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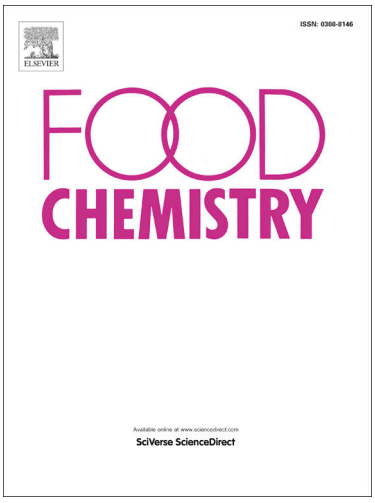

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Sustainable extraction of antioxidants from out-of-caliber kiwifruits

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Abstract

Valorisation of discarded kiwifruits is proposed by extracting bioactive compounds using sustainable solvents namely deep eutectic solvents (DES). A screening of fifteen DES and several hydrogen bonding donor solvents was carried out. Extraction efficiency was measured in terms of antioxidant activity using DPPH and FRAP tests. The influence of solvents characteristics in particular DES structure, presence of ethanol or water, and pH of DES/water mixture on the antioxidant properties of the extracts was studied. Results show that kiwi peels extracts obtained with DES based on carboxylic acids exhibit enhanced antioxidant activity compared to conventional solvents and alcohol-based DES with a maximum DPPH scavenging activity of 42.0 mg TE/g DW. Glycerol or ethylene glycol are also efficient at extracting antioxidant compounds with DPPH scavenging activity of 33.1 and 36.7 mg TE/g DW. Finally, a chemical analysis of extracts using HPTLC revealed that most active compounds extracted are polyphenolic compounds, presumably tannins.

Keywords Kiwifruit by-product; deep eutectic solvent; antioxidant activity; HPTLC analysis

1. Introduction

Industrial production of fruit leads to large quantities of residues discarded every year. For example, fruits that are too small or misshapen are usually discarded, and composting is their main use on an industrial scale, which is a poor way to valorise these by-products, considering the valuable compounds present in them. This problem occurs in particular in kiwifruits production. Kiwifruits are originating from North-Central and Eastern China and have gained over the last decades a worldwide production with growing numbers of cultivars and hybrids. The most commercially significant species are *Actinidia deliciosa* and *Actinidia chinensis* (S. Wang et al., 2021). In 2017, *Actinidia deliciosa* was the most commercialised crop with a total production of about 1.8 million tons (SM et al., 2017). The health benefits of kiwifruits such as antioxidant, anti-inflammatory, and antimicrobial have been described in details (S. Wang et al., 2021). Kiwifruits are known to contain various compounds of interest such as carbohydrates, lipids, fatty acids, proteins, vitamins, phenolic, flavonoids, chlorophylls and carotenoids (Xiong et al., 2021). The extraction of bioactive compounds from out-of-caliber discarded fruits appears attractive in the context of waste upcycling and circular economy.

Several extraction methods of natural compounds from kiwifruit have been reported in the literature. The solvents used for such methods are ethanol, hydroalcoholic mixtures (Aires & Carvalho, 2020; Kheirkhah et al., 2019; Salama et al., 2018; Yutang Wang et al., 2018) or subcritical water (Guthrie et al., 2020; Kheirkhah et al., 2019).

It is currently ongoing a very strong effort in the chemistry community in order to propose alternative and sustainable processes. In most cases, alternative and sustainable solvents are used in such processes. Among them, deep eutectic solvents (DES) are currently very promising candidates as replacements for convention organic solvents. DES are mixtures of hydrogen bond acceptors (HBA) and hydrogen bond donors (HBD) of different acidity (Smith et al., 2014) that, when mixed present a depression in the melting point larger than that expected from an ideal liquid mixture (Martins et al., 2018). The DES formation results from the stronger HB interactions established between the HBA and HBD than those present in the pure compounds. These interactions can be highlighted using Fourier Transform Infrared Spectroscopy (FTIR) (Gautam et al., 2020). DES of different natures were reported, and a classification in five types was proposed according to the chemical class of HBA and HBD (Smith et al., 2014)(Abranches et al., 2019). These solvents have been the focus of intense work in the last decade, and have been applied to a large range of fields, including extraction of natural products from plant raw material (Redha, 2021; Wils et al., 2021). In this field, most DES reported in the literature are type III DES. These are formed from a salt or a zwitterion containing an ammonium, phosphonium, or sulfonium cation, commonly choline chloride or

betaine, and hydrogen bond donors such as amines, carboxylic acids, or alcohols. Among other advantages, these DES are considered as environmentally friendly solvents, exhibit low production cost, are non-toxic and biodegradable. DES are also solvents with tunable properties and polarity because of the high number of HBA and HBD available (Wils et al., 2021) and were, in addition, reported as efficient solvents for breaking plant cell walls (Yinan Wang et al., 2020). All these characteristics made DES very interesting solvents for plant extraction. To the best of our knowledge, there has been no data reported on the production of kiwifruit extracts with DES.

Valorisation of agri-food wastes is currently the focus of numerous works for their potential application to cosmetics, nutraceuticals and even feed industry (Castrica et al., 2019; Fraga-Corral et al., 2021; Piccolella et al., 2019). In the field of cosmetic formulation, agri-food industry and emerging field of nutraceuticals, extracts are expected to exhibit a biological activity, among which antioxidant activity is the most widely looked for (Fraga-Corral et al., 2021).

Polyphenols are currently the most widely targeted antioxidant compounds (Quideau et al., 2011). After their extraction from natural raw material, analysis of polyphenols present in extracts is usually carried out by various analytical methods, including “Folin-Ciocalteu reagent based total phenolic content” (TPC), total flavonoid content, HPLC quantitative analysis of major polyphenols, or in-vitro bioactivity test of antioxidant activity. Up to twenty *in-vitro* methods have been reported to assess the antioxidant capacity (Alam et al., 2013). DPPH test (Brand-Williams et al., 1995), based on the 2,2-diphenyl-1-picrylhyldrazyl radical (DPPH●), and Ferric Reducing-Antioxidant Power (FRAP) assay are fast, widely used, and recognised as reference methods to assess the antioxidant activity. Moreover, they can be automatised and performed in 96-wells plates for fast throughput (Benzie & J, 1996; Plainfossé et al., 2020; Pohanka et al., 2012). Then, the evaluation of the chemical composition of the extracts and in particular the identification of antioxidant compounds extracted towards a DES can be lengthy and tedious, because of the large number of compounds extracted. GC-MS/FID is a powerful technique to identify and quantify the constituent of a complex mixture, but this technique is limited to the analysis of volatile compounds, that is, to say, compounds that can pass into the gas phase at a reasonable temperature and without degradation which is not the case with polyphenols which are often responsible for antioxidant activities. HPLC analysis is a classical method for polyphenols analysis, even though with DES extracts this method is very challenging. Due to the low volatility of these solvents, DES extracts are usually analysed without removing the solvent. Then impurities originating from the DES that are in large amounts in the extract might sorb irreversibly to the HPLC column, damaging it (Sherma, 2010). In addition, concentrating the extract without a specific preparation in order to remove

DES is impossible, which can be an issue to analyse the compounds in traces. Recent works (Agatonovic-kustrin et al., 2021; Nikolaichuk et al., 2020; Pedan et al., 2018) have reported the use of high-performance thin layer chromatography (HPTLC) as a promising method for characterising qualitatively and quantitatively the chemical families of compounds present on extracts and even on natural DES extracts (Liu et al., 2018). This technique is an enhancement of thin layer chromatography, notably in terms of compound resolution of the compounds to be separated. As the development of a method for separation of DES from antioxidants compounds would be difficult since their specific chemical nature was unknown at this stage, HPTLC is advantageous. The HPTLC plates, i.e., the HPTLC stationary phase, are not reused so the extracts could be deposed on the plate without worrying about irreversibly sorbed compounds. On the contrary in HPLC, a sample preparation is necessary to ensure that all the compounds injected would be eluted to not damage the column. Subsequently to the separation of families of compounds on the HPTLC plate, identification of chemical families is easily carried out using derivatisation reagents in particular to understand the chemical nature of the compounds responsible for the antioxidant activities. (Krebs et al., 1980).

