DETERMINATION OF POTENTIALLY TOXIC AND ESSENTIAL ELEMENTS IN COUSCOUS FROM ARABIC COUNTRIES USING ICP-MS AND ICP-OES

By

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Abstract

Couscous, which is made of semolina and flour, constitutes a primary food staple in many Arabic countries (such as Libya, Tunisia, Algeria, and Morocco), as well as the second most popular dish in France and other parts of the world. It is thus important to ensure its safety for consumption. This thesis looks at both potentially toxic (As, Se, Cd and Al) and essential elements (Cu, Zn, Fe, Mn, Mg, Mo, P, and S) in couscous, using an approach that can be used for realistic risk assessment. The analysis was carried out by inductively coupled plasma (ICP) optical emission spectrometry (OES) and mass spectrometry (MS), which were both used to assess the bio-accessibility of potentially toxic and essential elements in couscous. An ICP-MS instrument equipped with a collision-reaction interface (CRI) was used to mitigate the impact of polyatomic interferences. Closed vessel digestion was performed for the determination of total concentrations. A conventional batch method, involving artificial saliva, gastric juice and intestinal fluid as extracting agents, was used to provide a good approximation of the bioaccessibility of potentially toxic and essential elements in couscous. The results revealed that the majority of elements were released by gastric juice and then saliva. In contrast, no detectable amount of elements was released by artificial intestinal juice. Both the total and bio-accessible concentrations of elements, which are all at levels making couscous safe for consumption, vary greatly, likely depending on geographic location and perhaps on the several food processing steps.

Dedication

The thesis is dedicated to my loving late mother **Aisha** for her unconditional love and support. Without her love and prayers, it would not have been easy for me to come this far. I also would like to dedicate this work to my late father Piolet **Saeed** and late brother-in-law **Abdallah Mamlouk**, whom I have always looked up to. They are memorable people for me forever.

It is deep in my heart and extremely dedicated to my beloved wife **Mrs. Jamila Mamlouk** who gave me continuous support during this time. I love you more than you will ever know, thank you! Moreover my thanks go to all my sisters, especially **Suad**, brothers, family, mother-in-law, and children **Ritaj, Saeed, Mahmead, Mahmoud, Malik** and **Abdurrahman** who have been constant sources of joy and pleasure.

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List of Abbreviations

Algeria: DZA

As (III): Arsenite

As (V): Arsenate

CRI: Collision/reaction interface

CRC: Collision/reaction cell

CRM: Certified reference material

DDW: Doubly deionized water

DMA: Dimethylarsinic acid

EU: The European Union

EPA: Environmental Protection Agency (based in the United States)

GF-AAS: graphite furnace atomic absorption spectrometry

HG-AAS: hydride generation atomic absorption spectrometry

HG-AFS: hydride generation atomic fluorescence spectrometry

ICP-MS: inductively coupled plasma mass spectrometry

ICP-OES: optical emission spectrometry

IARC: International Agency for Research on Cancer

ICP-MS: Inductively coupled plasma mass spectrometry

LYA: Libya

LYAHB: handmade Libyan couscous B

DL: Detection limit

MMA: Monomethylarsonic acid

MS: Mass spectrometry

Mar: Morocco

PEEK: Polyether ether ketone

PTFE: Polytetrafluoroethylene

Tun: Tunisia

TPN: total parenteral nutrition

Chapter I

Introduction

1.1. Research overview

Food is the primary source of essential elements for humans, but can also be an important source of toxic elements. In this project, the goal is to investigate the value of essential elements (Cu, Fe, Mg, Mn, Mo, P, S, and Zn) and the level of potentially toxic elements (As, Cd, Se and Al) in couscous, which have not been studied before. A previous study indicated that rice [1-3], wheat, seafood [4, 5], vegetables [3], fruits [3] and other crops grown in soils contaminated with toxic heavy metals contained high concentrations of potentially toxic elements [6]. Potentially toxic elements in couscous may come from the food ingredients (semolina, water, flour, and salt). The samples used in this study have been collected from a local Libyan market. For accurate risk assessment, determination of the bio-available toxic elements in couscous should ideally be performed. "Bioavailability is defined as the fraction of an administered dose that reaches the central (blood) compartment from the gastrointestinal tract", [7] which is determined using in vivo methods. For the purpose of studying exposure to toxicants, or risk assessment, an in vitro method of analysis is preferred over in vivo because the in vivo method involves the determination of the toxicants within the blood stream [8], which is difficult to perform. On the other hand, an in vitro method looks into how much potentially toxic elements are leached by gastrointestinal juices from the sample, i.e. bioaccessibility. In the worst case scenario, when all that is dissolved in the gastro-intestinal tract ends up in the blood stream, the amount leached by these juices is equal to the amount that is bioavailable [9].

Metals and metalloids contaminated environments can pose serious threats to health and ecosystems. For example, arsenic, cadmium, lead, mercury, silver, etc. may cause conditions including

hypophosphatemia, heart disease, liver damage, cancer, neurological, cardiovascular diseases, central nervous system damage, and sensory disturbances.

National governments and international organizations play a major role in enforcing food safety standards through different ways, such as laws, regulations, inspections and the establishment of threshold values. Both regulating and renewing food safety guidelines help to improve the health of citizens of all nations. The European Union (EU), World Health Organization (WHO) and the United States Environmental Protection Agency (US EPA) are examples of organizations that have been regulating the contamination of food with toxic elements [12, 13]. Such a process notably increases people's awareness and knowledge about protecting themselves from contaminated food, water, etc. [10, 11]. The main goal of all of these regulations is to limit toxic intake, to improve the detection of problematic foods prior to their consumption and to provide realistic and valuable guidelines to protect public health. In this context, the level of essential and potentially toxic elements must be determined routinely in consumer food products. For this purpose, different atomic spectrometry techniques such as inductively coupled plasma (ICP) mass spectrometry (MS) and ICP optical emission spectrometry (OES) have been used [14].

1.2. Couscous

The term "couscous" refers to processed couscous semolina destined for direct human consumption. Couscous is culturally well-accepted by populations living in North Africa (such as Libya, Tunisia, Algeria and Morocco) as well as the second most popular dish in France and other parts of the world [15]. Historians have conflicting opinions regarding its origin. Today with numerous traditions and cultures covering several regions around the world, it continues to be one of the most delicious and attractive dishes [16].

To prepare couscous, the semolina is sprinkled with water and rolled by hand to form small pellets, sprinkled with dry flour to keep them separate, and then sieved. Any pellets that are too small to be finished granules of couscous fall through the sieve and are again rolled and sprinkled with dry semolina and rolled into pellets (figure 1.1). This process continues until all the semolina has been formed into tiny granules of couscous. This process is labor-intensive. In the traditional method of preparing couscous, groups of women came together to make large batches over several days, which were then dried in the sun and used for several months [17]. Couscous was traditionally made from the hard part of the durum, the part of the grain that resisted the grinding of the millstone [18, 19].

Figure 1.1: Couscous

 In modern times, couscous production is largely mechanized. Commercially-available couscous is similar to that produced by traditional preparation methods. Couscous preparation requires seven steps: (i) blending; (ii) agglomeration; (iii) shaping; (iv) steaming; (v) drying; (vi) cooling; (vi) grading; and (vii) storage. Kernel hardness and semolina protein, gluten, and particle size are important to the quality of couscous (Table 1) [15].

Table 1.1**:** Couscous semolina size [1].

Kernel/semolina size	Particle size range (µm)
Fine semolina	130-183
Medium semolina	183-475
Course semolina	475-700

To be safe for human consumption, couscous should not contain pathogenic micro-organisms and poisonous or deleterious substances, such as residues of hormones, antibiotics, pharmacologically-active chemicals, pesticides residues and toxic elements, in amounts that may represent a health hazard [15].

1.3. Bioavailability and Bioaccessibility

There are many different definitions of bioavailability in nutritional science. This should be taken as an indication of the complexity of the problem. The definitions and concepts of bioavailability and bioaccessibility are essentially important in evaluating the risks that are related to oral exposure to environmental contaminants [20]. Bioavailability means the rate and extent to which the active ingredient or active moiety is absorbed from a drug product and becomes available at the site of action. In nutritional science, the term "bioavailability" encompasses the sum of impacts that may reduce or foster the metabolic utilization of a nutrient [21]. Bioavailability in this sense can be quantified by the rate at which deficiency symptoms are cured or by the weight gain during growth.

Bioavailability fundamentally depends on bioaccessibility, which is defined as the fraction of the toxicant that is extracted from the matrix (dissolved) by saliva and digestive juices in the gastrointestinal tract and is, therefore, available for absorption [22]. Thus, determining the bioaccessibility of a contaminant from its matrix can be seen as a potential indicator for the maximum bioavailability of the

contaminant in the body and is, therefore, important in hazard assessment [23]. What is not absorbed by the body is excreted via renal-biliary or pulmonary processes [24].

1.3.1 Factors affecting bioavailability and bioaccessibility

Because the human body is controlled by complicated processes, bioavailability and absorption vary considerably depending on many factors. Exogenous factors include food matrix, the chemical form of a nutrient and co-ingested compounds, which can increase or decrease solubility, absorption and dosage. Endogenous factors include nutrient status, gastrointestinal disorders, and functional status of the gastrointestinal tract, systemic factors and liver and kidney functions. Most approaches used to measure bioaccessibility are static gastrointestinal assays, which mimic transit through the human digestive system. Because static models are easier to implement than dynamic models [25], only a few researchers have implemented dynamic gastrointestinal procedures, which simulate the gradual transit of extracted food mixtures through the simulated physiological conditions in the digestive tract of the human body. An example of the in-vitro method is the "Simulator of the Human Intestinal Microbial Ecosystem" (SHIME), which uses human gastro-intestinal microbiota to determine the bioaccessible fraction of a compound. The main concern with the different approaches that are available is the wide range of bioaccessibility values that result because of different laboratory conditions. Thus, research surrounding kinetics and metabolism of elements in both humans and animals is not simple or straightforward.

1.4 Bioavailability and bioaccessibility determination methods

There are numerous digestion techniques that are mentioned in the literature, which may lead to different results; care must be taken when comparing results where different digestion techniques have been used [26]. The main objectives of all bioavailability assays are identical, i.e. to obtain the best probable approximation of the amount of available toxins that may cause effects to the organisms [27].

Generally, a response is monitored after feeding the subject with a certain dose of contaminants, and the changes in concentration are measured in end products as a function of time [28]. This may include the determination of the contaminants in target organs [22]. Although, human studies are the best to assess the risk to humans, they are constrained by ethical considerations. Moreover, animal based bioavailability methods are time consuming and costly, not to mention that the selection of animals with comparable gastrointestinal tract, metabolism, etc. as those of humans is somewhat difficult [29]. Moreover, the probable interaction of contaminants with other components in the food and the variability between individuals are weaknesses of in vivo studies [30]. An in vitro study offers a simple, inexpensive, rapid and convenient means of evaluating bioaccessibility [31]. A major advantage of this technique is that there is no ethical constraint [32]. Nevertheless, the in vivo approach is considered as the most accurate method when assessing the actual chemical hazard to humans [27]. A number of different analytical approaches have been used to determine the overall levels of trace elements contamination in nonfoods and foods, including inductively coupled plasma mass spectrometry (ICP-MS), which is regarded as being appropriate for all elements; ICP optical emission spectrometry (OES) (often called atomic emission spectrometry); hydride generation atomic absorption spectrometry (HG-AAS) [33], hydride generation atomic fluorescence spectrometry (HG-AFS) [34], and graphite furnace atomic absorption spectrometry GF-AAS [35]. In the context of this research, ICP-MS and ICP-OES were used to determine the levels of potentially toxic and other trace elements in couscous. ICP-MS is a powerful method to quickly conduct trace and ultra-trace multi-elemental analysis [30], although there are instances where, due to cost or availability, other analysis methods have been used [36, 37]. Nonetheless, the usage of ICP-MS for routine analysis has increased because it offers advantages relative to other techniques. Importantly, ICP-MS is recognized as a high sensitivity multi-elemental technique offering very low detection limits for many elements in the Periodic Table, a large linear

dynamic range, simpler spectra compared to those in ICP-OES [38], and isotope measurement capability. These features allow the fast determination of numerous elements, thereby ensuring a high sample throughput [39, 40]. As a result, it has become the analytical technique of choice in a wide variety of application areas such as nuclear industry, environmental analysis, biomedical applications, food analysis, geochemical analysis, petrochemical industry, and metals analysis [39, 40].

1.5. Issues with inductively coupled plasma mass spectrometry

An ICP-MS instrument involves sample introduction, aerosol generation, and ion production in an argon plasma source, followed by separation of ions according to their mass-to-charge ratio (m/z) and their detection.

1.5.1 Spectroscopic interferences

One of the major problems in ICP-MS is the occurrence of spectroscopic interference that can prevent the trueness of some trace element analysis [41]. Spectroscopic interferences are caused by atomic and polyatomic ions with the same m/z as the ion of interest. They include isobaric interferences, polyatomic interferences and interference from doubly charged ions. These interferences are particularly problematic in the case of monoisotopic elements, such as As, as there is then no alternative isotope available for the measurement. It also leads to a significant decrease in sensitivity when the alternative isotope that is interference-free is much less abundant than the most abundant isotope (such as for V, K, etc.). Several ways can be used to overcome these interferences, such as using a high-resolution mass analyzer, which involves double focusing of the ion beam with both an electrostatic analyzer, which acts as mass filter, and a magnetic sector for m/z selection [38, 42]. A high-resolution mass analyzer can resolve several polyatomic interferences but not isobaric ones, where ions of different elements have the same m/z (such as Fe and Ni at m/z 58). The latter may be mitigated with sample pre-treatment if the

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analyte and isobaric interferent have different chemical properties. While double-focusing sector field ICP-MS may be the most straightforward for resolving several spectroscopic interferences, it has a high operational cost, which is more expensive than that of a quadrupole mass spectrometer, and requires more extensive maintenance [43, 44]. Table 1.2 gives examples of polyatomic interferences encountered on the most abundant isotopes of selected analytes determined during this work [45].

Table 1.2: Some examples of interferences in quadrupole-based ICP-MS.

