4°C until analysis.

All the glassware and sample cups for GFAAS were decontaminated from vestigial metals for at least 24 h in a 15% HNO_3 (v/v) solution, rinsed twice with distilled water and then twice with deionized water.

Atomic absorption spectrophotometry

Urinary Pb concentrations from the R group´ samples were determined by GFAAS (PerkinElmer AAnalyst™ 700) equipped with a Graphite Furnace, a programmable sample dispenser (AS 800 Auto Sampler) and WinLab 32 for AA software. Calibration curves were obtained with standard solutions of Pb and with $Mg(NO_3)_2$ (0.84 mol/L) used as a chemical modifier, which was added to blanks, standards and samples in equal volumes. The quantification limit (QL) was 3.8 µg Pb/L.

Results were expressed as mg Pb per g of urinary creat. Urinary levels of creat were determined by a colorimetric method with a Randox (CR510) commercial kit.

5.2.5 ALA analysis in urine

One mL of supernatant (2500 rpm, 10') from each sample (urine) was added to 1 ml of acetate buffer (pH 4.6) and 0.2 mL of acetoacetate. The samples were mixed (15'') and incubated (100 º C, 10') followed by the addition of 3 mL of ethyl acetate, agitation (15'') and centrifugation (2000 rpm, 3') to separate the two phases (organic and aqueous phase). A colorimetric reaction was started with Ehrlich reagent and the obtained organic phase. ALA concentrations were determined at 553 nm with a Hitachi spectrophotometer. Calibration curves were generated daily with ALA standard solutions (0.5, 1, 2.5, 5 and 10 mg/L). The QL was 0,07 mg ALA/L and the results are expressed as mg of ALA per g of urinary creat.

As it was mentioned before, urinary levels of creat were determined by a colorimetric method with a Randox (CR510) commercial kit.

5.2.6 Statistical analysis

Statistical analysis was performed with the SPSS 24.0 statistical package for Windows (SPSS, Inc., Chicago, IL, USA). Data are expressed as means ± SD. After verification of data adequacy for parametric methods, normal distribution by Kolmogorov-Smirnov test and homogeneity of variance with Levene's test, all the parameters were compared by ANOVA and post hoc Tuckey's test to assess differences between groups. The difference was considered when p-values were less than 0.05.

Discriminant analysis was also performed. This statistical tool allows: i- the identification of variables that better discriminate two or more different groups of individuals; ii- the use of these variables to create a discriminant function that represents in prudent manner the differences among the groups; and iii- the use of discriminant functions to classify new individuals in the distinct groups. This was achieved through the generation of classification functions where different function coefficients of each biomarker were obtained for each group of exposition. After applying all the functions to a subject under analysis, the group with the higher value of the classification function corresponds, according to the model, to the group where the subject belongs Discriminant analysis is

known to be very robust to data assumption violations, such as the lack of normality, homogeneity of variances among groups and the absence of multicollinearity (Marôco, 2011).

5.2.7 Combination of Biomarkers

Biomarkers were combined using discriminant analysis to create a model aiming to identify if an individual has a context of exposure according with a specific area/group.

5.3 Results

5.3.1 Biomarkers in urine

Detailed data of urinary porphyrins concentration and urinary ALA levels of each population's group analysed are presented at Table A3.1 in Appendix 3.

5.3.1.1 Urinary Porphyrin Profile

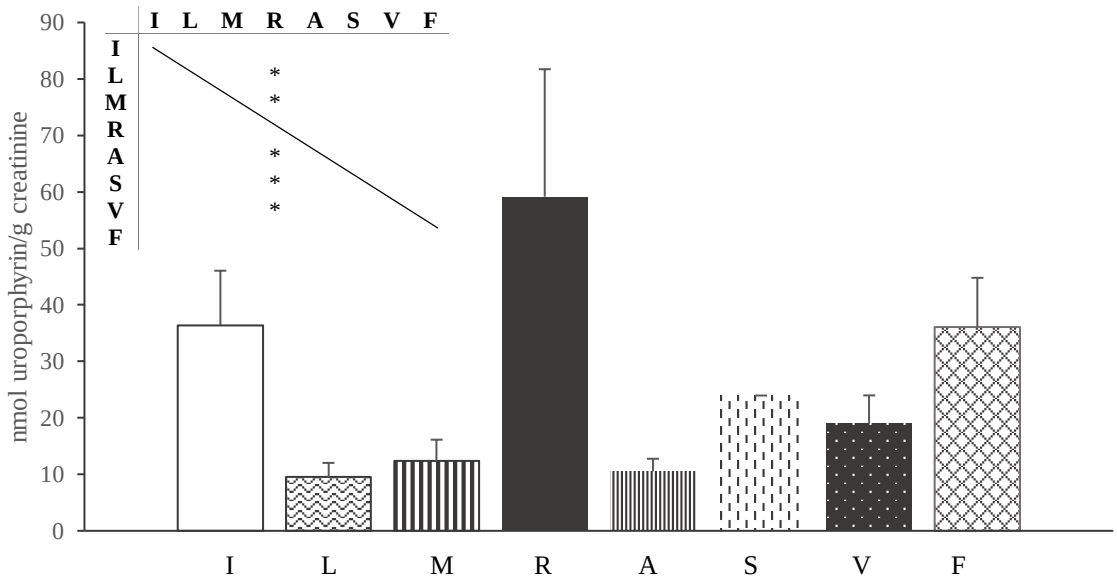

Fig.5.1 Urinary levels of uroporphyrin in each group (I, L, M, R, A, S, V and F). Data represent the mean ± SD. All the groups were compared by Tukey tests: when p<0,05 the difference is significative and marked with *. I- People living in an urban area of a Portuguese island; L- People living in a big urban area; M-Miners; A- People living in a rural area; R- X-Rays workers; S- People living in a non-industrialized urban area; V- People living in an industrialized urban area; F- Smokers.

The concentration of uro in the urine of the R subjects were significantly ($p<0.05$) higher than the ones determined in the L, M, A, S and V group (Fig. 5.1).

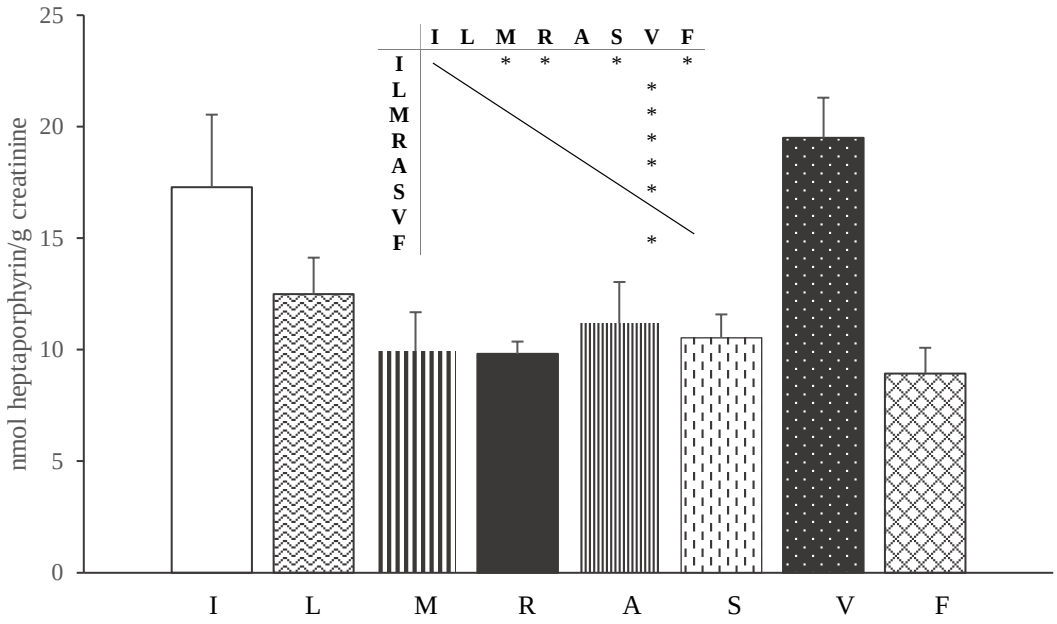

Fig. 5.2 Urinary levels of heptaporphyrin in each group (I, L, M, R, A, S, V and F). Data represent the mean ± SD. All the groups were compared by Tukey tests: when $p < 0.05$ the difference is significative and marked with *. I- People living in an urban area of a Portuguese island; L- People living in a big urban area; M-Miners; A- People living in a rural area; R- X-Rays workers; S- People living in a non-industrialized urban area; V- People living in an industrialized urban area; F- Smokers.

The I' hepta levels were significantly ($p<0.05$) higher than in M, R, S and F groups (Fig.5.2). The concentration in the V group was significantly increased compared with all the groups, except one, the I group.

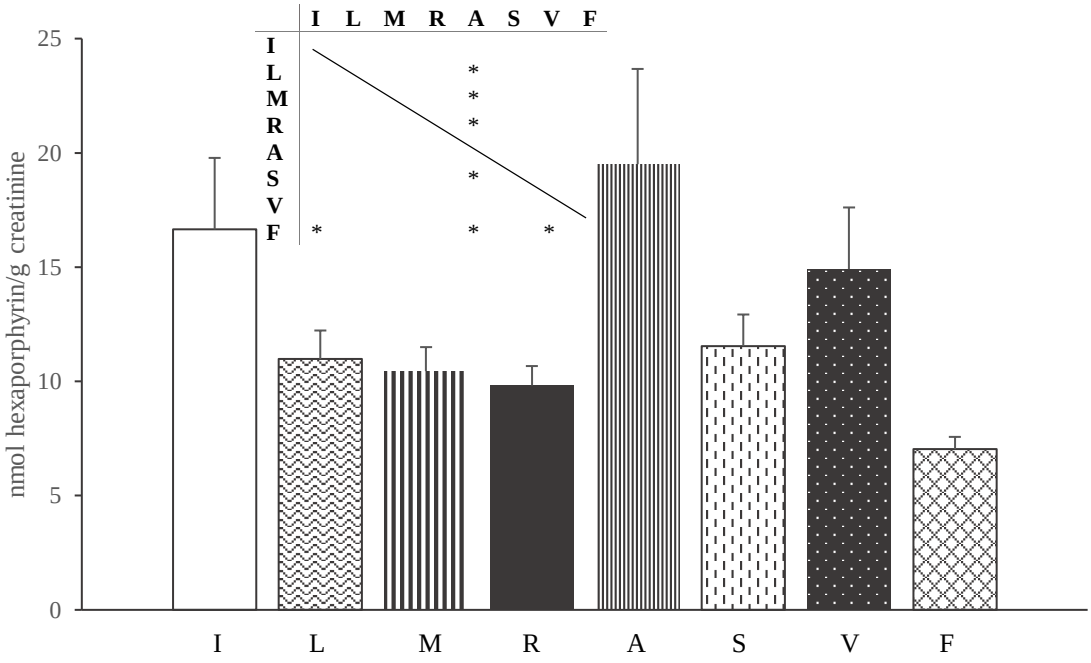

Fig.5.3 Urinary levels of hexaporphyrin in each group (I, L, M, R, A, S, V and F). Data represent the mean ± SD. All the groups were compared by Tukey tests: when $p < 0.05$ the difference is significative and marked with *. I- People living in an urban area of a Portuguese island; L- People living in a big urban area; M-Miners; A- People living in a rural area; R- X-Rays workers; S- People living in a non-industrialized urban area; V- People living in an industrialized urban area; F- Smokers.

