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Insights into the development of grapefruit nutraceutical powder by spray drying. Physical characterization, chemical composition and 3D intestinal permeability

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Abstract

The development of functional and nutraceutical foods comes from a greater awareness of the relationship between food and health by consumers. On the other hand, the idea of purifying and encapsulating bioactive compounds through techniques such as spray drying is well received in the food industry in order to improve bioactivity. The characterization and development of a grapefruit nutraceutical powder by spray drying adding biopolymers as encapsulating factors is of great interest on the basis that citrus are a source of different bioactive compounds. Physical properties such as water content, porosity, color, as well as the composition in total phenolic content (TPC), total flavonoid content (TFC) and antioxidant activity measured both by radical scavenging activity (DPPH) and ferric reducing antioxidant power (FRAP) were evaluated in the grapefruit powder. Besides, the bioavailability of the bioactive compounds was analyzed through a 3D intestinal model that used the combination of Caco-2 and HT29-MTX cell lines. The bioactive compounds theoretically assimilated by the digestive system were identified by LC-ESI-MS. Delphenidin-3-glucoside and hesperitin-7-*O*-glucoside presented a permeation higher than 50%, followed by hesperidin that was close to 30%. This work allows to establish that the formulation of grapefruit powder has a great potential as nutraceutical food.

Keywords: Grapefruit powder; bioactive compounds; LC-ESI-MS; 3D intestinal model, permeability.

1. Introduction

Citrus fruits are well known for their richness in ascorbic acid, also presenting considerable amounts sugar, calcium, phenols, phosphorus and vitamin B6, being currently employed in food and beverage industry as well as in cosmetic and pharmaceutical products (Lv *et al*. 2015). Within the family of citrus products, *Citrus paradise*, commonly known as grapefruit, is largely underestimate by consumers due to its bitter flavor (Obenland *et al*. 2018). However, different industries use grapefruit on a large variety of formulations due to the valuable compounds present in their skin, seed, pulp and juice (Uckoo *et al*. 2012). Recently, different studies focus on understanding the interactions of grapefruit bioactive compounds with the positive reduction of chronic diseases and the benefits that could be obtained for health (Hung *et al*. 2017; Lee *et al*. 2016; Hayanga *et al*. 2015; Gorinstein *et al.* 2005). *C. paradisi* has a characteristic taste, color and long shelf life, being of huge popularity in some European countries as well as in Asia and United States. The varieties of this citrus could be grouped in white and pigmented. Within the pigmented variety, the Star Ruby is remarkable, presenting an intense coloration, with scarce seeds and high yield in juice and bioactive compounds (Berk and Berk 2016). A recent review stated that the bioactive compounds of grapefruit juice are distributed in nine general groups of which flavonoids are the most relevant, being the major one naringin and eriocitrin, followed by carotenes and different types of acids, such as ascorbic or malic (Cristóbal-Luna *et al.* 2017). The flavonoids mainly appear in the glycosidated form, that takes place in position 7, in compounds such as rutinose or neohesperidose (Peterson *et al*. 2006). Other glucoside groups identified in grapefruit are nehoespiridine, didymin and poncirin (Kelebek 2010).

The formulation of a nutraceutical product in powdered form is of huge interest for consumers *(Moss et al*. 2018; Aditya *et al*. 2017; Accardi *et al*. 2016). For this reason, the selection and development of an appropriate matrix and technological process, able to maintain the active compound structure from production until consumer consumption and, simultaneously, guarantying the bioactive compounds delivery to the physiological target within the organism, is the most important step for the success of a specific nutraceutical food. Nevertheless, despite the richness in bioactive compounds that Star Ruby variety can offer, it is of huge importance to

identify the main bioactives compounds present as well as to understand their interaction with the digestive system. *In vitro* models are essential tools to evaluate the possible permeation of bioactive compounds in intestine and their possible health effects (Huang *et al*. 2018; Verhoeckx *et al*. 2015). The most commonly lineage used for the cell culture models is Caco-2, representing the intestinal line. However, this lineage has some limitations such as the lack of mucins production that have a great influence on absorption. Therefore, this cell line is normally complemented with HT29-MTX cell line, responsible for this property in order to improve the paracellular permeability of hydrophilic compounds in intestinal 3D models (Chen *et al*. 2010; Pereira *et al*. 2015).

The main aim of this paper was to characterize a grapefruit nutraceutical powder at the level of physical properties as well as its phenolic and antioxidant capacity. Indeed, the bioavailability of the bioactive compounds of grapefruit nutraceutical was assessed through a 3D intestinal model, identifying by LC-ESI-MS the bioactive compounds that could be assimilated by the digestive system.

2. Materials and methods

2.1 Preparation of feed mixture and spray drying conditions

The grapefruit powder was obtained by spray drying of grapefruit liquidized through addition of high molecular weight biopolymers as encapsulating factor. The powder formulation was composed of 9.4% of gum arabic (GA), 1.44% of whey protein isolate (WPI), 1.25% of maltodextrin (MD) and 87.95% of liquefied grapefruit (DeLonghi Ròbodiet Compact, Barcelona, Spain), based on the design of optimized response surface experiments (data not show). The fruits (*Citrus paradisi* variety Star Ruby) were obtained in a local supermarket in Valencia, Spain. The biopolymers GA and MD were supplied by Alfa Aesar® (Karlsruhe, Germany) and WPI LACPRODAN® DI-9212 was from Arla Foods Ingredients (Viby, Denmark). Once the mixture with all ingredients was prepared, the sample was spray dried in a Büchi-mini equipment (B-290, Flawil, Switzerland) under conditions of aspiration speed of 35

m^3/h, feed flow of 9 mL/min and an atomizer flow of 473 L/h at a maximum temperature of 148 °C and a pressure of $5 \cdot 10^5$ Pa. The powder was vacuum packed (Edesa vac-20 SL, Guipúzcoa, Spain) for further characterization steps.

2.2 Physical properties of the powder

The water content was determined, in triplicate, by a gravimetric method (AOAC 1992) in a vacuum oven (VACIOTEM, JP Selecta, Barcelona, Spain) at 60 °C until constant weight and expressed as g water/100 g powder.

The bulk density (ρ_a) was determined, in triplicate, based on the measure of the volume occupied by a known amount of sample (≈ 1 g) after being subjected to a stage of vibration at 1600 rpm for 10 s (Infrared Vortex Mixer, F202A0175, Spain) and applying Eq. (1).

$$\rho_a = m/v_f \quad (1)$$

where ρ_a (g/mL) is the bulk density, m is the mass (g) of powder and v_f is the volume after vibration (mL).

