New biomarkers of exposure to metals using exhaled breath condensate (EBC)

Pedro M. Félix

DEPARTMENT OF RADIATION, RADIONUCLIDES & REACTORS

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Pedro M. FÉLIX

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Prof. dr. H.Th. Wolterbeek
Prof. dr. Teresa Pinheiro

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Rector Magnificus, voorzitter
Prof. dr. H.Th. Wolterbeek, Technische Universiteit Delft, promotor
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Dr.ir. P. Bode, Technische Universiteit Delft

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Cover image: Optical micrograph (reflection microscopy) of a native sample of EBC containing particles.

The dose makes the poison

Paracelsus (1493-1541)

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Summary

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Occupational exposure to metals can result in a large variety of implications for human health. Industrial units, such as smelters, welding sites, or the plastic or battery industry present a potential hazardous exposure to their workers. At these industrial working places, especially the airborne particulates and its elemental composition, are known to imply health risks. Biological Monitoring, or biomonitoring (the measurement of a toxicant or its metabolite(s), in a biological compartment) is an important complementary measurement to environmental monitoring and is acknowledged as a measurement that provides more reliable results in assessing potential risk to the health and safety of workers than the direct measurements of toxic substances in the air, as in airborne particles.

Exhaled Breath Condensate (EBC) is a body fluid collected through the condensation of exhaled breath under conditions of tidal breathing, and is commonly used for the determination of oxidative biomarkers in airway inflammations. In this thesis, EBC is studied as possible non-invasive tool for the evaluation of exposure to metal aerosols, fumes and airborne particulate. The main goal was to investigate whether EBC is a suitable matrix to assess exposure to metals through the determination of these metals in EBC and to determine the applicability of EBC as a routine-based bioindicator in occupational settings.

In the study, workers from lead processing industries were selected, divided into groups of differently exposed workers and a non-exposed group, which were monitored for their exposure. The exposure was determined through the analysis of airborne contaminants at the workplace. For this, fine airborne particulate matter (APM) was collected with Gent samplers in two size ranges (PM2.5 and PM2.5-10) and its elemental composition was measured by Particle Induced X-Ray Emission (PIXE) and by Instrumental Neutron Activation Analysis (INAA). The studied APM characteristics served to demonstrate the exposure characteristics in the different work places and to establish a relation between the industrial processes and the APM properties resulting from those emissions.

EBC was collected from the participating workers, using the commercial device EcoScreen, and analysed by Total Reflection X-ray Fluorescence (TXRF) and Inductively Coupled Plasma Mass Spectrometry (ICP-MS). A first study effort on better understanding and characterizing of the EBC matrix, thereby using a Nuclear Microprobe (where a sample area is scanned by a proton beam), showed

the presence of PM and also indicated the sample heterogeneity, which made in clear that samples need to be pre-prepared to get representative results. To this end, methodological protocols were developed for the collection of samples, the sample preparation and analysis. An important step was the determination of the overall uncertainty of EBC analysis (Quality Control aspects), fundamental to the validation of the use of the biomarker.

The study showed EBC to reflect exposure to several metals, such as chromium (Cr), nickel (Ni), copper (Cu) and lead (Pb), present in the respiratory tract due to inhalation, and to discriminate between different levels of exposure, where workers exposed to larger amounts of airborne particles containing those metals revealed higher concentrations of the same elements in the EBC, in a linear relation. These outcomes indicate the potential of EBC as a matrix for new biomarkers of metal exposure. Lead (Pb) is the main contaminant at the studied work places, and regulations impose the monitoring of workers through the quantification of lead in the blood (Blood-Pb or B-Pb). The new biomarker EBC-Pb was compared to B-Pb results and advantages and limitations of both biomarkers are discussed. EBC-Pb has the advantage of being non-invasive, of providing a rapid response to Pb-exposure, it is less affected by confounders, and it shows a higher saturation level than B-Pb. The latter makes EBC-Pb largely suited for occupational exposure monitoring. The simple protocols for the sample collection and analysis allows the fast processing of large quantities of samples.

In conclusion, EBC is a biomarker that should be used in further study projects to achieve its application as a routine approach of monitoring of metal exposure in occupational working places.

Samenvatting

Nieuwe biomarkers voor blootstelling aan metalen gebruikmakend van uitgeademd luchtcondensaat (EBC)

Beroepsmatige blootstelling aan metalen kan resulteren in een grote verscheidenheid aan gezondheidseffecten. Industriële werkplekken zoals smelterijen, lasinrichtingen, of de plastic- of de accu-industrie zijn plekken met potentieel gevaarlijke blootstellingen voor de werkers. Op deze werkplekken zijn het met name de in de lucht aanwezige fijne deeltjes en hun elementsamenstelling die bekend staan als risicovol voor de gezondheid. Biologische monitioring, ofwel biomonitoring (de meting van een toxicant of zijn metaboliet(en) in een biologisch compartiment) is een belangrijke complementaire meting naast omgevingsmonitoring en wordt gezien als een meting die meer betrouwbare informatie oplevert ten aanzien van het potentiele risico voor de gezondheid en veiligheid van werkmensen dan de directe meting van toxische stoffen in de lucht, zoals in atmosferische deeltjes.

Uitgeademd luchtcondensaat (engelstalig: Exhaled Breath Condensate EBC), is een lichaamsvloeistof die verzameld wordt via de condensatie van uitgeademde lucht onder omstandigheden van "tidal" ademhaling (lucht uit een enkelvoudige ademhaling), en wordt veelal gebruikt voor de vaststelling van oxidatieve biomarkers van luchtweginfecties. In dit proefschrift is EBC onderzocht als mogelijke non-invasieve aanpak voor de evaluatie van de blootstelling aan metaalhoudende aerosolen, rook en fijne deeltjes in de lucht. Hoofddoel was het onderzoeken van EBC als bruikbare matrix voor de vaststelling van de blootstelling aan metalen via de analyse van deze metalen in EBC en het nagaan van de mogelijkheden om EBC te gebruiken als routinematige bio-indicator in beroepsmatige werkplekken.

In de studie zijn werkers geselecteerd uit de loodverwerkende industrie, verdeeld in groepen verschillend blootgestelde werkers en een niet-blootgestelde groep, die gevolgd werden, en van wie hun blootstelling werd vastgelegd. De blootstelling werd bepaald via de analyse van deeltjes in de lucht in de werkplekken. Hiertoe werden fijne deeltjes in de lucht (engelstalig: Airborne Particulate Matter APM of PM) verzameld met behulp van Gent luchtfilter-units, in twee grootteklassen (PM2.5 en PM2.5-10) en hun element-samenstelling werd gemeten via particle induced X-ray emission (PIXE) en Instrumentele neutronen aciveringsanalyse (INAA). Deze APM karakteristieken dienden voor de vaststelling van de

blootstellingskarakteristieken in de verschillende werkplekken en voor de vaststelling van de relatie tussen werkplek en APM-eigenschappen, veroorzaakt door deze emissies.

EBC werd verzameld van de deelnemende werkers, gebruikmakend van de commercieel verkrijgbare EcoScreen units, en geanalyseerd met behulp van total reflection X-ray fluorescence (TXRF) en inductively coupled plasma mass spectrometry (ICP-MS). Een eerste onderzoeksaanzet om de EBC matrix beter te begrijpen en te karakteriseren, gebruikmakend van een nuclear microprobe (waarbij een monster wordt gescanned met een protonbundel), gaf de aanwezigheid aan van PM, en liet ook de sampleheterogeniteit zien, waardoor het duidelijk werd dat monsters moesten worden voorbewerkt om tot representatieve resultaten te kunnen komen. Daartoe werden methodologische protocols ontwikkeld voor het verzamelen van de monsters, de monstervoorbereiding en de –analyse. Een belangrijke stap was ook de vaststelling van de overall onzekerheid van de EBC-analyse (Quality Control aspecten), fundamenteel benodigd voor de validatie van het gebruik van de biomarker.

De studie laat zien dat EBC de blootstelling aan diverse metalen reflecteert, zoals chroom (Cr), nikkel (Ni), koper (Cu) en lood (Pb), zoals die aanwezig zijn in het ademhalingssysteem als gevolg van inhalatie, en te discrimineren tussen verschillende blootstellingsniveaus, waar werkers die blootgesteld zijn aan hogere hoeveelheden deeltjes in de lucht in lineair verband hogere niveaus laten zien van deze elementen in hun EBC. Deze uitkomsten geven aan dat EBC in potentie geschikt is als nieuwe biomarker matrix voor de blootstelling aan metalen. Lood (Pb) is de hoofdcontaminant in de onderzochte werkplekken, en er bestaan voorschriften voor de noodzakelijke monitoring van werkers via de kwantificering van de lood-aanwezigheid in het bloed (bloed-Pb ofwel B-Pb). De nieuwe biomarker EBC-Pb is vergeleken met de B-Pb resultaten en voor- en nadelen van beide markers zijn bediscussieerd. EBC-Pb heeft als voordeel dat het non-invasief is, een snelle response op Pb-blootstelling geeft, weinig beïnvloed wordt door confounders, en een hoger verzadigingspunt heeft dan B-Pb. Dit laatste maakt EBC-Pb met name geschikt voor monitoring van beroepsmatige blootstelling. De simpele protocols voor het verzamelen van monsters en hun analyse maken het mogelijk grote hoeveelheden monsters te verwerken.

De conclusie uit de studie is dat EBC een biomarker is die ingezet zou moeten worden in verdere onderzoeksprojecten om te komen tot zijn toepassing als een routinematige aanpak van metaal-blootstellingsmonitoring in beroepsmatige werkplekken.

Chapter 1

1. Introduction

1.1 Biological monitoring

Environmental exposure to contaminants has long been recognized to have a critical and hazardous effect on human health (Bagatin and Kitamura, 2006). The knowledge and control of such exposure has been a concern ever since and efforts have been endeavoured to mitigate undesirable health outcomes on the population and individuals severely exposed, i.e., in industrial settings. The first line of action, still a rule today, was to identify and reduce the exposure levels. This process involves the quantification of contaminants in the environment, through environmental monitoring, thus, providing information of how much of the toxicant the subjects are exposed to. However, the determination of potential toxicants in the ambient air, although important, fails to give information about the amount of uptake by the organism and potential health influence of such toxicants. Environmental monitoring was followed by the measurement of the compounds in living compartments of the organism or the outcome of its interaction with the organism.

Biological monitoring, or biomonitoring, is the periodic measurement of a biomarker in an organism. The term is a natural adaptation to environmental monitoring, which aims to determine levels of concentration of toxicants in the workplace environment. Although both are important measures, biomonitoring provides information at the individual level that can vary naturally according to the variability associated to each biological system (rates of uptake, metabolism or susceptibility), giving valuable information that can lead to an estimation of target site concentration and dose-response (Zielhuis and Henderson, 1986; Christensen and Olsen, 1991; Christensen, 1995; Rainska et al., 2007). The National Research Council (NRC, 1989) defined biomarker of exposure as "an exogenous substance or its metabolite or the product of an interaction between a xenobiotic agent and some target molecule or cell that is measured in a compartment within an organism". Its real significance is based on how much of an exogenous agent is absorbed or accumulated. In fact, these common tools in biomonitoring, focus on the body burden or on the total dose absorbed (Mutti, 1999). This sets a difference from other approaches like biomarkers of effect that measures alterations within an organism as result of exposure to a toxicant agent and that is commonly found between end-points, usually after exposure and before a possible health effect (Mutti, 1995; NRC, 1989). Both approaches are complementary and both present

difficulties, either due to their sensitivity and specificity, i.e., the ability to avoid false negative or false positive results, respectively, and its correct use and interpretation (Manno et al., 2010). The ideal biomarker would have only one source and little or none confounders, otherwise, it may add unnecessary variability affecting its purpose and generating poorer results than the alternative of ambient atmospheric monitoring (Mutti, 1999). One of the major problems associated with biomarkers is their representativeness and inability to differentiate sources of exposure. A paradigmatic example is the use of Blood-Pb (B-Pb) to quantify chronic exposure to lead (Pb). This biomarker of exposure, although valid for medium-term exposure, not only has potential confounders, like age (Cory-Slechta et al., 1989), but also different sources, that depend on the health condition of the subject and lifestyle or hygiene habits (Garn et al., 1967; Silbergeld et al., 1988; Gulson et al., 1995). Also, depending on the subjects' condition, false positive results can be an outcome of iron deficiency episodes or low dietary calcium (Heard and Chamberlain, 1982; Blake and Mann, 1983; Mahaffey and Annest, 1986). Nonetheless, biomonitoring, whenever possible, constitutes a better alternative than environmental monitoring because it provides a more accurate assessment of individual exposure and potentially allows the determination of workload of recent and past exposures, health outcomes or individual susceptibility (Mutti, 1999; Manno et al., 2010).

Besides the difficulty on determining sources of exposure, biomonitoring has two other significant issues that are important to mention. On one hand there is the type of sample and its collection. The best sample has often a certain degree of invasiveness, such as blood draw, posing a problem to the subject himself and also raising the costs, due to the requirement of qualified personnel to collect the samples. Thus, whenever possible, non-invasive and easily collected samples are usually preferred (Henderson et al., 1989; Manno et al., 2010). On the other hand, there is the matter of individual variability. Inter- and intra-individual variability is a common trait on biological systems. However, although variability can sometimes be an analytical problem, in environmental health it can be considered a source of information, explained by toxicodynamic factors, kinetics and metabolism (Hattis, 1996; Mutti, 2001; Manini, 2007). Nonetheless, several uncontrollable aspects, such as physical condition, or diet, can induce biological variability (Droz, 1993), particularly in environmental and occupational monitoring campaigns, where all subjects are sampled. When failing to identify the possible sources of variation, increasing sample size becomes an important option and repetitive measures to help assess intra-individual variability. Also, a fundamental advantage to understand this variability and maintain variance as low as possible is the standardization of the collection procedures and analytical methodology (Watson and Mutti, 2004). Moreover, this would allow the inter-comparison of data and cooperation programs among research groups.

Overall, biomonitoring is an important instrument for assessing exposure, estimate the risk and minimizing it, controlling the subjects' exposure to a minimum and reducing health impacts.

1.2 Occupational exposure

The industrial production, boosted after de second half of the 18th century, as a result of the industrial revolution, brought, not only an important dynamic to the world's economy, but also a severe impact on human health. The industrial processes have always been a source of contaminants and have long been a concern (Legge et al., 1901; Hoffman, 1909; Blumgart, 1923). A particular focus has been given to metals which were soon recognized to have hazardous health effects. The documented manipulation of metals is dated as far back as 7000 BC. Metals were essentially used to produce objects and pigments and Pb, given its low melting point and, thus, easier to use, is one of the first documented metals in history to be used by man (Nriagu, 1983). However, the acknowledgement of metal toxicity and cure is documented since as early as 370 BC when Hippocrates described abdominal colic in a man who worked with metal extraction (Shanker, 2008). From antiquity to modern days, a long way has been travelled, regarding our knowledge of metals and its effect on human health. Since metal exposure was recognized as a health risk (late 19th century), there has been an effort to reduce this exposure and mitigate health consequences (Lesser, 1988). National and European legislation has come to slowly control and protect exposed subjects, with continuous revision of the policies for the implementation conduct rules and improvement of industrial facilities. The second half of the 20th century was significantly marked by the recognition and use of biomarkers in the context of human biomonitoring on subjects exposed to metals (Boogaard, 2007). Biomonitoring results, through the use of biomarkers, started assuming an important role in decision-making regarding health and economic impacts. Presently, the specificity and sensitivity of biomarkers used for occupational exposure assessment to metals have been rapidly improving, continuously refining the process of health effects mitigation in professional exposure.

1.2.1 *Industrial environment (an elemental perspective)*

Numerous industries have significant emissions of several elements in the form of gases, dust or fumes. Among the most studied cases of occupational exposure are the foundry industry, smelting, welding, mining and the recycling and production

battery industries, which present a high risk to their workers' health (Hernberg, 1973). Here, workers are required to follow rules of conduct and to wear protective equipment, which will reduce individual exposure to toxic agents, present at the work environment. However, the monitoring of these workers shows that there is still a significant uptake of toxicants by the organism (e.g. Goldoni et al., 2004; Nawrot et al., 2008; Gube et al., 2010). Although being a serious problem for human health, these toxicants are inseparable agents from industrial processes and are an integral part of human society, rarely without risk (IPCS, 1999; Hervé-Bazin, 2002).

In industrial settings, the airborne particulate matter (APM) resulting from the work activities, is a major concern and a larger problem than in the outdoor environment, due to the differences in the mass and elemental concentrations between each of them, highly increased in the industrial ambient air and highly diluted in the environment (Almeida et al., 2010). APM is a mixture of solid particles or liquid droplets in a gaseous medium (Seinfeld, 1986), present in the atmosphere and the indoor ambient air or the work environment. Its physical properties vary considerable depending on their origin and subsequent cycle. The dimension of the particles, given their commonly irregular geometry and variable composition, is expressed by its Aerodynamic Diameter (AD). It is equivalent to the diameter of a sphere with a density of $1 g.cm^{-3}$ with the same settling velocity as the particle of interest (Seinfeld e Pandis, 1998). Reference to the particles' dimension is made as PM_X, indicating particles inferior to X μm. Following the nomenclature used in health assessment, the particulate matter will be addressed in the present work as $PM_{2.5}$, $PM_{2.5-10}$ and PM_{10}. The work processes generate different sizes of particles. APM from combustion processes are characterized by being fine particles, opposing mechanical processes, for instance, that emit mainly coarser particles. (Hlavay et al., 1992; Seinfeld and Pandis, 1998; Wake et al., 2002; Cheng et al., 2008). The identification and distinction of both cases is of the utmost importance, given its differential influence on human health. Epidemiological data consistently suggest the increased health risks as a consequence of the increased exposure to fine particles (Pope et al., 2002, 2008; Zanobetti et al., 2009).

Therefore, the characterization of the work environment in terms of particle size and composition assumes a very important role, together with biomonitoring, in the assessment of occupational exposure. However, very few published studies characterize the industrial environment and in an elemental perspective they usually cover the obvious culprits, depending on the processes and materials used and the known causers of hazardous health effects, i.e., chromium (Cr) in chrome-plating, iron (Fe) and nickel (Ni) in welding and smelting or Pb in the battery-recycling industry (e.g. Karlsen et al., 1992; Edmé et al., 1997). There are a vast number of elements in the workplace environment in traceable concentrations that can have potential effects on the health of workers (e.g. Owoade et al., 2009). The

importance on characterizing the workplace's environment lies not only on the significance of identifying the toxic agents and the granulometric properties of the APM, but also, if possible, considering potential synergistic effects (Cross et al., 2001; Silins and Högberg, 2011).

1.2.2 *Effects of metals on human health*

Trace elements in general are a constant presence in the surrounding environment of our daily life. Metals in particular, as most trace elements, interact with biological systems due to their chemical properties, such as reduction and oxidation reactions under physiological conditions. Several of these interactions are harmful and disrupt the natural biological processes. However, some metal ions are essential in physiological processes and, in these circumstances, the same properties that make them functional are also the base of their toxicity, if present in excess (Shanker, 2008).

Once metals enter the organism, given their elementary nature, they endure as such. Until excretion, metals stay in the body where they can undergo chemical transformations into one more toxic or less toxic species (Friberg, 2007). In the present study, the subjects are exposed to airborne particulate and inhalation is the main route of intake. From the lung tissue, metals are absorbed and enter the systemic circulation and, depending on the element, the chemical species, and affinity to biomolecules, they can accumulate (e.g. Pb) or be transformed (e.g. Cr(VI) - Cr(III) reduction). These interactions dictate the half-life of the metal in the body, which may range from hours to decades, and therefore its rate of excretion. The celerity of this process also depends on the size of the inhaled particle, the solubility of the metal and the breathing effort of the subject (Baron, 2003). Metallic elements occur in all living organisms, with a variety of physiological functions. They may play the role of structural elements, stabilizers of biological structures, components of control mechanisms and, in particular, are activators or components of redox systems (Friberg, 2007). These are essential elements and their deficiency can result in an impairment of biological functions, but in excess can have a toxic effect (Becking et al., 2007). This is an obvious alert to the importance of assessment of exposure to all elements. Moreover, essential metals usually have a higher rate of uptake than toxic metals, as their absorption by cells is often done through carrier-mediated energy-dependent transport mechanisms (Bjerregaard and Andersen, 2007).

Specific effects on human health vary greatly according to the element and amount of uptake. Some metallic elements are classified according to their impact on human health. Several are now confirmed carcinogens, like Cd, As, Cr, Ni, possible carcinogens, like Co or probable carcinogens, which is the case of Pb

(IARC, 1990, 1993, 2006). There are, however, other health consequences to be considered as a result of an excess of essential metals or antagonistic effects from non-essential elements and all is dose dependent (Chen et al., 2005; Garrick et al., 2003). According to Valls and de Lorenzo (2002) metals can be classified into three categories, depending of their physiological role, i) essential, non-toxic ii) *essential but toxic above a given concentration iii) and toxic, causing health* impairments even at low concentrations. For toxic metals, effects can go from hypersensitivity reactions that may involve the skin, kidney, lung, hemopoietic system, and possibly the nervous system to teratogenic malformations, all the way through respiratory, gastrointestinal, hepatic, renal, reproductive or cardiovascular diseases (Kazantzis, 2007).

Pb has been, and continues to be, widely used in industrial processes. Workers of the lead processing industries are exposed to high concentrations of Pb which consequently made this element one of the most studied elements, concerning its impact on human health, and groups of exposed subjects are monitored according to legal regulations (Christensen and Kristiansen, 1994; Hernberg, 2000). This makes Pb an interesting model for biomonitoring studies in occupational settings, where workers are continuously exposed throughout their labouring period. Pb has no known biological function and it is primarily absorbed through the lungs (inhalation) or gastrointestinal tract (ingestion). After absorption Pb is transported by the bloodstream to all parts of the organism and although it is found in blood and soft tissues, it is mainly accumulated in the calcified tissues, like the bone that commonly stores around 94% of total body burden (Rabinowitz, 1991; O'Flaherty, 1995; Rust et al., 1999, Lowry et al., 2004). In blood, Pb is primarily bound to haemoglobin and can interact with several biological processes. Pb affects several enzymatic processes responsible for heme synthesis. Lead directly inhibits the activity of the cytoplasmic enzyme δ-aminolevulinic acid dehydratase (ALAD). Pb depresses coproporphyrinogen oxidase, resulting in increased coproporphyrin activity. Pb also interferes with the normal functioning of the intramitochondrial enzyme ferrochelatase, which is responsible for the chelation of iron by protoporphyrin. The failure to insert Fe into the protoporphyrin ring results in depressed heme formation and an accumulation of protoporphyrin, which in turn chelates zinc instead of Fe, to form zinc protoporphyrin. (Christensen and Kristiansen, 1994; Barbosa et al., 2005; ATSDR, 2007).

Chronic Pb poisoning, either from acute exposure or bone resorption (endogenous contamination), may result in a variety of symptoms depending on exposure levels. Acute exposures may produce colic, insomnia, shock, severe anaemia, nervousness, kidney damage and brain damage (Godish, 2003). These symptoms, that depend on the exposure level, can occur gradually. In a continuous exposure scenario, at B-Pb levels of 40$\mu g.dL^{-1}$, it may be observed a reduction of the sensory motor reaction time in male lead workers and some disturbance of

cognitive function. At over 50μg.dL^{-1}, visual/motor performance, memory, attention and verbal comprehension can be observed as subtle changes in neuropsychological function (Hernberg, 2000; Gidlow, 2004). Persistent exposures around 60μg.dL^{-1} B-Pb, can produce symptoms at the peripheral nervous system level, creating a reduced nerve conduction velocity and reduced dermal sensibility. If the neuropathy is severe the lesion may be permanent (Järup, 2003). For exposed women high levels of exposure potentially increase the risk of spontaneous abortion and even at low levels Pb exposure has been associated, not only with this outcome, but also with low pre-natal weights. In males, it has been associated with spermatogenesis related issues, causing low sperm counts, low motility and congenital malformations in offspring, but these latter results still present some inconsistencies, due to several confounders that can have a potential impact on the male reproductive ability (Gidlow, 2004; Bellinger, 2005). Lead poisoning, or saturnism, also produces visible signs. The classical picture includes a dark blue lead sulphide line at the gingival margin, also called the Burton line. In less serious cases, the most obvious sign of lead poisoning is disturbance of haemoglobin synthesis, and long-term lead exposure may lead to anaemia (Järup, 2003; Pearce, 2007).

As afore mentioned, apart from the impact of metallic elements on human health, the relevance of its compounds and chemical species, the particle size has itself a strong impact on the organism and consequent health outcomes depend greatly on this factor. Finer particles have a higher probability of reaching deeper regions of the lungs (respiratory bronchioles and alveoli), where they will remain until air is completely exchanged in the subsequent inspiratory/expiratory cycles, facilitating absorption and diffusion through the gas-capillary barrier and eventually cause adverse health effects (Beckett et al., 2007). Growing evidences link exposure to fine particles and hospital admissions for respiratory and cardiovascular diseases (Zanobetti et al., 2009; Pope et al., 2008). Earlier data suggested that rises of 10 μg.m^{-3} in $PM_{2.5}$ are accompanied by an increase in relative mortality risk of about 4%, including elevated risks from both cardiopulmonary mortality (6%) and lung cancer mortality (8%) (Pope et al., 2002).

In occupational settings, apart from the hazardous outcomes in the target organ(s), it is important to acknowledge the existence of diseases resulting from the contact of the toxic agent with the organ in the "front line" of the subjects' exposure – the respiratory system. Diseases such as chronic bronchitis, pulmonary emphysema, bronchial asthma or infections that frequently occur sooner than the episodes of chronic toxicity (Godish, 2003).

1.3 Exhaled Breath Condensate (EBC)

Exhaled breath condensate is assumed to reflect the composition of the airways' lining fluid (Knowles et al., 1997). The capability for non-invasive and easy access to the respiratory airways, contrasting with sputum induction or brochoalveolar lavage methods, represents a unique potential of EBC to assess lung exposure to pollutants. Through EBC it will be possible to quantify both the indicators of exposure and response, as well as markers of pathology (Effros et al., 2004; Corradi and Mutti, 2005; Montuschi, 2007). The lungs, which are responsible for gas-blood exchange, are directly exposed to airborne contaminants. In occupational settings where inhalation is the main route of intake of airborne toxicants, EBC opens a promising window to assess biomarkers of exposure for the organ of direct contact with the toxicant. Currently one of the problems associated with the use of EBC is the lack of standardization of its collection and analysis. Guidelines for EBC collection and measurement of response biomarkers have been proposed, although, similar directives for the analysis of metals and electrolytes in EBC have not yet been created (Horváth et al., 2005; Grob et al., 2008). The standardization of these procedures needs to be included in the first line of action for the use of EBC in order to assure that results are representative and that the methodology yields comparable results among the scientific community.

1.3.1 *Exhaled Breath Characteristics*

Exhaled breath consists of aerosols formed in the airways. During the breathing cycle the flux of air in and out of the lungs provides enough energy to generate lining fluid aerosol particles (Papineni and Rosenthal, 1997). It is mainly constituted by water vapour containing non-volatile compounds like cytokines, lipids, surfactant, ions, oxidation products, and adenosine, histamine, acetylcholine, and serotonin. In addition, EBC traps potentially volatile water-soluble compounds, including ammonia, hydrogen peroxide, and ethanol, and other volatile organic compounds. Also, EBC has readily measurable pH (reviewed in Hunt, 2002). The airway lining fluid may contain two kinds of components in terms of origin: those that enter the respiratory systems through inhalation, from the environment (exogenous); and those generated in the respiratory tissues and in the body, which diffuse through the alveoli and capillary membranes (endogenous) (Cao and Duan, 2006). Several endogenous compounds such as, H_2O_2, NO, CO, NH_4^+, isoprostanes, cytokines, acetone, have been reported to increase in pathological conditions and/or inflammatory processes. Many of these indicators have been successfully used as biomarkers in asthma, chronic obstructive pulmonary disease

(COPD), cystic fibrosis and tumours. Additionally, these biomarkers, may provide information about the disease mechanisms (Kharitonov 2001; Hunt, 2002; Goldoni et al., 2004; Cao and Duan, 2006; Gergelova et al., 2008; Chan et al., 2009). Henceforth, EBC becomes an attractive matrix and an interesting non-invasive tool for human biomonitoring.