In this work, we studied the extraction of natural compounds from kiwi peels and pomace. The fruits used in this study were out-of-caliber, i.e., too small or misshapen and were intended for composting. Such fruits were provided by OUI!GREENS, an association dedicated to collecting agricultural wastes directly from the producers and finding ways to valorize them. A range of 22 solvents, including three organic solvents, four hydrogen bonding donor compounds, namely glycerol, ethylene glycol, acetic acid, and lactic acid, and fifteen DES, based on either cholinium chloride, betaine, or betaine hydrochloride were used. These different HBA have been selected because (i) DES based on these HBA have been widely studied, (ii) cholinium chloride is currently forbidden in cosmetics application according to European regulation (*REGULATION (EC) No 1223/2009*), though accepted in the animal feed industry. Betaine is known for its moisturising properties is allowed (Ertel, 2000; Ship et al., 2007).

Structures and abbreviations are detailed in Tables S1 and 1. The extracts obtained here were analysed by measuring their antioxidant activities using both DPPH and FRAP tests. The influence of the co-solvent nature and amount was also assessed using DES diluted in ethanol or water in different proportions. Finally, HPTLC was used to study the nature of the compounds extracted from kiwi peels.

2. Material and methods

2.1. Plant raw material

Out-of-caliber kiwifruits (*Actinidia deliciosa* Hayward) were obtained from OUI!GREENS, an association that collected discarded kiwifruits. These fruits were initially purchased by OUI!GREENS from a local producer (EARL Le fruit d'Henri, Nimes, France). Peels were manually separated from the pulp and dried for a few hours at room temperature. The juice was separated from the pulp using a domestic centrifugal juice extractor (Aicok AMR516) to recover a pomace which was dried at room temperature overnight. Peels and pomace were then cryogrinded and stored in separated airtight containers in a freezer before conducting extraction experiments.

2.2. Chemicals

All chemicals including cholinium chloride (≥ 98% purity), betaine (≥ 98% purity), betaine hydrochloride (≥ 99% purity), glycerol (≥ 99,5% purity), ethylene glycol (99,8% purity), levulinic acid (≥ 97% purity), 1,2-propanediol (99% purity), D-sorbitol (99% purity), D-glucose (ACS reagent grade), lactic acid (85% FCC purity), citric acid (≥ 99,5% purity), malonic acid (99% purity), acetic acid (ACS reagent grade), urea (≥ 99% purity), 2,2-diphenyl-1-picrylhydrazyl, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (≥ 97% purity), 2,3,5-triphenyltetrazolium chloride (≥ 98% purity), iron (III) chloride hexahydrate (≥ 97% purity), hydrochloric acid (ACS reagent, 37%), formic acid (purity ≥96%), sulfuric acid (purity ≥95%), fast blue B salt (purity ≥95%), p-anisaldehyde (purity ≥98%), 2-aminoethyl diphenylborinate (purity ≥97.0%), ethanol (HPLC grade), methanol (HPLC grade), dimethyl sulfoxide (DNA and peptide synthesis grade), ethyl acetate (purity ≥99.7%) and dichloromethane (purity ≥99.8%) were purchased from Merck (Saint-Quentin-Fallavier, France), and used without further purification. Iron (II) sulphate heptahydrate (purity ≥98.5%) was purchased from Labkem and used without further purification. All abbreviations for all chemicals used in the antioxidants extraction are collected in Table S1, along with some of their physico-chemical properties.

2.3. Deep eutectic solvents (DES) preparation

The DES were prepared by heating the HBA-HBD mixture in an airtight bottle under continuous stirring at 70 °C until a homogenous transparent liquid was observed. For comparison purposes, when possible, a ratio of 1 to 2 mol equivalents of HBA and HBD, respectively was used. Only three DES were prepared using other molar ratios. More specifically, betaine:citric acid and cholinium chloride:malonic acid, were prepared using molar ratios of 2:3 and 1:1,

respectively (Abbott et al., 2006; Percevault et al., 2021). While for betaine hydrochloride : ethylene glycol, due to precipitation issues, different molar ratios (1:1, 1:2, 1:3, 1:5, 1:7 and 1:10) were tested, being choose the ratio of 1:10, since it was the only that did not yield any precipitation when mixed with 20 or 30%, w/w water. All abbreviations of the DES and their compositions used in this work are summarised in Table 1.

Table 1: List of DES used in this work.

Abreviation	Hydrogen bond acceptor (HBA)	Hydrogen bond donor (HBD)	Molar ratio (HBA:HBD)
ChCl:EG	Cholinium chloride	Ethylene glycol	1:2
ChCl :AcOHA	Cholinium chloride	Acetic acid	1:2
ChCl:Glyc	Cholinium chloride	Glycerol	1:2
ChCl:PropOH2	Cholinium chloride	1,2-Propanediol	1:2
ChCl:LevA	Cholinium chloride	Levulinic acid	1:2
ChCl:D-Sorb	Cholinium chloride	D-Sorbitol	1:1
ChCl:D-Gluc	Cholinium chloride	D-Glucose	1:1
ChCl:LacA	Cholinium chloride	Lactic acid	1:2
ChCl:CitA	Cholinium chloride	Citric acid	1:2
ChCl:Urea	Cholinium chloride	Urea	1:2
ChCl:MalA	Cholinium chloride	Malonic acid	1:1
BetHCl:EG	Betaine hydrochloride	Ethylene glycol	1:10
Bet:EG	Betaine	Ethylene glycol	1:2
Bet:CitA	Betaine	Citric acid	2:3
Bet:MalA	Betaine	Malonic acid	1:1

2.4. pH measurements

pH for all extracting phases, composed with 80%, w/w DES or HBD and 20%, w/w water were measured using a Bioblock WTW pH330 pH meter fitted with a WTW SenTix Precision pH electrode. The pH meter was calibrated with pH standard buffer solutions with pH=7.0 and 4.0.

2.5. Viscosity measurement

Viscosities of all DES containing 20%, w/w of water were measured using a Physica MCR 51 rotational Rheometer from Anton Paar fitted with a Plate Cone CP50-1. The measurements

were conducted at 25, 40 and 60 °C for DES/water mixture forming a homogeneous mixture at room temperature. Otherwise, the DES with 20%, w/w of water was stirred at 60 °C in an oil bath until a homogenous transparent liquid was observed, and the measurement was then conducted at 60 °C only. Before each measurement, the sample was equilibrated on the rheometer at the selected temperature and the stability of the temperature was checked for 1 min with a tolerance of ± 0.03 °C. The viscosities were measured for shear rate from 100 to 5000 s^{-1}. The shear rate was increased exponentially in 100 s, and one measurement point each per second was taken.

2.6. Solid-liquid extraction

For all solid-liquid extraction experiments, DES or HBD were diluted in 20%, w/w water, unless explicitly detailed. Kiwi peels or pomace (0.10 g) were then mixed with solvent (1 mL). Extractions were then carried out in a closed test tube, at 60 °C for 1 h under constant magnetic stirring at 500 rpm in an oil bath. For comparison purposes, neat water, water/ethanol mixture containing 20%, w/w water and neat methanol were also used as extracting solvents. After the extraction step, solvents were separated from peels by centrifugation (6000 rpm, 10 min) and the supernatant was filtered using a 0.45 µm syringe filter (WHATMAN, PVDF). 100 µL of the extracts were finally diluted in 900 µL dimethyl sulfoxide (DMSO) and stored in sealed vials at 4 °C before the antioxidant activity assays. All the extraction were performed in duplicates.

2.7. Antioxidant activity assays

The antioxidant activity assays (DPPH and FRAP) were performed in untreated 96-well plates (Greiner bio-one 96 well, PS, F-bottom, clear) using an automated pipetting system Eppendorf epMotion® 5075. Plates were sealed during incubation using adhesive films (Greiner EASYseal clear). All the samples were placed in 1.5 mL Eppendorf tubes, appropriate for the use of the automated pipetting system and analysed in triplicates. All neat solvents and DES used for solid-liquid extraction reported in this study were also analysed, revealing an absence of antioxidant activity (see supporting information, Table S2).

