Table 2 Iron species in corrosion product samples as determined by continuous-flow sequential extraction (n=3) (*ND* not detectable)

Sample ID	Extracted iron [mg Fe g^{-1} sample]				
	Water soluble phase	Acid soluble phase	Reducible phase	Oxidisable phase	Sum
001	ND	1.0±0.1	395.0±19.0	7.7±0.7	403.6±19.8
002	ND	58.4±3.9	334.6±8.7	3.8±0.9	396.7±12.4
003	3.9±0.2	22.1±1.9	185.2±18.2	1.7±0.5	213.0±20.5
004	0.1±0.0	0.7±0.1	318.8±21.4	ND	319.5±21.5
005	ND	42.4±5.0	367.3±23.1	1.5±0.0	411.3±28.1
006	ND	15.4±2.6	462.7±35.6	4.2±0.1	482.3±38.3
007	ND	2.8±0.2	371.9±8.2	8.9±0.5	383.5±8.7
008	ND	3.0±0.3	417.6±32.5	0.2±0.1	420.7±32.7
009	14.3±0.4	120.1±5.5	219.3±15.7	3.0±0.9	356.8±20.8
010	8.4±1.1	14.2±3.1	200.6±26.6	3.0±0.8	226.2±30.4
011	1.5±0.1	46.7±5.9	216.2±22.2	6.4±0.1	270.8±28.1

CH_3COOH in this extraction step. The concentrations of iron and co-extracted carbonate are used to evaluate the selectivity of the extractant. The results (Fig. 4) show that the amount of extracted iron increases at increasing concentrations of CH_3COOH. However, the co-extracted carbonate concentration becomes constant after 0.11 mol l^{-1} CH_3COOH, indicating that, at 0.11 mol l^{-1}, CH_3COOH is adequate to dissolve the carbonate phase. The increased amounts of iron at higher concentrations of CH_3COOH were due to partial dissolution of the Fe-(oxyhydr)oxides. For specific dissolution of $FeCO_3$, the molar concentrations of extracted iron and carbonate should be equal. The molar concentrations of iron and carbonate were found to be close to unity at 0.05 mol l^{-1} and 0.11 mol l^{-1} CH_3COOH, indicating selective leaching. Lower extractability at 0.05 mol l^{-1} CH_3COOH was due to incomplete dissolution. The higher extracted iron at CH_3COOH concentrations higher than 0.11 mol l^{-1} supported the aforementioned partial dissolution of Fe(oxyhydr)oxides and was further confirmed by the decreasing extracted iron in the reducible phase at increasing CH_3COOH concentrations. Therefore, the acid concentration of 0.11 mol l^{-1} was considered optimal for the acid soluble phase for corrosion product samples.

Application to corrosion product samples

The results presented above suggest that the extraction scheme developed can be used for evaluation of iron species in corrosion product samples. Results from its application to 11 samples (Table 2) showed the highest proportion of iron in the reducible fractions (61–99% of extractable iron). Fe-(oxyhydr)oxides appeared to be the major constituents of the corrosion products investigated, and, therefore, O_2 played an important role in the corrosion for these samples. Partial amounts of this phase may be transformed from $FeCO_3$. The study by Heuer and Stubbins [12] showed that $FeCO_3$ was unstable and quickly decomposed to Fe_2O_3 after contact with air. Very small proportions of iron were found in the water soluble and oxidisable fractions (0–4% of extractable iron). The sum of extracted and non-extracted iron (Table 3) from continuous-flow sequential extraction shows good agreement with the total concentration of iron obtained from acid

Table 3 Iron content in corrosion product samples as obtained from sequential extraction by continuous-flow system in comparison with acid digestion (n=3)

Sample ID	Extraction [%w/w]			Acid digestion [%w/w]
	Extracted[a]	Non-extracted (residue)	Sum	
001	40.4±2.0	1.6±0.7	41.9±1.3	42.9±0.4
002	39.7±1.2	0.0±0.0	39.7±1.2	39.1±1.6
003	21.3±2.0	1.2±0.2	22.5±1.9	24.1±0.2
004	32.0±2.1	16.7±3.9	48.6±1.8	49.6±0.4
005	41.1±2.8	12.1±0.4	53.3±2.4	55.5±1.6
006	48.2±3.8	0.3±0.4	48.5±3.4	50.0±3.8
007	38.4±0.9	4.7±1.0	43.1±0.6	44.5±2.0
008	42.1±3.3	4.8±1.2	46.9±2.3	47.1±0.3
009	35.7±2.1	6.4±0.4	42.0±2.1	44.2±0.2
010	22.6±3.0	13.4±1.4	36.0±2.1	36.2±0.8
011	27.1±2.8	5.4±1.2	32.5±2.3	33.1±0.3

[a] Summation of extracted iron from four extraction steps

digestion, with insignificant difference observed at the 95% confidence level. The non-extracted iron may be attributed to the iron associated with resistant complex species [25] that are insoluble in the extractants used. The residual or insoluble iron was difficult to dissolve and required aqua regia to facilitate solubilisation. The continuous-flow extraction method, with its collection of several subfractions, that we used in this work to obtain detailed extraction profiles could be tedious, and it required a large number of samples to be analysed. For practical use of this method, one fraction per step of sequential extraction is sufficient to obtain fractionation data. Additional advantages of the continuous-flow approach are the ease of operation and freedom from environmental and procedural contamination.

Conclusions

A continuous-flow sequential extraction was utilised to fractionate iron species in corrosion products from natural gas pipelines. Information on forms and quantities of iron in the samples can be obtained. The total concentration of iron was found to be in the range of 21–48% w/w. Iron is predominantly present in the reducible fraction. This indicates that corrosion products mostly result from O_2 (as gas contaminant) and water. Techniques such as ion chromatography and gas absorption with carbonate detection were applied to study the selectivity of extractants for the targeted iron phases. Ion chromatography was used to determine the amount of sulphate in the extracts of water soluble and oxidisable extraction steps to confirm the co-extraction of sulphate and sulphide. Simultaneous collection and determination of CO_2 generated from leaching of carbonate species revealed the association of iron and carbonate in the acid soluble fraction of corrosion products. The iron fractionation data can be used to understand the origins and mechanisms of corrosion and, consequently, to prevent or solve the corrosion problems in the petroleum and natural gas industries.

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Development of a simple extraction cell with bi-directional continuous flow coupled on-line to ICP-MS for assessment of elemental associations in solid samples†

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A continuous-flow system comprising a novel, custom-built extraction module and hyphenated with inductively coupled plasma-mass spectrometric (ICP-MS) detection is proposed for assessing metal mobilities and geochemical associations in soil compartments as based on using the three step BCR (now the Measurements and Testing Programme of the European Commission) sequential extraction scheme. Employing a peristaltic pump as liquid driver, alternate directional flows of the extractants are used to overcome compression of the solid particles within the extraction unit to ensure a steady partitioning flow rate and thus to maintain constant operationally defined extraction conditions. The proposed flow set-up is proven to allow for trouble-free handling of soil samples up to 1 g and flow rates ≤ 10 mL min^{-1}. The miniaturized extraction system was coupled to ICP-MS through a flow injection interface in order to discretely introduce appropriate extract volumes to the detector at a given time and with a given dilution factor. The proposed hyphenated method demonstrates excellent performance for on-line monitoring of major and trace elements (Ca, Mn, Fe, Ni, Pb, Zn and Cd) released when applying the various extracting reagents as addressed in the BCR scheme, that is, 0.11 M CH_3COOH, 0.1 $NH_2OH \cdot HCl$ and 30% H_2O_2, even when a well recognized matrix-sensitive detector, such as ICP-MS, is used. As a result of the enhanced temporal resolution of the ongoing extraction, insights into the breaking down of phases and into the kinetics of the metal release are obtained. With the simultaneous multielement detection capability of ICP-MS, the dynamic fractionation system presents itself as an efficient front-end for evaluation of actual elemental association by interelement comparison of metals leached concurrently during the extraction time. Thus, the intimate elemental association between Cd and Zn in contaminated soils could be assessed.

1. Introduction

At present, it is widely accepted that risk assessment of trace elements contaminating environmental solid substrates is a topic of utmost relevance in environmental studies. This information can be used for evaluation of the impact on biota and serves as a reference of environmental policy decisions. In soil analysis, knowledge about the chemical forms of elements is required, because of its close link to the mobility and bioavailability of the species, which cannot be assessed by merely measuring the total concentration of the elements.

Batch sequential extraction procedures are conventionally employed for the fractionation of metals according to their leachability.[1–7] This technique makes use of suitable chemical reagents, which are applied, in a given order, to the sample to sequentially attack metals associated with different solid compartments. However, the batch procedures suffer from several shortcomings, such as being tedious, time consuming and being prone to risk of contamination and to metal adsorption/re-distribution phenomena, and, more importantly, they are able to provide neither information about the kinetics of leaching nor a detailed insight of the bindings of the metals within the solid phases.

Recent trends in the soil analysis field have been towards the development of methods aimed at tackling these drawbacks and additionally at mimicking environmental events more accurately than their classical extraction counterparts. The dynamic (non-equilibrium)-based extraction approach has proven to constitute an appealing alternative for trace element partitioning.[8] Thus, a number of research groups have proposed singular flow-through extraction systems[9–16] based on continuous or discontinuous delivery of fresh portions of the leaching reagents through small column-devices containing the solid material. A notable limitation of the flow-through extraction approach is the small inner dimension of the solid containers, the use of high amounts of sample can therefore cause unstable extraction flow rates due to blockage of the in-

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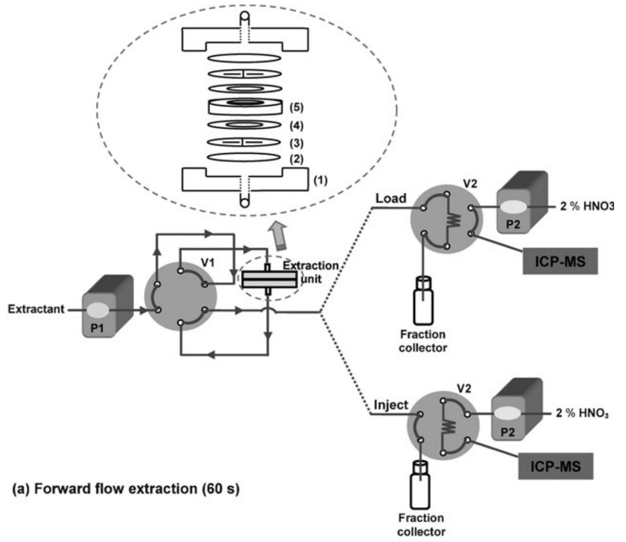
† The HTML version of this article has been enhanced with colour images.

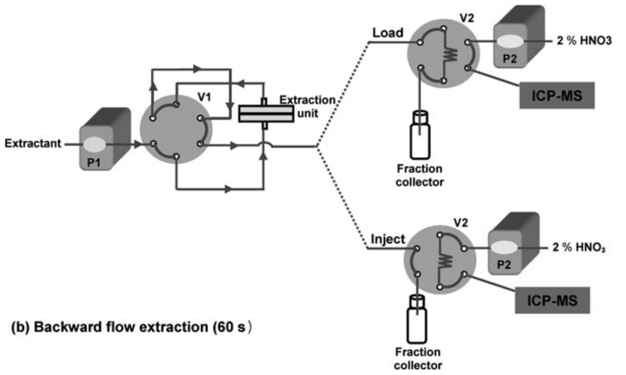
line filters by solid particles. Even though fast extraction and screening of trace element release can be accomplished by using small amounts of sample (typically $\leq$ 25 mg),[10,13–15] this does require that highly homogeneous environmental solids should be used.

By using flow-through approaches, on-line extraction measurements have become possible.[10,13–15,17–19] Chomchoei *et al.*[19] described the feasibility of coupling flow-through microcolumn extraction to classical FAAS. However, the simultaneous monitoring of metals was inherently restricted due to the single element detection nature of the detector. Therefore, hyphenated techniques based on the combination of dynamic extraction systems with a powerful multielement detector, *i.e.* ICP-OES or ICP-MS, should prove especially attractive for metal fractionation in environmentally relevant solids. Not only saving of analysis time per fraction but more accurate information on interelement interaction in solid phases should make it appealing for on-line fractionation/detection protocols. The on-line analysis of the extracts by ICP-OES measurements has been recently demonstrated by Fedotov *et al.*[18] who used a rotating coiled column as sample compartment coupled with the detector. Unlike in Fedotov's work, Jimoh *et al.*[15] exploited a flow injection manifold as an interfacing unit between the soil microcartridge and the detector to allow for a flexible manipulation of the leaching agents and to provide a universal means of coupling to different analytical instruments. The main shortcoming of the microcartridge approach is the small sample size (5 mg) used for fractionation.

In this work, the potential of flow-through sequential extraction hyphenated on-line with ICP-MS for exploration of elemental distribution and associations within soil phases is demonstrated. A simple, continuous-flow set-up based on bi-directional flow extraction is proposed for preventing the clogging of the membrane surfaces incorporated within the extraction module by solid particles. The ability to handle larger sample amounts and higher extraction flow rates as compared with earlier works will be also ascertained.



(a) Forward flow extraction (60 s)



(b) Backward flow extraction (60 s)

Fig. 1 Schematic diagram of the flow-through fractionation system coupled to ICP-MS for on-line elemental monitoring *via* the flow injection network; (a) forward and (b) backward extraction flow directions. P1 and P2, pumps; V1 and V2, 6 port rotary valves. (Top) Exploded view of the extraction unit comprising the following components: (1) extraction cap, (2) membrane filter, (3) filter support, (4) silicone gasket, (5) sample container. For operational details, see text.

2. Experimental

2.1 Preparation of standard solution and glassware

The chemical reagents used were of analytical grade. Ultrapure water from a Branstead water purification unit (Branstead International, IA, USA) was used throughout. Working standard solutions were prepared by appropriate dilution of the multi-element stock solution for ICP-MS (AccuStandard, Inc., CT, USA) with extracting reagents. All glassware and plastic containers were cleaned and soaked in 10% (v/v) HNO_3 for at least 24 h and rinsed with ultrapure water before use.

2.2 Extraction unit

The dedicated flow-through extraction unit employed in this work is shown in Fig. 1. It was machined out of polyoxymethylene that can tolerate the extractants used. The unit comprises two polyoxymethylene end-caps, glass microfibre membrane filters, PTFE membrane supports designed for maximizing extractant flow through the entire sample, silicone gaskets and a cylindrical sample container with an approximate inner volume of 2.8 mL. All parts were assembled according to the numbering order given in the figure. The amount of soil sample (up to 1.0 g) was placed in the sample container and sandwiched with glass microfibre filters (47 mm diameter, 1 μm pore size (Whatman, Maidstone, UK)) together with the PTFE membrane supports. The silicone gaskets were used to prevent leakage. The whole compartment was screwed tightly in position.

2.3 Soil material

The SRM 2711 from the National Institute of Standard and Technology was used for evaluation of accuracy and reliability of the proposed hyphenated technique and to allow for further comparison of results. The soil material was collected from the tilled layer of a wheat field (MT, USA). It is a moderately contaminated pasture soil that was oven-dried, sieved and blended to achieve a high degree of homogeneity, the total concentration being certified for a number of elements.

A top soil sample (0–10 cm) from Tak Province, Thailand was used for evaluation of the ability of the designed extraction unit to handle different ranges of soil particle sizes as well as the maximum soil amount applicable without causing an unstable extraction flow rate. Being an alkaline sandy clay loam soil (pH 7.48) having 3.17% organic matter, the soil sample was ground and sieved for obtaining soil particle sizes of <150, 150–250 and >250 μm, respectively.

2.4 Extracting reagents

The BCR three-step sequential extraction scheme[4] was conducted in a dynamic fashion. The extracting reagents utilized and the corresponding targeted phases are detailed as follows: (i) 0.11 M CH_3COOH (metals bound to carbonate, the so-called acid soluble fraction); (ii) 0.1 M $NH_2OH \cdot HCl$ in 25% CH_3COOH adjusted to pH 2 with HNO_3 (metals bound to Fe or Mn oxides, the so-called reducible fraction); (iii) 30% H_2O_2 (metals bound to organic matter, the so-called oxidizable fraction)

2.5 Dissolution of soil residues

A closed-vessel microwave digestion system (Milestone model MLS-1200 Mega, Bergamo, Italy) was employed for pseudo-total digestion of residues. The residue leftover after the flow-through sequential extraction scheme was transferred to a digestion vessel of 20 mL capacity together with concentrated HNO_3 (5 mL) and 30% H_2O_2 (5 mL). The vessel was then tightly sealed and subjected to a microwave digestion protocol. The microwave digestion program consists of 5 steps, using power levels and durations of /250/400/500/600/0 W and /10/5/10/5/5 min, respectively. After cooling, the clear digests were transferred and made up to an appropriate volume with deionized water and the content of metals was determined by ICP-MS.

2.6 System configuration

The configuration of the bi-directional continuous-flow extraction system devised for dynamic metal fractionation and on-line monitoring of the extracts is depicted in Fig. 1. A peristaltic pump (P1) (Micro-tube pump, MP-3N, EYELA (Tokyo Rikakikai Co., Ltd.)) was employed for continuously propelling the leaching reagents through the extraction unit at varying flow rates. The alternate change of extractant flow direction was performed by the aid of the rotary valve (V1) (Upchurch Scientific, Inc., WA). The interfacing of the extraction unit with the detection device was effected *via* a discrete loop incorporated within a 6 port injection valve (V2) (Upchurch Scientific, Inc., WA), which upon actuation allowed the content of the loop to be introduced into the ICP-MS *via* a second peristaltic pump (P2). 2%HNO_3 was used as carrier and was continuously pumped to the nebulizer of the ICP-MS (Sciex Elan 6000, Perkin Elmer, CT) by P2 (Gilson, France) at a flow rate of 1.2 mL min^{-1}. All outlets of the rotary valves were connected through PEEK ferrules with rigid PTFE tubing (0.8 mm id). All connections consisted of Tygon tubing of 1.2 mm (id). The extract loop was also made of Tygon tubing (26.5 cm long, 1.2 mm id) with an internal volume of *ca.* 300 μL.

The ICP-MS operating conditions are as follow: RF power 1350 W; nebulizer, auxiliary and plasma gas flow rate are 0.99, 0.9 and 15 L min^{-1}, respectively; isotopes monitored being ^{44}Ca, ^{55}Mn, ^{54}Fe, ^{60}Ni, ^{66}Zn, ^{111}Cd and ^{208}Pb.

2.7 Operational procedure for bi-directional continuous-flow sequential extraction and on-line ICP-MS detection

An amount of 0.25 g of soil sample was selected for on-line fractionation studies. Initially, V1 and V2 were switched to the forward and load positions, respectively, and P1 was started to continuously pump 0.11 M CH_3COOH at the flow rate of 5.0 mL min^{-1} through the extraction unit for allowing the leaching of elements from the solid sample to take place. At this stage, the valve loop was filled with extract containing the released elements, the surplus flowing into the sample collector of 30 mL capacity. After 30 s of extraction, valve V2 was switched to the inject position and the extract zone entrapped within the sample loop was by means of P2 transferred to the ICP-MS as propelled by the carrier solution (2% HNO_3). When the extract had been flushed out from the extract loop, V2 was returned to its original position. After a total of 60 s of extraction in the forward direction, V1 was activated to the backward position (Fig. 1b.), causing the extracting reagent to flow in the opposite direction through the extraction unit for 60 s before being turned to the forward position once again. During the 60 s of backward flow, sampling by V2 was effected in the same manner as described for the forward flow direction. In general, the forward and backward extractions are repeated until the signals of the elements under investigation are close to baseline. Thereafter, the next extracting reagent (*viz.*, 0.1 M $NH_2OH \cdot HCl$ or 30% H_2O_2) is introduced and the operation repeated until all three extraction steps have been completed.

Apart from online ICP-MS detection, the extracts collected in each sample collector were analyzed by ICP-MS for metal quantification. The results were used for confirmation of the reliability of the proposed technique.

3. Results and discussion

3.1 Performance of the bi-directional continuous-flow extraction approach for sequential extraction

In on-line fractionation/detection schemes, a critical issue to take into account for successful coupling of the extraction module with the detection instrument is the requirement of a steady flow rate. Actually, an unstable extraction flow rate due to blocking of the in-line filters by solid particles would impede the metering of accurate volumes of leachates, thus hindering the real-time monitoring of the extracts. Non-constant extraction flow rates were, in fact, pronounced in our first design, namely, a continuous-flow stirred-flow cell unit, involving uni-directional flow of the extractants.[20,21] Although this problem recently most elegantly was solved by the development of a new dynamic extraction approach[16] incorporating a bi-conical microcolumn within a sequential injection network, it requires more expensive, software-controlled instrumentation.

In order to provide a cost-effective flow-through partitioning system, the bi-directional continuous-flow extraction concept is therefore proposed. It is based on an alternate change of extractant's flow direction through the extraction chamber with two filtering membranes at both ends of the sample compartment. This approach prevents the compactness of solid particles on the membrane filter surface and at the same time favors an intimate sample–extractant contact. The bi-directional flow extraction system requires solely a peristaltic pump for continuous delivering of the extracting agents. Besides the cost effectiveness, simplicity and ease of operation are additional advantages. In order to assess the performance of the developed system, potential factors affecting the extraction flow rate were taken into consideration.

Fine solid particles as well as high delivery rates for the extractants are prone to cause the deterioration of the analytical performance of the ongoing extraction. Fine particles are easily retained within the membrane pores, while high pumping rates generate increased flow impedance. An unsteady extraction flow rate is especially noticeable whenever the extractant is continuously and unidirectionally pumped through the extraction vessel.[10,15,21] However, in the bi-directional continuous-flow system sample weights of 250 mg, soil particle sizes of even <150 μm, and extraction flow rates as high as 10 mL min^{-1} are admissible without any observable decrease of the extraction rate. As a result of the application of high extractant flow rates, the leaching behavior of readily mobilisable elements can be thoroughly investigated as opposed to previous reports where the extraction flow rate was ≤ 3 mL min^{-1}.[11,15–18]

A major issue in microcolumn fractionation approaches is the capability of handling environmentally representative sample amounts. Recent works reported the use of solid masses ≤ 25 mg,[10,13–15] which are only appropriate for highly homogeneous substrates. Beauchemin *et al.*[10] experienced significant flow back-pressure when scaling the sample size up from 25 to 250 mg in a microcolumn approach. By exploiting the devised extraction module with a top soil sample from Tak Province, Thailand (see Section 2.3), sample amounts as high as 1.0 g could be processed on-line without deterioration of the extraction flow rate when soil samples with particle sizes within the ranges of >250, 150–250 and <150 μm were analyzed at rates of ≤ 7.0 mL min^{-1}. Thus, soil particle size, extractant delivery rate and amount of sample had virtually no effect on the extraction flow rate.

3.2 Instrumental configuration of the hyphenated technique for on-line fractionation exploiting bi-directional flow

A bi-directional continuous-flow sequential extraction system hyphenated with ICP-MS *via* a flow injection network is here proposed for concurrently monitoring of the various trace and major metals released during the extraction protocols. This contrasts with recent publications from Beauchemin *et al.*[10] and Fedotov *et al.*[18] who directly coupled the extraction line with ICP-MS and ICP-OES, respectively. The interfacing of the extraction and detection streams *via* an FI manifold was selected in the present work because it offers several advantages, such as (i) independent control of the nebulization and the extraction flow rates, and (ii) the possibility of automatic on-line dilution of the extract matrix. Although ICP-MS has been considered as a powerful analytical tool for metal determinations as a result of its high sensitivity, wide linear range and multielemental capabilities, it is well known as a matrix-sensitive detector. The intrinsic low tolerance level of total dissolved solids[22,23] limits its potential use for metal determination in matrices containing high electrolyte concentrations. In fact, a dramatic decrease of the instrument's sensitivity was found within 1 h of operation when the CH_3COOH-extracts in the collected fractions were directly introduced into the nebulizer. The deposition of carbon and soil matrices on the sampler cone of the MS interface drastically affected the sensitivity and the stability of the signals. However, as a consequence of the dispersion/dilution of the minute extract volume within the carrier stream during transportation to the detector, the present set up expectedly yielded much fewer matrix interferences and generally did not give rise to problems for on-line detection of the various leachates. By using FI discrete sample introduction, the operational time can be extended significantly without observable reduction in sensitivity.

3.3 Data treatment and evaluation of the hyphenated bi-directional continuous flow sequential extraction set-up for the exploration of metal partitioning

Although membrane supports working as diffusers were inserted in the extraction unit to facilitate a close contact between soil particles and the leaching reagent, the extractability in the two flow directions was noticeably different, as can be observed in the raw signals illustrated in Fig. 2a. This happens regardless of the extraction unit orientation (horizontal or upright) or direction sequence (left to right or *vice versa*) of extraction. The occurrence was still observed when the internal volume of the extraction unit was reduced from 2.8 to 1.4 mL. This effect is very likely due to the different leachant fractions analyzed on-line, as a consequence of slight differences in the flowing paths of the leaching agents in both directions and the manual operation of the injection valves, while the influence of the flow direction on the fluidized bed-like extraction conditions might also play a role.

The raw extraction patterns were therefore processed in order to obtain a kinetic leaching profile. The final extractogram (Fig. 2b) was the result of averaging each pair of forward and backward peaks: each such average was plotted against the average exposure time. The quantification of the total extractable amount of elements was effected by integration of the processed extractogram.

The SRM 2711 was used to evaluate the accuracy and reliability of the hyphenated system. The extractable metal content obtained *via* on-line detection was compared with the off-line mode, involving the determination of metal concentrations in the fraction collectors and also with previously reported data of batchwise extraction.[24] As is apparent, both on-line and off-line modes in the proposed approach yielded

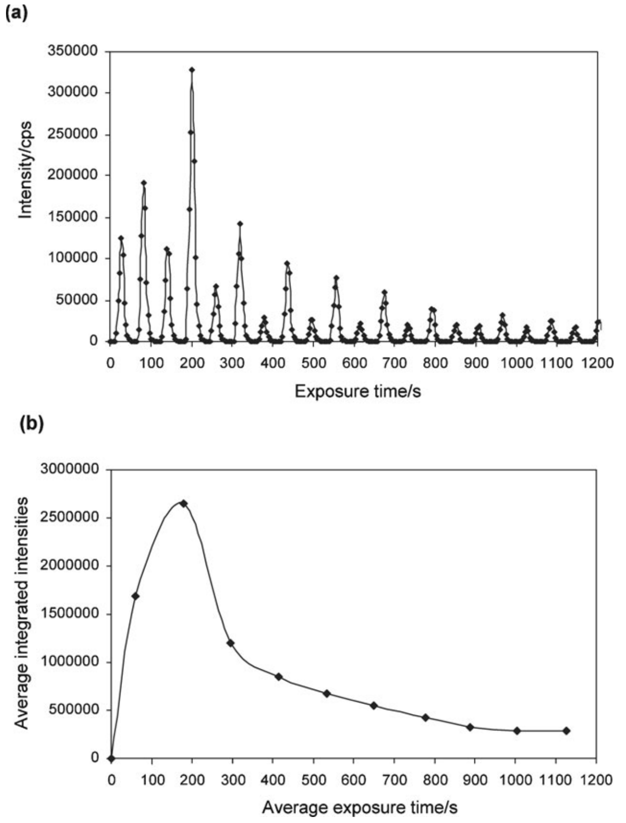


Fig. 2 Extraction profiles of Cd for the acid-soluble step, as obtained from the bi-directional continuous-flow extraction system coupled on-line to ICP-MS. (a) Raw signals showing alternate forward and backward peaks; and (b) processed profile obtained by averaging each pair of forward and backward peaks. Sample SRM 2711.

values which were insignificantly different at the significance level of 0.05 for every extraction step of all metals studied (see Table 1). Comparing batch and flow-through extraction, the percentage distribution of metals in the various fractions was significantly different. This is caused by the variable, operationally-defined, experimental conditions used in the two approaches. The redistribution/readsorption of leached metals onto the remaining solid substrate during a long extraction period, the different extraction times, and/or the incomplete metal leaching[25] in equilibrium-based extraction protocols may contribute to the different metal partitioning patterns. In contrast with batch extraction, the dynamic method minimizes the risk of readsorption due to the fact that the contact time between the solid and the liquid phases is minimized by the continuous delivery of fresh portions of leaching reagent through the soil container. However, the total concentrations of Cd, Ni, Pb and Zn obtained by summation of all fractions plus residue from the flow-through hyphenated technique as well as batch extractions are in good agreement with the certified concentrations in SRM 2711. However, those of Ca, Fe and Mn show lower recoveries probably due to the incomplete digestion in the pseudo-total analysis of the residual fraction.

3.4 Investigation of the metal soil phase associations

Apart from the detailed insight into metal distribution in the various phases, the extractogram of each element, as obtained by a graphical plot of the extracted concentration against the leaching time, can provide additional information. The appearance of leaching peaks in the extractrograms enables an examination of the selectivity of extracting agents for each nominal soil phase and the binding between trace elements and soil parent phases. An illustration of this study is shown in Fig. 3. In the first step of extraction, CH_3COOH was used for dissolving $CaCO_3$ and releasing metals bound to that phase. For the second step, $NH_2OH \cdot HCl$ was used for attacking oxides/hydroxides of Fe and Mn. Therefore, simultaneous monitoring of the stripping of trace metals and major elements in solid phases, *i.e.* Ca, Fe and Mn, can be used for demonstrating how trace elements are bound to the particular soil phases.

As seen in the extractrogram, peak positions and shapes between targeted metals (*viz.*, Cd and Pb) are all accompanied

Table 1 Comparison of extractable metal contents (mg kg^{-1}) for SRM 2711 as obtained by the bi-directional continuous-flow sequential extraction system with on-line and off-line ICP-MS detection modes and batchwise fractionation

		Step I	Step II	Step III	Residual	Sum	Certified value
Cd	This work (on-line)[a]	18.8 ± 0.5	13.7 ± 1.6	2.13 ± 0.25	2.82 ± 0.01	37.5 ± 1.7	41.70 ± 0.25
	This work (off-line)[a]	19.9 ± 0.5	14.5 ± 0.9	2.01 ± 0.1	2.82 ± 0.01	39.2 ± 1.0	
	Ho *et al.* (batch)[b]	28.6 ± 1.1	9.3 ± 0.6	2.4 ± 0.9	<1	40.2 ± 0.8	
Ni	This work (on-line)	2.7 ± 0.3	<D.L[c]	<D.L[c]	17.3 ± 2.0	20.0 ± 2.0	20.6 ± 1.1
	This work (off-line)	3.2 ± 0.1	<D.L	<D.L	17.3 ± 2.0	20.5 ± 2.0	
Pb	This work (on-line)	168 ± 11	517 ± 52	187 ± 15	149 ± 20	1021 ± 59	1162 ± 31
	This work (off-line)	187 ± 8	570 ± 24	212 ± 7	149 ± 20	1118 ± 33	
	Ho *et al.* (batch)	302 ± 27	349 ± 32	356 ± 85	97.9 ± 19.7	1100 ± 100	
Zn	This work (on-line)	94.9 ± 9.0	70.8 ± 1.7	129 ± 14	66.6 ± 2.3	361 ± 17	350.4 ± 4.8
	This work (off-line)	96.5 ± 6.9	73.8 ± 0.7	140 ± 7	66.6 ± 2.3	377 ± 10	
	Ho *et al.*(batch)	41.8 ± 1.2	62.2 ± 7.1	37.1 ± 13.3	206 ± 33	347 ± 34	
Ca	This work (on-line)	9630 ± 610	1910 ± 120	2100 ± 170	8500 ± 660	22 100 ± 900	28 800 ± 800
	This work (off-line)	9920 ± 430	2110 ± 50	2050 ± 60	8500 ± 660	22 600 ± 800	
Fe	This work (on-line)	301 ± 27	1771 ± 120	865 ± 76	18 270 ± 770	21 200 ± 800	28 900 ± 600
	This work (off-line)	310 ± 15	1900 ± 113	897 ± 54	18 270 ± 770	21 400 ± 800	
Mn	This work (on-line)	145 ± 4	211 ± 13	15.5 ± 1.3	159 ± 14	531 ± 20	638 ± 28
	This work (off-line)	157 ± 5	233 ± 5	15.4 ± 0.7	159 ± 14	564 ± 16	

[a] Results are expressed as the mean of 3 replicates ± standard deviation. [b] Former BCR scheme in batchwise extraction.[24] [c] The detection limits were 0.2 and 0.1 μg kg^{-1} for steps II and III of extraction, respectively.

