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Natural deep eutectic solvent (NADES): A strategy to improve the bioavailability of blueberry phenolic compounds in a ready-to-use extract

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ABSTRACT

This study investigated whether a ready-to-use extract obtained using a natural deep eutectic solvent (NADES) affects the pharmacokinetic profile of blueberry phenolic compounds compared to organic solvent (SORG)-extracted compounds. SORG extract was administered as an aqueous solution after solvent removal. Wistar rats received a single dose of crude extract of blueberry obtained using NADES (CE-NADES) or SORG (CE-SORG), followed by LC-DAD-MS/MS analysis of blood and cecal feces. Non-compartmental pharmacokinetic analysis revealed that CE-NADES increased the bioavailability of anthocyanins by 140% compared to CE-SORG. CE-NADES increased the stability of phenolic compounds during *in vitro* digestion by delaying gastric chyme neutralization. These results suggest that besides being an eco-friendly solvent for the extraction of phytochemicals, choline chloride:glycerol:citric acid-based NADES can be used as a ready-to-use vehicle for increasing oral absorption of bioactive compounds such as anthocyanins.

1. Introduction

Natural deep eutectic solvents (NADES) represent a new green strategy to overcome the toxicity and environmental disadvantages of conventional organic solvents, such as methanol, acetone and formic acid, for the extraction of plant phytochemicals (Misan et al., 2020). NADES are composed of a mixture of a hydrogen receptor (usually choline chloride, ChCl) and a natural hydrogen donor such as amino acids, carboxylic acids and sugars, among others (Paiva, Craveiro, Aroso, Martins, Reis, & Duarte, 2014) and exhibit high extraction efficiency for various natural compounds (Misan et al., 2020).

We have recently demonstrated that a choline chloride:glycerol:citric acid (0.5:2:0.5, molar ratio) NADES is as efficient as the conventional organic solvent for the extraction of anthocyanins from blueberries (Silva, Pauletto et al., 2020a). Moreover, the blueberry extract obtained using this NADES mixture exhibited a protective index of 94% against the formation of ethanol-induced gastric ulcers in rats (Silva, Rodrigues et al., 2020b). No solvent was removed before using the extract and no toxicity was observed for the NADES *per se*, demonstrating that this NADES mixture can be used to obtain ready-to-use extracts (Silva, Rodrigues et al., 2020b). Thus, NADES have become an attractive alternative to increase the nutraceutical products field, because they are composed of food-grade ingredients that apparently would not need to be removed before use (Dai et al., 2020; Panić et al., 2019; Silva, Rodrigues et al., 2020b).

Blueberry is particularly rich in phenolic compounds, such as anthocyanins, hydroxycinnamic acid derivatives and flavonols (Zhong, Sandhu, Edirisinghe, & Burton-Freeman, 2017). The phenolic compounds of blueberry, including anthocyanins, modulate various cellular processes and have been implicated in the inhibition of cancer cell proliferation and prevention of obesity, type 2 diabetes, inflammation and cardiovascular disease (Fang, 2014).

The systemic biological activity of most phenolic compounds is limited by their low bioavailability, which is around 0.2% for

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chlorogenic acids and ranges from 0.7% to 1.1% for anthocyanins and most hydroxycinnamic acids from blueberry (Rodriguez-Mateos et al., 2016; Zhong et al., 2017). In general, phenolic compounds are absorbed throughout the gastrointestinal tract, especially in the stomach, small intestine and colon (Teng & Chen, 2019). The portion of phenolic compounds that is not absorbed up to the small intestine can be extensively metabolized by the colonic microbiota into a series of simple phenolic acids that can reach the circulation and are likely implicated in many health benefits (Augusti et al., 2021).

Interestingly, the use of NADES as a vehicle has been demonstrated to increase the bioavailability of rutin, calycosin-7-O-β-D-glucoside, puerarin isoflavones, hydroxysafflor yellow A and anhydrosafflor yellow B in rodents (Dai et al., 2020; Faggian et al., 2016; Huang et al., 2021; Tong et al., 2021). Increased phenolic solubility has been suggested to be responsible for this effect that resulted in peak phenolic plasma levels that were 2–4 times greater when NADES vehicles were used compared to aqueous suspensions (Dai et al., 2020; Faggian et al., 2016; Tong et al., 2021). However, research concerning the effect of NADES on the bioavailability of plant phenolics *in vivo* is scarce. Only compounds belonging to the flavonol (rutin; Faggian et al., 2016), isoflavonol (calycosin-7-O-β-D-glucoside; Dai et al., 2020; and puerarin isoflavones; Huang et al., 2021) and quinochalcone C-glycosides (hydroxysafflor yellow A and anhydrosafflor yellow B; Tong et al., 2021) classes have been investigated. The expansion of bioavailability studies to other classes of phenolic compounds from phytochemical-rich plant sources such as blueberry will boost strategies for maximizing the potential health benefits of these phytochemicals by means of NADES.

We hypothesized that a food-grade NADES yields a ready-to-use blueberry extract exhibiting higher bioavailability of phenolic compounds than organic solvent-extracted phenolics. Thus, the objective of this study was to investigate the bioavailability of blueberry phenolic compounds extracted with NADES or conventional organic solvent, elucidating the differences in the pharmacokinetic profile and in the bioaccessibility between these different extracts.

2. Materials and methods

2.1. Chemicals, materials and fruit samples

Choline chloride (ChCl; 98%) and trifluoroacetic acid (TFA; 99%) were acquired from Sigma-Aldrich (St. Louis, MO, USA), glycerol was from Belga (Parana, Brazil) and citric acid was from Vetec (Rio de Janeiro, Brazil). Methanol, acetonitrile and formic acid (88%) were from J.T. Baker® (Center Valley, PA, USA). Pepsin, pancreatin and bile salts were acquired from Sigma-Aldrich (P7000, P7545 and C1254; St Louis, MO, USA).

Liquid chromatography (LC)-grade water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). Polytetrafluoroethylene syringe filter membrane (PTFE) and C18 cartridges for reversed-phase solid phase extraction (SPE; Sep-Pak® Vac 6 cc, 500 mg/6 mL) were from Waters (Milford, MA, USA). Analytical standards gallic acid (98%), quercetin (95%), ferulic acid (99%), vanillic acid (97%) and cyanidin 3-glucoside (95%) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Blueberry fruits (*Vaccinium corymbosum*) of the cultivar O'Neal were collected in an orchard belonging to the Brazilian Agricultural Research Corporation (EMBRAPA-Clima Temperado) located in Pelotas city (Rio Grande do Sul State) in November 2018. The fruits were lyophilized and ground in a mill in liquid nitrogen until obtaining a visually homogeneous powder; samples obtained were stored at −20 °C.

2.2. Determination of blueberry powder particle size

The particle size of blueberry powder samples obtained after lyophilization and grounding was evaluated using a laser diffraction particle size analyzer, equipped with a sonication control unit (model LS 13 230, Beckman Coulter, California, USA). Triplicate samples were added to the equipment until optimal obscuration was achieved in the software. Thereafter, the powders were dispersed in an aqueous medium and submitted to an ultrasound treatment for 1 min inside the equipment before analysis.

2.3. Preparation of NADES and blueberry extracts

The composition of NADES was chosen based on a previous study that investigated the best conditions for the extraction of blueberry anthocyanins (Silva, Pauletto et al., 2020a). NADES was prepared by mixing choline chloride, glycerol, and citric acid at a molar ratio of 0.5:2:0.5, plus 25% water according to Silva, Pauletto et al. (2020a). This NADES was previously characterized by our research group (Silva, Pauletto et al., 2020a) using Fourier-transformed infrared spectroscopy (FRI) and differential scanning calorimetry (DSC) analysis, which showed changes in the characteristic bands of the initial reagents and the presence of hydrogen bonds confirming the formation of deep eutectic properties after the preparation of this NADES mixture.

To obtain the crude extract using NADES (CE-NADES), 8 g of powdered blueberry samples (average particle size: 715.5 ± 12.3 μm) was extracted with 40 mL (8:40, w/v) of NADES, as previously described by Silva, Pauletto et al. (2020a). After solvent addition, samples were submitted to an ultrasonic bath (40 kHz, 50 min at room temperature) and then centrifuged at 2100 × *g* for 15 min. Sample temperature did not exceed 33 °C during this ultrasound-assisted extraction, which along with the low pH of the extraction solvent contributed to preserve phenolic compounds during extraction. To obtain enough extract for the whole animal experiment, this extractive procedure was repeated until approximately 1.5 L of crude blueberry extract was obtained, with a total of 0.3 kg of blueberry powder used. The supernatants were collected, combined and adjusted with a small volume of NADES to result in a solution containing exactly 1.10 mg of total phenolic compounds/mL of CE-NADES.

