Universidade de Aveiro Departamento de Química
2019

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MEDICAMENTOS GENÉRICOS CONTÊM MAIS IMPUREZAS?

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Química, realizada sob a orientação científica da Doutora Maria Eduarda da Cunha Pereira, Professora Associada no Departamento de Química na Universidade de Aveiro, Doutora Cláudia Maria Batista, Bolseiro de pós-Doutoramento na Universidade de Aveiro e Mestre Lina Carvalho, Técnica de ICP do Laboratório Central de Análises da Universidade de Aveiro.

Dedico este trabalho a todos os que sempre me ajudaram e apoiaram.

O júri

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Palavras-chave

Medicamentos genéricos; Espectrometria de massa por plasma acoplado indutivamente; Fármacos; Impurezas elementares; Validação de métodos

Resumo

Atualmente são comercializados em toda a união europeia medicamentos genéricos a um preço muito mais apelativo que os seus equivalentes de marca sendo este fator muitas vezes decisivo na escolha da marca do medicamento. No entanto, o facto da legislação referente aos genéricos estar atualmente facilitada em relação aos respetivos medicamentos de referência permite a produção de medicamentos genéricos com um custo mais baixo. Para isso, as farmacêuticas recorrem frequentemente a diferentes matérias primas e excipientes, por vezes, de menor qualidade que podem conter na sua matriz vários contaminantes considerados perigosos. Aliado a isto, existem ainda vários casos reportados sobre incidentes com medicamentos genéricos que apresentam diferentes efeitos secundários comparativamente aos seus medicamentos de referência, o que suscita muitas dúvidas e falta de confiança neste tipo de medicamentos tanto por parte dos profissionais de saúde como dos utentes. Com esta dissertação pretende-se adquirir conhecimento acerca da investigação já efetuada sobre esta temática, em específico, na quantificação de impurezas presentes em fármacos. Pretendeu-se também validar o método de quantificação de manganês, crómio e cobre em fármacos, bem como comparar impurezas presentes em medicamentos genéricos e nos respetivos medicamentos de referência.

Keywords

Generic drugs; Inductively Coupled Plasma Mass Spectrometry; Pharmaceutical drugs; Elemental Impurities; Method validation.

Abstract

Generic drugs are currently sold all over European union presenting a much more appealing price than their corresponding pharmaceutical drugs from other brands, which frequently influence the consumer regarding the choice of the drug brand. The lower prices are mostly due to the fact that the legislation applied to the generic drugs is currently simplified comparatively to the respective reference pharmaceutical drugs legislation, allowing that the pharmaceutical industry produce generic drugs at a lower cost. In order to reduce production costs even more, are often use different excipients and raw materials that sometimes present lower quality and may contain in their matrix several contaminants considered dangerous. In addition, there are several reported cases of incidents with generic drugs and generic pharmaceutical products with different side effects compared to their reference products, which raises many doubts and lack of trust in this type of pharmaceutical products both by health professionals and users. The aim of this dissertation is the acknowledgement of the studies published about the topic mentioned above, specifically, the determination and quantification of elemental impurities present in pharmaceutical drugs. It is also presented to validate the quantification method of manganese, chromium and cooper in pharmaceutical drugs, as well as, the determination and comparison of elemental impurities in both generic and respective reference pharmaceutical drugs.

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CHAPTER I: INTRODUCTION

1. PHARMACEUTICAL DRUGS

Pharmaceutical drugs are economically considered to be a merit asset as in the various Member States of the European Union its widespread availability to the population is seen as a priority. About two-thirds of the expenditure in pharmaceutical drugs is assumed by the State itself and this primary role of the State in the widespread availability of medicines combined with the scientific progress that has diversified the supply of treatments and increased their effectiveness has been an effective determinant of improving living conditions and average longevity (Pereira and Vilares, 2014).

The success achieved has led to a nominal growth of public expenses on pharmaceutical drugs substantially higher than the nominal growth of gross domestic product (GDP). Public expenses in the European Union Member States increased by an average of 76% between 2000 and 2009, translating into an annual growth of 5.8%, against just a nominal gross domestic product growth of 2.8% (Vogler et al., 2011).

Portugal is one of the Member States with the highest consumption of pharmaceutical drugs per capita and the tendency is to increase the numbers every year (**Table 1**).

Table 1: *Number of medicine packs sold and respective total values over the years in Portugal.*

Year	Nº medicine packs	Value at retail price (€)	NHS charges (€)	User charges (€)
2013	149 086 465	1 849 703 511	1 160 219 375	689 484 136
2014	153 020 413	1 873 043 848	1 170 352 630	702 691 219
2015	154 964 976	1 891 956 858	1 182 180 185	709 776 673
2016	155 972 138	1 887 107 629	1 189 820 191	697 287 438
2017	157 349 517	1 913 105 122	1 213 514 100	699 591 022

NHS – National Health Service

As can be seen in **Table 1**, over the last 5 years the number of pharmaceutical drug packs has increased although the user charges are apparently decreasing. This is due to the fact that the NHS is increasingly participating more and in a higher proportion the pharmaceutical drugs that are more commonly used reducing the pharmaceutical charges weight to users (Infarmed, 2018).

According to the Portuguese Association of the Pharmaceutical Industry (Apifarma), since 1990 the use of pharmaceutical drugs has allowed that two billion years of healthy life was added to the Portuguese population. In other words, the use of pharmaceutical drugs prevented more than 110 thousand deaths in Portugal and contributed to the increase of up to 10 years in the average life expectancy of patients.

The annual income of Portuguese families increased by 280 million euros due to the use of pharmaceutical drugs as the patients return to active life more quickly, and reduce the number of hospitalizations, ensuring savings to the health system (Apifarma, 2018).

According to the Apifarma study (2018), the economic benefit of these years of healthy life is higher than the economic investment in the production of pharmaceutical drugs. However, this increase in pharmaceutical drugs consumption has negative implications too, as it is a heavy burden for the National Health Service and it also implies risks to the patient lives in the case of polimedication (simultaneous consumption of five or more pharmaceutical drugs) that can have extremely serious consequences for the patient and is responsible for many hospitalizations.

Just in the first three months of 2018, the expenses of users with pharmaceutical drugs were 18,18 €/per capita and 48.2 % of the pharmaceutical's units dispensed were generic. **Table 2** shows the most consumed pharmaceutical drugs in the first three months of 2018 (Silva and Santos, 2014).

Table 2: *Nº of pharmaceutical packs sold and market weigh of each of the most sold pharmaceutical drugs from January to March of 2018.*

Pharmacotherapeutic classification	Nº of packs sold	Market weigh
Renin Angiotensin Axis Modifiers	3 284 702	8.1%
Antidystopia	2 955 374	7.2%
Anxiolytics, Sedatives and Hypnotics	2 646 239	6.5%
Antidiabetics	2 403 361	5.9%
Antidepressants	2 142 822	5.3%
Anticoagulants	1 787 835	4.4%
Gastric Secretion Modifiers	1 727 755	4.2%
Adrenergic Activity Depressants	1 464 031	3.6%
Analgesics and Antipyretics	1 392 312	3.4%

Antiepileptics and Anticonvulsants	1 084 863	2.7%
Other Subgroups	19 901 666	48.8%
Total	40 790 960	100%

2. GENERIC PHARMACEUTICAL DRUGS

A generic drug is a pharmaceutical product that has the same active substance, pharmaceutical form, and dosage as well as the same therapeutic indication as for the reference drug, a pharmaceutical drug that has already been authorised (EMA, 2012).

According to Decree-Law no. 176/2006, of 30th of August, the MAH (Marketing Authorisation Holder) of generic pharmaceutical drugs is not subject to the same legal provisions of other pharmaceutical drugs, being the presentation of pre-clinical and clinical trials not required as long as the bioequivalence is demonstrated by bioavailability. When these studies are not appropriate, therapeutic equivalence by means of appropriate clinical pharmacology studies (tests strictly in accordance with the Community standards) or others to be requested by INFARMED can be applied.

2.1. ADVANTAGES

2.1.1. SAFETY AND EFFECTIVENESS

European medicines agent (EMA) claims that as these pharmaceutical drugs have their active substances in the market for several years (10 years at least), they present a greater guarantee of effectiveness and allow a better knowledge of their safety profile. Therefore, European pharmaceutical drugs agent declares that through the bioavailability studies, generic drugs demonstrate their bioequivalence, being as safe and effective as the reference medicine but with a more appellative price for the user (Infarmed, 2016).

2.1.2. COSTS

In fact, generic pharmaceutical drugs are 20 to 35% cheaper than the reference pharmaceutical drugs with the same dosage and pharmaceutical form (Zarowitz, 2008), which considering our national economy, becomes an huge economic advantage for the users because these drugs are substantially cheaper than the reference pharmaceutical drugs. It is also an advantage for the NHS since it allows better management of available resources (Infarmed, 2016).

The lower cost of generic pharmaceutical drugs also has the potential to reduce the prices of their reference pharmaceutical products by creating a more competitive market because, as shown in some studies, generic pharmaceutical drugs do not present any inferiority relatively to their reference (Kesselheim et al., 2008).

2.2. QUALITY

Many generic pharmaceutical drugs are produced by the same manufacturer that already produced the respective reference pharmaceutical drugs when the patent of the reference ones expires. This ensures quality as it is the exact same product, however as the manufacturer just need to present bioequivalence tests, the pharmaceutical drugs price can be lowered (Peters et al., 2009).

That being said, one may think that generic pharmaceutical drugs are always undoubtedly the best choice, but it is not the case as they have some concerning disadvantages regarding testing and formulation (Lewek and Kardas, 2010).

2.3. DISADVANTAGES

2.3.1. BIOEQUIVALENCE

As stated by the World Health Organization (WHO), for the bioequivalence study of a generic pharmaceutical drug it is only needed 18 to 24 healthy adult volunteers (WHO Technical Support Series, 1996). The people who are selected as volunteer can't take medication or smoke at the time of the bioequivalence study. When an oral formulation is tested, the volunteers are also subject to a standardized meal to avoid the possibility of food coadministration interferences (US Food and Drug Administration, 2002). All of these precautions reduce the possibility of other interferences (like medication interference, current conditions and disease process) rather than by formulation ones.

Moreover, to decrease and try to avoid even more the nondrug-related variations, in the studies of bioequivalence usually, it is applied a crossover design. In this study format, half of the volunteers receive the brand-name pharmaceutical drug first and then the test drug with a washout period in between, while the other half receive the pharmaceutical products in the reverse order. Indeed, the study can be modified according to the drug that needed to be tested as is the case of extended release products and topical agents (Meyer, 1998).

The key point that is often questioned is whether all of these measures to minimize non-drug related effects and variations mimic the real world. In fact, expert's argument that these controlled environment does not represent at all the real world. They are worried that when generic products are taken by real patients that really have the illnesses, make use of other medications or have other medical conditions, the results may be dissimilar from the ones obtained in the equivalence studies in extremely controlled environments (Meredith, 2003). If this is the case, it may result in cancellation of the effect of the generic pharmaceutical drug or in more worrying cases, unwanted side effects that can greatly affect the patient's health.

2.3.2. DIFFERENCES IN FORMULATION

This is another disadvantage that is concerning the experts as it can affect the taking of generic pharmaceutical drugs by the patients. As already discussed, a generic pharmaceutical drug has to have exactly the same active ingredient as their reference in the same dose formulation and administrated by the same route of administration (Meredith, 2003). Non-the-less, although the generic pharmaceutical drug has to have a similar ratio of inert to active compound as their reference, the inert ingredients, also called the excipient, does not have to be the same (Meredith, 2003).

When there is a need for medicine taking in real life, usually the patient has to take several doses, however, the administration of the test drug in the bioequivalence tests is made in a single dose. Therefore, when the excipient in generic pharmaceutical drugs is different from the branded-drugs, critics wonder if, when taken for longer periods of time, the test drug serum concentration becomes too high or whether the excipient will affect any stage of the drug cycle like the absorption, distribution and metabolism (Lewek and Kardas, 2010).

2.3.3. REPORTED INCIDENTS

Taking generic pharmaceutical drugs instead of branded drugs for most medications usually poses no problem for the patients. However, in some drug classes, especially with narrow therapeutic range drugs the switch can be a problem as several cases of non-bioequivalent generic drugs among others unexpected effects are reported (Lewek and Kardas, 2010).

Antiepileptic drugs are one example in which the several studies reported differences between generic and their reference pharmaceutical drug (Crawford et al., 2006). For instance, it was reported that 1 of 3 generic formulations of carbamazepine compared was not bioequivalent (Silpakit et al., 1997). Moreover, other 3 generic formulations of the same active compound were tested and although they were all bioequivalent, it was reported a faster absorption than the brand-name drug (Silpakit et al., 1997).

Another example is the psychotropic agents in which there are reported cases of problems occurrence after the switch from one generic to another or from a reference pharmaceutical drug to the respective generic. Examples cited for the Food and Drug Administration (FDA) are amitriptyline/ perphenazine and venlafaxine (US Food and Drug Administration, 2018).

There are also doubts about levothyroxine (LT4) administration and generic substitution as there are still reported cases of generic LT4 adverse results being the reported events mostly associated with the substitution of reference pharmaceutical drugs for generic ones and from one generic to another generic pharmaceutical drug (Hennessey et al., 2010).

Overall, the safety, effectiveness, quality and mostly the significantly difference in retail price of generic pharmaceutical drugs and the huge advertising made for the use of generics appears to be overcoming all of the disadvantages associated with it, leading to a growing preference for generic pharmaceutical drugs rather than their reference ones as can be seen in the graphic presented by INFARMED in the monthly monitoring of drug consumption in the national health service outpatient clinic (**Figure 1**) (Infarmed, 2018).

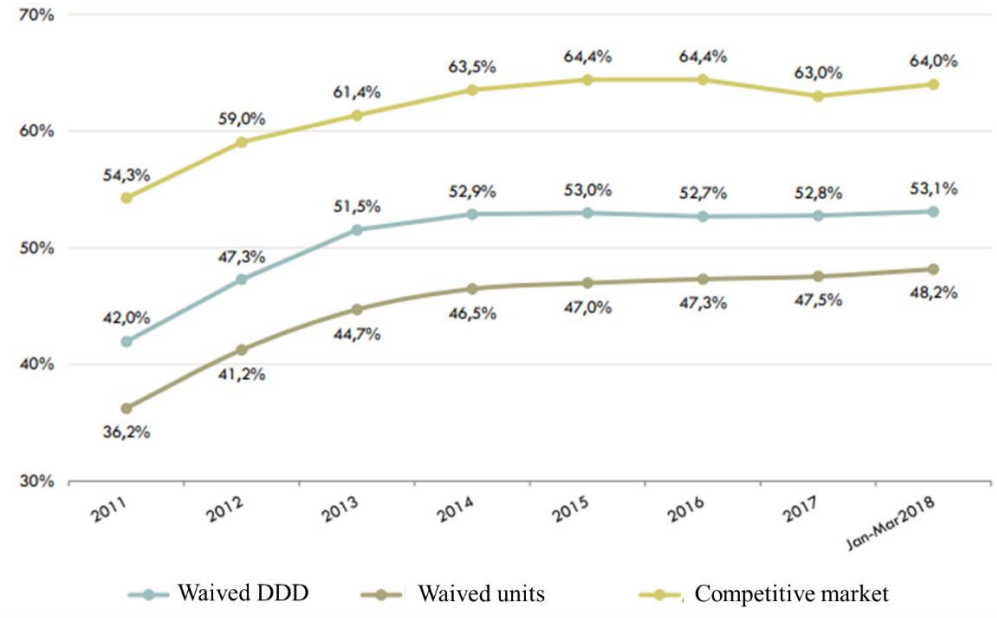

Figure 1: *Evolution of the sales of generic drugs over the years. DDD is a measuring unit assigned by the WHO (World Health Organization) that represents the average daily maintenance dose of a certain active substance in its main therapeutic indication for adults. A competitive market is the percentage of dispensed generic drugs.*

It is imperative to consider that this preference only highlights the importance of making sure that all generic pharmaceutical drugs are safe for usage. As the generic pharmaceutical drugs are often composed of different excipients it is extremely important to make sure that generic inert compounds have their elemental impurities under control.

3. PHARMACEUTICAL DRUGS IMPURITIES

Due to the fact that generic drugs have the whole process of obtaining the marketing authorisation holder simplified, they are exempt from the submission of expert reports on pharmacological, toxicological and clinical and preclinical tests according to the Decree-Law no. 176/2006, of 30 August and this fact turns important to question the quality of the generic pharmaceutical drugs concerning inorganic impurities (Infarmed, 2018).

Pharmaceutical drug pills are composed by a blended powder containing active pharmaceutical ingredients called APIs and a matrix of inactive organic compounds called excipients that helps to stabilize and delivery successfully the APIs (St-Onge et al., 2002). Excipients are substances that don't have therapeutic effects and are part of the pharmaceutical drugs to ensure their stability, physicochemical and organoleptic properties (Balbani et al., 2006; da Silva et al., 2017).

The film coating of capsules and tablets can also offer physiological advantages as they reduce the irritation that the exposure of the stomach to the high concentration of pharmaceutical drugs often present (Romero-Torres et al., 2006). These film coatings are mostly composed of plasticizers, pigments or cellulosic and acrylic polymers since they present good film coating properties (Sakata et al., 2006). And therefore, titanium dioxide and iron oxide are widely used for this type of coating (Luo et al., 2008).

As these types of elements, as well as others are part of the constitution of medicines, is imperative the determination of the impurities present in these pharmaceutical drugs because it is needed to ensure that all the impurities concentrations are below the legal maximum allowed. This is due to the fact that some elements in trace or even ultra-trace concentrations are toxic or can decrease the stability of active pharmaceutical ingredient.

There are some elements that are considered toxic in any concentration as is the case of cadmium (Cd), mercury (Hg) and lead (Pb) although their level of toxicity depends on the chemical form. Other concerning element is arsenic (As) that, however, it is not as toxic as the above mentioned, in concentrations higher than the allowed may have harmful consequences on human health (Balaram, 2016; Fliszar et al., n.d.; ICH Expert Working Group, 2014). Both arsenic and cadmium are considered by the International Agency for Research on Cancer (IARC) as carcinogenic in their inorganic forms and although mercury can't be considered carcinogenic (as it has low oral bioavailability), it can cause toxicological and hematopoietic effects, causing renal disorders and skin diseases (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2012). Lead, similar to mercury, cannot be considered carcinogenic but it causes neurological, reproductive, immune, cardiovascular and renal systems pathologies (ICH Expert Working Group, 2014).

Some inorganic impurities could also be indicators of non-adequate handling and storage (Zachariadis and Sahanidou, 2011) as many metallic ions besides putting at risk the human health security, also decreases the drug efficiency through the formation of stable metal-drug complexes (Incledon et al., 2003; Luo et al., 2008), or (under specific conditions) catalyse the degradation of the antibiotics (Romero-Torres et al., 2006; Sakata et al., 2006).

Commercial antibiotics usually contain APIs and several excipients (organic and inorganic matrixes) and therefore, residues of metals in trace amounts are not likely to be found. However, when catalysts are used during synthetic processes the pharmaceutical drug must be tested for the catalyst used (Circus et al., 2002). In other words, if a specific metal is used as a catalyst in the synthetic processes, there is a chance that it led to the presence of residues of this metal in the final product and a specific element assay should be executed to determine the concentration of these residues. The presence of trace impurities in the final product can also occur when natural substances are used or when various excipients, diluting agents, natural flavours and others are included without proper purification, or by drugs interaction with equipment, containers and surfaces (during processing) (Balaram, 2016; Fliszar et al., n.d.; Resano et al., 2015; Stoving et al., 2013).

As seen, most of the inorganic impurities found in trace or ultra-trace amounts can present toxicological risks and put human health at risk, being the effects of which very difficult to detect sometimes. Thus, there is a growing concern about monitoring the inorganic impurities, especially trace and ultra-trace elements that are of paramount importance. National and international legislation establish element specific daily exposures (PDEs) limits for finished pharmaceutical drug products as well as control standards and determination processes of these chemical elements, including analytical procedures and method validations.

As in pharmaceutical drugs, the inorganic impurities can be present in the final product by a numerous of sources and stages of the process of manufacturing (APIs, excipients, raw material, etc), not all of the 24-potential concerning elemental impurities considered have to be tested. In fact, depending on the route of administration (oral, parenteral or inhalation) different classes of elemental impurities need to be tested (Menoutis et al., 2018).

The International Conference on Harmonization (ICH) developed a global policy for limiting impurities in pharmaceutical drugs and ingredients back in 2009 and implemented an explicit guidance on specification limits for elemental impurities worldwide. This "Guidelines for Element Impurities" considered four different classes of elemental impurities and their permitted daily exposure as shown in **Table 3**.

Table 3: *Element impurity permitted daily exposures depending on the route of administration and respective classes of elemental impurities.*

Element	Class	Oral PDE (µg/day)	Parenteral PDE (µg/day)	Inhalation PDE (µg/day)
As	1	15	15	2
Cd	1	5	2	2
Hg	1	30	3	1
Pb	1	5	5	5
Co	2A	50	5	3
V	2A	100	10	1
Ni	2A	200	20	5
Tl	2B	8	8	8
Au	2B	100	100	1
Pd	2B	100	10	1
Ir	2B	100	10	1
Os	2B	100	10	1
Rb	2B	100	10	1
Ru	2B	100	10	1
Se	2B	150	80	130
Ag	2B	150	10	7
Pt	2B	100	10	1
Li	3	550	250	25
Sb	3	1200	90	20
Ba	3	1400	700	300
Mo	3	3000	1500	10
Cu	3	3000	300	30
Sn	3	6000	600	60
Cr	3	11000	1100	3

PDE – Permitted Daily Exposure

Class 1 are all the impurities that are significantly toxic to humans and have limited or no use in the manufacturing of pharmaceutical drugs. Usually, these impurities come from raw materials and need evaluation on the risk assessment performed in all potential sources of elemental impurities and routes of administration.

Impurities considered Class 2 are in general toxic to humans depending on the route of administration and are divided into two sub-classes. Sub-class 2A are elements that present the probability of occurrence in the pharmaceutical drugs quite high and therefore need to be tested in the risk assessment for all potential sources of elemental impurities and routes of administration. On the other hand, in the sub-class 2B there are elemental impurities that present low probability of occurrence in the pharmaceutical drugs as they have low potential to be co-isolated with other materials and low abundance and thus, except if they are added intentionally during any manufacture process step of the pharmaceutical drug, does not exist the need to test these elemental impurities in the risk assessment.