Interfered analyte	Polyatomic ion
$^{75}As^+$	$^{40}Ar^{35}Cl^+$, $^{38}Ar^{37}Cl^+$ and $^{38}Ar^{36}ArH^+$
$^{80}Se^+$	$^{40}A^{40}Ar^+$
$^{52}Cr^+$	$^{40}Ar^{12}C^+$, $^{36}Ar^{16}O^+$ and $^{35}Cl^{16}O^1H^+$
$^{56}Fe^+$	$^{40}Ar^{16}O^+$, $^{40}Ca^{16}O^+$
$^{27}Al^+$	$^{12}C^{15}N^+$, $^{12}C^{14+}H^+$
$^{40}Ca^+$	$^{40}Ar^+$
$^{51}V^+$	$^{35}Cl^{16}O^+$

Collision reaction cell (CRC), dynamic reaction cell (DRC) or collision reaction interface (CRI) are other approaches that can be used to minimize spectroscopic interference. Each technique has its own benefits and limitations. A CRC can be used to resolve an isobaric interference if only the interferent or the analyte reacts with the reaction gas to form an ion with m/z free of interference [46]. The CRI (used in this work) involves the introduction of reaction gas directly into hollow sampler and/or skimmer cones, as shown in Figure 1.2 [47]. This CRI technology reduces common polyatomic interferences on elements such as As, Se, Cr, V and Fe, thus achieving lower detection limits, even for samples with complex matrices. Unlike other interference management systems, the CRI does not use a pressurized quadrupole, hexapole or octopole prior to the mass analyzer. Instead, reaction and collision gases are

injected directly into the plasma through the tips of the interface cones to induce collisions or ion-molecule reactions with interfering ions. This innovative approach reduces argon-based polyatomic interferences before the interfering and analyte ions are extracted into the ion optics. Plasma conditions at the interface cone apertures favour collisions and reaction. The high plasma density and the high temperature lead to a high collision-reaction frequency between ions and the injected gases. As polyatomic ions are significantly larger than analyte ions, they undergo more collisions with the CRI gas. Hydrogen and helium are used as CRI gases, as these gases provide efficient interference attenuation, and avoid the need to use expensive or corrosive gases such as methane or ammonia. In this thesis work, a quadrupole-based ICP-MS instrument (Figure 2.1) was used for the determination of the bioaccessible fraction of As from couscous samples. Indeed, using H_2 as CRI gas was reported to efficiently curtail most of the carbon-argon or chlorine-argon based polyatomic interferences [48]. However, the CRI gas can also affect analyte ions, in particular their focusing. Therefore, a compromise CRI gas flow rate is vital in order to curtail interferences without endangering instrumental sensitivity for analyte measurement [49].
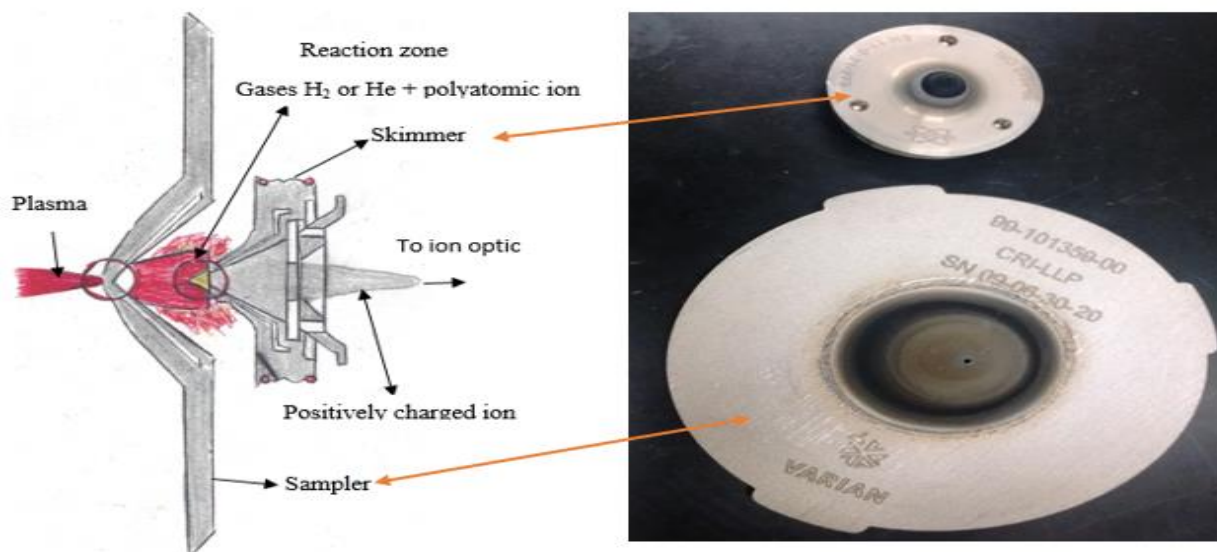
Figure 1.2: CRI system

1.6. Physical and chemical properties of trace elements

Heavy metals are present almost everywhere in the environment as a result of either natural processes or human activity [50]. Human beings are exposed to them through numerous pathways. Major sources of heavy metal contamination are wastewater irrigation, solid waste disposal, sludge applications, vehicle exhaust and unsafe industrial practices. Increased presence of heavy metals in agricultural soils does not only result in the contamination of soil but also ends up in an increased uptake by crops to elevated levels, which in turn creates a serious risk to food quality and human health. The presence of these metals in soils creates a serious potential risk to human health as intake of contaminated food is one of the major pathways into the human body [51]. There are 90 naturally occurring elements, which are heavy metals, but not all of them are biologically significant. Based on their solubility under physiological conditions, 17 heavy metals may be available for living cells and are of importance for organisms and ecosystems [52]. The heavy metals are defined as "metals with a density higher than 5 g cm^{-3}". The metalloids exert different toxic effects than the metals because they have different chemistries. Metals are predominantly present as cationic species and metalloids are predominantly present as anionic species. Elements such as Cu, Zn, and Fe are essential for normal plant growth and development since they are constituents of many enzymes and other proteins. However, elevated concentrations of both essential and nonessential metals can result in growth inhibition, and toxicity symptoms have classified metals into three categories on the basis of their biological functions and effects [53].

- The essential metals with known biological functions [viz., Na , K, Mg, Ca, V, Mn, Fe, Co, Ni, Cu, Zn, Mo, and W]

- The toxic metals [viz., Ag, Cd, Sn, Hg, Pb, Al] and metalloids [viz., Ge, As, and Sb]

- The nonessential, non-toxic metal with no known biological functions [viz., Rb, Cs, Sr, and Ti]. These metals are sometimes found in accumulated forms in the cells as a result of nonspecific sequestration and transport [4].

1.6.1 Arsenic

Arsenic is found widely in surface and ground waters in many parts of the world. It is a naturally occurring element in soil and minerals. Particularly, the substance is linked with those ores mined for metals, namely copper and lead, and therefore, during ore mining and smelting processes, the element may enter into the environment. Arsenic is one of the most toxic elements on earth and is listed as the third substance in the US Toxic Substance and Disease Registry after Pb and Hg [54]. It is an element that exists in several oxidation states: -3, 0, +3 or +5. The main uses are in agricultural chemicals, such as pesticides, herbicides, cotton desiccants and wood preservatives, and as additives to animal feeds, as well as in pharmaceutical products, all of which have a direct impact on the environment [55]. In fact, approximately 80% of the As in agricultural areas used to come from such supplements. Nowadays, this percentage has dropped for many reasons, such as people's awareness along with many regulations and policies [16]. In 1993, the World Health Organization (WHO) reduced the provisional guideline limit in food from 50 to 10 µg/L [56]. This was based on the observation that, at concentrations of 10 µg/L, there is a risk of 1 extra case of cancer per 100,000 individuals. Whereas the developed countries follow this standard, the developing countries still limit the groundwater As concentration at 50 µg/L. Arsenic primarily enters the human body through consumption of groundwater since it is tasteless and odor-free. Small mass transfer of As to water is required for As toxicity to be manifested in humans. A high level of As has been measured in the ground water of some countries: 60 − 100 million people in Bangladesh and India are exposed to inorganic arsenic contaminated drinking water, which in time may result in widespread illness in these areas. People living in these regions are experiencing the toxicity of As from

that source [17]. Similar situations exist in China, Taiwan, Vietnam, and Nepal [57]. Arsenic compounds are best known historically for their toxicity. Arsenic trioxide (As_2O_3) was used as a homicidal agent. Its popularity peaked in the fifteenth and sixteenth centuries, and this potent killer was described as the Poison of Kings and the King of Poisons, or Poudre de succession, "inheritance powder" [20]. The biological availability, toxicity, and transport mechanisms of arsenic depend markedly on the chemical form in which the element is present. The inorganic forms of As, which are the dominant forms in surface and groundwater, are the most toxic forms, while the organic forms common in fish products are much less toxic. Of all arsenic species, arsine (AsH_3) is known to be the most toxic, followed by inorganic arsenic forms [As (III) and As (V)], both of which are more toxic than organoarsenicals. With this taken into account, a number of vital systems within the body are affected by the accumulation of arsenic species, with chronic exposure to inorganic arsenic potentially causing various health-related problems in terms of cardiovascular system, gastrointestinal and respiratory tracts, hematopoietic system, liver, nervous system, and skin. Moreover, the levels of As species toxicity in human keratinocytes cultures were ranked in the following order: As (III) > monomethylarsine oxide MMAO (III) > complex dimethylarsinous acid with glutathione DMA (III) GS > DMA (V) > MMA (V) > As (V) [57]. Chronic exposure to excessive levels in drinking water or in arsenic-containing drugs is associated with an increased incidence of keratinization and pigmentation of the skin together with an increased risk of skin cancer [57].

1.6.2. Cadmium

Cadmium (Cd) is mainly present in the earth's crust, usually found combined with zinc, lead, and copper ores. It also occurs as a by-product of zinc, lead and copper extraction from different minerals. Cadmium is between Zn and Hg in the Periodic Table. It shares some properties with the essential element Zn, its lower atomic weight neighbor, and with the toxic element Hg, its higher atomic weight

neighbor. Cadmium is used as a yellow/orange pigment and as an anticorrosion agent in steel, as a plastic stabilizer, as an electrode material in batteries and as a semi-conductor in Cd-chalcogenide solar cells [58]. Notable levels of Cd entered soil as a direct result of agricultural and industrial activities [59]. Moreover, vapor from industrial operations [50] undergoes an immediate reaction producing cadmium oxide in the air, which then reacts with water vapor, carbon dioxide, and other gases to produce various cadmium salts. The main route of exposure is through nutrition and smoking or inhalation of particulate material. Dietary Cd is more concentrated in some food items such as shellfish, offal, grains, and seeds. Some crops, such as rice, soybeans, or wheat, are more likely to accumulate cadmium from polluted soils than others [60]. Cadmium is classified as a human carcinogen [59].

1.6.3 Selenium

Selenium (Se) is a non-metallic element that can be found as a red amorphous powder, a red crystalline material, and a grey crystalline material. It has a good conductivity of electricity and is used in photo and solar cells [61]. It is mainly used in the glass industry [62]. Some Se compounds are added to anti-dandruff shampoos. Both inorganic and organic Se compounds can be found in the environment. Se is found in soil as a result of human activities, such as mining, and volcano activity [63]. Although there is not a wealth of understanding concerning the metabolism of Se in the human body, it is nevertheless considered to be an essential element, as it is involved in proteins, i.e. selenoproteins [64]. Until recently, the only known metabolic role for Se in mammals was a component of the enzyme glutathione peroxidase, which, together with vitamin E, catalase, and superoxide dismutase, is a component of one of the antioxidant immune systems of the body [65]. On top of that, there is proof that an additional selenoenzyme protein is involved in the synthesis of the hormone triiodothyronine from thyroxine [66].

A deficiency in Se was first observed with Keshan disease, which was believed to be instigated through low Se levels in soil in Keshan (China) [67]. An increased incidence of Keshan disease has now been associated with low Se levels in staple cereals, resulting in a low intake of Se, sometimes lower than 10 μg Se/day [68]. High levels of Se can be toxic to people, although this ultimately depends on the species of Se. Chronic Se poisoning in people is characterized primarily by loss of hair and changes in fingernail morphology [69]. In some cases, skin lesions (redness, blistering) and nervous system abnormalities (paresthesia, paralysis, hemiplegia) are also observed. Toxicity is also linked with thyroid hormones syntheses [70]. The biochemical mechanisms of Se toxicity have not been clearly established.

1.6.4. Aluminum

Aluminum is among the most plentiful elements in the earth's crust, accounting for 8% of the total [71]. Exposure to aluminum increased markedly as its production increased rapidly in the 20th century, and may also have increased since both the solubility and the bioavailability to plants and aquatic life of environmental Al may have been increased by acid rain and industrial emissions [72]. Aluminum powders are used in pigments and paints, fuel additives, explosives, and propellants. Food-related uses include preservatives, fillers, coloring agents, anti-caking agents, emulsifiers and baking powders; soy-based infant formula can contain Al. There is no substantiated evidence that Al has any essential function in animals or humans [73]. Food is the main intake source of Al, followed by drinking water [74]. However, chronic use of antacids, buffered aspirins and other medical preparations would likely constitute the major uptake source, even when exposed at work. Locally increased concentrations of Al occur in the brain of patients with Alzheimer dementia [75].

1.6.5. Copper

Copper is a soft, malleable and ductile metal with very high thermal and electrical conductivity. It is found as a pure metal in nature. It is used as a conductor of heat and electricity. The major applications of copper are electrical wire, roofing and plumbing, industrial machinery, and as a constituent of various metal alloys, such as jewelry and coins [76]. Copper is essential to all living organisms as a trace dietary mineral because it is a key constituent of the respiratory enzyme complex cytochrome c oxidase. In humans, copper is found mainly in the liver, muscle, and bone [77]. Many of the well-established physiological functions of copper in the body arise directly from its role in a number of copper-containing metalloenzymes [78]. A variety of symptoms have been associated with Cu deficiency in animals, many of which are seen also in humans; they include hypochromic anaemia, neutropenia, hypopigmentation of hair and skin, abnormal borne formation with skeletal fragility and osteoporosis, vascular abnormalities and crimped or steely hair [77]. There is no single specific indicator of Cu in humans; acute Cu poisoning is rare and usually results from contamination of foodstuffs or beverages by Cu containers or from the accidental or deliberate ingestion of gram quantities of Cu salts [79]. Symptoms of acute Cu poisoning include salivation, epigastric pain, nausea, vomiting and diarrhea [80].