The crude extract obtained using organic solvent (CE-SORG) was extracted according to Pertuzatti et al. (2016) with the following modifications. Blueberry powder (8 g; average particle size: 715.5 ± 12.3 μm) was mixed with 20 mL of a methanol:water:formic acid mixture (50:48.5:1.5; v/v/v). Thereafter, the sample was subjected to an ultrasonic bath (40 kHz, 2 min) and centrifuged for 10 min at 1200×*g*. The supernatant was separated, whereas the residue was re-extracted once. The supernatants obtained were combined, totaling approximately 40 mL (8:40, w/v). This extraction procedure has been demonstrated to recover 99% of anthocyanins and 98% of non-anthocyanin compounds from blueberries (Pertuzatti et al., 2016; Pertuzatti, Barcia, Gómez-Alonso, Godoy, & Hermosín-Gutiérrez, 2021). In addition, this extractive procedure with organic solvent yielded nearly 99% of the phenolic amount obtained in the NADES extraction. The extractive procedure using organic solvent was repeated to extract up to 0.3 kg of blueberry powder yielding approximately 1.5 L of crude blueberry extract. Solvents were evaporated using a rotary evaporator (Bûchi-R3^{TM}, Flawil, Switzerland) at 38 ± 2 °C and the dry content was dissolved in approximately 1.5 L of distilled water to result in a solution containing exactly 1.10 mg of total phenolic compounds/mL of CE-SORG. The pH of this aqueous solution was < 3.0, which contributed to maintain anthocyanin stability. CE-NADES and CE-SORG extracts were stored at −20 °C until the end of the experiments.

2.4. Animals and pharmacokinetic study

Sixty-four adult male Wistar rats (200–250 g) were provided by the Central Animal House of Federal University of Santa Maria (UFSM, RS). All procedures adopted were approved by the Committee for Care and Use of Experimental Animal Resources/UFSM (protocol number 3253100219/2019). Animals were housed in standard polypropylene cages (four rats/cage) and kept under controlled temperature (22 ±

2 °C) and humidity (55 ± 5%) and a 12/12 h light/dark cycle with access to food and water *ad libitum*. Rats were allowed free access to water and food throughout the experimental period and the crude blueberry extract was administered in the morning. No fasting was conducted before or after extract administration. Animals were randomly divided into experimental groups as follows:

(I) Crude blueberry extract obtained using NADES (CE-NADES): received 9.1 mL of CE-NADES/kg body weight (b.w.) by gavage, amounting to 10.0 mg of total phenolic compounds/kg b.w.

(II) Crude blueberry extract obtained using organic solvent (CE-SORG): received 9.1 mL of CE-SORG/kg b.w. by gavage, amounting to 10.0 mg of total phenolic compounds/kg b.w.

This dose was chosen based on a previous study from our research group where CE-NADES at the dose of 10 mg of total phenolic compounds/kg b.w. was able to modulate the production of short-chain fatty acids by the rat gut microbiota (Silva, Rodrigues et al., 2020b). Animals were anesthetized with isoflurane and euthanized by exsanguination at 15, 30, 60, 120, 240, 360 or 720 min after CE administration (n = 4/per time and n = 28/per treatment). Intracardiac blood and cecal feces were collected from each animal. Cecal feces were stored in liquid nitrogen until analysis, whereas blood was immediately processed to obtain plasma samples. In order to obtain baseline values (t0), eight control rats were fed 2 mL of distilled water/kg b.w. by gavage and samples were similarly collected.

2.5. In vitro simulation of gastrointestinal digestion by INFOGEST method

The harmonized static *in vitro* digestion model proposed by INFOGEST was used to investigate the stability and bioaccessibility of phenolic compounds from CE-NADES and CE-SORG extracts during digestion. CE-NADES and CE-SORG were the same extracts used in the *in vivo* experiment, which means that they had the same phenolic concentration. CE-NADES was the NADES-containing extract, whereas CE-SORG was an aqueous solution (pH < 3.0). Human digestion was simulated by sequential static steps according to the guidelines proposed by Brodkorb et al. (2019), except that gastric lipase was omitted as the extracts had no lipids. To simulate the digestive process after gavage (similar to the *in vivo* assay) the oral phase was omitted. The gastric phase was simulated by incubating extracts (5 mL) with simulated gastric fluid (pH 3.0) and 2000 U/mL pepsin at 37 °C for 2 h under shaking (final volume 10 mL). No HCl was required to adjust gastric pH to 3.0. After gastric digestion, the simulated intestinal fluid was added to the gastric chyme and the initial intestinal pH was measured and adjusted to 7.0 using 5 M NaOH at varying amounts according to the samples. Thereafter, intestinal digestion was simulated by adding bile salts (10 mM) and pancreatin (100 U/mL) and incubating at 37 °C for 2 h under shaking (final volume 28 mL) (Brodkorb et al., 2019). At the end of incubation, the intestinal digesta was centrifuged at 1500×*g* for 10 min and two fractions were obtained: the supernatant, which represents the soluble/bioaccessible fraction of the digesta, and the sediment, which represents the insoluble/non-bioaccessible fraction.

Aliquots of 2 g were withdrawn at the end of the gastric phase and from each intestinal fraction and were snap frozen in liquid nitrogen and stored at − 80 °C until chromatographic analysis. After collection of the gastric phase sample, the amount of incubation medium remaining was assessed by weighing and taken into account to correct the amount of salts and enzymes to be added in the intestinal phase to maintain the protocol described by Brodkorb et al. (2019).

The bioaccessibility index of phenolic compounds was calculated as the amount of compounds recovered in the bioaccessible fraction after intestinal digestion (B fraction) relative to the amount of compounds in the undigested extract and was calculated using the following equation (1):

$$\text{Bioaccessibility}(\%) = (\text{B fraction}/\text{undigested extract}) \times 100$$

2.6. Extraction of phenolic compounds and their metabolites from plasma, cecal feces and gastrointestinal digesta

Fresh blood samples were collected in heparinized tubes and immediately centrifuged at 3000×*g* for 10 min. Plasma samples were extracted as described by He et al. (2006) and summarized below. Samples were mixed with an aqueous solution of 0.44 mol/L TFA (1:0.2, v/v) for acidification of this fluid and precipitation of proteins. Then, samples were centrifuged for 10 min at 3000×*g* and the supernatant was collected and stored in liquid nitrogen before SPE purification.

The sediment representing the non-bioaccessible fraction obtained after *in vitro* digestion was extracted with methanol:water:formic acid (85:15:0.5, v/v/v) as described by Quatrin et al. (2020), the solvent was evaporated and samples were purified by SPE as described below.

Deproteinized plasma samples, undigested extracts (CE-NADES and CE-SORG) and aliquots from gastric and intestinal phases (bioaccessible and non-bioaccessible fractions) were purified by C18 SPE according to He et al. (2006) with the following modifications. Samples of plasma (1.5 mL, after deproteinization), undigested extracts (0.2 mL) and gastric (0.2 mL) and intestinal phase fractions (0.8 mL) were applied to Sep-Pak® C18 cartridges (Waters, Massachusetts, USA) previously activated with methanol (6 mL) and preconditioned with 1% (v/v) TFA aqueous solution (12 mL). After sample loading, the cartridges were successively washed with 12 mL of 1% TFA aqueous solution to remove polar interfering compounds. Samples were eluted with two volumes of ethyl acetate to release the less polar phenolic compounds (hydroxybenzoate derivatives, flavonols, hydroxycinnamate derivatives and metabolites) and thereafter with methanol acidified with 1% (v/v) TFA to release anthocyanins. Solvents were evaporated using a rotary evaporator (Bûchi-R3^{TM}, Flawil, Switzerland) at 38 ± 2 °C and samples were adjusted to a known volume using acidified water (0.35% formic acid, v/v) for the anthocyanin fraction and 10% methanol in acidified water (0.1% formic acid; v/v) for the non-anthocyanin phenolic compound fraction. Before LC analysis, purified extracts were filtered through a 0.22 μm PTFE syringe filter (Millipore®, São Paulo, Brazil).

Anthocyanins and non-anthocyanin phenolic compounds were extracted from cecal feces according to He, Magnuson, and Giusti (2005) with the modifications described below. Feces samples (0.5 g) were extracted with 10 mL of a mixture containing methanol, water and TFA (60:40:1, v/v/v). Samples were subjected to an ultrasonic bath (40 kHz, 3 min) and centrifuged for 10 min at 2500×*g*. The supernatant was collected, and the pellet was re-extracted twice with 10 mL of extraction solvent following the same procedure described above. Supernatants were combined and evaporated in a rotary evaporator at 38 ± 2 °C. After evaporation, the solution was diluted with 4 mL of acidified water (0.1% formic acid, v/v) and then 2 mL was applied to an SPE cartridges for purification as described for deproteinized plasma samples.

