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Elemental bioavailability in whey protein supplements

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ABSTRACT

Whey protein (WP) as a dietary supplement for athletes and gym-users is characterized by a content of high-quality amino acids. The benefits of their consumption are well-known and mainly related to the improvements of strength and body composition. Nevertheless, there is a scarce information about the risk associated to their elemental composition. The aim was to evaluate the total content and bioavailability of twenty-five elements in twenty whey protein powder samples, using Inductively Coupled Plasma Mass Spectrometry, and to evaluate the possible risk associated to the excessive daily intake of such products. Results showed that Na, K, Ca and Mg were the most predominant elements. After Hierarchical Cluster Analysis three different groups of whey protein supplements were observed. ANOVA analysis indicates that the concentration of the Na, K and Mg above mentioned elements is the key for the observed classification. The average elemental bioavailable fraction in the stomach is 45 % whereas in the intestine is 64 %. Most of the elements tested (nineteen) show gastric bioavailability higher than 60 %, being Al the lowest (37 %) and Co the highest one (76 %). According to our results, previously published data and recommendations of producers, WP samples can be considered save from the elemental composition point of view.

1. Introduction

The rise of the protein supplement intake concerns athletes and gym-users. In general terms, more than 50 % of the adult population of the United States intake dietary supplements (Lieberman et al., 2010). Regarding sport and physical exercise, more than 40 % of athletes use or have used ergogenic supplements, mainly Whey Protein (WP) (Bianco et al., 2015, 2011), to improve their physical performance, gain muscle and improve body composition (Bergia et al., 2018; Davies et al., 2018). Whey Protein is rich in essential and branched-chain amino acids, being a high-quality source of protein, associated with rapid absorption and higher muscle protein synthesis in comparison with other protein sources (Bergia et al., 2018; Cengiz et al., 2017; Davies et al., 2018).

Several studies have demonstrated the benefits of WP consumption for improving cardiovascular, metabolic, antioxidant, immune, anti-inflammatory, carcinetic, hypoglicemic and body composition parameters when included as part of a training program (Bergia et al., 2018; Bumrungpert et al., 2018; Dos Santos et al., 2018; Flaim et al., 2017). Whey protein supplements are employed in sports nutrition in different commercial forms (baked goods, salad dressings, emulsifiers, bars, etc.) for increasing the average per kg protein intake in the diet (Callahan, 2013; Herda et al., 2013; Hoffman and Falvo, 2005; Jager et al., 2017). Among them, the most popular product consumed by athletes or gym-users are the protein drinks based on powders contained in over-sized bottles.

The choice of an specific supplement depends on the customer characteristics (training, sport, economy, aims) (Bianco et al., 2015) and also on the marketing strategies promoted by different protein supplement brands. Although the legislation should provide information to costumers about the usage, dose, security, market access and availability of the supplements (Martínez-Sanz et al., 2017), there is a high offer for their consumption without the appropriate supervision of health professionals (Goston and Toulson Davisson Correia, 2010; Morrison et al., 2004). Some global health organizations, such as the Food and Drug Administration (FDA), have recently imposed stringent standards for manufacturers and distributors of supplements, which should contain only what is labeled and declared, without any harmful or undesirable substances, including pesticides and heavy metals (Avula et al., 2011;

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Dolan et al., 2003; Liddle and Connor, 2013; Van Der Voet et al., 2008). Producers usually imply to consumers that their products are "natural" or "pure organic" products, but there is a lack of information about the elemental composition, although they can be contaminated by metals during the process, the chemical treatment or storage (Filipiak-Szok et al., 2015). Some elements are essentials but become harmful when found at high concentrations (i.e., iron, manganese or zinc), whereas others, such as arsenic, cadmium or lead, are necessarily toxic (Lu et al., 2018). Nowadays, 19 elements: 18 metals and one metalloid (arsenic) are included in the list of 150 national priorities of the Agency for the Register of Toxic Substances and Diseases (ATSDR) (Nordberg et al., 2007). Some of the elements present in the list are essential metals that have also toxic effects in case of overexposure or excessive daily intake, such as cobalt, zinc and manganese (Nordberg et al., 2007). This exposure can lead to chronic effects and genetic diseases (Nordberg et al., 2007; Turkez et al., 2012; Waalkes, 2000).

There is evidence that many athletes or gym-users are already consuming protein supplements at a level above the current guideline range of 1.5–2.5 g/kg/day (MacKenzie-Shalders et al., 2015), exceeding up to three times the generally recommended dose by manufacturers (usually a maximum daily intake of 30 g). Users must know the actual composition of the products, having their benefits explained to them, but also by drawing their attention to the consequences of their inappropriate use. It is to remark that the nutritional facts available on the WP labels do not include detailed elemental information and only the generic description of "salt" appears in some cases. Hence, although products are safe when using the recommended dose, they could become harmful when overdose.

As a consequence of the big international market associated to these products, some analytical works related to the detection of frauds in WP using molecular techniques have been done. Thus, Gong et al. (2019), used Raman Spectroscopy and Pereira et al., 2018 (Pereira et al., 2018), Stationary and Time Resolved Fluorescence Spectroscopy to this end. As regards elemental composition, Pinto et al. (2020), determined the total content of 26 essential and non-essential trace elements in 49 samples of WP from Portugal by using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). They show that WP can be considered an important source of some essential elements, mainly Mo and Se, and according to the Permitted Daily Exposure limits (PDE) can be considered as safe products (daily intake of 50 g). Elgammal et al. (2019), determined the concentration of 15 elements in samples from Egypt using Inductively Coupled Plasma Optical Emission Spectrometry (ICPOES) and Graphite Furnace Atomic Absorption Spectrometry (GFASS) and found that some elements (Mn, Fe, Cr, Na and Cu) are present in samples, but not defined on the label. According to the health risk assessment performed by those authors, the Hazard Index (HI) of WP (considering the ingestion rate recommended by the producer) samples are, in general, below the recommended limits but they conclude that it must be taking into consideration the concentrations found for some elements (Cu, Zn, Fe, Mn, Ni, Al, Sn and Pb) in other foods and their accumulation in the human body for a correct evaluation of the Hazard Quotient (HQ). Finally, they consider that additional studies should be carried out to ensure a higher safety profile of WP. In this way, Bioavailability (BA), defined as the fraction of the ingested element that reaches the systemic circulation from the gastrointestinal tract (bioavailable fraction) and which is available to promote this action in the organism (Reeder et al., 2006), should be employed for a more realistic risk assessment due to the presence of elements in WP products instead of their total content. Several methods involving sequential treatment of samples by solutions imitating the physiological conditions of the gastrointestinal tract, i.e., chemical composition of digestive fluid, pH and typical residence time for each step of the digestion process are proposed for elemental bioavailability evaluation. Those called *in-vitro* methods represent a good approximation to reality and show several advantages over the *in*-vivo methods: (i) good reproducibility; (ii) easy to control; (iii) simplicity; (iv) low cost and high precision and (v) rapidity (Moreda-Piñeiro et al., 2011).