1.6.6. Phosphorus

Phosphorus exists in two major forms, white phosphorus and red phosphorus [81]. Phosphorus is an essential and important macro-mineral. It normally makes up about 1% of total body weight. Phosphorus is required for normal functioning of every cell in the body but most of it, approximately 85%, is bound with calcium to form bones and teeth [82].White P is extremely toxic to humans, while order forms of P are much less toxic [83]. Most P is used in the production of phosphoric acid and phosphates, which are used in the fertilizers industry. Our bodies need a balance of Ca, P, and Mg. If this balance is not

maintained, our health is affected. Phosphorus is absorbed in the small intestine, and excess is excreted by the kidneys. Most P is essential for life [84]. Phosphates are a component of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), adenosine triphosphate (ATP), and the phospholipids, which form all cell membranes [85]. In medicine, phosphate deficiency is called hypophosphatemia, the symptoms of which include neurological dysfunction and disruption of muscle and blood cells due to lack of ATP, growth retardation, infertility, and possibly retention of placenta. Too much phosphate can lead to diarrhea and calcification (hardening) of organs and soft tissue, and can interfere with the body's ability to use Fe, Ca, Mg, and Zn. Ca needs P to build strong bones [86].

People can be exposed to phosphorus in the workplace by inhalation, ingestion, skin contact, and eye contact [92]. Acute oral exposure to high levels of white phosphorus in humans is characterized by vomiting, abdominal cramps, and pain, along with kidney, liver, cardiovascular effects [87, 88].

1.6.7. Molybdenum

Mo is a silvery white, very hard transition metal, but is softer and more ductile than W. It was often mixed with graphite and Pb ore. Some molybdenite is obtained as a by-product of W and Cu production. It has a high elastic modulus. Molybdenum has one of the highest melting points of all pure elements. Molybdenum is used in alloys, electrodes, and catalysts. Molybdenum is an essential trace element in plant nutrition. Some lands are barren for lack of this element in the soil. Molybdenum differs from the other micronutrients in soils in that it is less soluble in acidic soils and more soluble in alkaline soils, the result being that its availability to plants is sensitive to pH and drainage conditions. Molybdenum is essential to all species. As with other trace metals, though, what is essential in tiny amounts can be highly toxic at larger doses. Animal experiments have shown that too much Mo causes fetal deformities. Fodder with more than 10 mg/L of Mo would put most livestock at risk [90]. The amount of Mo

required is relatively small, and Mo deficiency usually does not occur in natural settings. However, it can occur in individuals receiving parenteral nutrition [91]. Parenteral nutrition, also known as intravenous feeding, is a method of getting nutrition into the body through the veins. While it is most commonly referred to as total parenteral nutrition (TPN), some patients need to get only certain types of nutrients intravenously. Parenteral nutrition is often used for patients with Crohn's disease, cancer, short bowel syndrome, and ischemic bowel disease. In animal experiments, too much Mo Leads to joint pains in the knees, hands and feet, articular deformities, erythema, and edema of the joint areas [92].

1.6.8. Magnesium

Magnesium is the eighth most abundant element, constituting about 2% of the Earth's crust by weight, and it is the third most plentiful element dissolved in seawater. Magnesium is silvery white and very light. It is used as a catalyst. Magnesium compounds are used as refractory material in furnace linings, for producing metals, glass, and cement and in agriculture [93]. Humans take in between 250 and 350 mg/day of Mg and need at least 200 mg, but the body deals very effectively with this element, taking it from food when it can, and recycling what we already have when it cannot [94]. Symptomatic Mg deficiency due to low dietary intake in otherwise healthy people is uncommon because the kidneys limit urinary excretion of this mineral [95]. Magnesium powder has a low toxicity and is not considered to be hazardous to health. There is no evidence that Mg produces systemic poisoning although persistent over-indulgence in taking Mg supplements and medicines can lead to muscles weakness, lethargy, and confusion [96]. There is very little information available on the environmental effects of magnesium oxide fume. Magnesium powder is not suspected of being highly harmful to the environment [107]. Symptoms of Mg toxicity, which usually develop after serum concentrations exceed 1.74–2.61 mmol/L, can include hypotension, nausea, vomiting, facial flushing, and retention of urine, ileus, depression, and

lethargy before progressing to muscle weakness, difficulty breathing, extreme hypotension, irregular heartbeat, and cardiac arrest [97]. The risk of magnesium toxicity increases with impaired renal function or kidney failure because the ability to remove excess magnesium is reduced or lost [97].

1.6.9 Sulphur

Elemental sulphur is produced all over the world. Canada is the largest exporter [98]. Sulphur is the primary source in the production of sulphuric acid, the world's most used chemical and a versatile mineral acid used as an essential intermediate in many processes in the chemical and manufacturing industries. Sulphuric acid is used by the fertilizer industry to manufacture primarily phosphates, and also nitrogen, potassium, and sulphate fertilizers[99] Sulphur is also used in many other industries including non-ferrous metals, pigments, fibers, hydrofluoric acid, carbon disulphide, pharmaceuticals, agricultural pesticides, personal care products, cosmetics, synthetic rubber vulcanization, water treatment, and steel pickling. Sulphur's unique properties improve the characteristics of asphalt [100]. Sulphur is also a vital nutrient for crops, animals, and people. Like nitrogen, phosphorus, and potassium, sulphur is one of the essential plant nutrients. It improves the use efficiency of other essential plant nutrients, particularly nitrogen and phosphorus. In general, sulphur has similar functions in plant growth and nutrition as nitrogen [101]. Sulphur deficiency is quite rare in nature [102]. Too much sulphur may reduce pulmonary function. Radiological examinations have revealed irregular opacities in the lungs and occasionally modulation has been reported, but not true nodular fibrosis [102]. Chronic exposure to elemental sulphur at low levels is generally recognized as safe. Epidemiological studies show that mine workers exposed to sulphur dioxide throughout their lives often had eye and respiratory disturbances, chronic bronchitis and chronic sinus effects. However, no known risks of oncogenic, teratogenic, or

reproductive effects are associated with the use of sulphur [103]. Also, sulphur has been shown to be non-mutagenic in microorganisms [104].

1.6.10. Zinc

Zinc (Zn) is an abundant element that can be found in soil, water, and food. It is a lustrous bluish-white metal, which is brittle and crystalline at ordinary temperatures [105]. Zinc is an important component to humans as it is involved in a large number of enzymes [106]. It plays an essential role in polynucleotide transcription and translation and thus in the processes of genetic expression [58]. The principal clinical features of severe Zn deficiency in humans are growth retardation, a delay in sexual and skeletal maturation, the development of orificial and acral dermatitis, diarrhea, alopecia, a loss of appetite and the appearance of behavioral changes [107]. An increased susceptibility to infections reflects the development of defects in the immune system. A healthy immune system requires Zn, which can also help to prevent skin problems [108]. There are various problems associated with a high intake of Zn, including diarrhea, dizziness, drowsiness, hallucinations, nausea, and weakness of the immune system. Long-term exposure to high Zn intakes substantially in excess of requirements has been shown to result in interference with the metabolism of other trace elements [109]. Copper utilization is especially sensitive to an excess of Zn. This copper/zinc interaction has been responsible for the inadvertent induction of Cu deficiency, but has also been deliberately exploited to control Cu accumulation in Wilson disease [109]. A Zn intake of as little as 50 mg/day has been shown to influence copper status [58].

1.6.11. Manganese

Manganese (Mn) is an abundant element in the earth's crust. It is present in soil, water and food. It is a pinkish-grey element and is chemically active. Manganese is a key component of low-cost stainless steel

formulations [110]. Both inorganic and organic Mn compounds are present in the environment, although the inorganic form is the most common [111]. For animal, human and plant life, Mn is recognized as fundamental, important for development, growth, and the preservation of good health, and is known to enter the environment through a number of different methods, such as via air and water [112]. Notably, in the aquatic environment, Mn occurs in the forms of Mn (II) and Mn (IV). Manganese has been identified as another element potentially causing health problems amongst Bangladeshi populations through water consumption, as Mn levels higher than the World Health Organization standard (0.400 mg/L) were measured in the groundwater of Bangladesh [113]. A number of fundamental metabolic functions in humans are facilitated by Mn, including energy metabolism, enzyme activation, immunological system and nervous system, blood clotting, the control of cellular energy, and tissue growth [114].

Signs of Mn deficiency include impaired growth, skeletal abnormalities, disturbed or depressed reproductive function, ataxia of the newborn, and defects in lipid and carbohydrate metabolism [115]. Manganese toxicity is most commonly the result of chronic inhalation of large amounts of airborne Mn in mines, steel mills, and some chemical industries [116]. In this regard, it is recognized that Mn toxicity has the potential to cause brain damage, with disease referred to as Manganese and recognized as being not dissimilar to Parkinson's disease [117].

1.7 Thesis Objectives

The aim of this thesis is to determine the levels of potentially toxic elements and essential elements in couscous samples from Arabic countries, which have not been studied extensively before, for quick risk assessment of its safety. This thesis investigates the potential of human exposure to potentially toxic elements (such as As) and evaluates the deficiency of essential elements (such as Zn) through the consumption of couscous samples. Objectives of this thesis are listed below:

- To measure the total concentrations of both potentially toxic and essential elements in different couscous samples by ICP-MS and ICP-OES.

- To determine the bioaccessibility of both potentially toxic and essential species in different couscous samples by using ICP-MS and ICP-OES.

- To compare the total concentration and bioaccessible concentrations with the available food safety regulations to assess the risk to human health.

Chapter II

Experimental

2.1 Instrumentation

For the determination of elements, inductively coupled plasma (ICP) mass spectrometry (MS) and ICP optical emission spectrometry (OES) were used.

2.1.1 Inductively coupled plasma mass spectrometry

A Varian 820MS quadrupole-based ICP-MS instrument equipped with a CRI (Varian Inc., Australia) (Figure 2.1) was used to determine $^{75}As^+$, $^{110}Cd^+$, $^{112}Cd^+$, $^{113}Cd^+$, $^{114}Cd^+$, $^{77}Se^+$, and $^{78}Se^+$. The sample introduction system consisted of a Micro Mist concentric nebulizer (Glass Expansion, Victoria, Australia) fitted into a Peltier-cooled Scott double-pass spray chamber (SCP Science, Quebec, Canada) maintained at 0°C via a computer-controlled Peltier cooling system, and a three channel peristaltic pump (shown in Figure 2.2) are respectively used for ion extraction and ion focusing, which result in high sensitivity and low background. A tuning solution containing 5 µg/L As, Ba and In was used to optimize the torch position, gas flow rates, and ions lens settings daily so as to maximize and stabilize the As signal while minimizing the level of oxide and doubly-charged ions. Typical operating conditions are listed in Table 2.1.

Figure 2.1: Varian 820 ICP-MS instrument
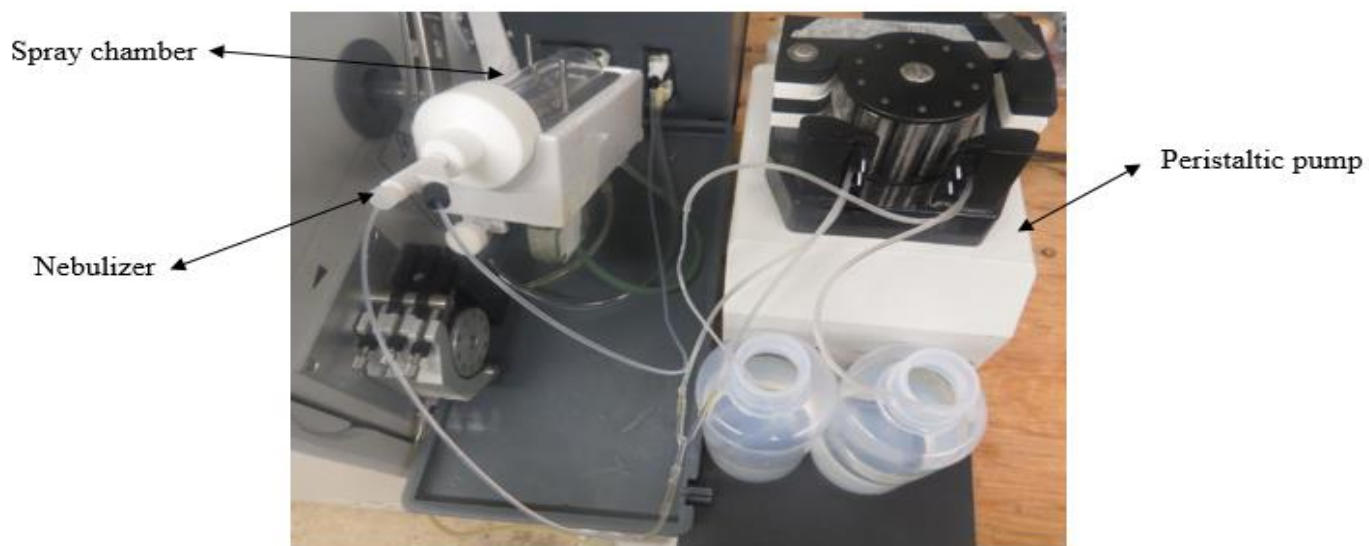
Figure 2.2: Sample introduction system of ICP-MS

2.1.2 Optimization of CRI conditions

Optimization of the CRI gas flow rate is important to reduce interference from polyatomic ions, as too low a flow rate will not effectively mitigate spectroscopic interferences whereas too high a flow rate can reduce the instrumental sensitivity. For the multi-elemental analysis of couscous, a multivariate optimization, of the H2 CRI gas flow rate and other instrumental parameters (shown in table 2.1) was carried out using Minitab 16 statistical software (Minitab Inc.) for the determination of As, in artificial gastric juice, as gastric juice contains higher concentration of carbon and chloride than other gastrointestinal reagents and will thus give rise to higher spectroscopic interference on As. The resulting to the signal of Mn^+ (m/z = 55), which was used as a replacement element that is close in mass-to-charge (m/z) ratio to the target analyte. A gastric juice solution containing 10 μg/L Mn was aspirated and the signal ratio of m/z 52 over m/z 55 was continuously monitored while adjusting the CRI H_2 flow rate. As the CRI gas flow rate was increased, the signals at both m/z 52 and m/z 55 decreased, but that at m/z 52 (from polyatomic interferences) was reduced to a greater extent than that of Mn, resulting in an improved signal-to-background ratio. The CRI H_2 gas flow rate corresponding to the maximum ratio was 80 mL/min, which was adopted for the remainder of this research.

