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

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Abstract

BACKGROUND: The development of functional and nutraceutical foods comes from a greater awareness of the relationship between food and health by consumers. In recent years, the idea of purifying and encapsulating bioactive compounds through techniques such as spray drying has been well received by the food industry. The development and characterization of a grapefruit (*Citrus paradisi*) nutraceutical powder obtained by spray drying is of great interest owing to the different bioactive compounds and the potential health effects.

RESULTS: The grapefruit powder was characterized by a low water amount (1.5 g water per 100 g powder) and a high porosity (75%). The color parameters were $L^* = 80.0 \pm 1.8$, $h_{ab}^* = 61.7 \pm 0.4$ and $C_{ab}^* = 11.4 \pm 0.6$. The IC_{50} values determined for the freeze-dried oxalic acid extract (FDOA) and the freeze-dried methanol–water extract (FDMW) were 0.48 and 0.72 mg mL^{-1} respectively, while the total phenolic content (TPC) ranged between 1274 and 1294 mg gallic acid equivalent (GAE) per 100 g dry basis (d.b.). Regarding total flavonoid content (TFC), FDOA presented the highest amount (6592 mg quercetin equivalent (QE) per 100 g d.b.). For both extracts, the cell viability in Caco-2 and HT29-MTX was above 90% at 100 μg mL^{-1}. The bioavailability of the bioactive compounds was analyzed through a 3D intestinal model. Delphenidin-3-glucoside and hesperitin-7-*O*-glucoside presented a permeation higher than 50%, followed by hesperidin which was close to 30%.

CONCLUSION: This work allows to establish that the formulation of grapefruit powder has great potential as a nutraceutical food, with spray drying being a good alternative technique in the food industry.

Keywords: grapefruit powder; bioactive compounds; LC/ESI-MS; 3D intestinal model; permeability

INTRODUCTION

Citrus fruits are well known for their richness in ascorbic acid, also presenting considerable amounts of sugar, calcium, phenols, phosphorus and vitamin B6.[1] Within the family of citrus products, *Citrus paradisi*, commonly known as grapefruit, is largely underestimated by consumers owing to its bitter flavor. However, the food and beverage industry as well as cosmetic and pharmaceutical products use grapefruit in a large variety of formulations owing to the valuable compounds present in its skin, seed, pulp and juice.[1,2] *Citrus paradisi* has a characteristic taste and color and a long shelf life, being of huge popularity in some European countries as well as in Asia and the USA. The varieties of this citrus fruit can be grouped into white and pigmented. Within the pigmented variety, Star Ruby is remarkable, presenting an intense coloration, with few seeds and a high yield of juice and bioactive compounds.[3] A recent review stated that the bioactive compounds of grapefruit juice are distributed in nine general groups, of which flavonoids are the most relevant, naringin (26.25 ± 0.39 mg per 100 mL) and eriocitrin (34.2 ± 1.06 mg L^{-1})

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being the major ones, followed by carotenes (α-carotene, 8–5 μg per 100 g; β-carotene, 14–603 μg per 100 g) and different types of acid such as ascorbic acid (13.44–16.76 mg per 100 mL) and malic acid (0.03–0.23 g per 100 mL).[4] The flavonoids mainly appear in glycosylated form, which takes place at position 7, in compounds such as rutinose and neohesperidose.[5] Other glucoside groups identified in grapefruit are neohespiridin, didymin and poncirin.[6] Recently, different studies have focused on understanding the interactions of grapefruit bioactive compounds with the positive reduction of chronic diseases and the health benefits.[7]

The formulation of a nutraceutical product in powdered form is of huge interest for consumers. For this reason, it is important (i) to ensure the selection and development of an appropriate matrix and technological process able to maintain the active compound structure from production until consumer consumption and (ii) to guarantee delivery of the bioactive compounds to the physiological target within the organism. Spray drying is the most popular encapsulation method not only in the food industry but also in pharmaceutical and agrochemical industries.[8] This method of drying consists of spraying a fluid in a stream of very hot air, causing the evaporation of water in a very short time, with a minimum negative effect on the properties of the food.[9] Spray drying has the advantage of generating high-quality powders, being a controllable, low-cost, scalable and continuous process that is applicable to both heat-sensitive and heat-resistant materials. However, some disadvantages related to the process yield can occur depending on the product composition. In the case of fruits, their high sugar and organic acid contents lead to a rubbery matrix when dehydrated. In order to increase the glass transition temperature (T_g) of the product to ensure its glassy state and avoid stickiness problems, high-molecular-weight polymers can be added.[10] Maltodextrin (MD), gum arabic (GA) and whey protein isolate (WPI) are examples of such carriers.

