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ChapterTitle	Heavy Metal Bioavailability and Bioaccessibility in Soil	
Chapter Sub-Title		
Chapter CopyRight - Year	Humana Press, a part of Springer Science+Business Media, LLC 2009 (This will be the copyright line in the final PDF)	
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Abstract	This chapter considers the use of a variety of approaches to assess either the bioavailability or the bioaccessibility of metals in soil. The bioavailability of metals from soils is considered with respect to a series of single-extraction methods, including the use of ethylenediaminetetraacetic acid (EDTA), acetic acid, diethylenetriaminepentaacetic acid (DTPA), ammonium nitrate, calcium chloride and sodium nitrate. Then, a procedure for the recovery of metals using a three-stage sequential extraction protocol is described. Two alternate approaches for assessing the environmental health risk to humans by undertaking in vitro gastrointestinal extraction (also known as the physiologically based extraction test, PBET) are considered. Finally, two acid digestion protocols that allow the pseudo-total metal content of samples to be assessed are provided.In all cases details of how the different approaches can be performed are provided, including the specific reagents required (and their preparation), details of the different extraction and acid digestion protocols to be followed and suitable analytical details to allow the measurement of metals by inductively coupled plasma mass spectrometry (ICP-MS) with/without a collision/reaction cell. A detailed Notes section provides experimental details to guide the reader through some of the practical aspects of the procedures. Finally, some experimental results are provided as evidence of the suitability of the approaches described including single-extraction data, using EDTA and acetic acid, for metals in CRM BCR 700. In addition, in vitro gastrointestinal extraction data are provided for metals in CRM SRM 1570A (spinach leaves). The influence of time on the intestinal fluid phase on the recovery of metals in CRM SRM 1570A (spinach leaves) and CRM INCT-TL-1 (tea leaves) is investigated, as well as the repeatability in terms of recovery of metals from soil over a 3-week period by in vitro gastrointestinal extraction.

Keywords (separated by '-')	Single-extraction methods - sequential extraction method - physiologically based extraction test (PBET) - in vitro gastrointestinal extraction - inductively coupled plasma mass spectrometry (ICP-MS)

Chapter 2

Heavy Metal Bioavailability and Bioaccessibility in Soil

John Richard Dean

Abstract

This chapter considers the use of a variety of approaches to assess either the bioavailability or the bioaccessibility of metals in soil. The bioavailability of metals from soils is considered with respect to a series of single-extraction methods, including the use of ethylenediaminetetraacetic acid (EDTA), acetic acid, diethylenetriaminepentaacetic acid (DTPA), ammonium nitrate, calcium chloride and sodium nitrate. Then, a procedure for the recovery of metals using a three-stage sequential extraction protocol is described. Two alternate approaches for assessing the environmental health risk to humans by undertaking in vitro gastrointestinal extraction (also known as the physiologically based extraction test, PBET) are considered. Finally, two acid digestion protocols that allow the pseudo-total metal content of samples to be assessed are provided.

In all cases details of how the different approaches can be performed are provided, including the specific reagents required (and their preparation), details of the different extraction and acid digestion protocols to be followed and suitable analytical details to allow the measurement of metals by inductively coupled plasma mass spectrometry (ICP-MS) with/without a collision/reaction cell. A detailed Notes section provides experimental details to guide the reader through some of the practical aspects of the procedures. Finally, some experimental results are provided as evidence of the suitability of the approaches described including single-extraction data, using EDTA and acetic acid, for metals in CRM BCR 700. In addition, in vitro gastrointestinal extraction data are provided for metals in CRM SRM 1570A (spinach leaves). The influence of time on the intestinal fluid phase on the recovery of metals in CRM SRM 1570A (spinach leaves) and CRM INCT-TL-1 (tea leaves) is investigated, as well as the repeatability in terms of recovery of metals from soil over a 3-week period by in vitro gastrointestinal extraction.

Key words: Single-extraction methods, sequential extraction method, physiologically based extraction test (PBET), in vitro gastrointestinal extraction, inductively coupled plasma mass spectrometry (ICP-MS).

1. Introduction

The release of metals from soil is normally accomplished using heat and concentrated acids (in a process termed acid digestion)

S.P. Cummings (ed.), *Bioremediation*, Methods in Molecular Biology 599,
DOI 10.1007/978-1-60761-439-5_2, © Humana Press, a part of Springer Science+Business Media, LLC 2010

(1). The aim of this approach is to destroy the soil matrix releasing metals into solution. In reality, depending upon the choice of acid (or acid combination) this may or may not be possible, but the approach is nevertheless used to determine the metal (pseudo)total in the soil matrix. Approaches to assess the metal bioavailability and bioaccessibility are available (2). In the case of metal bioavailability, the approaches are based on the use of selective chemical extractants to liberate the metals from the soil matrix by overcoming specific interactions. These approaches are based on single- or sequential extraction methods, which were originally developed by the Standard, Measurements and Testing Programme (SM & T – formerly BCR) of the European Union (3–5). Single-extraction methods are based on the use of ethylenediaminetetraacetic acid (EDTA), acetic acid or diethylenetriaminepentaacetic acid (DTPA) as well as some other reagents, whereas the sequential extraction method uses specific reagents to assess the exchangeable, reducible and oxidisable fractions of metals in soil. In the case of metal (oral) bioaccessibility, the approach is based on the use of reagents that seek to mimic the human digestive system (2). This method is often described as either in vitro (simulated) gastrointestinal extraction or the physiologically based extraction test (PBET). In each case the use of specific extraction scenarios to provide an estimation of the environmental risk to humans and plants from heavy metal contaminated soil is done.