1.3.2 *Sample collection equipment*

The collection of EBC relies on the cooling of the exhaled breath under conditions of tidal breathing that promotes the formation of droplets and allows the collection of a liquid sample (Hunt, 2002; Effros et al., 2004). For this purpose there are a variety of devices that fundamentally differ in efficiency. However, it must be also chosen according to the objective. The following aspects determine the major differences between the devices: i) materials used, depending if the routine's purpose is the assessment of trace metals, some may have materials that can contaminate the sample or are not well isolated from the surrounding ambient air; ii) volume of EBC collected; iii) and the circuit of the breathing apparatus that should promote the less contact possible between the exhaled and inhaled air.

Among several devices there are the commercially available EcoScreen (Jaeger, Germany) and R-tube (Respiratory Research Inc., Charlottesville, USA), the most commonly used, while some laboratories have used their own custom-made devices, like TURBODECCS (Italchill, Italy), which is now also commercialized. For the present work two EBC collecting apparatus were tested and only one selected to collect the samples. The one discarded, the TURBODECCS, had the advantage of being portable and, thus, greatly facilitating its transport. However, besides being unable to prevent mixing the exhaled and inhaled air, because the breathing apparatus consisted of a tube that ended in a collection case, it had (without any solution available) inefficient valves that didn't prevent the entry of air from the surrounding environment. The issue is not only the contamination of the sample and losses, but also the difficulty on keeping the exhaled air cooled. With this equipment the average volume collected was of 1mL/15min. The equipment used in the present study was the EcoScreen. A review article by Grob et al. (2008) identified the EcoScreen as the most popular EBC collection device with 27 studies published, followed by the R-tube with 12 studies and other condensing systems with 11 studies. Its main features were the condensation region, which is electrically cooled at -30 °C guaranteeing the aggregation of the droplets of the exhaled air and the two unidirectional valves that prevent inhaled and exhaled air from mixing in the collection tube, forming a closed circuit, and a saliva trap. During collection, a nose clip should be used to prevent air intake through the nostrils, thus maximizing the collection of exhaled air in the condensate (Figure 1). The average EBC volume collected was of 2mL for a collection time of 15min.

Since the purpose of the analysis was the determination of trace elements, all parts used for the breathing and sample collection were decontaminated in a solution of 50% HNO_3, before being thoroughly washed with ultrapure water at 18MΩ.cm (Milli-Q Element®, Millipore Corp., MA). This treatment implies that the materials must resist such procedures and, the non-disposable parts of EcoScreen are Teflon made.

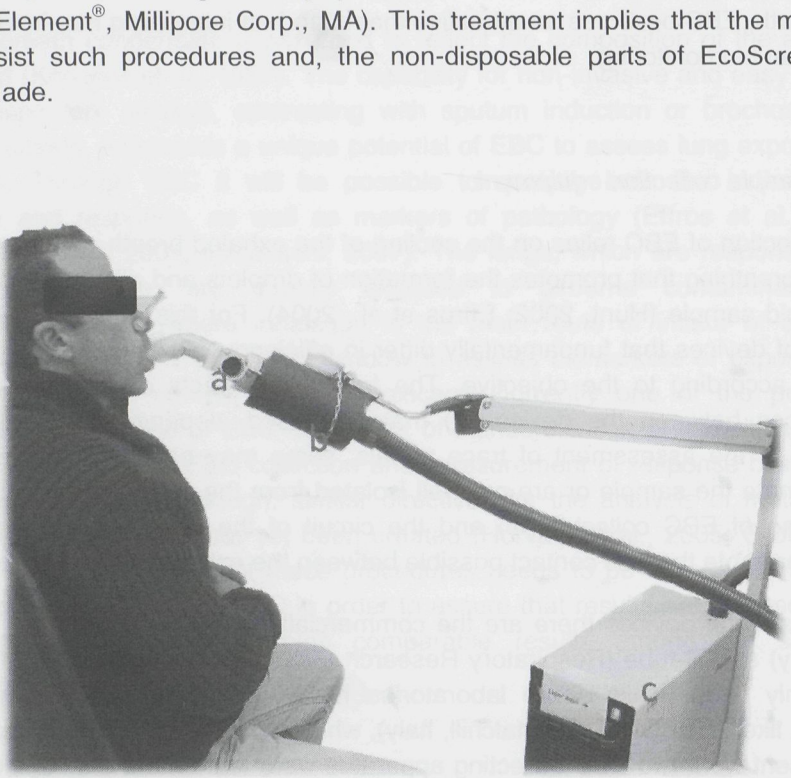

Figure 1 – Sample collection, using EcoScreen. (a) – breathing apparatus; (b) – cooling condenser; (c) – electric refrigerator.

1.3.3 *Trace metals in EBC*

An increasing number of biomarkers have been identified in EBC since the beginning of the use of breath analysis in occupational medicine, in the 1930s (Amorim and Cardeal, 2007). Until recently, the EBC was used mainly in clinical assessments of pulmonary pathobiology and physiological processes, as a promising alternative to invasive methods (Antczak and Gorski, 2002; Lemière, 2002; Chan et al., 2009; de Gennaro et al., 2010). Despite its early start, the use of EBC in occupational assessments has been considerably less recurrent. Few studies focusing exposure to metals have been published so far and even less studies report on the analysis of the metallic contents as a measurement of intake, revealing this a research field still unexplored. The published studies based on volunteers from the metal processing industry revealed that EBC could be a

promising tool to assess exposure to metal dust, gases and fumes. In a study carried out with chromium-plating workers it was demonstrated the adequacy of EBC matrix to investigate chromium (Cr) (Caglieri et al., 2006). Goldoni et al. (2004) reported on increased contents of tungsten (W) and cobalt (Co) in EBC of exposed workers, suggesting that the variations of these specific elements may express recent exposure. More recently, Hoffmeyer et al., (2011) evaluated the impact of different patterns according to the exposure conditions through the quantification of iron (Fe), nickel (Ni) and Cr in EBC of subjects of the welding industry.

Since the beginning of the use of breath analysis, the major limitation to its scientific advances was the collection and analysis techniques. Given its high water content, the current limitation of EBC measurements is the low concentration of many biomarkers so that their measurement is limited by the sensitivity of assays (Effros et al., 2004; Horvath et al., 2005). One additional drawback regarding EBC is that it's mass limited. Commonly, EBC sampling collects, on average, no more than 1 or 2 mL until it starts to become a discomfort to the donor and time consuming. For trace metal analysis, these limitations have been overcome with the development of multi-elemental techniques with very low detection limits. Inductively Coupled Plasma Mass Spectrometry (ICP-MS) has steadily gained ground to other techniques, such as Atomic Absorption or ICP-AES. The ICP-MS suitability to overcome all these difficulties of EBC analysis, relies not only on its multi-elemental analysis, but also due to its improved detection limits (in the order of magnitude of pg – 10^{-12} g) and its requirement for very low sample volumes, making it a suitable technique for trace metal quantification in exhaled breath.

1.4 Scope

Despite the technological requirements, imposed by safety regulations, that guarantee an improvement in indoor industrial air quality, workers continue to be excessively exposed to metals in their work environment. This occurs either due to the ineffectiveness or inexistence of equipment or negligence of the subject himself. Nevertheless, biomonitoring is an important way of assessing and controlling this exposure. There are several elements, usually airborne particulate, present at the industrial working sites, that are known to present a risk to human health, such as Lead (Pb), Arsenic (As), Chromium (Cr), Cobalt (Co), Nickel (Ni), Copper (Cu), Mercury (Hg), Antimony (Sb), Zinc (Zn), Manganese (Mn), Beryllium (Be), etc. There several ways of assessing elemental concentrations in the human body, but they are not evenly disseminated along the organs, so the usage of different samples/tissues should produce different results. Body fluids (urine, blood, milk, sweat or saliva) reflect short-term exposure, while soft and hard tissues (liver,

kidney, placenta, adipose tissue, bones, teeth, hair and nails) are good/better indicators of long-term exposure (Iyengar et al., 1998; Petersen et al., 2000; Apostoli 2002; Nordberg et al., 2007). One of the most common contaminants in occupational settings is Pb and until now the most used biomarker to assess its exposure, included in national regulations is Pb in whole blood (B-Pb). However, and despite having a semi-invasive method of collection has some disadvantages, many resembling other biomarkers of exposure used for other elements: Pb had already a major physiological interaction; it's a relatively conservative measure; it only provides information on the Pb in the systemic circulation; it is susceptible to the influence of several confounders; it is difficult to identify the source of contamination (endogenous or exogenous); and requires sample preparation and a high manipulation (Barbosa et al., 2005). In this scenario, EBC emerges as a potential tool for the assessment of occupational exposure to metals, with the prospective to overcome several of the mentioned limitations of other bioindicators as it can provide information on the deposited material in the lung lining fluid through inhalation, the main route of intake the workplace. Moreover, EBC is a non-invasive method of sampling the airways that can be repeated easily and is acceptable to patients. This is an advantageous feature for the monitorization on occupational settings, allowing continuous measurements of a subject whenever necessary.

1.5 Objectives and thesis outline

The present study aims to explore EBC for its capability to be used as a routine-based bioindicator for metal exposure in occupational settings. Therefore, biomonitoring using biomarkers in EBC will be complemented with the assessment of environmental conditions in the industry, in terms of elemental characterization. Thus, enabling the establishment of a relation between exposure and biomarkers in EBC. To comply with the objective, the work was conducted in order to meet a series of individual goals, dividing the central objective into:

- The characterization of the industrial environment in terms of airborne elemental concentrations, to determine levels of exposure in metal processing industries;
- The determination of elemental concentrations in the EBC of workers from those industries and the relationship between the measured concentrations and magnitude of exposure;
- The validation of the developed procedures involving sample collection, storage and analysis of EBC through a process of quality control;

- The potential of EBC-Pb as a biomarker of exposure and the advantages of EBC when compared to Blood-Pb, which is the current biomarker used for monitoring lead occupational exposures.

The process involved the collaboration of two industries that process lead, and have distinct environmental settings, and a non-exposed group, working at offices of the same geographical area. The search for these goals is described in the following chapters.

Chapter 2 describes the preliminary work that identified levels of exposure in an industrial site, by determining metal concentrations in the air particulate matter, and that confirmed the presence of particles in the exhaled breath condensate and characterized them as to its elemental composition and size, in a nuclear microprobe analysis of the EBC. This work demonstrated that EBC reflects exposure in terms of inhaled particulate matter and, particularly the knowledge on the EBC matrix's constituents, enabled to delineate methodological procedures suitable for occupational assessments.

Chapters 3 and 4 deal with the critical aspects concerning the validation of EBC as a biomarker of exposure to metals for the respiratory system. The chapters are based on a pilot study that was performed in groups of metal dust-exposed workers, using Pb processing industry as models, and a group of non-exposed individuals working in offices, both followed throughout a period of time. Chapter 3 develops procedures for the collection and analysis of EBC and studies the analytical figures of merit such as, trueness and reproducibility for different metals present in the work environment. Chapter 4 intended to understand the EBC matrix in terms of analysis and estimated the overall uncertainty, using two multi-elemental techniques, with different physical backgrounds (ICP-MS and TXRF). This allowed demonstrating that it is possible to discriminate between groups of individuals exposed to different levels of contaminants, using EBC, and is proof of EBC's potential in establishing new biomarkers of exposure to metals in an occupational scenario. This preliminary work created the bases for the following research on the assessment of EBC as a bioindicator of exposure.

Chapter 5 represents the first approach on the comparison between airborne particulate matter and the contents of EBC. On this cross-sectional study, using three levels of exposure to metallic elements, the work environment was characterized in terms of elemental composition, concentration for different particles size fractions and each factory's fingerprint was described. For each group of workers it was quantified the elemental composition of EBC and its contents were investigated in order to identify its representativeness in terms of intake, which was enabled by the existence of different work tasks in the same industry. Apart from Pb, the main contaminant of these industrial sites, four other metals were chosen, according to its presence in the work environment and the

high risk that it can represent to human health. This chapter evaluated the line of action that should be undertaken for a correct exposure assessment.

Chapter 6 investigates whether the determination of the parent toxicant in EBC (of the element itself, instead of its metabolites – EBC-Pb) is a suitable biomarker of exposure to lead in occupational exposure. It describes EBC-Pb in terms of context when compared to the common biomarker for exposure to lead, B-Pb, thus, discussing the applicability of EBC as a routine-based bioindicator, and the advantages of this non-invasive tool.

Finally, in Chapter 7, there is a final overview of the thesis and of how the objectives of the work were fulfilled, with comments on future prospects.

1.6 References

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Chapter 2

Particulate matter in Exhaled Breath Condensate: a promising indicator of human exposure

This chapter is a version based on the following publications: Almeida SM, Félix PM, Franco C, Freitas MC, Barreiros A, Alves L, Garcia SM, Pinheiro T, 2010. Using the exhaled breath condensate as a tool for non-invasive evaluation of pollutant exposure. *Int. J. Environment and Health*, 4: 293-304 and Pinheiro T, Barreiros MA, Alves LC, Félix PM, Franco C, Sousa J, Almeida SM, 2011. Particulate matter in Exhaled Breath Condensate: a promising indicator of environmental conditions. *Nucl. Instrum. Methods Phys. Res. B*, 269: 2404-2408.

Abstract

Assessing the retention of aerosol particles in the human lung, one of the most important pathways of absorption, is a demanding issue. At present, there is no direct biomarker of exposure for the respiratory system. The collection of exhaled breath condensate (EBC) constitutes a new non-invasive method for sampling from the lung. However, the heterogeneity of the sample due to particulate matter suspended in the condensed phase may influence the quality of analytical results in occupational assessments.

The main objective of the study was to identify levels of exposure in the workplace by determining metal concentrations in the air particulate matter and to confirm the presence of particles in the condensate, as well as to investigate how large the particles in suspension could be and to determine their elemental contents relative to those of EBC matrix.

This paper reports on preliminary nuclear microprobe data of particulate matter in EBC. The sizes and the elemental contents of particles suspended in EBC of workers of a lead processing industry and in EBC of non-exposed individuals were evaluated. Results demonstrated that EBC of workers contain large aerosol particles isolated and in agglomerates, contrasting with non-exposed individuals. The EBC particles contained high concentrations of several elements, including Cl,

Ca, Zn and Pb, that are elements associated to the production process, which were found in the air particulate matter at the workplace. These elements were also present in the EBC matrix although in much lower levels, suggesting that a fraction of the inhaled particulate matter was solubilised or their size-ranges were below the nuclear microprobe resolution. Therefore, the morphological characterization of individual particles achieved with nuclear microprobe techniques helped describing EBC constituents in detail, to comprehend their origin and enabled to delineate methodological procedures that can be recommended in occupational assessments. These aspects are critical to the validation of EBC as a biomarker of exposure to metals for the respiratory system.

2.1 Introduction

Assessing the retention of aerosol particles in the human lung, one of the most important pathways of absorption, is a demanding issue. The damage caused by these pollutants can be insidious and difficult to demonstrate.

Standard methods are often indirect or invasive, which limits their applicability to monitoring exposed populations. At present, there is no direct biomarker of exposure for the respiratory system. The collection of exhaled breath condensate (EBC) constitutes a new non-invasive method for sampling from the lung (Sidorenko et al., 1980; Kharitonov and Barnes, 2001; Hunt, 2002; Hoffmeyer et al., 2007). The method is easy and quickly collected, and does not induce an inflammatory reaction itself, what enables its use for repeated measurements (Rosias et al., 2010). The EBC is an aqueous suspension containing several ions, molecules and proteins (Hunt, 2002; Grob et al., 2008). Like blood and urine EBC is a matrix where a variety of ions, molecules, peptides and other constituents can be measured simultaneously. The measurement of some of these constituents proved to be useful in clinical assessments (Kharitonov and Barnes, 2001; Hunt, 2002; Hoffmeyer et al., 2007; Rosias et al., 2010).

EBC may also give information on the fraction of aerosol particles that are inhaled and interact with the pulmonary epithelial lining fluid and cells and therefore, about dose at the target organ level and airway susceptibility to inhaled metals and pollutants (Ädelroth et al., 2006; Caglieri et al., 2006; Do et al., 2008; Broding et al., 2009). However, it has to be demonstrated that EBC reflect environmental conditions and that methodologies developed are adequate (Horváth et al., 2005). This aspect is relevant as despite the multiple applications of EBC reported in literature, EBC chemical and biological characteristics are not well depicted and there are no standardised methods to analyse metabolites in EBC. Realistic EBC samples are usually collected during 15 min what corresponds to a volume of approximately 2 mL. Besides the small sample volume obtained, EBC is a diluted sample. Elemental concentrations are low (below $\mu g.L^{-1}$ level), therefore particulate matter suspended in the condensed phase may strongly influence the quality of quantitative results.

In this context, it was important to identify levels of exposure of workers to airborne particulate matter (APM) in the metal processing industry and to confirm, not only the presence of particulate matter in the condensate, but to investigate how large the particles in suspension could be and if possible to determine their composition. These aspects are of the utmost importance to characterize morphologically EBC

and to determine appropriate sample preparation for elemental determination purposes in bulk samples that can be recommended in occupational assessments.

For this purpose, nuclear microprobe techniques were used to investigate whether large size-fraction particulate matter is present in EBC and INAA and PIXE techniques for the quantification of APM (Almeida et al., 2010a). Results will help delineate appropriate methodologies for elemental determination in EBC that can be further recommended in exposure studies. Workers from a lead processing industry and a group of non-exposed individuals have been engaged in this study.

2.2 Materials and Methods

2.2.1 Study groups

The industrial site selected, is a smelting facility that recycles metal alloys, but essentially lead (Pb). The plant operates 24 h a day, on 8 h shifts for 5 days a week, with 2 intercalary resting days. Workers included in the study have been working for more than five years in the industry. They work in a confined area where smelting continuously operate, alternating between the different tasks that are performed, being therefore, identically exposed during the work shift. The company's policy imposes the use of protective and filtered mask, as well as appropriate suit, shoes and gloves at all times. Also, strict hygiene practices had to be followed by workers, on work pauses and on the end of the shift, such as shower, teeth brushing and change of clothes and shoes.

For the sampling of APM, two working places were selected: the inside of the smelting industry from which the subjects will be assessed and the offices of a Research Institute (both located in the same urban-industrialised area and characterized by significant different concentrations of heavy metals in the indoor air).

For the EBC sampling, eleven workers (mean age 41 [33-48]) exposed to Pb fumes and particles were enrolled in this study. Also, a reference group consisting of ten non-exposed (CTR) individuals (mean age 37 [26-47]), working in the same offices from where APM sampling was carried out, was also established for comparison purposes. All the individuals recruited to this study, work and live in the same geographic region of Lisbon in Portugal and were informed of the objectives of the study, giving their consent in study involvement.

2.2.2 Exhaled Breath Condensate – collection and sample preparation

The EBC was collected using portable equipment (EcoScreen, Viasys Healthcare GmbH), divided in aliquots, and stored at -80 °C. All the containers were in polypropylene and were thoroughly cleaned with suprapur HNO_3 (20 % v/v) before use. For particle morphological characterization and elemental analysis, an aliquot of 50 μL of the collected EBC was deposited on a polycarbonate thin foil, dried in vacuum and directly analyzed using nuclear microprobe techniques.

2.2.3 Analytical method for Exhaled Breath Condensate

Elemental maps were produced at the ITN Nuclear Microprobe (Alves et al., 2000). A 2.0 MeV proton beam is focused to diameters of 1 μm to 3 μm and a sample area scanned by means of a beam deflecting system. Typical currents of 100 pA were used. Simultaneous STIM, PIXE and RBS analyses were carried out to enable enhancement of sample's morphological features and quantitative micro-PIXE analysis, as described elsewhere (Aguer et al., 2005; Ynsa et al., 2006; Veríssimo et al., 2007). Briefly, PIXE, RBS and STIM spectra were collected for specific features identified in maps (point analysis) or data was extracted from regions of interest in order to obtain quantitative elemental data. The procedure is described elsewhere in detail (Ynsa et al., 2006; Veríssimo et al., 2007). Quantitative results for the elements detected with PIXE were obtained using mass density normalization from the RBS spectra using OMDAQ and DAN32 software (Grime and Dawson, 1995).

2.2.4 Airborne Particulate Matter – Sampling

APM was sampled inside the factory, inside the offices and in the outdoor environment near these two sites. In each working place (factory and offices), particles were collected with four low volume Gent samplers (Maenhaut, 1992) working in parallel. In the environment only one Gent sampler was used.

Gent samplers were equipped with a PM_{10} pre-impactor stage and with a Stacked Filter Unit (SFU). The SFU carried, in two different stages, two 47 mm Nuclepore polycarbonate filters. Air was sampled at a rate of 15-16 $L.min^{-1}$, which allowed the collection of particles with aerodynamic diameter (AD) between 2.5 and 10 μm in the first stage and particles with AD < 2.5 μm in the second stage. Data regarding sampling parameters are resumed in Table 1.

Table 1 - Sampling parameters: sampling time and sampled $PM_{2.5}$ and $PM_{2.5-10}$ mass, at a rate flow of 16L.min^{-1}. Average values and range (when applied). Sampling time was optimized in order to collect enough mass for the chemical analysis and to prevent the clogging of the filters.

	Sampling time (hour/sampler)	$PM_{2.5}$ Mass (μg)	$PM_{2.5-10}$ Mass (μg)
Offices	30	300 [250-370]	380 [350-400]
Environment	24	340 [15-630]	410 [92-2000]
Industry	1	440 [280-590]	1100 [400-1900]

2.2.5 Airborne Particulate Matter – gravimetric and chemical analysis

The filter loads were measured by gravimetry in a controlled clean room (ISO7). Nuclepore filters were weighted on a semi-micro balance. Filter mass before and after sampling was obtained as the average of three measurements, when observed variations were less than 5%.

Each filter was divided in four parts. For chemical identification one quarter was analyzed by Particle Induced X-Ray Emission (PIXE) and another quarter by Instrumental Neutron Activation Analysis (INAA). The remaining were stored for future analyses.

PIXE (Johansson et al., 1995) analysis was carried out at a Van de Graaff accelerator, in vacuum and two X-ray spectra were taken for each of the samples; one with a 1.2MeV proton beam and no absorber in front of the Si(Li) detector for low energy X-ray elements and another with a 2.4MeV proton beam and a 250 μm Mylar® filter to detect elements with atomic number higher than 20. The beam area at the target was 20 mm^2.

To perform the quality control of the PIXE procedure, previous to the chemical analysis, NIST Standard Reference Material 2783 (Air Particulate on Filter Media) was analysed and tests of reproducibility within the filters and between filters were carried out, using parallel sampling with two similar sampling units and measuring the particle species by PIXE. Blank filters for each granulometry were measured and treated the same way as regular samples (Almeida et al., 2003a).

For INAA (De Soete et al., 1972), the filter quarter was rolled up and put into a thin foil of aluminium and irradiated for 5-h at a thermal neutron flux of 1.03×10^{13} cm^{-2}s^{-1} in the Portuguese Research Reactor. After irradiation the sample was removed from the aluminium foil and transferred to a polyethylene container. For each irradiated sample, two gamma spectra were measured with a hyperpure

germanium detector: one spectrum 3 days after the irradiation and the other one after 4 weeks. The distance between the sample and detector was the smallest possible that tried to guarantee a dead time lower than 12%, however, typically 66.6mm for the first measurement (3 days after irradiation) and 11.6mm for the second (4 weeks after irradiation). The k_0-INAA method (De Corte, 1987) was used and 0.1% Au–Al discs were co-irradiated as comparators.

Blank Nuclepore filters were treated the same way as regular samples. All measured species were very homogeneously distributed; therefore concentrations were corrected by subtracting the filter blank contents.

In order to perform the quality control of the INAA process, each group of samples was irradiated with the National Institute of Standards and Technology (NIST) Standard Reference Material 1633a - Coal Fly Ash. Previous to the sampling campaign, tests of reproducibility within the filters and between filters were taken, using parallel sampling with two similar sampling units and measuring the particle species by INAA and PIXE. Results show that reproducibility between filters falls within 5-15%, providing strong support for the validity of the analytical techniques. The details of sampling and analytical control tests are given in Almeida et al. (2003a, 2003b). The trueness of analytical methods was evaluated with the NIST Standard Reference Material 2783 (Air Particulate on Filter Media), revealing results with an agreement of ±10% (Almeida et al., 2006).

2.2.6 Statistical Analysis

Data from particulate matter (PM) in the filters were graphically represented using mean concentration values and error, using Origin v7.5 (OriginLab).

EBC data was summarized using medians, minimum and maximum values. Differences between independent groups were calculated using Mann-Whitney and Kolmogorov-Smirnov non-parametric tests. The elemental concentrations were examined by cluster analysis to see if individual cases can be formed into any natural system of groups (Kirkwood and Sterne, 2005). Cluster analysis was carried out on normalized variables (z-score ≡ μ=0; SD=1) using between-groups linkage method, squared euclidean distance procedure to measure the similarities between items and results represented graphically by a dendrogram (Kirkwood and Sterne, 2005). The dendrogram is a visual representation of the steps in a hierarchical clustering solution that shows the clusters being combined and the values of the distance coefficients rescaled to numbers between 0 and 25, preserving the ratio of the distances between steps. Connected vertical lines

designate joined cases. SPSS Inc. statistical package (PAWS 18.0, IBM, SPSS Statistics) was used in the analysis.

2.3 Results and discussion

2.3.1 Airborne Particulate Matter

Figure 1 presents the total APM mass concentration measured inside the plant, inside the offices and in the local environment. It is clear that the average concentration in the industry was significantly higher than in the offices and in the local outdoor. PM_{10} and $PM_{2.5}$ average concentrations measured in the industry were 1400 $\mu g.m^{-3}$ and 420 $\mu g.m^{-3}$, respectively. $PM_{2.5}$ and $PM_{2.5-10}$ levels measured in the offices and in the environment didn't present significant differences. This fact was expected because offices have natural ventilation made by the open windows.

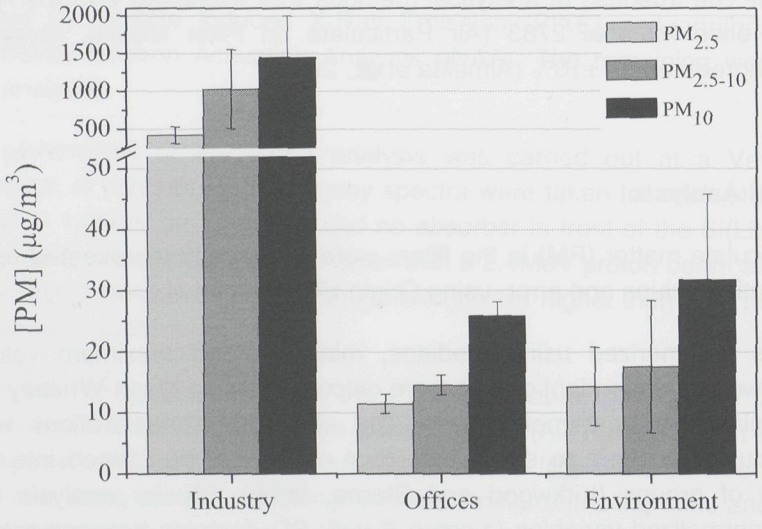

Figure 1 – Average (±SE) PM mass concentration in the industry, offices and environment (values in $\mu g.m^{-3}$).

Chemical analysis was performed in the particles collected in the industry, in the offices and in the environment. Figure 2 presents the element concentration for

$PM_{2.5}$ and $PM_{2.5-10}$ measured in these 3 sampling points. The elements which occurred with higher concentrations (> 10000 $ng.m^{-3}$) in the industry were Pb, Sb, Cl and Na, although several other elements are in much higher concentrations than those of offices and environment (e.g. Al, As, Ca, Fe, K, Si, Zn). These elements are associated with the process and materials used in the production.

