Elucidation of multi-origin Inula helenium L. for antimicrobial activity and heavy metal profiling as an exemplar for the use and regulation of plants as antibiotics.

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Elucidation of multi-origin *Inula helenium* L. for antimicrobial activity and trace element profiling as an exemplar for the use and regulation of plants as antibiotics

*A thesis presented for the award of:*

**Doctor of Philosophy**

*By*

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*Department of Biological Sciences*

*Munster Technological University, Cork, Ireland*

**Supervisors:**

Dr. Brigid Lucey (*Department of Biological Sciences*)

& Dr. Ambrose Furey (*Department of Physical Sciences*)

Submitted to Munster Technological University, May 2021.
Dedication

To my wonderful parents, Alice and Colman:

Thank you.
Acknowledgements

First and foremost, I would like to extend my sincere thanks and appreciation to my supervisors, Dr. Brigid Lucey and Dr. Ambrose Furey. Thank you for believing in me, and encouraging me to keep going, especially during the times when I couldn’t see the wood for the trees. Brigid, you have not only been a supervisor but a role model and friend. I am so grateful to have had the opportunity to explore elecampane a little more under your guidance. Ambrose, thank you for sharing your vast expertise and introducing me to the world of metallomics.

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I am forever blessed to have the kindest, most hard-working, and loving parents. Thank you, Mam and Dad, for absolutely everything you have done and continue to do for us. To my siblings - Conor, Eoghan, Joycie and Niamh - thank you for the unwavering encouragement, support and advice (and infinite vales of tea!) which brightened many a day in the “dungeon of study”.

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    - Ciara-Ruth
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# Abbreviations

## A

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AI/ADI</td>
<td>Average intake/ average daily intake</td>
</tr>
<tr>
<td>AL</td>
<td>Alantolactone</td>
</tr>
<tr>
<td>AMR</td>
<td>Antimicrobial resistance</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AST</td>
<td>Antimicrobial susceptibility testing</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>ATSDR</td>
<td>Agency for Toxic Substances and Disease Registry</td>
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## B

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BHP</td>
<td>British Herbal Pharmacopeia</td>
</tr>
<tr>
<td>BMDL</td>
<td>Benchmark dose level</td>
</tr>
<tr>
<td>BT</td>
<td>Botanical samples</td>
</tr>
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</table>

## C

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CDC</td>
<td>Centres for Disease Control and Prevention</td>
</tr>
<tr>
<td>CDI</td>
<td>Chronic daily intake</td>
</tr>
<tr>
<td>CITES</td>
<td>Convention on International Trade in Endangered Species</td>
</tr>
<tr>
<td>CLSI</td>
<td>Clinical &amp; Laboratory Standards Institute</td>
</tr>
<tr>
<td>CNS</td>
<td>Coagulase negative <em>Staphylococci</em></td>
</tr>
<tr>
<td>CT/CM</td>
<td>Cultivated/ Commercial <em>Inula helenium</em> L. samples</td>
</tr>
<tr>
<td>CUH</td>
<td>Cork University Hospital</td>
</tr>
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## D

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>DAD</td>
<td>Diode array detection</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DRV</td>
<td>Dietary reference value</td>
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## E

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>EC</td>
<td>European Commission</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>EDI</td>
<td>Estimated daily intake</td>
</tr>
<tr>
<td>EFSA</td>
<td>European Food Safety Authority</td>
</tr>
<tr>
<td>EMA</td>
<td>European Medicines Agency</td>
</tr>
<tr>
<td>EPA</td>
<td>Environmental Protection Agency</td>
</tr>
<tr>
<td>ESCOP</td>
<td>European Scientific Cooperative on Phytotherapy</td>
</tr>
<tr>
<td>EUCAST</td>
<td>European Committee on Antimicrobial Susceptibility Testing</td>
</tr>
<tr>
<td>FCM</td>
<td>Food contact material</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FoodEx2</td>
<td>Food classification &amp; description system for exposure assess.</td>
</tr>
<tr>
<td>FSAI</td>
<td>Food Safety Authority of Ireland</td>
</tr>
<tr>
<td>GACP</td>
<td>Good Agricultural and Collection Practices</td>
</tr>
<tr>
<td>GC</td>
<td>Gas Chromatography</td>
</tr>
<tr>
<td>GMP</td>
<td>Good Manufacturing Practices</td>
</tr>
<tr>
<td>HBGV</td>
<td>Health based guidance value</td>
</tr>
<tr>
<td>HD</td>
<td>Hydro-distilled</td>
</tr>
<tr>
<td>HI</td>
<td>Hazard Index</td>
</tr>
<tr>
<td>HMPC</td>
<td>Committee on Herbal Medicinal Products</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HPTLC</td>
<td>High performance thin layer chromatography</td>
</tr>
<tr>
<td>HQ</td>
<td>Hazard Quotient</td>
</tr>
<tr>
<td>HR-</td>
<td>High resolution-</td>
</tr>
<tr>
<td>HTS</td>
<td>High-throughput screening</td>
</tr>
<tr>
<td>IAL</td>
<td>Isoalantolactone</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
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</tr>
<tr>
<td>IARC</td>
<td>International Agency for Research on Cancer</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>Half maximal inhibitory concentration</td>
</tr>
<tr>
<td>ICH</td>
<td>International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use</td>
</tr>
<tr>
<td>ICP-SFMS</td>
<td>Inductively coupled plasma sector field mass spectrometry</td>
</tr>
<tr>
<td>IN</td>
<td><em>Inula helenium</em> samples</td>
</tr>
<tr>
<td>ITSD</td>
<td>Internal standard</td>
</tr>
<tr>
<td>JECFA</td>
<td>Joint FAO/WHO Expert Committee on Food Additives</td>
</tr>
<tr>
<td>LOD/LOQ</td>
<td>Limit of detection/ limit of quantification</td>
</tr>
<tr>
<td>MBC</td>
<td>Minimum bactericidal concentration</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
</tr>
<tr>
<td>ML</td>
<td>Maximum levels</td>
</tr>
<tr>
<td>MoA</td>
<td>Mechanism of action</td>
</tr>
<tr>
<td>MRL/MCL</td>
<td>Maximum residue level/ maximum concentration level</td>
</tr>
<tr>
<td>MRSA/VRSA</td>
<td>Methicillin-/vancomycin-resistant <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>N</td>
<td>$n =$ Number</td>
</tr>
<tr>
<td>NADH/NAD(PH)</td>
<td>Nicotinamide adenine dinucleotide (phosphate)</td>
</tr>
<tr>
<td>NCE</td>
<td>New chemical entity</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance ($^1$H = proton)</td>
</tr>
<tr>
<td>NOAEL</td>
<td>No adverse effect level</td>
</tr>
<tr>
<td><strong>P</strong></td>
<td><strong>Polish Academy of Sciences</strong></td>
</tr>
<tr>
<td>----------</td>
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<tr>
<td><strong>PAS</strong></td>
<td></td>
</tr>
<tr>
<td><strong>PFS</strong></td>
<td><strong>Plant food supplements</strong></td>
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<tr>
<th><strong>R</strong></th>
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<tbody>
<tr>
<td><strong>RACE</strong></td>
<td><strong>Rapid Analysis of Contaminant Exposure</strong></td>
</tr>
<tr>
<td><strong>RASFF</strong></td>
<td><strong>Rapid Alert System for Food and Feed</strong></td>
</tr>
<tr>
<td><strong>RDA</strong></td>
<td><strong>Recommended daily intake</strong></td>
</tr>
<tr>
<td><strong>REE</strong></td>
<td><strong>Rare earth elements</strong></td>
</tr>
<tr>
<td><strong>R/D (A) (P)</strong></td>
<td><strong>Reference dose (A = ‘acute’, P = ‘provisional’)</strong></td>
</tr>
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<td><strong>SMILES</strong></td>
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<td><strong>SML</strong></td>
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<td><strong>(Provisional)- tolerable monthly intake</strong></td>
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<td><strong>(Provisional)- tolerable weekly intake</strong></td>
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<td><strong>(T)HMP(D)</strong></td>
<td><strong>(Traditional) herbal medicinal products</strong></td>
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<td><strong>VOC</strong></td>
<td><strong>Volatile organic compound</strong></td>
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WHO
World Health Organisation

Units of Measurement

%  Percent
µg  Microgram
µL  Millilitre
µm  Micrometre
µM  Micromolar
h  Hour
Kg  Kilogram
L  Litre
m/z  Mass-to-charge
mg  Milligram
min  Minute
min⁻¹  Per minute
mL  Millilitre
mm:ss  Minute(s): second(s)
nm  Nanometre
°C  Degrees Celsius
w/v  Weight per volume

Solvents

CDCl₃  Deuterated chloroform or chloroform-d
DMSO  Dimethyl sulfoxide
EtOH  Ethanol
HNO₃  Nitric acid
MeCN  Acetonitrile
MeOH  Methanol
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Thesis Abstract

An impending post-antibiotic era looms, thus new sources of therapeutic chemical entities are crucial to sustain effective antimicrobial chemotherapy worldwide. Plant natural products are a source of accessible, structurally diverse compounds that provide therapeutic potential. The pharmacological applications of plants in medicine can be guided by the attestation of traditional use or the application of this knowledge to uncover new leads in the drug discovery process. The range of modified in vitro methods commonly used to investigate preclinical antimicrobial efficacy of plant-derived natural products, and the associated limitations and challenges in the provision of new antimicrobial drugs from plants is discussed. Currently, there are no approved guidelines, standards, or official recommendations governing in vitro antimicrobial methodologies for natural products of plant origin thus jeopardising transparency within this field of research. Standardisation of methodology will support future in vitro R&D and efficacy testing of plant-derived antibiotics. Evidence shows that the rate of new antimicrobial development is insufficient to meet our current and future needs globally. This, along with a weak drug portfolio pipeline, emphasises the importance – and relevance - of investigating prosperous sources of potentially new structures, such as plants.

*Inula helenium* L. (elecampane) is a perennial herb of the Asteraceae family, naturalised in Ireland. Irish ethnobotanical indications centre around respiratory, digestive, and dermal ailments. A key research objective of this project was to identify the natural product compound(s) attributing to the anti-staphylococcal activity of a traditional hydro-ethanolic extract of multi-origin elecampane root, previously observed within our laboratory. This thesis explores the application of a novel clean-up strategy and bioactivity-guided fractionation process which resulted in a subset of antibacterial or ‘active’ fractions. The phytochemical composition of these fractions was later analysed using a validated high-performance liquid-chromatography with diode array detection (HPLC-DAD) method supported by $^1$H nuclear magnetic resonance (NMR). The natural products attributing to the observed in vitro activity were identified as alantolactone, isoalantolactone, igalan(e), and an unseparated mixture of dugesialactone and alloalantolactone as major constituents. Another finding of this study was that the geographical origin of elecampane did not appear to influence either the chemical profile...
or the bioactivity of the analysed traditional root extracts. Elecampane clearly
demonstrates activity against *Staphylococcus* spp. and considering the occurrence
of antimicrobial resistance in Irish hospitals among this genus and the high prevalence of
MRSA, further investigation is warranted. This body of work therefore justifies
elecampane as a promising reservoir of antimicrobial compounds. Follow-on studies
could include large-scale purification or synthesis of the identified compounds followed
by *in vivo* analysis of the compounds, individually and in combination, and combinatorial
experimentation as potentiator or adjuvant compounds to support conventional antibiotic
treatment.

The concept of elemental impurification of plant material, the consequences of exposure
to human health, and the regulations that are currently in place to safeguard the general
European population, are explored in this thesis. Plants acquire metals from the
environment during cultivation and collection (geogenic and anthropogenic-derived
sources), and as elemental impurities during processing and manufacturing.
Toxicologically significant metals can thus arise as unsolicited and hazardous residues in
plants and products thereof, thus plants have been shown to be a risk factor for human
exposure to metals. While regulatory measures are enforced in European Member States,
there are apparent gaps in legislation concerning the presence of certain metals in plant-
derived food and phytopharmaceuticals. The adapted ICH Q3D guidelines published by
the EMA define limits for a total of twenty-four elements, however, these limits are not
currently applicable to herbs. In contrast, compendial (Ph. Eur.) limits for herbal products
exist for only Cd, Hg and Pb. From reviewing the literature, our investigation suggests
that two possible solutions to consider include extending the application of ICH Q3D
guidelines to cover herbal products, or alternatively, the establishment of a defined set of
general permissible limits for a greater suite of toxicologically significant metals
applicable to herbal products. Levels within plant-derived food and medicinal products
are not always routinely analysed, nor are patients’ samples routinely tested for metal
levels in clinical settings. Trace element interactions can influence nutritional status and
interfere with normal biological functions in humans. It is difficult to know the extent that
metal contaminants play in the symptoms or aetiology of chronic human disease,
therefore, harmonisation of quality requirements for food and medicinal products is a
necessity, particularly in the context of international trade and assurance of consumer
safety.
Two investigations were performed to determine the multi-elemental (metallomic) profiles of elecampane \((n = 27)\) and other commonly-used medicinal plant species \((n = 50)\), using a validated high-resolution inductively coupled plasma sector-field mass spectrometry (HR-ICP-SFMS) method following microwave acid-digestion. For the elecampane study, the research objective was to quantify for the first time, the novel multi-elemental (metallomic) profile of naturalised Irish and commercial samples of elecampane and investigate the risk of dietary exposure \textit{in silico} using the EFSA RACE tool to contextualise the toxicological significance of acute and/or chronic dietary exposure to metal contaminants.

Results from the analysis showed that chronic exposure to lead (Pb) at a maximum quantified concentration of 4617.42 µg.kg\(^{-1}\) is of potential risk to adult consumers in Ireland (18-65 y), at an estimated mean and 95\(^{th}\) percentile exposure of 0.049 and 0.189 µg-(kg BW)\(^{-1}\)d\(^{-1}\), respectively. Further investigation is advised, including soil and water analysis from the cultivation land to ascertain if Pb levels are within acceptable environmental limits, particularly if the farms are producing herbs, vegetables or crops for local communities. Additionally, 52\% of the elecampane samples were found to exceed European limits for Cd in food (200 µg.kg\(^{-1}\)), and one sample exceeded the compendial ML for Cd impurities in herbal material/drugs (> 1 mg.kg\(^{-1}\)). These findings illustrate the availability of non-compliant herbal substances, purchasable by local communities and online consumers.

Dietary exposure to Cd at the highest observed concentration (1285.97 µg.kg\(^{-1}\)) however, despite exceeding European regulatory and pharmacopeial permissible limits, was deemed “no risk” to Irish consumers when considering consumption of this single food category. The remaining elements (Li, Be, Mo, Sn, Ba, Hg, Tl, V, Cr, Co, Ni, Cu) were well below regulatory and pharmacopeial limits in all samples and thus dietary exposures to these elements are therefore of negligible concern to Irish consumers. Outputs from the RACE tool, however, focus on one contaminant in one food commodity at a time, and therefore background exposure from all other dietary sources needs consideration.

Another major finding of the elecampane investigation revealed a significant difference in element distribution between flowers – leaves (Be, Li, Ba, Cd, Bi), root – leaves (Mo, Sn, Co, Hg) and flowers – root (Ba and Tl) in naturalised elecampane samples. Considering the range of elements tested, the geographical distribution of the sampled
plants and the sufficient sample size, the findings of this investigation can be used in future studies as a control population for inter-study comparisons.

The multi-elemental (metallomic) profiles of fifty medicinal plant samples was investigated, many for the first-time, including arnica, bush vetch, sweet cicely, yellow rattle, bogbean, rock-tea and tufted catchfly. Highest quantified concentrations were observed in ox-eye daisy flower (Li: 3964 µg.kg⁻¹), dandelion leaf (Be: 122 µg.kg⁻¹; Cd: 325 µg.kg⁻¹; and Sn: 165 µg.kg⁻¹), dandelion root (Ti: 5827 µg.kg⁻¹), great mullein leaf (Mo: 4505 µg.kg⁻¹; and Tl: 91 µg.kg⁻¹), elderberry fruit (Ba: 4646 µg.kg⁻¹), hawthorn flower and leaf (Pt: (33 µg.kg⁻¹); Hg: (30 µg.kg⁻¹), Pb: 4248 µg.kg⁻¹; and Bi: 30 µg.kg⁻¹), comfrey root (V: 1758 µg.kg⁻¹), boldo leaf (Cr: 4534 µg.kg⁻¹), coltsfoot leaf (Co: 652 µg.kg⁻¹) and flower (Ni: 6060 µg.kg⁻¹), and lastly aerial bush vetch (Cu: 6340 µg.kg⁻¹).

Shorter-than-lifetime daily intakes were estimated using conservative ("worst-case") and realistic theoretical exposure scenarios. The non-carcinogenic risk assessment was subsequently evaluated using the Hazard Quotient (HQ) and Hazard Index (HI) mathematical equations. All botanical samples analysed (n = 50) were below the compendial limits for metal impurities in herbal substances/starting materials: Cd (≤ 1 mg.kg⁻¹), Hg (0.1 mg.kg⁻¹), and Pb (5 mg.kg⁻¹), however, Li, Mo, Tl, Pb, Co, and Ni were quantified at potentially unsafe levels at the theoretical worst-case exposure scenario. Furthermore, 42% of all samples tested, representing 16 different plant species (n = 30), were categorised as potentially unsafe to consumers (HI ≥ 1) with regards to the non-carcinogenic cumulative exposure to Cu, Cd, Hg and Pb, including: hawthorn, arnica, dandelion, marigold, nettle, yarrow, comfrey, borage, coltsfoot, birds foot trefoil, ox-eye daisy, yellow rattle, mugwort, great mullein, tufted catchfly, marshmallow. Major data gaps hindering the risk evaluation of plants and derived products are discussed. Intake and consumption data at European level and the availability of guidance in the absence of experimental data (e.g. metal transfer rates, exposure durations and frequency) to facilitate harmonised deterministic and probabilistic risk assessment criteria would help ensure botanical safety.

In conclusion, the work presented in this thesis aims to provide a basis for plant research methodology standardisation, and harmonisation or extension of inclusion criteria within pharmacognosy and the wider botanical sciences to assure botanical safety, quality and regulation – from initial screening to product commercialisation.
Chapter I

A post antibiotic era looms: can natural product research fill the void?

Chapter based on manuscript published in the ‘British Journal of Biomedical Sciences’:


(See Appendix I)
Abstract

Antimicrobial resistance is increasing among certain pathogenic bacteria to the extent that treatment efficacy is no longer always assured. According to the CDC, as few as six new antibiotics have been released for use over the past 30 years. Resistance has already been observed to each of these. Eleven plant natural products have been approved for therapeutic use during the same period – none of them being antimicrobial agents. We have learned through experience that some microorganisms will inevitably overcome antibiotic treatment in certain situations, and then spread, and it is clear that the rate of new antimicrobial development is insufficient to meet our current and future needs, which should be addressed in order to guarantee the effective future of antimicrobial chemotherapy. However, in recent years there has been an increase in the number of peer-reviewed reports of antimicrobial efficacy among plant-derived secondary metabolites. A limitation with these reports is the wide range of modified in vitro methods used to determine preclinical antimicrobial efficacy of these products, showing an absence of the type of standardisation that is the norm when testing the efficacy of single- or combined agent conventional antimicrobials in the laboratory, thereby making inter-study comparison difficult. Overall, despite the large diversity in preparation and testing strategies used currently for natural product plant-derived antimicrobials, our investigations suggest that the field shows promise in the provision of novel antimicrobial agents, as exemplified by our selected case-study: *Inula helenium* L. (elecampane).

Keywords: Antimicrobial Susceptibility Testing; Antimicrobial Resistance; Natural Product Research; Plant constituents; *Inula helenium*; Elecampane.
1.1 Introduction

Antimicrobial activity may apply to both commercially produced antibiotics and to certain bioactive natural products produced by living organisms found in nature, whether of plant, microbial or marine origin. Antimicrobial resistance to conventional antimicrobial agents is increasing among pathogens. For example, in 2013, approximately 480,000 new cases of multi-drug resistant tuberculosis were documented in over 100 countries (WHO, 2015). Significant global increase in drug-resistant infections will potentially result in 10 million preventable deaths worldwide and an enormous financial cost reaching $100 trillion by 2050 (O’Neill, 2014). The number of compounds that are currently being developed is still insufficient to control global threats of infectious disease (Bassetti and Righi, 2015).

This rise of antimicrobial resistance (AMR), particularly among significant pathogens, when considered in the light of few novel replacements over recent decades, suggests an urgent need to find new and effective antimicrobial agents. Only an estimated 15% of approximately 300,000 higher plant species have been investigated phytochemically – which suggests that plant-derived natural products provide potential for new agents (Cragg and Newman, 2013). The term ‘chemical space’ has been defined as the set of all possible molecular structures in one collection (Medina-Franco et al., 2008). Combinatorial compound diversity, chosen by pharmaceutical companies in recent times as a means to generate active compounds, has shown considerably less diversity than have natural product compounds (Feher and Schmidt, 2003). Structural diversity of natural products inevitably surpasses that from synthetic or combinatorial compounds (Mishra et al., 2008), thus suggesting that plant-derived natural products provide potential for new chemically diverse agents.

Atanasov et al. (2015) list all isolated plant natural products approved for therapeutic use in the last thirty years (1984 – 2014), including artemisinin, capsaicin, colchicine, dronabinol, cannabidiol, galantamine, ingenol mebutate, masoprocol, omacetaxine mepesuccinate, paclitaxel and solamargine - none of which are antimicrobial agents. Furthermore, according to the CDC (2015), just six commercial antibiotics have been developed in a similar timeframe (1985 – 2010); including imipenem and ceftazidime (1985), levofloxacin (1996), linezolid (2000), daptomycin (2003), and ceftaroline (2010). Linezolid and daptomycin, of these, are antibiotics with novel modes of action, but even
with these agents, bacterial resistance has been noted, despite their relatively recent development and release for use (Lellek et al., 2015). Nevertheless, natural products are a continuing source of novel drug possibilities to tackle AMR, requiring interdisciplinary collaborations and vigorous exploration of all approaches to drug discovery for the effective development of novel therapeutics (Cragg and Newman, 2013).

Natural products may be utilised in numerous modified and unmodified forms. Cragg et al. (2012) have analysed the major sources of new commercial drugs from 1981 – 2010, which may be summarised as follows:

- Unmodified natural (pure) compounds
- Modified natural compounds
- Semi-synthetic compounds
- Synthetic compounds with no natural product conception
- Synthetic compounds with natural product pharmacophore

‘Secondary metabolites’, whilst widely regarded as being synonymous with the term ‘natural product’, can be defined as organic compounds in the correct chiral configuration to exert bioactivity with no growth, development or reproductive function in an organism (Pichersky and Gang, 2000). These molecules oftentimes elicit protective and defence mechanisms for the plant in situ. Occurrence of molecularly diverse secondary plant metabolites can be restricted to particular higher (vascular) plant families, genera or singular species (Berkov et al., 2014). The term ‘bioactive compounds’ or ‘bioactive constituents’ are molecules which elicit confirmed biological effects in human or animal systems. Drug discovery and development involves the identification of therapeutically relevant new chemical entities (NCEs). Koehn (2008) describes advanced analytical and spectroscopic methods that allow the rapid identification and structural elucidation of complex natural products in crude or pre-fractionated plant extracts while profiling their bioactivity in a process coined ‘bioassay-guided-fractionation’. Advanced developments in the field of metabolite analysis have recently been reviewed by Wolfender et al. (2015).

Methods of evaluating antimicrobial activity of natural products often involve the modification and adaption of standard in vitro methods designed for single constituent compounds (i.e. pharmaceutical antibiotics), as shown in Figure 1.1. In this mini review, the advantages, limitations and comparisons between each current diffusion, dilution and
bioautographic method will be highlighted with illustration using the anti-staphylococcal plant *Inula helenium* L., commonly known as elecampane (Figure 1.3).

**Figure 1.1 Types of natural products derived from plants and categorisation of widely adopted in vitro antimicrobial methods.**

**1.2 *In vitro* antimicrobial methodology adaption in plant natural product research: what are the major issues?**

Figure 1.1 depicts the types of conventional antimicrobial assays available to researchers and since each method is not based on the exact same principle, the results obtained may be influenced profoundly by the method selected, inoculum size, choice of organisms, degree of sample solubility, compound stability, chemical complexity and stereochemistry (Mishra et al., 2008; Valgas et al., 2007). Currently available antimicrobial screening methods in natural product research may be divided into three categories:

1. Diffusion methods (qualitative)
2. Dilution methods (quantitative)
3. Chromatographic-coupled bioautographic methods (qualitative & quantitative)

Standardised procedures and approved guidelines (through agencies such as the European Committee on Antimicrobial Susceptibility Testing (EUCAST), or the US Clinical Laboratory and Standards Institute (CLSI)) are the accepted practice for the antimicrobial susceptibility testing of conventional single constituent chemotherapeutics; however, these regulated methodologies may be unsuited to the direct investigation of complex
multi-constituent agents (i.e. crude or fractionated plant extracts). Standard diffusion and
dilution techniques initially employed for single constituent agents have been modified
consequently (Mondello et al., 2009). The problem with such modification is that these
methods are not universally standardised for plant natural products. Incomplete
standardisation, redundant and out-dated methodology (Tan and Lim, 2015) and
inconsideration of all of the components of the extracts and their physiochemical
properties has led to a plethora of unsubstantiated reporting of results. This complicates
efforts of inter-laboratory comparison between different plant extracts by authors and
research groups worldwide (Tan and Lim, 2015). No single assay or combination of
assays is necessarily optimal given the diversity of complex multi-constituents present in
different extracts (Power et al., 2013), even where methods might possibly be
standardised between laboratories. Furthermore, evaluating the bioactivity of natural
products – whether of plant, marine or animal origin – requires interdisciplinary
collaboration rooted in both biology and chemistry specialties.

Rios et al. (1988) were among the first authors to review the application of standardised
conventional AST methods to natural product research. In addition to the antibacterial
assay selected, many other factors can affect the overall outcome in natural product
research, including environmental and climatic growing conditions, plant material,
extract preparation, extraction method and choice of solvent (Ncube et al., 2008), thereby
complicating efforts to standardise methods for natural product antimicrobial assessment.

1.3 Modified AST Methods used for the assessment of multi-
constituent antimicrobial agents

1.3.1 Diffusion Methods

1.3.1.1 Disk-diffusion method
Diffusion methods are among the most widely adopted conventional *in vitro* antibacterial
screening techniques used today which provide what is often described as a qualitative
(rather than quantitative) categorical interpretation of the degree of microbial
susceptibility to an antimicrobial agent (Hombach et al., 2013; Tan and Lim, 2015). The
methods are easy to perform, economical and require little or no specialised
instrumentation (Ogata et al., 2014; Osato, 2005). In the case of plant extract testing for
antimicrobial activity, the disk diffusion procedure is performed similarly to the standard method using antibiotic impregnated disks, whereby the method involves loading circular disks with a chosen plant extract which is then aseptically placed onto the surface of an inoculated agar medium. Minor variations have been reported in the literature including the following:

- Refrigeration of the prepared plates at 4°C for 1 – 2 hours to allow pre-diffusion of the plant extract into the inoculated medium prior to incubation (Lourens et al., 2004; Schmourlo et al., 2005; Tepe et al., 2004).
- Extended soaking of the sterilized disks with plant extract for two hours before placing on the inoculated medium (Mbata et al., 2008).
- Drying of the impregnated disks under laminar airflow overnight to facilitate solvent/diluent evaporation (Basri and Fan, 2005).

Disk-diffusion methods generate data suitable for preliminary bioactivity screening of novel extracts and cannot be manipulated for the generation of minimum inhibitory concentrations (MICs) or minimum bactericidal concentrations (MBCs) (Ncube et al., 2008). The compounds diffuse from the disk into the surrounding medium, any antibacterial activity is visually represented by the presence of a clear zone which depicts the interrupted growth of bacteria as a result of encounter with the agents (Lee et al., 2010). Qualitative results are recorded upon measuring the difference in circumference from the disk to the surrounding clear zones.

1.3.1.2 Agar-well (hole-plate) and cylinder diffusion

The agar-well and cylinder diffusion assays are similar to the disk-diffusion method, with minor exceptions regarding the type of reservoir used (Ncube et al., 2008). In the agar-well method, defined wells (6 – 8 mm) are aseptically perforated into the agar using a sterile cork-borer, allowing adequate (30 mm) space between adjacent wells. For the cylinder method, aluminium, glass, or porcelain reservoirs are required. Plant extracts or fractions of defined concentration (i.e. 0.01 - 10 mg.mL⁻¹) are introduced into the cylinders or newly formed wells (Janssen et al., 1987; Mbata et al., 2008). Valgas et al. (2007) claim that the agar-well method is more sensitive when testing plant samples in comparison to the disk-variant methods – the premise of which is rooted in the potential
constituent adsorption to the paper disks (Burgess et al., 1999) as described in Table 1.1 below.

Table 1.1 Limitations of diffusion methods in plant natural product research:

<table>
<thead>
<tr>
<th>Experimental Variable(s)</th>
<th>Associated Limitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qualitative screening</td>
<td>Diffusion methods cannot generate accurate MICs or distinguish between bactericidal and bacteriostatic activity (Parekh et al., 2006).</td>
</tr>
<tr>
<td>Comparability issues</td>
<td>Zones of inhibition are not always correlated with the strength of antibacterial activity – a common misconception. The results are preliminary (Tan and Lim, 2015).</td>
</tr>
<tr>
<td>Solubility issues</td>
<td>Diffusion and dilution-based methods are largely dependent on the availability of the active principle which is a function of the test compounds’ solubility (Ncube et al., 2008).</td>
</tr>
<tr>
<td>Varied diffusion rates</td>
<td>Variability is introduced with the presence of crude extracts or multi-constituent fractions (Ncube et al., 2008). Particle size, shape, and volume influence molecule diffusion rates (Valgas et al., 2007).</td>
</tr>
<tr>
<td>Compound adsorption</td>
<td>Cellulose filter paper is frequently used for disk preparation, (i.e. β-(1-4) glucose monomers). The presence of free hydroxyl groups on each glucose residue renders the disk surface hydrophilic. Cationic polar plant constituents would therefore be expected to adsorb to the disk surface thus influencing compound diffusion through the medium (Burgess et al., 1999).</td>
</tr>
<tr>
<td>Subjective interpretations</td>
<td>Greater dependence on the investigator to perform the procedures and interpret the results correctly in the absence of approved instrumentation and standardization (Hombach et al., 2013).</td>
</tr>
<tr>
<td>Choice of solvent and controls</td>
<td>Solvents used for both extraction and/or solubilisation of extracts should be non-toxic; ensuring no assay interference. (Ncube et al., 2008).</td>
</tr>
<tr>
<td>Factors influencing inhibitory zone diameter</td>
<td>Method sensitivity is influenced by microorganism species, growth medium pH and composition, agar thickness and disk adsorption (Pikkemaat et al., 2009; Scorzoni et al., 2007).</td>
</tr>
</tbody>
</table>

1.3.1.3 Vapour diffusion

Vapour diffusion methods can also be referred to in the literature as ‘micro-atmosphere diffusion’ or ‘disk volatilization’. Plant volatile oils are naturally occurring, highly complex organic compounds characterized by a potent aroma. These compounds are produced by aromatic plants as secondary plant metabolites (Bakkali et al., 2008). Some earlier studies have documented the evaluation of plant-derived volatile organic compounds (VOCs) (e.g. essential oils, phytoncides) through direct contact (diffusion) methods or liquid (dilution) methods; however, compound diffusion and solubility factors largely affect the feasibility of these methods (Tyagi and Malik, 2010).

Vapour diffusion is a modified agar diffusion procedure developed on the premise that VOC’s exert critical biological activity (Mondello et al., 2009). This method allows for a more uniform vapour distribution within the petri-dish headspace. Each individual constituent has individual volatility, therefore when the constituents are introduced into a
free, non-saturated state in a closed environment, the volatile constituents begin to disperse at differing rates until they reach equilibrium (Kloucek et al., 2012). At equilibrium the headspace composition is homogeneous, and this has led to the development of specifically designed air-tight chambers that facilitate the parallel assessment of antimicrobial activity using multiple solid agar media (Tyagi and Malik, 2011). A limitation of the method is vapour loss owing to insufficient or damaged sealing of the exposure chamber, and compound adsorption to the petri-dish material. Some authors have documented the addition of agar (≤ 5 mL) into the inner surface of petri dishes to help seal and prevent the adsorption of volatile vapours to the petri-dish material and thus preventing interference with the internal environment (Kloucek et al., 2012; Tyagi et al., 2012).

1.3.2 Dilution Methods

1.3.2.1 Agar dilution

This conventional method incorporates different concentrations of the plant-derived sample into an agar medium, followed by inoculation with a defined bacterial suspension. This method has similar disadvantages as other dilution methods (See Table 1.2), being, in addition, more laborious than the broth-based alternatives (Nasir et al., 2015; Tan and Lim, 2015).

1.3.2.2 Broth dilution: macrodilution and microdilution

Broth macrodilution is the precursor dilution method and is infrequently used in modern laboratories. It requires the manual preparation of serial dilutions using relatively high quantities of reagents and laboratory space. Disadvantages include the labour-intensive preparation of test solutions and the concurrent possibility of errors associated with such repeated preparations (Jorgensen and Ferraro, 2009).

The microdilution technique involves serial dilution of the sample, which is sequentially inoculated with bacterial suspension in a 96-well microtiter plate (King et al., 2008), with or without the addition of a colorimetric indicator. Notable merits include rapid quantitative generation of MIC and MBC values, real-time microbial kinetic analysis, enhanced reproducibility, method feasibility and convenience.
Miniaturization of the technique greatly reduces expenditure on reagents and laboratory space required for implementing the technique (Jorgensen and Ferraro, 2009). Precise aliquots of diluted samples in broth are dispensed into each singular reservoir using an automated multi-pipette. Quantitative endpoints (e.g. MICs) are measured post-incubation using automated systems which generate reports (Jorgensen and Ferraro, 2009). The merits of using automated instrumentation include higher throughput and the non-subjective standardisation of endpoints in comparison to manual readings; while also facilitating the analysis of large amounts of data, and monitoring of trends in resistance and susceptibility (Dipiro et al., 2011; Jorgensen and Ferraro, 2009) – although limitations do still exist (Jorgensen and Ferraro, 2009; Kulah et al., 2009; Swenson et al., 2009).

Overall, microdilution methods provide reliable and efficient assessment of most plant-derived antimicrobial samples, provided the sample is soluble in an inert diluent – a common challenge in botanical sciences (Ríos and Recio, 2005).

<table>
<thead>
<tr>
<th>Experimental Variable(s)</th>
<th>Associated Limitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-polar compound precipitation</td>
<td>Reduces contact with the bacterial suspension (Cushnie and Lamb, 2005).</td>
</tr>
<tr>
<td>Membrane filtration adsorption</td>
<td>Hydrophilic constituents and heat-labile water-based extracts can only be reliably sterilised via membrane filtration, which could potentially allow constituents to adsorb onto the membrane thus affecting bioactivity (EUCAST, 2003).</td>
</tr>
<tr>
<td>Ambiguous MIC determination</td>
<td>Interference by turbid or coloured extracts can obstruct accurate visual or spectrophotometric measurements of the microtiter plates post-incubation, suggesting an advantage to using colorimetric methods.</td>
</tr>
<tr>
<td>Subjective visual results</td>
<td>Visual assessment of turbidity, when testing a variety of agents can be subjective (Othman et al., 2011).</td>
</tr>
<tr>
<td>The Inoculum Effect (IE)</td>
<td>High inoculum can induce false resistance and low inoculant can result in false-susceptibility (Bidl, et al., 2008; Othman et al., 2011), in the absence of recommended guidelines by a competent authority.</td>
</tr>
<tr>
<td>Incomparability to other methods</td>
<td>Results obtained from the microdilution method are not always comparable to diffusion or bioautographic methods (King et al., 2008).</td>
</tr>
</tbody>
</table>

The principle of high-throughput screening (HTS) is the random and systematic evaluation of libraries of chemicals likely to modulate a specific biological target in cell-free-, phenotypic- or targeted cell-based-assays. Since the 1990s, HTS evaluation has accelerated in parallel with the HTS chemistry methods that initiated the development of synthetic compound libraries (David, B., Ausseil, 2011; David et al., 2014; Henrich and
Beutler, 2013). Guidelines for testing natural products for antimicrobial efficacy using these technologies have not been generated to date, however.

1.3.3 Chromatographic-coupled Bioautography

Bioautography is an analytical phytochemical screening tool which detects bioactive portions of a complex extract based on the principle of bioassay-guided fractionation (Choma and Jesionek, 2015; Nostro et al., 2000). In natural product research, bioautographic detection is a functional microbial screening method typically combined with planar chromatographic techniques including (high-performance) thin layer chromatography ((HP)-TLC), high-performance liquid chromatography (HPLC), over-pressured-layer chromatography (OPLC) and planar electro-chromatography (PEC) (Choma and Jesionek, 2015). Merits of these methods over modified diffusion and dilution assays are summarised below.

- **Easily applicable plant screening method**: hyphenated-bioautography remedies the issue of isolating antimicrobial compounds from complex crude plant extracts by offering a simple alternative isolation and identification process.

- **Reduced extract quantities**: minimised requirement for plant extract volume in comparison to other methods, which has both time- and budget-saving benefits.

- **Target-directed isolation of active constituents**: the localisation of active compounds in a complex matrix combined with *in situ* bioactivity determination enables the guided isolation of targeted constituents (Rahalison et al., 1991; Shahverdi et al., 2007).

- **Parallel bioactivity comparison between samples**: samples of varied origin can be assayed on the one TLC plate allowing a direct comparison between extracts (Suleiman et al., 2010).

- **Bio-guiding method**: bioautographic methods points towards active compounds that warrant further investigation via structural analysis (Choma and Jesionek, 2015).

- **Spectroscopic input**: provides a broad range of information regarding the bioactivity and even the structures of the target compound(s).
• **Preparative TLC Isolation**: separated components can be obtained directly from TLC plates by applying the sample in a wide band, which is scraped directly from the plate, eluted with appropriate solvents and structurally analysed via LC-MS, OPLC or other sophisticated methods for structural evaluation (Choma and Jesionek, 2015).

Three versions of TLC-coupled bioautographic methods are currently used in plant research: contact, direct and immersion variants, as shown in Figure 1.2, together with a listing of their disadvantages in Table 1.3.

![Figure 1.2 Schematic diagram of available (HP)-TLC Bioautographic methods](image)

1. **Contact (Agar Diffusion) Bioautography**: involves placing the developed chromatogram onto the pre-inoculated agar medium surface for a specific period to allow diffusion. The next step requires the removal of the chromatogram and the subsequent incubation of the inoculated agar layer. Clear zones indicate growth inhibition (Choma and Grzelak, 2011).
2. **Direct Bioautography**: involves the application of an antimicrobial agent directly onto developed TLC plates, development in an appropriate solvent system and the direct application of the suspended bacterial test strain onto the TLC plate. Nutrients from the broth medium adhere to the TLC plate surface - which acts as a nutrient source for the bacterial strains thus enabling the direct growth of the bacteria on to the plate surface (Choma and Grzelak, 2011). Similarly to the diffusion assays, clear inhibitory zones on the TLC plate indicate the presence of antimicrobial agents. Colorimetric indicators are used to visualise microbial growth, typically tetrazolium salts (Choma and Jesionek, 2015). Microorganisms convert tetrazolium salts into formazan via the presence of dehydrogenases (Choma and Grzelak, 2011).

3. **Immersion (agar-overlay) Bioautography**: This is a hybrid of both contact and direct procedures (Das et al., 2010). The technique requires total immersion of the pre-exposed TLC plate with seeded agar medium. To enhance diffusion rates (Shahverdi et al., 2007), it is suggested that plates should be kept at a low temperature for a few hours prior to incubation to allow greater diffusion of the antimicrobial agent into the medium, thus optimizing the procedure.

<table>
<thead>
<tr>
<th>Experimental Variable</th>
<th>Associated Limitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active zones close to the origin</td>
<td>Clear inhibitory zones in close proximity to the origin must be separated further with a mobile phase of greater polarity to reveal active sub-fractions suitable for further evaluation and to reject non-active fractions (Choma and Jesionek, 2015).</td>
</tr>
<tr>
<td>Active constituent(s) ‘synergy’ disruption</td>
<td>Some authors report the potential disruption of synergistic mechanisms between the active constituents in the plant extract thus potentially reducing the biological activity of the extract at large (Schmourlo et al., 2005; Sulciman et al., 2010).</td>
</tr>
<tr>
<td>Unsuitable for VOCs</td>
<td>Volatile fractions may be lost through evaporation from the chromatogram.</td>
</tr>
<tr>
<td>Non-standardized method parameters</td>
<td>Criteria including mobile phase composition, stationary phase adsorbent, test species, TLC plate pre-conditioning and visualisation methods are all varying parameters that may potentially affect output (Choma and Grzelak, 2011).</td>
</tr>
<tr>
<td>Mobile phase pH and/or composition</td>
<td>Strongly acidic or alkaline solvents may remain on TLC plates thus potentially inhibiting bacterial growth (Hamburger and Cordell, 1987).</td>
</tr>
</tbody>
</table>
## 1.4 Plant-derived Natural Product Drug Discovery: Current Challenges

Aside from poor methodological setup and analysis of data (Butterweck and Nahrstedt, 2012), the pre-clinical analysis of plant-derived natural products faces a variety of inherent challenges, a selection of which are outlined in Table 1.4 below. Ultimately, the purpose of addressing these challenges is to maximise accurate screening in plant natural product research (Liu, 2008).

### Table 1.4 Plant-derived Natural Product Drug Discovery: Current Challenges

<table>
<thead>
<tr>
<th>Specific Challenges</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Species-specific compounds</strong></td>
<td>Natural products occurring in many different plant species are favoured over species-specific compounds as this removes the supply constraints hindering successful research, development and commercialisation of natural product-derived drugs (Amirkia and Heinrich, 2014; Atanasov et al., 2015).</td>
</tr>
<tr>
<td><strong>Natural product compound Resupply</strong></td>
<td>Resupply from the original plant species is often unfeasible and economically non-viable to meet market demands upon commercialisation of plant-derived drugs. Emerging biotechnological and [total-, semi-] chemical synthesis strategies offer potential alternatives to overcome the precursor resupply obstacle (Atanasov et al., 2015).</td>
</tr>
<tr>
<td><strong>Illogical dosage/concentrations and erroneous endpoints</strong></td>
<td>The use of excessively high (unrealistic) concentrations of plant extracts for preclinical testing to obtain a dose-response effect have been documented in the literature (Butterweck and Nahrstedt, 2012; Cos et al., 2006). The need for stringent end-point criteria is imperative. Cos et al. (2006) recommend *IC50 values ≤ 100 µg.mL−1 for extracts and ≤ 25 µM for pure compounds as a relative starting point for standardised endpoints.</td>
</tr>
<tr>
<td><strong>Presence of nuisance compounds</strong></td>
<td>Contamination of plant material with metals can cause defective bioassay outcomes (Atanasov et al., 2015). Metal accumulating plant species can concentrate heavy metals (e.g. cadmium, zinc, copper, manganese, nickel, lead) up to 100 or 1000 times that of non-accumulating plants (Tangahu et al., 2011). Profiling of plant samples is advised where possible.</td>
</tr>
<tr>
<td><strong>Pro-drug evaluation</strong></td>
<td>Some natural products are categorised as [inactive] pro-drugs that require metabolic activation to confer its pharmacological activity (Butterweck and Nahrstedt, 2012), for example cyanogenetic glycosides (David et al., 2014). This complicates identification methods when effector compounds are not present in the starting plant material, thus undetectable through typical identification methods.</td>
</tr>
<tr>
<td><strong>General Inherent Challenges</strong></td>
<td>The World Health Organisation (WHO) released Industry Guidelines on Good Agricultural and Collection Practices (GACP) for medicinal plants to promote sustainable harvest and collection techniques and to reduce ecological issues pertaining to wild crafting of medicinal plants (Atanasov et al., 2015).</td>
</tr>
<tr>
<td><strong>Cultivation, harvest &amp; post-harvest neglect</strong></td>
<td>Unequivocal plant material identification requires genetic (-omic techniques) and chemical analysis, including hyphenation of GC or HPLC with various spectroscopic methods. Plant taxonomy and synonym issues can complicate this step further (David et al., 2014).</td>
</tr>
</tbody>
</table>
1.5 Case Study:

The antimicrobial potential of *Inula helenium* L. (Elecampane)

*I helenium*, which is a plant naturalised to Ireland, was selected for particular study in this review, both on the basis that it had been studied previously in our own laboratory and elsewhere, and that there have been several recent published studies of its antimicrobial efficacy.

![Images of plant parts](wildflowersofireland.net)

**Figure 1.3** Close-ups of aerial parts of native *Inula helenium* L. (elecampane) growing in Co. Clare; a - b = yellow central tubular disk bordered by ray flowers around the periphery, c = alternate leaf arrangement along the hairy stem, d = simple, wide elliptical and irregularly serrated leaves. Images sourced with permission from Zoë Devlin (wildflowersofireland.net).
From our earlier work (O’Shea et al., 2009), 100% inhibition of 200 staphylococci isolates encompassing methicillin resistant *Staphylococcus aureus* (MRSA), methicillin sensitive *S. aureus* (MSSA) and coagulase negative Staphylococci (CNS) were reported with MBC values of 0.9 – 9.0 mg.mL⁻¹ recorded for *I. helenium* crude extracts using a crude drop-test, whereby a specified volume of extract was pipetted on a freshly inoculated bacterial lawn.

Deriu *et al.* (2008) evaluated supercritical fluid (SCF) and hydro-distilled (HD) essential oil extracts of Italian *I. helenium* roots against several bacterial strains using disk-diffusion and microdilution methods. MICs ranged from 0.009 to >14 mg.mL⁻¹. An MIC of 0.6 mg.mL⁻¹ (HD extract) and 3.7 mg.mL⁻¹ (SCF extract) was reported against *S. aureus* (ATCC 29213); the streptomycin control MIC was 0.05 mg.mL⁻¹– significantly lower than both *I. helenium* extracts. *Pseudomonas aeruginosa* was the most resistant of all strains tested (MIC 14.8 mg.mL⁻¹). Both extracts significantly inhibited ampicillin-, erythromycin-, penicillin-, and tetracycline-resistant *Enterococcus faecium* strains (MIC >0.3 mg.mL⁻¹). The extracted oil was found to be more a potent antifungal against *Candida* spp. when compared to tea tree and bergamot oils. The authors remarked that the HD-extracted oil is more active than the SCF-extract and this increased potency is potentially attributable to synergism between the profile and concentration of compounds extracted via HD. This however remains to be proven at the time of writing.

A recent review by Seca *et al.* (2014) stated that alantolactone is one of the most widely tested isolated constituents from *I. helenium*. Stojanović-Radić *et al.* (2011) reported alantolactone, diplophyllin and isoalantolactone as the active anti-staphylococcal constituents of *I. helenium*.

Gökbulut *et al.* (2013) states that methanol *I. helenium* root extracts of Turkish origin were most active against Gram-positive bacteria in a CLSI modified agar-dilution method using a concentration range from 6.25 – 800 µg.mL⁻¹. Lowest MICs were generally observed for the *I. helenium* root extracts over either flower or leaf extracts: *Enterococcus faecalis* (ATCC: 29212; MIC 100 µg.mL⁻¹), *E. coli* (ATCC: 25922; MIC 200 µg.mL⁻¹), *S. aureus* (ATCC: 29213; MIC 100 µg.mL⁻¹) and *P. aeruginosa* (ATCC: 27853; MIC 400 µg.mL⁻¹). In agreement with Deriu *et al.* (2008), *P. aeruginosa* exhibited strongest *in vitro* resistance to *I. helenium*. 

16
Jiang et al. (2011) evaluated the antibacterial activity of isolated Chinese *I. helenium*-derived compounds against *B. cereus, E. coli, Erwinia carotovora, S. aureus* and *P. aeruginosa* using the broth microdilution method with reference to CLSI guidelines. A total of 16 extracted compounds were listed; 4α,15α-epoxyisoalantolactone demonstrated stronger activity (MIC 15.5 µg.mL⁻¹) to *B. cereus* than the positive ampicillin control, while isoalantolactone exhibited moderate antibiotic to *B. cereus* (MIC 31.3 µg.mL⁻¹). Compounds isoalantolactone, 4α,15α-epoxyisoalantolactone, macrophyllilactone, 4α,15-epoxyisoalantolactone, telekin and 3α-hydroxyeudesm-4,11-dien-12,8β-olide demonstrated weak inhibition (MICs 62.5 – 125 µg.mL⁻¹) to *E. coli*. Again, the compound 3α-hydroxyeudesm-4,11-dien-12,8β-olide demonstrated weak inhibition to *B. cereus* and isoalantolactone to *S. aureus*, while compounds isoalantolactone, macrophyllilactone, telekin and 3α-hydroxyeudesm-4,11-dien-12,8β-olide inhibited *E. carotovora* and compounds isoalantolactone and 3α-hydroxyeudesm-4,11-dien-12,8β-olide inhibited *B. subtilis*. All other [non-specified] compounds were inactive, showing MICs >250 µg.mL⁻¹. This study is an example of critical, comprehensive, and high-standard natural product research. The authors state that the bioactivity of the aforementioned compounds is characteristic of an eudesmane structure with an α,β-lactone and oxirane thus emphasising the link between molecular structures and configuration with constituent bioactivity – which further strengthens the importance of documenting plant chemical profiles in conjunction with bioassay evaluations in order to ascertain any claimed activity.

Stojanović-Radić et al. (2012) also demonstrated the structure-activity relationship of *I. helenium*, suggesting that the eudesmane core olefinic bonds together with the α, β-methylene-lactone moiety (See Figure 1.4) are vital structural features (motifs) necessary for anti-staphylococcal activity. Further conformational analysis demonstrated that the enhancement of potency for the compound diplophyllin was attributed to optimal lactone moiety interaction with the binding region of targets.

Qiu et al. (2011) concluded that isolated isoalantolactone did not exhibit anti-staphylococcal activity *in vitro* using the modified CLSI microdilution method. The authors did, however, note the compound’s ability to inhibit α-toxin expression in *S. aureus* at very low concentrations and its protection of mice against pneumonia *in vivo*. Alpha-toxin is a pore-forming toxin secreted by most *S. aureus* strains which is essential
for pneumonia pathogenesis. This is an example of where unremarkable *in vitro* antimicrobial results may be less significant than elucidation of [natural products’] bioactive mechanisms of action, which may actually provide more vital information toward their use as anti-infective agents, than the initial results suggest.

Radulović *et al.* (2014) also reported the inactivity of Serbian *I. helenium* root-derived 3-methyl-2-alkanones against *S. aureus* using the microdilution method *in vitro* (MIC > 3.70 mg.mL⁻¹) in comparison to the positive control nystatin (MIC = 0.78 µg.mL⁻¹). The authors imply that the bioactive sesquiterpene aldehydes of this fraction are still unidentified and that they occupy a mass spectrum resembling bicyclogermacrenal.

Stojakowska *et al.* (2005) reported moderate antibacterial activity of *I. helenium* root-derived compound, 10-Isobutyryloxy-8,9-epoxythymol isobutyrate, against *S. aureus* (MIC 50 – 250 µg.mL⁻¹), *E. faecalis* (MIC = 1000 µg.mL⁻¹), *E. coli* (MIC = 1000 µg.mL⁻¹) and *P. aeruginosa* (MIC 1000 µg.mL⁻¹) using the broth microdilution method *in vitro*. The authors comment on the role of thymol derivatives in the young roots of *I. helenium* potentially contributing to its antibacterial activity.

As an interesting sequel to the traditional use of *I. helenium* root as an antimycobacterial agent, Gautam *et al.* (2007) reviewed *in vitro* anti-mycobacterial screening methods, including dilution, diffusion, radiorespirometry and reporter gene assays when testing *I. helenium* and *I. racemosa*. Prior to this, Cantrell *et al.* (1998) had reported 100% inhibition of *Mycobacterium tuberculosis* (H382v; ATCC 27294) by crude organic *I. helenium* root extracts (MIC: 0.1 mg.mL⁻¹) prepared from mother plants of South, Central and North American origin, using an *in vitro* radiorespirometric assay. Percentage inhibition was unaffected by choice of extraction solvent used (hexane, dichloromethane and methanol).

Of the literature cited, it is clear that many authors in the field of natural products do not attempt to include vital information in their methodology, particularly descriptions of the plant material, extraction process, constituent profiling, MIC/MBC definitions, guidelines followed [if any] or verification of the assumed extract potency.

*In vitro* evaluation is important as a primary screen, but compounds exhibiting promising activity require further studies to validate or ascertain therapeutic potential. Confirmation of the pharmacological potential of, for example, *I. helenium* bioactive secondary
metabolites must be subjected to *in vivo* studies, such as the Zebrafish (*Danio rerio*) model. The Zebrafish model has emerged as a bio-medically relevant *in vivo* model for high content drug screening and the simultaneous determination of multiple efficacy parameters including selectivity and toxicity in the content of the whole organism (Hung et al., 2012).

An important parameter that appears to be often overlooked, is not only the correlation between *in vitro* and *in vivo* results, but the dose- and route-of-administration-associated toxicity of plant extracts and isolations. The majority of the literature reviewed for this review have cited compounds exhibiting high IC$_{50}$ values (µM) – thus representing perhaps unfeasibly high necessary dosages - when compared, for example, with commonly-used antibiotics such as amoxicillin, therapeutically quoted at nanomolar levels. Comprehensive toxicological study is therefore also a vital consideration within pre-clinical validation of potential therapeutic compounds from plants (Deriu et al., 2008).