2.7. Identification and quantification of polyphenols and their metabolites by LC-ESI-MS/MS and LC/DAD/UV–vis

The identification of anthocyanins, phenolic compounds, and their metabolites was performed in a high-performance LC connected in series to a diode array detector (DAD; SPD-M20A) and a mass spectrometer (MS) with a quadrupole-time-of-fly (QTOF) analyzer and an electrospray ionization (ESI) source (model micrOTOF-Q II, Bruker Daltonics, Bremen, Germany). The MS parameters and tentative identification were performed as described by Silva, Pauletto et al. (2020a) and Quatrin et al. (2019). The tentative identification was based on their MS/MS and DAD/UV/vis spectral data and their elution order compared to the available standards and literature data.

Anthocyanins were quantified using an LC (Shimadzu, Columbia, MD, USA) with a reverse-phase Symmetry C-18 column (2.6 μm particle size, 100 mm, 4.6 mm; Phenomenex, Torrance, CA, USA) and coupled to an UV–visible detector (SPD-20AV, Shimadzu, Columbia, MD, USA) in the analytical conditions validated by Silva, Pauletto et al. (2020a).

Anthocyanins in CE-NADES, CE-SORG, undigested extracts, gastric and intestinal phase fractions, plasma and cecal feces were quantified at 520 nm as equivalents of cyanidin 3-glucoside.

Due to the complex structure of non-anthocyanin phenolics and their metabolites, the quantification of these compounds was done using an LC (CBM-20A Prominence, Shimadzu, Kyoto, Japan) equipped with a degasser (DGU20A5 prominence, Shimadzu, Japan) and column oven (CTO-20A Prominence, Shimadzu, Japan) and coupled to a DAD (SPDM-20A Prominence, Shimadzu, Japan). Separation was performed in a reverse-phase C-18 Hypersil Gold column (5 μm particle size, 150 mm, 4.6 mm; Thermo Fisher Scientific, MA, USA) at 38 °C, according to the method previously validated by Quatrin et al. (2019) in our laboratory. In CE-NADES, CE-SORG, undigested extracts, and gastric and intestinal phase fractions, non-anthocyanin phenolic compounds such as hydroxybenzoate, hydroxycinnamate and flavan-3-ol derivatives were quantified at 280 nm as equivalents of catechin, whereas flavonol derivatives were quantified at 360 nm as equivalents of quercetin. In plasma and cecal feces, phenolic compounds such as hydroxybenzoic acid derivatives were quantified at 280 nm as equivalents of their respective parent phenolic acid standard (vanillic acid or gallic acid). Valerolactone compounds and hippuric acid in plasma and cecal samples were quantified at 280 nm as equivalents of gallic acid. Hydroxycinnamic acid derivatives were quantified at 320 nm as equivalents of their respective parent phenolic acid standard (p-coumaric acid or ferulic acid).

2.8. Accuracy and precision

The precision and accuracy assays followed the Brazilian Health Regulatory Agency Guidelines (ANVISA Agência Nacional de Vigilância Sanitária, 2017). Accuracy was assessed as the recovery after sample spiking with phenolic standards from two different classes. Vanillic acid was used to assess the recovery of hydroxybenzoic acid derivatives, whereas ferulic acid was used to assess the recovery of hydroxycinnamic acid derivatives. In this recovery test, samples of plasma and feces were fortified with the standards at a low, medium or high level, which corresponded to 80%, 100% and 120% of the amount of phenolic compounds found in samples. In addition, recovery was also determined by spiking plasma and fecal samples with an amount of CE-NADES to result in a known concentration of anthocyanins (1561.0 μg/mL for plasma and 791.3 μg/g for cecal feces) and of non-anthocyanin compounds (1516.8 μg/mL for plasma and 743.6 μg/g for cecal feces). The sum of peak areas for anthocyanin or non-anthocyanin compounds in spiked plasma and feces samples was compared with CE-NADES, after subtracting the area of blank plasma and feces samples. Recovery was assessed for triplicate determinations at each concentration level and was calculated as (total amount recovered/total amount added) × 100%; and showed acceptable values for the methods used (Supplementary Tables 1S and 2S).

The precision of the method was determined as the repeatability (intra-day) and intermediate precision (inter-day) at the same conditions used in the recovery assays. The precision of the method was calculated by measuring the areas of the peaks of vanillic and ferulic acids obtained from triplicate determinations of a sample fortified at three different concentration levels (low, medium or high) on the same day (repeatability) and in different days (intermediate precision) and was represented as the relative standard deviation (RSD%) from peak area values of phenolic compounds (Supplementary Tables 1S and 3S).

2.9. Statistical analysis

Pharmacokinetic parameters such as the maximum plasma concentration (C_{max}), time at which maximum plasma concentration is achieved (T_{max}), area under the plasma concentration–time curve ($AUC_{0\text{-}720min}$), half-life ($t1/2$), mean residence time (MRT), lambda_z and clearance (CL) were calculated by non-compartmental analysis using PKSolver software. Fractional bioavailability was calculated for anthocyanins based on parent compounds maintaining the parent C6-C3-C6 flavonoid structure in blood relative to the amount ingested through the extracts. The results were statistically analyzed using Statistica V.7 software (Statsoft Inc., Tulsa, OK, USA). Data were expressed as the mean ± standard error (SEM). Differences between means were analyzed by Student's t test and a significant difference was considered at the level of $p \leq 0.05$. Pearson's correlation was run to investigate the association between the initial intestinal pH or the amount of NaOH to adjust intestinal pH versus the content of phenolic compounds after intestinal digestion of CE-NADES and CE-SORG extracts.

3. Results

3.1. Composition of crude extracts CE-NADES and CE-SORG

Blueberry extracts obtained with NADES or organic solvent were adjusted to the same concentration of total polyphenols that amounted to 1.10 mg/mL of extract. The anthocyanin-rich fraction had 0.32 mg of cyanidin-3-glucoside equivalents/mL of extract, whereas the non-anthocyanin phenolic fraction had 0.31 mg of catechin equivalents (hydroxybenzoate, hydroxycinnamate and flavan-3-ol derivatives)/mL and 0.47 mg of quercetin equivalents (flavonol derivatives)/mL of extract.

The phenolic profiles of CE-NADES and CE-SORG extracts were elucidated by LC-ESI-MS/MS and LC/DAD/UV–vis (Supplementary Fig. 1S and Table 4S). The tentative identifications based on the characteristic DAD and MS/MS spectral data and the percentage of individual phenolic compounds based on UV/vis peak areas are presented in Supplementary Table 4S. The phenolic profile was similar between CE-NADES and CE-SORG extracts. Concerning the anthocyanin profile, for both extracts delphinidin-3-hexoside and malvidin-3-hexoside/delphinidin-3-acetylhexoside were the most abundant anthocyanins followed by cyanidin-3-galactoside, petunidin-3-hexoside, malvidin-3-pentoside, delphinidin-3-pentoside, petunidin-3-pentoside, malvidin 3-acetylhexoside, peonidin-3-pentoside, whereas other minor anthocyanins were found at concentrations lower than 1% of the total anthocyanin content. Among non-anthocyanin phenolic compounds, the chlorogenic acid isomer 3-caffeoylquinic acid was the major one followed by quercetin-3-galactoside, quercetin-3-glucoside, myricetin-3-hexoside, quercetin-3-pentoside, 5-caffeoylquinic acid (chlorogenic acid isomer), quercetin 3-(6″-acetyl)hexoside, catechin, laricitrin-3-hexoside, caffeoyl-*O*-hexoside, whereas other minor compounds were found at concentrations lower than 1% of the total non-anthocyanin phenolic compounds. It is noteworthy that the coefficient of variation for the relative amount of major phenolic compounds between CE-NADES and CE-SORG was lower than 10% of respective mean, indicating no relevant differences between the two extracts (Supplementary Table 4S). The only exception was the amount of peonidin-3-pentoside that had a 12.7% coefficient of variation between the two extracts, being slightly more abundant in the CE-NADES extract (Supplementary Table 4S).

3.2. Identification and chromatographic profile of polyphenols and its metabolites in plasma and cecal feces after CE-NADES or CE-SORG administration

After the administration of a single oral dose of CE-NADES or CE-SORG to rats, a total of 28 possible phenolic metabolites were identified, 21 of which were detected in plasma and 19 in cecal feces samples (Table 1). The identification was performed at the time of highest concentration in plasma (120 min for CE-NADES and 15 min for CE-SORG) and cecal feces samples (360 min for both extracts). The chromatographic profile of plasma and cecal feces is shown in Supplementary (Fig. 2S–5S).