The urinary levels of hexa were significantly higher (p<0,05) in the A group when compared whith the L, M, R, S and F group. The F group had hexa levels significantly lower than I, A and V groups (Fig. 5.3).

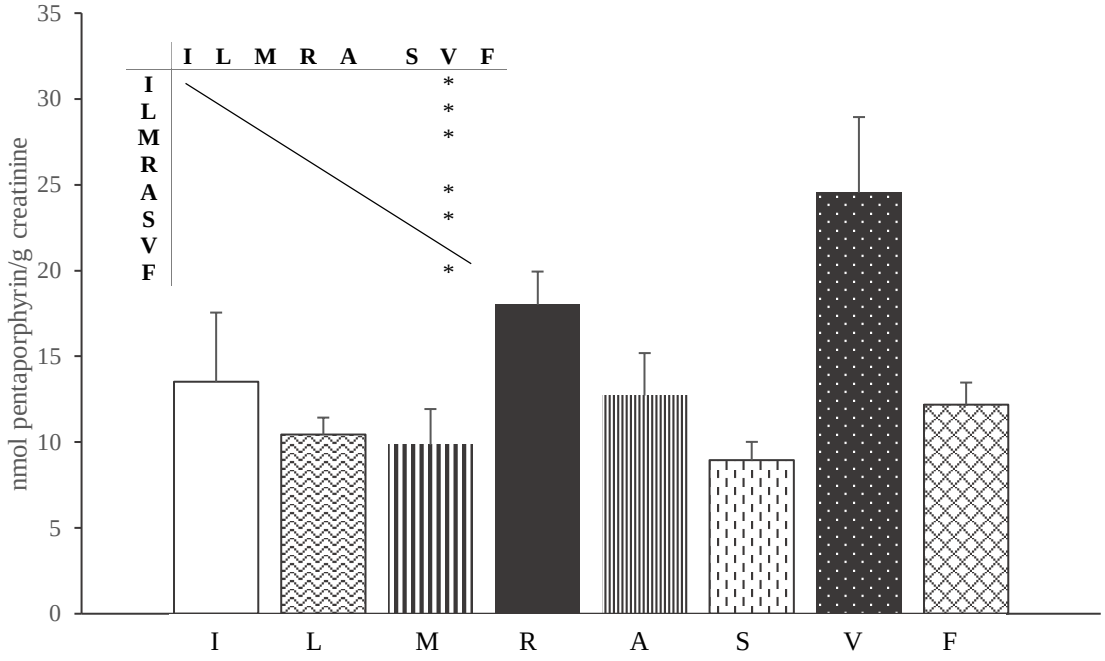

Fig. 5.4 Urinary levels of pentaporphyrin in each group (I, L, M, R, A, S, V and F). Data represent the mean ± SD. All the groups were compared by Tukey tests: when p < 0.05 the difference is significative and marked with *. I- People living in an urban area of a Portuguese island; L- People living in a big urban area; M-Miners; A- People living in a rural area; R- X-Rays workers; S- People living in a non-industrialized urban area; V- People living in an industrialized urban area; F- Smokers.

The concentration of penta in the urine of V subjects was significantly (p<0.05) higher than the determined in all the other groups (I, L, M, A, S and F) with exception of the R group (Fig. 5.4).

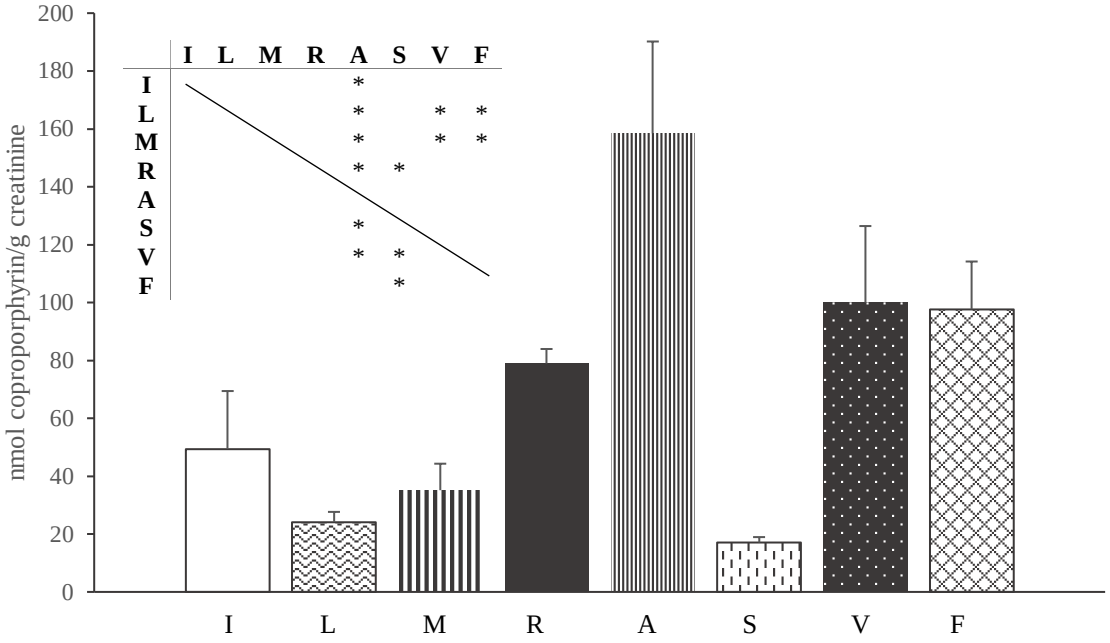

Fig. 5.5 Urinary levels of coproporphyrin in each group (I, L, M, R, A, S, V and F). Data represent the mean ± SD. All the groups were compared by Tukey tests: when $p < 0.05$ the difference is significative and marked with *. I- People living in an urban area of a Portuguese island; L- People living in a big urban area; M-Miners; A- People living in a rural area; R- X-Rays workers; S- People living in a non-industrialized urban area; V- People living in an industrialized urban area; F- Smokers.

The A group had the highest copro concentration in urine. The difference is significative ($p < 0,05$) when compared with the I, L, M, R, S and V group. The V and F groups had copro levels significantly higher than the ones obtained in L, M and S groups. S is significant lower than groups R, A, V and F (Fig.5.5).

To better visualize the comparison of the results summarized in Figures 5.1 to 5.5, we presented the levels of uro, hepta, hexa, penta and copro – the urinary porphyrin profiles- of each group (I, L, M, R, A, S, V and F) in the following figure (Fig.5.6).

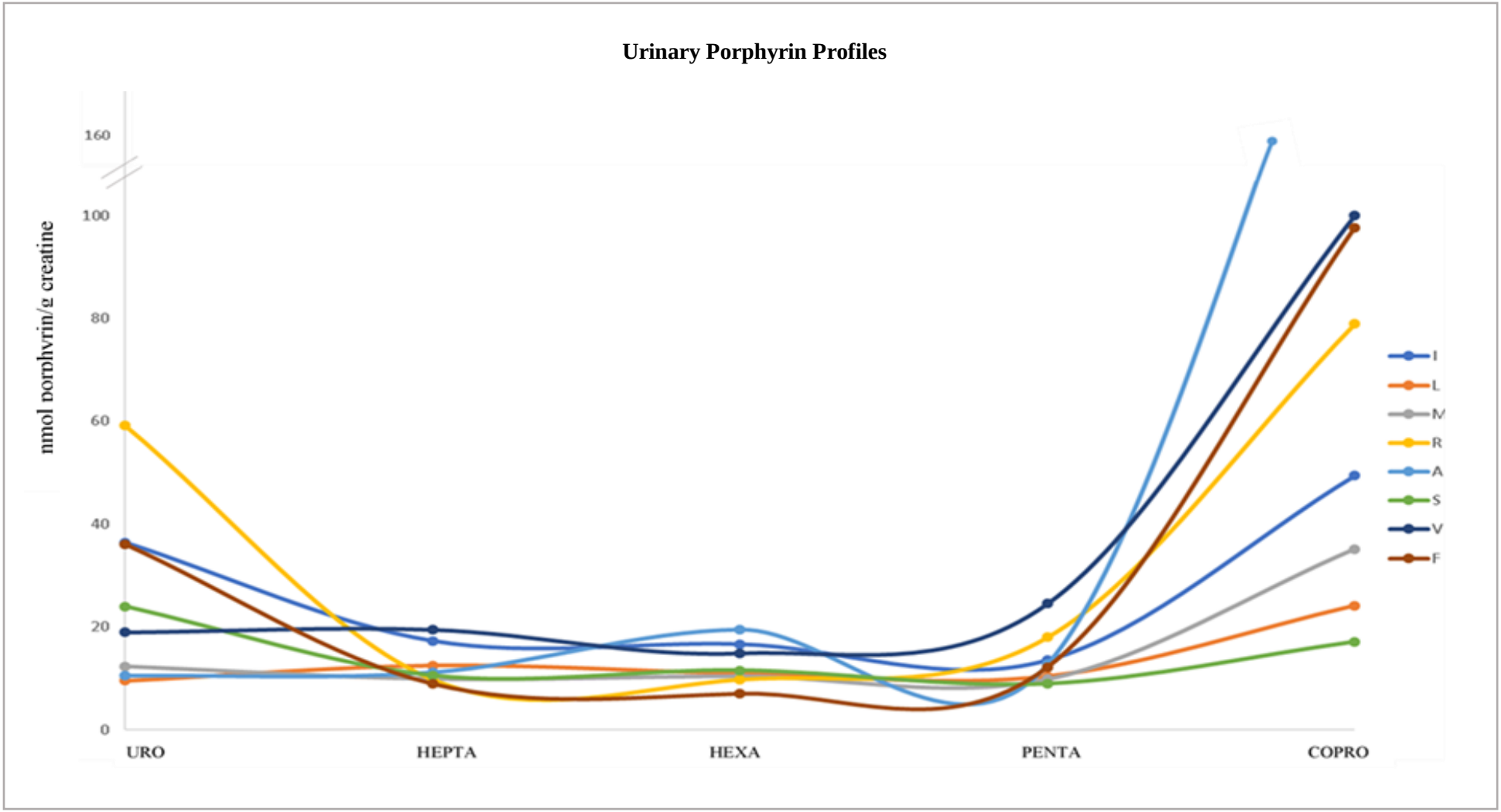

Fig. 5.6 Comparison between the urinary porphyrin profiles (levels of uro, hepta, hexa, penta and copro) in all the analysed groups (I, L, M, R, A, S, V and F). The concentration of each porphyrin is expressed in nmol/g creat. I- People living in an urban area of a Portuguese island; L- People living in a big urban area; M-Miners; A- People living in a rural area; R- X-Rays workers; S- People living in a non-industrialized urban area; V- People living in an industrialized urban area; F- Smokers.