To date and to the best of our knowledge, no studies about the bioavailability of elements in Whey Proteins supplements have been published. For this reason, the aim of this study is to evaluate the bioavailability of twenty-five elements, covering a wide range of concentrations (μg/Kg to mg/Kg) and atomic weights (^{7}Li to ^{209}Bi), in whey protein powder samples and to evaluate the possible risk associated to the daily intake of such products.

2. Materials and methods

2.1. Samples

A set of 20 whey protein powder samples was selected to be representative of the commercially available products in the European Union (labeled WP1 to WP20) was analyzed. The samples were stored in polyethylene bottles at room temperature until processed. The nutritional facts available on the labels do not include elemental information. Only the generic description of salt, ranging between 0.12 and 0.43 mg/serving size appears in eight samples.

Qualitative information about the presence of seventeen aminoacids, flavors, sweeteners, other additives and allergens are also included (Supplementary data).

2.2. Elemental analysis

Twenty-five elements (^{7}Li, ^{23}Na, ^{24}Mg, ^{27}Al, ^{39}K, ^{44}Ca, ^{51}V, ^{52}Cr, ^{55}Mn, ^{56}Fe, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{66}Zn, ^{71}Ga, ^{75}As, ^{78}Se, ^{88}Sr, ^{107}Ag, ^{111}Cd, ^{115}In, ^{118}Sn, ^{137}Ba, ^{208}Pb and ^{209}Bi) were determined by ICPMS (Agilent 7700x (Agilent Technologies, Santa Clara, USA)). Table 1 covers the ICPMS experimental conditions. Matrix matching calibration solutions were prepared by the appropriate dilution of standard solutions in the corresponding matrix: 1.5 % nitric acid in the case of the total content and the corresponding gastric or intestine simulant extraction matrix for the bioavailability assays. In addition, ^{186}Re, ^{73}Ge, ^{128}Te and ^{45}Sc (20 μg/L) where added as internal standards to assess the accuracy and improve the precision of the results. All calibration curves had correlation coefficients $R^2 > 0.995$ (twelve points) and cross-contamination overcome by analyzing blank solutions together with the samples. A 1000 mg/L multi-elemental standard solution ICP Merck-IV (Merck, Darmstadt, Germany) and mono-elemental 1000 mg/L standard solutions of As, Se V and Sn (Applichem, Panreac, Castellar del Valles, Spain) where employed. High-purity water with a resistivity > 18 MΩ cm obtained from a Milli-Q water Direct-Q3 purification system (Millipore Inc., Paris, France) was used to prepare the solutions employed throughout this work. Limits of detection (LODs) where estimated using three times the standard deviation of the blank signal (n = 10).

For the determination of the elemental total content samples were digested by using a microwave assisted acid digestion (model Start D, Milestone, S.r.l., Sorisole, Italy) and high-pressure digestion vessels (model SK-12 T) using the program recommended by the manufacturer for trace metal analysis of pharmaceutical matrices and dietary supplements (application note HPR-FO-18). Concentrated nitric acid (65 %)

Table 1
ICPMS experimental conditions.

Plasma forward power (W)	1400
Argon flow rate (L min^{-1})	
Plasma	15.00
Auxiliary	0.90
Nebulizer	1.0
Sample uptake rate (mL min^{-1})	0.500
Dwell time (ms)	15
Sweeps	100
Replicate measurements	3

and hydrogen peroxide (33 %) (Panreac, Castellar del Valles, Spain) where employed as reagents. Samples were analyzed in triplicate (n = 3).

2.3. In vitro gastro-intestinal digestion

In vitro gastro-intestinal digestions were carried out by a modified version of the method proposed by Moreda-Piñeiro et al. (2011) to assess the bioavailability of arsenic, selenium and mercury species in food samples.

The gastric step simulation was carried out using 2 g of whey protein and the gastric solution was prepared adding 0.3 mL of pepsin 1 % w/w (pH 3.4; isolated from porcine gastric mucosa, Sigma-Aldrich) to a 100 mL of 0.1 M of HCl solution (Hiperpur 35 % AppliChem, Panreac). The pH was adjusted to 2 by using concentrated hydrochloric acid, checked every 15 min and readjusted if necessary. Flasks were covered and incubated at 37 °C with orbital–horizontal shaking in hot-water bath at 70 rpm for 60 min. Then, the flasks were placed at 4 °C to stop the enzymatic reactions. At the end of the step an aliquot of 10 mL was taken from the solution for analysis (GP solution).

For the intestinal step, the pH of the gastric digest (GP) was raised to pH 7 by addition of concentrated sodium bicarbonate solution. After that, 0.5 mL of 3 % w/w pancreatin solution (Pancreatin from porcine pancreas secretions, Sigma-Aldrich) and 0.5 mL of 5 % w/w bile extract (Bile extract porcine, Sigma-Aldrich) were added. The mixture was diluted to 100 mL with water and incubated at 37 °C in hot-water bath during 120 min. The enzymatic reaction was stopped placing the flasks at 4 °C. Finally, a 10 mL aliquot was collected for analysis (IP solution).

Both solutions (GP and IP) were transferred to glass centrifuge tubes and centrifuged at 3500 rpm for 35 min to separate the soluble fraction. After that, solutions were clear, and no filtration was necessary. All experiments were carried out by triplicate and blanks were also analyzed in each batch of samples. The bioavailability was calculated as the fraction of each element present in the simulated digestion solutions (GP or IP) to the total amount of the element under consideration.

2.4. Methods validation

Due to the lack of certified materials for multielemental analysis of whey protein powder samples (only the total content of Ca and Mg appears in one material) European conformity guidelines for analytical methods of food contaminants were employed to validate the three methods employed (i.e., total content, gastric and intestine simulant procedures) (Vanhaecke et al., 2011). The accuracy was evaluated by means of recovery tests of the main elements using three randomly selected samples (WP4, WP7, WP15). ICPMS measurements were performed by matrix matching calibration and internal standard as mentioned above. Irrespective of the methodology employed, the recovery values for all the analytes were within − 20–10 % stablished by the European conformity guidelines thus confirming results accuracy (Supplementary data, Fig. 1). Multiple injections showed that the results are highly reproducible and showed low standard deviation (SD).