2. Materials

2.1. Extraction Reagents for Single-Extraction Methods

1. 50 mM ethylenediaminetetraaceticacid (EDTA): In a fume cupboard add 146 +/− 0.05 g of EDTA (free acid) to 800 +/− 20 mL of distilled water (*see* **Note 1**). To aid dissolution of EDTA, stir in 130 +/− 5 mL of saturated ammonia solution (prepared by bubbling ammonia gas into distilled water). Continue to add the ammonia solution until all the EDTA has dissolved. The resultant solution should be filtered, if necessary, through a filter paper of porosity 1.4–2.0 μm into a pre-cleaned 10 L polyethylene bottle and then diluted to 9.0 +/− 0.5 L with distilled water. Adjust the pH to 7.00 +/− 0.05 by addition of a few drops of either ammonia or concentrated hydrochloric acid, as appropriate. The solution should then be made up to 10 L with distilled water to obtain an EDTA solution of 50 mM. Analyse a sample of each fresh batch of EDTA solution for its metal impurity content (*see* **Notes 2 and 3**).

2. 0.43 M acetic acid: In a fume cupboard add 250 +/− 2 mL of glacial acetic acid (AnalaR or similar) to approximately 5 L of distilled water in a pre-cleaned 10 L polyethylene bottle and make up to 10 L with distilled water. Analyse a sample of each fresh batch of acetic acid solution for its metal impurity content (*see* **Notes 2 and 3**).
3. 5 mM diethylenetriaminepentaacetic acid (DTPA): In a fume cupboard dissolve 149.2 g triethanolamine (0.01 M), 19.67 g DTPA (5 mM) and 14.7 g calcium chloride in approximately 200 mL distilled water. Allow the DTPA to dissolve and then dilute to 9 L. Adjust the pH to 7.3 +/− 0.5 with concentrated HCl while stirring and then dilute to 10 L in distilled water. Analyse a sample of each fresh batch of DTPA solution for its metal impurity content (*see* **Notes 2 and 3**).
4. 1 M ammonium nitrate (NH_4NO_3): In a fume cupboard dissolve 80.04 g of NH_4NO_3 in water, then make up to 1 L with water. Analyse a sample of each fresh batch of NH_4NO_3 solution for its metal impurity content (*see* **Notes 2 and 3**).
5. 0.01 M calcium chloride: In a fume cupboard dissolve 1.470 g of $CaCl_2 2H_2O$ in water, then make up to 1 L with water. Verify that the Ca concentration is 400 +/− 10 mg/L by EDTA titration. Analyse a sample of each fresh batch of $CaCl_2$ solution for its metal impurity content (*see* **Notes 2 and 3**).
6. 0.1 M sodium nitrate ($NaNO_3$): In a fume cupboard dissolve 8.50 g of $NaNO_3$ in water, then make up to 1 L with water. Analyse a sample of each fresh batch of $NaNO_3$ solution for its metal impurity content (*see* **Notes 2, 3 and 4**).

2.2. Extraction Reagents for Sequential Extraction Method

1. Solution A: 0.11 M acetic acid. Add in a fume cupboard 25 +/− 0.1 mL of glacial acetic acid to approximately 0.5 L of water in a 1 L polyethylene bottle and make up to 1 L with water. Take 250 mL of this solution (acetic acid 0.43 M) and dilute to 1 L with water to obtain an acetic acid solution of 0.11 M. Analyse a sample of each fresh batch of solution A for its metal impurity content (*see* **Note 2**).
2. Solution B: 0.5 M hydroxylamine hydrochloride or hydroxyammonium chloride. Dissolve 34.75 g of hydroxylamine hydrochloride in 400 mL of water. Transfer to a 1 L volumetric flask and add 25 mL of 2 M HNO_3 (prepared by weighing from a concentration solution) (the pH should be 1.5). Make up to 1 L with water. Prepare this solution on the same day as the extraction is carried out. Analyse a sample of each fresh batch of solution B for its metal impurity content (*see* **Note 2**).

3. Solution C: (8.8 M hydrogen peroxide (300 mg/g). Use H_2O_2 as supplied by the manufacturer, i.e. acid-stabilized to pH 2–3. Analyse a sample of each fresh batch of solution C for its metal impurity content (*see* **Note 2**).
4. Solution D: (1 M ammonium acetate). Dissolve 77.08 g of ammonium acetate in 800 mL of water. Adjust to pH 2 +/− 0.1 with concentrated HNO_3 and make up to 1 L with water. Analyse a sample of each fresh batch of solution D for its metal impurity content (*see* **Note 2**).

2.3. Extraction Reagents for In vitro Gastrointestinal Extraction: Approach 1

1. Gastric solution: 1.25 g pepsin (1 Anson unit/g lactose as diluents), 0.5 g sodium malate, 0.5 g sodium citrate, 420 μL lactic acid and 500 μL acetic acid made up to 1 L with water, adjusted to pH 2.5 with concentrated HCl.
2. Intestinal solution: 52.5 mg bile salts (bovine) and 15 mg pancreatin (pig) added into the sample−gastric solution mixture and the pH adjusted to pH 7.0 with saturated $NaHCO_3$.

2.4. Extraction Reagents for In vitro Gastrointestinal Extraction: Approach 2 Simulated Saliva Fluid

1. First add 145 mg of α-amylase (bacillus species), 50.0 mg mucin and 15.0 mg uric acid to a 2 L HDPE screw-top bottle.
2. Separately add 896 mg of KCl, 888 mg NaH_2PO_4, 200 mg KSCN, 570 mg Na_2SO_4, 298 mg NaCl and 1.80 mL of 1.0 M HCl into a 500 mL volume container and make up to the mark with water (inorganic saliva components).
3. In a second 500 mL volume container, add 200 mg urea and make up to the mark with water (organic saliva components).
4. Then, simultaneously pour 500 mL of inorganic and 500 mL of organic saliva components into the 2 L HDPE screw-top bottle.
5. Shake the entire contents of the screw-top bottle thoroughly.
6. Measure the pH of this solution (gastric-simulated fluid). The pH should be 6.5 ± 0.5. If necessary, adjust the pH by adding either 1.0 M NaOH or 37% HCl.

2.5. Simulated Gastric Fluid

1. First add 1000 mg of bovine serum albumin, 3000 mg mucin and 1000 mg pepsin to a 2 L HDPE screw-top bottle.
2. Separately add 824 mg of KCl, 266 mg NaH_2PO_4, 400 mg $CaCl_2$, 306 mg NH_4Cl, 2752 mg NaCl and 8.30 mL of 37% HCl into a 500 mL volume container and make up to the mark with water (inorganic gastric components).
3. In a second 500 mL volume container, add 650 mg glucose, 20.0 mg glucuronic acid, 85.0 mg urea and

330 mg glucosaminehydrochloride and make up to the mark with water (organic gastric components).

