Minerals and vitamin B_9 in dried plants *vs.* infusions: assessing absorption dynamics of minerals by membrane dialysis tandem *in vitro* digestion

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Running title: Minerals and vitamin B_9 in dried plants vs. infusions: extractability and bioacessability

Abstract

Vitamins and mineral elements are among the most important phytochemicals due to their important role in the maintenance of human health. Despite these components had already been studied in different plant species, their full characterization in several wild species is still scarce. In addition, the knowledge regarding the *in vivo* effects of phytochemicals, particularly their bioaccessibility, is still scarce. Accordingly, a membrane dialysis process was used to simulate gastrointestinal conditions in order to assess the potential bioaccessibility of mineral elements in different preparations of *Achillea millefolium* (yarrow), *Laurus nobilis* (laurel) and *Taraxacum* sect. *Ruderalia* (dandelion). The retention/passage dynamics was evaluated using a cellulose membrane with 34 mm pore. Dandelion showed the highest levels of all studied mineral elements (except zinc) independently of the used formulations (dried plant or infusion), but yarrow was the only species yielding minerals after the dialysis step, either in dried form, or as infusion. In fact, the ability of each evaluated element to cross the dialysis membrane showed significant differences, being also highly dependent on the plant species. Regarding the potential use of these plants as complementary vitamin B_9 sources, the detected values were much lower in the infusions, most likely due to the thermolability effect.

Keywords: Vitamin B_9; Minerals; Infusions; Wild plants

1. Introduction

The interest for traditionally used plants is rising, since they are considered a valuable and reliable source of natural compounds with recognised health effects. Among those compounds, the study of vitamins and mineral elements is crucial, due to their important role in the maintenance of human health; in fact, the lack of vitamins can cause a number of diseases, and mineral trace elements have essential biochemical functions such as the activation of chemical components present in the organism (Rihawy et al., 2010). The possible applications of plants should be complemented by a complete chemical characterization (Leśniewicz et al., 2006). Despite the high number of scientific publications profiling chemical compounds in plants, some wild species are still lacking for comprehensive studies. *Achillea millefolium* L. (yarrow, Asteraceae), *Laurus nobilis* L. (bay leaves, Laureacea) and *Taraxacum* sect. *Ruderalia* (dandelion, Asteraceae) were scarcely studied for their mineral profile and vitamin B_9 composition, making them good candidates for this type of profiling studies.

Vitamin B_9 (folic acid/folates) is an important cofactor of many biochemical reactions in cells. The absence of this vitamin would lead to non-cell division, anaemia, cardiovascular disease and neural tube defects in infants. Common food sources of vitamin B_9 are vegetables, bread and cereals, which may contain various forms of folate depending on food processing and storage. In food, folates are naturally presented as polyglutamates (PteGlun), mainly as mono-, penta- and hexaglutamates (Scott et al., 2000), being the monoglutamate form absorbed in the intestinal tube (Scott, 1999) and further converted to tetrahydrofolate (the most bioactive form of this vitamin) (Bailey & Ayling, 2009).

Microelements such as iron (Fe), copper (Cu), manganese (Mn) and zinc (Zn) represent a group correlated with the prevention of cardiovascular diseases, and some of them display also important biological functions such as osmoprotection (Fe), mitochondrial respiration

(Cu), and energy production and maintenance of structural integrity of biomembranes (Zn) (Hänsch & Mendel, 2009). These elements, which are required by the body in low amounts, can be obtained (together with numerous organic compounds) in the infusions of medicinal plants, subsequently leading to different physiologic functions, toxicity and absorption rates (Mutaftchiev, 2001; Özcan, 2004). Macroelements such as calcium (Ca), phosphorus (P), magnesium (Mg), potassium (K) and sodium (Na) serve as structural elements of the tissues and modulate the metabolism and acid-base balance, being present in the body in higher amounts than microelements (Leśniewicz et al., 2006; Özcan, 2004). Within the same species, the concentration of micro and macroelements in plants is conditioned by geochemical characteristics, rainfall and agricultural practices (Łozak et al., 2002; Konieczyński & Wesołowski, 2007).