After observing Figure 5.6 we may highlight the following: the concentrations of hepta, hexa and penta did not reveal drastic differences between groups. The differences are mainly concerned with uro and copro. Thus, uro had the highest concentration in R group, followed by I and F; with regard to copro, we can say that the A group presented the highest value pursued by V, F and R groups.

5.3.1.2 ALA levels in urine

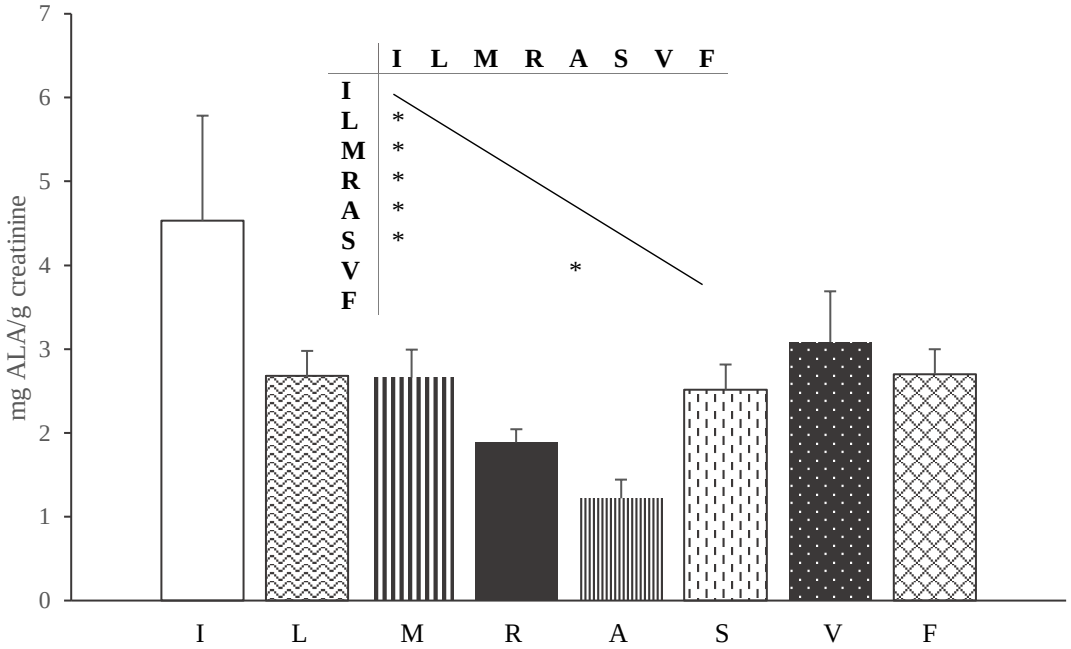

Fig. 5.7 Urinary concentrations of ALA in each group (I, L, M, R, A, S, V and F). Data represent the mean ± SD. All the groups were compared by Tukey tests: when $p < 0.05$ the difference is significative and marked with *. I- People living in an urban area of a Portuguese island; L- People living in a big urban area; M-Miners; A- People living in a rural area; R- X-Rays workers; S- People living in a non-industrialized urban area; V- People living in an industrialized urban area; F- Smokers.

Subjects from I group exhibited urinary concentrations of ALA significantly ($p<0.05$) increased when compared with the L, M, R, A and S groups. The A group revealed to have urinary ALA concentration significantly ($p<0.05$) lower than the I and V groups (Fig.5.7).

5.3.2 Combination of Biomarkers

5.3.2.1 Assessment of the exposure

Procedure I

In Procedure I, we used the values obtained for all the groups under study (L, M, R, A, S, V and F), with exception of the I group, concerning the application of biomarkers (Figure 5.8). We decided to remove the I group because the level of exposition in this area is very difficult to classify and the levels of porphyrin in urine and ALA oscillated inside the group.

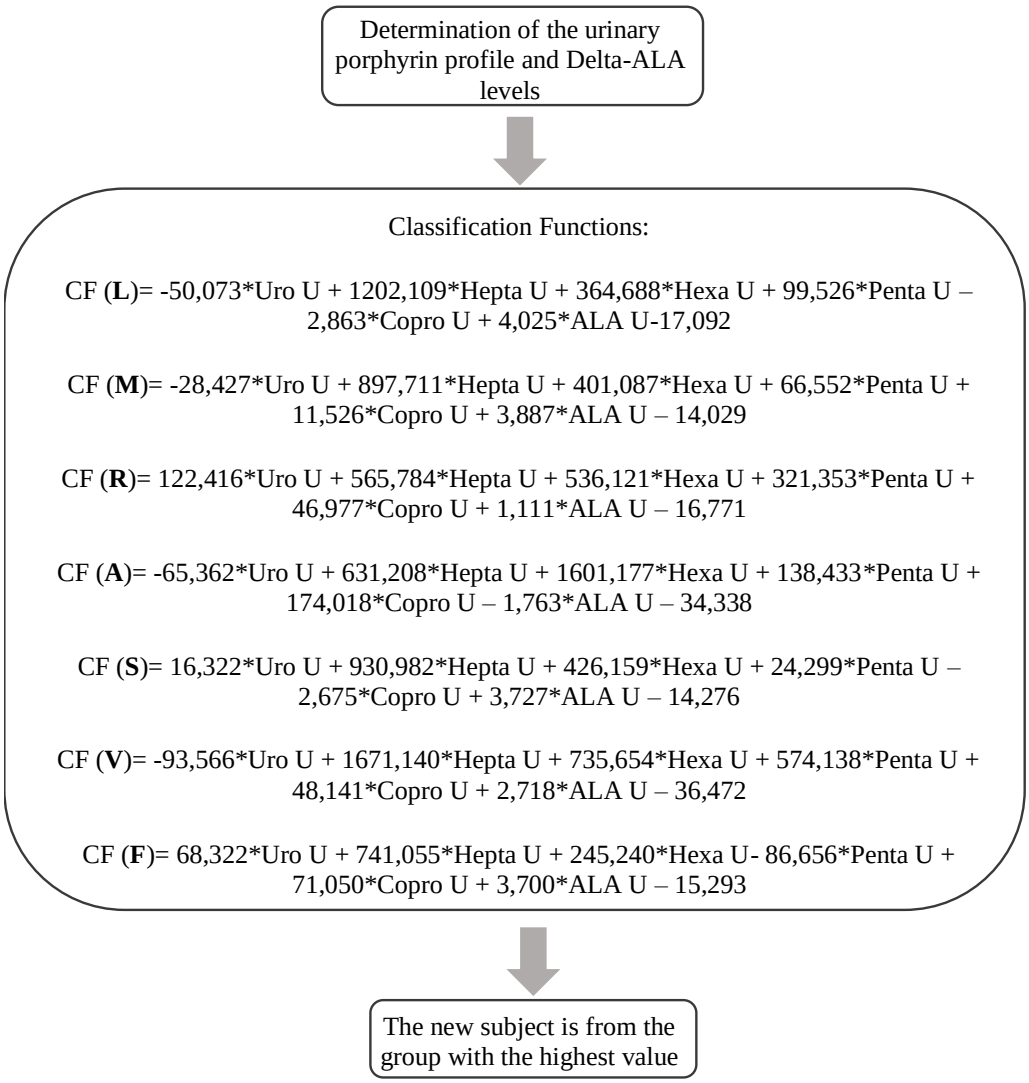

Fig. 5.8 Procedure proposed for individual assessment of the type of exposure in several exposed groups (L, M, R, A, S, V and F) through the determination of the urinary (U) porphyrin profile (levels of Uro-, Hepta-, Hexa-, Penta- and Coproporphyrins) and levels of ALA. Seven classification functions (CF) were generated: the CF with the highest value indicates the type of exposure predicted by the model. L- People living in a big urban area; M-Miners; A- People living in a rural area; R- X-Rays workers; S- People living in a non-industrialized urban area; V- People living in an industrialized urban area; F- Smokers.

Procedure II

In Procedure II, we used the biomarkers' concentrations of the groups exposed to an environmental/passive exposure (L, A, S, and V groups). This way, we can characterize these areas for the type of environmental exposure, and predict the type of environmental exposure of a new subject (Figure 5.9).

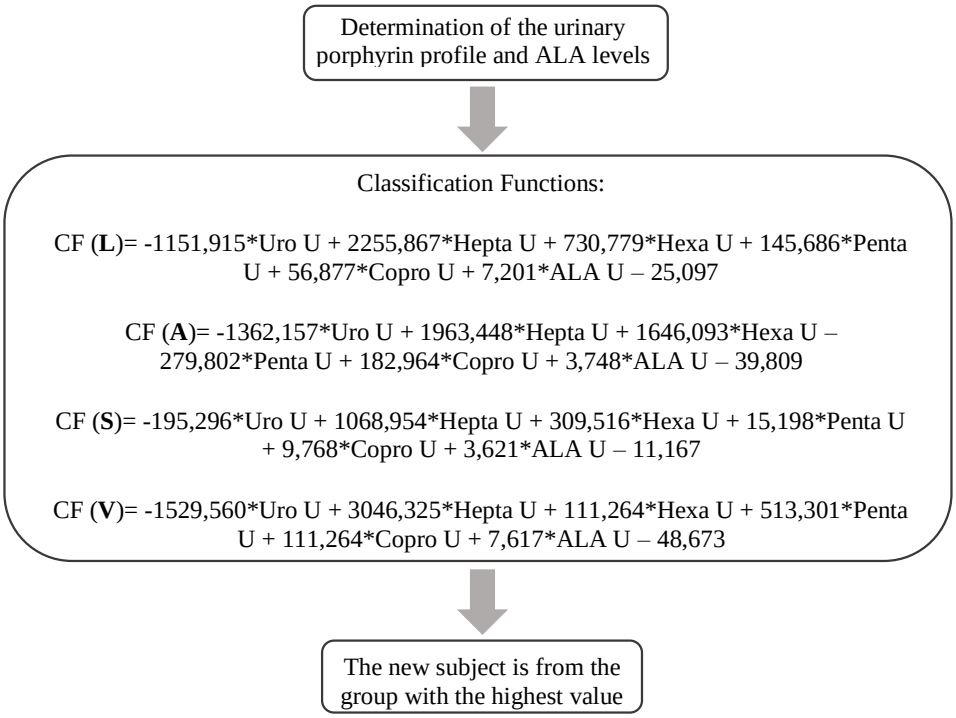

Fig. 5.9 Procedure proposed for individual assessment of the type of environmental exposure in several exposed groups (L, A, S and V) through the determination of the urinary (U) porphyrin profile (levels of Uro-, Hepta-, Hexa-, Penta- and Coproporphyrins) and levels of ALA. Four classification functions (CF) were generated: the CF with the highest value indicates the type of exposure predicted by the model. L- People living in a big urban area; A- People living in a rural area; S- People living in a non-industrialized urban area; V- People living in an industrialized urban area;

Procedure III

In Procedure III, we used the biomarkers levels of groups M, R and F, to characterize an active exposure to metals. Active exposure involves occupational exposure (miners and hospital workers) and personal habits (smoking). This way, we can predict the type of active exposure of a new subject (Figure 5.10).