Nevertheless, despite the richness in bioactive compounds that Star Ruby grapefruit can offer, it is important to identify the main bioactive 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 the intestine and their possible health effects.[11] The most commonly lineage used for cell culture models is Caco-2, representing the intestinal line. However, this lineage has some limitations that have a great influence on absorption, such as the lack of mucin production. Therefore this cell line is normally complemented with the HT29-MTX cell line responsible for this property in order to improve the paracellular permeability of hydrophilic compounds in intestinal 3D models.[12,13]

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

MATERIALS AND METHODS

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% GA, 1.44% WPI, 1.25% MD and 87.95% liquefied grapefruit (RoboDiet Compact, DeLonghi, Barcelona, Spain), based on the design of optimized response surface experiments.[14] The fruits (*C. paradisi* var. Star Ruby) were obtained in a local supermarket in Valencia, Spain. The liquidized grapefruit was sieved through a 0.7 mm mesh (CISA 200/50, Barcelona, Spain) to ensure the absence of any pulp. The biopolymers GA and MD were supplied by Alfa Aesar® (Karlsruhe, Germany), while WPI Lacprodan® DI-9212 was from Arla Foods Ingredients (Viby, Denmark). Once the mixture with all ingredients was prepared, the sample was spray dried (Mini Spray Dryer B-290, Büchi, Flawil, Switzerland) under conditions of an aspiration speed of 35 $m^3\ h^{-1}$, a feed flow of 9 mL min^{-1} and an atomizer flow of 473 L h^{-1} at a maximum temperature of 148 °C and a pressure of 5×10^5 Pa. The powder was vacuum packed (Edesa VAC-20 SL, Guipúzcoa, Spain) pending further characterization steps.

Physical properties of powder

The water content was determined in triplicate by a gravimetric method[15] in a vacuum oven (VACIOTEM, JP Selecta, Barcelona, Spain) at 60 °C until constant weight and expressed as g water per 100 g powder.

The bulk density (ρ_a) was determined in triplicate based on the measurement 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 (Fisher Scientific Infrared Vortex Mixer F202A0175, Madrid, Spain) and applying Eqn (1).

$$\rho_a = m/v_f \tag{1}$$

where ρ_a is the bulk density (g mL^{-1}), m is the mass of powder (g) and v_f is the volume after vibration (mL).

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

$$\varepsilon = \left(\rho - \rho_a\right)/\rho \tag{2}$$

$$\rho = 1/\left[x_w^p/\rho_w + \left(1 - x_w^p\right)/\rho_{CH}\right] \tag{3}$$

where ε is the porosity, ρ is the true density, ρ_a is the bulk density (Eqn (1)), ρ_w is the water density at 20 °C (0.9976 g mL^{-1}), ρ_{CH} is the carbohydrate density at 20 °C (1.4246 g mL^{-1}) and x_w^p is the water content of the powder (g water g^{-1} powder.

The color of the samples was measured in triplicate using a spectrocolorimeter (Minolta CM3600-D, Madrid, Spain) with D65 reference illuminant and 10° observer. The CIE $L^*a^*b^*$ coordinates were obtained, from which the hue angle (h_{ab}^*, Eqn (4)) and chroma (C_{ab}^*, Eqn (5)) were calculated.

$$h_{ab}^* = \tan^{-1}\left(b^*/a^*\right) \tag{4}$$

$$C_{ab}^* = \left(a^{*2} + b^{*2}\right)^{1/2} \tag{5}$$

Preparation of freeze-dried extracts

In order to find the maximal information from the bioactive composition of the grapefruit powdered product, two extraction solvents were used: (i) oxalic acid (Scharlab SL, Barcelona, Spain) with a concentration of 0.1% (w/v) in distilled water and (ii) methanol–water (Scharlab SL) in a proportion of 70:30 (v/v). A 1 g quantity 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,

followed by centrifugation (Eppendorf 5810R, Wesseling-Berzdorf, Germany) at 5867 × *g* 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 h), the temperature was kept at −55 °C in the condenser. Two extractions with each solvent were 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).

