

**Novel methods for the
assessment of chromium species
bioavailability**

ELLIOTT HAMILTON, MChem

Thesis submitted to the
University of Nottingham for the
degree of Doctor of Philosophy

May 2020

Abstract

Chromium (Cr) exists in the environment as two chemical species with very different physiological effects. The World Health Organization classifies hexavalent chromium (Cr^{VI}) as a carcinogen, whilst trivalent chromium (Cr^{III}) is essential for the regulation of glucose and lipid metabolism. Assessing the risk to human health from Cr^{VI} is partly dependent on a greater understanding of the species interconversions at the soil-to-plant interface. Vital to this objective is the validation of techniques capable of accurately determining Cr^{VI} in solid matrices such as soil and plant tissues.

This thesis aimed to develop, optimise and apply novel analytical methods to understand Cr species interconversions occurring at spatial and temporal scales relevant to plant uptake of Cr from soil. Initially, a modified speciated isotope dilution mass spectrometry (SIDMS) technique, incorporating microwave-assisted alkaline extraction and online species separation, was used to measure Cr^{VI} in agricultural soils exposed to elevated metal concentrations through dispersed mine tailings. The Cr^{VI} concentrations in all samples indicated a low exposure risk despite optimal conditions for preservation of Cr as the hexavalent species in the tailings material, highlighting the influence of soil characteristics on reduction of Cr^{VI} .

The mechanisms of Cr species uptake into spinach plants, and the species interconversions occurring prior to uptake, were assessed on a solution basis using isotopic tracers. The impact of sulphate, reported to compete with Cr^{VI} for active uptake, was also investigated. The results showed that Cr^{III} could be absorbed from solution into roots through active (symplastic) and passive (apoplastic) mechanisms, with reduction of Cr^{VI} observed both after uptake and in the tracer solutions. Sulphate promoted active uptake of Cr^{VI} , which was present in the symplast as Cr^{III} . These findings suggested that plants can tolerate Cr^{VI} through reduction processes before and after uptake, with evidence of a synergistic effect in the presence of sulphur.

The suitability of microdialysis for extracting Cr^{VI} from soil solution was appraised during the development of an online system, coupled to HPLC-ICP-QQQ. The setup was able to monitor changes in the concentration of labelled- $^{53}\text{Cr}^{\text{VI}}$, added to soil through direct spiking and homogenised mixing, at 15-minute intervals, far surpassing the temporal resolution of previous passive sampling techniques. Analytical figures of merit show that the system was sufficiently sensitive ($\text{LOD} \leq 0.3 \mu\text{g L}^{-1}$) and produced repeatable measurements ($\text{RSD of standards} < 2.5\%$), demonstrating that it is a powerful tool for providing further insight into the bioavailability of Cr^{VI} and its interactions with soil microbes and the rhizosphere.

This thesis contributes to a better understanding of plant-Cr species dynamics, and demonstrates the importance of developing and validating accurate, high-resolution analytical techniques to investigate existing and emerging research questions surrounding soil-to-plant transfer of Cr^{VI} .

Publications arising from part-time PhD programme

Hamilton EM, Young SD, Bailey EH and Watts MJ. "Chromium speciation in foodstuffs: A review." *Food Chemistry* 250 (2018): 105-112. doi: 10.1016/j.foodchem.2018.01.016

Hamilton EM, Lark RM, Young SD, Bailey EH, Sakala GM, Maseka KK, and Watts MJ. "Reconnaissance sampling and determination of hexavalent chromium in potentially-contaminated agricultural soils in Copperbelt Province, Zambia." *Chemosphere* 247 (2020): 125984. doi: 10.1016/j.chemosphere.2020.125984

Hamilton EM, Young SD, Bailey EH, Humphrey OS, and Watts MJ. "Assessment of chromium species dynamics in root solutions using isotope tracers." *Journal of Trace Elements in Medicine and Biology* (2020): 126514. doi: 10.1016/j.jtemb.2020.126514

Hamilton EM, Young SD, Bailey EH, Humphrey OS, and Watts MJ. "Online microdialysis-high performance liquid chromatography-mass spectrometry (MD-HPLC-ICP-MS) as a novel tool for sampling hexavalent chromium in soil solution." Under review in *Environmental Science and Technology*.

Co-author/publications arising during part-time PhD programme

Hamilton EM, Barlow TS, Gowing CJB, and Watts MJ. "Bioaccessibility performance data for fifty-seven elements in guidance material BGS 102." *Microchemical Journal* 123 (2015): 131-138. doi: 10.1016/j.microc.2015.06.001

Ander EL, Watts MJ, Smedley PL, **Hamilton EM**, Close R, Crabbe H, Fletcher T, Rimell A, Studden M, and Leonardi G. "Variability in the chemistry of private drinking water supplies and the impact of domestic treatment systems on water quality." *Environmental Geochemistry and Health* 38, no. 6 (2016): 1313-1332. doi: 10.1007/s10653-016-9798-0

Middleton DRS, Watts MJ, **Hamilton EM**, Ander EL, Close RM, Exley KS, Crabbe H, Leonardi GS, Fletcher T, and Polya DA. "Urinary arsenic profiles reveal exposures to inorganic arsenic from private drinking water supplies in Cornwall, UK." *Scientific Reports* 6 (2016): 25656. doi: 10.1038/srep25656

Middleton DRS, Watts MJ, **Hamilton EM**, Fletcher T, Leonardi GS, Close RM, Exley KS, Crabbe H, and Polya DA. "Prolonged exposure to arsenic in UK private water supplies: Toenail, hair and drinking water concentrations." *Environmental Science: Processes & Impacts* 18, no. 5 (2016): 562-574. doi: 10.1039/C6EM00072J

Joy EJM, Ander EL, Broadley MR, Young SD, Chilimba ADC, **Hamilton EM**, and Watts MJ. "Elemental composition of Malawian rice." *Environmental Geochemistry and Health* 39, no. 4 (2017): 835-845. doi: 10.1007/s10653-016-9854-9

Lark RM, **Hamilton EM**, Kaninga B, Maseka KK, Mutondo M, Sakala GM, and Watts MJ. "Planning spatial sampling of the soil from an uncertain reconnaissance variogram." *Soil* 3, no. 4 (2017): 235. doi: 10.5194/soil-3-235-2017

Palumbo-Roe B, Banks VJ, Bonsor HC, **Hamilton EM**, and Watts MJ. "Limitations on the role of the hyporheic zone in chromium natural attenuation in a contaminated urban stream." *Applied Geochemistry* 83 (2017): 108-120. doi: 10.1016/j.apgeochem.2017.02.011

Middleton DRS, Watts MJ, Beriro DJ, **Hamilton EM**, Leonardi GS, Fletcher T, Close RM, and Polya DA. "Arsenic in residential soil and household dust in Cornwall, south west England: potential human exposure and the influence of historical mining." *Environmental Science: Processes & Impacts* 19, no. 4 (2017): 517-527. doi: 10.1039/C6EM00690F

Lark RM, **Hamilton EM**, Kaninga B, Maseka KK, Mutondo M, Sakala GM and Watts MJ.

"Nested sampling and spatial analysis for reconnaissance investigations of soil: an example from agricultural land near mine tailings in Zambia." *European Journal of Soil Science* 68(5) (2017): 605-620. doi: 10.1111/ejss.12449

Wragg J, Cave M, **Hamilton EM**, and Lister TR. "The link between soil geochemistry in South-West England and human exposure to soil arsenic." *Minerals* 8, no. 12 (2018): 570. doi: 10.3390/min8120570

Middleton DRS, Watts MJ, **Hamilton EM**, Coe JD, Fletcher T, Crabbe H, Close R, Leonardi GS, and Polya DA. "Surface wipe and bulk sampling of household dust: arsenic exposure in Cornwall, UK." *Environmental Science: Processes & Impacts* 20, no. 3 (2018): 505-512. doi: 10.1039/C7EM00463J

Middleton DRS, McCormack VA, Munishi MO, Menya D, Marriott AL, **Hamilton EM**, Mwasamwaja AO, Mmbaga BT, Samoei D, Osano O, Schüz J, and Watts MJ. "Intra-household agreement of urinary elemental concentrations in Tanzania and Kenya: potential surrogates in case-control studies." *Journal of Exposure Science & Environmental Epidemiology* 29, no. 3 (2019): 335-343. doi: 10.1038/s41370-018-0071-8

Humphrey OS, Young SD, Bailey EH, Crout NMJ, Ander EL, **Hamilton EM**, and Watts MJ. "Iodine uptake, storage and translocation mechanisms in spinach (*Spinacia oleracea* L.)." *Environmental Geochemistry and Health* 41, no. 5 (2019): 2145-2156. doi: 10.1007/s10653-019-00272-z

Humphrey OS, Young SD, Bailey EH, Crout NMJ, Ander EL, **Hamilton EM**, and Watts MJ. "Investigating the use of microdialysis and SEC-UV-ICP-MS to assess iodine

interactions in soil solution." *Chemosphere* 229 (2019): 41-50. doi: 10.1016/j.chemosphere.2019.04.215

Johnson L, Montgomery J, Evans J, and **Hamilton EM**. "Contribution of strontium to the human diet from querns and millstones: an experiment in digestive strontium isotope uptake." *Archaeometry* 61, no. 6 (2019): 1366-1381. doi: 10.1111/arcm.12485

Watts MJ, Middleton DRS, Marriott AL, Humphrey OS, **Hamilton EM**, McCormack V, Menya D, Farebrother J, and Osano O. "Iodine status in western Kenya: a community-based cross-sectional survey of urinary and drinking water iodine concentrations." *Environmental Geochemistry and Health* (2019): 1-11. doi: 10.1007/s10653-019-00352-0

Nakaona L, Maseka KK, **Hamilton EM**, and Watts MJ. "Using human hair and nails as biomarkers to assess exposure of potentially harmful elements to populations living near mine waste dumps." *Environmental Geochemistry and Health* (2019): 1-13. doi: 10.1007/s10653-019-00376-6

Watts MJ, Middleton DRS, Marriott AL, Humphrey OS, **Hamilton EM**, Gardner A, Smith M, McCormack VA, Menya D, Munishi MO, Mmbaga BT, and Osano O. "Source apportionment of micronutrients in the diets of Kilimanjaro, Tanzania and Counties of Western Kenya." *Scientific Reports* 9, no. 1 (2019): 1-14. doi: 10.1038/s41598-019-51075-2

Manzeke MG, Mtambanengwe F, Watts MJ, **Hamilton EM**, Lark RM, Broadley MR, and Mapfumo P. "Fertilizer management and soil type influence grain zinc and iron concentration under contrasting smallholder cropping systems in Zimbabwe." *Scientific Reports* 9, no. 1 (2019): 1-13. doi: 10.1038/s41598-019-42828-0

Sach F, Yon L, Henley MD, Bedetti A, Buss P, de Boer WF, Dierenfeld ES, Gardner A, Langley-Evans SC, **Hamilton EM**, Lark RM, Prins HHT, Swemmer AM, and Watts MJ.

"Spatial geochemistry influences the home range of elephants." *Science of The Total Environment* (2020): 139066. 10.1016/j.scitotenv.2020.139066

Sach F, Dierenfeld ES, Langley-Evans SC, **Hamilton EM**, Lark RM, Yon L, and Watts MJ.

"Potential bio-indicators for assessment of mineral status in elephants." *Scientific Reports* 10, no. 1 (2020): 1-14.

Acknowledgments

I would like to take the opportunity to thank a number of people whose contribution to this thesis, and my personal development throughout the research as a whole, has been nothing short of invaluable. Firstly, my supervisors Michael Watts, Scott Young and Liz Bailey for your support, guidance, wisdom and humour throughout this journey. To in-country collaborators in my “second home” of Zambia- Godfrey Sakala (snake belt currently on the way), Prof. Maseka (bottle of Blue Label may or may not be on the way), Moola Mutundo, Belinda Kaninga, all the ZARI drivers- thank you for your contributions towards site selection, study design, fieldwork and your advice throughout the PhD.

I would like to extend my gratitude towards the Learning & Development team at BGS (Dee Flight, Theresa Mankelow *et al.*) for enabling me to balance PhD activities whilst remaining in employment. In addition, I would like to thank the many faces at BGS (current or former) who have made this experience more enjoyable or acted as a sounding board for ideas and frustrations, including Olivier Humphrey, Dan Middleton, Simon Chenery, Andy Marriott, Darren Beriro, Tom Kelly, Murray Lark, Andy Tye, and countless others who have helped along the way.

Finally, I would like to thank my parents, my sister and my partner Danielle, for your encouragement, support and understanding over the last six years; this thesis is dedicated to you.

Contents

Abstract	i
Publications arising from part-time PhD programme	ii
Acknowledgments	vii
Abbreviations and Acronyms	xi
List of Tables.....	xiii
List of Figures	xiii
Chapter 1. Introduction.....	1
Background	1
Research Aim	3
Thesis Structure	4
Chapter 2. Chromium speciation in foodstuffs: A review	6
Author list	6
Author contribution.....	6
Abstract.....	7
Introduction	7
Background	7
Chromium in soil-water systems	8
Analytical methods for chromium speciation in foodstuffs	11
Sample Pretreatment	25
Off-Line Analytical Techniques	26
On-Line Analytical Techniques.....	27
Reference Materials- Validation of Speciation Methodology	29
Disparity of results between off-line and on-line analytical techniques.....	29
Measurement of Cr(III)	30
Conclusion	31
Chapter 3. Methods	33
Introduction	33
Sample Collection, Preparation and Analysis	33
Quality Control.....	34
Speciated Isotope Dilution Mass Spectrometry (Chapter 4)	34
Introduction	34
Comparison of external calibration and SIDMS.....	35

Online microdialysis high performance liquid chromatography mass spectrometry (MD-HPLC-ICP-MS) (Chapter 6)	38
Introduction	38
Rationale for online MD method	38
Initial Setup- Continuous Sampling and Monitoring	39
Continuous Sampling and Semi-Continuous Monitoring	41
Chapter 4. Reconnaissance sampling and determination of hexavalent chromium in potentially-contaminated agricultural soils in Copperbelt Province, Zambia	43
Author list	43
Author contribution	43
Abstract	44
Introduction	44
Materials and Methods	47
Sample Preparation and Analysis	47
Study Area and Sampling	48
Reagents and Materials	50
Determination of Cr(VI)	51
Results and Discussion	52
Analytical Figures of Merit for HPLC-ICP-QQQ Speciation of Chromium	52
Processing of SIDMS Data	54
Total Cr and Cr(VI) in Mugala Village Soil Samples	54
Conclusions	56
Supplementary Material	57
Chapter 5. Assessment of chromium species dynamics in root solutions using isotope tracers	62
Author list	62
Author contribution	62
Abstract	63
Background	63
Methods	63
Results	63
Conclusion	64
Introduction	64
Materials and Methods	66
Preparation of $^{50}\text{Cr}^{\text{III}}$ and $^{53}\text{Cr}^{\text{VI}}$ stock solutions	66

Preparation of plant materials.....	67
Cr ^{III} and Cr ^{VI} transfer mechanisms	67
Influence of sulphate on Cr ^{VI} uptake mechanisms	68
Instrumentation and Data Analysis	68
Results and Discussion.....	69
Analytical figures of merit.....	70
Cr ^{III} and Cr ^{VI} interconversions in treatment solutions	70
Cr ^{III} and Cr ^{VI} transfer mechanisms at 200, 500 and 1000 µg L ⁻¹ Cr ^{III} and Cr ^{VI}	74
Impact of Sulphate on Cr ^{VI} Transfer Mechanisms	76
Conclusions.....	77
Chapter 6. Online microdialysis-high performance liquid chromatography-mass spectrometry (MD-HPLC-ICP-MS) as a novel tool for sampling hexavalent chromium in soil solution	79
Author list	79
Author contribution.....	79
Abstract.....	80
Introduction	80
Materials and Methods	84
Reagents	84
Instrumental Apparatus and Analysis.....	84
Soil Sampling and Incubation	86
Calibration of MD-HPLC-ICP-MS System in Stirred Solutions.....	87
Relative recovery of Cr ^{VI} in solution.....	89
Analytical Figures of Merit.....	90
Soil Solution Sampling- Homogenized Mixing	92
Future Prospects for Optimization and Implementation of Online MD.....	95
Chapter 7. Conclusions.....	99
Limitations of the study.....	104
Recommendations for future research	107
References	110

Abbreviations and Acronyms

C4SL	Category 4 screening level
CPE	Cloud point extraction
Cr	Chromium
Cr ^{III}	Trivalent chromium
CRM	Certified reference material
Cr ^{VI}	Hexavalent chromium
DAP	Days after planting
DDW	Distilled deionised water
DGT	Diffusive gradients in thin films
DL	Detection limit
DPC	Diphenylcarbazide
DRS-FTIR	Diffuse reflectance-Fourier transform infrared spectroscopy
EDTA	Ethylenediaminetetraacetic acid
ETAAS	Electrothermal atomic absorption spectroscopy
FAAS	Flame atomic absorption spectroscopy
HDPE	High-density polyethylene
HPLC	High performance liquid chromatography
ICP-MS	Inductively coupled plasma mass spectrometry
ICP-QQQ	Triple quadrupole inductively coupled plasma mass spectrometry
ISTD	Internal standard
K _d	Partition coefficient
LC	Liquid chromatography
LOI	Loss on ignition
MD	Microdialysis
MS	Mass Spectrometry
MWCO	Molecular weight cutoff
OD	Outer diameter
PEEK	Polyether ether ketone
PHE	Potentially harmful element
Q _p	Perfusate flow rate
R _d	Resistance component: dialysate
R _{ext}	Resistance component: external environment
R _m	Resistance component: probe membrane
ROS	Reactive oxygen species
RR	Relative recovery
RSD	Relative standard deviation
SIDMS	Speciated isotope dilution mass spectrometry
SOC	Soil organic carbon
SOM	Soil organic matter
SPE	Solid phase extraction
SPW	Soil pore water
TRIS	Trisaminomethane
US EPA	United States Environmental Protection Agency

WHC	Water holding capacity
XANES	X-ray adsorption near-edge spectroscopy
XRF	X-ray fluorescence

List of Tables

Chapter 2

Table 2.1	Extraction and quantification techniques for chromium speciation in foodstuffs. Table is ordered according to foodstuff.	Page 12
Table 2.2	Reported Cr(VI) concentrations in foodstuffs.	Page 21

Chapter 3

Table 3.1	Recoveries of SRM 2701 calculated from external calibration and SIDMS.	Page 36
-----------	--	---------

Chapter 4

Table 4.1	Isotopic composition of $^{53}\text{Cr(VI)}$ used throughout study.	Page 50
Table 4S.1	Elemental and speciation data for agricultural soils in Copperbelt Province, Zambia	Page 57

Chapter 6

Table 6.1	Average RR and relative standard deviation (RSD, $n = 5$) for replicate injections at perfusate flow rates of 1, 3, 5, 7.5 and $10 \mu\text{L min}^{-1}$.	Page 90
-----------	---	---------

List of Figures

Chapter 3

Figure 3.1	Time-resolved signal at m/z 52 for introduction and washout of $100 \mu\text{g L}^{-1} \text{Cr}^{\text{VI}}$ standard over 20 minutes. The microdialysis probe was initially placed in DDW to establish a background baseline before being placed in the Cr^{VI} standard. Internal standard signal has not been shown due to scaling.	Page 39
Figure 3.2	Instability in m/z 52 signal due to dialysate channel backflow. Signal acquired over 50 minute period.	Page 40
Figure 3.3	Chromatogram of $100 \mu\text{g L}^{-1} ^{53}\text{Cr}^{\text{VI}}$ standard, passively-sampled and injected using MD-HPLC-ICP-MS setup.	Page 41

Chapter 4

Figure 4.1	Study site with (i) sample points for nested sampling design (white circles) and (ii) kriging output created from total Cr data. Maps created using ArcGIS® software by Esri. ArcGIS® and ArcMap™ are the intellectual property of Esri and are used herein under license. Copyright © Esri. All rights reserved.	Page 48
Figure 4.2	Speciation of Cr(VI) and Cr(III) in a natural isotopic abundance standard ($10 \mu\text{g L}^{-1}$).	Page 52
Figure 4.3	Distribution of Cr(VI) concentrations in agricultural soil samples.	Page 54
Figure 4S.1	Locations of samples analysed for Cr(VI) in this study (see Table 4S.1 for corresponding sample names and additional elemental data). Map created using ArcGIS® software by Esri. ArcGIS® and ArcMap™ are the intellectual property of Esri and are used herein under license. Copyright © Esri. All rights reserved.	Page 56

Chapter 5

Figure 5.1	Speciation of Cr^{III} and Cr^{VI} , at m/z 50 and 53, in a $5 \mu\text{g L}^{-1}$ quality control solution.	Page 69
Figure 5.2	Species distributions for treatment solutions (both $^{50}\text{Cr}^{\text{III}}$ and $^{53}\text{Cr}^{\text{VI}}$) at different nominal concentrations after a 24 hr treatment period. [i] Treatment solutions were not exposed to plant roots. [ii] Treatment solutions contained plant roots. Values expressed as mean $\pm$ SD ($n = 3$).	Page 70
Figure 5.3	Species distribution for treatment solutions containing only $^{53}\text{Cr}^{\text{VI}}$ at $500 \mu\text{g L}^{-1}$ after a 24 hr treatment period. Values expressed as mean $\pm$ SD ($n = 3$).	Page 71
Figure 5.4	Speciation of chromium in [i] apoplastic and [ii] symplastic root solutions exposed to $^{50}\text{Cr}^{\text{III}}$ at 282, 565 and $1130 \mu\text{g L}^{-1}$ and $^{53}\text{Cr}^{\text{VI}}$ at 250, 500 and $1000 \mu\text{g L}^{-1}$ for 24 hr. Values expressed as mean $\pm$ SD ($n = 3$).	Page 73
Figure 5.5	$^{53}\text{Cr}^{\text{III}}$ and Cr^{VI} concentrations in apoplastic and symplastic root solutions exposed to $500 \mu\text{g L}^{-1}$ $^{53}\text{Cr}^{\text{VI}}$ only, $500 \mu\text{g L}^{-1}$ $^{53}\text{Cr}^{\text{VI}}$ + $150 \text{mg L}^{-1} \text{SO}_4^{2-}$ and $500 \mu\text{g L}^{-1}$ $^{53}\text{Cr}^{\text{VI}}$ + $250 \text{mg L}^{-1} \text{SO}_4^{2-}$ for 24 hr. Values expressed as mean $\pm$ SD ($n = 3$).	Page 75

Chapter 6

Figure 6.1	Speciation of $^{53}\text{Cr}^{\text{VI}}$ in a $5 \mu\text{g L}^{-1}$ solution.	Page 84
Figure 6.2	The effect of perfusate flow rate ($\mu\text{L min}^{-1}$) on relative recovery (%) of Cr^{VI} ($100 \mu\text{g L}^{-1}$) in stirred solutions ($n = 5$ for each flow rate). Exponential trendline and correlation coefficient (R^2) are displayed within the chart. Error bars indicate $\pm$ standard error from 5 replicates.	Page 88

Figure 6.3	Calibration curve for online MD-HPLC-ICP-MS system at nominal concentrations of 1, 10 and 100 $\mu\text{g L}^{-1}$. MD probes were immersed in 50 mL plastic beakers containing Cr^{VI} solution, stirred and perfused at 3 $\mu\text{L min}^{-1}$.	Page 90
Figure 6.4	Decrease in passively-sampled $^{53}\text{Cr}^{\text{VI}}$ added to soil through homogenized mixing method over 120 minutes, measured by online MD-HPLC-ICP-MS every 15 minutes following spike introduction.	Page 92
Figure 6.5	Changes in passively-sampled $^{53}\text{Cr}^{\text{VI}}$ added to soil through direct injection method over 120 minutes, measured by online MD-HPLC-ICP-MS every 15 minutes following spike introduction. Replicates were undertaken on consecutive days.	Page 93
Figure 6.6	Diffusion of solutes (blue circles) towards probes implanted in soil matrix. Reduced permeability (represented by diagonal fill) in right MD probe shifts the diffusion gradient towards the left MD probe, therefore the right MD probe will sample lower concentrations of solute.	Page 95

Chapter 1. Introduction

Background

Chromium (Cr) is a hard metal located in group 6 of the Periodic Table of the Elements; it is the sixth most abundant transition element in the Earth's crust. Its ground state electronic configuration is $[\text{Ar}] 3d^5 4s^1$, due to its lowest energy being the high spin configuration. The two most stable oxidation states of Cr are Cr^{III} and Cr^{VI} ; Cr^{III} is chemically basic and forms six-coordinate complexes with water and ammonia whilst Cr^{VI} is acidic, strongly oxidising and forms chromate $[\text{CrO}_4]^{2-}$ and dichromate $[\text{Cr}_2\text{O}_7]^{2-}$ ions (Bini et al., 2008). The oxidation state of Cr dictates its mobility, bioavailability and toxicity; Cr^{VI} is more mobile due to the negative charge of its compounds, and is a recognised carcinogen through inhalation (Stout et al., 2009). Conversely, Cr^{III} is essential for the regulation of glucose and lipid metabolism, and has been shown to reduce blood cholesterol concentrations through the removal of low-density lipoproteins (Mohan and Pittman, 2006). Due to these differences, a significant number of research articles have been published in recent years concerning the quantification of Cr species in environmental samples using a variety of off- and on-line speciation techniques (Séby and Vacchina, 2018).

Global discharge of total Cr in water, air and soil is 142,000, 30,000 and 896,000 metric tonnes per year respectively (Nriagu and Pacyna, 1988). Hexavalent chromium is predominantly used in industrial applications, including electroplating, wood preservation and tanning (Kimbrough et al., 1999). Groundwater contamination of Cr^{VI} is therefore significantly influenced by the proximity of effluent discharge and subsequent transfer to aquifers (Sun et al., 2015), although geogenic contributions are possible where ultramafic and mafic rocks are present in the underlying geology (Coyte et al., 2020). The predominant occupational exposure route for Cr^{VI} is through

inhalation, with non-occupational exposure occurring through consumption of food and water containing Cr (Shrivastava et al., 2002). Due to its biological role in humans, Cr^{III} is widely used in nutritional supplements as a putative aid to weight loss (Martone et al., 2013), which has led to an increase in studies concerning its oral genotoxicity in comparison to Cr^{VI} (Fang et al., 2014). Despite this research interest, few analytical techniques are able to determine total Cr^{III} in solid matrices, such as soil and edible plants, due to the differing chemical properties of the species and the regulatory focus on Cr^{VI} (Unceta et al., 2010).

Plants have no known function for Cr; uptake can result in deleterious effects depending on the species of plant and Cr concentration in the soil (Davies Jr et al., 2002). Mechanistically, the uptake of Cr^{VI} is an active process facilitated by sulphate carrier proteins (Cervantes et al., 2001) due to the absence of Cr-specific transfer mechanisms (Shanker et al., 2005); conversely, Cr^{III} uptake is a passive process which expends no energy on the part of the plant (Park, 2020). Plants have a number of Cr^{VI} detoxification mechanisms, the most significant of which is reduction to Cr^{III} and subsequent complexation with organic acids in root cells (Bluskov et al., 2005a, Shahid et al., 2017). However, it is still unclear whether this reduction process occurs in the lower pH environment of the rhizosphere (Zeng et al., 2008) or following uptake (Pradas del Real et al., 2014b), and how soil amendments (liming, organic reincorporation) influence soil-to-plant uptake of Cr species (Shahid et al., 2017).

The assessment of metal bioavailability to plants is best achieved using solution concentrations measured in soil pore water (SPW) as opposed to the total soil concentration (Vig et al., 2003). However, the determination of metal concentrations in SPW is prone to artefacts due to the limitations associated with extraction and analysis (Di Bonito et al., 2008). Chemical extractants (EDTA, calcium nitrate) are most

commonly employed due to ease of use and cost, but the results they provide are dependent on the concentration of the extractant solution, the amount of sample extracted and the extraction time (Young et al., 2005). There are numerous “physical” methods, both field and laboratory based, designed to (amongst others) separate the SPW from the solid phase (e.g. centrifugation), passively sample the soil solution (e.g. Rhizon samplers) or passively sample solutes within the soil solution through diffusion and chelation (e.g. diffusive gradients in thin films, DGTs) (Ernstberger et al., 2002). However, these methods result in disturbance of soil environments and are lacking in root-relevant spatial and temporal resolution, presenting a significant challenge when assessing the *in situ* concentration, speciation and availability of contaminants such as Cr^{VI} (Humphrey et al., 2019a). The development of analytical techniques capable of assessing the “real time” availability of Cr^{VI} to plants with minimal disturbance and at finer scales, alongside experiments designed to elucidate the uptake mechanisms for Cr^{III} and Cr^{VI}, would contribute to a greater understanding of the biogeodynamics of Cr.

Research Aim

The aim of this study was to improve the understanding of soil-to-plant transfer of chromium species through the development, optimisation and application of novel analytical methods. High-resolution, mechanistic approaches were taken, focusing on species transfer and redox changes in bulk soil, soil pore water and plant hydroponic systems. The objectives were:

1. Optimisation of reconnaissance sampling and analytical techniques to collect, extract and measure total Cr^{VI} in soil samples.

2. Assessment of short-term (24 h) transfer and interconversion of aqueous Cr^{III} and Cr^{VI} in the presence of plant roots using stable isotope tracers and isolation of active and passive transfer root solutions.
3. Development of simultaneous sampling and analysis of Cr^{VI} in soil solution at sub-hour scales to investigate species availability in near-real time.

Thesis Structure

The thesis is divided into the following chapters. Chapters 4 to 6 are presented in 'paper format' with all author contributions clearly outlined.

Chapter 1 is a general introduction to the thesis, including necessary background information on Cr geodynamics and soil-to-plant transfer.

Chapter 2 is a literature review concerning speciation of chromium in foodstuffs up to 2018 (year of publication of journal article), focussing on the different analytical strategies and metrological issues surrounding the separation and quantification of Cr^{VI} . This 'paper format' chapter is a continuation of the introduction, providing further theoretical background and summarising the analytical techniques used to undertake Cr speciation.

Chapter 3 is an account of the development and/or optimisation of the analytical methods used in Chapters 4-6.

Chapter 4 applied an optimised speciated isotope dilution mass spectrometry (SIDMS) technique to measure Cr^{VI} in agricultural soils, collected in the Copperbelt Province of Zambia using geostatistically-optimised sampling. This technique minimises the potential for species interconversion during sample extraction, increases the accuracy of Cr^{VI} data over offline separation/analysis and demonstrates the efficacy with which Cr^{VI} can be reduced in this soil type.

Chapter 5 investigated the solution transfer mechanisms of Cr^{III} and Cr^{VI} in spinach (*Spinacia oleracea*). Due to the differing mobilities and toxicities between Cr species, an understanding of the mechanisms governing the transport and ultimate fate of Cr into plants is essential when determining human exposure routes for Cr^{VI}.

Chapter 6 presented the development of a new analytical technique, using microdialysis coupled to HPLC-ICP-MS for the continuous passive sampling and simultaneous analysis of Cr(VI) in soil solution. Artefacts associated with the introduction of isotopically-enriched material to the soil were investigated. The technique allows for lower temporal resolution and near-real time analysis of species availability in soil solution, improving understanding of contaminant removal rates following addition events.

Chapter 7 is a general conclusion, evaluating how the novel tools developed and optimised in this study have contributed to a greater understanding of chromium dynamics in soil-plant systems. In addition, some limitations of the work and suggestions for future study are included.

Chapter 2. Chromium speciation in foodstuffs: A review

Paper published in *Food Chemistry* (Hamilton *et al.*, 2018. 250: 105-112)

DOI: 10.1016/j.foodchem.2018.01.016

Author list

Hamilton EM, Young SD, Bailey EH and Watts MJ

Author contribution

Review conceived by EMH and MJW

All tables and figures produced by EMH

Construction of the paper by EMH

All authors were involved during manuscript development

Abstract

Numerous critical reviews have evaluated exposure to toxic and carcinogenic hexavalent chromium (Cr(VI)) from a number of pathways; including workplace air, cement and packaging materials. The contribution of foodstuffs to dietary Cr(VI) has been increasingly under investigation, however no summary of this work has been carried out. The objective of this article is to review the last twenty years of chromium speciation research in foodstuffs. Alkaline extraction, used for chromium speciation in other solids, is the most widely-reported procedure. Previous measurement of Cr(VI) in foodstuffs is questionable due to the reducing power of organic matter and antioxidants, leading to the development of speciated isotope dilution mass spectrometry (SIDMS) techniques to monitor interconversions. Evaluation of the genotoxicity of trivalent chromium (Cr(III)), which acts through a different pathway to that of Cr(VI), requires reconsideration towards measurement of Cr(III), which is present at higher concentrations in foodstuffs following reduction of the more-bioavailable Cr(VI).

Introduction

Background

Chromium (Cr) is a transition metal that exists in the environment as Cr(III) (trivalent) and Cr(VI) (hexavalent) forms. These naturally occurring oxidation states differ significantly in their mobility, bioavailability and toxicity (Oliveira, 2012a). Trivalent Cr is present as cationic species and is considered to be essential for insulin regulation and glucose metabolism (Office of Dietary Supplements: National Institute of Health, 2013), whereas Cr(VI), which is largely anthropogenic in origin and exists as anionic species, is toxic and a known carcinogen through inhalation (McCarroll et al., 2010). The EH40/2005 Workplace Exposure Limit (WEP) assigned for Cr(VI) is 0.05 milligrams

per cubic metre of air averaged over an 8-hour period (Health and Safety Executive, 2013). However, there is evidence of genotoxicity of Cr(III) compounds (Fang et al., 2014); their use in nutritional supplements has been the subject of a number of research articles (Bailey, 2014).

The main applications of chromium are in the metallurgical and tanning industries (Stasicka, 2000). Owing to its hardness and resistance to corrosion, chromium is used to anodise aluminium (Kendig et al., 2001), as an additive in primer paint for aerospace and automobile refinishing (Basketter et al., 2000), and in the production of steel and alloys (Dhal et al., 2013a). Trivalent Cr salts are used to tan leather by cross-linking collagen fibres (Brown, 1997). In the United States, the mineral crocoite (PbCrO_4) was used as a yellow pigment for school buses because it does not degrade in light and has a strong colour, but this has declined due to environmental concerns over its disposal (Gettens, 1966). Lead chromate was historically used as a food adulterant to bulk flour and enhance the yellow colour in turmeric (Ellis et al., 2012). Chromium-containing pesticides, such as chromated copper arsenate, were utilised in the timber industry (Hingston et al., 2001), although their use in the UK has been highly restricted since 2004 (Mercer and Frostick, 2012). This widespread use has led to concern over Cr(VI) pollution of the environment (Zayed and Terry, 2003). Chromium is one of four priority soil-metal contaminants for the U.S. Department of Defense, owing to the use of Cr(VI) compounds for aircraft fuselages (Salatas et al., 2004).

Chromium in soil-water systems

When discussing the total concentration and speciation of Cr in foodstuffs, it is important to consider the sources of chromium and the chemical forms present in these sources prior to plant uptake. Whilst mitigating the issues associated with rapid urbanisation and water scarcity, it is well documented that the irrigation of crops with

untreated domestic and industrial effluents results in accumulation of heavy metals in soils (Stasinos and Zabetakis, 2013) which increases the potential for transfer into crops. Therefore, an understanding of the chemical behaviour of Cr in soil-water systems is essential for predicting species stability and the likelihood of transfer into foodstuffs.