Class 3 elements are impurities that possess low oral administration toxicity but in the case of inhalation or parenteral administration route, they must entail a risk assessment. Lastly, elements like Al, B, Ca, Fe, K, Mg, Mn, Na, W, and Zn doesn't present establish Permitted Daily Exposures (PDEs) in the document mentioned as their PDEs are different depending on regulations of each region due to their low toxicities. If these elemental impurities are present in pharmaceutical drugs, they must be addressed by the regional guidelines or regulations where these are going to enter the market (Pharma & Biopharma, 2013).

As seen, quality control is a crucial part of the manufacturing process of pharmaceutical drugs and therefore, the determination of potential impurities in the different stages of the manufacturing processes, especially in the final product, is obligatory.

This need for quality control is even more highlighted in the case of pharmaceutical drugs prescribed for continuous use, for example, in the treatment of chronic and/or degenerative diseases, for relieving pain, as contraceptives or even for the prevention of diseases as in these cases, the patient is permanently exposed to the elemental impurities present in the pharmaceutical drugs, that can be persistent and bioaccumulative in several human body parts (**Figure 2**). They may also generate unwanted and unknown pharmacological–toxicological effects (Balbani et al., 2006).

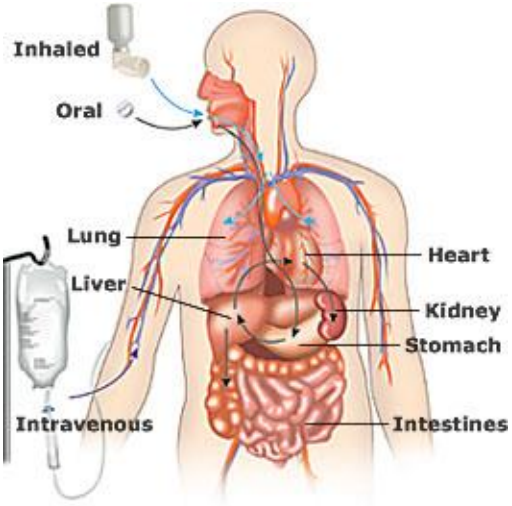

Figure 2*: Pharmaceutical drug cycle - Medicines are taken orally pass through the liver before they are absorbed into the bloodstream. Other forms of drug administration bypass the liver, entering the blood directly. Retrieved from: https://publications.nigms.nih.gov/medbydesign/chapter1.html, accessed on 11/10/2018*

The control of these possible impurities is typically performed using limit tests, performed by several existent techniques. Although few studies have been published about elemental impurities quantification on pharmaceutical drug pills by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) or other existing techniques, a search has been made and the keywords mentioned next were used in Web of Science, SCOPUS and b-on:

1. Elemental impurities
2. ICP OR GF-AAS OR F-AAS
3. Analysis OR quantification

Few articles that contain the focus subject were found in the literature, nonetheless, the most relevant studies were presented in **Table 4** mentioning all the conditions of the sample dissolution, treatment and analysis.

Table 4: *Techniques used for the quantification of elemental impurities and respective conditions used as reported in the literature.*

Pharmaceutical Drug	Type	Dissolution and treatment method	Sample weight	Sample volume	Analytical method	Quantified parameters	Obtained concentrations	Ref.
Amoxicillin	Antibiotic	Tablets were ground and sieved. <100 µm particle size fraction + 0.5 mol/L HNO_3 + Triton (0.5%, v/v) resulting in 200 µg/L slurry concentration. Stirring at 600 rpm and introduced during continuous stirring into the nebulization system using a peristaltic pump.	(a)	(a)	ICP-AES	7 elemental impurities	**(in µg/g)** Al-n.d Ca-8.2 Cu-n.d Fe-1.5 Mg-94 Mn-n.d Zn-0.81	(Zachariadis and Michos, 2007)
Arbidol (Umifenovir)	Antiviral	Microwave-assisted digestion in closed vessel: 100 mg of sample + 6 mL HNO_3 + 2 mL H_2O_2, 125 °C for 30 min. After cooling dilute with deionised water to 100 mL.	100 mg	100 mL	ICP-MS	6 elemental impurities	**(in µg/g)** Mn-0.96 Co-0.94 Ni-0.99 Cu-0.99 Pb-0.91 Cd-1.04	(Rudovica et al., 2014)

Aspirin	Non-Steroidal Anti-Inflammatory	Microwave-assisted digestion in closed vessel: 200 mg Aspirin + 3.0 mL HNO_3 + 1.0 mL H_2O_2 reacts for 10 min. Add 4.0 mL HNO_3 + 2.0 mL of HCl. Microwave heating program: 5 min ramp to 210 ºC, hold 5 min; 3 min ramp to 250 ºC, hold 6 min. After 20 min cooling, transfer to 50 mL volumetric flask, add internal standard and was then added, with a final concentration of 50 ug L^{-1} in the sample when diluted to volume with 1% nitric acid.	200 mg	50 mL	ICP-MS	17 elemental impurities	**(in µg/L)** Cd-1.91 Pb-2.04 As-5.36 Hg-11.67 Co-19.92 V-40.20 Ni-78.95 Tl-3.18 Au-40.55	Pb-39.40 Ir-40.97 Os-93.52 Rh-39.48 Ru-39.48 Se-56.72 Ag-60.60 Pt-39.95	(Menoutis et al., 2018)
Calcium Folinate	Adjuvant Chemotherapy	0.1 g of tablet ashed at 550 °C for 1–2 h in a quartz crucible. After cooling, add 1.0 mL of aqua regia. Heat and evaporated to dryness. Dissolved in 100 mL of 0.2 mol/L HNO_3 containing 10 ng/mL of indium as an internal standard.	100 mg	100 mL	ICP-MS	12 elemental impurities	**(in µg/g)** Be-<0.01 V-0.36 Mn-<0.01 Co-<0.07 Ni-<0.01 Cu-1.03	Zn-8.2 Mo<-0.23 Cd-<0.001 Sn-<0.001 Tl-<0.001 Pb-0.13	(Lásztity et al., 2002)
Carbamazepine	Anticonvulsant	Microwave-assisted digestion in closed vessel: 400 mg sample + 2 mL water + 2 mL of HNO_3 + 2mL HCl. Heating program: 85 °C for 8 min; 5 min ramp to 130 °C; 5 min ramp to 200 °C, 30 min hold at 200 °C. Transfer to a 20 mL volumetric flask and dilute with water. For ICP measurements dilute 1:10 with water.	400 mg	20 mL	ICP-OES and ICP-MS	17 elemental impurities	**(in µg/g)** Cd-<0.070 Co-<0.035 Ir-<0.002 Mn-<0.064 Mo-<0.055 Ni-<0.092 Pb-<0.050 Pd-<0.078	Pt-<0.001 Ru-<0.009 Rh-<0.001 Sn-<0.180 Sb-<0.050 V-<0.050 Cu-<1.0 Fe-<2.0 Zn-<0.5	(Wollein et al., 2015)

Diclofenac	Non-Steroidal Anti-Inflammatory	Treatment and dissolution identical to the Carbamazepine pharmaceutical drug.	400 mg	20 mL	ICP-OES and ICP-MS	17 elemental impurities	**(in µg/g)** Cd-<0.070 Co-<0.035 Ir-<0.002 Mn-<0.064 Mo-<0.055 Ni-<0.092 Pb-<0.050 Pd-<0.078	Pt-<0.001 Ru-<0.009 Rh-<0.001 Sn-<0.180 Sb-<0.050 V-<0.050 Cu-<1.0 Fe-<2.0 Zn-<0.5	(Wollein et al., 2015)
Enalapril (Enalapril maleate)	Antihypertensive	Treatment and dissolution identical to the Titrace pharmaceutical drug.	400 mg	25 mL	ICP-MS	3 elemental impurities	**(in ng/g)** Pd - <0.9 Pt - <4 Rh -<5		(Simitchiev et al., 2008)
Fingolimod	Immunomodulating	Microwave-assisted digestion in closed vessel: 200 mg sample + 4 mL of HNO_3 + 0.2 mL of $HClO_4$. Digest at 250°C–260 °C for 45 minutes in a high-pressure autoclave. After cooling add 0.1 mL of IS and transfer to a 10 mL volumetric flask and dilute with water.	200 mg	10 mL	ICP-OES	6 elemental impurities	Cu + Fe + Ni + Pb + Pd + Zn - < 5ppm		(Correale et al., 2014)
Heparin-Na (Intravenous Drug)	Anticoagulants	Lyophilization of samples. Residues acid digested at high pressure and evaporated to small volumes. V complexed with cupferron at a pH of 2 and extracted three times. After evaporation of the organic layers, the residues were dissolved in formic acid.	(a)	(a)	GF-AAS	1 elemental impurity	**(in µg/L)** V-4.9		(Heinemann and Vogt, 2000)

Isosulfan blue	Lymphatic imaging agent	UV photolysis assisted digestion: 75 mg sample + 3 mL HNO_3 reacts for 10 min. Then add 200 µL H_2O_2 + 2 mL water + UV irradiation at 85 ± 5 °C for 1 h. At every 10 min add 0.1 mL H_2O_2 + 1 mL HNO_3 during digestion until a maximum of 4 additions. Finally, dilute to 10 mL volumetric flask with water.	75 mg	10 mL	ICP-MS	5 elemental impurities	**(in µg/g)** Cr- 0.48 Cd- 0.46 Cu- 0.81 Sn- 1.67 Pb- 1.2	(Dash et al., 2011)
Levetiracetam	Antiepileptic	Microwave-assisted digestion in closed vessel: 1g sample + 15 mL HNO_3 + 2 mL H_2O_2 at 250ºC until obtain a clear solution and a reduction of volume to 5 mL. Then ramp of 10 °C, hold at 250 °C for 10 min. After cooling transfer to a 25 mL volumetric flask and dilute with Millipore water.	1 g	25 mL	ICP-OES	23 elemental impurities	**(in ppm)** Ag- <0.4 Au- <0.4 As- <0.4 Bi- <0.4 Cd- <0.4 Cr- <0.4 Cu- <0.4 Fe- <0.4 Hg- <0.4 Ir- <0.4 Mn- <0.4	Mo- <0.4 Ni- <0.4 Os- <0.4 Pb- <0.1 Pd- <0.4 Pt- <0.4 Rh- <0.1 Ru- <0.4 Sb- <0.4 Sn- <0.4 V- <0.4 Zn- <0.4	(Dash et al., 2011)
Levodopa	Antiparkinsonian	Dissolved in 0.2 mol/L HNO_3 to obtain the final concentration of 0.1%.	100 mg	100 mL	ICP-MS	12 elemental impurities	**(in µg/g)** Be-<0.01 V-0.47 Mn-<0.01 Co-<0.01 Ni-<0.01 Cu-<0.01	Zn-1.5 Mo-0.08 Cd-<0.001 Sn-0.27 Tl-<0.01 Pb-<0.01	(Lásztity et al., 2002)
Lisinipril	ACE Inhibitor	Treatment and dissolution identical to the Aspirin one.	200 mg	50 mL	ICP-MS	17 elemental impurities	**(in µg/L)** Cd-2.04 Pb-2.00	Au-41.27 Pb-42.80 Ir-40.62	(Menoutis et al., 2018)

							As-6.34 Hg-12.13 Co-20.63 V-40.75 Ni-82.57 Tl-3.22	Os-112.28 Rh-40.25 Ru-41.27 Se-65.55 Ag-60.07 Pt-41.05	
Metformin Hydrochloride	Biguanide Hypoglycemic Agent	Microwave-assisted digestion in triplicate closed vessel: 500 mg + 5.0 mL inverse aqua regia. Heating program: 2 min ramp to 170 °C, 5 min hold; 2 min ramp to 190 °C, 10 min hold; 2 min ramp to 210 °C, 20 min hold. After cooling dilute to 50 mL volumetric flask with distilled-deionized water. When containing residual solids: centrifuged for 5 min at 3500 rpm. After, dilute 0.1 mg of the solution with deionized water.	500 mg	50 mL	ICP-MS	4 elemental impurities	**(in µg/kg)** As-99 Cd-115 Hg-87 Pb-n.d		(da Silva et al., 2017)
Methotrexate	Anti-Rheumatics	Microwave-assisted digestion: Into the quartz vessel: 200 mg Methotrexate + 1 mL concentrated H_2SO_4 + 0.5 mL concentrated HNO_3. Outside quartz vessel: 3 mL concentrated HNO_3. 12 samples were heated at the same time using a microwave program. After cooling continue digestion using another heating program. After, sample dilution with ultrapure water into 20 mL volumetric flasks.	200 mg	20 mL	GF-AAS	2 elemental impurities	**(in µg/g)** Fe- 0.41 Pd-0.60		(Niemelä et al., 2004)

Naloxone	Opioid antagonist	Treatment and dissolution identical to the Carbamazepine pharmaceutical drug.	400 mg	20 mL	ICP-OES and ICP-MS	17 elemental impurities	**(in µg/g)** Cd-<0.070 Co-<0.035 Ir-<0.002 Mn-<0.064 Mo-<0.055 Ni-<0.092 Pb-<0.050 Pd-<0.078	Pt-<0.001 Ru-<0.009 Rh-<0.001 Sn-<0.180 Sb-<0.050 V-<0.050 Cu-<1.0 Fe-<2.0 Zn-<0.5	(Wollein et al., 2015)
Paracetamol	Analgesic and Antipyretic	Microwave-assisted digestion in closed vessel: 400 mg Paracetamol + 2 mL H_2O + 1 mL HNO_3 + 1 mL HCl. Heating program: 85 °C hold 8 min; 5 min ramp to 130 °C; 5 min ramp to 200 °C, 30 min hold. After cooling add H_2O to 20 mL volume. For ICP-MS measurements dilute solution to 1:10 with water.	400 mg	20 mL	ICP-MS and ICP-OES	21 elemental impurities	**(in ppm)** Mo-n.d Cu-n.d Cr-n.d Mn-0.25 Fe-25 Zn-n.d Co-n.d Ni-25 Pb-0.10 Cd-n.d	As-n.d Hg-n.d Pd-n.d Pt-n.d Ir-n.d Os-n.d Ru-n.d V-0.11 Sn-n.d Sb-n.d Rh-n.d	(Wollein et al., 2015)
Ramipril (Tritace)	Antihypertensive	Microwave-assisted digestion in a closed vessel: 0.4 g of sample + 4 mL HCl + 2 mL of H_2O_2, cooled down for 1 hour and digest 6 vessels with a heating program. After, transfer and dilute to a 25 mL volumetric flask and centrifuged for 15 min at 3000 rpm. Heating program: 2 min at 60% power, cooling for 5 min; 2 min at 60% power, cooling for 5 min; 2 min at 30% power, cooling	400 mg	25 mL	ICP-MS	3 elemental impurities	**(in ng/g)** Pd - <0.8 Pt - 77 Rh -<4		(Simitchiev et al., 2008)

		for 5 min; 2 min at 30% power, cooling for 30 min;							
Simvastatin	Antilipemic Agent	Microwave digestion: 3g simvastatin + 10 mL deionized water + 10 mL HNO_3 + 2 mL H_2O_2 and repeat 3 times. Then add 30mL HNO_3 + 6 mL H_2O_2. Digest until left 3 mL. After cooling transfer to a 50 mL volumetric flask, dilute with ultra-pure water. Take 5 mL into 10 mL volumetric flask + 0.5 mL HCl + 2 mL standard solution and dilute with ultra-pure water to the mark.	3 g	50 mL	AAS	1 elemental impurity	**(in µg/mL)** Li-<0.009		(Jia Tao, Hao Qian, n.d.)
Unknown	Antibiotic	Digestion: 0.1 g of sample + HNO_3 – HCl – HF solution + 2.00 mg L^{-1} of yttrium as internal standard.	100 mg	(a)	ICP-OES and ICP-MS	10 elemental impurities	**(in µg/g)** Ti- 0.854 Al- n.d Zn- n.d Mg- n.d Fe- n.d	Cu- n.d Mn- n-d Cr- n.d Pb-n.d B-n.d	(Zachariadis and Sahanidou, 2011)

n.d - All results indicated by "n.d." show levels lower than Limit of Quantitation.

(a) – Value not indicated in the correspondent pharmaceutical product literature.

The following factors were analysed based on the selected articles from the literature review presented above.

3.1. TYPE OF PHARMACEUTICAL PRODUCT

The pharmaceutical drugs most analysed are from the most variate types and coincident with the utmost used type of pharmaceutical drugs. The range goes from simple Non-Steroidal Anti-Inflammatory drug like Aspirin, Diclofenac (Menoutis et al., 2018) or Paracetamol (Wollein et al., 2015) that does not require a prescription, passing through the most consumed antibiotics like Amoxicillin (Zachariadis and Michos, 2007), anticoagulants (Heinemann and Vogt, 2000), anti-rheumatic like Methotrexate (Niemelä et al., 2004), biguanide hypoglycemic agent (da Silva et al., 2017), ACE inhibitor (Menoutis et al., 2018), antilipemic agent (Jia Tao, Hao Qian, n.d), antihypertensive (Simitchiev et al., 2008), antiepileptic (Dash et al., 2011), anticonvulsant (Wollein et al., 2015) or an antiviral drug (Rudovica et al., 2014) that already require a prescription. It was also reported the analysis of a more specific range of pharmaceutical drugs in which are included the antiparkinsonian Levodopa, chemotherapy adjuvants as is the case of Calcium Folinate (Lásztity et al., 2002), opioid antagonist (Wollein et al., 2015), lymphatic imaging agent (Dash et al., 2011) or an immunomodulating (Correale et al., 2014).

3.2. SAMPLE DISSOLUTION AND TREATMENT METHODS

Sample preparation is a fundamental step in inorganic pharmaceutical impurities detection and quantification tests as they present low detection limits (< 1μg/g) and difficult matrices. In general, as seen in the **Table 4**, if the pharmaceutical drug is water-soluble or miscible liquid substance, it can be simply dissolved in suitable acids, solvents or solvent mixture as it is the case of levodopa that it is simply dissolved in HNO_3 (Lásztity et al., 2002).

However, if the matrix is difficult to dissolve, the sample has to be digested, using UV photolysis assisted digested, microwave-assisted digested or directly introduced in the spray chamber in the form of a slurry. The last two techniques have the advantage of low risk of contamination and the last one has an even greater advantage that eliminates the time-consuming digestion step. There are two types of microwave digestion described in literature, wet sample digestion that it is the most common one and dry sample digestion that has been shown better results (Altundag and Tuzen, 2011). Nonetheless, concerning the determination and quantification of elemental impurities in pharmaceutical products, the only form of microwave-assisted digestion found in literature was wet digestion as shown in **Table 4.**

In all of the pharmaceutical drugs that presented sample wet microwave-assisted digestion, the digestion was in almost all cases carried out using HNO_3 in several concentrations (Correale et al., 2014; Dash et al., 2011; Jia Tao, n.d.; Menoutis et al., 2018; Niemelä et al., 2004; Rudovica et al., 2014; da Silva et al., 2017; Simitchiev et al., 2008; Wollein et al., 2015), H_2O_2 (Dash et al., 2011; Jia Tao, n.d.; Menoutis et al., 2018; Rudovica et al., 2014; Simitchiev et al., 2008), HCL (Jia Tao, n.d.; Menoutis et al., 2018; da Silva et al., 2017; Wollein et al., 2015) and H_2O (Jia Tao, n.d.; Wollein et al., 2015).

The digestion maximum temperatures varied between 125ºC (Rudovica et al., 2014) and 550ºC (Lásztity et al., 2002) using several microwave heating programs to obtain the optimal digestion temperature.

Regarding the direct introduction of the sample in the form of a slurry it was only used in the case of the antibiotic amoxicillin and the sample was diluted with HNO_3 (Zachariadis and Michos, 2007). UV photolysis assisted digestion with HNO_3, H_2O_2 and H_2O was used in the case of Isosulfan Blue (Dash et al., 2011), simple digestion in the case of the unknown antibiotic and it was performed with a mixture of HNO_3, HCl and HF (Zachariadis and Sahanidou, 2011). It was reported a lyophilization too for the sample treatment of Heparin-Na (intravenous drug) (Heinemann and Vogt, 2000).

3.3. SAMPLE WEIGHT

The sample weight varies from 75 mg to 3 g depending on the pharmaceutical drug. For Isosulfan Blue the sample weight was 75 mg (Dash et al., 2011) while for Calcium Folinate, Levodopa, Arbidol and an unknown antibiotic the sample weight was 100 mg (Lásztity et al., 2002; Rudovica et al., 2014; Zachariadis and Sahanidou, 2011). In the case of Aspirin, Fingolimod, Lisinipril and Methotrexate the sample weight was 200 mg (Correale et al., 2014; Menoutis et al., 2018; Niemelä et al., 2004), 400 mg for Paracetamol, Ramipril, Naloxone, Enalapril, Diclofenac and Carbamazepine (Simitchiev et al., 2008; Wollein et al., 2015) and 500 mg for Metformin Hydrochloride samples (da Silva et al., 2017). Finally, 1g in the case of Levetiracetam samples (Dash et al., 2011) and for Simvastatin the sample weight was 3 g (Jia Tao, n.d.).

For Amoxicillin and Heparin-Na, the sample weight is not known as the literature do not provide them (Heinemann and Vogt, 2000; Zachariadis and Michos, 2007).

3.4. SAMPLE VOLUME

In all the cases, the sample volume varies from 10 mL to 100 mL which is a relatively low sample volume. For Isosulfan Blue and Fingolimod the sample volume was 10 mL (Correale et al., 2014; Dash et al., 2011) while for Paracetamol, Naloxone, Enalapril, Diclofenac, Carbamazepine and Methotrexate the volume used was 20 mL (Niemelä et al., 2004; Simitchiev et al., 2008; Wollein et al., 2015). In the case of Ramipril and Levetiracetam the sample volume used was 25 mL (Dash et al., 2011; Simitchiev et al., 2008), for Aspirin, Lisinipril, Simvastatin and Metformin Hydrochloride the sample volume used in literature was 50 mL (Jia Tao, n.d.; Menoutis et al., 2018; da Silva et al., 2017), while for Calcium Folinate, Levodopa and Arbidol the volume used was 100 mL (Lásztity et al., 2002; Rudovica et al., 2014).

Regarding the unknown antibiotic, Amoxicillin and Heparin-Na, the sample volume is not provided by the literature (Heinemann and Vogt, 2000; Zachariadis and Michos, 2007; Zachariadis and Sahanidou, 2011).

