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RESEARCH

Characterization, Bioaccesibility and Antioxidant Activities of Phenolic Compounds Recovered from Yellow pea (*Pisum sativum*) Flour and Protein Isolate

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Abstract

This study focused on studying the bioaccesible phenolic compounds (PCs) from yellow pea flour (**F**) and protein isolate (**I**). Total phenolic contents (TPC), PCs composition and antioxidant activities were analysed in ethanol 60% extracts obtained by applying ultrasound assisted extraction (UAE, 15 min/40% amplitude). The preparation of **I** under alkaline conditions and the elimination of some soluble components at lower pH produced a change of PCs profile and antioxidant activity. After simulated gastrointestinal digestion (SGID) of both ingredients to obtain the digests **FD** and **ID**, notable changes in the PCs concentration and profiles could be demonstrated. **FD** presented a higher ORAC activity than **ID** (IC_{50} = 0.022 and 0.039 mg GAE/g dm, respectively), but lower $ABTS^{\bullet+}$ activity (IC_{50} = 0.8 and 0.3 mg GAE/g dm, respectively). After treatment with cholestyramine of extracts from **FD** and **ID** in order to eliminate bile salts and obtain the bioaccesible fractions $\mathbf{FD_b}$ and $\mathbf{ID_b}$, ROS scavenging in H_2O_2-induced Caco2-TC7 cells was evaluated, registering a greater activity for **ID** respect to **FD** (IC_{50} = 0.042 and 0.017 mg GAE/mL, respectively). These activities could be attributed to the major bioaccesible PCs: OH-tyrosol, polydatin, *trans*-resveratrol, rutin, (-)-epicatechin and (-)-gallocatechin gallate for **FD**; syringic (the most concentrated) and ellagic acids, *trans*-resveratrol, and (-)-gallocatechin gallate for **ID**, but probably other compounds such as peptides or amino acids can also contribute.

Keywords Yellow peas flour · Protein isolate · Ultrasound assisted extraction · Simulated gastrointestinal digestion · Phenolic compounds · Free radical scavenging · Intracellular ROS inhibition

Introduction

Legumes have a series of nutritional characteristics that make them very attractive as sources of ingredients for the development of innovative products. They are a good source of B vitamins, minerals and proteins, particularly rich in the essential amino acids lysine and leucine. Several health benefits have been attributed to components such as soluble and insoluble fibre, low digestible starch, prebiotic oligosaccharides and phenolic compounds (PCs) [1]. Different legumes have variable contents and types of PCs which can be free, esterified or linked to other components. Ferulic acid has been reported as the most abundant phenolic acid, while flavonol glycosides, anthocyanins and tannins, which primarily exist in the seed coat of pulses, are present in high or low concentrations depending on the pulse type and genotype [1, 2]. PCs have shown to protect the human body from the reactive oxygen species (ROS) damage which is associated with many diseases. However, several aspects should be taken into account when biological functionality of food matrices/ingredients containing PCs is evaluated: low stability during food processing and storage; modifications during digestion (by pH and enzymes) and interactions with other food components. The bioavailability of PCs is strongly dependent on their structures and only a part of the ingested PCs is bioavailable after oral administration. Some

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PCs need to undergo hydrolysis and be metabolized through the stomach/intestine environment and/or by the microbiota of the digestive tract before their absorption. The digestion process may change the PCs structure and, consequently, their antioxidant activity [3]. There is currently little literature on the characterization and antioxidant activity of the PCs of yellow peas [4–6], and even less on their bioaccessibility as well as on the study of these aspects on a derived and high-value ingredient such as protein isolate. The aim of this study was to evaluate the qualitative, quantitative and antioxidant activity profiles of the recovered PCs -ultrasound-assisted extraction (UAE) and a low-toxic solvent (ethanol/water)- from yellow pea flour and protein isolate, and their changes and bioaccesibility after SGID.

Materials and Methods

The detailed "Materials and Methods" section is provided as a supplementary material section (SM1).

Results and Discussion

TPC Content, Antioxidant Activity and PCs Composition of F and I

Ethanol 60% extracts from **F** and **I** were obtained applying UAE under conditions (15 min, 40% amplitude) previously optimized in our lab (unpublished). The TPC values registered for extracts from **F** (0.68 mg GAE/g **F** dm, Table 1) were within the range reported by other authors. Giusti et al. [7] performed extractions (0.25 mg/mL, 70% EtOH, pH 4, sonication) of different legumes flours reporting a value of 0.72 mg GAE/g for splitted green peas. Wu et al. [6] informed that the TPC of pea flours varied between 0.126 and 1.286 mg GAE/g which was significantly correlated with the colour and shape of seed coats. Compared with those results, the values of the present work are in the middle of this range. In the case of **I**, the TPC content of UAE extracts was 0.98 mg GAE/g **I** dm, significantly higher ($p < 0.05$) than that obtained for **F** (Table 1). Accordingly, a two-fold increase in the TPC content of the amaranth protein isolate compared to flour has been reported [8].

