Flow-through dynamic extraction system for automatic *in-vitro* assessment of chyme bioaccessibility in food commodities

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Abstract

An automatic flow-through dynamic extraction method is proposed for the first time for *in-vitro* exploration, with high temporal resolution, of the transit of the chyme from the gastric to the duodenal compartments using the Versantvoort's fed-state physiologically relevant extraction test. The flow manifold was coupled on-line to an inductively coupled plasma optical emission spectrometer (ICP OES) for real-time elucidation of the bioaccessible elemental fraction of micronutrients (viz., Cu, Fe and Mn) in food commodities across the gastrointestinal tract. The simulated intestinal and bile biofluid (added to the gastric phase) was successively pumped at 1.0 mL min^{-1} through a large-bore column (maintained at 37.0 ± 2.0 °C) initially loaded with a weighed amount of linseed (250 mg) using a PVDF filter membrane (5.0 μm pore size) for retaining of the particulate matter and in-line filtration of the extracts. The lack of bias (trueness) of the on-line gastrointestinal extraction method coupled to ICP OES was confirmed using mass balance validation following microwave assisted digestion of the residual (non-bioaccessible) elemental fraction. Mass balance validation yielded absolute recoveries spanning from 79 to 121% for the overall analytes and samples. On-line dynamic extraction was critically appraised against batch counterparts for both gastric and gastrointestinal compartments. Due to the lack of consensus in the literature regarding the agitation method for batch oral bioaccessibility testing, several extraction approaches (viz., magnetic stirring, end-over-end rotation and head-over-heels) were evaluated. Improved gastric extractability for Fe along with bioaccessible data comparable to the dynamic counterpart based on the continuous displacement of the extraction equilibrium

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was obtained with batchwise magnetic stirring, which is deemed most appropriate for ascertaining worst-case/maximum bioaccessibility scenarios.

1. INTRODUCTION

Linseed (*Linum usitatissimum L.*) is currently regarded as a functional food that serves as a source of omega-3 fatty acids, lignans, dietary fiber, proteins, carbohydrates, lipids and essential elements (e.g., Ca, Cu, Fe, K, Mn, P, Na and Zn). These components are important to maintain human body homeostasis, in addition to ensure beneficial effects on hormonal regulation in the prevention of diseases, such as cancer and diabetes [1,2]. The mere occurrence of nutrients in foodstuff does not guarantee by its own its availability by the human body after ingestion. To assess the actual pools of nutrients that are released into the gastrointestinal (GI) tract and are available for intestinal absorption, *in-vivo* methods have been developed over the past decades. However, *in-vivo* tests that use animal models are ethically controversial, time-consuming, cost expensive and require specialized and trained personnel [3,4]. Therefore, EU regulations (e.g., REACH) [5] suggested the replacement of *in-vivo* assays by *in-vitro* counterparts as a proxy for oral bioaccessibility with no need of animal models. The Bioaccessibility Research Group of Europe launched a standardized and validated operational procedure (so-called unified bioaccessibility method (UBM) [6,7] that harmonizes the various bioaccessibility tests for health risk assessment of metals from contaminated soils. *In-vitro* digestion methods dedicated to bioaccessibility studies in food samples are also available in the literature [8-10]. As is the case with the UBM method, the so-called Versantvoort's test [10] embraces two consecutive extraction steps to mimic two digestion compartments (i) the gastric compartment in which the gastric fluid and saliva are incorporated to the solid sample, and (ii) the GI compartment involving the addition of the bile and duodenal body fluids to the mock chyme. The extraction method is performed with biorelevant digestive fluid surrogates the composition of which resembles human physiology. As compared with the UBM test, which is performed mimicking fasted state, the Versantvoort's method capitalizes upon fed state conditions whereby operational modifications are undertaken based on the physiological modifications occurring within the GI tract with food components, such as change in pH and GI residence times, and the increase in the secretion of gastric acid, bile and pancreatic fluids [11]. It should be however noted that overly simplistic *in vitro* bioaccessibility methods [12-15], e.g., those based in

compendial body fluids endorsed by the United States Pharmacopeia (USP) [16], have been sometimes proposed in the literature for food commodities. USP-based digestive fluids do not properly simulate the real composition of the human GI tract, and predominantly are applied individually, that is, the food is first exposed and extracted with the gastric fluid and then the solid residue is exposed to the GI phase, whereby information about the chyme transit is lost. Two common limitations of conventional oral bioaccessibility tests based upon batchwise analysis are (i) the lack of immediate removal of desorbed compounds from the surface of the matrix, which is inherent to the bioaccessibility concept [17], and (ii) the absence of insight into the leaching kinetics of target species at real-time [18].

To tackle the above shortcomings, several teams performed *in vitro* dynamic bioaccessibility assays with the aid of flow setups that are able to bring fresh portions of body fluids (usually gastric fluid) through the solid sample as contained in a dedicated flow-through chamber. For example, Beauchemin's group used on-line dynamic extraction for the speciation of bioaccessible arsenic [19-21] and the identification of bioaccessible pools of Cr, As, Cd and Pb in bread [22]. Notwithstanding the simplification of the body fluids using USP recommendations, the authors were unable to analyse the duodenal phase because of clogging of the flow conduits [23]. Rosende *et al.* [24] investigated the risk exposure of metal species in soil materials under worst-case extraction scenarios with a flow-through system using UBM bio-relevant gastric phase, yet mucin that generates turbid and viscous milieu was omitted from the biofluid after statistical data processing. However, the enzyme might behave as a vital component of body fluid surrogates in the GI digestion of foodstuff. Further previous flow systems using small-scale column setups [18-23,25] were unable to handle sample amounts > 200 mg which might not assure the sample representativity in bioaccessibility assays of food commodities.

In this work, an automatic flow-through dynamic system is proposed for the first time for exploring the transit of the chyme from the gastric to the duodenal compartments based on the Versantvoort's fed-state method for on-line elucidation of the bioaccessible elemental fraction of food commodities with high temporal resolution. The proof of concept applicability of the novel flow approach hyphenated to an inductively coupled plasma optical emission spectrometer (ICP OES) was demonstrated by on-line analysis of bioaccessible concentrations of micronutrients (viz., Mn, Fe and Cu) in commercially available golden and brown linseed.

2. EXPERIMENTAL

2.1. Reagents, solutions and samples

All solutions were prepared from analytical reagents using Milli-Q water (18.2 MΩ·cm, Millipore Synthesis A10, Billerica, USA). A multi-element standard solution 5 (TraceCERT, Fluka, Sant Louis) was used for ICP OES calibration. Gastrointestinal/chyme extracts were analyzed by a matrix-matched protocol using dilute working solutions of the multi-elemental standard.

The polyethylene containers (Corning®) and glassware were soaked in 10% (v/v) nitric acid for ca. 14 h and rinsed three times with Milli-Q water pending use.

