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INTERACTIONS OF BLACKCURRANT POLYPHENOLS IN MODEL SYSTEMS

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INTERACTIONS OF BLACKCURRANT POLYPHENOLS IN MODEL SYSTEMS

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RESUMEN

Consumir frutas y verduras está recomendado por sus efectos beneficiosos en la salud, debidos a su contenido en fibra, vitaminas y compuestos fitoquímicos. Sin embargo, el nivel de consumo de este tipo de alimentos en España no es adecuado. Una forma de incrementar dicho consumo es enriqueciendo otros productos alimentarios como lácteos o bollería. Esta vía sirve también para aprovechar los subproductos generados en la industria y que no son aprovechados para el consumo directo. Por ejemplo, en la industria de zumos de frutos rojos se genera un bagazo al prensar las frutas. Un caso destacado es en la Grosella negra (*Ribes nigrum*), cuyo bagazo es rico en fibra y compuestos fitoquímicos como los polifenoles. Dado que estos compuestos son beneficiosos para la salud, resulta muy interesante estudiar la incorporación de los mismos en la dieta. Sin embargo, estos compuestos pueden presentar interacciones con otros componentes alterando sus mecanismos de acción. Por ello, este trabajo tiene como objetivo el diseño de sistemas modelo en los que poder estudiar las interacciones de los polifenoles presentes en el bagazo de Grosella negra con los macronutrientes más comunes en los alimentos: hidratos de carbono, lípidos, proteínas y un sistema conjunto de los tres. Los sistemas modelo han sido diseñados acorde con el consumo de los macronutrientes en la sociedad española. Los resultados muestran una interacción preferente de los polifenoles presentes con las proteínas, seguidas por los carbohidratos y en menor medida por los lípidos. La estructura química de los polifenoles y los métodos analíticos empleados juegan un papel fundamental en la comprensión de los resultados obtenidos.

PALABRAS CLAVE: bagazo, TPC, TAC, FRAP, antocianinas, actividad antioxidante, extracto.

RESUM

Consumir fruites i verdures està recomanat pels seus efectes beneficiosos en la salut, deguts al seu contingut en fibra, vitamines i compostos fitoquímics. No obstant això, el nivell de consum d'aquest tipus d'aliments a Espanya no és adequat. Una forma d'incrementar aquest consum és enriquint altres productes alimentaris com lactis o productes fornejats. Aquesta via serveix també per a aprofitar els subproductes generats en la

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indústria i que no són aprofitats per al consum directe. Per exemple, en la indústria de sucs de fruites roges es genera un bagàs en premsar les fruites. Un cas destacat és en la Grosella negra, sent el seu bagàs ric en fibra i compostos fitoquímics com polifenols. Atés que aquests compostos són beneficiosos per a la salut, resulta molt interessant estudiar la incorporació dels mateixos a la dieta. No obstant això, aquests compostos poden presentar interaccions amb altres components alterant els seus mecanismes d'acció. Per això, aquest treball té com a objectiu el disseny de sistemes model en què poder estudiar les interaccions dels polifenols presents en el bagàs de Grosella negra amb els macronutrients més comuns: hidrats de carboni, lípids, proteïnes i un sistema conjunt dels tres. Els sistemes model han estat dissenyats d'acord amb el consum dels macronutrients en la societat espanyola. Els resultats mostren una interacció preferent dels polifenols presents amb les proteïnes, seguides pels carbohidrats i en menor medida per lípids. L'estructura química dels polifenols i els mètodes analítics utilitzats juguen un paper fonamental en la comprensió dels resultats obtinguts.

PARAULES CLAU:, bagàs, TPC, TAC, FRAP, antocianines, activitat antioxidant, extracte.

ABSTRACT

Fruits and vegetables consume is recommended for its beneficial effects on health, due to its content in fiber, vitamins and phytochemicals. However, the level of consumption of this type of food in Spain is not sufficient. One way to increase its consumption is to enrich other food products such as dairy or baked products. This also serves to take advantage of the by-products generated in the industry that are not used for direct consumption. For instance, a pomace is generated in berry fruits juice industry. A prominent case is Blackcurrant (*Ribes nigrum*), whose pomace is rich in fiber and phytochemical compounds such as polyphenols. Due to these compounds are beneficial for health, it is very interesting to study their incorporation into the diet. Nevertheless, these compounds can present interactions with other components modifying their mechanisms of action. Therefore, the aim of this work is to design model systems to study the interactions between polyphenols presents in Blackcurrant pomace and the most common macronutrients in foods: carbohydrates, lipids, proteins and a joint system of these three components. Model systems are designed in accordance with the consumption of macronutrients for Spanish society. Results show a preferential interaction of polyphenols with proteins, followed by carbohydrates and far lower by lipids. The chemical structure of the polyphenols and the analytical methods used play a fundamental role to understand the results obtained in the work.

KEY WORDS: pomace, TPC, TAC, FRAP, anthocyanins, antioxidant activity, extract.

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1. INTRODUCTION

Fruits are a heterogeneous group of foods in colours, textures, farming and consumption. However, they have in common that they are rich in vitamins, minerals and fiber, which confers them the condition of healthy foods. In this sense, studies have shown that the best way to reduce the risk of suffering from some types of diseases is to maintain a varied diet in fruits and vegetables (Agudo and González, 2007; Hercberg et al., 2006). The consumption of fruits and vegetables recommended by national and international institutions and organizations is 400 g per day or five servings of fruits and / or vegetables per day (WHO, 2005).

Health benefits of fruit consumption are mainly due to the bioactive compounds present in them (Wang et al., 2011, Wootton-Beard and Ryan, 2011). Bioactive compounds exert a beneficial function for the organism, such as those that have an antioxidant function. The nature of these compounds is very diverse, and can be classified according to different parameters, such as their endogenous or exogenous origin.

Another way to classify them is according to their nutritional value for the organism. Among the antioxidants with nutritional value are vitamins, such as vitamins C, B and E, and minerals, such as Selenium. Several studies relate the antioxidant capacity of these compounds with prevention of certain types of cancer (Klein et al., 2011; Lippman et al., 2009) and other diseases such as type 2 diabetes (Song et al., 2009). On the other hand, there are antioxidants without nutritional value, so called phytochemicals because the body does not need them in the short and medium term. Phytochemicals are a varied group of secondary metabolites of plant origin, which are easily found in fruits, seeds and vegetables in general. Its main function is to contribute to plant growth, pigmentation, pollination and resistance against pathogens, predators and environmental stresses (Serra et al., 2018). This protective activity has led to the study of the benefits of its consumption for health (Grosso et al., 2017; Jain and Ramawat, 2013; Serra et al., 2018). In fruits, the most abundant phytochemicals are polyphenols and carotenoids (Huang et al., 2005).