Determination of total phenolic content, total flavonoid content and antioxidant activity

The total phenolic content (TPC) was determined spectrophotometrically according to the Folin–Ciocalteu[16] procedure with minor modifications.[17] 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), then mixed with distilled water (1:10, v/v) and 120 μL of 7.5% (w/v) Na_2CO_3 solution (Sigma-Aldrich) and incubated at 40 °C for 15 min. The mixture was then left for 30 min at room temperature protected from light, before its absorbance at 765 nm was determined using a Synergy HT Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA). Gallic acid (Sigma-Aldrich) was used as standard and a calibration curve was prepared (5–100 mg L^{-1}, $R^2 > 0.999$). The TPC of samples was expressed as mg gallic acid equivalent (GAE) per 100 g dry basis (d.b.).

The total flavonoid content (TFC) was determined by a colorimetric assay[18] 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%, w/v). After 5 min, 45 μL of $AlCl_3$ (5%, w/v) was added as well as 60 μL of NaOH (1 mol L^{-1}) and 45 μL of distilled water. The absorbance was determined at 510 nm using a Synergy HT Microplate Reader (BioTek Instruments, Inc.). A calibration curve was prepared with quercetin (5–300 mg mL^{-1}, $R^2 > 0.999$). The TFC of samples was expressed as mg quercetin equivalent (QE) per 100 g d.b..

Two different assays were used to screen the antioxidant properties: scavenging activity on the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH•) (measuring the decrease in DPPH• absorption after exposure to radical scavengers) and ferric reducing antioxidant power (FRAP) (measuring the conversion of an Fe^{3+}/ferricyanide complex to ferrous form (Fe^{2+})).

Different sample concentrations were prepared to determine the effective concentration of the antioxidant necessary to decrease the DPPH• concentration by 50% (IC_{50}).[19] The IC_{50} value 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) (6×10^{-5} mol L^{-1}) dissolved in methanol. The DPPH• reduction was determined by measuring the absorption at 525 nm in a Synergy HT Microplate Reader (BioTek Instruments, Inc.). A calibration curve for the standard Trolox (Sigma-Aldrich) was prepared (5–175 mg mL^{-1}, $R^2 > 0.999$). The results were expressed as mg mL^{-1} of DPPH• reduction.

FRAP analysis was carried according out according to the Benzie and Strain[20] procedure with minor modifications. Briefly, 35 μL of reconstituted sample in its respective extractor solvent was added to 265 μL of FRAP reagent (10 parts of 300 mmol L^{-1} sodium acetate buffer at pH 3.6, 1 part of 10 mmol L^{-1} tripyridyl-s-triazine (TPTZ) (Sigma-Aldrich) solution and 1 part of 20 mmol L^{-1} $FeCl_3 \cdot 6H_2O$ solution) and incubated at 37 °C for 30 min. The absorbance was measured at 595 nm in a Synergy HT Microplate Reader (BioTek Instruments, Inc.). A calibration curve was prepared with Trolox (25–500 μmol L^{-1}, $R^2 > 0.999$). The results were expressed as mmol Trolox equivalent (TE) per 100 g d.b.

