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Content and bioaccessibility of bioactive compounds with potential benefits for macular health in tiger nut products

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ABSTRACT

Clinical studies have associated the consumption of tiger nuts (*Cyperus esculentus* L.), and its derivates, with improved macular health, due to the presence of certain liposoluble compounds with antioxidant activity, especially to lutein and zeaxanthin. However, the benefits derived from ingestion of tiger nut products may vary depending on the release of these compounds from the food matrix to the digestive fluids and their eventual bioabsorption. Therefore, this study aims at evaluating the influence of the food matrix on the liberation and final bioaccessibility of bioactive compounds with positive impact on macular health in different tiger nut products (flour, oil, and milk with and without added sucrose). The *in vitro* digestibility of macronutrients and the release of vitamin E, lutein, zeaxanthin, β-carotene, and total polyphenols of tiger nut products was studied. In addition, the antioxidant activity of the bioaccessible fraction was determined by ABTS, DPPH and FRAP assays. Results showed that tiger nut milk resulted in the greatest proteolysis and total polyphenols bioaccessibility. Moreover, flour can contribute to hypoglycaemia and, alongside oil, were the best sources of carotenoids and vitamin E. Gastrointestinal digestion increased the antioxidant activity of milk, but the addition of sucrose decreased this effect. The food matrix was a highly relevant factor protecting or hindering the digestion and release of bioactive compounds in the digestive environment. Thus, tiger nut products are presented as ingredients with great potential to develop functional foods oriented to macular health.

1. Introduction

Ocular deficiencies such as glaucoma, dry eye syndrome, and age-related macular degeneration (AMD) are the most prevalent visual impairments affecting the quality of life in developed countries (Gehrs et al., 2006; Quillen, 1999). By 2050, the estimated number of people with age-macular degeneration is expected to be more than twice the current prevalence of 2.07 million (Klein & Klein, 2009; Age-Related Macular Degeneration (AMD) Data and Statistics | National Eye Institute., 2019). In Europe, the number of persons affected by AMD, in particular, is expected to increase to 18.8 million in 2040 (Kawasaki et al., 2010), along with life expectancy. AMD, which mainly affects central vision, is a consequence of the high oxidative stress level of photoreceptors and retinal pigment epithelium because of light exposure.

This exposure, together with other non-genetic (sex, age, lifestyle, among others) and genetic factors, modulate ADM. Among lifestyle factors, long-time use of LED screens and diet have been reported to play a crucial role on AMD. Nutrients with antioxidative properties, in particular lutein and zeaxanthin, zinc, vitamins C and E, and polyunsaturated fatty acids, have shown to be beneficial for macular integrity. Evidence for these beneficial effects stems from trials with high-dose supplementation and from reports on dietary intake (Khoo et al., 2019, p. 8; Rinninella et al., 2018, p. 167). Olivares-González et al. (2021), p. 103 reported the ability of antioxidant species in improving the function and morphology of the retina, besides reducing the redox and inflammatory situations in mouse models. Thus, the combination of lutein and zeaxanthin (known as macular pigments) with zinc has shown to be highly effective as it is closely related to the enzymes responsible for both pigmentation of the eye and maintenance of the retinal pigment epithelium (Bian et al., 2012; Pao et al., 2018). Similarly, several studies confirm that a diet rich in vitamins C and E, carotenoids and polyphenols can help reducing the risk of suffering from this disease (Coleman & Barnard, 2007). On the other hand, vitamin E improves the functionality

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of lutein, thus protecting the oxidation of the retinal epithelial pigment cells as blue filter (Feng et al., 2010). In this context, most of the current therapies and clinical treatments consist of the oral supplementation of these compounds. The supplement format ensures the recommended daily intake of the cited compounds, as otherwise they are present with low bioaccessibility in foods. Oral supplementation, however, can turn out expensive and difficult to incorporate to the daily lifestyle. This is why functional foods specifically designed for contributing to the macular health might be considered as an alternative to oral supplementation. Thus, certain plant foods such as dark green leafy vegetables, cabbage, nuts or roots have been postulated as good vehicles to maintain the macular pigment optical density and to help control dyslipidaemia and oxidative stress (Eisenhauer et al., 2017, p. 12). Among them, tiger nut (*Cyperus esculentus* L.*)* stands out by its nutritional point of view. This small tuber comes from the roots of the hazelnut sedge, it is very appreciated in the Spanish Mediterranean region, and also cultivated in other parts of the world, especially in the American regions of Chile and Brazil (Sánchez-Zapata et al., 2012). Tiger nut's fat content can represent 20% depending on the variety and cultivation conditions, being even higher than nuts like walnuts and peanuts. Its lipid profile is characterized by a high presence of polyunsaturated fatty acids, representing 80% approx., oleic acid being the most abundant (Roselló-Soto, Poojary, et al., 2018). Tiger nuts are also rich in vitamin E, specifically in α-tocopherol, which gives it great antioxidant properties. In this sense, the daily consumption of 30 g of soaked tiger nuts for three months have been proven to increase the volume of macular pigment until 50% in the clinical trial conducted by Valero-Vello et al. (2021), p. 1. Besides, the daily consumption of tiger nut was found to positively contribute to cardiovascular health with a decrease of low-density lipoproteins coupled with an increase of high density lipoproteins. Despite the mentioned benefits, oral mastication of the soaked tuber can result in uneasiness, especially for older people with teeth disorders. Nevertheless, the inclusion of tiger nut products such as liquid extract also called "tiger nut milk", oil or flour, alternatively to the direct consumption of the soaked tuber, could be taken into account in the development of functional foods for macular health. Thus, not only is the bioactive compound concentration in the different tiger nut fractions relevant for their selection, but also the release of the bioactive compounds from the food matrix to the digestive fluids, i.e., bioaccessibility, and thus their potential bioabsorption and impact on macular health (Perales-Vázquez et al., 2020). Regarding lipophilic compounds, such as carotenoids, vitamins A, E or D, the relationship between lipolysis extent and their bioaccessibility has been highlighted for the correct assimilation of carotenoids (Nagao et al., 2014; Rostamabadi et al., 2019). Similarly, proteins:lipids and carbohydrates:lipids ratios in food matrix also impact fats hydrolysis and carotenoids released (Calvo-Lerma, Asensio-Grau, et al., 2020; Laurent et al., 2007).