The Eq. (2) was used to obtain the porosity (ε). The true density (ρ) was calculated from the composition in water and carbohydrate of the samples, applying Eq. (3).

$$\varepsilon = \frac{\rho - \rho_a}{\rho} \quad (2)$$

$$\rho = \frac{1}{\frac{x_w^p}{\rho_w} + \frac{(1-x_w^p)}{\rho_{CH}}} \quad (3)$$

where ε is the porosity, ρ is the true density and ρ_a is bulk density (Eq. 1), ρ_w is the water density at 20 ºC (0.9976 g/mL) and ρ_{CH} is the carbohydrate density at 20 ºC (1.4246 g/mL) (Choi and Okos 1986) and x_w^p water content of the powder expressed as g water/ g powder.

The color of the samples was measured in triplicate by using a spectrocolorimeter (MINOLTA, CM3600-D, Spain, reference illuminant D65 and 10 ° observer). The CIE L*a*b* coordinates were obtained from which the hue angle (h^*_{ab}, Eq. 4) and the chroma (C^*_{ab}, Eq. 5) were calculated.

$$h^*_{ab}=\text{arctg}\left(b^*/_{a^*}\right) \quad (4)$$

$$C^*_{ab}=\sqrt{a^{*2}+b^{*2}} \quad (5)$$

2.3 Preparation of the Freeze-dried extracts

In order to find the maximal information from the bioactive composition of the grapefruit powdered product, two extraction solvents were used: (1) Oxalic Acid (Scharlab S.L, Barcelona, Spain) with a concentration of 0.1% (w/v) in distilled water and (2) Methanol-Water (Scharlab S.L, Barcelona, Spain) in a proportion of 70:30 (v/v). 1 g of the powder was mixed with 9 mL of each extraction solvent. The extraction was carried out with magnetic stirring of the mixture for 20 min, in darkness at room temperature and after being centrifuged (Eppendorf^{Tm} 5810R, Wesseling-Berzdorf, Germany) at 10000 rpm for 10 min at 4 °C. The supernatant was evaporated in a rotavapor (Büchi R-200, Postfach, Switzerland) and retained in a plastic container to be subsequently freeze-dried (Telstar® CRYODOS-80, Terrassa, Spain). During freeze-drying (72 hours), the temperature was kept at -55 ºC in the condenser. Two extractions which each solvent was carried out. The extraction yields were quantified by the weight of the freeze-dried Oxalic Acid extract (FDOA) or freeze-dried Methanol-Water extract (FDMW).

2.4 Determination of the Total Phenolic Content

The Total Phenolic Content (TPC) was spectrophotometrically determined according to the Folin-Ciocalteu procedure with minor modifications (Alves *et al*. 2010). Briefly, 30 µL of reconstituted sample in its respective extractor solvent (till the initial volume before dehydration) was mixed with 150 µL of Folin-Ciocalteu reagent (Sigma-Aldrich, Darmstadt,

Germany), and mixed with distilled water (1:10) and 120 µL of 7.5% Na_2CO_3 solution (Sigma-Aldrich, Darmstadt, Germany) and incubated at 40 ºC during 15 min. The mixture was then allowed 30 min at room temperature protected from light before the absorbance being determined at 765 nm using a Synergy HT Microplate Reader (BioTek Instruments, Inc., Winoosli, VT, USA). Gallic acid (Sigma-Aldrich, Darmstadt, Germany) was used as standard and a calibration curve was prepared (5-100 mg/L, $R^2 > 0.999$). The TPC of samples was expressed as mg of Gallic Acid Equivalents (GAE) per 100 g dry basis (mg GAE/100 g db).

2.5 Determination of the Total Flavonoid Content

The Total Flavonoid Content (TFC) was determined by a colorimetric assay (de Francisco *et al.* 2018) with minor modifications. Briefly, 30 µL of reconstituted sample in its respective extractor solvent was mixed with 75 µL of distilled water and 45 µL of $NaNO_2$ (1%). After 5 minutes, 45 µL of $AlCl_3$ (5%) was added as well as 60 µL NaOH (1M) and 45 µL of distilled water. The absorbance was determined at 510 nm using a Synergy HT Microplate Reader (BioTek Instruments, Inc., Winoosli, VT, USA). A calibration curve was prepared with Quercetin (5-300 mg/mL, $R^2 > 0.999$). $NaNO_2$, $AlCl_3$, NaOH and Quercetin were all purchased from Sigma-Aldrich (Darmstadt, Germany). The TFC of samples was expressed as mg of Quercetin Equivalents (QE) per 100 g dry basis (mg QE/100 g db).

2.6 Determination of antioxidant activity

Two different assays were used to screen the antioxidant properties: scavenging activity on DPPH radical (measuring the decrease in DPPH radical absorption after exposure to radical scavengers) and reducing power (measuring the conversion of a Fe^{3+}/ferricyanide complex to the ferrous form (Fe^{2+})).

2.6.1 DPPH free radical scavenging assay

Different sample concentrations were prepared to determine the effective concentration of the antioxidant necessary to decrease the DPPH$^{\bullet}$ (1,1-diphenyl-2-picrylhydrazyl) concentration by

50% (IC_{50}) (Barros *et al*. 2007). The value IC_{50} was calculated from the graph of radical scavenging activity (RSA) percentage against extract concentration. Briefly, 30 µL of reconstituted sample in its respective extractor solvent was mixed with 270 µL of DPPH (Sigma-Aldrich, Germany) radicals ($6x10^{-5}$ M) dissolved in methanol. The DPPH radical reduction was determined by measuring the absorption at 525 nm in Synergy HT Microplate Reader (BioTek Instruments, Inc., Winoosli, VT, USA). A calibration curve for the standard Trolox (Sigma-Aldrich, Darmstadt, Germany) was prepared (5-175 mg/mL, R^2>0.999). The results were expressed as milligram per milliliter (mg/mL) of DPPH radical reduce.

2.6.2 Ferric reducing antioxidant power (FRAP) assay

This analysis was carried according out according to Benzie and Strain (1999) procedure, with minor modifications. Briefly, an aliquot of 35 µL of reconstituted sample in its respective extractor solvent was added to 265 µL of FRAP reagent (10 parts of 300 millimol sodium acetate buffer at pH 3.6, 1 part of 10 millimol TPTZ (Sigma-Aldrich, Darmstadt, Germany) solution and 1 part of 20 millimol $FeCl_3 \cdot 6H_2O$ solution) and the reaction mixture was incubated at 37 ºC for 30 min. The absorbance was measured at 595 nm in a Synergy HT Microplate Reader (BioTek Instruments, Inc., Winoosli, VT, USA). A calibration curve was prepared with Trolox (25-500 µM, R^2>0.999). TPTZ, sodium acetate, $FeCl_3$ and trolox were all purchased from Sigma-Aldrich (Darmstadt, Germany). The results were expressed as millimol Trolox Equivalents (TE) per 100 g dry basis (mg TE/100 g db).