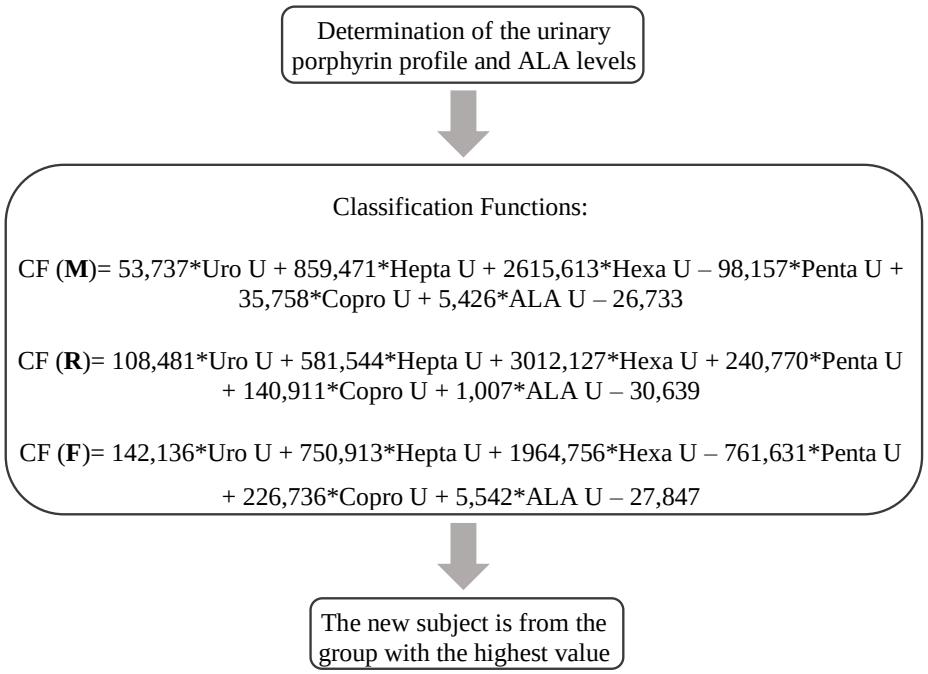

Fig. 5.10 Procedure proposed for individual assessment of the type of exposure in several exposed groups (M, R and F) through the determination of the urinary (U) porphyrin profile (levels of Uro-, Hepta-, Hexa-, Penta- and Coproporphyrins) and levels of ALA. Three classification functions (CF) were generated: the CF with the highest value indicates the type of exposure predicted by the model. M-Miners; R- X-Rays workers; F- Smokers.

5.3.2.2 Evaluation of the procedures

Procedure I

Procedure I was applied to access whether the type of exposure of each subject (N=56) could be correctly identified through the combination of individual urinary porphyrin profile and the concentration of ALA in urine, in seven different groups. After using this model we could observe that 76,8% of the cases were correctly identified. In the M group only 6 of the 12 subjects were correctly classified. In the F group, all subjects were properly classified (Table 5.2).

Table 5.2 Identification of exposed subjects (from L, M, R, A, S, V and F groups) using their individual urinary (U) porphyrin profile (levels of Uro-, Hepta-, Hexa-, Penta- and Coproporphyrin) and levels of ALA. Discriminant analysis was performed. The table represents the type of exposure predicted by the model and the real exposure (Group). L- People living in a big urban area; M-Miners; A- People living in a rural area; R- X-Rays workers; S- People living in a non-industrialized urban area; V- People living in an industrialized urban area; F- Smokers.

		Predicted Type of Exposure						
Group	**Total**	**L**	**M**	**R**	**A**	**S**	**V**	**F**
L	8	5	3	0	0	0	0	0
M	12	4	6	0	0	2	0	0
R	6	0	1	5	0	0	0	0
A	5	0	0	0	4	0	1	0
S	7	0	1	0	0	6	0	0
V	11	1	0	0	0	0	10	0
F	7	0	0	0	0	0	0	7

76,8% of the cases correctly classified

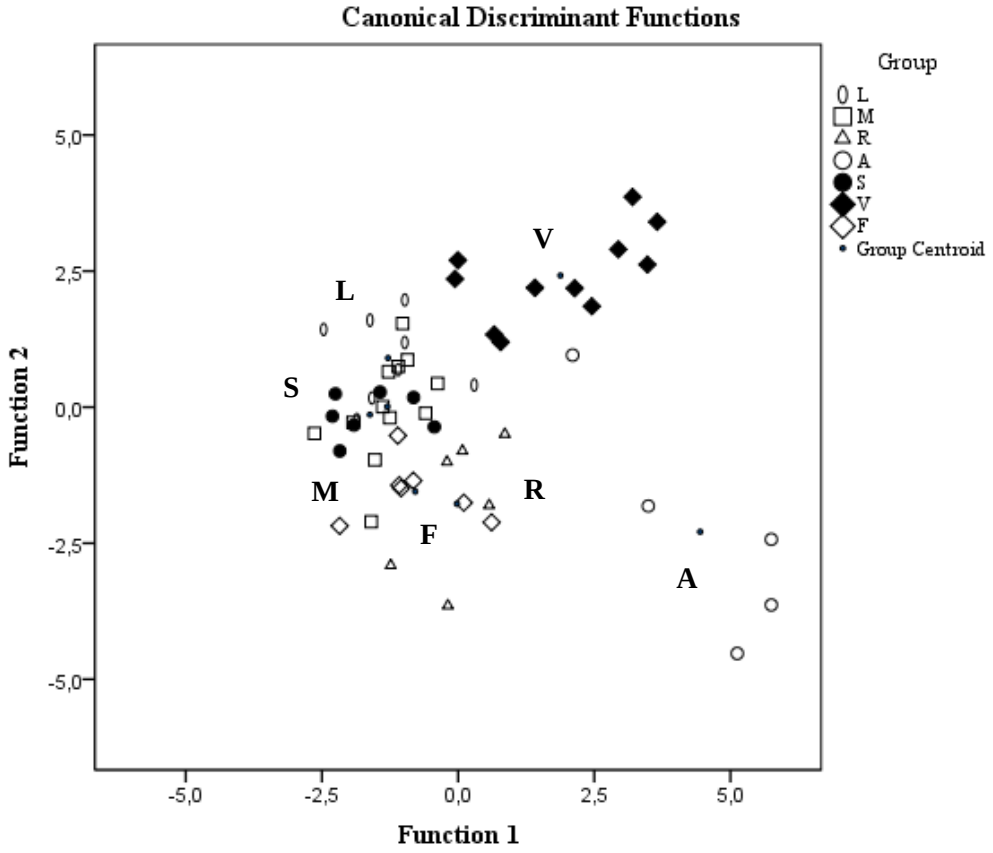

Fig. 5.11 Graphical representation of each subject's classification according to the type of exposure (L, M, R, A, S, V and F groups) applying their urinary (U) porphyrin profile and levels of ALA through discriminant analysis. A centroid value was calculated for each group and the results are plotted by the canonical discriminant functions. L- People living in a big urban area; M-Miners; A- People living in a rural area; R- X-Rays workers; S- People living in a non-industrialized urban area; V- People living in an industrialized urban area; F- Smokers.

Fig.5.11 shows that the areas of the seven groups overlapped, revealing a poor discrimination capability of Procedure I. However, group V and A were clearly separated from the rest of the groups.

Procedure II

The assessment of the type of environmental exposure through the application of Procedure II (to L, A, S and V groups) showed that 96,8% of the cases were correctly classified. In the A group only one subject was wrongly classified, as belonging to the V group (Table 5.3).

Table 5.3 Identification of exposed subjects (from L, A, S and V groups) using their individual urinary (U) porphyrin profile (levels of Uro-, Hepta-, Hexa-, Penta- and Coproporphyrin) and levels of ALA. Discriminant analysis was performed. The table represents the type of exposure predicted by the model and the real exposure (Group). L- People living in a big urban area; A- People living in a rural area; S- People living in a non-industrialized urban area; V- People living in an industrialized urban area;

		Predicted Type of Exposure			
Group	**Total**	**L**	**A**	**S**	**V**
L	8	8	0	0	0
A	5	0	4	0	1
S	7	0	0	7	0
V	11	0	0	0	11

96,8% of cases correctly classified

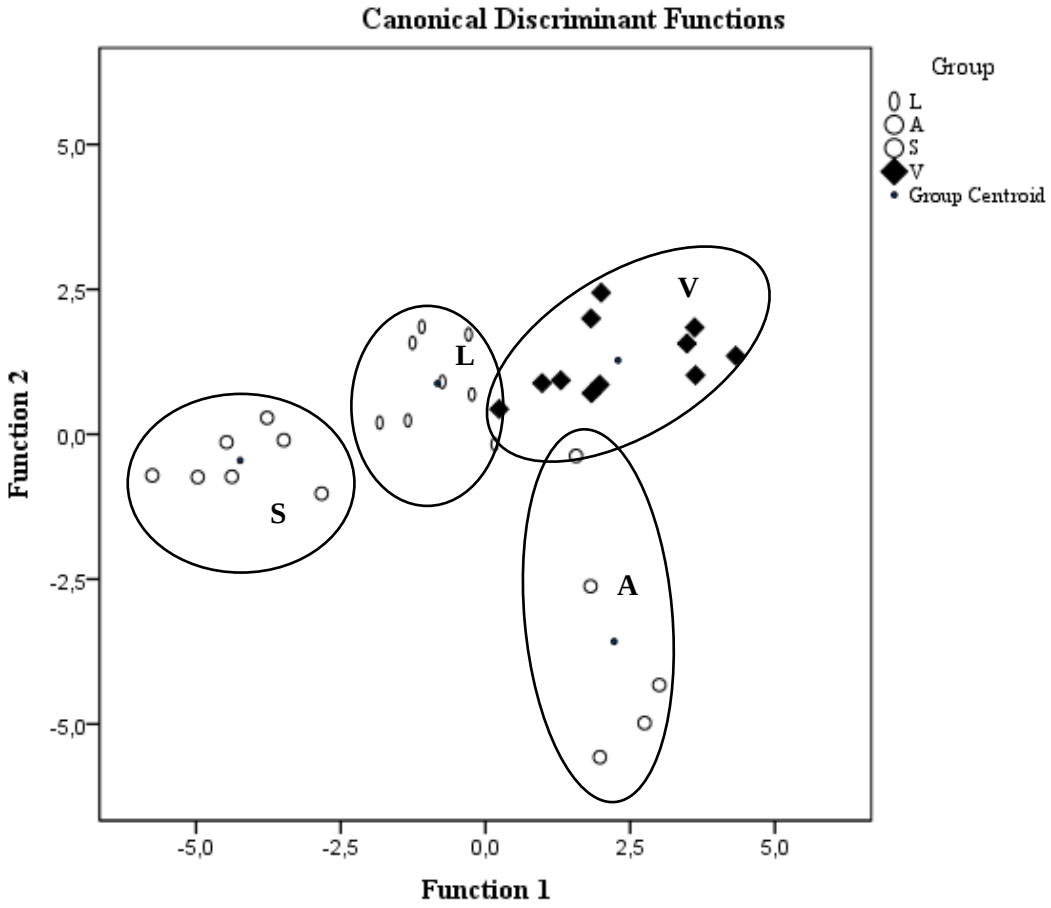

Fig. 5.12 Graphical representation of each subject's classification according to the type of environmental exposure (L, A, S and V groups), applying their urinary (U) porphyrin profile and levels of ALA through discriminant analysis. A centroid value was calculated for each group and the results are plotted by the canonical discriminant functions. L- People living in a big urban area; A- People living in a rural area; S- People living in a non-industrialized urban area; V- People living in an industrialized urban area;

The 4 groups, L, A, S and V, were clearly discriminated by Procedure II showing definitely separated areas. However, one subject from the A area was classified erroneously in the V environment group (Fig. 5.12).

