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Fractionated extraction of polyphenols from mate tea leaves using a combination of hydrophobic/ hydrophilic NADES

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ABSTRACT

A new methodology for the selective extraction of antioxidants from mate tea leaves (and decaffeinated mate tea leaves), using different natural deep eutectic systems (NADES), is reported in this paper. A fractionated extraction was carried out and the optimization of the extraction conditions such as solid/liquid ratio, temperature, time, stirring and the use of ultrasound assisted extraction (UAE) technology was performed. The results demonstrate that a sequential extraction using, in a first step, an hydrophobic system Men:Lau (2:1) and, in a second step, an hydrophilic lactic acid-based NADES, leads to two distinct extracts: the first one rich in pigments and the second one rich in polyphenols.

NADES systems were able to extract 30% more of the polyphenolic components of the mate tea leaves matrices, when compared with traditional solvents/techniques. Moreover, it has been shown that the incorporation of the extract in the NADES, compared to the same extract in aqueous medium was beneficial for the stabilization of the antioxidants. It maintains their functionality at least for three months, reaching 41% more versus the extracts obtained by traditional solvents/techniques. The absence of caffeine in the extracts did not shown to have any effects on the stability results.

1. Introduction

Superfoods have gained substantial reputation due to their vast benefits, and consequently, their consumption has become popular in a healthy lifestyle. The valuable properties of these type of functional foods are related with their bioactive ingredients, which can be reflected in the wealth of phenolics, protecting the human body from damage rising from oxidative stress, improving the quality and nutritional value of food (Ferlemi and Lamari, 2016; Gullón et al., 2016; Salo et al., 2021; van den Driessche et al., 2018). Mate tea leaves (*Ilex paraguariensis* St. Hil.) are an important source of natural compounds and their derivatives, recognized as phytochemicals, bring many health benefits (Gawron-Gzella et al., 2021). Their therapeutic properties (anti-inflammatory, anti-cancer, antioxidant, anti-obesity and cardioprotective), revolutionized the Mate market, which is a product commercialized worldwide (da Silveira et al., 2017; Gullón et al., 2018). According to literature, mate tea leaves extract has a very strong antioxidant ability, compared to even green tea, a powerful antioxidant product (Konieczynski et al., 2017).

Mate tea leaves extracts are mainly composed by phenolic compounds (chlorogenic acid), methylxanthines (caffeine, theobromine and theophylline), flavonoids (rutin, quercetin and kaempherol), saponins, amino acids, minerals and vitamins (Gómez-Juaristi et al., 2018; Heck C. I. and Mejia de E.G., 2007). The methylxanthines present in mate tea leaves, in particular caffeine, are responsible for limiting the consumption of this product by a part of the population. Caffeine is considered as the main active component of mate tea leaves. It acts on the central nervous system, stimulates heartbeat (leading to tachycardia in some cases) and originates dilation of peripheral vessels. It also acts on the basal metabolism and increases the production of gastric fluid. In small doses, caffeine decreases fatigue but when ingested in excess can cause, in some cases, negative effects such as irritability, anxiety, headache and insomnia. It is a substance with many applications, in functional food supplements, in pharmaceutical drugs, as well as in the composition of

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cosmetic products due to its biological activity and ability to penetrate the skin barrier (Herman and Herman, 2012; Zapata et al., 2020).

This has led to the development of processes for total or partial removal of this compound and the production of decaffeinated mate tea leaves. The most used technologies for decaffeination are the extraction processes by organic solvents (ethyl acetate and dichloromethane) and supercritical extraction, a method of separation and purification, that uses carbon dioxide (CO_2) and water as solvents (Cassel et al, 2010).

Another drawback of mate tea leaves is the fact that recently, they started to be associated to cancers cases, leading to questioning the real benefits of the tea (Dasanayake et al., 2010; Okaru et al., 2018; Ronco et al., 2017). However, it was proven that the actual cause for the cancer cases was the temperature at which the tea was consumed. One possible solution is to extract the beneficial compounds, e.g., polyphenols and alkaloids, from mate tea leaves and incorporate them into functional foods (Frizon et al., 2018). Phenolics are often extracted from agro-industrial products and incorporated in food products and nutraceutical supplements. To achieve a truly biocompatible and environmental benign process, phenolics and other bioactive compounds must be extracted with biocompatible and non-toxic solvents.

Deep eutectic systems (DES) are considered a new class of alternative solvents. DES were first reported by Abbott et al. (Abbott et al., 2004) as eutectic mixtures, formed by at least one hydrogen bond acceptor (HBA) and one hydrogen bond donor (HBD), that when combined at a certain molar ratio present a significant decrease in the melting point becoming liquid at, or near, room temperature. These components can be primary metabolites, namely, amino acids, organic acids, sugars, or choline derivatives, components that can be found in nature. Normally these DES are denominated as natural deep eutectic systems (NADES) (Paiva et al., 2014).

By changing the composition of the NADES it is possible to modulate the physico-chemical characteristics of solvents, but also to improve the biological activities of plants extracts (Panić et al., 2019). This is associated with the possibility of preparing NADES with compounds that possess the desired properties, for instance, some macromolecules, proteins and polysaccharides that are soluble in these solvents (Dai et al., 2013). Furthermore, since NADES components are included in our daily diet, as well as some food, e.g., vitamins, amino acids, etc, it is expected that the extract obtained by NADES can be directly used in food, pharmaceuticals, cosmetics without the need for the removal of the solvent, avoiding expensive downstream processing and purifications (Dai et al., 2013).

The increasing range of applications of these systems arises from the large combination of compounds potentially used to generate NADES, thus bestowing them a wide range of physical properties, such as polarity.

Although there is a market need for bioactive food, containing antioxidants, the lack of stability of these type of compounds still poses a challenge (Yousuf et al., 2016). Hence, the increase of the stability of these compounds during food processing is of utmost importance. Therefore, a new successfully extraction methodology for the key polyphenolic constituents of mate tea leaves was proposed, using NADES as extracting and stabilizing agents.

Previous studies demonstrated a higher solubilization ability of compounds such as rutin, one of the main phenolics in mate tea leaves, in choline chloride- and glycerol-based NADES when compared with water (Huang et al., 2017). In addition, the efficiency of using NADES to extract phytochemical compounds from different agro-industrial products has been previously demonstrated in several manuscripts. Yue and co-workers reported a successfully extraction of chlorogenic acid from *Artemisiae scopariae*, using proline:malic acid (1:1), reaching a concentration of 16.75 mg/mL (Yue et al., 2020). Another example explored was the extraction of rutin from Chokeberry (*Aronia melanocarpa*), using choline chloride:fructose:water (2:1:1) as solvent, a yield of 4.91 ± 0.33 mg_{rutin}/g of dry chokeberries was achieved (Razboršek et al., 2020). Caffeine and theobromine were identified in the extracts of coffee pulp and cocoa hush, promising results were obtained using choline chloride: lactic acid:water (1:2:1.5), with yields of 0.53 g of caffeine/100 g of coffee pulp and 0.65 g pf theobromine/100 g cocoa hush, respectively (Ruesgas-Ramón et al., 2020).