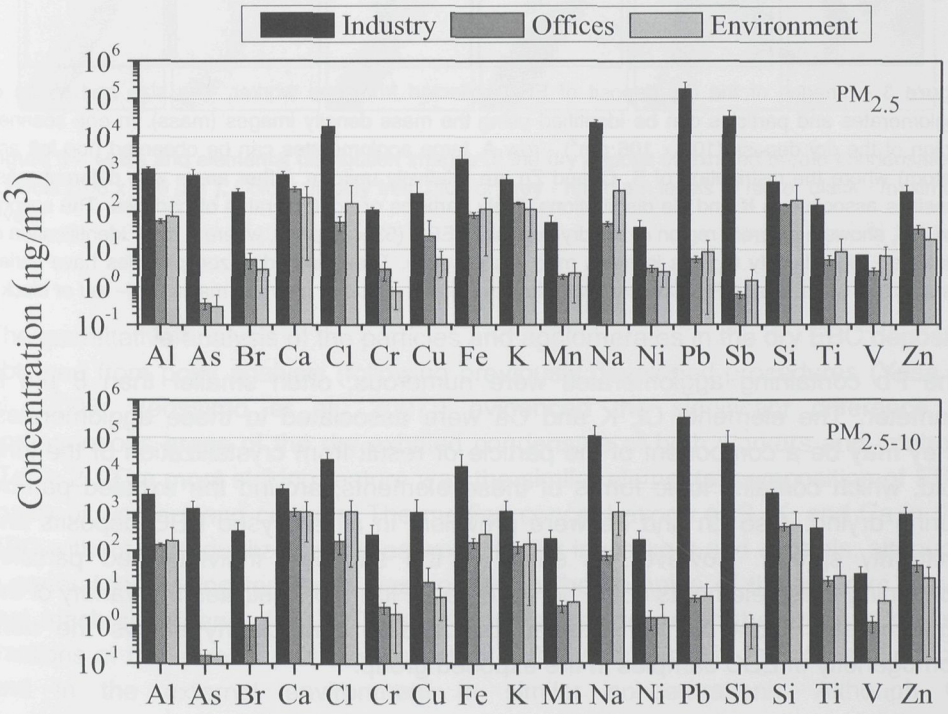

Figure 2 – Average (±SE) element mass concentration in PM sampled in the industry, offices and environment (values in $ng.m^{-3}$).

2.3.2 Exhaled Breath Condensate

Isolated particles (detectable masses determined/mapped above the resolution of the technique, ≈1μm) and agglomerates of particles were visualized in the dried deposit of EBC collected from workers. The size and shape of these particle agglomerates varied as well as elemental composition. As can be observed in Figure 3, transmission images obtained with STIM helped identifying agglomerates and particle boundaries, as density variations are more perceptive than elemental distributions (Aguer et al., 2005; Veríssimo et al., 2007).

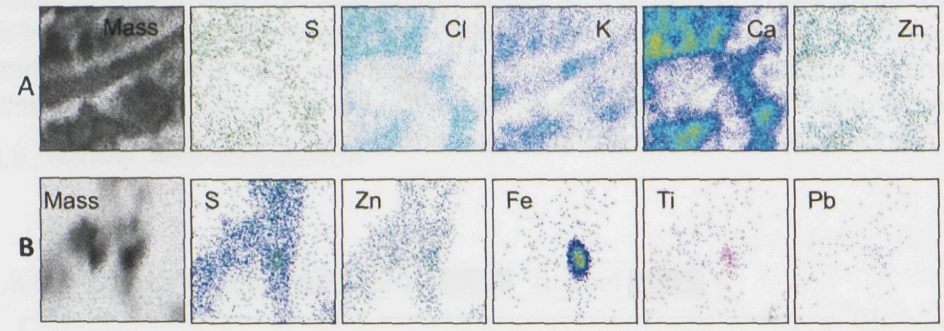

Figure 3 – Images of the dry deposit of EBC collected from one worker. The size and shape of agglomerates and particles can be identified using the mass density images (mass). In one scanned region of the dry deposit (106 x 106 μm^2) - row A, large agglomerates can be observed (top left and bottom) where the distribution of S, Cl and Zn are relatively uniform. Other areas with different mass densities associate to K and Ca distributions, likely particles or agglomerates of particles. The second row – B, shows a different region of the dry deposit of EBC (53 x 53 μm^2), where a clear identification of particles - high density regions in mass map - is possible. These individualized particles have varied composition of Fe, Ti and Pb. Content levels from minimum – blue or grey - to maximum – red or black.

The Pb containing agglomerates were numerous, often smaller than 8 μm in diameter. The elements Cl, K and Ca were associated to these agglomerates. They may be a component of the particle or result from crystallization of the lung fluid, which contains ionic forms of these elements, around the exhaled particle during drying. Also Zn and Fe were prevalent in all analysed EBC deposits and uniformly spread. However, in some of the samples, individualized particles containing these elements could be observed. Figure 4 illustrates the variety of the agglomerates found in the exhaled condensate and clearly shows the non-homogeneity of EBC samples in the exposed group.

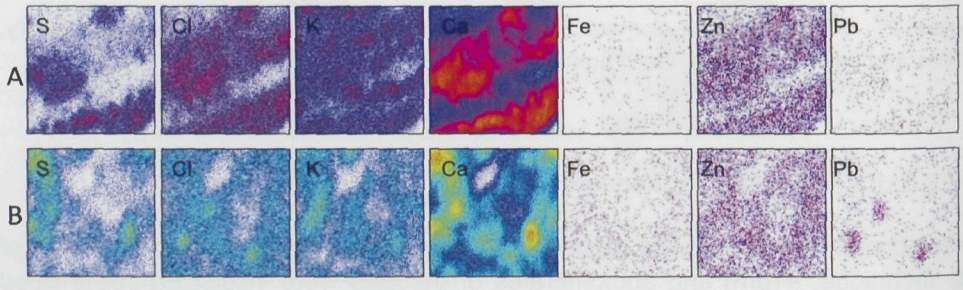

Figure 4 – Images of particles and agglomerates in small scanned areas of EBC dry deposits (53 x 53 μm^2). Rows A and B correspond to different aliquots from one EBC sample obtained from one worker. The elemental distributions clearly show their varied composition. In some regions the distributions of Fe, Zn and Pb are regular (row A), while in other regions these elements are strongly associated to particles (row B) with various shapes and dimensions. Content levels from minimum - blue or grey to maximum – red or black.

Opposite, EBC of non-exposed individuals (controls) presented a uniform distribution of S, Cl, K, Ca, Fe and Zn. No large particles or agglomerates could be identified (Figure 5).

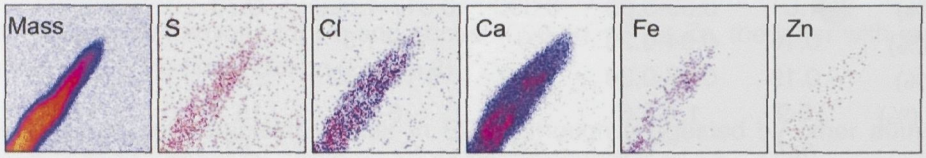

Figure 5 – Mass and elemental distribution images of the dry deposit of exhaled breath condensate of controls (106 x 106 μm^2). Colour display from blue or grey - minimum levels to red or black - maximum levels.

The quantitative analysis of the particles and agglomerates in the dry EBC deposits obtained from point analysis (following previously developed procedures (Ynsa et al., 2006; Veríssimo et al., 2007)) evidenced the significant difference to homogeneous areas of the dry exhaled condensate of both workers and controls (Table 2). The most striking feature was the similar elemental composition of EBC matrix of workers and controls. The median concentrations of S, K, and Ca in the EBC without individualized particles were similar in workers and controls, although a general increasing tendency was verified in the samples of the workers group that reach significance for Cl. These elements are present in the fine and coarse fractions of the aerosol particles collected in the workplace, in the offices (indoor) and in the external environment in similar concentrations. Although Cl concentration at the workplace was remarkably higher (above 10000 $ng.m^{-3}$) (Figure 2) as it is associated with the process and materials used in the production what may justify the increased level in the EBC of workers. Nevertheless, the Cl, K and Ca in EBC may also originate from respiratory fluids. Furthermore, the relatively steady levels of S in EBC deposits suggest an internal origin. Respiratory epithelium secretes surfactant proteins, which may contain S (Capote et al., 2003), to maintain the stability of pulmonary tissue by reducing the surface tension of fluids that coat the lung.

Therefore, the EBC matrix of both exposed and non-exposed individuals seems to reflect environmental characteristics and lung fluid constituents.

Table 2 – Median values of concentrations ($\mu g.g^{-1}$ except when indicated) obtained in the dry EBC deposit of non-exposed individuals (CTR) and of workers: EBC matrix without individualized particles (WithoutPtc) and with particles (Ptc). Minimum and maximum values are also indicated.

	CTR		***WithoutPtc***		***Ptc***	
	Median	*Min-Max.*	*Median*	*Min-Max.*	*Median*	*Min-Max.*
S (%)	1.01	0.03-3.05	0.093	0.017-1.628	0.70	0.09-3.82
Cl (%)	0.16	0.04-0.28	0.9 *	0.6-1.5	2.2 *†	0.2-9.1
K (%)	0.19	0.07-0.30	0.50	0.19-0.62	2.7 *†	1.0-6.2
Ca (%)	5.9	1.4-6.6	8	6-9	15 *†	11-18
Ti	*n.d.*		*n.d.*		81	52-208
Mn	*n.d.*		*n.d.*		85	25-355
Fe	330	10-670	64	38-126	254 †	129-1182
Cu	22	20-25	35	31-47	250 *†	147-739
Zn	312	63-448	1075	285-1272	16590 *†	10923-27680
Pb	*n.d.*		404	262-545	7380 †	1570-177624

* - significant difference to CTR $p<0.01$

† - significant difference to WPtc $p<0.01$

In controls Fe was only detected in 50% of the analysed regions, contrasting with Zn, which was always detected. As Fe levels in EBC were low, its variability may derive from high uncertainty in concentration determination. In the exposed group the EBC matrix had Fe always in detectable concentrations, increased Zn contents (marginally significant to controls, $p=0.06$) and Pb, which was not detectable in controls. Direct elemental analysis of the EBC samples previously carried out with Total Reflection X-Ray Fluorescence showed that Fe, Cu, Zn and Pb, among other elements, were detectable in the EBC of controls and workers and the differences between groups observed were consistent with the nuclear microprobe data reported in this paper: the average Pb concentrations in EBC of controls were very low when compared to workers (1.5 $ng.mL^{-1}$ in controls versus 23 $ng.mL^{-1}$ in workers); the Zn had the highest concentration level in EBC; and the largest variability was observed for Fe in both workers and controls (Almeida et al., 2010b). Therefore, the high Zn concentrations in EBC may reflect the solubility of this element in the condensed phase of the exhaled air and the Fe inconsistencies may reflect the non-homogeneous characteristics of EBC deriving from ultra-fine particles. At present the resolution range of the technique limits the identification of particle sizes below a few micrometers. Although, these ultra-fine particles cannot be visualized in the elemental distribution maps, the elemental concentrations obtained from selected regions of the dry deposit give information on their distribution.

The particle agglomerates had high and uneven concentrations of Cl, K, Ca, Fe, Zn and Pb and often associate other metals, such as Ti, Mn and Cu. (Table 2). The *increased elemental concentrations in particle agglomerates were highly significant* when compared to EBC matrix of both controls and workers. The high elemental contents measured in particles and agglomerates were consistent with the composition of the particulate matter collected in the workplace. The major constituents were Zn and Pb, which concentrations exceeded 10000 $ng.m^{-3}$ in both fine and coarse fractions for Pb and above 1000 $ng.m^{-3}$ for Zn, the latter with more *expression in the finer fraction* (Figure 2).

The presence of particulate matter in EBC although suggested by other authors (Caglieri et al., 2006; Broding et al., 2009) was never visualised. Fireman et al. (2008) reported data on particulate matter in sputum of welders. Scanning electron microscopy was used to identify isolated and agglomerates of particles in sputum. Their sizes ranged from sub-micrometer to 30 μm, what is consistent with our results.

Using a set of common variables, i.e., S, Cl, K, Ca, Fe and Zn, measured in most of the EBC samples, elemental concentrations could be classified in two main clusters that separate the EBC matrix of controls and regions without particles of workers EBC, from data on particles and agglomerates (Figure 6). These results confirm the similarity of the EBC matrices in the two groups and the diverse characteristics of particles in terms of elemental concentrations and distribution as can be observed in the maps of Figures 3, 4 and 5.

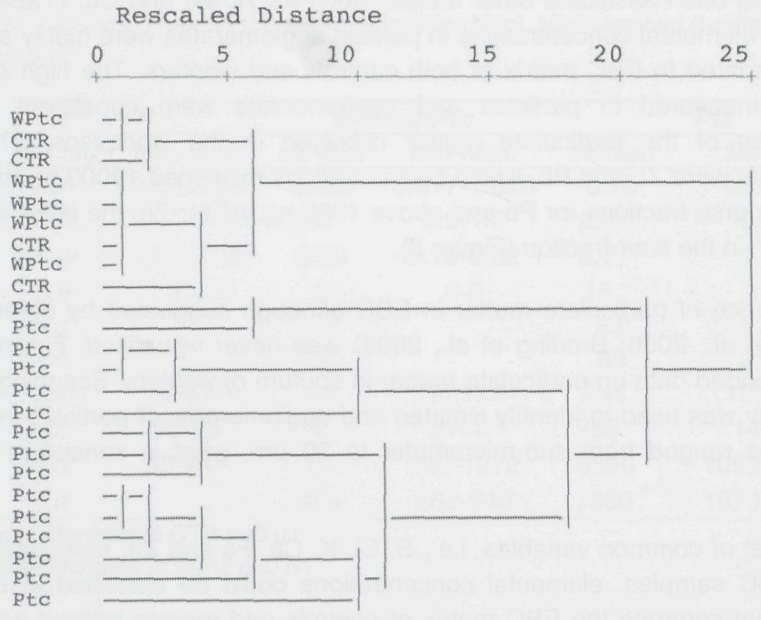

Figure 6 – Cluster analysis of EBC data from controls and from workers. The distances between the set of points considered are graphically represented by a dendrogram (details in Methods). Ctr – data corresponding to the EBC matrix of control samples; WPtc – data corresponding to EBC matrix without visible particles; Pct – data corresponding to individualised particles.

Results demonstrate that EBC contain respired aerosol particles and can reflect external environmental conditions. The more uniform distribution of metals in EBC matrix, even for Fe and Zn, suggest the association of these elements to fine and ultra-fine particulate matter (below the limit of spatial resolution of the technique) or a higher solubility of these elements in the condensed phase of exhaled breath. Likely, the presence of Pb in the matrix of EBC of workers may also reflect the fraction of very fine dust and/or fumes inhaled in the workplace.

Concerning Fe and Zn, despite their essentiality to biological systems and their unequivocal role in cells and tissues physiology, their release into lung fluids is very unlikely. These elements are always bound to proteins and confined in cells and specific body fluids. Therefore, the presence of elements in EBC, especially metals, may constitute a tag of the particulate matter that is dragged during inhalation into the lung.

2.4 Conclusions

This preliminary study demonstrated the levels of exposure of workers from the lead processing industry that were consistently reflected in the collected EBC. Furthermore, the heterogeneity of EBC was confirmed by nuclear microprobe techniques. EBC contained particles of different sizes, shapes and compositions, which may influence the quality of the results in bulk analysis. Therefore, in order to determine bulk elemental concentrations in EBC, granting reproducible and reliable results, sample homogenization, previous to analysis, is mandatory.

The use of nuclear microprobe allowed the visualization and identification of particulate matter in the EBC, the measurement of its elemental concentrations, as well as the elemental concentration of the matrix. These findings confirm that EBC contains inhaled particles and it could be regarded as a viable tool for occupational exposure assessment. Also, bearing this possibility in mind, these aspects are of the utmost relevance to describe EBC constituents in detail, to comprehend their origin, to help improving analytical methods as sample heterogeneity may hamper results quality and therefore to significantly contribute to the validation of EBC as a biomarker of exposure to metals for the respiratory system.

2.5 Acknowledgements

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Chapter 3

Biomarkers of exposure to metal dust in exhaled breath condensate: methodology optimization

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Abstract

In occupational assessments where workers are exposed to metal dust the liquid condensate of exhaled breath (EBC) may provide unique indication of pulmonary exposure. The main goal was to demonstrate the quality of EBC to biological monitoring human exposure. A pilot study was performed in a group of metal dust-exposed workers and a group of non-exposed individuals working in offices. Only metal dust-exposed workers were followed along the working week to determine best time of collection. Metal analyses were performed with ICP-MS. Analytical methodology was tested using an EBC sample pool for several occupationally exposed metals: potassium, chromium, manganese, copper, zinc, strontium, cadmium, antimony and lead. Metal contents in EBC of exposed workers were higher than controls at the beginning of the shift and remained augmented throughout the working week. The results obtained support the establishment of EBC as an indicator of pulmonary exposure to metals.

3.1 Introduction

Exhaled breath condensate (EBC) is a matrix in which numerous volatile and non-volatile substances can be detected, where biomarkers of effect (e.g., occupational assessments) and response (e.g., pulmonary pathophysiology assessments) have been described (Dwyer, 2004; Horváth et al., 2005; Conrad et al., 2007; Grob et al., 2008). EBC collection can be carried out easily and non-invasively by breathing into the condenser, using portable equipment in any out-setting, such as workplace, home, etc. As the procedure does not induce an inflammatory reaction it is especially suited for the sequential and longitudinal sampling of individuals at any age (Baraldi, 2003; Rosias et al., 2004; Epton et al., 2008).

The analysis of EBC might be extremely useful to biological monitoring. Most occupational approaches the exposure occurs by inhalation, therefore being the respiratory tract the primary organ of exposure for pollutants, such as metals. Blood and urine are the matrices currently used for the biological monitoring of workers exposed to metals. These two matrices do not provide direct information about lung tissue levels of metals, which may cause local damage and inflammation on the respiratory system. The exhaled air gives the exhaled dose which may reflect the lung dose responsible for eventual airway adverse effects.

EBC has been used in clinical studies, and several markers have been measured as indicators of pulmonary physiology and pathology (Antczak and Gorski, 2002; Lemière, 2002; Chan et al., 2008; de Gennaro et al., 2010). The use of EBC in occupational assessments has been used considerably less. Few studies focusing exposure to metals have been published so far. In chromium-plating workers EBC matrix was adequate to investigate chromium (Cr) exposure and oxidative stress markers (Caglieri et al., 2006). Goldoni et al. (2004) reported on increased contents of tungsten (W) and cobalt (Co) in EBC of exposed workers, suggesting that the measurement of these specific elements may quantify recent exposure.

The usefulness of EBC matrix in occupational exposure assessments and in clinical applications is not consensual (Hunt, 2002; Rosias et al., 2004; Sack et al., 2006; Broding et al., 2009). The main reason to the divergence of published results, as pointed out by several authors, may be the lack of standardization in collection and analysis of EBC (Horváth et al., 2005; Grob et al., 2008; Broding et al., 2009). Guidelines for EBC collection and measurement of response biomarkers (e.g. Horváth et al., 2005) were proposed. Similar directives for the analysis of metals in EBC have not yet been developed. The EBC matrix may be heterogeneous as the condensed droplets of lining fluid may contain miscellaneous particles in a variety of dimensions. These features may become relevant in occupational assessments, where workers are exposed to metal dust (Hlavay et

al., 1992; Almeida et al., 2010). Therefore, the representativeness of the sample will inevitably influence analytical results. A systematic study of the EBC matrix and of the analytical procedures should precede any occupational application. Validation of the methodology can provide confidence of results and indicate the validity of measurements. A reliable analysis of relevant individual metals in EBC is required to measure metal exposure, which can indicate potential adverse effects in the respiratory tract. These measurements will assist in the determination of maximum allowed exposures.

The main objectives of this study were to investigate the features of EBC matrix, to establish procedures for its collection and to study the analytical figures of merit such as, trueness and reproducibility for different metals useful in metal exposure assessments.

The applicability of the procedure was tested using EBC of workers of a lead recycling industry as a model, where previous air pollution studies have been carried out (Almeida et al., 2010). Occupationally relevant metals were identified in this industry apart from lead (Pb). Metals, such as potassium (K), Cr, manganese (Mn), copper (Cu), zinc (Zn), strontium (Sr), cadmium (Cd) and antimony (Sb) were also present in relevant concentrations in the work environment and most of them have toxic potential. Consequently, these metals were measured in EBC.

3.2 Materials and Methods

3.2.1 Study groups and sampling

Two groups with different exposure levels to particulate matter were selected for this study: a non-exposed group constituted by 22 individuals (mean age 37 [26-55]) working in offices (state department office); and an exposed group of 16 workers (mean age 41 [33-48]) working in a metal processing industry for more than 5 years.

The selected industrial site is a smelting industry where metal alloys containing Pb are recycled. The plant operates 24 hours/day, on 8-hour shifts, for 5 days/week and two resting days. The workers rotate in each shift between different tasks that are performed in a confined area where smelting continuously operates. The exposed workers were identically exposed during each work shift. The workers were assessed at four times (two times on the first day and two times on the last day) during the work week to evaluate the influence of exposure on measured

data: a - first day of the five day working period – before the beginning of the shift; b - first day of the five day working period – after the end of the shift; c – last day of the five day working period – before the beginning of the shift; d – last day of the five day working period – after the end of the shift.

The non-exposed subjects were controls for the EBC measurements. Samples were collected at no specific time during the working week, which is of 35 h, from Monday to Friday. All the individuals recruited to this study, work and live in the same geographic region of Lisbon, Portugal, and gave their informed consent to participate in the study.

3.2.2 EBC collection

EBC was collected using commercial equipment (EcoScreen, Jager, Germany). The main features of this equipment are the condensation region, which is cooled to -30 °C guaranteeing the aggregation of the droplets of the exhaled air. The collection device has two unidirectional valves that prevent inhaled and exhaled air from mixing in the collection tube and work as a saliva trap (Goldoni et al., 2004; Almeida et al., 2010). A nose clip was used to prevent air intake through the nostrils, thus maximizing the collection of exhaled air in the condensate.

The EBC collecting period of 15 min was a comfortable sampling time for the donor and an acceptable labour interval for the factory. This collection period provided enough volume of condensate for the analysis (2 mL).

The EBC samples were collected in controlled environmental conditions, differing from the daily working conditions. For the exposed group, EBC was collected at the occupational health unit located in an isolated building within the factory complex, distant 50 m from the working-place. In the beginning of the shift, EBC was collected before starting work; in the end of the shift EBC was collected immediately after stopping work and before hygiene requirements at the end of the work-shift. For the non-exposed group the EBC was collected in a clean room (ISO 7).

3.2.3 Instrumentation

To assess metal concentrations in whole EBC samples and to estimate the reliability of results produced, two techniques were used: Inductively Coupled Plasma Mass Spectrometry (ICP-MS) and Total Reflection X-ray Fluorescence (TXRF).

The ICP-MS equipment, ELAN DRCe (Perkin Elmer, SCIEX, USA) was operated at 1100 W, with argon gas flow of 15 $L.min^{-1}$ (litre per minute) and 0.85 aerosol $L.min^{-1}$ gas carrier using a Peltier-cooled cyclonic spray chamber. Data acquisition was done at peak-hopping mode with 50 ms dwell time, 20 sweeps/reading, 1 reading/replicate and 3 replicates. Quantitative analysis was carried out based on an external calibration using Y as an internal standard. Data was collected, processed and analysed with ELAN 3.4 software.

A TXRF EXTRA II-A spectrometer (ATOMIKA) equipped with two 2 kW fine focus X-ray tubes (Mo and W anodes) was used as reference technique. The TXRF technique is accredited for water analysis at LNEG, by the Portuguese Quality System following the regulations of ISO/IEC 17025 (Barreiros et al., 1997; ISO/IEC17025, 2005). Metal quantification was performed by the internal standard method using Ga as an internal standard and calculations were carried out using QXAS software package (Van Espen et al., 1986).

3.2.4 Reagents

Multi-element atomic spectroscopy standard solution Fluka 70008 (Sigma-Aldrich®) was used for calibration. Stock solutions of 1000 ± 10 $\mu g.L^{-1}$ of Y (AAS Specpure® Y solution, Alpha Aesar) and Ga (AAS Specpure® Ga solution, Alpha Aesar) were used for internal standardization and preparation of spiked samples. Ultrapure water of 18 MΩ.cm (Milli-Q Element®, Millipore Corp., MA) was used for dilution of stock solutions and to prepare blank solutions. Concentrated Suprapur® nitric acid (HNO_3) high-purity grade was obtained from Merck (Germany).

3.2.5 Sample preparation

For TXRF analysis, EBC samples were doped with Ga as an internal standard, in a concentration of 100 $\mu g.L^{-1}$. The homogeneous distribution of the internal standard in samples was attempted mixing thoroughly and 20 µL pipetted onto appropriate quartz sample carriers for TXRF measurements. From each sample at least two replicates were analysed.

For ICP-MS analysis, 500 μL of EBC samples were doped with Y as an internal standard in a concentration of 10 $\mu g.L^{-1}$ and diluted 5 fold with acidified 1 % v/v HNO_3 ultrapure water. A total volume of 2.5 mL is the minimum required to perform an ICP-MS granting adequate conditions of sample introduction and analysis.

3.2.6 Optimization of the analytical method

To validate the methodology and analytical procedure, ICP-MS and TXRF techniques were used as referred above. The approach had into account the metrologic characteristics of both techniques using known homogeneous samples to study EBC matrix and the need of further sample preparation before analysis.

Standard reference materials (SRMs) with an aqueous matrix and elemental levels similar to those of EBC were used. Two SRMs were chosen from the National Institute of Standards and Technology (NIST), i.e., Trace Elements in Natural Water SRM-1640 (analysed by TXRF) and Trace Elements in Water SRM-1643e (analysed by ICP-MS). The standards were repeatedly analysed in independent runs, together with EBC samples. Standard aliquots were prepared for ICP-MS and TXRF analysis following the criteria used in EBC, as described above. In the ICP-MS analysis aliquots were diluted 1:5 v/v in acidified ultrapure water, and in TXRF analysis no sample preparation was applied.

The heterogeneity of EBC was tested by comparing results obtained by both techniques without sample preparation and by analysing filtered and acidified samples. For the filtration of EBC samples a syringe filter (Millipore ™) with 0.22 µm pore size was used. To attempt the homogenization and dissolution of the material in suspension in EBC, acidification of samples at room temperature followed by sonication for 10 minutes was carried out. Two levels of acid concentration 1% v/v HNO_3 and 3% v/v HNO_3 were tested.

3.2.7 Statistical Analysis

All statistical analyses were performed using the SPSS statistical package (version 18.0, SPSS Inc., Chicago, IL) software. In the evaluation of SRMs data, the closeness of agreement between replicate measurements of the concentrations and the certified value was used to evaluate trueness (ISO5725-1, 1994; JCGM200, 2008). The repeatability achieved using TXRF and ICP-MS techniques was calculated as the relative standard deviation obtained in the total number of analyses carried (ISO5725-1, 1994; JCGM200, 2008). Differences between study groups - exposed and non-exposed groups - were assessed using the non-parametric Mann–Whitney test and the differences between work shifts - periods of sampling a, b, c, and d - were estimated by the non-parametric Wilcoxon signed pair-test. Statistical results were considered significant at $p<0.05$.

3.2.8 Analytical Performance

The study of EBC had into account its matrix concerning organic and particulate matter. The first criterion to assure was the performance of TXRF and ICP-MS techniques in the analysis of analogous samples in terms of elemental concentrations but without organic matrix and material in suspension. Standard reference materials (SRMs) with an aqueous matrix and elemental concentration levels similar to those of EBC, NIST 1643e and NIST 1640, were analysed by ICP-MS and TXRF, respectively. At least 10 independent analyses of the SRMs were carried out. K, Cr, Mn, Cu, Zn, Sr, Cd, Sb and Pb were the elements measured. The precision obtained in both techniques (relative standard deviation of the mean concentration values) was below 5 % for all of the metals (except 6 % for Cr in ICP-MS). The trueness (relative difference to the certified value) was below 5 % for most of the elements determined by ICP-MS and TXRF (Figure 1-A and -B). The major deviation to the certified value was observed for Zn (6 %) in ICP-MS analysis. For TXRF, deviations of 8.8 % observed for Mn and of 7.8 % for Pb, were in accordance with the 10% acceptable performance of the technique.