2.5. Data analysis

Multivariate data analysis was performed by means of Principal Components Analysis (PCA) and Hierarchical Cluster Analysis (HCA) using the *Past3* statistics software. Furthermore, comparison between groups (resulted from HCA) was performed using ANCOVA analysis including kcal as covariate and Bonferroni post-hoc test. Significant difference was considered when p value was < 0.05. Effect sizes were calculated using the partial eta-squared statistic (ηp^2) to establish the substantive meaningfulness of the differences found.

3. Results and discussion

3.1. Total content

The concentrations obtained for all samples are reported in Tables 2 and 3 (median±SD). Table 2 covers all the elements present in the mg/Kg range (trace elements) whereas Table 3 shows the elements present in concentrations in the μg/Kg range (ultratrace elements). As regards both tables, and as general result, it is to remark that the 25 elements were present in all samples and were determined. According to the results shown in Table 2, Na, K, Ca and Mg are the most predominant elements with average concentrations around few thousands of mg/Kg. Zn and Fe show similar average concentration (around 20 mg mg/Kg) and Ba and Ni are the elements present in lower concentration, (less than one mg/Kg). The high amount of Na, K, Ca and Mg found is directly related to the high concentration of these elements in biological samples. Nevertheless, some salts of them are also employed as additives in whey protein supplements with different purposes, thus increasing their natural concentrations. The sodium hydrophosphate is employed as a flavor enhancer, the potassium hydrophosphate and citrate are both employed as pH buffers whereas the chlorides of sodium and potassium are employed as electrolytes. Calcium phosphate is also added as anti-binding agent in powders.

Table 3 shows the concentration of the elements tested with concentration in the μg/Kg range. In this case, the most concentrated elements are Li, Cr, Se and Sn, showing average concentrations around one

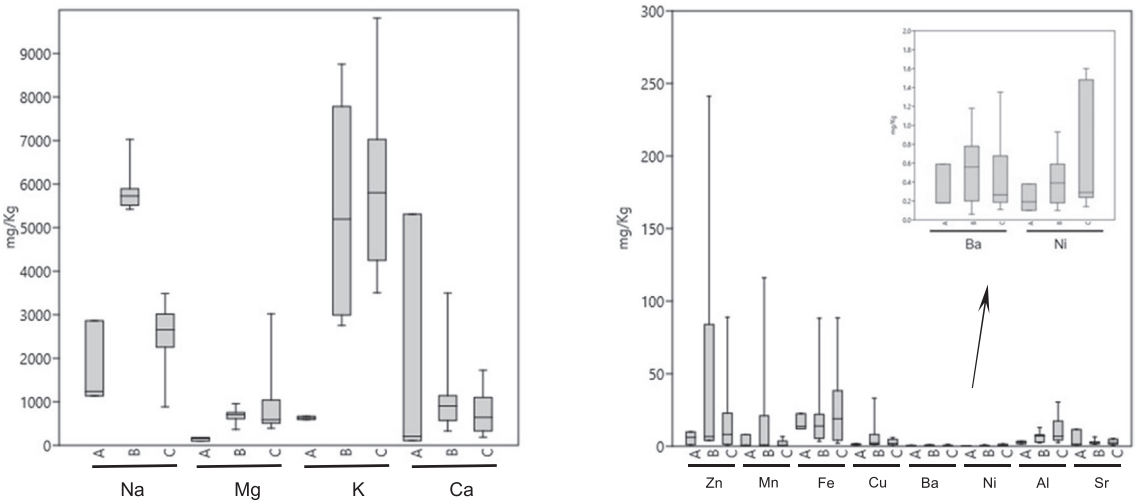

Fig. 1. - Box plot for elements in the mg/Kg range in groups A, B and C.

Table 2
Concentration of the elements (median±SD) present in the mg/Kg range in dry whey protein; n = 3.