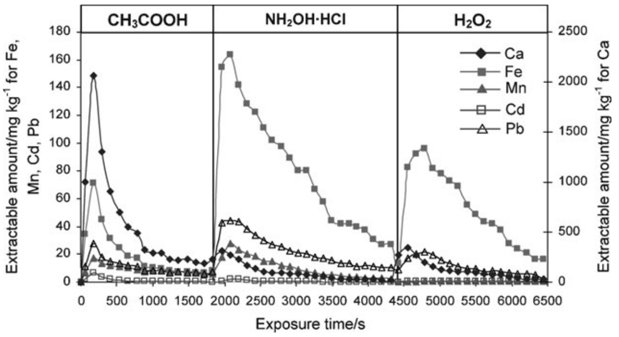


Fig. 3 Processed extractograms of trace and major elements in SRM 2711, as obtained from the three step BCR sequential extraction scheme performed in the proposed extraction system in hyphenation with on-line ICP-MS detection.

by a Ca peak in step I and Fe and Mn peaks in step II. This can be taken as evidence that the release of Cd and Pb by CH_3COOH and $NH_2OH \cdot HCl$ is dependent on the dissolution of soil solid phase material. This proves that the fraction of both metals retained by exchangeable soil sites is not as relevant as the Ca-bound fraction. Therefore, the dynamic fractionation method should be regarded as appealing approach for discrimination of soil phase associations taking into account the lack of selectivity of the three BCR extracting reagents for a defined soil compartment.

A detailed extractogram also provides further knowledge on the homogeneity of binding and mobility of metals in the nominal phases. The surface bound metals would dissolve earlier than the remaining in the same soil fraction and generate a distorted peak profile.[26] It is clear that the present approach provides a better way for exploration of metal soil phase associations that can be of utmost importance to assess the impact of readily accessible trace elements in environmental substrates.

3.5 Applicability of the proposed approach for the study of the elemental associations

Traditionally, metal associations in soil have been ascertained by investigation of their correlation in soil phases. Due to the restrictions of the batchwise approach that provides solely a single value of extractable metals in each fraction, the statistical comparison of the content of metals in a particular phase of a number of samples is commonly performed.[27–29] When the contents of the elements correlate well, they are considered to be closely associated. This basis overlooks the fact that elements extracted in the same extraction step may not have dissolved simultaneously but at a different timing during that step. The present approach may thus offer a better evidence of elemental associations and may more accurately be exploited for correlation investigations.

Shiowatana *et al.*[21] and Hinsin *et al.*[30] demonstrated the potential of overlayed extractograms obtained from batch analysis of extract fractions resulting from continuous-flow partitioning systems for investigation of metal associations. Two elements are considered as closely associated if their peak profiles and shapes coincide with time during a given extraction step. Elemental associations can be more accurately detected by on-line ICP-MS interelement comparison as a consequence of the high temporal resolution of the leaching profile, as exemplified in this work by overlapping the on-line extractograms for Cd and Zn. The extraction profiles illustrated in Fig. 4 reveal a close association between Cd and Zn in the acid soluble and reducible fractions, but not in the oxidizable fraction. The strong overlapping of peak profiles for Cd and Zn indicates the simultaneous release of these two elements from the same solid compartment. Extractograms are not merely applicable to the evaluation of elemental distribution and soil phase associations, but they can be extended for the investigation of the homogeneity of solid materials,[30] readsorption behavior of trace elements[25,31] as well as the degree of anthropogenic soil contamination.[26,32]

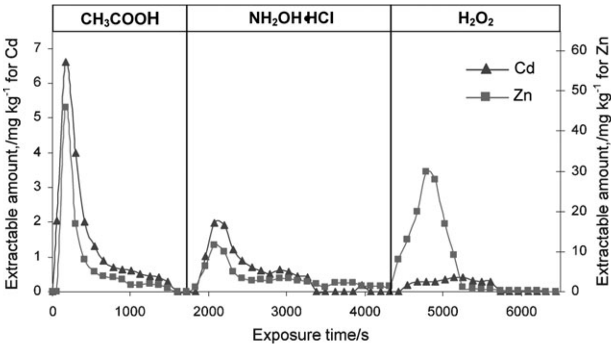


Fig. 4 Processed extraction profiles for Cd and Zn in SRM 2711.

4. Conclusion

The bi-directional continuous-flow hyphenated technique proposed herein has proven to be an appealing approach for conducting metal fractionation studies in environmental solid substrates. The non-steady flow rate during the fractionation protocol, as detected in former flow-through stirred cell systems, which hinders the coupling of the extraction units with atomic spectrometers, can be entirely circumvented by the alternate change of the flow direction of the extractant. Based on this concept, partitioning schemes can be carried out on-line with soil amounts of up to 1 g, thus assuring, as opposed to previous miniaturized microcolumn systems, sample representativeness and leaching flow rates of up to 10 mL min^{-1}. It can therefore be applied to various solid substrates for evaluation of metal accessibility under fast leaching conditions.

The hyphenated method offers not only information on trace element distribution but it constitutes itself as a powerful analytical tool for research in soil science in order to ascertain the modes of occurrence and accessibility of metals to biota as well as the detection of possible anthropogenic point sources. Thanks to the multielemental detection capacity of ICP-MS, interelemental comparison for exploration of metal associations in defined soil geological phases can be performed in a straightforward manner.

Although the alternate change of extractant flow direction through the extraction unit and the injection of discrete extract plugs into the ICP-MS were performed manually in this work, it is also feasible to develop a computer-controlled hyphenated set-up for fully automated on-line soil processing.

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On-line dynamic fractionation and automatic determination of inorganic phosphorus in environmental solid substrates exploiting sequential injection microcolumn extraction and flow injection analysis

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Abstract

Sequential injection microcolumn extraction (SI-MCE) based on the implementation of a soil-containing microcartridge as external reactor in a sequential injection network is, for the first time, proposed for dynamic fractionation of macronutrients in environmental solids, as exemplified by the partitioning of inorganic phosphorus in agricultural soils. The on-line fractionation method capitalises on the accurate metering and sequential exposure of the various extractants to the solid sample by application of programmable flow as precisely coordinated by a syringe pump.

Three different soil phase associations for phosphorus, that is, exchangeable, Al- and Fe-bound, and Ca-bound fractions, were elucidated by accommodation in the flow manifold of the three steps of the Hieltjes–Lijklema (HL) scheme involving the use of 1.0 M NH_4Cl, 0.1 M NaOH and 0.5 M HCl, respectively, as sequential leaching reagents. The precise timing and versatility of SI for tailoring various operational extraction modes were utilized for investigating the extractability and the extent of phosphorus re-distribution for variable partitioning times.

Automatic spectrophotometric determination of soluble reactive phosphorus in soil extracts was performed by a flow injection (FI) analyser based on the Molybdenum Blue (MB) chemistry. The 3σ detection limit was $0.02\,\mathrm{mg\,P\,L^{-1}}$ while the linear dynamic range extended up to $20\,\mathrm{mg\,P\,L^{-1}}$ regardless of the extracting media. Despite the variable chemical composition of the HL extracts, a single FI set-up was assembled with no need for either manifold re-configuration or modification of chemical composition of reagents.

The mobilization of trace elements, such as Cd, often present in grazed pastures as a result of the application of phosphate fertilizers, was also explored in the HL fractions by electrothermal atomic absorption spectrometry.

Keywords: Sequential injection; Dynamic fractionation; Inorganic phosphorus; Soil

1. Introduction

Phosphorus is an essential nutrient supporting the plant growth but it is also considered as a core factor for the eutrophication of rivers and lakes [1,2]. Accurate determination of inorganic phosphorus in natural waters and environmental solids is a current topic of major concern since this species is generally regarded as the limiting nutrient for biota growth. The quantitation of phosphorus in soils is aimed, for example, at the evaluation of the optimum soil phosphorus concentration required for plant growth; the determination of the phosphorus supplying capacity of soils according to the parental rocks; and most recently, the identification of scenarios where the prolonged application of superphosphate fertilizers might contribute to point source of trace element pollution.

In the geochemical, ecological and environmental fields, there has been an increasing interest for the chemical and physical characterization of the different forms of phosphorus in soils and sediments in order to determine the bioavailable fraction. A common route to estimate the stock of potentially accessible forms is to fractionate phosphorus according to the extractability by leaching reagents of increasing aggressiveness [3–8].

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Sequential extraction techniques are traditionally conducted in a batch end-over-end fashion, which is rather laborious, tedious, time consuming and it is subjected to several potential errors including risks of contamination due to sample manipulation and underestimation of given fractions due to re-adsorption phenomena. Although naturally occurring processes inherently always are dynamic, batchwise extraction are nevertheless routinely performed with particular leaching agents for extended periods of time to ensure the establishment of steady-state conditions between the solid and liquid phases.

In order to alleviate these drawbacks, recent trends have been focused on the development of on-line extraction protocols. A number of research groups have directed their efforts into the characterization of dynamic fractionation systems, mostly involving continuous flow or flow injection approaches [8–15], where fresh portions of extracting reagents are continuously delivered to small containers or microcartridges containing the solid material. This approach offers enhanced information on the fractionation procedure as regards to the kinetics of the ongoing leaching process, chemical associations between elements and size of pools available under environmentally changing scenarios.

Tiyapongpattana et al. [8] reported a continuous flow method for sequential fractionation of inorganic phosphorus based on the principle of stirred flow cells. Soil samples are extracted in a closed extraction chamber furnished with a glass microfiber filter by the action of leaching reagents that are continuously propelled forward by means of a peristaltic pump. Besides its simplicity, the continuous flow system features a significant reduction in extraction time as compared with conventional batch protocols. However, the proposed configuration is not sufficiently rugged for on-line dynamic sequential extractions owing to the instability of the flow rate occasioned by the clogging of the in-line filter. In addition, the large dimensions of the sample container hinder appropriate resolution of the extraction profile.

The inherent shortcomings of stirred flow cell configurations are to be overcome by exploiting sequential injection microcolumn extraction (SI-MCE) [16], that should be regarded as a simple, robust and expeditious technique for ascertaining the fractional distribution and binding sites of specific elements in solid samples. The automated sequence comprises the consecutive aspiration of the individual extractants from different external ports of the valve, which, via flow reversal, sequentially are exposed to the solid sample as contained in the microcartridge attached externally to one of the port positions. To the best of our knowledge, SI-MCE has been merely utilized for exploration of the mobility and availability of trace metals in soils [16–18], but, so far, no work has been reported on the potential applicability to macronutrient fractionation.

Amongst the various operationally defined sequential extraction procedures for the determination of phosphorus forms in environmental solids, the Williams et al. [19] and Hieltjes–Lijklema (HL) [3] schemes have been commonly adopted as routine methods. Even though the former has been taken as a working basis for the harmonized protocol of the Standard, Measurement and Testing (SM&T) Program of the Commission of the European Communities [20], its adaptability to flow systems for on-line fractionation is rather cumbersome because of the single extraction nature of the several steps. Hence, the sequential HL method, which is established as the standard within the field of limnology for phosphorus fractionation [21] was herein selected for on-line SI-MCE. This scheme addresses three inorganic forms of phosphate, that is: (i) the fraction retained by exchange sites, (ii) the fraction associated with aluminium and iron oxyhydroxides and (iii) the calcium-bound fraction, generally referred to as apatite-phosphate.

In dynamic extraction approaches, in contrast to conventional fractionation protocols, a plethora of extract solutions are generated, thereby rendering rather time consuming procedures when performing the extract analyses in a batchwise mode [8,9,11,16,17]. This work was therefore also aimed at the development and characterization of an FI system based on Molybdenum Blue (MB) complex formation for automatic and rapid analysis of HL extracts. It should be noted that FI has been primarily utilized as an analytical tool for monitoring dissolved reactive phosphorus in freshwater systems [22–30], yet only a few papers have dealt with soil extract analysis [31–34].

2. Experimental

2.1. Apparatus

An FIAlab-3500 flow injection/sequential injection system (Alitea, USA) equipped with an internally incorporated 10-port multiposition selection valve (SV) and a syringe pump (SP, Cavro, Sunnyvale, USA) with a capacity of 5 mL was used. The SI-system was computer controlled by the associated FIAlab software. The extraction microcolumn was connected within the SI-system as an external module as shown in Fig. 1a and b. All outlets of the SV were connected through PEEK ferrules with rigid PTFE tubing (0.5 mm i.d.). The central port of the SV was connected to the SP via a holding coil (HC1), which consisted of a 5 m long PTFE tubing (0.8 mm i.d.), with an inner volume of ca. 2.5 mL. The holding coil for the extracts in Fig. 1b was also made of PTFE (HC2, 2.5 m long, 1.0 mm i.d.).

The FI manifold devised for determination of orthophosphate in the SI-MC soil extracts is depicted in Fig. 2. The flow system is composed of two low-pulsation multichannel peristaltic pumps with rate selector (Type IPS-4, Ismatec, Zurich, Swizerland) used for reagents/sample propulsion and sample loading, respectively. The flow rates of the various streams as well as the optimum dimensions of the reaction coils for heteropolyacid complex formation and reduction by tin(II) chloride are shown in the figure. A minute volume of extract (viz., 25 μL) is injected into the flow network via a six-port rotary injection valve (Rheodyne, Type 5041). An UV–vis spectrophotometer (Jenway, Model 6300, UK) equipped with a 10 mm optical path flow-through cell was set at 690 nm and connected to a chart recorder (Radiometer, Model Rec 80 Servograph, Denmark) for monitoring of the molybdenum blue complex.

Determination of the concentration of cadmium in the HL fractions was performed by a Perkin-Elmer 2100 Electrothermal Atomic Absorption Spectrometer (ETAAS) equipped with

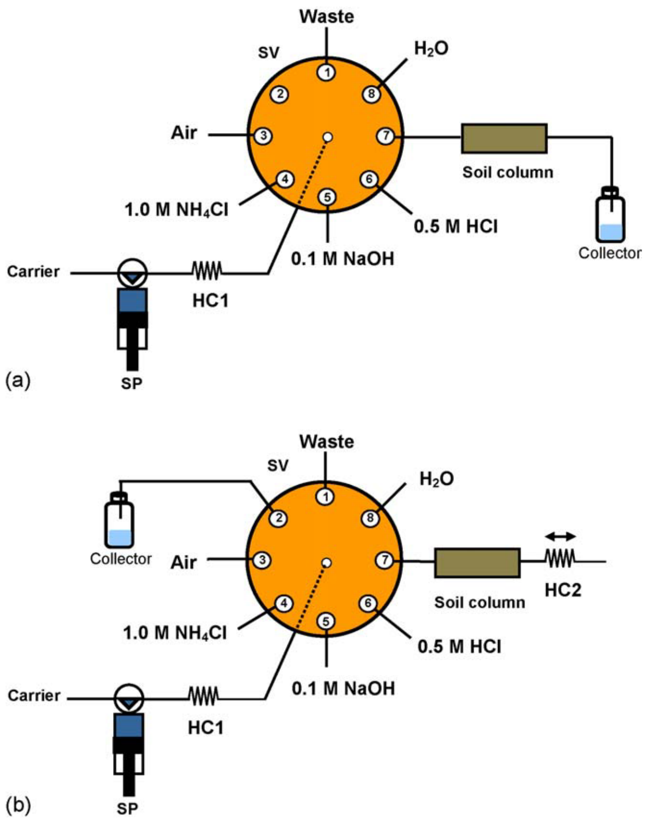

Fig. 1. Schematic diagrams of the sequential injection microcolumn set-ups for dynamic fractionation of inorganic phosphorus in soils as exploited for: (a) uni-directional and stopped flow and (b) bi-directional flow extraction. SP, syringe pump; SV, selection valve; HC1 and HC2, holding coils.

deuterium background correction, following manufacturer recommendations [35]. One microgram of $Pd(NO_3)_2$ was utilized as a matrix modifier.

2.2. Microcolumn assembly

The dedicated extraction microcolumn exploited in this work has been described in detail elsewhere [16,18]. Made of PEEK, it comprises a central dual-conical shaped sample container for facilitating fluidized-bed like mixing conditions. The entire unit is assembled with the aid of filter supports and caps at both ends. The membrane filters (Millipore, Fluoropore^{TM} filter, 13 mm diameter, 0.45 μm pore size) used at both ends of the extraction microcolumn allowed solutions and leachates to flow freely through but retained particulate matter.

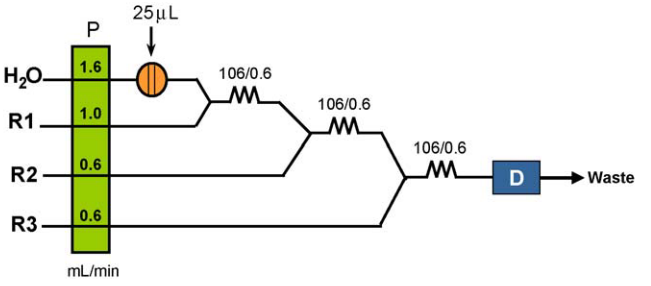

Fig. 2. Flow injection manifold for automatic monitoring of orthophosphate in soil extracts resulting from the Hieltjes–Lijklema scheme. R1, 2.4 g L^{-1} tartaric acid; R2, 12 g L^{-1} ammonium molybdate in 0.6 M H_2SO_4; R3, 0.3 g L^{-1} tin(II) chloride + 1.88 g L^{-1} hydrazinium sulphate in 0.5 M H_2SO_4.

2.3. Preparation of reagents

All glassware used was previously rinsed with 25% (v/v) concentrated nitric acid/water in a washing machine (Miehle, Model G 7735 MCU, Germany) and afterward rinsed with Milli-Q water. All chemicals were of analytical-reagent grade and Milli-Q water was used throughout. The various chemicals employed in this work are detailed as follows.

2.3.1. Standard solutions

A stock standard solution of orthophosphate (100 mg P L^{-1}) was prepared by dissolving 0.4392 g of KH_2PO_4 (Merck) in water and making up to a final volume of 1000 mL with Milli-Q water. The stock standard solution of cadmium (1000 mg L^{-1}) for AAS was purchased from Merck. Working standard solutions of P and Cd were obtained by stepwise dilution of the stock solutions.

2.3.2. Leaching reagents for sequential extraction

The chemicals utilized in the HL sequential extraction scheme are the following:

Step 1 1.0 M NH_4Cl adjusted at pH 7 with NH_4OH,
Step 2 0.1 M NaOH,
Step 3 0.5 M HCl,

which were aimed at stripping the labile, Fe- and Al-bound, and Ca-bound phosphorus fractions, respectively.

2.3.3. Reagents for flow injection analysis

According to the schematic diagram depicted in Fig. 2, R1 is composed of 2.4 g L^{-1} tartaric acid solution (Aldrich) in Milli-Q water, R2 involves 12 g L^{-1} ammonium molybdate (Riedel-de-Haën) in 0.6 M H_2SO_4 (Merck) and R3 comprises 0.3 g L^{-1} $SnCl_2$ (Merck) in 0.5 M H_2SO_4. The latter chemical also contains 1.88 g L^{-1} hydrazinium sulphate (J.T. Baker) for reagent preservation, whereby the combined solution is stable for, at least, 2 weeks.

2.4. Soil sample

A soil certified reference material from the National Institute of Standards and Technology, namely SRM 2711, was used for method validation. The soil material was collected from the tilled layer of a wheat field (MT, USA). It is a moderately contaminated pasture soil that was oven-dried, sieved and blended to achieve a high degree of homogeneity, the total concentration being certified for a number of elements, including phosphorus. The custom-built microcartridge was packed with 50 mg soil, thereby matching the sample weight endorsed in the batchwise HL scheme. Under these conditions, the free column volume (FCV) was estimated to be 250 μL.

2.5. Dissolution of soil residues

A closed-vessel microwave digestion system (Anton Paar Model Multiwave 3000, Austria) was used for digestion of residues after on-line fractionation. Residues from the extraction microcolumn were transferred to glass vessels as water slurries together with 2.0 mL of HNO_3 (65%, Merck) and 6.0 mL of HCl (30%, Merck). The vessels were then tightly sealed and subjected to a microwave digestion, according to manufacturer recommendations (1400 W, 30 min). After cooling, the digested solutions were made up to appropriate volume with Milli-Q water, and the content of orthophosphate was determined by spectrophotometry using a batchwise standard addition method.

2.6. Operational sequences for sequential injection microcolumn extraction

In this study, three applicable modes for the SI-MCE, that is, the uni-directional and stopped flow (see Fig. 1a) and the bi-directional flow (Fig. 1b), were investigated for inorganic phosphorus accessibility from soils. In all procedures, a maximum of 45 mL of each extractant was used. In order to avoid undue backpressure during extractant loading in those protocols involving flow reversal, merely 1 mL aliquots were manipulated at a time in the system using an air-sandwiched fashion. The complete sequential extraction procedure of the three approaches is described below.

2.6.1. Uni-directional flow

Before initialization of the extraction cycle, a 900 μL air plug is aspirated from port 3 of SV into HC1. Afterward, SP is set to aspirate 1.00 mL of 1.0 M NH_4Cl from port 4 into HC1 at a flow rate of 50 $\mu L\,s^{-1}$, whereupon the extractant and air plugs are pushed forward at the same rate through the microcolumn, allowing extraction to take place. For each five cycle runs, the extracts from the microcolumn are collected in a separate plastic vial, thus totally amounting to nine 5 mL subfractions for a complete set. Prior to continuing with the ensuing HL extraction step, a washing protocol is implemented by sequential aspiration of 900 μL of air and 1.00 mL of H_2O into HC1 which, via flow reversal, are delivered through the sample container into the fraction collector. This run is repeated five times. The amount of phosphorus leached in the cleansing step is summed to the content of the subfractions collected in the previous extraction. Thereafter, the next extractant (viz., 0.1 M NaOH or 0.5 M HCl) is automatically aspirated from the respective valve port and the collection of nine 5 mL subfractions repeated until all three leaching steps have been completed.

2.6.2. Stopped flow

The system operation was similar to that of the uni-directional flow procedure, except for the inclusion of an ancillary stopped flow stage. Here, a 900 μL air plug and 250 μL of extractant (that matches the free column volume) are consecutively pulled inwards into HC1 at 50 $\mu L\,s^{-1}$, the leaching reagent being dispensed afterwards via port 7 into the microcolumn where it remains halted for 30 s. Afterwards, the air segment in HC1 is dispensed forward to flush the leachant from the column into the fraction collector. The stopped flow mode is only programmed for the first step of the HL scheme, which estimates the loosely bound phosphorus fraction, in order to assess the possible re-adsorption of the target analyte on active soil sites. The extraction procedure was repeated with 20 cycle runs for each subfraction, thus amounting to a total of 9 subfractions for each set.

2.6.3. Bi-directional flow

SP is set to aspirate consecutively 900 μL of air and 1.00 mL of 1.0 M NH_4Cl into HC1 at a flow rate of 50 $\mu L\,s^{-1}$. The entire extractant plug is then delivered through the soil column at 50 $\mu L\,s^{-1}$ and becomes positioned in HC2. Thereafter, the extract is aspirated backwards through the column and via the central port into HC1, and then finally dispensed to the collection vial attached to port 2. Again, the leaching process is repeated with five cycle runs for each subfraction, and totally nine subfractions for each set. Just as the stopped flow approach, this operational mode is merely applied to the first step of the HL scheme.

The amount of orthophosphate collected in the various subfractions was determined by flow injection spectrophotometry using the manifold depicted in Fig. 2. Accurate determination of trace concentrations of cadmium released during application of HL method was conducted by ETAAS via direct injection of 20 μL extracts.

3. Results and discussion

3.1. Configuration of the sequential injection microcolumn system for fractionation of inorganic phosphorus

Although originally conceived for automated liquid phase analysis, the scope of SI has been recently expanded to the on-line handling of solid materials [16–18], which is a consequence of its inherent flexibility for accommodating external modules into the flow network. Yet, there is still place for the improvement for the various SI operational extraction modes, that is, uni-directional, bi-directional and stopped flow-based fractionation. Even though SI is a discontinuously operating flow approach, a truly forward flow fractionation scheme has been designed in this work. This is carried out by sandwiching metered volumes of extractant between discrete air plugs, thus preventing a portion of the leaching reagent to reside in the sample line in intimate contact with the packed soil during loading of the next leachant zone into HC1. Therefore, the contact time is identical for the entire segment in each run of the iterative multiple-step protocol. In addition, the inexistence of a concentration gradient in HC1 is assured by hindering the dispersion of the aspirated reagent plug into the carrier solution.

As opposed to earlier works [16,17], there is no need for re-configuration of the SI-manifold for the bi-directional flow approach. This is circumvented by replacement of the pre-valve microcolumn arrangement for an in-valve column attached configuration (see Fig. 1b). Improvement of the stopped flow mode is made by calculating the free column volume of the soil-packed

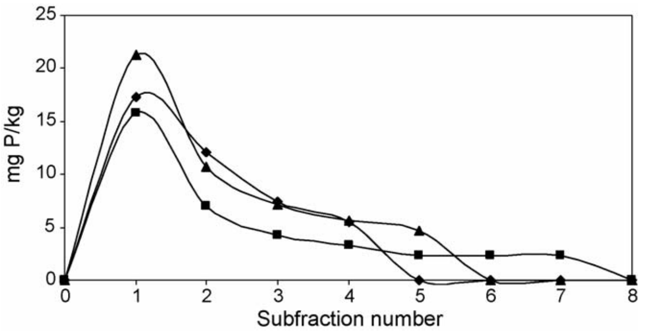

Fig. 3. Sequential injection extractograms of inorganic phosphorus in SRM 2711 as obtained from the application of three different operational modes for the step I of the Hieltjes–Lijklema scheme: uni-directional flow (♦); bi-directional flow (■); stopped flow (▲). Subfraction volume: 5 mL.

container and programming the liquid driver for accurate delivery of consecutive FCVs of extractant through the microcolumn prior to halting the flow in each single step of the analysis cycle.

In all cases, analysis of SI-MC extracts is conducted off-line because of the likelihood of effecting multiparametric sequential determinations of macronutrients and trace elements in each subfraction by spectrophotometry and atomic absorption spectrometry, respectively.

3.2. Sequential injection microcolumn for investigation of phosphorus re-adsorption

In sequential extraction techniques, aside from the non-selectivity of the extractant solutions, a crucial issue to be investigated is the extent of re-adsorption of leached elements onto the remaining solid phases during the time span of the extraction, which causes the underestimation of bioavailable fractions. Taking into account the versatility of movements of the syringe pump, the potential re-distribution of phosphorus during partitioning can be thoroughly studied by critical comparison of experimental data provided by the three aforementioned extraction modes for SI-MCE. It should be noted that the longer the intimate contact time between solid and leachant is, the higher becomes the probability that re-adsorption might occur. Therefore, the extraction time should be reduced to the extent possible [36], yet, a compromise should be reached regarding leaching kinetics for the targeted phases. Hence, the uni-directional flow, where the sample is continuously subjected to fresh extractant volumes ($t_{\text{ext}} = 5$ s) is expected to be free from re-adsorption as opposed to the bi-directional and stopped flow alternatives where the extracts remain longer ($t_{\text{ext}} = 10$ and 35 s, respectively) with the undissolved solid.

Fig. 3 shows the extractograms (graphical plots of extractable amounts versus subfraction number) recorded for the forward flow, bi-directional and stopped flow modes for the first step of the HL protocol. The investigation was restricted to the readily bioavailable phosphorus fraction because the re-distribution phenomenon is generally pronounced when employing mild extractants [37].

The fraction of exchangeable phosphorus determined by the uni-directional, bi-directional and stopped flow modes amounted to 0.0045 ± 0.0005, 0.0039 ± 0.0001 and 0.0043 ± 0.0007 mg P L^{-1}, respectively. The inexistence of significant differences at the significance level of 0.05 revealed that the re-adsorption was negligible for this particular solid material. This is attributed to the lack of selectivity of NH_4Cl, which is capable of removing partially carbonates from soil, thereby eliminating potential surfaces for phosphorus fixation [3,38]. The extraction patterns in Fig. 3 showed a dependence of the partitioning mode with the extraction rates, the highest rates being observed for the stopped flow approach.

3.3. Optimization of a flow injection system for monitoring of inorganic phosphorus in HL soil extracts

Two recent reports [31,39] have addressed the automatic determination of MB-reactive phosphorus in soil solutions resulting from batchwise fractionation via flow-based systems, namely FI and all injection analysis. However, Tiyapongpattana [39] claimed that it was a requirement to replace the carrier solution for each extractant medium to minimize the Schlieren effect, and Amornthammarong et al. [31] found that it was also required to adjust the concentration of sulphuric acid in the ammonium molybdate solution for the different leachant solutions. In this work, these flow manifolds were modified in order to design a simple set-up capable of admitting high electrolyte solutions as well as acidic and alkaline samples resulting from the HL scheme without changing neither the configuration of the system nor the chemical composition of the various streams. Schlieren effects were minimized by a 10-fold reduction of the sample volume used as compared to the earlier assembly [39] and a slight increase in residence time, thus promoting better axial mixing between the minute, well-defined volume of extract and the carrier solution (water) on their way towards the detector. On-line quantitation of soluble reactive phosphorus relies upon reaction of the target species with acidic molybdate to form molybdophosphoric acid which is subsequently reduced by tin(II) chloride to yield the blue-colored MB compound. Although the on-line formation of molybdosilicate is precluded by increasing the acidity of the reaction medium, the higher the acidity the lower the method's sensitivity due to the slow development of the reaction for molybdophosphate formation. Thus, the recommended concentration of sulphuric acid in the heteropolyacid forming reagent [39] was reduced from 1.0 to 0.6 M. The detection and determination limits for the optimized FI set-up at the 3 and 10σ levels were 0.02 and 0.09 mg P L^{-1}, respectively, regardless of the extraction medium, while the linear dynamic range extended up to 20 mg P L^{-1}. Actually, the tolerance of the flow injection method to metasilicate in 0.1 M NaOH, and in the presence of tartaric acid as a masking agent, is ca. 500 mg Si L^{-1}. Taking into account that silicate forms are hydrolysed, and thus mobilized, in alkaline solutions, and that the maximum available amount of silicon in SRM 2711 is merely 15 mg, the proposed method offers suitable selectivity for spectrophotometric monitoring of inorganic phosphorus in the selected material.

Table 1
Comparison of extractable amounts of inorganic phosphorus in SRM 2711 using the Hieltjes–Lijklema fractionation scheme as obtained with sequential injection microcolumn extraction and continuous flow stirred flow cell-based approaches

Flow-through method	Phosphorus content (%w/w)					
	1.0 M NH_4Cl	0.1 M NaOH	0.5 M HCl	Residue	Sum	Certified value
Continuous flow [8]	0.0189 ± 0.0006	0.0077 ± 0.0004	0.0413 ± 0.0006	0.0085 ± 0.0004	0.0764 ± 0.0011	0.086 ± 0.007
Sequential injection microcolumn[a] (this work)	0.0045 ± 0.0005	0.0093 ± 0.0010	0.0373 ± 0.0018	0.0275 ± 0.0048	0.0786 ± 0.0052	

[a] Results are expressed as the mean of five replicates ± standard deviation.

3.4. Accuracy of the proposed system and critical comparison of the analytical performance of SI-MCE with that of continuous flow systems

To evaluate the accuracy of the SI-MC system for phosphorus fractionation, a grazed soil with low organic phosphorus content, that is, the SRM 2711 Montana soil, was selected. Table 1 lists the amounts of orthophosphate extracted by on-line dynamic SI-MCE, which are compared with those recently reported for the same solid substrate using a continuous flow manifold with a stirred flow chamber [8]. The total phosphorus concentration obtained by summation of the entire set of fractions plus residue in SI-MCE showed a good agreement with the certified value and that obtained by fraction summation in the stirred flow cell method. Nevertheless, a significant difference in available phosphorus was distribution was found for the loosely bound fraction between the SI and the continuous flow method. This discrepancy is attributed to the different operationally defined extraction conditions for both procedures, such as, e.g., the partitioning time, and the non-selectivity of NH_4Cl for the targeted phase. In fact, the residence time of an extractant plug within the stirred chamber amounted to 120 s versus merely 5 s for the uni-directional SI-MC fashion. The shorter residence time in the proposed system would concurrently minimize the stripping of matrix ingredients which might potentially interfere in the determination step. Actually, while the standard addition method was mandatory for the determination of soluble reactive phosphorus in the NaOH and HCl extracts in the continuous flow system [8] owing to the poor performance of calibration protocols involving matrix matching with the pertinent extracting reagent, no multiplicative interferences were found in SI-MCE, whereby a straightforward external calibration approach with matrix matching was adopted.

The lack of selectivity of NH_4Cl for the exchangeable phosphorus fraction is occasioned by the capability of the reagent for attacking iron and aluminium hydroxy phosphate minerals, i.e., strengite and variscite, as a function of the contact time between solid and liquid phases [40]. This explains the overestimation of labile phosphorus and underestimation of aluminium- and iron-bound phosphorus in the continuous flow system as compared to SI-MCE (see Table 1).

