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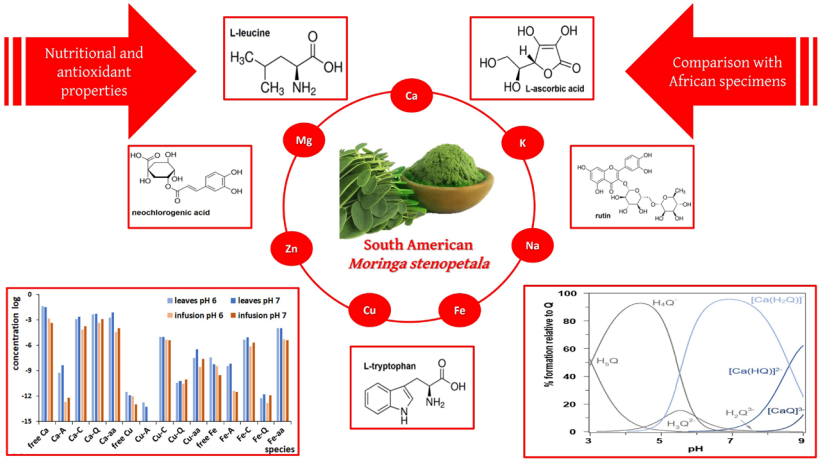
ARTICLE

Nutritional and Antioxidant Properties of South American *Moringa stenopetala*

Florencia Tissot[1], Maite Cuadrado[2], Jessica Santander[1], Julia Torres[2], Ignacio Machado[1]*

[1]*BIOESP, Área Química Analítica, Facultad de Química, Universidad de la República, Av. Gral. Flores 2124, 11800, Montevideo, Uruguay*

[2]*Área Química Inorgánica, Facultad de Química, Universidad de la República, Av. Gral. Flores 2124, 11800, Montevideo, Uruguay*



Moringa stenopetala is a tropical tree from the Moringaceae family, native to north-east Africa, that has widespread to many countries. It is highly valued for the associated nutritional and medicinal properties. The nutritional value of South American *Moringa stenopetala* was assessed in this work, giving some deeper insight into the expected biological accessibility of the main nutrients present in raw leaves and prepared infusions. Metal ions were determined by FAAS; L-ascorbic acid, leucine, and tryptophan by LC-DAD; rutin, isoquercitrin and neochlorogenic acid by LC-ESI-MS/MS. Metal ions content in analyzed specimens was in the same order of previously reported data, except for lower values for iron, probably associated to the different soil composition. Tested amino acids were also found in the previously reported range, while higher contents of L-ascorbic acid, rutin and neochlorogenic acid were determined. Interestingly, transfer factors to infusions resulted especially low for magnesium, calcium, iron, and L-ascorbic acid, indicating that the way of consumption exerts a strong influence on the nutritional value of the vegetal material. Chemical speciation modeling experiments to predict bioaccessibility showed that sodium, potassium, magnesium, and calcium ions to be predominantly free. On the other hand, the micronutrients are predicted to be partly associated to polyphenolic compounds. Finally, the antioxidant activity was studied both in the vegetal material and the prepared infusions by the ORAC assay, both showing relevant antioxidant activity probably associated to the high rutin content. This biomolecule, together with other polyphenolic compounds present, are expected to partially retain metal ions in solution, also contributing to the antioxidant beneficial properties of *Moringa stenopetala*.

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INTRODUCTION

Moringa stenopetala is a tropical tree that belongs to the *Moringaceae* family, which is native to north-east tropical Africa (some regions of Ethiopia, Kenya, and Somalia) but it is nowadays currently widespread to many tropical and sub-tropical countries.[1-3] It is used for human and animal feed, being highly valued as a source of minerals, carbohydrates, vitamins, crude protein, essential amino acids, and polyphenols.[2-10] Interestingly, the chemical composition of the different edible parts of *Moringa stenopetala* and the associated nutritional value is highly conserved throughout seasons. However, even though most studies are restricted to north-eastern African specimens, a geographical dependence has also been reported, which is mainly associated to the soil composition variation.[7,10,11]

Leaves, which are the most consumed part of *Moringa stenopetala* tree can be eaten directly in salads, but also grinded and incorporated into flours or extracted in infusions or soups.[2,6] *Moringa stenopetala* leaves stand out in the *Moringaceae* family as being rich in the macro nutrients potassium (K), magnesium (Mg) and calcium (Ca), in quantities similar to those found in foods usually recommended as a source of these metal ions like bananas, spinach and cow milk, respectively.[6-10,12] Essential micronutrients are also present in high content, especially iron (Fe), copper (Cu) and zinc (Zn).[7,8,10]

On the other hand, many relevant biomolecules are present in *Moringa stenopetala* including especially important vitamins such as L-ascorbic acid (H_2A in the neutral form), in levels similar to those found in oranges which are a good source of this nutrient.[6,12] The leaves are also a rich source of crude protein and many essential amino acids. Among the latter, L-leucine, (HL in the zwitterionic neutral form) shows the highest content.[8-10] Other essential amino acids, valine, isoleucine, phenylalanine, lysine, methionine, threonine, and tryptophan are also present in the leaves.[8-10] All of them share the same basic chemical structure but have different aliphatic chains attached to the anomeric carbon, which can lead to a different reactivity and function. Specially, L-tryptophan (HT in the neutral zwitterionic form), bearing an indole as side chain, has a more hydrophobic character than other amino acids and possibly offers a different acid-base character and reactivity towards metal ions.[13] Finally, *Moringa stenopetala* leaves are a natural source of polyphenols with antioxidant activity, among which rutin, (H_4R in the neutral form) is the most abundant, showing a similar content to that found in apples, which is a good source of polyphenols. The non-flavonoid polyphenol neochlorogenic acid (H_3N in the neutral form) is also present.[4,5]

The antioxidant activity, mostly associated to both L-ascorbic acid and the polyphenolic compounds present in *Moringa stenopetala* stands out as a highly beneficial nutritional value. Electronic transport systems using oxygen at cellular level have a high risk of generating unwanted byproducts called reactive oxygen species (ROS). These species, though vital for physiological processes, can lead to oxidative stress conditions when produced in excess.[14] The ability of *Moringa stenopetala* extracts to inhibit lipid peroxidation under oxidative stress conditions was previously reported.[15] The major antioxidant component of the leaves is rutin, while neochlorogenic acid appears in a second place.[5] Furthermore, the presence of these polyphenolic compounds together with L-ascorbic acid exerts a synergistic effect, providing a greater antioxidant protection.[16]

In this context, even though the nutritional and medicinal benefits of *Moringa stenopetala* have been previously assessed, some relevant related aspects are still not fully unveiled. First, the geographic influence on its chemical composition has been demonstrated only in a restricted geographical area, implying that further analysis of specimens grown in different locations is still needed to develop a better understanding on the matter.[10] Besides, since the different nutrients present in the leaves might chemically interact among each other, a more complete chemical study should be carried out in order to estimate biological accessibility of the different components both for the leaf direct intake and the infusion drinking. Furthermore, this chemical interaction must be considered for a full description of the antioxidant activity. Indeed, metal ion chelation can increase antioxidant activity, while the ability of some biomolecules to form

soluble stable coordination compounds can be relevant in decreasing Fenton reaction pathways, further increasing the observed antioxidant effect.[14,17,18]

We provide here in this work new information on the chemical composition of different samples of *Moringa stenopetala* leaves commercially available in Uruguay. The analytes assayed both on a dry basis and on infusions include the main macronutrients Na, K, Ca and Mg, the essential micronutrients Fe, Cu, and Zn, and some highly relevant biomolecules. The chemical interactions among the most relevant components were studied to give a deeper insight into the real nutritional value of this vegetal material. For this task, some new thermodynamic data, needed to model the interactions, were determined by potentiometry.[19] Finally, antioxidant activity was experimentally measured by means of the oxygen radical absorbance capacity (ORAC) assay, both on a dry basis and infusions.[20] Antioxidant activity was also interpreted in light of the chemical interaction among the most relevant nutrients.

