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Trace Elements Quantification in Beers Consumed in Portugal: arsenic, lead, cobalt, copper, iron and zinc

Mestrado em Biologia Humana e Ambiente

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Resumo

As bebidas alcoólicas incluindo a cerveja começaram a ser produzidas praticamente desde o início da agricultura há mais de 10 000 anos na região do Crescente Fértil. Egipto, Mesopetânia e Babilónia foram as populações que mais impulsionaram o fabrico e consumo de cerveja tendo passado o conhecimento para os Gregos que por sua vez o transmitiram aos Romanos. Estes últimos através das suas conquistas ao longo da expansão do seu Império acabaram por levar a cerveja por quase toda a Europa. Desde a Idade Média, passando pela Idade Moderna até aos dias de hoje foram desenvolvidos mais processos no fabrico de cerveja, novos ingredientes foram sendo adicionados e novas tecnologias revolucionaram o mundo da cerveja. Actualmente a cerveja é uma das bebidas mais consumidas em todo o mundo.

No fabrico da cerveja são utilizadas várias matérias-primas como água, sementes, leveduras e lúpulo. A composição final da cerveja depende em grande parte do produto pretendido e como tal das matérias-primas envolvidas. Há dois tipos de cerveja, *lager* e *ale*, em que a principal diferença ocorre no passo da fermentação em que leveduras da estirpe *Saccharomyces pastorianus* são usadas para obter as cervejas do tipo *lager* e leveduras da estirpe *Saccharomyces cerevisiae* para as cervejas do tipo *ale*. As cervejas do tipo *lager* são as mais produzidas correspondendo a uma produção de 90% da cerveja mundial.

Tendo em conta o seu grande consumo a nível mundial não é de admirar que a cerveja tenha um impacto determinante ao nível da economia, especialmente ao nível da indústria e da restauração devido aos postos de trabalho que directa ou indirectamente daí advêm.

Contudo com o aumento do consumo de cerveja vem também a preocupação com o impacto que esse elevado consumo possa ter no consumidor devido à exposição a contaminantes que podem ser prejudiciais à sua saúde. Em termos de contaminantes os metais são os que mais se destacam pois acima de determinadas concentrações podem provocar efeitos tóxicos para o ser humano. Os metais são constituintes naturais da crosta terrestre e encontram-se amplamente distribuídos na natureza e, como tal, podem entrar na constituição da cerveja em qualquer etada do seu fabrico. De facto os metais podem estar presentes nas próprias matérias-primas necessárias à produção da cerveja, seja por ocorrência natural ou pela actividade antropogénica (uso de pesticidas e fertilizantes, emissões provenientes do tráfico automóvel e indústrias). O próprio equipamento utilizado na produção de cerveja também pode

ser uma fonte de contaminação bem como os recipientes em que a cerveja é armazenada e transportada.

Tal como se verifica com vários alimentos, também há legislação própria para as cervejas, que em Portugal é estabelecida pela Portaria nº 1/96 de 3 de Janeiro. Esta Portaria regula vários parâmetros que influenciam a qualidade da cerveja como cor, cheiro, sabor, pH, acidez total, conteúdo alcoólico, entre outros. Para além disso também regula a presença de metais nesta bebida estabelecendo concentrações maximas para arsénio (0.1 mg/L), chumbo (0.2 mg/L), cobalto (0.05 mg/L) cobre (0.2 mg/L), ferro (0.3 mg/L) e zinco (1 mg/L).

Assim este estudo tem como objectivo determinar se esses limites são respeitados em diferentes marcas de cerveja comercializadas em Portugal. As amostras foram seleccionadas e recolhidas pela Autoridade de Segurança Alimentar e Económica (ASAE) de entre as marcas nacionais mais consumidas no mercado português.

Para a determinação dos metais seleccionados para o estudo recorreu-se a duas metodologias que foram desenvolvidas e optimizadas num estudo previamente realizado no Laboratório de Métodos Instrumentais de Análise da Faculdade de Farmácia da Universidade de Lisboa, onde também este estudo foi realizado. Assim, para analisar a presença de metais como chumbo, cobalto, cobre, e o ferro utilizou-se a Espectrofotometria de Absorção Atómica com Câmara de Grafite (GFAAS) e para o arsénio a Espectrofotometria de Absorção Atómica com Geração de Hidretos (HGAAS). Antes da análise as amostras de cerveja foram sujeitas a uma digestão pressurizada por microondas.

No presente estudo foi possível concluir que as amostras de cervejas seleccionadas pela ASAE apresentavam quantidades inferiores aos limites estabelecidos pela legislação Portuguesa no que respeita aos metais analisados. Assim, a presença dos referidos metais nas cervejas analisadas não constitui perigo para o consumidor.

Palavras-chave: Cervejas, metais, GFAAS, HGAAS

Abstract

Alcoholic beverages including beer began to be produced shortly after the onset of agriculture. Since then beer consumption has increased over the centuries and nowadays beer is one of the most consumed beverages in the world. Due to its high consumption, all studies conducted in order to assess and control the quality of this drink are of great importance not only to brewers but also to consumers. Since beer is obtained through the use of various raw materials and because of their dependence on variables introduced in the several steps involved in the brewing process, a wide variety of analysis can be performed for testing the quality of the different commercialized types of beers. Metals contained in both types of beers, *lager* and *ale*, may come from different sources such as natural ones like water, soil, cereal, and yeast, as well as environmental contamination with the use of fertilizers and pesticides. Brewing equipment itself and the different substances added during the brewing process to control fermentation and maturation processes are also sources of metal contamination in beers.

This study aims to evaluate the presence of metals referenced by the Portuguese legislation (Portaria nº 1/96 from 3rd January) in order to verify if their contents are according with established reference values. The elements specified in the aforementioned law are arsenic, cobalt, copper, iron, lead, and zinc, and their quantification is the focus of this study. A preliminary study was conducted in Laboratory of Instrumental Methods of Analysis, where under optimized conditions validation parameters were obtained for all target elements.

Analyzed samples were selected and collected in the Portuguese market by the Portuguese Food and Economic Safety Authority (ASAE), Department of Food Risks and Laboratories. Quantification of elements specified in the aforementioned law in beers was performed by Atomic Absorption Spectrometry (AAS) techniques after Microwave Pressure Digestion. Hydride Generation Atomic Absorption Spectrometry (HGAAS) was selected for arsenic analysis while Graphite Furnace Atomic Absorption Spectrometry (GFAAS) was selected for cobalt, copper, iron, and lead.

Analyzed samples previously selected by ASAE, using Portuguese consumers' most preferred beer brands, presented metal content below the maximum threshold allowed by the Portuguese legislation for all analyzed metals. Therefore, the presence of the analyzed metals in selected beers presents no danger to the consumers.

Keywords: Beers, metals, GFAAS, HGAAS.

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First of all I want to show my gratitude for Doctor Professor Maria Luísa Mateus for allowing to work at her Laboratory and collaborate in this project and for helping me in every step of the way. Thank you for always defending my best interests and for giving me a lot of freedom in my work.

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To my friends I refer my gratitude for supporting me during this important phase of my life. I would like to leave a special thanks to Ana, Cátia, Laura, and Rita for their friendship and for being there when I most needed them.

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List of Abbreviations

ASAE – Autoridade de Segurança Alimentar e Económica

ASS – Atomic Absorption Spectrometry

ANOVA – Analysis of Variance

CRM – Certified Reference Material

D.L. – Detection Limit

DMT-1 – Divalent Metal Transporter Protein

EDLs – Electrodeless Discharge Lamps

EU – European Union

FAAS – Flame Atomic Absorption Spectrometry

GFAAS – Graphite Furnace Atomic Absorption Spectrometry

HCLs – Hollow Cathode Lamps

HCP1 – Heme Carrier Protein-1

HGAAS – Hydride Generation Atomic Absorption Spectrometry

ICP-AES – Inductively Couple Plasma Atomic Emission Spectrometry

ICP-MS – Inductively Couple Plasma Mass Spectrometry

$NaBH_4$ – Sodium tetrahydroborate

Q.L. – Quantification Limit

RSD – Relative Standard Deviation

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1. Introduction

1.1. Beer

1.1.1. History

Beer is the product of a yeast alcoholic fermentation of extracts of grains such as einkorn, wheat and barley, to which hops are added (Pohl, 2008). However its origin, temporally speaking, is not so easy to determine. Alcohol as a beverage is mentioned to have originated in prehistory, maybe in Paleolithic (Hornsey, 2003). Beer *per se* appears to have been first described by the Sumerians more than 5 000 years ago (Pavsler and Buiatti, 2009a). The practice of brewing may have happened for the first time by chance through the discovery of a spontaneous fermentation of a cereal (APCV, 2015a). Nevertheless it is most likely beer was first produced in the early beginnings of agriculture in the Fertile Crescent mainly because due to the availability and diversity of grain, fire control and suitable brewing vessels (Meussdoerffer, 2009).

The Egyptian civilization was one of the first to produce beer. Egyptians believed beer was a gift from one of their Gods, Osiris. Barley was the most used grain to produce this alcoholic beverage but each tribe had its mode of preparation (Hornsey, 2003). Generally to produce beer, grains were firstly germinated and milled and then transformed into dough which would be molded into breads. In turn this bread was slightly cooked and then crumbled into a container with water and left in there for a few days in order to ferment. After fermentation, the product was filtered and stored in appropriated containers (e.g. amphora) (APCV, 2015a). The barley beer of Egypt was called *Zythos* and was consumed by all strata of society without exception (Meussdoerffer, 2009).

Alongside with the Egyptians the Babylonians (descendants of the Sumerian civilization) also produced and consumed beer. Unlike the Egyptian civilization, however, Babylonians had several different types of beers resultant from diverse combinations of aromatic plants combined with honey, barley or wheat (APCV, 2015a).

Greece under Alexander the Great conquered Egypt in 331 BC and introduced wine to the Egyptian civilization. Soon wine was preferred by the upper classes and beer was the drink of the lower strata (Meussdoerffer, 2009).

The Romans learned the brewing process from the Greeks. However it was considered a despicable drink and typical of barbarians (APCV, 2015a). Due to a grain crisis viticulture was prohibited in land where cereals could be sown and consequently

the Roman legions acquired provisions of beer. This allowed for the practice of brewing to spread throughout Europe, with beer becoming a strong competitor to wine (Meussdoerffer, 2009).

During the Middle Ages the brewing process evolved, thus leading to the introduction of a mixture of herbs which acted as beer preservatives. This mixture was called *gruyt* (APCV, 2015a). In the late Middle Ages, in Britain, Flanders workers introduced hops, a plant with aromatic properties, in the brewing process (Pavsler and Buiatti, 2009a). Hops give beer its characteristic aroma and flavor in addition to protecting it against microbiological contaminations (UNICER, 2015a). Actually in this period *ale* and *beer* had two different meanings - *ale* was described as a non-hopped malt beverage and was the main type of beer in Europe until the sixteenth century while *beer* was the hopper malt beverage (Pavsler and Buiatti, 2009a).

Despite the development of brewing process during the Middle Ages there was still room for improvement. In the Modern Age the discovery of the existence of bottom-fermenting yeasts by Emil Christian Hansen allowed greater consistency of taste and beer quality (APCV, 2015a; UNICER, 2015b). Another significant addition to the brewing process was the maintenance of fermentation tanks and the ability to keep storage cellars at low temperatures throughout the year (UNICER, 2015b).

Beer has a long history in Portugal but it was only since the seventeenth century that beer consumption started rising. In the eighteenth century brewing factories started appearing and in the nineteenth century those factories began to be grouped into major companies. Today two major groups are responsible for beer commercialization in Portugal – UNICER and Sociedade Central Cervejas e Bebidas (APCV, 2015a).

1.1.2. Raw materials

Beer is one of the most consumed alcoholic beverages worldwide. There is a wide variety of beers resulting from different brewing process but in all cases four main ingredients are required – water, malted cereals, hops, and yeasts (Harrison, 2009; Wunderlich and Back, 2009).

Water is the main ingredient in beer as it comprises 90-95% of the content of the finished product, which means the purity of water plays an important role in the brewing process (APCV, 2015b; Harrison, 2009). Its chemical characteristics such as pH and metal content (which may vary geographically) can dramatically affect beer color, flavor and aroma (Harrison, 2009; Wunderlich and Back, 2009).

Malted cereal originates from the germination of cereals under predetermined and controlled environmental conditions. After water, this is the second most important

ingredient in the brewing process. Malted cereals are an important source of enzymes that degrade several components of grain which are essential to yeast growth. This ingredient has a significant impact on the process because several grain components end up in final product influencing (both positively and negatively) various attributes of the mentioned beverage. Albumins, globulins, minerals, lipids and polyphenols are some of those components which will be mentioned later in 1.1.5. (Wunderlich and Back, 2009). Non-malted cereals or adjuncts (corn, rice, and wheat) are frequently used as ingredients as well in order to add fermentable carbohydrates necessary to yeast growth (Harrison, 2009).

Hop (*Humulus lupulus*) is an aromatic plant used in the brewing process since the late Middle Ages (APCV, 2015b). Generally hops are added in wort processing (explained further 1.1.4.) to alter beer flavor and aroma. In addition this ingredient prevents microbial contamination. Just like malted cereals, hops contents (resins, flavoring agents and polyphenols) also affect final beer characteristics and even provide antioxidant properties (Harrison, 2009; UNICER, 2015a; Wunderlich and Back, 2009).

Fermentation is the essential step in the brewing process. There are two fermentation organisms that can be used in this stage – bacteria and yeast. Yeast is more commonly used (Harrison, 2009). This organism in anaerobic conditions promotes alcoholic fermentation, a metabolic pathway which convert sugar from wort into ethanol and carbon dioxide (APCV, 2015b; Wunderlich and Back, 2009). Final beer characteristics are also influenced by yeast especially because there is a large variability of yeast strains (Harrison, 2009).

1.1.3. Beer types

Regardless of the enormous diversity of beer products, generally only two main categories are considered – *ale* and *lager*. Brewing processes are identical for both types except for the yeast species used in the fermentation step, since *ale* beers are produced with *Saccharomyces cerevisiae*, while *lager* beers are produced with *Saccharomyces pastorianus* (APCV, 2015c; Harrison, 2009; Pavsler and Buiatti, 2009a; Pavsler and Buiatti, 2009b). Despite their variety, yeast species are divided into two major groups as well – bottom-fermenting yeasts and top-fermenting yeasts. This yeast classification can be related to beer categories – bottom-fermenting yeasts are used to produce *lager* beers, while top-fermenting yeasts are used to produce *ale* beers (APCV, 2015c; Harrison, 2009; UNICER, 2015b).

As mentioned above *ale* beers' fermentation is carried out by *Saccharomyces cerevisiae*, a top-fermenting yeast. This means incubation temperature is in the

range of 18–27ºC for a period of time no longer than one week (Harrison, 2009). Cell migration to the surface also confers a wider range of aromas and flavors to *ale* beer (APCV, 2015c). Top-fermented beers usually have higher alcohol content when compared to bottom-fermented beers. Several sub-groups can be considered in *ale* beers such as Porter, Stout, India Pale Ale, Sour Ale, and so many others. Consumed widely until late Middle Ages, today it only represents about 10% of all beer produced worldwide (Pavsler and Buiatti, 2009a).

On the other hand *lager* beers' fermentation is carried out by *Saccharomyces pastorianus*, a bottom-fermenting yeast. While *ale* beers only have one fermentation step, *lagers* have two steps – primary and secondary. For primary fermentation, incubation temperature is in the range of 8-15ºC. During this step yeast tend to flocculate and migrate to the bottom. Primary fermentation is followed by a long secondary fermentation step at lower temperatures (between -1ºC and +4ºC) which can take up to a month or even more (Pavsler and Buiatti, 2009b). In olden days producing lager beers was too difficult because it was too hard to maintain such low temperatures for long periods of time (APCV, 2015a). However, with the development of refrigeration after the nineteenth century producing *lager* beer became possible and today it represents more than 90% of all beer produced worldwide (APCV, 2015c; Pavsler and Buiatti, 2009a). Several sub-groups can be considered in *lager* beers such as Pilsner, Dark Lager, Bock, Munchener, Dortmunder, and many others (Pavsler and Buiatti, 2009b).

1.1.4. Brewing process

Brewing process evolved throughout history, undergoing several changes, which still happens nowadays. There are several different beer products, many of those with only slight differences between them. However, in all cases the brewing process has the same main steps, although conditions in each step may vary depending on the produced beer. Malting, mashing, wort processing, fermentation and conditioning, and finishing are the main steps. Each procedure has its stages which may be different from beer to beer (Harrison, 2009). A brief explanation of each step will be presented in this section. A diagram related to the overall brewing process can be found in figure 1.

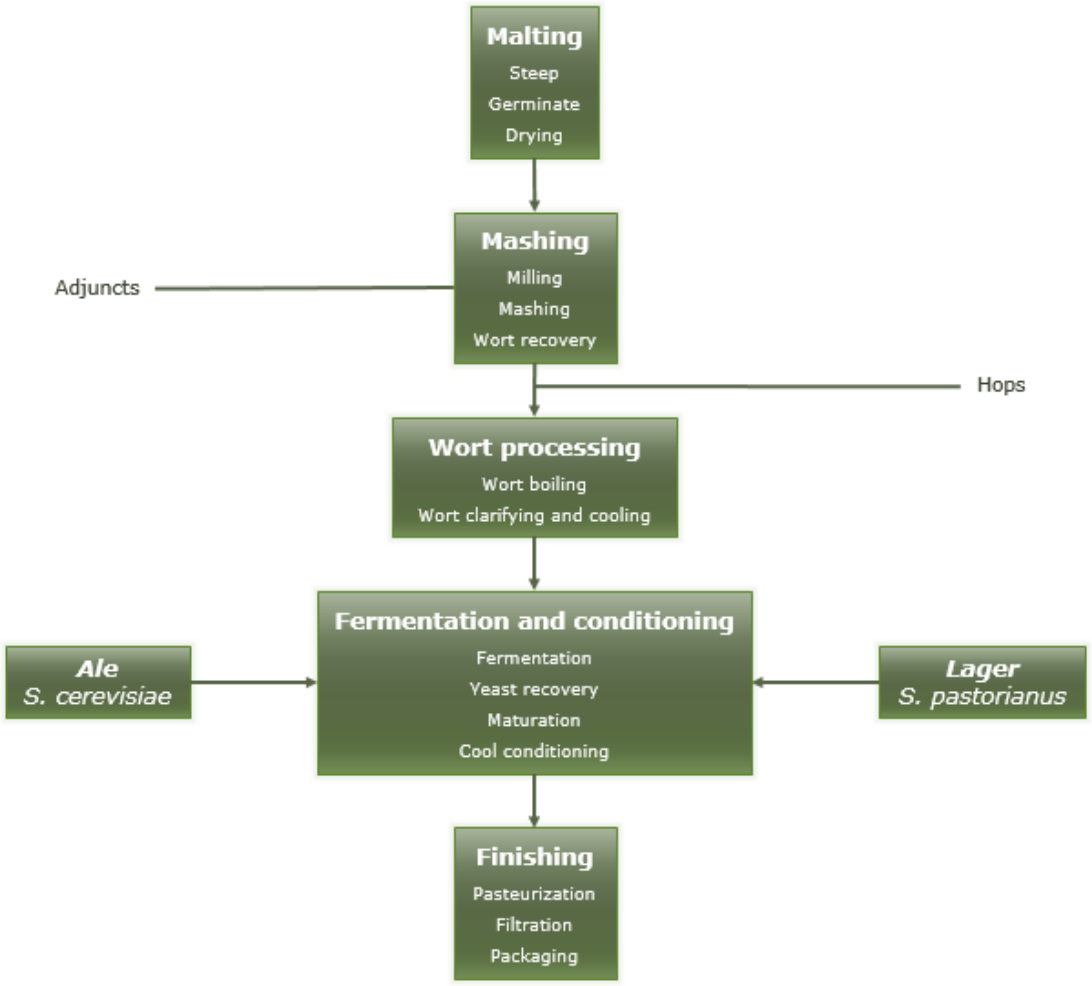


Figure 1 - General brewing process (adapted from Harrison, 2009).

The first step in the brewing process is malting, a process of controlled germination in order to induce biochemical changes (Wunderlich and Back, 2009). Those changes produce an ample supply of enzymes capable of degrading components of grain. This degradation will provide the necessary nutrients for yeast growth. Malting can be divided into three stages – steeping, germinating and drying (Harrison, 2009). Barley is the most common cereal used in brewing especially because it prospers even in adverse growing conditions. However, it still needs some basic conditions to germinate such as sufficient oxygen, heat, and humidity (Wunderlich and Back, 2009). To promote such conditions barley grains are steeped in 10-15ºC water until water content in grain reaches more than 40% and then germinated at 15-20ºC for almost one week. With germination the embryos' activity rises and several enzymes (amylase, protease and other hydrolases) are produced to degrade starch, proteins and other components of grain into substances (sugars, amino acids, fatty acids) needed to yeast growth. After germination, grains are

subjected to a drying process to drastically reduce water content which will stop the embryos' growth without inactivating the produced enzymes (Harrison, 2009; Wunderlich and Back, 2009). Water content may be reduced to 3.5-4% to produce pale malts or to 1.5-2% for dark malts (Wunderlich and Back, 2009).

Mashing is important for wort production and it can be split into three steps – milling, mashing and wort recovery (Harrison, 2009). Malt produced in the previous step must be milled first to remove dust and stones and thus prevent damage to the brewing equipment (Wunderlich and Back, 2009). Adjuncts can be added at this step to add fermentable carbohydrates necessary to yeast growth (Harrison, 2009). At the end flour (malted and non-malted cereals) is obtained (UNICER, 2015a). Water is added to flour which in turn is submitted to different variables such as time, pH and temperature. These conditions may vary depending on the type of beer to be produced. During mashing, enzymes present in malt promote the hydrolysis of more complex molecules into simpler ones (Harrison, 2009; UNICER, 2015a; Wunderlich and Back, 2009). Hydrolysis of all grain content occurs within a few hours and after that temperature is increased up to 75ºC leading to enzyme denaturation. However, pH of malt is not acidic enough for optimal enzyme activity, which means some adjustments have to be made. Lactic acid addition or bacterial fermentation allows for a reduction of pH to approximately 5.2 (suitable for *lager* production) or even lower (*ale* production) (Harrison, 2009). After the enzymes are inactivated a soluble phase and an insoluble one remain. The insoluble phase corresponds to the solid that settles out and the soluble phase is the wort which has the necessary compounds for yeast growth (Harrison, 2009; Wunderlich and Back, 2009). Nevertheless, wort must be separated from the insoluble phase through filtration at almost 80ºC for about 2 or 3 hours (Wunderlich and Back, 2009; UNICER, 2015a). Then wort is transferred into a brew kettle followed by the addition of hop in order to provide aroma and flavor to beer (Harrison, 2009).