	Element											
sample	Na	K	Ca	Mg	Zn	Mn	Fe	Cu	Al	Sr	Ni	Ba
WP1	2900 ± 90	5600 ± 200	1348 ± 13	3020 ± 90	18.0 ± 0.2	1.3 ± 0.1	2.0 ± 0.1	5.9 ± 0.2	4.6 ± 0.2	5.3 ± 0.2	0.29 ± 0.01	0.19 ± 0.01
WP2	5500 ± 110	8800 ± 300	1142 ± 11	960 ± 30	241 ± 2	21.0 ± 0.	3.4 ± 0.1	33.3 ± 0.3	2.4 ± 0.1	2.3 ± 0.1	0.25 ± 0.01	0.20 ± 0.01
WP3	5790 ± 60	2750 ± 60	3500 ± 40	703 ± 7	84 ± 1	0.8 ± 0.1	6.4 ± 0.2	1.0 ± 0.1	7.9 ± 0.2	2.5 ± 0.1	0.19 ± 0.01	0.29 ± 0.01
WP4	2300 ± 50	3980 ± 40	588 ± 6	520 ± 30	1.0 ± 0.1	0.1 ± 0.1	2.3 ± 0.1	0.2 ± 0.1	30.4 ± 0.3	4.7 ± 0.2	0.10 ± 0.01	0.18 ± 0.02
WP5	2300 ± 140	6380 ± 60	188 ± 2	1260 ± 30	90 ± 3	5.0 ± 0.2	88 ± 1	5.6 ± 0.1	6.2 ± 0.1	2.4 ± 0.1	1.47 ± 0.06	0.17 ± 0.01
WP6	5900 ± 200	5200 ± 50	693 ± 7	752 ± 8	4.0 ± 0.1	0.7 ± 0.1	14.0 ± 0.1	1.7 ± 0.1	7.8 ± 0.1	1.3 ± 0.1	0.28 ± 0.01	0.25 ± 0.01
WP7	2660 ± 80	3500 ± 40	467 ± 9	392 ± 8	1.0 ± 0.1	0.1 ± 0.1	4.8 ± 0.1	1.4 ± 01	2.5 ± 0.1	1.6 ± 0.1	0.38 ± 0.01	0.18 ± 0.01
WP8	2130 ± 40	8070 ± 80	923 ± 9	594 ± 6	11.2 ± 0.1	0.2 ± 0.1	17.4 ± 0.2	1.1 ± 0.1	19.8 ± 0.6	3.0 ± 0.1	0.29 ± 0.01	0.61 ± 0.01
WP9	2670 ± 130	5200 ± 200	1020 ± 20	580 ± 30	6.1 ± 0.1	0.1 ± 0.1	5.8 ± 0.1	1.3 ± 0.1	3.5 ± 0.1	1.0 ± 0.1	0.10 ± 0.01	0.54 ± 0.02
WP10	5580 ± 110	2990 ± 30	332 ± 3	363 ± 4	6.7 ± 0.1	116 ± 2	22.0 ± 0.2	2.5 ± 0.1	2.9 ± 0.1	2.9 ± 0.1	0.59 ± 0.02	0.69 ± 0.01
WP11	1240 ± 40	635 ± 6	5300 ± 50	93 ± 5	1.0 ± 0.1	0.7 ± 0.1	12.0 ± 0.6	0.8 ± 0.1	3.4 ± 0.1	11.5 ± 0.1	0.26 ± 0.01	0.28 ± 0.01
WP12	3490 ± 170	5960 ± 60	699 ± 7	551 ± 11	1.5 ± 0.1	0.1 ± 0.1	2.0 ± 0.1	0.0 ± 0.1	16.6 ± 0.2	2.4 ± 0.1	0.14 ± 0.01	0.11 ± 0.01
WP13	2650 ± 160	4340 ± 40	233 ± 2	463 ± 5	5.1 ± 0.2	3.0 ± 0.1	31.2 ± 0.3	2.3 ± 0.1	4.9 ± 0.1	2.0 ± 0.1	0.76 ± 0.03	0.20 ± 0.01
WP14	1140 ± 20	590 ± 20	205 ± 6	154 ± 5	9.9 ± 0.1	0.5 ± 0.1	13.7 ± 0.1	1.1 ± 0.1	2.7 ± 0.1	1.4 ± 0.1	0.17 ± 0.01	0.29 ± 0.01
WP15	3300 ± 100	9800 ± 100	363 ± 7	970 ± 30	10.2 ± 0.3	6.7 ± 0.1	39.0 ± 0.4	4.5 ± 0.1	12.4 ± 0.2	5.3 ± 0.1	1.60 ± 0.04	1.35 ± 0.03
WP16	2860 ± 30	665 ± 13	105 ± 5	182 ± 5	6.1 ± 0.1	8.0 ± 0.1	22.5 ± 0.5	1.6 ± 0.1	1.7 ± 0.1	0.8 ± 0.1	0.39 ± 0.01	0.56 ± 0.01
WP17	5730 ± 170	4520 ± 50	904 ± 9	612 ± 18	3.9 ± 0.1	0.1 ± 0.1	5.6 ± 0.1	1.2 ± 0.1	3.3 ± 0.1	3.0 ± 0.1	0.18 ± 0.01	0.78 ± 0.02
WP18	880 ± 20	6680 ± 130	1730 ± 50	889 ± 18	37.3 ± 0.4	2.5 ± 0.1	38.1 ± 0.4	3.6 ± 0.1	7.6 ± 0.1	0.6 ± 0.11	0.93 ± 0.02	1.18 ± 0.05
WP19	5420 ± 110	6110 ± 60	957 ± 11	682 ± 7	5.2 ± 0.1	0.5 ± 0.1	21.9 ± 0.7	2.2 ± 0.1	7.2 ± 0.1	1.7 ± 0.1	0.43 ± 0.02	0.06 ± 0.01
WP20	7000 ± 200	7780 ± 80	574 ± 2	740 ± 7	20 ± 1	6.3 ± 0.3	88.2 ± 0.9	8.1 ± 0.2	12.9 ± 0.5	6.4 ± 0.1	1.52 ± 0.08	0.88 ± 0.03
Max	7000	9800	5300	3020	241	116	88.2	33.3	30.4	11.5	1.60	1.35
Min	880	591	105	93	1.0	0.1	2.0	0.0	1.7	0.6	0.10	0.06
Average	3575	4978	1064	724	28.1	8.7	22.0	4.0	8.0	3.1	0.52	0.46

hundred μg/Kg whereas other well-known toxic elements such as Pb, Cd and As are found in concentrations around 10 μg/Kg. Indium shows the lowest concentration (1.2 μg/Kg).

Data shown in Tables 2 and 3 are, in general terms, comparable to those previously reported in the literature (Elgammal et al., 2019; Pinto et al., 2020) but, unfortunately, a full comparison is not possible because the elements determined, and the analytical techniques employed are different. Thus, Pinto et al. determined by ICPMS only ultratrace elements (Na, K, Ca and Mg are missing) whereas many of the ultratrace elements determined by Sherif et al., 2019 (Elgammal et al., 2019) show concentrations below the limits of detection or quantification achieved by ICPOES or GFAAS. As regard the concentration of a given element in different samples, it is worth noting the high variability observed, up to two orders of magnitude (i.e., Mg). Pinto et al. (2020), also observed a high variability in 26 samples from Portugal and conclude that is related to the raw materials composition as well as the manufacturing equipment employed. Finally, it is to remark that no effect of brand and/or flavor is observed.

Cobalt is present in all samples with an average concentration of 34.5 μg·mg/Kg (see Table 2). This element, as hypoxia-inducible factor activating agent, has been listed in the last annual Prohibited List of World Anti-Doping Agency (WADA). Nevertheless, the dosages found were not significant to exceed WADA limits (Heffernan et al., 2019) expected to report some benefits in sport performance in athletes (over 5 mg/day (Hoffmeister et al., 2019, 2018)). Then, the ingestion of WP by itself could not affect as doping fact, addressing the WADA postulate, which main purpose is to lead a collaborative worldwide movement for doping-free sport, and its activities focus on the responsibilities set out in the World Anti-Doping Code (Heuberger and Cohen, 2018).

Zn and Se are key micronutrients present in all samples related to cell membrane protection within antioxidant capacity function that are present in different concentration range. It is accepted that WP presents benefits, including improvement of body composition, when it is included as part of a weight loss program (Bergia et al., 2018), as well as immune system support and metabolism. In this sense, the activity of WP trace elements such as Zn and Se as key micronutrients should be considered.