MATERIALS AND METHODS

Chemicals

Standard solutions for calibration curves were prepared by serial dilution of 1000 mg L^{-1} stock solutions of Na, K, Mg, Ca, Fe, Cu, and Zn (Merck, Darmstadt, Germany) using ultrapure water.

The analyzed biomolecules were L-ascorbic acid, the essential amino acids L-leucine and L-tryptophan, and the major polyphenolic compounds rutin, isoquercitrin and neochlorogenic acid (Figure 1). For L-ascorbic acid determination, a 1000 mg L^{-1} standard solution prepared from 99% L-ascorbic acid (Sigma-Aldrich, St. Louis, USA) in ultrapure water was used. For the extraction, a 0.5% v/v H_3PO_4 solution prepared from 85% v/v H_3PO_4 (Merck, Darmstadt, Germany) was used. For L-leucine and L-tryptophan determination, 1000 mg L^{-1} standard solutions were prepared from 98% L-leucine (Sigma-Aldrich, St. Louis, USA) or 98% L-tryptophan (Sigma-Aldrich, St. Louis, USA) in 0.4 mol L^{-1} $HClO_4$. Quercetin 3-O-rhamnosylglucoside (rutin), quercetin 3-O-glucoside (isoquercitrin) and 5-caffeoylquinic acid (neochlorogenic acid) were used (Sigma-Aldrich, St. Louis, USA).

The metal ion solutions used for the potentiometric studies were prepared from $CaCl_2 \cdot 2H_2O$, $FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$ and $FeCl_3 \cdot 6H_2O$ (Sigma-Aldrich, St. Louis, USA) whereas the ligand solutions were prepared from 98% L-leucine (Sigma-Aldrich, St. Louis, USA), 98% L-tryptophan (Sigma-Aldrich, St. Louis, USA) and 3-caffeoylquinic acid (chlorogenic acid) (Sigma-Aldrich, St. Louis, USA). The acid stock solutions of the metal cations were standardized according to standard techniques.[21]

The standard HCl and NaOH solutions used in the potentiometric determinations were prepared by diluting Titrisol standard ampoules (Merck, Darmstadt, Germany) and standardized with Na_2CO_3 and $KC_8H_5O_4$ (potassium biphthalate) (Sigma-Aldrich, St. Louis, USA) respectively. The ionic strength was kept constant throughout the titrations by using solutions containing $NaClO_4$ (Sigma-Aldrich, St. Louis, USA). All the solutions were freed of carbon dioxide by argon (Ar) bubbling.

For the determination of the antioxidant activity, α,α′-azodiisobutyramidine dihydrochloride (AAPH) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were used (Sigma-Aldrich, St. Louis, USA).

For the optimization and validation of the analytical determinations, two certified reference materials (CRM) were used: apple leaves (NIST 1515) and brussels sprouts (BCR 431).

Ultrapure water of 18.2 MΩ cm resistivity (ASTM Type I) obtained from a Millipore™ DirectQ3 UV (Merck Millipore, São Paulo, Brazil) water purification system was used throughout this work.

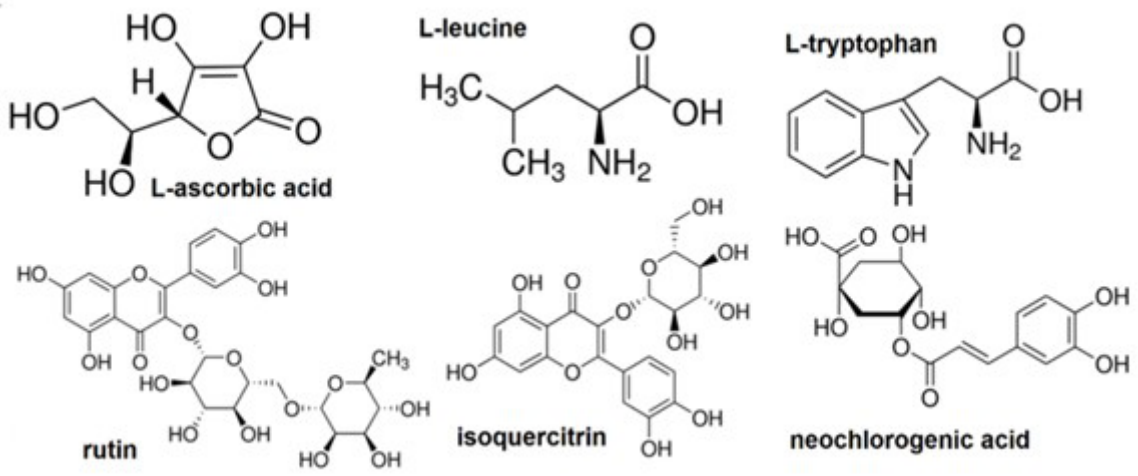

Figure 1. Chemical structures of *Moringa stenopetala* main biomolecules selected in this work.

Samples

A total of 27 samples of *Moringa stenopetala* leaves (50 g each) were purchased in local markets of Montevideo (Uruguay), originally cultivated in Brazil, Paraguay, and Venezuela. Leaves were identified by a botanist and a voucher specimen was deposited in the Herbarium of the Cathedra of Botany of the Faculty of Chemistry, Universidad de la República, Montevideo, Uruguay. Samples were washed with ultrapure water and dried at 70 °C using a Daihan ON-50 oven (Daihan Scientific, Seoul, South Korea). Afterwards, they were chopped using a blade mill and stored at 20 °C in the absence of light. In those cases where there were no CRM available, solid samples were spiked with appropriate volumes of standard solution before extraction and used for trueness evaluation by calculating the corresponding percentage recovery. Spiked samples were prepared with a concentration above the middle of the concentration range of each analyte.

