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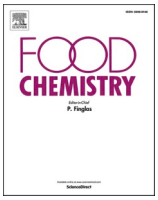

Assessing the effect of gastrointestinal conditions and solubility on the bioaccessibility of polyphenolic compounds from a white grape marc extract

Lorena G. Calvo [a], Maria Celeiro [b,c], Marta Lores [b,d], Ana G. Abril [a], Trinidad de Miguel [a,*]

[a] Department of Microbiology and Parasitology, Universidade de Santiago de Compostela, E-15782 Santiago de Compostela, Spain
[b] Laboratory of Research and Development of Analytical Solutions (LIDSA), Department of Analytical Chemistry, Nutrition and Food Science, Universidade de Santiago de Compostela, E-15782 Santiago de Compostela, Spain
[c] CRETUS, Department of Analytical Chemistry, Nutrition and Food Science, Universidade de Santiago de Compostela, E-15782 Santiago de Compostela, Spain
[d] i-Grape Laboratory, Via Isaac Pera 32, 15890, Santiago de Compostela, Spain

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ABSTRACT

This study investigates the bioaccessibility of phenolic compounds from a *Vitis vinifera* marc extract using an *in vitro* gastrointestinal model. Both undiluted and five-fold diluted extracts were digested to assess how solubility and gastrointestinal conditions impact polyphenol bioaccessibility. The extract was obtained using the environmentally friendly Medium Scale Ambient Temperature (MSAT) system. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis revealed that gastric digestion significantly increased polyphenolic content, particularly catechin, epicatechin, and procyanidins. Diluted extracts showed 30 % higher polyphenolic content and a 200 % increase in gallic acid compared to undigested samples. However, bioaccessibility decreased during intestinal digestion. Interaction tests with bile salts revealed 50 % polyphenol insolubility, suggesting that some compounds may remain in the residual fraction and serve as substrates for colonic microbiota fermentation. These findings emphasize the crucial role of gastrointestinal digestion in polyphenol bioaccessibility and highlight white grape marc extract as a potential source of bioactives for microbiota modulation and functional nutrition.

1. Introduction

In recent times, consumers have preferred a health-conscious well-rounded diet based on natural products due to its increasingly established connection with overall health. As a result, the utilization of bioactive compounds to produce functional foods has been gaining popularity. (García-Lomillo & González-San José, 2017). Polyphenols are the most abundant secondary metabolites present in the plant kingdom. They represent a large and diverse group of molecules including two main families, the flavonoids, and the non-flavonoids. Polyphenols have been at the front of new plant-based therapies research for the last two decades. Their large variety and composition endow them with plenty of properties, such as antioxidant capability, metabolism regulation, hormonal activity, antimicrobial activity, pH regulation or, anti-inflammatory activity. (Cory et al., 2018, Pandey & R, 2009). Among the varieties of polyphenolic dietary sources, grape constitutes one of the major providers of phenolic compounds. Significative polyphenolic differences have been noticed between grape varieties, being the absence of anthocyanidins in white grapes one of the most remarkable. Oenological practices and ripeness stage are also key factors involved in grape polyphenolic content (Yu & Ahmedna, 2013). Although this fruit is mainly used to produce wine, during the vinification process, a large quantity of by-products that turn out to be rich in bioactive compounds are generated. These include polyphenols, which are of great interest at the industrial level (Yu & Ahmedna, 2013). Interestingly, grape peel and seeds contain the biggest amount of polyphenols, in particular condensed tannins, flavanols, flavonols, and phenolic acids. Hence, wine by-products are currently being investigated as sources of nutraceutical products (López-Gutiérrez et al., 2016).

Nevertheless, one of the main topics concerning the beneficial effect of polyphenols is their low bioavailability and metabolic fate (Tain & Hsu, 2022). The bioavailability of a dietary compound is dependent

* Corresponding author.
E-mail address: trinidad.demiguel@usc.es (T. de Miguel).

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upon its digestive stability, its release from the food matrix, and its transepithelial absorption (Tagliazucchi et al., 2010). On the other hand, bioaccessibility depends on several factors, which include molecular size, glycosylation, hydrophobicity, and different pH-dependent transformations, as well as solubility and interactions between polyphenols and food components (Alminger et al., 2014). For example, quercetin presents different bioavailability rates if it is obtained from onions or apples, and glycoside forms of quercetin, such as quercetin-3-o-glucuronide and quercetin-3-o-rutinoside have shown diverse absorption rates (Manach et al., 2005). Hence, the most abundant polyphenols in our diet are not necessarily those leading to the highest concentrations of active metabolites.

All the abovementioned characteristics led researchers to find new ways of improving polyphenols' bioaccessibility. One of those manners involves conventional extractive techniques with different extraction solvents in order to obtain a liquid matrix where polyphenols are present. In fact, polyphenols in liquid matrices are directly available for absorption while those in solid matrices must first be extracted through mechanical, chemical, and enzymatic actions to make the absorption in the gastrointestinal tract easier (Cantele et al., 2020). Medium Scale Ambient Temperature (MSAT) system, represents a very suitable and green alternative to classic procedures to obtain extracts rich in bioactive compounds. As it is well known, the extraction solvent is the most critical experimental parameter to obtain extracts. Using MSATs, the extraction solvent can be easily modulated, enabling the obtention of multicomponent extracts (Castillo et al., 2022).

Hence, concentrated polyphenolic extracts obtained by MSAT from winemaking by-products are an interesting option not only to fight biomass waste in an ecological manner but also as a way of producing bioactive food-function extracts that will help consumers to complement their healthy dietary habits.

As already stated, another key factor involved in determining the bioavailability of polyphenols is their stability under gastrointestinal conditions. *In vitro* digestive assays are particularly useful to assess the influence of digestion conditions and to evaluate the effect of the food structure, composition, and processing on nutrients and the bioaccessibility of bioactive compounds (Wojtunik-Kulesza et al., 2020). Previous studies by Bermúdez-Soto et al. (2007) evaluating chokeberry polyphenol stability under digestive conditions, found no effect of the gastric conditions on the polyphenolic content, meanwhile, a sensible decrease was observed under mild alkaline conditions in the small intestine. The same results were assessed by Rueda et al. (2017) during the evaluation of the effect of gastrointestinal conditions on the polyphenolic composition of extra virgin argan oil. Domínguez-Rodríguez et al. (2022) evaluated the bioavailability of non-extractable polyphenols from cherry pomace and suggested that the digestive procedure helps the conversion of polyphenols into their monomeric forms. Evaluation of bioavailabilities and total absorbable fraction indicated that not all the bioaccessible polyphenols are bioavailable. Finally, A. Vaz et al. (2022) detected a decrease on the polyphenolic composition after *in vitro* digestion and colonic fermentation of dietary fiber, suggesting that non-soluble polyphenols may be transformed by gut microbiota.

