CAPÍTULO 8

ULTRASOUND-ASSISTED GREEN EXTRACTION OF ANTIOXIDANT COMPONENTS FROM THEOBROMA SPECIOSUM FLOWER USING NATURAL DEEP EUTECTIC SOLVENTS

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ABSTRACT: Background: This study investigates sustainable strategies for the extraction of bioactive compounds from T. speciosum flowers using natural deep eutectic solvents (NADES) as environmentally friendly alternatives to conventional solvents. **Methods:** Seven NADES systems (ST1 to ST7) were formulated using choline chloride (ChCl) as the hydrogen bond acceptor (HBA) and different hydrogen bond donors (HBD), including citric acid, lactic acid, malic acid, glucose, and glycerol. The solvents were prepared by heating at 50 °C and applying ultrasound at 60% amplitude. The antioxidant capacity of the extracts was evaluated through DPPH, ABTS, and FRAP assays. **Results:** Most NADES extracts showed enhanced or comparable radical scavenging capacity at lower concentrations than traditional solvents, particularly in the DPPH and ABTS assays, while also demonstrating effective ferric ion reduction in the FRAP assay. The pH was identified as a critical factor influencing extraction efficiency and compound stability, affecting the solubility and interaction between NADES and the target compounds. Among the systems tested, the NADES composed of ChCl and lactic acid (ST2) demonstrated the highest extraction efficiency for phenolic compounds and anthocyanins. **Conclusion:** NADES represent a sustainable, low-toxicity, and cost-effective alternative for the extraction of natural antioxidants. Their tunable properties and high performance suggest broad potential for application in food, pharmaceutical, and cosmetic industries, contributing to greener and more efficient bioprocesses.
KEYWORDS: Ultrasound, NADES, Antioxidant Components.

INTRODUCTION

New sources of healthy foods containing bioactive compounds are being investigated as adequate solutions for health and nutrition. The bioactive compounds can help prevent many diseases, including cancers, coughing, inflammatory, cardiovascular and oxidative stress diseases [1].

The phenolic compounds are one of the most popular bioactive compounds that have a wide range of biological activities and are widely used in food, cosmetic and pharmaceutical industries [2]. The extraction of the phenolic compounds from vegetal materials has got urge attention of these last decades. Generally, for the industrial-scale extraction of these bioactive compounds, conventional organic solvents such as hexane, chloroform, alcohols and ethyl acetate are used. However, the use of large quantities of these organic solvents has the drawbacks of having undesirable effects on the environment and leaving unacceptable residues in the extracts [3].

Water is an eco-friendly solvent, but it is very difficult to extract some non-polar and water-insoluble compounds owing to its intrinsic chemical properties. In terms of organic solvents, it is strongly discouraged in the food, cosmetic and pharmaceutical industries owing to its high toxicity, strong volatility, and flammability [4]. Natural deep eutectic solvents (NADES) are novel, eco-friendly solvents made by heating two or three natural components like sugars, organic acids, and polyols. Due to their low toxicity, broad polarity range, biodegradability, and low cost, NADES are widely used for extracting and separating various bioactive compounds such as phenolics, flavonoids, anthocyanins, pectin, and polysaccharides [5].

Natural deep eutectic solvent (NADES) is a novel class of green and sustainable solvent based on natural components such as carboxylic acids, choline chloride, urea, polyols and sugars. The application of NADES for the extraction of phenolic compounds has emerged as a greener approach, efficient and alternative to organic solvents [6]. NADES are formed by mixing two or more natural components that interact through hydrogen bonds, creating a eutectic mixture with a melting point much lower than that of the individual ingredients [7]. The hydrogen bond interactions take place between the hydrogen bond donor (HBD) and the hydrogen bond acceptor (HBA) [8].

NADES enable selective extraction thanks to their versatility, and when combined with modern methods like ultrasound, microwave, and high-pressure extraction, they effectively enhance the recovery of valuable compounds from plants and agricultural waste [9]

Ultrasound-assisted extraction is an emerging technology that merges ultrasonic and microwave techniques, using their combined high-energy effects to overcome drawbacks of traditional methods like long extraction times, poor temperature control, and uneven heating [10].

To the best of the authors' knowledge, no studies have explored using NADES to extract phenolic compounds from *Theobroma speciosum* ("cacauí") flowers. This study aimed to develop an efficient, eco-friendly method to extract anthocyanins from *T. speciosum* using NADES combined with ultrasound-assisted extraction. Seven different NADES formulations were tested, and antioxidant properties such as phenolic content and radical scavenging capacity were evaluated. The work focused on creating a sustainable solvent system that maximizes extraction efficiency and stabilizes compounds. This novel approach highlights the potential of acid-, sugar-, and alcohol-based NADES with ultrasound to enhance antioxidant extraction from *T. speciosum* flowers.