Chromium in water originates from natural sources such as the weathering of rocks and precipitation, with elevated concentrations attributed to wastewater from industrial sources (Stasicka, 2000). Chromium has a strong affinity with ultrabasic and basic rocks (peridotite, serpentinite, gabbro, dolerite and basalt), because some pyroxenes (e.g. kosmochlor) have undergone isomorphic substitution of Cr(III) for Al (Oze et al., 2004). Chromium in soils typically originates from fallout and washout of Cr-containing particles (Stasicka, 2000) through geochemical processes such as weathering, diagenetic reactions and volcanic eruptions (Prado et al., 2016). Agricultural use of sewage sludge as a fertiliser can also introduce Cr into soils (Loubna et al., 2015). Background concentrations of chromium in European soils are typically between 5 and 68 mg kg⁻¹ (Utermann et al., 2006). Disposal of industrial waste can lead to percent levels of Cr in soil, with the highest concentrations reported in India, East Africa, South America and China (Shahid et al., 2017).

Due to its presence as cationic species such as Cr³⁺, Cr(OH)²⁺ and Cr(OH)₂⁺, Cr(III) tends to form strong complexes with alumino-silicate clays, soil organic matter and Fe/Al hydrous oxides in soil, which restricts the mobility and bioavailability of Cr(III). The affinity of soil for Cr(III) increases with pH due to the increased negative charge on soil particles (Ritchie and Sposito, 2007). By contrast, the negative charge of Cr(VI) repels the predominantly-negatively charged clay minerals and humus in soil, accounting for the increased mobility and bioavailability of Cr(VI) as pH rises (Jardine et al., 2013). In

the form of CrO_4^{2-} or HCrO_4^- , Cr(VI) is adsorbed by Fe/Al hydrous oxides (James and Bartlett, 1983b). The oxidation of Cr(III) and reduction of Cr(VI) in soils is dependent on pH, oxygen concentration and the presence of appropriate redox coupling agents. Hexavalent Cr as CrO_4^{2-} and HCrO_4^- can be reduced by Fe(II) and sulphide (James and Bartlett, 1983b), with Fe(II) dominating at $\text{pH} > 5.5$ and sulphide dominating at lower pH (Fendorf et al., 2000). Oxidation of Cr(III) to Cr(VI) can occur in the presence of oxidised manganese as Mn(IV)O_2 (Bartlett and James, 1979).

The toxicity of chromium to plants has been extensively covered in a number of reviews (Shanker et al., 2005). Chromium has no essential role in plant metabolism (Mertz, 1969) and is poorly translocated within plants (Singh et al., 2013). This is likely due to Cr(III) binding to cell walls (Zayed et al., 1998b), leading to increased concentrations of chromium in the roots (Kabata-Pendias, 2010). Both Cr(III) and Cr(VI) can be absorbed, although preferential uptake of Cr(VI) through sulphate carriers has been observed (Banerjee et al., 2008). Ultimately Cr(VI) is reduced to Cr(III) in roots by Fe(III) reductase enzymes (Zayed et al., 1998b). Deleterious effects, including restricted root growth, biomass reduction and distortion of leaf appearance have been recorded for plants grown in media with excess Cr(III) and Cr(VI) (Dube et al., 2003).

The toxicity and greater mobility of Cr(VI) has led to a number of research articles concerned with quantitatively evaluating the Cr(VI) content in a variety of foodstuffs, including milk (Lameiras et al., 1998), mushrooms (Figueiredo et al., 2007), tea (Chen et al., 2014), bread (Soares et al., 2010) and beer (Vieira et al., 2014), utilising different sample preparation techniques and analytical methodologies. Meat, dairy products, bread and tea are the most studied as they constitute the main sources of Cr in the human diet (Lendinez et al., 2001). The key objective is to assess the oral genotoxicity of Cr(VI) from foodstuffs to reduce the risk to human health from this pathway

(Proctor et al., 2002). It is likely that any ingested Cr(VI) will be reduced to Cr(III) in the acidic conditions of the stomach (Milacic and Stupar, 1994), and any Cr(VI) transferred from soil to plant will also be reduced. However, there are other sources of Cr(VI) introduced during the manufacture and processing of foodstuffs (e.g. stainless steel vats) that justify the need to undertake chromium speciation. It is therefore vital to understand the limitations and interferences of each speciation technique. To the author's knowledge, this article represents the first literature report to compile and critically review the speciation of chromium in foodstuffs over the last twenty years.

Analytical methods for chromium speciation in foodstuffs

The approach to chromium speciation in foodstuffs can be broken down into two categories: off-line determination of chromium species following sample pre-treatment and on-line speciation of the pre-treated sample using hyphenated techniques. The pre-treatment step is designed to solubilise all forms of Cr(VI) in the sample without inducing oxidation of Cr(III) or reduction of Cr(VI) (Unceta et al., 2010). This is particularly important because, depending on the sample matrix, the concentration of Cr(III) can be between 10 and 1000 times greater than the concentration of Cr(VI) (Ndung'u et al., 1999). The use of solid-state speciation techniques for foodstuffs has not been reported in the literature and is therefore outside the scope of this review. Table 2.1 is a summary of the reported literature for the speciation of Cr in foodstuffs, with analytical merits indicated; the table is arranged according to foodstuff. A number of additional articles have summarised the speciation of Cr in other solid matrices (Unceta et al., 2010). Table 2.2 is a summary of the reported Cr(VI) concentrations for the literature in Table 2.1.

Table 2.1 Extraction and quantification techniques for chromium speciation in foodstuffs. Table is ordered according to foodstuff.

Foodstuff	Analytical Technique	Sample Preparation	Analytical Merits	Reference
Milk and Dairy Products				
UHT Milk	ETAAS	Selective extraction using Chromabond NH ₂ column following precipitation of proteins with acetic acid-sodium acetate buffer (pH 3.5).	Cr(VI) concentrations in milk approximately 2-4 times lower than total Cr. Detection limit of 0.15 µg L ⁻¹ Cr(VI).	(Lameiras et al., 1998)
Cow milk	ICP-MS	Selective extraction using Chromabond NH ₂ column following separation of proteins using centrifugation.	Detection limit of 0.085 µg L ⁻¹ Cr(VI). Reported Cr(VI) range of 1.31-3.28% compared to total Cr.	(Ambushe et al., 2009)

Powdered milk formula	ETAAS	Reconstituted milk selectively extracted using Chromabond NH ₂ .	Cr(VI) detection limit of 1.8 µg L ⁻¹ . Cr(VI) concentrations in commercial brands ranging between 10 and 75 µg kg ⁻¹ .	(Soares et al., 2000)
Dairy products, flour, chocolate, vegetables, fruits, meat, fish, eggs, beverages	HPLC-ICP-MS	Alkaline extraction using NH ₄ OH (pH 11.5).	No Cr(VI) found in any samples. Stability of Cr(VI) in cow milk investigated, organic matter found to induce reduction of Cr(VI).	(Vacchina et al., 2015)
Fungi				
Mushrooms	ETAAS	Cap and stalk removed, sample consisted of several portions of either cap or stalk dried at 30-	Detection limit of 0.17 µg L ⁻¹ Cr(VI). Mushrooms accumulate Cr(VI), potential use as	(Figueiredo et al., 2007)

		35°C for 3 days. Alkaline extraction using 0.01 M NaOH/1.0 M NH ₄ NO ₃ .	bioindicators for Cr(VI) soil pollution.	
Pulses				
Red lentils	FAAS	Selective extraction using Amberlite XAD-1180 resin.	Detection limit of 7.7 µg L ⁻¹ Cr(VI). Preconcentration factor of 75.	(Narin et al., 2008)
Bread and Cereals				
Bread	ETAAS	Melting durum flour and Cr(III) solution to simulate bread toasting and investigate possible oxidation of Cr(III) to Cr(VI) during this process.	No oxidation of Cr(III) to Cr(VI) occurred during simulated toasting procedure.	(Kovács et al., 2007)

Bread	ETAAS	Small portions dried at 30-35°C for 1 hour, approximately 10 g ground in agate mortar by hand. Alkaline extraction using 0.01 M NaOH/1.0 M NH_4NO_3 .	On average, 12% of total Cr in bread is Cr(VI). Estimated average daily intake of 0.57 $\mu\text{g/day}$ and 0.69 $\mu\text{g/day}$ for white and whole bread respectively.	(Soares et al., 2010)
Bread, breakfast cereals	HR-CS AAS	Alkaline extraction using 0.1 M Na_2CO_3 .	Between 33-73% of total Cr in bread found to be Cr(VI). Demonstrated oxidation of Cr(III) to Cr(VI) during toasting. Daily consumption of cereal and bread does not exceed EPA maximum acceptable	(Mathebula et al., 2017)

			concentration of 0.003 mg kg ⁻¹ bw day ⁻¹ .	
Beverages				
Tea leaves, bread	SIDMS	Tea samples (aqueous): alkaline extraction using 0.1 M Na ₂ CO ₃ . Double spiking using ⁵⁰ Cr(VI) and ⁵³ Cr(III) to monitor species interconversions. Bread samples: alkaline extraction using 0.01 M NaOH/1.0 M NH ₄ NO ₃ , double spiking.	No Cr(VI) detected in any extracts. No evidence of Cr(III) oxidation during sample preparation, evidence of Cr(VI) reduction in tea due to antioxidants and in bread due to high organic matter content. Emphasising requirement of speciation analysis.	(Novotnik et al., 2013)

Black, green and herbal teas	ETAAS	Alkaline extraction using 0.1 M Na ₂ CO ₃ .	Up to 17.5 µg L ⁻¹ Cr(VI) could be consumed per 200 ml cup of black tea.	(Mandiwana et al., 2011)
Tea leaves, tea infusions	ICP-MS	Selective solid phase extraction of Cr(III) using titanium dioxide nanotubes (TDNTs).	Cr(III) retained in pH range 5.0-8.0, Cr(VI) remains in solution. Detection limit of 0.0075 µg L ⁻¹ Cr(III).	(Chen et al., 2014)
Neem tea infusions	SIDMS	Alkaline extraction using 0.1 M Na ₂ CO ₃ /0.1 M MgCl ₂ to induce precipitation of Cr(III). Double spiking using ⁵⁰ Cr(VI) and ⁵³ Cr(III) to monitor species interconversions.	Cr(VI) reduced by antioxidants, below LOD (0.06 µg L ⁻¹).	(Novotnik et al., 2015)

Lager beer	ETAAS	Selective extraction using Chromabond NH ₂ column following precipitation of proteins.	Cr(VI) only detected in pale lager and low alcohol lager, estimated daily intake of 0.47 µg/day and 1.26 µg/day respectively.	(Vieira et al., 2014)
Miscellaneous				
Tobacco, eggplant	Activated carbon from tea-industry waste (TIWAC), FAAS	Extraction of Cr(III) using TIWAC, determination using FAAS	Detection limit for Cr(III) of 0.27 µg L ⁻¹ . Preconcentration factor of 50 when using sample volume of 200 ml.	(Duran et al., 2011)
Vinegar	Catalytic adsorptive stripping voltammetry	Reduction of Cr(VI) at electrode surface to Cr(III), complexation with rubeanic acid,	Detection limit of 0.002 µg L ⁻¹ .	(Abbasi and Bahiraei, 2012)

		voltammetric response measured.		
Edible animal oils (cattle, fish)	HPLC-ICP-MS	Microwave extraction with 0.4% v/v HF and 2% Triton X-100 in HPLC mobile phase (0.5 mmol L ⁻¹ TBAP and 0.3 mmol L ⁻¹ EDTA in 1% methanol adjusted to pH 6.9).	Detection limit of 0.045 µg L ⁻¹ for Cr(III) and 0.052 µg L ⁻¹ for Cr(VI). No Cr(VI) detected in any samples, unidentified peak observed during chromatographic separation. Spike recoveries suggest reduction of Cr(VI) to Cr(III) in oil matrix.	(Lin et al., 2016)
Turmeric	Cloud point extraction (CPE), diffuse reflectance-	Complexation of Cr(VI) with 1,5-diphenylcarbazide, enrichment of complex	Detection limit for Cr(VI) of 1.22 mg L ⁻¹ . Between 3.31	(Tiwari et al., 2017)

	Fourier transform infrared spectroscopy (DRS-FTIR)	using CPE, analysis of complex using DRS-FTIR.	and 5.41 mg L ⁻¹ Cr(VI) found in turmeric samples.	
Plants (Alyssum obovatum, Cochlearia arctica (Brassicaceae), Armeria scabra (Limoniaceae), Salix arctica (Salicaceae))	ETAAS	Alkaline extraction using 0.1 M Na ₂ CO ₃ .	Cr(VI) stable in extraction matrix for up to 6 months. Less than 10% of total Cr in samples found to be Cr(VI).	(Panichev et al., 2005)
Fish, white cheese, cow meat, black tea, boiled wheat	FAAS	Coprecipitation of Cr(III) using Ni ²⁺ /2-Nitroso-1-naphthol-4-sulfonic acid, subtraction of total Cr and Cr(III) to determine Cr(VI).	Detection limit for Cr(III) of 1.33 µg L ⁻¹ .	(Uluozlu et al., 2009)

Apple, parsley, wheat, marrow, quince	FAAS	Selective extraction of Cr(III) using synthesised chelating resin.	Detection limit of 1.11 $\mu\text{g L}^{-1}$. ¹ . Cr(VI) concentrations in foodstuffs investigated were below detection limit.	(Çimen et al., 2013)
---------------------------------------	------	--	---	----------------------

Table 2.2 Reported Cr(VI) concentrations in foodstuffs

Foodstuff	Reported Cr(VI)	Reference
Milk and Dairy Products		
UHT Milk	0.15-1.20 $\mu\text{g L}^{-1}$	(Lameiras et al., 1998)
Cow milk	0.61-1.44 $\mu\text{g L}^{-1}$	(Ambushe et al., 2009)
Powdered milk formula	<10-75 $\mu\text{g kg}^{-1}$	(Soares et al., 2000)
Dairy products, flour, chocolate, vegetables, fruits, meat, fish, eggs, beverages	Below detection limit	(Vacchina et al., 2015)
Fungi		

Mushrooms	<0.0085-0.580 mg kg ⁻¹ in cap, <0.0085-0.813 mg kg ⁻¹ in stalk	(Figueiredo et al., 2007)
Pulses		
Red lentils	Below detection limit	(Narin et al., 2008)
Bread and Cereals		
Bread	Below detection limit	(Kovács et al., 2007)
Bread	<5.60-18.80 µg kg ⁻¹ in white bread, <5.60-19.70 µg kg ⁻¹ in whole bread	(Soares et al., 2010)
Bread, breakfast cereals	19.25-63.86 µg kg ⁻¹ , 41.0- 470.4 µg kg ⁻¹ in breakfast cereal	(Mathebula et al., 2017)
Beverages		

Tea leaves, bread	Below detection limit	(Novotnik et al., 2013)
Black, green and herbal teas	0.03-3.15 mg kg ⁻¹ total, 0.025-1.75 mg kg ⁻¹ water soluble	(Mandiwana et al., 2011)
Tea leaves, tea infusions	3.39-4.41 µg L ⁻¹	(Chen et al., 2014)
Neem tea infusions	Below detection limit	(Novotnik et al., 2015)
Lager beer	<1.61-13.0 µg L ⁻¹	(Vieira et al., 2014)
Miscellaneous		
Tobacco, eggplant	Below detection limit	(Duran et al., 2011)
Vinegar	3.30 ± 0.11 µg L ⁻¹	(Abbasi and Bahiraei, 2012)
Edible animal oils (cattle, fish)	Below detection limit	(Lin et al., 2016)
Turmeric	3.19-5.41 mg L ⁻¹	(Tiwari et al., 2017)

Plants (Alyssum obovatum, Cochlearia arctica (Brassicaceae), Armeria scabra (Limoniaceae), Salix arctica (Salicaceae))	0.09-0.59 mg kg ⁻¹	(Panichev et al., 2005)
Fish, white cheese, cow meat, black tea, boiled wheat	Below detection limit	(Uluozlu et al., 2009)
Apple, parsley, wheat, marrow, quince	Below detection limit	(Çimen et al., 2013)

0

Sample Pretreatment

The extraction of Cr(VI) is most commonly undertaken in an alkaline medium due to the stability of Cr(VI) in high-pH matrices (Zhao et al., 2016). The U.S. Environmental Protection Agency (EPA) developed a number of extraction methods that are in common use; for example (1) Method 3060A Alkaline Digestion for Hexavalent Chromium (United States Environmental Protection Agency, 1996) and (2) Method 7196A Hexavalent Chromium by Colorimetry (United States Environmental Protection Agency, 1992). The latter is based on the reaction of Cr(VI) with diphenylcarbazide (DPC) to form a Cr-diphenylcarbazone complex which can be detected using UV-Vis Spectrophotometry at 540 nm. The main disadvantages of this technique are comparatively high detection limits (typically 0.5 mg L^{-1}) and susceptibility to interfering species including molybdenum, vanadium, mercury and iron. The release of humic compounds during the extraction can also bias determination of Cr(VI), as these also absorb at 540 nm (Pettine and Capri, 2005).

To account for this, and the possibility of Cr(III) oxidation under alkaline conditions, Method 3060A was developed; this involves extracting the sample using 0.28M Na_2CO_3 /0.5M NaOH at 90-95°C for 60 minutes. In cases of high Cr(III) concentrations the addition of Mg^{2+} can suppress oxidation of Cr(III) to Cr(VI). The resulting solution can then be analysed using a suitable off-line or on-line analytical technique (United States Environmental Protection Agency, 1996). The majority of Cr(III) in the sample precipitates out in the form of hydroxides, oxides and carbonates (Rahman et al., 2005) which can be filtered out of the solution prior to analysis. The species present in the insoluble solid can then be determined and a Cr mass balance employed to confirm the efficiency of the entire procedure (Wolle et al., 2014).

Ammonium nitrate (NH_4NO_3) (Soares et al., 2010) and ammonium hydroxide (NH_4OH) (Vacchina et al., 2015) have also been used for selective extraction of Cr(VI). Aside from alkaline extraction, solid phase extraction (SPE) columns can be used to selectively retain Cr(III) or Cr(VI); this method also enables pre-concentration of the retained species for improved detection limits (Ahmad et al., 1990). Solid phase extraction can utilize alumina columns (Mahmoud et al., 2008) or nanotubes (Chen et al., 2014), occasionally with a reduction step to convert Cr(VI) to Cr(III) for retention on the SPE medium.

Cloud point extraction (CPE) has recently been used by Tiwari *et al.* for speciation of chromium in turmeric to investigate food adulteration (Tiwari et al., 2017). Cloud point extraction has been used for speciation of Cr in waters (Kiran et al., 2008), (Zhu et al., 2005) and is considered to be a “greener” method of pre-concentration and speciation owing to the use of fewer harmful chemicals (Samaddar and Sen, 2014). Tiwari *et al.* used diffuse reflectance-Fourier transform infrared spectroscopy (DRS-FTIR) to analyse Cr(VI) complexed with 1,5-diphenylcarbazide, offering a lower cost alternative to other techniques at the expense of higher detection limits.

Off-Line Analytical Techniques

Off-line analytical techniques can be described as instrumentation with no separation technique at the point of analysis, requiring the separation of chemical species through selective extraction prior to determination of Cr concentration. Off-line techniques require reduced sample preparation and post-analysis processing. Up until c. 2013, the speciation of chromium in foodstuffs was undertaken using off-line techniques, with the most common detection method being electrothermal atomic absorption spectrometry (ETAAS) (Lameiras et al., 1998, Panichev et al., 2005, Figueiredo et al., 2007, Soares et al., 2010), followed by inductively coupled plasma

mass spectrometry (ICP-MS) (Ambushe et al., 2009) and flame atomic absorption spectroscopy (FAAS) (Uluozlu et al., 2009). ETAAS is a mono-elemental technique capable of detection limits lower than $1 \mu\text{g L}^{-1}$ using sample volumes less than $100 \mu\text{l}$ (Daftsis and Zachariadis, 2007), making it suitable for the sensitive determination of Cr. The larger linear dynamic range of FAAS is applicable to samples with high concentrations of Cr(VI) (Marqués et al., 2000).

Aside from spectrometric techniques, electroanalytical speciation of Cr in foodstuffs was reported by Abbasi *et al.* (Abbasi and Bahiraei, 2012) using catalytic adsorptive stripping voltammetry. The speciation of Cr(VI) in vinegar was achieved with a detection limit of $0.002 \mu\text{g L}^{-1}$. Electroanalytical speciation has also been used for speciation of Cr in soil samples (Grabarczyk et al., 2006). The main issue with this form of speciation is the underlying assumption that all of the chromium measured in the extract is in the form of Cr(VI), and that no species interconversions have taken place during and/or after the extraction procedure.

Prior to the development and implementation of speciated isotope dilution mass spectrometry (SIDMS), a number of research articles reported Cr(VI) concentrations in bread (Soares et al., 2010) and tea (Mandiwana et al., 2011) and estimated daily intakes of Cr(VI) based on these results (Novotnik et al., 2013). The organic content of bread and presence of antioxidants in tea is likely to induce reduction of Cr(VI) to Cr(III) (Vacchina et al., 2015), which would be highlighted by on-line separation techniques.

On-Line Analytical Techniques

The uncertainty surrounding off-line analytical techniques has led to a number of on-line methods incorporating pre-instrument separation and subsequent determination of Cr species. The ease of coupling chromatographic methods, such as high performance liquid chromatography (HPLC), to ICP-MS has led to an increase in the

number of published studies using this approach to undertake Cr speciation in foodstuffs. These methods also allow for isotopic labelling of Cr(III) and Cr(VI) for SIDMS applications, where species interconversions can be monitored throughout the digestion and analysis procedures. EPA Method 6800 (Samaddar and Sen, 2014) utilises the same alkaline extraction as Method 3060A, but during the preparation step known quantities of isotopically-labelled Cr(III) and Cr(VI) are added and equilibrated with the sample. By monitoring the isotope of each species, redox conversions can be corrected for through the application of deconvolution calculations (Meija et al., 2006).

The enriched Cr isotopes $^{50}\text{Cr(III)}$ and $^{53}\text{Cr(VI)}$ are most commonly used as isotopic spikes due to their ease of preparation, with a few laboratories offering bespoke products designed for SIDMS applications (Humphrey et al., 2019a). Single-spike isotope dilution mass spectrometry (IDMS) has also been explored, where samples are spiked with only one isotope corresponding to the species of interest. Guidotti *et al.* demonstrated reliable extraction and quantification of Cr(VI) in contaminated soils using $^{53}\text{Cr(VI)}$ and ethylenediaminetetraacetic acid (EDTA) extraction, with minimal reduction of Cr(VI) even in soils with high Fe and organic matter content (Guidotti et al., 2015).

Anion-exchange columns are most frequently used to separate chromium species (Sabty-Daily et al., 2002). If the sample pre-treatment step does not include a chelating agent, such as EDTA, Cr(VI) will be the only species attracted to the stationary phase of the column. The presence of polyatomic interferences associated with the extraction matrix, such as $^{40}\text{Ar}^{12}\text{C}^+$ and $^{35}\text{Cl}^{16}\text{O}^1\text{H}^+$, can be controlled using the collision or reaction cell of the ICP-MS; this is usually achieved by pressurising with NH_3 (Chang and Jiang, 2001) or He (Hagendorfer and Goessler, 2008).

Reference Materials- Validation of Speciation Methodology

One of the main limitations associated with elemental speciation is the verification of the accuracy of the speciation methodology and sample preparation procedures. Non-aqueous matrix-specific certified reference materials (CRMs) are difficult to produce due to potential species instability and interconversions during the extraction procedure (Oliveira, 2012a). Commonly the accuracy of these methodologies is determined through spike recoveries, but these fail to take into account the possibility for species interactions with matrix components (James et al., 1995). Although currently no CRM exists for speciation of Cr in foodstuffs, the National Institute of Standards and Technology (NIST) have produced the only CRM for Cr speciation in contaminated soils, Standard Reference Material® SRM 2701 (Nagourney et al., 2008). Data from EPA Method 6800 were used to produce a certified value for Cr(VI) in this material, with the conclusion that this CRM should be included as an external quality control sample to confirm the accuracy of Cr(VI) analyses in Cr-containing waste. The development of CRMs for Cr(VI) in a range of foodstuffs, particularly bread, dairy products and tea that may be adulterated by manufacturing and processing methods, is an important consideration for the validation of future work.

Disparity of results between off-line and on-line analytical techniques

As mentioned previously, the results obtained from off-line and on-line analytical techniques have led to conflicting conclusions regarding the content of Cr(VI) in foodstuffs. One particular example is the presence of Cr(VI) in bread. Prior to the implementation of SIDMS, Soares *et al.* investigated bread samples using alkaline extraction and ETAAS, reporting Cr(VI) as representing 12% of the total Cr concentration (Soares et al., 2010). Novotnik *et al.* repeated the experiment using SIDMS, showing that Cr(VI) is not stable in bread extracts and is partially reduced to

Cr(III) by organic matter within the sample, with the extent of reduction greater in wholegrain bread compared to white (Novotnik et al., 2013). This highlights the issue of reporting species concentrations without a speciation step; selective extraction alone cannot account for poor extraction efficiency or interconversions, and any assessments of dietary intake could be significantly skewed as a result. More recently, Mathebula *et al.* reported that between 33 and 73% of total Cr in bread samples from South Africa was Cr(VI) using selective extraction and high resolution continuum source atomic absorption spectrometry, although it was concluded that average consumption would not exceed maximum acceptable concentrations of Cr(VI) (Mathebula et al., 2017).

Measurement of Cr(III)

The majority of the analytical methods reviewed in this article prioritise measurement of Cr(VI) over Cr(III) due to the known genotoxicity of Cr(VI). However, the use of Cr(III) compounds in nutritional supplements has generated a number of research articles assessing the biological importance and potential toxicity of Cr(III) (Lukaski, 2007). The use of Cr(III)-containing supplements has increased over the last 15 years (Prado et al., 2016), with emphasis being placed on the putative ability of the supplements to control diabetes and aid in weight loss. There are a number of Cr(III) compounds used in nutritional supplements, with Cr picolinate being the most common due to its relatively high bioavailability (Zayed and Terry, 2003). Sales of Cr(III)-containing supplements are approximately \$100 million per year (Young et al., 2005), accounting for 6% of all supplement sales (Chen et al., 2014). Despite this large market it has been shown that, while Cr(III) may play a role in the management of type II diabetes (Bailey, 2014), there is no significant improvement or acceleration of weight loss that can be attributed solely to supplementation of Cr(III) (Dhal et al., 2013a). Recently, Wu *et al.*

found that adipocytes treated with Cr(III) displayed localised regions or “hotspots” of Cr(V) and Cr(VI), likely formed during cell signalling where oxidising agents such as hydrogen peroxide are created with the potential to oxidise Cr(III) (Wu et al., 2016).

Concerning the genotoxicity of Cr(III), a recent article by Fang *et al.* (Fang et al., 2014) reported that both Cr(III) and Cr(VI) induce genetic mutations and DNA damage in yeast cells, with the effect of Cr(III) being greater than that of Cr(VI). The mechanism of DNA damage is also different; Cr(III) interferes with base pair stacking whilst Cr(VI) intercalates within DNA. As the research focus shifts to Cr(III) genotoxicity, and the potential for oxidation to Cr(VI) following ingestion, the need to measure Cr(III) as well as Cr(VI) is likely to become more commonplace. The likelihood of Cr(VI) reducing to Cr(III) during transfer into staple crops could increase the concentration of Cr(III) within the foodstuff (Lytle et al., 1998), potentially leading to an increased risk of exposure to Cr(VI) following Cr(III) re-oxidation in cells. Therefore, more research is needed to explore this possible route for Cr(VI) exposure, with future speciation methods incorporating measurement of both Cr(III) and Cr(VI).

Conclusion

This article presented a review of the analytical techniques used to undertake Cr speciation in foodstuffs over the last twenty years. Selective extraction using an alkaline medium, commonly a mixture of NaOH/Na₂CO₃ or NaOH/NH₄NO₃, is the most widely used technique for liberating Cr(VI) from the foodstuff prior to determination using off-line (FAAS, ETAAS, ICP-MS) or on-line (HPLC-ICP-MS) analytical methods. For all techniques the main goal is to maximise the amount of Cr(VI) extracted from the foodstuff whilst minimising species interconversions. The introduction of SIDMS has highlighted problems with studies that utilised selective extraction with no on-line speciation step; despite this, researchers are continuing to publish data for Cr(VI) in

foodstuffs and estimating daily intakes of Cr(VI) using off-line techniques. In light of recent toxicological studies, future research should incorporate measurement of Cr(III) to better evaluate the health risks from consuming foodstuffs high in Cr.

Chapter 3. Methods

Introduction

This chapter presents the development and/or optimisation of the methods used in Chapters 4 and 6. Due to the use of ‘paper format’ for Chapters 4 and 6, the brevity of the individual “Materials and Methods” sections does not fully encapsulate the processes through which the published methods were confirmed to be suitable for addressing the objectives set out in the Research Aim. Within this, a number of the methods share the same fundamental sample preparation, instrumentation and quality control procedures; these are detailed below. Although Chapter 5 also uses ‘paper format’, the isolation of apoplastic and symplastic root solutions used in the chapter is a well-established process (Yu et al., 1999, Mitani and Ma, 2005, Humphrey et al., 2019b). Therefore, optimisation was primarily concerned with establishing suitable concentrations of $^{50}\text{Cr}^{\text{III}}$ and $^{53}\text{Cr}^{\text{VI}}$ for use in treatment solutions; following one pilot experiment, these concentrations were confirmed and employed as described in the chapter.

Sample Collection, Preparation and Analysis

The collection of soil samples, used in Chapters 4 and 6, followed a geostatistically-designed sampling scheme outlined in Chapter 4.

Due to the known clay content, soil samples were freeze-dried prior to manual disaggregation and sieved to ≤ 4 mm. Subsamples of sieved material were milled to approximately ≤ 53 μm using a planetary ball mill equipped with agate grinder pots and balls, prior to graphite hotblock dissolution using mixed acid ($\text{HNO}_3/\text{HF}/\text{HClO}_4$) digestion.

All analysis was undertaken within the Inorganic Geochemistry laboratories at the British Geological Survey, under a quality system compliant with the requirements of International Standards BS EN ISO/IEC 17025 and BS EN ISO 9001. Total elemental analysis was carried out using triple-quadrupole Inductively Coupled Plasma Mass Spectrometry (ICP-QQQ, Agilent 8900). Speciation analysis of alkaline extracts (Chapter 4), root solutions (Chapter 5) and passively sampled soil solution (Chapter 6) was achieved through the coupling of high performance liquid chromatography (HPLC) with the ICP-QQQ instrument.

Quality Control

Data quality was confirmed using in-house and/or commercially available materials.

These included:

- Matrix-matched, species-specific certified reference materials (CRM) for Cr^{VI} in contaminated soil. Full details of CRM performance are given in Chapter 4.
- In-house quality control (QC) check standards for Cr^{III} and Cr^{VI} speciation measurements. QC performance is documented in Chapter 5.

Speciated Isotope Dilution Mass Spectrometry (Chapter 4)

Introduction

The accurate measurement of Cr^{VI} in soil samples is essential when assessing the fate of Cr released into the environment through geogenic and/or anthropogenic sources. The United States Environmental Protection Agency (US EPA) has a number of standardised methods for the extraction and quantification of Cr^{VI} in soil samples (Malherbe et al., 2011). Cr^{VI} is most stable at high pH, therefore alkaline extractants are typically used to solubilise Cr^{VI} whilst minimising the oxidation of Cr^{III} (Séby et al., 2008). Cr^{III} determined in alkaline extractions is considered “soluble” as the alkaline

extraction procedure does not mobilise all forms of Cr^{III}; aged Cr(OH)₃ and Cr₂O₃ remain in the solid phase (Wolle et al., 2014). US EPA Method 3060A uses a 3% Na₂CO₃–2% NaOH extraction solution, with the addition of Mg²⁺ in phosphate buffer to inhibit oxidation of Cr^{III} (Drinčić et al., 2018). Ethylenediaminetetraacetic acid (EDTA), adjusted to pH 10.0 using NH₄OH, has previously been reported as an alternative extractant due to its ability to mobilise Cr^{VI} and chelate soluble forms of Cr^{III} to minimise the potential for interconversions (Fabregat-Cabello et al., 2012b). Despite this, there is still a risk of Cr^{III} oxidation if high concentrations of manganese oxides are present in the soil sample, and Method 3060A can only be regarded as semi-quantitative with respect to Cr^{VI} when compared to solid-phase speciation techniques such as XANES (Wazne et al., 2007). Therefore, a metrological system is required which not only accounts for species interconversions during sample preparation, but also relies on absolute measurement of abundance as a workaround for incomplete extraction of the target analyte.

One method of accounting for Cr species interconversions during the extraction procedure is to use SIDMS. (“Skip” Kingston et al., 1998). SIDMS follows the same principles as isotope dilution (ID), where the concentration of an analyte in a sample is calculated through the addition of a known amount of an enriched isotope and subsequent measurement of the altered isotope ratios (Rodríguez-González et al., 2005). Following addition of an isotopically-enriched spike with the same chemical speciation as the analyte of interest, any interconversions that occur after spiking can be corrected for. SIDMS can also be used as a diagnostic tool, through the addition of spikes at different stages of extraction and analysis to identify specific processes that induce interconversions (Rahman and Kingston, 2004).

Comparison of external calibration and SIDMS

Microwave-assisted extraction of SRM 2701 was undertaken according to previously-published methods with minor modifications (Fabregat-Cabello et al., 2012b, Guidotti et al., 2015); full details are given in Chapter 4. To determine the best approach for determination of Cr^{VI} in soils, aliquots of SRM 2701 were processed using external calibration (calibration curve consisting of Cr^{III} and Cr^{VI} at 1, 5, 10 and 100 µg L⁻¹) and SIDMS (spiked with ⁵³Cr^{VI}); in both cases, species were separated online using HPLC-ICP-MS (Table 3.1).

Table 3.1 Recoveries of SRM 2701 calculated from external calibration and SIDMS

	External Calibration		SIDMS	
Replicate	Conc. (mg kg ⁻¹)	Recovery (%)	Conc. (mg kg ⁻¹)	Recovery (%)
1	340	62	507	92
2	334	61	514	93
3	328	59	536	97

For external calibration, the average recovery of SRM 2701 was 61 ± 0.9%, indicating a significant negative bias. Previous literature have reported a similar negative bias (≈ 40%) for this CRM when using comparable alkaline extractants and external calibration (Nagourney et al., 2008). The reason for this is still unclear but could be attributed to incomplete digestion of the CRM due to the association of Cr^{VI} with a variety of host phases in chromite ore processing residue (COPR) (Hillier et al., 2007). The SIDMS method produced more accurate data, due to the tolerance of isotope dilution to incomplete sample dissolution (Fassett and Paulsen, 1989). Subsequent analysis of unknown samples and CRMs was undertaken using the SIDMS strategy (chapter 4).