Sample preparation procedure and analytical determinations

Determination of metal ions: Na, K, Mg, Ca, Fe, Cu and Zn

A microwave-assisted acid digestion was carried out using a CEM Mars 6 microwave oven (CEM, Charlotte, USA) provided with 12 Easy Prep Plus® vessels. For sample preparation, 0.5 g of the chopped sample was accurately weighed, and 10.0 mL of 4.5 mol L^{-1} HNO_3 were added into each vessel (three replicates per sample). The program set increased the power from 400 to 1800 W in a 15-minute ramp raising the temperature to 200 °C and then maintaining this temperature for 10 min; the maximum pressure was set at 500 psi.[22] All analytes were found at an adequate level to perform a simultaneous sample treatment. However, a further dilution with ultrapure water was required for some of the analytes: 1:25 (Na and K), 1:100 (Mg and Ca), 1:5 (Zn). Analytical determinations were performed by flame atomic absorption spectrometry (FAAS) using a Perkin Elmer AAnalyst 200 spectrometer (Perkin Elmer, Norwalk, USA). Lumina® hollow cathode lamps (Perkin Elmer, Norwalk, USA) were operated at the following analytical lines: 589.00 nm (Na), 766.49 nm (K), 285.21 nm (Mg), 422.67 nm (Ca), 248.33 nm (Fe), 324.75 nm (Cu), and 213.86 nm (Zn). A 10 cm burner was used. Flame gas composition was acetylene (2.5 L min^{-1}) - air (10.0 L min^{-1}). The same flame composition and observation height was used for the determination of all elements.[22]

Determination of L-ascorbic acid

In a typical experiment, 0.2 g of chopped sample was accurately weighed and 2.0 mL of 0.5% H_3PO_4 v/v (pH = 2.8) was added for extraction. The suspensions obtained were vortexed with a Vortex-5 (Qilinbeier,

Jiangsu, China) for 1 minute and then centrifuged in a Luguimac LC-15 centrifuge (Luguimac, Buenos Aires, Argentina) at 28000 *g* for 15 minutes. The obtained suspensions were filtered using Minisart® NY25 nylon filters of 0.45 µm (Sartorius, Göttingen, Germany).[23] Three replicates per sample were performed. No further dilution was needed. Analytical determinations of L-ascorbic acid were performed by liquid chromatography with diode array detection (LC-DAD) using an Agilent 1260 Infinity chromatograph (Agilent Technologies, Palo Alto, USA). The mobile phase consisted of a 0.5% H_3PO_4/acetonitrile mixture (95:5 v/v) at a flow rate of 0.7 mL min^{-1} in isocratic mode. A Zorbax Eclipse Plus C18 column (4.6 x 250 mm, 5 µm) (Agilent Technologies, Palo Alto, USA) was used as the stationary phase. Assays were performed at room temperature. Injection volume was 50 µL. The detection wavelength was 254 nm.[23] The total time of each chromatographic run was 10 min.

Determination of amino acids: L-leucin and L-tryptophan

For each L-leucine and L-tryptophan determination, 0.1 g of chopped sample was accurately weighed and 2.0 mL of 0.4 mol L^{-1} $HClO_4$ were added for extraction. The suspensions obtained were vortexed with a Vortex-5 (Qilinbeier, Jiangsu, China) for 5 minutes and then centrifuged in a Luguimac LC-15 (Luguimac, Buenos Aires, Argentina) at 28000 *g* for 10 minutes. The solutions were filtered using 0.45 µm Minisart® NY25 nylon filters (Sartorius, Göttingen, Germany).[24,25] Three replicates per sample were performed. Extracts were diluted 1:50 (L-tryptophan) and 1:100 (L-leucine). Analytical determinations of amino acids were performed by liquid chromatography with diode array detection (LC-DAD) using a Shimadzu LC-20AT (Shimadzu Corporation, Kyoto, Japan) equipment. A Phenomenex Luna C18 column (4.6 x 250 mm, 5 µm) (Phenomenex, Torrance, USA) was used as stationary phase. The mobile phase consisted of a water/acetonitrile mixture (90:10 v/v) at pH 2.7, in isocratic mode. The flow rate was 1.5 mL min^{-1} and the injection volume 20 µL. Assays were carried out at room temperature and the eluents were analyzed at 210 nm (L-leucine) and 273 nm (L-tryptophan), respectively.[24,25] Injection volume was 50 µL. The total time of each chromatographic run was 10 min.

Determination of polyphenolic compounds: rutin, isoquercitrin and neochlorogenic acid

To obtain the hydroalcoholic extract, 25 mL of methanol/water mixture (80:20, v/v) were added to 2.0 g of accurately weighed dried chopped leaves and magnetically stirred on a Daihan WHM12034 heating mantle (Daihan Scientific, Seoul, South Korea) for 30 min at 60 °C. The hydroalcoholic mixture was filtered through cotton and concentrated under reduced pressure in a Büchi RE111 rotary evaporator at 40 °C (Büchi Labortechnik, Flawil, Switzerland) and then freeze-dried in a Labotec 01.JLG lyophilizer (Labotec Group, Montevideo, Uruguay). All the extracts were transferred to amber flasks, sealed under nitrogen flow, and stored in the dark at -18 °C until analysis. Dried extracts were finally solubilized in 1.5 mL of methanol/water mixture (50:50, v/v). Three replicates per sample were performed. No further dilution was needed.[4,26] Analytical determinations of polyphenolic compounds were performed by liquid chromatography using an Agilent 1200 LC system (Agilent Technologies, Palo Alto, USA) equipped with a diode array detector, DAD, coupled to an AB Sciex 4000QTRAP quadrupole-linear ion trap (AB Sciex, Concord, Canada), operated in triple quadrupole MS/MS mode with electrospray ionization source (ESI). The separation was performed on a ZORBAX Eclipse XDB-C18 (150 mm x 4.6 mm, 5 µm) column (Agilent Technologies, Palo Alto, USA). The gradient as well as the column temperature, flow rate and injection volume were optimized to provide a good separation of the compounds. Solvent A was water/glacial acetic acid mixture (99:1 v/v) and solvent B was methanol. Flow rate was 1.0 mL min^{-1} and the gradient profile was: isocratic 10% B for 5 min, 10% to 30% B linear increase during 5 min, 30% B to 50% B linear increase during 10 min, 50% B to 70% B linear increase during 10 min, 70% B to 90% B linear increase during 5 min and finally linear decrease from 90% to 30% B during 10 min. The mass spectra were acquired with a scan range from *m/z* 100 to 1000. Injection volume was 10 µL. Tandem MS detection was performed using the multiple reaction monitoring (MRM) mode. The optimal MRM conditions for each analyte were optimized using direct injection in the ESI negative mode. Source

temperature was 350 °C, the ionization voltage was -4200 V, the nebulizer gas was air at 50 psi and curtain gas was nitrogen at 20 psi. The compounds were identified from the elution order, retention time and mass spectra as compared to pure standards analyzed under the same experimental conditions.[4,26]

Preparation of infusions

Infusions were prepared by accurately weighing 2.0 g of dried sample in a Pyrex® flask and adding 25.0 mL of ultrapure water at 100 °C. The mixtures were left at room temperature for 5 minutes and immediately filtered using a Whatman filter paper No. 541. These aqueous extracts were freeze-dried in a Labotec 01.JLG lyophilizer (Labotec Group, Montevideo, Uruguay), transferred to amber flasks, sealed under nitrogen, and stored in the dark at -18 °C until analysis. Extracts were finally solubilized in 1.5 mL of methanol/water mixture (50:50, v/v).[26]