In this study, we demonstrated a successful extraction methodology of the main constituents of mate tea leaves, showing at the same time the advantageous stabilization of the antioxidants from mate tea leaves extracts in NADES, and the extracts incorporated in NADES in aqueous medium, with the preservation of their activity over at least three months.

2. Materials and methods

2.1. Plant material

For the extraction experiments, the used mate tea leaves were supplied by Baldo S.A, an industrial processing plantation in São Mateus do Sul, Brazil. In the process step, the vegetal material used was recovery after the roasting (Isolabella et al., 2010). The mate tea leaves were crushed and the mean particle diameter obtain was 0.428 mm. The diameter of the particles was evaluated using 5 sieves from the Tyler series with mesh sizes ranging from 16/46 plus the pan (during 15 min of agitation). The water content of the mate leaves was 7.85 ± 0.80%.

For the mate tea leaves decaffeinated, the extraction of caffeine was done by supercritical CO_2. The process was carried out in Unit Operations Laboratory, School of Technology, PUCRS, (Porto Alegre, Brazil). The conditions used in the supercritical extraction were as follows: 300 bar, 60 °C, 5 h of extraction, 140 g of mate tea leaves with an average particle diameter of 0.427 mm, 1200 g/L of CO_2 flow rate and 5% of ethanol (in mass) in relation to CO_2 flow. The extraction apparatus and methodology are described in more detail elsewhere (Medeiros-neves et al., 2020). The water content of the mate decaffeinated tea leaves was 9.29 ± 0.79%.

2.2. Preparation of eutectic mixtures

The raw materials used for the preparation of the different DES were: betaine (≥99% purity), sucrose (≥99.5% purity), lactic acid (85% purity), (D)-(+) - glucose anhydrous), (DL)-menthol (≥95% purity), lauric acid (≥98% purity) all purchased from Sigma-Aldrich. L-proline (≥99.5% purity), choline chloride (≥98% purity) and glycine (98.5% purity, Alfa Aesar) were from Alfa-Aesar. The citric acid (≥99% purity) was from Panreac, (DL)-malic acid (≥99% purity) was from Scharlau and oxalic acid (98% purity) was from ACROS Organic. All systems were prepared by mixing the compounds at a defined molar ratio. The solutions were stirred and heated, until a clear and homogenous solution is achieved. All the chemicals were analytical grade and used as purchased without further treatment or purification.

2.3. Mate tea leaves water content determination

The water content of the mate tea leaves, was determined using a moisture analyser DAB 200–2 (Kern, Balingen, Germany). All the measurements were done in triplicate. The values presented are an average of three measurements.

2.4. Characterization of the NADES

2.4.1. NADES water content

The water content of each eutectic mixture was determined using a 831 Karl Fischer Coulometer with generator electrode (Metrohm). Each measurement was done in triplicate for each system. The values presented are an average of three measurements.

2.4.2. Polarity

The polarity was evaluated using Nile red (≥98% purity) from

Sigma-Aldrich as a solvatochromic probe. A stock solution was prepared, with 1 $g.L^{-1}$ of Nile red in ethanol (>96% purity, Carlo Erba) and stored at 4 °C. The polarity was measured by a standard experimental method reported in literature (Craveiro et al., 2016). The NADES samples were placed in a cuvette and for each system, a blank was recorded. After that, the Nile red solution was added into each sample of NADES (dilution 1:200) for a final volume of 1 mL. Ethanol was used a reference solvent. The polarity of acetonitrile, dichloromethane, ethanol and water was also assessed for comparison purposes. The UV spectra of each sample was immediately acquired at room temperature. The wavelength of maximum absorbance of Nile red was recorded. All measurements were performed in triplicate.

2.4.3. Viscosity/density measurements

Measurements of viscosity and density of the systems were carried out in the temperature range of 20–80 °C for Men:Lau (2:1) system and 20–60 °C for LA:Glu:Water (5:1:3) and LA:Gly:Water (3:1:3) at atmospheric pressure. The equipment used was an Anton Paar (model SVM 3001) automated rotational Stabinger viscometer-densimeter. The viscosities have been measured by a standard experimental procedure, which the details have been reported by our group in the literature (Haghbakhsh et al., 2021, 2022). The measurements presented are an average of three measurements and results are shown as average ± standard deviation.

2.5. Extraction of bioactive compounds from mate tea leaves

2.5.1. Screening of NADES systems and optimization of the extraction procedure

The following NADES were prepared: citric acid:betaine:water (1:1:6 M ratio), citric acid:proline:water (1:1:4 M ratio), choline chloride:citric acid:water (1:1:4 M ratio), choline chloride:sucrose (4:1 M ratio), lactic acid:glucose:water (5:1:3 M ratio), lactic acid:glycine:water (5:1:3 M ratio), malic acid:proline (1:1 M ratio), menthol:lauric acid (2:1 M ratio) and oxalic acid:betaine:proline (1:1:1 M ratio). These were tested in a first screening of the ability to extract polyphenols from mate tea leaves. Two extraction methods were compared, the first one using a stirring plate (150 rpm) and the second one using ultrasound assisted extraction (UAE) (100 W). In a first step a screening of several NADES was performed. Extraction of mate tea leaves was performed using UAE, with a S/L ratio of 1:20, 40 °C and 60 min (4 cycles of 15 min). For the NADES with which the highest yield in phenolic compounds was achieved, a further optimization of extraction parameters was achieved. For this purpose, three parameters were optimized: temperature, 40 and 60 °C, solid-liquid (S/L) ratio 1:10 and 1:20 and extraction time, 30 min (2 cycles of 15 min) and 60 min (4 cycles of 15 min).

2.5.2. Fractionated extraction

For the first step of the extraction process, was chosen the NADES Men:Lau (2:1) once it was able to extract the pigments, including chlorophylls. The liquid fraction was collected, and the remained solids washed with hexane (96% purity) from Carlo Erba and dried.

The second extraction step was prepared using the lactic acid based NADES and the dried solids collected from the first step. After the extraction, the mixture was centrifuged during 15 min at 12000 rpm. The supernatant was used for total phenolics quantification, through Folin-Ciocalteu method and HPLC.