Table 1 TPC and antioxidant activities (ORAC, ABTS) of UAE extracts[1] from the yellow pea flour (F), protein isolate (I), their simulated gastrointestinal digests (FD, ID), and their bioaccesible fractions (FD_b and ID_b)

Sample		TPC	ORAC	ABTS
		mg GAE/g dm	IC_{50} (mg GAE/g dm)	IC_{50} (mg GAE/g dm)
Non digested	**F**	0.68 ± 0.02^{a}	0.023 ± 0.002^{a}	0.5 ± 0.1^{ab}
	I	0.98 ± 0.04^{b}	0.017 ± 0.002^{a}	0.6 ± 0.1^{bc}
After SGID	**FD**	5.04 ± 0.07^{d}	0.022 ± 0.002^{a}	0.8 ± 0.1^{c}
	ID	13.50 ± 0.10^{f}	0.039 ± 0.001^{b}	0.3 ± 0.1^{a}
After cholestyramine (bioaccesible)	**FD_b**	3.10 ± 0.10^{c}	0.060 ± 0.010^{c}	0.8 ± 0.1^{c}
	ID_b	10.30 ± 0.10^{d}	0.060 ± 0.010^{c}	0.6 ± 0.1^{bc}

[1]UAE conditions: EtOH 60%, 15 min, 40% amplitude.

dm: dry matter. In the case of **FD** and **ID**, content are referred to the original **F** and **I.**

Different superscript letters in the same column indicate significant differences among extracts ($p < 0.05$).

PCs profiles of the UAE extracts were analysed by HPLC-DAD-FLD. The majority of extracted and detected PCs of **F** were flavonoids. Among them, the flavan-3-ol (-)-epigallocatechin was the major; the procyanidin B1 (dimer epicatechin- (4β → 8) -catechin) was also relevant, while (+)-catechin and (-)-epicatechin were in lower concentration (Table 2). The flavonols rutin (quercetin-3-O-rutinoside) and the less abundant kaempferol-3-glucoside and quercetin-3-glucoside were also detected; as well as the flavanones herperetin and naringenin (at low concentration). Regarding stilbenes family, the content of polydatin (resveratrol-3-β-mono-D-glucoside) was important in **F**, while *trans*-resveratrol was found in a significantly lower concentration. Among the phenolic acids, rosmarinic, p-coumaric, gallic, ferulic and ellagic acids were detected. Also, OH-tyrosol was found (Table 2). Some of the PCs detected in **F** were previously reported by other authors in different parts of the pea seed. Dueñas et al. [9] identified the phenolic acids *trans p*-coumaric, *cis p*-coumaric, *trans* ferulic and (-)-epigallocatechin in pea cotyledons, and gallic acid, (-)-epigallocatechin, (+)-catechin, *trans p*-coumaric and *trans* ferulic acids in the pea seed coat. Giusti et al. [7] detected caffeic, *p*-coumaric and ferulic acids in splitted green peas. Borges-Martinez et al. [10] reported the presence of gallic acid and (+)-catechin in pea seeds. Methanolic extracts of the free phenolic fraction of peas contained *p*-hydroxybenzoic, *p*-coumaric, caffeic and ferulic acids, while the protein bound fraction contained *p*-hydroxybenzoic, *p*-coumaric, ferulic and sinapic acids [2]. Although Leguminosae family is an important source of stilbenes, as far as we know, polydatin has not been reported in yellow peas until now. Kalogeropoulos et al. [11] reported the presence of *trans*-resveratrol in cooked yellow and green peas. Reported differences could result from multiple factors, such as plant species and part, growth and storage conditions, methodology (extraction and analytical procedure, degradation).

nd: not detected. Different superscript letters in the same raw indicate significant differences ($p < 0.05$).

Differences between PCs profile of **I** and **F** were detected. **I** contained a greater proportion of phenolic acids

Table 2 Phenolic compounds (PCs) profile of **F**, **I** and their simulated gastrointestinal digests (**FD** and **ID**)

