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ORIGINAL PAPER

Sustainable valorization of citrus by-products: natural deep eutectic solvents for bioactive extraction and biological applications of *Citrus sinensis* peel

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Abstract

Orange is one of the most consumed fruits worldwide, generating significant byproducts and wastes. By following the principles of green chemistry, the reuse of orange by-products can be made sustainable. In this context, natural deep eutectic solvents (NADES) have emerged as a promising alternative. The present study aims to analyze the physico-chemical characteristics and biological effects of orange peel extracts obtained with choline chloride-based NADES. An investigation was conducted on the extracts, including a chromatographic analysis of phenolic and volatile compounds, a spectroscopic FTIR analysis, as well as the evaluation of their antioxidant capacity, their antimicrobial activity on different pathogens, and their capacity to modulate the activity of digestive enzymes. Both chromatographic approaches and FTIR studies indicated that different NADES had various extraction efficiencies towards phenolic compounds, affecting the antioxidant capacity of extracts. The volatile profile of NADES extracts was primarily composed of alcohols, aldehydes, and terpenes, whereas the ethanolic extract (50% (v/v), used as a control) exhibited a higher abundance of terpenes. All extracts were demonstrated to enhance pepsin enzyme activity without affecting that of chymotrypsin. Only choline chloride: glycerol: citric acid gave an extract capable of inhibiting trypsin and amylase activity, as well as the proliferation of pathogenic microorganisms. In conclusion, choline chloride-based NADES may represent a sustainable method for reusing orange byproducts, as they are more effective in extracting valuable bioactive compounds from orange peel when compared to old-fashioned organic solvents.

Keywords Natural deep eutectic solvents · Orange Peel · Phenolic compounds · Infrared · Antimicrobial activity · Volatile profile · Digestive enzymes

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Abbreviations

NADES	Natural Deep eutectic solvents
ChChl:Fruc	Choline chloride:fructose
ChChlXyl	Choline chloride:xylose
ChChl:Gly	Choline chloride:glycerol
ChChl:Gly:CA	Choline chloride:glycerol:citric acid

Introduction

According to a recent estimate by the UN Food and Agriculture Organization, citrus fruit production in the world has reached 144 million metric tons and as increased significantly in the last few years [1]. Today, the production of orange juice results in a considerable quantity of solid and liquid residues (around 1 million tons/year, globally), which is still considered as waste or used as a complement in agriculture [2].

Wastes from citrus processing are a source of nutritionally valuable compounds, including polyphenols, pigments (chlorophylls and carotenoids), tocopherols, and volatile and aroma compounds [3]. These features make citrus waste an attractive potential source of ingredients for new and/or added-value products for medical, cosmetic, and food/feed products. Citrus products probably represent one of the richest edible sources of phenolic compounds, reportedly associated with positive health effects in animals and humans [4–8].

In contrast to the conventional linear model of "produce, use, and dispose of," agri-food waste utilization would give rise to the circular economy. This proposed regenerative economic model envisions a system in which products and materials are reused and revalued throughout their life cycle [9]. This approach, which is gaining ground in the citrus industry, encourages the full use of fruits. It is driven by the growing pressure to create sustainable waste management solutions and reduce the environmental impact of industrial activities.

Following the circular economy approach, recently, much focus has been placed on the recovery of bioactive compounds from agri-food waste and byproducts through environmentally friendly, efficient, cost-effective, and sustainable methods. The sustainable utilization of agri-food wastes and byproducts for producing value-added products also provides an opportunity for complying with Agenda 2023 and the Sustainable Development Goals [10]. In response to the imperative for environmentally responsible processes, novel trends are being explored within the food industry, including the utilization of non-thermal extraction techniques and unconventional natural solvents [11].

Natural deep eutectic solvents (NADES) are new promising solvents that are emerging as a replacement for ones based on water-miscible organic solvents, such as methanol, acetone, or acetonitrile [12]. NADES use two or more natural components - in a specific molar ratio– with one of the components acting as a hydrogen bond donor and the other as a hydrogen bond acceptor [13]. The resulting eutectic mixture can be tailored for a specific aim, such as the extraction of specific compounds, making these solvents a viable alternative for industry [14]. The advantages of NADES are biodegradability, recyclability, extremely low vapor pressure, low costs, and low toxicity of all their components [15, 16].

NADES selectivity may minimize the need for extract purification [17] and NADES can have a stabilizing effect on bioactive species and/or enhance their biological activity and bioavailability [18]. Bioactive species in NADES-based extracts have demonstrated the capacity to modulate digestive enzyme activity, to possess antimicrobial activity toward different bacterial strains, along with anticancer properties toward specific cell lines [19]. While there is a growing body of research examining the use of NADES in the extraction of bioactive compounds from food matrices [20–23] the majority of studies have focused on the isolation of carotenoids or phenolic substances, with minimal attention paid to other molecules or the evaluation of their biological effects. This limited scope of research highlights the need for a more comprehensive understanding of the aforementioned effects, which can be achieved through the improved chemical characterization of extracted compounds. This process should encompass parameters influencing the quality, efficacy, and efficiency of extracts from alternative solvents. This study aims to provide an overview of the physicochemical characteristics and biological effects of extracts obtained from orange peel by-products by using choline chloride-based NADES, with aqueous ethanol extraction serving as a comparative standard extractant. Polyphenols and volatile compound profiles and content in the extracts were evaluated by separation approaches, with spectroscopic methods used for identification of functional groups. The total antioxidant capacity of the various extracts was also evaluated, along with their ability to modulate digestive enzyme activity and inhibit the growth of foodborne pathogenic/spoiling bacteria.

Materials and methods

Chemicals and reagents

Unless otherwise specified, chemicals and solvents were of analytical grade from either VWR Chemicals (Leuven, Belgium) or Sigma-Aldrich (St. Louis, MO, USA).

NADES formulation

Four different types of NADES were formulated using choline chloride as a hydrogen bond acceptor (Table 1). All NADES were prepared following the method described by Dai et al. [14] with some modifications. The reagents were mixed in specific molar ratios and stirred at 80 ºC in a water bath until a transparent liquid was formed. To decrease viscosity and facilitate the migration of bioactive compounds from the matrix into the solvent, 25% (w/w) distilled water was added to each NADES formulation. A 1/1 (v/v) water/EtOH mixture was used for comparison.

Table 1 Natural deep eutectic solvents and control used, and their characteristics

Acronym	Hydrogen bond donor	Hydrogen bond acceptor	Molar ratio	Water content	Appearance	pH
ChChl: Fruc	Choline Chloride	Fructose	1.9:1	25% (w/w)	Transparent colorless liquid	4.2
ChChl: Xyl		Xylose	2:1			4.01
ChChl: Gly		Glycerol	1:2			4.28
ChChl: Gly: CA		Glycerol: citric acid	1:1:1			2.03

Sample Preparation and extraction

The oranges (*Citrus sinensis*, Navel cultivar, harvested in early November) were donated by a local agricultural cooperative in Carlet, Spain. After washing with distilled water, peels were manually separated from the pulp and crushed in a kitchen blender. Bioactive compounds were extracted by suspending 1 g of crushed fresh orange peel in 10 mL of NADES or 50% aqueous EtOH, followed by stirring at 45 °C for 30 min. The extracts were centrifuged (1250 *g*, 10 min, 10 °C), the supernatant (the extract) was recovered and stored at 4 ºC until use, the orange peel was discarded.