The analytical performance of both batchwise and flow-through dynamic methods for fractionation of inorganic phosphorus according to the HL scheme is detailed in Table 2. The totally enclosed continuous flow system speeds up the extraction process as a result of the steady renewal of the leaching solutions and minimizes the risk of personal errors and contamination due to the avoidance of manual phase separations. Yet, the major problem posed by the application of continuous fractionation using stirred flow cells is the large dead volume of the sample container. Thus, a five-fold increase in soil amount versus batch fractionation is necessitated for attaining detectable signals due to the inherent dilution of released species. In addition, a larger consumption of reagents is concomitantly entailed for extraction until completion (≥300 mL versus 50–100 and 25–30 mL in batch and SI-MCE, respectively, for a single fraction). In fact, the stirred flow cell method was not deemed sensitive enough to be coupled to FI, for which reason the extracts were analysed by batch spectrophotometry. Furthermore, the fraction volumes collected in the continuous flow system, namely 60 mL, were 12-fold higher than those of SI-MC fractionation. The temporal resolution of the former method is therefore inappropriate for a detailed insight into leaching kinetics and phosphorus distribution in the soil phases, and only averaged leachable concentrations can be estimated. Finally, the instability of flow rates in the flow cell arrangement as a result of the pulsed flow of the peristaltic pump and blockage of the membrane filter with colloid particles, especially in the NaOH extraction step, was circumvented in SI-MCE by the novel design of the sample container and the ruggedness of the liquid driver.

Table 2
Analytical performance of batchwise and dynamic extraction methods based on Hieltjes–Lijklema scheme for fractionation of inorganic phosphorus in soils

Condition/parameter	Batch	Dynamic extraction	
		Continuous flow	SI-MCE
Sample (g)/extractant (mL)	1/1000	1/40[a]	1/5[b]
Extractant volume (mL)			
NH_4Cl fraction	2 × 50	540	25
NaOH fraction	50	300	30
HCl fraction	50	300	30
Extraction time per sample (h)	45[c]	3.8	2.1
Operational time for extract analysis (min)	20[d]	120[e]	20[f]

[a] Calculated as the ratio between sample weight and container volume.

[b] Calculated as the ratio between sample weight and free column volume.

[c] The length of the manual solid–liquid separations is not included.

[d] Only three extracts are analysed for the three-step fractionation procedure.

[e] The extracts containing NaOH and HCl extracted P are analysed by the standard addition method.

[f] The entire set of extracts is analysed by external calibration.

3.5. Applicability of Hieltjes and Lijklema scheme for cadmium fractionation in soil

Phosphorus is added to pasture soils by direct application of either reactive phosphate or acidulated rocks in the forms of single superphosphate, triple superphosphate or diammonium phosphate. These fertilizers contain varying concentrations of Cd depending on the particular phosphate mineral used as a raw material for their manufacture [41]. The Cd content of phosphate rocks varies widely, with the phosphate rocks of sedimentary origin having a very high concentration of Cd, and those of igneous origin having very low concentrations. The continuous use of fertilizers over a long time period might cause an undesired accumulation of Cd to levels above the maximum permissible concentrations set by regulatory authorities concerning human health. The impact of such fertilizers contaminated with trace elements, such as Cd, on the environment can be ascertained by sequential extraction. A multitude of partitioning schemes have been launched for evaluating the origin, mode of occurrence and mobility of trace elements in natural environments [37,42–45]. The potential applicability of the HL scheme to elucidate soil phase chemical associations not only for phosphorus but also for trace elements is, however, for the first time, investigated in this work. In fact, the HL procedure confines certain analogies with the well-established three-step fractionation scheme of the SM&T of the EU [45]. The first and third steps of HL, that involve the stripping of the exchangeable pools and phosphorus bound to calcium or adsorbed on calcium carbonate, are comparable with the first step of the SM&T protocol (i.e., acid soluble fraction) in terms of the nature of the soil phases solubilized. In fact, the summation of extractable Cd in SRM 2711 in steps I and III of the HL scheme performed in an MCE uni-directional flow fashion amounted to $37.9 \pm 1.1\ \mathrm{mg\ Cd\ kg^{-1}}$, which correlates well with the acid soluble Cd (viz., $36.2 \pm 0.6\ \mathrm{mg\ Cd\ kg^{-1}}$) as obtained from the step I of the SM&T protocol effected in a batchwise mode [46]. This good agreement between batch and flow-through partitioning methods is expected for labile, readily leachable elements, such as Cd. It should be also noted that the HL scheme offers, as opposed to SM&T, the possibility of discrimination of geologically relevant fractions (viz., exchangeable and acid soluble) which are of particular interest for readily mobilisable elements. The fractional distribution and extraction profiles of cadmium and phosphate using the HL protocol are schematically illustrated in Fig. 4. From the extractograms, it is feasible to discern the weakly adsorbed cadmium, probably as a free metal ion or chlorocomplex on ionic sites (step I in HL) from the cadmium carbonate (step III in HL) resulting from the isomorphic substitution of Ca by Cd in calcite minerals. The current impact of other potentially toxic elements in rock phosphates [47] such as As, Pb, Cr, Zn, Hg and F, which can be made available concurrently with phosphorus under environmentally changing conditions, might analogously be assessed via the HL partitioning method effected in a dynamic fashion.

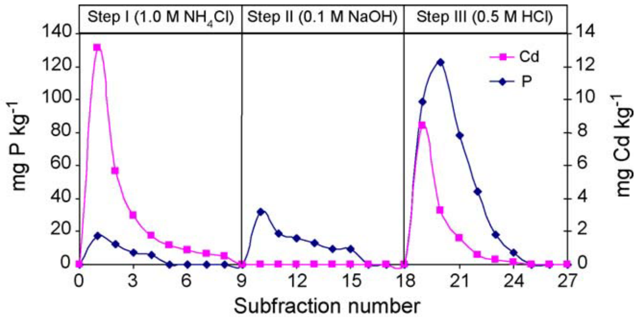

Fig. 4. Sequential injection extractograms of P and Cd in SRM 2711 as obtained from the application of Hieltjes–Lijklema scheme using the SI-MCE system in a uni-directional flow fashion. Subfraction volume: 5 mL.

4. Conclusion

The sequential injection microcolumn concept has been presented as a unique alternative to the batchwise and continuous flow counterparts for automatic fractionation of inorganic phosphorus in environmental solids. As opposed to previous flow-based approaches, the proposed SI-method features enhanced ruggedness and significant reduction of reagent consumption besides being characterized by rapidity, ease of operation and improved temporal resolution. The operational procedure and the configuration of the SI-MC system have been designed for on-line microfluidic handling of the HL leachant solutions and accurate control of partitioning time by exploiting programmable flow.

Bearing in mind the capability of SI to accommodate several operational fractionation modes in a dynamic fashion, i.e., uni-directional, bi-directional and stopped flow, the potential redistribution of phosphate for a particular extracting reagent can be assessed in a fully automated manner. In addition, a single flow injection system was assembled for facilitating the analyses of the HL extracts using the MB chemistry regardless of the composition of the extracting media.

The HL scheme, which offers enhanced phase resolution for readily mobilisable elements with respect to the harmonized SM&T protocol, was also utilized for the fractional distribution of cadmium in soils exposed to contamination via phosphate fertilizers. Experimental results revealed that both cadmium and phosphorus are predominantly bound to the calcium carbonate phase for the pasture soil (SRM 2711) analysed. Further research is being currently conducted in our group to exploit novel flow-based techniques, such as multicommutation flow analysis, multi-pumping flow analysis and multisyringe flow injection analysis, for on-line treatment of the multiple extracts obtained sequentially via dynamic SI-MC fractionation.

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PAPER

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Fractionation and elemental association of Zn, Cd and Pb in soils contaminated by Zn minings using a continuous-flow sequential extraction

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Fractionation and elemental association of Zn, Cd, and Pb in soils near Zn mining areas were studied using a continuous-flow sequential extraction approach. The recently developed sequential extraction procedure not only gave fractional distribution data for evaluation of the mobility or potential environmental impact of the metals, but also the extraction profiles (extractograms) which were used for study of elemental association. In addition, the elemental atomic ratio plot extractogram can be used to demonstrate the degree of anthropogenic contamination. Seventy-nine soil samples were collected in the vicinity of a Zn mine and were fractionated into 4 phases *i.e.* exchangeable (F1), acid soluble (F2), reducible (F3) and oxidizable (F4) phases. Most samples were contaminated with Zn, Cd and Pb. The reducible phase is the most abundant fraction for Zn and Pb ($>50\%$) while Cd is concentrated in the first 3 extraction steps. The distribution patterns of Cd were obviously affected by soil pH. 55% of Cd appears predominantly in the F1 fraction for acidic soils while in neutral and alkaline soils, it was mostly (70%) found in the F2 + F3 fractions. The extractograms obtained from the continuous-flow extraction system revealed close association between Zn, Cd, Pb and Fe in the acid soluble phase, Cd–Pb and Zn–Fe in the reducible phase for contaminated soils. A correlation study of the 3 metals using a correlation coefficient was also performed to compare the results with the elemental association revealed by the extractograms. Atomic ratio plot extractograms of Zn/Fe, Cd/Fe and Pb/Fe in the reducible phase, where contaminated metals are predominant, can be used to evaluate the degree of anthropogenic contamination. From the elemental atomic ratio plot, it is obvious that the contaminants Cd and Pb are mostly adsorbed on the surface of Fe oxides. Zn, which is present in an approximately 1 : 1 ratio with Fe in contaminated soils, does not show a similar trend to that found for Cd and Pb.

Introduction

To understand the issue of metal contamination and its effect on ecosystems and living organisms, not only its occurrence but also its behavior in soil has been of particular interest. At present, it is recognized that the distribution, mobility and bioavailability of heavy metals depends not only on their total concentration but also on their forms in the solid phase to which they are bound. A sequential extraction technique is widely used to fractionate metals in solid samples due to their leachability.[1–4] This technique makes use of suitable chemical reagents which are applied in a given order to the sample to sequentially leach metals of different phases. The reagents used are dependent on the goals pursued and on the physical characteristics of the sample.

Sequential extraction procedures are mostly performed in a batch procedure. Recently, our group has developed a continuous-flow extraction system for sequential extraction[5] which has shown many advantages compared to a batch method. The flow system has the benefits of simplicity, rapidity, less risk of contamination, possibility for automation and provision of kinetic leaching information. In this report, the system was applied to soil samples taken from near a Zn mining area in Tak Province, Thailand. The first objective of the study is to examine fractionation of metals in soil samples resulting from Zn mining activities. Since the continuous-flow extraction can provide extractograms of kinetic leaching of elements, it is the second aim of this work to use the extractograms to study the chemical associations in soil fractions and to evaluate the degree of anthropogenic contamination. Finally, the mobility and potential bioavailability of Cd in the soil was studied by investigation of the correlation between Cd in rice grain and Cd in specific fractions.

The environmental problems resulting from Zn mining have been reported in many parts of the world such as Derbyshire in central England[6] and in southern Poland.[7,8] In those areas, a number of elements associated with Zn or Pb mineralization have been found to be highly elevated in soil contaminated by mining operations. Soils from the vicinity of a Zn/Pb mining and smelting complex in Poland were found to contain high total concentrations of Zn, Pb and Cd: 234–12 400; 42–3570; 2–73.2 mg kg^{-1}, respectively.[8]

Associations of heavy metals with Zn ore have been reported. Zn, Cd and Pb minerals often occur together because they have similar chemical behavior and they are often found to combine with sulfur as primary minerals.[9] Other heavy metals found as common impurities in Zn ores are Mn, Hg, Ge, Ag in sphalerite; Fe, Co, Cu, and Mn, in smithsonite and Mn and Fe in zincite.[10,11]

Chemical partitioning of heavy metals (Pb, Zn and Cd) in soils contaminated by past Zn/Pb mining and smelting activities was investigated by Li and Thornton.[12] They found that the main chemical phases of Pb are carbonates (24–55%) and Fe–Mn oxides (30%). Zn was found to be strongly associated with the Fe–Mn oxides (30%) and residual fractions (50%). For Cd, the exchangeable, carbonate and Fe–Mn oxide phases account for more than 70% of the total Cd content.

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In metal bioavailability studies of contaminated soil, it is generally accepted that metal bioavailability in soils depends on many factors. These factors are not completely understood and simple relationships are seldom found in natural soil systems between plant metal levels and total metal concentrations in soil.[13–15] In some investigations, correlations between specific metal fractions and plant metal contents were found. For example, in soils that had been amended with composted or liquid sewage sludge it was found that Zn in exchangeable and oxidizable fractions had a strong correlation with Zn in the leaves of barley (*Hordeum vulgar*).[16] A simple relationship existed between Co in the exchangeable fraction and the Co concentration in winter wheat (*Triticum aestivum* L.) and alfalfa (*Medicago sativa* L.) while the adsorption of Ni, Cu and Pb by plants could be predicted by a stepwise multiple regression procedure.[17] These studies show the possibility for using sequential extraction data to evaluate the correlation between metals in soil and plant uptake. Therefore, the relationship between plant uptake of metals and the metal concentration in some fractions was also investigated.

Experimental

Chemicals and apparatus

All chemicals used were of analytical grade. Multi-element stock solution for ICP-MS (AccuStandard, Inc. CT, USA) has been used for the preparation of standard solutions. Before use, all glassware and plastic containers were cleaned and soaked in 10% (v/v) HNO_3 for at least 24 h, followed by rinsing three times with ultra pure water.

Inductively coupled plasma mass spectrometry (ICP-MS, Perkin Elmer ELAN 6000) was used for the elemental determination of extracts.

Sample under investigation

Seventy-nine top soil samples (0–10 cm) near a Zn mining area in Tak Province, Thailand were sampled. All the samples were then sieved through a 2 mm sieve and milled in an agate mortar to fine particles, to obtain good homogeneity, which were used in the chemical analysis. Soil pH, soil texture and % organic matter (%OM), determined by the Walkley and Black method,[18] of the samples investigated are given in Table 1. The soil samples studied were slightly acidic, neutral or alkaline. Most samples (69 samples) had a %OM between 1.5–3.5%. Their textures were sandy loam, loam, sandy clay loam or clay loam.

Fractionation scheme

The modified Tessier sequential extraction scheme[4,19] was carried out using the following solutions:

Step 1 (F1): 0.01 M $Ca(NO_3)_2$ (exchangeable fraction)

Step 2 (F2): 0.11 M CH_3COOH (acid soluble fraction)

Step 3 (F3): 0.04 M $NH_2OH \cdot HCl$ adjusted to pH 2 with HNO_3, 85 °C (reducible fraction)

Step 4 (F4): 8 : 3 v/v (30% H_2O_2 : 0.02 M HNO_3), 96 °C (oxidizable fraction)

Step 5 (F5): 1 : 1 v/v (conc. HNO_3 : 30% H_2O_2) (residual fraction)

Table 1 Some chemical and physical properties of the soil used in the study

pH	*n*	Texture	*n*	%OM	*n*
4.5–5.0 (Very acid)	7	Sandy loam	21	<1.5	1
5.1–6.0 (Acid)	12	Loam	16	1.5–3.5	69
6.1–6.5 (Slightly acid)	13	Sandy clay loam	20	>3.5	9
6.6–7.3 (Neutral)	12	Clay loam	22		
7.4–7.8 (Slightly alkaline)	21				
7.9–8.4 (Alkaline)	14				

n = Number of samples.

Continuous-flow sequential extraction system

Extraction chamber. The continuous-flow extraction system as previously reported[5] was used. The extraction chamber was designed to allow containment and stirring of a weighed sample. Extractants could flow sequentially through the chamber and leach metals from the targeted phases. The chamber and its cover was constructed from borosilicate glass to have a capacity of approximately 10 ml. The outlet of the chamber was furnished with a Whatman (Maidstone, UK) glass microfibre filter (GF/B, 47-mm diameter, 1 µm particle retention) to allow dissolved matter to flow through. Extractant was pumped through the chamber using a peristaltic pump (Micro tube pump, MP-3N, EYELA (Tokyo Rikakikai Co. Ltd)) at varying flow rates using Tygon tubing of 2.25 mm inner diameter. Heating of the extractant in steps III and IV at 85 °C and 96 °C was carried out by passing the extractant through a glass heating coil approximately 120 cm in length which was placed in a waterbath before the extraction chamber. The temperature of the extractant, either 85 °C or 96 °C, in the chamber was achieved when the heating coil was immersed in a thermostatted water bath controlled at 88 °C and 99 °C respectively, due to slight heat loss.

Extraction procedure. A weighed sample (approx. 0.25 g) was transferred to a clean extraction chamber together with a magnetic bar. A glass microfibre filter was then placed on the outlet followed by a silicone rubber gasket, and the chamber cover was securely clamped in position. The chamber was connected to the extractant reservoir and the collector vial using Tygon tubing and placed on a magnetic stirrer. The magnetic stirrer and peristaltic pump were switched on to start the extraction. The extracting reagents were sequentially flowed through the chamber and the extraction took place. The extract passing the membrane filter was collected at 30 ml volume intervals to obtain 6 subfractions for each extractant. Extraction was carried out until all four extraction steps were completed.

Total dissolution of sample and dissolution of residue. A closed-vessel microwave digestion system (Milestone model MLS-1200 Mega, Bergamo, Italy) was used for pseudo-total digestion of soil samples and residues. Weighed soil samples (0.25 g) or residue from the extraction chamber were transferred to the vessels together with concentrated HNO_3 (5 ml) and 30% H_2O_2 (5 ml). The vessel was then tightly sealed and subjected to a microwave digestion. After cooling, the digested solutions were made up to volume in volumetric flasks.

Analysis of heavy metals in soil extracts and digests

Cr, Mn, Fe, Co, Ni, Cu, Zn, Cd and Pb in soil extracts and digests were determined by ICP-MS. The contents of Cr, Mn, Fe, Co, Ni, and Cu were found to be in the normal range while concentrations of Zn Cd and Pb were significantly elevated with respect to the background concentrations reported by Soil Science Division, Thailand. The high concentrations of Zn, Cd and Pb detected in the soil extracts indicate that these sites have been contaminated by these three metals. Therefore, this report will focus on Zn, Cd and Pb.

Quality control of analytical data. A soil certified reference material, National Institute of Standards and Technology (NIST) SRM 2711, was used for method validation. Further-

Table 2 Comparison of metals contents in SRM 2711 soil as determined by continuous-flow sequential extraction procedure and certified values

	Concentration/mg kg^{-1}, $n = 2$						
Element	F1	F2	F3	F4	F5	Total	Certified value
Cd	0.24 ± 0.09	30.1 ± 1.2	2.2 ± 0.9	0.5 ± 0.1	0.01 ± 0.00	33.1 ± 0.2	41.70 ± 0.25
Zn	0.0 ± 0.00	68.9 ± 6.2	165.3 ± 10.4	157.6 ± 54.3	1.64 ± 0.001	393.4 ± 50.1	350.4 ± 4.8
Pb	0.0 ± 0.00	631.1 ± 105.7	461.2 ± 110.0	25.9 ± 2.5	0.54 ± 0.15	1118.7 ± 6.7	1162 ± 31

more, one-third of the total number of samples examined were also subjected to residual analyses to obtain total concentrations by summation of all fractions. The results were compared with those obtained by total digestion of the corresponding samples.

Blank analysis was performed frequently and every time a change of reagents or materials was used. High blank values were sometimes found, particularly for zinc, from some batches of glass fiber filters. However, this was found to be no problem because the levels of zinc in almost all samples studied were very high, such that extracted amounts were much higher than the blank values.

Results and discussion

Evaluation of the continuous-flow sequential extraction system

Evaluation of the continuous-flow sequential extraction system was performed by comparison of the summation data of all fractions with the certified values for total metal concentrations of the NIST SRM 2711 (Table 2). The total concentrations of Cd, Zn and Pb by summation showed good agreement with the total concentration from the certified values with slightly lower Cd and Pb values. This was also observed in our previous work, probably because the concentrations of elements in extracts were often lower in the continuous-flow system due to a dilution effect and some subfractions of the extract may not be detectable. Zn was found to be slightly higher probably from the higher blank values as a result of leaching contamination.

Fig. 1 presents correlation plots between the total metal contents of 24 samples determined by a single digestion of non-fractionated soil and those obtained by summation of metal amounts of all fractions from continuous-flow sequential extraction. The results show very good correlations with R^2 being higher than 0.99 for the three elements studied. The values from summation of fractionation data were slightly lower than those of total digestion as depicted by slopes being 0.87, 0.83 and 0.86 for Cd, Zn and Pb, respectively. This was also found in our previous work and was likely to be due to the dilution effect which caused poorer detectability for sequential extraction procedures. These levels of recoveries can be considered acceptable for this type of determination which involves summation of several data from subfractions and a dilution effect.

Distribution of Cd, Zn and Pb in soil samples

Soil samples studied were measured for their total concentrations by pseudo-total digestion and divided into 4 groups according to their total Cd, Zn and Pb concentrations; background level, slightly contaminated (2–5 times of background level), moderately contaminated (6–50 times of background level), and highly contaminated (more than 50 times background level). Most soil samples are contaminated with Cd and Zn in moderate and high levels (62 samples for Cd and 52 samples for Zn). For Pb, 46 of 79 soil samples analyzed are at background level while 26 samples are slightly and 7 samples moderately contaminated.

The concentrations of Cd, Zn and Pb in soils adjacent to the zinc mining area are distinctly elevated. Cd, Zn and Pb concentrations were found to decrease with distance from the mining area (data not shown) indicating the likeliness of zinc mining as the point source of contamination.

Fractionation of Pb, Zn and Cd in soil samples of different pHs

It is well known that many soil properties such as soil organic matter, cation exchange capacity (CEC) and soil pH can affect the distribution of heavy metals. It is interesting to investigate the fractional distribution of Pb, Zn and Cd in various soil pHs. Soil pH in this study falls in a very acid zone to an alkaline zone. The numbers of soil samples in each soil property group are shown in Table 1. To study the effect of soil pH on metal retention, the relationship between total

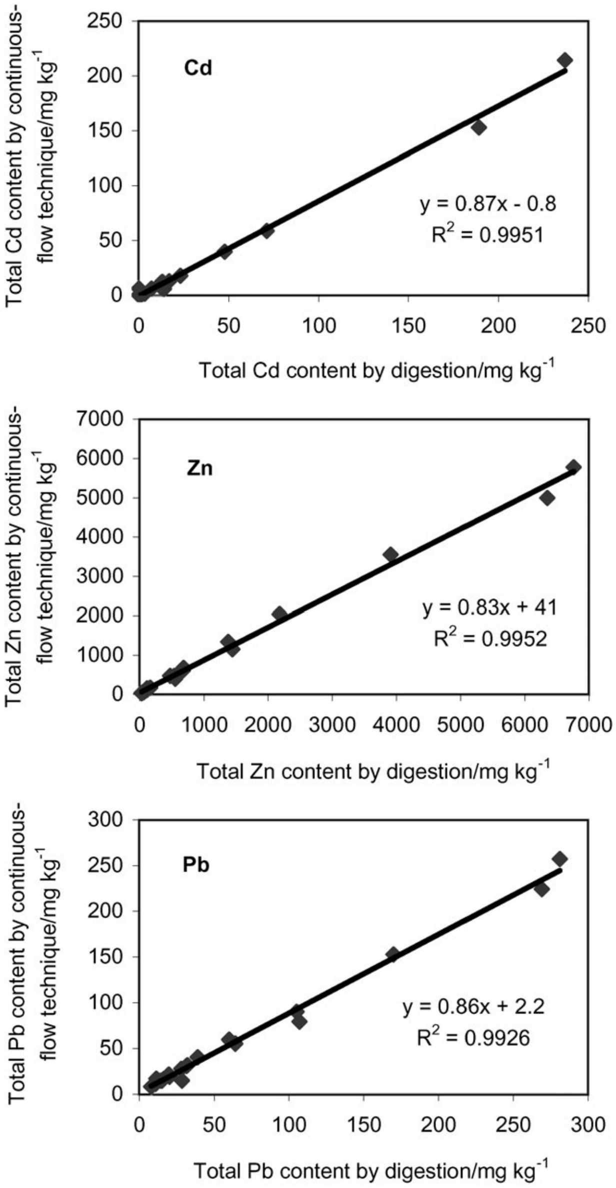

Fig. 1 Correlation plots of total metal contents determined by a single digestion of non-fractionated soil and by the continuous-flow sequential extraction procedure.

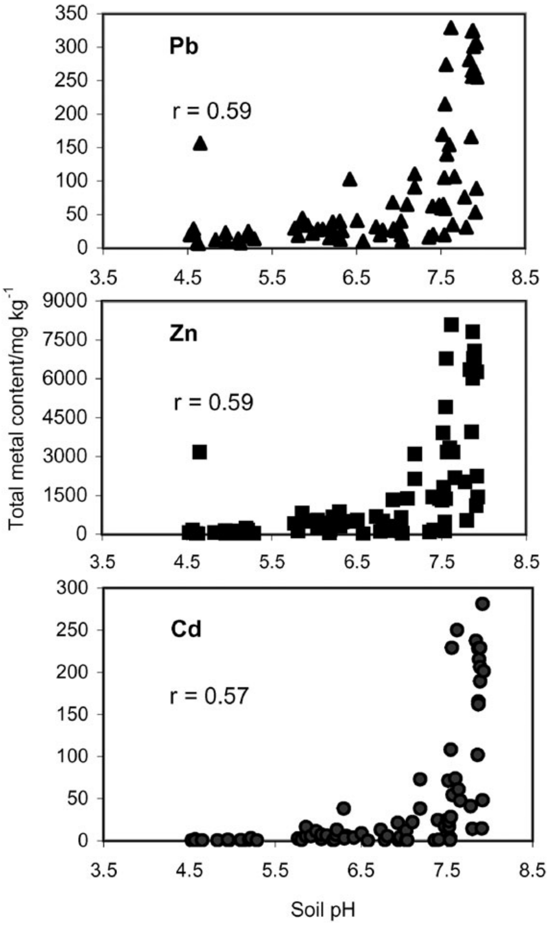

Fig. 2 Correlation plot between total metal contents and soil pH.

contents of Pb, Zn and Cd and soil pH was investigated. Fig. 2 shows clearly the pH dependence of metal retention in soil of the three elements. The three elements exhibit similar characteristics of pH dependence. Their total contents increase sharply with increasing pH from 7.0 to 7.9. Alkaline soils were proven to be a good scavenger of metals. This phenomenon is well-known and can be related to an increase of both specific and non-specific adsorption and to precipitation .[20]

The effect of soil pH on fraction distribution was also investigated. The average fraction distributions of Pb, Zn and Cd in 3 different soil pH zones, *i.e.* acidic (pH 4.5–6.5), neutral (pH 6.6–7.3) and alkaline (pH > 7.3) zones, are presented in Fig. 3. Samples with negligible amounts of extractable metals are not included in this investigation therefore the number of samples in acidic, neutral and alkaline zones are 32, 12 and 34 for Pb, 31, 12 and 34 for Zn, and 22, 8 and 32 for Cd, respectively.

For Pb, the most abundant fraction for the soils studied was found to be in the reducible phase (F3) regardless of soil pH. This fraction accounts for more than 70% of the extractable Pb content. The result agrees well with the observation of Li and Thornton[12] who found that a large amount of Pb in the soils at historical Pb mining and smelting areas was in the reducible fraction. The fraction distribution patterns of Pb are also very similar independent of the soil pH. The proportions of Pb were found to be in the order of: reducible (F3) > acid soluble (F2) > oxidizable (F4) > exchangeable (F1) fractions.

For Zn, about 50% of the extractable form was present in the F3 fraction for all three soil pH groups. Cadmium was predominantly (>55%) found in the first 2 fractions (F1 + F2) and mostly (>85%) in the first 3 fractions (F1 + F2 + F3). These results agree with many previous observations of Kuo *et al.*,[21] Hickey and Kittrick[22] and Xian[23] who reported that the organic/sulfide phase has only a minor role in binding Cd. The reducible phase was found to behave as an important scavenger of Pb and Zn and to a lesser degree for Cd.

In the case of Zn and Cd, soil pH shows a significant influence on the distribution patterns of these metals. Increasing soil pH caused a decrease in Cd and Zn contents in the exchangeable (F1) fraction but an increase in Cd and Zn contents in the acid soluble (F2) fraction. The effect of soil pH on the Cd distribution pattern has been explained[20,24] that as pH decreases, the solubility of the metal increases and metal is released in the forms of free or solvated ions. Therefore, lowering the pH of the soil can increase the concentration of metals in the soil solution.

Since exchangeable and acid soluble fractions (F1 + F2) are usually considered as mobile and having the most environmental impact, a comparison of their abundance in soil of different pHs was made. The Zn concentration in the mobile fraction (F1 + F2) in alkaline soils is higher than that in neutral soils and higher than that in acidic soils but that of Cd shows the opposite trend. The high Cd proportion in the mobile fraction (55–70%) clearly illustrates that Cd is more mobile and easily transported than Zn and Pb and it is therefore of particular concern, even without considering its higher toxicity.

The distribution of Zn and Cd in the oxidizable fraction (F4) is also affected by soil pH. Zn and Cd contents in this fraction seem to decrease with increasing soil pH. A clear explanation of changes in the percentage distribution of Zn and Cd in the F4 fraction is not possible in this study. Metal binding with organic matter *i.e.* humic acids was considered to be complex and dependent on many factors[25] such as pH and the presence of other metal ions.

Elemental associations in soil fractions using correlation study and extractograms

Generally, the association between metals in soils has been studied by investigation of their correlation in soil phases. This is performed by comparison of contents of two elements in a particular fraction of a number of samples. When the contents of two elements correlate well, they are considered to be closely associated. One of the problems when studying elemental association by this approach is the non-absolute evidence of elemental associations. Elements extracted in the same extrac-

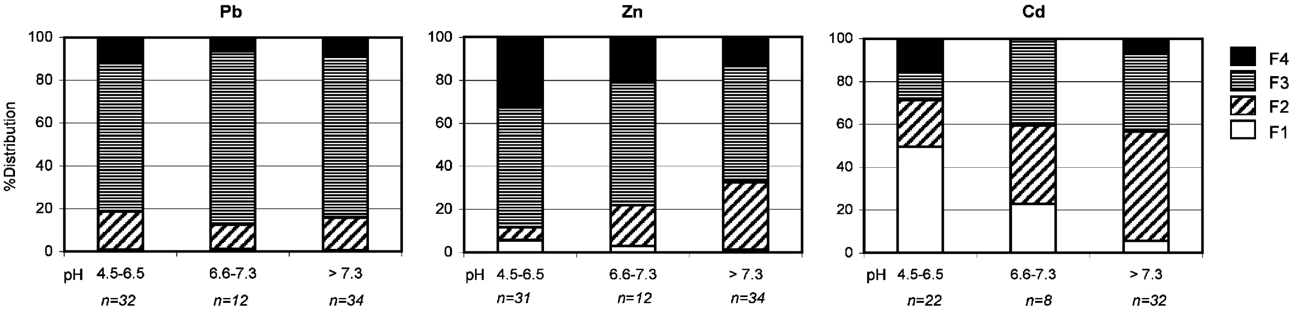

Fig. 3 Average % Pb, Zn and Cd distribution in soils of different soil pHs.

Table 3 Correlation coefficient between metal in soil fractions with other metal in corresponding soil fractions

	F1		F2		F3		F4	
	Cd	Zn	Cd	Zn	Cd	Zn	Cd	Zn
Group I (Cd < 0.15–0.75 mg kg^{-1}), $n = 17$								
Zn	0.000		0.577		0.714[a]		−0.067	
Pb	0.000	−0.087	−0.133	−0.167	0.562	0.576	0.876[a]	0.310
Group II (Cd > 0.75 mg kg^{-1}), $n = 62$								
Zn	0.372[a]		0.807[a]		0.848[a]		0.417[a]	
Pb	−0.019	0.109	0.752[a]	0.630[a]	0.851[a]	0.950[a]	0.396[a]	0.643[a]

[a] Correlation coefficient is significant at $p < 0.01$.

tion step may not have dissolved simultaneously but at a different time during that step. In other words, correlation of elements may not be a true elemental association. Such study of the relationship between metals in individual fractions by correlation study may be better referred to as 'elemental correlation'.