The present research analyses the bioaccessibility of individual polyphenols from a *V. vinifera* marc extract obtained by MSAT using a Generally Recognized as Safe (GRAS) organic solvent. Unlike the previous studies in the field, focused on red grape varieties (Elejalde et al., 2024; Tagliazucchi et al., 2010), this extract comes from white grapes of the Albariño variety, with a different qualitative polyphenolic composition. By utilizing the INFOGEST *in vitro* digestion model, the study provides unique insights into the polyphenolic behaviour under realistic gastrointestinal conditions, which are relevant for developing sustainable functional ingredients. Finally, the impact of the solubility and the presence of bile salts on the bioaccessibility of polyphenols during digestion was assessed, offering a comprehensive understanding of how these factors affect their stability.

2. Material and methods

2.1. Materials

The target polyphenols and their identification CAS numbers are summarized in Table S1. Methanol MS grade, water MS, formic acid (mobile phase components) and the Folin–Ciocalteu phenol reagent were purchased from Merck (Steinheim, Germany). Sodium carbonate was obtained from Scharlau (Barcelona, Spain).

Individual Polyphenol standard stock solutions (1,000–10,000 μg mL^{-1}) were prepared in methanol. Working solutions were weekly prepared by dilution in 50:50 (*v*/v) methanol/water. Stock and working solutions were stored in a freezer at −20 °C and protected from light. All solvents and reagents were of analytical grade.

For the *in vitro* digestions, $CaCl_2$, HCl, Pepsin (0,8 FIP U/mg), and Pancreatin (36,000 FIP U/g) were purchased from ITW reagents (Barcelona, Spain). Bile extract porcine from Sigma-Aldrich (USA) was added to the intestinal digestion.

2.2. Grape marc extract production

The grape marc extract was produced by i-Grape Laboratory S.L. using Albariño grape marc from Mar de Frades winery (Galicia, Northwest Spain). The extract was obtained through a MSAT system, under the patented procedure by Lores et al. (2013).

2.3. In vitro gastrointestinal digestion

In vitro gastrointestinal digestion was performed following the INFOGEST® 2.0 protocol (Brodkorb et al., 2019), summarized in Fig. 1. Considerations by Minekus et al., 2014 have been incorporated.

The oral phase was skipped because of the liquid nature of the extract. 25 mL of extract were mixed with simulated gastric fluids (1:1 *v*/v), the pH was adjusted to 3.0 with 1 N HCl and then 12.5 μL of 0.3 M $CaCl_2$, and 2000 U mL^{-1} of pepsin were added. Gastric phase digestion was carried out for 2 h under constant shaking (100 rpm) at 37 °C using an Innova 4340 incubator. Samples were taken at time zero and after digestion. The intestinal phase started with the resulting volume (20 mL), which was mixed with an equal volume of simulated intestinal fluid; the pH was adjusted to 7.0 and then 40 μL of 0.3 M $CaCl_2$, pancreatin (800 U mL^{-1}) and bile salts (10 mM mL^{-1} in the final mixture) were added. Aliquots of 5 mL were recovered before and after each phase. Digestates were filtered through 0.22 μm membranes, kept in ice in order to inactivate the enzymes, and subsequently analysed to obtain the total polyphenolic content (TPC) and by liquid chromatography-tandem mass spectrometry (LC-MS/MS) to quantify target polyphenols.

The same methodology was followed in order to evaluate diluted extract samples. The extract was previously mixed with distilled water (1:5 *v*/v) achieving final concentrations of 10 % and 5 % of the extract in the gastric and intestinal phases, respectively. Samples were taken before and after each digestive stage, processed as above mentioned, and immediately analysed by LC-MS. *In vitro* digestions were conducted in duplicate. Samples were taken in triplicate at each of the stages shown in Fig 1and subsequently processed for their chromatographic analyses in duplicate.

Finally, polyphenols bioaccessibility was calculated using Eq. 1. For each digestive phase, the accumulative presence of the compounds in the bioaccessible fraction was expressed as a percentage of the initial phenolic content.

$$\%\text{Bioaccessibility} = \frac{\text{X polyphenolic content (mg/L)}}{\text{X Initial polyphenolic content (mg/L)}} \times 100 \quad (1)$$

Equation 1: Bioaccessibility percentage is expressed as the concentration of each individual polyphenol (x) on the final intestinal digestion divided by the individual polyphenol concentration in the non-digested