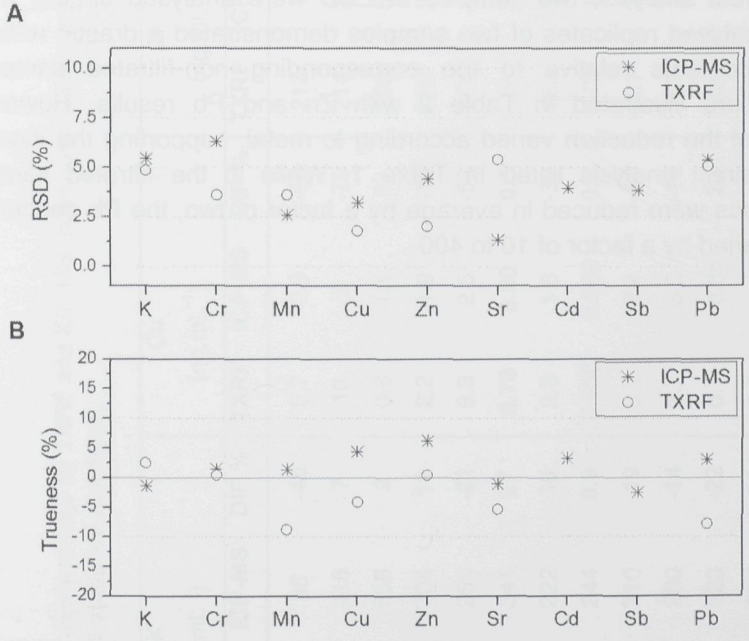

Figure 1 – Precision (A) and trueness (B) for TXRF (N=10) and ICP-MS (N=12) based on the analysis of SRM - NIST 1640 and SRM - NIST 1643e, respectively.

3.3 Results

Optimization of sample preparation

The direct analysis of EBC (untreated samples) did not provide acceptable results. The analysis of EBC samples by TXRF and ICP-MS showed a wide variability and the relative difference between the two techniques of concentrations measured for several metals was high and inconsistent. These discrepancies are shown in Table 1 for K, Cu, Zn, Sr and Pb concentrations measured in 12 different samples of EBC. The metal concentrations measured by TXRF and ICP-MS were not coherent varying independently of the sample and of the metal. The differences between techniques can be higher than 100 % as observed for Pb in one sample. These results suggest that particulate matter in suspension may be the cause of the high variability observed. The heterogeneity of EBC samples can be observed by optical microscopy as depicted in Fig. 2 a, b. To prove that heterogeneity in EBC samples prevents direct analysis, two samples of EBC were analysed directly and after filtration. Analysed replicates of two samples demonstrated a drastic reduction of elemental contents relative to the corresponding non-filtrated sample. The differences are illustrated in Table 2 with Zn and Pb results. However, the magnitude of the reduction varied according to metal, supporting the inconsistent results of direct analysis listed in Table 1. While in the filtrated samples Zn concentrations were reduced in average by a factor of two, the Pb concentrations decrease varied by a factor of 10 to 400.

Table 1 – Direct analysis of EBC by TXRF and ICP-MS techniques. Values are concentrations ($ng.mL^{-1}$) in 12 samples analysed. Relative differences (DIF) between techniques are also listed.

	K ($ng.mL^{-1}$)			Cu ($ng.mL^{-1}$)			Zn ($ng.mL^{-1}$)			Sr ($ng.mL^{-1}$)			Pb ($ng.mL^{-1}$)		
Sample	**TXRF**	**ICP-MS**	**DIF %**	**TXRF**	**ICP-MS**	**DIF %**	**TXRF**	**ICP-MS**	**DIF %**	**TXRF**	**ICP-MS**	**DIF %**	**TXRF**	**ICP-MS**	**DIF %**
1	161	96	**-40**	2.7	0.9	**-68**	212	61	**-71**	6	2	**-70**	16	6	**-62**
2	390	418	**7**	10	2	**-79**	211	217	**3**	14	15	**9**	170	168	**-1**
3	547	558	**2**	0.8	1.1	**33**	339	511	**51**	15	20	**30**	7.5	11	**48**
4	633	704	**11**	2.2	1.9	**-15**	208	287	**38**	14	18	**31**	33	89	**165**
5	322	255	**-21**	5.3	2.5	**-52**	764	466	**-39**	34	23	**-32**	16	8.1	**-49**
6	268	341	**27**	3.78	3.80	**0.5**	72	76	**5**	53	54	**2**	24	20	**-17**
7	171	222	**30**	2.9	1.8	**-39**	153	127	**-17**	61	58	**-5**	8.3	8.4	**2**
8	242	244	**0.9**	0.956	0.959	**0.3**	173	144	**-17**	56	52	**-8**	21	18	**-14**
9	260	210	**-19**	5.1	2.4	**-53**	621	336	**-46**	13	9	**-31**	44	26	**-42**
10	324	280	**-14**	3.7	3.3	**-10**	267	191	**-28**	26	24	**-7**	6.39	6.41	**0.28**
11	408	320	**-22**	0.7	0.8	**22**	42	36	**-13**	24	25	**3**	86	27	**-69**
12	203	292	**44**	0.9	1.3	**38**	55	105	**90**	35	40	**14**	6.4	8.3	**30**

DIF = [ICP-MS]-[TXRF])/ [TXRF]

Table 2 – Concentrations of Zn and Pb obtained by TXRF in replicates (3 to 6) of two EBC samples (1 and 2) non-filtrated (NF) and filtrated (F). Relative differences (DIF) between non-filtrated and filtrated samples are also listed.

	Zn ($ng.mL^{-1}$)			Pb ($ng.mL^{-1}$)		
Sample	**NF**	**F**	**DIF (%)**	**NF**	**F**	**DIF (%)**
EBC1-1	138	99	28	179	3.4	98
EBC1-2	174	95	45	79	2.5	97
EBC1-3	151	89	41	179	4.4	98
EBC2-1	18	13	28	65	3.4	95
EBC2-2	20	14	30	66	5.9	91
EBC2-3	32	13	59	1745	4.4	99.7
EBC2-4	20	12	40	43	2.1	95
EBC2-5	20	10	50	45	3.8	92
EBC2-6	23	10	56	70	2.9	96

DIF = [NF]-[F])/ [NF]

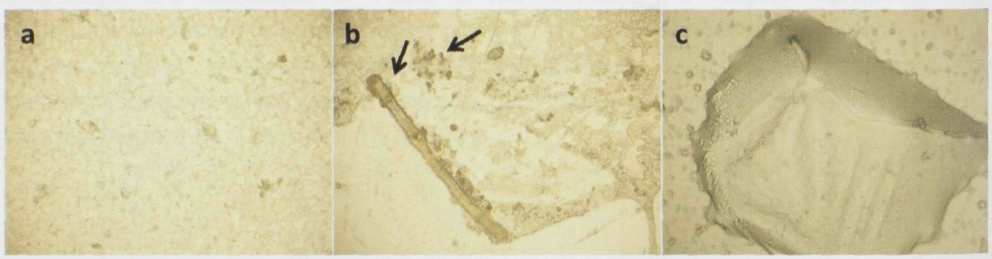

Figure 2 – Optical micrographs (reflection microscopy) of EBC. Native samples showing a) regular distribution of particles (200x) and b) cluster and large particle (200x) in droplets deposited onto the TXRF quartz support (arrows); c) Droplet of an acidified sample (1 % HNO_3) showing an uniform surface (200x). Acidification modifies surface tension of EBC and the droplet appears convex, as a round-shape object.

The non-homogeneous characteristics of the EBC sample, containing particles of different size, shape and composition influences the quality of TXRF and ICP-MS results. In the case of TXRF, the non-predictable presence of particles in aliquots of 20 μL prevented analysis from being representative. In the case of ICP-MS, the entrance of particles in the plasma can be hampered as they may not be nebulized

in the ICP-MS nebulisation chamber, producing unrepresentative concentration values of the total sample.

Once the direct elemental analysis of the total EBC sample did not yield acceptable results, a pre-treatment leading to the homogenization of EBC was tested by acidification with 1 % and 3% HNO_3 (v/v) as described previously, using a pool of EBC samples. For the lower level of acidification the concentrations measured increased significantly relative to direct analysis, but the agreement between the two techniques was still not satisfactory. Increases were of 50 % in TXRF and ranged from 20 - 80 % in ICP-MS (data not shown). The acidification level of 3% HNO_3 (v/v) rendered differences below 9 % between TXRF and ICP-MS except for Cu (15 %) (Table 3). This procedure provided reliable results as differences between both techniques were satisfactory (≤ 15 %) and the relative standard deviations were below 5.0 % for both ICP-MS and TXRF, which is in accordance with validation results carried out with SRM's (see section 3.2.8). The improvement in homogeneity could also be verified by optical microscopy (Fig. 2 c).

Table 3 – Average concentrations ($ng.mL^{-1}$) and relative standard deviations (RSD) measured by TXRF and ICP-MS in N replicates of a sample pool of EBC acidified with 3% HNO_3. The relative difference between techniques (DIF) is also indicated.

	TXRF N=20		ICP-MS N=20		
	X	RSD	X	RSD	DIF%
K	422	0.05	435	0.02	**3**
Cr	< QL		3.4	0.05	
Mn	< QL		1.9	0.04	
Cu	3.9	0.05	4.5	0.03	**15**
Zn	287	0.04	305	0.05	**6**
Sr	42	0.05	46	0.03	**9**
Cd	< MDL		0.4	0.05	
Sb	< MDL		2.0	0.03	
Pb	40	0.05	42	0.02	**5**

RSD = SD/mean value; DIF = [ICP-MS]-[TXRF])/ [TXRF]
QL = quantification limit (Cr = 3.6 $ng.mL^{-1}$; Mn = 2.9 $ng.mL^{-1}$)
MDL = minimum detection limit (Cd = 5 $ng.mL^{-1}$; Sb = 8 $ng.mL^{-1}$)

Collection methodology

The best time of EBC sampling for metal exposure assessment was evaluated on the basis of repeated measurements along the working week of each individual. Metal concentrations in EBC of workers and controls were determined by ICP-MS. Samples were acidified with 3% HNO_3 (v/v) as this was the procedure that provided adequate results. The measured concentrations in the EBC of non-exposed individuals and workers at the four collection times (sampling phases a, b, c and d) along the working week are listed in Table 4. The concentrations of K, Cr, Mn, Cu, Zn, Sr, Cd, Sb and Pb were determined. Differences for all the elemental concentrations between the two studied groups were observed, excluding Zn and Sr. The concentrations of these metals were importantly increased in workers relative to controls at the beginning of the shift and remained augmented through the whole working week, although significance was not always reached in all sampling phases as was observed for K, Cu and Cd. In the exposed group the variations in elemental concentrations along the working week did not significantly vary, with sporadic exceptions for Cd, Sb and Pb (pairtest, $p<0.05$). The concentration of Pb was decreased in the beginning of the working period, opposite to Cd concentrations that were increased in the beginning of the shift. The Sb concentration showed an increasing trend to the end of the working day although variations were only significant in the end of the first day of work (phase b).

As can be inferred from Table 4, the concentrations of Cr, Mn, and Cu in EBC of workers were 3 to 6 fold higher than controls, while the concentrations of Sb and Pb, the most abundant pollutants in the work environment (Almeida et al., 2010), were up to 30 times higher than controls. A moderate percentage increase of K and Zn concentrations of approximately 40% were observed, although their concentrations in the work environment were higher or of the same order of Cr, Mn and Cu.

Table 4 – Average elemental concentrations ($ng.mL^{-1}$) and respective standard errors (Mean ± SE) in EBC of non-exposed (N=22) and exposed (N=16) individuals, obtained by ICP-MS. For the exposed group, the concentrations measured in each sampling phase (a, b, c, d) are listed.

	Non-exposed Group ($ng.mL^{-1}$)	Exposed Group ($ng.mL^{-1}$)			
		a	b	c	d
	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
K	212 ± 13	344 ± 47 *	447 ± 62 *	327 ± 64	330 ± 52 *
Cr	0.52 ± 0.14	2.5 ± 0.9 *	3.0 ± 0.9 *	5.0 ± 1.8 *	3 ± 1 *
Mn	0.53 ± 0.11	1.9 ± 0.3 *	2.4 ± 0.6 *	1.3 ± 0.2 *	2.7 ± 0.8 *
Cu	2.4 ± 0.3	14 ± 9 *	15 ± 6 *	8 ± 4	10 ± 5 *
Zn	201 ± 31	342 ± 71	217 ± 55	155 ± 31	198 ± 38
Sr	32 ± 3	43 ± 9	30 ± 6	35 ± 8	36 ± 5
Cd	0.17 ± 0.03	0.57 ± 0.09 * [c]	0.42 ± 0.08 *	0.27 ± 0.05	0.38 ± 0.09
Sb	0.036 ± 0.005	1.1 ± 0.21 * [b]	1.7 ± 0.2 * [c,d]	0.9 ± 0.2 *	1.3 ± 0.2 *
Pb	1.05 ± 0.15	29 ± 5 * [b]	40 ± 8 *	35 ± 9 *	36 ± 7 *

* Significant difference to controls ($p<0.05$);

[b,c,d] Significant difference from sampling a (beginning of shift) to b, c, and d sampling phases; pair-test, $p<0.05$.

3.4 Discussion

The possibility of carrying out quick and reliable analysis of EBC for assessing metal exposure in occupational environments was evaluated, as in most environmental health assessments, conclusions are based on the analysis of large number of samples and therefore undemanding analytical procedures are encouraged. Various metals were studied which were i) important in physiological functions, such as K and Zn and at a certain extent Sr; ii) relevant in the occupational context, such as Pb; and iii) pollutants with toxicity potential also present in the work environment, such as, Cr, Mn, Cu, Cd and Sb.

Homogenization of EBC matrix is required to have a representative ICP-MS analysis of the elemental concentration in the total sample. The direct ICP-MS analysis of EBC elemental concentrations produced incoherent results being unrepresentative concerning the total EBC sample. Similar results were also obtained for TXRF analysis of untreated EBC. The results reflected sample heterogeneity as aliquots pipetted and nebulized may arbitrarily contain particulate matter in suspension. As EBC contains particle aggregates they may not be nebulized and sample metal measurements will underestimate the metal concentration. Data reported for size and composition of suspended material in EBC indicated that the EBC of workers contained particulate matter (Pinheiro et al., 2011) matching those collected in the work environment (Almeida et al., 2010). The analysis of filtrated EBC showed that different metals may preferentially associate to different particle sizes, e.g., Pb is likely to be associated to particles or aggregates larger than those that have Zn what is in accordance with published data (Pinheiro et al., 2011). These features hampered the analytical reliability and reproducibility of elemental concentrations measurements. As far as this type of matrix is concerned, EBC samples require pre-treatment. Additional sample treatments is not encouraged as they increase time of analysis and in most environmental health assessments conclusions are based on the analysis of large number of samples. Nevertheless, sample homogenization must be achieved in order to quantify in the total sample toxic metals present in indoor and occupational environment, if EBC has to be used as an exposure bio-indicator. Pre-treatment procedure providing homogenization in mass-limited samples such as EBC should substitute conventional acid digestion. The attempt of solubilizing the inorganic material in suspension in EBC by acidification at room temperature was positive. This procedure enabled matching the precision criteria obtained with SRMs in the elemental analysis of EBC.

So far there are no standardized methods for the analysis of electrolytes and metals in EBC. The task force for EBC created under the American Thoracic Society and the European Respiratory Society aimed at developing guidelines for EBC collection and measurement of exhaled biomarkers (Horváth et al., 2005). Characteristics of equipment used ensuring most adequate conditions of temperature stability for preservation of molecules and ions throughout collection (Rosias et al., 2004; Soyer et al., 2006), unidirectional valves realistically efficient in avoiding saliva contamination (Effros et al., 2006; Mutti et al., 2006) as used in this study, are some of the methodological issues addressed. The task force focused the analysis of volatile molecules, peptides and proteins, metabolites of lipid oxidation, and cytokines, but the measurement of metals and electrolytes was not addressed. Guidelines that are in the overall good laboratory practices ensuring the appropriate control of factors affection collection and analysis, were also proposed. The present study provides a practical contribution to this strategy, as it extends EBC guidelines to sample preparation and to elemental content analysis, proposing a feasible, fast and reliable procedure that can be applied in environmental health assessment.

A pilot study was also carried out aiming at checking a collection methodology suitable for occupational assessment, therefore, the variations of metal contents in EBC of workers along the 5-day working period was followed. Several metals, relevant in the occupational context, were measured in the EBC of workers and proved to reflect the environment in the factory. K, Cr, Mn, Cu, Cd, Sb and Pb concentrations in the EBC of workers were consistently different from non-exposed subjects, and the concentration levels remained relatively steady during the whole working week. The higher concentration values measured in exposed subjects when compared to non-exposed individuals were especially relevant for Pb and Sb. The levels of these two elements showed also an increasing trend towards the end of the working shift and were diminished in the beginning of the working week after non-exposed resting period, suggesting that the week variations of Sb and Pb concentrations in EBC are due to occupational exposure. In fact, Pb is associated with the process and materials used in the industry. The evaluation of the workplace environment revealed high contents of Sb and Pb in different fractions of particulate matter collected (Almeida et al., 2010). The concentrations of these two elements were found to be four orders of magnitude higher than the concentrations measured in outdoor and indoor (offices) environment. Cr, Mn and Cu were present in the work environment suggesting that the increased concentration of these elements in EBC were due to occupational exposure. Cd was not studied in the factory air, as it was not a characteristic signature of the emissions. Nevertheless, Cd was present in EBC of workers and its concentration augmented relative to non-exposed individuals, although its percentage increase is less

evident than those observed for Cr, Mn and Cu. As the factory recycles Pb containing materials, Cd may be present as a contaminant. The evaluation of the variability of Cd with smoking habits should be addressed in further occupational or environmental health assessments (Lin et al., 2011).

The concentrations of K, Zn and Sr in EBC were kept in a relatively narrow range of variation, independently of the levels of these elements in the environment. Reported data on Sr exposure is scarce with no recognized physiological role for this metal. The toxic effects of exposure to metal dust of K and Zn in human health have been indicated as relatively harmless (Plum et al., 2010). Thus, the moderate variations of these elements in workers suggest that their concentrations in EBC may reflect their physiological levels in the alveolar lining fluid. Electrolytes, such as K, are physiologically important in keeping water tension within airways and there are experimental evidences that its presence in EBC results from water vapour dilution of alveolar lining fluid (Bondesson et al., 2009). Also Zn has multiple biological roles of cell signalling, regulation and detoxification. Excess free Zn is quickly sequestrated inside cell specialized organelles, and tightly bound to metallothioneins and zinc finger proteins (Bell and Vallee, 2009; Plum et al., 2010).

Although few individuals were enrolled this prospective study, the presented results showed the potential use of EBC for occupational assessment. EBC identified exposure during the 5-day working period. Metal exposure can feasibly be assessed by evaluating EBC metal contents in the beginning and at the end of the labouring week.

The analytical protocol described in this study, suggest that EBC can measure exposures but must be followed in subsequent studies to solve the above problems. To standardize a methodology it must be assured that results are representative and the methodology yields comparable results. The proposed methodology for metal determinations in EBC using ICP-MS stimulates further studies to explore the EBC potential in exposure assessment, such as in toxicant bio-availability or estimation of dose.

3.5 Conclusions

The case study presented in this paper, using a Pb processing industry as a model, showed that EBC can be a promising indicator of human exposure to Pb and other metals present in the suspended particulate matter in the work environment.

The preparation of EBC samples was crucial in the analysis of elemental contents, as EBC can have significant amounts of particulate matter in suspension. A standardized procedure for collection and analysis of EBC was developed and method validation was carried out. The method enabled reliable results and the proof of EBC's potential in establishing new biomarkers of exposure to metals in an occupational scenario.

The characteristics of EBC, non-invasiveness and the undemanding method of collection, make of EBC an attractive matrix that can be applied to the study of metal exposure. Advances in the field and the establishment of new biomarkers for the respiratory system will eventually help to identify health risks of airborne pollutants in exposed groups.

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Chapter 4

Exhaled Breath Condensate as a biomonitor for metal exposure: A new analytical challenge

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Abstract

The study of Exhaled Breath Condensate (EBC) obtained by cooling exhaled air under conditions of spontaneous breathing is considered one of the areas with higher interest in respiratory health research. The use of EBC for elemental determination in occupational exposure requires a standard methodological procedure to implement its practice in occupational studies. EBC is an inhomogeneous sample with organic and particulate matter in suspension, which may hamper analytical results' reliability. Total Reflection X-ray Fluorescence (TXRF) and Inductively Coupled-Plasma Mass Spectrometry (ICP-MS) techniques were chosen as both are multielemental, require small sample volumes and have appropriate detection limits. Estimation of the overall uncertainty in both techniques was carried out using a pool of EBC collected from a group of workers of a lead processing industry to perform precision and trueness studies for K, Mn, Cu, Cd, Sb and Pb. Precision was estimated in terms of repeatability using the native EBC sample pool and trueness in terms of recovery obtained from spiking aliquots of the EBC pool with K, Mn, Cu, Cd, Sb and Pb at different concentrations. Recovery was the most significant contribution to total uncertainty. The overall uncertainties obtained for ICP-MS enabled to discriminate between groups of individuals exposed to different levels of contaminants. Therefore EBC proved to be useful in human biomonitoring.

4.1 Introduction

Exhaled Breath Condensate (EBC) is obtained by condensing the exhaled air into a cooled collection device by breathing tidally. The EBC is a matrix in which numerous volatile and non-volatile substances can be detected, enabling the assessment of biomarkers of effect (e.g., occupational assessments) and response (e.g., pulmonary pathobiology assessments) in real-time or in conditions close to real-time (Horváth et al., 2005). Measuring metals in EBC is a promising method of risk assessment, comparing with other common indicators (like blood), once it is non-invasive and quickly and easily collected (Dwyer, 2004; Grob et al., 2008).

In occupational assessments where workers are exposed to metal dust the EBC may provide unique indication of direct exposure (Goldoni et al., 2004; Caglieri et al., 2006; Broding et al., 2009). The emerging use of EBC for metal quantification in occupational exposure studies needs a standard methodological procedure (Horváth et al., 2005), based on the requirements of both method validation and uncertainty estimation, in order to implement its practice in exposure assessments, as an indicator of body status. The EBC sample is a water suspension, highly diluted, inhomogeneous, containing organic and significant amounts of particulate matter (Pinheiro et al., 2011). Moreover, an EBC sample has only a few millilitres of volume, which has to be enough to perform accurate analysis of occupationally relevant elements, usually present at the workplace in trace levels. These issues influence sample representativeness and raise analytical difficulties, limiting direct analysis of the sample and constraining the use of conventional pre-treatment methods. So, multielemental analysis of EBC samples constitutes a true analytical challenge mainly due to the degree of rigour required to biomedical or occupational studies.

In this work samples were collected from workers of the lead processing industry, where the most relevant contaminant was Pb though, Mn, Cu, Cd and Sb, among other metals, were also present in the work environment (Almeida et al., 2010). Total Reflection X-ray Fluorescence (TXRF) and Inductively Coupled-Plasma Mass Spectrometry (ICP-MS) were the techniques chosen to analyse selected elemental concentrations in EBC, i.e., K, Mn, Cu, Cd, Sb and Pb. Both techniques are appropriate since they are multielemental, requiring only very small sample volumes and presenting detection limits that fit for metal analysis in EBC samples. In addition, TXRF is a technique accredited for water analysis at LNEG by the Portuguese Quality System following the regulations of ISO/IEC 17025 that

assures the continuous validation of the analytical methodology and the reliability of results (Barreiros et al., 1997).

The validation and Quality Control process consisted of overall uncertainty estimation for both techniques to assess the fitness for purpose of the procedure and therefore to demonstrate the reliability of measurements. By comparing the overall uncertainties obtained the suitability of the analytical procedure can be clarified. The adequacy of each analytical method for occupational studies are discussed based on realistic and controlled uncertainties determined as a mean to ensure comparability of results and to discriminate between exposure levels.

4.2 Experimental

4.2.1 Sampling and sample preparation

EBC was collected with commercial equipment (EcoScreen, Jager, Germany) consisting of a trap cooled to -30 °C where exhaled air condenses and two unidirectional valves that prevent inhaled and exhaled air from mixing in the collection tube and work as a saliva trap (Goldoni et al., 2004; Almeida et al., 2010). The EBC collection period was of 15 min, providing an average volume of 2 mL of condensate.

The EBC samples were collected from workers of two industries exposed to different ambient levels of metal dust and non-exposed individuals working in offices. Three groups of exposure were constituted: 1) Low-level exposure consisting of 55 samples collected from non-exposed individuals; Intermediate-level exposure comprising 160 samples collected from workers of a battery assembling industry; and a high-level exposure comprising 50 samples obtained from workers of a Pb recycling industry. Particulate matter was collected in fine ($PM_{2.5}$) and coarse ($PM_{2.5-10}$) fractions and Pb concentrations determined particulate in both fractions: high-level, 182 ± 32 $\mu g.m^{-3}$ in $PM_{2.5}$ and 336 ± 82 $\mu g.m^{-3}$ in $PM_{2.5-10}$; intermediated-level, 4.4 ± 1.1 $\mu g.m^{-3}$ in $PM_{2.5}$, and 14.8 ± 3.1 $\mu g.m^{-3}$ in $PM_{2.5-10}$; low-level, 5.5 ± 0.5 $ng.m^{-3}$ in $PM_{2.5}$ and 5.3 ± 0.5 $ng.m^{-3}$ in $PM_{2.5-10}$.

To carry out uncertainty estimation an EBC pool was made by mixing individual EBC samples collected. The EBC sample pool was spiked with mono-elemental standard solutions of K, Mn, Cu, Cd, Sb and Pb (Certipure® Merck) in order to perform recovery tests. To guarantee realistic uncertainty estimation with both

TXRF and ICP-MS a pre-established amount of each element was added to the EBC pool according to the concentration level in the native pool.

For validation study two standard reference materials (SRMs) with an aqueous matrix and elemental levels similar to those of EBC were used. Two SRMs were chosen from the National Institute of Standards and Technology (NIST), i.e., Trace Elements in Natural Water SRM-1640 (analysed by TXRF) and Trace Elements in Water SRM-1643e (analysed by ICP-MS).

Detailed sample collection and preparation is described elsewhere (Félix et al., 2012). Briefly, EBC samples were acidified with 3% v/v HNO_3 at room temperature, sonicated for 10 minutes and stored at -80 °C.

For TXRF analysis, EBC samples were doped with Ga (AAS Specpure® Ga solution 1000 ±10 $\mu g.L^{-1}$ Alpha Aesar) as an internal standard, in a concentration of 100 $\mu g.L^{-1}$. Doped samples were strongly homogenized and 20 µL pipetted onto appropriate quartz sample carriers for TXRF measurements. For ICP-MS analysis, 500 µL of EBC samples were doped with Y (AAS Specpure® Y solution 1000 ± 10 $\mu g.L^{-1}$ Alpha Aesar) as an internal standard in a concentration of 10 $\mu g.L^{-1}$. Samples were diluted 5 fold in 18 MΩ.cm ultrapure water (Milli-Q Element®) acidified with 1 % v/v HNO_3 suprapur (Merck, Germany).

4.2.2 Analytical techniques

The ICP-MS equipment, ELAN DRCe (Perkin Elmer, SCIEX, USA) was operated at 1100 W, with argon gas flow of 15 $L.min^{-1}$. A Peltier-cooled cyclonic spray chamber was used with a flow of 0.85 $L.min^{-1}$. Quantitative analysis was carried out based on an external calibration. Data was collected, processed and analyzed with ELAN v3.4 software. The TXRF analyses were carried out in an EXTRA II_A spectrometer (ATOMIKA) and elemental quantification performed by the internal standard method using QXAS software package (Van Espen et al., 1986).

4.2.3 Uncertainty calculation

The method performance parameters, i.e., precision and trueness, were evaluated during method validation and used to estimate uncertainties. Two standard reference materials (SRMs) and a pool of EBC samples were analysed. Trueness was estimated in terms of overall recovery (the ratio between the observed and the expected value) obtained from spiking aliquots of the EBC pool at different concentrations for K, Mn, Cu, Cd, Sb and Pb (addiction of pre-determined

concentrations). Uncertainties were estimated according to methods currently used in analytical chemistry, which are based in whole method performance parameters (ISO/IEC5725-1, 1994; Barwick and Ellison, 2000):

$$U_{C_Z} = C_Z \times \left(k \times u_{(rel)C}\right)$$

where U_{CZ} is the expanded uncertainty for the C_Z concentration of the element "Z", (using a coverage factor of k=2 to provide and expanded uncertainty reflecting an approximate level of confidence of 95 %), and $u_{(rel)c}$ is the relative combined uncertainty, which is given by

$$u_{(rel)c} = \sqrt{RSD^2 + u^2_{(rel)rec}},$$

where RSD is the relative standard deviation of C_Z and $u_{(rel)rec}$ is the relative uncertainty in the recovery, which is calculated by two different equations, either estimated using a certified reference material (CRM – the NIST SRM in this study) or from the spike study. When a CRM is used, recovery is given by:

$$u_{(rel)rec} = \sqrt{\frac{s^2_{obs}}{n \times \overline{C}^2_{obs}} + \left(\frac{u_{CRM}}{C_{CRM}}\right)^2} \qquad (CRM),$$

where $\overline{C}_{obs}$ is the mean of the observed results in the replicate analyses of the CRM, s_{obs} is the respective standard deviation; u_{CRM} is the standard uncertainty in the certified value C_{CRM}, indicated in the CRM certificate. In the spike study, for each recovery experiment a bottom-top approach was used. The contribution of each recovery experiment (b_i) for the overall bias ($u_{(rel)rec}$) is considered. The relative uncertainty in the recovery from the spike study is estimated using:

$$u^2_{(rel)rec} = \frac{\sum_{i=1}^{n} b_i^2}{n} \qquad (spiking),$$

$$\text{with} \quad b_i = \frac{\left(C_{obs,i} - C_{exp}\right)}{C_{exp}} \quad \text{and} \quad C_{exp} = C_{pool} + C_{added}$$

where b_i is the relative bias for each recovery experiment, $C_{obs,i}$ is the observed concentration of each analysis, *n* the number of replicates and C_{exp} is the expected concentration of the spiked pool, that is the concentration in the pool plus the concentration added.