Physiologically-based digestive fluids were composed of organic reagents, enzymes and salts according to UBM [6], yet with the increase in enzyme concentrations by a factor of 4-10 (conservative conditions) throughout the various body fluids in the mimicry of the fed-state system, as previously suggested by Versantvoort's [10] and FOREhsT [11] methods. In short, the *in-vitro* saliva (pH 6.8 ± 0.2) is composed as follows: 298 mg L^{-1} NaCl, 200 mg L^{-1} KSCN, 896 mg L^{-1} KCl, 1694mg L^{-1} $NaHCO_3$, 570 mg L^{-1} Na_2SO_4, 888 mg L^{-1} NaH_2PO_4, 200 mg L^{-1} urea, 30 mg L^{-1} uric acid, 580 mg L^{-1} alpha amylase from *Bacillus sp* (1594 units mg^{-1} protein, Sigma, A-6814), and 50 mg L^{-1}, mucin from porcine stomach (type II, Sigma, M2378). The simulated gastric phase (pH 1.30 ± 0.02) consists of 2752 mg L^{-1} NaCl, 824 mg L^{-1} KCl, 266 mg L^{-1} NaH_2PO_4, 306 mg L^{-1} NH_4Cl, 400 mg L^{-1} $CaCl_2 \cdot 2H_2O$, 85 g L^{-1} urea, 20 mg L^{-1} glucuronic acid, 650 mg L^{-1} glucose, 330 g L^{-1} glucosamine hydrochloride, 6.5 ml HCl (37 %), 2000 mg L^{-1} albumin from bovine serum (BSA, Merck, 112018, Darmstadt, Germany), 5000 mg L^{-1} pepsin from porcine gastric mucosa (0.7 FIP-U mg^{-1}, Merck, 107185) and 6000 mg L^{-1} mucin. The *in-vitro* duodenal fluid (pH 8.1 ± 0.2) consists of 7012 mg L^{-1} NaCl, 564 mg L^{-1} KCl, 200 mg L^{-1} $CaCl_2 \cdot 2H_2O$, 50 mg L^{-1} $MgCl_2$, 3388mg L^{-1} $NaHCO_3$, 80 mg L^{-1} KH_2PO_4, 100 g L^{-1} urea, 180 µL of HCl (37 %), 2000 mg L^{-1} BSA, 18,000 mg L^{-1} pancreatin from porcine pancreas (24,000 FIP-U g g^{-1} lipase, 1400 FIP-U g^{-1} protease, 30,000 FIP-U g^{-1} amylase, Merck, 107133), 3000 mg L^{-1} lipase from porcine pancreas (114 units mg^{-1} protein, Sigma, L-3126). The surrogate bile fluid (pH 8.2 ± 0.2) contains 5260 mg L^{-1} NaCl, 376 mg L^{-1} KCl, 222 mg L^{-1} $CaCl_2 \cdot 2H_2O$, 5785mg L^{-1} $NaHCO_3$, 250 g L^{-1} urea, 150 µL of HCl (37 %), 3600 mg L^{-1} BSA, 60000 mg L^{-1} bile from porcine pancreas (Sigma, B-3883). All of the body fluids were subjected to agitation (100 rpm) for ca. 3 h aimed at the dissolution of the salts along with the organic and the enzyme components that were

kept overnight at room temperature. The occurrence of mucin, pancreatin and lipase in the body fluids accounts for the formation of weakly turbid aqueous suspensions. On the same day of analysis, the abovementioned oral fluids were heated to 37±2 °C for 1 h for activation of the enzymes.

The gastric extractant was prepared by mixing 6.6 ml of saliva and 13.4 ml of gastric fluid and the chyme/GI extractant was prepared by mixing the gastric extract with 13.4 ml of duodenal fluid, 6.6 ml of bile and 2.2 ml of $NaHCO_3$.

Nitric acid (69%, Sigma–Aldrich) was used for microwave-assisted digestion of linseed and extraction residues in mass balance validation studies.

Bulk golden and brown linseed were purchased from a local natural food store in Salvador, Bahia, Brazil and a pack of golden linseed was purchased from a local food store in Palma de Mallorca, Illes Balears, Spain.

Prior to the oral bioaccessibility assays, finely ground linseed samples were obtained using an analytical batch mill (IKA M20, KG. Staufen, Germany) for 10 s so as to mimic the grinding process in the mouth and to get insight into the maximum bioaccessible pools of target micronutrients, which is in good agreement with previous papers reporting bioaccessibility studies in foodstuff [12,13,21,22]. It should be taken into account that crushing of seeds and foodstuff is a common practice in recent years to take advantage of seed nutritional benefits.

2.2 Analytical instrumentation

2.2.1 Flow-through manifold

A diagrammatic illustration of the hyphenated flow system controlled by the Cocosoft freeware [26] for assessment of dynamic chyme bioaccessible concentrations of Fe, Mn and Cu in golden and brown linseed is shown in Fig. 1. The flow setup is composed of a standalone 3000-step bidirectional micro-syringe pump (SP; CAVRO XP3000, Männedorf, Switzerland) for manipulation of the digestive fluids and pumping of metered volumes by software control to the food commodity as contained in a dedicated chamber. The SP was furnished with a gas-tight 5 mL-glass syringe (Hamilton, Switzerland) and a three-way valve at the head, which permitted aspirating carrier (Milli-Q water) from the external reservoir or alternatively connecting with the flow setup. An 8-port multi-port rotary valve (SV; Crison Instruments, Spain) was employed for the automatic manipulation of the gastric and GI fluids, and the on-line gastric/chyme/gastrointestinal extracts as well. The peripheral ports of SV served also to connect the SP with (i) a large

bore column extractor (see below), (ii) an extract homogenizer made of a 5 mL pipette tip, (iii) ICP OES, (iv) air and (iv) waste using polytetrafluoroethylene (PTFE) tubing (1.5 mm i.d.) and polyetheretherketone flangeless nuts. The SP was connected to the SV central port using a holding coil (HC) made of a 3.0 m-long PTFE tubing with a capacity of 5.4 mL.

For accurate injection of metered volumes of chyme/gastrointestinal extracts by ICP OES, a 6-port injection valve (IV) was equipped with a 300 µL-loop of 0.76 mm i.d. PTFE. The SV and the IV valves communicated through a 110 µL-transfer line of 0.76 mm i.d. PTFE.

2.2.2 Membrane holder for on-line dynamic extraction tests

The dedicated flow-through extraction unit employed in this work consists of a large bore column (LBC). It comprises two threaded polypropylene filter holders ended with fitting for standard ¼-28 female to male luer connection, a membrane filter, and two PTFE gaskets. Both filter holders were drilled radially with a number of 18 rectangular apertures (5 mm long, 1 mm width), to allow free flow of the extractant while retaining the solid particles. The assembled flow-through extraction device has an inner volume of ~2.5 mL (height: 22 mm and diameter: 47 mm). The feasibility of LBC for on-line gastrointestinal extraction was compared against stirred flow chamber (SFC) configurations previously described for bioaccessibility tests of soil materials [27]. The primary differences between both dynamic extraction chambers (LBC vs SFC) capitalize upon the use of magnetic agitation in SFC and the distinct column void volumes. While SFC usually bears internal volumes of 10-15 mL [24,27], those of LBC are usually < 3 mL [28], which, in turn, allows high temporal resolution in the course of the dynamic extraction. Polyvinylidiene difluoride (PVDF, Merck Millipore, Spain) filters with pore sizes of 0.45 or 5.0 µm, and nylon filter membranes (GE Osmonics Labsto, USA) with pore size of 0.45 µm, each with a diameter of 47 mm, were assessed in LBC and SFC as a support to allow the dissolved linseed constituents to flow through but retain particles without pressure drop effects.

In the final configuration, the LBC system was interfaced between the SV and the extract container (see Fig. 1) using 20 cm of 1.5 mm i.d. PTFE tubing at the inlet, and 7 cm of 1.5 mm i.d. PTFE tubing followed by 53 cm of 0.8 mm i.d. PTFE tubing at the outlet. A given amount of linseed (either 250 or 500 mg) was weighed onto the LBC membrane

filter, and the overall parts of the sample-laden chamber were tighten pending incorporation in the flow setup.