![Figure 1.4 Putative pharmacophore of sesquiterpene lactones attributed to anti-staphylococcal activity: the α-methyl-γ-lactone moiety (Gach et al., 2015)](image)

**1.6 Conclusion and recommendations towards method transparency in plant natural product antimicrobial research**

At the time of writing, no approved guidelines, standards, or official recommendations exist, to our knowledge, for *in vitro* antimicrobial screening or susceptibility testing methodologies for natural products of plant origin. There are many considerations to be made here. These might be expected to include botanical certainty of the identity of the
plants used and their potential geographical and environmental variations. Further considerations include unification of extraction protocols, assay choice, inoculum densities, results interpretation, and inter-laboratory reproducibility in determination of MIC and MBC. The current challenges may be divided into two distinct areas – the first being the comprehensive identification of the constituents to be extracted for each species of plant, and a preliminary analysis of their respective concentrations, while seeking to maintain synergistic effects where they occur to the final production stage and testing thereafter. The second area concerns the *in-vitro* susceptibility testing of these products, where methods and interpretive guidelines may be established to determine efficacy for treatment of infections, ultimately, similar to the methods used currently for conventional commercial antimicrobial agents. The investment costs to reach this stage with any natural plant-derived product will undoubtedly be significant.

Overall, despite the large diversity in preparation and testing strategies used currently for natural product plant-derived antimicrobials, our investigations suggest that the field shows promise in the provision of novel antimicrobial agents -as exemplified by our selected case-study, *I. helenium*, in addition to other accepted natural sources (e.g. Manuka honey, tea tree oil) as efficacious topical antimicrobial agents. Although our review of the literature suggests that much work is needed in this field of research.

We have learned that microorganisms will inevitably overcome antibiotic treatment in some situations, and then spread, and it is clear that the rate of new antimicrobial development is insufficient to meet our current and future needs. This underlines the importance of investigating and developing promising antimicrobial agents to guarantee the effective future of antimicrobial chemotherapy.

**References**


https://doi.org/10.1016/j.foodres.2011.04.044


Chapter II

Using ethnobotany to uncover potential antibiotic therapeutics from *Inula helenium* L. (Elecampane): a comparison of Irish and International sources

Chapter findings presented at: The Institute of Chemistry Ireland (ICI) Symposium, online, September 2020 (first-place poster presentation award; see Appendix II).

Manuscript based on this chapter in preparation for publication.
Abstract

Introduction: An impending post-antibiotic era looms: and therefore, new sources of therapeutic chemical entities are crucial to sustain effective antimicrobial chemotherapy worldwide. Plant natural products are a source of accessible, structurally diverse compounds with the potential for use as antimicrobial agents. In Irish ethnomedical literature, *Inula helenium* L. (elecampane) is often indicated for respiratory and dermal ailments – the common name ‘Scabwort’ derived from the latter. This is the first assessment of antimicrobial sesquiterpene lactones from the roots of elecampane cultivated in Ireland.

Methods: Traditional hydro-ethanolic extracts of elecampane were prepared from commercially sourced and cultivated (Irish) plant material. A novel Sephadex clean-up strategy was employed and facilitated the bioactivity-guided fractionation of a subset of anti-staphylococcal fractions - the composition of which were investigated using high-performance liquid chromatography with diode array detection (HPLC-DAD) supported by $^1$H NMR.

Results: The natural products attributing to the bioactivity observed *in vitro* were identified as alantolactone, isoalantolactone, igalan(e), and an unseparated mixture of dugesialactone and alloalantolactone, as major constituents. The results suggest that the geographical origin of the plant does not appear to influence the anti-bacterial potency nor chemical composition of traditional elecampane root extracts.

Discussion/Conclusion: The pharmacological applications of plants in medicine can be guided by the attestation of traditional/indigenous use or the application of this knowledge to uncover new bioactive leads. Considering the prevalence of staphylococci-associated infections in Irish hospitals currently, further research is warranted into the usage of the identified sesquiterpene lactones as potential candidates in the control of staphylococcal carriage and infection.

Key words: *Inula helenium*; elecampane; antimicrobial; ethnobotany; compound identification; sesquiterpene lactones.
Research highlights

- The ethnobotanical use of *I. helenium* L. (elecampane) was explored.
- The aim of this study was to complete the first assessment of antimicrobial sesquiterpene lactones from elecampane cultivated in Ireland.
- A novel one-step size exclusion clean-up strategy was applied to facilitate the bioactivity-guided fractionation of antimicrobial sesquiterpene lactones in traditional (hydro)ethanolic root extracts of elecampane.
- Application of an HPLC-DAD method and $^1$H NMR led to the identification of the key compounds attributing to the bioactivity observed *in vitro*: viz. igalan(e), isoalantolactone, alantolactone and a mixture of compounds containing dugesialactone and alloalantolactone.
- Findings showed no observable differences in anti-staphylococcal activity from extracts of various origin.
2.1 Introduction

2.1.1 Ethnobotany in drug discovery

Challenges in antimicrobial chemotherapy are widespread. Most chemotherapeutic agents in clinical development today are modifications of known structures and thus cannot alleviate existing issues with cross- and pan-resistance among pathogens. The prevalence of antimicrobial resistance (AMR) combined with a lack of novel structural classes introduced to the antibacterial armamentarium in recent decades are at the foundation of this impending crisis. The lack of diverse and innovative leads is described by WHO (2017) as the main “bottleneck” in antibiotic discovery at present. Considering that there are approximately 80,000 species of plants in the Amazon alone (Schultes, 1994), the potential structural diversity in the Plant Kingdom is enormous, albeit underutilised.

Target-directed drug discovery models have outstanding merits, however, when it comes to microbial infection, focusing on one molecule and one target could be an ineffective method over time, since microorganisms evolve at a greater rate than we can create new drugs with new targets. This is evidenced by the fact that only six new antibiotics have been approved for therapeutic use in the last 30 years and resistance has already been observed to these (Kenny et al., 2015), combined with a weak pipeline for anticipated new antimicrobial agents (WHO, 2020). Thereby focusing on multi-compound- (combinatorial treatment), multi-targeted-, or potentiator/adjuvant- therapies (Abreu et al., 2017; Wu et al., 2019) are alternatives to this model which warrants investment.

Using an ethnobotanical approach, the pharmacological applications of plants in medicine can be guided by attesting traditional or indigenous use or by applying this knowledge to uncover natural products with new biological applications (Seca et al., 2014; Surh, 2011). This approach serves as an accessible starting point for pharmacological research and increases the probability of discovering medicinally useful compound(s) (Clapp and Crook, 2002). Plants, such as *I. helenium*, are a prosperous source of therapeutic chemical entities and may offer significant potential in the universal quest for effective infectious control.
2.1.2 Botanical description

The genus *Inula*, of the Asteraceae family, consists of over one hundred plant species (Seca et al., 2014). *I. helenium* is a distinctive perennial herb naturalised in Ireland. The plant parts used medicinally are the roots and rhizomes which are harvested in Autumn and early Winter months, optimally 2-3 years old. The primary root structure is thick and slightly branching with long cylindrical or cone-like projections. The upright stems are 1-2 metres in height, with dense short pubescence on the surface. The plant has an alternate leaf arrangement comprised of wide elliptical and irregularly serrated leaves with an amplexicaul cordate leaf-base. Leaves are covered in pubescent trichomes on both sides. Yellow capitulum inflorescence (flowers) in a corymb arrangement are a characteristic feature of the plant. The corolla is tubular at the centre and ligate at the border, with accompanying layers of 5-10 sepals arranged in a hemisphere calyx, and the fruits are prismatic achenes with ridges (Yoon et al., 2014).

2.1.3 Traditional preparations to regulated products

*I. helenium* is a reservoir of diverse phytochemical compounds with a long history of ethnomedicinal use with records from Minoan, Mycenaean, Egyptian, Assyrian and Serbian (Chilander Medical Codex) pharmacotherapy manuscripts circa 2700 – 1100 B.C. (Seca et al., 2014). The root extract features in many traditional medical systems including Tibetan, Ayurvedic and Traditional Chinese Medicine (TCM) where it is referred to as ‘Radix Inulae’ (Tu-Mu-Xiang, Zang-Mu-Xiang) (Seca et al., 2014). In the European pharmacopoeias it is referenced as ‘Aunée’ (France), ‘Radix Helenii’ (Netherlands), ‘Rhizoma Helenii’ (Germany) and ‘Helenii Rhizoma’ (U.K.) (Seca et al., 2014).

Cameron (1883) postulated that *I. helenium* likely originated, in Gaelic and Irish tradition, from the historic officinal name ‘*Inula campana*’, or ‘Helénula’ (‘Little Helen’). The plant has been referred in traditional Irish texts as ‘*aílleán*’, derived from ‘*aílle*’ meaning beautiful or lovely, and ‘*Ellea*’ derived from the Gaelic ‘*Eilidh*’ meaning Helen (Moloney 1919; Cameron, 1883). This association is thought to be rooted from Greek Mythology, as Helen of Troy favoured the flowers of *I. helenium* for their aesthetic splendour (Cameron, 1883); as legend recites the blossom of elecampane from her fallen tears upon abduction from her homeland (Grieves, 1972). The plant features in Celtic folklore as
'Elf-Dock' and 'Elf-Wort', and other common names include 'Horse-heal' and 'Scabwort'; the latter relating to its use as a topical agent (Grieves, 1972)

In ancient Irish literature, the medicinal use of *I. helenium* is often described for respiratory ailments (Ó Cuinn, 2019). In a translated version of Tadgh Ó Cuinn’s medieval Irish *Materia Medica* (circa 1415 A.D.), references to the use of *I. helenium* to treat respiratory organs, coughs and consumption [from tuberculosis], are documented. Respiratory preparations specified boiling the powdered herb with dilute barley water, liquorice, cinnamon and sugar; whilst digestive ailments (e.g. “ileus, colic and stranguria”) were treated with a plaster applied to the naval comprised of the herb boiled in wine and oil (Ó Cuinn, 2019). Celtic ethnobotany is somewhat historically neglected however, and as Moloney (1919) recites: “[we have] relegated to oblivion many a(n) herb”. The recent establishment of digital archives (e.g. Dúchas.ie, CELT.ie) will function to preserve and facilitate research of Irish traditional medicinal knowledge (Koay et al., 2020; Shannon et al., 2017).

The British Herbal Pharmacopoeia (BHP) lists the therapeutic actions of the herb as antitussive, antiseptic expectorant, diaphoretic and bactericidal (BHMA, 1983). Suggested indications include respiratory mucosal catarrh (tracheal, bronchial), cough and phthisis associated with pulmonary tuberculosis, bronchitis and whooping cough in infants (BHMA, 1983). The root is traditionally administered as a decoction. Decoctions are liquid preparations made by mixing comminuted herbal material with water, which is brought to the boil and strained before administration (Hoffmann, 2003). Dosage recommendations in the BHP range from 1.5 - 4 g decoction from the dried root/rhizome, or 1.5 – 4 mL liquid extract (i.e. 1:1 herbal tincture in 25% alcohol) thrice daily (BHMA, 1983). The plant can be combined with extracts of *Marrubium, Tussilago, Asclepias* or *Millefolium* (BHMA, 1983) however guidelines for such preparations are not detailed in the monograph.

In the 1980’s, the German Commission E published an official monograph disproving the therapeutic use of *I. helenium* root based on insufficient available evidence to support the efficacy of the herb and preparations thereof, and the risk of associated adverse effects (Blumenthal, 1998). Elecampane was later considered an “unapproved” medicinal herb, and consequently excluded in consecutive global monographs and compendial texts from thereon, including: the ESCOP monographs, WHO Selected Medicinal Herb monographs
Vols. 1-3), the British Herbal Compendium (Vols. 1 & 2), and the European Medicines Agency (EMA) official monographs. This outcome was a likely consequence of individual case reports documenting adverse inflammatory effects thought to be associated with *I. helenium* (Aberer and Hausen, 1990; Gil Mateo et al., 1995; Kim et al., 1988; Pazzaglia et al., 1995) and related species: *I. conyza* (Reinboth, 1967; Ulbrich et al., 1966), *I. viscosa* (Pinedo et al., 1987; Sertoli et al., 1978) and *I. graveolens* Desf. (Schneider and du Plessis, 1980). The findings of these earlier studies accept that the presence of causative allergens was likely family/genus related. Paulsen (2002) acknowledges that while *I. helenium* is suspected to be an inducer of Compositae (Asteraceae) allergic dermatitis, there is no epidemiological data available to support this. Paulsen (2017), in agreement with Amorim et al. (2013) later cautioned towards the systemic allergic dermatitis associated with sesquiterpene lactone (SL)-containing plants in general, however, emphasises the need to determine the pathogenesis for individual haptens per species. Regarding isolated compounds such as alantolactone, there are conflicting reports of sensitization (Alonso Blasi et al., 1992; Hausen and Vieluf, 1997; Mitchell et al., 1970) and opposing anti-allergenic properties (Lee et al., 2018; Wang et al., 2018). More recent evidence, however, infers support for further research into the therapeutic efficacy and safety of *I. helenium*-derived compounds, explored below (Sections 2.1.4 and 2.3).

In a recent randomised, double-blind, placebo-controlled clinical trial, a cough syrup (KalaboTUSS®) containing a traditional elecampane extract proved to be efficacious in relieving cough in children (Carnevali et al., 2021). There are no registered elecampane-containing Traditional Herbal Medicinal Products (THMPs) on the market in Ireland, at the time of writing.

2.1.4 Antimicrobial potential of elecampane-derived sesquiterpene lactones

Sesquiterpene lactones (SLs) are an important group of bioactive metabolites present in root extracts of *I. helenium* (Seca et al., 2015, 2014). A comprehensive list of known SLs’ identified in *I. helenium* root to-date are listed in Table 2.1. Research on many of these compounds has increased in recent years, specifically relating to the potential of
alantolactone, isoalantolactone, 5α-epoxyalantolactone, (iso)costunolide and igalan(e) in cancer therapeutics.

Olechnowicz-Stepien and Skurska (1960) first reported the antimicrobial activity of *I. helenium* root *in vitro*, while some of the earliest compounds isolated included alantopicrin (Von Gizycki, 1954) and dammaradienyl acetate (Yosioka and Yamada, 1963). A cascade of research followed in the late 1990’s investigating the antimicrobial activity of different extracts of *I. helenium* root against various pathogens (Blagojević and Radulović, 2012; Cantrell et al., 1999, 1998; Deriu et al., 2008; Gökbulut et al., 2013; Jiang et al., 2011; Mazzio et al., 2016; O’Shea et al., 2009; Qiu et al., 2011b; Radulović et al., 2014; Stojakowska et al., 2005; Stojanović-Radić et al., 2012).

Methicillin- and vancomycin-resistant *Staphylococcus aureus* (MRSA/VRSA) are among the listed pathogens of “medium-to-high priority” published by the WHO (2017), and at a national level, Ireland has some of the highest occurrences of *Staphylococci* and *Enterococci*-associated systemic bloodstream infections in all the European member states (Department of Health, 2017). *Staphylococcus aureus* was therefore selected as a suitable target organism in this present study because of its priority status and its clinical relevance in Irish hospitals. Furthermore, it is in line with ethnobotanical use and the microorganism has a known susceptibility to crude extracts of *I. helenium* as previously demonstrated amongst a suite of clinically relevant *S. aureus* isolates in our laboratory (O’Shea et al., 2009), and among other groups - as summarised previously (Kenny et al., 2015).

The aim of this study is to complete the first assessment of antimicrobial sesquiterpene lactones in a traditional (hydro)ethanolic root extracts of *I. helenium* L. naturalised to the Irish climate, with comparison to dried root sourced internationally. Documented is the application of a novel one-step clean-up strategy to facilitate the bioactivity guided fractionation of antimicrobial sesquiterpene lactones attributing to the anti-staphylococcal activity observed *in vitro*. A validated HPLC-DAD method was used to investigate the presence of igalan(e), isoalantolactone, alantolactone and a mixture of compounds containing dugesialactone and alloalantolactone – which could serve as potential antibiotic lead compounds. The results further showed no observed difference in anti-staphylococcal activity from extracts of Irish and international origin, suggesting the antimicrobial activity of elecampane is not dependent on the cultivation environment.
Table 2.1 Overview of known isolated sesquiterpene lactones from I. helenium root extracts to-date:

<table>
<thead>
<tr>
<th>No.</th>
<th>Identified compound</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Alantolactone</td>
<td>Gerhardt, 1840; Ružička et al., 1933; Tsuda et al., 1957; Asselineau and Bory, 1958; Marshall and Cohen 1964</td>
</tr>
<tr>
<td>2</td>
<td>Isoalantolactone</td>
<td>Kallen J, 1876; Ružička &amp; van Melsen, 1931; Wunderlich 1959</td>
</tr>
<tr>
<td>3</td>
<td>Dihydroalantolactone</td>
<td>Hansen, 1931a; Hansen 1931b; Kerimov and Chizhov 1974; Konishi et al., 2002</td>
</tr>
<tr>
<td>4</td>
<td>Dihydroisoalantolactone</td>
<td>Hansen, 1931a; Hansen 1931b; Kerimov and Chizhov 1974; Konishi et al., 2002</td>
</tr>
<tr>
<td>5</td>
<td>Tetrahydroalantolactone</td>
<td>Rosik et al., 1991</td>
</tr>
<tr>
<td>6</td>
<td>Alloalantolactone (= 1-Deoxyivangustin, = (+)-Diplophyllin)</td>
<td>Bohlmann et al., 1978</td>
</tr>
<tr>
<td>7</td>
<td>Bialantolactone</td>
<td>Jiang et al., 2011</td>
</tr>
<tr>
<td>8</td>
<td>Trinoralantolactone</td>
<td>Jiang et al., 2011</td>
</tr>
<tr>
<td>9</td>
<td>5α-Epoxyalantolactone</td>
<td>Konishi et al., 2002</td>
</tr>
<tr>
<td>10</td>
<td>4-Noralantolactone (= 4-oxo-5(6),11-eudesmadiene-8,12-olide)</td>
<td>Huo et al., 2008</td>
</tr>
<tr>
<td>11</td>
<td>4-Norisoalantolactone</td>
<td>Huo et al., 2008</td>
</tr>
<tr>
<td>12</td>
<td>1α-Hydroxy-11,13-dihydroisoalantolactone</td>
<td>Zhao et al., 2010</td>
</tr>
<tr>
<td>13</td>
<td>3α-Hydroxy-11,13-dihydroalantolactone</td>
<td>Zhao et al., 2010</td>
</tr>
<tr>
<td>14</td>
<td>Macrophyllilactone E</td>
<td>Zhao et al., 2010</td>
</tr>
<tr>
<td>15</td>
<td>4α,15α-Epoxyisoalantolactone</td>
<td>Jiang et al., 2011</td>
</tr>
<tr>
<td>16</td>
<td>4,5-seco-Eudesm-11(13)-en-4,5-dioxo-8β,12-olide (= Umbellifolide)</td>
<td>Jiang et al., 2011</td>
</tr>
<tr>
<td>17</td>
<td>11α-Hydroxyeudesm-5-en-8β,12-olide</td>
<td>Jiang et al., 2011</td>
</tr>
<tr>
<td>18</td>
<td>3α-Hydroxyeudesma-4,11-dien-8β,12-olide</td>
<td>Jiang et al., 2011</td>
</tr>
<tr>
<td>19</td>
<td>Telekin</td>
<td>Jiang et al., 2011</td>
</tr>
<tr>
<td>20</td>
<td>3-Oxo-eudesma-4(5),11-dien-8,12-olide</td>
<td>Huo et al., 2012</td>
</tr>
<tr>
<td>21</td>
<td>11α,13-Dihydro-α-cyclocostunolide</td>
<td>Ma et al., 2008</td>
</tr>
<tr>
<td>22</td>
<td>15-Hydroxy-11/12-eudesm-4-en-8β,12-olide</td>
<td>Ma et al., 2008</td>
</tr>
<tr>
<td>23</td>
<td>3α-Hydroxy-11/12-eudesm-5-en-8β,12-olide</td>
<td>Ma et al., 2008</td>
</tr>
<tr>
<td>24</td>
<td>2β,11α-Dihydroxy-eudesm-5-en-8β,12-olide</td>
<td>Ma et al., 2008</td>
</tr>
<tr>
<td>25</td>
<td>Isoheleproline</td>
<td>Zaima et al., 2013</td>
</tr>
<tr>
<td>26</td>
<td>11β-Hydroxy-13-chloro-eudesm-5-en-8β,12-olide</td>
<td>Ding et al., 2019</td>
</tr>
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<td></td>
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<tr>
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</tr>
<tr>
<td>27</td>
<td>5-epi-telekin</td>
<td>Ding et al., 2019</td>
</tr>
<tr>
<td>28</td>
<td>Racemosalactone A</td>
<td>Ding et al., 2019</td>
</tr>
<tr>
<td>29</td>
<td>Macrophyllilactone F</td>
<td>Ding et al., 2019</td>
</tr>
<tr>
<td><strong>Elemanolides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>Igalan(e) (= 1,3,11(13)-Elematrien-8β,12-olide)</td>
<td>Konishi et al., 2002</td>
</tr>
<tr>
<td><strong>Eremophilanolides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>Dugesialactone</td>
<td>Huo et al., 2010</td>
</tr>
<tr>
<td><strong>Guaianolides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>Dehydrocostus lactone</td>
<td>Ma et al., 2008</td>
</tr>
<tr>
<td>33</td>
<td>4α-Hydroxy-1β-guaia-11(13),10(14)-dien-12,8α-olide</td>
<td>Huo et al. 2012</td>
</tr>
<tr>
<td><strong>Germacranolides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>Germacrene-D-lactone (= Germacra-1(10),4(15),5(6),11(13)-tetraen-8,12-olide)</td>
<td>Bohlmann et al., 1978</td>
</tr>
<tr>
<td>35</td>
<td>4β,5α-Epoxygermacra-1(10),11(13)-dien-12,8α-olide</td>
<td>Konishi et al., 2002</td>
</tr>
<tr>
<td>36</td>
<td>Isocostunolide</td>
<td>Chen et al., 2007</td>
</tr>
<tr>
<td>37</td>
<td>(1(10)E)-5β-Hydroxygermacra-1(10),4(15),11(13)-trien-12,8α-olide</td>
<td>Huo et al., 2008</td>
</tr>
<tr>
<td>38</td>
<td>14-Hydroxy-11β,13-dihydrocostunolide/ 11β, 13-Dihydro-14-hydrocostunolide</td>
<td>Ma et al., 2008/ Seca et al., 2015</td>
</tr>
<tr>
<td>39</td>
<td>Costunolide</td>
<td>Ma et al., 2008</td>
</tr>
<tr>
<td>40</td>
<td>5β-Hydroxygermacra-1(10),4(15),11(13)-trien-12,8β-olide</td>
<td>Jiang et al., 2011</td>
</tr>
<tr>
<td>41</td>
<td>4α,5α-Epoxygermacra-1(10),11(13)-dien-12,8β-olide</td>
<td>Jiang et al., 2011</td>
</tr>
</tbody>
</table>
2.2 Experimental methods

2.2.1 Crude extract preparation

2.2.1.1 Sources of plant material

Cultivated *I. helenium* roots (CT) were collected from Bandon Medicinal Herbs Ltd. (West Cork, Ireland) (See Figure 2.1), and authenticated accordingly (O’Shea et al., 2009). The plant material was also identified against a voucher specimen deposited in the New York Botanical Gardens (NYBG) Steere Herbarium (Barcode: 2924501), and botanical key (Yoon et al., 2014). Commercial dried *I. helenium* root (CM) was purchased from a registered supplier; Herbs in a Bottle© Ltd. (U.K.). Product information as per label: Code #6202b; Batch #148845; Specification: Cut; Date of Manufacture: 18/06/2015; Issue #160575; Origin: China; Identification: Conforms.

![Figure 2.1 Harvested roots of I. helenium L. (Elecampane)](image)

2.2.1.2 Traditional maceration

Roots and rhizomes of *I. helenium* were collected in September 2015 from Bandon Medicinal Herbs, West Cork. The harvested roots were washed with ultrapure Milli-Q water (18.2 MΩ·cm) and left to dry naturally at room temperature. The dried roots were then powdered and stored in a sterile air-tight container protected from light.

To compare cultivated versus commercially sourced plant material, a total of four plant extracts were prepared at a concentration of 100 mg.mL⁻¹ in 50% aqueous ethanol (v/v) and absolute ethanol (See Table 2.2), as per O’Shea *et al.* (2009). The above extracts will be referred to as CT50 or CT100 (cultivated), and CM50 or CM100 (commercial), from herein. See Figure 2.2 for the schematic overview of the extraction process.
Table 2.2 Composition of the cultivated (CT) and commercial (CM) extracts and yields:

<table>
<thead>
<tr>
<th>Extract</th>
<th>Content</th>
<th>Total Yield* (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT50</td>
<td>Traditional extract containing the cultivated root powder in 50% ethanol (v/v)</td>
<td>36.3</td>
</tr>
<tr>
<td>CT100</td>
<td>Traditional extract containing the cultivated root powder in absolute ethanol</td>
<td>47.4</td>
</tr>
<tr>
<td>CM50</td>
<td>Traditional extract containing the commercially acquired root powder in 50% ethanol (v/v)</td>
<td>40.0</td>
</tr>
<tr>
<td>CM100</td>
<td>Traditional extract containing the commercially acquired root powder in absolute ethanol</td>
<td>38.4</td>
</tr>
</tbody>
</table>

*Yield measured by weighing the dried residue after evaporation.

The comminuted herbal material was introduced to the vessel containing the extraction solvent as per Table 2.2. Extracts were periodically mixed by gentle inversion. Maceration took place for a total of 28 days at room temperature protected from light. The crude macerate(s) were centrifuged in a Thermo Scientific IEC-CL30R for 15-20 minutes at 6500 RPM. The decanted supernatant(s) were subsequently vacuum filtered using a Büchner funnel lined with Whatman filter paper No. 1 discs for clarification (11 µm particle retention) followed by filter sterilisation using cellulose nitrate loaded syringes (0.45 µm). Retained samples of the crude extracts were stored at -20°C in a repository at Muster Technological University, while the test articles were stored at 2-8°C for use as an experimental control.

![Figure 2.2 Schematic overview of extract preparation for in vitro analysis in the current study](image-url)
2.2.2 Bioactivity-guided fractionation of antimicrobial compounds

2.2.2.1 Gravity-eluted size-exclusion chromatography

The solvent removal process was performed using a BÜCHI Rotavapor R-205 (BTX-RE2) with an attached BÜCHI vacuum controller V-800. The instrument parameters were set to (40°C; 175 mbar) and total evaporation time was approximately 90 minutes per 100 mL extract. Total extract yields are recorded in Table 2.2. Residues were stored at -20°C until required for further analysis.

The CT50 extract was retrieved from -20°C storage and let thaw at room temperature. One millilitre of methanol was added to resolubilise the viscous residue under sonication. The resuspended extract was transferred to a 2 mL amber vial and the solvent was removed under a gentle flow of nitrogen. Ethanol (extractant solvent) was used to resolubilise the extract as it is less potently toxic to cells in vitro. To maintain consistency, ethanol was further utilised as the mobile phase for subsequent chromatographic separation.

Sephadex LH-20 (25-100 µm) was prepared in methanol and left in solution for 24 hours at room temperature. All glassware was rinsed in methanol and air-dried before use. A glass column (height 90 cm; diameter 3.2 cm) was carefully packed with the Sephadex solution and secured with parafilm to facilitate deposition. The Sephadex was prevented from drying out in the column once packed by ensuring that the apex remained submerged in ethanol. The soluble crude extract (2 mL, 100 mg) was applied to the column as a thin solvent band using a glass pipette, and the band was allowed penetrate the top layer of Sephadex. Once completed, a reservoir of ethanol (300 mL) was introduced as the mobile phase. The column was eluted with 96% ethanol under gravitational flow, and fractions were manually collected in 10 mL aliquots. A total of seventy-two fractions were collected per extract over a period of 12 hours.

These steps were repeated for the remaining three extracts: CT100, CM100 and CM50. All fractions were immediately screened for their antimicrobial activity against *Staphylococcus aureus* using a modified in vitro agar-well method, detailed in Section 2.2.2.2 – 2.2.2.4.
2.2.2.2 Bacterial strains and media preparation

- Clinical diagnostic reference strain *Staphylococcus aureus* NCTC 6571 (cross-referenced in the American Type Culture Collection (ATCC) as ATCC 9144 (Kearns et al., 2006)).
- Eleven *S. aureus* clinical isolates from Cork University Hospital (CUH).
- Culture media prepared as per manufacturer guidelines: Mueller Hinton (MH) broth (Lab M; Lot: 141370/357) and agar (Lab M; Lot: 144209/172). Cation-adjusted Mueller Hinton II (MHII) broth (Sigma-Aldrich; Lot: BCBT9094) and agar (Sigma-Aldrich; Lot: BCBV4646).
- Sodium chloride (PanReac AppliChem; Lot: 0000893728).
- Glycerol solution, 84-88% (Sigma-Aldrich; Lot: SZBC010BV).
- Alantolactone standard (Sigma; Lot No. 125M4751V).
- Isoalantolactone standard (CliniSciences; HY-N0780/CS-3635; Batch No. 20994).

2.2.2.3 Preparation of *S. aureus* stocks

*S. aureus* was used as the target organism to guide the fractionation process. Stocks were maintained in glycerol at -80°C. Briefly, overnight cultures were centrifuged at 4000 RPM for 15-20 minutes. The supernatant was discarded, and the pellet re-suspended in MH broth. Aliquots were combined with sterile glycerol (1:2, v/v) under laminar flow. Working stocks were stored at -20°C and reference stocks at -80°C.

2.2.2.4 In vitro agar-well screening (Modified EUCAST disk-diffusion method)

Antimicrobial screening was performed following European Committee on Antimicrobial Susceptibility Testing (EUCAST) Guidelines (EUCAST, 2003, 2020), modified accordingly. Briefly: overnight cultures of *S. aureus* NCTC 6571 were adjusted to 0.5 McFarland standard in 0.85% sterile saline solution. The surfaces of MH agar plates were inoculated with the adjusted bacterial suspension using sterile cotton swabs. To prepare the wells or reservoirs, 8 mm holes were aseptically punched into the agar surface after inoculation. Seventy-five µl of each fraction were transferred to their corresponding well. Alantolactone (0.2 – 3.2 µg.mL⁻¹) and isoalantolactone (1 mg.mL⁻¹) were tested in tandem. Plates were left under laminar airflow for up to 60 minutes to facilitate solvent evaporation prior to incubation at 37°C. Mupirocin and crude *I. helenium* extracts were
used as positive controls, and sterile water as a negative control. Plates were prepared in
triplicate and incubated for 24 hours at 37°C. Antimicrobial activity was determined by
measuring zones of inhibition (mm; $\bar{x} \pm SD$ (standard deviation)) using a calibrated
Vernier callipers (Mitutoyo).

2.2.2.5 Solvent tolerance test
To assess the possibility of ethanol potentiating antimicrobial activity in the microdilution
method, a separate test was used to confirm inactivity using a solvent tolerance assay as
per Pando et al. (2017) with modifications. Eleven strains of clinical S. aureus isolates,
including Methicillin-Resistant S. aureus (MRSA), were used as the target organism. The
EtOH concentration range increased in 1% increments (1 – 30% EtOH). A 10% addition
of Alamar blue was added to the wells before measuring absorbance.

2.2.3 Structural investigation of bioactive fractions
2.2.3.1 Sample preparation
Each bioactive fraction was reduced to dryness using a Zymark TurboVap® LV
Concentration Evaporator system. Nitrogen flow rate was set to 20 psi for 20 mins and
increased incrementally to 50 psi as volume reduced. Each dried residue in the test tube(s)
was resolubilised in pure ethanol, sonicated and transferred to amber HPLC vial(s) (1 mL
in total). The samples were reduced under a gentle flow of nitrogen again and stored at -
20°C until required for further analysis.

2.2.3.2 Standards and reagents
External standards asperilin (purity > 95%) and isoalantolactone (purity > 90%) were
isolated from flowers of Telekia speciosa (Schreb.) Baumg, as it was described earlier
(Stojakowska et al., 2015). A mixture of alantolactone and isoalantolactone of lower
purity, isolated from I. helenium roots, was also used for identification purposes. Water
was purified by a Milli-Q system (Millipore Corp., Bedford, MA, USA). Methanol
(MeOH) and acetonitrile (MeCN) of gradient grade for liquid chromatography, were
purchased from Merck (Darmstadt, Germany).

2.2.3.3 HPLC-DAD analysis
Chromatographic separations were performed using Agilent 1200 Series HPLC system
(Agilent Technologies, Palo Alto, CA, USA) equipped with a Rheodyne manual sample
injector, quaternary pump, degasser, column oven and a diode array detector (DAD). Analytical separations were carried out using a Kinetex 5\(\mu\)m XB-C\(_{18}\) column (260 x 4.6 mm, 100Å pore size) from Phenomenex (Torrance, CA, USA), at 40 °C, with a gradient mode elution, as it was described elsewhere (Stojakowska et al., 2015). The mobile phase consisted of H\(_2\)O (A) and MeCN (B). Linear gradient from 12% B to 15% B in 5 min, 25% B in 5 min, 60% B in 5 min, 98% B in another 10 min was applied (stop time: 35 min, post time: 12 min). Flow rate was established at 1 mL min\(^{-1}\). Alternatively, a Zorbax Eclipse XDB-C\(_{18}\) column (150 x 4.6 mm, 5 \(\mu\)m) (Agilent, Santa Clara, CA, USA) with a mobile phase consisting of MeCN and H\(_2\)O (11:9, v/v) was used, as it was proposed by Huo et al. (2010). As similar chromatographic resolutions of the analysed compounds were achieved by both methods, the gradient elution system was used exclusively. This method is routinely used at the Phytochemistry Department of the Maj Institute of Pharmacology at the Polish Academy of Sciences (PAS), for quantification of sesquiterpene lactones in various extracts from plants of the Inuleae tribe.

Accurately weighted aliquots of the active samples were transferred into 1.5 mL Eppendorf tubes and dissolved in 1 mL of 70\% MeOH and MeCN mixture (1:1, v/v). The solution was centrifuged (11,340 g, 5 min) prior to HPLC analysis, and injected (5 \(\mu\)l) into the column. The detection wavelength was set at 205 nm. Quantification of compounds 1-3 was done by an external standard method (ESM), as it was described earlier (Stojakowska et al., 2015). The content of the mixture under Peak 4 (Figure 2.4) was assessed semi-quantitatively, with an assumption that the signal is generated by eudesmanolides.

### 2.2.3.4 Semipreparative HPLC separation

The active fractions of *I. helenium* extracts were dissolved in 90\% MeOH and injected into the Vertex Plus column (Eurospher II 100-5 C18, 250 x 8 mm) (Knauer, Berlin, Germany). The chromatographic separations were carried out in an isocratic mode (solvent flow rate 2 mL min\(^{-1}\)) using MeCN: H\(_2\)O (1:1, v/v) as an eluent. Fractions corresponding to the four major signals that showed absorption at 205 nm were collected.

### 2.2.3.5 \(^1\)H NMR spectroscopy (400.17 MHz, CDCl\(_3\), \(\delta\)-Scale)

Subfractions of the *I. helenium* extract obtained by semiprep. HPLC, were subjected to \(^1\)H NMR analysis as per Huo et al. (2010). Briefly, the subfractions were dissolved in CDCl\(_3\) containing TMS (tetramethylsilane) as an internal standard (ITSD). NMR spectra
were recorded in CDCl₃ on a Bruker AVANCE III HD 400 (resonance frequency 400.17 MHz) equipped with 5 mm probes @ 300K. Chemical shifts were given on the delta (δ) scale and referenced to the ITSD (Huo et al., 2010).

Canonical and isomeric SMILES (Simplified Molecular-Input Line-Entry System) were derived from PubChem and input to draw the chemical structures using the PubChem sketcher V2.4 tool (input: isomeric SMILES) (Figure 2.6).
Figure 2.3 Schematic overview of the phytochemical analysis process
2.3 Results and Discussion

There is widespread acceptance that global AMR presents alarming dangers, and the current clinical antimicrobial pipeline is insufficient to allay consequent threats in infectious control (WHO, 2017). This reality has resulted in a united global ambition in the pursuit of new therapeutic modalities for infectious disease. Plants documented as anti-infectives in historical literature, such as *I. helenium*, could offer potential as mono-/poly-therapeutics and adjuvant/potentiator agents in modern medicine (Abreu et al., 2017; Wu et al., 2019). The use of plants and Ethnobotanical principles as a starting point in the drug discovery process therefore warrants consideration. The aim of the present study was to investigate the key compounds from a traditional (hydro)ethanolic root extract of *I. helenium* attributing to its *in vitro* anti-staphylococcal activity.

An initial preliminary screen confirmed that the crude extracts were active against the gram-positives: Group-A *Streptococcus pyogenes*, Group-B *Streptococcus agalactiae*, *Listeria monocytogenes*, *Escherichia faecalis* ATCC 29212 and *Escherichia coli*, as well as *Mycobacterium tuberculosis* H37Ra (ATCC 25177) (data not shown). *S. aureus* was chosen as the target organism for the bioactivity-guided fractionation in this study based on previous results (O’Shea et al., 2009), and its clinical relevance in Irish hospitals currently (Department of Health, 2017).

An optimised extraction method involving the traditional maceration of the comminuted root with the extractant solvent was performed as before (O’Shea et al., 2009) to compare the activity between the root originating from a source plant naturalised to West Cork (Ireland) versus internationally sourced, commercially available dried root samples. Our findings suggest that the antimicrobial potency of the plant extract is not determined by its geographical origin or environmental conditions in this case, since all samples resulted in comparable activity (See Table 2.3) and major constituent profiles (See Figure 2.6).
Table 2.3 Average zone diameter (mm) and total yield (mg) per active fraction (n = 3):

<table>
<thead>
<tr>
<th>Extract</th>
<th>Active fraction No.</th>
<th>Inhibitory zone diameter ($\bar{x} \pm SD$; mm)</th>
<th>Total Yield ($\bar{x}$; mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT_50</td>
<td>F14</td>
<td>12.2 ± 0.2</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>F15</td>
<td>16.5 ± 0.3</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>F16</td>
<td>16.3 ± 0.5</td>
<td>47.7</td>
</tr>
<tr>
<td></td>
<td>F17</td>
<td>16.1 ± 0.3</td>
<td>56.1</td>
</tr>
<tr>
<td></td>
<td>F18</td>
<td>13.4 ± 0.6</td>
<td>55.7</td>
</tr>
<tr>
<td></td>
<td>F19</td>
<td>10.8 ± 0.5</td>
<td>56.3</td>
</tr>
<tr>
<td></td>
<td>F20</td>
<td>11.0 ± 0.3</td>
<td>76.3</td>
</tr>
<tr>
<td></td>
<td>F21</td>
<td>11.5 ± 0.6</td>
<td>82.8</td>
</tr>
<tr>
<td></td>
<td>F22</td>
<td>13.0 ± 0.5</td>
<td>96</td>
</tr>
<tr>
<td>CM_50</td>
<td>F16</td>
<td>15.0 ± 0.6</td>
<td>84.7</td>
</tr>
<tr>
<td></td>
<td>F17</td>
<td>15.6 ± 1.1</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>F18</td>
<td>15.0 ± 0.1</td>
<td>42.3</td>
</tr>
<tr>
<td></td>
<td>F19</td>
<td>13.4 ± 0.3</td>
<td>41.5</td>
</tr>
<tr>
<td></td>
<td>F20</td>
<td>12.3 ± 1.2</td>
<td>44.3</td>
</tr>
<tr>
<td></td>
<td>F21</td>
<td>13.1 ± 0.7</td>
<td>62.4</td>
</tr>
<tr>
<td></td>
<td>F22</td>
<td>15.1 ± 0.8</td>
<td>43.3</td>
</tr>
<tr>
<td></td>
<td>F23</td>
<td>16.2 ± 0.1</td>
<td>114.7</td>
</tr>
<tr>
<td></td>
<td>F24</td>
<td>14.0 ± 0.9</td>
<td>134.4</td>
</tr>
<tr>
<td>CT_100</td>
<td>F15</td>
<td>12.2 ± 0.5</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>F16</td>
<td>13.8 ± 0.1</td>
<td>57.1</td>
</tr>
<tr>
<td></td>
<td>F17</td>
<td>20.0 ± 0.1</td>
<td>102.5</td>
</tr>
<tr>
<td></td>
<td>F18</td>
<td>18.7 ± 0.4</td>
<td>165.7</td>
</tr>
<tr>
<td></td>
<td>F19</td>
<td>20.0 ± 0.3</td>
<td>84.3</td>
</tr>
<tr>
<td></td>
<td>F20</td>
<td>12.3 ± 1.0</td>
<td>54.1</td>
</tr>
<tr>
<td></td>
<td>F21</td>
<td>11.3 ± 0.2</td>
<td>50.5</td>
</tr>
<tr>
<td></td>
<td>F22</td>
<td>11.2 ± 0.3</td>
<td>57.4</td>
</tr>
<tr>
<td></td>
<td>F23</td>
<td>13.4 ± 0.3</td>
<td>57.6</td>
</tr>
<tr>
<td>CM_100</td>
<td>F16</td>
<td>18.7 ± 0.7</td>
<td>46.1</td>
</tr>
<tr>
<td></td>
<td>F17</td>
<td>17.4 ± 0.6</td>
<td>101.3</td>
</tr>
<tr>
<td></td>
<td>F18</td>
<td>17.7 ± 1.6</td>
<td>61.7</td>
</tr>
<tr>
<td></td>
<td>F19</td>
<td>14.7 ± 0.7</td>
<td>13.9</td>
</tr>
<tr>
<td></td>
<td>F20</td>
<td>13.1 ± 0.1</td>
<td>9.7</td>
</tr>
<tr>
<td></td>
<td>F21</td>
<td>16.5 ± 0.5</td>
<td>12.7</td>
</tr>
</tbody>
</table>

As a follow-on from previous work (O’Shea et al., 2009), we investigated the bioactive composition of the crude extract. This was achieved using an *in vitro* bioactivity-guided fractionation strategy applied to fractions generated from a single chromatographic clean-up step using Sephadex LH-20 as the stationary matrix. This model could serve as a starting point for process development if considering the general extraction of antimicrobial compounds from this plant. Sephadex LH-20 was the matrix of choice based on the desire to minimize compound loss via *in situ* sorption effects/phenomena. Sephadex LH-20 has been used for the isolation of compounds from other *Inula* species (Ding et al., 2016; Guo et al., 2007; Hua et al., 2012; Zheng et al., 2015), however this is the first account of its use for the initial fractionation of bioactive compounds from crude extracts of *I. helenium*. Results from the agar-well screening suggested that the
composition of the active fractions contained a range of compounds that were physiochemically related and hence co-eluted within a narrow range, e.g. F16-24 (See Table 2.3).

Table 2.4 Composition of active fractions isolated from hydroethanolic extracts of I. helenium root - expressed as % of the total weight, i.e. g/100 g sample:

<table>
<thead>
<tr>
<th>Extract</th>
<th>Fraction</th>
<th>1(^a)</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>Total Yield(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT_50</td>
<td>F14</td>
<td>1.64</td>
<td>9.72</td>
<td>15.91</td>
<td>3.52</td>
<td>30.79</td>
</tr>
<tr>
<td></td>
<td>F15</td>
<td>2.85</td>
<td>14.62</td>
<td>21.07</td>
<td>4.07</td>
<td>42.62</td>
</tr>
<tr>
<td></td>
<td>F16</td>
<td>2.89</td>
<td>12.52</td>
<td>16.32</td>
<td>3.51</td>
<td>35.24</td>
</tr>
<tr>
<td></td>
<td>F17</td>
<td>0.79</td>
<td>3.81</td>
<td>5.87</td>
<td>1.10</td>
<td>11.56</td>
</tr>
<tr>
<td></td>
<td>F18</td>
<td>1.87</td>
<td>8.93</td>
<td>14.32</td>
<td>2.66</td>
<td>27.78</td>
</tr>
<tr>
<td></td>
<td>F19</td>
<td>1.34</td>
<td>5.92</td>
<td>9.24</td>
<td>1.86</td>
<td>18.35</td>
</tr>
<tr>
<td></td>
<td>F20</td>
<td>0.20</td>
<td>1.10</td>
<td>1.75</td>
<td>0.34</td>
<td>3.40</td>
</tr>
<tr>
<td></td>
<td>F21</td>
<td>0.40</td>
<td>1.68</td>
<td>2.35</td>
<td>0.52</td>
<td>4.95</td>
</tr>
<tr>
<td></td>
<td>F22</td>
<td>1.64</td>
<td>9.72</td>
<td>15.91</td>
<td>3.52</td>
<td>30.79</td>
</tr>
</tbody>
</table>

| CM_50   | F16      | 1.21   | 11.34 | 20.73 | 1.99 | 35.27          |
|         | F17      | 2.42   | 20.48 | 21.16 | 2.83 | 46.89          |
|         | F18      | 1.60   | 11.47 | 15.43 | 1.88 | 30.37          |
|         | F19      | 1.31   | 9.06  | 10.69 | 1.44 | 22.50          |
|         | F20      | 0.67   | 5.28  | 5.29  | 0.72 | 11.96          |
|         | F21      | 0.49   | 3.34  | 4.23  | 0.58 | 8.64           |
|         | F22      | 1.25   | 9.93  | 10.04 | 1.45 | 22.67          |
|         | F23      | 0.68   | 5.50  | 4.79  | 0.73 | 11.70          |
|         | F24      | 0.36   | 2.32  | 2.22  | 0.35 | 5.25           |

\(^a\) Igalane (Huo et al., 2010; not unequivocally confirmed by \(^1\)H NMR due to substantial amounts of lipids in the subfraction); 2: Isoalantolactone (confirmed with standard); 3: Alantolactone (confirmed with standard); 4: unseparated mixture of dugesialactone and alloalantolactone (Huo et al., 2010; alloalantolactone presence confirmed by \(^1\)H NMR).

\(^b\) Except for the eudesmanolides, fatty acids and, as it was shown by \(^1\)H NMR analysis, complex mixture of lipids without UV/Vis absorption may constitute the sample.

Further elucidation of the composition of the active fractions was performed following methods routinely used at the Phytochemistry Department of the Maj Institute of Pharmacology for quantification of sesquiterpene lactones in various extracts from plants of the Inuleae tribe (Stojakowska et al., 2015). Figures 2.4 and 2.5 outline the HPLC chromatograms for fraction F16 (CM50) following the Huo et al. (2010) and Stojakowska et al. (2015) methods, respectively. Since both methods produced comparable spectra, the Stojakowska et al. (2015) was used exclusively. All samples, regardless of their source location, similarly contained a mixture of closely related eudesmanolides (i.e. helenin) with alantolactone (AL) and isoalantolactone (IAL) as major signals – annotated in Figures 2.4 and 2.5 as chromatographic peaks 2 and 3; and confirmed with comparison to external standard samples as per Stojakowska et al. (2015).
Quantitatively, the mixture of the eudesmanolides constituted up to 50% of the sample weight (See Table 2.4). To confirm the identification of the partially overlapping minor lactones (Peak 4, Figure 2.5), $^1$H NMR was performed as per Huo et al. (2010). Peak assignments were consistent with those available in the literature (Huo et al., 2010), however analyses of subfractions were influenced by the presence of lipids/fatty-acids (Table 2.4). The content of the mixture under Peak 4 was assessed semi-quantitatively, with an assumption that the signal is generated by eudesmanolides.

Sesquiterpene lactones (SLs), depending on the structure of their carbon skeleton, can be classified into several groups, including germacranolides, eudesmanolides, guaianolides and pseudoguanianolides. A comprehensive list of known sesquiterpene lactones identified in *Inula helenium* root to-date can be found in Table 2.1. Several reviews discuss the extensive pharmacological potential of metabolites from the *Inula* genus (Seca et al., 2015; Seca et al., 2014; Tavares and Seca, 2019; Wang et al., 2014). Recent research on the pharmacological activity of the identified compounds in the literature are discussed briefly below.
Crude extracts have exerted diverse activity in pre-clinical literature. Antineoplastic (Dorn et al., 2006) and anti-cancer activity was demonstrated in multiple cell lines including brain (Koc et al., 2018), pancreatic (Zhang et al., 2018) and breast (Chun et al., 2018) cancers. Other studies reported reduced inflammatory responses in in vivo sepsis models (Mazzio et al., 2016; Park et al., 2013), rheumatoid arthritis (Gao et al., 2017) and suppressed neutrophil-mediated inflammation in acute bronchitis by down-regulating the β2-integrin (Gierlikowska et al., 2020), following exposure to the crude extract. Alantolactone has been reported to exert anti-tumour activity in a number of cancer models, including Jurkat T-lymphocyte cells (Dirsch et al., 2001), β-cell acute lymphoblastic leukaemia (Xu et al., 2019), chronic myelogenous leukaemia (Yang et al., 2013) gastric (He et al., 2019), colon (Shi et al., 2011; Zhang et al., 2013), liver (Kang et al., 2019; Lei et al., 2012), lung (Liu et al., 2019; Maryam et al., 2017; Wang et al., 2019) pancreatic (He et al., 2018) and breast cancers (Cui et al., 2018; Liu et al., 2018). Moreover, the compound inhibited lipopolysaccharide (LPS)-induced nitric oxide
synthesis in murine macrophages (RAW 264.7), the activity of which is attributed to the presence of the \(\alpha\)-methylene-\(\gamma\)-lactone moiety - a key structural motif central to the bioactivity of many identified sesquiterpene lactones (Dirsch et al., 2000).

Isoalantolactone exerts activity against a number of cancer cell lines \textit{in vitro} (Lawrence et al., 2001), including squamous cell carcinoma (head, neck) (Wu et al., 2013), oesophageal cancer (Lu et al., 2018), chronic myelogenous leukaemia (Cai et al., 2014) and breast cancer cell lines (Wang et al., 2016). The compound inhibits LPS- and Phorbol-12-myristate-13-acetate (PMA)-induced inflammatory response \textit{in vitro} and \textit{in vivo} (He et al., 2017; Hehner et al., 1998). In pulmonary models, isoalantolactone exerts anti-inflammatory effects in Acute Lung Injury (ALI) (Ding et al., 2019) and inhibits \(\alpha\)-toxin, an important virulence factor secreted by \textit{S. aureus} which potentiates pneumonia pathogenesis (Qiu et al., 2011). Isoalantolactone, alantolactone and alloalantolactone have comparable anti-tumour effects in pancreatic cell lines (Yan et al., 2019). Alantolactone (Seo et al., 2008), isoalantolactone (Seo et al., 2009) and 5\(\alpha\)-epoxyalantolactone were reported as potential chemopreventative agents (Lim et al., 2007). While 5\(\alpha\)-epoxyalantolactone exhibits antiproliferative effects in acute myelogenous leukaemia progenitor cells (Ding et al., 2019). Igalan, albeit less studied, shows promise as a protective and detoxifying agent in HepG2 cells (Lee et al., 2019) and as an anti-inflammatory agent in a simulated atopic dermatitis model (Dao et al., 2020).

And lastly costunolide and isocostunolise, neither of which were identified in this study, also show promise for the anti-proliferative, anti-metastatic and neuroprotective effects \textit{in vivo} (Cai et al., 2019; Chen et al., 2007; Peng et al., 2019). Our results, and the above research exemplifies the diversity and potential of \textit{I. helenium} derived compounds, including non-traditional applications such as anti-cancer and anti-inflammatory agents.

There are however limitations to this study. Natural product extracts are complex - comprised of multiple compounds of unknown molecular weight and variable characteristics (e.g. polarity, solubility, viscosity, stability, toxins, fluorophores, pigments) that can cause bioassay interference in manual and automated screening platforms (Schmid et al., 1999; Wilson et al., 2020). Furthermore, the unavailability of standardised antimicrobial breakpoints to guide AST of natural products such as plant compounds, is a recognised limitation in this area of research (Kenny et al., 2015). The crude extract of \textit{I. helenium} ranges from green (aqueous-ethanolic extract) to dark brown (ethanolic extract). Both the plant extract pigmentation and the extractant solvent are
central to the experimental issues our lab observed when performing dilution-based antimicrobial methods, including microdilutions (i.e. MIC determination) and biofilm assays (data not included in this report). Pigmentation in extracts or fractions can interfere with spectral quantification of microbial turbidity and biofilm staining irrespective of the use of colorimetric indicators. Some authors report the pre-treatment of coloured plant extracts prior to testing such as the multiple sequential centrifugation of the crude filtrate (Cowan, 1999), or the addition of activated carbon decolourisation steps for the removal of non-polar pigments (Petkova et al., 2014). Any pre-treatment in the sample preparation phase would need to be considered when interpreting the results to avoid adversely affecting intra- and inter-laboratory reproducibility (EUCAST, 2003).