4.3 Results and discussion

The validation of the analytical method used to determine elemental concentrations in EBC included the estimation of the performance parameters, precision and trueness. By measuring CRM samples these parameters were evaluated for natural water analysis. In the absence of appropriate certified samples, similar to EBC matrix, the precision was determined by repeated measures of a pool constituted of EBC samples and the trueness of the method was obtained from a spike study, where the recovery estimation was determined for the concentrations measured in the spiked EBC pool.

The SRM samples were repeatedly analysed in 12 independent runs under reproducibility conditions. Mean elemental concentrations and relative standard deviations (RSD) obtained are presented in Table 1 together with the corresponding values of the relative uncertainty in the recovery and the mean recovery $\overline{C}_{obs}/C_{ref}$. The contribution of the uncertainty due to precision, expressed by RSD, was <6 % for all elements and both techniques. The bias obtained was ≤9 % and relative uncertainty in the recovery ($u_{(rel)rec}$) was ≤2 %.

However the uncertainty budget increased, as the recovery obtained for Cd using ICP-MS and Mn, Cu, and Pb using both TXRF and ICP-MS was statistically different from 1 (Barwick and Ellison, 2000). Thus, for these elements, the correction factor Δ was applied using:

$$\Delta = \frac{\left(\overline{C}_{obs} - C_{CRM}\right)}{C_{CRM}}$$

In this particular case, the relative combined uncertainty is given by:

$$u_{(rel)c} = \sqrt{RSD^2 + u^2_{(rel)rec} + \Delta^2}$$

The increased recovery relative uncertainty reaches 9 % for Mn (TXRF), 7 % for Cu and Cd (ICP-MS) and Pb (TXRF), 4 % for Pb (ICP-MS) and Cu (TXRF). The calculated expanded uncertainty (U) for ICP-MS and TXRF was ≤17% and ≤19%, respectively, and shown in Table 2.

The analysis of the native EBC pool enabled to evaluate precision. In Table 3, the mean elemental concentrations ($\overline{C}_{obs}$), relative standard deviations (RSD) and detection limits (LD), obtained in the analysis of 20 replicates for ICP-MS and TXRF are indicated. In this case, the contribution to the combined uncertainty from precision was ≤ 5% for ICP-MS and TXRF for all elements, except for Mn in the latter. The poor precision obtained for Mn determination with TXRF can be justified as the Mn concentration in EBC is between the LD and the quantification limit (2.9 $\mu g.L^{-1}$) of the method.

The results obtained in the analysis of the spiked EBC pool are shown in Figure 1, where expected (native + spike) and observed concentrations are compared. Differences to expected values are ≤ 13 % for TXRF and ≤ 15 % for ICP-MS. Table 4 allows the comparison between uncertainty contributions from precision (RSD) and trueness ($u_{(rel)rec}$), presenting, as well, combined and expanded uncertainties. As can be inferred from Table 4 the values of $_{(rel)rec}$ obtained in the spiking study represent the most relevant contribution to total uncertainty.

Table 1 – Elemental concentrations ($\mu g.L^{-1}$), relative standard deviations and relative uncertainty in the recovery obtained with ICP-MS and TXRF in 12 replicates of SRM 1643e and SRM 1640. C_{CRM} – certified concentration; u_{CRM} – standard uncertainty in the certified value; $\overline{C}_{obs}$ - mean concentration observed; RSD_{obs} - relative standard deviation observed; $u_{(rel)rec}$ – relative uncertainty in the recovery.

	SRM 1643e		ICP-MS			SRM 1640		TXRF			$\overline{C}_{obs}/C_{CRM}$	
	C_{CRM}	u_{CRM}	$\overline{C}_{obs}$	RSD_{obs}	$u_{(rel)rec}$	C_{CRM}	u_{CRM}	$\overline{C}_{obs}$	RSD_{obs}	$u_{(rel)rec}$	ICP-MS	TXRF
K	2034	14	2054	0.058	0.013	996	14	1021	0.048	0.020	1.01	1.03
Mn	38.97	0.23	39.5	0.026	0.008	121.5	0.55	111	0.036	0.012	1.01	0.91
Cu	22.76	0.15	24.4	0.026	0.009	85.2	0.6	81.7	0.018	0.009	1.07	0.96
Cd	6.57	0.04	7.06	0.043	0.010	22.79	0.48	-	-	-	1.07	-
Sb	58.30	0.31	58.43	0.031	0.008	13.79	0.21	-	-	-	1.00	-
Pb	19.63	0.11	20.56	0.048	0.012	27.89	0.07	25.7	0.051	0.016	1.05	0.92

Table 2 – Expanded uncertainty (U) based on ICP-MS and TXRF analysis of SRM's. C_Z – Concentration of element "Z" (K, Mn, Cu, Cd, Sb, Pb).

	ICP-MS U	TXRF U
K	$C_K \times 0.119$	$C_K \times 0.105$
Mn	$C_{Mn} \times 0.061$	$C_{Mn} \times 0.191$
Cu	$C_{Cu} \times 0.156$	$C_{Cu} \times 0.093$
Cd	$C_{Cd} \times 0.172$	-
Sb	$C_{Sb} \times 0.064$	-
Pb	$C_{Pb} \times 0.137$	$C_{Pb} \times 0.189$

Pb	C_{Pb} x 0.137	C_{Pb} x 0.189

Table 3 – Mean elemental concentrations ($\overline{C}_{obs}$) in μg/L and relative standard deviations (RSD) obtained with ICP-MS and TXRF in 20 replicates of the native EBC pool. Detection limits (LD) are also indicated.

	ICP-MS (n=20)			TXRF (n=20)		
	$\overline{C}_{obs}$	RSD	LD	$\overline{C}_{obs}$	RSD	LD
K	435	0.016	27	422	0.048	6.4
Mn	1.87	0.038	0.034	1.4	0.167	0.9
Cu	4.49	0.026	0.039	3.8	0.054	0.4
Cd	0.44	0.050	0.003	-	-	-
Sb	1.96	0.032	0.011	-	-	-
Pb	42	0.021	0.029	40	0.050	0.7

Table 4 – Uncertainty estimation based on ICP-MS and TXRF analysis of the EBC pool. Uncertainty of precision (RSD) and recovery ($u_{(rel)rec}$), combined uncertainty ($u_{(rel)c}$) and expanded uncertainty (U).

	ICP-MS				TXRF			
	RSD	$u_{(rel)rec}$	$u_{(rel)c}$	U	RSD	$u_{(rel)rec}$	$u_{(rel)c}$	U
K	0.016	0.156	0.157	C_K x 0.313	0.048	0.084	0.097	C_K x 0.195
Mn	0.038	0.073	0.083	C_{Mn} x 0.166	0.167	0.148	0.223	C_{Mn} x 0.446
Cu	0.026	0.141	0.144	C_{Cu} x 0.287	0.054	0.137	0.147	C_{Cu} x 0.294
Cd	0.050	0.111	0.121	C_{Cd} x 0.243	-		-	-
Sb	0.032	0.104	0.109	C_{Sb} x 0.219	-		-	-
Pb	0.021	0.082	0.085	C_{Pb} x 0.169	0.050	0.086	0.100	C_{Pb} x 0.201

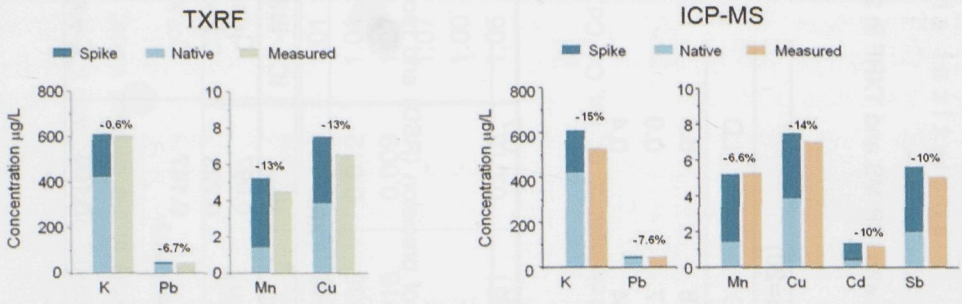

Figure 1 – *Expected (native + spike) vs. observed concentrations in spiked EBC pool for* TXRF and ICP-MS. Numbers on top of columns are relative differences (%) between *observed (measured) and expected concentrations.*

The combined uncertainty, $u_{(rel)c}$, calculated in the spike study, varied between 8% and 15.6% for ICP-MS and between 10% to 22 % for TXRF. Therefore the expanded uncertainties at 95% confidence (coverage factor of 2) were < 25% for the majority of elements.

The estimated uncertainties in EBC analysis by TXRF were somewhat larger than those obtained with ICP-MS. Incomplete sample homogenization may be the cause of uncertainty differences between both techniques, calling the attention for the need of further improve analytical methodology.

Nevertheless, the adequacy of the method to assess occupational exposure can be demonstrated by applying the uncertainty values estimated to the elemental concentrations measured in the EBC of non-exposed individuals and exposed workers. The procedure was applied to ICP-MS results of Cd, Sb and Pb concentrations in EBC collected from individuals exposed to different levels of contaminants. The work environment characterization was carried out previously (Almeida et al., 2010) enabling the establishment of three levels of exposure based on particulate matter Pb concentration. Two groups of workers of the lead industry, exposed to metal dust containing relevant concentrations of Pb in different amounts (intermediate and high level groups), and one group of non-exposed subjects (working in offices) were used. The concentration of Cd in EBC was also selected, due to the toxicological relevance of this element, although their presence in the environment cannot be directly associated to the industry processes. As can be depicted in Figure 2 the concentration intervals obtained for the Sb and Pb mean concentrations having into account the expanded uncertainty

value at 95% confidence (see Table 4) were completely separated. The Cd concentrations in EBC cannot distinguish between low-level and intermediate-level of exposure, but can discriminate the high level of exposure. The result suggests that Cd may exist as a contaminant of the materials processed in the workplace.

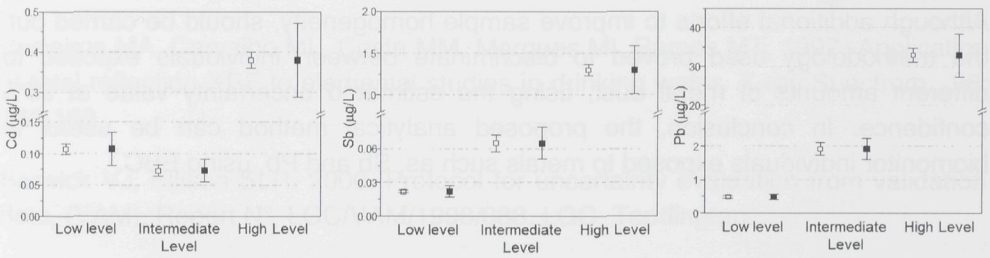

Figure 2 – Elemental concentrations in EBC determined with ICP-MS for the three exposure level groups. Mean values and the respective standard deviations (white squares) and deviations calculated with expanded uncertainty U (black squares).

Therefore, we can conclude that the procedure proved to be useful to distinguish exposure groups, meaning that the total uncertainty of the method was appropriate to discriminate exposure levels.

The study was focused on the concentrations of K, Mn, Cu, Cd, Sb and Pb. These elements were selected to provide a wide range of concentration levels (from $mg.L^{-1}$ to $\mu g.L^{-1}$), and to obtain data for relevant elements in biological fluids as K (Effros et al., 2002) and confirmed pollutants, such as Mn, Cu, Cd, Sb and Pb, some of them also playing essential physiological roles (i.e., Mn and Cu). Although changes in recognized toxicants are important to assess exposure risk, essential elements may provide unique information on physiological imbalances caused by acute or chronic exposures (Rosias et al., 2004). In this context the knowledge of total uncertainties that can be attributed to the analytical method used will be extremely important in order to assure the significance of concentration changes observed.

4.4 Conclusions

It was demonstrated that the use of two different analytical techniques, ICP-MS and TXRF, enabled the validation of EBC analysis. The analysis of spiked EBC samples showed that recovery is the most relevant contribution to total uncertainty. Although additional efforts to improve sample homogeneity, should be carried out; the methodology used proved to discriminate between individuals exposed to different amounts of metal dust, using the estimated uncertainty value at 95% confidence. In conclusion, the proposed analytical method can be useful to biomonitor individuals exposed to metals such as, Sb and Pb, using EBC.

4.5 Acknowledgements

The authors gratefully acknowledge Fundação para a Ciência e Tecnologia (FCT) for funding the project PTDC/AMB/65828/2006 - Exhaled breath condensate: a tool for noninvasive evaluation of pollutant exposure?

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Chapter 5

Assessment of exposure to metals in lead processing industries

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Abstract

Inhalation of particulate matter in industrial environments has been associated with respiratory symptoms and lung diseases, which continues to lead to long- and short-term hazardous health effects on exposed subjects. The main objectives of this study were a) to determine the dust exposure of workers from the lead industry in different operations and b) to evaluate if the Exhaled Breath Condensate (EBC) can be used as a non-invasive tool to evaluate this exposure. Therefore, this cross-sectional study not only measured the exposure to Airborne Particulate Matter (APM) and to the associated elements but also analysed the EBC elemental composition. APM was collected in Industry1, Industry2, Offices and outdoor with Gent samplers, which delivers two size fractions: fine particulate matter (< 2.5μm), and coarse particulate matter (between 2.5 and 10μm). EBC samples were collected from the workers and from a non-exposed group working in Offices. The techniques INAA and PIXE were used for the APM element characterization and ICP-MS for EBC elemental content. The $PM_{2.5}$ and $PM_{2.5-10}$ mass concentrations were significantly higher in the industries studied than in Offices and in the environment. At the industrial sites surveyed the coarse fraction dominated and both factories had different fingerprints: APM elements with higher expression were Pb, Sb, Na, Cl and Fe in Ind1 and Pb, Si, Br, Ca, Al, Cl and Na in Ind2. Most of these elements revealed a gradient of concentration where Ind1 > Ind2 > Offices and EBC revealed a clear translation of this exposure, suggesting the latter to be a potential good indicator of exposure to metals in occupational settings. Pb in EBC presented the most representative results. Even though EBC was found to reflect predominantly the inhaled coarser fraction it is more related to concentration levels of exposure than to the predominance of APM fraction.

The present study demonstrated not only the ability of EBC to reflect environmental exposure to metals but also the importance of measuring and characterizing different fractions of APM for a correct assessment.

5.1 Introduction

Epidemiological studies have consistently shown an association between Airborne Particulate Matter (APM) pollution and the number of deaths from cancer, cardiovascular and respiratory diseases (Brook et al., 2010; Pope, 2007). There is also evidence linking particulate air pollution, especially the fine particle fraction ($PM_{2.5}$, denoting PM < 2.5 μm diameter size), and increases in hospital admissions for respiratory and cardiovascular diseases (Zanobetti et al., 2009; Pope et al., 2008). Several studies demonstrated that particles can induce alveolar inflammation, with release of mediators capable of causing exacerbations of lung diseases and increasing blood coagulability in susceptible individuals, thus, also explaining the observed increases in cardiovascular deaths associated with urban pollution episodes (Brook et al., 2010). Epidemiological data from the USA suggested that rises of 10 $\mu g.m^{-3}$ in $PM_{2.5}$ are accompanied by an increase in relative mortality risk of about 4%, including elevated risks from both cardiopulmonary mortality (6%) and lung cancer mortality (8%) (Pope et al., 2002).

In industrial scenarios, particles are a major concern. Firstly, dust concentrations inside industries are very high comparing with the environment. Almeida et al. (2010) showed that PM_{10} concentrations in a foundry industry reached values 50 times higher than in the outdoor air. Secondly, there are more toxic compounds in the composition of the particles sampled inside the industries. In the same study, Almeida et al. (2010) measured concentrations of lead 35000 times higher in PM_{10} sampled in the foundry industry than in the atmosphere. Thirdly, the majority of the people spend more time in the workplace (33% of the day) than in the outdoor (10% of the day) (USEPA, 1989; Oliveira Fernandes et al., 2009), therefore, exposure to pollutants in the workplace is more relevant than outdoor pollution.

In industrial environments, particle size appears to be dependent on the source type. Among metal processing industries, the foundry/smelting and welding processes contribute mostly to the particles with lower granulometries (Wake et al., 2002), whereas cleaning processes, handwork and cut emit mainly coarse particles (e.g. Hlavay et al., 1992; Cheng et al., 2008). Moreover, the type of processes and the raw material used are associated with characteristic emission regarding the combination of metals and chemical compounds in each particle size fraction. Karlsen et al. (1992) showed the differences between the chemical composition and the morphology of particles provided from welding fumes and gridding dust.

Despite the technological requirements, imposed by safety regulations, that guarantee an improvement in indoor industrial air quality, workers continue to be excessively exposed to APM (e.g. Goldoni et al., 2004; Nawrot et al., 2008; Almeida et al., 2010; Félix et al., 2012). This occurs, not only due to the

ineffectiveness or inexistence of equipment that promotes the extraction of particles or protects the workers individually, but also to the negligence of the subject himself.

Exhaled Breath Condensate (EBC) has been progressively considered a potential bioindicator of exposure especially fitted to occupational assessments due to several features: (1) it is non-invasive; (2) quickly collected; (3) representative of the organ of direct contact with the toxicant (the lungs); (4) it is not aggressive for the subject, allowing the possibility of repeated collections; (5) and easily analysed, without complex protocols of sample preparation (Hunt, 2002; Goldoni et al., 2004; Caglieri et al., 2006; Cao and Duan, 2006; Almeida et al., 2010; Gube et al., 2010; Hoffmeyer, et al., 2011; Félix et al., 2012).

The aim of the present study was to determine the exposure level of workers in their workplace in two lead processing factories, by determining airborne elemental concentrations, and evaluate the potential of EBC as medium for the quantification of biomarkers of such exposure. For that, metal concentrations were measured both in the APM collected in different sites at the workplace and in the EBC of workers.

5.2 Materials and Methods

5.2.1 Industry and study group

The present study was performed with the collaboration of two industries that process lead: Industry 1 (Ind1) that recycles batteries and Industry 2 (Ind2) that produces batteries. Although dealing with the same contaminant, both factories have distinct features, such as, physical processes and fabrication, number of employees, air extraction requirements and architectonics. The foundry industry (Ind1) has two contiguous areas in an open space, one with two furnaces for lead meltdown, and a second where workers re-melt lead with additives for refinement. The area of the factory is not a closed area due to material transfer requirements. The facility has fumes and dust extractors running, according to national regulations. There are no fixed work-posts. The second industry (Ind2) is a battery assembling facility where workplaces are divided into two categories: lead plates cut (automatic and manual); and assembling lines. Each workplace has air extractors for particle removal originated from the work activities. Workers alternate frequently in their workplace between automatic and manual cuts and within the several posts in the assembling line, but not between the two main categories of manufacturing. In both factories, air quality and appropriate personal safety

measures are implemented as imposed by national regulations and all personnel is required to wear protective clothes, glasses and masks.

Ind1 labours 24h a day, on three 8h shifts and Ind2 operates only on two 8h shifts. Workers from both factories labour five days a week with two intercalary resting days.

In Ind1, 17 workers participated in the study whereas in Ind2 83 workers were enrolled, all labouring for more than five years in the factory. For reference purposes, a group of 54 volunteers working in Offices and not exposed to gases, dusts or fumes in their working activity was also constituted. All subjects gave their informed consent to participate in the study and filled a questionnaire reporting on age, smoking habits, gender and past respiratory diseases (the selected did not report any respiratory pathologic history).

5.2.2 Airborne Particulate Matter

Sampling

APM was collected with low volume Gent samplers (Maenhaut, 1992). Gent samplers were equipped with a PM_{10} pre-impactor stage and with a Stacked Filter Unit (SFU) of two stages, carrying 47 mm Nuclepore™ polycarbonate filters. Air was sampled at 15-16 $L.min^{-1}$, which allowed the collection of particles with aerodynamic diameter (AD) between 2.5 and 10 μm in the first stage and particles with AD < 2.5 μm in the second stage.

Sampling was carried out in factories using several Gent samples working in parallel. Samplers were operated during labouring period at 1.6 m high, which corresponds to the breathing height of workers, thereby ensuring the best representativeness of working conditions.

In Ind1 four samplers were used in parallel distributed throughout the factory, in representation of the whole labouring area. In Ind2, where activities are divided in different rooms and/or areas, four samplers were distributed along each manufacturing line (manual and automatic plates cut and assembly).

For reference purposes, sampling was also carried out in Offices placed in the same geographical area of the studied factories (30 km maximum distance) and in outdoor environment. In Offices one Gent sampler was used per flat. Also, in the outdoor environment only one Gent sampler was used (Almeida et al., 2005; Almeida et al., 2006a). Data regarding sampling parameters are resumed in Table 1.

Table 1 - Sampling parameters: sampling time and sampled $PM_{2.5}$ and $PM_{2.5-10}$ mass, at a rate flow of 16L.min^{-1}. Average values and range (when applied). Sampling time was optimized in order to collect enough mass for the chemical analysis and to prevent the clogging of the filters.

	Sampling time (hour/sampler)	$PM_{2.5}$ Mass (μg)	$PM_{2.5-10}$ Mass (μg)
Offices	30	300 [250-370]	380 [350-400]
Environment	24	340 [15-630]	410 [92-2000]
Industry 1	1	440 [280-590]	1100 [400-1900]
Industry 2	4	160 [110-210]	250 [150-390]

Chemical analysis

The filter loads were measured by gravimetric means in a clean room (ISO7). Nuclepore filters were weighted using a 0.1 μg sensitivity balance (Mettler Toledo UMT5). Filter mass before and after sampling was obtained as the average of at least three measurements, assuring that the variation coefficient < 5%.

Each filter was divided providing portions suited for elemental analysis by Particle Induced X-Ray Emission (PIXE) and by Instrumental Neutron Activation Analysis (INAA). The techniques are multi-elemental and complementary in terms of detected elements (Almeida et al., 2006b). The elements As, Br, Cd, Cr, Na, Sb, Se and Sn were determined by INAA and Al, Ca, Cl, Cu, Mn, Ni, Pb, Si, Ti and V by PIXE. K, Fe and Zn concentrations were measured by both techniques with a good agreement and therefore an average of both techniques was calculated for each sample.

Due logistic reasons, INAA analysis (De Soete et al., 1972) of air sampling filters collected at Ind1 was performed in the Portuguese Research Reactor of ITN; and air sampling filters from Ind2 were analysed at the Higher Education Reactor of TU Delft, The Netherlands.

At ITN, the portion of the filter to be analysed by INAA was rolled up and put into a thin foil of aluminium and irradiated for 5-h at a thermal neutron flux of 1.03×10^{13} cm^{-2}s^{-1}. After irradiation the sample was removed from the aluminium foil and transferred to a polyethylene container. For each irradiated sample, two gamma spectra were measured with a hyperpure germanium detector: one spectrum 3 days after the irradiation and the other one after 4 weeks. The distance between the sample and detector was the smallest possible that tried to guarantee a dead

time lower than 12%, however, typically 66.6mm for the first measurement (3 days after irradiation) and 11.6mm for the second (4 weeks after irradiation). The k_0-INAA method (De Corte, 1987) was used and 0.1% Au–Al discs were co-irradiated as comparators. The software k_0-IAEA was used to interpret the spectra and to calculate the concentrations.

In Delft, the irradiation procedure consisted of packing the filter in high purity polyethylene capsules and irradiating for 5-h at a thermal neutron flux of 5.10×10^{12} $cm^{-2}s^{-1}$. After irradiation three gamma spectra were measured with a hyperpure germanium well-type detector: the first spectrum was measured after 2 days, the second after 4 days and the third one after 3 weeks. The k_0-INAA method was also used and Zn was co-irradiated as comparator. The software k_0-IAEA was used to interpret the spectra and to calculate the concentrations.

PIXE (Johansson et al., 1995) analysis was carried out at ITN Van de Graaff accelerator, in vacuum. A proton beam of 2.4 MeV was used and X-ray spectra were detected using a Si(Li) detector. Filters were mounted on appropriate Teflon™ sample holders and analysed with no further treatment. The beam area at the target was 20 mm^2.

Previous to the sampling campaign, tests of reproducibility within the filters and between filters were taken, using parallel sampling with two similar sampling units. The details of sampling and analytical control tests are given in Almeida et al. (2003) and Ammerlaan and Bode (2009).

5.2.3 Exhaled Breath Condensate

Sampling

EBC was collected with commercial equipment EcoScreen (Jager, Germany) that consists of an electric refrigerated system with an extendable arm that allows the subject to sit upright on a chair and exhale into the cooled chamber. The equipment had two unidirectional valves that prevent inhaled and exhaled air to mix in the collection tube. The valve system was designed to act also as a saliva trap. The temperature of the condenser was kept constant throughout the collection period, which guarantees the efficiency of the device on aggregating the droplets of the exhaled air.

Subjects were asked to clean the oral cavity with water, rinsing and spitting out repeatedly, before collection, and to breathe tidally for 15 min, forming a seal around the mouthpiece with their lips. A nose clip was used to prevent air intake through the nostrils. Sample collection was carried out within the factory site at

occupational health units, which are located in different buildings away from the workplace. EBC of the non-exposed group was collected in a clean room (ISO7). The sampling periods for workers were: A- before the beginning of the shift, on the first day of the week; and B- after the end of the shift on the last day of the week. The two sampling periods were chosen as the most representative of the exposure through the 5-day period of work. EBC aliquots were pipetted into polypropylene containers, previously cleaned with HNO_3 suprapur (20% v/v) and acidified with 3% of the same solution, prior to storage at -80°C, according to the method described in Félix et al. (2012).

Chemical analysis

Elemental concentrations in EBC samples were determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS). The ICP-MS equipment (ELAN DRC-e, PerkinElmer SCIEX, USA) was operated at 1100 W (RF power) and with a nebulizer gas flow of 0.85 $L.min^{-1}$. The ICP-MS was equipped with nickel cones and a Peltier-cooled quartz cyclonic spray chamber, fitted for low volume samples. Data was collected, processed and analysed with ELAN software v3.4.

Quantitative analysis was carried out based on an external calibration. The calibration standards were prepared with an ICP-MS multielement standard solution Certipur® 11355 (Merck) and several dilutions were prepared to match the necessary range of concentrations, depending on the element. Ultrapure water of 18MΩ.cm was obtained from a Milli-Q Element® water system (Millipore Corp., MA) and used for dilution of stock solutions and to prepare blanks. An Yttrium (Y) solution, containing ^{89}Y (AAS Specpure® Y solution 1000 ± 10 $mg.mL^{-1}$ Alpha Aesar) was used as an internal standard and HNO_3 suprapur grade (Merck) was used for the acidification of solutions. The ICP-MS elemental analysis measured the most abundant natural isotopes of each element. For Pb quantification the isotopes ^{206}Pb, ^{207}Pb and ^{208}Pb were measured to account for Pb isotope ratio variation in each sample.

The EBC samples were spiked with Y (10 $\mu g.L^{-1}$) and diluted 1:5 (v/v) in acidified water at 1% (v/v). For blanks acidified water (1% (v/v)) was used, spiked with the same concentration of internal standard as the EBC samples.

5.2.4 Statistical Analysis

Mann-Whitney U-tests were used for the determination of differences between EBC samples from exposed and non-exposed group and between the APM

elemental concentrations from Industries and Offices. Correlation analyses were performed in APM samples collected using the Spearman's rank correlation coefficient. The linear correlation between Pb in APM and EBC-Pb was evaluated through the Pearson's correlation coefficient, for both phases (*a* and *b*) and using Offices, Ind1 and both isolated areas of Ind2 (manual and automatic plates cut and assembly), as workers do not alternate between these two main categories of manufacturing.