Table 2.1: Optimal ICP-MS and ICP-OES operational parameters used for analysis.

Parameter	ICP-MS	ICP-OES
Ar plasma gas flow rate (L/min)	18	12
Ar auxiliary gas flow rate (L/min)	1.8	1
Ar sheath gas flow rate (L/min)	0.05	-
Ar nebulizer flow rate (L/min)	1.05	1
Sampling depth (mm)	5.8	10
RF Power (kW)	1.4	1.4
CRI skimmer gas	H_2	-
Sample uptake rate (mL/min)	0.8	1
Skimmer gas flow rate (mL/min)	80	-
Dwell time(ms) / Integration time	10	10

2.1.3 Inductively coupled plasma optical emission spectrometry

An ARCOS ICP-OES instrument (SPECTRO Analytical Instruments, Kleve, Germany) fitted with a concentric nebulizer and baffled cyclonic spray chamber (shown in figure 2.3) was used to determine Al, Cu, Fe, Mg, Mn, Mo, P, S, and Zn. The ICP-OES analysis of macro nutrients was performed in lateral viewing mode. Instrumental conditions are listed in Table 2.1.Quantitative analysis was performed using two to five different spectral lines for each element. The following wavelengths (nm) were used: Al: 167.078, 396.152, and 394.401, Fe: 259.941, 238.204, 239.562, and 234.349, Cu:

324.754, 327.396, 224.700, and 219.226, Mg: 279.553, 280.270, 285.213, 277.983, and 279.800, Mn: 257.611, 259.373, 260.569, and 293.930, Mo: 202.095, 203.909, and 204.664, P: 177.495, and 178.287, S: 180.731, and 182.856, Zn: 213.856, 206.200, and 202.613. The concentration was the average of several replicates at a given wavelength. More than one wavelength was monitored for each element in order to verify that they was no unforeseen spectroscopic interference [118, 119].
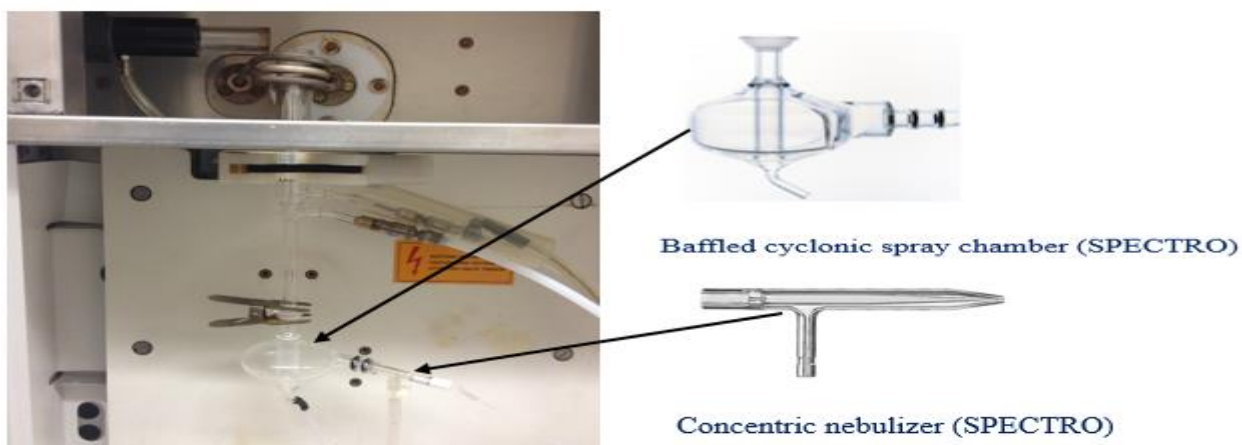
Figure 2.3: Sample introduction system of ICP-OES

2.2 Material and methods

2.2.1 Samples

The samples used in this study were collected from a Libyan (four samples) and Tunisian (four samples) local markets. Two of them were made by traditional method (handmade from Libya), and the rest were produced by different companies from Libya, Tunisia, Algeria, and Morocco (figure 2.4). Couscous samples were stored in clean plastic containers (all containers cleaned before use by rinsing three times with HNO_3 (10% v/v) and three times with DDW. Than kept in clean place at room temperature for further experimental use.

Figure 2.4: Sources of couscous samples (picture taken from Google)

2.2.2 Reagents and standards preparation

Artificial saliva, gastric juice, and intestinal fluid were prepared as follows. For artificial saliva, 6.8 g of KH_2PO_4 (ACS grade; Fisher Scientific, NJ, USA) were mixed with 77 mL of 0.2 mol/L NaOH (ACS grade; BioShop, Burlington, Canada) and diluted to 1 L using doubly deionized water (DDW) (18.2 MΩ·cm), with pH adjusted to 6.5 using 0.2 mol/L NaOH. Artificial gastric juice was prepared by mixing 2.0 g of NaCl (ACS grade; BioShop, Burlington Canada), 3.2 g of pepsin (Sigma-Aldrich, Oakville, Canada) and 7.0 mL of sub-boiled HCl (ACS grade; Fisher Scientific, Ottawa, Canada) and diluting to 1 L using DDW (pH=1.2). For artificial intestinal fluid, 6.8 g of KH_2PO_4, 10 g of pancreatin (Sigma-Aldrich, St. Louis, USA), 77 mL of 0.2 mol/L NaOH were mixed and diluted to 1 L using DDW, with pH adjusted to 6.8. All HNO and HCl were purified prior to use by a DST-1000 sub-boiling distillation system (Savillex, Minnetonka, USA). For the digestion of couscous and residues from the bioaccessibility studies, sub-boiled HNO_3 and H_2O_2 (J.T. Baker, Phillipsburg, USA) were utilized. Standard solutions and an in internal standard solution were prepared from 1000 mg/L stocks (SCP Science, Baie d'Urfé, Quebec, Canada). A 5 µg/L tuning solution in 5% (v/v) nitric acid was prepared by dilution of a 10 mg/L multi-element (Ba, Be, Ce, Co, In, Mg, Pb, Th and Tl) standard solution. All these standard solutions were diluted to 10 mg/L and stored at 4°C in the dark to prevent decomposition or oxidation. For external calibration, standard solutions of 5, 15, 20, 25, 30, 50 and 100 µg/L were made daily by appropriate dilution.

2.2.3 Total digestion

1 g of couscous was mixed with 2.5 mL of distilled concentrated nitric acid and 0.5 mL of hydrogen peroxide and digested for 2 hours at approximately 50˚C, diluted to 25 mL with DDW and finally analyzed by ICP-MS and ICP-OES by continuous nebulization using 5 standard solutions prepared in the same final matrix as that of the diluted digest.

2.2.4 Batch method

About 1 g of couscous sample was placed in a falcon tube and 6 mL of artificial saliva was added. The falcon tube was then put in an automatic shaker for 10 minutes at human body temperature (37°C). The supernatant was decanted off. The procedure was repeated using gastric and intestinal juices each for 2 hours. Figure 2.5 shows a flow chart of the batch method. The shaking time was set to mimic the human digestive tract. These supernatants were quantitatively analyzed by external calibration with internal standardization. Standard solutions were prepared in the corresponding matrix while the internal standard solution (5 ppb In) was prepared in 4% HNO_3. These solutions were mixed on-line through a Y-connector.

1. One gram of couscous sample

2. Add 7 mL saliva
3. Shake and Heat (37 ˚C) for 10 minutes
4. Centrifuge and decant

5. Add 24 mL gastric juice
6. Shake and Heat (37˚C) for 120 minutes
7. Centrifuge and decant

8. Add 24 mL intestinal juice
9. Shake and Heat (37 ˚C) for 120 minutes
10. Centrifuge and decant

11. Add 2.5 mL of HNO_3 and 1 mL H_2O_2
12. Heat (50˚C)
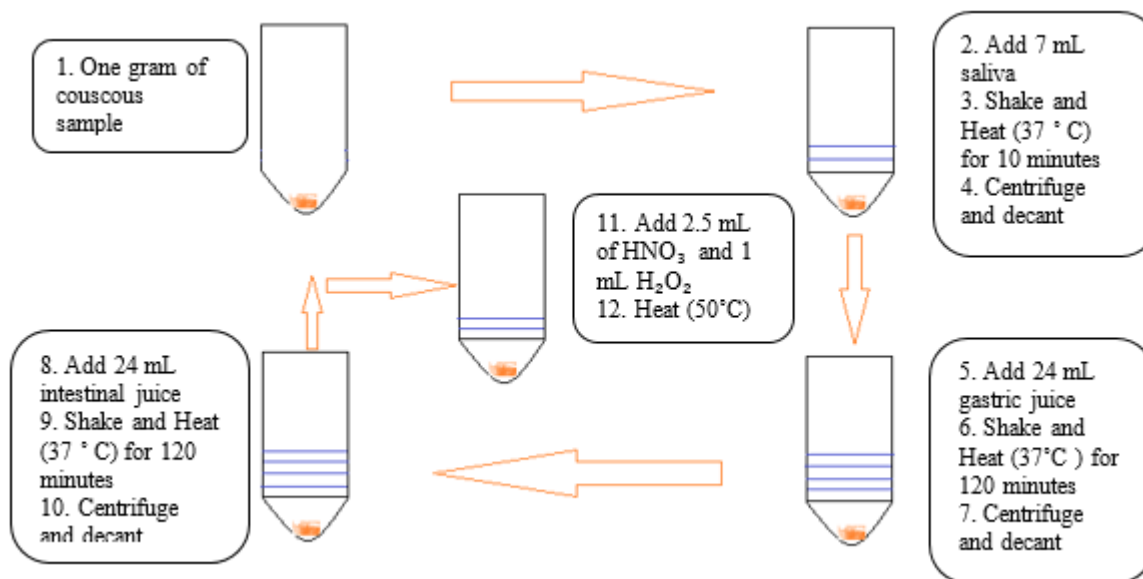
Figure 2.5 Schematic representation of the batch leaching method.

2.2.5 Verification of mass balance

A mass balance was performed on batch residues. This step is important to ensure that the sum of bio-accessible fraction and that remaining in the residue is the same as the total concentration measured separately in the couscous samples. To verify mass balance, 2.5 mL of concentrated sub-boiled nitric acid and 0.5 mL of hydrogen peroxide were used to digest the residue from the batch method. Digestion was done at 50°C for 2.5 hour by using a digestion vessel (Savillex). Each digest was then diluted to 25 mL with DDW. A blank was prepared following the same procedure but without a sample. Standard solutions used for external calibration were prepared in the same matrix. Internal standardization was done through the on-line addition of 5 ppb In through a Y connector.

2.2.6 Quality assurance

All the vessels and flasks were cleaned before use by rinsing three times with HNO_3 (10% v/v) and three times with DDW. All the analyses of couscous samples were repeated five times and the resulting relative standard deviation ranged from 2 to 10%. To monitor contamination during experimental procedures, blanks were used. The series of standard solutions used to establish calibration curves also allowed an assessment of linearity. A standard solution was aspirated after every two samples and at the end to monitor for instrumental drift. External calibration was used for quantitation of total elements concentration

Chapter III

Risk assessment of the safety of couscous

This chapter focuses on the total and bioaccessible concentrations of As and other trace elements in couscous originating mainly from North African countries. One main objective of this study is to assess the variability and trend in As, Cd, Se, Al, and essential elements concentration in couscous. Previous studies have not focused on couscous from this region. .Different varieties of couscous from Libya, Tunisia, Morocco, and Algeria (n=9), were investigated. After the couscous sample was exposed to the various fluids, the residue was digested to verify if any remained. Mass balance was checked by comparing the total amount leached plus that left in the residue with the expected total concentration determined by digestion of the couscous, using a Student's t- test at the 95% confidence level.

3.1. Detection limits and sensitivity

Detection limits for trace elements were calculated based on ten replicate determinations of the reagent blank as three times the standard deviation of the average blank signal divided by the corresponding sensitivity, sensitivity being the slope of the calibration curve. The square of the correlation coefficient was better than 0.997 over the investigated concentration range. Table 3-1 compares the detection limits and sensitivities in different matrices. The reason why there is not a greater difference between the sensitivities of ICP-OES and ICP-MS is the use of CRI in ICP-MS to reduce spectroscopic interferences from polyatomic ions negatively impacts the sensitivity.