Solvents are employed for the initial extraction, chromatographic separation and resolubilisation of dried plant residues. In this study, ethanol was chosen as the extractant solvent based on traditional usage (O’Shea et al., 2009) and similar studies evaluating ethanol extracts of *I. helenium* (Huo et al., 2008; Jiang et al., 2011; Trendafilova et al., 2010; Y. M. Zhao et al., 2010; Zhao et al., 2015). In dilution-based methods, solvents can interfere with activity – either potentiating or falsely depicting antimicrobial activity. Increased dilutions ranges using assay media as the diluent are therefore necessary which can sometimes lead to an underrepresentation of activity because water as a medium is an inefficient vehicle for certain compound solubility (Camp et al., 2020). Solubilisation of the test articles in an inert or biologically inactive solvent across a wide range of polarities and miscible with assay media is preferred (Klančnik et al., 2010). Cyrene™ (dihydrolevoglucosenone), an aprotic dipolar solvent, has recently been proposed as a green bio-based alternative to DMSO offering comparable solubility properties, low toxicity and no evidence of antioxidant or radical scavenging properties unlike DMSO (Camp et al., 2020). Validation of its use in plant research would be valuable to the field.

A negative control containing the solvent, or carrier, used to extract or dissolve the test article(s) was included in order to confirm inactivity and non-toxicity against the target microorganism. We performed a solvent tolerance test and confirmed that our target organism tolerated ethanol up to 20% per well in the microdilution method (data not shown). With the diffusion-based assay, the ethanol content of the test articles (e.g. crude extracts, fractions or standards) did not adversely affect the assay as the plates were left under laminar flow to evaporate the ethanol prior to incubation. Similarly, the pigment was retained within the well, and did not diffuse throughout the agar. The evaluation of
dilution and diffusion methods used to determine the antimicrobial activity of plant extracts has been reviewed elsewhere (Kenny et al., 2015; Klančnik et al., 2010). EUCAST guidelines, while directed for single-constituent or conventional antibiotics, encompass the core criteria to perform AST including inoculum preparation, media preparation and dilution schemes and were therefore used for the current natural product study also, in the absence of any standardised methods for antimicrobial assessment of plant extracts. Outside conventional AST, methods exploring other aspects of bacterial weaponry constitute a relatively untapped market for novel targets to complement anti-infective chemotherapeutic strategies such as methods targeting virulence factors (e.g. adhesins, toxins, effectors, ion chelators, destructive enzymes, secretory and signalling molecules), structural assembly, and biofilms compositions (Rasko and Sperandio, 2010).

Figure 2.6 Structures of the identified compounds: (a) Igalan(e); (b) Isoalantolactone; (c) Alantolactone; (d) Dugesialactone and (d) Alloalantolactone. Structures drawn using the online PubChem sketcher V2.4 tool (Section 2.2.3.5).
Our results demonstrate that in vitro anti-staphylococcal efficacy of *I. helenium*. As mentioned earlier (Section 2.1), traditional uses of *I. helenium* are generally indicated for conditions of the respiratory, gastrointestinal and integumentary systems (BHMA, 1983; Ó’Cuinn, 2019). Its use in dermal (topical) applications is scarcely explored in recent scientific literature, which is a likely consequence of earlier anecdotal case reports. Given the prevalence of *Staphylococci*-associated infections in Irish hospitals, further research into the usage of the identified compounds (alone and in combination) is necessitated as an alternative treatment in the control of *Staphylococcal* carriage and infection.

### 2.4 Conclusion

The natural product compounds attributing to the anti-staphylococcal activity of a traditional hydro-ethanolic extract of the root of *I. helenium* L. (elecampane), previously observed within our laboratory, were investigated in this study. A novel clean-up strategy resulted in a subset of active fractions – the composition of which were later analysed using a HPLC-DAD method supported by $^1$H NMR. The compounds identified using HPLC were the eudesmanolides alantolactone and isoalantolactone, the elemanolide igalan(e), and an unseparated mixture of the eremophilanolide and eudesmanolide; dugesialactone and alloalantolactone, respectively, as major constituents. Alloalantolactone was later confirmed following $^1$H NMR analysis. Furthermore, our findings suggest that the geographical origin of the plant did not appear to influence either the chemical profile or the bioactivity of the extract.

**Declarations of interest**

Declarations of interest: none.

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Chapter III

Adverse human health effects of metal contaminants in terrestrial plant derived food and phytopharmaceuticals

Chapter published in the IFTS Advances in Food Science book titled ‘Herbs Spices and Medicinal Plants: Processing, Health Benefits & Safety’:


(See Appendix III)
Abstract

Plants acquire heavy metals from the environment as a result of natural and human-directed activities. Metals thus arise as unsolicited residues in our food and medicinal products leading to a plethora of adverse health effects. The Agency for Toxic Substances and Disease Registry (ATSDR) lists aluminium (Al), arsenic (As), cadmium (Cd), chromium (Cr), mercury (Hg), and lead (Pb) as top priority metals of human concern, and these are further classified as human carcinogens by the International Agency for Research on Cancer (IARC). This review discusses the many avenues through which we as humans are exposed to non-essential, potentially toxic-level metal contaminants in plant-derived food and phytopharmaceuticals. We discuss how metals enter plant tissues from the environment, the biological consequences of exposure, the regulations that are currently in place to safeguard the general [non-occupationally exposed] European population and highlight gaps in legislation.

Key words: Metals; contamination; plant-derived food; phytopharmaceuticals; regulations; bioaccumulation; adverse effects.
3.1 Introduction

Plants can be described as sessile transitional reservoirs through which trace elements (metals, metalloids) from soil, water, and air transfer to humans via nutritional or medicinal avenues. Metals are ubiquitous in nature and can therefore occur as unsolicited residues in plants due to both natural (non-anthropogenic) and human-directed (anthropogenic) processes, with energy conversion and mineral consumption as chief sources of accelerated metal pollution within the biosphere. Metal contaminants can also enter our food during processing, packaging, storage or preparation (Chan, 2003; EFSA, 2017a). Metals are non-biodegradable by nature and cause toxicity to eukaryotic organisms even at very low concentrations (Memon & Schröder, 2009), thus ingestion of contaminated plant material poses a threat to consumers. Contamination can compromise the purity of plant material thus affecting overall quality, safety and perhaps even their nutritional or medicinal efficacy (Jordan et al. 2010).

Metals can enter plant tissue via (above-ground) atmospheric deposition or uptake through the soil (below-ground). Metal concentration in plant-derived foodstuffs (e.g. food crops, vegetables, herbs, spices, teas) is affected by the metal species involved, the plant species itself (e.g. non- or hyper-accumulator), its cultivation environment (e.g. soil profile, irrigation conditions), agrochemical usage and surrounding anthropogenic contamination levels. Increased metal-uptake by plants grown either for direct human consumption or as feed-plants for animals contributes to the bioaccumulation, biomagnification and persistence of hazardous metals throughout the food-chain. The geographical location of terrestrial produce cultivation directly affects their metal content, further suggesting the need for monitoring. This is important owing to globalisation of plant-based food and medicinal products.

Bioaccumulation and eukaryotic toxicity are the major health threats associated with environmentally persistent metal pollution (Beek, 1999; Kabata-Pendias, 2010). It is widely accepted that metals above safe threshold concentrations, can cause acute toxicity and a range of adverse effects in humans (Tchounwou et al., 2012). Diet is therefore a major factor concerning (oral) human exposure to potentially hazardous metals, whether as a single acute dose or through accumulation. The Agency for Toxic Substances and Disease Registry (ATSDR) lists aluminium (Al), arsenic (As), cadmium (Cd), chromium (Cr), lead (Pb) and mercury (Hg) as top priority metals of human concern; which are
further classified as human carcinogens by the International Agency for Research on Cancer (IARC).

Acute toxicity from heavy metal exposure via food (dietary consumption) is an unlikely fate in Europe, presently, based on current exposure assessment data released by the European Food Safety Authority (EFSA). The likelihood of sub-acute or chronic toxicity from continual exposure to low levels of metals and their associated compounds, however, is uncertain (Hartwig & Jahnke, 2017) and therefore cannot be ruled out; particularly in vulnerable subgroups. While efforts have been made to establish maximum levels in some foods, recent data suggest that the possibility of adverse effects arising from contaminated foods cannot be excluded. The quality of phytopharmaceuticals is a critical determinant of product safety (Verma, 2016). The World Health Organisation (WHO) states that metal exposure from the administration or consumption of phytopharmaceuticals or herbal medicinal products (HMP’s) is largely insignificant in terms of acute exposure, however, as reflected by the EFSA, chronic exposure contributing to the total bioburden or accumulation of these metals over time cannot be excluded based on current evidence (Hartwig and Jahnke, 2017). Minimizing exposure to these metals where possible is therefore a necessary approach to protect public health.

In addition to food, plants are utilised for medicine. There are variable definitions of permissible levels of heavy metals in plants and plant-derived products (medicinal or foodstuff) across geographical locations, because of non-uniformity of legislation and regulatory standards, currently. Although the European Union and several other countries have developed region-specific guidelines, their regulation in developing countries is rarely enforced (Tripathy et al., 2015). To achieve regulatory approval in the EU, all drugs/medicines must be authorised before reaching the market to protect public health and ensure the availability of safe, high-quality, and effective medicines. Phytopharmaceuticals can be marketed upon successful authorisation from one of the following application routes: full-, well-established use-, or traditional use-application, the later relating to the ‘Herbal Directive’ scheme. General quality requirements are universal regardless of the application route except in the case of metal or elemental impurities and (pre)-clinical efficacy data requirements. The International Conference on Harmonisation (ICH) Q3D guidelines have been implemented verbatim as a replacement to metal impurity specifications for individual monographs in the latest revision of the European Pharmacopoeia (Ph. Eur.; 9th Ed.). These guidelines however are not legally
applicable to certain product categories, including herbal or plant-derived products. Several key issues challenging the quality control of phytopharmaceuticals include the lack of product classification or categorisation criteria and metal impurity limits. They also include non-enforceable Good Agricultural and Collection Practices (GACP) guidelines and the unregulated practice of wildcrafting (wild plant collection) in non-European countries.

This review discusses the many avenues through which we as humans are exposed to non-essential, potentially toxic, metal impurities and contaminants in plant-derived food and phytopharmaceuticals. We discuss how metals enter plant tissues, the biological consequences of exposure to selected metals (Al, As, Cd, Cr, Hg and Pb) and the regulations that are currently in place to safeguard the general non-occupationally exposed European population.

3.2 Sources of environmental metal impurities contributing to plant material contamination

Plant biomass has the capacity to sequester metals from the environment (environment > plants > humans). Heavy metals, even at trace levels, pose substantial ecological and public health risks due to their toxic and non-biodegradable nature (Singh et al., 2011). Metal bioavailability to plants (phytoavailability), albeit a non-quantitative concept, refers to an element which is in a chemically acceptable form for plant uptake or absorption (Roberts et al, 2005), and which is influenced by numerous factors including metal species and climatic conditions. In addition to natural (non-anthropogenic) processes, exposure to trace metals has increased in the advent of global anthropogenic activity via agricultural, rural and industrial pollution. The widespread environmental persistence of heavy metals is widely reported in the literature (Alexander et al., 2009b; Singh et al., 2016). The following is a list of common sources of anthropogenic-derived metal contamination and the resulting metal pollutants commonly associated with each (Gautam et al., 2014):

- Agrochemicals: As, Cd, Cr, Mo, Pb, U, V, Zn (Nicholson et al., 2003; Otero et al., 2005);
• Waste manures/sewage effluent/sludge: Zn, Cu, Ni, Pb, Cd, Cr, As, Hg (Schmiermund, 2004; Walter et al. 2006);
• Landfill leachate: Zn, Cu, Cd, Pb, Ni, Cr, Hg (Fernández et al., 2005; Kjeldsen et al., 2002);
• Metalliferous dust: Cd, Cu, Ni, Cr, Co, Zn, As (Harris et al., 2011);
• Batteries: Pb, Sb, Zn, Cd, Ni, Hg (Naiya et al., 2009);
• Electroplating: Cr, Ni, Zn, Cu (Álvarez-Ayuso et al. 2003; Castelblanque & Salimbeni, 2004);
• Electronics: Pb, Cd, Hg, Pt, Au, Cr, As, Ni, Mn (Vegliò et al., 2003);
• Paints and pigments: Pb, Cr, As, Ti, Ba, Zn (Monken, 2010);
• Paper and pulp: Zn, Cu, Cd, Pb, Ni, Fe, Mn (Hakeem & Bhatnagar, 2010); and
• Specialist alloys and steels: Pb, Mo, Ni, Cu, Cd, As, Te, U, Zn (Cheng, 2003; Rule et al., 2006).

Consequent contamination of high-value plant material (i.e. crops, vegetables, medicinal and aromatic herbs) arises during plant cultivation, processing, preparation and/or storage. The following section gives an overview of the origins of environmental metal impurities contributing to plant material contamination: soil, water, and air (see Figure 3.1).
3.2.1 Soil and surrounding rhizosphere

Soils contain trace elements of lithogenic (calculi formation) and pedogenic (soil forming) origin. The complexity of soil results largely from its inherent microbiome and trace element diversity (Geldner & Salt, 2014). Soil acts as a natural buffer which contributes to elemental transport to the atmosphere, biota and hydrosphere. Trace elemental concentrations in plants are highly correlated with the chemical makeup of their growth media (i.e. soil and surficial sediments) (Kabata-Pendias, 2011); the trace element content of which largely depends on the parent material from which it was derived (Mason and Moore, 1982). Subsequent leaching and nutrient cycling (soil-plants-animals) creates horizons exhibiting both depletion and enrichment. The behaviour and phytoavailability of each element differs as to their origin – however anthropogenic-derived elements are more mobile and thus more phytoavailable than lithogenic and pedogenic elements (Kuo et al. 1983).
Heavy metals introduced to soil via non-anthropogenic routes are usually related to parent rock weathering and pedogenesis (soil formation) (Ghiyasi et al., 2010). Agrochemical application (fertilizers & pesticides) and sewage-derived products are examples of long-range aerial sources of trace elements which have a capacity to contribute to soil pollution (Bhandari, 2014). Elemental mobilisation via seepage waters and deposition of organic or inorganic airborne particulates of natural, industrial or rural origin are predominant sources of surface soil contamination (Eshel and Beeckman, 2013; Pacyna, 1995).

Within soil solutions, metals can occur as free ions, or complexed to organic and/or inorganic ligands creating metal-ligand complexes (Roberts et al., 2005). Plant roots can only access total bioavailable metals in soluble metal fractions. Trace metal solubility is often expressed as a function of pH, affected by the type and quantity of organic matter present (Kabata-Pendias, 2011). Soil factors which govern the metal proportion in soil solutions, and consequently affect their phytoavailability, include pH, redox conditions, organic matter, clay, moisture, cation exchangeable capacity and microbiome composition (Micó et al., 2008). Fundamental soil processes which determine the fate of metals in soil include microbial absorption, dissolution, diffusion, (organic & inorganic) complexation, migration, occlusion, precipitation and volatilisation – among others (Kabata-Pendias, 2011).

The rhizosphere (endorhizosphere, rhizoplane and ectorhizosphere) encompasses the area (1 – 2 mm) of soil around plant roots inhabited by a heterogeneous microbial population which is influenced by the root exudates (i.e. ions, free oxygen and water molecules, enzymes and metabolites) (McNear, 2013). Beneficial rhizobacteria can increase root surface area or root hair production thus directly affecting elemental absorption capacity (Dutta & Podile, 2010) – a phenomenon triggered by the production of plant growth regulators and ethylene-degrading enzymes to inhibit growth (Lugtenberg & Kamilova, 2009), or the acidification of the rhizosphere by rhizobacteria thus producing chelating agents that can enhance metal solubility (Eshel and Beeckman, 2013; Rajkumar et al., 2012).

Phytoavailability is influenced by both soil and rhizosphere composition and is strongly correlated to metal speciation, metal solubility, metal mobility (transport, translocation, distribution) surrounding edaphic (soil) factors and microbial associations, plant morphological traits (e.g. root architecture) and biochemical secretion (e.g. root
exudates). Soil properties are inversely proportional to metal bioavailability to plant (roots) (Dghaim et al., 2015). Natural organic matter in the solid phase (e.g. humic substances) strongly retain metals thus lowering their bioavailability for root uptake (Ross, 1994). General phytoavailability of root-acquired trace elements depend on their behavioural properties in soil; where trace element availability to plant roots is strongly correlated with cationic concentration of the liquid soil portion (Kabata-Pendias, 2011).

### 3.2.2 Air

The atmospheric deposition of metal particulates contributes to global biosphere contamination, affecting water, soil and, consequently, plant systems.

- ‘*Atmospheric particulates*’ are defined as complex mixtures of air-suspended solid and liquid particles of organic and inorganic substances (WHO, 2007a); and
- ‘*Particulate Matter*’ (PM): are solid or liquid particles in the air classified in relation to particle size or aerodynamic diameter, for example PM$_{10}$ refers to particles with an aerodynamic diameter of $\leq 10$ µm.

The ubiquitous existence of trace elements suspended within the atmosphere originate from both anthropogenic activities (e.g. metallurgical dust, automobile emissions, combustion emissions, industrial and nuclear processes) and natural sources (e.g. mineral aerosol, bio-aerosol, lithic dusts and volcanic debris) (Pacyna, 1995)

- ‘*Primary aerosols*’: refer to those that are directly emitted in particulate form, such as automobile emissions; and
- ‘*Secondary aerosols*’: refer to particles formed from vapour condensation or chemical reactions.

Organic and inorganic aerosol particles (e.g. dust, fume, smoke, fog, smog) range in diameter from 0.001 – 100 µm in size (Pacyna, 1995). Trace elements are absorbed by organic and inorganic particles - the diameter of which affects the overall atmospheric persistence of the pollutant. Larger particles tend to precipitate rapidly near their discharge source, and low-density particles tend to persist and translocate within the atmosphere for varying periods of time (Kabata-Pendias, 2011).

Plant foliage acquire metals from atmospheric sources via aerial deposition of gaseous, dissolved or air-soluble particulate matter, referred to as ‘*foliar uptake*’ (Kannan, 1980; Schreck et al., 2012). Absorption of foliar deposited particulates occurs predominantly
through stomatal and cuticular openings, ectodesmata/ectocythodes (channels) positioned in the outer epidermal wall, and lenticels in woody stems (Franke, 2012). Particulates therefore tend to enter plant tissue as a result of cuticular or stomatal deposition on leaf surfaces followed by upper adaxial sequestration or underground (shoot-to-root) translocation via xylem or phloem vasculature (Shahid et al., 2017). Metabolic routes of entry permit the accumulation of metals against a concentration gradient within plant tissues or organs, referred to as metabolic association. Non-metabolic cuticular penetration (passive diffusion) routes permits the entry of metals through direct superficial contact to exposed aerial foliage (Kabata-Pendias, 2011).

### 3.2.3 Water (ground-, surface- and waste-water)

Water plays a fundamental role in aerial deposition of trace metals to plants (via rain or irrigation > foliage) and facilitating root acquisition of metals (via groundwater > soil). Both natural processes, including geochemical weathering of rocks and soil leaching, and anthropogenic processes resulting in wastewater effluents (e.g. mining, atomic/nuclear, industrial, clinical and municipal waste) steadily contribute to the increased elemental concentration in the hydrosphere (Gautam et al., 2014). Most trace elements, chiefly trace metals, exist predominantly as suspended colloids or are fixed by organic/mineral substances in water. Volatile elements, however, can vaporise under favourable climatic conditions. Furthermore, microbial alkylation of certain metalloids, including As, Hg, Se, Sn, Te chiefly occur in water as sediments or suspensions (Bentley and Chasteen, 2002). Biogeochemical mechanisms involving microorganisms play a vital role in the transformation between soluble and insoluble phases of metals/metalloids (White et al. 1997).

### 3.2.4 Processing, packaging, storage, and preparation

Plant material (starting material, intermediate product or finished product) is vulnerable to post-harvest contamination throughout industrial processing and packaging (Morgan, 1999). Sources of contamination include the transfer from manufacturing infrastructure and equipment used (i.e. metal grinders, reactors, conveyors, rubber septum or glassware), storage vessels (e.g. lead-lined containers), and decontamination procedures utilised on-site (WHO, 2007b). Cross-contamination from the uncontrolled exposure to residues or the release of metallic particulates, gases or vapours in-process is a constant risk (WHO, 2007b).
As an example, most plant material undergoes mechanical grinding processes to produce cut (e.g. tea leaves) or powdered material (e.g. for enhanced extraction efficiency of medicinal plants (Azwanida, 2015)). Li and Qian (2017) reported the migration of heavy metals, including Al, As and Pb, from pharmaceutical-grade rubber-stoppers during drug manufacture and preparation. Manufacturers are therefore required to comply with specified instructions, details of the process, apparatus specifications and allowable limits for residues. Manufacturing and storage facilities must also undergo periodic environmental monitoring to ascertain the absence of contaminating elemental particulate matter (WHO, 2007b, 2007a).

Furthermore, certification of anti-migration packaging and food-contact material (FCM) is becoming increasingly important. Bruna et al. (2015) reported the Cu, Cr, and Ni transfer from various packaging materials of carbonated soft drinks; exemplifying the influence of packaging material composition and the potential risk of contaminant transfer. In Europe, general requirements and Good Manufacturing Practices (GMP) for FCMs are laid out in the Commission Regulation (EC) No. 1935/2004 and Regulation (EC) No. 2023/2006 (Muncke, 2014), respectively. Such legislation prevents the transfer of contaminants from food packaging, utensils and conveyor belts that may endanger the health of consumers. In-process metal contamination of plants however, at the time of writing, is certainly not widely reported in scientific literature.

### 3.3 Overview of metal uptake systems in plants

Plants adopt variable and selective absorption patterns. Access is correlated to both the metal bioavailability and the source of uptake (e.g. air- or soil-borne) (Alford et al., 2010). The elemental uptake (absorption) efficiency of plants has been shown to differ between plant species (Schönherr & Luber, 2001). Hyperaccumulators, for example, function to maintain characteristic high metal concentrations and sustained metal accumulation in plant aerial tissue thus are becoming increasingly lucrative for their eco-toxicological uses such as phytoremediation, phytomining, biomonitoring and rhizofiltration (Krämer, 2010; Rascio and Navari-Izzo, 2011). Modifications in elemental concentrations in plants can change depending on several other variable factors, including variations (Baxter, 2009) in:
• Site of metal acquisition (i.e. root or aerial)
• Soil-chemical profile and surrounding environmental conditions
• Plant morphological and physiological states
• Plant development stages (i.e. vegetative or reproductive stage of growth)
• Plant uptake capacity (i.e. relating to transporter channels or cell wall composition)
• Chelator accumulation in plant tissues and organs
• Intracellular element compartmentalisation and subsequent concentration (e.g. vacuolar or mitochondrial sequestration)

Many factors influence the fate of absorbed metals within plant tissue, as summarised in Table 3.1.
### Table 3.1 Factors affecting the various metal fates in plants both pre- and post-entry.

<table>
<thead>
<tr>
<th>Metal Uptake Systems</th>
<th>Key points</th>
<th>References</th>
</tr>
</thead>
</table>
| **Metal availability** (Phyto-availability) | **Soil Composition** | • Parent material composition  
• Soil pH  
• Organo-metallic complex formation  
• Oxidation-reduction state  
• Metal sorption reactions | (Ross, 1994; Rieuwerts et al., 1998; Michalke, 2003; Roberts et al. 2005; Dghaim et al., 2015) |
| | **Rhizosphere Composition** | • Rhizobacterial and mycorrhizal enhanced root architecture  
• Metal (de-)mobilising root exudates (e.g. oxalic and malic acids, glomalin secretion) | (Leyval et al., 1997; Hall, 2002; Carter, 2008; Lugtenberg & Kamijo, 2009; Dutta & Podile, 2010; Rajkumar et al., 2012; Eshel & Beeckman, 2013; McNear, 2013; Kushwaha et al., 2016) |
| **Metal Absorption** | **Air-Foliage** | • Environmental temperature & humidity  
• Leaf morphology  
• Cuticular and stomatal permeability  
• Ectodesmata (ectocythodes)  
• Phyllosphere microbiota interactions | (Kannan, 1980; Schreiber & Schönheit, 1990; Prasad, 2004; Eichert & Goldbach, 2008; Birbaum et al., 2010; Eichert & Fernández, 2012) |
| | **Soil-Root** | • Root branching  
• Selective foraging  
• Root hair formation  
• Root morphological modulation  
• Root exudate and Membrane transporter expression | (Alford et al., 2010; Tangahur et al., 2011; Atkinson et al., 2014; Giehl & von Wirén, 2014; Zelazny & Vert, 2014; Montiel-Rozas et al., 2016) |
| **Translocation/Mobility/Distribution** | **Xylem (Apoplastic)** | • Cation complexation with organic acids facilitate root-to-shoot translocation | (Marschner, 1995; Prasad, 2004; Riesen & Feller, 2005; Colangelo & Guerinot, 2006; Liang et al., 2009; Rascio & Navari-Izzo, 2011; Page & Feller, 2015; Singh et al., 2016) |
| | **Phloem (Symplastic)** | • Metal-binding compounds (e.g. nicotianamine) facilitate aerial-to-source organ (e.g. senescing leaves) or -sink organ translocation (e.g. maturing fruit) | |
| | **Metal Transporter Proteins (MTP)** | • Govern metal uptake, efflux, translocation/distribution | |
| **Biomolecular interactions** | **Complexation**  
**Chelation**  
**Immobilisation**  
**Sequestration**  
**Compartmentalisation** | • Low molecular mass (e.g. organic acids, amino acids) and high molecular mass ligands (e.g. proteins, polysaccharides)  
• Categorised as oxygen-, sulphur-, or nitrogen-donor based ligands | (Baker et al., 2000; Haydon & Cobbett, 2007; Husted et al., 2011; Timerbaev, 2012; Leitenmaier & Kupper, 2013; Mehes-Smith et al., 2013; Alvarez-Fernández et al., 2014) |
## Adaptive Responses to Metal Stress

### Hyperaccumulation
- Ability to retain high metal concentrations without exhibiting phytotoxicity
- Enhanced metal uptake, translocation and compartmentalisation
- Upregulation of metal transport genes

(White et al., 2002; Alford, Pilon-Smits & Paschke, 2010; Krämer, 2010; Rascio & Navari-Izzo, 2011; Eshel & Beeckman, 2013; Leitenmaier & Kupper, 2013; Giehl & von Wirén, 2014; Viehweger et al., 2014; Kushwaha et al., 2016; Singh et al., 2016)

### Homeostatic Maintenance
- Selective resource acquisition via root absorption stimulation or suppression
- Rhizospheric, physiochemical and microbial adaptations
- Metal sequestration, precipitation and exclusion

(Assunção et al., 2013; Clemens, 2001; Eshel and Beeckman, 2013; Haydon et al., 2011; Leitenmaier and Kupper, 2013; Puig and Peñarrubia, 2009)

### Detoxification Strategies
- **Uptake restrictions (pre-entry):** avoidance phenomena, translocation restriction, exudate immobilisation, rhizospheric pH alteration, mycorrhizal association, membrane alterations
- **Defence strategies (post-entry):** complex formation, sequestration, efflux pump activation, cell wall modification, apoplastic exclusion, aerial leaching, phytovolatilization, redox mechanisms, hormone-mediated tolerance

(Hall, 2002; Haydon & Cobbett, 2007; González-Mendoza & Zapata-Pérez, 2008; Kabata-Pendias, 2011; Mehes-Smith, et al., 2013; Ernanzverdian et al., 2015)
3.4 Human exposure to metals in plant-derived food and associated regulation

Long-term, low-dose exposure is a more likely route of metal exposure through dietary means, resulting in systemic accumulation over-time (Goyer et al., 2004). Evaluating plant-derived food as sources of human exposure to non-essential metals, their associated compounds, and the formation of reactive metabolites contributes to a more unified understanding of the effects of aggregated exposure (Thomas & Bradham, 2016) to multiple contaminants. The severity of the toxicity varies depending on the metal species involved, chronicity and exposure, gender, genetics and lifestyle (Tchounwou et al., 2012). Nutritional status plays an irrefutable role in metal toxicity. Nutrient deficiency predisposes people to increased risk of toxicity from non-essential metals (Peraza et al., 1998). Adequate nutrient levels can decrease the bioavailability of certain metals from food in humans thus reducing the risk of toxicity (e.g. cadmium in iron deficiency), and vice versa. The species (chemical form/ oxidation state/ valency) of metals needs consideration, as some metal species are more potently toxic than others (e.g. hexavalent chromium (Cr(VI)) is more toxic than its trivalent (Cr(III)) form. Molecular or ionic mimicry describes situations where a metal complexes with an endogenous intracellular ligand and the end-product of this complex mimics the behaviour of the normal substrate but alters its typical function (Goyer et al., 2004; Bridges & Zalups, 2005). Such interactions are crucial to the understanding of metal uptake, kinetics and ultimately metal toxicity in humans (Goyer et al., 2004).

When interpreting metal toxicity data from food, it is vital to include the rate of metal absorption and intake frequency as it may not be the food commodities with the highest metal level(s) but the foods which are more regularly consumed in larger quantities which pose the greatest risk to consumers over time. Dietary exposure is assessed based on the metal concentration present in foods and associated food consumption estimates which includes both the consumption patterns of the food/ingredient and the probability of the consumer ingesting the food (Kroes et al., 2002). Various exposure assessment methodologies used to estimate potential health impacts of residues and contaminants in food have been reviewed elsewhere (EFSA, 2011; Kroes et al., 2002). Examples of calculation used to identify the transfer risk of plant-derived metals to humans include the daily dietary intake indices such as the Chronic or Estimated Daily Intakes (CDI,
EDI), Hazard Index (HI) and Hazard Quotient (HQ) (Gall et al., 2015). Such techniques are necessary to quantify the extent of exposure to consumers in relation to health-based guidance values (EFSA, 2011).

Where genetic predisposition to micronutrient-associated disease (e.g. hereditary haemochromatosis) and/or metal storage disorders (e.g. Wilson’s Disease), the concentration of certain metals in food and medicine can adversely impact certain subpopulations more aggressively – an important consideration not to be overlooked from a pharmacovigilance and food safety perspective. Routine clinical testing of human samples does not include the assessment of the concentration of heavy metals except in exceptional circumstances. Therefore, the contribution of these elements to individual human pathologies is almost always unknown. Nevertheless, the limits allowable through regulation, combined with what has been reported in the scientific literature of the pathological effects of these individual elements have provided us with considerable information, which is reviewed below. The authors suggest that clinical metal toxicity should be diagnosed through laboratory testing, particularly among patients lacking a diagnosis while suffering from chronic symptoms.

Table 3.2 summarises the target systems for As, Cd, Hg and Pb toxicity and the current carcinogenic classification of these metals. It is necessary to control the levels of metal contaminants or impurities in our food and medicinal products to safeguard the public from exposure to potentially harmful metals.
<table>
<thead>
<tr>
<th>Hazardous Metal</th>
<th>Symptoms of Exposure</th>
<th>Dietary limits (EU)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aluminium (Al)</strong></td>
<td>Not documented</td>
<td><strong>Acute</strong></td>
<td><strong>Chronic</strong></td>
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<tr>
<td>**Arsenic (As)⁺</td>
<td>Gastrointestinal</td>
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<td></td>
<td>Haematological</td>
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<td>Neuropathy</td>
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<td>Encephalopathy</td>
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<td>**Cadmium (Cd)⁺</td>
<td>Pneumonitis</td>
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<td>Renal damage</td>
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<td>Steroidogenesis alteration</td>
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<td><strong>Chromium (Cr)</strong></td>
<td>Gastrointestinal haemorrhage</td>
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<td>Haemolysis</td>
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<td>Reproductive</td>
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<td>Dermal effects</td>
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<td>**Mercury (Hg)⁺</td>
<td>Renal necrosis</td>
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<td>Caustic gastroenteritis</td>
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<td>Parasthesia</td>
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<tr>
<td>**Lead (Pb)⁺</td>
<td>Gastrointestinal effects</td>
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<td>Encephalopathy</td>
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<td>Renal and hepatic damage</td>
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<td>Acute psychosis</td>
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IARC classifies As (IARC, 2012) and Cd (IARC, 2012) as Group 1 carcinogens (carcinogenic to humans); Pb as Group 2A carcinogen (probably carcinogenic to humans) (IARC, 2006); Me-Hg as Group 2B carcinogen (possibly carcinogenic to humans); and Inorganic Me-Hg as Group 3 carcinogens (unclassifiable as to its carcinogenicity to humans) (IARC, 1993).

*BW = body weight

### 3.4.1 Aluminium (Al)

#### 3.4.1.1 Human health effects

Al (3+ oxidation state) occurs naturally in our environment and is the most abundant metal component of the earth’s crust (ATSDR, 2008; Jaishankar et al., 2014). It is frequently combined with fluorine (aluminium fluoride), oxygen (aluminium oxide) and...
silicon for industrial uses. Despite being an experimentally demonstrated neurotoxin (Tomljenovic & Shaw, 2011), it is commonly used in medical and personal care products such as antacids, astringents, antiperspirants, buffered aspirin, food additives (ATSDR, 2008), vaccine adjuvants (e.g. al-hydroxyphosphate and boehmite) (Lindblad, 2004) and FCM’s (Klotz et al., 2017). The nervous system is a well-known target for Al toxicity, in addition to the musculoskeletal and respiratory systems (ATSDR, 2008). While Al could be a potentially toxic co-factor in neurodegenerative conditions, one must acknowledge that these diseases are likely the result of multiple aetiologies (Tomljenovic & Shaw, 2011), and further research is required. Al is non-carcinogenic in vitro however human trials have not yet been conducted.

3.4.1.2 Dietary exposure: occurrence, prevalence, and regulatory limits in plant-based food

While most unprocessed products contain ≤5 mg.kg⁻¹ of Al (FSAI, 2016), EFSA have reported that tea leaves, herbs and spices, soy and cocoa products contain high concentrations of Al (EFSA, 2008), which is interesting since its accumulation in terrestrial plants is thought to be unlikely (ATSDR, 2008). Bakery foodstuffs (e.g. cereals, cakes, pastries, breads) containing Al food-additives, such as sodium aluminium phosphate, similarly contain higher Al concentrations (FSAI, 2016). Recent restrictions on the use of Al-containing food additives were introduced by the European Commission (Commission Regulation (EU) No 380/2012) to reduce consumer exposure (FSAI, 2016). Considering a mean dietary exposure of ≤2.3 mg.kg⁻¹, EFSA established a Tolerable Weekly Intake (TWI) of 1 mg-(kg BW)⁻¹wk⁻¹ for Al in unprocessed food, with higher permissible concentrations in baked goods (EFSA, 2008). This is lower than the provisional TWI of 2 mg.kg⁻¹ established by the Joint Food and Agriculture Organisation/World Health Organisation Expert Committee on Food additives (JECFA) (JECFA, 2011). The U.S. Food and Drug Administration (FDA) regards Al as safe for use as food additives (e.g. in cereals and cheese products), food-contact materials or medicinal excipients, e.g. buffered analgesics and antacids (ATSDR, 2008). A Total Diet Survey (TDS) conducted by the FDA estimated average Al intake ranges from 0.7 mg.day⁻¹ in infants, 11.5 mg.day⁻¹ in male teens, 8-9 mg.day⁻¹ in male adults and 7 mg.day⁻¹ in female adults (Pennington & Schoen, 1995). The U.S. Environmental Protection Agency (EPA) recommends a secondary maximum contaminant level (SMCL) of 0.05 – 0.2 mg.L⁻¹ for Al in potable water, while EU limits are 200 μg.L⁻¹ for Al
Dietary Al exposure is within the TWI range; however, while average exposure is below the TWI, it is still close to the TWI for Al. While orally ingested Al is poorly absorbed, once within the body, it has a whole-body half-life of approximately 50 years (FSAI, 2016). Taking this into account, an attempt should be made to reduce Al exposure, particularly for infants, toddlers and children (Hartwig & Jahnke, 2017).

### 3.4.2 Arsenic (As)

#### 3.4.2.1 Human health effects

As is a naturally occurring metalloid present in groundwater, soil and air. It exists in organic and inorganic structural forms and various oxidation states (-3, 0, +3, +5) (Hughes, 2002). Natural sources include volcanic emissions, weathering of rocks and natural springs (Zhao et al., 2010). Historically As has been used as a therapeutic agent in products such as Fowler’s solution (potassium arsenite: AsKO₂) and Salvarsan, and in modern medicine as an approved chemotherapeutic agent (arsenic trioxide: As₂O₃) in acute promyelocytic leukaemia (Murgo, 2001; Tchounwou et al., 2012a). Evidence of the environmental persistence of arsenate residues as a result of previously-accepted agricultural practices are reported in the literature (Hood, 2006), following the application of arsenic-based agrochemicals such as Paris-green insecticides (copper acetoarsenite: Cu(C₂H₃O₂)₂·3Cu(AsO₂)₂) and pesticides (lead hydrogen arsenate: PbHAsO₄; monosodium methanearsonate: CH₄AsNaO₃; cacodylic acid: Cu₃(AsO₄)₂). Other environmental sources of As include copper smelting industries, wood preservation (chromated copper arsenate: Cu₃(AsO₄)₂), glass and semi-conductor production (Hughes, 2002; Hughes et al., 2011). Three further classes of arsenical compounds of toxicologic concern, predominantly present in seafood, are the complex organic arsenicals (Borak & Hosgood, 2007; Thomas & Bradham, 2016): arseno-lipids, -sugars and trimethylarsonium. These compounds are characterised by the occurrence of a di- or tri-methylated As-containing moiety in an aliphatic or aromatic molecule, and contribute to the aggregate exposure to As in the human population (Thomas & Bradham, 2016). Primary dietary sources include fish, fish oil supplements and macroalgae (i.e. seaweed).
The toxic potencies of As compounds depend on its chemical form, oxidation state and effects of mammalian metabolism, including reduction to a trivalent state and oxidative methylation to a pentavalent state (Thomas et al., 2001; Hughes, 2002). In essence, As metabolism involves a cascade of events from pentavalency to trivalency (reduction) catalysed by As$^{13}$-methyltransferase, and back to pentavalency (oxidative methylation) (Hughes et al., 2011). Toxicologically relevant forms of inorganic As (see Figure 3.2) are pentavalent arsenate (AsO$_4^{3-}$) and trivalent arsenite (AsO$_3^{3-}$) (Tchounwou et al., 2012). Monomethylarsonic acid (MMA), dimethylarsinic acid (DMA) and trimethylarsine oxide are stable methylated mammalian metabolites of inorganic As (Hughes, 2002), often referred to as the ‘organic’ forms of As.

Genetic polymorphisms associated with increased susceptibility to inorganic As-induced disease have been reported (De Chaudhuri et al., 2008; McCarty et al., 2007; Porter et al., 2010; Steinmaus et al., 2007; Wang et al., 2009). Genotypic variation in the As$^{3+}$MT (As$^{3+}$ Methyltransferase) genotype can alter methylation profiles for inorganic As, referred to as the As methylation phenotype (Hughes et al., 2011). Studies show that persistently high urinary MMA concentrations associated with the $M287T$ (Methionine 287 Threonine) genotype, which affects the As methylation phenotype, is potentially linked
with increased risk of As-induced adverse health effects (Chung et al., 2009; Fujihara et al., 2009; Rodrigues et al., 2012; Valenzuela et al., 2009).

Exposure to As compounds are explicity linked to cancer; As compounds are classified as Group 1 carcinogens (carcinogenic in humans) by the IARC and EPA (IARC, 2012). Consumption of As-contaminated foodstuffs is a potential contributor to the global cancer epidemic (Oberoi et al., 2014; Thomas & Bradham, 2016). Associated adverse effects are well-established. Acute toxicity arising from trivalent As exposure is greater than that arising from pentavalent ion (Hughes, 2002), due to its reactivity with sulfur and cellular generation of reactive oxygen species (ROS) (Hughes et al., 2011). Methylated trivalent intermediates of As are more acutely (geno)toxic then arsenite (Mass. et al., 2001). Symptoms of acute As toxicity are centred around gastrointestinal (GIT) disorders, whereas symptoms of chronic As toxicity affect multiple systems including the integumentary, cardiovascular, nervous, excretory (renal) and endocrine systems (Hughes, 2002). Adverse effects associated with long-term consumption of inorganic As in humans are abnormal glucose metabolism, diabetes, cancer, cardiovascular disease, developmental toxicity, neurotoxicity and skin lesions (EFSA-CONTAM, 2009). The development of skin lesions characterised by hyper-keratosis, hypo- and hyper-pigmentation, are a distinctive characteristic of chronic oral exposure to As (Hughes, 2002; Paul et al., 2015). Based on frequency, toxicity and likelihood of human exposure, As ranks first on the priority list of hazardous substances published by the ATSDR, ahead of Pb and Hg (ATSDR, 2017).

The toxicological profile of As compounds are discussed in detail by the ATSDR (ATSDR, 2007a). The mechanism(s) of action (MoA) responsible for these effects however, are complex and still poorly understood (Shen et al., 2013). Potential MoA for As compounds has been extensively reviewed in the literature (Kitchin, 2001; Hughes, 2002; Tapio & Grosche, 2006; Platanias, 2009; Kitchin & Conolly, 2010; Singh et al., 2011). The toxicity of As-contaminated potable water is acknowledged worldwide (NRC, 1999; Zhao et al., 2010; Thomas & Bradham, 2016), and further elucidation of this is outside the scope of this review. Dietary exposure to As through food is thought to be just as prevalent, however (Kurzius-Spencer et al., 2014; Wilson, 2015; Thomas & Bradham, 2016).
3.4.2.1.1 Interactions with endogenous oxyanions: sulfur and phosphate

The hydrogen arsenate ion (As\(\text{H}_2\text{O}_4^{2-}\)) is a molecular analogue of the hydrogen phosphate ion (HPO\(\text{4}_2^{2-}\)). Structural similarities and comparable dissociation constants thereby permit inorganic arsenate to compete for phosphate anion transporters (Shen et al., 2013) and replace phosphate in biochemical reactions such as the *in vitro* uncoupling of ATP; coined “arsenolysis”. Arsenolysis weakens the *in vitro* formation of ATP at both substrate (glycolysis) or mitochondrial levels (oxidative-phosphorylation) by substituting phosphate with arsenate in enzymatic reactions creating unstable arsenate anhydrides (Crane and Lipmann, 1953; Dixon, 1996; Gresser, 1981). Ultimately, the binding of pentavalent arsenicals in phosphate-utilising enzymes that typically alkylate, acylate or phosphorylate the phosphate (Dixon, 1996), confers interfering biological alterations (Shen et al., 2013). The similarities between these two oxyanions may explain its prevalence in marine organisms. Evidence suggests that marine algae cannot distinguish between them and unintentionally uptake arsenate instead of the essential nutrient phosphate which subsequently leads to the production of toxic methylated organo-arsenic compounds which bioaccumulate throughout the food-chain. Studies have also demonstrated arsenite as a functional mimic of estrogen (estradiol) which could suggest its potential role in the aetiology of breast cancer or hormone-related disease (Bridges & Zalups, 2005).

3.4.2.1.2 Altered glucose metabolism

The cellular binding location of As species is an important determinant of toxicity. The integration of trivalent arsenicals to specific functional groups within (co)-enzymes or receptors would likely result in adverse metabolic function, whereas binding to non-essential protein sites may be a necessary cellular detoxification mechanism (Aposhian, 1997). MMA\(\text{III}\) inhibits pyruvate dehydrogenase (PDH) activity by binding to the lipoic acid moiety which contains vicinal dithiols (Petrick et al., 2001), thereby interfering with pyruvate oxidation to acetyl-coA in the citric-acid cycle (Hughes, 2002). This enzymatic interference may cause decreased ATP production and altered glucose metabolism. Recent studies suggest that exposure to inorganic As can worsen diabetogenic effects in type-2 patients (Liu *et al.*, 2014). The results of one study showed that inorganic As altered lipid metabolism and pancreatic \(\beta\)-cell dysfunction, increased gluconeogenesis and oxidative hepatic damage in mice, and additionally worsened glucose tolerance in diabetic mice (Liu *et al.*, 2014).
3.4.2.1.3 Oxidative stress and ROS-generation

Cellular ROS generation is a prominent inducer of toxicity and disease in humans (Jomova et al., 2011). Arsenic-associated ROS species include: As-centred and As-peroxyl radicals, hydrogen peroxide, hydroxyl radical, superoxide anion and reactive nitrogen species (Kitchin, 2001). Chronic, low-dose As alters oxidative stress and inflammation-associated genes and proteins and transcriptional regulators of the resulting altered genes are redox-sensitive (ATSDR, 2007a). As-induced oxidative stress has been confirmed both in vitro and in vivo, and is often implicated in the inhibition of numerous enzymes (Shen et al., 2013). Arsenite constrains PDH activity upon both the direct binding to inherent vicinal sulfhydryl groups within the enzyme, and/or by generating ROS which ultimately inactivates the enzyme (Samikkannu et al., 2003). As-induced-ROS play a role in a number of hypothesized MoA such as altered signal transduction, altered DNA repair, cell proliferation and genotoxicity (Hughes et al., 2011); however, the mechanisms by which As form ROS is poorly understood. Proposed metabolic events leading to ROS generation include the metabolic oxidation of arsenite to arsenate (Del Razo et al., 2001), the production of arsenic metabolites (Yamanaka and Okada, 1994), NADH or NADPH oxidase activation (Chou et al., 2004), thiol chelation/complexation in dithiol-containing enzymes (Shen et al., 2013), and ferritin iron release in the presence of ascorbic acid (Ahmad et al., 2000). Oxidative stress may be involved in As-induced cytotoxicity (Yedjou et al., 2006; Jomova et al., 2011), while ROS-mediated genomic mutation is a likely initiation step of As-induced carcinogenicity (Li and Chen, 2016; Wei et al., 2005).

3.4.2.1.4 Carcinogenicity

As is classified as a Group 1 carcinogen (IARC, 2012) based on several epidemiological studies directly confirming the carcinogenicity of As compounds. Current evidence from MoA studies suggest that As may be a co-carcinogen, a promoter or a progressor of carcinogenesis (Tchounwou et al., 2012). Proposed mechanisms of carcinogenesis include altered cell proliferation and DNA methylation, co-mutagenesis (Hughes et al., 2011), genotoxicity, oxidative stress and tumour protection (Hughes, 2002; Martinez et al., 2011). As-induced epigenetic effects, such as DNA methylation, posttranslational histone modification and microRNA interference, play a role in As toxicity (Bjørklund et al., 2017a; Li and Chen, 2016; Tchounwou et al., 2012). Key pathways involved in As-induced carcinogenesis have reportedly been modulated as a result of such epigenetic
alterations. Research has shown that As alters genomic methylation profiles within human cells; leading to anomalous gene expression resulting in various pathophysiological outcomes including genomic instability and oncogenesis (Paul et al., 2017). There is emerging evidence to support the hypotheses that the interactions of As compounds with chemical compounds (Pershagen et al., 1984), or UV radiation (Rossman et al., 2001) causes a co-carcinogenic effect (Hughes, 2002). As can interfere with cell signalling pathways implicated in tumour progression, e.g. suppression of the TP53 gene (Tchounwou et al., 2012a), intracellular kinases and transcription factor signalling pathways (Huang et al., 2004). The ability of As species to modulate gene expression contributes to the carcinogenic potential of these compounds (Tchounwou et al., 2012a). Given the carcinogenic potential of As and As compounds, it is vital to minimize human exposure where possible - starting with food.

3.4.2.2 Dietary exposure: occurrence, prevalence, and regulatory limits in plant-based foods
Predominant As compounds routinely present in food are MMA, DMA\textsuperscript{V}, arsobetaine, arsenoeholine, arsenosugars and arsenolipids, which are of course dependent on the food type (Hughes et al., 2011). Organic As species, such as arsobetaine and arsenosugars, are most frequently present in seafood while inorganic As forms predominate in plant-derived foods, including As(V), As(III), methylarsonate, methylarsenite and DMA (EFSA, 2014). Contaminated water used in agricultural irrigation of dietary crops accelerates the introduction of As into the food-chain via plant uptake. Plant-derived food products generally contain low levels of total and inorganic As ($\leq$0.25 mg.kg$^{-1}$). Foods such as rice, seafood, algae and animal offal are the main dietary contributors (EFSA-CONTAM, 2009). Dietary intake of inorganic As from plants is generally low except for populations who consume rice as a dietary staple (Schoof et al., 1999).

EFSA lists processed grain-based products, such as rice grains and derivatives, wheat bread, dairy and potable water as the chief sources of As exposure for the general European population (European Commission, 2017a). Other specific food groups, including fish, vegetables, algae, coffee and special dietary food are also significant contributors to the daily dietary exposure of the general European population to inorganic As (European Commission, 2017a). Estimates of dietary inorganic As intake levels vary worldwide (Hughes et al., 2011). Recently, EFSA estimated that dietary-intake of As for average consumers in European populations ranged from 0.2 – 1.37 µg·(kg

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among infants and young children, with the 95th percentile estimates ranging from 0.36 – 2.09 µg·(kg BW)⁻¹·d⁻¹ (EFSA, 2014). Average dietary exposure among the adult population was much lower, ranging from 0.09 – 0.38 µg·(kg BW)⁻¹·d⁻¹, with the 95th percentile estimates ranging from 0.14 – 0.64 µg·(kg BW)⁻¹·d⁻¹ (EFSA, 2014).

Currently, no maximum levels (MLs) or tolerable intake values have been defined for As compounds in dietary foodstuffs (Sun et al., 2008), except for rice and rice-based products (see Table 3.3). In 2009, the European Commission revoked the PTWI (Provisional TWI) for inorganic As of 15 µg·(kg BW)⁻¹, established by the JECFA, based on reported data confirming the respiratory-, skin- and urinary-carcinogenic potential of inorganic As at exposures lower than the current PTWI (European Commission, 2015). The CONTAM panel recommends a Benchmark Dose (Lower Limit) BMDL₀¹ values of 0.3 – 8 µg·(kg BW)⁻¹·d⁻¹ to be used in place of a singular reference point in risk characterisation for inorganic As. In 2011, JECFA established BMDL₀.₅ values µg·(kg BW)⁻¹·d⁻¹ of 3 (lung cancer), 5.2 (bladder cancer) and 5.4 (skin lesions) (FSAI, 2016) The EFSA scientific opinion concluded that the estimated dietary exposure to inorganic As for European consumers, while within the range, is close to the BMDL₀₁ values. Thus there is little or no safe margin of exposure and the possibility of a risk to some consumers cannot be excluded (European Commission, 2015). For As compounds, bench-mark calculations suggest that increased risk of cancer related to the dietary intake of inorganic As cannot be ruled out taking the current exposure levels into account (EFSA-CONTAM, 2009; CONTAM, 2014; Hartwig and Jahnke, 2017).

Children (≤3 years) are the most exposed subpopulation to inorganic As (European Commission, 2017a), with an estimated 2- to 3-fold higher exposure rates to adults (EFSA-CONTAM, 2009; European Commission, 2015). Based on available evidence, vegetarians are not at an increased risk of dietary exposure to As compared to the general population, unless large amounts of algae-derived products are consumed (European Commission, 2017a). Alternating patterns of food usage has been suggested as a strategy to decrease exposure to vulnerable subpopulations (Gundert-Remy et al., 2015). Alternatively, As limits for specific, frequently consumed food commodities should exist where possible.

As-accumulation in marine organisms, including fish, algae and seaweed is widely reported in the literature (Thomas & Bradham, 2016); with increasing interest in algae as
a source of bioactive natural products for use in pharmaceuticals, nutraceuticals, dietary supplements, novel and functional foods (Abdul et al., 2016; Cardoso et al., 2015; Fan et al., 2014). Levels in the starting materials of these marine-derived products should be monitored to ensure consumer safety. In a bid to reduce As accumulation in plants, the use of phosphorous fertilizers has been employed as a semi-successful treatment. Phosphate competes with arsenate for root uptake and adsorption on Fe oxides and/or hydroxides (Zhao et al., 2010) thereby limiting the success of these fertilisers. An alternative is to increase cellular concentration of phosphorous as a detoxification mechanism (Zhao et al., 2010).


<table>
<thead>
<tr>
<th>Food commodities</th>
<th>Permissible limit(s) of As (mg kg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milled rice (non-parboiled, polished/white)</td>
<td>0.20</td>
</tr>
<tr>
<td>Parboiled and husked rice</td>
<td>0.25</td>
</tr>
<tr>
<td>Rice cakes/crackers/waffles/wafers</td>
<td>0.30</td>
</tr>
<tr>
<td>Rice intended for infant/children food products</td>
<td>0.10</td>
</tr>
</tbody>
</table>

*Wet weight

3.4.2.2.1 Rice and rice-based products

It is becoming increasingly acknowledged worldwide that inorganic As accumulation in plant-based dietary staples, such as rice (*Oryza sativa* L.), poses a tangible risk to populations with high levels of daily rice consumption (Zhao et al., 2010), particularly in susceptible subpopulations such as infants and young-children (Signes-Pastor et al., 2017). Rice is a staple foodstuff for more than half the world’s population (Muthayya et al., 2014). Kitchen preparation techniques have been explored as a means of reducing As load in foods such as rice (Carey et al., 2015). Undissociated arsenite and methylated As species enter plant tissues through nodulin 26-like (NIP) aquaporin channels, whereas arsenate enters via phosphate transporters. Silic acid and arsenous acid share similarities which permit the entry of the latter via the silicon transport pathway. Arsenite enters plant tissue via the silicon transport pathway which then leads to arsenite mobilisation. Arsenate can be reduced to arsenite, which complexes with thiol-rich peptides such as phytochelatins and/or vacuolar sequestration as a means of cellular detoxification *in planta*. Complexation with sulfur may restrict As mobility from the root as a detoxification mechanism (Zhao et al., 2010)
A recent study showed that 73% of rice-based products targeted for infants and young children exceeded the stipulated EU limit of 0.1 mg.kg\(^{-1}\) for inorganic As (Signes-Pastor et al., 2017). As for all contaminants, actual risk is based on mg.kg\(^{-1}\) daily intake (Sun et al., 2008), therefore based on infants’ size alone, they are more sensitive to exposure to elevated levels of inorganic As in comparison to children or adults. This means that they are relatively exposed to higher levels of inorganic As from the same food item on a body-weight basis. This study illustrates non-compliance of rice-based products on the current EU market thus justifying current advise against the consumption of rice-based products (e.g. non-dairy infant formula, milk, puddings, crackers/cakes) in infants and young children (Signes-Pastor et al., 2017).