The dry weight of orange peel has also been calculated, giving a 73% humidity (w/w), where 1 g of fresh orange peel equivale to 0.2 g of dry weight (the orange peel was dried in an oven at 100 ± 0.2 ºC to constant weight). To be able to understand the dry matter of the extract, and the different extraction efficiency of each NADES and the control it has been calculated by direct weight, the calculation has been performed on an analytical balance (Kern, ABT 120-5DM. ± 1 mg) by weighing 1 mL of the extracts in triplicate, giving the following results: ChChl: Fru 11.2 ± 0.6 mg/mL, ChChl: Xyl 12.7 ± 0.4 mg/mL, ChChl: Gly 10.6 ± 0.3 mg/mL, ChChl: Gly: CA 14.53 mg/mL, 50% EtOH 9.3 ± 0.1 mg/mL.

Determination of phenolic compounds

Separation of phenolic compounds in orange peel extracts was carried out on a C-18 column (Poroshell 120, SB-C18, 3.0 × 100 mm, 2.7 µm; Agilent Technologies, Palo Alto, CA, USA). Briefly, 20 mL of Milli-Q water containing 0.1% HCOOH was used to dilute a 5 mL NADES extract. Following preconditioning, samples were allowed to stand in the cartridges for two minutes. The cartridges were rinsed with 5 mL of 0.1% HCOOH in Milli-Q water after loading. Three milliliters (0.1% HCOOH) in MeOH were used to recover the polyphenols. After being evaporated with a nitrogen stream, all samples were reconstituted in 1 mL of MeOH/HCl (0.6 mol/L) (75:25, v/v). Chromatographic separations were carried out using a TripleTOFTM 5600 LC/MS/MS system (SCIEX) mounting an ACQUITY UPLC BEHC 18 column (2.1 × 50 mm I.D., 1.7 µm; Waters, Milford, Massachusetts, USA), as reported by Anticona et al. [24],. The following elution binary gradient was used, with a flow rate of 0.4 ml min-1: 5–12 min. linear from 70 to 5% A; 12–18 min. isocratic 5% A; 18–18.5 min. linear from 5 to 70% A; and 18.5–25 min. isocratic 70% A. 0–5 min. isocratic 70% solvent A: water/formic acid 99.9/0.1 (v/v), 30% solvent B: methanol/formic acid, 99.9/0.1 (v/v). The injection had a 5 l volume. The following instrument settings were used to capture data in negative mode over a mass/charge range of 100–950 m/z: Ion source gases 1 and 2 are each 50 psi; curtain gas 1, 25 psi; 450 ºC; ion spray voltage, −4500 V. An external calibrant delivery system (CDS), which injects a calibration solution before the entry of the sample, was used to carry out automated calibration. Polyphenol standards with concentrations ranging from 1 to 5 g/ml were employed for external calibration. The sample dilution was applied when the sample concentration was higher than the calibration curve. The information-dependent acquisition (IDA) approach was employed by the mass spectrometer (MS) with the survey scan type (TOF-MS) and the dependent scan type (production) utilizing collision energy of 30 V. PeakViewTM software was used to qualitatively analyze the data, and MultiquantTM-3.0.3 software was utilized to quantify the phenolic chemicals found. Chromatographic parameters, matrix effect, LOD, and LOQ are provided in Table 2. The matrix effect percentage (ME%) was determined according to the following formula:

$$\mathrm{ME}\ \% = [(\mathrm{A1} - \mathrm{A2})\,/\mathrm{A0}] \times 100$$

where: A1 is the spiked sample area, A2 is the sample area and A0 is the standard area.

Identification parameters are shown in supplementary material Table S1.

TEAC method

Trolox-equivalent antioxidant capacity (TEAC) was carried out according to Re et al., (1999). Briefly, Potassium persulfate (140 mM) was used to create the ABTS radical (ABTS•+), and ethanol was used to dilute the solution until its absorbance at 734 nm was 0.70 ± 0.02. After measuring the absorbance of produced ABTS•+, 100 µL of samples were added. After incubation at 30 °C for 3 min, the response was measured. Equation 1 was used to obtain the percentage of inhibition (%I). Trolox standard in the range of 0–250 µM was used for the calibration curve. Results were expressed as µM TE.

Table 2 Phenols content in orange Peel extracts

Compounds	LOD (µg/ mL)	LOQ (µg/mL)	Matrix Effect (%)	ChChl: Fru (µg/mL)	ChChl: Xyl (µg/mL)	ChChl: Gly (µg/mL)	ChChl: Gly: CA (µg/mL)	50% EtOH (µg/mL)
Phenolic acids								
p-Coumaric acid	0.074	0.092	117	16.4 ± 1.1[a]	13.4 ± 0.0[ab]	11.8 ± 1.4[b]	6.31 ± 0.2[c]	5.11 ± 0.0[c]
Ferulic acid	0.001	0.003	120	118.0 ± 1.0[a]	53.7 ± 1.2[c]	91.5 ± 0.4[b]	29.1 ± 0.0[d]	29.2 ± 0.2[d]
Total phenolic acids				134.4 ± 0.1[a]	67.1 ± 1.2[c]	103.3 ± 1.8[b]	35.4 ± 0.2[d]	34.4 ± 0.2[d]
Flavanones								
Hesperidin	0.095	0.123	96	2827 ± 25.5[b]	3388 ± 3.1[a]	3259 ± 137.5[a]	3442 ± 54.6[a]	3205 ± 143.0[a]
Rutin	0.01	0.03	116	2.46 ± 0.03[a]	1.45 ± 0.02[c]	1.93 ± 0.02[b]	0.87 ± 0.00[d]	1.98 ± 0.00[b]
Narirutin	0.146	0.291	104	242.7 ± 1.2[ab]	251.0 ± 6.0[a]	225.3 ± 0.1[bc]	221.0 ± 5.6[c]	160.5 ± 6.4[d]
Total flavanones				3072 ± 22.3[c]	3641 ± 7.3[a]	3487 ± 137.4[b]	3664 ± 60.2[a]	3367 ± 149.4[bc]
Total phenolic compounds				3207 ± 22.2[c]	3708 ± 8.6[a]	3590 ± 139.2[b]	3700 ± 60.1[a]	3402 ± 149.2[bc]

Data are expressed as µg/mL extract and are mean ± SD of three chromatographic analyses. Statistical analysis was by one-way ANOVA (all: $p < 0.005$) with Tukey's post hoc test (different letters indicate significant differences). CA: citric acid; ChChl: choline chloride; Et-OH: ethanol; Fru: fructose; Gly: glycerol; Xyl: xylose

$$\%\,inhibition = \left(1 - \frac{A1}{A0}\right) * 100 \quad (1)$$

Where A_1 is the absorbance in the presence of the sample, and A_0 is the absorbance of the control (ABTS•+).

DPPH method

DPPH method was performed according to the method described by Brand-Williams et al., (1995). 50 µL of adequate sample dilution at 1.45 mL DPPH (0.06 mM) is added. The sample is incubated for 30 min at room temperature. Absorbance is measured at the wavelength of 515 nm in a Lambda 365 spectrophotometer (Perkin Elmer ® Massachusetts, USA). The %I is calculated using the Trolox standard (Eq. 1) prepared in the range of 0–500 µM for the calibration curve and the results are expressed as µM TE.

Ferric reducing antioxidant power (FRAP)

FRAP was measured as reported by Gomez-Urios, et al. [25]. A volume of 8 µL of NADES extract was added to 192 µL of 1.7 mM Fe (III)/0.8 mM tripyridyltriazine, monitoring the absorbance increase at 595 nm for 30 min. The operative model consisted in a mixture of 900 µl of FRAP solution, 90 µl of distilled water, and 30 µl of suitably diluted sample for incubation in amber glass tubes at 37 °C. Absorbance was determined on a Lambda 365 spectrophotometer (Perkin Elmer ® Massachusetts, USA). The FRAP standard calibration curve was done under the same conditions as the samples, in a range of concentration of 0–250 µM. Data were compared to the concentration-response curve of a standard solution of Trolox and expressed as mmol Trolox equivalents.