Polyphenols are characterized by the presence of one or more hydroxyl groups linked to a benzene ring. More than 8000 polyphenols have been identified (Ou et al., 2019). Polyphenols vary in their chemical structure and properties, ranging from simple molecules, such as phenolic acids, to highly polymerized molecules, for example proanthocyanidins (Landete, 2012). Based on their structure, they are divided into two main groups: flavonoids and nonflavonoids (Serra et al., 2018). Flavonoids are composed of 15 carbons with two aromatic rings connected by a three-carbon bridge. The flavonoids group is divided into numerous subgroups, including flavones, flavonols, anthocyanidins, chalcones, dihydroflavonols, flavanones and flavan-3-ols (Crozier et al., 2009; Cutrim and Cortez, 2018; Grosso et al., 2017). Most flavonoids appear in plants in their glycoside forms and a few of them in their aglycone form (Anouar et al., 2012). Nonflavonoids include

phenolic acids, which are derivatives of benzoic acid and hydroxycinnamic acid, lignans and stilbenes (Landete, 2012; Serra et al., 2018).

The multiple functions of polyphenols have increased their consumption and production. The industrial extraction of polyphenols reached 16,380 tons in 2015 and is anticipated to increase to 33,880 tons by the 2024 (Ou et al., 2019). Its industrial utilization is dominated by functional beverages (44%) and enriched foods (33%) (Adebooye et al., 2018). One of the main factors of this increase is the fact that most of the sources of polyphenols are by-products of other industries as the remains of squeezed fruit, also known as pomaces. Pomaces consist of the remains of skins and seeds, which are rich in fiber, essential oils and bioactive compounds such as polyphenols. The further use of pomace in food formulation adds value to a material that is usually treated as waste, and therefore contributes to an improved sustainability of the agri-food processing chain. Inside of possible candidates, berry pomaces are especially interesting because of its high content of polyphenols (Reißner et al., 2018).

Consequently, the great variety, its effects on health (Chen et al., 2015; Karas et al., 2017) and its relative ease in obtaining make polyphenols ideal bioactive compounds to produce functional foods. However, the efficacy in the use of these bioactive compounds is affected by several factors, such as food matrix, solubility, digestibility, bioaccessibility, molecular structures or metabolizing enzymes. The physiological activity of polyphenols does not depend directly on their prevalence in the human direct (Karas et al., 2017). Many studies researched the interactions between polyphenols and other biochemical compounds, such as proteins, carbohydrates or lipids (Karas et al., 2017; McDougall et all., 2005; Sengul et al., 2014) or foods as meat, juices, breads and others (Kamiloglu and Capanoglu, 2014; Ou et al., 2019; Sengul et al., 2014; Swieca et al., 2013; Xu et al., 2019) and their relationships with digestibility. The results from these works show great differences, which are mainly due to three factors: the type of polyphenol, the matrix in which they are found and the digestive process.

The objective of this work was to study the polyphenols and macronutrients interactions in food model systems. In order to study that, polyphenols, which were obtained from Blackcurrant by-product, were added to the model systems as dry pomace or as powder extract.

2. MATERIALS AND METHODS

2.1. Materials

The ingredients used in the preparation of the model systems were: Blackcurrant (BC) pomace (*Ribes nigrum*), supplied by the Institute of Natural Materials Technology (Technische Universität Dresden, Germany) and prepared drying fresh blackcurrant pomace at 70°C for 2 h and milling it in a ZM 100 ultracentrifugal mill (Retsch GmbH, Haan, Germany) at 14 000 rpm using a 1 mm sieve; olive oil (Consum, Valencia, Spain); native wheat starch

(C☆Gel, Cargill BV, Amsterdam, Netherlands) and whey protein isolate from milk (Harrison Sport Nutrition S.L, Granada, Spain).

2.2. Pomace polyphenol extraction and powder extract obtention

The extraction of the polyphenols from the pomace was carried out for 120 min at 60°C in 60% ethanol, in agitation and darkness with a 1:8 ratio of sample and solvent (MohdMaidin et al., 2019). After the extraction, a vacuum filtering was carried out and the ethanol was eliminated by means of a rotary evaporator. After that, the resulting aqueous extract was freeze-dried to obtain a polyphenol rich extract powder (PE). This powder extract was used to compare with the dry pomace (BP) in the model system experiments.

2.3. Model Systems preparation

The consumption data in Spain of the different macronutrient are 5.5 g of carbohydrates, 4 g of fat and 4 g of protein per 100 g of consumption (ANIBES, 2013). According to this information, five types of model system were prepared with the following ingredients: water as control, wheat starch as standard for carbohydrates, olive oil as standard for fats, whey protein as standard for proteins, and a fifth model combining wheat starch, olive oil and whey protein. Five g additional dry pomace (BP) or 0.275 g of polyphenols rich extract (PE) (corresponding to the equivalent amount of polyphenols in 5 g of pomace) were added to the different model systems.

A total of 10 model systems were studied. Table 1 summarizes the composition of all of them:

TABLE 1. Composition of the model systems.

	Dry pomace (BP)	Polyphenols rich extract (PE)
Water (w)	BP-w	PE-w
Starch (s)	BP-s	PE-s
Oil (o)	BP-o	PE-o
Whey protein (wp)	BP-wp	PE-wp
Complete model (cm)	BP-cm	PE-cm

The models were prepared as follows. The water model system was prepared by dissolving 5 g of BP or 0.275 g of PE in 100 mL of distilled water for 30 min. For the carbohydrate model, 5.5 g of starch were gelatinized in distilled water (up to 100 mL) for 40 min at 65°C and constant agitation and BP or PE were added. For the fat model, 4 g of olive oil were mixed with the indicated quantity of BP or PE, and after that, 96 g of distilled water were added and mixed 1 min in ultraturrax at 13000 rpm. For the protein model, 4 g of whey protein were dissolved in distilled water (up to 100 mL) and BP or PE were added. Finally, for the fifth model system or complete model, 5.5 g

of starch were gelatinized in 70 mL of distilled water as described before. When it was cooled, 4 g of oil and 4 g of whey protein were added in distilled water up to 100 mL. Lastly, the solution was mixed 1 min in ultraturrax at 13000 rpm and BP or PE were added.