*In situ* distribution and localisation of As within the actual rice grain should not be overlooked (Zhao et al., 2010) as it is a factor contributing to the bioavailability of As in the food chain (Lombi et al., 2009). Elemental mapping of rice grain shows prominent As accumulation alongside the margin of the rice grain, which corresponds to the ovular vascular trace containing vascular tissue responsible for nutrient transport (Meharg et al., 2008; Lombi et al., 2009; Zhao et al., 2010). Studies have shown that rice bran is more enriched with As than the polished endosperm (Rahman et al., 2007; Sun et al., 2008), with some studies reporting As concentrations 10-fold higher in whole-grain bran (brown rice) at 1.9 mg.kg\(^{-1}\) As compared to the processed endosperm (white rice) at 0.21 mg.kg\(^{-1}\) As (Sun et al., 2008). Considering a recommended serving of 20 g.day\(^{-1}\); this equates to 0.012 – 0.038 mg dietary intake of inorganic As from one food source. Furthermore, these findings are a concern where such products, rice hull (husk) or soluble rice bran (milled hull), are marketed as “superfoods” in developed counties, as a source of soluble fibre (Sun et al., 2008). Even more concerning, is the use of stabilised rice bran as a staple dietary supplement for malnourished children through humanitarian food-aid programs (Meharg et al., 2008; Sun et al., 2008; FedEx, 2009; Kinyuru et al., 2015).

### 3.4.3 Cadmium

#### 3.4.3.1 Human health effects

The ATSDR ranks Cd as the seventh leading contaminant on its priority list of hazardous substances, ahead of benzo(α)pyrene and polycyclic aromatic compounds (ATSDR, 2017). Chief industrial and manufacturing sources that contribute to the anthropogenic
Cd burden in our environment include: non-ferrous metal mining (Cu, Pb, Zn), phosphate agri-fertilizers, alkaline (Ni-Cd-hydroxide) rechargeable batteries (electrode component), Cd-based pigments (Jaishankar et al., 2014) and anti-corrosion applications (Jarup, 2003). Indirect food-associated sources of Cd include Cd-coated utensils, pottery, ceramics and as a plastic stabilizer in FCMs (Galal-Gorchev, 2009). Volcanic activity and weathering of rocks account for the natural emission of inorganic Cd into the environment (EFSA, 2012a). Cd binds to organic matter within soil matrices which enhances Cd uptake in plants roots (ATSDR, 2012a). Soil contamination thus leads to increased Cd-uptake by food crops and vegetables grown either for direct human consumption (Jarup, 2003), or as feed-plants for animals thereby contributing to bioaccumulation of this metal within the food-chain. Cd-uptake (via plant roots) is further enhanced at acidic soil pH (Järup et al., 1998).

Cd is classified by the IARC as a Group 1 carcinogen based on increased human risk of bladder, breast, endometrium and lung cancer (IARC, 2012). Proposed mechanisms of Cd carcinogenesis include induced oxidative stress, inactivation of DNA-repair proteins, enzymes, altered cell proliferation and signal transduction, tumour-suppressor protein inactivation (e.g. p53), upregulation of ROS tolerance genes and epigenetic alterations (Stohs et al., 2001; Potts, et al., 2003; Waisberg et al., 2003; Joseph, 2009; Wang et al., 2012; Rani et al., 2014; Venza et al., 2015; Vilahur et al., 2015). The generation of ROS and oxidative damage is associated with the inhibition of antioxidant enzymes (IARC, 2012; Stohs et al., 2001; Valko et al., 2006).

Dietary (oral) exposure through food is the most likely route of exposure for the general population (ATSDR, 2012a). Major food sources are fruits and vegetables due to its soil-to-root transfer (Satarug et al., 2010). Risk of Cd exposure through the food supply is secondary for smokers, and primary for non-smokers (ATSDR, 2012a). Direct consumption of Cd-containing food and water accounts for 1-10% of the total exposure to Cd (ATSDR, 2012a). Cd levels vary depending on the food group in question, and is influenced by soil and irrigation conditions, agrochemical usage and atmospheric deposition rates in the surrounding environment. Potatoes and grains, soybeans, leafy vegetables, soybeans and oilseeds (e.g. sunflower) generally contain high levels of Cd (ATSDR, 2012a). Cd has a low intestinal absorption rate in humans (approx. 6.5 %) (Horiguchi et al., 2004), or ≤10 % from food (Flanagan et al., 1978).
The kidneys and bones are the primary sites of oral toxicity resulting from chronic, low-levels of exposure to Cd (ATSDR, 2012a), with a long half-life (10 – 30 years) in renal organs (kidneys) and skeletal tissue (bones) (Mamtani et al., 2011). Renal sensitivity to Cd is related to the de novo synthesis of metallothionein in the kidneys (ATSDR, 2012a). Acute oral toxicity causes severe GIT disturbances (Liu et al., 2014) and even death, depending on the dosage (ATSDR, 2012a). Other adverse effects include haematological-, hepatic-, reproductive- and immunological-effects, and nephropathy (ATSDR, 2012a). Chronic, low-level exposure to Cd in food combined with inhalation from cigarettes can cause asymptomatic accumulation in target organs leading to the onset of non-reversible adverse effects, such as renal dysfunction or osteoporosis, over time (Goyer et al., 2004; Järup et al., 1998). Low level exposure causes nephrotoxicity for certain sub-groups; particularly infants and children (Hartwig and Jahnke, 2017) and sub-groups at risk of co-exposure with tobacco smoking (ATSDR, 2012a).

The ATSDR state that the toxicological properties of Cd salts and oxides are similar to those of elemental Cd. Not all forms of Cd are equally absorbed in humans; and this variation in absorption and distribution consequently leads to differences in biological effects (ATSDR, 2012a). Ionic Cd is more readily absorbed from the GIT compared to Cd-bound complexes in food (ATSDR, 2012a). Soluble Cd exists as the Cd$^{+2}$ ion, irrespective of the original salt. Cd exists in the free-ionic form in water, while in food matrices the Cd ion complexes with a number of organic ligands, such as metallothionein (Stillmanso et al., 1987; McKenzie-Parnell et al., 1988).

The nutritional status of an individual is an important parameter not to be overlooked when interpreting toxicity data from food. Individuals deficient in calcium (Ca) or iron (Fe) are more susceptible to Cd toxicity (Hartwig & Jahnke, 2017). The relationship between Cd-absorption and Fe deficiency has been experimentally confirmed (Alexander et al., 2009). Fe deficiency upregulates the expression of Divalent Metal Transporter 1 (DMT1), which also has a high affinity for Cd (Kim et al., 2007; Kayaaltı et al., 2015), which may explain the enhanced intestinal absorption of Cd in Fe-deprived states. As exemplified by Reeves & Vanderpool, (1997), high habitual intakes of sunflower kernels not only increased Cd intake, but also Cu and phytate – the presence of which reduces the bioavailability (and thus toxicity) of the ingested Cd. Thus adequate nutrient levels, such as Fe, can decrease the bioavailability of Cd from food in humans (ATSDR, 2012a).
Conversely, micronutrient-associated disease can also affect the bioavailability and thus resulting toxicity from ingested Cd.

3.4.3.1.1 Renal nephropathy
Toxicity is dependent on renal Cd concentrations within the kidney(s) (ATSDR, 2012a). The development of kidney stones, related to increased urinary excretion of Ca, has been demonstrated in numerous studies (Hossny et al., 2001). Studies have established an association between Cd exposure and (chronic) End-Stage Renal Disease (ESRD) (Hellström et al., 2001). Events of acute oral toxicity are rare; however renal nephropathy can occur in conditions of chronic low-level exposure to Cd and Cd-compounds (Hartwig & Jahnke, 2017), because accumulation can lead to eventual renal failure.

Cd binds to metallothionein (via the blood), undergoes glomerular filtration and reabsorption in the proximal tubule, within which the Cd-metallothionein-complex is degraded resulting in the release of unbound Cd which stimulates metallothionein synthesis in the tubular cells (Dorian et al., 1992). Unbound Cd levels above 50 – 300 µg (wet weight) initiate tubular damage (ATSDR, 2012a). The cellular uptake of Cd may be related to ionic mimicry of Zn (Goyer et al., 2004). Both Zn and Cd induce metallothionein-synthesizing genes through MTF-1 regulation (Alexander et al., 2009).

There are seven Zn atoms shared between two clusters of the metallothionein monomer, and Cd can replace Zn in either of these clusters (Nordberg et al., 1971; Foulkes, 1978; Alexander et al., 2009), which consequently prevents free-radical scavenging activity (Ruttkay-Nedecky et al., 2013) of metallothionein, among other effects. Further proposed MoAs include the inactivation of metalloenzymes, activation of calmodulin, and ROS-induced cellular damage (ATSDR, 2012a).

The secretion of low molecular weight proteins (e.g. β2-microglobulin, α1-microglobulin (pHC) and retinol binding protein) are known proteinaceous biomarkers indicative of Cd-induced kidney damage in humans (Jarup, 2003). Other indicative signs include the increased urinary concentration of the intercellular tubular enzyme N-acetyl-β-glycosaminidase (NAG), amino-acids, albumin, cysteine-rich (sulfur) metallothioneins and increased excretion of Ca, sodium and potassium (Nordberg et al., 1971; Alexander et al., 2009; ATSDR, 2012a). Cd-sulfhydryl complexes interfere with MAP kinases and Na⁺-K⁺-ATPase activity (Chunhabundit, 2016). There is some evidence to suggest that diabetic (Type II) subgroups may be more sensitive to renal nephropathy associated with
Cd-toxicity (Buchet et al., 1990; Åkesson et al., 2005; Haswell-Elkins et al., 2008; Edwards & Prozialeck, 2009), and may benefit from Zn supplementation (Anetor et al., 2016). Studies have also shown that Cd-induced hepatoxicity can occur in the general population at urinary Cd-levels of 2-3 µg.g⁻¹ (creatinine) (Buchet et al., 1990; Jarup, 2003; Jarup et al., 2000).

3.4.3.1.2 Skeletal abnormalities
Cd-induced renal dysfunction is directly linked with adverse skeletal damage. Altered renal metabolism of Vitamin D, and the reduced assimilation of Ca and phosphate compounds accelerates the onset of bone disease (ATSDR, 2012a; Hossny et al., 2001; Jarup, 2003). Itai-Itai disease is a Cd-induced illness prevalent in nutrient-deprived populations with high consumption of Cd-contaminated rice and water (ATSDR, 2012a). Factors such as multiple pregnancies and nutrient deficiencies (i.e. Ca, Vitamin D, Fe and Zn) as a result of inadequate diet, contribute to the aetiology of this disease (ATSDR, 2012a). The first reports of Itai-Itai disease were recorded in Japan from Cd-contaminated water intake. Recent studies have indicated that chronic Cd exposure causes skeletal abnormalities as evidenced by bone demineralisation and fractures (Alfvén et al., 2000; Nordberg et al., 2002; Staessen et al., 1999). Cd-induced hypercalciuria from renal tubular dysfunction indirectly decreases bone density (Chunhabundit, 2016). Other bone-related effects include osteomalacia, osteoporosis, increased risk of fractures and decreased bone-densities (ATSDR, 2012a). Proposed MoAs include signal transduction pathways (e.g. inositol polyphosphate formation), elevated cystolic Ca levels and Ca channel disruption (Tchounwou et al., 2012).

3.4.3.2 Dietary Exposure: occurrence, prevalence, and regulatory limits in plant-based foods
Low levels of Cd are present in meat and marine organisms with the exception of certain shellfish and organ meats (e.g. kidney, liver) which accumulate Cd (IARC, 2012). High levels of Cd have been observed in crustaceans, edible offal, fungi, oilseeds (e.g. sunflower), seaweed, water molluscs, algae-derived and cocoa-based products (including bitter-chocolate) and identified as the leading dietary sources of Cd in the general European population (EFSA, 2012a). It is important to note that it may not be the food commodities with the highest Cd levels but the foods which are more regularly consumed.
(intake frequency) in large quantities which pose the greatest risk of Cd exposure over time (EFSA, 2012a). By analysing consumption data, the major food groups which contribute to dietary exposure were determined. A recent scientific report by the EFSA (2012a), listed the following food commodities as the principal dietary sources of Cd in adults: cereals/grains and associated products, potatoes and tubers, and vegetables. Major dietary sources for children and adolescents were similar, with the addition of confectionary as a primary source of dietary exposure. For children and infants, major food sources were the same as the adult population with the addition of dairy products and infant formulae (milk and soya). The EFSA concluded that dietary contribution to oral Cd exposure is spread out over a variety of different food groups (EFSA, 2012a; European Commission, 2014), which agrees with the recommendations of Gundert-Remy et al. (2015) for alternating patterns of food consumption as a strategy to reduce the risk of accumulative dietary exposure.

In a scientific opinion on Cd in food, mean dietary exposures to Cd for the general European population are between 2.5 – 4.8 µg.kg⁻¹ (adults); which are close to or slightly above the TWI of 2.5 µg.kg⁻¹.bw established by the EFSA CONTAM Panel (Alexander et al., 2009). In the report, mean dietary exposure to Cd for children and adults (smokers) currently exceeds the TWI. The CONTAM panel suggested a reduction in the exposure to Cd at the population level (Alexander et al., 2009).

Maximum levels of Cd for individual food groups were therefore established by the European Commission in Regulation (EC) No. 488/2014 (“Maximum levels of Cadmium in foodstuffs”) (European Commission, 2014), as modified in Table 3.4, as a step towards reducing exposure. The risk of adverse effects for the general population through dietary exposure is unlikely because the TWI is based on early biomarkers of toxicity; however a reduction in Cd exposure at the population level is encouraged by the CONTAM panel (EFSA, 2017a). A limit of 0.3 mg.L⁻¹ for Cd is permitted to transfer from ceramic food contact materials (FCMs) or 0.1 mg.L⁻¹ from cooking utensils, packaging or storage vessels (≥ 3 L capacity), as outlined in the Directive 84/500/EEC (FSAI, 2016). In contrast, JECFA established a provisional tolerable monthly intake (PTMI) of 25 µg⋅(kg BW)⁻¹month⁻¹ (JECFA, 2013), which is considerably higher than EFSA’s acceptable limit.
Immobilised Cd uptake from contaminated soil and water can accumulate in plants and organisms, consequently entering the food supply (ATSDR, 2012a). Cd-concentration in soils is not the sole determinant of Cd uptake in plant roots, as Cd concentration in plant-derived foodstuffs is affected by the Cd species involved, the plant species itself (e.g. non- or hyper-accumulator), its cultivation environment (e.g. soil profile, irrigation), agrochemical usage (e.g. phosphate fertilizer application) and surrounding anthropogenic contamination levels (e.g. soil and water (IARC, 2012)).

It has been suggested that because Cd accumulates predominantly in the liver and kidneys and is poorly absorbed in the intestines and muscles of vertebrate that the risk of biomagnification may therefore be an insignificant risk to human health. Nevertheless, the ATSDR state that there is strong evidence to prove Cd bioaccumulation throughout the food chain, which has important implications for human exposure (ATSDR, 2012a).

Table 3.4 Limits of Cd in a range of terrestrial plant-derived food commodities reported in Regulation (EC) No. 488/2014 (as amended) (European Commission, 2014).

<table>
<thead>
<tr>
<th>Food commodities</th>
<th>Permissible limit(s) of Cd (mg kg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Leafy vegetables (e.g. leafy Brassica)</td>
<td></td>
</tr>
<tr>
<td>• Celery (Apium graveolens) and celeriac (A. graveolens var. rapaceum)</td>
<td></td>
</tr>
<tr>
<td>• Parsnip (Pastinaca sativa)</td>
<td>0.20</td>
</tr>
<tr>
<td>• Salsify (Tragopogon porrifolius)</td>
<td></td>
</tr>
<tr>
<td>• Horseradish (Armoracia rusticana)</td>
<td></td>
</tr>
<tr>
<td>• Fresh culinary herbs</td>
<td></td>
</tr>
<tr>
<td>Root and tuber vegetables (excluding the above)</td>
<td>0.10</td>
</tr>
<tr>
<td>Vegetables and fruit (excluding all the above, stem vegetables and seaweed)</td>
<td>0.05</td>
</tr>
<tr>
<td>Cereal grains (excluding rice, wheat)</td>
<td>0.10</td>
</tr>
<tr>
<td>Wheat, rice, rice-bran, soybeans</td>
<td>0.20</td>
</tr>
</tbody>
</table>

*Wet weight

3.4.4 Chromium (Cr)

3.4.4.1 Human health effects

Cr is an abundant element of the earth’s crust which exists in multiple oxidation or valence states, with trivalent [Cr(III)] and hexavalent Cr [Cr(VI)] being the most biologically relevant in terms of human dietary exposure (Tchounwou et al., 2012). Industrial sources of Cr include metallurgy, electroplating (Jaishankar et al., 2014), wood preservation (Cu-dichromate), leather tanning (Cr-sulfate), textiles and stainless steel production (ATSDR, 2012b). Cr(III) is naturally present in many fresh foods,
Cr(VI) is considered a toxic environmental pollutant present in terrestrial-derived foods only as a consequence of anthropogenic contamination (EFSA-CONTAM, 2014).

The ATSDR ranks Cr(VI) as the seventeenth contaminant on its priority list of hazardous substances (ATSDR, 2017). The IARC classifies Cr(VI) as a Group 1 carcinogen based on its ability to form respiratory-targeted cancers (EFSA-CONTAM, 2014; IARC, 2012). Unlike Cr(III), a TDI for Cr(VI) could not be established based on its associated carcinogenicity. Cr(III) is postulated as an essential nutrient required for macromolecular metabolism and insulin efficiency (e.g. chromodulin) (Pechova & Pavlata, 2007), although its essentiality as a dietary nutrient has been questioned based on a lack of appropriate evidence of any physiological function or symptoms of dietary deficiency (Di Bona et al., 2011; Stearns, 2000; Vincent and Love, 2012). Regardless, EFSA established a TDI of 0.3 mg.kg⁻¹ bw for Cr(III) to ensure consumer safety. Dietary exposure to Cr(III) in the general European population is well below the established TDI for all age groups, thus Cr(III) is of negligible concern for public health, according to EFSA experts (EFSA-CONTAM, 2014).

3.4.4.1.1 Cr absorption and metabolism

Cr(VI) can enter cells via non-specific anion channels whereas Cr(III) cannot (Sun et al., 2015; Witt et al., 2013). Cr(III) generally presents lower dietary toxicity compared to Cr(VI) (Goyer et al., 2004; Jennette, 1981). In general Cr has a low intestinal absorption rate - less than 10% of the ingested dose, which is largely dependent on its oxidation state and the presence of ligands (e.g. phytate, oxalate, amino acids) (EFSA-CONTAM, 2014). The onset of adverse effects depends on whether the ingested Cr(VI) is intra- or extracellularly reduced; the efficacy of which essentially determines the outcome of oral Cr-toxicity. The extracellular reduction of Cr(VI) to Cr(III) in saliva or gastric fluid in the stomach (Guertin, 2014; Sun et al., 2015) reduces its bioavailability, and consequently its potential to confer toxicity, since the reduced Cr(III) cannot gain entry to the cell. Remaining water-insoluble chromate by-products are phagocytosed while water-soluble chromates are taken up by sulfate channels based on their iso-structural similarity to phosphate and sulfate (Costa, 1997; IARC, 2012). Both the entry of Cr(VI) into the cell and the location of cellular reduction is crucial when considering the likelihood of Cr toxicity via food. Recent evidence however suggests that while the biological effects of Cr(VI) oral exposure are alleviated upon extracellular gastrointestinal reduction, a percentage of the ingested Cr(VI) evades this detoxification and reaches target organs.
(Witt et al., 2013) of the haematological, hepatic, renal and gastrointestinal systems. If ingested Cr(VI) enters the cell, it is intracellularly reduced by ascorbate, reduced GSH and cysteine (Zhitkovich, 2005, 2011; Sun et al., 2015) creating reactive intermediates which alter cellular function (O’Brien et al., 2003; De Mattia et al., 2004; Tchounwou et al., 2012). Studies have shown that intracellular Cr(III) may contribute to the genotoxicity of Cr(VI) (Zhitkovich, 2005; Nickens et al., 2010; Sun et al., 2015). Uncertainty prevails regarding the rate, extent and location of Cr(VI) metabolism; as the possibility cannot be excluded that a small percentage of ingested Cr(VI), even at low dosages, may penetrate cellular membranes and confer adverse biological effects (EFSA-CONTAM, 2014).

Intracellular Cr(III) binds to the Fe circulating protein apo-transferrin (Vincent & Love, 2012). There is evidence, albeit contradictory, to suggest that Cr(III) may interfere with Fe absorption (Stearns, 2000) caused by antagonistic competitive binding to apo-transferrin (Ani & Moshtaghie, 1992; Bjørklund et al., 2017). While the haematological effects of ingested Cr remains to be determined, caution should be exercised in patients presenting with hypoferremia and Fe-deficiency anaemia on the possibility that trace-metal interactions could potentially affect Fe status (Bjørklund et al., 2017).

3.4.4.2 Dietary Exposure: occurrence, prevalence, and regulatory limits in plant-based foods

The leading source of Cr exposure in the general population is from food and Cr-containing dietary-supplements, followed by drinking water. Typical levels of Cr in food ranges from <10 – 1300 µg.kg⁻¹ (Ettinger et al., 2009; Wilbur et al., 2012). In a 2014 study published by the CONTAM panel, the food groups exhibiting the highest levels of detectable Cr were vegetables and fungi, vegetable/animal fats and oils, herbs, spices, condiments and confectionary foods (EFSA-CONTAM, 2014). All analytical results in the above food samples were considered as Cr(III) and the panel concluded that the presence of Cr(VI) in foods is uncommon (EFSA-CONTAM, 2014). Estimated chronic dietary exposure levels ranged from 0.6 – 5.9 µg.(kg BW)⁻¹d⁻¹. Toddlers displayed highest chronic exposure levels out of all the age-groups, averaging 2.3 – 5.9 µg.(kg BW)⁻¹d⁻¹. The main contributors to elevated Cr(III) exposure was infant- and follow-on formulae, milk and dairy products and grain products. Food preparation may also contribute to the total Cr content in foods (CONTAM 2014).
stainless steel via harvesting, processing or cooking utensils to acidic foods has been documented in the literature (Anderson, 1997; Kamerud et al., 2013; Offenbacher and Pi-Sunyer, 1983; Wilbur et al., 2012).

Cr(VI) exists under oxidizing conditions as CrO$_4^{2-}$ and HCrO$_4$; both of which are mobile within soil matrices (James et al., 1997). Cr(III) however is the predominant species found in soils but has limited mobility which explains the inefficient root-to-shoot translocation of Cr in plants. The bioaccumulation of Cr in the aerial plant parts, and subsequent biomagnification of Cr along the food chain is therefore unlikely (Langård, 1982; Petruzzelli et al., 1987).

Maximum Levels (MLs) of Cr in foodstuffs have not been established (EFSA-CONTAM, 2014). The EFSA current opinion on Cr(VI) in food is that the hexavalent ion is of negligible concern to the general population – however more speciation data is required on the Cr(III) and Cr(VI) content in food and drinking water across Member States and greater insight into the gastrointestinal detoxification of Cr(VI) at doses relevant for human exposure (EFSA-CONTAM, 2014).

### 3.4.5 Lead (Pb) and Mercury (Hg)

Lead (Pb) and mercury (Hg) are both significant environmental contaminants and are ranked second and third, respectively, on the ATSDR’s priority list of hazardous substances. The exclusion of a discussion on Pb and Hg in this chapter, however, is based on their unlikeliness to exceed safe (oral) limits in plant-derived food.

A recent Scientific Opinion published by EFSA (EFSA-CONTAM, 2011) concluded that cereals/grains, leafy vegetables and (peeled) potatoes were the main plant-derived contributors to dietary Pb exposure to the general European population. Maximum levels for Pb permitted in various terrestrial-derived food range from 0.05 – 0.30 mg.kg$^{-1}$ wet weight, as per Commission Regulation (EU) 2015/1005 (Amending Regulation (EC) No 1881/2006) (European Commission, 2015). The original PTWI of 25 µg·(kg BW)$^{-1}$d$^{-1}$ established by the JECFA was withdrawn based on evidence of developmental neurotoxicity (children), cardio-, renal- and nephron-toxicity (adults) associated with Pb exposure (EFSA CONTAM, 2010). EFSA established a BMLD$_{01}$ and BMDL$_{10}$ of 1.5 and 0.6 µg·(kg BW)$^{-1}$d$^{-1}$, respectively. The Margins of Exposure (MoE) calculated by the
CONTAM panel suggested that the possibility of adverse effects from Pb cannot be excluded; particularly in young children (EFSA CONTAM, 2010). As a result, a BMDL$_{01}$ of 50 µg-(kg BW)$^{-1}$d$^{-1}$ was developed to ensure the safety of young children. By implementing a lower BMDL$_{01}$; EFSA are safeguarding children against the risk of neurodevelopmental effects which is a sequential protective measure for all other adverse effects in all other populations (EFSA CONTAM, 2010). Nonetheless, EFSA also established a BMLD$_{01}$ (cardiovascular) and BMDL$_{10}$ (nephrotoxicity) of 1.5 and 0.63 µg-(kg BW)$^{-1}$d$^{-1}$, respectively, for the adult population (EFSA CONTAM, 2010). In Europe, mean dietary intake is within the range of the calculated benchmark dose and acute toxicity to Pb has been reported as being improbable (Hartwig & Jahnke, 2017) – however neurotoxicity (in foetuses, infants and children) cannot be ruled out even at current exposures. Pb levels should be limited at all stages of life but most importantly during pregnancy and early childhood (EFSA, 2017a).

For Hg compounds, no maximum permissible concentrations have been set in terrestrial-derived foods since they typically contain low concentrations (ATSDR, 1999; Francesconi, 2007), supported by recent data indicating that the average dietary exposure is within the TWI of 1.6 – 4 µg-(kg BW)$^{-1}$d$^{-1}$for Hg (and Hg compounds) across all age groups (EFSA-CONTAM, 2012). The current opinion adopted by EFSA concludes that the occurrence of Hg in foods, except for marine-soured organisms, are of negligible concern to the European population.

3.5 Metals in phytopharmaceuticals and associated regulation

3.5.1 Phytopharmaceuticals

Phytopharmaceuticals is a collective term for botanical drugs, herbal drugs or herbal medicines intended for human use. Official definitions of phytopharmaceutical products include (EMA, 2010):

- ‘Herbal substance’: all whole, fragmented or cut starting material in an unprocessed (fresh or dried) form (e.g. leaf, root, berry, inflorescence). Defined by the binomial system (genus, species, variety, and author) and plant part used
• ‘Herbal preparation’: obtained by subjecting herbal substances to treatments such as solvent extraction, purification, expression, concentration, distillation, or fermentation (e.g. comminuted/powdered material, extracts, essential oils)

• ‘Herbal Medicinal Product’ (HMP): [finished herbal drug]: any medicinal product, exclusively containing as active agents, one or more herbal substance(s) or herbal preparation(s) individually or in combination.

3.5.2 Current European regulatory guidelines: The Herbal Directive Scheme

To achieve regulatory approval in the EU, all drugs must be authorised before reaching the market to protect public health and ensure the availability of safe, high-quality and effective medicines (EMA, 2016a). The EU regulatory system for human medicines is collaboratively governed by the European Commission (EC), European member states and the European Medicines Agency (EMA) whom control and monitor both the initial market authorisation and post-authorisation of human medicines, including phytopharmaceuticals, for sale in European member states. Harmonisation is achieved by implementing and enforcing the same set of rules and regulations.

In the European system, phytopharmaceuticals can be marketed upon successful authorisation from one of the following application routes: full-, well-established use- or traditional use-application (Poveda, 2015). General quality requirements are universal regardless of the application route with the exception of metal or elemental impurities (EMA, 2016a, 2016b) and (pre)-clinical efficacy data requirements, which differ in the dossier depending on the application route. The pre-clinical, or non-clinical, stages refer to laboratory-based in vitro and in vivo studies, including compound screening, pharmacological (pharmacodynamic and pharmacokinetic) and toxicity data. The clinical development stages refer to human studies or trials intended to prove the safety of the drug in humans (Hughes et al., 2011).

Products outside of the well-established or traditional-use categories must comply with all relevant quality, safety and efficacy criteria that are required for any drug product. For traditional products, a simplified registration process has been adopted by the European Committee since 2004, known as the Herbal Directive (Directive 2004/24/EC) (European
Commission, 2017b). This scheme was established to overcome issues encountered in the application of pharmaceutical legislation to THMPD, by providing a simplified regulatory approval process for the authorisation of THMPD onto the EU market (European Commission 2017). Prior to this, no formal authorisation process existed in the EU and consequently products were regulated at a national level which introduced disharmony in the market.

The Committee for Herbal Medicinal Products (HMPC) was established by the EMA in accordance with the Herbal Directive and Regulation (EC) 726/2004 to support harmonisation within the European market, thereby replacing the former Committee for Proprietary Medicinal Products’ (CPMP) working party on Herbal Medicinal Products. By evaluating available scientific data, the HMPC is responsible for preparing the EMA’s scientific opinions on herbal substances, preparations and combinations thereof, with the intention of establishing official (EU) herbal (formerly community) monographs and a comprehensive (EU) list of herbal substances with a long tradition of recommended and safe usage (EMA, 2017a).

The Herbal Directive is aimed at HMP that have either a long tradition of use or have a well-established record of use. In other words, the entry of HMP deficient of published scientific evidence, tests for safety and efficacy, preclinical and clinical trial data is permitted based on sufficient (ethno)pharmacological evidence of medicinal use throughout a period of ≥30 years, including ≥15 years in the European Community, i.e. a product which is generally considered safe under normal conditions of use and without medical supervision (European Commission, 2017). As per Article 16(a)(1), there are several conditions which must be met for a product to be granted authorisation. The medicinal indication of use must not require medical intervention (i.e. non-prescription only); only oral, dermal and/or inhalation route of administration are permitted; the posology and strength must be specified and derived from traditional documents (e.g. monographs); and the safety/efficacy must be justified based on long-term use in Europe.

This scheme therefore justifies less stringent regulation of THMPD (Moshiuzzaman and Choudhary, 2008), in comparison to conventional pharmaceuticals, by permitting an exemption of pharmacological, toxicological and clinical data because their safety and efficacy can be deduced from their long-standing use under the specified conditions of use (Qu et al., 2014), following careful assessment of the quality and credibility of the
ethnopharmacological or bibliographic evidence provided. In addition to scientific literature, monographs established by The European Scientific Cooperative on Phytotherapy (ESCOP), WHO and the German Commission E are used as a source of bibliographic evidence on various medicinal plants (Verma, 2016). For HMP that fall outside the category of THMPD, like any other pharmaceutical drug, must acquire market authorisation or registration from a Competent Authority as a pre-requisite for entry to the EU market, in compliance with the official legal frameworks established by the EC which govern medicines for human use in the EU.

3.5.2.1 Non-European traditional products (e.g. Ayurvedic, Anthroposophic, Chinese, Korean, Thai, Tibetan, Unani, Vietnamese Medicines)

The Herbal Directive has been a vital initiative to protect public health against unauthorised phytopharmaceuticals by clarifying the status of THMPD. By harmonizing registration rules and regulations, the directive has consequently eased the importation and exportation of such products within Member States (European Commission, 2017). While the scheme has demonstrated success, challenges regarding the registration of non-European THMPD, e.g. Chinese or Ayurvedic traditions, has received some criticism particularly among the alternative and complementary therapy communities (Qu et al., 2014). A strict requirement of the directive is to provide evidence of safe medicinal use over a minimum 15-year period in the EU as per Article 16a (1d and 1e). The absence of this ultimately impedes the successful registration of a non-European THMPD. The rationale behind the exclusion related to incidences of non-compliant formulations (e.g. mineral, animal or metal ingredients) and/or route of administration (e.g. injection), the requirement for supervised administration and a lack of bibliographic or expert evidence (EMA, 2014).

Current export data for the sale of traditional Chinese HMPs to the EU suggest that unauthorised non-European THMPD remain on the EU market without appropriate legal compliance, which is a potential concern for the public who have access to these unauthorised products (Qu et al., 2014). Caution is expressed concerning the procurement of unlicensed or unregulated products online. Consumers are encouraged to familiarize themselves with the official list of approved online vendors monitored by the EMA (EMA, 2017b).
The European Commission (EC) Rapid Alert System for Food and Feed (RASFF) database collates relevant information for every notification of non-conformance throughout Europe (Leuschner et al., 2013). As an example, Table 3.5 lists all documented notifications of heavy metal contaminants present in herbs and spices between 1994 – 2019, which includes cases of As, Cd, Pb, Hg and Pb-oxide contamination. The following criteria were searched on the RASFF Database on 03/05/2019 (Product category = herbs and spices; Hazard Category = Heavy Metals) (European Commission, 2019).

Table 3.56 Record of cross-border “herbs and spices” product refusals in Europe identified as potentially hazardous to consumers (1994 – 2019).

<table>
<thead>
<tr>
<th>Plant material</th>
<th>Heavy Metal(s) Identified &amp; Concentration (mg kg⁻¹)</th>
<th>Country of Origin</th>
<th>Notifying Country</th>
<th>Date of case</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arctium lappa radix (burdock root)</td>
<td>Pb (11)</td>
<td>Bulgaria</td>
<td>France</td>
<td>15/09/2017</td>
</tr>
<tr>
<td>Organic Curcuma longa (turmeric)</td>
<td>Pb (58)</td>
<td>India</td>
<td>U.K.</td>
<td>02/05/2017</td>
</tr>
<tr>
<td>Garcinia spp. powder</td>
<td>Pb (2.4)</td>
<td>Sri Lanka</td>
<td>Finland</td>
<td>05/07/2016</td>
</tr>
<tr>
<td>Dried urtica dioica (nettle)</td>
<td>Pb (9.9)</td>
<td>Germany</td>
<td>Finland</td>
<td>31/01/2014</td>
</tr>
<tr>
<td>Myristica fragrans (nutmeg) powder</td>
<td>Hg (0.21)</td>
<td>Indonesia via Italy</td>
<td>Greece</td>
<td>24/03/2011</td>
</tr>
<tr>
<td>Zanthoxylum Spp. (Szechuan pepper)</td>
<td>As (10.2); Cd (0.35); Pb (76.8)</td>
<td>Singapore</td>
<td>U.K.</td>
<td>21/01/2011</td>
</tr>
<tr>
<td>Artemisia absinthium (common wormwood)</td>
<td>Cd (0.113); Pb (0.6)</td>
<td>Serbia</td>
<td>Italy</td>
<td>02/10/2007</td>
</tr>
<tr>
<td>Panax ginseng (ginseng root)</td>
<td>Pb (0.208 and 0.334)</td>
<td>China</td>
<td>Italy</td>
<td>19/09/2007</td>
</tr>
<tr>
<td>Capsicum spp. (Peppers)</td>
<td>Pb (1.3)</td>
<td>Turkey</td>
<td>U.K.</td>
<td>04/06/2007</td>
</tr>
<tr>
<td>Table Salt</td>
<td>Pb (0.42; 0.59)</td>
<td>Netherlands</td>
<td>Belgium</td>
<td>30/06/2006</td>
</tr>
<tr>
<td>Spirulina platensis</td>
<td>Cd (24)</td>
<td>China</td>
<td>Germany</td>
<td>14/06/2005</td>
</tr>
<tr>
<td>Allium sativum (garlic) powder</td>
<td>Pb (2.926)</td>
<td>China via the U.K.</td>
<td>Belgium</td>
<td>18/03/2005</td>
</tr>
<tr>
<td>Meat &amp; vegetable masala seasoning mix</td>
<td>Pb (62; 38)</td>
<td>Bangladesh</td>
<td>U.K.</td>
<td>11/05/2004</td>
</tr>
<tr>
<td>Table Salt</td>
<td>Cu (0.3)</td>
<td>Malta</td>
<td>Italy</td>
<td>18/10/2002</td>
</tr>
<tr>
<td>Herbal products (unspecified)</td>
<td>Unspecified</td>
<td>Unspecified</td>
<td>Italy</td>
<td>24/09/1998</td>
</tr>
<tr>
<td>Capsicum annuum (paprika) powder</td>
<td>Pb-oxide (Unspecified concentration)</td>
<td>Unspecified</td>
<td>Germany</td>
<td>03/10/1994</td>
</tr>
</tbody>
</table>
3.5.3 Current quality criteria for metal impurities

Included in the quality criteria for pharmaceuticals is the control of inorganic elemental impurities in both the starting materials and the finished product. All manufactured pharmaceuticals can become contaminated with metals throughout the process in numerous ways. Potential origins of contamination include the raw materials, air-borne particulates, metal reagents or catalysts used during synthesis, excipients, or from vessels, equipment or probes (Wang et al., 2000). Monitoring of both the starting material, process intermediates and/or finished product is therefore necessary to ensure the manufacture of quality medicinal products (Balaram, 2016; EMA, 2011; Wang et al., 2000). Compendial texts, such as the European Pharmacopoeia, detail legally binding quality standards which include specifications, general texts and general methods.

In the latest edition (Ph. Eur., 9th Edition) at the time of writing, individual monographs no longer specify heavy metal tests; instead implementation of International Conference on Harmonisation (ICH) principles is now enforced. Advantages and challenges of these newly addressed changes are reviewed elsewhere (Balaram, 2016; Pohl et al., 2017). General methods for quality and control of impurities that have been recently revised in the European Pharmacopoeia (9th Edition; Ph. Eur.) include chapter 2.4.20: Detection of metal impurities (formerly ‘Detection of metal catalysts or metal reagent residues’), and chapter 5.20: Elemental impurities (formerly ‘Metal catalysts or metal reagent residues’) which replaces the EMA Guideline on specification limits for residues of metal catalyst and metal reagents (EMA, 2008) with the principles of the harmonised guideline: ICH Q3D for elemental impurities (EMA, 2016b; ICH, 2014). The current guideline adopted by the EMA ICH Q3D: elemental impurities is legally enforceable from 2017-12 onwards for both new marketing authorisation applicants and authorised medicinal products. This document details the assessment and control of elemental impurities in drugs using the ICH principles of risk management (ICH Q9). The document, however, does not apply to herbal products. The ICH commented that herbal products are commonly subject to region-specific regulations rather than harmonised guidance. The ICH state that the application of the Q3D to herbal products will be addressed by regional regulatory authorities in the future (ICH, 2016).
3.5.3.1 Starting materials (herbal substances)

Considering the ability of many plant species to (hyper)accumulate metals within their tissue, either directly from the soil or rhizosphere, air or water, it seems logical to enforce environmental monitoring specifications for plant cultivators. The metal content of plant material is therefore directly influenced by the environment in which they are cultivated. Geographical location, soil profile, cultivation methods, harvesting and post-harvesting conditions not only affects the metabolite profile (active constituent(s)) between batches but also the purity of the resulting intermediate or final product (Wah et al., 2012). The first step in reducing public risk and eliminating the possibilities of human exposure to medicinal plants (and product thereof) containing harmful metal impurities is the quality control of the starting material. This is not a straightforward task and requires agricultural and environmental monitoring (WHO, 2007b).

Consequently, The World Health Organisation (WHO) originally established the following technical documents: “Good Agricultural and Collection Practices (GACP) guidelines for medicinal plants” (WHO, 2003), “Assessing the quality of plant materials with reference to contaminants and residues” (WHO, 2007b), and “Quality control (QC) methods for medicinal plant materials” (WHO, 1998), among others. The WHO states that the GACP should be implemented only as guidelines in national and/or regional quality control of herbal medicines (WHO, 2007b). Unfortunately, GACP is therefore not currently enforceable by law, it is simply an optional checklist (Verma, 2016). Both GACP and GMP compliance is critical for the manufacture of high-quality medicinal plant products, from cultivation to the final product (Heinrich, 2015; WHO, 2007b).

There are no universal limits for metal impurities in medicinal plants or products thereof. Acceptance criteria differ based on both the matrix (e.g. soil, water, starting materials, finished products) in question and the regulations implemented at a regional and/or national level (see Table 3.6). Countries including Canada, China, India, Malaysia, Singapore and Thailand, all implement their own national limits to assure safety and quality of medicinal plants (WHO, 2007c; Sahoo et al., 2010; Tripathy et al., 2015). The legal diversity between national laws jeopardises the harmonisation of the quality of medicinal plants and products thereof (Govindaraghavan & Sucher, 2015) and could ultimately threaten the international trade of high-value plants. Unifying regulatory requirements will result in the production of globally acceptable, safe products for human
consumption (Sharma, 2015). This, however, is a complex task and it should be noted that uniformity may never be achieved.

Table 3.6 Various national maximum permissible limits for As, Cd, Pb and Hg in medicinal plants (Tripathy et al., 2015).

<table>
<thead>
<tr>
<th>Location</th>
<th>As (mg kg⁻¹)</th>
<th>Cd (mg kg⁻¹)</th>
<th>Pb (mg kg⁻¹)</th>
<th>Hg (mg kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WHO</td>
<td>10.0</td>
<td>0.30</td>
<td>10.0</td>
<td>1.00</td>
</tr>
<tr>
<td>FDA</td>
<td>10.0</td>
<td>0.30</td>
<td>10.0</td>
<td>1.00</td>
</tr>
<tr>
<td>Ph. Eur.</td>
<td>-</td>
<td>1.00</td>
<td>5.00</td>
<td>0.10</td>
</tr>
<tr>
<td>Singapore (HAS*)</td>
<td>5.00</td>
<td>0.05</td>
<td>20.0</td>
<td>0.50</td>
</tr>
<tr>
<td>Ph. China</td>
<td>2.00</td>
<td>0.30</td>
<td>5.00</td>
<td>0.50</td>
</tr>
<tr>
<td>India (AYUSH†)</td>
<td>10.0</td>
<td>0.30</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Canada</td>
<td>5.00</td>
<td>0.30</td>
<td>10.0</td>
<td>0.20</td>
</tr>
<tr>
<td>Malaysia (FoSIM)‡</td>
<td>5.00</td>
<td>-</td>
<td>10.0</td>
<td>0.50</td>
</tr>
<tr>
<td>Thailand</td>
<td>4.00</td>
<td>0.30</td>
<td>10.0</td>
<td>-</td>
</tr>
</tbody>
</table>

*Health Sciences Authority (HAS); †Ministry of Ayurveda, Yoga and Naturopathy, Unani, Siddha and Homeopathy (AYUSH); ‡ Food Safety Information System of Malaysia (FoSIM).

3.5.3.2 Extracts (herbal preparations) and HMPs (finished products)

Current European guidelines for metal impurities in herbal products are outlined in Heavy metals in herbal drugs and fatty oils [8th Ed.; Chapter 2.4.27], which describes recommended tests. Included are general maximum limits in herbal products for Cd, Hg and Pb at 1.0, 0.1 and 5 ppm, respectively (Bouin & Wierer, 2014). Limited exceptions include willow bark, tormentil, fumitory, kelp and linseed oil. Recommended maximum or permissible limits are clearly deficient from compendial texts. An extended suite of limits for a greater number of toxicologically significant elements in herbal products is therefore desirable. As per EMA guidelines, however, the testing of HMPs for metal impurities is not required if metal impurity levels of the herbal substance(s) and/or preparation(s) used to prepare HMP (final product) are satisfactory (EMA, 2011). This consequently excludes the possibility that extracts or HMPs may become contaminated with metal residues throughout the various stages of processing and/or packaging. The potential contamination of unregulated herbal products and preparations formulated by herbal medicine practitioners for sale in clinical settings is acknowledged, however, the topic is outside the scope of this chapter. Acquisition of starting material from reputable and compliant medicinal plant suppliers is encouraged amongst practitioners, however, use of wild-collected plants for use in herbal products and preparations is not currently monitored (Kenny et al., 2015).
3.5.4 Key issues

3.5.4.1 Product classification or categorisation
The classification of a health product ultimately determines the route of entry for that product to the European market, and thus the quality requirements. Health products that are plant-derived, can essentially be marketed as either herbal medicines, food supplements, functional foods or novel ingredients in different regions (Chan et al., 2009), as exemplified by *Ginkgo biloba* L. Ginkgo is registered as a food supplement or traditional medicine in the U.K., as a dietary supplement in the U.S., as a medicine in Germany and as a listed medicine in Australia (Heinrich, 2015). The herbal medication market is more stringently monitored than food supplements which may encourage enterprises to seek food supplementation status for plant-derived products over herbal registration (Wah et al., 2012). Both the EFSA and EMA clearly state that it is not their role to determine the classification of a botanical as either a medicine or food supplement (EFSA, 2017b). Without clear requirements for product classification however, difficulty in distinguishing between a dietary supplement versus a medicinal product of plant origin exists (Wah et al., 2012; Ekor, 2014). Product categorisation is primarily dependent on the product’s claims, whether health-related or otherwise. Products devoid of any claim to prevent disease or ailment can be regulated as a food (including spices) or dietary supplement (Wah et al., 2012).

3.5.4.2 Non-universal metal impurity limits
Food and pharmaceutical safety standards within the EU are stringently controlled, thanks to the suite of EU legislation implemented. Quality, however, remains a key concern with respect to plant material outside the EU (Mukherjee et al., 2015). Our analysis of data collected from the RASFF database, concluded that the China, India and the U.S. were the top three countries from which repeated cases of metal contamination of plant products originated, between 1994 and 2019 (European Commission, 2019). This highlights the issue of quality-related problems inherent throughout the supply chain, resulting in plant material and plant-derived products which do not meet the quality standards of similar commodities produced under EU legislation (Mukherjee et al., 2015). Over time this non-uniformity may result in trading constraints between countries.

Recommendations for the implementation of an international monitoring body for the surveillance of contamination (e.g. biological, chemical or metal) have been previously...
proposed (Tripathy et al., 2015), however the cost-effectiveness of such a programme is questionable. Furthermore, some authors have expressed concern that legally imposing more stringent regulations in developing countries may not be feasible due to insufficient resources or expertise in the area (Wah et al., 2012).

As stated in the WHO “Guidelines for Assessing Quality of Herbal Medicines with reference to contaminants and residues”; it would be “desirable to harmonize limits for toxic metals and standards, as this would have many benefits including the facilitation of global trade”. To initiate change, the establishment of universal acceptance criteria and universal analytical detection methods for elemental impurities in phytopharmaceuticals needs to be agreed upon and legally enforced. The expansion of international trade further emphasises the necessity of global standards for human medicines (Rose, 2016).

### 3.5.4.3 Non-enforceable GACP guidelines

Manufacturing facilities are legally obliged to conform to GMP for the authorised manufacture of medicinal plant products. GACP guidelines, however, are non-enforceable by law for cultivators/farmers for manufacturers (Wah et al., 2012). At a minimum, mandatory plant cultivation criterion such as selection of non-hazardous soil profiles and use of irrigation water free from or within the acceptable limits of toxicologically relevant metals needs to be enforced in lieu of full GACP conformance.

### 3.5.4.4 Non-regulated wildcrafting practices

The gathering of botanicals from the wild (i.e. wildcrafting), without either the implementation of GACP guidelines or on-site expertise in botanical taxonomy, immediately compromises plant quality in terms of correct species authentication and traceability (Govindaraghavan & Sucher, 2015). Evidence suggests that wild-collected plant material is more likely to contain higher levels of contaminants than their selectively cultivated counterparts (Harris et al., 2011). For example, organic powdered turmeric root sourced from India reached the U.K. where is was found to contain 58 mg.kg⁻¹ Pb (European Commission, 2019), which is 5 times the recommended limit as per WHO guidelines (see Table 3.6).

In India alone, 90% of plant materials and their derivatives are collected from the wild (Mukherjee et al., 2015), i.e. non-regulated environments. Additionally, wildcrafting in India by untrained personnel has consequently endangered 20 – 25% of existing plant species due to over-collection of economically and medicinally valuable plant material.
biomass (Laloo et al., 2006; Mosihuzzaman and Choudhary, 2008). Ethical sourcing of plants and maintenance of biodiversity is regulated by the Convention on International Trade in Endangered Species of Wild Fauna (CITES) (Mosihuzzaman et al., 2008). CITES also provides details on prohibited or restricted botanicals and information concerning necessary export/import permits (Mosihuzzaman et al., 2008). Encouraging responsible farming will have a positive outcome on the quality of raw starting materials before reaching the manufacturing and processing stages of production while also safeguarding plant biodiversity.

3.6 Metal contaminants in supplements: an overview

As with any health-care product, the degree of regulatory control imposed depends on how the product is originally classified, which is based on any direct product health claims (Wah et al. 2012). In the U.S., herbal products are generally marketed as food supplements, and specific health claims must be authorised by the FDA (Verma, 2016). In Europe, the quality requirements for food supplements are based on food legislation, which is less stringent than medicinal or pharmaceutical legislation in terms of composition and quality requirements (Heinrich, 2015). Marketing claims on botanical ingredients in food supplements are not yet harmonised under EU law. Consequently, the regulation of therapeutic claims is monitored at a national level which, similarly to the lack of uniform metal levels in products, leads to under- and over-regulation between countries. BELFRIT, a draft decree, has been developed by joint efforts from Belgium, France, and Italy to provide a harmonised list of \([\geq 1000]\) established botanicals approved for use in food supplements. The use of health-maintaining (i.e. non-therapeutic) claims on food supplement labels or packaging requires authorisation by the European Commission through EFSA, provided satisfactory data is presented to substantiate said claim(s) (Miroddi et al., 2013). The EC and EFSA have not yet established maximum limits of elemental impurities in dietary supplements, fortified foods, novel foods or food ingredients – which highlights a further gap in legislation.

Additionally, the EC has not yet established a universal authorisation process for the use of botanicals or derived preparations (i.e. plants, algae, fungi or lichens) in dietary (food) supplements, such as St. John’s Wort, ginseng, ginkgo, garlic or echinacea (EFSA, 2017b). EFSA provides science-based safety criteria to organisations involved in the
safety assessment of botanical ingredients to establish the safe usage of botanicals or derived preparations used in dietary (food) supplements. EFSA published a “toolkit” for risk assessors and food manufacturers which provides guidance documents, reports and a ‘Compendium of botanicals’ database (EFSA, 2012b). Nevertheless, dietary food supplements containing botanical ingredients must comply with European food supplement legislation (Directive 2002/46/EC) and general European Food Law (Regulation (EC) No 178/2002) (EFSA, 2017b). The use, safety and permitted health claims of botanical ingredients in dietary supplements is currently under review by EFSA (EFSA, 2017b).

3.7 Conclusion

Metals can enter plant tissue from the environment. Plants are consecutively consumed as dietary foodstuffs and/or phytopharmaceuticals containing plants as active ingredients. Because the levels of metals within these foods or medicinal products are not routinely analysed, it is difficult to know if we are subjecting ourselves to any form of acute or chronic metal exposure – symptomatic or asymptomatic. Diet has been shown to be a risk factor for human exposure to heavy metals, whether as a single dose or through accumulation. Above safe threshold concentrations, metals can cause a range of deleterious adverse effects in humans. Stringent regulatory controls are currently in place in European Member States to safeguard the general population against exposure to hazardous heavy metals in food. While regulatory measures are enforced, there are apparent pitfalls since current dietary exposure data published by scientific expert groups at the EFSA acknowledge that the risks of adverse health effects from certain metals in food cannot be excluded. The authors suggest that maximum limits for frequently consumed foodstuffs should be established where possible to safeguard public health. Furthermore, advocating alternate patterns of food consumption by introducing variety and implementing safe kitchen preparation techniques are recommended to reduce metal load in food. Regarding phytopharmaceuticals, the adapted ICH Q3D guidelines published by the EMA define limits for a total of twenty-four elements however these limits are not currently applicable to phytopharmaceuticals. In contrast, compendial (Ph. Eur.) limits for herbal products exist for only Cd, Hg and Pb. Two possible solutions to consider include extending the...
application of ICH Q3D guidelines to phytopharmaceuticals, or alternatively, establishing a defined set of general permissible elemental limits for a greater suite of toxicologically significant metals applicable to herbal products. Harmonisation of quality requirements for these products is a necessity, particularly in the context of international trade, to reduce human exposure to potentially dangerous metal impurities.

**Declaration of interest**

The authors declare that there is no conflict of interest in this work.

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Chapter IV

Dietary exposure assessment of inorganic metal contaminants relevant to Irish consumers in naturalised Irish (wild, cultivated) and commercial herbal samples using high-resolution inductively coupled plasma sector field mass spectrometry (HR-ICP-SFMS)

Manuscript based on this chapter in preparation for publication.
Abstract

High-resolution inductively coupled plasma sector-field mass spectrometry (HR-ICP-SFMS) was used for the multi-elemental analysis (metallomic profiling) of microwave acid-digested raw herbal material, *Inula helenium* L. (elecampane), for the first time. Statistical analysis revealed a significant difference in element distribution between flowers – leaves (Be, Li, Ba, Cd, Bi), root – leaves (Mo, Sn, Co, Hg) and flowers – root (Ba and Tl) within naturalised elecampane. Maximum limits (MLs) for metal contaminants in herbal material/drugs are limited and exist only for Cd, Hg and Pb. In food matrices however, toxicological health-based guidance values (HBGVs) are available for a greater suite of elements, which are applicable to plant-derived foods too.