Many exogenous (food matrix and compound structure) and endogenous (active transport, metabolism and excretion in the human body) factors affect the entrance of compounds in the lumen and therefore its bioavailability. As a part of the concept of bioavailability, bioaccessibility is defined as the amount of a food constituent that is present in the gut as a consequence of its release from the solid food matrix, and may be able to pass through the intestinal barrier and be potentially bioavailable (Saura-Calixto et al., 2007). *In vitro* gastrointestinal models provide a very useful methodology to screen food ingredients (*e.g.*, minerals, vitamins, phenolic compounds, among others) for their bioavailability. These system provide a great amount of results in a short period of time, allowing the study of matrices with different compositions and structures, simultaneously overcoming the complexity of *in vivo* studies (Hur et al., 2011).

The content of mineral elements was already determined by atomic absorption spectroscopy methods in *A. millefolium* (Chizzola et al., 2003; Konieczyński & Wesołowski, 2007; Divrikli et al., 2006), *L. nobilis* (Özcan, 2004; Divrikli et al., 2006; Sekeroglu et al., 2008; Zengin et

al., 2008) and *Taraxacum obovatum* (Willd.) DC. basal leaves (García-Herrera et al., 2014) samples from different locations. Nevertheless, to our knowledge, there are no reports of the content of vitamin B_9 in yarrow or bay leaves. A particular species of dandelion, *Taraxacum obovatum* (Willd.) DC., was previously studied for the vitamin B_9 content in its basal leaves (Morales et al., 2014). Nevertheless, to our knowledge, there are no studies on the vitamin B_9 content of yarrow and laurel, nor on the *in vitro* bioaccessibility of mineral elements from the plants studied herein. Therefore, the main objective of the present work was to characterize vitamin B_9 and minerals profile in dried material and infusions of wild samples of *A. millefolium*, *L. nobilis* and *Taraxacum* sect. *Ruderalia*. Furthermore, an *in vitro* gastrointestinal model was applied to provide a preliminary study of mineral elements bioaccessibility in these food matrices.

2. Materials and methods

2.1. Samples and infusions preparation

The wild samples of yarrow (inflorescences and upper leaves), laurel (leaves; before flowering) and the vegetative parts of wild *Taraxacum* sect. Ruderalia were collected in Bragança (Portugal). Voucher specimens of yarrow (nº 9623 BRESA), laurel (nº 9634 BRESA) and dandelion (nº 9686) were deposited at the Herbarium of the Escola Superior Agrária de Bragança (BRESA) (Dias et al., 2013; Dias et al., 2014a; Dias et al., 2014b). Morphological key characters from the Flora Iberica (Castroviejo, 1986-2012) were used for plant identification. The wild samples were lyophilized (FreeZone 4.5, Labconco, Kansas, USA) and stored at 4ºC until analysis.

The infusions were prepared according to the traditional procedure used to prepare tea (1 bag with ~1 g dry material, and 1 teapot with ~200 mL); therefore, each sample (1 g) was added to 200 mL of boiling distilled water and left to stand at room temperature for 5 min, and then

filtered under reduced pressure. The obtained infusions were frozen, lyophilized and stored at -6 ºC until analysis.