3.5. ANALYTICAL METHOD

The analytical methods found in the literature that reported elemental impurities in pharmaceutical formulations were both ICP-OES and ICP-MS for Paracetamol, Naloxone, Diclofenac, Carbamazepine, Metformin Hydrochloride and an unknown antibiotic (da Silva et al., 2017; Wollein et al., 2015; Zachariadis and Sahanidou, 2011). For Aspirin, Lisinipril, Calcium Folinate, Ramipril, Metformin Hydrochloride, Isosulfan Blue, Enalapril, Arbidol and Levodopa it was only reported the use of ICP-MS (Dash et al., 2011; Lásztity et al., 2002; Menoutis et al., 2018; Rudovica et al., 2014; da Silva et al., 2017; Simitchiev et al., 2008), while for Amoxicillin, Levetiracetam and Fingolimod was reported the exclusive use of ICP-OES (Correale et al., 2014; Dash et al., 2011; Zachariadis and Michos, 2007).

Other older techniques were used as was the case of Simvastatin that used AAS (Jia Tao, n.d.) and Heparin-Na (Intravenous Drug) and Methotrexate that used GF-AAS (Heinemann and Vogt, 2000; Niemelä et al., 2004).

3.6. QUANTIFIED PARAMETERS AND OBTAINED CONCENTRATIONS

In all cases, the parameters quantified were elemental impurities. These impurities were different depending on the pharmaceutical drug because as seen on **CHAPTER I** topic **3.PHARMACEUTICAL IMPURITIES**, not all of the 24-potential concerning elemental impurities considered have to be tested, as it depends on the route of administration, materials and reagents used in the several stages of the manufacturing stages.

The obtained concentrations described in the literature were in most cases below the detection limit and therefore are presented as n.d or with a sign (<) followed by the quantification limit of the elemental impurity. The element concentration obtained were expressed in µg/L in the case of Aspirin, Lisinipril and Heparin-Na (Heinemann and Vogt, 2000; Menoutis et al., 2018) and in µg/mL in the case of Simvastatin (Jia Tao, n.d.). For Paracetamol, Leveteracitam and Findolimod the concentrations were presented in ppm (Correale et al., 2014; Dash et al., 2011; Wollein et al., 2015) and for Metformin Hydrochloride in µg/kg (da Silva et al., 2017). In the case of Amoxicillin, Methotrexate, Calcium Folinate, Levodopa, Naloxone, Isosulfan Blue, Diclofenac, Carbamazepine, Arbidol and the unknown antibiotic the elemental concentrations were expressed in µg/g (Dash et al., 2011; Lásztity et al., 2002; Niemelä et al., 2004; Rudovica et al., 2014; Wollein

et al., 2015; Zachariadis and Michos, 2007; Zachariadis and Sahanidou, 2011). In addition, the results of Ramipril and Enalapril were presented in ng/g (Simitchiev et al., 2008).

4. HYPOTHESIS OF WORK AND OBJECTIVES

With the already said increasing tendency to consume generic pharmaceutical drugs instead of the reference medicine and according to the reported data on determination of elemental impurities on pharmaceutical drugs present on **Table 4**, the limited information in this area of study and the lack of literature comparing elemental impurities of generic drugs with their reference medicines using ICP are prominent. Therefore, the overall aim of this research is to develop and validate rapid and sensitive ICP methods for the determination of elemental impurities in generic pharmaceutical drugs and compare the results with their reference medicines. Specific objectives are:

- To successfully dissolve and treat pharmaceutical product samples based on the processes described in literature;
- To analyse and validate a methodology for pharmaceutical drugs by ICP-MS;
- To analyse, compare and evaluate major differences regarding elemental impurities both in generic and reference pharmaceutical drugs;

Additionally, a proposed sample treatment and digestion method based on the literature found will be presented.

CHAPTER II: METHODS AND MATERIALS

1. SOLUTIONS AND REAGENTS

It was used class 1 water for the material washing, and ultra-pure water for dilutions and preparation of all the standard solutions. The ultra-pure water was obtained using an ultra-purification Helix system coupled with a Mili-Q filter, assuring a resistivity of 18,2 MΩ.cm and a total organic carbon concentration of approximately 1 μg/L.

All the reagents used throughout the work had at least p.a. puriss. More in detail, it was used nitric acid with a puriss of at least 65% (v/v), hydrochloric acid fuming with 37% (v/v) and Perdrogen (hydrogen peroxide) with at least 30% (w/w) for the sample digestions and in the blank solution used for the instrument background.

For the daily optimization of the ICP-MS equipment sensibility, it was used a solution with a 10 mg/L concentration, named Tune A, containing a matrix that matches to the one present in the samples. This match minimizes the matrix effects and corrects the background. As internal standard it was used a solution of Indium 115 with a concentration of approximately 23 μg/L. The washing solution used between different sample reading is a solution of 1% (v/v) of nitric acid.

Given that there aren't certified reference materials for pharmaceutical drugs, the quantification limit verification standard solutions could not be prepared (PVLQ). Therefore, it were only prepared the calibration curve verification standard solutions (PVRC).

It is important to enhance that all the PVRC were prepared by dilution of a mother-solution with an element concentration of 10 mg/L. This primary solution was also prepared using a multielement commercial solution. All the solutions were prepared and stored in plastic flasks.

2. MATERIAL WASHING

In the determination of elemental impurities, it is extremely important to prevent the contamination of the analyte. As it is a technique with high sensibility, the smallest add or loss of analyte will influence immensely the results obtained. Therefore, all the material has to be properly washed to prevent contamination of the analyte with dust in the laboratory environment or material impurities.

In order to accomplish the best washing, before each use, all the equipment used was washed with a solution of 96% (v/v) of ethanol and dried. The microwave Teflon vessels had a specific washing treatment, they were washed with the laboratory detergent, tap water and dried. Then, the Teflon vessels were submitted to a microwave washing program, specific for the vessels in use, with a solution of 50% (v/v) of nitric acid. Lastly, all the Teflon vessels were washed 10 times with ultrapure water (mili-Q water) and dried with compressed air.

3. SAMPLE PREPARATION METHOD

Giving the lack of certified reference material (CRM) regarding pharmaceutical products, there were only analysed standard and pharmaceutical drugs samples during the work. All the pharmaceutical samples were prepared following the same method, being each pharmaceutical drug, grounded in a mortar to homogenize the sample and then, all the samples were weight and microwaved-assisted digested. In more detail, 6 pills of each pharmaceutical drug used were ground in a mortar and stored in a plastic test tube, then, weighted 200 mg of the pharmaceutical sample directly to a Teflon vessel. To minimize the static electricity that causes material dispersion, every Teflon vessel was coated with aluminium foil when weighing. It was then added to the Teflon vessel 1.5 mL of nitric acid, 0.5 mL of hydrochloric acid and 0.1 mL of hydrogen peroxide. This process was repeated for each pharmaceutical drug sample.

For the validation of the quantification method, the process just described was performed 9 times for the sample pharmaceutical drugs and then, in one of the 9 samples, 1.0 mL of an auxiliary standard added to the matrix. In an empty Teflon vessel, a blank solution was also prepared, adding just the (1.5 mL of nitric acid, 0.5 mL of hydrochloric acid and 0.1 mL of hydrogen peroxide).

Subsequently, all the vessels were properly closed, accommodated in a carousel, placed in the CEM - MDS-81D microwave oven equipped with 10 Teflon vessels and undergone a microwave program with a ramp of 10 minutes to 170 ºC and holding 10 minutes at 170 ºC. Ended the program, the vessels were let to cold, reach the room temperature and then opened. The samples were transferred to a properly identified plastic test tube, diluted to the mark (25 mL) with ultrapure water and weighed.

It were prepared seven standard solutions to perform the calibration curves with concentrations of 0; 0,1; 0,5; 2,0; 10; 50; 100 µg/L. In order to prepare these standard solutions, it was prepared a mother solution with elements concentration of 10 mg/L. Aliquots of 0; 0,5; 2,5; 10; 50; 250 and 500 µL were pipetted form the mother solution to plastic volumetric flasks of 50 mL. The volumetric flasks were properly identified, and the volumes diluted to the mark with a solution of 1% of HNO_3.

4. TECHNIQUES FOR THE DETERMINATION OF ELEMENTAL IMPURITIES IN PHARMACEUTICAL DRUGS

In literature, several analytical methods are reported for the determination of trace and ultra-trace elements in pharmaceutical drugs. Typically, these methods require sample preparation, which are normally wet-acid digestion of samples or introduced in the form of a slurry in the equipment although there are several others sample preparation techniques (Zachariadis and Sahanidou, 2011) as mentioned before.

The chapters of the European Pharmacopoeia (2.4.8), for the determination of trace and ultra-trace elements, describes as limit tests, the precipitation of the element of interest as sulphide, followed by visual examination comparing with lead standard solutions. Never-the-less, this practice seems no longer be adequate to the actual demanding requirements of the pharmaceutical regulation (da Silva et al., 2017). In addition, the main disadvantages of these techniques are the non-specific and non-sensitive determination of the analytes and also the time-consuming digestion or ignition procedures and therefore there is a rising tendency to replace the simple trace elements test for modern instrumental techniques (Wang et al., 2003; Zachariadis and Kapsimali, 2006).

Atomic absorption spectrometry is typically applied for single element analysis (Kelkó-Lévai et al., 1999; Van Staden and Hattingh, 2000; Wang et al., 2003; Zachariadis and Kapsimali, 2006), as well as atomic emission spectrometry (Wang et al., 2003, 1999) although the last one requires digestion of the pharmaceutical drug (Zachariadis and Michos, 2007). There are also different spectrometric and spectroscopic techniques like graphite furnace atomic absorption spectrometry (GF-AAS) or flame atomic absorption spectroscopy (F-AAS). Nonetheless, all of these techniques have a major disadvantage as they only analyze one element at the time.

In other hand, inductively coupled plasma atomic emission spectrometry (ICP-AES) (Nölte, 2003a; Thomas, 2004), and mass spectrometry (ICP-MS) (Amaral et al., 2015; Lewen, n.d.; Volker Thomsen, Debbie Schatzlein, 2003) have also been reported for the analysis of pharmaceutical drugs having the advantage of presenting multielement analysis and allowing the quantification of much lower concentration values even for ultra-trace elemental impurities. This fact is of major importance since the pharmaceutical regulatory framework forces to quantify impurities present in bulk preparations and in finished drug products and, ensure that the concentration of the analysed elements are below the permitted daily exposure. However, most of the times these elements are in trace or ultra-trace amounts (da Silva et al., 2017).

Apart from the inductively coupled plasma with mass spectrometry technique that is explored in more detail in the topic **6.1.1** below, all the other techniques (inductively coupled plasma atomic emission spectrometry, flame atomic absorption spectroscopy and graphite furnace atomic absorption spectrometry) are approach in more detail in **ANNEX I**.

4.1. INDUCTIVELY COUPLED PLASMA

About the same time that flame-based instruments like flame atomic emission or absorption spectroscopy became outstanding, S.Greenfield and associates developed, in the mid-1960s, the first plasma-based instruments using direct current or microwave-induced systems. Non the less, due to the interference effects and plasma instability problems associated with the plasma-based instruments, flame-based spectrometry instruments dominated the analytical market for metals analysis (Greenfield et al., 1964).

Years later, the problems associated with plasma-based spectrometry were overcome by using instead, an inductively coupled plasma (ICP) system. The possibility of sequential multielement analysis coupled with high sensitivity and shorter analysis time made this technique quite popular in the 1980s (Greenfield et al., 1964).

In an ICP system, there are three gases flowing into a torch (**Figure 3**) that is comprised of three concentric tubes typically made of quartz. An ICP gas flows in the outer tube, this gas is also known as cool gas, it flows in the range of 13–17 L/min and his primary role is to shape the plasma and ensure that the high temperature of the plasma does not melt the torch. The second gas flows into the central tube and is usually named as plasma gas, it flows in the range of 1 L/min and is the one that in fact is ionised and form the plasma. The last one is called carrier gas, it flows in the innermost ring and is responsible for carrying the sample into the plasma.

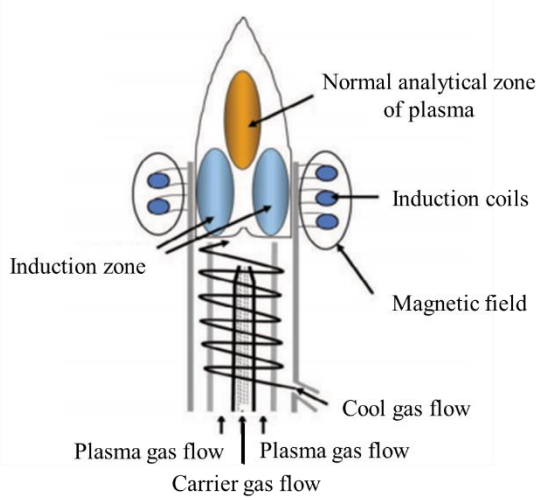

Figure 3: *Scheme of an ICP torch and respective components.*

The end of the torch is placed inside an induction coil (as seen in **Figure 3**) and supplied with a radio-frequency electric current that flows through the coil to create an electromagnetic field in which the plasma gas (usually argon) flows through. A high voltage discharge (electric spark) is applied to introduce free electrons into the gas stream. When these electrons pass through the electromagnetic field applied, they gain energy and they are accelerated. When they collide with the plasma gas, they transfer their energy and ionise the gas. This creates a set of ion-electron pairs that are, in turn, energized in the presence of the electromagnetic field creating a cascading effect as schematized in **Figure 4** that outcomes in the formation of a plasma that is maintained as long as the radio-frequency current is at a sufficient intensity and the gas flow are preserved (Abou-Shakra, 2003).

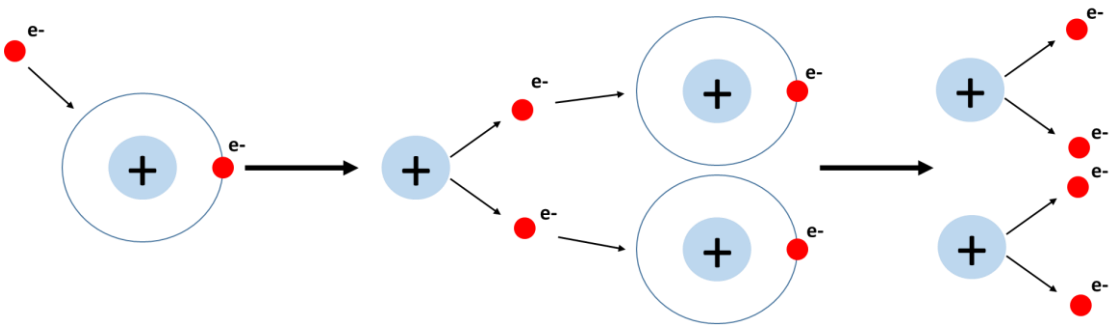

Figure 4: *Schematic representation of the cascading effect that originates and sustains the plasma.*

Argon plasma is characterized by a bluish-white emission colour due to the combination of the argon atomic spectrum and the continuous spectrum of ion-electron recombination's that are taking place in the plasma. As the plasma also produces ultraviolet light, it is not suitable to look at it directly.

The combined cooling systems and the central tube for the carrier gas provide plasma with a shape that allows the sample to be introduced without disturbing considerably the plasma or changing its composition (Abou-Shakra, 2003). It also affords a maximum temperature of approximately 10 000 K in the hottest region and an analytical zone around 6 000 to 7 000 K in the cone-shaped region outside the quartz torch as seen in **Figure 5** (Thomas, 2001).

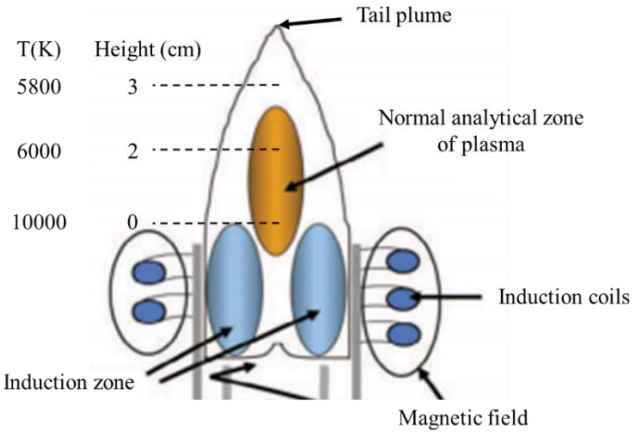

Figure 5: *ICP plasma representation and respective temperatures zones.*

Nowadays, the ICP is commonly coupled with an atomic emission spectrometer (OES) or a mass spectrometer (MS). Given the nature of the analysed samples and the concentration of the elements present in them, throughout this work, it is only going to be used an ICP coupled with a mass spectrometer.

ICP offers the advantages of a low sample size requirement, screening capability, element-specific information, quantitation and rapid sample throughput (Lewen et al., 2004). Both ICP-OES and ICP-MS also present a multielement capability which is a major feature regarding elemental impurities in pharmaceutical products. The low detection limits are typically in μg/L range for ICP-OES and up to ng/L range for ICP-MS (Nölte, 2003a; The United States Pharmacopeial Convention, 2013; Thomas, 2004).

Although ICP is a really useful technique in the multielement analysis, the use of organic solvents (Montaser, 1998) or high amount of total dissolved solids (TDS) can put in jeopardy the ICP analysis and therefore, compromises have to be made so that a suitable analytical technique could be chosen (Niemelä et al., 2004).

4.1.1. INDUCTIVELY COUPLED PLASMA – MASS SPECTROMETRY

If ICP-OES has become one of the election techniques, ICP-MS is not far behind. In the past three decades, since the first unit sale back in 1983, it has become the most accurate choice in the detection and quantification of many trace and ultra-trace elements.

This system, as shown in **Figure 6**, consists of an ion source coupled with a plasma sampling interface and a mass spectrometer. The ions produced by the plasma torch are extracted from the atmospheric pressure through an often-made Ni or Cu water-cooled sampling cone with a 0.5 - 1.0 mm orifice that is placed in the plasma, to a second more sharply angled cone. This second cone is called skimmer and in there, the pressure is lowered to 1 Torr through a rotary pump.

Then, the ions pass to the ion lenses that are usually cylinders held at appropriate voltages that has the mission of focus ions into the mass spectrometer, which is usually a quadrupole system operating at 10^{-5} to 10^{-6} Torr. There, the ions are separated based on their mass-to-charge ratio and then, a detector receives the ion signal that are proportional to their concentration (Olesik, 1991).

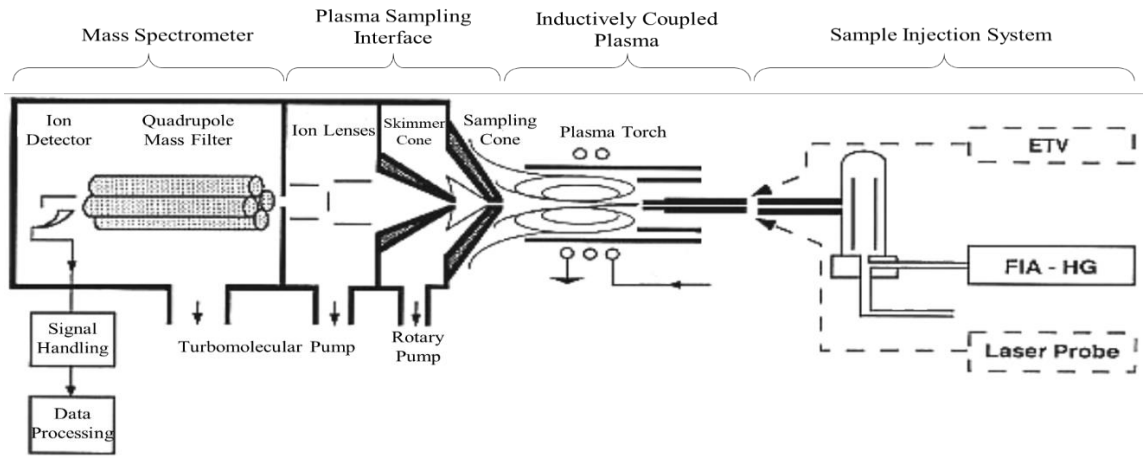

Figure 6: *Typical quadrupole ICP-MS system with various sample introduction options: electrothermal vaporization (ETV), laser probe or ablation (LA), and flow injection analysis-hydride generation (FIA-HG).*

Then, the sample concentration is determined through calibration with reference material that is certified. This certified material can be single or multi-element reference standards depending on what is analysed.

There are other types of mass spectrometers like high-resolution ICP-MS nonetheless when speaking of trace elements analysis, quadrupole ICP-MS is preferred as it is very reliable and present the possibility of an unattended and overnight operation (Sneddon and Vincent, 2008).

As reported, this technique has been increasingly used as a substitute for the out-dated limit test of trace and ultra-trace elements prescribed by the United States, European and British Pharmacopoeia for pharmaceutical drug substances, intermediates, and raw materials. An example is an article where is reported a multi-element inductively coupled plasma-mass spectrometry survey method as an alternative for the old test where the technique allows the results to be presented until the lows µg/g (Wang et al., 2000).

4.1.1.1. ADVANTAGES OF ICP-MS

ICP-MS offers analysis speed as it can scan a mass spectrum in just a few seconds, depending on exact instrument settings. This technique is also able to move from mass to mass with high precision degree allowing the *peak hopping* analysis technique. *Peak hopping* consists in obtaining only one single point of data at the top of the peak of each element during the analysis.

Additionally, ICP-MS has high sensibility archiving to detect trace quantity levels, well below a part per billion (ppb) or ng/g. These detection limits are up to 3 orders of magnitude superior to those archived with an ICP-OES device, much due to the fact that there is no fundamental source of continuum background in ICP-MS.

ICP-MS quadrupole systems may present a high background noise caused by the ion optics of the quadrupole mass analyser, making it susceptible to background noise on the detector. Nevertheless, high-resolution ICP-MS possess a design that prevents any stray photon from reach the detector and has extremely high ion transport efficiency resulting in extremely low backgrounds (less than 0.2 counts per second). It also has the potential to minimize or even eliminate the molecular argon inconvenient using variable resolution.

In the case of metals detection, their characteristics provide a very low detection limit of <1ppq or less than 1 fg/g and by variating the resolution of the system it is even possible to analyse each metal in such a resolution that eliminates any interferences without over solving it. For instance, the element ^{56}Fe has it most common interference $^{40}Ar^{16}O$ due to the fact that this molecule has a very big peak that tails into the Fe peak, but with a 2500 resolution this interference is reduced or eliminated.