Before fermentation, wort must be processed through boiling. This step kills all microorganisms, including lactic acid bacteria added to lower pH during mashing, denatures remaining enzymes, extracts essential oils and resins from hops, promotes precipitation of substances responsible for cloudiness, enhances color development, removes undesirable volatiles and concentrates wort (Harrison, 2009; UNICER, 2015a; Wunderlich and Back, 2009).

After being boiled wort is inoculated with yeasts through a process called pitching or seeding. Yeasts used for inoculation depend on the type of beer to be produced - *Saccharomyces cerevisiae* for *ale* beers and *Saccharomyces pastorianus* for *lager* beers. Temperature range and time are also different depending on the type of beer

as mentioned in 1.1.3. (Harrison, 2009; Wunderlich and Back, 2009). A constant temperature and humidity must be achieve to maintain the desired growth rate for the yeast. To growth yeasts need to consume essential nutrients (sugars, amino acids, fatty acids) present in malt and available after mashing process. For instance, sugars are converted into alcohol and carbon dioxide (Harrison, 2009; UNICER, 2015a; Wunderlich and Back, 2009). There is a decrease of pH during fermentation which increases fermentation stability and decreases the possibility of contamination. During fermentation, bottom-fermenting yeasts tend to flocculate and settle to the bottom of the brew kettle. Top-fermenting yeasts tend to form small clumps of cells that are carried to the top. Either way, yeasts may be collected from the bottom or from the surface, respectively, and reused in the next fermentation (Harrison, 2009).

At the end of the fermentation there is a residual extract composed by non-fermentable sugars, sugars which were not converted into another compound and should be left in final beer. This residual extract will allow carbon dioxide formation during maturation (Wunderlich and Back, 2009). Some volatile compounds like aldehydes and sulphur are produced during fermentation and generally have a negative impact on aroma and flavor of final beer (UNICER, 2015a; Wunderlich and Back, 2009). Maturation step aims to remove those compounds by trapping them into carbon dioxide bubbles formed by residual extract fermentation (Wunderlich and Back, 2009). Maturation occurs at lower temperatures (0-2ºC) for several weeks allowing the beer clarification and stabilization (Harrison, 2009; UNICER, 2015a; Wunderlich and Back, 2009).

After maturation beer is cloudy and presents suspended particles (yeasts, bacteria, colloids). Pumping beer through a suitable filter or centrifuge it will remove suspended particles and beer will appear clear (UNICER, 2015a; Wunderlich and Back, 2009). After filtration or centrifugation some bacteria and yeast residues may remain in beer. Those residues are removed through pasteurization, a thermal treatment. Pasteurization can be carried out by heat or cold (Harrison, 2009; UNICER, 2015a). In heat pasteurization beers are subjected to almost 75ºC for a period ranging between 15 and 30 seconds. Nevertheless, such temperatures may have a negative impact on flavor. On the other hand cold pasteurization involves the use of chemical agents for preservation or filtration through membrane filters avoiding flavor loss. Finally, in aseptic conditions beer is packed into appropriate containers (cans, bottles, barrels, kegs) which are generally pasteurized as well (Harrison, 2009).

1.1.5. Beer composition

Beers have several compounds in its final composition. Such compounds may be derived from raw materials or be a result of the brewing process (Harrison, 2009).

Quantitatively water is the most important ingredient in the brewing process since it represents more than 90% of the beer composition. Thus, water quality plays an important role and must be potable, pure, and free of pathogens. However, water mineral content may affect beer properties and flavor. Depending on the concentration in which metals are found, they may have negative or positive effects on beer quality. Some of those are extremely toxic even at lower concentrations but trace amounts can be crucial for yeast growth during the brewing process (Buiatti, 2009). Metals may be provided by malts but water is the main source (Wunderlich and Back, 2009).

Starch, cellulose and hemicellulose are the most important grain components. These polysaccharides are hydrolyzed into smaller carbohydrates which will be converted into carbon dioxide and alcohol (mainly ethanol) (Wunderlich and Back, 2009). Both carbon dioxide and ethanol are flavor enhancers. Ethanol may counteract with the bitterness effect introduced by hop's resins. However, some carbohydrates such as glucose, fructose, and maltose, are present in final because they are unfermented carbohydrates and consequently were not converted into carbon dioxide and ethanol. Despite its lower concentrations, unfermented carbohydrates may have a significant influence on beer taste (Buiatti, 2009).

Proteins are hydrolyzed into its fundamental units, amino acids, during malting. Amino acids are essential for yeast growth but not all of them are required. Unutilized amino acids such as proline are not converted into another compounds and corresponds to nitrogenous compounds present in beer. About 80-85% of those amino acids came from malts and 10-15% from yeasts (Buiatti, 2009). Beyond amino acids a tiny fraction of proteins such as albumins and globulins may also be found in final beer. Those proteins are important for foam and colloidal characteristics (Wunderlich and Back, 2009).

During fermentation many amino acids are subjected to deamination and transamination reactions carried out by yeasts converting those monomeric units into organic acids, aldehydes, alcohols and esters.

Hops contain a range of chemical species which may influence beer properties – essential oils and resins. Essential oils from hops are a complex mixture which can be divided into two major fractions – the group of hydrocarbons without oxygen and the group of hydrocarbons with oxygen including esters, aldehydes, ketones, acids,

and alcohols. The latter group is responsible for modifying beer aroma. Resins are hops' derived compounds which grant bitterness to beer (Buiatti, 2009). Besides bitterness resins also enhance beer physiological digestibility, foam stability, and bacteriostatic nature (Wunderlich and Back, 2009).

Both grains and hops are important sources of polyphenols, a substance with a high impact in brewing in many ways. It generally has a positive on influence color, foam, taste, and haze formation. Polyphenols also have some properties such as antioxidant ones which enhances beer quality (Wunderlich and Back, 2009).

Generally lipids are not present in beer because they are eliminated during the brewing process. Nevertheless, when present lipids affect negatively beer giving it a cardboard taste (Buiatti, 2009).

Another group of compounds present in beer are vitamins. These substances are usually present in the embryos' and aleurone layer (Wunderlich and Back, 2009). Although they do not have any effect on beer characteristics, vitamins (mainly B-group) are crucial as growth factor for yeast (Buiatti, 2009).

1.1.6. Beer consumption, production and its impact

Despite the world economic crisis that in recent years beer consumption has remained constant at least in the countries of the European Union (EU) (see table 1) (The Brewers of Europe, 2014).

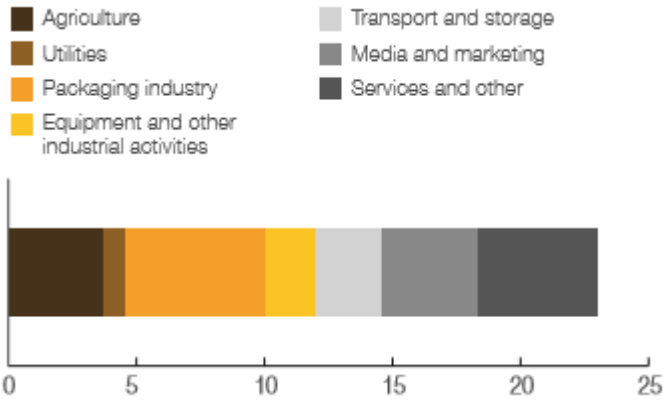
Table 1 – Total beer consumption per capita between 2008 and 2013 (in 1000 hl) in EU (adapted from The Brewers of Europe, 2014).

	2008	2009	2010	2011	2012	2013
Average EU 28	78	75	73	73	73	71

Today beer still remains one of the most widely consumed alcoholic beverages and to cope with its large consumption, production has to keep pace with demand. In the last years beer production has succeeded to satisfy demand but by a close range (The Brewers of Europe, 2014).

A high beer production has a huge impact especially at an economic level. In this matter suppliers, employment, government revenues and hospitality are of particular interest (The Brewers of Europe, 2013).

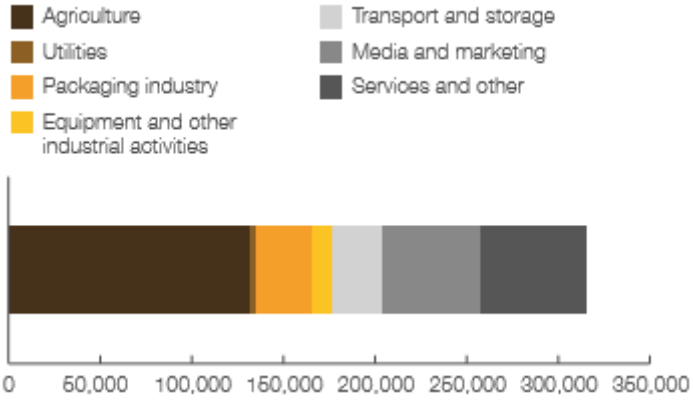
Several sectors are involved in beer production from raw materials provided by agriculture until it reach the consumers through marketing corresponding to a move 23 billion of euros in European Union (EU) just in 2012 (The Brewers of Europe, 2013).



Graphic 1 - EU purchases of goods and services by brewing sector in 2012 (adapted from Brewers of Europe, 2013).

As it can be seen in graphic 1 almost a quarter of breweries' purchases concerned packaging. In fact beer is storage in recipients purchased to packaging industry and then sold to consumers. Packaging industry provide to the brewing sector different kinds of recipients. About 50% of beer is sold in glass bottles (returnable and non-returnable) and about 30% in can. There is also kegs, casks and bulk tanks. (The Brewers of Europe, 2013).

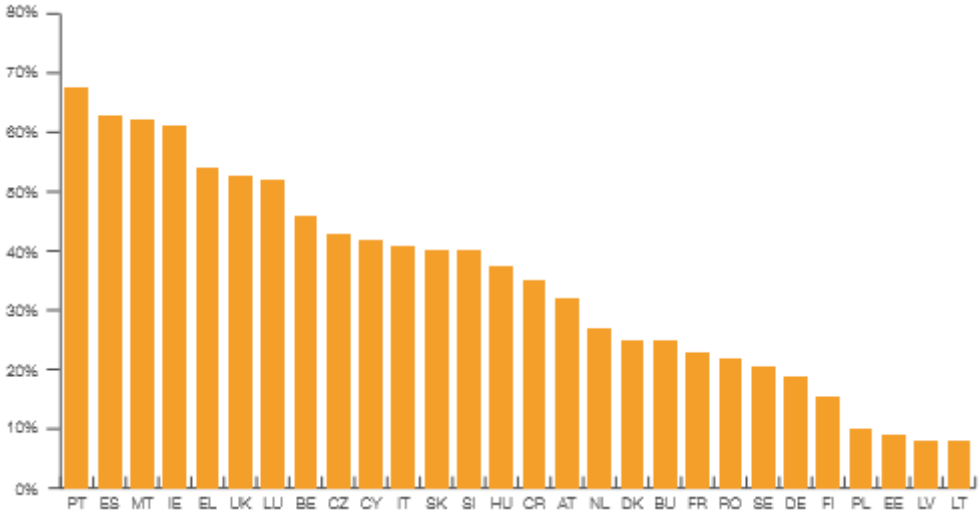
Brewing sector has a strict relationship with the industries which means brewing development supply industry by creating more jobs. Above all its indirect employment is higher in agriculture but this also occurs in the other industry sectors as shown in graphic 2. In 2012 the brewing sector helped create more than 300 000 jobs in other industry sectors in EU (The Brewers of Europe, 2013).



Graphic 2 - Indirect employment related to beer in the EU in 2012 (adapted from The Brewers of Europe, 2013).

Hospitality sector also benefits from brewing sector since beers are available in bars, pubs, and restaurants. Yet about 75% of the EU Member States present a higher beer consumption at home than in hospitality facilities. This explains why beer

is sold more in bottle than in other containers. Just out of curiosity in 2012 Portugal registered the higher beer consumption in the hospitality sector in all EU as shown in graphic 3 (The Brewers of Europe, 2013).



Graphic 3 - Beer consumption in the hospitality sector, as a percentage of total beer consumption per EU Member State in 2012 (adapted from The Brewers of Europe, 2013).

Government of each country also benefits from brewing sector since they get revenues from beer products. There are three major categories of tax revenues due to beer. The first one is income, payroll taxes and security contributors which are paid by employers and employees involved in all sector related to beer. The second one is valued added tax (VAT) which is levied on the turnover of beer sales in retail and hospitality (The Brewers of Europe, 2013). The third one is called excise duty which is an indirect tax on the sale or use of specific products and is applied in all EU Members States. Alcohol (includes beer) is one of those products alongside with tobacco and energy (European Commission, 2015). This tax can represent up to one fifth of total government revenues due to beer (The Brewers of Europe, 2013).

In Portugal total beer consumption dropped about 20% between 2008 and 2012 but a considerable amount is still being consumed. Like other EU Member States, beer consumption at home (beer consumption off-trade) in Portugal also increased as shown in table 2 (The Brewers of Europe, 2013).

Table 2 - Basic characteristics of beer market in Portugal between 2008 and 2012 (adapted from The Brewers of Europe, 2013).

	2008	2009	2010	2011	2012	Δ 2008-2012
Total consumption (in hectoliters)	6 200 000	6 100 000	5 900 000	5 320 000	4 927 000	-20.5%
Total consumer spending (in million Euro)	2 687	3 133	3 001	2 808	2 636	-1.9%
Consumption of beer per capita (in liters)	61	60	59	53	49	-19.7%
Beer consumption on-trade (hospitality)	70%	70%	69%	69%	67.5%	-2.5%
Beer consumption off-trade (retail)	30%	30%	31%	31%	32.5%	2.5%
Average consumer price in on-trade (1 liter, including taxes) (in Euro)	6.00	6.60	6.60	6.85	7.04	17.3%
Average consumer price in off-trade (1 liter, including taxes) (in Euro)	1.56	1.72	1.72	1.78	1.84	18.3%

In 2012 Portugal was one of the top 5 countries to export beer especially to countries out of EU (The Brewers of Europe, 2013).

1.1.7. Metal contaminants

In all beers metal presence is verified although it is not added intentionally. Its presence is due in large part to raw materials (water, cereals, barley, and hops) that are used in brewing corresponding to endogenous sources of metals in beers. Metal content is variable and depends on the substrates used, the type of beer produced, and the country of origin of beer. However metal presence in beers is not explained only by the raw materials used in their manufacture - metals may be added inadvertently during fermentation and maturation processes. Brewing equipment itself as well containers in which beer is stored and transported are possible exogenous metal sources (Donadini et al., 2008; Pohl, 2008).

1.1.8. Legislation

The large consumption of beer and the possibility of contamination with substances that may be harmful to consumers' health raises great concerns. Hereupon, as many products on the market for human consumption, also beers are regulated by law. In Portugal beers are regulated by Portaria nº 1/96 from 3rd January defining what is meant by beer, what kinds of beer are allowed and permitted

additives, marketing and labelling. It refers also what features must be controlled such as color, smell, taste, acidity, and pH. Concerning metals, limits are set for some of them – arsenic (0.1 mg/L), cobalt (0.05 mg/L), copper (0.2 mg/L), iron (0.3 mg/L), lead (0.2 mg/L) and zinc (1 mg/L), the target metals of this study (Portaria nº 1/96).

1.2. Metals referred in legislation

Metals are present in all kind of beverages, alcoholic or not. Some of them are essential in the brewing process others not so much. Still metals may affect beer characteristics in a positive or negative way and may also be a risk for the consumer's health.

In this study metal quantification will be performed to six elements and all of them are regulated by Portuguese law – arsenic, cobalt, copper, iron, lead, and zinc. Characteristics as well as human exposure, toxicokinetics and toxicology for each of the aforementioned metals will be briefly described in this section.

1.2.1. Arsenic

Arsenic is a steeled grey solid material widely distributed in the Earth's crust. Chemically it has metal and non-metal characteristics, therefore is classified as a metalloid. However, in environment arsenic is not found in a solid state but combined with other elements (ATSDR, 2007a). There are several oxidation states but the most common ones for arsenic are III and V (Lindo and Silvestre, 2010; Valko et al., 2005). Depending to each element is combined with arsenic can be classified into two categories - organic (carbon and hydrogen) and inorganic (oxygen, chlorine and sulphur) and both forms may occur in environment and in human body (ATSDR, 2007a). Inorganic arsenic is considerably more toxic and more common than the organic form and it is even considered as a human carcinogen by IARC (included in Group I) (Donadini et al., 2008).

The lowest exposure possible to arsenic is the best preventive measure to follow. Nonetheless, people may be exposed to this metalloid through food, water and air especially because arsenic does not have smell neither taste (ATSDR, 2007a). Among food sources arsenic is mainly found in fish and agriculture products. Arsenic is a naturally occurring element which means drinking water from geological structures turns out to be another source of exposure. Nevertheless, much of exposure to arsenic is due to anthropogenic activity – traffic fumes, uncontrolled industrialization and massive use of pesticides in agriculture. Much of arsenic from anthropogenic activity accumulates in soil and water and consequently in grains used to produce beer which means this beverage (as other beverage and products derived from

agriculture) could easily introduce arsenic in human body (Donadini et al., 2008). Apparently it does not alter chemical and physical properties of beer.

There are at least three main pathways of exposure through which arsenic may enter in human body – inhalation, oral and dermal. Tough skin is a potential route of exposure to arsenic almost none of this element is absorbed through the mentioned organ. After reach the lung through inhalation, arsenic accumulates on the lung surface and then absorbed (ATSDR, 2007a). Arsenic is easily absorbed in the gastrointestinal tract when the element intake is due to oral exposure – about 80 to 90% of ingested arsenic is absorbed. From there arsenic is transported to every organ in the human body, yet it seems to be more accumulated in hair, nails and skin (Lindo and Silvestre, 2010).

Once present in human organism arsenic can undergo two different metabolic processes – reduction/oxidation and methylation. Reduction/oxidation process interconvert As (III) and As (V). Methylation is a more complex process (biotransformation) where arsenate (arsenic with an oxidation state of V) is reduced to arsenite (arsenic with an oxidation state of III) and then subjected to a series of methylation reactions producing methylarsonous acid (MMA) and dimethylarsinic acid (DMA) (ATSDR, 2007a). Methylation pathway has being considered as a detoxification but recently evidences seem to show the opposite – methylated and dimethylated arsenicals in the trivalent oxidation state appear to be more cytotoxic and genotoxic than the ones in the pentavalent oxidation state. Thereby, arsenic methylation may actually be a pathway for arsenic potential activation instead of diminishing its toxicity (Liu et al., 2008; Thomas et al., 2001 *in* Valko et al., 2005), especially because arsenicals in the trivalent oxidation state are thiol-reactive, thus inhibiting enzymes and/or altering proteins by reacting with proteinaceous thiol groups (Liu et al., 2008).

As regards to its excretion arsenic may be expelled from the organism through skin, feces and urine (ATSDR, 2007a). Skin is the major organ where arsenic accumulates, therefore it can be excreted through skin desquamation and sweat (Liu et al., 2008). Only small amounts of arsenic are excreted with feces (ATSDR, 2007a). Urine is the primary pathway of arsenic excretion – about 10% is excreted in inorganic acid form, 10 to 20 % MMA and 60 to 80% DMA (Lindo and Silvestre, 2010).

Exposure to arsenic (organic and inorganic forms) may have extremely adverse effects in humans and it may even be fatal if ingested in larger doses (70 – 180 mg) (Liu et al., 2008). Several systems like respiratory, cardiovascular, nervous and hematopoietic ones are negatively affected by this element (Lindo and Silvestre, 2010; Liu et al., 2008). It is also associated to cancer development in some organs

such as lung, skin, liver, bladder, and kidney (Donadini et al., 2008; Valko et al., 2005).

1.2.2. Cobalt

Cobalt is a natural occurring element with some properties similar to iron like magnetism (Liu et al., 2008). It can be found all over the environment – rocks, soil, water, plants, animals, and even in meteorites (ATSDR, 2004a). It has several applications especially in painting since its salts are commonly used in paint driers and as ingredients of colored pigments. Other applications include corrosion and wear resistance and magnetism (Liu et al., 2008; Valko et al., 2005). Cobalt is an essential element since cobalamin (a form of cobalt) is a critical component of vitamin B_{12}, an important substance to human being since it is required for the production of red blood cells (Liu et al., 2008; Simonsen et al., 2012).

Cobalt exposure is inevitable especially due to diet which is the largest source of cobalt intake (ATSDR, 2004a). Humans cannot synthetize vitamin B_{12} so it must be ingested to fulfil nutritional requirements and with it cobalt in cobalamin form (Valko et al., 2005). However, occupational exposure it is also relevant especially in plant processing, metallurgical and ceramic industries (ATSDR, 2004a; Simonsen et al., 2012).

Inhalation, oral and dermal exposures are the reported cobalt intakes into the human body. The need to ingest vitamin B_{12} make oral exposure the most relevant one. Cobalt absorption occurs in gastrointestinal tract (ATSDR, 2004a). Some studies from almost three decades reported cobalt absorption depends on the compound to which cobalt is associated when ingested. For instance, there is a 30% cobalt absorption from cobalt chloride while absorption from cobalt oxide is only 5%. Cobalt absorption also has a huge variation from individual to individual and can go from 5 up to 45% (Elinder and Friberg, 1986 *in* Liu et al., 2008; Patrick et al., 1989 *in* ATSDR, 2004a). After absorption cobalt is transported to every organs through bloodstream, especially organs that required vitamin B_{12} such as liver and kidneys. Cobalt metabolism is most of all related to vitamin B_{12} metabolism (ATSDR, 2004a). Vitamin B_{12} is an essential cofactor in methyl transfer reactions during the conversion of homocysteine to methionine (Ansari et al., 2014). As regards to its excretion most of absorbed cobalt is expelled through urine (80%) and feces (15%) (Liu et al., 2008).

Cobalt mechanisms of toxicity are still unclear but several potential ones have been identified (ATSDR, 2004a; Simonsen et al., 2012). Cobalt may inhibit crucial enzymes especially when they are composed with amino acids such as cysteine and

methionine because cobalt has high affinity for sulfhydryl groups. Cobalt is also a calcium antagonist which means cobalt can compete with calcium for intracellular calcium binding proteins and even inhibit calcium signaling based processes (Simonsen et al., 2012). Cobalt may also interfere with DNA repair processes and induce DNA damage through oxidative stress. This effects on DNA are a result of several oxidant-based and free radical-based processes where cobalt is involved and capable of generating oxygen radicals like superoxide and thus leading to oxidative stress (ATSDR, 2004a; Simonsen et al., 2012; Valko et al., 2005).