Compound	F	I	FD	ID
OH-tyrosol	1.7 ± 0.1^{b}	1.3 ± 0.1^{a}	13.6 ± 0.1^{c}	nd
Phenolic acids				
Ellagic acid	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1^{a}	5.1 ± 0.1^{b}
Gallic acid	0.8 ± 0.1^{a}	0.8 ± 0.1^{b}	nd	nd
Syringic acid	nd	nd	nd	220.0 ± 9.0
Caffeic acid	2.1 ± 0.5^{b}	6.0 ± 0.5^{c}	0.7 ± 0.2^{a}	nd
p-coumaric acid	1.5 ± 0.1^{b}	2.1 ± 0.1^{c}	1.1 ± 0.1^{a}	nd
Ferulic acid	0.5 ± 0.1^{a}	4.1 ± 0.1^{c}	0.8 ± 0.1^{b}	nd
Rosmarinic acid	5.2 ± 0.4^{c}	9.3 ± 0.1^{d}	3.3 ± 0.1^{b}	0.1 ± 0.1^{a}
Total phenolic acids	**10.0 ± 1.0**	**22.5 ± 0.1**	**6.1 ± 0.2**	**225.0 ± 9.0**
Stilbenes				
Polydatin	26.1 ± 0.1^{b}	41.9 ± 0.5^{c}	23.2 ± 0.3^{a}	nd
trans-resveratrol	2.6 ± 0.1^{a}	5.4 ± 0.1^{b}	7.5 ± 0.1^{c}	5.0 ± 1.0^{b}
Total stilbenes	**28.6 ± 0.1**	**47.3 ± 0.5**	**30.7 ± 0.3**	**5.0 ± 1.0**
Flavonoids				
Procyanidin B1	13.0 ± 6.0^{b}	4.6 ± 0.1^{a}	nd	nd
Rutin	5.2 ± 0.4^{a}	nd	6.1 ± 0.2^{a}	nd
Quercetin-3-glucoside	0.9 ± 0.1^{b}	0.4 ± 0.1^{a}	nd	nd
Kaempferol-3-glucoside	2.3 ± 0.3^{b}	6.2 ± 0.0^{c}	0.8 ± 0.1^{a}	nd
Daidzein	nd	0.3 ± 0.1	nd	nd
Genistein	nd	0.4 ± 0.1	nd	nd
(+)-catechin	1.1 ± 0.1^{b}	0.6 ± 0.1^{a}	nd	1.5 ± 0.1^{c}
(-)-epigallocatechin	59.7 ± 0.2^{b}	35.2 ± 0.9^{a}	nd	nd
(-)-epicatechin	0.6 ± 0.1	nd	27.0 ± 5.0^{a}	nd
(-)-gallocatechin gallate	nd	nd	6.3 ± 0.2^{a}	12.3 ± 0.7^{b}
Naringenin	0.3 ± 0.1	nd	nd	nd
Hesperetin	0.7 ± 0.1^{b}	0.5 ± 0.1^{a}	nd	nd
Myricetin	nd	nd	nd	2.5 ± 0.1
Total flavonoids	**84.0 ± 6.0**	**49.0 ± 1.0**	**40.0 ± 5.0**	**16.0 ± 1.0**
Total	**125.0 ± 6.0**	**119.0 ± 1.0**	**90.0 ± 5.0**	**246.0 ± 10.0**

Contents are expresed as µg/g d.m. In the case of **FD** and **ID**, content are referred to the original **F** and **I**. dm: dry matter

and stilbenes, and a lower proportion of flavonoids than **F**, with similar content of total PCs (sum of quantified compounds by HPLC-DAD-FLD, Table 2**).** Regarding individual PCs, OH-tyrosol and the flavonoids (+)-catechin, (-)-epicatechin, (-)-epigallocatechin, and procyanidin B1 (flavanols); rutin, quercetin-3-glucoside (flavonols), and hesperetin (flavanone) presented a lower content in **I**. During the preparation of **I**, some PCs originally present in **F** in a soluble form may be lost, and some compounds such as catechins could be degraded at the alkaline pH condition of the protein extraction [12], in correlation with the decrease of these compounds in **I**. In other way, there was an increase of polydatin (which was the most abundant PC in **I**), *trans*-resveratrol, kaempferol-3-glucoside, gallic acid, caffeic acid, *p*-coumaric acid, ferulic acid and rosmarinic acid, and the appearance of daidzein and genistein (isoflavones) in **I** (Table 2). PCs that were part of the bound fraction could be released since the alkaline treatment could break the interactions of PCs with proteins or fiber [8].

Antioxidant activity by ORAC and $ABTS^{\bullet+}$ scavenging assays were performed; IC_{50} values are shown in the Table 1. The extract obtained from **I** showed greater ORAC potency compared to **F**, while difference was no significant ($p > 0.05$) for the $ABTS^{\bullet+}$ method (Table 1). Zhao et al. [13] analysed the correlation between TPC and the antioxidant activity of 10 varieties of legumes. They reported that the antioxidant activity differed significantly between the legume extracts but in general a significant positive correlation was observed between TPC and the total antioxidant activity, the DPPH scavenging activity and the total reducing power of legume extracts. In our case, an increase in the TPC content also generated a small increase in the antioxidant activity. However, it is important to take into account the previously described changes in the PCs profile of **I** respect to **F** to explain the differences in antioxidant activity, especially in the capacity to scavenge peroxyl radicals (ORAC). In addition, it was possible that other types of compounds were present in the extracts. To analyse this, FPLC gel filtration chromatography of the UAE extracts was performed. The chromatogram corresponding to **F** is shown in Fig. 1A; a relevant presence of molecules with MW > 1 kDa and even > 6.5 kDa. Therefore, this ethanolic extract contains molecules of higher MW than identified PCs, probably peptides and polypeptides that could contribute both to the TPC value and to the antioxidant activities. Similar result was obtained for **I**, but with differences in the intensity of some of the peaks respect to **F** (Fig. 1A).