2.4. Analytical determinations

To determine the content of phenolic compounds, the different model systems were centrifuged for 20 min at 5000G at 4°C (Chan et al., 2018), filtering the supernatant with a paper filter. The extract obtained will be used also to determine total anthocyanins content and the antioxidant activity.

2.4.1. TOTAL PHENOLIC CONTENT (TPC)

Total phenolic content (TPC) was determined by Folin-Ciocalteu assay according to the procedure described in González-Peña et al. (2013) and Fernández-Jalao et al. (2017). This method was based on the ability of phenols to react with oxidizing agents. One mL aliquot of the extracts was taken and mixed with 6 mL of bidistilled water in test tubes. For the blank, 7 mL of bidistilled water were placed. Then, 0.5 mL of the Folin-Ciocalteu reagent were added. Mix well and wait 3 min from the addition of the Folin-Ciocalteu reagent to the first test tube. When time was up, 1 mL of saturated Na_2CO_3 to 20% and 1.5 mL of bidistilled water were added, homogenizing with the help of a vortex after adding each reagent. After adding all the reagents, they were stored in the dark for 90 min at room temperature. When finished, the absorbance of the samples was measured at a wavelength of 765 nm. The absorbances obtained were related to a calibration curve, which was prepared using different concentrations of gallic acid. The results were expressed as grams of gallic acid per 100 g of sample, which are the model systems.

2.4.2. TOTAL ANTHOCYANINS CONTENT (TAC)

The assay was determined according to Lee et al. (2005) with slightly modifications. Two test tubes per sample were used. First, 2 mL of extract were added to each test tube. In a tube, 8 mL of buffer pH 1 and 8 mL of buffer pH 4.5 were added to the other test tube. With the help of a vortex they were homogenized. After that, the test tubes were kept for 20 min in the dark at room temperature. Once the time was over, it was measured the absorbance of each tube at wavelength 520 nm and 700 nm, using distilled water as reference. To calculate the content of anthocyanins, expressed in mg/L, the next equation (1) was used:

$$\text{Total anthocyanins (mg/L)} = \frac{A \times Mw \times FD \times 10^3}{\varepsilon \times 1} \quad (1)$$

Where:

- $A = (Abs_{520} - Abs_{700})_{pH1} - (Abs_{520} - Abs_{700})_{pH4,5}$

- Mw (molecular weight) = 449.2 g/mol for cyanidin-3-glucoside.
- FD = dilution factor of the extract in the buffer.
- 10^3 = conversion g to mg.
- Ɛ = 26900 → Molar extinction coefficient of cyanidin-3-glucoside.
- 1 = path length of the cuvette (cm).

2.4.3. ANTIOXIDANT ACTIVITY (FRAP)

The methodology described by Benzie and Stain (1996) and Pulido et al. (2000) with slightly modifications was used. Following the order described below, 30 µL distilled water, 30 µL extract and 900 µL FRAP reagent were added in 1.5 mL cuvettes. As blank, distilled water was used. The cuvettes were incubated in a bath at 37°C for 30 minutes and the absorbance at wavelength of 595 nm was measured. The absorbances obtained were related to a calibration curve, which was prepared using different concentrations of Trolox. The result was expressed in micromoles equivalent of Trolox per g of sample, which are the model systems.

2.5. Microstructure

A Nikon ECLIPSE 80i (Nikon Co., Ltd, Tokyo, Japan) light microscope was used. An aliquot of each model system was placed on a glass slide and observed at 20× magnification using bright field and fluorescence modes. The fluorescence of the samples was observed while using a mercury arc lamp with a TRICTC filter as excitation source. A camera (ExWaveHAD, model no. DXC-190, Sony Electronics Inc, Park Ridge, New Jersey, USA) was attached to the microscope and connected to the video entry port of a computer. The images were captured and stored at 1280×1024 pixels using the microscope software (NIS-Elements F, Version 4.0, Nikon, Tokyo, Japan). The software interfaced directly with the microscope, enabling image recording control.

2.6. Statistical Analysis

A categorical multifactorial experimental design with two factors (BC type and model system type) was used. Analysis of variance was performed on the data using the Statgraphics Centurion XVII, version 17.2.00, software package (Statistical graph Co., Rockville, MD). Least significant difference (LSD) Fisher's test was used to evaluate mean value differences ($P<0.05$) using XLSTAT 2014 statistical software (Addinsoft, New York, NY, USA).

3. RESULTS AND DISCUSSION

3.1. Analytical determinations

3.1.1. TOTAL PHENOLIC CONTENT (TPC)

A two-way ANOVA was performed on the results, showing significant interactions (P<0.05) between the factors type of Blackcurrant (BP or PE) and model system. The results can be seen in Figure 1.

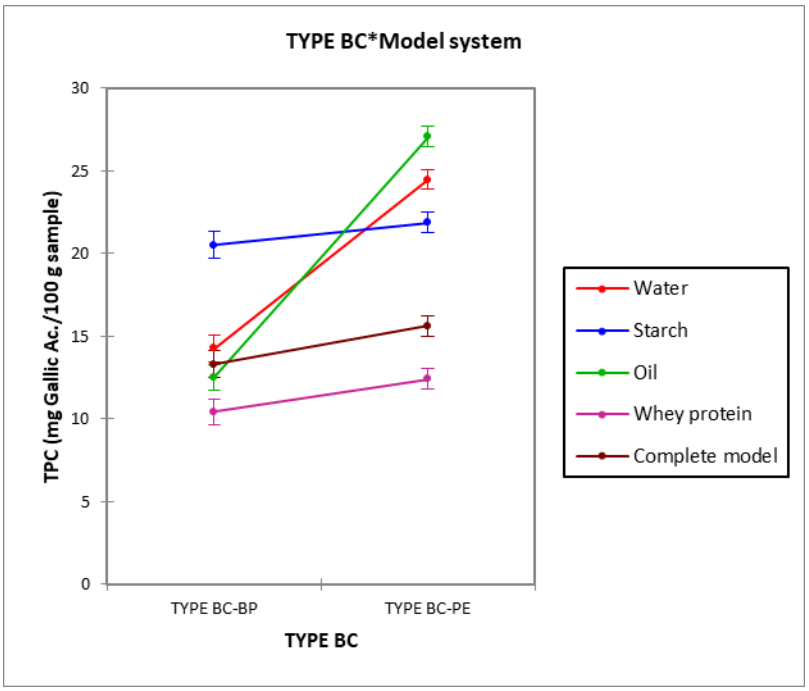

FIGURE 1. Interaction Plot between type of Blackcurrant and type of Model system for Total phenolic content (TPC). BP: Blackcurrant pomace; PE: polyphenol rich extract.