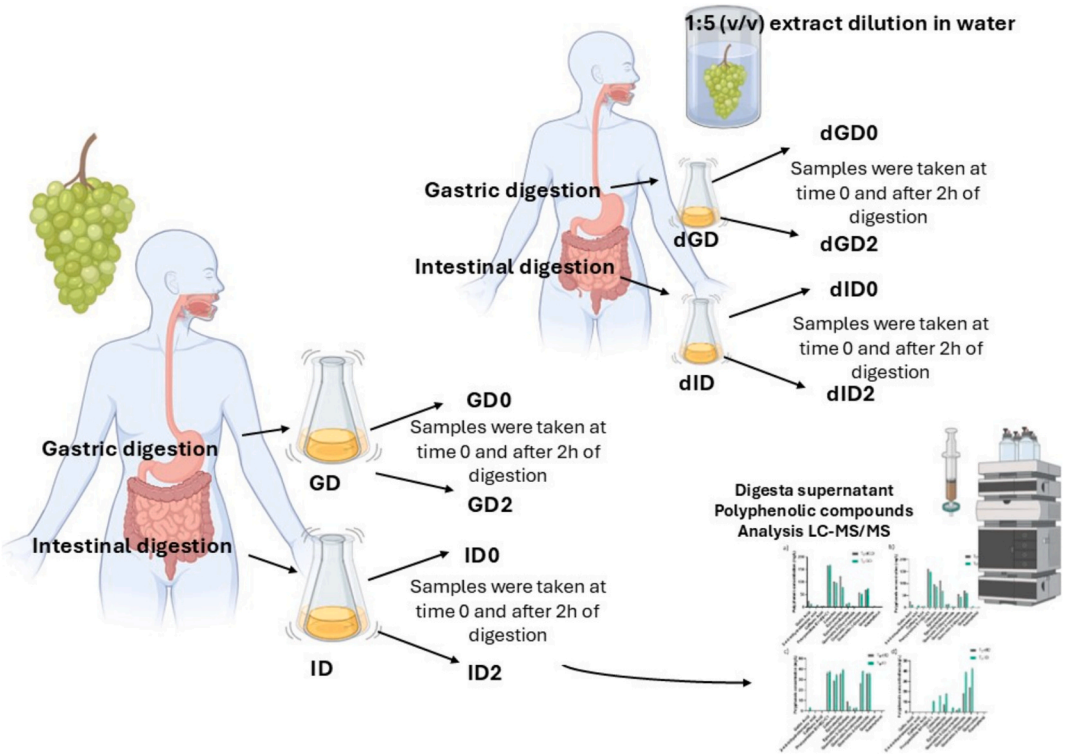

Fig. 1. *In vitro* gastrointestinal digestion of white grape marc extract. Non-digested extract was used as a control. INFOGEST protocol was followed. Extract was exposed to gastric and intestinal conditions. Samples were analysed at time zero and two hours after exposure to each digestive conditions. GD0: gastric digestion time 0. GD2: gastric digestion time 2 h. ID0: intestinal digestion time 0. ID2: intestinal digestion time 2 h. Diluted extract was exposed to the same digestive conditions in order to elucidate the effect of water solubility on the bioaccessibility of polyphenols. dilGD0: gastric digestion time 0. dilGD2: gastric digestion time 2 h. dilID0: intestinal digestion time 0. dilID2: intestinal digestion time 2 h. Figure created using Biorender (https://biorender.com/).

grape marc extract.

2.4. Total phenolic content (TPC)

The total phenolic content (TPC) was measured for the grape marc extract before and after treatment with bile salts to show if any interaction occurred. TPC was determined by the Folin-Ciocalteu method following Rubio et al., 2022 guidelines for microtitration in 96-well plates employing a microplate reader (BMG LABTECH, Ortenberg, Germany). Briefly, 20 μL of the obtained extract was diluted and subsequently mixed with 100 μL of Folin-Ciocalteu reagent (1:10, *v*/v) and 80 μL of sodium carbonate solution (7.5 g L^{-1}). The mixture was shaken and kept in the dark for 30 min. Then, the absorbance was measured at 760 nm. To express the TPC index, calibration curves of gallic acid covering a concentration range of 20–160 mg L^{-1} were employed. TPC was expressed as milligrams of gallic acid equivalent per liter of extract (mg GAE L^{-1}).

2.5. LC-MS/MS analysis

The quantification of the target polyphenols in the extracts and in the digestates was performed by LC-MS/MS using a Thermo Scientific (San Jose, CA, USA) instrument based on a TSQ Quantum Ultra™ triple quadrupole mass spectrometer equipped with a heated electrospray ionization (HESI) source, and an Accela Open autosampler with a 20 μL loop. Optimal instrumental conditions were previously optimized by Celeiro et al., 2019 to obtain the best chromatographic separation of the target polyphenols. The chromatographic separation was performed employing a Kinetex C18 column (100 mm, 2.1 μm, 100 Å) obtained from Phenomenex (Torrance, CA, USA). Column temperature was set at 50 °C. The mobile phase was composed of water (A) and methanol (B), both with 0.1 % formic acid. The chromatographic gradient was set at 5 % B, reaching 90 % B in 11 min and kept constant for 3 min. Initial conditions were achieved in 6 min. The total run time was 20 min. Injection volume was 10 μL at a flow rate of 0.2 mL min^{-1}. Compounds identification and quantification were performed employing selected reaction monitoring (SRM) working mode, scanning simultaneously in both negative and positive modes and monitoring two or three MS/MS transitions for each compound (see Table S1). Other HESI source parameters were the spray voltage: 3000 V, vaporizer temperature: 350 °C, sheath gas pressure: 35 au (arbitrary units), and ion sweep and auxiliar gas pressure: 0 and 10 au, respectively, and the capillary temperature: 320 °C. The system was operated by Xcalibur 2.2. and Trace Finder 3.1. software. External calibration was used for the quantification of polyphenols. Linearity was evaluated in a wide range of concentrations from 0.01 to 10 μg mL^{-1} (8 levels and 3 replicates per level), employing standard solutions prepared in water/methanol (50:50 *v*/v). The obtained coefficients of determination (R^2) were, in all cases, higher than 0.9900.

2.6. Statistical analysis

In vitro experiments and analytical determinations were performed as explained in the previous sections. The concentration of target polyphenols in the samples was calculated as the mean of the concentrations present in the replicates and expressed in mg/L of extract. Standard deviation (SD) of *in vitro* digestion replicates is included in the graphs. All experimental data were subjected to analysis of variance (ANOVA) with a 95 % confidence level, followed by mean comparison tests, including Tukey and Dunnett tests ($p < 0.05$). Pearson's correlation study between polyphenols variation during *in vitro* digestive procedure and dilution was performed. All statistical tests were conducted using the software Graphpad Prism 9.0.

3. Results and discussion

3.1. Phenolic compounds profile

Twelve different polyphenolic compounds were detected in the characterized white grape marc extract, whose concentrations are collected in Table 1. Three different groups of polyphenols were identified, being flavan-3-ols the most abundant (208 mg/L), followed by flavonols (128 mg/L), and finally phenolic acids (18 mg/L). Procyanidins sum (Σ B1 + B2 + C1) is the predominant polyphenols with a total concentration of 93 mg/L, followed by the glycoside form of quercetin, quercetin-3-glucoside (68 mg/L) and catechin (64 mg/L). All the mentioned flavonoids have been previously studied and related with a reduction of cardiovascular risk, (Kanazawa & Osakabe, 2013) and inflammatory signalling, as well as with antimicrobial activity (Chen et al., 2022). On the other hand, low concentrations of quercetin and kaempferol have been detected in the extract, probably linked to the low polarity of these molecules (Vendramin et al., 2022). Similar results had previously been obtained by Manso et al. (2023) during the characterization of a white grape marc extract obtained by MSAT using a hydro-organic solvent (Manso et al., 2023). Nevertheless, when comparing this white grape marc extract with the one described in the above-mentioned publication, significant differences in the composition of flavonols as well as in phenolic acids were found. This variability exhibited by extracts obtained from a similar raw matter, underscores the relevance of the solvents as a determinant of the extract's composition.