Effect of SGID on TPC Content, Antioxidant Activity and PCs Composition of F and I

After SGID, both digests (**FD** and **ID**) presented a significant ($p < 0.05$) and notable increase in the TPC content compared to **F** and **I**. The increment was about 7.5 times for **FD** and about 13.5 times for **ID** (Table 1). For the UAE extract of the digestion reagent blank (**BR**) a concentration of 0.022 ± 0.002 mg GAE/mL was obtained. That represented about a 12 and 10% of the TPC of the **FD** (0.181 mg GAE/mL) and **ID** (0.229 mg GAE/mL) extracts, respectively, showing a negligible contribution of the digestion reagents to the TPC values of digests. SGID produced a significant release of compounds of **F** and **I** soluble in EtOH 60% and quantifiable by the Folin-Ciocalteau. Beyond this TPC increase in TPC, when the samples were analysed by

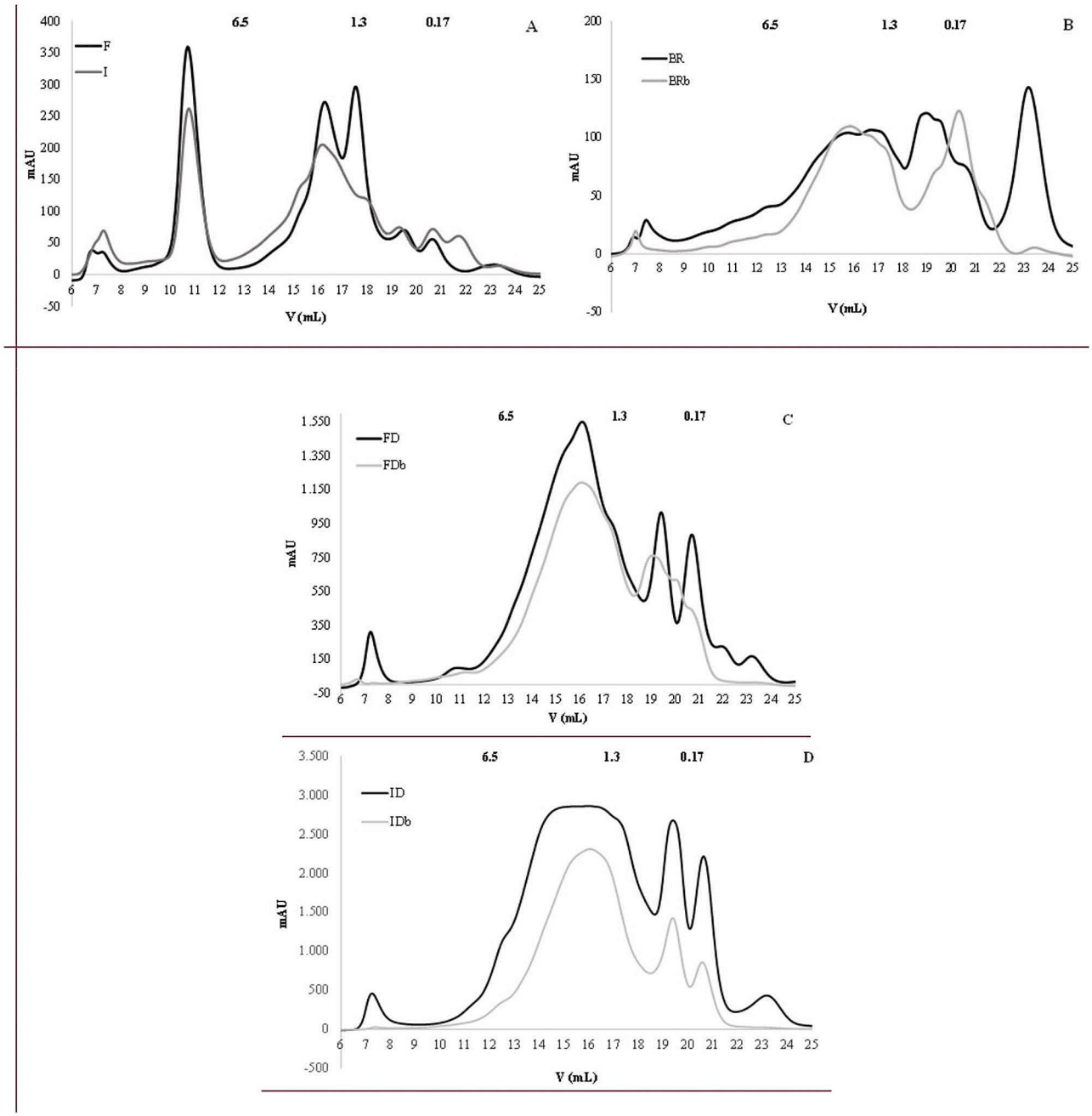

Fig. 1 Gel filtration FPLC (Superdex 30 column) of UAE 60% ethanol extracts (solubilized in PBS) from: **A**) yellow pea flour (**F**) and protein isolate (**I**); **B**) reagent blanks of SGID (**BR**) and their bioaccesible fraction ($\mathbf{BR_b}$); **C**) SGI digest of F (**FD**) and its bioaccesible fraction ($\mathbf{FD_b}$); **D**) SGI digest of **F** (**ID**) and its bioaccesible fraction ($\mathbf{ID_b}$). Bioaccesible fractions were obtained by cholestyramine treatment. Molecular weight markers (kDa) are indicated at the top of figures