The conditions of extraction for both steps were: solvent ratio of 1:20 (w/v, g/mL), 60 min (4 cycles of 15 min), using an UAE at 40 °C.

2.5.3. Hydroalcholic extraction

A mixture of mate tea leaves and a preheated hydroalcoholic solution of methanol (≥99.8% purity) from Sigma-Aldrich, 70% (v/v), at 70 °C was prepared in a solid to solvent ratio of 1:20 (w/v, g/mL). The mixture was stirred for 20 min, under the same temperature conditions. Then, the extracts were collected and centrifuged at 3500 rpm for 15 min. The supernatant was collected and stored at 4 °C in order to be analyzed through Folin-Ciocalteu method. The remaining solids were dried and weighted. The extractions were performed in triplicate.

2.5.4. Methanol Soxhlet extraction

2 g of mate tea leaves were placed in a Soxhlet apparatus (50 mL of volume), using methanol as solvent refluxed for 3 h. After the extraction process, the solvent was evaporated and the extract was recovered in the powder form. The extracts were weighed. The extractions were performed in triplicate.

2.6. Total phenolic content (TPC) - Folin-Ciocalteu method

Before determination of phenolics, it was necessary to perform a step of protein precipitation as they interfere with phenolics quantification. To 800 μL of sugar-rich liquor were added 120 μL of 100% (w/v) trichloroacetic acid (99.5% purity) from Scharlau. The mixture was stirred well, and stored for 5 min at −20 °C, and then at 4 °C for 15 min. After centrifugation (12000 g, 15 min) (Heraeus sepatech, Biofuge 13 Centrifuge), the precipitate was discarded (Sivaraman et al., 1997).

The TPC of the different extracts was determined by a colorimetric method reported in literature with slight modifications (Waterhouse, 2001), which uses the Folin-Ciocalteu reagent from Panreac. A more detailed analytical technique description can be found elsewhere (Everette et al., 2010). In brief, 20 μL of sample extraction (previously diluted in 1:10) were transferred to a glass test tube, and 1.58 mL of deionized water and 100 μL of Folin-Ciocalteu's reagent were added. The solution was mixed in vortex during a few seconds and incubated for 5 min 300 μL of sodium carbonate solution (20 g in 100 mL) were added, mixed in vortex and incubated 30 min at 40 °C. The absorbance was measured by UV–Vis spectrophotometry and maximum value was read at ~750 nm. A calibration curve was set by using gallic acid standard solutions with concentrations of 50, 100, 150, 250, 500 mg/L. Therefore, the content of extract phenolic compounds is expressed as the weight of gallic acid equivalent (GAE).

2.7. Identification and quantification of phenolic compounds by HPLC

High performance liquid chromatography analysis were performed using a Thermo HPLC Dionex Ultimate 3000, equipped with a quaternary pump, solvent degasser, auto sampler and column oven, coupled to a Photodiode Array Detector Thermo Dionex DAD-3000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA.). The column used was a LiCrospher® 100 RP-18, 250 × 4 mm; 5 μm; Merck®. The column temperature was 35 °C and the autosampler temperature was set at 12 °C. The injection volume was 20 μL and the flow rate of 0.6 mL/min.

The chromatographic separation was performed through a gradient elution, using two mixtures, with different ratios of formic acid (HCOOH), acetronitrile (ACN) and Milli-Q water. Eluent A (0.5% formic acid in Milli-Q water) and eluent B (90% acetonitrile + 0.5% formic acid in Milli-Q water) respectively, following the program: the solvent gradient started with 94.4% A and 5.6% B, reaching 80% A after 15 min, 60% A after 22 min, isocratic during 10 min, 0% A after 45 min, isocratic during 5 min, followed by a return to initial conditions in 5 min and an isocratic step until the end of the run.

The DAD detector was set to monitor channels at 280, 320, 360 nm for identification of phenolic compounds The detection wavelength was 280 nm for chlorogenic acid (≥95% purity), caffeine (99% purity), ferulic acid (99% purity), rutin (≥95% purity), theobromine (≥99% purity), theophylline (≥99% purity) and 360 nm for quercetin (≥95% purity) and kaempferol (≥90% purity). The HPLC analysis was done, following the procedure reported in literature (Anacleto et al., 2020), with slight modifications. All the standards were purchased from Sigma-Aldrich. The quantification of the compounds was calculated based on the calibration curve of pure external standards.

2.8. Antioxidant capacity determination

2.8.1. DPPH assay

To evaluate the antioxidant activity of the mate tea leaves extracts, the DPPH method was used. The determination of DPPH radicals scavenging activity (RSA) was estimated with the method used by Brand-Williams et al. (Brand-Williams et al., 1995), with some modifications. DPPH is a stable free radical which possesses a deep purple colour and a strong absorption around 517 nm. The scavenging of DPPH radicals causes a color shift from purple to yellow or transparent, therefore, it is possible to quantify the ability of antioxidants to quench the DPPH radical.

First, a 24% (w/v) of 1,1-diphenyl-2-picrylhydrazyl solution was prepared in methanol and stored at −20 °C, for at least 2 h, before use. The stock solution was then diluted for a 4.4% (v/v) with methanol. 4 mL of the stock solution was added 150 μL of water (blank) or sample. After reacting in the dark for 40 min, the solutions were well mixed and the absorbance measured at 517 nm. The antioxidant capacity, or the percentage of inhibition, is determined using the following equation:

$$\%\ inhibition = \frac{A_{517nm}blank - A_{517nm}sample}{A_{517nm}blank} \times 100 \quad \text{(Eq. 1)}$$

For EC_{50} parameter determination a range of concentrations between 1 and 100 μg/mL were used.

The pure LA-based systems were also submitted to the DPPH assay.

2.8.2. Stability of extracts

To determine the stability of the extracts obtained from fractionated extraction from NADES, Soxhlet and hydroalcoholic extractions were stored protected from light and at room temperature. Two different samples were tested: the original extracts and the same extracts diluted in water to a concentration equal to the EC_{50} of each extract. In the case of Soxhlet and hydroalcoholic extraction, the extracts were dried with a stream of nitrogen to remove the solvent. In the end, a powder extract was obtained and redissolved to a concentration equal to the EC_{50} of that same extract.

All the samples were left during 90 days under the conditions mentioned above. During this period, a sample was taken at 1, 3, 7, 15, 30, 60 and 90 days and the evaluation of antioxidant capacity was determined by the DPPH method previously described. The procedure was adapted from (Dai et al., 2014), with some modifications.

All measurements were performed in triplicate and the results are presented as the average ± standard deviation.