3.2. Effect of gastrointestinal digestion on total and individual polyphenolic content

To determine the potential bioavailability of polyphenols from the white grape marc extract, *in vitro* simulated digestions were carried out, Fig. 1. The extract was exposed to gastric and intestinal conditions in order to evaluate the effect of digestion on bioactive polyphenols' bioaccessibility. The results reported in Table 2, show that gastric digestion increases the bioaccessibility of total polyphenols, improving their total content by 27 % by the end of the gastric phase. A significative variation was already observed between the undigested extract and the extract at the initial gastric digestive conditions, where TPC increased by 45 %. Variability in the concentrations of the target polyphenols was observed throughout the gastric phase, with significant differences ($p < 0.05$), as shown in Table 2. However, no significant changes were noted in the concentrations of caftaric acid, quercetin, and kaempferol during gastric digestion. Similar results were reported in a study where whole grapes were also exposed to digestive conditions (Tagliazucchi et al., 2010). Increased amounts of polyphenols were associated with a food-matrix polyphenols extraction by the gastric chemical environment. Grape marc is mainly composed of grape peel, which contains high concentrations of flavonoids, main polyphenols presented after gastric phase, suggesting that this increase is related to the liberation of non-bioaccesible polyphenols from previously undigested grape fiber. When the same authors exposed individual polyphenols to gastric conditions, they observed a very slight decrease after 2 h of digestion, revealing a good stability of these substances at acidic pH. Although most of the polyphenols of the extract used in the present work are in solution, we have observed the formation of a precipitate due to the presence of a rich composition that includes sugars, proteins and other compounds that could form insoluble complexes with the polyphenols. During the gastric digestion, enzymes, low pH and motility facilitate polyphenols liberation and bioaccessibility (Padayachee et al., 2013). Besides, the flavan-3-ols can exist as dimeric or trimeric forms. As reported by Odriozola-Serrano et al. (2023) the effect of the gastric digestion varies depending on the structure and the polymerization degree of the polyphenols. These authors observed that monomeric flavan-3-ols can be released from the oligomeric compounds due to the acidic pH reached in the stomach.

In the present work, a notable increase of flavan-3-ol (procyanidins, catechin, and epicatechin) was assessed under gastric conditions, as

Table 1
Concentration (mg/L) of the main phenolic compounds detected in the white grape marc extract.

Phenolic group	Phenolic compound	Concentration (mg/L)
	Gallic Acid	9
Phenolic acids	2,4,6-trihydrobenzoic acid	5
	Caftaric Acid	4
	Total Phenolic acids	18
	Procyanidine B1 + B2 + C1	93
Flavan-3-ols	Catechin	64
	Epicatechin	40
	Epicatechin gallate	10
	Total Flavan-3-ols	208
	Quercetin-3-glucuronide	2
	Quercetin-3-rutinoside	49
Flavonols	Quercetin-3-glucoside	68
	Quercetin	8
	Kaempferol	1
	Total Flavonols	129
Total Polyphenols		355

Table 2
(A) White grape marc extract polyphenols concentration (mg/L) variation during *in vitro* digestions. Control: non-digested extract. GD0: gastric digestion time zero. GD2: gastric digestion time 2 h. ID0: intestinal digestion time zero. ID2 intestinal digestion time 2 h. Dilution factors were applied so that the values among different conditions could be comparable (x2 for GD0 and GD2 and x4 for ID0 and ID2). BI: intestinal bioaccessibility, calculated as the % of the initial concentration of compound that is detected after the gastrointestinal digestion. (B) Polyphenolic concentration of 5× diluted extract (mg/L) Dilution factors were applied so that the values among different conditions could be comparable (x10 for dGD0 and dGD2 and x20 for dID0 and dID2). Different letters in the same row indicate that the mean values are significantly different ($p < 0.05$); nd: not detected.

Non-diluted						
Phenolic compound	Control	GD0	GD2	ID0	ID2	BI
Gallic Acid	9^{a}	12^{b}	12^{b}	3^{c}	nd	nd
2,4,6-trihydrobenzoic acid	5^{a}	8^{b}	7^{c}	nd	nd	nd
Caftaric Acid	4^{a}	5^{b}	5^{b}	nd	nd	nd
Procyanidine B1 + B2 + C1	93^{a}	166^{b}	149^{c}	37^{d}	10^{e}	11 %
Catechin	64^{a}	96^{b}	86^{c}	34^{d}	15^{e}	24 %
Epicatechin	40^{a}	78^{b}	67^{c}	39^{a}	18^{d}	44 %
Epicatechin gallate	10^{a}	16^{b}	13^{c}	4^{d}	4^{d}	42 %
Quercetin-3-glucuronide	2^{a}	4^{b}	3^{c}	3^{c}	4^{b}	162 %
Quercetin-3-rutinoside	49^{a}	52^{b}	43^{c}	38^{d}	39^{d}	79 %
Quercetin-3-glucoside	68^{a}	73^{b}	60^{c}	36^{d}	42^{e}	61 %
Quercetin	8^{a}	4^{b}	5^{b}	nd	nd	nd
Kaempferol	1^{a}	2^{b}	2^{b}	nd	nd	nd
Total	355	516	452	195	132	37 %
Diluted						
Phenolic compound	Control	dGD0	dGD2	dID0	dID2	BI
Gallic Acid	9^{a}	24^{b}	24^{b}	nd	nd	nd
2,4,6-trihydrobenzoic acid	5	nd	nd	nd	nd	nd
Caftaric Acid	4	4	4	nd	nd	nd
Procyanidine B1 + B2 + C1	93^{a}	164^{b}	160^{c}	36^{d}	nd	nd
Catechin	64^{a}	100^{b}	95^{c}	29^{d}	nd	nd
Epicatechin	40^{a}	122^{b}	111^{c}	35^{d}	7^{e}	18 %
Epicatechin gallate	10^{a}	11^{b}	12^{c}	9^{d}	nd	nd
Quercetin-3-glucuronide	2^{a}	4^{b}	4^{b}	3^{c}	2^{a}	79 %
Quercetin-3-rutinoside	49^{a}	58^{b}	55^{c}	26^{d}	18^{e}	37 %
Quercetin-3-glucoside	68^{a}	68^{a}	70^{b}	36^{c}	24^{d}	35 %
Quercetin	8	nd	nd	nd	nd	nd
Kaempferol	1	nd	nd	nd	nd	nd
Total	355	613	589	173	59	17 %

shown in Table 2. This is probably due to the low pH in the stomach that degrades oligomers and complex molecules to smaller units and, as a result of this degradation, a higher polyphenolic content can be determined.