Exposure to cobalt may have some adverse effects to humans. Those effects may include problems at thyroid gland (ATSDR, 2004a; Simonsen et al., 2012), lung related conditions such as asthma, pneumonia, and wheezing (Simonsen *et al*., 2012; Valko et al., 2005), allergic contact dermatitis (Simonsen et al., 2012) and even cardiomyopathy (Liu et al., 2008).

1.2.3. Copper

Like the previous elements copper is a natural occurring element as well (ATSDR, 2004b). It has several properties such as has malleability, ductility, and electrical and thermal conductivity whereby this element has several applications like coins, electrical wiring, water pipes, metal products and many others (ATSDR, 2004b; Lindo and Silvestre, 2010). Copper is an essential microelement which plays several important roles. For instance, it is a fundamental component in several enzymes; acts as a reductant in the enzymes that reduce molecular oxygen, scavenging and neutralizing free radicals; participates on hemoglobin, myelin, and melanin production; is involved in the development of numerous tissues and participates in both iron and energy metabolism (Fraga, 2005; Lindo and Silvestre, 2010; Liu et al., 2008; Osredkar and Sustar, 2011).

Exposure to copper is inevitable because it is everywhere – rocks, soils, sediments, water and even air. Although copper plays vital roles in the human body, an exaggerated exposure to this element is not advisable in order to minimize toxic effects. Exposure to copper may occur by inhalation of copper-containing dust but oral exposure is much more common (ATSDR, 2004b). Actually there is a recommended daily intake of 1 mg per day for young adults (Fraga, 2005; Osredkar and Sustar, 2011). Sea food, liver, cereals, and seeds are excellent sources of copper (Osredkar and Sustar, 2011; Romaña et al., 2011). Water ingestion is also one of the bigger sources for copper intake especially tap water since copper is commonly used in water pipes (ATSDR, 2004b).

Although small amounts of copper are absorbed in the stomach, most of it is absorbed by the gastrointestinal tract, primarily in the duodenum (Liu et al., 2008; Romaña et al., 2011; Valko et al., 2005). Copper absorption in the gastrointestinal tract may be negatively affected by other substances such as iron, zinc, and fructose (Liu et al., 2008). Once absorbed copper may be transported to the liver bounded to albumin. In the liver copper may be stored in hepatocytes (Osredkar and Sustar, 2011; Valko et al., 2005). From the liver copper is transported in the bloodstream to the other organs by being an integral part of ceruloplasmin, a protein responsible for catalyzing the oxidation of minerals such as iron (ATSDR, 2004; Osredkar and Sustar, 2011; Valko et al., 2005). Ceruloplasmin is synthetized in the liver (ATSDR, 2004).

With so many sources of exposure to this element especially by ingestion, copper daily ingestion may exceed the recommended. However, copper levels are maintained within sustainable limits through control of excretion. Although is not an excretion process, copper storage is a way to remove the element from the bloodstream maintaining physiological levels of plasmatic copper. Copper may be excreted through sweat, urine, feces, and menses, but the major route of copper excretion is the bile representing about 80% of total excretion (Liu et al., 2008; Romaña et al., 2011; Valko et al., 2005). About 10-15% copper excreted from bile is reabsorbed (Romaña et al., 2011).

Exposure to high levels of copper can lead to hemolytic anemia, liver injuries, vomiting, and epigastric pain (Lindo and Silvestre, 2010; Liu et al., 2008). As mentioned before copper is important to neutralizing free radicals. However, in excess it may have the opposite effect because this element has the potential to act has an oxidant. Cupric ion (Cu^{2+}) may be reduced by ascorbic acid or by glutathione-S-transferase to cupric ion (Cu^{+}) which is capable of catalyzing the formation of reactive radicals through the decomposition of hydrogen peroxide. Hereupon, in excess copper is capable of generating free radicals which will lead to oxidation stress and consequently DNA damage and even low-density lipoprotein (LDL) oxidation. LDL oxidation may have several consequences especially because it promotes atherogenesis (Valko et al., 2005).

There are also two copper related genetic disorders worth mentioning – Menkes and Wilson Diseases. In Menkes Disease there is a deficiency on the carrier protein (albumin) responsible for transporting copper from the enterocytes to the liver. In Wilson Disease copper is normally transported to the liver but it accumulates in this organ due to a poor incorporation in ceruloplasmin. Also in Wilson Disease there is a deficient excretion through bile leading to copper accumulation in other organs (Liu et al., 2008; Romaña et al., 2011; Osredkar and Sustar, 2011).

Unlike arsenic and cobalt, copper does has influence in beer qualities and it is a parameter to take into account during the brewing process. Copper plays an important role in aerobic beer ageing and flavor stability during storage. However, it may be toxic to yeasts and lead to an irreversible beer haze. To prevent haze formation and yeast mutagenesis an upper limit of 0.1 mg/L is recommended (Buiatti, 2009; Pohl, 2008).

1.2.4. Iron

Iron is one of the most abundant elements in the Earth's crust. In its pure form has a silver color and is highly reactive especially in the presence of oxygen which leads to iron oxidation and thus generating iron oxide (Lindo and Silvestre, 2010). There are several forms of iron oxide but magnetite, maghemite and hematite are the most common ones. These oxides are very important at a technology level mainly due to its magnetic properties. Among the possible applications are magnetic seals and inks, magnetic recording media, catalysts, ferrofluids and even contrast agents for magnetic resonance imaging and therapeutic agents for cancer treatment (Teja and Koh, 2009). Such as copper also iron plays vital roles in human body. It is essential in erythropoiesis and is a fundamental component of several proteins such as hemoglobin, myoglobin (Fe-heme proteins), and Fe-containing and/or Fe-activated enzymes (succinate dehydrogenase, alcohol dehydrogenase) (Fraga, 2005; Liu et al., 2008).

Iron deficiency causes anemia. A total iron intake ranging between 14.4 and 20.2 mg/day is enough to prevent iron deficiency related problems (Fraga, 2005). There are two forms of iron – heme Fe and non-heme Fe – and both of them are obtainable through diet. Examples of sources of heme Fe are meat, poultry, and fish, while cereals, seed of leguminous plants, fruits, vegetables, and dairy products are good sources of non-heme Fe (Fraga, 2005; Liu et al., 2008).

Iron homeostasis is regulated through several processes including absorption, storage and excretion. Most of the dietary iron is absorbed in the duodenum (Grotto, 2008; Liu et al., 2008; Valko et al., 2005). Some factors such as acidity, and solubilizers enhance iron absorption. The amount of iron absorbed by the body is regulated by the need of the latter (Grotto, 2008). This element is absorbed in the apical membrane of the intestinal epithelial cells (Lindo and Silvestre, 2010). Different protein transporters are involved in heme Fe and non-heme Fe. After been reduced from trivalent iron to divalent iron, dietary free iron (non-heme Fe) is transported into the enterocytes by the divalent metal transporter protein (DMT-1) (Valko et al., 2005). Dietary heme Fe is internalized by the heme carrier protein-1 (HCP1). HCP1 is expressed in the liver and kidneys and its regulation occurs according

to intracellular iron level – transport dietary heme Fe when necessary and avoids it when the organism do not need more iron preventing accumulation. Intestinal iron absorption is also influenced by other protein, hepcidin whose expression is regulated at transcriptional level. When iron levels are high there is no need for iron absorption at intestinal level whereby hepcidin expression inhibits DMT-1. If iron absorption is required there is no hepcidin expression (Grotto, 2008).

Once in the enterocytes iron can be stored by ferritin and hemosiderin or transported to the plasma by ferroportin. Then iron bounds to transferrin. However, this protein is not selective for divalent iron which means divalent iron must be converted to trivalent iron. Ceruloplasmin and hephaestin promote divalent iron oxidation to trivalent iron (Valko et al., 2005; Grotto, 2008). Once bounded to transferrin iron is transported and distributed to different tissues. Nevertheless, to enter in a cell a transferrin receptor must exist on the cell membrane, otherwise iron incorporation do not occur. If there is a transferrin receptor on the cell membrane, iron binds to that receptor followed by endocytosis (Grotto, 2008; Liu et al., 2008; Valko et al., 2005).

Besides diet iron may be acquired through recycling senescent blood red cells. Blood red cells have an average life span of 120 days. After that they are no longer functional and must be removed. Such removal is carried out by macrophages which will phagocytose and degrade blood red cells. A great portion of iron is incorporated in the heme group of those cells so this recycling is an important source of the element. After being recovered iron (current oxidation state is II) may be stored in the macrophage itself in ferritin form or exported through ferroportin and oxidized by ceruloplasmin. Finally iron is transported mainly to bone marrow to participate in the production of new red blood cells (Grotto, 2008).

Iron levels are also maintained through excretion. Excretion through gastrointestinal tract is the most common way but iron can also be excreted through urine and skin (Liu et al., 2008).

Accidental ingestion of mineral supplements may lead to acute iron poisoning (Liu et al., 2008). Chronic iron intoxication may also occur especially if there is a high dietary iron intake or if the individual took repeated blood transfusions (Fraga, 2005). Hemochromatosis is probably the most common cause of chronic intoxication. Hemochromatosis is a genetic disorder which enhances iron absorption from the gut leading to the accumulation of this element (Fraga, 2005; Valko et al., 2005). Similarly to copper, iron excess may lead to the generation of free radicals and consequently to serious damage in the organism, including DNA damage due to oxidative stress, lipid and protein peroxidation (Valko et al., 2005).

Iron has influence in beer qualities and it is a parameter to take into account during the brewing process. Iron is responsible for beer quality and flavor stability. It may also influence beer color by darkening it. However, in high quantities beer acquires an unpleasant harsh and metallic taste, foam appears greyish, yeast activity is reduced, and there is haze formation (Buiatti, 2009; Pohl, 2008). There is a recommended upper limit which is the same for copper – 0.1 mg/L (Pohl, 2008).

1.2.5. Lead

Lead is a naturally occurring element and probably the most common environmental pollutant. Normally lead is not found in its pure form, as a metal but combined with more elements such as lead oxides (ATSDR, 2007b; Lindo and Silvestre, 2010; Sharma et al., 2011). It can be found in two forms – organic (e.g. gasoline) and inorganic (e.g. paints, pipes) (Donadini et al., 2008). This element has many applications in several areas. It is used as a pigment in paints and dyes, and in the manufacture of batteries, accumulators, and conduits (ATSDR, 2007b; Lindo and Silvestre, 2010).

Due to its numerous applications lead exposure is quite high. Besides that lead contamination through anthropogenic activities must be considered because lead may be present in air and food (Donadini et al., 2008). Bad habits such as smoking is also a form of exposure because cigarettes contain small amounts of lead. Exposure to lead may happen through inhalation and ingestion (ATSDR, 2007b).

After inhalation of air contaminated with lead or aerosols containing this element, lead particles are deposited in the respiratory tract. Amounts and patterns of such deposition is mainly affected by particles size and breathing patterns which may or may not be related to the individual age. Absorption of deposited lead is influenced by particle size and respective solubility (ATSDR, 2007b; Sharma et al., 2011). It should be noted that most of lead particles inhaled have very small dimensions so almost of them are absorbed (Liu et al., 2008). Only a tiny fraction of ingested lead is absorbed and even less is retained. However, this is not exactly true for children since in this case about half of ingested lead is absorbed and one third is retained (Liu et al., 2008).

Regardless the route of exposure, after being absorbed lead enters in the bloodstream and most of it (about 99%) is bounded to hemoglobin and only about 1% on serum. The half-life of lead in blood is about 30 days. Only lead present is serum is distributed. First it goes to soft tissues such as liver and kidneys and then is redistributed to skeleton and hair (ATSDR, 2007b; Liu et al., 2008; Sharma et al., 2011). The half-time of lead in soft tissues is about 40 days (Lindo and Silvestre,

2010). Once lead reaches skeleton it tends to accumulate and its level increases with age. The half-time of lead in skeleton is about 20% which is a significant source of endogenous exposure (Liu et al., 2008).

Lead excretion is also independent of the route of exposure. Absorbed lead may be excreted through several ways but the main ones are urine and feces (ATSDR, 2007b; Liu et al., 2008). Lead may also be excreted through sweat, saliva, hair, and nails (ATSDR, 2007b).

Lead is considered one of the most dangerous toxic metals especially due to the amount and severity of the effects that it can cause. Those effects are dependable on the dose and duration of exposure (Lindo and Silvestre, 2010; Liu et al., 2008). Nervous system, bone marrow and kidneys are probably the most affected by lead but this element may also damage hematopoietic and reproductive systems (Lindo and Silvestre, 2010; Liu et al., 2008; Sharma et al., 2011). Similarly to copper and iron, lead is also capable to cause oxidative stress by inducing the generation of reactive oxygen species (Sharma et al., 2011).

Relatively to its carcinogenicity lead in inorganic form is classified as a probable human carcinogen while lead in organic form has not yet been classified (Donadini et al., 2008).

1.2.6. Zinc

Zinc is one of the most common elements in the Earth's crust and it can be found just about everywhere from air, water, soil up to the most varied foods (ATSDR, 2005). In its pure form zinc is a silvered solid which reacts violently when exposed to moist air (ATSDR, 2005; Lindo and Silvestre, 2010). This metal has several applications especially in metallurgic industry where it is used to produce alloys (ATSDR, 2005; Liu et al., 2008). Zinc is usually found combined with other elements forming oxides, chlorides and sulphide compounds which can be used to make paints, rubber, drugs and others (ATSDR, 2005). It is an essential element due to its biological functions in the human body. Is an important component of hundreds of enzymes and transcription factors being required for protein and DNA synthesis, cell division and it can act as an antioxidant. Zinc is also necessary in cell membrane integrity, bone and teeth mineralization, normal taste and wound healing. It also supports normal growth and development during pregnancy, childhood and adolescence (Fraga, 2005; Liu et al., 2008; Osredkar and Sustar, 2011; Valko et al., 2005). Unlike copper and iron, zinc does not participate in redox reactions (Lindo and Silvestre, 2010).

Zinc exposure occurs mainly through ingestion. Red meat, seafood (especially oysters) and dairy products are some examples of good zinc sources (Fraga, 2005; Osredkar and Sustar, 2011). However, occupational exposure may also occur especially by inhalation of zinc dust or fumes from zinc-smelting or zinc-welding operation (ATSDR, 2005).

Zinc is absorbed in the duodenum and jejunum through passive diffusion which involves some carrier-mediated processes (Lindo and Silvestre, 2010; Liu et al., 2008). Zinc absorption may be affected by several compounds. For instance, it can be enhanced amino acids and prostaglandins while it can be reduced by dietary fibers, calcium and phosphorus (Liu et al., 2008). Zinc absorption may also be inhibited by metallothionenin action which competes with cysteine-rich intestinal protein, a diffusible intracellular zinc carrier (ATSDR, 2005; Lindo and Silvestre, 2010). Once absorbed zinc is transported to the liver where is stored. From the liver this element will be distributed to the other tissues. Besides liver zinc may also be stored in muscles and bones. Most of the zinc (almost 80%) is bounded to albumin in plasma but it can be bounded to transferrin as well (ATSDR, 2005; Lindo and Silvestre, 2010; Liu et al., 2008).

Intestinal excretion is the main route of excretion of zinc followed by urine. There are other routes of excretion such as saliva, hair loss, and sweat but these are only the minor ones (ATSDR, 2005).

When compared with the previous metals zinc may be considered as relatively harmless. However, at considerable high concentrations or intakes of 150-450 mg per day may have toxic effects. Vomiting, diarrhea, nausea, gastrointestinal irritation, loss of appetite, abdominal cramps, and headaches are among those effects. Long-term zinc may decrease copper absorption and pancreatic injuries (Lindo and Silvestre, 2010; Osredkar and Sustar, 2011).

Zinc has influence in beer qualities and it is a parameter to take into account during the brewing process. Since this element acts as a cofactor to many enzymes it plays an important role in yeast growth and metabolism. However, at higher concentrations zinc is toxic to yeast and inhibits enzymes and also affects negatively fermentation and colloidal stability of beer. To affect positively beer quality and improve yeast growth and metabolism a 0.15-0.5 mg/L concentration is recommended (Buiatti, 2009; Pohl, 2008).

1.3. Digestion methods

Three digestion processes are mentioned several times in the literature with some changes from article to article - dry ashing, wet digestion and microwave

pressure digestion (Doner and Ege, 2004; Enders and Lehmann, 2012; Korn et al., 2008; Lajunen and Peramaki, 2004; Momen et al., 2006; Pohl, 2008). Dry ashing and wet digestion process will be briefly described. Microwave pressure digestion was the chosen method to digest beer samples in this work which is why it will have a more detailed description.

1.3.1. Dry-ash digestion

This method allows removal of organic matter from a sample and it is indicated for toxic and nutrient elements digestion and quantification (Momen et a*l*., 2006). Many protocols are described for this digestion method but the use of a muffle furnace (or oven) is a common step (Doner and Ege, 2004; Enders and Lehmann, 2012; Korn et al., 2008; Lajunen and Peramaki, 2004; Momen et al., 2006; Pohl, 2008).

Generally the first step is weighting (if solid) or pipetting (if liquid) the sample into a ceramic crucible. In case of liquid samples the ceramic crucible is placed on a hot plate at a certain temperature until a solid residue remains (no more than 150 ºC). Ceramic crucible is placed into an oven and then heated at a temperature that can go from 400 to 700 ºC for a certain period (it can be two/three periods of eight hours each, for example) (Doner and Ege, 2004; Enders and Lehmann, 2012; Lajunen and Peramaki, 2004). In the end white ashes should be obtained. Afterwards, concentrated HNO_3 is added to the ashes and placed on a hot plate until total evaporation. After cooling down H_2O_2 may also be added to aid the digestion process, especially when the sample has a very complex matrix (Enders and Lehmann, 2012; Momen et al., 2006). Lastly, the result is solubilized with deionized water and stored into an appropriate recipient (Enders and Lehmann, 2012).

However, this method has several disadvantages. Volatilization loss of some elements such as arsenic, lead and zinc may occur due to temperatures reached inside the muffle furnace. To reduce volatilization losses and secondly accelerate ash formation ashing aids such as magnesium nitrate and ammonium dihydrogen phosphate may be added (Doner and Ege, 2004; Hoening, 2001 *in* Korn et al., 2008; Lajunen and Peramaki, 2004). Since dry ashing method is an open acid digestion, risk of contamination is always a possibility (Korn et al., 2008; Lajunen and Peramaki, 2004). In addition this method has other drawbacks such as being time consuming with high chemical consumption (Doner and Ege, 2004).

1.3.2. Wet digestion

This digestion method is indicated for food materials digestion mainly due to its versatility. The procedure can be changed when necessary regarding to sample weights and digestion conditions in order to get better results and minimize

volatilization losses (Momen et al., 2006). In the literature several similar procedures are published for this method (Doner and Ege, 2004; Enders and Lehmann, 2012; Korn et al., 2008; Lajunen and Peramaki, 2004; Momen et al., 2006; Pohl, 2008).

While in dry ashing digestion is carried out either in ceramic crucible, wet digestion is carried out in glass or Teflon vessels. From those two materials, Teflon is better since it is less susceptible to contamination (Lajunen and Peramaki, 2004). The start is similar to dry ashing – weighting or pipetting the sample into a glass or Teflon vessel. An acid mixture is added to the sample (that mixture may involve two or more from the following – HNO_3, H_2SO_4 or even an oxidation reagent such as H_2O_2). Shortly after an initial reaction between reagents and sample, vessel contents are gently mixed and heated – first at low temperatures and then at higher temperatures until there is no fume liberation – this step can take between 2 and 15 hours or even more (Doner and Ege, 2004; Enders and Lehmann, 2012; Momen et al., 2006). A glass filter funnel may be used to reflux acid fumes generated during digestion (Momen et al., 2006). Afterwards, deionized water is added to each vessel until a certain percentage of acid concentration is achieved (normally 5%) (Enders and Lehmann, 2012). If there is a residue in the end, it is advisable to add HCl to dissolve it before addition of deionized water (Momen et al., 2006).

Like dry ashing digestion method, wet digestion is also an open acid digestion which means that both methods share some disadvantages such as risk of contamination and volatilization losses. This method is also time consuming (Doner and Ege, 2004). Other disadvantages reported in literature about this method are co-precipitation of sparingly soluble compounds, incomplete digestion of organic compounds and formation of insoluble compounds (Feng et al., 1999 *in* Momen et al., 2006; Wu *et al*, 1996 *in* Momen et al., 2006).

1.3.3. Microwave pressure digestion

This digestion method can be applied to a wide variety of samples. Also microwave pressure digestion has huge advantages when compared with the two methods described in 1.3.1. and 1.3.2.

- Usually sample preparation is the most time consuming step. With microwave pressure digestion method time spent in sample preparation is dramatically reduced especially when compared with the two digestion methods described in 1.3.1. and 1.3.2. – with this method digestion programs usually take less than 60 minutes (Bellido-Milla et al., 2004 *in* Pohl, 2008; Berghof, 2014a; Lajunen and Peramaki, 2004;).

- Since a few years digestion vessels are being entirely manufactured of high quality TFM-PTFE (polytetrafluoroethylene with additional modifier perfluorpropylvinylether), a material with optimized surface characteristics and reduced absorption and adsorption effects. This means no contamination risk from digestion vessels and consequently no memory effects by using the same vessels for other digestions (Berghof, 2014a).
- In microwave pressure digestion, samples are heated directly by absorption of microwave radiation which means there is a rapid and simultaneous heating of all vessels. Since this is a close acid digestion as temperature increases the same happens to pressure making this digestion method extremely efficient (Berghof, 2014a). This rapid heating process allows an increase of sample/acid solution temperature without interact with the vessel walls reinforcing the memory effects absence mentioned in the last point (Buldini et al., 2002). Since samples are heating, fast exothermic reactions may occur. Fortunately pressure and temperature reaction parameters are monitored by sensor systems. This sensors are not incorporated in digestion vessels so they are not a possible contamination source (Berghof, 2014a).
- Other advantage of this method is being a closed acid digestion which means contaminant risks from air are minimized. Volatilization losses are also completely avoided (Berghof, 2014a; Lajunen and Peramaki, 2004).
- Reduced consumption of reagents is other aspect worth mentioning as an advantage (Bellido-Milla et al., 2004 in Pohl, 2008; Berghof, 2014a; Lajunen and Peramaki, 2004).
- Finally, its versatility makes microwave pressure digestion more desirable when compared to conventional digestion methods. The amount of necessary reagents can be easily adapted depending on the sample to be digested. Operator can control pretty much all the variables which can influence the digestion process – temperature, rate and pressure (Doner and Ege, 2004).

