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STUDY OF TRACE ELEMENTS AND ORGANIC COMPOUNDS OF THE TOXICOLOGICAL INTEREST IN TEA SAMPLES

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Study of Trace Elements and Organic Compounds of the Toxicological Interest in Tea Samples

D. Alá Salem Ali Alnaimat

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Dra. María Carmen Barciela Alonso
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بِسْمِ اللَّـهِ الرَّحْمَـٰنِ الرَّحِيمِ

رَبِّ أَوْزِعْنِيٓ أَنْ أَشْكُرَ نِعْمَتَكَ ٱلَّتِيٓ أَنْعَمْتَ عَلَيَّ وَعَلَىٰ وَٰلِدَيَّ وَأَنْ أَعْمَلَ صَٰلِحًا تَرْضَىٰهُ وَأَدْخِلْنِي بِرَحْمَتِكَ فِي عِبَادِكَ ٱلصَّٰلِحِينَ النمل: 19

"*¡Oh, Señor mío! Haz que sepa agradecerte los favores que nos has concedido, tanto a mí como a mis padres, y que pueda realizar obras buenas que Te complazcan. Concédeme una descendencia piadosa. Me arrepiento a Ti [de mis pecados] y soy de los musulmanes***" {***Capítulo: An-Naml 27***: 19}**

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From Santiago de Compostela to Al-Farthakh, from Spain to Jordan, this thesis is dedicated to the soul of my father, the soul of my best friend "Basheer" and to my lovely mother, brother, and sister wherever they reside

الى أمي الغالية و الى روح أبي الطاهرة ... رحمهما الله كما ربوني صغيراً .....إبنكم: علاء

علاء سالم النعيمات
سانتياغو دي كومبوستيلا – إسبانيا
25 يونيو 2020

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ABBREVIATIONS

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Abbreviations

A	
AAPH	**2,2'-A**zobis-**(2-A**midino-**P**ropane**)** Dihydrochloride
AA	**A**ntioxidant **A**ctivity
ABCN	**1,1′-A**zobis**(C**yclohexanecarbonitrile**)**
ABTS	**2,2`-A**zino-**B**is**(3-E**thylbenzthiazoline**-6-S**ulphonic **A**cid
ACN	**A**cetonitrile
AD	**A**lzheimer's **D**isease
AFS	**A**tomic **F**luorescence **S**pectroscopy
AIBN	**2,2´-** Azobisisobutyronitrile
AIDS	**A**cquired **I**mmunodeficiency **S**yndrome
AIMN	**2,2´-** Azobis**(M**ethylbutyronitrile**)**
allyl-β-CD	**A**llylic **B**romine**-C**yclodextrin
ATR	**A**ttenuated **T**otal **R**eflection
ATSDR	**A**gency for **T**oxic **S**ubstances and **D**isease **R**egistry
B	
BBP	**B**utyl **B**enzyl **P**hthalate
BP	**M**onobutyl **P**hthalate
BPA	**B**isphenol **A**
BPB	**B**isphenol **B**
BW	**B**ody **W**eight
C	
C	**C**atechin
C. Sinensis	***Camellia Sinensis***
CE	**C**apillary **E**lectrophoresis
CP	**4-C**umylphenol
CPE	**C**loud**-P**oint **E**xtraction
CRM	**C**ertified **R**eference **M**aterial
CVD	**C**ardiovascular **D**isease
CW-DVB	**C**arbowax**-D**ivinylbenzene

D	
DAD	**D**iode **A**rray **D**etector
DAP	**D**iamyl **P**hthalate
DBP	**D**ibutyl **P**hthalate
DCHP	**D**icyclohexyl **P**hthalate
DCM	**D**ichloromethane
DCP	**2,4-B**is-**(D**imethylbenzyl**)P**henol
DDT	**D**ichlorodiphenyltrichloroethane
DEHA	**D**i**(2-E**thylhexyl**) A**dipate**)**
DEHP	**D**i**-2-E**thylhexyl **P**hthalate
DEP	**D**iethyl **P**hthalates
DES	**D**iethylstilbestrol
DIDP	**D**iisodecyl **P**hthalate
DIMs	**D**ummy **I**mprinted **M**icrospheres
DINA	**D**i**-I**sononyl **A**dipate
DINCH	**1,2-C**yclohexanedicarboxylic **A**cid **D**iisononyl **E**ster
DINP	**D**iisononyl **P**hthalate
DiOP	**D**iicooctyl **P**hthalate
DLLME	**D**ispersive **L**iquid-**L**iquid **M**icroextraction
DMP	**D**imethyl **P**hthalate
DNA	**D**eoxyribonucleic **A**cid
DNOP	**D**ioctyl **P**hthalate
DOP	**D**i**-N-O**ctylphthalate
DP	**D**eclustering **P**otential
DPeP	**D**ipentyl **P**hthalate
DPPH	**D**iphenyl picrylhydrazyl
DSMIP	**D**ummy **S**urface **M**olecularly **I**mprinted **P**olymer
d-SPE	**D**ispersive-**S**olid **P**hase **E**xtraction
E	
EC	**E**picatechin
ECD	**E**lectron **C**apture **D**etector

ECG	**E**picatechin-**3**-**G**allate
ECHA	**T**he **C**hemical **A**gency of the **E**uropean **U**nion
ED	**E**ndocrine **D**isruptor
EDAI	**E**stimated **A**verage **D**aily **I**ntake
EDMA	**E**thylene **G**lycol **D**imethacrylate
EFSA	**E**uropean **F**ood **S**afety **A**ssociation
EGC	**E**pigallocatechin
EGCG	**E**pigallocatechin-**3**-**G**allate
EP	**E**nhance **P**otential
EPA	**E**nvironmental **P**rotection **A**gency
EPSFs	**N**-**E**thyl-**P**yrrolidinone **S**ubstituted **F**lavan-3-ols
ETAAS	**E**lectrothermal **A**tomic **A**bsorption **S**pectrometry
EU	**E**uropean **U**nion
F	
FAAS	**F**lame **A**tomic **A**bsorption **S**pectrometry
FAES	**F**lame **A**tomic **E**mission **S**pectrometry
FAO-IGG	**F**ood **and** **A**griculture **O**rganization-**I**ntergovernmental **G**roup
FBPA	**F**luorinated **B**isphenol **A**
FCR	**F**olin-**C**iocalteu **R**eagent
Fe(III)-TPTZ	**F**erric **T**ripyridyltriazine
FID	**F**lame **I**onization **D**etector
FLD	**F**luorescence **D**etection
FP	**F**ocusing **P**otential
FRAP	**F**erric **A**ntioxidant **P**ower
FTIR	**F**ourier **T**ransform **I**nfrared
G	
GAE	**G**allic **A**cid **E**quivalent
GC	**G**allocatechin
GC-MS	**G**as **C**hromatography-**M**ass **S**pectrometry
GF-AAS	**G**raphite **F**urnace **A**tomic **A**bsorption **S**pectrometry

GI	**G**astrointestinal
GID	**G**astrointestinal **D**igestion
GIT	**G**astrointestinal **T**ract
GTCs	**G**reen **T**ea **C**atechins
H	
HAT	**H**ydrogen **A**tom **T**ransfer
HF-LPME	**H**ollow-**F**iber **L**iquid **P**hase **M**icroextraction
HPLC-UV	**H**igh-**P**erformance **L**iquid **C**hromatography - **U**ltraviolet
HQ	**H**azard **Q**uotient
HQI	**H**it **Q**uality **I**ndex
HS-SPME	**H**ead-**S**pace **S**olid **P**hase **M**icroextraction
I	
iBP	**I**sobutyl **P**hthalate
ICP-AES	**I**nductively **C**oupled **P**lasma **A**tomic **E**mission **S**pectrometry
ICP-MS	**I**nductively **C**oupled **P**lasma **M**ass **S**pectrometry
K	
kDa	**K**ilo **D**alton
kg	**K**ilogram
L	
L	**L**iter
LC-MS	**L**iquid **C**hromatography-**M**ass **S**pectrometry
LLE	**L**iquid-**L**iquid **E**xtraction
LLME	**L**iquid-**L**iquid **M**icroextraction
LOD	**L**imit of **D**etection
LOQ	**L**imit of **Q**uantification
LPME	**L**iquid **P**hase **M**icroextraction
M	
µg	**M**icrogram
µM	**M**icromolar
MAA	**M**ethacrylic **A**cid

MAE	**M**icrowave-**A**ssisted **E**xtraction
mBnP	**M**onobenzyl **P**hthalate
mBP	**M**onobutyl **P**hthalate
MCE	**M**ixed **C**ellulose **E**sters
MEHP	**M**ono**(2-E**thylhexyl**) P**hthalate
mEP	**M**onoethyl **P**hthalate
MIP	**M**olecularly **I**mprinted **P**olymer
MNP	**M**agnetic **N**anoparticle
MS	**M**ass **S**pectrometry
MTBE	**M**ethyl-**T**ert-**B**utyl-**E**ther
MW	**M**icrowaves
MWCNTs	**M**ulti-**W**alled **C**arbon **N**anotubes
MWCO	**M**olecular **W**eight **C**ut **O**ff
N	
NP	**N**onylphenol
NRs	**N**uclear **R**eceptors
O	
OP	**4-O**ctylphenol
ORAC	**O**xygen **R**adical **A**bsorbance **C**apacity
P	
PA	**P**olyacrylate
PAEs	**P**hthalate **E**sters
PDMS	**P**olydimethylsiloxane
PDMS-DVB	**P**olydimethylsiloxane-**D**ivinylbenzene
PEG	**P**olyethylen**e G**lycol
PET	**P**oly **(E**thylene **T**erephthalate**)**
PIPES	**P**iperazine-**N,N'-B**is**(2-E**thane-**S**ulfonic **A**cid**) D**isodium **S**alt
PLA	**P**olyactic **A**cid
PLE	**P**ressurized **L**iquid **E**xtraction
PPO	**P**olyphenol **O**xidase
PRLP-S	**S**tyrene–**D**ivinylbenzene **C**opolymer
PS-DVB	**P**olystyrene-**D**ivinylbenzene
PTFE	**P**olytetrafluoroethylene
PVC	**P**olyvinyl **C**hloride

Q	
QuEChERS	**Q**uick, **E**asy, **C**heap, **E**ffective, **R**ugged, **A**nd **S**afe
R	
REEs	**R**are **E**arth **E**lements
RF	**R**ed **F**ruits
RfD	**R**eference **D**ose level
ROS	**R**eactive **O**xygen **S**pecies
RR	**R**ed **R**ooibos
RSD	**R**elative **S**tandard **D**eviation
RTKs	**R**eceptor **T**yrosine **K**inases
S	
SBSE	**S**tir **B**ar **S**orptive **E**xtraction
SD	**S**tandard **D**eviation
SDB-XD	**O**ctadesyl-**C**oated **S**tyrene **D**ivinylbenzene **P**olymer
SDE	**S**imultaneous **D**istillation **E**xtraction
SDME	**S**ingle **D**rop **M**icroextraction
SDVB-MA	**S**tyrene-**D**ivinylbenzene **M**ethacrylate
SET	**S**ingle **E**lectron **T**ransfer
SLE	**S**olid-**L**iquid **E**xtraction
SML	**S**pecific **M**igration **L**imit
SPE	**S**olid **P**hase **E**xtraction
SPME	**S**olid-**P**hase **M**icroextraction
SWCNTs	**S**ingle-**W**alled **C**arbon **N**anotubes
T	
T4CE	**T**etrachloroethylene
TBBPA	**T**etrabromobisphenol **A**
t-BP	**T**ertbutylphenol
TDI	**T**olerable **D**aily **I**ntake
TEAC	**T**rolox **E**quivalent **A**ntioxidant **C**apacity
TFs	**T**heaflavins
TNO	**T**he **N**etherlands **O**rganization
TOTM	**T**rioctyl **T**rimellitate
TPC	**T**otal **P**henolic **C**ontent

TRE	**T**rolox **E**quivalent
TRIM	**T**rimethacrylate
TRs	**T**hearubigins
U	
UK	**U**nited **K**ingdom
UPLC	**U**ltra-**P**erformance **L**iquid **C**hromatography
USA	**U**nited **S**tate **of A**merica
USEPA	**U**nited **S**tate **E**nvironmental **P**rotection **A**gency
UV	**U**ltraviolet
V	
VL	**V**erbena **L**eaves
VP	**4-V**inylpyridine
W	
WHO	**W**orld **H**ealth **O**rganization

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ABSTRACT

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Abstract

Tea, the most popular drink in the world after water, has become one of the most widely investigated beverages due to its health benefits for human body. Tea, produced from *Camellia Sinensis* (*C. Sinensis*) leaves, was discovered approximately 5000 years ago in the ancient Chinese civilization, and spread over all the world to be cultivated in more than 30 countries. Tea is usually consumed as a hot or cold infusion prepared by soaking young leaves and buds of *C. Sinensis* in water for a period of time. Different varieties of tea have been commercialized including the three most popular tea types are black tea (78%) of the total global tea consumption, green tea (20%) and oolong tea (2%), alongside other varieties like white and yellow teas. All these varieties are originated from the same plant source (*C. Sinensis*) but subjected to different production processes including withering, rolling, fermentation, and drying. Once harvested, leaves are dried immediately and rolled to elaborate green tea by minimizing the oxidation process. Partial withering followed by partial oxidation treatment to elaborate oolong varieties while full oxidation produces black variety. Yellow tea production involves a yellowing stage of the dried green leaves while white tea is produced from the immature delicate young leaves and buds.

The popularity of tea comes from its beneficial effect on human health owing to its chemical composition. Fresh *C. Sinensis* leaves are rich in polyphenols, mainly flavanols and their dimers and trimers. Unprocessed leaves contain up to a 30% (w/w)catechins. Catechins are thought to be responsible for the chemical function of tea leaves. Catechins usually include (+)-catechin (C), (-)-epicatechin (EC), (-)-gallocatechin (GC), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG), (-)-epigallocatechin-3-gallate (EGCG). EGCG contributes to 50% of the total weight of catechins. Alongside catechins, fresh tea leaves contain alkaloids (4%) such as caffeine,

theobromine, and theophylline, and methylxanthines (8%) that include lignin, and organic acids, pigments like chlorophyll, free amino acids carbohydrates, proteins, volatile compounds, minerals, and trace elements. The chemical composition of tea changes according to its respective variety. For instance, green tea contains higher contents of catechins than oxidized varieties (e.g. black and oolong teas) due to the partial or full conversion of tea catechins into other chemicals named theaflavins (TFs) and thearubigins (TRs) due to the enzymatic oxidation by polyphenol oxidase.

Because of its polyphenols, tea has been considered a medicinal plant since ancient times. Extensive investigations on the effects of tea on human health are increasing accompanied by the growing need to find naturally healthy diets which include plant-derived polyphenols. Thus, several studies have revealed the antioxidative properties of tea polyphenols. The antioxidative effect of tea polyphenols is associated with their ability to prevent the oxidative stress caused by reactive oxygen species (ROS) and chelate metal ions involved in the formation of free radicals. Several studies have reported that tea polyphenols have anti-heart-disease and anticancer activity in humans, antiallergic action, anti-inflammatory, and antimicrobial properties.

Although of the popularity of tea and the diversity of its composition, tea may also pose other harmful risks to human health. These risks are mainly associated with the presence of many toxic elements (Cd, Hg, As, Cr, … etc.) and toxic organic components. These toxic materials come usually either by leaching from the contaminated soil where the plant is cultivated, or by contaminated air with heavy metals and air-traveling organics, especially when the cultivation zone is near to the industrial zone.

This thesis has focused on the determination of harmful organic toxicants, major and trace elements, and tea antioxidants. This thesis has been divided into four main parts: (1) determination of endocrine disruptors (EDs), (2) total determination of major and trace elements in tea leaves and their infusions, (3) total determination of antioxidant activity, and (4) the bioavailability of tea elements and tea antioxidants.

1. Determination of endocrine disruptors: phthalate esters (PAEs) and bisphenol A (BPA)

Endocrine disruptors are adverse chemical compounds that tend to affect the endocrine system by mimicking the naturally occurring hormones leading to serious health problems to the living organisms. PAEs and BPA are among the most widely used EDs in plastic industry as plasticizers, therefore, it is necessary to determine their levels in food and drinks to avoid their risk. Since these substances may be presented at trace levels in tea samples, a highly sensitive technique for analyzing preceded by a sample preparation step is required.

Through this part, dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP), butyl benzyl phthalate (BBP) and BPA were determined in tea infusions using high-performance liquid chromatography-electrospray ionization coupled to mass spectrometry (HPLC-ESI-MS). PAEs analyzed were preconcentrated by solid-phase extraction (SPE) using a molecularly imprinted polymer (MIP) as a sorbent.

The first step in this part was the optimization of the ESI-MS conditions to achieve high resolution and sensibility at the selected mass-to-charge (*m/z*) for each compound (DMP (163.25), BBP (91.15), DEP and BBP (149.05), and BPA (227). ESI-MS conditions include ion source potentials, nebulizer and curtain gas and temperature. The interface potentials were declustering potential (DP), focusing potential (FP) and enhance potential (EP). ESI-MS conditions were optimized by the direct injection of a PAEs mixture (500 µg/L) standard to the MS working in positive mode and, separately, 500 µg/L BPA standard working in negative mode. The optimum interface potentials as well as the m/z for the determination of PAEs and BPA are summarized in **Table 1**.

The optimum values of the ion-source voltage, pressure of nebulizer and curtain gas, and temperature for introducing the analytes to the MS were as follows: 5500 V, 14 psi, and 450°C for PAEs determination, and -4500 V, 12 psi, and 450°C for BPA determination.

Table 1. Optimum conditions for mass spectrometer

Compound	*m/z*	Potentials (V)			Mode
		DP	FP	EP	
DMP	163.25	40.38	73.87	8	Positive
BBP	91.15	25	225	6	
DEP, DBP	149.05	25	290	8.5	
BPA	227	-125	-195	-9	Negative

Once the MS conditions were optimized, the HPLC parameters were optimized to achieve the best separation of the analytes of interest. HPLC parameters were the selection of the chromatographic column, the composition of the mobile phase, the flow rate, and the mode of elution (i.e., gradient or isocratic). **Table 2** summarizes the HPLC optimum conditions.

Table 2. Optimum conditions for the HPLC separation

Parameter	PAEs	BPA
Mobile Phase	CH_3CN : H_2O (0.1%(v/v) acetic acid)	CH_3CN : H_2O (0.8% NH_3)
Column	Zorbax Eclipse XDB-C8, 50x2.1 mm, particle size 3.5 µm)	
Flow Rate (µL/min)	200	250
Injection Volume/µL	10	5
Elution Mode	Gradient	Isocratic

Tea infusions were prepared according to the recommendations of the manufacturers. PAEs and BPA may be found at trace levels in tea infusions, therefore, a preconcentration stage was needed to increase the sensitivity of the analysis. MIPs were used for selective and specific preconcentration of PAEs and BPA from tea infusions. The preparation of MIPs for both analytes was carried out using precipitation polymerization. Methacrylic acid (MAA) as a monomer, ethylene glycol dimethacrylate (EDMA) as a crosslinking agent, 2,2′-azobisisobutyronitrile (AIBN) as an initiator, and acetonitrile as a solvent were used for MIP synthesis. However, the templates were

different; DBP and BPA were used as templates for PAEs and BPA preconcentration, respectively. Once the polymerization finished, the MIPs were filtered and washed thoroughly with methanol, dichloromethane, methanol, and methanol/water (1:1, v/v) to remove the unreacted reagents and remove the templates. Removing the templates is required to activate the MIP to be able to retain the analyte of interest. The prepared MIPs were used as SPE sorbents by being packed in a glass cartridge and preconditioned with methanol followed by ultrapure water before each extraction process.

The SPE was optimized to obtain a high recovery of analytes. Sample loading and elution flow rates as well as the effect of pH were optimized using, separately, standards of PAEs mixture and BPA (100 µg/L) at different flow rates, pH values and elution volumes. The SPE was performed at a flow rate of 1.0 mL/min for both PAEs and BPA pre-treatment while the optimum pH was at neutral level. Elution volumes (with methanol) were 3 mL and 4 mL for phthalates and BPA, respectively.

Once the MIP-SPE was optimized and the whole method was validated for the linearity, sensitivity, precision and accuracy in terms of analytical recovery, the preconcentration and determination of PAEs and BPA using MIP-SPE-HPLC-ESI-MS were applied to tea infusions. On the other hand, the optimized method was also applied to the infusions of tea-free bags to evaluate the contribution of the bags themselves to the total amounts of PAEs and BPA migrated to the infusions. Alongside the migration study, the composition of tea-free bags was studied using Fourier-transform infrared spectroscopy. For PAEs determination, the optimized method was sensitive (detection limit < 2 µg/L), precise (RSD <10%) and accurate with recovery percentages ranging from 84% to 97%. Among PAEs studied, DEP levels were lower than the detection limit, while DBP was the most abundant phthalate in all tea samples analyzed. BBP and DMP were detected in some samples. The migration study proved that a fraction of PAEs comes from the bag itself. The migration percentages varied from 1.8 to 93.5%.

BPA was only detected in two of the samples analyzed. Moreover, a migration study of BPA was carried out using the tea-free

bags. The results obtained in the migration study showed that the bag contributed in a 14% and 62% of BPA present in the infusion in two samples.

2. *Total determination of major and trace elements in tea leaves and their infusions*

Tea leaves and processed teas are a rich source of different elements including major, minor and trace elements. The element levels differentiate from one type of tea to another due to different factors including geographical factors such as climate, soil composition, and agricultural practice, and processing factors like drying or fermentation. Some elements (e.g., Ca, Co, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, Se, V and Zn) that contribute to the nutritional value of tea are considered essential to the human health, while other elements are unessential and even toxic elements, such as Pb, As, Hg, and Cd, are originated from atmospheric dusts, rainfalls or fertilizers used during stages of plant growth or from manufacturing processes. Considering different properties of elements (i.e., essential, nonessential, and toxic) and the substantial contribution of tea to the daily consumption of tea as an infusion, the total determination of elements in tea leaves and their infusions seems of a great importance.

In this part of the thesis, the total concentrations of 33 elements in different types of tea were determined in both, tea leaves and tea infusions. A microwave assisted acid digestion method was used for total element determination in tea leaves. The leaves were accurately weighed (0.5000 g) and transferred into Teflon vessels. A volume of 3 mL of HNO_3, 1 mL of H_2O_2 and 4 mL ultrapure water were then added, and the vessels were then closed and introduced to a microwave oven and subjected to a three-step temperature program for digestion. The digestion began by linearly heating the reactors to 90°C alongside increasing the power to 1000 W for 2.5 min. The temperature then increased to 140°C while holding the power at 1000W for 60 min, the third step involved increasing the temperature to 180°C over 11 min followed by a final cooling step. Once finished, the obtained clear solutions were transferred to volumetric flasks, diluted with ultrapure water to 25 mL, and stored till analysis.

The analysis was carried out using three spectrometric techniques (i.e. ICP-MS, ICP-OES, and FAAS) according to the concentration of each element, which was estimated by a fast ICP-MS screening. The analytical performance of the method (i.e., digestion and detection) was validated in terms of linearity, sensitivity, precision and accuracy. The accuracy of the method was evaluated using two certified reference materials (Tea Leaves INCT-TL-1 and Rye Grass). The validated method was applied to the analysis of 35 tea samples including black, green, red, and white teas.

Moreover, the total element concentrations in tea infusions were also assessed. Tea infusions were prepared according to the manufacturer's recommendations by soaking 1.5 g of tea leaves in 150 mL boiled ultrapure water for 5 min. The infusions were left to cool, filtered and stored in capped polyethylene bottles at 4˚C until the analysis. The method was validated and found to be sensitive, accurate and precise with analytical recoveries ranging between 92 and 115 %. The validated method was applied to the determination of total element concentration in 35 tea infusions.

Among the elements studied, K was the most abundant element in tea leaves and tea infusions in almost all samples followed by Ca, Mg, and P. On the other hand, the extraction efficiencies from tea leaves into tea infusions were also evaluated. Elements were classified into three categories: highly extractable elements (>50%): Cs, Tl, Ni, and Rb, moderately extractable elements (10-50%): Li, Be, Cu, Ti, Co, As, Al, Cr, P, Mg, Mn, Si, Zn and K, and poorly extractable elements (<10%): Ga, Cd, Pb, V, Ba, Fe, Ca, and Sr, while some elements like Mo, Hg, Pt, Ag, Se, Sn, and Sb show a very poor extraction behavior and their concentrations were very low or even non-detectable.

3. Total antioxidant activity in tea infusion

Tea is one of the most remarkable sources of bioactive compounds with antioxidant activity including polyphenols, flavonoids, phenolic compounds, etc. Green tea is the most powerful antioxidant tea due to the presence of large amounts of catechins. Many studies reported the important role of tea consumption that affects human health, especially reducing or preventing the deleterious

consequences of oxidative stress that may cause cancer, neurodegenerative diseases and cardiovascular diseases. Since tea is used in the daily diet in many countries around the world as an infusion, this part of the thesis is an intent to evaluate the total antioxidant activity of different types of tea such as green, white, black and red varieties. Therefore, tea infusions previously prepared during the total element concentration section were divided into two parts: one for elemental study and the other for antioxidant study.

The antioxidant activity of tea polyphenols was evaluated using spectrophotometric methods: total phenolic content (TPC) was measured using the Folin-Ciocalteu reagent (FCR) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. For TPC determination, an aliquot of each tea infusion was diluted (40-fold) with ultrapure water and mixed with 100 µL of FCR and 1.0 mL of 20 %(w/v) Na_2CO_3. The mixture was diluted to 5 mL with ultrapure water and incubated in the dark for 30 min at room temperature. The absorbance of the developed blue color was monitored at 760 nm. TPC was expressed in terms of mg of gallic acid equivalents per liter (mg GAE/L). The TPC in 34 tea samples was evaluated using the standard addition method prepared with gallic acid standard solutions covering the range of 1 – 15 mg/L and showing good linearity (r = 0.999). The detection limit was 23 mg GAE/L. Results have shown that the TPC in the tea infusions was in the range of 87 – 1580 mg GAE/L.The variation in TPC among the same types of tea may be attributed to tea origin, age, processing, temperature, and time of brewing. Green and white teas show, relatively, higher antioxidant activity than black and red teas, which may be attributed to the production process.

For DPPH assay, a diluted aliquot of tea infusion (1:20) was mixed with 2.9 mL of 0.1 mM DPPH. The mixture was then vortexed vigorously and kept in the dark for 30 min at room temperature. The reduction of the absorbance was measured using the spectrophotometer at 517 nm. The antioxidant activity was evaluated by plotting the absorbance of the resulting solutions against Trolox, and results were expressed in mmol Trolox equivalents per liter of the infusion (mM TRE).

The standard calibration method (0.05 – 0.8 mM TRE) was used for the DPPH assay, and the detection limit was 0.05 mM TRE. In general, green tea possesses, relatively, the highest antioxidant activity among all the tea samples analyzed. The antioxidant activity ranged from 1 to 9.9 mM TR. There was a good correlation between the total phenolic concentration and the antioxidant activity with a correlation coefficient of 0.9247 for green tea samples.

4. Bioavailability of trace elements and antioxidant in tea infusions

Brewed teas are a rich source of antioxidants as well as essential elements. Element analysis of tea infusions is commonly focused on the total concentration determination of various metals to assess the quality and the safety of this ubiquitous beverage or to estimate recommended daily intakes (RDIs) for nutritionally relevant or toxic metals. However, antioxidant analysis from tea is overwhelmingly directed to the determination of the total antioxidant activity, the determination of the contribution of individual tea polyphenols to the total antioxidant activity or the medicinal benefits of tea consumption. Both lines (elemental analysis and antioxidant activity determination) consider that elements and phenolic compounds released into tea infusions are 100% bioaccessible for nutritional purposes. Understanding the nutritional benefits, or even possible harmful risks, of consuming tea infusions to human health requires simulation protocols to mimic the bioprocessing that occurs in the gastrointestinal tract. Traditionally, a two-step protocol with a mixture of gastric solutions (pepsin and intestinal solutions (pancreatin and bile salts)) are commonly used to simulate gastrointestinal digestion. However, other parameters should be considered when simulating the gastrointestinal digestion such as body temperature, digestion time, and gastrointestinal peristalsis movements. Subjecting a nutrient to a simulated gastrointestinal digestion model gives an idea about the bioavailable fraction of that nutrient for human body. Several models have been reported for assessing the bioavailability of a nutrient to human body such as solubility, dialyzability, and Caco-2 cell line models. The most realistic approach is Caco-2 cell lines since the

procedure involves the use of living cells like those found in the intestine. Meanwhile solubility and dialyzability involves the same procedure, in general, but the dialyzability is better than solubility due to the use of dialysis membrane that mimic the intestine walls providing an idea about nutrient transport through the wall.

In this part of the thesis, the bioavailability of tea elements and tea polyphenols released into infusions was evaluated using a dialyzability approach. The bioavailability study uses tea infusions prepared in section "Total determination of major and trace elements in tea leaves and their infusions". Hence, the dialyzability protocol was divided into two stages: gastric and intestinal stage to simulate the gastrointestinal digestion. During the gastric stage, a volume of 20 mL of a tea infusion was placed in a 100 mL Erlenmeyer flask and adjusted to a pH of 2.0 with 6.0 M HCl. A mass of 0.15 g of gastric solution (freshly prepared by dissolving 6% (m/v) pepsin in 6.0 M HCl) was subsequently added and the flasks were sealed and incubated in an orbital-horizontal shaker for 2 h at 37˚C spinning at 150 rpm. The gastric digestion stage was then ended by immersing the flasks into an ice bath. The gastrointestinal digestion was started again with the intestinal simulation step by introducing 5 mL of freshly prepared intestinal solution (prepared by dissolving bile salts 2.5% and pancreatin 4.0% (m/v) in 0.1 M $NaHCO_3$) into the flasks alongside dialysis membranes of MWCO of 10 kDa containing 20 mL of a 0.15 N PIPES solution (pH 7.5, adjusted with HCl). Flasks were then incubated for 2 h at 37˚C and 150 rpm. Intestinal digestion was stopped introducing the flasks into an ice bath. Dialyzates, as well as non-dialyzate fractions, were then stored in polyethylene tubes at -20˚C until the analysis of bioavailable tea elements and tea polyphenols. Blanks of the digestion procedure were obtained applying the same protocol to 20 mL of ultrapure water. The *in vitro* bioavailability approach was applied for 18 tea samples (9 black teas, 4 green teas, 3 red teas, and 2 white teas).

For tea elements bioavailability, the analysis was carried out as described in section "Total determination of major and trace elements in tea leaves and their infusions" the analysis method was validated for linearity, sensitivity and precision, while the accuracy of the

method was evaluated in term of mass balance assay. The LODs for dialyzable elements varied from 0.018 to 142 µg/L. In general, Cs and Zn were the most dialyzable elements up to 76% and 84%, respectively, followed by Si and Ca that show moderate to high dialyzability percentages.

For the bioavailability of tea polyphenols, the antioxidant activity in terms of TPC and DPPH assays was evaluated for the dialyzable fraction from the gastrointestinal digestion. After the *in vitro* gastrointestinal simulation, TPC was reduced by 76 – 94 % of the original TPC in tea infusions, and approximately 99 % of the antioxidant activity was lost. The reduction of the antioxidant character of tea polyphenols may be attributed to various factors such as complexation of polyphenols with metal ions like Al and Fe.

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Resumen

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Resumen

El té, la bebida más popular en el mundo después del agua, se convierte en una de las bebidas ampliamente investigadas debido a sus efectos beneficiosos para el cuerpo humano. El té, producido por las hojas de *C. Sinensis*, fue descubierto hace unos 5000 años en la antigua civilización china, y se extendió por todo el mundo cultivándose en más de 30 países. Generalmente, el té se consume como una infusión preparada en agua fría o caliente, por lo cual, las hojas jóvenes y los brotes de la planta *C. Sinensis* se infusionan en agua durante un período de tiempo. Hoy en día, se comercializan diferentes variedades de té, incluidos los tres tipos de té más populares: el té negro (78%) del consumo global, el té verde (20%) y el té oolong (2%), junto con otras variedades como el té blanco y el amarillo. Todas estas variedades se prodecen de la misma planta (*C. Sinensis*) pero se distinguen por el proceso de producción que incluyen etapas de marchitamiento, laminación, fermentación, y secado. Una vez cosechadas, las hojas se secan inmediatamente y se enrollan en tiras minimizando el proceso de oxidación para elaborar el té verde. Con el marchitamiento parcial seguido de la oxidación parcial se obtiene el té oolong, mientras que la oxidación completa produce el té negro. La producción de té amarillo implica una etapa de amarillamiento de las hojas verdes secas, mientras que el té blanco se produce a partir de las hojas y brotes jóvenes, delicados e inmaduros.

La popularidad del té proviene de su efecto beneficioso para la salud humana debido a su composición química. Las hojas frescas de *C. Sinensis* se consideran como una fuente rica en polifenoles, principalmente flavanoles y sus dímeros y trímeros. Las hojas frescas contienen hasta 30% en peso de catequinas. Se cree que las catequinas son responsables de los beneficios atribuidos al consumo el té debido a sus propiedades químicas. Las catequinas incluyen catequina (C), epicatequina (EC), gallocatequina (GC), epigalocatequina (EGC), epicatequina-3-galato (ECG), epigalocatequina-3-galato (EGCG). EGCG contribuye al 50% del peso total de las catequinas. Junto a las catequinas, las hojas frescas de té contienen alcaloides (4%) como cafeína, teobromina y teofilina, metilxantinas (8%) que incluyen

lignina y ácidos orgánicos, pigmentos como la clorofila, aminoácidos libres, carbohidratos, proteínas, clorofila, compuestos volátiles, minerales y elementos trazas. La composición química del té varía según el tipo de té. Por ejemplo, el té verde contiene un mayor contenido de catequinas que las variedades oxidadas (p. ej., el té negro y el té oolong) debido a la conversión parcial o total de las catequinas en otras sustancias químicas llamadas teaflavinas (TFs) y tearubiguinas (TRs) producida por la reacción con el polifenol oxidasa.

Gracias a sus polifenoles, el té ha sido considerado como una planta medicinal desde la antigüedad. En los últimos años han aumentado los estudios sobre los efectos del consumo de té en la salud humana, acompañada de la creciente necesidad de encontrar dietas naturalmente saludables que incluyan polifenoles derivados de plantas. El efecto antioxidante de los polifenoles del té está asociado a su capacidad para prevenir el estrés oxidativo causado por las especies reactivas de oxígeno (ROS) y los quelatos metálicos involucrados en la formación de radicales libres. Varios estudios indican que los polifenoles del té funcionan como anticancerígenos en humanos, y tienen acción antialérgica, propiedades antiinflamatorias y antimicrobianas.

A pesar de la popularidad del té debido a sus propiedades antioxidantes y su contenido en elementos traza esenciales, el té pueda contener otros componentes que pueden producir riesgos para nuestra salud. Estos riesgos se asocian principalmente a la presencia de elementos tóxicos (p. ej., Cd, Hg, As, Cr) y compuestos orgánicos tóxicos. Estos compuestos generalmente provienen del suelo donde se cultiva la planta, del aire especialmente cuando la zona de cultivo está en las proximidades de una zona industrial o del proceso de producción.

Esta tesis se ha centrado en la determinación de compuestos orgánicos tóxicos, elementos mayoritarios y elementos traza, y los antioxidantes presentes en té. La tesis se ha dividido en cuatro partes principales: (1) la determinación de disruptores endocrinos (EDs), (2) la determinación total de elementos mayoritarios y elementos traza en las hojas de té y sus infusiones, (3) la determinación total de la

actividad antioxidante del té, y (4) estudio de la biodisponibilidad de elementos traza y antioxidantes en muestras de té.

A. Determinación de disruptores endocrinos: ésteres del ácido ftálico (PAE) y bisfenol A (BPA)

Los disruptores endocrinos (EDs) son compuestos químicos adversos que interfieren en el sistema endocrino humano imitando las hormonas naturales. EDs pueden provocar graves problemas a la salud de los organismos vivos. Los PAEs y BPA se encuentran entre los EDs ampliamente utilizados en el sector industrial del plástico como plastificantes. Con el fin de evitar riesgos para la salud, es necesario determinar los niveles de estos compuestos en los alimentos y bebidas que estén en contacto con estos materiales. Dado que estas sustancias pueden presentarse en niveles traza en la muestra de té, se requieren técnicas altamente sensibles para el análisis, precedidas por una etapa de preparación de la muestra.

En este apartado de la tesis se han determinado cuatro ftalatos: dimetilo ftalato (DMP), dietilo ftalato (DEP), dibutilftalato (DBP), y butilbencil ftalato (BBP) y BPA en infusiones de té. La técnica de análisis utilizada en este trabajo fue la cromatografía líquida de alta resolución acoplada a la espectrometría de masas (HPLC-MS). Los analitos se preconcentraron mediante la extracción en fase sólida (SPE) utilizando como sorbentes polímeros de impronta molecular (MIP).

El primer paso en esta parte fue optimizar las condiciones del MS para lograr una mayor resolución y sensibilidad a la relación masa/carga (m/z) seleccionada para cada compuesto (DMP (163,25), BBP (91,15), DEP y BBP (149,05) y BPA (227)). Los potenciales en la interfase del MS son: el potencial de declustering (DP), el potencial de enfoque (FP) y el potencial de entrada (EP). Los parámetros de la fuente se optimizaron por inyección directa de una mezcla de estándares de 500 µg/L de ftalatos trabajando en modo positivo. Del mismo modo se optimizaron las condiciones de medida para la determinación de BPA, utilizando una disolución estándar de 500 µg/L de BPA y trabajando en modo negativo. La **tabla 1** resume los

potenciales óptimos de la interfase, así como la relación m/z seleccionada para la detección de los ftalatos y BPA.

Los parámetros optimizados para la fuente de iones son el voltaje del ion spray, presión del gas de nebulización (N_2) y del gas cortina (N_2) así como la temperatura de la fuente. Los valores óptimos del voltaje de la fuente de iones, la presión del gas de nebulización y el gas cortina (N_2), y la temperatura para introducir los analitos al MS fueron de 5500 V, 14 psi y 450 ℃, respectivamente para la determinación de los ftalatos, y -4500V, 12 psi y 450 ℃, para la determinación de BPA.

Tabla 1. Condiciones óptimas de medida en el espectrómetro de masa

Compuesto	*m / z*	Potenciales (V)			Modo
		DP	FP	EP	
DMP	163,25	40,38	73,87	8	
BBP	91,15	25	225	6 6	Positivo
DEP, DBP	149,05	25	290	8,5	
BPA	227	-125	-195	-9	Negativo

Una vez optimizadas las condiciones de la fuente, se optimizan los parámetros de HPLC para obtener la mejor separación de los analitos de interés. Los parámetros de HPLC son la columna cromatográfica, la composición de la fase móvil, el flujo y el modo de elución (es decir, en modo gradiente o isocrático). La **tabla 2** resume las condiciones óptimas para la separación cromatográfica.

Tabla 2. condiciones óptimas para la separación cromatográfica para la determinación de ftalatos y BPA.

Parámetro	PAEs	BPA
Fase móvil	CH_3CN: H_2O (ácido acético al 0,1% (v/v))	CH_3CN: H_2O (0,8% NH_3)
Columna	Zorbax Eclipse XDB-C8, 50x2,1 mm, tamaño de partícula 3,5 µm)	
Flujo fase móvil (µL/min)	200	250
Volumen de inyección (µL)	10	5
Modo de elución	Gradiente	Isocrático

Para la determinación de estos compuestos en las muestras de té, se prepararon las infusiones teniendo en cuenta las recomendaciones de los fabricantes. Debido a que estos compuestos se pueden encontrar en niveles traza en las infusiones, es necesario realizar una etapa de preconcentración para aumentar la sensibilidad del análisis. El procedimiento de preconcentración seleccionado es la extracción en fase sólida utilizando polímeros de impronta molecular, (MIP) sintetizados para la preconcentración selectiva y específica de los ftalatos y BPA. El procedimiento de síntesis llevado a cabo en este apartado ha sido realizado mediante una reacción de polimerización por precipitación. La síntesis de los dos polímeros se realizó usando ácido metacrílico (MAA) como monómero, etilenglicoldimetacritalo (EDMA) como entrecruzante, 2,2′-azobisisobutironitrilo (AIBN) como iniciador y acetonitrilo como disolvente. Sin embargo, las plantillas fueron diferentes; DBP se usa como plantilla para la síntesis del MIP para la preconcentración de los ftalatos y BPA se usa como plantilla para la síntesis del MIP para la preconcentración de BPA. Una vez finalizada la polimerización, los polímeros se filtraron y se lavaron con metanol, diclorometano, metanol y metanol/agua (1:1, v/v) para eliminar las plantillas. Se requiere eliminar la plantilla para activar el polímero para ser utilizado para la extracción del analito de interés. Los polímeros sintetizados se empacaron en cartuchos de vidrio y se acondicionaron pasando a través de ellos metanol seguido de agua ultrapura antes del proceso de extracción.

El proceso de extracción se optimizó para obtener una alta recuperación de los analitos. Las variables estudiadas fueron el flujo de carga y elución de la muestra, pH de la muestra, volumen de eluyente. El proceso de optimización se realizó utilizando una disolución patrón de mezcla de ftalatos (100 µg/L), para el MIP de ftalatos y una disolución patrón de BPA (100 µg/L) para el proceso de extracción del BPA. Las condiciones optimizadas para el proceso de extracción son las siguientes: el flujo de carga de muestra óptimo es de 1,0 mL/min tanto para los ftalatos como para BPA, mientras que el pH óptimo de la muestra estaba en un nivel neutro. El volumen de eluyente (metanol) seleccionado fue de 3 mL y 4 mL para los ftalatos y BPA, respectivamente.

Una vez optimizado el proceso de extracción, se realizó la validación de los métodos estudiando la linealidad, sensibilidad, precisión y exactitud (recuperación analítica). Los métodos optimizados fueron sensibles (límite de detección < 2 μg/L para los ftalatos y 72 ng/L por BPA) precisos (RSD <10%) y con porcentajes de recuperación entre 84% y 97% para los ftalatos y 101 % para el BPA. Finalmente, los métodos optimizados se aplicaron para la determinación de estos compuestos en infusiones de muestras de té comercializadas en bolsas. Por otro lado, se realizó un estudio de migración de ftalatos y BPA del material de las bolsas de té para evaluar la contribución de las propias bolsas a las cantidades totales de ftalatos y BPA presentes en la infusión.

Junto con el estudio de migración, se estudió la composición del material de las bolsas de té utilizando espectroscopía infrarroja con transformada de Fourier. Los resultados obtenidos indican que las bolsas están compuestas mayoritariamente de ácido poliláctico, celulosa y el poli(tereftalato) de etileno

Entre los ftalatos estudiados, los niveles de DEP fueron inferiores al límite de detección, mientras que DBP fue el más abundante en todas las muestras. También se han detectado BBP y DMP en dos y tres muestras de té, respectivamente. El estudio de migración demostró que una fracción de PAEs presente en las infusiones proviene de la bolsa de té. Los porcentajes de migración ftalatos varían entre 1,8 y 93,5 %.

En el caso de la determinación de BPA en las infusiones, se ha detectado el BPA en dos muestras de las 24 muestras analizadas. Por otro lado, se ha realizado un estudio de migración de BPA por el análisis de las infusiones realizadas a partir de las bolsas del té vacías. Los resultados del estudio de migración indican que las bolas de té contribuyen en un 14% y 62% de BPA para las dos muestras en las que se había cuantificado.

B. Determinación del contenido total de elementos mayoritarios y trazas en las hojas de té y sus infusiones.

Las hojas de té y los tés procesados son una fuente rica de elementos que incluyen elementos mayoritarios, minoritarios y traza. Los niveles de estos elementos en las muestras son debidos a factores

geográficos como el clima, la composición del suelo y la práctica agrícola del lugar donde se cultivó el té, y de los métodos de procesamiento de las hojas como el secado, la fermentación, etc. Algunos elementos son esenciales para la salud humana (p. ej., Ca, Co, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, Se, V y Zn), mientras que otros elementos pueden ser tóxicos, como Pb, As, Hg y Cd. Estos elementos pueden proceder del suelo de cultivo, del polvo atmosférico, precipitaciones, fertilizantes utilizados durante el cultivo de las plantas o de los procesos de fabricación. Teniendo en cuenta las diferentes propiedades de los elementos (esenciales, no esenciales y tóxicos) y debido al alto consumo de té en algunos países, es importante su determinación tanto en las hojas de té como en su infusión.

En esta parte de la tesis, se determinaron las concentraciones totales de 33 elementos en distintos tipos de té, tanto en hojas como en infusiones. Para la determinación de los elementos en las hojas de té, se realizó una digestión ácida asistida por microondas de las muestras de té. Las hojas se pesaron con exactitud (0,5000 g) y se transfirieron a reactores de teflón junto con 3 mL de HNO_3, 1 mL de H_2O_2 y 4 mL de agua ultrapura. La digestión se realizó mediante un programa de tres pasos. La digestión comenzó por el calentamiento de los reactores variando linealmente la temperatura desde temperatura ambiente hasta 90°C durante 2,5 min a una potencia de 1000 W. Luego se aumentó la temperatura a 140°C mantenido la potencia a 1000W durante 60 minutos, seguido por otro incremento de temperatura hasta 180°C durante 11 minutos. Una vez finalizado el proceso, las soluciones (a temperatura ambiente) obtenidas se transfirieron a matraces volumétricos, se diluyeron con agua ultrapura hasta 25 mL y se guardaron en frascos de polietileno hasta el momento del análisis.

El análisis se realizó utilizando tres técnicas espectrométricas ICP-MS (Li, Be, Cr, Ti, Cu, Ga, Ag, Hg, Cd, Cs, Co, Pt, Tl, Pb, As, Ni, V, Se, Sn and Sb), ICP-OES (Al, Ba, Ca, Fe, Mg, Mn, Mo, P, Rb, Si, Zn, and Sr), y FAAS (K). El método analítico (digestión y detección) propuesto fue validado, estudiando el intervalo de linealidad, sensibilidad, precisión y exactitud. La exactitud del método se evaluó utilizando dos materiales de referencia certificados (Tea Leaves INCT-TL-1 y Rye Grass), así como mediante la recuperación

analítica. El método validado se aplicó para el análisis de 35 muestras de té, incluyendo té negro, verde, rojo y blanco.

Por otro lado, se determinaron las concentraciones totales de elementos en infusiones de té. Las infusiones se prepararon según las recomendaciones del fabricante, sumergiendo 1,5 g de hojas de té en 150 mL de agua ultrapura hervida durante 5 minutos. Las infusiones se dejaron enfriar, se filtraron y se guardaron en frascos de polietileno a 4 °C hasta el análisis. El método fue validado y resultó ser sensible, preciso y exacto con recuperaciones analíticas entre 92 y 115%. El método validado se aplicó para el análisis de 35 muestras de té.

Entre los elementos estudiados, K fue el elemento más abundante tanto en las hojas de té como a las infusiones, seguido de Ca, Mg y P. Por otro lado, también se evaluaron las eficiencias de extracción de los elementos de las hojas de té en las infusiones de té. Los elementos se clasificaron en tres categorías: elementos altamente extraíbles (> 50%): Cs, Tl, Ni y Rb, elementos moderadamente extraíbles (10 - 50%): Li, Be, Cu, Ti, Co, As, Al, Cr , P, Mg, Mn, Si, Zn y K, y elementos poco extraíbles (< 10%): Ga, Cd, Pb, V, Ba, Fe, Ca y Sr, mientras que algunos elementos como Mo, Hg, Pt, Ag, Se, Sn y Sb muestran una extracción muy débil y sus concentraciones fueron muy bajas o incluso no detectables.

C. *La actividad antioxidante de las infusiones de té*

El té es una importante fuente de compuestos bioactivos con actividad antioxidante, entre los cuáles se encuentran los polifenoles, flavonoides, compuestos fenólicos, etc. El té verde tiene una gran actividad antioxidante debido a la presencia de una gran cantidad de catequinas. Numerosos estudios han demostrado que los polifenoles de té tienen un gran efecto para la salud humana reduciendo o previniendo las consecuencias perjudiciales del estrés oxidativo que pueden causar cáncer, enfermedades neurodegenerativas y enfermedades cardiovasculares. Dado que las infusiones de té se consumen diariamente en muchos países del mundo, esta parte de la tesis se ha enfocado a la evaluación de la actividad antioxidante de diferentes tipos de té (verde, blanco, negro y rojo). Las muestras incluidas en este estudio son las mismas que las analizadas para la

determinación de elementos traza. Por lo tanto, las infusiones de té preparadas dividieron en dos partes: una para el estudio elemental y la otra para el estudio antioxidante.

Se evaluó la actividad antioxidante de los polifenoles del té utilizando métodos espectrofotométricos: determinación de fenoles totales (TPC) usando el método espectrofotométrico desarrollado por Folin y Ciocalteu (FC) y el método de captura del radical DPPH (2,2- difenil-1-picrilhidrazilo).

Para la determinación de fenoles totales, una alícuota de cada infusión de té se diluyó (40 veces) con agua ultrapura y se mezcló con 100 µl del reactivo de FC y 1 mL de 20% Na_2CO_3. La mezcla se diluyó a 5 mL con agua ultrapura y se incubó en la oscuridad durante 30 minutos a temperatura ambiente. La absorbancia del color azul desarrollado se mide a 760 nm. Los resultados se expresan en mg equivalentes de ácido gálico por litro de infusión (mg GAE/L). El contenido total de los fenoles se determinó utilizando el método de adición estándar preparado con soluciones estándar de ácido gálico que cubren el rango de 1 - 15 mg/L, presentando una buena linealidad ($r = 0,999$). El límite de detección obtenido fue de 23 mg GAE/L. Los resultados obtenidos muestran que el contenido total de fenoles está entre 87 – 1580 mg GAE/L. La variación en TPC entre muestras del mismo tipo de té puede atribuirse al origen del té, la edad, el método de procesamiento, la temperatura y tiempo de infusión. Los tés verdes y blancos contienen niveles, relativamente, más altos de fenoles que los negros y rojos debido al proceso de producción.

Para el ensayo DPPH, se mezcló una alícuota diluida de infusión de té (1:20) con 2,9 mL de 0,1 mM DPPH. La mezcla se agitó vigorosamente en vortex y se mantuvo en la oscuridad durante 30 minutos a temperatura ambiente. La reducción de la absorbancia se midió a 517 nm y los resultados se expresan como actividad equivalente a Trolox (mM TRE). El ensayo DPPH se realizó utilizando el método de calibración externa (0,05 - 0,8 mM TRE), y el límite de detección obtenido fue 0,05 mM TRE. En general, el té verde posee la mayor actividad antioxidante entre las muestras de té analizadas. Los resultados indican que la capacidad antioxidante en las infusiones de té se encuentra en el rango de 1 - 9,9 mM TRE.

Estadísticamente, existe una buena correlación entre los fenoles y la capacidad antioxidante del té, especialmente el té verde mostrando un coeficiente de correlación de 0,9247.

D. Biodisponibilidad de elementos traza y antioxidantes en las muestras de té.

El té es una fuente rica en antioxidantes y elementos esenciales. Sin embargo, para comprender los beneficios nutricionales, o incluso posibles riesgos del consumo de té sobre la salud humana requiere estudiar la cantidad de nutriente liberado de la matriz que está disponible para su absorción en el intestino. La biodisponibilidad incluye la digestión gastrointestinal, la absorción, el metabolismo y la bioactividad de la fracción de nutriente ingerido dentro del organismo vivo. Otro término utilizado por los científicos para referirse a la biodisponibilidad de un componente es la bioaccesibilidad. La bioaccesibilidad de un componente se define como la fracción de este componente que está lista para ser absorbida. Para ello, se han desarrollado varios protocolos simulando el proceso de digestión en el tracto gastrointestinal.

Para simular la digestión gastrointestinal se utiliza generalmente un protocolo de dos pasos con una mezcla de disoluciones gástricas (pepsina) y disoluciones intestinales (pancreatina y sales biliares). Al hacer la simulación es necesario tener en cuenta parámetros como la temperatura corporal, el tiempo de digestión y el movimiento peristáltico del tracto gastrointestinal. Someter un nutriente a un modelo simulación de digestión gastrointestinal da una idea sobre la fracción biodisponible de ese nutriente en el cuerpo humano. Se han utilizado varios modelos para evaluar la biodisponibilidad de un nutriente en el tracto gastrointestinal como la solubilidad, la dializabilidad y modelos de línea celular Caco-2. Después de hacer la digestión gastrointestinal, la disolución digerida se centrifuga o se filtra para separar el sobrenadante del sólido como en el caso de la solubilidad, o se realiza un proceso de diálisis a través membranas como en el caso de la dializibilidad. El modelo más realista es el uso de la línea celular Caco-2, ya que el procedimiento implica el uso de

células vivas similares a las que se encuentran en el intestino. La solubilidad y la dializabilidad utilizan el mismo procedimiento inicial, pero la dializabilidad es preferible a la solubilidad debido al uso de una membrana de diálisis que imita las paredes del intestino dando una idea sobre el transporte de nutrientes a través de la pared.