2.2. Standards and reagents

Micro (Fe, Cu, Mn and Zn) and macroelements (Ca, Mg, Na and K) standards (> 99% purity), as well $LaCl_2$ and CsCl (> 99% purity) were purchased from Merck (Darmstadt, Germany). Standards of 5-CH_3-H_4folate monoglutamate (ref. 16252; Schircks Laboratories, Jona, Switzerland) and pteroyl diglutamic acid (ref. 16235; Schircks Laboratories, Jona, Switzerland), pancreatic chicken homogenate (Pel Freeze, Arkansas), rat serum, $NaBH_4$, formaldehyde and octanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile fluorescence grade was bought from Fisher Scientific (Madrid, Spain). All other general laboratory reagents were purchased from Panreac Química S.L.U. (Barcelona, Spain). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.3. Vitamin B_9 (folic acid/folates)

Vitamin B_9 content was determined according to the methodology previously described by Morales et al. (2015), using HPLC-FL system, consisted of a Beta 10 (Ecom, Prague, Czech Republic) gradient pump with Gastorr Degasser HPLC Four Channel BR-14 (Triad Scientific, New Jersey, USA) as degassing device, joined to an AS-1555 automatic injector (Jasco, Easton, MD, USA), and to a FP-2020 Plus Fluorescence detector (Jasco, Easton, MD, USA) with RP 18 endcapped Lichrospher 100 column (Merck, Darmstadt, Germany; 250 × 5 mm; 5 μm). Quantification was performed by comparison of the area of the peaks recorded with calibration curves obtained from commercial standards (5-CH_3-H_4folate mono and diglutamate), and expressed as total folates (from the sum of both compounds) per 100 g plant (dw) or per 100 mL infusion.

Chromatographic parameters, namely limit of detection (LOD), limit of quantification (LOQ), linearity, recovery, repeatability and reproducibility were accepted as previously assessed (Morales et al., 2015).

2.4. Mineral and trace elements content

Mineral elements analysis was performed according to the method 930.05 of AOAC procedures for ash obtention, and then following the methodology previously described by Fernández-Ruiz, Olives, Cámara, Sánchez-Mata & Torija (2011). All measurements were performed in atomic absorption spectroscopy (AAS) with air/acetylene flame in Analyst 200 Perkin Elmer equipment (Perkin Elmer, Waltham, MA, USA), comparing absorbance responses with > 99.9% purity analytical standard solutions for AAS made with $Fe(NO3)_3$, $Cu(NO_3)_2$, Mn $(NO_3)_2$, Zn $(NO_3)_2$, NaCl, KCl, $CaCO_3$ and Mg band. Limit of detection (LOD), limit of quantification (LOQ), linearity, recovery, repeatability and reproducibility were accepted as previously assessed (Sanchez-Mata, 2000).

2.5. In vitro gastrointestinal model (dialysis)

The *in vitro* model applied consisted of an initial simulation phase of intraluminal digestion, followed by an intestinal absorption using a dialysis model (Ramírez-Moreno et al., 2011). Thus, minerals bioaccessibility was estimated using 25 mL of aqueous solutions prepared from dry material (20 mg/mL) or lyophilized infusion (20 µg/mL), Gastric digestion was simulated, adjusting the pH of each sample to 2, adding 150 µL of a pepsin solution (40 mg/mL of HCl 0.1M), and incubating the mixture in a water bath at 37^{o}C for 2 h with stirring (60 osc/min). The intestinal processes were then simulated, adding to the digested product a pancreatin/bile solution (5/25 mg of pancreatin/bile per 1 mL of 0.1M $NaHCO_3$). The mixture was then transferred to dialysis membranes (Medicell 7000/2, width 34 mm, 7000 MW cut

off), previously boiled in distilled water for 15 min. The dialysis membranes/mixture was then placed into a flask containing 250 mL of $NaHCO_3$ pH 7.5 and incubated in a water bath at room temperature for 3 h with stirring (60 osc/min). After dialysis, the obtained final solution of $NaHCO_3$ pH 7.5 was frozen and lyophilized for further assays.

2.6. Statistical analysis

For each plant material, three samples were used and all the assays were carried out in triplicate. When evaluating macroelements bioaccessibility, the results were expressed as mean values±standard deviation (SD) and differences were analysed using a *t*-student test, since there were fewer than 3 groups.