2.7.1. 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical

DPPH assays were carried out as previously reported (Plainfossé et al., 2020). Briefly, 75 µL of an 0.1 M acetate buffer solution at pH 5.4 and 75 µL of ethanol were added into each well, together with 7.5 µL of sample. Accordingly, negative and positive controls were prepared by replacing the sample by dimethyl sulfoxide or a solution of 1 mg/mL of Trolox in DMSO. The plate was vortexed for 2 min, and a first optical density reading (OD) was then performed at

517 nm (OD_{blank}) using a microplate reader (Spectramax Plus 384, Molecular Devices). Data were acquired with SoftMaxPro software (Molecular Devices). Then, 100 µl of a DPPH solution (386.25 µM in EtOH, analytical grade) was distributed in each well. The plate was sealed, vortexed for 2 min and incubated in the dark at room temperature for 30 min. The final OD for a sample (OD_{Final}) was then recorded at 517 nm in order to assess antioxidant activity. Optical densities of samples (OD_{sample}) or controls ($OD_{control}$) were calculated as the final optical density corrected with the blank using the following expression:

$$OD_{sample} \text{ or } OD_{control} = OD_{final} - OD_{blank} \quad (1)$$

Standard curves were obtained by measuring activities of 10 to 275 mg /L Trolox solutions. The antioxidant activity (AA_{DPPH}) of an extract was expressed on a dry kiwi peel or pomace weight basis in milligrams of Trolox equivalents per gram of dry weight (mg TE/g DW) and calculated as follows:

$$AA_{DPPH} = \left[\frac{ODneg\_control - ODsample}{ODneg\_control}\right] \times \frac{V_{extraction}}{V_{analysed} \times w_{peels}} \times A \times V_{sample} \quad (2)$$

with $OD_{neg\_control}$ the final OD of the negative control corrected with the blank, $V_{extraction}$ the volume of extraction solvent (1 mL), $V_{analysed}$ the volume of extract in the sample analysed (100 µL), V_{sample} the volume of the sample (1 mL), w_{peels} the dry weight of kiwi peels extracted (0.1 g) and A the coefficient obtained with the standard curves:

$$C_{Trolox} = A \times \left[\frac{ODneg\_control - ODpos}{ODneg\_control}\right] \quad (3)$$

with C_{trolox} the Trolox concentration, $OD_{pos\_control}$ the final OD of the positive control corrected with the blank, A = 0.576 mg/mL, R^2 = 0.9905

2.7.2. Ferric ion reducing antioxidant potential (FRAP)

FRAP assays were adapted from previously reported procedures (Benzie & J, 1996; Pohanka et al., 2012). FRAP reagent was freshly prepared by mixing 25 mL acetate buffer (0.3 M, pH = 3.6), 2.5 mL TPTZ solution (10×10^{-3} M 2,4,6-Tris(2-pyridyl)-s-triazine in 40 mM HCl), and 2.5 mL of 20×10^{-3} M $FeCl_3$ solution. 150µL of an acetate buffer (0.3 M pH = 3.6) was distributed in each well, together with 7.5 µL of sample. An Iron (II) sulphate solution of 1 mg/mL $FeSO_4 \cdot 7H_2O$ in water and neat DMSO were used respectively as positive and negative controls. The plate was vortexed for 2 min and a first optical density (OD) reading was then performed at 593 nm (OD_{blank}). Then, 100 µL of the FRAP reagent was distributed in each well.

The plate was sealed, vortexed and the final OD at 593 nm was then recorded after 4 min in order to assess antioxidant activity. Optical densities of samples (OD_{sample}) or controls ($OD_{control}$) were calculated using eq. (1). Standard curves were obtained by measuring activities of 12.5 to 333 × 10^{-6} M $FeSO_4$. For clarity reasons, antioxidant activities (AA_{FRAP}) of extracts were reported in micromoles of ferro equivalent per gram of dry weight of kiwi peel (10^{-6} mol Fe^{2+} eq./g DW) and calculated as follows:

$$AA_{FRAP} = (OD_{sample} - OD_{neg\_control}) \times \frac{V_{extraction}}{V_{analysed} \times w_{peels}} \times A \times V_{sample} \quad (4)$$

with $OD_{neg\_control}$ the final OD of the negative control corrected with the blank, $V_{extraction}$ the volume of extraction solvent (1 mL), $V_{analysed}$ the volume of extract in the sample analysed (100 µL), V_{sample} the volume of the sample (1 mL), w_{peels} the weight of dry kiwi peels extracted (0.1 g) and A the coefficient obtained with the standard curves:

$$C_{Fe^{2+}} = A \times (OD_{pos\_control} - OD_{neg\_control}) \quad (5)$$

with C_{Fe}^{2+} the $FeSO_4 \cdot 7H_2O$ concentration, $OD_{pos\_control}$ the final OD of the positive control corrected with the blank.

Note that the FRAP analysis of the extracts obtained with citric or malonic acid was not possible to be performed. As the assays were performed in a buffer at pH = 3.6, and these two acids have a pK_a below 3.6 (see supporting information, Table S1), it means that there is competition between the formation of the ferrous-tripyridyltriazine complex and iron citrate or malonate complexes, being therefore impossible to perform the analysis.

2.7.3. Data analysis

Antioxidant activity data were compiled and analysed using Microsoft® Excel® for Windows (Version 2206). Average and standard deviations between replicates were calculated using the functions AVERAGE and STDEV, respectively. This last function estimated the standard deviation using the “n-1” method as follows:

$$\sigma = \sqrt{\frac{\sum(x - \bar{x})^2}{n - 1}} \quad (6)$$

With σ the standard deviation, x a sample, $\bar{x}$ the average value and n the number of samples. The data were then represented using a clustered column chart showing values in 2D columns. The height of each column represents the average value of replicates with error bars representing twice the standard deviation.

2.8. High performance thin layer chromatography (HPTLC)

HPTLC analyses were performed using Merck (0.20 mm) silica gel 60 F254 (20 cm × 10 cm) glass HPTLC analytical plate using a Camag (Muttenz, Switzerland) HPTLC system equipped with an automatic TLC sampler (ATS 4), an automatic developing chamber ADC2 with humidity control and a visualiser controlled with WinCATS software.

2.8.1. *Sample application and chromatography*

All HPTLC analyses were carried out as previously reported (Do et al., 2014) with a few modifications. First, HPTLC plates were developed from the lower edge of the plate until 70 mm. Before the development of the plates, humidity was monitored within the development chamber and kept between 33 and 38% of relative humidity, for 20 min. Second, the solvent was added and left for 20 min in order to reach saturation within the chamber. Visual check of the plate was carried out under 254 nm and 366 nm and white light in order to confirm the absence of impurities or contaminants. All solutions were applied in triplicates on the same plate, band wise. First track was applied to start at a distance of 15 mm from the edge of the plate. Each track was of 8 mm length, distance between each track was 2.6 mm and spraying speed was 50 nL/s. A mobile phase, previously reported for polyphenols analysis (Shivatare et al., 2013), composed with 100, 11, 11, and 26 mL of ethyl acetate, formic acid, acetic acid and water, respectively, was used for plate development. The plates were then dried for 5 min and recording of the developed plates were carried out under 254 nm and 366 nm and white light. The plate was finely cut into three identical plates using a glass cutter in order to be submitted to different derivatisation.

2.8.2. Post-chromatographic derivatization

Flavanols, further phenolics and tanning agents were visualised with the fast blue B salt derivatising reagent as previously reported (Pedan et al., 2018). With this derivatising reagent these compounds develop a reddish colour on a colourless background. To that end, a dried HPTLC plate was heated at 100 °C for 2 min on a TLC plate heater (CAMAG, Muttenz, Switzerland) and cooled down to room temperature for 1.5 min prior to derivatisation. The derivatisation solution was prepared by dissolving 140 mg fast blue salt B in a mixture of 140 mL methanol, 10 mL water, and 50 mL dichloromethane. Derivatisation was done in the CAMAG TLC Immersion Device III (vertical speed 5 cm/s, dwell time 0 s, CAMAG, Muttenz, Switzerland). After immersion, the plate was dried for 30 s with a stream of cold air using a hair dryer and documented under white light.