2.7 Cell viability assay

2.7.1 Cell lines and culture conditions

Caco-2 (ATCC HTB-37, passage 31-34) was purchased from the American Type Culture Collection (ATCC, USA). Dr. T. Lesuffleur (INSERM U178, Villejuif, France) kindly provided HT29-MTX (passage 40-41) cell line. Cells were grown separately in tissue culture of 75 cm^2 flasks (Orange Scientific) in Dulbecco's modified Eagle's medium (DMEM) supplemented with

10% (v/v) of inactivated fetal bovine serum (FBS), 1% (v/v) of non-essential aminoacids (NEAA) and 1% (v/v) of antibiotic/antimitotic mixture (100 U/mL penicillin and 100 U/mL of Streptomycin). Cells were preserved in a humidified atmosphere containing 5% CO_2 / 95% air at 37 ºC (MCO-18ACUV-PE IncuSafe, Panasonic, Kadoma, Japan), and supplied with fresh medium and washing with Hank's Balanced Salt Solution (HBSS) every 48 hours. The cells were harvested at 90-95% confluence using trypsin. DMEM, FBS, NEAA, antibiotic/antimitotic, HBSS and trypsin were from Invitrogen (Carlsbad, CA, USA). All the cell related procedures were done in a Thermo Scientific™ MSC-Advantage™ Class II Biological Safety Cabinets (Darmstadt, Germany).

2.7.2 MTT assay

Cell were cultured in 96-well micro titer plates at a density of 25 $x10^3$ cells per mL culture medium for 24 h. Then, cells were washed with HBSS and incubated with different extracts (FDOA, FDMW) concentrations (0.1, 1, 10, 100 and 1000 µg/mL) previously dissolved in DMEM. A positive (cell plus DMEM) and a negative (Triton X-100, 1% w/v) control were used. After this period the extracts were removed and the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was added and incubated for 4 h. Dimethylsulfoxide (DMSO) was used to dissolve the MTT crystals and the absorbance was measured at 590 nm with a background subtraction at 630 nm. MTT and DMSO were purchased from Promega (Madison, WI, USA), Triton X-100 was purchased from Boehringer (Mannheim, Germany). The different concentrations were carried out in triplicates in three diverse experiences.

2.8 3D Intestinal permeability assay

The permeability study was carried out through a co-culture model with 90% of Caco-2 and 10% of HT29-MTX, according to Araújo and Sarmento (2013). The experiments were performed 21 days after seeding the cells. During this period, the transepithelial electrical resistance (TEER) was monitored to evaluate the cell monolayer integrity. In the last day, cell monolayers were pre-equilibrated with fresh HBSS, pH 7.4 at 37 ºC during 30 min. Afterwards,

0.5 mL of FDOA (100 mg/mL) concentration prepared in HBSS was added to the apical side of the co-culture monolayers and 1.5 mL of HBSS to the basolateral side. Samples were withdrawn from receptor side at 15, 30, 60, 90, 120, 150 and 180 minutes to determine the bioactive compounds transported across the monolayer. At the same times the TEER was evaluated. After each sampling time, the basolateral side was replaced with the same HBSS volume. Samples were conserved at -20 ºC for subsequent LC-ESI-MS analysis.

The permeability results (Almeida *et al*. 2015) were expressed in relative percentage, using as a base the apparent permeability (P_{app}), which was calculated using Eq.6.

$$P_{app} = \frac{\Delta Q}{A \times C_0 \times \Delta t} \quad (6)$$

where C_0 is the initial concentration in the apical compartment (μg/mL), A is the surface area of the insert (cm^2), Δt is the time during which the experiment occurred (seconds) and ΔQ is the amount of compound detected in the basolateral side (μg).

2.9 LC-ESI-MS analysis

To analyze the flavones that potentially crossed the 3D intestinal model, the methodology developed by Teixeira *et al*. (2018) was employed. Samples (FDOA) were analyzed by Liquid Chormatography-Electrospray Ionization-Mass spectrometry (LC-ESI-MS) performed in a Finnigan Surveyor Plus HPLC fitted with a PDA Plus detector, an auto-sampler Plus and a LC quaternary pump plus coupled to a Finnigan LCQ Deca XP Plus mass detector equipped with an ESI source and an ion trap quadrupole. The stationary phase was a Thermo Finnigan Hypersil Gold column (150 × 4.6 mm i.d., 5 μm) at 25 °C. The mass spectrometer was operated in the negative-ion mode with source, with a capillary temperature of 275 °C and capillary voltages of 4.5 kV. The mass spectra were recorded between 250 and 2000 m/z.

The mobile phase was composed by solvent A, 1 % (v/v) formic acid, and solvent B, 100 % (v/v) acetonitrile. The flow rate was 0.50 mL/min and the gradient method started with a linear

gradient ranging from 90 % A to 50 % A in 50 min, then reaching 100 % B in 10 min, a final isocratic gradient of 100% B during 5 min and a final re-equilibration isocratic gradient of 90 % A for 5 min.

2.10 Statistical analysis

All the results were expressed as mean ± standard deviation. To study the possible significant differences between the samples, analyzes of the unifactorial variance (ANOVA) and multifactorial (MANOVA) were performed, with a confidence level of 95% ($p < 0.05$). Pearson correlations were also obtained between the antioxidant activity and the bioactive compounds analyzed. The Statgraphics Centurion XVI program was used to perform the analysis.

3. Results and discussion

3.1 Nutraceutical product characterization

The spray drying process involves complex interactions that influence the final product quality. However, spray drying has been frequently described as a harsh drying method due to its often high-temperature operation (Murugesan and Orsat 2012). The physicochemical properties of the final product mainly depend on the feed flow rate, particle size, viscosity, food matrix, spray dryer inlet and outlet temperatures, pressure and type of equipment (Costa *et al*. 2015). One of the properties influenced by the process of spray drying is the water content of the obtained powder. This property influences other characteristics, such as porosity, compaction or flowability, also affecting the electrochemical and biological properties. Thus, food, pharmaceuticals and chemical industries always take into account this characteristic (Karam *et al*. 2016). The obtained grapefruit powder presented 1.5 ± 0.2 g water/100 g powder. The low water content of a nutraceutical product leads to a longer shelf life, minimizing the microbial growth and chemical deterioration (González et al. 2018). Also, it is more convenient to use and cheaper to transport because of its reduced weight and volume (Murugesan and Orsat 2012). The porosity is related with the free-flowing properties of the powder, the greater the ε value the

greater the flowability (Agudelo et al., 2017). The porosity of the obtained powder was set in 75% ± 0.12.