Oxygen radical absorbance capacity (ORAC)

ORAC was assessed based on the inhibition of fluorescein oxidation by oxygen radicals generated by 2,2′-azobis(2-amidinopropan) dihydrochloride (AAPH) [26]. It was carried out using the Wallac 1420 VICTOR2 multilabel counter equipment (Perkin Elmer, USA) with fluorescence filters, at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

In brief, 50 µl of the NADES extract was combined with 78 nM of fluorescein, and the reaction was initiated with the addition of 25 µl of 221.2 mM AAPH. The measurements (λex = 485 nm, λem = 535 nm) were taken in cycles of five minutes until the relative fluorescence intensity dropped below 5% of the starting value. Trolox was used as standard, and ORAC values were expressed as mmol/mL.

ORAC values expressed in µM Trolox equivalents (µM TE) were calculated by applying Eq. 2 shown below:

$$ORAC\ (\mu\mathrm{M\,TE}) = \frac{C\,Trolox \cdot (AUC\ sample - AUC\ Blank) \cdot k}{(AUC\,Trolox - AUC\ Blank)} \quad (2)$$

Where, CTrolox is the Trolox concentration (µM), k is the sample dilution factor and AUC is the area under the fluorescence decay curve, Sample, blank, and Trolox standard respectively, and calculated by applying Eq. 3 set out below:

$$AUC = 0.5 + \frac{f5}{f0} + \frac{f10}{f0} + \ldots + fn + 5/f0) \cdot 5 \quad (3)$$

Where f_0 is the initial fluorescence and f_n is the fluorescence at time n.

FTIR spectra acquisition

Fourier-transform near-infrared spectroscopy (FTIR) spectra were collected using a single reflection ATR module on a Vertex 70 spectrometer (Bruker Optics, Milan, Italy) with a spectral range of 4000–600 cm^{-1}, a resolution of 4 cm^{-1}, and 24 scans, by at around 25 °C [27]. A background was collected before the analysis of each NADES extract. The acquisition system was controlled by OPUS software (Bruker Optics, Milan, Italy). For data visualization and exploration, the carbon dioxide absorption band was removed (2500–2000 cm^{-1}).

Determination of volatile molecule profiles

Separation of volatile molecules from extracts was carried out by solid-phase microextraction followed by gas chromatography (GC; 6890 N Network GC System) coupled with mass spectrometry (MS) 5970 MSD (Hewlett-Packard, Geneva, Switzerland), as described by Siroli et al. [28] was based on a comparison with the NIST 11 (National Institute of Standards and Technology) mass spectral database. The relative quantity of identified species is expressed as a percentage of the total area.

Pepsin, Trypsin, chymotrypsin and α-amylase activity

Pepsin, trypsin, and chymotrypsin activities were determined according to Borgonovi et al. [29], with slight modifications. Extracts corresponding to 0.2 mM (final concentration, f.c.) of hesperidin equivalent were added to a final volume of 500 µL of hemoglobin (f.c. 2.5% w/v in water, pH 2 for pepsin and pH 7 for trypsin and chymotrypsin). The reaction was started with 100 µL of a solution of the appropriate enzyme (30 µg/mL in 0.15 M NaCl, 0.0115 M $CaCl_2$, pH 2 for pepsin, and pH 7 for trypsin and chymotrypsin) in 2 mL plastic tubes. After 5–10 min, the reaction was stopped by adding 1 mL of 20% (w/v) trichloroacetic acid (TCA). Uncleaved proteins were separated by centrifugation (12,000 x g for 10 min), and TCA-soluble peptides released from the substrate were spectrophotometrically detected at 280 nm. Alpha-amylase activity was evaluated using the Alpha-amylase Assay Kit (Megazyme, Wicklow, Irland), with slight modifications. Extracts corresponding to a 0.2 mM (f.c.) of hesperidin equivalent were added to 200 µL of substrate solution containing blocked p-nitrophenyl maltoheptaoside and excess levels of amyloglucosidase and α-glucosidase. The reaction was started with the addition of 0.1 mL α-amylase (30 µg/mL in 0.15 M NaCl, 0.0115 M $CaCl_2$, pH 7). After 10 min at 40 °C, the reaction was stopped by adding 3 mL of the kit-provided stop solution and the absorbance read at 400 nm. The addition of the extracts did not result in a substantial alteration of the pH level in the working solution across all assays. In all cases, an enzyme unit corresponded to an absorbance change of 0.001/min. Activity data were normalized for the enzyme amount (mg) in each assay.

Antimicrobial activity

The antimicrobial activity of extracts was determined through an agar well diffusion assay against foodborne bacteria responsible for pathogenicity or spoilage (*Listeria monocytogenes* Scott A; *Escherichia coli* 555, and *Staphylococcus aureus* 231, and bacteria associated to contamination of human skin, *Staphylococcus epidermidis* E6), according to Siroli et al. [30]. The extracts were tested as such or after 50% dilution in sterile distilled water. In these tests, the very low pH of ChChl: Gly: CA (pH of 1.03) extracts was adjusted to 4.2 by adding 1 M NaOH. The antimicrobial activity was based on the observed diameter of growth inhibition areas: "-", < 8 mm; "+", 8–11 mm; "++", 11–14 mm; "+++", 14–18 mm; "++++",> 18 mm.

Statistical analysis

The extraction was performed in triplicate, and the analysis was done in triplicate. Statistical differences were determined by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test using Prism software ver. 7.0 (GraphPad, San Diego, CA, USA). Different letters indicate significant differences ($p<0.05$). Principal component analysis (PCA) was performed by to explore the multivariate nature of the volatile profile data and FT-IR spectra. Before PCA, volatile profile data, expressed as peak areas were normalized and scaled, whereas FT-IR spectra were reduced in the spectral range (4000–670 cm^{-1}) and the carbon dioxide absorption band was removed (2500–2000 cm^{-1}). Several pre-treatments were performed to assess which one could efficiently correct the signal and reduce noise: baseline correction (automatic weighted least squares), smoothing (Savitzky–Golay, filter width 11 points; 3rd polynomial order), and first and second derivatives, in combination or alone. Data exploration was performed in the MATLAB environment (v. 2017b, Mathworks, Inc., Natick, MA, USA) using the PLS toolbox (v. 8.5, Eigenvector Research, Inc., Seattle, WA, USA).

Results

Polyphenols profile, content, and antioxidant activity

Orange peel is a rich source of phenolic compounds, including flavonoids, phenolic acids, tannins, and coumarins [31]. Table 2 summarizes the most abundant phenolics found in various extracts from orange peel. Phenolic compounds identified by LC/MS are grouped into two classes (two phenolic acids and three flavanones). Flavanones were the most representative free phenolic class, accounting for approximately 96–99% of total free phenolic content. Hesperidin is the most abundant free polyphenol, accounting for 88–94% of total polyphenols, followed by narirutin (4–8%) and rutin (2–4%).

Table 2 makes it evident that the total extraction yield (of the compounds analyzed) and levels of the various phenolic classes and of individual compounds depend on the type of extractant. ChChl: Xyl and ChChl: Gly: CA resulted in the highest extraction of total phenolic compounds and flavanones, whereas ChChl: Fru gave a good extraction yield for phenolic acids but a modest one for hesperidin, which was most effectively extracted by ChChl: Gly and by 50% EtOH.