A Canonical Correspondence Analysis (CCA) was used to determine patterns in the characteristic emissions of workplaces and its correlation with EBC. CCA is a multivariate ordination technique that has the advantage of establishing a direct relation between observations/measurements and environmental gradients, based on correlation coefficients (Ter Braak, 1986; Ter Braak and Prentice, 1988). This eigenvector technique was designed to explore patterns between response variables (APM concentrations in each work-post) and explanatory variables or predictors (workers' elemental concentration levels in EBC) as a co-variable data matrix. This method of direct analysis has an advantage towards other commonly used methods, like regression analysis, due to its aptitude to plot and relate a large number of variables, which in the latter separate analyses may become impractical and difficult to combine, in order to get a reasonable overview.

For this ordination design, Cr, Ni, Cu, Sb and Pb were the analytes used (the descriptors), following the criteria of elements determined with a good degree of confidence in EBC (Félix et al., 2012) and the analytes common to both analyses (APM in filters and EBC).

Statistical results were considered significant at $p<0.05$. Tests were run in Statistica 8.0 (Statsoft®), Origin v7.5 (OriginLab®) and Canoco v4.5 (Biometris®) for the CCA analysis.

5.3 Results

5.3.1 APM levels

Figure 1 presents the $PM_{2.5}$ and $PM_{2.5-10}$ mass concentration measured in Ind1, Ind2, Offices and Environment. APM levels in both industrial sites were notably higher than in Offices and in Environment. Nonetheless, PM_{10} (sum of both fractions) and $PM_{2.5}$ average concentrations measured in Ind1 (1440 $\mu g.m^{-3}$ and 420 $\mu g.m^{-3}$, respectively) and in Ind2 (125 $\mu g.m^{-3}$ and 60 $\mu g.m^{-3}$, respectively) did not exceed the limit value for respirable particles - PM_4 (3000 $\mu g.m^{-3}$) established by the Portuguese NP1796 (Occupational Exposure Limits to Chemical Agents). In

Offices, Environment and Ind2, the coarse fraction was slightly higher than 50% of total mass. In Ind1, this $PM_{2.5-10}$ fraction contributed with over 70% for PM_{10} (Figure 1).

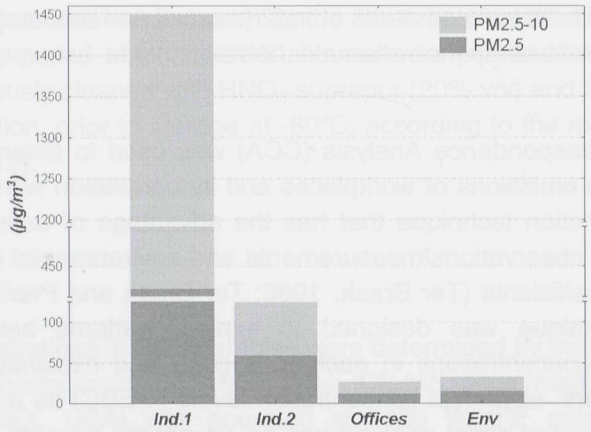

Figure 1 – Particulate matter concentration ($\mu g.m^{-3}$) in the four sampling sites for both fractions (fine and coarse). The total length of the bars represents PM_{10} or total mass. Scale-breaks to each fraction were made in the same percentage, so the illustration proportion would sustain.

Ind1 presented significantly higher $PM_{2.5}$ and $PM_{2.5-10}$ mass concentrations than Ind2 ($p=0.0001$). In Ind2 significant differences occur between different work places for $PM_{2.5}$ ($p=0.04$) with higher mass concentrations at the assembling line sections (Figure 2) and $PM_{2.5-10}$ had higher levels in the cut section, shown in Figure 2 ($p=0.05$).

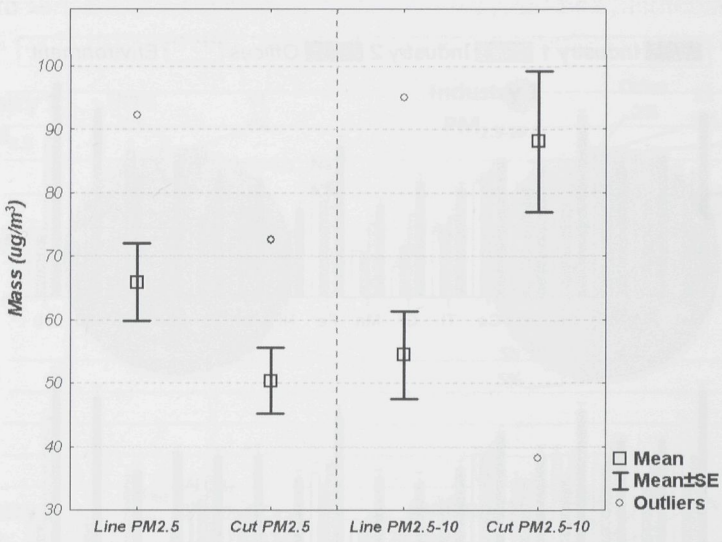

Figure 2 – Average mass concentrations ($\mu g.m^{-3}$) in APM sampled in both Assembling Line and Cut sections of Industry 2.

5.3.2 APM chemical composition

Figure 3 presents the average element concentration measured in the 4 sampling sites. The industrial sites were easily identified for their significantly higher concentrations of Br, Sb, Pb and Fe. In the industries, Pb concentration in PM_{10} was $4x10^4$ (Ind1) and $2x10^3$ (Ind2) times higher than in Offices and Environment.

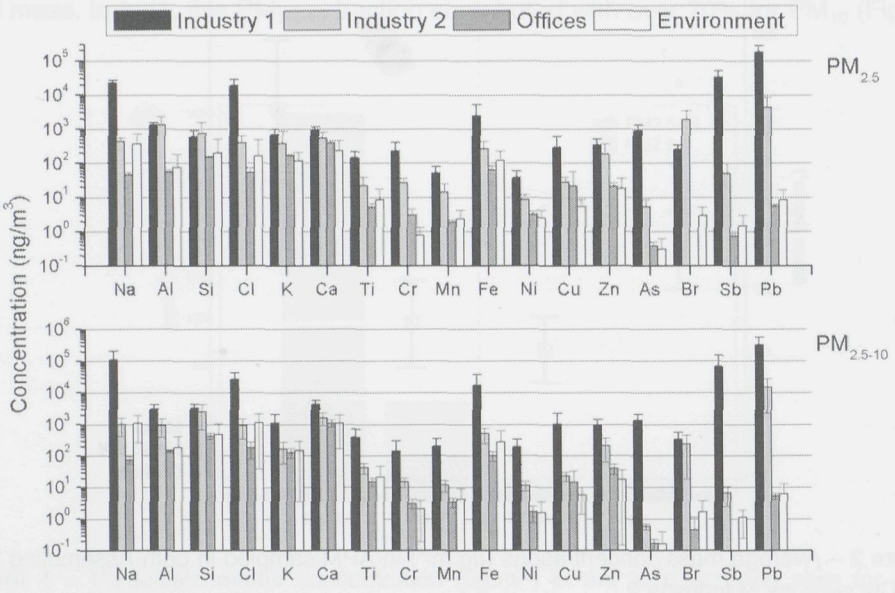

Figure 3 – Average element mass concentration ($ng.m^{-3}$ ±SE) in APM sampled in the Industry 1, Industry 2, Offices and Environment.

The contribution of the most represented elements for $PM_{2.5}$ and $PM_{2.5-10}$ can be depicted in Figure 4 (only elements with a contribution higher than 1% to the total mass were presented individually). The elements with higher expression for $PM_{2.5}$ (Fig.4A) in the combustion and refining processes in the recycling industry (Ind1) were Pb, Sb, Na, Cl. For $PM_{2.5-10}$ (Fig.4B), adding to the previous elements, also Fe had a contribution higher than 1% to the total mass. For the battery production industry (Ind2), Pb, Al, Br, Si and Ca for $PM_{2.5}$ (Fig.4C) and Pb, Si, Ca, Na, Cl, and Al for $PM_{2.5-10}$ (Fig.4D) were the major elements. The remaining mass fraction in Figure 4 corresponds to non-analysed elements and compounds that contributed to total mass. In both industries, the most represented element in $PM_{2.5}$ and $PM_{2.5-10}$ was Pb. The relative contribution of this element in Ind1 was always above Ind2, for both PM fractions. In Ind1 Pb in $PM_{2.5}$ was higher than in $PM_{2.5-10}$ (41% over 32%, respectively), whereas in Ind2 this tendency was inverted (8% under 19%, respectively), where $PM_{2.5-10}$ showed higher values of Pb. In Ind1 Sb contribution for APM levels was also very relevant. Na and Cl concentrations in this site were associated with additives used for lead refinement in the crucibles. Cl showed a strong correlation with Pb ($r=0.96$; $p<0.0001$ for $PM_{2.5}$).

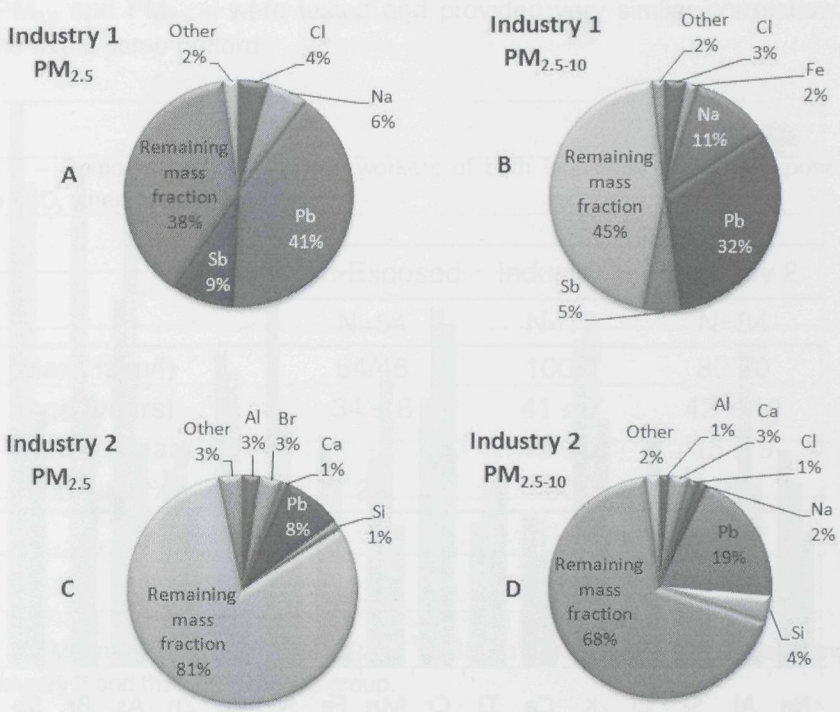

Figure 4 – Contribution of each element measured (percentage of total mass). "Other" refers to the sum of elements with contributions lower than 1%.

Although respirable particles' concentration did not exceed legal exposure limits, the Pb concentration in Ind1 (500 $\mu g.m^{-3}$) significantly exceeds the limit value (50 $\mu g.m^{-3}$) established by the Portuguese NP1796 for this element.

Figure 5 shows the ratio $PM_{2.5}/PM_{10}$ for the measured elements. Regarding the industrial sites, except for Pb, Cl and Ti, Ind1 had the lowest ratios, revealing that the coarse fraction for the remaining elements was more represented in this site than in Ind2. In Ind2, the elemental concentrations of Al, K, Cr, Mn, Cu, As, Br and Sb were more represented in the fine fraction. This fact could be explained by the association of these elements with some of the welding processes carried out in Ind2. In contrast, Pb was more associated with the coarse fraction when compared to Ind1. This could be due to the fact that in Ind2 the processes are mostly physical and mechanical (machining, abrasion and cut of Pb), whereas in Ind1 smelting

processes dominate, therefore Pb fumes are generated in the combustion, which can originate secondary fine particles.

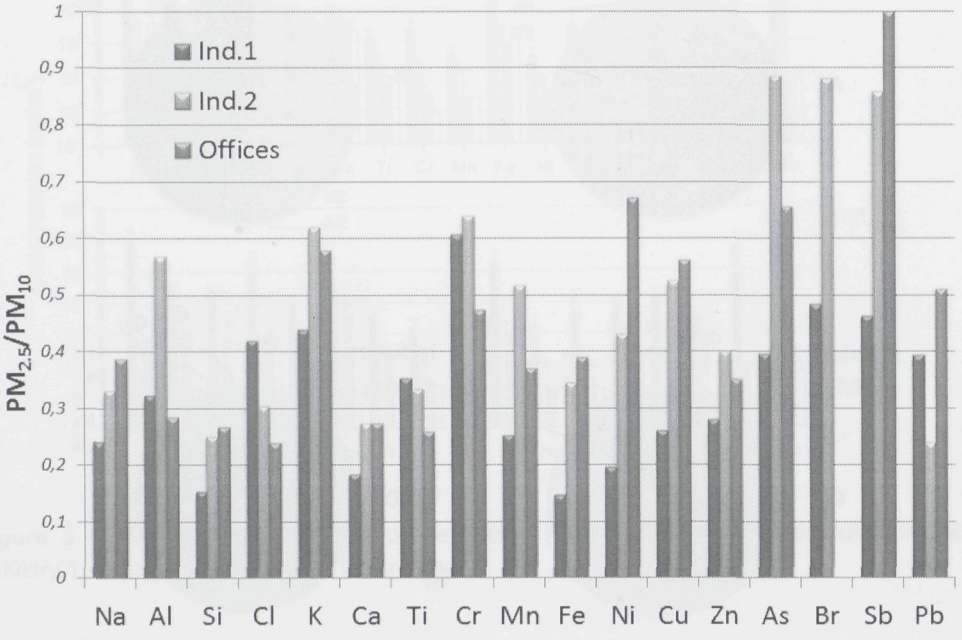

Figure 5 – Ratio between $PM_{2.5}$ and PM_{10} for Industry 1, Industry 2 and Offices.

5.3.3 EBC

The data characterizing workers and the non-exposed group is represented in Table 2. Table 3 presents the mean Cr, Ni, Cu, Sb, and Pb concentrations measured in the EBC of workers from Ind1 and Ind2 and of non-exposed individuals. EBC results showed a significant difference between workers and non-exposed individuals for all elements, although with a less evident difference for Sb and Cu in Ind2. Except for Cr, elemental concentrations from Ind1 workers were significantly higher than from Ind2, reflecting the influence of exposure to high levels of these pollutants according to the APM elemental concentrations measured (see Figure 3). No influence of the possible confounders such as smoking habits, gender and age was found. For Pb, the main pollutant in these industries there was a positive linear correlation between Pb in APM and the biomarker EBC-Pb, either with sampling phase *a* and phase *b*, showing a distance between both fits (Figure 6). This distance is more evident in the plot for the two

higher exposure levels in Ind2 - cut section - and Ind1 (higher exposure level). Correlation was made with PM_{10}, representing full exposure, although correlations with $PM_{2.5}$ and $PM_{2.5-10}$ were tested and provided very similar correlation results and the exact same pattern.

Table 2 – Demographic data on the workers of both industries and non-exposed group (mean ±SD, when applicable).

	Non-Exposed	Industry 1	Industry 2
	N=54	N=17	N=84
Sex (% m/f)	54/46	100/0	80/20
Age (years)	34 ± 8	41 ± 7	42 ± 10
Working years	-	13 ± 7	15 ± 9
Smokers (%)	24	30	38

Table 3 – Mean concentration values ($\mu g.L^{-1}$ ± SE) in EBC from workers of both Industry 1 and Industry 2 and the non-exposed group.

	Cr	Ni	Cu	Sb	Pb
Industry 1	3.1 ± 0.5*	5.5 ± 0.7*,§	5.4 ± 0.9*,§	1.5 ± 0.2*,§	34 ± 3*,§
Industry 2	2.3 ± 0.2*	1.63 ± 0.10*,§	1.64 ± 0.12¥,§	0.08 ± 0.02¥,§	2.2 ± 0.3*,§
Non-Exposed	0.83 ± 0.09	0.43 ± 0.07	1.4 ± 0.2	0.04 ± 0.007	0.9 ± 0.2

Significant difference with non-exposed group: * for $p < 0.0008$ and ¥ for $p = 0.02$; § Significant difference between Ind.1 and Ind.2.

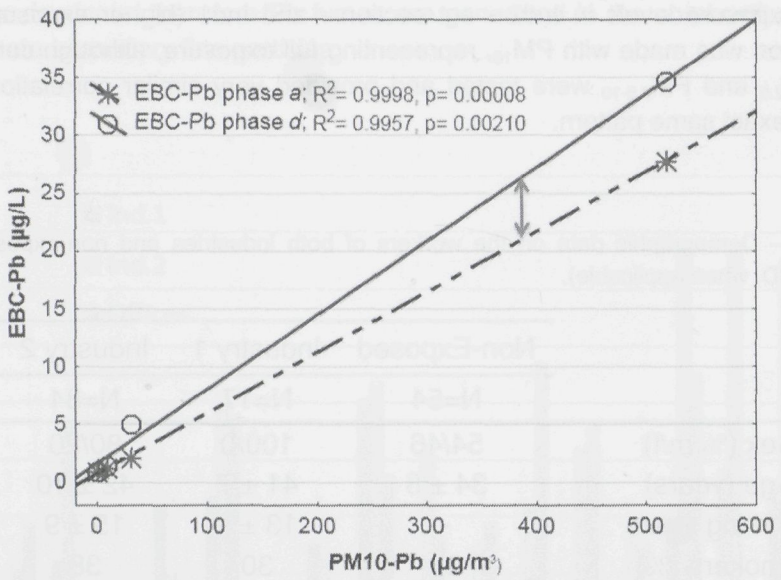

Figure 6 - Multiple correlation and linear fit between average Pb in PM_{10} and EBC-Pb (phases *a* and *b*). Data from Offices, Ind1 and Ind2. The latter with to sampling sites - assembling line and plate cut. Double arrow indicating the distance between both linear fits. Pearson correlation parameters (R^2 and p-value) represented.

5.3.4 Impact of workplace emissions on the EBC

At Ind2 the distribution of workers between two main activities (assembling lines and Pb plates cut section) allowed assessing the impression of the workplace emissions on the EBC, based on the known differences of APM in both sites (see Figure 2).

On the CCA diagram obtained for both sections of Ind2, the first two axes accounted for 80.2 % of total variance (APM concentration data) and the EBC data had a good correlation with both axes, r=0.953 with the first and r=0.951 with the second (Figure 7). In this diagram sites are represented by dots (different shapes represent attributed classes for each site, for easy distinction) and EBC represented by arrows. The axes are extracted (orthogonally) explaining the maximum variance and the graphical representation depicts the dispersion based on the composition of each site, in terms of elements present (Cr, Ni, Cu, Sb and Pb) and the relative importance of these elements within each site and for each PM fraction. In this representation, proximity of the dots implies similarity. Regarding the arrows, representing the EBC values, the position of the arrow head depends on the eigenvalues of the axes and the intraset correlations of these variables with

the axes. The relation between EBC values (arrows) and APM levels (dots) is read by an extension of the arrow in both directions (imagining or drawing) and connect it to each dot by perpendicular lines. Regardless of distance from the dot to the arrow line, proximity to the head of the arrow indicates large positive correlation, whereas an opposite projection of the arrow indicates negative correlation.

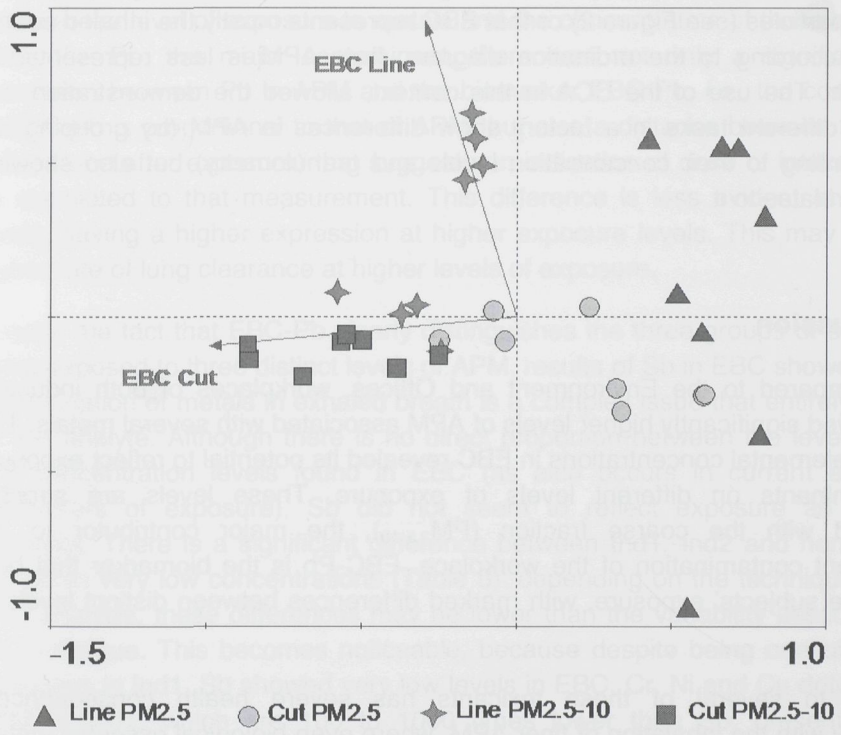

Figure 7 – Canonical Correspondence Analysis of the assembling line and cut sections in APM of Industry 2 and EBC from workers of each section (vectors). Analysis carried out using the elemental concentrations of Cr, Ni, Cu, Sb and Pb.

In Figure 7, it is possible to distinguish two main groups along the first axis (xx, which explains the most variance): one in the right section of the diagram corresponding to the finer fraction and a second to the left composed mainly by the coarser fraction (of both sections). The second axis (yy), although with less explained variance, essentially distinguishes the workplace "line" (from the centre up) from "cut" sections (from the centre down). This indicates that each site is characteristic and distinguishable by its APM fraction and elemental composition. The two dots close to the centre (Cut $PM_{2.5}$), typically indicates the presence of

homogeneous concentration levels of every element (the descriptors) in the analysis, meaning that these particular samples do not show a marked distinction.

The vectors representing EBC from workers, both from the assembling line and cut sections have a strong association with $PM_{2.5-10}$, suggesting that higher values measured in EBC occur in subjects working in environments with larger proportion of coarse particles (see Figure 2) or that EBC represents mostly the inhaled coarse fraction. According to the ordination diagram, finer APM is less represented in exhaled air. The use of the CCA in this context, allowed the demonstration, not only, that different tasks in a factory show differences in APM (by grouping the sites according to their concentration levels and granulometry) but also showing how EBC relates to it.

5.4 Discussion

When compared to the Environment and Offices, workplaces of both industrial sites showed significantly higher levels of APM associated with several metals. The analysed elemental concentrations in EBC revealed its potential to reflect exposure to contaminants on different levels of exposure. These levels are strongly associated with the coarse fraction ($PM_{2.5-10}$), the major contributor to the environment contamination of the workplace. EBC-Pb is the biomarker that best reflects the subjects' exposure, with marked differences between distinct levels of exposure.

Exposure to several of these toxicants has severe health consequences, particularly with the inhalation of finer APM, where even biological essential metals can have a toxic and carcinogenic effect depending on several factors, including dose and route of intake (Santamaria et al., 1990; Godish, 2003; ATDSR, 2007; Beyersmann & Hartwig, 2008). Thus, the determination of different APM fractions in the workplace, along with their proportion, is key for a correct assessment of exposure. Particularly for Pb, exposure can have long term consequences due to its preferential accumulation in the bone, where its half-life is longer as a consequence of its slow turn-over rate. This phenomenon can induce endogenous contamination, even long after exposure ceased (reviewed in Barbosa et al., 2005). Both Pb processing industries, although dealing with the same raw material, do not have the same 'fingerprint'. The elemental mass concentrations showed that the combustion processes had higher emissions of combustion related elements than manual processes for both fine and coarser particles. Apart from Pb, the major pollutant, there were observable variations: Sb had a considerable expression in Ind1, but not in Ind2 and higher concentrations of Si were measured in Ind2. The Pb-Cl correlation, found in Ind1 for $PM_{2.5}$, was in agreement with studies that state

this as a common phenomenon in combustion processes and that usually occurs in smaller particles (Winchester et al., 1967; Paciga et al., 1975).

EBC revealed workers were significantly exposed, presenting higher concentration values in Ind1, for all analysed elements and revealing a gradient of exposure (Ind1 > Ind2 > N-Exposed). These values showed a coherent correspondence between the level of exposure to metals and the concentration of those analytes in EBC. For Pb, the major contaminant, there was not only a significant linear correlation between Pb in APM and the biomarker EBC-Pb, *i.e.*, the concentration of EBC-Pb is proportional to that of APM, but also a distance between fit *a* and *b* (before and after exposure) that suggests an indication of dose of intake and may be correlated to that measurement. This difference is less evident at the lower levels, having a higher expression at higher exposure levels. This may indicate a higher rate of lung clearance at higher levels of exposure.

Despite the fact that EBC-Pb clearly distinguishes the three groups of subjects as being exposed to three distinct levels of APM, results of Sb in EBC showed that the determination of metals in exhaled breath is a complex issue that entirely depends on the analyte. Although there is no direct proportion between the levels of APM and concentration levels found in EBC (as also occurs in current established biomarkers of exposure), Sb did not seem to reflect exposure as did other elements. There is a significant difference between Ind1, Ind2 and non-exposed, but within very low concentrations (Table 3): depending on the technique used for EBC analysis, these differences may be lower than the variability associated with the technique. This becomes noticeable, because despite being one of the main emissions in Ind1, Sb showed very low levels in EBC. Cr, Ni and Cu determined in APM of Ind1, which are 100 to 1000 times lower than Sb, presented higher concentration levels in EBC. Given the low solubility of Sb and its inorganic compounds in the pulmonary surface as well as its slow lung clearance (Felicetti et al., 1974; Gerhardsson et al., 1982; Leffler et al., 1984; Gebel, 1997), it would be intuitive to conclude that non-absorbed Sb should be available to aerosolise in the respiratory airways, especially in a scenario of continuous exposure, but this is apparently not so. Even given the potential mechanisms of retention like specific protein binding, such as to metallothioneins, or to globulins (specially alpha-globulin family), which act as scavengers, this would also be true for other elements, like Pb which occurs on its free ionized state only in a residual fraction (Quintanilla-Vega et al., 1995; ATSDR, 2007; Tylenda and Fowler, 2007) and is still markedly present in EBC.

Hence, one of the difficulties on determining the representativeness of EBC from what is deposited in the lung lining fluid is the section of the lungs where EBC is

formed. The distribution of aerosols in to 2 main fractions (coarse and fine) in Ind2, revealed relatively constant and correlated values within each fraction, as shown in the CCA. This indicates a relatively steady exposure of the workers labouring at those workplaces. The correlation found between EBC and coarse fraction indicated that $PM_{2.5-10}$ was more represented in EBC than $PM_{2.5}$. Under the assumption that finer particles penetrate deeper into the respiratory tract (Santamaria et al., 1990; Godish, 2003; ATDSR, 2007), this could point towards the preferential area of aerosol formation in the lungs. A recent study (Bondesson et al., 2009) revealed that aerosols that constitute EBC are preferentially formed in the central area of the lungs and secondarily in the alveolar lining fluid, revealing an agreement with results presented in this study. However, despite EBC may predominantly reflect the inhaled coarser fraction, this does not seem to cancel its ability as a matrix for biomarkers of exposure. Still, the total elemental concentration measured in the EBC will necessarily depend on the amount and proportion of each fraction.

Thus, even though EBC presents a consistent reflection of exposure it would appear that its levels on subjects exposed to a higher proportion of finer APM for a given element would be underestimated. When comparing different levels of exposure (Ind1, Ind2 and Offices), sites with similar ratios for a given element (Figure 5) apparently correspond to a lesser distance between EBC values of workers from those sites, e.g., Cr in Ind1 vs. Ind2, Cu and Sb in Ind2 vs. non-exposed. Nonetheless, these differences between occupational environments are still evident and significant and, when this is the case, in spite of the similarity between ratios, EBC concentrations still express exposure variations. This can be substantiated by the fact that even though the ratios of Cr at the referred sites are equivalent, there are differences in exposure levels, which EBC clearly reflects. Thus, showing that the biomarkers studied are more related to the levels of exposure, than to the predominance of an APM fraction. This is more evident in Pb where APM fraction has a negligible influence. EBC-Cu in Ind2 and non-exposed, on the other hand, have not only approximate ratios but also closer environmental concentration levels and this explains the similar EBC values.