Nine anthocyanins were identified in plasma samples in their intact

Table 1
Anthocyanins, phenolic acids and other metabolites identified in rat plasma and feces after administration of crude extracts of blueberry (CE-NADES or CE-SORG).

Compounds	Sample	Rt (min)[a]	λmax (nm)[b]	Experimental MS[c]	Experimental fragments	Identification
Anthocyanins						
Delphinidin 3-hexoside	P, F	3.6	220; 521	465.0862	303.0410	MS/MS PUB
Delphinidin 3-pentoside	P, F	5.4	220; 520	435.0781	303.0500	MS/MS PUB
Cyanidin 3-galactoside[d]	P	5.8	220; 510	449.0946	287.0458	MS/MS PUB
Petunidin 3-hexoside	F	7.5	220; 521	479.1021	317.0561	MS/MS PUB
NI	P	8.0	221; 506	–	–	–
NI*	F	9.5	n.d	–	–	–
NI*	F	10.3	n.d	–	–	–
NI*	F	10.5	n.d	–	–	–
Petunidin 3-pentoside	P, F	11.8	202; 522	449.0948	317.0552	MS/MS PUB
Malvidin 3-hexoside	P	13.3	220; 526	493.1186	331.0707	MS/MS PUB
Peonidin 3-pentoside	P	13.7	220; 521	433.0983	301.0597	MS/MS PUB
Malvidin 3-pentoside	P	15.1	220; 528	463.1101	331.0714	MS/MS PUB
Petunidin 3-acetylhexoside	P	16.3	220; 527	521.1142	317.0557	MS/MS PUB
Malvidin 3-acetylhexoside	P	17.4	220; 528	535.1293	331.0719	MS/MS PUB
Hydroxybenzoic acid derivatives						
Gallic acid	P, F	3.8	207; 270	169.0124	125.0225	STD
Protocatechuic acid	P, F	6.9	259; 293	153.0221	109.0315; 108.0233	STD
NI[e]	P, F	10.9	214; 277	181.0518	135.0415	–
Vanillic acid	P, F	14.5	259; 292	167.0335	152.0134; 108.0176	STD
Hippuric acids						
Hippuric acid	P, F	13.6	195; 247	178.0519	134.0619	MS/MS PUB
Hydroxycinnamic acid derivatives						
NI[e]	F	16.6	201; 307	207.0675	102.9992	–
Dihydrocoumaric acid	P, F	25.9	217; 288	165.0563	119.0472; 121.0560	MS/MS PUB
Dihydroferulic acid	F	31.4	222; 276	195.0673	136.0503	MS/MS PUB
Ferulic acid	P, F	34.4	264; 321	193.0500	134.1061	STD
NI	F	37.6	n.d	243.1251	163.1116; 119.0507	–
Phenylpropionic acids						
3-(3-Hydroxyphenyl) propanoic acid	P, F	30.4	216; 274	165.0557	121.0520	MS/MS PUB
Amino acids						
Tyrosine[e]	P	3.3	195; 274	180.0678	163.0444; 147.0449; 112.9875	MS/MS PUB
Phenylalanine	P, F	5.9	193; 276	164.0728	147.0429; 119.0407	MS/MS PUB
Tryptophan	P, F	9.7	216; 278	203.0840	116.0518; 142.0655; 159.0957	MS/MS PUB
N-Phenylacetylglycine[e]	P	17.5	194	192.0203	121.0299	MS/MS PUB
Phenylacetic acids						
3́-Hydroxyphenylacetic acid	F	14.2	213; 275	151.0501	107.0440	MS/MS PUB
Valerolactones						
5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone	F	15.3	216; 289	206.0462	162.0551; 147.0322; 118.0287	MS/MS PUB
Carboxylic acids						
Citric acid[e]	P	2.9	194; 208	191.0223	111.0112	MS/MS PUB
Indole-2- carboxylic acid	F	11.5	201; 256; 336	160.0416	159.0331; 131.0378	MS/MS PUB
5-Hydroxyindole-3-acetate	F	24.3	n.d	190.0515	146.0640	MS/MS PUB
NI	P	36.6	n.d	204.0683	–	–
Azelaic acid	F	52.4	n.d	187.0980	125.0967	MS/MS PUB
Others organic compounds						
NI	P	27.1	n.d	283.0851	–	–

[a] Retention time on the C_{18} Phenomenex (2.6 μm; 100 × 4.6 mm) column for anthocyanins (elution gradient of 3% formic acid in water and pure acetonitrile) and on the C_{18} Thermo Fisher Scientific (5 μm; 150 × 4.6 mm) column for non-anthocyanin compounds (elution gradient of 5% methanol in acidified water (0.1% formic acid) and acetonitrile with 0.1% formic acid). [b] Wavelength of maximum UV–vis absorption. [c] Experimental MS corresponds to *m/z* $[M]^+$ for anthocyanins and *m/z* $[M-H]^-$ for non-anthocyanin compounds. [d] Galactoside sugar identified by comparison with the retention time of cyanidin-3-glucoside standard. [e] Compounds that were found in t0 (baseline) samples and did not undergo concentration changes after CE-NADES or CE-SORG administration. CE-NADES: crude extract of blueberry obtained using NADES. CE-SORG: crude extract of blueberry obtained using organic solvent. P: plasma. F: cecal feces. STD: identification by comparing mass spectra and retention time to available standard. MS/MS PUB: tentative identification by prediction of chemical formula from exact mass and confirmation by comparing fragmentation mass spectra and UV–vis spectra with literature, in addition to the observed elution order relative to the available standards. NI: not identified compound. NI*: not identified compound that was formed during the metabolism of phenolics because it did not appear at t0. nd: not detected.

form after the administration of crude blueberry extract: delphinidin-3-hexoside, malvidin-3-hexoside, cyanidin-3-galactoside, malvidin-3-pentoside, delphinidin-3-pentoside, malvidin 3-acetylhexoside, peonidin-3-pentoside, petunidin-3-pentoside and petunidin-3-acetylhexoside (Table 1). No anthocyanins were found in plasma samples at the baseline time (t0) (Supplementary Fig. 2S, panel A). Among metabolites identified in the plasma samples at the baseline time and after the administration of blueberry extracts we found amino acids (tyrosine, phenylalanine, tryptophan and *N*-phenylacetylglycine), phenolic acids resulting from the degradation of anthocyanins (gallic acid, vanillic acid and protocatechuic acid), compounds that are likely bacterial metabolites or products of intestinal esterases (dihydrocoumaric acid, ferulic acid, (3-(3-hydroxyphenyl) propanoic acid and hippuric acid) and organic acids (citric acid) (Table 1 and Supplementary Fig. 4S). Although some metabolites were already found in baseline plasma samples, the levels of most of these metabolites changed after the administration of blueberry extracts. In fact, only citric acid, tyrosine and *N*-phenylacetylglycine did not undergo changes in plasma samples after administration of CE-NADES or CE-SORG.

Only four anthocyanins were identified in their intact form in cecal

feces after the administration of crude blueberry extracts: delphinidin-3-hexoside, delphinidin-3-pentoside, petunidin-3-hexoside and petunidin-3-pentoside (Table 1 and Supplementary Fig. 3S). As observed for plasma samples, anthocyanins were not detected in feces samples at t0 (Supplementary Fig. 3S, panel A). Among the 15 remaining metabolites found in cecal samples, we identified gallic acid, hippuric acid, vanillic acid, protocatechuic acid, dihydrocoumaric acid, ferulic acid, dihydroferulic acid, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, 3-(3-hydroxyphenyl) propanoic acid and 3′ -hydroxyphenyl acetic acid as degradation products of anthocyanins and possible bacterial metabolites (Table 1). In addition, we found carboxylic acids, namely indole-2-carboxylic acid, 5-hydroxyindole-3-acetate and azelaic acid, and amino acids such as tryptophan and phenylalanine (Table 1, Supplementary Fig. 5S). Some of these identified fecal metabolites underwent increases after administration of CE-NADES or CE-SORG as presented in section 3.3.

3.3. Pharmacokinetic analysis and determination of phenolic compounds and their metabolites in cecal feces after CE-NADES or CE-SORG administration

After a single oral dose (10 mg of total polyphenols/kg b.w.), anthocyanins reached the maximum concentration in plasma 15 min after CE-SORG administration, while for the CE-NADES group the maximum plasma concentration was reached at 120 min ($p \leq 0.05$; Fig. 1A). The plasma chromatographic profiles at t0 (baseline), t120 for CE-NADES and t15 for CE-SORG are shown in Supplementary Fig. 2S. The pharmacokinetic parameters of phenolic compounds were estimated by applying a non-compartmental model (Table 2). The T_{max}, mean half-life (t1/2), $AUC_{0\text{-}720\ min}$ and MRT of anthocyanins were significantly higher for CE-NADES than for CE-SORG ($p \leq 0.05$). Concerning the mean values estimated for the pharmacokinetic parameters of elimination, CL and Lambda_z were significantly smaller for CE-NADES than for the CE-SORG group ($p \leq 0.05$). Although the maximum plasma concentration of anthocyanins (C_{max}) did not differ between the two extracts, the bioavailability of anthocyanins from CE-NADES was 140% greater when compared to the CE-SORG group (0.82 vs. 0.34%, $p \leq 0.05$, respectively; Table 2).