2.2.3 Apparatus and detection techniques

A magnetic stirring and heating device combined with a digital thermoregulator (VELP Scientifica, Italy) was used for setting and monitoring the temperature of a water bath, which incorporated the gastric and GI extractants, and the sample-laden LBC extraction device, at 37.0 ± 2.0 °C (see Fig. 1). The stirrer was affixed to 300 rpm to afford well dispersed composite gastric and GI extractants.

Automatic pH measurements of the GI extracts were continuously recorded using a small-volume pH meter (Double pore slim electrode, Hamilton, Reno, U.S.A) connected to a digital potentiometer (Eutech Instruments, model PC2700, Toronto, Canada).

The chyme/GI fluid extracts (bioaccessible micronutrients), the residual (non-bioaccessible micronutrients) pools, and the raw linseed samples after microwave-assisted digestion (total micronutrient content) were analyzed by ICP OES (PerkinElmer Optima 5300DV). Further description of the microwave digestion procedure, and the ICP OES analysis along with synchronization of the detection system with the on-line dynamic extraction method is available in SI.

2.3. Analytical procedure for on-line chyme bioaccessibility measurements

The LBC extractor was initially loaded with a metered amount of golden or brown linseed sample using a membrane filter (47 mm diameter) for trapping of food particles The peristaltic pump of the ICP OES was programmed to bring a steady flow of carrier to the spectrometer using the IV (see Fig. 1) switched to load mode. The ICP OES was herein fed with Milli-Q water in lieu of 2% nitric acid to avoid precipitation of proteins from the chyme/GI extracts into the flow conduits and nebulizer as well. The automatic analytical protocol is composed of four distinct steps as detailed in the following:

I) Gastric dynamic extraction procedure: The operational procedure starts by filling the 60 cm-PTFE tubing line of 0.8 mm i.d. connecting with the saliva + gastric fluid composite (SV, port 6 in Fig. 1), and the HC with gastric fluid and Milli-Q water, respectively. The automatic analytical procedure continues with drawing 100 µL of air (port 2 of the SV, see Fig. 1) into HC aiming at overcoming the mixing of the biorelevant digestive fluid with the carrier. After this, a metered volume of mock-gastric fluid (viz., 4900 µL) was aspirated from port 6 (see Fig. 1) at 10 mL min^{-1} toward HC. The SP was

then programmed to perfuse the linseed sample containing LBC extractor (SV, port 5 in Fig. 1) with the extractant volume at a fixed flow rate of 0.5 mL min^{-1}, yet keeping the air plug in the HC. The gastric extract was collected into the polypropylene pipette tip, acting as extract container, attached to one of the ports of SV (port 4 in Fig. 1). The extract container permits collecting the gastric extracts and the removal of gas bubbles evolved during the linseed digestion under acidic conditions. A volume of 1.0 mL of air was brought up-flow into the extract container at 10 mL min^{-1} to aid in the homogenization of the gastric extract. The container was then emptied by withdrawal of the entire extract volume into HC, and the SP was then programmed to pump the first 4.9 mL-gastric leachate toward the extract reservoir in port 8 of SV. The above procedure was threefold repeated using a gastric extractant volume of 4900 µL each and one more time with a surrogate gastric extractant volume of 400 µL to obtain a final gastric phase of 20.00 mL, so as to match the experimental results from the batch mode extraction as explained below in Results and Discussion. Thereafter, 1000 µL of air were brought from SV (port 2 in Fig. 1) into the HC followed by aspiration of 500 µL of the gastric extract (port 8 of SV in Fig. 1) at 10 mL min^{-1}. The SP was then activated to dispense the 500 µL-gastric leachate toward the injection loop of the IV at 5 mL min^{-1} so as to collect 300 µL for further analysis, the surplus of air being delivered at 10 mL min^{-1} to waste. The ICP OES was then relay-triggered and the IV turned to 'inject' for recording of the transient signal of the overall bioaccessible gastric pools of micronutrients. The ICP OES analysis was in all instances synchronized with the sampling of the gastric/chyme/GI fractions.

II) Automatic generation of the gastrointestinal extractant:

Next, 100 µL of air and 4900 µL of the simulated duodenal + bile composite + HCO_3^-, for pH adjustment (port 7, Fig. 1) were consecutively drawn into the HC at 10 mL min^{-1}. After flow reversal the surrogate intestinal fluid was mixed with the gastric leachate in the extract reservoir at port 8 of the SV. This step was threefold repeated by aspiration of 4900 µL of duodenal + bile + HCO_3^- composite and one more time with 2620 µL to add a total volume of intestinal fluid of 22.22 mL for maintaining the gastric to intestinal phase volume ratio as endorsed by the Versantvoort's method [10]. The GI extractant composed of the gastric extract plus the duodenal + bile composite and HCO_3^- for pH adjustment, was then automatically generated ad hoc and was ready for use. To the best of our knowledge, this is the first article reporting a fully automatic dynamic GI extraction

method performed in additive mode as recommended by physiologically based extraction tests [6,10,11].

III) On-line analysis of chyme/GI bioaccessible micronutrients in seeds:
For dynamic assessment of the chyme/GI bioaccessibility of the target elements, 100 µL of air (port 2 in Fig. 1) and 650 µL of as-prepared GI composite biofluid were consecutively aspirated at 10 mL min^{-1} into HC. By reversing the flow, the GI extractant perfused the linseed-laden LBC extractor filled with the gastric fluid at 1 mL min^{-1} (0.5, 1.0 or 1.5 mL min^{-1} in preliminary tests), while keeping the air volume in HC. The content of the gastric phase in the flow lines and LBC extractor was discarded by flushing with GI extractant. The first chyme extract was then collected into the extractant container and homogenized by bubbling of 1.0 mL of air plug at 10 mL min^{-1}. After the homogenization step, the pH value was automatically measured and the total content of the container was dispensed in a backward-forward mode toward the injection loop of IV at 5 mL min^{-1}. The ICP OES was then triggered and the IV switched to injection for recording of the transient signals. The above automatic procedure was repeated 19 times (viz., 19 fractions) with an extractant volume of 650 µL each for investigation of the kinetics of nutrient bioaccessibility from the chyme in the transit to the duodenum with high temporal resolution. The remaining volume of gastric + duodenal composite extractant was on-line processed through the sample so as to obtain 10 additional fractions (ca. 3 mL each) of GI bioaccessible nutrients. After the on-line oral bioaccessibility assay, the flow conduits and LBC extractor were washed with 5 mL of 2% (v/v) nitric acid followed by ca.10 mL of Milli-Q water to circumvent sample cross-contamination.

IV) Automatic on-line calibration of the ICP OES based method:
A five-point matrix-matched external calibration (in GI medium filtrated through 5.0 µm PVDF membrane) was used for the determination of chyme/GI bioaccessible micronutrients in golden and brown linseed. Hereto, a set of 5 multi-elemental standards (0, 20, 50, 100 and 200 µg L^{-1} of Mn and Cu, and 0, 50, 100, 300, 500 µg L^{-1} for Fe) were aspired from port 4 of the SV and analyzed by on-line ICP OES.

2.4. Batch method

The batchwise physiologically based extraction assay by Versantvoort *et al.* [10] was used to evaluate the effect of several stirring modes upon the magnitude of elemental

bioaccessible pools method was conducted. In brief, 6 mL of saliva were added to 0.5 g of ground linseed and the mixture was incubated at 37 ± 2 °C for 5 min. Thereafter, 12 mL of gastric fluid were added, and the tree types of stirring, that is, magnetic agitation, head-over-heels rotation and end-over end rotation, all at 55 rpm at 37 ± 2 °C for 2 h were compared. Finally, 6 mL of bile, 12 mL of duodenal fluid, and 2.0 of mL of 1.0 mol L^{-1} sodium hydrogen carbonate were consecutively added, and the mixture was agitated for another 2 h at the same temperature using orbital shaking at 55 rpm. After the *in vitro* extraction test, the digestion tubes were centrifuged at 2750g for 5min, so as to isolate the supernatant, that is the GI extract, from the digested matrix (the pellet). Finally, the GI extracts were filtrated through 0.45 μm PVDF syringe filters, acidified with 2% (v:v) HNO_3 to avoid metal hydrolysis, and stored at -20°C pending analysis.