NADES selectivity was reported in the literature [21, 32–34], and related to the physicochemical properties of these solvents. Each NADES possesses distinctive characteristics, including polarity, pH, and conductivity [13, 35, 36], all playing an important role in the extraction yield achieved. It is also possible to modify the NADES formulation to obtain a solvent system specifically tailored to the extraction of a specific compound [14]. Water addition helps to decrease viscosity, moreover, with a water content of less than 50%, NADES are still considered eutectic mixtures, which help maintain their extractive properties [37, 38].

All the phenolics identified in these extracts are known to have antioxidant activity [39], reportedly exerted through a variety of mechanisms [40]. Table 3 compares the antioxidant activity of the various NADES extracts, as evaluated by four distinct methodologies. Water/ethanol extracts had the highest antioxidant capacity, as determined by the ABTS, FRAP, and ORAC assays. ChCl: Gly: CA extracts had the highest antioxidant capacity in the DPPH and ORAC assays, but the lowest in FRAP assays. These discrepancies are quite common and have been related to the different nature of the chemical reactions in each assay, as well as to electron transfer kinetic issues and the solubility of individual phenols in each reaction medium [41].

For instance, FRAP cannot detect species that act by a radical quenching mechanism [42], whereas the antioxidant behavior in the ORAC assay is dominated by the number and type of substituents and not by the Bors criteria, at least in the absence of steric hindrance effects [43]. These results can be compared with previous results, where, Gomez-Urios, et al. [36] studied the different antioxidant capacities of fourteen NADES systems, showing different results depending on the radical used. Unlike the peroxide and ABTS radicals, the DPPH radical is soluble in organic solvents because it is hydrophobic. Due to steric inaccessibility, antioxidants that react quickly with the latter may be slow or inactive toward DPPH [44]. The overall activity of antioxidant compounds is lower than that of individual compounds because of steric hindrance [45].

FT-IR

As reported in Fig. 1A, FTIR spectra show distinctive absorption peaks of ChChl: Fru, ChChl: Xyl, ChChl: Gly, ChChl: Gly: CA, and 50% EtOH extracts corresponding to the functional groups present. Notably, the broad intense absorption band between 3000 and 3600 $cm^{-1,}$ present for all the extracts, can be associated with the oscillation of the–OH groups of water with slight differences among the extracts due to the formation of intermolecular hydrogen bonds in the NADES mixtures [13]. In the region 2873–2972 cm^{-1} the extracts differed in peaks: ChChl: Gly, ChChl: Gly: CA showed two peaks at 2950 and 2890 cm^{-1}, resulting in unique broadband for ChChl: Xyl and one visible peak at 2950 cm^{-1} for ChChl: Fru which can be assigned to νC–H (–H2) vibrations [46].

At 1480 cm^{-1}, the CH_2 oscillation mode band can be noticed, characteristic for ChChl: Fru, ChChl: Xyl, ChChl: Gly, ChChl: Gly: CA [47]. The ChChl: Gly has a similar profile to the one recorded by Wang et al. [48]. The ChChl: Gly: CA extract showed the highest absorbance at the two peaks at 2950 and 2890 cm^{-1} as well as in the region

Table 3 Total antioxidant capacity of orange Peel extracts

	ChChl: Fru	ChChl: Xyl	ChChl: Gly	ChChl: Gly: CA	50% EtOH
ABTS	2.13 ± 0.01^c	2.32 ± 0.01^b	1.84 ± 0.01^d	1.06 ± 0.02^e	2.76 ± 0.05^a
DPPH	1.04 ± 0.03^b	0.73 ± 0.12^c	0.57 ± 0.01^d	1.44 ± 0.02^a	0.81 ± 0.05^c
FRAP	2.03 ± 0.02^c	2.29 ± 0.04^b	1.97 ± 0.07^c	0.39 ± 0.02^d	3.20 ± 0.09^a
ORAC	11,341 ± 1052^c	8072 ± 457^c	18,293 ± 488^b	21,314 ± 2104^a	25,422 ± 4559^a

Data are expressed as mmol TE/mL extract and are means ± SD of three determinations. Statistical analysis was by one-way ANOVA (all: $p < 0.005$) with Tukey's post hoc test (different letters indicate significant differences). CA: citric acid; ChChl: choline chloride; Et-OH: ethanol; FRAP: ferric reducing antioxidant power; Fru: fructose; Gly: glycerol; ORAC: oxygen radical antioxidant capacity; Xyl: xylose

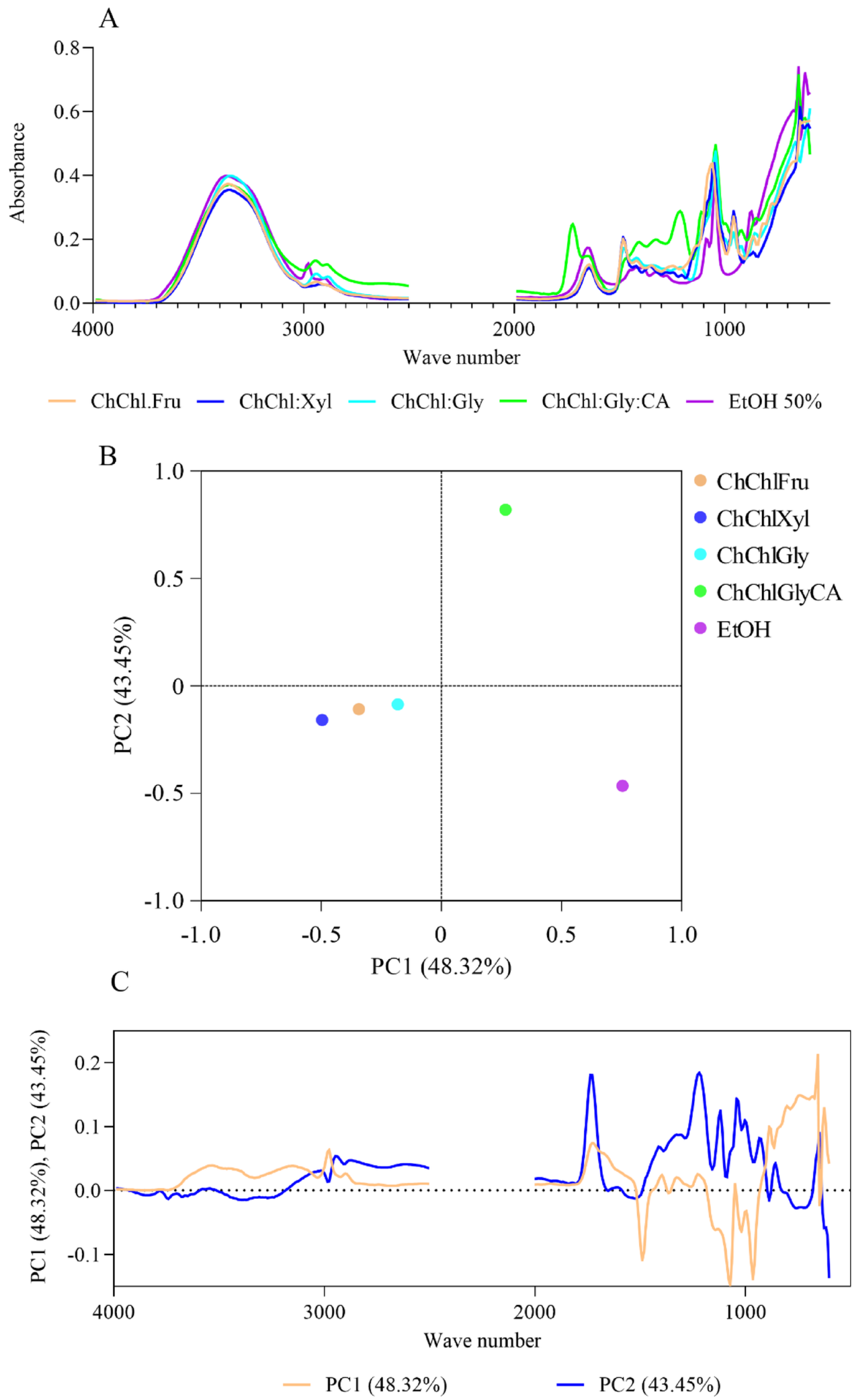

Fig. 1 Representative FT-NIR spectrum (A), PCA scores plot (B), and loadings plot (C) on the whole spectra dataset collected in orange peel NADES and 50% EtOH extracts. *Data are expressed as absorbance and are mean of three spectroscopic analyses. CA: citric acid; ChChl: choline chloride; EtOH: ethanol; Fru: fructose; Gly: glycerol; Xyl: xylose*