Procedure III

Procedure III (employing individuals from M, R and F groups) showed that the type of exposure of each subject could be correctly identified through the combination of individual porphyrin profiles and the concentration of ALA in urine. All the subjects were correctly classified by the model (Table 5.4).

Table 5.4 Identification of active exposed subjects (from M, R and F groups) using their individual urinary (U) porphyrin profile (levels of Uro-, Hepta-, Hexa-, Penta- and Coproporphyrin) and levels of ALA. Discriminant analysis was performed. The table represents the type of exposure predicted by the model and the real exposure (Group). M-Miners; R- X-Rays workers; F- Smokers.

	Predicted Type of Exposure			
Group	**Total**	**M**	**R**	**F**
M	12	12	0	0
R	6	0	6	0
F	7	0	0	7

100% of cases correctly classified

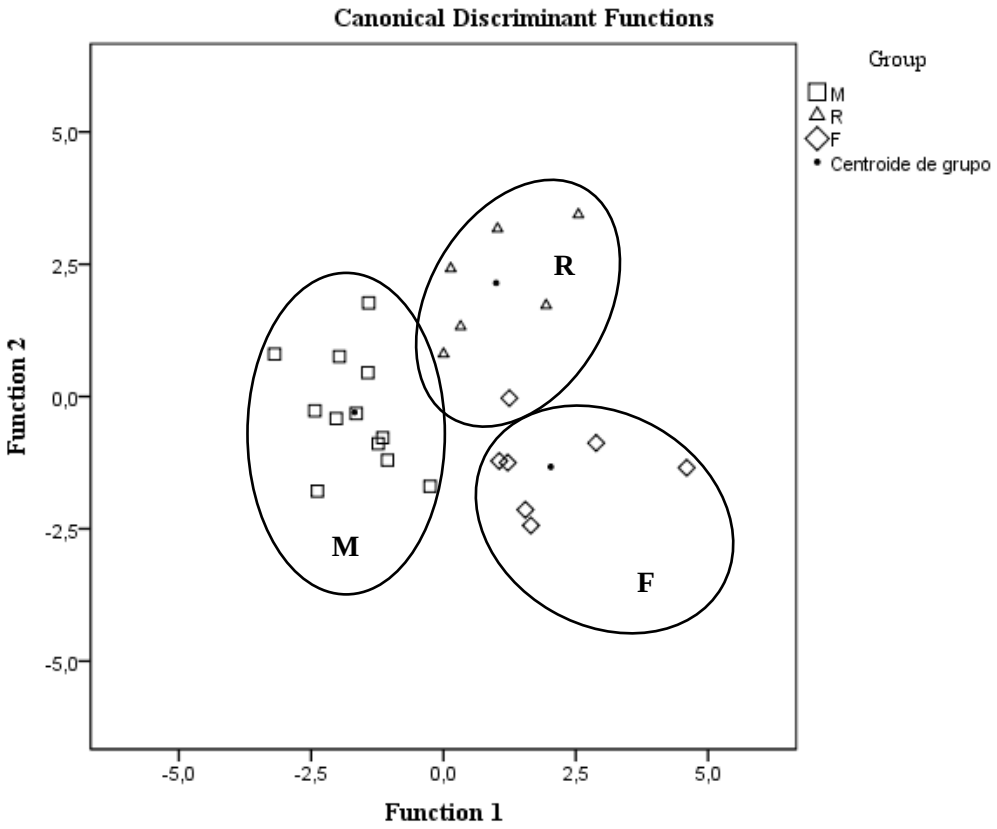

Fig. 5.13 Graphical representation of each subject's classification according to the type of active exposure (M, R and F groups), applying their urinary (U) porphyrin profile and levels of ALA through discriminant analysis. A centroid value was calculated for each group and the results are plotted by the canonical discriminant functions. M-Miners; R- X-Rays workers; F- Smokers.

Fig. 5.13 shows that the areas of the three groups are clearly separated, showing a good discriminant capability of Procedure III.

5.3.3 Determination of urinary Pb in x-rays workers

The urinary concentration of Pb in group R is showed in Table 5.5. The values ranged from 0,9 until 31,4 µg Pb/g creat. There is no observable correlation between the levels of Pb and the levels of ALA.

Table 5.5 Comparison between urinary concentration of Pb (µg/L and µg/g creat) and urinary levels of ALA (mg/L and mg/g creat), in a group of x-rays workers.

µg Pb/L	**µg Pb/ g creat**	**ALA mg/L**	**ALA mg/g creat**
11,28	4,6	5,0	1,9
39,12	10,8	11,0	3,1
67,5	25,6	4,2	1,4
18,3	31,4	3,8	2,3
1,74	0,9	5,7	2,1
nd	nd	5,7	1,8

nd= not determined

5.4 Discussion

The interest in the biological effects of toxic metals such as Pb, As and Mn, has increased during the last decades (Wong et al., 2006). The main reason is that large amounts of toxic including carcinogenic elements have been released into the environment, especially in industrial areas (Yousaf et al., 2016).

Occupational, as well as environmental exposure to these elements may pose both short-term and long-term health risks. It concerns workers and subpopulations living in urban and industrial zones or in areas with industrial impact (Wong et al., 2006).

The consequences of occupational and environmental exposure on health populations is usually estimated by the measurement of the air pollutants or by biomonitoring. However, the extent to which airborne trace element levels reflect true external exposure is doubtful, and it may be considered that adverse health effects of these trace elements are not only related to the species concerned and total dose but also to peaks of exposure and time of exposure (Christensen, 1995).

The uses of biomonitoring data to assess exposure, absorption and possible health risks have been discussed. The use of an adequate selected combination of biomarkers may lead to a better risk assessment (Kakkar and Jaffery, 2005). Urinary porphyrin profiles´ measurements may be used as a biomarker of metal exposure and effect in human subjects (Woods, 1996).The urinary level of ALA, a precursor of porphyrins synthesis in heme metabolism, has also been accepted in occupational health

as a marker of Pb biochemical effect (Higashikawa et al., 2000). Surveillance programmes using biomarkers have to be carried out for health protection of the general population as well as exposed workers to assess environmental and occupational exposures to heavy metals (Christensen, 1995).

Our goal was to use a combination of biomarkers (urinary porphyrins and ALA) to identify whether each subject was living in an industrialized/non-industrialized/big urban area or in a rural area, was occupationally exposed in mines or to x-rays or was an exposure derived from lifestyle, like smoking.

5.4.1 Biomarkers in urine

5.4.1.1 Urinary Porphyrin Profile

We determined urinary porphyrin profiles and levels of ALA in spot urinary samples of 63 individuals from Portuguese populations, differently exposed to heavy metals. Urinary excretion of porphyrins in spot urinary samples, after creat correction, has been shown to reflect total daily excretion, avoiding the problems of collecting 24-h specimens and facilitating comparison with values in the literature (Alves et al., 2009).

We will consider each porphyrin alone, and confront the obtained values with the urinary porphyrin profile of normal subjects, with 20.7 nmol/g creat of uro, 4.1 nmol/g creat of hepta, 0.89 nmol/g creat of hexa, 4.45 nmol/g creat of penta and 195 nmol/g creat of copro (Kern et al., 2011).

The concentration of uro in the urine of x-ray workers were significantly ($p<0.05$) higher than the ones determined in other groups (Fig. 5.1). The mean value of uro concentration of x-ray workers (59,2 nmol/g creat) is higher than the reference value (20.7 nmol/g creat). However, this also happened to subjects living in an urban area from an island, non-insdustrialized urban area and smokers. Significant increase of total urinary uro has been observed in high As-exposed subjects. Through this fact, we can suppose that x-ray workers, people living in an urban area from this island, in a non-industrialized urban area and smokers had high exposition to As. In general, As concentrations in urban areas are higher than those in non-urban areas (Chirenje et al., 2003), which explains the high uro levels in groups living in urban areas (I and S). Cigarette smoking lowers elimination of As from the body, which can induce alterations of the heme metabolism, like increase of uro levels (Marchiset-Ferlay et al., 2012). However, we can't find evidences of x-ray workers with As.

Subjects living in the Portuguese island presented the highest levels of hepta in urine (17,3 nmol/g creat), above the reference average (4.1 nmol/g creat). The urinary levels of hexa were significantly higher ($p<0,05$) in the rural group (19,5 nmol/g creat), when compared with the rest of the groups and the literatures' value (0,89 nmol/g creat). The increase of hepta and hexa may lead to the rise of total porphyrin concentration. In fact, two persons from the rural group presented higher total porphyrin levels than normal (> 225 nmol/g creat) (Kern et al., 2011).Usually, increase of total porphyrin excretion is a sign of heavy metal exposure, like As. In rural environments, the exposure to heavy metals may be lower than in urban and industrial areas, however rural soils can have high As concentrations (Reis et al., 2007).

The concentration of penta in the urine of subjects living in an industrialized area (24,5 nmol/g creat), were significantly higher than the ones determined in all the other groups (Fig. 5.4), and higher than the normal value according to Kern´s study (4,45 nmol/g creat). Hg body-burden has been demonstrated to be associated with elevations in urinary penta (Geier and Geier, 2007), this can indicate that subjects living in an industrial environment may also be exposed to mercury. Based on Dinis and Fiúza research (2011), Hg (and compounds) air emissions in Portugal (2010) from industrial activity, exceeded 1,7 t.

The rural group had the highest copro concentration in urine (158,5 nmol/g creat), however this value is within the normal range for copro concentration (195 nmol/g creat) (Fig. 5.5). On the contrary, people living in urban areas (I, L and S) and mine workers had lower values of copro in urine (<74 nmol/g creat) (Kern et al., 2011). These results can indicate exposure to Pb, because Pb exposure produced a prominent decrease of copro. This effect can be explained mainly by inhibition of the enzyme ALAD, resulting in a decreased monopyrrole supply for porphyrin biosynthesis, and probably by inhibition of the enzyme uroporphyrinogen decarboxylase (Quintanilla-Vega et al., 1995)**.**

In Figure 5.6, we showed that it is possible to obtain specific porphyrin profiles, resulting from changes in urinary porphyrins excretion patterns, for representative groups exposed to metals. These results support the utility of urinary porphyrin profiles to infer about a probable metal exposure and potential health effects in human subjects.

In summary, we conclude that urinary porphyrin profile can be used to inform about the exposure to heavy metals, so we support the assumption that porphyrins can be used as biomarkers of exposure. We hope to have soon more collected samples representative of each sup-population. We analysed a total of 63 subjects, so our conclusions must be confirmed in a future study with a bigger dimension.