A dietary risk assessment was performed *in silico* to determine the level of risk to Irish consumers by comparing levels of exposure to the available toxicological references. The Rapid Assessment of Contaminant Exposure (RACE) tool developed by the European Food Safety Authority (EFSA) is a useful platform to rapidly assess risk and contextualise quantitative data. An analytical value exceeding regulatory limits could be of concern for one food type, but not another since dietary exposure is modelled on consumer intake patterns specific to the food type. Results showed that chronic exposure to lead (Pb) at a maximum quantified concentration of 4617.42 µg.kg⁻¹ in edible plant material is a potential risk to adult consumers in Ireland (18-65 y), at an estimated mean and 95th percentile exposure of 0.049 and 0.189 µg.kg⁻¹ per kg body weight (BW) per day, respectively. Several samples exceeded maximum limits for Cd applicable to food and herbal material as per the European food and pharmacopeial regulatory criterion, respectively. Estimated exposure at the highest Cd concentration (1285.97 µg.kg⁻¹), however, was deemed of no risk to consumers. Dietary exposure to the remaining elements (Li, Be, Mo, Sn, Ba, Tl, Hg, V, Cr, Co, Ni, Cu) were well below the toxicological references and are therefore of negligible concern to Irish consumers, considering the intake level and type of food commodity analysed. Platinum (Pt) and bismuth (Bi) are currently excluded on the list of available substances on the RACE platform, their addition in forthcoming versions is advised, considering increased environmental prevalence, due to technological advancements and subsequent anthropogenic-derived pollution.

**Key words:** ICP-SFMS; metals; herbal; plant-derived food; RACE exposure assessment
4.1 Introduction

*Inula helenium* L. (elecampane) is a perennial herb belonging to the Asteraceae family which demonstrates a diverse range of prospective high-value nutritional, environmental and biotechnological applications. Ethnobotanical indications for the medicinal use of elecampane roots are mostly targeted towards respiratory and digestive ailments (Seca et al. 2014). Current bioactivity research has explored the antimicrobial, anthelmintic, anti-inflammatory, and anti-cancer properties of isolated phytochemicals (Wang et al. 2014; Seca et al. 2015; Tavares and Seca 2019). Among other tubers such as chicory, dahlia, yacón and Jerusalem artichoke; elecampane is an excellent source of tuber- or root-derived inulin, a naturally-occurring reserve carbohydrate gaining popularity as a sustainable, economical and abundant functional food or novel ingredient, coffee substitute, biorefinery source and vaccine adjuvant (Chi et al. 2011; Kumar and Tummala 2013; Li et al. 2013; Wang et al. 2016; Hughes et al. 2017). The derived sugars, inulooligosaccharides (IOS) and fructo-oligosaccharides, are predominantly utilised as functional sweeteners and soluble dietary fibre with prebiotic and immunomodulating properties (Cho et al. 2001; Wang et al. 2016). The bifidogenic effect of both inulin and its oligofructose derivatives on the gut microflora has been well-characterised (Meyer and Stasse-Wolthuis 2009). Other food applications of inulin and its derivatives include the production of ultra-high fructose syrup and utilisation as a texture modifier in fat-reduced foods (Niness 1999). Inulin-type fructans resist human digestion based on the configuration of the β-linkages between the fructose monomers (Li et al. 2013) contributing to its popularity as a functional health food. Few studies, however, have yet explored elecampane as a commercial source of inulin similar to chicory (Bell and Palmer 1952; Petkova et al. 2015). Plants are multi-purpose organisms, and elecampane is clearly no exception.

Medicinal and aromatic plants are abundantly utilised as raw materials or ingredients in the pharmaceutical, nutraceutical, cosmetic, agricultural and food industries. Terrestrial plants acquire abiotic elements from the surrounding environment, seventeen of which are considered vital for optimal plant nutrition (Marschner 1995; Broadley et al. 2001). Carbon (C), hydrogen (H) and oxygen (O) are the most abundant elements in plants, followed by the macronutrients calcium (Ca), magnesium (Mg), potassium (K), nitrogen (N), phosphorous (P) and sulphur (S), the latter of which are found in most plants at concentrations ≤0.1% (dry weight). The essential micronutrients boron (B), copper (Cu),
chlorine (Cl), iron (Fe), molybdenum (Mo), manganese (Mn), nickel (Ni) and zinc (Zn) are found at concentrations ≤0.01% (dry weight) (Singh et al. 2017). Mineral elements are implicated in the biosynthesis and regulation of metabolites of medicinal or aromatic importance (Singh et al. 2017). By influencing cellular metabolism, plant nutrition ultimately governs overall health, biomass yield, and the range and concentration of metabolites produced thus directly impacting both the commercial and beneficial value of a plant in terms of medicinal efficacy or aromatic flavour composition (Singh et al. 2017). Plant species evolution can occur independently to taxonomy (i.e. Genus) (Broadley et al. 2001), resulting in the existence of, for example, metallophyte, halophyte and calciotropic phenotypic traits. For this reason, the use of plants in eco-toxicological endeavours (i.e. phytoremediation) has been gaining approval worldwide (Lee 2013).

Adequate nutrition is synonymous to optimal health – for both human and plants alike. The safety of plant-derived foods is not only determined or influenced by the phytochemical composition of a plant, but also the levels of residues or contaminants present in raw plant material and subsequent plant-derived products (European Commission 2017). Safety issues related to product contamination, adulteration and illicit addition exists (Di Lorenzo et al. 2018). The presence of non-essential or excess metal and metalloid trace elements, referred to as metals herein, can occur as unsolicited contaminants in food plants and products derived thereof (Kenny et al. 2020) which subsequently could be a potential risk to both the plant and the end consumer. Furthermore, the presence of metals in planta may in turn influence the bioactivity of medicinal plants, considering the effects of certain metals in metabolic pathways (Singh et al. 2017). Minimizing exposure of humans and animals to acute and chronic levels of bioaccumulated metal contaminants in edible plant food products is therefore necessary to ensure safe, high-quality raw material for commercial purposes (Kenny et al. 2020).

Dietary exposure assessment integrates the analytical quantification or concentration of a substance present in a single food commodity with the quantity of the commodity consumed in a specific population. Rapid Assessment of Contaminant Exposure (RACE) is a risk evaluation tool established by the European Food Safety Authority (EFSA) which estimates acute and/or chronic exposure of European population groups to chemical contaminants in a single food commodity. The estimated exposure results are compared with relevant health-based guidance values (HBGVs) or toxicological reference points, where available. This process facilitates the risk-based classification of chemical
contaminants and supports the decision to notify analytical results of legitimate concern or non-compliance in the European Rapid Alert System for Food and Feed (RASFF) (EFSA et al. 2019).

This study set out to quantify for the first time, the concentration of the selected elements: lithium (7Li), beryllium (9Be), molybdenum (95Mo), cadmium (111Cd), tin (118Sn), barium (137Ba), platinum (195Pt), gold (197Au), mercury (202Hg), thallium (205Tl), lead (208Pb), bismuth (209Bi), magnesium (24Mg), aluminium (27Al), titanium (47Ti), vanadium (51V), chromium (52Cr), manganese (55Mn), iron (56Fe), cobalt (59Co), nickel (60Ni) and copper (63Cu) in naturalised Irish (wild, cultivated) and commercial herbal samples of elecampane using a validated HR-ICP-SFMS method (Ring et al., 2021). Furthermore, the RACE tool is implemented in this study to translate analytical findings into a quantifiable level of risk, which will provide an insight into the estimated intake of metal contaminants originating from raw herbal material in the Irish (adult) diet.

4.2 Materials and methods

4.2.1 Reagents and materials

Ultrapure milli-Q water (15.0 MΩ·cm); trace-metal grade nitric acid (HNO₃) (PlasmaPure, 67 – 69% v/v, SCP Science); Tune-Up solution (Thermo Scientific, USA; 1 µg.L⁻¹). Multi-elemental standard solutions including lithium (7Li), beryllium (9Be), molybdenum (95Mo), cadmium (111Cd), tin (118Sn), barium (137Ba), platinum (195Pt), gold (197Au), mercury (202Hg), thallium (205Tl), lead (208Pb), bismuth (209Bi), magnesium (24Mg), aluminium (27Al), titanium (47Ti), vanadium (51V), chromium (52Cr), manganese (55Mn), iron (56Fe), cobalt (59Co), nickel (60Ni) and copper (63Cu) (Agilent, USA) were used in this study. The internal standards (ISTDs) used in this study were gallium (71Ga), scandium (45Sc), rhodium (103Rh), iridium (193Ir) and again these were certified standards traceable to NIST reference materials, sourced from SCP Science. Polymethylpentene (PMP) beakers, volumetric flasks, graduated cylinders, and pipettes were sourced from VWR International Ltd. (Blanchardstown, Dublin 15, Ireland).

4.2.2 Instrumentation

The MARS-6™ microwave-assisted digestion system was used for sample preparation (CEM Corporation, USA). The Thermo Scientific Element 2™ ICP-SFMS (Thermo
Scientific, USA) was coupled with an ESI autosampler and was used for multi-elemental analysis (metallomic profiling) of samples.

4.2.3 Herbal plant material (IN samples)

Naturalised cultivated *I. helenium* samples (IN_01-24) were kindly donated by several Irish enterprises. All wild and cultivated samples (i.e. root, leaf, stem, flowers/inflorescence) were collected in August, during the flowering stage of growth. Commercial samples were all sourced online from U.K. registered suppliers (IN_25-27), and their source location was not specified by the supplier and therefore classified as “unknown” (See Table 4.1).

Table 4.1 List of naturalised and commercial *I. helenium* L. (elecampane) samples analysed:

<table>
<thead>
<tr>
<th>Sample I.D.</th>
<th>Source/ Location</th>
<th>Plant part(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IN_01</td>
<td>Sligo, Ireland</td>
<td>Root (F)</td>
</tr>
<tr>
<td>IN_02</td>
<td>Sligo, Ireland</td>
<td>Leaves (F)</td>
</tr>
<tr>
<td>IN_03</td>
<td>Cork, Ireland</td>
<td>Root (D)</td>
</tr>
<tr>
<td>IN_04</td>
<td>Cork, Ireland</td>
<td>Leaves (F)</td>
</tr>
<tr>
<td>IN_05</td>
<td>Dublin, Ireland</td>
<td>Flower, stem (F)</td>
</tr>
<tr>
<td>IN_06</td>
<td>Dublin, Ireland</td>
<td>Leaves (F)</td>
</tr>
<tr>
<td>IN_07</td>
<td>Clare, Ireland</td>
<td>Roots (F)</td>
</tr>
<tr>
<td>IN_08</td>
<td>Clare, Ireland</td>
<td>Leaves (F)</td>
</tr>
<tr>
<td>IN_09</td>
<td>Clare, Ireland</td>
<td>Flower, stem (F)</td>
</tr>
<tr>
<td>IN_10</td>
<td>Down, Northern Ireland</td>
<td>Root (F)</td>
</tr>
<tr>
<td>IN_11</td>
<td>Down, Northern Ireland</td>
<td>Leaves (F)</td>
</tr>
<tr>
<td>IN_12</td>
<td>Down, Northern Ireland</td>
<td>Flower, stem (F)</td>
</tr>
<tr>
<td>IN_13</td>
<td>Cork, Ireland</td>
<td>Root (F)</td>
</tr>
<tr>
<td>IN_14</td>
<td>Cork, Ireland</td>
<td>Leaves (F)</td>
</tr>
<tr>
<td>IN_15</td>
<td>Cork, Ireland</td>
<td>Flower, stem (F)</td>
</tr>
<tr>
<td>IN_16</td>
<td>Leitrim, Ireland</td>
<td>Root (F)</td>
</tr>
<tr>
<td>IN_17</td>
<td>Leitrim, Ireland</td>
<td>Leaves (F)</td>
</tr>
<tr>
<td>IN_18</td>
<td>Leitrim, Ireland</td>
<td>Flower, stem (F)</td>
</tr>
<tr>
<td>IN_19</td>
<td>Cork, Ireland</td>
<td>Root (F)</td>
</tr>
<tr>
<td>IN_20</td>
<td>Cork, Ireland</td>
<td>Leaves (F)</td>
</tr>
<tr>
<td>IN_21</td>
<td>Cork, Ireland</td>
<td>Flower, stem (F)</td>
</tr>
<tr>
<td>IN_22</td>
<td>Cork, Ireland</td>
<td>Root (F)</td>
</tr>
<tr>
<td>IN_23</td>
<td>Cork, Ireland</td>
<td>Leaves (F)</td>
</tr>
<tr>
<td>IN_24</td>
<td>Cork, Ireland</td>
<td>Flower, stem (F)</td>
</tr>
<tr>
<td>IN_25</td>
<td>Unknown (Commercial)</td>
<td>Whole plant (D)</td>
</tr>
<tr>
<td>IN_26</td>
<td>Unknown (Commercial)</td>
<td>Whole plant (D)</td>
</tr>
<tr>
<td>IN_27</td>
<td>Unknown (Commercial)</td>
<td>Root (D)</td>
</tr>
</tbody>
</table>

Note: F = collected fresh; D = obtained dried

4.2.4 Sample preparation

Fresh plant samples (Figure 4.1) were washed with ultrapure deionised water and categorised into root, stem and leaves, flowers, and seeds. The fresh plant material (10.0
15.0 g) was oven-dried at 70°C for 24-48 hours (Fu et al. 2018). The dried samples were ground to a fine powder, sieved (US mesh size 100), and stored in airtight sterile plastic containers at room temperature until required for analysis.

4.2.4.1 Microwave-assisted acid digestion

4.2.4.2 Vessel preparation
All experimental MARS Xpress vessels were rinsed in triplicate with deionised water before undergoing the Mars6 Xpress cleaning cycle. Ten millilitres of 5% HNO₃ (v/v) was added to each vessel before initiating the pre-programmed OneTouch “Express Clean” programme (Stages: 1; Power: 100-1800; Ramp Time: 15 mins; Hold Time: 10 mins; Temp.: 150°C; Temp. Guard: Off) (CEM, 2020). On completion of the cleaning cycle, vessels were again rinsed in triplicate with clean deionised water before being allowed to air dry.

4.2.4.3 Pre-digestion of herbal samples
Samples (0.5 g; dry weight) were accurately weighed and transferred into a pre-cleaned MARS Xpress digestion vessel (Material: TFM; maximum vessel volume: 55 mL; operation pressure and temperature: medium). For the pre-digestion step, concentrated trace-metal grade HNO₃ (v/v; 67-69%; 10 mL) was added to the vessel and gently swirled before securing the inner lid and allowed to stand for 15 minutes at room temperature.
Any gas produced during the pre-digestion step was manually released before securing the vessel and placing it into the MARS-6 carousel.

4.2.4.4 Programmed digestion of herbal samples
The pre-programmed CEM OneTouch “Plant Material” method was selected (Stage: 01; Power: 1030 – 1800 W; Ramp time: 20 – 25: 00 mm/ss; Hold time: 10:00 mm/ss; Pressure: 800 psi; Temperature: 200°C; Temp. Guard: off; Stirring: off) (CEM, 2020). After cooling, the digestates were transferred to sterile 15 mL PMP sample tubes. The tubes were gently inverted and vented multiple times to release gaseous build-up before storage at -24°C. The above steps were repeated for all Inula samples (IN_01-27).

4.2.5 Validated ICP-SFMS Multi-Elemental Analysis (Metallomic Profiling)

4.2.5.1 Multi-elemental standard and control preparation
Calibration standards (in the range of 0.001 – 50 µg.L⁻¹) and controls were prepared as described in Ring et al. (2021). To prepare the matrix-spiked controls, sample IN_10 was diluted 1:10,000 using 2% HNO₃ before being spiked with standard and ISTD stock solutions. The final concentrations of these controls were as follows: A (0.2 µg.L⁻¹), B (1 µg.L⁻¹), C (5 µg.L⁻¹), D (15 µg.L⁻¹) and E (40 µg.L⁻¹).

4.2.5.2 Instrumentational analysis
A volume of the initial digested sample (5.0 mL) was diluted to 25 mL in 2% HNO₃ and spiked with the internal standard (to a final ISTD concentration of 2.5 µg.L⁻¹). The samples were placed on the ESI autosampler rack, and analysis of all samples by ICP-SFMS was performed as per the procedure described in Ring et al. (2021).

Heavy metals and trace elements present in the digested herbal samples were analysed using a high-resolution inductively coupled plasma sector-field mass spectrometer (ICP-SFMS); (Thermo Scientific™ Element 2™ High-Resolution ICP-MS). Certified calibration standards (traceable to NIST reference materials), controls (calibration verification standards) and blanks were run prior to sample injections. The diluted sample results determined at the instrument were expressed in parts per billion (ppb = µg.L⁻¹) and the final concentrations were obtained by calculating back to the original solid sample that was initially weighed out (µg.kg⁻¹). The following element isotopes were quantified
in this study as previously described in Ring et al. (2021): $^7$Li, $^9$Be, $^{95}$Mo, $^{111}$Cd, $^{118}$Sn, $^{137}$Ba, $^{195}$Pt, $^{197}$Au, $^{202}$Hg, $^{205}$Tl, $^{208}$Pb, $^{209}$Bi, $^{24}$Mg, $^{27}$Al, $^{47}$Ti, $^{51}$V, $^{52}$Cr, $^{55}$Mn, $^{56}$Fe, $^{59}$Co, $^{60}$Ni and $^{63}$Cu. Matrix-spiked controls were analysed at five levels spanning the calibration range (0.2, 1, 5 15 and 40 µg.L$^{-1}$) after every 20 samples. Calibration readback QCs (made up in 2% HNO$_3$) were also ran at the end of the analytical sequence to verify the calibration line and instrument performance (Ring et al., 2021).

4.2.5.3 Calibration and quality assurance

In Table 4.2, a summary of the calibrations for each element of interest is presented, as well as the method limit of detection/limit of quantification (LOD/LOQ) for each analyte. All elements analysed achieved acceptable linearity ($R^2 \geq 0.995$) across their respective working ranges. These calibrations were used to interpolate the concentrations of samples and matrix-spiked controls.

Table 4.2 Summary of the calibrations of each element:

<table>
<thead>
<tr>
<th>Analyte Isotope</th>
<th>ISTD</th>
<th>Equation of the Line</th>
<th>Linear Range (µg.L$^{-1}$)</th>
<th>No. of calibration points</th>
<th>Correlation Coefficient ($R^2$)</th>
<th>LOD (ng.L$^{-1}$)</th>
<th>LOQ (ng.L$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^7$Li</td>
<td>$^7{\text{Ga}}$</td>
<td>$y = 661.3x + 17.879$</td>
<td>0.001 - 35</td>
<td>11</td>
<td>$R^2 = 0.9999$</td>
<td>&lt;1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>$^9$Be</td>
<td>$^{103}$Rh</td>
<td>$y = 50.303x - 1.1758$</td>
<td>0.001 - 50</td>
<td>10</td>
<td>$R^2 = 0.9999$</td>
<td>0.36</td>
<td>1.18</td>
</tr>
<tr>
<td>$^{95}$Mo</td>
<td>$^{103}$Rh</td>
<td>$y = 125.66x - 3.494$</td>
<td>0.005 - 50</td>
<td>10</td>
<td>$R^2 = 0.9999$</td>
<td>1.64</td>
<td>5.40</td>
</tr>
<tr>
<td>$^{111}$Cd</td>
<td>$^{193}$Ir</td>
<td>$y = 0.0393x - 0.0011$</td>
<td>0.001 - 50</td>
<td>12</td>
<td>$R^2 = 0.9999$</td>
<td>0.28</td>
<td>0.94</td>
</tr>
<tr>
<td>$^{118}$Sn</td>
<td>$^{103}$Rh</td>
<td>$y = 218.16x - 5.0398$</td>
<td>0.005 - 50</td>
<td>14</td>
<td>$R^2 = 0.9999$</td>
<td>1.64</td>
<td>5.42</td>
</tr>
<tr>
<td>$^{137}$Ba</td>
<td>$^{103}$Rh</td>
<td>$y = 136.42x + 2.7994$</td>
<td>0.010 - 50</td>
<td>11</td>
<td>$R^2 = 0.9999$</td>
<td>1.96</td>
<td>6.46</td>
</tr>
<tr>
<td>$^{195}$Pt</td>
<td>$^{193}$Ir</td>
<td>$y = 0.1414x + 0.0016$</td>
<td>0.005 - 25</td>
<td>11</td>
<td>$R^2 = 0.9999$</td>
<td>0.43</td>
<td>1.42</td>
</tr>
<tr>
<td>$^{197}$Au</td>
<td>$^{193}$Ir</td>
<td>$y = 385.37x - 27.221$</td>
<td>0.025 - 50</td>
<td>8</td>
<td>$R^2 = 0.9999$</td>
<td>11.52</td>
<td>25.00</td>
</tr>
<tr>
<td>$^{202}$Hg</td>
<td>$^{193}$Ir</td>
<td>$y = 102.4x - 2.6221$</td>
<td>0.010 - 35</td>
<td>11</td>
<td>$R^2 = 0.9997$</td>
<td>11.79</td>
<td>38.91</td>
</tr>
<tr>
<td>$^{205}$Tl</td>
<td>$^{71}$Ga</td>
<td>$y = 1.19x - 0.0274$</td>
<td>0.001 - 35</td>
<td>11</td>
<td>$R^2 = 0.9999$</td>
<td>0.02</td>
<td>0.07</td>
</tr>
<tr>
<td>$^{208}$Pb</td>
<td>$^{103}$Rh</td>
<td>$y = 613.49x + 30.858$</td>
<td>0.005 - 50</td>
<td>8</td>
<td>$R^2 = 0.9999$</td>
<td>2.52</td>
<td>5.00</td>
</tr>
<tr>
<td>$^{209}$Bi</td>
<td>$^{103}$Rh</td>
<td>$y = 942.11x - 16.505$</td>
<td>0.001 - 50</td>
<td>12</td>
<td>$R^2 = 0.9999$</td>
<td>0.39</td>
<td>1.30</td>
</tr>
<tr>
<td>$^{24}$Mg</td>
<td>$^{45}$Sc</td>
<td>$y = 0.2058x + 0.3627$</td>
<td>0.250 - 50</td>
<td>7</td>
<td>$R^2 = 0.9993$</td>
<td>25.00</td>
<td>100.00</td>
</tr>
<tr>
<td>$^{27}$Al</td>
<td>$^{45}$Sc</td>
<td>$y = 0.285x + 0.6584$</td>
<td>0.025 - 50</td>
<td>7</td>
<td>$R^2 = 0.9998$</td>
<td>10.00</td>
<td>25.00</td>
</tr>
<tr>
<td>$^{47}$Ti</td>
<td>$^{45}$Sc</td>
<td>$y = 0.0262x + 0.0015$</td>
<td>0.100 - 50</td>
<td>7</td>
<td>$R^2 = 0.9999$</td>
<td>14.49</td>
<td>47.83</td>
</tr>
</tbody>
</table>

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The validity of results was assured through the analysis of matrix-spiked controls at five concentration levels spanning the entire calibration range of the method (0.2 µg.L⁻¹, 1 µg.L⁻¹, 5 µg.L⁻¹, 15 µg.L⁻¹ and 40 µg.L⁻¹). Percent recovery (% recovery) was used as the parameter to evaluate calibration/instrument performance, with an acceptance tolerance of 100 ± 25% recovery (i.e. 75 – 125% of the assigned value for each control concentration). As can be seen in Table 4.3, all elements achieved acceptable recoveries across each concentration level examined. The acceptable performance of the controls indicated that the calibration was fit for purpose and could be used to accurately determine element concentrations in the samples (IN_1 to IN_27).

Table 4.3 Average % recoveries for matrix-spiked controls analysed by ICP-SFMS (n = 5)

<table>
<thead>
<tr>
<th>Element(s)</th>
<th>0.2 µg.L⁻¹</th>
<th>1 µg.L⁻¹</th>
<th>5 µg.L⁻¹</th>
<th>15 µg.L⁻¹</th>
<th>40 µg.L⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lithium (7Li)</td>
<td>116.5</td>
<td>105.3</td>
<td>110.4</td>
<td>107.8</td>
<td>105.8</td>
</tr>
<tr>
<td>Beryllium (9Be)</td>
<td>116.6</td>
<td>103.6</td>
<td>106.0</td>
<td>107.0</td>
<td>108.6</td>
</tr>
<tr>
<td>Molybdenum (95Mo)</td>
<td>113.0</td>
<td>89.3</td>
<td>88.9</td>
<td>91.8</td>
<td>97.1</td>
</tr>
<tr>
<td>Cadmium (111Cd)</td>
<td>114.2</td>
<td>96.4</td>
<td>101.1</td>
<td>100.4</td>
<td>97.4</td>
</tr>
<tr>
<td>Tin (118Sn)</td>
<td>105.7</td>
<td>95.2</td>
<td>94.2</td>
<td>93.7</td>
<td>94.9</td>
</tr>
<tr>
<td>Barium (137Ba)</td>
<td>109.2</td>
<td>92.4</td>
<td>88.9</td>
<td>90.6</td>
<td>93.0</td>
</tr>
<tr>
<td>Platinum (195Pt)</td>
<td>106.7</td>
<td>98.3</td>
<td>101.7</td>
<td>96.9</td>
<td>95.7</td>
</tr>
<tr>
<td>Mercury (202Hg)</td>
<td>121.0</td>
<td>99.1</td>
<td>103.6</td>
<td>96.9</td>
<td>92.5</td>
</tr>
<tr>
<td>Thallium (205Tl)</td>
<td>94.5</td>
<td>85.1</td>
<td>80.4</td>
<td>79.9</td>
<td>80.8</td>
</tr>
<tr>
<td>Element</td>
<td>77.8</td>
<td>90.3</td>
<td>87.2</td>
<td>85.0</td>
<td>87.9</td>
</tr>
<tr>
<td>------------------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>Lead ((_{208})Pb)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bismuth ((_{209})Bi)</td>
<td></td>
<td></td>
<td>87.4</td>
<td>85.1</td>
<td>87.6</td>
</tr>
<tr>
<td>Titanium ((_{47})Ti)</td>
<td></td>
<td>92.2</td>
<td>89.3</td>
<td>89.9</td>
<td></td>
</tr>
<tr>
<td>Vanadium ((_{51})V)</td>
<td>109.1</td>
<td></td>
<td>90.7</td>
<td>89.5</td>
<td></td>
</tr>
<tr>
<td>Chromium ((_{52})Cr)</td>
<td>96.0</td>
<td>90.4</td>
<td>89.9</td>
<td>89.4</td>
<td>90.9</td>
</tr>
<tr>
<td>Cobalt ((_{59})Co)</td>
<td>104.2</td>
<td>96.4</td>
<td>91.0</td>
<td>89.7</td>
<td>92.8</td>
</tr>
<tr>
<td>Nickel ((_{58})Ni(^+))</td>
<td>100.5</td>
<td>93.1</td>
<td>89.1</td>
<td>91.6</td>
<td></td>
</tr>
<tr>
<td>Copper ((_{63})Cu(^+))</td>
<td>106.7</td>
<td>92.9</td>
<td>90.8</td>
<td>92.2</td>
<td></td>
</tr>
</tbody>
</table>

Note: \(^*\)Nickel and copper have LOQs of 0.25 \(\mu g.L^{-1}\), which is above the lowest (0.2 \(\mu g.L^{-1}\)) control level, hence they have not been assessed at the 0.2 \(\mu g.L^{-1}\) level.

The concentrations of elements in each sample were determined by ICP-SFMS and a summary of the results (expressed in \(\mu g.kg^{-1}\)) can be found in Table 4.8. In some samples, element concentrations were found to be outside the instrument calibration range (Mo, Ba, Tl, Pb, Ti and Cu), and because the samples could not be further diluted and repeated, these values are reported as ‘NR’ in the table. In the cases of Au, Al, Fe, Mg and Mn, all samples tested yielded concentrations that lay outside the calibration range and because further dilution was not possible, these elements have been removed from the table entirely. Where sample concentrations were below the LOQ, final concentrations have been reported as <LOQ.

4.2.6 Non-Parametric Statistical Analysis

As the dataset contained repeated samples (i.e. roots, leaves and flowers were analysed from the same plant) for a number of plants, it was evident that a repeated measures procedure was necessary. The assumption of normality was not satisfied for a parametric repeated measure analysis of variance (ANOVA) to be performed therefore a repeated samples Friedman two-way ANOVA using ranks was conducted.

Using a level of significance of 0.05, it was determined whether the distribution of some elements (Li, Be, Mo, Cd, Sn, Ba, Pt, Hg, Tl, Pb, Bi, Ti, V, Cr, Co, Ni and Cu) between

\(^1\) Principal Component Analysis (PCA) will be considered in resulting publications,
plant parts (i.e. root, leaf, flower) were significantly different. If level of significance is >0.05, there is no significant difference in element distribution across the plant parts. If level of significance is <0.05, there appears to be a difference between element distribution across the root, leaves and flowers. A pairwise comparison was then performed to determine where the difference is (i.e. between root and leaves, root, and flowers, or leaves and flowers). These significance values were then adjusted by the Bonferroni correction for multiple tests, as presented in Table 4.4 below.

All analyses were performed using IBM SPSS Statistics 26. Heavy metals and trace elements that were unable to be quantified following ICP-SFMS (Al, Au, Fe, Mg, Mn), and the commercial samples (\( n = \leq 3 \) for commercial root and whole samples) were excluded from statistical assessment.

**Table 4.4 Summary of the statistical outputs:**

<table>
<thead>
<tr>
<th>Element(s)</th>
<th>Significance Value(s)</th>
<th>Pairwise Comparison (Adjusted Significance Value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beryllium (Be)</td>
<td>0.016</td>
<td>Flowers-Leaves (0.012)</td>
</tr>
<tr>
<td>Lithium (Li)</td>
<td>0.030</td>
<td>Flowers – Leaves (0.028)</td>
</tr>
<tr>
<td>Molybdenum (Mo)</td>
<td>0.009</td>
<td>Root – Leaves (0.012)</td>
</tr>
<tr>
<td>Barium (Ba)</td>
<td>0.005</td>
<td>Flowers – Root (0.028)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Flowers – Leaves (0.028)</td>
</tr>
<tr>
<td>Cadmium (Cd)</td>
<td>0.002</td>
<td>Flowers – Leaves (0.002)</td>
</tr>
<tr>
<td>Platinum (Pt)</td>
<td>0.846</td>
<td>-</td>
</tr>
<tr>
<td>Tin (Sn)</td>
<td>0.009</td>
<td>Root – Leaves (0.012)</td>
</tr>
<tr>
<td>Bismuth (Bi)</td>
<td>0.009</td>
<td>Flowers – Leaves (0.012)</td>
</tr>
<tr>
<td>Chromium (Cr)</td>
<td>0.311</td>
<td>-</td>
</tr>
<tr>
<td>Cobalt (Co)</td>
<td>0.030</td>
<td>Root – Leaves (0.028)</td>
</tr>
<tr>
<td>Copper (Cu)</td>
<td>0.735</td>
<td>-</td>
</tr>
<tr>
<td>Mercury (Hg)</td>
<td>0.030</td>
<td>Root – Leaves (0.028)</td>
</tr>
<tr>
<td>Lead (Pb)</td>
<td>0.070</td>
<td>-</td>
</tr>
<tr>
<td>Nickel (Ni)</td>
<td>0.115</td>
<td>-</td>
</tr>
<tr>
<td>Thallium (Tl)</td>
<td>0.006</td>
<td>Flowers – Root (0.004)</td>
</tr>
<tr>
<td>Titanium (Ti)</td>
<td>0.513</td>
<td>-</td>
</tr>
<tr>
<td>Vanadium (V)</td>
<td>0.223</td>
<td>-</td>
</tr>
</tbody>
</table>

**4.2.7 Comparison of Cd, Hg, Pb levels quantified versus maximum levels (MLs) outlined in national and European food and pharmacopeial regulations**

Limited ML’s exist for metal contaminants in plant-derived food and phytopharmaceuticals, and those that are available can differ between countries and regions (Kenny et al. 2020). Table 4.5 summarises the ML’s for the following metal
contaminants in food and herbal material/drugs: As, Cd, Hg, Pb, and Sn. Figures 4.3 – 4.5 represent these ML’s, where applicable, within the context of our data.

Table 4.5 7Maximum levels (MLs) of metal contamination in plant-derived food and phytopharmaceuticals according to European and Irish regulations currently in force:

<table>
<thead>
<tr>
<th>Element</th>
<th>Maximum Level(s)</th>
<th>Food</th>
<th>Herbal material/Herbal Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>European Legislation</td>
<td>National Legislation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(mg.kg⁻¹; wet weight)</td>
<td>(Ireland)</td>
<td></td>
</tr>
<tr>
<td>As</td>
<td>Rice only*</td>
<td>5 mg.kg⁻¹</td>
<td>N/A</td>
</tr>
<tr>
<td>Cd</td>
<td>0.20</td>
<td>N/A</td>
<td>1.0 mg.kg⁻¹</td>
</tr>
<tr>
<td>Hg</td>
<td>0.10</td>
<td>N/A</td>
<td>0.1 mg.kg⁻¹</td>
</tr>
<tr>
<td>Pb</td>
<td>Food</td>
<td>0.10 – 0.30**</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Food Supplements</td>
<td>3.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Herbal material/Herbal Drugs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sn</td>
<td>N/A***</td>
<td>200 µg.kg⁻¹</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Note(s): *Limits applicable to rice and rice-products only. **Applicable to vegetables, leafy Brassica, leafy veg., and salsify only – excludes fresh herbs, seaweed, fruiting veg. and certain fungi as per Reg. (EU) No. 2015/1005. ***Inorganic Sn ML is applicable to tinned food only and therefore not applicable to raw plant material.

4.2.8 Dietary exposure risk assessment and evaluation in plant food matrices

4.2.8.1 Food classification for exposure assessment

The EFSA’s standardised food classification and description system - FoodEx2 (Food classification and description system for Exposure assessment) – and MTX exposure hierarchy are catalogues used to precisely select the most relevant food category/item to include in risk assessment models, i.e. the closest matrix within which the analyte was detected (EFSA 2015; EFSA et al. 2019). Raw plant material and/or herbal ingredients are complex because they can fit into several categorises (See Table 4.6) listed in the FoodEx2 catalogue. The choice of category will likely influence the resulting exposure estimates. Since there was no specific food category for Inula species, a higher level (L2) FoodEx2 category was chosen to model exposure risk. For this current study, the selected category from the RASFF exposure hierarchy input for all exposure analyses was ‘herbs and edible flowers’.
Table 4.6 FoodEx2 classification/descriptors applicable to plant-derived food and products thereof:

<table>
<thead>
<tr>
<th>Level 1 Classification</th>
<th>Level 2 – 3 Group(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vegetables and vegetable products (not specified)*</td>
<td>Herbs and edible flowers*</td>
</tr>
<tr>
<td>Legumes, nuts, oils, seeds and spices</td>
<td>Spices:</td>
</tr>
<tr>
<td></td>
<td>• Dried herbs</td>
</tr>
<tr>
<td></td>
<td>• Roots and rhizomes</td>
</tr>
<tr>
<td></td>
<td>• Flowers [or parts]</td>
</tr>
<tr>
<td>Fruit and vegetable juices and nectars (including concentrates)</td>
<td>Extracts of plant origin</td>
</tr>
<tr>
<td>Coffee, cocoa, tea and infusions</td>
<td>Ingredients for coffee, tea, and herbal infusions</td>
</tr>
</tbody>
</table>

Note: *Used for all analyses in this study (see Step IV; Section 4.2.8.3).

4.2.8.2 Sources of toxicological information

Toxicological information is necessary to support the construction of a risk-assessment decision tree (See Figure 4.2). Toxicological reference points (RF) and reference values (RV) were sourced from the EFSA OpenFoodTox database (https://www.efsa.europa.eu/en/microstrategy/openfoodtox - ‘hazard characterisation’) and recent EFSA Scientific Opinions. Other relevant scientific literature was sourced (via PubMed, Science Direct, Google Scholar) where proposed or provisional reference points/values were available. Information on the genotoxicity status of the analytical compounds (i.e. heavy metals, elements, metalloids) were also sourced from the OpenFoodTox database. Table 4.7 outlines the most current toxicological RP/RV’s for each element analysed in this current study, where derived.
Table 4.7 Current HBGV’s and toxicological RP/RV’s applicable in the risk assessment of metal contaminants in food matrices, and reported genotoxicity status of the metals analysed in this study

<table>
<thead>
<tr>
<th>Element</th>
<th>Genotoxicity</th>
<th>RP/RV/HBGV</th>
<th>Value</th>
<th>Unit</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lithium (Li)</td>
<td>N/A</td>
<td>pR/D</td>
<td>2.0</td>
<td>µg·(kg BW)^-1·d^-1</td>
<td>(U.S. EPA 2008)</td>
</tr>
<tr>
<td>Beryllium (Be)</td>
<td>N/A</td>
<td>TDI</td>
<td>2.0</td>
<td>µg·(kg BW)^-1·d^-1</td>
<td>(ATSDR 2002; WHO 2009)</td>
</tr>
<tr>
<td>Molybdenum (Mo)</td>
<td>N/A</td>
<td>AI</td>
<td>65.0</td>
<td>mg·d^-1</td>
<td>(EFSA 2017a)</td>
</tr>
<tr>
<td>Cadmium (Cd)</td>
<td>N/A</td>
<td>TWI</td>
<td>2.5</td>
<td>µg·(kg BW)^-1·wk^-1</td>
<td>(EFSA-COMTAM 2011; EFSA 2012)</td>
</tr>
<tr>
<td>Tin (Sn)</td>
<td>Ambiguous</td>
<td>PTWI</td>
<td>14.0</td>
<td>mg·(kg BW)^-1·wk^-1</td>
<td>(JECFA, 2006)</td>
</tr>
<tr>
<td>Barium (Ba)</td>
<td>N/A</td>
<td>TWI</td>
<td>0.21</td>
<td>mg·(kg BW)^-1·wk^-1</td>
<td>(WHO 2016)</td>
</tr>
<tr>
<td>N/A</td>
<td></td>
<td>R/D</td>
<td>0.005</td>
<td>mg·(kg BW)^-1·d^-1</td>
<td>(U.S. EPA 1992)</td>
</tr>
<tr>
<td>N/A</td>
<td></td>
<td>NOAEL</td>
<td>0.9</td>
<td>mg·(kg BW)^-1·d^-1</td>
<td>(EFSA SCF &amp; NDA Panel 2006)</td>
</tr>
<tr>
<td>N/A</td>
<td></td>
<td>UL</td>
<td>0.01</td>
<td>mg·(kg BW)^-1·d^-1</td>
<td></td>
</tr>
<tr>
<td>Inorganic Mercury (Hg)</td>
<td>Ambiguous</td>
<td>TWI</td>
<td>4.0</td>
<td>µg·(kg BW)^-1·wk^-1</td>
<td>(EFSA-COMTAM 2012)</td>
</tr>
<tr>
<td>Methyl-Mercury (MeHg)</td>
<td>N/A</td>
<td>TWI</td>
<td>1.3</td>
<td>µg·(kg BW)^-1·wk^-1</td>
<td></td>
</tr>
<tr>
<td>Thallium (Tl)</td>
<td>N/A</td>
<td>(p)R/D</td>
<td>0.08</td>
<td>µg·(kg BW)^-1·d^-1</td>
<td>(BfR, 2004)</td>
</tr>
<tr>
<td>Lead (Pb)</td>
<td>Ambiguous</td>
<td>BMLD₀₁ (Dev. Neurotoxicity)</td>
<td>0.5</td>
<td>µg·(kg BW)^-1·d^-1</td>
<td>(EFSA CONTAM 2010; Steinkellner 2020)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BMLD₀₁ (Blood Pressure)</td>
<td>1.5</td>
<td>µg·(kg BW)^-1·d^-1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>BMLD₁₀ (Kidneys)</td>
<td>0.63</td>
<td>µg·(kg BW)^-1·d^-1</td>
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<tr>
<td>Bismuth (Bi)</td>
<td>N/A</td>
<td>NOAEL (alopecia)</td>
<td>0.4</td>
<td>mg·(kg BW)^-1·d^-1</td>
<td>(Downs et al. 1960)</td>
</tr>
<tr>
<td>Magnesium (Mg)</td>
<td>N/A</td>
<td>AI</td>
<td>350.0</td>
<td>mg·day^-1</td>
<td>(EFSA 2017b)</td>
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<tr>
<td>Aluminium (Al)</td>
<td>N/A</td>
<td>TWI</td>
<td>1.0</td>
<td>mg·(kg BW)^-1·wk^-1</td>
<td>(EFSA-AFC 2008)</td>
</tr>
<tr>
<td>Titanium (Ti)</td>
<td>N/A</td>
<td>NOAEL (TiO₂)</td>
<td>2250.0</td>
<td>mg·(kg BW)^-1·d^-1</td>
<td>(EFSA-ANS et al. 2018)</td>
</tr>
<tr>
<td>Vanadium (V)</td>
<td>Positive</td>
<td>R/D (Vanadium (V) oxide)</td>
<td>9.0</td>
<td>µg·(kg BW)^-1·d^-1</td>
<td>(U.S. EPA 1988)</td>
</tr>
<tr>
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<td></td>
<td>UL (USA)</td>
<td>1.8</td>
<td>mg·day^-1</td>
<td>(U.S. IOM (Institute of Medicine) 2001)</td>
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<tr>
<td>Chromium (Cr)</td>
<td>Ambiguous (Cr(III))</td>
<td>TDI</td>
<td>300</td>
<td>µg·(kg BW)^-1·d^-1</td>
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<tr>
<td></td>
<td>Positive (Cr(VI))</td>
<td>BMDL₀₅ (Haematology)</td>
<td>0.2</td>
<td>mg·(kg BW)^-1·d^-1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>BMDL₁₀ (Histo-non-neoplastic)</td>
<td>0.1 - 1</td>
<td>mg·(kg BW)^-1·d^-1</td>
<td></td>
</tr>
<tr>
<td>Element</td>
<td>Reference Point</td>
<td>Value</td>
<td>Unit</td>
<td>Source</td>
<td></td>
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<td>----------------</td>
<td>-------</td>
<td>------</td>
<td>--------</td>
<td></td>
</tr>
<tr>
<td>Manganese (Mn)</td>
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<td>AI</td>
<td>3.0</td>
<td>mg.day⁻¹</td>
<td>(EFSA-NDA 2013)</td>
</tr>
<tr>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Iron (Fe)</td>
<td>N/A</td>
<td>AR</td>
<td>6.0</td>
<td>mg.day⁻¹</td>
<td>(EFSA 2017a)</td>
</tr>
<tr>
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<td></td>
<td>PRI</td>
<td>11.0</td>
<td>mg.day⁻¹</td>
<td></td>
</tr>
<tr>
<td>Cobalt (Co)</td>
<td>N/A</td>
<td>TDI</td>
<td>1.4</td>
<td>µg/(kg BW)⁻¹.d⁻¹</td>
<td>(EFSA FEEDAP 2012)</td>
</tr>
<tr>
<td>Nickel (Ni)</td>
<td>Positive</td>
<td>TDI</td>
<td>13.0</td>
<td>µg/(kg BW)⁻¹.d⁻¹</td>
<td>(EFSA-CONTAM et al. 2020)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R/D (water-soluble Ni salts)</td>
<td>20.0</td>
<td>µg/(kg BW)⁻¹.d⁻¹</td>
<td>(Ambrose et al. 1976; EFSA-CONTAM et al. 2020)</td>
</tr>
<tr>
<td>Copper (Cu)</td>
<td>N/A</td>
<td>AI</td>
<td>1.6</td>
<td>mg.day⁻¹</td>
<td>(EFSA 2017a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>UL</td>
<td>0.6</td>
<td>mg.day⁻¹</td>
<td>(EFSA SCF &amp; NDA Panel 2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NOAEL</td>
<td>10</td>
<td>mg.day⁻¹</td>
<td></td>
</tr>
</tbody>
</table>

Note(s): Reference point (RP) is defined as the “estimated dose (on a body mass basis) or the concentration of an agent to which an individual may be exposed over a specified period without appreciable risk” (EFSA et al. 2019) (e.g. Acceptable Daily Intake (ADI), Average Intake (AI), Upper Intake/Level (UL), Tolerable Daily Intake (TDI), Tolerable Weekly Intake (TWI), Provisional/Acute-Reference Dose (pRD/AR/D). Reference values (RV), synonymous to the Point of Departure (USA), are established by applying an uncertainty/safety factor to the RP; “defined points on an experimental dose-response relationship for the critical effect” (EFSA et al. 2019) (e.g. NOAEL (No Observed Adverse Effect Level), BMDL (Lower Bound of Benchmark Dose lower Limit)).

4.2.8.3 RACE Tool Analysis (Accessed 17-04-21)

Analyses performed as per ‘Appendix J’ of EFSA’s official technical document (EFSA et al. 2019). The steps (I - VI) of the process were repeated for each element and are outlined below, briefly:

- **Step I** involved the construction of a decision tree as illustrated in Figure 4.2.

- **Steps II - III** involved the insertion of a new analysis online, de-selecting “match case” in the search box and inputting the substance (i.e. food substance/contaminant) to be analysed. One search is permitted per substance/contaminant. The following food contaminants were searched on 14-04-2021 [date accessed]: Li, Be, Mo, Cd, Sn, Ba, Hg (total and inorganic), Tl, Pb, Ti, V, Cr, Co, Ni and Cu. The elements Au, Al, Fe, Mg, Mn exceeded the calibration range and could not be accurately determined therefore excluded from the analyses. Pt and Bi are unavailable to assess using the RACE platform currently, and thus excluded also.

- **Step IV** involved selecting the most appropriate food item/category from the RASFF Exposure Hierarchy derived from FoodEx2 as outlined in Table 4.6. In this study, Level 1 ‘vegetable and vegetable products’ > Level 2 ‘herbs and edible
flowers’ were selected for each run. The risk evaluation run is then saved, and the user returns to the RACE homepage.

- **Step V** involved opening the selected risk evaluation (from steps I - IV) and inputting the analytical value and available toxicological acute and/or chronic values, as outlined in Tables 4.7 and 4.8, respectively. The maximum quantifiable concentration for each metal across all *Inula* samples (*n* = 27) was input as the analytical value. The final step (VI) was to run the analysis and export data (.xml format).

The results are expressed by the comparison of dietary exposure from single foods to a relevant toxicological end-point (acute/chronic), and are classified as: (a) no risk; (b) low probability of adverse health effects; (c) low concern for public health; (d) potential risk; or (e) risk (EFSA et al. 2019). All elements were classified as “no risk”, except for Pb (see Table 4.9). Cd is the only element that exceeded applicable national and European ML’s (see Figure 4.4) yet was still classified as no risk at the highest concentration detected.
Figure 4.2 Hazard Characterisation decision tree for metal contaminants in food matrices, adapted from EFSA (2019).

Note:**Threshold for Toxicological Concern (TTC) Approach (Steps 5-7, EFSA (2019)) not applicable for excluded substances, such as inorganic substances/metals/organometallics, in situations where a reference point is unavailable.
4.3 Results and discussion

4.3.1 Multi-elemental (metallomic) profiling results

This current study is, to the best of our knowledge, the first metallomic profiling of various *I. helenium* (root, flower, leaf, stem) samples using high-resolution ICP-SFMS. Figure 4.3 below illustrates the average elemental profile for the multi-origin elecampane samples analysed. It is worthwhile noting that some of the isotopes quantified are not the most abundant isotope(s) [of the element] found in nature, such as $^{95}$Mo, $^{111}$Cd, $^{118}$Sn, $^{47}$Ti and $^{60}$Ni. Availability of standard solutions at the time of analysis was a limiting factor in this regard.

![Average Metalomic Profile of Multi-Origin Elecampane](image)

*Figure 4.3 Average element isotope levels across all *Inula* samples (n = 27) graphed on logarithmic scale (log10). Error bars represent the standard deviation (SD).*

Other studies have explored the metal content of *I. helenium* following automatic absorption spectrometry (AAS) (Ražić et al. 2008; Tamakhina et al. 2018). The study by Ražić et al. (2008) lists “*Inulae Radix***” in the materials, which is *I. helenium* root, however, misidentifies the Latin binomial as *I. britannica*. Other related *Inula* species have been analysed, including *I. viscosa* (Swaileh et al. 2004; Hunaiti et al. 2007; Gisbert et al. 2008; Barbaferi et al. 2011; Baycu et al. 2015; Christou et al. 2017), *I. japonica* (Wang et al. 2004), *I. germanica* (Shallari et al. 1998), *I. ensifolia* (Turnau et al. 2010),
and *I. cuspidata* C. B. Clarke (Kumar-Paliwal et al. 2017). Interest in *I. viscosa* appears to be credited to its potential phytoremediation applications, attributable to the characteristics of the leaf morphology (Freitas 1993). Studies have explored the species as an exemplar for environmental metal contaminant biomonitoring (Swaileh et al. 2004), phytostabilization and phytoextraction (Cd, Pb) (Barbafieri et al. 2011; Christou et al. 2017), as well as arsenic (As) accumulation (Gisbert et al. 2008).