1500–1150 cm^{-1}, which might be related to the highest presence of total phenolic compounds (3700 ± 60 µg/mL) in the extract.

The 50% EtOH showed a different profile all along the IR region with characteristic peaks linked to ethanol at around: 3400 cm^{-1} due to O–H bond stretching, 2980 cm^{-1} due to C–H bond stretching, from 1350 to 1260 cm^{-1} due to O–H bending deformation band, at around 1055–1100 cm^{-1}, with two peaks linked to C–O bond stretching.

The fingerprint region (1100–850 cm^{-1}) is more complex to be specifically described for each extract as it is typical for the signal related to the combination of both NADES and extracted phenolic compounds.

PCA on data pre-treated by smoothing gave the most informative exploration of the FT-IR spectra, highlighting the relation among the samples and the influence of the variables, i.e. the wavenumbers. The scores plot of PC1 (explained variance: 48.32%) vs. PC2 (explained variance: 43.45%) (Fig. 1B) confirmed the observed spectral differences for ChChl: Gly: CA and 50% EtOH concerning ChChl: Fru, ChChl: Xyl, ChChl: Gly. In detail the ChChl: Gly: CA showed positive PC1 and PC2 scores, the 50% EtOH extract is located in the IV quadrant, and ChChl: Fru, ChChl: Xyl, ChChl: Gly are grouped in the III quadrant. The differences along PC1 (Fig. 1C) are linked to the positive loadings of PC1 at 2978 cm^{-1}, characteristic of 50% EtOH extract, together with the absorbance at 1720, 1390, 1211, 1219, and 1040 cm^{-1} which are characteristic of citric acid extract. Furthermore, ChChl: Fru, ChChl: Xyl, ChChl: Gly differed from ChChl: Gly: CA along PC2 due to the signals in the region 1500–1000 cm^{-1}, linked to fructose, xylose, and glycerol absorbance.

Volatile compounds profile and relative content

This study identified 50 volatile compounds in orange peel extracts. Table 4 summarises the volatile compounds, divided into six classes: terpenes (13), aldehydes (10), alcohols (18), ketones (5), organic acids (2), and esters (2). As for phenolics, the levels of the volatile classes and individual components were found to depend on the type of extraction solvent. In NADES extracts, the most representative classes were alcohols, aldehydes, and terpenes, which accounted for approximately 95% of the total volatile compound content. In contrast, terpenes were the most representative class in 50% EtOH extracts, accounting for about 99% of the total volatile compounds. The profile of volatile molecules in the extracts is mainly related to the polarity of the solvent. Choline chloride-based NADES have a hydrophilic nature and exhibit higher polarity than water [49]. This leads to greater extraction of polar and high molecular weight molecules concerning more hydrophobic terpenes, which were most abundant in extracts using 50% EtOH, which has lower polarity [50].

As for individual volatile components, the most typical compounds in ChChl: Fru, ChChl: Xyl, and ChChl: Gly were linalool > limonene > 1-octanol, which accounted for nearly 54% of the total compounds. In ChChl: Gly: CA, the most characteristic volatile compounds were limonene > α-terpineol > 1-octanol, accounting for approximately 55% al compounds. In the 50% EtOH extract, the most prominent volatile compounds were limonene >> β-myrcene, which together accounted for almost 94% of the total. Ethanol's lower polarity compared to the other solvent mixtures likely favors the extraction of more hydrophobic terpenes, such as limonene and β-myrcene [51], whereas the presence of both polar and moderately hydrophobic volatile compounds in NADES extracts indicates a balance in extracting a range of volatiles with varying polarities.

All the major volatile compounds detected in this study have been reported to be components of citrus essential oil [52]. However, ChChl: Gly extract was different from those obtained using ChChl: Fru and ChChl: Xyl, which showed fairly similar flavor profiles. The extract obtained using ChChl: Gly: CA was very different from the others, including ChChl: Gly, likely because of the acidic pH of the extractant containing citric acid, which could lead to the conversion of some terpenoids. For example, the conversion of nerol and geraniol to alpha-terpineol has been found to occur in an exceedingly acidic environment [53]. Literature data indicate that terpenoids such as α-terpineol and linalool are more effective antimicrobials compared to terpenes such as limonene and β-myrcene [54].

A PCA on the volatile profile data was performed to highlight the differences among the samples based on the complexity of the data (Fig. 2). The scores plot of PC1 (explained variance: 50.95%) vs. PC2 (explained variance: 30.02%) confirmed clear differences among the samples. The PCA scores (Fig. 2A) displayed a clear separation of the NADES extract in the factor plane, except for the extracts obtained with ChChl: Fru and ChChl: Xyl, which clustered together. The latter were well separated from ChChl: Gly and from the 50% EtOH extract mainly along PC1, which explains 50.95% of the total variance. On the contrary, ChChl: CA extracts were well separated from the ChChl: Fru and ChChl: Xyl ones and from the 50% EtOH extract both along PC1 and PC2 (which explains 30.02% of the total variance) and from the ChChl: Gly extract mainly along PC2. The PCA loadings (Fig. 2B) indicate that terpenes emerged as the characteristic volatile molecules of the phenolic extracts, allowing their separation along PC1. Conversely, alcohols, esters, aldehydes, ketones, and acids were the molecules characterizing the NADES extracts and their separation along PC1 and PC2.