The use of the extract (PE) gave significant (P<0.05) higher values for TPC if compared to pomace (BP) for the different model systems. This difference was especially noticeable when the water or oil were used. When PE was added, the TPC content followed the next order: oil model > water model > starch model > whey protein model > complete model, and significant differences were observed (P<0.05) among all the samples. When BP was added to the systems, no differences (P>0.05) were detected between BP-o and BP-cm and between BP-w and BP-cm; starch model showed the highest values (P>0.05) and the lowest values (P>0.05) were obtained for protein model system. Possible causes of these results will be discussed later.

3.1.2. TOTAL ANTHOCYANINS CONTENT (TAC)

For TAC, the two-way ANOVA also showed significant interactions ($P<0.05$) between the factors type of Blackcurrant (BP or PE) and model system (Figure 2).

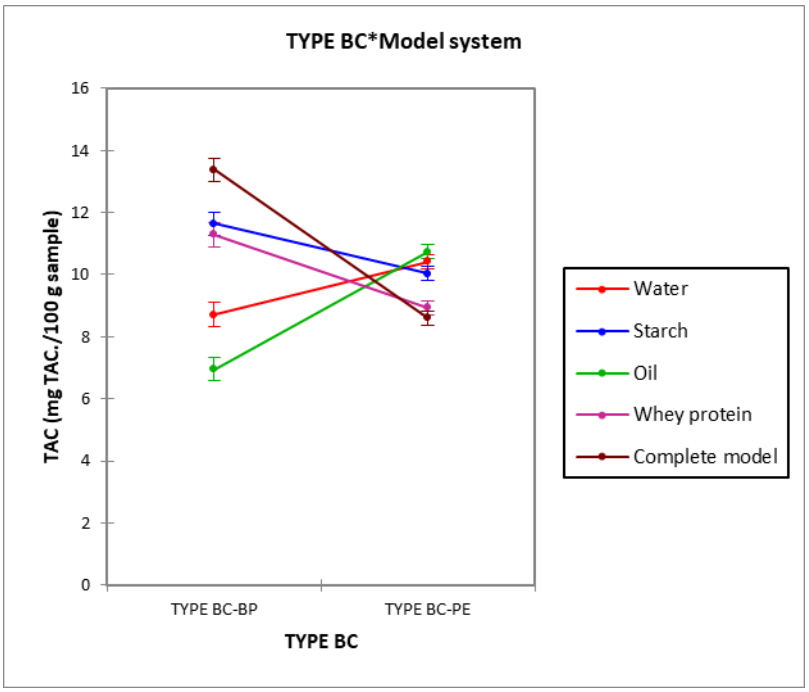

FIGURE 2. Interaction Plot between type of Blackcurrant and type of Model system for Total Anthocyanin content (TAC). BP: Blackcurrant pomace; PE: polyphenol rich extract.

TAC (Figure 2) does not follow the same trend as observed in Figure 1 for TPC. When PE was added to the different model systems, TAC content decreased significantly ($P<0.05$) in the starch, protein and complete model systems, meanwhile it increased in the water and oil model systems ($P<0.05$) if compared to the model systems with added pomace (BP). No significant differences were observed ($P>0.05$) between PE-wp and PE-cm, and between PE-w and PE-o. When BP was added to the different model systems, TAC content followed the next trend: complete model > starch model > whey protein model > water model > oil model. Significant differences were observed ($P<0.05$) among all the samples. As in the case of TPC, possible causes of these results will be discussed later.

3.1.3. ANTIOXIDANT ACTIVITY (FRAP)

The two-way ANOVA also showed significant interactions ($P<0.05$) between the factors for antioxidant activity measured by FRAP method. The results can be seen in Figure 3.

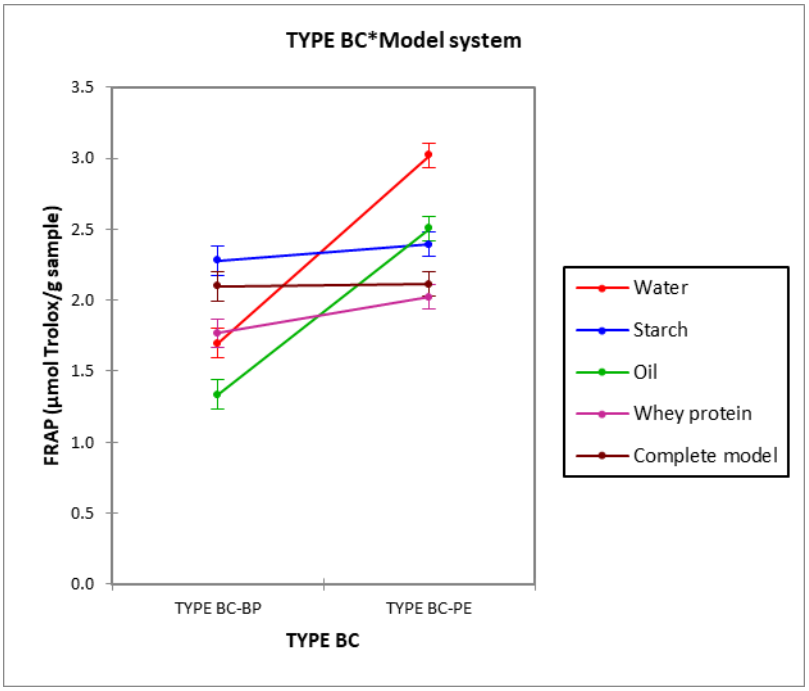

FIGURE 3. Interaction Plot between type of Blackcurrant and type of Model system for Antioxidant Activity (FRAP). BP: Blackcurrant pomace; PE: polyphenol rich extract.

As it can be observed, Figure 3 followed the trend described in Figure 1. When comparing BP and PE models, those prepared with PE exhibited higher values ($P<0.05$) than those prepared with BP, except for the starch and complete model systems, where not significative differences ($P>0.05$) were detected. When PE was used, no significant differences were observed between PE-s and PE-o, and between PE-wp and PE-cm ($P>0.05$), being the highest values for water model system (PE-w). When BP was used, starch model system showed the highest values ($P<0.05$) and the oil model system the lowest ($P<0.05$). No significant differences were observed between BP-w and BP-wp ($P>0.05$).