The plasma curves of five selected phenolic acids are presented in Fig. 1 (panels B to F). Gallic acid, vanillic acid, ferulic acid, hippuric acid and dihydrocoumaric acid were detected in plasma at the baseline time (t0) probably due to the phytochemical composition of the animal feed (Supplementary Fig. 4S, panel A). For these compounds, it was not possible to calculate pharmacokinetic parameters such as t1/2, MRT, lambda_z, and CL by PKSolver software because they were detected at fewer than three time points along the elimination terminal phase and the concentration in this phase showed an ascending tendency.

Among the analyzed parameters, there was no significant difference in T_{max} for gallic acid and hippuric acid between CE-NADES and CE-SORG groups. However, the T_{max} obtained in the CE-NADES group was significantly higher for vanillic and ferulic acids when compared to that for the CE-SORG group. Only for dihydrocoumaric acid, T_{max} was higher in the CE-SORG than CE-NADES group (720.0 vs. 52.5 min, $p \leq 0.05$). No significant differences were observed in C_{max} of phenolic acids between the CE-NADES and CE-SORG groups. Only the $AUC_{0\text{-}720\ min}$ of gallic acid was higher for CE-SORG in comparison to the CE-NADES group ($p \leq 0.05$), whereas the other phenolic acids showed no differences in $AUC_{0\text{-}720\ min}$ between the CE-NADES and CE-SORG groups.

The fecal concentration of anthocyanins after a single administration of CE-NADES or CE-SORG is presented in Fig. 2A. Anthocyanins were first detected in cecal feces only at 120 min after extract administration for the CE-NADES group and at 240 min for the CE-SORG group (Fig. 2A,

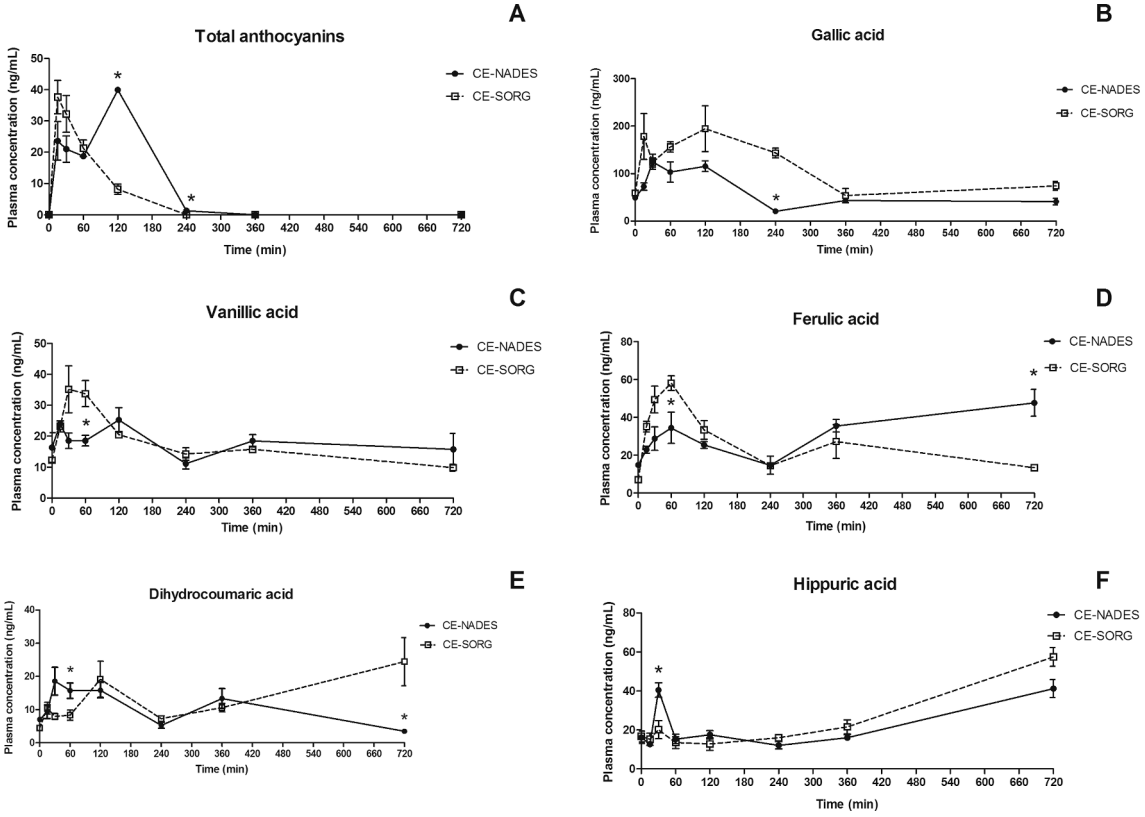

Fig. 1. Plasma profile for total anthocyanins, phenolic acids and their metabolites after a single oral administration of CE-NADES or CE-SORG extract to rats: total anthocyanins (A); gallic acid (B); vanillic acid (C); ferulic acid (D); dihydrocoumaric acid (E); hippuric acid (F). Data are presented as mean ± SEM (n = 4). *Indicates difference compared to the CE-SORG group at the same time point, $p \leq 0.05$ (Student's *t* test). CE-NADES: crude extract of blueberry obtained using NADES. CE-SORG: crude extract of blueberry obtained using organic solvent.

Table 2
Pharmacokinetic parameters of phenolic compounds following oral administration of CE-NADES or CE-SORG extracts.

Pharmacokinetic parameter	CE-NADES	CE-SORG
Total anthocyanins		
T_{max} (min)	120.0 ± 0.0*	15.0 ± 0.0
C_{max} (ng/mL)	40.0 ± 0.8	44.6 ± 3.3
*t*1/2 (min)	141.6 ± 3.2*	45.1 ± 9.2
$AUC_{0\text{-}720\ min}$ (ng/mL × min)	5351.0 ± 267.2*	2599.0 ± 178.0
MRT (min)	162.6 ± 1.1*	76.8 ± 13.1
Lambda_z (1/min)	0.005 ± 0.000*	0.015 ± 0.003
CL [(mg/kg)/(ng/mL)/min]	0.0005 ± 0.0000*	0.0009 ± 0.0000
Bioavailability (%)	0.82 ± 0.05*	0.34 ± 0.02
Phenolic acids		
Gallic acid		
T_{max} (min)	82.1 ± 0.2	108.8 ± 10.5
C_{max} (ng/mL)	145.5 ± 10.9	230.5 ± 49.3
$AUC_{0\text{-}720\ min}$ (ng/mL × min)	36058.0 ± 267.2*	74453.4 ± 6576.1
Vanillic acid		
T_{max} (min)	120.0 ± 0.0*	56.3 ± 10.2
C_{max} (ng/mL)	27.0 ± 2.5	33.9 ± 5.2
$AUC_{0\text{-}720\ min}$ (ng/mL × min)	6458.9 ± 390.6	6772.9 ± 464.8
Ferulic acid		
T_{max} (min)	720.0 ± 0.0*	60.0 ± 0.0
C_{max} (ng/mL)	51.6 ± 4.1	58.4 ± 4.0
$AUC_{0\text{-}720\ min}$ (ng/mL × min)	23837.1 ± 1110.7	15954.8 ± 1423.8
Hippuric acid		
T_{max} (min)	720.0 ± 0.0	720.0 ± 0.0
C_{max} (ng/mL)	43.9 ± 3.6	57.4 ± 4.9
$AUC_{0\text{-}720\ min}$ (ng/mL × min)	16235.7 ± 1149.7	20011.7 ± 628.2
Dihydrocoumaric acid		
T_{max} (min)	52.5 ± 25.9*	720.0 ± 00.0
C_{max} (ng/mL)	21.6 ± 0.9	24.4 ± 7.3
$AUC_{0\text{-}720\ min}$ (ng/mL × min)	8349.2 ± 459.1	9713.6 ± 1140.5

Data are presented as mean ± SEM, n = 4.
CE-NADES: crude extract of blueberry obtained using NADES. CE-SORG: crude extract of blueberry obtained using organic solvent. T_{max}: time at which maximum plasma concentration is achieved. C_{max}: maximum plasma concentration. *t*1/2: elimination half-life. $AUC_{0\text{-}720\ min}$: area under the concentration vs. time curve. MRT: mean residence time. Lambda_z: individual estimate of the terminal elimination rate constant. CL: clearance. Bioavailability is the relative/fractional bioavailability and was calculated using $AUC_{0\text{-}720\ min}$. * Indicates difference compared to the CE-SORG group, $p \leq 0.05$ (Student's *t* test).