Tamakhina *et al.* (2018) reported the hyperaccumulation of Cd, Cu, Mo, Pb, and Zn in *Inula* species from Russia, with levels of Cd and Pb twice exceeding national [Russian] regulatory limits of 1000 and 6000 µg.kg⁻¹, respectively, in the above-ground parts – similar to *I. viscosa*; a related Pb and Cd phytostabiliser (Christou et al. 2017). Levels in the root were much lower (Tamakhina et al. 2018). Both Pb and Cd are known environmental persisters (Broadley et al. 2001). Reported mean averages in the below-ground part ranged as follows (µg.kg⁻¹.g.kg): Cu (4210 – 8860), Zn (16830 – 18750), Pb (2140 – 5030), Cd (280 – 450) and Mo (6530 – 8120), compared to the higher levels in the above-ground parts (µg.kg⁻¹.g.kg) for Cu (15400 – 29900), Zn (20620 – 23480), Pb (5460 – 12510), Cd (1530 – 2060) and Mo (6530 – 8120) (Tamakhina et al., 2018). Similarly, Ražić *et al.* (2008) quantified the following levels in *Inula* of Serbian origin (µg.kg⁻¹.g.kg): Cu (9880), Zn (20500), Mn (5.3 x10⁴), Fe (3.25x10⁵), Ni (3540), Cd (0120), Pb (3650), Cr (7780), K (2.623x10⁶), Ca (2.617 x10⁶) and Mg (1852 x10⁶). The previous results reported for Cd, Cu, Mo, Ni, and Pb in *Inula* spp. are in line with the levels detected in this current study (See Table 4.8).
Table 4.8 Concentration (μg kg\(^{-1}\))** of each element in samples IN-1 to 27 following analysis by ICP-SFMS.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Li</th>
<th>Be</th>
<th>Mo</th>
<th>Cd</th>
<th>Sn</th>
<th>Na</th>
<th>Ti</th>
<th>V</th>
<th>Cr</th>
<th>Co</th>
<th>Ni</th>
<th>Cu</th>
</tr>
</thead>
<tbody>
<tr>
<td>IN-1</td>
<td>50.31</td>
<td>11.55</td>
<td>122.12</td>
<td>155.89</td>
<td>9.95</td>
<td></td>
<td>5.05</td>
<td>5.34</td>
<td>13.61</td>
<td>462.51</td>
<td>10.77</td>
<td></td>
</tr>
<tr>
<td>IN-2</td>
<td>20.44</td>
<td>3.98</td>
<td>308.71</td>
<td>248.86</td>
<td>16.77</td>
<td>NR*</td>
<td>11.60</td>
<td>7.75</td>
<td>3.53</td>
<td>57.63</td>
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</tr>
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<td>59.12</td>
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<td>NR*</td>
<td>8.50</td>
<td>5.77</td>
<td>14.59</td>
<td>289.14</td>
<td>60.35</td>
<td>3743.1</td>
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<tr>
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<td>5.82</td>
<td>950.66</td>
<td>472.23</td>
<td>24.02</td>
<td>NR*</td>
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<td>11.89</td>
<td>4.40</td>
<td>80.86</td>
<td>8.16</td>
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<td>15.07</td>
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<td>2.79</td>
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**Note:** NR* indicates not reported.
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<td>2046.5</td>
<td>5</td>
<td>128.07</td>
<td>449.96</td>
<td>27.12</td>
<td>186.83</td>
<td>4724.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IN-20</td>
<td>375.8</td>
<td>5</td>
<td>11.16</td>
<td>2066.1</td>
<td>0</td>
<td>144.32</td>
<td><em>NR</em></td>
<td>8.18</td>
<td>20.88</td>
<td>5.76</td>
<td><em>NR</em></td>
<td>25.59</td>
<td>2684.6</td>
<td>8</td>
<td>316.58</td>
<td>941.60</td>
<td>132.60</td>
<td>464.66</td>
<td><em>NR</em></td>
<td></td>
</tr>
<tr>
<td>IN-21</td>
<td>39.34</td>
<td>3.86</td>
<td>548.28</td>
<td>39.38</td>
<td>28.28</td>
<td>3434.6</td>
<td>8</td>
<td>7.15</td>
<td>5.64</td>
<td>3.13</td>
<td>1646.0</td>
<td>8</td>
<td>503.07</td>
<td>44.78</td>
<td>177.87</td>
<td>45.96</td>
<td>597.71</td>
<td><em>NR</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IN-22</td>
<td>20.23</td>
<td>2.02</td>
<td>284.41</td>
<td>38.88</td>
<td>9.18</td>
<td>3176.7</td>
<td>4</td>
<td>3.83</td>
<td>3.57</td>
<td>12.41</td>
<td>328.49</td>
<td>4.82</td>
<td>1718.4</td>
<td>0</td>
<td>21.48</td>
<td>129.09</td>
<td>12.83</td>
<td>157.88</td>
<td><em>NR</em></td>
<td></td>
</tr>
<tr>
<td>IN-23</td>
<td>204.2</td>
<td>3</td>
<td>1295.6</td>
<td>5</td>
<td>90.76</td>
<td>104.86</td>
<td><em>NR</em></td>
<td>6.54</td>
<td>11.71</td>
<td>7.90</td>
<td><em>NR</em></td>
<td>31.99</td>
<td>1659.2</td>
<td>6</td>
<td>119.42</td>
<td>534.54</td>
<td>113.91</td>
<td>354.89</td>
<td><em>NR</em></td>
<td></td>
</tr>
<tr>
<td>IN-24</td>
<td>115.9</td>
<td>0</td>
<td>614.27</td>
<td>28.71</td>
<td>57.20</td>
<td>2920.2</td>
<td>7</td>
<td>2.07</td>
<td>3.62</td>
<td>5.20</td>
<td><em>NR</em></td>
<td>22.90</td>
<td>2694.6</td>
<td>0</td>
<td>172.75</td>
<td>812.68</td>
<td>95.89</td>
<td>625.32</td>
<td><em>NR</em></td>
<td></td>
</tr>
<tr>
<td>IN-25</td>
<td>911.6</td>
<td>7</td>
<td>68.60</td>
<td>227.55</td>
<td>168.88</td>
<td>82.96</td>
<td><em>NR</em></td>
<td>6.48</td>
<td>5.85</td>
<td>9.61</td>
<td><em>NR</em></td>
<td>2310.9</td>
<td>1</td>
<td>12.44</td>
<td><em>NR</em></td>
<td>3649.7</td>
<td>3</td>
<td>246.19</td>
<td><em>NR</em></td>
<td></td>
</tr>
<tr>
<td>IN-26</td>
<td>838.3</td>
<td>4</td>
<td>65.06</td>
<td>178.12</td>
<td>189.36</td>
<td>73.60</td>
<td><em>NR</em></td>
<td>5.30</td>
<td>5.54</td>
<td>9.80</td>
<td>796.93</td>
<td>10.17</td>
<td><em>NR</em></td>
<td>1245.3</td>
<td>7</td>
<td>1099.7</td>
<td>3</td>
<td>234.50</td>
<td><em>NR</em></td>
<td></td>
</tr>
<tr>
<td>IN-27</td>
<td>435.0</td>
<td>5</td>
<td>18.79</td>
<td>409.41</td>
<td>108.31</td>
<td>10.89</td>
<td><em>NR</em></td>
<td>8.33</td>
<td>8.23</td>
<td>25.09</td>
<td>1251.8</td>
<td>6</td>
<td>11.55</td>
<td><em>NR</em></td>
<td>762.81</td>
<td>2256.0</td>
<td>1</td>
<td>232.34</td>
<td><em>NR</em></td>
<td></td>
</tr>
</tbody>
</table>

*Note(s): NR* - Element concentrations which exceeded the highest calibration standard and could not be accurately quantified. The concentrations of Al, Au, Fe, Mg, and Mn in all samples tested were outside the calibration range and could not be accurately determined; and thus, they are excluded from this table. **Highest quantified concentrations of the corresponding element(s) are highlighted in grey. These values were input into the risk assessment model described in Section 4.2.8.3.*
4.3.2 Risk assessment and evaluation

In the absence of a specific food category and consumption data for Inula species, intake of all herbs and edible flowers was considered by selecting a higher level (L2) FoodEx2 category to model exposure risk (See Table 4.6). It is important to note, that when a category is selected on a general and non-specific basis, there is a higher risk for the over- or under-estimation of exposure, the former being more probable (EFSA, 2019). When estimating dietary exposure from residues in a single commodity or food that is less frequently or rarely consumed (i.e. herbs) in comparison to staple foods (e.g. milk, bread), it is recommended to use data reported as “consumers only” or “consuming days only” instead of the “total population” statistics, in other words, food consumption data specific to the subjects or data for which consumption of the single food/commodity was reported (EFSA, 2019). There are limitations to using consumer-only data namely the possible over-estimation of long-term intake values, which should be taken into consideration when interpreting results. It is safe/realistic to assume that consumption of Inula derived products (e.g. extract, tisane, candied root, derived inulin) is more likely to be classified as intermittent or occasional in the Irish diet, as opposed to, for example, staple foods such as bread, potatoes, or rice. Another limitation to consider is the condition of the final product versus the state when analysed (EFSA, 2019); herbal material can be used fresh or dry for culinary uses or as part of an extract and/or herbal tea thus preparation methods could influence the analyte concentrations prior to consumption. A dilution factor, for example, could therefore be applied in such scenarios to improve accuracy. The analyses using the RACE tool focuses on one contaminant in one food commodity at a time, thus background or aggregate exposure from all other dietary sources needs consideration. Although herbs and edible flowers are considered minor contributors to the overall consumer exposure, consumer intake risk cannot be excluded in this food category, currently (EFSA, 2020).
Table 4.9 Estimated (consumers-only) dietary exposure for the adult, elderly and very elderly Irish population following RACE analysis (accessed: 17-04-21):

<table>
<thead>
<tr>
<th>Statistical Output per Population Group (n = number of consumers)</th>
<th>Element (µg.kg⁻¹)d,e</th>
<th>Estimated Acute Exposure (µg-(kg BW)⁻¹d⁻¹)</th>
<th>Estimated Chronic Exposure (µg-(kg BW)⁻¹d⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Li (912)</td>
<td>Be (69)</td>
<td>Mo (2698)</td>
</tr>
<tr>
<td>Adult (n=444)</td>
<td>Mean</td>
<td>0.031</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>p95 c</td>
<td>0.125</td>
<td>0.009</td>
</tr>
<tr>
<td>Elderly (n=40)</td>
<td>Mean</td>
<td>0.026</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>p95 c</td>
<td>0.108</td>
<td>0.008</td>
</tr>
<tr>
<td>Very Elderly (n=27)</td>
<td>Mean</td>
<td>0.043</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>p95 c</td>
<td>0.146</td>
<td>0.011</td>
</tr>
</tbody>
</table>

\(a = \text{mg (kg BW)}^{-1}\text{d}^{-1}; \ b = \text{mg.day}^{-1}; \ c = 95\text{th Percentile, representing “above-average” consumers;} \ d = \text{Bi, Pt, Ti, Al, Au, Mg, Mn and Fe excluded from analysis due to unavailability on RACE platform and/or concentrations not quantified.} \ e = \text{Maximum concentration detected in parentheses, expressed as } \mu\text{g.kg}^{-1}.\)
In this present study, the maximum concentration of each element detected was input to the exposure assessment platform, EFSA’s RACE tool, which represent a “worst-case” scenario (see Table 4.9). The RACE tool utilises food consumption data from the EFSA Comprehensive European Food Consumption Database to calculate acute and chronic exposure estimates in a single food category by comparing toxicological reference points/values (EFSA et al., 2019) (see Tables 4.7 & 4.8).

The results of each metal are discussed separately below (4.3.2.1 – 4.3.2.19) in terms of their estimated toxicological significance in the Irish diet, and comparison to regional levels reported in Irish soils.

The soil data was derived from spatial distribution maps in the ‘Soil Geochemical atlas of Ireland’ (Fay et al. 2007) and ‘Guide to the Tellus Data’ (Young and Donald 2013) for the Republic and North of Ireland, respectively. The Tellus geochemical survey of N. I. revealed large variation in the levels and distribution of trace elements in soil across sampling areas, exemplifying how soil composition is influenced by the underlying bedrock geology, superficial (Quaternary) deposits, mineralisation, agricultural (i.e. intensive farming) and other environmental factors (Young et al. 2016). Furthermore, root elemental uptake is dependent on soil pH, a property of soil which also varies widely across survey areas in Ireland (Young et al. 2016). Such variations make interpretations complex. Nevertheless, data on the elemental content of Irish soil could help contribute towards a greater understanding of the high or low occurrence of metals in terrestrial-derived plants and food – as advocated by the Food Safety Authority of Ireland (FSAI) (FSAI 2016a).

In future work, soil sampling in accordance to validated guidelines (e.g. Geochemical Baseline Survey of the Environment (G-BASE)), would ideally be investigated in tandem to the metallomic profiling of terrestrial plant samples. This would facilitate the identification of soil-to-plant metal transfer rates and the bioconcentration of potential contaminants in situ. Such data would also further provide a basis and contribute to more extensive research in the emerging discipline of medical geology, which investigates the relationship between element abundance and epidemiology of disease in the survey area(s), as exemplified by McKinley et al. (2013) who studied the geocoded incidences of various types of cancers in relation to the surrounding geochemistry in parts of South-Eastern N.I. Outputs of such integrated research endeavours could influence regional
public health agendas, for example the implementation of focused screening programmes for GIT cancers in areas of elevated and bioaccessible arsenic in Ireland (McKinley et al. 2016). Another interesting direction in this field, would be the mathematical assessment of final “consumer-ready” products or final preparations. Considering the prospective use of elecampane root extract as a topical anti-Staphylococcal agent (Kenny et al., 2015), mathematical modelling of dermal trace element absorption patterns warrants consideration in future studies.

4.3.2.1 Lithium (\(^7\)Li)
Li is utilised in the manufacture of batteries, glass (optics), ceramics, plastics and psychopharmaceuticals (Kabata-Pendias 2011). The psychopharmacological application of Li compounds for the treatment of depression, schizophrenia and bipolar disorder is well-known (Szklarska and Rzymski 2019). Li is readily absorbed by plants and has demonstrated both stimulatory and retardation of tissue growth, affecting biomass yields (Kavanagh et al. 2017). A median distribution of 19700 µg.kg\(^{-1}\) is reported in Irish soils (Fay et al., 2007; FSAI, 2016). Higher Li concentrations in above-ground parts are expected (Kabata-Pendias 2011), however, our results do not reflect this. Levels of Li varied widely across all samples in this study, ranging from 5.20 (IN_18; flowers) to 911.67 µg.kg\(^{-1}\) (IN_25; whole). Of the samples tested, it is evident that all commercial elecampane samples (IN_25-27) resulted in higher concentration. The sample size \(n = \leq 3\) was insufficient to formally analyse the statistical significance of this observed trend in the commercial sample cohort. Shahzad et al. (2016) reported average Li concentrations of 300 – 4600 µg.kg\(^{-1}\) g.kg in dark-green leafy vegetables levels considerably higher than those observed in this present study. Conversely, Filippini et al. (2020) reported much lower values ranging from 0.95 – 35.66 µg.kg\(^{-1}\) in various (unspecified) plant foods. Li levels vary significantly between plant parts of naturalised elecampane (0.03), notably between the flowers and leaves (0.028) (see Table 4.4).

A specific migration limit (SML) of 600 µg.kg\(^{-1}\) in food is established for Li migration via food contact materials however, there are no established HBGV’s for dietary Li (EFSA SCF & NDA Panel 2006). While it is not considered an essential dietary nutrient currently, Schrauzer (2002) proposed a provisional RDA of 1000 µg.kg\(^{-1}\), corresponding to 14.3 µg.(kg BW\(^{-1}\)) for a 70 Kg adult. The U.S. EPA later established a provisional (sub)chronic reference dose (p-R\(_{FD}\)) of 2 µg.(kg BW\(^{-1}\).d\(^{-1}\)) based on data pertaining to the
lower-bound serum Li concentration associated with observed adverse effects reported during therapeutic regimes (Pearson and Ashmore 2020). As outlined in Table 4.9, estimated acute exposures to Li, at the maximum observed concentration, were estimated at 0.031 and 0.125 µg·(kg BW)⁻¹·d⁻¹ for the average and above average (95th percentile) adult Irish consumers representing 1.5 and 6.2 percentage contribution to the AR/D, respectively. The data suggests that dietary exposure to Li in edible plants and herbs is of no risk to Irish adult consumers from this food category.

4.3.2.2 Beryllium (9Be)

Be is utilised in electronic and electrical components, and in precision instrumentation (Kabata-Pendias 2011). Be concentrations ranged from 2.02 (IN_22; root) to 68.60 µg µg.kg⁻¹ (IN_25; whole). Similarly to Li, the commercial samples (IN_25-27) resulted in up to 30-fold higher Be concentrations compared to the lowest value of 2.02 µg.kg⁻¹ in naturalised counterparts. Significant differences between plant parts (0.016), particularly the flowers and leaves (0.012) were observed for naturalised elecampane (see Table 4.4). Lower levels are expected in aerial tissues, given its poor translocation capacity in planta (Kabata-Pendias 2011). McGrath and Fleming (2007) stated that Be likely accumulates in the B-horizon (subsoil), however, it is excluded in the most recent national soil survey of Ireland (Fay et al. 2007), and thus comparisons between plant and soil elemental levels cannot be made.

WHO (2009) published a TDI/MRL of 2 µg·(kg BW)⁻¹·d⁻¹ for Be. Estimated acute exposures of 0.002 and 0.009 µg·(kg BW)⁻¹·d⁻¹ were calculated for the average and 95th percentile adult Irish consumers which represents 0.08 and 0.3 percentage contribution to the AR/D. Estimated chronic exposures were 0.001 and 0.003 µg·(kg BW)⁻¹·d⁻¹ for the average and 95th percentile adult Irish consumers, which represents negligible percentage contribution to the TDI. The data suggests that dietary exposure to Be in edible plants and herbs is of no risk to Irish adult consumers.

4.3.2.3 Molybdenum (95Mo)

Mo acts as a co-factor of redox (metallo-flavoproteins) enzymes in humans, including sulphite-, aldehyde- and xanthine-oxidases, and mitochondrial amidoximes reducing component (mARC). Molybdenosis, common in ruminants, is caused by the conversion of Mo to thiomolybdates in the rumen and consequently binding to sulphide and Cu forming complexes which can cause gastroenteritis and systemic Cu deficiency (Hall
The levels of Mo and sulphur in pastures and feed and the subsequent uptake of Mo by terrestrial plants can be problematic for ruminants grazing on Mo-rich vegetation, the outcome of which can result in Cu-deficiency (Fay et al. 2007). Mo is categorised as an essential trace element in the human diet. EFSA (EFSA SCF & NDA Panel 2006) derived an UL of \(10 \mu g \cdot (kg \text{ BW})^{-1} \cdot d^{-1}\) (equivalent to 600 µg per person/day), and later established an AI and NRV of 65 and 50 µg.d\(^{-1}\), respectively, for the average adult male population (EFSA 2017a).

In this study, higher Mo concentrations were significantly higher for the naturalised aerial leaf samples compared to most of the root (0.012) (see Table 4.4). Despite undergoing a 1:5 dilution prior to analysis by ICP-SFMS, the concentrations of Mo in 11% of samples were above the calibration range and further dilution was not possible. Therefore, concentrations of Mo could not be accurately determined in these leaf samples. A quantifiable range of 122.12 (IN_01; root) – 2697.93 µg.kg\(^{-1}\) (IN_11; leaves) was observed. These values are lower than those reported for common herbage species grown in Irish soil, such as (µg.kg\(^{-1}\)) White Clover (85,000 g.kg), Yorkshire Fog (63,000) and Timothy (7000g.kg) (Mcgrath and Fleming 2007). Considering a median distribution of 910 µg.kg\(^{-1}\) in Irish soils (Fay et al. 2007; FSAI 2016a), this data could suggest that plants accumulate or sequester this element \textit{in planta}. Dublin and Leitrim have some of the highest Mo soil levels in Ireland (>1800 µg.kg\(^{-1}\) g.kg\(^{-1}\)) (Fay et al., 2007), which is reflected in our results.

The output of the RACE analysis revealed estimated acute exposures of <0.0001 mg.(kg BW\(^{-1}\)d\(^{-1}\) for the average and 95\(^{th}\) percentile adult Irish consumers, representing 1.8 and 7.4 percentage contribution to the AR/D. Estimated chronic exposures of 0.002 and 0.008 mg.day\(^{-1}\) were calculated for the average and 95\(^{th}\) percentile adult Irish consumers which represents 0.4 and 1.3 percentage contribution to the UL. In comparison, dietary exposure at the highest concentration detected in this study, 2697.93 µg.kg\(^{-1}\), in a plant-derived staple food item (e.g. potatoes) would be deemed a “potential risk” for above-average Irish consumers (data not shown). Our data suggests that dietary exposure to Mo in edible plants and herbs as a single food commodity however is well below the available toxicological endpoints and therefore of no risk to Irish adult consumers for this food category.
4.3.2.4  Cadmium (111Cd)

Cd is a Group 1 carcinogen based on an increased risk of bladder, breast, endometrium, and lung cancer development in humans (IARC 2012). The human health effects, including Cd-induced renal nephropathy and skeletal abnormalities, as well as dietary exposure to Cd in plant-derived food for human consumption, has been previously reviewed (Kenny et al. 2020). The Republic of Ireland has been flagged as a priority area at risk of heavy metal soil pollution, predominantly around the coastal regions of the country (Tóth et al. 2016). Cd is abundant in the limestone shale belt in Irish soils (Mcgrath and Fleming 2007; Tóth et al. 2016), commonly co-existing in topsoil with Sb and Pb (Toth et al., 2016). A median distribution of 326 µg.kg⁻¹ is reported in Irish soils, with reports of 15% of agricultural Irish soils exceeding >1000 µg.kg⁻¹ higher than most mainland European countries (Fay et al. 2007; FSAI 2016a). In this present study, Cd concentrations were highest in the cohort of naturalised elecampane samples derived from Leitrim (IN_16-18) and leaves from Dublin (IN_6; 933.53 µg.kg⁻¹). High soil Cd levels are correlated with topography, low pH, drainage, organic matter, and sulphide mineral soil content (Young and Donald 2013). Higher levels in soils around Dublin is likely attributable to the underlying impure limestone bedrock (Fay et al. 2007).

![Figure 4.4](image)

Evidence suggests that Cd is mobile in planta and localises in both above and below-ground parts. Cd levels in the root (IN_16) and flowers (IN_18) from Leitrim are within
the estimated range distributed in the soil, however the leaves were >2-fold higher. Cd levels between plant parts of elecampane are statistically significant (0.002), particularly between flowers and leaves (0.002) (see Table 4.4). Cd concentrations ranged from 28.71 (IN_24; flowers) to 1285.97 µg.kg⁻¹ (IN_17; leaves), with 52% of the samples exceeding European limits for Cd in food (200 µg.kg⁻¹), and one sample (IN_17 leaves) exceeding the ML for herbal material/drugs (1000 µg.kg⁻¹; Ph. Eur., 2013) as shown in Figure 4.4. Analytical values exceeding limits in routine testing would “trigger” a risk evaluation, warranting further investigation. These results, however, are in line with Tamackhina et al. (2018) who reported a maximum Cd concentration of 1530 µg.kg⁻¹ in the aerial parts of elecampane. Both Tamackhina et al. (2018) and Ražić et al. (2008) observed higher Cd levels in above-ground parts of *Inula* spp.

Data from the most recent Irish Total Diet Survey (TDS) (2012 – 2014), showed upper and lower-bound Cd concentrations of 120 and 90 µg.kg⁻¹ in herbs and spices. General estimated exposure from all food groups contributed to mean adult daily intakes of 1.1 – 1.5 µg·(kg BW)⁻¹wk⁻¹ (FSAI 2016b), which is below the TWI of 2.5 µg·(kg BW)⁻¹wk⁻¹. Cereals and vegetables were the major contributing sources of dietary Cd from the TDS (FSAI, 2016). In this current study, despite the high Cd concentrations observed (IN 2, 4, 6, 8, 10, 11, 13-14, 16-19, 26), estimated chronic exposures of 0.10 and 0.37 µg·(kg BW)⁻¹d⁻¹ were calculated for the average and 95th percentile adult Irish consumers at the highest observed concentration (1285.97 µg.kg⁻¹). This represents an estimated 3.9 and 14.7 percentage contribution to the TWI, and thus demonstrates no appreciable risk to Irish consumers from this single food commodity. In agreement with the FSAI (2016b), the levels of Cd in this food category do not present unacceptable risk to the adult Irish consumer. It is worthwhile to consider however, aggregate Cd exposure from other food categories and lifestyle factors such as smoking, as well as individuals deficient in calcium or Fe whom are more susceptible to Cd toxicity (Hartwig and Jahnke 2017), and thus extra consideration should be given to such groups.

### 4.3.2.5 Tin (¹¹⁸Sn)

Sn occurs naturally in foods (stannous-, stannic-salts, stannous chloride) and is a legal food additive (E512). Average intakes in the U.K. are estimated within the range of 1.8 - 6 mg.day⁻¹ (EFSA SCF & NDA Panel 2006). Sn is poorly absorbed in the GIT thus poses low risks for systemic toxicity (EFSA, 2006). There is evidence to suggest that
concentrations >250 mg [canned food] could influence the absorption of Cu, Fe and Zn. Absorbed Tn is therefore thought to indirectly contribute to the manifestation of symptoms of other trace element deficiencies in humans, e.g. femoral epiphysis, or pancreatic atrophy, as opposed to causing adverse effects directly (EFSA, 2006). Sn concentrations in this study ranged from 7.41 (IN_9; flowers) to 125.45 µg.kg⁻¹ (IN_20; leaves). Maximum limits for Sn (inorganic) in food exist only for tinned products according to European regulations, however, national limits in Ireland are set at 200 µg.kg⁻¹ food as per Statutory Instrument (SI) No. 389/1993 (see Table 4.5). In this current study, levels of Sn in all elecampane samples are well below this limit.

A median distribution of 1680 µg.kg⁻¹ is reported in Irish soils (Fay et al., 2007; FSAI, 2016). In agreement with Fay et al. (2007), higher Sn levels were found in samples from around Cork City which could be due to localised anthropogenic enrichment. Widespread anthropogenic anomalies at soil sampled at locations of prolonged human activity (i.e. road intersections) around N.I., was also reported in Young & Donald (2013). The authors further suggest that the widespread variation in anthropogenic Sn could be independent of population and spatial extent of urbanisation. Based on our data, levels of Sn differ significantly between plant parts of cultivated elecampane (0.009), particularly between root and leaf samples (0.012) (see Table 4.4). Following RACE analysis, the overall contributions of Sn to the PTWI were <0.0001% and therefore chronic dietary exposure via herbs and edible flowers is of negligible concern to Irish adult consumers.

4.3.2.6 Barium (¹³⁷Ba)

Industrial and medical uses of Ba include material and paint production, rubber synthesis (Kabata-Pendias 2011) and as a contrast agent in gastrointestinal medical imaging (i.e. X-rays, CT scans). Orally ingested Ba-sulfate, while non-toxic, can persist in the colon for several weeks. There are reports of barium fecaliths, colonic stasis and appendicitis related to Ba residues in the gut (Aronson 2016). A median distribution of 230.2 mg.day⁻¹ is reported in Irish soils (Fay et al. 2007; FSAI 2016a), which is relatively high. Ba levels varied significantly between plant parts derived from naturalised elecampane (0.005); particularly between flowers to root (0.028) and flowers to leaves (0.028) (see Table 4.4). Ba could not be estimated in 67% of the root and leaf samples analysed because the diluted samples (1:5) had concentrations above the calibration range and subsequent dilution was not possible at the time of analysis. Similarly, Pearson and
Ashmore (2020) reported 98% of food samples >LOD for Ba. Levels in flowers ranged from 1782.22 (IN_12; flowers) – 4463.62 µg.kg⁻¹ (IN_19; root). These values align with those reported by Filippini et al. (2020) (µg.kg⁻¹): cereals (700 – 1400µg.kg⁻¹), vegetables (80 – 500µg.kg⁻¹) and nuts, seeds, and dried fruit (600 – 2400µg.kg⁻¹). Following RACE analysis, estimated acute and chronic exposures for adults were ≤0.001 mg·(kg BW)⁻¹·wk⁻¹ from herbs and edible flower intake, which represents negligible percentage contribution to the HBGV’s for average and above-average adult consumer. The data suggests that dietary exposure to Ba in this food category is of no risk to Irish adult consumers.

### 4.3.2.7 Platinum (¹⁹⁵Pt)

Environmental Pt levels are rising as a result of the increased usage of Pt in medicine and automobile engineering (Ravindra et al. 2004). Average soil Pt levels of 1.8 µg.kg⁻¹ reported in Co. Down (Young and Donald 2013) are in line with previously reported ranges (2 – 40 µg.kg⁻¹) (Kabata-Pendias 2011). Distribution of Pt in soils of the Republic of Ireland has not yet been explored. Variation in the distribution of Pt within plant parts of naturalised elecampane are not statistically significant (0.846) (see Table 4.4). The levels quantified in this study ranged from 2.07 (IN_22; flowers) to 16.34 µg.kg⁻¹ (IN_14; leaves) which reflects the average ranges expected in non-contaminated soils. An oral Permitted Daily Exposure (PDE) of 108 µg.day⁻¹ in pharmaceuticals was calculated based on an estimated NOAEL of 146 g.kg⁻¹ or 1080 µg·(kg BW)⁻¹·d⁻¹ (EMA 2019), however there is currently insufficient data to support the establishment of HBGV recommendations for Pt in food. Pt and Bi were excluded from the RACE analysis due to unavailability in the search function at present. Research into the presence, distribution, and health effects of Pt in plant-derived foods is needed in order to merge the data gap within the literature for this element.

### 4.3.2.8 Gold (¹⁹⁷Au)

The occurrence, exposure and effects of dietary Au is reviewed elsewhere (Dolara 2014). From this analysis, Au levels could not be determined because the analytical values were below the calibration range and were too low to be quantified except for leaves sampled from Dublin and N.I. (IN_6 and IN_11; 11.92 and 11.63 µg.kg⁻¹) (data not shown). Within root vascular systems, Au is readily translocated to aerial tissues (Kabata-Pendias 2011). A level of 5.7 µg.kg⁻¹ was reported for soils in Co. Down (Young and Donald 2013), and
a spatial distribution of 10 µg.kg\(^{-1}\) was recorded regionally (Young et al. 2016). This could
demonstrate a correlation between soil-plant Au levels and warrants further investigation.
Areas of soil with Au deposits are often associated with high antimony (Sb) and arsenic (As) levels (Fay et al. 2007). Such associations can be valuable indicators for the prospector (Young et al. 2016). On the other hand, Au levels could indicate the presence of other potentially hazardous elements such as As, especially considering the ability of other *Inula* species to accumulate As (Gisbert et al. 2008). Cu commonly co-exists with Au in soils (Anderson et al. 2005; Wilson-Corral et al. 2012). Plants do not typically accumulate Au, however certain species have been shown to secrete lixiviant-type exudates that mobilise Au in soil thus permitting root uptake (Anderson et al. 2005). There is evidence to suggest plants that uptake Au subsequently deposit Au-nanoparticles *in planta* (Wilson-Corral et al. 2012), as exemplified by the presence of nano-Au-bearing particles, consisting of Au, Cu, O and Cl, identified in *Erigeron canadensis* and *Boehmeria nivea* following stomatal uptake (Luo and Cao 2018). The phenomenon is also displayed by Pt and other Platinum-Group Metals (Harumain et al. 2017).

An oral Permitted Daily Exposure (PDE) of 134 µg.day\(^{-1}\) is recommended for Au
impurities in pharmaceuticals (EMA 2019). In food, Au can be employed as a food additive (E175), but similar to Pt, there is currently insufficient data to support the establishment of HBGV recommendations (EFSA-ANS 2016). Estimated dietary exposures could therefore not be assessed using the RACE tool in this study.

### 4.3.2.9 Mercury (\(^{202}\text{Hg}\))

The human health effects and dietary exposure to Hg in plant-derived raw materials for human consumption has been previously reviewed (Kenny et al. 2020). A median distribution of 86 µg.kg\(^{-1}\) is reported in Irish soils, with higher soil-Hg levels recorded in Co. Wicklow (Fay et al. 2007; FSAI 2016a). Reported background levels of Hg in various fruit and vegetables are low, ranging from 0.6 – 86 µg.kg\(^{-1}\) (Kabata-Pendias 2011). Low levels were observed in this current study too; and all samples (\(n = 27\)) analysed were well below the European ML’s (100 µg.kg\(^{-1}\)) permissible in food, including dietary supplements (see Figure 4.5).
Quantified Hg concentrations ranged from 3.57 (IN_22; root) to 20.88 µg.kg$^{-1}$ (IN_20; leaves). Estimated acute exposures to inorganic-Hg and total-Hg, at the maximum observed concentration observed (20.88 µg.kg$^{-1}$), were estimated at 0.002 and 0.006 µg⋅(kg BW)$^{-1}$d$^{-1}$ for the average and above average (95th percentile) adult Irish consumers representing 0.04 and 0.2 percentage contribution to the TWI, respectively. This data suggests, in agreement with The EFSA-CONTAM Panel (2012), that dietary exposure to inorganic-Hg in edible plants and herbs is of no risk to consumers.

4.3.2.10 Thallium (205Tl)

Adverse effects from Tl exposure in humans are varied and include renal impairment and hair follicle atrophy (e.g. alopecia) (U.S. EPA, 2009). Tl is thought to accumulate in multiple tissues and organs, and its structural similarity to potassium offers opportunity for mechanisms of ionic mimicry (NFI et al. 2020). One sample (IN_15; flowers) could not be estimated because the analytical values of the 1:5 diluted samples had concentrations below the calibration range. Quantified Tl concentrations ranged from 2.48 (IN_12; flowers) to 30.94 µg.kg$^{-1}$ (IN_16; root). These values are in line with those reported by Filippini et al. (2020) for (unspecified) plant-based foods (0.001 – 2.002 µg.kg$^{-1}$). A median distribution of 430 µg.kg$^{-1}$ is reported in Irish soils (Fay et al. 2007;
FSAI 2016a), which is higher than the levels reported in all samples. Uptake via stomatal transpiration does not occur (Pallaghy 1972; WHO 1996) and Tl is thought to be poorly distributed from root to shoot in planta – which could explain the significantly lower (0.006) Tl concentrations in aerial samples, compared to root observed in this current study – particularly between flowers and root (0.004) (see Table 4.4).

EPA (2012) discusses the complexity surrounding derivatisation of oral toxicological references for Tl (metallic Tl and Tl salts). The report outlines (p)-RfD’s for Tl-salts ranging from 1 – 5 x 10⁻⁵ mg.kg⁻¹.day⁻¹ (EPA, 2012), and a sub-chronic NOAEL (alopecia) for Tl-(I)-acetate of 0.4 mg·(kg BW)⁻¹·d⁻¹(Downs et al. 1960). In the absence of official reference values/points, a surrogate RfD for mineral water inferred by the German Federal Institute for Risk Assessment (BfR, 2004), and sub-chronic NOAEL (alopecia) were used to model exposure risk in this study (see Table 4.7). Estimated acute exposures of 0.001 and 0.004 µg·(kg BW)⁻¹·d⁻¹ for the average and 95th percentile adult Irish consumers representing 1.3 and 5.2 percentage contribution to the ARfD, respectively. Estimated chronic exposure were >10,000 (SF) in this food category, and thus deemed “no risk” to Irish adult consumers. The data thus demonstrates that both acute and chronic Tl exposure from this single food commodity is of no risk to adult Irish consumers. A recent review by the National Food Institute (Tech. University of Denmark) published in the EFSA Journal (NFI et al. 2020) urges the collection of occurrence data of Tl, tellurium and rare earth elements (REEs) in plant-based foodstuffs considering the increased environmental prevalence of these substances, as a result of technological advancements.

4.3.2.11 Lead (²⁰⁸Pb)

The human health effects and dietary exposure to Pb in plant-derived raw materials for human consumption has been previously reviewed (Kenny et al. 2020). Pb is a Group 2A (probable) human carcinogen (IARC 2006), and benchmark dosages were calculated for use as toxicological reference points (Steinkellner 2020).

Pb concentrations ranged from 12.60 (IN_09; flowers) to 4617.42 µg·kg⁻¹ (IN_219; root) and the Pb concentration could not be estimated in 11% of the samples analysed (IN_20, 23 (leaves) and IN_24 (flowers)) since the analytical values of the 1:5 diluted samples had concentrations above the calibration range and subsequent dilution was not possible. Pb is considered less mobile within plant systems, indicating that root-Pb levels likely
originates from the surrounding soil/rhizosphere, while its occurrence in aerial counterparts is putatively derived from aerial deposition (e.g. vehicle exhaust emissions) (Ražić et al. 2008). In agreement, Swaileh et al. (2004) demonstrated a positive correlation between Pb content in *I. viscosa* leaves and distance from a roadside in Palestine. A recommendation by Swaileh et al. (2004) is to collect leaves of the similar size for consistency, following statistically significant differences in Pb and Fe levels in *I. viscosa* leaves of varying sizes.

Pb, Bi and Tl are daughter isotopes of nuclear decay series (e.g. actinium, thorium, uranium /radium series) and their presence in terrestrial-derived food could be an indicator of either primordial (natural) radionuclide decay within soil matrices, or the transformation of anthropogenic (artificial) by-products originating from nuclear fallout – such as the Chernobyl nuclear power plant accident. Radionuclide contamination of food, however, is considered “outside the remit” of the EFSA currently (EFSA 2011).

As outlined in Table 4.5, the pharmacopeial ML for Pb permitted in herbal material/herbal drugs is 5000 µg kg⁻¹ as per Ph. Eur. (2013), and 10,000 µg kg⁻¹ in dried herbs and spices according to national ML’s in Ireland (SI No. 44/1992 amend. 44/1972). All *Inula*
samples analysed were below these ML’s for Pb. There are no ML’s specified for fresh and/or dried herbs for use as food or food ingredients in European legislation, at the time of writing. Commission Reg. (EC) No. 1881/2006 (‘Setting maximum levels for certain contaminants in foodstuffs’), as amended by Commission Reg. (EC) No. 629/2008, applies to metal contaminants in food, including dietary supplements. Commission Reg. (EU) 2015/1005 amends the criteria for lead (Pb) in foodstuffs (‘Maximum levels of lead in certain foodstuffs’) (European Commission 2006, 2008, 2015). Within this document, the consumption of herbal tea and infusions is acknowledged as a relevant contributor to dietary Pb exposure and calls for the establishment of specific ML(s) in future amendments. It is clearly stated in the document, that a lack of data on dry plant parts (i.e. herbs or herbal ingredients, and tea leaves) is primarily why ML’s have not yet been established for this food category. An ML of 100 g.kg100 µg.kg-1 is specified for Pb in “vegetables excluding leafy brassica, salsify, leaf vegetables and fresh herbs, fungi and fruiting vegetables”. A higher ML of g.kg300 µg.kg-1 is specified for Pb in “leafy brassica, salsify and leaf vegetables excluding fresh herbs and the following fungi...”. And an ML of 3000 µg.kg-1 g.kg is specified for Pb in food supplements (unspecified; “as sold” or final product format). In the absence of a specified ML for herbs, the aforementioned [vegetable] ML’s of 0.1 and 3000 µg.kg-1 g.kg, respectively, were used as surrogate values in this present study because they represent the most applicable limits to terrestrial-derived herbal material such as elecampane. Figure 4.6 illustrates the number of samples which would theoretically exceed these ML’s if applicable to herbs, which as shown in the figure, is approximately 59% of the samples analysed. It is clear that more data, including occurrence and consumption data, is required to critically evaluate the risk of Pb in herbs/herbal ingredients.

In terms of dietary risk, estimated chronic exposures of 0.049 and 0.189 µg·(kg BW)−1·d−1 for the average and 95th percentile adult Irish consumers were recorded, and deemed a “potential risk” since the RP/exposure is <1,000 (EFSA et al. 2019). The data suggest that dietary exposure to Pb in edible plants and herbs food is of potential risk to Irish adult consumers, and further analysis is advised. The value this exposure estimate is modelled on is elecampane root sourced from Cork City (IN_19). The accompanying aerial leaf samples collected from the same parent plant along with other aerial samples from Cork City (IN_23-24) could not be quantified because the Pb concentrations in the samples exceeded the highest point on the calibration range.
The commercial samples contained notably high Pb levels, the statistical significance of which could not be formally assessed due to insufficient samples size. The commercial samples were acquired from a U.K. supplier and the origin or source location was not declared on the packaging thus making product traceability impossible. The findings for Pb indicate a potential risk of adverse health effects following chronic consumption and further investigation is advised, including soil and water analysis from the cultivation land - particularly if the farms are producing vegetables or crops for local communities. When interpreting exposure data, it is however important to note that it may not be the food commodities with the highest metal levels, but the foods which are more regularly consumed in larger quantities (i.e. intake frequency and duration) which pose the greatest risk over time (Kenny et al. 2020).

4.3.2.12 Bismuth (\(^{209}\)Bi)

The occurrence, exposure and toxic effects of dietary Bi is reviewed elsewhere (Dolara 2014). Bi-salt complexes are applied pharmaceutically as gastrointestinal agents acting as antimicrobial and mucosal protectants in conditions such as Travellers’ Diarrhoea (TD), ulcers and \(Helicobacter pylori\) infection (Alkim et al. 2017). An NOAEL of 16 mg-(kg BW)\(^{-1}\)d\(^{-1}\)was recommended by the European Scientific Community on Consumer Safety (SCCS) for safety calculations based on a study of the effects of sub-chronic (>90 days) oral toxicity in rats (SCCS, 2013). An earlier study by Sano et al. (2005) reported an NOAEL of 1000 mg.kg\(^{-1}\) for Bi from an acute oral (single) and 28-day (repeated) administration study in rats. There is no data available for chronic toxicity (>12 months), and therefore a HBGV for Bi in food is yet to be derived. In this study, Bi concentrations ranged from 3.60 (IN_09; flowers) to 168.02 µg.kg\(^{-1}\) (IN_06; leaves). A statistical significance was observed for Bi distribution in samples of naturalised elecampane, notably flowers and leaves (0.012). Dietary exposure of Bi is considered unlikely to be of toxicological concern; however, this has not yet been evaluated by the EFSA (Dolara 2014). Inclusion of Bi and Pt in future versions of the RACE platform is recommended.

4.3.2.13 Aluminium (\(^{27}\)Al)

The human health effects and dietary exposure to Al in plant-derived raw materials for human consumption has been previously reviewed (Dolara 2014; Kenny et al. 2020). Al levels could not be estimated, except for flowers collected in Co. Leitrim (IN_18; 3680 µg.kg\(^{-1}\)) (data not shown), since the analytical values of the 1:5 diluted samples had
concentrations above the calibration range and subsequent dilution was not possible for any samples. Lower Al levels were expected in samples from the West of Ireland, in accordance to soil data reported by Fay et al. (2007). High Al in above- and under-ground parts of *I. viscosa* were similarly reported (>1000 mg.kg⁻¹) (Christou et al. 2017).

**4.3.2.14 Titanium (⁴⁷Ti)**

Levels of Ti varied widely between samples in this study, ranging from 140.45 (IN_06; leaves) to 4988.71 µg.kg⁻¹ (IN_10; root). Similarly, Filippini et al. (2020) reported variable Ti concentration in (unspecified) plant-derived foods ranging from 0.07 - 6670 µg.kg⁻¹. In this study, differences in Ti levels between plant parts of naturalised elecampane were revealed however the difference was not statistically significant (see Table 4). Levels in commercial *Inula* samples (IN_25-27) exceeded the calibration range and could not be accurately determined. There are currently no HBGV’s derived for Ti and so RACE analysis was not performed.

**4.3.2.15 Vanadium (⁵¹V)**

V is widely used in industry from electrochemical storage systems to photographic development and colouring agents (Treviño et al. 2019). V is reported as a genotoxic substance (EFSA 2004), and its essentiality to humans has not been demonstrated (EFSA SCF & NDA Panel 2006). Thus, there are no established DRV’s/HBGV’s in Europe (EFSA 2004, 2008). The U.S. IOM (Institute of Medicine, 2001) derived an UL, which was used as a surrogate RV in this study. V concentrations were higher than those previously reported (Filippini et al. 2020) ranging from 14.99 (IN_05; flowers) to 1383.85 µg.kg⁻¹ (IN_25; whole). There is no clear regularity to the distribution of V in plant organs (Kabata-Pendias 2011) which our data reflects. Differences in V levels between plant parts of naturalised elecampane was not statistically significant (see Table 4.4). Estimated acute exposures of 0.05 and 0.19 µg.(kg BW)⁻¹d⁻¹ for the average and 95th percentile adult Irish consumers. Estimated chronic exposures of 0.001 and 0.004 mg.day⁻¹ for the average and 95th percentile adult Irish consumers which represents 0.06 and 0.23 percentage contribution to the UL. The data suggests that dietary exposure to V in edible plants and herbs is of no risk to Irish adult consumers.

**4.3.2.16 Chromium (⁵²Cr)**

The human health effects and dietary exposure to Cr in plant-derived raw materials for human consumption has been previously reviewed (Kenny et al. 2020). A wide variation
in Cr levels were quantified in this study, ranging from 89.19 (IN_18; flowers) to 3649.73 µg.kg⁻¹ (IN_25; whole). Underlying greywackes, igneous rock, and basalt attribute to higher Cr levels in soils in the Republic of Ireland (Fay et al. 2007). Palmer et al. (2016) describes total Cr and Ni in North-Eastern soils of N.I. as “unusually high”, which is likely linked to the basalts of the Antrim Plateau. This is reflected in our results, with roots from N.I. containing the highest Cr levels out of all the collected samples (IN_10). Soil-acquired Cr tends to sequester in the root (Kabata-Pendias 2011) and while there is evidence of an increasing trend in root-Cr levels in our data; statistical analysis revealed there to be no significance in Cr distribution between plant parts of naturalised elecampane (see Table 4.4).

Cr levels are markedly higher in the commercial samples (IN_25-27) which were obtained prior to analysis in either comminuted (IN_26) or cut (IN_25 and 27) format. It is plausible to suggest that Cr contamination from the manufacturing process may have contributed to the higher Cr content in these products, compared to the cultivated and wild-collected counterparts. Similar findings have been reported in the literature for Cr transfer from industrial equipment to plant-based products, such as tea leaf processing (Seenivasan et al. 2008).

It is assumed that the Cr present in terrestrial-derived food is Cr(III) (EFSA-CONTAM 2014). The highest concentration of Cr detected in this study (3649.73 µg.kg⁻¹) is 10X the TDI of Cr(III), however, dietary exposure through consumption of a plant-derived staple food item (e.g. potatoes), which is consumed more frequently than herbs and edible flowers in the Irish diet, is still deemed “no risk” to Irish consumers (data not shown). An estimated chronic exposure of 0.04 and 0.15 µg·(kg BW)⁻¹·d⁻¹ for the average and 95th percentile Irish adults was determined from the survey data. This data represents a ≤0.05% contribution to the TDI and thus demonstrates no risk to Irish adult consumers.

4.3.2.17 Cobalt (⁵⁹Co)

The occurrence, exposure and effects of dietary Co is reviewed elsewhere (Dolara 2014). An ARfD has not yet been derived for Co by either EFSA or the U.S. EPA, however a provisional R/D (30 µg.kg⁻¹.day⁻¹) has been postulated by Finley et al. (2012). In this present study, Co concentrations ranged from 12.83 (IN_22; root) to 246.19 µg.kg⁻¹ (IN_25; whole). These findings are in line with those reported for cereal grains (5 – 270 µg.kg⁻¹) and similar to concentrations in various vegetables (8 – 170 µg.kg⁻¹) (Kabata-
Soil texture reportedly controls Co levels in plants (Sillanpää et al. 1992), as well as the effects of podzolization. Co levels varied significantly between plant parts of naturalised elecampane (0.030), particularly between root and leaves (0.028). Estimated chronic exposure was ≤0.01 µg·(kg BW)\(^{-1}\)d\(^{-1}\). The data suggests that Co exposure through this food category represents no risk to Irish adult consumers.

### 4.3.2.18 Nickel (\(^{60}\)Ni)

The IARC (1990) categorised Ni as a Group 1 Human Carcinogen based on evidence of carcinogenesis in respiratory tissues. Average daily Ni intakes of 20-406 µg.day\(^{-1}\) have been estimated (Dabeka and McKenzie 1995). Oral intakes of >500 µg.kg\(^{-1}\) (approx. 8 µg·(kg BW)\(^{-1}\)d\(^{-1}\)) reportedly triggered dermal symptoms in Ni-sensitized subjects. Average concentrations in foodstuffs are within 10 – 100 µg.kg\(^{-1}\) (IARC, 2012). The occurrence, exposure and effects of dietary Ni is reviewed elsewhere (Dolara 2014). Ni concentrations analysed in this study ranged from 157.88 (IN_22; root) to 3909.26µg.kg\(^{-1}\) (IN_02; leaves), which is in line with levels reported in various vegetables, fruits and grains (60 – 2000 µg.kg\(^{-1}\)) (Kabata-Pendias 2011). In this present study, notably higher concentrations were observed in samples of the flowers and stems of naturalised elecampane – however, these variations were not statistically significant (see Table 4.4). This observed foliar prevalence of Ni is consistent with previous findings (Halstead et al. 1969).

The essentiality of Ni in the human diet has not been demonstrated (SCF, 1993), and no maximum contaminant or impurity limits in foodstuffs are yet derived (EFSA-COMTAM et al. 2020). EFSA’s CONTAM Panel (2015) established a TDI of 2.8 µg·(kg BW)\(^{-1}\)d\(^{-1}\), which has recently been increased to 13 µg·(kg BW)\(^{-1}\)d\(^{-1}\). Derivation of an AR/FD is not yet supported by EFSA due to insufficient data (EFSA-COMTAM et al. 2020). A surrogate R/D (20 µg·(kg BW)\(^{-1}\)d\(^{-1}\)) was therefore used for this study, as per Filippini et al. (2020). Estimated acute exposures of 0.13 and 0.53 µg·(kg BW)\(^{-1}\)d\(^{-1}\), and estimated chronic exposures of 0.42 and 0.16 µg·(kg BW)\(^{-1}\)d\(^{-1}\), for the average and 95\(^{th}\) percentile adult Irish consumers were calculated. This data therefore suggests that Ni exposure from herbs and edible flowers as a single food commodity is of no risk to adult Irish consumers.

### 4.3.2.19 Copper (\(^{63}\)Cu)

Cu could not be estimated for 74% of the samples tested since the analytical values exceeded the calibration range (3450 µg.kg\(^{-1}\)) and subsequent dilution was not possible.
Estimated chronic exposures at the highest quantified value (5305.85 µg.kg\(^{-1}\)) were 0.004 and 0.016 mg.day\(^{-1}\) for the average and 95\(^{th}\) percentile adult Irish consumers - which represents a 0.7 – 2.6% percentage contribution to the UL, and therefore is of no risk to adult Irish consumers.

### 4.4 Non-parametric statistical analysis results

For statistical assessment, the twenty-four naturalised Irish (wild, cultivated) elecampane samples were grouped based on relatedness and could not be analysed separately considering the existence of repeated measures within the dataset (i.e. 2 – 3 samples derived from the same mother plant). This resulted in nine representative samples with at least two values for each (i.e. two out of root, leaves or flowers) – as outlined in Table 4.4. The commercial samples were excluded from assessment because the sample size was insufficient to formally test (i.e. \(n \leq 3\) for commercial root and whole samples). The element concentration was not normally distributed for differences between plant parts (i.e. root and leaves, leaves, and flowers, root, and flowers) thus a repeated samples Friedman two-way ANOVA using ranks was performed. Using a level of significance (0.05), it was determined that the distribution of element concentrations is significantly different between plant parts in elecampane for Be, Li, Mo, Ba, Cd, Sn, Bi, Co, Hg, and Tl. Conversely, distribution of Pt, Cr, Cu, Pb, Ni, Ti, and V is not significantly different by plant part. A pairwise comparison was then performed to determine where the difference is (i.e. between root and leaves, root, and flowers, or leaves and flowers). As shown in Table 4.4, a significant difference (<0.05) was observed for Be, Li, Ba, Cd, Bi (flowers – leaves), Mo, Sn, Co, Hg (root – leaves) and Ba and Tl (flowers – root). These results could contribute to a greater understanding on element distribution and accumulation within Inula species.

### 4.5 Conclusion

This study explored the novel metallomic profiling of multi-origin *I. helenium* L. (elecampane) samples. The isotopes were quantified (\(^7\)Li, \(^9\)Be, \(^{95}\)Mo, \(^{111}\)Cd, \(^{118}\)Sn, \(^{137}\)Ba, \(^{195}\)Pt, \(^{197}\)Au, \(^{202}\)Hg, \(^{205}\)Tl, \(^{208}\)Pb, \(^{209}\)Bi, \(^{24}\)Mg, \(^{27}\)Al, \(^{47}\)Ti, \(^{51}\)V, \(^{52}\)Cr, \(^{55}\)Mn, \(^{56}\)Fe, \(^{59}\)Co, \(^{60}\)Ni and \(^{63}\)Cu) using a validated high-resolution ICP-SFMS method following microwave acid-digestion of the herbal samples. Statistical analysis revealed a significant difference
in element distribution between flowers – leaves (Be, Li, Ba, Cd, Bi), root – leaves (Mo, Sn, Co, Hg) and flowers – root (Ba and Tl) in naturalised elecampane.

The mathematical RACE tool established by EFSA was used to contextualise the toxicological significance of acute and/or chronic dietary exposure to metal contaminants in a specific food matrix (i.e. ‘herbs and edible flowers’) relevant to Irish consumers. Results showed that chronic exposure to lead (Pb) at a maximum quantified concentration of 4617.42 µg.kg⁻¹ in edible plant material is of potential risk to adult consumers in Ireland (18-65 y), at an estimated mean and 95th percentile exposure of 0.049 and 0.189 µg·(kg BW)⁻¹·d⁻¹, respectively. Further investigation is advised, including soil and water analysis from the cultivation land to ascertain if Pb levels are within acceptable environmental limits, particularly if the farms are producing vegetables or crops for local communities. The findings show, with the exception of Pb, that there is a low probability of adverse health effects to Irish consumers following intake of herbs and edible flowers at the element levels quantified in this study. Furthermore, at the levels quantified, a RASFF notification would not be required in a routine risk evaluation for metal contaminants.

Dietary exposure to Au, Al, Fe, Mg, and Mn could not be estimated since these elements exceeded the calibration range and could not be accurately determined. Exposure to Pt and Bi could not be assessed either because they are currently excluded on the list of available substances on the RACE platform – their addition in forthcoming versions is advised, considering their increased environmental prevalence as a result of technological advancements and subsequent anthropogenic-derived pollution.

Fifty-two % of the herbal samples exceeded European limits for Cd in food, and one sample exceeded the European pharmacopeial ML for Cd impurities in herbal material/drugs. Dietary exposure to Cd at the highest observed concentration (1285.97 µg.kg⁻¹) despite exceeding European regulatory and pharmacopeial permissible limits was deemed “no risk” to Irish consumers when considering consumption of this single food category. These results do not however take into account the aggregate dietary and environmental exposure risk to Cd and should be interpreted with caution. The remaining elements (Li, Be, Mo, Sn, Ba, Hg, Tl, V, Cr, Co, Ni, Cu) were well below regulatory and pharmacopeial limits in all samples of naturalised and commercial elecampane. Dietary exposures to these elements are therefore of negligible concern to Irish consumers based on the exposure assessment of the maximum concentration quantified within a cohort of
27 samples. Outputs from the RACE tool, however, focus on one contaminant in one food commodity at a time, and therefore background exposure from all other dietary sources needs consideration. Furthermore, the findings are based on an “aggressive” acid-extraction, which would not reflect the traditional use of *Inula* spp. in medicinal formulations (e.g., tinctures, decoctions, lozenges etc.) or culinary recipes. The metal transfer rate, from the raw starting material to the final product or preparation, is therefore an important consideration for future metallomic profiling studies in this area of research.

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Declaration of interest

The authors declare that there is no conflict of interest in this work.

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Chapter V

Multi-Elemental profiling and the mathematical non-carcinogenic risk assessment of fifty plant matrices for use in herbal medicinal products and botanical dietary supplements using high-resolution inductively coupled plasma sector field mass spectrometry (HR-ICP-SFMS)

Manuscript based on this chapter in preparation for publication.
Abstract

Plant material is often used as ingredients in (traditional) herbal medicinal products (T)HMPs), plant-food supplements (PFS) and herbal teas/infusions – governed in Europe by either food legislation or the (EC) Herbal Directive. In this study, the multi-elemental profile(s) of fifty botanical matrices commonly used in botanical preparations - including hawthorn, elderflower, lavender, St. John’s Wort, and peppermint - were investigated using a validated high-resolution inductively coupled plasma sector field mass spectrometry (HR-ICP-SFMS). Plants provide an avenue for the acquisition of essential minerals in the diet - nevertheless, contamination with non-essential or toxic levels of inorganic metals can impurify raw material during cultivation, processing, and storage.