Currently, there is scarce evidence of the impact of tiger nut structural matrix on the hydrolysis and release of macro and micronutrients along gastrointestinal digestion. *In vitro* standardized digestion models have gained special attention in the field of food science and technology as they present clear advantages compared to *in vivo* models (Brodkorb et al., 2019; Minekus et al., 2014) to determine the food matrix behaviour at gastrointestinal level under a controlled and comparable environment. In this context, the goal of this study was to analyse the role of four tiger nut matrices on macronutrients digestibility and release of antioxidant compounds with potential benefits for macular health after *in vitro* simulated digestion.

2. Materials and methods

2.1. Raw material

Commercial tiger nut products used in this study were whole flour, oil and milk with and without added sucrose, all of them supplied by a local company (Mon Orxata, Valencia, Spain). Milk with added sucrose was also considered because tiger nut milk is commonly consumed with added sucrose and this fact could affect other macro and micronutrients digestion.

2.2. Chemicals

The following reagents were used for the preparation of simulated digestive fluids: porcine gastric mucosal pepsin (3200–4500 U/mg), porcine pancreatin (8 × USP), bovine bile, potassium chloride, potassium dihydrogen phosphate, sodium hydrogen carbonate, sodium chloride, magnesium chloride, ammonium carbonate, calcium chloride, sodium hydroxide and hydrochloric acid, all obtained from Sigma-Aldrich (St. Louis, MO, USA).

For analytical determinations, bovine serum albumin, gallic acid, Trolox, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,3,5-triphenyltetrazolium chloride (TPTZ), iron (III) chloride hexahydrate, sodium acetate trihydrate, acetic acid, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), potassium persulfate, trichloroacetic acid (TCA), ethanol (HPLC grade, ≥99.9%), methanol (HPLC grade, ≥99.9%), tetrahydrofuran (HPLC grade, ≥99.9%), ascorbic acid, pyrogallol acid (HPLC grade, ≥98%), lutein (HPLC grade, ≥96%), zeaxanthin (HPLC grade, ≥95%), β-carotene (HPLC grade, ≥95%) and α-tocopherol (HPLC grade, analytical standard), deuterated chloroform (99.8 atom % D, containing 0.03% (v/v) tetramethylsilane (TMS) as internal standard), all obtained from Sigma-Aldrich (St. Louis, MO, USA). In addition, acetonitrile (JT-Baker, Center Valley, PA, USA) and *n*-hexane, ethyl acetate and potassium hydroxide, all purchased from Scharlau (Barcelona, Spain).

2.3. Static in vitro digestion

In vitro digestion of the tiger nut products was carried out in a static system according to the INFOGEST protocol published by Minekus et al. (2014) and Brodkorb et al. (2019) with some modifications. The simulation of gastrointestinal digestion entails the oral, gastric and small intestine stages, and relies on the simulation of the conditions of pH, enzyme and bile concentrations, temperature, agitation and residence time in each stage, among other variables. The formulated digestive fluids of each phase are a mixture of electrolytes and enzymes which are specific to each stage. The simulated gastric (SGF) and intestinal (SIF) fluids were prepared fresh daily from stock solutions and the enzymatic activity of the enzymes was tested before each experiment as recommended by Minekus et al. (2014) and Brodkorb et al. (2019).

In vitro digestion was carried out as follows:

Oral stage: Saliva extracted from a healthy adult (5 mL) was added to sample (5 g) to obtain a final volume ratio of 1:1 (v/v). This mixture was incubated for approximately 2 min at 37C° to achieve optimal action of the amylase enzyme present in the saliva.

Gastric stage: Simulated gastric fluid (SGF) (10 mL) was added to the bolus resulting from the previous step. pH of 3 was reached by addition of HCl (1N) and then it was incubated at 37C° with continuous agitation for 2 h in an incubation chamber (JP Selecta SA, Barcelona) and a head shaker (Intelli-Mixer RM-2, Elmi Ltd, Riga) at 55 rpm. In order to ensure optimal performance of the pepsin present in the SGF, the pH was monitored every 40 min and readjusted by addition of HCl (1N) during these 2 h. After gastric digestion, the pH of digests was adjusted to 7 (to inactivate pepsin and to prepare the environment of the small intestine).

Intestinal stage: simulated intestinal fluid (FIS) was added to the simulated chyme from the previous stage and the pH was adjusted to 7 with HCl (1N). It was then incubated under the same conditions as in the previous stage, also controlling and adjusting the pH every 40 min, in order to ensure adequate enzymatic activity. After 2 h the pH was adjusted to ~5.5 and kept in an ice bath for 10 min to inhibit the enzymatic reactions.

Once the enzyme activity in each stage was diminished, aliquots were taken for subsequent analytical determinations. Where needed, the

separation of the liquid fraction from the undigested remaining solids was done using a centrifuge at 4000×*g* (5810R, Eppendorf, Hamburg, Germany) for 5 min at 10 °C to obtain the supernatant.

2.4. Analytical determinations

2.4.1. Proximate composition of tiger nut products

Moisture, ash, fat, fibre and crude protein (using a Kjeldahl factor of 6.25) contents were characterized in the samples according to the AOAC official methods 934.01, 942.05, 920.39, 962.09 and 960.52 (Association of Official Analysis Chemists International (AOAC) (2000), respectively. Sugars were determined quantifying glucose using the DNS colorimetric method according to Hernández-Olivas, Muñoz-Pina, et al. (2021). Ethanolic extracts were prepared by mixing undigested tiger nut products (1 g) with pure ethanol (4 mL) for 30 min. The extracts (50 μL) were added to deionised water (250 μL) and DNS mixture (750 μL containing a 1:1:5 mixture of 0.5 mg/mL glucose:4 M NaOH:DNS reagent (10 g/L 3,5-dinitrosalicylic acid, containing 300 g potassium sodium tartrate and 16 g NaOH)). The reaction was catalysed by heating at 100 °C for 15 min. Then, cold deionised water (4 mL) was added and the absorbances were measured at 530 nm using a UV-VIS spectrophotometer (DU ® 730, Beckman Coulter Inc., Brea, CA, USA). Glucose was used to obtain a calibration curve (from 0 to 10 mg/L). Total starch content was determined using the Megazyme total starch assay kit (K-TSTA, Megazyme, Co. Wicklow, Ireland). Briefly, starch was hydrolyzed to glucose during several stages using enzymes (α-amylase and amyloglucosidase). The glucose was then reacted colourimetrically with a glucose oxidase/peroxidase reagent (GOPOD) and the absorbance was measured at a wavelength of 510 nm in a spectrophotometer (DU ® 730, Beckman Coulter Inc., Brea, CA, USA).