For investigation of elemental association, the overlayed extractograms obtained from a continuous-flow extraction can be used. Shiowatana *et al.*[26] and Hinsin *et al.*[27] used extractograms for evaluating the elemental associations in soils and iron hydroxide precipitates. The elemental associations for different elements can be studied by comparing detailed peak profiles and peak shapes of extractograms. Such detailed comparison is impossible using batch fractionation data alone, where the only possible comparison is between the elements extracted in a particular phase. The elemental correlation by considering the correlation coefficient and elemental association from extractograms are discussed using the data obtained in this study as follows:

The elemental correlations between Zn–Cd, Zn–Pb and Cd–Pb in individual fractions of soils are shown in Table 3. Because soils with high and low Cd contents may show different elemental correlations and in order to have a large enough number of samples in each group for correlation study, samples are divided into 2 groups based on the total content of Cd. In Table 3, the first group represents data from soils containing a total Cd content of <0.15–0.75 mg kg^{-1} (uncontaminated to slightly contaminated soil). The second group contains >0.75 mg kg^{-1} (moderately to highly contaminated soil).

The correlations between metals in the two groups of soil samples are different. For group I, correlation between Cd–Zn, Cd–Pb was found only in F3 ($r = 0.714$) and F4 ($r = 0.876$), respectively but significant correlations between Zn, Cd and Pb were found in group II for F2, F3 and F4 where contaminated metals were predominant.

Considering the elemental association in a particular fraction by using the extractograms, Fig. 4 shows the extractograms of Zn, Cd and Pb in highly, moderately contaminated and uncontaminated soil. The results show close associations between Zn, Cd and Pb and Fe in the acid soluble phase for the highly contaminated soil (Fig. 4a). In the reducible fraction, Cd and Pb appear to dissolve at the same time and earlier than Zn and Fe which appear together in the later part of the leaching. This indicates a strong association of Zn with Fe oxides which are important constituents of soil in the reducible fraction. The results of the relationship between metals in individual fractions from the two methods are obviously different because the two methods were based on different approaches as previously indicated.

Elemental atomic ratio plots for evaluation of the degree of anthropogenic contamination

The degree of contamination can be studied by the continuous-flow sequential extraction. This is obtained by a graphical plot of the elemental atomic ratio of metals of interest *versus* the subfraction number in a particular phase. The atomic ratio plots of Cd/Fe, Pb/Fe and Zn/Fe for highly contaminated, moderately contaminated and uncontaminated soils are shown in Fig. 5.

The extent of Cd, Pb and Zn adsorption on Fe oxides in the reducible fraction can be clearly observed in Fig. 5. For Cd in highly contaminated soil, high ratios of Cd/Fe were found in the early subfractions and decreased with subfraction number to approach a constant value. The atomic ratio of Cd/Fe in moderately contaminated soil differs from highly contaminated soil in that the atomic ratio is very low and almost constant for all subfraction numbers. Cd contents in uncontaminated soil

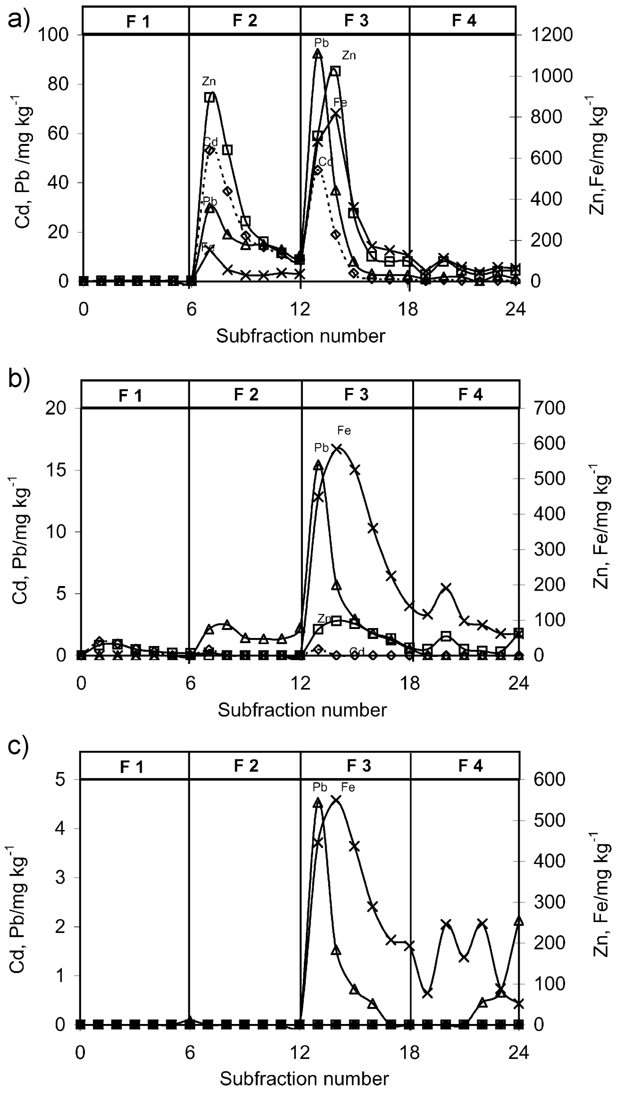

Fig. 4 Extractograms for Zn, Cd, Pb and Fe for highly (a), moderately (b) and uncontaminated (c) soils.

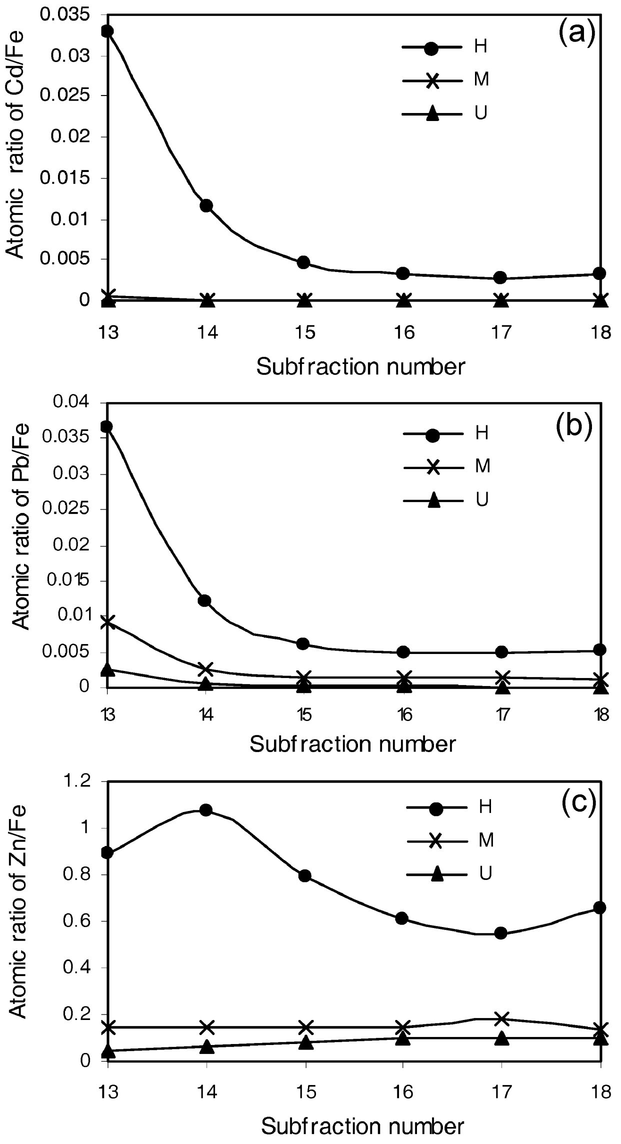

Fig. 5 Elemental atomic ratio of Cd/Fe (a), Pb/Fe (b) and Zn/Fe (c) plotted against subfraction number in the reducible phase, H = highly, M = moderately and U = uncontaminated soils.

are too low to be detectable; therefore, atomic ratio of Cd/Fe of the soil could not be determined. The results indicate that the contaminated Cd is mostly adsorbed on the surfaces of Fe oxides. The atomic ratio plots of Pb/Fe (Fig. 5b), show the same tendency as those of Cd. It appears that the atomic ratio plot can be used as a tool for identification of anthropogenic origin and degree of contamination. The atomic ratio of Zn/Fe in highly contaminated soil varied in the range 0.6–1.1 (Fig. 5c) while that of moderately contaminated and uncontaminated soil are lower than 0.2. The atomic ratio plot of Zn/Fe in highly contaminated soil does not show a similar trend as found for Cd and Pb.

Correlation between metal in rice grain and metal fractions

Heavy metal uptake by plants in contaminated soils has been extensively studied. The sequential extraction method has been used in an attempt to evaluate the relationship between the bioavailable fraction of metal in soil and the metal content in plant. Mench *et al.*[28] found that Cd and Ni uptake by maize was correlated with their contents in the exchangeable fraction. In this study, correlations between metal in rice grain, total metal contents, exchangeable (F1), acid soluble (F2) fractions and the summation of exchangeable and acid soluble (F1 + F2) fractions were studied. Pb in soil samples was mostly found near background levels or slightly contaminated therefore Pb is not included in this study. The correlation coefficients between metal in rice grain and their different fractions in soil collected from the corresponding field of rice grain samples are listed in Table 4.

Table 4 Correlation coefficient between metals in rice grain, total metal contents, exchangeable (F1), acid soluble (F2) and the summation of exchangeable and acid soluble (F1 + F2) fractions

	Metals in soil			
Metal in rice grain	Total	F1	F2	F1 + F2
Cd	0.341	0.680[a]	0.266	0.298
Zn	0.115	0.133	0.301	0.172
Fe	N.A.	0.567	0.079	0.034

N.A. = Not available.[a] Correlation coefficient is significant at $p < 0.01$.

A significant correlation was found between Cd in the F1 fraction and Cd in rice grain ($r = 0.680$). The result from fractionation shows the predominant Cd content in F1 for acidic soils. This implies that rice will uptake more Cd in acidic soil than neutral and alkaline soils. In other word, Cd uptake can be regulated by pH control of the soil. The present study agrees with previous reports which found that the uptake of Cd into crops grown on contaminated soils was correlated with Cd in the exchangeable fraction which was governed by soil pH.[7,29]

Total metal contents were poorly correlated with metal concentrations in rice grains ($r = 0.341$, 0.115 for Cd and Zn, respectively). These results agree with previous studies.[13,30–32] The different physical and chemical properties of soil have prevented the obtainment of direct relationships between the total metal contents in soil and plants.

The correlations between Fe and Zn in rice grain, total metal contents, concentrations of F1, F2 and F1 + F2 were also studied. The contents of Fe and Zn in the soil fractions do not correlate with the metal contents in rice grain. No significant correlation for these two elements was found.

Conclusions

A continuous-flow sequential extraction was utilized to study fractionation and elemental association of Zn, Cd and Pb in soil contaminated from zinc mining activities. Zn and Pb are predominantly present in the reducible fraction while Cd is concentrated in the first three fractions. Soil pH strongly affects the distribution of Cd but has no effect on Pb. The elemental associations were investigated from extractograms obtained. The results show close associations between Zn, Cd, Pb and Fe in the acid soluble phase for the contaminated soil. In the reducible fraction, Cd and Pb appear to dissolve at the same time and earlier than Zn and Fe, which appear together in the later part of the leaching. The extractograms provide detailed information that is not possible to obtain using a batch extraction technique. The data from elemental atomic ratio plot extractograms of Cd/Fe and Pb/Fe and Zn/Fe in the reducible phase confirm the information obtained from extractograms that contaminated Cd and Pb is adsorbed on the surface of Fe oxides. The higher the degree of contamination, the higher the Cd/Fe and Pb/Fe atomic ratio in the earlier subfractions of extraction. The two additional advantages of the continuous-flow extraction, *i.e.* using extractograms to study elemental association and use of elemental atomic ratio plots as a tool for identification of the anthropogenic origin and degree of contamination, have proved the continuous-flow extraction as an efficient system for detailed investigation of soil contamination. However, the continuous-flow extraction does have some drawbacks concerning the larger numbers of analyses of subfractions required and the dilution effect from the flowing stream of extractant.

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Automated Sequential Injection-Microcolumn Approach with On-Line Flame Atomic Absorption Spectrometric Detection for Implementing Metal Fractionation Schemes of Homogeneous and Nonhomogeneous Solid Samples of Environmental Interest

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An automated sequential injection (SI) system incorporating a dual-conical microcolumn is proposed as a versatile approach for the accommodation of both single and sequential extraction schemes for metal fractionation of solid samples of environmental concern. Coupled to flame atomic absorption spectrometric detection and used for the determination of Cu as a model analyte, the potentials of this novel hyphenated approach are demonstrated by the ability of handling up to a 300 mg sample of a nonhomogeneous sewage amended soil (viz., CRM 483). The three steps of the endorsed Standards, Measurements, and Testing sequential extraction method have been also performed in a dynamic fashion and critically compared with the conventional batchwise protocols. The ecotoxicological relevance of the data provided by both methods with different operationally defined conditions is thoroughly discussed. As compared to traditional batch systems, the developed SI assembly offers minimal risks of sample contamination, the absence of metal redistribution/readsorption, and dramatic saving of operational times (from 16 h to 40−80 min per partitioning step). It readily facilitates the accurate manipulation of the extracting reagents into the flow network and the minute, well-defined injection of the desired leachate volume into the detector. Moreover, highly time-resolved information on the ongoing extraction is given, which is particularly relevant for monitoring fast leaching kinetics, such as those involving strong chelating agents. On-line and off-line (for Cu, Pb, and Zn) single extraction schemes are also proven to constitute attractive alternatives for fast screening of metal pollution in solid samples and for predicting the current, rather than the potential, element bioavailability by the assessment of the readily mobilizable metal forms.

Sequential injection (SI) analysis has already been established as a powerful, fully automated computer-controlled sample pretreatment tool for wet chemical analysis.[1−4] Basically, SI encompasses a multiposition selection valve with a central communication line that can be made to address each of its peripheral ports (refer to Figure 1), and which, by means of a syringe pump, is used for sequentially aspirating well-defined segments of the various constituents of the assay, situated at the individual ports, and placing them into a holding coil. Afterward, the segments are, via flow reversal, propelled toward the detector, the manifold allowing appropriate unit operations to be executed by incorporation of packed column reactors, reaction coils, dilution chambers, digestion or extraction units, or gas diffusion/dialysis modules.[5,6] The system, moreover, allows metering of minute volumes (down to a few tenths of microliters), and the use of a syringe pump readily and reproducibly permits automatic microfluidic handling and operation at variable flow rates at will.[7]

Despite the recognized advantages of SI with respect to the first generation of flow injection (FI),[8−9] manipulation of solid materials into the microchannels of the SI network remains a challenge for analytical chemists. Yet, this flow approach entails inherent potentials for on-line processing of solid samples, as recently demonstrated in our research group,[10] which should

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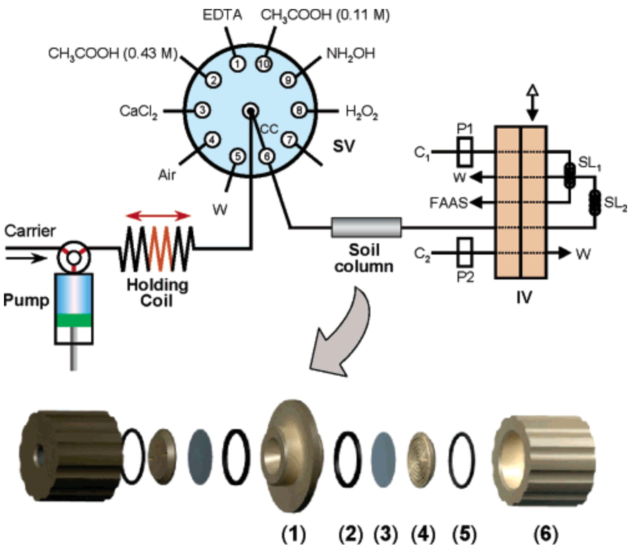

Figure 1. (Top) Schematic diagram of the hybrid FI/SI system with on-line FAAS detection for metal partitioning studies, comprising a syringe pump, a 10-port selection valve (SV) with a central communication channel (CC), a holding coil, a soil column, and a two-position injection valve (IV) accommodating sample loops SL$_1$ and SL$_2$. C$_1$ and C$_2$, carrier solutions; P1 and P2, peristaltic pump; W, waste. (Bottom) Exploded view of the extraction microcolumn, comprising the following components: (1) dual-conical sample container, (2) silicone gasket, (3) membrane filter, (4) filter support, (5) O-ring, and (6) end screw cap. The total volume of the sample container is ca. 400 μL. Bottom figure adapted with permission from ref 10. Copyright 2004 Elsevier Science Publishers.

attract the interest of practitioners in other scientific fields, such as soil science. In this discipline, fractionation analysis is currently regarded as a powerful tool for the identification and quantification of trace pollutants (e.g., anthropogenic metal species) bound to predefined geological phases in the soil sample. The batchwise procedures conventionally used are effected in a single or sequential extraction fashion under operationally defined conditions by attacking pollutant soil phase associations with solely one chemical reagent or by the sequential action of various extractants of increasing aggressiveness to leach the target species from particular soil compartments. Single extractions are mostly exploited to evaluate the exchangeable fraction of trace elements in soils, to estimate the fraction of total metal content available for root uptake,[11] and to predict pools of contaminants mobilizable via acidification or complexation processes.[12] Sequential extractions, though more laborious and time-consuming, provide enhanced information on the leachability of metal forms in different targeted phases. The ultimate aim of these fractionation schemes is to acquire knowledge on items of major environmental concern such as the origin, mode of occurrence, bioavailability, potential mobility, and the transport of the elements in natural scenarios.[13−17]

Although both single and sequential extraction protocols, such as the well-established three-step scheme from the Standards, Measurements and Testing (SM&T) Program of the European Commission[18,19] (formerly BCR), originally were devised to simulate natural conditions, researchers have more recently realized that naturally occurring processes are, in fact, always dynamic,[20,21] rather than static as they are identified by the traditional equilibrium-based methods. In addition, these batch ("end-over-end") extraction schemes suffer from several limitations regarding practicability (e.g., lack of automation, potential risks of contamination, labor-intensive and time-consuming protocols) and the significance of information provided, due to the inherent phase overlapping as a consequence of phase transformations, readsorption, and re-distribution of extracted elements.

In recent years, a number of groups have developed clever dynamic fractionation methods capitalizing on the advantageous features of FI schemes to circumvent the abovementioned drawbacks.[20−25] In all cases, the solid sample, as contained in small containers or microcartridges, is continuously subjected to the effect of fresh portions of a given leaching agent provided in a semiautomated fashion. These strategies have already opened new avenues for exploring the kinetics of the leaching processes as a function of exposure time and for obtaining a more realistic insight into metal lability from the different soil fractions. However, the manipulation of the extractant into the FI network to allow for increasing contact times with the solid substrate is rather cumbersome due to the intrinsic rigidity of the FI manifold and the progressive aging of the flexible tubes of the peristaltic pumps demanding a high frequency of maintenance. Although hyphenated microanalytical techniques for on-line detection of released species have been proposed recently, metal fractionation studies have merely been performed by increasing the concentration of the nitric acid supplied to the packed sample.[21,23] To the best of our knowledge, the application of a complete sequential extraction scheme in a fully automated mode along with on-line detection has not yet been reported.

In this paper, SI is exploited for the first time as a versatile and automated approach for the implementation of both single and sequential metal extraction/fractionation protocols in a single, compact module furnished with a dedicated dual-conical microcolumn, which can be hyphenated with atomic spectrometers (e.g., ICP-AES, ICP-MS, or AAS) for on-line leachate measurements. While it would be preferable to use a multielement detection device, a flame-AAS (FAAS) instrument was used in this investigation to exploit the principle applicability of the methodology.

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Detailed information about the kinetics of the ongoing extraction process and the current mobility of the various chemical forms of the target elements may thus be obtained. By taking advantage of the multiposition selection valve and the accurate microfluidic handling of the syringe pump, the different and well-accepted SM&T single and sequential extraction solutions can be precisely aspirated and dispensed through the soil microcolumn, whereupon the stripped target species are immediately injected into the detection device via a straightforward FI interface. Controllable on-line dilution to account for the variability of metal concentrations in the various extracts is readily accomplished by programming the portion of the FI sample loop to be filled by the leachate.

To ascertain the potentials of the hyphenated technique, a nonhomogeneous metal-contaminated complex sample was employed, namely, the sewage amended CRM 483 soil, with certified metal extractable contents for single extraction procedures (acetic acid and ethylenediaminetetraacetic acid (EDTA)), using Cu as the model of an anthropogenic metal ion of major concern, for instance in mining areas.[26] The dynamic information obtained with the microanalytical automated flow system is also critically compared with that previously reported for batch fractionations.

EXPERIMENTAL SECTION

Instrumentation. A FIAlab-3500 flow injection/sequential injection system (FIAlab, USA) equipped with an internally incorporated ten-port selection valve (SV), and a syringe pump (SP, Cavro, Sunnyvale, CA) with a capacity of 5 mL was used. The SI system was computer-controlled by the associated FIAlab software. The extraction microcolumn was incorporated within the SI system, and the outlet of the sample container was connected to a ten-port two-position injection valve (IV), acting as interface to a flame atomic absorption spectrometer (FAAS, AAnalyst 100, Perkin-Elmer), as depicted schematically in Figure 1. All ports of the SV and IV were connected through PEEK ferrules with rigid PTFE tubing (0.5 mm i.d./1.60 mm o.d.). The central port of the SV was connected to the holding coil (HC), which consisted of PTFE tubing (1.32 mm i.d./1.93 mm o.d.), the length being 110 cm, corresponding to a volume of 1.5 mL. The lengths of the PTFE tubing from port 6 of SV to the microcolumn and from the outlet of the microcolumn to IV were each 20 cm, corresponding to a volume of 40 μL. The IV was furnished with two sample loops (SL_1 and SL_2) each of 175 μL capacity. The connecting line between the IV and the FAAS nebulizer, which contributes to leachate dilution, was 19 cm long. The carrier solutions (C_1, and C_2; Milli-Q water) were pumped by means of a multichannel peristaltic pump (Ismatec, SA 8031) furnished with Tygon tubing. The peristaltic pump (in Figure 1 for graphical reasons depicted as two separate pumps, P1 and P2) was set at a slightly higher flow rate (viz., 6.2 mL min^{-1}) than the uptake rate of the FAAS (viz., 6.0 mL min^{-1}) to deliver the extract into the nebulizer by a positive pressure.

For on-line FAAS detection of Cu the wavelength and slit width were 324.5 nm and 0.7 nm (except for the extracts obtained in the EDTA single extraction method, where 216.5 nm and 0.2 nm were used). The same conditions were used in the off-line measurements of Cu, while the analytical wavelengths and slit widths for Pb and Zn were fixed at 283.3 nm and 0.7 nm, and 213.9 nm and 0.7 nm, respectively. Electrothermal atomic absorption spectrometry (ETAAS, Perkin-Elmer 2100) was exploited as a detection system to determine Pb in the acetic acid subfractions. The temperature program was executed according to the manufacturer's recommendations,[27] yet a 0.17 M $NH_4H_2PO_4$ solution was used as a modifier to increase the pyrolysis temperature to 850 °C.

Microcolumn Assembly. The purpose-made extraction microcolumn exploited in this work (Figure 1, bottom) was made of PEEK and comprised a central dual-conical shaped sample container with an inner volume of approximately 400 μL.[10] The entire unit is assembled with the aid of filter supports and caps at both ends. The membrane filters (Millipore, Fluoropore membrane filter, 13 mm diameter, 1.0 μm particle retention) used at both ends of the sample holder retains efficiently the solid particles while allowing solutions and leachates to flow freely with no backpressure effects.

Soil Sample. A certified reference material from the Standards, Measurements and Testing Program, namely, CRM 483 soil, was used to evaluate both the EDTA and the acetic acid single extractions, and the endorsed SM&T three-step sequential extraction method.[19] The solid material (sewage sludge amended soil) containing high levels of organic matter (viz., 12%) was collected from Great Billings Sewage Farm (Northampton, U.K.), as reported elsewhere.[28]

It should be stressed that the pretreatment steps for the preparation of this reference material involved sieving through a 2 mm round-hole sieve and mixing thoroughly by rolling for several days. The resulting material is, thus, characterized by a poor homogeneity, so that when the conical microcolumn was packed with the 300 mg soil, used in all experiments herein, it was guaranteed to constitute a representative fraction of the CRM 483 material.[29] It should be stressed that the selected soil cannot be handled reliably with previously reported FI/continuous microcartridge extraction techniques utilizing minute quantities of solid sample (5−25 mg).[21,23,25]

Reagents and Solutions. All chemicals were, at least, of analytical-reagent grade, and Milli-Q water was used throughout. Working standard solutions of metal ions were prepared by appropriate dilution of 1000 mg L^{-1} stock standard solutions (Merck) with 0.1 M nitric acid. The various chemicals employed in this work are detailed as follows: glacial acetic acid (Merck), EDTA free acid (Sigma), hydroxylammonium chloride (Merck), hydrogen peroxide (30%; Merck), calcium chloride dihydrate (Merck), Suprapur ammonia (25%; Merck), Suprapur nitric acid (Merck), Suprapur perchloric acid (Merck), and hydrofluoric acid (Merck).

Prior to use, all glassware was rinsed with 25% (v/v) concentrated nitric acid/water in a washing machine (Miehle, Model G 7735 MCU, Germany) and afterward rinsed with Milli-Q water.

Single Extraction and Modified SM&T Sequential Extraction Schemes. The reagents used in the single and sequential

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extraction schemes according to the SM&T recommendations are presented in the following sections:

Single Extraction Scheme. A 0.05 mol L^{-1} amount of EDTA was prepared as the ammonium salt solution by adding in a fume cupboard 14.61 g of EDTA free acid to 80 mL water and by partially dissolving it by stirring in 13 mL of 25% ammonia solution. The addition of ammonia was continued until all the EDTA was dissolved. The obtained solution was filtered through filter paper into a 1.00 L polyethylene volumetric flask and diluted with water to 0.9 L. The pH was adjusted to 7.00 ± 0.05 by addition of a few drops of ammonia or hydrochloric acid as appropriate. Finally, the solution was diluted with water to 1.00 L, well-mixed, and stored in a stoppered polyethylene container.

A 0.43 M solution of acetic acid was prepared by adding in a fume cupboard 25 mL of glacial acetic acid to about 500 mL of water in a 1.00 L polyethylene volumetric flask. The solution was diluted with water to 1.00 L volume, well-mixed, and stored in a stoppered polyethylene container.

A 0.05 M aqueous solution of calcium chloride was prepared by weighing 7.35 mg of the dried salt and then diluting it to 1.00 L volume with water.

Three-Step Modified Sequential Extraction Scheme. Step I (acid soluble fraction): A 0.11 M acetic acid solution was obtained by appropriate dilution of the 0.43 M acetic acid stock solution prepared previously.

Step II (reducible fraction): A 0.5 M hydroxylamine solution was prepared daily by dissolving 3.48 g of hydroxylamine hydrochloride in the minimum volume of water (ca. 10 mL) to which was added 1.6 mL of 2 M HNO_3. The solution was finally diluted to 100 mL with water.

Step III (oxidizable fraction): A 30% hydrogen peroxide solution was used as supplied by the manufacturer.

Dissolution of Residues. The residues from the extraction microcolumn were transferred to a PTFE vessel and, following addition of 6.0 mL of concentrated nitric acid and 5.0 mL of concentrated hydrofluoric acid, then heated gently to near dryness in a sand bath, with the temperature not exceeding 200 °C. Upon complete dissolution, the samples were cooled and 5 mL of perchloric acid was added, whereafter they were heated again to near dryness and diluted with 0.1 M HNO_3 to 50 mL prior to atomic absorption spectrometric measurements.

General Procedure for On-Line Single and Sequential Extraction. Initially, the holding coil was filled with the carrier solution (Milli-Q water) and all tubing from the peripheral SV ports were filled with the respective leaching solutions, while an air segment was delivered to the SL_2 interface via the assembled soil containing microcartridge. Since the total volume of the conduits between the holding coil (HC) and the FAAS (including microcolumn and sample loop) was 655 μL, SP was set to aspirate an equivalent volume of the desired extractant into HC and, then, the entire extractant plug was dispensed (10 $\mu L\ s^{-1}$) from HC directly to the microcolumn, allowing soil phase dissolution to take place, along with leachate collection into loop SL_2. After a delay time of 3 s, during which the pump was stopped, IV was switched (downward direction of the arrow in Figure 1) to make SL_2 part of the conduit between P2 and the FAAS and the extract was injected into the detector by means of carrier C_2. The 3 s delay time was incorporated first to engage the FAAS measurement and second to avoid creation of a pressure change during the valve shift.

Then SP was set to aspirate consecutively 100 μL of air from port 4 and 1050 μL of extractant into HC (at the rate of 100 $\mu L\ s^{-1}$). The role of the air segment is to prevent dispersion of the leaching reagent into the carrier solution. Solely 175 μL of the extractant plug (i.e., equal to the volume of the loops SL) was dispensed (10 $\mu L\ s^{-1}$), at each time, through the microcolumn into the SI−FAAS interface, in this case loop SL_1. The IV was again turned and the leachate, supplied to the nebulizer by carrier C_1, was analyzed on-line. The described procedure, alternately filling loops SL_1 and SL_2, was continued with a given reagent until no detectable amount of metal was leached (i.e., the signal was below LOD), or the increase was lower than 5% of the total metal leached. For elements continuously leaching from slowly accessible pools, as occurring in the acetic acid single extraction, a sample weight to extractant volume ratio similar to that of the batch method was selected.

The quantification of target analytes was done by peak integration (6 s) and summing up the peak areas of the particular metal fraction. All determinations were made using external calibration with standards prepared in diluted nitric acid. Although a common practice in batchwise fractionation is the use of calibration protocols based on matrix matching with the pertinent extracting reagent,[11,30] in our case, under the operationally selected conditions for CRM 483 partitioning, no multiplicative matrix interferences were encountered, as revealed by the application of the method of standard additions. This is a consequence of the discrete injection of minute, well-controlled volumes of extract into a continuously flowing carrier stream prior to FAAS detection, thus ensuring a high wash-to-sample exposure time, which diminishes risks of burner blockage.

RESULTS AND DISCUSSION

SI Analyzer for Single and Sequential Extraction Schemes. Dynamic extraction procedures for monitoring metal ion release from solid samples can be categorized into continuous[20−23] and discontinuous leaching processes[10] by exploiting the first (FI) and second (SI) generations of flow injection analysis, respectively. SI is more advantageous than FI in terms of accurate handling of microvolumes at predefined uniform flow rates through the solid substrate, because the flows are precisely controlled via the syringe pump with no need for frequent system recalibration. Furthermore, back-pressure or clogging effects in SI assemblies housing solid samples can, if called for, efficiently be alleviated by accommodating bidirectional flow approaches.

In our case, however, the SI setup with unidirectional flow fashion was entirely free from increase in flow resistance, even after long-term use. This is a result of the optimum hydrodynamic features of the dedicated biconical shaped microcolumn design (refer to Figure 1), which, in fact, accepts considerable amounts of solid material (up to 300 mg), so that solid materials lacking homogeneity can, also, be pretreated fully automatically without problems.

With regard to on-line detection, the discontinuous operational nature of SI must be taken into account for appropriate hyphen-

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ation with the detection instrument. Electrothermal atomic absorption spectrometry (ETAAS) is inherently the most attractive detector for trace metal monitoring following SI extraction. Yet, the accommodation volume of the graphite tube (<50 μL) is unnecessarily small even for monitoring fast leaching kinetics, and the extraction schemes can therefore be excessively prolonged as a consequence of the resulting low measurement frequency of ETAAS.

Although ICP-AES and ICP-MS nowadays have been consolidated as routine tools for metal determinations because of their high sensitivity and multielemental capabilities, their intrinsic low tolerance to total dissolved salts[31,32] limits their potential uses. For ICP-MS, spectral interferences arising from isobaric overlapping, and the presence of polyatomic ions, further makes its application troublesome.[33]

From an operational point of view and despite its single-element capability, FAAS should be regarded as an attractive alternative for on-line analysis of metal contaminated soil samples using the well-accepted SM&T extractants. Not the least because the moderate dilution required to bring the analyte concentrations into the linear dynamic range can be readily performed in the microconduits of the flow setup. Furthermore, this detection principle has proven to be compatible with direct FI injection of the leaching agents with negligible matrix interferences.

To make compatible techniques of different nature, an external FI device was required, thereby yielding a SI−FI hybrid approach. It should be stressed that while the free uptake rate of FAAS usually ranges from 4 to 10 mL min^{-1}, the packed microcolumn accepts maximum delivery flow rates of ca. 1.0 mL min^{-1}. As can be seen in Figure 1, a leachate injector furnished with two sample loops was used to ensure optimum performance of the SI and FAAS systems, and allowing consecutive measurements to be effected, that is, the leaching process itself became the limiting step of the overall procedure.