Cell viability assay

Cell lines and culture conditions

Caco-2 (ATCC HTB-37, passage 31–34) cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, 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, Braine-l'Alleud, Belgium) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) inactivated fetal bovine serum (FBS), 1% (v/v) non-essential amino acids (NEAA) and 1% (v/v) antibiotic/antimitotic mixture (100 U mL^{-1} penicillin and 100 U mL^{-1} streptomycin). Cells were preserved in a humidified atmosphere containing 5% CO_2/95% air at 37 °C (MCO-18ACUV-PE IncuSafe, Panasonic, Kadoma, Japan) and supplied with fresh medium and washing with Hank's Balanced Salt Solution (HBSS) every 48 h. 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 cell-related procedures were done in a Thermo Scientific™ MSC-Advantage™ Class II Biological Safety Cabinet (Darmstadt, Germany).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cell were cultured in 96-well micro titer plates at a density of 25×10^3 cells mL^{-1} culture medium for 24 h, then washed with HBSS and incubated with different extract (FDOA, FDMW) concentrations (0.1, 1, 10, 100 and 1000 μg mL^{-1}) 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 MTT was added and incubated for 4 h. Dimethyl sulfoxide (DMSO) was used to dissolve the MTT crystals and the absorbance at 590 nm was measured 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 triplicate in three diverse experiments.

3D intestinal permeability assay

The permeability study was carried out through a co-culture model with 90% Caco-2 and 10% HT29-MTX according to Araújo and Sarmento.[21] 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. On the last day, cell monolayers were pre-equilibrated with fresh HBSS (pH 7.4) at 37 °C for 30 min. Afterwards, 0.5 mL of FDOA (100 mg mL^{-1}) 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 the receptor side at 15, 30, 60, 90, 120, 150 and 180 min 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 °C for subsequent LC/ESI-MS analysis.

The permeability results were expressed as relative percentage of transport.

LC/ESI-MS analysis

To analyze the flavones that potentially crossed the 3D intestinal model, the methodology developed by Teixeira *et al.*[22] was employed. Samples (FDOA) were analyzed by LC/ESI-MS using a Finnigan Surveyor Plus HPLC system (Thermo Fisher Scientific, Waltham, MA, USA) fitted with a PDA Plus detector, an auto-sampler Plus and an LC quaternary pump 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 mm × 4.6 mm i.d., 5 μm) at 25 °C. The mass spectrometer was operated in negative ion mode with source, with a capillary temperature of 275 °C and a capillary voltage of 4.5 kV. The mass spectra were recorded between *m/z* 250 and 2000.

The mobile phase was composed of solvent A, 1% (v/v) formic acid, and solvent B, 100% (v/v) acetonitrile. The flow rate was 0.50 mL min^{-1} and the gradient method started with a linear gradient ranging from 90 to 50% A in 50 min, then reaching 100% B in 10 min, a final isocratic gradient of 100% B for 5 min and a final re-equilibration isocratic gradient of 90% A for 5 min.

Statistical analysis

All results were expressed as mean ± standard deviation. To study possible significant differences between the samples, analyses of unifactorial (ANOVA) and multifactorial (MANOVA) variance 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.

RESULTS AND DISCUSSION

Nutraceutical product characterization

The spray drying process involves complex interactions that influence the final product quality. According to Murugesan and Orsat,[23] spray drying has been frequently described as a harsh drying method owing to its often high-temperature operation. 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.[24] One of the properties influenced by the process of spray drying is the water content of the obtained powder. Water content influences other characteristics such as porosity, compaction and flowability, also affecting the electrochemical and biological properties. Thus food, pharmaceutical and chemical industries always take into account this characteristic.[25] The obtained grapefruit powder presented 1.5 ± 0.2 g water per 100 g powder. The low water content of a nutraceutical product leads to a longer shelf life, minimizing the microbial growth and chemical deterioration.[26] Also, it is more convenient to use and cheaper to transport owing to its reduced weight and volume.[23] The porosity is related to the free-flowing properties of the powder: the greater the ε value, the better the flowability.[27] The porosity of the obtained powder was 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 consumer acceptability. The grapefruit powder showed $L^* = 80.0 \pm 1.8$, $h_{ab}^* = 61.7 \pm 0.4$ and $C_{ab}^* = 11.4 \pm 0.6$. These color values fall within the range of those reported by González *et al.*[26] and Telis and Martínez-Navarrete[28] 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 essential but also, most importantly, the benefits for the consumer must be guaranteed. In the case of a nutraceutical, the biodisponibility of the bioactive compounds should be assured. As almost all food products are complex mixtures of vitamins, sugars, lipids, fibers and phytochemicals, the extraction of bioactive compounds from the food matrix is a matter of interest.[29] 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. In 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 FDOA and FDMW respectively.