2.5. Evaluation of operational and analytical variables

A thorough investigation of the flow-system configuration including mini-column design (LBC or SFC) and on-line interface to ICP OES along with physicochemical variables, viz., sample amount (250-500 mg), flow rate of digestive fluids (0.5-1.0 mL min^{-1}), filter type (nylon or PVDF), pore dimensions (0.45 or 5.0 μm), extraction temperature (ambient or 37ºC), volume of gastric and gastrointestinal fluids, liquid to solid ratio, and number of fractions, is pinpointed in the ensuing sections so as to assure highly resolved temporal extraction profiles for automatic on-line monitoring of micronutrient chyme bioaccessibility in linseed.

3. RESULTS AND DISCUSSION

3.1. System configuration

Preliminary results obtained with SFC in the absence of food sample (only gastrointestinal fluids) demonstrated that leakage and build-up of pressure occurred at flow rates as low as 0.5 mL min^{-1} for filters of either nylon or PVDF, regardless of the pore size (0.45 and 5.0 μm), which resulted in the halting of the syringe pump that operates as a liquid driver. It should be borne in mind that the surrogate digestive fluids by Versantvoort's method [10] are complex solutions composed by large amounts of organic species, electrolytes and enzymes, which lead to suspended colloidal dispersions. Hereto, automatic handling of physiological fluids (in particular duodenal and bile fluids) throughout the flow system is proven troublesome as a consequence of the gradual

clogging of the tubing of the flow manifold and membrane of the SFC extractor by suspended matter. In fact, previous continuous-flow extraction systems for determination of oral bioaccessible contaminants made use of overly simplistic body fluid-like milieus [19-22] in which insoluble enzymes are frequently eliminated [24].

The same behavior was observed whenever a 0.45 μm nylon filter was used as a holder in the LBC configuration. On the contrary, no increment of pressure drop was identified with the use of a 0.45 μm-PVDF filter in the absence of linseed sample for 40 mL of GI extractant brought to the LBC at 0.5 mL min^{-1}. It is important to note that PVDF membranes bind far less protein than nylon filters and that their exploitation for in-line filtration of biomimetic body fluids has been reported earlier [29]. Unfortunately, after loading 500 mg of food sample, only 20 ml of GI fluid could be handled at 0.5 mL min^{-1} without jeopardizing the stability of the flow system because of blocking of the membrane pores by the GI digests. However, negligible clogging effects and backpressure issues were observed in the course of the overall *in-vitro* dynamic GI digestion for 40 mL of GI fluid with the use of 5.0 μm PVDF membranes, without and with foodstuff. Notwithstanding the increase of the filter pore size, the dynamic GI extracts were in all instances clear, without the occurrence of solid particles or colloidal matter, which in turn fostered direct on-line ICP OES analysis of the GI digests with no need of further extract treatment. Therefore, the combination of LBC and 5.0 μm-PDVF membranes was selected for the remainder of the work.

The differential behavior of LBC against SFC for in-line oral bioaccessibility tests is attributed to their own configuration and flow dynamics. Though the nominal diameter of the filter is in both cases 47 mm, the actual effective surface area amount to 4.9 cm^2 for SFC against 11.3 cm^2 for LBC. In addition, the top cover above the filter membrane of glass SFC (cfr. [27]) is usually made to contain a mere 8 outlet holes of overall 0.78 mm^2 whilst the polymeric LBC used herein features 18 rectangular outlet apertures with overall surface area of 90 mm^2 for free flow of the filtrated digests.

3.2. Investigation of the experimental parameters

3.2.1. Investigation of the liquid to solid ratio and shaking method of batch bioaccessibility assays

As a consequence of the lack of harmonization and standardization of the different operational conditions in oral bioaccessibility procedures for metal species in solid materials [30,31] including foodstuff, preliminary studies were undertaken in this work

to evaluate the effect of GI bioaccessibility upon the liquid-to-solid (L/S) ratio in a batchwise mode. In fact, two distinctly different sample amounts were handled in the literature by previous authors (viz., 3.0 g [32] and 0.5 g [10]) following Versantvoort's method, which afforded L/S ratios of 12.6 and 76, respectively. The GI bioaccessibilities of the three elements were < 12 % for L/S=12.6 against GI bioaccessibilities > 20% for L/S=76, indicating that low L/S ratios (< 12) should be avoided to prevent saturation of the GI fluids, and thus do not serve for estimation of maximum bioaccessibility of micronutrients. To this end, a sample weight of 0.5 g was affixed for the static extraction method so as to assure the exhaustive extraction of the micronutrients under physiologically relevant gastrointestinal conditions. At this juncture, it should be pointed out that previous batchwise physiologically based extraction methods for environmental solids normally employed sample amounts spanning from 300-600 mg [6,12].

The shaking method is another yet operationally defined parameter that might notably affect the GI bioaccessibility of nutrients in food [33]. Therefore, Versantvoort's-based gastric extraction of BGL was performed in a batchwise mode by resorting to a variety of shaking approaches (viz., magnetic stirring, end-over-end rotation and head-over-heels rotation) at 55 rpm and 37ºC for 2 h. As can be seen in Fig. 2, gastric bioaccessibility of Cu in BGL is independent from the sample agitation method. For the gastric bioaccessibility of Mn magnetic agitation and end-over-end rotation afforded virtually identical extraction efficiencies. Conversely, statistically significant differences were encountered for Fe bioaccessibility. While head-over-heels and end-over-end rotation approaches rendered Fe gastric bioaccessibility <17 mg kg^{-1}, magnetic agitation afforded superior bioaccessibility, viz., 31.9 ± 0.4 mg kg^{-1}. Because dynamic extraction capitalizes upon the continuous pumping of fresh portions of digestive fluids across the solid sample with the consequent displacement of the extraction equilibrium to the liquid phase, thus fostering enhanced metal extractability [34], magnetic stirring affording the highest bioaccessibility of Fe was selected as a reference shaking method to get insight into the maximum gastric phase extractability under static conditions.

3.2.2. Investigation of the liquid to solid ratio of on-line dynamic bioaccessibility assays

To equate dynamic gastric bioaccessibility to the batchwise counterpart, the effect of the L/S ratio under flow-through dynamic extraction was thoroughly investigated for 500 mg of sample by increasing the volume of saliva + gastric phase composite pumped through

the linseed-laden LBC setup within the range of 18-45 mL at 0.5 mL min^{-1}. For BGL, no statistically significant differences were encountered at the 0.05 significance level for any of the target micronutrients between the two extraction modes (batch vs dynamic) for an extraction volume of 40 mL (see Table 1). This also holds true for most of the micronutrients analyzed in the brown linseed (BL) and Spanish golden linseed (SGL) samples. Hereto, a volume of 40 mL of saliva + gastric (G) fluid composite, standing for a L/S=80, which is in good agreement with the preliminary tests reported above, was selected as the gastric phase of the flow-through dynamic extraction system for exploring chyme/GI bioaccessibility of micronutrients in seeds.