2.4.2. Digestibility of macronutrients

2.4.2.1. Protein. The protein digestibility was evaluated by quantifying the trichloroacetic acid (TCA) soluble protein fraction. Aliquots taken at the end of the gastric and intestinal phase of the different samples were used. The digested samples (500 μL) were mixed with a TCA solution to achieve a concentration of 12% and allowed to react for 15 min in a mixer (Eppendorf Thermomixer, Germany). The samples were then centrifuged (Eppendorf MiniSpin Plus, Germany) for 5 min at 10000 rpm and the resulting supernatant was diluted in buffer (50 mM EDTA and 8 M Urea at pH 10). Absorbance was measured in quartz cuvette at 280 nm. Bovine serum albumin (BSA) was used as standard for a calibration line between 0 and 200 mg/L. The TCA soluble protein obtained from the bioaccessible fraction and the crude protein measured in undigested products were used to calculate the extent of proteolysis, expressed as g of TCA-soluble protein/100 g of protein (Equation (1)).

$$Extent\ of\ proteolysis\ (\%) = \frac{g\ TCA\ s.p.\ in\ the\ bioaccessible\ fraction}{g\ crude\ protein\ in\ undigested\ food} \times 100 \quad (1)$$

2.4.2.2. Lipids. The lipid profiles of the different tiger nut products (tiger nut flour, tiger nut milks and tiger nut oil) were obtained before and after *in vitro* digestion by means of proton nuclear magnetic resonance (1H NMR) (Bruker, model 400/R), according to Nieva-Echevarría et al. (2018). The molar percentages of triglycerides (TG), diglycerides (1,2-DG and 1,3-DG), monoglycerides (1-MG and 2-MG), and free fatty acids (FFA) were determined in the samples. Equations (2)–(4) were used to calculate the absorbable fraction, the non-absorbable fraction and the extent of lipolysis.

$$Absorbablelipidfraction(\%) = AG_{2-MG}\ \%\ + AG_{1-MG}\%\ + FA \quad (2)$$

$$Non-absorbablelipidfraction(\%) = AG_{1,2-DG}\ \%\ + AG_{1,3-DG}\% \quad (3)$$

$$Extent\ of\ lipolysis\ (\%) = AG_{1,2-DG}\ \%\ + AG_{1,3-DG}\% + AG_{2-MG}\ \% + AG_{1-MG}\%\ +\ FA \quad (4)$$

where AG correspond to the acyl groups supported on the different glyceryl backbone structures (TG, 1,2-DG, 1,3-DG, 2-MG, 1-MG) and fatty acids (FA).

2.4.2.3. Carbohydrates. The extent of glycolysis was estimated by measuring the reducing sugars released as a consequence of starch digestion (monosaccharides). For this, the bioaccessible fraction was used for a further analysis with a colorimetric method using dinitrosalicylic acid (DNS) after a secondary enzymatic digestion using invertase and amyloglucosidase (Armellini et al., 2019; Hernández-Olivas, Muñoz-Pina, et al., 2021). An aliquot of 1 mL of the bioaccessible fraction was mixed with 4 mL of absolute ethanol to prepare an extract. The ethanolic extract (50 μL) were added to 250 μL of the enzymatic solution (1% amyloglucosidase + 1% invertase in acetate buffer, pH 5.2) and incubated at 37 °C for 10 min. The DNS mixture (750 μL containing a 1:1:5 mixture of 0.5 mg/mL glucose:4 M NaOH:DNS reagent (10 g/L of 3,5-dinitrosalicylic acid, containing 300 g potassium sodium tartrate and 16 g NaOH)) were added and heated for 15 min at 100 °C. Then, 4 mL of cold deionised water were added and absorbances measured at 530 nm using an UV-VIS spectrophotometer (DU ® 730, Beckman Coulter Inc., Brea, CA, USA). Glucose was used to obtain a calibration curve (from 0 to 10 mg/L). The extent of glycolysis was calculated using equation (5):

$$\textbf{\textit{Extent of glycolysis}}\ (\%) = \frac{(\textbf{\textit{g glucose Eq. in bioaccessible fraction}})}{(\textbf{\textit{g starch in undigested food}})} \times 100 \quad (5)$$

2.4.3. Release of bioactive compounds

Vitamin E, carotenoids and total polyphenols were determined in order to estimate their bioaccessibility (%) (as the percentage of a given bioactive compound solubilized in the digested sample with respect to its total content in the non-digested sample). Determinations were done in undigested samples and in their correspondent bioaccessible fraction at the end of the intestinal phase.

For undigested samples, extraction with methanol was necessary for total polyphenols determination. Samples were homogenized using a 1:10 food:methanol ratio (w/v) for 2 min at 9000 rpm in UltraTurrax (Ultra-Turrax T25D, IKA, Germany) and left in continuous agitation for 2 h. In the case of the bioaccessible fraction of digested samples, aliquots at the end of intestinal phase, previously stored and frozen, were diluted with methanol in 1:1 (v/v) ratio for analytical determinations.

The evaluation of total polyphenol content was carried out using the Folin-Ciocalteu method with the modifications reported by Muñoz-Pina et al. (2022). For this, methanol extracts (digested and undigested samples) (0.03 mL) were mixed with double distilled water (2.37 mL) and Folin-Ciocalteu's reagent (0.15 mL). The mixture was stirred and allowed to stand in the dark for 8 min, after which time 20% sodium carbonate (0.03 mL) was added and incubated at room temperature for 2 h. The absorbance of the samples was measured at 765 nm and the results were expressed in mg of Gallic Acid Equivalent (GAE) per g of dry basis.