Table 1 summarizes the TPC, TFC and antioxidant activity of the freeze-dried extracts. In general terms, there were significant differences ($P < 0.05$) in antioxidant capacity between the extracts.

No statistical differences ($P > 0.05$) were observed in the TPC of both freeze-dried extracts, while TFC was higher ($P < 0.05$) in FDOA. Haminiuk *et al.*[30] reported different methodologies of phenolic compound extraction, the values obtained in the present study being similar to those of strawberry, açaí and fig. Polyphenols have been reported to be responsible for the antioxidant activity of citrus fruits owing to their redox characteristics.[31] Therefore the high values of TPC and TFC of the grapefruit product provide high expectations in its role of chemopreventive properties as well as its antioxidant, anti-inflammatory and antimicrobial activity for human health. The greatest antioxidant capacity ($P < 0.05$) expressed as IC_{50} was obtained in FDOA, 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 mandarin, orange, grapefruit and lemon, among other citrus fruits.[32–34] These results indicated that the powdered product, despite being exposed to changes in its structure, still has similar values to different fresh products.[35,36] Although it is true that the main advantage of the method established for the quantification of antioxidant activity is its 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 radical production.[37] There is some controversy about the influence of the bioactive compounds present in fruits and vegetables on their antioxidant capacity.[38] 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.[39] 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).

A negative or positive Pearson correlation was observed between TPC or TFC and IC_{50} or FRAP respectively. These results indicate that phenolic compounds in general and flavonoids in particular were linked to high antioxidant capacity. The antioxidant properties conferred by these compounds are due to the phenolic hydroxyl groups attached to ring structures, as they can act as reducing agents such as hydrogen donators, singlet oxygen quenchers, superoxide radical scavengers and even metal chelators.[31]

Table 1. Total phenolic content (TPC), total flavonoid content (TFC), radical scavenging activity (IC_{50}) and antioxidant activity (FRAP) of extracts

Sample	IC_{50} (mg mL^{-1})	FRAP (mmol TEper 100 g d.b.)	TPC (mg GAEper 100 g d.b.)	TFC (mg QEper 100 g d.b.)
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*

TE, Trolox equivalent; d.b., dry basis; GAE, gallic acid equivalent; QE, quercetin equivalent; FDOA, freeze-dried oxalic acid extract; FDMW, freeze-dried methanol–water extract.
Values are expressed as mean ± standard deviation ($n = 9$). Different letters in the same column indicate significant differences between mean values ($P < 0.05$).

Table 2. Pearson correlation coefficients among TPC, TFC, IC_{50} and FRAP

Correlation	FDOA	FDMW
FRAP *vs* TPC	0.5262	0.5392
FRAP *vs* TFC	0.7449*	0.6739*
TPC *vs* IC_{50}	−0.8399*	−0.7352*
TFC *vs* IC_{50}	−0.4654	−0.8843*

*$P < 0.05$, indicating 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.

These results were expected, since grapefruit is mainly known for its richness in citric acid and ascorbic acid.[40] Both organic acids provide enzymatic browning and contribute to the antioxidant capacity. Nevertheless, grapefruit also presents high amounts of flavonoids, phytochemicals related to anti-inflammatory, antimicrobial and anticancer properties, as already reported by different authors.[2,41,42] Despite the potential health benefits of grapefruit due to its high content of phenols and flavonoids, it should be highlighted that narigin as well as other bioactive compounds present in grapefruit may cause important drug interactions, considering the inhibitory effects on cytochrome P450 3A4 enzyme already reported by different authors.[43,44]

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 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^{-1}). 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 FDMW presented the best result for Caco-2 (135%). However, in both extracts the cell viability was above 90%.

The MANOVA 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 (Fig. 1), it is possible to observe that the cell viability remains above 90% in all extracts from 0.1 to 100 μg mL^{-1}.