3. Results and discussion

The most efficient extraction method should maximize the recovery of the target compounds with the minimal influence on their bioactive properties. In this sense, the optimization of the fractionated extraction requires several steps, which include, in a first approach, the choice of the most suitable NADES to be used as a solvent. For this choice, we have chosen a combination of amino acids, sugars, organic acids and alcohols, once these compounds are biocompatible, biodegradable, and do not influence the stability of the resultant extract and consequently does not affect the bioactivity of the extract.

3.1. Screening the potential NADES as extraction media for mate tea leaves

Nine different NADES, listed in Table S1 (supplementary material), were prepared and tested as possible extraction solvents. In this preliminary work, only mate tea leaves were used.

Right after the preparation of all systems, the choline chloride based NADES, the MA:Pro (1:1) and the Oxa A:Bet:Pro (1:1:1) presented a high viscosity, and were discarded, not being tested as extraction solvents. The high viscosity is a strong limitation in the solvent choice, since it may hinder mass transfer.

The other five systems were tested as extraction medium and compared in terms of extraction efficiencies. Two methods were compared, stirring plate (150 rpm) and UAE (100 W). S/L ratio, temperature and extraction time were kept constant at 1:20 (w/v, g/mL), 40 °C and 60 min (4 cycles of 15 min for UAE), respectively. The extraction efficiency was compared in terms of the TPC of the extract, in mg GAE/100 mg mate tea leaves (see Table S2, supplementary material).

After the extractions, in both citric acid-based systems (CA:Bet:Water (1:1:6) and CA:Pro:Water (1:1:4), TPC was not detected, being both discarded.

The highest values of TPC were obtained, 11 ± 1.0% (mg GAE/100 mg mate leaves), 8.0 ± 0.9% (mg GAE/100 mg mate leaves) and 7.0 ± 0.5% (mg GAE/100 mg mate leaves), for CA:Pro:Water (1:1:4), LA:Glu:Water (5:1:3) and LA:Gly:Water (3:1:3) respectively, using the stirring method.

In the case of LA:Glu:Water (5:1:3) and LA:Gly:Water (3:1:3) the use of UAE, revealed an increase of 35 and 48 in the % (mg GAE/100 mg mate), respectively, when compared to the stirring method.

On the other hand, in the system Men:Lau (2:1) no polyphenols were identified by HPLC. This was to be expected due to the hydrophobic nature of the NADES.

To obtain a phenolic-rich extract without the interference of chlorophylls, it is necessary to perform a preliminary extraction with a DES that is highly selective towards chlorophylls. Some chlorophylls have a long apolar chain, promoting interactions in hydrophobic media (Agostiano et al., 2002), which results in a stronger affinity for hydrophobic solvents such as Men:LA (2:1). Therefore, Men:Lau (2:1) was used to extract chlorophylls without extracting the phenolics. After the complete extraction of the chlorophylls, the lactic acid-based NADES were used in a second extraction step, once they demonstrate promising results in extracting the phenolic compounds. Herein, the solid obtained in the first extraction step with Men:Lau (2:1), was washed, dried, and used in the second extraction step, (with LA-based systems).

For that, the lactic acid-based NADES (LA:Glu:Water (5:1:3) and LA:Gly:Water (3:1:3)) and the Men:Lau (2:1) extracts were analyzed for the presence of chlorophyll. The chlorophyll in the extracts was determined through UV/Vis and compared with literature (Lim et al., 2015). Chlorophyll has two absorption maxima peaks, the first one at approximately 420 nm and the second at around 645 nm (Lim et al., 2015).

As can be seen in Fig. S1 (Supplementary material) in lactic acid-based NADES extracts (2^{nd} extraction), no chlorophyll was detected, as the typical peak from chlorophyll does not appear in the extracts from lactic acid systems. Therefore, it is possible to conclude that chlorophylls and phenolics can be separated in different steps of the extraction, resulting in different extracts. All extractions were made under the same conditions (temperature 40 °C, S/L ratio 1:20 and time (60 min (4 cycles of 15min each) and using UAE, for the three systems.

Thus, we validated the hypothesis of a successful fractionated extraction of high-added value compounds from mate, demonstrating the versatility of NADES.

After this first screening we focused our study on the systems Men:Lau (2:1), LA:Glu:Water (5:1:3) and LA:Gly:Water (3:1:3) and the results suggest that a fractionated extraction of the mate tea leaves can be envisaged. For this reason, the characterization of some of the physicochemical properties of these three systems were determined.

3.2. NADES characterization

3.2.1. Water content

The water content of the systems (LA:Glu:Water (5:1:3), LA:Gly:Water (3:1:3) and Men:Lau (2:1) was measured. The water content is a very important parameter to characterize a NADES system. For each system, the water content present in each DES was determined by the Karl-Fischer method and the following results were obtained: 0.03 ±

0.01 (wt %) for Men. Lau (2:1), 15.93 ± 0.88 (wt %) for LA:Glu:Water (5:1:3) and 23.29 ± 0.27 (wt %) for LA:Gly:Water (3:1:3), respectively. For lactic acid-based NADES, water is an intrinsic part of the system; on the contrary, the system with menthol, being hydrophobic presents a very low water content in its composition. The water percentage can change the interactions between the constituent components of the NADES and decreases the viscosity (Dai et al., 2013).

3.2.2. Polarity

The polarity of the different NADES was determined recurring to the Nile red polar parameter (E_{NR}) values, using the Equation (2):

$$E_{NR} = 28591 / \lambda_{máx} \quad \text{(Eq. 2)}$$

The results are showed in Fig. 1. These measurements provide a relative scale, in order to compare our NADES with conventional organic solvents and we can observe that in fact the system Men:Lau (2:1 M ratio) is more similar to less polar solvents, such as acetonitrile or dicloromethane and the hydrophilic systems based on lactic acid are more polar. Solvents with higher polarity results in low values of E_{NR}. It was already reported by Dai and her co-workers that the values of NADES with water usually fell between an E_{NR} of 45–50 kcal/mol (Dai et al., 2013). In this study, the values of our NADES with water had polarity values ranging from 47 to 49 kcal/mol, which are similar to the value of water (48.79 kcal/mol). In fact LA:Glu:Water (5:1:3) (47.84 kcal/mol) has higher polarity than the LA:Gly:Water (3:1:3) (48.65 kcal/mol) and Men:Lau (2:1) (53.48 kcal/mol). This is in agreement with the results already reported (Dai et al., 2013), which referred that organic acid-based NADES, as lactic acid systems are the most polar, followed by amino-acid based NADES and pure sugar based NADES. In our case the difference between the two systems with lactic acid are the glucose and glycine and the amount of water, which is higher in the case of LA:Gly:Water (3:1:3). Moreover, glucose is more polar than glycine, which could be the explanation for the behavior of the systems.