Regarding the evaluation of the effects of plant species (*A. millefolium*, *L. nobilis* or *T.* sect. *Ruderalia*) and formulation (dried plant or infusion), an analysis of variance (ANOVA) with type III sums of squares was performed using the Repeated Measures Analysis procedure of the General Linear Model. Since the independence of variables could not be assumed, it was need to verify the sphericity criterion, which evaluates if the correlation between treatments is the same, assuming that variances in the differences among conditions are equal. Sphericity was evaluated trough the Mauchly's test; every time the sphericity assumption was violated, the Greenhouse-Geisser correction was applied.

All the statistical analysis were carried out using SPSS v. 22.0 program (IBM Corp., Armonk, NY, USA).

3. Results and discussion

In the evaluated parameters, it was intended to verify the effects of plant species, independently of the used formulation, and the differences among formulations, regardless of the plant species. Accordingly, results were compared by a 2-way ANOVA, following the

generalized linear model coupled to the repeated measures analysis technique. In this analysis, it is important to check for the homogeneity of variances in the measures done for each of the factors' levels. Since the independence of variables cannot be assumed, the former requisite was evaluated by the Mauchly's sphericity test.

The results obtained for the infusions (which were prepared using ~1 g of dried plant material) were converted to be expressed in 100 g of dried plant basis to allow their direct comparison with those obtained from the direct analysis of the dried plant.

3.1. Effects on microelements

The results for iron, copper, manganese and zinc are given in Table 1. The evaluated factors, plant species (PS) and formulation (F) showed a significant interaction (PS×F) in all cases, indicating that the yields in microelements that can be obtained from the dried plant or its infusion are highly dependent of the used plant species (and *vice-versa*). This occurrence hampers the possibility of indicating the best plant (independently of the formulation) and the formulation with highest suitability to be used for microelements obtention (independently of the plant species). Nevertheless, the effect of each individual factor *per se* was also significant in all cases, allowing to indicate specific trends: dandelion seemed to be the best source of iron (29.3 mg/100 g dw), copper (1.87 mg/100 g dw) and manganese (5.1 mg/100 g dw), while laurel gave the highest contents in zinc (9.1 mg/100 g dw). The results obtained for yarrow samples are in the same range as those quantified previously, despite the higher zinc levels (6.61 mg/100 g) in Polish samples (Konieczyński & Wesołowski, 2007) and the lower levels of iron (2.65 mg/100 g). On the other hand, Divrikli et al. (2006) reported higher levels of iron and copper (31.67 and 1.76 mg/ 100 g, respectively), but similar concentrations of manganese and zinc (4.23 and 2.54 mg/ 100 g, respectively). In a study conducted in Turkish laurel samples, the levels of copper, iron and manganese were also detected in higher

amounts (Divrikli et al. 2006; Özcan, 2004; Zengin et al., 2008).

Among the formulations, using the powdered plant directly, instead of its infusion, would be the right option to maximize the yield in microelements. In fact, the extraction percentages for each microelement were quite dissimilar Mn<<<Zn<Cu<<Fe.

3.2. Effects on macro-elements

The results for calcium, magnesium and potassium are given in Table 2. The elements detected in highest amount in the samples of yarrow, laurel and dandelion were potassium, calcium and magnesium. In line with the observed for microelements, the interaction (PS×F) was significant ($p < 0.05$) in all cases. Nevertheless, the significant differences found for each factor (except for the effect of the formulation on the magnesium levels) allowed the identification of some overall trends. Dandelion showed the highest values in macro-elements (Ca: 882 mg/ 100 g dw, Mg: 223 mg/ 100 g dw; K: 2851 mg/ 100 g dw), while laurel gave the lowest (Ca: 283 mg/ 100 g dw, Mg: 88 mg/ 100 g dw; K: 484 mg/ 100 g dw), independently of their quantification in dried samples or their infusions. Chizzola et al., (2003) described lower values of mineral elements in a yarrow sample from Austria, while Özcan (2004) reported higher calcium content (1076.1 mg/100 g), but similar values for potassium (493.7 mg/ 100 g dw) in Turkish laurel samples. The powdered plants allowed higher macro-elements yields when compared to the samples prepared by infusion, but the extraction yields (particularly for magnesium and calcium) were higher than those achieved for the microelements. The concentration of mineral elements in infusions strongly depends on the type of bound formed with the plant cells, but also on its solubility in the solvent used for the extraction. In addition, the heat treatment may also have some influence in the final concentration of specific minerals in the infusions, since it can influence the extraction yield of these elements, breaking its connection with cell constituents (Pytlakowska et al., 2012).