En esta parte de la tesis, se evaluó la biodisponibilidad de los elementos traza y los polifenoles en infusiones de té utilizando el protocolo de dializabilidad. El estudio de biodisponibilidad se realizó con las infusiones de las muestras analizadas para el contenido total de elementos traza.

El protocolo de dializabilidad se dividió en dos etapas: etapa gástrica y otra intestinal para simular la digestión gastrointestinal. Durante la etapa gástrica, se introdujo un volumen de 20 mL de infusión de té en un matraz Erlenmeyer de 100 mL y se ajustó a pH 2,0 con HCl 6,0 M. Posteriormente se añadieron 0,15 g de solución gástrica (previamente preparada disolviendo pepsina al 6% (m/v) en HCl 6,0 M), los matraces se taparon con Parafilm y se incubaron en un agitador orbital horizontal durante 2 h a 37ºC girando a 150 rpm. Transcurrido ese tiempo se sumergieron los matraces en un baño de hielo para detener la etapa de digestión gástrica. Se continua con la etapa intestinal añadiendo a cada matraz 5 mL de solución intestinal recién preparada (sales biliares al 2,5% y pancreatina al 4,0% (m/v) en $NaHCO_3$ 0,1 M), y se introduce la membrana de diálisis (Cut Off 10 kDa) que contienen 20 mL de solución de PIPES 0,15N (pH 7,5, ajustado con HCl). Se incuban los matraces durante 2 horas 37 ° C y 150 rpm. Finalizada la etapa de digestión intestinal, se introducen los matraces en un baño de hielo para parar el proceso de digestión. Los dializados, así como las fracciones no dializadas, se guardaron en tubos de polietileno a -20 ° C hasta el análisis. Se realizaron blancos de digestión realizando el mismo procedimiento con 20 mL de agua ultrapura. El protocolo de biodisponibilidad *in vitro* se aplicó a 18 muestras de té (9 tés negros, 4 tés verdes, 3 tés rojos y 2 tés blancos).

El análisis de los elementos en la fracción dializada se realizó por ICP-MS y ICP-OES. El método de análisis se validó estudiando la linealidad, sensibilidad y precisión. La exactitud del método se evaluó realizando un balance de masas. Los límites de detección obtenidos

para la determinación de los elementos en la fracción dializable variaron de 0,018 a 142 µg/L. En general, Cs y Zn fueron los elementos más dializables hasta 76%, 84%, respectivamente, seguidos de Si y Ca que muestran porcentajes de dializabilidad de moderados a altos.

En el estudio de la biodisponibilidad de los polifenoles del té, se evaluó la actividad antioxidante en términos de ensayos de TPC y DPPH para la fracción dializable de la digestión gastrointestinal. Después de la simulación gastrointestinal *in vitro*, el contenido total de los fenoles de té se redujo en un 76 - 94% del contenido original en las infusiones de té, y se perdió alrededor del 99% de la actividad antioxidante. La reducción del carácter antioxidante de los polifenoles del té puede atribuirse a diversos factores, como la formación de complejos de polifenoles con iones metálicos como Al y Fe.

CHAPTER 1

Introduction

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Introduction

1.1 TEA AS A SAMPLE

1.1.1 History

Tea, an evergreen shrubby member of the Theaceae family, is the most consumable drink in the world after water. Tea, called as "The Diving Healer" by the Chinese emperor Shen Nung, was discovered around 5000 years ago in 2737 B.C. Several legends addressed the discovery of the tea; among these legends the one which mentioned the story of the Chinese emperor who was boiling water when leaves of a nearby tree fell into the boiled water producing a pleasant and intense flavored drink. The emperor declared the birth of a new beverage and called it "heaven-sent". Another myth speaks about that Buddhist monk who struggled to stay awake in a prolonged meditation by cutting his eyelids and flinging them to the ground, as a result of this devotion, the gods recompensed him by turning his eyelids into tea plants. The myth claimed that the monk was able to stay awake for seven years only by chewing the tea leaves. Despite what preceded, the ancient Chinese culture used to consider tea as a medicinal herb with more than 200 claimed uses for curing and alimentation [1]. In the 13th century, tea was imported to Japan, and 3 centuries later, it reached Europe and North America and later on to Africa and other parts of the world [2]. Nowadays, tea is cultivated across the world in more than 40 countries including China, India, Sri Lanka, the United Kingdom, North and South America, etc.[3].

1.1.2 Cultivation

Tea cultivation requires humid tropics and subtropics environments, and even though, it shows a wide adaptability for growing in wide a geographical zone (i.e., from north 42˚ N (Russia) in the north to south 27˚S (Argentina) longitude), and across altitudes ranging from sea level to 2200 m above sea level. The optimum climatic conditions for tea plantation are 2500 – 3000 mm annual rainfall and 18 – 20 ˚C. However, it is possible to cultivate tea in those areas with minimum precipitation of 1200 mm and an average temperature of 12 – 30˚ [4]. The wide geographical area for tea

cultivation means that soils differ from one zone to another; thus, the most important parameter for tea growth is soil pH. Acidic soil with a pH ranged between 4.5 – 5.6 is considered as the optimal environment for cultivation. Other parameters related to the soil are recommended such as being well-drained, well aired, deep and with more than 2% organic nutrients [4,5].

Tea shows unprecedented growth in domestic demands in the period of 2007 – 2016, where the production reached 5.73 million tons by 2016, with an annual increase of 4.4%. In 2016, China was the largest tea producer in the world with an output of 2.44 million tons (about 42.6% of the world tea production), followed by India (1.27 million tons). Kenya and Sri Lanka produced around 475300 tons and 295300 tons, respectively. Tea exports raised by 1.4% over the period 2007 -2016 reaching 1.75 million tons due to the increase in the domestic demands and the larger shipments from Kenya [6].

Since its discovery, tea was consumed for its medicinal benefits. Tea industry converted into a global industry, especially after noticing the stimulant properties of this magic drink because of its caffeine contents. Considering the consumption, tea is the second consumed drink in the world behind water. The Food and Agriculture Organization - Intergovernmental Group (FAO-IGG) reported that annual world tea consumption was increased by 4.5% (2007 – 2016) to reach 5.5 million tons by 2016. This growth was underpinned by the elevated incomes and production efforts that aim to produce diverse combinations of fruit extracts and flavored teas especially in China, India and other developing countries [6]. Turkey was the leading global tea consumer in 2016 with an approximate per capita tea consumption of 3.16 Kg/year, followed by Ireland 2.19 kg per capita, the UK, etc. while average per capita consumption in Spain stood at 0.05 kg/year [7,8].

Tea is classified into two main varieties: *C. Sinensis* variety assamica (Assam variety) and *C. Sinensis* variety *Sinensis* (China variety) [9]. Assam variety is known by its large-leaves, tall and fast-growing tree. However, China-variety is a slow-growing shrub with vertical small-leaves. Another less common tea variety is Cambod tea

(*C. assamica – lasiocalyx* Planch), which is a hybrid type between Assam and China teas [10].

Tea varieties can be classified according to the manufacturing operations involved in the production lines. For the production, the top buds and young leaves are harvested either by selective handpicking or using machines. Since the hand plucking is an expensive laborious method, machines were adopted in many countries to save money and time [11]. However, machine picking leads to the lack of quality since the harvest is not selective and some mature leaves may be included and leaves may get damaged which affects the quality and taste of tea beverages [12]. Using delicate hand plucking, up to 30 kg/day per plucker is picked. The plucking is made at regular intervals of the year; generally, every 4 – 10 days. The picked leaves must be saved loosely and immediately taken to the factory to prevent leaf damaging and avoid any possible uncontrolled fermentation.

1.1.3 Production process

Many chemical and physical changes are involved during the manufacturing process, where the leaves pass through different stages. These stages including withering, rolling, fermentation, and drying, determine the quality and the variety of tea needed.

1.1.3.1 Withering

Processing starts with withering, where the surface moisture of the green leaves is reduced. Withering can be done outdoors (natural withering) or indoors (artificial withering). Natural withering is carried out by exposing the leaves to air under suitable humidity and temperature. Artificial withering uses a stream of hot air generated in a hot room that is mixed with the outside air by fans. These fans are arranged carefully so the air temperature is maintained below 35˚C. The time of withering and rate of moisture removal during this stage is influenced by tea leaves type, air drying capacity, though load and whether the leaves are wet or dry. Usually, 12 - 18 hours are needed to reduce 10-15% of the moisture. The taste and the organic compounds (especially caffeine) are being intensified during this stage. After withering, leaves look flaccid and soft enough for the next stage.

1.1.3.2 Rolling

Withered leaves were then subjected to the rolling stage. During rolling, tea leaves are crushed into small pieces insuring the mixing of the cell components. Before rolling, the polyphenol oxidase (PPO) found in the cytoplasm and polyphenols are spatially separated, thereby crushing the leaves aims to activate the biochemical reformations by mixing PPO with polyphenols in the presence of oxygen [13].

Withering and rolling stages involve physical and chemical changes of tea, which affect the tea contents. Some contents decrease like moisture removal, the permeability of the cell membrane, grass-like odor, polyphenols, lipids, glycoside, etc. Other contents increase due to their chemical nature such as caffeine, free amino acids, enzyme activity, carotenoids, etc.[13].

1.1.3.3 Fermentation

Fermentation is an enzymatic oxidization stage of tea polyphenols. The oxidation activates the polyphenols, which are known to be responsible for the color, flavor and benefits popularly associated with tea consumption. The taste profile of the fermented tea leaves is different from those who do not undergo the fermentation process. During the fermentation stage, active enzymes, mainly PPO, convert tea catechins into higher molecular weight compounds like theaflavins and thearubigins [14]. For specific flavor intensities, tea producers control the oxidation process by adjusting several factors including fermentation temperature, time, humidity levels, and oxygen availability. Changing these factors leads to get different contents of theaflavins and thearubigins which, in turn, affects the color and taste of the fermented tea leaves. Thus, less oxidized leaves keep their green color partially due to the lower degree of polyphenols. Semi-oxidized tea has a brown aspect and produces yellow-amber extract once infused. On the other hand, wholly oxidized tea, where the amino acids and lipids are entirely broken down, gives a blackish-brown leaf [15, 16].

1.1.3.4 Drying

The main objective of this stage is to inhibit the enzymatic oxidation and minimize the water content of the fermented tea leaves to about 3%. Drying intensifies the tea's taste and pro-longs its self-life. It is crucial to control the time and temperature during the drying process; otherwise, tea quality may be deteriorated.

1.1.4 Types of tea

Based on their processing as well as levels of oxidation and fermentation, different types of tea can be produced and classified. Alongside the manufacturing processes, the plant variety, the quality of the leaves harvested, the location, and the harvest seasons have an important role in identifying the type of tea produced and as a result, influence its taste and aroma profile. The most common tea varieties (types) are black, green, oolong, yellow and white teas.

1.1.4.1 Green tea

Non-fermented *C. Sinensis* leaves with a minimal level of oxidation are used to produce green tea. Fresh buds and young leaves are immediately withered, rolled, steamed in baking pans and packaged. Steaming aims to prevent polyphenol degradation by deactivating the enzymatic oxidation, so the original amount of the biologically active compounds is considerably retained from degradation [17]. Green tea is known for its potent antioxidant activity among other types of tea, and this power comes from the fact that green tea contains high levels of polyphenols especially catechins which represent up to 25-35% of the dry weight of tea leaves. Green tea also contains other polyphenols like flavanols, quercetin, phenolic acids, caffeine, myricetin, etc. [18].

1.1.4.2 White tea

White tea is sourced from the *C. Sinensis* plant. It is produced from the delicate young leaves and buds. The name of "white" is a descriptive term that stands for the silky white needles that cover the immature buds and leaves. White tea has a sweet and delicate taste different from the unique flavor of green tea. White tea is plucked once a year in spring especially in a sunny morning so the remaining moisture on the leaves and buds is dried naturally. Without too much

processing, white tea is steamed and dried; thus, neither oxidation nor rolling is included. White tea is more expensive than the other types of tea due to the minimal processing, least labor and short time needed [19].

1.1.4.3 Oolong tea

Historically, oolong tea was introduced during the Ming dynasty in the Fujian province of China [20]. This type of tea is mainly consumed in southern China, Taiwan, and most Eastern countries and represents 2% of world tea consumption [21]. It is prepared by a partial fermentation of withered *C. Sinensis* leaves followed by roasting in baking pans. Different types of oolong tea can be produced depending on the degree of oxidation. For instance, green oolong is prepared at 20% oxidation, while a darker one is obtained once the oxidation reached 60% [22]. The slightly fermented oolong tea retains most of its bioactive components, which means that this type of tea possesses antioxidants activity towards radical oxidative species. Also, oolong tea has other pharmacological effects like anti-cancer, anti-obesity, protective effects against atherosclerosis, etc. [22, 23].

1.1.4.4 Black tea

Black tea is the most produced and consumed tea in the world. Black tea is made of *C. Sinensis* plant, just like green, white and oolong teas. However, black tea is a fully fermented type. During the fermentation process, secondary polyphenols are formed by the enzymatic oxidation, where catechins are converted into theaflavins and thearubigins. Theaflavins are responsible for the astringency, brightness, and briskness of the tea infusion, while the red-brown color and the intensity of the mouth-feel is attributed to the thearubigins [24,25]. In contrary to the fact that theaflavin levels in tea rise to a certain level before starting to drop, thearubigins contents increases during the whole fermentation process. Therefore, the manufacturing process must be controlled to get the proper ratio between theaflavins and thearubigins and to increase the theaflavin levels. Practically, the color change (from green to copper) and the

developed aroma are used to decide the completion of the fermentation [26].

1.1.4.5 Pu-erh tea

Pu-erh or Pu'er tea is a naturally aged variety tea produced from *C. Sinensis* var. Assamica Kitamura. Originating from Yunnan province in China, Pu-erh tea may be treated in two ways, either like green tea where the leaves are partially heated and left to be aged or like a post-fermented black tea where the fermentation occurs with the help of microorganisms in a hot humid ambient. Freshly plucked tea buds (commonly two or three buds) are partially dried by sun for 8 h. PPO enzyme activity is then partially inactivated by heating the tea leaves in a container at a low temperature compared to that used in the case of green tea [27]. The tea leaves were then rolled for a short time so the cell breakage rate will be lower, dried again by exposing them for the sun for 3 – 5 h at 30˚C at least. The post-fermentation process is a microbial process where the extracellular enzymes formed by microorganisms catalyze the dried leaves to be oxidized by the condensation and degradation of the chemical components [28].

1.1.4.6 Yellow tea

The production process of the yellow tea is like that used in the case of green tea; however, a new stage called “sealed yellowing” is involved. Yellow tea has a sweet taste compared to the grassy taste obtained from green tea [29]. Mainly, the sealed yellowing step is affected by the water content and the treatment temperature; thus, faster yellowing can be achieved by applying higher water content and higher temperature. During the yellowing step, many microorganisms are included to catalyze the macromolecules decomposition by the action of extracellular enzymes. Comparing to pu-erh tea aging time (up to years), a shorter time (up to a few days) is needed to perform the yellowing step [30]. **Figure 1.1** shows the production steps of different types of tea.

Among these different types of tea, black tea is the most consumed one especially in Western countries, accounts for 78% of the produced teas in the world. However, green tea, mostly consumed in Asian and Northern African countries, account for 20% of the

global consumption followed by oolong tea (2%) which is consumed in Eastern countries (southern China, Taiwan, etc.) [22]. Pu-erh tea, mainly consumed in China, accounts for 58.2% of the total tea produced there [28].

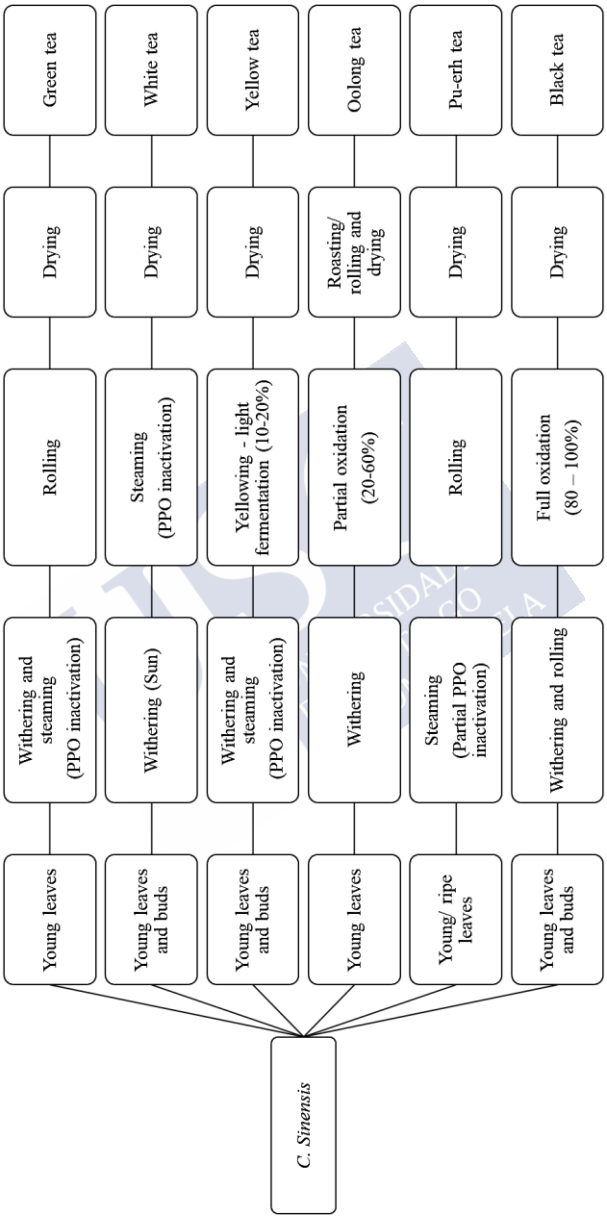

Figure 1.1 Production steps of the different types of tea (Figure adapted from Xu et al. [30])

1.1.5 Chemical composition

As explained in the preceding section, several types of tea can be obtained using different manufacturing pathways. The different production processes lead to obtain a distinct chemical composition of the tea produced. This diversity includes the presence organic acids, proteins, carbohydrates, minerals, polyphenols, vitamins, fibers and caffeine [31, 32]. Fresh *C. Sinensis* leaves are rich in polyphenols dominated by flavanols and their dimers and trimers. Catechins form up to 30% of the unprocessed tea leaves weight which predominate the chemical function of tea leaves [33]. Catechins usually include (+)-catechin (C), (-)-epicatechin (EC), (-)-gallocatechin (GC), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG), (-)-epigallocatechin-3-gallate (EGCG). EGCG contributes to 50% of the total weight of catechins [34]. Moreover, *C. Sinensis* buds and leaves contain also a nonprotein amino acids responsible for the umami taste of the beverage (i.e., pleasant savory taste caused by the glutamates and nucleotides). Among these amino acids; theanine is the most abundant amino acid in fresh tea leaves accounts for 1.5% of the dry weight of the leaves [35]. On the other hand, degradation products of theanine is hypothesized to produce some derivatives called N-ethyl-pyrrolidinone substituted flavan-3-ols (EPSFs). These derivatives have been separated and identified from white, yellow, post-fermented (Pu-erh) and black teas [36–39]. Nevertheless, it still unclear whether EPSFs are biosynthesized in the plant or formed by nonenzymatic interaction between theanine and catechins.

Alongside catechins, fresh tea leaves contain approximately 4% of stimulant alkaloids such as caffeine (3.5%), theobromine (0.2%), and theophylline (0.04%), and methylxanthines that include 6.5% lignin, 1.5% organic acids, pigments like chlorophyll (0.5%) and 5.5% free amino acids of the total dry weight [3]. Moreover, tea leaves contain trace amounts of elements (about 5% of the dry weight) including: K, Mg, Ca, P, Cu, Fe, Zn, Mo, Al, Na, Co, Sr, Ni, F, Mn, etc. Also trace amounts of vitamins B, C, and E, volatile organic compounds (Esters, aldehyde, lactones, etc.), and flavonol aglycones (quercetin, kaempferol, myricetin), etc. [40].

During the fermentation process, the chemical composition is changed. For instance, simple catechins are easily transferred into other higher molecular weight derivatives like theaflavins (TFs) and thearubigins (TRs) due to the enzymatic oxidation by polyphenol oxidase. This transformation leads to decrease the catechins content depending on the degree of oxidation. For example, polyphenols content in green tea ranges between 30 and 40% while black tea contains from 3 to 10 % [41]. TFs and TRs contribute to the distinctive bright color of the black tea and represent 35% of the dry weight of black tea [24]. **Table 1.1** lists the contents of these compounds in six types of tea.

In conclusion, the chemical composition of tea differs from one type to another according to the original levels in the leaves, the origin, cultivation conditions, and different degrees of fermentation during processing [42, 43].

Table 1.1 The chemical profile of some bioactive compounds reported in six types of tea.

Chemical	*White tea*	*Green tea*	*Yellow tea*	*Oolong tea*	*Black tea*	*Pu-erh tea*	*Ref.*
Caffeine [a]	27.2 ± 5.4	34.86 ± 4.32	33.32 ± 7.1	19.67 ± 3	28.5 ± 3.7	31.8 ± 4.9	[44]
	16.8	16.28	-	19.31	17.73	-	[45]
	-	-	24.5 ± 1.4	-	-	-	[36]
Catechins (Total) [a]	54.6	112.72	118.55	75.2	7.22	6.25	[44]
	56.6 ± 12.5	105.9 ± 35.7	-	86.9 ± 23.5	10.2 ± 6.7	5.01 ± 1.8	[46]
Theaflavins (Total) [a]	-	0.88-5.6	-	0.66-3.6	10.7 - 17.3	-	[47]
L-theanine [a]	6.26	6.56	-	6.09	5.13	-	[45]
Amino acid [b]	-	213.4 - 328.7	-	111.51	15.88	6.47	[48]
Quercetin [c]	-	175 ± 48	-	130	210 ± 76	52	[49]
Kaempferol [c]	-	130 ± 34	-	90	132 ± 47	23	[49]
Myricetin [c]	-	101 ± 43	-	49	25 ± 21	40	[49]
Polysaccharides [d]	-	0.13	0.36	-	0.28	-	[50]

a: in mg/g, b: in µg/g, c: in mg/100g, d: mg/mL

1.1.6 Tea consumption and its health benefits

Extensive researches have been carried out to evaluate the beneficial effects of tea compounds. To date, tea is known for its health-promoting influence on human. Many experimental, clinical and epidemiological studies have been indicated that tea and its bioactive components are responsible for the vast beneficial effects on health. Tea consumption, especially green tea possesses antimicrobial, antihypertension, anti-inflammatory, antioxidative and cholesterol-lowering properties. These properties are positively associated with the prevention of many diseases like cardiovascular disease (CVD), arthritis, obesity, diabetes, cancer, etc. [22].

The antioxidative character of tea polyphenols attributes to the scavenging of the reactive oxygen species (ROS) that cause oxidative stress. ROS, such as superoxide radicals, singlet oxygen, nitric oxide, hydroxyl and peroxyl radicals, etc., are oxygen free radicals formed as side-products of the biological metabolism. The presence of such free radicals contributes to the damage of macromolecules like DNA, lipids and proteins in the cell-free systems. The oxidative stress contributes to the development of some chronic diseases including Alzheimer's disease (AD), diabetes, aging, brain edema, etc. [51]. Many studies reported the free radical scavenging of tea polyphenols. Among tea catechins, EGCG was reported to be as the most potent antiradical with an antioxidant capacity of approximately 50% of the total capacity of green tea [52]. Alongside their scavenging capacity towards ROS, tea catechins also indirectly enhance the detoxifying function of some detoxification enzymes such as glutathione reductase, glutathione peroxidase, glutathione-S-transferase, catalase and quinine reductase in small intestine, liver and lungs [53]. The antioxidant activity of tea catechins is also due to their ability to chelate metal ions involved in the formation of free radicals [54]. Chantre et al. [55] studied the effect of green tea extract consumption containing 25% EGCG on the body weight. After 3 months, they noticed a decrease of 4.6% in the body weight and 4.48% in the waist circumference. Another Japanese study reported that the daily consumption of tea containing 690 mg catechins for 12 weeks decrease body fat [56].

On the other hand, several studies were conducted to investigate the relation between tea intake and the CVDs [57–59]. For instance, a Japanese study involving 40000 adults reported that the CVD mortality shows an inverse relationship to green tea consumption. The study found that women who drank ≥ 5 cups per day had 31% lower chance of dying due to CVDs and stroke than those who drank less than one dup per day [57].

The unlimited benefits of tea have been studied for long time. Protective effects of tea against all types of cancer were discussed extensively in many papers trying to investigate the role of tea as an anticancer agent. The investigations focused on black and green teas demonstrate inhibitory activities of tea polyphenols against tumorigenesis in various organ sites [60]. Tea, particularly green tea, was evidenced to decrease cancer incidence in mouth [61], esophagus [62], lungs [63, 64], pancreas [65], stomach [62], prostate [66, 67], liver [68] and colon [69, 70].

1.1.7 Other effect of tea consumption

Despite of the tea benefits mentioned in the preceding section, tea was reported to have adverse effects. Samman et al. [71] determined the effects of phenolic extracts from green tea on nonheme-iron absorption, and they concluded that the dietary iron is reduced by the chelation occurs between iron and tea phenolic components. Moreover, phenolic compounds which are thought to be beneficial to health may act as pro-oxidants and produce ROS which may cause a damage for DNA and biological molecules [72]. Tea catechins have also been reported to restrict the transport and metabolism of enzymes associated to drugs [73] and may bind to the drug components and affect their bioavailability [74]. On the other hand, tea may contain some toxic elements like Al, Cd, Pb, Hg, As, etc. [75]. Therefore, tea consumption may contribute to adverse effects related to the presence of such toxic elements leading to long-term toxicity, especially when impaired absorption or excretion of these toxic elements in the body [76]. Also, organic contaminants are another main source for the adverse effects of tea consumption.

1.2 ENDOCRINE DISRUPTORS – PHTHALATES AND BISPHENOL A

Endocrine disruptors (EDs) are chemical substances that can be chemically synthesized (e.g. phthalates, bisphenol A, etc.) or naturally produced (e.g. phytoestrogens). These adverse substances affect the endocrine system by competing with naturally occurring hormone either by mimicking them, resulting prevention of their actions, or even by affecting their synthesis or elimination [77].

Many mechanisms were proposed to describe the action of EDs in the living organism. Among these mechanisms, receptor-mediated and non-receptor-mediated mechanisms. Regarding the receptor-mediated mechanisms, EDs usually target nuclear receptors (NRs) and act like ligands for steroid hormone NRs, particularly androgen (the female sex hormone), estrogen (the female sex hormone), and thyroid hormone receptors. According to this mechanism, EDs bind to the receptors and inhibit the binding of other molecules have similar or close structures [78]. On the other hand, non-receptor-mediated mechanisms are based on the interfering of EDs with the function of enzymes involved in steroid synthesis and metabolism and the ability of EDs to regulate gene expression without changing the DNA sequence. The latter effect are permanent once the change occurs [79, 80].

In recent years, attention has been raised towards the harmful health impacts of EDs since they induce adverse changes in the endocrine system in human and animals. These materials can enter the body by different pathways including ingestion, inhalation, or skin contact with products contain ED chemicals. EDs are associated with different diseases related to the reproductive system [81], central nervous system [82], metabolic disorders [83, 84], and thyroid gland function [85, 86].

EDs include a vast variety of chemical substances like pharmaceutical additives (Diethylstilbestrol (DES)), pesticides (e.g. chlorpyrifos, methoxychlorine, dichlorodiphenyltrichloro-ethane (DDT), etc.), plasticizers (e.g. Phthalate ester, and Bisphenol A) and other industrial stuff utilized in daily routine like fungicides and lubricants (dioxins) [87]. Phthalate esters (PAEs) and bisphenol A (BPA) will be discussed in detail through this PhD thesis.

Endocrine disruptors like BPA and PAEs (**Figure 1.2)** are widespread chemical compounds in the environment [88]. (PAEs) are a class of synthetic compounds formed by esterification reaction of one mole of phthalic acid with two moles of monohydric alcohol molecules. BPA is also a synthetic compound formed commercially by a condensation reaction of two mole of phenol with one mole of acetone. In principle, BPA is used in plastic industry such as polyvinyl chloride (PVC), epoxy resins, building materials, printed circuit boards, medical devices and dental fillings [89]. PAEs are also involved in the industry of children's toys, medical devices, personal-care products, paints, food packaging materials and insecticides [90]. For instance, di-2-ethylhexyl phthalate (DEHP), diisononyl phthalate (DINP), and diisodecyl phthalate (DIDP) account for more 85% of all PVC plasticizers contribution [91].

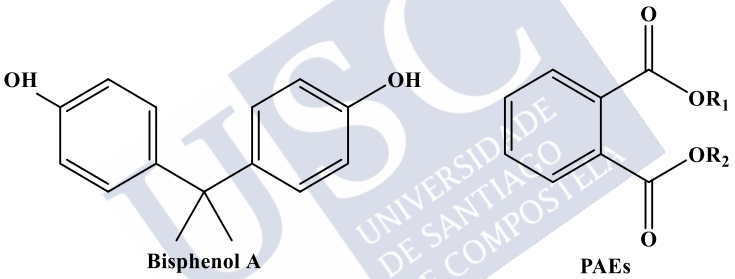

Figure 1.2 General structure of PAEs and BPA.

PAEs, especially DEHP, are added to the rigid PVC in the medical devices production to make it more flexible. Flexible PVC accounts for more than 30% of plasticized medical devices including blood bags, urine bags, tubing for dialysis, infusion, and feeding, and medical gloves. PVC is used in medical devices due to its chemical stability, transparency, biocompatibility, mechanical resistance and durability. However, the European authorities raise the concern about the vast use of PAEs in medical devices since a significant exposure for leachable PAEs was observed. Also, the European authorities recommend using alternative plasticizers in PVC production (e.g. replacing DEHP by Trioctyl trimellitate (TOTM), (Di(2-ethylhexyl)adipate) (DEHA), (1,2-Cyclohexanedicarboxylic acid diisononyl ester) (DINCH), DINP, etc.) [92]. On the other hand, other

phthalates like DINP are used as plasticizers for PVC in children's products and toys. In reference to food and drinks, PAEs are used in the packaging materials that are daily used by millions of people around the world. PAEs like butyl benzyl phthalate (BBP), dibutyl phthalate (DBP), di-isononyl adipate (DINA), DEHP, DEHA, and DINP were found in different food stuff [93]. In cosmetics industry, diethyl phthalates (DEP) is the most used phthalates in cosmetics as a fragrance ingredient [94].

1.2.1 Human exposure

Human is expose to BPA and PAEs by different pathways. Diet is the most important route of BPA and PAEs by feeding on contaminated food and drinks [95, 96]. Moreover, inhalation of volatile PAEs from the workspace or air, and prolonged skin contact (especially via personal-care products in the case of PAEs and thermal receipt paper in the case of BPA) are also other source of exposure [97]. BPA and PAEs can migrate from the container materials to food and drinks due to their weak bonding which can be disrupted by heat and solvent [98].

The alimentary exposure of BPA and PAEs was confirmed in several previous studies that indicates their migration from food packaging materials to food and drinks. For example, Saito et al. [93] studied fifty samples of processed food and found that DBP, DEHP, DINP, DEHA, DINA, BBP migrated from the wrapping film to the samples analyzed, and higher migration was observed when food got wrapped immediately after frying compared to wrapping after standing for 5 and 30 min. Another study done by Yang et al. [99] on 283 convenience foods and the plastic packaging materials reported that the migration of PAEs from packaging materials to food and drinks increases with time and temperature. Fasano et al. [100] evaluated the migration of different PAEs from food packaging (including: yogurt packaging, polystyrene, oil and natural tuna cans, marmalade cap, teat, bread bag, cans, etc.), and they found that the migration was lower than the specific migration limit (SML) set by the European Union (EU) for DBP (0.3 mg/kg), BBP (30 mg/kg), and DEHP (1.5 mg/kg), but even though, PAEs should be monitored in food containers. On the other hand, BPA enters the body through the

oral route, where it is absorbed and hepatic metabolized to produce several metabolites like BPA monosulfate, BPA disulfate and BPA glucuronide [101], and then excreted in the feces and urine [95].

Despite of the large surface area of skin interfacing the environment, it is considered as first protective barrier against pathogens and chemical compounds. Human can expose to PAEs via dermal contact with personal-care products or plastics. Short-term exposure for products like shampoos, soaps, etc. may cause little or no harm for the health. However, long-term frequent exposure to cosmetics may lead to increase the absorption rate of PAEs by skin. Dermal absorption of PAEs is thought to be low or even negligible, however, the skin capacity to metabolize PAEs was previously studied. For instance, Sugino et al. [102] evaluated the risk of dermal exposure to PAEs (DBP and BBP) and their metabolites in hairless rat skin by applying an *in vitro* skin permeation method. They found that the permeation of PAEs and their metabolites depends on the activity of esterase enzyme presented in skin. Serine esterase enzymes hydrolyzed the lipophilic dialkyl phthalates (DBP and BBP) to their relatively hydrophilic monoester metabolites; monobutyl phthalate (mBP) and monobenzyl phthalate (mBnP), during the skin permeation. The same behavior was also reported by Hopf et al. [103] who studied the permeation rate of DEHP via excised human viable skin. The authors reported that DEHP was metabolized during the *in vitro* permeation in viable human skin to form mono-(2-ethylhexyl) phthalate (MEHP) metabolite.

Biedermann et al. [104] studied the dermal exposure of BPA due to the use of thermal paper. They found that BPA concentration was ranged between 8 and 17 mg/g in 11 out 13 thermal paper samples used in Switzerland. Moreover, they reported that 0.2 – 0.6 µg of BPA was passed to middle finger and forefinger of a normal skin by touching the thermal papers for 5 s.

Traditionally, ingestion is considered as the most important route of PAEs exposure. However, inhalation of contaminated air or dust, especially in industrial workplaces, plays an important role in this line. PAEs released from PVC materials was reported to be associated with asthma and allergies [91, 105, 106]. There have been several

epidemiological studies about the relationship between PAEs exposure and children's airways diseases. For instance, a case-control studies reported the association between PVC flooring materials and bronchial obstruction in children [107, 108]. Human can expose to BPA via inhalation routes such as smoking since tobacco filters have up to 25% BPA [109]. Moreover, human can be exposed to BPA via indoor air and workplace air. For comparison, the average levels of BPA in a children care-center in North Carolina, USA were lower than 1 ng/m^3 [110], while BPA measured in a plastic workplace reached 208 ng/m^3 [111].

1.2.2 Regulations

Considering the health risk caused by PAEs, several regulations were issued to control the use of PAEs in industry, toys, food, etc. The European Food Safety Association (EFSA) established a tolerable daily intake (TDI) of five PAEs used in food contact materials (DBP, BBP, DEHP, DINP, and DIDP) as follows: 50 µg/kg body weight (bw)/day for DBP, BBP, DEHP, DINP and far below 150 µg/kg body weight (bw) per day for DIDP [112]. In 2005, the EU banned the use of DEHP, DINP, DIDP, dioctyl phthalate (DNOP), BBP, and DBP in children´s toys and pediatric materials that could be mouthed for children under the age of three years (Directive 2005/84/European Commission) [113]. In 2013, the EU classified DEHP as a priority hazardous substance (Directive 2013/39/EC) [114], while the maximum acceptable concentration of DEHP established in drinking water, for example, by WHO and the United State Environmental Protection Agency (USEPA) is 8 µg/L and 6 µg/L, respectively [115]. The European Commission also prohibited the use of DEHP and DBP in cosmetics [116].

Canada was the world leading country to list BPA as a toxic chemical compound and, as a result, banned its use in baby bottles [117]. The European Food Safety Authority (EFSA) and the U.S. Environmental Protection Agency (EPA) have established the maximum (TDI) of 50 µg BPA/kg bw/day [118, 119]. The EFSA panel states that the temporary (t-TDI) is below 4 µg/kg bw/ day for the highest susceptible classes, which includes infants, children and adolescents concluding that there is no health concern by

alimentary exposure [120]. The European established the SML of BPA from materials come into contact with food and beverages as 0.6 mg/kg [121]. In 2011, the EU prohibited the use of BPA in the production of polycarbonate infant feeding bottles, and also prohibited the importation of infant bottles contain BPA into the EU (Commission Regulation No. 321/2011 of 1 April 2011) [122].

1.2.3 Phthalate esters and bisphenol A in tea

Since *C. Sinensis* plant is cultivated in very large growth zones around the world, it can easily absorb organic contaminants that can be transferred by atmosphere over long distances or those comes from contaminated soil or water before accumulating in leaves. Two studies were carried out to investigate the uptake of DEHP from spiked contaminated soils by eleven plants [123] and woodchips by ten vegetables [124]. The results show that plants and vegetables can absorb and accumulate DEHP from soil and the woodchips used in field agriculture. Contamination of tea leaves can be attributed to the production process (i.e., transport, transformation, flavoring, and packaging) which may contain a contact with plastic materials [125]. Moreover, insecticides contain some PAEs like DBP may also contribute to the tea exposure for such contaminants [126]. The migration of PAEs from packaging material to food and drinks has been reported in several studies [127–130]. Tea infusions may be found in detectable levels of PAEs due to the migration of PAEs from contaminated filters or plastic teabags to the infusion [131]. Migration of PAEs into foodstuff depends on the pH, alcohol content, contact time, lipid content of the matrix and temperature [128]. Since tea is usually served hot, the leaching rate of PAEs from contaminated leaves or bags into the infusion is increased [132]. BPA is primarily introduced to food and drinks by migration from containers and epoxy coating materials [133] and bags used as filters [121, 134].

1.2.4 Analysis of phthalate esters and bisphenol A

Several techniques have been reported in literature for the determination of PAEs and BPA in different matrix samples including biological samples, water, air, soil, medical devices, cosmetics, and sludge. The analysis is usually carried out using high-performance

liquid chromatography (HPLC) and gas chromatography (GC) alongside different detectors like ultraviolet detector (UV), electron capture detector (ECD), flame ionization detector (FID) and all type of mass spectrometry (MS).

Analyzing PAEs and BPA is usually preceded by a pretreatment step to clean up the sample and to obtain a more concentrated pure analyte. Several pretreatment methods have been used for the extraction of PAEs from different samples, such as solid-phase extraction (SPE), solid phase microextraction (SPME), liquid-liquid extraction (LLE), and stir bar sorptive extraction (SBSE).

1.2.4.1 Sample preparation

The determination of PAEs and BPA normally requires separation and preconcentration steps previous the instrumental analysis. Several preconcentration methods have been used in this way including SPE, LLE, LPME, SPME, etc.

- Liquid-Liquid extraction (LLE)

Several LLE protocols have been applied to separate and preconcentrate PAEs and BPA from food and beverages. LLE is based on the relative distribution of the analyte or solute between two immiscible phases: aqueous phase (normally water) and organic phase (organic solvents such as dichloromethane, hexane, ether, chloroform, etc.). After mixing the two phases, the organic layer is separated, and the solvent is evaporated to concentrate the analyte.

LLE is widely applied in sample clean up and analyte enrichment in a wide range of samples including biological samples (e.g., urine, blood, etc.) food and drinks, plants, etc. For instance, several studies have been carried out for PAEs separation using LLE in milk, serum, plasma, urine, oily food [135], water [136], olive oil [137], vegetable oils [138], tobacco [139], honey and royal jelly [140]. Guo et al. [141] extracted PAEs from foodstuffs applying LLE. Among beverages studied, green tea was extracted with hexane, and the extracts were evaporated and concentrated prior their analysis by GC-MS.

DEHP was the most abundant compound in most of the sample as well as green tea. González-Castro et al. [129] applied LLE

for PAEs and BPA extraction from vegetable cans, baby bottles and microwaveable containers from the Mexican market. The authors used water as a food simulant, and they carried out the extraction using three organic solvents (Hexane, hexane: ethylic ether (1:1), and chloroform). **Table 1.2** includes some applications of LLE for the pre-treatment of PAEs and BPA under different conditions.

Although LLE is a simple inexpensive method for sample preparation, its drawbacks are obvious because it is inappropriate for ultra-trace analysis, time-consuming process and environmentally harmful solvents. Therefore, some improved LLE methods have been proposed to overcome these drawbacks such as single drop microextraction (SDME) [142], cloud-point extraction (CPE) [143], liquid-liquid microextraction (LLME) [144], dispersive liquid-liquid microextraction (DLLME) [145], etc.

DLLME is used to preconcentrate and extract organic compounds from sample matrices. During the DLLME, a mixture of microliters of extracting solvent and dispersive solvent is introduced quickly to the sample solution utilizing a syringe. The dispersion leads to rapid extraction of the analyte from the sample. The dispersion is then extracted by centrifugation and withdrawn by micro-syringe prior the analysis [146]. DLLME was also used to extract PAEs and BPA from different samples such as water [147, 148], plastic water containers [149], urine [150, 151], etc. In a recent study, De Oliveira et al. [146] used DLLME combined with LC-MS for EDs determination including BPA in human saliva. López-Darias et al. [142] determined several EDs (including BPA) in seawater using SDME and DLLME in combination with HPLC-UV. The authors compared these two methods and concluded that DLLME is more efficient than SDME offering higher enrichment factors than SDME, good recoveries and lower detection limits.

In the recent years, hollow-fiber liquid phase microextraction (HF-LPME) was introduced as an alternative to the classical LLE. HF-LPME offers good preconcentration factors and higher sensitivity than the classical ones, and it also enhances the automation possibilities. HF-LPME is based on the removal of analytes from aqueous matrices (donor phase) by acceptor phase (e.g.,

an organic or aqueous solution) filled inside the lumen of the HF membrane [152]. This method has been applied to determine BPA and PAEs in aqueous samples. Fernandez et al. [153] developed a method for the analysis of PAEs metabolites and BPA in women's urine samples using this separation method combined with GC-MS for detection.

- Solid liquid extraction (SLE)

Since the extraction of analytes of interest is harder in the case of solid samples, SLE technique is usually selected for this purpose. SLE is normally carried out using different solvents like hexane, acetone, methanol, or even a mixture of different solvents. Soxhlet extraction is the commonly used set for this purpose. However, other alternatives may be used like ultrasonic extraction and microwave assisted extraction. Several studies have been reported in the literature for PAEs extraction from different samples using different solvents. For instance, ethyl acetate was used to extract PAEs from packaging [166], methanol and acetone from sediments [167, 168], and dichloromethane from dump soil [169]. Moreover, BPA was isolated from plastic packaged baby food using acetonitrile as a solvent followed by dispersive-SPE [170], from sewage sludge using methanol/water mixture [171], and from infant formula powders using 50% ethanolic solution [172].

Table 1.2. Applications of LLE for PAEs and BPA preconcentration.

Sample	Analyte	Solvent	Detection	Ref.
Milk	DBP, BBP, DEHP, DINP, DIDP	Tert-butyl methyl ether and hexane	LC/ESI-MS/MS	[154]
Human milk, consumer milk, and infant formula	Phthalate monoesters	Ethyl acetate : cyclohexane (95:5)	LC-MS-MS	[155]
Bottled water	DMP, DEP, DBP, BBP, DEHP, DOP	Methylene chloride: petroleum ether (20:80)	GC-MS	[136]
Parenteral nutrition	DMP, BBP, DEP, DBP, DPeP, DEHP, DOP	Hexane	HPLC-MS	[156]
Serum	DEHP	LLE: 2 mL ACN and 2 mL NaOH	HPLC-UV	[157]
Sediments	BPA	5 mL methanol/water (5:3, v/v)	HPLC-MS/MS	[158]
Parenteral nutrition and plasma	DEHP	NaOH: hexane (1: 2 mL)	HPLC-UV	[159]

Table 1.2. (continued)

Sample	Analyte	Solvent	Detection	Ref.
Cosmetics	DMP, DEP, DPP, DiBP, BBP, DBP, DEHP,	Ultrasonic extraction LLE ethanol-water (90:10 v/v)	HPLC-UV	[160]
Foodstuff	EDs including BPA	DLLME: acetonitrile and water	HPLC–FLD	[161]
Liquid food matrices	BPA and BPB	DLLME: T4CE (extractant), ACN (dispersant),	GC-MS	[162]
Seawater	BPA, 4-CP, t-BP, OP, and NP	SDME: 2.5 µL of decanol in 5 mL of seawater DLLME: 150 µL of a mixture acetonitrile: decanol (ratio 15:7, v/v) in 5 mL seawater	HPLC-UV	[142]
Urine	BPA and six analogues (S, F, Z, P, AF, AP)	DLLME: acetone (dispersant), 1,2-dichloroethane (extractant)	LC- MS/MS	[163]
Bottles carbonated beverages	BPA and 8 analogues (C, AF, E, F, G, M, S, and Z)	DLLME: 2-dodecanone (extractant), acetone (dispersant)	GC-MS	[164]
Polycarbonate baby bottles	BPA	LLE: ACN followed by SPE	HPLC-FLD	[165]

DPeP: dipentyl phthalate, ACN: acetonitrile, BPB: bisphenol B, T4CE: tetrachloroethylene, 4-CP: 4-cumylphenol, t-BP: 4-tertbutylphenol, OP: 4-octylphenol, NP: 4-n-nonylphenol, DPP: dipropyl phthalate, DIBP: di-isobutyl phthalate

- Solid phase extraction (SPE)

SPE is a separation and preconcentration process where the matrix containing the analyte of interest is passed through a sorbent material. The sorbent retains the analytes depending on the interaction between them and the analytes is then eluted by suitable solvent. SPE is normally achieved by applying four simple steps. Firstly, the cartridge (containing the sorbent) is preconditioned to interact with the analyte of interest. The matrix (containing the analyte) is then passed through the sorbent where the analyte is retained. The rest of matrix that may contain the interferences is eluted (but not the analyte) using a suitable solvent. Finally, the analyte is eluted using a specific solvent. Several sorbents can be used in SPE including silica based C18 [173], polystyrene-divinylbenzene (PS-DVB) [174], octadesyl-coated styrene divinylbenzene polymer (SDB-XD) [175], polytetrafluoroethylene (PTFE) [176], multi-walled carbon nanotubes (MWCNTs) [177], etc. for PAEs and BPA extraction from different matrices including water, wine, biological samples, etc. **Table 1.3** lists some applications of SPE for preconcentration PAEs and BPA from different sample matrices. Molecularly imprinted polymers (MIPs) are a class of SPE sorbents for the selective recognition of template-like molecules. MIPs will be explained in detail in the next section.

Recently, BPA and PAEs have been also preconcentrated using QuEChERS method. QuEChERS (Quick, easy, cheap, effective, rugged, and safe) is powerful sample preparation tool based on SPE. QuEChERS was used for extraction of contaminants from food samples. This method is simple to apply, cost effective and provides high-quality analytical results. QuEChERS consists of two steps; extraction of contaminants with acetonitrile in presence of salt to remove water, followed by cleaning up step utilizing dispersive SPE (d-SPE) [178]. Regarding analysis of PAEs and BPA in tea samples, Yin et al. [179] developed a modified QueChERS method to determine 16 PAEs in 105 tea samples by GC-MS/MS. The authors found that all samples were contaminated with PAEs, especially DMP, DEP, DiBP, DBP and DEHP. Guanwei et al. [180] applied QueChERS method to extract and analyze BPA and its derivative

tetrabromobisphenol A (TBBPA) in different types of tea samples using LC-MS/MS. In this study, BPA was quantified in tea samples in the following order of concentration: smoked black tea > non-smoked black tea > oolong tea > green tea. The authors explained this behavior by the manufacturing process of different types of tea.

Table 1.3. Applications of SPE for PAEs and BPA preconcentration procedures from several sample matrices.

Sample	Analyte	Sorbent	Conditioning solvent	Elution	Detection	Ref.
Tea	DMP, DEP, DBP, DEHP, DINP	Oasis HLB	6 mL methanol and 6 mL water	6 mL methanol	GC-MS	[181]
Wine	DMP, DEP, DEHP, iBP, DBP, BBP	C18	10 mL DCM and 2.5 mL methanol	5 mL DCM	GC-MS	[182]
Water	DEP, DPP, DiBP, DCHP	MWCNTs	5 mL methanol and 5 mL water	5 mL ACN	HPLC - UV (DAD)	[183]
Ham sausages	DMP, DEP, DBP, BBP, DEHP, DNOP,	Oasis MAX	1 mL ACN and 1 mL water	2 mL ACN	GC-MS	[184]
Milk	DMP, DEP, DBP, BPA, BBP, DEHP,	C18	12 mL (DCM: hexane, (4:1) 12 mL methanol and 12 mL water	Fraction A: 12 mL DCM: hexane, (4:1, v/v) Fraction B: ethyl acetate	GC-MS	[173]

Table 1.3. (continued)

Sample	Analyte	Sorbent	Conditioning solvent	Elution	Detection	Ref.
Human urine	15 PAEs metabolites	SDVB-MA copolymer	1 mL ACN and 1 mL, 0.14 M NaH_2PO_4 in 0.85% H_3PO_4) (pH=2)	1 mL ACN followed by 1 mL ethyl acetate	HPLC-ESI-MS/MS	[186]
Water	DBP, BBP, DEHP	Online SPE: PLRP-S	50 % methanol	Ethyl acetate	GC-MS	[187]
Wastewater	BPA and chlorinated BPA, DMP, DEP, DBP, DOP, BBP, DEHP	LiChrolut RP-18	5 mL diethyl ether, 5 mL methanol, and 5 mL water	6 mL (diethyl ether: methanol) (9:1, v/v)	GC-MS	[188]
Human urine	BPA, NP, and OP	OASIS HLB	5 mL DCM: methanol (9:1), 5 mL methanol, 5 mL water (pH=3.0-3.5 with HCl)	5 mL of DCM: methanol (9:1)	UPLC-MS/MS	[189]
Breast milk	BPA	Glass Oasis HLB	6 times: 4 mL MTBE, 3 mL methanol, 5 mL water	4 mL MTBE	LC-MS/MS	[190]

Table 1.3. (continued)

Sample	Analyte	Sorbent	Conditioning solvent	Elution	Detection	Ref.
Soft drinks	BPA and 4 analogues	On-line SPE: C18	Methanol: water (5:95, v/v)	2 mL Methanol: water (15:85, v/v)	LC-MS/MS	[191]
Beverages (including tea)	BPA	Bond Elut C18	13 mL methanol and 13 mL water	6.5 mL ACN: water (50:50, v/v)	LC-MS/MS	[192]
Beverages (including tea)	BPA and 10 of its analogues	Oasis HLB, Oasis MAX, Oasis MCX	5 mL ethyl aetate, 5 mL methanol, 5 mL water/formic acid (99:1, v/v)	10 mL methanol: ethyl acetate: formic acid (8:91:1, v/v)	LC-MS/MS	[193]

iBP: isobutyl phthalate, DCM: dichloromethane, DCHP: dicyclohexyl phthalate, MWCNTs: multi-walled carbon nanotubes, DAD: diode array detector, MNP: magnetic nano-particle, mMP: m mBP: monobutyl phthalate, mEP: monoethyl phthalate, mBP: mono n-butyl phthalate, mEHP: mono-(2-ethylhexyl) phthalate, SDVB-MA: styrene-divinylbenzene methacrylate, PRLP-S: styrene–divinylbenzene copolymer, NP: Nonylphenol, OP: Octylphenol, UPLC: Ultra performance liquid chromatography, MTBE: methyl-tert-butyl-ether.

- Molecularly imprinted polymers (MIPs)

MIPs are artificially synthesized substances known by their high selectivity, excellent stability to the extreme pH, organic solvent, and temperature, which improve the separation of analytes [194]. As early as 1994, Sellergren [195] designed a new SPE sorbent called MIP to extract pentamidine, a medication used in acquired immunodeficiency syndrome (AIDS)-related pneumonia therapy, from urine samples. Since then, several reports were published regarding the use of MIPs as a highly selective SPE sorbent to preconcentrate compounds from complex samples including biofluids, food and beverages, plants and environmental samples. Moreover, their selectivity leads not only to preconcentrate the target analytes, but also to eliminate the matrix effect [196].

MIPs are conventionally synthesized by, firstly, mixing the template and monomer(s) in a selected solvent to produce a stable pre-polymerization complex, followed by adding a suitable crosslinker alongside a proper initiator. Once the polymerization is over, the template is removed by consecutive washing with a suitable solvent to activate the active sites of the MIP [197]. Several methods were used to prepare MIP including bulk polymerization, precipitation polymerization, suspension polymerization, swelling polymerization, mini-emulsion polymerization, and core-shell emulsion polymerization [196, 198]. During the preparation of MIP, the selection of the template depends on the target analyte. Thus, the template used needs to be chemically inert during the polymerization and structurally compatible with the target analyte.