Various factors may influence the levels of heavy metals in the human body: factors associated with the workplace (kind of work, performed operations, use of technical and individual protective devices); lifestyle (feeding habits, water quality, smoking, consumption of alcohol beverages, personal hygiene products, hobbies, chores, place of residence); and other factors like age, sex, pregnancy and genetic factors (Kozlowska et al., 2003).

Since different metals can have different effects on porphyrin biosynthesis, and various intrinsic factors may influence the levels of heavy metals in human subjects, drawing conclusions about the exposure to a specific metal and the level of exposure can be difficult.

5.4.1.2 ALA levels in urine

Subjects living in an urban area of a Portuguese island, presented concentrations of ALA significantly increased when compared with the other groups (Fig.5.7). The rural group had the lowest ALA levels (1,2 mg/g creat). The limits for urinary ALA are set at 0,3-2,85 mg/g creat for normal subjects. The mean value of urinary ALA for Pb workers was 8.7 ±10.6 mg/g creat according with (Oishi et al., 1996). All the groups had mean values below the level for Pb workers, however, the island (4,5 mg/g creat) and industrialized area group (3,1 mg/g creat) had levels above the range for normal subjects.

Pb exposure promotes the activity of ALAS and inhibits the activity of ALAD. The effect of ALAS induction and ALAD inhibition result in an increased urinary excretion of urinary ALA (Quintanilla-Vega et al., 1995). Thus, we can assume that people living in an urban area of a Portuguese island and in an industrialized area are more exposed to Pb than the other groups, while people living in a rural area had the lowest Pb exposure. It was suggested that air pollution contributes up to 25% of the blood Pb levels in urban areas, while contributes about 12% in rural areas (Christensen, 1995).

In Portugal, Pb and Pb compounds emissions were generated, in majority, from the production and processing metals (66%). Energy sector and mineral industry were also contributors representing 17% and 14% respectively of total 2007 air emissions. Waste and the wastewater treatment sector were the main responsible for water emissions (78%) followed by production and processing metals sector (14%) (Dinis and Fiúza, 2011). We may suggest that the populations in the urban area from a Portuguese island and in the industrialized area may be subjected to emissions of these Pb´s sources, which contributed for the increase of their ALA urinary levels.

5.4.2 Combination of Biomarkers

The principal aim of this chapter was to use urinary porphyrin profiles and ALA levels in urine, as biomarkers of exposure or effect, to characterize and discriminate samples of Portuguese populations exposed, in different contexts, to heavy metals. To reach our goal we used three different procedures: one that distinguished seven groups of subjects supposed to be exposed to heavy metals, the groups under study, one that distinguished four populations subjected to environmental exposure but in different sites, and the third that separated three groups of subjects actively and differently exposed to heavy metals.

Procedure I was quite promising, since it intended to distinguish seven groups of subjects with different contexts of exposure to heavy metals. Even so, this procedure was able to correctly classify 76.8% of the population related with the source group (Table 5.2). Figure 5.11 shows that this procedure had not a good separation capability among groups, especially between subjects living in a big urban area and mine workers. Only 50% of the mine workers were correctly classified (Table 5.6). After all, these results may suggest that the population of the big urban area has a similar exposure to metals that the mine works under study. However, we must say that we have a small sample. A larger sample should be analysed, to see if this trend is or not confirmed.

Procedure II was applied to assess the type of environmental exposure in four of the previous groups living in different contexts. This procedure allowed to correctly classify 96,8% of the cases, with only 1 of 31 subjects incorrectly identified as living in an industrial area, when in fact it was a person from the rural group (Table 5.3). This subject was quite distant from its original group (Fig.5.12). No data was available concerning the history of the subject and thus, it is not possible to conclude if it was a failure of the procedure to classify the subject, or if this person had some intrinsic factors that altered the absorption or metabolism of heavy metals.

Procedure III showed that it was possible to discriminate groups with different types of active exposure to heavy metals, through the combination of individual porphyrin profiles and the concentration of ALA in urine. All the subjects were correctly classified by the model (Table 5.4). The areas of the three groups are clearly separated, showing a good discriminative capability of Procedure

III to discriminate mine workers, from x-ray workers and smokers (Fig.5.13). This result is important to evaluate, for example, a person who is a mine worker and smoker, which is the predominant way of exposure to heavy metals.

After reaching our conclusions, it will be important to analyse the applicability of these procedures to other exposed persons in similar contexts. This study can be considered and developed in future works.

5.4.3 Determination of urinary Pb in x-rays workers

X-rays are widely used both in medicine and in industry. These rays can cause physical damage to people who are exposed to these radiations, being necessary to enclose or shield the x-rays generating units with a material which resists to x-rays penetration. Since the impenetrability of the shielding material is a function of its density, Pb is usually the most economical material for such shielding applications (Lead Industries Association, 1984).

Hospitals, factories and laboratories require a mobile barrier which will effectively shield the operating personnel. The thickness of the barriers varies with the type and number of examinations that are performed and the distance between the x-ray emitter and the diagnostic technician. In the rooms where x-rays examinations are performed, the walls are covered with Pb with a thickness between 2 and 3 mm. The operator, during the examination, is housed in a cabinet, which is also covered with Pb (Lead Industries Association, 1984).

It was supposed that x-rays workers are exposed to Pb, by dermal contact and inhalation, making them a group occupationally exposed to Pb. Due to this fact, we analysed the urinary concentration of Pb in these subjects (Table 5.5)

The levels of urinary Pb for all the workers were within a range of concentrations described for urban populations (4-270 μg/g creat) (Agency for Toxic Substances and Disease Registry, 2005).

We compared the average levels of Pb from R group (14.64 μg / g creat) with the levels presented by Coelho et al., (2012) for a group of workers from Panasqueira mine (4.54 μg / g creat). Our levels were higher than the levels obtained for the mine workers, which can indicate that x-ray workers were exposed to higher levels of Pb. However, the results obtained from this group are within the range considered normal in urban population by ATSDR. In addition, the number of individuals studied is small, so, these results must be confirmed with a greater sample.

The limits for urinary ALA are set at 5-15 mg/L for workers in general (Higashikawa et al., 2000). Indeed, x-ray workers presented a level of 6,6 mg/L, indicating occupational exposure to Pb. Urinary ALA is lower than 6 mg/L in normal adults not Pb exposed, and urinary ALA levels of 3 mg/g creat are proposed as a threshold to detect levels of Pb in blood equal or greater than 20 μg/L for which adverse health effects including neurological alterations are described (Abadin et al., 2007). Only one subject in the analysed x-ray workers presented ALA levels above 3 mg/g creat, which suggest a level of blood Pb equal or greater than 20 μg/L.

There was not a direct correlation between urinary Pb levels and levels of ALA in urine in this workers' group (Table 5.5). One possible explanation for this, is the existence of genes that make an

individual more vulnerable to Pb exposure, like ALAD and VDR genes (Makino et al., 2000). Also, Pb levels in urine can be influenced by many factors, like subject's lifestyle, diet and health status. In addition, the samples were not collected on the same day of the week.

The x-rays workers presented indeed high levels of urinary ALA, but Pb levels were within the normal range. This result could eventually be influenced by the method chosen for ALA determination, because colorimetry methods tended to give two or more times higher values than fluorometric HPLC (Higashikawa et al., 2000).

It also would be desirable that Pb in urine could be a valid substitute to blood Pb levels, as a simpler and non-invasive method. However, urinary Pb may lead to some problems: in low level exposures, urinary Pb levels are close to the detection limit of the analytical methods, Pb urinary excretion reflects mainly recent exposure, and the determination of Pb in urine is further complicated by the decrements in kidney function, in association with nephrotoxic Pb effects in cases of high Pb exposure (Abadin et al., 2007).

Chapter 6

Concluding remarks and future perspectives

6.1 Concluding Remarks

In chapter 3, our goal was to study and explore different sample storage conditions over time in order to have confidence on the results obtained of urinary porphyrin profiles of subjects. In this regard, we developed an experimental design, which consisted of analysing the concentration of porphyrins in urine samples, subject to different conditions like at which point the analysis is made (7, 30 and 90 days), temperature of storage (4º, -20 º and -80º C), method of storage (aliquots or pool) and addition or not of preservatives (HCl or Na_2CO_3).

Summarizing the results attained in this chapter, we conclude that:

- Copro can be preserved at -20 ºC and hepta at -80 ºC for 90 days, without addition of preservative.

- When in the presence of Na_2CO_3, we can preserve copro at -20 ºC and -80 ºC and hexa at -80 ºC, until 90 days.

- Preservation of uro at -80 ºC is guaranteed for 90 days when HCl is the selected preservative.

- We suggest the addition of a preservative, like HCl or Na_2CO_3, to samples of urine, for a better preservation of porphyrins, particularly uro and copro.

- The urine samples should be storage in aliquots.

We have tried to achieve our objectives for this chapter however porphyrins are very unstable molecules, varying with light, pH, ionic strength, matrix and solvents, making it hard to understand if porphyrins losses or gains are derived from storage conditions or simply changes in their medium. Because of this, defining the best storage conditions of urinary porphyrins can be a difficult task.

Although, we believe that our work is useful to standardize storage conditions of urinary porphyrins, which may improve consistency and reliability of the results between laboratories and enhance analysis accuracy and adequate diagnosis.

The main objectives of **chapter 4**, were to provide insights about spectrophotometric and fluorometric methods commonly used for porphyrin determination.

The principal conclusions we have achieved, are:

- Uro I is not ether-soluble and its solubility in organic solvents is compromised. Spectrophotometric methods that use ether to extract porphyrins from urine are not effective for uro quantification. However, these methods can be used for copro analyses.

- Total porphyrins can be detected in acidified urine by spectrophotometry, and estimated semi-quantitatively, provided a correction is made for background absorbance.

- HPLC should be the method of choice for a correct quantification of total porphyrins in urine, because the various porphyrins can be separated and individually quantified.

- Spectrophotometric methods can be used to perform fast and preliminary analyses of total urinary porphyrins, but increased concentrations require HPLC to identify the individual porphyrins.

This chapter provides important conclusions for the understanding and application of methodologies used for porphyrins determination. We believe that this is a valid work for the improvement and optimization of methods currently applied in the laboratory, especially in research laboratories, allowing for more accurate and reliable results in clinical or scientific studies.

In chapter 5, our main goal was to use a multi-biomarker approach to identify whether each individual belonged to a particular group in different contexts of metal exposure. In such a way, the groups were associated with a big/industrialized/non-industrialized urban area, an urban area on a Portuguese island, a rural setting, occupationally exposed in mines or to x-rays or an exposure derived from life habits, like smoking.

For this chapter we can highlight:

- Urinary porphyrin profiles informed about a possible exposure to heavy metals, specially, Pb and As, in the analysed sub-populations.

- The urinary ALA levels may indicate that subjects living in an urban area on a Portuguese island and in industrial areas are more exposed to Pb than persons living in a rural environment.

- The combination of urinary porphyrin profiles and ALA levels could be used to classify the type of exposure to heavy metals in the studied subpopulations.

- Through an integrative multi-biomarker approach, it was possible to discriminate the exposure level of populations environmentally exposed with 96,8% of success.