The goal of this work is related to the elemental bioavailability, nevertheless it is necessary to know as much as possible about the total composition of samples before bioavailability assays. To go further in data analysis, multivariate analysis was carried out. First, a Principal Components Analysis was performed, and no samples grouping was observed. A model made up to 7 components is necessary for a total explained variance higher than 80 %. After that, a new PCA analysis when adding the samples main characteristics shown in the Supplementary data (table: organic components and other additives) and the flavor and brand as group variable to the elemental data set (Tables 2 and 3) was also performed. Again, no samples grouping was observed,

Table 3
Elemental composition (median±SD) of the elements present in the μg/Kg range in dry whey protein samples; n = 3.

	Element (μg/Kg ± SD)												
sample	Pb	Cd	As	Cr	Sn	V	Co	Li	Se	In	Bi	Ga	Ag
WP1	18.4 ± 0.4	8.6 ± 0.2	19.3 ± 0.2	50.2 ± 0.5	10.0 ± 0.2	34.1 ± 0.2	17.7 ± 0.1	213.9 ± 0.1	254.9 ± 1.3	1.0 ± 0.1	19.4 ± 0.4	1.3 ± 0.1	5.9 ± 0.1
WP2	5.1 ± 0.1	8.5 ± 0.1	30.0 ± 0.9	53.6 ± 1.3	192 ± 2	5.7 ± 0.3	20.0 ± 0.1	263.4 ± 0.8	99.2 ± 1.2	10 ± 0.1	4.4 ± 0.1	1.9 ± 0.1	6.0 ± 0.1
WP3	19.0 ± 0.8	7.7 ± 0.2	25.9 ± 0.3	50.0 ± 0.6	282 ± 2	70.4 ± 1.0	19.1 ± 0.1	84.1 ± 1.1	355 ± 2	2.8 ± 0.1	43.5 ± 1.1	1.4 ± 0.1	5.3 ± 0.1
WP4	0.6 ± 0.1	3.2 ± 0.1	8.0 ± 0.3	10.1 ± 0.2	20.0 ± 0.6	8.9 ± 0.3	7.3 ± 0.1	64.8 ± 1.1	183 ± 2	1.1 ± 0.1	4.5 ± 0.2	0.4 ± 0.1	4.7 ± 0.1
WP5	16.0 ± 0.5	28.0 ± 0.3	10.0 ± 0.3	550 ± 30	110 ± 3	51 ± 2	75.3 ± 0.3	138 ± 3	60.7 ± 1.3	0.7 ± 0.1	4.1 ± 0.2	2.0 ± 0.1	4.8 ± 0.1
WP6	4.4 ± 0.2	14.9 ± 0.4	10.8 ± 0.5	74.6 ± 0.4	80.2 ± 0.6	10.1 ± 0.2	25.3 ± 0.1	127 ± 5	386 ± 1	01 ± 0.1	3.26 ± 0.1	0.4 ± 0.1	4.7 ± 0.1
WP7	4.7 ± 0.2	6.9 ± 0.1	19.3 ± 1.0	58.9 ± 0.4	290.1 ± 0.9	322 ± 6	17.8 ± 0.1	116 ± 4	227 ± 6	1.3 ± 0.1	3.9 ± 0.1	0.3 ± 0.1	4.1 ± 0.1
WP8	4.4 ± 0.1	6.6 ± 0.1	10.0 ± 0.2	90.4 ± 0.4	190.0 ± 1.3	7.1 ± 0.3	24.3 ± 0.1	153 ± 6	167 ± 3	0.5 ± 0.1	13.6 ± 0.3	2.0 ± 0.1	4.7 ± 0.2
WP9	2.1 ± 0.1	4.2 ± 0.1	11.1 ± 0.4	14.0 ± 0.2	310 ± 10	5.5 ± 0.1	12.5 ± 0.1	56.9 ± 1.4	306 ± 6	1.3 ± 0.1	5.2 ± 0.2	0.4 ± 0.1	2.8 ± 0.1
WP10	9.7 ± 0.4	15.0 ± 0.1	11.0 ± 0.1	326 ± 3	110 ± 3	11.1 ± 0.3	47.5 ± 0.5	162.1 ± 0.2	113.3 ± 1.6	0.2 ± 0.1	12.6 ± 0.1	3.4 ± 0.1	4.4 ± 0.2
WP11	7.3 ± 0.3	11.3 ± 0.1	8.7 ± 0.4	104.5 ± 1.4	670 ± 20	17.2 ± 0.7	29.4 ± 0.4	43.9 ± 0.2	29.5 ± 0.2	0.1 ± 0.1	1.9 ± 0.1	1.6 ± 0.1	6.3 ± 0.3
WP12	19.0 ± 0.4	3.9 ± 0.1	9.8 ± 0.6	31.9 ± 0.3	110.0 ± 0.8	4.9 ± 0.1	5.5 ± 0.1	46.0 ± 0.1	79 ± 5	1.8 ± 0.1	2.2 ± 0.1	0.4 ± 0.1	4.5 ± 0.2
WP13	22.2 ± 0.7	36.2 ± 0.4	19.4 ± 1.0	261 ± 5	80.0 ± 1.6	147.7 ± 1.3	82.6 ± 0.7	144 ± 5	56.6 ± 0.6	2.5 ± 0.1	2.7 ± 0.1	9.8 ± 0.1	27.2 ± 0.4
WP14	1.9 ± 0.1	1.8 ± 0.1	23.7 ± 0.5	20.1 ± 0.1	280 ± 2	33 ± 2	3.25 ± 0.1	86.7 ± 4	94.5 ± 1.4	1.9 ± 0.1	1.5 ± 0.1	0.4 ± 0.1	3.5 ± 0.1
WP15	11.2 ± 0.6	16.9 ± 0.7	18.3 ± 0.5	285.7 ± 1.1	180.0 ± 1.6	16.1 ± 0.6	57.9 ± 0.7	144 ± 5	144 ± 1	1.0 ± 0.1	0.5 ± 0.1	2.9 ± 0.1	2.7 ± 0.1
WP16	12.0 ± 0.1	10.7 ± 0.5	9.3 ± 0.5	20.5 ± 0.2	580 ± 30	10.8 ± 0.6	14.7 ± 0.1	170 ± 12	46.3 ± 0.6	1.9 ± 0.1	2.6 ± 0.1	2.6 ± 0.1	9.7 ± 0.3
WP17	2.5 ± 0.1	3.2 ± 0.1	9.3 ± 0.4	65.9 ± 0.6	100 ± 5	9.3 ± 0.1	9.13 ± 0.1	36.9 ± 0.1	275 ± 5	1.4 ± 0.1	2.9 ± 0.1	0.4 ± 0.1	4.6 ± 0.2
WP18	13.5 ± 0.5	17.0 ± 0.2	10.3 ± 0.5	326 ± 3	750 ± 14	36.9 ± 0.8	84.1 ± 1.3	38.5 ± 0.4	204.2 ± 0.8	0.6 ± 0.1	24.0 ± 0.5	4.3 ± 0.2	5.2 ± 0.1
WP19	3.8 ± 0.2	10.4 ± 0.5	9.8 ± 0.6	153 ± 3	385 ± 11	12.9 ± 0.1	44.4 ± 0.2	75.1 ± 1.1	301 ± 2	1.74 ± 0.1	3.23 ± 0.1	2.1 ± 0.1	4.8 ± 0.1
WP20	37.3 ± 0.4	42.6 ± 0.9	24.3 ± 1.2	247 ± 2	90.3 ± 1.5	58 ± 1	91.7 ± 0.3	207 ± 5	366 ± 1	0.4 ± 0.1	8.88 ± 0.1	9.0 ± 0.2	2.3 ± 0.1
Max	37.3	42.6	30.0	548.3	750.0	322.4	91.7	263.4	386.6	2.8	43.5	9.8	27.2
Min	0.6	1.8	8.0	10.1	10.0	4.9	3.3	36.9	29.5	0.1	0.5	0.3	2.4
Average	10.8	12.9	14.9	139.6	241.0	43.7	34.5	118.5	187.5	1.2	8.2	2.3	5.9