In order to maintain the volume of G phase and duodenal + bile (GI) phase at the ratio of 1:1 as endorsed by Versantvoort and co-workers, the volume of duodenal juice and bile composite was adjusted to 40 mL (plus ca. 4 mL of bicarbonate), which in turn resulted in a final GI fluid volume of ca. 84 mL for exploring chyme and GI bioaccessibility. Unfortunately, pressure drop was proven to be noticeable for GI volumes above 60 mL, whereby the sample amount and the G and GI volumes and bicarbonate were twofold reduced. Notwithstanding the fact that the reduction of the sample size from 500 to 250 mg could affect the representativity of the assays, repeatability values of GI bioaccessibility of the target elements, given as relative standard deviation, were in all instances $\leq$ 9.2%, regardless of the sample amount, demonstrating that reliable dynamic extraction data should be obtained with 250 mg of homogenized sample.

3.2.3. Investigation of the extraction temperature and extractant flow rate of on-line dynamic bioaccessibility tests

The effect of the flow-rate of the GI composite fluid and the extraction temperature on the dynamic leachability of the target nutrients in BGL was also studied. To this end, three discrete GI extractant flow rates, *viz*., 0.5, 1.0 and 1.5 mL min^{-1} were tested. The highest flow rate was discarded because of occasional backpressure effects, yet bioaccessibility values were statistically identical (α=0.05) for 0.5 and 1.0 mL min^{-1}. A flow rate of 1.0 mL min^{-1} was thus chosen to expedite the automatic assays. As to the extraction temperature, room temperature, that is, 25 ºC, and physiologically relevant temperature, that is 37ºC, for the column system and body fluids were initially assessed using a flow rate of 1.0 ml min^{-1} for the digestive fluids. Unfortunately, GI extractability at room temperature could not be eventually investigated because of the increase in pressure drop. It should be borne in mind that the activity and solubility of enzymes are

affected by temperature [35] whereby potential denaturation and precipitation of proteins occurred at room temperature. Hence, the temperature of the water bath was affixed to 37 ± 2°C, throughout. In a previously reported biomimetic system [24] the temperature was deemed not to be a significant variable under dynamic leaching conditions, however, this was merely investigated for the gastric phase.

3.3. Analytical performance

The analytical performance of the automatic flow-through method was evaluated based on intermediate precision, dynamic range, trueness and limit of detection (LOD). The dynamic linear range was assessed at five concentration levels and spanned from 20 to 200 µg L^{-1} for Cu and Mn and 50 to 500 µg L^{-1} for Fe using matrix-matched calibration curves to avoid the dependence of GI components upon the nebulization efficiency of the target species. The surrogate linseed-free matrix was obtained by filtration across 5-µm PVDF membrane of the GI fluid composite (salivary + stomach + duodenal+ bile fluids + bicarbonate). The matrix matched standard solutions were on-line injected directly into the ICP OES nebulizer via one of the ports (no. 4) of the selection valve of the hyphenated flow system. In-line acidification of the GI extracts is deemed unfeasible as a result of the precipitation of the filtrated GI proteins, which in turn accounts for the use of Milli-Q water as ICP OES carrier instead of dilute HNO_3. The linear relationship of peak area (I) against analyte concentration (X in µg L^{-1}), *viz*., I =620X + 1081; (p=0.99), I=383 X + 1216; (p=0.99) and I =3451 X +5324; (p=0.95) for Cu, Fe, and Mn, respectively, over the range 20-200 µg L^{-1} for Cu and Mn, and 50-500 µg L^{-1} for Fe, using a matrix-matched calibration, was demonstrated at the 95 % confidence level. P-values of the lack-of-fit test of the regression models were in all instances ≥ 0.05. The LOD values of micronutrients in GI extracts, calculated based on the criterion of $3S_{blank}$ [36] of ten blank replicates of the GI fluid, and referred to the discrete volume of 300 µL of GI extract analyzed on-line in every fraction, were 0.011 µg g^{-1}, 0.057 µg g^{-1} and 0.008 µg g^{-1} for Cu, Fe and Mn, respectively.

Relative standard deviations of the overall dynamic bioaccessible micronutrient concentrations in the GI phase, evaluated from inter-day replicates of BGL, SGL and BL (n=3), ranged from 9 to 39% for Cu, 16 to 26 % for Fe and 8 to 24 % for Mn. Our dynamic flow-through system affords similar intermediate precision for GI bioaccessibility of Mn and better RSD for Fe as compared with a previous batchwise UBM-based GI protocol

in transgenic soya beans with RSDs up to 20% for Mn and within the range of 22 to 40% for Fe [37].

The lack of bias (trueness) of the flow-through GI extraction method for determination of chyme bioaccessibility of Fe, Cu and Mn in linseed was ascertained using mass balance validation for BGL, BL and SGL (n=3). The average of the sum of the GI bioaccessible concentrations plus the residual (non-extractable) fraction (X-axis) for the overall elements was statistically compared against the total metal concentration as obtained by microwave acid digestion (Y-axis) using a least-squares regression method [38]. The regression line was fitted to $Y = (1.1 \pm 0.2)\ X - (2.2 \pm 7.4)$ ($R^2 = 0.97$). The correlation between values was evaluated via statistical hypothesis tests for comparison of the slope and intercept with reference to the optimal scenario of slope of 1 and zero intercept. The statistics *t* of the intercept and slope were calculated, respectively, as follows: $t = (a - 0)/s_a$ and $t = (b - 1)/s_b$ where *a* and *b* stand for the intercept and slope values, respectively, and s_a and s_b for the standard deviation of the intercept and the slope, respectively. As the statistics *t* of the slope (viz., 0.46) and the intercept (viz.,0.34) were below than the critical *t*-value (2.36) for 7 degrees of freedom, no significant differences were encountered at the 0.05 significance level between the sum of bioaccessible plus residual fractions for the overall target elements and the total microwave concentrations. The reliability of the GI bioaccessible data as determined by the automatic dynamic method is thus demonstrated, which makes the use of the method of the standards addition unnecessary for the analysis of the GI and chyme extracts. In fact, relative recoveries (mass balance validation) for the three elements (see Table 2) spanned from 84 to 115%, 79 to 121% and 87 to 115% for SGL, BL and BGL, respectively.

Using the proposed automatic biomimetic extraction method with high temporal resolution, insight into the in-vitro chyme bioaccessibility of elements is obtained for the first time in this work. This is accomplished by pumping the GI composite extractant through the extraction column system containing the foodstuff surrounded by the gastric fluid (viz., chyme) to mimic the pH gradient across the GI tract (See Fig. 3). In fact, the chyme with a pH of approximately 2 is expelled by the stomach into the duodenum wherein by mixing with intestinal fluid undergoes a pH increase up to 6.8-7.0 [39]. Previously reported dynamic oral bioaccessibility procedures with on-line atomic spectrometry detection [19,20,23] do not properly mimic chyme conditions because gastric and intestinal fluids are used discretely, as is the case with sequential extraction procedures [40]. Batchwise physiologically based extraction procedures (e.g., UBM)

would neither allow for chyme bioaccessibility assessment because of thorough mixing of the food-laden gastric extract with duodenal and bile solutions prior to initiate the *in-vitro* GI digestion.

In our system, the time-course analysis of GI bioaccessible pools was readily undertaken using peak area integration of the transient ICP OES signals of the in-line GI extracts followed by the graphical representation of the bioaccessible pools against the cumulative GI volume (see Fig. S1). Similar trends were observed in the leaching patterns of Fe regardless of the linseed variety. The extraction of the micronutrient took place within the G phase (54-68% from total Fe, see Table 1), and the GI bioaccessibility dropped significantly to baseline within the first 5 or 6 fractions (0.65 mL each) of the GI composite fluid (see Fig. S1). Because the column void volume was estimated as 2.8 mL, the overall GI bioaccessibility of Fe ranging from 6-7% (see Table 2) is measured for chyme under extremely acidic conditions, which is attributed to the generation of low-solubility iron oxyhydroxides at near-neutral pH [29].