HPLC-DAD-FLD, a significant decrease of total PCs was found in the case of **FD**, where about a 72% of PCs amount was recovered after SGID. However, an increment of about 2 times of the total PCs was registered in the case of **ID** (Table 2), showing a clear difference in the behaviour of PCs contained in each ingredient during the SGID. This fact can be explained taken into account that the digests contained other components such as polypeptides, peptides or free amino acids that could be extracted in EtOH/water and can increase the TPC values.

Molecules with MW > 1 kDa could be observed in the gel filtration chromatograms of **FD** (Fig. 1C) and **ID** (Fig. 1D).

Important changes with respect to **F** and **I** (Fig. 1A) were evident, such as the disappearance of the molecules with MW between 6.5 and 10 kDa, the increment of molecules with MW > 10 kDa and with MW < 6.5 kDa, including small molecules (< 0.17 kDa). As can be observed, the low MW fractions increased in both matrices, but mostly in the **ID**. The reagent blank (**BR**) contributed in a lower proportion with this kind of molecules, especially the smaller ones in a greater amount (Fig. 1B).

The HPLC-DAD-FLD profiles showed that many of the PCs present in **F** were not detected in **FD**, especially some non-abundant flavonoids compounds (quercetin-3-glucoside, naringenin, hesperetin, (+)-catechin) and the most abundant ones (procyanidin B1 and (-)-epigallocatechin). However, (-)-epicatechin increased and (-)-gallocatechin gallate appeared in **FD** (Table 2). These facts suggested the occurrence of dimers hydrolysis, modification, and isomerization of catechins during the SGID. Previous studies carried out on green tea catechins demonstrated a loss of this kind of compounds after in vitro digestion. It was attributed to the formation of semiquinone free radicals in the pyrogallol residue of the B ring at almost neutral pH, presenting epigallocatechin (a major compound in **F**) a high tendency to this modification [14]. Also, it has been demonstrated that procyanidins are unstable at the gastric pH, and degradation of procyanidins and epimerization have been observed in the mild alkaline intestinal environment [15]. In other way, the flavonoid rutin did not show significant changes after SGID (Table 2). The stilbene polydatin presented a significant but small reduction together with an increase in the content of *trans*-resveratrol (Table 2). Also, OH-tyrosol and ferulic acid increased while caffeic, *p*-coumaric and rosmarinic acids decreased in **FD** with respect to **F**. These results suggested that SGID produced several effects on the PCs of peas including chemical instability, chemical modifications but also the release of some compounds from the matrix. In this way, PCs profile of **FD** was significantly different from those of **F**. In agreement with the present results, Ma et al. [5] studied the PCs of red and yellow pea hull after SGID observing that TPC, total flavonoid content and individual PCs (the last ones only determined in red peas hull) decreased along the sequential digestion steps. A reduction between 52 and 75% of PCs was informed after SGID of two varieties of beans which was attributed to instability at high pH values [16]. In this way, different studies reported different stability of legume PCs during salivary, gastric and intestinal digestion. Regarding SGID of **I**, of the 17 PCs detected in this sample, only 4 were identified in **ID**: increased ellagic acid and (+)-catechin, decreased rosmarinic acid, and *trans*-resveratrol without significant change in it concentration. In addition, (-)-gallocatechin gallate (as in **FD**), myricetin (a flavonol) and a notorious amount of syringic acid (a hydroxybenzoic acid) appeared in **ID** (Table 2). These results showed differences in the PCs composition of **FD** and **ID**. Cao et al. [17] reported a poor stability after SGID for several PCs of passion fruit peel extracts, such as quercetin 3-glucoside (as in **FD** and **ID**), naringenin (as in **FD**), rutin (no modified in our digests), and polydatin (slightly decreased in **FD** and disappeared in **ID**). Differences in the stability of some PCs during SGID could be related to the matrix, even between **F** and **I**.