A diverse range of concentrations were quantified for each metal analyte across all samples: $^7\text{Li}$ (17.51 – 3964.03 µg.kg$^{-1}$); $^9\text{Be}$ (2.95 – 121.50 µg.kg$^{-1}$); $^{95}\text{Mo}$ (74.62 – 4504.50 µg.kg$^{-1}$); $^{111}\text{Cd}$ (4.75 – 325.33 µg.kg$^{-1}$); $^{118}\text{Sn}$ (6.39 – 165.15 µg.kg$^{-1}$); $^{137}\text{Ba}$ (746.66 – 4645.63 µg.kg$^{-1}$); $^{195}\text{Pt}$ (2.05 – 32.68 µg.kg$^{-1}$); $^{202}\text{Hg}$ (4.67 – 29.87 µg.kg$^{-1}$); $^{205}\text{TI}$ (2.64 – 90.82 µg.kg$^{-1}$); $^{208}\text{Pb}$ (11.58 – 4248.07 µg.kg$^{-1}$); $^{209}\text{Bi}$ (2.42 - 29.64 µg.kg$^{-1}$); $^{47}\text{Ti}$ (130.96 – 5827.41 µg.kg$^{-1}$); $^{51}\text{V}$ (14.81 – 1757.80 µg.kg$^{-1}$); $^{52}\text{Cr}$ (99.64 – 4534.43 µg.kg$^{-1}$); $^{59}\text{Co}$ (20.68 – 651.76 µg.kg$^{-1}$); $^{60}\text{Ni}$ (229.94 – 6060.33 µg.kg$^{-1}$) and $^{63}\text{Cu}$ (1910.27 – 6340.13 µg.kg$^{-1}$). Highest quantified concentrations were observed in ox-eye daisy flower (Li), dandelion leaf (Be, Cd, Sn) and root (Ti), great mullein leaf (Mo), elderberry fruit (Ba), hawthorn flower and leaf (Hg, Pb, Bi), comfrey root (V), boldo leaf (Cr), coltsfoot (Ni) flower and aerial bush vetch (Cu).

Shorter-than-lifetime daily intakes were estimated for each analyte in all sample matrices using conservative and realistic theoretical exposure scenarios. The non-carcinogenic risk assessment for exposure to Cd, Cu, Hg and Pb was evaluated using Hazard Quotient (HQ) and Hazard Index (HI) estimations. All botanical samples analysed were below the compendial ML’s for metal impurities in herbal substances/starting materials: Cd (≤ 1 mg.kg$^{-1}$), Hg (0.1 mg.kg$^{-1}$), and Pb (5 mg.kg$^{-1}$), however, Li, Mo, Ti, Pb, Cr, Co, and Ni were quantified at potentially unsafe levels (e.g. high EDI[@ 200 g.day-1] > HBGV) at the theoretical worst-case exposure scenario. Furthermore, 16 (n = 21 out of 50) of the plant species analysed were categorised as potentially unsafe to consumers (HI ≥ 1) with regards to the non-carcinogenic cumulative exposure (HI ≥ 1).
A major data gap hindering the risk evaluation of plants and derived products, is the lack of intake and consumption data at European level and the availability of guidance in the absence of experimental data (e.g. in the form of generic input parameters such as metal transfer rates, exposure durations, exposure frequency) to facilitate harmonised deterministic and probabilistic risk assessment criteria that would help ensure botanical safety.

**Key words:** ICP-SFMS; herbs; botanicals; medicinal plants; dietary exposure assessment; toxicity
5.1 Introduction

Plants serve a dual role in medicine and food. With reference to regulatory legislation, plant materials used as ingredients in dietary supplements are increasingly described as “botanicals”, whereas plants used as active ingredients in medicinal herbal products are more commonly referred to as “herbs”, regardless, the shared denominator in all such products, are plants (Trovato & Ballabio, 2018). Plants are essentially wildcards which are not universally governed by a singular regulatory (EU) framework. In the absence of a harmonised process at European level, their intended use determines their route of regulation, not their phytochemical composition or toxicological properties, as one might expect. The diverse range of food products in which they can be used as ingredients, are distinguishable primarily by labelling and health benefits claimed by the manufacturer. Plants and products thereof can be regulated, depending on the proposed use and recommended intake, in accordance to various legislative categories – be it food (general-, novel-, fortified- or genetically modified-food), pharmaceutical, herbal (i.e. Herbal Directive) or cosmetics.

As it currently stands, in the absence of clarity, the same product can be marketed as a foodstuff in one country and a medicinal product in another (Kenny et al., 2020). This is further complicated with the application of the “principle of mutual recognition”, whereby any legally marketed product in one European Member State can be sold in other Member States (Larrañaga-Guetaria, 2012). The E.C. insists that is not feasible to pursue harmonisation of botanicals and conditions of usage until further scientific data is available (Coppens & Pettman, 2018). Furthermore, the application of approved health claims regulated under (EC) 1924/2006 to botanical-containing products has resulted in a legal moratorium (Coppens & Pettman, 2018), largely related to conflicting opinions on the level of scientific rigor required to substantiate such claims (FSAI, 2020). Currently, the BELFRIT [Belgium, France, Italy] list is the only existing amalgamation of accepted “safe” botanicals for use in supplements (Trovato & Ballabio, 2018) and while it serves as a good starting point towards harmonisation, its use is not legally enforceable in Member States. In Ireland, the Food Safety Authority of Ireland (FSAI) has rendered the BELFRIT list(s) as unsuitable for adoption in the regulatory risk management of botanicals on the Irish market. This decision was based on “non-transparency” in the methodologies used (FSAI, 2020), however the FSAI agree that the use of the BELFRIT list(s) in conjunction with the available EFSA guidance
documentation and Compendium of Botanicals (CoB) are useful preliminary resources for the risk assessment and management of botanical ingredients (FSAI, 2020). The EFSA (2009) acknowledges the market volume expansion for plant-based products and the subsequent need for improved characterisation of an increasing botanical product portfolio and overall harmonisation of the risk assessment process. A recurring opinion among governing bodies is the lack of data in the realm of botanical sciences.

The human health effects and regulation of metal contaminants in plant-derived food and phytopharmaceuticals has been previously reviewed (Kenny et al., 2020). In brief, existing gaps include a lack of (Coppens & Pettman, 2018; FSAI, 2020; Garcia-Alvarez et al., 2018):

- Prospective population intake/consumption data
- Nutrient and elemental profiles for medicinal plants
- Specification data including permissible or maximum limits for a greater suite of metal contaminants/impurities
- List of permitted plant species regarded as safe for oral consumption
- List of restricted plant species regarded as unsafe for oral consumption
- Assessed health claims for medicinal plants
- Toxicological risk assessments for medicinal plants regarding phytochemical composition
- Advisory labelling statements
- Global monitoring system(s)

In summary, concerns with regards to metal contaminants in botanical ingredients or herbal substances (i.e. starting or raw material) include the unregulated cultivation of medicinal plants, non-enforceable Good Agriculture and Collection Practices (GACP) for raw material of plant origin, and the absence of general specifications and acceptance criteria (i.e. maximum residue limits) (Kenny et al., 2020). Maximum permissible limits or maximum levels (MLs) for elemental impurities in medicinal plants can vary, in some cases significantly, between countries and organisations (Kenny et al., 2020), such as: Pb (3.0 – 30.0 mg.kg\(^{-1}\)); As (0.6 – 5.0 mg.kg\(^{-1}\)); Cd (0.2 – 4.0 mg.kg\(^{-1}\)); Hg (0.02 – 1.0 mg.kg\(^{-1}\)) and Cu (10.0 – 150.0 mg.kg\(^{-1}\)) (Luo et al., 2021). Currently, there are no universal limits for inorganic metal impurities in medicinal plants or products thereof – and uniformity may never be achieved. Two plausible solutions to consider are the application of the ICH
Q3D guidelines to phytopharmaceuticals, or alternatively, the establishment of an extended suite of toxicologically significant specifications for the control of inorganic metal contaminants, for both MLs and instrumental procedures in plant-derived products (Kenny et al., 2020). Exceedance of such limits would not automatically affirm the presence of risk, but more so act as a “trigger” that warrants further investigation. Some authors acclaim that in processes whereby herbal substances (raw plant material) are found in exceedance of threshold limits for elemental impurities, justification should be waived provided compliance is assured in the final [consumer-ready] product (Guédon et al., 2008). Others assume the probability of exceeding As, Co, Ni and V limits in herbal drugs is low (using ICH Q3D limits as guidance) and thus general limits for the aforementioned in future editions of the European Pharmacopeia (Ph. Eur.) is not a necessity (Albert et al., 2018).

A major data gap hindering the risk evaluation of plants and derived products, is the lack of intake and consumption data at European level and the availability of guidance in the absence of experimental data. Such critical information is required to facilitate harmonised deterministic and probabilistic risk assessment criteria with the outlook of ensuring botanical safety. We later discuss the proposition of such generic input parameters for use when risk assessing plant matrices, including metal transfer rates, exposure durations and frequency data. There is an explicit need for further data within the botanical sciences (i.e. phytotherapy, pharmacognosy) and it is hoped that studies, such as the present investigation, can help contribute knowledge in this field.

The purpose of this investigation was to analyse the multi-elemental profiles of fifty common medicinal and aromatic plants sourced from 12 countries and mathematically assess their potential non-carcinogenic risk to human health via oral consumption. The following is a list of the analysed botanical matrices, with the botanical common name listed in parentheses: *Crataegus laevigata* (hawthorn), *Taraxacum officinalis* (dandelion), *Arnica montana* (arnica), *Sambucus nigra* L. (elder) *Sambucus nigra* fruct. (elderberry), *Sambucus nigra* flos. (elderflower), *Calendula officinalis* (marigold), *Aesculus hippocastanum* (horse chestnut), *Urtica dioica* (nettle), *Achillea millefolium* (yarrow), *Symphytum officinale* (comfrey), *Borago officinalis* (borage), *Tussilago farfara* (coltsfoot), *Vicia sepium* (bush vetch), *Lotus corniculatus* (birds foot trefoil), *Leucanthemum vulgare* (ox-eye daisy), *Myrrhis odorata* (sweet cicely), *Rhinanthus*
minor (yellow rattle), Menyanthes trifoliata (bogbean), Artemisia vulgaris (mugwort), Verbascum thapsus (great mullein), Jasonia glutinosa (rock tea), Silene saxifraga L. (tufted catchfly), Salvia officinalis L. (Sage), Glycyrrhiza glabra (liquorice), Althaea officinalis (marshmallow), Lavandula angustifolia (lavender), Hypericum perforatum (St. John’s Wort), Melissa officinalis (lemon balm), Santolina chamaecyparissus (cotton lavender), Mentha × piperita (peppermint) and Peumus boldus Molina (boldo). Shorter-than-lifetime daily intakes were estimated for each analyte in all sample matrices using conservative and realistic theoretical exposure scenarios. And the non-carcinogenic risk assessment for exposure to Cd, Cu, Hg, and Pb was evaluated using theoretical Hazard Quotient (HQ) and Hazard Index (HI) estimations.

This is the first account of the elemental profiling of arnica, bush vetch, sweet cicely, yellow rattle, bogbean, rock-tea and tufted catchfly to-date.

5.2 Materials and methodology

5.2.1 Reagents and materials

Ultrapure milli-Q water (15.0 MΩ·cm); trace-metal grade nitric acid (HNO₃) (PlasmaPure, 67 – 69% v/v, SCP Science); Tune-Up solution (Thermo Scientific, USA; 1 µg.L⁻¹). Multi-elemental standard solutions including lithium (⁷Li), beryllium (⁹Be), molybdenum (⁹⁵Mo), cadmium (¹¹¹Cd), tin (¹¹⁸Sn), barium (¹³⁷Ba), platinum (¹⁹⁵Pt), gold (¹⁹⁷Au), mercury (²⁰²Hg), thallium (²⁰⁵Tl), lead (²⁰⁸Pb), bismuth (²⁰⁹Bi), magnesium (²⁴Mg), aluminium (²⁷Al), titanium (⁴⁷Ti), vanadium (⁵¹V), chromium (⁵²Cr), manganese (⁵⁵Mn), iron (⁵⁶Fe), cobalt (⁵⁹Co), nickel (⁶⁰Ni) and copper (⁶⁵Cu) (Agilent, USA) were used in this study. The internal standards (ISTDs) used in this study were gallium (⁷¹Ga), scandium (⁴⁵Sc), rhodium (¹⁰³Rh), iridium (¹⁹³Ir) and again these were certified standards traceable to NIST reference materials, sourced from SCP Science. Polymethylpentene (PMP) beakers, volumetric flasks, graduated cylinders, and pipettes were sourced from VWR International Ltd. (Blanchardstown, Dublin 15, Ireland).

5.2.1.1 Instrumentation

The MARS-6™ microwave-accelerated reaction system was used for sample digestion (CEM Corporation, USA). The Thermo Scientific Element 2™ ICP-SFMS (Thermo
Scientific, USA) was coupled with an ESI autosampler and was used for multi-elemental analysis (metallic profiling) of samples.

### 5.2.1.2 Sources of dried raw plant material

Botanical samples were sourced in raw bulk format (dried, and: cut, fragmented, powdered, or whole) from registered commercial suppliers, wild collections, and cultivated sources (See Table 5.1). These samples at large represent raw starting materials or herbal/botanical ingredients and are not considered consumer-ready products.

**Table 5.1 List of botanical samples (n = 30 species) analysed in this study.**

<table>
<thead>
<tr>
<th>Sample I.D.</th>
<th>Latin name</th>
<th>Common Name</th>
<th>Plant Part; Specification</th>
<th>Origin</th>
<th>Source</th>
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<tbody>
<tr>
<td>BT 01</td>
<td>Crataegus laevigata</td>
<td>Hawthorn</td>
<td>Flower &amp; Leaf; Cut</td>
<td>China</td>
<td>Commercial</td>
</tr>
<tr>
<td>BT 02</td>
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<td>Eastern Europe</td>
<td>Commercial</td>
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<td>Wild</td>
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<td>Wild</td>
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<tr>
<td>BT 06</td>
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<tr>
<td>BT 12</td>
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<td>Flower; Whole; Rubbed</td>
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<td>BT 13</td>
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<td>BT 17</td>
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<td>Comfrey</td>
<td>Stem; Cut</td>
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<td>BT 26</td>
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<td>Borago officinalis</td>
<td>Borage</td>
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<td>Germany</td>
<td>Commercial</td>
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<tr>
<td>BT 28</td>
<td>Tussilago farfara</td>
<td>Coltsfoot</td>
<td>Flowers; Cut</td>
<td>Albania</td>
<td>Commercial</td>
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<td>BT 29</td>
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<td>Coltsfoot</td>
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<td>Poland</td>
<td>Commercial</td>
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<td>BT 30</td>
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<td>Bush Vetch</td>
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<td>Ireland</td>
<td>Wild</td>
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<tr>
<td>BT 31</td>
<td>Lotus corniculatus</td>
<td>Birds Foot Trefoil</td>
<td>Aerial; Cut</td>
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<td>BT 32</td>
<td>Leonanthemum vulgaris</td>
<td>Ox-Eye Daisy</td>
<td>Flower; Whole</td>
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<td>Wild</td>
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<tr>
<td>BT 33</td>
<td>Artemisia vulgaris</td>
<td>Sweet Cicely</td>
<td>Aerial; Cut</td>
<td>Ireland</td>
<td>Wild</td>
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<td>BT 34</td>
<td>Myrrhis odorata</td>
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<td>Aerial; Cut</td>
<td>Ireland</td>
<td>Wild</td>
</tr>
<tr>
<td>BT 35</td>
<td>Rhinanthus minor</td>
<td>Yellow Rattle</td>
<td>Aerial; Cut</td>
<td>Ireland</td>
<td>Wild</td>
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<tr>
<td>BT 36</td>
<td>Menyanthes trifoliata</td>
<td>Bogbean</td>
<td>Aerial; Cut</td>
<td>Ireland</td>
<td>Wild</td>
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<tr>
<td>BT 37</td>
<td>Artemisia vulgaris</td>
<td>Mugwort</td>
<td>Flower &amp; Leaf; Cut</td>
<td>Ireland</td>
<td>Wild</td>
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<td>Verbascum thapsus</td>
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<td>Sligo</td>
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<td>Jasminum officinatum</td>
<td>Rock Tea</td>
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<td>Tufted Catchfly</td>
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<td>Salvia officinalis L.</td>
<td>Sage</td>
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<td>BT 42</td>
<td>Glycyrrhiza glabra</td>
<td>Liquorice</td>
<td>Root; Cut</td>
<td>Spain</td>
<td>Cultivated</td>
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<tr>
<td>BT 43</td>
<td>Althaea officinalis</td>
<td>Marshmallow</td>
<td>Root; Cut</td>
<td>Spain</td>
<td>Cultivated</td>
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<tr>
<td>BT 44</td>
<td>Lavandula angustifolia</td>
<td>Lavender</td>
<td>Flowers; Whole</td>
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<td>BT 45</td>
<td>Hypericum perforatum</td>
<td>St. John’s Wort</td>
<td>Aerial; Whole</td>
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<td>BT 46</td>
<td>Melissa officinalis</td>
<td>Lemon Balm</td>
<td>Aerial; Whole</td>
<td>Spain</td>
<td>Cultivated</td>
</tr>
</tbody>
</table>
5.2.1.3 Sample preparation

All samples were acquired in dried format. The dried samples were ground to a fine powder, sieved, and stored in airtight sterile plastic containers at room temperature until required for analysis.

5.2.2 Microwave-assisted acid digestion

5.2.2.1 Vessel preparation

All experimental MARS Xpress vessels were rinsed in triplicate with deionised water before undergoing the Mars6 Xpress cleaning cycle. Ten millilitres of 5% HNO₃ (v/v) was added to each vessel before initiating the pre-programmed OneTouch “Express Clean” programme (Stages: 1; Power: 100-1800; Ramp Time: 15 mins; Hold Time: 10 mins; Temp.: 150°C; Temp. Guard: Off) (CEM, 2020). On completion of the cleaning cycle, vessels were again rinsed in triplicate with clean deionised water before being allowed to air dry.

5.2.2.2 Pre-digestion of botanical samples

Samples (0.5 g; dry weight) were accurately weighed and transferred into a pre-cleaned MARS Xpress digestion vessel (Material: TFM; maximum vessel volume: 55 mL; operation pressure and temperature: medium). For the pre-digestion step, concentrated trace-metal grade HNO₃ (v/v; 67-69%; 10 mL) was added to the vessel and gently swirled before securing the inner lid and allowed to stand for 15 minutes at room temperature. Any gas produced during the pre-digestion step was manually released before securing the vessel and placing it into the MARS-6 carousel.

5.2.2.3 Programmed digestion of botanical (BT) samples

The pre-programmed CEM OneTouch “Plant Material” method was selected (Stage: 01; Power: 1030 – 1800 W; Ramp time: 20 – 25: 00 mm/ss; Hold time: 10:00 mm/ss; Pressure: 800 psi; Temperature: 200°C; Temp. Guard: off; Stirring: off) (CEM, 2020). After cooling, the digestates were transferred to sterile 15 mL PMP sample tubes. The tubes were gently inverted and vented multiple times to release gaseous build-up.

<table>
<thead>
<tr>
<th>Code</th>
<th>Name</th>
<th>Description</th>
<th>Origin</th>
<th>Cultivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT_47</td>
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<td>Cotton Lavender Flowers; Whole</td>
<td>Spain</td>
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<td>Elderberry Aerial; Whole</td>
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<td>Cultivated</td>
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<td>BT_49</td>
<td>Mentha × piperita</td>
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<td>Spain</td>
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<td>BT_50</td>
<td>Peumus boldus Molina</td>
<td>Boldo Leaves; Cut</td>
<td>Spain</td>
<td>Cultivated</td>
</tr>
</tbody>
</table>

*Certified organic plant material*
before storage at -24°C. The above steps were repeated for all botanical samples (BT-01 to BT-50).

5.2.3 Validated ICP-SFMS Multi-Elemental Analysis (Metallomic Profiling)

Method performed as per Ring et al. (2021); previously validated in our laboratory (Dept. Physical Sciences, MTU Ireland). Standard/control preparation, instrumental analysis and QC measures outlined briefly below.

5.2.3.1 Multi-elemental standard and control preparation

Calibration standards (in the range of 0.001 – 50 µg.L⁻¹) and controls were prepared as described in Ring et al. (2021). To prepare the matrix-spiked controls, a sample was diluted 1:10,000 using 2% HNO₃ before being spiked with standard and ISTD stock solutions. The final concentrations of these controls were as follows: A (0.2 µg.L⁻¹), B (1 µg.L⁻¹), C (5 µg.L⁻¹), D (15 µg.L⁻¹) and E (40 µg.L⁻¹).

5.2.3.2 Instrumentational analysis

A volume of the initial digested sample (5.0 mL) was diluted to 25 mL in 2% HNO₃ and spiked with the internal standard (to a final ISTD concentration of 2.5 µg.L⁻¹). The samples were placed on the ESI autosampler rack, and analysis of all samples by ICP-SFMS was performed as per the procedure described in Ring et al. (2021).

Heavy metals and trace elements present in the digested botanical samples were analysed using a high-resolution inductively coupled plasma sector-field mass spectrometer (ICP-SFMS); Thermo Scientific™ Element 2™ High-Resolution ICP-MS. Certified calibration standards (traceable to NIST reference materials), controls (calibration verification standards) and blanks were run prior to sample injections. The diluted sample results determined at the instrument were expressed in parts per billion (ppb = µg.L⁻¹) and the final concentrations were obtained by calculating back to the original solid sample that was initially weighed out (µg.kg⁻¹). The following element isotopes were quantified in this study as previously described in Ring et al. (2021): ⁷Li, ⁹Be, ⁹⁵Mo, ¹¹¹Cd, ¹¹⁸Sn, ¹³⁷Ba, ¹⁹⁵Pt, ¹⁹⁷Au, ²⁰²Hg, ²⁰⁵Tl, ²⁰⁸Pb, ²⁰⁹Bi, ²⁴Mg, ²⁷Al, ⁴⁷Ti, ⁵¹V, ⁵³Cr, ⁵⁵Mn, ⁵⁶Fe, ⁵⁹Co, ⁶⁰Ni and ⁶³Cu. Matrix-spiked controls were analysed at five levels spanning the calibration range (0.2, 1, 5, 15 and 40 µg.L⁻¹) after every 20 samples. Calibration readback
QCs (made up in 2% HNO₃) were also ran at the end of the analytical sequence to verify the calibration line and instrument performance (Ring et al., 2021).

### 5.2.3.3 Calibration and quality assurance

In Table 5.2, a summary of the calibrations for each element of interest is presented, as well as the method limit of LOD/LOQ for each analyte. All elements analysed achieved acceptable linearity ($R^2 \geq 0.995$) across their respective working ranges. These calibrations were used to interpolate the concentrations of samples and matrix-spiked controls.

<table>
<thead>
<tr>
<th>Analyte Isotope</th>
<th>ISTD</th>
<th>Equation of the Line</th>
<th>Linear Range (µg.L⁻¹)</th>
<th>No. of calibration points</th>
<th>Correlation Coefficient ($R^2$)</th>
<th>LOD (ng.L⁻¹)</th>
<th>LOQ (ng.L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^7$Li</td>
<td>$^{71}$Ga</td>
<td>$y = 661.3x + 17.879$</td>
<td>0.001 - 35</td>
<td>11</td>
<td>$R^2 = 0.9999$</td>
<td>&lt;1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>$^9$Be</td>
<td>$^{101}$Rh</td>
<td>$y = 50.303x - 1.1758$</td>
<td>0.001 - 50</td>
<td>10</td>
<td>$R^2 = 0.9999$</td>
<td>0.36</td>
<td>1.18</td>
</tr>
<tr>
<td>$^{95}$Mo</td>
<td>$^{101}$Rh</td>
<td>$y = 125.66x - 3.494$</td>
<td>0.005 - 50</td>
<td>10</td>
<td>$R^2 = 0.9999$</td>
<td>1.64</td>
<td>5.40</td>
</tr>
<tr>
<td>$^{111}$Cd</td>
<td>$^{193}$Ir</td>
<td>$y = 0.0393x - 0.0011$</td>
<td>0.001 - 50</td>
<td>12</td>
<td>$R^2 = 0.9999$</td>
<td>0.28</td>
<td>0.94</td>
</tr>
<tr>
<td>$^{116}$Sn</td>
<td>$^{101}$Rh</td>
<td>$y = 218.16x - 5.0398$</td>
<td>0.005 - 50</td>
<td>14</td>
<td>$R^2 = 0.9999$</td>
<td>1.64</td>
<td>5.42</td>
</tr>
<tr>
<td>$^{117}$Ba</td>
<td>$^{101}$Rh</td>
<td>$y = 136.42x + 2.7994$</td>
<td>0.010 - 50</td>
<td>11</td>
<td>$R^2 = 0.9999$</td>
<td>1.96</td>
<td>6.46</td>
</tr>
<tr>
<td>$^{193}$Pt</td>
<td>$^{193}$Ir</td>
<td>$y = 0.1414x + 0.0016$</td>
<td>0.005 - 25</td>
<td>11</td>
<td>$R^2 = 0.9999$</td>
<td>0.43</td>
<td>1.42</td>
</tr>
<tr>
<td>$^{197}$Au</td>
<td>$^{193}$Ir</td>
<td>$y = 385.37x - 27.221$</td>
<td>0.025 - 50</td>
<td>8</td>
<td>$R^2 = 0.9999$</td>
<td>11.52</td>
<td>38.91</td>
</tr>
<tr>
<td>$^{202}$Hg</td>
<td>$^{193}$Ir</td>
<td>$y = 102.4x - 2.6221$</td>
<td>0.010 - 35</td>
<td>11</td>
<td>$R^2 = 0.9997$</td>
<td>11.79</td>
<td>38.91</td>
</tr>
<tr>
<td>$^{205}$Pb</td>
<td>$^{71}$Ga</td>
<td>$y = 661.3x + 17.879$</td>
<td>0.001 - 35</td>
<td>11</td>
<td>$R^2 = 0.9999$</td>
<td>0.02</td>
<td>0.07</td>
</tr>
<tr>
<td>$^{209}$Bi</td>
<td>$^{101}$Rh</td>
<td>$y = 613.49x + 30.858$</td>
<td>0.005 - 50</td>
<td>8</td>
<td>$R^2 = 0.9999$</td>
<td>2.52</td>
<td>5.00</td>
</tr>
<tr>
<td>$^{24}$Mg</td>
<td>$^{45}$Sc</td>
<td>$y = 0.2058x + 0.3627$</td>
<td>0.250 - 50</td>
<td>7</td>
<td>$R^2 = 0.9993$</td>
<td>25.00</td>
<td>100.00</td>
</tr>
<tr>
<td>$^{27}$Al</td>
<td>$^{45}$Sc</td>
<td>$y = 0.285x + 0.6584$</td>
<td>0.025 - 50</td>
<td>7</td>
<td>$R^2 = 0.9998$</td>
<td>10.00</td>
<td>25.00</td>
</tr>
<tr>
<td>$^{47}$Ti</td>
<td>$^{45}$Sc</td>
<td>$y = 0.0262x + 0.0015$</td>
<td>0.100 - 50</td>
<td>7</td>
<td>$R^2 = 0.9999$</td>
<td>14.49</td>
<td>47.83</td>
</tr>
<tr>
<td>$^{51}$V</td>
<td>$^{45}$Sc</td>
<td>$y = 0.2958x - 0.007$</td>
<td>0.010 - 35</td>
<td>14</td>
<td>$R^2 = 0.9999$</td>
<td>1.91</td>
<td>6.29</td>
</tr>
<tr>
<td>$^{52}$Cr</td>
<td>$^{45}$Sc</td>
<td>$y = 0.2815x + 0.0353$</td>
<td>0.025 - 50</td>
<td>11</td>
<td>$R^2 = 0.9999$</td>
<td>10.23</td>
<td>33.76</td>
</tr>
<tr>
<td>$^{55}$Mn</td>
<td>$^{45}$Sc</td>
<td>$y = 0.3524x + 0.0535$</td>
<td>0.025 - 50</td>
<td>12</td>
<td>$R^2 = 0.9999$</td>
<td>4.18</td>
<td>13.79</td>
</tr>
<tr>
<td>$^{56}$Fe</td>
<td>$^{45}$Sc</td>
<td>$y = 0.2916x + 0.5195$</td>
<td>0.250 - 50</td>
<td>8</td>
<td>$R^2 = 0.9997$</td>
<td>25.00</td>
<td>100.00</td>
</tr>
<tr>
<td>$^{58}$Co</td>
<td>$^{45}$Sc</td>
<td>$y = 0.2896x - 0.0018$</td>
<td>0.025 - 45</td>
<td>13</td>
<td>$R^2 = 0.9997$</td>
<td>7.13</td>
<td>23.54</td>
</tr>
<tr>
<td>$^{60}$Ni</td>
<td>$^{45}$Sc</td>
<td>$y = 0.0686x + 0.0432$</td>
<td>0.250 - 50</td>
<td>8</td>
<td>$R^2 = 0.9998$</td>
<td>10.00</td>
<td>189.82</td>
</tr>
<tr>
<td>$^{60}$Cu</td>
<td>$^{45}$Sc</td>
<td>$y = 0.1351x + 0.0259$</td>
<td>0.250 - 50</td>
<td>8</td>
<td>$R^2 = 0.9996$</td>
<td>10.00</td>
<td>250.00</td>
</tr>
</tbody>
</table>
The validity of results was assured through the analysis of matrix-spiked controls at five concentration levels spanning the entire calibration range of the method (0.2 µg.L⁻¹, 1 µg.L⁻¹, 5 µg.L⁻¹, 15 µg.L⁻¹ and 40 µg.L⁻¹). Percent recovery (%) was used as the parameter to evaluate calibration/instrument performance, with an acceptance tolerance of 100 ± 25% recovery (i.e. 75 – 125% of the assigned value for each control concentration). As can be seen in Table 5.3, all elements achieved acceptable recoveries across each concentration level examined. The acceptable performance of the controls indicated that the calibration was fit for purpose and could be used to accurately determine element concentrations in the samples (BT-1 to BT-50).

**Table 5.3 Average % recoveries for matrix-spiked controls analysed by ICP-SFMS (n = 5).**

<table>
<thead>
<tr>
<th>Element(s)</th>
<th>Average % Recovery (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.2 µg.L⁻¹</td>
</tr>
<tr>
<td>Lithium (7Li)</td>
<td>116.5</td>
</tr>
<tr>
<td>Beryllium (9Be)</td>
<td>116.6</td>
</tr>
<tr>
<td>Molybdenum (95Mo)</td>
<td>113.0</td>
</tr>
<tr>
<td>Cadmium (111Cd)</td>
<td>114.2</td>
</tr>
<tr>
<td>Tin (118Sn)</td>
<td>105.7</td>
</tr>
<tr>
<td>Barium (137Ba)</td>
<td>109.2</td>
</tr>
<tr>
<td>Platinum (195Pt)</td>
<td>106.7</td>
</tr>
<tr>
<td>Mercury (202Hg)</td>
<td>121.0</td>
</tr>
<tr>
<td>Thallium (205Tl)</td>
<td>94.5</td>
</tr>
<tr>
<td>Lead (208Pb)</td>
<td>77.8</td>
</tr>
<tr>
<td>Bismuth (209Bi)</td>
<td>89.8</td>
</tr>
<tr>
<td>Titanium (47Ti)</td>
<td>108.5</td>
</tr>
<tr>
<td>Vanadium (51V)</td>
<td>109.1</td>
</tr>
<tr>
<td>Chromium (52Cr)</td>
<td>96.0</td>
</tr>
<tr>
<td>Cobalt (59Co)</td>
<td>104.2</td>
</tr>
<tr>
<td>Nickel (60Ni)</td>
<td>-</td>
</tr>
<tr>
<td>Copper (64Cu)</td>
<td>-</td>
</tr>
</tbody>
</table>

Note: *Nickel and copper have LOQs of 0.25 µg.L⁻¹, which is above the lowest (0.2 µg.L⁻¹) control level, hence they have not been assessed at the 0.2 µg.L⁻¹ level.

The concentrations of elements in each sample were determined by ICP-SFMS and a summary of the results (expressed in µg.kg⁻¹) can be found in Table 5.8. In some samples, element concentrations were found to be outside the instrument calibration range (Mo, Ba, Tl, Pb, Ti and Cu), and because the samples could not be further diluted and repeated, these values are reported as ‘NR’ in the table. In the cases of Au, Al, Fe, Mg and Mn, all
samples tested yielded concentrations that lay outside the calibration range and because further dilution was not possible, these elements have been removed from the table entirely. Where sample concentrations were below the LOQ, final concentrations have been reported as <LOQ.

### 5.2.4 Mathematical modelling of human risk to metal exposure through medicinal plant consumption

The following table summarises the key input data required for the mathematical risk assessments (See Equation # 1 – 4): The Estimated Daily Intake (EDI), Chronic Daily Intake (CDI), (Target) Hazard Quotient ((T)HQ) and Hazard Index (HI).

*Table 5.49 List of input data for the evaluation of exposure and risk (Equation # 1 - 4).*

<table>
<thead>
<tr>
<th>Input Parameter(s)</th>
<th>Abbreviation</th>
<th>Value</th>
<th>Unit(s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estimated Daily Intake</td>
<td>EDI</td>
<td>-</td>
<td>mg-(kg BW)⁻¹d⁻¹</td>
<td>Chen et al. (2019) and Luo et al. (2021)</td>
</tr>
<tr>
<td>Concentration</td>
<td>C</td>
<td>-</td>
<td>mg kg⁻¹</td>
<td>Chen et al. (2019) and Luo et al. (2021)</td>
</tr>
<tr>
<td>Body Weight (Adult; European default value)</td>
<td>BW</td>
<td>70</td>
<td>Kg</td>
<td>EFSA, 2012</td>
</tr>
<tr>
<td>Ingestion/Intake Rate</td>
<td>IR Low</td>
<td>0.0002</td>
<td>Kg.day⁻¹</td>
<td>Chen et al. 2019</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>0.2</td>
<td></td>
<td>CP, 2020</td>
</tr>
<tr>
<td>Exposure frequency</td>
<td>EF</td>
<td>90</td>
<td>days</td>
<td>Chen et al. (2019) and Luo et al. (2021)</td>
</tr>
<tr>
<td>Exposure Duration (Shorter-than-lifetime)</td>
<td>ED</td>
<td>20</td>
<td>years</td>
<td>Chen et al. (2019) and Luo et al. (2021)</td>
</tr>
<tr>
<td></td>
<td>ED (Average Lifetime)</td>
<td>70</td>
<td>years</td>
<td>Chen et al. (2019) and Luo et al. (2021)</td>
</tr>
<tr>
<td>Transfer Rate (Metal)</td>
<td>t Cd</td>
<td>14</td>
<td>%</td>
<td>Chen et al. (2019) and Luo et al. (2021)</td>
</tr>
<tr>
<td></td>
<td>Cu</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hg</td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>All Other elements</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average Exposure Time</td>
<td>AT</td>
<td>25,550</td>
<td>days</td>
<td>Chen et al. (2019) and Luo et al. (2021)</td>
</tr>
<tr>
<td>(Average lifetime (ED) = 365 days x 70 years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reference Dose (Oral)</td>
<td>RFD</td>
<td></td>
<td>mg.kg⁻¹.day⁻¹</td>
<td>Orosun et al. (2020)</td>
</tr>
<tr>
<td></td>
<td>Cd (1.00 x 10⁻³)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cr (3.00 x 10⁻³)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cu (4.00 x 10⁻²)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mn (4.6 x 10⁻²)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ni (2.00 x 10⁻²)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pb (3.50 x 10⁻³)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
5.2.4.1 Calculation of the Estimated Daily Intakes (EDI) and Chronic Daily Intakes (CDI) of metal contaminants from the theoretical consumption of herbal/medicinal plants (dry weight)

5.2.4.1.1 Estimated Daily Intake (EDI)

The EDI (µg (kg BW)^-1 d^-1) for each metal was calculated using the following Equation #1 as per Chen et al. (2019) and Luo et al. (2021), with adaptations. As outlined in Table 5.4; “C” represents the analyte concentration detected per sample (mg.kg^-1) (See Table 5.5), “IR” (or ingestion dose) refers to the daily ingestion or intake rate (Kg.day^-1), and “BW” refers to the default adult body weight proposed by EFSA (2012) of 70 kg.

\[
EDI = \frac{C \times IR}{BW}
\]

(Equation #1)

*aDosage information provided by the manufacturer/supplier can be input for the IR in this equation; however, for this study, the sample matrices are raw plant material (i.e. not in final format) and thus recommended daily intakes are not possible. Generic IR(D)’s of 200 and 500 g.day^-1, signifying the mean and 95th percentile (maximum daily dosage) of Chinese Herbal Medicinal Products, respectively, was proposed in the 2020 Chinese Pharmacopeia (ChP, 2020) and used in recent health risk assessments of herbal preparations (Zuo et al., 2020; Luo et al., 2021).

b In the absence of a validated European equivalent; 200 g.day^-1 was surrogated in this study to represent a theoretical maximum or “conservative” IR (CP, 2020) and a theoretical minimum IR of 200 mg.day^-1 (Chen et al., 2019) to represent a more “realistic” exposure scenario.

5.2.4.1.2 Chronic Daily Intake (CDI)

The CDI (shorter-than lifetime exposure scenarios) (Ćwieląg-Drabek et al., 2020) was estimated by inputting the relevant data outlined in Table 5.4 into the following Equation #2 recommended by the U.S. EPA (2011) with adaptations (Zuo et al., 2020).

\[
CDI = \frac{C \times IR \times EF \times ED \times t}{AT \times BW}
\]

(Equation #2)

2 Principal Component Analysis (PCA) will be considered in resulting publications.
5.2.4.2  Hazard Quotient (HQ) evaluation for non-carcinogenic risk

The non-carcinogenic risk was determined following estimation of the theoretical target Hazard Quotient (HQ) – a unitless ratio involving the comparison of the exposure level (i.e. EDI or CDI) over a specified period with a reference dose derived for a similar exposure period (Sawut et al., 2018). HQ is a deterministic risk-assessment expression that allows a level of risk to be demonstrated but cannot estimate probabilistic risks to exposed populations above the maximum threshold (Ćwieląg-Drabek et al., 2020). The U.S. EPA (1989; 2011) proposed evaluation of the HQ by dividing exposure by the relevant reference dose (RfD), as shown in Equation #3 below. ISO oral RfD’s for traditional Chinese medicines (TCM) were used in this equation as per Luo et al. (2021) (mg⁻¹.Kg⁻¹.day): Cd (0.0005); Cu (0.04); Hg (0.0003); Pb (0.0035). An HQ < 1 is considered ‘no risk’; conversely an HQ ≥ 1 is considered a ‘potential non-carcinogenic risk’.

\[
HQ = \frac{Exposure \ (CDI \ or \ EDI)}{RfD}
\]

(Equation #3)

5.2.4.3  Hazard Index (HI)

HI is defined as the sum of HQ obtained, as shown in Equation #4 (Orosun et al., 2020; U.S. EPA, 2011). The non-carcinogenic HI was established to evaluate human health risk to exposure to more than one metal at a time, i.e. simultaneous or cumulative exposure. Similarly, HI < 1 is considered ‘no risk’; conversely, HI ≥ 1 is considered as a ‘potential non-carcinogenic risk’. Ultimately, the higher the HQ or HI the greater the risk to consumers.

\[
HI = \Sigma(HQ)
\]

(Equation #4)
5.3 Results and discussion

5.3.1 Multi-elemental analysis (metallomic profiling) results

This is the first multi-elemental analysis of arnica, bush vetch, sweet cicely, yellow rattle, bogbean, rock-tea and tufted catchfly to-date, to the best of our knowledge. A diverse range of concentrations were quantified for each element across all sample matrices in this present study: $^7$Li (17.51 – 3964.03 µg.kg$^{-1}$); $^9$Be (2.95 – 121.50 µg.kg$^{-1}$); $^{95}$Mo (74.62 – 4504.50 µg.kg$^{-1}$); $^{111}$Cd (4.75 – 325.33 µg.kg$^{-1}$); $^{118}$Sn (6.39 – 165.15 µg.kg$^{-1}$); $^{137}$Ba (746.66 – 4645.63 µg.kg$^{-1}$); $^{195}$Pt (2.05 – 32.68 µg.kg$^{-1}$); $^{202}$Hg (4.67 – 29.87 µg.kg$^{-1}$); $^{205}$Tl (2.64 – 90.82 µg.kg$^{-1}$); $^{208}$Pb (11.58 – 4248.07 µg.kg$^{-1}$); $^{209}$Bi (2.42 – 29.64 µg.kg$^{-1}$); $^{47}$Ti (130.96 – 5827.41 µg.kg$^{-1}$); $^{51}$V (14.81 – 1757.80 µg.kg$^{-1}$); $^{52}$Cr (99.64 – 4534.43 µg.kg$^{-1}$); $^{59}$Co (20.68 – 651.76 µg.kg$^{-1}$); $^{60}$Ni (229.94 – 6060.33 µg.kg$^{-1}$) and $^{63}$Cu (1910.27 – 6340.13 µg.kg$^{-1}$). Au, Mg, Mn, Al, and Fe (data not shown) were outside the calibration range and could not be quantified (i.e. “NR”: not reported). Levels of $^{137}$Ba, $^7$Ti, and $^{63}$Cu were above the calibration range for 92-, 78- and 48-percent of all samples, respectively. The wide range of elements observed between samples, regardless of botanical relatedness, is not considered unusual (Filipiak-szok et al., 2015) considering that the elemental composition of plant tissues and organs is largely influenced by edaphic conditions, soil geochemistry and ecophysiological factors (Bonari et al., 2019).
Table 5.510 Concentration (µg kg⁻¹) of each element in sample (BT-1 to -50) following ICP-SFMS multi-elemental analysis (metallic profiling).

<table>
<thead>
<tr>
<th>ID # (BT-1)</th>
<th>²⁷Li</th>
<th>²⁷Re</th>
<th>⁷⁹Me</th>
<th>³¹Cd</th>
<th>⁴⁰Sr</th>
<th>⁴¹Ba</th>
<th>⁴²Kr</th>
<th>⁴⁰He</th>
<th>⁴⁰Ti</th>
<th>⁴⁰Ph</th>
<th>⁴⁰Rn</th>
<th>⁴⁰Ti</th>
<th>⁴¹V</th>
<th>⁴²Cr</th>
<th>⁴³Ca</th>
<th>⁴⁴Ni</th>
<th>⁴⁴Cu</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1459.04</td>
<td>47.28</td>
<td>584.02</td>
<td>68.11</td>
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<td>38</td>
<td>228.26</td>
<td>11.39</td>
<td>4504.50</td>
<td>51.05</td>
<td>133.44</td>
<td>NR</td>
<td>8.59</td>
<td>13.83</td>
<td>90.82</td>
<td>2047.83</td>
<td>28.37</td>
<td>NR</td>
<td>4138.97</td>
<td>1023.23</td>
<td>1883.72</td>
<td>106.47</td>
<td>985.80</td>
</tr>
<tr>
<td>39</td>
<td>911.24</td>
<td>11.18</td>
<td>411.12</td>
<td>51.82</td>
<td>31.98</td>
<td>NR</td>
<td>9.73</td>
<td>8.98</td>
<td>13.61</td>
<td>383.87</td>
<td>4.03</td>
<td>NR</td>
<td>370.05</td>
<td>871.08</td>
<td>78.19</td>
<td>628.80</td>
<td>NR</td>
</tr>
<tr>
<td>40</td>
<td>725.13</td>
<td>54.29</td>
<td>90.70</td>
<td>70.28</td>
<td>41.47</td>
<td>NR</td>
<td>6.73</td>
<td>17.67</td>
<td>11.26</td>
<td>1004.57</td>
<td>14.10</td>
<td>NR</td>
<td>5500.88</td>
<td>629.59</td>
<td>1108.20</td>
<td>541.49</td>
<td>1177.67</td>
</tr>
</tbody>
</table>

230
| (cont.) | $^{7}$Li | $^{9}$Be | $^{9}$Be | $^{11}$Cd | $^{11}$Cd | $^{13}$Na | $^{13}$Na | $^{13}$Br | $^{13}$Br | $^{20}$He | $^{20}$He | $^{20}$Ti | $^{20}$Ti | $^{38}$Ph | $^{38}$Ph | $^{38}$Rb | $^{38}$Rb | $^{44}$Ti | $^{44}$Ti | $^{55}$V | $^{55}$V | $^{55}$Cr | $^{55}$Cr | $^{55}$Co | $^{58}$Ni | $^{64}$Cu |
|--------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| 41     | 1044.78 | 51.59   | 430.67  | 12.33   | 59.08   | NR      | 6.02    | 17.26   | 7.32    | 598.07  | 8.72    | NR      | 953.66  | 1546.24 | 166.51  | 532.01  | 2318.00 |
| 42     | 617.44  | 16.91   | 1129.19 | 25.03   | 15.37   | NR      | 7.32    | 8.72    | 25.17   | 167.88  | 3.50    | NR      | 1117.97 | 2568.35 | 337.17  | NR      | 3695.86 |
| 43     | 191.97  | 11.56   | 265.31  | 214.19  | 6.39    | NR      | 9.09    | 6.80    | 7.85    | 474.40  | 4.65    | 3394.64 | 321.29  | 1012.27 | 135.85  | 1228.32 | NR      |
| 44     | 405.54  | 22.74   | 447.92  | 8.64    | 35.94   | NR      | 5.75    | 8.67    | 5.84    | 321.35  | 5.71    | 4323.13 | 381.75  | 1209.22 | 92.94   | 887.69  | 3746.49 |
| 45     | 100.32  | 5.04    | 728.99  | 129.62  | 12.45   | NR      | 8.24    | 8.62    | 4.87    | 194.33  | 3.04    | 1238.44 | 138.19  | 534.89  | 273.12  | NR      | NR      |
| 46     | 301.43  | 14.87   | 1292.97 | 24.54   | 53.87   | NR      | 7.89    | 21.74   | 5.93    | 732.89  | 6.38    | 4282.37 | 376.90  | 549.99  | 93.86   | 724.70  | NR      |
| 47     | 566.17  | 4.67    | 373.96  | 19.04   | 10.41   | 1817.19 | 7.47    | 6.77    | 3.56    | 48.01   | 3.00    | 576.06  | 62.61   | 190.89  | 43.64   | 879.70  | NR      |
| 48     | 241.70  | 8.39    | 928.82  | 23.39   | 30.83   | NR      | 4.38    | 5.36    | 5.73    | 307.48  | 3.34    | 2205.42 | 159.13  | 608.89  | 33.20   | 717.33  | NR      |
| 49     | 562.12  | 22.55   | 495.47  | 8.07    | 21.73   | NR      | 9.63    | 13.20   | 4.64    | 259.96  | 4.48    | 1921.74 | 473.70  | 1286.46 | 113.17  | 782.23  | 4903.02 |
| 50     | 181.91  | 20.90   | 446.21  | 11.76   | 28.69   | NR      | 8.61    | 15.72   | 3.50    | 129.26  | 3.21    | NR      | 292.01  | 4534.43 | 50.81   | 678.60  | 2408.17 |

Note(s): NR represents the element concentrations which exceeded the highest calibration standard and could not be accurately quantified. The concentrations of As, Al, Fe, Mg, and Mn in all samples tested were outside the calibration range and could not be accurately determined; and thus, they are excluded from this table. *Highest quantified concentrations of the corresponding element(s) are highlighted in grey.
5.3.2 Results of the mathematical modelling of the non-carcinogenic human risk to metal exposure through medicinal plant consumption

None of the samples analysed (n = 50) exceeded the ML’s for metal impurities in herbal substances or [raw] herbal starting materials laid out in the European Pharmacopeia (Ph. Eur., 2013): Cd (≤ 1 mg.kg⁻¹), Hg (0.1 mg.kg⁻¹), and Pb (5 mg.kg⁻¹). Furthermore, using European HBGV’s and/or toxicological endpoints for elements in food, all calculated chronic exposure estimates (e.g. CDIs; see Table 5.6) are well within the acceptable ranges from a dietary perspective. All EDI’s derived from the lower IR (200 mg.day⁻¹) are well below the regulatory limits and so too are the majority of the EDI’s derived from the higher IR (200 g.day⁻¹); thus, dietary exposure to the analysed botanical ingredients at the theoretical intakes used in this study, are of negligible concern to consumers. The exceptions are, however, Li, Mo, Tl, Pb, Co, and Ni at the highest IR, which are represented in grey in Table 5.6.

An EDI range of 0.05 – 11.33 µg·(kg BW)⁻¹d⁻¹ was calculated for Li, which exceeds the pR/D of 2 µg·(kg BW)⁻¹d⁻¹(U.S. EPA, 2008). An EDI range of 0.21 – 12.87 µg·(kg BW)⁻¹d⁻¹ was calculated for Mo, which exceeds the UL of 10 µg·(kg BW)⁻¹d⁻¹(EFSA SCF & NDA Panel, 2006). An EDI range of 0.01 – 0.26 µg·(kg BW)⁻¹d⁻¹ was calculated for Tl, which exceeds the pR/D of 0.08 µg·(kg BW)⁻¹d⁻¹(BfR, 2004). An EDI range of 0.03 – 12.14 µg·(kg BW)⁻¹d⁻¹ was calculated for Pb, which exceeds the BMDL’s for Pb, which are (µg·(kg BW)⁻¹d⁻¹): 0.5 (BMDL₀₁ [Developmental neurotoxicity]), 1.5 (BMDL₀₁ [blood pressure]) and 0.63 (BMDL₁₀ [kidneys]) (EFSA CONTAM, 2010; Steinkellner, 2020). An EDI range of 0.06 – 1.86 µg·(kg BW)⁻¹d⁻¹ was calculated for Co, which exceeds the TDI of 1.4 µg·(kg BW)⁻¹d⁻¹(EFSA FEEDAP, 2012). And lastly, an EDI range of 0.06 – 17.32 µg·(kg BW)⁻¹d⁻¹ was calculated for Ni, which exceeds the recently updated TDI of 13 µg·(kg BW)⁻¹d⁻¹(EFSA-CONTAM et al., 2020). Interstudy comparison to previous studies in the literature was challenging in the absence of standardisation or official guidance, i.e. variations in methodology, equations and input criteria, as discussed later in this chapter.
Table 5.6 Summary of the range (minimum to maximum) short-term Estimated Daily Intake (EDI) and Chronic Daily Intake (CDI) for each metal across all BT samples representing the worst-case exposure scenario at a high theoretical ingestion rate (i.e. 200 g.day\(^{-1}\) (CP, 2020)) of medicinal herbs or preparations (\(n = 50\)) and corresponding European dietary limits.

<table>
<thead>
<tr>
<th>Element</th>
<th>High EDI Range (mg·(kg BW(^{-1}))·day(^{-1}))</th>
<th>High CDI Range (mg·(kg BW(^{-1}))·year(^{-1}))</th>
<th>European Dietary Limits</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Min.</td>
<td>Max.</td>
<td>Min.</td>
</tr>
<tr>
<td>Li</td>
<td>0.05</td>
<td>11.33</td>
<td>&lt;0.00</td>
</tr>
<tr>
<td>Be</td>
<td>0.01</td>
<td>0.35</td>
<td>&lt;0.00</td>
</tr>
<tr>
<td>Mo</td>
<td>0.21</td>
<td>12.87</td>
<td>&lt;0.00</td>
</tr>
<tr>
<td>Cd</td>
<td>0.01</td>
<td>0.93</td>
<td>&lt;0.00</td>
</tr>
<tr>
<td>Sn</td>
<td>0.02</td>
<td>0.47</td>
<td>&lt;0.00</td>
</tr>
<tr>
<td>Pb</td>
<td>0.01</td>
<td>0.09</td>
<td>&lt;0.00</td>
</tr>
<tr>
<td>Hg (Inorganic)</td>
<td>0.01</td>
<td>0.09</td>
<td>&lt;0.00</td>
</tr>
<tr>
<td>Bi</td>
<td>0.01</td>
<td>0.08</td>
<td>&lt;0.00</td>
</tr>
<tr>
<td>Ti</td>
<td>0.01</td>
<td>0.26</td>
<td>&lt;0.00</td>
</tr>
<tr>
<td>Pb</td>
<td>0.03</td>
<td>12.14</td>
<td>&lt;0.00</td>
</tr>
<tr>
<td>Bi</td>
<td>0.01</td>
<td>0.08</td>
<td>&lt;0.00</td>
</tr>
<tr>
<td>Ti</td>
<td>0.37</td>
<td>157.17</td>
<td>&lt;0.00</td>
</tr>
<tr>
<td>V</td>
<td>0.04</td>
<td>5.02</td>
<td>&lt;0.00</td>
</tr>
<tr>
<td>Cr (Cr(III))</td>
<td>0.28</td>
<td>12.96</td>
<td>&lt;0.00</td>
</tr>
<tr>
<td>Cu</td>
<td>0.06</td>
<td>1.86</td>
<td>&lt;0.00</td>
</tr>
<tr>
<td>Ni</td>
<td>0.66</td>
<td>17.32</td>
<td>&lt;0.00</td>
</tr>
<tr>
<td>Cu</td>
<td>5.46</td>
<td>18.11</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Calculation of each individual analytical results not shown in table. Element(s) highlighted in grey are in exceedance of accompanying dietary limits. \(^a\) Tolerable Weekly Intake (TWI) = mg·(kg BW\(^{-1}\))·wk\(^{-1}\); \(^b\) Benchmark dose levels (BMDL’s) for Pb (\(\mu\)g·(kg BW\(^{-1}\))·d\(^{-1}\)), 0.5 (BMDL\(_{01}\) [developmental neurotoxicity]), 1.5 (BMDL\(_{01}\) [blood pressure]) and 0.63 (BMDL\(_{01}\) [kidneys]) (EFSA CONTAM, 2010); \(^c\) No adverse Effect Level (NOAEL) = mg·(kg BW\(^{-1}\))·d\(^{-1}\); \(^d\) Tolerable Daily Intake (TDI); \(^e\) Average Intake (AI) = mg·day\(^{-1}\).

