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Analytical Methods

Development of a reproducible method of analysis of iron, zinc and phosphorus in vegetables digests by SEC-ICP-MS

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ABSTRACT

Vegetables contain iron, zinc and phosphorus as complexes with phytates limiting their availability from a vegetarian diet, meaning non-haem iron deficiency anaemia and zinc deficiency immune malfunction are a risk. Although these elements have been analysed previously in biological fluids and cereal using LC-ICP-MS, there is no method suitable for analysing iron, zinc and phosphorus simultaneously in vegetables because of their complex matrix. In this study, we analysed iron, zinc and phosphorus in cabbage, broccoli, pepper, spinach, kale and rocket after a simulated gastrointestinal digestion using a newly optimised SEC-ICP-MS method. Ammonium nitrate, as the mobile phase, and a suitable rinsing regime, allowed good reproducibility and maintenance of the equipment. The method showed good reproducibility and can be easily adapted to other vegetables, as required.

1. Introduction

Vegetarian diets have become more common in developed countries where people are advised to eat more vegetables to reduce the incidence of non-communicable diseases including obesity (Turner-McGrievy, Mandes, & Crimarco, 2017), diabetes (Pawlak, 2017) and cardiovascular diseases (Keung & Owen, 2004) as well as environmental concerns associated with meat production. Although the beneficial effects of a vegetarian diet are well recognised, there is a risk of non-haem iron deficiency anaemia (Mawani, Ali, Bano, & Ali, 2016; Cairo, Silva, Bustani, & Marques, 2014). Iron deficiency leads to inflammation (Nunes & Tátá, 2017), impaired immune (Hassan et al., 2016) and endocrine (Maldonado-Araque et al., 2018) functions. Zinc is also important for optimal immune function as well as a normal pregnancy and child growth (Wessells & Brown, 2012), and deficiency is associated with diarrhoea and respiratory infections in children (Bailey, 2015). Iron and zinc are strongly bound to phytate (myo-inositol phosphates) (Abdel-Gawad, 2016; Veiga et al., 2015), as well as proteins, peptides and other organic compounds, making them less bioavailable.

Several methods have been used for the analysis of iron, zinc and phosphorus in food. Liquid chromatography-inductively coupled plasma mass spectrometry (LC-ICP-MS) (Kretschy, Koellensperger, & Hann, 2012; Bettmer, Jakubowski, & Prange, 2006), liquid chromatography-mass spectrometry (LC-MS) (Lane, 2005) and inductively coupled plasma mass spectrometry (ICP-MS) (Ornatsky et al., 2008; Careri, Elviri, Mangia, & Mucchino, 2007) have been used widely to determine speciation of metals in antibodies, biomolecules and proteins (Zhang, Zhang, Xing, & Zhang, 2004; Bettmer et al., 2006; Hann, Koellensperger, Obinger, Furtmüller, & Stingeder, 2004; Lothian & Roberts, 2016), and reversed phase liquid chromatography coupled with tandem mass spectrometry (RPLC-MS-MS) (Van de Meent & de Jong, 2011) and size-exclusion chromatography inductively coupled plasma mass spectrometry (SEC-ICP-MS) (Fingerová & Koplík, 1999) have been used for legumes.

The most widely used mobile phases for SEC of cereals, legumes and proteins are: Tris-HCl buffer (Koplík, Komínková, et al., 2004; Koplík, Mestek, Komínková, Borková, & Suchánek, 2004; Persson, Hansen, Laursen, Schjoerring, & Husted, 2009), Tris-HNO3 (Fingerová & Koplík, 1999) and ammonium acetate (Persson et al., 2006; Hann et al., 2004) with different regimes being used to wash the columns between runs and batches.

Separation on a cross-linked agarose and dextran column has been reported using an automatic injection program with five repetitive 20 mL injections of a 5 mM EDTA–50 mM ammonium acetate solution (pH = 7.5) with a 3 mins delay between each injection (Persson et al., 2006). Separation on the same column, by injecting an aliquot of 0.002 M EDTA solution and washing with the mobile phase (0.02 M Tris–HCl pH = 7.5) for 1 h between two consecutive replicates, has also been reported (Koplík, Komínková, et al., 2004). Methods using this type of column report injecting 100 μL aliquots of 20 mM EDTA/50 mM Tris-HCl buffer (pH 7.5) between sample analyses and a mixture of

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Table 1
Reproducibility of the retention time, concentration and molecular size of iron-bound compounds in different vegetables; (average of seven samples each). ± standard error. n.d. = not detected.