The transition from the acidic gastric to the mild alkaline intestinal environment caused a decrease in the amount of bioaccessible total polyphenols. Individual polyphenolic variation during digestion is shown in Fig. 2. A drop of 63 % of the total polyphenolic content after initial intestinal digestion was observed. This abrupt decrease is linked to the flavan-3-ols drop. Procyanidins, epicatechin gallate, catechin, and epicatechin are the most affected molecules, suffering a content reduction of 75 %, 68 %, 60 %, and 42 %, respectively. Significant differences were observed among flavan-3-ols concentration values along the digestive phases (Table 2). Some studies have shown that the stability of procyanidins is related to the environmental pH, being higher at acidic pH than under neutral or alkaline conditions (Odriozola-Serrano et al., 2023). Catechin instability under intestinal digestive conditions has been previously reported by Green et al. (2007) during their *in vitro* assessment of tea catechins' recovery rate. They reported an 80 % loss of.

total catechins, being epigallocatechin and epigallocatechingallate the most affected. These authors point out that upper small intestine conditions are particularly favourable for catechin degradative reactions. The elevated pH, the residual oxygen in solution, and the likely presence of reactive oxygen species from normal digestive function may facilitate several reactions including epimerization and auto-oxidation in the intestinal lumen. Besides, elevated pH conditions promoted autoxidation reactions which facilitates homo- and hetero-dimerization, preventing the detection of polyphenol.

Quercetin is a powerful antioxidant widely distributed in edible plants but mostly as glycoside forms. Its low bioavailability has been reported by Rocha-Amador et al. (2017). In this study, quercetin was not detected at the intestinal stage. On the contrary, a stable concentration of glycosylated quercetin forms during all the digestive stages (Fig. 2) was detected. This stability depends on the glycoside form, being quercetin-3-glucuronide the only one that maintains and even improves its concentration during the alkaline intestinal stage, where the rest of the polyphenol's concentration drops. On the other hand, during the intestinal digestion, quercetin-3-glucoside shows a reduction of 40 % with respect to the final gastric digestion value. Surprisingly, at the end of the intestinal phase the concentration of this molecule experiences a slight increase. No salient effect has been observed on the concentration of quercetin-3-rutinoside during the intestinal digestive process, although significant differences were detected during the gastric phase (Table 2). Quercetin's low stability in digestive conditions could be related to its hydroxyl substitution, which tends to bond a glycoside group. Rocha-Amador et al. (2017) suggested that glycosylation gives stability and improves quercetin bioaccessibility. The results presented in the current research support these observations.

Flavan-3-ols' concentration keeps dropping during the intestinal digestion, being procyanidins, catechin, and epicatechin concentrations near 50 % of the initial one at this digestive stage. The concentration of epicatechin gallate drops at the beginning of the intestinal digestion but keeps stable during the rest of the process (Fig. 2C).

Finally, phenolic acids have mainly disappeared at the beginning of the intestinal stage. The only remaining acid, gallic acid, shows a decrease of 73 % with respect to the final gastric value, finally disappearing completely after two hours of intestinal digestion.

3.3. *Effect of solubility on the stability of polyphenols under gastrointestinal digestion*

White grape marc extract was five-fold diluted in distilled water in order to evaluate polyphenol water solubility and its effect on stability during digestive conditions. Since other grape marc extracts have already shown promising antipathogenic results, (Rama et al., 2021, Manso et al., 2023) the assessment of the effect of solubility on the bioaccessibility of these extracts is an important step to fit an adequate

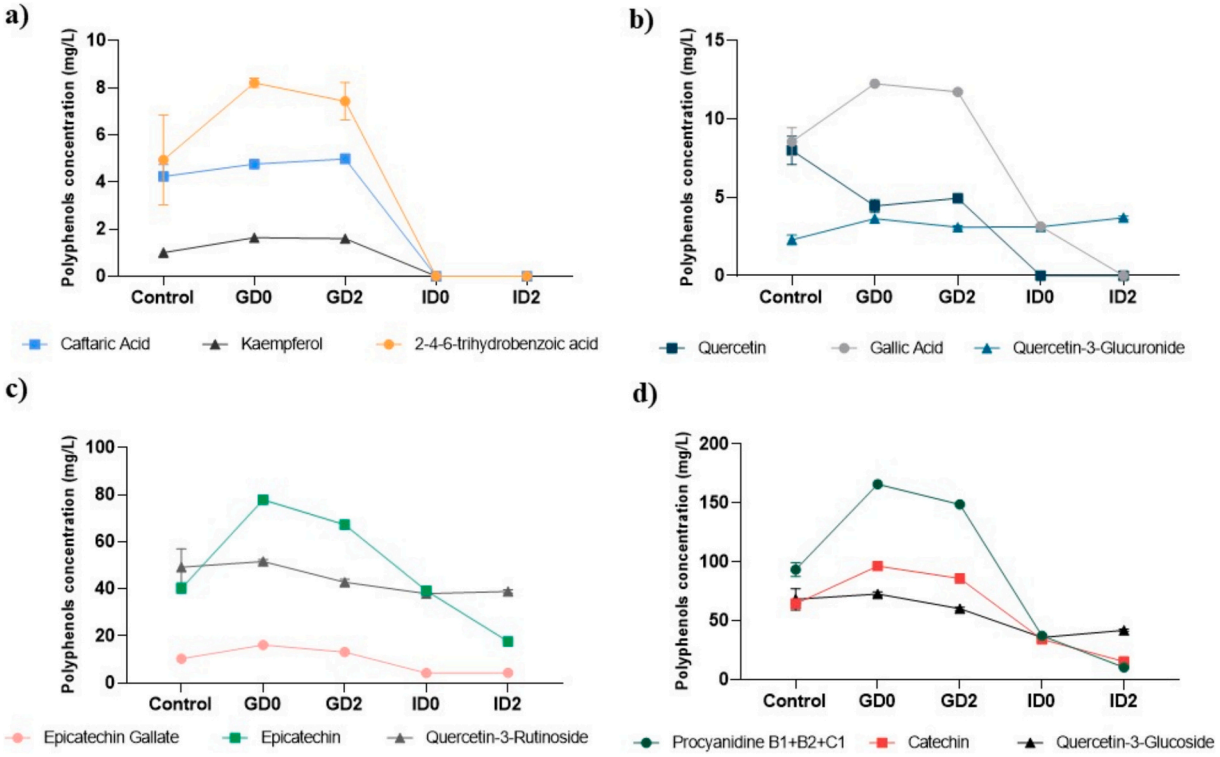

Fig. 2. White grape marc extract polyphenols variation during *in vitro* simulated digestions. Control: non-digested extract. GD0: gastric digestion time 0. GD2: gastric digestion time 2 h. ID0: intestinal digestion time 0. ID2: intestinal digestion time 2 h. Data are represented as average concentration values per volume of extracts. Standard deviations are represented.

concentration when used as antimicrobials.