Visualisation of flavanols, phenols and further natural compounds was done using NPA solution followed by a p-anisaldehyde derivatisation as previously reported (Pedan et al., 2018) with slight modifications. First, the plate was immersed (vertical speed 5 cm/s, dwell time 0 s) into a NPA solution (1 g 2-aminoethyl diphenylborinate in 200 mL of ethyl acetate), dried for 1 min in a stream of warm air and documented under UV 366nm. Second, the same plate was immersed with a dwell time of 0 s and 5 cm/s dipping into the anisaldehyde derivatisation reagent (0.5 mL anisaldehyde, 10 mL acetic acid, 85 mL methanol, and 10 mL sulphuric acid), warmed at 100°C for 5min and documented under UV 366 nm.

The post-chromatographic derivatisation with DPPH was carried out as previously described (Sethiya et al., 2013). The plate was immersed with a dwell time of 0 s and 5 cm/s dipping into a DPPH solution (0.4 g 2,2-diphenyl-1-picrylhydrazyl in 200 mL of methanol). After immersion, plate was incubated in dark for 30 min before documentation under white light. Using this method, antioxidants appear yellow white on a purple background.

3. Results

A first set of extraction experiments was conducted on out-of-caliber kiwifruits considering two kiwi by-products: peels and pomace – residue of the extraction of juice obtained from kiwi pulp using a centrifugal juice extractor. Both by-products were dried at room temperature before cryogrinding. A first comparison of the extraction from these two raw materials was performed using four different solvents. Methanol was used as a conventional organic solvent, and ethylene glycol as a neat hydrogen bonding donor. ChCl:EG and ChCl:MalA, mixed with 20%,w/w water, were used as typical DES containing cholinium chloride and either a simple polyol or a carboxylic acid HBD compound, accordingly. Since the objective of this work is to obtain biologically active samples, extraction efficiency was measured in terms of antioxidant activity, using DPPH assay. Results are presented in supporting information, Figure S3. Pomace extracts showed very small antioxidant activities when compared to peel extracts, even with the increase of the plant/solvent ratio from 1/10 to 1/4. The best antioxidant activity obtained using kiwi pomace was only of 2.6 mg TE / g DW using ChCl:EG and a plant/solvent ratio 1/10. For comparison, the activities of kiwi peel extracts ranged from 11.4 to 42.0 mg TE / g DW. These results are consistent with recently published data showing that kiwi peels exhibit higher antioxidant activities than pulp (Dias et al., 2020). Another study reported that kiwi seeds contain antioxidant and polyphenolic compounds (Deng et al., 2016). Comparison between seeds and peel has also revealed that the latter contains the highest concentrations of flavonoids, polyphenols and the highest antioxidant activities (Yutang Wang et al., 2018). In our work, it has been decided not to separate seeds from the rest of the pomace because this

task is tedious, and seeds represent only a small fraction of the pomace. During cryogrinding, some seeds were not efficiently ground and pulverized, while some were found intact. This explains the low antioxidant activity of the resulting extracts. Pomace is edible and can be straightforwardly valorized in foods or beverages, and it exhibits low antioxidant properties, thus, the study reported hereafter will focus on kiwi peels.

3.1. Extraction of antioxidants from kiwi peels

Results for the extraction from out-of-caliber kiwi peels carried out using 22 different solvents are presented in Figure 1. In agreement with the literature, all extraction experiments involving DES or an HBD were carried out using a mixture of the corresponding and 20%, w/w water. As before, extraction efficiencies were measured in terms of antioxidant activity, using DPPH and another widely accepted test, the FRAP test. As shown in Figure 1, the results of both tests are in good agreement. It is worth noticing here that all neat solvents' antioxidant activity was measured. Results are presented in supporting information and reveal that solvents exhibit negligible antioxidant activity. Therefore, all data reported here are related to the extracted compounds and not the solvent used.

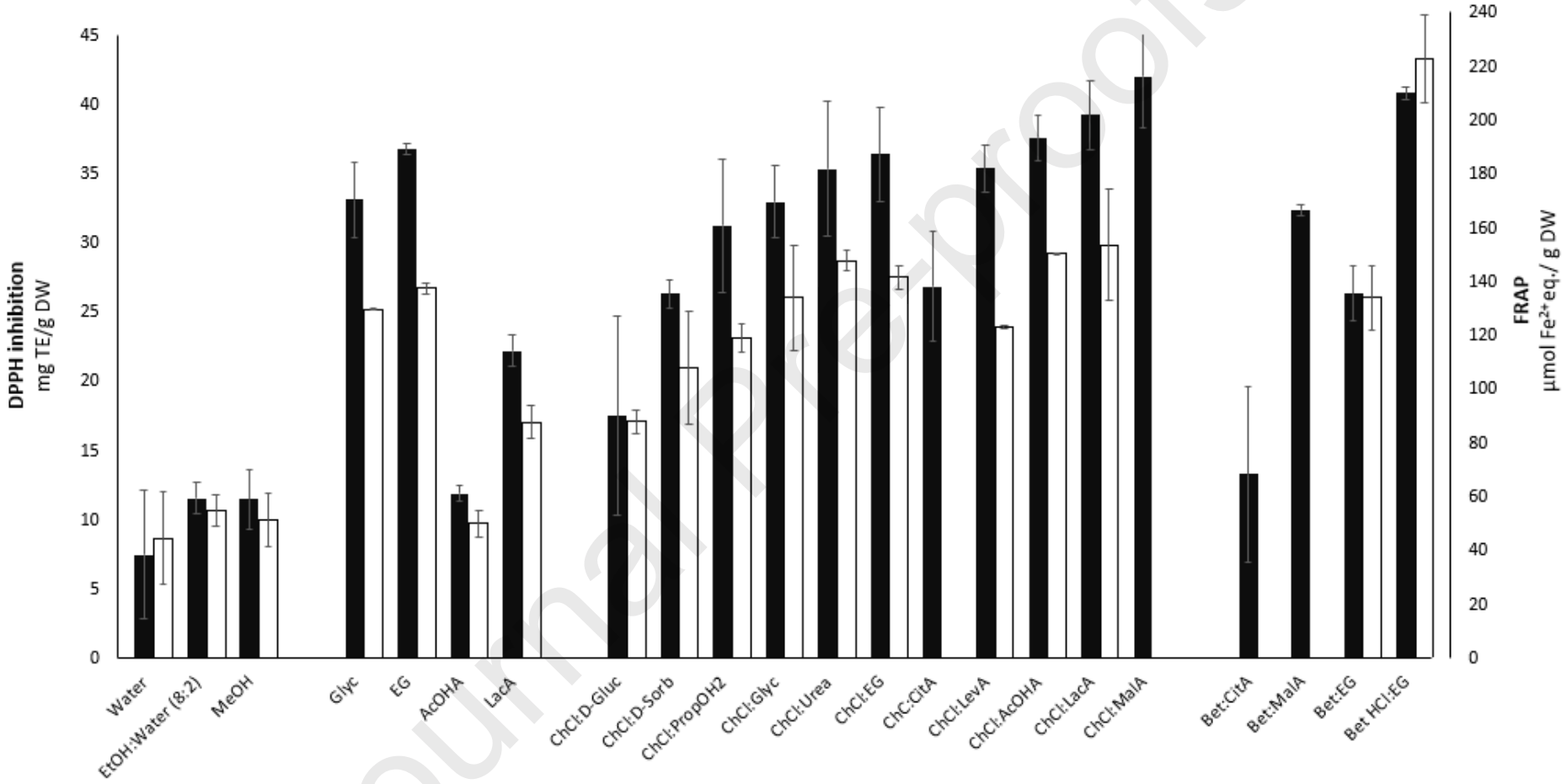


Figure 1: Antioxidant activities measured using DPPH test (black) and FRAP test (white) of the kiwi peels extract according to the solvent used.