and a new model made up to 8 components is necessary for a total explained variance higher than 80 %. Hence, according to this result and the aim of this study, only the elemental composition was used in the rest of this work.

After that, a Hierarchical Cluster Analysis (HCA) was also performed. When using HCA, a cluster tree (dendogram) is used to represent data, where each group (cluster) links to two or more successor groups. The groups are nested and shown as a tree. Each node in the cluster tree contains a group of similar samples. Clusters at one level join with clusters in the next level up, using a degree of similarity; The process carries on until all nodes are in the tree, which contains the whole set. The number of clusters is not determined in advance. In this case samples are classified in three different groups (Supplementary data Fig. 2): The first one (A) includes the samples number 11, 14 and 16. The second group (B) includes the samples number 2, 3, 6, 10, 17, 19, and 20, whereas the rest of samples (1, 4, 5, 7, 8, 9, 12, 13, 15 and 18) are included in the third group (C). For the analysis of this information the three groups obtained by HCA were characterized by means of a box and whiskers plot. Thus, Fig. 1 shows the box plot graph for elements in the mg/Kg range (major and minor). Although the high variability observed in the sample composition, when regarding major elements the group A shows the lowest values of Na, K and Mg (up to 6 and 9 times lower) whereas the group B shows the highest Na value (2–3 times higher). Samples of Group B can also be differentiated from groups A and C because in all cases the presence of L-glutamine is declared in their labels (253 mg/30 g). There are no clear differences between the rest of element concentrations in the three groups. Similar situation can be observed when comparing ultratrace elements (see Supplementary data Fig. 3). In general terms, there is no significant differences between groups. Nevertheless, it is to note that Se and Li concentrations are higher than the rest of elements except for Cr and V due to the high variability observed in those elements. Grouping is usually governed by variables with higher variability between groups irrespective their absolute values, since are normalized before analysis. According to Pinto et al. (2020), the high variability observed between groups should be related to the original whey composition, the additives and the manufacturing equipment employed. Data shown in Fig. 1 seems to agree with this statement. Nevertheless, considering that there is not enough previous information about this kind of samples in the literature, an ANCOVA analysis was also done. Thus, ANCOVA comparison between groups showed significant differences between A and B or C, being the A lower at variables K (A vs B: $p = 0.009$; A vs C: $p = 0.003$; effect size ($\eta p^2 = 0.513$) and Mg (A vs B: $p = 0.031$; A vs C: $p = 0.003$; effect size ($\eta p^2 = 0.496$), and higher at Sn (A vs B: $p = 0.008$; A vs C: $p = 0.002$; effect size ($\eta p^2 = 0.536$). Moreover, Na comparison analysis presented a lower significant quantity from B in comparison with A or C groups (B vs A: $p < 0.001$; B vs C: $p < 0.001$; effect size ($\eta p^2 = 0.877$). Therefore, the classification in three groups is mainly related to the

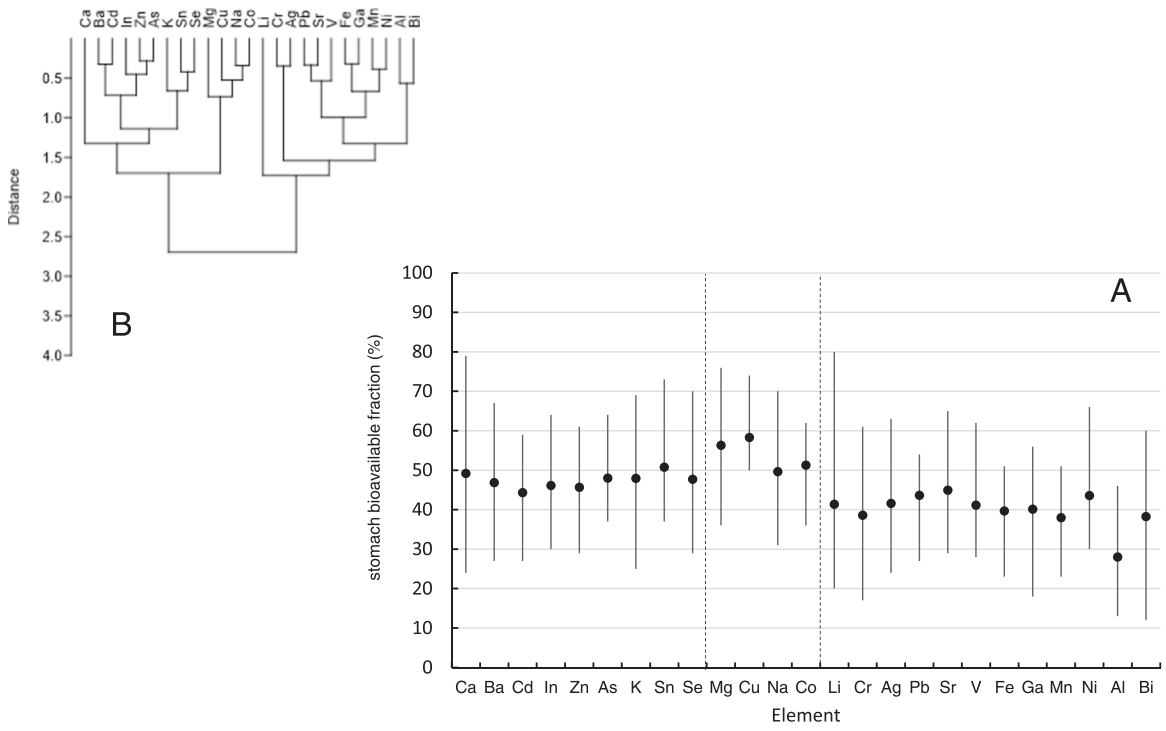

Fig. 2. - Stomach in vitro samples bioavailability and dendogram.