1.4. Trace elements quantification

Normally in food products metals are present in low quantities. In beers that presence is in the order of µg/L (Matusiewicz and Mikolajczak, 2001; Pohl, 2008). To detect such low levels of metals certain techniques are required, especially Atomic Spectrometry ones (Bolann et al., 2007; Pohl, 2008). Atomic Spectrometry techniques can be divided in two major groups – absorption and emission. In both groups samples are introduced into an atomizer and converted into free atoms and/or ions (Bolann et al., 2007). Some Atomic Spectrometry techniques (absorption and emission) were already reported in literature as common measure for trace elements

quantification in beers. For example, Flame Atomic Absorption Spectrometry (FAAS) (Bolann et al., 2007; Doner and Ege, 2004; Korn et al., 2008; Onate-Jaen et al., 2006 *in* Pohl, 2008) and Graphite Furnace Atomic Absorption Spectrometry (GFAAS) are the most common AAS techniques performed in this field (Bolann *et al*., 2007; Korn et al., 2008; Llobat-Estelles et al., 2006 *in* Pohl, 2008). Hydride Generation techniques (an AAS technique) are more desirable to quantify volatile elements such as arsenic, mercury and selenium (Bolann et al., 2007; Lajunen and Peramaki, 2004; Matusiewicz and Mikolajczak, 2001). Atomic Emission Spectrometry techniques such as Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) and Inductively Coupled Plasma Mass Spectrometry (ICP-MS) require higher expertise to operate and have high operating costs but they too are widely used in trace elements quantification (Bolann et al., 2007; Donadini et al., 2008; Enders and Lehmann, 2012; Momen et al., 2006; Sedin, 2006 *in* Pohl, 2008). In figure 2 a general diagram of the most Atomic Spectrometry techniques commonly used in trace elements quantification can be consulted.

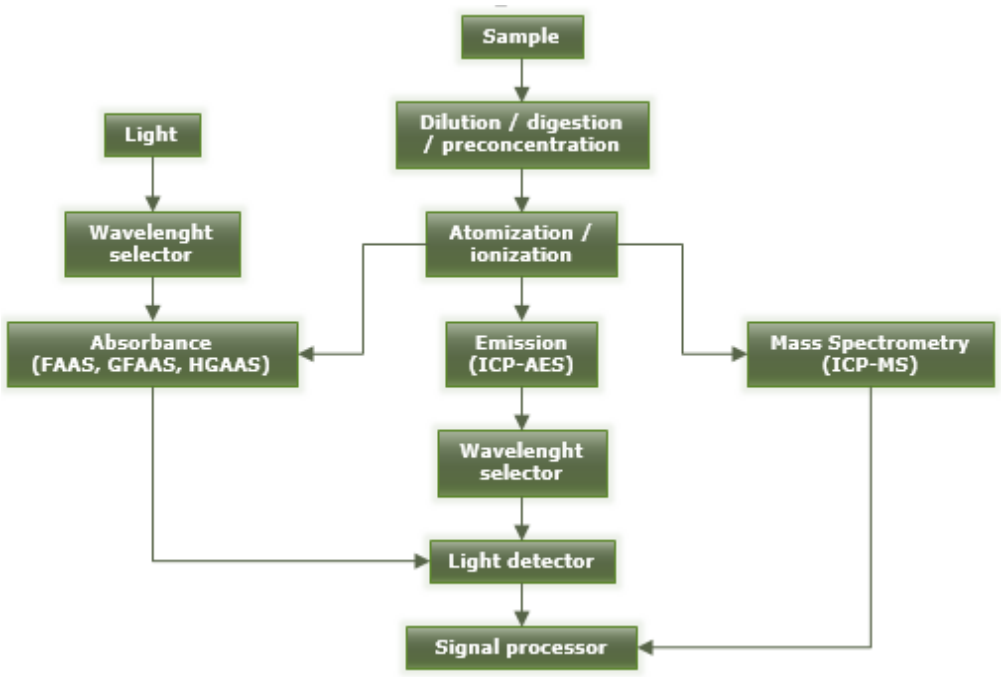


Figure 2 - General diagram of Atomic Spectrometry techniques (adapted from Bolann et al., 2007).

With so many Atomic Spectrometry techniques available it can be a huge challenge to choose the most appropriate one. Several criteria must be taken into account before making the final decision. Among these criteria are detection limits, working range, sample volume available, analytical interferences and many others. Other important factor is the laboratory itself – does it have the necessary equipment

(or funding to buy one if needed), specialized staff or how often that kind of analysis is requested? (Bolann et al., 2007).Relevant technical parameters about some Atomic Spectrometry techniques can be consulted in table 3.

Table 3 - Technical parameters of Atomic Spectrometry techniques commonly used in trace elements quantification (adapted from Bolann et al*.,* 2007).

Parameter	FAAS	HGAAS	GFAAS	ICP-AES	ICP-MS
Detection limit (µg/L)	0.1 - 100	0.01 - 10	< 0.01 - 10	0.1 - 100	< 0.001 - 10
Dynamic range (orders of linearity, power of 10)	2 - 3	2 - 3	2 - 3	5 - 7	5 - 7
Operational time	Fast	Slow	Slow	Fast	Fast
Cost	Low	Low	Intermediate	High	High
Automation	Yes	Yes	Yes	Yes	Yes
Element analysis	Sequential	Sequential	Sequential	Simultaneous and sequential	Simultaneous and sequential
Ease of use of instrument	User friendly	User friendly	Requires some expertise	Requires some expertise	Requires high expertise
Interferences	Well characterized	Well characterized	Fairly characterized	Well characterized	Well characterized
Internal standard	Not used	Not used	Not used	Used	Used

Elements to be analyzed must be taken into account as well. An accurate measure for some elements can be achieved using a specific technique instead of another. Hereupon, some techniques may be used more often than orders to quantify certain elements (Bolann et al., 2007). Information regarding the elements analyzed in this study can be found in table 4.

Table 4 - Methods used in the analysis of the elements targeted in this study. + - technique used in some biological material; ++ - technique used; +++ - technique most commonly used (adapted from Bolann et al*.,* 2007).

Element	FAAS	HGAAS	GFAAS	ICP-AES	ICP-MS
As		++	+		++
Co			+++	+	++
Cu	+++		++	+++	++
Fe	+++		++	+++	++
Pb	+		+++	+	+++
Zn	+++		++	+++	++

1.4.1. Radiation sources for Atomic Absorption

AAS may be regarded as the study of radiant energy absorption in UV-Vis by neutral atoms in ground state. When hit by UV-Vis radiation neutral atoms can absorb that radiation. However, an element cannot absorb all radiation. Only when hit by radiation of a specific wavelength can an atom absorb a photon and enter into an excited state. In order to return to its ground state an atom can emit energy from the photon producing an electronic transition. This transition is usually called resonance line. This means Atomic Absorption is highly specific because electronic transitions are specific for each element and consequently resonance lines are too narrow (10^{-3} to 10^{-2} nm) (Lajunen and Peramaki, 2004).

There is a component in Atomic Absorption, the monochromator, capable to isolate resonance lines. Nevertheless, it is not capable, *per se*, to isolate such narrow resonance lines. This will only be possible if there is a source that emits the desirable element resonance line with sufficient radiation intensity and stability to allow accurate absorption measurements. Actually at least four kinds of radiation sources for Atomic Absorption can be considered – hollow cathode lamps (HCLs), electrodeless discharge lamps (EDLs), microwave EDLs and radiofrequency EDLs (Gonçalves, 2001; Lajunen and Peramaki, 2004). In this study HCLs will be used for cobalt, copper, iron, lead and zinc analysis and EDLs for arsenic analysis which is why only these two radiation sources are described in the following paragraphs.

HCLs are the most common radiation source in Atomic Absorption. These lamps consist of a glass cylinder, an anode and a cathode. Glass cylinder is filled with an inert gas, usually argon, because it does not react with the cathode and its resonance lines are different from the analyte elements (Gonçalves, 2001; Lajunen and Peramaki, 2004). The anode is composed by only a tungsten, nickel or zirconium wire (Lajunen and Peramaki, 2004). The cathode may be regarded as the prime component since it is made of the analyte element (or filled with it) which means a different lamp is required for each metal. HCLs functionality is relatively simple. All starts when a voltage is applied between the anode and cathode. That voltage leads to a discharge which ionizes the filler gas liberating positive ions. Then, those ions accelerate toward the cathode heating it and atomic spectrum of the corresponding metal is emitted (Bolann et al., 2007; Gonçalves, 2001; Lajunen and Peramaki, 2004). Simultaneously a beam emitted by the radiation source is modulated into a signal which passes through the sample atomic vapor. In this vapor analyte free atoms absorb radiation of the line-like radiation source. Since resonance lines are specific to each element, the desirable one is selected by the monochromator and the isolate analyte line falls onto a detector to convert the light signal into an electrical

one. Finally, that signal is amplified and recorded by a readout device giving a quantitative measure of the element concentration (Broekaert, 2005 *in* Bolann et al., 2007; Lajunen and Peramaki, 2004). HCLs are recommended for GFAAS (Gonçalves, 2001; Lajunen and Peramaki, 2004).

EDLs consist of sealed quartz tubes which are filled with a few milligrams of analyte metal or with a metal halite, and with argon at low pressure. Quartz tube is introduced into a coil of a high frequency generator and an electric discharge is produced due to a microwave frequency electromagnetic shield. These lamps are not so used as HCLs especially due to their reduce lifetime and long heating period. On the other side they are the only ones that can be used to quantify elements in the UV region. They are also the most recommendable for volatile elements quantification mainly due to their high intensity of radiation which may be several orders of magnitude higher than HCLs. There is also a better signal to noise ratio and consequently a lower detection limits making EDLs ideal for arsenic quantification in this study using HGAAS (Gonçalves, 2001; Lajunen and Peramaki, 2004).

1.4.2. Graphite Furnace Atomic Absorption Spectrometry (GFAAS)

In this Absorption technique only a small amount of sample is needed (no more than 100 µL). In this study samples were introduced into a graphite tube with an autosampler allowing a greater repeatability and precision when compared to manual injection. After being introduced the sample undergoes by some heating steps but for that a temperature program must be developed. This is a critical step because the same temperature program cannot be applied to all elements. To complicate even further matrix the sample matrix must be taken into account as well. Normally equipment supplier gives suggestions about the recommended temperature in each step and its respective period. Generally GFAAS has four steps – drying, thermal pre-treatment (or pyrolysis), atomization and cleaning (Bolann et al., 2007; Gonçalves, 2001; Harris, 2007; Lajunen and Peramaki, 2004).

At all times, except during atomization, furnace is purged with argon to prevent graphite oxidation and remove volatile material (Harris, 2007).

On the first step, drying, solvent is removed from the sample by evaporation. In order to prevent sample splashing and/or expelling drying must be a slow and steady step using a temperature slightly higher than solvent boiling point. Desirable temperature must be achieved gradually and maintained for a certain period (this is valid for all steps in GFAAS) (Gonçalves, 2001; Lajunen and Peramaki, 2004).

In thermal pre-treatment (or pyrolysis) the sample matrix is decomposed. (Harris, 2007). However, this step can be somewhat challenging. The sample matrix

must be decomposed before atomization process otherwise interferences will occur and absorption signal measurement will be impossible. Temperature selection is critical here. Normally, temperatures in this step are much higher than in drying. Sometimes a higher temperature is required to decompose the sample matrix compared to analyte atomization which leads most likely to analyte loss. Preventing this situation is possible by making the matrix more volatile which can be done by adding a substance called matrix modifier. Using matrix modifiers to turn sample matrices more volatile enables the use of lower temperatures and consequently less analyte loss (Harris, 2007; Lajunen and Peramaki, 2004).

Atomization is a very short step. A few seconds are required to reach the desirable atomization temperature and atomization itself take place within a few seconds. Once atomization temperature is reached analyte volatilization occurs generating a vapor where analyte free atoms can absorb radiation emitted by the radiation source (Lajunen and Peramaki, 2004).

Sometimes, during atomization the sample is not totally volatilized. If not cleaned, the next result will not be precise because previous sample residue will interfere in that result. This is called memory effect. The purpose of the cleaning step is to prevent memory effects. In order to do that graphite tube must be subjected to a temperature of 2500ºC for a period no longer than 5 seconds (Gonçalves, 2001).

1.4.2.1. Interferences

Such as all quantitative determination techniques also in GFAAS interferences may occur. The most critical ones occur during the atomization step. Generally those interferences can be divided into two distinct groups – physical, spectral, and chemical interferences (Lajunen and Peramaki, 2004).

Physical state of the samples is the major concern regarding physical interferences. Depending on samples viscosity and their surface, tension differences may occur at pipetting consequently affecting its precision (Lajunen and Peramaki, 2004).

The most common spectral interferences are background absorption and scattering effects due to the presence of other species which absorb radiation in the same wavelength as the analyte (this interference can be easily removed out of the equation by having a background corrector system such as deuterium lamp which was used in this study). Analyte response enhancement in later analysis due to memory effects can be easily treatable by increasing atomization temperature and/or adding a cleaning step in the temperature program (Gonçalves, 2001; Lajunen and Peramaki, 2004).

Regarding chemical interferences the most critical one is analyte loss. That analyte loss may occur even before atomization step especially if that analyte is volatile at pyrolysis temperature. Matrix components may convert the analyte into a volatile form leading to analyte loss as well. Analyte loss may be reduced by adding matrix modifiers which makes the analyte less volatile than the sample matrix (Lajunen and Peramaki, 2004).

1.4.2.2. Advantages and disadvantages

GFAAS, as all analytical techniques, has its advantages and disadvantages. Probably the biggest advantage of this method is to allow metal quantification in the concentration of the order of µg/L alongside with small amounts of sample required to do that quantification (Bolann et al., 2007; Lajunen and Peramaki, 2004). On the other hand some expertise is required when performing this technique. Despite autosampler injection is available, sample injection into a graphite furnace may be a challenge – the droplet must contact the graphite furnace floor and remain in a small area; it cannot be injected too high or too low otherwise poor precision may be experienced (Harris, 2007).

1.4.3. Hydride Generation Atomic Absorption Spectrometry (HGAAS)

Some elements such arsenic, mercury and selenium may be difficult to quantify using GFAAS due to those elements volatility. These elements have a common characteristic – they form volatile, covalent hydrides. Besides that they have high oxidation state (e.g. arsenic oxidation state is V). To quantify elements with such characteristics hydride generation techniques are usually performed (Bolann et al., 2007; Lajunen and Peramaki, 2004). Hydride generation usually implies the reduction of the volatile element from a higher oxidation state to a lower one (e.g. from V to III which happens in arsenic analysis) leading to the separation and enrichment of the analyte and interference reduction (Abdul-Majeed and Zimmerman, 2012; Gonçalves, 2001).

Generally, HGAAS has three successive steps – hydride generation, hydride transfer to atomizer, and hydride decomposition. In this study a flow injection technique was used, therefore all HGAAS steps took place in a fully automated closed system. These steps allow separation of volatile elements from the sample matrix before their introduction into the detection device preventing volatilization loss (Lajunen and Peramaki, 2004).

Summarizing, firstly sample and reagents – a reductant and a carrier - are pumped separately and continuously blended in a junction leading to hydride generation (Lajunen and Peramaki, 2004).

Several chemical substances can be selected as a reductant in hydride generation but sodium tetrahydroborate ($NaBH_4$) is the most commonly used. From all reductants this one presents more advantages – has a fast reaction time, may be added in solution form and may be employed to several elements to produce its respective volatile metal hydrides (Lajunen and Peramaki, 2004).

When an element solution is blended with a $NaBH_4$ alkaline solution in acidic medium, hydroborate anion oxidation to boric acid occurs (Abdul-Majeed and Zimmerman, 2012). Following equation represents the reactions that lead to the formation of the hydride element:

$$\mathrm{NaBH_4\ (aq) + HCl\ (aq) + 3H_2O\ (aq) \rightarrow H_3BO_3\ (aq) + NaCl\ (aq) + 8}H^{\cdot} \xrightarrow{\mathrm{A^{m+}}} \mathrm{AH_n + H_2\ (excess)}$$

where; A represents the analyte, m^+ the oxidation state of the analyte, n the oxidation state of the hydride, and $H^{\cdot}$ the nascent hydrogen (Abdul and Zimmerman, 2012).

Once generated, the hydride is transported by a carrier (HCl) to a gas-liquid separator. An argon current transfers the resultant gas to a quartz cell at 900ºC leading to hydride in gaseous form to vaporize, generating a vapor where analyte free atoms can absorb radiation emitted by the radiation source (Lajunen and Peramaki, 2004).

1.4.3.1. Interferences

Similar to GFASS, HGAAS is prone to many interferences as well such as kinetic, oxidation state and chemical ones. Spectral interferences are not considered because the element is transferred to the atomizer in gaseous hydride form meaning possible interferences remain in aqueous phase (Lajunen and Peramaki, 2004).

Regarding kinetic interferences the most critical one is the different release rates of the hydride solution. Those release rates are influenced by the sample matrix and sample volume – hydrides are released more easily from smaller sample volumes than from larger ones. Kinetic interferences may be minimized by peak area measurement instead of the peak height (Gonçalves, 2001; Lajunen and Peramaki, 2004).

HGAAS is the only Atomic Absorption technique where sensibility is highly dependable of the element oxidation state. Generally HGAAS has higher sensibility at lower oxidation states (Gonçalves, 2001; Lajunen and Peramaki). As mentioned further in 3.6. HGAAS was the selected technique for arsenic analysis. In this

particular case the sensitivity of As^{V} is about 70-80% of the sensitivity of As^{III}, meaning a pre-reduction before hydride generation is required (Lajunen and Peramaki, 2004).

Chemical interferences only have a minor influence in element quantification except when an element have a strong dependence with pH (e.g. arsenic, lead and selenium). In those cases element quantification occurs in buffered solution. Hydrides from different elements may interfere with each other leading to chemical interference as well (Gonçalves, 2001).

1.4.3.2. Advantages and disadvantages

Separate the element from the matrix and therefore canceling matrix effects is probably the biggest advantage of HGAAS alongside with the lower detection limits in the µg/L order (Gonçalves, 2001; Lajunen and Peramaki, 2004).

However this technique has more drawbacks than benefits. It is only applicable to a few elements and to every one of them (except bismuth) oxidation state must be taken into account. Also pre-reduction before hydride generation, waiting time for lamp and cell quartz heating are some disadvantages of this method (Gonçalves, 2001; Lajunen and Peramaki, 2004).

2. Objectives

Metal content in beers is taken into account by the Portuguese legislation (Portaria nº 1/96 from 3rd January). Aforementioned law establish limits for metal presence in beers for some elements such as arsenic (0.1 mg/L), cobalt (0.05 mg/L), copper (0.2 mg/L), iron (0.3 mg/L), lead (0.2 mg/L), and zinc (1 mg/L).

Therefore, this study aims to evaluate the presence of those metals in samples previously selected by the Portuguese Food and Economic Safety Authority (ASAE) from the most consumed ones and verify if their contents are within legal limits established by the Portuguese legislation.

Beer samples will be digested through microwave pressure digestion with an equipment purchased for this purpose. Optimal digestion conditions will be assessed to obtain a compete digestion. This method will be also validated in order to demonstrate that in the selected conditions where it is performed digestion is effective.

Quantification of such elements will be performed through Atomic Absorption techniques. GFAAS was selected for cobalt, copper, iron, lead, and zinc analysis while HGAAS was selected for arsenic analysis. Validation of these two techniques will not be an aim of this study because it was already performed in other study conducted in the same Laboratory as this one. However, linearity, analytical thresholds, and accuracy will be checked.

3. Materials and Methodologies

3.1. Samples

Seventeen beers from twelve brands manufactured in Portugal and commercialized in the Portuguese market were selected and collected by the Portuguese Food and Economic Safety Authority (ASAE), Department of Food Risks and Laboratories. Those samples represent the most common brands manufactured and consumed in Portugal and are sold in can and glass recipients (see table 5). Samples from four of the twelve brands came both in can and glass recipients – A and G represent one brand and the same is valid for D and H; E and F; I, J and K.

Table 5 - Description of the analyzed samples.

Samples	Recipient
A, B, D, F, J, K, L, M, N, O, P, Q	Bottle
C, E, G, H, I	Can

3.2. Reagents and standard solutions

Table 6 - Reagents used in sample analysis.

Reagents		Brand
Standard Solutions	Arsenic (1000 µg/mL)	SCP Science (Quebec, Canada)
	Cobalt (1000 µg/mL)	Merck (Darmstadt, Germany)
	Copper (1000 µg/mL)	SCP Science (Quebec, Canada)
	Iron (1000 µg/mL)	Fluka Analytical (Switzerland)
	Lead (1000 µg/mL)	SCP Science (Quebec, Canada)
	Zinc (1000 µg/mL)	SCP Science (Quebec, Canada)
Ascorbic acid		Merck (Darmstadt, Germany)
Hydrochloric acid 37%		Sigma-Aldrich (Steinhen, Germany)
Nitric acid 65%		Panreac (Barcelona, Spain)
Nitric acid 67%		Prolabo
Sodium borohydride		Merck (Darmstadt, Germany)
Sodium hydroxide		Merck (Darmstadt, Germany)
Potassium iodide		Panreac (Barcelona, Spain)
Certified reference material (TM -24.3; TM-26.3)		Environment Canada
Chemical modifier ($NH_4H_2PO_4$) 100±2 g/L		Merck (Darmstadt, Germany)
Chemical modifier ($Mg(NO_3)_2$) 2% Mg		SCP Science (Quebec, Canada)
Hydrogen peroxide		Merck (Fluka Analytical, Switzerland)

Standard solutions were prepared in acidic medium (nitric acid at 5% (V/V)) by dilution of single element standard stock (1000 µg/mL). Reagent water was de-ionized water (18.2 MΩcm resistance) obtained by a Milli-Q system from Millipore.

Nitric acid (67%) and hydrogen peroxide (30%) were suitable for trace analysis and used to sample digestion. All the other used reagents had high purity and thus suitable for sample analysis. Low concentration solutions were prepared immediately before use. All reagents and standard solutions used are represented in table 6.

3.3. Materials and equipment

As mentioned in 1.1.7. one possible explanation for metal presence in beers may be due to contamination by the brewery equipment. Trace elements quantification in beers may be affected by this too which means metal analysis in beers could not be entire realistic, especially when smaller analyte concentrations are present. In order to minimize this all material went to a decontamination process whereupon all material was immersed on a nitric acid 15% (V/V) solution for 24 hours. At the end the material was washed several times with deionized water 18.2 MΩcm, dried and stored, protected from dust and other possible sources of contamination. In table 7 all materials and equipment necessary for this study are described.

Table 7 - Used equipment.