The quantification of the parent compounds in EBC, such as Cr, Ni, Cu and Pb was demonstrated to correspond to the levels of exposure. Especially the successful EBC-Pb results offer an important contribute to the establishment of the latter as a biomarker of exposure. In exposure settings where inhalation is the main route of intake, the use of biomarkers determined at the lung level significantly contributes to the improvement of human biomonitoring. Not only opens a window to the determination of dose of intake, which in turn can lead to an estimation of total body burden for continuously exposed subjects and the establishment of reliable

levels of maximum exposure, but also to the hazardous effects on the organ itself and its direct relation to dose. Determination of dose is a crucial next step in the validity of this biomarker, filling an important gap posed by Blood-Pb, the current established biomarker for Pb exposure, which has the limitation of quantifying systemic lead. The whole blood Pb not only does not reflect tissue accumulated Pb (the highest percentage of absorbed Pb), but it is also biased by the release of stored Pb (Hu et al., 1998; ATSDR, 2007). Moreover, the use of a non-invasive method such as the EBC collection allows continuous, repetitive measurements, without adverse reactions to the donor, breaking the boundaries associated with invasive methods, such as broncho-alveolar lavage or even blood draw. This is not only a positive trait for the study of human biomonitoring, but also fitted for occupational settings and its demanding requirements, like number of samples and analytical costs (Félix et al., 2012). Also, it is important and advisable that, for an informative monitorization, the profile of the environmental concentration levels in the workplace are evaluated. It should involve the determination of APM fraction in order to know their proportions.

5.5 Conclusions

This study demonstrated the ability of EBC to reflect environmental exposure to metals such as Pb and potentially for Cr, Ni and Cu. The limitations found in the determination of Sb in EBC, do not allow us to consider it as a reliable marker of exposure. The three levels of exposure and their determination of the environmental concentration levels along with its fractioning, not only substantiated and clarified the EBC's effectiveness as a matrix for biomarkers of exposure to metals, but also stressed the importance to know these features – the industry's 'fingerprint'. For the establishment of such biomarkers of exposure, the course of the research on EBC for occupational settings should lead to the determination of dose of intake, suggested to be measurable in EBC.

5.6 Acknowledgements

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Chapter 6

Exhaled Breath Condensate: a suitable matrix to assess occupational exposure to lead?

This chapter is submitted as Félix PM, Almeida SM, Franco, C, Almeida AB, Wolterbeek HTh, Pinheiro T, 2012. Exhaled Breath Condensate: a suitable matrix to assess occupational exposure to lead?

Abstract

Occupational exposure to lead (Pb), although currently lessened, still presents a hazard on human health and, subsequently, current industrial workers need a continuous monitorization, in order to mitigate short- and long-term effects. The search for a good biomarker aims for several aspects, not only concerning its applicability to industrial settings (high number of samples, analytical procedures and level of invasiveness), but also its intrinsic characteristics, like variability, response to exposure, reduced influence of confounders or sensitivity. Exhaled breath condensate (EBC) has been demonstrated to be an emerging tool with a potential to be a reliable matrix to assess exposure to metals. The aim of the present study was to investigate whether the determination of the parent toxicant in EBC (EBC-Pb) is a suitable biomarker of exposure to lead in occupational exposure.

A non-exposed group, not exposed to gases, dusts or fumes in their working activity, and workers from two Pb processing industries, with different levels of exposure were studied. EBC-Pb was quantified and compared to whole blood Pb (B-Pb), the current most used biomarker of chronic exposure, from the same workers.

EBC was demonstrated to be a suitable matrix to assess occupational exposure to Pb and ready to initiate trial research programmes for its implementation, presenting important features required in a biomarker and with advantages regarding the common used biomarker of exposure.

6.1 Introduction

Lead (Pb) is one of the most commonly used heavy metals and it has had wide applications since ancient times due to its ductile and noncorrosive properties (Heskel, 1983). Although present Pb exposures in working and general environments are undoubtedly lower than several decades ago, occupational and environmental exposures to Pb continue to be among the most significant public health problems (Rempel, 1989; Levin and Goldberg, 2000; Gurer-Orhan et al., 2004; Grover et al., 2010). Pb can have adverse effects on many organ systems and exposure to this metal can cause haematological, gastrointestinal, rheumatologic, endocrine, neurological, renal and thyroid problems in humans (Pagliuca et al., 1990; Singh et al., 2000; Dundar et al., 2006; Pekcici et al., 2010).

In industries dealing with Pb, the assessment of occupational exposure is frequently carried out to determine potential risks to the workers' health. This evaluation is performed in two ways: 1) measuring Pb in the workplace air using direct monitoring; and 2) via biological monitoring. Results from direct measuring can be misleading given the various mechanisms of intake and absorption of toxic substances into the human body. Therefore, more reliable results can be determined by biological monitoring, which can lead to an estimation of target site concentration and dose-response (Zielhuis and Henderson, 1986; Christensen and Olsen, 1991; Christensen and Kristiansen, 1994; Christensen, 1995; Rainska et al., 2007).

From its intake, absorption leads Pb through different pathways, commonly bound to proteins and other structures (e.g. Ong and Lee, 1980; Al-Modhefer, 1991; Quintanilla-Vega et al., 1995; Fowler, 1998). Most Pb is accumulated into calcified structures, like bone and teeth, where it can have a half-life from 10 up to 30 years, accounting for over 94% of total body burden in adults (Rabinowitz, 1991; O'Flaherty, 1995; Rust et al., 1999, Lowry et al., 2004; Gwiazda et al., 2005; Karahalil et al., 2007). For the remaining Pb, a significant part is accumulated in other tissues like the liver, skeletal muscle, skin, connective tissue, fat, kidney, brain, and others (Schroeder and Tipton, 1968; DuVal and Fowler, 1989; Fowler, 1989). In blood, about 98% of its Pb load (around 2% of total body burden) binds to several intracellular proteins of erythrocytes (Schütz et al., 1996; Bergdahl et al., 1997, 1998, 1999; Hernandez-Avila et al., 1998; Bannon et al., 2000; Manton et al., 2001; Smith et al., 2002) and only the remaining is found in the plasma, most forming complexes with several of its constituents and simply 1/5000th as free ionized Pb (Pb^{2+}) (Al-Modhefer et al., 1991). A study with exposed subjects to Pb, by Chamberlain et al. (1975) determined that, within 24h, blood Pb (B-Pb) reached

equilibrium (maximum stabled value) for both of those who received the dose intravenously and by inhalation of fine aerodynamic particles. Conclusions such as these lead to the determination of blood as a good mirror of intake and, in fact, whole blood has come to be the most used matrix to assess exposure to Pb. However, in occupational exposed subjects, where inhaled particles vary considerably (Almeida et al., 2010; Pinheiro et al., 2011; Félix et al., 2012a, 2012b), other factors have to come into consideration. Inhaled finer particles are relatively easily absorbed than coarser particles which deposit higher in the respiratory system. This latter fraction, also represented in the exhaled breath (Bondesson et al., 2009; Félix et al., 2012b) is more likely to enter the digestive system, through sputum secretion (that retains particles), removed by ciliated epithelia and reflections like sneezing or cough, than to be absorbed through the lungs, entering the systemic system through potentially different absorption pathways (Guyton and Hall, 2000). Moreover, B-Pb reflects only the systemic Pb, neglecting to provide enough information to estimate a reasonably value of dose. Although we can find variations of B-Pb if an individual reduces or increases exposure (e.g. Araújo et al., 1999), not only the time-lag is high (Pb in erythrocytes with a half-life of approximately 120 days, related to the red blood cells turn over) (Chamberlain et al. 1978; Griffin et al. 1975; Rabinowitz et al. 1976), but also false positives are likely. Episodes of iron (Fe) deficiency, low dietary calcium (Ca), bone reabsorption or osteoporosis can increase B-Pb levels (Mahaffey and Annest 1986; Marcus and Schwartz 1987; Blake and Mann 1983; Heard and Chamberlain 1982). One of the hazards of assessing exposure through the current biomarkers is Pb's accumulation in tissues that is unknown and can last until long after the worker ceased exposure and here lies a possible confounder. With advancing age in exposed workers, and reduction in bone mass (Garn et al., 1967) the levels B-Pb are potentially and increasingly biased by the release of Pb accumulated in bone (Silbergeld et al., 1988; Cory-Slechta et al., 1989; O'Flaherty 1991, 1993; Gulson et al., 1995, 1996). Thus, B-Pb levels can have two simultaneous sources: absorbed Pb and endogenous Pb.

One other common biomarker used to assess Pb exposure is Zinc-protoporphyrin (ZPP). Its accentuated occurrence in the organism results from the inhibition of the enzyme ferrochelatase by Pb. Ferrochelatase catalyses the insertion of Fe into the porphyrin rings in the heme biosynthesis pathway. The result is erythrocytes containing protoporphyrin IX which chelate Zn, forming ZPP internalized for the entire lifetime of the cell (Lamola and Yamane, 1974; Zhang, 1993; Labbé, et al., 1999). For this reason, this biomarker also shows a significant time-lag in variation due to its dependence on the turn-over of the erythrocytes. In fact, ZPP has a higher time-lag in the response to exposure than B-Pb, being also susceptible to endogenous contamination and individual metabolism, resulting in an inter-individual variability (Grunder and Moffitt, 1982; Lerner et al., 1982; Grandjean et

al., 1991; Froom et al., 1998). Furthermore, and similarly to B-Pb, ZPP also has other potential confounders like iron deficiency and even sickle cell anaemia or hyperbilirubinemia that may result in false positives (CDC, 1985; Mahaffey and Annest 1986; Marcus and Schwartz 1987).

The usefulness of a biomarker depends greatly on the target purpose. For occupational exposed subjects, the estimation of dose and the determination of its effect are crucial information in health related issues, especially for Pb that readily accumulates in tissues and that tracking of body burden is easily an undeterminable matter. Recently, Exhaled Breath Condensate (EBC), a body fluid commonly used for determination of oxidative biomarkers in airway inflammations (e.g. Hunt, 2002; Griese et al., 2003; Rahman and Kelly, 2003; Corradi et al., 2004; Garey et al., 2004; Horvath et al., 2005) has been gathering growing evidence of its potential as a tool for the evaluation of pollutant exposure to metal aerosols, airborne particulate and fumes (e.g. Mutlu et al., 2001; Goldoni et al., 2004, 2006, 2008; Caglieri et al., 2006; Félix et al., 2012b). This method allows the collection of inhaled particles that deposit on the surface of the respiratory airways through inhalation (Bondesson et al., 2009), through a simple, non-invasive and quick sampling procedure and, for metal analysis, does not require a complex sample preparation which simplifies the process and reduces the risk of contamination and allows continuous, repetitive measurements, all significant for biomonitoring in occupational settings (Félix et al., 2012a). Pb measurements in EBC were never used for the assessment of workers exposure to this agent and previous studies revealed promising results that enabled the use of EBC-Pb as a future biomarker of exposure to this agent in occupational scenarios (Almeida et al., 2010; Pinheiro et al., 2011; Félix et al., 2012a, 2012b).

The aim of the present study was to investigate whether EBC-Pb is a suitable biomarker of exposure to lead in occupational exposure, to determine the applicability of EBC as a routine-based bioindicator, and the advantages of this matrix regarding the common used bioindicator, whole blood, in a scenario of professional exposure.

6.2 Materials and Methods

6.2.1 Industry and study group

This study was developed with the collaboration of two industries that process lead: Industry 1 (Ind1) that recycles batteries and Industry 2 (Ind2) that produces batteries. Workers from these industries have different levels of exposure to Pb, offering a good base for the study of this element in EBC (Félix et al., 2012b). The

foundry industry (Ind1) has two contiguous areas in an open space, one with two furnaces for lead meltdown, and a second where workers re-melt lead with additives for refinement. There are no fixed work-posts and the facility is constantly opened to the outdoor, due to material transfer needs. The second industry is a battery assembling facility (Ind2) where workplaces have air extractors, for particle removal originated from the work activities. In both facilities, all personnel is required to wear protective clothes, glasses and masks.

Ind1 labours 24h a day, on three 8h shifts and Ind2 operates only on two 8h shifts. Workers from both factories labour five days a week with two intercalary resting days.

In Ind1, 15 workers participated in the study whereas in Ind2, which is a larger factory, there was a collaboration of 77 volunteers. A non-exposed group, constituted 52 volunteers and working in offices, not exposed to gases, dusts or fumes in their working activity, was selected to provide a baseline for EBC measurements. Samples from the latter group were collected during the working week, which is of 35 h, from Monday to Friday.

All the individuals recruited to this study, work and live in the same geographic region of Lisbon, Portugal, and gave their informed consent to participate in the study.

6.2.2 EBC sampling and clinical evaluation

For the EBC sampling procedure, it was used a commercial equipment (EcoScreen, Jager, Germany) that consists of an electric refrigerated system with an extendable arm that allows the subject to sit upright on a chair and exhale into the cooled chamber. The equipment had two unidirectional valves that prevent inhaled and exhaled air to mix in the collection tube and a functional saliva trap (Rosias et al., 2004; Broding et al., 2009). The temperature was kept constant throughout the collection period, which guarantees the efficiency of the device on aggregating the droplets of the exhaled air.

Subjects were asked to clean the oral cavity with water, rinsing and spitting out repeatedly, before collection, and to breathe tidally for 15 min, forming a seal around the mouthpiece with their lips. A nose clip was used to prevent air intake through the nostrils. Sample collection was carried out within the factory complex, protected from the working conditions at its occupational health units. EBC of the non-exposed group was collected in a clean room (ISO7). The sampling periods for workers were: A- before the beginning of the shift, on the first day of the week; and

B- after the end of the shift on the last day of the week. All EBC samples were pipetted into polypropylene containers, previously cleaned with HNO_3 suprapur (20% v/v) and acidified with 3% of the same solution, prior to storage at -80ºC, according to the method described in Félix et al. (2012a). The workers were also asked to fill a questionnaire reporting on demographics, smoking habits and clinical history concerning respiratory or other diseases. They were also clinically evaluated and the respiratory function assessed using a Vitalograph Compact II spirometer (Vitalograph, Ennis, Ireland) and the exhaled nitric oxide (NO) using a portable device NIOX MINO (Aerocrine, Solna, Sweden).

6.2.3 Chemical analysis

Exposure to suspended particles with diameter lower than 10 μm (PM_{10}) and to Pb in PM_{10} was assessed in the three workplaces. PM_{10} was sampled with a low volume Gent sampler and filters loads were measured by gravimetry in order to determine the PM_{10} mass concentration. Pb concentrations were measured by Particle Induced X-Ray Emission (PIXE). Details about the sampling and analysis of particles were presented in Félix et al. (2012b).

Pb concentrations in EBC samples were determined by Inductively Coupled Plasma Mass Spectrometer (ICP-MS). ICP-MS equipment (ELAN DRC-e, PerkinElmer SCIEX, USA) was operated at 1100 W (RF power) and with a nebulizer gas flow of 0.85 $L.min^{-1}$. The ICP-MS was equipped with nickel cones and a Peltier-cooled quartz cyclonic spray chamber, fitted for low volume samples. Data was collected, processed and analysed with ELAN software v3.4.

The calibration standards were prepared with an ICP multi-element standard solution Certipur® 11355 (Merck) and several dilutions were prepared to match the necessary range of concentrations, covering the Pb concentrations present in the EBC of the subjects in the present study. Ultrapure water of 18MΩ.cm was produced by a Milli-Q Element® water system. An Yttrium (Y) solution (AAS Specpure® Y solution 1000 ± 10 $\mu g.mL^{-1}$ Alpha Aesar) was used as an internal standard.

The EBC samples were diluted 1:5 in acidified water 1% v/v and 10 $\mu g.L^{-1}$ of Y was added to each sample, before ICP-MS analysis. For the blank solution it was used ultrapure H_2O containing HNO_3 suprapur (Merck) 1% v/v spiked with the same concentration of internal standard as the EBC samples.

6.2.4 Blood-Pb

As Pb is the major pollutant in the studied factories, the levels of this element in whole blood are regularly measured in the exposed personnel following the National Regulations (National Decree 274/89, 1989). Therefore, for each worker, the industries provided us the blood Pb concentration of the closest date to the EBC collection (20 days maximum difference). Pb concentrations in the blood were measured by Atomic Absorption Spectrometry (AAS). The B-Pb concentration value for a non-exposed group was selected from Reis et al. (2007) that characterises the population of the same geographical area as this study in the same conditions presently observed, namely, the same regional industrial facilities. This measurement (4.3 $\mu g.dL^{-1}$) was included for reference and extrapolation purposes.

6.2.5 Statistical Analysis

Mann-Whitney U-tests were performed to determine differences in EBC-Pb and B-Pb between industries and exposed vs. non-exposed, as well as to determine the influence of confounders (smokers, gender, age, working years). The Wilcoxon routine tested the differences between collection phase (a vs. b). The latter non-parametric tests and all descriptive and exploratory statists were run in Statistica, Statsoft®. Curve estimates were performed to fit the distribution of B-Pb against EBC-Pb, the dependent variable, using SPSS v17, IBM®. The fits explored the possible relations between EBC-Pb and B-Pb and only the best fit was included in the presentation of the results. Relations of EBC-Pb with B-Pb were made with the collection phase *b*, corresponding to the after-exposure period (after the last day's shift). All statistical tests were considered significant at $p<0.05$.

6.3 Results

Table 1 represents the data characterizing workers and non-exposed group. The ages of all groups were equivalent as well as the years of exposure (working years) of subjects from both Ind1 and Ind2. Only differences in sex were found, due to the non-existence of female workers in industry 1. Respiratory symptoms were significantly increased in Ind1 and significant differences between mean values of FEV1 and FVC were also found, although, not in the Tiffeneau index

(Table 2). NO and pH levels measured in EBC did not present significant differences between the studied groups.

Table 1 – Demographic data on the workers of both industries and non-exposed group (mean ±SD, when applicable).

	Non-Exposed	Industry 1	Industry 2
	N=52	N=15	N=77
Sex (m/f)	29/23	15/0	64/17 *
Age (years)	34 ± 8	40 ± 8	42 ± 10
Working years	-	13 ± 8	14 ± 9
Smokers (%)	24	24	38

* Significant differences between both industries ($\chi^2 < 0.001$)

Table 2 – Clinical assessment of the workers of both industries and non-exposed group. Values include mean ±SD (when applicable). Respiratory complaints refer to occasional cough, wheezing and sputum production; FEV1 - mean percentage of Forced Expiratory Volume in the 1st second; FVC - the Forced Vital Capacity; Tiffeneau index = (FEV1/FVC); NO - exhaled nitric oxide.

	Non-Exposed	Industry 1	Industry 2
Allergic complaints (%)	-	20	30
Respiratory symptoms (%)	-	60	16 *
FVC (%)	-	89 ± 13	97 ± 29 *
FEV1 (%)	-	90 ± 14	105 ± 16 *
Tiffeneau índex	-	0.80 ± 0.04	0.83 ± 0.05
NO (ppb)	20 ± 14	18 ± 13	15 ± 10
pH (EBC)	7.5 ± 0.4	7.2 ± 0.9	7.2 ± 0.3

* Significant differences between both industries (Mann-Whitney and χ^2 for continuous and categorical variables, respectively.

Table 3 shows that the groups studied in this work were exposed to three different levels of exposure to particles with diameter lower than 10 μm (PM_{10}) and to Pb in PM_{10}. Workers from Ind1 were exposed to the highest levels of PM_{10} (1440 $\mu g.m^{-3}$) and Pb (518 $\mu g.m^{-3}$) whereas the control group was exposed to the lowest levels of PM_{10} (25 $\mu g.m^{-3}$) and Pb (0.01 $\mu g.m^{-3}$).

Table 3 – Workplace characterization: PM_{10} concentration and Pb concentration in PM_{10} (Félix et al., 2012b).

	Offices (low level)	Industry 2 (intermediate level)	Industry 1 (high level)
PM_{10} ($\mu g.m^{-3}$) ±SE	25 ± 1	125 ± 9	1440 ± 193
Pb - PM_{10} ($\mu g.m^{-3}$) ±SE	0.0108 ± 0.0006	19 ± 4	518 ± 103

Table 4 represents the Pb concentrations in EBC and whole blood from all groups, where it is possible to depict three levels of exposure in EBC-Pb (Ind1, Ind2 and non-exposed) and an increment of concentration values after the shift, at the last day of the working week, but not significant. B-Pb also showed a significant higher mean value in Ind1. Both industries were significantly different from the non-exposed group. No significant influence of the possible confounders smoking habits, gender, age and working years was found for each of the industries' workers or non-exposed group, either for EBC-Pb or B-Pb. However, a slight trend was observed in the increase of B-Pb with increasing age, although not statistically significant (ρ=0.19, p=0.079).

Table 4 – Average lead (Pb) concentrations in EBC ($\mu g.L^{-1}$ ± SE) in workers from Industry 1 (N=15), Industry 2 (N=77) and non-exposed group (N=52); and Blood ($\mu g.dL^{-1}$ ± SE) in workers from Industry 1 and Industry 2. B-Pb of the non-exposed is the mean value of a group of not occupationally exposed subjects of the same geographical area of Lisbon, under similar conditions of the non-exposed group of the present study, from Reis et al. (2007). For EBC-Pb: (a) before shift, first day of the week; (b) after shift, last day of the week.

	a X ($\mu g.L^{-1}$) ± SE	***b*** X ($\mu g.L^{-1}$) ± SE	**B-Pb** X ($\mu g.dL^{-1}$) ± SE
Industry 1	28 ± 5 *¥	36 ± 7 *¥	43 ± 4 ¥
Industry 2	1.6 ± 0.2 *¥§	2.3 ± 0.3 *¥§	19 ± 0.8 ¥
Non-Exposed	0.97 ± 0.25		4.3 ± 0.3 †

* Significant differences to non-exposed; ¥ between industries; and § between collection phase a and b (p<0.001). † From Reis et al. (2007).

No significant correlations were found between B-Pb and EBC-Pb for each Ind1 or Ind2 (Figure 1), although Ind1 showed a different dispersion behaviour and a closer proximity to a significant correlation. Nonetheless, a correlation between B-Pb and EBC-Pb for all workers of both industries was found ($p<0.0001$) in all tested fits. This may indicate that B-Pb might reflect the transport between body pools, in this case, between lungs and other organs and find a pattern between EBC and the transport mechanism of Pb. Therefore, it was inspected if this relation between B-Pb and EBC-Pb could be modelled by a statistical function.

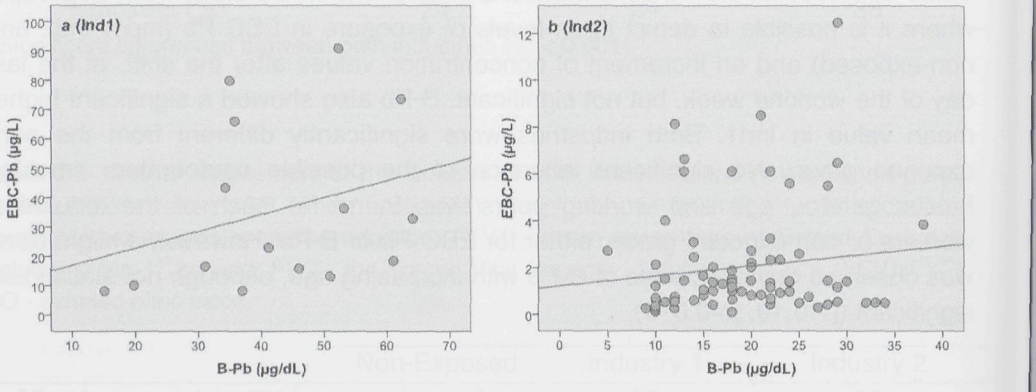

Figure 1 – Scatter plot depicting (a) Industry 1 and (b) Industry 2 values of B-Pb ($\mu g.dL^{-1}$) against EBC-Pb ($\mu g.L^{-1}$) and respective trend lines.

Figure 2 depicts the best fit for this distribution, from all that were tested (linear, exponential and logistic). The linear fit did not reveal acceptable results, with a y-interception at a negative value, and the logistic fit showed very similar results with the exponential fit, indicating that the data is comprised within the exponential area of the curve. This is true regardless of the logistic's upper bound levels tested. The exponential fit had an R^2 of 0.397 with a p-value of 0.0001. Besides the significance of the fits, the ability of the models to predict a B-Pb reference value was also verified based on the 4.3 $\mu g.dL^{-1}$ of a similar group to the non-exposed of the present study, from Reis et al. (2007). Using the exponential model for parameter estimation, the obtained EBC-Pb concentration, based on the non-exposed subjects' B-Pb average value (4.3 $\mu g,dL^{-1}$), was 0.51 $\mu g.L^{-1}$, which is slightly below the lower confidence interval calculated for the EBC-Pb mean value listed in Table 4 (mean 0.97 $\mu g.L^{-1}$ ±0.25). In turn, considering this mean value as

input in the exponential model equation, a B-Pb concentration of 12.9 $\mu g.dL^{-1}$ was obtained, which is closer to Ind2 average value but below maximum values reported by Reis et al. (2007) for the Portuguese population (0.1- 19.9 $\mu g.dL^{-1}$).

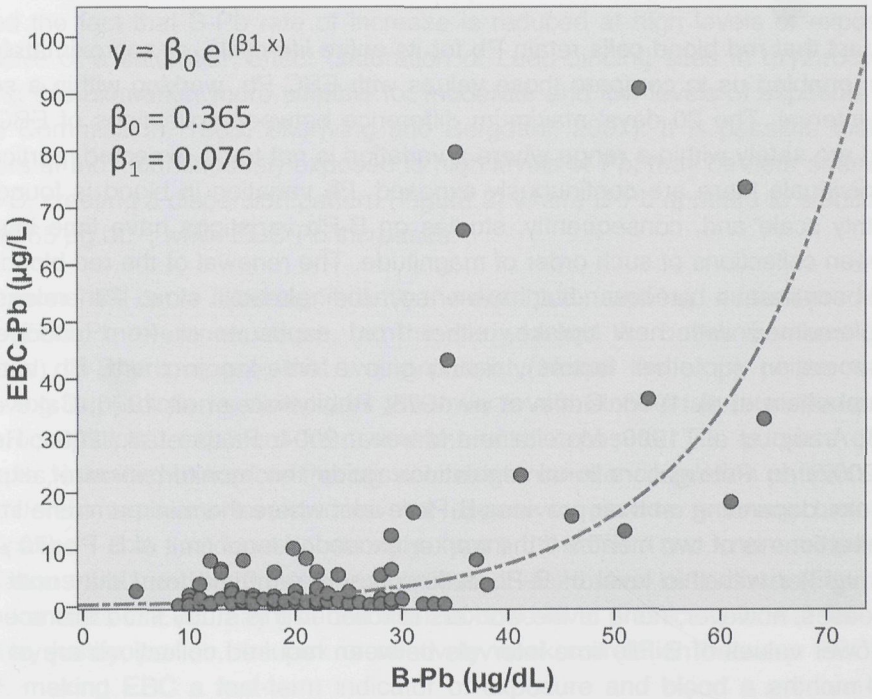

Figure 2 – Exponential curve fitted to all workers of Industry 1 and Industry 2, B-Pb ($\mu g.dL^{-1}$) against EBC-Pb ($\mu g.L^{-1}$).

6.4 Discussion

The present work, demonstrated the ability of EBC to assess professional exposure to Pb, despite the concentrations to which workers are exposed to, as both industrial facilities show distinct levels of pollutant emissions (Table 3). All workers enrolled in this study, from both industries, had a similar clinical condition, not displaying any abnormal complaints or past respiratory diseases that could prevent or constrain the study and, although values of pulmonary function in Ind2 were above Ind1, both were within the reference interval, for normal respiratory function. Ages were also similar and since Ind2 had female workers, it allowed the

comparison between both sexes and the influence of possible confounders was also rejected. The lower pH of the workers' EBC, when compared to the non-exposed group, may be related to the inhalation of acid compounds in the workplace. Also, the acidification of organic fluids, and particularly the airways, is common in toxicity and inflammation episodes and in scenarios of chronic exposure to pollutants (Do et al., 2008).