Therefore, the differences found in the released percentage of minerals in the infusions could be explained by the obvious biological and botanical differences existing in the tissues of each one of the plants, which could modulate the extraction of mineral elements from the plant cells. When comparing the results obtained in the powdered plants and in the infusions, it might be concluded that manganese and potassium were, respectively, the micro- and macro-element that were most retained by the plants during the infusion process. In general, these results indicate higher extraction efficiency of mineral elements to infusions than the obtained by Zengin et al. (2008), despite the different solid to solvent ratios (1:200 in our case, 1:20 in the research reported by Zengin et al. (2008).

3.3. Vitamin B_9 in dry plant and infusions

Once again, the differences among the yields obtained using dried plant or its infusion depend on the assayed plant species (*i.e.*, the interaction PS×F was significant, Fig. 1). Regardless of the formulation, the highest amounts of vitamin B_9 were quantified in yarrow (257 μg/100 g dw), followed by dandelion (91 μg/100 g dw) and laurel, in which this vitamin was nearly absent (0.082 μg/100 g dw). In fact, the potential of vegetables to act as sources of vitamin B_9 varies greatly; some examples such as asparagus, spinach and okra are considered excellent, but others like as celery, kale, broccoli and even lettuce, contain very limited levels of this vitamin (Suitor & Bailey, 2000).

When comparing the dried plants with the corresponding infusions, a ~10-fold difference was detected (powder: 210 μg/100 g dw; infusion 22 μg/100 g dw). This can be explained by the fact that vitamin B_9 has high solubility and reactivity, being susceptible to degradation in many processing steps, including the high temperatures used for the infusions preparation (Scott et al., 2000). Furthermore, the potential retention of the vitamin B_9 native form by the

vegetal matrices, due to its interaction with other plant constituents that effectively could influence its bioavailability, is a well-known fact, which might also explain this difference.

The vitamin B_9 levels detected in yarrow and dandelion might offer new possible applications for these plant species. It has been stated that a rich vitamin B_9 diet reduces the risk of chronic diseases, such as cardiovascular problems. Several international organizations, and particularly the Food and Nutrition Board (Trumbo et al., 2002), have Recommended Dietary Allowance (RDA) of 400 μg of folic acid, with particular relevancy among pregnant women (Krawinkel et al., 2014). Moreover, according to the Regulation (EC) No. 1169/ 2011 (Regulation (EC) No. 1169/ 2011) of the European Parliament and of the Council, of 25 October 2011, on the provision of food information to consumers, it is necessary an intake of at least 7.5 and 15% of de NRV (Nutritional References Values) of this vitamin (200 μg/day) to consider the studied infusions and plants as "sources of vitamin B_9". The detected levels of vitamin B_9, despite relevant among natural sources, did not allow considering these plants as the sole daily source of this vitamin.