Several studies have been reported regarding the extraction of PAEs and BPA from complex samples. Bai et al. [199] synthesized a MIP by bulk polymerization using di-n-octylphthalate (DOP) as a template, methacrylic acid (MAA) as a monomer, ethylene glycol dimethacrylate (EDMA) as crosslinker and 2,2'-azobisisobutyronitrile (AIBN) as an initiator. The obtained MIP was used as a SPE sorbent to preconcentrate and determine DOP, DBP, and DMP in the bottled sprites by HPLC. Shaikh et al. [200] also used suspension polymerization to synthesize a MIP using DEHP as a template, methacrylamide as a functional monomer and N,N′-methylene-bis-

acrylamide as a cross-linker. This MIP was used to preconcentrate DEHP from an aqueous sample in combination with GC-FID. On the other hand, Yan et al. [201] synthesized dummy imprinted microspheres (DIMs) using DINP as a dummy template in term of precipitation polymerization. The authors used the DIMs as a SPE sorbent to isolate and determine DEP, DBP, BBP, DIOP, and DNOP in plastic bottled beverages using GC-FID [201].

Table 1.4 summarizes other studies that used MIP-SPE to preconcentrate and determine PAEs in different type of samples.

The use of MIP as a SPE sorbent for the extraction of BPA from different matrices was also reported in literature. Wu et al. [202] used BPA as a template to synthesize MIP to extract BPA from vegetables and juice samples. For the synthesis, the authors used 4-vinylpyridine (4-VP) as a monomer, and AIBN as an initiator and trimethylolpropane trimethacrylate (TRIM) as a cross-linker applying precipitation polymerization. In another study, Herrero-Hernández et al. [203] determined several phenolics including BPA, bisphenol F and 4-nitrophenol in honey samples. In this study, the MIP was prepared via precipitation polymerization using BPA as a template, 4-VP as a monomer, EDMA as a crosslinking agent and AIBN as an initiator. The extracted analytes were analyzed using LC coupled to diode array UV detector. Zhang et al. [204] developed a magnetic MIP based on multiwalled carbon nanotubes for the extraction of BPA from water.

Table 1.4. Synthesized MIPs for the determination of PAEs and BPA from different sample matrices.

Analyte	Template/monomer/Crosslinker/initiator/solvent	Sample	Detection	Ref.
PAEs	DBP/MAA/EDMA/AIBN/chloroform	Soybean milk	GC-MS	[205]
DMP, DEP, DBP, DAP, DNOP	DBP/MAA/EDMA/AIBN/chloroform	aqueous sample	GC-MS	[206]
DBP, BBP, DEP, DMP	DBP/MAA/EDMA/AIBN/acetonitrile	Water and wine	HPLC-MS	[207]
DEP, DBP, BBP, DIOP, DNOP	DINP/AM/DVB/AIBN/ (toluene: acetonitrile, 4:26 mL)	Plastic bottle	GC-FID	[201]
DMP, DEP, DBP, DAP, DNOP	DBP/MAA/EDMA/AIBN	Water	GC-MS	[208]
DMP, DEP, DBP, DPP, DEHP, DNOP	DPeP/allyl-ß-CD and (MAA)/EDMA/AIBN	Cow milk	GC-MS	[209]

Table 1.4. (continued)

Analyte	Template/monomer/Crosslinker/initiator/solvent	Sample	Detection	Ref.
BPA	BPA/4-VP/EDMA/ABCN/toluene	Milk	HPLC-FLD	[210]
BPA analogues	Commercial AFFINIMIP SPE bisphenol A cartridges	Beverage and canned food	LC-MS/MS	[211]
BPA	BPA/MAA, 4-VP/EDMA, TRIM/AIMN/ methanol, acetonitrile, toluene	Soils, aqueous canned foods.	HPLC-FLD	[212]
BPA	FBPA/4-VP/TRIM/AIBN/acetonitrile	Water	HPLC-UV	[213]
BPA	BPA/MAA/EDMA/AIBN/acetonitrile	Tea	HPLC-MS	[121]
BPA	BPA/4-VP/EDMA/AIBN/toluene and dodecanol	River water	Online SPE-HPLC-UV	[214]
BPA	BPA/4-VP/TRIM/AIBN/acetonitrile	wastewater, water, soil, shrimp, and human urine	CE-UV	[215]

ABCN: 1,1′-azobis(cyclohexanecarbonitrile), FLD: fluorescence detection, FBPA: fluorinated bisphenol A, AIMN: 2,2´- azobis(methylbutyronitrile), CE: capillary electrophoresis, allyl-ß-CD: allylic bromine-cyclodextrin, DAP: diamyl phthalate.

- Solid phase microextraction extraction (SPME)

In 1989, Pawliszyn and his co-workers [216] developed a SPME method for preconcentrating organic compounds from water using chemically modified fused silica fiber as a solid phase absorbent. In one single solvent-free step, SPME combines all pretreatment steps that involves sampling, extraction, concentration and introduction of the analyte of interest. This protocol reduces the contamination risk and make the analysis easier and efficient and automatable. Carbowax [217], polyaniline [218], polydimethylsiloxane-divinylbenzene (PDMS-DVB) [219], carbowax-divinylbenzene (CW-DVB) [220], polyacrylate (PA) [221] and polydimethylsiloxane (PDMS) [219] are widely used fiber coatings for the extraction of analytes from different environmental and nutritional matrices. SPME has been used for vast range of food applications such as fats and oils, meat, wine, non-alcoholic drinks (including tea), and fruits and vegetables [222, 223]. Du et al. [223] investigated the aroma composition of Pu-erh tea using head-space (HS-SPME). Among esters identified in this study, diisobutyl phthalate was one of the most abundant esters in all samples analyzed. Rastkari et al. [224] developed a method for the extraction of BPA and bisphenol F in canned food using SPME prior the detection using GC-MS. The authors used single-walled carbon nanotubes (SWCNTs) as a headspace SPME adsorbent to preconcentrate the bisphenols from canned food. Amayreh et al. [225] extracted DEP, BBP, DBP, and BPA from blood and seawater samples using an electro-enhanced SPME method combined with GC-MS. In this method, the SPME fiber and an inert metallic wire were immersed together in the sample matrix solution. The difference in the voltage between the SPME holder and the metallic wire leads increase the transport of the charged analyte to the fiber through electrophoresis properties.

- Stir bar sorptive extraction (SBSE)

Based on the same idea of SPME, SBSE method can separate and enriching the organic compounds in liquid samples. It is based on immersing a coated stainless-steel bar in the liquid phase and stirring continually for a certain period of time. During the gentle

stirring, the stir bar collects and retains the analyte of interest by partition [226]. Comparing to SPME, the stir bar contains higher amount of PDMS than the SPME fiber resulting higher recoveries and capacities as well as better sensitivity [227]. SBSE offers a solvent-free sample preparation to isolate the analyte of interest from samples. Extracted analytes by SBSE are commonly analyzed by GC-MS and HPLC-MS [228]. In this sense, this method was used extensively to preconcentrate PAEs and BPA from different matrices including water [228–230] vegetables and foodstuff [231, 232], body fluid [233], personal care products [230], etc.

1.2.4.2 Separation and detection techniques

Liquid chromatography (LC) and gas chromatography (GC) combined with different detectors are commonly used to analyze PAEs and BPA in different types of samples like food samples, biological samples, and environmental samples. Normally, GC requires a derivatization step prior the analysis, which, in turn, makes LC more favorable over GC.

- Gas chromatography

GC technique provides usually better sensitivity over LC technique. The separation between analytes in GC occurs due the difference in their volatility, and the interaction with the stationary phase. For the analysis of PAEs and BPA, GC is usually combined with different detector such as flame ionization detector (FID), electron capture detection and mass spectrometry detectors (MS). The analysis of analytes presented in trace levels using such detectors may lead to positive false results resulting from the cross-contamination during the analysis. In this way, MS has been become an essential tool for daily laboratory analysis routine. **Table 1.5** lists some applications for BPA and PAEs determination of in different matrices.

Table 1.5 GC determination of BPA and PAEs in different samples.

Sample	Analyte	Chromatographic column	Detector	LOD	Ref.
Bottled water	DMP, DEP, DBP, BBP, DEHP, DOP	Fused capillary SPB-5 column (30 m × 0.25 mm, 0.25 µm)	MS: *m/z* 50 to 400	< 0.1 µg/L	[136]
Tea	27 plasticizers including PAEs and BPA	Supelco SPB-5MS (30 m × 0.25 mm, 0.25 µm)	MS *m/z*: 163 for DMP and 149 for the other PAEs BPA: 213	PAEs: 0.005 - 3.752 mg/L BPA: 0.436 mg/L	[181]
Wine	DMP, DEP, DEHP, iBP, DBP, BBP	Restek RTX-5MS (30 m × 0.25 mm, 0.25 µm)	MS: *m/z*: 163 for DMP and 149 for the others	15 – 18 ng/L	[182]
Ham sausages	DMP, DEP, DBP, BBP, DEHP, DNOP	Fused silica capillary column DB-5MS (30 m × 0.25 mm, 0.25 µm)	MS *m/z*: DMP (163), DEP (177), DBP (150), BBP (149), DEHP (167), DNOP (279)	016 – 0.54 ng/g	[184]
Milk	DMP, DEP, DBP, BPA, BBP, DEHP,	HP 5MS fused-silica capillary column (30 m × 0.25 mm, 0.25 µm)	MS *m/z*: DMP: 163 For the other PAEs: 149 BPA: 213	PAEs: 0.06 -0.12 µg/kg BPA: 0.15 µg/kg	[173]
Human urine	mMP, mEP, mBP, mEHP, mBnP	BPX5 5% phenyl column (30 m, 0.25 mm, 0.25 µm)	MS *m/z*: mMP (237), mEP (251), mBP (223), mEHP (221), mBnP (179)	0.025 – 0.05 µg/L	[185]

Table 1.5 (continued)

Sample	Analyte	Chromatographic column	Detector	LOD	Ref.
Water	DBP, BBP, DEHP	HP-5 MS (30 m, 0.25 mm, 0.25 µm)	MS *m/z*: 149 for all PAEs	0.1 – 7 ng/L	[187]
Water	DMP, DEP, DBP, DAP, DNOP	DB-17 capillary column (30 m×0.25 mm, 0.25 µm)	MS *m/z*: DMP: 163, 194; DEP: 149, 177; DBP: 149, 223; DAP: 149, 237; and DOP: 149, 279	5.49 – 21.6 ng/L	[208]
wastewater	BPA, DMP, DEP, DBP, DOP, BBP, DEHP	ZB-5 MS Zebron (30 m × 0.25 mm, 0.25 µm)	MS *m/z*: BPA: 372, DMP: 194, DEP: 177, DBP:223, BBP: 205, DEHP and DOP:279	-	[188]
Soybean milk	DMP, DEP, DBP, DAP, DOP	DB-5 capillary column (30 m × 0.25 mm, 0.25 µm)	MS m/z DMP: 163, 194; DEP: 149, 177; DBP: 149, 223; DAP: 149,237; DOP: 149, 279	0.013 - 0.022 µg/mL	[205]
Plastic bottle	DEP, DBP, BBP, DIOP, DNOP	KB-1 (30 m × 0.25 mm, 0.25 µm)	FID	0.85–1.38 µg/L	[201]
Cow milk	DMP, DEP, DBP, BBP, DPP, DEHP, DNOP	DB-5 capillary column (60 m × 0.32 mm,1.00 µm)	MS *m/z*: DMP 163/77, DEP 149/177, DBP 149/223, BPP 149/237, DEHP 149/167, DOP 149/279	-	[209]

Table 1.5 (continued)

Sample	Analyte	Chromatographic column	Detector	LOD	Ref.
Cow milk	DMP, DEP, DBP, BBP, DBP, DEHP, DNOP	DB-5 capillary column (30 m × 0.25 mm, 0.25 µm)	MS *m/z*: DMP 163/77/194, DEP 149/177/104, DBP 149/223/104, BBP 149/91/206, DEHP 149/167/279, DOP 149/279/104	For 10.8% fat: 0.72 – 4.4 ng/g	[220]
Vegetable oil	DMP, DEP, DBP, BBP, DEHP, DOP	DB-35 (30 m × 0.25 mm, 0.15 µm)	ECD	LOQ: 0.2 - 0.5 mg/kg	[234]
Food packaging films	DMP, DEHP, DMTP, DPP, DNP, DEP, DBP, BBP	Supelco column MDN-5 (30m x 0.25mm, 0.25 µm)	FID	13.88 - 87.6 mg/L	[166]
Liquid food matrices	BPA and BPB	DB-5HT (5 m × 0.32 mm, 0.10 µm)	MS *m/z*: BPA 270, BPB 213	BPA < 60 ng/L BPB < 30 ng/L	[162]

Table 1.5 (continued)

Sample	Analyte	Chromatographic column	Detector	LOD	Ref.
Bottles carbonated beverages	BPA and 8 analogues (C, AF, E, F, G, M, S, and Z)	DB-5 MS 5% Phenyl and 95% dimethyl arylene siloxane	MS/MS *m/z:* 50 – 500	0.021 –0.104 ng/L	[164]
Drinking bottled water	BPA	Elite-5-MS (30 m x 0.25 nm, 0.5 µm)	MS	-	[235]
Prawn	BPA, 4-CP, 2,4-DCP	Thermo TR-5 ms SQC (30 m × 0.25 mm, 0.25 µm)	MS *m/z:* BPA 213, 4-CP 197, 2,4-DCP 237	1.5 – 36.4 ng/kg	[236]
Foods	BPA	ZB-5MS (30 m ×0.25 mm, 0.25 µm)	MS/MS 357.2 > 191.2, 372.2 > 357.2, 357.2 > 207.2, 357.2 > 341.1	0.03–0.1 µg/kg	[237]

2,4-DCP: 2,4-bis-(dimethylbenzyl)phenol, LOD: limit of detection, LOQ: limit of quantification, DMTP: Dimethyl terephthalate, DNP: Dinonyl phthalate

- Liquid chromatography

Liquid chromatography (LC) is another technique for the analysis of PAEs and BPA in a wide range of sample matrices. LC can be used in combination with different detectors such as UV, fluorescence detectors (FLD), and MS. The main advantage of using LC over GC is that derivatization step is no necessary. Among the detectors, MS is the widely used one to identify the contaminants' identities due to its high sensitivity. Nowadays, HPLC-MS and HPLC-MS/MS are the mainly used for quantitative analysis of PAEs and BPA in different matrices including biological samples, food and beverage samples, and environmental samples. The separation of PAEs and BPA is usually carried out using reversed phase LC column (mainly C18 and C8), but other phenyl-based columns were also used for the same purpose [238, 239]. **Table 1.6** summarizes some applications of HPLC technique coupled to different detectors for the determination of PAEs and BPA.

Table 1.6 HPLC methods for BPA and PAEs determination in different samples.

Sample	Analyte	Chromatographic column	Mobile phase	Detection	LOD	Ref.
Seawater	BPA, 4-CP, t-BP, OP, and NP	C18 Res Elut HPLC (5 µm, 150 mm × 4.6 mm i.d.)	ACN and H_2O at flow rate: 1.0 mL/min A linear gradient was used from 50 to 80% of ACN over 8 min, and then 80% of acetonitrile was maintained for 9 min.	UV λ 228 nm	4 – 9 µg/L	[142]
Milk	DBP, BBP, DEHP, DINP, DIDP	Luna C5 100A (5mm, 50 mm × 2.0 mm i.d.)	2.0% (v/v) H_2O in methanol/ACN (1:1) Flow rate: 200 µL/min Isocratic elution	MS/MS	5 – 9 µg/kg	[154]
Human milk, consumer milk, and infant formula	Phthalate monoesters	Betasil Phenyl (3 µm, 100 mm × 2.1 mm i.d.)	H_2O (A) and ACN (B) (both containing 0.1% acetic acid) at flow rate: 350 µL/min A nonlinear gradient elution from 96% A to 100% B.	MS/MS	10 – 500 ng/L	[155]
Parenteral nutrition	DMP, BBP, DEP, DBP, DPeP, DEHP, DOP	ZORBAX Eclipse XDB-C8 (3.5 µm, 50 mm × 2.1 mm i.d.)	H_2O and ACN at flow rate: 200 µL/min A linear gradient elution from 5% of ACN to 75% in 5 min. This composition was maintained for 27 min before returning to the initial conditions	MS	< 44.1 µg/L	[156]
Serum	DEHP	C18 HYPERSIL BDS (5 µm, 150 mm× 4.6 mm i.d.)	ACN buffer (triethylamine 0.2% adjusted to pH 2.5 with phosphoric acid) mixture (90:10, v: v) at flowrate: 1.5 mL/min Isocratic elution	UV λ 222 nm	-	[157]
Parenteral nutrition and plasma	DEHP	Waters Spherisorb C18 (5 µm, 150 mm × 4.6 mm i.d.)	H2O and ACN 0.08% triethylamine adjusted to pH 2.8 with 1M phosphoric acid) mixture (88:12, v/v), flow rate: 1.0 mL/min, Isocratic elution	UV λ 202 nm	LOQ: 20 µg/L	[159]

Table 1.6 (continued)

Sample	Analyte	Chromatographic column	Mobile phase	Detection	LOD	Ref.
Cosmetics	DMP, DEP, DPP, DiBP, BBP, DBP, DEHP,	ZORBAX Eclipse XDB-C18 (3.5 µm, 150 mm × 4.6 mm i.d.)	Ethanol and H_2O at flow rate: 1.0 mL/min A linear gradient elution starting from 50% up to 95% ethanol in 30 min. This composition was maintained for 10 min before re-equilibrating the column	UV λ 254 nm	0.5 µg/mL	[160]
Sediment	BPA	Betasil C18 column (5 µm, 100 mm × 2.1 mm i.d.)	Methanol and H_2O at flow rate: 300µL/min. A gradient elution starting from 15 % up to 50% methanol in 13 min. This composition was maintained for 3 min before reverting to 15 and held for 5 min	MS/MS	LOQ 0.25 – 1.0 ng/g	[158]
Foodstuff	EDs including BPA	Hypersil C18 (5 µm, 200 mm × 4.6 mm i.d.)	H_2O containing 5% ACN (A) and acetonitrile (B) at flow rate of 1 mL/min A linear gradient elution by increasing the amount of mobile phase B as follows: 0–3 min (65–78%), 3–10 min (78–80%), 10–18 min (80–100%), 18–21 min (100–100%)	FLD $\lambda_{exc.}$ 279 nm $\lambda_{emm.}$ 380 nm.	30 ng/L	[161]
Urine	BPA and six analogues (S, F, Z, P, AF, AP)	Supelco Ascentis Express C18 (2.7 µm, 75 mm × 2.1 mm i.d.)	Methanol and water (containing 0.1% of ammonium hydroxide (v/v)) Flow rate: 500 µl/min Gradient elution	MS/MS	0.005 – 0.1 µg/L	[163]

Table 1.6 (continued)

Sample	Analyte	Chromatographic column	Mobile phase	Detection	LOD	Ref.
Polycarbonate baby bottles	BPA	Hypersil C18 (5 µm, 250 mm × 4.6 mm i.d.)	The mobile phase was a gradient prepared by mixing (A) water and (B) acetonitrile delivered at 900 µl/min . The gradient employed was: 0–2 min, 25% B; 2–15 min, 25–45% B; 15–38 min, 45–70% B; 38–45 min, 70–100% B; 45–50 min, 100% B; 50–52 min, 25% B	FLD λ 227 nm	4 – 7 ng/g	[165]
Soft drinks and canned food	BPA	Discovery C18 (5 µm, 250 mm × 4.6 mm i.d.)	Water and methanol at flow rate: 600 µl/min	MS	0.6 ng/g	[240]
Breast milk	BPA	BEH C18 (1.7 µm, 100 mm × 21 mm i.d.)	ACN and H2O (both containing 0.1 % acetic acid), starting with 30 % ACN for 0.5 min, then increased linearly to 61 % to 6.5 min, held for 2.5 min before returned to 30% over 1 min Flow rate: 433 µl/min	MS/MS	0.22 µg/L	[190]
Soft drinks	BPA and 4 analogues	C18 Fused-Core ™ (2.7 µm, 50 mm×2.1 mm i.d.) On-line SPE	Methanol/water at flow rate: 600 µL/min Elution: 0 min 15% MeOH; from 0 to 3 min a linear gradient elution up to 80% MeOH followed by an isocratic step of 3.5 min	MS/MS	25 – 50 ng/L	[191]

Table 1.6 (continued)

Sample	Analyte	Chromatographic column	Mobile phase	Detection	LOD	Ref.
Beverages (including tea)	BPA and 10 of its analogues	Pentafluoro phenylpropyl Ascentis Express F5 (100 mm × 2.1 mm i.d., 2.7 µm)	(A) H_2O/formic acid (99.8:0.2, v/v) and (B) ACN/H_2O/formic acid (97.8:2:0.2, v/v/v) at flow rate: 240 µL/min Linear gradient: 0 min, 45% B; 0.5 min, 45% B;9.5 min, 75% B; 10.5 min, 98% B; 12.0 min, 98% B; 12.5 min, 45% Band 18 min, 45% B	MS/MS	1.6 – 27.9 ng/L	[193]
Beverages (including tea)	BPA	Ascentis C18 (15 cm × 4.6 mm i.d., 5 µm)	60% acidified H_2O (1% acetic acid), 35% ACN and 5% methanol Flow rate: 950 µl/min Isocratic elution	FLD $\lambda_{exc.}$ 275 nm $\lambda_{emm.}$ 305 nm	0.18 µg/L	[192]
Water and wine	BBP, DEP, DBP, and DMP	Zorbax Eclipse XDB-C8 (50 mm × 2.1 mm i.d., 3.5µm)	H_2O and ACN (both contain 0.1 % acetic acid) at flow rate: 200 µl/min Linear gradient elution: starting with 5% ACN, increased to 75% in 5 min, held for 8 min before returning to the original composition.	MS	For H_2O: < 0.84 µg/L For wine: < 0.68 µg/L	[207]
Tea	BPA	Zorbax Eclipse XDB-C8 (50 mm × 2.1 mm i.d., 3.5µm)	Acetonitrile-ultrapure water (40:60) containing 0.8% (v/v) of 1 mM NH_4OH flow rate: 250 mL/min Isocratic elution	MS	72 ng/L	[121]

$\lambda_{exc.}$ excitation wavelength, $\lambda_{emm.}$ emission wavelength.

- Capillary electrophoresis

Capillary electrophoresis (CE) is a promising separation technique that is still under developing. This technique offers several advantages including time saving, high sensitivity and efficiency to separate analytes of interest. Sun et al. [241] developed a method based on CE to separate DMP, DEP, and DEHP in environmental samples. The LODs obtained were: 4.06, 3.07, and 2.07 μg/L for DMP, DEP and DEHP, respectively. Mei et al. [215] developed a method for the determination of BPA in wastewater, river water, soil, and tap water using CE. In this study, BPA was extracted and preconcentrated using SPE using MIP as a sorbent. The LOD obtained in this study was 0.3 mg/L. This technique shows some drawbacks such as low sensitivity and small injection volume (nano-liters) which cause high margin of error, especially for trace level measurements, which, in turn, require an intensive sample pre-treatment [241].

1.2.5 Contamination problems

The extensive use of PAEs and BPA in industry results the elevation of their presence in water, solvents, air, etc. Therefore, cross-contamination, caused by this vast usage, is the main problem that can mislead the analysis of such substances resulting false positive results. PAEs and BPA may get adsorbed on glassware and other materials used for sample preparation and analysis. Since the contamination may vary from one laboratory to another according to different factors related to the workplace ambient like ventilation, precautions must be considered to minimize or even avoid the cross-contamination [242]. The systematic contamination may occur during the sample preparation and/or the analysis stage. Contamination occurred during sample preparation may be explained using contaminated glassware and materials. Reducing the number of sample preparation steps and glassware used and avoiding the use of any plastic material (like pipette tips, plastic syringe, and plastic filters) can reduce the risk of the sample contamination. On the other hand, the contamination during the analysis is related mainly to the autosampler vials cap. To overcome this problem, single injection per vial should be performed.

Several cleaning protocols have been suggested to minimize or even avoid the cross-contamination during sample preparation and analysis stages. The use of highly pure solvent such as acetonitrile and acetone for rinsing the glassware was the most important feature of theses protocols [121, 243, 244]. Pérez Feás et al. [244] used the Environmental Protection Agency (EPA) recommendations (Method 506) [245] to clean glassware used during the analysis of PAEs in physiological saline solutions. The authors washed the glassware with hot water and soap followed by rinsing with ultrapure water and acetone, and finally, the washed glassware was dried, sealed with aluminum foil and saved prior to used.

1.3 ELEMENTS – GENERAL OVERVIEW

Elements are found in various forms in nature, and these elements possess an essential role to operate different functions in the body. Trace elements are very critical for cell functions at different pathways, including chemical, biological, and molecular streams. Trace element is a term used to define those elements presented in the living organism at low concentration (µg/g or less) [246]. Despite their low levels, trace elements play a crucial role in several specific biological functions. The World Health Organization (WHO) has named 19 trace elements, including Zn, Cu, and Se, for their importance to human health [247]. While trace elements are essential for enzymatic function, they can be toxic for the same biological function at high concentration. Therefore, the essentiality of the element for the living organism is generally related to its absence or deficiency from the diet that causes either physiological or systemic alternation leading to producing diseases [248]. Trace elements in tea may have both beneficial and adverse functions on humans. For example, Al accumulation in tea is contributed to Alzheimer's disease [249]. On the other hand, tea leaves may contain some toxic elements like Cd, Cr, Pb, Hg, and As [250].

1.3.1 Role of elements in the human body

Human body mass consists approximately of 98% major elements (mainly non-metallic elements: O, C, N, and H), 1.4 % of Ca, while the rest is constituted of 44 other elements (primarily semi-major

elements like Na, K, Mg, and trace elements such as Cu, Zn, Co, Se, etc.) [251]. Nevertheless, this tiny percentage possesses an important effect on overall body functions. While major elements form an essential part of covalent bonds formed in the tissues, semi-major elements like K and Na are involved in living body functions by controlling the membrane's potential and osmotic pressure [252]. Moreover, Ca plays a vital function in bone building, blood clotting, muscle contraction, and nerve impulse transmission [253]. Lack of major elements may contribute to metabolic and nutritional defects, while excessive abundant may cause obesity. On the other hand, trace elements, including Zn, Cu, Fe, Cr, Mn, Mo, Co, and Se, that contribute to less than 0.02% of the total body weight serves as catalysts and/or cofactors for several enzymes and as stabilizing centers for building structures in enzymes and proteins. The reduction of enzyme activity is the main result of trace element deficiency, while their excess state may contribute to some severe disease and body poisoning. For instance, the lack of Cu leads to anemia, leukopenia, and bone defects, while the excessive ingestion causes diarrhea, vomiting and nausea [254]. Selenium excessive intake may lead to an increase in the concentration of reactive oxygen species (ROS) in the body while its deficiency leads to cardiomyopathy and lower extremities weaknesses [255]. Zn is another trace element that plays a vital role in nucleic acid metabolism, tissue repair and growth. The dietary sources of Zn include shellfish, red meat, eggs, milk, etc. however, the lack of Zn may lead to taste disorder, vomiting, growth retardation and anorexia [256], and the excessive dose may lead to gastroenteritis, microcytosis and relative neutropenia [257].

1.3.2 Elements in tea

As mentioned before, about 5% of the dry weight of tea leaves is constituted of minerals and trace elements [258]. Tea elements are divided into two main categories, macro-elements such as K, P, Ca, S, and Mg, and micro-elements (trace elements) including Fe, Zn, Ca, Se, Mn, and Mo. Several studies have been published in the literature regarding the elemental content of tea. The element levels varied from mg/g as in the case of Ca, K, Na, Mg, and Mn to a few μg/g for elements like Fe, Co, Cr, Ni, Zn, Cu, and Cd [259]. The content of

each element may also vary from one sample to another according to different factors like origin, tea type, cultivation and harvest method, age, climate, soil, and seasonal variation [260]. The presence of trace elements in tea plants is attributed to the highly acidic soil where tea is cultivated, which enhances the bioavailability of such elements from soil to the plant [261]. Kumar et al. [262] studied the concentration of Na, K, Mn, Cu, and Br in different brands of tea leaves (15 Indian brands and 7 American brands). The results showed that Mn had shown a narrow range of concentration (371-758 μg/g), while the American brands were widely ranged between 79 -768 μg/g with a mean of 329 ± 231 μg/g. Cu and Na levels were extended over a wide range for both brands. Meanwhile, both brands possess similar K concentrations. Br was absent in the American brands.

The daily intake of elements also varies from one element to another. The recommended daily intake of essential elements like Mn, found at highest levels in cereals, grains and nuts, is ranged between 2 and 5 mg [263]. However, its deficiency reduces serum cholesterol and causes growth retardation, while its high intake leads to Parkinson's syndrome [264].

Since tea is the second most consumable drink in the daily diet overall the world after water and considering the valuable benefits on its minerals to the body, assessment of its essential, trace and toxic elements is of great interest from several perspectives, including quality, health, nutrition, and pollution. Therefore, elemental contents in tea leaves and their infusion were extensively studied in the literature [265–272]. The total evaluation of tea elements in the leaves as well as the infusion is usually performed by atomic spectrometry techniques like graphite furnace atomic absorption spectrometry (GF-AAS), flame atomic absorption spectrometry (FAAS), inductively coupled plasma atomic emission spectrometry (ICP-AES) and mass spectrometry (MS) technique (i.e., ICP-MS). Prior to the analysis, tea samples are pre-treated according to the purpose of the study, which means whether the study aims to determine the element contents in tea leaves or tea infusion. Sample preparation and analysis techniques will be explained in the next two sections.

1.3.3 Sample preparation prior to analysis

Elemental determination in tea samples is commonly dealing with the evaluation of elements either in the tea leaves (digestion of tea leaves) or in tea infusion (brewing of tea leaves). Several methods have been developed to prepare tea leaves for the total element determination, such as dry ashing, wet acid digestion (including conventional and microwaves methods), and UV-photolysis. On the other hand, tea infusion is generally prepared by brewing tea leaves according to the traditional way and following the manufacturer's instructions.

1.3.3.1 Digestion of tea leaves

Several methods have been applied to digest tea leaves, including dry ashing digestion, wet digestion using microwaves (MW) assisted digestion, UV-photolysis assisted digestion, and conventional wet digestion using a hot plate.

Ashing digestion of tea leaves can be carried out following two strategies: dry or combined dry and wet digestion. During the dry ashing digestion, tea leaves (0.5 – 5 g) are accurately weighed and placed in a crucible for ashing at different fixed temperatures like 450℃ [273], 480℃ [274], 500℃ [275], 550 ℃ [276], and 600 ℃ [277] or stepwise increase of temperature using muffle furnace [278]. A solution of HCl (different concentrations were used in literature) [273, 274, 279], aqua regia [278], or a concentrated solution of HNO_3 [276], were added to the ashed leaves inside the crucible and heating continues to acquire a clear solution. The resulting solution is diluted with water and filtered prior to the analysis to remove any particles [279]. Costa et al. [280] proposed a combination of dry ashing and wet digestion to determine Al, Ca, Mg, and Mn in tea leaves. The procedure started by ashing 1.0 g of tea leaves at 500 ℃ for an hour, followed by acid treating (concentrated HNO_3). The acid was then dried off, and the residual was heated for 15 min at 500 ℃. Finally, the resulting ash was wetted with water, dissolved in concentrated HCl, and diluted with water. Despite ashing digestion show a precise and accurate method for sample preparation, it is time-consuming protocol, and volatile elements like Cd, Pb and Hg may be lost during the preparation at high temperatures [281].

Nowadays, wet digestion is preferable over ashing methods in the daily routine sample preparation. This method is usually performed in closed systems using microwave oven or in an open systems using conventional hot plates for heating, using a minimum mass of sample (0.05 – 2.0 g) [282]. The most typical solvent for wet digestion is a concentrated solution of HNO_3, its mixtures with other solvents like $HClO_4$ [283], H_2O_2 [284], HCl [285], and its complex mixtures with more than one solvent such as HNO_3 with (H_2O_2 and HF) [286], HNO_3 with (HCl and HF) [287], HNO_3, $HClO_4$, H_2O_2 and HF [288], and HNO_3 with (HCl and $HClO_3$) [262]. Wet digestion is usually carried out at lower temperatures than those applied in ashing methods (i.e., temperatures range between 90 – 190 °C) [282]. After digestion, the solution is diluted to the needed volume with water and filtered to remove any solid particulates. **Table 1.7** shows more applications of sample preparation using dry and wet digestion methods. Generally, MW-assisted acid digestion is favorable over conventional heating since it is faster, use less volume of solvents, small amount of sample, and minimize the loss of analyte [281].

UV-photolysis is another sample preparation method based on a radical decomposition of sample materials. The radical digestion involves several intermediate reactive species produced by exciting a radical generating reagent like hydrogen peroxides, superoxide ions, hydroxyl radicals, etc. The sample is suspended and pre-dissolved in proper radical generating reagent and then placed in quartz tubes. The tubes are then loaded in the UV-digester and irradiated for a certain time. The resulting solution is diluted to the required volume prior to the analysis [289]. Dash et al. [290] used this method to determine Al, Ca, Cr, Cu, Fe, K, Mg, Mn, Na, Pb and Rb in tea and tulsi or holy basil (*Ocimum sanctum*) samples. The method was efficient and reliable.

1.3.3.2 The brewing of tea leaves (tea infusion)

Regarding the preparation of tea in term of brewing, the leached amount of elements from tea (loose or bagged leaves) to the infusions relies mainly on several factors such as sample-to-water ratio (w/v) [291], brewing temperature [292], brewing time [76], quality of water [293], type of tea [294], number of times of infusion [295], and

agitation applied [280]. The preparation of tea infusion is usually achieved by steeping 1 – 5 g of tea leaves or tea bags in beakers in a boiling de-ionized [274], distilled [278], or ultrapure [296] water at different mass-to-volume (g/mL) ratios from 1:12 up to 1:100 or even more [291,293]. The agitation of tea leaves or bags before or during brewing ensures the wetting of leaves to extract more analytes and homogenizing the infusion [274, 295]. Several brewing times were reported in the literature starting from a minute [294] up to hours including 2 min [297], 2.5 min [298], 3 min [296], 5 min [295], 6 min [299], 10 min [300], 15 min [293], 20 min [301], 30 min [286], 60, 120 up to 360 min [287]. After brewing, tea infusion is filtered to remove leaves and settled solid using filter paper or membrane filter [283]. Centrifugation, followed by filtration or decantation, has also been reported in the literature [290,302]. Elements in the infusion are analyzed by atomic spectrometry techniques by introducing the prepared infusion (diluted, undiluted or acidified HNO_3) [267, 283, 294] to the spectrometric and/or mass analyzers.

Table 1.7 Applications of dry and wet digestion procedures for the preparation of tea leaves

Sample Preparation	Type of tea	Procedure	Element analyzed	Ref.
Dry ashing	Black, green	Ashing in a muffle at 450 ℃ for 3 h, then 5 mL of 6M HCl was added followed by heating on a hot plate. The residue was dissolved in 0.1 M HNO_3 and diluted to 50 mL	Cu, Ni, Pb, Cd, Cr, Zn, Mn	[303]
	Oolong, green, black	Ashing at 500 ℃ for 6 h, followed by dissolving with 2 mL 6M HCl and diluted to 25 mL	Al, B, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Na, P, Pb, Zn	[272]
	Black, green, white, oolong	Ashing in a furnace muffle at 500 ℃ for 16 h, then heating for 10 min at 100 ℃ in 3 mL Aqua regia and diluted to 20 mL	Al, B, Cu, Fe, Mn, P, Zn	[304]
Wet digestion (open vessel)	Black	Tea was digested with concentrated HNO_3 + $HClO_4$ (3 + 2 mL) at 130℃ for 1 h and diluted to 25 mL	Al, As, Ca, Cd, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Zn	[283]
	Black, green, oolong	Tea was digested with concentrated HNO_3 + $HClO_4$ (14 + 1 mL) at 180 ℃ for 3 h and diluted to 50 mL	Ba, Bi, Cd, Ce, Co, Cu, Eu, Ga, La, Nb, Ni, Pb, Pr, Rb, Sr, Ti, Tl, Y	[305]
	Pu-erh tea and tea leaves	0.3 g in 8 mL HNO_3 + 0.5 -1 mL $HClO_4$ (if needed) at 120 – 130 ℃ overnight	Al, As, Cd, Cu, Hg, Pb, Zn	[306]
	Black, green, white	1 g in 7 mL HNO_3 (overnight) followed by drying and heating with 8 mL $HClO_4$ at 200 ℃	Cu, Fe, K, Mn, Na, Ni, Zn	[307]

Table 1.7 (continued)

Sample Preparation	Type of tea	Procedure	Element analyzed	Ref.
Wet digestion (MW-assisted digestion)	Black, green, oolong	0.5 g in 5 mL HNO_3 was digested using MW at 170 °C for 10 min and diluted to 25 mL	As, Cd, Co, Cr, CU	[308]
	Black, green, oolong	2 g in 20 mL HNO_3 was digested using 2-step program: 190 °C over 30 min and then maintenance at 190 °C for 40 min. The digested solutions were filtered and diluted to 50 mL	As, Cd, Co, Cr, Cu, Fe, Mg, Pb, Se, Zn	[250]
	Black, green, oolong, pu-erh, white	0.25 g in HNO_3 + H_2O_2 (6 + 1 mL), in 5-step MW digestion: 0-2 min (250 W), 2-4 min (0W), 4-10 (250W), 10 - 15 min (400W), 15-23 min (800W), then diluted to 25 mL	Al, Ba, Ca, Cu, Fe, K, Mg, Mn, Na, Ni, P, S, Sr, Zn	[271]
	Black	1 g in HNO_3 + HCl (3 + 1 mL), 3-step digestion program: 3min (180W), 5min (360W), 3min (180W), then diluted to 25 mL	Cd, Co, Cr, Cu, Mg, Ni, Pb, Zn	[309]

1.3.4 Analysis of elements in tea samples

The study of tea composition is crucial to understand its effect on human health, judge its nutritional benefits, and estimate its possible harmful impact due to high concentrations. Analysis of elements in tea leaves and their infusions require sensitive, accurate, precise, reliable, and versatile techniques. Several analytical methods have been applied for element determinations in tea; the most used techniques are based on absorption or emission atomic spectroscopy and atomic mass spectrometry.

1.3.4.1 Atomic spectroscopy

Atomic absorption spectroscopy (AAS), atomic emission spectroscopy (AES) and Atomic fluorescence spectroscopy (AFS) are reported in the literature for trace element determination in tea samples. Graphite furnace AAS (GF-AAS) and Flame AAS (FAAS) were applied in several studies to determine the trace elements in tea samples. Zhong et al. [310] determined Pb, Cd, Cr, Cu, and Ni in 25 digested tea samples of different varieties, including black, green, white, yellow, Pu'erh, oolong, and jasmine teas using GF-AAS. The method shown high precision and good analytical recoveries.

Al-Othman et al. [61] used FAAS for Cd, Pb, Mn, Zn, Ni, Co, Fe, Cr and Cu determination in teas [311]. A recent study performed by Zhang et al. [284] used GF for AFS determination of hydride-forming elements (Bi, As, Te, and Se) in tea leaves. This combination was found to be sensitive, fast, easy to operate, and cheap. Although the analysis using FAAS and GF-AAS techniques is performed element by element, which is considered as an inconvenience, these techniques are still applied for such types of determinations.

The inductively coupled plasma atomic emission spectrometry is advantageous over AAS techniques since a multi-elemental analysis can be achieved at the same time. This technique is extensively used for elements determination in tea. Szymczycha-Madeja et al. [312] determined Ba, Ca, Cd, Cr, Cu, Fe, Mg, Mn, Ni, P, Pb, Sr and Zn in slim tea products using ICP-AES achieving a limit of detection (LOD) between 0.15 and 98.4 ng/L. More applications of AAS and ICP-AES are summarized in **Table 1.8**.

1.3.4.2 Atomic mass spectrometry

Since inductively coupled plasma mass spectrometry (ICP-MS) was first introduced in 1980, it has become a universal technique for the determination of elements in biological samples. Comparing to the atomic spectroscopic techniques, ICP-MS is more sensitive with LOD in the range of ng/L with a capability of simultaneous analysis of almost all elements with easier spectra to interpret, and wider linear dynamic range [313].

Table 1.8 lists some applications using ICP-MS for tea analysis. For instance, Troisi et al. [125] quantified Al, As, B, Ba, Cd, Co, Cr, Cu, Fe, Hg, Li, Mn, Mo, Ni, Pb, Sb, Se, Sr, V, and Zn in black and green teas using ICP-MS, and LODs were ranged between 0.05 µg/L (Hg) to 2.57 µg/L (Fe).

Table 1.8 Applications of elemental analysis in different types of tea using atomic and mass spectrometric techniques.

Technique	Sample	Analyzed element	LOD	Ref.
ICP-MS	Black, green, oolong	AS, Cd, Co, Cu, Fe, Mg, Pb, Se, Zn	<50 ppt	[250]
	Black, green	Ce, Co, Cr, La	-	[268]
	Green	As, Ba, Cd, Co, Cu, Cr, Ni, Pb, Se, V, Zn	0.5 – 5.55 µg/L	[314]
	Black, green, pu'erh	Li, Cr, Co, Ni, Cu, Zn, Ga, As, Se, Mo, Cd, Sb,Cs, Pb, Mg, Al, Mn, REEs*	< 0.6 µg/g	[315]
	Black, green, oolong	Ba, Bi, Cd, Ce, Co, Cr, Cs, Cu, Eu, Ga, La, Nb, Ni, Pb, Pr, Rb, Sr, Ti, Tl, Y	-	[305]
	Pu'erh tea and tea leaves	Al, As, Cd, Cu, Hg, Pb, Zn	0.011 - 0.4 µg/L	[306]
	Not specified teas	Mg, Al, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Se, Sr, Sb, Ba, As, Cd, Hg, Pb	-	[316]
	Black, green	Al, Ca, Cd, Cr, Cu, Fe, Hg, K, Mg, Mn Na, Ni, Pb, Zn	-	[317]
ICP-AES	Black, green	Ba, Ca, Ce, Cu, Fe, K, Mg, Mn, Na, Ni, P, Sr, Zn	-	[268]
	White, green, black, Oolong, Pu-erh	Al, Ba, Ca, Cu, Fe, K, Mg, Mn, Na, Ni, P, S, Sr, Zn	-	[271]
	Black	Al, As, Ca, Cd, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Zn	2 – 50 ng/g	[283]
	Black, green	Cu, Fe, Mn, Pb, Zn	<10.3 µg/L	[285]

Table 1.8 (continued)

Technique	Sample	Analyzed element	LOD	Ref.
ICP-AES	Pu'erh tea	As, Cd, Cr, Cu, Pb		[318]
	Black, green, oolong, other teas	As, Cd, Co, Cr, Cu	70, 0.5, 1, 1, and 1.3 µg/L, respectively	[308]
	Not specified teas	Se, Zn, Ni, Mn, Cr, Pb, Mg, Ca, Cu, Al, Na, K	0.002 – 0.018 µg/L	[319]
	Black, green	Al, Ba, Cd, Co, Cr, Cu, Ni, Pb, Sr, Zn	LOQ: 1.4 – 11 µg/L	[320]
	Black, green	Al, Ba, Ca, Cu, Fe, Mg, Mn, Ni, Sr, Zn	-	[321]
	Black	Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Zn	-	[322]
	Tea leaves (green)	Ca, Cu, K, Mg, Zn	-	[323]
GF-AAS	Black	Cu, Cr, Ni, Cd, Pb	-	[273]
	Green, white, yellow, black, oolong, Pu'erh, jasmine tea	Pb, Cd, Cr, Cu, Ni	0.005 - 4.4 mg/L	[310]
	Black, green, rooibos, tea with seaweed	Ca, Co, Cu, Fe, Mn, Ni, Na, Zn	2.3 - 36.2 µg/L	[324]
	green, black, Oolong, and scented teas	Cu, Pb	-	[325]

Table 1.8. (continued)

Technique	Sample	Analyzed element	LOD	Ref.
FAAS	Not specified	Ca, Cd, Co, Cu, Fe, K*, Na* Mg, Mn, Pb, Zn	4 - 74 µg/L	[259]
	Tea leaves (green)	Al	-	[323]
	Black, green	Cu, Ni, Zn	-	[281]
	Black	Mn, Cr, Pb, Ni, Cd, Cu, Co, Zn	0.09 - 0.11 mg/L	[52]
	Black, green	Ca, Fe, K, Mg, Mn, Na	LOQ: 6.3 - 75 µg/L	[320]
	Black, green, white, herbal teas	Cu, Fe, K*, Mn, Na*, Ni, Zn	0.06 - 0.6 mg/L	[326]
	Green	Na, K	Na:0.3 mg/L K: 0.04 mg/L	[327]

REEs: rare earth elements, *: using emission mode or a flame photometer, LOQ: limit of quantification.

1.4 ANTIOXIDANTS IN PLANTS

The plants kingdom is a huge factory of wide varieties of secondary metabolites. Terpenoids, alkaloids and phenolic compounds form the majority classes of these metabolites. Among these classes, phenolic compounds have been reported to possess several biological impacts like antioxidant activity [328]. These substances possess a crucial function in plants through different pathway such as growth and protection against harsh conditions like UV radiation and pathogens. Moreover, phenolic compounds contribute to other characters like color, dietary properties and sensory profile of plants [329].

1.4.1 Role of antioxidants in human health

During the past few decades, the scientific efforts have been focused towards including plant food in the diet considering their high contents of antioxidants that is thought to have a vital role in the prevention of particular human diseases and oxidative stress [330]. Dietary antioxidants, such as phenolic compounds, vitamins C and E, flavonoids, coumarins and tannins, acts as a free radical scavenger by stopping the radical chain reactions and inhibiting the formation of reactive species in the living organism [331]. These properties, alongside to the antimicrobial and anti-inflammatory properties, make the use of plant extracts trending especially in food industry. For instance, tea extract (especially green tea) is rich in polyphenols, alkaloids, polysaccharides, vitamin C, lipids, amino acids, minerals and other components that can inhibit lipid oxidation, scavenge free radicals and chelate metal ions (e.g. Cu and Fe) [332]. Several studies have been concluded that a regular consumption of tea leads to improve the antioxidant status in living organisms, which, in turn, leads to decrease the risk of some chronic diseases like some types of cancer, stroke, coronary heart disease, and atherosclerosis [333], decrease inflammation and mutagenicity [334, 335], protect against Alzheimer's disease [336], and increase the sensitivity of insulin [337]. In a public-based study, 40000 middle-aged Japanese were involved to investigate the relationship between consuming green tea and mortality caused by cancer, cardiovascular disease (CVD), and all-cause mortality [57]. The study concluded that the mortality due to

CVD was reduced by 22 – 33% for those who daily drank more than two cups of green tea compared to those who daily consumed less than a half-cup of green tea. In the same study, the authors reported that drinking green tea is not associated with mortality reduction due to cancer. In a cross-sectional study, Yang et al. [338] examined the effect of tea consumption on hypertension. This study claimed that hypertension was reduced by 46% and 65% for those who consumed 120 – 599 mL tea per day and more than 600 mL tea per day, respectively. Kim et al. [339] suggested that 8 g of green tea (powder) per day for 2 weeks improves the flow mediated blood vessels expansion in chronic smokers, which, in turn, leads to avoid future cardiovascular attacks in smokers.

The action of tea antioxidants is almost regulated by their antioxidant activity, free radicals scavenging power and metal chelating function. Among different tea varieties, green tea is thought to have the highest potent antioxidant activity owing to its high contents of catechins. Among these catechins, epigallocatechin gallate (EGCG) is claimed to be the most efficient antioxidant [340]. The potency of EGCG comes from the fact that it contains multi hydroxy groups. The doubled three adjacent -OH groups provide the EGCG with higher capacity to chelate metal ions like Fe and Cu [341]. Grey and Adlercreutz [342] reported that the chelating capacity of catechins is better than other antioxidant like vitamin C, and they proposed that catechin antioxidative character is based on its chelating ability rather than radical scavenging mechanism alone. On the other hand, black tea can act as antioxidant due to the presence of theaflavins which inhibits the activation of extracellular signal-regulated protein kinases [343], inhibits DNA single strand cleavage and regulates formation of nitric oxide synthase [344].

Tea is typically consumed as infusion where, for example, green tea infusion contains 30 – 40 % of catechins by weight while brewed black tea contributes of 3 – 10 % catechins by weight. However, higher percentages of tea phenols can be extracted efficiently using organic solvents [345]. Nevertheless, several factors may affect the released percentage of tea phenols such as tea variety, weight of tea leaves or bag, age of the leaves and extracting technique (brewing or

organic solvent). The addition of tea extract or even tea leaves themselves to foodstuff has been reported to improve their dietary value of food. For example, Tsong-Ming et al. [346] reported that when preparing sponge cake that contains up to 20% of its ingredients as green tea powder, the antioxidant activity was enhanced comparing to cake prepared with 100% cake flour. Mitsumoto et al. [347] reported that tea catechins inhibited lipid oxidation when added to raw beef (200 or 400 mg/kg) better than vitamin C.

1.4.2 Determination of antioxidant activity

Since antioxidants play a vital role as free radical scavengers and metal chelating agents, it is worthy to mention the mechanism of how they react with such active harmful species. According to Prior et al. [348], antioxidants can react with free radicals via two main mechanisms: hydrogen atom transfer (HAT) and single electron transfer (SET). HAT-based mechanism deals with the ability of antioxidant (AH) to donate a hydrogen atom to the free radical ($X^{\bullet}$):

$$AH + X^{\bullet} \longrightarrow XH + A^{\bullet}$$

HAT is works independently of the solvent used and pH of the solution, and the reaction is completed in short time (seconds or minutes) [349]. However, SET measures the ability of the antioxidant to reduce any compound (i.e., radicals, metals (M), and carbonyl) by transferring one electron:

$$X + AH \longrightarrow X^{-} + AH^{\cdot+}$$

$$AH \underset{}{\overset{H_2O}{\rightleftharpoons}} A + H_3O^{+}$$

$$X + H_3O^{+} \longrightarrow XH + H_2O$$

$$M^{+n} + AH \longrightarrow AH^{+} + M^{+(n+1)}$$

SET mechanism is slow, pH-dependent, and require longer time to reach the endpoint. Both mechanisms leads to the same results, and the main difference between them is related to kinetics and the side-reaction's potential [348]. The endpoint of the reaction can be observed once the color changes indicating that the antioxidant has quenched the oxidant, and the degree of color developed during the

reaction is associated to the concentration of antioxidant in the sample [349].

Using these mechanisms, several methods have been developed to estimate the antioxidant activity in a sample. In this section, we will consider the most common assays used to measure tea antioxidant activity. Among these assays, HAT-based assays such as oxygen radical absorbance capacity (ORAC), SET-based assays such as Trolox equivalent antioxidant capacity (TEAC), ferric antioxidant power (FRAP), total phenolic content (TPC) using Folin-Ciocalteu reagent and HAT and/or SET-based mechanism such as diphenylpicrylhydrazyl (DPPH) assay.

1.4.2.1 Total phenolic content using Folin-Ciocalteu reagent

Folin-Ciocalteu reagent (FCR) has been used for many decades to determine the total phenolics in different natural products. It is a SET-based reaction where single electrons transfer from the antioxidant phenolic compound to a molybdenum to produce a blue complex which can be spectrophotometrically monitored from 750 to 765 nm. Even that this assay is sensitive, rapid, simple and precise, it was slow in acidic medium and the reagent reacts non-specifically with sample components. Thus, Singleton and Rossi [350] optimized the assay to obtain more reliable data. The optimization included several parameters such as using a proper alkali-to-FCR ratio, optimization the reaction temperature and time until the color develops, measuring the color developed at 765 nm, and using gallic acid as a reference standard phenol to represent the data obtained. This assay offers a simple, reproducible, and reliable measurements, but also suffers from sample's components interference like aromatic amines, sugar, metals, etc. [348]. TPC using FCR is normally expressed as mg gallic acid equivalent (GAE) per amount of sample (weight or volume).

1.4.2.2 Trolox equivalent antioxidant capacity (TEAC)

In this assay, 2,2`-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS) is used as peroxidase substrate that is converted into $ABTS^{\bullet+}$ giving an intense colored solution when it is oxidized by an oxidant in presence of hydrogen peroxide (H_2O_2).