In contrast to Fe, the overall bioaccessibility of Mn (sum of fractions) changed to a lesser extent in the transit from the stomach into the duodenal extraction conditions (see Fig. S1) with a decrease from 68 % to 54% and 71% to 45% for BL and BGL, respectively, and remained invariable (56%) for SGL (see Tables 1 and 2). In fact, the formation of oxyhydroxides seems to be partially offset by the element leachability under GI conditions. Experimental results in Fig. S1 demonstrated that the leachability of Mn is steady throughout time after reaching intestinal pH conditions (> fraction 12). Notwithstanding the differences in linseed variety and origin, similar extractograms were encountered for Mn. Only the bioaccessible leaching profiles of Cu exhibit appreciable differences across samples. Whereas Cu was merely leached out in the course of chyme transit for SGL and BL with GI/chyme bioaccessibility of 21 and 18 %, respectively, the behavior of Cu in BGL was akin to Mn, with a marked bioaccessibility drop during the chyme transit followed by steady leaching in the duodenal phase, which in turn lead to a 2.5-fold GI bioaccessibility increase compared with that of the chyme phase. In our case, the most and least bioaccessible micronutrients in linseed obtained under dynamic flow-through GI extraction conditions were Mn and Fe, respectively, which is in good agreement with earlier biomimetic extraction tests in pumpkin sunflower seeds [41].

3.4. Comparison of dynamic chyme/GI bioaccessible pools of nutrients against conventional batchwise extraction counterparts

To evaluate the reliability of the analytical data provided by the Versantvoort's extraction method for evaluation of bioaccessible pools of micronutrients in foodstuff, the three linseed samples (BGL, BL, SGL) analyzed using either the conventional batchwise protocol or the proposed flow-through LBC-based method (see Experimental). The batchwise method, as is the case with the proposed dynamic procedure, was validated by mass balance assessment as applied to individual target micronutrients (see Table 3 and significance tests as SI). As can be seen in Fig. 4, the two extraction methods afforded distinctly contrasting GI bioaccessibility data as a result of the dissimilarity in the underlying fundamental principles of both approaches, as previously observed in dynamic extraction procedures of trace elements from solid waste incineration bottom ashes[42] and contaminated soils [43].

With the proposed dynamic method increased Mn bioaccessibility was observed for the overall linseed samples as compared with the batchwise counterpart, which is in agreement with a previous article evaluating gastric bioaccessibility of Pb from a contaminated soil [34]. Dynamic extraction is based on the continuous renewal of the extractant (herein oral fluids) with the subsequent displacement of the extraction equilibria, which in turn gives rise to meaningful insight into the maximum amount of bioaccessible micronutrient. On the contrary, bioaccessible elemental concentrations measured by batchwise protocols are limited by the solubility product constant of salts in the GI medium [34].

In contrast to Mn, the batchwise extraction method afforded larger amounts of bioaccessible Fe with 2.6, 3.1, and 3.3-fold GI bioaccessibility increase for the SGL, BL and BGL, respectively, in comparison to the dynamic method. The same behavior was observed for the GI bioaccessibility of Cu in BGL and BL, but in this case the increment was 4.6 and 1.4-fold, respectively. It should be borne in mind that phytic acid, which occurs in linseed at a concentration level of about 1% [44], forms preferably water-soluble complexes of monoferric phytate [45] and Cu-phytate [46] in the physiological intestinal environment (pH=6.8). Hereto, the batch method most likely overestimates the GI bioaccessibility and potential bioavailability of Cu and Fe because the larger extraction times against dynamic counterparts foster competing reactions of quelation of Cu and Fe with phytate against hydrolytic reactions under GI conditions. As a result, water-soluble Cu-phytate [46] and monoferric phytate [45] complexes are primarily generated, which in turn make nutrients bioaccessible, notwithstanding the fact that the metal quelates are known to hinder nutrient bioavailability.

4. CONCLUSIONS

In this paper, a dynamic flow-through extraction system featuring unique temporal resolution is herein proposed for the first time for mimicking the transit of the chyme from the gastric environment to the duodenum compartment. Elucidation by ICP OES of the bioaccessible concentrations in the gastrointestinal tract of Mn, Fe, and Cu from linseed was selected as a proof-of-concept applicability. Using the proposed flow assembly, the Versantvoort's fed-state method was fully automated in a dynamic format. In brief, the gastric extract was at-line mixed with fresh duodenal + bile fluids; the chyme pH was *in-situ* monitored throughout the kinetic method; and the GI bioaccessible fractions were continuously analyzed by smart hyphenation of the flow-through extraction system with ICP OES.

The experimental results led us to conclude that the Versantvoot's method in a batchwise format overestimates the potential GI bioavailability of Cu and Fe against dynamic methods because the larger extraction times used in the former trigger the formation of water-soluble complexes of Cu and Fe with phytate rather than non-soluble oxyhydroxides under GI conditions. Notwithstanding the fact that metal complexes are non-bioavailable, they are deemed bioaccessible by the batch protocol.

Further research is underway to expand the scope of the automatic dynamic flow-through system for the assessment of the chyme and GI bioaccessibility of organic emerging contaminants in foodstuff as a front end to LC-MS detection.

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Table 1. Comparison of the gastric bioaccessible concentrations of Fe, Cu and Mn as obtained by the flow-through dynamic physiologically based extraction system against conventional batchwise extraction mode

Gastric bioaccessibility (mg kg $^{-1}$)					
Samples	Element	Batch (Magnetic stirring)	Dynamic (0.5 mL min^{-1})	Recovery (%)	t_{exp} *
SGL	Cu	11.6 ± 0.3	10.1 ± 0.4	87	3.39
	Fe	28 ± 2	26 ± 2	93	1.58
	Mn	16 ± 2	15 ± 1	94	1.35
BL	Cu	12.3 ± 0.3	13 ± 1	108	1.53
	Fe	27 ± 1	30 ± 4	111	0.91
	Mn	15.5 ± 0.3	13 ± 1	84	3.42
BGL	Cu	10 ± 1	9 ± 1	90	0.99
	Fe	31.9 ± 0.4	32 ± 4	100	0.07
	Mn	24 ± 3	25 ± 2	104	0.54

Results are expressed as the mean of three replicates ± standard deviation.
* t_{crit}= 2.77 (d.f. =4)

Table 2.Chyme/GI bioaccessible concentrations of Mn, Cu and Fe from linseed samples and mass balance validation of the on-line physiologically based extraction system

Sample	Element	GI bioaccessibility (mg kg $^{-1}$)	Chyme bioaccessibility (mg kg $^{-1}$)	Residue (mg kg^{-1})[a]	Total (mg kg $^{-1}$)[b]	Total microwave digestion (mg kg $^{-1}$)	Recovery (%)
SGL	Cu	2.0 ± 0.8 (21%)	2.0 ± 0.8 (21%)	6 ± 1	8 ± 1	9.5 ± 0.3	84 ± 11
	Fe	3.0 ±0.5 (6%)	3.0 ±0.5 (6%)	51 ± 7	54 ± 7	48 ± 2	115 ± 15
	Mn	15 ± 1 (56%)	5.8 ±0.5 (21%)	10 ± 1	25.2 ± 0.3	27 ± 1	95 ± 1
BL	Cu	3± 1 (21%)	2.5 ± 1.0 (17%)	9 ± 3	11 ± 2	14.5 ± 0.1	79 ± 15
	Fe	3.0 ± 0.8 (7%)	3.0 ± 0.8 (7%)	47 ± 10	50 ± 10	44 ± 6	121 ± 24
	Mn	10 ± 1 (54%)	4 ± 1 (22%)	9 ± 1	19 ± 1	19 ± 1	103 ± 7
BGL	Cu	11.1 ± 0.9 (74%)	4.5 ± 0.5 (30%)	6 ± 2	17 ± 1	15 ± 1	115 ± 6
	Fe	4.0 ± 0.7 (7%)	4.0 ± 0.7 (7%)	55 ± 16	59 ± 16	58 ± 4	101± 28
	Mn	15 ± 4 (45%)	7 ± 3 (21%)	14 ± 4	28.6 ± 0.3	33.1 ± 0.8	86.6 ± 0.8