4.1.1.2. DISADVANTAGES OF ICP-MS

If ICP-MS has great advantages, it also presents a couple of not so good qualities. Concerning the analysis of elements with ICP-MS quadrupole systems, it has the inability of resolving some target isotopes from molecular interferences as the instruments are only able to resolve a mass spectrum in one-unit resolution. Taking the last example, an ICP-MS quadrupole although it can differentiate ^{56}Fe and ^{57}Fe, it would not be able to differentiate ^{56}Fe (mass 55.9349) from the ^{40}Ar ^{16}O molecular species (mass 55.9573), which is very easily formed in an argon plasma.

This disadvantage can be solved using the technique called “cold plasma” but it would compromise the sensitivity. “Cold plasma” consists in lowering the radiofrequency power to low the plasma temperature. This way, the number of ionized argon particles is diminished, and the interference reduced (Sneddon and Vincent, 2008).

The background noise is also an issue as it can be produced by ions that pass around the quadrupole but still reach the detector (stray ions) or ions that are precisely on axis in the quadrupole and therefore not mass selected. Photons can also reach the detector and produce a signal. Elements with ionization potentials lower than 9 eV exist mainly as singly charged ions in the plasma, yet as there is a poor transmission of ions from the atmospheric pressure plasma to the low-pressure spectrometer, the number of ions reaching the detector is limited. For instance, a solution of Mn with 10 ppm concentration, in plasma produces around 1.8 X 10^{13} $Mn^{+,}$ but only 1 in 6 X 10^{7} ions or less are detected, leading to a count rate of about 3 X 10^{5} Hz (Golightly and Montaser, 1992). This background noise results in degraded or reduces detection limits as it is directly proportional to variations in the background noise.

Regarding the high-resolution ICP-MS analysers, as it uses an electromagnet to separate the masses, it has to change the magnetic field strength for each mass which takes significantly more time than changing the electrical field as it’s the case of quadrupole ICP-MS, resulting in a relatively slow analysis speed (Olesik, 1991).

Another disadvantage of the high resolution ICP-MS analysers is the mass drift. Due to the fact that at high resolutions the peaks are narrow and it turns much more difficult for the magnet to move exactly to the same mass every time a metal is determined, it is necessary to scanning across the entire peak which is more time-consuming than the “peak hopping” use in the quadrupole system (Sneddon and Vincent, 2008).

4.1.2. ICP-OES VERSUS ICP-MS

Comparing the two inductively coupled plasma techniques, it can be said that both techniques are used for similar goals, i.e., for the determination and quantification of trace and ultra-trace elements in several solutions including pharmaceutical drugs. The choice between this two is based on a commitment between the one that best fits the needs of the analysts and the costs that the technique involves. In **Table 5** is shown a comparison of the main aspects of each technique (Georgiou and Danezis, 2015).

Table 5: *Comparison between the analytical techniques ICP-MS and ICP-OES.*

ICP-OES	ICP-MS
ICP is used as the excitation source	ICP is used as an ionization source
Detection limits of sub-µg/mL (µg/g)	Detection limits of sub-µg/L (ng/g)
Uses peak intensity at a wavelength for quantitative determination	Uses m/z values at wavelengths for quantitative determination
Initial cost moderate, $75 + K for basic system	Initial cost expensive, $150 + K for basic system
Relatively straightforward to operate and moderate routine maintenance	More complex to operate and a high degree of maintenance required
Sequential and simultaneous capability	Sequential and simultaneous capability
Capable of direct solid sampling	Capable of direct solid sampling
Spectral interferences well documented and often avoidable	Isobaric interferences well documented and often avoidable
Chemical interferences well understood	Chemical interferences well understood

4.2. ICP VERSUS OTHER TECHNIQUES

To facilitate the comparison between the several techniques described above on topic **6.1.1** and in the **ANNEX I**, a summary table of the main characteristics of the techniques is presented next (**Table 6**).

Table 6: *Comparison of the four more used analytical techniques in pharmaceutical drugs impurities quantifications.*

Technique Characteristic	F-AAS	GF-AAS	ICP-OES	ICP-MS
Detection Limits	Very good for some elements	Excellent for some elements	Very good for most elements	Excellent for most elements
Sample throughput	10 to 15 s per element	3 to 4 min per element	<3 min per 60 elements	<3 min per 73 elements
Dynamic Range	10^3	10^2	10^6	10^8

Semiquantitative analysis	No	No	Yes	Yes
Isotopic Analysis	No	No	No	Yes
Ease of use	Very Easy	Moderate	Easy	Moderate
Method Development	Easy	Moderate	Moderately Easy	Moderate
Unattended Operation	No	Yes	Yes	Yes
Sample Volumes Required	Large	Very small	Medium	Very small to medium
Interferences				
Spectral	Few	Few	Many	Few
Chemical Matrix	Many	Many	Very Few	Some
Physical Matrix	Some	Very Few	Very Few	Some
Dissolved solids	0.5-5%	>20%	0-20%	0.1-0.4%
Costs				
Capital Costs	Low	Medium	High	Very High
Running Costs	Low	Medium	High	High
Cost per Analysis				
Few elements/high throughput	Low	High	Medium	Medium
Many elements/high throughput	Medium	High	Low to Medium	Low to Medium

Offering detection limits in the range of the subparts per trillion (10^8), ICP-MS presents the lowest detection limits of all the techniques, making it a very attractive system when compared to other systems like F-AAS, GF-AAS or even ICP-OES.

ICP-MS also presents clear advantages concerning it analysis speed, isotopic capability, multielement analysis ability characteristic and high versatility as it can be used with several hybrid techniques or coupled with other techniques like liquid chromatography (LC-ICP-MS) (Rao and M. V. N., 2007).

Comparing now the general costs of the equipment's it is here that it is found the great disadvantages of the ICP-mass spectrometry. Comparing to all the other technique presented in the table above, the costs for purchase and running the instrument at consumables level (argon, super pure acids…), ICP-MS is the less attractive one as presents the highest costs. In addition, it is necessary highly trained and formed employees to carry the analytical analysis.

Finally, when analysing complex matrices or samples, ICP-MS can generate great amounts of data that is very time-consuming to analyse and process as, although there are several softwares written to serve the purpose, it is vital a visual inspection of the analytical results by an experienced specialist (Cubadda, 2007; Hou and Jones, 2000; Thomas, 2004).

5. ELEMENTAL IMPURITIES QUANTIFICATION

Previous to any analysis on ICP-MS, it is always performed an optimization and verification of the proper functioning of the equipment. In the case of the inductively coupled plasma with mass spectrometry, the calibration is made daily before the analysis through *auto-tune*. The *auto-tune* choses the ideal conditions for the day, maximizing the internal standard signal (in this case the isotope ^{115}In) and screening the reasons $^{140}Ce^{16}O/^{140}Ce$ and $^{69}Ga/^{138}Ba$ in order to minimize the formation of oxides and double charge species. Only then the isotopes of the to be analysed elements and the possible interferents are chosen and the analytical calibration initiated. The analytical calibration is performed through the analysis of the blanks and standard solutions referred on topic **3** of **CHAPTER II** and the calibration function defined with the blanks and standard solutions that presented less than 10% of relative error. After the calibration, the analysis is carried on, interpolating the sample analysis the analysis of the washing solution (1% (v/v) of nitric acid) to ensure the validity of the results.

6. EQUIPMENT USED

For the determination of elemental impurities, it was used an inductively coupled plasma with a mass spectrometer *Thermo X Series (Thermo Scientific) ICP-MS* equipped with a 3-channel peristaltic pump, a *Burgener* nebulizer and nickel cones. In the determination of each elemental impurity was used a different isotope with a specific atomic mass unit as described in **Table 7.**

***Table 7**: Atomic mass units set for the determination of each element.*

Element	Atomic mass units
Cr	52
Cu	65
Mn	55

CHAPTER III: RESULTS AND DISCUSSION

1. VALIDATION OF QUANTIFICATION METHODS FOR PHARMACEUTICAL DRUGS

When dealing with quantification of compounds of interest through analytical methods, as it is the case of the quantification of trace and ultra-trace elements in pharmaceutical drugs, there is a need to evaluate both the quality of the instruments used and the quality of the results obtained. This evaluation is achieved by performing the validation of the testing method by running several tests of well establish parameters. The results obtained in this validation must correspond to the quality control established by the laboratory (RELACRE, 2000).

In order to produce valid results, there is a need to follow some rules as the use of methods and equipment which have been tested to ensure that they are suitable for the analytical measurements. These measurements should always be carried out by qualified and competent staff for the proper execution of the method and the quality control and quality assurance of the procedures must be always well-defined. An independent evaluation of the laboratory performance should be periodically executed and the analytical measurements results carried out in the evaluated laboratory must be consistent with those carried out in other laboratories (eurachem, 2014).

1.1. METHOD PERFORMANCE PARAMETERS

To ensure the laboratory quality, performance parameters to evaluate have to be defined in order to acquire an adequate level of confidence in the results. It is also important to highlight that there has to be a balance between technical possibilities, costs and risks, as declared for the NP ISO / IEC 17025 (IPQ, 2005).

Therefore, the method performance parameters that were studied in LCA (Laboratório Central de Análises) are presented next.

1.1.1. SPECIFICITY AND SELECTIVITY

The terms specificity and selectivity are often used misguidedly and therefore it is important to differentiate them. In one hand, specificity refers to a method that produces a single response to a single analyte. On the other hand, selectivity refers to a method that provides several responses to various analytes that can be differentiated from each other or not. In other words, a method can be considered selective if the response to the analyte can be distinguished from the responses of the other constituents of the sample.

The specificity and selectivity of the method are evaluated through an interference study where the interferences can be either isobaric (additives) or matrix (multiplicative). Isobaric interferences are evaluated through possible elementary or molecular interferents study, while matrix interferences are evaluated through recovery tests (NATA, 2013).

1.1.1.1. MATRIX INTERFERENCES

Recovery tests were performed in order to determine this parameter. These tests consisted of the addition of analyte volumes with well-known concentrations to the samples that are going to be analysed by ICP-MS. The **Equation 1** presented next is then applied to the results and the recovery % calculated:

$$Recovery\ (\%) = \frac{[(C_f \times V_f) - (C_a \times V_a)]}{C_{adic} \times V_{aded}} \times\ 100$$

Equation 1

Where:

C_f – Analyte concentration in the fortified sample;

C_a – Analyte concentration in the sample;

C_{adic} – Analyte concentration in the fortification solution;

V_f – Volume of the fortified sample;

V_a – Sample volume;

V_{aded} - Volume added of fortification solution.

1.1.1.2. ISOBARIC INTERFERENCES

When analysing samples by ICP-MS, this parameter is evaluated by considering the respective interferences of the isotopes that are going to be analysed as described in ISO 17294. A mono-elementary standard solution containing the possible interferent is analysed and it is verified if the intensity of the interferent in the analyte signal is higher or lower than the detection limit. In other words, it is verified if the interference is significant, and if so, the interference factor (f), a function that relates the analyte and interference signals is calculated and applied. It is considered to have been successfully applied when it is found that the analyte concentration in the mono-elementary standard solution containing the interferent is lower than the detection limit (ISO, 2004, 2003).

1.1.2. LIMIT OF DETECTION AND QUANTIFICATION

There are different forms to perform the calculation of the detection and quantification limits that are recommended by the International Union of Pure and Applied Chemistry. One is the experimental determination in which the mean and standard deviation of *n* test readings performed on the blank sample is calculated. In this case, the analysis shall be performed under intermediate precision conditions, which shall be independent, and the following **Equation 2** and **3** should be applied for the calculation of the limits of detection (LOD) and quantification (LOQ) (respectively):

$$LOD = 3s_0$$

Equation 2

$$LOQ = 10s_0$$

Equation 3

Where,

s_0- Standard deviation associated with the *n* blank solution readings values.

Another way is to admit that the quantification limit value corresponds to the value of the lowest standard solution concentration of the calibration curve. In this case, the value of the detection limit should be one-third of the quantification limit value.

When defining both the limit of quantification and the limit of detection, it should be paid attention to the type of sample and the equipment capacity as, although for most equipment's the coefficient of variation and the average relative error associated with the limit of quantification are in the order of 10%, these values may vary and be higher. This

is so important because the uncertainties associated with the quantifications must be consistent with the coefficient of variation and relative error mean values (eurachem, 2014).

1.1.3. WORKING RANGE

Working range is defined as the analyte concentrations range in which the analytical method can be applied for analysis. Within the working range, the method is able to return values with an uncertainty value in accordance with the quality criteria accepted and estimated by the laboratory responsible for carrying out the analyses. This range usually corresponds to the linear zone of the calibration function and the upper and lower limits are usually defined by the concentration at which the equipment ceases to give a linear response (saturation) and by the quantification limit, respectively (eurachem, 2014).

1.1.3.1. CALIBRATION CURVE

For the proper analyte quantification, it is imperative the construction of calibration curves. This calibration curves are composed by, at least, 5 standard solutions throughout the working range. It must be calculated, a total of 5 independent calibration curves in 3 independent days. It is only considered for the construction of the calibration curve, the standard solutions that present a standard deviation below the maximum limit stablished by the laboratory quality control.

1.1.3.2. SENSITIVITY

Sensitivity is defined as the ability to generate variation in the value of the studied property by the analytical method used. This variation of the response signal in function of the analyte variation, in practice, translates into the slope of the calibration curve. If the calibration curve is linear, the sensitivity will be constant and independent of the concentration of the analyte, if the calibration curve is not linear, then the sensitivity will be dependent of the analyte concentration and therefore not constant. The method sensitivity is higher, the higher the slope of the calibration curve, which means that with the same variation of analyte concentration a bigger response (signal) variation is obtained (Skoog et al., 2014).

1.1.3.3. CORRELATION COEFFICIENT

The correlation coefficient measures the correlation degree between two variables x and y. The correlation coefficient should present a value higher than 0.995 accordingly to the LCA (Laboratório Central de Análises) quality control parameters established.

1.1.3.4. LINEARITY

The ability of an analytical method to demonstrate that the results obtained are directly proportional to the analyte concentration is referred to as linearity. This criterion can be evaluated using the Mandel test, applying the **Equation 4**:

$$F_{test} = \frac{(N-2) \times s_{e,2}^2 - (N-3) \times s_{e,3}^2}{s_{e,2}^2}$$

Equation 4

Where,

N – Number of standards used in calibration;

$s_{e,x}$ - Residual standard deviation associated with the linear calibration function and the quadratic calibration function.

The value of the F_{test} is compared with the value of the Fisher distribution (*F*) for a 95% confidence level and if the value of F_{test} is lower than *F*, then the best calibration function is the linear as the quadratic calibration function does not lead to a significantly better adjustment.

This test evaluates whether the variances associated with a linear function and a linear quadratic function are significantly different or not. Linearity deviations may occur in specific cases such as the lack of linearity of the detector response by saturation or due to chemical effects derived from the sample matrix. To avoid affecting the quality of the results, the upper working range limit value is defined as the point from which the slope of the calibration function deviates from the linear behaviour, and therefore it is advisable not to work above it (Skoog et al., 2014).

1.1.4. TRUENESS, PRECISION AND UNCERTAINTY

In quality control is important to ensure the agreement degree between the measured value and the true value of an analyte (IPQ, 2012). This parameter is evaluated by trueness and precision which are by their turn, related to total error that are likely to occur during an analytical assessment, namely systematic and random errors, as best exemplified in **Figure 7** (eurachem, 2014).

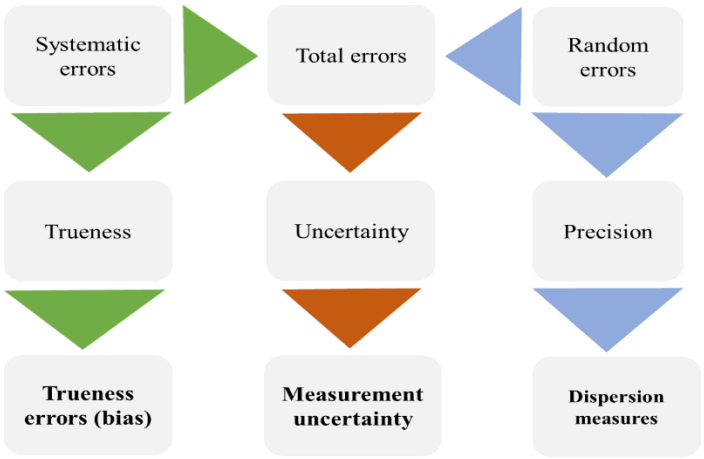

Figure 7: *Relationship between types of errors, performance characteristics and their quantitative expression.*

1.1.4.1. <u>TRUENESS</u>

The approximation between the mean of a number of values repeatedly measured and the reference value is defined as trueness. This parameter is usually evaluated by calculating the relative error or in terms of bias. However, given the nature of the analyte to be analysed in this work and has said before, there is no certified reference material to use in this parameter's calculation, and therefore, it is only possible to apply the process of standard addition (recovery tests). For the standard addition method, it is necessary to add an aliquot of a multielement solution to the samples. The multielement solution is of well know concentration and contains all the elements that we want to analyse.

This parameter is estimated by calculating the standard uncertainty associated with the trueness using the values of the recovery tests performed and applying the **Equations 5** and **6** (RELACRE, 2018).

$$\bar{R} = \frac{\overline{C_{obs}}}{C_{fortif}}$$

Equation 5

$$\mu_{ver}(\bar{R}) = \bar{R}\sqrt{\left(\frac{s_{obs}^2}{n \times \overline{c_{obs}}^2}\right) + \left(\frac{\mu(c_{fortif})}{c_{fortif}}\right)^2}$$

Equation 6

Where,

$\overline{C_{obs}}$ - estimated mean of the concentration of n tests of fortified samples;

C_{fortif} – Element concentration in the fortification sample added;

s_{obs}- Standard deviation of the series of n test results of fortified samples;

n - Number of fortified sample tests;

$\mu(c_{fortif})$ - standard uncertainty associated with the element concentration in the fortification sample.

1.1.4.2. PRECISION

Precision is defined as the level of agreement, under specific conditions, between values obtained by repeating measurements between similar samples or the same sample. It is evaluated through studies that use several replicates of different samples or patterns that fit within the working range. These studies are usually measurement reproducibility, repeatability and intermediate precision (eurachem, 2014).

1.1.4.2.1. REPLICATION

This parameter is analysed using duplicate analysis. Control charts are also shown for the relative difference between duplicate readings whether they are from the same sample or not. The following expression (**Equation 7**) is used to calculate the relative difference between duplicate analyses:

$$Relative\ difference\ (\%) = \frac{C_2 - C_1}{\bar{C}} \times 100$$

Equation 7

Where,

$C_2\ and\ C_1$- Concentrations obtained for the sample reading and the duplicate, respectively;

$\bar{C}$- Mean of the duplicate readings concentration.

This parameter allows evaluating drifts that may occur during the analyte quantification process as the sample is analysed at the beginning of the analysis and the duplicate at the end of the run.

1.1.4.2.2. RELATIVE STANDARD DEVIATION

The sample variation coefficient is obtained by calculating the relative standard deviation of three reading for the same sample using **Equation 8**. This coefficient also permits the determination of the concentration from which the desired analyte can be quantified since it tends to increase as the analysed samples concentrations are closer to the quantification limit.

$$RSD = \frac{s}{\bar{x}}$$

Equation 8

Where,

s - Standard deviation associated with the three reading for the same sample;

$\bar{x}$ – Mean of the three readings of the same sample.

1.1.4.2.3. REPEATABILITY

Repeatability corresponds to the dispersion of the results obtained for the same sample or standard, under the same conditions and expresses the lowest variation associated with the results. The repeatability limit allows us to know whether, under repeatability conditions, the difference between duplicates of a sample is significant or not.

For the evaluation of this parameter, the sample are prepared and analysed in-between of other samples in order to evaluate the variability of the digestion and preparation of the samples and the variability associated with the quantification process.

The estimated repeatability variation is determined using **Equation 9**:

$$s_{ri}^2 = \left[\frac{\sum(x_i - \bar{x})}{n-1}\right]^2$$

Equation 9

Where,

x_i - Individual values considered;

$\bar{x}$ – Mean of the individual values considered;

n - number of readings performed for each sample.

Then, for a 95% confidence level, the limit of repeatability (r) is calculated following the **Equation 10** and the variation coefficient (*VCr*) determined, in percentage, for each sample or standard following the **Equation 11**:

$$r = 2.8 \times \sqrt{s_{ri}^2} \qquad CV_r = \frac{s_{ri}^2}{\bar{x}} \times 100$$

Equation 10 *Equation 11*

1.1.4.2.4. INTERMEDIATE PRECISION

Intermediate precision quantifies the variation associated with the results of the same sample or standard evaluated in the same laboratory by the same method and operator but on independent days. Intermediate precision (S_i) can be determined by combining the within-group variance (associated with repeatability) and the variation between groups obtained using the **Equation 12**. Within-group variance can be obtained directly from an ANOVA table corresponding to the sum of the squares mean within the group (MS_w) (eurachem, 2014) and the variation between groups with **Equation 13**.

$$S_i = \sqrt{MS_w + S_{between}^2} \qquad S_{between} = \sqrt{\frac{MS_b + MS_w}{n}}$$

Equation 12 *Equation 13*

Where,

$S_{between}$- Standard deviation associated with the between-group variation;

MS_b- Sum of the between-groups squares mean;

MS_w - Sum of the within-group squares mean;

n – Number of sample readings.

1.1.4.3. <u>UNCERTAINTY</u>

The uncertainty can be defined as a non-negative measure that characterizes the dispersion of the values obtained from the analyte analysis (IPQ, 2012). In order to calculate the estimated uncertainty, the data obtained in the validation process are used among the **Equation 14**.

$$\mu_{R_w} = \sqrt{\mu_{R_w,stand}^2 + \mu_{r,range}^2}$$

Equation 14

Where,

$\mu_{R_w,stand}$- Uncertainty component of the results from the standard solution;

$\mu_{r,range}$- Uncertainty component from the range control chart.

It is important to highlight that for the estimation of both uncertainty contributions it is needed a minimum of eight measurements.

1.2. VALIDATION OF THE Mn QUANTIFICATION METHOD FOR PHARMACEUTICAL DRUGS

For the validation of the quantification methods process, it was used two different samples. For Brufen sample, it was validated the quantification methods of Cr and Mn while for Maalox sample, it was validated the quantification methods of Cr, Mn and Cu. In all the cases the elements quantification was performed using an inductively coupled plasma - mass spectrometry. For that, it was followed the DQ.PSQ.019 lab internal methods validation procedure.