Color is one of the principal attributes of foods. Although it does not necessarily reflect nutritional, flavor or functional values, the color of powdered foods may be associated with the original food and determines the acceptability by consumers. The grapefruit powder showed L*=80.0 ± 1.8, h^*_{ab}= 61.7 ± 0.4 and a C^*_{ab} = of 11.4 ± 0.6. These color values fall within the range of those studied by González *et al*. (2018) and Telis and Martínez-Navarrete (2010) in a similar grapefruit product obtained by spray-drying and freeze-drying, respectively.

In every process of product creation not only the physical appearance is important but, mostly, the benefits for consumer must be guarantee. In the case of a nutraceutical, the biodisponibility of the bioactive compounds should be guaranteed. As almost all the food products are a complex mixture of vitamins, sugar, lipids, fibers, and phytochemicals, among others, to extract the bioactive compounds from the food matrix (Chemat *et al*. 2017) may be a matter of interest. Different extraction methodologies that use solvents in different proportions are available and each one has advantages and disadvantages, which can be exploited according to the interest of the bioactive compound studied. Trying to find the maximal information from the bioactive composition of the grapefruit powdered product, two extraction solvents were used in this study. Afterwards the corresponding liquid extracts were freeze-dried. The yield of this process was 85% ± 2 and 51.0% ± 1.2 for the FDOA and the FDMW extracts, respectively.

Table 1 summarizes the TPC, TFC and antioxidant activity of the freeze-dried extracts. In general terms, there were significant differences ($p<0.05$) between the antioxidant capacity of the extracts obtained both by IC_{50} and FRAP .

- Insert Table 1 -

There were no significant differences ($p>0.05$) in TPC of both freeze-dried extracts, while TFC was higher ($p<0.05$) in FDOA. Haminiuk *et al*. (2012) reported different methodologies of extraction phenolic compounds, being the values obtained in the present study similar to those of strawberry, açaí and fig, among others. Polyphenols have been reported as responsible for the antioxidant activity of citrus fruits due to their redox characteristics (Carocho and Ferreira,

2012), therefore, the high values of TPC and TFC of the grapefruit product provide high expectations in its role of chemo preventive properties, as well as its antioxidant, anti-inflammatory and antimicrobial activity for human health (Gioxari et al. 2015). The greatest antioxidant capacity ($p<0.05$) expressed as IC_{50} was obtained in the FDOA extract, while FDMW showed more ability to reduce Fe^{3+} to Fe^{2+} ($p<0.05$). The antioxidant capacity of the nutraceutical product was similar to those found in other citrus fruits studied by Klimczak *et al*. (2007), Chen *et al*. (2014) and Álvarez *et al*. (2014). These results indicated that the powdered product, despite being exposed to changes in its structure, still has similar values to different fresh products (Özlem *et al*. 2017; Oboh and Ademosun 2012; Diab 2016; Ghasemi *et al*. 2009). Although it is true that the main advantage of the methods established for the quantification of antioxidant activity is it simplicity, the biggest disadvantage is that the results can be influenced by many factors, such as the interaction of the antioxidants in the sample, reagents, pH, times or free radicals production (Shahidi and Zhong 2015). There is some controversy about the influence of the bioactive compounds present in fruits and vegetables with their antioxidant capacity (Guo et al. 2003). Chemical interactions affecting free radical scavenging properties between phytochemicals have not been extensively reported in fruits and vegetables, yet both synergistic and antagonistic interactions may affect antioxidant capacity (Talcott et al. 2003). In this sense, in order to better understand the interactions of TPC and TFC with the antioxidant capacity of the extracts, a Pearson correlation was performed (Table 2). According to Table 2, the Pearson correlation coefficient between FRAP (-0.9830, oxalic acid solvent extract) and IC_{50} (-0.9306, methanol and water solvent extract) is higher, validating that both techniques are complementary.

- Insert Table 2 –

A similar correlation was produced between TPC or TFC and antioxidant capacity (IC_{50}), but with a negative tendency according to Pearson. These results indicate that the phenolic capacity was linked to a high or low antioxidant capacity. Opposite results were found when TPC, TFC were evaluated in FRAP. There was a positive tendency according to Pearson. However, it must be taken into account that the changes in the trend in the interactions of bioactive compounds

can be given by the extractive agent. The interactions that occur with oxalic acid can be explained based on its strong acidity, being soluble in water and alcoholic compounds and presenting a great power of interaction in the presence of strong oxidative agents (due to the presence of the carboxyl group in its composition). In addition, the antioxidant properties are conferred to flavonoids by the phenolic hydroxyl groups attached to ring structures as they can act as reducing agents, like hydrogen donators, singlet oxygen quenchers, superoxide radical scavengers and even as metal chelators (Carocho and Ferreira 2012). These characteristics, together with the interactive power of the extract, generate the relations in a positive or negative way.

These results were expected, since grapefruit is mainly known for its richness in citric and ascorbic acid (La Cava and Sgroppo 2015). Both organic acids provide enzymatic browning and contribute to the antioxidant capacity. Nevertheless, grapefruit is also rich in flavonoids, phytochemicals related to anti-inflammatory, antimicrobial, anticancer properties already reported by different authors (Uckoo *et al*. 2012; Di Majo *et al*. 2005; Zou *et al*. 2015).

3.2 Cell viability assay

In order to determine if the bioactive compounds of the nutraceutical product could lead to negative effects on cell proliferation or direct cytotoxic properties that eventually lead to cell death, cell viability assays were performed. In this case, FDOA and FDMW were evaluated in different concentrations (0.1 – 1000 µg/mL). Figure 1 summarize the obtained results.

- Insert Figure 1 –

As it is possible to observe, initially there were significant differences ($p < 0.05$) between the different extraction solvents. The highest viability in HT29-MTX cells was obtained with FDOA (130%), while for Caco-2 the FDMW presented the best result (135%). However, in both extracts the cell viability was above 90%.

The MANOVA analysis indicated that the main effects that influence cell viability ($p < 0.05$) were the different solvent extractors and their respective concentrations. Analyzing the effect of

concentrations (Figure 2), it is possible to observe that the cell viability remains above 90% in all extracts from 0.1 to 100 µg/mL.