As opposed to unidirectional continuous extraction protocols in FI systems, the hybrid SI−FI extraction technique allows all of the extract to be subjected to analysis, and hence an almost continuous extraction profile can be recorded (whenever required, in-line dilution can be realized via the software-controlled injection of a well-defined microvolume of leachate into the carrier of the SI−FAAS interface). Thus, by using volumes equal to only 175 μL of extract per injection, detailed knowledge on the leaching kinetics is gained, which otherwise could not be obtained, as off-line FAAS detection in the continuous-mode requires more than 1000 μL solution for reliable measurements.

In the following, discussions of the two extraction schemes with on- or off-line detection are offered in more detail.

Single Extraction Schemes with Off-Line Detection. Weak acid and strong complexing solutions are commonly used to assess trace-element availability in soil sciences. Complexing agents extract both carbonate and organically bound fractions, whereas weak acids mimic the effect of an acid input, such as acid rainfall or an anthropogenic spill, onto solid substrates of environmental origin.[12,13] Therefore, one of the aims of the present work was critically to compare the analytical results from the proposed dynamic method as achieved with the CRM 483 soil with those obtained under equilibrium conditions. The SI extraction system with off-line detection was assembled for fractionation explorations of three trace elements of different recognized lability (viz., Pb, Cu, and Zn). While Zn is considered as a typical mobile element, Cu is regarded as a metal of intermediate mobility and Pb as a fixed element.[12] The automated extraction was performed in the unidirectional flow mode as reported before,[10] yet at a perfusate flow rate of 10 μL min^{-1} with collection of 1 and 2 mL per subfraction for EDTA and CH_3COOH, respectively.

Table 1. Extractable Contents of Trace Metals (mg kg^{-1}) in CRM 483 As Obtained by the Single Extraction Procedure Using the SI Extraction System with Off-Line Detection[a]

	acetic acid ($n = 3$)		EDTA ($n = 3$)	
element	obtained	certified value	obtained	certified value
Pb	1.4 ± 0.1	2.10 ± 0.25	206 ± 9	229 ± 8.0
Cu	19 ± 2	33.5 ± 1.6	227 ± 11	215 ± 11
Zn	637 ± 38	620 ± 24	617 ± 5	612 ± 19

[a] Soil sample, 300 mg; extraction flow rate, 10 μL s^{-1}; subfraction volume/total extractant used, 1 mL/50 mL and 2 mL/12 mL for EDTA and CH_3COOH, respectively.

Table 1 is a compilation of the extractable amounts of Pb, Cu, and Zn from the CRM 483 soil as obtained by resorting to the SI single extraction schemes with off-line detection. For the CH_3COOH extraction of Pb and Cu, the total volume of the extractant was fixed at 12 mL to maintain a soil mass/volume ratio identical to that in the batchwise method. For these two nonreadily accessible metal ions, a nearly constant, continuous leaching profile was detected, due to the nonselectivity of the reagent,[34] which is also capable of releasing different metal pools prone to acidification processes. On the other hand, Zn was rapidly leached from the sewage amended soil. The CH_3COOH extraction pattern was monitored until the increase of metal leached in five consecutive subfractions was less than 5% of the accumulated amount. As shown in Table 1, the extractable content of Zn via SI−CH_3COOH extraction is statistically comparable to the certified value, thereby indicating that both, conceptually different methods yield similar results whenever applied to weakly retained forms. This also holds true for reagents with a strong capacity for metal mobilization and stabilization, such as EDTA, thus minimizing readsorption and re-distribution drawbacks. In fact, several researchers have employed chelating agents aimed at limiting precipitation and sorption of solubilized forms during batch procedures.[35] In this context it should be born in mind that EDTA is not merely capable of mobilizing carbonate and organic metal forms by competing complexing reactions but also metals entrapped in hydrous oxides of iron because of the high stability constant of the Fe−EDTA chelates (viz, log $K = 25$). This explains the good agreement between the SI and the batch results for the EDTA extractable fractions of the entire set of target elements.

In contrast, the leachable content under dynamic mode for less labile forms, such as acid soluble Pb and Cu, is appreciably lower

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Table 2. Extractable Contents of Trace Metals (mg kg^{-1}) for CRM 483 As Obtained by the Single Extraction Procedure and the Modified SM&T Three-Step Sequential Extraction Procedure Using the SI Extraction System with On-Line FAAS Detection

		single extraction ($n = 3$)		sequential extraction ($n = 3$)				
element	method	acetic acid	EDTA	step I	step II	step III	residue	total (I + II + III + residue)
Cu	proposed system (automated)	26 ± 1	221 ± 5	12 ± 1	198 ± 8	39 ± 3	112 ± 6	361 ± 11
	reported value (batchwise)	33.5 ± 1.6[a]	215 ± 11[a]	16.8 ± 1.5[b]	141 ± 20[b]	132 ± 29[b]	43.3 ± 3.8[b]	335 ± 35[b] 362 ± 12[c]

[a] Certified value. [b] Taken from ref 30: soil sample, 300 mg; extraction flow rate, 10 μL s^{-1} (except step III, 5 μL s^{-1}); subfraction volume, 175 μL; total extractant used, 12 and 18 mL for CH_3COOH and EDTA (single extraction) and 12, 24, and 12 mL for steps I, II, and III (sequential extraction), respectively. [c] Total aqua regia digestion value.[30]

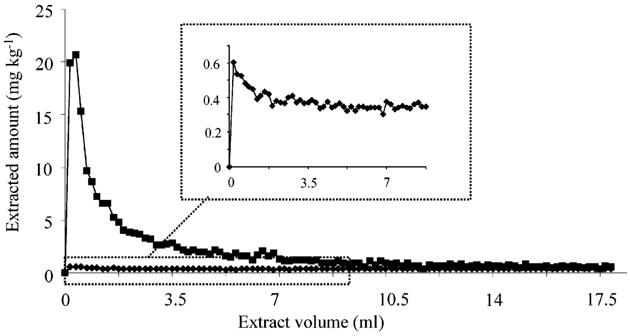

Figure 2. Extractograms for Cu in CRM 483, as obtained from single extraction on-line FAAS detection: (■) EDTA extraction; (◆) CH_3COOH extraction; subfraction volume, 175 μL; extraction flow rate, 10 μL s^{-1}. In the insert is shown a close-up of the extractogram within the first 8 min using CH_3COOH.

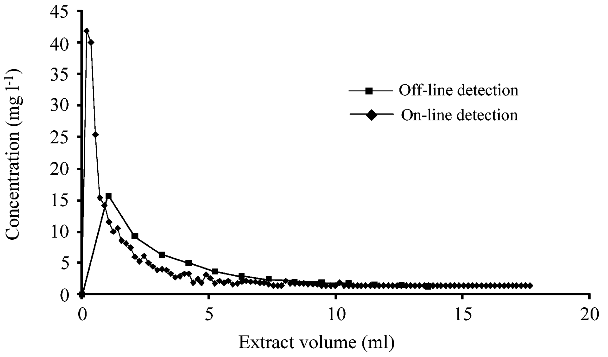

Figure 3. Extractograms for Cu with EDTA extraction, comparing the concentration of Cu in each subfraction when using off-line (1.0 mL per collected subfraction) and on-line FAAS detection (175 μL per subfraction).

than that obtained under a steady-state regime. This is a consequence of the inherently longer intimate contact time between soil and solution in the batchwise method. Actually, the results provided by both procedures are expected to differ considerably whenever the leaching agent attacks soil phases containing large available pools of slowly accessible elements. Yet, when increasing the acidic extractant volume in the SI method up to 50 mL, the leached amount for Pb and Cu was 3- and 2-fold higher, respectively, than the certified values due to the continuous shift of the metal distribution equilibria. Therefore, it is possible to conclude that the automated fractionation studies are better suited than their traditional counterparts for predicting actual risks associated with soil contamination.

Automated Single and Sequential Extraction Schemes with On-Line Detection. Microcolumn-based single and sequential extraction schemes were equally well accommodated in the fully automated SI setup for element partitioning studies. Besides preventing contamination risks, the totally enclosed environment reduces auxiliary sample manipulations dramatically, as neither separation nor dilution steps are needed. Very importantly, the microanalytical hyphenated technique ensures highly time-resolved information on the ongoing extraction process. Hence, fast leaching processes, such as those involving EDTA, can be appropriately monitored as depicted in Figure 2. For the particular reference material under investigation, more than 45% of the Cu(II) potentially prone to complexation is released in the first 2 mL of EDTA solution. Figure 3 clearly illustrates the different kinetic information rendered by the on-line analysis of minute volumes of leachate and, for comparison, measurements after manual dilution of 1 mL subfractions. The averaged concentrations of the semiautomated procedure are, thus, inadequate for short-term assessment of the ecological impact of pollutants liberated under the action of soluble ligands.

For the first time, the use of the three reagents in the well-established (sometimes termed standard[36]) SM&T extraction scheme[19] has been effected in a dynamic flow-through mode. According to previous researchers,[20] the third step of the procedure was realized at room rather than at elevated temperature, yet the flow rate was half (viz. 5 μL s^{-1}) of that for the other extractants in order to increase the contact time between the oxidizing agent and the sample. It should be emphasized that the methodology applied in the standard scheme for organic matter degradation solely is useful to estimate the total metal content associated with oxidizable soil phases. The oxidation at room temperature is a more realistic measure of the current, rather than potential, availability of metal ions by changes in oxidizing conditions or microbial activity in real-life samples, as metals associated with refractory organic compounds included in the mineral soil compartments are assumed to remain in the sample matrix for longer periods.[13]

As shown in Table 2, the fully automated single CH_3COOH extraction method renders, similarly to the off-line detection scheme, lower Cu recoveries than the batch protocol. Yet, the on-line available fraction (i.e., 26 mg kg^{-1} Cu) differs from that achieved under nonequilibrium regime but with fraction collection

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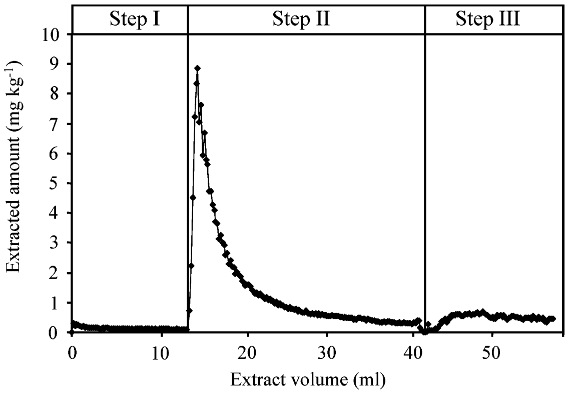

Figure 4. Extractogram for Cu in CRM 483 using the three-step sequential extraction scheme in the proposed SI system with on-line FAAS detection. Subfraction volume, 175 μL.

(i.e., 19 mg kg^{-1} Cu). These discrepancies are the result of the differences of the two operational procedures, since the delay time per subfraction required in the on-line method between extraction and measurement causes longer extractant−sample contact times (viz., 42 s/(mL of reagent)). The leaching patterns are obviously unaffected by the detection mode, as deduced from Figure 2 and the comments in the above section.

To compare critically both the dynamic and the batch extraction techniques, it should be noted that the contact time between the soil and the solution in the latter one is 16 h, so that metal re-distribution processes most likely take place. Gómez-Ariza et al.[37] reported that the percentages of readsorption of metals were strongly dependent upon the Fe−Mn oxide and organic matter content. In this context, Chomchoei et al.[38] demonstrated that the higher the organic matter content, the higher the readsorption of leached divalent metals, such as Cu, was encountered. Therefore, when the manual sequential extraction method is applied to soils containing high levels of organic matter, the first two steps are expected to be influenced by re-distribution processes. The higher recovery of Cu associated with easily reducible phases (mostly, manganese oxyhydroxides) for the sewage amended CRM 483 soil using the hybrid SI/FI system (see Table 2) as compared with the standard procedure is, hence, to be explained by the contribution of this phenomenon from step II to step III. The metal content liberated from oxidizable phases, cannot, however, be strictly compared because of the variable operational conditions of both methods.

In Figure 4 is illustrated the three-step Cu extractogram for the CRM 483 using the proposed flow system with on-line FAAS detection. The extraction profile renders an additional insight into the chemical associations of the elements within the soil compartments and into the leaching kinetics through continuous monitoring of the overall partitioning protocol.

As detailed in Table 2, the accuracy of the proposed approach is assessed by comparing statistically the summation of the extracted concentrations in each operationally defined phase plus the residue fraction with the reported total concentrations using aqua regia digestion. No significant differences existed between the total metal contents provided by the two methodologies. Thus, the extractable amount of Cu obtained by summation of steps I, II, and III (i.e., 249 mg kg^{-1}) is fairly similar to that mobilized using the EDTA single extraction method (i.e., 221 mg kg^{-1}). Therefore, the EDTA scheme can be regarded as a valuable tool for fast screening of metal pollution and measurement of readily bioavailable forms.

The most easily accessible pool of Cu from the CRM 483 was also monitored by resorting to the McLaren−Crawford scheme, originally developed for copper fractionation in soils.[39] The first two steps of this procedure, involving 0.05 M $CaCl_2$ for mobilization of water-soluble and exchangeable fractions and 0.43 M CH_3COOH for the acid soluble phases (or metals weakly bound to specific sites), were compared with the SM&T acetic acid single extraction scheme. The metal concentrations found in the exchangeable and acid soluble fractions were 1.8 ± 0.2 and 25 ± 2 mg kg^{-1}, respectively. The summation of these two fractions is statistically comparable to that of the SM&T method (viz., 26 ± 1 mg kg^{-1}), thereby demonstrating that single extractions are time and cost-effective alternatives to the sequential procedures for empirical assessment of the ecotoxicological significance of a given element in environmentally relevant solid samples, as also pointed out by several researchers.[13,36]

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Sequential injection system incorporating a micro-extraction column for automatic fractionation of metal ions in solid samples Comparison of the extraction profiles when employing uni-, bi-, and multi-bi-directional flow plus stopped-flow sequential extraction modes

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Abstract

Recently a novel approach to perform sequential extractions (SE) of elements in solid samples was developed by this group, based upon the use of a sequential injection (SI) system incorporating a specially designed extraction microcolumn. Entailing a number of distinct advantages as compared to conventional batch methods, this fully automated approach furthermore offers the potentials of a variety of operational extraction protocols. Employing the three-step sequential extraction BCR scheme to a certified homogeneous soil reference material (NIST, SRM 2710), this communication investigates four operating modes, namely uni-, bi- and multi-bi-directional flow and stopped-flow, allowing comparison of the metal fractionation profiles. Apart from demonstrating the versatility of the novel approach, the data obtained on the metal distribution in the various soil phases might offer valuable information as to the kinetics of the leaching processes and chemical associations in different soil geological phases. Special attention is also paid to the potentials of the microcolumn flowing technique for automatic processing of solid materials with variable homogeneity, as demonstrated with the sewage amended CRM483 soil which exhibits inhomogeneity in the particle size distribution.

Keywords: Sequential extraction; Sequential injection; Soil; Leaching kinetics; Microcolumn; Metal fractionation

1. Introduction

Sequential extraction is one of the most important tools to assess the impact of trace elements in solid samples (namely, soils, sediments, solid wastes, sludges, airborne particulates, biological tissues and foodstuffs) [1]. The results offer detailed information about the origin, mode of occurrence, bioavailability, potential mobility, and the transport of the elements in the natural environment [1–5]. When sequential extraction is practiced in the batch mode, there is, however, a high risk of sample contamination. Moreover, the classical manual procedures are tedious, time consuming, labor intensive and subject to several potential errors.

Exploiting the benefits of the manipulatory advantages of sequential injection (SI) [6–9], this group recently developed a novel, robust approach to perform sequential extraction of element in solid soil samples by using an SI-system incorporating a specially designed extraction microcolumn [10]. In this context, it was especially useful that SI-systems are based on using programmable, bi-directional discontinuous

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flow as precisely co-ordinated and controlled by a computer. Therefore, the flow network is easy and simple to reprogram from one application to another, notable advantages being that it allows the exact metering of even small volumetric volumes and that it readily and reproducibly permits flow reversals. Furthermore, sample/reagent consumption, operating times, as well as risks of contamination and analyte loss due to manipulation are considerably reduced in comparison with conventional batchwise systems. In this context, it should be stressed that the latter operationally-defined procedures are performed under pseudo-equilibrium conditions, so that kinetic information on the mobility of a given element is lost.

Based on the operation of the syringe pump, four operational modes are potentially feasible, comprising uni-directional flow, bi-directional flow, multi-bi-directional flow and stopped-flow, which are critically compared in this work aimed at metal fractionation explorations in solid samples. Sequential injection analysis is, thus, used as a powerful automated technique for accurate handling of micro-volumes of extracting solutions through the soil microcolumn in whatever desired sequence, and also as a promising tool in looking into the kinetics of the leaching processes as a function of exposure time, and into the chemical associations of the different components of the solid materials in the various leaching protocols.

In order to ascertain the results, the flow system was tested on two different certified reference materials with variable particle size distribution, namely, the homogeneous SRM 2170 pasture soil using the three-step BCR extraction scheme, and the heterogeneous sewage amended CRM 483 soil with single extraction protocols. It should be mentioned that the latter soil cannot be handled with reported flow injection/continuous microcartridge extraction techniques utilising minute amounts of solid sample [11–13], since the packed material cannot necessarily be considered as representative of the bulk medium. Though the emphasis herein is more on the potentials of the SI-microextraction technique as a novel operational concept able to integrate extraction schemes for metal fractionation in samples of different complexity, an interpretation of the behavior of the individually extracted metal species in the various operational modes is also provided for the SRM 2710 soil.

2. Experimental

2.1. Instrumentation

A FIAlab-3500 flow injection/sequential injection system (Alitea, USA) equipped with an internally incorporated 10-port selection valve (SV), and a syringe pump (SP, Cavro, Sunnyvale, USA) with a capacity of 10 ml was used. The SI-system was computer controlled by the associated FIAlab software. The extraction microcolumn was connected within the SI-system as shown in Fig. 1. All outlets of the SV were connected through PEEK ferrules with rigid PTFE tubing (0.5 mm i.d./1.60 mm o.d.) (Bohlender, Germany). The central port of the SV was connected to the holding coil (HC), which consisted of PTFE tubing (1.32 mm i.d./1.93 mm o.d.) (Cole-Parmer Instrument Company, USA), the length being 110 cm, corresponding to a volume of 1.5 ml.

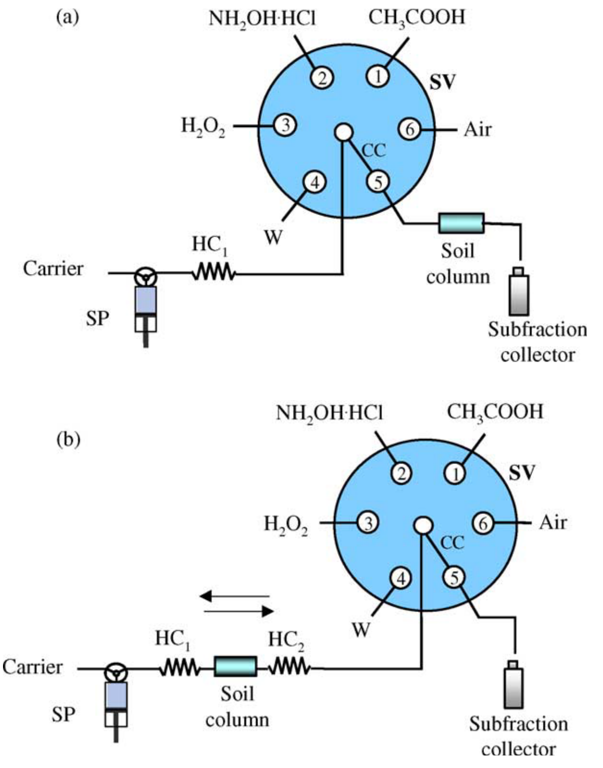

Fig. 1. Schematic diagram of the SI/SE systems used: (a) uni-directional flow and stopped-flow systems; (b) bi-directional flow and multi-bi-directional flow system. SV, selection valve; CC, central communication channel; SP, syringe pump; HC_1 and HC_2, holding coils number 1 and 2; W, waste; carrier, Milli-Q water (from Ref. [10] by permission of Elsevier Science Publishers).

Determination of the concentrations of the individual metal species was performed by using a Perkin-Elmer Analyst 100 flame atomic absorption spectrometer (FAAS) equipped with deuterium background correction.

When required, heating of extractants was made by means a thermostated hotplate allowing the solutions to obtain the required temperature before aspiration into the system.

2.2. Apparatus and procedure

The extraction microcolumn employed in this work has been described in detail previously [10]. Made of PEEK, and comprising a central bi-conical shaped sample container, with a volume of ca. 359 μl, and equipped with filters and filter supports and caps at both ends, the entire unit is ca. 43 mm long when fully assembled. The membrane filters (Millipore, Fluoropore^{TM} membrane filter, 13 mm diameter, 1.0 μm particle retention) used at both ends of the extraction microcolumn allowed dissolved matter to flow freely through.

Table 1
Experimental details of the three-step BCR sequential extraction scheme performed in the flow-through automated fashion

Step	Extracting reagent	Fraction
I	0.11 mol l^{-1} acetic acid, room temperature	Acid soluble
II	0.1 mol l^{-1} hydroxylamine hydrochloride, pH 2.0, 65 ± 5 °C	Reducible
III	8.8 mol l^{-1} hydrogen peroxide, pH 2.0, 85 ± 5 °C	Oxidisable

In this study, the three-step sequential extraction scheme of the Community Bureau of Reference (BCR) [14] was carried out as shown in Table 1.

2.3. Soil sample

A soil certified reference material from the National Institute of Standards and Technology (NIST), SRM 2710, was used. The soil material was collected from a pasture land (Montana, USA) affected by the deposition of creek sediments. It is a highly contaminated soil, the certified total concentration values of a number of elements being given. According to the certification report, this material was carefully prepared to achieve a high degree of homogeneity, its primary mission in this work being to provide a convenient way for evaluation of the accuracy of the proposed schemes. The extracts obtained automatically from the highly contaminated soil in the different extraction steps of the various operational SI-modes were processed by FAAS.

In this investigation, the dedicated microcolumn was packed with merely 25 mg of SRM 2710 soil. However, acceptable results when working with such low amounts of homogeneous solid materials have been verified previously [10] and also reported in the literature [11–13,15].

As a model solid sample to assess the potentials of the automated SI-extraction system, another certified reference material was also used, i.e., CRM 483 from BCR, which is a heterogeneous sewage sludge amended soil containing high levels of organic matter.

2.4. Preparation of reagents

All reagents were of analytical grade. Milli-Q water was used throughout. Working standard solutions were prepared by diluting 1000 mg l^{-1} stock standard solutions (Merck).

Other chemicals employed in the experiments were Suprapur nitric acid (65%, Merck), Suprapur perchloric acid (70%, Merck), hydrofluoric acid (40%, Merck), hydrogen peroxide (30%, Merck), glacial acetic acid (100%, Merck), and hydroxylammonium chloride (Merck).

Prior to use all glassware was rinsed by 25% (v/v) concentrated nitric acid in a washing machine (Miehle, Model G 7735 MCU, Germany) and afterwards cleaned with Milli-Q water.

2.5. Dissolution of residues and determination of total concentrations of metals

Residues from the extraction column were transferred to a PTFE vessel and then 3.0 ml of nitric acid (65%) and 3.0 ml of hydrofluoric acid (40%) were added and heated gently to near dryness in a sand bath, with temperature not exceeding 140 °C, as reported elsewhere [16]. The samples were cooled and 1 ml of perchloric acid was added, thereafter they were heated again to near dryness and finally diluted with 2% nitric acid. All the sample solutions were then further properly diluted to make the analyte concentrations within the linear dynamic range of the FAAS instrument.

2.6. Operating procedures

In this study, four applicable modes of the sequential injection/sequential extraction (SI/SE) procedure were investigated, that is, the uni-directional flow and the stopped-flow as shown in Fig. 1(a), and the bi-directional flow and triple bi-directional flow as depicted in Fig. 1(b). In all procedures, a total of 50 ml of each extractant was used. However, in order to ensure appropriate temperature control of the solutions, and to avoid back-pressure effects during extractant loading in those protocols involving flow reversal, only 1 ml aliquots were manipulated at a time in the system. The complete sequential extraction procedure of the four approaches thus runs through the following sequences.

Uni-directional flow. SP was set to aspirate consecutively 300 μl of air from port 6 and 1.00 ml of 0.11 M CH_3COOH from port 1 into HC_1 (at a rate of 100 μl s^{-1}). The role of the air segment is to prevent dispersion of the leaching reagent into the carrier solution. The entire extractant plug was then dispensed (50 μl s^{-1}) from HC_1 directly to port 5 and then passed through the microcolumn, allowing extraction to take place. For each five cycle runs, the extracts from the microcolumn were collected in a separate plastic vial, thus totally amounting to ten 5 ml subfractions for a complete set. Thereafter, the next extractant was automatically aspirated from the respective valve port and the collection of ten 5 ml subfractions repeated until all three leaching steps had been completed.

Stopped-flow. The system operation was similar to that of the uni-directional flow procedure, except that the procedure included the extra stopped-flow step. After aspiration of the air and of the extractant segment into HC_1, 800 μl of the leaching agent was dispensed (50 μl s^{-1}) from HC_1 directly to port 5 to wash out the former extract and stop the next fraction volume into the microcolumn, the stop period being affixed to 2 min. Afterwards, the remaining extractant in HC_1 was dispensed to pass through the column, thereby transporting totally 1.00 ml to the subfraction collector. The stopped-flow step was performed only in step I (acid soluble fraction) to assess the possible re-adsorption of the various elements on the soil particle surface, as well as the influence on the metal extractability by application of the ensuing reducing and oxidising leaching agents. The extraction procedure was repeated with five cycle runs for each subfraction and totally ten 5 ml subfractions for each set. Extractions with the following two extractants were made as described in the uni-directional flow procedure above.

Bi-directional flow. SP was set to aspirate 1.00 ml of 0.11 M CH_3COOH ($50\,\mu l\,s^{-1}$) from port 1 to pass through the microcolumn to HC_1. To ensure that all of the extractant actually had been passed through the column, the central communication channel (CC) in the SV was immediately, after the loading of the extractant, switched to port 6 and SP made to aspirate 1.00 ml of air ($50\,\mu l\,s^{-1}$) which hence became positioned in HC_2. The extract was then dispensed backward ($50\,\mu l\,s^{-1}$) through the column, HC_2, port 5 and finally collected in the subfraction collector. Again, the leaching process was repeated with five cycle runs for each subfraction, amounting to a total of 10 subfractions for each set. Identical protocols were executed for the consecutive steps of the sequential extraction scheme.

Triple bi-directional flow. The operation of the system was similar to that of the bi-directional flow procedure, except that the extractant in each cycle run was passed through the extraction microcolumn backward and forward totally six times (3× bi-directional). The triple bi-directional step was performed merely in the first step of the SE protocol, so that the extraction yields of the acid soluble fraction are to be compared for the various SI-extraction modes handled, that is, the following extractants were used as described in the bi-directional flow procedure.

The extracts were subjected to FAAS measurement after the overall automated extraction steps were completed. All determinations were made using external calibration without matrix matching, as this previously has been found unnecessary for this particular reference material [10]. The total metal concentrations were obtained for each extraction mode by summation of the metal content in the soil phase associations sensitive to acidification and redox processes, and the residual fraction, allowing statistical comparison with the certified values.

3. Results and discussion

3.1. Extraction by the SI/SE system

By exploiting the SI/SE microcolumn set-up illustrated in Fig. 1, and taking advantage of the versatility of the movements of the syringe pump, four modes of extraction are practicable, that is, uni-directional flow, bi-directional flow, multi-bi-directional flow, and stopped-flow, being also readily applicable to single extraction schemes.

In the uni-directional flow the extractant plugs are delivered only once through the microcolumn. This approach is simple, rapid and easy to perform. Moreover, in contrast to the other extraction protocols, and to batch-mode extractions, the uni-directional multiple-step scheme minimizes the problem of readsorption and redistribution of released species during extraction, because the extractant is being continuously renewed, and the contact time between the leachant solution and soil sample is, to a great extent, reduced. Thus, this dynamically operating mode can be regarded as an elegant solution to reduce the readsorption effects, which may dramatically affect trace metal extractability in the earlier steps of sequential extraction protocols, as reported by different researchers [17–19].

Yet, in order to obtain additional kinetic insight and valuable information on the various metal pools available, longer contact time between the extractant and the soil sample might be profitable, which can readily be achieved by exploiting the stopped-flow and the forward–backward flow approaches. The bi-directional flow mode must also be considered as a suitable alternative whenever back-pressure effects are encountered in the uni-directional approach due to the progressive tighter packing of the solid material. In the present application, however, the uni-directional operation turned out to be entirely trouble-free, and neither flow resistance nor tendency of clogging was observed even after long-term use at the moderately high flow rates applied (namely $3.0\,ml\,min^{-1}$), which is attributed to both the minute amounts of solid material employed and the optimum hydrodynamics of the custom-built microcolumn.

3.2. Comparison of the extractable amount of metals by different modes of extraction

One of the aims of the present work was to critically compare the analytical performance of the various SI-extraction procedures performed in an automated fashion in terms of extractability and acceleration of the operationally-defined SE schemes, thus reducing the number of subfractions. As a consequence of the longer contact times between the extractant plugs and the packed soil sample in the triple bi-directional flow and stopped-flow with respect to the uni- and bi-directional approaches, higher extraction efficiencies might be expected. On the other hand, it should be also kept in mind that problems due to metal readsorption/redistribution processes might emerge. If that is the case, the extractable amount of target metals by using the two former modes should decrease in step I and increase in either step II or III. Therefore, in this study, triple bi-directional flow and stopped-flow were executed solely in step I.

It is important to emphasise the existence of a correlation between the contact time and the extent of the redistribution/readsorption phenomenon, as the larger the extraction time the larger influence of this phenomenon is expected. However, it is possible to discern between both effects by comparison of the metal leachable contents obtained for the stopped-flow and forward–backward approaches for a given extraction time. In fact, the stopped-flow mode provides relevant information on the readsorption process as a consequence of the stagnant nature of the leaching solution, whereas the effect of the contact time may be

Table 2
Extractable amounts of Pb, Cu, Zn, and Mn for the various operationally-defined automated extraction modes using the SI/SE microcolumn set-up

Element	Mode	Amount extracted (mg g^{-1}) ± S.D. (n = 3)				Total (mg g^{-1})	Certified values (mg g^{-1})
		Step I	Step II	Step III	Residue		
Pb	Uni-directional	1.0 ± 0.1	2.11 ± 0.03	<0.36[a]	2.3 ± 0.1	5.5 ± 0.1	5.53 ± 0.08
	Bi-directional	0.97 ± 0.02	2.6 ± 0.1	0.5 ± 0.1	1.45 ± 0.09	5.5 ± 0.2	
	Stopped-flow	1.04 ± 0.01	2.48 ± 0.01	< 0.36[a]	2.10 ± 0.05	5.62 ± 0.05	
	Triple bi-directional	0.9 ± 0.1	2.39 ± 0.06	0.6 ± 0.1	1.5 ± 0.1	5.5 ± 0.2	
Cu	Uni-directional	1.38 ± 0.09	0.53 ± 0.02	0.15 ± 0.01	0.75 ± 0.07	2.8 ± 0.1	2.9 ± 0.1
	Bi-directional	1.46 ± 0.06	0.65 ± 0.05	0.14 ± 0.06	0.71 ± 0.06	3.0 ± 0.1	
	Stopped-flow	1.56 ± 0.09	0.73 ± 0.02	0.22 ± 0.04	0.40 ± 0.09	2.9 ± 0.1	
	Triple bi-directional	1.6 ± 0.1	0.70 ± 0.09	0.17 ± 0.08	0.4 ± 0.1	2.9 ± 0.2	
Zn	Uni-directional	1.62 ± 0.02	1.4 ± 0.1	1.1 ± 0.1	2.7 ± 0.2	6.9 ± 0.2	6.95 ± 0.09
	Bi-directional	1.65 ± 0.04	1.48 ± 0.03	1.27 ± 0.06	2.5 ± 0.3	6.9 ± 0.3	
	Stopped-flow	1.7 ± 0.1	1.52 ± 0.03	1.04 ± 0.03	2.7 ± 0.1	6.9 ± 0.1	
	Triple bi-directional	1.75 ± 0.09	1.53 ± 0.05	1.04 ± 0.01	2.6 ± 0.2	6.9 ± 0.2	
Mn	Uni-directional	1.9 ± 0.2	0.44 ± 0.02	0.63 ± 0.03	6.9 ± 0.7	9.9 ± 0.7	10.1 ± 0.4
	Bi-directional	0.95 ± 0.03	1.62 ± 0.04	0.46 ± 0.05	6.8 ± 0.8	9.9 ± 0.8	
	Stopped-flow	1.78 ± 0.05	1.5 ± 0.1	0.6 ± 0.1	6.2 ± 0.1	10.0 ± 0.2	
	Triple bi-directional	1.92 ± 0.07	1.6 ± 0.3	0.55 ± 0.02	5.9 ± 0.1	10.0 ± 0.3	

Sample, SRM 2710 soil; S/C ratio, 1:14; extraction flow rate, 50 μl s^{-1}.