$p \leq 0.05$). Thus, the CE-NADES group had higher fecal concentration of anthocyanins than the CE-SORG group at 120 min ($p \leq 0.05$) but no differences between groups were detected at the other time points. The chromatographic profile of anthocyanins in cecal feces at t0 (baseline), t360 for CE-NADES and t360 for CE-SORG is shown in Supplementary Fig. 3S).

Just like in plasma, phenolic compounds were detected in cecal feces at t0 (Supplementary Fig. 5S, panel A). In general, most non-anthocyanin phenolic compounds showed a higher fecal concentration in the CE-NADES group than in the CE-SORG group (Fig. 2, panels B–E). Compared to the CE-SORG group, the CE-NADES group had a significantly higher fecal concentration of vanillic acid (at 360 min after administration; Fig. 2B), ferulic acid (at 30, 60, 240 and 360 min after administration; Fig. 2C) and hippuric acid (at 60 min after administration; Fig. 2E) but a significantly lower concentration of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (at 30, 60 and 720 min after administration; Fig. 2D). Dihydro ferulic acid and 3′ -hydroxyphenyl acetic acid were detected in cecal feces but were below the limit of quantification.

3.4. *In vitro gastrointestinal digestion of phenolic compounds from CE-NADES and CE-SORG blueberry extracts*

An *in vitro* sequential gastrointestinal digestion assay was conducted to investigate differences in the stability and bioaccessibility of phenolic compounds between CE-NADES and CE-SORG (Fig. 3). Before digestion, CE-NADES and CE-SORG extracts showed a similar content of anthocyanins (290.1 vs 301.2 μg equivalents of cyanidin-3-glucoside/mL of extract) and non-anthocyanin phenolic compounds (949.6 vs 945.8 μg sum of catechin and quercetin equivalents/mL of extract, respectively) (Fig. 3, panels A and C). Anthocyanins underwent degradation during gastric digestion (Fig. 3A), but there was a higher degree of degradation of non-anthocyanin phenolics during gastric digestion (Fig. 3C). However, no differences were observed in the content of anthocyanin or non-anthocyanin phenolic compounds between CE-NADES and CE-SORG after simulation of gastric phase digestion (Fig. 3, panels A and C).

Interestingly, after adding simulated intestinal fluid, the amount of NaOH required to adjust to pH 7.0 was about 16 times higher for CE-NADES than for CE-SORG (3.09 ± 0.04 vs. 0.18 ± 0.02 mL of 5 M NaOH, respectively; $p \leq 0.05$). After intestinal digestion there was extensive degradation of anthocyanin and non-anthocyanin phenolic compounds (Fig. 3, panels A and C). Moreover, after intestinal digestion, the content of anthocyanin and non-anthocyanin phenolic compounds was significantly higher for CE-NADES than for CE-SORG in the bioaccessible and non-bioaccessible intestinal fractions (Fig. 3, panels B and D). The intestinal bioaccessibility of phenolic compounds was remarkably increased in CE-NADES compared to CE-SORG, being about 35-fold higher for anthocyanins (21.6 ± 0.1% vs 0.6 ± 0.2%, respectively; $p \leq 0.05$) and 5-fold higher for non-anthocyanin phenolics (20.5 ± 5.5% vs 3.3 ± 0.1%, respectively; $p \leq 0.05$).

Pearson's correlation analysis revealed that a higher content of phenolic compounds at the end of intestinal digestion was associated with a lower initial intestinal pH (R^2 = -0.9985 for anthocyanins and −0.8951 for non-anthocyanin phenolics, $p \leq 0.05$) and a higher amount of NaOH required to reach intestinal pH 7.0 (R^2 = 0.9994 for anthocyanins and 0.8771 for non-anthocyanin compounds, $p \leq 0.05$) (Supplementary Table 5S).

4. Discussion

In the present study, we demonstrated that the extraction of blueberry polyphenols using NADES produces a ready-to-use extract that has higher oral bioavailability for anthocyanins in rats when compared to the extract obtained using organic solvent. These findings confirm the hypothesis of the study and reveal a great advantage of NADES extracts over those obtained using organic solvents, which additionally require removal of solvent and resuspension in water before human or animal use. Such effect was obtained for the NADES mixture composed of choline chloride:glycerol:citric acid (0.5:2:0.5, molar ratio) plus 25% water, which was recently demonstrated to be as effective as organic solvents into extracting blueberry anthocyanins (Silva, Pauletto et al., 2020a). However, it is important to highlight that these results cannot be directly generalized to all NADES because of the large differences in NADES composition. In addition, subsequent studies should evaluate whether the effect of NADES on the bioavailability of phenolic compounds is dependent on the dose of NADES or phenolic compounds.

Intact glycosylated forms of anthocyanins were detected in the plasma of rats after administration of CE-NADES or CE-SORG, which is in line with previous reports for blueberry (Baron et al., 2017; Ichiyanagi, Shida, Rahman, Hatano, & Konishi, 2006). The non-compartmental pharmacokinetic parameters of anthocyanins were significantly different between CE-NADES and CE-SORG groups. Animal model studies indicate that bilberry anthocyanins are rapidly absorbed, appearing in the bloodstream within few minutes (6 to 20 min) after consumption (Ichiyanagi et al., 2006). In the CE-SORG group, where the previously dried extract was redissolved in water before administration to rats, the T_{max} of anthocyanins in plasma was 15 min, which is in accordance with Baron et al. (2017). However, the CE-NADES group displayed a biphasic profile for anthocyanin absorption that was characterized by a low plasma peak at 15 min followed by a high peak at 120

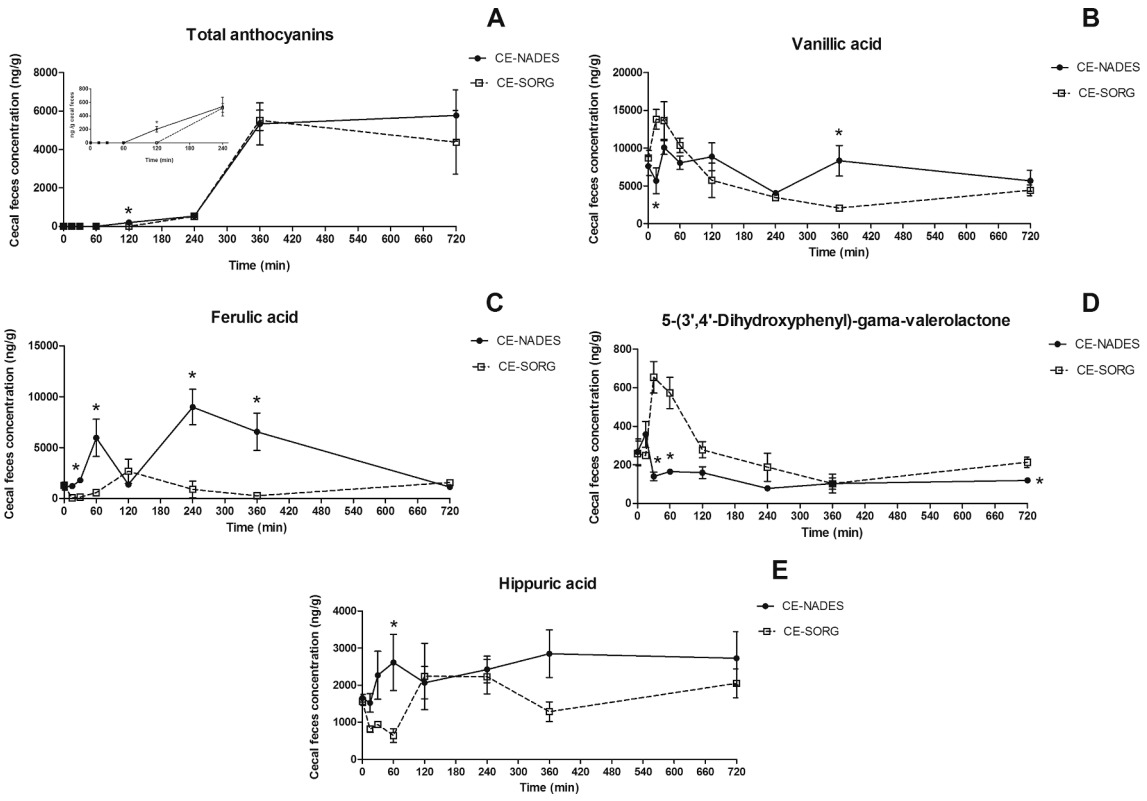

Fig. 2. Cecal feces concentrations of total anthocyanins, phenolic acids and their metabolites after a single oral administration of CE-NADES or CE-SORG extract to rats: total anthocyanins (A); vanillic acid (B); ferulic acid (C); 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (D); hippuric acid (E). Data are presented as mean ± SEM (n = 4). *Indicates difference compared to the CE-SORG group at the same time point, $p \leq 0.05$ (Student's *t* test). CE-NADES: crude extract of blueberry obtained using NADES. CE-SORG: crude extract of blueberry obtained using organic solvent.