- Insert Figure 2 –

At high concentrations, the cell viability decreases, probably due to the extract composition. Nevertheless, in the maximum concentration tested (1000 µg/mL), Caco-2 cells showed a significant ($p < 0.05$) viability decrease, not exceeding 80% survival in FDMW and up to 50 % survival in FDOA. Conversely, HT29-MTX cells in this concentration exceed 100%. However, within the same concentration in the different extractor solvent, significant differences ($p < 0.05$) were observed in Caco-2 and HT29-MTX cells. These results are in accordance with Laitinen *et al.* (2004) that evaluated the extracts effects of food supplements and food fractions in Caco-2 cells, obtaining a greater cell viability at lower concentrations (0.02 and 0.2 mg/mL) and finding a minimum viability of 77% in all samples. Similar results were reported by Xu *et al.* (2003) in a study focused on citrus juices, in which grapefruit juice did not affect the viability of Caco-2 cells. Equally, Chen and Kitts (2017) found good results in orange peel in Caco-2 cells using 7.5% ethanol as extract solvent. In fact, FDOA an FDMW did not lead to a toxic effect in both cell lines ($p < 0.05$). This could indicate a protective effect of these extracts (in concentrations between 0.1 and 100 µg /mL) in both cell line, since instead of causing damage to the cell, it keeps it in good condition (Groh and Muncke, 2017; Kaindl *et al*., 2008). Nevertheless, a more detailed study has to be performed to support this protective effect.

3.3 3D Intestinal permeability assay

3.3.1 Identification of bioactive compounds

Citrus fruits are rich in bioactive compounds, especially the Star ruby variety (García-Martínez *et al.* 2018). Flavonoids are among these compounds, being characterized by a skeleton of 15 carbons, mostly linked to one or more sugar molecules (Zhang *et al*. 2017). According to Theile *et al*. (2017), grapefruit presents high flavonoid glycosides contents, being characterized by the

presence of rutinoside (such as hesperidin and narirutin) and neohesperidoside flavonoids (namely naringin and neohesperidin). Figure 3 show the phenolic profile obtained for grapefruit in FDOA, indicating the presence of flavonoids, particularly between 20 and 35 minutes.

- Insert Figure 3 –

Table 3 summarize the different phenolic compounds identified.

- Insert Table 3 -

In the analysis of the initial sample, different compounds were identified depending on the wavelength evaluated, the retention times and the maximum fragments that the mass spectrometer can detect. A possible identification was performed based on these characteristics. Compound 1 could be an anthocyanin (delphinidin-3-glucoside), according to the molecular weight and the fragments obtained (465, 303 m/z). However, taking into account its wavelength and retention time, it could also be a flavonone (hesperitin-7-*O*-glucoside). Compounds 2 and 3, due to their closeness in molecular weights (595 m/z) and fragments (287 m/z) with differences in retention time (23.23 and 24.25 min) and wavelengths, could be a flavonone of 7-*O*-glucoside group. In this case, compound 2 would be neoeriocitin (eriodictyol-7-*O*-neoheperidiside) and compound 3 eriocitrin (eriodictyol-7-*O*-rutinoside). According to the literature, compound 4 is probably a hesperidin or a neohesperidin (Abad-García *et al.* 2014; Londoño-londoño *et al.* 2010; Dugo *et al.* 2005; Vaclavik *et al.* 2012; Kim *et al.* 2012; Yan *et al.* 2007; Cheigh *et al.* 2012; Durand-Hulak *et al.* 2015; Tong *et al.* 2018). In what concerns to compounds 5 and 6 that present similar molecular weight and fragments (595, 433 and 287 m/z) are probably didymin, poncirin or saponarin, while compounds 7, 8 and 9 were identified by their molecular weight and respective fragments. In the case of compounds 7 and 8 (581, 419 and 273 m/z) a naringin or a narirutin are probably present. Compound 9 could be more accurately hesperidin (611, 449 m/z), being similar to compound 4 and differing in the fragments obtained.

3.3.2 3D Intestinal permeability

In order to ensure the intestinal permeability, it is necessary to monitor the co-culture TEER. TEER is a very sensitive and reliable method to confirm the integrity and permeability of cells culture, being a non-invasive method that can be applied to monitor living cells during various stages of growth and differentiation (Pereira et al. 2015). Figure 4 shows the TEER measurements during the 21 days.

- Insert Figure 4 –

Similar TEER measurements were made during the permeability experience to guarantee the viability process. The values confirm the integrity of the 3D model, presenting comparable TEER to the one reported by Pereira *et al*. (2015).

Once the 180 minutes of permeation in the co-culture was finished, different results were obtained regarding permeability (Figure 5). Figure 5 shows the two working wavelengths and the compounds that were detected initially, at time zero of the experience. Also, the permeation graph expressed in relative percentage of release, taking into account the apparent permeability that was calculated as the ratio of the original relative percentage permeated through the monolayer, between the apical chamber (time cero min) and the basolateral chamber (time 180 min).

- Insert Figure 5 –

Regarding all the bioactive compounds identified, only didymin, poncirin or saponarin (compounds 5 and 6) were not detected. Nevertheless, a high permeation was achieved for compound 1 (delphenidin-3-glucoside or hesperitin-7-*O*-glucoside) and compound 9 (hesperidin), with compound 1 presenting a permeation higher than 50%, followed by hesperidin that was close to 30%. Naringin or narirutin presented a permeability lower than 25% as well as the compound identified as neohesperidin or hesperidin. Tian *et al*. (2009) found similar permeation result with flavonoid compounds, namely hesperetin, eriodictyol and naringenin, obtaining values not greater than 60% in Caco-2 cells.

A number of factors interfere with the transport of bioactive compounds present in nutraceutical products, such as the concentration used, the extraction form, the molecule size, the permeation time or even the TEER variability (Gioxari et al. 2015). In addition another factor to take into account is the matrix that protects the bioactive compounds, which refers to the biopolymers (GA, MD and WPI) added during the formulation of the grapefruit nutraceutical product, which can be barriers against permeability (Alminger et al. 2014). Oxidative stress may be the main cause in the transference of bioactive compounds, since this mechanism is activated during cell permeation (Chen *et al*. 2012). The compounds that were not transported in their entirety were probably retained within the cell model. This phenomena may be due to the fact that bioactive compounds of citrus origin can be used as elements of cell cytoprotection, reducing the oxidative stress, which is in accordance with Cilla *et al*. (2018).

According to the obtained results, compound 1, 4 and 9 were easily transported. This may be due to the microencapsulation process carried out by means of spray draying. Compounds 1 and 9 are the bioactive with the best results. Delphenidin-3-glucoside (compound 1) was extensively studied as a suppressive element in cancer cells (Yang *et al*. 2016). However, it can also be hesperitin-7-*O*-glucoside and hesperidin (compound 9) that agree to be the main bioactive compounds of citrus fruits presenting various pharmacological activities, such as antioxidant, antibacterial, anti-inflammatory or anticancer (Wang *et al*. 2016).