- The discrimination of subjects actively exposed to heavy metals, occupationally and through tobacco smoking, was achieved with a classification of 100%.

We believe that the application of the selected biomarkers, can be a useful tool to predict the type of exposure and the magnitude of toxic effects induced by metals, in exposed populations. However, the study population consisted of only 63 subjects, therefore requiring further studies.

6.2 Future Perspectives

Porphyrins are fascinating molecules with biological significance and potential for a wide variety of applications. To continue the development of our knowledge in this theme, and proceed our study, we present some directions for future works.

We intend to:

- Perform a new experimental study of porphyrin preservation in the same used storage conditions, but using porphyrin standards.

- Optimize the spectrophotometric methods for porphyrin determination, currently used in our laboratory.

- Explore whether the semiquantitative method for total porphyrins determination, can indeed analyse, and with accuracy, the uro, hepta, hexa, penta and copro fractions.

- Implement an HPLC method for the fluorometric determination of ALA in our laboratory.

- Test if our multi-biomarker approach can be applied to other exposed populations in similar contexts.

- Continue the research on the potential of porphyrins and their precursors, as biomarkers of exposure and effect for heavy metals exposure.

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Appendices

Appendix 1

Table A1.1 Concentration of Uro-, Hepta-, Hexa-, Penta- and Coproporphyrin along time (0, 7, 30 and 90 days) in urine samples, aliquots or pool, preserved at different temperatures (4º, -20º and -80º C), without additional preservative. Data represent the mean of triplicate assays.

		4 ºC					-20 ºC					-80 ºC				
	Time (Days)	**Uro**	**Hepta**	**Hexa**	**Penta**	**Copro**	**Uro**	**Hepta**	**Hexa**	**Penta**	**Copro**	**Uro**	**Hepta**	**Hexa**	**Penta**	**Copro**
	0	0,046	0,015	0,011	0,011	0,087	0,090	0,009	0,007	0,011	0,093	0,037	0,010	0,011	0,011	0,060
Aliquots	**7**	0,060	0,011	0,011	0,020	0,039	0,079	0,011	0,013	0,018	0,125	0,067	0,009	0,007	0,009	0,015
	30	0,071	0,014	0,012	0,014	0,088	0,043	0,014	0,010	0,012	0,088	0,053	0,009	0,011	0,009	0,085
	90	0,061	0,013	0,010	0,012	0,021	0,023	0,014	0,009	0,011	0,088	0,016	0,010	0,007	0,008	0,093
Pool	**7**	0,091	0,013	0,008	0,018	0,044	0,091	0,012	0,009	0,017	0,071	0,097	0,012	0,008	0,010	0,044
	30	0,080	0,011	0,015	0,009	0,124	0,045	0,011	0,011	0,016	0,132	0,244	0,012	0,009	0,013	0,121
	90	0,038	0,008	0,007	0,010	0,021	0,213	0,014	0,013	0,014	0,064	0,048	0,011	0,011	0,008	0,105

Table A1.2 Concentration of Uro-, Hepta-, Hexa-, Penta- and Coproporphyrin along time (0, 7, 30 and 90 days) in urine samples, aliquots or pool, preserved at different temperatures (4º, -20º and -80º C), with Na_2CO_3, as preservative. Data represent the mean of triplicate assays.

		4 ºC					-20 ºC					-80 ºC				
	Time (Days)	**Uro**	**Hepta**	**Hexa**	**Penta**	**Copro**	**Uro**	**Hepta**	**Hexa**	**Penta**	**Copro**	**Uro**	**Hepta**	**Hexa**	**Penta**	**Copro**
	0	0,113	0,013	0,008	0,013	0,101	0,026	0,015	0,008	0,008	0,061	0,023	0,013	0,010	0,012	0,057
Aliquots	**7**	0,060	0,009	0,011	0,013	0,021	0,051	0,013	0,010	0,011	0,078	0,043	0,013	0,008	0,012	0,067
	30	0,248	0,010	0,010	0,012	0,066	0,112	0,019	0,011	0,012	0,114	0,061	0,009	0,008	0,010	0,095
	90	0,024	0,012	0,009	0,013	0,038	0,027	0,010	0,008	0,009	0,073	0,038	0,013	0,008	0,008	0,060
Pool	**7**	0,073	0,012	0,008	0,013	0,030	0,029	0,009	0,008	0,010	0,059	0,025	0,010	0,010	0,009	0,054
	30	0,268	0,015	0,007	0,010	0,123	0,182	0,012	0,008	0,009	0,077	0,026	0,012	0,009	0,008	0,057
	90	0,044	0,014	0,007	0,009	0,017	0,024	0,011	0,010	0,007	0,052	0,036	0,013	0,011	0,009	0,046

Table A1.3 Concentration of Uro-, Hepta-, Hexa-, Penta- and Coproporphyrin along time (0, 7, 30 and 90 days) in urine samples, aliquots or pool, preserved at different temperatures (4º, -20º and -80º C), with HCl, as preservative. Data represent the mean of triplicate assays.

		4 ºC					-20 ºC					-80 ºC				
	Time (Days)	**Uro**	**Hepta**	**Hexa**	**Penta**	**Copro**	**Uro**	**Hepta**	**Hexa**	**Penta**	**Copro**	**Uro**	**Hepta**	**Hexa**	**Penta**	**Copro**
	0	0,298	0,016	0,011	0,013	0,050	0,082	0,013	0,008	0,009	0,061	0,082	0,016	0,008	0,009	0,040
Aliquots	**7**	0,167	0,012	0,009	0,011	0,022	0,166	0,011	0,008	0,018	0,044	0,056	0,010	0,008	0,009	0,115
	30	0,101	0,012	0,012	0,014	0,112	0,033	0,015	0,015	0,008	0,179	0,217	0,011	0,011	0,008	0,100
	90	0,040	0,011	0,011	0,009	0,019	0,044	0,012	0,017	0,008	0,066	0,022	0,018	0,012	0,008	0,019
Pool	**7**	0,040	0,010	0,008	0,015	0,025	0,149	0,012	0,009	0,015	0,028	0,042	0,011	0,009	0,009	0,024
	30	0,082	0,013	0,008	0,009	0,061	0,063	0,013	0,011	0,011	0,034	0,044	0,008	0,008	0,009	0,055
	90	0,017	0,010	0,007	0,007	0,010	0,033	0,009	0,008	0,008	0,027	0,052	0,013	0,009	0,008	0,021

Table A1.4 Standard-Deviation of Uro-, Hepta-, Hexa-, Penta- and Coproporphyrin' concentrations along time (0, 7, 30 and 90 days) in urine samples, aliquots or pool, preserved at different temperatures (4º, -20º and -80º C), without additional preservative. Data represent the mean of triplicate assay

		4 ºC					-20 ºC					-80 ºC				
	Time (Days)	**Uro**	**Hepta**	**Hexa**	**Penta**	**Copro**	**Uro**	**Hepta**	**Hexa**	**Penta**	**Copro**	**Uro**	**Hepta**	**Hexa**	**Penta**	**Copro**
	0	0,004	0,000	0,002	0,001	0,001	0,000	0,001	0,000	0,000	0,021	0,003	0,002	0,001	0,001	0,009
Aliquots	**7**	0,025	0,002	0,001	0,001	0,019	0,010	0,000	0,001	0,001	0,027	0,065	0,001	0,000	0,002	0,009
	30	0,041	0,002	0,001	0,001	0,022	0,010	0,000	0,001	0,000	0,016	0,006	0,000	0,001	0,000	0,004
	90	0,004	0,000	0,001	0,000	0,001	0,011	0,000	0,001	0,000	0,012	0,007	0,000	0,000	0,000	0,014
Pool	**7**	0,051	0,000	0,001	0,001	0,020	0,028	0,000	0,001	0,001	0,033	0,079	0,003	0,001	0,001	0,020
	30	0,010	0,001	0,000	0,003	0,026	0,029	0,004	0,003	0,005	0,071	0,010	0,000	0,001	0,000	0,006
	90	0,028	0,001	0,001	0,001	0,004	0,074	0,002	0,001	0,005	0,025	0,017	0,001	0,001	0,001	0,012

Table A1.5 Standard-Deviation of Uro-, Hepta-, Hexa-, Penta- and Coproporphyrin' concentrations along time (0, 7, 30 and 90 days) in urine samples, aliquots or pool, preserved at different temperatures (4º, -20º and -80º C), with Na_2CO_3 as preservative. Data represent the mean of triplicate assays.

		4 ºC					-20 ºC					-80 ºC				
	Time (Days)	**Uro**	**Hepta**	**Hexa**	**Penta**	**Copro**	**Uro**	**Hepta**	**Hexa**	**Penta**	**Copro**	**Uro**	**Hepta**	**Hexa**	**Penta**	**Copro**
	0	0,011	0,001	0,001	0,001	0,009	0,005	0,001	0,001	0,001	0,037	0,008	0,001	0,003	0,002	0,030
Aliquots	**7**	0,027	0,001	0,001	0,001	0,010	0,003	0,001	0,000	0,000	0,006	0,004	0,001	0,001	0,001	0,008
	30	0,243	0,002	0,001	0,001	0,041	0,035	0,000	0,002	0,002	0,032	0,007	0,001	0,001	0,000	0,003
	90	0,003	0,000	0,001	0,003	0,005	0,006	0,000	0,002	0,001	0,007	0,029	0,001	0,002	0,000	0,006
Pool	**7**	0,009	0,001	0,001	0,002	0,014	0,005	0,000	0,001	0,000	0,006	0,005	0,000	0,001	0,001	0,005
	30	0,019	0,001	0,000	0,001	0,002	0,021	0,001	0,001	0,002	0,018	0,001	0,001	0,000	0,001	0,022
	90	0,000	0,002	0,001	0,003	0,000	0,013	0,000	0,000	0,001	0,013	0,008	0,001	0,001	0,001	0,007

Table A1.6 Standard-Deviation of Uro-, Hepta-, Hexa-, Penta- and Coproporphyrin' concentrations along time (0, 7, 30 and 90 days) in urine samples, aliquots or pool, preserved at different temperatures (4º, -20º and -80º C), with HCl as preservative. Data represent the mean of triplicate assays.

		4 ºC					-20 ºC					-80 ºC				
	Time (Days)	**Uro**	**Hepta**	**Hexa**	**Penta**	**Copro**	**Uro**	**Hepta**	**Hexa**	**Penta**	**Copro**	**Uro**	**Hepta**	**Hexa**	**Penta**	**Copro**
	0	0,013	0,000	0,001	0,002	0,016	0,035	0,002	0,001	0,002	0,006	0,050	0,001	0,001	0,002	0,011
Aliquots	**7**	0,149	0,002	0,002	0,000	0,008	0,061	0,001	0,001	0,001	0,010	0,012	0,000	0,001	0,000	0,020
	30	0,058	0,005	0,003	0,004	0,057	0,016	0,000	0,002	0,001	0,025	0,212	0,001	0,000	0,002	0,036
	90	0,004	0,001	0,003	0,002	0,007	0,007	0,001	0,001	0,001	0,023	0,002	0,002	0,001	0,000	0,003
Pool	**7**	0,027	0,000	0,001	0,000	0,003	0,002	0,001	0,001	0,000	0,001	0,016	0,001	0,000	0,002	0,007
	30	0,035	0,002	0,001	0,002	0,006	0,004	0,005	0,003	0,003	0,004	0,008	0,000	0,001	0,002	0,000
	90	0,015	0,002	0,000	0,000	0,003	0,000	0,001	0,002	0,001	0,004	0,042	0,000	0,001	0,002	0,004

Appendix 2

Table A2.1 Concentration of urinary coproporphyrin (µmol copro/g creat) and total urinary porphyrin concentration (µmol porphyrins/g creat) of subjects, analysed by different methods: Soulsby Method (coproporphyrin), Elder Method (total porphyrin) and HPLC method (copro and total porphyrins).