4. Then, simultaneously pour 500 mL of inorganic and 500 mL of organic components into the 2 L HDPE screw-top bottle.
5. Shake the entire contents of the screw-top bottle thoroughly.
6. Measure the pH of this solution (gastric-simulated fluid). The pH should be within the range 0.9–1.0. If necessary, adjust the pH to this range (0.9–1.0) by adding either 1.0 M NaOH or 37% HCl.
7. Check that the combination of mixed saliva fluid (1 mL) and gastric fluid (1.5 mL) is in the pH 1.2–1.4. If the combined mixture is not within this range, it is necessary to adjust the pH of the gastric fluid by adding either 1.0 M NaOH or 37% HCl.
8. Re-check that the combination of mixed saliva fluid (1 mL) and gastric fluid (1.5 mL) is in the pH 1.2–1.4.

2.6. Simulated Duodenal Fluid

1. First add 200 mg of $CaCl_2$, 1000 mg bovine serum albumin, 3000 mg pancreatin and 500 mg lipase to a 2 L HDPE screw-top bottle.
2. Separately add 564 mg of KCl, 80 mg KH_2PO_4, 50.0 mg $MgCl_2$, 5607 mg $NaHCO_3$, 7012 mg NaCl and 180 μL of 37% HCl into a 500 mL volume container and make up to the mark with water (inorganic duodenal components).
3. In a second 500 mL volume container, add 100 mg urea and make up to the mark with water (organic duodenal components).
4. Then, simultaneously pour 500 mL of inorganic and 500 mL of organic duodenal components into the 2 L HDPE screw-top bottle.
5. Shake the entire contents of the screw-top bottle thoroughly.
6. Measure the pH of this solution (simulated duodenal fluid). The pH should be within the range 7.4 ± 0.2. If necessary, adjust the pH of the duodenal fluid by adding either 1.0 M NaOH or 37% HCl.

2.7. Simulated Bile Fluid

1. First add 222 mg of $CaCl_2$, 1800 mg bovine serum albumin and 6000 mg bile to a 2 L HDPE screw-top bottle.
2. Separately add 376 mg of KCl, 5785 mg $NaHCO_3$, 5259 mg NaCl and 180 μL of 37% HCl into a 500 mL volume container and make up to the mark with water (inorganic bile components).

3. Into a second 500 mL volume container, add 250 mg urea and make up to the mark with water (organic bile components).
4. Then, simultaneously pour 500 mL of inorganic and 500 mL of organic bile components into the 2 L HDPE screw-top bottle.
5. Shake the entire contents of the screw-top bottle thoroughly.
6. Allow the solution to stand for approximately 1 h, at room temperature, to allow for complete dissolution of solid reagents.
7. Measure the pH of this solution (simulated bile fluid). The pH should be within the range 8.0 ± 0.2. If necessary, adjust the pH of the duodenal fluid by adding either 1.0 M NaOH or 37% HCl.
8. Check that the combination of saliva fluid (1.0 mL), gastric fluid (1.5 mL), 3.0 mL duodenal fluid and 1.0 mL bile fluid is in the pH 6.3 ± 0.5. If the combined mixture is not within this range, it is necessary to adjust the pH of the duodenal fluid by adding either 1.0 M NaOH or 37% HCl.
9. Re-check that the combination of saliva fluid (1.0 mL), gastric fluid (1.5 mL), 3.0 mL duodenal fluid and 1.0 mL bile fluid is in the pH 6.3 ± 0.5.

2.8. Instrumentation for Metal Analysis

1. All metal measurements were made using an inductively coupled plasma mass spectrometer (ICP-MS, XSeries II, Thermo Electron Corporation, Cheshire, UK).
2. A multi-element standard is used for K, Ca, Mg, Na, Cr, Mn, Fe, Ni, Cu, Zn, Mo, Cd and Pb and internal standard solutions for Sc, In and Tb (SPEXCertiPrep, Middlesex, UK).

3. Methods

3.1. Chemical-Selective Extraction for Single-Extraction Methods

Chemical-selective extractions of the soil are carried out in order to assess the metal bioavailability. The main procedures identified for the extraction of metals using single-extraction methods are based on the use of ethylenediaminetetraacetic acid, acetic acid or diethylenetriaminepentaacetic acid. However, other reagents are also used and include the use of ammonium nitrate, calcium chloride and sodium nitrate. The extraction protocols, using EDTA, CH_3COOH and $CaCl_2$, used are based on those developed by the Standard, Measurements and Testing Program (formerly BCR) of the European Community (3–5) and subsequently re-evaluated for EDTA and acetic acid (6).

3.1.1. Ethylenediaminetetraacetic Acid Extraction

1. 2 g of soil sample is weighed into a 50 mL Sarstedt extraction tube and 20 mL of 0.05 M EDTA (pH 7.0) is added (*see* **Note 5**).
2. The mixture is shaken in an end-over-end shaker at 30 rpm for 1 h at ambient temperature (20 ± 2°C) (*see* **Note 6**).
3. Then centrifuge the mixture for 10 min at 3000*g*.
4. Remove the supernatant with a pipette and store in a polyethylene bottle at 4°C.
5. Prior to analysis, re-homogenise the sample by manually shaking for 5 min.
6. Analyse by ICP-MS (*see* **Notes 10 and 11**).

1. Example results for the EDTA extraction of nine elements from a certified reference material (BCR 700) are shown in **Table 2.1**.