Results are expressed as the mean of three replicates ± standard deviation.
In parenthesis, the percentage of GI and chyme bioaccessibility is referred to the total amount of micronutrient.
[a] Concentration of non-bioaccessible micronutrient is determined by microwave acid digestion of the filtered linseed residue (see SI)
[b] Sum of GI bioaccessible pools and residual concentration

Table 3. Concentration of GI bioaccessible pools and mass balance validation of Cu, Fe and Mn in linseed samples using the batchwise Versantvoot's method

Sample	Element	GI bioaccessibility (mg kg $^{-1}$)	Residue (mg kg $^{-1}$)[a]	Total (mg kg $^{-1}$)[b]	Total microwave digestion (mg kg $^{-1}$)	Recovery (%)
SGL	Cu	5.2 ± 0.4 (58%)	5.1 ± 0.2	10.52 ± 0.06	9.5 ± 0.3	109.6 ± 0.6
	Fe	10.5 ± 0.3 (22%)	34.0 ± 0.5	45 ± 6	48 ± 2	93± 12
	Mn	9.9 ± 0.5 (37%)	11 ± 1	21 ± 1	27 ± 1	80 ± 4
BL	Cu	6.1 ± 0.1 (42%)	9.7 ± 0.8	15.8 ± 0.8	14.5 ± 0.1	112 ± 6
	Fe	9.4 ± 0.3 (52%)	21.4 ± 1	31 ± 3	44 ± 6	70 ± 3
	Mn	7.0 ± 0.4 (37%)	7.4 ± 0.5	14.4 ± 0.4	19 ± 1	76 ± 2
BGL	Cu	9.2 ± 0.7 (61%)	5.7 ± 0.5	14.9 ± 0.4	15 ± 1	99 ± 3
	Fe	9.9 ± 0.6 (17%)	52 ± 2	62 ± 2	58 ± 4	107 ± 4
	Mn	8.0 ± 0.4 (24%)	29 ± 8	36 ± 8	33.1 ± 0.8	110 ± 25

Results are expressed as the mean of three replicates ± standard deviation.
In parenthesis, the percentage of GI bioaccessibility is referred to the total amount of micronutrient.
[a] Concentration of non-bioaccessible micronutrient is determined by microwave acid digestion of the filtered linseed residue (see SI)
[b] Sum of GI bioaccessible pool and residual concentration

Figures captions

Fig. 1. Schematic illustration of the hyphenated flow system with on-line ICP OES analysis for automatic monitoring of the *in-vitro* GI extraction of Mn, Fe and Cu in golden and brown linseed. W: Waste, HC: Holding coil (5.3 mL), IV: Injection valve, IC: injection coil (300 µL), PP: Peristaltic pump, SV: Multi-position selection valve, C: Carrier: Milli-Q water, ICP OES: Inductively coupled plasma optical emission spectrometer. Large bore-column dimensions, height: 22 mm, diameter: 47 mm, and internal volume: 2.8 mL.

Fig 2. Comparison of different shaking modes for assessment of the gastric bioaccessibility of Cu, Fe and Mn in golden linseed from Brazil (n=3). Error bars stand for standard deviation.

Fig. 3. Illustration of the pH gradient across the surrogate gastrointestinal tract of *in-vitro* digests of Spanish Golden (SGL), Brown (BL) and Brazilian golden linseed (BGL) as obtained by flow-through dynamic physiologically based extraction tests

Fig. 4. Comparison of GI bioaccessible pools of Mn, Cu and Fe as obtained by the dynamic flow-through method against the conventional batchwise Versantvoort's method for Spanish Golden (SGL), Brown (BL) and Brazilian golden linseed (BGL). Error bars indicate standard deviation.

Fig. 1

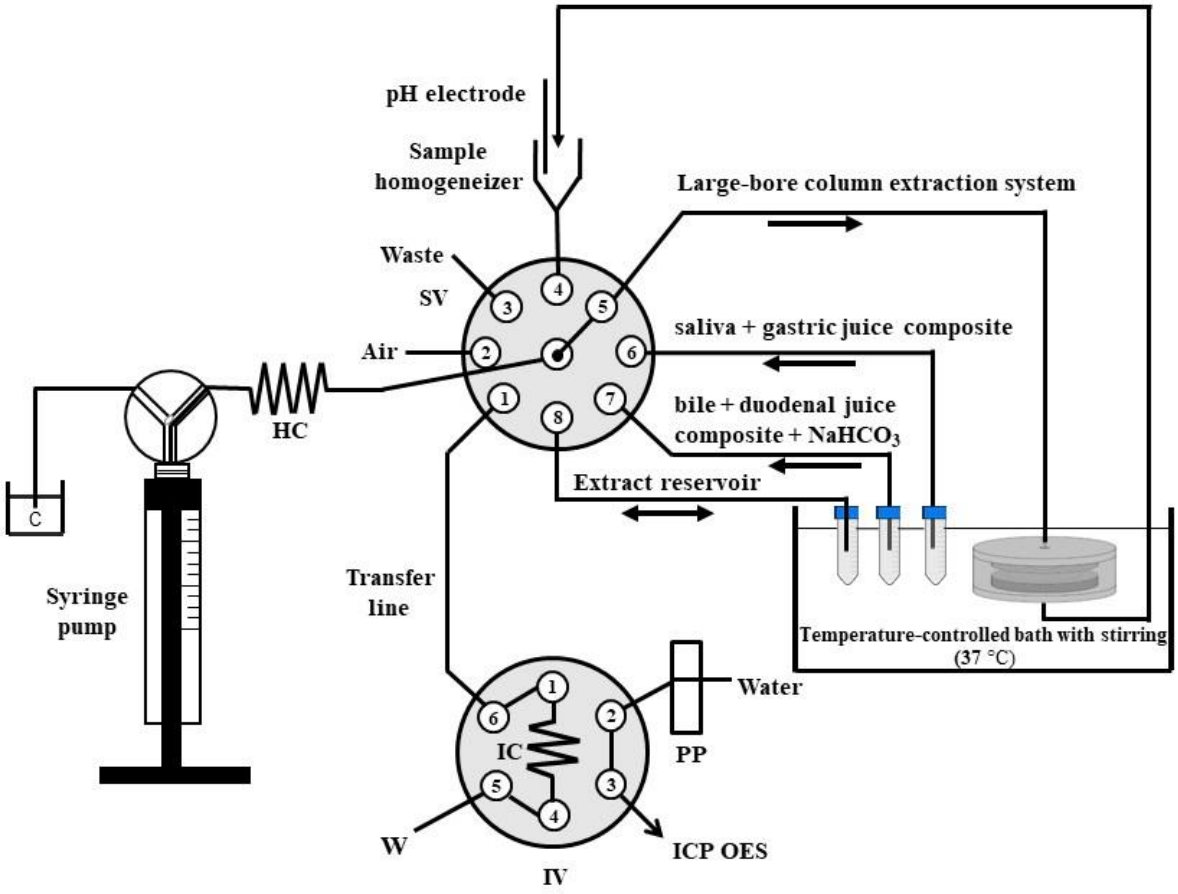

pH electrode
Sample homogeneizer
Large-bore column extraction system
Waste
SV
Air
saliva + gastric juice composite
HC
bile + duodenal juice composite + NaHCO3
Extract reservoir
C
Syringe pump
Transfer line
Temperature-controlled bath with stirring (37 °C)
Water
IC
PP
W
ICP OES
IV