Results from the mathematical non-carcinogenic exposure assessment using the EDI values, as presented in Table 5.7, show that all BT samples (1-50) at a theoretical IR of 200 mg·day\(^{-1}\) are considered safe and of no risk to consumers, when considering Cd, Cu, Hg, and Pb exposure. Similarly, HQ’s calculated at the lower EDI (200 mg·day\(^{-1}\)) and both high and low CDI’s (200 mg·day\(^{-1}\) and g·day\(^{-1}\)) for all botanical samples (\(n = 50\)) were HQ ≤ 0.01 and HI ≤ 0.05, respectively, and thus considered to be of no potential risk to consumers. At a theoretical “worst-case” exposure scenario of 200 g·day\(^{-1}\), as recommended by the Chinese Pharmacopeia (ChP, 2020), 16% and 8% of samples are
considered potentially unsafe (HQ ≥ 1) regarding Pb and Cd exposure, respectively. Cu and Hg exposure, even at a high ingestion rate, are still considered safe and of no risk to consumers. This is expected for Hg since levels are generally low in terrestrial plants (Kenny et al., 2020), however the results for Cu may not be entirely representative per se considering that 48% of the samples were above the calibration range and could not be quantified and therefore excluded from the risk assessments, resulting in a smaller sample size for this element.

As shown in Table 5.7, a total of 42% of samples are categorised as potentially unsafe (HI ≥ 1) with regards to the cumulative exposure to Cu, Cd, Hg and Pb, representing 16 different plant species in total, including: hawthorn, arnica, dandelion, marigold, nettle, yarrow, comfrey, borage, coltsfoot, birds foot trefoil, ox-eye daisy, yellow rattle, mugwort, great mullein, tufted catchfly, marshmallow.

<table>
<thead>
<tr>
<th>Sample I.D.</th>
<th>HQ (Cd)</th>
<th>HQ (Hg)</th>
<th>HQ (Pb)</th>
<th>HQ (Cu)</th>
<th>HI [High EDI]</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT_01</td>
<td>0.39</td>
<td>0.28</td>
<td>3.47</td>
<td>0.32</td>
<td>4.46</td>
</tr>
<tr>
<td>BT_02</td>
<td>0.69</td>
<td>0.15</td>
<td>0.61</td>
<td>-</td>
<td>1.46</td>
</tr>
<tr>
<td>BT_03</td>
<td>0.29</td>
<td>0.23</td>
<td>0.15</td>
<td>-</td>
<td>0.68</td>
</tr>
<tr>
<td>BT_04</td>
<td>0.23</td>
<td>0.13</td>
<td>0.04</td>
<td>-</td>
<td>0.39</td>
</tr>
<tr>
<td>BT_05</td>
<td>0.37</td>
<td>0.12</td>
<td>0.20</td>
<td>-</td>
<td>0.69</td>
</tr>
<tr>
<td>BT_06</td>
<td>0.16</td>
<td>0.06</td>
<td>0.07</td>
<td>0.32</td>
<td>0.61</td>
</tr>
<tr>
<td>BT_07</td>
<td>0.09</td>
<td>0.09</td>
<td>0.03</td>
<td>0.31</td>
<td>0.52</td>
</tr>
<tr>
<td>BT_08</td>
<td>1.22</td>
<td>0.09</td>
<td>0.60</td>
<td>-</td>
<td>1.91</td>
</tr>
<tr>
<td>BT_09</td>
<td>1.07</td>
<td>0.07</td>
<td>0.16</td>
<td>-</td>
<td>1.30</td>
</tr>
<tr>
<td>BT_10</td>
<td>1.86</td>
<td>0.15</td>
<td>1.05</td>
<td>0.32</td>
<td>3.38</td>
</tr>
<tr>
<td>BT_11</td>
<td>0.06</td>
<td>0.11</td>
<td>0.07</td>
<td>-</td>
<td>0.23</td>
</tr>
<tr>
<td>BT_12</td>
<td>0.12</td>
<td>0.10</td>
<td>0.36</td>
<td>-</td>
<td>0.58</td>
</tr>
<tr>
<td>BT_13</td>
<td>0.05</td>
<td>0.08</td>
<td>0.26</td>
<td>0.26</td>
<td>0.65</td>
</tr>
<tr>
<td>BT_14</td>
<td>0.06</td>
<td>0.09</td>
<td>0.02</td>
<td>0.25</td>
<td>0.42</td>
</tr>
<tr>
<td>BT_15</td>
<td>0.03</td>
<td>0.06</td>
<td>0.03</td>
<td>0.25</td>
<td>0.36</td>
</tr>
<tr>
<td>BT_16</td>
<td>0.06</td>
<td>0.08</td>
<td>0.16</td>
<td>0.29</td>
<td>0.58</td>
</tr>
<tr>
<td>BT_17</td>
<td>0.14</td>
<td>0.06</td>
<td>0.79</td>
<td>0.24</td>
<td>1.23</td>
</tr>
<tr>
<td>BT_18</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
<td>0.37</td>
<td>0.49</td>
</tr>
<tr>
<td>BT_19</td>
<td>0.14</td>
<td>0.13</td>
<td>0.82</td>
<td>-</td>
<td>1.09</td>
</tr>
<tr>
<td>BT_20</td>
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<td>0.10</td>
<td>0.22</td>
<td>0.31</td>
<td>0.84</td>
</tr>
<tr>
<td>BT_21</td>
<td>1.77</td>
<td>0.11</td>
<td>0.24</td>
<td>-</td>
<td>2.12</td>
</tr>
<tr>
<td>BT_22</td>
<td>0.66</td>
<td>0.12</td>
<td>1.15</td>
<td>-</td>
<td>1.93</td>
</tr>
<tr>
<td>BT_23</td>
<td>0.59</td>
<td>0.16</td>
<td>0.67</td>
<td>-</td>
<td>1.41</td>
</tr>
<tr>
<td>BT_24</td>
<td>0.24</td>
<td>0.27</td>
<td>0.04</td>
<td>0.38</td>
<td>0.94</td>
</tr>
</tbody>
</table>

Table 5.7 Theoretical Hazard Quotient (HQ) and resulting Hazard Indices (HI) estimated for Cd, Hg, Pb, and Cu - representing the worst-case exposure scenario only (i.e. Short-term EDI @ 200 g day⁻¹ consumption)³,⁴.
5.3.3 Discussion on sampled botanicals

The following section covers the quantified element concentrations in each plant species \( (n = 30) \) with comparison to reported elemental profiles in the literature, and results of the subsequent non-carcinogenic risk assessments performed in this current study.

5.3.3.1 Crataegus laevigata (Hawthorn)

Element concentrations appeared lowered in hawthorn fruit/berry samples (BT-06 and 07) than the flower and leaf (BT-01 to -05); however a comparison to the available literature could not be made since the leaf and flower samples analysed were acquired pre-mixed, and available studies analysed flower, leaf, berry and seed separately (Juranović Cindrič et al., 2015). Our findings for Cd, Co, Cr, Cu are largely within the
ranges reported previously; with the exception of Li, Mo, Ni (Juranović Cindrić et al., 2015) and V (Özcan et al., 2005).

Hawthorn flower and leaf (BT-1) sourced from China presented with the highest non-carcinogenic risk (HI = 4.46) out of all the samples tested (see Table 5.7), based on the EDI at an IR of 200 g.day⁻¹, as recommended by the Chinese Pharmacopeia (ChP, 2020). The same sample contains potentially unsafe levels of Pb (HQ(Pb) = 3.47) at a quantified concentration of 4248.07 µg.kg⁻¹, which is 300 times higher than the lowest concentration detected in this study (BT-25; comfrey stem), and much higher than data previously reported in similar studies for the flowers (Juranović Cindrić et al., 2015) and fruits (M. Özcan et al., 2005). Additionally, BT-1 presented with the highest Bi levels out of all the samples tested, 29.64 µg.kg⁻¹ – although at this concentration, no risk (HQ < 1) was determined at an EDI and CDI of 0.014 and <0.0001 µg⋅(kg BW)^⁻¹d⁻¹, respectively. Both Pb and Bi - in addition to primordial and anthropogenic-derivatisation - are also daughter isotopes of nuclear decay chains (e.g. actinium, thorium, uranium /radium series). Their presence in aerial samples of hawthorn, if theoretically derived from radionuclide decay in soil matrices, could indicate the successful root to aerial transfer of these elements in planta. There is no experimental data in the literature currently to support this.

The same sample (BT-1) also presented with the highest Hg concentration (29.87 µg.kg⁻¹) out of all the samples tested. No risk was detected for the oral consumption at both high and low theoretical exposure scenarios investigated (HQ(Hg) and HI <1). The sample also contained levels of Ti above the calibration range, and therefore could not be quantified – while the hawthorn sample wild-collected in Co. Cork (BT-4) contained the lowest Ti levels out of all the samples tested. Hawthorn flower and leaf (BT-2) sourced from Eastern Europe (BT-2) also presented as a potential non-carcinogenic risk for cumulative exposure to Cu, Cd, Hg, and Pb (HI = 1.46), based on the EDI derived from the higher IR. Furthermore, an aerial (flower and leaf) sample collected from a farm in West Cork (Ireland) (BT-3) presented with the highest Pt level (32.68 µg.kg⁻¹) out of all the samples tested. No risk was detected for the oral consumption at both high and low theoretical exposure scenarios investigated (HQ’s and HI <1). The remaining hawthorn samples (BT-2 to -7) were also considered safe and of no risk to consumers, based on the results of the exposure assessments detailed earlier.
5.3.3.2  *Arnica montana* (Arnica)
Our findings are, to the best of our knowledge, the first account of the multi-elemental (metallomic) profiling of *arnica* using ICP-SFMS. Levels of Ba, Ni, and Cu were not quantifiable in arnica flowers. Based on the results of the exposure assessment using EDI’s derived from the higher IR (See Table 5.6), a potential risk of unacceptable Cd exposure (HQ = 1.22) and subsequent risk of cumulative exposure to Cd, Cu, Hg, Pb (HI = 1.91) was detected for the oral consumption of arnica flowers (BT-8). Conversely, no risk was detected when considering the EDI derived from the lower IR, and CDI’s.

5.3.3.3  *Taraxacum officinale* (Dandelion)
Dandelion is known to accumulate metals in aerial tissues better than underground organs (Nadgórska–Socha et al., 2017), which was reflected in our results where we observed highest levels of Be (121.50 µg.kg⁻¹), Cd (325.33 µg.kg⁻¹) and Sn (165.15 µg.kg⁻¹) across all fifty samples in certified-organic dandelion leaf (BT-10) sourced from the Netherlands. An exception was Ti (5827.41 µg.kg⁻¹) in organic root from France (BT-9), which exhibited the highest Ti concentrations across all samples – however, the Ti content in the leaf could not be quantified as it was above the calibration range. From the results of the exposure assessment, dandelion leaf (BT-10) represents the second highest non-carcinogenic risk (HI = 3.38) out of all the samples tested (see Table 5.7), based on the EDI at an IR of 200 g.day⁻¹. The same sample also contains potentially unsafe levels of Cd (HQ(Cd) = 1.86) and Pb (HQ(Pb) = 1.05). These results are in accordance with Luo et al. (2021) who reported an EDI(Pb) of 4 µg.kg⁻¹.day⁻¹ for dandelion aerial parts at a higher IR (0.5 Kg.day⁻¹), and a HI > 1. The root (BT-9) contains potentially unsafe levels of Cd too (HQ(Cd) = 1.07), and a cumulative risk of toxicity to consumers (HI = 1.30).

Our findings did not demonstrate higher accumulation of Cd than Ni as previously reported (Kováčik et al., 2019) but were within the lower ranges (<0.4 and <0.2 mg.kg⁻¹) reported by Lisiak-Zielińska et al. (2021). We observed similar Cu (Kováčik et al., 2019), but lower Cr levels than previously reported (Lisiak-Zielińska et al., 2021). Odigie et al. (2019) described a benchmark level of 200 µg.kg⁻¹ for Pb in dandelion leaves – a concentration that is more in line with the root (BT-09; 202.06 µg.kg⁻¹) compared to the elevated leaf concentration (BT-10; 1283.91 µg.kg⁻¹) in this study. Levels of Pt and Pb are more reflective of those reported by Djingova et al. (2003). The authors observed the accumulation of traffic emission-related Platinum Group Elements (PGE’s) in dandelion,
with profiles positively correlated with the PGE pollution profile of environmental street dust sampled at the same time. The increased prevalence of PGE’s in plants growing along motorways since the introduction of catalytic converters was acknowledged (Djingova et al., 2003), leading to further research focusing on dandelion as a promising biomonitoring and remediation tool for urban environmental pollution (Kováčik et al., 2019). Studies concerning the phytoextraction of Rare Earth Elements (REEs) using dandelion (Mleczez et al., 2018) and other species is increasing and is likely to continue to do so considering the projected global initiatives supporting widespread use of electric motor vehicles; the design of which utilise REE’s in current models.

5.3.3.4 *Sambucus nigra* L., *S. nigra flos* and *S. nigra fruct* (Elder, Elderflower, Elderberry)

Wild-collected elderberry fruit (BT-14) contained the lowest levels of Ni. Another wild-collected elderberry sample (BT-15) from Co. Cork (Ireland) contained notably lower concentrations of Cd (4.75 µg.kg⁻¹), Tl (2.64 µg.kg⁻¹), and Co (20.68 µg.kg⁻¹) compared to the other samples; and the highest quantified concentration of Ba (4.7 mg.kg⁻¹). Ninety-two % of the samples tested were above the calibration range for Ba and consequently could not be quantified, and therefore this is not entirely representative of the sample set per se. Similar levels of Cd and Hg were reported by Schulzki *et al.* (2017) compared to an average Cd and Hg concentration of 12.6 and 8.5 µg.kg⁻¹, respectively, across all elder-derived samples in this study (BT-11 to 16 and BT-48). Pace *et al.* (2020) observed higher Pb levels in aerial tissues (8.7 – 13.7 mg.kg⁻¹) compared to the fruit (0.9 mg.kg⁻¹) of elder sampled from a Pb-contaminated site. Our results demonstrate a wide variation in Pb concentration among the elder-derived samples (26 – 437 µg.kg⁻¹), with levels in wild-collected samples from Co. Cork (Ireland) measurably lower than commercial and cultivated counterparts. Based on the output of the exposure assessment (see Table 5.7), no risk was detected for the oral consumption of any of the elder-derived samples when considering both the high and low theoretical exposure scenarios modelled in this study (i.e. 200 mg.day⁻¹ and 200 g.day⁻¹).

5.3.3.5 *Calendula officinalis* (Marigold)

Studies on the phytoremedial potential of *C. officinalis* seedlings (Fan *et al.*, 2016), hydroponic cultures (Shao *et al.*, 2019), and the aerial phytostabilization of Cd by *C. officinalis* (Liu *et al.*, 2008; Thongchai *et al.*, 2019) and related species *C. calypso* (Farooq
et al., 2020), has been explored; yet the multi-element (metallomic) profiling of marigold raw materials appears infrequently in the literature. Levels of Ba and Ti were above the calibration range and could not be quantified. This current study revealed a potential risk of cumulative exposure to Cd, Cu, Hg, Pb (HI = 1.23) from marigold flower consumption based on the EDI derived from the higher IR (200 g.day⁻¹).

Levels of Pb (970.03 µg.kg⁻¹) in the flowers were measurably lower than those previously reported in the inflorescences (9.34 mg.kg⁻¹) and leaves (11.57 mg.kg⁻¹) collected near a motorway. The proximity to the motorway may have influenced the higher Pb levels observed by Meos et al. (2011) – who further advised against the collection of the leaf [for subsequent analysis] during or directly after rain showers. In agreement, Deljanin et al. (2014) observed a 30% reduction in Pb load after rinsing plant material before analysis. These are examples of collection parameters that could be considered, for example, in the WHO GACP guidelines or equivalent.

5.3.3.6 *Aesculus hippocastanum* (Horse Chestnut)

Several studies in the literature report the analysis of horse chestnut leaf composition (Deljanin et al., 2016; Tomašević et al., 2011); yet few regarding seed composition exist. Levels of Li, Be, Ba, Hg, Bi were lowest in horse chestnut seed (BT-18) out of all the samples analysed in this study. Levels of Ni in seed (BT-18) sourced from the UK were comparable to those reported by Čukanović et al. (2020), however Cr was notably higher, and Cu much lower in our study. Levels of Cd, Hg and Pb were also much lower compared to those previously reported by Caldas & Machado (2004). Based on the output of the exposure assessment (see Table 5.7), no risk was detected for the oral consumption of horse chestnut seed when considering both the high and low theoretical exposure scenarios modelled in this study (i.e. 200 mg.day⁻¹ and 200 g.day⁻¹).

5.3.3.7 *Urtica dioica* (Nettle)

Nettle leaf and root are reportedly good biomonitoring indictors for Cr, Pb and Zn (Murphy et al., 2000). Our findings for both nettle samples (BT-19 to 20) were lower for Cd, Cr, Tl and Pb, and within the lower range of concentrations for Co, Ni and V reported by Jabłońska-Czapla et al. (2020). Hg concentrations are comparable to Fischer et al. (2017). In agreement with Mihaljev et al. (2014), higher Mo content was observed in the leaf (4216.93 µg.kg⁻¹) than root (259.08 µg.kg⁻¹) (see Table 5.5). Similarly, levels of Pb and Ni were higher in the leaf than root. Ba and Fe were poorly leached from nettle
infusions (Lozak et al., 2002), indicating a poor transfer rate from this matrix. Based on the output of the exposure assessment (see Table 5.7), no risk was detected for the oral consumption of certified-organic nettle root (BT-19) when considering both the high and low theoretical exposure scenarios modelled in this study (i.e. 200 mg.day\(^{-1}\) and 200 g.day\(^{-1}\)). Conversely, a potential non-carcinogenic risk for the cumulative exposure following consumption (i.e. high EDI) of the [non-organic] leaf was calculated (HI = 1.09).

5.3.3.8 *Achillea millefolium* (Yarrow)

Comparable Ni (Ražić et al., 2008) and Pb levels (Arpadjan et al., 2008) were observed in this study and lower Sn (20 vs 3000 µg.kg\(^{-1}\)) and Mo (490 vs 2300 µg.kg\(^{-1}\))(Mihaljev et al., 2014). Elevated Cd (310 vs 76 µg.kg\(^{-1}\)), Co (256 vs 21 µg.kg\(^{-1}\)), and Cr (896 vs 490 µg.kg\(^{-1}\)) levels were observed in our analysis of yarrow flowers (BT-21) compared to concentrations in Zeiner et al. (2015). Based on the results of the exposure assessment using EDI’s derived from the higher IR (See Table 5.7), a potential risk of unacceptable Cd exposure (HQ\(_{\text{Cd}}\) = 1.77) and subsequent risk of cumulative exposure (HI = 2.12) was detected for the oral consumption of yarrow flowers (BT-21).

5.3.3.9 *Symphytum officinale* (Comfrey)

Few studies qualitatively describe the composition of comfrey (Ozyigit et al., 2018; Stanojković-Sebić et al., 2017), and the *in vitro* modelling of Pb-tannin chelation *in planta* (Chin et al., 2009). In a cluster containing coltsfoot too, comfrey demonstrated measurably higher Fe levels than other medicinal plants, as well as Zn and Cr (Ozyigit et al., 2018). In this current study, the stem (BT-25) contained the lowest Mo, Pb, and V compared to all the samples analysed (n = 50). The commercial root (BT-22), conversely, had the highest quantified V level of all the samples. Based on the results of the exposure assessment using EDI’s derived from the higher IR (See Table 5.7), a potential risk of unacceptable Pb exposure (HQ\(_{\text{Pb}}\) = 1.15) for the consumption of comfrey root (BT-22) was detected. A subsequent non-carcinogenic risk of cumulative exposure was detected for the oral consumption of commercial root (BT-22) from Bulgaria, and leaf (BT-23) from Hungary (HI = 1.93 and 1.41). The remaining comfrey samples (BT-24 and 25) collected from a farm in Co. Cork (Ireland) contained notably lower Cd, Pb, Ti, V, and Co than the commercial samples and were considered safe for consumer consumption (HQ and HI <1).
5.3.3.10 *Borago officinalis* (Borage)
A recent study profiled the phytochemical composition of borage flowers, excluding mineral nutrients (Fernandes et al., 2020). Another study by Volpe *et al.* (2015) reported higher Cu and Li concentrations compared to those quantified for BT-25 and -26 in this study. Based on the results of the exposure assessment using EDI’s derived from the higher IR (See Table 5.7), a potential risk of unacceptable Cd exposure was detected for the consumption of powdered (BT-26; HQ\(_{(Cd)} = 1.03\)) and cut (BT-27; HQ\(_{(Cd)} = 1.11\)) aerial borage material from Germany. A subsequent non-carcinogenic risk of cumulative exposure was detected for the oral consumption of both samples (BT-22, HI = 1.93; and BT-23, 1.41).

5.3.3.11 *Tussilago farfara* (Coltsfoot)
The hyperaccumulating potential of coltsfoot was recently investigated (Jakovljević *et al.*, 2020; Wechtler *et al.*, 2019). In a recent analysis of medicinal plants, coltsfoot contained the highest levels of Cr, Fe, K, Ni, and the lowest concentration of Pb (Ozyigit *et al.*, 2018). In agreement, Petukhov *et al.* (2020) noted highest Fe accumulation in coltsfoot in addition to Mn and Zn, levels of which cannot be compared to since these elements were not quantifiable in this current study. Comparable levels of Cd, Cr, Ni and Pb - in line with Wechtler *et al.* (2019) - were observed for coltsfoot flowers from Albania (BT-28) and leaves from Poland (BT-29). The flowers contained the highest Ni (6060.33 µg.kg\(^{-1}\)) levels; and the leaves contained the highest Co (651.76 µg.kg\(^{-1}\)) levels out of all fifty samples analysed in this current study. Coltsfoot leaf (BT-29) was the only sample where Li was above the calibration range and could not be quantified. Based on the results of the exposure assessment using EDI’s derived from the higher IR (See Table 5.7), both samples displayed safe HQ values (i.e. <1). The non-carcinogenic risk of cumulative exposure, however, was detected for the oral consumption of both samples (BT-28, HI = 1.36; and BT-29, 1.53). These results are in-agreement with Luo *et al.* (2021) who reported HI > 1 for coltsfoot flower.

5.3.3.12 *Vicia sepium* (Bush Vetch)
This is the first multi-elemental (metallomic) profiling of bush vetch to the best of our knowledge. Other studies have investigated related species, for example the *in situ* phytostabilisation of Cd, Pb and Zn in *Vicia sativa* (Brown *et al.*, 2003), induced Hg accumulation in *Vicia villosa* (hairy vetch) (Moreno *et al.*, 2005) and accumulated levels
of Cu, Fe, Pb and Zn in *Vicia cracca* (wild bird vetch) (Petukhov et al., 2020). Based on the results from the mathematical risk assessment (see Table 5.7), there was no risk detected (i.e. HQ and HI < 1) for the oral consumption of aerial bush vetch wild-collected in Ireland, at the theoretical exposure scenarios modelled in this study (i.e. 200 mg.day\(^{-1}\) and 200 g.day\(^{-1}\)).

5.3.3.13  *Lotus corniculatus* (Birds Foot Trefoil)

Babincev (2017) reported elevated levels of Pb (87 – 254 mg.kg\(^{-1}\)) and Cd (3 – 11 mg.kg\(^{-1}\)) in bird’s-foot trefoil compared to our findings (BT-31) of 403.10 and 47.97 µg.kg\(^{-1}\), respectively – otherwise, data is limited in the literature for this plant. Based on the results of the exposure assessment using EDI’s derived from the higher IR (See Table 5.7), an aerial sample of bird’s foot-trefoil wild-collected in Ireland displayed safe HQ values (i.e. <1). The non-carcinogenic risk of cumulative exposure, however, was detected for the theoretical oral consumption of the sample (HI = 1.12), at the higher IR.

5.3.3.14  *Leucanthemum vulgare* (Ox-Eye Daisy)

An earlier study by Badino *et al.* (1998) observed Pb and Zn accumulation in ox-eye daisy; however, no other information is available in the literature concerning its elemental composition. The highest Li concentration out of all fifty samples was observed in the flower (BT-32) at 3964.03 µg.kg\(^{-1}\), which is over 200 times more than the lowest value observed for horse-chestnut seed (BT-18). The flower (BT-32) typically contained higher levels of all elements analysed, except for Ni, when compared to the leaf (BT-33) sampled from the same parent plant which was wild-collected in Ireland. As a result, despite demonstrating safe HQ values (i.e. <1), the non-carcinogenic risk of cumulative exposure was detected for the flower (BT-32, HI = 2.12) but not the leaf (see Table 5.7).

5.3.3.15  *Myrrhis odorata* (Sweet Cicely); *Rhinanthus minor* (Yellow Rattle); *Menyanthes trifoliata* (Bogbean); *Jasione glutinosa* (Rock Tea):

This is the first multi-elemental (metallomic) profiling of sweet cicely, yellow rattle, bogbean and rock tea, to the best of our knowledge. Bogbean (BT-36; aerial) contained lowest levels of Pt (2.05 µg.kg\(^{-1}\)) and Cr (99.64 µg.kg\(^{-1}\)) out of all fifty samples analysed. Levels of Ti and Mo were above the calibration range and could not be quantified for yellow rattle and rock tea, respectively. Out of the 4 novel samples, Cu was only quantifiable in bogbean (BT-36; aerial) at 3904.57 µg.kg\(^{-1}\). Based on the results from the mathematical risk assessment (see Table 5.7), there was no risk detected (i.e. HQ and HI
< 1) for the oral consumption of the aerial samples of wild Irish sweet cicely (BT-34) and bogbean (BT-36), or rock tea from Spain (BT-39), at the theoretical exposure scenarios modelled in this study (i.e. 200 mg.day\(^{-1}\) and 200 g.day\(^{-1}\)). A non-carcinogenic risk of cumulative exposure was detected for wild-collected yellow rattle from Ireland (BT-35; HI = 1.05), however based on the EDI derived from the high IR (i.e. 200 g.day\(^{-1}\)) or “worst-case” exposure scenario.

5.3.3.16 **Artemisia vulgaris** (Mugwort):

Data for mugwort is limited, with reports concerning Cd-accumulation (Rebele & Lehmann, 2011) and related species, i.e. *Artemisia arborescens* (wormwood) only (El Hamiani et al., 2015). Based on the results of the exposure assessment using EDI’s derived from the higher IR (See Table 5.7), a potential risk of unacceptable Cd exposure (HQ\(_{(Cd)}\) = 1.10) and subsequent risk of cumulative exposure (HI = 1.66) was detected for the oral consumption of mugwort flower and leaf (BT-37) wild-collected in Ireland.

5.3.3.17 **Verbascum thapsus** (Great Mullein)

There are several studies on related species *V. olympicum* Boiss. (Arslan et al., 2010; Güleryüz et al., 2006) and *V. speciosum* (Malayeri et al., 2013). Data is limited, however, on the multi-elemental composition of *V. thapsus*. Recent studies have investigated its Cd accumulation (Čudić et al., 2016) and Cu phytoextraction efficiencies (Kavousi et al., 2021). Another study quantified Pb levels in the root and shoot (1342 and 995 mg.kg\(^{-1}\)) that were much higher than our findings for BT-38 (2047.83 µg.kg\(^{-1}\)) (Yildirim & Sasmaz, 2017). Wild-collected great mullein from Co. Sligo (Ireland) contained highest levels of Mo (4504.50 µg.kg\(^{-1}\)) and Tl (90.82 µg.kg\(^{-1}\)) in this current study. Based on the results of the exposure assessment using EDI’s derived from the higher IR (See Table 5.7), a potential non-carcinogenic risk of unacceptable Pb exposure (HQ\(_{(Pb)}\) = 1.67) and subsequent risk of cumulative exposure (HI = 2.10) was detected for the oral consumption of great mullein leaves (BT-37).

5.3.3.18 **Silene saxifraga** L. (Tufted Catchfly)

Analyses of related species are found in the literature, such as Tl accumulation in *S. latifolia* (Escarré et al., 2011), As accumulation in *Silene vulgaris* (Schmidt et al., 2004), and Cu tolerance in *S. paradoxa* (Colzi et al., 2015). This current study is however, the first multi-elemental (metallomic) profiling of this species. The lowest quantifiable levels of Cu were detected in tufted catchfly (BT-40) at 1910.27 µg.kg\(^{-1}\). Based on the results
from the mathematical risk assessment (see Table 5.7), there was no risk detected (i.e. HQ and HI < 1) for the oral consumption of the aerial samples of the leaf and stem sample of tufted catchfly (BT-40) at the theoretical exposure scenarios modelled in this study (i.e. 200 mg.day\(^{-1}\) and 200 g.day\(^{-1}\)). A non-carcinogenic risk of cumulative exposure however was detected (HI = 1.53), based on the EDI derived from the high IR (i.e. 200 g.day\(^{-1}\)) or “worst-case” exposure scenario.

5.3.3.19  \textit{Salvia officinalis} L. (Sage)

Metals tend to accumulate in the aerial parts of sage, including the inflorescences (Angelova et al., 2006). In previous studies, mean Cu content in sage leaf was 1.4 mg.kg\(^{-1}\) (Pytlakowska et al., 2012) and 10.5 mg.kg\(^{-1}\) (Juranović Cindrić et al., 2013) in comparison to 2318 µg.kg\(^{-1}\) (BT-41). Our findings are similar for Co, lower for Cd, Ni, Pb, and higher for Cr and V when compared to Thabit et al. (2020). Levels of Cu, Cd and Pb were also lower than those reported in other studies using AAS (Dghaim et al., 2015) and FAAS (Kilicel et al., 2017). There was no risk detected (i.e. HQ and HI < 1) for the oral consumption of aerial sage material sourced from Spain at the theoretical exposure levels modelled in this study.

5.3.3.20  \textit{Glycyrrhiza glabra} (Liquorice)

Elevated levels of Cr, Cu and Pb analysed via TRXF were reported previously (Khuder et al., 2009). Similarly, higher levels were observed for Cd (720 vs 25.03 µg.kg\(^{-1}\)) (Özcan & Akbulut, 2008) and Li (1.8 vs 0.6 mg.kg\(^{-1}\)) (Zengin et al., 2008) compared to our findings. Other studies analysed liquorice stem and leaf only which is outside the scope of this study (Kulhari et al., 2013). There was no risk detected (i.e. HQ and HI < 1) for the oral consumption of liquorice root sourced from Spain at the theoretical exposure levels modelled in this study.

5.3.3.21  \textit{Althaea officinalis} (Marshmallow)

Our findings for Co and Ni in marshmallow root are in good accordance to the data published by Mihaljev et al. (2014) – however, Mo and Sn content in BT-43 was lower. Sn concentration in the root (BT-43) was the lowest out of all samples analysed in this current study (6.39 µg.kg\(^{-1}\)). Data is limited in the literature for this plant. Based on the results of the exposure assessment using EDI’s derived from the higher IR (See Table 5.7), a potential risk of unacceptable Cd exposure (HQ\(_{(Cd)}\) = 1.22) and subsequent non-
carcinogenic risk of cumulative exposure (HI = 1.68) was detected for the oral consumption of the root (BT-43) sourced from Spain.

5.3.3.22  **Lavandula angustifolia (Lavender)**
Our findings for Pb (Bozhanov et al., 2007), Cr and Ni (S. S. Ražić et al., 2006) are in line with previous results. Data for Co, Cu and V are measurably lower than previous reports (Bozhanov et al., 2007; Haidu et al., 2017). Interestingly, (Zheljazkov and Nielsen (1996) further observed a positive correlation between the Cu concentration in the inflorescence and the resulting essential oil derived – but not for Cd, Pb, Mn, Fe, Zn. This agrees with a later study of trace element transfer [flower to oil] in *L. angustifolia* (Bozhanov et al., 2007). Sierra *et al.* (2012) also describes lavender as a Hg tolerant excluder, and the presence of Hg and Mn influences Pb uptake in lavender. There was no risk detected (i.e. HQ and HI < 1; see Table 5.7) for the oral consumption of lavender flower sourced from Spain at the theoretical exposure levels modelled in this study.

5.3.3.23  **Hypericum perforatum (St. John’s Wort)**
Leaf Cu (Pytlakowska et al., 2012), Zn (Tokalioglu, 2012), and Ni (Filipiak-Szok et al., 2015) could not be compared as they were above the calibration range in this study and thus could not be quantified. Our findings for Co, Cr (Bonari et al., 2019), Cd (Filipiak-Szok et al., 2015) and Pt (Owen et al., 2016) were lower than previously reported. Owen *et al.* (2016) reports that elevated plant Cr concentrations in St. John’s Wort medicinal products could be due to contamination from metal alloys in the manufacturing process. There was no risk detected (i.e. HQ and HI < 1) for the oral consumption of an aerial sample of St. John’s Wort (BT-45; see Table 5.7) sourced from Spain at the theoretical exposure levels modelled in this study.

5.3.3.24  **Melissa officinalis (Lemon Balm)**
Our findings for Cr, Cu, Ti, V and Ni were lower than those reported previously (Özcana et al., 2008; Sussa et al., 2016; Tokalioglu, 2012); except for Co (Sussa et al., 2016) and Pb (Tokalioglu, 2012). Cd-Zn interactions have been shown to alter Cu, Pb and Mn uptake in lemon balm (Adamczyk-Szabela et al., 2019, 2020). Cd was shown to reduce essential oil yield in lemon balm seedlings (Kilic & Kilic, 2017), thus demonstrating that cultivation parameters can impact the medicinal value of a plant. In this study, there was no risk detected (i.e. HQ and HI < 1) for the oral consumption of an aerial sample of
lemon balm (BT-46 see Table 5.7) sourced from Spain at the theoretical exposure levels modelled.

5.3.3.25  *Santolina chamaecyparissus* (Cotton Lavender)

Element profiling data is limited in the literature for this plant. Zekri et al. (2019) describes this plant as a ‘*Pb excluder’*, which might explain the lower Pb concentration (48.01 µg.kg⁻¹) observed in cotton lavender flowers (BT-47) compared to the majority other samples tested. In this study, there was no risk detected (i.e. HQ and HI < 1; see Table 5.7) for the oral consumption of cotton lavender flowers sourced from Spain at the theoretical exposure levels modelled.

5.3.3.26  *Mentha × piperita* (Peppermint)

Co, Cr and Li concentrations are in good accordance with Lozak et al. (2002). Similarly, Cd is within the lower range of previous reports (Arpadjan et al., 2008; Milani et al., 2019; Rubio et al., 2012). Cu, Pb and Zn distribution in peppermint decreased accordingly: roots > leaves > stem > flower, and for Cd: roots > flowers > leaves > stem (Angelova et al., 2006). The elevated levels observed in this study (BT-49) when compared to previous findings, could be related to the fact we analysed the comminuted whole aerial plant parts (leaves, stem, flowers) – not the isolated part(s). This emphasises the importance of specifying the plant part analysed, and not just the species, to ascertain variations between plant tissues and organs. There was no risk detected (i.e. HQ and HI < 1; see Table 5.7) for the oral consumption of peppermint (BT-49) sourced from Spain at the theoretical exposure levels modelled.

5.3.3.27  *Peumus boldus* Molina (Boldo)

Boldo leaf presented with the highest levels of Cr (4534.43 µg.kg⁻¹) out of all fifty samples analysed in this present study – which is higher than levels observed previously (Milani et al., 2019; Silva et al., 2016). Levels of Cd (Santos et al., 2017) are in accordance with previous reports. Our findings for Pb (129.26 µg.kg⁻¹) are in line with Milani et al. (2019), but much lower for Co, Hg and V compared to Silva et al. (2016). Based on the risk assessments in this current study, no risk was detected (i.e. HQ and HI < 1; see Table 5.7) for the oral consumption of boldo leaves sourced from Spain at the theoretical exposure levels modelled.
5.3.4 Recommendations to facilitate botanical safety assessments

Health risk is primarily associated with duration (Ćwieląg-Drabek et al., 2020), and the rates of ingestion (Harris et al., 2011). A current, major data gap in Europe is the considerable lack of intake (consumption, occurrence) survey data for medicinal herbs and botanicals ingredients (Restani, 2018), despite evidence of increasing popularity among consumers (Colombo et al., 2020). Consumer intake data directly influences actual exposure and corresponding risk assessment predictions. It is vitally important with regards to the actual contributions and realistic exposure scenarios (Schulzki et al., 2017). Risk assessment methodologies often account for lifelong daily use which may not be representative for herbal/botanical preparations (Chen et al., 2019). Shorter-than-lifetime use are often more reflective of real-life scenarios, considering intermittent, non-consecutive usage of herbal- or plant-food supplements, herbal medicinal products (HMPs) or herbal beverages (Chen et al., 2019). Intake patterns are variable, from a few days, to a few years, to daily consumption (Colombo et al., 2020), which makes interpretation challenging. Integrating measures of prospective intake in national dietary surveys, albeit complex, would provide crucial data for botanical safety assessments at European level (García-Alvarez et al., 2014).

The inclusion of realistic exposure scenarios can also help contextualise analytical findings. Generic IR(D)’s of 200 and 500 g day\(^{-1}\), signifying the mean and 95\(^{th}\) percentile (maximum daily dosage) of Chinese Herbal Medicinal Products, respectively, was proposed in the 2020 Chinese Pharmacopeia (ChP, 2020) and validated in recent health risk assessments of herbal preparations (Luo et al., 2021; Zuo et al., 2020). These intake values are considerably higher than those [infrequently] quoted by European counterparts regarding medicinal herb consumption (Chen et al., 2019; Mihats et al., 2017; Schulzki et al., 2017). According to a National Food Consumption Survey (Germany), an herbal tea intake of 0.093 g (kg BW\(^{-1}\)d\(^{-1}\)) was estimated, equating to an average 6.5 g day\(^{-1}\) (adult; 70 kg BW) for high consumers (Schulzki et al., 2017), corresponding to the preparation of a water-based herbal tea/infusion (10 g plant material per 1L). Another study assumed realistic and worst-case scenario daily intakes of 95.4 mL and 363 mL (children), 194.7 mL and 1 L (adult; female), 114 mL and 600 mL (adult; male) (Mihats et al., 2017), again for herbal tea/infusions only. Recently, Chen et al. (2019) surrogated a lifetime exposure of 200 mg day\(^{-1}\) in their risk assessment of herbal products, modelled on data described by EFSA (2016). Considering the absence of a validated European
equivalent, we therefore opted to implement 200 g.day\(^{-1}\) to represent a theoretical maximum or “conservative” IR(D) (ChP, 2020) and a theoretical minimum IR(D) of 200 mg.day\(^{-1}\) (Chen et al., 2019) to represent a more “realistic” exposure scenario, in the risk assessment equations outlined earlier (Section 5.2.4).

An additional consideration for the analysis of herbal preparations (e.g. teas, decoctions, tinctures) is **metal ion solubility** and the associated **metal ion transition rate** (%). Estimations involve the comparison of the metal concentration in the raw (fresh or dried) plant material to the final preparation at a specific volume as described by Schulzki *et al.* (2017), and prior referred to as the “leaching efficiency” by Harris *et al.* (2011). Some authors suggest that the metal transition rates in herbal teas/infusions are influenced by the matrix (i.e. plant species), origin, grade (i.e. tea leaf grade), particle size, processing techniques and mode of preparation (i.e. infusion duration, water temperature) (Schulzki *et al.*, 2017). Milani *et al.* (2019) categorised Al, As, Ba, Sc, Cr, Fe, Pb and Se as poorly extractable and Cu, Mn, Ni and Zn, as moderately extractable in herbal infusions. Two hypothetical exposure scenarios proposed by Harris *et al.* (2011) were referred to as the “most likely” and the “most conservative”, referring to acute exposure with 10% leaching (i.e. from plant material to final product/ preparation), and chronic exposure at 100% leaching, respectively. Alternatively, a low, medium, and high (i.e. worst-case) theoretical transition rate of 10, 50 and 100% could be implemented in calculations to have a more representative suite of [metal-to-preparation] transition rates.

In the absence of a either a standardised/theoretical universal transfer rate or an experimental transfer rate specific to each metal and plant matrix analysed, generic assumptions have been applied in studies however this may not be truly representative and could lead to over- or under-estimations. Transition rates are highly variable between analytes and samples - Zuo *et al.* (2020) reported that the average metal transfer rate for Chinese Herbal Medicinal Products is ≤10%; while Schulzki *et al.* (2017) and Luo *et al.* (2021) reported transfer rates of 16 - 92.2% (Cu) and 13.1 – 50.0% (Al); and 14% (Cd, Cu and Pb), 35% (As) and 24% (Hg), respectively. Development of a universal default transfer (or bioavailability) rate for risk assessment would facilitate further inter-study comparisons. The transfer rates and other relevant mathematical input criteria applied in this study are outlined in Table 5.4.
The carcinogenic risk (CR) assessment allows for the estimation of the possibility of a population developing cancer following exposure to a carcinogen (Orosun et al., 2020). Some studies report the carcinogenic risk of Cd, Cr, Pb and Ni using the incremental lifetime cancer risk (ILCR) equation, which is a probabilistic assessment of carcinogenic risk involving the multiplication of the estimated chronic daily intake over a lifetime (e.g. 70 years) by the corresponding cancer slope factor (CSF) for the carcinogenic substance (i.e. Cd, Cr, Pb and Ni), as shown in Table 5.4 (Orosun et al. 2020, Ssempijja et al., 2020; CalEPA, 2019; IRIS Database, 2012). Level of risk can be categorised based on the Delphi method from $<10^{-6}$ (extremely low risk) to $>10^{-3}$ (extremely high risk) (Orosun et al., 2020). CR assessment was however excluded from this current study due to the unavailability of a validated method and the generally unexplained variations in the equation used in many studies assessing the CR of carcinogenic metals in botanical or herbal products (Luo et al., 2021; Orosun et al., 2020; Zuo et al., 2020). Additionally, if considering supplementation or treatment with PFS and/or HMPs, intermittent exposure scenarios may be more representative and thus guidance on the estimated frequency and duration (EF, ED) is necessary to ensure robust estimations. Considering that the IARC classifies Be, Cd, Cr(VI) and Ni as Group 1 compounds (carcinogenic to humans), Pb as Group 2A (probable carcinogens) and Co as Group 2B (possible carcinogens) (IARC, 1990b, 1990a, 1993, 2006), the standardised assessment of the carcinogenic risk of these hazardous contaminants is essential in the context of public health.

Standardisation and/or official guidance on risk assessment input parameters and criteria would critically support future inter-study comparison in this area of research, and thus, help assure botanical safety.

5.4 Conclusion

This study aimed to quantify the concentration of twenty-two selected elements in fifty medicinal plant matrices from various geographical locations using ICP-SFMS, and to contextualise the toxicological significance of non-carcinogenic human exposure at defined theoretical intake rates. All analysed samples were below the European pharmacopeial ML’s for metal impurities in herbal substances/starting materials: Cd ($\leq 1 \text{ mg.kg}^{-1}$), Hg ($0.1 \text{ mg.kg}^{-1}$), and Pb ($5 \text{ mg.kg}^{-1}$). Findings from the mathematical modelling showed that all calculated dietary exposure estimates were well within the
acceptable ranges, except for Li, Mo, Tl, Pb, Cr, Co, and Ni. The authors further discuss key points pertaining to the risk assessment of botanicals, with a research outcome of influencing policy at a national and/or international level. This work demonstrates progress in the metallomic profiling of plants and provides a range of novel evidence for the metal composition of common botanicals (medical and culinary). In summary, there must be a concerted effort to build on observational and quantitative data within this field, especially with regards to safety.

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**Declaration of interest**

The authors declare that there is no conflict of interest in this work.
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Chapter VI

Thesis Summary
**Thesis Summary**

The work presented in this thesis explored *Inula helenium* L. (elecampane) as an exemplar for the use and regulation of plants as antimicrobial agents. It is hoped that the topics presented in this thesis will both support and re-ignite interest in botanical sciences. The scope of this thesis encompassed *in vitro* bioactivity-guided drug discovery processes, multi-elemental (metallomic) profiling of plant matrices, regulation, and consumer safety. Key contributions to the field of pharmacognosy and phytotherapy are listed below, followed by a summary of the research aims and key findings from each individual chapter:

2. Critical evaluation of gaps in plant research methodologies, method standardisation and governing regulation of raw plant material (herbal substances) and final consumer-ready products (e.g. herbal medicinal products, herbal teas/infusions, botanical ingredients, phytopharmaceuticals and plant-derived foods).

Chapter I reviewed a range of modified *in vitro* methods commonly employed to determine preclinical antimicrobial efficacy of plant-derived natural products, and the associated limitations and challenges in the provision of new antimicrobial drugs from plants. Currently, there are no approved guidelines, standards, or official recommendations governing *in vitro* antimicrobial screening or susceptibility testing methodologies for natural products of plant origin thus jeopardising transparency within the field. Research shows the rate of new antimicrobial development is insufficient to meet our current and future needs globally. This, along with a weak drug portfolio pipeline, emphasises the importance – and relevance - of investigating prosperous sources of potentially new structures, such as plants. This chapter introduced the experimental model used throughout this body of work, *Inula helenium* L. (elecampane) and justifies elecampane as a promising reservoir of antimicrobial compounds.

As shown in chapter II, a key research question of this project was to identify the natural product compound(s) attributing to the anti-staphylococcal activity of a traditional hydro-ethanolic extract of multi-origin elecampane root, previously observed within our
laboratory. Application of a novel clean-up strategy and bioactivity-guided fractionation process resulted in a subset of antibacterial or ‘active’ fractions. The phytochemical composition of these fractions was later analysed using a validated HPLC-DAD method supported by $^1$H NMR, in collaboration with the Polish Academy of Sciences. The target compounds associated with the observed activity were identified as alantolactone, isoalantolactone, igalan(e), and an unseparated mixture of dugesialactone and alloalantolactone as major constituents. Another finding of this study was that the geographical origin of elecampane did not appear to influence either the chemical profile or the bioactivity of the analysed root extracts. Elecampane clearly demonstrates activity against *Staphylococcus* spp. and considering the prevalence of antimicrobial resistance in Irish hospitals among this genus and the high prevalence of MRSA, further investigation is warranted. Follow-on studies could include large-scale purification or synthesis of the identified compounds followed by *in vivo* analysis of the compounds, individually and in combination, and combinatorial experimentation as potentiator or adjuvant compounds to conventional antibiotic treatment.

Chapter III reviewed the current literature and introduced the concept of elemental impurification of plant material, the consequences of exposure to human health, and the regulations that are currently in place to safeguard the general European population. Plants acquire toxicologically significant metals from the environment during cultivation and collection, and processing/manufacturing. Metals can thus arise as unsolicited residues in our food and medicinal products leading to a plethora of acute and/or chronic adverse health effects. The ATSDR lists aluminium (Al), arsenic (As), cadmium (Cd), chromium (Cr), mercury (Hg), and lead (Pb) as top priority metals of human concern, which are further classified as human carcinogens by the IARC. While regulatory measures are enforced in European Member States, there are apparent gaps in legislation concerning the presence of certain metals in food. Regarding phytopharmaceuticals, the adapted ICH Q3D guidelines published by the EMA define limits for a total of twenty-four elements, however, these limits are not currently applicable to herbs or phytopharmaceuticals. In contrast, compendial (Ph. Eur.) limits for herbal products exist for only Cd, Hg and Pb. Two possible solutions to consider include extending the application of ICH Q3D guidelines to cover herbal products, or alternatively, the establishment of a defined set of general permissible limits for a greater suite of toxicologically significant metals applicable to herbal products. Trace element
interactions could influence nutritional status and interfere with normal biological functions in humans, therefore, harmonisation of quality requirements for food and medicinal products is a necessity, particularly in the context of international trade and assurance of consumer safety.

Chapter IV documents the novel elemental profiling of multi-origin elecampane \( (n = 27) \). The aim of this study was to quantify for the first time, the multi-elemental (metallomic) profile of naturalised Irish and commercial samples of elecampane and investigate the risk of dietary exposure \textit{in silico}. The herbal samples were analysed using a validated ICP-SFMS method following microwave acid-digestion. The EFSA RACE tool was used to contextualise the toxicological significance of acute and/or chronic dietary exposure to metal contaminants in a specific food matrix (i.e. ‘herbs and edible flowers’) in relation to Irish consumers (adults, elderly and very elderly). Results showed that chronic exposure to Pb at a maximum quantified concentration of 4617.42 µg.kg\(^{-1}\) in edible plant material is of potential risk to adult consumers in Ireland (18-65 y), at an estimated average and above-average exposure of 0.049 and 0.189 µg \((\text{kg BW})^{-1}\text{d}^{-1}\), respectively. Further investigation is advised, including soil and water analysis from the sampling location to ascertain if Pb levels are within acceptable environmental limits. Additionally, 52% of the herbal samples were found to exceed European limits for Cd in food (200 µg.kg\(^{-1}\)), and one sample exceeded the compendial ML for Cd impurities in herbal material/drugs (> 1 mg.kg\(^{-1}\)). These findings illustrate non-compliance of herbal substances, available to purchase by local communities in Ireland and online consumers.

Dietary exposure to Cd at the highest observed concentration (1285.97 µg.kg\(^{-1}\)) in the elecampane samples, despite exceeding regulatory limits, was categorised as no risk to Irish consumers when considering consumption of this single food category. The remaining elements (Li, Be, Mo, Sn, Ba, Hg, Tl, V, Cr, Co, Ni, Cu) were well below ML’s, thus dietary exposures to these elements are of negligible concern. Outputs from the RACE analysis, however, focus on one contaminant in one food commodity at a time, and therefore background or aggregate exposure from all other dietary sources needs consideration – an important factor to consider when interpreting risk assessment data. Another major finding of this study revealed a statistically significant difference in element distribution between flowers – leaves (Be, Li, Ba, Cd, Bi), root – leaves (Mo, Sn, Co, Hg) and flowers – root (Ba and Tl) in naturalised elecampane. The data presented in
chapters IV and V will contribute to knowledge in the areas of phytoremediation, nutritional analysis, impurity profiling and consumer safety.

Chapter V examined the multi-elemental (metallomic) profile(s) of fifty botanical matrices commonly used in botanical preparations using a validated ICP-SFMS method. This work is the first to report the metallomic profiling of arnica, bush vetch, sweet cicely, yellow rattle, bogbean, rock-tea and tufted catchfly to date. Shorter-than-lifetime daily intakes were estimated using conservative (“worst-case”) and realistic theoretical exposure scenarios. The non-carcinogenic risk assessment for Cd, Cu, Hg and Pb exposure was subsequently evaluated using HQ and HI estimations.

All botanical samples analysed ($n = 50$) were below the compendial ML’s for metal impurities in herbal substances/starting materials: Cd ($\leq 1 \text{mg.kg}^{-1}$), Hg (0.1 mg.kg$^{-1}$), and Pb (5 mg.kg$^{-1}$), however, Li, Mo, Tl, Pb, Co, and Ni were quantified at potentially unsafe levels (e.g. high EDI$_{(200 \text{g.day}^{-1})}$ > HBGV) at the theoretical worst-case exposure scenario. Furthermore, 42% of all samples tested representing 16 different plant species ($n = 30$) were categorised as potentially unsafe to consumers (HI $\geq 1$) with regards to the non-carcinogenic cumulative exposure to Cu, Cd, Hg and Pb, including: hawthorn, arnica, dandelion, marigold, nettle, yarrow, comfrey, borage, coltsfoot, birds foot trefoil, ox-eye daisy, yellow rattle, mugwort, great mullein, tufted catchfly and marshmallow. These findings suggest a potential risk associated with the consumption of several herbal substances (raw plant material) available on the EU market currently, which further justifies the necessary monitoring of these products to protect consumers. Additional findings from Chapter V established that pertinent data including intake consumption data and frequency rates at European level, are vitally needed to facilitate robust risk assessment and ensure botanical safety for consumers. Both the disclosure and record of plant-derived product(s) intake should therefore be incorporated in national dietary surveys where possible. Standardisation and/or the availability of official guidance on risk assessment input parameters would critically support future inter-study comparison in this area of research. The work presented in Chapter IV - V demonstrates progress in the metallomic profiling of plants and provides a range of novel evidence for the metal composition of common botanicals (medical and culinary). Overall, there must be a concerted effort to build on observational and quantitative data within this field, especially with regards to safety.
In conclusion, plants, whether utilised as food or medicine, should meet acceptable safety and quality standards. Research methodology standardisation and validation is encouraged to increase transparency within the field, particularly in the provision of novel therapeutics such as antibiotics. Greater knowledge of element concentrations in plants (and extracts or products thereof) is necessitated to minimize risk to consumers and ensure the future of botanical safety.
Written Declaration

I hereby declare that the submitted Ph.D. thesis is my own work, and to the best of my knowledge, it contains no materials previously published or written by another person. None of the material presented in this thesis has been submitted for an award at another institution.

The research presented complies with MTU’s Code of Good Practice in Research. Any contribution to the research by others, with whom I have worked, is acknowledged in the thesis. I also state that the intellectual content of the work presented herein is the product of my own efforts, however, I may have received assistance from others on the project design, style, presentation, and language expressions.

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