Table 2.1
Example results for selected single-extraction protocols

	EDTA extraction		CH_3COOH extraction	
Element	CRM organic-rich soil (BCR 700) (mg/kg)	Concentrations (mg/kg) Mean ± SD, *n* = 6	CRM organic-rich soil (BCR 700) (mg/kg)	Concentrations (mg/kg) Mean ± SD, *n* = 6
Cr	10.1 ± 0.9	9.2 ± 0.2	19.0 ± 1.1	20.5 ± 0.7
Mn	na	146 ± 6	na	266 ± 19
Fe	na	1224 ± 95	na	33.0 ± 1.8
Ni	53.2 ± 2.8	51.5 ± 1.0	99.0 ± 5.1	102.8 ± 2.6
Cu	89.4 ± 2.8	91.9 ± 1.3	36.3 ± 1.6	37.3 ± 2.6
Zn	510 ± 17	455 ± 5	719 ± 24	715.7 ± 55.5
Mo	na	1.10 ± 0.08	na	0.06 ± 0.01
Cd	65.2 ± 3.5	65.7 ± 5.1	67.5 ± 2.8	67.1 ± 2.5
Pb	103 ± 5	101.9 ± 0.9	4.85 ± 0.38	4.82 ± 0.44

na = not available

3.1.2. Acetic Acid Extraction

1. 1 g of soil sample is weighed into a 50 mL Sarstedt extraction tube and 40 mL of 0.43 M CH_3COOH is added (*see* **Note 5**).
2. The mixture is shaken in an end-over-end shaker at 30 rpm for 16 h at ambient temperature (20 ± 2°C) (*see* **Note 6**).
3. Then centrifuge the mixture for 10 min at 3000*g*.
4. Remove the supernatant with a pipette and store in a polyethylene bottle at 4°C.

5. Prior to analysis, re-homogenise the sample by manually shaking for 5 min.
6. Analyse by ICP-MS (*see* **Notes 10 and 11**).

Example results for the acetic acid extraction of nine elements from a certified reference material (BCR 700) are shown in **Table 2.1**.

3.1.3. Diethylenetriaminepentaacetic Acid Extraction

1. 2 g of soil sample is weighed into a 50 mL Sarstedt extraction tube and 4 mL of 0.005 M DTPA is added (*see* **Note 5**).
2. The mixture is shaken in an end-over-end shaker at 30 rpm for 2 h at ambient temperature (20 ± 2°C) (*see* **Note 6**).
3. Then centrifuge the mixture for 10 min at 3000*g*.
4. Remove the supernatant with a pipette and store in a polyethylene bottle at 4°C.
5. Prior to analysis, re-homogenise the sample by manually shaking for 5 min.
6. Analyse by ICP-MS (*see* **Notes 10 and 11**).

3.1.4. Calcium Chloride Extraction

1. 2 g of soil sample is weighed into a 50 mL Sarstedt extraction tube and 20 mL of 0.01 M $CaCl_2$ is added (*see* **Note 5**).
2. The mixture is shaken in an end-over-end shaker at 30 rpm for 3 h at ambient temperature (20 ± 2°C) (*see* **Note 6**).
3. Decant 12 mL into a centrifuge tube and centrifuge for 10 min at 3000*g*.
4. Analyse extracts immediately by ICP-MS (*see* **Notes 10 and 11**).

3.1.5. Ammonium Nitrate

1. 2 g of soil sample is weighed into a 50 mL Sarstedt extraction tube and 5 mL of 1.0 M NH_4NO_3 is added (*see* **Note 5**).
2. The mixture is shaken in an end-over-end shaker at 50–60 rpm for 2 h at ambient temperature (20 ± 2°C) (*see* **Note 6**).
3. Then, pass the supernatant through an acid-washed filter paper into a 50 mL polyethylene bottle (discard the first 5 mL of the filtrate). Stabilise by adding 1 mL of concentrated HNO_3.
4. If solids remain, centrifuge or filter through a 0.45 μm membrane filter.
5. Analyse extracts immediately by ICP-MS (*see* **Notes 10 and 11**).

3.1.6. Sodium Nitrate Extraction

1. 2 g of soil sample is weighed into a 50 mL Sarstedt extraction tube and 5 mL of 0.1 M $NaNO_3$ is added (*see* **Note 5**).

2. The mixture is shaken in an end-over-end shaker at 120 rpm for 2 h at ambient temperature (20 ± 2°C) (*see* **Note 6**).
3. Then centrifuge the mixture for 10 min at 4000*g*.
4. Remove the supernatant with a syringe and filter through a 0.45 μm membrane filter into a 50 mL polyethylene bottle. Add 2 mL of concentrated HNO_3 to a 50 mL volumetric flask and make up to volume with the filtered extract.
5. Analyse extracts immediately by ICP-MS (*see* **Notes 10 and 11**).

3.2. Chemical-Selective Extraction for Sequential Extraction Method

The procedure adopted for the sequential extraction of metals from soil/sediments is based on three distinct stages (6). In stage 1 (exchangeable fraction), the metals released are representative of those that are the most bioavailable (and hence most mobile). They include those metals that are weakly absorbed on the sediment/soil surface by relatively weak electrostatic interaction, metals that can be released by ion exchange processes or metals that can be co-precipitated with carbonates present in many sediments/soils. Any changes in the ionic composition, influencing adsorption–desorption reactions, or lowering of pH could cause mobilisation of metals from this fraction. In stage 2 (reducible fraction), the metals bound to iron/manganese oxides are identified; they are therefore unstable under reduction conditions. Changes in the redox potential (E_h) could induce the dissolution of these oxides, leading to their release from the soil/sediment. Finally, in stage 3 (oxidisable fraction), those metals bound to organic matter within the sediment/soil matrix are released into solution. The residual fraction is then acid-digested (*see* **Section 6**).

3.2.1. Stage 1 Extraction

1. 1 g of soil sample is weighed into a 80–100 mL PTFE centrifuge tube and 40 mL of acetic acid (0.11 M) – Solution A – is added (*see* **Note 5**).
2. The mixture is shaken in an end-over-end shaker at 30 rpm for 16 h at ambient temperature (22 ± 5°C) (*see* **Notes 6 and 7**).
3. Centrifuge at 3000*g* for 20 min.
4. Remove the supernatant with a pipette and store in a polyethylene bottle at 4°C.
5. Analyse extracts by ICP-MS (*see* **Notes 10 and 11**).
6. Wash the residue with 20 mL of water by shaking for 15 min.
7. Centrifuge the residue for 20 min at 3000*g* and discard the supernatant. Take care not to lose any of the solid residue.