	Retention time average (min)							%CV						
	Peak 1	peak 2	peak 3	peak 4	Peak 5	Peak 6	Peak 7	Peak 1	peak 2	peak 3	peak 4	Peak 5	Peak 6	Peak 7
Cabbage	n.d.	n.d.	n.d.	n.d.	28.5 ± 0.0	n.d.	32.3 ± 0.1	n.d.	n.d.	n.d.	n.d.	0.1	n.d.	0.6
Broccoli	n.d.	n.d.	n.d.	28.0 ± 0.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.1	n.d.	n.d.	n.d.
Spinach	n.d.	n.d.	n.d.	n.d.	28.5 ± 0.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.1	n.d.	n.d.
Kale	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	30.8 ± 0.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.1
Pepper	n.d.	13.1 ± 0.0	n.d.	n.d.	28.4 ± 0.0	n.d.	30.6 ± 0.1	n.d.	0.4	n.d.	n.d.	0.1	n.d.	0.5
Rocket	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	30.7 ± 0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.6
	Concentration average (μg/L)													
Cabbage	n.d.	n.d.	n.d.	n.d.	523 ± 4.3	n.d.	2145 ± 52.5	n.d.	n.d.	n.d.	n.d.	4.1	n.d.	12.1
Broccoli	n.d.	n.d.	n.d.	456 ± 22.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.3	n.d.	n.d.	n.d.
Spinach	n.d.	n.d.	n.d.	n.d.	326 ± 22.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	11.2	n.d.	n.d.
Kale	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	716 ± 0.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.6
Pepper	n.d.	5913 ± 6.5	n.d.	n.d.	12889 ± 2.6	n.d.	103299 ± 94.8	n.d.	0.5	n.d.	n.d.	0.1	n.d.	0.2
Rocket	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	248 ± 14.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.6
	Molecular weight average (Da)													
Cabbage	n.d.	n.d.	n.d.	n.d.	257 ± 6.5	n.d.	53.4 ± 3.0	n.d.	n.d.	n.d.	n.d.	4.3	n.d.	4.6
Broccoli	n.d.	n.d.	n.d.	344 ± 4.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.0	n.d.	n.d.	n.d.
Spinach	n.d.	n.d.	n.d.	n.d.	257 ± 2.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	7.1	n.d.	n.d.
Kale	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	52.6 ± 1.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.5
Pepper	n.d.	148790 ± 152	n.d.	n.d.	268 ± 7.6	n.d.	53.4 ± 3.6	n.d.	1.0	n.d.	n.d.	5.3	n.d.	5.2
Rocket	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	53.0 ± 1.4	n.d.	n.d.	0.0	n.d.	n.d.	n.d.	3.8

pepsin (1 mg mL^{-1}), phytase (2 mg mL^{-1}), NaCl (0.5 M), and acetic acid (10%) between batches of samples. In addition, 10 mM EDTA/50 mM Tris-HCl buffer (pH 7.5) was run through the column for 40 min each day to remove any residual metals retained by the column (Eagling et al., 2014; Xue et al., 2014). Although SEC-ICP-MS has been used successfully for speciation in cereal and legumes, as mentioned above, relatively few studies have focused on establishing conditions for the analysis of metals in vegetables because of their complex matrix. Using SEC-ICP-MS, information about molecular weight of the fractions, type of metal, and concentrations can be achieved in a short time. This leads to the hypothesis that an optimised method would provide reproducible analyses of complex vegetable systems while maintaining good performance of the analytical instrumentation. Thus, the aim of this study was to develop a robust method for the determination of iron, zinc and phosphorus concentrations in vegetables extracts and the molecular weight of their fractions, which also allow good maintenance of the column and ICP-MS equipment.

2. Experimental

2.1. Material

Tri-glycine, vitamin B_{12}, cytochrome, apoferritin, blue dextran, ammonium hydroxide, pepsin, bile, pancreatin and ammonium nitrate were purchased from Sigma-Aldrich (Poole, Dorset, UK). Vegetable digests from savoy cabbage, broccoli, spinach, kale, green pepper and rocket were provided by the Norwich Medical School, University of East Anglia, Norwich Research Park, Norwich, UK. The stock ICP standard solution was purchased from Fisher Scientific (Loughborough, Leicestershire, UK).

2.2. Sample preparation

Vegetables were purchased from a local supermarket, boiled (except for pepper which was used raw), frozen, freeze-dried, and subjected to in vitro gastrointestinal digestion in a solution of 140 mM NaCl and 5 mM KCl at pH 2.0 in the presence of pepsin (0.04 g/mL) and incubated for 90 min on a rolling table at 37 °C. Ascorbic acid (AA) was added to the digestion (10:1 AA:Fe), as described by Moore et al. (2018). Bile (0.007 g/mL) and pancreatin (0.001 g/mL) were added, the pH adjusted to pH 7 and the samples incubated for 60 min at 37 °C as described by Rodriguez-Ramiro et al. (in press). Each vegetable digest was then diluted (1:10) with NH_4NO_3 buffer (200 mM pH 7.6) and passed through a 0.2 μm Millipore filter.