The nutraceutical product derived from the grapefruit and obtained by means of the spray drying process has a great potential as a nutraceutical. The results support that the bioactive compounds were able to be encapsulated and the behavior in cell viability and permeability test showed that there is a likelihood that compounds such as delphinidin-3-glucoside, hesperitin-7-*O*-glucoside, hesperidin, neohesperidin reach the body target, being a source of oxidative protection.

4. Conclusion

In the last decade, functional and nutraceutical foods have obtained a great demand among consumers due to the potential health benefits that they can offer. In the present study, a grapefruit nutraceutical powder has been obtained by spray drying and characterized regarding

different physical and chemical parameters as well as in its intestinal permeability. The obtained results support the good stability in what concerns to moisture and porosity, also presenting an attractive grapefruit color, as well as antioxidant capacity and high content of phenols and flavonoids. The main conclusion of this study is the bioavailability that this product offers to encapsulate the most important *C. paradise* bioactive compounds, such as delphinidin-3-glucoside, hesperitin-7-*O*-glucoside, hesperidin or neohesperidin, that showed a permeation up to 50% in the 3D intestinal model. Thus, spray drying technique can be classified as a great alternative in food industry for these products as well as the biopolymers employed in this process.

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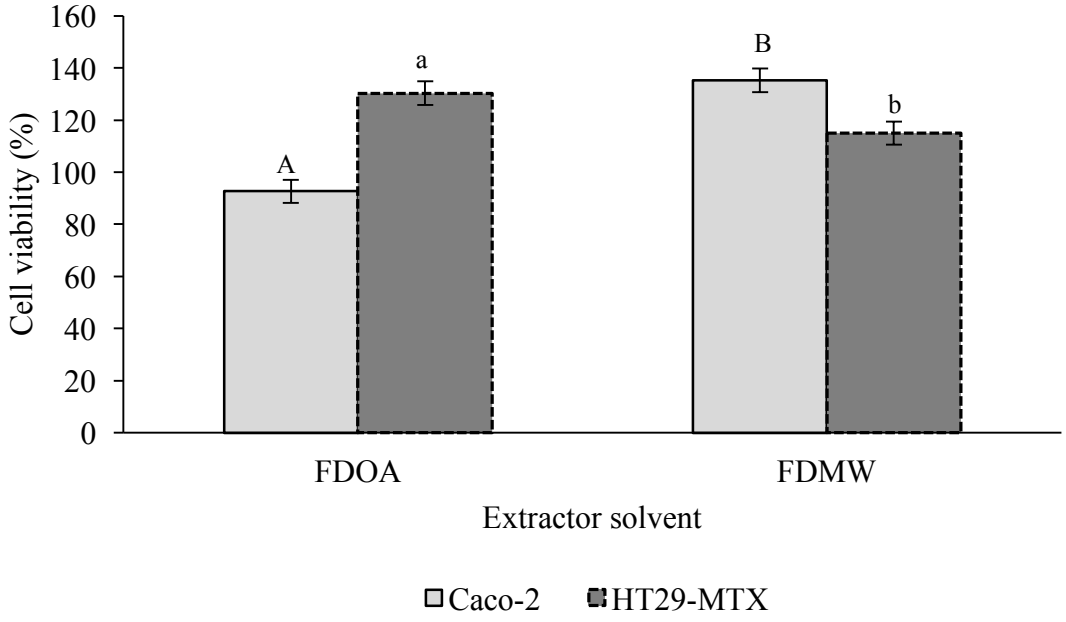

Figure 1. Interactions of cell line and extractor solvent. Freeze-dried acid oxalic extract (FDOA), freeze-dried Methanol-Water extract (FDMW). Different letters (A-B, a-b) indicate significant differences between mean values of extractor solvent and cell line ($p < 0.05$).

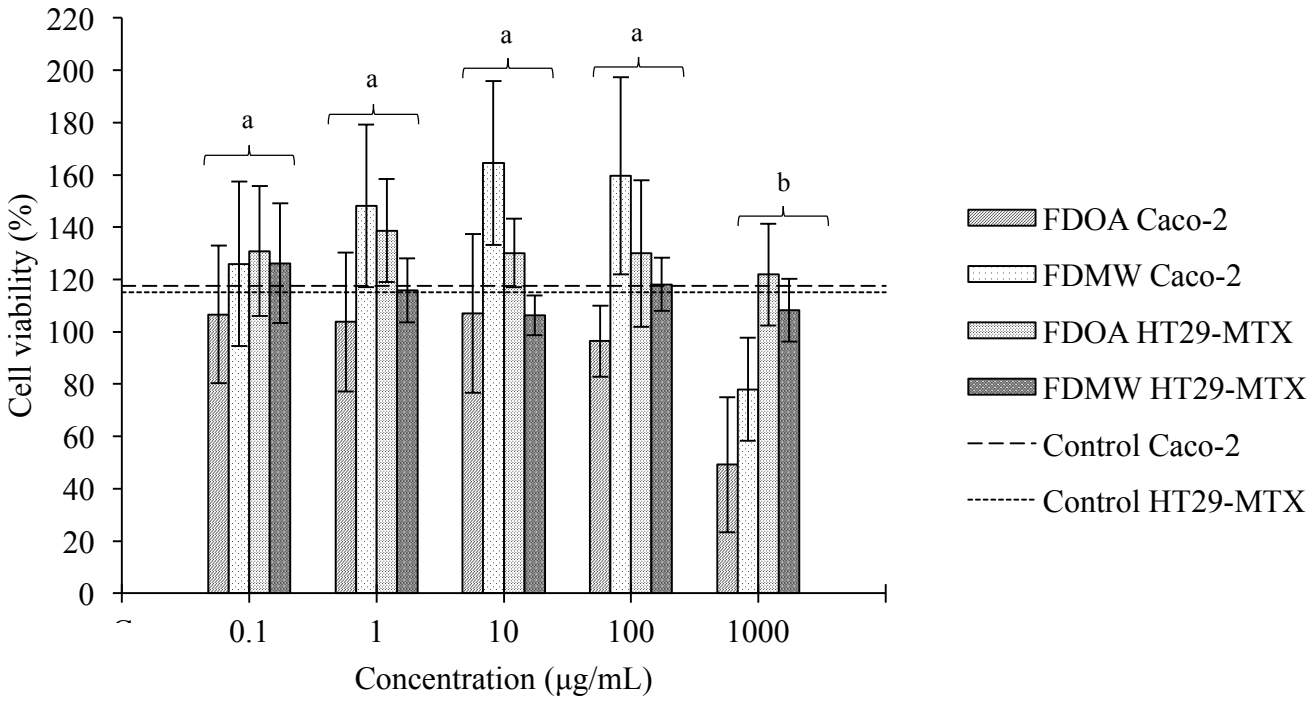

Figure 2. Cell viability in different concentrations according to the extracts in Caco-2 and HT29-MTX cell lines. Freeze-dried acid oxalic extract (FDOA), freeze-dried Methanol-Water extract (FDMW) and positive Control (dashed lines). Different letters (a-b) indicate significant differences between mean values to concentrations ($p < 0.05$).