8. Break the "cake" formed during centrifugation prior to stage 2.

3.2.2. Stage 2 Extraction

1. Add 40 mL of hydroxylammonium chloride (0.1 M, adjusted to pH 2 with nitric acid) – Solution B – to the residue from stage 1.
2. The mixture is shaken in an end-over-end shaker at 30 rpm for 16 h at ambient temperature (22 ± 5°C) (*see* **Note 6**).
3. Centrifuge at 3000*g* for 20 min.
4. Remove the supernatant with a pipette and store in a polyethylene bottle at 4°C.
5. Analyse extracts by ICP-MS (*see* **Notes 10 and 11**).
6. Wash the residue with 20 mL of water by shaking for 15 min.
7. Centrifuge the residue for 20 min at 3000*g* and discard the supernatant. Take care not to lose any of the solid residue.
8. Break the "cake" formed during centrifugation prior to stage 3.

3.2.3. Stage 3 Extraction

1. Add carefully, to avoid losses due to any violent reaction, 10 mL of hydrogen peroxide (8.8 M) – Solution C – to the residue from stage 2.
2. Allow the sample to digest for 1 h with occasional manual stirring. Ensure the container is covered with a watch glass (or similar).
3. Continue the digestion by heating the sample to 85 ± 2°C, with occasional manual stirring for the first 30 min, for 1 h in a water bath or similar.
4. Reduce the volume of liquid to 2–3 mL by further heating, after removal of the watch glass.
5. Add a further 10 mL of hydrogen peroxide (Solution C) and heat to 85 ± 2°C for 1 h in a water bath (with occasional manual stirring for the first 30 min).
6. Remove the watch glass and reduce the volume of liquid to approximately 1 mL by further heating.
7. Add 50 mL of ammonium acetate (1.0 M) – Solution D – to the cooled, moist residue.
8. The mixture is shaken in an end-over-end shaker at 30 rpm for 16 h at ambient temperature (20 ± 5°C).
9. Centrifuge at 3000*g* for 20 min.
10. Remove the supernatant with a pipette and store in a polyethylene bottle at 4°C.
11. Analyse extracts by ICP-MS (*see* **Notes 10 and 11**).

3.3. Physiologically Based Extraction Test or In vitro Gastrointestinal Extraction

In vitro gastrointestinal extraction consists of two sequential processes, a gastric and an intestinal digestion, each one carried out employing simulated human conditions (enzymes, pH and temperature) (2). Several distinct approaches for performing in vitro gastrointestinal extraction are available (7, 8); however, two are considered in this chapter.

3.3.1. Approach 1: Gastric Extraction

1. 0.3 g (accurately weighed) of sample is placed into a 50 mL screw-cap Sarstedt tube and treated with 30 mL of gastric juice.
2. The mixture is then shaken at 100 rpm in a thermostatic water bath maintained at 37°C.
3. After 1 h, the solution is centrifuged at 3000 rpm for 10 min and a 5 mL aliquot is removed and filtered through 0.45 μm filter disk.
4. The extracts are analysed by ICP-MS (*see* **Notes 10 and 11**).
5. 5.0 mL of the original gastric solution is then backflushed through the filter into the sample tube to retain the original solid:solution ratio, i.e. 0.3:30 g/mL.

3.3.2. Approach 1: Intestinal Extraction

1. Intestinal juice (52.5 mg bile salts and 15 mg pancreatin) is added into the sample tube and the mixture is adjusted to pH 7.0 with saturated $NaHCO_3$.
2. The sample is shaken at 100 rpm in a thermostatic water bath maintained at 37°C for a further 2 h.
3. A 5.0 mL aliquot is removed and filtered and analysed by ICP-MS.
4. After an additional 2 h, a second 5.0 mL extract aliquot is removed, filtered and analysed by ICP-MS (*see* **Notes 10 and 11**).
5. The second intestinal aliquot is used to check that the small intestinal equilibrium has been reached (9).

Example results for the in vitro gastrointestinal extraction of nine elements from two certified reference materials (INCT-TL-1 and SRM 1570a) are shown in **Table 2.2**. Data indicating that the additional 2 h equilibration period (*see* **Section 3.3.2**, Step 4) had no significance at the 95% confidence interval are shown in **Table 2.3** for the two certified reference materials. The repeatability of the in vitro gastrointestinal extraction for the recovery of eight elements from a contaminated soil digest on three separate occasions is shown in **Table 2.4**.

3.3.3. Approach 2: "Stomach" Extraction

1. 0.6 g (accurately weighed) of sample is placed into a 50 mL screw-cap Sarstedt tube and treated with 9 mL of simulated saliva fluid (*see* **Note 12**).

Table 2.2
Example results for in vitro gastrointestinal extraction using approach 1
(A)

Element	Certified values of tea leaves (INCT-TL-1) (mg/kg) Mean ± SD	Concentration (mg/kg) Gastric stage Mean ± SD ($n = 3$)	%	Intestinal stage Mean ± SD ($n = 3$)	%	Residual stage Mean ± SD ($n = 3$)	%	Σ Total stages Mean ± SD ($n = 3$)
Cr	1.91 ± 0.22	0.67 ± 0.13	32.57	0.73 ± 0.09	35.73	0.65 ± 0.09	31.70	2.04 ± 0.11
Mn	1570 ± 110	998 ± 298	58	356 ± 231	21	360 ± 32	21	1714 ± 105
Fe	(432)	1 ± 1	0.2	6 ± 2	1.5	429 ± 46	98.3	437 ± 43
Ni	6.12 ± 0.52	2.68 ± 0.57	39.82	2.43 ± 0.24	36.05	1.63 ± 0.46	24.13	6.74 ± 0.43
Cu	20.4 ± 1.5	3.7 ± 1.0	17.3	7.2 ± 0.5	33.3	10.7 ± 1.1	49.5	21.7 ± 0.4
Zn	34.7 ± 2.7	17.0 ± 2.8	40.8	10.9 ± 1.3	26.2	13.7 ± 2.6	32.9	41.7 ± 4.9
Mo	Na	0.005 ± 0.003	6.13	0.024 ± 0.005	27.20	0.058 ± 0.002	66.67	0.087 ± 0.003
Cd	0.030 ± 0.004	0.016 ± 0.013	41.69	0.004 ± 0.003	9.91	0.018 ± 0.020	48.40	0.038 ± 0.012
Pb	1.78 ± 0.24	0.13 ± 0.02	7.45	0.20 ± 0.02	11.51	1.40 ± 0.01	81.04	1.73 ± 0.05