Originally, the first protocol set by Miller et al. [351] involved the generation of ferrylmyoglobin by reacting myoglobin with H_2O_2, which then brought to react with ABTS to produce $ABTS^{\bullet+}$. However, this approach was then modified by Re et al. [352] to take into account the order of the addition since the original approach involved the sample in the reaction before generating the radical ion $ABTS^{\bullet+}$. The revised approach requires the generation of the radical ion before adding the sample to avoid overestimating of antioxidant capacity. The increase of the absorbance is plotted against the concentration of Trolox and the antioxidant capacity is expressed as Trolox equivalent per amount of sample (weight or volume). This assay is reliable, fast and the radical ion can be solubilized in organic and aqueous medium, but the fixed reaction time (4 – 6 min) may lead to misestimating the antioxidant capacity due to incomplete reaction, and the radical ion must be generated by chemical reaction or enzyme [348, 353]. TEAC is applied to determine the antioxidant capacity in different sample matrices.

1.4.2.3 DPPH radical scavenging activity assay

DPPH offers a wide range of measuring the antioxidant activity in different sample matrices since it can work in both mechanisms (HAT and SET). This assay is based on measuring the disappearing of DPPH color (purple) to pale yellow hydrazine (DPPH derivative) at 515 nm. DPPH assay can be expressed in term of millimole Trolox equivalent (mmol TRE) per amount of sample (weight or volume). The advantages of this approach are mainly its simplicity, low-cost, and the ability of determining rapidly the antiradical activity. On the other hand, overlapping between the spectra of some antioxidants like carotenoids with the spectra of DPPH at 515 nm and the lack of DPPH solubility in aqueous medium are the main disadvantages of this approach [348, 354]. DPPH assay was used to determine the antiradical activity in several plants, drinks and foodstuff.

1.4.2.4 Ferric reducing antioxidant power (FRAP) assay

FRAP assay is a SET-based assay measures the ability of the antioxidants to reduce ferric tripyridyltriazine complex (Fe(III)-TPTZ)

(yellow color) to ferrous complex (Fe(II)-TPTZ) (navy blue color). Originally, this assay was used to evaluate the reducing power in plasma, but it was improved to measure the antioxidant activity in botanicals [355]. Once the reaction reached the endpoint (blue color), the solution is monitored spectrophotometrically at 593 nm to measure the amount of Fe(III) reduced which is related to the amounts of antioxidants in the sample. Several standards can be used as antioxidant references such as Trolox and ascorbic acid [356, 357]. Both TEAC and FRAP are SET-based mechanisms, and there is not much difference between them except the medium where the redox reaction occurs. TEAC is performed in neutral medium while FRAP assay requires acidic medium (pH=3.6) to maintain the solubility of iron. FRAP assay is cheap, simple, reliable and rapid [348]. However, positive false results may be obtained because the presence of any electron-donating species with lower redox potential than that for Fe^{+3}/Fe^{+2} even without antioxidant character may contribute to the FRAP results [358].

1.4.2.5 Oxygen Radical Absorbance Capacity (ORAC) assay

In this assay, the antioxidant capacity is evaluated by measuring the inhibition of peroxyl radical, induced by 2,2'-azobis-(2-amidino-propane) dihydrochloride (AAPH). Basically, thermal decomposition of AAPH produces peroxyl radicals which react with an oxidizable protein substrate (fluorescent probe). When the endpoint is reached, the fluorescent probe turns into nonfluorescent substrate and the loss of fluorescence can be easily monitored and quantified [331]. Trolox can be used as an antioxidant reference and the antioxidant capacity can be expressed as mmol Trolox equivalent per amount of sample. ORAC has been used to measure the antioxidant capacity in biological and botanical samples. ORAC assay is the only approach that can combine both degree and time of inhibition into a single quantity [359].

Many studies were published in the way of assessing the antioxidant activity in different matrices such as fruit and vegetable extracts, soft beverages, alcoholic beverages, coffee, spices, nuts, pulses, cereals and sweets [356, 360], dried fruits [361], Cocoa beans [362], mushrooms [363], wine [364], fruits [364] and tea [365],

utilizing one or more of these above-mentioned assays. For instance, Jiménez-Zamora et al. [366] measured the antioxidant capacity in 36 selected traditionally plants used as infusion including white, green, red, and black teas, using FCR, ABTS, DPPH and FRAP methods. In this study, green tea showed the highest antioxidant capacity in all assays among all samples analyzed. Results obtained from DPPH, ABTS, and FRAP were comparable for the same sample and the order of the antioxidant capacity for tea samples was as follows: green > white > red > black, which is expected according to the degree of oxidation in the processing. Zhang et al. [367] used ORAC assay to study the effect of factors related to the cultivation process of black and green teas. The authors compared the results obtained from the total ORAC assay with the individual determination of theaflavin and catechins. The results indicated that black and green teas were affected by geographical location and these results were correlated with those obtained by analyzing catechins and theaflavin.

Finally, these assays serve for the total analysis of phenolic compounds in tested sample, which means that it provides us with an estimation about the total antioxidant content but not possible to determine individually the amounts of the antioxidant in a sample.

1.5 BIOAVAILABILITY STUDY

When one consumes food or drink, the contained nutrients liberated from the matrix diffuse into the bloodstream and then move towards their respective final destinations. Nevertheless, not all nutrients behave in the same way during the digestion process, which means that their potential health effect is different. Therefore, it is important to understand the effect of the digestion process on the ingested nutrient, its bioactivity, and its stability. The information obtained about nutrient's bioavailability can help to establish nutritional recommendations of the ingested nutrient.

The bioavailability of a nutrient and/or a toxic component is defined as the fraction of this ingested nutrient and/or toxic that reaches the systematic circulation [368]. Bioavailability includes gastrointestinal digestion (GID), absorption, metabolism, and the bioactivity of the ingested nutrient and/or toxic component inside the

living organism. However, the bioactivity of a bioavailable component is practically quite difficult to measure, so bioavailability is commonly defined as the fraction of a component or its metabolite that is introduced into the bloodstream. Another term used by different scientists to address the bioavailability of a component is the bioaccessibility. The bioaccessibility of a component is defined as the fraction of this component that is ready to be absorbed and metabolized after releasing from the matrix (e.g., food, beverage, and plants) [369]. Therefore, element bioaccessibility itself does not provide a direct measure of element bioavailability, since not all soluble element species are absorbable. Several factors may affect bioavailability including the nutrient itself and host factors such as genotype, chronic disease states, physiological state (e.g., pregnancy and obesity), age, and intrinsic factors [370].

The bioavailability of a nutrient is related to the physicochemical and physiological conditions in the living organism. Therefore, it is necessary to establish a protocol that includes these conditions once one studies the nutrient's bioavailability in the human gastrointestinal tract (GIT). Different models have been developed to simulate GIT conditions such as *in vivo* and *in vitro* models. The more realistic model to study the bioavailability is the *in vivo* model. *In vivo* models are usually performed using animals such as rats [371], pigs [372], and dogs [373], or human. *In vivo* models provide closer representative conclusions about the nutrient bioavailability, since they include all digestive processes occur for the nutrient until its arrival to the systematic circulation, and even allow studying the bioactivity of the bioavailable nutrient. However, *in vivo* models suffer from lower throughput, lack of certified reference standards, high cost, slowness, the complexity of the functional system, low reproducibility, and ethical constraints [374]. Therefore, *in vitro* models have been proposed as an alternative to the *in vivo* models when studying the nutrient bioavailability. *In vitro* models are faster, less expensive, capable of controlling the experimental variables, and offer to study the bioavailability of toxic components, which cannot be studied using the *in vivo* models. Even though, the *in vitro* methods cannot replace the *in vivo* methods since not all physicochemical and

physiological conditions are considered during the simulation, which makes these methods as an approximation and should be treated as a screening or categorizing method [375].

In vitro models are classified into two main categories: static and dynamic models. When using static models, digestion stages in the mouth, stomach and intestine are simulated at fixed pH, time, and concentrations of salivary, gastric, and intestinal solutions. Peristaltic movements are mimicked by simple agitation. On the other hand, dynamic models consist of a series of compartments where each stage of the digestive process is separately mimicked, and the resultant digest from each stage is gradually transited to the next compartment and so on. The physicochemical changes (e.g., pH, temperature, concentrations of salts and enzymes, and agitation) in dynamic models are controlled using software. As an example of a commercial dynamic model, The Netherlands organization (TNO) developed a gastrointestinal model (TIM) where the conditions of the human digestive system are simulated. TIM consists of two computer-controlled compartments called TIM1 (represents the digestion occurs in the stomach and small intestine) and TIM2 (represents the large intestine to mimic the fermentation occurs in the colon) [376].

1.5.1 *In vitro* methods

Bioavailability is measure using three main methods: solubility, gastrointestinal dialyzability and Caco-2 method. The simulation of the GID using these methods is commonly performed through two-step digestion: gastric and intestinal digestions. During the gastric digestion, pepsin solution (from the porcine stomach) is mixed with a pre-adjusted sample with pH 2 (to mimic the adult's gastric pH) or 4 (to mimic the infant's gastric pH). The pH adjustment is important since pepsin at pH ≥ 5 starts to denature itself resulting in a loss of its activity. Before intestinal digestion, the gastric digest is neutralized to pH 5.5 – 6 followed by adding the intestinal solution, pancreatin (which contains a mixture of pancreatic enzymes like pancreatic amylase, ribonuclease, lipase, and trypsin) and bile salts as emulsifiers. The mixture is then readjusted to pH 6.5 – 7. Sometimes, three-step digestion is carried out by introducing a salivary digestion step before gastric digestion. Salivary digestion simulates the

digestion that happens in the mouth using an α-amylase enzyme which is responsible for the hydrolysis of alpha bonds of large polysaccharides such as starch, producing glucose and maltose [370].

For the solubility method, the digested fraction is usually centrifuged or filtered, obtaining a precipitate and a supernatant which contains the soluble analyte [377, 378]. The nutrient in question, separated in a soluble form in the supernatant, is then analyzed using spectrophotometric and/or mass spectrometric methods such as atomic absorption spectrophotometry (AAS), inductively coupled plasma atomic emission spectroscopy (ICP-AES), and inductively coupled plasma mass spectrometry (ICP-MS), or chromatographic methods such as high-performance liquid chromatography (HPLC), gas chromatography (GC) and capillary electrophoresis (CE). Solubility assay is a simple, inexpensive, and easy to perform, but cannot evaluate the uptake absorption which leads to obtaining nonreliable conclusion about nutrient bioavailability. *In vitro* solubility has been used to study the bioavailability of different nutrients in several matrices such as polyphenols in olive oil [379], cocoa liquor [380], raspberries [381], calcium, iron, and zinc in rice-based products [382].

In 1981, Miller et al. [383] introduced the dialyzability approach to evaluate iron bioavailability from foods. This approach follows the general procedure of the GID where sample is predigested with gastric solution followed by intestinal digestion, but the main improvement was the introduction of dialysis bag/tubing/membrane of a specific molecular weight cut off (MWCO) filled with buffer solution such as sodium bicarbonate. The dialysis bag is introduced before carrying out the intestinal digestion, so the buffer solution diffuses slowly out of the bag to neutralize the digest. After adding the intestinal solution, the whole mixture is incubated and the analyte of interest diffuses into the bag, named dialyzate. The diffusion of the analyte into the bag depends on the MWCO of the dialysis bag where equilibrium dialysis is established. This model offers more realistic idea than solubility model since the dialysis membrane mimics the small intestine and the dialyzate represents the amount of dialyzable nutrient available for the organisms. Dialyzability model has been used to study the bioavailability of great number of nutrients

such as calcium, magnesium, polyphenols, etc. Many digestion parameters have been incorporated into this model such as body temperature, peristalsis, and mixing. Moreover, continuous-flow dialysis modes were also applied to remove the dialyzable components during the GID [384]. Several studies have been performed to evaluate the bioavailability, in terms of dialysis assay, of several nutrients in different food and beverage matrices such as Fe, Cu and Zn from infant formula [385, 386], As, Co, Cr, Cu, Fe, Ni, Se and Zn from edible seaweed [387], trace metals in marine foodstuff [388], metals in edible nuts [389], polyphenols from nuts and seed [390], apple varieties [391], etc. The in vitro dialyzability methods suffers from its inability to mimic bioprocesses occur in the living organism, lack of hormonal control, peristaltic movements, the contribution of the immune system [392], the influence of applying fixed pH and enzymes concentration [393].

Caco-2 cell model is considered the most used in vitro model for bioavailability assessment of nutrients since Caco-2 cells, from epithelial cell lines, are derived from human colon rectal carcinoma [394, 395]. The intestinal-like permeability behavior of these cells allows studying transepithelial transport of bioavailable nutrients via their permeability through a monolayer polarized cell [396]. Caco-2 cells grown on a permeable filter (plastic dish or transwells) become differentiated and polarized into a monolayer with tight junctions that resembles the phenotype of the enterocytes lining the small intestine. Once the GID is accomplished, the digested fraction is filtered or centrifuged and transferred to the grown Caco-2 monolayer. The absorption occurs through the apical membrane to the basolateral membrane to collect the bioavailable fraction. Caco- cell model offers many advantages including short experimental time, the ability to analyze a large number of metabolic compounds alongside the ability of studying the efficiency of digestion and transport mechanism of the bioavailable component [374]. However, it also possesses several significant limitations such as the difference of epithelium density in vivo is many times higher than Caco-2 monolayer which causes a significant difference in the permeability. Artursson et al. [397] found that the transport of compounds via the

Caco-2 monolayer is 20 and 100-fold slower than that via the small and large intestine, respectively. When performing the *in vitro* Caco-2 cell, the analyte is applied as a native of enriched form, which is neither the form nor the concentration found in the tissue or plasma of the living organism [398]. Several bioavailability studies of bioactive compounds and elements in food matrices, beverages, human milk and infant formula, and the pharmaceutical industry have been carried out using the Caco-2 cell model [399–403].

Being the second most-consumed drink in the world, tea has been received more attention regarding its beneficial health effects. Generally, beneficial health effects tea consumption is associated with its antioxidant properties since it is a rich source of polyphenols [404]. Moreover, tea also contains high concentrations of dietary minerals such as sodium, potassium, magnesium, calcium, phosphorous, alongside trace levels of essential elements like zinc, cobalt, selenium, manganese and toxic elements including lead, cadmium, arsenic, and mercury [32, 405]. Considering all these chemical compounds with their beneficial and/or toxic effects, the scientific efforts have been focused on studying their bioavailability to the human body. The next sections will focus on the *in vitro* assessment of bioavailable polyphenols and elements in tea.

1.5.2 Bioavailability of elements in tea samples

Few studies have been revealed in the scientific field regarding the assessment of bioavailable elements in tea [263, 406–411]. Basically, there are two main *in vitro* protocols for the determination of element bioavailability from tea samples using GID: solubility and dialyzability. Most of the published work evaluated the element's bioavailability in terms of solubility where the GID is followed by centrifugation and/or filtration of the soluble fraction [263, 406, 407, 409, 410]. Nevertheless, the dialysis approach was utilized twice applying static [411] and continuous [408] modes. Edemir et al. [409, 410] determined the in vitro solubility of Mg, Mn, Fe and Li from black, green, and Early gray flavored black tea. Samples were steeped at different time intervals (2, 5, and 10 min). The infusions were then subjected to a gastric digestion phase using pepsin (pH 1.9) and then

incubated for 2 h at 37°C. The gastric digests were then cooled, and the intestinal digestion phase was initiated by adjusting the pH to 6.9, followed by introducing pancreatin and bile salts and incubated at 37°C for 2 h. Once the intestinal digestion finished, the digests were cooled and centrifuged, and the supernatant was analyzed by ICP-MS. The detection limits obtained were in the range of 0.01 – 0.42 μg/g. The bioavailability/bioaccessibility of the elements analyzed showed different behavior (increase or decrease) when varying the steeping time. The authors attributed this behavior to the manufacturing process and the enzyme activity during tea brewing. In another study, Powell et al. [263] evaluated the bioaccessibility of Al, Ca, Cu, Fe, K, Mg, Mn, Na, and Zn from one black tea infusion. The infusions were incubated with human gastric juice (collected from patients using routine gastroscopy) to mimic the digestion phase at 37°C for an hour, and then, the pH was adjusted to 6.5 to mimic the intestinal pH phase. The obtained digests were then centrifuged and ultra-filtrated over 3, 10 and 30 kDa MWCO membranes. The resultant solutions were diluted (1:1), acidified with HNO_3 and analyzed by ICP-OES. The results showed that Fe could not diffuse through all membranes concluding that it is not bioaccessible. The authors explained that this behavior is due to the presence of tea polyphenols that form insoluble complexes with Fe. On the other hand, the bioaccessibility of the other elements was reduced after adjusting the pH to simulate the intestinal pH, and the ultra-filterability was in the order of $M^+ > M^{2+} > M^{3+}$. Aluminum bioaccessibility was measured from green, black, and oolong tea [406, 407] in terms of solubility. Both studies showed low bioaccessibility of Al (15 % and 5.1%) from analyzed teas, which was explained by the low solubility of Al at pH 6 – 8 and the presence of ligands in the intestine that chelated Al and prevent its absorption.

In a recent study, Szymczycha-Madeja et al. [411] assessed the bioavailability of Al, Ba, Ca, Cu, Fe, Mg, Mn, Ni, Sr, and Zn from black and green teas using static dialyzability model. In this study, samples of infusion were incubated with gastric pepsin solution (after adjusting the pH to 2) for 2 h at 37°C. The gastric digestion was then stopped, followed by adding the intestinal solution (pancreatin and bile salts), and a dialysis membrane of 12.4 kDa MWCO filled with

PIPES ((piperazine-NN-bis(2-ethane-sulfonic acid) disodium salt)) was placed inside the digested fraction for incubation at 37°C for 2 h. Once finished, the dialyzable fractions were analyzed by FAAS and ICP-OES. Among elements analyzed, Ca showed the highest bioaccessibility and Al was the lowest bioaccessible from all teas analyzed.

Robberecht et al. [408] evaluated silicon bioavailability in food and drinks (including tea infusion) using a continuous flow dialysis model. In this study, the dialysis protocol was divided into two compartments: gastric and intestinal compartments. During the gastric phase, the samples were adjusted to pH 2 and before incubating with the gastric solution at 37°C for 2 h in a water-bath at 120 strokes/min. The gastric digest was then passed to the intestinal compartment which consists of a stirred cell, membrane bag MWCO 10-12 kDa (containing $NaHCO_3$) and a dialysis membrane (MWCO of 1000 Da). The acidic pH of the gastric digest was increased gradually over 30 min to neutral by the dialysis bag (filled with 1M $NaHCO_3$). Next, the intestinal digestion started by adding pancreatin and bile salt, and the incubation continued for another 2 h. During the intestinal phase, the dialyzate was removed continuously via the dialysis membrane (1000 Da MWCO) and the Si concentration was determined by AAS. Silicon bioaccessibility in tea infusion was found to be 20 ± 2 %.

1.5.3 Bioavailability of tea polyphenols

The main *in vitro* approaches used to study the bioavailability of tea polyphenols are the solubility [412–414] and Caco-2 cell [415–419] model. Tenore et al. [417] assessed the bioaccessibility and bioavailability of tea polyphenols using solubility and monolayer Caco-2 cell methods, respectively. Firstly, tea infusions (white, green, and black teas) were subjected to three-steps GID procedure (salivary, gastric and intestinal phases), the GI digests were then centrifuged and the supernatant was divided into two part: one for direct analysis by HPLC/diode-array detector (DAD) and the other part was used for the evaluation of transepithelial permeability by Caco-2 cell. After preconditioning the grown Caco-2 cell monolayer, the intestinal digests were introduced and incubated for 4 h at 37°C. The apical and basal solution were then collected and analyzed by HPLC. A very low

bioavailability was obtained by solubility (about 8%) and Caco-2 cell line (2-15%) methods ascribing, according to the authors, to the neutral intestinal pH (6.5) resulting epimerization and auto-oxidation of tea catechins. Furthermore, the authors indicated that the low transepithelial permeation of catechins through Caco-2 monolayer may be related to the presence of efflux transporters on the apical membrane of intestinal cells and/or the instability of tea catechins at neutral. Peters et al. [415] reported similar results indicating the reduction of green tea polyphenols bioavailability (using GI solubility and Caco-2 cell line method) compared to non-digested tea infusion. Moreover, Peters et al. [50] found that the bioavailability of tea catechins enhanced when mixing green tea extracts with ascorbic acid and/or sucrose. Conversely, Coe et al. [420] found that bioavailability of green, white, and black teas polyphenols enhanced in the gastric and the intestinal digests after performing an *in vitro* GI solubility method. In a recent study, Annunziata et al. [421] assessed the antioxidant activity in term of TPC, DPPH and ABTS assay after performing an *in vitro* GID followed by simulated colon digestion. The colon digestion was carried out by incubating the intestinal digests with Pronase E solution (pH 8) for 1 h, followed by Viscozyme L (pH 4) for 16 h. The analysis of the GI and colon digested fractions showed that the antioxidant activity was enhanced after the colon phase compared to the gastrointestinal phase. The TPC was increased by 5-fold for green and black teas and 9-fold for white tea in colon stage compared to the duodenal stage. This elevating in the antioxidant activity was explained by the activity of the gut microbiota which metabolize tea polyphenols and generate metabolites possess greater antioxidant activity [56].

Limited number of studies (only two studies) have been carried out applying an *in vitro* dialyzability approach [422, 423]. Although these two studies were developed to evaluate the iron bioavailability and its effect on the antioxidant activity of tea polyphenols, the results obtained may provide a good idea about the interactions of dietary tea polyphenols with iron during the GID and its effects on the bioactivity of tea polyphenols. Both studies applied a similar *in vitro* protocol regarding gastric and intestinal solutions. However, Alexandropoulou

et al. [47] studied the total antioxidant capacity of dialyzates (in term of FRAP and TPC (using Folin–Ciocalteu reagent) assays) after GID of mixtures of green tea and dietary iron, ascorbic acid, meat and casein using dialysis membranes of 6000 – 8000 Da MWCO. On the other hand, Matsingou et al. [423] studied the antioxidant activity of black tea dialyzates in the presence of dietary iron using dialysis membranes of 14 kDa. Both studies reported a significant decrease in the antioxidant capacities of tea polyphenols in the presence of iron.

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OBJECTIVES

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Objectives

The popularity of tea as a second most consumed drink in the world raises the attention to investigate its dietary beneficial properties and increases the awareness of any possible risk to the human health.

Therefore, the present Doctoral thesis aims to:

1. Develop an analytical method for phthalate esters determination in several tea infusions using solid phase extraction with molecularly imprinted polymers (MIPSPE) and HPLC-MS.

2. Synthesize a molecularly imprinted polymer for BPA preconcentration from tea infusion samples.

3. Develop an analytical method for bisphenol A determination in several tea infusions by MIPSPE-HPLC-MS

4. Evaluate the percentage of migration of phthalates and bisphenol A from the tea bags' materials to tea infusions.

5. Determine the total element concentration in tea leaves and their infusions and to evaluate the bioavailability of these elements from tea infusion using an *in vitro* gastrointestinal digestion model.

6. Evaluate the total content of tea antioxidants and to assess their bioavailability for the human.

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CHAPTER 3

Development of a sensitive method for the analysis of four phthalates in tea samples: tea bag contribution to the total amount in tea infusion

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Development of a sensitive method for the analysis of four phthalates in tea samples: tea bag contribution to the total amount in tea infusion

3.1 Abstract

A sensitive, precise and selective method for the analysis of butyl benzyl phthalate (BBP), diethyl phthalate (DEP), dibutyl phthalate (DBP), and dimethyl phthalate (DMP) in tea samples has been applied. Molecularly Imprinted Polymer-Solid Phase Extraction (MIP-SPE) has been used for the separation and preconcentration of these compounds. Phthalates extracted by SPE were analyzed by high-performance liquid chromatography-electrospray ionization-mass spectrometry (HPLC-ESI-MS). The method was sensitive (LOD < 2 μg/L), precise (RSD <10%) and accurate with recovery percentages ranging from 84% to 97%. Finally, the developed method was applied for the analysis of these phthalates in several tea samples marketed in bags. Migration studies were also performed to evaluate the concentration of phthalates released from the bags into the infusions, and teabag filters were analyzed by Fourier-transform infrared spectroscopy. The migration study shows that tea filter bags contribute to the total phthalate's concentration in tea infusion, and this contribution varies between 1.8 to 93.5 % of the total phthalates' concentrations. Tea filter bags release higher DBP than BBP, DMP, and DEP.

3.2 Introduction

Phthalates are a class of phthalic acid esters used as plasticizers to make the high molecular weight plastics more flexible. Di(2-ethylhexyl) phthalate (DEHP) and butyl benzyl phthalate (BBP) are considered the most abundant phthalates in the environment because of their extensive usage in industrial applications. Due to the stability, fluidity and low volatility of higher molecular weight phthalates, they are widely used as plasticizers in several industrial products such as

insecticides, pesticides, cosmetics, paints, lubricants, children's toys, medical devices and personal care products [1].

The interest of studying phthalates comes from the fact that they pose numerous risks to human health, specifically to the reproductive, endocrine and respiratory systems leading to serious issues especially in children such as birth defects and obesity [2]. Phthalates are used extensively in the plastic industry, and since they are not covalently bound to plastic, they can be liberated from plastic containers into foods and drinks [3, 4].

The Chemical Agency of the European Union (ECHA) declares that BBP, DBP (dibutyl phthalate) and DEHP are being classified as very highly concerned reproductive toxicants and not to be used without specific authorization. The European Commission established the specific migration limits (SML) for certain phthalates (COMMISSION REGULATION (EU) No 10/2011), taking into account the tolerable daily intakes (TDIs) established by The European Food Safety Authority (EFSA). According to EFSA, DBP concentrations higher than or equivalent to 300 μg/kg (SML) were considered high, while concentrations between zero and 50 μg/kg of food were considered low (migration at this level poses low potential for exposure); however, concentrations above 50 and below 300 μg/kg were designated as medium levels [5–7].

Analysis and detection of phthalates in real samples in a routine analysis laboratory usually require a rapid, sensitive and reliable technique. The measurement of phthalate concentrations is commonly performed by HPLC or gas chromatography (GC) in combination with different detectors. HPLC may be coupled to ultraviolet UV [8, 9] Mass -Spectroscopy MS, or MS/MS [10, 11] while GC is coupled to electron capture detector ECD [12], flame ionization detector FID [13] and MS [14–16] or MS/MS [17]. HPLC may be the best choice because analysis by GC requires a derivatization step prior to the analysis.

Extremely low levels of phthalates in some samples require a preconcentration step before the analysis. Several analytical methods have been used to preconcentrate these compounds, including solid-phase extraction (SPE) [9, 18, 19], solid-phase micro-extraction

(SPME) [20, 21], micro-dispersive solid phase extraction (μ-dSPE) [10,22], liquid-liquid extraction (LLE) [23–25], dispersive liquid-liquid extraction (DLLME) [26], the quick, easy, cheap, effective, rugged, and safe (QuEChERS) methods [27] and stir bar sorptive extraction SBSE [28, 29].

There is no single optimal sorbent for all analytes; therefore, the type of sorbent chosen depends on the nature of the analyte, functional groups, the polarity of the sorbent and the analyte. Several sorbents can be used for SPE; one of these specific sorbents is the Molecular Imprinted Polymer (MIP). MIPs are widely used in SPE due to their high selectivity, excellent stability to the extreme pH, organic solvents, and temperature, which improves the separation of the analytical technique [30, 31]. MIP sorbents have been prepared using different phthalate compounds as templates. Jin et al. [32] synthesized a MIP using di-n-octylphthalate (DOP) as a template to preconcentrate and determine the DOP, DBP, and DMP in bottled beverages. Shaikh et al. [33] synthesized a MIP using di(2-ethylhexyl) phthalate DEHP as a template to preconcentrate DEHP from an aqueous sample. Yan et al. [34] prepared an imprinted microsphere of diisononyl phthalate (DiNP) as a dummy template for determining DEP, DBP, BBP, diisooctyl phthalate (DiOP), and DOP from plastic bottled beverages. Barciela-Alonso et al. [35] synthesized a MIP using DBP as a template for phthalate determination in water and wine samples. Jiaojiao et al. [16] used a MIP based on carbon nanotubes for a selective determination of DOP from beverage samples.

Cross-contamination is the main inconvenience to determine phthalate esters, which may interfere with the pretreatment steps. This contamination problem can be minimized using protocols reported in the literature [36, 37].

In some countries, tea is usually commercialized in tea filter bags made of materials such as paper and a variety of plastics. These materials may contain phthalates that can be released into the solution during the preparation of the tea at high temperatures, that may pose risk for human health.

Being the second most consumed beverage in the world, tea has been received more attention regarding the presence of health risky

substances such as phthalates. Moreover, the use of synthetic materials as filter bags for packaging the leaves increases the need to investigate the additive contribution of these materials when migrated into the tea infusion. Tea samples investigated here were selected as the frequently consumed types by the population including *C. Sinensis* (green and black teas) and non-*C. Sinensis* varieties (verbena leaves, red fruits and red rooibos teas). Since phthalates are expected to presents in very low concentration in tea infusion, the present work aims to developed a method for phthalate preconcentration (BBP, DBP, DMP, and DEP) from the infusions by SPE using a selective MIP as a sorbent. These phthalates were selected based on their solubility in water. A sensitive analytical method using a combination of high-performance liquid chromatography and mass spectrometry was validated and applied for the detection of these phthalates. An FT-IR scanning study was also performed to study the composition of the filter bags used for packaging the tea leaves.

3.3 MATERIALS AND METHODS

3.3.1 Reagents and standards

Dibutyl phthalate (DBP) and Diethyl phthalate (DEP) were purchased from Riedel-de Haën (Seelze, Germany). Butyl benzyl phthalate (BBP) and Dimethyl phthalate (DMP) were obtained from Supelco (Bellefonte, PA, USA). The purity of these reagents was over 98%. Ultrapure (resi-analyzed) water was obtained from Fluka (Steinhelm, Germany). Methanol and Lichrosolv gradient grade acetonitrile from Merck (Darmstadt, Germany). Technical-grade acetone and glacial acetic acid were purchased from Panreac (Barcelona, Spain).

Stock standard solutions of 1000 mg/L of each phthalate ester were prepared in methanol. A working standard solution containing all phthalates at a concentration of 100 mg/L in methanol was prepared from these solutions. The latter working standard solution was used to prepare the daily standard solutions by suitable dilution with methanol.

To remove the possible cross-contamination, all glassware was washed before the analysis considering the recommendations stated in U.S. EPA Method 506 [37]. This protocol included a first washing step with hot water and soap. The materials were then rinsed with tap water followed by ultrapure water. Finally, the clean glassware was rinsed with acetone, coated with aluminium foil and stored.

3.3.2 Instrumentation

A Series 1100 liquid chromatography from Agilent Technologies (Waldbronn, Germany) was used for phthalates separation. These compounds were analyzed using an API 150EX single quadrupole Mass Spectrometer (Applied Biosystems, Concord, Canada) equipped with a Turbo Ionspray™ ionization source operated in positive ion mode. A Zorbax Eclipse XDB-C8 column (3.5 μm, 2.1 mm×50 mm) from Agilent Technologies was used for phthalates separation. Analyst Software 1.4.2 (Applied Biosystems) was used for data acquisition and processing. The polymerization was carried out using a Boxcult incubator situated on a Rotabit orbital-rocking platform shaker (J.P. Selecta, Barcelona, Spain). The SPE was carried out in column mode using a peristaltic pump (Perkin Elmer, Norwalk, Connecticut, USA). Varian Agilent 670-IR Fourier Transform Infrared (FTIR) instrument was used to study the composition of teabags.

3.3.3 Molecular printed Polymer-Solid Phase Extraction procedure

Phthalates were separated and preconcentrated from the tea infusion using the MIP-SPE procedure. MIPs were synthesized by precipitation polymerization using DBP as a template, according to previous studies performed in our research group [35].

A MIP cartridge was prepared by introducing 200 mg of MIP in a glass syringe between two Teflon discs. The cartridge was preconditioned by washing the MIP sorbent with 15 mL methanol following by 15 mL of Milli-Q® water. 25mL of tea sample was then passed through the column (Flow rate of 1 mL/min), followed by a washing step using 1 mL of methanol: acetonitrile (1:1, v/v). The retained analytes were eluted with methanol (3 mL) and analyzed by

LC-ESI-MS. The preconcentration factor obtained using this procedure was 8.3.

3.3.4 Chromatographic and mass spectrometry conditions

The chromatographic conditions were optimized in previous work [36]. Phthalates were separated using a ZORBAX Eclipse XDB-C8 column (2.1 mm × 50 mm, and particle size of 3.5µm). A volume of 10 µL of the sample was injected into the column. The compounds were then separated using acetonitrile and ultrapure water, both containing 0.1% (v/v) acetic acid, as a mobile phase (flow rate of 200 µL/min). Separation in the chromatographic column was performed using gradient mode beginning with 5% acetonitrile which increased by 5 min to 75%. The composition remains the same for the next 8 min, followed by column equilibration for more 10 minutes using 5% acetonitrile.

Positive ion mode with a needle voltage of 5500V was used for electrospray ionization. Nitrogen gas as a nebulizer and a curtain gas was set at a pressure of 14 psi. The optimal conditions of the LC-ESI-MS are shown in **Table 3.1.**

Table 3.1. LC-ESI-MS parameters

Analyte	Acronym	*m/z* ratio	RT (min)	Potential (V)		
				DP	FP	EP
Butyl benzyl phthalate	BBP	91.15	10.1	25	225	6
Diethyl phthalate	DEP	149.05	8.12	25	290	8.5
Dibutyl phthalate	DBP	149.05	10.4	25	290	8.5
Dimethyl phthalate	DMP	163.25	7.3	40.38	73.87	8

Nebulizer and curtain gas (N_2): 14 psi
Heater gas: 7,000 cm^3/min
ES temperature: 450ºC
Ionspray voltage: 5500 V, Mode: positive
DP: declustering potential, *FP:* focusing potential, *EP:* enhance potential

3.4 ANALYSIS OF TEA SAMPLES

The method was applied to determine BMP, BBP, DEP and DBP in nine tea samples marketed in bags acquired from local supermarkets. The tea samples infusions were prepared according to the manufacturers' instructions by soaking them for 5 min in 150 mL of

boiled ultrapure water inside a glass beaker pre-cleaned according to the U.S. EPA Method 506 mentioned earlier. The infusions were then cooled to room temperature and filtered (0.45 µm cellulose acetate syringe filters) to remove any suspended materials. The infusions were then subjected to the optimized SPE protocol in duplicate. The analysis of phthalates in the extracts was carried out by LC-MS in triplicate. Standard addition calibration methods were used in concentrations ranging from 10 to 500 µg/L, for all compounds studied. A tea sample extract collected after applying the SPE protocol was used to prepare the calibration.

Contamination is one of the main problems that may affect the determination of phthalates. Since there is no way to get zero method blanks to analyze phthalates, the contamination level was minimized by using high-quality solvents as well as a strict glassware cleaning protocol using ultrapure water and acetone. Moreover, blank samples were also analyzed using the optimized SPE protocol and the results obtained for the blanks were subtracted to correct experimental values for the samples.

3.5 FT-IR ANALYSIS OF TEA BAGS

Tea bag materials were analyzed using an Infrared spectrometer to determine their chemical composition. The analysis was conducted using Varian 670 FT-IR spectrometer and the IR spectra were registered between 4000 and 400 cm^{-1}.

3.6 RESULTS AND DISCUSSION

MIP-SPE combined with LC-ESI-MS was applied for the analysis of four phthalates (DMP, DEP, BBP, and DBP) in tea samples. The conditions used for the MIP-SPE procedure were developed in a previous work performed in our laboratory [35] and described in the section "*Molecular imprinted polymer-solid Phase extraction procedure*". The working conditions for LC-ESI-MS determination are described in the section "*Chromatographic and mass spectrometry conditions*".

- Calibration

External calibration in methanol and standard addition calibration methods (10 to 500 µg/L) were used to evaluate the influence of the sample matrix on the sensitivity of the method. As can be seen in **Table 3.2**, both calibration graphs showed good linearity, with correlation coefficients (r) higher than 0.9972 for all the compounds studied. The slopes of both calibrations were examined using a t-test (95% significance level) [38] and significant differences were found for BBP, DEP and DMP. Because of the matrix effect observed, standard addition method was used for the analysis of these compounds in tea samples.

- Sensitivity

The instrumental limit of detection (LOD) and limit of quantitation (LOQ) were calculated using ten injections of a blank sample extract. The blank sample extract was obtained using ultrapure water as a sample applying the MIP-SPE procedure. LODs and LOQs considering the sample preparation (preconcentration factor: 8.3) were also calculated. The results obtained are shown in **Table 3.2.** The LODs obtained were between 0.23 and 1.18 µg/L. The LODs obtained in this study for BBP, DEP, and DBP are almost similar or even lower than those found in other previous works. Yan et al. [34] determined these phthalates in plastic bottled beverages by GC-FID obtaining LODs of 0.85, 1.18, and 1.01 µg/L for BBP, DEP, and DBP, respectively. Bai et al. [32] determined DBP and DEP in bottled beverages by HPLC-UV where LODs for DEP and DBP were 8 and 12 µg/L, respectively. Yin et al. [39] also determined the concentration of 16 phthalates in tea samples using a modified QuEChERS method and the LODs were found to be 0.6- 36.0 µg/ kg. Recently, Du et al. [40] used simultaneous distillation extraction (SDE) coupled with GC-MS to determine the concentration of five phthalates in teas extracts (using water-hexane mixture) and tea infusions with limits of detection in

the range of 0.24–3.72 µg/kg (2.4-37.2 µg/L, calculated taking into account the procedure described in this work). However, our LODs are higher than those obtained by Jiao-Jiao Zhang et al. [41] for the determination of six phthalates (DMP, DEP, DBP, BBP, DEHP and DOP) in herbal tea using SPE combined with dispersive liquid-liquid microextraction (DLLME) where the detection limits were between 0.02 and 0.05 ng/mL. LODs obtained in the present study can be improved by conducting the SPE procedure using larger volumes of tea samples.

- Precision and recovery

The precision of the method in terms of reproducibility and the repeatability was evaluated. The repeatability was evaluated using an extract of tea samples' mixture spiked with 25 µg/L before applying the SPE procedure and analyzed 12 times by LC-ESI-MS. The relative standard deviations (RSD, in percentage) obtained were around 3.5% for all the compounds. The reproducibility was studied using a mixture of tea samples spiked with 25 µg/L of BBP, DEP, DBP and DMP. The SPE protocol was applied for six aliquots of this sample. The extracts collected from the SPE were analyzed in triplicate by LC-ESI-MS. The RSDs calculated were 8.7, 8.8, 9.9 and 10.3 % for BBP, DEP, DBP and DMP, respectively. Based on the results obtained, we can conclude that the method is precise.

The accuracy of the method was evaluated in terms of analytical recovery. Accordingly, a mixture of tea samples spiked with two different concentration levels of phthalates (12 and 36 µg/L) was extracted using the optimized SPE in triplicate, and then analyzed by LC-ESI-MS in duplicate.

Table 3.2 shows the recovery percentages for each concentration level as well as the mean recoveries. The mean recoveries percentages were in a range from 87% (DEP) to 97 % (DBP).

3.7 STUDY OF PHTHALATE LEVELS IN TEA SAMPLES

The proposed analytical method was applied to assess the presence of the studied phthalates in nine commercial tea samples and determine their concentrations. Samples (3 black tea, 3 green tea, 2 red teas and one verbena leaves) of different brands were acquired in local marked in Spain and the information of each sample are summarize in **Table 3.3**. All these tea samples were commercialized in tea bags and the chemical composition of the tea bags was studied by FTIR spectroscopic analysis. Bio-Rad library was used to compare the spectra and to calculate the Hit Quality Index (HQI). The FT-IR analysis of the bags summarized in **Table 3.3** reveals that the main components of the bags are either cellulose-based compounds, polyester materials, poly (ethylene terephthalate) (PET) or polylactic acid (PLA)-based compounds. **Figure 3.1** shows the spectra obtained by FTIR analysis of tea bags 3, 6 and 7 related to PLA, PET and cellulose-based compounds, respectively.

Table 3.2. Slopes and correlation coefficients using external calibration and the standard addition method in extracts of tea samples, LODs and LOQs (µg/L) obtained for BBP, DEP, DBP and DMP in tea samples, and analytical recovery % [Mean ± SD, (n=3)].

Compound	*Direct Calibration*		*Addition Calibration*		*Sensitivity*				*Analytical Recovery (%)*		
	Slope	R	Slope	R	LOD[a]	LOQ[a]	LOD[b]	LOQ[b]	12 µg/L	36 µg/L	Mean Recovery
BBP	14359	0.9986	21228	0.9972	1.88	6.28	0.23	0.75	100 ± 5	89 ± 5	94 ± 8
DEP	4224	0.9991	5823	0.9993	13.90	46.34	1.18	3.95	94 ± 7	80 ± 5	87 ± 9
DBP	14646	0.9999	13577	0.9998	7.67	25.56	0.92	3.07	103 ± 5	90 ± 1	97 ± 5
DMP	7713	0.9998	9438	0.9991	5.04	16.79	0.6	2.0	95 ± 7	92 ± 1	93 ± 3

a: LOD and LOQ (instrumental), b: LOD and LOQ (referred to the sample), R: correlation Coefficient.

Table 3.3. Major contents of tea-bags analyzed by FT-IR

Sample	Type	Origin	IR-Prediction	Hit Quality Index (%)
1	Black tea with bergamot flavor	Sri Lanka	α-Cellulose	99.7
			Cellulose-N,N-diethylamonioethyl ether	99.1
2	Green tea with ginger and cardamom flavors	India	Polylactic acid (PLA) triethoxysilane	96.5
			α-hydroxy-ω-vinyl ether Polyethylene glycol-block-PLA	96.4
3	Green tea with mint flavoring	China	PLA triethoxysilane	96.6
			α-hydroxy-ω-vinyl ether PEG-block-PLA	96.5
4	Red fruits	UK	α-Cellulose	99.2
			Cellulose	99
5	Verbena leaves	EU	Poly (ethylene terephthalate)	99.1
			Poly ester film	96.8
6	Black tea with lemon flavoring	EU	Poly (ethylene terephthalate)	98.2
			Poly ester film	96.6
7	Red rooibos	South Africa	Cellulose 100 micron	99.2
			α-Cellulose	99
8	Green tea with mint flavoring	Sri Lanka	Cellulose 50 micron	99.2
			α-Cellulose	99.2
9	Black tea	Sri Lanka	α-Cellulose	99.2
			Cellulose 100 Micron	99.1

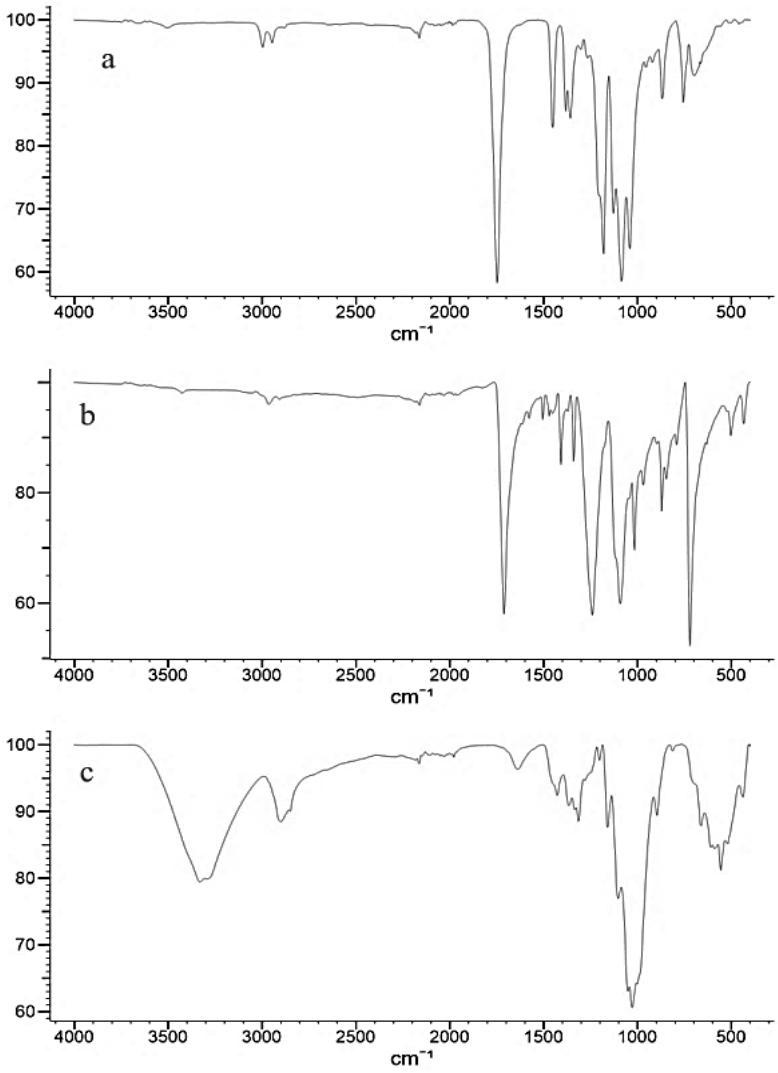

Figure 3.1 IR spectra; a) tea bag 3 containing PLA-based compounds, b) tea bag 6 containing PET-based compounds and c) tea bag 7 containing cellulose containing compounds

Tea infusions were prepared according to the manufacturers' instructions by soaking them for 5 min in 150 mL of boiled ultrapure water and aliquots of tea infusion were preconcentrated in duplicate using the optimized SPE protocol. The extracts were injected in triplicate to the LC-ESI-MS. **Table 3.4** shows the concentration of the targeted phthalates in tea infusions. **Figure 3.2** shows the chromatogram obtained for tea sample 1. Our findings indicate that DEP levels in the tea samples were lower than the LOD for all samples analyzed. DBP was the compound present at the highest concentrations in all the infusions studied, with concentrations ranging from 32.7 to 562.2 μg/L. BBP was detected in tea samples 1 and 5 with concentrations of 0.44 and 0.62 μg/L, respectively. DMP was detected in tea samples 1, 2 and 7 (concentrations ranging from 3.1 to 51.9 μg/L). The presence of these compounds in tea infusions could be due to the release from the materials of the tea bags in the soaking process. Thus, an additional study was performed to assess the contribution of the filter bags used for packaging the tea leaves.

3.8 MIGRATION STUDY

The migration of phthalates from the tea bags during the soaking process was also evaluated. This study was carried out using the same sample preparation procedure for the teabag itself. Therefore, the bag was soaked in 150 mL of boiled ultrapure water for 5 min, and then 25 mL of the cold bag infusion was filtered and delivered to the SPE column. The results of the analysis of phthalates in the infusions of tea bags (infusion of bag without tea) are shown in **Table 3.4**. DEP was not detected in any of the infusion of tea bags analyzed. BBP was only detected in samples 1 and 5 in concentrations of 0.4 and 0.22 μg/L, respectively. DBP was detected in all the infusions of tea bags in a concentration range from 12.6 to 51.7 μg/L. Finally, DMP was detected in samples 1, 2 and 7 with concentrations of 1.3, 2.4 and 2.9 μg/L, respectively. Results indicate that a fraction of phthalates found in the infusion was released from the bag.

Table 3.4. Phthalate concentrations (µg/L) ± SD (n=3) found in different tea samples (bags included) and tea bags infusions as well as the released percentage (%) of PAEs from tea bag to the infusion.

Sample	[BBP]			[DBP]			DMP		
	Tea	Bag	% Released	Tea	Bag	% Released	Tea	Bag	% Released
1	0.44 ± 0.3	0.4 ± 0.2	90.9	86.4 ± 42	49.7 ± 2	57.5	15.0 ± 1	1.3 ± 0.1	8.7
2	< LOD	< LOD	-	58.9 ± 1	51.7 ± 1	87.7	51.9 ± 2	2.4 ± 0.1	4.6
3	< LOD	< LOD	-	244.78 ± 51	27.9 ± 0.1	11.4	< LOD	< LOD	-
4	< LOD	< LOD	-	562.2 ± 23	10.0 ± 0.2	1.8	< LOD	< LOD	-
5	0.62 ± 0.1	0.22 ± 0.1	35.4	43.9 ± 6	22.8 ± 1	51.9	< LOD	< LOD	-
6	< LOD	< LOD	-	42.4 ± 3	12.6 ± 0.2	29.6	< LOD	< LOD	-
7	< LOD	< LOD	-	48.4 ± 20	13.6 ± 0.1	28.1	3.1 ± 0.4	2.9 ± 0.3	93.5
8	< LOD	< LOD	-	32.7 ± 5	13.0 ± 0.8	39.8	< LOD	< LOD	-
9	< LOD	< LOD	-	37.2 ± 10	21.5 ± 0.1	57.8	< LOD	< LOD	-

CBC: Cellulose-Based Compound, PLA-BC: Polylactic Acid-Based Compound, PET-BC: Poly (ethylene terephthalate)-Based Compound.

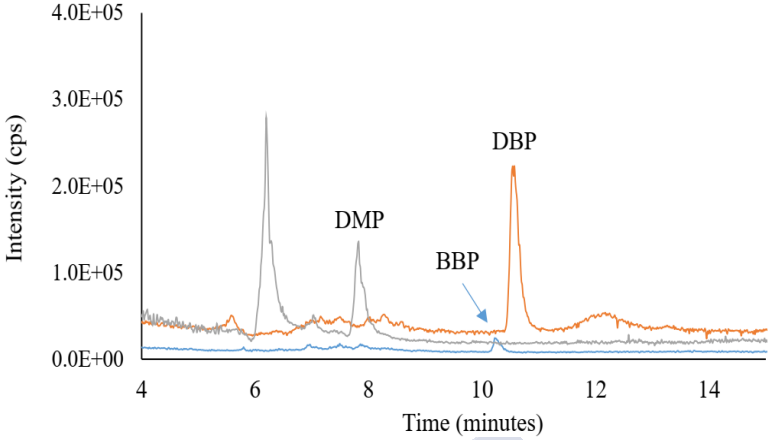

Figure 3.2 LC-ESI-MS ion chromatogram obtained for the extract of tea sample 1.

The released percentages (%) of phthalates, calculated as the ratio of phthalate concentrations in the tea bag infusion to that in the tea infusion (**Table 3.4**) varied from 1.8 to 93.5%. These results show a high phthalate release from the bags into the tea infusion of samples 1, 2, 5 and 7. DBP shows the highest contribution of the total concentration of phthalates among the compounds studied in the tea infusions.

The results show that all the tea bag materials released DBP. The highest amount of DBP released from the filter bag was found in sample 2 (87.7%) where the bag was made of polylactic acid-based compounds (according to the IR analysis). Moderate contribution of the bag was obtained in the case of tea sample 1 and 5 – 9 in the range of 28.1 – 57.8%. On the other hand, tea filter-bags 3 (11.4 %) and 4 (1.8%) have shown a low contribution to the total DBP released to their tea infusions where the filter bag was made of PLA-based compounds and cellulose-based compounds, respectively. BBP was only detected at low concentrations in sample 1 ad 5, with tea bags based on cellulose and Poly (ethylene terephthalate) compounds, respectively. DMP was released from the tea bag of samples 1, 2 and 7, with bag compositions based on cellulose (samples 1 and 7), polylactic acid (sample 2). Low contribution of DMP released from the tea bag 1 and 2 as 8.7 and 4.6%, respectively, while the major

DMP released to the infusion 7 comes from the filter bag itself (93.5%). Considering the results obtained, we cannot find a direct relationship between the migration percentage of phthalates from tea bags with the composition of these bags. The contamination of teabag filters with phthalates can occur during the production of the bags because of using contaminated materials or even storage and transporting stages.

On the other hand, migration study indicated that a fraction of phthalates comes from the tea leaves themselves, especially if one considers the small fraction of some phthalates found in the infusions prepared with bags themselves. Mohammad et al. [26] analyzed phthalates in three tea bag infusions (black, green and white teas) and their respective bags using GC-MS. The authors reported that phthalate levels in tea-free bags were not detectable. Alhough, the findings showed in the present study revealed that tea-free bags contributes to the total phthalates´ amounts in the tea infusion, however, the main source of some phthalates is tea leaves. Tea leaves may be contaminated by accumulating phthalates from the air, water and soil [42] or during production lines [26]. Moreover, according to previous studies reported in the literature, the presence of phthalates in the tea infusions could be due to essential oil, synthetic essences, colorants and flavours added in the production lines [43, 44].

3.9 RISK ASSESSMENT

The risk of health effects from phthalate intake from daily tea consumption was evaluated as previously reported by Troisi et al. [45]. The estimated average daily intake (EADI) of phthalates in tea samples was calculated considering Spanish tea consumption of 114 mL/day (Caini et al. 2019) while the body weight was established as 70 kg. The EADI obtained was compared with their reference dose levels (RfD derived by the US Environmental Protection Agency (USEPA) or US Agency for Toxic Substances & Disease Registry (ATSDR), which are given as follows: BBP of 0.2, DBP of 0.1, DMP of 0.2, and DEP of 0.8 mg/kg bw/day from RfD chronic exposure [46]. The hazard quotient (HQ) given as (HQ = EADI/RfD) obtained in this study were below 1, which indicates that the daily intake of tea

with these phthalates´ concentrations do not pose risk for human health.