2.3. Instruments and procedure

Samples were analysed using a HPLC (PerkinElmer LC 200 Series HS, Seer Green, UK) composed of an injector, a Flexar UV/VIS detector operating at 280 nm and a high-pressure peristaltic pump equipped of PEEK tubing (0.17 mm id) and operating at a flow rate of 0.6 mL min^{-1} in isocratic mode. Samples (100 μL) were separated, at room temperature, by molecular size on a Superdex Peptide 10/300 GL column (10 × 300 mm, GE Healthcare Bio-Sciences, Sweden) with an optimum separation range between 100 and 7000 Da. The column was mass calibrated with tri-glycine (0.189 kDa), vitamin B_{12} (1.35 kDa), cytochrome (12.4 kDa), apoferritin (443 kDa) and blue dextran (2000 kDa) dissolved in an appropriate amount of mobile phase and filtered.

The mobile phase (NH_4NO_3 200 mM, pH 7.6) was prepared daily by dissolving 16 g of NH_4NO_3 in 1 L of Purite ultrapure water and the pH adjusted with ammonium hydroxide. The solution was degassed by vacuum filtration using 0.2 μm filters (Millipore, Watford, UK). Samples were analysed using an ICP-MS (PerkinElmer NexION300XX) equipped with a glass Meinhard nebulizer, a quadrupole mass spectrometer and a collision cell. The ICP-MS settings were: gas flow 1 L/min; auxiliary gas flow 1.2 L/min; plasma flow 18 L/min; RF power 1600 Watts; cell gas flow He 3.9 mL/min. Detection and quantification of elements were achieved using Chromera (PerkinElmer v. 4.1.0). The digest solution only and the same digest solution containing $FeSO_4$ were used as a blank and a control, respectively. External calibration for quantification was performed by injecting the elemental standards (Fe, Zn and P), dissolved in a solution of 2% HNO_3, into the ICP-MS via HPLC but without the column, which were detected as Fe^{56}, Zn^{64} and P^{31} isotopes. After five runs, a series of five repetitive 20 μL aliquots of water was injected over 20 min, without delay between injections, followed by one 100 μL aliquot of NH_4NO_3 (200 mM at pH 7.6) to re-equilibrate the column. The same series of five repetitive 20 μL aliquots of water was injected at the end of the batch, but this time using water as the mobile phase. The method was validated on both LC/ICP-MS systems.

Table 2
Reproducibility of the retention time, concentration and molecular size of phosphorus-bound compounds in different vegetables; (average of seven samples each. ± standard error. n.d. = not detected.

	Retention time average (min)							%CV						
	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6	Peak 7	Peak 1	peak 2	peak 3	peak 4	Peak 5	Peak 6	Peak 7
Cabbage	n.d.	n.d.	27.3 ± 0.0	n.d.	n.d.	29.7 ± 0.0	32.3 ± 0.0	n.d.	n.d.	0.1	n.d.	n.d.	0.0	0.0
Broccoli	12.0 ± 0.0	13.0 ± 0.0	27.4 ± 0.0	n.d.	n.d.	29.4 ± 0.0	32.3 ± 0.0	0.2	0.3	0.0	n.d.	n.d.	0.0	0.1
Spinach	12.2 ± 0.0	13.0 ± 0.0	27.3 ± 0.0	n.d.	n.d.	29.6 ± 0.2	32.3 ± 0.0	0.1	0.2	0.2	n.d.	n.d.	1.8	0.1
Kale	n.d.	n.d.	27.3 ± 0.0	n.d.	n.d.	29.4 ± 0.3	32.2 ± 0.0	n.d.	n.d.	0.1	n.d.	n.d.	2.6	0.1
Pepper	n.d.	n.d.	27.2 ± 0.0	n.d.	n.d.	29.3 ± 0.3	32.2 ± 0.0	n.d.	n.d.	0.1	n.d.	n.d.	2.6	0.0
Rocket	n.d.	n.d.	27.4 ± 0.0	n.d.	n.d.	n.d.	32.2 ± 0.0	n.d.	n.d.	0.1	n.d.	n.d.	1.9	0.0
	Concentration average (μg/L)													
Cabbage	n.d.	n.d.	8331 ± 45.1	n.d.	n.d.	57943 ± 564.0	128048 ± 712.3	n.d.	n.d.	1.3	n.d.	n.d.	2.8	1.6
Broccoli	5285 ± 24.3	8904 ± 103	12035 ± 90.6	n.d.	n.d.	36579 ± 204.0	118262 ± 52.7	0.7	3.2	2.1	n.d.	n.d.	1.6	0.1
Spinach	3810 ± 0.0	6756 ± 0.0	10957 ± 4.0	n.d.	n.d.	22298 ± 29.9	184145 ± 224.3	0.0	0.0	0.1	n.d.	n.d.	0.4	0.3
Kale	n.d.	n.d.	13430 ± 210.4	n.d.	n.d.	12945 ± 35.4	71762 ± 105.6	n.d.	n.d.	4.5	n.d.	n.d.	0.7	0.4
Pepper	n.d.	n.d.	5643 ± 74.2	n.d.	n.d.	1214589.7	114221 ± 723.2	n.d.	n.d.	3.4	n.d.	n.d.	1.9	1.6
Rocket	n.d.	n.d.	5136 ± 0.4	n.d.	n.d.	n.d.	47736 ± 539.4	n.d.	n.d.	0.2	n.d.	n.d.	0.0	3.3
	Molecular weight average (Da)													
Cabbage	n.d.	n.d.	420 ± 1.0	n.d.	n.d.	157 ± 0.9	53.4 ± 0.6	n.d.	n.d.	0.4	n.d.	n.d.	0.7	0.9
Broccoli	238739 ± 14.1	157793 ± 90.9	406 ± 4.9	n.d.	n.d.	177 ± 2.4	53.4 ± 0.8	0.0	0.4	1.8	n.d.	n.d.	1.7	1.4
Spinach	219523 ± 0.0	157445 ± 0.0	414 ± 0.7	n.d.	n.d.	159 ± 0.6	53.4 ± 0.1	0.0	0.0	0.2	n.d.	n.d.	0.4	0.2
Kale	n.d.	n.d.	397 ± 1.4	n.d.	n.d.	169 ± 0.4	53.4 ± 0.2	n.d.	n.d.	0.5	n.d.	n.d.	0.3	0.3
Pepper	n.d.	n.d.	419 ± 22.0	n.d.	n.d.	173 ± 2.1	53.4 ± 0.2	n.d.	n.d.	9.5	n.d.	n.d.	1.4	0.3
Rocket	n.d.	n.d.	388 ± 4.9	n.d.	n.d.	n.d.	53.0 ± 0.7	n.d.	n.d.	1.7	n.d.	n.d.	n.d.	1.0