On the other hand, undigested and fractions obtained after centrifugation of digested samples (bioaccessible, retained in solid phase and retained in oily phase) were subjected to saponification and extraction of vitamin E (α-tocopherol) and carotenoids (lutein/zeaxanthin and β-carotene) (Hernández-Olivas, Muñóz-Pina, et al., 2021). Quantification of fat-soluble compounds was carried out in an RP-HPLC (Waters e2695 Separation Module, Waters, Milford, MA, USA) using a Kinetex™C18 column, 5 μm, 100 Å, 150 × 4.6 mm (Phenomenex, Torrance, CA, USA). Compounds were detected using a photodiode array detector (Waters PDA 2996, Waters, Milford, MA, USA) at 292 and 450 nm for

vitamin E and carotenoids, respectively. Isocratic separation was performed using acetonitrile (15%), water (7%) and methanol:tetrahydrofuran (90:10 v/v, 78%) for 30 min at a flow rate of 1 mL/min. The sample injection volume was 20 μL. α-tocopherol, lutein, zeaxanthin and β-carotene were used as standards to construct calibration lines.

2.4.4. Antioxidant activity

Antioxidant activity was also determined for undigested and bioaccessible fraction of digested samples at the end of the intestinal phase, using the same methanolic extract for total polyphenols.

The antioxidant activity was evaluated by three different methods (DPPH, FRAP and ABTS) based on the protocols published by Thaipong et al. (2006) and Asensio-Grau et al. (2020) using a spectrophotometer (DU ® 730, Beckman Coulter Inc., Brea, CA, USA). Trolox was used to elaborate a calibration line between 0 and 200 mg/L for the quantification of antioxidant activity in the three methods. The results were expressed as mg TE/g on a dry basis.

2.5. Statistical analysis

All the tests described were performed at least by triplicates. The results obtained were processed using the Statgraphics Centurion 18 Software, version 18.1.13 (Statgraphics Technologies, Inc. The Plains, VA, USA). Specifically, analysis of variance, or simple ANOVA, with a 95% confidence interval (p-value<0.05) was performed to determine the statistical significance of the type of food matrix on macronutrients digestibility and bioaccessibility of the bioactive compounds. Homogeneous groups were identified by least significant difference (Fisher's LSD). In addition, principal component analysis (PCA) was performed to understand the descriptive relationship among digestion-end-parameters (e.g., digestibility of macronutrients, bioaccessibility of polyphenols, vitamin E and carotenoids, as well as the antioxidant capacity) of the four different tiger nuts products.

3. Results and discussion

3.1. Tiger nut products proximal composition and antioxidant properties

Table 1 gathers the nutritional composition of the four tiger nut products and antioxidant capacity. Results are expressed in dry basis to enable easy comparison of the composition and antioxidant activity among the products despite their different water content (see Table 2).

With respect to carbohydrates, the flour stands out as the best source of fiber among the studied food matrices, while the milk with added sucrose had higher sugar content due to the external sucrose addition. Results provide evidence that tiger nut milks presented higher content in proteins, polyphenols and minerals than the flour, since they are hydrophilic compounds passing into the liquid phase fraction during solid-liquid extraction for milk preparation. Moreover, tubers were subjected to soaking before milk extraction, thus enhancing milling and water-soluble nutrients diffusion (Ejoh et al., 2006). Likewise, based on the polyphenol content of the oil, tiger nut would contain a fat-soluble fraction of polyphenols extracted during oil separation, leaving the water-soluble fraction retained in the cake. Ezeh et al. (2016) reported that non-conventional oil extraction techniques, such as overpressuring or supercritical fluid application, allow obtaining oils with significantly higher polyphenolic content than with conventional conditions. These techniques entail a greater degradation of the cell wall facilitating the release of phenolic compounds (Roselló-Soto, Barba, et al., 2018, p. 1).

In relation to the fat-soluble compounds of interest for macular health, statistically significant differences ($p < 0.05$) were detected among the tiger nut products. As expected, oil presented significantly higher contents of α-tocopherol, lutein + zeaxanthin and β-carotene than the rest of the products, followed by the flour. Of note, the content of these compounds increases as lipid content does in the different food matrices due to their liposoluble nature.

On the other hand, liposoluble compounds are highly sensitive to heat, oxygen, light and enzymatic degradation compounds. As reported by Zhang et al. (2019), lutein and zeaxanthin are the most thermoresistant compounds, while β-carotene and α-tocopherol are quite sensitive to temperature. In turn, these compounds have high antioxidant activity (Marques et al., 2021), having vitamin E the highest responsibility for this activity in the case of tiger nut (Zulueta et al., 2007). The antioxidant activity of the substrates was determined with three different methods, two of them based on radical-based scavenging assays (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays) and one on non-radical redox potential-based assay (FRAP). ABTS and DPPH radicals are usually quenched by two mechanisms, i.e., by transferring either an electron (ET) or a hydrogen atom (HAT) to transform the radical to a more stable species; while FRAP only involved ET chemical species. FRAP assay is based on antioxidants to reduce the ferric 2,4,6-tripyridyl-*s*-triazine complex $[Fe^{3+}-(TPTZ)_2]^{3+}$ from ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) in acidic medium. The reaction detects compounds with redox potentials lower than 0.7 V. Because the redox potential of $[Fe^{3+}-(TPTZ)_2]^{3+}$ is closed to ABTS•+ potential (0.68 V), similar compounds react in both the FRAP and ABTS assays (Prior et al., 2005). Results showed that flour and oil exhibited higher FRAP activity, while milks higher capacity to block the ABTS free radical. Accordingly, HAT-antioxidant species are predominant in tiger nut milks; while ET are in oil and flour. Of note, the higher the polyphenols content, the higher the ABTS antioxidant capacity (Zulueta et al., 2007). Phenolic

Table 1
Proximate composition (g/100 g d.b.), polyphenol content (mg GAE/g d.b.), α-tocopherol content, lutein + zeaxanthin and β-carotene (μg/g d.b.) and antioxidant activity (mg TE/g d.b.) measured by DPPH, ABTS and FRAP assays of tiger nut products (flour, oil, milk and milk with added sucrose).