3.10 Conclusion

A MIP-SPE method coupled to LC-ESI-MS was developed for the determination of BBP, DEP, DBP, and DMP in tea samples. The method established is fast, selective, sensitive, and precise. The advantage of using MIP as a sorbent is the high selectivity for the analytes of interest. The LODs obtained were in the same order of or even better than those found in the literature. The pre-treatment procedure was designed to minimize the cross-contamination problems during the analysis of these compounds.

Nine commercial tea samples were analyzed using the proposed method. DBP was detected in all the samples analyzed, DEP was not detected while BBP and DMP were detected in two and three of the nine samples studied, respectively. The migration studies conducted for these compounds demonstrate that a fraction of phthalates found in the infusion was released from the tea bag (Migration percentages ranged from 1.8 to 92.6%). The IR study reveals that the tea bags were mainly based on cellulose-based material, polylactic acid and poly (ethylene terephthalate). There was no relation between the migration percentage of phthalates from tea bags with the composition of these bags. The presence of phthalates in the teabag infusions could be due to the presence of minor portions of the materials used in the manufacturing of the bags and to other compounds (essential oil, synthetic essences, colorants and flavours) used in the tea production lines. Although the phthalates´ concentrations found in this study not pose a risk for human health, the control of the materials used to manufacture these filter bags and the tea is necessary to avoid exposure to these toxic compounds and reduce the risk for human health.

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CHAPTER 4

Determination of Bisphenol A in Tea Samples by Solid Phase Extraction and Liquid Chromatography coupled to Mass Spectrometry

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Determination of Bisphenol A in Tea Samples by Solid Phase Extraction and Liquid Chromatography coupled to Mass Spectrometry

4.1 Abstract

A simple, fast, sensitive and selective method for the extraction of Bisphenol A (BPA) from tea samples has been developed. Due to the low concentration of the analyte, sample preparation using Solid Phase Extraction (SPE) with a Molecularly Imprinted Polymer (MIP) as a sorbent was used to preconcentrate BPA from samples. The MIP was synthesized by the precipitation polymerization using BPA as a template, methacrylic acid (MAA) as a monomer, ethylene glycol dimethacrylate (EDMA) as a crosslinking agent, 2,2´-azobisisobutyronitrile (AIBN) as an initiator, and acetonitrile as a solvent. The optimized SPE process was applied loading 100 mL of the sample through the MIP sorbent at a flow rate of 1.0 mL/min. The retained BPA was then eluted with 4 mL of methanol. Finally, the extract was analyzed by High-Performance Liquid Chromatography-Electrospray Ionization Coupled to a Mass Spectrometry (HPLC-ESI-MS) working in negative mode. The method showed good recovery and precision (% RSD of 7.3). The limits of detection and quantification were 0.072 µg/L and 0.24 µg/L, respectively. The optimized method was applied to BPA determination in several commercial tea samples.

4.2 Introduction

Bisphenol A (BPA) is an organic compound widely distributed in the environment because, the larger number of industrial applications such as, the manufacture of polycarbonate plastics and epoxy food-can coatings. Food and beverage may be expected to contain traces of BPA due to migration from packaging materials [1, 2]. BPA is extensively used in industry due to its hardness, resistance to acids, transparency, and its high strength across a wide range of temperatures (-40 – 145°C). It is used in the production of thermal

receipt paper [3], printed circuit boards, building materials, compact discs, medical devices and dental fillings [4]. European legislation has established 0.6 mg/kg as the migration limit for BPA in material intended to come into contact with food and drinks [5]. The tolerable daily intake (TDI) set by the European Union (EU) Commission is 0.05 mg BPA/kg body weight/day[6]. BPA is one of the most frequently detected endocrine disruptors in the environment [7]. Because of its high production-volume, extensive use, and endocrine disrupting and toxic properties, several analytical methods have been published regarding BPA determination in real samples. BPA has been analyzed mainly by high-performance liquid chromatography (HPLC) or gas chromatography (GC) coupled to a mass spectrometer (MS). A derivatization step is recommended for GC analysis to improve sensitivity in MS, which increases analysis time. HPLC has been used as an alternative technique in recent years [8,9]. HPLC coupled to different detectors, such as MS, MS/MS, UV detector, electrochemical detector (ED), or fluorescence detection has been used for BPA determination [4, 10–13]. Several sample preparation techniques have been reported in the literature for BPA separation and preconcentration. Solvent-based extraction procedures have been used to clean-up BPA from the sample matrix. These include Liquid-Liquid Extraction (LLE) [14, 15], Microwave-Assisted Extraction (MAE) [16, 17] and Pressurized Liquid Extraction (PLE) [18]. Solid-based extraction techniques have also been used for the same purpose. The C18 SPE procedure was used by Fasano et al. [12] to pre-concentrate BPA from soft drinks and other beverages (including canned tea) before analysis by HPLC-fluorescence and LC-MS/MS. Regueiro et al. [19] used an Oasis HLB cartridge for SPE of BPA and its analogues from alcoholic and non-alcoholic beverages (including tea samples) prior to analyzing by LC-MS/MS. A Molecularly Imprinted Polymer (MIP) is one of the most selective solid-based absorbents used in SPE protocol. Yang et al. [20] used an MIP provided by Polyintell (Val de Reuil, France) for BPA extraction from beverage samples (including tea) before determination by LC-MS. Wu et al. [10] synthesized MIP by precipitation polymerization for selective SPE of BPA from vegetables and juice samples. An MIP was

synthesized using BPA as a template, 4-vinylpyridine (4-VP) as a monomer, trimethylolpropane trimethacrylate (TRIM) as a cross-linker and AIBN as an initiator. Li Jin et al. [21] prepared a dummy surface molecularly imprinted polymer (DSMIP) by emulsion polymerization. DSMIP was prepared using Tetrabromobisphenol A (TBBPA) as a template, 4-VP as a monomer, EDMA as a crosslinking agent, and AIBN as an initiator. This DSMIP was used to extract BPA from drinks and fruits, and the enriched extract was analyzed by HPLC coupled to a UV detector. Zhang et al. [22] prepared a magnetic MIP based on multiwalled carbon nanotubes for magnetic extraction of BPA from water. Mei et al. [23] developed an analytical method to determine BPA in complex samples such as wastewater, soil, shrimps and human urine using a highly selective MISPE coupled to a highly effective capillary electrophoresis (CE). The MIP was prepared via precipitation polymerization using BPA as a template, and TRIM as a crosslinking monomer.

In the present study, a selective analytical method has been developed for BPA determination in commercial tea samples using MIP-SPE coupled to LC-ESI-MS.

4.3 Materials and Methods

4.3.1 Reagents

Bisphenol A (BPA) was obtained from Sigma-Aldrich (Steinheim, German); the purity was over 99%. 2,2'-azobisisobutyronitrile (AIBN) was purchased from Fluka (Steinheim, Germany). Methacrylic acid (MAA) 99%, and Ethylene glycol dimethacrylate (EDMA) 98% were purchased from Sigma Aldrich (St. Louis, USA).

All other reagents used were analytical reagent-grade. Ultrapure (re-analyzed) water was obtained from Fluka (Steinheim, Germany). Lichrosolv gradient grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Technical-grade acetone was purchased from Panreac (Barcelona, Spain), and ammonia from Scharlau (Barcelona, Spain).

Special attention was paid to avoid the contact of reagents and solvents with plastic materials. Glassware was cleaned with soap and hot water, followed by tap-water and ultrapure water, and finally rinsed with acetone to eliminate any potential background BPA.

A stock solution of BPA (1000 mg/L) was prepared in methanol, kept in an Amber Teflon-capped glass vial and stored at 4 °C. A standard working solution of 10 mg/L was prepared by stepwise dilution of this stock solution with methanol. Diluted working standard solutions were prepared daily by appropriate dilution of the 10 mg/L working solution with methanol for analysis.

4.3.2 Instrumentation

A High-Performance Liquid Chromatography (HPLC) Agilent 1100 Series with auto-sampler, quaternary pump system of solvent and a binary pump was coupled with an API 150EX™ single quadrupole Mass Spectrometer (Applied Biosystems, Concord, Canada) equipped with a Turbo IonsprayTM ionization source. Data acquisition and processing were performed using Analyst Software 1.4.2 (Applied Biosystems). A Zorbax Eclipse XDB-C8 column (3.5 µm, 2.1 mm×50 mm) from Agilent Technologies was used for the separation. A Boxcult incubator situated on a Rotabit orbital-rocking platform shaker (J.P. Selecta, Barcelona, Spain) was used for the polymerization process. A peristaltic pump (Perkin Elmer, Norwalk, Connecticut, USA) was used for the SPE procedure. Varian 670-IR Fourier Transform Infrared (FTIR) from Agilent was used for the analysis of tea bags.

4.3.3 Preparation of the MIPs

The MIP was prepared based on previous studies performed by Alenazi et al. [24] and Barciela-Alonso et al. [25] for phthalate determination in water and wine samples. In the current study, BPA was used as template and MMA as functional monomer. Thus, 1 mmol of BPA and 4 mmol of MMA were dissolved in 10 mL acetonitrile in a glass tube. The mixture was vortexed for a few minutes and placed in an ice bath at 0ºC for 30 min. Afterward, EDMA crosslinking agent (20 mL) and initiator AIBN (30 mg) were dissolved in 15 mL acetonitrile and added to the template mixture and

vortexed for a few minutes. The glass tube was then sealed under Ar stream and placed horizontally on a Roller shaker inside an incubation chamber that was programmed to attain a gently rotational motion of 30 rpm and a temperature of 60˚C for 48 h. Finally, the polymer was separated by vacuum filtration, and the template was removed by washing the polymer with 50 mL of methanol, followed by 50 mL of dichloromethane, 50 mL methanol, and 50 mL methanol/water (1:1, v/v). The washing procedure was repeated until no BPA trace was detected by LC-ESI-MS. A non-imprinted polymer was prepared according to the above-mentioned procedure but without using the template.

4.3.4 Tea sample preparation

Tea samples commercialized in tea bags were purchased from local markets in several countries, including Portugal, Spain, Jordan, India and Azerbaijan. Various samples types were: black tea (12 sample), green tea (5 samples), red tea (2 samples) and white tea (2 samples), where also sample 4, 5, and 7 were red fruits (RF), verbena leaves (VL) and red rooibos (RR) infusions; respectively. **Table 4.1** shows the origin and types of samples analyzed. The infusions were prepared, following manufacturer´s instructions, by soaking the teabags for 5 min in 150 mL of boiled water of LC-MS grade. The infusion was then cooled and filtered to remove any suspended materials.

4.3.5 FT-IR analysis

All the tea bags of the samples were analyzed using Infrared spectrometer to check their major contents. Infrared spectra of these bags were acquired using a Varian 670 FT-IR spectrometer with an optical element of diamond in the range from 4000 to 400 cm^{-1}. ATR-FTIR spectrometer was controlled by the Resolutions Pro software package. An overview of all analyzed samples is presented in **Table 4.1.**

4.3.6 Solid Phase Extraction using a Molecular Imprinted Polymer

BPA was preconcentrated from tea samples by MIP-SPE. A total of 200 mg of dry BPA-imprinted polymer was packed between two

Teflon frits into a glass cartridge. The cartridge was preconditioned by eluting 15 mL of methanol followed by 15 mL Milli-Q® water through the packed sorbent.

Afterward, 100 mL of tea infusion was passed through the conditioned cartridge at a flow rate of 1 mL/min, followed by 1mL of acetonitrile-methanol (1:1, v/v) to remove any possible impurities that could have been retained by the MIP. Finally, the retained BPA was eluted with 4 mL of methanol prior to analyzing by LC-ESI-MS. The SPE procedure was performed at a flow rate of 1.0 mL/min. The preconcentration factor was 25.

Table 4.1 Types and origins of analyzed samples.

Tea Sample	Type	Origin	Tea Sample	Type	Origin
1	Black	Sri Lanka and Kenya	13	Black	-
2	Green	India	14	Black	-
3	Green	China	15	Red	-
4	RF	UK	16	Green	Sri Lanka and Kenya
5	VL	EU	17	Black	China
6	Black	EU	18	Green	India
7	RR	South Africa	19	Black	Sri Lanka
8	Green	Sri Lanka	20	Black	Ceylon and Kenya
9	Black	Sri Lanka	21	Black	India
10	White	China	22	White	China
11	Red	China	23	Black	Sri Lanka
12	Black	India	24	Black	Sri Lanka

RF: red fruits, VL: verbena leaves, RR: red rooibos, UK: United Kingdom, EU: European Union.

Table 4.2 Major contents of tea-bags analyzed by FT-IR

Sample	Type	Prediction	Hit Quality Index (%)	Sample	Type	Prediction	Hit Quality Index (%)
1	Black	α-cellulose	99.7	**8**	Green	Cellulose 50 micron	99.2
		Cellulose-N,N-diethylamonioethyl ether	99.1			α-Cellulose	99.2
2	Green	PLA triethoxysilane	96.5	**9**	Black	α-cellulose	99.2
		α-hydroxy-ω-vinyl ether PEG-block-PLA	96.4			Cellulose 100 Micron	99.1
3	Green	PLA triethoxysilane	96.6	**10**	White	Cellulose	95.7
		α-hydroxy-ω-vinyl ether PEG-block-PLA	96.5			α-cellulose	94.8
4	RF	α-cellulose	99.2	**11**	Red	Cellulose composite	98.5
		Cellulose	99			Cellulose	94.1
5	VL	Poly (ethylene terephthalate)	99.1	**12**	Black	Cellulose (20 Micron)	94.7
		Poly ester film	96.8			Cellulose	94.6
6	Black	Poly (ethylene terephthalate)	98.2	**13**	Black	α-cellulose	99.3
		Poly ester film	96.6			Cellulose 100 Micron	99.2
7	RR	Cellulose 100 micron	99.2	**14**	Black	Cellulose	99.2
		α-cellulose	99			α-cellulose	99

Table 4.2. (Continued)

Sample	Type	Prediction	Hit Quality Index (%)	Sample	Type	Prediction	Hit Quality Index (%)
15	Red	α-cellulose	99.2	**20**	Black	α-cellulose	99.2
		Cellulose 50 Micron	98.9			Cellulose (50 Micron)	99.2
16	Green	α-cellulose	99.2	**21**	Black	Cellulose (20 Micron)	98.6
		Cellulose	99			Cellulose	98.4
17	Black	Cellulose	95.3	**22**	White	α-cellulose	98.5
		Cellulose (20 Micron)	94.7			Cellulose	98.3
18	Green	Cellulose (20 Micron)	97.3	**23**	Black	cellulose	95.4
		Cellulose	97.1			Cellulose (20 Micron)	94.7
19	Black	α-cellulose	99.3	**24**	Black	Cellulose	95.4
		Cellulose	99			Cellulose (20 Micron)	94.7

4.3.1 LC-MS analysis

The target analyte was separated by an Agilent Liquid Chromatography system. The analytical column was a Zorbax Eclipse XDB-C8 column (3.5 μm, 2.1 mm×50 mm). The mobile phase consisted of acetonitrile-ultrapure water (40:60) containing 0.8% (v/v) of 1.0 mM NH_4OH. The separation was performed using isocratic mode at a flow rate of 250 μL/min, with an analysis time of 6 min. The sample injection volume was 5 μL.

An API 150EX single quadrupole Mass Spectrometer was used to determine analyte concentrations. **Table 4.3** Shows the conditions for the ESI-MS operating in a negative-ion mode.

Table 4.3 ESI-MS instrumental parameters used for the BPA determination

Analyte	Acronym	m/z ratio	RT /min	Potential/V		
				DP	FP	EP
Bisphenol A	BPA	227	3.2	-195	-125	-9
Nebulizer and curtain gas (N_2): 12 psi						
Heater gas: 7.000 cm^3/min; ES temperature: 450ºC						
Ion spray voltage : -4500 V; Mode: negative						
DP: Declustering Potential FP: Focusing Potential EP: Enhance Potential						

RT: retention time, psi: pounds per square inch, V: volt.

4.4 RESULTS AND DISCUSSION

4.4.1 ESI-MS optimization

ESI-MS parameters including ion source potentials, nebulizer and curtain gas and temperature were optimized. The ion source potentials [Declustering potential (DP), focusing potential (FP) and enhance potential (EP)] were optimized automatically by infusing 500 μg/L of BPA solution directly into the MS. The obtained potentials were -125 V, -195 V, and -9 V for FP, DP, and EP, respectively. Nebulizer gas, curtain gas, and temperature were also studied, and the optimum conditions are shown in **Table 4.3.**

4.4.2 Optimization of chromatographic parameters

The chromatographic parameters of the mobile phase (i.e., the mobile phase composition, pH of the mobile phase, mode of elution and flow rate) were optimized after optimizing the detection conditions. A standard of 100 µg/L BPA was used to carry out the optimization of the chromatographic parameters. Starting with the mobile phase component, two mobile phases were prepared: 1) ultra-pure water-methanol; and 2) ultra-pure water-acetonitrile. The water: methanol mixture showed a bad analytical response where the signal-to-noise ratio was very low. However, the water-acetonitrile mobile phase showed better results, so it was selected for the study. After choosing the mobile phase, two different elution modes were evaluated for better analytical response: gradient and isocratic modes. Different compositions were tested for the mobile phase containing acetonitrile and water in gradient mode elution. **Table 4.4** shows one of the gradient composition tests used. The results obtained working in gradient mode show low sensitivity and bad peak shape. Therefore, isocratic mode using several varied compositions of the mobile phase were tested, starting with 10:90 of water-acetonitrile with increments of 5% of water. The best results were obtained using a composition of 40:60 of water-acetonitrile.

Table 4.4. HPLC gradient elution program

Time (min)	Flow rate (µL/min)	% Water	% Acetonitrile
0	200	90	10
3	200	50	50
7	200	25	75
18	200	25	75
21	200	90	10
28	200	90	10

The analysis was performed using negative mode, where ammonia was added to the mobile phase components. The influence of the ammonia concentration was evaluated, and a series of mobile phases were spiked with 1mM ammonia to get a final concentration ranging from 0.5 to 1.5% (v/v). The highest sensitivity was obtained with

0.8% of ammonia. The mobile phase flow rate was also evaluated. Two flow rates were studied: 200 and 250 µL/min. Changing the flow rate of the mobile phase leads to changes in the retention time but not in the sensitivity of the analysis, so the analysis was carried out using a flow rate of 250 µL/min to reduce the time of analysis. Finally, the analysis was performed using an isocratic mode at a flow rate of 250 µL/min for 6 min. The sample injection volume was 5 µL.

Figure 4.1 shows a chromatogram obtained for a 100 µg/L of BPA standard solution using the optimized parameters. Under these conditions the BPA retention time was 3.2 min. **Figure 4.2** shows the LC-MS chromatogram obtained for a tea extract spiked with 250 µg/L of BPA.

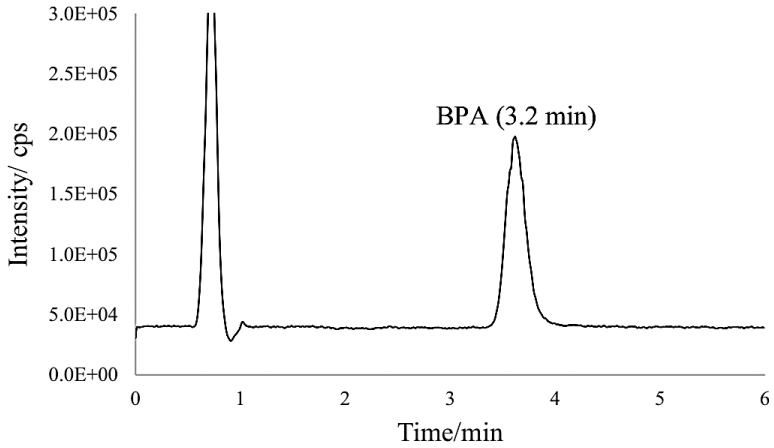

Figure 4.1 Chromatogram of 100 µg/L BPA analyzed by optimized LC-MS.

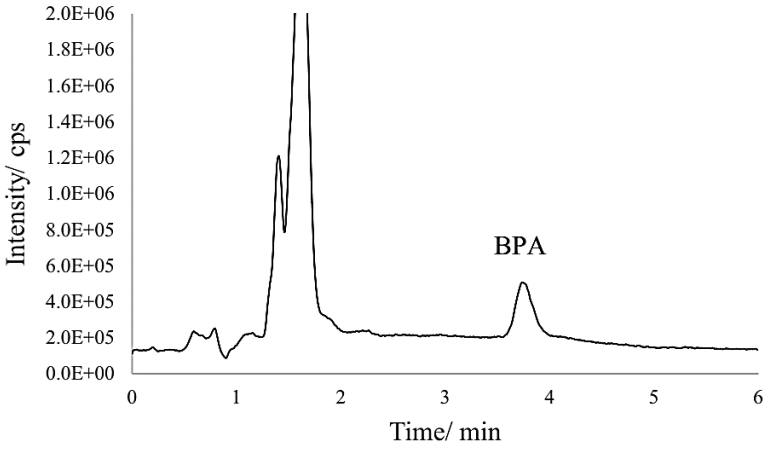

Figure 4.2 Tea extract spiked with 250 µg/L of BPA.

4.4.3 SPE optimization

The objective of the present work was to pre-concentrate BPA from tea samples by MISPE protocol to be analyzed by LC-ESI-MS. Previous studies reported the use of 4-VP as a functional monomer for synthesizing MIP sorbent for BPA extraction [21, 26, 27]. In the current study, the MIP was synthesized as explained in the "Preparation of the MIPs" section. The MIP was prepared, by precipitation polymerization, based on previous studies performed by Alenazi et al. [24] and Barciela-Alonso et al. [25] for phthalate determination in water and wine samples, using BPA as a template and MMA as a functional monomer. This polymerization method diminishes the number of steps necessary for MIP preparation compare with previous studies reported in the literature, using bulk polymerization [26, 29].

The SPE cartridge was prepared using a glass syringe which was filled with 200 mg of MIP or NIP sorbent packed between two Teflon frits. The SPE procedure followed four steps, starting with conditioning the absorbent by passing 15 mL methanol, followed by 15 mL of water.

Afterward, 100 mL of tea sample was loaded into the cartridge at a flow rate of 1 mL/min, followed by 1mL of acetonitrile: methanol (1:1) to remove any interferences. Finally, the retained BPA was

eluted using 4 mL of methanol. Parameters related to the SPE procedure, such as sample loading and elution solvent flow rate, solvent elution volume, and pH of the sample were evaluated using 100 µg/L BPA standard solution. This solution was subjected to the SPE procedure in triplicate at flow rates of 0.55, 1 and 1.35 mL/min. The extracts were then analyzed in duplicate by LC-ESI-MS and the peak areas were measured. The results obtained are shown in **Figure 4.3**. No significant differences (95% significance level) [28] of the analytical response were observed between the three flow rates, even though sample loading at a flow rate of 1.0 mL/min shows a higher analytical response. The sample loading flow rate was thus set at 1.0 mL/min.

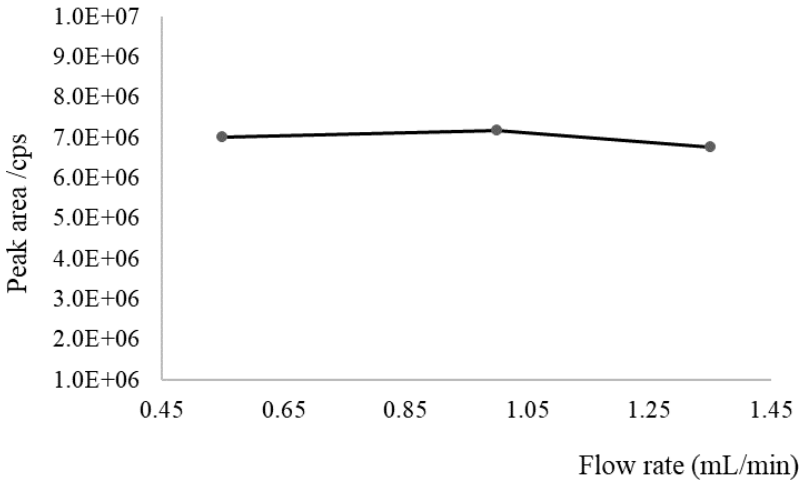

Figure 4.3 Influence of sample loading flow rate on the SPE procedure.

Figure 4.4 shows the results related to the elution flow rate where the analytical signal has no significant difference between the eluting flow rate of 1.0 and 1.35 mL/min, while it was lower when 0.55 mL/min was used. Taking into account these results and the reproducibility of the measurements, an elution flow rate of 1.0 mL/min was selected.

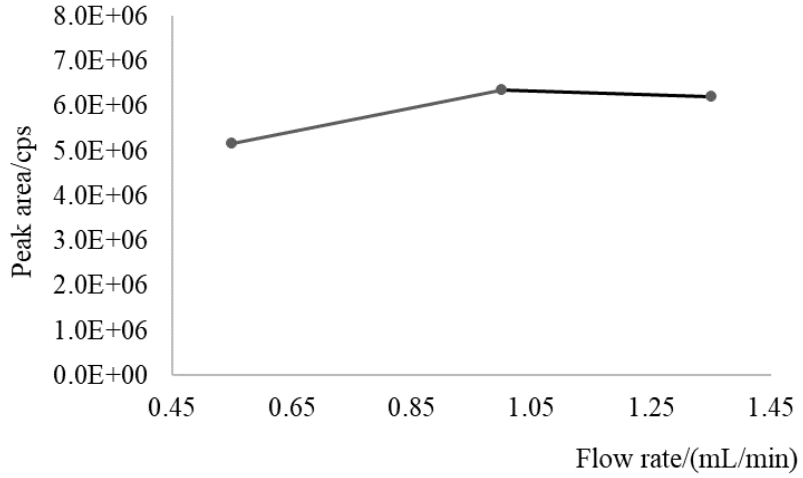

Figure 4.4 Influence of elution flow rate on the SPE procedure.

The influence of the sample pH on the SPE procedure was also evaluated. Four aliquots of a BPA solution (100 µg/L) with pH of 3, 5, 7 and 9 were used. The pH adjustment was made using diluted HCl and NaOH solutions. After adjusting the pH, all aliquots were subjected to the SPE procedure, and the extracts were analyzed in duplicate by LC-ESI-MS. Results obtained are shown in **Figure 4.5.** The highest values were obtained when the pH was 7. This value is the pH of the tea samples prepared in the laboratory. Therefore, the samples were preconcentrated without any pH-adjustment.

Methanol was used in this study for BPA elution; therefore, the elution volume was also studied. Several volumes of methanol ranging from 3 to 6 mL were evaluated. Results show that recovery improved up till 4 mL and then remained constant while increasing the volume of methanol. Thus, 4 mL of methanol was selected for BPA elution

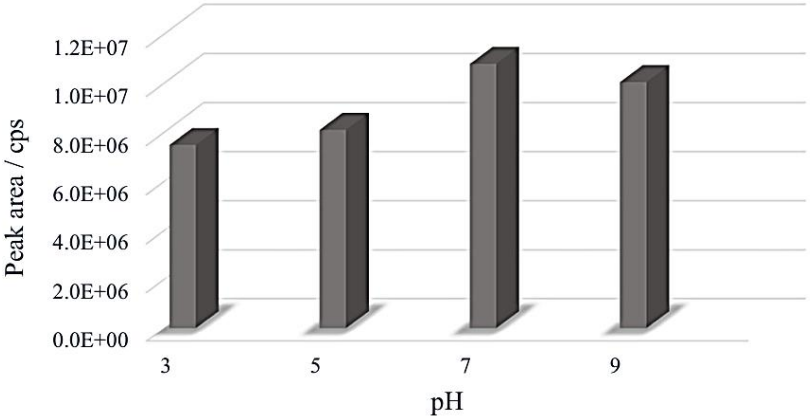

Figure 4.5 Influence of the pH on the SPE procedure.

4.4.4 Analytical Performance

- Calibration

The linearity of the method was examined using the direct calibration method at six concentration levels of BPA ranging from 0 to 500 µg/L prepared in methanol. Each concentration level was analyzed in triplicate, and a calibration curve was obtained by plotting the peak area against the concentration. The standard addition curve was also prepared at the same concentration levels using an extract of a pool of tea samples subjected to the SPE protocol. Both calibration methods show good linearity with a correlation coefficient (r) of 0.9997 and 0.9990 for direct and addition calibration. The equations obtained for each calibration are as follows:

Direct calibration: PA = 20401C + 35113
Addition method: PA = 31325C + 258757

Where PA is the peak area and C is the concentration in µg/L.

The slopes of direct and addition calibration were compared using a t-test (95% significance level). Results show a significant difference between slopes, which means that the sample matrix affects the sensitivity of the method. The standard addition method was thus used to determine BPA in tea samples.

- Sensitivity

The sensitivity of the method was determined by estimating the limit of detection (LOD) and limit of quantitation (LOQ). LOD was calculated by means of the standard deviation (SD) of a blank sample and the slope of the addition curve (m) as 3SD/m; while LOQ was calculated by 10SD/m. LOD and LOQ were estimated analysing twelve times by LC-ESI-MS an extract of a blank (ultrapure water) sample subjected to SPE. The instrumental LOD and LOQ obtained for BPA were 1.79 and 5.98 µg/L, respectively; while LOD and LOQ referred to the sample, taking into account the preconcentration factor (25), were 0.072 and 0.24 µg/L, respectively. The limits obtained in the present study were better than those obtained by Li, Jin [21] for determination of BPA in drinks such as green tea and herbal tea by DSMIP, where LOD was 3 µg/L. The LOD obtained in this study was also lower than the LOD obtained by San Vicente et al. [29] (1 ng/mL), for BPA determination in river water using a continuous MISPE, followed by subsequent analysis by HPLC with fluorescence detection.

- Precision

The precision of the method was evaluated by the repeatability and reproducibility, using a tea pool sample spiked with 1 µg/L of BPA solution. For the reproducibility study, six aliquots of the tea pool sample were subjected to the SPE procedure and analyzed in triplicate by LC-ESI-MS. The relative standard deviation (% RSD) obtained was 7.3%. The repeatability of the method was evaluated by analysing one of these extracts 12 times by LC-ESI-MS, obtaining an RSD of 5.7%. The RSD values obtained are below 10%, so it can be concluded that the method is precise.

- Analytical recovery

To evaluate the accuracy of the method regarding analytical recovery, aliquots of a tea pool sample were spiked with five concentration levels (1, 2, 4, 8 and 12 µg/L) of BPA. Afterward, the SPE procedure was carried out in triplicate, and each extract was analyzed in duplicate by LC-ESI-MS. The recoveries were 94, 97, 104, 103 and 108% for concentrations of 1, 2, 4, 8 and 12,

respectively. The method shows a good mean recovery of 101 ± 11 for BPA.

4.4.5 Analysis of BPA in tea samples

The optimized analytical method has been used to check the presence of BPA in 24 commercial tea samples. Tea infusions were prepared according with the procedure described in the section 2.4 (Tea sample preparation). Each tea sample was then extracted according to the optimized SPE procedure in triplicate using a volume of 100 mL, and each extract was analyzed by LC-ESI-MS in triplicate. The analysis shows that the concentration of BPA was lower than the LOD in 22 tea samples, while tea samples 4 (with flavored fruits infusion) and 14 (black tea) shows a detectable level of BPA with a concentration of 13.3 ± 0.3 and 2.1 ± 0.1 µg/L (tea samples acquired in Portugal and Spain, respectively). The presence of BPA in these samples could be attributed to the migration from the tea bags.

4.5 MIGRATION STUDY

Migration studies were carried out to assess the presence of BPA in the tea bags of samples that present high BPA levels. To know that, these two samples (samples 4 and 14) were used for further analysis by applying the same sample preparation process as well as LC-MS analysis for the tea-bag itself. The bag was soaked for 5 min in 150 mL of boiled water of LC-MS grade. The bag infusion was then cooled and 100 mL of the infusion was subjected to the optimized SPE, finally the extract was analyzed by LC-MS. The result shows that the concentration of BPA in the tea bags were 1.8 ± 0.03 and 1.3 ± 0.04 µg/L, respectively. According to these values, the migration percentages of BPA from the tea bags to the tea infusion were 14% and 62%, respectively. Migration percentage was calculated by dividing the BPA concentration in the bag infusion by the total BPA concentration multiplied by 100.

Although the manufacturer´s instructions for tea preparation is to infuse the tea bag for 3-5 minutes, experiments have been carried out to assess the effect of different steep times on the concentration of BPA leached from the tea sample and the tea bag. Therefore, samples 4 and 14 were prepared by steeping the tea sample and the tea bag for

5, 10 and 20 min in 150 mL of boiled water. These samples were subjected in duplicate to the SPE procedure and the extract analyzed in triplicate by LC-MS. The results obtained are shown in Figure 4.6. As can be seen in the figure, BPA concentration in the tea solution increased with the soaking time for both samples studied. Results obtained for sample 14 showed that there is not statistically significant difference between the BPA concentration obtained from 10 min to 20 min at the 95.0% confidence level. This means that the whole amount of BPA was almost leached within a period of 10 min of brewing. On the other hand, results obtained for sample 4 shown statistically significant difference at the times studied, increasing the BPA concentration in the period of time studied. On the other hand, FTIR spectroscopic analysis was also applied to these bags to determine their chemical composition. The obtained IR spectra were compared to those of the Bio-Rad library, and the Hit Quality Index (HQI), that provides the best correspondence to identify compounds, was calculated.

Table 4.2 lists the major chemical contents of the tea bags while **Figure 4.7** shows the IR spectra of tea bags 2, 4, and 5, considering the best matches of the library polylactic acid (PLA), cellulose-based compounds, and poly(ethylene terephthalate), respectively. The composition of tea bags that released BPA (4 and 14) was based in cellulose in both cases, similarly to other samples such as samples from 7-23. Therefore, based on the results of IR analysis of tea bags and the results obtained from the migration study, we can conclude that the trace amount of BPA detected in the tea bags infusion might be due to other minor components added in the manufacturing process of the bags' materials.

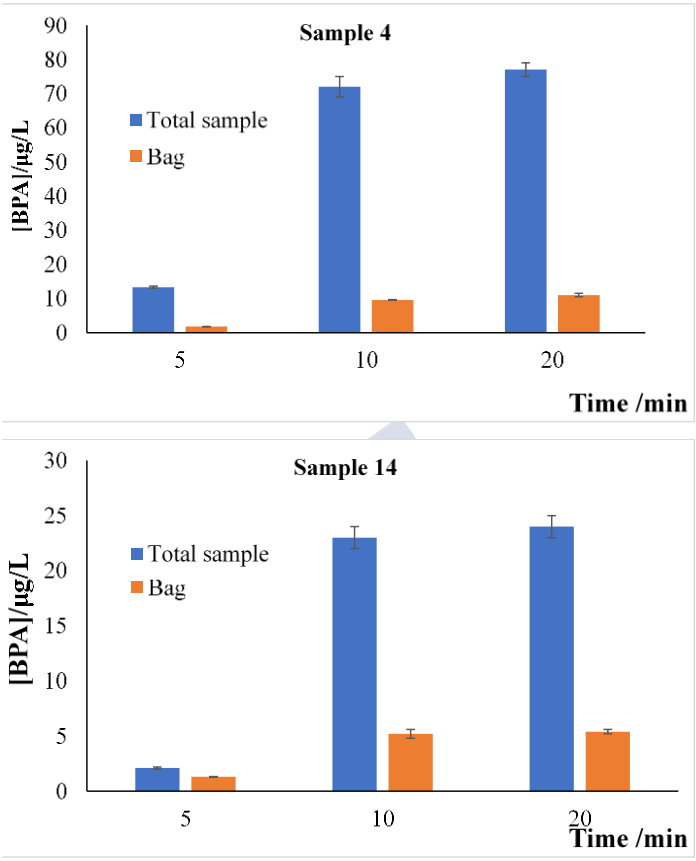

Figure 4.6 Influence of the soaking time on the BPA concentration.

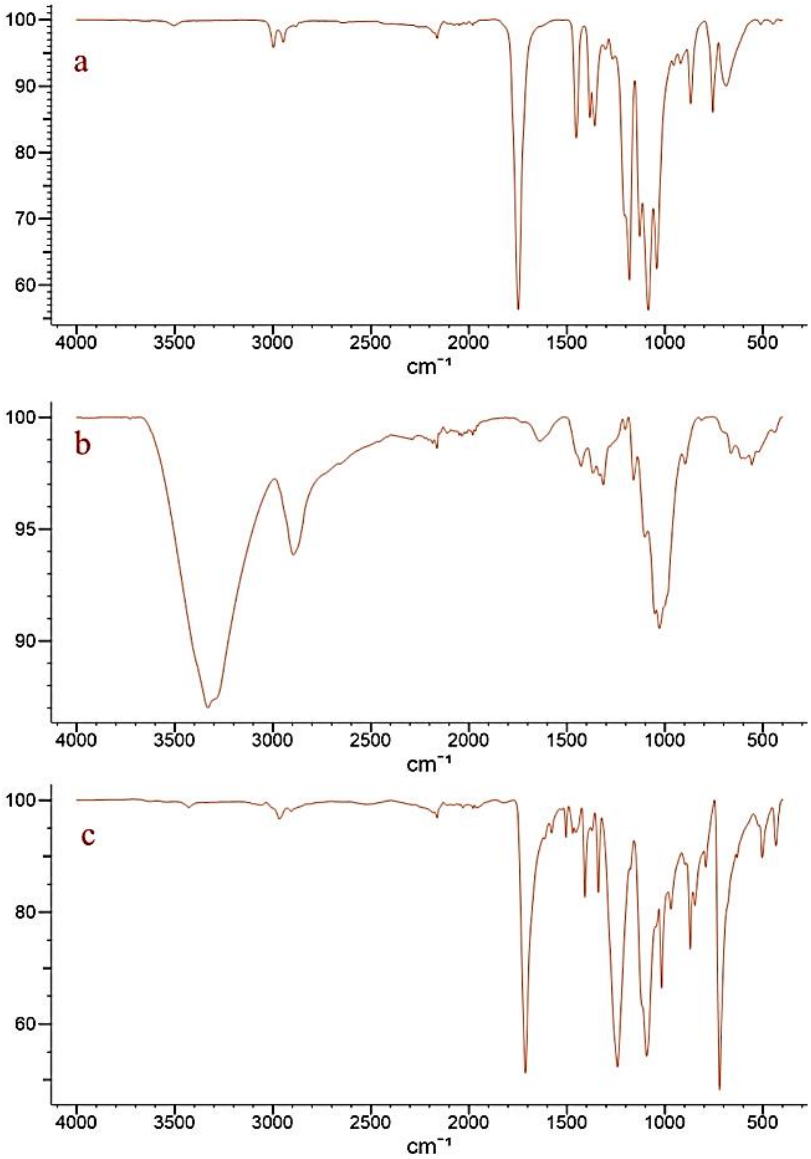

Figure 4.7 FT-IR spectra: a) tea bag 2 (based on PLA triethoxysilane), b) tea bag 4 (based on Cellulose) and c) tea bag 5 (based on poly(ethylene terephthalate)).

4.6 Conclusion

In this study, an analytical method has been described for determining BPA in tea samples using a selective MIP-SPE technique. The MIP was prepared by precipitation polymerization and was used as an SPE sorbent to preconcentrate BPA from tea samples. The collected extracts were analyzed by optimized LC-MS. The method shows good precision and high sensitivity. The high accuracy of the method was evaluated using the recovery. The optimized method was applied to the determination of BPA in commercial tea samples in plastic bags acquired at a local market. Two samples out of 24 tea samples showed a detectable level of BPA. The migration test of BPA from the bags into the infusion shows that a part of BPA present in the tea infusion migrated from the tea bag materials. Moreover, the BPA concentration in the tea infusion increased with the steep time. The IR study indicates that the major contents of the tea bag were cellulose-based material, and polyester-based materials.

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CHAPTER 5

In vitro assesment of major and trace element bioavailability in tea samples

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In vitro assessment of major and trace element bioavailability in tea samples

5.1 ABSTRACT

Tea is considered as a good source of many essential substances with biologically active properties such as trace elements. The high consumption of tea as infusion around the world makes it a daily source of such nutrients. However, the beneficial content of elements in tea for the human body depends mainly on their bioavailability. Previous studies reported in the literature applied solubility and bioaccessibility approaches to evaluate element availability in tea samples without considering element absorption and transportation. This study aims to assess the bioavailable content of trace and major elements in tea samples using a dialyzability protocol. An *in vitro* dialysis procedure was used to evaluate the bioavailable fraction of trace and major elements in tea infusions. The *in vitro* protocol used here simulates the gastrointestinal digestion utilizing dialysis membranes (10 kDa) to mimic intestine walls where the absorption of nutrients happens. A pepsin solution was used for gastric digestion, and a solution of pancreatin and bile salts was used for intestinal digestion during the *in vitro* study. The total content of elements in tea leaves, their infusions and dialyzable fractions were determined by spectrometric methods (ICP-MS, ICP-OES, and FAES). The analytical performances of the methods were studied. The LODs for elements determined in tea leaves were in a range of 0.11 – 656 ng/g and 0.02 - 145.6 µg/g for trace and major elements, respectively. The LODs for tea infusions ranged between 0.23 – 399.9 ng/L and 0.2 – 1248 µg/L for trace and major elements, respectively and dialyzable elements varied from 0.018 to 142 µg/L. The accuracy of the method for total element determination was evaluated using two certified reference materials (Tea Leaves INCT-TL-1 and Rye Grass CRM 281). Analytical recoveries were also assessed for elements analyzed in digested tea leaves and their infusions showing good recoveries (92-115 %). The accuracy of the dialysis process was studied using a mass-balance assessment. Among the elements studied, K was the most abundant element in tea leaves and tea infusions in almost all

samples followed by Ca, Mg, and P. The *in vitro* simulation revealed that Cs and Zn were the most dialyzable elements up to 76% and 84%, respectively, followed by Si, Ca, and K that show moderate to high dialyzability percentages.

5.2 INTRODUCTION

Tea is the most popular non-alcoholic beverage consumed in different parts of the world after water. Tea plants are cultivated in tropical and subtropical areas with adequate rainfall and acid soils with excellent drainage, like China, India, Sri Lanka, Kenya, etc. [1, 2]. Tea, produced from *C. Sinensis* plant, is classified into four main types: green tea, black tea, oolong tea and white tea based on their manufacturing techniques [3]. Tea has attracted much attention because of its benefits, such as antimicrobial, anticarcinogenic, antioxidant, and anti-inflammatory properties [4, 5]. Additionally, tea consumption is related to lowering serum cholesterol, preventing low-density lipoprotein oxidation, minimizing the risk of cardiovascular disease and cancer [6].

Tea contains major elements that are considered essential nutrients like Ca, K, Na, and Mn. Moreover, it may contain toxic elements such as Cd, As, Hg, etc. The main sources of elements to tea plants are their growth ambient conditions like nutrient concentrations, soil type and its pH, in addition to the usage of pesticides and fertilizers [7–9]. Although tea has several beneficial properties, some studies showed that the ingestion of tea alongside food might inhibit the absorption of some elements like iron in human bodies [10]. Due to the importance of evaluating the toxicological and nutritional values of elements in tea, several studies were conducted to determine their levels in tea leaves and their infusions.

Various spectrometric techniques were used to determine these elements in tea including electrothermal atomic absorption spectrometry (ETAAS)[11, 12], flame atomic absorption spectrometry (FAAS) [13, 14], inductively coupled plasma optical emission spectrometry (ICP-OES) [13, 15], and inductively coupled plasma mass spectrometry (ICP-MS) [16].

Several sample preparation methods were used to prepare tea samples for total content analysis including dry ashing [17], conventional wet acid digestion [18, 19], microwave acid digestion [16, 20], and other methods like UV photolysis-assisted digestion [21], and slurry sampling [22, 23].

The human body requires several essential elements for nutritional purposes that make their deficiency or excess a severe issue for human health. When studying the role of elements in human health, their availability should be considered; thus, elements must be liberated from the food and drink matrices and assimilated into the gastrointestinal tract (GIT). Therefore, it is crucial to investigate the influence of the digestion process on element availability.

Different digestion models have been proposed by the scientific community to simulate the human GIT, considering the physicochemical/physiological conditions of the digestion process. Bioaccessibility and bioavailability approaches are used to express the availability of elements for the living organisms. While bioaccessibility is defined as the fraction of nutrients released from the food/drink in the GIT and turned into an available fraction for absorption [24], bioavailability is defined as the fraction of nutrient absorbed by the body and used for its functions [25]. Bioavailability studies can be performed using different approaches including *in vivo* models and *in vitro* models. *In vivo* assessment of elements, using living organisms like humans or animals is more realistic than *in vitro* assessment, but it is still challenging and expensive, and it is only possible at a small scale [26].

Most *in vitro* studies, that aim to determine element bioavailability in tea samples, are based on solubility and bioaccessibility approaches to simulate the *in vitro* gastrointestinal digestion using similar conditions found in a living organism (temperature, pH, enzymatic and chemical conditions, etc.). Powell et al. [27] applied an *in vitro* assessment to evaluate the potential bioavailability of Mn, Ca, Fe, Cu, Mg, K, Zn and Na from tea infusions. They simulated gastrointestinal digestion by incubating tea infusions with human gastric juice at 37 °C at pH 6.5 for an hour. The samples were then centrifuged through ultrafilters with molecular

weight cut-offs (MWCO) of 3, 10 and 30 kDa, and analyzed by ICP-OES. Lin et al. [28] studied the bioaccessibility of Al from different tea samples using a gastrointestinal digestion protocol by mixing the tea infusion with gastric juice at room temperature for 2 h followed by the addition of the intestinal solution and incubation for other 2 h at room temperature. Afterward, Al content was analyzed by atomic absorption spectrometry.

To the best of our knowledge, there is only one study reported in the literature about silicon bioavailability in tea samples using an *in vitro* continuous flow dialysis model with MWCO of 1000 Da [29]. Therefore, the aim of this work is to study the bioavailability of trace and major elements in tea samples using an *in vitro* dialysis protocol. The total content of elements in tea leaves and their infusions was determined, and the leaching efficiencies of elements from tea leaves into tea infusions were also evaluated.

5.3 MATERIALS AND METHODS

5.3.1 Instrumentation

Total trace elements in acid digested tea samples, tea infusions and dialyzate fractions were determined using a Perkin Elmer NexIon 300X ICP-MS instrument (Waltham, MA, USA) equipped with a SeaFast SC2 DX autosampler (Elemental Scientific, Omaha, NB, USA). Total major elements were analyzed using an Optima 3300 DV inductively coupled plasma atomic emission spectrometer (Perkin Elmer) equipped with an autosampler AS 91 (Perkin Elmer) and a Gem-Cone crossflow nebulizer type (Perkin Elmer). K was determined in acid-digested samples with a Perkin Elmer flame atomic absorption spectrometer (FAAS-3110) using emission mode. A Boxcult incubator situated on a Rotabit orbital-rocking platform shaker (J.P. Selecta, Barcelona, Spain) was used for temperature control during the gastrointestinal digestion procedure. Microwave-assisted acid digestion was carried out using an Ethos Plus microwave lab-station (Milestone, Sorisole, Italy) with 100 mL closed Teflon vessels and Teflon covers, HTC adapter plate and HTC safety springs (Milestone). pH-adjustment was performed using a Basic 20 pH-meter with a glass-calomel electrode (Crison, Barcelona, Spain).

5.3.2 Reagents

A multi-element standard solution containing 10 mg/L of As, Ag, Be, Cd, Co, Cs, Cr, Cu, Ga, Li, Ni, Pb, Se, Tl and V, a standard solution of 5000 mg/L containing K, Ca and Mg, individual standard solutions of 1000 mg/L of Ti, Hg, P, Rb, Sr, Sn, Fe, and Sn and internal standards of Ge, Y and Rh of 10 mg/L each, were from Perkin Elmer. Individual stock solutions of 1000 mg/L of Mo, Ba, Au and Sb were supplied by Merck (Poole, Dorset, UK). 1000 mg/L of each standard of Al and Mn were purchased from Scharlau (Scharlab, Spain). AnalaR nitric acid 69 % (w/v), hydrogen peroxide 33 % (w/v) and Pt (1000 mg/L) were from Panreac (Barcelona, Spain). Ultrapure water of resistance 18 MΩ cm was obtained from a Milli-Q purification system (Millipore Co., Billerica, MA, USA). Sodium hydrogen carbonate and 37 % (w/v) hydrochloric acid from Merck were used to prepare the intestinal solution and the gastric solution, respectively. A stock standard of Si (1000 mg/L), digestive enzymes (porcine pepsin, p-7000, porcine pancreatin, P-1750), bile salts (approximately 50% sodium cholate and 50 % sodium deoxycholate), and piperazine-NN-bis(2-ethanesulphonic acid) di-sodium salt (PIPES), were obtained from Sigma Chemicals (St. Louis, MO, USA). CRM 281 Rye Grass (Brussels, Belgium) and Tea leaves (INCT-TL-1) from the Institute of Nuclear Chemistry and Technology (Warsaw, Poland) were used as certified reference materials.

The *in vitro* dialysis protocol was carried out using Cellu Sep® H1 high grade regenerated cellulose tubular membranes (MWCO 10 kDa, 50 cm length, 25.5 mm diameter dried, and a volume to length ratio of 5.10 mL/cm) purchased from Membrane Filtration Products Inc. (Seguin, TX, USA).

A special protocol was used to clean the glassware and plasticware prior to the analysis. All the material used were washed with liquid soap followed by rinsing with ultrapure water and soaked in 10 % (v/v) nitric acid for 48 hrs. The material was then rinsed several times with ultrapure water, dried and stored until use.

5.3.3 Tea samples

Thirty-five tea samples were purchased at different markets in Spain and other countries like India, Jordan, Portugal, Azerbaijan, and

Yemen. These samples include: 19 black (B) teas, 8 green (G) teas, 3 red (R) teas, 2 white (W) teas and red fruits (RF), verbena leaves (VL) and red rooibos (RR) used to flavor tea infusions. **Table S1 (Supplementary data-Annex I)** shows the types, origins and additive flavors of the samples studied.

5.3.4 Microwave-assisted acid digestion procedure

Samples studied were digested according to previous studies performed by Barciela-Alonso et al. [30] with minor modifications. Tea samples (0.5000 g) were accurately weighed and transferred into 100 mL Teflon vessels. Volumes of 3 mL of HNO_3, 1 mL of H_2O_2 and 4 mL of ultrapure water were then added. Subsequently, the vessels were introduced into the microwave oven and subjected to a three-steps temperature program for digestion. Firstly, the temperature was linearly elevated from room temperature to 90˚C by increasing the power up to 1000 W for 2.5 min. The temperature was then increased to 140˚C at 1000W in 6 min, followed by the third increase of the temperature up to 180 ˚C for 11 minutes. Finally, digestion ends with a cooling step to room temperature. The digestion procedure was carried out in triplicate for each tea sample. Blank digestion was also prepared in the same way without introducing the tea sample into the Teflon vessels. The digested samples were then diluted to 25 mL with ultrapure water and kept in capped polyethylene bottles until analysis.

5.3.5 Preparation of tea infusions

The preparation of tea infusions was carried out in triplicate according to the manufacturers' recommendations and considering drinking habits. A mass of 1.5000 g of leaves was soaked into 150 mL of boiled ultrapure water for 5 minutes, left to cool and then filtrated using a Mixed Cellulose Esters (MCE) membrane syringe filter (0.45µm). Tea infusions were stored in capped polyethylene bottles at 4˚C until analysis.

5.3.6 *In vitro* dialyzability protocol

The *in vitro* gastrointestinal digestion was carried out following the procedure described elsewhere by García-Sartal et al. [31] with minor modifications. In this study, Concisely, the gastric digestion stage was carried out as follows: a volume of 20 mL of a tea infusion

was introduced into 100 mL Erlenmeyer flasks and the pH was adjusted to 2.0 with a 6.0 M HCl solution. A mass of 0.15 g of a freshly prepared gastric solution (6.0 % (w/v) pepsin dissolved in 6.0 M HCl) was then added. The flasks were covered and placed in the incubation chamber at 37˚C with an orbital–horizontal shaking at 150 rpm for 120 min. The enzymatic digestion was stopped by placing the flasks in an ice-water bath. The gastrointestinal digestion was then resumed by adding 5 mL of the intestinal solution (pancreatin 4.0% (m/v) and bile salts 2.5% (m/v) dissolved in 0.1 M sodium hydrogen carbonate) to the gastric digest. At this moment, the dialysis membranes of MWCO of 10 kDa filled with 20 mL of a 0.15 N PIPES solution (pH 7.5 adjusted with hydrochloric acid) were introduced inside the flasks. The flasks were incubated at 37˚C and 150 rpm of an orbital–horizontal shaking for 2 hours. Finally, the intestinal digestion was stopped by placing the flasks in an ice-water bath. Membranes; containing the dialyzate were then taken out of the flasks, washed their outer surface with ultra-pure water. Dialyzable fractions and non-dialyzable fractions obtained from this procedure were stored in polyethylene vials at -20˚C till the analysis. Blanks were also prepared applying the same procedure, but tea infusion was replaced by 20 mL of ultrapure water. The procedure was carried out in triplicate for each sample.