n.d. = not detected.

Table 3
Reproducibility of the retention time, concentration and molecular size of zinc-bound compounds in different vegetables; (average of seven samples each). ± standard error. n.d. = not detected.

	Retention time average (min)							%CV						
	Peak 1	peak 2	peak 3	peak 4	Peak 5	Peak 6	Peak 7	Peak 1	peak 2	peak 3	peak 4	Peak 5	Peak 6	Peak 7
Cabbage	n.d.	n.d.	n.d.	28.1 ± 0.0	n.d.	n.d.	32.7 ± 0.0	n.d.	n.d.	n.d.	0.1	n.d.	n.d.	0.1
Broccoli	n.d.	n.d.	n.d.	28.0 ± 0.0	n.d.	n.d.	32.6 ± 0.0	n.d.	n.d.	n.d.	0.0	n.d.	n.d.	0.2
Spinach	n.d.	n.d.	n.d.	28.0 ± 0.0	n.d.	n.d.	32.9 ± 0.0	n.d.	n.d.	n.d.	0.2	n.d.	n.d.	0.3
Kale	n.d.	n.d.	n.d.	27.6 ± 0.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.2	n.d.	n.d.	n.d.
Pepper	n.d.	n.d.	n.d.	27.5 ± 0.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.0	n.d.	n.d.	n.d.
Rocket	n.d.	n.d.	n.d.	27.5 ± 0.0	n.d.	n.d.	32.2 ± 0.1	n.d.	n.d.	n.d.	0.4	n.d.	n.d.	0.9
	Concentration average (μg/L)													
cabbage	n.d.	n.d.	n.d.	1232 ± 25.4	n.d.	n.d.	111 ± 5.4	n.d.	n.d.	n.d.	5.5	n.d.	n.d.	8.0
Broccoli	n.d.	n.d.	n.d.	1565 ± 27.9	n.d.	n.d.	69 ± 2.9	n.d.	n.d.	n.d.	4.7	n.d.	n.d.	11.1
Spinach	n.d.	n.d.	n.d.	568 ± 6.5	n.d.	n.d.	87 ± 2.3	n.d.	n.d.	n.d.	2.0	n.d.	n.d.	3.7
Kale	n.d.	n.d.	n.d.	593 ± 24.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	8.9	n.d.	n.d.	n.d.
Pepper	n.d.	n.d.	n.d.	1212 ± 30.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.8	n.d.	n.d.	n.d.
Rocket	n.d.	n.d.	n.d.	1153 ± 0.1	n.d.	n.d.	23 ± 0.0	n.d.	n.d.	n.d.	0.0	n.d.	n.d.	0.9
	Molecular weight average (Da)													
Cabbage	n.d.	n.d.	n.d.	340 ± 0.8	n.d.	n.d.	53.4 ± 0.2	n.d.	n.d.	n.d.	0.4	n.d.	n.d.	0.4
Broccoli	n.d.	n.d.	n.d.	344 ± 1.7	n.d.	n.d.	53.4 ± 0.5	n.d.	n.d.	n.d.	0.8	n.d.	n.d.	1.0
Spinach	n.d.	n.d.	n.d.	397 ± 0.4	n.d.	n.d.	53.4 ± 0.6	n.d.	n.d.	n.d.	0.2	n.d.	n.d.	1.2
Kale	n.d.	n.d.	n.d.	347 ± 7.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.3	n.d.	n.d.	n.d.
Pepper	n.d.	n.d.	n.d.	388 ± 5.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.2	n.d.	n.d.	n.d.
Rocket	n.d.	n.d.	n.d.	366 ± 1.6	n.d.	n.d.	53.0 ± 1.7	n.d.	n.d.	n.d.	0.7	n.d.	n.d.	3.7

n.d. = not detected.