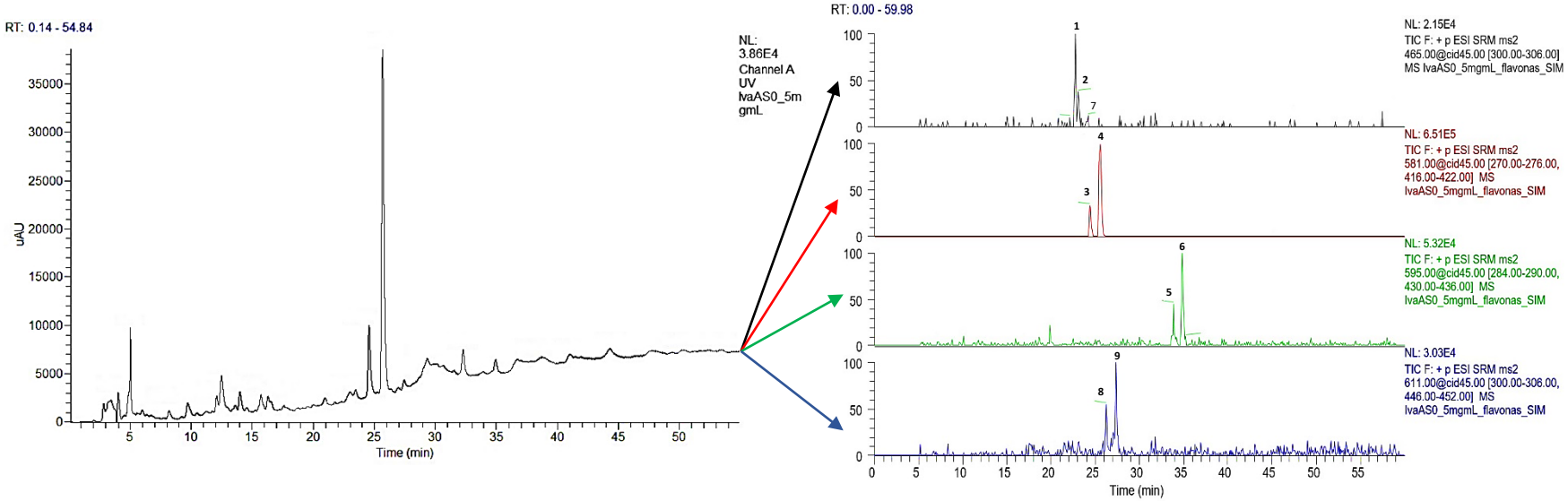

Figure 3. Example of the LC-ESI-MS analysis of the FDAO in its different wavelengths. 1: Delphinidin-3-glucuside or herperitin-7-*O*-glucodide; 2: Eriodictyol-7-*O*-neohesperidiside; 3: Eriodictyol-7-O-rutinoside; 4: Hesperidin or neohesperidin; 5 and 6: Didymin, poncirin or saponarin; 7 and 8: Naringin or narirutin; 9: Hesperidin.

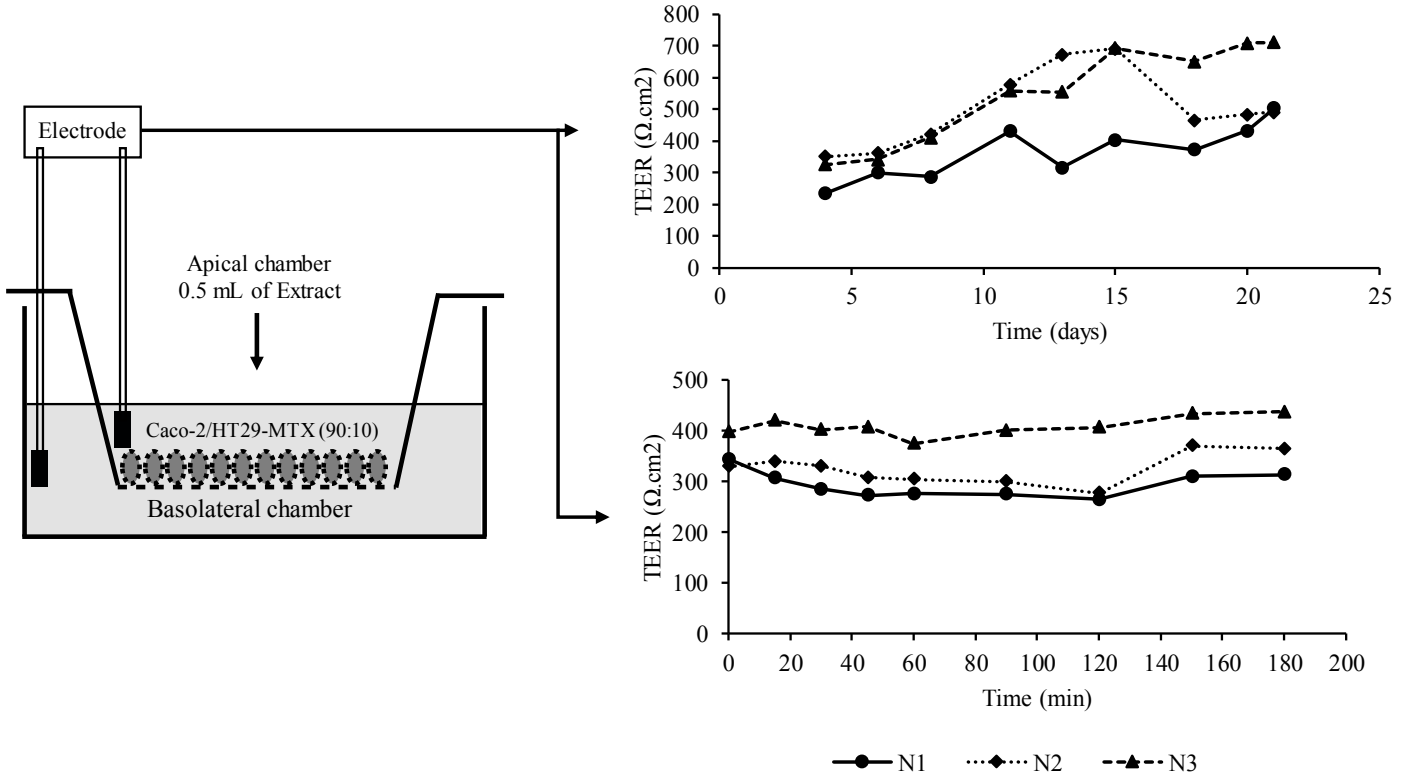

Figure 4. Transepithelial electrical resistance (TEER) measurements of coculture cells (90% Caco-2 and 10% HT29-MTX) during the 21 days and 180 minutes of permeability assay. N1, N2, N3: number of repetitions made.