5.3.7 Total element determination by ICP-MS, ICP-OES and FAES

Multi-element determinations (Li, Be, Cr, Ti, Cu, Ga, Ag, Hg, Cd, Cs, Co, Pt, Tl, Pb, As, Ni, V, Se, Sn and Sb) were conducted by ICP-MS using the operating conditions shown in **Table S2.** Calibration measurements were performed using the standard addition method in the concentration range of 0 -100 µg/L except for Hg calibration which ranged between 0 and 5 µg/L. A standard solution of 200 µg/L of Au was added to all standards and samples analyzed to stabilize Hg and prevent its deposition in the ICP-MS sample introduction system. A multi-elemental solution of 10 µg/L of yttrium (Y), germanium (Ge) and rhodium (Rh) was used as an internal standard. Al, Ba, Ca, Fe, Mg, Mn, Mo, P, Rb, Si, Zn, and Sr were analyzed by ICP-OES under the operating conditions and using the emission wavelengths given in

Table S3, and the standard addition calibration in the range of 0 - 50 mg/L.

A Perkin-Elmer model 3110 (Norwalk, CT, USA) atomic absorption spectrometer equipped with acetylene-air flame was used for K determination in digested tea samples and their infusions at 766.5 nm operating at emission mode. The calibration was performed by the standard addition method (0 – 50 mg/L calibration range).

5.4 Results and Discussion

5.4.1 Total element determination in tea leaves

Microwaves-assisted acid digestion was used for tea leaves sample preparation before analysis by ICP-MS, ICP-OES and FAES. The sample digestion method was described in section 2.4. The method was validated and applied to tea samples acquired at different markets.

5.4.1.1 Analytical performance

- Calibration

Total elements determination in tea samples was carried out by ICP-MS and ICP-OES using the operating conditions mentioned in **Table S2** and **Table S3**, respectively. K was analyzed by FAES at 766.5 nm. Matrix effect was evaluated by comparing the slopes of the direct calibration method and those obtained by applying the standard addition method in the calibration range of 0 – 100 µg/L for ICP-MS, 0 – 50 mg/L for ICP-OES as well as FAES. The slopes were compared using t-test (95% significance level) showing that there was a statistically significant difference between slopes due to the matrix effect for almost all elements studied. Hence, the analysis was carried out using the standard addition method for all elements.

- Sensitivity

Limits of detection (LODs) and limits of quantification (LOQs) were calculated based on 3SD/m and 10SD/m, respectively, where SD indicates the standard deviation of twelve measurements of a blank and m is the slope of the addition curve**. Table 5.1** and **Table 5.2** show the instrumental LODs and LOQs, as well as those referred

to the sample considering the dilution factors and the mass of sample used in the preparation. The LODs obtained ranged from 0.11 to 8.1 ng/g for all the trace elements except for Se which showed the highest LOD (0.66 µg/g). The LODs for major elements ranged between 0.02 and 16.6 µg/g with the exception of K (LOD = 0.146 mg/g).

- Precision

Precision (expressed by the relative standard deviation, % RSD) was evaluated in terms of intra-day (repeatability) and inter-day (reproducibility) measurements. The RSDs obtained for all the elements analyzed in the digested tea leaves and tea infusions are shown in **Figure 5.1** The method showed good precision with % RSD values around 10% for all the elements studied.

Table 5.1 Limits of detection (LOD) and Limits of quantification (LOQ) of elements in digested tea leaves, their infusions and the dialyzate fractions analyzed by ICP-MS

Element	Total				Infusion				Dialyzate			
	Instrumental		Related to sample		Instrumental		Related to sample		Instrumental		Related to sample	
	LOD (ng/L)	LOQ (ng/L)	LOD (ng/g)	LOQ (ng/g)	LOD ng/L)	LOQ (ng/L)	LOD (ng/g)	LOQ (ng/g)	LOD (µg/L)	LOQ (µg/L)	LOD (µg/L)	LOQ (µg/L)
Li	2.6	8.6	1.3	4.3	1.6	5.2	1.9	6.5	0.014	0.047	0.14	0.47
Be	6.3	21.0	3.1	10.5	0.4	1.3	0.5	1.6	0.002	0.008	0.02	0.08
Ti	5.7	19.0	2.8	9.5	108.3	360.9	135.4	451.2	0.3	1.1	3	11
Cu	1.2	3.9	0.6	1.9	44.6	148.8	55.8	186.0	0.078	0.260	0.78	2.60
Ga	0.6	2.0	0.3	1.0	11.4	37.9	14.2	47.4	0.107	0.358	1.07	3.58
Ag	0.8	2.8	0.4	1.4	18.3	61.0	22.9	76.3	0.024	0.082	0.24	0.82
Hg	7.4	24.8	3.7	12.4	30.6	101.8	38.2	127.3	0.2	0.8	2.4	8.0
Cd	3.5	11.6	1.7	5.8	1.1	3.8	1.4	4.8	0.004	0.013	0.04	0.13
Cs	0.2	0.8	0.1	0.4	1.1	3.6	1.3	4.5	0.003	0.010	0.03	0.10
Pt	2.0	6.5	1.0	3.3	0.2	0.6	0.2	0.8	0.059	0.198	0.59	1.98
Tl	0.6	1.8	0.3	0.9	0.2	0.6	0.2	0.8	0.014	0.045	0.14	0.45
Pb	0.3	0.9	0.1	0.5	8.8	29.2	10.9	36.5	0.015	0.049	0.15	0.49
As	3.6	11.8	1.8	5.9	10.5	35.0	13.1	43.8	0.754	2.514	7.54	25.14

Table 5.1 (continued)

Element	Total				Infusion				Dialyzate			
	Instrumental		Related to sample		Instrumental		Related to sample		Instrumental		Related to sample	
	LOD (ng/L)	LOQ (ng/L)	LOD (ng/g)	LOQ (ng/g)	LOD (ng/L)	LOQ (ng/L)	LOD (ng/g)	LOQ (ng/g)	LOD (µg/L)	LOQ (µg/L)	LOD (µg/L)	LOQ (µg/L)
Cr	5.2	17.5	2.6	8.7	7.5	24.9	9.3	31.1	6.1	20.3	61	203
Co	0.2	0.7	0.1	0.3	0.8	21.8	1.0	27.2	0.002	0.006	0.02	0.06
Ni	16.1	53.7	8.1	26.8	17.0	56.7	21.2	70.8	0.057	0.189	0.57	1.89
V	0.6	1.8	0.3	0.9	3.1	10.3	3.8	12.8	0.015	0.052	0.15	0.52
Se	1313	4375	656	2187	319.9	1066.4	399.9	1333	0.610	2.032	6.10	20.32
Sn	7.7	25.8	3.9	12.9	7.0	23.3	8.7	29.2	0.806	2.686	8.06	26.86
Sb	9.7	32.2	4.8	16.1	9.4	31.4	11.8	39.3	0.017	0.058	0.17	0.58

Table 5.2 Limits of detection (LOD) and Limits of quantification (LOQ) of elements in digested tea leaves, their infusions and the dialyzate fractions analyzed by ICP-OES and FAES.

Element	Total				Infusion				Dialyzate			
	Instrumental		Related to sample		Instrumental		Related to sample		Instrumental		Related to sample	
	LOD (µg/L)	LOQ (µg/L)	LOD (µg/g)	LOQ (µg/g)	LOD (µg/L)	LOQ (µg/L)	LOD (µg/L)	LOQ (µg/L)	LOD (µg/L)	LOQ (µg/L)	LOD (µg/L)	LOQ (µg/L)
Al	8.73	29.10	0.87	2.91	9.3	31.1	46.7	155.6	0.174	0.581	1.7	5.8
Ba	0.48	1.59	0.05	0.16	0.7	2.3	3.4	11.3	0.030	0.100	0.3	1.0
Rb	8.53	28.44	0.85	2.84	11.7	38.9	58.3	194.4	7.5	24.9	37.4	124.6
Fe	0.90	3.00	0.09	0.30	0.4	1.2	1.8	6.1	0.607	2.024	6.1	20.2
Zn	1.35	4.50	0.67	2.25	0.4	1.4	2.2	7.2	0.9	3.1	4.7	15.7
P	33.2	110.5	16.6	55.3	13.9	46.5	69.7	232.3	28.4	94.7	142.0	473.5
Si	10.41	34.71	1.04	3.47	1.3	4.5	6.7	22.7	6.1	20.4	30.6	101.9
Ca	0.76	2.52	0.19	0.63	1.0	3.2	4.8	15.9	3.8	12.6	18.9	63.1
Mg	0.27	0.88	0.07	0.22	0.3	0.9	1.3	4.4	0.8	2.6	3.9	13.1
Mn	0.09	0.28	0.02	0.07	0.0	0.1	0.2	0.6	0.2	0.5	0.8	2.5
Mo	4.51	15.04	1.13	3.76	3.7	12.5	18.7	62.4	6.6	21.9	32.8	109.3
Sr	0.09	0.31	0.02	0.08	0.1	0.4	0.5	1.8	0.01	0.05	0.1	0.5
K*	14.56	48.52	145.6	485.2	256.8	856.0	1284.0	4280	14.5	48.3	72.5	241.6

*: K was analyzed using FAES.

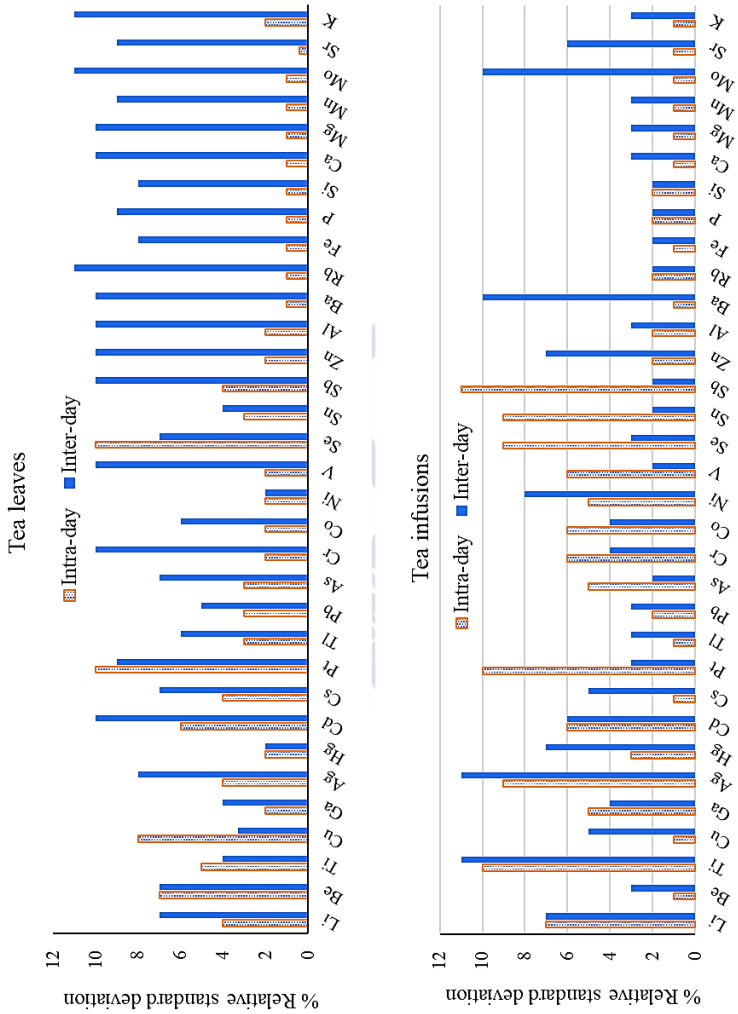

Figure 5.1 The intra- and inter-day precisions (in % RSD) of the total element determination in tea leaves.

- Accuracy

The accuracy of the method was studied using two certified reference materials (CRM) Tea Leaves INCT-TL-1 (including certified elements: As, Cd, Co, Cr, Cs, Cu, Ni, Pb, V, Zn, Tl, Ba, Rb, Ti, Sb, Fe, P, Al, Ca, K, Mg, and Mn) and CRM 281 Rye Grass (including certified elements: As, Cd, Cr, Cu, Ni, Pb, Zn, Sb, Se, Sn, Mn, and Mo). The CRMs were digested using the same method described in section 2.1 "Microwave-assisted acid digestion of tea samples". The digested CRMs were analyzed and the results obtained are shown in **Figure 5.2** alongside the certified element concentrations. The results obtained were compared with the certified values using a t-test (95% confidence level) [32]. The comparison shows a good agreement between the certified and experimental values, and no statistically significant differences were found. In the case of Ti, Sb, Fe, and P, informative values are given in Tea Leaves CRM: 30, 0.05, 432, and 1810 µg/g, respectively, for each element. The measured concentration of Sb (0.05 ± 0.002 µg/g) and Fe (432 ± 21 µg/g) were in good agreement with the informative values. Ti showed a concentration of 22.7 ± 0.8 µg/g which is lower than the informative value, while P showed a higher concentration of 2040 ± 68 µg/g (9 % higher than the informative value).

Analytical recoveries were also assessed for acid-digested tea samples. A digested tea sample was spiked with three concentration levels (1, 25, and 100 µg/L) in the case of the elements measured by ICP-MS, and four concentration levels (0.1, 0.25, 1, and 3 mg/L) in case of elements measured by ICP-OES and FAES. **Figure 5.3** shows the mean recoveries for all the elements in tea leaves which ranged from 95 to 114%.

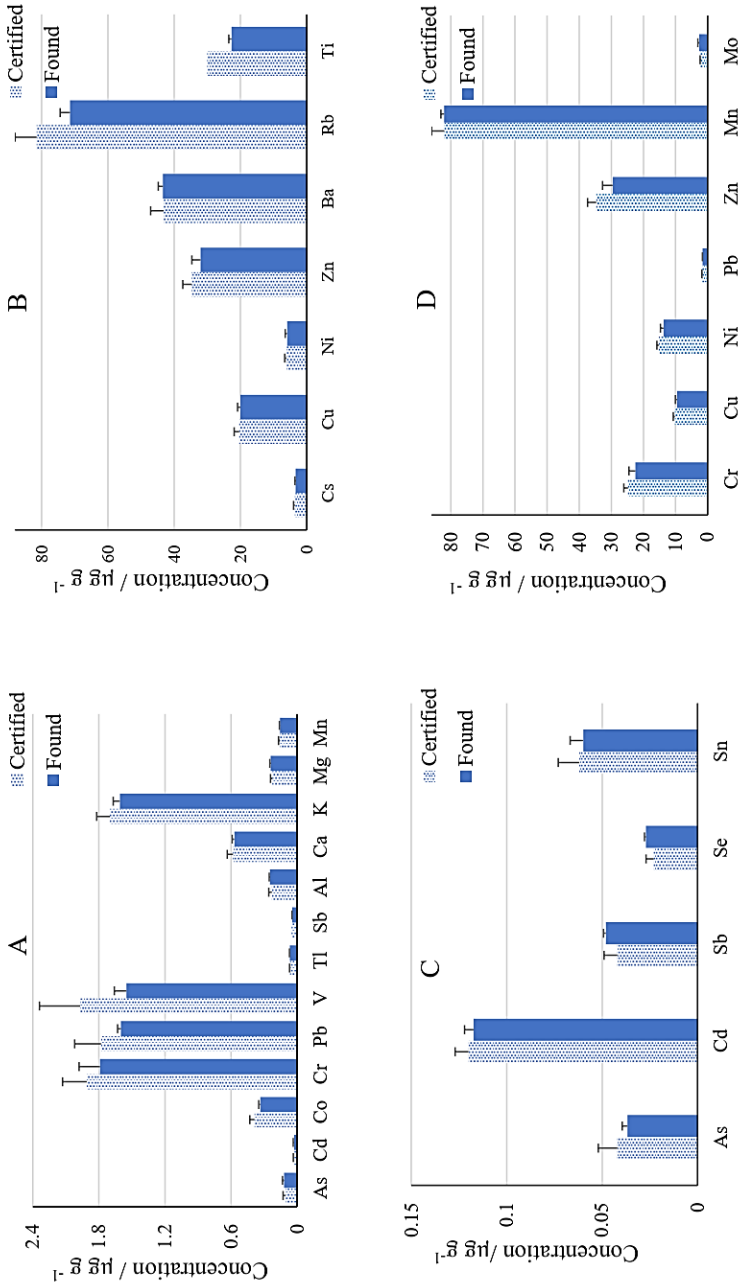

Figure 5.2 Comparison between certified element contents in CRM and the obtained elements' concentrations A and B: elements certified in Tea Leaves CRM (Concentrations of Al, Ca, K, Mg, and Mn are expressed in (w/w)%), C and D: elements certified in CRM Rye Grass

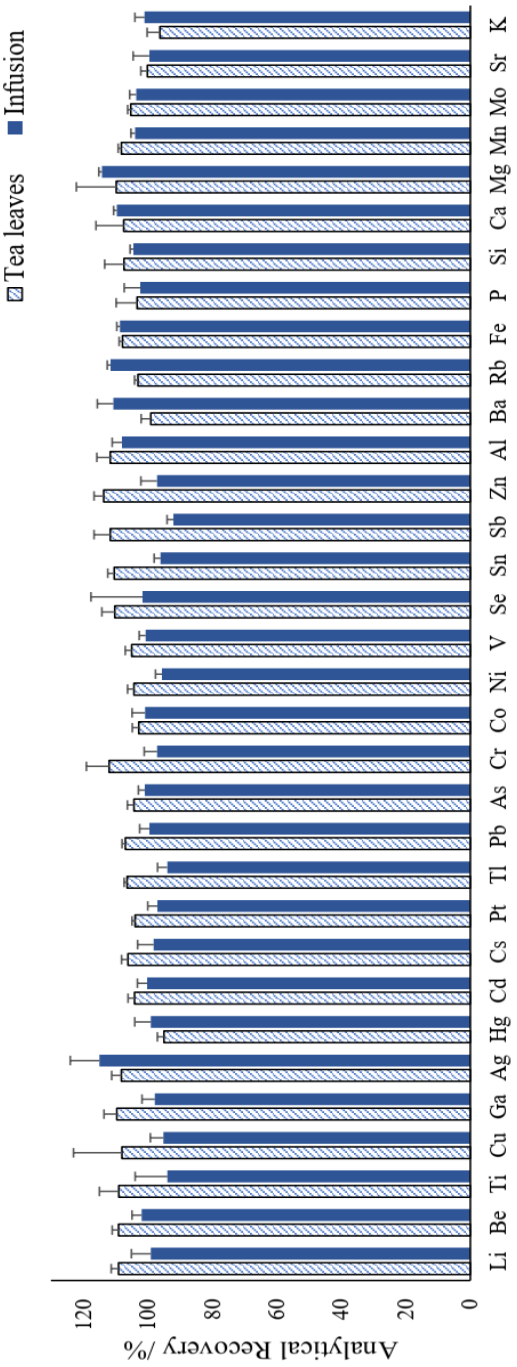

Figure 5.3 Analytical recoveries (in percentage, %) for the elements studied in tea leaves and tea infusions

5.4.1.1 Analysis of digested tea samples

The validated method was applied to determine the total element concentrations in 35 tea samples by ICP-MS, ICP-OES and FAES after microwave-assisted acid digestion. **Table S4** lists element concentrations for the analyzed tea samples expressed as the arithmetic mean and their standard deviation.

Results show that Pt concentration was lower than the LOD in all the samples analyzed while Se and Mo were below the LOQ. High concentrations of macronutrient elements like K, Ca, Mg and P were found in the ranges of 6.4-69.8, 3.9-15.4, 1.4- 3.7, and 0.7- 4.7 mg/g, respectively. These concentrations agree with the results achieved in previous studies [17, 33] except K in green tea samples that showed higher levels. Aluminum concentrations were in the range of 206 - 2474 µg/g. These levels agree with those reported by De Silva et al. [34] (274 – 3134 µg/g). Average Al contents in black and green tea were 946 and 1116 µg/g, respectively. These averages are comparable with those results reported by Matsuura et al. [33] with higher Al contents in green tea than in black tea. Essential trace element contents (Mn, Fe, Cu, Zn and Ni) in tea leaves were arranged as follows: Mn (58.0 - 2031.9 µg/g), as the most abundant element followed by Fe (80.6 - 766.4 µg/g), Cu (13.1 - 42.8 µg/g), Zn (4.9 - 28.6 µg/g) and Ni (0.8 - 13.9 µg/g). The obtained results agree with those reported by Salahinejad et al. [35]. On the other hand, toxic trace elements such as Cr, Cd, Pb, As and Hg varied as follows: concentration values < LOD - 0.07 µg/g for Hg, 0.01- 0.19 µg/g for Cd, 0.1-1.7 µg/g for Pb, 0.01- 0.25 µg/g for As, and < LOD – 21.2 µg/g for Cr. The higher concentration of Pb in tea leaves could be attributed to the dust particles accumulated during the production process, the use of leaded fuel and the industrial activities [36]. Arsenic levels reported here were 19-fold lower than those levels reported by Chungang Yuan et al. [37]. Levels of Li, Be, Ga, Co, V, and Sr ranged as follow: Li (0.04 - 1.53 µg/g), Be (< LOD - 0.05 µg/g), Ga (0.3 - 1.44 µg/g), Co (0.06 - 1.03 µg/g), V (0.1 - 1.5 µg/g), and Sr (14.17 - 196.04 µg/g). These concentrations were lower than those reported by Li et al. [38] in the case of Ga, Co and V, while Be shows comparable levels. On the other hand, Sr shows a higher

content than those previously reported [38] in Tibet tea. Barium contents were in the range from 6.2 to 217.6 µg/g. The amount of Ba was higher than other results previously reported [18, 33].

5.4.2 Total element determination in tea infusions

In the present section, the total element contents in tea infusions were evaluated to assess their leaching percentage from tea leaves to their infusions. Tea infusions were prepared according to the manufacturer's recommendations. The sample preparation step, as well as the method of analysis for total element determinations, was also validated.

5.4.2.1 Analytical performance

The total contents of leachable elements were measured using ICP-MS, ICP-OES and FAES. The linearity, sensitivity, precision, and accuracy of the analytical method were studied. Linearity was examined for ICP-MS (ranges between 0 and 100 µg/L), ICP-OES and FAES (ranged between 0 and 50 mg/L). The analysis was performed using the standard addition method due to the presence of matrix effect. The sensitivity, precision and accuracy of the method were evaluated, showing a good sensitivity considering the LODs and LOQs listed in **Table 5.1** and **Table 5.2,** good precision (RSD around 10% as shown in **Figure 5.1** and analytical recoveries (**Figure 5.3**) in the range of 92 - 115%.

5.4.2.2 Analysis of elements in tea infusions

The total concentrations of the elements analyzed in tea infusions are summarized in **Table S5.** Results show that Ag, Se, Pt, Mo, and Hg concentrations (in all samples except B1, G1, G2 and RF) were lower than the LOQ. K was the most abundant element with concentrations ranging from 2.8 to 26.1 mg/g followed by Mg (0.22 – 2.9 mg/g), Ca (0.05 – 2.4 mg/g) and P (0.2 – 2.1 mg/g). Calcium shows the highest levels in tea-flavored infusions like red fruits (RF), red rooibos (RR) and verbena leaves (VL). Aluminum concentrations were the highest among the trace elements and were in the range of 8 – 938 µg/g. The level of Fe in tea infusion was very low and varied from 0.4 – 18.5 µg/g leading to conclude that Fe is slightly soluble in tea infusion. Despite having very low levels of Fe, red tea (R1 and

R2), red fruits (RF), and red rooibos (RR) show a highly significant concentration of Fe compared to green, black and white tea. Moreover, RR has the highest level of Fe which corresponds to the lowest concentration of tannins that tend to bind Fe strongly [56]. Toxic elements (As, Cd, Cr, and Pb) contents varied from 0.01 – 0.11 µg/g, 0.002 – 0.02 µg/g, 0.02 – 1.17 µg/g and < LOD - 0.1 µg/g for As, Cd, Cr, and Pb, respectively. As level was within the range of the study reported by Chungang et al. [37]. Among the micronutrients (Mn, Zn, and Cu), Mn shows the highest concentrations (5.7 – 667.3 µg/g). Zn and Cu were found at low concentrations. Zinc levels (3 – 14 µg/g) were approximately two-fold Cu levels (1 – 7.9 µg/g). The levels of other microelements (Co, Ni and V) varied over a wide range of concentrations through the analyzed samples. Nickel showed higher levels than V and Co which were ranged from non-detectable levels to 0.03 µg/g, and between 0.02 and 0.5 µg/g, respectively. Levels of the non-essential elements Sr and Rb varied from 0.21 - 19.7 µg/g (Sr), and 6.1 – 172 µg/g (Rb). Barium concentrations varied between 0.3 and 16.4 µg/g, which are comparable to those results previously reported by Başgel [39]. Be, Tl, Li, Ga and Ti were found at very low concentrations ranged as: <LOD – 0.03 µg/g for Be, 0.002 – 0.12 µg/g for Tl, 0.02 – 1.38 µg/g for Li, 0.04 – 1.44 µg/g for Ga and 0.72 – 6.07 µg/g for Ti. Sn and Sb levels were not detected in 10 and 25 samples, respectively, and their concentrations were lower than 0.07 µg/g for Sn, and 0.05 µg/g for Sb. Silicon concentrations varied between 8.3 – 324.5 µg/g.

5.4.3 Leaching efficiency

The dietary intake of mineral nutrients in tea leaves is commonly related to the concentrations of elements leached into the infusions. Therefore, the leaching efficiency (%), estimated as the ratio of element concentration in tea infusions to its total concentration in tea leaves, has been calculated. According to the results in **Table S6**, elements could be classified into three groups; highly extractable elements (> 50%): Cs, Tl, Ni, and Rb, moderately extractable elements (10-50%): Li, Be, Cu, Ti, Co, As, Al, Cr, P, Mg, Mn, Si, Zn and K, and poorly extractable elements (< 10%): Ga, Cd, Pb, V, Ba, Fe, Ca, and Sr. Some elements like Mo, Hg, Pt, Ag, Se, Sn, and Sb

show a very poor extraction behavior and their concentrations were very low or even non-detectable. These results show a good agreement with previous studies reported by Szymczycha-Madeja et al. [40] and Polechońska et al. [41]. The extraction behavior is related to the organic components of tea samples. Thus, the higher the organic contents (e.g., tannins, polyphenols, flavanols, etc.) present in the sample, the stronger the binding to the element it is, which reduces the leachability of elements [35, 40, 42]. According to the poor extraction efficiencies of Fe (< 4%), tea infusions cannot be considered as a beneficial dietary source of such an essential element.

5.4.4 Bioavailable element contents in tea samples

In vitro models have been proposed to predict the bioavailability of different nutrients for living organisms. These models are considered as simple, reproducible, and economical tools to investigate the stability of the digestive system, the transport and metabolism of the intestine. However, the *in vitro* models must be validated and optimized to control all those factors that may affect the bioavailability of nutrients at different stages of the GIT in living organisms.

In the present study, the bioavailability of elements in tea samples was studied by simulating the following factors: the human body temperature (37˚C), the mechanical muscular contractions of human body using the Rotabit orbital-rocking platform shaker, the gastric and intestinal conditions by using pepsin enzyme, pancreatin enzyme and bile salts, and by controlling the pH of the gastric and intestinal stages.

The dialysis method was applied for 18 tea samples with the highest element concentrations quantified in infusions. The calibration showed a linear range of 1 – 50 µg/L for these elements analyzed by ICP-MS, and a linear range of 1 – 50 mg/L for those elements analyzed by ICP-OES. Instrumental LODs and LOQs, as well as those related to the dialyzate, are listed in **Table 5.1** and **Table 5.2.**

Table S7 shows element concentrations in dialyzates, expressed as the mean of three replicates for each sample, recovered after simulating the gastrointestinal digestion process. Mercury was not detected in any of the dialyzate fractions analyzed. Macronutrient elements K, Ca, Mg and P were within the following ranges: 40.0 –

115.0, 0.14 – 5.64, 1.20 – 8.37 and 1.72 – 5.14 mg/L; respectively. Sr, Si, Ba, Rb, Ni, Zn, Mn, Al, Co and Cu were in concentrations ranging from < LOD – 67, < LOQ – 442.6, < LOQ – 15.6, 177 – 714.6, < LOD – 28, 42.3 – 102.4, 101.3 – 1117.9, 6.8 – 157, 0.1 – 1.4, 0.5 – 9.9 and 4 – 17 µg/L, respectively. Lithium was only quantified in the green tea sample (G4) with a concentration of 1.02 ± 0.07 µg/L. Beryllium was also quantified only in the black tea sample (B6) as 0.123 ± 0.042 µg/L. Ti, Ga, Hg, Cd, Pb, As, Cr, V, Sn, and Sb concentrations were lower than the LOD in the dialyzate fractions while Tl concentrations were lower than the LOQ. Fe showed non-detectable levels in all dialyzate fractions, and this result may be attributed to the inhibition of Fe absorption caused by the presence of polyphenols in tea samples [43].

Dialyzability percentages (%), given as the ratio of the element concentration in the dialyzate fraction to that in tea infusions, were calculated for each element and listed in **Table 5.3**. According to Moreda-Piñeiro et al. [44], element dialyzability percentages can be classified into three categories: high (> 40%), moderate (10 – 40%) and low (< 10%). Potassium, the most abundant element in tea leaves and their infusions, showed moderate to high dialyzability percentages ranging between 28 and 54%. Ca, Mg and P showed also elevated concentrations in tea leaves and tea infusions, but they are moderately dialyzable except Ca from green tea, which showed highly dialyzability levels (47 – 100%). High dialyzability percentages were found in the case of Cs (52 – 76%) and Zn (40 - 84%). Silicon also showed a relatively high dialyzability (24 – 86%) in black tea samples, while green, white and red tea dialyzates show moderate dialyzability. Robberecht et al. [29] reported that Si shows a moderate dialyzability (20%) from tea infusion samples using an *in vitro* continuous flow gastroduodenal simulation protocol. Low dialyzability percentages were found for Al and Mn (<1.2% and < 15%, respectively). Barium shows moderate dialyzability ratios (10 – 28%) in green tea samples and low dialyzability ratios in the other samples. Moderate bioavailability ratios were found in case of Cu (10 – 30%), Co (12 - 33%), P (14 – 26%), and Rb (20 – 37%) except B9 which showed a highly dialyzability (57 ± 2 %) and G4 which is not

detectable in the infusions studied. Strontium showed a wide range of dialyzability percentages ranging from 3 to 68%.
Nickel showed a relatively moderate to high dialyzability percentages ranging between 16 and 60% for those dialyzates with detectable levels of the element.

As mentioned before, to the best of our knowledge, the previously published studies related to element bioavailability in tea samples were conducted by solubility models and bioaccessibility approaches using ultrafilters, except for Si [29]. However, the present study proposes a method for such determination using dialysis membranes for GI simulation. As expected, the results obtained in our study were lower than those obtained in previous studies reported in the literature using bioaccessibility for Al, Mn, Mg, Ca, Fe, Cu, K, and Zn [27, 28]. For other elements like Ba, Sr, Co, Cs, Ni and Rb, there are no published studies that demonstrate their bioavailability tea samples.

5.4.5 Mass balance study

The accuracy of the gastrointestinal protocol was evaluated using a mass balance study. A black tea (B6) sample was selected as a representative sample to perform a mass balance test since it showed the highest levels of almost all the elements in tea infusions. For the dialyzable element, the total concentration in both fractions (the sum of dialyzable and non-dialyzable) was statistically compared with its total concentration determined in tea infusions. **Table 5.4** lists the total contents (in μg) of bioavailable elements in tea infusions, the total contents in the dialyzable fraction, the contents in the non-dialyzable fraction and the sum of dialyzable and non-dialyzable fractions. Mean values for total contents and the sum of dialyzable and non-dialyzable fractions were statistically compared by applying a t-test (95% confidence level) using STATGRAPHICS Centurion XVI software. P-values obtained by comparing the means of the two contents were also listed in **Table 5.4**. P-values were greater than 0.05 (95% confidence level) implying that there is no statistically significant difference between the means values, indicating a good recovery of the gastrointestinal digestion protocol.

Table 5.3 Elements' dialyzability percentages (mean value ± SD, % (n=3)) from tea infusions after the *in vitro* dialysis.

Code	B1	B2	B3	B6	B7	B9	B10	B16	B17
Cu	22 ± 2	18 ± 1	23 ± 2	12 ± 1	14 ± 1	27 ± 2	30 ± 1	22 ± 2	15 ± 2
Cs	68 ± 2	72 ± 3	66 ± 5	63 ± 2	63 ± 2	65 ± 2	68 ± 6	62 ± 2	60 ± 3
Co	27 ± 1	22 ± 3	27 ± 1	21 ± 2	12 ± 1	25 ± 1	33 ± 3	23 ± 3	21 ± 3
Ni	60 ± 14	-	-	26 ± 1	47 ± 1	19 ± 2	-	-	29 ± 10
Al	0.6 ± 0.01	0.2 ± 0.01	0.3 ± 0.02	1.2 ± 0.02	0.4 ± 0.05	0.4 ± 0.1	0.4 ± 0.1	0.2 ± 0.001	0.2 ± 0.1
Ba	14 ± 1	7 ± 0.4	6 ± 0.1	3 ± 0.4	6 ± 0.2	-	10 ±1	7 ± 2	7 ± 2
Rb	30 ± 2	20 ± 1	29 ± 2	30 ± 1	37 ± 1	57 ± 2	30 ± 3	32 ± 1	36 ± 1
Zn	72 ± 4	60 ± 6	50 ± 3	77 ± 7	67 ± 3	60 ± 1	63 ± 3	57 ± 4	40 ± 4
P	26 ± 1	16 ± 1	17 ± 1	25 ± 2	22 ± 2	22 ± 2	23 ± 1	25 ± 3	14 ± 1
Si	79 ± 4	86 ± 15	37 ± 6	-	24 ± 2	53 ± 4	48 ± 2	66 ± 1	-
Ca	41 ± 6	22 ± 3	18 ± 3	53 ± 6	41 ± 2	25 ± 1	19 ± 1	81 ± 18	13 ± 3
Mg	30 ± 2	14 ± 1	15 ± 1	20 ± 1	24 ± 1	25 ± 1	27 ± 1	24 ± 1	17 ± 1
Mn	14 ± 1	5 ± 0.3	8 ± 0.1	8 ± 0.3	12 ± 1	9 ± 1	11 ± 1	8 ± 1	5 ± 0.3
Sr	35 ± 2	16 ± 1	21 ± 1	9 ± 0.4	25 ± 0.3	12 ± 0.4	21 ± 2	-	-
K	46 ± 2	31 ± 1	35 ± 2	42 ± 1	32 ± 1	34 ± 1	50 ± 4	43 ± 1	51 ± 2

Table 5.3 (Continued)

Code	G1	G2	G4	G5	R1	R2	R3	W1	W2
Cu	18 ± 1	11 ± 1	20 ± 3	17 ± 1	11 ± 1	-	10 ± 2	20 ± 1	12 ± 1
Cs	58 ± 3	64 ± 4	61 ± 3	61 ± 1	76 ± 1	62 ± 5	56 ± 7	71 ± 2	52 ± 1
Co	22 ± 1	23 ± 1	24 ± 2	23 ± 2	24 ± 1	15 ± 2	19 ± 1	31 ± 1	14 ± 1
Ni	-	33 ± 1	32 ± 3	16 ± 1	18 ± 1	18 ± 1	16 ± 1	27 ± 3	22 ± 1
Al	0.7 ± 0.1	0.4 ± 0.1	0.7 ± 0.04	0.4 ± 0.02	0.5 ± 0.01	0.6 ± 0.06	-	0.8 ± 0.2	0.4 ± 0.01
Ba	-	28 ± 1	24 ± 1	20 ± 1	4 ± 0.2	5 ± 0.3	-	10 ± 1	13 ± 1
Rb	23 ± 2	26 ± 1	-	32 ± 2	26 ± 2	26 ± 2	24 ± 1	21 ± 1	29 ± 1
Zn	66 ± 4	58 ± 1	63 ± 3	70 ± 2	38 ± 3	68 ± 4	84 ± 6	82 ± 1	51 ± 3
P	28 ± 2	21 ± 1	17 ± 1	23 ± 1	15 ± 1	17 ± 1	19 ± 3	22 ± 1	17 ± 2
Si	34 ± 1	27 ± 1	17 ± 1	42 ± 8	20 ± 1	-	29 ± 11	40 ± 1	-
Ca	58 ± 13	100 ± 5	47 ± 4	36 ± 3	11 ± 2	12 ± 1	43 ± 4	28 ± 1	34 ± 7
Mg	28 ± 2	26 ± 1	20 ± 1	26 ± 1	13 ± 1	19 ± 2	22 ± 2	23 ± 1	18 ± 2
Mn	12 ± 1	15 ± 2	11 ± 1	12 ± 1	5 ± 0.01	9 ± 1	9 ± 0.4	8 ± 0.2	8 ± 1
Sr	15 ± 1	68 ± 2	30 ± 1	31 ± 3	3 ±1	8 ± 1	-	17 ± 1	18 ± 2
K	49 ± 3	39 ± 1	31 ± 2	37 ± 2	28 ± 1	34 ± 3	43 ± 1	54 ± 3	35 ± 1

Table 5.4 Mass balance study for the gastrointestinal protocol of brewed tea sample B6.

Element	Total content in infusion	Dialyzable content	Non dialyzable content	Dialyzable + non-dialyzable content	P value
Cu	1.3 ± 0.004	0.157 ± 0.009	1.11 ± 0.086	1.26 ± 0.086	0.3493
Cs	0.33 ± 0.001	0.173 ± 0.006	0.17 ± 0.008	0.34 ± 0.01	0.0591
Co	0.024 ± 0.001	0.006 ± 0.002	0.017 ± 0.002	0.023 ± 0.002	0.2697
Ni	1.02 ± 0.003	0.262 ± 0.004	0.79 ± 0.053	1.06 ± 0.053	0.4365
Al	226.6 ± 0.7	3.1 ± 0.3	224.9 ± 16.9	228.1 ± 16.7	0.884
Ba	1.06 ± 0.003	0.036 ± 0.004	1.02 ± 0.064	1.06 ± 0.065	0.4408
Rb	25.8 ± 0.1	10.7 ± 3.5	15.8 ± 2.9	26.5 ± 4.6	0.8052
Zn	1.2 ± 0.04	0.9 ± 0.12	0.4 ± 0.03	1.3 ± 0.12	0.2833
P	87.2 ± 1.5	26.1 ± 7.9	57.6 ± 2.6	83.7 ± 8.3	0.512
Si	14.4 ± 0.04	6.8 ± 1.04	6.8 ± 1.2	13.7 ± 1.5	0.4644
Ca	43.5 ± 1.9	22.9 ± 2.8	26.7 ± 1.9	49.5 ± 3.4	0.0689
Mg	282.3 ± 0.9	71.2 ± 19.2	202.4 ± 13.8	273.6 ± 23.6	0.5581
Mn	67.9 ± 0.2	4 ± 0.003	66.4 ± 4.9	70.4 ± 4.9	0.4271
Sr	0.36 ± 0.001	0.031 ± 0.001	0.34 ± 0.03	0.37 ± 0.033	0.5116
K	2387 ± 326	997 ± 13	1591 ± 90	2588 ± 91	0.3648

5.5 CONCLUSIONS

Precise, accurate and sensitive methods were used for total determination of 33 elements (Li, Be, Ti, Ga, Cu, Ag, Hg, Cd, Cs, Pt, Tl, Pb, As, Cr, Co, Ni, V, Se, Sn, Sb, Rb, Ba, Al, Fe, Zn, Si, Ca, Mg, Mn, Mo, Sr, P and K) in digested tea samples, their infusions as well as dialyzate fractions by ICP-MS, ICP-OES and FAES. To the best of our knowledge, this is the first study that uses an *in vitro* dialyzability approach to evaluate the bioavailability of trace elements in tea samples.

K was the most abundant element in both digested tea samples and their infusions followed by Ca, Mg and P. The leaching efficiencies of the elements from tea leaves to infusions were evaluated and elements were classified according to their leaching

efficiency to high, moderate, and low extractable elements. Elements present at high concentrations in tea leaves (K, Ca, Mg and P) show either moderate (K, Mg, and P) or poor (Ca) leaching efficiencies. The low extractability of some elements such as Ca and Fe could be related to the organic compounds (polyphenols, tannins, etc.) present in tea samples. Iron low extractability leads to non-detectable levels in the dialyzable fractions; thus, tea infusions cannot be considered as a good source of this element for nutritional purposes. Tea could be considered as a rich dietary source of K, P, Mg, Mn, Ca, Si and Rb since they showed the highest bioavailability percentages, especially K and Ca followed by Mg. On the other hand, toxic elements (As, Cd, Hg, and Pb) are present at very low concentrations in tea leaves and were not detected in the dialyzate fraction. Therefore, considering the element concentrations in the tea infusions analyzed, as well as the results obtained in the dialyzability study, we can conclude that the consumption of these tea samples does not pose a risk for consumer's health and provide the organism of some the essential elements.

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CHAPTER 6

Bioavailability of Tea Polyphenols: an *in vitro* dialyzability study

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Bioavailability of Tea Polyphenols: an *in vitro* dialyzability study

6.1 Abstract

An *in vitro* gastrointestinal (GI) protocol was performed to determine the bioavailable phenolics compounds in commercial tea samples including black, green, red, and white teas. The GI simulation was employed using dialysis membranes modeling. The total phenolic content (TPC) in tea infusions and the dialyzable fractions was evaluated using Folin-Ciocalteu spectrophotometric protocol. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay was used to assess the antioxidant activity (AA) before and after the GI dialysis. Results have shown that the TPC and the AA in the tea infusions were in the range of 87 – 1580 mg GAE/L and 1 – 9.9 mM TRE, respectively. TPC in the dialyzate fractions of the selected tea samples was ranged between 62 – 160 mg GAE/L. TPC was reduced after the GI digestion and 76 – 94 % of the original TPC in tea infusions was lost. A mass balance study was performed to test the trueness of the GI simulation

6.2 Introduction

C. Sinensis infusion, commonly known as tea, is the second most consumed beverage in the world after water [1]. It is one of the most remarkable sources for bioactive compounds (i.e., antioxidants like polyphenols, flavonoids, phenolic compounds, etc.). Antioxidants are known for their important role that affects human health, especially those issues related to reducing or preventing the deleterious consequences of oxidative stress [2].

Tea is classified into four main types (green, white, oolong, and black) according to its degree of oxidation before drying [3]. Green tea is produced of young leaves after the rolling and steaming process to reduce the oxidation [4]. Green tea usually contains the highest level of antioxidants, among them catechin (C) and its primary

compounds like epicatechin (EC), epicatechin gallate (ECG), gallocatechin (GC), epigallocatechin (EGC), catechin gallate (CG) and epigallocatechin gallate (EGCG) [5]. On the other hand, black tea is the fully fermented one with the lowest contents of antioxidants. The production process of black tea involves bringing the phenolic compounds of the rolled young leaves into contact with polyphenol oxidase, where catechins are oxidized to theaflavins and thearubigins [6]. White tea, recognized by its buds coated with delicate white hair, is produced by light oxidation of young leaves by exposing the buds to sunshine withering [7]. Oolong tea (semifermented tea) is commonly consumed in China and Japan [8], it is produced by partial oxidation of phenolic compounds to form dimeric and oligomeric phenolic polymers like theasinensins [9]. Tea composition is affected by season, leaves' age, horticultural practices, climate, etc. [10].

Studying the bioavailable fraction of nutrients (in this case, antioxidants) for the body and their metabolic fate is essential to understand their possible beneficial effects for human health. Before becoming bioavailable, nutrients should be leached from the food/beverage matrix into the gastrointestinal tract (GIT). The fraction of nutrients released from the food matrix into the GIT is called the bioaccessible fraction [11]. The bioaccessible fraction of antioxidants is controlled by different factors related to the antioxidants such as their initial concentration, solubility, stability against the GIT conditions, permeability and metabolic transformations. The food matrix may also affect the bioaccessibility of antioxidants [12]. Many studies have been conducted to evaluate the bioaccessibility of antioxidants in tea infusions after performing an *in vitro* GI digestion, as well as the impact of additives, including sweeteners and ascorbic acid [13-15]. However, bioaccessibility gives a less realistic indication about the bioprocesses (including absorption and metabolism of nutrients) that happen in the real digestion stage once it is available. Therefore, bioavailability is proposed to understand what happens to the fraction of ingested nutrients that reaches the bloodstream. Thus, bioavailability already includes the bioaccessibility function. Bioavailability can be carried out using either *in vivo* (using living organisms) or *in vitro (*GI simulation) methods. *In vitro* assessment

studies established on dialysis were reported for polyphenol assessments in food and drinks such as nuts and seeds [16], apple [17], soluble coffee [18], wheat [19], fruit wines [20]. Matsingou et al. [21] and Alexandropoulou et al. [22] studied the effect of iron and other dietary factors on the antioxidant activity in black and green teas using an *in vitro* gastrointestinal digestion in terms of dialysis model.

Both spectrophotometric techniques and/or chromatographic methods are commonly used to perform the antioxidant evaluation. Spectrophotometric techniques are typically used for estimating the total contents of antioxidants, while chromatographic techniques [8, 23, 24] were used for the quantitative analysis of individual antioxidant.

To the best of our knowledge, the published reports available in the literature used to describe the bioavailability of tea polyphenols in terms of solubility and bioaccessibility approaches. The present work proposes a more realistic approach for evaluating the bioavailability of polyphenols in several types of tea based on an *in vitro* dialysis protocol to simulate the gastrointestinal digestion. Thus, the total phenolic contents in tea samples and the antioxidant activity were evaluated before and after the gastrointestinal digestion.

6.3 MATERIALS AND METHODS

6.3.1 Instrumentation

Spectrophotometric measurements were conducted using UV-Visible spectrophotometer Hitachi Model U-2100. *In vitro* bioavailability assessment was carried out using a Boxcult incubator chamber situated on a Rotabit orbital-rocking platform shaker (J.P. Selecta, Barcelona, Spain). Cellu Sep® H1 high-grade regenerated cellulose tubular membranes (10 kDa molecular weight cut off, 50 cm in length, 25.5 mm in dried diameter, and volume to length ratio of 5.10 mL/cm) from Membrane Filtration Products Inc. (Seguin, TX, USA) were used to perform the dialyzability procedure. Ultrapure water of resistance 18 MΩ cm was obtained from a Milli-Q purification device (Millipore Co., Billerica, MA, USA).

6.3.2 Reagents

In vitro dialyzability procedure was carried out using porcine pepsin p-7000, porcine pancreatin P-1750, bile salts (approximately 50% sodium cholate and 50% sodium deoxycholate) and piperazine-NN-bis (2-ethanesulphonic acid) di-sodium salt (PIPES) which were obtained from Sigma Chemicals (St. Louis, MO, USA). Sodium hydrogen carbonate and hydrochloric acid (37%) from Merck (Poole, Dorset, UK) were used to prepare the intestinal solution and gastric solution, respectively. Folin-Ciocalteu Reagent (FCR) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were acquired from Sigma-Aldrich. (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) from sigma-Aldrich was used to evaluate the AA. Gallic acid monohydrate (Sigma-Aldrich) was used to prepare a stock standard solution of (5000 mg/L) in methanol. This stock solution was used to prepare the daily working solutions by appropriate dilution with water. Stock standard solutions were kept away from light and stored at - 20 °C.

6.3.3 Tea samples preparation

Tea samples acquired at different markets were prepared according to the manufacturer's instructions. Several *C. Sinensis teas* including 19 black teas (B), 7 green teas (G), 3 red teas (R), 2 white teas (W), and non-*C. Sinensis* teas including verbena leaves (VL), red fruits (RF), and red rooibos (RR) were analyzed. Table 6.1 shows tea samples studied including their types, origins and additive flavors.

Tea infusions were prepared by steeping 1.5 g of each tea sample in 150 mL boiled ultrapure water for 5 min. The infusions were left to cool, filtered to remove the leaves and stored at -20˚C in capped polyethylene bottles until the analysis.

6.3.4 *In vitro* dialyzability protocol

The *in vitro* GI simulation was conducted according to the procedure outlined by Moreda-Piñeiro et al. [16] with minor modifications, where 20 mL of tea infusion was subjected to the *in vitro* digestion protocol. Blank solutions, using ultrapure water instead

of tea infusion, were also subjected to the *in vitro* protocol to control the possible contamination. Dialyzates, and non-dialyzate fractions, were stored in polyethylene tubes at -20˚C until the analysis. The *in vitro* bioavailability approach was applied for 18 tea samples out of 34 samples (9 black teas, 4 green teas, 3 red teas, and 2 white teas) in triplicate.

6.3.5 Total phenolic content

Total phenolic content (TPC) in tea infusions and dialyzates was determined using Folin-Ciocalteu (FC) spectrophotometric protocol [25]. Briefly, in the case of tea infusion, an aliquot of 250 µL of previously diluted tea sample (40-fold)) was mixed with 100 µL Folin-Ciocalteu Reagent (FCR) and 1 mL of 20 % (w/v) Na_2CO_3. The mixture was diluted to 5 mL with ultrapure water and incubated for 30 min at room temperature in the dark. Ultrapure water was used as a blank reagent. The analysis was performed in triplicate.

The same procedure was also applied in the case of the dialyzate fractions but using 500 µL of dialyzate without the dilution step. The absorbance of the developed blue color was monitored at 760 nm. TPC was expressed in terms of mg of gallic acid equivalents per liter (mg GAE/L) for both tea infusions and dialyzates.

6.3.6 DPPH radical-scavenging activity

DPPH scavenging activity was evaluated using the method of Brand-Williams et al. [26] with slight modifications. A volume of 100 µL of diluted tea sample (1:20) was mixed with 2.9 mL of 0.1 mM DPPH. The mixture was then vortexed vigorously and kept in the dark for 30 min at room temperature. The reduction of the absorbance was measured using the spectrophotometer at 517 nm. The AA was evaluated by plotting the absorbance of the resulting solutions against Trolox (0.05 – 0.8 mM), and results were expressed in mmol Trolox Equivalents per liter of the infusion (mmol TRE/L).

For the dialyzate fractions, a volume 500 µL of each dialyzate was brought to react with 2.5 mL of 0.1 mM DPPH for 30 min and the absorbance of the resultant solutions was measured at 517 nm. The calibration curve obtained by plotting the absorbance against Trolox

(1 – 25 µM) was used to calculate the AA (in µM TRE) after performing the *in vitro* dialysis protocol.

Table 6.1 Types, origins, additives and other characters of tea samples studied.

Code	Origin	Additives/other characters	Code	Origin	Additives/other characters
B1	Sri Lanka	Bergamot	**B18**	Azerbaijan	Bergamot
B2	EU	Tea Limon	**B19**	Sri Lanka	Bergamot
B3	Sri Lanka	-	**G1**	India	Cinnamon, Cardamom, ginger, cloves, and pepper
B4	China	-	**G2**	China	Mint
B5	India	Bergamot	**G3**	Sri Lanka	Mint
B6	-	-	**G4**	China	Mint
B7	Sri Lanka	Bergamot/ Decaffeinated	**G5**	-	-
B8	India	-	**G6**	China	Mint
B9	Sri Lanka	-	**G7**	China	-
B10	Sri Lanka	-	**R1**	China	-
B11	India	Cinnamon	**R2**	-	-
B12	Georgia	-	**R3**	India	-
B13	Yemen	-	**RF**	UK	Hibiscus, Apple, blackberry, orange peels
B14	India	-	**RR**	South Africa	Rooibos, Orange, Hibiscus/Decaffeinated
B15	India	-	**VL**	EU	Verbena
B16	India	Cinnamon, Cardamom	**W1**	China	-
B17	Sri Lanka	-	**W2**	China	-

6.4 RESULTS AND DISCUSSION

6.4.1 Total phenolic contents in tea samples

Total polyphenols contents were estimated in 34 tea samples, including *C. Sinensis* and non-*C. Sinensis* teas, using the Folin-Ciocalteu spectrophotometric method developed by Singleton and Rossi [25]. TPC was evaluated using standard addition calibration prepared with gallic acid standard solutions covering the range of 1 – 15 mg/L and showing good linearity (r = 0.999). Limit of detection (LOD) and limit of quantification (LOQ) were 23 mg GAE/L and 77 mg GAE/L, respectively. TPC was expressed as an arithmetic mean of mg equivalent of gallic acid per liter of infusion (mg GAE/L) and their standard deviation. TPC obtained for the analyzed samples are listed in **Table 6.2**.