At high concentrations, the cell viability decreases, probably owing to the extract composition. Nevertheless, in the maximum concentration tested (1000 μg mL^{-1}), 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 solvents, significant differences ($P < 0.05$) were observed in Caco-2 and HT29-MTX cells. These results are in accordance with Laitinen *et al.*[45] who evaluated the extract 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^{-1}) and finding a minimum viability of 77% in all samples. Similar results were reported by Xu *et al.*[46] in a study focused on citrus juices, in which grapefruit juice did not affect the viability of Caco-2 cells. Equally, Chen and Kitts[47] reported good results in orange peel in Caco-2 cells using 7.5% (v/v) ethanol as extract solvent. In fact, FDOA and 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^{-1}) in both cell lines, since instead of causing damage to the cells, they keep them in good condition.[48,49] Nevertheless, a more detailed study has to be performed to support this protective effect.

3D intestinal permeability assay

Identification of bioactive compounds

Citrus fruits are rich in bioactive compounds, especially the Star Ruby variety.[50] Flavonoids are among these compounds, being characterized by a skeleton of 15 carbons, mostly linked to one or more sugar molecules. According to Theile *et al.*,[51] grapefruit presents high flavonoid glycoside contents, being characterized by the presence of rutinoside (such as hesperidin and narirutin) and neohesperidoside (namely naringin and neohesperidin) flavonoids. Figure 2 show the phenolic profile obtained for grapefruit in FDOA, indicating the presence of flavonoids, particularly between 20 and 35 min.

Table 3 summarizes the different phenolic compounds identified.

In the analysis of the initial sample, different compounds were identified depending on the wavelengths 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 (*m/z* 465, 303). However, taking into account its wavelength and retention time, it could also be a flavonone (hesperitin-7-*O*-glucoside). Compounds 2 and 3, owing to their closeness in molecular weights (*m/z* 595) and fragments (*m/z* 287) with differences in retention times (23.23 and 24.25 min) and wavelengths, could be flavonones of the 7-*O*-glucoside group. In this case, compound 2 would be neoeriocitin (eriodictyol-7-*O*-neohesperidiside) and compound 3 eriocitrin (eriodictyol-7-*O*-rutinoside). According to the literature, compound 4 is probably a hesperidin or a neohesperidin.[52–54]

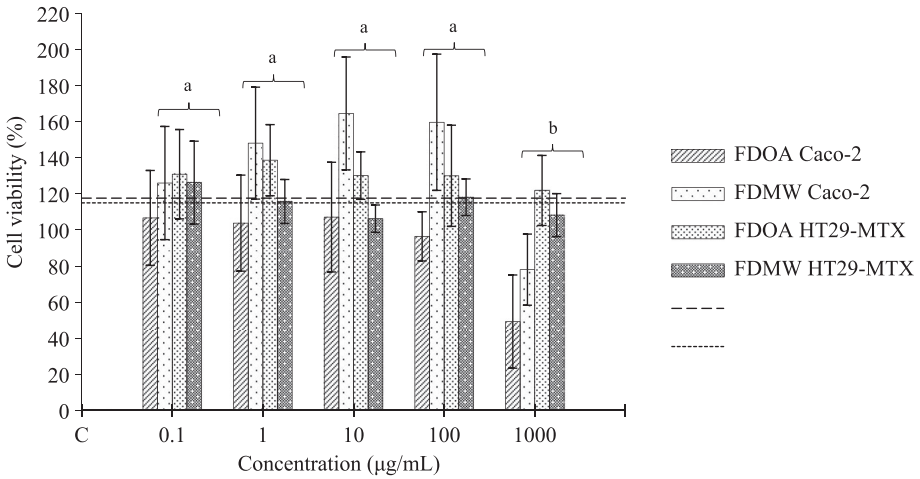

Figure 1. Cell viability in different concentrations of extracts in Caco-2 and HT29-MTX cell lines. Freeze-dried acid oxalic extract (FDOA), freeze-dried methanol–water extract (FDMW) and positive control. Different letters indicate significant differences in mean values between concentrations ($P < 0.05$).