		Flour	Oil	Milk	Milk with added sucrose
Water	g/100 g d.b.	8.54 ± 0.02^{a}		911.33 ± 0.07^{c}	521 ± 3^{b}
Protein	g/100 g d.b.	10.4 ± 0.4^{b}		15.5 ± 0.2^{c}	8.39 ± 0.12^{a}
Lipids	g/100 g d.b.	28.36 ± 0.14^{b}	100^{c}	27 ± 3^{b}	19.1 ± 0.2^{a}
Ashes	g/100 g d.b.	1.97 ± 0.02^{a}		3.94 ± 0.12^{c}	2.11 ± 0.05^{b}
Sugars	g glucose eq./100 g d.b.	15.8 ± 1.3^{a}		25.3 ± 0.5^{b}	41 ± 4^{c}
Starch	g/100 g d.b.	22.36 ± 0.13^{a}		45 ± 2^{c}	26.4 ± 0.8^{b}
Fiber	g/100 g d.b.	20.2 ± 0.2^{c}		4.0 ± 0.2^{b}	2.17 ± 0.12^{a}
Total polyphenols	mg GAE/g d.b.	1.20 ± 0.15^{b}	0.34 ± 0.03^{a}	7.71 ± 0.12^{d}	4.7 ± 0.2^{c}
Vitamin E	μg α-Tocopherol/g d.b.	34 ± 4^{b}	56.3 ± 0.4^{c}	15 ± 2^{a}	16.2 ± 1.3^{a}
Lutein + zeaxanthin	μg/g d.b.	0.77 ± 0.02^{a}	1.42 ± 0.07^{c}	1.46 ± 0.04^{c}	0.87 ± 0.03^{b}
β-Carotene	μg/g d.b.	1.368 ± 0.109^{b}	4.06 ± 0.13^{c}	1.49 ± 0.03^{b}	0.88 ± 0.02^{a}
DPPH antioxidant activity	mg TE/g d.b.	0.84 ± 0.03^{b}	0.29 ± 0.06^{a}	2.93 ± 0.02^{d}	1.9 ± 0.2^{c}
FRAP antioxidant activity	mg TE/g d.b.	3.46 ± 0.10^{d}	1.60 ± 0.10^{a}	2.9 ± 0.2^{c}	1.96 ± 0.03^{b}
ABTS antioxidant activity	mg TE/g d.b.	1.74 ± 0.03^{b}	0.760 ± 0.009^{a}	11.2 ± 0.3^{d}	6.1 ± 0.2^{c}

[abc] Different lowercase letters in the same row indicate statistically significant differences between the matrices at the 95% confidence level. Data shown are mean values from triplicates and the standard deviation.

Table 2
Molar percentages of acyl groups (AG) supported on the different glyceryl backbone structures (TG, 1,2-DG, 1,3-DG, 2-MG, 1-MG) and free fatty acids (FFA), present in non-digested (ND) and gastrointestinal *in vitro* digested (D) tiger nut products (flour, oil, milk and milk with added sucrose).

		AG_{Tg} (%)	$AG_{1,2\text{-}DG}$ (%)	$AG_{1,3\text{-}DG}$ (%)	$AG_{2\text{-}MG}$ (%)	$AG_{1\text{-}MG}$ (%)	FFA (%)	Absorbable fraction (%)	Non-absorbable fraction (%)	Lipolysis extent (%)
Flour	**ND**	86.0 ± 0.4^{a}	1.0 ± 0.5^{b}	4.02 ± 0.05^{c}	0.02 ± 0.02^{abc}	0.40 ± 0.13^{b}	8.55 ± 0.17	61 ± 2^{b}	13.5 ± 0.5^{c}	74 ± 3^{a}
	D	26 ± 2^{b}	11.2 ± 0.3^{c}	2.31 ± 0.13^{c}	1.9 ± 0.2^{b}	1.52 ± 0.09^{b}	57 ± 2^{b}			
Oil	**ND**	99.1 ± 0.6^{b}	0 ± 0	1.49 ± 0.08^{b}	0.015 ± 0.002^{b}	0.262 ± 0.006^{a}	0 ± 0	46 ± 2^{a}	28 ± 2^{d}	74 ± 5^{a}
	D	26 ± 2^{b}	24.66 ± 1.08^{d}	2.75 ± 0.16^{d}	4.3 ± 0.2^{c}	2.26 ± 0.07^{c}	39 ± 2^{a}			
Milk	**ND**	99.5 ± 0.3^{b}	0.14 ± 0.04^{a}	0.62 ± 0.15^{a}	0.011 ± 0.002^{a}	0.30 ± 0.09^{ab}	0 ± 0	77 ± 4^{c}	8 ± 2^{b}	85 ± 5^{b}
	D	15 ± 5^{a}	7.1 ± 1.4^{b}	1.10 ± 0.08^{b}	0.63 ± 0.16^{a}	0.6 ± 0.2^{a}	75 ± 3^{c}			
Milk with added sucrose	**ND**	98.7 ± 0.7^{b}	0.3 ± 0.5^{ab}	0.79 ± 0.15^{a}	0.034 ± 0.007^{c}	0.3 ± 0.2^{ab}	0 ± 0	89 ± 3^{d}	5.2 ± 0.6^{a}	94 ± 3^{c}
	D	6 ± 4^{a}	4.4 ± 0.6^{a}	0.822 ± 0.009^{a}	0.6 ± 0.4^{a}	1.4 ± 0.3^{b}	87 ± 2^{d}			

^{abcd} Different minor letters represent statistical differences ($p < 0.05$) among tiger nut products in the same lipid condition (e.g., non-digested or digested samples). Data shown are mean values from triplicates and the standard deviation. Absorbable fraction is composed of monoglycerides (2-MG and 1-MG) and free fatty acids (FFA). Non-absorbable fraction includes diglycerides (1,2-DG and 1,3-DG). Lipolysis extent represent the sum.

compounds are good oxygen radical scavengers, since their electron reduction potential is lower than that of oxygen radicals and also phenoxy radicals are less reactive than oxygen radicals. Based on literature, phenolics compounds exert HAT from the hydroxyl group(s) attached to their benzene rings (Simić et al., 2007). In turn, vitamin E and β-carotene higher in flour and oil would be responsible sample's ability to reduce the ferric 2,4,6-tripyridyl-*s*-triazine complex $[Fe^{3+}\text{–}(TPTZ)_2]^{3+}$.