Extracts obtained using conventional organic solvents exhibit the lowest antioxidant activities measured in this study. Among these, the highest antioxidant activity was obtained using a mixture of 80 and 20%, w/w of ethanol and water, respectively. Values of 11.4 mg TE/g DW and 54.7 × 10^{-6} mol Fe^{2+}eq./g DW by DPPH and FRAP assays, respectively, were obtained.

Extraction of kiwi peels carried out with four typical HBD, namely lactic acid, acetic acid, ethylene glycol or glycerol diluted with 20%, w/w of water, revealed that extraction using lactic and acetic acid exhibited low antioxidant activities, similar to or up to twice as active as those obtained with an ethanol/water mixture. On the opposite, glycerol and ethylene glycol exhibited a high antioxidant activity of 33.1 and 36.7 mg TE/g DW and 129.4 and 137.2 × 10^{-6} mol Fe^{2+}eq./g DW for DPPH and FRAP, respectively. All extracts obtained with DES exhibit activities much higher than those obtained using conventional organic solvents. Values for the activities of all DES extracts measured using DPPH and FRAPP ranged between 17 and 42 mg TE/g DW and between 87 and 220 × 10^{-6} mol eq. Fe^{2+}eq./g DW, respectively. Interestingly, for all DES based on cholinium chloride and a cyclic or linear polyol, antioxidant activity is generally lower or similar to those obtained using glycerol or ethylene glycol alone questioning the interest of using cholinium chloride in order to extract antioxidant compounds from kiwifruit. Only ChCl:Urea and ChCl:EG yields extracts with similar antioxidant activities.

On the contrary, when cholinium chloride is used with a carboxylic acid, the antioxidant activities of the resulting extracts are significantly higher than those obtained using just lactic or acetic acid. Furthermore, activities of ChCl:MalA extracts measured using DPPH are higher than those obtained using ChCl:EG, implying a synergistic or at least an additional effect between cholinium chloride and a carboxylic acid extracting antioxidant compounds from kiwi peels. The activities measured by the FRAP assay show a somewhat different behaviour. The activity of the extract obtained using ChCl:LevA is lower than that obtained using ChCl:PropOH2, whereas ChCl:LacA and ChCl:AcOHA exhibit activities similar to ChCl:EG. ChCl:MalA and ChCl:CitA were not tested with the FRAP assay because of complexation issues occurring between iron ions from FRAP and malonate or citrate ions, accordingly.

In order to study the influence of the nature of HBA on extraction of antioxidant compounds from kiwi peels, five additional DES based on betaine or betaine hydrochloride were tested. Citric acid, malonic acid, glycerol, and ethylene glycol were used as HBD. Results show that the presence of betaine yields extracts exhibiting lower activities than those obtained using cholinium chloride homologues.

DES based on betaine and citric acid, ethylene glycol and malonic acid exhibited antioxidant activities of respectively 13.2, 26.3 and 32.3 mg TE/g DW. These values are systematically lower than those of their cholinium chloride homologues. Nevertheless, it appears that changing from betaine to betaine hydrochloride, yields a significant improvement in the activity of the extract. A value of 40.8 mg TE/g DW is obtained for BetHCl:EG, which slightly exceeds

that obtained for the cholinium chloride-based homologue. The latter yields an extract exhibiting the highest antioxidant activity obtained in this study using the FRAP assay. This shows that some specific additional effects are occurring when a chloride salt is used as an HBA.

Properties of all DES based on choline chloride used in the study were previously reported in the literature (Rodríguez-Llorente et al., 2020). Many of them were characterised using FTIR to reveal some specific interactions occurring within a DES. Such interactions were evidenced by the appearance of bands that are not existing in neat HBA or HBD.(Delgado-Mellado et al., 2018) In addition, when FTIR was used together with NMR and quantum chemistry calculations the results revealed that the formation of DES based on choline chloride and polyols is due to hydrogen bond interaction between the chloride anion of choline chloride and a hydrogen atom in the hydroxyl groups of polyols (H. Wang et al., 2019). Similar interaction was found between ChCl and carboxylic acid. In addition, for such carboxylic acid containing DES, hydrogen bond formation was observed between a double bonded oxygen in carboxylic acids and the hydroxyl group of choline chloride, along with weak hydrogen bonds between hydrogen atoms in methyl groups of choline chloride and the oxygen of the acid group.(Gautam et al., 2020) The improvement in antioxidant activity of extracts using DES compared to conventional organic solvents could be due to these particular interactions occurring between DES components and to surprisingly high solubility in DES of natural compounds as already reported in the literature (Sepúlveda-Orellana et al., 2021; Soares et al., 2017; Vieira et al., 2018). In the work form Sepúlveda-Orellana et al., an aqueous solution of DES based on cholinium chloride and levulinic acid, ethylene glycol or glycerol, accordingly, was shown to improve the solubility of gallic acid compared to its solubility in water or in an aqueous solution containing only its corresponding HBD. Soares et al. (2017) described important solubility enhancements of polyphenolic compounds in DES aqueous solutions. Authors studied the solubility of monomeric compounds from lignin, such as syringaldehyde, syringic acid, vanillic acid and ferulic acid in DES:Water solutions and reported a hydrotropic mechanism for the dissolution of such compounds in DES solutions, related to the presence of dispersive interactions. Finally, Viera et al, reported similar improvements during extractions of phenolic compounds from *Juglans regia L.*. In this study, the highest extraction yield of phenolic compounds has been obtained using DES containing choline chloride and butyric or phenylpropionic acid in mixture with 20%w/w of water. DES were also reported to be more efficient at breaking and penetrating plant matrix compared to organic solvents. Such a phenomenon has already been reported in the literature using scanning electron microscopy (Yinan Wang et al., 2020).

For the same HBA, namely cholinium chloride, it appears that the number of alcohol and carboxylic acid groups on a HBD has a significant influence on the solubility of antioxidant compounds, hence the activity of the extract. Glucose and sorbitol, containing five and six

hydroxyl groups, respectively, yielded extracts poorer in antioxidant compounds, when compared with the extract obtained with ethylene glycol or glycerol. Similarly, citric acid containing three carboxylic groups yield solvent that is poorer than malonic acid or lactic acid at extracting antioxidants. These results are in fair agreement with previous works (Hong et al., 2020; Soares et al., 2017). Solubility of syringic acid within a DES was reported to decrease with the number of carboxylic groups on a HBD and with HBD polarity (Soares et al., 2017). The solubility of lignin, a polyphenol-based polymer, was found to decrease with the number of hydroxyl or carboxylic groups. Furthermore, it was also claimed that an increase in H-bond interactions occurring within a DES due to the presence of hydroxyl groups hinders interactions with a solute and its related solubilisation (Hong et al., 2020).

Finally, as stated previously, viscosity was reported to be related to the strength of the H-bond network within ad DES and to have, to some extent an influence on the extraction efficiency of antioxidants towards polyphenols (Hong et al., 2020). Viscosity values of all DES studied here were thus measured at 60 °C, the extraction temperature. Data are collected in Table S6. Plotting DPPH scavenging activities of extracts as a function of the viscosity of the extracting phase, as shown in Figure S5, reveals that the larger the amount of carboxylic acids, such as in citric acid, or hydroxyl groups, such as glucose and sorbitol the higher the viscosity and the lower the antioxidant activity of the corresponding extract. On the opposite, when DES exhibits viscosity values below 20 mPa.s, no straightforward correlation was observed between viscosity and antioxidant activity. This shows that the extraction of antioxidant compounds is most probably limited by mass transfer when DES exhibits a viscosity typically above 20 mPa.s and by solvation, dominated by other factors such as structure, pH or solvent polarity, below this value.