Table 3.1: Detection limit (µg/L) and sensitivity (cps/µg/L) for the determination of analytes in different matrices by ICP-MS (As, Cd, and Se) and ICP-OES (Al, Cu, Fe, Mg, Mn, and Zn)

Method	Batch			Total digestion
Matrix	Saliva	Gastric	Intestinal	10% HNO$_3$
As DL	0.04	0.02	0.10	0.20
Sensitivity	300±4	700±16	206±8	800±9
Cd DL	0.02	0.02	0.02	0.05
Sensitivity	5050±50	44100±30	2700±70	9000±90
Se DL	0.02	0.01	0.2	0.3
Sensitivity	200±3	680±10	190±9	720±8
Al DL	0.01	0.001	0.001	0.01
Sensitivity	$(3.0±0.4)×10^4$	$(6.00±0.04)×10^4$	$(6.0±1.2)×10^4$	$(5.0±0.5)×10^4$
Cu DL	0.005	0.01	0.01	0.06
Sensitivity	$(1.0±0.1)×10^5$	$(2.00±0.01)×10^5$	$(2.00±0.02)×10^5$	$(1.00±0.02)×10^5$
Fe DL	0.005	0.004	0.03	0.02
Sensitivity	$(5.0±0.5)×10^4$	$(1.00±0.01)×10^5$	$(1.0±0.2)×10^5$	$(6.0±0.1)×10^4$
Mg DL	0.0006	0.0002	0.0004	0.001
Sensitivity	$(2.00±0.01)×10^5$	$(2.00±0.01)×10^6$	$(2.00±0.04)×10^6$	$(2.00±0.06)×10^5$
Mn DL	0.0004	0.0002	0.0004	0.001
Sensitivity	$(3.00±0.04)×10^5$	$(4.00±0.01)×10^5$	$(4.00±0.02)×10^5$	$(4.00±0.03)×10^5$
Zn DL	0.005	0.002	0.005	0.01
Sensitivity	$(1.50±0.14)×10^5$	$(3.00±0.01)×10^5$	$(3.0±0.5)×10^5$	$(1.3±0.1)×10^4$

3.2. Total concentration of potentially toxic and of essential elements in couscous

A total digestion of couscous using nitric acid and hydrogen peroxide was first conducted to ensure the content of elements being determined was high enough to be detected and to be of concern. The results are summarized in Figure 3-1. These differences between couscous samples likely reflect differences in purity of the ingredients (semolina, flour) grown in different geographical areas, and on whether urea and phosphate-based fertilizer were used. It is well known that phosphate-based fertilizers can contain high levels of different toxic elements including Cd [120].
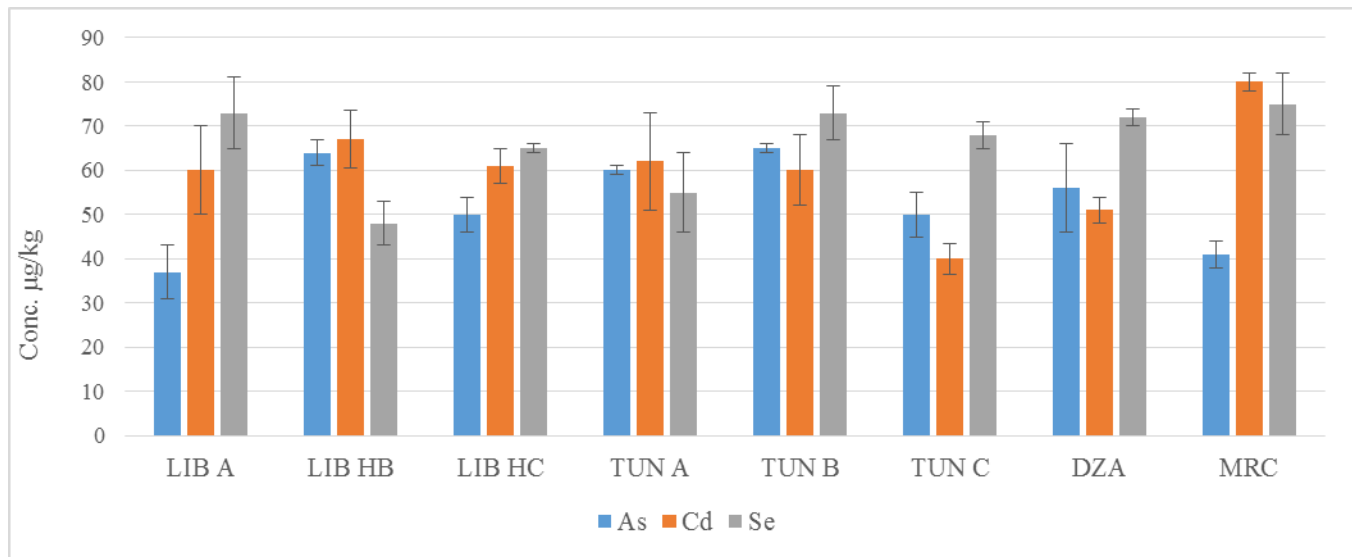
LIB = Libya   DZA = Algeria   TUN = Tunisia   MRC = Morocco

Figure 3.1. Average total concentration (error bar = standard deviation; n=5) of potentially toxic and essential elements in couscous samples from Libya, Tunisia, Algeria, and Morocco

Table 3.2: Total concentration in (mean ± standard deviation, n=5) of Mo and % of P and S in couscous

Sample	Mo µg/kg	%P	%S
LIB A	600 ± 30	0.20	0.14
LIB HB*	410 ± 35	0.16	O.11
LIB HC*	280 ± 10	0.20	0.12
TUN A	261 ±17	0.15	0.12
TUN B	135 ± 15	0.16	0.13
TUN C	310 ± 22	0.40	0.16
DZA	400 ± 51	0.14	0.13
MRC	500 ± 44	0.12	0.12

LIB = Libya   DZA = Algeria   TUN = Tunisia   MRC = Morocco

H*: Handmade couscous

3.3 Bioaccessibility of toxic and essential elements

This is the first bioaccessibility study on potentially toxic elements in couscous samples, which was determined by a batch method. The batch method tries to mimic human gastrointestinal conditions. For realistic risk assessment, it is important to assess the worst case scenario, as if a food is safe under those conditions, it will be safe for everybody. Artificial saliva, gastric juice and intestinal fluid were used as extracting agents to provide a good approximation of the bioaccessibility of potentially toxic and essential elements in couscous. The majority of the bioaccessible fraction of all elements was released

by gastric juice, as evident in Figure 3.2 (A, B, C, D, and E) and Table 3-2. This is not surprising because it is similar to digestion in the human stomach, where the residence time for food is relatively long (a couple of hours) and the low pH of gastric juice significantly increases metal solubility [121]. In contrast, no detectable amount of elements was released by artificial intestinal juice.

Only a portion of the total concentration was found to be bioaccessible for several elements. For example, between 20 to 60% of As was bioaccessible from Tunisia and Morocco couscous, while Se bioaccessible values were within 35 to 68% for the same samples. In contrast, the Al bioaccessible values ranged between 60 to 100 %. Different behaviors were noticed for essential elements. Interestingly, Cu and Zn were only leached by gastric juice but not by saliva and intestinal juice. In contrast, Mn and Mg were released by all fluids. The amount of Mn, and Mg extracted gradually decreased in the order gastric juice, saliva, and intestinal juice. Like several other elements, Fe was released more by gastric juice than by saliva and intestinal juice. It has been previously reported in the literature that the process of absorption of dietary Fe in the body is a dynamic process and that the portion absorbed is very low in comparison to the ingested amount [122]. This process depends on the type Fe and its source. The portion of absorbed Fe typically ranges from 5 to 35 % [123]. In this work, the bioaccessible percentage of Fe ranged between 8 to 40%, which is in good agreement with the above-mentioned range for absorbed Fe. In addition, the amount of absorbed Fe may be influenced by Al, which interacts with a number of elements, such as Ca, F, Fe, Mg, P, and S and, when ingested in excess, can reduce their absorption [124]. Finally, there are clear differences when considering individual leaching agents. There were different ranges for potentially toxic and essential elements concentration in couscous, which clearly show the effect of locations.
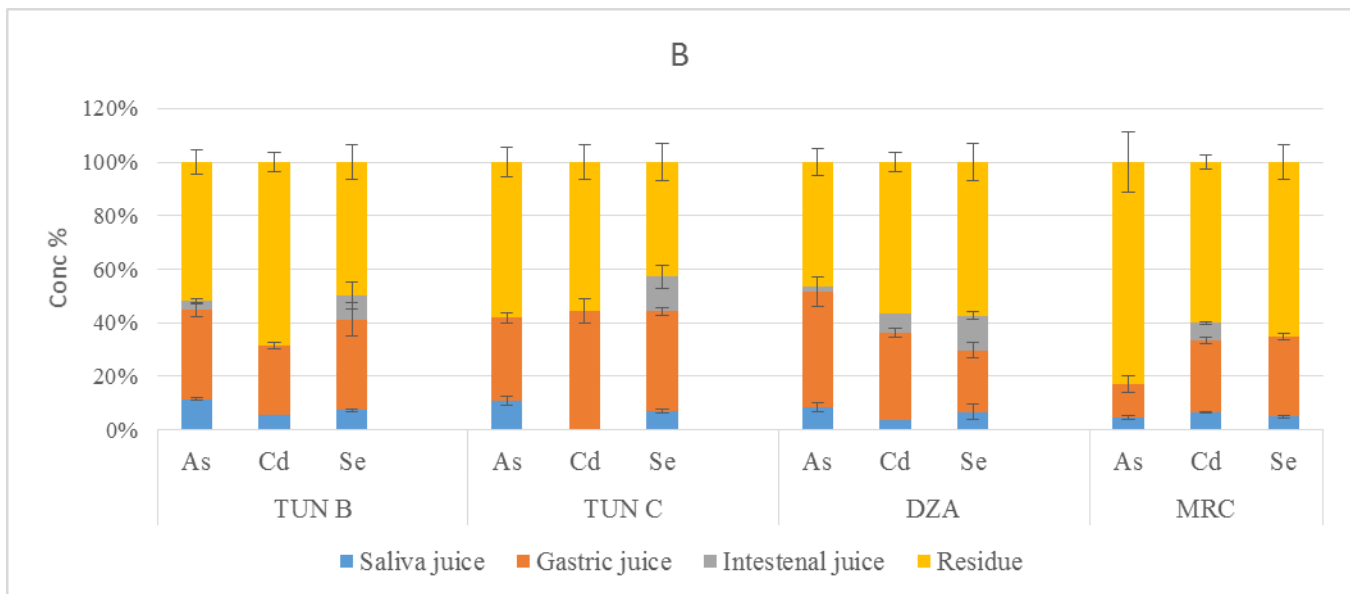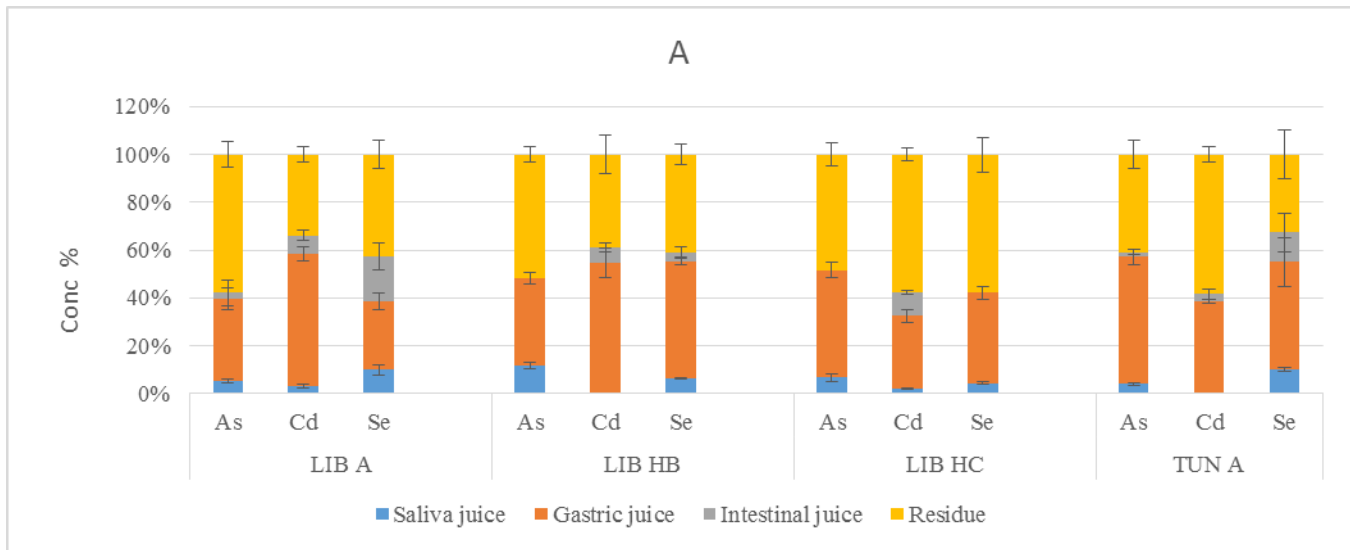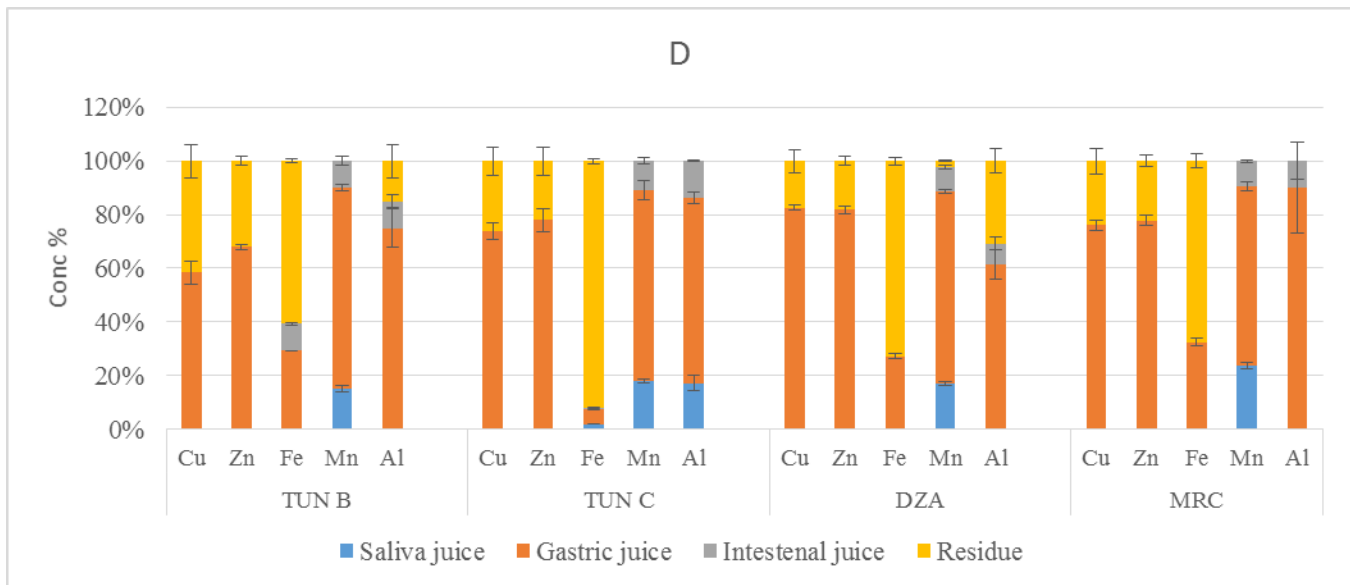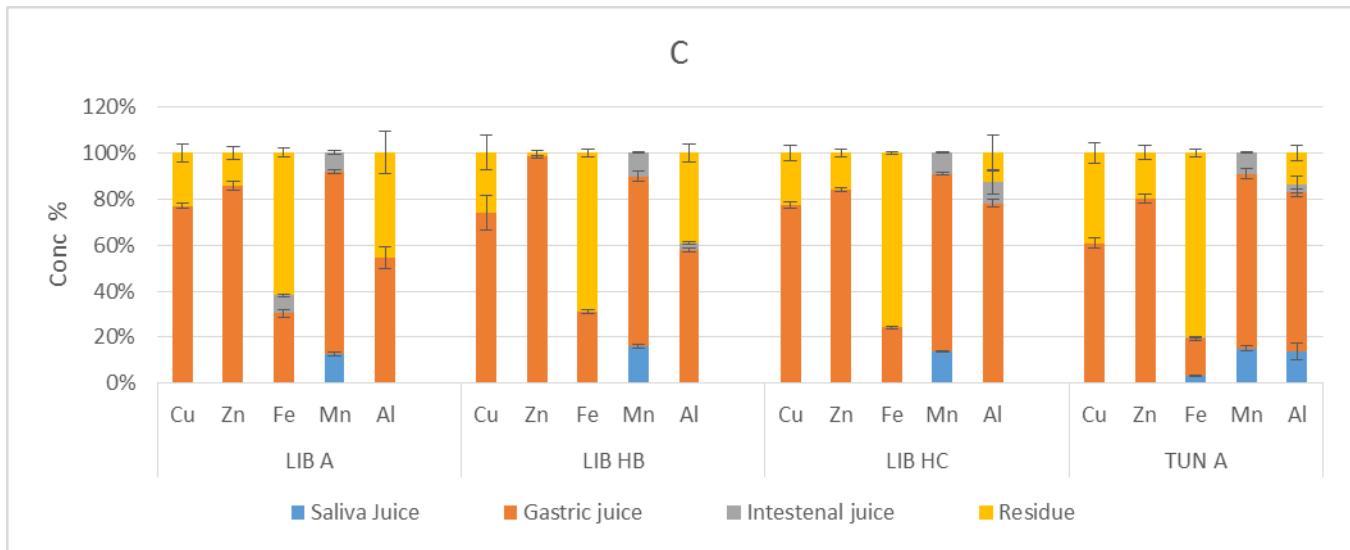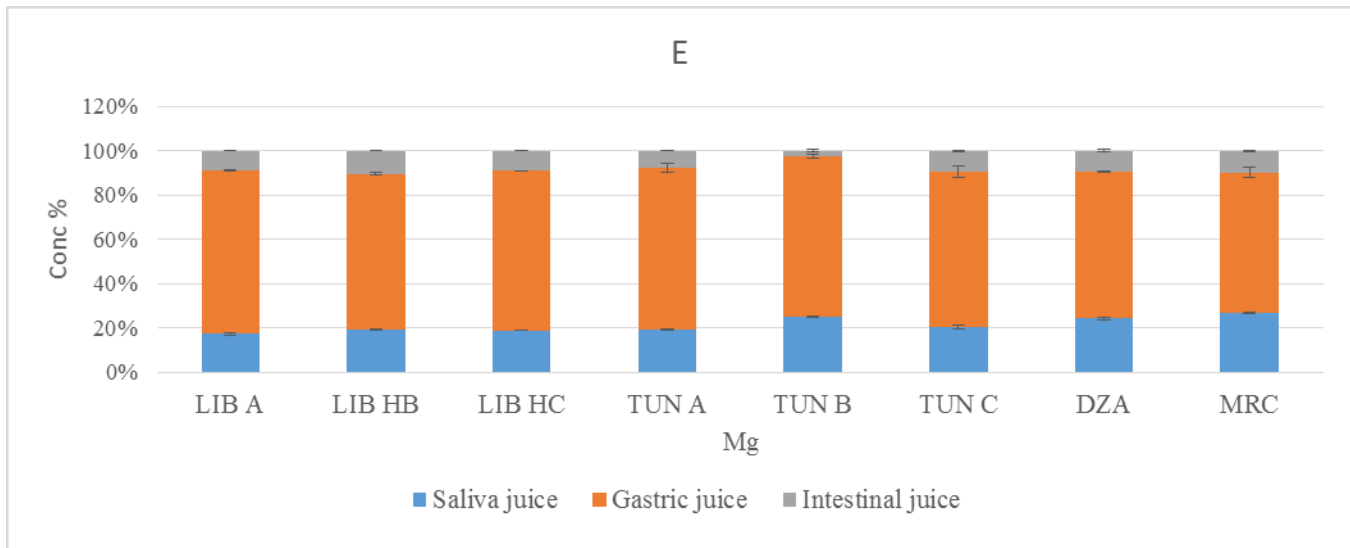
Figure 3.2 Distribution of elements as percentage that is bioaccessible or not in couscous samples