TPC (Figure 1), TAC (Figure 2) and FRAP (Figure 3) results allow separating the systems in two groups. On the one side, the model systems with Blackcurrant with water and with oil (w and o). On the other side, the model systems with starch, whey protein and complete (s, wp and cm). The difference between these two groups will be discussed in depth later.

3.1.4. DISCUSSION ON ANALYTICAL DETERMINATIONS

Polyphenols may interact with macromolecules in reversible and irreversible ways (Jakobek, 2015; Le Bourvellec and Renard, 2012). To understand the possible mechanisms of interaction between polyphenols and macronutrients, it is necessary to know the nature of the polyphenols presents in the source, in this case Blackcurrant.

Most polyphenols, specifically anthocyanins, are compartmentalized primarily in vacuoles, which are encapsulated by the cell-wall plant (González-Aguilar et al., 2017). This maintains polyphenols separated of other components which they can interact with, such as cell-wall compounds and enzymes (Renard et al., 2017). However, when cells are disrupted in food processing, for example by grinding and pressing, these compounds come in contact. Three mechanisms can be jointly responsible for formation of polyphenol and cell-walls complexes: non-covalent adsorption of polyphenols to the cell-wall matrix, covalent bonding with products of the enzymatic activity and covalent bonding with reactive products made under acidic conditions (Renard et al., 2017).

In our work, the raw material used was processed thermally and mechanically to become a dry powder (Reißner et al. 2018). So, the effects of enzymatic activity are minimized. In addition, there is no strong acidic conditions during the processes of drying and milling or during the elaboration of the model systems to induce the formation of reactive carbocations that could interact with cell-wall compounds (Renard et al., 2017). In contrast, non-covalent forces have been reported between polyphenols and cell-wall compounds and macromolecules, both polysaccharides and proteins (Bordenave et al., 2014; González-Aguilar et al., 2017; Jakobek, 2015; Le Bourvellec and Renard, 2012; Renard et al., 2017), which will be described later. Hydrogen bonding, hydrophobic bonding and van der Waals forces are usually implicated when the complexation is reversible (Le Bourvellec and Renard, 2012).

The scope of these forces will depend on the reactivity of polyphenols. Polyphenol reactivity can be explained by structural factors, including the molecular weight, the conformational mobility and flexibility, projection of the hydroxyl groups, presence of galloyl groups or the affinity of the polyphenol for water (Le Bourvellec and Renard, 2012). To better explain these hypotheses, the chemical structures of the polyphenols found in greater quantities in Blackcurrant according to Reißner et al. (2018) are shown below in Figure 4.

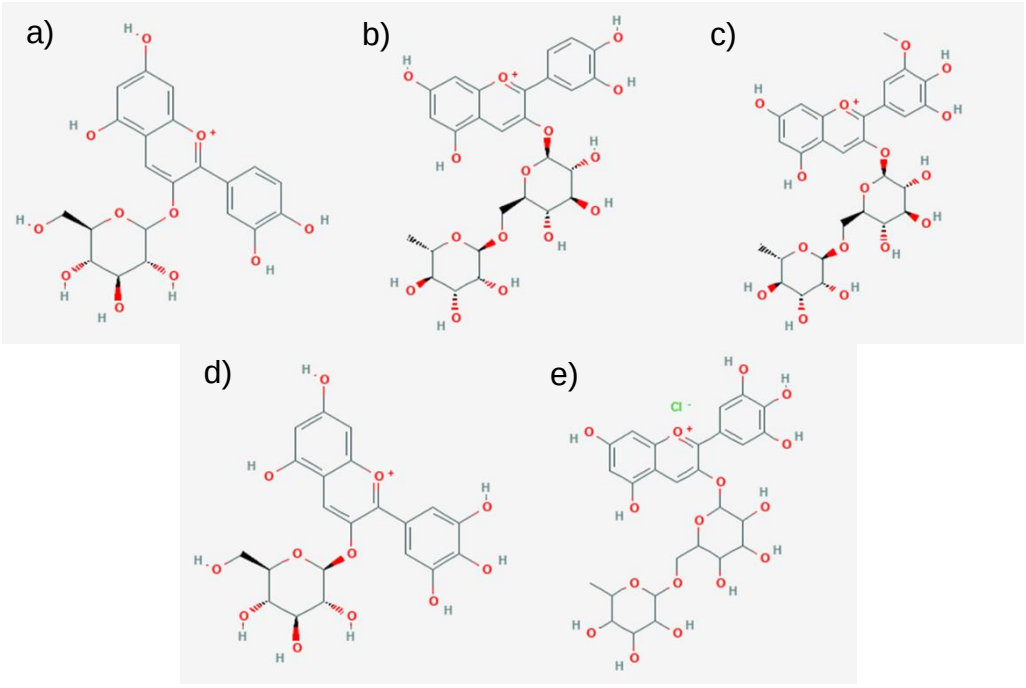

FIGURE 4. Molecular structures of main polyphenols in Blackcurrant (Reißner et al., 2018). a) Cyanidin 3-O-glucoside; b) Cyanidin 3-O-rutinoside; c) Petunidin 3-O-rutinoside; d) Delphinidin 3-O-glucoside and e) Delphinidin-3-O-rutinoside (Pubchem, 2019).

Besides the distinctive aromatic rings, the abundant presence of hydroxyl groups can be seen in Figure 4. These chemical groups are highly polar and can favour interactions with other molecules, especially by hydrogen bonds. This high polarity affects the solubility in water and the interaction with other macromolecules (Le Bourvellec and Renard, 2012; Vasserot et al., 1997).

Although the presence of hydrogen bonds may influence the experimental results obtained, this influence seems to be higher in the results for the model systems with water (w) and with oil (o) (figures 1, 2 and 3). This could be due to the high hydrophilicity of polyphenols. When the extract was added to the model system PE-o, no interaction with the oil was observed. However, in the case of BP-o (figures 1, 2 and 3), oil interacts with fiber compounds by hydrophobic forces (Le Bourvellec and Renard, 2012). At the same time, polyphenols interact with fiber compounds by hydrogen bonds, being both covered by oil and making more difficult the extraction of polyphenols to determinate. This effect gave lower values for BP-o than PE-o systems in TPC, TAC and FRAP analysis when oil model system was studied.