[a] Below the FAAS detection limit.

ascertained via the bi-/multi-bi-directional modes since the diffusion layer of the extractant solution in contact with the soil matrix—containing the highest concentration of released metal species—is renewed for each extraction step.

Table 2 shows the extractable amounts of Pb, Cu, Zn and Mn for the SRM 2710 soil as obtained with the SI/SE system exploiting various automated extraction protocols. Despite the uni-, bi-, triple bi-directional and stopped-flow intrinsically give rise to different extraction times (namely, 7, 14, 48 and 120 s, respectively, for a loading flow rate of 50 μl s^{-1}), the extractability of trace metals from each soil phase association is by and large similar. However, the contribution of re-distribution processes can be detected for Pb and Cu due to the appreciable increase in the extractability for the reducing agent using the backward–forward (ca. 18 and 28%, respectively) and stopped-flow (ca. 17 and 38%, respectively) modes in comparison with the uni-directional flow. In fact, the phenomenon of re-distribution for the batchwise SE schemes has been found particularly relevant for Cu and Pb, the manganese oxide and humic acids being mostly responsible for this effect [2].

Moreover, as opposed to Pb, the SI-extraction yields for Cu using the chemical extractant with the lowest leachant strength, i.e., 0.11 M acetic acid, improved to some extent ($\leq$15%) by increasing the leaching time, which can be explained according to the different mobility of both metal ions: While Cu is regarded as an element of intermediate mobility, with pools of the readily leachable carbonate bound phase, Pb is considered as a typical fixed element [5]. In addition, whereas the Pb fraction susceptible of oxidation processes is not significantly different for the bi- and triple bi-directional flow, both the uni-directional and stopped-flow modes yielded lower extraction yields. This behavior might be explained by the difference of the extracting operations, because the uni-directional flow and the stopped-flow are basically based on the same operating approach, just as are the bi- and the triple bi-directional flow procedures.

The behavior of manganese was somewhat different from that of the remaining metals explored. It should, however, be taken into account that Mn, as opposed to Pb, Cu and Zn, is a major element in soil matrices. Although hydrous oxides of manganese are commonly described as easily reducible soil phases [2], manganese should be regarded in SRM 2710 as a non-labile element, being strongly associated to the residual fraction. According to Table 2, the extractable amounts associated to carbonates for the bi-directional flow, and the reducible fraction for the uni-directional flow are lower than those attained by the other modes of extraction. This is probably caused by the insufficient contact time between soil and extractant.

3.3. *Kinetic leaching and chemical associations in the soil sample*

Apart from the information on metal distribution in the various phases, extractograms of each element [20] as obtained by a graphical plot of extracted concentration and subfraction number can provide an additional useful insight into the kinetics of the leaching processes and the chemical associations of the elements within the solid phases.

According to the extractograms of Pb, Cu, Zn, and Mn resulting from the application of the different SI/SE operational modes to the SRM 2710 soil (see Fig. 2 for further details), the most labile elements, i.e. Zn and Cu, are specially sensitive to acidification processes, so that more than 25% of the total metal content is bound to carbonates.

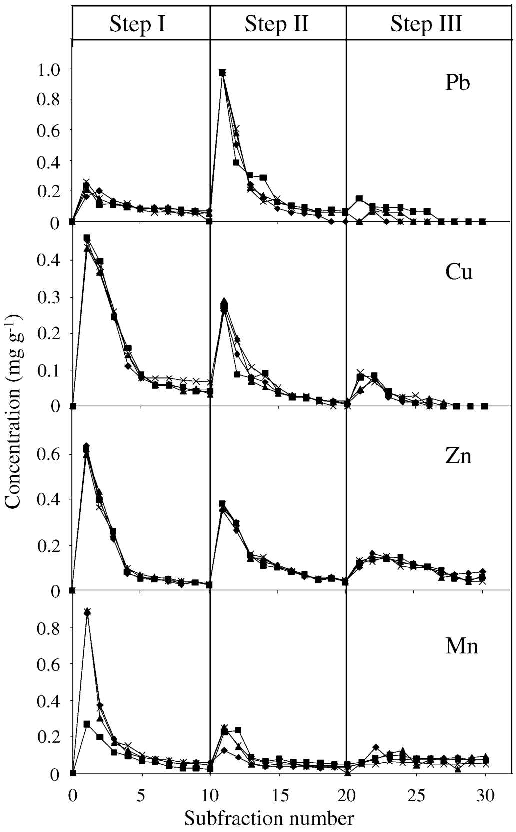

Fig. 2. SI/SE extractograms of metals in the SRM 2710 reference soil resulting from the application of different operational modes: uni-directional flow (♦); bi-directional flow (■); stopped-flow(▲); and triple bi-directional flow (×). Sample, 25 mg; subfraction, 5 ml; extraction flow rate, 50 μl s^{-1}.

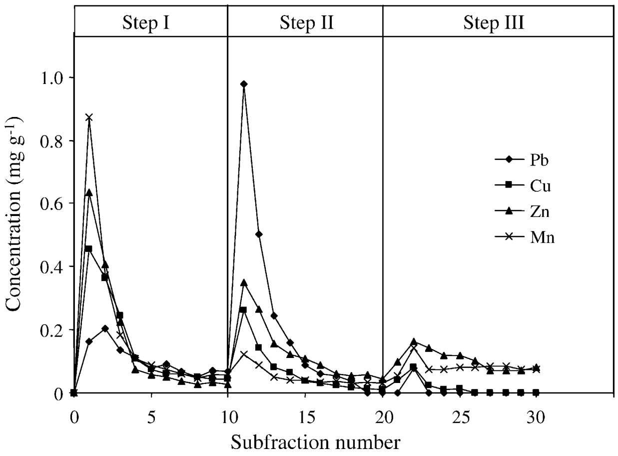

Fig. 3. Extractograms of metals in the SRM 2710 reference soil resulting from the application of the BCR three-step sequential extraction protocol using the SI-microanalytical system in a uni-directional fashion. Sample, 25 mg; extraction flow rate, 50 μl s^{-1}; subfraction volume, 5 ml.

Moreover, the target phases are rapidly leachable, since more than 80% of the maximum metal available is obtained in the first four subfractions. On the other hand, lead is highly affected by reduction processes since the maximum extractability (ca. 45%) was obtained in step II of the SE scheme. This result agrees with previous researchers [21,22] who found that fixed elements, such as lead, are bound to easily or moderately reducible fractions (namely, manganese oxyhydroxides and amorphous iron oxides).

When comparing the leaching patterns of the various metals in SRM 2710 for the uni-directional mode (Fig. 3), it is noticeable that the kinetics of metal release from the solid material follow the same trends for all trace metals: each target analyte is mostly leached in the first subfraction (>30% for Cu, and >40% for Zn and Pb), and the concentration in solution rapidly decreases in the following subfractions. Similar pictures are encountered when exploiting the remaining dynamic extraction sequences, and virtually the same leaching profiles are obtained.

Another outstanding asset of the SI/SE system is the feasibility of comparing both the peak positions and the profile shapes between elements to identify pollutant–soil phase interactions. In order to gain more detailed information of the leaching kinetics and elemental association, the detailed, continuous extractograms are to be explored. This can either be effected by collection of smaller volumes of extract per subfraction, or, as this group presently is engaged in, by interfacing the SI-microcolumn set-up directly with an appropriate flow-through detector, thereby allowing on-line determination of the individual metals.

3.4. Particle size and soil homogeneity

The proposed SI/SE concept with automatic handling of solutions can be regarded as a versatile tool and supplementary technique to the flow injection/continuous flow microcolumn schemes [11–13,23] that have recently attracted special interest for on-line fractionation of metal ions in solid materials and assessment of leaching kinetics to receive valuable information on metal mobility and availability to biota.

Although the use of minute amounts of solid samples, even lower than 10 mg [11], have been reported recently, it should be born in mind that the reliability of the leaching assays is to be strongly influenced by the particle size distribution, which, in turn, depends on the sample preparation protocols effected prior to initialisation of the extraction tests. As suggested by Beauchemin et al. [12], the treatment of solid materials with poor homogeneity by automated microextraction techniques could be troublesome due to the limited capacity of the sample container and the increase of flow resistance. Yet, the feasibility of handling heterogeneous soil samples into flow manifolds is a current subject of exploration in our research group. To this end, the sewage sludge amended CRM 483 soil with certified release concentrations of anthropogenic metals following standardised single extraction procedures [24] is being used as a model of heterogeneous material with particle size up to 2 mm, as compared with <74 μm in the SRM 2710 pasture soil. The experiments have been scaled up to evaluate the maximum sample amount to be accommodated in the dedicated microcolumn. Sample size is proven to be readily enlarged up to 300 mg with minimum back-pressure increase, because of the favourable hydrodynamics of the dual-conical shaped column. The perfusing extractant flow rate should, however, be tailored to the dimensions of the column for appropriate long-term performance of the micro-analytical system. Thus, the coefficients of variation for lead in consecutive uni-directional single extractions for CRM 483 have been reduced from 35 to 5% by increasing the sample mass from 25 to 300 mg, rendering R.S.D. values comparable, and even better, to those of the homogeneous SRM 2710 soil (see Table 2 for further information).

As a result, the proposed configuration has opened new avenues for automatic extraction schemes of environmentally relevant parameters in soils with different morphology and variable homogeneity. Current research is also being focused on the implementation of single and sequential extraction protocols in a single, compact and miniaturised SI-manifold aiming at developing a robust tool with inherent capabilities for fast screening of soil pollution and at-site monitoring of soil phase associations.

3.5. *Validation of the proposed system*

The accuracy of the proposed approach was evaluated by analyzing the SRM 2710 certified reference material, and comparing statistically the summation of extracted concentrations in each operationally-defined phase and residue fraction with the certified total concentrations detailed in Table 2. The *t*-test for mean comparison was applied as a significance test [25]. Since the experimental *t*-values for Pb, Zn, Cu and Mn were lower than the critical value of $|t|$ (viz., 2.23, $P = 0.95$) no significant differences were encountered between the certified values and the sum of concentrations at the 5% significance level. As a consequence, the SI/SE method is free from both additive and multiplicative matrix interferences, making the application of the method of the standard additions unnecessary.

The correlation between the uni-directional flow (UNI) and the bi-directional (BI), triple bi-directional (TR) and stopped (ST) flow modes was assessed via statistical treatment of the regression curves obtained from the overall results listed in Table 2 for trace elements. The correlation lines were as follows: $\mathrm{UNI} = (1.16 \pm 0.22)\,\mathrm{BI} - (0.09 \pm 0.28)$; $\mathrm{UNI} = (1.10 \pm 0.20)\,\mathrm{TR} - (0.04 \pm 0.27)$ and $\mathrm{UNI} = (1.09 \pm 0.21)\,\mathrm{ST} + (0.003 \pm 0.27)$.

As deduced from the above equations, the calculated slopes and intercepts do not differ significantly from 1 and 0, respectively, thus confirming the inexistence of systematic differences between the various operational modes for the SRM 2710 reference material. It can be concluded from these results, that this homogeneous, highly contaminated soil contains large pools of easily mobilisable metal fractions, so that the uni-directional flow provides extraction yields which are statistically comparable with that of the other automated protocols. However, the application of operational modes involving longer extraction times should be desirable to enhance the extraction efficiency in soils, sediments or sludges with large fractions of slowly accessible metals, as currently being investigated in our laboratory exploiting both single and sequential extraction approaches in an automated flow fashion.

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Assessment of lead availability in contaminated soil using isotope dilution techniques

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Abstract

Isotope dilution methods using a stable isotope tracer (^{207}Pb) were developed for the determination of Pb availability in contaminated soils. The methods included determination of E values (isotopically exchangeable pool), L values (plant labile pool) and isotopic exchange kinetics (IEK). Isotopically exchangeable Pb was monitored at different exchange times based on measurement of the ^{207}Pb/^{208}Pb ratio in soil solution following addition of the tracer. The rate of decrease in the ^{207}Pb/^{208}Pb ratio in solution could be described by using the same IEK equation as used previously with radioisotope tracers. The amounts of isotopically exchangeable Pb in Pb-contaminated soils estimated from long-term IEK parameters were in good agreement with directly determined E values up to 15 days. However, values of some of the fitted IEK parameters cast doubts on the validity of using the IEK approach with ^{207}Pb, most probably as a result of irreversible fixation of some of the spike by reactive surfaces in the soils. Estimation of isotopically exchangeable Pb using short-term kinetics data was unsuccessful, substantially underestimating E values. Results for the control (uncontaminated) soil were highly variable, most probably as a result of fixation of tracer by the soil and poor analytical precision due to low solution Pb concentrations. A compartmental analysis of the variation in E values with time indicates a good potential for estimating bioavailable Pb in contaminated soils. The amounts of available Pb obtained from summation of the $E_{1\ \mathrm{min}}$ and $E_{1\ \mathrm{min}\text{–}24\ \mathrm{h}}$ pools ($E_{(\mathrm{available})}$), accounting for an average of 57.62% of total soil Pb, were significantly correlated with both the L values and with Pb extracted from soil with EDTA.

Keywords: Isotopic exchange kinetics; Lead; Bioavailability

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1. Introduction

The present amount of lead (Pb) in the environment is a result of release by natural processes and a long history of anthropogenic use of Pb. The increasing uses of Pb have resulted in recurring

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environmental contamination in developing and industrialised areas of the world. Contamination of soil with Pb from the main recognised sources is well documented, i.e. mining and smelting, automotive emissions, Pb-based paints and industrial activity (e.g. Haack et al., 2003; Wong and Li, 2004; Ettler et al., 2004; Monastra et al., 2004). Accumulation of lead in surface soils can impact on environmental health and can affect food quality and human health. It is widely recognized that the mobility and bioavailability of metals in the environment depend not just on their total concentrations. Hence, determination of metal availability is important for the assessment of metal toxicity in contaminated soils. In response to this concept, there has been a great deal of research conducted to find suitable methods for the determination of available Pb in contaminated soils (e.g. McLaughlin et al., 2000; Bacon et al., 2004; Bäckström et al., 2004).

In relation to the mobility and plant uptake of elements, isotope dilution techniques have been used for investigating elemental dynamics in the soil–plant system (Tiller et al., 1972; Fardeau, 1993; Singh and Pandeya, 1998; Gérard et al., 2000). These techniques are based on the assumption that isotopic tracers added to the soil solution will exchange with the potentially available and mobile forms of elements present in the soil solid phase (often referred to as the labile pool). In principle, the isotopic exchange procedure can be performed using both radioactive and stable isotope as tracers. In previous studies, isotopic exchange with radioactive isotopes has been used mainly for the determination of labile forms of metals such as Cd, Zn and Ni in soils (e.g. Echevarria et al., 1998; Hamon et al., 1998; Smolders et al., 1999). There are two main types of labile pool measurements by isotopic exchange, i.e. the determination of E and L values. The E value is a direct measurement of the isotopically exchangeable metal pool obtained by equilibrating soil suspension with a radioisotope spike, followed by measurement of the specific activity of the soil solution after equilibration. For the determination of L values, plants are grown in soils spiked and pre-equilibrated with the radioisotope. Measurement of the specific activity of the isotope in the plants is then used to compute the labile pool in the soil. Some studies have suggested that E and L values produce different estimates of the labile pool. (Tiller et al., 1972; Smolders et al., 1999; Gray et al., 2001). In contrast, Echevarria et al. (1998) found that E and L values for Ni were similar. Errors may arise with L values because the specific activity of the exchangeable metal as sampled by plants can be affected by many complex chemical and biological processes in the soil.

For additional information, a method has been developed to study isotopic exchange as a function of time. This approach is usually referred to by the term isotope exchange kinetics (IEK) (Fardeau et al., 1985). The IEK method has been successfully applied to describe the kinetic transfer of radioactive phosphate ions ($^{32}PO_4$) from the soil solution to the solid phase (Fardeau, 1996; Frossard and Sinaj, 1997). It has been shown that the IEK technique is very useful in describing P availability in terms of the concepts of intensity, quantity and capacity factors. More recently, the IEK technique has been applied successfully to study Zn exchangeability in soils (Sinaj et al., 1999), and Echevarria et al. (1998) used IEK to assess Ni phytoavailability in Ni polluted soils. Gérard et al. (2000) and Gray et al. (2004) have used the IEK technique to investigate Cd availability in cadmium-contaminated soils.

All the above studies of IEK have used radioisotopes tracers. However, procedures employing radioisotopes generally require special facilities for the safe handling and final disposal of radioactive materials. In addition, for Pb there is no radioisotope tracer that can be used for isotopic exchange studies. In contrast to use of radioisotopes, there have been only a handful of studies that have used stable isotopes to measure labile metals in soils. Given some of the advantages that stable isotopes offer over radioisotopes as outlined by Vanhaecke et al. (1997), and the increasing availability and sophistication of mass spectrometry (MS) instruments, there is now increased interest in using analytical techniques that rely on stable tracers to measure labile fractions of heavy metals. For example, in recent years, analytical techniques using inductively coupled plasma-mass spectrometry (ICP-MS) coupled with using stable isotopes have been developed and applied to study the bioavailability (labile fraction) of metals in soils by Gäbler et al. (1999), Ahnstrom and Parker (2001), Ayoub et al. (2003) and by Gray et al. (2003). The labile fraction is defined as that portion of an element in a soil that rapidly exchanges with an added spike which contains a known quantity of the element of interest, artificially enriched with one of the isotopes of the element. In the case of Pb, the determination of ^{207}Pb and ^{208}Pb can be easily carried out using quadrupole-based ICP-MS instruments (ICP-QMS) (Becker, 2002).

The labile pool of a metal in soil, whether determined by radioactive or stable isotopes, can be considered as a relatively unambiguous assessment of the chemical and biological reactivity of that metal in soil compared with fractions isolated by chemical extractants (e.g. Nakhone and Young, 1993). However, it should be recognised that isotopic exchange determinations are based on the assumption that the added spike (radioisotope or stable isotope) remains 100% available for exchange. Examination of the equations for the calculation of E values shows that fixation ('irreversible' sorption) of some of the spike by reactive soil phases results in an overestimate of the size of the labile pool. In the case of relatively mobile elements such as Cd and Zn, the assumption that 100% of the spike remains available for exchange appears reasonable, although, in some cases, some fixation of the spike may occur even with these elements. For example, Tiller et al. (1972) reported evidence of fixation of spiked radioactive Zn by soils. They found quite erratic overestimates of isotopically exchangeable Zn for alkaline soils, sometimes even exceeding the total amount of soil Zn. In the case of Pb, fixation of the spike could potentially be a considerable problem since Pb is known to be very strongly bound by soil surfaces. Indeed, examination of the results of Gäbler et al. (1999) suggests that fixation may be a real issue. Their values for isotopically exchangeable Pb, determined in water or 1M NH_4NO_3, were actually higher than the concentration of EDTA-extractable Pb determined for their study soil. This would seem a somewhat unlikely situation. In addition, several recent isotope studies examining isotopic signatures of different soil Pb pools show that there is considerable Pb isotopic heterogeneity between pools, and at best, exchange between fractions and homogenisation of isotopic ratios must be extremely slow (e.g. Emmanuel and Erel, 2002; Wong and Li, 2004; Bacon et al., 2004).

In the present study we have examined the use of an enriched stable Pb isotope to determine the pool of isotopically exchangeable Pb in soils (E value). We have also investigated whether it is possible, using the IEK approach, to predict isotopically exchangeable Pb for long-term exchange times, i.e. up to 15 days, from short-term data (1–100 min). Our study also includes the measurement of isotopically exchangeable Pb based on plant uptake (L value). With all methods, we examine the data for evidence that fixation of the spike may limit or invalidate their usefulness. Finally we compare the different measurements of labile Pb with a single soil extraction with ethylenediaminetetraacetate disodium salt (EDTA).

2. Materials and methods

2.1. Description of study site and soil samples

Surface soil samples (0–10 cm) were sampled from the area surrounding a small lead smelter located near the town of Rangsit, approximately 110 km north of Bangkok, Thailand. The smelter, has been operating for the last 10 years and is used essentially to re-cycle lead (Pb) from old batteries. Sampling was carried out at distances of between 0 and 2.5 km from the smelter by taking several sub samples at each location with a trowel from an area of approximately 0.5 m^2 and bulking. A preliminary sampling of the site was carried out and the total concentrations of Pb in these samples were determined. The results showed that total Pb concentrations varied in the range of 20–250 mg/kg Pb. Five samples were chosen for use in this study to represent a range from background to high concentrations of Pb. The background soil was sampled at a distance of 2 km from the smelter. The chemical and physical properties of these five soils are shown in Table 1. Soil samples were dried at 30 °C in an oven for 5 days. All the samples were then ground and sieved through a 2 mm stainless steel sieve. All soils were stored in a desiccator prior to laboratory analysis. Soil pH was measured in a water suspension using a soil/solution ratio of 1:2.5 after the suspensions were shaken for 24 h on a reciprocating shaker at 20 °C (Blakemore et al., 1987). Total carbon content was determined by LECO CNS 2000 Analyser. Soil texture was measured by the Malvern

Table 1
Properties of the experimental soils

Soil	Total Pb (mg/kg)	pH	Sand (%)	Silt (%)	Clay (%)	Org. C (%)	Total Fe (%)	Total Al (%)
C1	21.2	5.54	21.3	56.9	21.8	2.23	5.1	1.8
S1	69.9	6.51	20.5	63.6	15.9	2.89	7.6	1.2
S2	99.2	6.17	24.6	61.9	13.5	1.47	4.7	0.8
S3	143.0	5.91	17.1	65.1	17.8	1.81	3.3	1.1
S4	246.6	5.73	20.5	61.4	18.1	2.65	3.4	1.1

Laser Sizer method (Singer et al., 1988). Total Pb, Fe and Al were determined by acid digestion as described by Kovács et al. (2000), followed by atomic absorption spectrophotometry or ICP-QMS using a Perkin Elmer SCIEX model ELAN 6000.

2.2. Reagents

All chemicals used were of Analytical Reagent grade and obtained from BDH Chemicals (Poole, Dorset, UK). High purity water was used throughout with a metered resistivity ≥18 MΩ. Elemental Pb enriched with ^{207}Pb (≥94.6% ^{207}Pb) was purchased from Novachem Pty. Ltd. (Australia). The enriched Pb was initially dissolved in HNO_3 and then diluted with water to give a stock solution of approximately 1800 mg/L.

2.3. Theory

2.3.1. Isotopically exchangeable Pb

When a spike solution enriched with ^{207}Pb is added to a soil, it is assumed to equilibrate with all forms of soil Pb that constitute the overall labile pool. At equilibrium, the Pb isotope ratios of the solution and solid isotopically exchangeable phases should be the same (in our case we measured the ^{207}Pb/^{208}Pb ratio). The amount of isotopically exchangeable metal, at equilibrium time t (E_t) in the soil can be calculated using Eq. (1) (Gäbler et al., 1999).

$$E_{(t)} = \frac{\mathrm{V_{spike}} \cdot \mathrm{C_{spike-solution}} \cdot \mathrm{M} \cdot (\mathrm{h^{light}_{spike}} - \mathrm{r} \cdot \mathrm{h^{heavy}_{spike}})}{\mathrm{m_{sample}} \cdot \mathrm{N_A} \cdot (\mathrm{r} \cdot \mathrm{h^{heavy}_{sample}} - \mathrm{h^{light}_{sample}})} \cdot 10^6 \tag{1}$$

Where V_{spike} is the volume of the added spike solution (mL), $C_{spike\ solution}$ is the concentration of the element in the spike solution (atom/mL), M is the molar mass (g/mol) of the element, N_A is Avogadro's constant, m_{sample} is the mass of the sample (g), h is the isotopic abundance of the lighter (^{207}Pb) or heavier (^{208}Pb) isotope in the spike solution or the natural isotopic abundance in the sample, r is the measured ^{207}Pb/^{208}Pb isotope ratio in solution after isotopic exchange. The natural isotopic abundance of Pb occurring in each contaminated soil sample, h_{sample} was determined for each sample by ICP-QMS. Eq. (1) can also be used to calculate L values from plant uptake studies. In this case, r is the ^{207}Pb/^{208}Pb ratio measured in plants grown in spiked soil.

2.3.2. Isotopic exchange kinetics

The principle of IEK employing a radioactive tracer has been described in a number of recent papers (Echevarria et al., 1998; Sinaj et al., 1999; Gray et al., 2004). When radioactive ions are added carrier free to a soil solution system at a steady state, the quantity of radioactivity in solution (r_t) decreased as a function of time, t (min) for the duration of the isotopic exchange according to the following Eq. (2) (Fardeau et al., 1985).

$$r_{(t)}/R = \left[r_{(1)}/R\right]\left\{t + \left[r_{(1)}/R\right]^{1/n}\right\}^{-n} + r_{(\infty)}/R \tag{2}$$

Where R is the total introduced radioactivity (MBq) and $r_{(1)}$ and $r_{(\infty)}$ are the radioactivity (MBq) remaining in the solution after 1 min and an infinite exchange time, respectively. The value n is a parameter describing the rate of disappearance of the radioactive tracer from the solution for times longer than 1 min of exchange.

The present IEK study was carried out using a stable isotope as a tracer. In this case, the rate of decrease in ^{207}Pb/^{208}Pb ratio in solution, as a result of ^{207}Pb exchange with Pb on the solid phase, was assumed to follow the same behaviour as previously observed with radioactive tracers (Eq. (2)). Therefore, with reference to Eq. (2), $r_{(t)}$ is defined as the ^{207}Pb/^{208}Pb ratio in solution at time (t) and R is the ^{207}Pb/^{208}Pb ratio of the spike solution. The $r_{(1)}$ *and* $r_{(\infty)}$ values are the ^{207}Pb/^{208}Pb ratios in the solution after 1 min and an infinite exchange time, n is a parameter describing the rate of decrease in ^{207}Pb/^{208}Pb ratio in solution for times longer than 1 min of exchange.

2.4. Procedure for determination of E values and IEK

Generally, for isotope exchange determinations, tracers (radioactive or stable) are added to soil suspensions already at chemical equilibrium. However, the length of time taken for soils to achieve chemical equilibrium for metals in water, or weak salt solutions can be quite variable, ranging from a few hours to several days (e.g. Tiller et al., 1972; Sinaj et al., 1999; Gray et al., 2004). We selected a 3-day equilibrium period before adding tracer to the soil suspension. An important factor of concern when using stable isotope tracers is the amount of spiking tracer added. Ideally, the amount of tracer added should ensure a significant and detectable change in the isotope ratio in the soil solution (^{207}Pb/^{208}Pb) from that in unspiked samples, even after isotopic equilibration has taken place. Gäbler et al. (1999) successfully determined amounts of isotopically exchangeable Pb in soils using a spike of 10^{16} atom/g soil. Since the total soil concentrations of Pb in our study were in the same range as those reported by Gäbler et al. (1999), we also added the stable isotope tracer at a rate of 10^{16} atom/g soil.

Samples of soil (3.00 g) were equilibrated with 30 mL deionized water on an end-to-end shaker for 3 days. At this time (t=0 min), 60 μL of an enriched ^{207}Pb solution (^{207}Pb; 94.6% and ^{208}Pb; 2.9%) was spiked into the equilibrated soil suspension (2.148×10^{16} atom/g soil). The soils were then re-suspended and shaken further. At intervals of 1, 10, 40 and 100 min, 1, 2, 3, 7, 11 and 15 days, duplicate samples were removed from the shaker, centrifuged (10 000 rpm for 10 min) and filtered through a 0.45 μm cellulose acetate membrane. Concentrations of ^{207}Pb and ^{208}Pb were determined in the filtrates by ICP-QMS.

2.5. Procedure for determination of L values

Five soils were spiked with a ^{207}Pb-enriched solution for use in a plant growth study. A 40 mL volume of ^{207}Pb-enriched solution was added to 400 g of sieved dried soil to give a final mean soil atom of approximately 1.597×10^{16} atom/g soil. The soils and ^{207}Pb spiking tracer were then mixed manually together with an additional 56 mL of a mixed nutrient solution of KNO_3 (6.5 mM), NH_4NO_3 (7.5 mM), $MgSO_4$ (2 mM) and KH_2PO_4 (0.6 mM). After mixing, the soil was divided into four and placed into acid washed plastic pots, lined with perforated plastic bags. Each spiked soil (approximately 125 g moist soil at 80% of the water holding capacity) was covered and left to incubate for 1 week.

Seeds of wheat (*Triticum aestivum* L. cv. Monad) were then placed just below the soil surface, at a sowing density of 9 seeds per pot, and thinned to 6 plants following seedling development to remove late germinating and unhealthy seedlings. Four replicates of each soil were arranged in a randomized block design and the plants were grown in a controlled environment growth chamber (15/20 °C, 8 h dark/16 h light). The moisture content was maintained at 80% of the water holding capacity by watering to weight with de-ionized water daily. Every 2 weeks, a further addition of the mixed nutrient solution was made. Plant shoots were harvested after 6 weeks, at which time there was sufficient plant material for analysis. Plants were rinsed in deionized water and then dried at 80 °C for 48 h, and the dry weights recorded. Then the dried plants were finely ground and digested in 10 mL HNO_3 over 7 h. Following HNO_3 digestion, the lead isotope ratio of ^{207}Pb/^{208}Pb in the digested solution was determined by ICP-QMS.

2.6. Single extraction with 0.04 M EDTA

EDTA-extractable Pb was determined by extraction of 10 g of soil with 25 mL of 0.04 M EDTA at 20 °C for 2 h on an end-over-end shaker. After extraction the soil suspension was centrifuged at 10 000 rpm for 10 min and then filtered through Whatman 42 filter paper before Pb measurements by ICP-QMS. The filtered solutions were diluted 50 times with deionized water and spiked with ^{115}In as an internal standard prior to ICP-QMS measurements. The extractable amounts of ^{207}Pb and ^{208}Pb isotopes provided the 'natural' isotopic abundance of ^{207}Pb and ^{208}Pb for each soil sample prior to addition of the ^{207}Pb enriched spike. In fact, there was no significant difference in this value between the five soils.

2.7. Data analysis

For all five soil samples, long-term isotopic kinetics was carried out. The IEK procedure was carried out for 1, 10, 40 and 100 min, and then for a further 1, 2, 3, 7, 11 and 15 days of isotopic exchange. The ^{207}Pb/^{208}Pb ratio in the soil solution was then determined by ICP-MS at each interval. At each sampling interval for these five soils, $r_{(t)}$ values were used to calculate directly the isotopically exchangeable Pb, $E_{(t)}$ using Eq. (1) (up to 15 days). We refer to these data as measured E-values (E_{meas}).

To calculate long-term kinetic parameters, data for $r_{(t)}/R$ at any time, t (min) was fitted to Eq. (2) using on iterative nonlinear regression algorithm (REGRESSION WIZARD, SigmaPlot, Version 6.0) to obtain $r_{(1)}/R$, n and $r_{(\infty)}/R$ kinetic parameters. The fitting procedure constricted the value for $r_{(\infty)}/R$ to values equal to or above the value obtained assuming complete dilution of the spike by the total Pb in the soil sample (see short-term calculation below). This was done because, in reality, no values for $r_{(\infty)}/R$ can be smaller than the estimates made assuming that all the soil Pb is ultimately isotopically exchangeable. Using the fitted parameters, $E_{(t)}$ values were then calculated from Eqs. (2) and (1). We refer to these calculated values as long-term predicted E-values ($E_{\text{long-pred}}$).

For relatively short periods of exchange (we used 100 min), Eq. (1) simplifies to (Fardeau et al., 1991):

$$r_{(t)}/R = r_{(1)}/R[t]^{-n} \qquad (3)$$

So the values of $r_{(1)}/R$ and n can be estimated from a linear regression plot between log $r_{(t)}/R$ and log t. The value of $r_{(\infty)}/R$ can be estimated by assuming complete dilution of the spike by the total Pb in the soil sample.

$$r_{(\infty)}/R = \frac{(94.6 \times 7.4 + \mathrm{Pb}_t \times 22.2)}{(2.9 \times 7.4 + \mathrm{Pb}_t \times 52.3)} \times 1/32.62 \qquad (4)$$

Where Pb_t=total soil lead concentration (mg/kg), The values of 94.6 and 2.9 are the percentage abundances of ^{207}Pb and ^{208}Pb in the added spike. The values of 22.2 and 52.3 are the measured natural abundance of ^{207}Pb and ^{208}Pb in the soils. The value of 32.62 is the ^{207}Pb/^{208}Pb ratio (R) in the spiking solution. The estimated values of $r_{(1)}/R$, $r_{(\infty)}/R$ and n were then used to calculate $E_{(t)}$ values for periods up to 15 days (Eqs. (1) and (2)). We refer to these values as short-term predicted E-values ($E_{\text{short-pred}}$).