(error bar = standard deviation; n=5) from Libya, Tunisia, Algeria, and Morocco

Table 3-3: Concentration in ng/g (mean ± standard deviation, n=5) of bioaccessible elements and verification of mass balance through Student's t test between the sum of bioaccessible and residue concentrations and that expected i.e. the total concentration measured following digestion

Element	Sample	Saliva	Gastric Juice	Intestinal Juice	Residue	Bioaccessible + residue	Expected	t_{found}
	LIB A	2.0 ±0.3	13 ±2	1.0 ±0.3	24±1	40 ±2	37 ±6	1.06
	LIB HB***	7 ±1	22 ±1	ND**	31±2	60 ±2	64 ±3	2.48
	LIB HC***	4 ±1	27 ±2	ND	29 ±2	60 ±3	50 ±4	4.47
As	TUN A	2.0 ±0.3	26 ±2	1.0 ±0.5	20±2	50 ±3	60 ±1	7.07
	TUN B	8 ±1	23 ±1	2.4 ±2.0	36±2	70 ±3	65 ±1	3.53
	TUN C	6 ±1	17 ±3	ND	32 ±1	55 ±3	50 ±5	1.91
	DZA	5.0 ±0.3	26 ±1	1.0 ±0.5	28±5	60 ±5	56 ±10	0.8
	MRC	2.0 ±0.1	6 ±5	ND	37±2	45 ±5	41 ±3	1.53

Element	Sample	Saliva	Gastric Juice	Intestinal Juice	Residue	Bioaccessible + residue	Expected	t_{found}
Cd	LIB A	2.0 ±0.5	36 ±2	5 ±2	22 ±4.5	65 ±5	60 ±10	1.0
	LIB HB	ND	35 ±4	4 ±1	26±3	65 ±5	67 ±6.5	0.54
	LIB HC	1.5 ±0.1	21 ±2	7 ±1	40 ±3	70 ±4	61 ±4	3.55
	TUN A	ND	25 ±1	2 ±1	38±4	65 ±4	62 ±11	0.57
	TUN B	3 ±1	14 ±2	ND	27 ±2	55 ±3	60 ± 8	1.30
	TUN C	ND	20 ±1	ND	25±2	45 ±2	40 ±4	2.77
	DZA	2.0 ±0.2	18 ±1	4 ±0.3	31±2	55 ±2	51 ±3	2.48
	MRC	5 ±1	20 ±2	5 ±1	45±2	75 ±3	80 ±2	3.10
Al	LIB A	ND	600±50	ND	500±90	1100±100	1000±104	1.54
	LIB HB	ND	1500±24	80±10	1020±100	2600±100	2500±200	1.00
	LIB HC	ND	2000±36	240±135	360±140	2600±200	2400±300	1.24
	TUN A	400±100	2000±50	100±100	400±50	2900±100	3000±70	1.83
	TUN B	ND	1500±140	200±45	200±50	1900±200	1800±160	0.87
	TUN C	500±80	2000±60	400±10	ND	2900±100	2700±150	2.48
	DZA	ND	800±70	100±30	400±60	1300±100	1200±50	2.00
	MRC	ND	900±169	100±70	ND	1000±200	600±170	3.40

Element	Sample	Saliva	Gastric Juice	Intestinal Juice	Residue	Bioaccessible + residue	Expected	t_{found}
Se	LIB A	7±2	20±2	13±4	30±3	70±4	73±8	0.75
	LIB HB	3.0±0.1	24±1	2±1	20±2.5	50±2	48±5	0.83
	LIB HC	3.0±0.4	26±2	ND	40±4.5	70±5	65±1	2.19
	TUN A	5.0±0.4	22±5	6 ±4	16 ±4	50±7.5	55±9	0.95
	TUN B	6±1	27±1	7 ±3	40 ±4	80±5	73±6	2.0
	TUN C	5 ±2	26±2	9 ±1	30 ±4	70±5	68±3	0.76
	DZA	4.7±0.3	16±1	9±3	40±4	70±5	72±2	0.83
	MRC	4.0±0.3	24 ±2	ND	42 ±5	80±2	75±7	1.53
Zn	LIB A	ND	12000±260	ND	2000±300	14000±400	14400±1100	0.76
	LIB HB	ND	17700±100	ND	300±100	18000±200	17000±300	6.20
	LIB HC	ND	16000±160	ND	3000±250	19000±300	20000±100	7.07
	TUN A	ND	8000±200	ND	2000±225	10000±300	10600±700	1.76
	TUN B	ND	8500±130	ND	2000±140	12500±200	13000±10	5.58
	TUN C	ND	7400±400	ND	2100±300	9500±500	10000±200	2.07
	DZA	ND	9000±150	ND	2000±110	11000±200	10500±400	2.50
	MRC	ND	7000±180	ND	2000±90	9000±200	8400±230	4.40

Element	Sample	Saliva	Gastric Juice	Intestinal Juice	Residue	Bioaccessible + residue	Expected	t_{found}
Cu	LIB A	ND	2000±30	ND	600±95	2600±100	2500±100	1.58
	LIB HB	ND	2000±200	ND	700±30	2700±200	2800±10	1.11
	LIB HC	ND	2400±40	ND	700±90	3100±100	3000±40	2.07
	TUN A	ND	1400±50	ND	900±85	2300±100	2400±170	1.13
	TUN B	ND	1400±100	ND	1000±110	2400±150	2300±150	1.05
	TUN C	ND	1400±60	ND	500±80	1900±100	1800±50	2.0
	DZA	ND	1900±20	ND	400±95	2300±100	2200±60	1.91
	MRC	ND	1600±40	ND	500±90	2100±100	2000±40	2.07
Fe	LIB A	ND	5000±250	1300±80	10200±145	16500±300	16000±500	1.91
	LIB HB	ND	5300±170	ND	11700±250	17000±300	16000±1000	2.14
	LIB HC	ND	4600±80	ND	16400±200	21000±215	22000±400	4.92
	TUN A	400±40	2000±100	100±30	10000±170	12500±200	12000±500	2.07
	TUN B	ND	3800±20	1300±80	7900±290	13000±300	12000±1000	2.19
	TUN C	400±0.01	1300±100	74±0.09	20700±170	22500±200	22000±600	1.76
	DZA	ND	3000±110	ND	8000±100	11000±150	10700±300	2.0
	MRC	ND	2600±130	ND	5400±150	8000±200	8100±290	0.60

Element	Sample	Saliva	Gastric Juice	Intestinal Juice	Residue	Bioaccessible + residue	Expected	t_{found}
Mn	LIB A	1300±70	8100±90	860±90	ND	10260±100	9800±600	1.69
	LIB HB	4000±200	18000±500	2500±30	ND	24500±500	18000±100	28.5
	LIB HC	2900±30	16000±160	1900±90	200	20800±200	21000±600	0.70
	TUN A	1000±80	5000±150	600±20	100	6600±200	6700±450	0.45
	TUN B	1000±80	5000±80	650±120	ND	6550±200	6500±100	0.50
	TUN C	1000±40	4000±200	600±70	ND	5600±200	5200±180	3.32
	DZA	1500±60	6300±45	800±55	ND	9600±100	7500±200	21
	MRC	1600±90	4500±110	630±30	ND	6730±200	6200±185	4.34
Mg	LIB A	$(1.05\pm0.04)\times10^5$	$(5.00\pm0.03)\times10^5$	$(5.40\pm0.05)\times10^4$	ND	$(6.10\pm0.05)\times10^5$	$(5.5\pm0.2)\times10^5$	5.75
	LIBHB	$(1.40\pm0.02)\times10^5$	$(5.20\pm0.04)\times10^5$	$(8.00\pm0.02)\times10^4$	ND	$(7.3\pm0.4)\times10^5$	$(5.0\pm0.2)\times10^5$	22.89
	LIBHC	$(1.200\pm0.001)\times10^5$	$(4.60\pm0.01)\times10^5$	$(6.0\pm0.1)\times10^4$	ND	$(6.30\pm0.01)\times10^5$	$(6.1\pm0.1)\times10^5$	3.72
	TUN A	$(7.1\pm0.2)\times10^4$	$(3.00\pm0.04)\times10^5$	$(3.00\pm0.03)\times10^4$	ND	$(4.00\pm0.08)\times10^5$	$(4.00\pm0.01)\times10^5$	2.78
	TUN B	$(1.100\pm0.004)\times10^5$	$(3.2\pm0.1)\times10^5$	$(1.05\pm0.30)\times10^4$	ND	$(4.40\pm0.05)\times10^5$	$(4.0\pm0.1)\times10^5$	4.63
	TUN C	$(7.00\pm0.03)\times10^5$	$(2.30\pm0.09)\times10^5$	$(3.0\pm0.1)\times10^4$	ND	$(3.3\pm0.1)\times10^5$	$(3.1\pm0.1)\times10^5$	3.81
	DZA	$(1.20\pm0.03)\times10^5$	$(3.300\pm0.004)\times10^5$	$(5.0\pm0.3)\times10^4$	ND	$(5.00\pm0.04)\times10^5$	$(4.00\pm0.02)\times10^5$	45
	MRC	$(1.100\pm0.001)\times10^5$	$(3.00\pm0.10)\times10^5$	$(4.0\pm0.1)\times10^5$	ND	$(4.1\pm0.1)\times10^5$	$(3.3\pm0.1)\times10^5$	13.55

LIB = Libya   DZA = Algeria   TUN = Tunisia   MRC = Morocco
* The Table value for Student's t at the 95% confidence level is 2.306
**ND = Not detected.   ***H indicates handmade couscous

3.4. Correlations between elements in couscous

Correlations between potentially toxic and essential metals could be a useful tool to get some information on the sources of metals [125]. Correlations of As, Cd, Se, Al and essential elements in couscous were investigated. SPSS software (Statistical Package for the Social Sciences) was used for the analysis of the couscous data. Paired elements correlation was used. The mathematical formula for computing the Pearson's correlation coefficient, **r,** is:

$$r = \frac{n\sum xy - \left(\sum x\right)\left(\sum y\right)}{\sqrt{n\left(\sum x^2\right) - \left(\sum x\right)^2}\sqrt{n\left(\sum y^2\right) - \left(\sum y\right)^2}}$$

Where *n* is the number of pairs of data [126].