Table 4 Volatile compounds relative content in orange Peel extracts

Compounds	ChChl: Fruc	ChChl: Xyl	ChChl: Gly	ChChl: Gly: CA	50% EtOH
Terpenes					
α-Thujene	n.d.	n.d.	n.d.	n.d.	0.15 ± 0.05[a]
β-Thujene	n.d.	n.d.	n.d.	n.d.	1.31 ± 0.35[a]
3-Carene	n.d.	n.d.	n.d.	n.d.	0.35 ± 0.07[a]
β-Myrcene	0.59 ± 0.09[bc]	0.51 ± 0.08[cd]	0.40 ± 0.01[d]	0.79 ± 0.13[b]	5.82 ± 0.76[a]
Isocineole	n.d.	n.d.	n.d.	0.53 ± 0.14[a]	n.d.
Limonene	17.23 ± 0.66[c]	16.43 ± 0.84[c]	17.27 ± 0.46[c]	38.15 ± 1.87[b]	88.38 ± 2.12[a]
β-Phellandrene	n.d.	n.d.	n.d.	n.d.	1.12 ± 0.36[a]
β-Cimene	n.d.	n.d.	n.d.	0.80 ± 0.08[a]	0.06 ± 0.02[b]
Terpinolen	n.d.	n.d.	n.d.	2.16 ± 0.12[a]	0.14 ± 0.04[b]
α-Pinene	0.43 ± 0.04[ab]	0.36 ± 0.06[b]	0.49 ± 0.06[ab]	0.50 ± 0.05[a]	n.d.
Carvone	0.51 ± 0.05[a]	0.49 ± 0.07[a]	0.49 ± 0.05[a]	0.42 ± 0.07[a]	n.d.
Dimethylstyrene	n.d.	n.d.	n.d.	2.87 ± 0.31[a]	n.d.
Total terpenes	**18.76 ± 0.76[c]**	**17.79 ± 0.75[c]**	**18.65 ± 0.36[c]**	**46.22 ± 1.51[b]**	**98.70 ± 0.28[a]**
Aldehydes					
Hexanal	5.31 ± 0.55[b]	6.95 ± 0.94[a]	4.17 ± 0.29[c]	0.76 ± 0.12[d]	n.d.
Octanal	4.21 ± 0.32[b]	4.98 ± 0.77[b]	15.35 ± 1.09[a]	2.14 ± 0.14[c]	0.44 ± 0.10[d]
Nonanal	n.d.	0.29 ± 0.08[b]	0.91 ± 0.10[a]	0.47 ± 0.12[b]	0.06 ± 0.01[c]
Decanal	n.d.	0.22 ± 0.03[c]	0.97 ± 0.15[a]	0.48 ± 0.07[b]	0.47 ± 0.09[b]
2-Hexenal	5.68 ± 0.53[a]	5.92 ± 0.46[a]	3.78 ± 0.27[b]	3.12 ± 0.42[b]	n.d.
Butanal	0.19 ± 0.02[a]	0.18 ± 0.03[a]	n.d.	n.d.	n.d.
Neral	2.05 ± 0.16[a]	1.77 ± 0.31[a]	2.20 ± 0.19[a]	n.d.	0.04 ± 0.02[b]
Geranial	1.67 ± 0.13[b]	1.78 ± 0.34[ab]	2.30 ± 0.32[a]	n.d.	0.05 ± 0.02[c]
Perillal	0.29 ± 0.06[b]	0.29 ± 0.08[b]	0.37 ± 0.11[ab]	0.50 ± 0.10[a]	n.d.
Benzaldehyde	0.23 ± 0.03[a]	n.d.	n.d.	n.d.	n.d.
Total aldehydes	**19.62 ± 0.27[c]**	**22.38 ± 1.04[b]**	**30.05 ± 1.40[a]**	**7.46 ± 0.70[d]**	**1.06 ± 0.22[e]**
Alcoholes					
Eucalyptol	n.d.	n.d.	0.31 ± 0.09[b]	2.06 ± 0.18[a]	n.d.
trans-2-Hexenol	1.91 ± 0.13[a]	1.53 ± 0.45[ab]	0.55 ± 0.07[c]	1.04 ± 0.17[b]	n.d.
2-Buten-1-ol, 3-methyl-	n.d.	n.d.	n.d.	0.58 ± 0.05[a]	n.d.
1-Hexanol	2.78 ± 0.16[a]	2.59 ± 0.42[a]	0.95 ± 0.10[c]	1.41 ± 0.13[b]	n.d.
3-Hexen-1-ol, (Z)-	0.71 ± 0.10[a]	0.41 ± 0.06[b]	0.28 ± 0.05[c]	0.31 ± 0.02[c]	n.d.
1-Hexanol, 2-ethyl-	0.45 ± 0.09[a]	0.32 ± 0.10[a]	n.d.	n.d.	n.d.
Linalol	29.41 ± 0.66[a]	27.83 ± 1.62[a]	30.25 ± 0.87[a]	1.77 ± 0.61[b]	0.16 ± 0.03[c]
1-Octanol	9.23 ± 0.35[b]	10.07 ± 0.87[ab]	9.19 ± 0.37[b]	10.67 ± 0.62[a]	0.04 ± 0.02[c]
4-Terpineol	1.47 ± 0.12[b]	1.47 ± 0.19[b]	0.97 ± 0.15[c]	2.64 ± 0.10[a]	n.d.
Verbenol	0.49 ± 0.04[b]	0.57 ± 0.27[abc]	0.28 ± 0.05[c]	0.93 ± 0.09[a]	n.d.
Menthol	n.d.	n.d.	n.d.	2.07 ± 0.18[a]	n.d.
α-Terpineol	5.97 ± 0.16[b]	4.60 ± 0.39[c]	3.28 ± 0.33[d]	17.90 ± 1.69[a]	0.03 ± 0.02[e]
β-Citronellolo	0.65 ± 0.10[a]	0.71 ± 0.27	0.56 ± 0.12[a]	n.d.	n.d.
Nerol	1.56 ± 0.15[a]	1.26 ± 0.15[a]	0.91 ± 0.07[b]	n.d.	n.d.
2-Cyclohexen-1-ol, 1-methyl-4-(1-methylethenyl)-, trans-	0.26 ± 0.01[a]	0.28 ± 0.05[a]	n.d.	n.d.	n.d.
cis-Carveol	0.36 ± 0.05[a]	0.38 ± 0.15[a]	n.d.	n.d.	n.d.
Geraniol	0.69 ± 0.08[a]	0.89 ± 0.22[a]	0.28 ± 0.10[b]	n.d.	n.d.
p-Mentha-1(7),8(10)-dien-9-ol	0.61 ± 0.05[a]	0.38 ± 0.03[b]	0.26 ± 0.04[c]	0.39 ± 0.12[bc]	n.d.
Total alcohols	**56.54 ± 0.06[a]**	**53.30 ± 0.79[b]**	**48.07 ± 2.03[c]**	**41.76 ± 0.21[d]**	**0.24 ± 0.06[e]**
Ketones					
2-Propanone, 1-Hydroxy	n.d.	n.d.	0.69 ± 0.13[a]	0.49 ± 0.06[a]	n.d.
Camphor	n.d.	n.d.	0.19 ± 0.02[a]	n.d.	n.d.
Cyclopentanone, 2-ethyl	0.64 ± 0.11[a]	0.57 ± 0.15[ab]	0.36 ± 0.05[b]	0.33 ± 0.08[b]	n.d.
Isopiperitenon	0.99 ± 0.06[a]	0.90 ± 0.08[a]	0.29 ± 0.04[b]	1.23 ± 0.28[a]	n.d.
Total ketones	**1.63 ± 0.17[ab]**	**1.47 ± 0.23[b]**	**1.52 ± 0.09[b]**	**2.05 ± 0.30[a]**	**n.d.**
Organic acids					
Glycolic Acid	2.24 ± 0.41[b]	3.64 ± 0.58[a]	1.41 ± 0.19[c]	2.08 ± 0.25[b]	n.d.
Fumaramic acid	0.40 ± 0.03[a]	0.58 ± 0.16[a]	n.d.	n.d.	n.d.