3.4. Bioaccessibility studies

After *in vitro* digestion only a few minerals were detected in all plant samples as it can be seen in Fig. 2. The majority of mineral found were macroelements (calcium, magnesium and potassium), despite the presence of low amounts of manganese. *A. millefolium* was the only plant that presented dialyzable minerals in both formulations, dried plant and infusion. Potassium and manganese were detected in the dried plant of yarrow (433.31mg/100g and 0.14mg/100 g, respectively, data no shown), which represented 26% and 2%, respectively of minerals that passed through the dialysis membrane. In the yarrow infusion, the only detected element was calcium (2.25 mg/100 mL, data not shown) that reached 76% of mineral passing through the membrane. *L. nobilis* only showed dialyzable minerals in the infusion form,

particularly potassium and calcium (1.33 mg/ 100 mL in both minerals), corresponding to 48% of mineral that passed after dialysis. On the other hand, no micro or macroelements were detected in laurel dried material after *in vitro* digestion. Probably, these elements were below the limit of detection of the AAS technique (usually limited to the ppm range).

In *T.* sect *Ruderalia* the dialyzable minerals were only detected in the dried plant. In this case, magnesium, calcium and manganese were not completely retained, yielding 5% (0.9 mg/100 g), 25% (214.7 mg/100 g) and 4% (7.9 mg/100 g) of their global amounts.

4. Conclusion

Dandelion showed the highest levels of all studied micro (except zinc, which showed the highest content in laurel) and macroelements, independently of the used formulation. On the other hand, yarrow gave the highest content in vitamin B9. Dried plants, as expected, allowed higher contents in all analytes when compared to the corresponding infusions; nevertheless, the extraction yields for mineral elements varied greatly, being higher for the macroelements: Mg>Ca>K>Fe>Cu>Zn>Mn. The levels of vitamin B_9 were much lower in the infusions, most likely due to the degradation induced by using boiling water.

Regarding the bioaccessibility, the elements with best performance in the dialysis process were calcium and potassium.

Overall with this preliminary study, the studied plant species, especially if used directly in the dried form, might be considered in the development of novel food formulations.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Table 1 Composition in micro-elements of powdered material and infusions (mg/100 g) of the studied wild samples. Results are presented as estimated marginal mean±standard error.

		Micro-elements			
		Fe	Cu	Mn	Zn
Plant species	Yarrow	4.8±0.1	0.79±0.01	3.8±0.1	2.3±0.1
	Laurel	5.9±0.1	1.22±0.03	1.2±0.1	9.1±0.1
	Dandelion	29.3±0.5	1.87±0.01	5.1±0.1	4.8±0.1
Mauchly's test of sphericity (*p*-value)		0.105 (<0.001)	0.496 (0.086)	0.132 (0.001)	0.062 (<0.001)
p-value[a]		<0.001	<0.001	<0.001	<0.001
Formulation	Powder	17.0±0.4	2.06±0.02	6.5±0.1	9.0±0.1
	Infusion	9.7±0.2	0.52±0.01	0.17±0.01	1.8±0.1
Mauchly's test of sphericity		1.000	1.000	1.000	1.000
p-value[a]		<0.001	<0.001	<0.001	<0.001
PS×F interaction					
Mauchly's test of sphericity (*p*-value)		0.024 (<0.001)	0.361 (0.028)	0.248 (0.008)	0.097 (<0.001)
p-value[a]		<0.001	<0.001	<0.001	<0.001

[a]Significance value for the tests of between subjects effects. When sphericity assumption was not met ($p<0.05$), the *p*-value was obtained from the Greenhouse-Geisser correction.

Table 2 Composition in macro-elements of dried material and infusions (mg/100 g) of the studied wild samples. Results are presented as estimated marginal mean±standard error.

		Macro-elements		
		Ca	Mg	K
Plant specie	Yarrow	395±5	172±5	1267±10
	Laurel	283±2	88±1	484±7
	Dandelion	882±8	223±2	2851±52
Mauchly's test of sphericity (*p*-value)		0.141 (0.001)	0.221 (0.005)	0.193 (0.003)
p-value[a]		<0.001	<0.001	<0.001
Formulation	Powder	564±5	167±4	1889±36
	Infusion	476±3	156±2	1178±8
Mauchly's test of sphericity		1.000	1.000	1.000
p-value[a]		<0.001	0.051	<0.001
PS×F interaction				
Mauchly's test of sphericity (*p*-value)		0.893 (0.673)	0.612 (0.180)	0.548 (0.122)
p-value[a]		<0.001	<0.001	<0.001

[a]Significance value for the tests of between subjects effects. When sphericity assumption was not met ($p<0.05$), the *p*-value was obtained from the Greenhouse-Geisser correction.