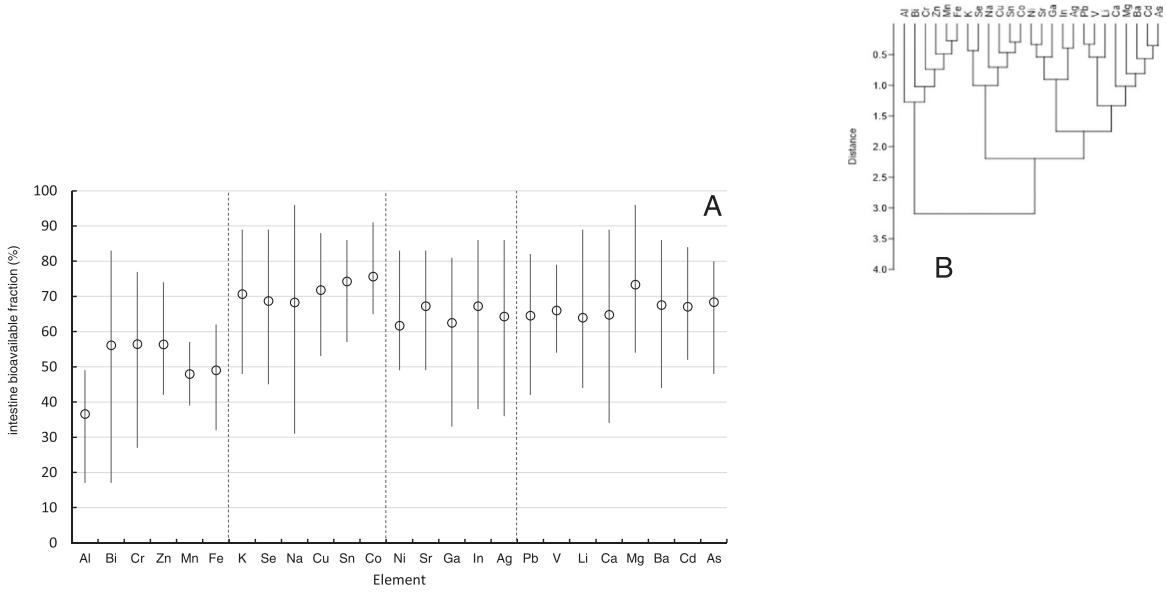

Fig. 3. - Intestin in vitro samples bioavailability and dendogram.

presence of Na, K and Mg.

3.2. Bioavailability

3.2.1. Stomach

Fig. 2 shows the bioavailability (average, maximum and minimum values) of the 25 elements in the twenty samples studied following the in vitro digestion procedure described previously (Fig. 2. A) and the dendogram obtained using Hierarchical Cluster Analysis (Fig. 2. B).

Regarding the stomach bioavailability of essential elements separately, is worth to highlight the results of some elements, presented as mean and it range (minimum to maximum). Ca presents a mean of 49.20 and a range between 24 % and 79 %, Cu presents a mean of 58.30 and a range between 50 % and 74 %, Fe presents a mean of 39.70 and a range between 23 % and 51 %, K presents a mean of 47.95 and a range between 25 % and 69 %, Mg presents a mean of 56.35 and a range between 36 % and 76 %, Na presents a mean of 49.65 and a range between 31 % and 70 %, and Zn presents a mean of 45.70 and a range between 29 % and 61 %. Furthermore, is important to show in deep detail the stomach bioavailability of these four toxic elements (As, Cd Ni and Pb), potentially dangerous for health. As presents a mean of 48.00 and a range between 37 % and 64 %, Cd presents a mean of 44.30 and a range between 27 % and 59 %, Ni presents a mean of 43.60 % and a range between 30.00 and 66.00, Pb presents a mean of 43.65 and a range

between 27 % and 54 %.

The average bioavailability of the elements presents in WP during the gastric step simulated digestion is 45 %, ranging between 12 % for Bi in WP8 and 80 % for Li in WP1 (see Supplementary data). In the case of the four most concentrated elements (Na, K, Ca and Mg) their bioavailability is similar, 53 % on average. Zn, Mn and Fe are also present in elevated concentration (around 100 mg mg/Kg) and show a moderate bioavailability (41 % on average) with a maximum of 54 % and a minimum of 25 %. On the contrary, Cu, that is present in concentrations around 30 mg mg/Kg, shows higher values, ranging from 50 % to 75 %. The so called "toxic elements": Pb, Cd, As, Ba, Ni, Al, Sr, and Sn show fractions ranging from 28 % to 60 % and the rest of considered elements varies between 25 % and 66 %.

No clear correlation between samples and elements bioavailability is observed (see Supplementary data). This is not an unexpected result because once the WP is ingested the concepts brand or flavor have no sense and only the elements should be considered. The amount of a given element solved during the simulation process mainly depends on its chemical form, but also on the matrix composition. For this reason, a Principal Component Analysis, similar to that performed previously with the total content has been carried out and no grouping was observed. Again, a model made up to 7 components is necessary for a total explained variance higher than 80 %. On the contrary, the Hierarchical Cluster Analysis (HCA) shows two groups at a re-scaled distance of 2.0 (Fig. 2. B). The first one is formed by all the elements with bioavailability fractions higher than 45 % (Ca, Ba, Cd, In, Zn, As, K, Sn, Se, Mg, Cu, Na and Co) with a sub-group constituted by the four elements with average values higher than 50 % (Mg, Cu, Na and Co) and a second one that includes the elements for which the bioavailable fractions are below 45 % (Li, Cr, Ag, Pb, Sr, V, Fe, Ga, Mn, Ni, Al, and Bi).