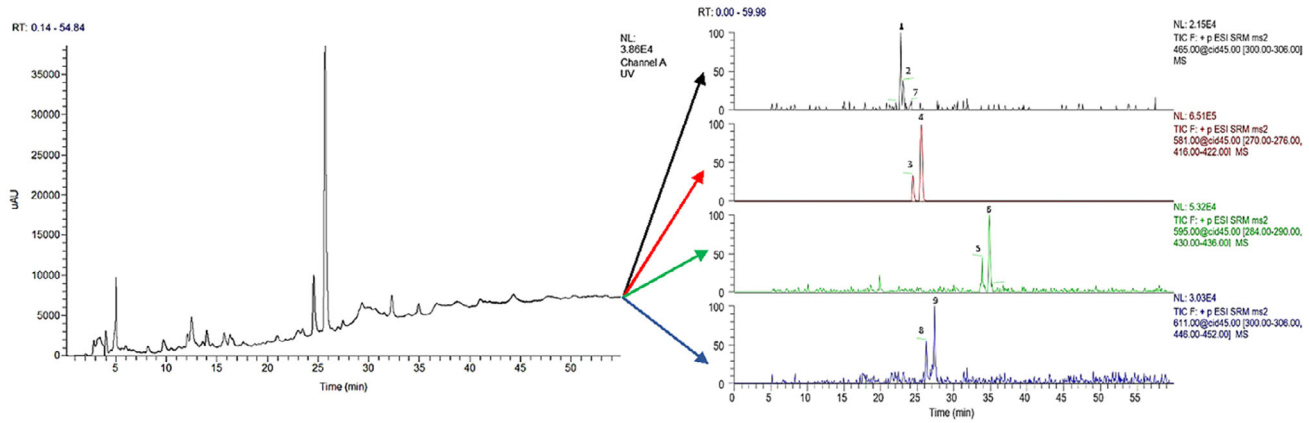

Figure 2. Example of chromatography analysis at different wavelengths: 1, delphinidin-3-glucoside or hesperitin-7-*O*-glucoside; 2, eriodictyol-7-*O*-neohesperidiside; 3, eriodictyol-7-*O*-rutinoside; 4, hesperidin or neohesperidin; 5 and 6, didymin, poncirin or saponarin; 7 and 8, naringin or narirutin; 9, hesperidin.

Compounds 5 and 6 present similar molecular weights and fragments (*m/z* 595, 433, 287) and are probably didymin, poncirin or saponarin, while compounds 7, 8 and 9 were identified by their molecular weights and respective fragments. In the case of compounds 7 and 8 (*m/z* 581, 419, 273), a naringin or a narirutin is probably present. Compound 9 could be more accurately a hesperidin (*m/z* 611, 449), being similar to compound 4 but differing in the fragments obtained.

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 cell cultures, being a non-invasive method that can be applied to monitor living cells during various stages of growth and differentiation.[13] Figure 3 shows the TEER measurements during the 21 days.

Table 3. Tentative identification of grapefruit nutraceutical powder main compounds. Retention time (RT), molecular ion with negative charge [MS] (*m/z*), fragments of ions [MS^2] [MS^3] (*m/z*), wavelength at maximum visible absorption (λ_{max})

Compound	RT (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)	1,2,4–6,9,18
4	26.22	611	449	303	280, 320	Hesperidin/neohesperidin	7–11,14,15,17,20
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	11,15–17,19,20
9	20–35	611	449		280, 320	Hesperidin	15,17,20