Equipment		Brand/model
Microwave digestion system		Berghof Speedwave Two
Water purification system		Direct-Q UV3, Millipore - Bedford
Balance		Mettler Toledo
Atomic Absorption Spectrophotometer		PerkinElmer instruments Analyst 700 equipped with deuterium background corrector
Graphite chambers with L'vov platform		PerkinElmer
Automatic sampler		PerkinElmer FIAS 100 Flow Injection System
Hydride Generation system		(EDL) Sys 2 - PerkinElmer
Lamps	Arsenic (λ = 193,7 nm; I = 380 mA)	EDL - PerkinElmer
	Cobalt (λ = 240,7 nm; I = 15 mA)	HCL - PerkinElmer
	Copper (λ = 324,8 nm; I = 12 mA)	HCL - PerkinElmer
	Iron (λ = 248,3 nm; I = 30 mA)	HCL - PerkinElmer
	Lead (λ = 283,3 nm; I = 12 mA)	HCL - PerkinElmer
	Zinc (λ = 213,9 nm; I = 10 mA)	HCL - PerkinElmer

3.4. Sample preparation

Metal content in beer samples was determined using Atomic Absorption techniques. Normally in this kind of techniques samples must be in a liquid form which is the case (Lajunen and Peramaki, 2004). However, beer samples cannot be introduced directly in Atomic Absorption apparatus. Besides metals, in its contents

beers have considerable amounts of organic compounds such as carbohydrates and proteins (which can be from raw materials or originated during the brewing process) and considerable amounts of carbon dioxide (Buiatti, 2009; Pohl, 2008). Hereupon, beer samples have a very complex matrix which can be a source of matrix effects and influence the obtained results. Prior to analysis, metals must be released from the sample matrix (Bolann et al., 2007; Nascentes et al., 2005 *in* Pohl, 2008). This can be done by subjecting beers samples to a treatment using strong acids and extreme conditions in a process called digestion (Bolann et al., 2007; Lajunen and Peramaki, 2004).

Nevertheless, to be digested an exact sample volume for analysis must be taken. In this case that can be a problem because it is difficult to pipette beer due to its carbon dioxide content – when pipetted there is bubble formation making impossible to do it. Degassing allows an accurate pipetting. There are many methods described in the literature to do that. For example, it can be added some concentrated HNO_3 to the beer sample and leave it loosely capped for a period of 24 hours (Sharpe and Williams, 1995 *in* Pohl, 2008) but less time consuming methods should be considered. Beer samples may be filtered with a specific pore size membrane filter (Pohl, 2008) or even been exposed to microwaves in a pressurized closed vessel microwave oven which will perform the degassing (Bellido-Milla et al., 2004 *in* Pohl, 2008). None of this methods were chosen by us to degas beer samples. The chosen method was to put beer samples in an ultrasonic bath for a 30 minutes period. After that period no bubbles were formed at pipetting, and an exact sample amount can be measured. Only after an exact amount of sample is taken the digestion process can start (Pohl, 2008).

3.5. Digestion method

Laboratory of Instrumental Methods of Analysis has the necessary conditions to perform the digestion methods previously described in 1.3. but the chosen one to digest beer samples was the microwave pressure digestion mainly due to the huge advantages when compared to the other methods.

Beer samples digestions were performed with a microwave digestion system Speedwave Two from Berghof (see figure 3). Information regarding digestion vessels materials, safety measures and technical specifications about this equipment can be found in Appendix A (Berghof, 2014b).

As in conventional digestion method there are several protocols that may be used to digest beer samples using microwave pressure digestion. In the following paragraph adapted protocol to digest beer samples after optimization is presented.

Beforehand beer samples were degassed through ultrasonic bath as described before in 3.4. After degassing 5 mL of sample were pipetted to digestion vessels. Subsequently 0.5 mL of concentrated nitric acid (67%) and 1 mL of 30% (m/v) hydrogen peroxide (in this order) were added to the sample and allowed to react for about thirty minutes. Then digestion vessels were closed and transferred to a digester. The digestion process was started using an application recommend by the equipment provider (Berghof) which is present below in table 8.

Table 8 - Beer digestion program.

Step	1	2	3	4
Ramp	5	1	1	1
T [ºC]	140	160	190	75
Power [%]	90	90	90	90
Time [min]	10	10	10	10

Once finished the digestion process and after cooling down, digestion vessels were opened and the solution was transferred into 10 mL volumetric flasks and the volume completed with deionized water and stored into a decontaminated tube for later analysis.

Berghof recommends in its applications different reagent amounts to be added to digest beer samples - 6 mL of HNO_3 and 2 mL of H_2O_2 (microwave digestion of original wort (beer)) (Berghof, 2014c). Different attempts were made to reduce the volume of reagents used specially the volume of HNO_3 until it represents no more than 5% of the final solution. This allow to protect the graphite tube increasing its life time. At the end of the digestion process solutions obtained appeared to be completely digested since they were colorless and clear. Other important factor which confirmed solutions were completely digested was the absence of precipitate during the reduction reaction used in hydride generation.

Figure 3 - Berghof Microwave Digestion System Speedwave Two (adapted from Berghof, 2014b).

3.6. Trace elements quantification

The aim of this study is trace element quantification of arsenic, cobalt, copper, iron, lead, and zinc in beer samples consumed in Portugal. It is clear that the best way to do it is using the Atomic Spectrometry techniques mentioned in table 3. From those Laboratory of Instrumental Methods of Analysis only have the means to perform the absorption ones. A similar study using a different digestion method was conducted before in this laboratory. At that time chosen techniques were HGAAS and GFAAS and its validation was performed too. For that reason those techniques were selected for this study as well. Hereupon, after sample digestion, HGAAS was selected for arsenic analysis, and GFAAS to quantify cobalt, copper, iron, lead, and zinc content. However, technical issues made impossible zinc analysis. HCLs for GFAAS and EDLs for HGAAS were the selected radiation sources.

3.6.1. Graphite furnace Atomic Absorption Spectrometry (GFAAS)

Selected conditions for trace elements quantification were already been established before in the Laboratory by testing the temperature programs recommend by the software for each metal and adjusted when necessary. Those conditions are presented below in table 9.

Table 9 - Analysis parameters of metals by GFAAS.

Parameters	Cobalt	Copper	Iron	Lead	Zinc
Wavelength (λ) (nm)	240.7	324.8	248.3	283.3	213.9
Pyrolysis temperature (˚C)	1400	1000	1400	1100	700
Atomization temperature (˚C)	2500	2300	2400	1600	1800
Inert gas	Argon 250 mL/min				

A matrix modifier was added to all standard solutions and analyzed samples. The matrix modifier for each metal and respective concentration is shown in table 10.

Table 10 - Matrix modifiers utilized in metals analysis by GFAAS.

Element	Modifier	Concentration (mg per 5 µL)
Cobalt	$Mg(NO_3)_2$	0.05
Copper	$Mg(NO_3)_2$	0.01
Iron	$Mg(NO_3)_2$	0.05
Lead	$NH_4H_2PO_4$	0.2

Zinc is not present in table 10 because due to technical problems zinc analysis was not possible. Arsenic is not present as well because its analysis was performed by HGAAS, therefore no matrix modifier was required. The Atomic Absorption Spectrometer model used in this study is represented in figure 4.

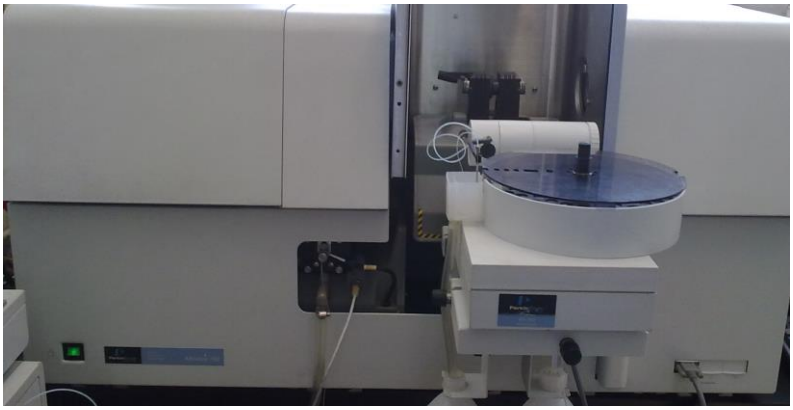


Figure 4 - Atomic Absorption Spectrometer, PerkinElmer Instruments AAnalyst 700.

3.6.2. Hydride Generation Atomic Absorption Spectrometry (HGAAS)

Arsenic quantification was performed through HGAAS instead of the GFAAS used for cobalt, copper, iron, and lead. Matusiewicz and Mikolajczak (2001) reported a study from Cervera et al. (1989) regarding arsenic analysis where GFAAS was applied and the respective D.L. was too high to enable arsenic to be determined at very low concentration levels in beer. The same authors reported a study from Segura et al. (1999) where HGAAS was applied and the arsenic D.L. was lower than the described in Cervera et al. (1989) enabling arsenic to be determined at very low concentration levels. Taking this into account and the information referred in 1.4.2. HGAAS was selected for arsenic analysis.

After digestion samples were subjected to a reduction reaction to reduce arsenic from an oxidation state V to III and the same reaction was performed to standard

solutions. In this reaction, and by this order, 1 mL of concentrated hydrochloric acid, 1 mL of potassium iodide 5% (p/v) and 1 mL of ascorbic acid 5% (p/v) were added to 1 mL of sample in a 10 mL volumetric flask and the volume completed with deionized water. Solutions were allowed to stay at room temperature for at least 45 minutes before analysis. During analysis 500 μL of sample were added to 0.2% $NaBH_4$ and 0.05% NaOH solution (reductant agent) and to 10% HCl solution (carrier solution). The quartz cell was at 900ºC. The Hydride Generation System model used in this study is represented in figure 5.

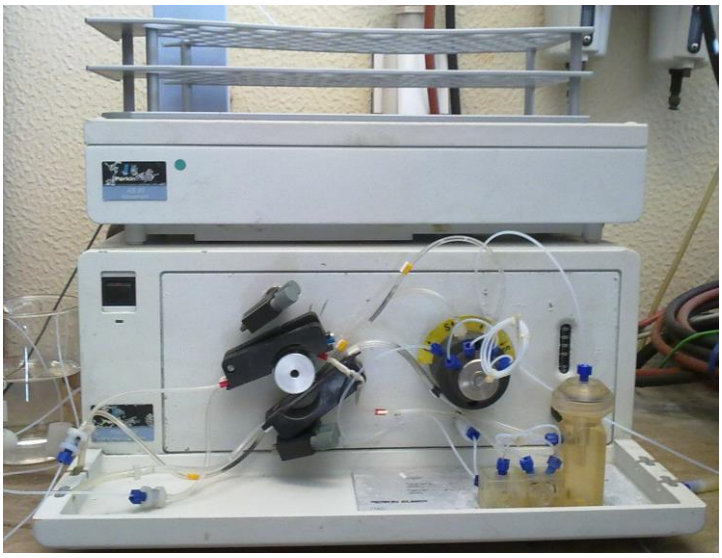


Figure 5 - Hydride Generation System, PerkinElmer FIAS 100 Flow Injection System.

3.7. Method validation

Any outcome may be influenced significantly by systematic and/or random errors which can be accumulated due to various manipulations required in some analytical methods. According to Guia Relacre 13, method validation aims to standardize criteria used to demonstrate that an internal test method has the appropriate features to obtain results with the required quality in the conditions where it is practiced (Guia Relacre 13, 2000).

Every time a Laboratory execute internal test methods a validation process of those methods must be performed. Internal test method validation must be adapted to each case but it always implies minimum requirements variable to each case and includes the study of some parameters such as working range, linearity, analytical thresholds (detection and quantification), selectivity/specificity, precision and accuracy (Guia Relacre 13, 2000).

Validation process involves the study of parameters for direct and indirect evaluation. Determination of such parameters for each metal was performed according to Guia Relacre 13.

All validation for each metal in this study was already performed in a previous study conducted in the Laboratory of Instrumental Methods of Analysis for GFAAS and HGAAS but not for the digestion method (Guerreiro, 2014). To validate microwave pressure digestion method, study of selectivity/specificity and precision parameters is required. Every day a calibration curve was necessary to perform metal analysis whereby linearity, analytical thresholds and accuracy parameters were also study despite previous work.

Data regarding working range and precision GFAAS (repeatability and intermediate) from a previous work conducted in the Laboratory of Instrumental Methods of Analysis can be consulted in the Appendix B.

3.7.1. Indirect evaluation

3.7.1.1. Selectivity/specificity

Selectivity is the ability of a method to identify and distinguish a particular analyte without interferences from other components.

Generally samples have more than one component beyond the target analyte. A method can only be considered specific if it allows to discriminate the analyte relatively to those other components present in the sample. In other words a method is specific when the measurement concerns only the desirable analyte.

Multi-component sample should be used to assess selectivity/specificity. Possible interferences from different component present in a sample may be evaluated through a recovery test where an analyte in study is intentionally added at a known concentration. Samples should have the same matrix and be analyzed in duplicates. An analytical method may be considered applicable (selective and specific) when recovery rate is closer to 100%. In this study a recovery rate between 80 and 120% was accepted.

Beer samples were spiked with a solution containing all target metals. After spiking, beer samples had a 10 µg/L metal increment.

Recovery rate can be calculated by the following equation

$$\text{Recovery } (\%) = \left(\frac{C_1 - C_2}{C_3} \right) \times 100$$

where C_1 is the quantified concentration on the spiked sample, C_2 the quantified concentration on the non-spiked sample and C_3 the sample known concentration.

3.7.1.2. Linearity

Linearity may be evaluated using a statistical model according to ISO 8466-1 standard. This standard states that a calibration curve must have at least five points evenly distributed in the working range (the recommended number is ten).

In quantitative analysis, calibration indicates the process by which a measurement system response is related to an amount or a concentration of a known substance. Establishing a calibration curve (equipment signal as a function of concentration) will allow to quantify the analyte concentration in the samples.

From ordered set of pairs provided by the calibration curve, linear calibration and nonlinear calibration curves may be calculated as well as the corresponding residual standard deviation, $S_{y/x}$ and S_{y2}, respectively.

The difference of the variances (DS^2) may be determined by the following formula:

$$DS^2 = (N - 2) * S^2_{y/x} - (N - 3) * S^2_{y2}$$

where N is the number of calibration standards.

Calculate test value, PG, is the next step:

$$PG = \frac{DS^2}{S^2_{y2}}$$

Test value is then compared with the tabulated value of the Snedecor/Fisher distribution F.

- If PG ≤ F, calibration function is linear
- If PG ≥ F, calibration function is nonlinear

3.7.1.3. Analytical thresholds

There are several ways to calculate analytical thresholds. Generally, in method validation, those thresholds correspond to detection and quantification limits. Both can be calculated through a blank series and/or a linear calibration. In this study linear calibration was used to determine such thresholds.

Detection limit (D.L.) is the smallest measured amount from which it is possible to detect the presence of an analyte with reasonable statistical certainty. This analytical threshold correspond to a smaller amount of analyte that can be detected

in a sample but not necessarily quantitated as exact value. A measurement below the detection limit does not mean the analyte is absence from the sample. It can only be stated that a certain analyte could be present at a concentration lower than the detection limit.

Through linear calibration detection limit can be obtained by

$$D.L. = \frac{\lfloor 3.3 * S_{y/x} \rfloor}{b}$$

where $S_{y/x}$ is the calibration curve residual standard deviation and *b* the calibration curve slope.

Quantification limit (Q.L.) is the smaller measured concentration from which the quantification of the analyte is possible with a certain accuracy and precision.

Through linear calibration quantification limit can be obtained by

$$Q.L. = \frac{\lfloor 10 * S_{y/x} \rfloor}{b}$$

where $S_{y/x}$ is the calibration curve residual standard deviation and *b* the calibration curve slope.

3.7.1.4. Precision

Precision intends to evaluate the dispersion of the results from independent trials repeated on the same sample, similar samples or standards under specified conditions. There are two extreme measures to assess this dispersion called repeatability and reproducibility. Between these two extreme precision measurements there is another measure termed intermediate precision or intra-laboratory variability.

Repeatability was already performed in a previous work conducted on the Laboratory and data about it can be consulted in the Appendix B. Reproducibility was not performed in the previous work mentioned before neither in the present study because it would involve different laboratories, analysts and equipments. Intermediate precision was already performed for GFAAS and HGAAS but not for microwave pressure digestion methods.

Intermediate precision refers to precision evaluated in the same sample, similar samples or standards using the same method in the same laboratory but defining the conditions which vary. Those conditions could be different analysts, different equipments, and different days. This precision measurement is recognized as the most representative of the variability of the results in a laboratory. In this study

selected condition was different days (three was the number of days) and in each day ten measurements were performed on the same sample.

3.7.2. Direct evaluation

This evaluation aims to know method's accuracy. It is defined as the correlation between the result of a test and the reference value accepted as true. Certified reference materials (CRM), inter-laboratory trials and comparative tests can be applied to assess accuracy. In this study accuracy could only be assessed by CMR.

A CMR has a concentration value for each parameter and an associated uncertainty. MCR acquisition must be made to a recognized and credible supplier. For this study CMR were acquired from National Research Council Canada.

The proper use of a CRM consists in its analysis to evaluate the laboratory performance. Data obtained from CRM analysis must be compared with the certified value, determining the error and the accuracy of analysis. Z-score factor was the chosen process to evaluate data obtained from CRM analysis. Z-score factor is calculated through the following equation:

$$Z = \frac{(Xlab - Xv)}{S}$$

where Xlab is the value obtained by the Laboratory, Xv the reference value accepted as true (CMR certified value) and S the deviation unity which may be the CMR uncertainty.

Evaluation can be made according to the following rating scale:

- $|Z| \leq 2$ – Satisfactory
- $2 < |Z| \leq 3$ – Questionable
- $|Z| > 3$ – Incorrect

3.8. Statistical analysis

All graphics and statistical treatment including one-way ANOVA with $\alpha = 0.05$ significance level were performed using the basic tools from Microsoft Excel.

4. Results and Discussion

Before presenting the results, first a summary of what was written in materials and methodologies section about the sample digestion procedure and further sample analysis is presented.

Prior to analysis, all beer samples were degassed using an ultrasonic bath for 30 minutes and then transferred to a digestion vessel Teflon like material. 0.5 mL of nitric acid (67%) and 1 mL of 30% (m/v) hydrogen peroxide (in this order) were added to 5 mL of degassed sample and mixed. Shortly after, the mixture went into a microwave pressure digestion following the temperature program described in table 6. At the end, the digestion result was transferred to a 10 mL volumetric flask and finally stored into a decontaminated tube for later analysis.

AAS techniques were performed to quantify the metals in study. GFAAS was selected for cobalt, copper, iron and lead analysis while HGAAS was selected for arsenic analysis. Zinc analysis was planned too but due to technical problems such analysis was not possible.

4.1. Validation

Following Guia Relacre 13 recommendations several parameters were taken into account for method validation in order to ensure that the methods used have the necessary characteristics to obtain results with the required quality. As mentioned before all validation for each metal in this study was already performed in a previous study conducted in the Laboratory of Instrumental Methods of Analysis for GFAAS and HGAAS but not for the current digestion method.

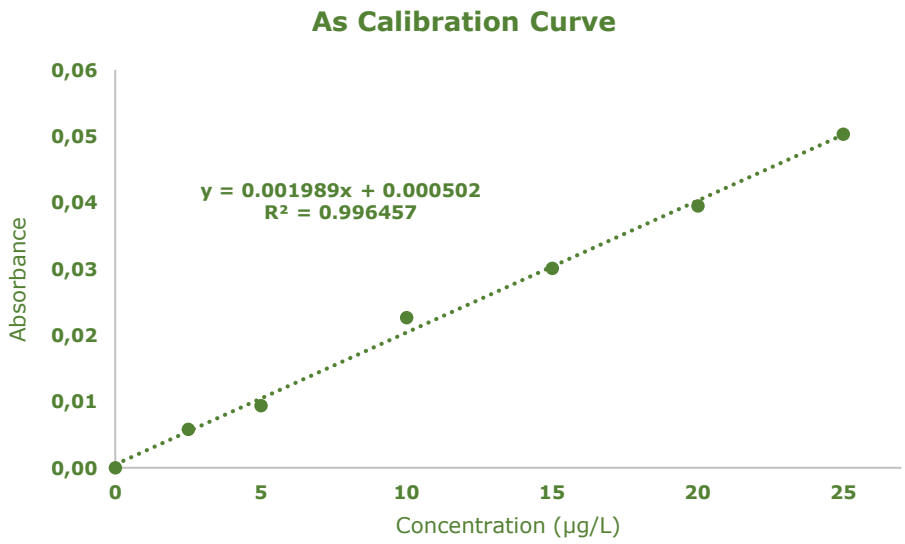
Calibration curves were performed for each element, being subsequently used for determination of the concentrations of various metals in each sample. In each day a calibration curve with at least six concentration points including blank was performed before starting analyzing. All standard solutions were prepared in acidic medium (nitric acid at 5% (V/V)) and a matrix modifier was added to all standards and samples analyzed by GFAAS.

In this section linearity, analytical thresholds and accuracy parameters will be presented. Parameters regarding microwave pressure digestion method validation will be presented as well (specificity/selectivity). Data relating working range and precision GFAAS (repeatability and intermediate) from previous work conducted on Laboratory of Instrumental Methods of Analysis can be consulted in Appendix B.

4.1.1. Linearity

Linearity was verified by calculating linear calibration function and nonlinear calibration function as well as the respective standard deviations $S_{y/x}$ and S_y^2. Test value (PG) was calculated and then compared with the tabulated value of the Snedecor/Fisher distribution F with 95% confidence.

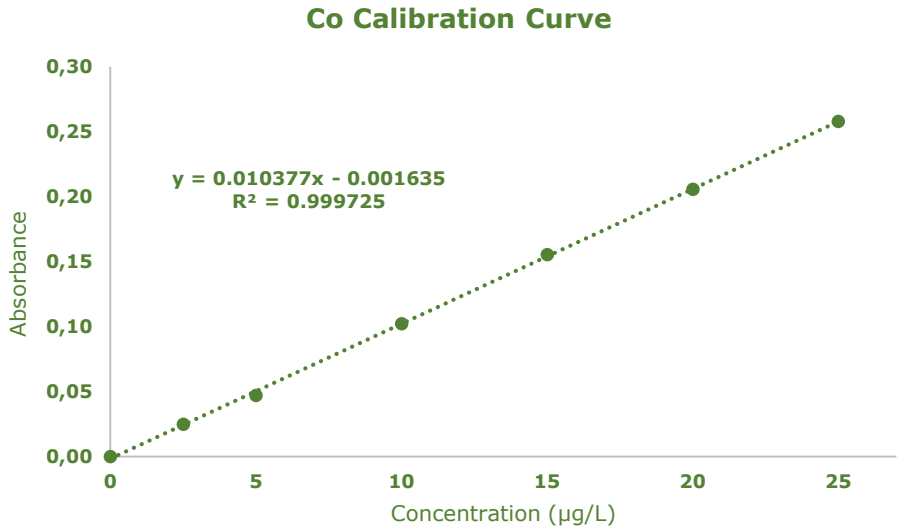
From graphic 4 to graphic 8 is presented a representative calibration curve for each metal. Statistic data regarding linearity study is presented from table 11 to table 15 for each metal as well.