min after CE-NADES administration. It is not possible to definitively explain this behavior. However, the first peak (15 min) could be likely attributed to the gastric absorption of anthocyanins, whereas the second peak at 120 min could be interpreted as enterohepatic recirculation, which is characterized by the secretion of compounds retained in the bile into the duodenum and their subsequent reabsorption (Fang, 2014; Zhong et al., 2017). This phenomenon has already been reported for intact anthocyanins (Stalmach, Edwards, Wightman, & Crozier, 2012) and monoglucuronide metabolites (Zhong et al., 2017), but this is the first study showing that NADES seems to favor a biphasic absorption profile for phenolic compounds.

Based on the pharmacokinetic characteristics, namely *t*1/2, MRT, lambda_z and CL, CE-NADES increased the extent to which anthocyanins were absorbed and delayed their elimination. AUC is the most reliable measure of a compound's bioavailability because it is directly proportional to the total amount of an unaltered analyte that reaches the systemic circulation (Le, 2017). While the time versus concentration curves demonstrated that CE-NADES and CE-SORG groups achieved the same maximum plasma concentration of anthocyanins, the $AUC_{0\text{-}720\ min}$ and consequently the bioavailability of anthocyanins were remarkably higher in the CE-NADES group. These differences may be attributed to the delayed elimination and longer plasma permanence of anthocyanins (*t*1/2, MRT, lambda_z and CL). Similar data were found when a proline: glutamic acid NADES was used as a vehicle for rutin administration to rats (Faggian et al., 2016), whereas honey that has NADES properties has been demonstrated to increase the plasma peak of calycosin-7-O-β-d-glucoside from *Astragali radix* (Dai et al., 2020). Another NADES mixture based on L-proline-malic acid also increased the bioavailability of isoflavones from the root of *Pueraria lobata* (Huang et al., 2021), whereas L-proline:acetamide has been shown to increase plasma peak of quinochalcone C-glycosides from *Carthamus tinctorius* L. (Tong et al., 2021). However, the present study is the first one to assess the effect of NADES on the bioavailability of phenolic compounds in a ready-to-use fruit extract. Moreover, the influence of NADES on the bioavailability of anthocyanins and phenolic acids was also investigated for the first time.

Compared to other flavonoid groups, the bioavailability of anthocyanins is very low (Hanske et al., 2013). The systemic bioavailability of anthocyanins ranges between 0.26% and 1.80% in animal studies and amounts to 1.09% in humans (Fang, 2014; Ichiyanagi et al., 2006; Zhong et al., 2017). Interestingly, we demonstrated that the bioavailability of total anthocyanin compounds maintaining their C6-C3-C6 structure was 140% higher in the CE-NADES group than in the CE-SORG group. Since CE-NADES and CE-SORG had a similar phenolic composition and concentration, the improvement of absorption kinetics, together with the biphasic response profile can be attributed to the type of vehicle used, i.e. NADES for CE-NADES vs. water for CE-SORG. Sut et al. (2017) and Faggian et al. (2016) demonstrated that the increase in the bioavailability of berberine (alkaloid) and rutin is related to the better solubilization properties of different eutectic mixtures compared to aqueous suspensions. In fact, we have data showing that the solubility of anthocyanin compounds was increased by 20% in the NADES used in the present study when compared to water (Supplementary Fig. 6S). This finding suggests that the best solubility of anthocyanins in NADES may contribute to the increased bioavailability of anthocyanins.

Moreover, we attribute the longer plasma permanence of anthocyanins to the sustained intestinal absorption due to increased stability of anthocyanins during gastrointestinal digestion in the CE-NADES group, when compared to CE-SORG. This hypothesis is supported by the greater stability and bioaccessibility of CE-NADES phenolics during *in vitro* intestinal digestion. In addition, CE-NADES group had higher fecal concentration of intact anthocyanins than CE-SORG group at 120 min, which is the time at which plasma levels of anthocyanins peak in the CE-NADES group. In vitro studies also corroborate that other NADES mixtures, namely a lactic acid–glucose–based NADES, are able to improve

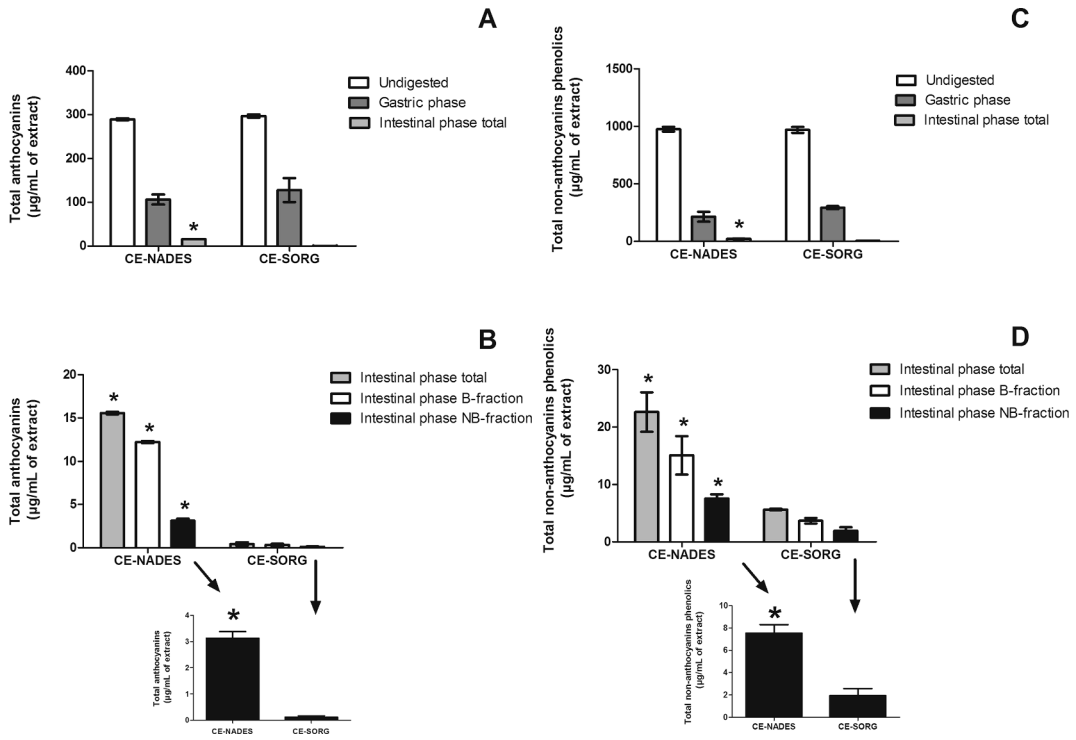

Fig. 3. Changes in total anthocyanin (A, B) and non-anthocyanin phenolic compound (C, D) content during *in vitro* digestion of crude extracts of blueberry obtained using NADES (CE-NADES) or organic solvent followed by drying and reconstitution in water (CE-SORG). Samples assessed before (undigested samples) and after in vitro digestion (gastric and intestinal phases) are shown in panels A and C, whereas panels B and D show the distribution of compounds after intestinal digestion. B-fraction: bioaccessible fraction; NB-fraction: non-bioaccessible fraction; intestinal phase total: sum of bioaccessible (B-fraction) and non-bioaccessible (NB-fraction) fractions. Data are presented as mean ± SEM (n = 3). *Indicates difference compared to the CE-SORG group, $p \leq 0.05$ (Student's *t* test). Total anthocyanins are the sum of all anthocyanins (μg of cyanidin-3-glucoside equivalents), whereas non-anthocyanin phenolic compounds are expressed as the sum of hydroxybenzoate, hydroxycinnamate and flavan-3-ol derivatives (μg of catechin equivalents) and flavonol derivatives (μg of quercetin equivalents).

the stability of cyanidin (Dai, Rozema, Verpoorte, & Choi, 2016). In fact, although anthocyanins are quite stable under the acid gastric pH, they have been shown to be extensively degraded under conditions that simulate small intestine digestion (Quatrin et al., 2020), as also observed in our *in vitro* digestion assay. Interestingly, the choline chloride:glycerol:citric acid-based NADES reduced the initial intestinal pH and increased the amount of NaOH required to reach intestinal pH 7, which likely contributed to increased stability and bioaccessibility of phenolics. Accordingly, correlation analysis revealed that the lower the initial intestinal pH, the higher the concentration of phenolic compounds recovered after intestinal digestion.