Table 4
Molecular weight distribution determined by the polydispersity index (Mw/Mn)*.

	Iron-bound fractions						
	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6	Peak 7
Cabbage	–	–	–	–	1.0014	–	1.0015
Broccoli	–	–	–	1.0001	–	–	–
Spinach	–	–	–	–	–	1.0024	–
Kale	–	–	–	–	–	–	1.0004
Pepper	–	1.0001	–	–	–	1.0019	1.0019
Rocket	–	–	–	–	–	–	1.0007
	Phosphorus-bound fractions						
Cabbage	–	–	1.0000	–	–	1.0001	1.0002
Broccoli	1.0000	1.0000	1.0005	–	–	1.0001	1.0006
Spinach	1.0000	1.0000	1.0000	–	–	1.0000	1.0000
Kale	–	–	1.0001	–	–	1.0000	1.0000
Pepper	–	–	1.0185	–	–	1.0004	1.0347
Rocket	–	–	1.0336	–	–	1.0000	1.0116
	Zinc-bound fractions						
Cabbage	–	–	–	1.0000	–	–	1.0000
Broccoli	–	–	–	1.0000	–	–	1.0001
Spinach	–	–	–	1.0000	–	–	1.0001
Kale	–	–	–	1.0006	–	–	
Pepper	–	–	–	1.0003	–	–	
Rocket	–	–	–	1.0000	–	–	1.0006

* Mn = Number Average Molecular Weight, Mw = Weight Average Molecular Weight.

2.4. Data analysis

Concentrations of elements in vegetable digests were determined using the area ratio between analyte and external standard. The area ratio was compared to a calibration line consisting of six concentrations of Fe, Zn and P standards (0, 0.050, 0.5, 5, 10 and 100 mg L^{-1}). A linear weighting factor was applied. Data are presented as the average of seven intra- and inter-day analyses. The limit of detection was 0.3 μg/L. Variation was determined as a range between maximum and minimum averages of seven values, CV, standard error, and the 95% confidence interval.

Data analysis was performed using one-way analysis of variance (ANOVA) using GenStat, 18th ed., (VSN International, Hemel Hempstead, UK). Molecular sizes of the fractions were determined using a log – linear regression curve derived from a plot of molecular size versus coefficient of distribution (K_d) for the standards according to the equation: $y = -0.2824x + 1.5995$. Because of the varied matrices, recovery was determined as the area ratio of elution buffer and neat standards.

3. Results and discussion

The complex matrix of vegetables tissues, even after enzymatic digestion, can make analysis challenging. Apart from the general requirements on robustness, precision, accuracy and reproducibility, an analytical method must be able to cope with a wide range of different matrices. Also, determination of recoveries and matrix effects are of limited value and can vary significantly. In theory, recoveries might be between 94.6% and 100.6%, but this can vary with real samples due to binding of matrix material. For examples, do Nascimento Silva et al. (2015) described recoveries ranging between 70% and 102.9%.

3.1. Precision and reproducibility

Precision and reproducibility for intra- and inter-batch were confirmed, for our method in all the vegetable, by the coefficients of variation (CVs). CVs for retention times, concentrations and molecular sizes, for iron fractions, were between 0.1 and 0.6%, between 0.2 and 12.1% and between 0 and 7.1% respectively (Table 1); between 0 and 2.6%, between 0 and 3.4% and between 0 and 9.5% respectively, for phosphorus fractions (Table 2); between 0 and 0.9%, between 0 and 11.1% and between 0.2% and 3.7% respectively, for zinc fractions (Table 3). Among the fractions of the six vegetables, elution times, concentrations and molecular sizes were significantly different ($p < 0.001$).

3.2. Molecular weight distribution

As the identities of the components were unknown, and each fraction was likely to contain a mixture of molecules with different molecular weights, a more precise approach was undertaken calculating the