Graphic 4 - Representative arsenic calibration curve. Data refers to average, n=2.

Table 11 - Equations of arsenic linear and nonlinear calibration curves and statistical data for linearity assessment.

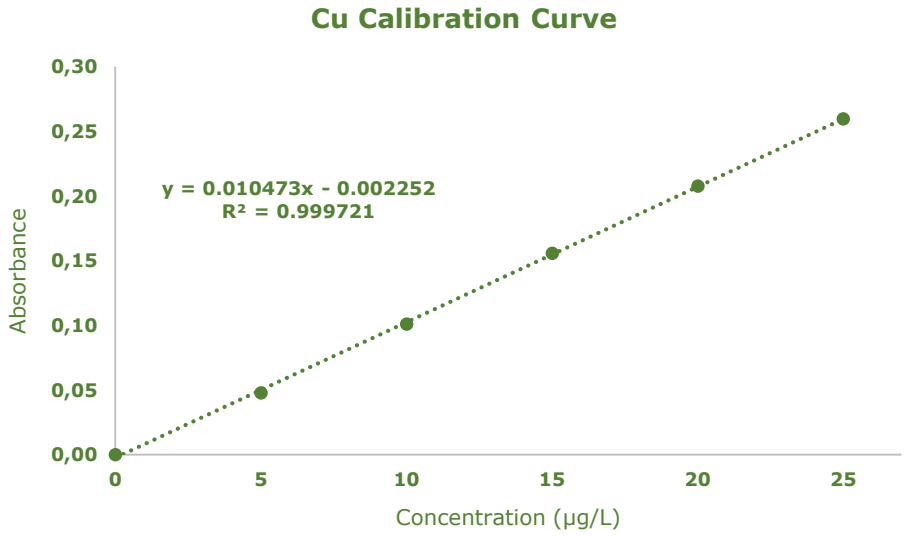
Linear equation	y = 0.001989x + 0.000502
Coefficient of determination (R^2)	0.996457
Residual standard deviation of linear equation	0.0012
Non-linear equation	$-0.000005x^2 + 0.002120x + 0.000105$
Residual standard deviation of non-linear equation	0.0013
Difference of variances (DS^2)	8.750E-07
Test value (PG)	0.52
Tabulated value of the F distribution with α = 0.05 significance level	5.05



Graphic 5 - Representative cobalt calibration curve. Data refers to average, n=4.

Table 12 - Equations of cobalt linear and nonlinear calibration curves and statistical data for linearity assessment.

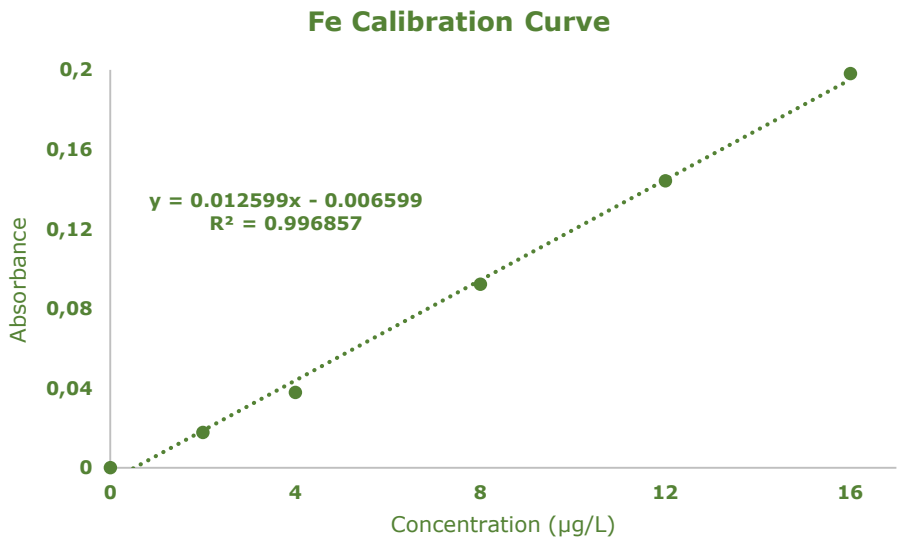
Linear equation	y = 0.010377x – 0.001635
Coefficient of determination (R^2)	0.999725
Residual standard deviation of linear equation	0,0018
Non-linear equation	$y = 0.000005x^2 + 0.010261x – 0.001281$
Residual standard deviation of non-linear equation	0,0019
Difference of variances (DS^2)	1.178E-06
Test value (PG)	0.32
Tabulated value of the F distribution with α = 0.05 significance level	5.05



Graphic 6 - Representative copper calibration curve. Data refers to average, n=7.

Table 13 - Equations of copper linear and nonlinear calibration curves and statistical data for linearity assessment.

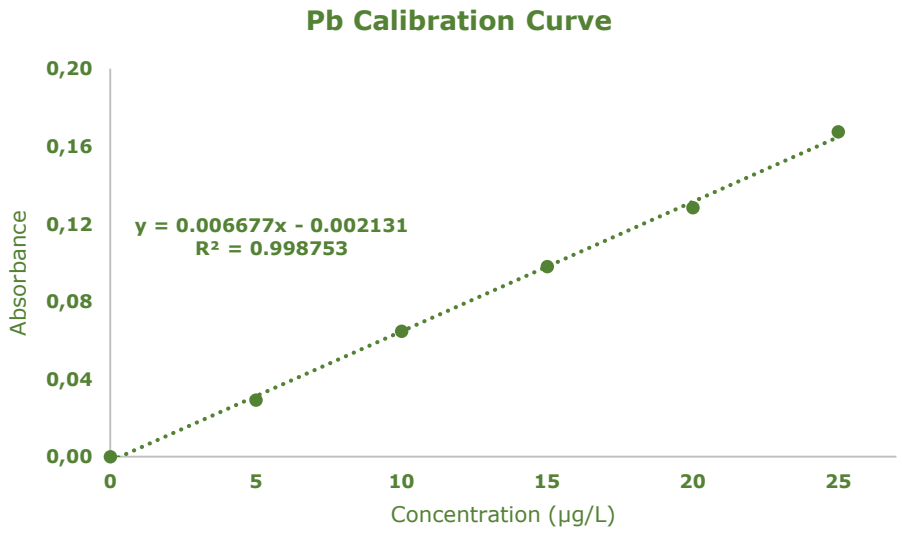
Linear equation	y = 0.010473x – 0.002252
Coefficient of determination (R^2)	0.999721
Residual standard deviation of linear equation	0.0018
Non-linear equation	$y = 0.000012x^2 + 0.010185x – 0.001291$
Residual standard deviation of non-linear equation	0.0019
Difference of variances (DS^2)	3.183E-06
Test value (PG)	0.93
Tabulated value of the F distribution with α = 0.05 significance level	6.39



Graphic 7 - Representative iron calibration curve. Data refers to average, n=7.

Table 14 - Equations of iron linear and nonlinear calibration curves and statistical data for linearity assessment.

Linear equation	y = 0.012599x – 0.006599
Coefficient of determination (R^2)	0.996857
Residual standard deviation of linear equation	0.0049
Non-linear equation	$y = 0.000132x^2 + 0.010505x – 0.002569$
Residual standard deviation of non-linear equation	0.0033
Difference of variances (DS^2)	4.952E-05
Test value (PG)	4.54
Tabulated value of the F distribution with α = 0.05 significance level	6.39



Graphic 8 - Representative lead calibration curve. Data refers to average, n=4.

Table 15 - Equations of lead linear and nonlinear calibration curves and statistical data for linearity assessment.

Linear equation	y = 0.006677x – 0.002131
Coefficient of determination (R^2)	0.998753
Residual standard deviation of linear equation	0.0025
Non-linear equation	$y = 0.000021x^2 + 0.006164x – 0.000420$
Residual standard deviation of non-linear equation	0.0022
Difference of variances (DS^2)	8.590E-06
Test value (PG)	1.78
Tabulated value of the F distribution with α = 0.05 significance level	6.39

Correlation coefficient (R) is commonly used to assess linearity of calibration curves. There is usually a tendency to believe that a particular linear calibration curve shows linearity when correlation coefficient is considerable high (e.g. 0.995). However, such high correlation coefficient may also be verified in nonlinear calibration curves given a false idea of linearity when nonlinear curves are not linear. Although it is commonly used, correlation coefficient is not suitable for assessing the linearity of calibration curves (Van Loco et al., 2002).

Based on data obtained from the linearity study it is clear that the calibration function is linear for all metals analyzed since test value is always less than the tabulated value of the Snedecor/Fisher distribution F with 95% confidence (see Appendix C).

4.1.2. Analytical thresholds

Analytical thresholds, detection and quantification limits, for each metal in this study were determined using parameters of a linear calibration curve. Data obtained is present in table 16.

Table 16 - Analytical thresholds regarding analyzed metals.

Metals	D.L. (µg/L)	Q.L. (µg/L)
Arsenic	2.0130	6.1000
Cobalt	0.5595	1.6956
Copper	0.5763	1.7463
Iron	1.2771	3.8699
Lead	1.2195	3.6955

Data obtained shows that for all analyzed metal D.L. is lower than the concentration of the first point of the calibration curves. However, the same is not true regarding Q.L. regarding arsenic and iron. For arsenic Q.L. is greater than the two first points of the calibration curves and for iron is greater than the first one.

Determination of analytical thresholds is important because it allows the operator to know the extent to which results are feasible. When the result is below the Q.L. there is no guarantee that the value correspond to the real concentration of a certain metal present in a sample.

4.1.3. Intermediate precision

In order to evaluate dispersion results between independent assays relatively to microwave pressure digestion an intermediate precision study for each metal was performed. For this study a beer sample was used in ten repetitions with the same procedure and in the same Laboratory in three non-consecutive days. Those results are presented in table 17. Precision studies (intermediate and repeatability) for GFAAS were already performed before on Laboratory of Instrumental Methods of Analysis and corresponding data can be consulted in Appendix B.

Table 17 - Mean values (n=10) of intermediate precision for three different days and respective relative standard deviation percentage (RSD (%)).

Metal	Day	Mean (µg/L)	RSD (%)
Copper	1	62.33	2.85
	2	59.27	2.82
	3	58.36	3.34
	Mean (µg/L)	59.99	
	RSD (%)	3.46	

Metal	Day	Mean (µg/L)	RSD (%)
Iron	1	71.16	12.72
	2	75.28	12.46
	3	73.41	13.84
	Mean (µg/L)	73.28	
	RSD (%)	2.82	

Data regarding arsenic, cobalt and lead are not shown because concentration values were below quantification limit.

Relative standard deviation calculated for copper in each day is lower than 5% and RSD of the three days is also lower than 5% which is an acceptable intermediate precision.

Intermediate precision for iron is not as linear as for copper. Relative standard deviation calculated in each day is above 10% showing result dispersion. However such dispersion appears to be steady on non-consecutive days. RSD from the three days is lower than 5% due to this steadiness making this an acceptable intermediate precision for iron.

4.1.4. Pool

Sampling was composed by seventeen beers from twelve different brands manufactured and commercialized in the Portuguese market. Volume of each sample was about one liter. Except for samples A and F all samples had their volume distributed between two and six recipients. Each sample's recipient came from the same allotment. Since for each sample those recipients came from the same allotment it is expected that metal content would be similar for all recipients within the same sample. To confirm this beer contained in each recipient within the same sample was analyzed regarding its metal content. After that a pool was made with beer from all recipients from the same sample. This pool was composed by equal parts from beer contained in each recipient within a sample. Three samples were chosen at random and results can be consulted in table 18.

Table 18 - Metal content comparison between different recipients from the same sample and respective pool (n=3).

Samples (µg/L)		Copper	Iron
B	Can 1	26.69	52.03
	Can 2	25.51	62.97
	Can 3	22.79	58.48
	Can 4	27.01	50.13
	Mean	25.50	57.82
	Pool	24.84	54.13
E	Can 1	43.66	69.56
	Can 2	45.02	88.56
	Can 3	46.00	111.98
	Mean	44.89	90.03
	Pool	47.08	104.69
I	Can 1	44.33	40.14
	Can 2	44.75	36.90
	Mean	44.54	38.52
	Pool	45.96	46.90

Average copper content from different recipients from the respective samples (B, E and I) are very similar to the respective copper content in pool. Iron content is not so similar between the average content and pool as copper was but it can be still considered close enough. Since copper and iron average content from the recipients is pretty similar it is not necessary to analyze metal content from all recipients from all samples. Instead a pool can be made for each sample and the respective analysis will give similar results to those that would be obtained if the content of all recipients were analyzed individually. At the end analyzing pools turns out to be less expensive and time consuming. Hereupon for metal content analysis pool construction was selected over the individual recipients.

Data regarding arsenic, cobalt and lead are not shown because concentration values were below quantification limit.

4.1.5. Accuracy

Method accuracy was assessed using certified reference materials (CRM). Data obtained from CRM analysis was compared with the certified value and for assessment result Z-score was calculated. Data obtained can be consulted below in table 19.

Table 19 - CRM values, data obtained and Z-score.

Metal	Certified value (µg/L)	Experimental value (µg/L)	Z-score
Cobalt	8.1 ± 1 (TM-26.3)	7.219	0.8810
Copper	6.79 ± 0.64 (TM-24.3)	6.455	0.5234
Iron	15.4 ± 4.2 (TM-24.3)	14.51	0.2119
Lead	5.82 ± 0.45 (TM-24.3)	5.572	0.5511

Observing the data it can be concluded that the experimental values are within the range of the certified value for each of the analyzed metals. Regarding to Z-score values they are always lower than 2 which according to rating scale is satisfactory. Arsenic data is not shown because certified value (5.21 ± 0.53 µg/L) is lower than the calculated Q.L. (6.1 µg/L).

4.1.6. Microwave pressure digestion validation

To validate microwave pressure digestion two similar doses (5 mL) were taken from a beer sample. One of the doses was spiked with a solution containing all metals in study (in this solution the concentration of each metal was 10 µg/L) and nothing was added to the other dose. Both doses were digested using the same procedure. Data obtained can be consulted in table 20.

Table 20 - Recovery rate (%) from the addition of cobalt, copper, iron, and lead standards.

Metal	Standard added (µg/L)	Metal concentration (µg/L)	Standard recovery (µg/L)	Recovery rate (%)
Cobalt	10.0	< L.Q.	8.55	85.50
Copper	10.0	55.58	64.92	93.40
Iron	10.0	98.60	108.67	100.70
Lead	10.0	1.82	13.73	119.09

Recovery rate for copper and iron are very close to 100%. Recovery rate for cobalt and lead are furthest from 100% when compared to copper and iron. Cobalt concentration from the non-spiking dose is below the detection limit while lead concentration from the very same sample is below the quantification limits. These facts can be a possible explanation for such difference from 100% recovery rate. However for this study a recovery rate between 80 and 120% was accepted so it can be said that the digestion method was effective. An alternative could be using a beer sample with a quantifiable cobalt and lead content but no beer sample in this study shown such content (only five beer samples had quantifiable cobalt content but still very close to the quantification limit which can be consulted in tables 21 and 22). Using standards is also a possibility but solutions would not present such a complex

matrix as verified in beers. Arsenic data is not shown because all values were below Q.L.

4.2. Trace element quantification

After digestion target metals content were quantified through AAS – GFAAS for cobalt, copper, iron, and lead; HGAAS for arsenic.

Beer packaging were either can or glass are results are presented taken such characteristic into account – table 21 for canned beer and table 22 for bottled beer. In both tables results presented correspond to the average of three readings for each sample. Whenever a result expresses a concentration below the quantification limit it is indicated as < Q.L. (below quantification limit). Q.L. used as reference for each metal can be consulted further back in table 16.

Table 21 - Trace element quantification in beer samples contained in can package (n=3).

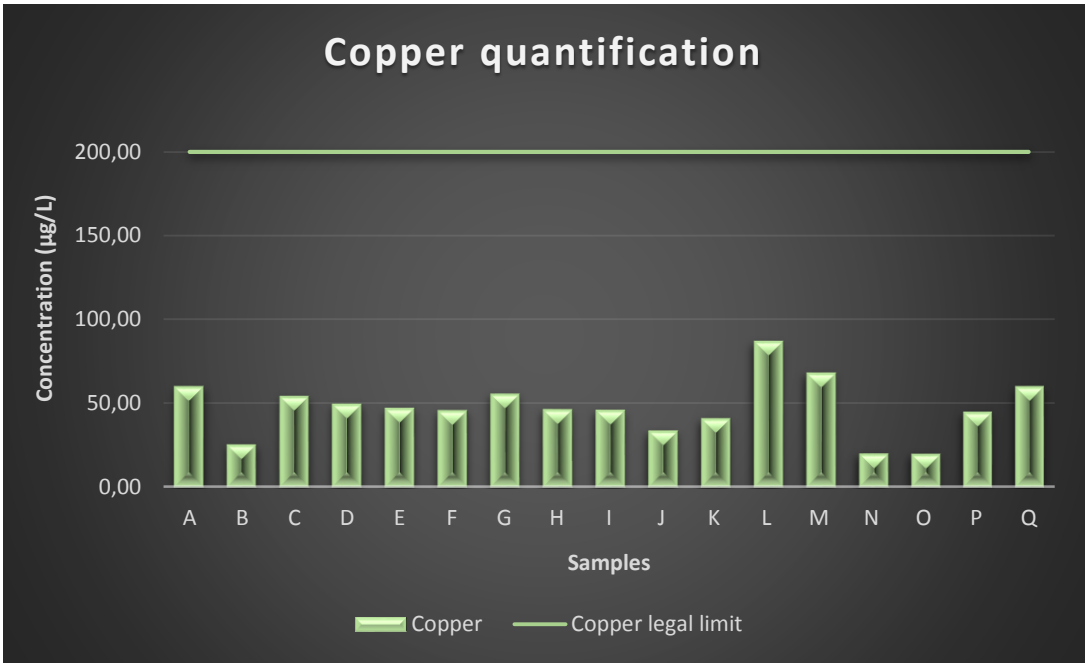
Sample	Metals (µg/L)				
	Arsenic	Cobalt	Copper	Iron	Lead
C	< L.Q.	< L.Q.	54.17	28.31	< L.Q.
E	< L.Q.	< L.Q.	47.08	104.69	< L.Q.
G	< L.Q.	< L.Q.	55.49	44.43	< L.Q.
H	< L.Q.	< L.Q.	46.39	115.86	< L.Q.
I	< L.Q.	< L.Q.	45.96	46.90	< L.Q.

Table 22 - Trace element quantification in beer samples contained in glass package (n=3).

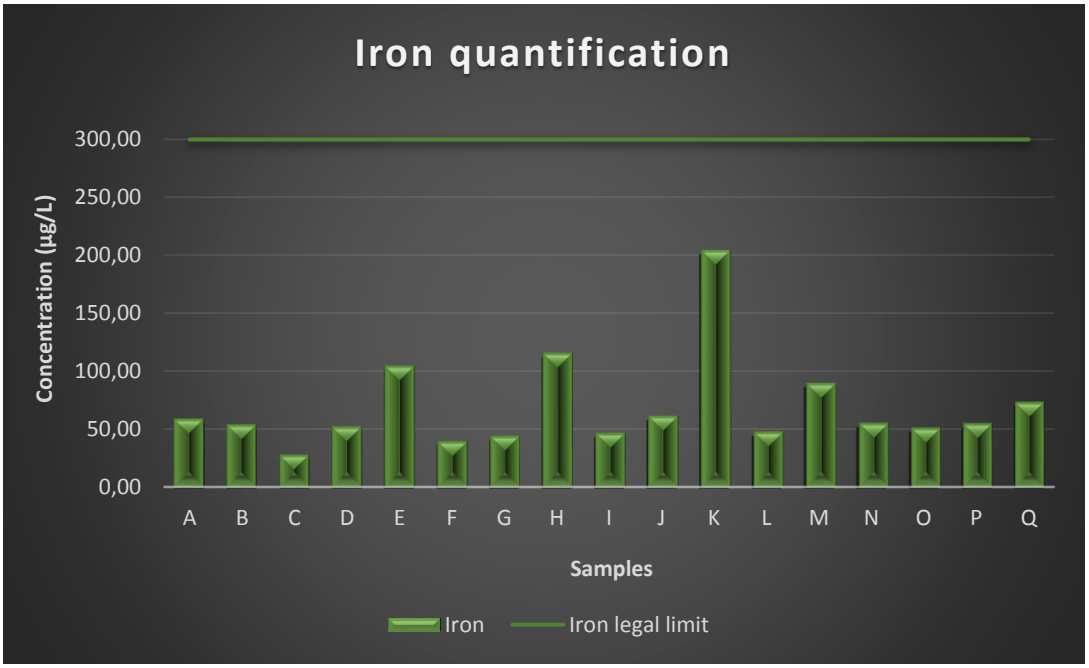
Sample	Metals (µg/L)				
	Arsenic	Cobalt	Copper	Iron	Lead
A	< L.Q.	< L.Q.	59.99	59.27	< L.Q.
B	< L.Q.	< L.Q.	25.56	54.13	< L.Q.
D	< L.Q.	< L.Q.	49.52	52.49	< L.Q.
F	< L.Q.	< L.Q.	45.84	39.95	< L.Q.
J	< L.Q.	< L.Q.	33.71	61.60	< L.Q.
K	< L.Q.	< L.Q.	40.91	203.99	< L.Q.
L	< L.Q.	< L.Q.	86.67	48.19	< L.Q.
M	< L.Q.	3.83	67.97	89.96	< L.Q.
N	< L.Q.	3.68	20.18	55.49	< L.Q.
O	< L.Q.	3.97	19.84	51.50	< L.Q.
P	< L.Q.	4.72	44.81	55.36	< L.Q.
Q	< L.Q.	4.70	59.86	73.67	< L.Q.

Analyzing the results present at tables 21 and 22 it becomes apparent that measurable concentrations were not detected regarding arsenic and lead. In relation to cobalt content it was measurable only in five samples (M, N, O, P, and Q).