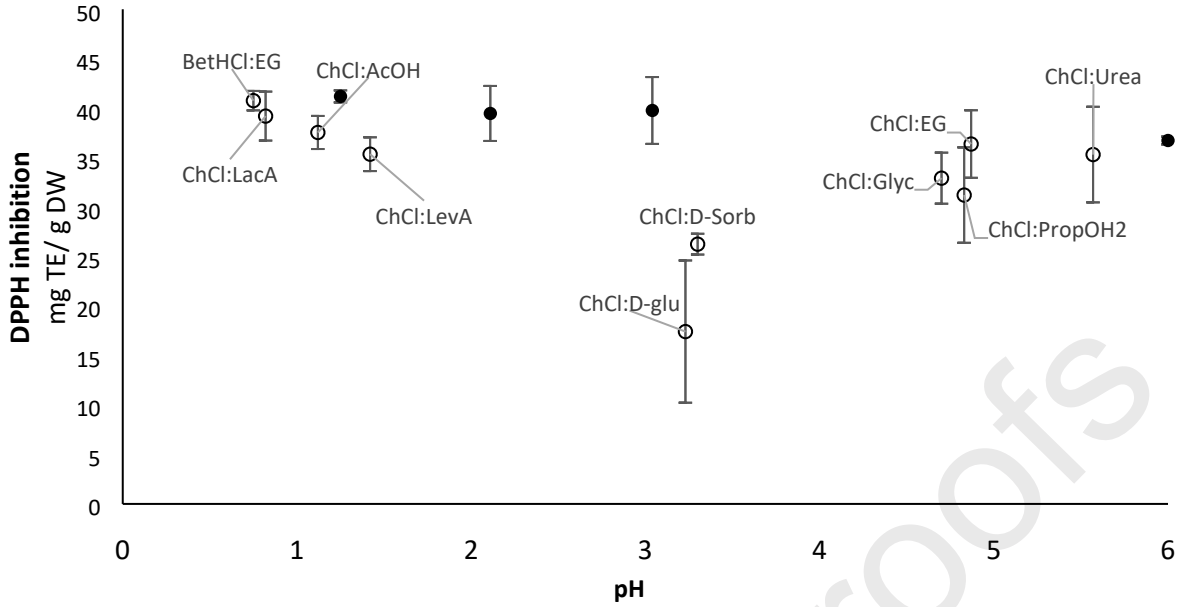
Overall, it appears that DES are promising extracting solvents when prepared using a carboxylic acid such as lactic or acetic acid. Because cholinium chloride is regulated and because DES based on betaine hydrochloride present antioxidant properties similar to or higher than those from cholinium chloride-based extracts, these DES present a good alternative to cholinium chloride. Furthermore, extracts obtained using ethylene glycol or glycerol exhibited antioxidant activities similar to those obtained with DES-based extracts. Considering the toxicity of ethylene glycol (Brent, 2001) and the fact that glycerol is currently used in cosmetics products as a humectant, providing moisturising and healing qualities to the skin (Overgaard Olsen & Jemec, 1993), the latter therefore appears to be a very good candidate as well for extracting antioxidant compounds from kiwi peels.

3.2. Influence of pH and co-solvent within DES

Further insights into the reasons for such increases in antioxidant activity using carboxylic acid-based DES were investigated by measuring the pH of different extracts based on cholinium

chloride or betaine hydrochloride. Because the influence of solvent pH on extraction yield was reported previously, pH values for DES/water mixture were measured and reported in table S5. Such pH measurement for DES using a simple pH meter was previously reported (Chen et al., 2019; Omar & Sadeghi, 2020). As shown in Figure 2, acidic DES exhibit pH values ranging from 1 to 3, lower than those measured for non-acidic homologues. pH values follow pKa values for the corresponding acids except for acetic acid and levulinic acid for which an inversion is observed. It also appears that the lower the pH, the higher the antioxidant activity, even though this increase is small. When ethylene glycol, mixed with 20%, w/w water containing various concentrations of HCl was used as an extracting phase, the resulting antioxidant activity of these acidified extracts decrease with pH. Antioxidant activity of ethylene glycol at pH 1 appears to be very similar to that of ChCl:LacA, a DES also exhibiting a pH of 1. The assumption of such a pH effect on the extraction of antioxidant compounds is in agreement with previous reports revealing that the optimal conditions for extraction of kiwifruit residues using ethanol or subcritical water is obtained at pH 2 (Aires & Carvalho, 2020; Guthrie et al., 2020). This is probably because acidic conditions increase the stability or the solubility in the Water/DES extracting phase of polyphenolic compounds, the main compounds expected to be responsible for the antioxidising activity of kiwifruit peel extracts. In the case of BetHCl:EG, the enhanced activity observed here appears to be due to the combined effects of i) the molecular structure of betaine hydrochloride, which embeds a COOH functional group and a chloride anion, and ii) the pH of the extract, since BetHCl:EG exhibited the lowest pH measured here.

Overall, these results suggest that two extraction mechanisms may play in such systems: if the viscosity of DES is high, this parameter limits the extraction efficiency of this solvent. On the contrary, if the solvent's viscosity is sufficiently low, typically below 20 mPa.s, pH seems to be the parameter controlling the antioxidant extraction.



517

518 **Figure 2:** Plot of the antioxidant activity measured using DPPH test for a given extract obtained
519 with a DES containing 20%, w/w water as a function of the solvent pH. Black dots represent
520 extracts obtained with EG and 20%, w/w water at different pH (see text for details).

521

522 As stated previously and in agreement with the literature, all extraction experiments were
523 carried out using 20%, w/w of water. In order to study the influence of this co-solvent on the
524 extraction abilities of DES, water was first replaced with ethanol. This solvent was chosen
525 because (i) it is a sustainable solvent produced from renewable resource and biodegradable
526 (ii) ChCl-based DES are soluble in ethanol, (iii) this solvent has properties significantly different
527 from water in particular in terms of polarity and (iv) it is a solvent classically used for plant
528 extraction and acceptable in food or cosmetic applications (Lachenmeier, 2008). To that end,
529 four DES, that gave significant antioxidant activities, namely ChCl:EG, ChCl:LacA, ChCl:Gly
530 and ChCl:Urea, were used. Results are reported in Figure 3, along with those obtained using
531 water.

532

533

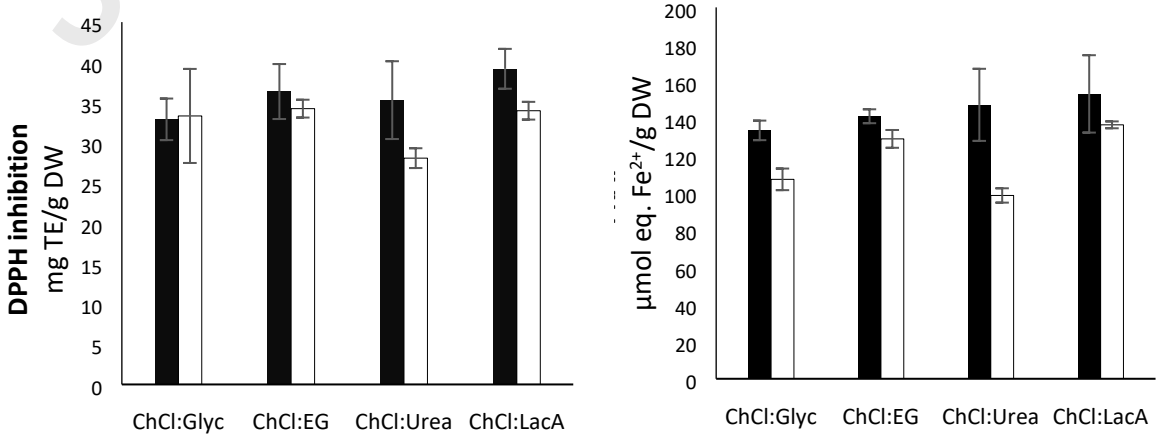
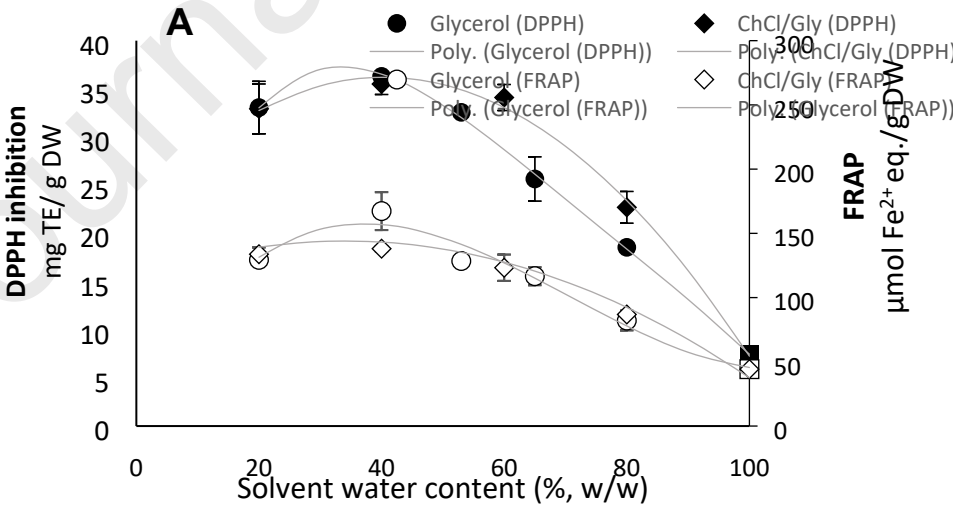