The analysis of tea samples shows that white and green teas contain relatively higher levels of TPC than black and red teas. However, non-*C. Sinensis* teas had a relatively lower TPC than *C. Sinensis* teas. The variation in TPC between the same type of tea may be attributed to tea origin, age, processing, temperature and time of brewing.

TPC in black and green teas was ranged between 238 – 1316 and 571- 1524 mg GAE/L, respectively. These findings were lower than those reported by Khokhar and Magnusdottir [27] and Fatemeh et al. [28]. However, the average TPC in black tea (936 ± 260 mg GAE/L) and in green tea (1099 ± 359 mg GAE/L) was slightly higher than those obtained by Manzocco et al. [29] (801 and 953 mg GAE/L for black and green teas, respectively).

TPC in white teas W1, and W2 was 701 ± 13 and 1580 ± 30 mg GAE/L, respectively, with an average value of 1141 ± 30 mg GAE/L that corresponds to that obtained by Venditti et al. [30] (ca. 1120 mg GAE/L), but slightly lower than those obtained by Castiglioni et al. [31] (ca. 1540 – 3222 mg GAE/L).

Table 6.2. TPC and AA in tea samples (mean ± standard deviation, n=3).

Code	TPC (mg GAE /L)	AA (mM TRE)	Code	TPC (mg GAE /L)	AA (mM TRE)
B1	876 ± 17	7.8 ± 0.1	**B18**	1082 ± 6	2.3 ± 0.1
B2	705 ± 24	4.5 ± 0.2	**B19**	238 ± 6	1.97 ± 0.1
B3	1061 ± 10	7.3 ± 0.1	**G1**	571 ± 7	6.1 ± 0.2
B4	1050 ± 14	5.3 ± 0.2	**G2**	1264 ± 13	9.1 ± 0.1
B5	978 ± 19	6.0 ± 0.2	**G3**	1524 ± 18	9.9 ± 0.1
B6	776 ± 28	4.8 ± 0.04	**G4**	1158 ± 32	9.2 ± 0.3
B7	1277 ± 34	9.3 ± 0.05	**G5**	650 ± 10	7.9 ± 0.03
B8	1149 ± 36	5.1 ± 0.1	**G6**	1145 ± 3	9.3 ± 0.1
B9	1316 ± 11	8.3 ± 0.1	**G7**	1379 ± 10	9.7 ± 0.1
B10	653 ± 10	8.1 ± 0.1	**R1**	751 ± 24	3.4 ± 0.1
B11	1055 ± 18	5.2 ± 0.1	**R2**	410 ± 7	3.2 ± 0.2
B12	975 ± 6	3.8 ± 0.2	**R3**	543 ± 20	4.1 ± 0.2
B13	968 ± 16	8.8 ± 0.1	**RF**	87 ± 6	2.0 ± 0.02
B14	710 ± 5	3.3 ± 0.1	**RR**	178 ± 6	0.98 ± 0.1
B15	1014 ± 11	4.3 ± 0.1	**VL**	366 ± 17	2.3 ± 0.1
B16	689 ± 65	5.4 ± 0.1	**W1**	701 ± 13	6.4 ± 0.2
B17	1215 ± 22	9.9 ± 0.1	**W2**	1580 ± 30	9.5 ± 0.1

TPC in white teas W1, and W2 was 701 ± 13 and 1580 ± 30 mg GAE/L, respectively, with an average value of 1141 ± 30 mg GAE/L that corresponds to that obtained by Venditti et al. [30] (ca. 1120 mg GAE/L), but slightly lower than those obtained by Castiglioni et al. [31] (ca. 1540 – 3222 mg GAE/L).

Red tea, a partially oxidized class of *C. Sinensis*, samples analyzed herein R1, R2, and R3 contain TPC of 751 ± 24, 410 ± 7, and 543 ± 20 mg GAE/L, respectively. Red rooibos tea (RR), derived from *Aspalathus linearis* had TPC of 178 ± 6 mg GAE/L. Almajano et al. [32] obtained higher amounts of TPC in red tea and red rooibos infusions of 825 ± 117 and 881 ± 85.2 mg GAE/L, respectively.

Verbena leaves (VL), a *non-C. Sinensis* plant known for its therapeutic and health benefits, was also studied to assess the TPC. TPC in VL (366 ± 17 mg GAE/L, ca. 41.8 ± 1.9 mg GAE/g) was higher than that obtained by Shikanga et al. [33] who found values in the range of 8.7 - 14.8 mg GAE/g. Red fruits commonly contain berry fruit alongside other fruits like apple, orange, and hibiscus. According to Hidalgo et al. [34], red fruits are considered as a rich antioxidant source; however, TPC obtained here for RF infusion was very low comparing to the other infusions analyzed (88 ± 6 mg GAE/L).

6.4.2 DPPH radical scavenging activity in tea samples

The AA was estimated using the DPPH radical scavenging assay, in which the stable purple-colored DPPH radical is reduced to a yellow-colored diphenylpicrylhydrazine (nonradical derivative) by hydrogen donating antioxidant. The AA in tea infusions was quantified utilizing the Trolox calibration curve and expressed as mmol Trolox Equivalents per liter of tea extract (mM TRE). LOD and LOQ were 0.05- and 0.2-mM TRE, respectively. **Table 6.2** lists the AA for tea samples analyzed.

The AA of green teas was ranged between 6.1 – 9.9 mM TRE, while white tea W1 and W2 show activity of 6.4 ± 0.2, and 9.5 ± 0.1 mM TRE, respectively. The results correspond to other previous studies reported by Rusak et al. [4] and Venditti et al. [30], but lower than those reported by Fatemeh et al. [28] in case of green tea (98.2 µg/mL, ca. 393 mM TRE). Also, black teas have a relatively similar AA to that reported by Venditti et al. [30] but lower than those found by Fatemeh et al. [28]. Meanwhile, the AA in red tea infusions (3.2 – 4.1 mM TRE) and red rooibos infusion (0.98 ±0.1 mM TRE) were lower than those reported by Almajano et al. [32] (1215 ± 91.7 and 746 ± 12.9 mM TRE, respectively) using 2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assay (TEAC). The antiradical activities of non-*C. Sinensis* tea RF and VL were 2 ± 0.02, and 2.3 ± 0.1 mM TRE, respectively.

Previous works [4, 31, 35] reported that the AA increases with increasing the TPC in the infusion. In the present study, a statistical analysis was performed between TPC and AA using STATGRAPHICS 18 package. A moderately strong positive

correlation with R= 0.7533 (at 95% confidence level) was found between TPC and AA in all samples studied. Moreover, green teas show a strong relationship between the TPC and AA (R = 0.9247 at 95% confidence level). The presence of different antioxidant varieties in the sample that act in different mechanisms of scavenging may explain the lack or presence of correlation between TPC and AA [36].

6.5 *IN VITRO* BIOAVAILABILITY OF TEA POLYPHENOLS

Several factors may affect the bioavailability of polyphenols such as their chemical structures, concentration in the dietary nutrients, intestinal and systemic factors related to the host body [37].

Bioavailability of tea polyphenols was evaluated by simulated GI digestion according to the procedure described by Moreda-Piñeiro et al. [16] and described in section (*In vitro* dialyzability protocol*).* The intestinal absorption was assessed by using dialysis membranes that allow the diffusion of specific molecular weight species. Dialysis membranes act as an efficient control of chemical interactions and give a more realistic view of the processes that take place in the intestine. TPCs (mean ± standard deviation, n = 3) in the dialyzable fractions were listed in **Table 6.3.** LOD and LOQ of TPC in the dialyzate were 1.3 and 4 mg GAE/L, respectively.

Table 6.3 lists the TPC in the dialyzable fractions and the calculated average of dialyzable TPC obtained from different tea samples after the GI simulation. For all samples analyzed, TPC varied between 62 – 160 mg GAE/L. Results show that green and white teas had predominant levels of TPC (80 ± 13 and 85 ± 35 mg GAE/L, respectively) over black and red teas (69 ± 29 and 37 ± 10 mg GAE/L, respectively) considering that the number of sample of each tea type was not similar for all. Considering the initial TPC amounts in tea infusions, TPC was reduced after the simulated GI digestion in all dialyzates. The TPC reduction was reported in other previous studies [35, 38] applied to black and green teas. According to Record et al. [35], the reduction of TPC may attribute to the change of pH. The authors simulated the GI digestion by only mimicking the pH of the stomach and the intestine and without using digestive enzymes. They

found that TPC was noticeably reduced after incubating green and black teas at slightly basic pH.

Table 6.3. Antioxidant activities of bioavailable TP in dialzsates (mean ± standard deviation, n=3)

Code	TPC (mg GAE/L)	AA (μM TRE)	% Decrease in the AA
B1	127 ± 3	87 ± 5	-98.9
B2	66 ± 1	38 ± 2	-99.2
B3	122 ± 5	72 ± 4	-99
B6	96 ± 10	34 ± 3	-99.6
B7	87 ± 1	93 ± 2	-99
B9	79 ± 1	109 ± 4	-98.7
B10	126 ± 2	78 ± 4	-99
B16	62 ± 1	53 ± 3	-99
B17	83 ± 1	66 ± 1	-99.3
G1	70 ± 1	70 ± 1	-98.9
G2	160 ± 12	99 ± 14	-98.9
G4	145 ± 10	71 ± 6	-99.2
G5	75 ± 1	75 ± 3	-99.1
R1	88 ± 3	39 ± 1	-98.9
R2	96 ± 10	29 ± 1	-99.2
R3	93 ± 3	48 ± 4	-98.9
W1	110 ± 6	60 ± 5	-99.1
W2	91 ± 1	123 ± 12	-98.8
Black tea dialyzates (mean)	94 ± 25	69 ± 29	
Green tea dialyzates (mean)	113 ± 47	80 ± 13	
Red tea dialyzates (mean)	92 ± 4	37 ± 10	
White tea dialyzates (mean)	101 ± 13	85 ± 35	

The dialyzability percentages were calculated using the following equation:

Dialyzability percentage (%) = $[TPC]_{dial}$ / $[TPC]_{inf}$ x100

Where $[TPC]_{dial}$ and $[TPC]_{inf}$ were the TPC in dialyzable fraction and the TPC in tea infusion expressed in mg GAE/L of the dialyzate and infusions, respectively. TPC bioavailability expressed in terms of dialyzability percentages (in %) was also shown in **Figure 6.1.**

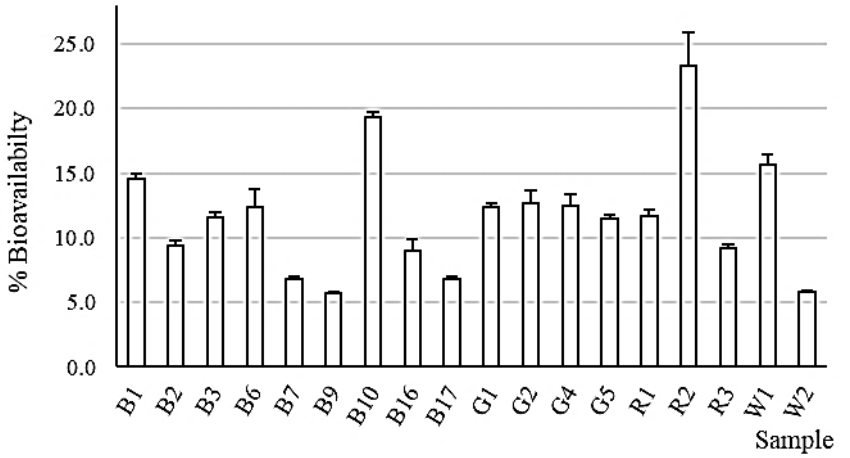

Figure 6.1 Bioavailability percentages of tea antioxidants in different types of tea.

Low bioavailability percentages ranged between 6 – 24% were observed for all tea samples analyzed. Black tea shows a significant variation in the bioavailability percentages between 6 – 20%, while green tea shows the lower variability of bioavailability percentages (i.e., the average percentage of 12.5 ± 0.2 %). Bioavailability percentages of red teas R1, R2, and R3 were 13 ± 1, 24 ± 3, and 9 ± 1%, respectively. TPC dialyzability percentages for white teas W1 and W2 were 17 ± 1 and 6 ± 1%, respectively. Thus, poor bioavailability percentages of phenolic compounds in all tea samples were obtained, which means that 76 – 94 % of native phenolics were lost during the GI digestion. TPC reduction was reported in tea samples [39] and soluble coffee samples [18]. They reported that the dialyzed coffee samples showed 5-fold lower TPC levels than the samples themselves.

To the best of our knowledge, this is the first study of tea polyphenols bioavailability using dialysis membranes to simulate the intestine walls. On the other hand, Tenore et al. [39] studied the bioavailability of individual tea phenolics (C, EC, ECG, EGC, CG, GC, and EGCG) by applying an *in vitro* GI model using Caco-2 cell lines to simulate the absorption in the intestine. They found that most of the original tea phenolics (91.8%) were lost after the GI digestion of different types of tea (white, green, and black). These findings agree with the results reported in our study.

On the other hand, other studies evaluate the bioavailability of tea phenols in terms of solubility and bioaccessibility. Considering these reports, the bioaccessible fraction is generally higher than the bioavailable one. For instance, Jilani et al. [40] studied the bioaccessibility of black and green tea phenolic compounds. These authors reported high bioaccessibility percentages (85% for black tea and 96% for green tea). The main difference is attributed to the approach used for bioavailability assessment; the reported data were achieved after applying a bioaccessibility approach, which is related to the maximum amount of nutrients that can be liberated to be bioavailable for the GIT.

Usually, bioavailability depends on the stability of nutrients toward the GIT conditions. Since phenolic compounds are gastro-sensitive substances, they suffer from degradation in a mild basic medium, which leads to poor absorption in the intestinal tract [39]. According to Green et al. [13], degradation of tea catechins may occur mainly during the intestinal phase, especially in the small intestine where the pH range is 6-8 and in the presence of reactive dissolved oxygen formed by the normal digestive process. These factors facilitate the degradation of polyphenols through epimerization and auto-oxidation [35]. Moreover, Green et al. [13] also pointed out that the pH conditions are responsible for tea catechins sensitivity rather than the digestive enzyme do. The bioavailability of black tea pigments (theaflavins and thearubigins) tends to be low due to their high MW and large polar surface area that leads to lowering their permeability through the membranes[41].

Moreover, other studies were conducted to determine the TPC bioavailability using the dialysis model in other types of food such as Chilean maqui berry [42] and Pomegranate juice [43]. The results obtained in these studies report the reduction of TPC after the GI digestion to lower than 29% of the initial amounts of TPC.

Finally, the effect of the *in vitro* model on the AA was evaluated by DPPH assay and the results were listed in **Table 6.3** LOD and LOQ were 0.8 and 2.6 μM TRE, respectively. The AA in dialyzate fractions was ranged between 29 – 123 μM TRE. The dialyzate fractions show a strong decline in the AA compared to the initial levels obtained before the GI digestion where about 99% of the AA was lost after the GI simulation. The reduction in the AA after the GI digestion might be greatly influenced by TPC levels, which were notably dropped after the GI digestion. This pattern (i.e., AA decline) was reported in other studies using the *in vitro* digestion protocol to evaluate the AA in different types of foods [20, 44, 45]. Donlao et al. [46] evaluated the AA of green tea in terms of bioaccessibility and reported that the total decrease in the AA was ranged between 16.0% to 25.7% of the original AA after the *in vitro* digestion. Previous works suggested that structural transformations in polyphenolic compounds might be induced during the intestinal environment leading to reduce AA [47, 48].

6.6 MASS BALANCE STUDY

The trueness of the GI dialysis protocol was assessed by employing mass balance measurements. Hence, TPC was analyzed in non-dialyzates fractions of tea infusion B6 saved in the section "*In vitro* dialyzability protocol".

TPC in the non-dialyzate fractions was 14.1 ± 0.6 mg GAE, while tea infusion B6 itself contains 15.8 ± 0.6 mg GAE, and the dialyzate fraction contains 2.0 ± 0.2 mg GAE. Both dialyzable and non-dialyzable fraction have a TPC of 16.2 ± 0.6. TPC in the original tea infusion was statistically (at confidence level 95%) compared with that calculated by summing up TPC in the dialyzable and non-dialyzable fractions. The comparison provided a p-value of 0.4923, which is higher than 0.05 (at 95% confidence level), which indicates

that TPC in the original infusion is statistically similar to that found in both dialyzable and non-dialyzable fractions. The mass study demonstrates the trueness of the *in vitro* approach.

6.7 CONCLUSIONS

The present work evaluated the amounts of phenolic compounds and their antioxidant activity in commercialized tea samples before and after an *in vitro* simulated gastrointestinal digestion using dialysis membranes. TPC in the green, white and black tea infusions show a higher level than those found in red tea and non-*C. Sinensis* infusions. The antioxidant activity was as follows: red teas and non-*C. Sinensis* infusions < black < green and white tea infusions. This study also shows a positive correlation between the TPC and the antiradical properties for the infusions analyzed. TPC was reduced after performing the dialysis study compared to that found in the original tea infusions. Low TPC bioavailability expressed in terms of dialyzability percentage was obtained for all samples analyzed. Green tea shows low bioavailability variation, while black tea shows a wide range of bioavailability percentages. The antioxidant activity in the bioavailable fraction was also reduced.

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CONCLUSION

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Conclusion

This thesis has focused on the determination of harmful endocrine disruptors (phthalate esters and bisphenol A), major and trace elements, tea antioxidants as well as the bioavailability of tea elements and tea polyphenols.

- Phthalate esters (PAEs) in tea infusions

Dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP), and butyl benzyl phthalate (BBP) were determined in tea infusions using high-performance liquid chromatography-electrospray ionization coupled to mass spectrometry (HPLC-ESI-MS). PAEs analyzed were preconcentrated by solid-phase extraction (SPE) using molecularly imprinted polymer (MIP) as a sorbent. The optimized MIP-SPE-LC-ESI-MS method show good sensitivity, precision, and high analytical recoveries.

Among PAEs studied, DEP levels were lower than detection limit in all the samples analyzed, while DBP was the most abundant phthalates in all tea samples. BBP and DMP were detected in some samples. The migration study proved that a fraction of PAEs comes from the bag itself. The contribution of tea-free bags to the total amounts of PAEs in tea infusions varied between 1.8 to 93.5%.

The composition of the bags was analyzed using Infrared spectroscopy indicating that the main components of the bags are either cellulose-based compounds, polyester materials, poly (ethylene terephthalate) (PET) or polylactic acid (PLA)-based compounds. When comparing the results obtained from the migration study and those related to the composition of the bags, we can conclude that the amount of PAEs released is not related to the composition of the bag. However, these phthalates found in tea infusions as well as tea-free bag infusion may be due to a possible contamination during the manufacturing process such as the use tea additives (colorants, synthetic essences, and flavor).

After the risk assessment study, we can conclude, considering the average Spanish tea consumption per day that the daily intake of tea containing the levels reported here has no risk for human health.

- Bisphenol A (BPA) in tea infusions

A sensitive, accurate and precise method based on MIP-SPE coupled to HPLC-ESI-MS has been developed to determine BPA from tea samples and their respective tea-free bag infusions.

The validated method was applied for the determination of BPA from the infusions of 34 tea samples and their respective bags. Only two tea samples showed a detectable level of BPA. The migration study (made by analyzing tea-free bag infusion) indicates that part of the detected BPA was released from the bag itself. The migration percentages of BPA from the tea bags to the tea infusion were 14% and 62%. Moreover, the BPA concentration in the tea infusion increased with the steep time.

Based on these results, we can conclude that the trace amount of BPA detected in the tea bags infusion might be due to other minor components added in the manufacturing process of the bags materials

- Major and trace elements in tea leaves and their infusions

The total concentrations of 33 elements, including major elements (Rb, Ba, Al, Fe, Zn, Si, Ca, Mg, Mn, Mo, Sr, P and K) and trace elements (Li, Be, Ti, Ga, Cu, Ag, Hg, Cd, Cs, Pt, Tl, Pb, As, Cr, Co, Ni, V, Se, Sn and Sb), from different types of tea were determined in both, tea leaves and tea infusions. The analysis was carried out using ICP-MS (Li, Be, Cr, Ti, Cu, Ga, Ag, Hg, Cd, Cs, Co, Pt, Tl, Pb, As, Ni, V, Se, Sn and Sb), ICP-OES (Al, Ba, Ca, Fe, Mg, Mn, Mo, P, Rb, Si, Zn, and Sr), y FAAS (K). The analytical performances of the methods were validated in terms of linearity, sensitivity, precision, and accuracy. Certified reference materials (Tea Leaves INCT-TL-1 and Rye Grass) were used to evaluate the accuracy of the elemental determination in the digested tea leaves and no statistical differences were found between the experimental (at 95% confidence level) and certified value. Moreover, the method shows good analytical recoveries for elements analyzed in digested tea leaves (95 – 114 %) and their infusions (92 – 115%).

Among the elements studied in the 35 samples tested here, K was the most abundant element in tea leaves in all samples followed by Ca, Mg, and P. The levels of toxic trace elements such as Cr, Cd, Pb, As and Hg were very low or even not detected in some samples. For tea infusions, Ag, Se, Pt, and Mo were not quantified in any tea samples analyzed, while Hg was quantified in four samples including one black teas, two green teas, and one herbal tea. K was the most abundant element followed by Mg, Ca and P. Very low concentrations of some elements like Fe, and Al were noticeably very low comparing to their level in the leaves which may attribute the complexation occurs between these elements and tea organic compounds like polyphenols.

On the other hand, the extraction efficiencies of elements from tea leaves into tea infusions were also evaluated. Elements were classified into three categories: highly extractable elements (>50%): Cs, Tl, Ni, and Rb, moderately extractable elements (10-50%): Li, Be, Cu, Ti, Co, As, Al, Cr, P, Mg, Mn, Si, Zn and K, and poorly extractable elements (<10%): Ga, Cd, Pb, V, Ba, Fe, Ca, and Sr, while some elements like Mo, Hg, Pt, Ag, Se, Sn, and Sb show a very poor extraction behavior and their concentrations were very low or even non-detectable

- Total antioxidant activity in tea infusion

The antioxidant activity of tea polyphenols was evaluated using spectrophotometric methods: total phenolic content (TPC) using Folin-Ciocalteu reagent (FCR) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay.

The TPC in 34 tea samples was evaluated using standard addition method prepared with gallic acid standard solutions covering the range of 1 – 15 mg/L, showing good linearity (r = 0.999) and the detection limit was 23 mg GAE/L.

Herbal teas possess, relatively, lower phenolic compounds than teas produced from *C. Sinensis* plant. Considering the TPC levels and the antioxidant activity of the samples analyzed, green tea possesses, relatively, the highest antioxidant activity, which is attributed to the minimal oxidation of phenolic compound during the production process. The antioxidant activity was in the order of red teas and non-

C. Sinensis infusions < black < green and white tea infusions. A moderate positive correlation (R = 0. 0.7533 at 95% confidence level) between TPC and antioxidant activity in all teas analyzed was found, while green teas showed a strong positive correlation between TPC and the antioxidant activity with correlation coefficient R= 0.9247 (at 95% confidence level).

- Bioavailability of tea elements and tea antioxidants.

The bioavailability of elements and tea polyphenols of the infusion was evaluated using dialyzability approach. The study was applied for 18 tea samples (9 black teas, 4 green teas, 3 red teas, and 2 white teas).

The method used for element determination in the dialyzable fraction was validated in terms linearity, sensitivity, and precision, while the accuracy of the method was evaluated in term of mass balance assay. Only fifteen elements out of 33 target elements were dialyzable from all or some tea samples analyzed, and these elements were Cu, Cs, Co, Ni, Al, Ba, Rb, Zn, P, Si, Ca, Mg, Mn, Sr, and K. Elements were classified into three main categories: highly dialyzable (> 40%) such as Cs and Zn, moderately dialyzable (10 – 40%) such as Cu, Co, P, and Rb, and low dialyzable (< 10%) such as Al and Mn. However, other elements show moderate to high dialyzability percentage such as K and Si in black teas. Ca shows a moderate dialyzability in all tea samples analyzed except in green tea where it shows high dialyzability. Iron was non-dialyzable in all samples analyzed which may attribute to its low leaching efficiency, the possibility of forming complexes with tea polyphenols, or even forming insoluble compounds in the basic medium of the intestine. Toxic elements like As, Cd, Hg, and Pb were present at very low concentrations in tea leaves and were not detected in the dialyzate fraction. Therefore, the presence of these elements in tea leaves does not pose a risk for human health. Tea could be considered as a rich dietary source of K, P, Mg, Mn, Ca, Si and Rb due to their considerable bioavailability percentages considering their total concentrations in the infusions, especially K and Ca followed by Mg.

Bioavailable tea polyphenols in the dialyzable fractions were evaluated in terms of TPC and DPPH assays. After the *in vitro*

gastrointestinal simulation, TPC was reduced by 76 – 94 % of the original TPC in tea infusions. Green and white teas show predominant levels of TPC over black and herbal teas. Moreover, tea lost approximately 99 % of the its antioxidative activity after performing the gastrointestinal simulation. The reduction of the antioxidative character of tea polyphenols may be attributed to various factors such as complexation of polyphenols with metal ions like Al and Fe, and the gastrointestinal sensitivity of the phenolic compounds during the gastrointestinal digestion, especially the change of the pH from acidic (gastric stage) into slightly basic (intestinal stage).

As a conclusion, the bioavailability of the elements present in tea infusion may be affected by the presence of phenolic compounds in tea infusion and vice versa.

As a conclusion of this thesis, we have observed that the concentrations of phthalates, bisphenol A and toxic elements in tea are not a risk to human health. The results also indicate that only a small fraction of trace elements and antioxidants from tea is bioavailable except for some essential elements such as K, Ca, Mg and Mn.

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Annex I
Supplementary data

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Annex 1. Supplementary data

Table S1 Types, origins, additives and other characters of the sample studied.

Code	Origin	Additives/other characters	Code	Origin	Additives/other characters
B1	Sri Lanka	Bergamot	**B18**	Azerbaijan	Bergamot
B2	EU	Tea Limon	**B19**	Sri Lanka	Bergamot
B3	Sri Lanka		**G1**	India	Cinnamon, Cardamom, ginger, cloves and pepper
B4	China		**G2**	China	Mint
B5	India	Bergamot	**G3**	Sri Lanka	Mint
B6	-		**G4**	China	Mint
B7	Sri Lanka	Bergamot/ Decaffeinated	**G5**	-	
B8	India		**G6**	China	Mint
B9	Sri Lanka		**G7**	China	
B10	Sri Lanka		**R1**	China	
B11	India	Cinnamon	**R2**	-	
B12	Georgia		**R3**	India	
B13	Yemen		**RF**	UK	Hibiscus, Apple, blackberry, and orange peels
B14	India		**RR**	South Africa	Rooibos, Orange, Hibiscus/Decaffeinated
B15	India		**VL**	EU	Verbena
B16	India	Cinnamon, Cardamom	**W1**	China	
B17	Sri Lanka		**W2**	China	

Table S2. ICP-MS Operating conditions

ICP-MS Operating conditions	
Nebulizer	PFA-ST Microflow Nebulizer
Spray Chamber	PC^3 Peltier Cooler- Quartz cyclonic
Triple Cone Interface Material	Nickel
Plasma Gas Flow	16.0 L /min
Auxiliary Gas Flow	1.2 L /min
Nebulizer Gas Flow	0.91 L /min
Sample Uptake Rate	400 µg /min
RF Power	1600 W
Integration Time	50 ms
Replicates per Sample	3
Mode of Operation	KED 1ml /min He and KED 4 ml /min He
Internal standards	10 µg/L ^{74}Ge, ^{89}Y, ^{103}Rh
***m/z* ratio**	KED1: ^{7}Li, ^{9}Be, ^{65}Cu, ^{63}Cu, ^{98}Ga, ^{110}Ag, ^{133}Cs, ^{195}Pt, ^{205}Tl, ^{208}Pb, ^{111}Cd, ^{202}Hg.
	KED4: ^{75}As, ^{52}Cr, ^{59}Co, ^{51}V, ^{47}Ti, ^{60}Ni, ^{77}Se, ^{118}Sn, ^{121}Sb

KED: Kinetic Energy Discrimination

Table S3. ICP-OES Operating conditions

Component/Parameter	Type/Value/Mode
Radiofrequency power/W	1300
Nebulizer type	Crossflow
Plasma flows (L/min)	16.0
Auxiliary flows(L/min)	0.5
Nebulizer flows (L/min)	0.80
Sample uptake rate/mL /min	1.5 mL/min
Integration time/s	5 s
Stabilization delay/s	40
Number of replicates	5
Working mode	Axial except for Ba and Sr in Radial
Detection wavelengths/nm	Al *308*, Ba *233*, Ca *317*, Fe *239*, Mg *285*, Mn *257*, Mo *203*, P *213*, Si *212*, Zn *206* Sr *421*, Rb *780, K 766 (in dialyzate)*

Table S4. Elements levels in tea leaves (Mean ± SD in µg/g, n = 3)

Code	*Li*	*Ti*	*Cu*	*Ga*	*Cs*	*Pb*	*Cr*
B1	0.32 ± 0.02	14.1 ± 0.4	14.7 ± 0.4	3.5 ± 0.1	0.1 ± 0.001	0.24 ± 0.02	2.08 ± 0.17
B2	0.42 ± 0.05	10.02 ± 0.9	15.5 ± 0.3	3.6 ± 0.4	0.25 ± 0.03	0.46 ± 0.05	0.89 ± 0.01
B3	0.35 ± 0.03	13.2 ± 0.9	11.2 ± 0.7	2 ± 0.1	0.12 ± 0.01	0.1 ± 0.01	2.8 ± 0.16
B4	0.21 ± 0.006	10.2 ± 0.6	21.5 ± 1.9	2.17 ± 0.03	0.41 ± 0.02	0.4 ± 0.02	0.44 ± 0.01
B5	0.3 ± 0.007	8.6 ± 0.6	13.5 ± 1.1	2.3 ± 0.2	0.17 ± 0.02	0.17 ± 0.003	5.49 ± 0.34
B6	0.33 ± 0.02	10.0 ± 0.21	19.9 ± 1.7	3.5 ± 0.2	1.22 ± 0.06	0.25 ± 0.03	1.81 ± 0.18
B7	0.36 ± 0.01	8.6 ± 0.6	17.9 ± 0.7	3.4 ± 0.1	0.17 ± 0.01	0.39 ± 0.04	5.95 ± 0.1
B8	0.37 ± 0.02	14.2 ± 0.5	12.6 ± 0.3	2.57 ± 0.05	0.066 ± 0.001	0.26 ± 0.01	3 ± 0.02
B9	0.29 ± 0.01	8.68 ± 0.1	23.6 ± 0.8	1.55 ± 0.03	0.09 ± 0.004	0.51 ± 0.01	3.07 ± 0.17
B10	0.29 ± 0.01	14.9 ± 0.3	15.3 ± 0.4	3.6 ± 0.1	2.23 ± 0.12	0.24 ± 0.005	11.33 ± 0.96
B11	0.25 ± 0.02	9.74 ± 0.7	20.4 ± 0.5	2.7 ± 0.2	2.23 ± 0.12	0.58 ± 0.02	0.24 ± 0.01
B12	0.28 ± 0.02	9.2 ± 0.4	15.7 ± 0.6	1.2 ± 0.02	0.24 ± 0.02	0.17 ± 0.01	< LOD
B13	0.28 ± 0.01	22.1 ± 2.1	12.5 ± 0.9	1.6 ± 0.1	0.41 ± 0.02	0.08 ± 0.004	1.82 ± 0.12
B14	0.37 ± 0.003	11.8 ± 0.5	16.5 ± 0.3	3.7 ± 0.1	0.1 ± 0.001	0.2 ± 0.02	13.52 ± 1.34
B15	0.31 ± 0.01	12.0 ± 0.4	17.9 ± 0.4	3 ± 0.2	0.08 ± 0.01	0.26 ± 0.02	10.59 ± 0.72
B16	0.43 ± 0.03	14.1 ± 0.8	17.3 ± 0.9	3.8 ± 0.1	0.24 ± 0.02	0.36 ± 0.03	21.15 ± 2.2
B17	0.06 ± 0.003	9.9 ± 0.1	27.9 ± 0.7	2.8 ± 0.1	0.08 ± 0.001	0.74 ± 0.03	0.41 ± 0.003
B18	0.04 ± 0.001	9.7 ± 0.02	17.4 ± 0.7	1.07 ± 0.03	0.16 ± 0.004	0.3 ± 0.03	0.12 ± 0.01
B19	0.09 ± 0.004	15. 1± 0.4	14 ± 0.3	2.37 ± 0.03	0.25 ± 0.01	0.17 ± 0.001	4.93 ± 0.19
G1	0.46 ± 0.03	8.3 ± 0.4	10.9 ± 0.4	1.9 ± 0.2	0.26 ± 0.01	0.82 ± 0.06	0.31 ± 0.03
G2	0.7 ± 0.06	17.4 ± 0.97	15 ± 0.8	1.6 ± 0.1	0.41 ± 0.03	1.55 ± 0.26	0.71 ± 0.06
G3	0.36 ± 0.03	14.5 ± 0.5	11.6 ± 0.2	1.85 ± 0.03	0.99 ± 0.03	0.33 ± 0.03	1.4 ± 0.08
G4	0.56 ± 0.03	32.6 ± 0.9	16.4 ± 0.6	3.5 ± 0.1	0.35 ± 0.02	1.22 ± 0.07	2.06 ± 0.11
G5	0.39 ± 0.02	9.2 ± 0.2	18.4 ± 0.6	3.37 ± 0.04	0.19 ± 0.003	1.22 ± 0.02	0.38 ± 0.01

Table S4. Elements levels in tea leaves (Mean ± SD in µg/g, n = 3) (Continued)

Code	*Li*	*Ti*	*Cu*	*Ga*	*Cs*	*Pb*	*Cr*
G6	0.33 ± 0.01	6.7 ± 0.7	12.3 ± 0.7	5.1 ± 0.4	2.71 ± 0.27	0.21 ± 0.01	1.24 ± 0.09
G7	0.3 ± 0.02	7.47 ± 0.1	17.3 ± 1.1	2.8 ± 0.1	0.02 ± 0.001	0.46 ± 0.05	0.25 ± 0.02
G8	0.3 ± 0.02	27.2 ± 1.8	9.4 ± 0.1	0.35 ± 0.02	0.01 ± 0.001	0.2 ± 0.01	0.02 ± 0.01
R1	0.49 ± 0.03	21.5 ± 1.1	21.4 ± 1.6	2.8 ± 0.1	0.51 ± 0.02	1.29 ± 0.04	2.74 ± 0.3
R2	0.52 ± 0.05	18.9 ± 0.7	20.5 ± 1.4	4 ± 0.4	0.69 ± 0.06	1.65 ± 0.08	2.28 ± 0.15
R3	0.34 ± 0.01	11.1 ± 0.1	19 ± 1.6	3.8 ± 0.1	0.09 ± 0.001	0.18 ± 0.01	9.48 ± 0.77
RF	0.65 ± 0.007	12.7 ± 0.4	5 ± 0.2	5.9 ± 0.2	0.05 ± 0.001	0.91 ± 0.07	1.2 ± 0.07
RR	1.53 ± 0.1	10.9 ± 0.3	4.9 ± 0.2	1.04 ± 0.03	0.03 ± 0.002	0.38 ± 0.02	2.03 ± 0.13
VL	0.44 ± 0.03	12.0 ± 1.4	12.6 ± 0.8	9 ± 0.7	0.16 ± 0.02	0.24 ± 0.001	1.33 ± 0.1
W1	0.48 ± 0.02	11.9 ± 0.3	28.6 ± 0.6	3.1 ± 0.1	0.14 ± 0.003	1.52 ± 0.06	1.11 ± 0.04
W2	0.3 ± 0.004	11.8 ± 0.1	18 ± 0.6	2.21 ± 0.01	1.14 ± 0.02	0.97 ± 0.09	0.29 ± 0.01

Table S4. Elements levels in tea leaves (Mean ± SD in µg/g, n = 3) (Continued)

Code	*Ni*	*V*	*Sn*	*Sb*	*Al*	*Ba*	*Rb*	*Fe*
B1	5.5 ± 0.28	0.18 ± 0.005	0.06 ± 0.004	0.027 ± 0.001	764.2 ± 1	46 ± 0.5	100.2 ± 2.2	171.9 ± 6
B2	7 ± 0.18	0.2 ± 0.022	0.12 ± 0.021	0.076 ± 0.004	1020.1 ± 30	47.5 ± 5	54.8 ± 3.6	151.1 ± 2
B3	4.4 ± 0.28	0.19 ± 0.011	< LOD	< LOD	874.3 ± 48	38.1 ± 2.2	102.3 ± 5.3	214 ± 16
B4	4.7 ± 0.28	0.14 ± 0.017	0.27 ± 0.017	< LOD	815.5 ± 78	40.5 ± 0.9	66.2 ± 1.5	103.2 ± 8
B5	6.8 ± 0.71	0.27 ± 0.06	0.06 ± 0.002	< LOD	983.9 ± 94	42 ± 5.6	38.5 ± 1.5	205.4 ± 24
B6	6.4 ± 0.28	0.52 ± 0.066	0.12 ± 0.018	< LOD	2474.1 ± 138	68.7 ± 2.5	96.4 ± 4.6	274 ± 29
B7	12.4 ± 0.09	0.18 ± 0.015	0.18 ± 0.01	0.017 ± 0.001	1013.7 ± 88	47.7 ± 5.1	225 ± 3	166.7 ± 13
B8	9.9 ± 0.1	0.07 ± 0.003	0.05 ± 0.004	< LOD	1085.6 ± 22	34.8 ± 0.5	143.8 ± 3.4	211.3 ± 5
B9	8.8 ± 0.26	0.21 ± 0.008	0.27 ± 0.015	< LOQ	879.7 ± 25	20.7 ± 0.5	33.1 ± 1.1	161.2 ± 11
B10	13.9 ± 0.33	0.35 ± 0.017	0.07 ± 0.003	0.041 ± 0.006	896.5 ± 20	49.4 ± 1.2	82.1 ± 1.2	268.3 ± 13

Table S4. Elements levels in tea leaves (Mean ± SD in µg/g, n = 3) (Continued)

Code	*Ni*	*V*	*Sn*	*Sb*	*Al*	*Ba*	*Rb*	*Fe*
B11	2.6 ± 0.11	0.18 ± 0.019	0.29 ± 0.00002	0.013 ± 0.001	1396.8 ± 48	34.1 ± 1.9	71.6 ± 2.1	90.8 ± 5
B12	1.8 ± 0.04	0.1 ± 0.02	0.11 ± 0.01	< LOD	541.6 ± 24	16.6 ± 0.7	64.4 ± 3.9	80.6 ± 4
B13	3.7 ± 0.3	0.19 ± 0.005	< LOD	< LOD	565.2 ± 21	21.6 ± 0.7	122.2 ± 5.2	237.4 ± 8
B14	8.6 ± 0.39	0.22 ± 0.018	< LOD	< LOD	758.7 ± 9	48.2 ± 0	77.3 ± 5.1	210.5 ± 10
B15	9 ± 0.43	0.22 ± 0.021	0.04 ± 0.002	< LOD	695.4 ± 38	38.6 ± 1.3	75.1 ± 6.4	218.3 ± 20
B16	10.9 ± 0.54	0.4 ± 0.028	< LOD	< LOD	1145.2 ± 17	49.1 ± 2.4	80.9 ± 5.8	360.7 ± 34
B17	6.4 ± 0.52	0.21 ± 0.021	0.4 ± 0.04	< LOD	877.2 ± 3	37 ± 0.9	40 ± 1.7	135.2 ± 6
B18	3.1 ± 0.04	0.11 ± 0.009	0.07 ± 0.004	< LOD	580.9 ± 7	14.1 ± 0.5	46.3 ± 0.1	106.1 ± 6
B19	5.3 ± 0.08	0.21 ± 0.005	< LOD	< LOD	609.7 ± 2	31 ± 0.3	85.9 ± 1.2	211.1 ± 6
G1	5.7 ± 0.4	0.19 ± 0.003	< LOD	< LOQ	892 ± 54	32.5 ± 2.8	77.3 ± 5.7	124.6 ± 2
G2	5.1 ± 0.43	0.65 ± 0.146	< LOD	0.02 ± 0.003	1044.8 ± 78	28.2 ± 2.8	126.8 ± 13.6	454.3 ± 51
G3	3.8 ± 0.3	0.27 ± 0.01	< LOD	< LOD	1320.6 ± 27	35.3 ± 0.6	114.5 ± 1.6	265.1 ± 61
G4	9.2 ± 0.1	1.45 ± 0.06	0.13 ± 0.004	0.062 ± 0.008	1716.3 ± 77	46.1 ± 1.5	51.1 ± 1.3	766.4 ± 6
G5	9.5 ± 0.07	0.28 ± 0.045	0.15 ± 0.008	0.04 ± 0.002	872 ± 24	45 ± 0.3	68.7 ± 1.9	164.4 ± 0.5
G6	9.1 ± 0.57	0.22 ± 0.05	0.07 ± 0.003	< LOQ	1908.1 ± 158	68.8 ± 5.5	93.6 ± 7.8	149.9 ± 6
G7	9.9 ± 0.25	0.12 ± 0.017	0.24 ± 0.017	< LOQ	782.6 ± 79	37.6 ± 1.9	6.2 ± 0.4	106.7 ± 14
G8	4.2 ± 0.01	0.73 ± 0.087	0.05 ± 0.001	< LOD	391.6 ± 42	4.8 ± 0.4	25.7 ± 2.2	361.9 ± 0.3
R1	5.9 ± 0.37	0.92 ± 0.107	0.21 ± 0.02	0.076 ± 0.008	1772.7 ± 126	53.6 ± 2.4	88.2 ± 2.8	752.2 ± 86
R2	11.8 ± 1.1	0.91 ± 0.152	0.28 ± 0.01	0.081 ± 0.001	1939.4 ± 74	59.7 ± 2.8	104.6 ± 7.3	639 ± 56
R3	8 ± 0.1	0.17 ± 0.014	< LOD	< LOD	730.6 ± 6	48.6 ± 0.7	74.1 ± 2.3	185.5 ± 11
RF	2.7 ± 0.03	0.75 ± 0.157	0.07 ± 0.006	< LOD	557.7 ± 64	103.3 ± 1.6	30.3 ± 0.6	495.7 ± 64
RR	4.2 ± 0.29	0.49 ± 0.037	0.1 ± 0.009	0.019 ± 0.001	260.4 ± 27	17.5 ± 0.4	7.1 ± 0.2	524.3 ± 50
VL	0.8 ± 0.09	0.59 ± 0.07	< LOD	< LOD	395.7 ± 28	201.7 ± 19.5	25.9 ± 1.7	323.7 ± 26
W1	8.3 ± 0.19	0.54 ± 0.011	0.27 ± 0.013	0.028 ± 0.002	1683.4 ± 83	59.3 ± 2.4	69.1 ± 3.2	257.6 ± 3
W2	5.7 ± 0.27	0.15 ± 0.005	0.16 ± 0.015	0.082 ± 0.013	778.7 ± 64	28 ± 0.7	217.7 ± 2	153.3 ± 10

Table S4. Elements levels in tea leaves (Mean ± SD in µg/g, n = 3) (Continued)

Code	*P*	*Si*	*Ca*	*Mg*	*Mn*	*Sr*	*Zn*	*K*
B1	2995 ± 22	206 ± 14	4628 ± 0.2	2128 ± 0.1	762 ± 22	35 ± 2.9	26 ± 1.1	20277 ± 1219
B2	2173 ± 201	170 ± 12	5364 ± 0.2	1541 ± 0.1	882 ± 61	23 ± 2.8	26 ± 2.6	16015 ± 966
B3	3147 ± 185	197 ± 8	5316 ± 0.1	2707 ± 0.02	1511 ± 5	69 ± 0.9	24 ± 1	19493 ± 1253
B4	3060 ± 145	104 ± 10	7960 ± 0.6	3273 ± 0.2	946 ± 39	68 ± 4.4	30 ± 1.2	24966 ± 509
B5	2126 ± 229	328 ± 35	5381 ± 0.1	1432 ± 0.03	878 ± 25	47 ± 3.2	22 ± 2.4	15585 ± 1929
B6	1846 ± 70	193 ± 18	9378 ± 0.7	3573 ± 0.2	1786 ± 111	48 ± 1.3	13 ± 1	17769 ± 514
B7	2621 ± 275	141 ± 10	5916 ± 0.2	2556 ± 0.1	992 ± 24	22 ± 2.7	30 ± 2.2	35234 ± 3215
B8	3277 ± 69	329 ± 27	5090 ± 0.2	2409 ± 0.1	2032 ± 121	47 ± 1.8	17 ± 0.5	28165 ± 527
B9	2659 ± 81	103 ± 4	5324 ± 0.1	2028 ± 0.1	305 ± 2	16 ± 0.7	34 ± 2	21554 ± 7
B10	3030 ± 14	258 ± 14	5548 ± 0.5	2437 ± 0.2	814 ± 66	32 ± 4.7	26 ± 0.4	22319 ± 846
B11	2057 ± 75	80 ± 5	8531 ± 0.4	2322 ± 0.2	411 ± 37	73 ± 5.3	20 ± 0.9	17387 ± 174
B12	2736 ± 123	72 ± 5	4714 ± 0.3	2490 ± 0.1	460 ± 36	25 ± 3	23 ± 0.5	22241 ± 1445
B13	3566 ± 137	204 ± 10	4063 ± 0.6	1952 ± 0.3	1045 ± 166	56 ± 10.4	30 ± 1.1	22776 ± 1959
B14	2920 ± 21	162 ± 4	5150 ± 0.4	2544 ± 0.2	870 ± 52	26 ± 2.8	30 ± 1.5	23918 ± 1212
B15	3065 ± 33	145 ± 13	5034 ± 0.1	2804 ± 0.03	1006 ± 41	22 ± 2	34 ± 2.1	24944 ± 1859
B16	2589 ± 16	283 ± 13	5729 ± 0.1	2292 ± 0.1	956 ± 22	24 ± 1.9	30 ± 1	21078 ± 47
B17	2676 ± 24	410 ± 42	4529 ± 0.2	1913 ± 0.1	497 ± 26	17 ± 0.3	38 ± 0.7	19031 ± 1084
B18	2843 ± 59	67 ± 2	3929 ± 0.2	2022 ± 0.1	443 ± 18	14 ± 0.9	25 ± 0.6	19897 ± 587
B19	2963 ± 16	200 ± 2	4766 ± 0.1	2065 ± 0.1	717 ± 10	37 ± 1.7	27 ± 0.2	18608 ± 1815
G1	2276 ± 112	178 ± 13	4648 ± 0.5	1706 ± 0.1	1296 ± 125	23 ± 0.3	26 ± 1.5	17076 ± 1039
G2	2771 ± 62	642 ± 11	7137 ± 0.4	3090 ± 0.1	921 ± 87	60 ± 2.6	29 ± 1.3	42458 ± 1898
G3	2592 ± 79	313 ± 22	6701 ± 0.4	2135 ± 0.2	1267 ± 22	59 ± 9.1	20 ± 0.2	31826 ± 1336
G4	2165 ± 82	1202 ± 21	8402 ± 0.2	3420 ± 0.9	881 ± 44	73 ± 1	23 ± 0.8	69796 ± 2527
G5	2478 ± 15	287 ± 29	4098 ± 0.1	1906 ± 0.2	1074 ± 91	48 ± 2.6	35 ± 0.7	16493 ± 99
G6	1747 ± 112	160 ± 14	6365 ± 0.5	2445 ± 0.2	1228 ± 82	34 ± 2.6	21 ± 0.9	37482 ± 1893

Table S4. Elements levels in tea leaves (Mean ± SD in µg/g, n = 3) (Continued)

Code	*P*	*Si*	*Ca*	*Mg*	*Mn*	*Sr*	*Zn*	*K*
G7	2582 ± 98	98 ± 9	5114 ± 0.5	1756 ± 0.1	537 ± 31	23 ± 1.9	34 ± 1.3	20208 ± 833
G8	4673 ± 501	1407 ± 135	11070 ± 0.7	3253 ± 0.2	107 ± 6	45 ± 3.2	43 ± 4.6	48887 ± 1772
R1	2575 ± 84	707 ± 46	8243 ± 0.4	3394 ± 0.2	1580 ± 102	44 ± 4.3	31 ± 0.7	18133 ± 1643
R2	2674 ± 138	667 ± 73	6785 ± 0.5	2848 ± 0.1	1362 ± 69	36 ± 3.5	34 ± 2.9	50967 ± 1961
R3	2950 ± 10	126 ± 3	5876 ± 0.3	2725 ± 0.1	897 ± 57	23 ± 0.2	35 ± 0.01	23594 ± 1438
RF	1288 ± 57	957 ± 65	9501 ± 0.003	1989 ± 0.1	379 ± 16	58 ± 1.2	29 ± 0.3	15589 ± 887
RR	737 ± 24	771 ± 60	10764 ± 0.8	1997 ± 0.1	126 ± 6	95 ± 3	15 ± 1.3	6411 ± 850
VL	1956 ± 170	1180 ± 133	15413 ± 0.9	3676 ± 0.1	58 ± 5	196 ± 27.1	22 ± 1.7	13670 ± 1200
W1	2242 ± 103	538 ± 13	8544 ± 0.5	3216 ± 0.2	1836 ± 119	57 ± 3	31 ± 0.6	17719 ± 601
W2	3046 ± 93	128 ± 6	4453 ± 0.2	2150 ± 0.1	739 ± 4	17 ± 1.4	41 ± 0.7	22461 ± 1120

Table S4.Elements levels in tea leaves (Mean ± SD in ng/g, n = 3)

Code	*Be*	*Co*	*Ag*	*Hg*	*Cd*	*As*	*Tl*
B1	14± 1	210 ± 2	10 ± 0.5	30 ± 3	20 ± 1	40 ± 3	45 ± 1
B2	< LOQ	440 ± 60	20 ± 5	20 ± 1	30 ± 1	120 ± 3	143 ± 4
B3	15 ± 1	180 ± 4	< LOD	< LOD	20 ± 1	40 ± 4	8 ± 1
B4	< LOQ	180 ± 10	< LOD	20 ± 2	20 ± 2	40 ± 2	89 ± 3
B5	< LOQ	340 ± 70	< LOD	< LOD	20 ± 3	30 ± 3	25 ± 2
B6	26 ± 2	250 ± 60	< LOD	< LOD	10 ± 1	6 ± 3	55 ± 3
B7	13 ± 1	370 ± 40	< LOD	60 ± 2	40 ± 1	40 ± 4	73 ± 7
B8	45 ± 2	70 ± 2	2 ± 0.4	50 ± 9	40 ± 1	80 ± 1	20 ± 1
B9	< LOQ	160 ± 3	4 ± 0.1	30 ± 1	20 ± 1	40 ± 3	64 ± 2
B10	25 ± 2	330 ± 10	11 ± 0.1	10 ± 2	30 ± 1	50 ± 2	80 ± 5

Table S4.Elements levels in tea leaves (Mean ± SD in ng/g, n = 3) (Continued)

Code	Be	Co	Ag	Hg	Cd	As	Tl
B11	< LOD	60 ± 2	6 ± 2	30 ± 8	40 ± 2	40 ± 3	276 ± 18
B12	< LOQ	100 ± 10	< LOD	< LOD	10 ± 1	10 ± 1	237 ± 21
B13	11 ± 1	170 ± 10	10 ± 1	< LOD	20 ± 1	30 ± 6	15 ± 1
B14	17 ± 1	310 ± 4	< LOD	< LOD	40 ± 1	50 ± 1	79 ± 3
B15	13 ± 0.3	330 ± 13	< LOD	< LOD	40 ± 1	60 ± 5	59 ± 1
B16	28 ± 2	450 ± 10	< LOD	< LOD	50 ± 1	80 ± 5	91 ± 5
B17	< LOQ	260 ±10	< LOD	30 ± 5	60 ± 4	40 ± 5	42 ± 1
B18	< LOD	150 ± 3	< LOD	20 ± 6	10 ± 1	20 ± 1	126 ± 3
B19	11 ± 1	230 ± 1	3 ± 0.1	10 ± 1	30 ± 1	30 ± 1	59 ± 1
G1	45 ± 1	1000 ± 110	4 ± 0.3	10 ± 1	80 ± 6	110 ± 1	56 ± 7
G2	36 ± 2	390 ± 30	< LOD	30 ± 4	70 ± 6	250 ± 15	54 ± 5
G3	16 ± 1	150 ± 10	< LOD	< LOD	20 ± 1	80 ± 5	123 ± 5
G4	36 ± 1	460 ± 30	10 ± 1	30 ± 1	60 ± 1	140 ± 8	55 ± 1
G5	18 ± 1	320 ± 10	10 ± 0.1	10 ± 3	60 ± 1	120 ± 1	44 ± 1
G6	17 ± 1	200 ±10	< LOD	30 ± 6	20 ± 2	60 ± 2	161 ± 15
G7	< LOQ	460 ± 40	19 ± 5	20 ± 3	10 ± 1	20 ± 1	10 ± 1
G8	12 ± 1	750± 90	15 ± 1	70 ± 6	19 ± 1	140 ± 14	4 ± 0.5
R1	32 ± 3	350 ± 20	< LOD	20 ± 1	40 ± 1	240 ± 11	40 ± 2
R2	34 ± 3	410 ± 70	10 ± 1	30 ± 2	60 ± 2	210 ± 5	69 ± 6
R3	14 ± 1	310 ± 3	< LOD	< LOD	50 ± 4	50 ± 1	77 ± 2
RF	52 ± 1	270 ± 20	< LOQ	20 ± 3	130 ± 5	160 ± 13	6 ± 1
RR	16 ± 1	300 ± 30	4 ± 0.2	40 ± 3	40 ± 3	140 ± 15	3 ± 0.1
VL	< LOD	120 ± 10	10 ± 1	< LOQ	30 ± 3	190 ± 9	10 ± 10
W1	36 ± 4	420 ± 20	2 ± 0.1	30 ± 2	60 ± 5	130 ± 10	30 ± 2
W2	< LOQ	330 ± 10	11 ± 1	50 ± 4	40 ± 3	100 ± 4	224 ± 8