3.2. Macronutrients in vitro digestibility in the different tiger nut products

Macronutrients hydrolysis through digestion may be conditioned by the physico-chemical characteristics of foods such as structure or nutrients interactions (Heredia et al., 2021). At the same time, hydrolyzed macronutrients could affect the release and the incorporation of the bioactive compounds with health interest into the digestive fluids and, therefore, their bioaccessible fraction. Consequently, proteolysis, lipolysis and glycolysis extents of the different tiger nut products are discussed in this section. Firstly, Fig. 1 shows proteolysis extent (g of TCA soluble protein/100g of initial protein) of flour and both tiger nut milks, with and without added sucrose. Results show that proteins begin to be hydrolyzed in stomach by pepsin action under acidic pH. However, proteolysis extent did not exceed 27%, corresponding this value to milk with added sucrose. Even if statistical differences ($p < 0.05$) were found among samples in terms of gastric proteolysis, values were very close in the different tiger nut products, being these differences more evident at the end of digestion. In particular, TCA soluble protein (mainly short-chain peptides and free amino acids) in tiger nut milk was higher than in flour (21%) and, at the same time, higher in tiger nut milk with higher sucrose content (27%) than in tiger nut milk without added sucrose (16%). The highest percentage of absorbable protein was achieved in the intestine, since only proteins that are directly hydrolyzed to dipeptides or tripeptides can be intactly absorbed in the stomach. The remaining peptides must pass to the intestine where they continue the digestive process (Amigo & Hernández-Ledesma, 2020, p. 4). This fact demonstrates the relevance of the later stage and, therefore, the action of pancreatic peptidases (trypsin and chymotrypsin) to continue protein hydrolysis at intestinal phase (Hernández-Olivas et al., 2022). On the other hand, according to results, the access of the enzymes to the substrate was favored in the liquid matrices compared to the solid (flour). The influence of the structural food matrix on digestibility has been reported in different studies (Fernández-Jalao et al., 2017; Calvo-Lerma, Paz-Yépez, et al., 2020; Hernández-Olivas et al., 2020, 2021b; Capuano

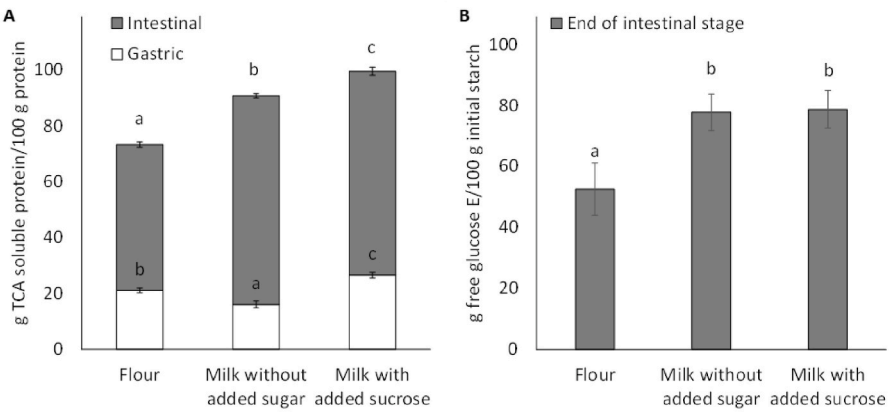

Fig. 1. A) Extent of proteolysis (g TCA soluble protein/100 g protein) and **B)** extent of glycolysis (g free glucose E/100 g initial starch) of the tiger nut products (flour, oil, milk and milk with added sucrose) at the end of gastric (only for proteins) and intestinal digestion. ^{abc} Different minor letters represent statistical differences ($p < 0.05$) among tiger nut products in the same digestive stage. Data shown are mean values from triplicates and the standard deviation.

& Janssen, 2021). So, in solid matrices with low humidity such as flour, there is greater packaging and interaction between macronutrients, which could hinder their solubility and access by digestive enzymes to the substrate, compared to liquid matrices, in spite of being an unstructured matrix.

A similar trend was found for starch and lipids digestibility whom extent was higher in tiger nut milks than in the other products. Glycolysis was found to be around 78% for liquid matrices and 53% for the flour. Thus, tiger nut whole flour would be more recommendable than milk for individuals suffering of hyperglycemia and therefore, for functional food formulations addressed to diabetics.

In turn, lipolysis extent in digested samples, obtained by 1H NMR analysis, was 94, 85, 74% in milk with added sucrose, milk and finally oil and flour, respectively. Of note, ~14% of absorbable lipid species (e.g., monoglycerides and free fatty acids) was found in flour before digestion, so only 50% of flour-triglycerides were hydrolyzed throughout the digestive process. This fact could be related to the qualitative and quantitative changes that occur over time of food storage, such as changes in free fatty acids, the generation of lipid oxidation products and consequently, changes in organoleptic properties (Kumar & Mishra, 2004; Nina et al., 2020; Vázquez et al., 2013). Thus, an increase in the final absorbable fraction could have occurred in flour compared to oil. Besides, even if absorbable fraction in all samples was mostly composed by free fatty acids, the percentage of monoglycerides ($AG_{1\text{-}MG}$ and $AG_{2\text{-}MG}$) in this fraction was significantly higher in oil than in the others. Likewise, oil had a higher fraction of non-absorbable lipids, being the $AG_{1,2\text{-}DG}$ lipid species the responsible with ~25%.

Fibre content, being much higher in flour (20%) than in milks (2–4%), could be also responsible of the lower protein and starch digestibility found in flour compared to milks. Dietary fibre has been reported to limit nutrient digestion in plant-foods due to their capacity of increasing viscosity in the gut lumen (Grundy et al., 2016) with the consequent reduction of the interactions between enzymes and substrates. In addition, dietary fibre present as solubilized polysaccharide chains may reduce macronutrient hydrolysis by direct binding to digestive enzymes and/or by physical interaction (binding) with hydrophilic substrate surfaces. Indeed, previous studies have suggested that guar galactomannan inhibits the rate of starch digestion by one or both mechanisms (Brennan et al., 1996; Slaughter et al., 2002). Similarly, soluble fibre fraction has been found to reduce the ileal digestibility of protein and amino acids as well as lipids in animal studies (Dégen et al., 2007). Finally, another reported mechanism of fiber inhibiting macronutrient hydrolysis relates to its capacity to increase the viscosity of the digestion medium, which contributes to impede enzymes accessibility to their substrates (Calvo-Lerma et al., 2020b, 2022, p. 410; Hernández-Olivas, Muñoz-Pina, et al., 2021).