Given that all the methods validated undergone an identical analysis and treatment process, it is only going to be shown the validation process of the Mn quantification for Brufen and Maalox as an example. All the others validation methods processes are shown in more detail in **ANNEX II.**

1.2.1. SPECIFICITY AND SELECTIVITY

Both specificity and selectivity were evaluated through interference studies.

1.2.1.1. RECOVERY TESTS - MATRIX INTERFERENCES EVALUATION

For each pharmaceutical drug there were performed on 3 independent days, 9 recovery tests each day. In each day, the same amount of analyte was added to the samples to ensure fortified one. The control charts of the Mn recovery test percentages in Brufen and Maalox matrices are shown in **Figure 8** and **Figure 9,** respectively. Both charts presented several sample recovery % that can be from the same day of analysis or not. Thus, some tendencies may seem to occur, however, it does not mean that the process is out of control (RELACRE, 1996).

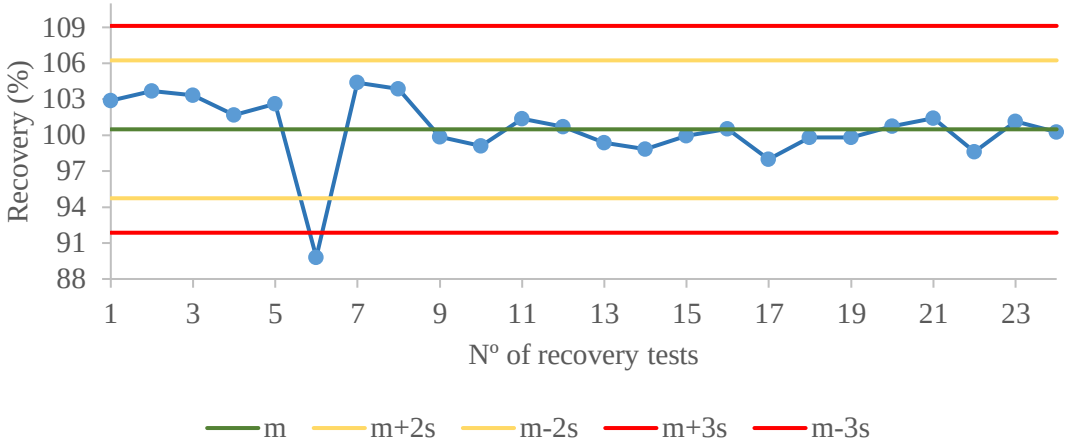

Figure 8: *Control chart of Mn recovery tests in Brufen samples.*

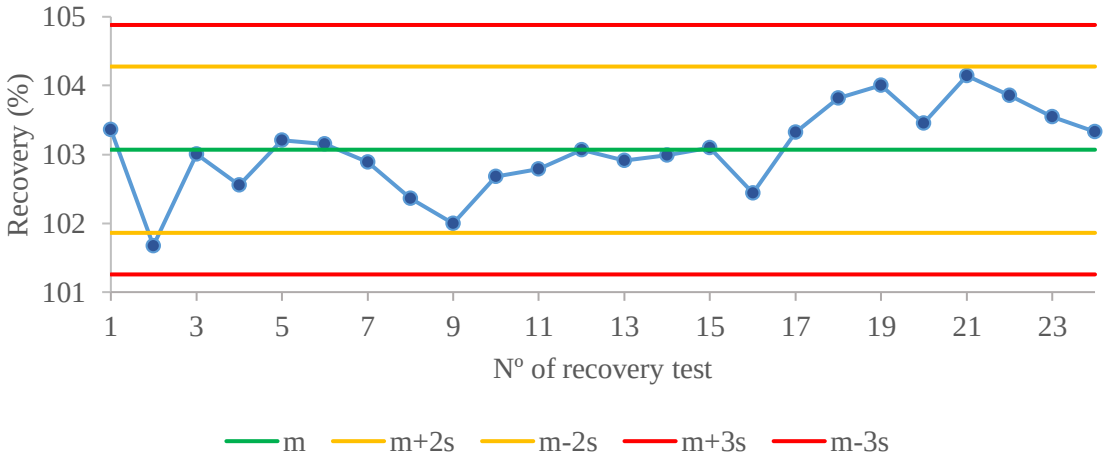

Figure 9: *Control chart of Mn recovery tests in Maalox samples.*

Figure 8 and **Figure 9** allow to verify that medium recovery percentage of Mn for Brufen samples is 100% while for Maalox is 103%. In both cases, there were made 24 recovery tests and in all of the Brufen samples, the recovery percentages obtain were between 98% and 104% with the exception of the 6th recovery test that presents a recovery percentage of 90%. In Maalox samples, the recovery percentages verified were between 102 and 104%. Since that the acceptance criteria range for recovery tests establishes by the laboratory (LCA) is between 80% and 120%, it can be said that all the recovery tests obtained for both pharmaceutical drugs are within the quality limits required.

The control charts referring to the validation process of all the other elements (Cr for Brufen and Cr and Cu for Maalox) are presented in **ANNEX II**. They present similar results to that obtained above for Mn, being that all the recovery percentages obtained are within the acceptance criteria range defined by the laboratory. The recovery percentages for Brufen samples are between 98% and 110% for Cr. For Maalox samples were obtained recovery percentages between 108 and 113% for Cr (with the exception of the 8th recovery test that presents a recovery percentage of 102%) and within 90% and 93% for Cu.

As already said, the 6th test of Mn recovery tests in Brufen presents a recovery percentage of 90% and the 8th recovery test of Cr in Maalox presents a recovery percentage of 102%, which are, in both cases, within the quality standards defined by the laboratory. Nonetheless, by further analysis of the respective control charts, it can be notice that the recovery tests mentioned are out of control. Therefore, the 6th and 8th recovery test mentioned were considered outliers and excluded from the remain performance parameters calculations. With the exception of the cited recovery tests, all of the other recovery tests obtained for the analysed elements in both Brufen and Maalox are under control and within the quality control limits stablished by the laboratory. Thus, it may be concluded that, for the analysed elements, there weren't registered significant matrix interferents.

Regarding the isobaric interferences, all of the interferent concentrations detected were lower than the quantification limit and therefore no interference corrections were performed. Given that all of the recovery tests were within the quality control standard defined by LCA and there weren't registered isobaric interferences, it can be said that the quantification methods tested are all selective and specific.

1.2.2. LIMIT OF DETECTION AND QUANTIFICATION

Figure 10 and **Figure 11** presented the charts for the obtained absolute values of the blanks concentrations regarding the quantification of Mn. It is represented as well, in each chart, the estimated limit of detection (yellow line) and limit of quantification (red line).

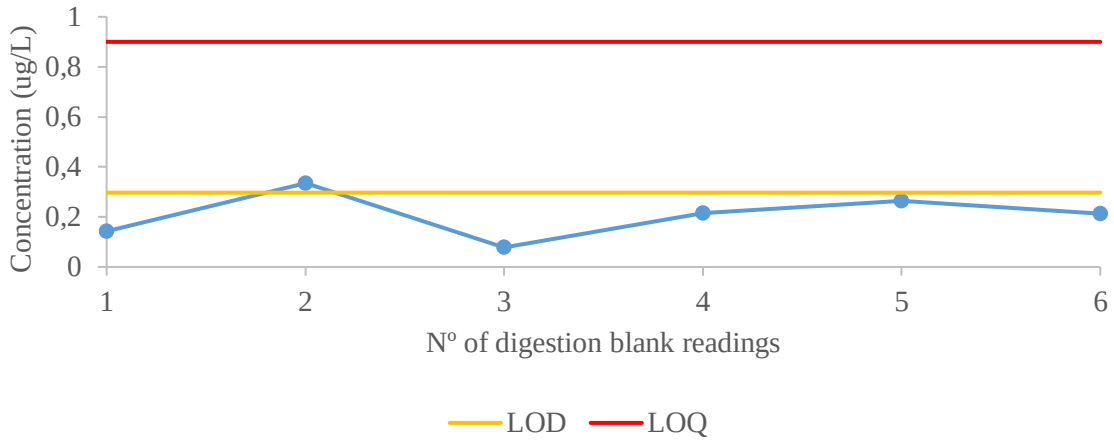

Figure 10: *Verification chart of the values obtained after digestion of blank solutions concerning the determination of Mn and respective limit of detection (LOD) and limit of quantification (LOQ).*

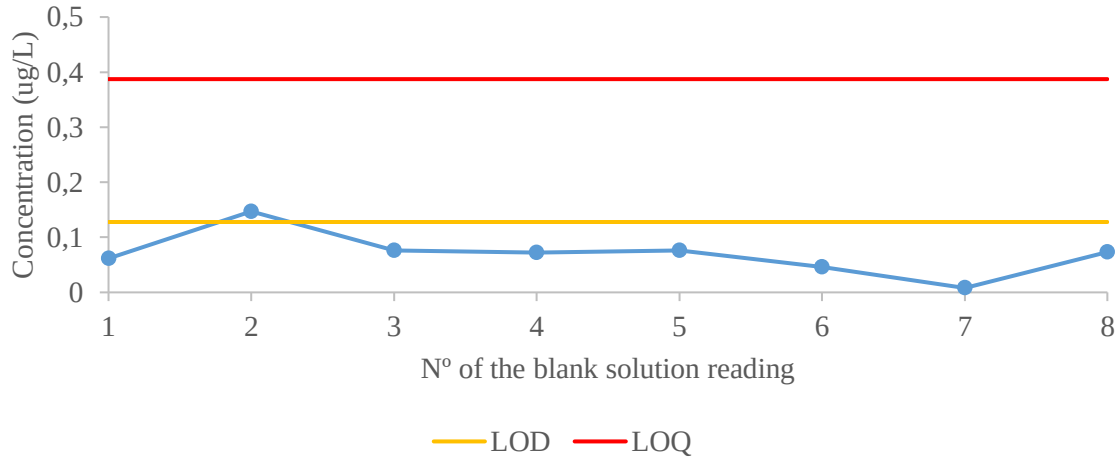

Figure 11: *Verification chart of the blanks solution values concerning the determination of Mn and respective limit of detection (LOD) and limit of quantification (LOQ).*

The LOD and LOQ lines represented in both charts were calculated following the **Equations 2** and **3** presented in topic **1.1.2** of **CHAPTER III**. Both equations used and the values obtained are presented in **Table 8**.

Table 8: *Equations and respective values of limit of detection (LOD) and limit of quantification (LOQ) for digestion blank and blank solutions regarding Mn quantification.*

Equation	Digested blank	Blank solutions
$\boldsymbol{LOD = 3s_0}$	2,97E-01µg/L	1,28E-01µg/L
$\boldsymbol{LOQ = 10s_0}$	9,00E-01µg/L	3,87E-01 µg/L

Table 8 shows that the values of LOD and LOQ are higher in the case of the digested blanks than in the blank solution. This registered difference could be due to a small portion of analyte that is being introduced inadvertently during the blanks preparation and digestion. In these cases, the limits that are established are always the highest values and therefore, the limit of detection is establish as 2,97E-01 µg/L and the limit of quantification establish as 9,00E-01µg/L, for Mn quantification.

The detection limit established for Cr is 1,22E+00 µg/L and 1,11E+00 µg/L for Cu. The quantification limits determined are 3,70E+00 µg/L for Cr and 3,37E+00 µg/L for Cu. All the calculation of the LOD and LOQ of the two elements just mentioned (Cr and Cu) are presented in more detail in **ANNEX II**.

The LOQ value cannot be experimentally confirmed or either subjected to the trueness error evaluation as the PVLQ (quantification limit verification standards) cannot be prepared due to the lack of certified reference materials for pharmaceutical products.

1.2.3. WORKING RANGE

Previously to each analysis, a calibration curve is calculated and established by the equipment software. Nonetheless, for academical purposes, the calibration curves calculations were performed for Mn and are presented in **Tables 9** to **11. Figure 12** represents the obtained function that validates the equipment software calculations.

All of the calculations and verification of the calibration curves, as well as all the quantification parameters for Cr and Cu, are presented with more detail in **ANNEX II**.

Table 9: *Parameters and respective values for the construction of the linear calibration function for Mn.*

Standard	Conc. (µg/L)	Signal (cps)	s_i	s_i^2	s_i^{-2}
P 0,0	0,000	7,17E+02	0,00	2,00E-06	5,00E+05
P 0,1	0,100	8,99E+02	0,00	2,00E-06	5,00E+05
P 0,5	0,500	1,59E+03	0,01	1,62E-04	6,17E+03
P 2,0	2,00	4,22E+03	0,05	2,05E-03	4,88E+02
P 10	10,0	1,86E+04	0,09	8,45E-03	1,18E+02
P 50	50,0	9,29E+04	0,61	3,72E-01	2,69E+00
P 100	100	1,83E+05	0,37	1,39E-01	7,17E+00
Sum	1,63E+02	3,02E+05	1,14E+00	5,22E-01	0,00E+00
Mean	2,32E+01	4,31E+04	1,62E-01	7,46E-02	0,00E+00

Table 10: *Parameters and respective values for the construction of the linear calibration function for Mn - Continuation.*

Standard	Conc. (µg/L)	w_i	w_i*x_i	w_i*y_i	$w_i*x_i*y_i$	$w_i*x_i^2$
P 0,0	0,000	3,48E+00	0,00E+00	0,00E+00	2,49E+03	6,21E+06
P 0,1	0,100	3,48E+00	3,48E-01	1,21E-01	3,13E+03	9,77E+06
P 0,5	0,500	4,29E-02	2,15E-02	4,60E-04	6,82E+01	4,65E+03
P 2,0	2,00	3,39E-03	6,79E-03	4,61E-05	1,43E+01	2,05E+02
P 10	10,0	8,23E-04	8,23E-03	6,77E-05	1,53E+01	2,34E+02
P 50	50,0	1,87E-05	9,34E-04	8,72E-07	1,73E+00	3,01E+00
P 100	100	4,99E-05	4,99E-03	2,49E-05	9,12E+00	8,32E+01
Sum	1,63E+02	7,00E+00	3,90E-01	1,21E-01	5,73E+03	1,60E+07
Mean	2,32E+01	1,00E+00	5,57E-02	1,74E-02	8,18E+02	2,28E+06

Table 11: *Calibration standards residual error value (%) for Mn.*

Standard	Conc. (µg/L)	Calculated Signal (cps)	Residual	Residual Error (%)	Calculated [Mn] (µg/L)	|Relative Error| (%)
P 0,0	0,000	7,17E+02	1,24E-01	0,020	6,80E-05	-
P 0,1	0,100	8,98E+02	5,09E-01	0,060	1,00E-01	0,280
P 0,5	0,500	1,62E+03	-3,69E+01	-2,33	4,80E-01	4,07
P 2,0	2,00	4,35E+03	-1,27E+02	-3,01	1,93E+00	3,50
P 10	10,0	1,89E+04	-2,82E+02	-1,52	9,84E+00	1,55
P 50	50,0	9,15E+04	1,34E+03	1,44	5,07E+01	1,47
P 100	100	1,82E+05	5,12E+02	0,280	1,00E+02	0,280

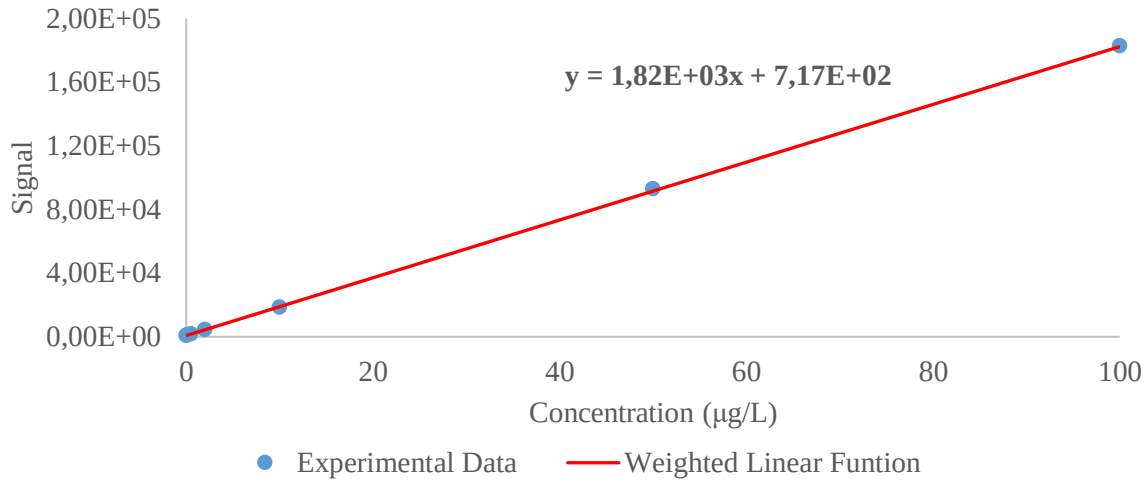

Figure 12: *Experimental data collected and respective linear calibration function for Mn.*

Table 12 shows the weighted calibration function parameter, namely the slope, y-intercept and correlation coefficient. For all of the elements validated in this work, the calibration function was calculated with, at least, 5 standard solutions distributed through the working range. For all of the weighed calibration functions obtained were verified a correlation coefficient not less than 0,999985.

***Table 12**: Weighted calibration function parameters for Mn.*

b_w	a_w	r
7,17E+02	1,82E+03	0,999948

Based on the results obtained, it can be settled that the instrumental working range for the Mn quantification is defined by the limit of quantification and the value of the higher standard solution concentration, in other words, the working range is 0,9 to 100 µg/L. **Figure 13** shows the residual error, in percentage, for the Mn calibration function.

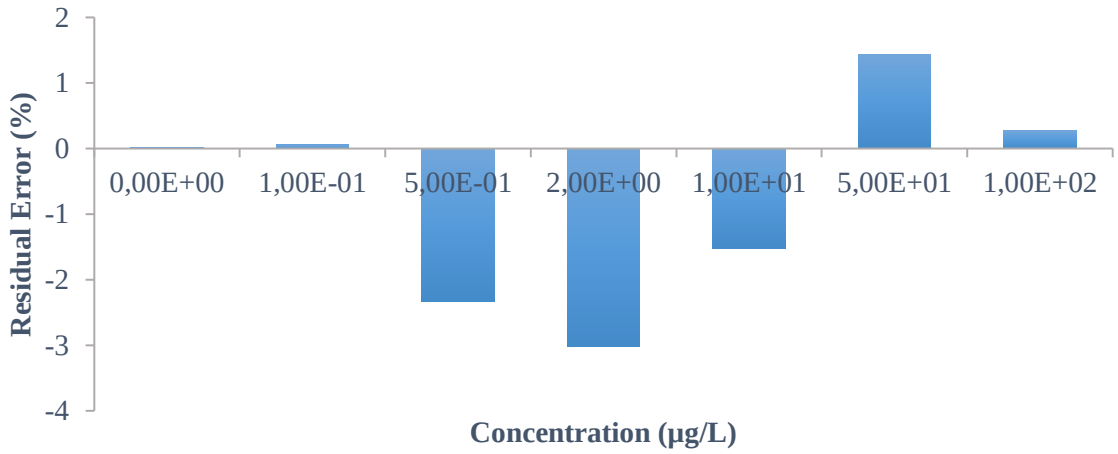

***Figure 13:** Residual error (%) for Mn quantification.*

LCA quality standards consider that the maximum acceptable percentage for residual error is 10%. Analysing **Figure 13** it can be concluded that all of the calibration standard solutions analysed have less than 3% of residual error. The high correlation coefficient and the acceptable residual errors confirm that the weighted calibration linear function calculated for Mn is suitable for the obtained results. By further analysis of the figure, it can also be noticed that there aren't more than 3 positive or negative consecutive residual error percentages. This means that the residual error does not present tendencies corroborating, even more, the suitability of the weighed linear function.

As described in **ANNEX II,** it was obtained a working range from 3,7 and 100 µg/L for Cr and for Cu from 3,4 to 100 µg/L. In all the cases the residual error (%) calculated was always below 10% and it was not verified more than 3 positive or negative consecutive residual error percentages. Therefore, it can be concluded that the residual error does not present tendencies in any of the cases and all of the weighted linear function obtained are suitable for the results obtained.

1.2.4. TRUENESS, PRECISION AND UNCERTAINTY

1.2.4.1. TRUENESS

Trueness was evaluated through the control chart of the bias associated with the calibration curves verification standards (PVRCs). It was only calculated the bias associated with the element quantification using PVRCs as it is not possible to estimate trueness due to the lack of certified reference material. **Figure 14** shows control chart of the bias associated with the quantification of Mn. The bias control charts referring Cr and Cu are presented in **ANNEX II.**

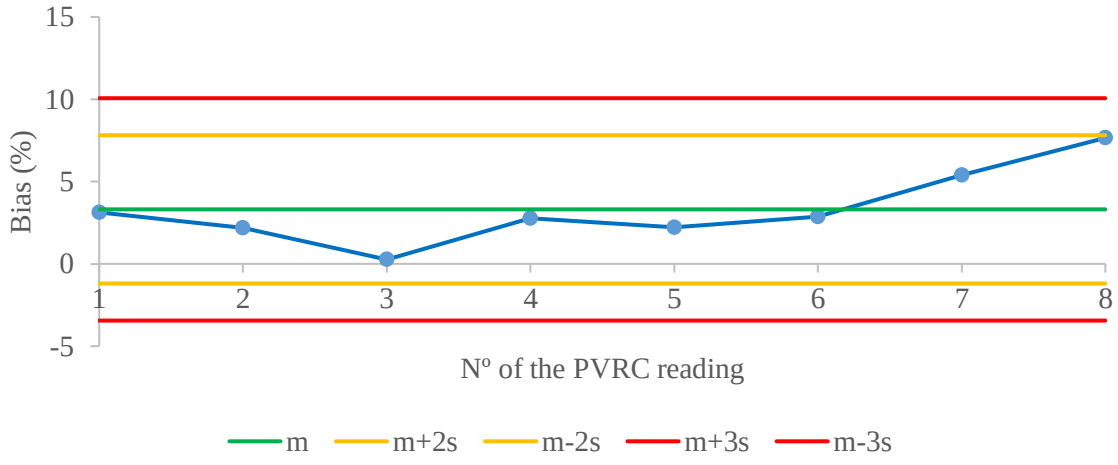

Figure 14: *Control chart of bias (%) for Mn quantification.*

Figure 14 shows that in all cases the bias is lower than 8%. Given that the maximum value accepted for trueness by the laboratory is 10%, it is possible to conclude that the quality requirements stipulated are fulfilled and therefore, the LCA quality standards are suitable for the quantification of Mn in pharmaceutical drugs. It can also be concluded that the method is under control and there isn't any significant error in the digestion process that affects significantly the trueness of the method.