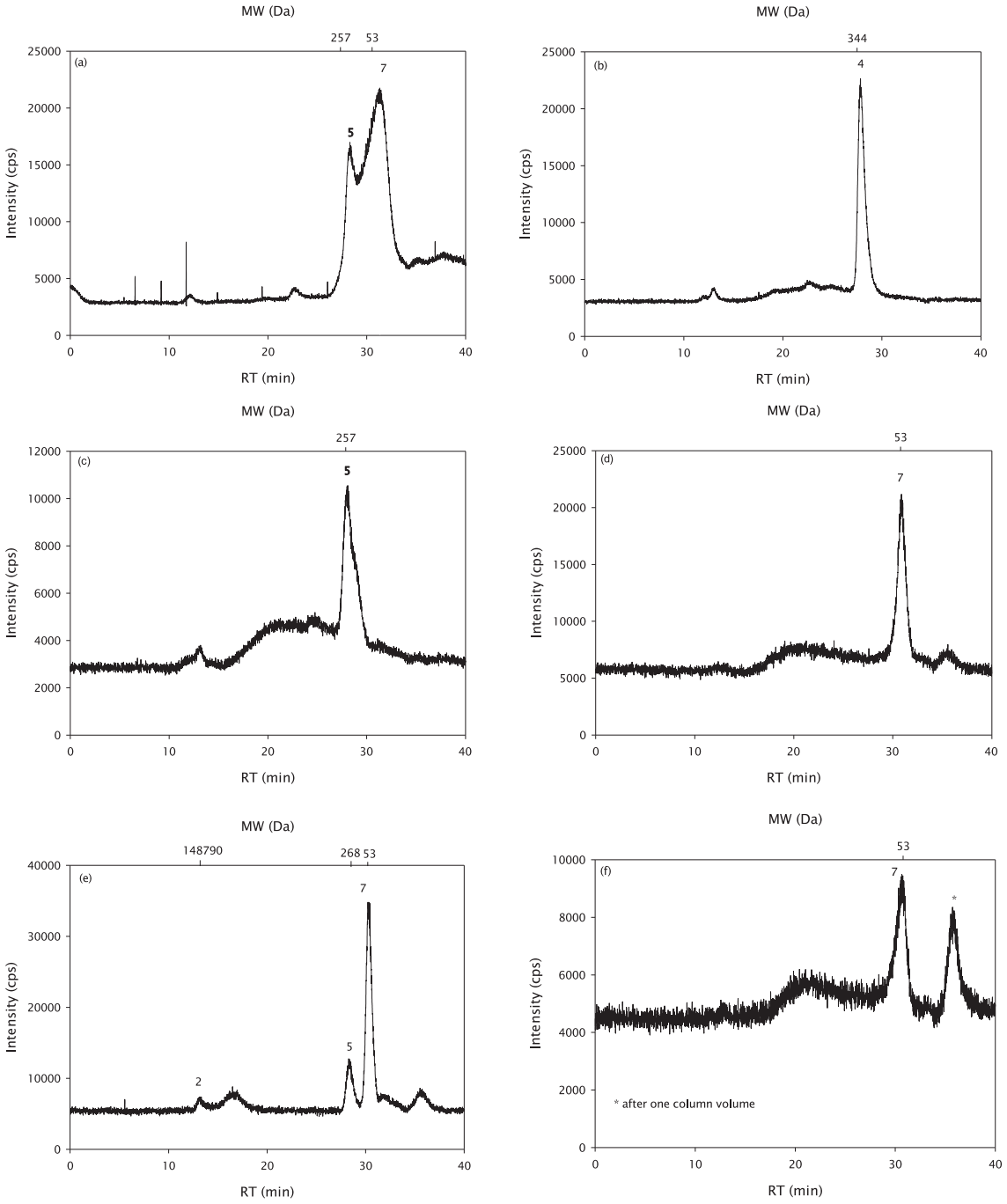

Fig. 1. Fe^{56} in (a) cabbage, (b) broccoli, (c) spinach, (d) kale, (e) pepper and (f) rocket extracts detected by SEC-ICP-MS.

molecular weight distributions in each fraction for each vegetable sample (Table 4). Molecular weight distributions were determined using the polydispersity index (Mw/Mn), where Mw is the number average molecular weight and Mn is the weight average molecular weight. The polydispersity index is used to determine whether the molecular weight distributions are narrow (Mw/Mn = 1.0) or broad (Mw/Mn > 1). The results showed an Mw/Mn = 1.0 (i.e. Mw distributions were narrow) for each iron, phosphorus and zinc in all the vegetables (Table 4).

3.3. Accuracy

Determination of accuracy is more difficult, because it is impossible to obtain a sample with a known amount of these elements due to a large variation of minerals content. Comparison with published data is not appropriate because contents of minerals in vegetables depend on different factors including environment, geographic area, fertilizers, harvesting time and so on (Marles, 2017).