*Online microdialysis high performance liquid chromatography mass spectrometry
(MD-HPLC-ICP-MS) (Chapter 6)*

Introduction

Microdialysis (MD) is a passive sampling technique involving the use of a probe fitted with a semi-permeable membrane (typically 10 mm long and 100 kDa molecular weight cut-off for soil applications), behind which a solution (known as “perfusate”) is pumped. This establishes a concentration gradient between the perfusate and the external medium being sampled (e.g. soil solution), through which solutes can diffuse across the probe membrane. Due to the continuous delivery of perfusate, the sampled solutes are pumped out of the probe and the resulting solution (known as “dialysate”) can be analysed using a suitable offline or online technique.

The efficacy of solute sampling is dependent on the ability of the solute to diffuse through the sampled medium and across the semi-permeable membrane (Kho et al., 2017). The mathematical framework of MD is well-established, with quantitative recovery defined as a function of resistance to the movement of solutes imposed by external environment, probe membrane, dialysate and perfusate flow rate (Bungay et al., 1990). In the context of MD sampling, recovery is most commonly defined as the proportion of solute in the dialysate relative to the external concentration in the sampled medium (relative recovery), but can also refer to the physical flux of solutes across the membrane (absolute recovery) (Buckley et al., 2020).

Rationale for online MD method

The coupling of MD, a minimally invasive sampling method, with a highly sensitive analytical detector such as ICP-MS presents an unparalleled tool for monitoring the removal of inorganic contaminants from soil solution. To date, online MD has been

applied to *in vivo* studies (mercury transition in rat brains following administration of thimerosal), the assessment of nitrogen availability in soil, and as a separation tool to avoid lengthy sample preparation procedures (determination of anthropogenic organic compounds in wastewater, beneficial metabolites in plants and seeds). Currently there are no publications concerning the “real-time” monitoring of inorganic species removal from soil solution through fixation and/or species interconversion and immobilisation; the successful implementation of such a method would provide an unmatched level of spatial and temporal resolution, and avoid the common pitfalls of offline MD such as low volume sample handling, sample storage and time-consuming analyses.

Initial Setup- Continuous Sampling and Monitoring

The aim of the initial setup was to develop a system that was capable of continuously monitoring the isotopic signal of the dialysate being introduced into the ICP-MS instrumentation, to track changes in passively-available Cr^{VI} in real time at sub-minute resolution. The setup was as follows:

Syringe Pump 1: DDW water as perfusate delivered into CMA 20 MD probe, inserted into Cr^{VI} solution. Resulting dialysate flows into T-piece. Max 10 $\mu\text{L min}^{-1}$ flow rate.

Syringe Pump 2: DDW/Rh carrier/ISTD solution pumped into T-piece at 40 $\mu\text{L min}^{-1}$ flow rate.

Nebuliser: 50 $\mu\text{L min}^{-1}$, PFA.

Solution: $^{52}\text{Cr}^{\text{VI}}$, 100 $\mu\text{g L}^{-1}$.

Initial experiments yielded stable m/z 52 signals (>10 min monitoring, Fig. 3.1) with fast response times (signal generated approx. 1.5 min after probe insertion) and reasonable sensitivity (2000 cps at 100 ppb, 10 cps as background).

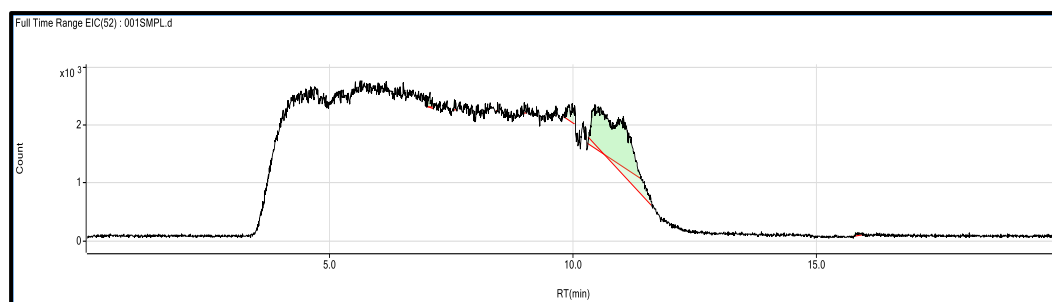


Figure 3.1. Time-resolved signal at m/z 52 for introduction and washout of $100 \mu\text{g L}^{-1}$ Cr^{VI} standard over 20 minutes. The microdialysis probe was initially placed in DDW to establish a background baseline before being placed in the Cr^{VI} standard. Internal standard signal has not been shown due to scaling.

The “dip” in signal at 10 minutes is due to the air gap created in the carrier/ISTD solution when switching from Cr^{VI} solution to DDW to initiate rinsing of MD probe. Despite initial success, issues began to arise with nebuliser blockages due to the internal diameter of the introduction capillary. In addition, the flow rate of the carrier/ISTD solution was found to be causing backflow into the dialysate flow path, leading to ultrafiltration and sharp troughs in the signal. The use of a lower carrier/ISTD flow rate resulted in unstable signal, due to the self-aspiration rate of the nebuliser ($50 \mu\text{L min}^{-1}$) being too high for the volume of sample delivered (Fig. 3.2). Therefore, despite the significant benefits of an online continuous monitoring system, a nebuliser with a much lower flow rate would be required to achieve a stable baseline and allow for reproducible passive-sampling of solution.

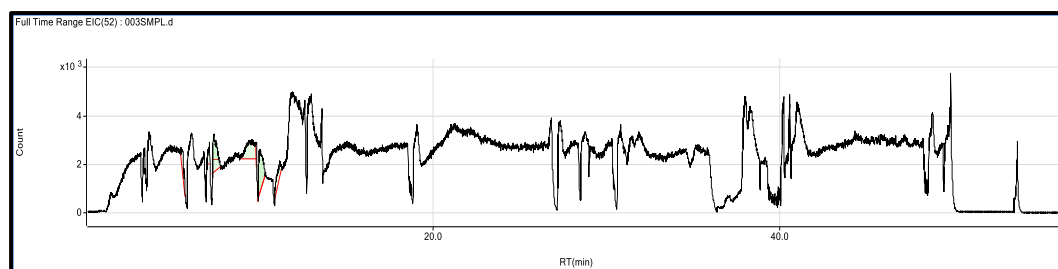


Figure 3.2. Instability in m/z 52 signal due to dialysate channel backflow. Signal acquired over 50 minute period.

Continuous Sampling and Semi-Continuous Monitoring

Further literature searching highlighted the combination of MD and a manual injector as a suitable alternative to continuous monitoring (Jin et al., 2008). This setup also allows for measurement of the speciation of the dialysate through the use of an LC pump and column, although the temporal resolution (dependent on perfusate flow rate) is decreased as a result of the need to fill a loop prior to injection into the LC flow path. The setup was as follows:

Syringe Pump: DDW water as perfusate delivered into CMA 20 MD probe, inserted into Cr^{VI} solution/soil. Flow rate $3 \mu\text{L min}^{-1}$.

Manual Injector and LC: Rheodyne 7125 6-port injector with $20 \mu\text{L}$ loop. Dionex GP50 Gradient Pump, Hamilton PRP-X100 anion exchange column ($250 \text{ mm} \times 4.6 \text{ mm} \times 5 \mu\text{m}$). Flow rate 1.2 mL min^{-1} .

To interface the MD probe and the manual injector, an FEP adaptor was connected to a #22 gauge LC needle and inserted directly into the Rheodyne injection port. Prior to each injection, the loop was filled with a minimum of two loop volumes of dialysate ($40 \mu\text{L}$) to ensure good reproducibility. Initial injections of dialysate from a stirred $100 \mu\text{g L}^{-1} \text{Cr}^{\text{VI}}$ solution ($10 \mu\text{L min}^{-1}$ flow rate) showed that the MD probe was able to passively sample the Cr^{VI} solution and that this was resolved on the LC column within

5.5 minutes (Fig. 3.3). Tests were undertaken using a shorter anion exchange column (50 mm) with the aim of reducing the HPLC-ICP-QQQ analysis time but, due to the Cr^{VI} peak eluting too close to the solvent front, the peak resolution was inconsistent and so the 250 mm column was used for all subsequent development.

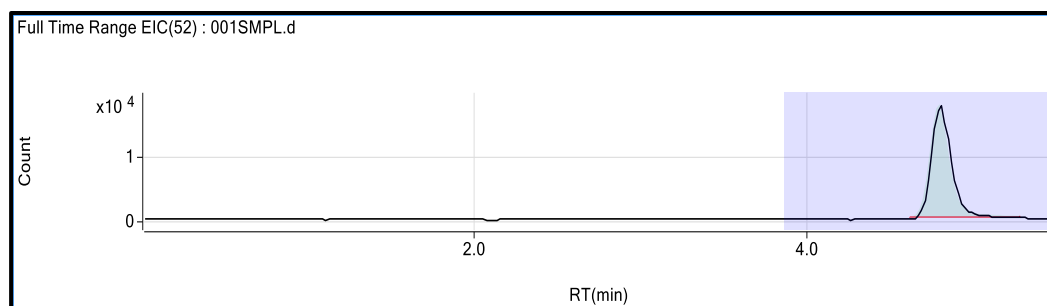


Figure 3.3. Chromatogram of $100 \mu\text{g L}^{-1} {}^{53}\text{Cr}^{\text{VI}}$ standard, passively-sampled and injected using MD-HPLC-ICP-MS setup.

Subsequent development steps, including flow rate calibration, precision, linearity of response and assessment of detection limit, are reported in Chapter 6.

Chapter 4. Reconnaissance sampling and determination of hexavalent chromium in potentially-contaminated agricultural soils in Copperbelt Province, Zambia

Paper published in *Chemosphere* (Hamilton *et al.*, 2020. 247: 125984)

DOI: 10.1016/j.chemosphere.2020.125984

Author list

Hamilton EM, Lark RM, Young SD, Bailey EH, Sakala GM, Maseka KK and Watts MJ

Author contribution

Study conceived by EMH, RML, SDY, EHB, GMS, KKM and MJW

Experiments and analysis performed by EMH and RML

All tables and figures produced by EMH

Construction of the paper by EMH

All authors were involved in the study and manuscript development

Abstract

The contamination of agricultural soils in the Copperbelt Province of Zambia through significant historical and current mining activity is inadequately monitored, with high concentrations of potentially harmful elements (PHEs) and their species present for uptake into staple crops. One such element is chromium (Cr), which warrants investigation due to the differing mobilities and toxicities of its species; trivalent [Cr(III)] and hexavalent chromium [Cr(VI)]. In addition, the possibility of species interconversions requires the implementation of robust methods that can correct for changes at the point of sampling, extraction and analysis. This work presents the application of speciated isotope dilution mass spectrometry (SIDMS) to accurately quantify Cr(VI) in agricultural soils within close proximity to a mine tailings dam in the Copperbelt Province of Zambia. Interpolated plots of total Cr, produced from data collected through a nested sampling design, were used to optimise the sampling across the spatial domain. Extraction of Cr(VI) was undertaken using a microwave assisted reaction system (80°C for 5 minutes) with 50 mM EDTA, to complex Cr(III) and reduce the likelihood of oxidation during the extraction. Isotopically-enriched $^{53}\text{Cr(VI)}$ was added to each sample prior to extraction to account for species interconversions. The accuracy of the method was confirmed using NIST SRM 2700 and 2701. Cr(VI) concentrations in the soil samples ranged between 0.03 and 0.29 mg kg⁻¹, significantly lower than the residential UK screening value for Cr(VI) of 21 mg kg⁻¹. The data indicate that this site poses a low environmental/human health risk with respect to Cr(VI) exposure.

Introduction

Chromium (Cr) is a naturally-occurring element that exists in the environment primarily as two chemical forms; trivalent chromium (Cr(III)) is considered non-toxic

and important for regulation of glucose and lipid metabolism (Krzysik et al., 2011), whereas hexavalent chromium (Cr(VI)) is toxic and a known carcinogen through inhalation (Langård and Costa, 2007). Chromium-containing compounds have a range of uses in industrial applications, including electroplating, steel manufacturing, wood preservation and leather tanning (Dhal et al., 2013b), which has increased both concerns and restrictions over the anthropogenic release of Cr(VI) into the environment (Oh et al., 2007).

Due to its presence as a positively-charged ion, Cr(III) is less mobile in soil-water systems than Cr(VI) (James and Bartlett, 1983a) and is therefore less likely to be transferred into plants grown in contaminated soil (Shanker et al., 2009). The majority of Cr(VI) in the environment can be attributed to anthropogenic activity from industrial processes such as leather tanning and metal finishing (Oliveira, 2012a). Geogenic Cr(VI) occurs as a result of weathering of ultramafic and serpentinite rocks (Oze et al., 2007); groundwater concentrations of Cr(VI) in ultramafic areas can range from 0.2 to 180 $\mu\text{g L}^{-1}$ (Chrysochoou et al., 2016).

The speciation of Cr in solid sample matrices has been reviewed in a number of articles (Hamilton et al., 2018) (Séby and Vacchina, 2018). The main analytical challenge to address is ensuring accurate determination of Cr(VI) in the sample without causing interconversion of species (Pettine and Capri, 2005), which may lead to under/over-reporting of Cr(VI) (Nagourney et al., 2008) and an insufficient assessment of the associated risk to human health (Novotnik et al., 2015). Speciated isotope dilution mass spectrometry (SIDMS) was developed to correct for changes in speciation which may occur at the point of analysis ("Skip" Kingston et al., 1998). This variation on conventional isotope dilution uses species-specific isotopically enriched spikes, added to the sample prior to digestion or extraction, to accurately determine species

concentrations in solid matrices and reduce analytical/species conversion errors that may occur through external calibration (Martone et al., 2013).

The Copperbelt Province of Zambia has been the site of extensive mining operations for over 100 years (Weissenstein and Sinkala, 2011), which had led to an increase in surface concentrations of potentially harmful elements (PHEs) such as Cr, arsenic (As), cobalt (Co), lead (Pb) and zinc (Zn) (Křibek et al., 2010). Numerous studies have evaluated the mobility of these PHEs (Ettler et al., 2011) (Kaninga et al., 2019) and the risk to human health through consumption of groundwater (von der Heyden and New, 2004) and staple crops grown in contaminated soil (Křibek et al., 2014); outside of the Copperbelt Province, the irrigation of crops with contaminated water and subsequent accumulation of PHEs in soil is well documented (Stasinou and Zabetakis, 2013). However, few studies have investigated the extent of Cr(VI) contamination in tailings-contaminated soil, despite the likelihood of increased preservation of Cr(VI) if the tailings are limed prior to pumping to neutralise and precipitate metals (Tang et al., 2016).

The objective of this study was to use previously-reported reconnaissance work to inform more detailed sampling across potentially-contaminated agricultural land within half a kilometre of a large tailings dam in the Copperbelt Province of Zambia. Single-spike speciated isotope dilution mass spectrometry (SIDMS) was then employed to quantify Cr(VI) in 50 soils over this area, to establish whether a human health risk could exist through exposure to Cr(VI) from soil contaminated with tailings material. To the best knowledge of the authors, this work presents the first occasion of SIDMS being used to quantify Cr(VI) in potentially-contaminated soil samples collected from the Copperbelt Province of Zambia.

Materials and Methods

Sample Preparation and Analysis

Samples were dried at 40°C, disaggregated using a pestle & mortar and sieved to ≤ 2 mm. From this sieved fraction, subsamples were milled to ≤ 53 μm using a planetary ball mill (Retsch GmbH, Germany) for total Cr and Cr(VI) analyses. Samples for measurement of total Cr were prepared using an in-house mixed acid ($\text{HF}/\text{HNO}_3/\text{HClO}_4$) open vessel hotblock digestion (Watts et al., 2019). A microwave assisted reaction system (MARS One, CEM Corporation, UK) was used for alkaline extraction of Cr(VI) from soil samples.

Determination of total Cr was performed by ICP-MS using an Agilent 7500cx instrument (Agilent Technologies, Tokyo, Japan) using previously reported operating conditions (Hamilton et al., 2015); measurement of Cr(VI) in alkaline extractions was undertaken using an Agilent 8900 ICP-QQQ instrument. Chromatographic separation employed an Agilent 1260 Infinity II Bio-Inert Liquid Chromatography (HPLC) System equipped with a 100 μL injection loop and a PRP-X100 anion exchange column (PEEK, 250 mm x 4.6 mm x 5 μm) (Hamilton Company, USA) connected to the nebuliser of the ICP-QQQ using a single piece of 1/16" OD PEEK tubing. The ICP-QQQ instrument was optimised prior to connection of the LC system using a 1 $\mu\text{g L}^{-1}$ tuning solution and operated in helium (He) collision mode at a flow rate of 5.5 mL min^{-1} to minimise the impact of polyatomic interferences such as $^{40}\text{Ar}^{12}\text{C}^+$. Cr was monitored at m/z 52 for total Cr analysis and m/z 50, 52, 53 and 54 for HPLC analysis. Soil pH measurements were undertaken on ≤ 2 mm subsamples using a solid body combined pH electrode in a 0.01 M CaCl_2 slurry (solid to solution ratio of 1:2.5). Loss-on-ignition (LOI) was measured on a 1 g subsample of milled material.

Study Area and Sampling

The study area comprises a village within 1 km of a tailings dam used by Mopani Copper Mines in Kitwe, Zambia (-12.800346, 28.118721, Figure 4.1); the mine has been in operation for 28 years. Initially, reconnaissance sampling of agricultural land was undertaken; this process has been outlined by Lark *et al.* (Lark et al., 2017b). Briefly, a nested sampling design was implemented to assess the spatial variation of chromium across the agricultural land within the village, which involved collecting samples along transects with sample main stations at loose intervals between 100 m and 200 m with substations in random directions a further 100 m, 10 m and 1 m from the initial main stations (Figure 4.1i). At each main station and substation, a topsoil sample (0–15 cm depth) was collected from a composite of 5 sub-samples using a Dutch auger. The data on total Cr were then used to produce interpolated values at locations on a fine grid across the study area by the method of ordinary kriging as implemented in the gstat package for the R platform (Pebesma, 2004, R Core Team, 2014). The interpolated values were then used to produce a map of total Cr with the visualization tools of the ESRI ArcGIS® software (Figure 4.1ii). The same method was used to interpolate values of soil pH and LOI at the same grid nodes.

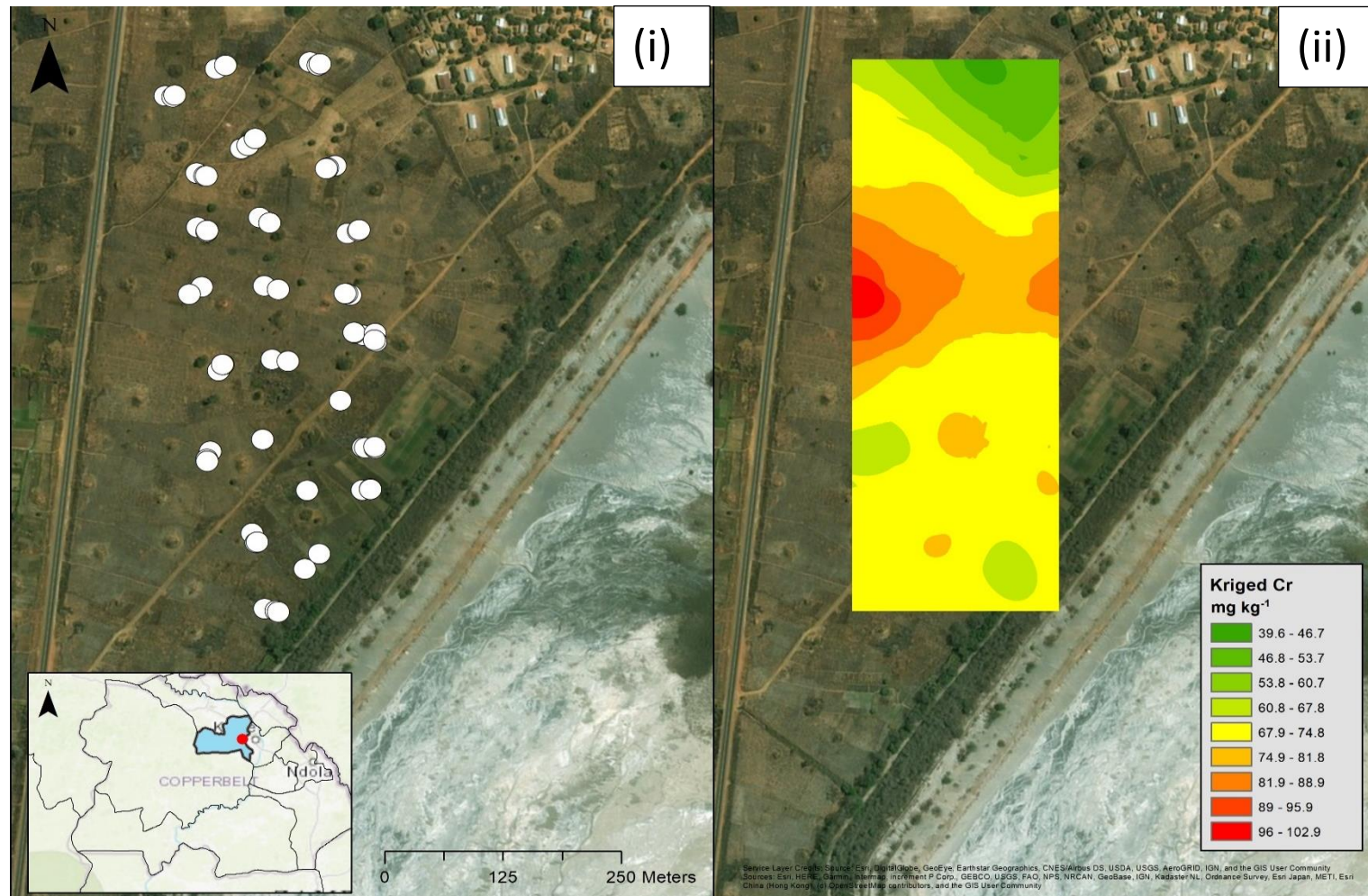


Figure 4.1. Study site with (i) sample points for nested sampling design (white circles) and (ii) kriging output created from total Cr data. Maps created using ArcGIS® software by Esri. ArcGIS® and ArcMap™ are the intellectual property of Esri and are used herein under license. Copyright © Esri. All rights reserved.

The selection of sample points for this study was done with the cube algorithm of Deville and Tillé (2004) as implemented in the `BalancedSampling` library for the R platform (Grafström and Lisic, 2016). The objective of this method is to draw a sample by random sampling according to pre-determined set of inclusion probabilities, in this case for the nodes of the fine grid for which interpolated values of Cr, pH and LOI were available, the sample was therefore unbiased. At the same time the algorithm achieved spatial balance; the mean coordinate values of the sample points are close to the mean coordinate values of all points on the sample site, and are also spread in the feature space defined by the interpolated values of Cr, LOI and pH. The sample therefore covers the range of these values. A total of 50 sample locations were selected in this way, and their coordinates were exported to the open source application “maps.me” for subsequent location and sampling.

Reagents and Materials

All solutions were prepared in 18.2 MΩ cm ultrapure water. The natural abundance standard of $^{52}\text{Cr(VI)}$ was purchased as a 1000 mg L⁻¹ solution in water (High Purity Standards, SC, USA), no further preparation was required. $^{53}\text{Cr(VI)}$ solution was prepared from the isotopically-enriched oxide (ISOFLEX, CA, USA) according to instructions outlined in EPA Method 6800 (2014). The concentration of $^{53}\text{Cr(VI)}$ in the stock and spiking solutions were verified before analysis using reverse isotope dilution; the isotopic composition of the spike solution is given in Table 4.1. The natural isotopic abundances used in the SIDMS calculations are those reported by the International Union of Pure and Applied Chemistry (IUPAC) (Weights, 2013).

Table 4.1. Isotopic composition of $^{53}\text{Cr(VI)}$ used throughout study.

Isotope	Abundance (%)
50	Not detected
52	2.8 ± 0.3
53	97.2 ± 0.4
54	Not detected

Ethylenediaminetetraacetic acid (di-ammonium salt, $\text{NH}_4\text{-EDTA}$), trisaminomethane (TRIS) and ammonium nitrate (NH_4NO_3) (Sigma Aldrich, UK) were used for the preparation of the chromatographic mobile phase. The certified reference materials (CRMs) SRM 2700 Hexavalent Chromium in Contaminated Soil (Low Level) and SRM 2701 Hexavalent Chromium in Contaminated Soil (High Level) (NIST, USA) were used to verify the accuracy of the extraction procedure.

Determination of Cr(VI)

Samples for measurement of Cr(VI) were prepared using microwave-assisted single-spike speciated isotope dilution mass spectrometry (SIDMS) according to the method outlined by Guidotti *et al.* with minor modifications (Guidotti *et al.*, 2015). Briefly, a 0.2 g subsample of the dried milled material was added to the microwave vessel, followed by 10 ml of 50 mM $\text{NH}_4\text{-EDTA}$ (pH 10.0). The vessel was then spiked with 0.4 ml of $^{53}\text{Cr(VI)}$ at a concentration sufficient to double the natural $^{53}\text{Cr(VI)}$ present in the sample. The use of a single isotopically-enriched spike was considered appropriate due to the reducing capacity of the soils (low pH, high concentrations of iron and aluminium), minimising the risk of conversion of Cr(III) to Cr(VI) during the extraction procedure. The use of $\text{NH}_4\text{-EDTA}$ as an extractant has also been shown to complex

solubilised forms of Cr(III), further reducing the likelihood of oxidation (Mamatha et al., 2014). The sample was then subjected to a 5 minute heating programme at 80°C (8 minute ramp time), cooled and centrifuged at 4000 min⁻¹ for 20 minutes to separate the supernatant from the extracted solid material. Prior to chromatographic separation, the sample was diluted with ultrapure water (2-fold for samples, 10- or 100-fold for CRMs) to ensure all analyses were within the pulse-counting mode of the electron multiplier (EM). In addition to the samples, at least two CRM replicates (either NIST SRM 2700 or 2701), three reagent blanks and two duplicate samples were extracted in each microwave batch.

The efficacy of this approach, with respect to minimising oxidation of Cr(III) to Cr(VI), was confirmed by spiking a subset of the samples (n = 6) with a known volume of ⁵⁰Cr(III) and processing as described above. The oxidation factor, defined as the percentage of added ⁵⁰Cr(III) oxidised to ⁵⁰Cr(VI) over the course of the extraction procedure, ranged between 1.3 and 3.9 % and was in good agreement with previous literature that used EDTA as an alkaline extractant (Fabregat-Cabello et al., 2012a). Therefore, the oxidation of Cr(III) to Cr(VI) can be considered negligible when employing the microwave heating programme outlined in this section.

Results and Discussion

Analytical Figures of Merit for HPLC-ICP-QQQ Speciation of Chromium

Speciation of Cr(VI) and Cr(III) in alkaline extractions was achieved in 7.5 minutes using an isocratic elution programme (100% 40 mM NH₄NO₃/50 mM TRIS Buffer/5 mM NH₄-EDTA, pH 7.0) at a flow rate of 1.2 mL min⁻¹. An example chromatogram at m/z 52 is shown in Figure 4.2.

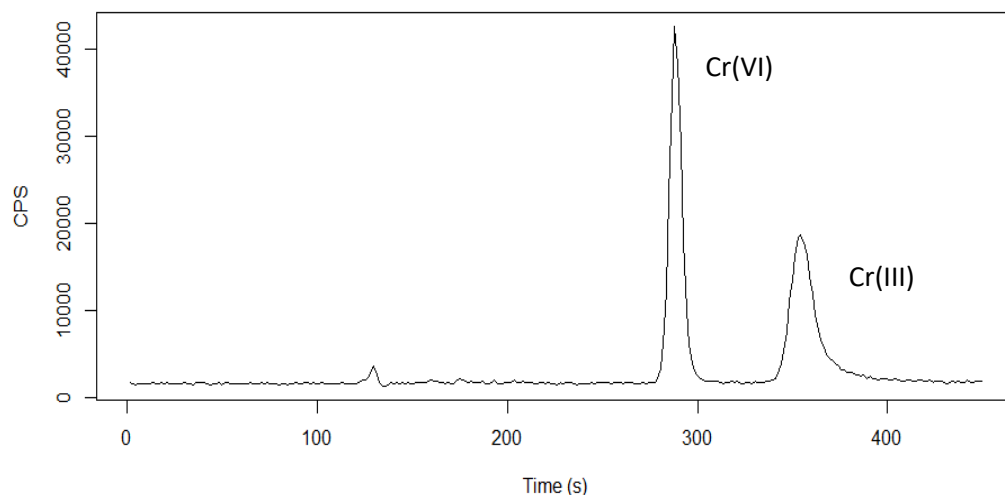


Figure 4.2. Speciation of Cr(VI) and Cr(III) in a natural isotopic abundance standard ($10 \mu\text{g L}^{-1}$).

The Cr(III) determined in the alkaline extractions is considered “soluble” Cr(III) as the alkaline extraction procedure does not mobilise all forms of Cr(III); aged Cr(OH)_3 and Cr_2O_3 remain in the solid phase (Wolle et al., 2014). The method limit of detection (LOD), calculated as three times the standard deviation of the $^{52}\text{Cr(VI)}$ concentration measured in ten reagent blanks spiked with $^{53}\text{Cr(VI)}$, was 0.03 mg kg^{-1} .

NIST SRM 2700 (Hexavalent Chromium in Contaminated Soil Low Level, certified value $14.9 \pm 1.2 \text{ mg kg}^{-1}$) demonstrated good accuracy with an average recovery of $109 \pm 3 \%$ across four replicates; SRM 2701 (Hexavalent Chromium in Contaminated Soil High Level, certified value $551.2 \pm 34.5 \text{ mg kg}^{-1}$) indicated negative bias with an average recovery of $86 \pm 6 \%$ across the same number of replicates. The higher concentration of Cr(VI) in SRM 2701 made it difficult to achieve a 1:1 spiking ratio with the $^{53}\text{Cr(VI)}$ isotopically-enriched spike used in this work, which could have resulted in poorer isotopic mixing and equilibration for this reference material (Vogl, 2007); the lower Cr(VI) concentration in SRM 2700 is more representative of the samples analysed in this study.

Processing of SIDMS Data

The microwave-assisted extraction method was applied to the 50 agricultural soil samples collected from Mugala Village in the Copperbelt Province of Zambia, followed by LC analysis to separate the extracted forms of Cr(VI) and Cr(III). Integrated peak areas for m/z 50, 52, 53 and 54 were extracted from the instrument data processing software and used to calculate abundances and the $^{53}\text{Cr(VI)}/^{52}\text{Cr(VI)}$ isotope ratio. Mass bias correction factors were calculated from a natural standard analysed over the course of the analytical run at regularly bracketed intervals (Rousseau et al., 2013). Following data processing, Eq. (4.1) (Huang et al., 2004) was used to calculate Cr(VI) concentrations in the soil samples:

$$Cx = Cs \frac{Mx \ Ws \ As - RBs}{Ms \ Wx \ RBx - Ax} \quad (4.1)$$

where Cx is the concentration of Cr(VI) in the sample, Cs is the concentration of Cr(VI) in the $^{53}\text{Cr(VI)}$ spike solution (in $\mu\text{g l}^{-1}$), Mx is the natural relative atomic mass (^{52}Cr), Ms is the isotopically-enriched relative atomic mass (^{53}Cr), Ws is the mass of the spike solution (in g), Wx is the mass of the soil sample (in g), As is the abundance of ^{52}Cr in the spike solution, R is the $^{52}\text{Cr(VI)}/^{53}\text{Cr(VI)}$ isotope ratio, Bs is the abundance of ^{53}Cr in the spike solution, Bx is the abundance of ^{53}Cr in the unspiked sample and Ax is the abundance of ^{52}Cr in the unspiked sample.

Total Cr and Cr(VI) in Mugala Village Soil Samples

Total Cr, Cr(VI), soil pH and selected element concentrations for the 50 agricultural soil samples are presented in Table 4S.1 of the Supplementary Material. The total Cr ranged from 53 to 82 mg kg^{-1} , with a median concentration of 70 mg kg^{-1} . Although these concentrations are higher than previously reported data for topsoil Cr in this region (Křibek et al., 2010), the study site is within close proximity to a large tailings

dam and is therefore more likely to have elevated topsoil concentrations through the deposition of wind-blown dust (Middleton et al., 2017, Nakaona et al., 2019).

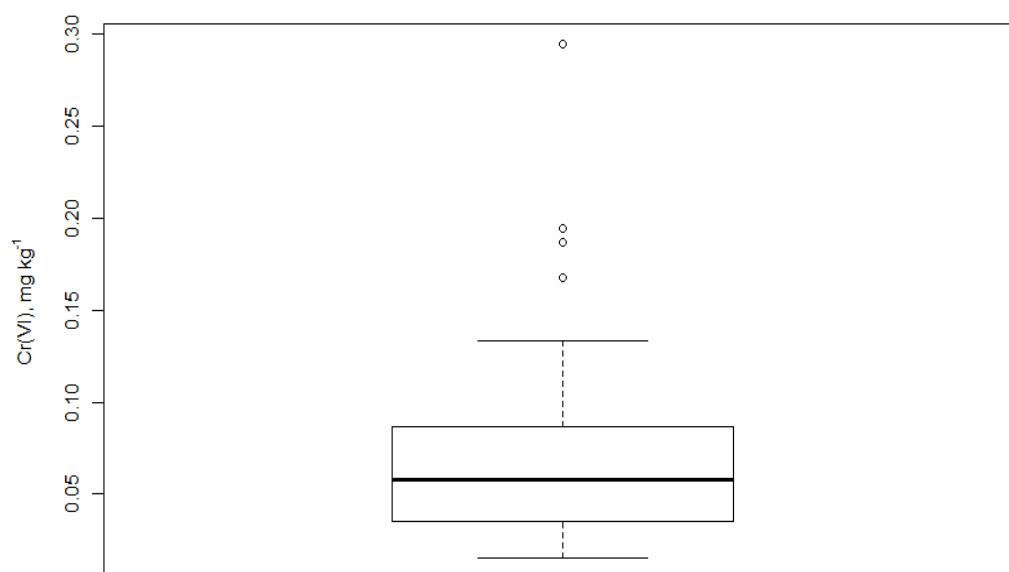


Figure 4.3. Distribution of Cr(VI) concentrations in agricultural soil samples.

Cr(VI) concentrations in the soil samples ranged from 0.03 to 0.29 mg kg⁻¹ (Figure 4.3), corresponding to between 0.04 and 0.44% of the total Cr in the soil samples. At the time of writing, there is no screening or guidance value for Cr(VI) in agricultural soil for Zambia. Therefore, the UK's provisional category 4 screening level (C4SL) for Cr(VI) was used to assess the significance of these concentrations. Based on the land-use of the study site (residential with consumption of homegrown produce), none of the soil samples exceeded the C4SL of 21 mg kg⁻¹ Cr(VI). This is likely due to the lateritic nature of the soils, with the presence of high concentrations of reducing components such as Fe and Al. In addition, the agricultural management strategies in place at the site, including reincorporation of crop residues following cultivation, are likely to increase soil organic carbon (SOC) (Zhang et al., 2019) leading to greater reduction of Cr(VI).