(continued)

Table 2.2 (continued)
(B)

Element	Certified values of spinach leaves (SRM 1570a) (mg/kg)	Concentration (mg/kg)							
		Gastric stage		Intestinal stage		Residual stage		Σ Total stages	
	Mean ± SD	Mean ± SD ($n = 3$)	%	Mean ± SD ($n = 3$)	%	Mean ± SD ($n = 3$)	%	Mean ± SD ($n = 3$)	
Cr	na	0.15 ± 0.02	9.64	0.29 ± 0.07	18.54	1.11 ± 0.07	71.82	1.54 ± 0.08	
Mn	75.9 ± 1.9	39.0 ± 0.6	47.0	31.0 ± 4.9	37.3	13.0 ± 2.0	15.7	83.0 ± 4.0	
Fe	NA	38 ± 3	20.2	63 ± 3	33.3	88 ± 5	46.5	189 ± 6	
Ni	2.14 ± 0.10	0.87 ± 0.06	42.13	0.73 ± 0.09	35.53	0.46 ± 0.18	22.33	2.06 ± 0.20	
Cu	12.2 ± 0.6	6.4 ± 0.1	44.7	5.7 ± 0.4	40.4	2.1 ± 0.5	14.9	14.2 ± 0.4	
Zn	82 ± 3	52 ± 2	57.1	30 ± 1	32.2	10 ± 0.4	10.7	92 ± 3	
Mo	na	0.206 ± 0.041	37.07	0.312 ± 0.052	55.99	0.039 ± 0.011	6.94	0.557 ± 0.086	
Cd	2.89 ± 0.07	1.02 ± 0.19	37.44	0.64 ± 0.11	23.42	1.07 ± 0.08	39.14	2.73 ± 0.37	
Pb	na	0.120 ± 0.068	30.68	0.110 ± 0.075	28.15	0.161 ± 0.083	41.17	0.392 ± 0.076	

na = not available

Table 2.3
Example results for the extraction equilibrium of the intestinal fluid phase

	Bioaccessible metals (mg/kg) – tea leaves (INCT-TL-1)						Bioaccessible metals (mg/kg) – spinach leaves (SRM 1570a)					
	Intestinal stage IIA		Intestinal stage IIB				Intestinal stage IIA		Intestinal stage IIB			
Element	Mean ($n = 3$)	SD	Mean ($n = 3$)	SD	*t*-stat	*P*-value	Mean ($n = 3$)	SD	Mean ($n = 3$)	SD	*t*-stat	*P*-value
Cr	0.730	0.093	0.760	0.100	–0.512	0.660	0.286	0.073	0.302	0.098	–0.794	0.511
Mn	356.020	230.635	324.751	201.412	1.737	0.225	30.972	4.873	30.086	3.757	0.946	0.444
Fe	6.415	1.901	5.990	1.912	5.899*	0.028*	62.857	3.005	59.936	1.364	2.752	0.111
Ni	2.429	0.236	2.208	0.101	1.247	0.339	0.733	0.091	0.720	0.117	0.873	0.475
Cu	7.212	0.465	7.147	0.985	0.162	0.886	5.741	0.431	5.923	0.601	–0.773	0.520
Zn	10.934	1.264	10.832	1.304	0.191	0.866	28.621	1.011	28.731	2.432	–0.109	0.923
Mo	0.024	0.005	0.020	0.003	3.417	0.076	0.312	0.052	0.278	0.059	3.687	0.066
Cd	0.004	0.003	0.003	0.001	1.153	0.368	0.639	0.115	0.603	0.131	2.705	0.114
Pb	0.199	0.024	0.215	0.038	–1.019	0.415	0.110	0.075	0.115	0.078	–1.982	0.186

Note: *t*-critical (two-tail) is 4.303 and *P*-values are reported at 5% significance level.
*1% significance level giving *t*-critical = 9.925.
Intestinal stage IIA refers to **Section 3.3.2**, Step 1–3, while intestinal stage IIB refers to **Section 3.3.2**, Step 4.

Table 2.4
Example results for in vitro gastrointestinal extraction using approach 1
(a) Data from week 1

	Aqua regia digest of soil (mg/kg)	Concentration (mg/kg)							
		Gastric stage		Intestinal stage		Residual stage		Σ Total stages	
Element	Mean ± SD	Mean ± SD ($n = 3$)	%	Mean ± SD ($n = 3$)	%	Mean ± SD ($n = 3$)	%	Mean ± SD ($n = 3$)	
Cr	130.2 ± 4.8	7.46 ± 0.15	5.2	9.89 ± 0.32	6.8	127.0 ± 12.9	88.0	144.3	
Mn	4980 ± 207	2269 ± 99	46.2	1349 ± 104	27.5	1293 ± 23	26.3	4911	
Ni	69.1 ± 3.3	7.80 ± 0.22	11.9	5.80 ± 0.21	8.9	51.8 ± 1.5	79.2	65.4	
Cu	25.0 ± 2.8	1.78 ± 0.03	8.9	5.25 ± 0.09	26.5	12.8 ± 0.3	64.6	19.8	
Zn	133.4 ± 3.8	19.8 ± 1.4	14.2	4.86 ± 0.11	3.5	114.6 ± 4.7	82.3	139.2	
Mo	4.2 ± 0.4	0.43 ± 0.01	12.3	0.62 ± 0.01	17.7	2.45 ± 0.02	70.0	3.5	
Cd	0.91 ± 0.02	0.14 ± 0.00	17.1	0.04 ± 0.01	4.9	0.64 ± 0.01	78.0	0.82	
Pb	59.8 ± 0.4	0.67 ± 0.06	1.6	0.50 ± 0.03	1.2	41.8 ± 0.8	97.2	43.0	