The polyphenolic concentrations in the digested diluted samples are collected in Table 2. TPC of the diluted extract after 2 h of gastric digestion reached values of 589 mg/L, 66 % higher than in the undigested control extract, and it shows a 30 % increase in comparison to the non-diluted sample at the same stage. This polyphenol enhancement is notable at the initial stage of the gastric digestion, where a huge increase of flavan-3-ols (procyanidins, catechin, and epicatechin) is detected. Previously, Barchan et al., 2014 reported the effect of solvents on polyphenol extraction, proving that using polar solvents such as methanol and water the TPC recovered was higher than using less polar ones. Phenolic acids are also affected by the water dilution especially during gastric digestion, being gallic acid the most abundant acidic phenol with a concentration almost three times higher to that in the control extract, and double that of the non-diluted digested extract (Table 2). Gallic acid is highly soluble in water due to the big number of hydroxyl groups, which leads to increased association interactions with water. (Mota et al., 2008). The rest of phenolic acids have not shown the same tendency. The concentration of caftaric acid was unaffected by the acidic water solubility and the 2,4,6-trihydroxybenzoic acid was neither detected at this preliminary digestive stage nor in the rest of the phases (Fig. 3A, B). Quercetin and kaempferol, both present in the extract in very low concentrations were not detected in the diluted digestive process. These flavonoids present low solubility in water which suggests that they might have precipitated. On the other hand, the previously observed stability of quercetin glycoside forms during gastric digestion is also found for the diluted extract during gastric digestion. Nevertheless, significant differences were observed for quercetin-3-rutinoside and quercetin-3-glucoside between the diluted and undiluted assays (Fig. 3A, B), suggesting a detectable effect of the solubility on their concentrations. Mild alkaline intestinal conditions also affect the presence of polyphenols during the intestinal digestion in a similar manner than that described for the undiluted samples. The slightly basic pH of the intestinal fluids and their oxygenation may influence the

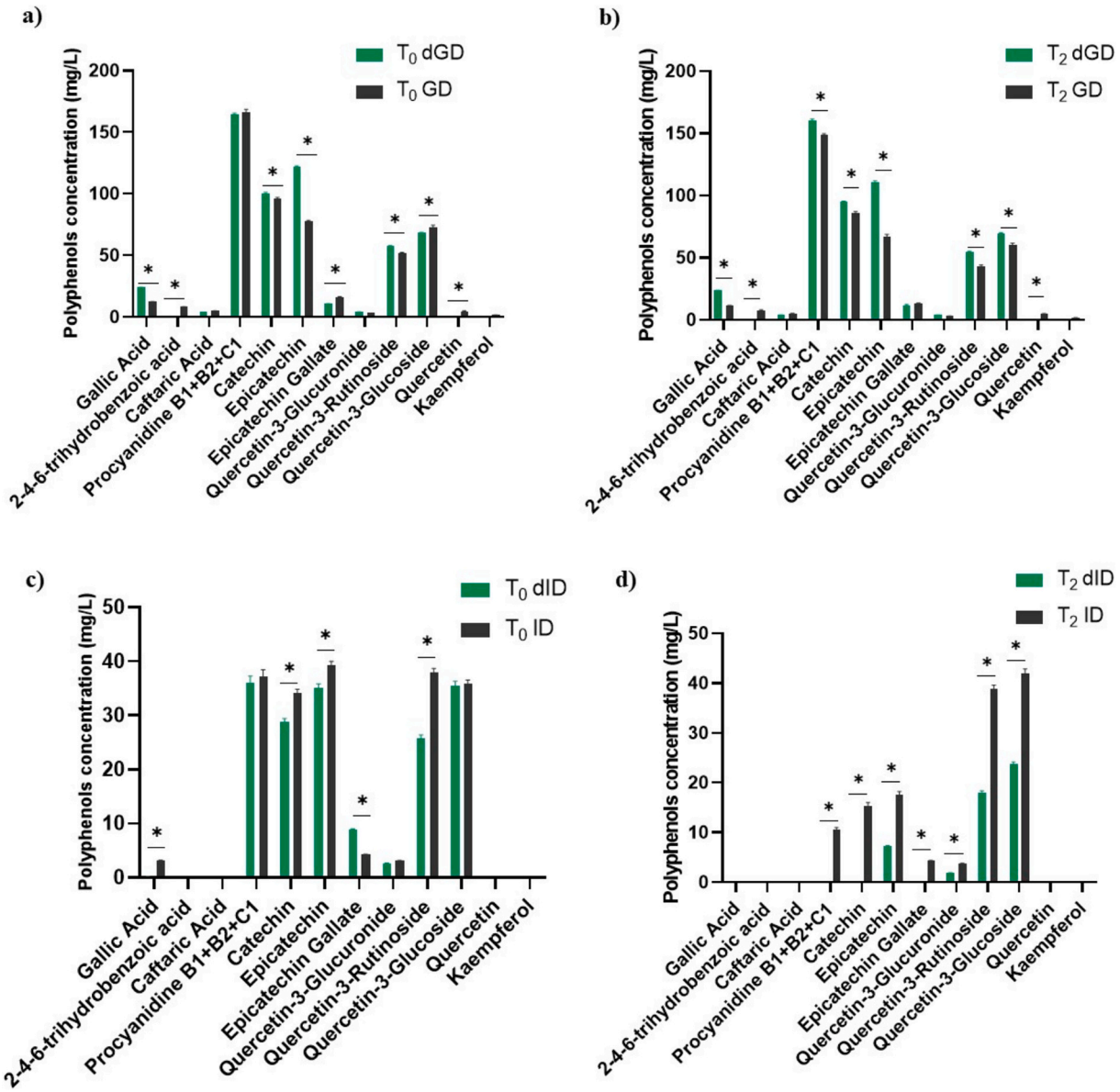

Fig. 3. Comparative polyphenols variation during *in vitro* digestion of diluted and non-diluted extract samples. GD0: gastric digestion time 0. GD2: gastric digestion time 2 h. ID0: intestinal digestion time 0. ID2: intestinal digestion time 2 h. dGD0: diluted-gastric digestion time 0. dGD2: diluted-gastric digestion time 2 h. dID0: diluted-intestinal digestion time 0. dID2: diluted-intestinal digestion time 2 h. Data are presented as average concentration per extract volume and standard deviation. Significant differences between diluted and non-diluted target polyphenols are represented * $p < 0.001$.