From the measured speciation data, it can be concluded that Cr(VI) poses a relatively low environmental and/or human health risk at this site, either through direct soil-to-

mouth transfer or from indirect exposure through wind-blown dust deposition onto staple crops.

Conclusions

The analysis of the agricultural soil samples indicate that a Cr(VI) exposure risk is relatively low in this area of the Copperbelt Province. Through the implementation of robust extraction and analytical methods, Cr(VI) was accurately quantified in the soil samples. Taking into consideration the total Cr concentrations in the samples, this study has once again highlighted the importance of speciation analysis to fully understand and evaluate the risk to environment and human health.

Although none of the analysed soil samples exceeded either the European total Cr threshold value (100 mg kg^{-1}) (Tóth et al., 2016) or the UK Cr(VI) C4SL (21 mg kg^{-1}), there may still be an exposure risk from compounding factors not investigated in this study. The dietary intake for the inhabitants of the village is dependent on subsistence agriculture, resulting in a much lower diversity of source. Due to the proximity of the mine tailings, there may be increased exposure to potentially harmful elements (PHEs) at concentrations close to threshold values, which themselves may have been derived without taking into account different pathways of exposure (oral ingestion, inhalation, crop consumption) and differences in bioaccessibility depending on source (Ljung et al., 2007). Therefore, wider health studies are required to address the significance of chronic sub-threshold PHE exposure on the health of people living within close proximity to mine tailings, which could feed into refinement of threshold value derivation and improvement of soil management and/or remediation strategies on a site-specific basis.

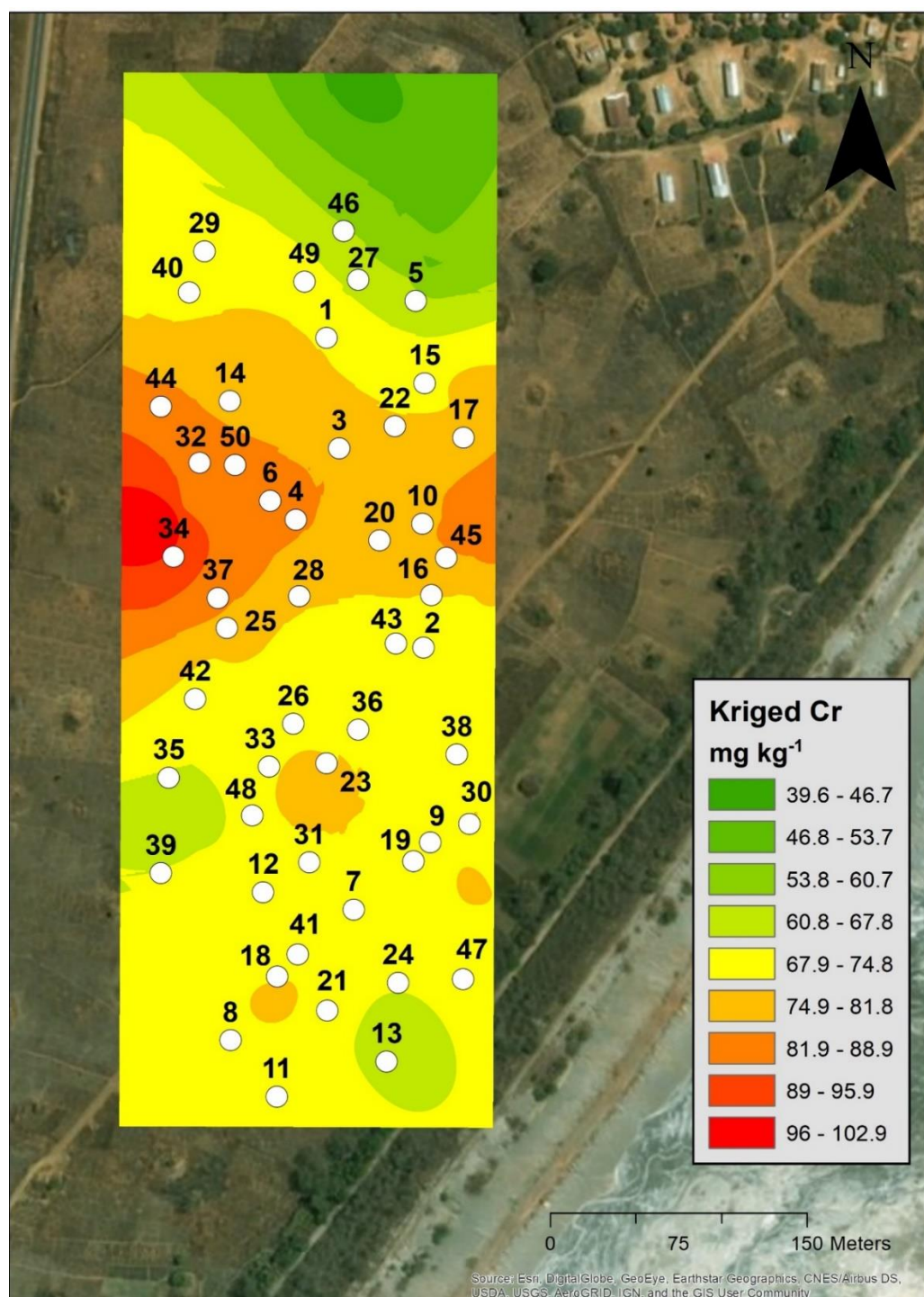


Figure 4S.1. Locations of samples analysed for Cr(VI) in this study (see Table 4S.1 for corresponding sample names and additional elemental data). Map created using ArcGIS® software by Esri. ArcGIS® and ArcMap™ are the intellectual property of Esri and are used herein under license. Copyright © Esri. All rights reserved.

Table 4S.1. Elemental and speciation data for agricultural soils in Copperbelt Province, Zambia

Sample Number	Latitude	Longitude	Loss-on-ignition	Soil pH	Al (mg/kg)	Cr (mg/kg)	Cr(VI) (mg/kg)
1	-12.78817	28.10294	5.53	5.5	67851	64.1	<0.03
2	-12.78982	28.10347	5.95	6.0	72019	70.1	<0.03
3	-12.78876	28.10301	5.03	5.6	72099	71.2	<0.03
4	-12.78914	28.10278	5.37	6.0	68056	71.2	<0.03
5	-12.78797	28.10342	5.41	5.4	56262	60.3	<0.03
6	-12.78904	28.10264	5.15	5.5	71995	70.2	<0.03
7	-12.79122	28.10310	6.62	6.4	69094	72.0	0.19
8	-12.79192	28.10244	5.39	7.5	64477	65.4	0.29
9	-12.79086	28.10351	4.45	7.0	71757	72.5	0.19
10	-12.78916	28.10346	4.38	6.4	74611	73.6	0.04
11	-12.79222	28.10269	4.38	6.7	68868	61.3	0.05
12	-12.79113	28.10261	4.77	6.6	73096	72.2	0.17
13	-12.79203	28.10328	5.56	6.9	72686	73.7	0.10
14	-12.78851	28.10242	5.80	5.9	78521	74.2	0.03
15	-12.78841	28.10347	3.75	5.6	50985	59.2	0.03
16	-12.78954	28.10351	4.47	6.2	68727	79.5	0.05
17	-12.78870	28.10368	6.22	5.7	67914	72.0	0.03
18	-12.79158	28.10269	6.22	6.4	78803	72.6	0.06
19	-12.79096	28.10342	5.62	6.5	68350	66.6	0.06
20	-12.78925	28.10323	5.48	5.6	71732	70.3	0.07
21	-12.79176	28.10296	5.89	6.3	73253	69.5	0.04
22	-12.78864	28.10331	5.25	5.3	67018	70.9	0.09
23	-12.79044	28.10295	4.33	6.5	70802	66.2	0.09
24	-12.79161	28.10334	5.30	6.8	65488	64.4	0.12
25	-12.78972	28.10241	7.50	5.6	70125	65.6	0.05
26	-12.79023	28.10277	4.60	6.2	81637	75.5	0.04

Sample Number	Latitude	Longitude	Loss-on-ignition	Soil pH	Al (mg/kg)	Cr (mg/kg)	Cr(VI) (mg/kg)
27	-12.78786	28.10311	4.09	5.3	54567	55.3	0.07
28	-12.78955	28.10280	6.84	5.7	71919	75.6	0.03
29	-12.78771	28.10228	3.83	5.0	53841	53.0	<0.03
30	-12.79076	28.10372	5.17	6.7	63764	73.9	0.09
31	-12.79097	28.10286	6.77	5.9	78275	79.2	0.10
32	-12.78884	28.10226	5.48	5.4	83219	80.2	0.03
33	-12.79046	28.10264	6.07	5.8	81022	82.4	0.07
34	-12.78934	28.10212	5.39	5.4	71140	75.5	0.05
35	-12.79052	28.10210	4.23	5.8	65725	65.0	0.07
36	-12.79026	28.10312	5.24	6.2	71795	66.5	0.04
37	-12.78956	28.10236	6.07	5.6	76054	76.0	0.07
38	-12.79039	28.10365	4.14	6.5	63365	67.3	0.09
39	-12.79103	28.10206	6.19	6.0	68261	63.0	0.07
40	-12.78793	28.10220	4.87	5.1	57581	68.3	0.08
41	-12.79146	28.10280	5.48	6.1	59934	62.5	0.06
42	-12.79010	28.10224	5.72	6.2	60551	64.6	0.10
43	-12.78980	28.10332	5.44	5.8	70491	66.5	0.08
44	-12.78854	28.10205	5.08	5.1	86130	78.3	0.05
45	-12.78934	28.10359	4.86	5.8	66076	65.8	0.05
46	-12.78760	28.10303	4.43	5.2	47379	52.8	0.06
47	-12.79159	28.10369	4.98	6.4	62303	68.8	0.13
48	-12.79072	28.10255	4.85	6.0	69762	65.0	0.08
49	-12.78787	28.10282	5.05	5.0	69359	61.4	0.05
50	-12.78885	28.10245	6.22	4.9	80374	73.1	0.03

Sample Number	Fe (mg/kg)	Co (mg/kg)	Ni (mg/kg)	Cu (mg/kg)	Zn (mg/kg)	Ba (mg/kg)	Pb (mg/kg)
1	32464	44.6	33.0	1030	40	214	11.0
2	34180	52.5	34.8	967	26	271	9.52
3	35009	51.0	36.9	944	29	231	10.2
4	34282	47.6	36.7	838	27	221	9.27
5	28072	45.5	28.8	1000	28	196	9.99
6	35989	44.1	39.6	874	29	215	9.91
7	32142	68.0	40.5	896	25	217	9.35
8	28842	71.6	38.9	968	26	209	9.85
9	34205	54.7	40.7	882	26	212	9.36
10	35031	36.6	39.9	537	28	237	8.19
11	28862	46.4	40.5	679	25	215	8.67
12	31538	55.8	43.7	899	28	219	10.0
13	33655	117	39.1	1261	29	313	10.3
14	37162	43.3	42.9	792	31	234	9.82
15	27392	32.9	24.8	805	26	181	8.79
16	34795	48.0	35.8	970	28	237	9.70
17	33353	40.7	34.9	857	30	240	9.42
18	33213	66.9	46.0	979	29	228	10.3
19	32406	59.1	39.5	924	27	213	9.73
20	34389	44.2	37.0	946	27	237	9.69
21	31957	74.4	43.2	979	31	229	11.4
22	33552	41.6	35.5	840	32	218	10.8
23	33056	56.8	39.5	1004	28	238	10.9
24	28822	78.4	38.8	975	24	245	9.37
25	31971	59.9	38.3	1321	34	234	13.5
26	35466	37.8	47.4	512	24	384	7.62
27	27756	39.9	28.3	772	27	201	8.49
28	36280	49.9	39.7	942	26	236	9.36
29	26842	37.4	29.8	666	24	156	7.80

Sample Number	Fe (mg/kg)	Co (mg/kg)	Ni (mg/kg)	Cu (mg/kg)	Zn (mg/kg)	Ba (mg/kg)	Pb (mg/kg)
30	32911	59.7	36.5	857	24	213	8.50
31	36083	61.7	44.0	1108	30	248	10.9
32	40256	45.8	48.0	927	31	230	10.7
33	36937	57.0	45.8	1058	31	240	10.9
34	34480	44.0	38.7	920	27	231	10.4
35	31012	36.0	36.2	697	26	189	8.58
36	33720	42.0	39.3	735	25	214	8.23
37	37228	50.6	42.7	1110	29	227	11.2
38	31717	47.1	33.8	766	22	197	8.16
39	28994	54.9	37.4	1153	34	222	12.3
40	32499	38.4	35.2	879	32	188	11.2
41	28262	44.8	40.9	790	21	189	9.49
42	32353	44.0	38.2	1029	28	197	12.9
43	36685	51.6	38.9	1235	31	260	11.6
44	43659	46.2	54.2	1074	34	250	12.6
45	34308	41.3	37.7	946	27	240	9.82
46	27297	37.0	26.7	973	28	173	9.93
47	32118	92.0	36.8	981	26	269	9.08
48	33474	48.7	43.6	934	24	219	10.4
49	33961	45.0	38.9	1048	33	218	11.2
50	40896	51.7	47.9	1254	33	252	12.9

Chapter 5. Assessment of chromium species dynamics in root solutions using isotope tracers

Paper published in *Journal of Trace Elements in Medicine and Biology* (Hamilton *et al.*, 2020. 61: 126514)

DOI: 10.1016/j.jtemb.2020.126514

Author list

Hamilton EM, Young SD, Bailey EH, Humphrey OS and Watts MJ

Author contribution

Study conceived by EMH, SDY, EHB, OHS and MJW

Experiments and analysis performed by EMH

All tables and figures produced by EMH

Construction of the paper by EMH

All authors were involved in the study and manuscript development

Abstract

Background

Chromium (Cr) exists in the environment in two chemical forms; Cr^{III} is an essential micronutrient for glucose and lipid metabolism, whereas Cr^{VI} is toxic and a recognised carcinogen through inhalation. Numerous studies have attempted to evaluate their transfer mechanisms from soil and solution media into plants, usually with respect to the hyperaccumulation, detoxification and tolerance of the plant to Cr^{VI}.

Methods

Isotopically enriched species of Cr, added as ⁵⁰Cr^{III} and ⁵³Cr^{VI}, were used to investigate transfer from solution into the root systems of *Spinacia oleracea*. In addition the effect of sulphate (SO₄²⁻), as a competitor for Cr^{VI} uptake, was investigated. Separation of ⁵⁰Cr^{III} and ⁵³Cr^{VI} was undertaken using HPLC-ICP-QQQ following isolation of root solutions using freeze/thaw centrifugation.

Results

Irrespective of supplied Cr^{VI} concentration (250, 500 or 1000 µg L⁻¹), the dominant species in both apoplastic (routed through cell wall and intercellular space as a passive mechanism) and symplastic (routed through cytoplasm as an active mechanism) root solutions was Cr^{III}. There was evidence for Cr^{VI} reduction in the rhizosphere prior to uptake as an additional detoxification mechanism. Sulphate promoted uptake of Cr^{VI} through the active pathway, although increases in SO₄²⁻ concentration did not yield a proportional increase in Cr symplastic solution concentration; Cr^{III} was also the dominant species in these root solutions.

Conclusion

The results indicate that *Spinacia oleracea* plants can effectively reduce Cr^{VI} to Cr^{III} and that the uptake pathways for both Cr^{III} and Cr^{VI} are more complex than previously reported. Further work is required to understand the physiological processes that result in the reduction of Cr^{VI} prior to, and during, uptake. The efficacy of sulphate to augment existing agricultural management strategies, such as liming and organic reincorporation, also requires further investigation to establish suitable application rates and applicability to other environmental contaminants.

Introduction

Chromium (Cr) is an element with a long history of research concerning how its chemical species behave in soil-water-plant systems. This is due to its ability to exist as both non-toxic Cr^{III} and toxic Cr^{VI} species depending on environmental conditions such as pH, organic matter content and metal oxide concentration (Gomes et al., 2017), as well as anthropogenic activities such as chrome plating and leather tanning (Séby and Vacchina, 2018). Trivalent Cr^{III} is considered to be an essential micronutrient for glucose and lipid metabolism, which has led to its widespread use as a weight-loss supplement (Lukaski et al., 2007). Conversely, Cr^{VI} is toxic and a known carcinogen through inhalation (Nickens et al., 2010), and is more mobile and bioavailable in soil-water systems than Cr^{III} (Chai et al., 2019). Trivalent Cr^{III} can be oxidised to Cr^{VI} in the presence of manganese oxide, whilst Cr^{VI} is reduced to Cr^{III} by soil organic matter (Jobby et al., 2018).

Plants have no known requirement for Cr; both Cr^{III} and Cr^{VI} are considered phytotoxic and promote the formation of reactive oxygen species (ROS), leading to oxidative stress which causes damage to DNA, proteins and pigments (Choudhury and Panda, 2005). The majority of Cr in plants is transferred through carrier proteins that are

specific to essential ions for plant metabolism (Huang et al., 2018). The dominant species of Cr in plants is Cr^{III} (Zayed et al., 1998a), which has been shown to concentrate in, and on, the roots of plants in a variety of different forms, including acetate (Bluskov et al., 2005b) and hydroxide (Howe et al., 2003). These processes act as detoxification steps to either immobilise the Cr or aid its transfer to other plant compartments to mitigate potential damage. Transfer of Cr^{III} is a passive process that expends no energy (Shanker et al., 2005), whereas transfer of Cr^{VI} into the plant is an active process that occurs primarily through sulphate (SO₄²⁻) transporters due to the structural similarities of SO₄²⁻ and Cr^{VI} as chromate (CrO₄²⁻) (de Oliveira et al., 2016). The activation of phosphate-specific transporters in the presence of Cr^{VI} has also been reported in *Arabidopsis thaliana* seedlings, with an increase in phosphate leading to a decrease in the uptake of Cr^{VI} (López-Bucio et al., 2014). Currently there are no European Union legislated maximum permissible levels for either species of Cr in plant-derived foods (Séby and Vacchina, 2018).

Despite the current understanding of Cr transport pathways, there is still a lack of clarity concerning the mechanisms by which Cr species are transferred, compartmentalised and detoxified in plants (Shahid et al., 2017), and the species interconversions that may occur before and during transfer. In addition, recent work (Fang et al., 2014) demonstrating the genotoxicity of Cr^{III} suggests that, if plants are reducing Cr^{VI} to Cr^{III} and there is sufficient translocation to edible plant compartments, these plants could still pose a human health risk through consumption (Hamilton et al., 2018, Kaninga et al., 2019).

Therefore, the aim of this work was to elucidate the pathways through which Cr^{III} and Cr^{VI} are transferred from solution into plants, with the following objectives:

- (i) assess the mechanisms of transfer of Cr^{III} and Cr^{VI} into plants using stable isotope species as tracers and the extraction of apoplastic and symplastic root solutions;
- (ii) evaluate the species interconversions that occur before and after uptake;
- (iii) assess the effect of SO_4^{2-} , considered a competing ion for Cr^{VI} uptake, on these mechanisms.

Materials and Methods

Preparation of $^{50}\text{Cr}^{\text{III}}$ and $^{53}\text{Cr}^{\text{VI}}$ stock solutions

Isotopically enriched $^{50}\text{Cr}^{\text{III}}$ and $^{53}\text{Cr}^{\text{VI}}$ stock solutions were prepared from the high purity metal and oxide materials respectively (ISOFLEX, USA), according to previously outlined methods (EPA, 2008). Briefly, the $^{50}\text{Cr}^{\text{III}}$ stock solution was prepared by dissolving the metal in dilute HCl (Romil, UK) and heating to near-dryness before reconstitution in dilute HNO_3 (Romil, UK). The $^{53}\text{Cr}^{\text{VI}}$ was prepared by dissolving the oxide in concentrated HClO_4 (Romil, UK) and oxidising the solution with concentrated H_2O_2 (Romil, UK) and NH_4OH (Sigma Aldrich, UK). The solution was heated to near-dryness to ensure removal of H_2O_2 prior to transfer and dilution. The concentration of Cr^{III} and Cr^{VI} in the solutions were determined by reverse isotope dilution mass spectrometry (reverse IDMS) (Trešl and Quétel, 2005), and were $226 \pm 0.5 \text{ mg L}^{-1}$ and $41 \pm 0.2 \text{ mg L}^{-1}$ respectively. The pH of the stock solutions, measured using a solid body combined pH electrode (Thermo Scientific, UK), were 1.1 ± 0.1 for $^{50}\text{Cr}^{\text{III}}$ and 4.5 ± 0.2 for $^{53}\text{Cr}^{\text{VI}}$ ($n = 3$).

Preparation of plant materials

Plants were grown at the Sutton Bonington glasshouses at The University of Nottingham. To ensure sufficient sample mass, 32 plants per treatment replicate were used with each treatment repeated in triplicate. Spinach (*Spinacia oleracea*) was chosen for ease of handling and turnaround time from sowing to maturity. Germinated spinach seeds were transferred to cell trays filled with silver sand, to minimise Cr backgrounds in harvested plants, and watered daily with a nutrient-enriched water solution (HortiMix Standard; NPK % 15:7:30, Hortifeed, Lincoln, UK). Following harvesting, plants were transferred to the Inorganic Geochemistry laboratories at the British Geological Survey and triple-rinsed with DDW to remove residual silver sand from roots and leaves prior to treatment.

Cr^{III} and Cr^{VI} transfer mechanisms

Twenty-one days after planting (DAP), spinach plants were harvested and triple-rinsed with DDW. Plant roots were exposed to treatment solutions for 24 hr, containing either ⁵⁰Cr^{III}, ⁵³Cr^{VI} or a combination of both species at a nominal concentration of 250, 500 or 1000 µg L⁻¹. Due to time constraints the reverse IDMS was undertaken on the ⁵⁰Cr^{III} stock solution after the treatment solutions had been prepared; therefore, the actual treatment levels for Cr^{III} were 282, 565 and 1130 µg L⁻¹. Root solutions were extracted through centrifugation using a method outlined by Humphrey *et al.* (Humphrey *et al.*, 2019c). Briefly, washed root tips (n = 32 per treatment replicate, 3 replicates) were removed from plants, placed cut-surface down into Spin-X Centrifuge Tubes containing 0.22 µm cellulose acetate filters (Corning, USA) and centrifuged at 2000 g for 15 minutes to extract the apoplastic (through cell) solution. Root tips were then frozen for 24 hr, thawed and centrifuged again at 2000 g for 15 minutes to extract the symplastic (through cytoplasm) solution. Root solutions were stored frozen prior

to Cr speciation analysis. The potential for retention of Cr species on the tube filter material was tested by centrifuging 5 separate aliquots of a solution containing both Cr^{III} and Cr^{VI} at $100 \mu\text{g L}^{-1}$; percentage recovery (measured species concentration divided by nominal concentration, multiplied by 100) was $99 \pm 1.5 \%$ and $97 \pm 1.5 \%$ for Cr^{III} and Cr^{VI} respectively, indicating no loss of either species of Cr during filtering.

Influence of sulphate on Cr^{VI} uptake mechanisms

Growth and processing of plant samples followed the same steps as previously described, with the exception of the treatment solutions used. Following harvest, plants were exposed to treatment solutions containing a fixed concentration of $^{53}\text{Cr}^{\text{VI}}$ ($500 \mu\text{g L}^{-1}$) with the addition of 150 or 250 mg L^{-1} sulphur added as ammonium sulphate ($(\text{NH}_4)_2\text{SO}_4$, Fisher Scientific, UK); the duration of exposure (24 hr) was the same for both concentrations of SO_4^{2-} . Ammonium sulphate was chosen over the more commonly used sodium sulphate to improve plasma robustness and reduce the likelihood of matrix suppression during analysis (Tan and Horlick, 1987). A lower SO_4^{2-} treatment concentration of 50 mg L^{-1} was attempted but only one replicate yielded enough solution for analysis and this treatment was therefore discounted. Due to previously reported competition between Cr^{VI} and SO_4^{2-} (Kaszycki et al., 2005), Cr^{VI} was the only species used; in addition, the acidic matrix of the $^{50}\text{Cr}^{\text{III}}$ stock solution could contribute to the reduction of the $^{53}\text{Cr}^{\text{VI}}$ when the two species are present in the same solution.

Instrumentation and Data Analysis

Speciation of Cr^{III} and Cr^{VI} was achieved using an Agilent 1260 Infinity II Bio-Inert Liquid Chromatography (HPLC) system (Agilent Technologies, Tokyo, Japan) equipped with a $100 \mu\text{l}$ injection loop, alongside a PRP-X100 anion exchange column ($250 \text{ mm} \times 4.6 \text{ mm} \times 5 \mu\text{m}$) (Hamilton Company, USA). The column was connected directly to the

nebuliser of an Agilent 8900 inductively coupled plasma triple quadrupole mass spectrometer (ICP-QQQ) using a single piece of 1/16" OD PEEK tubing. The ICP-QQQ instrument was optimised prior to analysis using a 1 µg L⁻¹ tuning solution, and operated in collision cell mode using helium (He) gas at a flow rate of 5.1 ml min⁻¹ to reduce the impact of polyatomic interferences (e.g. ³²S¹⁸O⁺, ³⁷Cl¹⁶O⁺) on *m/z* 50 and 53 (Hamilton et al., 2020a). The separation of Cr^{III} and Cr^{VI} was undertaken in 7.5 min using an isocratic elution programme (100% 40 mM ammonium nitrate (NH₄NO₃)/50 mM TRIS Buffer/5 mM ethylenediaminetetraacetic acid (di-ammonium salt, NH₄-EDTA), pH 7.0, 1.2 ml min⁻¹ flow rate). Prior to injection, the samples were diluted with 50 mM NH₄-EDTA (pH 10.0) and heated in an oven at 70°C for 1 hr to derivatize Cr^{III} for retention on the anion exchange column (Gürleyük and Wallschläger, 2001). Compound Independent Calibration (CIC) in Agilent's MassHunter software was used to quantify the ⁵⁰Cr^{VI} and ⁵³Cr^{III} present in the samples from the peak areas of their respective isotopic calibration standards (⁵⁰Cr^{III} and ⁵³Cr^{VI}). The correlation coefficients (R²) for ⁵⁰Cr^{III} and ⁵³Cr^{VI} calibration curves were 1.0000 and 0.9998 respectively. Quality control check standards (n = 6), containing ⁵⁰Cr^{III} and ⁵³Cr^{VI} at 5 µg L⁻¹, were analysed at the start and end of each analytical run and after no more than 10 samples (⁵⁰Cr^{III} recovery was 95%; ⁵³Cr^{VI} recovery was 95%).

Statistical analyses were undertaken using Microsoft® Excel® 2016 (version 16.0.4927.1000) alongside the "SPC For Excel" statistical analysis package. Significant differences between means were assessed using Student's t-test. Differences in medians between groups were assessed using Kruskal-Wallis Test with Pairwise Comparison. Results were considered to be statistically significant where P < 0.05 and are expressed as the mean of treatment replicates (n = 3) ± standard deviation (SD).

Results and Discussion

Analytical figures of merit

The limits of detection (LOD) for $^{50}\text{Cr}^{\text{III}}$ and $^{53}\text{Cr}^{\text{VI}}$, calculated as three times the standard deviation of ten analytical blanks (processed in the same way as samples in section 2.5), were $0.04\ \mu\text{g L}^{-1}$ and $0.03\ \mu\text{g L}^{-1}$ respectively. An example chromatogram at m/z 50 and 53 is shown in Fig. 5.1.

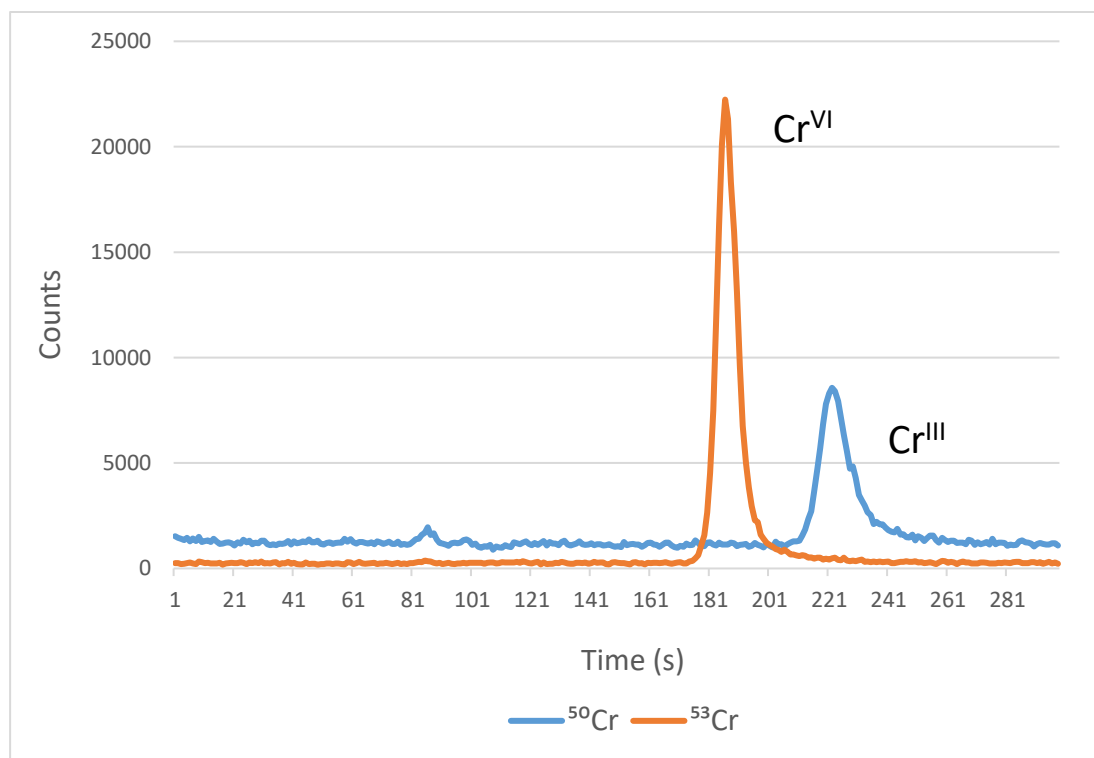


Figure 5.1. Speciation of Cr^{III} and Cr^{VI} , at m/z 50 and 53, in a $5\ \mu\text{g L}^{-1}$ quality control solution.

Cr^{III} and Cr^{VI} interconversions in treatment solutions

Treatment solutions ($^{50}\text{Cr}^{\text{III}}$ and $^{53}\text{Cr}^{\text{VI}}$ at nominal 250, 500 and $1000\ \mu\text{g L}^{-1}$, actual $^{50}\text{Cr}^{\text{III}}$ concentrations of 282, 565 and $1130\ \mu\text{g L}^{-1}$) used for accumulation of $^{50}\text{Cr}^{\text{III}}$ and $^{53}\text{Cr}^{\text{VI}}$ in *Spinacia oleracea* were subjected to speciation analysis to confirm the concentrations of the Cr species in the initial treatments and to investigate how the presence of the plant roots changed the speciation over the course of the treatment

period (Fig. 5.2). The pH of the treatment solutions were 3.6 ± 0.2 ($250 \mu\text{g L}^{-1}$), 3.3 ± 0.1 ($500 \mu\text{g L}^{-1}$) and 3.0 ± 0.2 ($1000 \mu\text{g L}^{-1}$) ($n = 3$).

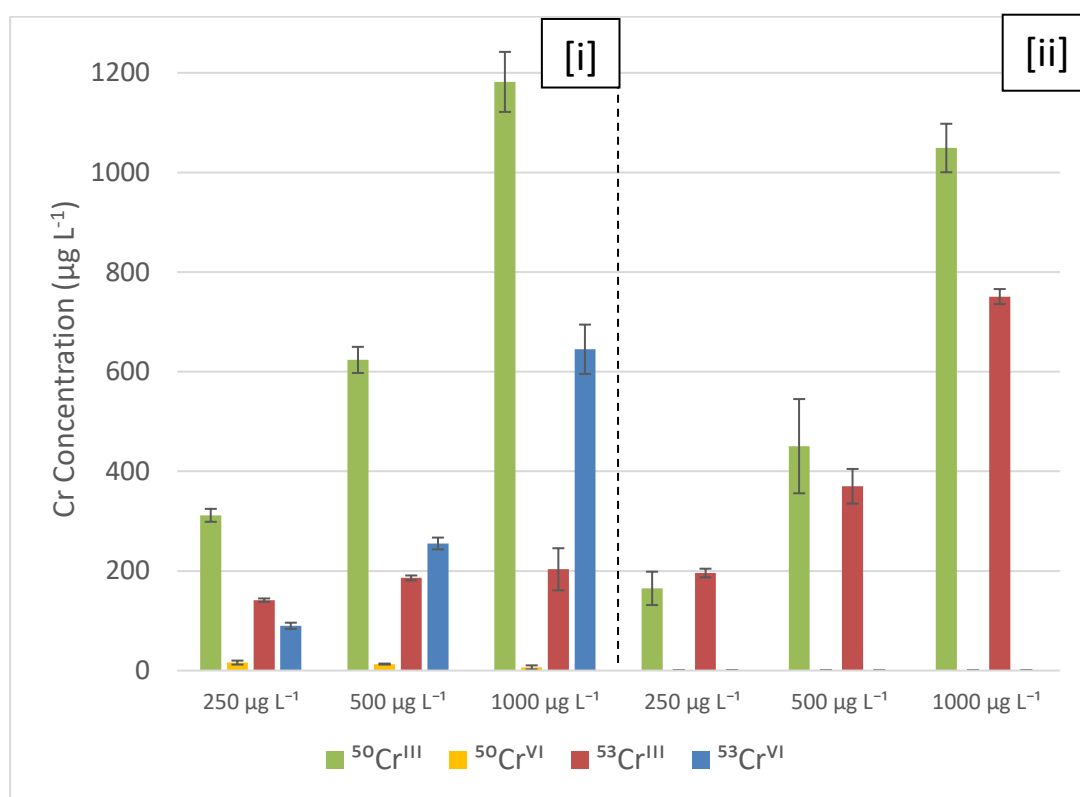


Figure 5.2. Species distributions for treatment solutions (both $^{50}\text{Cr}^{\text{III}}$ and $^{53}\text{Cr}^{\text{VI}}$) at different nominal concentrations after a 24 hr treatment period. [i] Treatment solutions were not exposed to plant roots. [ii] Treatment solutions contained plant roots. Values expressed as mean $\pm$ SD ($n = 3$).

There was no significant oxidation (5 % conversion to $^{50}\text{Cr}^{\text{VI}}$, yellow bar in Fig. 5.2) of the $^{50}\text{Cr}^{\text{III}}$ spike over the treatment period. Due to the pH of the treatment solutions, it is unlikely that this oxidation could be attributed to the presence of trace H_2O_2 following preparation of the $^{53}\text{Cr}^{\text{VI}}$ solution (Novotnik et al., 2012). In addition, the presence of the plant roots (Fig. 5.2 [ii]) did not influence the $^{50}\text{Cr}^{\text{III}}$ speciation. The decrease in $^{50}\text{Cr}^{\text{III}}$ concentration between [i] and [ii] in Fig. 5.2 can be attributed to transfer of $^{50}\text{Cr}^{\text{III}}$; the $^{50}\text{Cr}^{\text{VI}}$ formed over the course of the treatment was also transferred.