Table S5. Elements levels in tea infusions (Mean ± SD in ng/g, n = 3)

Code	*Li*	*Be*	*Tl*	*Pb*	*Ga*	*Hg*	*Cd*
B1	40 ± 1	10 ± 1	21 ± 1	12 ± 0.1	100 ± 1	20 ± 2	2 ± 0.1
B2	50 ± 1	< LOQ	51 ± 1	23 ± 0.3	130 ± 2	< LOQ	1 ± 0.3
B3	40 ± 1	10 ± 1	8 ± 1	31 ± 0.3	150 ± 3	< LOD	3 ± 0.3
B4	50 ± 3	< LOQ	63 ± 1	74 ± 0.1	80 ± 2	< LOD	2 ± 0.2
B5	50 ± 1	< LOQ	16 ± 1	9 ± 0.3	220 ± 3	< LOD	2 ± 0.1
B6	130 ± 4	30 ± 1	37 ±1	52 ± 0.4	400 ± 3	< LOD	2 ± 0.2
B7	130 ± 1	30 ± 2	8 ± 1	54 ± 0.1	130 ± 1	< LOD	4 ± 0.2
B8	90 ± 1	10 ± 1	20 ± 1	18 ± 0.3	200 ± 1	< LOD	3 ± 0.1
B9	40 ± 1	< LOQ	30 ± 1	36 ± 0.1	90 ± 1	< LOD	2 ± 0.4
B10	80 ± 1	10 ± 1	34 ± 1	32 ± 0.1	130 ± 3	< LOD	2 ± 0.2
B11	50 ± 2	< LOD	118 ± 2	41 ± 0.2	90 ± 1	< LOD	1 ± 0.1
B12	50 ± 1	< LOQ	109 ± 1	8 ± 0.2	40 ± 1	< LOD	1 ± 0.3
B13	30 ± 1	4 ± 0.1	7 ± 0.5	< LOD	60 ± 1	< LOD	1 ± 0.2
B14	100 ± 2	10 ± 0.2	31 ± 1	49 ± 0.1	100 ± 1	< LOD	3 ± 0.4
B15	80 ± 3	12 ± 1	25 ± 1	12 ± 0.1	110 ± 1	< LOD	2 ± 0.3
B16	110 ± 1	11 ± 1	39 ±	102 ± 0.3	150 ± 3	< LOD	3 ± 0.2
B17	50 ± 1	< LOQ	22 ± 1	86 ± 1	170 ± 3	< LOD	5 ± 0.5
B18	50 ± 1	< LOD	63 ± 2	10 ± 0.3	50 ± 1	< LOD	1 ± 0.1
B19	70 ± 2	10 ± 0.2	44 ± 1	5 ± 0.1	150 ± 1	< LOD	4 ± 0.5
G1	100 ± 1	10 ± 1	36 ± 1	113 ± 1	110 ± 1	10 ± 2	7 ± 0.7
G2	130 ± 5	11 ± 0.2	27 ± 1	68 ± 1	140 ± 1	20 ± 1	5 ± 0.3
G3	80 ± 2	10 ± 1	76 ± 2	22 ± 0.3	120 ± 3	< LOQ	3 ± 0.5
G4	170 ± 4	13 ± 0.1	21 ± 1	47 ± 1	190 ± 1	< LOD	4 ± 0.1
G5	120 ± 2	10 ± 1	20 ± 1	57 ± 1	200 ± 2	< LOD	5 ± 0.4
G6	140 ± 1	11 ± 1	117 ± 2	7 ± 0.2	410 ± 4	< LOD	2 ± 0.1

Table S5. Elements levels in tea infusions (Mean ± SD in ng/g, n = 3) (Continued)

Code	*Li*	*Be*	*Tl*	*Pb*	*Ga*	*Hg*	*Cd*
G7	20 ± 1	< LOQ	5 ± 0.1	27 ± 1	90 ± 2	< LOD	1 ± 0.2
G8	1050 ± 23	10 ± 0.1	2 ± 0.2	38 ± 1	40 ± 1	< LOQ	9 ± 0.1
R1	100 ± 2	11 ± 1	16 ± 1	100 ± 1	370 ± 2	< LOD	4 ± 0.1
R2	110 ± 2	10 ± 1	19 ± 2	110 ± 1	360 ± 7	< LOD	4 ± 0.3
R3	130 ± 2	11 ± 1	34 ± 1	56 ± 1	190 ± 3	< LOD	3 ± 0.4
RF	70 ± 2	12 ± 1	2 ± 0.1	71 ± 1	1440 ± 21	20 ± 2	24 ± 0.2
RR	1380 ± 2	11 ± 0.5	2 ± 0.2	34 ± 1	640 ± 10	< LOQ	18 ± 0.1
VL	20 ± 1	< LOD	13 ± 1	10 ± 0.2	1020 ± 17	< LOD	3 ± 0.2
W1	180 ± 3	20 ± 1	17 ± 1	84 ± 1	220 ± 2	< LOD	4 ± 0.3
W2	70 ± 1	< LOQ	109 ± 2	55 ± 1	120 ± 2	< LOD	4 ± 0.3

Table S5. Elements levels in tea infusions (Mean ± SD in ng/g, n = 3) (Continued)

Code	*As*	*Cr*	*Co*	*V*	*Sn*	*Sb*
B1	6.9 ± 0.8	420 ± 20	62.2 ± 1.6	<LOQ	80 ± 17	73 ± 17
B2	41.2 ± 1.4	170± 4	101 ± 0.6	4.5 ± 0.4	< LOD	13 ± 0.8
B3	5.7 ± 0.1	360 ± 13	58.5 ± 2.2	<LOQ	< LOD	< LOD
B4	11 ± 1.2	110 ± 6	48.6 ± 0.9	1.8 ± 0.2	10 ± 0.9	< LOD
B5	6.7 ± 0.3	360 ± 7	94.2 ± 2.2	3.5 ± 0.5	8 ± 0.5	< LOD
B6	9.8 ± 0.8	920 ± 26	107.1 ± 2.8	3.4 ± 0.3	10 ± 0.1	< LOD
B7	8.7 ± 0.2	420 ± 6	54.2 ± 2	<LOQ	7 ± 0.9	79 ± 0.2
B8	19.4 ± 0.5	560 ± 4	139.4 ± 2.6	3 ± 0.4	45 ± 0.1	< LOD
B9	8.7 ± 0.9	170 ± 3	45.7 ± 0.5	1.3 ± 0.3	9 ± 0.5	< LOQ
B10	7.7 ± 0.6	1010 ± 16	72.1 ± 1.9	<LOQ	6 ± 0.2	45 ± 0.1
B11	11.5 ± 1.3	30 ± 2	20.8 ± 0.4	3.9 ± 0.9	6 ± 0.7	47 ± 0.6

Table S5. Elements levels in tea infusions (Mean ± SD in ng/g, n = 3) (Continued)

Code	*As*	*Cr*	*Co*	*V*	*Sn*	*Sb*
B12	6.7 ± 0.7	< LOD	30.5 ± 1.6	<LOQ	9 ± 0.2	< LOD
B13	6.2 ± 1.2	260 ± 3	41.8 ± 0.3	<LOQ	< LOD	< LOD
B14	20 ± 2.4	690 ± 7	71.4 ± 3.1	1.6 ± 0.1	< LOD	< LOD
B15	25 ± 1.2	1170 ± 2	71.7 ± 0.3	2 ± 0.4	41 ± 0.1	< LOD
B16	21 ± 1.4	840 ± 1	100.9 ± 0.9	2.2 ± 0.4	< LOD	< LOD
B17	10.6 ± 0.4	140± 3	112.1 ± 2	10.1 ± 0.8	7 ± 0.1	< LOD
B18	6.7 ± 0.1	50 ± 3	35.3 ± 0.4	1.6 ± 0.4	16 ± 0.1	< LOD
B19	19.5 ± 1.4	491 ± 15	95.5 ± 3.5	2.1 ± 0.1	< LOD	< LOD
G1	40.7 ± 2.3	71 ± 1	497.5 ± 4	6.8 ± 0.2	< LOD	< LOQ
G2	112.5 ± 2.6	62 ± 2	215.8 ± 3.2	25 ± 0.7	< LOD	109 ± 5
G3	14.1 ± 0.6	181 ± 2	82 ± 1.4	8.1 ± 0.8	< LOD	< LOD
G4	35.9 ± 2	200 ± 5	136 ± 1.1	32.1 ± 0.5	5 ± 0.2	29 ± 0.7
G5	43.1 ± 2.5	81 ± 3	193.8 ± 1.4	4.6 ± 0.9	18 ± 0.1	31 ± 0.2
G6	19.1 ± 1.8	630 ± 9	156.8 ± 3.4	4.5 ± 0.8	13 ± 0.7	< LOQ
G7	8.6 ± 0.7	72 ± 1	322.1 ± 1.9	2.2 ± 0.2	13 ± 0.9	< LOQ
G8	34.7 ± 3.8	30 ± 2	260.7 ± 4.3	15.3 ± 1.1	8 ± 0.5	< LOD
R1	33.9 ± 2.7	450 ± 6	107 ± 2.4	9.9 ± 0.5	10 ± 0.9	76 ± 0.2
R2	60.3 ± 1.6	411 ± 7	134 ± 0.8	13.8 ± 0.8	13 ± 0.2	100 ± 2
R3	29.3 ± 2	971 ± 10	105.3 ± 3.2	4 ± 0.2	< LOD	< LOD
RF	16.5 ± 1.6	43 ± 1	56.7 ± 1.9	21.3 ± 0.4	68 ± 0.5	< LOD
RR	45.8 ± 0.7	420 ± 7	114.8 ± 3	34 ± 2.4	30 ± 0.2	16 ± 2
VL	25.5 ± 0.9	30 ± 2	40.1 ± 0.6	9.7 ± 1.5	< LOD	< LOD
W1	29.1 ± 3.3	270 ± 4	149.7 ± 2.9	9.6 ± 0.2	30 ± 0.3	10 ± 0.1
W2	44.6 ± 4.4	80 ± 2	212.8 ± 4	2 ± 0.3	14 ± 0.1	7 ± 0.9

Table S5. Elements levels in tea infusions (Mean ± SD in µg/g, n = 3)

Code	*Cu*	*Cs*	*Ti*	*Ni*	*Al*	*Ba*	*Rb*	*Fe*
B1	3.5 ± 0.02	0.11 ± 0.04	2.16 ± 0.04	2.88 ± 0.15	252.5 ± 0.7	1.1 ± 0.1	101.4 ± 5.8	0.94 ± 0.01
B2	3.7 ± 0.04	0.27 ± 0.01	1.05 ± 0.01	3.38 ± 0.03	291.7 ± 13	1.3 ± 0.1	60.4 ± 0.9	1.14 ± 0.09
B3	3.7 ± 0.04	0.12 ± 0.08	2.03 ± 0.08	2.54 ± 0.03	395.1 ± 25.1	1.8 ± 0.1	96.3 ± 5.6	0.99 ± 0.01
B4	4.9 ± 0.03	0.51 ± 0.04	1.8 ± 0.04	2.35 ± 0.01	297.3 ± 16.4	0.6 ± 0.01	51.8 ± 5.5	0.56 ± 0.01
B5	4.5 ± 0.03	0.25 ± 0.04	1.47 ± 0.04	4.04 ± 0.01	510.8 ± 12.7	1.9 ± 0.2	33.1 ± 2.8	1.16 ± 0.004
B6	5.9 ± 0.1	1.46 ± 0.01	1.04 ± 0.01	4.54 ± 0.04	937.9 ± 22.1	3.6 ± 0.3	83.7 ± 6.6	2.12 ± 0.005
B7	3.1 ± 0.03	0.05 ± 0.08	2.66 ± 0.08	3.25 ± 0.08	439.4 ± 11.8	1.6 ± 0.1	130.8 ± 1.6	2.81 ± 0.04
B8	5.2 ± 0.04	0.21 ± 0.08	2.74 ± 0.08	4.95 ± 0.06	419.2 ± 7.4	1.6 ± 0.1	38.2 ± 1.2	0.97 ± 0.03
B9	4.9 ± 0.05	0.1 ± 0.05	2.13 ± 0.05	2.4 ± 0.04	347.4 ± 3.9	1.13 ± 0.04	22.2 ± 1.7	1.24 ± 0.04
B10	2.9 ± 0.01	0.12 ± 0.02	2.66 ± 0.02	2.33 ± 0.003	343.6 ± 8.5	1.6 ± 0.04	82.1 ± 4.8	0.9 ± 0.08
B11	5.9 ± 0.1	2.45 ± 0.02	1.42 ± 0.02	1.53 ± 0.03	558.6 ± 6.5	0.78 ± 0.05	76.1 ± 12	0.98 ± 0.04
B12	2.3 ± 0.1	0.36 ± 0.06	3.54 ± 0.06	1.47 ± 0.02	167.6 ± 2.3	0.41 ± 0.02	33.6 ± 2.7	0.47 ± 0.005
B13	2.3 ± 0.01	0.45 ± 0.07	3.1 ± 0.07	1.55 ± 0.03	126.2 ± 4.1	0.46 ± 0.03	96.3 ± 6.8	0.52 ± 0.06
B14	3.4 ± 0.03	0.12 ± 0.05	2.91 ± 0.05	2.82 ± 0.04	258.1 ± 2.5	1 ± 0.1	55.1 ± 5.3	0.7 ± 0.06
B15	3.4 ± 0.04	0.09 ± 0.03	2.99 ± 0.03	3.36 ± 0.07	245.5 ± 3.6	1.2 ± 0.1	57.4 ± 7.5	0.72 ± 0.01
B16	5 ± 0.1	0.1 ± 0.05	2.62 ± 0.05	3.27 ± 0.05	236.5 ± 0.7	1.36 ± 0.1	49.4 ± 0.7	0.9 ± 0.05
B17	6.9 ± 0.1	0.1 ± 0.04	2.47 ± 0.04	4.23 ± 0.06	319.9 ± 7.8	1.7 ± 0.1	39.5 ± 0.5	2.81 ± 0.06
B18	2 ± 0.1	0.23 ± 0.08	2.74 ± 0.08	1.43 ± 0.03	147.6 ± 1.2	0.45 ± 0.03	23.6 ± 5.7	1.06 ± 0.1
B19	4.9 ± 0.1	0.35 ± 0.07	3.15 ± 0.07	3.57 ± 0.07	50.8 ± 0.6	0.35 ± 0.01	35.2 ± 0.5	0.37 ± 0.01
G1	3.7 ± 0.1	0.21 ± 0.05	1.36 ± 0.05	4.5 ± 0.02	282.2 ± 14.8	0.8 ± 0.1	72 ± 2.9	2.91 ± 0.07
G2	4.9 ± 0.1	0.27 ± 0.01	2.17 ± 0.01	4.95 ± 0.05	202.2 ± 5.4	2.4 ± 0.1	114.6 ± 4.3	3.8 ± 0.23
G3	4.4 ± 0.1	0.9 ± 0.05	1.79 ± 0.05	2.69 ± 0.04	452.9 ± 12.5	0.97 ± 0.02	109.6 ± 1.5	2.16 ± 0.15
G4	3.2 ± 0.03	0.3 ± 0.04	1.82 ± 0.04	3.49 ± 0.01	663.3 ± 29.6	4.5 ± 0.2	5.9 ± 7.7	7.02 ± 0.41
G5	7.9 ± 0.1	0.2 ± 0.02	2.12 ± 0.02	5.28 ± 0.03	186.8 ± 2.9	1.6 ± 0.2	58.1 ± 0.4	1.84 ± 0.01
G6	5.9 ± 0.1	3.87 ± 0.03	1.58 ± 0.01	6.3 ± 0.08	724 ± 9.9	2.2 ± 0.2	68.7 ± 18.4	2.55 ± 0.004

Table S5. Elements levels in tea infusions (Mean ± SD in µg/g, n = 3) (Continued)

Code	*Cu*	*Cs*	*Ti*	*Ni*	*Al*	*Ba*	*Rb*	*Fe*
G7	4.6 ± 0.1	0.03 ± 0.05	2.07 ± 0.05	6.56 ± 0.07	214.2 ± 0.9	0.63 ± 0.03	12.2 ± 1.6	1.15 ± 0.04
G8	3.9 ± 0.1	0.01 ± 0.001	6.07 ± 0.17	4.39 ± 0.08	9.8 ± 0.6	0.3 ± 0.1	10.1 ± 0.5	1.6 ± 0.03
R1	5.4 ± 0.2	0.46 ± 0.03	2.05 ± 0.03	4.53 ± 0.08	608.9 ± 11.4	4.7 ± 0.05	81.9 ± 0.4	8.96 ± 0.05
R2	3 ± 0.1	0.68 ± 0.08	2.6 ± 0.08	5.22 ± 0.06	371.5 ± 5.8	3.6 ± 0.1	96.6 ± 1.2	7 ± 0.08
R3	4 ± 0.2	0.21 ± 0.02	1.93 ± 0.02	3.99 ± 0.01	323.9 ± 4.7	1.8 ± 0.1	76.7 ± 1.3	1.21 ± 0.04
RF	1 ± 0.1	0.01 ± 0.02	0.72 ± 0.02	0.66 ± 0.003	10.1 ± 0.4	16.4 ± 0.4	8 ± 2	8.54 ± 0.08
RR	2.2 ± 0.1	0.02 ± 0.02	1.1 ± 0.02	1.89 ± 0.01	10.3 ± 0.9	3.5 ± 0.1	6.1 ± 0.1	18.51 ± 0.08
VL	6.7 ± 0.1	0.15 ± 0.03	1.18 ± 0.03	0.48 ± 0.01	7.8 ± 0.3	9.3 ± 0.7	11.7 ± 0.9	1.78 ± 0.04
W1	7 ± 0.1	0.13 ± 0.02	1.4 ± 0.02	6.76 ± 0.19	405.5 ± 15	1.1 ± 0.1	64.3 ± 1.2	1.27 ± 0.01
W2	7.9 ± 0.1	1.4 ± 0.04	2.85 ± 0.04	6.1 ± 0.06	291.2 ± 5.9	1.31 ± 0.02	172.9 ± 3.7	2.42 ± 0.07

Table S5. Elements levels in tea infusions (Mean ± SD in µg/g, n = 3) (Continued)

Code	*P*	*Si*	*Ca*	*Mg*	*Mn*	*Sr*	*Zn*	*K*
B1	1172 ± 1	18 ±1	54 ± 1	981 ± 1	187 ± 2	0.5 ± 0.04	8.7 ± 0.1	11408 ± 648
B2	830 ± 2	36 ± 1	75 ± 5	584 ± 1	147 ± 9	0.5 ± 0.01	5.8 ± 0.3	10411 ± 432
B3	1839 ± 1	29 ± 2	73 ± 4	1405 ± 2	449 ± 5	1.1 ± 0.04	10.7 ± 0.6	14214 ± 324
B4	1374 ± 1	28 ± 1	53 ± 3	1085 ± 7	163 ± 9	0.4 ± 0.02	8.1 ± 0.4	8354 ± 1080
B5	1048 ± 2	43 ± 1	113 ± 3	704 ± 2	226 ± 6	1 ± 0.04	6.5 ± 0.3	12281 ± 286
B6	388 ± 6	49 ± 1	193 ± 8	1040 ± 4	256 ± 2	1.2 ± 0.09	5.1 ± 0.2	8104 ± 1096
B7	1671 ± 10	45 ± 1	93 ± 3	1211 ± 4	667 ± 9	1.1 ± 0.05	9 ± 0.2	19388 ± 923
B8	993 ± 9	27 ± 1	98 ± 3	933 ± 4	208 ± 2	0.4 ± 0.01	8.3 ± 0.1	15959 ± 708
B9	1226 ± 20	19 ± 1	127 ± 14	1144 ± 2	99 ± 1	0.5 ± 0.02	10.6 ± 0.7	18889 ± 495
B10	1342 ± 3	27 ± 1	88 ± 5	1196 ± 4	230 ± 9	0.6 ± 0.04	8.6 ± 0.2	12303 ± 534
B11	727 ± 8	25 ± 1	228 ± 18	714 ± 2	64 ± 3	1.04 ± 0.05	4.5 ± 0.1	8696 ± 476

Table S5. Elements levels in tea infusions (Mean ± SD in µg/g, n = 3) (Continued)

Code	*P*	*Si*	*Ca*	*Mg*	*Mn*	*Sr*	*Zn*	*K*
B12	1299 ± 15	12 ± 1	62 ± 3	887 ± 1	110 ± 1	0.2 ± 0.02	6.8 ± 0.2	13587 ± 86
B13	1163 ± 4	11 ± 1	67 ± 4	777 ± 2	260 ± 8	0.7 ± 0.03	8 ± 0.2	9239 ± 171
B14	1104 ± 11	27 ± 2	65 ± 2	977 ± 2	190 ± 4	0.3 ± 0.01	8.3 ± 0.1	11512 ± 476
B15	1423 ± 17	30 ± 3	81 ± 3	1125 ± 3	241 ± 8	0.3 ± 0.01	10.1 ± 0.2	12995 ± 617
B16	993 ± 4	26 ± 2	74 ± 3	935 ± 3	180 ± 1	0.3 ± 0.04	8.4 ± 0.2	11117 ± 534
B17	1325 ± 13	39 ± 1	117 ± 3	946 ± 3	138 ± 4	0.6 ± 0.02	12.6 ± 0.3	10475 ± 1151
B18	972 ± 1	12 ± 2	52 ± 2	751 ± 3	93 ± 3	0.2 ± 0.01	6 ± 0.2	10574 ± 171
B19	269 ± 3	8 ± 2	50 ± 2	223 ± 1	51 ± 3	0.2 ± 0.01	3 ± 0.2	2816 ± 86
G1	447 ± 5	27 ± 2	52 ± 3	422 ± 3	175 ± 7	0.3 ± 0.05	5.4 ± 0.01	7356 ± 216
G2	1193 ± 9	57 ± 1	384 ± 18	2100 ± 6	233 ± 8	7 ± 0.42	11.2 ± 0.5	13590 ± 108
G3	1001 ± 22	35 ± 1	132 ± 6	1080 ± 4	368 ± 1	1.6 ± 0.1	8.3 ± 0.2	9476 ± 657
G4	1892 ± 2	115 ± 4	1024 ± 51	2925 ± 15	411 ± 8	14.4 ± 0.7	11.2 ± 0.4	26059 ± 471
G5	830 ± 8	38 ± 1	82 ± 3	650 ± 2	197 ± 8	0.9 ± 0.04	8.5 ± 0.3	8291 ± 432
G6	381 ± 5	33 ± 1	156 ± 7	773 ± 1	202 ± 6	0.8 ± 0.04	4.4 ± 0.2	8977 ± 324
G7	680 ± 4	20 ± 1	69 ± 4	510 ± 2	95 ± 5	0.3 ± 0.01	9.4 ± 0.4	8745 ± 148
G8	1993 ± 43	100 ± 1	1409 ± 54	930 ± 2	12 ± 1	3.7 ± 0.11	4.3 ± 0.1	15317 ± 428
R1	2077 ± 52	74 ± 1	157 ± 2	1131 ± 2	286 ± 4	1.6 ± 0.02	8.4 ± 0.1	13154 ± 601
R2	1327 ± 19	53 ± 1	124 ± 2	640 ± 1	161 ± 3	1.2 ± 0.01	5.3 ± 0.1	10162 ± 471
R3	837 ± 3	34 ± 1	78 ± 2	621 ± 1	151 ± 3	0.5 ± 0.02	5.7 ± 0.1	9141 ± 309
RF	228 ± 1	46 ± 1	1496 ± 40	604 ± 1	105 ± 2	8.7 ± 0.17	6.2 ± 0.1	4676 ± 374
RR	218 ± 7	22 ± 1	2428 ± 57	962 ± 2	40 ± 1	19.7 ± 0.53	4.6 ± 0.1	2805 ± 187
VL	447 ± 9	325 ± 8	1607 ± 69	1159 ± 5	6 ± 1	14 ± 1	4.1 ± 0.1	6982 ± 389
W1	644 ± 15	47 ± 2	87 ± 6	585 ± 3	171 ± 3	0.5 ± 0.03	5.5 ± 0.3	5143 ± 601
W2	1159 ± 2	22 ± 1	98 ± 2	1137 ± 2	256 ± 6	0.4 ± 0.02	14.2 ± 0.4	13637 ± 513

Table S6. Elements leaching percentages (%) from tea leaves to their infusions.

Code	*Li*	*Be*	*Ti*	*Cu*	*Ga*	*Hg*	*Cd*	*Cs*	*Tl*	*Pb*
B1	13 ± 1	46 ± 3	15 ± 1	24 ± 1	3 ± 0.1	77 ± 33	11 ± 1	101 ± 1	47 ± 1	5 ± 0.3
B2	12 ± 1	-	10 ± 1	24 ± 1	4 ± 0.4	-	5 ± 0.1	86 ± 1	36 ± 1	5 ± 0.1
B3	12 ± 1	68 ± 3	15 ± 1	33 ± 2	7 ± 1	-	14 ± 2	101 ± 4	104 ± 8	30 ± 3
B4	23 ± 1	-	18 ± 1	23 ± 2	4 ± 0.1	-	8 ± 1	97 ± 8	71 ± 3	18 ± 1
B5	17 ± 1	-	17 ± 1	33 ± 3	10 ± 1	-	14 ± 1	66 ± 7	63 ± 5	5 ± 0.2
B6	39 ± 3	98 ± 6	10 ± 0.2	30 ± 3	11 ± 1	-	12 ± 2	46 ± 2	67 ± 4	21 ± 2.4
B7	35 ± 1	108 ± 8	31 ± 3	18 ± 1	4 ± 0.2	-	11 ± 0.5	32 ± 3	11 ± 1	14 ± 1
B8	25 ± 2	21 ± 2	19 ± 1	41 ± 1	8 ± 0.1	-	6 ± 0.3	109 ± 1	100 ± 5	7 ± 0.3
B9	12 ± 0.4	-	24 ± 1	21 ± 1	6 ± 0.1	-	9 ± 2	107 ± 5	47 ± 2	7 ± 0.2
B10	26 ± 1	60 ± 6	18 ± 0.4	19 ± 0.5	4 ± 0.1	-	7 ± 1	104 ± 4	43 ± 3	13 ± 0.4
B11	19 ± 2	-	15 ± 1	29 ± 1	3 ± 0.2	-	3 ± 0.2	101 ± 9	43 ± 3	7 ± 0.3
B12	16 ± 1	-	38 ± 2	14 ± 1	3 ± 0.1	-	15 ± 1	47 ± 3	46 ± 4	4 ± 0.3
B13	12 ± 1	34 ± 5	14 ± 1	18 ± 1	4 ± 0.2	-	6 ± 1	36 ± 1	49 ± 5	-
B14	26 ± 1	54 ± 4	25 ± 1	21 ± 0.4	3 ± 0.05	-	7 ± 1	42 ± 0.3	40 ± 2	27 ± 1
B15	27 ± 1	46 ± 1	25 ± 1	19 ± 1	4 ± 0.2	-	6 ± 1	52 ± 3	43 ± 1	5 ± 0.4
B16	26 ± 2	33 ± 3	19 ± 1	29 ± 2	4 ± 0.2	-	7 ± 1	44 ± 4	42 ± 2	29 ± 2
B17	95 ± 5	-	25 ± 1	25 ± 1	6 ± 0.2	-	9 ± 1	102 ± 2	52 ± 2	12 ± 0.5
B18	63 ± 3	-	28 ± 1	12 ± 1	4 ± 0.1	-	11 ± 1	55 ± 2	50 ± 2	4 ± 0.4
B19	79 ± 5	46 ± 4	21 ± 1	35 ± 1	6 ± 0.1	-	12 ± 2	46 ± 2	75 ± 2	3 ± 0.001
G1	21 ± 1	52 ± 17	16 ± 1	34 ± 1	6 ± 1	90 ± 15	9 ± 1	79 ± 1	64 ± 7	14 ± 1
G2	18 ± 2	36 ± 2	12 ± 1	33 ± 2	8 ± 1	49 ± 13	7 ± 1	65 ± 4	50 ± 5	4 ± 0.7
G3	21 ± 2	77 ± 8	12 ± 1	38 ± 1	7 ± 0.2	-	14 ± 2	91 ± 3	62 ± 3	7 ± 0.6
G4	30 ± 2	33 ± 1	6 ± 0.2	19 ± 1	5 ± 0.1	-	8 ± 0.3	78 ± 4	39 ± 1	4 ± 0.2
G5	32 ± 2	54 ± 6	23 ± 1	43 ± 1	6 ± 0.1	-	9 ± 1	103 ± 1	46 ± 1	5 ± 0.1
G6	42 ± 2	50 ± 3	24 ± 2	48 ± 3	8 ± 1	-	11 ± 1	89 ± 3	73 ± 7	4 ± 0.2

Table S6. Elements leaching percentages (%) from tea leaves to their infusions (Continued)

Code	Li	Be	Ti	Cu	Ga	Hg	Cd	Cs	Tl	Pb
G7	7 ± 1	-	28 ± 1	27 ± 2	3 ± 0.1	-	11 ± 2	55 ± 2	54 ± 4	6 ± 0.6
G8	101 ± 6	4 ± 1	22 ± 2	42 ± 1	12 ± 1	-	5 ± 1	30 ± 0.3	61 ± 6	19 ± 1
R1	20 ± 1	37 ± 5	10 ± 0.5	25 ± 2	13 ± 1	-	8 ± 0.3	77 ± 4	39 ± 2	8 ± 0.3
R2	21 ± 2	34 ± 5	14 ± 1	15 ± 1	9 ± 1	-	7 ± 1	86 ± 1	28 ± 3	7 ± 0.3
R3	38 ± 1	71 ± 7	17 ± 0.2	21 ± 2	5 ± 0.1	-	7 ± 1	93 ± 1	44 ± 2	31 ± 1
RF	11 ± 0.4	23 ± 2	6 ± 0.2	20 ± 1	24 ± 1	78 ± 34	18 ± 1	27 ± 1	27 ± 3	8 ± 1
RR	90 ± 6	34 ± 4	10 ± 0.3	44 ± 2	62 ± 2	-	42 ± 4	66 ± 5	57 ± 5	9 ± 0.4
VL	6 ± 0.5	-	10 ± 1	53 ± 3	11 ± 1	-	10 ± 1	97 ± 9	61 ± 6	4 ± 0.1
W1	36 ± 2	49 ± 3	12 ± 0.3	25 ± 1	7 ± 0.2	-	7 ± 1	96 ± 2	56 ± 3	6 ± 0.2
W2	24 ± 0.3	-	24 ± 0.4	44 ± 2	6 ± 0.1	-	10 ± 1	102 ± 2	49 ± 2	6 ± 0.5

Table S6. Elements leaching percentages (%) from tea leaves to their infusions (Continued)

Code	As	Cr	Co	Ni	V	Sn	Sb	Al	Ba	Rb
B1	8 ± 1	20 ± 2	29 ± 1	52 ± 4	-	66 ± 16.3	30 ± 7	33 ± 0.1	2 ± 0.1	101 ± 6
B2	17 ± 1	20 ± 0.5	21 ± 1	48 ± 1	2 ± 0.03	12 ± 2.3	17 ± 1	29 ± 1.5	3 ± 0.3	110 ± 7
B3	7 ± 1	13 ± 1	32 ± 1	58 ± 4	-	-	-	45 ± 3.8	5 ± 0.4	94 ± 7
B4	14 ± 1	26 ± 1	27 ± 1	50 ± 3	1 ± 0.04	4 ± 0.5	-	36 ± 4	2 ± 0	78 ± 8
B5	11 ± 1	7 ± 0.4	31 ± 3	60 ± 6	2 ± 0.2	14 ± 1.3	-	52 ± 5.1	5 ± 0.7	86 ± 8
B6	8 ± 0.4	54 ± 2	31 ± 2	71 ± 3	1 ± 0.03	8 ± 1.8	-	38 ± 2.3	5 ± 0.5	87 ± 8
B7	10 ± 1	7 ± 0.2	15 ± 2	26 ± 1	-	4 ± 0.9	53 ± 3	43 ± 3.9	3 ± 0.4	58 ± 1
B8	12 ± 0.1	19 ± 0	108 ± 4	50 ± 1	5 ± 0.2	97 ± 8.8	-	39 ± 1	5 ± 0.2	27 ± 1
B9	11 ± 1	6 ± 0.3	28 ± 1	27 ± 1	1 ± 0.03	3 ± 0.3	-	39 ± 1.2	5 ± 0.3	67 ± 5
B10	8 ± 0.3	9 ± 1	22 ± 1	17 ± 0.4	-	9 ± 0.7	110 ± 17	38 ± 1.3	3 ± 0.1	100 ± 6

Table S6. Elements leaching percentages (%) from tea leaves to their infusions (Continued)

Code	*As*	*Cr*	*Co*	*Ni*	*V*	*Sn*	*Sb*	*Al*	*Ba*	*Rb*
B11	14 ± 1	14 ± 1	36 ± 1	58 ± 3	2 ± 0.2	2 ± 0.3	41 ± 2	40 ± 1.4	2 ± 0.2	106 ± 17
B12	38 ± 5	-	31 ± 3	84 ± 2	-	8.5 ± 0.7	-	31 ± 1.4	2 ± 0.2	52 ± 5
B13	12 ± 2	14 ± 1	24 ± 1	42 ± 4	-	-	-	22 ± 1.1	2 ± 0.2	79 ± 7
B14	20 ± 0.2	7 ± 1	23 ± 1	33 ± 2	1 ± 0.1	-	-	34 ± 0.5	2 ± 0.1	71 ± 8
B15	21 ± 2	11 ± 1	22 ± 1	37 ± 2	1 ± 0.03	102 ± 6.9	-	35 ± 2	3 ± 0.2	76 ± 12
B16	13 ± 1	4 ± 0.4	22 ± 1	30 ± 2	1 ± 0.04	-	-	21 ± 0.3	3 ± 0.3	61 ± 4
B17	14 ± 2	34 ± 1	43 ± 2	66 ± 5	5 ± 0.5	2 ± 0.2	-	36 ± 0.9	5 ± 0.2	99 ± 4
B18	21 ± 1	41 ± 3	23 ± 1	46 ± 1	1 ± 0.1	24 ± 2.6	-	25 ± 0.4	3 ± 0.2	51 ± 12
B19	31 ± 1	10 ± 0.5	41 ± 2	67 ± 2	1 ± 0.05	-	-	8 ± 0.1	1 ± 0.03	41 ± 1
G1	18 ± 1	23 ± 2	48 ± 5	79 ± 6	4 ± 0.1	-	-	32 ± 2.5	3 ± 0.3	93 ± 8
G2	22 ± 1	8 ± 1	56 ± 4	97 ± 8	3 ± 0.3	-	19 ± 3	19 ± 1.5	9 ± 0.9	90 ± 10
G3	8 ± 1	13 ± 1	55 ± 4	71 ± 6	3 ± 0.3	-	-	34 ± 1.2	3 ± 0.1	96 ± 2
G4	9 ± 0.3	10 ± 1	29 ± 2	38 ± 0.4	2 ± 0.1	4 ± 0.4	44 ± 8	39 ± 2.4	10 ± 0.5	-
G5	18 ± 0.1	21 ± 1	61 ± 1	56 ± 1	1 ± 0.1	12 ± 1.1	80 ± 6	21 ± 0.7	4 ± 0.4	85 ± 2
G6	17 ± 1	51 ± 4	78 ± 4	69 ± 4	2 ± 0.03	20 ± 1.8	-	38 ± 3.2	3 ± 0.1	73 ± 21
G7	23 ± 1	29 ± 2	70 ± 6	66 ± 2	2 ± 0.1	5 ± 0.6	-	27 ± 2.8	2 ± 0.1	< LOQ
G8	12 ± 1	98 ± 29	33 ± 4	91 ± 2	2 ± 0.03	16 ± 1.9	-	2 ± 0.1	6 ± 1	< LOQ
R1	7 ± 0.4	17 ± 2	31 ± 2	77 ± 5	1 ± 0.05	5 ± 0.7	22 ± 5	34 ± 2.5	9 ± 0.4	93 ± 3
R2	14 ± 0.5	18 ± 1	24 ± 0.02	44 ± 4	2 ± 0.2	5 ± 0.1	6 ± 0.1	19 ± 0.8	6 ± 0.3	92 ± 7
R3	32 ± 1	10 ± 1	34 ± 1	50 ± 1	2 ± 0.2	-	-	44 ± 0.7	4 ± 0.2	103 ± 4
RF	5 ± 0.4	4 ± 0.2	21 ± 2	25 ± 0.3	3 ± 0.3	97 ± 12.5	-	2 ± 0.2	16 ± 0.5	-
RR	16 ± 2	21 ± 1	39 ± 4	46 ± 3	7 ± 0.7	29 ± 3.5	86 ± 12	3 ± 0.1	20 ± 1	-
VL	7 ± 0.2	2 ± 0.2	34 ± 3	61 ± 7	2 ± 0.05	-	-	2 ± 0.2	5 ± 0.3	-
W1	11 ± 1	24 ± 1	35 ± 2	81 ± 3	2 ± 0.05	11 ± 1.3	37 ± 7	24 ± 1.5	2 ± 0.2	93 ± 5
W2	23 ± 1	27 ± 1	64 ± 2	100 ± 5	1 ± 0.05	9 ± 1.5	8 ± 2	37 ± 3.2	5 ± 0.1	79 ± 2

Table S6. Elements leaching percentages (%) from tea leaves to their infusions (Continued)

Code	*Fe*	*P*	*Si*	*Ca*	*Mg*	*Mn*	*Sr*	*Zn*	*K*
B1	1 ± 0.02	39 ± 0.3	9 ± 0.6	1 ± 0.02	46 ± 0.4	25 ± 1	1 ± 0.2	34 ± 1	56 ± 5
B2	1 ± 0.1	38 ± 4	21 ± 2	1 ± 0.1	38 ± 2	17 ± 2	2 ± 0.4	22 ± 2	65 ± 5
B3	0.5 ± 0.04	58 ± 5	15 ± 1	1 ± 0.1	52 ± 4	30 ± 0.4	2 ± 0.1	45 ± 3	73 ± 5
B4	0.5 ± 0.04	45 ± 4	27 ± 3	1 ± 0.03	33 ± 2	17 ± 1	1 ± 0.1	27 ± 2	35 ± 1
B5	1 ± 0.1	49 ± 5	13 ± 1	2 ± 0.1	49 ± 1	26 ± 1	2 ± 0.2	29 ± 3	79 ± 10
B6	1 ± 0.1	21 ± 1	25 ± 2	2 ± 0.1	29 ± 1	14 ± 1	3 ± 0.2	39 ± 3	46 ± 6
B7	2 ± 0.1	64 ± 7	32 ± 2	2 ± 0.1	47 ± 1	67 ± 2	5 ± 0.8	30 ± 2	55 ± 6
B8	0.5 ± 0.02	30 ± 1	8 ± 1	2 ± 0.1	39 ± 0.2	10 ± 1	1 ± 0	49 ± 2	57 ± 3
B9	1 ± 0.1	46 ± 2	18 ± 1	2 ± 0.3	56 ± 1	32 ± 0.4	3 ± 0.2	31 ± 3	88 ± 2
B10	0.3 ± 0.03	44 ± 1.2	11 ± 1	2 ± 0.1	49 ± 2	28 ± 3	1 ± 0.3	33 ± 1	55 ± 3
B11	1 ± 0.1	35 ± 1	31 ± 2.3	3 ± 0.2	31 ± 1	16 ± 2	1 ± 0.1	23 ± 1	50 ± 3
B12	0.6 ± 0.03	48 ± 2	17 ± 1.2	1 ± 0.1	36 ± 0.4	24 ± 2	1 ± 0.2	30 ± 1	61 ± 4
B13	0.2 ± 0.02	33 ± 1	6 ± 0.4	2 ± 0.1	40 ± 1	25 ± 3.2	1 ± 0.2	27 ± 1	41 ± 4
B14	0.3 ± 0.03	38 ± 0.5	17 ± 1	1 ± 0.04	38 ± 1	22 ± 1	1 ± 0.1	28 ± 1	48 ± 3
B15	0.3 ± 0.03	46 ± 1	21 ± 2	2 ± 0.1	40 ± 1	24 ± 1	2 ± 0.2	30 ± 2	52 ± 5
B16	0.3 ± 0.03	38 ± 0.3	9 ± 0.4	1 ± 0.1	41 ± 0.1	19 ± 0.4	2 ± 0.003	28 ± 1	53 ± 3
B17	2 ± 0.1	50 ± 1	10 ± 1	3 ± 0.1	49 ± 1	28 ± 2	4 ± 0.1	33 ± 1	55 ± 7
B18	1 ± 0.1	34 ± 1	18 ± 1	1 ± 0.04	37 ± 1	21 ± 1	2 ± 0.1	24 ± 1	53 ± 2
B19	0.2 ± 0.01	9 ± 0.1	4 ± 0.1	1 ± 0.03	11 ± 0.4	7 ± 0.4	1 ± 0.05	11 ± 1	18 ± 2
G1	2 ± 0.1	20 ± 1	15 ± 1	1 ± 0.1	25 ± 2	14 ± 1	1 ± 0.2	20 ± 1	43 ± 3
G2	1 ± 0.1	43 ± 1	9 ± 0.2	5 ± 0.3	68 ± 2	25 ± 3	12 ± 1	39 ± 3	32 ± 1
G3	1 ± 0.2	39 ± 1	11 ± 1	2 ± 0.1	51 ± 2	29 ± 1	3 ± 0.4	42 ± 1	30 ± 2
G4	1 ± 0.1	87 ± 3	10 ± 0.4	12 ± 1	86 ± 4	47 ± 2	20 ± 1	49 ± 2	37 ± 2
G5	1.1 ± 0.01	34 ± 1	13 ± 1	2 ± 0.1	34 ± 1	18 ± 2	2 ± 0.1	24 ± 1	50 ± 3
G6	2 ± 0.1	22 ± 1	21 ± 2	2 ± 0.1	32 ± 0.4	16 ± 1	2 ± 0.2	21 ± 1	24 ± 1

Table S6. Elements leaching percentages (%) from tea leaves to their infusions (Continued)

Code	*Fe*	*P*	*Si*	*Ca*	*Mg*	*Mn*	*Sr*	*Zn*	*K*
G7	1 ± 0.1	26 ± 1	20 ± 2	1 ± 0.1	29 ± 1.3	18 ± 1	1 ± 0.1	27 ± 2	43 ± 2
G8	0.4 ± 0.01	43 ± 5	7 ± 1	13 ± 0.5	29 ± 1	11 ± 1	8 ± 0.6	10 ± 1	31 ± 1
R1	1 ± 0.1	81 ± 3	10 ± 1	2 ± 0.02	33 ± 1	18 ± 1	4 ± 0.4	27 ± 1	73 ± 7
R2	1 ± 0.1	50 ± 3	8 ± 1	2 ± 0.03	22 ± 0.4	12 ± 1	3 ± 0.3	16 ± 1	20 ± 1
R3	0.7 ± 0.04	28 ± 0.1	27 ± 1	1 ± 0.03	23 ± 0.3	17 ± 1	2 ± 0.1	16 ± 0.1	39 ± 3
RF	2 ± 0.2	18 ± 1	5 ± 0.3	16 ± 0.4	30 ± 0.7	28 ± 1	15 ± 0.4	21 ± 0.2	30 ± 3
RR	4 ± 0.3	30 ± 1	3 ± 0.2	23 ± 0.5	48 ± 1	31 ± 2	21 ± 1	31 ± 3	44 ± 6
VL	1 ± 0.05	23 ± 2	28 ± 3.2	10 ± 0.5	32 ± 1	10 ± 1	10 ± 1	19 ± 1	51 ± 5
W1	0.5 ± 0.01	29 ± 1	9 ± 0.3	1 ± 0.1	18 ± 1	9 ± 1	1 ± 0.1	18 ± 1	29 ± 4
W2	2 ± 0.1	38 ± 1	17 ± 1	2 ± 0.05	53 ± 1	35 ± 1	3 ± 0.2	34 ± 1	61 ± 4

Table S7. Elements concentrations in the dialyzates (mean ± SD in µg /L, n = 3).

Code	*Cu*	*Cs*	*Co*	*Ni*	*Al*	*Ba*	*Rb*	*Zn*
B1	10.3 ± 1.3	1 ± 0.01	0.2 ± 0.002	24 ± 6.9	21.3 ± 0.6	2.8 ± 1.4	421 ± 33	86 ± 4
B2	7.8 ± 0.4	2.3 ± 0.2	0.3 ± 0.02	< LOD	6.8 ± 0.5	1.3 ± 0.1	178 ± 14	50 ± 3
B3	10.9 ± 0.6	1 ± 0.03	0.2 ± 0.01	< LOD	15.2 ± 0.6	1.4 ± 0.1	378 ± 10	74 ± 3
B6	7.5 ± 0.4	9.9 ± 0.3	0.2 ± 0.02	12.5 ± 0.4	157 ± 0.8	1.7 ± 0.2	356 ± 19	43 ± 5
B7	5.9 ± 0.3	0.5 ± 0.01	0.1 ± 0.01	20.9 ± 0.6	26 ± 2	1.3 ± 0.1	682 ± 24	84 ± 4
B9	17.9 ± 0.9	0.9 ± 0.05	0.2 ± 0.01	6 ± 0.3	18.2 ± 1.4	< LOQ	181 ± 6	87 ± 4
B10	11.8 ± 0.1	1 ± 0.1	0.3 ± 0.02	< LOD	19 ± 4	2.1 ± 0.3	347 ± 27	75 ± 3
B16	11.4 ± 0.2	0.6 ± 0.03	0.2 ± 0.03	< LOD	6.9 ± 1.3	1.4 ± 0.5	228 ± 11	69 ± 7
B17	13.9 ± 1.4	0.8 ± 0.1	0.3 ± 0.1	9.7 ± 3.3	9.2 ± 3.3	1.7 ± 0.3	213 ± 22	76 ± 2
G1	8.1 ± 0.4	1.5 ± 0.1	1.4 ± 0.04	< LOD	29.4 ± 2.1	1.2 ± 0.1	238 ± 17	54 ± 5
G2	7 ± 0.7	2.3 ± 0.2	0.7 ± 0.05	21.8 ± 1.3	12.4 ± 3.8	9.5 ± 0.04	415 ± 7	91 ± 4
G4	13.2 ± 1.5	4.6 ± 0.3	0.7 ± 0.1	28 ± 3.6	69 ± 5.4	15.6 ± 0.3	< LOQ	101 ± 6
G5	13.2 ± 0.7	1.2 ± 0.04	0.5 ± 0.01	8.4 ± 0.5	9.1 ± 0.6	4.3 ± 0.1	246 ± 16	79 ± 2
R1	5.6 ± 0.2	3.5 ± 0.1	0.3 ± 0.01	8 ± 0.4	41.3 ± 0.5	2.2 ± 0.2	290 ± 23	42 ± 4
R2	< LOD	4.4 ± 0.1	0.2 ± 0.01	9.6 ± 0.5	29 ± 0.9	2.3 ± 0.3	328 ± 29	53 ± 3
R3	4 ± 0.8	1.2 ± 0.2	0.2 ± 0.003	6.7 ± 0.1	< LOD	< LOD	258 ± 20	68 ± 5
W1	12 ± 0.4	0.8 ± 0.05	0.4 ± 0.01	15 ± 1.4	43.4 ± 7.9	1.4 ± 0.2	180 ± 2	61 ± 1
W2	10.3 ± 0.3	8.3 ± 0.2	0.3 ± 0.02	13.1 ± 1.3	16.7 ± 0.6	2.4 ± 0.2	715 ± 26	101 ± 4

Table S7. Elements concentrations in the dialyzates (mean ± SD in µg /L, n = 3) (Continued)

Code	*P*	*Si*	*Ca*	*Mg*	*Mn*	*Sr*	*K*
B1	4214 ± 23	189 ± 17	251 ± 20	4112 ± 48	371 ± 13	2 ± 0.3	73378 ± 5619
B2	1954 ± 52	443 ± 54	197 ± 22	1203 ± 42	101 ± 4	1 ± 0.01	46675 ± 3896
B3	4334 ± 135	150 ± 20	145 ± 31	2948 ± 35	444 ± 23	3 ± 0.1	67077 ± 2662
B6	1030 ± 58	< LOQ	1092 ± 133	2922 ± 56	298 ± 31	1 ± 0.1	47630 ± 1197
B7	5136 ± 261	154 ± 15	441 ± 13	4107 ± 186	1118 ± 69	4 ± 0.1	88123 ± 2443
B9	3801 ± 181	137 ± 3	353 ± 8	3878 ± 98	128 ± 9	1 ± 0.1	88447 ± 6247
B10	4286 ± 160	185 ± 15	186 ± 12	4425 ± 132	353 ± 19	2 ± 0.1	86606 ± 6272
B16	3503 ± 319	236 ± 7	989 ± 228	3113 ± 32	200 ± 13	< LOQ	67015 ± 1438
B17	2835 ± 95	< LOQ	136 ± 23	2442 ± 83	113 ± 0	< LOQ	82759 ± 497
G1	1785 ± 180	130 ± 6	357 ± 67	1704 ± 174	335 ± 23	1 ± 0.1	51952 ± 4434
G2	3464 ± 42	221 ± 19	5558 ± 477	7831 ± 43	479 ± 42	67 ± 0.4	74218 ± 2386
G4	4661 ± 428	281 ± 17	5640 ± 297	8371 ± 488	676 ± 32	62 ± 0.6	114976 ± 7674
G5	2548 ± 50	213 ± 45	328 ± 31	2308 ± 59	321 ± 22	4 ± 0.4	41602 ± 1112
R1	4087 ± 15	202 ± 3	181 ± 38	1926 ± 7	196 ± 2	1 ± 0.1	48524 ± 644
R2	3377 ± 174	< LOQ	173 ± 25	1744 ± 42	199 ± 1	1 ± 0.3	49019 ± 3174
R3	2244 ± 320	143 ± 54	391 ± 29	1959 ± 148	193 ± 6	< LOD	56445 ± 2264
W1	1959 ± 113	256 ± 0.1	274 ± 8	1804 ± 59	192 ± 6	1 ± 0.04	39991 ± 2678
W2	2784 ± 185	< LOD	340 ± 85	2866 ± 255	356 ± 32	1 ± 0.1	67546 ± 2007

Annex II
List of publications

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

List of publications

- Alá S. Alnaimat; Barciela-Alonso, María Carmen; and Pilar Bermejo-Barrera. (2019) **Determination of bisphenol A in tea samples by solid phase extraction and liquid chromatography coupled to mass spectrometry**. *Microchemical J.* 147, 598-604. https://doi.org/10.1016/j.microc.2019.03.026

- Alá S. Alnaimat; Barciela-Alonso, María Carmen; and Pilar Bermejo-Barrera. (2020) **Development of a sensitive method for the analysis of four phthalates in tea samples: Tea bag contribution to the total amount in tea infusion**. *Food Additives and Contaminants: Part A*, (n.d) https://doi.org/10.1080/19440049.2020.1786170

- Alá S. Alnaimat, María Carmen Barciela-Alonso, Paloma Herbello-Hermelo, Raquel DomínguezGonzález, and Pilar Bermejo-Barrera. ***In vitro* assessment of major and trace element bioavailability in tea samples** (**In press**).

- Alá S. Alnaimat, María Carmen Barciela-Alonso and Pilar Bermejo-Barrera. **Bioavailability of Tea Polyphenols: an *in vitro* dialyzability study** (**In press**).