3.2.2. Intestine

Fig. 3 shows the bioavailability (average, maximum and minimum values) of the 25 elements in the twenty samples studied following the in vitro digestion procedure described previously (Fig. 3. A) and the dendogram obtained using Hierarchical Cluster Analysis (Fig. 3. B).

Data shown in Fig. 3. A range from 37 % of Al up to 76 % of Co. Nineteen elements show values higher than 60 % and only Fe, Mn and Al show average values below 50 %. A detailed analysis about elemental chemical form and bioavailability is out of the scope of this work, but in some cases (extreme values) data shown in the figure can be explained. Thus, the high bioavailability shown by cobalt seems to be directly related with its presence in the whey as cianocobalamine (B12 hydrosoluble vitamin). On the contrary, aluminium shows the lowest value, as previously observed in the stomach digestion step, and this behavior should be related with its presence in form of insoluble inorganic salt.

Concerning the intestine bioavailability of the previous essential elements observed at stomach. The results show an increment in relation to the stomach bioavailability, presented as mean (increment with respect to the stomach) and it range (minimum to maximum). Ca presents a mean of 64.80 (increment of 15.60) and a range between 34 % and 89 %, Cu presents a mean of 71.80 (increment of 13.50) and a range between 53 % and 88 %, Fe presents a mean of 49.00 (increment of 9.3) and a range between 32 % and 62 %, K presents a mean of 70.65 (increment of 22.70) and a range between 48 % and 89 %, Mg presents a mean of 73.40 (increment of 17.05) and a range between 54 % and 96 %, Na presents a mean of 68.35 (increment of 18.7) and a range between 31 % and 96 %, and Zn presents a mean of 56.35 (increment of 10.65) and a range between 42 % and 74 %.

In addition, as in the case of bioavailability in the stomach, it has been considered appropriate to highlight individually the results of some toxic elements such as those listed below, presented as well as mean (increment with respect to the stomach) and it range (minimum to maximum). As presents a mean of 68.40 (increment of 20.40) and a range between 48 % and 80 %, Cd presents a mean of 67.05 (increment of 22.75) and a range between 52 % and 84 %, Ni presents a mean of 61.70 (increment of 18.10) and a range between 49 % and 83 %, and Pb presents a mean of 64.65 (increment of 20.9) and a range between 42 % and 82 %.

The dendogram obtained using Hierarchical Cluster Analysis (HCA) shows three main groups at a re-scaled distance of 2 (Fig. 3. B). The first one formed by the elements with the lowest values (below 60 %) Al, Bi, Cr, Zn, Mn, and Fe whereas the second one is formed by K, Se, Na, Cu, Sn and Co, that are the elements with the highest bioavailability. Finally, a third group formed by the elements with values ranging 60 %– 70 % (except Mg).

In general, the elemental bioavailable fraction in the stomach or gastric part (GP) is, on average, a 19 % lower than the intestinal part (IP) (45 % versus 64 %). These data are consistent to that previously reported by other authors when evaluating different foods (da Silva et al., 2013; Stelmach et al., 2016; Vitali et al., 2008). Nevertheless, though the final fraction bioavailable is similar for most of the elements tested, a detailed analysis reveals that the fraction of Fe, Zn and Cu bioavailable in stomach is the 81 % of the total absorbed (stomach+intestine). As, Ni, Na, Ca, Al, Mg and Mn are in the 70 %– 79 % range and the rest between 65 % and 69 %. Only V and Ga show values lower than 65 %.

Regarding the concentration levels, macroelements such as Na, Mg, Ca and K have similar average bioavailability (between 69 % and 73 %), being Mg and Na the elements with the highest observed values, up to 96 %. On the contrary, Fe, Zn and Mn (concentrations 10–20 mg mg/Kg) are elements with low available fractions. Thus, the average bioavailability of Fe is 49 %, 56 % for Zn and 48 % in the case of Mn. The Al is the last element with concentrations around 10 mg mg/Kg and shows the lowest average value, 37 %. Cu and Sr are present in the samples with concentrations around 3–4 mg mg/Kg and their average bioavailability is similar, 72 % and 67 % respectively.

The rest of elements are presents in concentrations below 1 mg mg/Kg and shows an average availability of 66 %. Only Co and Sn show values higher than 70 %.

According to the results shown above, previously published data and recommendations of producers, WP samples can be considered save from the elemental composition point of view. Nevertheless, it is necessary to point out that not all the substances present in protein supplements, as well as the specific amount of many of them, are shown in the labeling of protein supplements.

3.3. Limitations and future research

A comparison of the bioavailable amount of elements in whey protein dietary supplements and the necessary of safe quantity of minerals would be interesting in future research, connecting the determined elements in the examined food products and potentially consumed dose of minerals.

4. Conclusions

The total content and the bioavailability of twenty-five elements were determined in twenty samples of whey protein supplements using Inductively Coupled Plasma Mass Spectrometry.

Results showed that Na, K, Ca and Mg were the most predominant elements. After Hierarchical Cluster Analysis three different groups of whey protein supplements were observed. ANOVA analysis indicates that the concentration of the four above mentioned elements is the key for the observed classification. This is the first time that elemental bioavailability is evaluated in whey protein supplements. In general, the average elemental bioavailable fraction in the stomach or gastric part are 45 % and 64 % respectively. Nineteen elements show gastric bioavailability values higher than 60 %, ranging from 37 % of Al up to 76 % of Co.

Finally, companies of sports supplementation products should provide reliable information on their labeling so as not to mislead the consumer. In this sense, either by omission of substances present or by

alterations or errors in their analysis, consumer fraud could be taking place. In addition, the potential alterations in the consumption of substances by athletes could alter their health and performance, as well as even incur in strategies not permitted by the WADA. That is why, in any case, the consumption of supplements should be prescribed and supervised by a health professional competent in the matter, who justifies the need for their use and evaluates their safety, efficacy and legality.

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CRediT authorship contribution statement

All authors contributed to the study conception and design. **Fatima Zohra Guefai:** Software, Investigation, Data curation, Writing – original draft. **Alejandro Martínez-Rodríguez:** Validation, Resources, Data curation, Writing – original draft, Visualization. **Guillermo Grindlay:** Methodology, Validation, Investigation, Writing – original draft. **Juan Mora:** Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration. **Luis Gras:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – review & editing, Visualization, Supervision, Project administration. All authors have read and agreed to the published version of the manuscript."

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Data Availability

Raw data can be provided upon email request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jfca.2022.104696.

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