Subject nº	Soulsby µmol copro/g creat	HPLC µmol copro/g creat	Elder µmol porphyrins/g creat	HPLC µmol porphyrins/g creat
1	0,0229	0,0679	0,2244	0,1365
2	0,0295	0,0247	0,1822	0,0820
3	0,0127	0,0114	0,0921	0,0396
4	0,0170	0,0057	0,0677	0,0944
5	0,0352	0,0494	0,3255	0,2396
6	0,0298	0,1204	0,2909	0,4550
7	0,0616	0,0662	0,1525	0,0499
8	0,0673	0,0150	0,1272	0,0515
9	0,0357	0,0139	1,5107	0,3705
10	0,0480	0,0259	0,1925	0,0707
11	0,0471	0,0213	0,5787	0,1188
12	0,0761	0,0241	0,2860	0,0613
13	0,0337	0,0330	0,1524	0,0673
14	0,0361	0,0301	0,3221	0,0765
15	0,0401	0,0298	0,1727	0,1774
16	0,0459	0,0295	0,1560	0,2884
17	0,0521	0,0450	0,1009	0,1434
18	0,0225	0,0379	0,1603	0,1311
19	0,0382	0,0336	0,1618	0,1010
20	0,0231	0,0518	0,0664	0,0382
21	0,0286	0,0144	0,0516	0,0545
22	0,0332	0,0264	0,0880	0,1090
23	0,0320	0,0410	0,0802	0,1426
24	0,0323	0,0774	0,2456	0,0748
25	0,0436	0,0191	0,0453	0,0774
26	0,0135	0,0368	0,2355	0,3644
27	0,0332	0,0090	0,4349	0,4518
28	0,0536	0,0938	0,6851	0,5453
29	0,0511	0,0743	5,4287	0,2244
30	0,0502	0,0789	1,0422	0,1453
31	0,0499	0,0799	0,8762	0,1052
32	0,0580	0,0832	0,0878	0,2493
33	0,0364	0,0635	0,1155	0,4341
34	0,0728	0,1944	0,0704	0,2280
35	0,0842	0,2048	0,1298	0,1224
36	0,0564	0,1855	0,1366	0,3523
37	0,0539	0,0492	0,0914	0,0289

38	0,0934	0,1585	0,1033	0,1031
39	0,0261	0,0154	0,2291	0,1167
40	0,0187	0,0158	0,1503	0,4992
41	0,0051	0,0171	0,1129	0,1430
42	0,0209	0,0104	0,1785	0,3856
43	0,0250	0,0216	0,1007	0,0370
44	0,0197	0,0198	0,0422	0,0350
45	0,0153	0,0195	0,2275	0,2009
46	0,0623	0,0600	0,1833	0,1357
47	0,0328	0,1173	0,1716	0,2268
48	0,0227	0,0728	0,3667	0,2623
49	0,0337	0,1000	0,2860	0,3392
50	0,0194	0,1375	0,0183	0,0919
51	0,0449	0,0188	0,0142	0,2379
52	0,0390	0,1004	0,1257	0,1575
53	0,0309	0,0514	0,0964	0,2835
54	0,0458	0,0779	0,1079	0,1806
55	0,0416	0,1601	0,0452	0,0560
56	0,0373	0,2040		
57	0,0555	0,0977		
58	0,0490	0,0676		
59	0,0508	0,0606		
60	0,0618	0,1501		
61	0,0639	0,1336		
62	0,0391	0,0887		
63	0,0684	0,0854		

Table A2.2 Correlation test and mean value of results obtained for the samples analysed by the Soulsby and HPLC method.

		Soulsby	**HPLC**
Soulsby	**Pearson's Correlation**	1	,491**
	Sig. (bilateral)	-	,000
	N	63	63
HPLC	**Pearson's Correlation**	,491**	1
	Sig. (bilateral)	,000	-
	N	63	63
	Mean (µmol/g creat)	0,04	0,05
**. The correlation is significant at the 0.01 level (bivariate).			

Table A2.3 Correlation test and mean value of results obtained for the samples analysed by the Elder and HPLC method.

		Elder	HPLC
Elder	**Pearson's Correlation**	1	,293*
	Sig. (bilateral)	-	,032
	N	55	55
HPLC	**Pearson's Correlation**	,293*	1
	Sig. (bivariate)	,032	-
	N	55	55
	Mean (µmol/g creat)	0,228	0,181
*. The correlation is significant at the 0.05 level (bivariate).			

Appendix 3

Table A3.1 Concentration of Uro-, Hepta-, Hexa-, Penta- and Coproporphyrin (nmol/g creat) and ALA levels (mg/g creat) from 63 subjects of Portuguese sob-populations exposed in different contexts to heavy metals. Mean and SD of each group are also presented.

		nmol/g creat					mg/g creat
Group	**Subject**	**Uro**	**Hepta**	**Hexa**	**Penta**	**Copro**	**ALA**
I (people living in an urban area from an island)	I1	25,90	9,10	25,50	8,00	67,90	5,69
	I2	21,90	10,00	16,30	9,10	24,70	2,65
	I3	13,40	15,80	18,70	14,90	11,40	1,73
	I4	72,60	17,30	4,50	4,90	5,70	2,14
	I5	41,60	22,40	16,60	14,60	49,40	8,69
	I6	43,00	27,40	19,10	29,80	120,40	4,93
	I7	36,40	19,20	15,70	13,20	66,20	5,89
	Mean	36,40	17,30	16,60	13,50	49,40	4,53
	SD	19,30	6,50	6,30	8,10	40,10	2,51
L (people living in a big urban area)	L1	7,00	9,70	8,80	9,40	15,00	2,12
	L2	9,50	12,50	9,10	9,70	13,90	3,42
	L3	12,10	13,80	12,70	11,30	25,90	3,65
	L4	11,30	12,90	12,10	13,10	21,30	2,69
	L5	19,70	19,00	11,00	10,40	24,10	2,40
	L6	4,50	8,40	8,30	7,10	33,00	2,68
	L7	4,00	10,20	9,90	13,00	30,10	2,59
	L8	8,10	13,40	15,80	9,40	29,80	1,89
	Mean	9,50	12,50	11,00	10,40	24,10	2,68
	SD	5,00	3,30	2,50	2,00	7,00	0,60
M (Mine workers)	M1	16,50	16,50	12,00	10,40	29,50	2,79
	M2	4,70	10,70	13,10	12,20	45,00	2,76
	M3	3,70	6,50	5,60	11,40	37,90	2,57
	M4	7,00	12,50	11,10	9,40	33,60	2,70
	M5	14,50	7,40	11,10	14,60	51,80	3,66
	M6	11,80	3,60	9,90	5,00	14,40	1,57
	M7	4,20	11,50	8,20	4,30	26,40	1,56
	M8	30,90	11,30	10,20	15,60	41,00	2,06

	M9	16,10	13,60	9,30	13,00	77,40	3,53
	M10	11,00	9,90	12,90	12,90	19,10	3,02
	M11	15,80	8,30	11,60	5,00	36,80	2,70
	M12	12,40	7,40	10,40	4,40	9,00	3,03
	Mean	12,40	9,90	10,40	9,80	35,20	2,66
	SD	7,50	3,50	2,10	4,20	18,30	0,66
R (x-rays workers)	R1	121,80	9,20	10,50	23,40	93,80	1,87
	R2	105,50	9,80	7,00	19,70	74,30	1,89
	R3	59,20	10,70	10,40	18,70	78,90	1,35
	R4	27,00	9,40	9,80	16,60	79,90	2,29
	R5	28,90	11,50	12,10	18,00	83,20	2,08
	R6	12,50	8,30	9,20	11,70	63,50	1,84
	Mean	59,20	9,80	9,80	18,00	78,90	1,89
	SD	45,20	1,10	1,70	3,90	10,00	0,31
A (people living in a rural area)	A1	6,70	6,70	19,50	6,90	194,40	0,72
	A2	13,00	10,70	22,50	9,00	204,80	1,17
	A3	5,90	10,30	6,80	19,50	185,50	0,95
	A4	10,50	17,00	18,80	14,40	49,20	1,36
	A5	16,60	11,20	29,90	13,80	158,50	1,89
	Mean	10,50	11,20	19,50	12,70	158,50	1,22
	SD	4,40	3,70	8,30	5,00	63,50	0,45
S (people living in a non-industrialized urban area)	S1	19,70	10,30	9,00	9,30	15,40	2,82
	S2	14,90	11,00	12,10	9,10	15,80	1,27
	S3	34,80	10,90	13,70	7,40	17,10	3,07
	S4	24,00	10,50	11,50	5,30	10,40	2,90
	S5	36,90	13,80	16,00	10,40	21,60	2,66
	S6	16,40	6,50	7,70	8,90	19,80	2,38
	S7	20,90	10,70	10,80	12,20	19,50	2,52
	Mean	24,00	10,50	11,50	8,90	17,10	2,52
	SD	8,70	2,10	2,80	2,20	3,70	0,60
V (people living in an industrialized urban area)	V1	9,10	16,20	11,70	19,70	60,00	3,31
	V2	26,90	22,40	11,90	22,90	117,30	2,37
	V3	21,90	19,40	22,70	38,20	72,80	3,50
	V4	17,10	12,60	9,60	25,90	100,00	3,08
	V5	4,80	19,50	5,40	24,50	137,50	1,49
	V6	4,70	18,20	18,20	6,30	18,80	1,68
	V7	19,00	16,40	13,30	24,40	100,40	5,02
	V8	24,20	19,30	23,50	17,20	51,40	3,52
	V9	21,50	21,40	16,40	29,50	77,90	1,87
	V10	20,90	24,70	14,90	24,60	160,10	2,98
	V11	38,60	24,30	16,50	36,80	204,00	5,09
	Mean	19,00	19,50	14,90	24,50	100,00	3,08
	SD	10,00	3,60	5,40	8,80	52,80	1,21

F **(smokers)**	F1	27,90	11,20	6,40	11,80	97,70	3,22
	F2	45,70	11,80	6,20	11,40	67,60	1,54
	F3	66,60	8,30	7,00	11,30	60,60	2,70
	F4	36,10	9,90	6,10	13,60	150,10	2,37
	F5	18,10	5,20	6,90	16,80	133,60	3,15
	F6	16,50	7,20	7,40	8,40	88,70	2,83
	F7	41,60	8,90	9,20	12,00	85,40	3,11
	Mean	36,10	8,90	7,00	12,20	97,70	2,70
	SD	17,50	2,30	1,10	2,60	33,00	0,59