Peroxyl radical scavenging assay

The ROO• scavenging capacity was measured by monitoring the effect of the tested extracts on the fluorescence decay resulting from ROO• induced oxidation of fluorescein and expressed as the ORAC assay.[27] ROO• was in turn generated by thermal decomposition of AAPH. Reaction mixtures in the sample wells contained fluorescein (60 nM), *Moringa stenopetala* freeze dried extracts described above (7 - 1000 µg freeze dried extract/mL) and AAPH (20 mmol L^{-1}); final volume was 200 µL. The mixture was pre-incubated in a BioTek Synergy HT microplate reader (BioTek, Winooski, USA) for 15 min at 37 °C. The fluorescence signal was then monitored every minute at the emission wavelength of 528 nm with excitation at 485 nm until total decay of fluorescence. Trolox (0.7 – 7.0 µg mL^{-1}) was used as control standard in each assay. The net protection provided by the putative antioxidant sample was calculated from the difference between the area under the fluorescence intensity decay curve (AUC) determined in the presence and absence of the sample. Regression equations for net AUC *vs*. *Moringa stenopetala* concentration (expressed as: freeze dried extracts/mL) were calculated. The relative ROO• scavenging capacity was then calculated as the ratio between the AUC *vs*. concentration slopes (S) for each sample (S Sample) and Trolox (S Trolox).[26] The AUC values were calculated using GraphPad Prism 7 software. Analogous assays using quercetin, rutin and L-ascorbic acid were also carried out for comparison as positive controls.

Interaction study among components

For thermodynamic studies of the interaction among the most relevant nutrients, a 0.15 M ionic strength and 37.0 °C were selected as the more representative conditions resembling the physiological duodenal conditions.[28] To carry out the thermodynamic studies, data previously reported in similar representative conditions for selected metal ions and biomolecules were used and are shown in Table I.[29]

Table I. Thermodynamic reported data used for speciation calculations

Biomolecule	Equilibrium	log *K*	Conditions
L-ascorbic acid (H_2A in the neutral form)	$A^{2-} + H^+ \rightarrow HA^-$ $A^{2-} + 2H^+ \rightarrow H_2A$	10.35 14.31	37 °C; 0.15 M $NaClO_4$
	$Ca^{2+} + A^{2-} \rightarrow [CaA]$	0.19	25 °C; 0.16 M NaCl
	$Cu^{2+} + A^{2-} + H^+ \rightarrow [Cu(HA)]^+$ $Cu^{2+} + A^{2-} + 2H^+ \rightarrow [Cu(H_2A)]^{2+}$	12.67 18.25	25 °C; 0.1 M KNO_3
	$Fe^{2+} + A^{2-} \rightarrow [FeA]$	7.09	37 °C; 0.15 M $NaClO_4$
	$Fe^{3+} + 2HA^- \rightarrow [Fe(HA)_2]^+$	27.06	25 °C; 0.05 M NaCl

(continues on the next page)

Table I. Thermodynamic reported data used for speciation calculations (continuation)

Biomolecule	Equilibrium	log *K*	Conditions
L-leucine (HL in the zwitterionic form)	$Ca^{2+} + L^- \rightarrow [CaL]^+$	3.96	25 °C; 0.1 M KNO_3
	$Cu^{2+} + L^- \rightarrow [CuL]^+$ $Cu^{2+} + 2L^- \rightarrow [CuL_2]$ $Cu^{2+} + L^- + H_2O \rightarrow [Cu(OH)L] + H^+$	7.902 14.533 2.324	37 °C; 0.15 M $NaClO_4$
L-Tryptophan (HT in the zwitterionic form)	$Ca^{2+} + T^- \rightarrow [CaT]^+$	2.01	35 °C; 0.1 M KNO_3
	$Cu^{2+} + T^- \rightarrow [CuT]^+$ $Cu^{2+} + 2T^- \rightarrow [CuT_2]$	8.23 15.78	37 °C; 0.15 M $NaClO_4$
Quercetin (H_5Qin the neutral form)	$Q^{5-} + H^+ \rightarrow HQ^{4-}$ $Q^{5-} + 2H^+ \rightarrow H_2Q^{3-}$ $Q^{5-} + 3H^+ \rightarrow H_3Q^{2-}$ $Q^{5-} + 4H^+ \rightarrow H_4Q^-$ $Q^{5-} + 5H^+ \rightarrow H_5Q$	10.06 19.8 27.4 33.36 36.4	37 °C; 0.15 M $NaClO_4$
	$Ca^{2+} + Q^{5-} \rightarrow [CaQ]^{3-}$ $Ca^{2+} + Q^{5-} + H^+ \rightarrow [Ca(HQ)]^{2-}$ $Ca^{2+} + Q^{5-} + 2H^+ \rightarrow [Ca(H_2Q)]^-$	5.4 15.1 23.7	
	$Cu^{2+} + Q^{5-} \rightarrow [CuQ]^{3-}$ $Cu^{2+} + Q^{5-} + H^+ \rightarrow [Cu(HQ)]^{2-}$ $Cu^{2+} + Q^{5-} + 2H^+ \rightarrow [Cu(H_2Q)]^-$	11.9 18.5 25.7	
	$Fe^{2+} + Q^{5-} \rightarrow [FeQ]^{3-}$ $Fe^{2+} + Q^{5-} + H^+ \rightarrow [Fe(HQ)]^{2-}$ $Fe^{2+} + Q^{5-} + H_2O \rightarrow [Fe(OH)Q]^{4-} + H^+$	4.3 13.9 -6.2	
	$Fe^{3+} + Q^{5-} + H^+ \rightarrow [Fe(HQ)]^-$ $Fe^{3+} + Q^{5-} + H_2O \rightarrow [Fe(OH)Q]^{3-} + H^+$	26.3 8.6	
Chlorogenic acid (H_3C in the neutral form)	$C^{3-} + H^+ \rightarrow HC$ $C^{3-} + 2H^+ \rightarrow H_2C$ $C^{3-} + 3H^+ \rightarrow H_3C$	12.5 20.7 24.05	25 °C; 0.2 M KCl
	$Cu^{2+} + C^{3-} \rightarrow [CuC]^-$ $Cu^{2+} + C^{3-} + H^+ \rightarrow [Cu(HC)]$ $Cu^{2+} + 2C^{3-} \rightarrow [CuC_2]^{4-}$	12.39 22.12 16.65	25 °C; 0.15 M NaCl

In order to complete this thermodynamic data, potentiometric studies of the following systems were carried out in 0.15 M $NaClO_4$ and at 37.0 °C: Fe(II)-leucine, Fe(III)-leucine, Fe(II)-tryptophane, Fe(III)-tryptophane, Ca-chlorogenic acid, Fe(II)-chlorogenic acid and Fe(III)-chlorogenic acid. For leucine, tryptophane and chlorogenic acid, protonation constants were also determined under the same experimental conditions.

All potentiometric experiments were performed in a high-resolution automated Mettler Toledo T50 Graphix titrator (Mettler Toledo, Greifensee, Switzerland) with a 50 mL thermostated cell. After thermal equilibrium was reached, hydrogen ion concentrations were determined by successive readings, each performed after a small incremental addition of 0.1 mol L^{-1} NaOH titrant standard solution. The equipment was controlled through LabX® Titration software (Mettler Toledo, Greifensee, Switzerland). A combined glass membrane pH electrode and Ag/AgCl reference electrode were used.