Another aspect to support the hypothesis of the hydrogen bonds is the difference between pomace (BP-w) and the polyphenol rich extract (PE-w). As explained, the adsorption of polyphenols to the cell wall affects the obtained results. The adsorption is affected by the ionic strength, the temperature and the dissolvent used in the extraction process. Adsorption to the cell wall may increase with the increasing ionic strength and decrease with increasing temperature (Le Bourvellec and Renard, 2012). The

adsorption of procyanidins, a precursor of anthocyanins (the main polyphenol compounds of BC) show a decrease when it is in combination with urea, dioxane, and ethanol (Le Bourvellec et al., 2004; Renard et al., 2001). Thus, due to the extraction process, which implied the use of temperature (60ºC) and ethanol, the results for PE-w were higher than for BP-w. This indicated that the adsorption mechanism between polyphenols and cell-wall compounds involves the establishment of weak energy bonds, like hydrogen bonds (Le Bourvellec and Renard, 2012).

Nevertheless, these types of interactions are not only produced with cell-wall compounds. Numerous studies described the interactions between polyphenols and polysaccharides (Amoako and Awika, 2016; Arts et al., 2002; Bordenave et al., 2014; Gallo et al., 2013; Jakobek, 2015; Viljanen et al., 2005). Regarding the model systems, starch showed interactions with polyphenols (Amoako and Awika, 2016; Camelo-Méndez et al., 2016; Zhu, 2015). In figures 1, 2 and 3 it can be seen the effect of starch when it is combined with pomace (BP-s) or polyphenol rich extract (PE-s). All the determinations showed higher values ($P<0.05$) for BP-s in comparison with BP-w, and PE-s showed lower values ($P<0.05$) in comparison with PE-w. These differences may be due to weak interactions between starch and pomace and the high solubility of the polyphenols in water when the extract is used.

Although polyphenols structure is important to explain their interactions with other macromolecules, the structure of these macromolecules also affects their interactions, being very interesting in case of starch (Le Bourvellec and Renard, 2012; Zhu, 2015). Starch consists of two major types of α-glucans on the molecular level: the linear amylose and the branched amylopectin. On the granular level, starch is in form of semicrystalline granules. When it is heated in the presence of water, the gelatinization of the granules is produced, disrupting the granular structure, leading to granule swelling and the hydration and solubilization of starch molecules. When the gelatinized starch is cooled, the disordered chains undergo re-association through hydrophobic interactions and hydrogen bonding in a process called retrogradation (Zhu, 2010). The state of starch granules conditions their interactions with polyphenols (Zhu, 2015).

The molecular interactions between diverse starches and a range of polyphenolic compounds have been evidenced by the shift of the UV-spectrum of the native phenolics upon the addition of starch in solution (Takahama and Hirota, 2010; Takahama et al., 2013). Starch addition increased the absorbance of vignacyanidin (a cyanidin-catechin pigment) and shifted the absorption maxima to longer wavelengths, which was explained by its binding to starch (Takahama et al., 2013).

Zhu (2015) describes two types of interactions between amylose and polyphenols, a V-type amylose inclusion complex, which is driven by hydrophobic interactions and an interaction through hydrogen bounds. However, V-type amylose inclusion complex may not be possible because of chemical structure of main polyphenols in BC (Figure 4). As some authors

explain (Chai et al., 2013; Zhang et al., 2011), a bulky size of polyphenols, the lack of hydrophobicity and the limitation of the size of the cavity in the amylose helix may not allow its formation. For example, rutin (quercetin-3-O-rutinoside) was not able to form the V-type inclusion complex with high-amylose maize starch (Zhu, 2015). Same studies describe a possible hydrogen bounding based in an increased hydrodynamic radius of amylose (Chai et al., 2013) or changes in gelatinization and retrogradation properties (Wu et al., 2011). Therefore, the interactions between the polyphenols and the starch are probably due to hydrogen bonding.

So, it seems that amylose has the main interactions with polyphenols, especially with the ones with elevated molecular weight (Amoako and Awika, 2016). This interaction can be seen in the results (figures 1, 2 and 3), giving more credibility to the hypothesis related to the increase effect of starch in polyphenols determination.

In contrast proteins are, in the light to the results, the macronutrients which interact the most with polyphenols, for both pomace (BP-wp) and polyphenols rich extract (PE-wp) (figures 1, 2 and 3). Several studies reported the interactions between proteins and different polyphenols (Arts et al., 2002; Bordenave et al., 2014; Gallo et al., 2013; Jakobek, 2015; Le Bourvellec and Renard, 2012; Ribnicky et al., 2014; Viljanen et al., 2005; Yildirim and Kemal, 2017). These interactions are more studied because their consequences are more visible than in other nutrients. They affect to sensorial taste, form precipitates and affects bioavailability of essential amino acids and polyphenols. For instance, high attention has been paid to the perception of astringency of various food products such as fruits, coffee or tea, which is due to the interactions between proanthocyanidins and proline-rich salivary proteins (Bordenave et al., 2014; Jakobek, 2015). Another characteristic example is the interaction between polyphenols and milk proteins, such as caseins and whey protein (Arts et al., 2002; Gallo et al., 2013; Viljanen et al., 2005; Yildirim and Kemal, 2017).

The nature of these interactions can be both non-covalent and covalent forces (Gallo et al., 2013; Jakobek, 2015; Le Bourvellec and Renard, 2012; Yildirim and Kemal, 2017). Studies of non-covalent interactions converge towards a mechanism that involves weak associations, specifically a combination of hydrogen bonds and hydrophobic interactions. Hydrophobic interactions would involve aromatics rings of polyphenols and hydrophobic sites of proteins, meanwhile hydrogen bonding occurs between hydrogen-acceptor sites of the proteins and the hydroxyl groups of the polyphenols. These hydrophobic and hydrophilic interactions will be determined by the molecular weight, the conformational mobility and flexibility and the relation between donor/acceptor hydrogen bounds in proteins and polyphenols (Jakobek, 2015; Le Bourvellec and Renard, 2012). The proposed mechanisms of non-covalent interactions between polyphenols and proteins are showed in Figure 5 (Le Bourvellec and Renard, 2012). In addition, protein and polyphenols can bind with covalent bonds, which proposed mechanisms are enzyme, thermal or oxidative formation of o-quinones which eventually react with nucleophilic groups of proteins such as -NH_2 and -SH.