Table 4 (continued)

Compounds	ChChl: Fruc	ChChl: Xyl	ChChl: Gly	ChChl: Gly: CA	50% EtOH
Total organic acids	**2.64 ± 0.38^{b}**	**4.22 ± 0.74^{a}**	**1.41 ± 0.19^{c}**	**2.08 ± 0.25^{b}**	**n.d.**
Esters					
Methoxyacetic acid, cyclopenthylethyl ester	0.39 ± 0.12^{a}	0.30 ± 0.09^{a}	n.d.	n.d.	n.d.
Butanoic acid, 3-methylphenyl ester	0.40 ± 0.08^{ab}	0.56 ± 0.10^{a}	0.30 ± 0.01^{b}	0.43 ± 0.05^{a}	n.d.
Total esters	**0.79 ± 0.20^{a}**	**0.85 ± 0.02^{a}**	**0.30 ± 0.01^{c}**	**0.43 ± 0.05^{b}**	**n.d.**

Data are expressed as relative percentage and are means ± SD of three chromatographic analyses. Statistical analysis was done by one-way ANOVA (all: $p < 0.005$) with Tukey's post hoc test (different letters indicate significant differences). CA: citric acid; ChChl: choline chloride; EtOH: ethanol; Fru: fructose; Gly: glycerol; n.d.: not detected; Xyl: xylose

Effect on modulation of digestive enzymes

The effect of orange peel NADES and ethanolic extracts on the activity of pepsin, trypsin, and α-amylase is reported in Fig. 3A-C, respectively. In these assays, the final concentration of substrate and polyphenols was selected according to the standardized static in vitro digestion INFOGEST protocol [55] and to the predicted gut polyphenols concentration [56].

All the extracts were able to increase pepsin activity concerning the control condition (absence of extract) (Fig. 3A), but none of them affected chymotrypsin activity (data not shown). The ChCh: Gly: CA extract was found to decrease significantly the activity of trypsin and amylase (Fig. 3B and C, respectively).

While we are not aware of any studies that have evaluated the effect of the NADES on digestive enzymes activity, there seems to be some conflicting evidence on the impact of polyphenols and polyphenol-rich foodstuffs on it. Polyphenol-rich balsamic vinegar was found to decrease pepsin activity, with no effects on trypsin [57]. Flavonoids and stilbenes have been shown to improve pepsin activity by increasing the enzyme V_{max} [58]. Similarly, Tantoush et al. reported that pepsin-resistant proteins, such as β-lactoglobulin and Ara h 1 and Ara h 2, degrade faster in the presence of catechin-enriched green tea polyphenols [59]. Moreover, Borgonovi et al. recently conducted a comparative study to evaluate the modulatory effect of 25 bioactive compounds on the activity of digestive proteases. This investigation utilized an integrated in vitro and in silico approach, revealing that bioactive compounds may exhibit contradictory effects on proteolytic activity, contingent on the substrate/enzyme combination and the configuration of the bioactivities [60]. Although polyphenols may contribute to blood glucose control through various mechanisms, including the reduction of dietary sugars in the intestine [61], numerous studies have demonstrated that this class of bioactive compounds can inhibit pancreatic amylases and glucosidase activity through a structure-dependent effect [62, 63]. The diverse effects may stem from the binding of polyphenols to either the enzyme or its substrate(s), as dictated by their structure. Enzymes can also be inhibited when their structure is modified by ligands that affect the correct positioning and orientation of substrate and/or catalytic residues [64]. In contrast, polyphenols may act as allosteric effectors or modify enzyme conformation (stabilization) to improve the protein/substrate interaction [65]. In addition, not only do polyphenols reduce glucose absorption "per se", but their microbial secondary metabolites have also confirmed an anti-glycemic effect. As demonstrated very recently by Wu et al. [66], the metabolic conversion of hesperidin by gut microflora produces the aglycone, hesperitin, which has been shown to have significant anti-diabetic properties [67].

Antimicrobial activity

Table 5 compares the antimicrobial activity of NADES and ethanol extracts. The antimicrobial activity of the extracts was found to be similar to that of ethanolic extracts against selected strains of common foodborne pathogens, namely *E. coli*, *S. aureus*, and *S. epidermidis*. None of the extracts had an inhibitory effect on the growth of *Listeria*. An example of the original results of inhibition studies is provided in the Supplementary Material (Figure S1).

Regarding antimicrobial activity of both NADES and NADES-based extracts it is reported that it can be highly variable, depending on the NADES used, extraction matrix, the target microorganism, and the method employed to assess antimicrobial activity [68].

However, extracts obtained with ChChl: Gly: CA had the highest antimicrobial activity towards sensitive species, and retained it after 1/1 dilution of the extract in water (at difference with most other NADES extracts). Noteworthily, the antimicrobial activity of the ChChl: Gly: CA extract was retained after correcting the pH of the original undiluted extract (slightly above 1) to a value close to that of the other NADES extracts (pH 4.2).

This is in agreement with literature data indicating that the antimicrobial activity of NADES and related extracts is greater and more efficient when NADES are based on organic acids, particularly citric acid [68, 69].

Extracts are rich in terpenes and terpenoids, including limonene, linalool, and alpha-terpineol, which are known

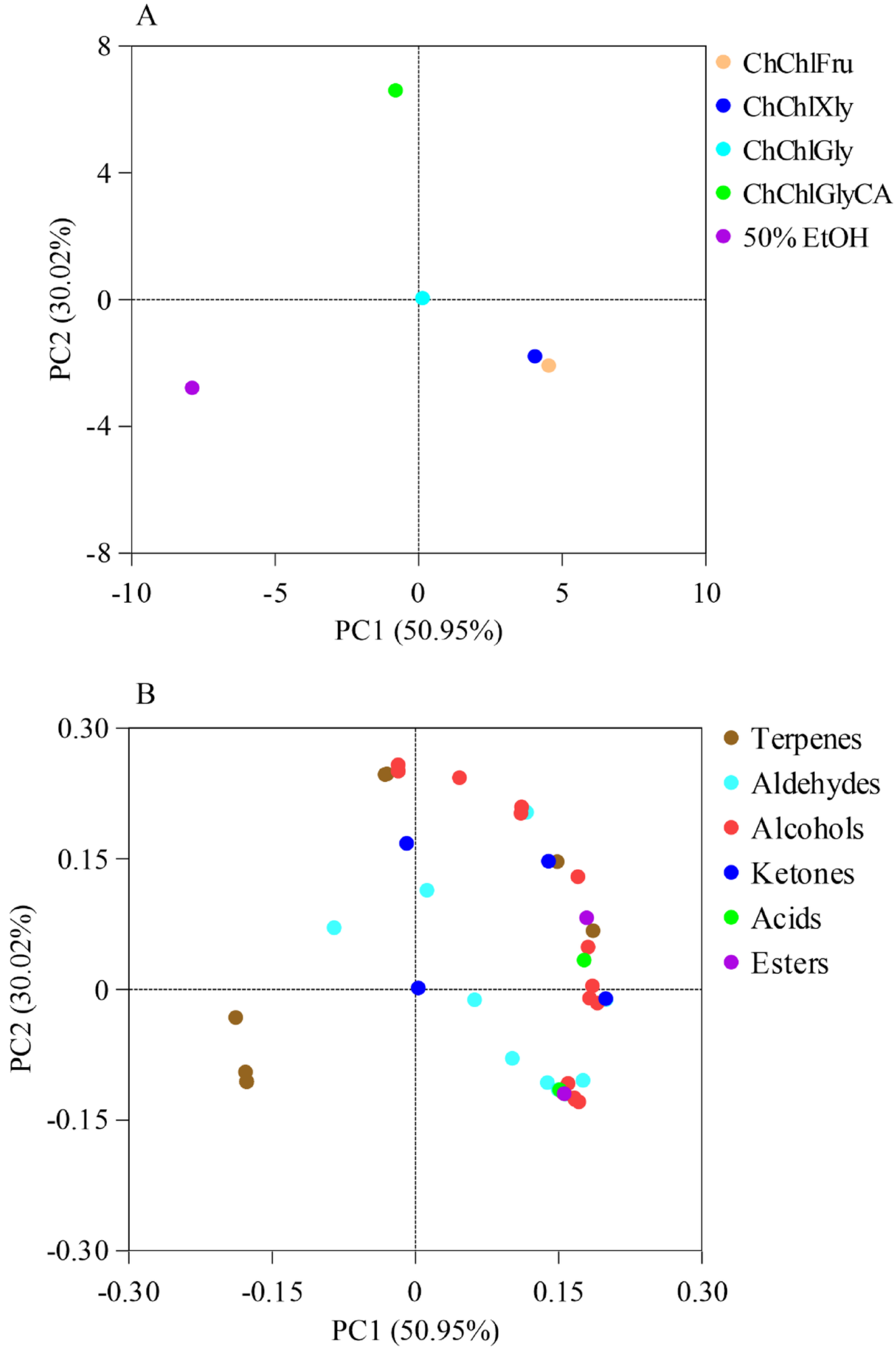

Fig. 2 Representative PCA scores (A) and loadings plot (B) of the whole volatile in the various orange peel extracts. *CA: citric acid; ChChl: choline chloride; EtOH: ethanol; Fru: fructose; Gly: glycerol; Xyl: xylose*