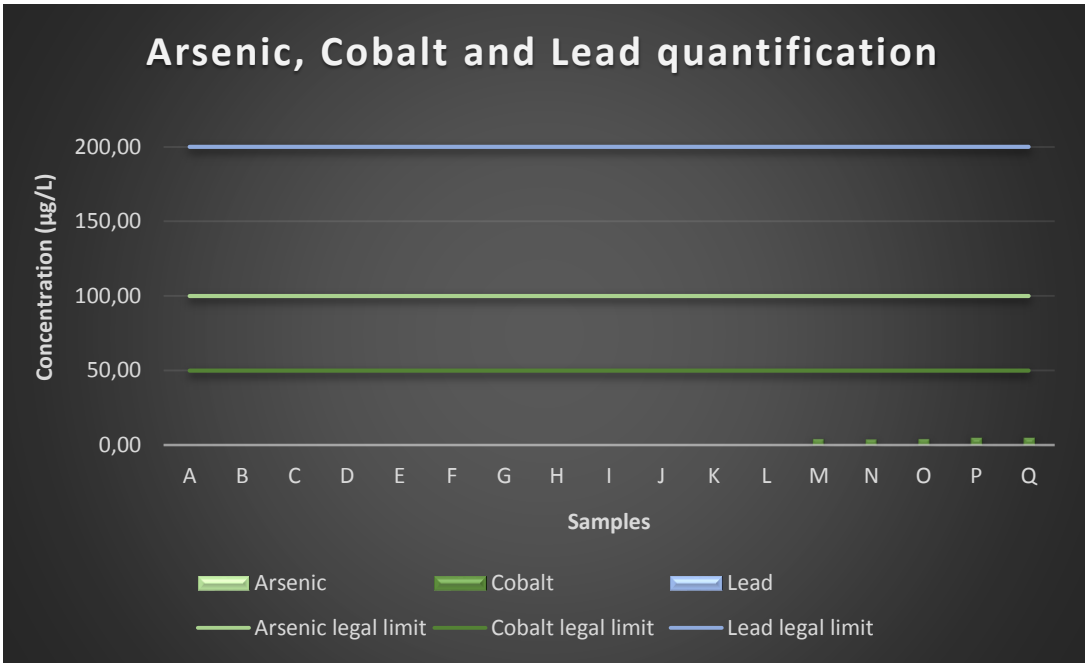
For a better visualization of data shown in the previous two tables same data can be consulted in graphics 9 (copper), 10 (iron), and 11 (arsenic, cobalt, and lead). In those graphics beer packaging was not considered so to know what kind of package correspond to a certain sample, tables 5, 21, and 22 should be consulted. In each graphic there is continuous line which represents the legal limit established by the Portuguese legislation (Portaria nº 1/96 from 3rd January) for the concentration of a particular metal present in beer.



Graphic 9 - Copper quantification in beer samples (n=3).



Graphic 10 - Iron quantification in beer samples (n=3).



Graphic 11 - Arsenic, cobalt, and lead quantification in beers (n=3).

According to Portuguese legislation (Portaria nº 1/96 from 3rd January) limits regarding its maximum concentration are set for arsenic (0.1 mg/L), cobalt (0.05 mg/L), copper (0.2 mg/L), iron (0.3 mg/L), and lead (0.2 mg/L). As it can be seen in the previous graphics none of the aforementioned metals is above the legal limits which was the prime objective of this study – verify if beers manufactured in Portugal

and commercialized in Portuguese market respected the limits established by Portuguese legislation regarding metal content.

It is possible to observe in graphics 9 and 10 a considerable variation regarding copper and iron content being more evident for iron. In fact relative standard deviation is quite high in both cases (35.91% for copper and 59.42%) indicating a great dispersion on the content of these two metals between the different analyzed beer samples.

Metal content of Portuguese beers not only differs between the brands and/or domestic products. There are also differences in relation to beers from other countries. Pohl (2008) made a data compilation regarding major, minor, and trace metal content in beers from different countries. In table 23 a segment from that compilation can be consulted.

Table 23 - Concentration of trace metals in beers of different country of origin.

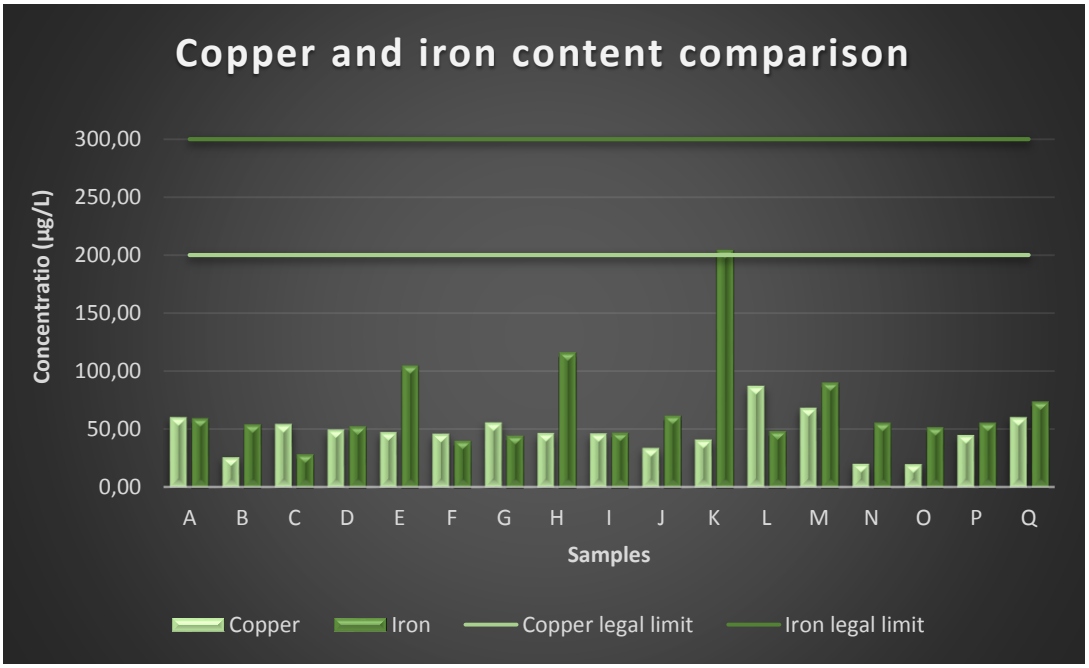
	Concentration (µg/L)					
Metals	**British**	**Dutch**	**German**	**Norwegian**	**Polish**	**Spanish**
Copper	80 - 800	32 - 68	19 - 800	29 - 50	29 - 150	24 - 80
Iron	67 - 500	64 - 430	40 - 1550	36 - 93	15 - 530	96 - 920
Lead			3 - 24			1 - 6

As it can be seen copper and iron content in Portuguese beers are similar to Norwegian ones. Copper content in Portuguese beers is also similar to Dutch ones. Although lead content was not detected in Portuguese beers, small amounts of this metal are present in German and Spanish beers but at minimum levels. However in most cases copper (British, German, and Polish) and iron (British, Dutch, German, Polish, and Spanish) content can be considerable higher when compared to Portuguese beers even have gone well beyond the legal limits allowed in Portugal.

Regarding arsenic content in beers Donadini et al. (2008) were able to quantify small amounts of arsenic (2 to 25 µg/L) in beers from the Italian market and with origin in different countries (Italy, Belgium, Germany, Ireland, Scotland, and others) while in this study it was not possible to quantify it. Nevertheless arsenic content reported in beers from the Italian market is within the legal limits allowed in Portugal. Obviously legal limits established by Portuguese law are not the same in other countries and high metal content as described in table 23 could even be allowed in those countries (but not in Portugal).

4.2.1. Copper and iron content comparison

Observing tables 21 and 22, copper and iron content appear to be similar in some samples. Graphic 12 highlights this observation. In that matter it should be interesting to discover if there are significant differences between the content of these two metals on the analyzed samples.

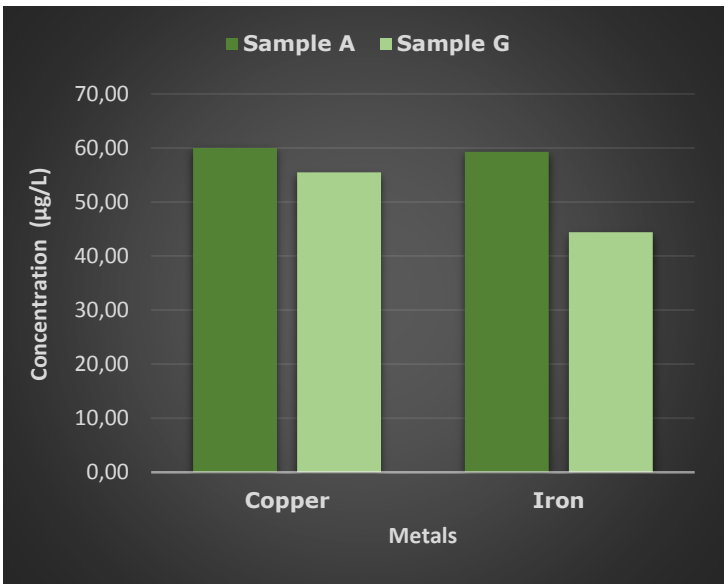


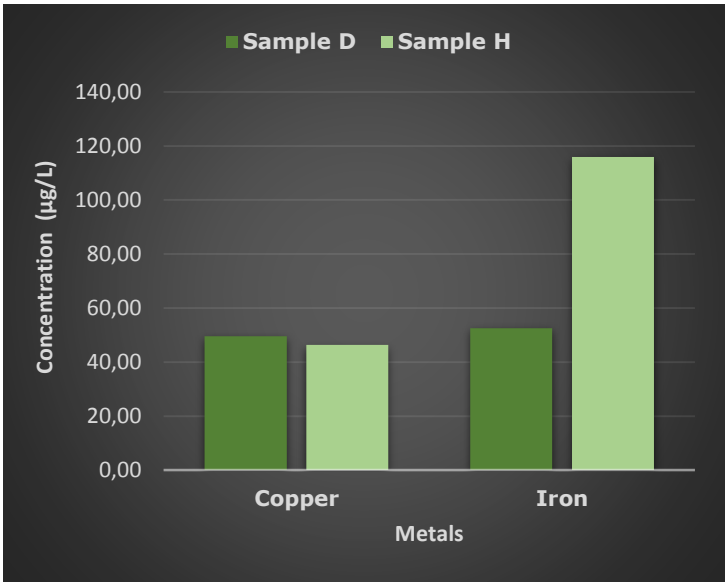
Graphic 12 - Comparison between copper and iron content in beers.

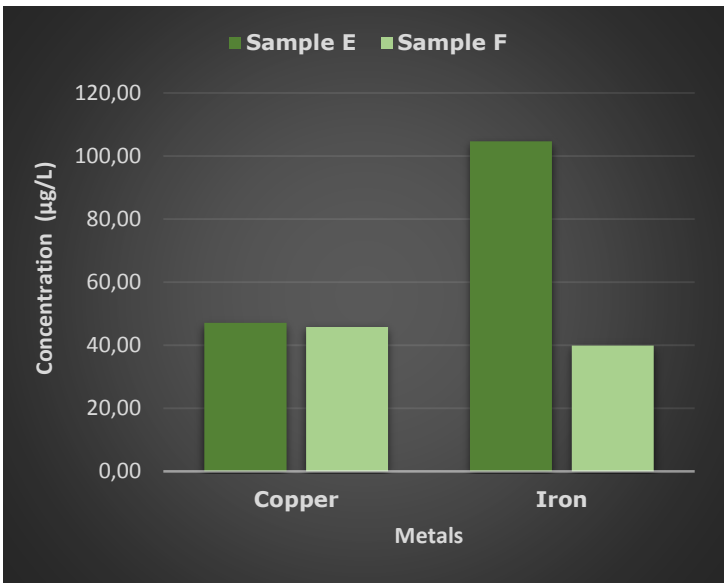
Despite the apparent similarity regarding copper and iron content highlighted by graphic 12 about almost half of the samples only three of them show no significance differences regarding the content of these two metals. After performing one-way ANOVA analysis with $\alpha=0.05$ significant level only samples A, D, and I present a similar copper and metal content (*p-value* > 0.05). Necessary data required for performing one-way ANOVA may be consulted in Appendix D and through the same data is possible determine copper and iron content present on tables 21 and 22.

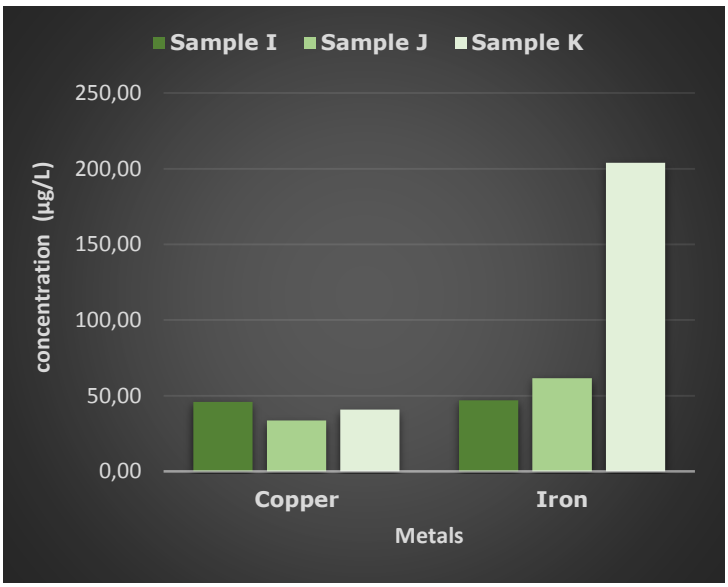
4.2.2. Packaging influence on metal content in beer

In this study seventeen beer samples were analyzed and they represent twelve brands commercialized in the Portuguese market. As mentioned in 3.1. samples from four of the twelve brands came both in can and glass recipients – A and G represent one brand and the same is valid for D and H; E and F; I, J and K (see table 5). Will beers samples from the same brand but stored in different containers have significant differences regarding copper and iron content? In graphic 13 there are represent four charts each one corresponding to two or three different samples from the same brand.









Graphic 13 - Comparison between copper and iron content in beer samples from different recipient types within the same brands.

Watching the charts present in graphic 13 is possible to verify that are significant differences regarding iron content between samples from the same brand. Actually performing one-way ANOVA confirm such fact since *p-value* is lower than 0.05. On the other hand copper content was significant different between samples A and G, and between samples I, J, and K. No significant difference was found regarding copper content between samples D and H, and E and F (*p-value* higher than 0.05).

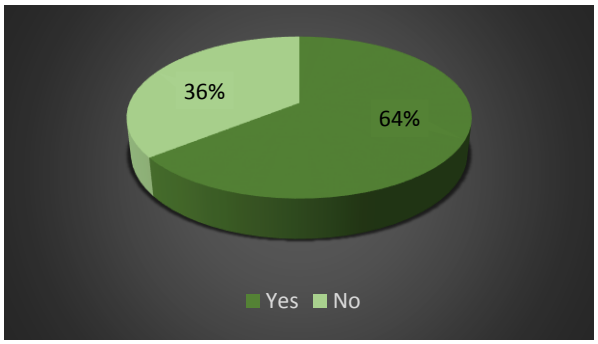
Despite the results it is not possible to draw conclusions because those samples from the same brand actually correspond to different products. Obviously different brand products are manufactured differently which in brewing means different grains, different yeast strains and other modifications in the brewing process. Such

modifications will lead to different compositions in the final product so comparing beers from the same brand in different container but corresponding to different products do not allow to reach any plausible conclusions. To do that it will be need to compare the exact same product from a brand stored in a glass and in a can container.

4.3. Questionnaire

In order to better understand the behavior of the population of the Faculty of Pharmacy, University of Lisbon in relation to beer consumption a questionnaire was made. Target public were students from the second and fourth year from the Integrated Master's Degree in Pharmaceutical Sciences. Target public was composed by 129 students (102 female students and 27 male students). Since target public is mostly female (almost 80%) comparison between those groups is not possible in this case. A copy of the questionnaire (in Portuguese) can be consulted in Appendix E.

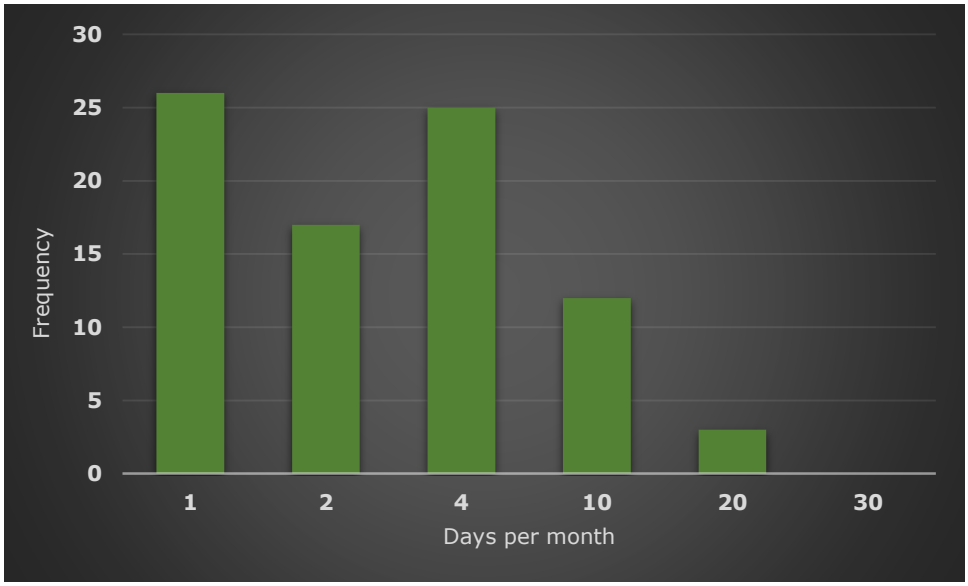
An overview of the answers to some of the questions can be consulted in the following charts.



Graphic 14 - Beer consumption habits.

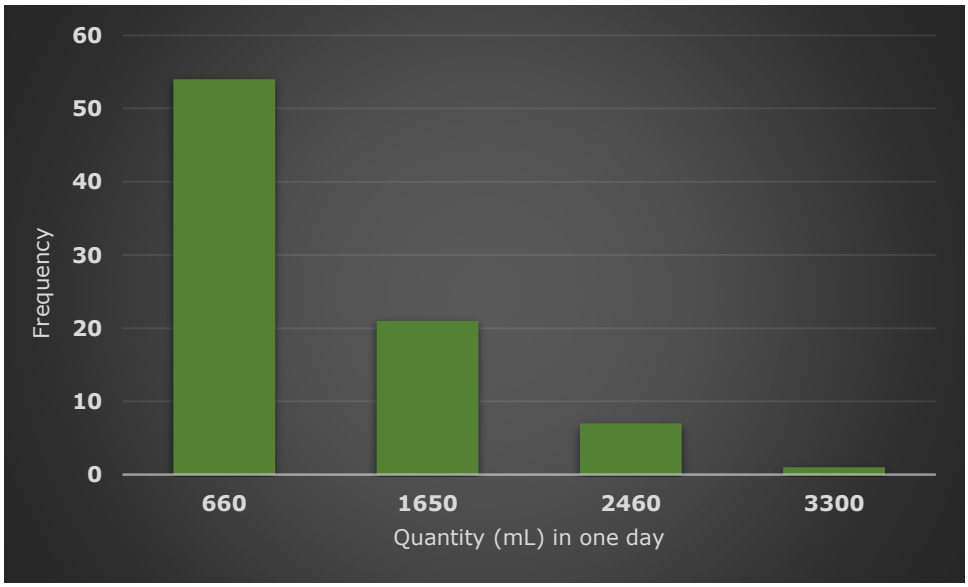
About two thirds (83 individuals) of the inquiries claimed to consume beer as opposed to the other third (49 individuals) who claimed not to consume beer (see graphic 14).

Following charts (see graphics 15, 16, and 17) and commentaries are only related to beer consumption habits of the inquiries (83 individuals). How often they consume beer, what is the higher amount they can consume in one day, reasons of why buying a beer from a brand instead of another, and preferential national brands are the main questions that this questionnaire tried to answer.



Graphic 15 - Frequency of beer consumption per month.

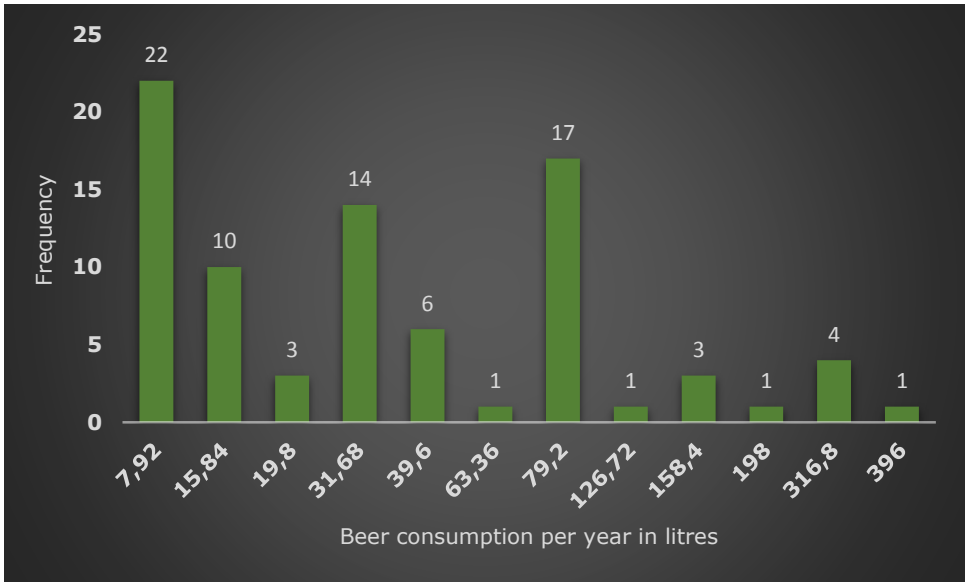
Pretty much of all the inquiries do not show an exaggerated frequency of beer consumption and it seems that consumption occurs mostly at the weekends.



Graphic 16 - Higher quantity (in liters) of beer consumed in one day.

Graphic 16 shows a moderate beer consumption for most of the inquiries but about a third of them appear to have a tendency to exaggerate.

Graphic 17 shows an estimate of the amount of beer consumed per inquiry at the end of the year using the data from the two previous graphics.



Graphic 17 – Estimate of beer consumption (in liters) per year.

Beer consumption per capita (in liters) has been declining in Portugal since 2008 and in 2012 was about 49 liters (see table 2). Taken this value into account and comparing it with data presented in graphic it can be concluded that about one third is consuming more beer than the average revealing what may be called bad habits related to beer consumption.