We propose that the buffering effect of citric acid in NADES delayed the neutralization of gastric chyme by intestinal fluid and attenuated the pH-triggered degradation of phenolic compounds when passing from the gastric to the intestinal phase. Citric acid, which amounted to 50 mM in the intestinal phase, has buffering properties in the acidic range due to the presence of three carboxyl groups, whose pKa values adjusted to 37 °C are 3.1, 4.71 and 6.42 (Martell & Smith, 1977). While food acidulants have recently been demonstrated to increase the bioaccessibility of some polyphenols from grains (Hithamani & Srinivasan, 2017), this is the first study describing the effect of a NADES on the bioaccessibility of phenolic compounds and proposing that organic acids increase the stability and bioaccessibility of phenolic compounds.

The metabolic fate of anthocyanins is complex, and metabolites produced after anthocyanin absorption may return to the gut lumen via the enterohepatic circulation (in bile) (Zhong et al., 2017). Thus, one cannot rule out the hypothesis that CE-NADES also promotes higher and earlier gastric absorption of anthocyanins followed by bile excretion and subsequent recycling via enterohepatic circulation. Together with the non-absorbed anthocyanins, systemic anthocyanin metabolites excreted through the bile will reach the colon where all these compounds can be converted into absorbable phenolic acids by the gut microbiota, or be directly excreted in the feces (Han et al., 2019).

The intestinal microbiota plays an important role in the metabolism of polyphenols (Wang et al., 2015). Fig. 4 summarizes a proposal for the biotransformation of blueberry polyphenols during digestion and highlights the pathways favored by the NADES vehicle based on the present study. The fecal profile of anthocyanins was similar between CE-NADES and CE-SORG groups and revealed that only delphinidin-3-pentoside, cyanidin-3-galactoside and petunidin-3-hexoside remained intact after blueberry extract passed through the gastrointestinal tract. The unidentified compounds found in feces are likely anthocyanin catabolites that may have originated during passage through the gastrointestinal tract or by the intestinal microbiota itself. After intestinal cleavage, anthocyanins are converted into aglycones that were not detected in the present study as they are highly unstable under physiological intestinal conditions, and therefore are rapidly metabolized into phenolic acids and phenolic aldehydes (Han et al., 2019; Hanske et al., 2013).

Overall, CE-NADES resulted in a greater amount of phenolic acids in the feces of rats than CE-SORG. We cannot attribute a greater elimination of phenolic acids to the CE-NADES group since feces were collected from the cecum, a site of intense microbial activity and where these compounds may be highly subject to resorption before feces are eliminated (Carabano, Badiola, Licois, & Gidenne, 2006). The changes in the phenolic composition of fecal samples triggered by NADES were not related to changes in fecal moisture. Although NADES contained glycerol, a compound that can increase the amount of water in the intestinal lumen, facilitating fecal evacuation and consequently the elimination of water-soluble compounds (Wapnir, Sia, & Fisher, 1996), the moisture of the cecal content was not different between the CE-NADES an CE-SORG groups (78.9 ± 1.1% vs 79.2 ± 1.9%; $p > 0.05$). Most phenolic acids in cecal feces, returned to their initial concentration after 720 min in both

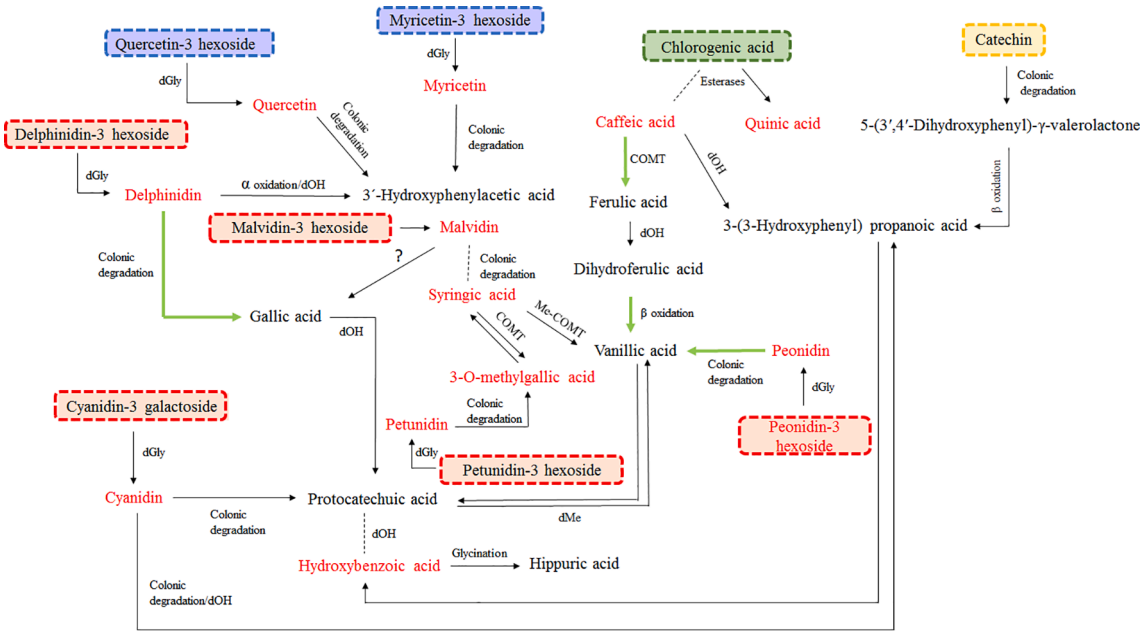

Fig. 4. Proposed metabolic pathways for the transformation of phenolic compounds after the ingestion of blueberry crude extract. The compounds found in the crude extract of blueberry administered to rats are shown in colored boxes (red: anthocyanins; blue: flavonols, green: hydroxycinnamic acid derivates; yellow: flavan-3-ol derivatives) whereas the metabolites identified in plasma or cecal feces are written in black font outside boxes. Green arrows indicate the biotransformation pathways favored by NADES. Dashed lines and red font indicate undetected compounds. SULF: sulfatases; UGT: uridine 5′-diphosphate-glucuronosyltransferase; dGly: deglycosylation; dOH: dehydroxylation; dCOOH: decarboxylation; Me: methylation; dMe: demethylation; COMT: catechol-O-methyl transferase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

groups, which may be attributed to their biotransformation by the colonic microbiota into hippuric acid.

Despite the appreciable amount of chlorogenic acid in crude blueberry extracts, this compound was not detected in the plasma or fecal samples of rats regardless of the extraction solvent. This finding indicates extensive degradation of chlorogenic acid during digestion and is in line with previous data showing low bioavailability for this phenolic acid (around 0.22%), which has been demonstrated to be immediately cleaved into caffeic acid and quinic acid after reaching the large intestine (Zhong et al., 2017). Caffeic acid may have been rapidly methylated to yield ferulic acid, which is metabolized to dihydroferulic acid in the colon (de Ferrars et al., 2014). CE-NADES appeared to have delayed the elimination of ferulic acid, since plasma peak levels were attained 660 min after those for the CE-SORG group. This finding may be related to the intestinal metabolism of caffeic acid, yielding sustained ferulic acid production in the CE-NADES group.

Hippuric acid, which has been described as one of the main end products of anthocyanin and non-anthocyanin phenolic acid metabolization (Zhong et al., 2017), showed a large increase in plasma at 720 min after the administration of CE-NADES or CE-SORG. By this time, most other phenolic acids had returned to baseline plasma levels, suggesting that metabolites such as gallic and vanillic acids are likely metabolized to benzoic acid and subsequently conjugated to glycine to form hippuric acid (de Ferrars et al., 2014). Thus, hippuric acid is not only a baseline metabolite originating from animal feed phytochemicals but is also a late metabolite produce from blueberry phenolic compounds.

5. Conclusion

Compared to an organic solvent extract, the NADES-based crude extract of blueberry showed increased bioavailability of anthocyanins. This effect was related to increased intestinal stability of phenolic compounds by delayed neutralization of gastric chyme in the presence of NADES, which favors a biphasic absorption profile and increases the plasma permanence of anthocyanins. Thus, in addition to being biocompatible, choline chloride:glycerol:citric acid NADES has great potential to yield ready-to-use extracts that increase the bioavailability of fruit phenolic compounds. This finding reveals NADES as a powerful tool for the development of functional foods, nutraceuticals and pharmacological formulations.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.130370.

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