Figure 3: Antioxidant activities measured using DPPH test (histogram on the left) and FRAP test (histogram on the right) of the kiwi peels extracts with DES/water (black) or DES/EtOH (white)

In all cases, ethanol yielded antioxidant activities similar to or slightly lower than those obtained using water as a co-solvent. At a percentage of 20%, w/w, the cosolvent effect is small. Therefore, water was kept as co-solvent for the rest of this study.

Finally, the influence of water concentration on the extraction yield was evaluated. Extraction experiments using several aqueous solutions of DES with various water concentrations were carried out here. Glycerol, ChCl:Glyc , lactic acid and ChCl:LacA mixed with 20, 40, 50, 60 or 80%, w/w water were used as extracting solvents for kiwi peels. Results are shown in Figure 4, along with those obtained using pure water as an extracting solvent. It was found that activity, both with DPPH and FRAP, slightly increases when changing from 20 to 40%, w/w water before decreasing continuously down to the values of the extracts obtained using pure water. On the contrary, activity at 20 or 40%, w/w water in ChCl:Glyc appears to be similar whatever the test used. Similar results are obtained for ChCl:LacA. The only discrepancy between DPPH and FRAP is observed with lactic acid. In the former, activity is held constant between 20 and 50%, w/w, after which it decreases. In the latter case, activity appears to increase significantly between 20 and 50%, w/w of water and then steeply decrease. Above 40-50%, w/w of water, the system is changing from a hydrated DES to an aqueous solution of DES with interactions, as well as solvation capability more akin to that of water than DES.



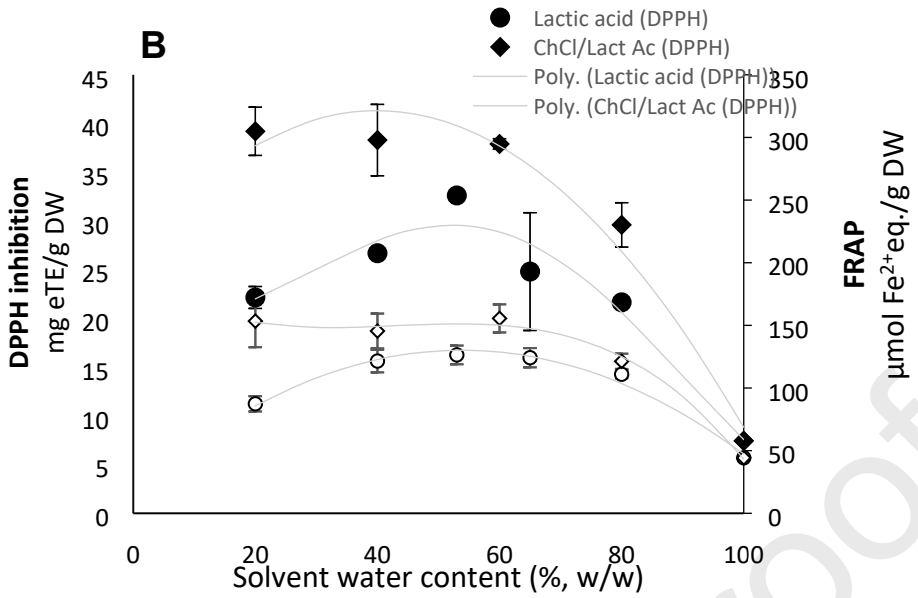


Figure 4: Antioxidant activities for kiwi peels extracts as a function of the amount of water in the extracting phase. Full black symbols correspond to DPPH test results. Empty symbols correspond to FRAP test results. **(A)** Extract obtained using glycerol (circle) or ChCl:Glyc (diamond). **(B)** Extract obtained using lactic acid (circle) or ChCl:Lac acid (diamond). Grey lines are guides to the eye.

These results are in agreement with several studies regarding the nature of the interactions occurring in DES:water mixtures and whether such solutions can be considered as an eutectic solvent or a simple aqueous mixture. Study of the nanostructure of a system cholinium chloride/urea/water by neutron scattering revealed that the eutectic mixture remains as such until 42%, w/w of water. Above that concentration, the mixture is similar to an aqueous mixture (Hammond et al., 2017). Another study of the structure of the ChCl:Urea, ChCl:Glyc and ChCl:EG by NMR techniques (such as ^{1}H, ^{13}C, correlation experiments, NOESY, ROESY, and diffusion experiments) showed that the system retains an eutectic character when diluted with 10 wt% of water, compared to 90%, w/w water (Delso et al., 2019). These results support the idea that a supramolecular structure still exists corresponding to a DES even when a reasonable amount of water is added. Our results are in surprisingly good agreement with these previous literature data, since our data suggest a modification of the properties of the extract when water exceeds 40%, w/w. The existence of such a supramolecular structure, or at least specific hydrogen bonding interactions typical of those found in eutectic solvents, most presumably accounts for these enhanced extraction properties of DES, when up to 40%, w/w water is added to the extracting phase.

3.2. Chemical composition of extracts

In order to obtain information about the chemical nature of the compounds responsible for the antioxidant activities, selected extracts were analysed using high performance thin layer chromatography (HPTLC). To that end, four detection reagents were used, namely natural product reagent A (NPA) solution for flavonoids and vegetable acids, p-anisaldehyde for sugars and terpenes, fast blue B salt for phenolics and tanning agents and DPPH for the compounds responsible for the antioxidant activities (Pedan et al., 2018; Sethiya et al., 2013). For comparison purposes, a track has been devoted to the deposition of a mixture of polyphenols, namely gallic acid, protocatechuic acid, (+)-catechin, chlorogenic acid, caffeic acid, (-)-epicatechin, ferrulic acid, quercetin and rutin because they are common antioxidants. The resulting profiles are reported in Figure 5.