In vitro antioxidant activity by ORAC and $ABTS^{\bullet+}$ assays were performed for extracts of the digests and compared to the non-digested samples (Table 1). SGID did not produce changes in the ORAC potency of **F**, since no significant difference ($p > 0.05$) between de IC_{50} values of **F** and **FD** were obtained. The $ABTS^{\bullet+}$ values showed a significantly lower activity of **FD** respect to **F**. In the case of **ID**, SGID produced a significant ($p < 0.05$) decrease of ORAC and increase of $ABTS^{\bullet+}$ activity. According to the present results, although the SGID of **F** and **I** released extractable components that increased the TPC value, the free radical scavenging activities were not necessarily the highest. **ID** showed the greatest activity measured by $ABTS^{\bullet+}$ but the lowest one by the ORAC assay, while **FD** presented an intermediate ORAC activity and the lowest $ABTS^{\bullet+}$ activity. Many studies in different plant-based products reported a decrease in antioxidant activity after SGID. Cao et al. [17] showed that SGID had a negative effect on the DPPH and FRAP activities of extracts of passion fruit peel flour; however, the $ABTS^{\bullet+}$ scavenging ability was improved. In the case of yellow peas hulls, a good correlation existed between the $ABTS^{\bullet+}$ scavenging activity and the TPC, both reduced after SGID, but a poor correlation was found between this activity and the total flavonoids content [5]. The observed behaviours would be related to the different PCs profiles of each sample, previously described. It is clear that the antioxidant activities are the result of the contribution of all the compounds present in each extract. However, in order to try to explain the differences, we can consider the major components in each digest: OH-tyrosol, polydatin, *trans*-resveratrol, rutin, (-)-epicatechin and (-)-gallocatechin gallate in **FD**; syringic and ellagic acids, *trans*-resveratrol and (-)-gallocatechin gallate in **ID**. Platzer et al. [18] did not found a clear correlation among the PCs structure and the outcome of the ORAC assay (based in a hydrogen atom transfer -HAT- mechanism), comparing to those previously reported from the $ABTS^{\bullet+}$, DPPH and TPC assays (three single electron transfer –SET- reactions), suggesting that they are influenced by different structural properties. The antioxidant behaviour of PCs is dominated by the substituents, whereas their backbone plays a minor role. The number of hydroxyl groups present in PCs had the highest influence on ORAC activity, except for molecules

with two or more hydroxyl groups next to each other, probably related to steric hindrance. Sugar residues at C-3 or C-5 reduced the antioxidant effect. Among the different PCs subgroups, flavonols presented the greatest mean ORAC activity. Ferreyra et al. [19] established that the flavanol (-)-epicatechin -found in **FD** but not in **ID-** presented a good correlation with the $ABTS^{\bullet+}$ activity but a bad correlation with the ORAC activity. Grzesik et al. [20] demonstrated that catechins showed the highest stoichiometry of $ABTS^{\bullet+}$ reduction comparing with other PCs among which were some found in our digests such as flavonols (for example rutin), and *trans*-resveratrol. In addition, (-)-gallocatechin gallate –found in **FD** as well as in **ID-** presented high $ABTS^{\bullet+}$ activity, being somewhat lower for (-)-epicatechin [21]. The stilbenes *trans*-resveratrol –present in **FD** and **ID-** and polydatin –present only in **FD-** have demonstrated good ORAC and $ABTS^{\bullet+}$ activity [22]. The OH-tyrosol (a relevant component of **FD**) showed a poor correlation with both antioxidant activities [19]. These authors also informed that syringic acid –the most abundant component of **ID-** had strong correlation with the $ABTS^{\bullet+}$ activity. In this way, the high content of syringic acid in **ID** could, at least partially, explain the greatest $ABTS^{\bullet+}$ activity of this digest. Luo et al. [23] informed a high bioaccessibility of this compound in three sesame seed varieties during the digestion and faecal reaction, giving the oxidation of lignin as a possible reason for syringic acid generation. In addition to PCs, the presence of small size peptides, which seems to be higher in **ID**, has been associated to increases in antioxidant activities of protein hydrolysates of diverse sources and particularly of yellow peas [24]. Results suggest potential additive or synergic effect between PCs and protein components.

In order to achieve a closer approximation to the potential in vivo antioxidant activity of PCs of **FD** and **ID**, cellular assays were carried out. Since the presence of bile salts was evident in the ethanolic extracts of digests and taking into account that they have demonstrated to have cytotoxic and oxidant power [26], a treatment with cholestyramine was performed to obtain the named bioaccesible fractions, $\mathbf{FD_b}$ and $\mathbf{ID_b}$. TPC, ORAC and ABTS activity were analysed for them (Table 1). TPC diminished after cholestyramine treatment in a 39% for $\mathbf{FD_b}$ and 24% for $\mathbf{ID_b}$, and was undetectable for $\mathbf{BR_b}$. Also, the antioxidant potency decreased: ORAC and $ABTS^{\bullet+}$ for $\mathbf{FD_b}$ and only ORAC for $\mathbf{ID_b}$. Rodríguez and Tironi [25] demonstrated that the treatment with cholestyramine of aqueous soluble fractions of digests of amaranth products resulted in an important reduction of bile acids, but also of compounds of protein nature. In our case, the comparison of the gel filtration chromatograms of **FD** and $\mathbf{FD_b}$ showed that after cholestyramine treatment of the ethanolic extracts, molecules with MM > 10 kDa and those with MM < 0.17 kDa were mainly lost, while it was a partial loss of molecules with MW between 0.17 and 6.5 kDa (Fig. 1C), in which bile salts will be included. Similar behaviour was observed for $\mathbf{ID_b}$ with a greater loss of these molecules (Fig. 1D). The loss of some PCs after treatment with cholestyramine cannot be ruled out.