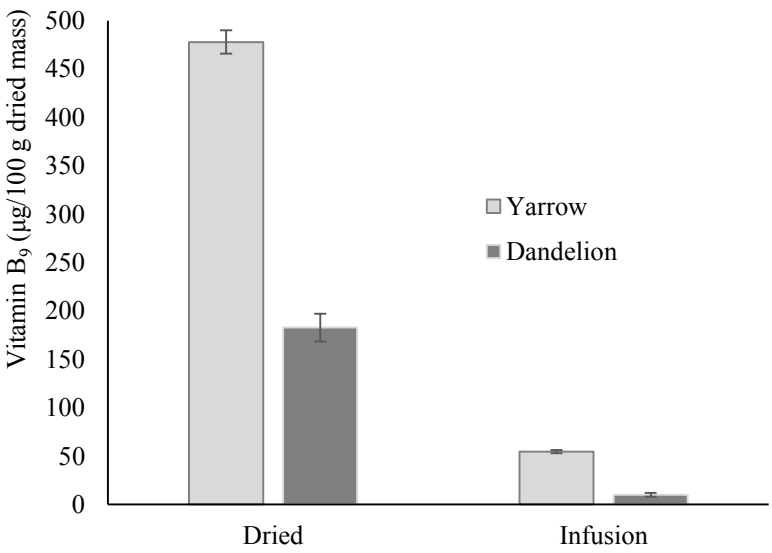

Fig.1. Estimated marginal mean plots representing the effect of plant species and formulation on vitamin B_9 levels. Bars corresponding to laurel samples were supressed due to their low magnitude (vitamin B_9 was nearly absent in laurel).

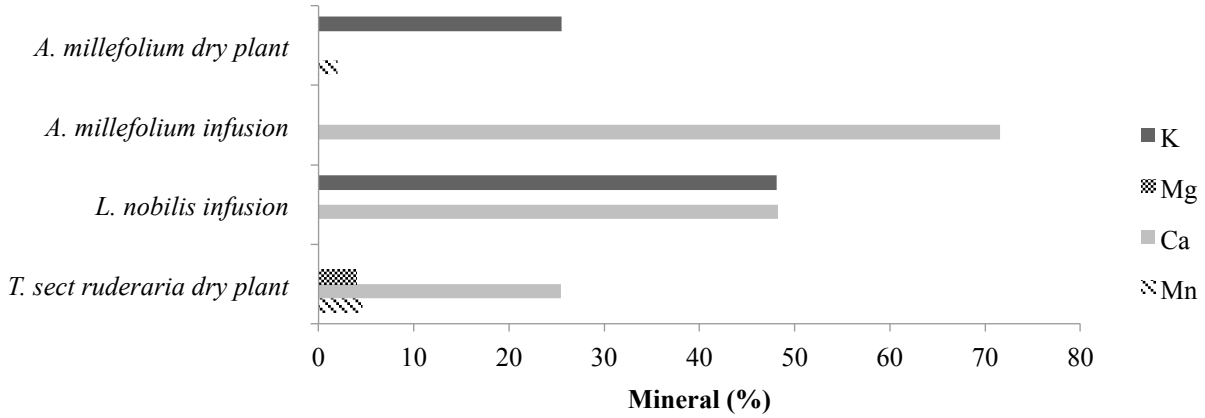

Fig. 2. Macro and microelements bioaccessibility percentages in *Achillea millefolium* L., *Laurus nobilis* L. and *Taraxacum* sect. *Ruderalia* infusions, after *in vitro* gastrointestinal digestion.