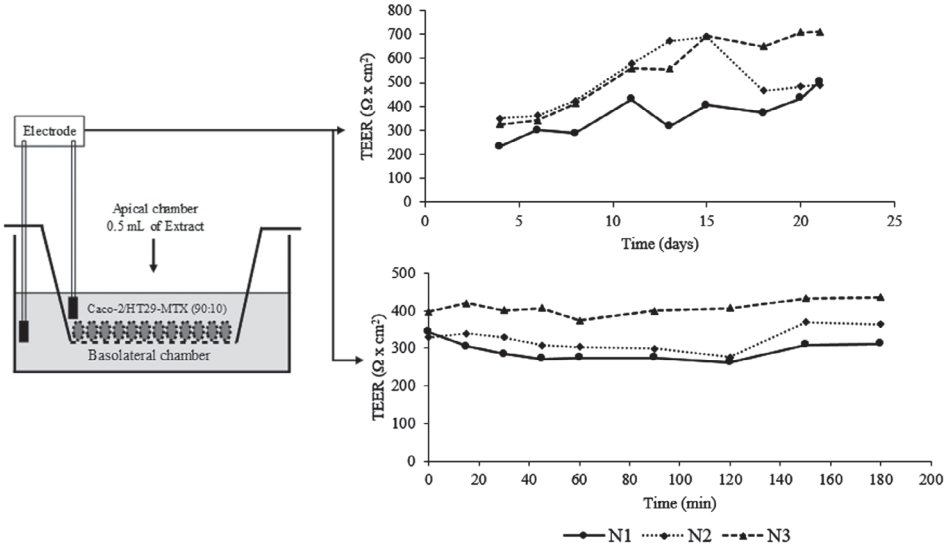

Figure 3. Transepithelial electrical resistance (TEER) measurements of co-culture cells (90% Caco-2 and 10% HT29-MTX) during 21 days and 180 min of permeability assay: N1, N2 and N3, number of repetitions made.

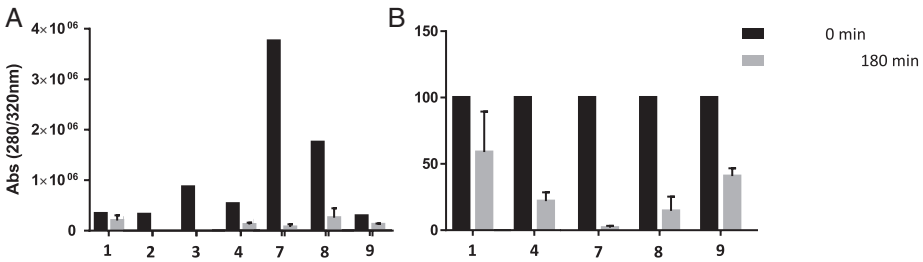

Figure 4. (A) Work wavelengths and (B) permeability of bioactive compounds at 0 min and after 180 min: 1, delphinidin-3-glucoside or hesperitin-7-*O*-glucoside; 2, eriodictyol-7-*O*-neohesperidiside; 3, eriodictyol-7-*O*-rutinoside; 4, hesperidin or neohesperidin; 7 and 8, naringin or narirutin; 9, hesperidin.

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.*[13]

Once the 180 min of permeation in the co-culture was finished, different results were obtained regarding permeability (Fig. 4). Figure 4 shows the two working wavelengths and the compounds that were detected initially at time zero of the experiment, 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 0 min) and the basolateral chamber (time 180 min).

Regarding all bioactive compounds identified, only didymin, poncirin or saponarin (compounds 5 and 6) were not detected. Nevertheless, a high permeation was achieved for compound 1 (delphinidin-3-glucoside or hesperitin-7-*O*-glucoside) and compound 9 (hesperidin), with compound 1 presenting a permeation higher than 50%, followed by hesperidin which 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.*[55] found similar permeation results 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 and even the TEER variability. 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.[56] Oxidative stress may be the main cause of the transference of bioactive compounds, since this mechanism is activated during cell permeation.[57] The compounds that were not transported in their entirety were probably retained within the cell model. This phenomenon 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.*[58]

According to the obtained results, compounds 1, 4 and 9 were easily transported. This may be due to the encapsulation process carried out by means of spray drying. Compounds 1 and 9 are the bioactive compounds with the best results. Delphinidin-3-glucoside (compound 1) has been extensively studied as a suppressive element in cancer cells.[59] 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 and anticancer.[60]

The nutraceutical product derived from the grapefruit and obtained by means of the spray drying process has 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 tests 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.

CONCLUSION

In the last decade, functional and nutraceutical foods have obtained a great demand among consumers owing 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 its intestinal permeability. The obtained results support good stability regarding 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 viability that this product offers to encapsulate the most important *C. paradisi* bioactive compounds such as delphinidin-3-glucoside, hesperitin-7-*O*-glucoside, hesperidin and neohesperidin, which showed a permeation up to 50% in the 3D intestinal model. Thus the spray drying technique can be classified as a good alternative in the food industry for these products as well as the biopolymers employed in this process.

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