The control charts of the trueness associated with the element quantification for Cr and Cu are presented in detail in **ANNEX II**. The maximum value of bias estimated for Cr is 8%, while for Cu is 9%. In sum, the quality standards of LCA are suitable for the quantification of all the elements mentioned and the quantification stage for all of the elements is under control, since the trueness were within the quality requirements established by the laboratory (10%).

1.2.4.2. PRECISION

1.1.4.2.5. RELATIVE STANDARD DEVIATION

Each sample used in the validation process was analysed in 3 independent days and the relative standard deviation (RSD) was calculated. **Figure 15** and **Figure 16** presented the control chart of the relative standard deviation (%) for the Mn quantification in Brufen and in Maalox, respectively. The RSD control chart for Cu quantification in Brufen and Cu and Cr in Maalox are presented in **ANNEX II.**

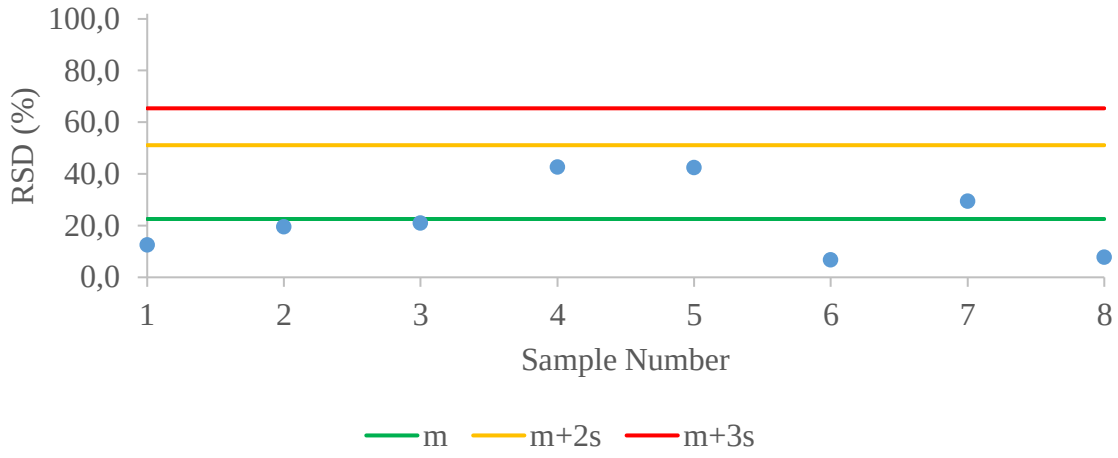

Figure 15: *Control chart of the relative standard deviation (RSD, %) for Mn quantification in Brufen.*

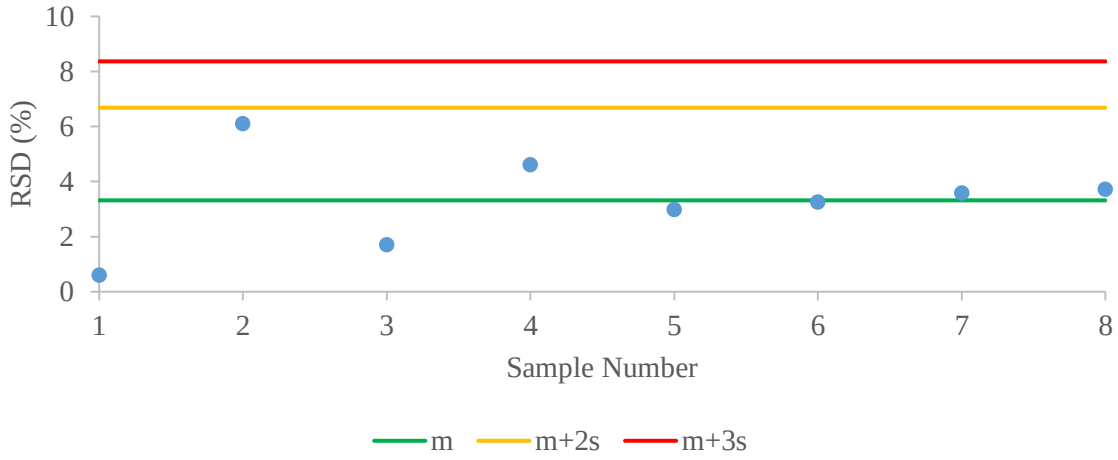

Figure 16: *Control chart of the relative standard deviation (RSD, %) for Mn quantification in Maalox.*

The relative standard deviation maximum value, in percentage, for the quantification of Mn in Brufen was 43% and for the quantification of Mn in Maalox was 6%. In general, the RSD values are high, meaning that the values obtain have high dispersion in the sample quantification process. For the quantification of Cr was obtained a relative standard deviation maximum value of 43% for Brufen and 8% for Maalox. In the case of Cu quantification, the RSD maximum value obtained for the quantification of Cu in Maalox was 24 %. Based on the high values obtained for RSD, it can be concluded that there are drifts in the quantification process.

The quality standards for methods validation suggests that RSD should be around 10 %, but this value might be superior depending on the analytical methodologies and the quantified parameters. Thus, the maximum quality criteria value for the RSD (%) should be altered for the maximum value mentioned in each case.

1.1.4.2.6. REPEATABILITY

Table 13 and **14** presented the repeatability variation coefficient of all the samples analysed in each day. It also shows calculated $HORRAT_r$ values for Mn quantification in Brufen and Maalox, respectively.

Table 13: *Estimated CV_r (%) and $HORRAT_r$ values for Mn quantification in Brufen.*

	Nº of readings	CV_r (%)	$HORRAT_r$
Day 1	7	78,9	36,6
Day 2	8	20,4	8,94
Day 3	8	34,7	14,8

Table 14: *Estimated CV_r (%) and $HORRAT_r$ values for Mn quantification in Maalox.*

	Nº of readings	CV_r (%)	$HORRAT_r$
Day 1	8	2,9	1,6
Day 2	8	2,1	1,1
Day 3	8	1,7	0,9

As it can be seen in **Table 13** the $HORRAT_r$ values are all higher than 2, which means that the method repeatability is not satisfactory. This may be due to the fact that Mn concentrations obtained were close to the LOQ which difficult repeatability evaluation as it leads to a higher result dispersion than expected. In the case of Mn quantification in Maalox (**Table 14)**, the $HORRAT_r$ are in all the cases lower than two meaning that in this case the repeatability was satisfactory.

Tables showing the values of CV_r(%) and $HORRAT_r$ for the quantification of Cr for Brufen and Maalox and Cu for Maalox are presented in **ANNEX II.** In all cases the method repeatability was not satisfactory.

1.1.4.2.7. INTERMEDIATE PRECISION

Table 15 presents the several CV_{SI} (%) obtained for Mn quantification in Brufen and Maalox and also the number of groups evaluated (p), the number of readings executed and the calculated $HORRAT_{SI}$ values.

Table 15: *Estimated values of CVSI (%) and HORRATSI for Mn quantification in Brufen and Maalox.*

Pharmaceutical Drug	CV_{SI} (%)	p	n	$HORRAT_{SI}$
Brufen	66,3	3	23	29,5
Maalox	3,8	3	23	2,09

$HORRAT_{SI}$ values are in both cases superior to 2, although in the case of Maalox the value is very close to 2. This occurrence shows the precision between days is not satisfactory. This is probably manly due to the low concentration of analyte as it difficult the element quantification and leads to higher variation coefficients. It can also be due to the high complexity of the matrices.

For other elements, results were similar, being in all the cases the value of $HORRAT_{SI}$ higher than 2. The estimated values of CV_{SI} (%) and $HORRAT_{SI}$ for Cr in Maalox and Brufen and Cu in Maalox are presented in **ANNEX II**. In sum, in all cases the precision between days was not satisfactory and it should be performed more tests to estimate the intermediate precision.

1.3. SUMMARY OF METHODS VALIDATION FOR PHARMACEUTICAL DRUGS

It was possible to validate the quantification method of the elemental impurities Mn and Cr in the pharmaceutical drugs Brufen and Maalox and Cu in Maalox. The performance parameters evaluated, and respective results are summarized in **Tables 16** to **18**.

Table 16: *Performance parameters summary for Mn quantification in pharmaceutical drugs.*

Validation Parameters		Required	Mn in Brufen	Mn in Maalox	Observations
			Obtained Value	Obtained Value	
Specificity Selectivity	**Matrix Interferences**	80-120%	98-104 %	102-104 %	Minimum and maximum value obtained
LOD LOQ	**Limit of Detection**	n.a.	9,27E-01 µg/L 1,28E-01 µg/L		Obtained with digestion and blank solutions, respectively
	Limit of Quantification	n.a.	9,00E-01µg/L 3,87E-01 µg/L		Obtained with digestion and blank solutions, respectively
Working Range	**Correlation Coefficient**	≥ 0,995	0,999948		n.a.
	Sensitivity/Slope	n.a.	1,71E+03		n.a.
	Working Range	n.a.	9,00E-01 to 100 µg/L		n.a.
Precision	**Trueness (bias)**	≤ 10 %	3,7 %		Maximum value obtained
	Sample Variation Coefficient	≤ 10 %	≤ 43%	≤ 6%	Maximum value obtained
	Repeatability Variation Coefficient	≤ 10 %	34,7%	2,9%	Maximum value obtained in digested sample replicas reading
	$HORRAT_r$	< 2	14,8	1,6	Maximum value obtained
	Intermediate Precision	≤ 10 %	26,5%	3,8%	Maximum value obtained
	$HORRAT_{SI}$	< 2	11,5	2,1	Maximum value obtained

Table 17: *Performance parameters summary for Cr quantification in pharmaceutical drugs.*

Validation Parameters		Required	Cr in Brufen	Cr in Maalox	Observations
			Obtained Value	Obtained Value	
Specificity Selectivity	**Matrix Interferences**	80-120%	98-110 %	108-113 %	Minimum and maximum value obtained
LOD LOQ	**Limit of Detection**	n.a.	2,53E-01 µg/L		Obtained with digestion and blank solutions, respectively
	Limit of Quantification	n.a.	7,68E-01µg/L		Obtained with digestion and blank solutions, respectively
Working Range	**Correlation Coefficient**	≥ 0,995	0,999997		n.a.
	Sensitivity / Slope	n.a.	1,75E+02		n.a.
	Working Range	n.a.	3,70 to 100 µg/L		n.a.
Precision	**Trueness (bias)**	≤ 10 %	7,87 %		Maximum value obtained
	Sample Variation Coefficient	≤ 10 %	≤ 43 %	≤ 8 %	Maximum value obtained
	Repeatability Variation Coefficient	≤ 10 %	19,6 %	5,1 %	Maximum value obtained in digested sample replicas reading
	$HORRAT_r$	< 2	10,4	2,8	Maximum value obtained
	Intermediate Precision	≤ 10 %	28,6 %	5,9 %	Maximum value obtained
	$HORRAT_{SI}$	< 2	14,8	3,2	Maximum value obtained

Table 18: *Performance parameters summary for Cu quantification in pharmaceutical drugs.*

Validation Parameters		Required	Cu in Maalox	Observations
			Obtained Value	
Specificity Selectivity	**Matrix Interferences**	80-120 %	90-93 %	Minimum and maximum value obtained
LOD LOQ	**Limit of Detection**	n.a.	9,22E-01 µg/L 1,1E+00 µg/L	Obtained with digestion and blank solutions, respectively
	Limit of Quantification	n.a.	2,79E+00 µg/L 3,37E+00 µg/L	Obtained with digestion and blank solutions, respectively
Working Range	**Correlation Coefficient**	≥ 0,995	0,999985	n.a.
	Sensitivity/Slope	n.a.	1,71E+03	n.a.
	Working Range	n.a.	3,37E+00 to 100 µg/L	n.a.
Precision	**Trueness (bias)**	≤ 10 %	9,4 %	Maximum value obtained
	Sample Variation Coefficient	≤ 10 %	≤ 24 %	Maximum value obtained
	Repeatability Variation Coefficient	≤ 10 %	15,7 %	Maximum value obtained in digested sample replicas reading
	$HORRAT_r$	< 2	6,8	Maximum value obtained
	Intermediate Precision	≤ 10 %	18,1 %	Maximum value obtained
	$HORRAT_{SI}$	< 2	7,6	Maximum value obtained

2. GENERIC AND REFERENCE PHARMACEUTICAL DRUGS ELEMENTAL IMPURITIES COMPARISON

Samples of several generic pharmaceutical drugs and respective reference drugs were analysed and the elemental impurities V, Cr, Mn, Cu, Zn and Pb determined (**Table 19)**. Although the quantification methods were not validated for all of the elemental impurities shown in the table below, it was still considered pertinent to show these values.

Table 19: *Concentration in µg/g of V, Cr, Mn, Cu, Zn and Pb in generic and reference pharmaceutical drugs.*

Pharmaceutical drug	Cr	Mn	Cu	Zn	Pb
	µg/g	µg/g	µg/g	µg/g	µg/g
Proton pump inhibitors					
Proton	0,46	0,28	< LOQ	1,29	0,02
Proton Capsule	0,81	0,37	0,80	1,06	0,09
Proton (Total)	**1,27**	**0,65**	**0,80**	**2,34**	**0,11**
Omeprazol Generis	0,34	0,28	< LOQ	0,87	0,10
Omeprazol Generis Capsule	0,52	0,30	0,42	0,64	0,11
Omeprazol Generis (Total)	**0,85**	**0,58**	**0,42**	**1,50**	**0,21**
Omeprazol Ratiopharm	0,88	0,16	0,25	1,27	0,16
Omeprazol Ratiopharm Capsule	1,90	0,24	0,99	1,07	0
Omeprazol Ratiopharm (Total)	**2,78**	**0,40**	**1,25**	**2,34**	**0,29**
Pantoprazol Teva	**0,53**	**1,04**	**< LOQ**	**0,95**	**0,35**
Lansoprazol Teva	0,98	0,10	0,19	0,57	0,08
Lansoprazol Teva Capsule	1,45	0,28	1,42	1,40	0,32
Lansoprazol Teva (Total)	**2,42**	**0,38**	**1,60**	**1,98**	**0,40**
Non-steroidal anti-inflammatory drugs					
Brufen	0,69	0,29	0,14	0,38	0,04
Ibuprofeno Azevedos	0,80	0,65	< LOQ	1,47	0,14
5-alpha reductase inhibitors					
Combodart	0,35	0,77	1,27	0,58	0,03
Durasterida Teva	0,25	0,18	0,24	1,99	0,02

Table 19 allow to verify that elemental impurities concentrations varies from one pharmaceutical drug to another. This finding corroborates the fact that the generic pharmaceutical drugs do not contain the same excipient as explained in topic **2.3.2** of **CHAPTER I**.

In the case of the inhibitor of the proton bomb, it can be observed that in all of the pharmaceutical drugs analysed, the greatest amount of Cr, Mn and Cu is introduced in the pharmaceutical drug by the capsules as the concentrations of these elemental impurities in the capsules are substantially higher than in the her contents. In the cases of the Lansoprazol and Proton, in addiction of the already said elemental impurities, the greatest amount of Pb is also introduce by their capsules.

By looking at the non-steroidal anti-inflammatory pharmaceutical drugs, one can see that, regarding the analysed elemental impurities, the generic pharmaceutical drug contains high elemental impurities concentration that their respective reference pharmaceutical drug. Nonetheless, it cannot be concluded that generic non-steroidal anti-inflammatory pharmaceutical drugs contain always more impurities than their reference drugs as it were only compared one generic with one reference pharmaceutical drug.

Regarding the 5-alpha reductase inhibitors, with the exception of V and Zn, it is observed that the reference drug, Combodart, presents higher elemental impurities concentrations than the generic pharmaceutical drug Durasterida Teva.

In all cases, even with the maximum intake of the pharmaceutical drug daily dosage recommended by the laboratory, the amount of elemental impurities ingested is well below the maximum permitted daily exposure to the elemental impurity (**Table 3**). Summing up, although the elemental impurities in generic pharmaceutical drugs are not safeguarded by the law, in the cases of the analysed pharmaceutical drugs there is any concerning situation.

3. CONCLUSIONS

This work had as fundamental goals, develop and validate ICP-MS methods for the determination of elemental impurities in generic pharmaceutical drugs and compare the results with their reference drugs.

The work allowed to propose a methodology to successfully dissolve and microwave digest the pharmaceutical drug samples. It was also accomplished the validation of the quantification methods by ICP-MS of Cr and Mn in the pharmaceutical drug Brufen and Cr, Mn and Cu in the pharmaceutical drug Maalox.

Also, it was compared the differences regarding elemental impurities both in generic and reference pharmaceutical drugs;

For all that has been said above, it can be concluded that the overall aim of this investigation, as well as, all of the specific goals described in the beginning of this work were successfully archived.

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ANNEX I: OTHER ELEMENTAL IMPURITIES DETERMINATION TECHNIQUES

1. FLAME ATOMIC ABSORPTION SPECTROSCOPY

Flame atomic absorption spectroscopy is a quantitative analytical technique that measures the light absorption of free, ground state atoms.

In F-AAS, the sample is aspirated through a capillary tube into the nebulizer where the sample solution is converted into an aerosol (similarly to ICP) and then, drops with diameter < 20 µm are carried to the burner head as schematized in **Figure 17**.

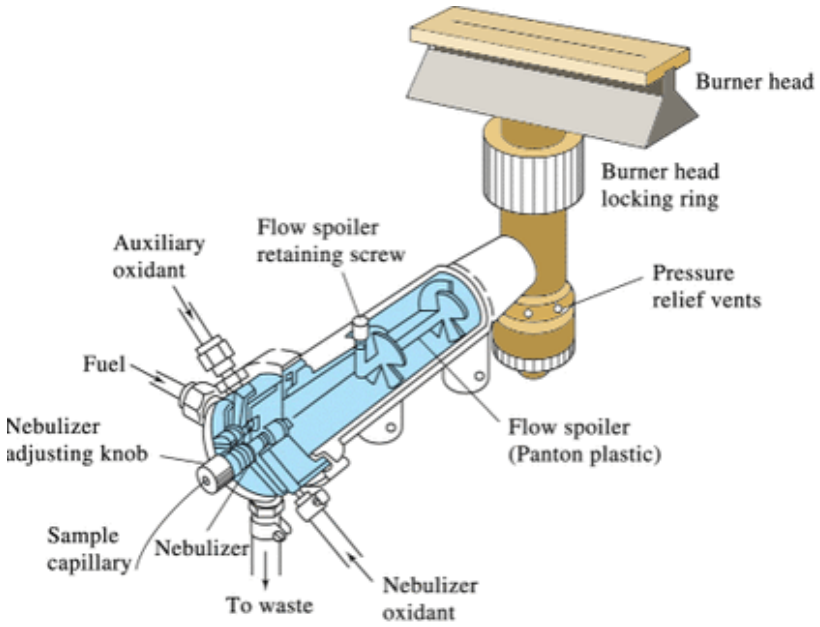

***Figure 17**: Injection system and burner head of a F-AAS device.*

Into the burner head, the sample is burned, and the temperature of the flame is optimized depending on the targeted element. Into the flame occurs several reactions and processes. Firstly, the water molecules are evaporated and therefore removed from the sample (Lebedev, 2013). Then, the organic and inorganic dehydrated complexes are degraded into gaseous atomic ground states. In the hotter parts of the flame occur the absorption of photons by the ground state gaseous atoms that due to this absorption get excited.

To excite the atoms, it is needed a light source that has to be pure to avoid excessive instrumental noise. Usually, as light source it is used a hollow cathode lamp that is specific for each element analysed and a double-beam technology. This technology consists in the splitting of the light beam into two paths, one of them do not passes through the sample and therefore is used to measure the interferences caused by lamp intensity fluctuations and other

sources not connected to the sample. The other beam is used to measure the actual radiation that interacts with the sample. As the lamp is specific for the element to analyse (only emits light in the wavelength that the element to analyse absorb) only the atoms of this element are going to absorb radiation and excite. This excitation results in a reduction in the intensity of radiation leaving the sample cell. This wicked radiation intensity is reflected by a series of mirrors into the monochromator and finally, the wavelength at which the element absorb is focused and amplified through the photomultiplier and the signal processed by the computer (Dimitrov Dakashev et al., 2012). A representation of the F-AAS is presented in **Figure 18** for the better idealization of the process described above.

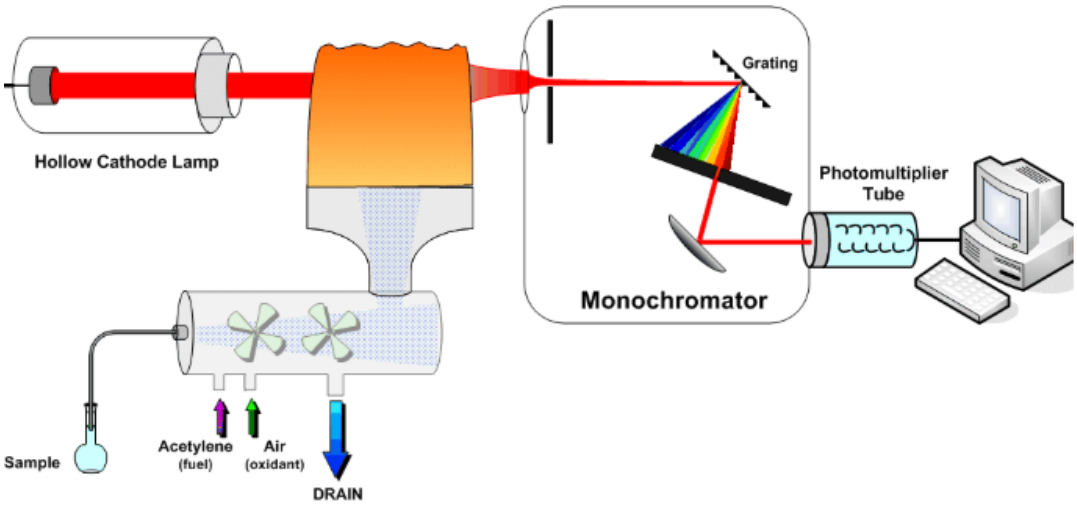

***Figure 18**: Scheme of the setup of a typical F-AAS device.*

This technique was applied in many investigations reported by numerous articles. One example is an article that reported the used of flame atomic absorption spectroscopy to develop a method to rapid quantitative analysis of magnesium stearate in pharmaceutical powders and solid dosage forms (Sugisawa et al., 2009).