precipitation or degradation of the free polyphenols. The diluted samples present a drop of 71 % of the total polyphenolic content at the beginning of the intestinal digestion, in comparison with the final gastric composition. This percentage of decrease was 13 % higher than in the non-diluted assay at the same stages.

The most abundant polyphenols −160 mg/L of procyanidins, 95 mg/L of catechin, and 111 mg/L of epicatechin suffered a dramatic concentration decrease in the diluted intestinal digestive assay (Table 2). Their concentration drops to values of around 30 mg/L, which means a reduction of 77 % procyanidins, 70 % catechin, and 68 % epicatechin. No relevant effect was observed on epicatechin gallate.

Interestingly, whereas a decrease on the total polyphenolic concentration was assessed after 2 h intestinal digestion for both diluted and non-diluted extracts, this was not very significant in the case of non-diluted digestion assays. A drop of 70 % of total polyphenolic content was observed at the final intestinal stage of diluted samples in comparison with the initial intestinal stage (173–59 mg/L), whilst a decrease of only 30 % (195–132 mg/L) was assessed on the non-diluted samples (Fig. 3D). Flavan-3-ols were the most affected polyphenols, since they almost completely disappeared during the intestinal digestion. Quercetin glycosides also suffer a content decrease, which conclude with quercetin-3-glucoside being the most abundant polyphenol in the final diluted digestive stage with a concentration of 24 mg/L. The results collected in Table 2 and Fig. 3, suggest that solubility influences the bioaccessibility of some of the polyphenols detected in the grape marc extract. An increase on the TPC during the gastric digestion proves that water performed an extra extractive process, allowing polyphenol liberation from complex molecular structures in which polyphenols can be trapped. Water effect was also seen in the intestinal digestion, where polyphenols initially dropped similarly to the undiluted assay, but

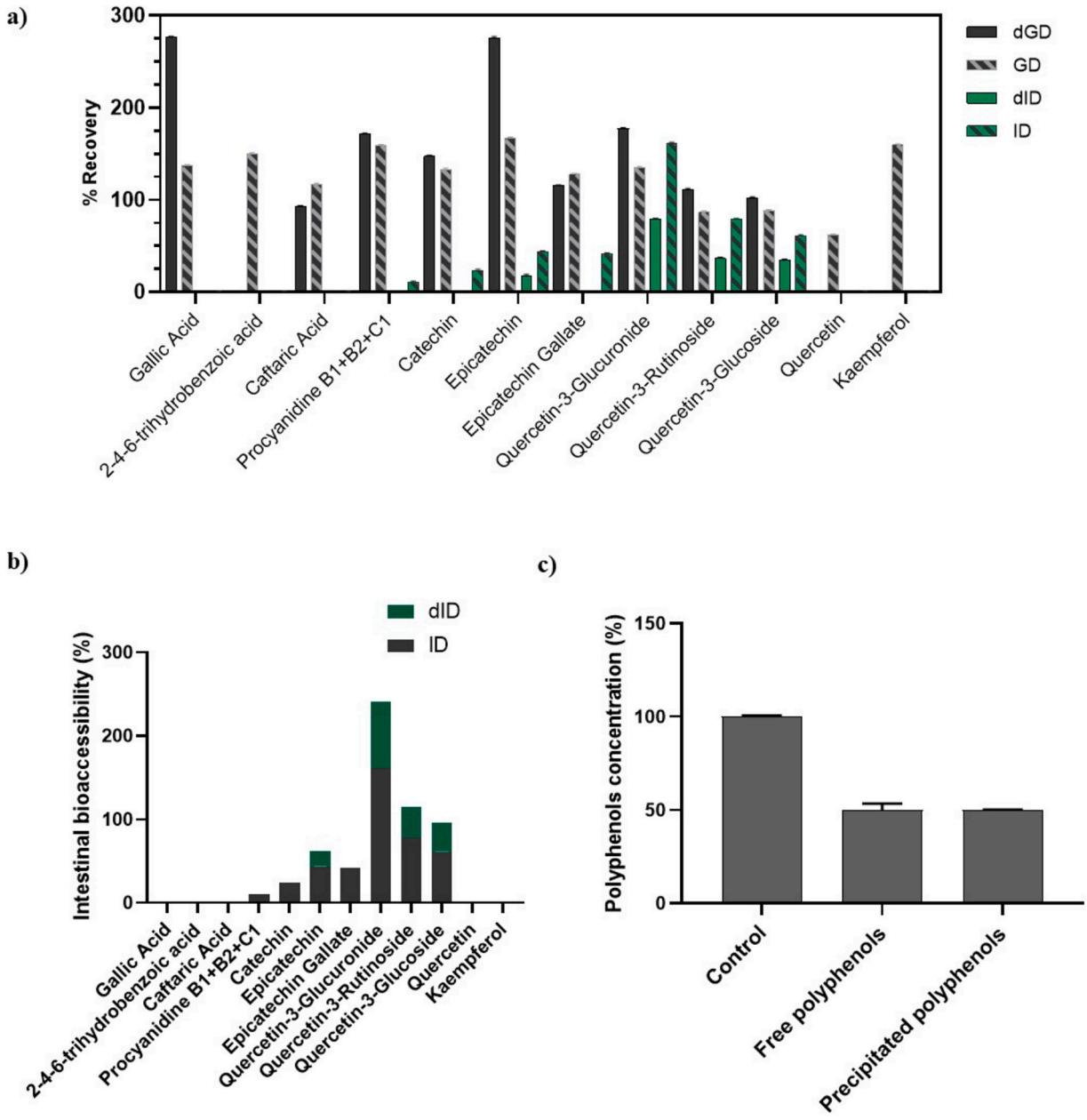

Fig. 4. Bioaccessibility percentage of individual polyphenols obtained after *in vitro* gastrointestinal digestions. a) Recovery rate of each polyphenol in comparison with control concentration during each digestive stage. b) Intestinal bioaccessibility, potential bioavailable polyphenols, on diluted (dID) and non-diluted (ID) samples. c) Percentage of polyphenols remaining in suspension and % of polyphenols precipitated after bile salts interaction. Control refers to the total extract polyphenolic content.

finally, after two hours of exposition to the mild alkaline conditions of the intestine, polyphenol content decreased dramatically, probably linked to the pH effect, oxidative conditions (Green et al., 2007), and interaction with bile salts.