Due to the acidic matrix of the $^{50}\text{Cr}^{\text{III}}$ stock solution ($\text{pH } 1.1 \pm 0.1$), there existed the potential for an increase in Cr^{VI} reduction when both species were used in the same treatment solution. Therefore, additional plant treatment experiments were also undertaken using only $^{53}\text{Cr}^{\text{VI}}$ at $500 \mu\text{g L}^{-1}$ to examine this artefact (Fig. 5.3). For reference, the pH of the treatment solution containing only $^{53}\text{Cr}^{\text{VI}}$ was 4.5 ± 0.2 ($n = 3$).

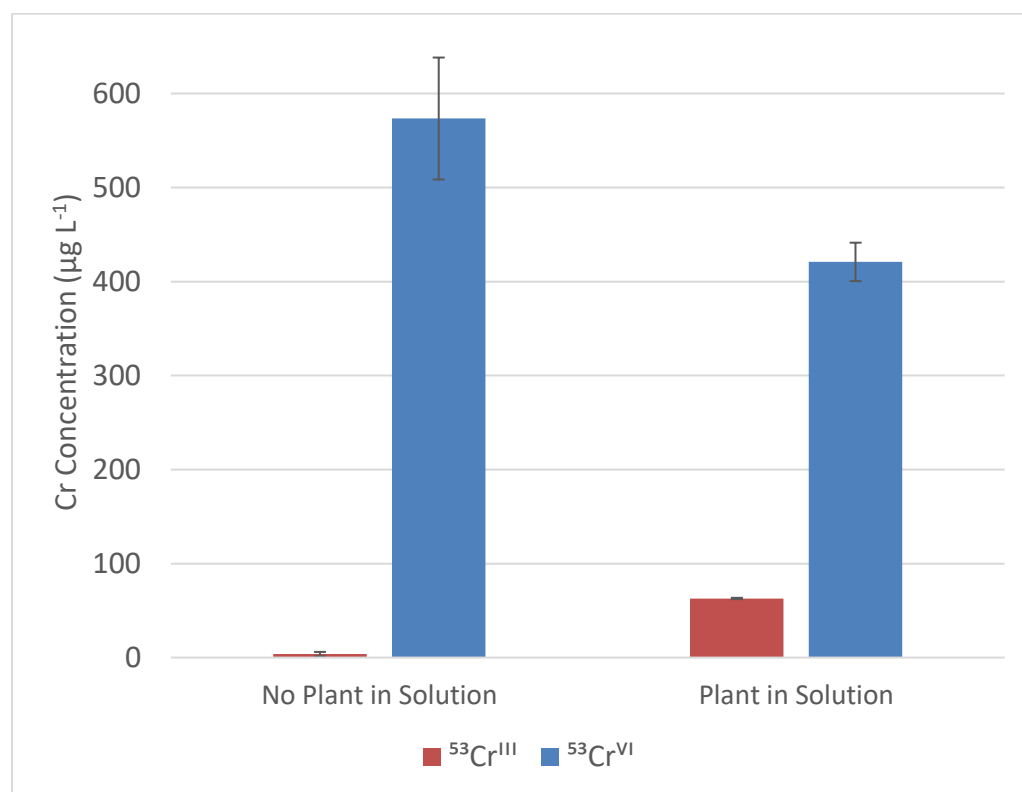


Figure 5.3. Species distribution for treatment solutions containing only $^{53}\text{Cr}^{\text{VI}}$ at $500 \mu\text{g L}^{-1}$ after a 24 hr treatment period. Values expressed as mean $\pm$ SD ($n = 3$).

In the case of the added $^{53}\text{Cr}^{\text{VI}}$, there was evidence of reduction to $^{53}\text{Cr}^{\text{III}}$ over the treatment period (presence of red bars in Figs 5.2 and 5.3). In the “ $^{53}\text{Cr}^{\text{VI}}$ only” treatment (Fig. 5.3), 13 % of the added $^{53}\text{Cr}^{\text{VI}}$ was reduced to Cr^{III} ; when both $^{50}\text{Cr}^{\text{III}}$ and $^{53}\text{Cr}^{\text{VI}}$ were present in the same solution, the extent of $^{53}\text{Cr}^{\text{VI}}$ reduction increased to between 20 and 60 % and was dependent on the $^{53}\text{Cr}^{\text{VI}}$ concentration added. This can be attributed to the acidic matrix of the $^{50}\text{Cr}^{\text{III}}$ stock solution ($\text{pH } 1.1 \pm 0.1$) contributing to increased reduction of $^{53}\text{Cr}^{\text{VI}}$, and/or the exudation of organic acids leading to

reduction of Cr^{VI} (Srivastava et al., 1999) and subsequent complexation as a labile Cr^{III} organic complex (Srivastava et al., 1998). Novotnik *et al.* (2012) previously reported artefactual reduction of Cr^{VI} due to the presence of trace H_2O_2 , commonly used during the preparation of spiking solutions to ensure complete oxidation of isotopically-enriched starting material (Novotnik et al., 2012). However, the highest $^{53}\text{Cr}^{\text{III}}$ concentrations were measured in the treatment solutions containing both species of Cr (red bars in Fig. 5.2 [i]), indicating no significant levels of H_2O_2 remained in the spiking solution prior to preparation of the treatment solutions. Reduction of Cr^{VI} was therefore a function of the lower pH from the $^{50}\text{Cr}^{\text{III}}$ solution and/or the root organic acids.

The presence of the plant roots can be seen to cause almost complete reduction of $^{53}\text{Cr}^{\text{VI}}$ (no blue bars in [ii] of Fig. 5.2), which is possibly indicative of a detoxification step prior to uptake to mitigate the phytotoxicity of Cr^{VI} . Reduction of Cr^{VI} within the rhizosphere has been observed before (Pradas del Real et al., 2014a), although the mechanisms are still unclear; as mentioned previously, it is thought that the low pH and presence of organic carbon compounds in the rhizosphere may promote reduction to Cr^{III} (Bluskov et al., 2005b, Zeng et al., 2008).

Cr^{III} and Cr^{VI} transfer mechanisms at 200, 500 and 1000 µg L⁻¹ Cr^{III} and Cr^{VI}

Species distribution of Cr^{III} and Cr^{VI} in apoplastic and symplastic root solutions are shown in Fig. 5.4 [i] and [ii] respectively.

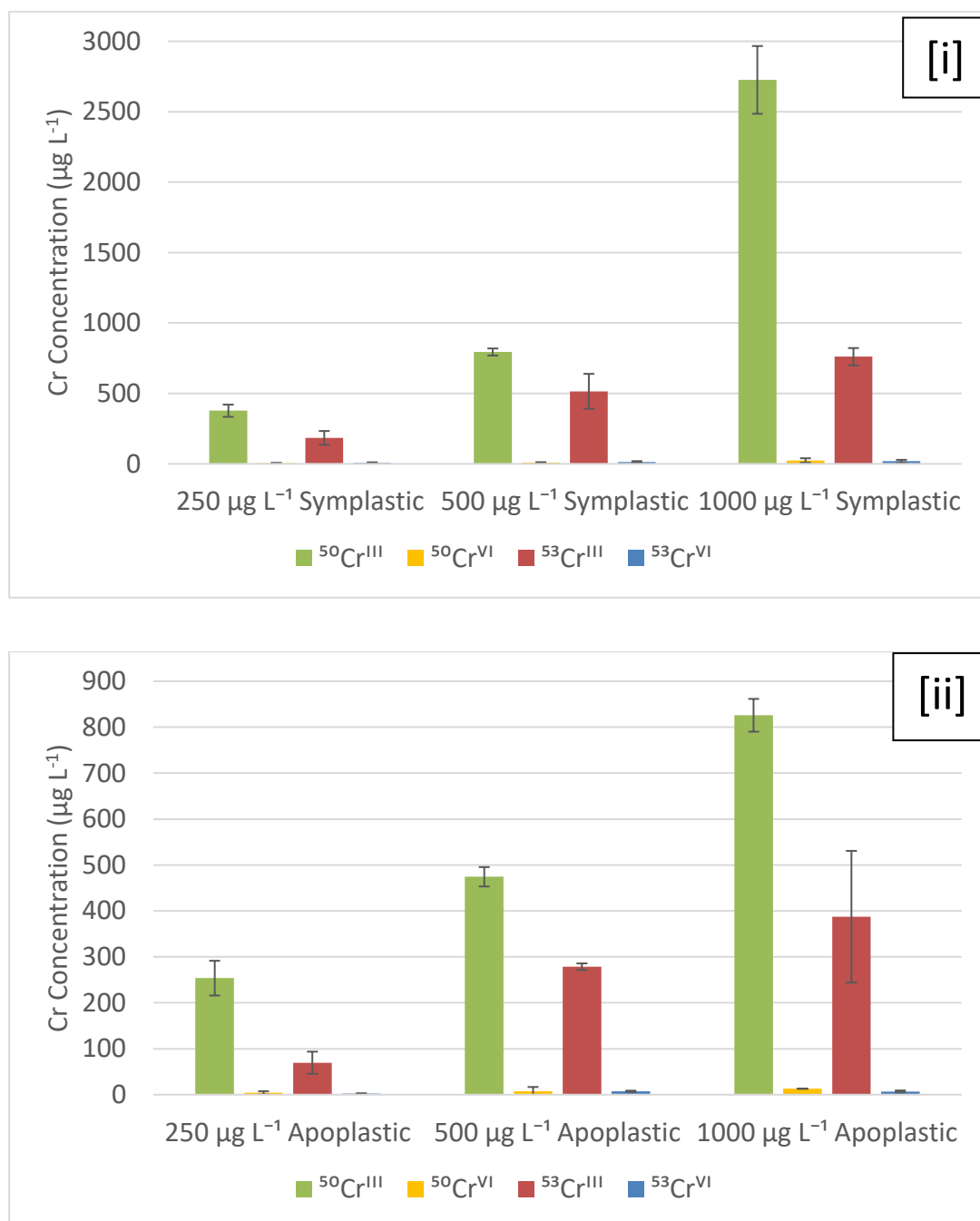


Figure 5.4. Speciation of chromium in [i] apoplastic and [ii] symplastic root solutions exposed to ⁵⁰Cr^{III} at 282, 565 and 1130 µg L⁻¹ and ⁵³Cr^{VI} at 250, 500 and 1000 µg L⁻¹ for 24 hr. Values expressed as mean ± SD (n = 3).

Quantitative mass balance of the system was difficult to achieve because root solutions from a number of plants undergoing the same treatment had to be combined to produce sufficient sample volume for analysis. This was further compounded by evidence of preconcentration in the symplastic solutions: due to the smaller volume extracted through centrifugation relative to the volume of treatment solution that was transferred by the plant during the experiment, the concentrations measured in the root solutions were higher than the supplied treatment concentration.

In both apoplastic and symplastic root solutions, the dominant measured species was Cr^{III} irrespective of the initial treatment solution speciation. The reduction of Cr^{VI} to Cr^{III} during plant uptake has been observed on numerous occasions (Cervantes et al., 2001) and is believed to be an active process occurring either through Fe^{III} reductase enzymes (Han et al., 2004) or interaction with organic matter in the vacuole (Vázquez et al., 1987). Although present in chromium-resistant bacteria (Thatoi et al., 2014), chromium reductase enzymes have yet to be identified in plants (Lytle et al., 1998). In this experiment, $^{50}\text{Cr}^{\text{III}}$ was transferred both actively and passively with no evidence of significant oxidation to Cr^{VI} . As also seen in the species distribution in the treatment solutions, the presence of $^{53}\text{Cr}^{\text{III}}$ was indicative of Cr^{VI} reduction prior to or following uptake, with evidence of active and passive transfer. It can be inferred that the uptake mechanisms for Cr^{III} and Cr^{VI} are more complex than previously thought, as both species display active and passive uptake pathways that follow a linear trend with increasing concentration.

Impact of Sulphate on Cr^{VI} Transfer Mechanisms

Due to the reduction artefacts highlighted in section 3.1, all experiments assessing the impact of SO₄²⁻ on Cr species plant uptake used ⁵³Cr^{VI} only at a fixed concentration of 500 µg L⁻¹ (Fig. 5.5).

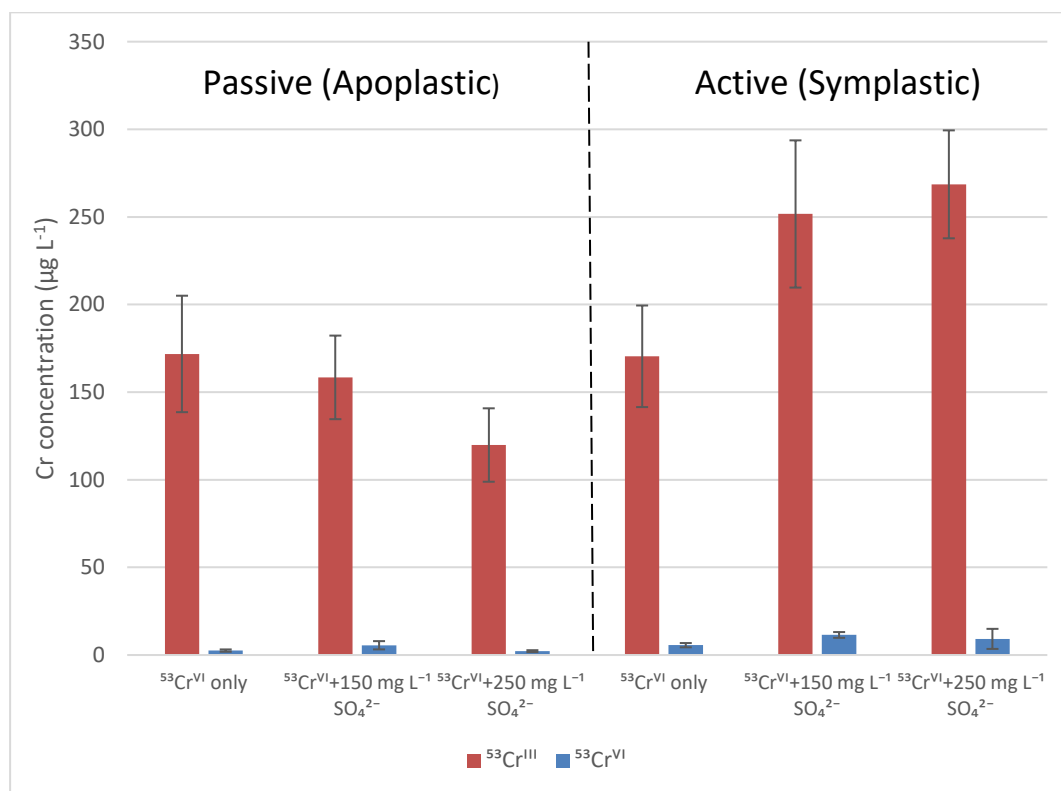


Figure 5.5. ⁵³Cr^{III} and Cr^{VI} concentrations in apoplastic and symplastic root solutions exposed to 500 µg L⁻¹ ⁵³Cr^{VI} only, 500 µg L⁻¹ ⁵³Cr^{VI} + 150 mg L⁻¹ SO₄²⁻ and 500 µg L⁻¹ ⁵³Cr^{VI} + 250 mg L⁻¹ SO₄²⁻ for 24 hr. Values expressed as mean ± SD (n = 3).

There was no trend of increasing ⁵³Cr^{III} in the symplastic solutions with increasing SO₄²⁻ as confirmed by Kruskal-Wallis test (P = 0.39). The concentration of SO₄²⁻ in the root solutions did not increase with increasing SO₄²⁻ treatment and did not differ significantly from background SO₄²⁻ in the control (data not included), indicating no promotion of SO₄²⁻ in the presence of Cr^{VI}.

There was a significant difference between the apoplastic and symplastic means ($P = 0.03$ for $150 \text{ mg L}^{-1} \text{ SO}_4^{2-}$, $P = 0.003$ for $250 \text{ mg L}^{-1} \text{ SO}_4^{2-}$), suggesting that the presence of SO_4^{2-} results in increased active transfer of Cr^{VI} . Previous studies have yet to reach a consensus on the impact of SO_4^{2-} on the extent of Cr^{VI} transfer; de Oliveira *et al.* (2016) investigated the effects of Cr^{VI} and SO_4^{2-} on uptake and translocation of both ions within the fern species *Pteris vittata*, a hyperaccumulator of arsenic (As), and demonstrated both an increase in Cr root concentrations and subsequent reduction of Cr^{VI} to Cr^{III} with increasing SO_4^{2-} concentration (de Oliveira *et al.*, 2016). Conversely, Ding *et al.* (2019) showed that sulphur (S, supplied as sodium sulphate) reduced the enrichment of Cr in *Arabidopsis thaliana* roots (Ding *et al.*, 2019). Despite this disparity, all previous work has concluded that the presence of S does mitigate the phytotoxicity of Cr^{VI} . Ding *et al.* (2019) observed a reduction in formation of malondialdehyde- a common marker of oxidative stress- with the addition of S, whilst Wu *et al.* (2018) suggest that S metabolites such as cysteine and reduced glutathione can reduce oxidative stress that may result from the formation of ROS during reduction of Cr^{VI} (Kováčik *et al.*, 2014, Wu *et al.*, 2018). In addition, Singh *et al.* (2017) proposed that the combination of Cr^{VI} and S increases the activity of S-assimilating enzymes and therefore protects the plant from Cr^{VI} toxicity (Singh *et al.*, 2017). This study provides evidence for the promotion of Cr uptake through an active pathway in the presence of SO_4^{2-} , although it still remains unknown whether this is due to the oxidative stress relief processes previously outlined or an additional mechanism.

Conclusions

The isolation of apoplastic and symplastic root solutions using the technique described in this article provides a powerful, inexpensive and easily adaptable tool to investigate plant uptake mechanisms. Although this technique is limited by its inability to achieve

full system stoichiometry, it is capable of elucidating basic transfer pathways and the species interconversions that occur throughout the process. In *Spinacia oleracea*, Cr^{III} and Cr^{VI} were shown to be transferred both actively (through the symplast) and passively (through the apoplast), with Cr^{III} being the dominant species following transfer. In the presence of SO₄²⁻, there was a higher concentration of reduced Cr^{VI} in the active pathway root solutions relative to the corresponding passive pathway solution. However, increases in SO₄²⁻ concentration did not result in statistically significant increases or decreases in reduced Cr^{VI} concentration within individual pathway solutions. Further investigation is required to understand both the cause and location of this reduction process. This suggests that SO₄²⁻ is promoting active transfer of Cr^{VI} as opposed to competing with Cr^{VI} uptake. The results also suggest two distinct detoxification pathways- prior to transfer in the rhizosphere and post-uptake in the root solutions- although additional experimental and analytical work (e.g. longer treatment duration, use of a metabolic inhibitor, gene expression, measurement of oxidative stress response) is needed to understand the physiological reasons for these reduction steps.

Further analytical work includes the development of solution-based extraction and analytical methods to assess how Cr^{III} and Cr^{VI} bind to organic ligands, such as acetate and oxalate, in plants. Currently the predominant techniques for achieving this are μ -XRF and XANES, which require specialist access to instrumental facilities. Development of a solution-based method would allow for a more accessible way to evaluate ligand complexation with inorganic species, helping to mitigate the limitations in progress highlighted by recent reviews of elemental speciation (Ardini et al., 2020). The use of a passive sampling technique with higher temporal resolution (e.g. microdialysis) would also improve understanding of the kinetics of time-sensitive interconversions such as those seen in this work.

Chapter 6. Online microdialysis-high performance liquid chromatography-mass spectrometry (MD-HPLC-ICP-MS) as a novel tool for sampling hexavalent chromium in soil solution

Paper as submitted to *Environmental Science and Technology*

Author list

Hamilton EM, Young SD, Bailey EH, Humphrey OS and Watts MJ

Author contribution

Study conceived by EMH, SDY, EHB, OHS and MJW

Experiments and analysis performed by EMH

All tables and figures produced by EMH

Construction of the paper by EMH

All authors were involved in the study and manuscript development

Abstract

Conventional soil solution sampling of species-sensitive inorganic contaminants, such as hexavalent chromium (Cr^{VI}), may induce interconversions due to disruption of system equilibrium. The temporal resolution that these sampling methods afford may also be insufficient, or require time-consuming and expensive analysis. Microdialysis (MD) is emerging as a minimally invasive passive sampling method in soil science, permitting the determination of solute fluxes and concentrations at previously unobtainable spatial scales and timeframes. This article presents the first use of MD coupled to HPLC-ICP-MS for the continuous sampling and simultaneous detection of isotopically-enriched Cr^{VI} added to soil. The performance criteria of the system were assessed using stirred solutions; good repeatability of measurement ($\text{RSD} < 2.5\%$) was obtained for Cr^{VI} , with a detection limit of $0.3 \mu\text{g L}^{-1}$. The online MD-HPLC-ICP-MS setup was applied to the sampling of isotopically-labelled Cr^{VI} , using two methods of addition (homogenized mixing and direct injection) to examine methodological biases. The system sampled and analyzed fresh soil solution at 15 minute intervals, offering improved temporal resolution and a significant reduction in analysis time. Simple modifications to the chromatographic conditions could resolve additional analytes, offering a powerful tool for the study of solute fluxes in soil systems to inform the study of nutrient availability or soil-to-plant transfer of potentially harmful elements.

Introduction

The separation and quantification of trivalent (Cr^{III}) and hexavalent (Cr^{VI}) chromium (Cr) in soil is a burgeoning area of research motivated by the significant differences in toxicity and mobility between the two oxidation states (Li et al., 2016). Due to the negative charge of its compounds, typically chromate (CrO_4^{2-}) and dichromate ($\text{Cr}_2\text{O}_7^{2-}$) (Deng et al., 2020), Cr^{VI} is more mobile and bioavailable in soil-water systems than

Cr^{III} (Costa, 2003), and is therefore more likely to be transferred from contaminated soil to drainage water and into plants (Shanker et al., 2009). The measurement of total Cr^{VI} in solid matrices presents a metrological challenge due to the potential for species interconversions during extraction and analysis (Hamilton et al., 2018), which is further compounded when measuring changes in the bioavailable pool of Cr^{VI} in soil-pore water systems. This usually involves specialized extractions and/or separation steps which not only cause significant disruption to the equilibrium of the system (Matern and Mansfeldt, 2016), but also produce large numbers of samples which are more susceptible to artefactual errors.

The kinetics of Cr species interconversions in soil-pore water systems are of particular importance when there is the potential for transportation of Cr^{VI} into groundwater and/or sediment systems. (Hellerich and Nikolaidis, 2005). Attenuation of Cr in these systems is dependent on the physiochemical properties of the water/soil and can be attributed to the formation of Cr^{III} following reduction of Cr^{VI} . Trivalent Cr is significantly limited in solubility due to its adsorption to mineral phases or co-precipitation with iron (Fe) (oxy-)hydroxides (Ball and Nordstrom, 1998). The removal of Cr^{VI} in soil-pore water systems is a slower process than for Cr^{III} , resulting in order-of-magnitude lower partition coefficients (K_d) (Hassan and Garrison, 1996). However, this process is accelerated through decreases in pH and increases in concentrations of soil organic carbon (SOC) and reducing inorganic components such as Fe(II) and sulfides (Palumbo-Roe et al., 2017). Previously, the exchange kinetics between soil solution and mineral phases have been measured using diffusive gradients in thin-films (DGT) (Ernstberger et al., 2005). This passive sampling technique involves the chelation of labile analytes on a resin implanted onto moist soil, causing depletion around the DGT device and a shift in system equilibrium to resupply the soil solution from the solid phase (Harper et al., 1998). This allows for the measurement of a range

of kinetic parameters, including distribution coefficients (K_{dl}), remobilization fluxes and adsorption/desorption rate constants (Ernstberger et al., 2002). Despite its advantages, the technique suffers from spatial limitations (a large sampling area, of the order of cm^2 , is required for successful device deployment), induces significant disruption to the equilibrium of the sampled soil and requires time-consuming offline sample preparation and analysis (Dufka and Dočekal, 2018). Depending on the temporal resolution required, a number of devices may need to be deployed which adds to the processing time, analytical requirements and overall cost (Almås et al., 2017).

Microdialysis (MD) is another passive sampling technique that has been garnering increasing interest within the field of soil science (Shaw et al., 2014, Humphrey et al., 2019a, Buckley et al., 2019, Inselsbacher et al., 2011) due to its high spatial and temporal resolution, and its preservation of the *in situ* dynamics of the system undergoing sampling (Buckley et al., 2020). Microdialysis uses a probe containing a semipermeable membrane with a specific molecular weight cut off (MWCO); the pumping of a perfusate solution into the probe creates a diffusion gradient within the sampled medium causing solutes to diffuse across the membrane (Miró and Frenzel, 2005). The solution exiting the probe (dialysate), containing the sampled solutes, can then be analyzed using a suitable analytical technique (Korf et al., 2010). The minimal disruption to the soil, coupled with the ability of the technique to sample soil solution at representative water contents ($\sim 50\%$ water holding capacity (%WHC) and higher) (Miró et al., 2010) makes MD a very attractive tool to increase understanding of small-scale inorganic solute availability in soil.

The majority of MD sampling is undertaken offline through the collection of discrete samples over varying timescales (typically in the order of minutes to hours) (Buckley

et al., 2020), although recent articles have demonstrated the potential for hyphenating MD with analytical detectors such as electrothermal atomic absorption spectrometry (ETAAS) (Miró et al., 2005) and high performance liquid chromatography (HPLC) (Jen et al., 2001). Online MD sampling and simultaneous analysis, depending on the analytical technique being used, has the potential to overcome one of the biggest compromises in MD sampling- relative recovery (RR) versus perfusate flow rate (Kho et al., 2017). The RR of a system can be defined as the ratio of the solute concentration in the dialysate to the solute concentration in the medium undergoing MD sampling, and is a function of the resistances that impede solute transport imposed by the external environment (R_{ext}), the MD probe membrane (R_m), the dialysate (R_d) and the perfusate flow rate (Q_p) (Bungay et al., 1990). Therefore the lower the Q_p , the greater the likelihood of reaching a steady-state between the solute concentration in the external solution and the probe membrane, leading to RR values close to 100 % depending on the analyte being studied (Miró and Frenzel, 2004). However, flow rates less than $5 \mu\text{L min}^{-1}$ are not practical for the majority of MD applications due to the increased time required to collect sufficient sample for analysis, and the subsequent impact on temporal resolution. Online systems, with careful optimisation of liquid handling steps, can allow for lower Q_p , increased RR and immediate analysis without adding additional time or cost constraints. The coupling of MD sampling to mass spectrometry was first conceived over 25 years ago (Deterding et al., 1992), but has seen limited application in soil since with no focus on inorganic solutes (Warren, 2018).

The aim of this study was to couple MD to HPLC-ICP-MS (henceforth referred to as online MD-HPLC-ICP-MS) for continuous passive soil solution sampling and simultaneous speciation analysis. The objectives were:

to undertake online MD calibration using stirred solutions of Cr^{VI};

- (i) to assess common performance characteristics (linearity, precision, limit of detection);
- (ii) to apply the MD-HPLC-ICP-MS method to the sampling of isotopically-enriched Cr^{VI} added to soil and;
- (iii) to understand some primary limitations and artefacts associated with the method.

Materials and Methods

Reagents

All solutions were prepared in 18.2 MΩ cm ultrapure water (DDW, Merck Millipore, UK). Natural abundance ⁵²Cr^{VI} standards were prepared through dilution of a commercially available solution (Greyhound Chromatography, UK). Isotopically-enriched ⁵³Cr^{VI} was prepared according to previously-outlined methods (Hamilton et al., 2020a, Hamilton et al., 2020b). Ethylenediaminetetraacetic acid (di-ammonium salt, NH₄-EDTA), trisaminomethane (TRIS) and ammonium nitrate (NH₄NO₃) (Sigma Aldrich, UK) were used for the preparation of the chromatographic mobile phase.

Instrumental Apparatus and Analysis

Speciation of Cr^{VI} was undertaken using a Dionex GP50 Gradient Pump (Dionex Corporation, USA) and a PRP-X100 anion exchange column (Hamilton Company, USA) coupled to an Agilent 8900 Triple Quad inductively coupled plasma mass spectrometer (ICP-MS) (Agilent Technologies, Tokyo, Japan). A Rheodyne 7125 injector/switching

valve (IDEX Corporation, USA) equipped with a 20 μL loop was used to interface the dialysate flow from the microdialysis probe with the column. The column was connected directly to the nebulizer of the ICP-MS instrument using a single piece of 1/16" OD PEEK tubing.

The speciation of Cr^{VI} was achieved through isocratic elution using a mobile phase consisting of 40 mM NH_4NO_3 , 50 mM TRIS buffer and 5 mM $\text{NH}_4\text{-EDTA}$, adjusted to pH 7.0 using concentrated nitric acid (HNO_3 , Romil, UK). Mobile phase was introduced into the injector/switching valve at a flow rate of 1.2 mL min^{-1} , resolving Cr^{VI} within 5 min (Fig. 6.1). The ICP-MS instrument was operated in collision cell mode, with the cell pressurized using helium (He) gas at a flow rate of 5.1 mL min^{-1} , to reduce the impact of polyatomic interferences on m/z 52 (e.g. $^{40}\text{Ar}^{12}\text{C}^+$).

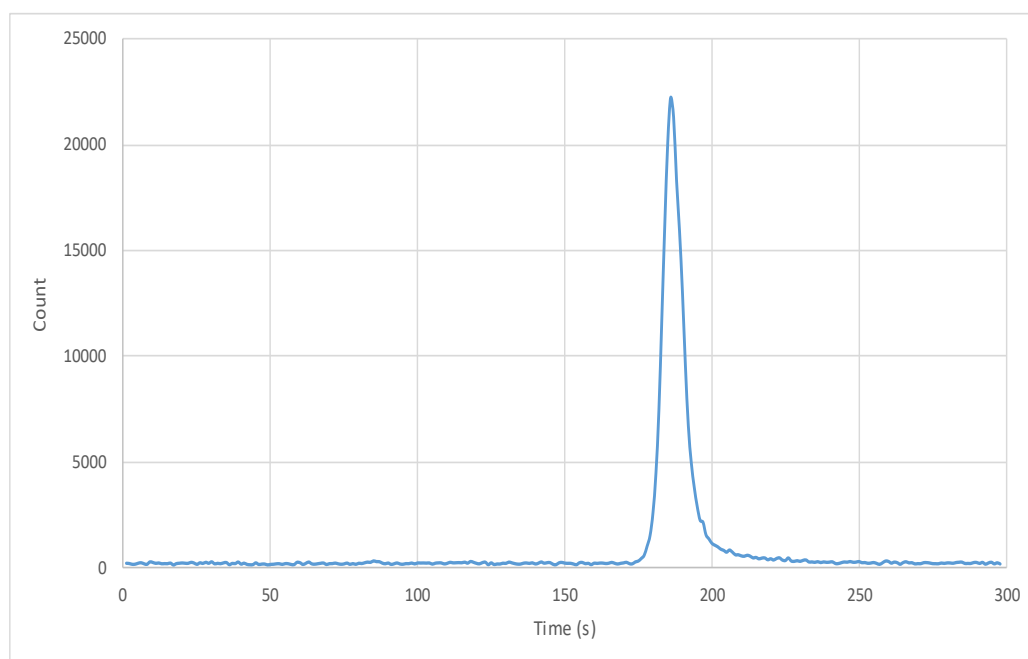


Figure 6.1. Speciation of $^{53}\text{Cr}^{\text{VI}}$ in a $5 \mu\text{g L}^{-1}$ solution.

Soil Sampling and Incubation

The collection, preparation and elemental analysis of the agricultural soil used in this study has been described previously; briefly, soil was collected from an area of cultivated land within 1 km of a mine tailing in the Copperbelt Province of Zambia (Lark et al., 2017a). The soil is allocated to the following units as classified by the FAO: chromi-haplic Acrisol with gleyi-haplic Acrisol and partly skeletal phase dystic Leptosol, with a clay-loam texture and an increasing clay content with depth (UNESCO. and FAO., 1974). Soil samples were freeze-dried and sieved to ≤ 4 mm (Hamilton et al., 2020a). Maximum percentage water holding capacity (% WHC) was determined on 50 g subsamples according to previously-outlined methods (Veihmeyer and Hendrickson, 1949). Approximately 500 g of soil was pre-incubated with DDW at 60% WHC for 5 days before being packed into 15 mL wide-mouth HDPE bottles (Nalgene, USA), henceforth referred to as microcosms, for online microdialysis sampling.

Previous articles have presented a number of different approaches for incubating soil with natural or isotopically-enriched spikes to monitor changes in the availability of solutes over time using MD (Humphrey et al., 2020, Warren, 2018). Homogenizing a bulk quantity of wetted soil with the spike ensures even distribution amongst the soil matrix, but results in a loss of early-stage temporal resolution due to the time delay between mixing, transportation of the sample and installation of MD probes for sampling (Humphrey et al., 2020). Injecting the spike directly into the packed microcosm removes this artefact, but reduces the likelihood of full dispersion of the spike within the microcosm and creates artificially-enriched pores in which the spike is the predominant medium being sampled.

To understand these artefacts, microcosms were incubated with $^{53}\text{Cr}^{\text{VI}}$ using two different isotopic spike introduction methods:

- (i) **Direct Injection.** Prior to isotopic spiking, an MD probe was implanted into the center of the microcosm through an incision hole, made using a syringe needle covered with pre-split fluorinated ethylene propylene (FEP) tubing. The MD probe was perfused at $3\ \mu\text{L}\ \text{min}^{-1}$ for 30 minutes (equivalent to two loop injections on the MD-HPLC-ICP-MS setup) to equilibrate the perfusate and microcosm. The spike ($^{53}\text{Cr}^{\text{VI}}$, $7.5\ \text{mg}\ \text{L}^{-1}$) was introduced into the soil through a 150 mm point-end needle with four radial holes, injected in $125\ \mu\text{L}$ aliquots at four points situated in a square approximately 10 mm around the MD probe. The needle was slowly extracted during injection to ensure even distribution of applied $^{53}\text{Cr}^{\text{VI}}$.
- (ii) **Homogenized Mixing.** 100 g of the 60% WHC soil was mixed with 3 mL spike ($^{53}\text{Cr}^{\text{VI}}$, $45\ \text{mg}\ \text{L}^{-1}$) using a hand blender. The isotopic spike was dispersed on the surface of the soil prior to mixing using the same needle as in (i). After mixing, the soil was packed into a microcosm and an MD probe was implanted according to (i). The time delay between mixing and probe installation was approximately 6 minutes.

Calibration of MD-HPLC-ICP-MS System in Stirred Solutions.

Prior to application of the online MD-HPLC-ICP-MS setup to soil solution sampling, the RR for a range of perfusate flow rates was calculated in stirred solutions to determine the optimum perfusate flow rate for the system. The Cr^{VI} solution ($100\ \mu\text{g}\ \text{L}^{-1}$) was perfused with distilled deionized water (DDW, $18.2\ \text{M}\Omega\ \text{cm}$ at $25\ ^\circ\text{C}$, Millipore Merck, UK) at flow rates of 1, 3, 5, 7.5 and $10\ \mu\text{L}\ \text{min}^{-1}$, with each flow rate replicated 5 times.