Groceries and hypermarkets are the preferential places to buy beers and flavor the characteristic with higher influence to choose what kind of beer to drink. Sagres and Super Bock are the preferred brands by the inquiries and more than half of them never tried handmade beer or without alcohol (data not shown).

5. Conclusions

Metal content from seventeen different beer samples from twelve brands manufactured and commercialized in the Portuguese market was analyzed in this study. Six metals were the targets of this analysis – arsenic, cobalt, copper, iron, lead, and zinc. However, zinc analysis was not possible due to technical problems.

Before analyzing metal content from beer samples some validation was required. A complete validation regarding metal quantification was already done in a previous study conducted on Laboratory of Instrumental Methods of Analysis**.** Nevertheless, microwave pressure digestion validation was necessary. Firstly digestion conditions had to be defined to digest beer samples. HNO_3 and H_2O_2 were the reagents selected for digestion process. Here the goal was to use the minimal volume of reagents and obtain a colorless, clear, and completely digested samples. These conditions were achieve with 0.5 mL of HNO_3 and 1 mL of H_2O_2. This digestion was an important step to remove possible matrix interferences.

Through validation it was possible to conclude that the methods used were suitable for obtaining credible and adequate results. In this validation some parameters such as linearity, analytical thresholds, intermediate precision and accuracy were studied.

Regarding linearity it was possible to conclude that linear equation determined for each analyzed metal shown linearity (in all cases test value was less than the tabulated value of the Snedecor/Fisher distribution F with 95% confidence).

Analytical thresholds proved to be different depending on the metal. As it can be seen in table 16 cobalt and copper have a quantification limit below 2 µg/L (1.7 µg/L for both of them) and iron and lead below 4 µg/L (3.9 and 3.7 µg/L respectively). Arsenic was the only metal analyzed through HGAAS and has the higher quantification limit of them all (6.1 µg/L).

Intermediate precision for microwave pressure digestion was only possible for copper and iron because beer samples selected to study this parameter had no detectable arsenic, cobalt, and lead content. Average copper and iron content was similar in three non-consecutive days which suggests an appropriate intermediate precision (RSD < 5%). To analyze this parameter for the other metals spiking them would be necessary especially because none of the samples had detectable arsenic, cobalt or lead content.

Analyzing pool from a sample was preferred over recipients' individual content because metal content proved to be similar and it was less expensive and time consuming.

Finally regarding validation studies accuracy was evaluated using CRM. With this study it was concluded that the respective experimental values for all analyzed metals were within the margin of CRM. Arsenic data is not shown because CRM value was below quantification limit (CRM for arsenic is 5.21 ± 0.53 µg/L and the calculated Q.L. for this metal is 6.1 µg/L).

To test microwave pressure digestion efficiency a recovery study was performed. Recovery rate for copper and iron are very close to 100% (93.4% for copper and 100.7 for iron). Recovery rate for cobalt and lead are furthest from 100% (85.5% for cobalt and 119.1 for lead) when compared to copper and iron but still within the established recovery limits (80% – 120%). It was thus shown that no significant losses were verified in the digestion process.

At the end sample treatment by microwave pressure digestion proved to be an easy, rapid and efficient method to destroy the organic matter present in the different beers.

After carrying out all the necessary studies for the required validation, analysis of metal content from the seventeen beer samples was performed. Portuguese legislation establish through Portaria nº 1/96 from 3rd January limits for metal content in beers – arsenic (0.1 mg/L), cobalt (0.05 mg/L), copper (0.2 mg/L), iron (0.3 mg/L), lead (0.2 mg/L) and zinc (1 mg/L).

The analyzed samples previously selected by ASAE present levels below the maximum allowed by the Portuguese legislation. No arsenic and lead content was found in beer samples and small amounts of cobalt were detected only in five samples (M, N, O, P, and Q) and below 5 µg/L. Copper and iron content was more variable and detected in considerable amounts but as mentioned before always below the legal limits. As mentioned before, zinc analysis was not possible due to technical problems. Therefore, it can be concluded that the presence of the analyzed metals in selected beer brands do not pose any risk to the consumer. Moderate beer consumption is always advisable otherwise the opposite can be true.

There are several possibilities worth mentioned as future studies. For example a similar study could be conducted using a different Atomic Spectrometry technique such as ICP-AES and/or ICP-MS to compare the results obtained to see if they are similar.

Probably a more interesting study would be to study the influence of the recipient material on the beer metal content. Such thing was tried in this study but the results were inconclusive. The problem was that those samples did not correspond to the exact same product but to different ones within the same brand. A proper evaluation of the container influence can only be made if the exact same product is stored in containers with different characteristics. In addition a migration study regarding recipient material could be made.

Besides metal content Portuguese legislation takes into account many other characteristics in beers offering different quality control perspectives. For instance the Portuguese legislation (Portaria nº 1/96 from 3rd January) also regulates total acidity content, pH and alcohol content.

The last three paragraphs only represent a few examples but there are several more possible studies which can be performed regarding beers.

Beer is one of the most consumed beverages in the world. Studies like this one are of great importance to both brewers and consumers. For brewers because analyzed metals play an important role on beer fermentation and maturation and they can influence both positively and negatively those process. For consumers because metals, depending on its concentration, may be essential or toxic to humans. Summarizing studies like this one allow to assess and control the quality of beers.

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7. Appendices

7.1. Appendix A

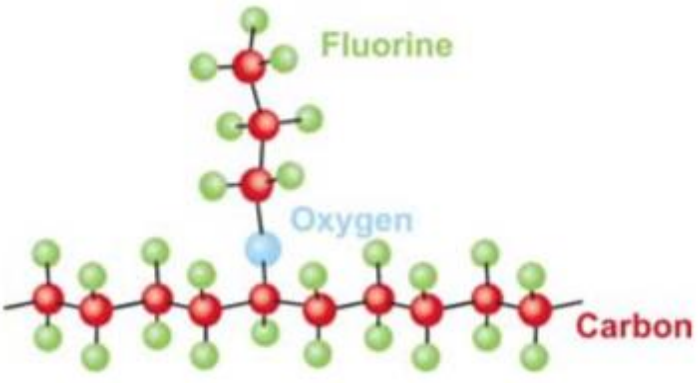
7.1.1. Safety of Microwave digestion system Speedwave Two

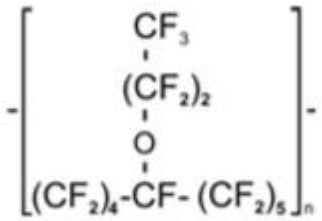
The sensor technology of the Speedwave Two and its temperature-controlled active microwave regulation reduces the risk of spontaneous reaction during microwave digestion to a minimum

- Temperature monitoring of all samples in real time
- Effective excess pressure relief
- Integrated gas collection system prevents emissions
- Shut-down in critical operating states
- Excess temperature cut-outs protect the oven chamber and magnetron

7.1.2. TFM™-PTFE (digestion vessels material) characteristics

- Perfluorated plastic with perfluoroalkoxy side chain (< 1% by weight)
- Melting temperature, approx. 300ºC
- Can be used at temperatures up to + 260ºC
- Nearly universal chemical resistance
- High purity
- Improved surface structure compared with PTFE





7.1.3. Speedwave Two – Technical specifications

Power supply	230 V / 50 Hz / 1350 W
Magnetron performance	1000 W (DAP-60K : 100 W per digestion vessel)
Frequency	2450 MHz
Weight/dimensions	Basic unit approx. 14 Kg / 520 x 460 x 330 mm (w x d x h) Control unit: approx. 0.5 Kg / 188 x 35 x 114 mm (w x d x h)
Oven diameter	Approx. 27 L / 350 x 340 x 215 mm (w x d x h)
Noise level	< 60 dB
Control unit	Colored touchscreen, 7" TFT-LCD Panel, 800 x 400 Pixel
Storage	1 GB SD card
Interfaces	USB, RS-232, Ethernet
Language	German, English, French, Italian, Spanish
Temperature measurement	Patented DIRC thermometer, measurement range 50-260ºC, 1ºC precision at 200ºC
Vessel material	Isostatically-molded TFT™-PTFE
Vessel types	DAP-60K: 60 mL capacity, 40 bar (580 psi), 10 vessels per sample turntable
Safety tests	CE conformity, fulfils EN 335-25, DIN EN 61010-1, DIN EN 61326-1, DIN EN 61326-2
Warranty	12 months, including digestion vessels

7.2. Appendix B

7.2.1. Working range

To assess the working range variances homogeneity test was performed in order to check for significant differences between them. Six calibration points (blank included) were used where the standard of lowest and highest concentration were analyzed in ten replicates. To check the adjustment of the working range test value (PG) was compared to the tabulated value of the Snedecor/Fisher distribution F with 95% confidence (see annex 3) (Guerreiro, 2014).

Table 24 - Ten replica data from the standard of lower and higher concentration of the calibration curve for each analyzed metal.

Arsenic		
Data	**2.5 (µg/L)**	**25 (µg/L)**
Mean	2.5722	25.2700
SD	0.0707	0.0867
Variance	0.0050	0.0075
Test Value (PG)	1.5043	
Tabulated value of the F distribution with a α = 0.05 significance level	3.18 > PG	

Cobalt		
Data	**2.5 (µg/L)**	**25 (µg/L)**
Mean	2.5420	25.2680
SD	0.0365	0.0625
Variance	0.0013	0.0039
Test Value (PG)	2.9193	
Tabulated value of the F distribution with a α = 0.05 significance level	3.18 > PG	

Copper		
Data	**5 (µg/L)**	**25 (µg/L)**
Mean	5.3802	25.1420
SD	0.1303	0.1891
Variance	0.0170	0.0358
Test Value (PG)	2.1043	
Tabulated value of the F distribution with a α = 0.05 significance level	3.18 > PG	

Iron		
Data	**2.5 (µg/L)**	**20 (µg/L)**
Mean	2.5043	20.0390
SD	0.1248	0.1885
Variance	0.0156	0.0355
Test Value (PG)	2.2806	
Tabulated value of the F distribution with a α = 0.05 significance level	3.18 > PG	

Lead		
Data	**5 (µg/L)**	**25 (µg/L)**
Mean	5.1944	25.2520
SD	0.1141	0.1812
Variance	0.0130	0.0328
Test Value (PG)	2.5195	
Tabulated value of the F distribution with a α = 0.05 significance level	3.18 > PG	

Taken into account that test value (PG) is always higher than tabulated value of the F distribution with α = 0.05 significant variances differences are not significant and the working range is well adjusted para all metals (Guerreiro, 2014).

7.2.2. GFAAS and HGAAS intermediate precision

In order to evaluate dispersion results between independent assays relatively to GFAAS an intermediate precision study for each metal was performed. For this study standard solutions of lower and higher concentration from the calibration curve of each metal were analyzed in replica of ten (Guerreiro, 2014).

Table 25 - Mean values (n=10) of intermediate precision for three different days and respective relative standard deviation percentage (RSD (%)).

Metal	Day	Mean (µg/L)	RSD (%)
Arsenic (2.5 µg/L)	1	2.5722	2.7471
	2	2.5270	3.9987
	3	2.5461	1.0703
Cobalt (2.5 µg/L)	1	2.5255	1.8343
	2	2.5291	1.1430
	3	2.2510	0.9551
Copper (5 µg/L)	1	5.4376	4.0492
	2	5.3623	3.1924
	3	5.3802	2.4227
Iron (2.5 µg/L)	1	2.5643	3.0323
	2	2.5020	3.1809
	3	2.4945	3.5402
Lead (5 µg/L)	1	5.1944	2.1972
	2	5.1985	1.7324
	3	5.3255	3.2755

Calculated values for the relative standard deviation was always lower than 5% for each metal which demonstrates that the methods used show a good intermediate precision (Guerreiro, 2014).

7.2.3. GFAAS and HGAAS repeatability

In order to evaluate method repeatability GFAAS and HGAAS standard solutions of the lowest concentration from the calibration curve of each metal were analyzed in replica of ten from the same day (Guerreiro, 2014).

Table 26 - Mean values (n=10) and respective relative standard deviation percentage (RSD (%)) obtained in repeatability study.

Arsenic			**Cobalt**		
	2.5 (µg/L)	**25 (µg/L)**		**2.5 (µg/L)**	**25 (µg/L)**
Mean	2.5722	25.270	**Mean**	2.5420	25.268
RSD (%)	2.7471	0.3430	**RSD (%)**	1.4391	0.2474
Copper			**Iron**		
	5 (µg/L)	**25 (µg/L)**		**2.5 (µg/L)**	**20 (µg/L)**
Mean	5.3802	25.142	**Mean**	2.5043	20.039
RSD (%)	2.4227	0.7520	**RSD (%)**	4.9834	0.9405
Lead					
	5 (µg/L)	**25 (µg/L)**			
Mean	5.1944	25.252			
RSD (%)	2.1972	0.7174			

Calculated values for the relative standard deviation was always lower than 5% for each metal which demonstrates that the methods used show a good repeatability (Guerreiro, 2014).

7.3. Appendix C

7.3.1. Snedecor/Fisher distribution F with 95% confidence

ν_2 (dén.)	ν_1 (numérateur)																			
	1	2	3	4	5	6	7	8	9	10	20	30	40	50	60	80	100	200	500	1 000
1	161.45	199.50	215.71	224.58	230.16	233.99	236.77	238.88	240.54	241.88	248.02	250.10	251.14	251.77	252.20	252.72	253.04	253.68	254.06	254.19
2	18.51	19.00	19.16	19.25	19.30	19.33	19.35	19.37	19.38	19.40	19.45	19.46	19.47	19.48	19.48	19.48	19.49	19.49	19.49	19.49
3	10.13	9.55	9.28	9.12	9.01	8.94	8.89	8.85	8.81	8.79	8.66	8.62	8.59	8.58	8.57	8.56	8.55	8.54	8.53	8.53
4	7.71	6.94	6.59	6.39	6.26	6.16	6.09	6.04	6.00	5.96	5.80	5.75	5.72	5.70	5.69	5.67	5.66	5.65	5.64	5.63
5	6.61	5.79	5.41	5.19	5.05	4.95	4.88	4.82	4.77	4.74	4.56	4.50	4.46	4.44	4.43	4.41	4.41	4.39	4.37	4.37
6	5.99	5.14	4.76	4.53	4.39	4.28	4.21	4.15	4.10	4.06	3.87	3.81	3.77	3.75	3.74	3.72	3.71	3.69	3.68	3.67
7	5.59	4.74	4.35	4.12	3.97	3.87	3.79	3.73	3.68	3.64	3.44	3.38	3.34	3.32	3.30	3.29	3.27	3.25	3.24	3.23
8	5.32	4.46	4.07	3.84	3.69	3.58	3.50	3.44	3.39	3.35	3.15	3.08	3.04	3.02	3.01	2.99	2.97	2.95	2.94	2.93
9	5.12	4.26	3.86	3.63	3.48	3.37	3.29	3.23	3.18	3.14	2.94	2.86	2.83	2.80	2.79	2.77	2.76	2.73	2.72	2.71
10	4.96	4.10	3.71	3.48	3.33	3.22	3.14	3.07	3.02	2.98	2.77	2.70	2.66	2.64	2.62	2.60	2.59	2.56	2.55	2.54
20	4.35	3.49	3.10	2.87	2.71	2.60	2.51	2.45	2.39	2.35	2.12	2.04	1.99	1.97	1.95	1.92	1.91	1.88	1.86	1.85
30	4.17	3.32	2.92	2.69	2.53	2.42	2.33	2.27	2.21	2.16	1.93	1.84	1.79	1.76	1.74	1.71	1.70	1.66	1.64	1.63
40	4.08	3.23	2.84	2.61	2.45	2.34	2.25	2.18	2.12	2.08	1.84	1.74	1.69	1.66	1.64	1.61	1.59	1.55	1.53	1.52
50	4.03	3.18	2.79	2.56	2.40	2.29	2.20	2.13	2.07	2.03	1.78	1.69	1.63	1.60	1.58	1.54	1.52	1.48	1.46	1.45
60	4.00	3.15	2.76	2.53	2.37	2.25	2.17	2.10	2.04	1.99	1.75	1.65	1.59	1.56	1.53	1.50	1.48	1.44	1.41	1.40
70	3.98	3.13	2.74	2.50	2.35	2.23	2.14	2.07	2.02	1.97	1.72	1.62	1.57	1.53	1.50	1.47	1.45	1.40	1.37	1.36
80	3.96	3.11	2.72	2.49	2.33	2.21	2.13	2.06	2.00	1.95	1.70	1.60	1.54	1.51	1.48	1.45	1.43	1.38	1.35	1.34
90	3.95	3.10	2.71	2.47	2.32	2.20	2.11	2.04	1.99	1.94	1.69	1.59	1.53	1.49	1.46	1.43	1.41	1.36	1.33	1.31
100	3.94	3.09	2.70	2.46	2.31	2.19	2.10	2.03	1.97	1.93	1.68	1.57	1.52	1.48	1.45	1.41	1.39	1.34	1.31	1.30
200	3.89	3.04	2.65	2.42	2.26	2.14	2.06	1.98	1.93	1.88	1.62	1.52	1.46	1.41	1.39	1.35	1.32	1.26	1.22	1.21
300	3.87	3.03	2.63	2.40	2.24	2.13	2.04	1.97	1.91	1.86	1.61	1.50	1.43	1.39	1.36	1.32	1.30	1.23	1.19	1.17
500	3.86	3.01	2.62	2.39	2.23	2.12	2.03	1.96	1.90	1.85	1.59	1.48	1.42	1.38	1.35	1.30	1.28	1.21	1.16	1.14
1 000	3.85	3.00	2.61	2.38	2.22	2.11	2.02	1.95	1.89	1.84	1.58	1.47	1.41	1.36	1.33	1.29	1.26	1.19	1.13	1.11
2 000	3.85	3.00	2.61	2.38	2.22	2.10	2.01	1.94	1.88	1.84	1.58	1.46	1.40	1.36	1.32	1.28	1.25	1.18	1.12	1.09

7.4. Appendix D

7.4.1. Quantification results (µg/L)

A		B		C		D	
Copper	**Iron**	**Copper**	**Iron**	**Copper**	**Iron**	**Copper**	**Iron**
63.21	66.67	25.35	61.21	55.53	29.65	64.91	55.72
62.47	66.67	24.58	51.54	53.99	29.23	41.68	50.46
61.63	67.32	24.58	49.64	53.00	26.07	41.96	51.30
63.00	63.22	**E**		**F**		**G**	
60.99	52.43	**Copper**	**Iron**	**Copper**	**Iron**	**Copper**	**Iron**
64.23	50.06	47.73	104.76	44.40	40.79	52.83	47.94
58.85	48.76	47.37	103.92	47.72	39.53	56.39	44.99
65.27	49.19	46.15	105.39	45.40	39.53	57.27	40.37
61.65	54.59	**H**		**I**		**J**	
62.00	51.35	**Copper**	**Iron**	**Copper**	**Iron**	**Copper**	**Iron**
60.57	69.71	46.37	115.21	45.13	48.17	34.63	60.13
61.24	65.91	47.52	114.72	47.59	42.67	34.29	61.39
61.07	58.31	45.27	117.65	45.17	49.86	32.19	63.29
59.80	55.77	**K**		**L**		**M**	
58.24	73.31	**Copper**	**Iron**	**Copper**	**Iron**	**Copper**	**Iron**
59.24	55.35	41.23	194.66	87.15	48.06	67.01	88.00
56.49	65.49	40.51	204.80	84.87	47.04	64.55	82.53
59.85	51.33	40.99	212.51	87.99	49.48	72.36	99.36
56.65	53.87	**N**		**O**		**P**	
59.56	55.98	**Copper**	**Iron**	**Copper**	**Iron**	**Copper**	**Iron**
58.00	71.70	24.36	55.15	21.77	51.91	45.11	51.71
59.01	66.23	19.60	56.37	18.38	51.10	44.47	54.34
62.13	54.25	16.59	54.95	19.36	51.50	44.85	60.02
57.63	54.04	**Q**					
56.85	70.36	**Copper**	**Iron**				
56.01	50.25	60.69	72,80				
56.99	61.39	59.71	73,00				
58.60	49.83	59.17	75,23				
57.25	56.98						
61.16	67.70						

7.5. Appendix E

7.5.1. Questionnaire

QUESTIONÁRIO SOBRE O CONSUMO DE CERVEJA

O presente questionário pretende completar o estudo de uma dissertação de mestrado, orientada pela Prof. Luísa Mateus.

O seu preenchimento é voluntário e anónimo.

Idade: _____

Sexo: ☐ Feminino

☐ Masculino

Costuma beber cerveja:

☐ Não. Se selecionou esta opção o preenchimento deste questionário acaba aqui. MUITO OBRIGADA POR PARTICIPAR.

☐ Sim.

Com que frequência consome cerveja:

☐ Diariamente
☐ De 4 a 6 vezes por semana
☐ De 2 a 3 vezes por semana
☐ 1 vez por semana
☐ A cada 15 dias
☐ 1 vez por mês

Considerando o dia que mais consome cerveja, qual a quantidade aproximada?

☐ de 1 a 5 latas de 350 mL, ou de 1 a 3 garrafas de 600 mL

☐ de 6 a 10 latas de 350 mL, ou de 4 a 6 garrafas de 600 mL

☐ de 11 a 15 latas de 350 mL, ou de 7 a 9 garrafas de 600 mL

☐ mais de 15 latas de 350 mL, ou mais de 9 garrafas de 600 mL

Em que local costuma comprar cerveja quando pretende consumir em sua casa ou em casa de outras pessoas?

- ☐ Supermercado
- ☐ Lojas de conveniência
- ☐ Cafés, pastelarias
- ☐ Outros. Quais? _____

Na sua opinião, quais os <u>3 itens</u> mais importantes para a sua seleção do tipo de cerveja? Enumere de 1 a 3, sendo o 1 o mais importante.

- ☐ Sabor
- ☐ Preço
- ☐ Marca
- ☐ Cerveja consumida por amigos e familiares

Qual a sua preferência quanto à marca de cerveja? Se tiver mais do que uma, enumere sendo 1 a mais preferida.

- ☐ Sagres
- ☐ Super Bock
- ☐ Cergal
- ☐ Marina
- ☐ Imperial
- ☐ Outra. Qual? _____
- ☐ Outra. Marca estrangeira. Qual? _____

Qual a sua preferência quanto ao tipo de cerveja?

- ☐ Pilsner
- ☐ Bohemian Pilsner
- ☐ Bock
- ☐ Ale
- ☐ Outra. Qual? _____

Já consumiu cerveja artesanal?

- ☐ Já e gostou
- ☐ Já e não gostou
- ☐ Não

Já consumiu cerveja sem álcool?

- ☐ Já e gostou
- ☐ Já e não gostou
- ☐ Não

OBRIGADA PELA SUA COLABORAÇÃO!