Cytotoxicity of $\mathbf{FD_b}$,$\mathbf{ID_b}$, and $\mathbf{BR_b}$ was evaluated in terms of the LDH release % in the supernatant of Caco2-TC7 cells treated with different dilutions of the samples, as an indicator of cellular damage (Table 3). It was possible to observe that, after treatment with cholestyramine, $\mathbf{BR_b}$ presents an undetectable TPC content but a high cytotoxicity (74%), probably related to remaining bile salts, which decreased with the dilution of this sample. $\mathbf{FD_b}$ presented a high but lower (56%) cytotoxicity value that also decreased as the dilution. In the case of $\mathbf{ID_b}$, the cytotoxicity values were even lower though it had a higher content of TPC. These results suggested that the cytotoxicity of the bioaccessible fractions is given by the remnants of digestion reagents and that it is attenuated by the presence of PCs (or other compounds) from **FD** and **ID**.

Table 3 Cytotoxicity as LDH activity and intracellular ROS scavenging of Caco2-TC7 cell treated with bioaccesible fractions ($\mathbf{FD_b}$, $\mathbf{ID_b}$**and** $\mathbf{BR_b}$) from UAE extracts of digests (**FD**, **ID, BR**)

	TPC		
Sample	C (µg GAE/mL)[1]	LDH (U/I)	%ROS scavenging[2]
$\mathbf{FD_b}$	140 ± 5	56 ± 8^{c}	66 ± 4^{c}
	28	26 ± 2^{b}	48 ± 2^{b}
	7	12 ± 4^{a}	22 ± 7^{a}
$\mathbf{ID_b}$	360 ± 4	33 ± 14^{b}	83 ± 5^{d}
	72	11 ± 1^{a}	67 ± 3^{c}
	18	11 ± 4^{a}	50 ± 3^{b}
$\mathbf{BR_b}$	8e-2 ± 1	74 ± 2	-
	1e-2	55 ± 8	-
	4e-3	23 ± 4	-

[1]concentration corresponding to direct, 1/5 and 1/20 dilution of the original bioaccesible fractions.

[2]calculated with respect to the corresponding dilution of BR. Different superscript letters in the same raw indicate significant differences ($p < 0.05$).

Intracellular ROS were measured using DCFH-DA. It diffuses through the cellular membrane and is enzymatically hydrolysed by intracellular esterase to DCFH, which can be oxidized to the fluorescent DCF. Fig. S1 (Supplementary material S2) shows the evolution of the fluorescence for the control system (C1: maximal oxidation, C2: basal state) as well as for $\mathbf{BR_b}$, $\mathbf{FD_b}$ and $\mathbf{ID_b}$, all in the same dilution (1:5). The pre-treatment of cells with $\mathbf{BR_b}$ induced a much greater increment of fluorescence that in case of C1. This effect has been previously reported and could be mainly associated to bile acids which are able to induce ROS generation in unpolarised Caco-2 cells [25]. Since the increment of ROS respect to C1 was dependent on the concentration of $\mathbf{BR_b}$, the

% ROS inhibition of each dilution of $\mathbf{FD_b}$ or $\mathbf{ID_b}$ (Table 3) was calculated using the corresponding dilution of $\mathbf{BR_b}$ as maximum oxidation control. Both, $\mathbf{FD_b}$ and $\mathbf{ID_b}$, presented a % inhibition of ROS that was dependent on the TPC concentration. **ID** presented a greater ROS scavenging potency since the IC_{50} values obtained from the data presented in Table 3 were 0.042 and 0.017 mg GAE/mL for $\mathbf{FD_b}$ and $\mathbf{ID_b}$, respectively. These results show EtOH 60% soluble compounds from FD and ID wer able to inhibit intracellular ROS in H_2O_2-induced Caco2-TC7 cells. The compounds responsible of this activity could be PCs although the presence in these extracts of other kind of active compounds such as peptides is highly probable, as previously discussed.