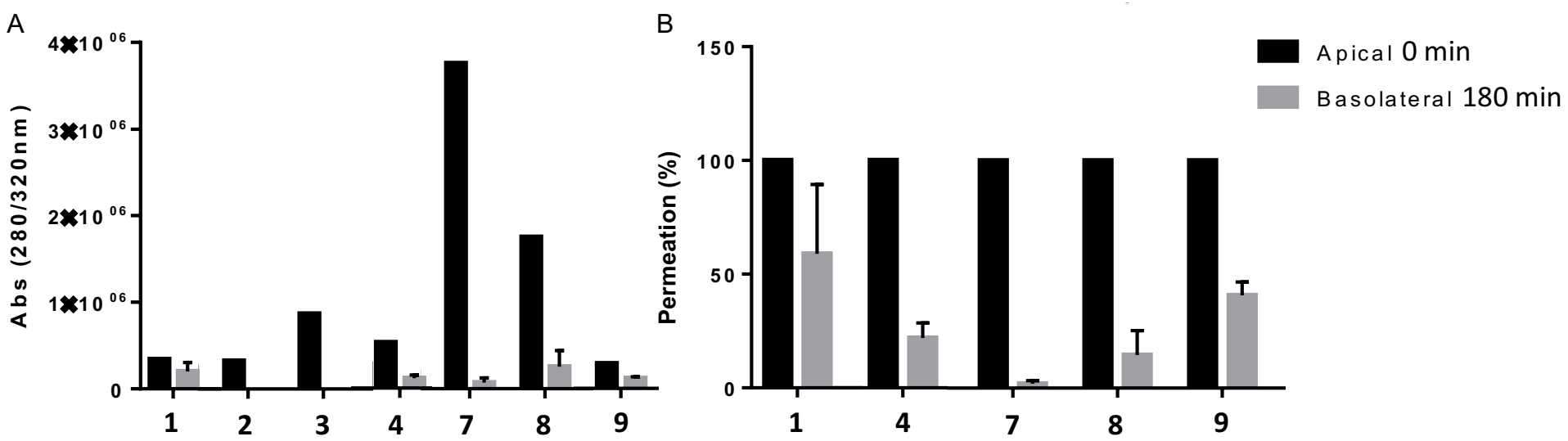

Figure 5. A: Work wavelengths. B: Permeability of bioactive compounds at time 0 and after 180 minutes. 1: Delphinidin-3-glucuside or herperitin-7-*O*-glucodide; 2: Eriodictyol-7-*O*-neohesperidiside; 3: Eriodictyol-7-*O*-rutinoside; 4: Hesperidin or neohesperidin; 7 and 8: Naringin or narirutin; 9: Hesperidin.

Figure Captions

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Table Captions

Table 1. Total Phenolic Content (TPC), Total Flavonoid Content (TFC), radical scavenging activity IC_{50} values and antioxidant activities based on their abilities to reduce ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) (FRAP analysis) of the extracts. Values are expressed as mean ± standard deviation (n = 9). Different letters (a, b) in the same column indicate significant differences between mean values ($p < 0.05$).

Table 2. Pearson correlation coefficients among total Phenolic Content (TPC), total Flavonoid Content (TFC), radical scavenging activity IC_{50} values and antioxidant activities based on their abilities to reduce ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) (FRAP analysis).

Table 3. Tentative identification of grapefruit nutraceutical powder main compounds. Time retention (tr), molecular ion with negative charge [MS] (m/z), fragments of ions [MS2] [MS3] (m/z), wavelength at maximum visible absorption (λmax).

Table 1

Sample	IC_{50} (mg/mL)	FRAP (mmol TE/100 g db)	TPC (mg GAE/100 g db)	TFC (mg QE/100 g db)
FDOA	0.48±0.04[a]	10.3±0.6[a]	1274±47.6[a]	6592±626.7[a]
FDMW	0.72±0.16[b]	12.7±0.6[b]	1294±98.2[a]	4314±518.9[b]

GAE, gallic acid equivalents. QE, quercetin equivalents. TE, trolox equivalents. FDOA, freeze-dried oxalic acid extract. FDMW, freeze-dried methanol-water extract. db, dry basis.

Table 2.

	FDOA	FDMW
FRAP vs. TPC	0.5262	0.5392
FRAP vs. TFC	0.7449*	0.6739*
FRAP vs. IC_{50}	-0.9830*	-0.9306*
TPC vs. TFC	0.4348	0.8003*
TPC vs. IC_{50}	-0.8399*	-0.7352*
TFC vs. IC_{50}	-0.4654	-0.8843*

Freeze-dried oxalic acid extract (FDOA), Freeze-dried methanol-water extract (FDMW), Total Phenolic Content (TPC), Total Flavonoid Content (TFC), radical scavenging activity IC_{50} values and FRAP antioxidant activity. *$p < 0.05$ indicate statistically significant correlations at the 95% confidence level. These correlation coefficients range between -1 and +1 and measure the strength of the linear relationship between the variables.

Table 3.

Compound	t_r (min)	[MS]	MS^2	MS^3	λ_{max} (nm)	Possible Compounds	References
1	22.79	465	303		247; 328	Delphinidin-3-glucoside/hesperitin-7-*O*-glucoside	12, 13, 19, 21
2	23.23	595	287		280	Neoeriocitrin (Eriodictyol-7-*O*-neohesperidiside)	2, 3, 5,9, 19,21
3	24.15	595	287		283; 325	Eriocitrin (Eriodictyol-7-*O*-rutinoside)	1, 2, 4,5, 6, 9, 18
4	26.22	611	449	303	280; 320	Hesperidin/Neohesperidin	7, 8, 9, 10, 11, 14, 15, 17, 20
5	33.88	595	433	287	280	Didymin/Poncirin/Saponarin	11, 17, 20
6	35.05	595	433	287	289; 320	Didymin/Poncirin/Saponarin	11, 17, 20
7	20-35	581	419	273	280; 320	Naringin/Narirutin	11, 15, 17, 19, 20
8	20-35	581	419	273	280; 320	Naringin/Narirutin	11, 15, 16, 17, 19, 20
9	20-35	611	449		280; 320	Hesperidin	15, 17, 20