The ionic strength was kept constant throughout the titrations by using solutions containing 0.15 mol L^{-1} $NaClO_4$ and a relatively low concentration of metal ions. Pre-saturated Ar was bubbled through the solutions during titrations to eliminate the adverse effect of atmospheric CO_2. Temperature was set at (37.0 ± 0.2) °C and monitored by means of a DT1000 temperature sensor (Mettler Toledo, Greifensee, Switzerland). The

cell electrode potential E^0 and the acidic junction potential were determined from independent titrations of the strong acid with the titrant solution.[30] Data were analyzed using the HYPERQUAD program.[31]

At least 3 potentiometric titrations (*ca.* 150 experimental points each) were performed for the determination of L-leucin, L-tryptophan and chlorogenic acid protonation constants in a concentration interval ranging from 0.1 to 6.0 mmol L^{-1}, covering pH values between 2 and 10. In general, the initial pH of the ligand solution was adjusted to *ca.* 2 by addition of 0.1 mol L^{-1} HCl stock solution and then titrated with 0.1 mol L^{-1} NaOH stock solution. The behavior of the ligand in the presence of each metal ion was then analyzed under identical experimental conditions. At least 3 potentiometric titrations (*ca.* 150 experimental points each) of metal ions acid stock solutions (Ca(II), Fe(II) or Fe(III)) were carried out in the presence of the ligand (concentration ranging from *ca.* 0.1 to 5 mmol L^{-1}), using different metal to ligand molar ratios (1:1 to 1:3). For Fe(II), the potentiometric titrations were carried out until the oxidation to Fe(III) was observed, which is favored as pH values increase. In the case of Fe(III) the potentiometric titrations were carried out until the formation of a solid was observed due to hydrolysis of the metal cation. So, for Fe(II) or Fe(III) containing systems, only data collected in the acid interval were used for calculation. The previously reported formation constants of soluble hydroxo species of Fe(III) (log *K1 = -2.88; log *β2 = -5.56)[32] were taken into account in the calculation of the formation constants.

Simulations were carried out using both previously reported values and the stability constant values determined in this work. Species distribution diagrams were produced using the HySS program.[33]

RESULTS AND DISCUSSION

Determination of metal ions

The microwave-assisted method was validated for each metal ion based on the recommendations of Eurachem Guide.[34] For trueness evaluation, a comparative study using a Student's *t*-test was performed to establish whether there was a difference between the obtained values and the certified values of the apple CRM samples. It was concluded that, at the 95% confidence level, the concentrations did not differ significantly from the certified values. Precision (repeatability) expressed as RSD (%) for the analysis of the CRM samples (n = 6) was better than 10% for all elements. So, the accuracy of the method was ensured. The main figures of merit are shown in Table II.

Table II. Main figures of merit obtained for each analyte after validation

Nutrient	Linear range (mg L^{-1})	LOD (mg kg^{-1}, dry basis) (3σ; n=10)	LOQ (mg kg^{-1}, dry basis) (10σ; n=10)	RSD (%) (n = 6)	Certified or spiked concentration (mg kg^{-1})	Determined concentration (mg kg^{-1})
Na	0.094 - 1.0	0.56	1.87	3.7	24.4 ± 2.1[(a)]	25.1 ± 0.9
K	0.099 - 1.0	0.59	1.97	3.5	16080 ± 210[(a)]	16402 ± 574
Mg	0.014 - 0.5	0.08	0.27	2.9	2710 ± 120[(a)]	2737 ± 79
Ca	0.15 - 3.0	0.87	2.90	3.2	15250 ± 100[(a)]	15403 ± 492
Fe	0.017 - 3.0	0.10	0.34	2.5	82.7 ± 2.6[(a)]	81.0 ± 2.0
Cu	0.027 - 4.0	0.16	0.54	2.3	5.69 ± 0.13[(a)]	5.63 ± 0.13
Zn	0.013 - 1.0	0.08	0.26	2.1	1245 ± 0.43[(a)]	1220 ± 25
L-ascorbic acid	0.13 - 40.0	0.40	1.30	4.1	4.83 ± 0.24[(b)]	4.68 ± 0.19
L-leucine	0.045 - 4.0	0.27	0.90	6.9	20102 ± 1005[(c)]	20303 ± 1401
L-tryptophan	0.25 - 3.5	0.15	0.50	4.8	2205 ± 110[(c)]	2183 ± 105

(continues on the next page)

Table II. Main figures of merit obtained for each analyte after validation (continuation)

Nutrient	Linear range (mg L^{-1})	LOD (mg kg^{-1}, dry basis) (3σ; n=10)	LOQ (mg kg^{-1}, dry basis) (10σ; n=10)	RSD (%) (n = 6)	Certified or spiked concentration (mg kg^{-1})	Determined concentration (mg kg^{-1})
Rutin	0.001 - 0.25	0.005	0.025	12.7	40203 ± 2010[(c)]	38193 ± 4850
Isoquercitrin	0.002 - 0.25	0.010	0.050	15.8	4505 ± 225[(c)]	4099 ± 648
Neochlorogenic acid	0.001 - 0.25	0.005	0.025	10.5	15707 ± 785[(c)]	14607 ± 1534

(a) Apple leaves (NIST 1515); (b) Brussels sprouts (BCR 431); (c) Spiking procedure.

Linearity ranges were determined by visual inspection of the calibration curves, the correlation coefficient values (r^2) and the randomness study of the residuals. The correlation coefficients of the linear regressions obtained after performing the calibration curves were greater than 0.99 for all the analytes under study. The limits of detection (LOD) and quantification (LOQ) were expressed as the content of the analyte corresponding to 3 and 10 times the standard deviation of a blank, respectively, divided by the slope of the calibration curve.

Table III shows the mean concentration obtained for each nutrient in commercial samples of *Moringa stenopetala* leaves, analyzed using the previously validated methodologies. The macronutrients Na, K, Mg and Ca median total contents are consistent with previously found values for samples grown in different locations of East Africa (Table III).[7-10,12,35] Notwithstanding, K and Mg show lower median values for South American samples than the reported median figures for African specimens, even though similarly low values have also been previously found both in a specific location in Africa and also in Israel.[12,36] On the other hand, median Na and Ca content are higher for South American samples.

Table III. Concentration obtained for each analyte in the analyzed samples (dry basis)

Nutrient	Concentration (mg g^{-1})*	Transfer factor to infusion (%)
Na	5.5 ± 1.0	65.0 – 96.8
K	6.3 ± 0.6	22.9 – 27.9
Mg	3.4 ± 0.8	5.2 – 9.4
Ca	19.3 ± 1.8	4.2 – 5.4
Fe	0.057 ± 0.010	5.6 – 6.9
Cu	0.0068 ± 0.0008	41.2 – 62.3
Zn	0.022 ± 0.003	49.8 – 54.7
L-ascorbic acid	0.39 ± 0.06	0.5 – 1.0
L-leucine	15.9 ± 2.1	37.2 – 44.8
L-tryptophan	1.79 ± 0.35	31.4 – 38.5
Rutin	34.7 ± 5.8	49.5 – 60.0
Isoquercitrin	3.9 ± 0.7	33.3 – 47.3
Neochlorogenic acid	10.6 ± 1.8	79.6 – 87.9

*Concentration expressed as: mean value ± standard deviation (n = 27).