3.2.3. Viscosity and density of NADES

The viscosity and density of Men:Lau (2:1), LA:Glu:Water (5:1:3) and LA:Gly:Water (3:1:3) was measured as a function of temperature. These properties are very important since they greatly affect the mass transference phenomena. As expected, the viscosity of all systems decreased with increasing temperature. The measures of the lactic acid based systems were carried out at a narrower temperature range (20–60 °C) because amino acids and sugars can easily degrade at higher temperatures. To confirm the accuracy of the data generated, the system Men: Lau (2:1) was studied under the same range of temperature (20–80 °C) as already presented by Ribeiro et al. (Ribeiro et al., 2015). The obtained values for density and viscosity were in the range 0.896–0.851 g/cm^3 and 32.030–2.895 mPa s, respectively. The results obtained are given in Table S3 (supplementary material), showing good agreement between our experimental results and those of Ribeiro et al. with a range of 0.897–0.853 g/cm^3 for density and 33.058–2.859 mPa s for viscosity (Ribeiro et al., 2015).

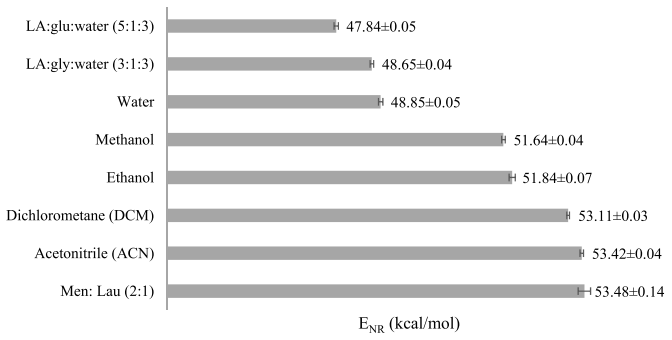

Fig. 1. E_{NR} values obtained for the NADES. Acetonitrile, dichloromethane, ethanol, methanol and water were measure for study comparison. Data expressed as mean ± S.D.

The experimental values obtained for the density of the lactic acid based systems (see Table S4, supplementary material) are very similar, with values between the range 1.265–1.233 g/cm^3 for LA:Glu:Water (5:1:3) and 1.234–1.203 g/cm^3 for LA:Gly:Water (3:1:3), with a very small decrease on the density as the temperature increase. Comparing these values with the system Men:Lau (2:1) the density is higher, probably due to the presence of water in both systems (see Fig. S2) (supplementary material). Between the lactic acid based systems, the effect of temperature is more relevant for the two parameters.

Regarding viscosity (Fig. S3) (supplementary material), it is observed a significant decrease of the viscosity as the temperature increases for both systems, the LA:Glu:Water (5:1:3), which ranges between 185.137 and 18.283 mPa s and LA:Gly:Water (3:1:3), starting with a lower value of 78.794 and decreasing up to 12.051 mPa s These systems have a similar behavior but when compared with the system Men:Lau (2:1) the decrease of viscosity is less pronounced for the last one. The system with glucose is more viscous, probably because this compound is a sugar and can contribute to the increase of viscosity. The system with glycine present lower values of viscosity, this could be related with the fact that has more water in is composition.

3.3. Characterization of the mate tea leaves extracts

3.3.1. Extraction of pigments (chlorophylls and betacarotenes)

As it was observed in the screening experiments, Men: Lau (2:1) was able to extract pigments while no polyphenol was detected. Therefore, this NADES can be used for the fractionation of chlorophylls from polyphenols as a first extraction step. Similar works in literature, such as (Wils et al., 2021), report NADES as efficient agents for extraction processes of pigments.

3.3.2. Extraction of phenolic compounds using LA-based DES

In order to achieve the optimal extractions conditions in the second extraction step with LA-based systems, different conditions of solid/liquid ratio (w/v, g/mL), temperature and time were tested.

The results were express in TPC (mg GAE/100 mg mate tea leaves) for each extract. All the experiments were done using UAE.

The release of secondary metabolites from the plant matrix to the solvent is promoted with an increase in temperature due to the decrease in solvent viscosity and an increase in diffusivity at higher temperatures (Liu et al., 2019).

As it can be seen from the results presented in Table 1, at 40 °C there is an increase in the yield of TCP, as a function of extraction time, which was observed for all S/L ratios and both NADES. Regarding to S/L ratio, the variations observed in yield were within the experimental error.

At 60 °C, there is a decrease in viscosity which will also decrease mass transfer limitations, but, on the other hand, at higher temperatures degradation of polyphenolic compounds may start to occur. In both NADES studied, the differences in yield at different extraction times are not significant, meaning that the maximum yield was achieved after 30 min. The slight decrease in TPC concentration in the extract with LA: Glu:Water (5:1:3) after 60 min of extraction may be due to some degradation that is starting to occur. However, this decrease is within the experimental error.

The effect of temperature is better observed at 30 min of extraction time for S/L ratios of 1:10 and 1:20. It is clear that the extraction kinetics is much higher at 60 °C with extraction yields at least 40% higher than at 40 °C. At 60 °C it is possible to achieve extraction yields similar to those obtained at 40 °C but in with half the extraction time. As said before the lower viscosity at 60 °C, reduces mass transfer limitation, this increasing extraction kinetics.

S/L ratio also plays an important role in extraction kinetics. At high S/L ratios a more concentrated extract is obtained, but if it is close to the solubility of the extracted compounds the diffusion driving forced is

Table 1
Summary of the results obtained for the optimization of the extraction parameters in terms of temperature, ratio S/L and time. All values are presented in mg GAE/100 mg mate tea leaves. Data is expressed as mean ± S.D.

NADES	40 °C 1:10		40 °C 1:20		60 °C 1:10		60 °C 1:20	
	30 min	60 min	30 min	60 min	30 min	60 min	30 min	60 min
LA:Glu:Water (5:1:3)	8.4 ± 1.4	12.6 ± 0.3	6.7 ± 2.4	12.2 ± 1.2	11.8 ± 1.6	10.2 ± 0.5	10.4 ± 0.9	9.3 ± 0.9
LA:Gly:Water (3:1:3)	7.4 ± 0.7	11.5 ± 1.2	5.7 ± 0.9	13.5 ± 1.6	12 ± 1.3	12.3 ± 0.6	11.6 ± 1.5	11.8 ± 1.3

reduced, thus slowing down the extraction process. At lower S/L ratios, the driving force for the diffusion of polyphenols from the solid matrix to the solvent is higher, but a more diluted extract is obtained. Experiments were carried out at 40 °C and 60 °C, to compare the different S/L ratios (1:10 and 1:20 g/mL). When the results are compared, there are no significant variations, with the S/L ratio (at the same temperature), with the exception of the LA:Gly:Water (3:1:3) system, which is observed a decreasing after 30 min, however, and taking into account the experimental error and the values obtained at 60 min, this difference is not significant. Considering that the differences were not so relevant at 60 °C, we decided to choose 60 min of extraction time and the temperature of 40 °C to ensure no degradation of the polyphenolic compounds, S/L of 1:20, avoiding high amounts of solvents. At 40 °C, the better yield values were reached. As previously said in the introduction, the increase of stability implicit avoidance of utmost importance in the processes of extraction (Yousuf et al., 2016), which justifies the use of lower temperatures, and guaranties more reliable applications.