The results obtained from the IEK technique can be interpreted using compartment analysis (Fardeau et al., 1991). A compartment is defined as a homogenous unit pool in which all the ions have the same kinetic properties and exchange at the same rate of exchange with the same ions present in other compartments of the system (Sheppard, 1962; Atkins, 1973). We have chosen to analyze soil isotopically exchangeable Pb with a three-pool model consisting of $E_{1\ \mathrm{min}}$, $E_{1\ \mathrm{min}\text{–}24\ \mathrm{h}}$ and $E_{>24\ \mathrm{h}}$.

2.8. Quality control

For the isotope ratio measurements of ^{207}Pb and ^{208}Pb, the ICP-QMS measurement parameters were daily optimized before use according to the manufacturers recommendation using a mixed standard solution containing Ba, Ce, Mg, Pb and Rh, and also

Table 2
ICP-MS operating parameters

Plasma conditions	
RF power	1050–1100 W
Nebulizer flow rate	1.0 mL/min
Auxiliary gas flow rate	0.9 mL/min
Plasma gas flow rate	15 L/min
Mass spectrometer setting	
Analog stage voltage	−2500 V
Pulse stage voltage	1400 V
AC rod offset	−5 V
Sampler cone	Nickel
Skimmer cone	Nickel
Measurement parameters	
Mode	Peak hopping
Resolution	Normal
Dwell time (isotope)	50 ms (Pb-207 and Pb-208)
Number of sweeps per reading	40
Number of replicate	3
Sample introduction	
Sample uptake flow rate	1.0–1.2 mL/min

including mass tuning to obtain separate well-defined peaks between ^{207}Pb and ^{208}Pb (Reference: Perkin-Elmer mass spectrometry manual instruction). The general operating conditions of the ICP-MS are shown in Table 2. The resolution of mass separation with the quadruple mass detector used in this work is 1 amu, with general well-defined peak widths of 0.7 amu each. The concentrations of ^{207}Pb and ^{208}Pb were determined together with an internal standard ^{115}In (Indium). The limit of detection for Pb was 0.05 μg/L. For a 'natural' Pb standard of 10 μg/L, the measured ^{207}Pb/^{208}Pb was 0.423+0.005 (n=10).

2.9. Statistical analysis

All statistical analyses were performed using the statistics program in MS-Excel'97. Long-term, n, $r_{(1)}/R$ and $r_{(\infty)}/R$ parameters were obtained using the nonlinear regression algorithm (REGRESSION WIZARD, Sigma Plot, Version 6.0).

3. Results and discussion

3.1. Change in E-value with time

The quantity of isotopically exchangeable Pb (E_{meas}) was calculated for each sampling period during the 15 day study. For all of the contaminated soils, the calculated E values increased rapidly during the first 24 h (1440 min) of exchange, followed by much slower rates of exchange (Fig. 1). The E values appeared to plateau at between 3 and 7 days (4000–10 000 min), indicating that isotopic equilibrium had

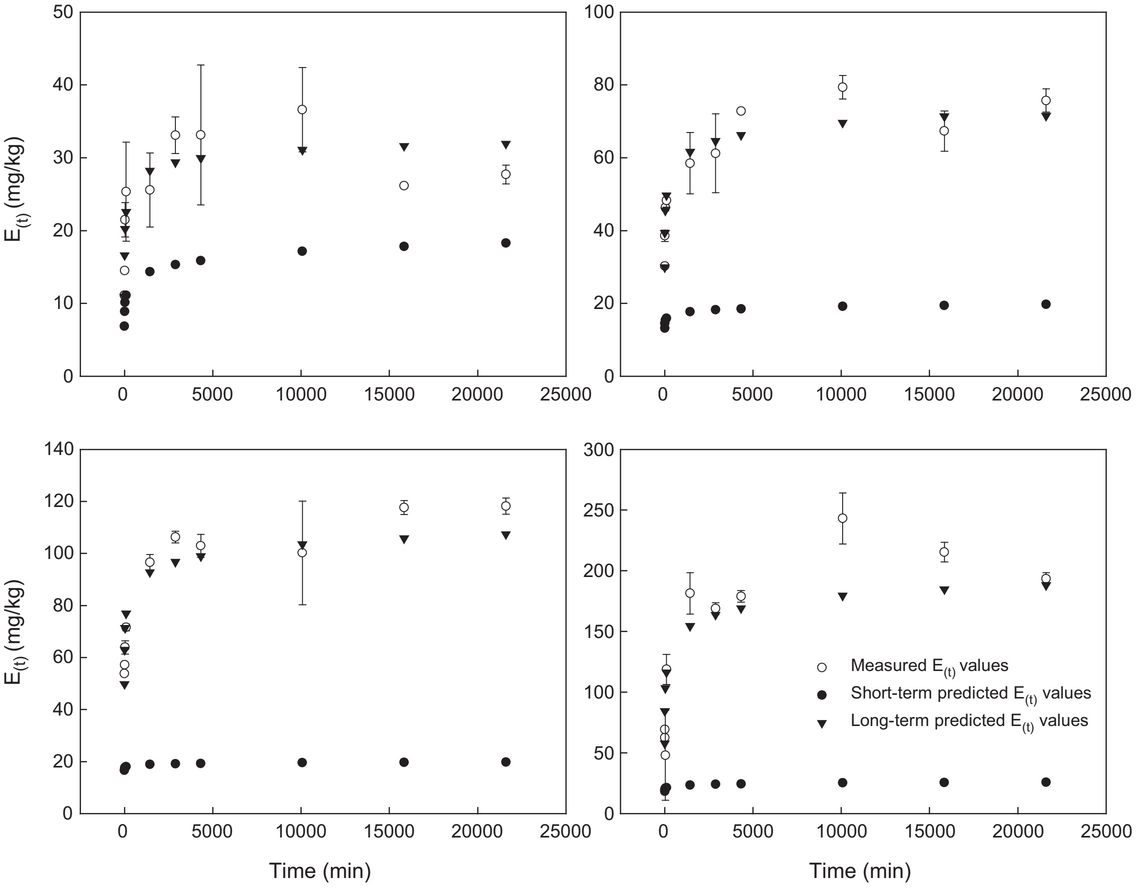

Fig. 1. Comparison between measured, long-term predicted, and short-term predicted $E_{(t)}$ values for contaminated soils S1 to S4. Error bars for measured $E_{(t)}$ values are ±S.E. of duplicate determinations.

largely been achieved by this stage. For soils S2, S3 and S4 in particular, the $E_{(\text{meas})}$ values lay on relatively smooth curves with increasing time (Fig. 1). In contrast, for the control (C1) soil (Fig. 2), and for contaminated soil S1, the data points between 3 and 7 days showed considerable fluctuation. The reasons for this may be related to a combination of some of the analytical and fixation problems discussed elsewhere in this paper.

Our results appear similar to previous reports for Zn and Cd (Sinaj et al., 1999; Gray et al., 2004). Sinaj et al. (1999) investigated labile Zn in both polluted and non-polluted soils, their results showing an increase in the quantity of isotopically exchangeable Zn during 15 days with a fast exchange in the early stages. Gray et al. (2004) found that the isotopic exchange process for Cd is relatively fast during the first 24 h of exchange. These results are similar to those found in the current work, which clearly indicated that the isotopic exchange of Pb is initially a relative fast process (1 min–1 day), followed by a period of much slower exchange.

In an attempt to examine the use of the IEK approach for a stable isotope, Eq. (2) was used to model the decrease in the ^{207}Pb/^{208}Pb ratio in solution with time after addition of the spike. For the contaminated soils (S1, S2, S3 and S4), Eq. (2) provided a reasonable fit to the experimental data as shown in Fig. 1 (R^2>0.91) and the long-term kinetics parameters; $r_{(1)}/R$, n and $r_{(\infty)}/R$ could be estimated from the equation fitting procedure (Table 3). Statistical tests demonstrated reasonable good agreement between values of $E_{(\text{meas})}$ and the corresponding E values ($E_{\text{long-pred}}$) calculated from the IEK parameters. The results indicated that the behavior of isotopically exchangeable Pb was similarly to that of Zn, Cd and phosphate ions that have previously been successfully fitted to Eq. (2). In contrast, for the control soil (C1),

Table 3
Comparison of kinetic parameters using IEK data between short-term and long-term exchange

Soil	$r_{(1)}/R$	n	$r_{(\infty)}/R$	R^2
Short-term				
Control C1	0.153	0.228	0.032	0.931
Soil S1	0.053	0.115	0.019	0.970
Soil S2	0.026	0.047	0.017	0.987
Soil S3	0.021	0.020	0.016	0.907
Soil S4	0.020	0.039	0.015	0.896
Long-term				
Control C1	0.125	0.448	0.040	0.919
Soil S1	0.025	0.289	0.024	0.931
Soil S2	0.0094	0.179	0.017	0.979
Soil S3	0.0054	0.163	0.016	0.914
Soil S4	0.0054	0.226	0.015	0.926

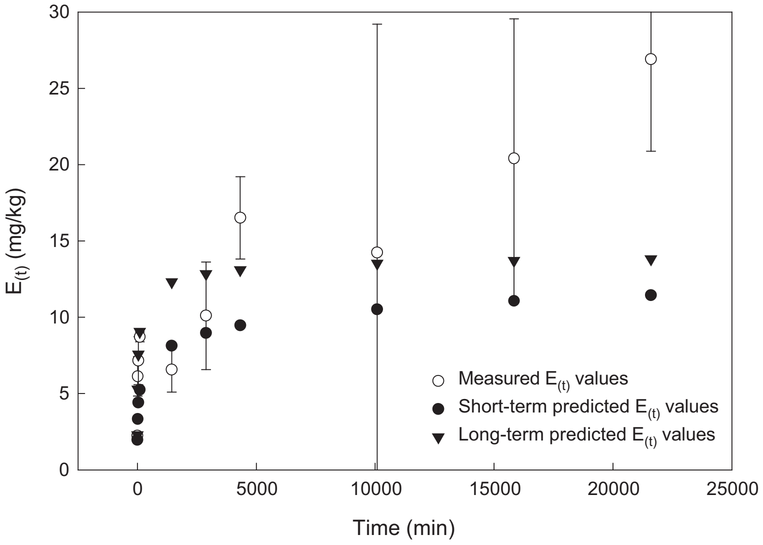

Fig. 2. Comparison between measured, long-term predicted, and short-term predicted $E_{(t)}$ values for the control soil (C1). Error bars for measured $E_{(t)}$ values are ±S.E. of duplicate determinations.

although the R^2 obtained from long-term kinetics fitting is relatively high (0.919) (Table 2), agreement between the $E_{(\text{long-pred})}$ and $E_{(\text{meas})}$ values is generally poor. It seems most likely that the poor fitting of the control soil data is related to the analytical errors for this soil, caused by the very low concentrations of Pb involved.

Attempts to use short-term data (up to 100 min) to predict E values ($E_{\text{short-pred}}$), for periods up to 15 days were not successful (Fig. 1). It was found that the $E_{\text{short-pred}}$ values were underestimated compared to the $E_{(\text{meas})}$ values for all soil samples. Our short-term kinetics approach shows that it is not possible, in the soil samples studied, using short-term parameters (1–100 min of isotopic exchange) to predict the amounts of isotopically exchangeable Pb for longer-term period (up to 15 days). To a certain extent, these large errors may be due to a lack of precision in the values used for time (t). For short equilibrations the length of time taken to separate solution from soil by centrifugation and filtration could have introduced substantial errors into the first two data points (1 and 10 min). However, Gray et al. (2004) also found that data from short-term kinetics data were only successful in predicting long-term exchangeable Cd for two out of six soils, even when they filtered soil suspensions immediately after sampling. For other soils, short-term IEK could predict exchangeable Cd only up to 24 h of exchange. After 24 h, $E_{\text{short-pred}}$ values were overestimated. In contrast, Sinaj et al. (1999) demonstrated that the results derived from a short-term IEK experiment (1–100 min) allowed for a precise prediction of exchangeable Zn up to 15 days in some soil samples. However, this was not possible for soils with high pH and low total Zn contents. From all of these studies, it would seem that short-term IEK procedures have some limitations for predicting long-term E values.

3.2. Validity of the IEK approach

Although as described above, Eq. (2) appeared to provide good fits to the experimental data (high R^2 values, Table 3), a closer examination of some of the parameters derived from the fitting process raises doubts about its validity for use in this study. In the case of three of the soils (S2, S3 and S4), the fitted values for $r_{(\infty)}/R$ are the minimum values possible, implying that ultimately all Pb in the soils is isotopically exchangeable. This seems extremely unlikely. More importantly the fitted $r_{(1)}/R$ values are lower than the $r_{(\infty)}/R$ values, which is of course an impossibility. This implies that Eq. (2) is inappropriate for modelling the isotopic exchange process, or that in this study the decrease in $^{207}\text{Pb}/^{208}\text{Pb}$ ratio in solution is not due solely to isotopic exchange. Fixation of some of the ^{207}Pb spike in non-isotopically exchangeable forms could account for this problem, and additional evidence of fixation occurring in this study is described below. In view of this issue, the use of Eq. (2) in this study should be regarded simply as a mathematical curve-fitting procedure enabling the 'smoothing' of the experimental data. On this basis it is probably unwise to pay much attention to the fitted values for the various parameters (Table 3) or make comparisons with values obtained for other metals such as Zn, Cd and Ni obtained in previous studies (e.g. Echevarria et al., 1998; Sinaj et al., 1999; Gérard et al., 2000; Diesing et al., 2002).

3.3. Compartment analysis of isotopically exchangeable lead

Previous studies have demonstrated that availability of metals is not a simple matter of labile and non-labile pools (Sinaj et al., 1999; Gray et al., 2004). In this present study, a three-compartment model was used to describe isotopically exchangeable Pb in our soil samples. The three compartments are $E_{1\ \text{min}}$, $E_{1\ \text{min–24 h}}$ and $E_{>24\ \text{h}}$ pool, respectively. The results of compartment analysis estimated from the long-term kinetics data are summarized in Table 4. They indicate that the $E_{1\ \text{min}}$ pool contained between 10.75% and 34.82% of the total soil Pb. This pool

Table 4
Isotopically exchangeable Pb in designated pools predicted from long-term IEK data

Soil	Pool 1: $E_{(1\ \text{min})}$ (mg/kg)	Pool 2: $E_{(1\ \text{min–24 h})}$ (mg/kg)	Pool 3: $E_{(>24\ \text{h})}$ (mg/kg)
Control C1	2.28 (10.75)	10.04 (47.36)	8.80 (41.89)
Soil S1	10.92 (15.92)	17.29 (24.74)	41.69 (59.64
Soil S2	29.98 (30.22)	31.63 (31.89)	37.59 (37.89)
Soil S3	49.79 (34.82)	43.02 (30.08)	50.19 (35.10)
Soil S4	57.66 (23.38)	96.78 (39.25)	92.16 (37.37)

Figures in parenthesis show % of total Pb in each pool.

represents Pb that is isotopically exchangeable during the first minute of exchange and corresponds to the instantaneously isotopically exchangeable Pb ions. The Pb present in this pool is composed of Pb^{2+} in the soil solution and adsorbed or chelated Pb that is very rapidly exchangeable. This pool must be considered as the pool of Pb ions directly available for plants. The size of the $E_{1\ \mathrm{min}\text{–}24\ \mathrm{h}}$ pool ranges between 24.74% and 47.36% of the total soil Pb. According to our data, the amounts of isotopically exchangeable Pb increased rapidly during the first day of exchange (Figs. 1 and 2), so that it is highly likely that Pb in the $E_{1\ \mathrm{min}\text{–}24\ \mathrm{h}}$ pools is also relatively plant available. Finally, the pool containing Pb that is either slowly exchangeable (i.e. between 24 h and 15 days), or not exchangeable with the spike, for instance Pb occluded in minerals or strongly adsorbed onto soil particles, is in the $E_{>24\ \mathrm{h}}$ pool. Lead in this pool ranges between 35.10% and 59.64% of total soil Pb. For the five soil samples, the proportions of Pb present in the $E_{1\ \mathrm{min}}$, $E_{1\ \mathrm{min}\text{–}24\ \mathrm{h}}$ and $E_{>24\ \mathrm{h}}$ pools were on average 22.96%, 34.66% and 42.38%, respectively.

3.4. L values from plant uptake study

As mentioned in the introduction, there is no previous report examining the use of the L value for determination of available lead in soils. In our work, L values have been determined by spiking of soils with a stable isotope tracer (^{207}Pb) and measuring the $^{207}Pb/^{208}Pb$ ratio in plants grown in the soils. The L values determined are shown in Table 5. The L value for the control soil (C1) shows a totally unrealistic value vastly exceeding the total content of soil Pb. This overestimation may result from the fixation at strongly binding sites in the soil. Fixation is considered to be a much more likely problem to occur in the control soil. In the current study, the fixation problem may be related to the high Fe content of our soils (Table 1). In the contaminated soils, fixation also probably occurred but may have had much less overall influence on the isotopic exchangeable process than in the control soil. In the contaminated soils, the most strongly fixing sites on the soil oxide surfaces are more likely to be already occupied by anthropogenic Pb prior to spiking. In our procedure for determination of the L value, Pb fixation could have occurred during the period of incubation with ^{207}Pb for 7 days, before seeding.

Table 5
Comparison of amounts of available Pb determined using different methods

Soil	$E_{(\mathrm{available})}$[a] (mg/kg)	L value (mg/kg±S.D.)	EDTA-extractable Pb (mg/kg)
Control C1	12.32	184.1±57.7	3.78
Soil S1	28.21	55.2±15.5	18.50
Soil S2	61.61	73.1±11.9	53.21
Soil S3	92.81	93.9±26.2	76.83
Soil S4	154.44	133.8±25.3	130.87

[a] $E_{(\mathrm{available})}=E_{(1\ \mathrm{min})}+E_{(1\ \mathrm{min}\text{–}24\ \mathrm{h})}$.

3.5. Comparison of available Pb determined by different methods

Estimates of labile pools of Pb are generally regarded as providing an indication of plant available Pb in the soil. The mean E values estimated from the long-term IEK fitting procedure were compared with the amounts of labile Pb determined using two other techniques, i.e. L values and single extraction with EDTA. Table 5 shows the comparison of the estimates of available Pb determined using the different methods. The $E_{(\mathrm{available})}$ values consist of Pb present in the $E_{1\ \mathrm{min}}$ plus $E_{1\ \mathrm{min}\text{–}24\ \mathrm{h}}$ pools. For the three most highly contaminated soils (S2, S3 and S4) there is relatively good agreement between the $E_{(\mathrm{available})}$ pool estimated from long-term IEK data and the corresponding L values. However, because of fixation of Pb as discussed in the previous section, there is no agreement between the two types of estimate for the control soil. The L value for the least contaminated soil (S1) is also much higher than the corresponding $E_{(\mathrm{available})}$ value, suggesting that Pb fixation may also have been an issue with this soil.

The amounts of available Pb estimated from a single EDTA extraction are generally lower than those estimated by $E_{(\mathrm{available})}$ or L values, again suggesting that fixation of the Pb spike may have resulted in an overestimate of labile/available Pb. However, in the case of the three most highly contaminated soils, the differences are not large. Many laboratory studies have shown that EDTA is effective in removing Pb, Zn, Cu and Cd from contaminated soils (Elliotte and Brown, 1989; Brown and Elliott, 1992). EDTA is a very commonly used soil extractant because of its strong chelating ability for different heavy metals. In spite of the potential fixation issue, labile Pb as predicted from both *E* and *L* values (*L* value for control (C1) soil excluded) is highly correlated with EDTA extractable soil Pb (Fig. 3). On this basis, EDTA would appear to be an effective extractant for estimating the amounts of labile Pb in contaminated soils.

Some previous studies have reported the correlation between the IEK-derived estimates of plant availability and plant uptake of elements. Frossard et al. (1992) have reported that P exchangeable between 1 min and 24 h correlated with root uptake of P from soils. Echevarria et al. (1998) have successfully used the IEK approach to assess Ni phytoavailability in Ni polluted soils. Gray et al. (2004) reported that the IEK compartment analysis showed clear differences in the distribution of Cd between exchange pools between soils where Cd was derived from phosphate fertilizer and those where Cd was derived from biosolids, especially Cd located in the $E_{1\ \text{min–}24\ \text{h}}$ pool. The results suggested that biosolids-derived Cd was more bioavailable than that derived from phosphate fertilizer. It can therefore be concluded that the proposed IEK approach is a useful method for characterizing the availability of these metals. However, because of the potential fixation of the Pb spike by soils, the characterization of Pb availability using the IEK approach appears to have some limitations.

4. Conclusions

Initially, our results indicated that the IEK equation used for radio-isotopic exchange processes also appeared to be an effective model for kinetics studies using a stable isotope. The fitted curves illustrate that isotopic exchange of Pb was a relatively fast process in the early stages (<24 h), followed by a much slower rate of exchange. The results found in our study were similar to those found in previous studies for Zn and Cd. However, a close examination of some of the IEK parameters derived from the fitting process raises doubts about its validity for use with the stable Pb isotope. Some limitations of the isotopic exchange approach have been observed where irreversible fixation of added Pb has appeared to occur. This fixation significantly affects the basic assumptions underlying this approach. Fixation of Pb coupled with errors associated with low concentrations of Pb in soil solution lead to unrealistically high *E* and *L* values, in particular for the control soil. It was also not possible to use short-term kinetics parameters (derived from 1–100 min) to predict the isotopically exchangeable Pb in the long-term (up to 15 days). Clearly, fixation of the added tracer by soil requires further investigation. It may well have been exacerbated in the current study by using soils with such high Fe oxide contents.

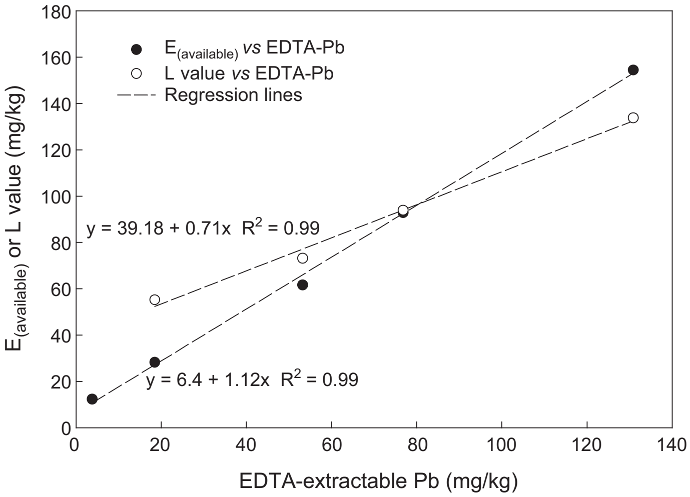

Fig. 3. Relationships between $E_{(\text{available})}$ and *L* values and EDTA-extractable Pb concentrations.

In spite of problems with using the IEK approach, other than as a simple mathematical curve-fitting exercise, the amounts of available Pb (*E* values), determined during 24 h of exchange were significantly correlated with the plant uptake method (*L* values), and with EDTA-extractable Pb for all contaminated soils.

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กรดอินทรีย์ในอาหารไทยเพิ่มการนำไปใช้ได้ของแคลเซียม

ORGANIC ACIDS IN THAI FOODS CAN ENHANCE CALCIUM BIOAVAILABILITY

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ABSTRACT

Vegetable is an important source of dietary calcium. Unfortunately, they contain substances, i.e., dietary fiber, phytate, and oxalate which can inhibit the bioavailability of calcium. Two vegetables were selected for study: kale and spinach which are representative of vegetables with high and low calcium bioavailability, respectively. The effect of adding these vegetables in different dishes on calcium bioavailability were investigated using an *in vitro* dialysis method. Four dishes: fish Tom Yam, fish Kaeng Som, fish soup, and fish soup with lime juice were prepared to study the effect of ingredients on calcium *in vitro* dialyzability of vegetables.

A novel continuous-flow dialysis method with flame atomic emission spectrometric detection (flame AES) was applied to estimate the dialyzability of minerals in dishes prepared. The method involves a simulated gastric digestion with pepsin, followed by dialysis occurring during a continuous flow of dialyzing solution ($NaHCO_3$). Using the proposed system, the enhancement of calcium dialyzability for kale was observed for fish Tom Yam (28%), and fish soup with lime juice (15%). For fish soup and fish Kaeng Som, no significant enhancement in calcium dialyzability for kale was found in these dishes. For spinach, the depression of calcium dialyzability was found in all dishes. The enhancement of bioavailability found could be attributed to the organic acids, especially citric acid in the dishes containing lime juice.

Key words : *In vitro* method, bioavailability, calcium, Thai dishes.

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บทคัดย่อ

ผักเป็นแหล่งของแคลเซียมที่สำคัญในอาหาร แต่ผักบางชนิดประกอบด้วยสารซึ่งสามารถยับยั้งการนำไปใช้ของแคลเซียม เช่น เส้นใยอาหาร ไฟเตต และออกซาเลต งานวิจัยนี้ได้ทำการศึกษาการนำไปใช้ได้ของแคลเซียมในตัวอย่างผักสองชนิด คือ ผักคะน้า และผักปวยเล้ง ซึ่งเป็นตัวแทนของผักที่มีแคลเซียมที่ร่างกายดูดซึมได้สูงและต่ำตามลำดับ และศึกษาผลของการนำผักเหล่านี้มาปรุงเป็นอาหาร 4 ชนิด ได้แก่ ต้มยำปลา แกงส้มปลา แกงจืดปลา แกงจืดปลาใส่น้ำมะนาว เพื่อศึกษาผลของส่วนประกอบในอาหารที่มีต่อการนำไปใช้ได้ของแคลเซียมโดยใช้วิธีในหลอดแก้วในการประเมินความสามารถในการนำไปใช้ได้ของแคลเซียม

วิธีในหลอดแก้วแบบไหลต่อเนื่องแนวใหม่ โดยการตรวจวัดด้วยเทคนิคอะตอมมิคอิมิสชันแบบเปลวไฟ ได้นำไปศึกษาปริมาณแร่ธาตุที่ไดอะไลส์ได้โดยเลียนแบบการย่อยในกระเพาะอาหารด้วยเอนไซม์เปปซินตามด้วยกระบวนการไดอะไลซิสภายในหลอดไดอะไลซิส โดยการผ่านสารละลายโซเดียมไบคาร์บอเนตอย่างต่อเนื่อง จากการใช้ระบบที่เสนอ พบการเพิ่มขึ้นของปริมาณที่ไดอะไลส์ได้ของแคลเซียมในคะน้า เมื่อนำคะน้าไปปรุงในอาหารที่ใส่น้ำมะนาว ได้แก่ ต้มยำปลา (28%) และแกงจืดปลาใส่น้ำมะนาว (15%) ในขณะที่แกงจืดปลาและแกงส้มปลา ไม่พบว่ามีการเพิ่มขึ้นของปริมาณที่ไดอะไลส์ได้ของแคลเซียม สำหรับอาหารข้างต้นที่เติมผักปวยเล้ง พบการลดลงของปริมาณที่ไดอะไลส์ได้ของแคลเซียมในอาหารที่ศึกษา การเพิ่มขึ้นของปริมาณที่ไดอะไลส์ได้ คาดว่าเป็นผลมาจากกรดอินทรีย์ โดยเฉพาะอย่างยิ่งกรดซิตริกในอาหารที่มีการเติมน้ำมะนาว ส่วนการลดลงน่าจะเป็นผลจากสารยับยั้งต่าง ๆ เช่น ออกซาเลต และไฟเตต เป็นต้น

บทนำ

โรคกระดูกพรุนจัดเป็นปัญหาสำคัญในทางสาธารณสุข เกิดจากการได้รับแคลเซียมไม่เพียงพอ ซึ่งมักพบในผู้ป่วยสูงวัย เนื่องด้วยเป็นวัยที่มีอัตราการสลายของธาตุแคลเซียมสูง เพื่อลดอุบัติการณ์ของภาวะกระดูกพรุน การรณรงค์ให้บริโภคอาหารที่มีแคลเซียมอย่างเพียงพอตั้งแต่วัยเยาว์ เพื่อเสริมสร้างกระดูกและลดอัตราการสูญเสียแคลเซียมเมื่อสูงวัยเป็นสิ่งสำคัญ อย่างไรก็ดีนอกจากปริมาณแคลเซียมที่บริโภคแล้ว ความสามารถในการนำแคลเซียมไปใช้ได้ในอาหารชนิดนั้นก็เป็นสิ่งที่ควรจะนำมาพิจารณา

อาหารที่พบแคลเซียมในปริมาณสูง ตัวอย่างเช่น นม และผักชนิดต่าง ๆ โดยเฉพาะอย่างยิ่งผักใบเขียวจัดเป็นแหล่งที่สำคัญที่สุดแหล่งหนึ่งของแคลเซียม ค่าความสามารถของร่างกายในการนำแคลเซียมไปใช้ได้ในผักเหล่านี้อยู่ในช่วงระหว่าง 5-50% (Sottimai *et al.*, 2003) โดยผักบางชนิดแม้จะมีปริมาณของแคลเซียมสูง แต่ร่างกายนำไปใช้ได้ต่ำมาก ทั้งนี้เพราะผักเหล่านี้มีสารบางชนิดเช่น ไฟเตต ออกซาเลต และไฟเบอร์ ซึ่งสามารถรวมตัวกับแคลเซียมเกิดเป็นสารประกอบที่ร่างกายไม่สามารถดูดซึมได้ (Lombardi-Boccia *et al.*, 1998; Weaver *et al.*, 1999; Kamchan *et al.*, 2004) เนื่องจากแคลเซียมเป็นแร่ธาตุที่สำคัญ จึงได้มีผู้ทำการศึกษาหาแนวทางการเพิ่มปริมาณการนำไปใช้ได้ของแร่ธาตุ ตัวอย่างเช่น การใช้อาหารเสริมในรูปยาเม็ด การเสริมธาตุที่ต้องการในอาหารที่บริโภคโดยตรง หรือแม้กระทั่งการใช้ปุ๋ย เพื่อส่งเสริมให้พืชสะสมธาตุอาหารที่ต้องการได้มากขึ้น หรือลดสารยับยั้งบางชนิดลง รวมถึงการใช้กระบวนการพันธุวิศวกรรมปรับปรุงพันธุ์พืช (Frossard *et al.*, 2000)

ในหลายปีที่ผ่านมาได้มีการศึกษาเกี่ยวกับส่วนประกอบในอาหารที่อาจส่งผลต่อการนำแคลเซียมไปใช้ โดยสามารถแบ่งออกได้เป็น 2 ประเภท คือ ปัจจัยที่ส่งเสริมการนำแคลเซียมไปใช้ เช่น น้ำตาลแลคโตส วิตามินดี วิตามินเค กรดอินทรีย์บางชนิด และปัจจัยที่ลดการนำแคลเซียมไปใช้ เช่น ไฟเบอร์ ไฟเตต ออกซาเลต โปรตีนบางชนิด ไขมัน และเกลือ (Gueguen *et al.*, 2000; Kennefick *et al.*, 2000) จากการศึกษาเบื้องต้นของคณะวิจัยก็พบ

ผลของกรดอินทรีย์ชนิดต่าง ๆ ที่สามารถเพิ่มการนำแคลเซียมไปใช้ได้ โดยพบว่ากรดซิตริก กรดทาร์ทาริก กรดมาลิก และกรดแอสคอร์บิกเพิ่มการนำไปใช้ได้ของแคลเซียมในตัวอย่างผักที่มีค่าการนำไปใช้ได้ต่ำ (Sottimai *et al.*, 2003) อย่างไรก็ตามโดยทั่วไปเราไม่ได้บริโภคผักโดด ๆ แต่จะปรุงเข้ากับส่วนประกอบอื่น ๆ งานวิจัยนี้จึงมีวัตถุประสงค์ในการศึกษาถึงอิทธิพลของกรดอินทรีย์ที่มีในเครื่องปรุงของอาหารไทยที่มีต่อการดูดซึมได้ของแคลเซียมในผัก โดยเลือกอาหารไทยที่มีรสเปรี้ยวจากการมีกรดอินทรีย์ในส่วนประกอบ เช่น ต้มยำมีกรดซิตริกจากน้ำมะนาว แกงส้มมีกรดทาร์ทาริกจากน้ำมะขามเปียก การศึกษาได้นำเอาวิธีในหลอดแก้วแบบไหลต่อเนื่องแนวใหม่ที่พัฒนาขึ้นครั้งแรกจากคณะวิจัยไปใช้ในการศึกษาการนำแคลเซียมไปใช้ในตัวอย่างผักต่าง ๆ และในอาหารไทย โดยผักที่เลือกศึกษามี 2 ชนิดคือ ผักคะน้า และปวยเล้งซึ่งเป็นผักที่มีแคลเซียมที่ร่างกายดูดซึมได้สูงและต่ำตามลำดับ โดยนำมาทำการปรุงเป็นอาหารไทย 4 ชนิด ได้แก่ ต้มยำปลา แกงส้มปลา แกงจืดปลา และแกงจืดปลาใส่น้ำมะนาว เพื่อทำการศึกษาผลของเครื่องปรุงในอาหารเหล่านี้ที่มีต่อการนำไปใช้ได้ของแคลเซียม

อุปกรณ์และวิธีการ

1. การเตรียมตัวอย่างผักและอาหาร

ผักสด (ผักคะน้าและปวยเล้ง) ซื้อจากตลาดสด และห้างสรรพสินค้าในเขตกรุงเทพมหานคร โดยนำมาล้างด้วยน้ำปราศจากไอออน ทำการอบให้แห้งที่อุณหภูมิ 65°C จนกระทั่งน้ำหนักคงที่ ทำการปั่นให้ละเอียด และเก็บไว้ในโถอบแห้ง สำหรับไว้ใช้ตลอดการศึกษา

อาหาร (ต้มยำปลา แกงส้มปลา แกงจืดปลา และแกงจืดปลาใส่น้ำมะนาว) เตรียมได้จากวิธีการปรุงในตำราอาหารไทย (มูลนิธิการแพทย์แผนไทยพัฒนา, 2544; วัลยา ภู่ภิญโญ, 2544) โดยนำผักคะน้าหรือปวยเล้ง (A) ประมาณ 80 กรัม ปรุงด้วยเครื่องปรุงอื่น ๆ (B) เป็นอาหาร 4 ชนิด ดัง Table 1.