Solutions were stirred to remove the resistance contribution from the external environment (R_{ext}) (Bungay et al., 1990).

The RR for each perfusate flow rate was calculated using Equation 6.1:

$$RR (\%) = 100 \times \frac{C_{dial}}{C_{std}} \quad (6.1)$$

where C_{dial} is the concentration ($\mu\text{g L}^{-1}$) of the analyte in the dialysate and C_{std} is the concentration ($\mu\text{g L}^{-1}$) of the analyte in the perfused solution.

Online Microdialysis Sampling.

The MD system consisted of a CMA 4004 syringe pump (CMA, Stockholm, Sweden) delivering perfusate (DDW) through a 10 mL syringe (BD Plastipak, US) into a CMA 20 microdialysis probe with a polyethersulfone membrane (10 mm length, 0.5 mm OD, 100 kDa MWCO). The dialysate flow was connected to the needle port of the injector/switching valve using a tubing adaptor (CMA, Stockholm, Sweden) and a #22 gauge square-cut end syringe needle. New probes were perfused at $10 \mu\text{L min}^{-1}$ with DDW. Prior to installation in soil microcosms, the online MD-HPLC-ICP-MS system was calibrated through perfusion and injection of stirred calibration standards containing $^{53}\text{Cr}^{VI}$ at 1, 10 and $100 \mu\text{g L}^{-1}$. A more concentrated calibration standard (between 1 and 2 mg L^{-1}) was perfused at the end of soil sampling to extend the linear dynamic range whilst mitigating washout issues that could arise when considering the low perfusate flow rate and narrow diameter of the inlet and outlet probe tubing.

Fifteen minutes after probe installation- the total time taken to fill the volume of the outlet tubing and the $20 \mu\text{L}$ loop- the valve was switched to “inject” and the time resolved analysis (TRA) sequence was initiated on the ICP-MS software to begin data acquisition. The injector remained in this position until ten sample volumes of mobile phase had been pumped through the loop (equivalent to 10 seconds with a $20 \mu\text{L}$

loop), before being switched back to “load” to collect freshly-sampled dialysate for the next injection. These steps were repeated over a period of 2 hours, giving a total of 8 injections of passively-sampled soil solution. Due to the manual nature of the MD-HPLC-ICP-MS setup, exploitation of the maximum temporal resolution was chosen over replication; however, the “direct injection” spike sampling was carried out in duplicate to examine artefactual errors.

Results and Discussion

Relative recovery of Cr^{VI} in solution

The RR of Cr^{VI} (stirred solutions) displayed a non-linear decrease with increasing flow rate (Fig. 6.2); perfusate was delivered into the MD probe at flow rates of 1, 3, 5, 7.5 and 10 $\mu\text{L min}^{-1}$ with collection and injection of the dialysate from each flow rate replicated 5 times.

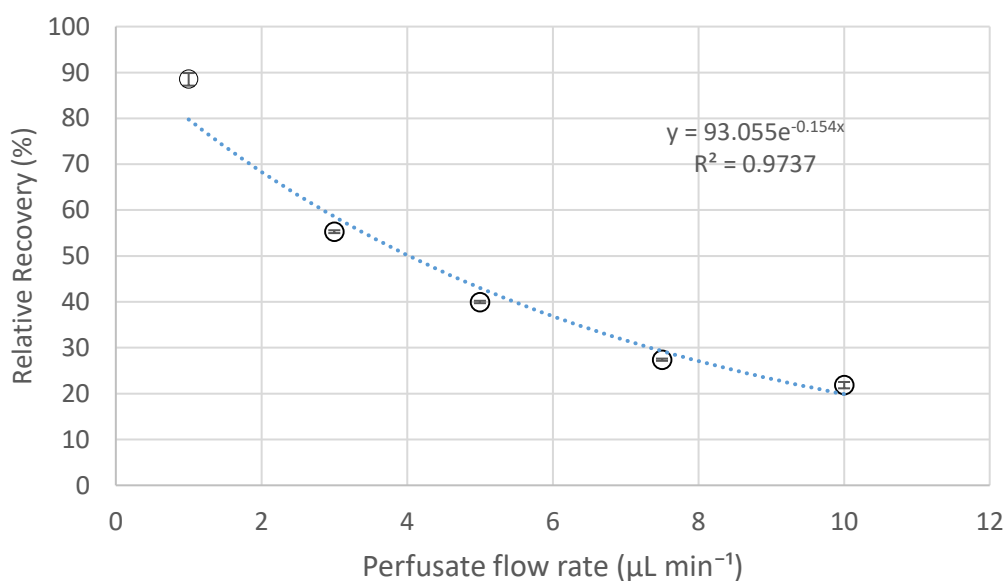


Figure 6.2. The effect of perfusate flow rate ($\mu\text{L min}^{-1}$) on relative recovery (%) of Cr^{VI} ($100 \mu\text{g L}^{-1}$) in stirred solutions ($n = 5$ for each flow rate). Exponential trendline and correlation coefficient (R^2) are displayed within the chart. Error bars indicate $\pm$ standard error from 5 replicates.

Subsequent solution optimization and soil sampling were undertaken using a perfusate flow rate of 3 $\mu\text{L min}^{-1}$. This flow rate represented the best compromise between RR (approximately 55%) and the frequency with which freshly sampled soil solution could be injected into the HPLC column. The resolution of the online MD-HPLC-ICP-MS system at this flow rate was 15 minutes, a significant improvement in temporal capability over conventional offline MD.

Previous studies have reported variability in RR due to inherent differences in probe structure arising from the manufacturing process (Torto et al., 2002, Humphrey et al., 2019a). A significant advantage of the online MD-HPLC-ICP-MS method is that, once flow rate calibration has been carried out for the analyte of interest, a single injection of a perfused calibration standard prior to soil sampling can identify any variability or reduction in the performance of the probe before soil is incubated with potentially expensive isotopically-enriched material.

Analytical Figures of Merit

The method detection limit (DL) was determined according to previously-outlined methods (Ripp, 1996). Briefly, 5 replicate injections of a perfused 0.5 $\mu\text{g L}^{-1}$ Cr^{VI} standard were undertaken using the online MD-HPLC-ICP-MS setup. The DL was calculated using Equation 6.2:

$$DL = \frac{(t)(RSD)(C_{std})}{100\%} \quad (6.2)$$

where t is a confidence factor using Student t -distribution with $\alpha = 0.99$ and $n-1$ degrees of freedom, RSD is the relative standard deviation of the peak areas for the Cr^{VI} standard and C_{std} is the nominal concentration of the injected Cr^{VI} standard. The DL was calculated as 0.3 $\mu\text{g L}^{-1}$ Cr^{VI} .

The precision of the online MD-HPLC-ICP-MS setup was assessed at the same time as the RR. Across each flow rate (1, 3, 5, 7.5, 10 $\mu\text{L min}^{-1}$), the 5 replicate injections displayed good precision, demonstrating the repeatability of the technique for solution sampling (Table 6.1).

Table 6.1. Average RR and relative standard deviation (RSD, $n = 5$) for replicate injections at perfusate flow rates of 1, 3, 5, 7.5 and 10 $\mu\text{L min}^{-1}$.

Flow Rate ($\mu\text{L min}^{-1}$)	Average RR (%)	RSD (%)
1	89	2.2
3	55	1.3
5	40	1.3
7.5	27	2.0
10	22	1.4

The linearity of response was determined through injections of perfused Cr^{VI} standards at nominal concentrations of 1, 10 and 100 $\mu\text{g L}^{-1}$. There was a strong positive linear correlation between peak area counts and Cr^{VI} concentrations in stirred solutions (Fig. 6.3).

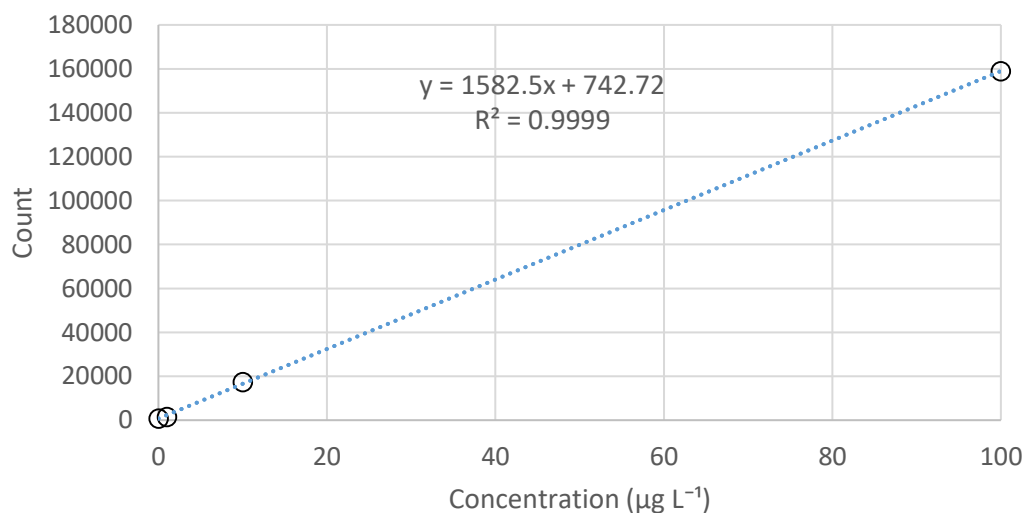


Figure 6.3. Calibration curve for online MD-HPLC-ICP-MS system at nominal concentrations of 1, 10 and 100 $\mu\text{g L}^{-1}$. MD probes were immersed in 50 mL plastic beakers containing $^{53}\text{Cr}^{\text{VI}}$ solution, stirred and perfused at 3 $\mu\text{L min}^{-1}$.

Soil Solution Sampling- Homogenized Mixing

The online MD-HPLC-ICP-MS setup was applied to the sampling of soil solution labelled with $^{53}\text{Cr}^{\text{VI}}$ through homogenized mixing (Fig. 6.4), using the perfusate flow rate ($3\ \mu\text{L min}^{-1}$) and injector timings established during solution calibration. From an initial concentration (15 minutes after spiking) of $276\ \mu\text{g L}^{-1}$, the concentration of Cr^{VI} decreased by 75% over the 120 minute sampling period, to $71\ \mu\text{g L}^{-1}$. This decrease is likely due to the formation of a depletion zone around the MD probe from continuous sampling, as opposed to the reduction of Cr^{VI} to Cr^{III} with subsequent adsorption to the soil solid phase (Castro-Rodríguez et al., 2015). Similar depletion profiles have been reported for offline MD studies employing continuous sampling, and could be an informative artefact as nutrient uptake by plant roots is also governed by depletion and formation of diffusion gradients within the soil (Leitner et al., 2010). The isotopic labelling of microcosms could also be considered analogous to the release of solutes within the rhizosphere or at locations of microbial activity (Sulyok et al., 2005); depending on probe placement, the online MD-HPLC-ICP-MS setup would therefore be sensitive enough to detect these events.

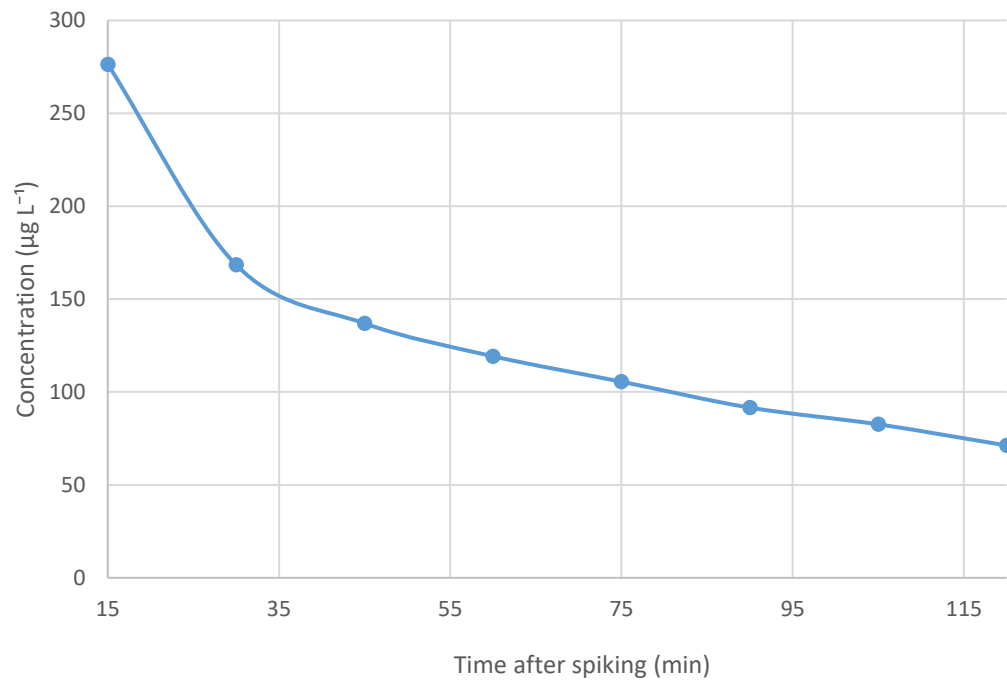


Figure 6.4. Decrease in passively-sampled $^{53}\text{Cr}^{\text{VI}}$ added to soil through homogenized mixing method over 120 minutes, measured by online MD-HPLC-ICP-MS every 15 minutes following spike introduction.

Soil Solution Sampling- Direct Injection.

One advantage of the direct injection method is that it allows for a true 'zero' tie-point to be sampled prior to introduction of the isotope; concentrations of $^{53}\text{Cr}^{\text{VI}}$ in both microcosm replicates were below DL ($0.3 \mu\text{g L}^{-1}$) at $t = 0 \text{ min}$ (Fig. 6.5). The concentration of $^{53}\text{Cr}^{\text{VI}}$ in the second dialysate injection (15 minutes after spiking) was higher than expected, notwithstanding the total volume (0.5 mL) and initial concentration (7.5 mg L^{-1}) of the spike introduced into the microcosm (average concentration of $1536 \mu\text{g L}^{-1}$). By the end of the 120 minute sampling period, the $^{53}\text{Cr}^{\text{VI}}$ concentration in the dialysate had decreased to between 2 and 3% of the initial concentration ($16\text{--}48 \mu\text{g L}^{-1}$), representing a more rapid decrease in passively-available Cr^{VI} than through homogenized mixing.

These observations can be attributed to the creation of localized solution-filled channels during direct injection of the $^{53}\text{Cr}^{\text{VI}}$ solution, limiting the successful dispersion of the spike through the microcosm. During injection of the spike it is likely that there was some expulsion of solution into the dead volume around the MD probe, creating an area of high Cr^{VI} concentration which allowed for unimpeded diffusion across the probe membrane. Depletion of solutes around the MD probe would then have occurred over the next 45 minutes, with resistance from the soil matrix limiting the diffusion of Cr^{VI} from other areas of the microcosm to replenish this depletion (Tinker and Nye, 2000).

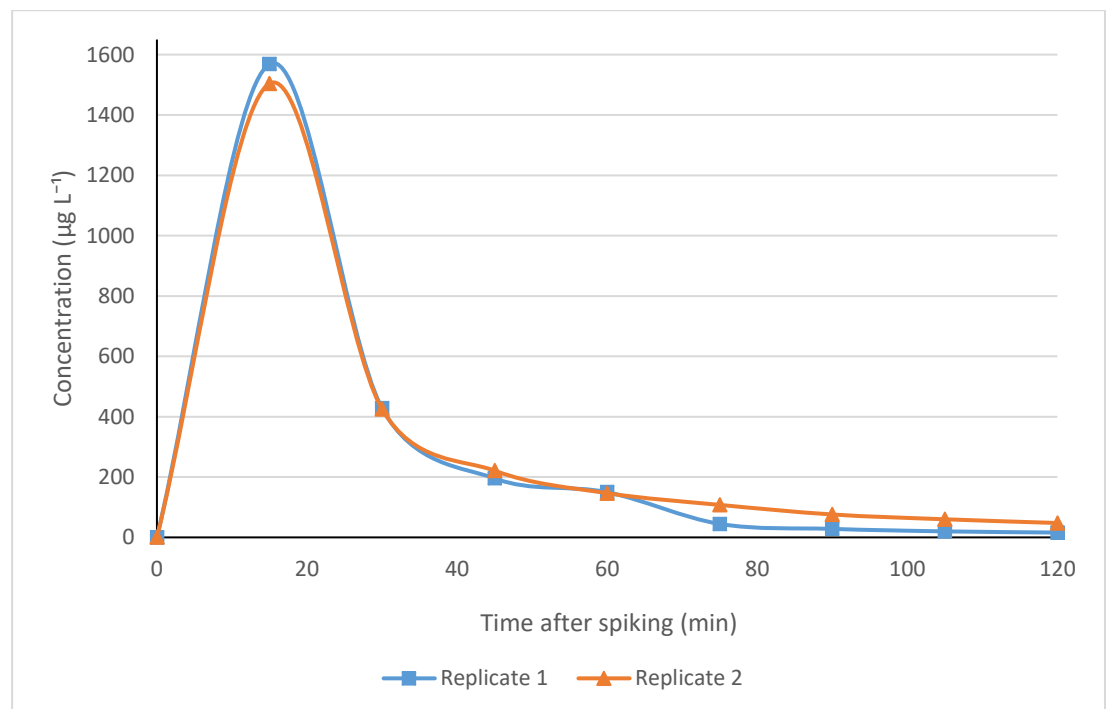


Figure 6.5. Changes in passively-sampled $^{53}\text{Cr}^{\text{VI}}$ added to soil through direct injection method over 120 minutes, measured by online MD-HPLC-ICP-MS every 15 minutes following spike introduction. Replicates were undertaken on consecutive days.

Numerous publications have reported the efficacy of the direct injection spiking method (Pörtl et al., 2007, Inselsbacher et al., 2009, Warren, 2018). Therefore, it is possible that the clay-loam texture soil used in this study was the reason for the

insufficient spike dispersion. Despite the limitations, there was good agreement between replicate spike injections and sampling across consecutive days, further demonstrating the precision of the technique. This artefact serves to highlight the importance of soil type/texture when considering how best to distribute an isotopically-enriched spike within a microcosm. The online MD-HPLC-ICP-MS setup was still able to sample Cr^{VI} from the microcosm, but the depletion zone around the probe was more significant than with the homogenous mixing method.

Future Prospects for Optimization and Implementation of Online MD.

The use of MD for soil solution sampling is still an emerging technique (the first comprehensive review was published in early 2020) and so, to a certain extent, the future prospects and discussion points for online and offline MD are complementary (Buckley et al., 2020). One important consideration in the continuous sampling of soil solution is the increased likelihood of depletion zones forming around the probe due to removal of solute from solution (Brackin et al., 2016). The recharge of this zone is dependent on a number of factors, including the ability of the solute to diffuse from un-sampled areas within the medium, the concentration of solute within the medium and the diffusive resistance imposed by the soil. These factors may have significant ramifications for the interpretation of solute diffusive flux data when multiple MD probes are deployed. The proximity of one MD probe to its neighbor could create competing diffusion gradients and lead to a situation where probes with reduced permeability- due to manufacturing defects, continued use or implantation in heterogeneous portions of soil- would sample lower solute concentrations (Fig. 6.6). Depletion zones are a well-known component of MD, but a more empirical investigation is required to fully understand how they impact both diffusive flux measurements and the efficacy of probes that are in close proximity to each other.

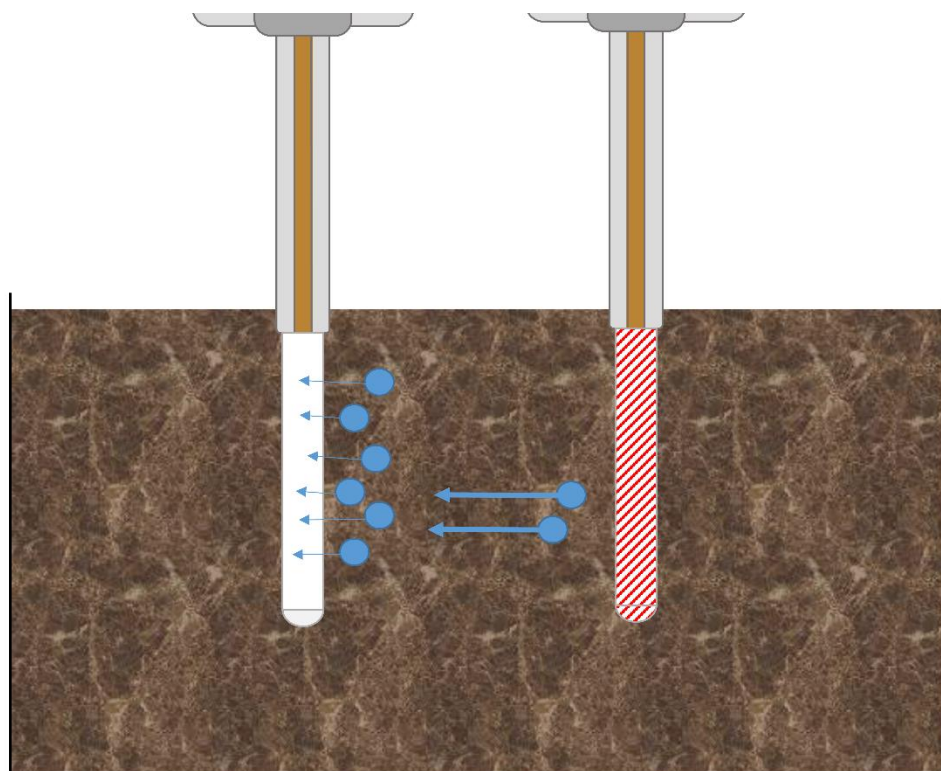


Figure 6.6. Diffusion of solutes (blue circles) towards probes implanted in soil matrix. Reduced permeability (represented by diagonal fill) in right MD probe shifts the diffusion gradient towards the left MD probe, therefore the right MD probe will sample lower concentrations of solute.

The temporal resolution of the online MD-HPLC-ICP-MS setup in this article represents a significant improvement over offline MD; further optimization of the instrumental setup could reduce this to sub-minute sampling frequencies. In recent years, the use of total consumption nebulizers has allowed sample volumes in the order of microliters to be introduced in to ICP-MS instruments (Schaumlöffel et al., 2003). The flow rates commonly used in MD are ideally suited to these sample introduction systems, with the potential for the outlet tubing from the MD probe to be interfaced directly with the nebulizer to monitor transient signals in real-time. Such a setup would also reduce the level of operator supervision required, as the only ‘hands-on’ task

would be the installation of the probe into the microcosm prior to time-resolved analysis.

The spatial resolution of MD, combined with the greater sampling frequency afforded by online coupling to analytical systems, would allow for the investigation of solute turnover/removal at root- and microbe-relevant scales in near real-time. Microbial reduction of Cr^{VI} has been reported on numerous occasions, with incubation times varying from 45 min to 42 days (Shahid et al., 2017) due to the significant variation in Cr^{VI} reduction efficiency between different strains (Dhal et al., 2010). Undertaking online MD-HPLC-ICP-MS on sterile and non-sterile soil could provide more information on the impact of microbial communities on Cr^{VI} reduction, with the ability to investigate parameters such as temperature, pH and soil type with greater replication than through batch experiments. Online monitoring would also allow for termination of the experiment once passively-sampled analyte concentrations reached DL, potentially saving days of time-consuming and costly experimentation and analysis (Jin et al., 2008).

Understanding the mechanisms governing rapid soil fixation and speciation changes, for important redox-active micronutrients such as iodine (I) and selenium (Se), previously limited in terms of temporal resolution, may now be possible (Humphrey et al., 2020) (Bowley et al., 2019, Phiri et al., 2019). The use of stable and radio-isotope trials have confirmed that the removal of I and Se from soil solution, primarily through incorporation into the solid phase or immobilization by soil organic matter (SOM), is a rapid process which significantly reduces the bioavailability of these micronutrients (Ligowe et al., 2020, Shetaya et al., 2012). Humphrey, et al (Humphrey et al., 2020), in (at the time of the writing) the only application of offline MD to investigate I dynamics in soil solution, showed that adsorption was more rapid than previously reported.

Increased frequency of sampling through online MD could further refine knowledge of the period over which soluble forms of I and Se are available for uptake by crops, leading to improvements in biofortification strategies intended to alleviate the prevalence of deficiency diseases.

The online MD-HPLC-ICP-MS system was only evaluated for Cr^{VI}, but through simple modification of the chromatographic conditions (column, mobile phase composition) the setup could be applied to the sampling and determination of other common inorganic species of interest, including compounds of arsenic, thallium and mercury, to better inform hazard assessment investigations. The soil solution dynamics of inorganic nutrients essential to human health (e.g. iodine, selenium) could be investigated at unprecedented temporal and spatial scales, allowing for more thorough assessments of the efficacy of staple crop biofortification strategies that are essential for the billions of people at risk of micronutrient deficiencies worldwide.

Chapter 7. Conclusions

Despite a general understanding in the literature of how species stability and plant uptake are influenced by soil properties and rhizosphere interactions, there is a greater need for methods that are capable of investigating speciation processes *in situ* at finer scales and with minimal disruption to the equilibrium of the system. The aim of this research was to improve the understanding of soil-to-plant transfer of Cr species through the development, optimisation and application of novel analytical methods. This was achieved through the use of pioneering analytical techniques, including high-resolution soil solution sampling and simultaneous analysis using microdialysis and the assessment of Cr^{III} and Cr^{VI} plant uptake mechanisms using species-specific isotopic tracers. This chapter summarises the results of the research, highlights some limitations of the study and includes recommendations for future work.

The disparity of results obtained from analytical methodologies designed to measure “total” Cr^{VI} in soils and other solid matrices is of concern, such as the reporting of false-positive Cr^{VI} concentrations in food matrices that are less likely to harbour the toxic species (Novotnik et al., 2015). This issue was highlighted in the comprehensive literature review undertaken in Chapter 2, and can be attributed to (i) the use of off-line extraction and analysis methods with no chromatographic procedure to separate Cr^{VI} from Cr^{III}, and (ii) incomplete recovery of Cr^{VI} when using alkaline extraction techniques (Wazne et al., 2007). It is encouraging to see an increase in the use of SIDMS, in part due to the wider availability of species-specific spikes and the change in perception of higher value on-line analytical instrumentation (HPLC-ICP-MS) as necessary investments to ensure full metrological traceability (Markiewicz et al., 2015).

Chapter 4 fulfilled the first objective of the thesis, outlining the implementation of SIDMS to extract and measure total Cr^{VI} in agricultural soil samples collected in the Copperbelt Province, Zambia through geostatistically-optimised sampling and analytical methods developed partly in Chapter 3. The interpolation of total Cr through ordinary kriging, made possible through the nested sampling design applied at the study site, enabled the identification of hotspots for further sampling. Although the original intention behind the second sampling campaign was to collect soils that captured the spatial variation of total Cr, soil pH and LOI for glasshouse experiments, there was sufficient dispersion of the sample points across the interpolated spatial domain to achieve a satisfactory assessment of Cr^{VI} soil concentrations across the cultivated land. Despite high concentrations of total Cr in the soil samples (average 70 mg kg⁻¹), the Cr^{VI} concentrations measured by SIDMS were close to detection limit (0.03 mg kg⁻¹) in all but a handful of cases, which themselves were two orders of magnitude below UK legislative thresholds for Cr^{VI}. These results, alongside additional spiking experiments included in Chapter 4 demonstrating the minimal oxidation of Cr^{III} during the extraction procedure, indicate that the SIDMS method was successfully applied to agricultural soils with no significant analytical artefacts.

The concentration of Cr within plants is dependent on both lithogenic and anthropogenic factors, with industrial effluent discharge and wastewater irrigation causing significant soil contamination and the potential for plant uptake (Khan et al., 2008). However, the mechanisms through which Cr^{III} and Cr^{VI} are transferred from soil to plants remain largely unknown, with contradictory information in the literature on how the presence of necessary nutrients (phosphate, sulphate) and exudation of organic acids within the rhizosphere compete with, or promote uptake of, Cr^{VI} (Shahid et al., 2017). In Chapter 5, root solutions corresponding to specific uptake pathways in *Spinacia oleracea* were isolated following short-term exposure to both Cr species. This

specificity extended to the measurement of the oxidation and reduction processes occurring before and after uptake, using isotopic treatments with distinguishable speciation ($^{50}\text{Cr}^{\text{III}}$ and $^{53}\text{Cr}^{\text{VI}}$), and marks the first occasion in the literature of this method being applied to assess the plant uptake mechanisms of metal species. The dominant species of Cr in apoplastic (passive) and symplastic (active) root solutions was Cr^{III} , with evidence for reduction of Cr^{VI} within the rhizosphere followed by passive uptake of resulting Cr^{III} . There was no preferential uptake pathway for Cr^{III} ; indeed, the highest Cr^{III} concentrations were measured in the active pathway root solutions, contrary to previous literature (Oliveira, 2012b), implying that Cr species uptake mechanisms are more complicated than previously thought. The concentration of $^{50}\text{Cr}^{\text{III}}$ transferred through both active and passive pathways was greater than the $^{53}\text{Cr}^{\text{III}}$ formed from reduction of $^{53}\text{Cr}^{\text{VI}}$, suggesting a rate-limiting step in uptake of Cr^{VI} due to reduction in the lower pH conditions of the rhizosphere. The presence of sulphate led to a statistically-significant increase in reduced Cr^{VI} through the active relative to the passive uptake pathway, indicating that sulphate and Cr^{VI} do not compete with each other and that Cr^{VI} may be taken into plants through transporters other than sulphate carriers (Schiavon et al., 2012). The mitigation of Cr^{VI} plant toxicity by sulphur is accepted in the literature, although the specific mechanisms controlling this mutualistic relationship have yet to be determined (de Oliveira et al., 2016). The results in Chapter 5 highlight the ability of *Spinacia oleracea* to tolerate Cr^{VI} , through reduction processes occurring in the low pH environment of the rhizosphere or following uptake into root systems, where the Cr is immobilised as Cr^{III} complexed to organic acids in root cells (Han et al., 2004). This further explains why low concentrations of Cr are measured in edible compartments of plants, and suggests that plants are equipped with the detoxification mechanisms required to tolerate Cr^{VI} .

The features of MD that aid in its application to neurochemical and pharmacokinetic studies also make it an attractive tool in the field of soil science, where processes occurring at micro-scales (e.g. aggregation of SOM) have a significant impact at the macro-scale, but may not be sufficiently investigated using the limited resolving power of existing methods (Lehmann et al., 2007). Chapter 6 demonstrated the coupling of MD to HPLC and ICP-MS, creating an online system capable of passively sampling and analysing Cr^{VI} from soil solution every 15 minutes. Previously, MD has been coupled to both chromatographic separation systems and analytical detectors, including mass spectrometry (MS) and ETAAS; the method presented in Chapter 6 marks the first use of MD-HPLC-ICP-MS to monitor the availability of Cr^{VI} following an addition event, alongside an investigation of the potential sources of analytical bias arising through different methods of introducing isotopically-labelled Cr^{VI}. Before application to soil sampling, the selectivity of the setup with respect to Cr^{VI} was confirmed through perfusate flow rate calibration; additional figures of merit (detection limit, linearity of response) were also assessed. The setup was sensitive enough to detect changes in passively-available Cr^{VI} with each sampling interval, with analytical artefacts confined to the method of isotopic labelling. The concentration of Cr^{VI} in microcosms were significantly above detection limit even after sampling for 120 minutes, suggesting that any decreases during sampling were due to the formation of depletion zones around the probe membrane (Buckley et al., 2020). The partition coefficient for Cr^{VI} is lower than that for Cr^{III}, leading to its accumulation in soil pore water and removal from solution at a slower rate than Cr^{III} (Hassan and Garrison, 1996). The online MD results support this observation, suggesting that the resolving power of the online setup is greater than what is required when considering the physicochemical properties of Cr species in solution. Nevertheless, the setup shows significant promise for evaluating fine scale, short-term Cr species availability within soil solution.

The online MD setup is not without its drawbacks; the cost of operating an online MD-HPLC-ICP-MS system renders the technique more expensive than analysing discrete samples collected using offline MD. However, the temporal resolution of the online system is improved significantly due to the lower volume of dialysate required for each analysis. The online system can therefore be considered context-specific as opposed to a total replacement for offline MD; when high-resolution sampling of solutes with fast turnover (e.g. removal from soil solution through complexation with SOM) is required, it is advisable to focus on optimising an online setup. The online setup is also limited by its requirement to be deployed in the laboratory, which restricts its application to theoretical and/or synthetic environmental systems as opposed to sampling of *in situ* species dynamics. When considering the issue of preserving soil solution Cr speciation when sampling using offline MD, there is the potential to couple the dialysate flow to an appropriately-packed micro solid-phase extraction (μ SPE) system to allow for field-based Cr species retention without the need to freeze samples. This could both reduce the logistical complications of maintaining species integrity (particularly when conducting overseas fieldwork), and minimise the introduction of low-volume liquid handling errors.

The methods presented in this thesis have contributed to a greater understanding of Cr^{VI} biogeodynamics, whilst also emphasising the importance of implementing validated analytical methodologies in soil-to-plant transfer studies. All too often, the biggest hindrance in the advancement of a particular field is the absence of methods or instrumentation capable of addressing the research question at scales relevant to the situation being investigated, with minimal introduction of artefacts. For example, triple-quadrupole ICP-MS technology now makes it possible to undertake analysis of radioisotopes (e.g. ¹²⁹I) which were previously fraught with analytical issues due to isobaric interference and abundance sensitivity overlaps (Humphrey et al., 2019c),

increasing their scope for soil-plant bioavailability studies. Microdialysis is refining and redefining our understanding of nutrient fluxes, at scales that only a few years ago were considered inconceivable to sample. Recent studies have confirmed the efficacy of MD probes to act as root simulators, with the potential for varying perfusion rates and the use of push-pull MD to mimic different plant uptake rates and hyperaccumulation. The differing chemistries and toxicities of Cr species and their compounds present ongoing research interest and metrological challenges. Responding adequately requires the use of validated analytical frameworks, ensuring any decisions made on remediation of contaminated land or mitigation of anthropogenic release of Cr are supported by robust data.

Limitations of the study

The scope and focus of the study had to be modified during the first half of the research period due to a number of limitations. Initially, the research aim was to assess the impact of agricultural amendments, such as liming and organic reincorporation, on the transfer of Cr^{VI} from tailings contaminated soil into staple crops. Prior to the measurement of Cr^{VI} in the agricultural soils collected in Chapter 4, glasshouse experiments were attempted with soil subsamples to establish translocation factors for Cr using *Spinacia oleracea*. Due to a lapse in watering over a holiday period, the plants died; the soils were subsequently wetted to field capacity to allow for easier removal of plants, but further attempts at plant growth failed as the soil structure had been irreparably altered. Following completion of the SIDMS analysis, the Cr^{VI} concentrations in the soil samples were determined to be significantly lower than current legislative criteria. Therefore, the site was not suitable for field or glasshouse trials with respect to the original research aim, which was then revised to encompass the development and/or optimisation of novel analytical methods to understand the

species interconversions occurring at spatial and temporal scales relevant to soil-to-plant uptake.