3.3. Release of compounds with potential for macular health and other antioxidant characteristics throughout gastrointestinal digestion

Table 3 gathers the content of liposoluble compounds (vitamin E, β-carotene, lutein and zeaxanthin) with potential on macular health at the end of *in vitro* digestion and in three different fractions: micellar bioaccessible, retained in solid phase and retained in oily phase (this latter only for flour and oil).

Results show the low incorporation of fat-soluble compounds to the bioaccessible fraction, as well as to the solid and oily phases. Moreover, it should be highlighted that none of the carotenoids were detected in the bioaccessible fractions of milks, and only β-carotene was retained in the solid phase. Even if higher lipolysis extent was achieved in tiger nut milks in comparison with flour or oil, the net amount of end-digestion lipidic products (free fatty acids and monoglycerides) which move to the micellar phase and contribute to the incorporation of other fat-soluble substances at the same time (Ozturk et al., 2015; Wilde & Chu, 2011), would be higher in flour and oil.

If carotenoids bioaccessibility values (%) are compared among tiger nut products, higher values were found in flour than in oil for lutein + zeaxanthin. It has been reported that carotenoids are highly sensitive to the acidic environment in gastric digestion (García-Hernández et al., 2018; Marques et al., 2021). Flour seems to exert a protective role against gastric pH, compared to oil, evidencing the contribution of the food matrix to this fact (Courraud et al., 2013). On the other hand, lutein + zeaxanthin presented higher bioaccessibility than β-carotene. These macular pigments have been reported to be less lipophilic than

Table 3
Vitamin E (α-tocopherol), lutein + zeaxanthin, β-carotene and total polyphenols contents in the different obtained fractions (bioaccessible, retained in solid phase and retained in oily phase) at the end of the intestinal stage of the *in vitro* digestion of tiger nut products (flour, oil, milk and milk with added sucrose).

		Bioaccessible fraction	Fraction retained in solid phase	Fraction retained in oily phase
Vitamin E (α-Tocopherol) (μg/g d.b.)	**Flour**	6.9 ± 0.6^{b} ($20.0 \pm 1.7\%$)	7.8 ± 0.4^{c} ($23.0 \pm 1.2\%$)	4.9 ± 0.7^{b} ($14 \pm 2\%$)
	Oil	11.2 ± 1.0^{c} ($20.0 \pm 1.8\%$)	1.30 ± 0.13^{a} ($2.0 \pm 0.2\%$)	3.0 ± 0.2^{a} ($5.0 \pm 0.3\%$)
	Milk	6.9 ± 0.6^{b} ($29 \pm 3\%$)	3.81 ± 0.12^{d} ($24 \pm 2\%$)	–
	Milk with added sucrose	3.97 ± 0.17^{a} ($25.00 \pm 1.07\%$)	3.6 ± 0.6^{b} ($22 \pm 4\%$)	–
Lutein + zeaxanthin (μg/g d.b.)	**Flour**	0.142 ± 0.013^{a} ($18.0 \pm 1.6\%$)	0.144 ± 0.005^{b} ($19.0 \pm 0.7\%$)	0.143 ± 0.005^{a} ($19.0 \pm 0.7\%$)
	Oil	0.135 ± 0.010^{a} ($10.0 \pm 0.7\%$)	0.128 ± 0.012^{a} ($9.0 \pm 0.8\%$)	0.136 ± 0.004^{a} ($10.0 \pm 0.3\%$)
	Milk	–	–	–
	Milk with added sucrose	–	–	–
β-Carotene (μg/g d.b.)	**Flour**	0.127 ± 0.012^{a} ($9.0 \pm 0.9\%$)	0.30 ± 0.02^{b} ($22.0 \pm 1.5\%$)	0.25 ± 0.04^{a} ($18 \pm 3\%$)
	Oil	0.27 ± 0.03^{b} ($7.0 \pm 0.7\%$)	0.070 ± 0.003^{a} ($2.00 \pm 0.09\%$)	1.29 ± 0.13^{b} ($32 \pm 3\%$)
	Milk	–	0.63 ± 0.02^{c} ($42.0 \pm 1.3\%$)	–
	Milk with added sucrose	–	0.39 ± 0.02^{b} ($45 \pm 2\%$)	–
Total polyphenols (mg GAE/g d.b.)	**Flour**	6.4 ± 0.4^{c} ($210 \pm 10\%$)	–	–
	Oil	4.9 ± 0.3^{d} ($160 \pm 10\%$)	–	–
	Milk	50 ± 3^{a} ($640 \pm 40\%$)	–	–
	Milk with added sucrose	28.8 ± 1.3^{b} ($260 \pm 10\%$)	–	–

[abc] Different minor letters by column in the same bioactive compound indicate significant differences ($p < 0.05$) among tiger nut products. Data shown are mean values from triplicates and the standard deviation. Values in brackets represent the percentage contained in fractions with respect to the non-digested samples. The bioaccessibility index is the amount of bioactive compounds solubilized at the end of the intestinal stage during *in vitro* digestion with respect to total content in the non-digested tiger nut products.

β-carotene, thus showed increased their solubilization in micelles and the subsequent absorption in the intestinal wall (Reboul et al., 2006).

With respect to vitamin E, a slightly higher bioaccessibility (%) was found in tiger nut milks than in flour or oil. Tiger nut milks, being liquid and water-based foods, favour greater solubilization of the released compounds. On the other hand, flour and oil have a similar amount of absorbable vitamin E. However, considering the sum of the fractions (bioaccessible and non-bioaccessible), recovery percentages of 27, 47, 57 and 53% were found for oil, milk with added sucrose, flour and milk without added sucrose, respectively. The oil presented the lowest recovery value, which suggests lower stability and/or extractability during digestion. However, considering the high concentration of vitamin E in oil, it could be considered a good source of α-tocopherol.

Table 3 also contains the total phenolic content (mg GAE/g dry basis) and the antioxidant activity in the bioaccessible fraction of the different tiger nut products can be found in Table 4. The results evidenced that water-based food matrices (e.g., tiger nut milks), compared to flour or oil, favored polyphenols migration to the digestive fluids and incorporation in the bioaccessible fraction in a greater extent than solid-matrices. This fact resulted in the higher antioxidant activity of the bioaccessible fraction these matrices compared to flour or oil. A positive correlation between phenols content and antioxidant activity has been reported in several studies (Lu et al., 2007; Turumtay et al., 2014). The water-solubility of the majority of the phenolic compounds, together with the greater molecular mobility found in milks, could be responsible of these results. Sucrose addition, however, seems to negatively interfere in phenols solubilization as bioaccessibility of these compounds were drastically reduced compared to milk without added sucrose ($p < 0.05$).