Table 1. Ingredients for samples of fish Tom Yam, fish Kaeng Som, fish soup, and fish soup with lime juice.

Ingredients	Fish Tom Yam (B)	Fish Kaeng Som (B)	Fish soup (B)	Fish soup with lime juice (B)
Boiled fish (g)	120.60	119.84	120.59	120.59
Tom Yam Chilli paste (g)	31.46	-	-	-
Kaeng Som Chilli paste (g)	-	40.30	-	-
Lime juice (g)	15.00	-	-	20.00
Tamarind juice (g)	-	15.50	-	-
Fish sauce (g)	5.50	10.00	5.48	5.48
Sugar (g)	-	4.00	-	-
Deionized water (mL)	250	300	250	250

อาหารที่เตรียมได้ จะนำมาปั่นด้วยเครื่องปั่นอาหาร และทำการอบให้แห้งที่อุณหภูมิ 65°C จนกระทั่งน้ำหนักคงที่ ทำการปั่นให้ละเอียดอีกครั้ง และเก็บไว้ในโถอบแห้งจนกระทั่งวิเคราะห์ โดยสามารถทำการทดลองกับตัวอย่างซ้ำเมื่อต้องการ

2. การวิเคราะห์หาปริมาณแคลเซียมทั้งหมดในตัวอย่าง

ตัวอย่างอาหารแห้งที่ได้ประมาณ 0.5 กรัม ชั่งโดยละเอียด นำมาย่อยด้วยสารละลายเข้มข้นของ HNO_3: H_2O_2 3:2 (v/v) ปริมาตร 10 mL โดยวิธีการย่อยด้วยไมโครเวฟ สารละลายใสที่ได้นำมาทำการเจือจางด้วยน้ำปราศจากไอออนให้มีปริมาตรเป็น 50.0 mL แล้วทำการตรวจวิเคราะห์ปริมาณโดยใช้ Flame AES

3. การวิเคราะห์หาปริมาณที่ไดอะไลส์ได้ของแคลเซียม

ปริมาณการนำแคลเซียมไปใช้ได้ในตัวอย่างผักและอาหาร ศึกษาได้จากวิธีในหลอดแก้วแบบไหลต่อเนื่องแนวใหม่ (Sottimai *et al.*, 2003) ซึ่งประกอบด้วยขั้นตอนหลัก ๆ 3 ขั้นตอนได้แก่ การจำลองสภาพการย่อยในกระเพาะอาหาร การหาความเข้มข้นที่เหมาะสมของสารละลาย $NaHCO_3$ และการจำลองสภาพการย่อยในลำไส้เล็ก

3.1 การจำลองสภาพการย่อยในกระเพาะอาหาร ทำได้โดยวิธีของ Miller

3.2 การหาความเข้มข้นที่เหมาะสมของสารละลาย $NaHCO_3$ ทำได้โดยหาค่าสภาพกรดของตัวอย่าง (titratable acidity) ที่ได้จากการไทเทรตสารผสมของ pepsin digestate (2.5 g) กับ pancreatin bile extract mixture (625 µL) ด้วย 0.1 M NaOH ค่าที่ได้นี้สามารถนำไปใช้ในการคำนวณหาความเข้มข้นที่เหมาะสมของ $NaHCO_3$ เพื่อใช้ปรับ pH ของอาหารที่ผ่านการย่อยในกระเพาะอาหารซึ่งมีค่าประมาณ 2.0 ให้เป็น 5.0 ซึ่งเป็น pH ในลำไส้เล็กภายในเวลา 30 นาที โดยพบว่า ความเข้มข้นที่เหมาะสมของ $NaHCO_3$ มีความสัมพันธ์เป็นเส้นตรงกับค่า titratable acidity ซึ่งหาได้จากสมการดังต่อไปนี้ สำหรับกรณีอาหารที่ศึกษาในงานวิจัยนี้

$$\boxed{\text{Optimum } NaHCO_3 \text{ concentration} = (\text{Titratable acidity in Molarity})/36}$$

3.3 การจำลองสภาพการย่อยในลำไส้เล็ก ทำได้โดยการบรรจุ pepsin digestate 2.5 กรัม ลงในถุงไดอะไลซิส (MWCO 12-14 kDa) นำไปติดตั้งตาม Figure 1. จากนั้นทำการผ่านสารละลาย $NaHCO_3$ ความเข้มข้นที่เหมาะสม ด้วยอัตราการไหล 1 mL/min แล้วทำการเก็บสารที่ไดอะไลส์ได้ในขวดเก็บสาร โดยภายหลัง 30 นาทีนับจากเริ่มต้นเก็บ ทำการฉีด pancreatin bile extract mixture 625 µL เข้าไปในถุงไดอะไลซิสผ่านวาล์วฉีดสาร และทำการผ่านสารละลาย $NaHCO_3$ อย่างต่อเนื่องอีก 2 ชั่วโมง ทำการเก็บสารที่ไดอะไลส์เป็นระยะ ๆ (10 mL/ fraction) จากนั้นจะทำการตรวจวัดด้วยเทคนิค flame AES ต่อไป

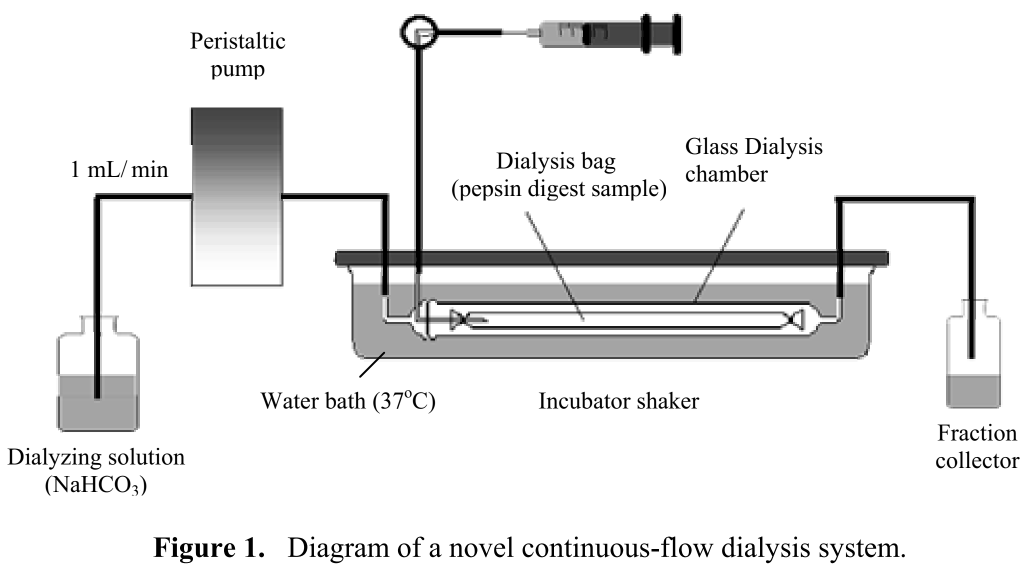

Figure 1. Diagram of a novel continuous-flow dialysis system.

4. การประเมินค่าความสามารถในการไดอะไลส์ได้ของแคลเซียม

ค่าความสามารถในการไดอะไลส์ได้ของแคลเซียม อาจใช้ในการชี้บอกถึงความสามารถในการนำแคลเซียมไปใช้ได้ของอาหารชนิดนั้น ซึ่งหาได้จากสมการ

$$\boxed{\text{Dialyzability (\%)} = \frac{D \times 100}{W \times A}}$$

โดยที่ D = ปริมาณของแคลเซียมที่ไดอะไลส์ได้ทั้งหมดในเวลา 150 นาที เมื่อหักลบออกจากค่าที่ได้จากการทำแบลงก์ (μg)

W= น้ำหนักตัวอย่างอาหารแห้ง (g)

A = ความเข้มข้นของแคลเซียมในตัวอย่างอาหารแห้ง (μg/g)

5. การประเมินค่าการเพิ่มขึ้นหรือลดลงของแคลเซียมที่ไดอะไลส์ได้ เมื่อนำผักไปประกอบเป็นอาหาร

$$\boxed{\text{Dialyzability Enhancement (or Depression) (\%)} = \frac{[D_{AB} - (D_A + D_B)] \times 100}{(D_A + D_B)}}$$

เมื่อ D_{AB} = ปริมาณการไดอะไลส์ได้ของแร่ธาตุ เมื่อนำผัก (A) และเครื่องปรุงอื่น ๆ (B) มาปรุงเข้าด้วยกันและทำให้สุก

D_A, D_B = ปริมาณการไดอะไลส์ได้ของแร่ธาตุในผัก (A) หรือเครื่องปรุงอื่น ๆ (B) เมื่อทำให้สุกโดดๆ

ถ้าค่าการเพิ่มขึ้นหรือลดลงของแคลเซียมที่ไดอะไลส์ได้ดังกล่าวมีค่าสูงกว่าหรือต่ำกว่ามากกว่า 10% จึงจะถือว่าเครื่องปรุงอื่น ๆ ในอาหารนั้นส่งผลให้มีการไดอะไลส์ได้เพิ่มขึ้นและลดลงของผัก

ผลและวิจารณ์

1. ผลการศึกษาปริมาณการนำแคลเซียมไปใช้ได้ในตัวอย่างผัก

ผักคะน้า

ผักปวยเล้ง

ผักที่เลือกทำการศึกษามี 2 ชนิด ได้แก่ ผักคะน้าและปวยเล้ง ซึ่งเป็นตัวแทนของผักที่มีความสามารถในการนำแคลเซียมไปใช้ได้สูงและต่ำตามลำดับ จากการศึกษาโดยใช้วิธีในหลอดแก้วแบบไหลต่อเนื่อง ผลที่ได้ใน

กราฟแสดงการเปลี่ยนแปลงของการไดอะไลส์ของแคลเซียมตามเวลาของการไดอะไลส์ และค่าการเปลี่ยนแปลงของ pH ในระหว่างการไดอะไลซิส (Figure 2.)

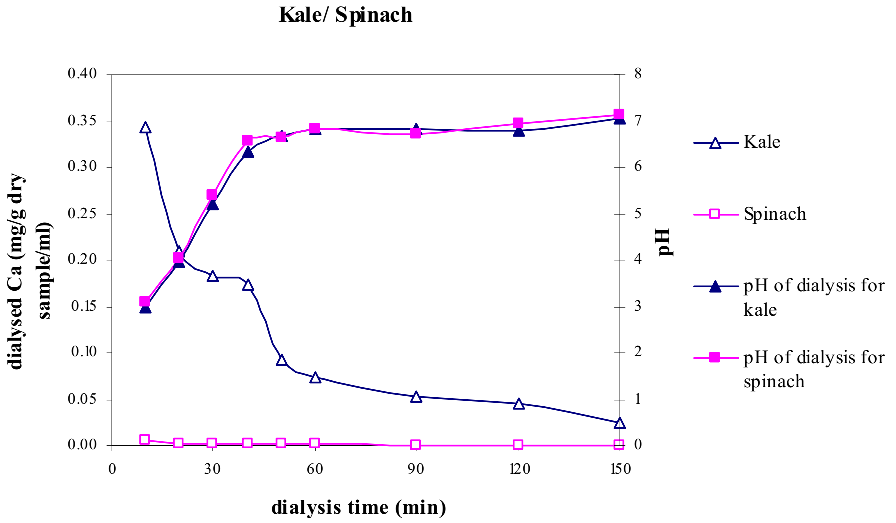

Figure 2. The profile of dialyzed calcium and pH change during dialysis for kale and spinach by continuous-flow dialysis method.

จากผลแสดงให้เห็นว่าวิธีที่เสนอขึ้นนี้ให้ค่าการเปลี่ยนแปลงของ pH ในระหว่างการไดอะไลซิสสอดคล้องกับสภาพ pH ในระบบทางเดินอาหารของมนุษย์ โดยมีการเปลี่ยนแปลง pH ในกระเพาะอาหารจาก 2.0 เป็น 5.0 ซึ่งเป็น pH ในลำไส้เล็กภายในเวลา 30 นาที สำหรับผลการเปรียบเทียบค่าความสามารถในการนำแคลเซียมไปใช้ของผักคะน้าและปวยเล้ง แสดงไว้ใน Table 2.

Table 2. The comparison of calcium bioavailability for kale and spinach by the continuous-flow dialysis and *in vivo* method.

Method	% Ca bioavailability	
	Kale	Spinach
In vitro method		
Continuous-flow dialysis (this study)	42.2 ± 0.2	2.89 ± 0.17
Continuous-flow dialysis (Sottimai *et al.*, 2003)	52.9 ± 1.1	4.6 ± 0.5
In vivo method		
Dual isotope tracer (Weaver *et al.*, 1994)	58.8	5.1
Dual isotope tracer (Weaver *et al.*, 1999)	49.3	5.1
Dual isotope tracer (Heaney *et al.*, 1990)	40.9	4.55

จากตารางจะเห็นว่าค่าที่ได้จากการศึกษาด้วยวิธีนี้ให้ค่าที่ใกล้เคียงกับการศึกษาด้วยวิธีในร่างกาย ความแตกต่างของค่าเหล่านี้ที่อาจมีบ้าง อาจเกิดจากหลายปัจจัยด้วยกัน เช่น อายุของผัก สิ่งแวดล้อม และสภาพดินที่ปลูกผักนั้น เหตุที่ผักปวยเล้งมีค่าการนำแคลเซียมไปใช้ต่ำมาก เป็นเพราะการมีปริมาณของออกซาเลตสูง ซึ่ง

สามารถรวมตัวกับแคลเซียมแล้วเกิดเป็นเกลือแคลเซียมออกซาเลตที่ร่างกายไม่สามารถนำแคลเซียมไปใช้ได้ (Reykdal *et al*., 1991; Savage *et al*., 2000) ในทางตรงข้ามคะน้าเป็นผักที่มีออกซาเลตต่ำ เช่นเดียวกับบรอคโคลี่ และ กะหล่ำปลี ทำให้ผักเหล่านี้มีค่าการนำแคลเซียมไปใช้สูง (Heaney *et al*., 1990; Heaney *et al*., 1993)

2. ผลการศึกษาความสามารถในการนำแคลเซียมไปใช้ได้ จากการนำผักคะน้าปรุงเป็นอาหารไทยต่างชนิดกัน

จากการศึกษาเบื้องต้น ถึงอิทธิพลของกรดอินทรีย์ต่าง ๆ เช่น กรดซิตริก กรดทาร์ทาริก กรดมาลิก และ กรดแอสคอร์บิกในการเพิ่มการนำแคลเซียมไปใช้ได้ในผักโขม พบว่าให้ผลดัง Table 3. ซึ่งแสดงผลการเพิ่มขึ้นของแคลเซียมที่ไดอะไลส์ได้ เมื่อมีกรดอินทรีย์ชนิดต่าง ๆ ในความเข้มข้นที่ต่างกัน

Table 3. The percent increase of calcium dialyzability in the presence of organic acid for amaranth as determined by continuous-flow dialysis method.

Organic acid	% increase of dialyzability in the presence of organic acid		
	1%	2.5%	5%
Ascorbic acid	0	15.1	29.1
Citric acid	32.8	54.5	65.7
Malic acid	0	33.2	46.1
Tartaric acid	26.6	41.6	55.8

การศึกษานี้จึงสนใจที่จะศึกษาดูว่ากรดอินทรีย์ที่มีอยู่ในอาหารไทยมีผลช่วยเพิ่มความสามารถในการดูดซึมของแคลเซียมได้หรือไม่ โดยเลือกผักคะน้าทำการปรุงเป็นอาหารไทยทั้งชนิดที่มีและไม่มีส่วนผสมของกรดอินทรีย์ โดยทำการศึกษาในอาหาร 2 ชนิดคือ ต้มยำปลาใส่ผักคะน้า (มีกรดซิตริกจากมะนาว) และแกงส้มปลาใส่ผักคะน้า (มีกรดทาร์ทาริกจากมะขาม) นอกจากนี้ยังได้ศึกษากรณีของแกงจืดปลาใส่คะน้าและแกงจืดปลาใส่คะน้าและน้ำมะนาวด้วยเพื่อยืนยันผลของน้ำมะนาวที่มีต่อการนำไปใช้ได้ของแคลเซียม จากการศึกษาพบว่าให้ผลเป็นดัง Table 4. Figure 3-4. เป็นกราฟแสดงการเปลี่ยนแปลงของการไดอะไลส์ของแคลเซียมตามเวลาของการไดอะไลส์ และค่าการเปลี่ยนแปลงของ pH ในระหว่างการไดอะไลซิส

Table 4. Calcium dialyzability of kale alone(A) and in different dishes(AB) and enhancement (depression) effect from ingredients.

Menu	**% organic acid**	**Total calcium (mg/serving)**	**Dialyzed Calcium (mg/serving), n=3**	**% enhancement (or depression)**
1. Fish Tom Yam with kale				
- Boiled kale alone(A)	(0.1)	55.1 ± 4	25.4 ± 0.6 (46.0%)	+28%
- Fish Tom Yam alone(B)	0.61^{a}	39.5 ± 3.6	19.0 ± 2.1 (48.1%)	
- Fish Tom Yam with kale(AB)	0.50^{a}+(0.1)	109 ± 1	56.7 ± 1.9 (52.0%)	
2. Fish Kaeng Som with kale				
- Boiled kale alone(A)	(0.1)	55.1 ± 4	25.4 ± 0.6 (46.0%)	+7% Insignificant effect
- Fish Kaeng Som alone(B)	0.10^{b}	71.7 ± 4.9	35.6 ± 1.1 (49.7%)	
- Fish Kaeng Som with kale(AB)	0.08^{b}+(0.1)	132 ± 13	65.1 ± 2.5 (49.4%)	
3. Fish soup with kale				
- Boiled kale alone(A)	(0.1)	82.8 ± 0.7	41.3 ± 0.9 (49.9%)	+6%
- Fish soup alone(B)	0.00	8.78 ± 0.53	3.71 ± 0.20 (42.4%)	

- Fish soup with kale(AB)	0.00+(0.1)	90.8 ± 3.6	47.5 ± 3.1 (52.3%)	Insignificant effect
4. <u>Fish soup with lime juice with kale</u> - Boiled kale alone(A) - Fish soup with lime juice alone(B) - Fish soup with lime juice with kale(AB)	 (0.1) 0.40^a 0.33^a+(0.1)	 79.3 ± 0.7 10.7 ± 1.2 86.6 ± 1.6	 39.6 ± 0.8 (49.9%) 4.98 ± 0.25 (46.6%) 51.2 ± 0.8 (59.1%)	+15%

* Values in brackets are percent dialyzability.

[a] Citric acid containing in the dish.
[b] Tartaric acid containing in the dish.

จากการศึกษานี้พบว่ากรดอินทรีย์ในส่วนประกอบของอาหารเพิ่มความสามารถในการนำแคลเซียมไปใช้ได้ ซึ่งชี้ให้เห็นว่ากรดซิตริกจากน้ำมะนาวเพิ่มการนำไปใช้ของแคลเซียมที่ดีกว่ากรดทาร์ทาริกจากน้ำมะขามเปียก แต่อย่างไรก็ตามค่าการเพิ่มขึ้นนี้ก็ยังขึ้นอยู่กับปริมาณของกรดอินทรีย์ที่อยู่ในอาหารด้วย โดยเปอร์เซ็นต์การเพิ่มขึ้นของแคลเซียมที่ไดอะไลส์ได้ เพิ่มตามความเข้มข้นของกรดอินทรีย์ที่มีในอาหารซึ่งเป็นแนวโน้มเดียวกับการศึกษาในเบื้องต้นดัง Table 3 โดยกรดอินทรีย์นั้นทำให้แคลเซียมอยู่ในรูปที่สามารถไดอะไลส์ได้มากขึ้น (Benway *et al.*, 1993; Lyon *et al.*, 1984) ในการยืนยันผลการเพิ่มขึ้นของการไดอะไลส์เนื่องจากน้ำมะนาว ได้ทำการศึกษาเปรียบเทียบอาหาร 2 ชนิด คือ แกงจืดปลาใส่ผักคะน้า และแกงจืดปลาใส่ผักคะน้าใส่น้ำมะนาว พบว่าน้ำมะนาวซึ่งมีกรดซิตริก สามารถเพิ่มการไดอะไลส์ของแคลเซียมได้จริง ซึ่งไม่เพียงแต่กรดซิตริกจะช่วยเพิ่มการไดอะไลส์ได้ของแคลเซียมในผักคะน้าเท่านั้น แต่ยังช่วยเพิ่มการไดอะไลส์ได้ในเนื้อปลาด้วย (42% เพิ่มเป็น 47% เมื่อพิจารณาค่า B ของแกงจืดปลาและแกงจืดปลาใส่น้ำมะนาวใน Table 4.)

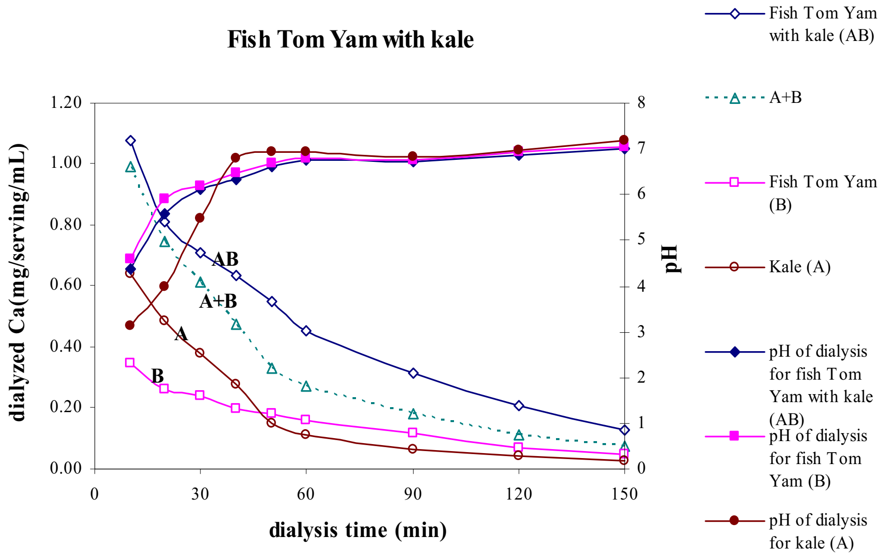

Figure 3. The profile of dialyzed calcium and pH change of fish Tom Yam with kale by continuous-flow dialysis method.

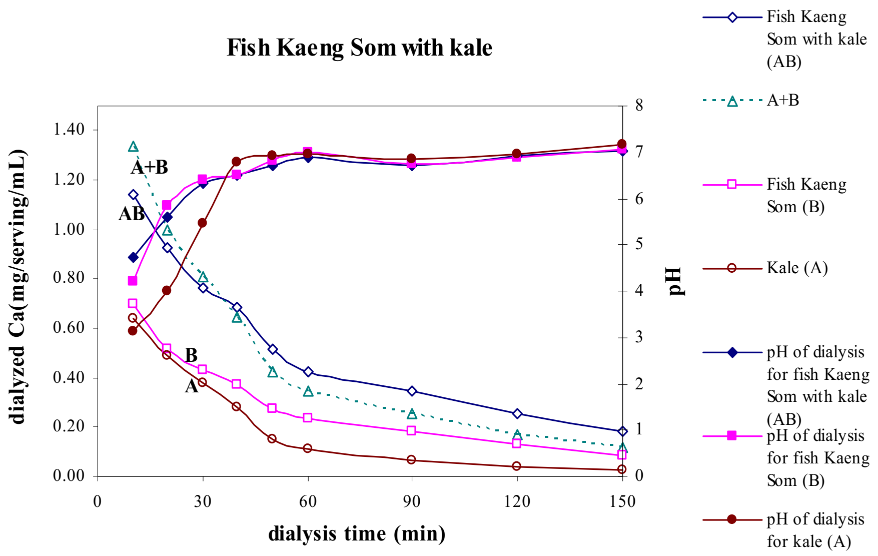

Figure 4. The profile of dialyzed calcium and pH change of fish Kaeng Som with kale by continuous-flow dialysis method.

จาก Figure 3-4 จะเห็นได้ว่า อาหารบางชนิดให้การเปลี่ยนแปลงของ pH ในระหว่างการไดอะไลซิสในชั่วโมงแรกช้ากว่า ทั้งนี้อาจเป็นเพราะ การมีองค์ประกอบบางอย่างในอาหารที่อาจไปขัดขวางการปล่อยออกของกรดสู่ภายนอกเมมเบรน ทำให้มีการเปลี่ยนแปลงของ pH ที่ช้ากว่า

3. ผลการศึกษาความสามารถในการนำแคลเซียมไปใช้ได้ จากการนำผักปวยเล้งปรุงเป็นอาหารไทยต่างชนิดกัน

ในทำนองเดียวกับการศึกษากับผักคะน้า การศึกษานี้ใช้ผักปวยเล้งทำการปรุงเป็นอาหาร 2 ชนิดคือ ต้มยำปลาใส่ผักปวยเล้ง แกงส้มปลาใส่ผักปวยเล้ง โดยผลการศึกษาเป็นไปดัง Table 5.

Table 5. Calcium dialyzability of spinach alone(A) and in different dishes(AB) and enhancement (depression) effect from ingredients.

Menu	% organic acid	Total calcium (mg/serving)	Dialyzed Calcium* (mg/serving), n=3	% enhancement (or depression)
1. <u>Fish Tom Yam with spinach</u> - Boiled spinach alone(A) - Fish Tom Yam alone(B) - Fish Tom Yam with spinach(AB)	 0.00^a 0.61^a 0.52^a	 34.1 ± 1.6 58.5 ± 4.2 100 ± 4	 0.98 ± 0.06 (2.89%) 27.1 ± 0.1 (46.3%) 6.14 ± 0.12 (6.14%)	-78%
2. <u>Fish Kaeng Som with spinach</u> - Boiled spinach alone(A) - Fish Kaeng Som alone(B) - Fish Kaeng Som with spinach(AB)	 0.00^b 0.10^b 0.08^b	 29.5 ± 1.4 96.1 ± 5.0 128 ± 2	 0.85 ± 0.05 (2.89%) 39.2 ± 2.0 (40.8%) 11.1 ± 0.4 (8.70%)	-72%

* Values in brackets are percent dialyzability.

[a] Citric acid containing in the dish.

[b] Tartaric acid containing in the dish.

จากตารางแสดงให้เห็นว่าปริมาณการนำแคลเซียมไปใช้นั้นลดลงอย่างมากในอาหารที่ศึกษา ผลเหล่านี้เกิดจากการมีสารยับยั้งโดยเฉพาะอย่างยิ่งออกซาเลตในรูปที่ละลายน้ำได้ในผักปวยเล้งที่พบในปริมาณสูง อาจถึง

737 ± 20 mg/100g (Brogren *et al*., 2003) ซึ่งนอกจากออกซาเลตในผักสามารถรวมตัวกับแคลเซียมในตัวมันเองแล้ว ยังรวมถึงแคลเซียมในส่วนประกอบของอาหารอื่น ๆ ส่งผลให้การนำไปใช้ของแคลเซียมที่อยู่ในส่วนประกอบอาหารลดลงด้วย ผลเหล่านี้สอดคล้องกับการศึกษาในมนุษย์ ซึ่งพบว่า การดูดซึมแคลเซียมในนมเมื่อบริโภคร่วมกับอาหารเช้านั้นมีค่า 27.6% แต่เมื่อบริโภคผักปวยเล้งร่วมกับอาหารเช้าพบว่าเหลือเพียง 5.1% เท่านั้น (Heaney *et al*., 1988) และจากการศึกษาในหนูทดลอง โดยเมื่อให้การดูดซึมของแคลเซียมในนมเป็น 100% พบว่าการดูดซึมสัมพัทธ์ของผักปวยเล้งมีค่า 47% และเมื่อบริโภคผักปวยเล้งร่วมกับนมจะมีค่าเพียง 52% (Poneros-Schneier *et al*., 1989)

การศึกษานี้แสดงให้เห็นว่ากรดอินทรีย์ในอาหารไม่สามารถต้านอิทธิพลของสารยับยั้งทำให้การไดอะไลส์ของแคลเซียมลดน้อยลงอย่างมาก (78% และ 72% ในต้มยำและแกงส้ม) อาจเป็นเพราะปริมาณของกรดอินทรีย์ไม่มากพอที่จะเอาชนะผลของออกซาเลตซึ่งมีอยู่ในปริมาณที่มากได้

สรุป

วิธีในหลอดแก้วแบบไหลต่อเนื่อง สามารถใช้ศึกษาแนวโน้มการนำแร่ธาตุไปใช้ได้ โดยเป็นเครื่องมือที่มีประโยชน์ในการประมาณค่าการนำไปใช้ได้ของแร่ธาตุในอาหาร หลักการวิธี *in vitro* นี้ สามารถใช้ศึกษาเบื้องต้นถึงปัจจัยที่มีผลต่อการนำแคลเซียมไปใช้ ก่อนทำการศึกษาจริงในมนุษย์เพื่อยืนยัน เพราะวิธี *in vitro* เป็นวิธีที่ง่ายทำให้รวดเร็วและค่าใช้จ่ายน้อย

จากการศึกษานี้ชี้ให้เห็นว่า ส่วนประกอบในอาหารสามารถส่งผลต่อการนำแคลเซียมไปใช้ โดยกรดอินทรีย์ในอาหารไทย เช่นกรดซิตริกจากน้ำมะนาวสามารถเพิ่มการนำแคลเซียมไปใช้ได้ในผักคะน้า ในการเสริมธาตุแคลเซียม แหล่งของแคลเซียมเป็นสิ่งสำคัญที่ควรพิจารณา โดยจะต้องพิจารณาทั้งปริมาณแคลเซียมที่มีในอาหารนั้นและสารที่เป็นปัจจัยเสริมหรือเป็นปัจจัยยับยั้งการดูดซึมด้วย จากการศึกษานี้พบว่าผักคะน้าเป็นแหล่งของแคลเซียมที่ดี ถ้าบริโภคร่วมกับส่วนประกอบที่มีกรดอินทรีย์สูงและไม่มีสารยับยั้ง

อย่างไรก็ตามเนื่องจากหลักการนี้ไม่สามารถที่จะเลียนแบบให้เหมือนกับกระบวนการเมทาบอลิสมต่าง ๆ ในร่างกายมนุษย์โดยสมบูรณ์ จึงควรนำไปใช้อย่างพิเคราะห์

กิตติกรรมประกาศ

ขอขอบพระคุณโครงการพัฒนาบัณฑิตศึกษาและการวิจัยทางเคมี (PERCH) ทุนวิจัยจากสำนักงานกองทุนสนับสนุนการวิจัยที่ได้สนับสนุนค่าใช้จ่ายต่าง ๆ ตลอดการศึกษาครั้งนี้ และภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล ที่เอื้ออำนวยความสะดวกในด้านเครื่องมือในการวิเคราะห์

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