Evidence emerged from recent works, that LA based NADES with water in their composition reveled excellent results in extraction (Macchioni et al., 2021) (Macchioni et al., 2021) (Fernándezde los Á. et al., 2018), corroborating our results.

3.3.3. *Extraction of phenolic compounds of mate tea leaves and decaffeinated mate tea leaves - comparison of different solvents and extraction techniques*

After the optimized extraction conditions, we carried out the extraction of decaffeinated mate tea leaves. The mate tea leaves had been previously subjected to supercritical CO_2 extraction to remove caffeine (Santo et al., 2021) and our aim is to evaluate if the absence of caffeine would lead to significant differences in the composition and in antioxidant power of the matrix. Furthermore, we also wanted to evaluate if the pre-treatment of the matrix with supercritical carbon dioxide would lead to any changes and, therefore if the extraction of the other components of mate tea leaves would be favored. Table 2 shows the comparison between the TPC (mg GAE/100 mg) in mate tea leaves and decaffeinated mate tea leaves, obtained for each extraction method:

The results reveal that the results obtained for mate tea leaves and decaffeinated mate tea leaves are similar in terms of TPC content. Only very small variations were observed, which may be attributed to the raw material itself rather than the effect of caffeine.

The main bioactive constituents of mate tea leaves and decaffeinated mate tea leaves were identified and quantified by HPLC (Table 3). The analysis confirm the presence of five major compounds, namely chlorogenic acid, ferulic acid, caffeine, rutin and theobromine. It is not straightforward the comparison of the composition of mate tea leaves with literature, since the species *Ilex paraguariensis* is native from different countries such as Argentina, Brazil, Uruguay and Paraguay and the mate composition changes, depending on the region, harvesting season, weather, among others.

It is reported, for instance, that the content of chlorogenic acid present in mate tea leaves is between 2 and 3 wt% and caffeine between 0.7 and 2.3 wt %, the relative amounts of other compounds reported in the literature vary significantly, depending of the extraction method used (Assis Jacques et al., 2006; Burris et al., 2012; Filip et al., 2001; Pagliosa et al., 2010).

Comparing all the extracts, the quantities of each compound extracted were much higher using NADES as solvents. The traditional methods presented lower values for all components, except for caffeine, where the value is within the range of values obtained by the methods using NADES. The decaffeination process with supercritical CO_2 yielded 2.1 g caffeine/100 g mate tea leaves (Santo et al., 2021), similar to the values obtained with both LA-based. As we expected, no traces of caffeine were detected in the extracts of decaffeinated mate tea leaves.

The LA-based NADES extracts showed very similar results, showing that these systems were successful in extracting chlorogenic acid, when compared with the traditional ones. Rutin appear at lower concentration, but with consistent values between the mate tea leaves with and without caffeine. A lower concentration of ferulic acid appears in the decaffeinated mate tea leaves, which could be related to the supercritical extraction of caffeine, which can also extract some phenolic compounds. Theobromine was detected in all extracts from both matrices, with a very small difference, but still noticeable, with lower values for the decaffeinated mate tea leaves in all extraction methods. Quercetin, kaempferol and theophylline were not detected in any sample.

In the Men:Lau (2:1) extracts, none of the analyzed compounds was detected.

The total of the principal phenolic compounds (chlorogenic acid, caffeine, rutin, ferulic acid and theobromine) obtained from HPLC analysis is lower than that obtained by colorimetric total phenolic compounds (TPC) determination, which can be explained by the heterogeneity of the matrix, not all compounds occurring in mate tea leaves were quantified, only the ones that exist in major quantity.

Table 2
Results of the content of polyphenols in mg GAE/100 mg mate tea leaves and decaffeinated mate tea leaves for each extraction method (40 °C, 1:20 S/L ratio, 60 min (4 cyles of 15 min)). Data expressed as mean ± S.D.

Extraction method	mg GAE/100 mg mate tea leaves	mg GAE/100 mg decaffeinated mate tea leaves
LA:Glu:Water (5:1:3)	12.2 ± 1.0	10.0 ± 0.9
LA:Gly:Water (3:1:3)	13.5 ± 1.0	12.7 ± 0.3
MeOH (70% v/v)	12 ± 1.2	10.6 ± 1.2
Soxhlet (MeOH)	9.4 ± 1.8	9.0 ± 0.2

3.4. *Antioxidant activity*

The antioxidant activity of the extracts was evaluated by the DPPH radical scavenging method. Phenolic compounds, or polyphenols, have a major contribution to antioxidant activity. Mate tea leaves contain large amounts of polyphenolic antioxidants, such as chlorogenic, and ferulic acids, as confirmed by HPLC analysis.

The EC_{50} represents the concentration at which a substance exerts half of its maximal response to have an antioxidant effect, therefore the lower the EC_{50} value the higher the antioxidant activity. Through Eq. (1) it was possible to determine the EC_{50} for both LA:Glu:Water (5:1:3) and LA:Gly:Water (3:1:3) extracts and for the extracts recovered from hydroalcoholic and Soxhlet extractions, obtained at the same extraction conditions of temperature (40 °C), time (60 min) and S/L ratio (1:20 (w/v, g/mL). The values are presented in Table 4.

The best result (lowest EC_{50}) was achieved by the LA:Gly:Water

Table 3
Chemical composition of mate tea and decaffeinated mate tea leaves extracts obtained with NADES, hydroalcoholic and Soxhlet extractions at 40 °C, 1:20 S/L ratio, 60 min (4 cyles of 15 min). Data expressed as mean ± S.D.