(continued)

Table 2.4 (continued)
(b) Data from week 2

	Aqua regia digest of soil (mg/kg)	Concentration (mg/kg)						
		Gastric stage		Intestinal stage		Residual stage		Σ Total stages
Element	Mean ± SD	Mean ± SD ($n = 3$)	%	Mean ± SD ($n = 3$)	%	Mean ± SD ($n = 3$)	%	Mean ± SD ($n = 3$)
Cr	130.2 ± 4.8	6.31 ± 0.13	4.5	10.78 ± 0.28	7.7	122.9 ± 8.9	87.8	139.9
Mn	4980 ± 207	1554.3 ± 50.2	29.6	802.0 ± 18.2	15.2	2900 ± 70	55.2	5256
Ni	69.1 ± 3.3	6.20 ± 0.40	11.1	7.59 ± 0.37	13.7	41.8 ± 3.1	75.2	55.6
Cu	25.0 ± 2.8	1.62 ± 0.05	6.3	6.00 ± 0.21	23.2	18.2 ± 1.1	70.5	25.8
Zn	133.4 ± 3.8	24.4 ± 4.9	19.0	4.44 ± 0.23	3.5	99.6 ± 3.6	77.5	128.4
Mo	4.2 ± 0.4	0.51 ± 0.01	10.5	0.76 ± 0.03	15.6	3.59 ± 0.06	73.9	4.9
Cd	0.91 ± 0.02	0.13 ± 0.01	14.3	0.06 ± 0.06	6.6	0.72 ± 0.01	79.1	0.9
Pb	59.8 ± 0.4	0.29 ± 0.03	0.5	0.62 ± 0.06	1.1	55.7 ± 1.5	98.4	56.6

(continued)

Table 2.4 (continued)
(c) Data from week 3

	Aqua regia digest of soil (mg/kg)	Concentration (mg/kg)						
		Gastric stage		Intestinal stage		Residual stage		Σ Total stages
Element	Mean ± SD	Mean ± SD ($n = 3$)	%	Mean ± SD ($n = 3$)	%	Mean ± SD ($n = 3$)	%	Mean ± SD ($n = 3$)
Cr	130.2 ± 4.8	8.05 ± 0.43	7.6	9.62 ± 0.57	9.1	88.3 ± 8.5	83.3	106
Mn	4980 ± 207	1591.7 ± 34.5	30.8	606.6 ± 14.2	11.8	2963 ± 101	57.4	5161
Ni	69.1 ± 3.3	6.64 ± 0.30	11.7	3.64 ± 0.14	6.4	46.5 ± 2.2	81.9	56.8
Cu	25.0 ± 2.8	1.38 ± 0.10	5.8	2.60 ± 0.14	10.8	20.0 ± 1.3	83.4	24.0
Zn	133.4 ± 3.8	18.83 ± 0.83	13.0	1.90 ± 0.27	1.3	124.6 ± 2.3	85.7	145.3
Mo	4.2 ± 0.4	0.60 ± 0.03	13.2	0.62 ± 0.01	13.6	3.32 ± 0.18	73.2	4.5
Cd	0.91 ± 0.02	0.24 ± 0.01	32.9	0.04 ± 0.00	5.5	0.45 ± 0.01	61.6	0.73
Pb	59.8 ± 0.4	0.33 ± 0.03	0.6	0.88 ± 0.06	1.5	58.8 ± 6.1	98.0	60.0

2. With the screw cap closed, manually shake the soil–fluid mixture.
3. After 5–15 min, add 13.5 mL of simulated gastric fluid.
4. The mixture is then shaken on an end-over-end shaker maintained at 37 ± 2°C.
5. After 1 h check the pH of the soil suspensions; the pH should be 1.2–1.7 (*see* **Note 13**).
6. The solution is centrifuged at 3000 rpm for 5 min and a 1.0 mL aliquot of supernatant is removed.
7. To the supernatant add 9.0 mL of 0.1 M HNO_3.
8. The sample is then stored at <8°C prior to analysis by ICP-MS (*see* **Notes 10 and 11**).

3.3.4. Approach 2: "Stomach + Intestine" Extraction

1. 0.6 g (accurately weighed) of sample is placed into a 50 mL screw-cap Sarstedt tube and treated with 9 mL of simulated saliva fluid (*see* **Note 12**).
2. With the screw cap closed, manually shake the soil–fluid mixture.
3. After 5–15 min, add 13.5 mL of simulated gastric fluid (*see* **Note 12**).
4. The mixture is then shaken on an end-over-end shaker maintained at 37 ± 2°C.
5. After 1 h check the pH of the soil suspensions; the pH should be 1.2–1.7 (*see* **Note 13**).
6. Then, add 27.0 mL of simulated duodenal fluid and 9.0 mL of simulated bile fluid (*see* **Note 12**).
7. With the screw cap closed, manually shake the soil–fluid mixture.
8. Adjust the pH of the resultant suspension to 6.3 ± 0.5 by the drop-wise addition of 37% HCl, 1 M or 10 M NaOH, as required.
9. The mixture is then shaken on an end-over-end shaker maintained at 37 ± 2°C for 4 h.
10. Remove the soil suspension.
11. Measure (and record) the pH of the soil suspension; pH should be 6.3 ± 0.5.
12. The soil suspension is then centrifuged at 3000 rpm for 5 min and a 1.0 mL aliquot of supernatant is removed.
13. To the supernatant is added 9.0 mL of 0.1 M HNO_3.
14. The sample is then stored at <8°C prior to analysis by ICP-MS (*see* **Notes 10 and 11**).

3.4. Method: Soil Digestion Procedure

An acid digestion procedure is used to provide pseudo-total metal analysis.