Phosphorus is the most abundant element in these types of samples, and it formed low molecular weight fractions (peak 7) with iron in all the samples (except in spinach and broccoli) and with zinc (except in kale and pepper) (Tables 1–3). However, phosphorus was not present in peaks 4 and 5 in which the Fe and Zn are presumably complexed with other components. Phosphorus was present in high molecular weight iron- and zinc-free compounds (peaks 1 and 2) in broccoli and spinach while in pepper peak 2 contained iron but not other minerals. Concentrations of iron were higher in low molecular weight fractions while concentrations of zinc were higher in medium molecular weight

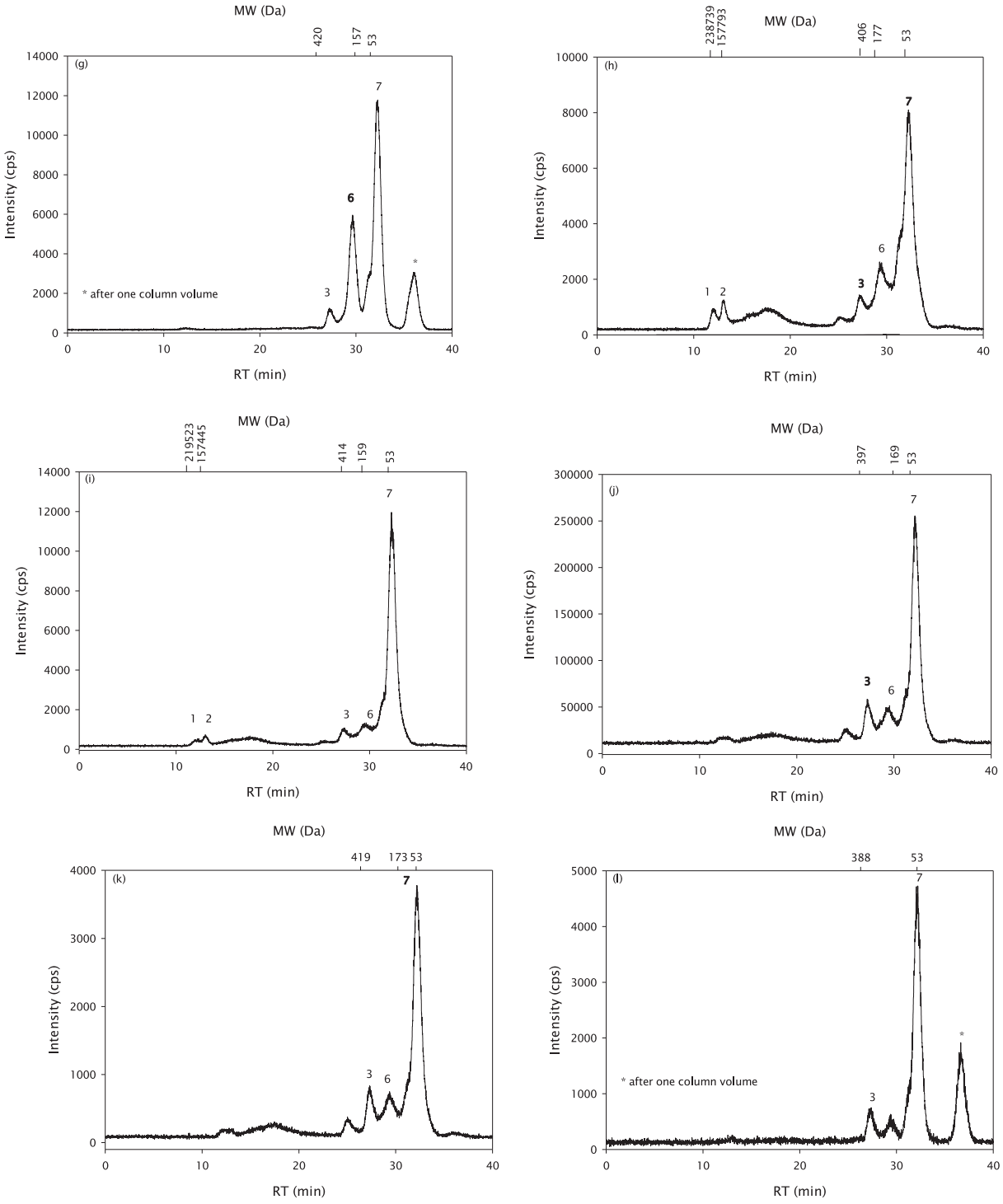

Fig. 2. P^{31} in (g) cabbage; (h) broccoli; (i) spinach; (j) kale; (k) pepper; (l) rocket extracts detected by SEC-ICP-MS.

fractions.

Initially, we used Tris/HCl in the mobile phase, because it is good buffer for cereal samples (Eagling et al., 2014; Xue et al., 2014), but it did not give the same performance for vegetable samples. Ammonium nitrate was chosen instead, because the background noise was more stable and elution times more reproducible. The rinsing procedure devised in this experiment gave excellent reproducibility of retention times, concentrations, and molecular sizes of the fractions (Figs. 1–3) as well as offering a good cleaning regime for the HPLC column and ICP equipment. These results were not achieved using Tris-HCl buffer (50 mM, pH 7.5), due to the instability of retention times.

Originally, the rinsing method was modified from one used for speciation in barley (Persson et al., 2009, 2006). However, because the method has been modified specifically for the analysis of vegetables, it would need to be re-evaluated for other types of matrix.

During analysis, the ICP cone and injector easily became dirty, depending on the sample injected and eluent used, and/or precipitate formed. The tubing, nebulizer, torch, and even the lens can also become dirty and blockages occur. Depending on the type of instrument used, dismantling, cleaning, and re-assembling can be very time consuming. However, using this method, we were able to achieve robust analytical performance and good maintenance of the instruments.