Due to restricted access, it was not possible to obtain a sample of the tailings heap from the site surveyed in Chapter 4. The liming of tailings, undertaken as a waste management strategy to reduce metal mobility, creates an ideal environment for the stabilisation of Cr^{VI} . The application of the SIDMS method to this material would have helped to confirm whether the physicochemical characteristics of the agricultural soil were the reason for the low measured Cr^{VI} concentrations in the soil samples.

Time constraints were the biggest limitations for Chapters 5 and 6. For Chapter 5, the number of plants that were required to achieve sufficient replication and sample volume contributed to a very time-consuming process, particularly when taking into account the time required for the plants to reach maturity in addition to harvesting, solution treatment exposure, root tip excision and isolation of root solutions through freeze-thaw centrifugation. Within Chapter 5, supplementary analyses such as measurement of oxidative stress markers and organic acid exudates (e.g. citrate, oxalate) could have provided evidence towards the physiological reasons for the observed increase in active transfer of reduced Cr^{VI} , when in the presence of sulphate. In addition, a metabolic inhibitor could have been employed to reduce the efficiency of uptake for a specific pathway (e.g. carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP) for inhibiting symplastic uptake) and further confirm the uptake pathways observed for Cr^{III} and Cr^{VI} in this chapter.

It is also worth noting that the root solution isolation method presented in Chapter 5 can only provide a snapshot in time with respect to the uptake pathways that Cr^{III} and Cr^{VI} take from the external solution into cortical cells in the spinach plant. For example, the radial transport of Cr species through these pathways can change depending on

the water status of the plant. The permeability of root cells to water and dissolved nutrients can vary to both facilitate uptake and subsequent xylem loading, and minimise loss of nutrients and photosynthesised compounds into soil (Zarebanadkouki et al., 2019), which will ultimately have an impact on the Cr species concentrations within root cells and other plant compartments. The identities of apoplastic and symplastic pathways can also interchange depending on the requirements of the plant; plasmodesmata (PDs), narrow membrane-lined channels which connect cells to each other to assist in movement of nutrients, are also integral to the transportation of signalling molecules to provide positioning cues and determine the identity of plant cells following cell division (Epel, 1994). PDs mediate transport between adjacent cells and have a molecular weight cut-off, but the mechanisms dictating which molecules are selected for transfer and which are excluded are not fully understood. Depending on the response of the plant to the supplied Cr^{III} and Cr^{VI} , there is the potential for primary and secondary PDs (formed during cell divisions and in already-established cell walls respectively) to form different structures to increase transport of transcription factors to tolerate Cr toxicity, or impede transport through a particular pathway to minimise cellular damage (Stahl and Simon, 2013). Although the isolation of apoplastic and symplastic root solutions is useful for providing a generalised assessment of species uptake pathways, it is important to remember that plants are dynamic systems with numerous tolerance mechanisms and homeostatic processes, and that the elemental speciation observed for any one experiment does not apply to all possible scenarios.

Chapter 6 presented the first use of online microdialysis, coupled to HPLC-ICP-MS, for the continuous monitoring and semi-continuous analysis of passively sampled $^{53}\text{Cr}^{\text{VI}}$ added to soil. The original aim was to achieve continuous sampling and analysis of soil solution, through direct coupling of the dialysate flow with the nebuliser of the ICP-

MS instrument. At the time of development, the uptake rates of all available nebulisers ($0.05\text{--}0.4\text{ ml min}^{-1}$) were too high for the perfusate flow rate ($3\text{ }\mu\text{L min}^{-1}$), resulting in significant signal instability. The commercial availability of sample introduction systems capable of handling low flow rates are improving as the field of Micro-Liquid Chromatography ($\mu\text{-LC}$) develops. However, the technique is currently still too esoteric to justify routine purchase by the majority of laboratories and such nebulisers suffer from back pressure issues if tubing diameters are not adequately matched (Wind et al., 2002). Therefore, the online MD setup was modified to incorporate a manual injector, allowing for the use of conventional nebulisation and the addition of column separation (HPLC) for *in situ* measurement of Cr^{VI} .

The temporal resolution of the online MD-HPLC-ICP-MS system was restricted by the loop size of the manual injector, and the requirement for the loop to be filled with the equivalent of two loop volumes ($40\text{ }\mu\text{L}$) to ensure maximum injection reproducibility (Kjellström et al., 2000). The use of smaller loop sizes, or loops integrated into the rotor seal of the injector, would reduce the time between MD sampling and injection and subsequently improve the temporal resolution of the system; in addition, this would allow for the use of lower perfusate flow rates, improving the relative recovery of Cr^{VI} .

Recommendations for future research

Due to emerging research concerning the genotoxicity of Cr^{III} , driven in part by its prevalence in weight-loss supplements, there is a need for speciation methods to account for the measurement of both Cr species. This is particularly important when considering that the dominant species of Cr in plants following uptake is Cr^{III} , with the potential for translocation to edible compartments as a detoxification mechanism (Lytle et al., 1998). Accurate speciation analysis, taking into account both forms of Cr

in a sample matrix, would allow for better assessment of the human health risk associated with consumption of plants grown in soil contaminated with Cr.

The sampling of analytes using microdialysis is made possible due to the movement of solutes down a diffusion gradient, from high concentrations in the sampled medium to the perfusate solution pumped behind the probe membrane. However, it is possible to reverse this principle through “retrodialysis”, using the perfusate solution to deliver solutes to a localised area within the implanted medium (Kho et al., 2017). Retrodialysis has been used to examine the pH-dependent release of metals (Fe, Pb) and essential nutrients (inorganic P) within the rhizosphere using perfusates designed to mimic organic exudates (Demand et al., 2017, Miró et al., 2005), but is more commonly used as a secondary assessment of relative recovery during MD calibration (Hsu et al., 2016). Investigating the dynamics of oxidative stress responses using microdialysis sampling within the rhizosphere, combined with retrodialysis to deliver the antagonist compound (e.g. Cr^{VI}), would offer an unmatched level of temporal and spatial resolution whilst maintaining the integrity of the soil-plant environment and minimising sampling artefacts. This technique could offer greater insight into the mechanisms through which plants tolerate Cr^{VI}, and the role that soil components (nutrients, microbes, SOM) may play in mitigating plant damage.

Improving the understanding of Cr species soil-to-plant transfer is significantly limited by the widespread availability of techniques capable of assessing species movement at micro- and nanometre scales. NanoSIMS (Secondary Ion Mass Spectrometry) is a familiar technique in the field of cosmochemistry, and is emerging as a powerful tool for, amongst other applications, the analysis and imaging of isotopically-labelled species in heterogeneous soil environments (Mueller et al., 2013). In one of the small number of publications concerning NanoSIMS in soil, during which wheat roots

(*Triticum aestivum*) were exposed to ^{15}N for 24 h, it was possible to visually identify accumulation of the isotope in individual microorganisms within different compartments of the rhizosphere, and confirm this accumulation through measurement of $^{15}\text{N}/^{14}\text{N}$ ratios (Clode et al., 2009). The application of such a technique to evaluate Cr species dynamics in the rhizosphere would provide unprecedented spatial resolution and quantitative assessment of Cr species assimilation within individual microorganisms, with the potential for supplementation with existing analytical techniques (MD, XANES) to identify endmembers for Cr species in soil-plant systems.

References

2014. EPA Method 6800: Elemental and Molecular Speciated Isotope Dilution Mass Spectrometry.
- “SKIP” KINGSTON, H. M., HUO, D., LU, Y. & CHALK, S. 1998. Accuracy in species analysis: speciated isotope dilution mass spectrometry (SIDMS) exemplified by the evaluation of chromium species. *Spectrochimica Acta Part B: Atomic Spectroscopy*, 53, 299-309.
- ABBASI, S. & BAHIRAEI, A. 2012. Ultra trace quantification of chromium(VI) in food and water samples by highly sensitive catalytic adsorptive stripping voltammetry with rubeanic acid. *Food Chemistry*, 133, 1075-1080.
- AHMAD, S., MURTHY, R. C. & CHANDRA, S. V. 1990. Chromium speciation by column chromatography using a direct current plasma atomic emission spectrometer. *Analyst*, 115, 287-289.
- ALMÅS, Å. R., SÆVARSSON, H. T. & KROGSTAD, T. 2017. The partitioning of P in soil determines the fluxes and deliveries of labile P in soil solution. *Geoderma*, 306, 135-143.
- AMBUSHE, A. A., MCCRINDLE, R. I. & MCCRINDLE, C. M. E. 2009. Speciation of chromium in cow's milk by solid-phase extraction/dynamic reaction cell inductively coupled plasma mass spectrometry (DRC-ICP-MS). *Journal of Analytical Atomic Spectrometry*, 24, 502-507.
- ARDINI, F., DAN, G. & GROTTI, M. 2020. Arsenic speciation analysis of environmental samples. *Journal of Analytical Atomic Spectrometry*.
- BAILEY, C. H. 2014. Improved Meta-Analytic Methods Show No Effect of Chromium Supplements on Fasting Glucose. *Biological Trace Element Research*, 157, 1-8.
- BALL, J. W. & NORDSTROM, D. K. 1998. Critical evaluation and selection of standard state thermodynamic properties for chromium metal and its aqueous ions, hydrolysis species, oxides, and hydroxides. *Journal of Chemical & Engineering Data*, 43, 895-918.
- BANERJEE, A., NAYAK, D., CHAKRABORTTY, D. & LAHIRI, S. 2008. Uptake studies of environmentally hazardous ⁵¹Cr in Mung beans. *Environmental Pollution*, 151, 423-427.
- BARTLETT, R. & JAMES, B. 1979. Behavior of Chromium in Soils: III. Oxidation1. *Journal of Environmental Quality*, 8.
- BASKETTER, D., SLODOVNIK, D., MERIMES, S., TRATTNER, A. & INGBER, A. 2000. Investigation of the threshold for allergic reactivity to chromium. *Contact Dermatitis*, 44, 70-74.
- BINI, C., MALECI, L. & ROMANIN, A. 2008. The chromium issue in soils of the leather tannery district in Italy. *Journal of Geochemical Exploration*, 96, 194-202.
- BLUSKOV, S., AROCENA, J., OMOTOSO, O. & YOUNG, J. 2005a. Uptake, distribution, and speciation of chromium in Brassica juncea. *International Journal of Phytoremediation*, 7, 153-165.
- BLUSKOV, S., AROCENA, J. M., OMOTOSO, O. O. & YOUNG, J. P. 2005b. Uptake, Distribution, and Speciation of Chromium in Brassica Juncea. *International Journal of Phytoremediation*, 7, 153-165.
- BOWLEY, H. E., YOUNG, S. D., ANDER, E. L., CROUT, N. M. J., WATTS, M. J. & BAILEY, E. H. 2019. Iodine bioavailability in acidic soils of Northern Ireland. *Geoderma*, 348, 97-106.
- BRACKIN, R., NASHOLM, T., ROBINSON, N., GUILLOU, S., VINALL, K., BUCKLEY, S., LAKSHMANAN, P., SCHMIDT, S. & INSELSBACHER, E. Microdialysis—a new

- technology for investigating soil nitrogen fluxes in the rhizosphere. 7th International Nitrogen Initiative Conference, 2016.
- BROWN, E. M. 1997. A Conformational Study of Collagen as Affected by Tanning Procedures. *Journal of the American Leather Chemists Association*, 92, 225-233.
- BUCKLEY, S., ALLEN, D., BRACKIN, R., JÄMTGÅRD, S., NÄSHOLM, T. & SCHMIDT, S. 2019. Microdialysis as an in situ technique for sampling soil enzymes. *Soil Biology and Biochemistry*, 135, 20-27.
- BUCKLEY, S., BRACKIN, R., JÄMTGÅRD, S., NÄSHOLM, T. & SCHMIDT, S. 2020. Microdialysis in soil environments: Current practice and future perspectives. *Soil Biology and Biochemistry*, 143, 107743.
- BUNGAY, P. M., MORRISON, P. F. & DEDRICK, R. L. 1990. Steady-state theory for quantitative microdialysis of solutes and water in vivo and in vitro. *Life Sciences*, 46, 105-119.
- CASTRO-RODRÍGUEZ, A., CARRO-PÉREZ, M. E., ITURBE-ARGÜELLES, R. & GONZÁLEZ-CHÁVEZ, J. L. 2015. Adsorption of hexavalent chromium in an industrial site contaminated with chromium in Mexico. *Environmental Earth Sciences*, 73, 175-183.
- CERVANTES, C., CAMPOS-GARCÍA, J., DEVAR, S., GUTIÉRREZ-CORONA, F., LOZATAVERA, H., TORRES-GUZMÁN, J. C. & MORENO-SÁNCHEZ, R. 2001. Interactions of chromium with microorganisms and plants. *FEMS Microbiology Reviews*, 25, 335-347.
- CHAI, L., DING, C., LI, J., YANG, Z. & SHI, Y. 2019. Multi-omics response of *Pannonibacter phragmitetus* BB to hexavalent chromium. *Environmental Pollution*, 249, 63-73.
- CHANG, Y.-L. & JIANG, S.-J. 2001. Determination of chromium species in water samples by liquid chromatography-inductively coupled plasma-dynamic reaction cell-mass spectrometry. *Journal of Analytical Atomic Spectrometry*, 16, 858-862.
- CHEN, S., ZHU, S., HE, Y. & LU, D. 2014. Speciation of chromium and its distribution in tea leaves and tea infusion using titanium dioxide nanotubes packed microcolumn coupled with inductively coupled plasma mass spectrometry. *Food Chemistry*, 150, 254-259.
- CHOUDHURY, S. & PANDA, S. K. 2005. Toxic Effects, Oxidative Stress and Ultrastructural Changes in Moss *Taxithelium Nepalense* (Schwaegr.) Broth. Under Chromium and Lead Phytotoxicity. *Water, Air, and Soil Pollution*, 167, 73-90.
- CHRYSOCHOOU, M., THEOLOGOU, E., BOMPOTI, N., DERMATAS, D. & PANAGIOTAKIS, I. 2016. Occurrence, Origin and Transformation Processes of Geogenic Chromium in Soils and Sediments. *Current Pollution Reports*, 2, 224-235.
- ÇİMEN, G., TOKALIO LU, ERIFE, ÖZENTÜRK, I. & SOYKAN, C. 2013. Speciation and preconcentration of chromium from water and food samples by synthesized chelating resin. *Journal of the Brazilian Chemical Society*, 24, 856-864.
- CLODE, P. L., KILBURN, M. R., JONES, D. L., STOCKDALE, E. A., CLIFF, J. B., HERRMANN, A. M. & MURPHY, D. V. 2009. In Situ Mapping of Nutrient Uptake in the Rhizosphere Using Nanoscale Secondary Ion Mass Spectrometry. *Plant Physiology*, 151, 1751-1757.
- COSTA, M. 2003. Potential hazards of hexavalent chromate in our drinking water. *Toxicology and Applied Pharmacology*, 188, 1-5.
- COYTE, R. M., MCKINLEY, K. L., JIANG, S., KARR, J., DWYER, G. S., KEYWORTH, A. J., DAVIS, C. C., KONDASH, A. J. & VENGOSH, A. 2020. Occurrence and distribution

- of hexavalent chromium in groundwater from North Carolina, USA. *Science of The Total Environment*, 711, 135135.
- DAFTSIS, E. J. & ZACHARIADIS, G. A. 2007. Analytical performance of ETAAS method for Cd, Co, Cr and Pb determination in blood fractions samples. *Talanta*, 71, 722-730.
- DAVIES JR, F., PURYEAR, J., NEWTON, R., EGILLA, J. & SARAIVA GROSSI, J. 2002. Mycorrhizal fungi increase chromium uptake by sunflower plants: influence on tissue mineral concentration, growth, and gas exchange. *Journal of Plant Nutrition*, 25, 2389-2407.
- DE OLIVEIRA, L. M., GRESS, J., DE, J., RATHINASABAPATHI, B., MARCHI, G., CHEN, Y. & MA, L. Q. 2016. Sulfate and chromate increased each other's uptake and translocation in As-hyperaccumulator *Pteris vittata*. *Chemosphere*, 147, 36-43.
- DEMAND, D., SCHACK-KIRCHNER, H. & LANG, F. 2017. Assessment of diffusive phosphate supply in soils by microdialysis. *Journal of Plant Nutrition and Soil Science*, 180, 220-230.
- DENG, X., CHEN, Y., WEN, J., XU, Y., ZHU, J. & BIAN, Z. 2020. Polyaniline-TiO₂ composite photocatalysts for light-driven hexavalent chromium ions reduction. *Science Bulletin*, 65, 105-112.
- DETERDING, L. J., DIX, K., BURKA, L. T. & TOMER, K. B. 1992. Online coupling of in vivo microdialysis with tandem mass spectrometry. *Analytical Chemistry*, 64, 2636-2641.
- DHAL, B., DAS, N. N., THATOI, H. N. & PANDEY, B. D. 2013a. Characterizing toxic Cr(VI) contamination in chromite mine overburden dump and its bacterial remediation. *Journal of Hazardous Materials*, 260, 141-149.
- DHAL, B., THATOI, H., DAS, N. & PANDEY, B. D. 2010. Reduction of hexavalent chromium by *Bacillus* sp. isolated from chromite mine soils and characterization of reduced product. *Journal of Chemical Technology & Biotechnology*, 85, 1471-1479.
- DHAL, B., THATOI, H. N., DAS, N. N. & PANDEY, B. D. 2013b. Chemical and microbial remediation of hexavalent chromium from contaminated soil and mining/metallurgical solid waste: A review. *Journal of Hazardous Materials*, 250-251, 272-291.
- DI BONITO, M., BREWARD, N., CROUT, N., SMITH, B. & YOUNG, S. 2008. Overview of selected soil pore water extraction methods for the determination of potentially toxic elements in contaminated soils: operational and technical aspects. *Environmental Geochemistry*. Elsevier.
- DING, G., JIN, Z., HAN, Y., SUN, P., LI, G. & LI, W. 2019. Mitigation of chromium toxicity in *Arabidopsis thaliana* by sulfur supplementation. *Ecotoxicology and Environmental Safety*, 182, 109379.
- DRINČIĆ, A., ZULIANI, T., ŠČANČAR, J. & MILAČIČ, R. 2018. Determination of hexavalent Cr in river sediments by speciated isotope dilution inductively coupled plasma mass spectrometry. *Science of The Total Environment*, 637-638, 1286-1294.
- DUBE, B. K., TEWARI, K., CHATTERJEE, J. & CHATTERJEE, C. 2003. Excess chromium alters uptake and translocation of certain nutrients in *citrullus*. *Chemosphere*, 53, 1147-1153.
- DUFKA, M. & DOČEKAL, B. 2018. Characterization of urban particulate matter by diffusive gradients in thin film technique. *Journal of analytical methods in chemistry*, 2018.
- DURAN, C., OZDES, D., GUNDOGDU, A., IMAMOGLU, M. & SENTURK, H. B. 2011. Tea-industry waste activated carbon, as a novel adsorbent, for separation,

- preconcentration and speciation of chromium. *Analytica Chimica Acta*, 688, 75-83.
- ELLIS, D. I., BREWSTER, V. L., DUNN, W. B., ALLWOOD, J. W., GOLOVANOV, A. P. & GOODACRE, R. 2012. Fingerprinting food: current technologies for the detection of food adulteration and contamination. *Chemical Society Reviews*, 41, 5706-5727.
- EPA, U. S. 2008. Method 6800: elemental and speciated isotope dilution mass spectrometry. *Test Methods for Evaluating Solid Waste, Physical/Chemical Methods (SW-846)*.
- EPEL, B. L. 1994. Plasmodesmata: composition, structure and trafficking. *Signals and Signal Transduction Pathways in Plants*. Springer.
- ERNSTBERGER, H., DAVISON, W., ZHANG, H., TYE, A. & YOUNG, S. 2002. Measurement and Dynamic Modeling of Trace Metal Mobilization in Soils Using DGT and DIFS. *Environmental Science & Technology*, 36, 349-354.
- ERNSTBERGER, H., ZHANG, H., TYE, A., YOUNG, S. & DAVISON, W. 2005. Desorption kinetics of Cd, Zn, and Ni measured in soils by DGT. *Environmental science & technology*, 39, 1591-1597.
- ETTLER, V., MIHALJEVIČ, M., KŘÍBEK, B., MAJER, V. & ŠEBEK, O. 2011. Tracing the spatial distribution and mobility of metal/metalloid contaminants in Oxisols in the vicinity of the Nkana copper smelter, Copperbelt province, Zambia. *Geoderma*, 164, 73-84.
- FABREGAT-CABELLO, N., RODRÍGUEZ-GONZÁLEZ, P., CASTILLO, Á., MALHERBE, J., ROIG-NAVARRO, A. F., LONG, S. E. & ALONSO, J. I. G. 2012a. Fast and Accurate Procedure for the Determination of Cr(VI) in Solid Samples by Isotope Dilution Mass Spectrometry. *Environmental Science & Technology*, 46, 12542-12549.
- FABREGAT-CABELLO, N., RODRÍGUEZ-GONZÁLEZ, P., CASTILLO, A. N., MALHERBE, J., ROIG-NAVARRO, A. F., LONG, S. E. & ALONSO, J. I. G. 2012b. Fast and accurate procedure for the determination of Cr (VI) in solid samples by isotope dilution mass spectrometry. *Environmental Science & Technology*, 46, 12542-12549.
- FANG, Z., ZHAO, M., ZHEN, H., CHEN, L., SHI, P. & HUANG, Z. 2014. Genotoxicity of Tri- and Hexavalent Chromium Compounds In Vivo and Their Modes of Action on DNA Damage In Vitro. *PLOS ONE*, 9, e103194.
- FASSETT, J. D. & PAULSEN, P. J. 1989. Isotope dilution mass spectrometry for accurate elemental analysis. *Analytical Chemistry*, 61, 643A-[649A].
- FENDORF, S., WIELINGA, B. W. & HANSEL, C. M. 2000. Chromium Transformations in Natural Environments: The Role of Biological and Abiological Processes in Chromium(VI) Reduction. *International Geology Review*, 42, 691-701.
- FIGUEIREDO, E., SOARES, M. E., BAPTISTA, P., CASTRO, M. & BASTOS, M. L. 2007. Validation of an Electrothermal Atomization Atomic Absorption Spectrometry Method for Quantification of Total Chromium and Chromium(VI) in Wild Mushrooms and Underlying Soils. *Journal of Agricultural and Food Chemistry*, 55, 7192-7198.
- GETTENS, J. R. 1966. *Painting Materials: A Short Encyclopaedia*, Courier Dover Publications.
- GOMES, M. A. D. C., HAUSER-DAVIS, R. A., SUZUKI, M. S. & VITÓRIA, A. P. 2017. Plant chromium uptake and transport, physiological effects and recent advances in molecular investigations. *Ecotoxicology and Environmental Safety*, 140, 55-64.
- GRABARCZYK, M., KOROLCZUK, M. & TYSZCZUK, K. 2006. Extraction and determination of hexavalent chromium in soil samples. *Analytical and Bioanalytical Chemistry*, 386, 357-362.

- GRAFSTRÖM, A. & LISIC, J. 2016. Balanced and Spatially Balanced Sampling,. R package version 1. 5. 2.
- GUIDOTTI, L., ABAD, S. Q., RODRÍGUEZ-GONZÁLEZ, P., ALONSO, J. I. G. & BEONE, G. M. 2015. Quantification of Cr(VI) in soil samples from a contaminated area in northern Italy by isotope dilution mass spectrometry. *Environmental Science and Pollution Research*, 22, 17569-17576.
- GÜRLEYÜK, H. & WALLSCHLÄGER, D. 2001. Determination of chromium(II) and chromium(VI) using suppressed ion chromatography inductively coupled plasma mass spectrometry. *Journal of Analytical Atomic Spectrometry*, 16, 926-930.
- HAGENDORFER, H. & GOESSLER, W. 2008. Separation of chromium(III) and chromium(VI) by ion chromatography and an inductively coupled plasma mass spectrometer as element-selective detector. *Talanta*, 76, 656-661.
- HAMILTON, E. M., BARLOW, T. S., GOWING, C. J. B. & WATTS, M. J. 2015. Bioaccessibility performance data for fifty-seven elements in guidance material BGS 102. *Microchemical Journal*, 123, 131-138.
- HAMILTON, E. M., LARK, R. M., YOUNG, S. D., BAILEY, E. H., SAKALA, G. M., MASEKA, K. K. & WATTS, M. J. 2020a. Reconnaissance sampling and determination of hexavalent chromium in potentially-contaminated agricultural soils in Copperbelt Province, Zambia. *Chemosphere*, 247, 125984.
- HAMILTON, E. M., YOUNG, S. D., BAILEY, E. H., HUMPHREY, O. S. & WATTS, M. J. 2020b. Assessment of chromium species dynamics in root solutions using isotope tracers. *Journal of Trace Elements in Medicine and Biology*, 126514.
- HAMILTON, E. M., YOUNG, S. D., BAILEY, E. H. & WATTS, M. J. 2018. Chromium speciation in foodstuffs: A review. *Food Chemistry*, 250, 105-112.
- HAN, F. X., SRIDHAR, B. B. M., MONTS, D. L. & SU, Y. 2004. Phytoavailability and toxicity of trivalent and hexavalent chromium to *Brassica juncea*. *New Phytologist*, 162, 489-499.
- HARPER, M. P., DAVISON, W., ZHANG, H. & TYCH, W. 1998. Kinetics of metal exchange between solids and solutions in sediments and soils interpreted from DGT measured fluxes. *Geochimica et Cosmochimica Acta*, 62, 2757-2770.
- HASSAN, S. M. & GARRISON, A. W. 1996. Distribution of chromium species between soil and porewater. *Chemical Speciation & Bioavailability*, 8, 85-103.
- HEALTH AND SAFETY EXECUTIVE. 2013. *Chromium and you. Working with chromium – are you at risk?* [Online]. Available: <http://www.hse.gov.uk/pubns/indg346.pdf> [Accessed].
- HELLERICH, L. A. & NIKOLAIDIS, N. P. 2005. Studies of hexavalent chromium attenuation in redox variable soils obtained from a sandy to sub-wetland groundwater environment. *Water Research*, 39, 2851-2868.
- HILLIER, S., LUMSDON, D. G., BRYDSON, R. & PATERSON, E. 2007. Hydrogarnet: A Host Phase for Cr(VI) in Chromite Ore Processing Residue (COPR) and Other High pH Wastes. *Environmental Science & Technology*, 41, 1921-1927.
- HINGSTON, J. A., COLLINS, C. D., MURPHY, R. J. & LESTER, J. N. 2001. Leaching of chromated copper arsenate wood preservatives: a review. *Environmental Pollution*, 111, 53-66.
- HOWE, J. A., LOEPPERT, R. H., DEROSE, V. J., HUNTER, D. B. & BERTSCH, P. M. 2003. Localization and Speciation of Chromium in Subterranean Clover Using XRF, XANES, and EPR Spectroscopy. *Environmental Science & Technology*, 37, 4091-4097.
- HSU, K.-C., HSU, P.-F., CHEN, Y.-C., LIN, H.-C., HUNG, C.-C., CHEN, P.-C. & HUANG, Y.-L. 2016. Oxidative stress during bacterial growth characterized through

- microdialysis sampling coupled with HPLC/fluorescence detection of malondialdehyde. *Journal of Chromatography B*, 1019, 112-116.
- HUANG, W., JIAO, J., RU, M., BAI, Z., YUAN, H., BAO, Z. & LIANG, Z. 2018. Localization and Speciation of Chromium in *Coptis chinensis* Franch. using Synchrotron Radiation X-ray Technology and Laser Ablation ICP-MS. *Scientific Reports*, 8, 8603.
- HUANG, Z.-Y., YANG, C.-Y., ZHUANG, Z.-X., WANG, X.-R. & LEE, F. S. C. 2004. Determination of trace lead in the high salt matrix of comestible CaCO₃ by an isotope dilution method with detection by inductively coupled plasma-mass spectrometry. *Analytica Chimica Acta*, 509, 77-82.
- HUMPHREY, O., YOUNG, S., BAILEY, E., CROUT, N., ANDER, E., HAMILTON, E. & WATTS, M. 2019a. Investigating the use of microdialysis and SEC-UV-ICP-MS to assess iodine interactions in soil solution. *Chemosphere*, 229, 41-50.
- HUMPHREY, O., YOUNG, S., BAILEY, E., CROUT, N., ANDER, E., HAMILTON, E. & WATTS, M. 2019b. Iodine uptake, storage and translocation mechanisms in spinach (*Spinacia oleracea* L.). *Environmental Geochemistry and Health*, 41, 2145-2156.
- HUMPHREY, O. S., YOUNG, S. D., BAILEY, E. H., CROUT, N. M. J., ANDER, E. L., HAMILTON, E. M. & WATTS, M. J. 2019c. Iodine uptake, storage and translocation mechanisms in spinach (*Spinacia oleracea* L.). *Environmental Geochemistry and Health*, 41, 2145-2156.
- HUMPHREY, O. S., YOUNG, S. D., CROUT, N. M. J., BAILEY, E. H., ANDER, E. L. & WATTS, M. J. 2020. Short-Term Iodine Dynamics in Soil Solution. *Environmental Science & Technology*, 54, 1443-1450.
- INSELSBACHER, E., ÖHLUND, J., JÄMTGÅRD, S., HUSS-DANELL, K. & NÄSHOLM, T. 2011. The potential of microdialysis to monitor organic and inorganic nitrogen compounds in soil. *Soil Biology and Biochemistry*, 43, 1321-1332.
- INSELSBACHER, E., RIPKA, K., KLAUBAU, S., FEDOSYENKO, D., HACKL, E., GORFER, M., HOOD-NOVOTNY, R., VON WIRÉN, N., SESSITSCH, A. & ZECHMEISTER-BOLTENSTERN, S. 2009. A cost-effective high-throughput microcosm system for studying nitrogen dynamics at the plant-microbe-soil interface. *Plant and Soil*, 317, 293-307.
- JAMES, B. R. & BARTLETT, R. J. 1983a. Behavior of Chromium in Soils: V. Fate of Organically Complexed Cr(III) Added to Soil. *Journal of Environmental Quality*, 12, 169-172.
- JAMES, B. R. & BARTLETT, R. J. 1983b. Behavior of Chromium in Soils: VII. Adsorption and Reduction of Hexavalent Forms¹. *Journal of Environmental Quality*, 12.
- JAMES, B. R., PETURA, J. C., VITALE, R. J. & MUSSOLINE, G. R. 1995. Hexavalent Chromium Extraction from Soils: A Comparison of Five Methods. *Environmental Science & Technology*, 29, 2377-2381.
- JARDINE, P. M., STEWART, M. A., BARNETT, M. O., BASTA, N. T., BROOKS, S. C., FENDORF, S. & MEHLHORN, T. L. 2013. Influence of Soil Geochemical and Physical Properties on Chromium(VI) Sorption and Bioaccessibility. *Environmental Science & Technology*, 47, 11241-11248.
- JEN, J.-F., CHANG, C.-T. & YANG, T. C. 2001. On-line microdialysis-high-performance liquid chromatographic determination of aniline and 2-chloroaniline in polymer industrial wastewater. *Journal of Chromatography A*, 930, 119-125.
- JIN, G., CHENG, Q., FENG, J. & LI, F. 2008. On-Line Microdialysis Coupled to Analytical Systems. *Journal of Chromatographic Science*, 46, 276-287.

- JOBBY, R., JHA, P., YADAV, A. K. & DESAI, N. 2018. Biosorption and biotransformation of hexavalent chromium [Cr(VI)]: A comprehensive review. *Chemosphere*, 207, 255-266.
- KABATA-PENDIAS, A. 2010. *Trace Elements in Soils and Plants, Fourth Edition*, CRC Press.
- KANINGA, B. K., CHISHALA, B. H., MASEKA, K. K., SAKALA, G. M., LARK, M. R., TYE, A. & WATTS, M. J. 2019. Review: mine tailings in an African tropical environment—mechanisms for the bioavailability of heavy metals in soils. *Environmental Geochemistry and Health*.
- KASZYCKI, P., GABRYŚ, H., APPENROTH, K. J., JAGLARZ, A., SEDZIWIY, S., WALCZAK, T. & KOLOCZEK, H. 2005. Exogenously applied sulphate as a tool to investigate transport and reduction of chromate in the duckweed *Spirodela polyrhiza*. *Plant, Cell & Environment*, 28, 260-268.
- KENDIG, M., JEANJAQUET, S., ADDISON, R. & WALDROP, J. 2001. Role of hexavalent chromium in the inhibition of corrosion of aluminum alloys. *Surface and Coatings Technology*, 140, 58-66.
- KHAN, S., CAO, Q., ZHENG, Y., HUANG, Y. & ZHU, Y. 2008. Health risks of heavy metals in contaminated soils and food crops irrigated with wastewater in Beijing, China. *Environmental Pollution*, 152, 686-692.
- KHO, C. M., ENCHE AB RAHIM, S. K., AHMAD, Z. A. & ABDULLAH, N. S. 2017. A Review on Microdialysis Calibration Methods: the Theory and Current Related Efforts. *Molecular Neurobiology*, 54, 3506-3527.
- KIMBROUGH, D. E., COHEN, Y., WINER, A. M., CREELMAN, L. & MABUNI, C. 1999. A Critical Assessment of Chromium in the Environment. *Critical Reviews in Environmental Science and Technology*, 29, 1-46.
- KIRAN, K., KUMAR, K. S., PRASAD, B., SUVARDHAN, K., LEKKALA, R. B. & JANARDHANAM, K. 2008. Speciation determination of chromium(III) and (VI) using preconcentration cloud point extraction with flame atomic absorption spectrometry (FAAS). *Journal of Hazardous Materials*, 150, 582-586.
- KJELLSTRÖM, S., LINDBERG, S., LAURELL, T. & MARKO-VARGA, G. 2000. Development of a push-pull microdialysis sampling technique for the quantitative determination of proteins. *Chromatographia*, 52, 334-339.
- KORF, J., HUININK, K. D. & POSTHUMA-TRUMPIE, G. A. 2010. Ultraslow microdialysis and microfiltration for in-line, on-line and off-line monitoring. *Trends in Biotechnology*, 28, 150-158.
- KOVÁČIK, J., BABULA, P., HEDBAVNY, J. & KLEJDUS, B. 2014. Hexavalent chromium damages chamomile plants by alteration of antioxidants and its uptake is prevented by calcium. *Journal of Hazardous Materials*, 273, 110-117.
- KOVÁCS, R., BÉNI, Á., KAROSI, R., SÓGOR, C. & POSTA, J. 2007. Investigation of chromium content in foodstuffs and nutrition supplements by GFAAS and determination of changing Cr(III) to Cr(VI) during baking and toasting bread. *Food Chemistry*, 105, 1209-1213.
- KŘÍBEK, B., MAJER, V., KNĚSL, I., NYAMBE, I., MIHALJEVIČ, M., ETTLER, V. & SRACEK, O. 2014. Concentrations of arsenic, copper, cobalt, lead and zinc in cassava (*Manihot esculenta* Crantz) growing on uncontaminated and contaminated soils of the Zambian Copperbelt. *Journal of African Earth Sciences*, 99, 713-723.
- KŘÍBEK, B., MAJER, V., VESELOVSKÝ, F. & NYAMBE, I. 2010. Discrimination of lithogenic and anthropogenic sources of metals and sulphur in soils of the central-northern part of the Zambian Copperbelt Mining District: A topsoil vs. subsurface soil concept. *Journal of Geochemical Exploration*, 104, 69-86.