3.4.1. Acid Digestion Procedure

1. Approximately 1 g of soil sample is accurately weighed into a digestion tube (250 mL volume).
2. Add 0.5–1.0 mL of water to obtain a slurry.
3. Then add, while mixing, 7 mL of 12.0 M HCl, followed by 2.3 mL of 15.8 M HNO_3 (drop by drop, if necessary to reduce foaming) (*see Note 8*).
4. Add 15 mL of 0.5 M HNO_3 to the reaction vessel and connect to a water-cooled reflux condenser.
5. Allow to stand for 16 h at room temperature to allow slow oxidation of the organic matter of the soil.
6. Raise the temperature of the reaction mixture until reflux conditions are achieved and maintain for 2 h.
7. Allow to cool slowly to room temperature.
8. Rinse the contents of the condenser into the reaction vessel with 10 mL of 0.5 M HNO_3.
9. Quantitatively transfer the contents of the reaction vessel to a 100 mL volumetric flask. Rinse the vessel with 0.5 M HNO_3 and transfer as well. Make up to the mark with water, stopper and shake.
10. Allow the undissolved matter to settle and then analyse the supernatant solution by ICP-MS (*see* **Notes 10 and 11**).

3.4.2. Alternate Acid Digestion Procedure

1. Approximately 1 g of soil sample is accurately weighed into a digestion tube and 10 mL of 1:1 v/v concentrated HNO_3:water is added.
2. The mixture is then heated at 95°C on a heating block for 15 min without boiling.
3. After cooling at room temperature for 5 min, 5 mL concentrated HNO_3 is added and the sample is heated at 95°C for 30 min.
4. An additional 5 mL of concentrated HNO_3 is added until no brown fumes are given off.
5. Evaporate the solution to <5 mL.
6. After cooling, 2 mL of water and 3 mL of 30% H_2O_2 are added and heated (<120°C) until effervescence subsides and the solution cools. Additional H_2O_2 is added until effervescence ceased (but add no more than 10 mL H_2O_2). This stage is continued for 2 h.
7. Evaporate the solution to <5 mL.

8. After cooling, add 10 mL of concentrated HCl and heat (<120°C) for 15 min.
9. After cooling, filter the sample through a Whatman No. 41 filter paper into a 100 mL volumetric flask, and then make up to the mark with water.
10. Analyse by ICP-MS (*see* **Notes 10 and 11**).

3.5. Method: Sample Analysis by ICP-MS

ICP-MS measurement conditions are optimised daily using the built-in PlasmaLab software procedure. Samples of the soil extracts/digests are analysed by ICP-MS using an external calibration technique. Sc, In and Tb internal standards (10 μg/L) are added to all samples, blanks and standard solutions. A blank is analysed with each analytical batch (*see* **Note 9**).

3.5.1. ICP-MS Operating Conditions: Standard Mode

1. In standard mode the following elements can be analysed: >90 amu
2. Forward power, 1400 W; coolant gas flow, 13.0 L/min; auxiliary gas flow, 0.90 L/min; nebuliser gas flow, 0.80 L/min; quadrupole bias, −1.0 V; hexapole bias, 0.0 V; dwell time per isotope, 10 ms.

3.5.2. ICP-MS Operating Conditions: Collision/Reaction Cell Mode

1. In collision/reaction cell mode the following elements can be analysed: <90 amu
2. Forward power, 1400 W; coolant gas flow, 13.0 L/min; auxiliary gas flow, 0.90 L/min; nebulizer gas flow, 0.80 L/min; collision cell gas, 4.50 L/min of 7% H_2/93% He; quadrupole bias, –14.0 V; hexapole bias, −15.0 V; dwell time per isotope, 10 ms.

4. Notes

1. Unless otherwise stated, all solutions should be prepared in water that has a resistivity of 18.2 $M^{\Omega} \times$ cm. This standard is referred to in the text as "water".
2. All laboratory ware should be made of borosilicate glass, polypropylene, polyethylene or PTFE, except for the centrifuge tubes, which should be made of borosilicate glass or PTFE.
3. All vessels in contact with samples or reagents should be cleaned in HNO_3 (4 mol/L) for at least 30 min, then rinsed with distilled water, cleaned with 0.05 mol/L EDTA and rinsed again with distilled water. Alternatively clean all

vessels by immersing in HNO_3 (4 mol/L) overnight and then rinse two to three times with water.

4. When extracting with sodium nitrate ($NaNO_3$), it is necessary to correct the results for the difference in final volume, i.e. 2 mL of HNO_3 was added to 48 mL of extract to give a final volume of 50 mL.
5. When using sequential extraction methods for the analysis of sediment or soil samples, a separate sub-sample should be dried (in a layer of approximately 1 mm depth) in an oven at 105 +/− 2°C for 2–3 h, transferred to a desiccator and allowed to cool prior to weighing.
6. Ensure that the sample, i.e. sediment/soil, does not form a "cake" during the extraction procedure. If a cake is formed, either adjust the shaking speed to ensure that the suspension is maintained or mechanically break the solid "cake" with a pre-cleaned glass rod. It is important that the sample remain in complete suspension during the extraction process.
7. In sequential extraction the mechanical shaker, preferably of the end-over-end type, should be operated at a speed of 30 +/− 10 rpm and a temperature of 22 +/− 5°C. All samples should be centrifuged at 3000*g* for 20 min.
8. The combination of 12.0 mol/L HCl and 15.8 mol/L HNO_3 in a volume ratio of 3:1, respectively, is known as aqua regia.
9. Calibration solutions for ICP-MS should be prepared with the appropriate extraction solution, i.e. use matrix-matched calibration solutions.
10. It is important to prepare a sample blank for every batch of extractions, i.e. prepare a container with no sediment/soil, but treated in the same manner as though it contained the sample.
11. It is recommended for ICP-MS that all extracts be filtered (0.45 μm) prior to analysis.
12. Simulated gastrointestinal fluids are stored at room temperature overnight prior to use. Prior to their use for bioaccessibility studies, the fluids need to be heated to 37°C at least 2 h before their use on the day following their preparation.
13. If the pH of a sample suspension is not within the guideline of 1.2–1.7, the sample should be discarded and sub-samples re-extracted. Before re-extracting, however, add an additional amount of 37% HCl (up to a maximum of 1.0 mL).

Acknowledgements The author would like to thank Dr Marisa Intawongse for evaluation of some of the methods described. Also, the technical assistance of Mr. Gary Askwith is acknowledged.

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