Fig. 2

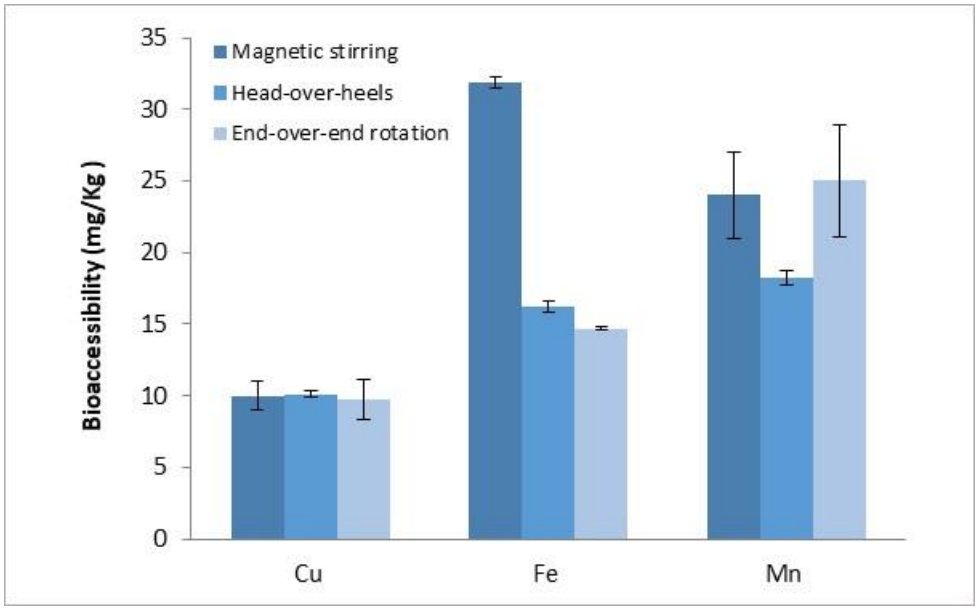

Bioaccessibility (mg/Kg)
35
30
25
20
15
10
5
0
Magnetic stirring
Head-over-heels
End-over-end rotation
Cu
Fe
Mn

Fig. 3

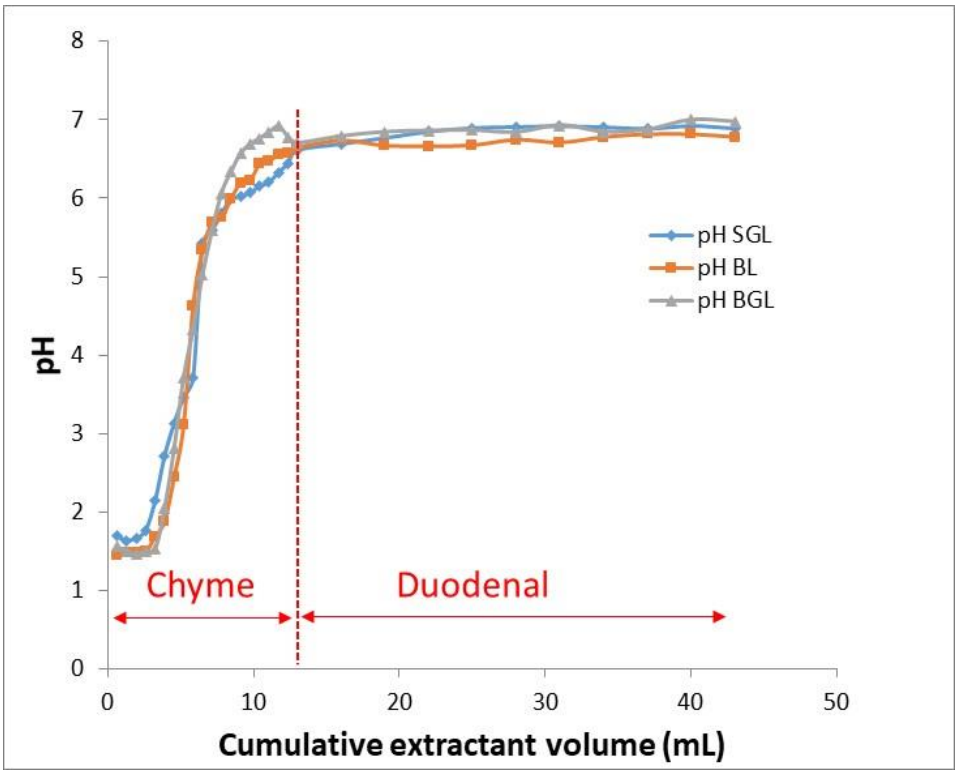
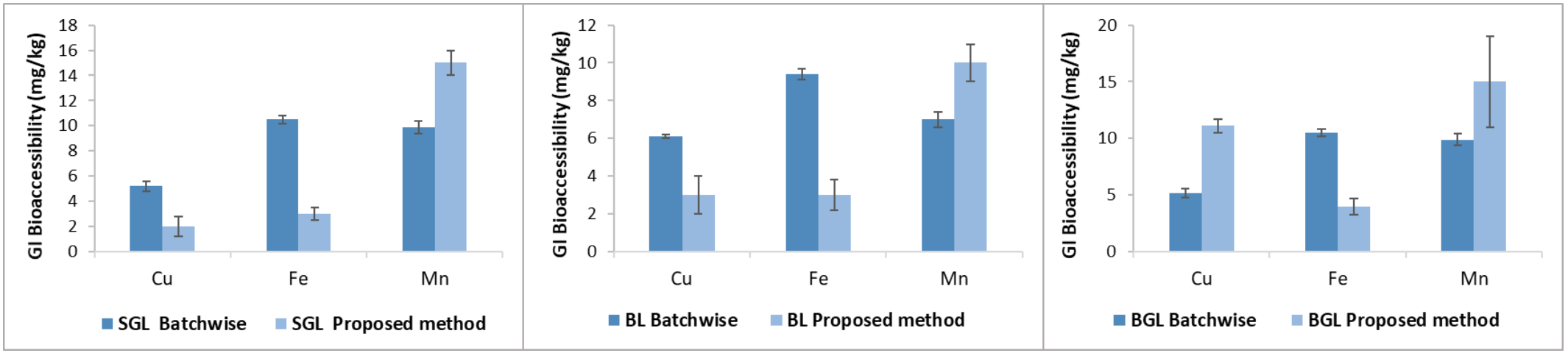

8
7
6
5
4
3
2
1
0
pH
0
10
20
30
40
50
Cumulative extractant volume (mL)
pH SGL
pH BL
pH BGL
Chyme
Duodenal

Fig. 4.


GI Bioaccessibility (mg/kg)
18
16
14
12
10
8
6
4
2
0
Cu
Fe
Mn
SGL Batchwise
SGL Proposed method
GI Bioaccessibility (mg/kg)
12
10
8
6
4
2
0
Cu
Fe
Mn
BL Batchwise
BL Proposed method
GI Bioaccessibility (mg/kg)
20
15
10
5
0
Cu
Fe
Mn
BGL Batchwise
BGL Proposed method

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