The fact that red blood cells retain Pb for its entire life-cycle, of approximately 120 days, enabled us to compare those values with EBC-Pb, working within a secure time interval. The 20 days' maximum difference between collections of EBC and blood are safely within a range where a variation is not to be expected, particularly in individuals there are continuously exposed. Pb variation in blood is found in a monthly scale and, consequently, studies on B-Pb variations have time intervals between collections of such order of magnitude. The renewal of the red blood cells is a continuous process but, even so, the already slow Pb release is complemented with new uptake, either from exposure or from endogenous contamination (or other factors), resulting in a time-lag in the B-Pb variation (Chamberlain et al. 1978; Griffin et al. 1975; Rabinowitz et al. 1976; Cake et al., 1996; Araújo et al., 1999; Moreira and Moreira, 2004; Prista et al., 2004; Reis et al., 2007). In Portugal, national regulations guide the monitorization of exposed workers, depending on their previous B-Pb levels, where the minimum time interval of collections is of two months if the worker exceeded legal limit of B-Pb (70 $\mu g.dL^{-1}$). A worker with this level of B-Pb is temporarily removed from duty until B-Pb decreases, however, none of the workers enrolled in this study fitted this scenario. For lower values of B-Pb, time intervals between required collections are of 6, 12 or 24 months.

Mean concentration values of EBC-Pb from the studied populations indicate that this biomarker reflects exposure, revealing three levels and, thus, reproducing the differences previously found in Pb measured in the PM_{10} from each workplace. Despite not significant, there was an increment between collection phase *a* and *b*. This reveals that EBC provides information on the intake of the week, but two resting days are not enough for total or substantial absorption of the Pb deposited in the surface of the respiratory tract. The comparison of EBC-Pb to B-Pb assuming exposure of all workers (both Ind1 and Ind2) to different concentrations of Pb appears to offer a significant fit to a non-linear curve, showing an apparent rise in the EBC-Pb only after B-Pb (reflecting absorption) reaches a value of about 20-30 $\mu g.dL^{-1}$ which then might eventually reach a saturation level. Moreover, the similarity of both non-linear curves (exponential and logistic) consistently indicates that if there is a saturation level, EBC-Pb concentration measurements in exposed workers are still in the exponential section of the curve. Possibly, reaching an

upper level of EBC-Pb will be more difficult to attain than reaching a saturation level of Pb in blood (Skerfving et al., 1993; Fleming et al., 1997). Pb concentration in EBC may contain always the signature of the external environment which in principle is not limited. Although the legal maximum value of B-Pb is of 70 $\mu g.dL^{-1}$, and none of the workers presented such levels, Figure 2 suggests, in fact, a trend towards saturation of B-Pb, while EBC-Pb continues to increase. It has been argued the fact that B-Pb rate of increase is reduced at high levels of exposure, indicative of a saturation effect (saturation of Lead-binding sites in erythrocytes), making this biomarker more suitable for moderate and low levels of exposure (UK Royal Commission, 1983; Skerfving and Bergdahl, 2007). It is possible that the workers at Ind1, continuously exposed to high levels of Pb, may be near saturation of Pb-B, creating a dispersion pattern (Figure 2) where B-Pb appears to stabilize at around 65 $\mu g.dL^{-1}$, while EBC-Pb increases.

The exponential curve fitted significantly the distribution and had a close prediction of the EBC-Pb reference values, but the study lacked the baseline values (non-exposed) for this purpose, that would possibly shape the slope of the curve, improving its prediction sensitivity for the lower levels, since it failed to differentiate lower occupational exposure from environmental exposure. The purpose of the curve fits, intended to explore the possible existence of a correlation pattern using two different exposure levels, does not appear to provide a strong biological significance and evidences strongly point towards two non-comparable populations: Ind1 and Ind2 that showed different distribution behaviours (Figure 1). Measurements in EBC may give the internal dose level practically in real time and this may justify, *per se*, the lack or relatively week correlation between EBC and blood, making EBC a fast-term indicator of exposure and blood a medium-term indicator, with several contributions for a time-lag between exposure and the Pb found in blood. It has previously been demonstrated that emissions in these industries reveal a characteristic and consistent pattern, with differences in the particle size dominance (Félix et al., 2012b). These results in B-Pb may, therefore, indicate a consequence of the natural biologic variability that ends in different absorption rates and kinetic processes involving Pb uptake. Morrow et al. (1980) verified equivalent lung retention of Pb, regardless of chemical species, that may differ depending on the origin of Pb, which in the present study vary according to the work processes of each factory. Absorption, however, depends not only on the chemical species but also on particle size and solubility (ATSDR, 1999) and once absorbed into blood its half-life depends greatly on intrinsic individual characteristics (Gulson et al. 1996; Manton et al. 2000; Cerná et al., 2012). Given the different nature of the processes in both industrial facilities (foundry and manufacturing) which have distinct emissions, the subjects should be treated as two different populations for B-Pb analysis, due to the influence of the mentioned

factors on the Pb absorption, and, thus, the correlation of both biomarkers should not include different exposure settings. Nonetheless, Ind1 was statistically closer to a correlation between B-Pb and EBC-Pb than Ind2, which could be confirmed by including more subjects of the foundry industry in the study. However, this is conceivably related to particle size which is a characteristic that distinguishes both factories. Particle size does not hamper the EBC-Pb analysis, which, despite this, clearly translates exposure and allows its use on different occupational settings (Félix et al., 2012b), but may influence exposure assessment through B-Pb. The fact the finer particles are easily absorbed to the blood through the lungs (Snee et al., 1985; ATSDR, 1999) may offer a faster response of B-Pb to the exposure and, thus, get closer to the values obtained through EBC, hence, the different distribution behaviour in the scatter-plot and closer proximity to a correlation with EBC-Pb. This is in agreement with the previously mentioned work of Chamberlain et al. (1975) that tested inhalation of fine particles. Moreover, since coarser particles have a high probability of being removed through a different pathway and enter the digestive system (Guyton and Hall, 2000), the total of inhaled Pb is represented in blood with a time-lag, and different rates of absorption, between coarse and fine particles, which will depend on the relative abundance of both particle fractions.

EBC-Pb may also overcome the potential problem of endogenous and dietary contamination. Evidences point towards the fact that most systemic Pb is bound to different compounds and protein structures, and only a negligible portion of Pb is in its free ionized state (Al-Modhefer et al., 1991; Schütz et al., 1996; Bergdahl et al., 1997, 1998, 1999; Fowler, 1998; Hernandez-Avila et al., 1998; Bannon et al., 2000; Manton et al., 2001; Smith et al., 2002; Gonick, 2011). Thus, although presently no known published work studied the phenomenon back-deposition of Pb into the lungs, the current knowledge on Pb bioavailability strongly suggests that the routes for this metal exclude blood-to-lungs transition, as schematised in Figure 3, portraying EBC-Pb as biomarker with a single source, eliminating endogenous confounders.

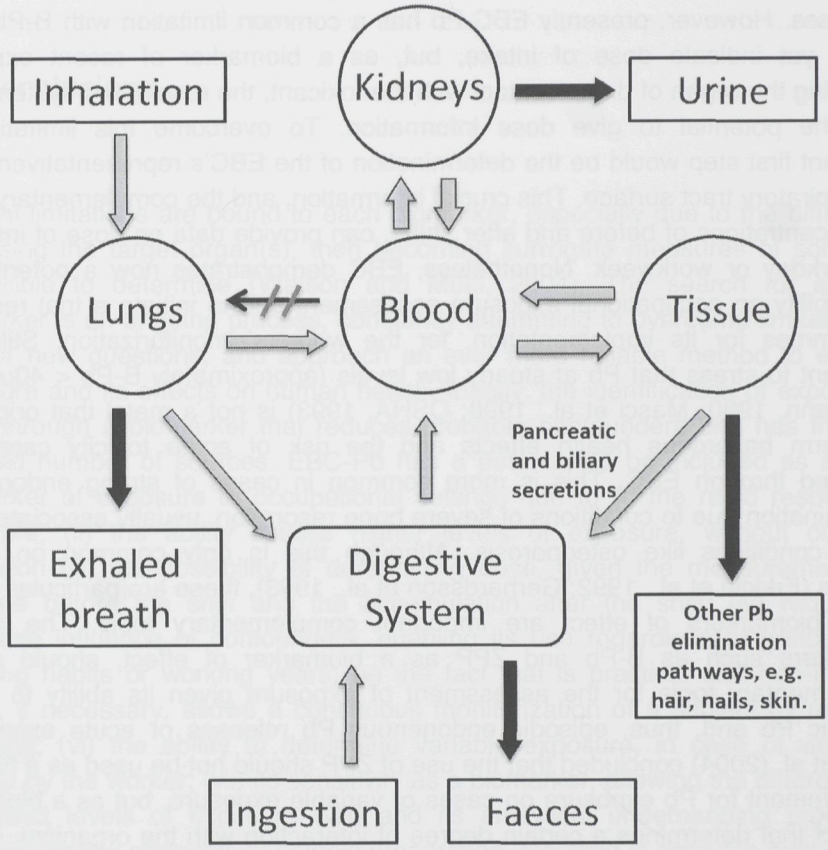

Figure 3 – Basic schematic design of the Pb pathways, based on O'Flaherty (1998), including main routes of excretion. Strikes over the arrow indicate an inexistent or negligible pathway.

The non-influence of the confounders indicates that EBC is sensitive enough to variations in exposure, without influence of the potential effects of the factors smoking habits, gender, age and working years. Also, considering other factors that may induce false positives in B-Pb, like diet and endogenous contamination, EBC is apparently a matrix with less probability for bias than whole blood. In fact, a peak in B-Pb does not necessarily mean a strong and recent deposit in the lungs, but an increased absorption. On the other hand, a low B-Pb does not mean that the subject is not highly exposed at the moment (OSHA, 1993; ATSDR, 2007). EBC-Pb responds rapidly to exposure, but collection should be done immediately after shift, under the risk of obtaining less accurate results, due lung clearance

processes. However, presently EBC-Pb has a common limitation with B-Pb, as it cannot yet indicate dose of intake, but, as a biomarker of recent exposure assessing the organ of direct contact with the toxicant, the respiratory system, may have the potential to give dose information. To overcome this limitation an important first step would be the determination of the EBC's representativeness of the respiratory tract surface. This crucial information, and the complementary EBC-Pb concentrations of before and after shifts, can provide data on dose of intake of the workday or workweek. Nonetheless, EBC demonstrates now a potential for applicability on occupational exposure assessment and to initiate a trial research programmes for its implementation, for the workers' monitorization. Still, it is important to stress that Pb at steady low levels (approximately B-Pb < 40μg.dL^{-1}) (Lippmann, 1990; Masci et al., 1998; OSHA, 1993) is not a metal that originates short-term hazardous health effects and the risk of acute toxicity cannot be assessed through EBC. This is more common in cases of strong endogenous contamination due to conditions of severe bone resorption, usually associated with health conditions like osteoporosis. Although this is only common on retired workers (Erkkilä et al., 1992; Gerhardsson et al., 1993), these are particular cases, where biomarkers of effect are important complementary tools. The use of biomarkers such as B-Pb and ZPP as a biomarker of effect, should act as complementary tools for the assessment of exposure given its ability to reflect systemic Pb and, thus, episodic endogenous Pb releases or acute exposures. Prista et al. (2004) concluded that the use of ZPP should not be used as a first line measurement for Pb exposure on cases of variable exposure, but as a biomarker of effect that determines a certain degree of interaction with the organism, should be used as a complementary measure. Moreover, due to its ability to reflect endogenous exposure and its low complexity and low cost technique (reviewed in Prista et al., 2004) ZPP is a strong candidate, not only as a complementary measure for EBC-Pb, but also on long-term monitorization of retired workers. Nonetheless, the aim of EBC-Pb is ultimately to determine and quantify the Pb intake, rather than its health effects and, in chronic, long-term exposure, it is of the utmost importance to assess the amount of Pb uptake which, in turn, can lead to acute toxicity episodes. The possibility of EBC being able to help quantify intake, considering the amount of time the worker has been exposed to Pb, may contribute to predict temporal limits of exposure and prevent hazardous health long-term outcomes, relatively common in chronic Pb exposure.

6.5 Conclusions

Several limitations are bound to each biomarker, especially due to the difficulty of assessing the target organ(s), then becoming surrogate measures of something impossible to determine (Watson and Mutti, 2004). The search for an ideal biomarker is an ongoing process, constantly attempting to overcome limitations, to answer new questions, and approach an ever more reliable method to evaluate exposure and its effects on human health. Ideally, the identification of exposure is done through a biomarker that reduces probable confounders and has the most reduced number of sources. EBC-Pb has a potential to be included as a viable biomarker of exposure to occupational settings due to (i) the rapid response to exposure; (ii) the ability assess higher levels of exposure, without observed saturation (iii) the possibility of determining dose, given the measurement of a baseline before the shift and the accumulation after the shift; (iv) reduced or negligible influence of confounders, enabling its use regardless of gender, age, smoking habits or working years; (v) the fact that is practical and non-invasive, which, if necessary, allows a continuous monitorization of a subject by repetitive sampling; (vi) the ability to determine variable exposure, in case of work-post change by the worker; (vii) its sensitivity as a biomarker, allowing the differentiation of distinct levels of exposure (viii) and its analytical undemanding processes, without complex protocols of sample preparation, resulting in expedite analysis and little sample manipulation, as demonstrated in Félix et al. (2012a). These features and the results of Félix et al., (2012a, 2012b) have been continuously listed as fundamental to the validity of a biomarker and its suitability for exposure to Pb, particularly in occupational settings (Wilcosky, 1993; Mutti, 1999; IPCS, 2001; Watson and Mutti, 2004).

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Chapter 7

7. Concluding remarks

Throughout the present thesis, exhaled breath was investigated and explored in search of a suitable, non-invasive, new bioindicator of exposure in occupational settings. A matrix in which it is possible to quantify exposure to metals and the follow-through of a process that demonstrated the ability to produce reliable results.

The experimental design included two lead processing industries, different in the nature of their work processes, with workers performing distinct tasks and the outdoor environment and offices, where workers were not exposed to metals. These features allowed a more complete assessment, not only of the exposure levels, measured through the quantification of elemental concentrations in airborne particles, but also on how EBC reflected such exposure. The characterisation of the industrial workplaces, offices and outdoor environment, demonstrated that workers were exposed to different levels of airborne contaminants, whose size fractions and relative abundances allowed the portrayal of a fingerprint characteristic of each site.

It was clearly demonstrated that EBC's elemental contents reflected exposure in both industries and that it distinguishes, not only between workers and non-exposed subjects, but also between workers from both industrial sites. The pilot study described in Chapter 2, scrutinized the composition of the EBC matrix, as well as the visualization of inhaled particles confirming that EBC is a heterogeneous sample inappropriate for direct analysis. It was demonstrated here that particles are only present in exposed workers, suspended in a common matrix, similar to all subjects. The design of a protocol for sample collection, preparation and analysis was validated, filling the gap pointed out by several authors of implementing standardized procedures in order to assure the representativeness and traceability of results. This was the first attempt of validating a procedure for the analysis of metals in EBC. As far as EBC analysis is concerned, a set of good practice rules was proposed for the collection and measurement of response biomarkers (e.g. Horváth et al., 2005), although no guidelines have been established so far. This is a probable consequence of the embryonic stage in which metal analysis in EBC presently is. The validated protocol for metal analysis in EBC established the following procedures:

i) Sample collection should be made after the shift on the last day of the labouring week, guaranteeing representativeness of prior exposure;

ii) Collection time of 15 min, for an average volume of 2mL, using the collection device EcoScreen. However, since the amount of sample required for elemental analysis, using a multielemental technique like ICP-MS, is of 500 μL, only a 5 minute sampling time is required to collect a comfortable 500 μL of EBC volume (on average).

iii) Samples should be transferred into previously decontaminated polypropylene containers, using a solution of H_3NO_3 50% (v/v), acidified with 3% (v/v) with H_3NO_3 of suprapur grade, for homogenization and dissolution of the material in suspension. For all dilutions it should be use ultrapure water (18 MΩ.cm);

iv) Storage at -80 °C and thaw only for analysis;

v) After thawing the samples, it is advisable a 10 min sonication, before dilution for analysis;

vi) For elemental analysis, ICP-MS proved to be an adequate technique, with low detection limits and requiring small sample volume.

The standardization of such a procedure can, hopefully, yield comparable results among the scientific community, favouring common policies, data share and the establishment of cooperation programmes, which would greatly enrich and improve the quality of biomonitoring research (Smolders et al., 2008; Thomsen et al., 2008). Also, quality assurance is a key issue for the validation of a biomarker of exposure, a matter whose importance is commonly highlighted (e.g. Aitio and Apostoli, 1995; Watson and Mutti, 2004) and explored in the present study through recovery tests and estimation of overall uncertainty, thus, demonstrating the adequacy of the method.

In this thesis, by studying the EBC's characteristics and analytical performance, a significant progress was achieved towards the demonstration of the usefulness of EBC matrix to assess biomarkers of exposure. The proof that EBC's metal content reflects the subjects' exposure, paves the way for its use in occupational settings.

Different biomarkers for the assessment of exposure of metals were validated in EBC. However, only Pb was more exhaustively investigated, by contextualizing it and comparing it to B-Pb, the common used biomarker to assess exposure and monitor workers of the lead processing industry. EBC-Pb proves its viability as a biomarker of exposure in occupational settings, and a surrogate of B-Pb in the

front-line of the workers biomonitoring. EBC has essential characteristics, which makes it an effective alternative in occupational health programmes:

i) The celerity of the process due to the production needs. The short collection time produces enough sample for analysis and the labour time interval at a maximum of 15 minutes is acceptable for the factory.

ii) Relatively low costs. When compared to blood draw, the collection of EBC does not require the same level of qualifications among the technical staff, reducing its costs.

iii) Reliable results. EBC requires few sample manipulation, which minimizes the risk of sample contamination.

iv) Quick analysis of large quantities of samples is possible. The EBC's analytical undemanding processes, without complex protocols of sample preparation, and the possibility of using ICP-MS, which is a gold-standard technique for metal analysis, result in an expedite analysis and large sample output.

This ongoing search for an ideal biomarker proves yet to have several limitations as do the all biomarkers currently used. As surrogate measures of an unachievable dimension, this search intends always to attain a balance between the feasibility of the whole monitoring process and the representativeness of the results. An important remark by Watson and Mutti (2004) states that an ideal biomarker of exposure should be specific for a particular chemical, detectable and quantifiable in very low quantities, measurable by non-invasive techniques, inexpensive and associated with prior exposure. Moreover, the determination of concentrations of the parent compound in the biological media, are generally preferable to the measurement of metabolites, which may be generated from different compounds. In some cases such as in solvent exposure, the parent compound correlates better with exposure when compared to intermediate products of metabolic processes. EBC proved to be a suitable matrix to measure the parent compound. As far as Pb exposure is concerned, in this study it was demonstrated that EBC can represent the organ of direct contact with the toxicant and reflected the level of exposure. A further added value to the use of EBC as an indicator of lung exposure to inhaled fumes and particulate matter containing metals is the elimination of endogenous confounders.

Future research requires, on a subsequent approach to this study, the unequivocal relation between biomarkers in EBC and exposure to contaminants. These future trials should begin with the study of Pb, the element more thoroughly studied in the present work. For that the following steps are crucial:

1. Increasing data, extending the study to other population groups, exposed to different Pb concentrations in ambient air;
2. For group analysis, it is advised a large sample size, above one hundred subjects;
3. Individual assessment, using selected groups of subjects, each exposed to different concentrations of Pb and carry out individual exposure tests with individual samplers. Determinations of EBC-Pb would have to be carried out on previously determined end-points of the subject's exposure to assess the relation between APM and EBC and intra- and inter-individual variability. This approach would also provide information on the calculation of dose on intake, due to the differences of concentrations measured between the end-points.

On a following step, it would be essential to explore the determination of the dose of intake and bioavailability of metal compounds. To achieve these goals it is critical to improve models of deposition and diffusion in the airways, which will account for the representativeness of the lung lining fluid in EBC and for routes of uptake of inhaled particles and metals. These aspects are strongly bound to effective particle sizes and composition, and consequently, to the regions of the respiratory airways that those particles can reach. This differential deposition has a direct implication on the uptake and an influence on the rate of this uptake, since the process of lung clearance varies depending on the site. In the upper respiratory system there is a higher probability for particles to be trapped in the mucus and be removed through ciliated movements towards the digestive system, and through cough reflexes, where the absorption is lower. In the lower respiratory system particles are more likely to move through the alveolar membrane and enter the systemic circulation, where the rate of absorption is higher. Consequently, both the body burden and the effect of particles (or its associated elements) in the human organism may vary according to particle properties, as it depends on how much is absorbed. This highlights the importance to know to origin of particles determined in EBC. In addition, particles can also be internalized by epithelial cells, if they succeed to diffuse into cells, and removed by macrophages, entering the lymphatic circulation, where they are destroyed and excreted or may be involved in immunity processes (Godish 2003; Beckett et al., 2007).

Although EBC reflects the inhalation of particles with different sizes, how much of each size fractions is retained in the lungs is an information still lacking and not easily achieved. Nonetheless, in the matter of the representativeness of EBC, the work of Bondesson et al. (2009) took a step forward in determining the central part of the lung (till bronchi and bronchiole) as the preferential anatomical site of origin of aerosol collected in EBC. In the present study results obtained seem to corroborate these findings as was described in Chapter 6.

The solubility of the particle also plays an important role in the rate of uptake. The solubility of metal compounds is of great toxicological importance, because it is one of the major factors influencing the availability and absorption of metals. Generally, the outcome of a higher solubility is a more efficient absorption into the systemic circulation. Solubility depends on many factors, such as the metallic element itself, chemical species and the presence of other ions, like H^+ or H_3O^+ (pH) or particle size (Cornelis and Nordberg, 2007; Ke et al., 2007). Determination of metal solubility levels in the lung is also a key issue as far as the rate of uptake is concerned and, eventually, to bioavailability and toxicity due to metal speciation – transitions of metallic elements to other ionic states – that contribute to the uptake process (Beckett et al., 2007). Biological fluids are a complex medium and this is essentially the reason why experimental data on the solubility of metals in biological fluids are limited (Cornelis and Nordberg, 2007). An attempt to mimic the lung lining fluid would provide a significant advance on the filling of such gap.

Overall, there are two main factors that need to be determined in order to calculate the representativeness of EBC regarding the lung lining fluid: i) the dilution factor ii) and the site of aerosol formation in the airways. This first approach in the determination of the relation between EBC concentration values and the airways, could be modelled by first determining the hydration level of the subject, ideally enabling its relation to the volume of EBC collected in a given period of time – the dilution factor – and, secondly, by including a weighting factor, determined from the probability of aerosol formation in each site of the respiratory airways. Undoubtedly, both factors are also influenced by the subjects' breathing pattern, which should not be overlooked and that most certainly accounts a great deal to the inter-subject variability. The answers to such questions will enable us to calculate dose.

Despite, the unsolved questions linked to its origin, the EBC proved to be a suitable matrix to assess occupational exposure. The procedures developed are a strong support to initiate trial research programmes for EBC implementation in occupational health surveillance, as EBC presents unquestionable unique characteristics regarding other common used biomarkers of exposure, which make of EBC an exceptional biomarker.

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Curriculum vitae

Pedro M. Félix was born on December 8th, 1977, in Lisbon, Portugal. In June 2006 he finished his licentiate degree in biology applied to marine animal resources, at the Faculty of Sciences, University of Lisbon. In the same year, he continued his education at the same faculty and in 2008 finished his master's degree in fisheries and aquaculture. While a student of this university, he collaborated, between 2002 and 2005, with the Institute of Oceanography at the Faculty of Sciences of the University of Lisbon working on the morphologic and biometric analysis of the otoliths of fish species. He was then a fellow at Nuclear and Technological Institute (IST/UTL), with a research grant, working in the group of Biomedical Studies, under the supervision of Prof. Dr. Teresa Pinheiro and Dr. Marta Almeida. This work involved the collaboration of several Portuguese institutions and the Radioisotopes for Health Section of the Faculty of Radiation, Radionuclides and Reactors from Delft University of Technology (TUDelft), where he started his PhD thesis under the supervision of Prof. Dr. H.Th. Wolterbeek and the previous supervisors. Here he worked on biomarkers of exposure and received training on the analytical technique ICP-MS and immunoassay techniques like ELISA and Flow Cytometry. In 2011 he returned to the Marine Zoology group of the Institute of Oceanography and started a research program on the study of the ecology and biodiversity of the fauna of coastal lagoons and the influence of metals in the fish species of coastal lagoons. Since 2009 he is also a certified trainee and lectured courses in marine biology and environment. His current interests comprise ecotoxicology, metal contamination and bioaccumulation and endocrine disruption of fish species.

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Oral presentations by invitation

2010 – Centre of Nuclear Physics of the University of Lisbon (CFNUL), Portugal: **Félix PM**. New biomarkers of exposure to metals using exhaled breath condensate (EBC).

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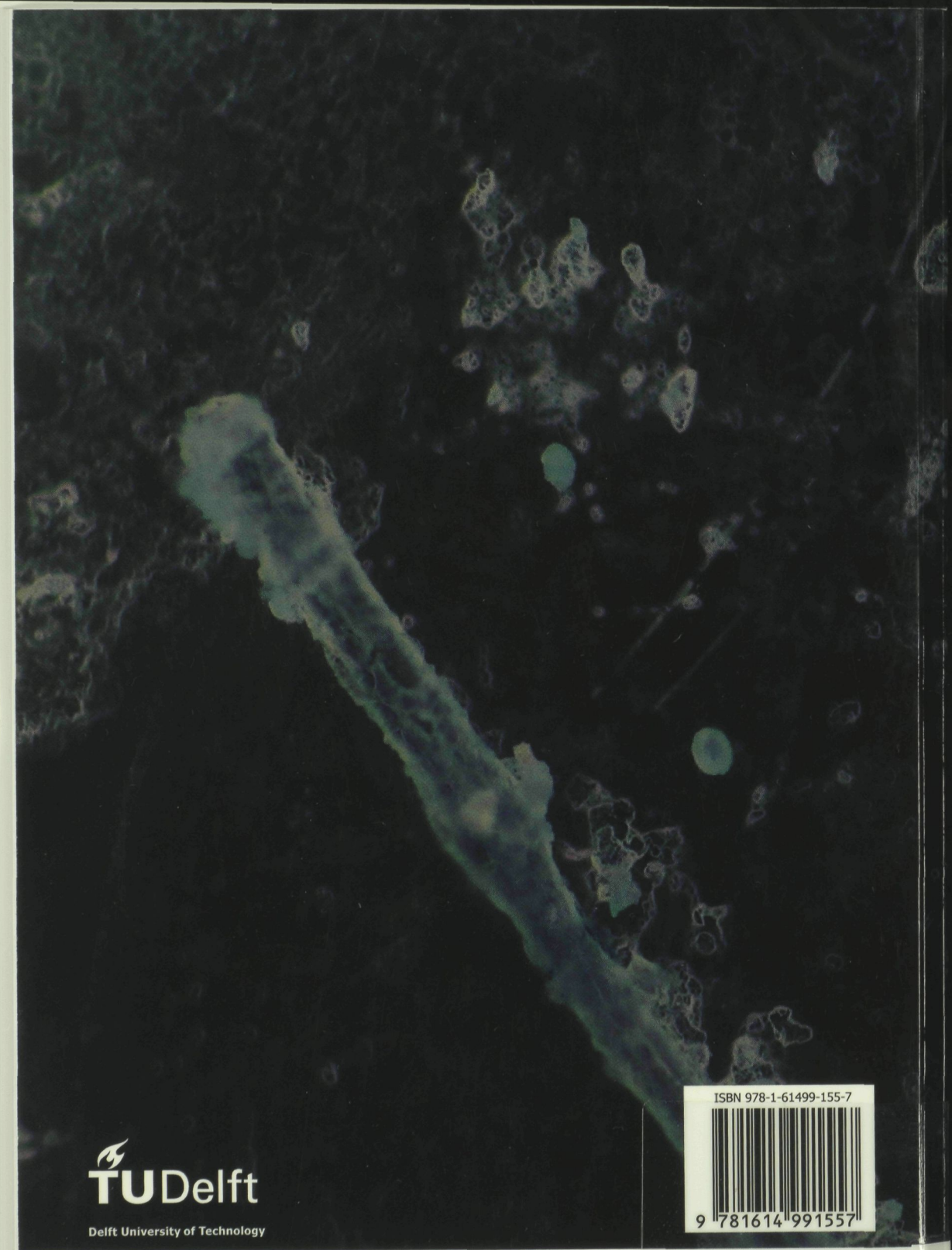

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