Regarding the antioxidant activity of the bioaccessible fraction, values were higher than those obtained in the undigested food matrices, except for the mean values obtained by the FRAP method in flour. The hydrolysis of antioxidant compounds along digestion process would be responsible for these results (Ortega et al., 2011). This is the case of phenolic compounds, most of them linked to esters, glycosides or associated with complex molecules (carbohydrates or proteins), making their extraction and subsequent absorption difficult (Quiñones et al., 2012). Digestive process takes on special relevance, since the chemical, mechanical and enzymatic reactions promote the hydrolysis, release and bioaccessibility of compounds with special relevance for human health (Ketnawa et al., 2021).

3.4. Analysis of structure-digestion relation

Finally, a principal component analysis (PCA) (Fig. 2) was applied to better summary and establish relationships among the obtained results. As it can be observed, the two principal dimensions or components (PC1 and PC2) of the PCA explained the 92.013% of the variability of the results. On one hand, PC1 (80.494%) clearly distinguishes between water-based matrices (i.e., tiger nut milks) from the oil and flour. Therefore, it can be concluded the structural matrix of tiger nut products is the most relevant food-factor determining macronutrients' digestibility and bioactive compounds' bioaccessibility and related properties. While liquid matrices favour macronutrients' hydrolysis together with polyphenols and α-tocopherol release; macular compounds (lutein + zeaxanthin) and β-carotene bioaccessibilities, for which the oil content of tiger nut products is determinant, were higher in oil and flour. PC1 also shows that phenols and α-tocopherol are mainly responsible for ABTS, DPPH and FRAP antioxidant activities.

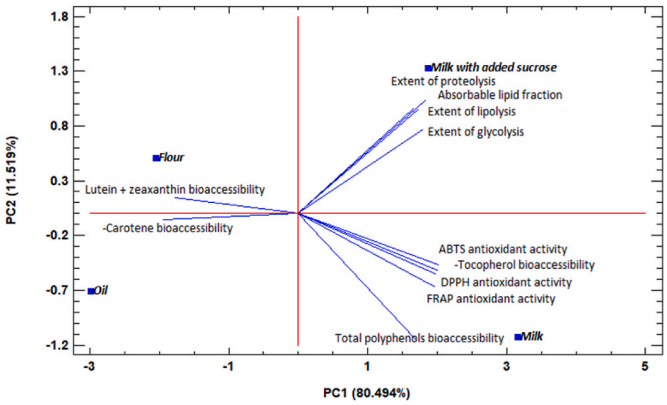

Fig. 2. Biplot resulting from a principal components analysis (PCA) applied to macronutrients' extent, bioactive compounds bioaccessibility and antioxidant activity of digesta and their relationship with the different tiger nut products (flour, oil, milk and milk with added sucrose).

On the other hand, PC2, unless with low percentage (11.519%), sets apart milk with added sucrose from milk as they locate in the upper and the bottom right-quadrants, respectively. According to that, sucrose addition for tiger nut milk implies higher macronutrients' hydrolysis, but negatively interferes the bioaccessibility of polyphenols and α-tocopherol, and thus decreases antioxidant activity of digesta.

4. Conclusions

Tiger nut products (flour, oil, milk with and without added sucrose) presented important contents of bioactive compounds of interest for macular health.

Higher protein digestibility resulted in tiger nut milks than in flour, while the latter would be suitable for dietary recommendations for individuals suffering hyperglycaemia. Therefore, flour may be a good choice as an ingredient for functional food formulations targeting diabetics.

With regard to total polyphenols content, the tiger nut milk with no added sucrose exhibited the highest content and bioaccessibility of total polyphenols. In contrast, although flour and oil were less bioaccessible than the water-based tigernut products, they represent a good alternative as net suppliers of carotenoids and vitamin E.

Gastrointestinal digestion favours the release of polyphenols and bioactive compounds linked to antioxidant activities in the bioaccessible fraction, being higher in tiger nut milks than in flour or oil. The negative impact of the addition of sucrose on the functional quality of tiger nut milk should be highlighted.

Once again, this study confirms the relevance of the food matrix in the release and subsequent bioaccessibility of bioactive compounds, sometimes exerting a protective effect of bioactive compounds against the digestive environment (like in the tiger nut flour), but in others it represents a hindrance for the hydrolysis and release in the digestive medium.

Finally, considering both the concentration and bioaccessibility of bioactive compounds, tiger nut products have the potential to be used in the design of functional foods for human health promotion, particularly for macular preservation and regeneration.

Table 4
Antioxidant capacity measured by DPPH, ABTS and FRAP assays (mg TE/g d.b.) in the bioaccessible fractions of tiger nut products (flour, oil, milk and milk with added sucrose).

	DPPH	FRAP	ABTS
	(mg TE/g d.b.)	(mg TE/g d.b.)	(mg TE/g d.b.)
Flour	2.97 ± 0.08^{c}	2.4 ± 0.2^{c}	2.89 ± 0.02^{c}
Oil	1.5 ± 0.3^{d}	1.5 ± 0.3^{d}	2.1 ± 0.2^{d}
Milk	11 ± 3^{a}	11.91 ± 1.02^{a}	25.6 ± 0.9^{a}
Milk with added sucrose	6.31 ± 1.12^{b}	6.3 ± 0.5^{b}	15.6 ± 1.6^{b}

^{abc} Different minor letters in the same column indicate significant ($p < 0.05$) differences between the tiger nut products. Data shown are mean values from triplicates and the standard deviation.

CRediT authorship contribution statement

Ever Hernández-Olivas: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Writing - Review & Editing, Visualization. Andrea Asensio-Grau: Writing - Review & Editing. Joaquim Calvo-Lerma: Writing - Review & Editing. Jorge García-Hernández: Conceptualization, Validation, Writing – Review & Editing, Visualization. Ana Heredia: Conceptualization, Formal analysis, Investigation, Resources, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision, Project administration and Funding acquisition. Ana Andrés: Conceptualization, Formal analysis, Investigation, Resources, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision, Project administration and Funding acquisition.

Declaration of competing interest

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

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