for their high antimicrobial efficacy [70], but also in polyphenols, which are present in high concentrations in the NADES extracts and reportedly exhibit notable antimicrobial activity [71].

Most literature data on the antimicrobial activity of fruit extracts deal with ethanolic and methanolic extracts [72]. This study found the antimicrobial efficacy of NADES extracts similar to that of 50% EtOH extracts, despite the differing composition of the extracts. The available literature data regarding the comparison between NADES and ethanolic extracts are quite variable. For instance, García-Roldán et al. [73] demonstrated greater antimicrobial activity in NADES extracts from spent coffee grounds compared to ethanolic extracts. However, the observed higher

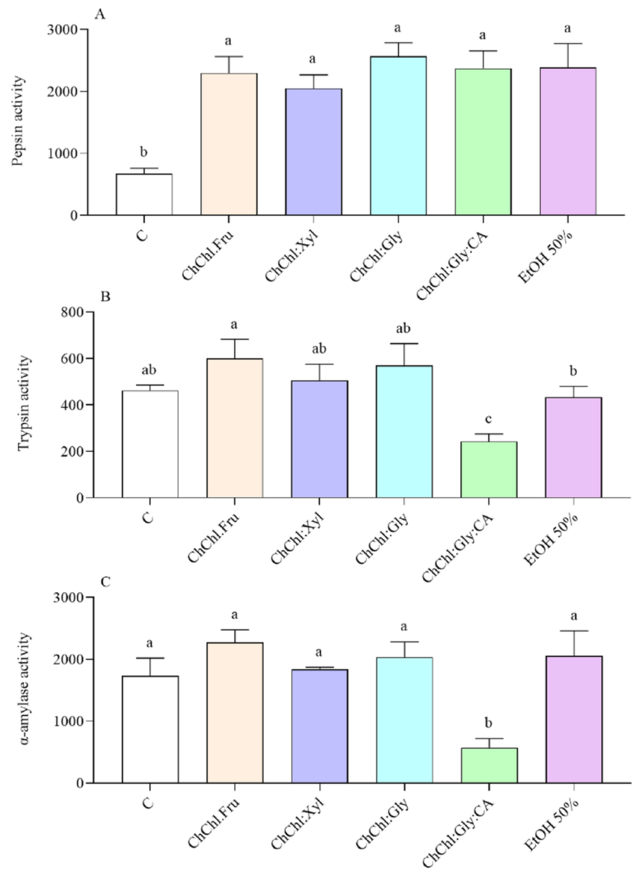

Fig. 3 Pepsin (A), trypsin (B), and α-amylase (C) activity in the absence/presence of orange peel NADES and 50% EtOH extracts. Data are expressed as U/mg enzymes and are means ± SD of at least three replicates. Statistical analysis was by the one-way ANOVA (all: $p < 0.05$) with Tukey's post hoc test. Different letters in the same row indicate significant difference (at least $p < 0.05$). CA: citric acid; ChChl: choline chloride; EtOH: ethanol; Fru: fructose; Gly: glycerol; Xyl: xylose

antimicrobial activity was attributed more to the composition of the NADES themselves rather than the phenolic compounds extracted. Conversely, other authors have reported greater antimicrobial activity in ethanolic extracts compared to NADES-based ones [74].

The sensitivity of microorganisms to natural antimicrobials is reported to be strain-dependent [75]. This finding is corroborated by the results of the present study, which demonstrated that the strain of *L. monocytogenes* used exhibited resistance to all the antimicrobial solutions tested. In contrast, the other strains, comprising both Gram-positive and Gram-negative bacteria, displayed comparable sensitivity to all the antimicrobials tested.

Conclusions

This study shows the promising potential of choline chloride-based NADES as sustainable solvents for the extraction of biologically active compounds from orange by-products, offering a green alternative to traditional organic solvents. The NADES-based extracts exhibited a high phenolic content and a distinctive volatile, indicating their potential as functional and sensorial food enhancers. Furthermore, NADES extracts, particularly ChChl: Gly: CA, showed significant bioactivity, including inhibition of digestive enzymes such as trypsin and amylase activities, suggesting their potential application in developing nutraceuticals. Moreover, the antimicrobial activity shown by the ChChl: Gly: CA extract suggests its suitability as a natural antimicrobial agent in food preservation.

In conclusion, using choline chloride-based NADES to extract bioactive compounds from orange peel exemplifies an innovative and environmentally friendly approach that aligns with the principles of green chemistry and contributes to the sustainable valorization of orange by-products. The findings of this research could suggest that NADES could

Table 5 Antimicrobial activity of orange Peel extracts as determined by the agar well diffusion method

NADES	pH		*L. monocytogenes*	*E. coli*	*S. aureus*	*S. epidermidis*
		Conc.	Inhibition	Inhibition	Inhibition	Inhibition
ChChl: Fruc	4.2	100%	-	+	+	+
		50%	-	+	+	+
ChChl: Xyl	4.01	100%	-	+	+	+
		50%	-	+	+	+
ChChl: Gly	4.28	100%	-	+	-	+
		50%	-	+	-	+
ChChl: Gly: CA	4.03	100%	+	++	++	++
		50%	-	+	+	+
EtOH 50%	4.98	100%	-	+	+	+
		50%	-	+	+	+

Scores are assigned based on the diameter of the inhibition halo: "-", < 8 mm; "+", 8–11 mm; "++", 11–14 mm; "+++", 14-18 mm; "++++", > 18 mm. CA: citric acid; ChChl: choline chloride; EtOH: ethanol; Fru: fructose; Gly: glycerol; Xyl: xylose be applied in food functionalization and preservation, and cosmetic products, thus addressing critical needs for sustainable and non-toxic alternatives in these industries, but further studies should be performed. Future research should prioritize optimizing NADES compositions to enhance extraction efficiency and scalability. Additionally, exploring potential synergistic interactions between NADES and specific bioactive compounds could unlock new applications and favor the transition of this technology to industrial-scale processes, paving the way for broader adoption and impactful commercial outcomes.

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Author contributions CG-U: Formal analysis, investigation, Writing - Original Draft, LS: formal analysis, visualization, Writing - Original Draft, SG: formal analysis, Writing - Original Draft, FP: Writing - Review & Editing, Supervision. JB: Data curation, visualization. MJE: Project administration, funding acquisition, supervision, conceptualization. RL: funding acquisition, project administration. AF: project administration. SI: funding acquisition, project administration, manuscript editing. MDN: Methodology, supervision, project administration, Conceptualization.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Conflict of interest The authors declare no competing interests.

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