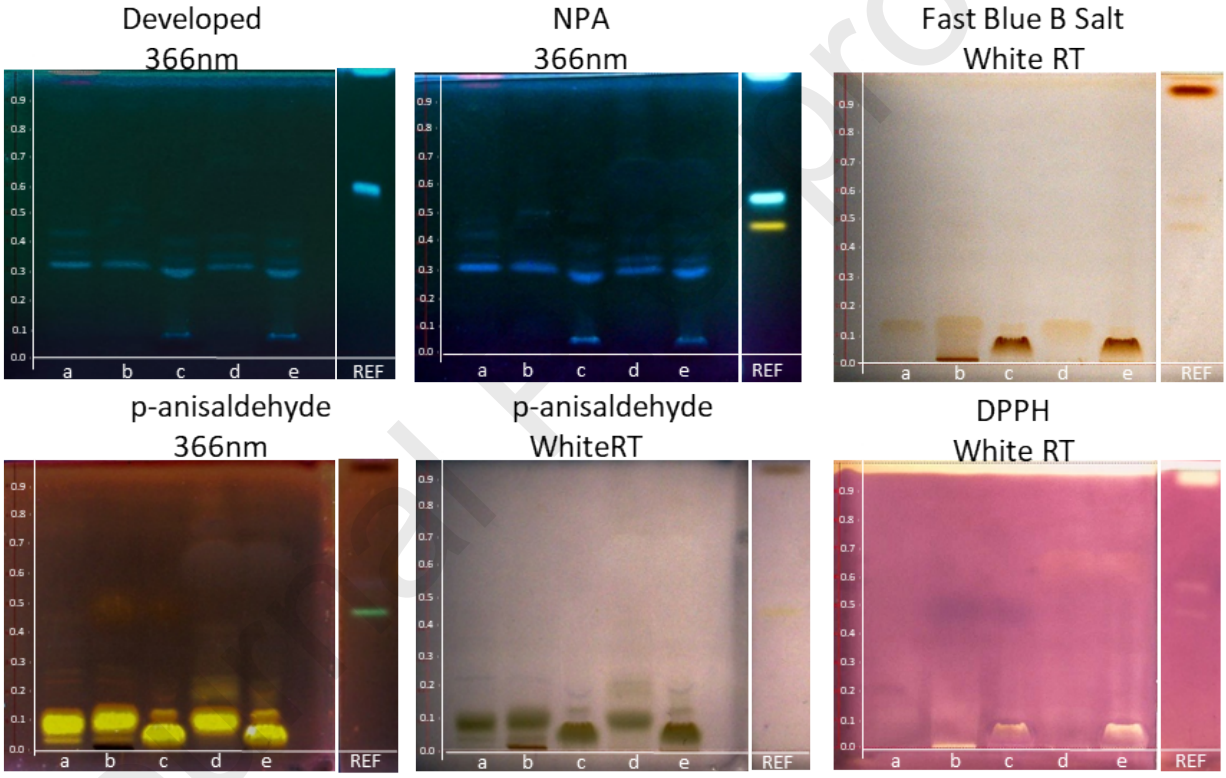


Figure 5: HPTLC profiling of kiwi peels extracts. For each snapshot: (a) EtOH/water mixture (80%,w/w ethanol). (b) Ethylene glycol (EG). (c) ChCl:EG. (d) LacA. (e) ChCl:LacA. (REF): mixture of gallic acid, protocatechuic acid, (+)-catechin, chlorogenic acid, caffeic acid, (-)-epicatechin, ferrulic acid, quercetin, rutin

With NPA, whatever the extract, no orange spot was observed. Such a result reveals the absence of flavanols in the extracts. Chlorophylls, revealed by a red colouration under NPA derivatisation, were only present in the ethanolic extract. No other extract showed the presence of chlorophyll, indicating that the DES used are selective to the target compounds. Using

p-anisaldehyde, the absence of blue or purple spots in our extracts under white light revealed an absence of terpenes. Green diffuse spots observed under white light and yellow spots observed under 366 nm wavelength light at retention factor (Rf) values ranging between 0.05 to 0.15, though still subject to the discussion are expected to correspond to sugars or polyphenolic groups (Ashraf et al., 2021). Derivatisation using fast blue B salt reveals phenolics, amines and tanning agents under the form of reddish orange spots (Pedan et al., 2018). For all samples, a band at the bottom of the plate gave an orange spot, implying the presence of compounds of these chemical families in the extracts.

Finally, derivatisation using DPPH helps identify which compounds are responsible for the antioxidant activities of the extracts. Such an activity is revealed as white/yellow spots on a purple background (Sethiya et al., 2013). Results show that extract **a**, namely the hydroethanolic extract, exhibits no spot, but a slight decolouration throughout the plate. For extracts **b** and **d**, antioxidant activities are located on the bottom of the plate, at Rf values below 0.05. This value is low compared to the position of model polyphenols, as found in the so-called reference sample. This suggests that the extracted antioxidant compounds are different in nature from the model molecules used here. In the case of extracts **c** and **e**, namely those containing cholinium chloride, the Rf value for antioxidants are located between 0.05 and 0.1. When a plate prepared using ChCl:EG and ChCl:LacA, was developed under the same conditions as that used for our extracts, a diffuse spot was observed under 254 nm between Rf values of 0.05 and 0.1. Because such DES are not active with DPPH, the spot observed using samples **c** and **e** are probably due to antioxidant compounds eluting with DES, and therefore expected to be located below 0.1 in the absence of DES. The nature of these compounds is thus expected to be similar to that observed in samples b and d. Comparing spot intensities (I) obtained upon DPPH derivatization, the following trend is observed: $I_{\mathbf{e}} > I_b > I_{\mathbf{c}} > I_d > I_{\mathbf{a}}$. This trend is in full agreement with the FRAP or DPPH antioxidant activities measured previously.

Finally, the spots observed using fast blue and DPPH derivatisation are found to be identical. One can therefore ascertain that the compounds responsible for the antioxidant activity of extracts studied here are the same as those revealed by fast blue, a derivatisation agent revealing the presence of polyphenolic compounds. Because the spot observed for extracts **b** to **e** are much lower than those observed for model phenolic compounds, polyphenolic compounds present in extracts are heavier and more hydrophilic than model compounds selected here. Furthermore, polyphenolic compounds exhibiting a low Rf value under our experimental and reacting with fast blue have been previously reported as a specific category of polyphenols, namely tannins (Asha & Lizzy, 2017). The latter has also been reported to exhibit antioxidant activity using classical DPPH tests (Ricci et al., 2019). In addition, the presence of tannins is supported by an experiment carried out using Vanillin-HCl test for tannin.

Briefly, condensed tannins exhibit a characteristic red colour in the presence of a solution of vanillin and chlorhydric acid. Details can be found in the supporting information file and in Figure S7. The vanillin-HCl test was carried out on two extracts, namely those obtained with ethanol-water or ChCl:AcOHA solvents, accordingly. Both extracts exhibited this characteristic red colour, confirming the presence of condensed tannins.

Overall, the extract tested seems to contain similar compounds namely sugars, polyphenols and a significant amounts of tanning agents as major antioxidant compounds. Only the ethanolic extract seems to contain different compounds namely chlorophylls. Further investigations into the chemical compositions of extracts and, in particular, the extracted compounds' structures will be carried out shortly.

4. Conclusion

The results reported in this work show that DESs are promising solvents for extracting antioxidant compounds from out-of-caliber kiwi peels. A screening of 15 DES based on cholinium chloride, betaine or betaine hydrochloride as HBA and polyols or carboxylic acids or urea as HBD was carried out. The highest antioxidant activities were obtained for extracts based on DES containing a carboxylic acid or betaine hydrochloride. Extracts obtained with glycerol or ethylene glycol exhibited antioxidant activities similar to those of their cholinium chloride-based homologues. This result raises questions related to the relevance of using cholinium chloride along with non-acidic and liquid at room temperature HBD compounds. The study of the influence of water on extraction revealed that up to 40%, w/w water could be added to the system without a negative impact on the antioxidant activity of an extract. Replacing water by ethanol did not have an influence on the antioxidant activity of the extracts.

Based on these results, the application of DES to extract active ingredients for nutraceutical or animal feed applications appears feasible. In the case of cosmetic applications because cholinium chloride is forbidden in cosmetic products (*REGULATION (EC) No 1223/2009*), and should be stripped from the extract prior to its use in cosmetics formulations. Furthermore, results obtained with neat ethylene glycol or glycerol, reveal that these compounds are very promising extracting solvents for obtaining active extracts usable in all applications mentioned above. Because of its interesting properties and because it is available as a food grade and not regulated in cosmetics applications, glycerol might be the most interesting extracting solvent reported here.

Finally, a preliminary study on the nature of extracted compounds was carried out using HPTLC method. For all samples, the active compounds are similar, and according to our results, these are most probably tanning agents, compounds from the polyphenols family. Phytochemical characterisation of the extracts in order to precisely identify and quantify active compounds within extracts will be carried out shortly using HPLC/HRMS. This will allow to

identify accurately active ingredients responsible for the antioxidant activities of an extract and gain better insight into the extraction mechanisms at play within a DES. Finally, further evaluation of the biological activities of extracts will be carried out using more specific tests such as collagenase, hyaluronidase, tyrosinase, elastase or lipoxygenase inhibition tests.

Conflict of interest

The authors have declared no conflicts of interest.

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Highlights

Recycling process for an agri-food waste: discarded kiwifruits

Sustainable extraction from kiwi peels

875 Extracts obtained using edible deep eutectic solvents

876 Extracts exhibiting high antioxidant activities

877 Optimisation of extraction parameters

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