Table IV. Comparative nutritional value of *Moringa stenopetala* leaves and infusions

M	South American median values (mg g^{-1})	African median values (mg g^{-1})[(a)]	Daily requirement (mg)[(b)]	% Dietary requirement (100 g leaves)[(c)]	% Dietary requirement (100 g leaf infusion)[(d)]
Na	4.520	2.968	978	46.2	44.7
K	6.300	16.750	1099	57.3	16.0
Mg	3.200	4.970	79	405.1	21.1
Ca	18.370	14.900	595	308.7	13.0
Fe	0.0575	0.162	17.8	32.3	1.8
Cu	0.0064	0.0051	0.892	71.7	29.6
Zn	0.020	0.022	12.5	16.0	8.0

(a) Calculated from: Abuye *et al*., 2003;[12] Debela and Tolera, 2013;[35] Kumssa *et al*., 2017;[7] Melesse *et al*., 2009;[8] Melesse, 2011;[9] Melesse *et al*. 2012.[10] (b) Values taken from Stadtlander and Becker, 2017.[36] (c) Calculated from median values obtained in this work and shown daily requirement values. (d) Calculated as (c) but considering the lowest obtained value for transfer factor in Table III.

The micronutrients Fe, Cu and Zn show lower total concentration values than the more abundant alkali and alkaline-earth metal ions, as expected. Interestingly, Cu and Zn show similar median values to those previously reported in East African samples (measured median values are within ± 20% of previous median value), while the values found for Fe in South American specimens are lower: measured median value is 33% lower than the previous figure for East African samples (Table IV).[7,10] Again, the same two already mentioned previous reports for an African and an Israelian specimens show similarly low Fe contents.[12,36] These differences could be related to geographic conditions, soil composition, time of sowing and collection, etc. Indeed, in a previous study on *Moringa stenopetala* leaves, a significant correlation was found for Fe content and soil nutrients.[7] It is worth mentioning in this context that the median soil African Fe content is higher than the South American corresponding value: 3.831% and 0.980%, respectively.[37] Also, in line with soil composition dependence on Fe leaves content, a high variability in the elemental content of Fe, a fact again probably related to the regional soil composition variation,[7] was observed in South American samples.

Moringa stenopetala analyzed samples can be considered as a good source of minerals for the recommended daily intake, especially for Mg, Ca, and Cu (Table III).[36] However, despite the high mineral content, low infusion transfer factors were obtained especially for Ca, and in a lower extent for Mg, possibly due to the way in which these elements bind to the matrix.[12] Indeed, if we consider the obtained median values and we compare them with the nutritional daily requirements (Table III), Ca content in an infusion prepared from 100 g of *Moringa stenopetala* leaves contains only a low percentage of the recommended daily value for Ca intake. On the other hand, for the micronutrient Cu, near 30% of daily recommended intake of this essential mineral remains available in the infusion of 100 g of *Moringa stenopetala* leaves.

Determination of L-ascorbic acid

The validation of the analytical method was performed according to Eurachem Guide recommendations.[34] Figures of merit are shown in Table II. Trueness evaluation was performed using the Brussels sprouts CRM (n = 6). After performing a Student´s *t*-test, the experimental value of *t* was found below the theoretical value of *t* (0.05, 5) = 2.57. Therefore, it could be concluded, with 95% significance level, that the concentration values obtained using the developed method did not differ significantly from those of the certified value. Precision (repeatability) expressed as RSD (%) for the analysis of the CRM samples (n = 6) was 4.1%. The LOD and LOQ were expressed as the content of the analyte corresponding to 3 and 10 times the standard deviation of a low concentration standard, respectively.

The validated method was then applied to the analysis of commercial samples of *Moringa stenopetala*. Table III shows the obtained results; median value was 0.365 mg g^{-1}, a value slightly higher than the unique previously reported value of 0.280 mg g^{-1} in an African sample.[12]

However, transfer factors of L-ascorbic acid to infusions are extremely low, a fact already observed in other *Moringa* species, due to the well-known decomposition at high temperature of this biomolecule.[38]

Determination of polyphenolic compounds

The LC–ESI–MS/MS method was validated for simultaneous quantification of three major polyphenolic compounds in *Moringa stenopetala* samples. The selectivity of the proposed method was assessed by MS/MS after the chromatographic separation, that showed no interference in the selected transitions. As shown in Table II, repeatability expressed as RSD% was below 20%. The method proved to be reliable for the determination of the studied polyphenolic compounds, being the recoveries within the recommended acceptable limits (70–110%).

The LOQ was estimated as the lowest concentration at which an acceptable precision was achieved (signal/noise ratio ≥ 10 and RSD ≤ 20%) while the LOD was estimated as the lowest concentration distinguishable from zero (signal/noise ratio ≥ 3). These limits were established based on the mean and relative standard results obtained for six independent replicates by spiking *Moringa stenopetala* samples with appropriate concentrations (Table II).

Rutin, isoquercitrin and neochlorogenic acid obtained concentrations are presented in Table III. Median values obtained for rutin, neochlorogenic acid and isoquercitrin were 31.9, 9.7 and 3.1 mg g^{-1}, respectively. The levels obtained are higher than previous reported values for rutin (23.4 and 3 mg g^{-1}) and neochlorogenic acid (6.2 mg g^{-1}).[4,5] No previous report is available for isoquercitrin in *Moringa sp*. for comparison.

Transfer factors to infusions of the most abundant polyphenols rutin and neochlorogenic acid were high, as shown in Table III. These results are in line with previous findings.[39] Isoquercitrin showed lower results.

Determination of amino acids

The validation of the analytical method was performed following Eurachem Guide recommendations.[34] Obtained figures of merit are shown in Table II. Trueness evaluation recovery studies (spiking experiments) were performed to give an indication of the bias level. The relative spike recovery R (%) was calculated by comparing the difference between the mean spiked value and the mean value of the sample, with the added concentration. The method proved to be reliable for this application, being R (%) within the recommended acceptable limits (70–110%).[22] Repeatability expressed as RSD (%) was 6.9% (L-leucine) and 4.8% (L-tryptophane), respectively. The LOD and LOQ were expressed as the content of the analyte corresponding to 3 and 10 times the standard deviation of a low concentration standard, respectively.

Table III shows the concentrations obtained for these two selected essential amino acids. Median obtained values for L-leucine and L-tryptophan are 17,3 and 1,56 mg g^{-1}, respectively. These values agree with results previously reported for *Moringa stenopetala*.[8-10,36] For the most abundant amino acid leucine having more previous reports, South American median value is slightly higher than East African corresponding value of 13.2 mg g^{-1}.[8-10,36]

Transfer factors for these essential amino acids are relatively high, L-tryptophan showing a slightly lower value, as expected from its lower solubility in water.

Chemical insight to the complex interaction of nutrients

The nutritional value of any food is affected by the possible in solution interaction among nutrients during the preparation, consumption, and travel across the gastrointestinal tract up to the point of absorption, normally at the duodenum. In this path, an intertwined set of chemical reactions can occur.[40] Here we estimated the maximum free concentration of each relevant nutrient, considering the other components present in the highest concentrations, as well as their chemical reactivity and their expected interactions from a thermodynamic point of view. Focus will be put on the duodenal conditions, since previous processes

occurring in the upper tract are mainly responsible for food disintegration and nutrient solubilization, but duodenum is where the most relevant part of absorption takes place.[40,41] Conditions are accordingly set at 37.0 °C, 0.15 M strength and a pH value of *ca.* 6.5 will be focused according to the more representative reported values.[28] This strategy allows us to give a first estimate of the maximum biological accessibility of the main nutrients.