Mate tea leaves	% (g chlorogenic acid/g mate)	% (g caffeine/g mate)	% (g rutin/g mate)	% (g ferulic acid/g mate)	% (g theobromine/g mate)
LA:Glu:Water (5:1:3)	2.25 ± 0.71	2.77 ± 0.96	0.87 ± 0.26	2.48 ± 0.97	0.39 ± 0.05
LA:Gly:Water (3:1:3)	4.28 ± 0.68	2.69 ± 0.87	1.03 ± 0.33	4.52 ± 0.76	0.53 ± 0.09
MeOH (70% v/v)	1.91 ± 0.26	1.69 ± 0.16	0.54 ± 0.04	1.5 ± 0.26	0.35 ± 0.01
Soxhlet (MeOH)	1.50 ± 0.10	2.18 ± 0.17	0.66 ± 0.05	0.95 ± 0.15	0.37 ± 0.01
Decaffeinated mate tea leaves	**% (g chlorogenic acid/g mate)**	**% (g caffeine/g mate)**	**% (g rutin/g mate)**	**% (g ferulic acid/g mate)**	**% (g theobromine/g mate)**
LA:Glu:Water (5:1:3)	3.77 ± 0.75	n.d	0.82 ± 0.13	1.87 ± 0.42	0.35 ± 0.02
LA:Gly:Water (3:1:3)	6.35 ± 0.80	n.d	0.81 ± 0.14	2.64 ± 0.39	0.42 ± 0.02
MeOH (70% v/v)	2.41 ± 0.49	n.d	0.49 ± 0.07	0.69 ± 0.22	0.32 ± 0.01
Soxhlet (MeOH)	2.15 ± 0.18	n.d	0.49 ± 0.05	0.61 ± 0.04	0.33 ± 0.01

n.d – not detected.

Table 4
EC_{50} (μg GAE/mL) values obtained for all extracts.

Extraction method	EC_{50} (μg GAE/mL)	
	Mate tea leaves extracts	Decaffeinated mate tea leaves extracts
LA:Glu:Water (5:1:3)	4.02 ± 0.53	4.67 ± 1.25
LA:Gly:Water (3:1:3)	1.06 ± 0.9	1.17 ± 0.7
MeOH (70% v/v)	6.42 ± 2.9	8.35 ± 2.1
Soxhlet (MeOH)	9.12 ± 1.4	8.44 ± 2.3

(3:1:3) extract, for both matrices, followed by the LA:Glu:Water (5:1:3), (MeOH 70%) and finally the Soxhlet (MeOH). This is in agreement with the results of the TPC for each extraction method, which reflects a relation between the amount of phenolics present in each extract and its antioxidant activity. However, in this work, the resultsdemonstrate that the presence or absence of caffeine in the extracts, does not seem to make a significant difference in the antioxidant activity. These outcomes are aligned with previous studies with mate tea (Santo et al., 2021) which also reported the same antioxidant activity behavior, concerning caffeinated and decaffeinated extracts. This can be related to the fact that caffeine is a pro-antioxidant compound, which is already disclosed in literature (Anesini et al., 2012). It is also reported by these authors that the EC_{50} for the mate tea aqueous extracts are 12.2 ± 1.1 μg/mL, which are in line when comparing with the values obtain in this work for the traditional extractions (hydroalcoholic and Soxhlet). The percentage of inhibition of the NADES alone (without the extract) was also tested, to determine if it has any influence in the antioxidant activity. The highest percentage of inhibition obtain for the pure NADES was 8%, which revealed that, in terms of comparison with the extracts, did not had a high significant contribution.

3.4.1. *Stability of the extracts*

One of the main objectives of this work was also to study the behavior of the mate tea leaves and decaffeinated mate tea leaves matrices in the NADES extracts. For this purpose, a study on the stability of the extracts was carried out over 90 days. The stability behavior of the extracts obtained with conventional techniques during the same time were used as control.

The NADES extracts were stored and used as they were obtained (after extraction), and the Soxhlet and hydroalcoholic extracts were dried. The powder was dissolved in the correspondent solvent to the desired EC_{50} concentration.

The behavior of the extracts in both NADES reflected the stabilization ability of the extracts incorporated in these systems. For both matrices, in the mate tea leaves and the decaffeinated mate tea leaves, the antioxidant capacity remains stable over the 90 days. When compared with the extracts obtained by conventional techniques, no notable differences were observed in the antioxidant activity during approximately 15 days, with exception for the Soxhlet extract, that already revealed a slight decrease in the % of inhibition. After that, a decrease in the antioxidant activity, is noted for both extracts of conventional techniques, but more pronounced for Soxhlet mate tea leaves extract at day 30, leading to a loss of antioxidant activity of about 42% at day 90. The stability performance was very similar in both mate tea leaves and decaffeinated mate tea leaves, showing that the absence of caffeine does not affect the stability of the extract. These results can be observed in Fig. 2 a) and b).

Several studies already reported that NADES promote stabilization of phenolic compounds. Dai and her co-workers reported that the lactic acid based systems provide an evidence in the stability of phenolic compounds, reveling similar behavior when compared with this work (Dai et al., 2016).

3.4.2. *Stability of the extracts in aqueous medium*

The stability of NADES extracts in aqueous medium was also studied during 90 days and compared with the extracts obtained by hydroalcoholic (MeOH 70%) and Soxhlet extractions.

Samples of the different extracts were dissolved in water at a concentration that would correspond to the EC_{50}.

The behavior of the NADES extracts remained constant throughout the three months. For the hydroalcoholic and Soxhlet extracts, the same was not observed; the loss of antioxidant capacity started to occur from the beginning, and after three months, the antioxidant capacity reaches almost 45% of the initial value. The performance of the two matrices is similar during the three months, as can be observed in Fig. 2c) and d). The presence of caffeine does not show to have any influence in the behavior of the antioxidant activity over the three months.

Our results reveal that mate tea leaves extracts and mate decaffeinated mate tea leaves extracts are more stable when incorporated in NADES than in other traditional solvents. Moreover, even when the extracts were in aqueous medium, the same behavior was observed. So, it has been demonstrated that the NADES had a protective effect in the extracts, avoiding the degradation of the phenolic compounds, and promoting the stability over time. Sugars, as glucose were already been reported to play this special effect in the phenolic extracts (Dai et al., 2014).

These experiments intend to validate if the NADES extracts could be incorporated in aqueous drinks, as an alternative to the traditional way of mate tea leaves consumption at high temperatures, that have already been proven to be harmful to health, and at the same time maintaining the antioxidant potential over at least three months.