2. GRAPHITE FURNACE ATOMIC ABSORPTION SPECTROSCOPY

In the past it was called as flameless atomic absorption (FAA) however, nowadays it is officially designated as electrothermal atomic absorption spectrometry (ET-AAS) by the IUPAC (International Union of Pure and Applied Chemistry) but is usually referred to as graphite furnace atomic absorption spectrometry or GF-AAS.

This analytical technique (**Figure 19**) has a graphite tube of about 2.5 to 3 cm long and 4 to 6 mm of inside diameter and it is composed of graphite with a pyropgraphite coating that is quite impermeable. This tube has a circular opening in the centre of approximately 1 to 2 mm of diameter used to introduce the sample.

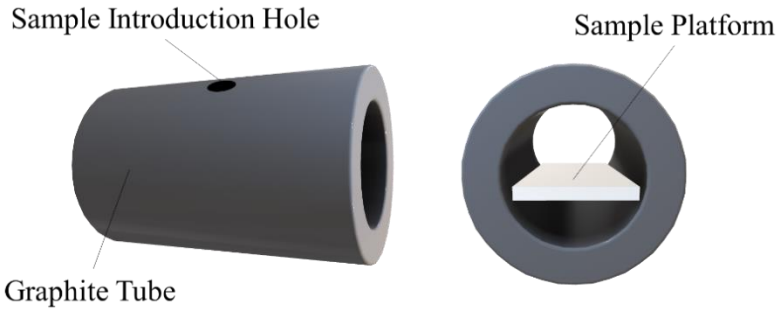

Figure 19: *3D schematic representation of a graphite furnace tube.*

Usually, the sample volume used is 10 to 20 µL, but the graphite furnace has the capacity to hold an aliquot up to 50 µL. Then, the graphite furnace tube is placed inside the graphite chamber and subjected to a pre-programmed progressive heating. This heating program has four phases (**Figure 20**) and the temperatures dependent on the element analysed. During this process, the chamber is protected from the surrounding atmosphere through an Ar (Argon) flow around the furnace to prevent oxidation processes that would affect the quantification.

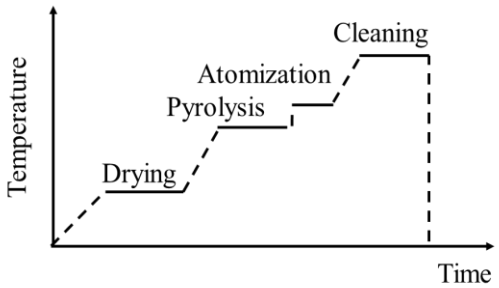

Figure 20: *Schematic representation of the four phases of the heating program of a GF-AAS in function of time.*

The first step of the heating program is the drying one, in which the solvent is smoothly evaporated so that no sample projections occur to increase precision. Then, in pyrolysis the matrix components are mineralized. This step should be carried at the highest temperature possible without losing analyte.

The next step is atomization, where is produced an atomic vapour that must be obtained at the lowest temperature possible that maximizes the atomization of the element analysed to obtain maximum sensitivity. Finally, in the last step, a flow of inert gas flow passes through the graphite chamber to eliminate matrix vapours (Inczédy et al., 1998).

Also, during the heating process, a light source passes through the tube and at the end of the circuit a detector measures the analyte absorbance signal during the whole process. At that point, through a Beer–Lambert's law, the absorbance signal is related to the element concentration. It is important to highlight that the light source is different depending on the element analysed as it is wanted the maximum absorption of the lamp radiation by the element so that the measurements can be accurate.

This technique present detection limits in the order of low parts per billion or low ρg/L range and therefore is still currently one of the most sensitive techniques for elemental analysis. The fact that it is capable of analysing elements in sample micro amounts with complex matrices combining with the relatively low running cots made GF-AAS a very attractive option when a single element needs to be analysed, the sample volume available is low or the sample matrix is complex (Holcombe and Borges, 2010).

One example that clearly illustrates what has been said is an article where is developed a graphite furnace atomic absorption spectroscopy method for the analysis of palladium (Pd) content in bulk pharmaceutical drugs. The results reported are all in low parts per billion as the lowest value is 2 ppb (part per billion) (Jia et al., 2001).

3. INDUCTIVELY COUPLED PLASMA - ATOMIC EMISSION SPECTROSCOPY

Inductively coupled plasma-atomic emission spectrometry (ICP-AES) also known as inductively coupled plasma-optical emission spectrometry (ICP-OES) was first commercialized in the mid-1970s and quickly became one of the election techniques for the determination of elements with low abundance in a wide range of samples types.

As schematized in **Figure 21**, the elements are aspirated into the spray chamber where the sample is converted into an aerosol (as explained with more detail in topic **4.1** of **CHAPTER II**). Then, the aerosol is conducted into the plasma where occurs the sample atomization. Due to the energy provided by the plasma high temperatures, the molecules are degraded into atoms. However, as the plasma temperature is so high, the energy that it provides also excites the atoms. When the excited atoms reach a colder part of the plasma, they emit the energy and return to the ground state and is this emission that a diffraction grating (placed near the plasma) resolves into its component radiation for the measurement of light intensity by the photomultiplier tube. In other words, the light emitted by the atoms when they return to the ground state is converted to an electrical signal to be quantitatively measured.

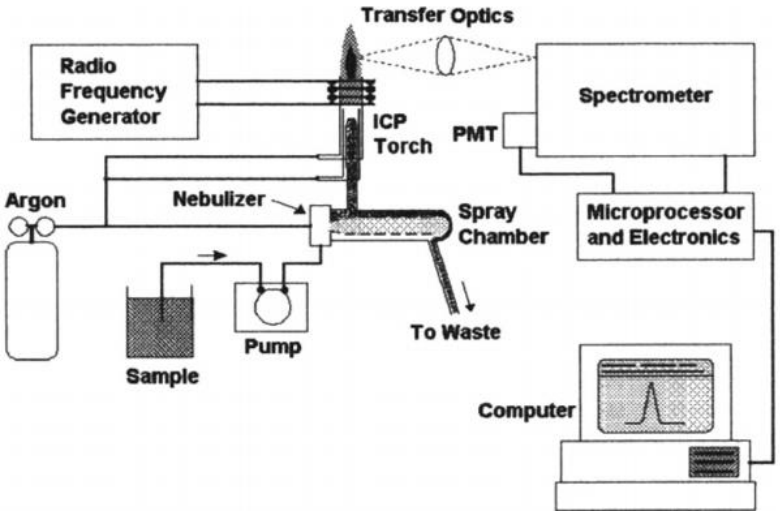

Figure 21: *Scheme of a typical inductively coupled plasma-optical emission spectrometry system.*

Since the first sold device, ICP has undergone a tremendous evolution both in the evaluation and understanding of the equipment with numerous studies on the system mechanisms and characterization and on the instrumentation level: the replacement of photomultiplier tubes by charge transfer devices for multielement determination, rotating the ICP through 90° in order to improve the detection limits and various sample introduction systems such as laser ablation and electrothermal vaporization (ETV) for solid sampling.

ICP-OES analysis always involves sample preparation (Sneddon et al., 2006) and the use of calibration curves obtained through the analysis of standard samples (Dean, 2005; Hill, 2006; Nölte, 2003b; Thomas, 2004). The use of ICP-OES for elemental analysis has been increasing and one example of the usefulness of this technique is the article that reports the development of a slurry introduction method for multi-element analysis of antibiotics by inductively coupled plasma atomic emission spectrometry using various types of spray chamber and nebulizer configurations. This technique could quantify elemental impurities present in the antibiotics down to the μg/g level (Zachariadis and Michos, 2007).

ANNEX II: INFORMATION ON METHODS VALIDATION

1. VALIDATION OF THE QUANTIFICATION METHOD FOR CR

1.1. SPECIFICITY AND SELECTIVITY

1.1.1. RECOVERY TESTS – MATRIX INTERFERENCES

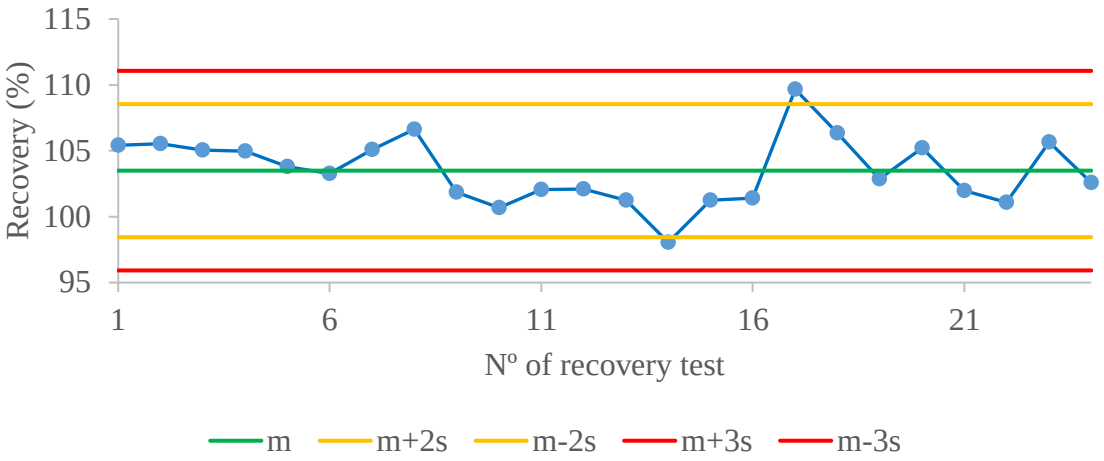

Figure 22: *Control chart of Cr recovery tests in Brufen samples.*

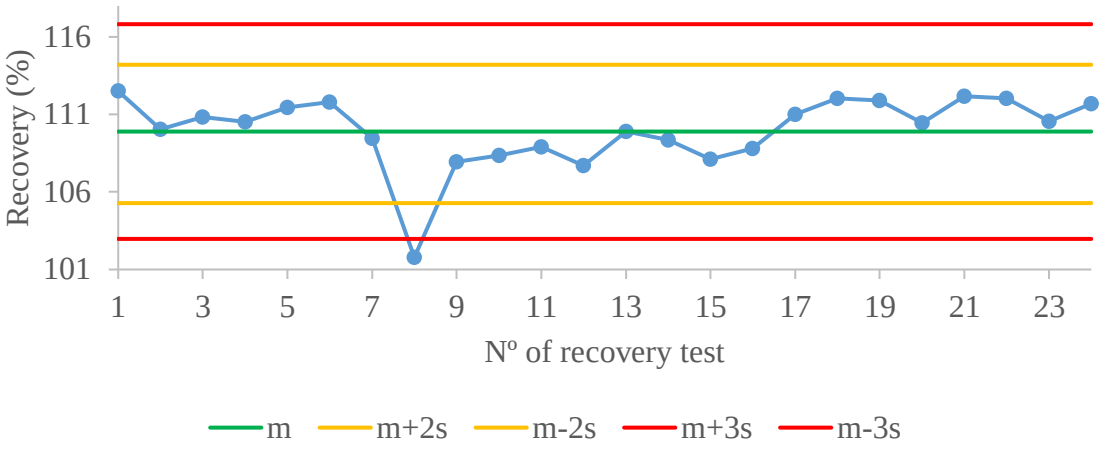

Figure 23*: Control chart of Cr recovery tests in Maalox samples.*

1.2. LIMITS OF DETECTION AND QUANTIFICATION

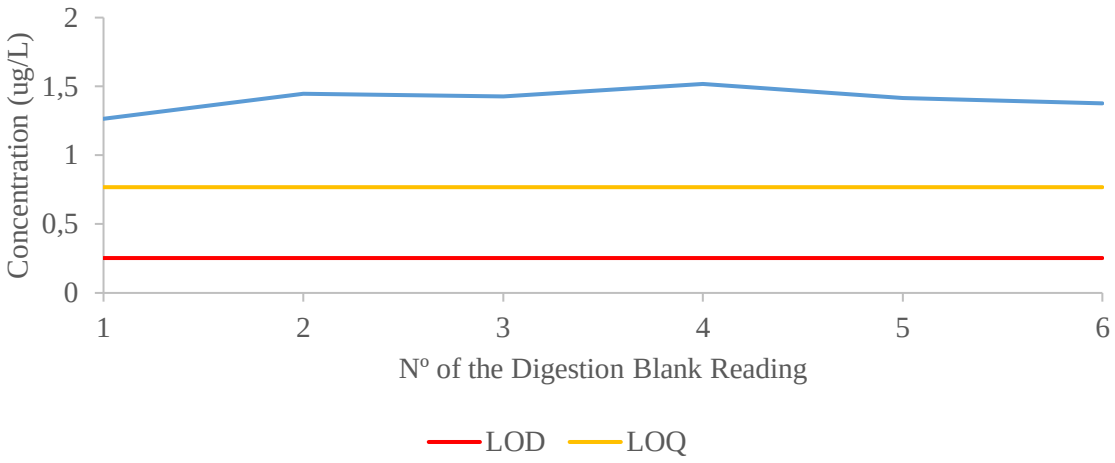

Figure 24: *Verification chart of the values obtained after digestion of blank solutions concerning the determination of Cr and respective limit of detection (LOD) and limit of quantification (LOQ).*

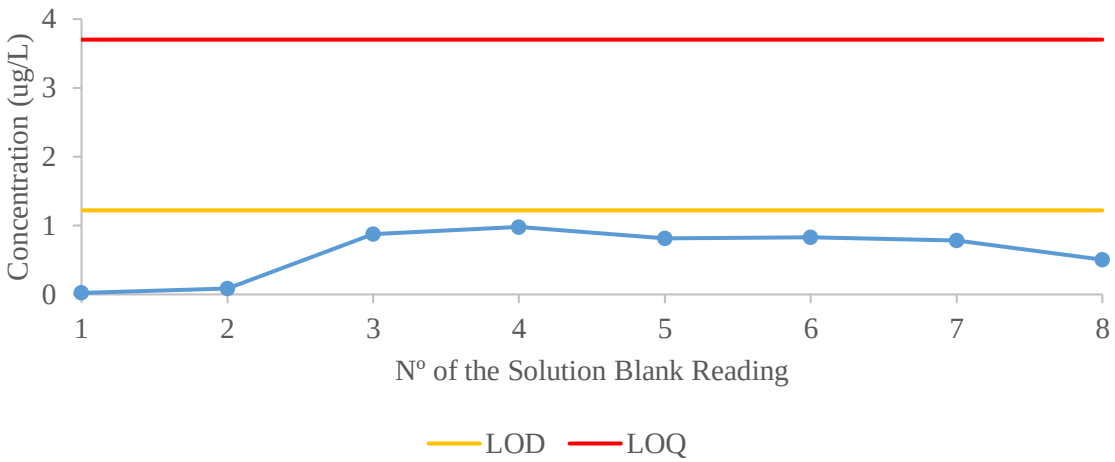

Figure 25: *Verification chart of the blanks solution values concerning the determination of Cr and respective limit of detection (LOD) and limit of quantification (LOQ).*

Table 20: *Equations and respective values of limit of detection (LOD) and limit of quantification (LOQ) for digestion blank and blank solutions regarding Cr quantification*

Equation	Digested blank	Blank solutions
$\boldsymbol{LOD = 3s_0}$	2,53E-01µg/L	1,22E+00 µg/L
$\boldsymbol{LOQ = 10s_0}$	7,68E-01µg/L	3,70E+00 µg/L

1.3. WORKING RANGE

Table 21: *Parameters and respective values for the construction of the linear calibration function for Cr.*

Standard	Conc. (µg/L)	Signal (cps)	s_i	s_i^2	s_i^{-2}
P 0,0	0,000	1175	0,00	5,00E-07	2,00E+06
P 0,5	0,500	1,67E+03	0,01	3,20E-05	3,12E+04
P 2,0	2,00	3,10E+03	0,02	4,80E-04	2,08E+03
P 10	10,0	1,09E+04	0,06	4,14E-03	2,42E+02
P 50	50,0	5,06E+04	0,33	1,10E-01	9,09E+00
P 100	100	9,92E+04	0,08	1,10E-01	9,09E+00
Sum	1,63E+02	1,67E+05	5,08E-01	2,25E-01	2,03E+06
Mean	2,71E+01	2,78E+04	8,46E-02	3,74E-02	3,39E+05

Table 22: *Parameters and respective values for the construction of the linear calibration function for Cr - Continuation.*

Standard	Conc. (µg/L)	w_i	w_i*x_i	w_i*y_i	$w_i*x_i*y_i$	$w_i*x_i^2$
P 0,0	0,000	5,90E+00	0,00E+00	6,93E+03	0,00E+00	0,00E+00
P 0,5	0,500	9,22E-02	4,61E-02	1,54E+02	7,71E+01	2,30E-02
P 2,0	2,00	6,14E-03	1,23E-02	1,91E+01	3,81E+01	2,46E-02
P 10	10,0	7,13E-04	7,13E-03	7,75E+00	7,75E+01	7,13E-02
P 50	50,0	2,68E-05	1,34E-03	1,36E+00	6,79E+01	6,71E-02
P 100	100	4,24E-04	4,24E-02	4,21E+01	4,21E+03	4,24E+00
Sum	1,63E+02	6,00E+00	1,09E-01	7,16E+03	4,47E+03	4,42E+00
Mean	2,71E+01	1,00E+00	1,82E-02	1,19E+03	7,44E+02	7,37E-01

Table 23: *Calibration standards residual error value (%) for Cr.*

Standard	Conc. (µg/L)	Calculated Signal (cps)	Residual	Residual Error (%)	Calculated [Cr] (µg/L)	|Relative Error| (%)
P 0,0	0,000	1,18E+03	-7,57E-02	-0,010	-7,72E-05	-
P 0,5	0,500	1,67E+03	7,62E+00	0,460	5,08E-01	1,55
P 2,0	2,00	3,14E+03	-3,23E+01	-1,04	1,97E+00	1,65
P 10	10,0	1,10E+04	-9,92E+01	-0,910	9,90E+00	1,01
P 50	50,0	5,02E+04	4,04E+02	0,800	5,04E+01	0,820
P 100	100	9,92E+04	4,94E+00	0,000	1,00E+02	0,010

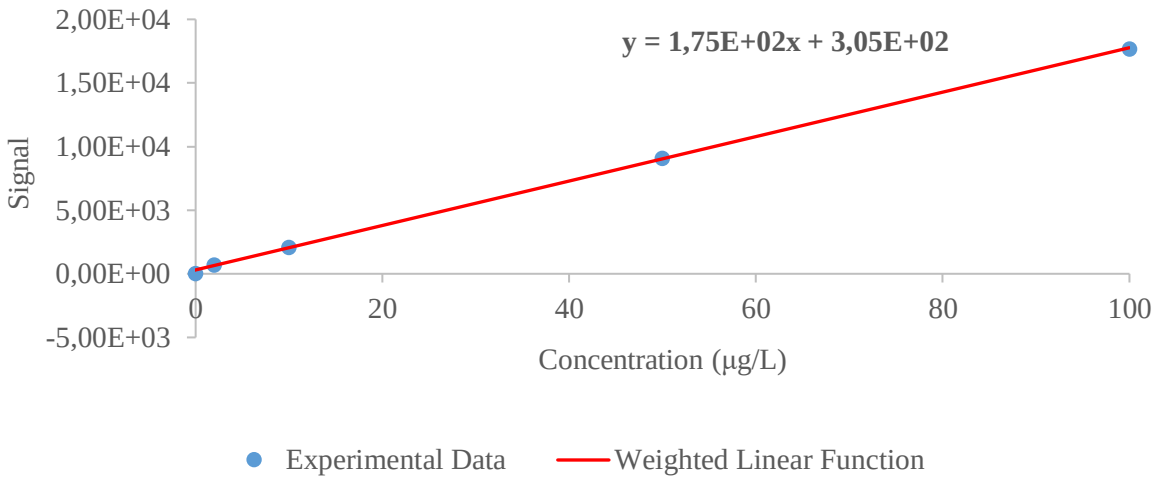

Figure 26: *Experimental data collected and respective linear calibration function for Cr.*