In this approach we will consider the metal ions K, Ca, Fe(II), Cu and Fe(III) as representative of the most relevant metal ions present in the leaves of *Moringa stenopetala*. This choice is primarily based on two main facts: i) metal ion oxidation state determines the strength of the interaction, and ii) the higher the individual concentration of a metal ion is, the stronger the influence of this ion in the final speciation becomes. In the case of +2 oxidation state, an alkaline earth, Ca, and two transition metal ions, Fe and Cu, were selected in order to cover the different reactivities. Among biomolecules, L-ascorbic acid, L-leucine, L-tryptophan, quercetin and chlorogenic acid, were also chosen as representative ligands. L-ascorbic acid is present in high concentration in the raw material. L-leucine is also an abundant biomolecule and L-tryptophane accounts for an amino acid with a slightly different reactivity due to the presence of the indol ring in the structure. Quercetin was selected because it is the basic flavonoid present in the tested glycosides rutin and isoquercitrin and the three molecules present remarkably similar reactivity towards metal ions.[17] Chlorogenic acid is used to model the less accessible optic isomer neochlorogenic acid. Considering all thermodynamic data, the general trend shows that +1 ions normally show extremely weak interactions with biomolecules due to their low charge, while +2 and especially +3 metal ions are expected to interact in solution to a higher extent.[29]

Results of potentiometric studies carried out to complete the thermodynamic data necessary for modeling all possible equilibria at 0.15 M $NaClO_4$ and 37.0 °C are shown in Table V. Results are in good agreement with previously determined data under similar conditions, when available.[29]

Table V. Logarithms of the overall formation constants determined in 0.15 mol L^{-1} $NaClO_4$ at 37.0 °C

Biomolecule	Equilibrium	log *K*	Σ
Leucine	$L^- + H^+ \rightarrow HL$ $L^- + 2H^+ \rightarrow H_2L^+$	9.33(1) 11.81(3)	1.7
	$Fe^{2+} + L^- \rightarrow [FeL]^+$	8.7(1)	0.4
	$Fe^{3+} + L^- \rightarrow [FeL]^{2+}$	10.1(1)	0.4
Tryptophan	$T^- + H^+ \rightarrow HT$ $T^- + 2H^+ \rightarrow H_2T^+$	9.67(1) 13.32(2)	1.1
	$Fe^{2+} + T^- \rightarrow [FeT]^+$	9.33(3)	0.2
	$Fe^{3+} + T^- \rightarrow [FeT]^{2+}$	11.69(3)	2.5
Chlorogenic acid	$L^{3-} + H^+ \rightarrow HL^{2-}$ $L^{3-} + 2H^+ \rightarrow H_2L^-$ $L^{3-} + 3H^+ \rightarrow H_3L$	10.74(2) 18.81(3) 22.20(4)	1.0
	$Ca^{2+} + C^{3-} \rightarrow [CaC]^-$ $Ca^{2+} + C^{3-} + H^+ \rightarrow [Ca(HC)]$	4.4(1) 14.1(2)	0.5
	$Fe^{2+} + C^{3-} \rightarrow [FeC]^-$ $Fe^{2+} + C^{3-} + H^+ \rightarrow [Fe(HC)]$	11.29(5) 17.58(5)	0.1
	$Fe^{3+} + C^{3-} \rightarrow [FeC]$ $Fe^{3+} + C^{3-} + H_2O \rightarrow [Fe(OH)C]^- + H^+$	17.63(7) 14.6(1)	0.3

HL and HT represent the zwitterionic form, while H_3C represents the neutral form. Values given in parentheses are the 1σ statistical uncertainties in the last digit of the constant. For the Fe(III) containing system, hydrolysis constants determined under the same experimental conditions ($\log_{10} {}^*K_1$ = - 2.88, $\log_{10} {}^*\beta_2$ = - 5.56)[32] were included in the Hyperquad input.

Results of the simulations carried out using the median total contents of the selected nutrients for 100 g leaves intake (either as raw material or after preparing an infusion) and both available and here determined thermodynamic data (Tables I and IV) are summarized in Figure 2. It is worth mentioning that previously determined concentrations coming from duodenal biological content were not summed up since they are always much lower than those calculated for 100 g of *Moringa stenopetala* ingestion, even considering the dilution of the sample up to 1 L before arriving to the duodenum.[42,43] Results show that for 100 g intake of raw leaves, Ca, being more abundant, remains predominantly free: 70-80% is predicted to be as the aqua ion in the pH interval 6-7. On the other hand, transition metal ions forming more stable species and being less abundant are expected to be almost totally associated with the biomolecules present, depending on their coordination chemistry preferences. For example, at pH 6.5, Cu is predicted to be almost totally associated to chlorogenic acid, whereas Fe(II) forms complexes in high percentage with amino acids, especially with the more abundant L-leucine (Figure 2a).

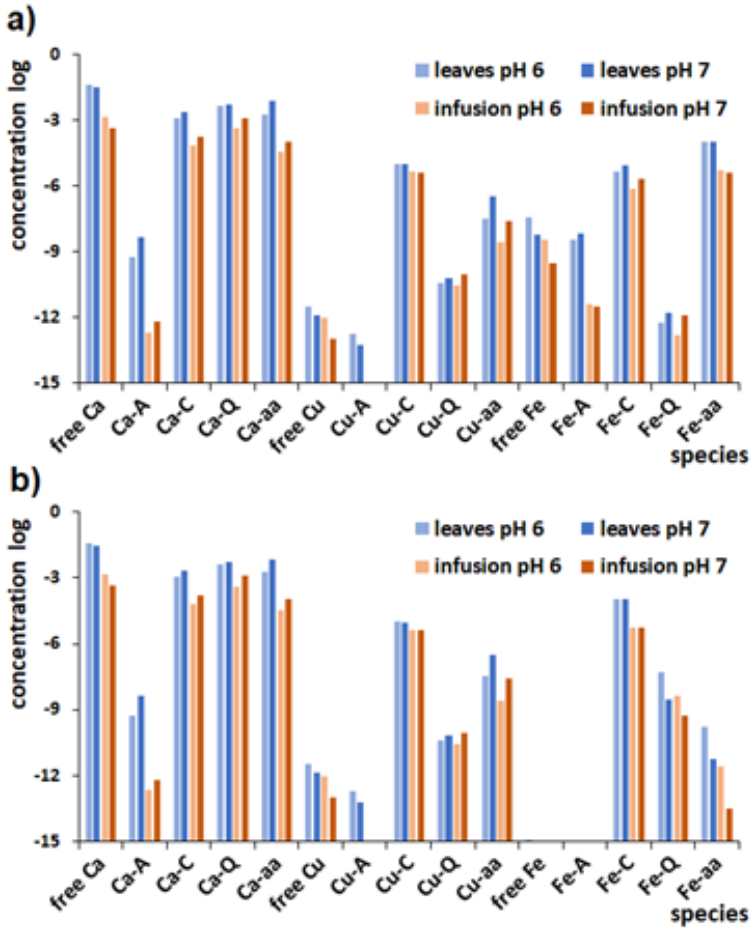

Figure 2. Results of simulations of nutrient interactions using median determined values for total concentrations and transfer factors as well as data from Tables I and IV. Letters A, C, Q and aa represent L-ascorbic acid, chlorogenic acid (as a model of neochlorogenic acid), quercetin (modeling both rutin and isoquercitrin) and amino acids (summing up species of leucine and tryptophane). M-L in each case represents the sum of species containing both the metal ion and each ligand in various protonation degrees. Molar concentration logarithms of each species are shown (if higher than 10^{-15} mol L^{-1}). a) Fe is reduced. b) Fe is oxidized.