4. Conclusions

In this work, a new methodology is proposed, and different NADES are used to perform a fractionated extraction from mate tea leaves. In

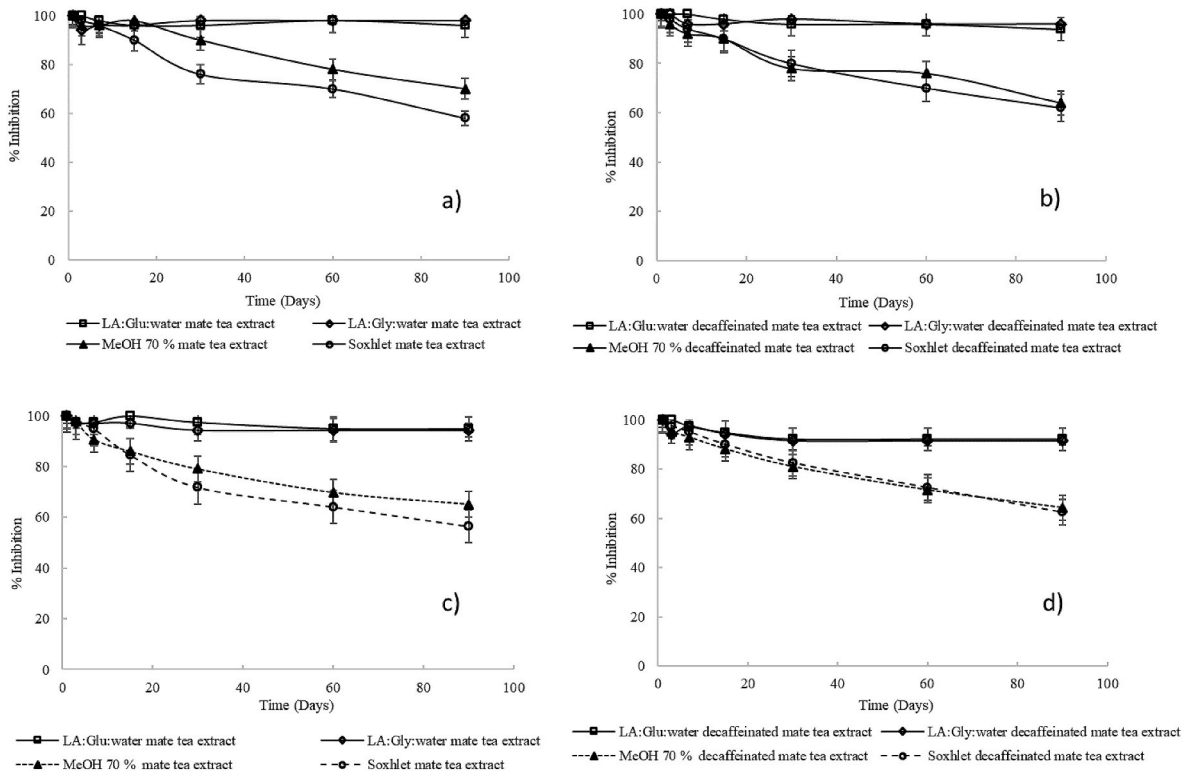

Fig. 2. % Inhibition for the mate tea leaves (a) and c)) and decaffeinated mate tea leaves (b) and d)) extracts obtained from NADES, hydroalcoholic (MeOH 70%) and Soxhlet extractions at room temperature during 90 days. The extracts c) and d) are obtain in aqueous medium.

this sense, the first step of the extraction is performed with a menthol-based system, which selectively extracts chlorophyll, while a second extraction step from the remaining residue is carried out by LA:Glu: Water (5:1:3) and LA:Gly:Water (3:1:3) to obtain a rich phenolic fraction. Chlorophylls were analyzed through UV/Vis spectroscopy, being only detected in the extracts from the first extraction step, with Men:Lau (2:1), and through HPLC it was proven that no phenolic compounds were extracted using this NADES system. In the case of phenolic extraction, various conditions were tested, namely, different NADES, different extraction temperatures and the use of ultrasounds on the yield of extraction was also evaluated. The highest concentration of phenolic compounds in the extracts was obtained using the solvents of high polarity relative to the other solvents, LA:Glu:Water (5:1:3) and LA:Gly: Water (3:1:3), with the values 12.2 $\pm$ 1.0 and 13.5 $\pm$ 1.0% (mg GAE/ 100 mg mate tea leaves), respectively.

Lactic acid-based system showed to be more efficient in extracting phenolics and it was also observed that the optimal extraction conditions were: solid/liquid ratio 1:20 (w/v, g/mL), during 60 min at 40 °C. When the extraction is performed with ultrasounds, the efficiency is greatly improved, yielding higher values, in less time.

The comparison between the extractions using NADES and traditional solvents with mate tea leaves and decaffeinated mate tea leaves reached similar values for the TPC and for EC_{50}. The presence of caffeine does not have a significant influence in these results. Regarding to stability studies, the extracts in NADES and the extracts incorporated in NADES in aqueous medium remained stable during at least three months.

The results obtained with this study can be very beneficial when applied in industries that use mate tea leaves as their raw material, once they can increase the efficiency of extractions, obtain extracts with more quality and the stability is preserving. The advantage of using NADES goes beyond their biocompatibility. It is expected that NADES will prevent the oxidation of the phenolics extracted, extending their shelf life. Furthermore, the main advantage is the fact that all the extract (NADES with bioactive compounds) can be used without any further step of purification or separation. These benefits make NADES the most suitable solvents to be used in extraction processes, in several areas, such as food industry, cosmetic or pharma. In the near future, it is expectable that NADES replace other traditional solvents, in a general and increasing way.

CRediT authorship contribution statement

Sílvia Rebocho: Conceptualization, Methodology, Writing – original draft, preparation, Visualization, Writing – review & editing, All authors have read and agreed to the published version of the manuscript. All authors discussed the results and commented on the manuscript. All authors have read and agreed to the published version of the manuscript. **Francisca Mano:** Conceptualization, Methodology, Writing – original draft, preparation, All authors have read and agreed to the published version of the manuscript. All authors discussed the results and commented on the manuscript. All authors have read and agreed to the published version of the manuscript. **Eduardo Cassel:** Writing – review & editing, All authors have read and agreed to the published version of the manuscript. All authors discussed the results and commented on the manuscript. All authors have read and agreed to the published version of the manuscript. **Beatriz Anacleto:** (HPLC analysis), Methodology, All authors have read and agreed to the published version of the manuscript. All authors discussed the results and commented on the manuscript. All authors have read and agreed to the published version of the manuscript. **Maria do Rosário Bronze:** Supervision, All authors have read and agreed to the published version of the manuscript. All authors discussed the results and commented on the manuscript. All authors have read and agreed to the published version of the manuscript. **Alexandre Paiva:** Conceptualization, Writing – review & editing, Supervision, All authors have read and agreed to the published version of the manuscript. All authors discussed the results and commented on the manuscript. All authors have read and agreed to the

published version of the manuscript. **Ana Rita C. Duarte:** Conceptualization, Writing – review & editing, Visualization, Supervision, All authors have read and agreed to the published version of the manuscript. All authors discussed the results and commented on the manuscript. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

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

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Appendix A. Supplementary data

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