3.4. Bioaccessibility and polyphenols interaction with bile salts

Bioaccessibility, expressed as the fraction of phenolic compounds freed from its matrix into the gastrointestinal tract and, therefore, accessible for intestinal absorption, has been calculated using Eq. 1.

Variation of the bioaccessibility along the complete process for non-diluted and diluted samples is collected in Fig. 4A. Intestinal bioaccessibility of polyphenols was higher in the non-diluted samples, with values twice higher than those of the diluted ones (Table 2). The observed low intestinal recovery is related with the low mild alkaline bioaccessibility of flavan-3-ols, primarily epicatechin gallate and catechin, which were not detected after intestinal digestion of the diluted samples (Fig. 3C). Interestingly, quite good bioaccessibility rates have been obtained for flavonols, especially for quercetin-3-glucuronide on both diluted and non-diluted digestions. This polyphenol shows an intestinal bioaccessibility of 79 % in the diluted digestion, and an improvement of its total content in the non-diluted samples, reaching a bioaccessibility fraction 60 % higher than those of the non-digested extract. The rest of glycosylated quercetin forms have obtained good bioaccessibility values, especially on the non-diluted digestive assays. Probably this recovery rate is related with the low solubility of the quercetin forms and the harmful effect of the intestinal conditions on its stability.

The drop of the polyphenols concentration at the beginning of the intestinal stage has a huge effect on the final bioaccessibility rate (Fig. 4B). In order to elucidate the main reason for this decrease, the interaction between bile salts at mimicked intestinal concentrations and the extract was assessed. During the experiment, immediate formation of precipitate was observed, suggesting that the decreased bioaccessibility of polyphenols may be linked to their insolubilization in the presence of bile salts. Specifically, half of the polyphenols in the extract precipitate in the presence of bile salts, meanwhile the other half remains bioavailable (Fig. 4C). These results fit the previous observed loss of free phenolic content at the beginning of the intestinal phase and even they can explain the major decrease in the diluted samples, where a higher interaction between bile salts and free polyphenols during digestion may occur. Besides, the affected polyphenols are the same during both, diluted and non-diluted assays.

Finally, Pearsons's correlation analysis was performed. A strong correlation in most phases, particularly between the gastric stages (GD0 and GD2, $r = 0.999$; dG0 and dGD2, $r = 0.999$) and between diluted and non-diluted gastric samples (dGD0 and dGD0, $r = 0.970$) was observed. Regarding intestinal conditions, moderate correlation has been observed between stages in both undiluted and diluted experiments (ID0 and ID2, $r = 0.811$; dID0 and dID2, $r = 0.575$). These results indicate that polyphenols follow similar trends during gastric digestion, even though their absolute concentrations decrease significantly, particularly during the intestinal phase. Additionally, a low correlation between gastric and intestinal phases ($r = 0.473$) was observed, which reflects a greater variability in the intestinal environment and a low polyphenols rate recovery.

Hence, the low bioaccessibility of polyphenols is not dependent on their degradation by the digestive enzymes. Tagliazucchi et al. (2010) reported the same polyphenolic content recovery during enzymatic and non-enzymatic digestion assays, proving that polyphenol degradation is a chemical environment-dependent process. Moreover, previous studies reported the inactivation of digestive enzymes by polyphenols, especially by glycosylated forms, suggesting substrate-enzyme binding (Alminger et al., 2014). These results could be quite interesting in order to control diet-metabolic chronic diseases as type 2 diabetes and cholesterol. (Tamargo et al., 2023).

Moreover, polyphenols retained in the intestinal tract could function as a fermentable substrate, which may be transformed by the gut microbiota into bioactive absorbable metabolites (Plamada & Vodnar, 2022), thereby having a valuable impact on the overall health.

4. Conclusions

In vitro bioaccessibility assays are a proficient way to assess the behaviour of bioactive compounds under digestive conditions. In this research a rich polyphenolic extract obtained from *Vitis vinifera* has shown an interesting phenolic profile, being rich in flavan-3-ols and flavonols with a high bioaccessibility rate, especially for quercetin glycosylated forms. It has been proved that gastric conditions perform extractive activity on polyphenols, enhancing their concentration. The results obtained during the initial intestinal phase show a dramatic decrease in polyphenol concentration. An additional experiment proved the interaction between bile salts and polyphenols, with the precipitation of 50 % of the phenolic compounds. This result suggests that the quantified decrease is not motivated by polyphenol degradation but by insolubilization. In order to assess the effect of the extract's dilution on polyphenol stability during digestive conditions, white grape marc extract was five-fold diluted in distilled water. The results obtained after the bioaccessibility experiments suggest that solubility has a beneficial effect on phenolic acids' bioaccessibility, especially for gallic acid, during gastric digestion, but no positive effect was seen after alkaline intestinal conditions. Finally, polyphenol recovery was influenced by the mild alkaline intestinal conditions, dropping drastically especially in the diluted samples. The interaction between polyphenols and bile salts detected during this study suggests that the insolubilized polyphenols can remain longer in the gut, acting as prebiotic compounds for the microbiota. This research lays the foundation for future studies focused on the use of white wine by-products as potential nutraceuticals and also highlights the importance of designing formulations aimed at improving the bioaccessibility of polyphenols.

CRediT authorship contribution statement

Lorena G. Calvo: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Conceptualization. **Maria Celeiro:** Software, Methodology, Investigation, Formal analysis. **Marta Lores:** Investigation, Funding acquisition. **Ana G. Abril:** Writing – review & editing, Supervision. **Trinidad de Miguel:** Writing – review & editing, Visualization, Supervision, Funding acquisition, Conceptualization.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2025.143810.

Data availability

No data was used for the research described in the article.

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