(Note that r is a dimensionless quantity; that is, it does not depend on the units employed.)

- The value of r is such that $-1 \leq r \leq +1$. The + and − signs are used for positive linear correlations and negative linear correlations, respectively.

- If *x* and *y* have a strong positive linear correlation, r is close to +1. An r value of exactly +1 indicates a perfect positive fit. Positive values indicate a relationship between *x* and *y* variables such that as values for *x* increase, values for *y* also increase.

- If *x* and *y* have a strong negative linear correlation, r is close to -1. An r value of exactly -1 indicates a perfect negative fit. Negative values indicate a relationship between *x* and *y* such that as values for *x* increase, values for *y* decrease.

- If there is no linear correlation or a weak linear correlation, r is close to 0. A value near zero means that there is a random, nonlinear relationship between the two variables.

The Pearson's correlation coefficient r between metals in couscous are given in Table 3-4, and Figure 3-3. A significant correlation (negative correlation) was observed with Mo (r value is -0.743, P= 0.05) and with Cd, Se, Fe, Mg, S, and P for all types of couscous. Se only has a significant negative correlation with Al (r= -0.741, P= 0.05). Cd showed significant negative correlations with S and P (r = -0.771 and -0.756 P= 0.05 respectively) in Figure 3.4. No significant correlations were observed between Cd and other metals, which may indicate different sources of Se and Cd from other metals. Interestingly, the highest strong positive correlations were found for Zn with Mn, Cu, Mg, and Fe (r= 0.933, 0.921, 0.902, and 0.586 (P=0.01) respectively). The strong correlations may imply common sources of these metals. In addition, a significant correlation (r=0.831, P=0.05) was found between the S and P. Finally, significant positive correlations were found between Mn and Mg (r=0.825, P=0.05) and between Fe and P (r=0.763, P=0.05).

Table 3.4: Correlation r values between the total concentrations of elements in couscous samples, with significant ones highlighted in green (positive correlations) or red (negative correlations)

	Mg	S	P	Mo	Al	Mn	Fe	Cu	Zn	As	Cd	Se
Mg	1											
S	-0.321	1										
P	-0.188	0.831*	1									
Mo	0.178	0.019	-0.126	1								
Al	0.022	0	0.421	-0.62	1							
Mn	0.825*	-0.527	-0.137	0.031	0.271	1						
Fe	0.436	0.408	0.763*	-0.149	0.561	0.505	1					
Cu	0.895**	-0.633	-0.365	-0.037	0.262	0.906**	0.309	1				
Zn	0.906**	-0.364	-0.05	-0.094	0.3	0.933**	0.586	0.922**	1			
As	-0.151	-0.349	-0.171	-0.743*	0.583	0.105	-0.092	0.202	0.142	1		
Cd	0.128	-0.771*	-0.756*	0.295	-0.403	0.212	-0.557	0.288	0.064	-0.142	1	
Se	-0.169	0.492	0.053	0.177	-0.741*	-0.497	-0.228	-0.51	-0.39	-0.564	-0.08	1

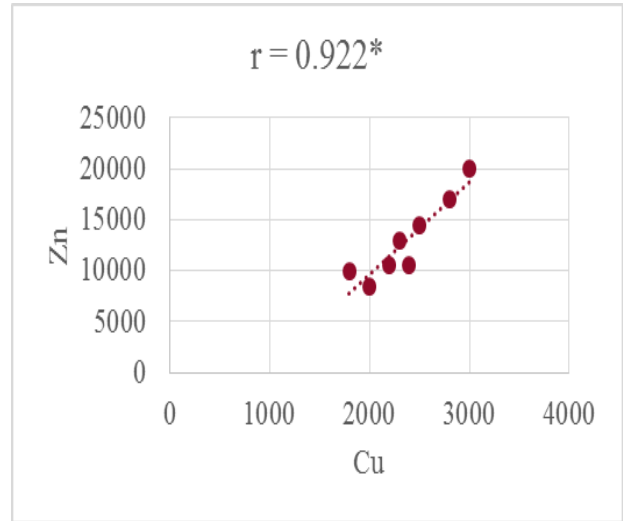* Correlation significant at the 0.05 level   ** Correlation significant at the 0.01 level
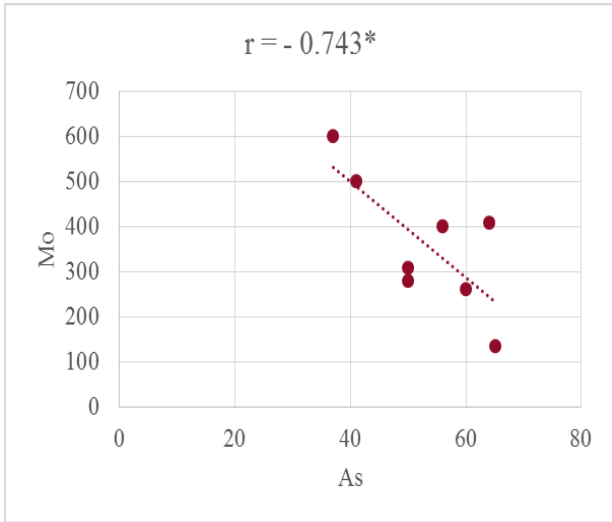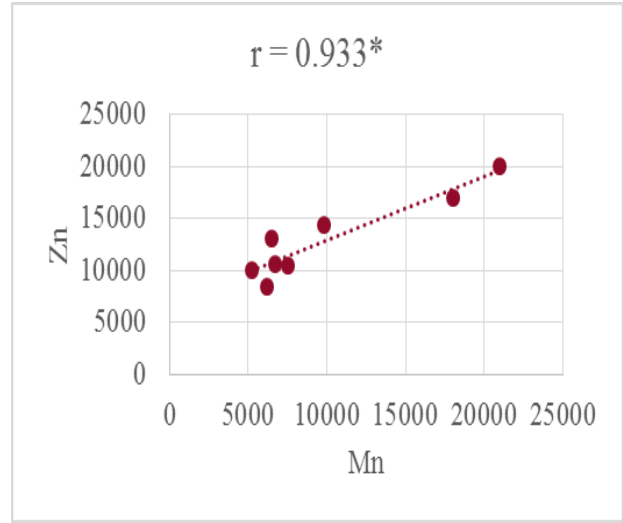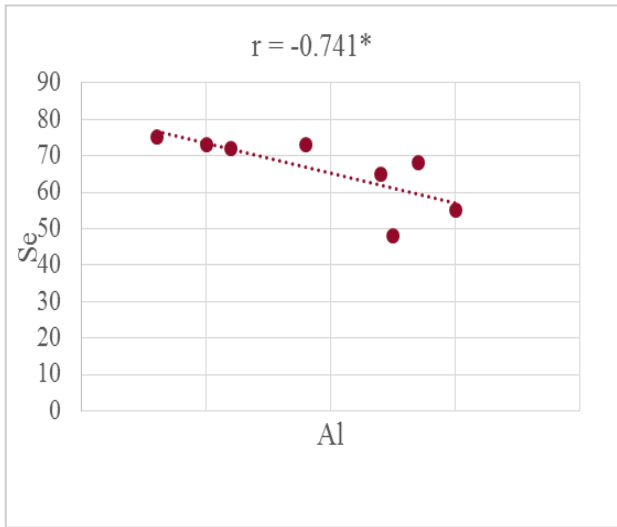
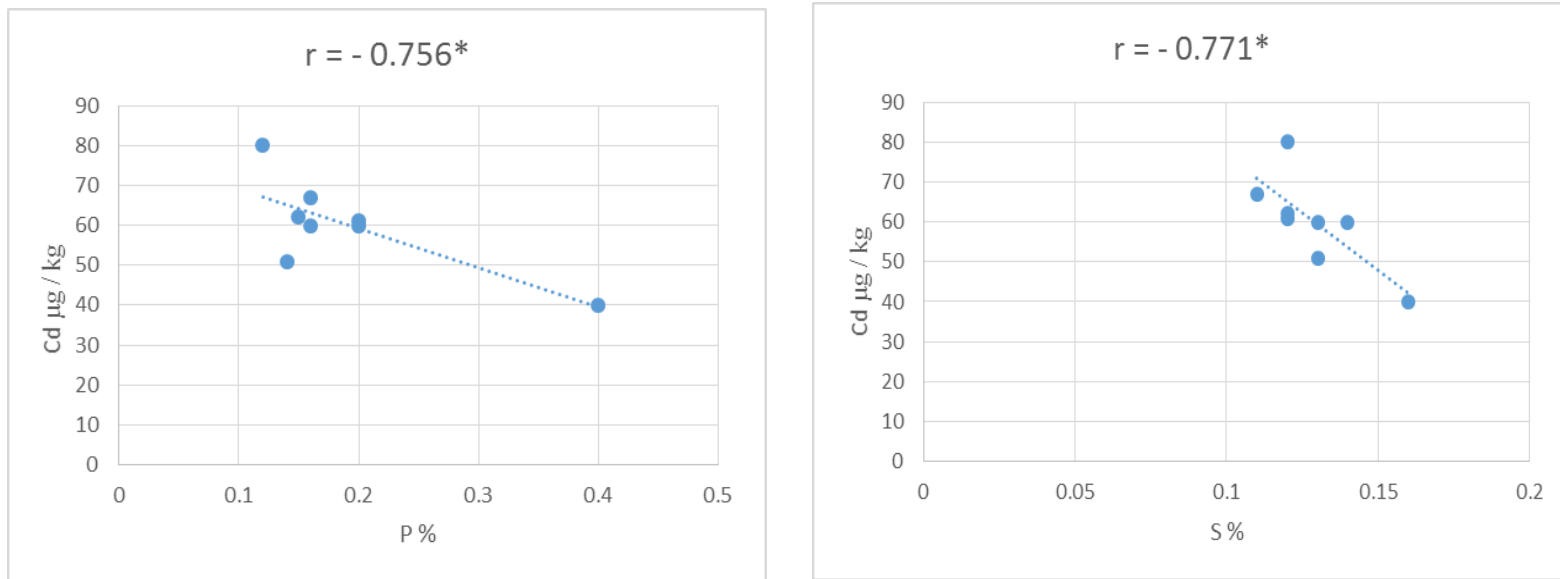Figure 3.3**:** Correlation r between some elements (As, Mo, Mn, Zn, Al, Se, and Cu)

Figure 3.4: Correlation r between Cd and each elements P, and S

3.5 Bioaccessibility correlation between the elements

To further support the assertion that Zn and Cu show a strong correlation between their bioaccessibility

 (r = 0.815), as shown in Figure 3.4. In addition, significant strong positive correlations were observed

for Zn with Mn (r= 0.960, P=0.01) and with Mg (r= 0.815, P = 0.05) figure 3.4. For Cu, significant

positive correlations were found with each of Mn and Mg (r= 0.876, and 0.863 (P = 0.01) respectively).

In addition, Cd showed positive Moderate correlations (r= 0.5 – 0.6) with elements Cu, Zn, Fe, Mn, and

Mg. Moreover Fe showed a significant correlation with Mg only (r= 0.771, P = 0.05) unlike in total

concentration Fe showed weak correlation with Mg. Finally, Mn showed significant correlation with Mg

(r= 0.831, P = 0.05). Table 3.5 summarizes bioaccessibility correlation.

Table 3.5: Correlation values between bioaccessible concentrations of elements in couscous samples.