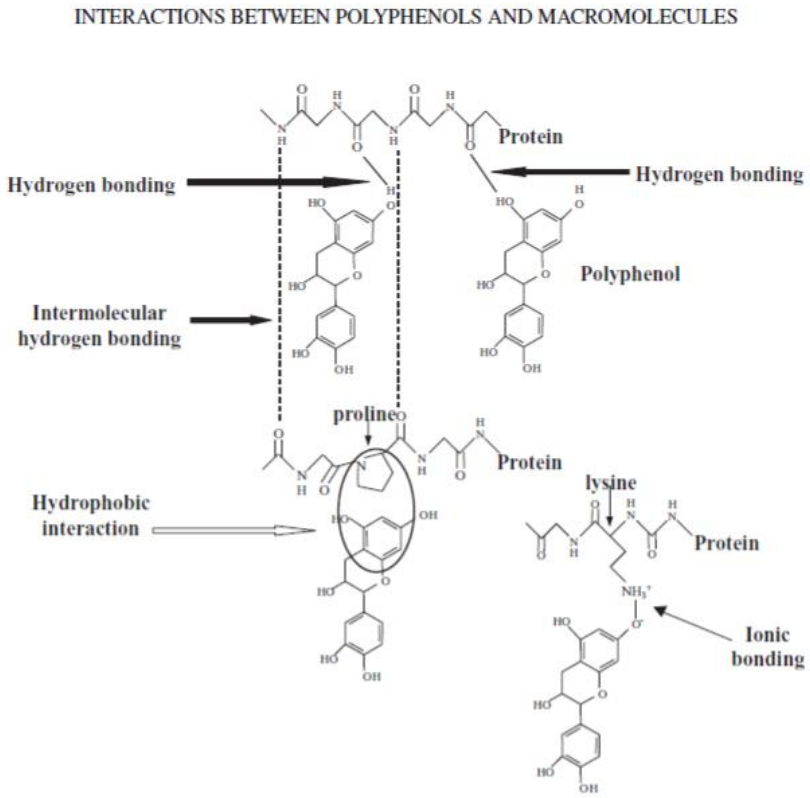

FIGURE 5. Mechanisms of non-covalent interactions between proteins and polyphenols (Le Bourvellec and Renard, 2012).

Milk proteins structure is essential to understand its interactions with polyphenols. Milk proteins are divided into two classes, namely caseins and whey proteins. In the case of whey protein, which has been used as model protein in experiments, it mainly consists of β-lactoglobulin, α-lactalbumin, immunoglobulins and bovine serum albumin, which are globular proteins. Most binding forces between polyphenols and milk proteins are non-covalent in nature and mostly stabilized by hydrogen bounds (Yildirim and Kemal, 2017).

This type of binding forces may be responsible for the results obtained experimentally (figures 1, 2 and 3). The polyphenols in BP and PE have great molecular weight and high polarity given the numerous hydroxyl groups (Figure 4). These characteristics promote the interaction between polyphenols and whey proteins. For instance, the interaction between polyphenols and whey proteins (PE-wp) gave place to significant ($P<0.05$) lower differences for TPC, TAC and FRAP in comparison with the water model system (PE-w), which may be due to the high availability of polyphenols to interact with protein structure, trough the hydroxyl groups. However, when BP was used, each determination had a different tendency. In Total Polyphenolic Content (TPC) (Figure 1), BP-wp shows the lowest value of all the model systems analysed. In contrast, Total Anthocyanins Content (TAC) (Figure 2) shows higher values ($P<0.05$) for BP-wp in comparison with BP-w or BP-o. Finally, in the antioxidant activity assay

(FRAP) (Figure 3) no significant differences between BP-wp and BP-w were found ($P>0.05$). When BP-wp and PE-wp are compared, it is important to point out that for TAC, BP-wp shows significant higher values ($P<0.05$) than PE-wp, meanwhile in TPC and FRAP a different trend is observed being PE-wp values higher than BP-wp ones.

The differences between these results may be due to the fundaments of the analytical methods used. Both TPC and FRAP assays are based on methods that use oxidation and reduction reactions (Benzie and Stain, 1996; Fernández-Jalao et al., 2017; González-Peña et al., 2013; Pulido et al., 2000), while TAC uses a method based on acid-base reactions (Lee et al., 2005). TPC and FRAP assays require the formation of complexes to be measured by means of spectrophotometry. In contrast, TAC requires structural changes but not the formation of complexes, since anthocyanins change their colour naturally with pH. Therefore, the interaction of proteins with polyphenols could be preventing the formation of the complexes needed to be measured in TPC and FRAP assays but it would not prevent the measurement in the case of TAC since changes are due to pH.

Once causes of interactions between BC and the macronutrients have been described individually, the possible causes in the complete model (cm) will be described. As it can be seen in figures 1, 2 and 3, the complete model followed similar trends to those observed in the protein model (wp). Thus, the hypothesis of the whey protein being the macronutrient that interacts most with polyphenols is strengthened. However, significant differences ($P<0.05$) between both models (protein and complete model) are shown (figures 1, 2 and 3). In case of TAC (Figure 2) and FRAP (Figure 3), no significant differences were observed ($P>0.05$) between PE-cm and PE-wp, while BP-cm showed significant higher values than the rest of models ($P<0.05$) for TAC (Figure 2). To explain these results, two hypotheses are suggested. In the one hand, the presence of starch in the complete model could explain an increase in BP-cm in TPC, TAC and FRAP in comparison to BP-wp, as it was described above. In the other hand, complete model presents an emulsion structure due to its preparation process (2.3 section), where protein is acting as stabilizer between phases. As a result, the emulsion process would contribute to have less protein available to interact with polyphenols, which would explain the slight increasing tendency of BP-cm and PE-cm compared to the protein model (BP-wp and PE-wp).

3.2. Microstructure

In order to complement the previous results, model systems were observed using light and fluorescence microscopy (Figure 6).

BP-w model system, as it can be seen in Figure 6a, is composed of pomace particles of different sizes and shapes. These particles are mostly reddish and brownish, and sometimes bluish. Besides, traces of cellular tissue can be seen. Blackcurrant is rich in polyphenols, mainly anthocyanins (Reißner et al., 2018). It is known that some anthocyanidins, the aglycone form of anthocyanins, are auto fluorescencent (Drabent et al., 1999; Nifli et al., 2007;

Rakic et al., 2015). The reddish coloration seen in the figure obtained by fluorescence (Figure 6b) is due to its high content of anthocyanidins.