As has been widely demonstrated, various PCs have clear in vitro antioxidant properties, since they can act, depending on their chemical structures, inhibiting the radical chain reaction, neutralizing free radicals or inhibiting their formation. While many of their biological actions have been attributed to such properties, accumulating evidence indicates that PCs exhibit several additional actions in complex biological systems. It has been reported that phenolic antioxidants can influence the expression of the antioxidant-responsive-element (ARE)-dependent genes through the activation of MAPK proteins, probably involved in the stabilization of the transcription factor nuclear factor erythroid 2 (Nrf2) through its phosphorylation, a pathway that ultimately leads to the stimulation of transcription of the antioxidant and detoxification defence systems. The different efficiency shown by the structures of the PCs clearly indicates a strong structure-activity relationship that may be related to the antioxidant capacity of each compound or to the different capacity to act as receptor ligands [26]. In this sense, the gastrointestinal digests of **F** and **I** were able to exert a direct free radical scavenging activity by HAT and SET mechanisms as well as an intracellular ROS scavenging activity. The scavenging of ROS in H_2O_2-induced Caco2-TC7 cells pre-treated with the bioaccesible fractions of **FD** or **ID** could be due to compounds that enter the cell and exert direct ROS neutralization mechanisms, and/or compounds that enter the cell or interact with the plasma membrane producing an effect on signalling pathways that lead to the induction of enzymes or antioxidant compounds. One of the majority PCs in **FD** was the (-)-epicatechin. The antioxidant efficacy of catechins is exerted through direct mechanisms scavenging ROS as previously discussed, but also, they can act by indirect mechanisms through the signalling cell pathway previously mentioned, inducing antioxidant enzymes, inhibiting pro-oxidant enzymes, and producing phase II detoxification enzymes and antioxidant enzymes. Catechins can interact with membranes via adsorption or penetration into the lipid bilayers [27]. These authors also demonstrated beneficial effects of (-)-epicatechin and its derivatives by direct modulation of cardiac mitochondrial functions. (-)- Gallocatechin gallate was in a lower concentration in **FD**, and was also found in **ID** but it was not the major one. The other majority PC in **FD** was polydatin (only found in **FD**). This compound as well as the *trans*-resveratrol (present in lower concentration in **FD** and **ID**) provided protection against oxidative damage in HepG2 cells by increased catalase activity, superoxide dismutase activity, and glutathione content, and decreasing generation of ROS, LDH level, and malondialdehyde content [22]. It is considered that polydatin exerts significantly protective and curative effects on oxidative stress-associated liver diseases via various molecular mechanisms, including the previously mentioned [28]. It is possible that this compound also exerts an antioxidant effect on the Caco2-TC7 cells used in the present study. Other potential biological activities predominantly through the modulation of signalling pathways involved in inflammation and apoptosis in addition to oxidative stress have been described for polydatin [29]. The third major compound in **FD** was the OH-tyrosol (not detected in **ID**). It has been reported that the antioxidant effect of this compound does not depend only on the capacity of scavenging oxidant chemical species, but it also depends on the ability to stimulate the activity and synthesis of antioxidant enzymes, DNA-repair proteins or phase II detoxifying enzymes, among other interesting biological activities [30]. Finally, in **ID** the syringic acid (hydroxybenzoic family) was widely majority. Syringic acid has been reported in many vegetables, fruits, and spices, including pumpkin, olives, grapes, acai palm, red wine, rice, rye, wheat, oats, maize, barley, sorghum, sugar cane, and even honey, but not in peas. Pure syringic acid or extracts containing this PC up regulated biochemical pathways involved in the production of endogenous antioxidant compounds such as Nrf-2, in cell culture and animal models, as well as other beneficial bioactivities [31]. Shahzad et al. [32] reported that the content of ROS, level of lipid, and protein oxidation diminished while antioxidant defence enhanced on peripheral blood mononuclear cells of myocardial infarction patients treated with syringic acid.

Conclusions

Different PCs composition and antioxidant activities of UAE extracts from **F** and **I** were reported. Qualitative and quantitative changes of PCs during the preparation of **I** can be the result of lost by solubilisation, chemical modification in the alkaline media, and/or absorption in the insoluble fraction. Also, notable changes in the PCs concentration, profiles and antioxidant activities after SGID of both yellow peas' ingredients were demonstrated for the first time. Both,

the PCs concentration and composition as well as the antioxidant activities in **FD** and **ID** were different. After SGID, the major bioaccesible PCs were OH-tyrosol, polydatin, *trans*-resveratrol, rutin, (-)-epicatechin and (-)-gallocatechin gallate for **FD**. For **ID**, syringic (the most concentrated) and ellagic acids, *trans*-resveratrol and (-)-gallocatechin gallate were the most bioaccessible. Several of these compounds have not been previously reported in peas, so this work add new knowledge related to phytochemical profile of this matrix and potential correlation with bioactivities. **FD** presented a higher ORAC but lower $ABTS^{\bullet+}$ and intracellular ROS scavenging activies than **ID**. Some relations among the detected PCs and the activity were analysed and the presence of other compounds that could differently contribute to antioxidant activity, for example peptides or amino acids, was demonstrated. The peptide fractions of the **FD** and **ID** are currently being studied in greater depth. This first approximation to the PCs bioaccesibility showed an interesting potential of both **F** and **I** as functional antioxidant ingredients. Further studies will be necessary to deepen the antioxidant mechanisms as well as to understand the effects of the faecal microbiota on these PCs.

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Data Availability Data is provided within the manuscript or supplementary information files.

Declarations

Competing Interests The authors declare no competing interests.

Ethical Approval Not applicable.

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