	As	Cd	Se	Al	Cu	Zn	Fe	Mn	Mg
As	1								
Cd	-0.387	1							
Se	0.065	-0.215	1						
Al	0.394	-0.496	0.209	1					
Cu	0.085	0.596	-0.442	-0.313	1				
Zn	0.276	0.645	-0.301	-0.066	**0.815**[*]	1			
Fe	0.116	0.608	0.198	-0.476	0.539	0.662	1		
Mn	0.269	0.534	-0.407	0.0376	**0.863**[**]	**0.960**[**]	0.540	1	
Mg	0.208	0.606	-0.098	-0.214	**0.876**[**]	**0.815**[*]	**0.771**[*]	**0.831**[*]	1

* Correlation significant at the 0.05 level   ** Correlation significant at the 0.01
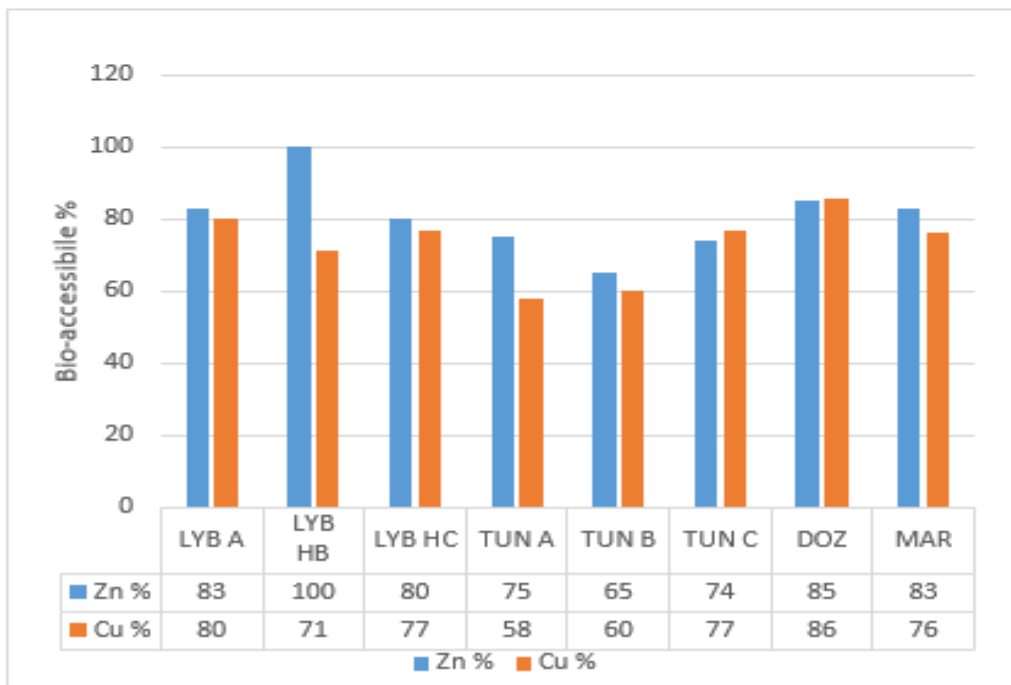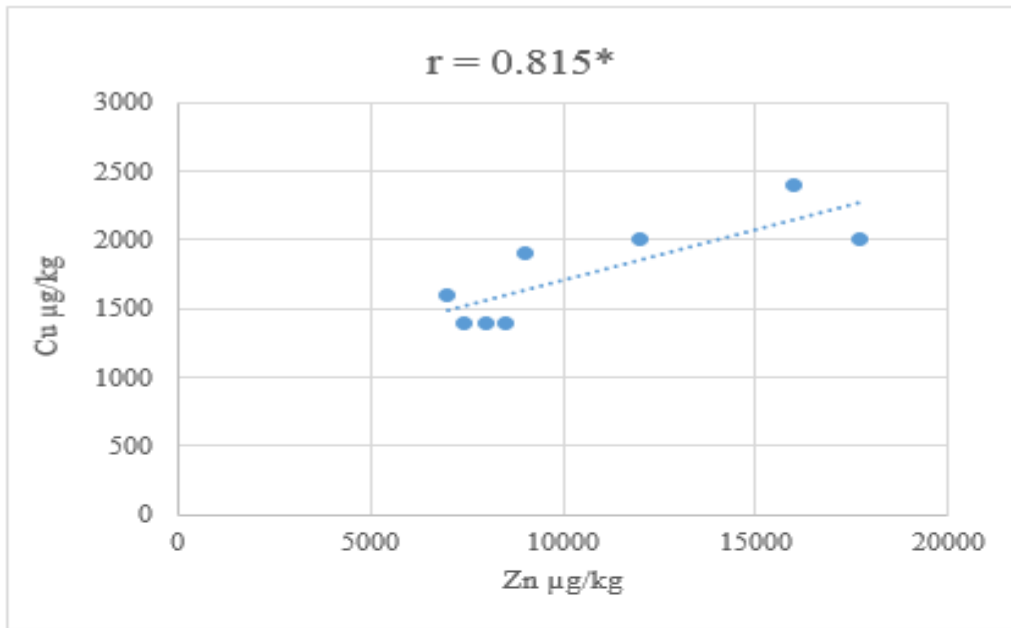
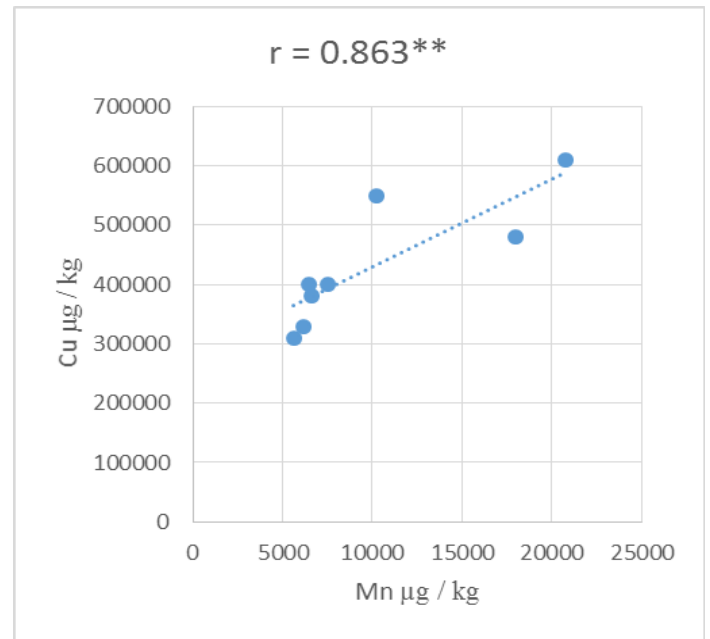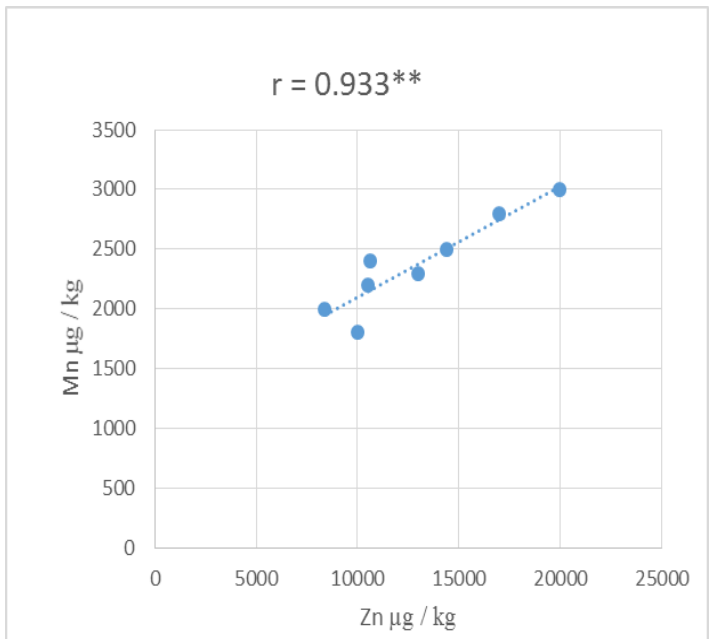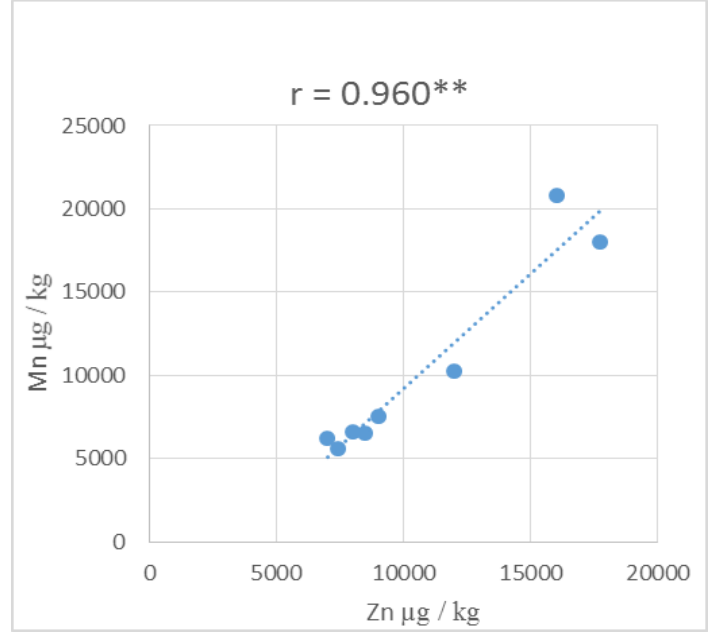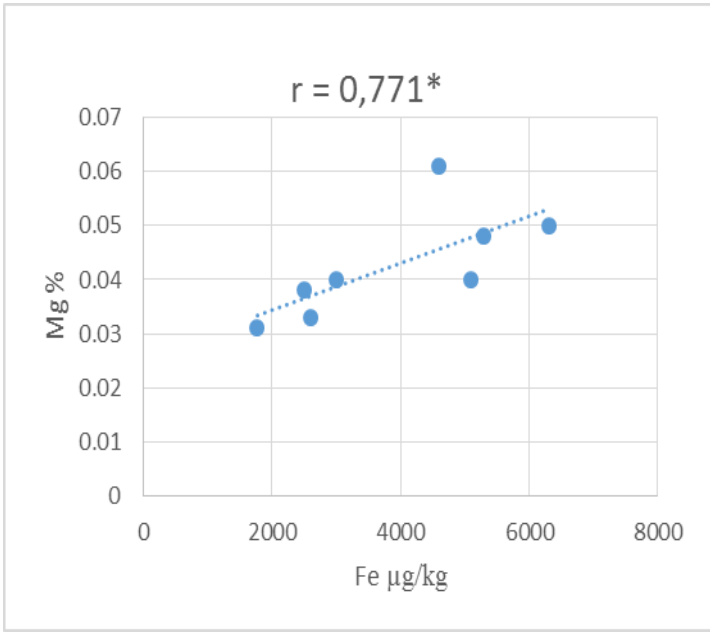Figure 3.5: Correlations between the bioaccessibilities of Zn and Cu

Figure 3.6:   **C**orrelations between the bioaccessibilities of some elements (Cu, Mg, Mn, Fe, and, Zn)

Chapter IV

Conclusion and future work

4.1 Conclusion

In this work, the bioaccessibility of potentially toxic metals in couscous was investigated, for the first time, by using a batch method. Bioaccessibility was low for all toxic elements except Al. Its source may be from irrigation water (Al salts are widely used in water treatment) or food additives [74]. It has been hypothesized that aluminum exposure is a risk factor for the development or acceleration of onset of Alzheimer disease in humans [75]. Although Al was reported to interact with a number of other elements, including Ca, F, Fe, Mg, P and S, and, when ingested in excess, can reduce their absorption [127], no significant correlation was observed between Al and Fe, Mg or P in this work. On the other hand, there was a negative correlation between Al and Se, The results in this work imply that the extraction of elements from couscous begins in the oral cavity. As food proceeds to the stomach, more extraction occurs due to a longer residence in an acidic solution. The majority of elements were leached out by gastric juice. In contrast, small amounts were leached by saliva, with very little leaching observed in intestinal juice.

The fact that relatively low levels of potentially toxic elements were measured in both total concentration and bioaccessible fraction means that speciation analysis is not required for these particular samples of couscous. All the determined elements remain under the maximum recommended levels by international organisms. For instance, the Food and Agricultural Organization (FAO)/World Health Organization established the provisional tolerable daily intake (PTDI) for As, Cd, Se and Al to 2.1 µg/kg, 0.82 µg/kg, 1 µg/kg 1 mg/kg body weight per day, respectively [128]. Based on total

concentration results of toxic elements in couscous, an adult weighing 60 kg would need to consume

more than 2 kg, 600 g, 800 g and 20 kg of couscous per day to reach the PTDI level for As, Cd , Se,

and Al respectively (Table 3.6). A 5-year old child (~ 18 kg), would have to consume 30% of the above

amount for an adult to reach the PTDI levels. Based on bioaccessibility results, couscous seem entirely

safe for all ages. This suggests that couscous consumption is within the safety limits.

Table 3.6: Amount of couscous that can be consumed daily by a 60-kg adult and 18-kg child based on

PTDI levels.

Element	Weight of person	PTDI Level*	Total Concentration	Bioaccessible concentration
As	60 kg (Adult)	2.10 µg/kg/bw/day	2 kg	3.6 kg
	18 kg (5 years old child)		0.6 kg	1.1 kg
Se	60 kg (Adult)	1 µg/kg/bw/day	0.8 kg	1.5 kg
	18 kg (5 years old child)		0.3 kg	0.5 kg
Cd	60 kg (Adult)	0.82 µg/kg/bw/day	0.6 kg	1.2 kg
	18 kg (5 years old child)		0.2 kg	0.4 kg
Al**	60 kg (Adult)	1000 µg/kg/bw/day	20 kg	24 kg
	18 kg (5 years old child)		6 kg	7.2 kg

* [128] ** [129]

In general, there were different r correlations (positive and negative) between the metals for various couscous. This might be attributed to a variety of sources of heavy metals in couscous. Interestingly, the highest significant correlation in this work was found to be between Zn and Mn for both total digestion and the batch method to measure bioaccessibility. In addition, a significant correlation between four elements (Zn, Cu, Mn, and Mg) in both methods was observed as well. Also a significant negative correlation between Cd, P, and S was detected. Finally, Zn and Cu showed high significant correlation in both methods and were leached only by gastric juice in all the analyzed samples. Unlike Cu and Zn, the Mn and Mg were leached by saliva, gastric and intestinal juices in all samples.

This work showed that the total concentration of potentially toxic elements and the fraction that is bio-accessible in couscous can differ largely between countries. There are many factors that may play a role in increasing the levels of potentially toxic and essential elements in wheat, which is used for the preparation of couscous, such as the type of irrigation, type and quality of fertilizer, pesticide, land and industrial process used. In addition, the different particle size of couscous may have an impact on bioaccessibility, as smaller particles have a larger surface area than larger particles [130]. This works shows that element-pair analysis is useful to reveal elements that are correlated.

4.2 Future work

So far, a conventional batch method has been tested on couscous. Future work could involve the online leaching method [101] to see if similar results can be obtained faster. Other potentially toxic and essential elements, such as Cr and Ca, could also be investigated in couscous. A batch method without centrifugation step could be compared with the original method with centrifugation, and any difference between them could be investigated. This investigation would be of particular interest because we know that a centrifuge does not actually mimic the human body and it would be interesting to compare the results with and without centrifuging of couscous. Additionally, artificial saliva containing amylase enzyme should be used to see if the enzyme has a significant effect on bioaccessibility. Using this enzyme would definitely make the artificial saliva more representative of actual human saliva. Moreover, the method should be applied to a broader range of samples. In addition, the bioaccessibility of different elements (potentially toxic and essential) in new couscous samples should also be studied, along with the effect of cooking on their release. As the particle size influences many properties of particulate materials and is a valuable indicator of quality and performance [130], future work will investigate the effect of particle size on the bioaccessible amount of potentially toxic and essential elements in couscous.

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