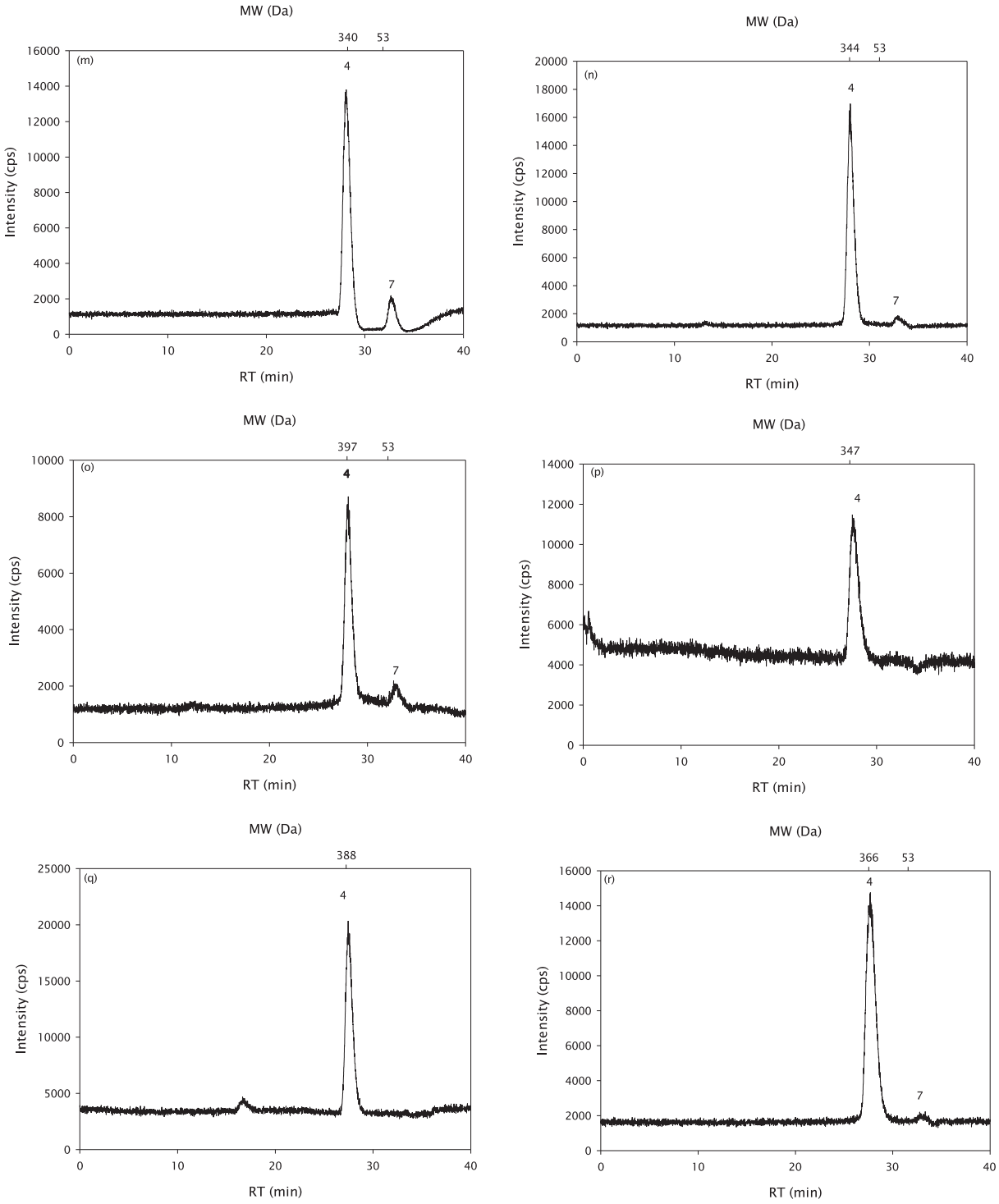

Fig. 3. Zn^{64} in (m) cabbage; (n) broccoli; (o) spinach; (p) kale; (q) pepper; (r) rocket detected by SEC-ICP-MS.

4. Conclusion

Here, we described a method of analysis to determine iron, zinc, and phosphorus in different vegetable digests. This method showed good precision and reproducibility, and allowed analysis of these elements in different vegetable matrices. Previous studies relied on Tris/HCl as the mobile phase and long rinsing methods were used, mainly in the analysis of metals in cereal. These methods are time consuming and not suitable for vegetables, due to instability of the analytical system and varied matrices of vegetables. The approach described here is the first generic method used for iron, zinc and phosphorus in vegetable digests, and can be used in studies where the determination of small amounts of elements in vegetables is required for nutritional purposes. Finally, this method contributed to good maintenance of the instruments, with minimal time spent cleaning.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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