When pomace is part of the model system with starch (BP-s) (Figure 6c) gelatinized starch granules constituting the continuous phase are observed. Some of these granules have a reddish coloration, which seems to indicate there are interactions between starch and pomace components, as it was described previously. In the model system with oil (BP-o) (Figure 6e), interactions between pomace compounds and oil can also be observed, as some oil globules appear coloured and they seem to retain pomace particles within. In other hand, protein model system (BP-wp) (Figure 6g) shows a continuous fluid phase in which pomace and small protein particles are distributed. No remarkable interaction between pomace and protein is observed in the picture, in contrast as it was suggested by analytical assays. When pomace is a part of the complete model system (BP-cm) (Figure 6i) it can be seen a continuous phase of gelatinized starch granules and fat globules, which are distributed homogeneously. However, both the granules and the globules appear less coloured than when the pomace is part of the models BP-s and BP-o, which could suggest there are lower interactions between polyphenols and starch and oil in the complete model than in BP-s and BP-o.

The pictures obtained by fluorescence indicate that the intensity of the intrinsic fluorescence of the pomace phenolic compounds is maintained when it is part of the BP-s and BP-o model systems, but it seems to decrease its intensity when it is part of the BP-cm and especially the BP-wp (Figure 6b, 6d, 6f, 6h and 6j). This can be related to the low analytical results obtained in these models, which causes have been described previously.

Regarding the polyphenol rich extract, as it can be seen in Figure 6, it is fully dissolved in the system, apart from small dispersed particles. These particles show a slight fluorescence (Figure 6l) but it is smaller than that observed in the pomace. Same trend is repeated in the different model systems (Figure 6n, 6p, 6r, 6t). In PE-s and PE-o systems starch and oil seem to be interacting with the PE in a lesser degree than when BP is used as less coloured granules and globules are observed. In the case of oil, it can be due to the high polarity of the polyphenols present in BC (Figure 4), as it was above mentioned. In protein model system, only small protein particles are observed, such as in case of pomace. Finally, the complete model (Figure 6s) shows the three components distributed by the system. The fluorescence in these model systems is minimal, which may be due to the high dispersion of polyphenols in the system.

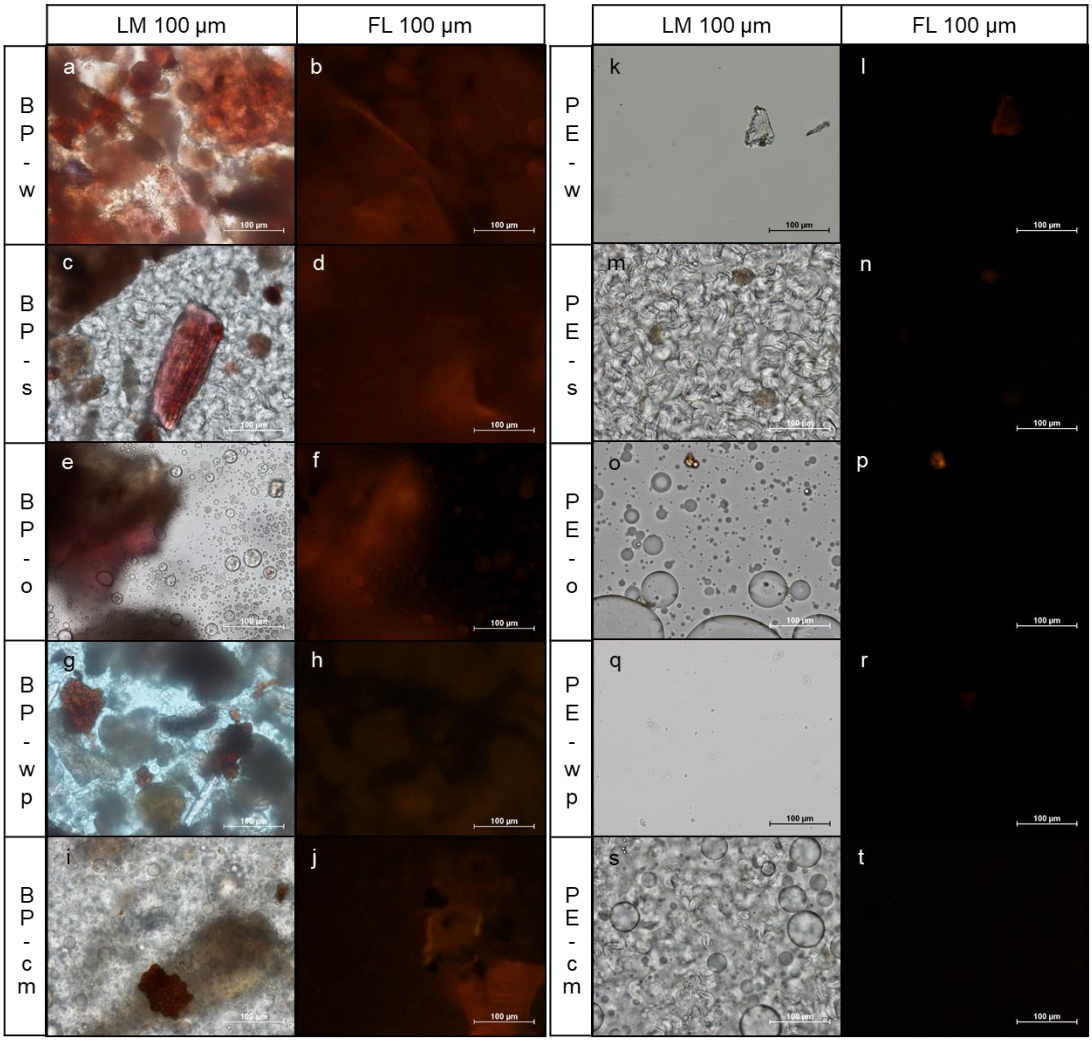

FIGURE 6. Light microscopy and fluorescence pictures of model systems at 100 µm. BP-w (a, b); BP-s (c, d); BP-o (e, f); BP-wp (g, h); BP-cm (i, j); PE-w (k, l); PE-s (m, n); PE-o (o, p); PE-wp (q, r); PE-cm (s, t). BP: Blackcurrant pomace; PE: polyphenol rich extract.

4. CONCLUSIONS

Pomace fiber plays an important role in the interactions between the polyphenols and the macronutrients in model systems. Moreover, the chemical structure, size and polarity of the polyphenols are key factors in their predisposition to interact with macronutrients. The high polarity of the polyphenols present in BC makes them interact very little with the oil and much more with starch and proteins, being proteins the macronutrients that most interact with polyphenols, decreasing their extraction. Besides, the fundaments of analytical determinations can influence the interpretation of the results. Fluorescence is highly observed in pomace, being almost imperceptible in polyphenols rich extract, which may be due to is complete dispersion in the different model system.

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