Table 24*: Weighted calibration function parameters for Cr.*

b_w	a_w	r
3,05E+02	1,75E+02	0,999997

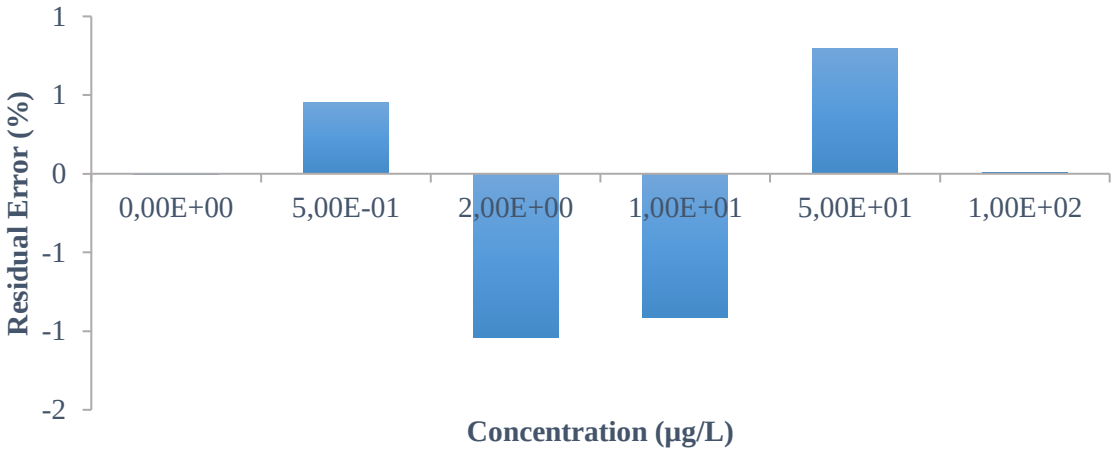

Figure 27: *Residual error (%) for Cr quantification.*

1.4. TRUENESS, PRECISION AND UNCERTAINTY

1.4.1. TRUENESS

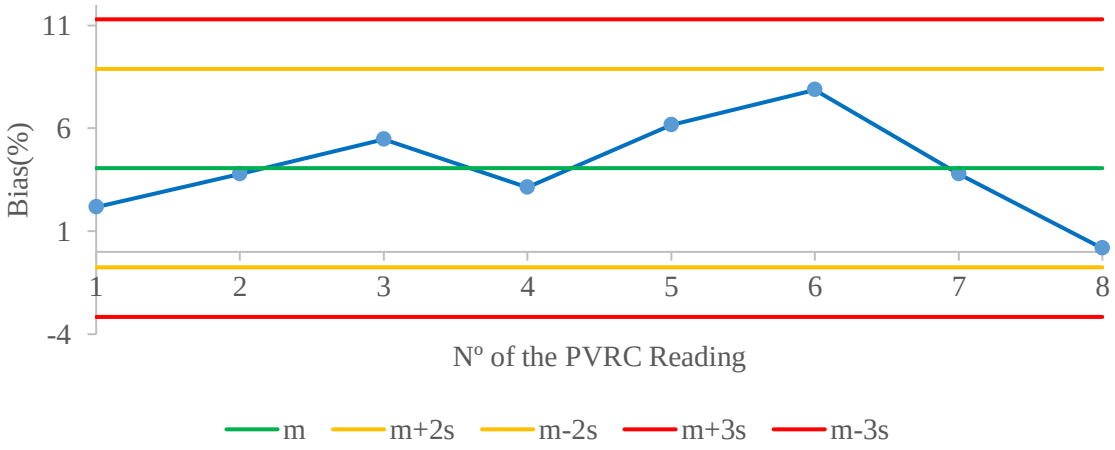

Figure 28: *Control chart of bias (%) for Cr quantification.*

1.4.2. PRECISION

1.4.2.1. SAMPLE VARIATION COEFFICIENT (RELATIVE STANDARD DEVIATION)

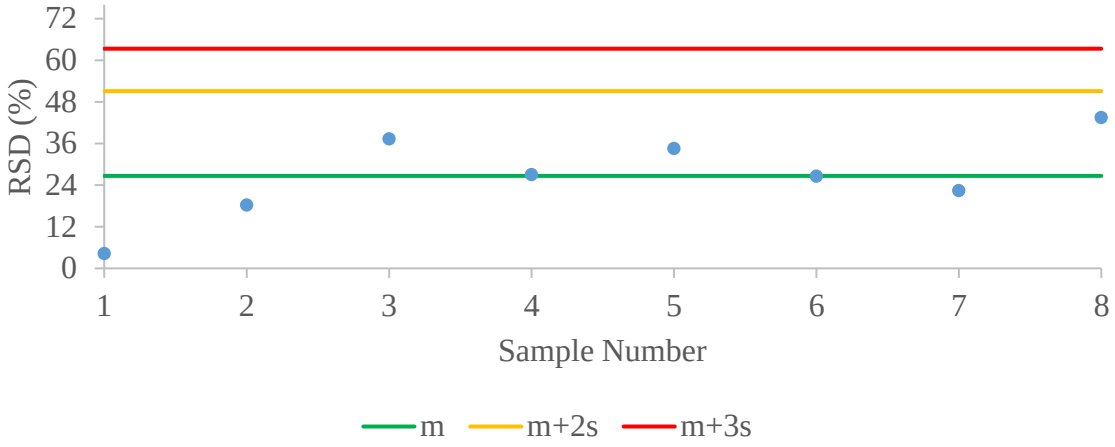

Figure 29: *Control chart of the relative standard deviation (RSD, %) for Cr quantification in Brufen.*

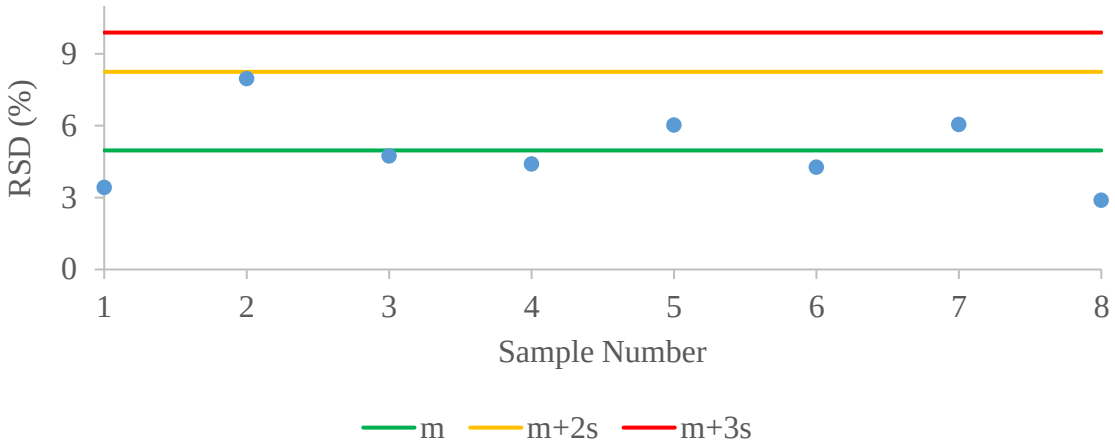

Figure 30: *Control chart of the relative standard deviation (RSD, %) for Cr quantification in Maalox.*

1.4.2.2. REPEATABILITY VARIATION COEFFICIENT

Table 25: *Estimated CV_r (%) and $HORRAT_r$ values for Cr quantification in Brufen.*

	Nº of readings	**CV_r (%)**	**$HORRAT_r$**
Day 1	8	11,8	5,8
Day 2	7	5,2	2,6
Day 3	8	19,6	10,4

Table 26: *Estimated CV_r (%) and $HORRAT_r$ values for Cr quantification in Maalox.*

	Nº of readings	**CV_r (%)**	**$HORRAT_r$**
Day 1	7	5,1	2,8
Day 2	8	4,1	2,3
Day 3	8	3,3	1,8

1.4.2.3. INTERMEDIATE

Table 27: *Estimated values of CVSI (%) and HORRATSI for Cr quantification in Brufen and Maalox.*

Pharmaceutical Drug	**CV_{SI} (%)**	**p**	**n**	**$HORRAT_{SI}$**
Brufen	29,2	3	22	15,0
Maalox	5,9	3	22	3,2

2. VALIDATION OF THE QUANTIFICATION METHOD FOR CU

2.1. SPECIFICITY AND SELECTIVITY

2.1.1. RECOVERY TESTS – MATRIX INTERFERENCES

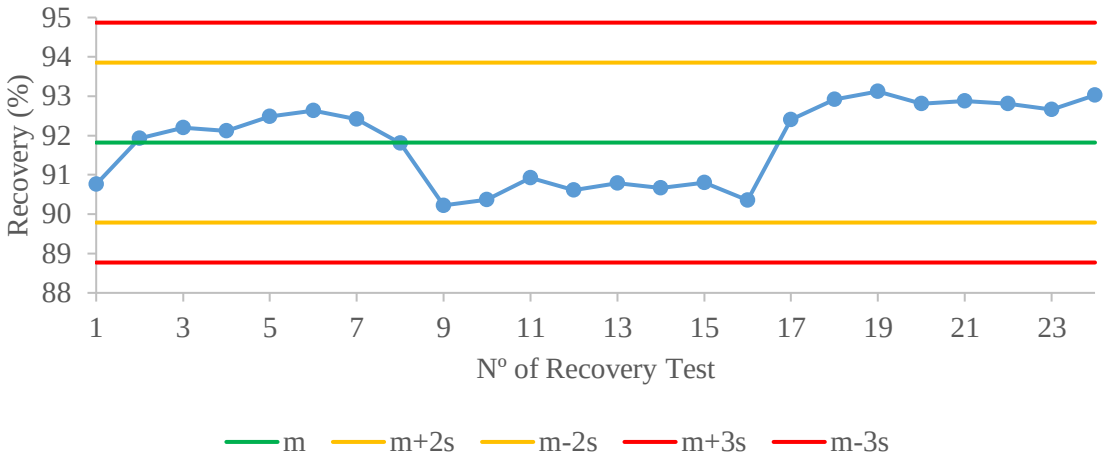

Figure 31: *Control chart of Cu recovery tests in Maalox samples.*

2.2. LIMITS OF DETECTION AND QUANTIFICATION

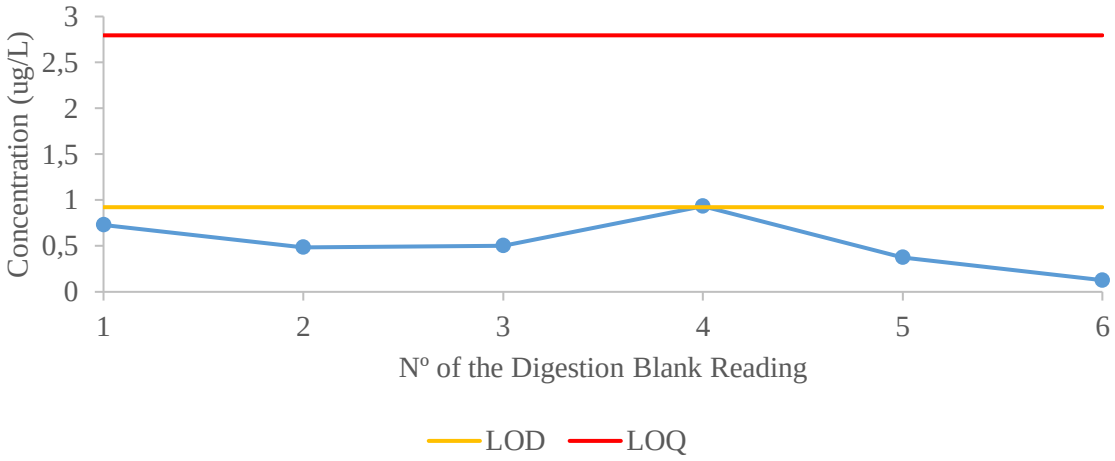

Figure 32: *Verification chart of the values obtained after digestion of blank solutions concerning the determination of Cu and respective limit of detection (LOD) and limit of quantification (LOQ).*

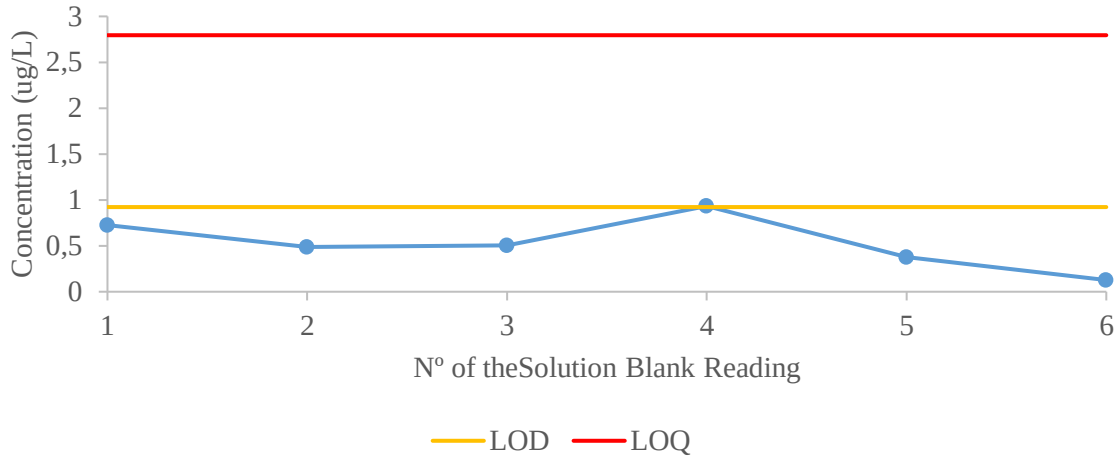

Figure 33: *Verification chart of the blanks solution values concerning the determination of Cu and respective limit of detection (LOD) and limit of quantification (LOQ).*

Table 28: *Equations and respective values of limit of detection (LOD) and limit of quantification (LOQ) for digestion blank and blank solutions regarding Cu quantification*

Equation	Digested blank	Blank solutions
$\boldsymbol{LOD = 3s_0}$	9,22E-01µg/L	1,11E+00 µg/L
$\boldsymbol{LOQ = 10s_0}$	2,79E+00 µg/L	3,37E+00 µg/L

2.3. WORKING RANGE

Table 29: *Parameters and respective values for the construction of the linear calibration function for Cu.*

Standard	Conc. (µg/L)	Signal (cps)	s_i	s_i^2	s_i^{-2}
P 0,0	0,000	3,70E+02	0,11	1,26E-02	7,91E+01
P 2,0	2,00	6,73E+02	0,06	3,28E-03	3,05E+02
P 10	10,0	2,07E+03	0,03	1,10E-03	9,05E+02
P 50	50,0	9,04E+03	0,33	1,09E-01	9,21E+00
P 100	100	1,76E+04	0,25	6,20E-02	1,61E+01
Sum	1,62E+02	2,98E+04	7,81E-01	1,88E-01	1,31E+03
Mean	3,24E+01	5,96E+03	1,56E-01	3,75E-02	2,63E+02

Table 30: *Parameters and respective values for the construction of the linear calibration function for Cu - Continuation.*

Standard	Conc. (µg/L)	w_i	w_i*x_i	w_i*y_i	$w_i*x_i*y_i$	$w_i*x_i^2$
P 0,0	0,000	3,01E-01	0,00E+00	1,11E+02	0,00E+00	0,00E+00
P 2,0	2,00	1,16E+00	2,32E+00	7,80E+02	1,56E+03	4,64E+00
P 10	10,0	3,44E+00	3,44E+01	7,14E+03	7,14E+04	3,44E+02
P 50	50,0	3,50E-02	1,75E+00	3,17E+02	1,58E+04	8,76E+01
P 100	100	6,14E-02	6,14E+00	1,08E+03	1,08E+05	6,14E+02
Sum	1,62E+02	5,00E+00	4,46E+01	9,43E+03	1,97E+05	1,05E+03
Mean	3,24E+01	1,00E+00	8,93E+00	1,89E+03	3,94E+04	2,10E+02

Table 31: *Calibration standards residual error value (%) for Cu.*

Standard	Conc. (µg/L)	Calculated Signal (cps)	Residual	Residual Error (%)	Calculated [Mn] (µg/L)	|Relative Error| (%)
P 0,0	0,000	3,41E+02	2,94E+01	7,95	1,70E-01	-
P 2,0	2,00	6,87E+02	-1,39E+01	-2,06	1,92E+00	4,00
P 10	10,0	2,07E+03	1,99E+00	0,10	1,00E+01	0,11
P 50	50,0	9,00E+03	4,22E+01	0,47	5,02E+01	0,49
P 100	100	1,77E+04	-1,80E+01	-0,10	9,99E+01	0,10

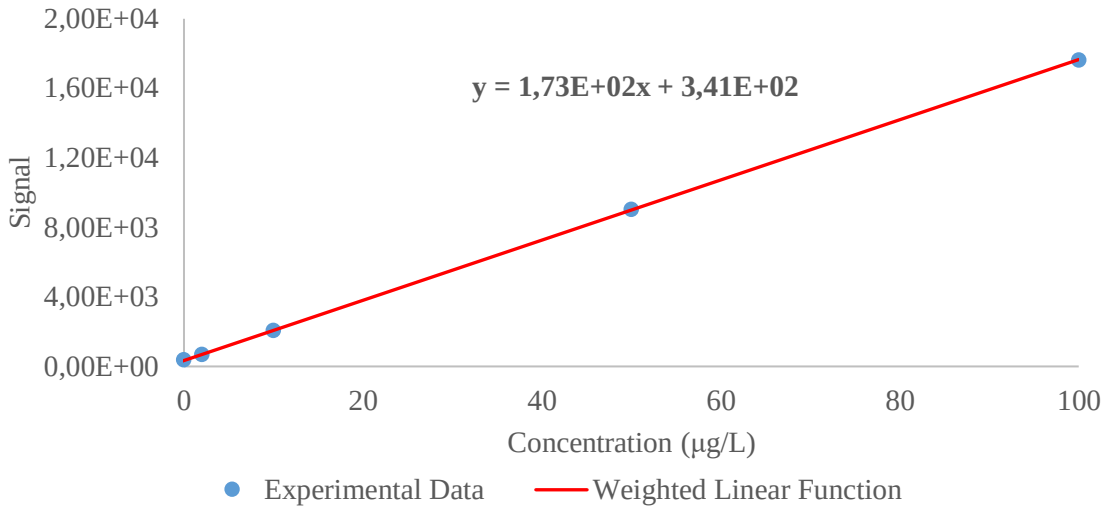

Figure 34: *Experimental data collected and respective linear calibration function for Cu.*

Table 32*: Weighted calibration function parameters for Cu.*

b_w	a_w	r
3,41E+02	1,73E+02	0,999985

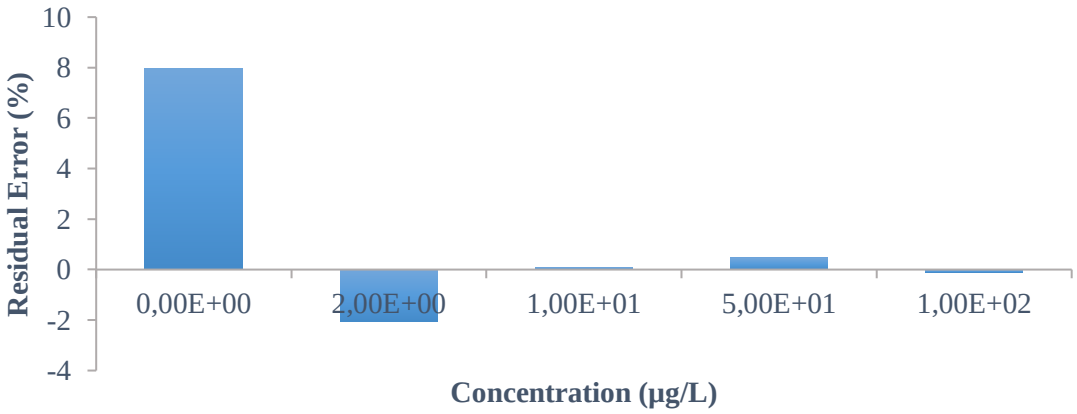

Figure 35*: Residual error (%) for Cu quantification.*

2.4. TRUENESS, PRECISION AND UNCERTAINTY

2.4.1. TRUENESS

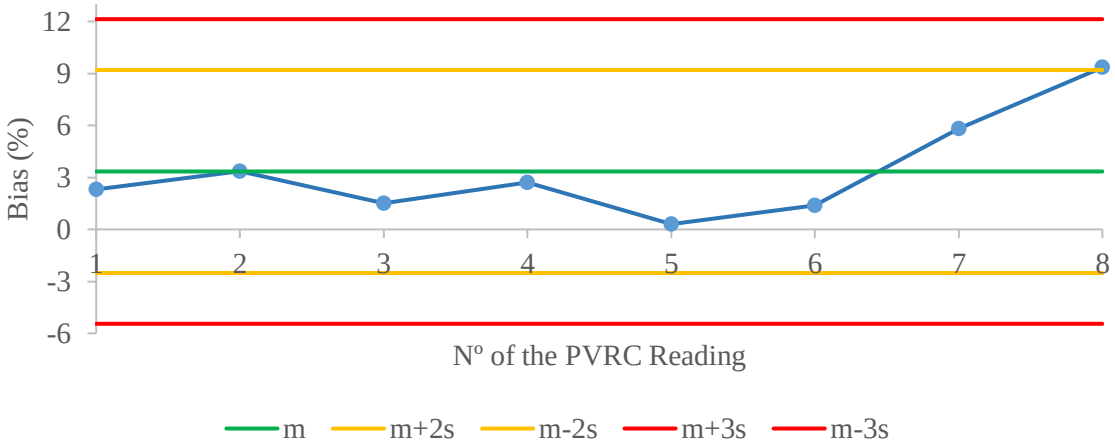

Figure 36: *Control chart of bias (%) for Cu quantification.*

2.4.2. PRECISION

2.4.2.1. SAMPLE VARIATION COEFFICIENT (RELATIVE STANDARD DEVIATION)

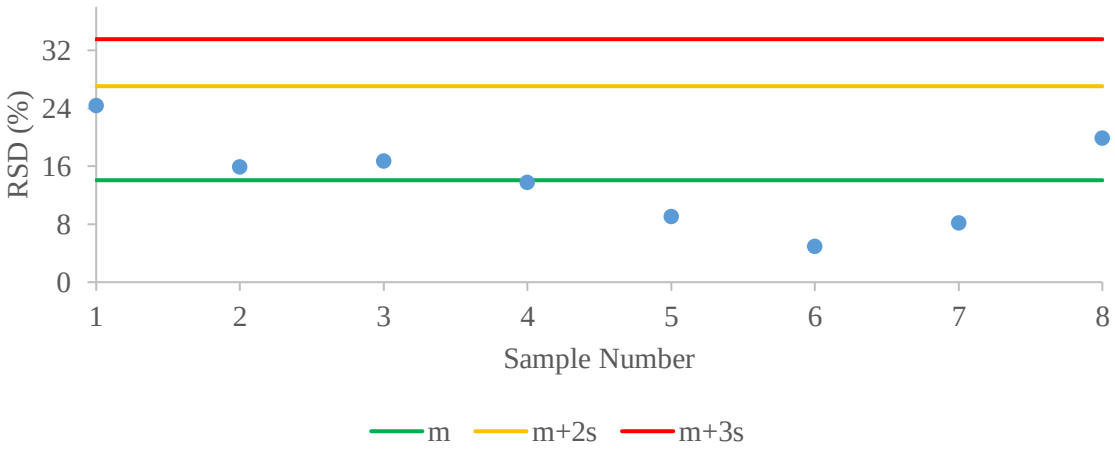

Figure 37*: Control chart of the relative standard deviation (RSD, %) for Cu quantification in Maalox.*

2.4.2.2. REPEATABILITY VARIATION COEFFICIENT

Table 33: *Estimated CVr (%) and HORRATr values for Cu quantification in Maalox.*

	Nº of readings	**CV_r (%)**	**$HORRAT_r$**
Day 1	8	15,7	6,8
Day 2	8	8,1	3,4
Day 3	8	7,8	3,2

2.4.2.3. INTERMEDIATE PRECISION

Table 34: *Estimated values of CVSI (%) and HORRATSI for Cu quantification in Maalox.*

Pharmaceutical Drug	**CV_{SI} (%)**	**p**	**n**	**$HORRAT_{SI}$**
Maalox	18,1	3	23	7,61