- KRZYSIK, M., GRAJETA, H., PRESCHA, A. & WEBER, R. 2011. Effect of cellulose, pectin and chromium(III) on lipid and carbohydrate metabolism in rats. *Journal of Trace Elements in Medicine and Biology*, 25, 97-102.
- LAMEIRAS, J., ELISA SOARES, M., LOURDES BASTOS, M. & FERREIRA, M. 1998. Quantification of total chromium and hexavalent chromium in UHT milk by ETAAS. *Analyst*, 123, 2091-2095.
- LANGÁRD, S. & COSTA, M. A. X. 2007. CHAPTER 24 - Chromium. In: NORDBERG, G. F., FOWLER, B. A., NORDBERG, M. & FRIBERG, L. T. (eds.) *Handbook on the Toxicology of Metals (Third Edition)*. Burlington: Academic Press.
- LARK, R., HAMILTON, E., KANINGA, B., MASEKA, K., MUTONDO, M., SAKALA, G. & WATTS, M. 2017a. Nested sampling and spatial analysis for reconnaissance investigations of soil: an example from agricultural land near mine tailings in Zambia. *European Journal of Soil Science*, 68, 605-620.
- LARK, R. M., HAMILTON, E. M., KANINGA, B., MASEKA, K. K., MUTONDO, M., SAKALA, G. M. & WATTS, M. J. 2017b. Nested sampling and spatial analysis for reconnaissance investigations of soil: an example from agricultural land near mine tailings in Zambia. *European Journal of Soil Science*, 68, 605-620.
- LEHMANN, J., KINYANGI, J. & SOLOMON, D. 2007. Organic matter stabilization in soil microaggregates: implications from spatial heterogeneity of organic carbon contents and carbon forms. *Biogeochemistry*, 85, 45-57.
- LEITNER, D., KLEPSCH, S., PTASHNYK, M., MARCHANT, A., KIRK, G., SCHNEPF, A. & ROOSE, T. 2010. A dynamic model of nutrient uptake by root hairs. *New Phytologist*, 185, 792-802.
- LENDINEZ, E., LORENZO, M. L., CABRERA, C. & LÓPEZ, M. C. 2001. Chromium in basic foods of the Spanish diet: seafood, cereals, vegetables, olive oils and dairy products. *Science of The Total Environment*, 278, 183-189.
- LI, S.-G., HOU, J., LIU, X.-H., CUI, B.-S. & BAI, J.-H. 2016. Morphological and transcriptional responses of *Lycopersicon esculentum* to hexavalent chromium in agricultural soil. *Environmental Toxicology and Chemistry*, 35, 1751-1758.
- LIGOWE, I. S., YOUNG, S. D., ANDER, E. L., KABAMBE, V., CHILIMBA, A. D. C., BAILEY, E. H., LARK, R. M. & NALIVATA, P. C. 2020. Selenium biofortification of crops on a Malawi Alfisol under conservation agriculture. *Geoderma*, 369, 114315.
- LIN, Y.-A., JIANG, S.-J., SAHAYAM, A. C. & HUANG, Y.-L. 2016. Speciation of chromium in edible animal oils after microwave extraction and liquid chromatography inductively coupled plasma mass spectrometry. *Microchemical Journal*, 128, 274-278.
- LJUNG, K., OOMEN, A., DUIT, M., SELINUS, O. & BERGLUND, M. 2007. Bioaccessibility of metals in urban playground soils. *Journal of Environmental Science and Health, Part A*, 42, 1241-1250.
- LÓPEZ-BUCIO, J., HERNÁNDEZ-MADRIGAL, F., CERVANTES, C., ORTIZ-CASTRO, R., CARREÓN-ABUD, Y. & MARTÍNEZ-TRUJILLO, M. 2014. Phosphate relieves chromium toxicity in *Arabidopsis thaliana* plants by interfering with chromate uptake. *BioMetals*, 27, 363-370.
- LOUBNA, E. F., HAFIDI, M., SILVESTRE, J., KALLERHOFF, J., MERLINA, G. & PINELLI, E. 2015. Efficiency of co-composting process to remove genotoxicity from sewage sludge contaminated with hexavalent chromium. *Ecological Engineering*, 82, 355-360.
- LUKASKI, H. C. 2007. Chapter 4 - Effects of chromium(III) as a nutritional supplement*,† A2 - Vincent, John B. *The Nutritional Biochemistry of Chromium (III)*. Amsterdam: Elsevier.

- LUKASKI, H. C., SIDERS, W. A. & PENLAND, J. G. 2007. Chromium picolinate supplementation in women: effects on body weight, composition, and iron status. *Nutrition*, 23, 187-195.
- LYTLE, C. M., LYTLE, F. W., YANG, N., QIAN, J.-H., HANSEN, D., ZAYED, A. & TERRY, N. 1998. Reduction of Cr(VI) to Cr(III) by Wetland Plants: Potential for In Situ Heavy Metal Detoxification. *Environmental Science & Technology*, 32, 3087-3093.
- MAHMOUD, M. E., YAKOUT, A. A., AHMED, S. B. & OSMAN, M. M. 2008. Speciation, selective extraction and preconcentration of chromium ions via alumina-functionalized-isatin-thiosemicarbazone. *Journal of Hazardous Materials*, 158, 541-548.
- MALHERBE, J., ISAURE, M.-P., SÉBY, F., WATSON, R. P., RODRIGUEZ-GONZALEZ, P., STUTZMAN, P. E., DAVIS, C. W., MAURIZIO, C., UNCETA, N. & SIEBER, J. R. 2011. Evaluation of hexavalent chromium extraction method EPA method 3060A for soils using XANES spectroscopy. *Environmental Science & Technology*, 45, 10492-10500.
- MAMATHA, P., VENKATESWARLU, G., SWAMY, A. V. N. & SAHAYAM, A. C. 2014. Microwave assisted extraction of Cr(iii) and Cr(vi) from soil/sediments combined with ion exchange separation and inductively coupled plasma optical emission spectrometry detection. *Analytical Methods*, 6, 9653-9657.
- MANDIWANA, K. L., PANICHEV, N. & PANICHEVA, S. 2011. Determination of chromium(VI) in black, green and herbal teas. *Food Chemistry*, 129, 1839-1843.
- MARKIEWICZ, B., KOMOROWICZ, I., SAJNÓG, A., BELTER, M. & BARAŁKIEWICZ, D. 2015. Chromium and its speciation in water samples by HPLC/ICP-MS – technique establishing metrological traceability: A review since 2000. *Talanta*, 132, 814-828.
- MARQUÉS, M. J., SALVADOR, A., MORALES-RUBIO, A. & GUARDIA, M. D. L. 2000. Chromium speciation in liquid matrices: a survey of the literature. *Fresenius' Journal of Analytical Chemistry*, 367, 601-613.
- MARTONE, N., RAHMAN, G. M. M., PAMUKU, M. & KINGSTON, H. M. S. 2013. Determination of Chromium Species in Dietary Supplements Using Speciated Isotope Dilution Mass Spectrometry with Mass Balance. *Journal of Agricultural and Food Chemistry*, 61, 9966-9976.
- MATERN, K. & MANSFELDT, T. 2016. Chromium Release from a COPR-Contaminated Soil at Varying Water Content and Redox Conditions. *Journal of Environmental Quality*, 45, 1259-1267.
- MATHEBULA, M. W., MANDIWANA, K. & PANICHEV, N. 2017. Speciation of chromium in bread and breakfast cereals. *Food Chemistry*, 217, 655-659.
- MCCARROLL, N., KESHAVA, N., CHEN, J., AKERMAN, G., KLIGERMAN, A. & RINDE, E. 2010. An evaluation of the mode of action framework for mutagenic carcinogens case study II: Chromium (VI). *Environmental and Molecular Mutagenesis*, 51, 89-111.
- MEIJA, J., YANG, L., CARUSO, J. A. & MESTER, Z. 2006. Calculations of double spike isotope dilution results revisited. *Journal of Analytical Atomic Spectrometry*, 21, 1294-1297.
- MERCER, T. G. & FROSTICK, L. E. 2012. Leaching characteristics of CCA-treated wood waste: A UK study. *Science of The Total Environment*, 427-428, 165-174.
- MERTZ, W. 1969. Chromium occurrence and function in biological systems. *Physiological Reviews*, 49, 163-239.

- MIDDLETON, D. R. S., WATTS, M. J., BERIRO, D. J., HAMILTON, E. M., LEONARDI, G. S., FLETCHER, T., CLOSE, R. M. & POLYA, D. A. 2017. Arsenic in residential soil and household dust in Cornwall, south west England: potential human exposure and the influence of historical mining. *Environmental Science: Processes & Impacts*, 19, 517-527.
- MILACIC, R. & STUPAR, J. 1994. Simultaneous determination of chromium(III) complexes and chromium(VI) by fast protein anion-exchange liquid chromatography-atomic absorption spectrometry. *Analyst*, 119, 627-632.
- MIRÓ, M., FITZ, W. J., SWOBODA, S. & WENZEL, W. W. 2010. In-situ sampling of soil pore water: Evaluation of linear-type microdialysis probes and suction cups at varied moisture contents. *Environmental Chemistry*, 7, 123-131.
- MIRÓ, M. & FRENZEL, W. 2004. Implantable flow-through capillary-type microdialyzers for continuous in situ monitoring of environmentally relevant parameters. *Analytical Chemistry*, 76, 5974-5981.
- MIRÓ, M. & FRENZEL, W. 2005. The potential of microdialysis as an automatic sample-processing technique for environmental research. *TrAC Trends in Analytical Chemistry*, 24, 324-333.
- MIRÓ, M., JIMOH, M. & FRENZEL, W. 2005. A novel dynamic approach for automatic microsampling and continuous monitoring of metal ion release from soils exploiting a dedicated flow-through microdialyser. *Analytical and Bioanalytical Chemistry*, 382, 396-404.
- MITANI, N. & MA, J. F. 2005. Uptake system of silicon in different plant species. *Journal of Experimental Botany*, 56, 1255-1261.
- MOHAN, D. & PITTMAN, C. U. 2006. Activated carbons and low cost adsorbents for remediation of tri- and hexavalent chromium from water. *Journal of Hazardous Materials*, 137, 762-811.
- MUELLER, C. W., WEBER, P. K., KILBURN, M. R., HOESCHEN, C., KLEBER, M. & PETTRIDGE, J. 2013. Advances in the analysis of biogeochemical interfaces: NanoSIMS to investigate soil microenvironments. *Advances in Agronomy*. Elsevier.
- NAGOURNEY, S. J., WILSON, S. A., BUCKLEY, B., KINGSTON, H. M. S., YANG, S.-Y. & LONG, S. E. 2008. Development of a standard reference material for Cr(vi) in contaminated soil. *Journal of Analytical Atomic Spectrometry*, 23, 1550-1554.
- NAKAONA, L., MASEKA, K. K., HAMILTON, E. M. & WATTS, M. J. 2019. Using human hair and nails as biomarkers to assess exposure of potentially harmful elements to populations living near mine waste dumps. *Environmental Geochemistry and Health*.
- NARIN, I., KARS, A. & SOYLAK, M. 2008. A novel solid phase extraction procedure on Amberlite XAD-1180 for speciation of Cr(III), Cr(VI) and total chromium in environmental and pharmaceutical samples. *Journal of Hazardous Materials*, 150, 453-458.
- NDUNG'U, K., DJANE, N.-K., MALCUS, F. & MATHIASSEN, L. 1999. Ultrasonic extraction of hexavalent chromium in solid samples followed by automated analysis using a combination of supported liquid membrane extraction and UV detection in a flow system. *Analyst*, 124, 1367-1372.
- NICKENS, K. P., PATIERNO, S. R. & CERYAK, S. 2010. Chromium genotoxicity: A double-edged sword. *Chemico-Biological Interactions*, 188, 276-288.
- NOVOTNIK, B., ZULIANI, T., MARTINČIČ, A., ŠČANČAR, J. & MILAČIČ, R. 2012. Preparation of Cr(VI) and Cr(III) isotopic spike solutions from ⁵⁰Cr and ⁵³Cr enriched oxides without the use of oxidizing and/or reducing agents. *Talanta*, 99, 83-90.

- NOVOTNIK, B., ZULIANI, T., SCANCAR, J. & MILACIC, R. 2013. Chromate in food samples: an artefact of wrongly applied analytical methodology? *Journal of Analytical Atomic Spectrometry*, 28, 558-566.
- NOVOTNIK, B., ZULIANI, T., ŠČANČAR, J. & MILAČIČ, R. 2015. Content of trace elements and chromium speciation in Neem powder and tea infusions. *Journal of Trace Elements in Medicine and Biology*, 31, 98-106.
- NRIAGU, J. O. & PACYNA, J. M. 1988. Quantitative assessment of worldwide contamination of air, water and soils by trace metals. *Nature*, 333, 134-139.
- OFFICE OF DIETARY SUPPLEMENTS: NATIONAL INSTITUTE OF HEALTH. 2013. *Dietary Supplement Fact Sheet: Chromium* [Online]. Available: <https://ods.od.nih.gov/factsheets/Chromium-HealthProfessional/> [Accessed].
- OH, Y. J., SONG, H., SHIN, W. S., CHOI, S. J. & KIM, Y.-H. 2007. Effect of amorphous silica and silica sand on removal of chromium(VI) by zero-valent iron. *Chemosphere*, 66, 858-865.
- OLIVEIRA, H. 2012a. Chromium as an Environmental Pollutant: Insights on Induced Plant Toxicity. *Journal of Botany*, 2012, 8.
- OLIVEIRA, H. 2012b. Chromium as an Environmental Pollutant: Insights on Induced Plant Toxicity. *Journal of Botany*, 2012, 375843.
- OZE, C., BIRD, D. K. & FENDORF, S. 2007. Genesis of hexavalent chromium from natural sources in soil and groundwater. *Proceedings of the National Academy of Sciences*, 104, 6544.
- OZE, C., FENDORF, S., BIRD, D. K. & COLEMAN, R. G. 2004. Chromium geochemistry in serpentinized ultramafic rocks and serpentine soils from the Franciscan Complex of California. *American Journal of Science*, 304, 67-101.
- PALUMBO-ROE, B., BANKS, V. J., BONSOR, H. C., HAMILTON, E. M. & WATTS, M. J. 2017. Limitations on the role of the hyporheic zone in chromium natural attenuation in a contaminated urban stream. *Applied Geochemistry*, 83, 108-120.
- PANICHEV, N., MANDIWANA, K., KATAEVA, M. & SIEBERT, S. 2005. Determination of Cr(VI) in plants by electrothermal atomic absorption spectrometry after leaching with sodium carbonate. *Spectrochimica Acta Part B: Atomic Spectroscopy*, 60, 699-703.
- PARK, J. H. 2020. Contrasting effects of Cr(III) and Cr(VI) on lettuce grown in hydroponics and soil: Chromium and manganese speciation. *Environmental Pollution*, 266, 115073.
- PEBESMA, E. J. 2004. Multivariable geostatistics in S: the gstat package. *The R Journal* 8(1) 204-218.
- PETTINE, M. & CAPRI, S. 2005. Digestion treatments and risks of Cr(III)–Cr(VI) interconversions during Cr(VI) determination in soils and sediments—a review. *Analytica Chimica Acta*, 540, 231-238.
- PHIRI, F. P., ANDER, E. L., BAILEY, E. H., CHILIMA, B., CHILIMBA, A. D., GONDWE, J., JOY, E. J., KALIMBIRA, A. A., KUMSSA, D. B. & LARK, R. M. 2019. The risk of selenium deficiency in Malawi is large and varies over multiple spatial scales. *Scientific reports*, 9, 1-8.
- PÖRTL, K., ZECHMEISTER-BOLTENSTERN, S., WANEK, W., AMBUS, P. & BERGER, T. W. 2007. Natural ^{15}N abundance of soil N pools and N_2O reflect the nitrogen dynamics of forest soils. *Plant and Soil*, 295, 79-94.
- PRADAS DEL REAL, A. E., PÉREZ-SANZ, A., LOBO, M. C. & MCNEAR, D. H. 2014a. The Chromium Detoxification Pathway in the Multimetal Accumulator *Silene vulgaris*. *Environmental Science & Technology*, 48, 11479-11486.

- PRADAS DEL REAL, A. E., PÉREZ-SANZ, A., LOBO, M. C. & MCNEAR JR, D. H. 2014b. The chromium detoxification pathway in the multimetal accumulator *Silene vulgaris*. *Environmental Science & Technology*, 48, 11479-11486.
- PRADO, F. E., HILAL, M., CHOCOBAR-PONCE, S., PAGANO, E., ROSA, M. & PRADO, C. 2016. Chapter 6 - Chromium and the Plant: A Dangerous Affair? A2 - Ahmad, Parvaiz. *Plant Metal Interaction*. Elsevier.
- PROCTOR, D. M., OTANI, J. M., FINLEY, B. L., PAUSTENBACH, D. J., BLAND, J. A., SPEIZER, N. & SARGENT, E. V. 2002. IS HEXAVALENT CHROMIUM CARCINOGENIC VIA INGESTION? A WEIGHT-OF-EVIDENCE REVIEW. *Journal of Toxicology and Environmental Health, Part A*, 65, 701-746.
- R CORE TEAM 2014. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing.
- RAHMAN, G. M. M. & KINGSTON, H. M. S. 2004. Application of Speciated Isotope Dilution Mass Spectrometry To Evaluate Extraction Methods for Determining Mercury Speciation in Soils and Sediments. *Analytical Chemistry*, 76, 3548-3555.
- RAHMAN, G. M. M., KINGSTON, H. M. S., TOWNS, T. G., VITALE, R. J. & CLAY, K. R. 2005. Determination of hexavalent chromium by using speciated isotope-dilution mass spectrometry after microwave speciated extraction of environmental and other solid materials. *Analytical and Bioanalytical Chemistry*, 382, 1111-1120.
- RIPP, J. 1996. Analytical detection limit guidance & laboratory guide for determining method detection limits. 1996: Wisconsin Department of Natural Resources. *Laboratory Certification Program*.
- RITCHIE, G. S. P. & SPOSITO, G. 2007. Speciation in Soils. *Chemical Speciation in the Environment*. Blackwell Science Ltd.
- RODRÍGUEZ-GONZÁLEZ, P., MARCHANTE-GAYÓN, J. M., GARCÍA ALONSO, J. I. & SANZ-MEDEL, A. 2005. Isotope dilution analysis for elemental speciation: a tutorial review. *Spectrochimica Acta Part B: Atomic Spectroscopy*, 60, 151-207.
- ROUSSEAU, T. C. C., SONKE, J. E., CHMELEFF, J., CANDAUDAP, F., LACAN, F., BOAVENTURA, G., SEYLER, P. & JEANDEL, C. 2013. Rare earth element analysis in natural waters by multiple isotope dilution – sector field ICP-MS. *Journal of Analytical Atomic Spectrometry*, 28, 573-584.
- SABTY-DAILY, R. A., LUK, K. K. & FROINES, J. R. 2002. The efficiency of alkaline extraction for the recovery of hexavalent chromium (CrVI) from paint samples and the effect of sample storage on CrVI recovery. *Analyst*, 127, 852-858.
- SALATAS, J. H., LOWNEY, Y. W., PASTOROK, R. A., NELSON, R. R. & RUBY, M. V. 2004. Metals that Drive Health-Based Remedial Decisions for Soils at U.S. Department of Defense Sites. *Human and Ecological Risk Assessment*, 18.
- SAMADDAR, P. & SEN, K. 2014. Cloud point extraction: A sustainable method of elemental preconcentration and speciation. *Journal of Industrial and Engineering Chemistry*, 20, 1209-1219.
- SCHAUMLÖFFEL, D., RUIZ ENCINAR, J. & ŁOBIŃSKI, R. 2003. Development of a Sheathless Interface between Reversed-Phase Capillary HPLC and ICPMS via a Microflow Total Consumption Nebulizer for Selenopeptide Mapping. *Analytical Chemistry*, 75, 6837-6842.
- SCHIAVON, M., GALLA, G., WIRTZ, M., PILON-SMITS, E. A. H., TELATIN, V., QUAGGIOTTI, S., HELL, R., BARCACCIA, G. & MALAGOLI, M. 2012. Transcriptome profiling of genes differentially modulated by sulfur and chromium identifies potential targets for phytoremediation and reveals a

- complex S–Cr interplay on sulfate transport regulation in *B. juncea*. *Journal of Hazardous Materials*, 239-240, 192-205.
- SÉBY, F., CASTETBON, A., ORTEGA, R., GUIMON, C., NIVEAU, F., BARROIS-LOUDIN, N., GARRAUD, H. & DONARD, O. 2008. Development of analytical procedures for the determination of hexavalent chromium in corrosion prevention coatings used in the automotive industry. *Analytical and Bioanalytical Chemistry*, 391, 587-597.
- SÉBY, F. & VACCHINA, V. 2018. Critical assessment of hexavalent chromium species from different solid environmental, industrial and food matrices. *TrAC Trends in Analytical Chemistry*, 104, 54-68.
- SHAHID, M., SHAMSHAD, S., RAFIQ, M., KHALID, S., BIBI, I., NIAZI, N. K., DUMAT, C. & RASHID, M. I. 2017. Chromium speciation, bioavailability, uptake, toxicity and detoxification in soil-plant system: A review. *Chemosphere*, 178, 513-533.
- SHANKER, A. K., CERVANTES, C., LOZA-TAVERA, H. & AVUDAINAYAGAM, S. 2005. Chromium toxicity in plants. *Environment International*, 31, 739-753.
- SHANKER, A. K., DJANAGUIRAMAN, M. & VENKATESWARLU, B. 2009. Chromium interactions in plants: current status and future strategies. *Metallomics*, 1, 375-383.
- SHAW, R., WILLIAMS, A. P. & JONES, D. L. 2014. Assessing Soil Nitrogen Availability using Microdialysis-Derived Diffusive Flux Measurements. *Soil Science Society of America Journal*, 78, 1797-1803.
- SHETAYA, W. H., YOUNG, S. D., WATTS, M. J., ANDER, E. L. & BAILEY, E. H. 2012. Iodine dynamics in soils. *Geochimica et Cosmochimica Acta*, 77, 457-473.
- SHRIVASTAVA, R., UPRETI, R. K., SETH, P. K. & CHATURVEDI, U. C. 2002. Effects of chromium on the immune system. *FEMS Immunology & Medical Microbiology*, 34, 1-7.
- SINGH, H. P., MAHAJAN, P., KAUR, S., BATISH, D. R. & KOHLI, R. K. 2013. Chromium toxicity and tolerance in plants. *Environmental Chemistry Letters*, 11, 229-254.
- SINGH, M., KUSHWAHA, B. K., SINGH, S., KUMAR, V., SINGH, V. P. & PRASAD, S. M. 2017. Sulphur alters chromium (VI) toxicity in *Solanum melongena* seedlings: Role of sulphur assimilation and sulphur-containing antioxidants. *Plant Physiology and Biochemistry*, 112, 183-192.
- SOARES, M. E., BASTOS, M. L. & FERREIRA, M. 2000. Selective Determination of Chromium (VI) in Powdered Milk Infant Formulas by Electrothermal Atomization Atomic Absorption Spectrometry after Ion Exchange. *Journal of AOAC International*, 83, 220-223.
- SOARES, M. E., VIEIRA, E. & BASTOS, M. D. L. 2010. Chromium Speciation Analysis in Bread Samples. *Journal of Agricultural and Food Chemistry*, 58, 1366-1370.
- SRIVASTAVA, S., PRAKASH, S. & SRIVASTAVA, M. M. 1999. Studies on mobilization of chromium with reference to its plant availability – Role of organic acids. *Biometals*, 12, 201-207.
- SRIVASTAVA, S., SRIVASTAVA, S., PRAKASH, S. & SRIVASTAVA, M. M. 1998. Fate of trivalent chromium in presence of organic acids: a hydroponic study on the tomato plant. *Chemical Speciation & Bioavailability*, 10, 147-150.
- STAHL, Y. & SIMON, R. 2013. Gated communities: apoplastic and symplastic signals converge at plasmodesmata to control cell fates. *Journal of Experimental Botany*, 64, 5237-5241.
- STASICKA, J. 2000. Chromium occurrence in the environment and methods of its speciation. *Environmental Pollution*, 92, 263-283.

- STASINOS, S. & ZABETAKIS, I. 2013. The uptake of nickel and chromium from irrigation water by potatoes, carrots and onions. *Ecotoxicology and Environmental Safety*, 91, 122-128.
- STOUT, M. D., HERBERT, R. A., KISSLING, G. E., COLLINS, B. J., TRAVLOS, G. S., WITT, K. L., MELNICK, R. L., ABDO, K. M., MALARKEY, D. E. & HOOTH, M. J. 2009. Hexavalent Chromium Is Carcinogenic to F344/N Rats and B6C3F1 Mice after Chronic Oral Exposure. *Environmental Health Perspectives*, 117, 716-722.
- SULYOK, M., MIRÓ, M., STINGEDER, G. & KOELLENSPERGER, G. 2005. The potential of flow-through microdialysis for probing low-molecular weight organic anions in rhizosphere soil solution. *Analytica Chimica Acta*, 546, 1-10.
- SUN, H., BROCATO, J. & COSTA, M. 2015. Oral Chromium Exposure and Toxicity. *Current Environmental Health Reports*, 2, 295-303.
- TAN, S. H. & HORLICK, G. 1987. Matrix-effect observations in inductively coupled plasma mass spectrometry. *Journal of Analytical Atomic Spectrometry*, 2, 745-763.
- TANG, H., WANG, X., SHUAI, W. & LIU, Y. 2016. Immobilization of Rare Earth Elements of the Mine Tailings Using Phosphates and Lime. *Procedia Environmental Sciences*, 31, 255-263.
- THATOI, H., DAS, S., MISHRA, J., RATH, B. P. & DAS, N. 2014. Bacterial chromate reductase, a potential enzyme for bioremediation of hexavalent chromium: A review. *Journal of Environmental Management*, 146, 383-399.
- TINKER, P. B. & NYE, P. H. 2000. *Solute movement in the rhizosphere*, Oxford University Press.
- TIWARI, S., DEB, M. K. & SEN, B. K. 2017. Cloud point extraction and diffuse reflectance-Fourier transform infrared spectroscopic determination of chromium(VI): A probe to adulteration in food stuffs. *Food Chemistry*, 221, 47-53.
- TORTO, N., MWATSETEZA, J. & SAWULA, G. 2002. A study of microdialysis sampling of metal ions. *Analytica Chimica Acta*, 456, 253-261.
- TÓTH, G., HERMANN, T., DA SILVA, M. R. & MONTANARELLA, L. 2016. Heavy metals in agricultural soils of the European Union with implications for food safety. *Environment International*, 88, 299-309.
- TREŠL, I. & QUÉTEL, C. R. 2005. Low Uncertainty Reverse Isotope Dilution ICP-MS Applied to Certifying an Isotopically Enriched Cd Candidate Reference Material: A Case Study. *Journal of the American Society for Mass Spectrometry*, 16, 708-716.
- ULUOZLU, O. D., TUZEN, M. & SOYLAK, M. 2009. Speciation and separation of Cr(VI) and Cr(III) using coprecipitation with Ni²⁺/2-Nitroso-1-naphthol-4-sulfonic acid and determination by FAAS in water and food samples. *Food and Chemical Toxicology*, 47, 2601-2605.
- UNCETA, N., SÉBY, F., MALHERBE, J. & DONARD, O. F. X. 2010. Chromium speciation in solid matrices and regulation: a review. *Analytical and Bioanalytical Chemistry*, 397, 1097-1111.
- UNESCO. & FAO. 1974. *Soil Map of the World: 1: 5000000*, Unesco.
- UNITED STATES ENVIRONMENTAL PROTECTION AGENCY 1992. Chromium, Hexavalent (Colorimetric). Washington, D.C.
- UNITED STATES ENVIRONMENTAL PROTECTION AGENCY 1996. Alkaline Digestion for Hexavalent Chromium. Washington, D.C.
- UTERMANN, J., DÜWEL, O. & NAGEL, I. 2006. Background values in European soils and sewage sludges. Results of a JRC-coordinated study on background values

- (Part II). European Commission DG-JRC., EUR 22265 EN. Luxembourg:Office for Official Publications of the European Communities.
- VACCHINA, V., DE LA CALLE, I. & SÉBY, F. 2015. Cr(VI) speciation in foods by HPLC-ICP-MS: investigation of Cr(VI)/food interactions by size exclusion and Cr(VI) determination and stability by ion-exchange on-line separations. *Analytical and Bioanalytical Chemistry*, 407, 3831-3839.
- VÁZQUEZ, M. D., POSCHENRIEDER, C. & BARCELÓ, J. 1987. Chromium VI Induced Structural and Ultrastructural Changes in Bush Bean Plants (*Phaseolus vulgaris* L.). *Annals of Botany*, 59, 427-438.
- VEIHMEYER, F. J. & HENDRICKSON, A. H. 1949. Methods of measuring field capacity and permanent wilting percentage of soils. *Soil Science*, 68, 75-94.
- VIEIRA, E., SOARES, M. E., KOZIOR, M., KREJPCIO, Z., FERREIRA, I. M. P. L. V. O. & BASTOS, M. L. 2014. Quantification of Total and Hexavalent Chromium in Lager Beers: Variability between Styles and Estimation of Daily Intake of Chromium from Beer. *Journal of Agricultural and Food Chemistry*, 62, 9195-9200.
- VIG, K., MEGHARAJ, M., SETHUNATHAN, N. & NAIDU, R. 2003. Bioavailability and toxicity of cadmium to microorganisms and their activities in soil: a review. *Advances in Environmental Research*, 8, 121-135.
- VOGL, J. 2007. Characterisation of reference materials by isotope dilution mass spectrometry. *Journal of Analytical Atomic Spectrometry*, 22, 475-492.
- VON DER HEYDEN, C. J. & NEW, M. G. 2004. Groundwater pollution on the Zambian Copperbelt: deciphering the source and the risk. *Science of The Total Environment*, 327, 17-30.
- WARREN, C. R. 2018. Development of online microdialysis-mass spectrometry for continuous minimally invasive measurement of soil solution dynamics. *Soil Biology and Biochemistry*, 123, 266-275.
- WATTS, M. J., MIDDLETON, D. R. S., MARRIOTT, A. L., HUMPHREY, O. S., HAMILTON, E. M., GARDNER, A., SMITH, M., MCCORMACK, V. A., MENYA, D., MUNISHI, M. O., MMBAGA, B. T. & OSANO, O. 2019. Source apportionment of micronutrients in the diets of Kilimanjaro, Tanzania and Counties of Western Kenya. *Scientific Reports*, 9, 14447.
- WAZNE, M., JAGUPILLA, S. C., MOON, D. H., JAGUPILLA, S. C., CHRISTODOULATOS, C. & KIM, M. G. 2007. Assessment of calcium polysulfide for the remediation of hexavalent chromium in chromite ore processing residue (COPR). *Journal of Hazardous Materials*, 143, 620-628.
- WEIGHTS, S. A. 2013. Commission on Isotopic Abundances and Atomic Weights.
- WEISSENSTEIN, K. & SINKALA, T. 2011. Soil pollution with heavy metals in mine environments, impact areas of mine dumps particularly of gold- and copper mining industries in Southern Africa. *Arid Ecosystems*, 1, 53.
- WIND, M., EISENMENGER, A. & LEHMANN, W. D. 2002. Modified direct injection high efficiency nebulizer with minimized dead volume for the analysis of biological samples by micro- and nano-LC-ICP-MS. *Journal of Analytical Atomic Spectrometry*, 17, 21-26.
- WOLLE, M. M., RAHMAN, G. M. M., SKIP KINGSTON, H. M. & PAMUKU, M. 2014. Optimization and validation of strategies for quantifying chromium species in soil based on speciated isotope dilution mass spectrometry with mass balance. *Journal of Analytical Atomic Spectrometry*, 29, 1640-1647.
- WU, L. E., LEVINA, A., HARRIS, H. H., CAI, Z., LAI, B., VOGT, S., JAMES, D. E. & LAY, P. A. 2016. Carcinogenic Chromium(VI) Compounds Formed by Intracellular

- Oxidation of Chromium(III) Dietary Supplements by Adipocytes. *Angewandte Chemie International Edition*, 55, 1742-1745.
- WU, S., HU, Y., ZHANG, X., SUN, Y., WU, Z., LI, T., LV, J., LI, J., ZHANG, J., ZHENG, L., HUANG, L. & CHEN, B. 2018. Chromium detoxification in arbuscular mycorrhizal symbiosis mediated by sulfur uptake and metabolism. *Environmental and Experimental Botany*, 147, 43-52.
- YOUNG, S., ZHANG, H., TYE, A., MAXTED, A., THUMS, C. & THORNTON, I. 2005. Characterizing the availability of metals in contaminated soils. I. The solid phase: sequential extraction and isotopic dilution. *Soil Use and Management*, 21, 450-458.
- YU, Q., TANG, C., CHEN, Z. & KUO, J. 1999. Extraction of apoplastic sap from plant roots by centrifugation. *The New Phytologist*, 143, 299-304.
- ZAREBANADKOUKI, M., TRTIK, P., HAYAT, F., CARMINATI, A. & KAESTNER, A. 2019. Root water uptake and its pathways across the root: quantification at the cellular scale. *Scientific Reports*, 9, 12979.
- ZAYED, A., LYTLE, C. M., QIAN, J.-H. & TERRY, N. 1998a. Chromium accumulation, translocation and chemical speciation in vegetable crops. *Planta*, 206, 293-299.
- ZAYED, A., LYTLE, M. C., QIAN, J.-H. & TERRY, N. 1998b. Chromium accumulation, translocation and chemical speciation in vegetable crops. *Planta*, 206, 293-299.
- ZAYED, A. M. & TERRY, N. 2003. Chromium in the environment: factors affecting biological remediation. *Plant and Soil*, 249, 139-156.
- ZENG, F., CHEN, S., MIAO, Y., WU, F. & ZHANG, G. 2008. Changes of organic acid exudation and rhizosphere pH in rice plants under chromium stress. *Environmental Pollution*, 155, 284-289.
- ZHANG, Y., LI, X., GREGORICH, E. G., MCLAUGHLIN, N. B., ZHANG, X., GUO, Y., GAO, Y. & LIANG, A. 2019. Evaluating storage and pool size of soil organic carbon in degraded soils: Tillage effects when crop residue is returned. *Soil and Tillage Research*, 192, 215-221.
- ZHAO, X., SOBECKY, P. A., ZHAO, L., CRAWFORD, P. & LI, M. 2016. Chromium(VI) transport and fate in unsaturated zone and aquifer: 3D Sandbox results. *Journal of Hazardous Materials*, 306, 203-209.
- ZHU, X., HU, B., JIANG, Z. & LI, M. 2005. Cloud point extraction for speciation of chromium in water samples by electrothermal atomic absorption spectrometry. *Water Research*, 39, 589-595.