The oxidation state of Fe under the conditions of absorption can be assumed to be +2 (as assumed in Figure 2a) due to the reducing medium of duodenal environment and the fact that L-ascorbic acid is also present in the simulated intake in high amounts. In the case of partial oxidation, Fe(III) would be expected to be associated almost completely to polyphenolic compounds (Figure 2b).

Other biomolecules that might be also present in the gastrointestinal tract after each meal could also compete for these ions, giving place to the formation of other competing complexes. This intertwined set of equilibria can change the concentration of the soluble species formed by each metal ion, influencing their accessibility. The pH value has also some influence on speciation, even within the possible short interval 6-7 normally expected for absorption conditions (Figure 2).

On the other hand, if we focus on infusions, considering the determined transfer factors, metal ion contents of the simulated intake are much lower. Besides, the observed degradation of L-ascorbic acid and the retention of polyphenolic compounds might favor Fe oxidation.[18] Indeed, results under that conditions show that Ca appears mainly associated to quercetin (mainly because other competing metal ions are in much lower concentrations) whereas Cu and Fe(III) remain mostly associated to polyphenolic compounds (Figure 2b).

Scavenging capacity against ROS

Table VI shows the obtained results. Both the hydroalcoholic extracts and the infusions have very efficient scavenging capacity compared to Trolox, accounting for a high antioxidant activity of the analyzed samples. Comparison with rutin, quercetin and L-ascorbic acid as positive controls, shows the high antioxidant power of *Moringa stenopetala* extracts. It is important to note that the high antioxidant activity of the extracts could not be attributed just to one specific phenolic compound but rather to the agonist action of the mixture of several bioactive compounds. However, according to the literature, the major antioxidant principle of the leaves is rutin while neochlorogenic acid appears in a second place.[14] It could be speculated that the high scavenging activity of *Moringa stenopetala* against ROO• is related to its high rutin level, which is the most abundant phenolic compound in the samples. This is supported by the fact that a positive correlation between antioxidant potency of *Moringa stenopetala* leaves extracts and rutin content has been previously stated.[14] For instance, studies have demonstrated that the antioxidant potential of *Moringa stenopetala* is significantly higher than that of *Moringa oleifera*, primarily due to differences in rutin concentration.[44] Flavonoids such as rutin, are characterized by their ability to stabilize free radicals by two different mechanisms: (1) transfer of a hydrogen atom from the antioxidant to the free radical (˙R) and (2) transfer of individual electrons where only the donation of an electron to the radical (˙R) is established.[45] Also, another important characteristic of flavonoids is its ability to chelate metal ions, such as Ca and Fe, as previously mentioned.[17] All these properties give these compounds a high free radical stabilizing ability and thus a high antioxidant capacity.

Table VI. Peroxyl radical (ROO•) scavenging capacities of *Moringa stenopetala* extracts

Extract	ROO• (S_{sample}/S_{Trolox})
dry leaves	0.42 ± 0.07
infusion	0.27 ± 0.04
quercetin	1.39 ± 0.09
rutin	0.97 ± 0.09
L-ascorbic acid	1.51 ± 0.08

Indeed, the same approach previously used could be used to predict the main species present both in the hydroalcoholic extracts and infusions to give some chemical rationalization of the results. Assuming that all nutrients are extracted in the cold hydroalcoholic solution, at neutral pH neochlorogenic acid is expected to be free whereas the more abundant flavonoids are expected to be associated with metal ions, especially to Ca which is the most abundant (Figure 3a). On the other hand, in the case of infusions, a lower content of antioxidant species is expected to be present due the low transfer factors determined. Besides, flavonoids are expected to be partly free due to the lower metal ion content (Figure 3b), also in line with a lower antioxidant activity. Apart from this, L-ascorbic acid is almost depleted during infusion, which is another factor that could negatively influence the antioxidant activity of the infusion.

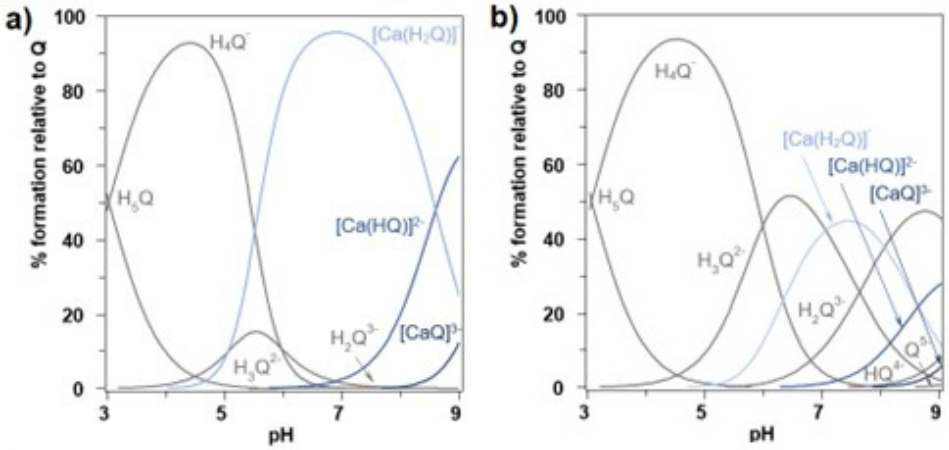

Figure 3. Distribution species diagrams produced by HySS software using data from Tables I and IV. a) Simulation total median concentrations of nutrients present in 100 g raw leaves extracted to solution. b) Simulation considering transferred concentrations to the prepared infusion.

CONCLUSIONS

Simple methods of sample preparation for the extraction and subsequent determination of metal ions, L-ascorbic acid, the amino acids L-leucine and L-tryptophan and major polyphenolic compounds were optimized and validated for *Moringa stenopetala* leaves. The nutritional value of South American samples confirms this vegetal as a good source of minerals, especially of Mg, Ca, and Cu. On the other hand, high contents of L-ascorbic acid and the polyphenolic compounds rutin and neochlorogenic acid account for a high antioxidant beneficial effect, which was also assessed. Transfer factor of main nutrients to infusion show that the way of consumption might markedly influence the nutritional value of *Moringa stenopetala*, especially in the case of Fe and L-ascorbic acid. Thermodynamic simulations show that the intertwined chemical interaction among the most abundant components might influence the biological accessibility of nutrients as well as their antioxidant activity.

Disclosure statement

The authors declare that there is no conflict of interest regarding the publication of this article.

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