MATERIAL AND METHODS

Preparation of NADES

Choline chloride (ChCl), used as the hydrogen bond acceptor, was pre-dried at 40 °C for 30 minutes. It was then mixed with one or more hydrogen bond donors in specific molar ratios and heated at 80 °C with stirring for 30 minutes. The mixture was diluted with 30% distilled water to obtain a clear, homogeneous solution without crystals [10]. The NADES abbreviations and molar ratios are shown in Table 1.

Serial number	Natural deep eutectic solvents	Molar ratio
ST1	Choline chloride: Citric acid	1: 1
ST2	Choline chloride: Lactic acid	1: 1
ST3	Choline chloride: Malic acid	1: 1
ST4	Choline chloride: Glucose	1: 1
ST5	Choline chloride: Glycerol	1: 2
ST6	Choline chloride:Citric acid: Glucose	1: 1: 1
ST7	Choline chloride:Citric acid: Glycerol	1: 1: 1

Table 1: NADES (30%, v/v) abbreviations and mole ratios.

Ultrasound-assisted NADES extraction

Flowers of *T. speciosum* were collected in Manaus, Brazil (3°6′26″S, 60°1′34″W; SisGen registration A26CD5E), carefully rinsed with distilled water, freeze-dried, and ground into a fine powder for subsequent use. An aliquot of freeze-dried *T. speciosum* flowers (0.5 g) was mixed with each NADES at a solid-to-liquid ratio of 1:15 (w/v), following the protocol described by Trivisiol et al., (2020).

All NADES formulations were prepared via a combination of sonication and heating at 50 °C, using a power amplitude of 60%, which corresponds to an ultrasonic energy density of 2.7 kJ·cm^{-3}. The sonication was performed for 10 minutes in an ice bath to prevent thermal degradation, as outlined by Sayago et al., 2023. During ultrasonic homogenization, the system was treated with a 25 mm diameter probe operating at 20 kHz and 750 W nominal power (VibraCell VCX 750, Sonics, Shawnee, OK, USA), as described by Mar et al., 2023. Each prepared system was followed by filtration, and the solution was centrifuged to remove small sample particles. The resulting concentrate was collected and stored at -18 °C [10].

Antioxidant capacity assessment

Antioxidant activity was assessed using DPPH• and ABTS•$^{+}$ radical scavenging assays, ferric reducing antioxidant power (FRAP), and total phenolic content, following previously described methods with slight modifications, using an Epoch 2 microplate reader

(BioTek, USA). Additionally, antioxidant capacity was evaluated through the β-carotene/linolenic acid co-oxidation system [14].

Determination of total phenolic content of extracts (TPC)

The total phenolic content was quantified using the Folin–Ciocalteu colorimetric method. Samples were first reacted with the Folin–Ciocalteu reagent for 5 minutes, followed by the addition of 6% sodium bicarbonate solution. The mixture was then incubated for 90 minutes at room temperature. Absorbance was measured at 750 nm using a microplate reader. Gallic acid was used as the calibration standard ($y = 0.0029x + 0.2008$, $R^2 = 0.9915$), and results were expressed as milligrams of gallic acid equivalents per milliliter of sample (mg GAE/mL) [13].

Total Anthocyanin Content

The total anthocyanin content in *T. speciosum* was determined using a pH differential spectrophotometric method. This technique quantifies anthocyanins based on their structural transformation under different pH conditions, with results expressed in milligrams of anthocyanins per 100 grams of sample. An extinction coefficient of 982 (at pH 2.0) was applied, following established methodology. Absorbance measurements were performed at 535 nm using a microplate reader (Epoch 2, BioTek, Winooski, VT, USA) [15].

Carotenoid Content

The determination of carotenoid content in *T. speciosum* samples was performed using a spectrophotometric method, in which 1 mL of NADES sample was mixed with 6 mL of distilled water and 5 mL of hexane, followed by vigorous vortexing for 1 minute. The resulting supernatant (hexane phase), containing the lipid fraction, was analyzed at 452 nm using a microplate reader (Epoch 2, BioTek, Winooski, VT, USA), with hexane as the blank. Results were expressed as carotenoid content, determined by referencing a previously constructed calibration curve using a β-carotene standard [15].

Color analysis

Color measurements were performed using a DeltaVista spectrophotometer (450G, DeltaColor, Brazil), evaluating the CIELAB coordinates L*, a*, and b* under the following conditions: LED light source, D65 illumination, and a 10° standard observer, as described by de Souza Carvalho et al., 2020. The reference white sample comprised tea unaffected by any temperature influence.

Thermogravimetric Analysis (TGA) of NADES

The runs were performed on a TA Instruments SDT Q600. Samples weighing approximately 10 mg were used, with a heating rate of 10 °C/min from room temperature to a final temperature of 700 °C, under a nitrogen gas flow (N_2 5.0) of 40 mL/min. The crucible used in the tests was a 90 μL platinum pan without a lid.

Statistical analysis

Statistical analysis was carried out using analysis of variance (ANOVA) in R software (version 3.5.1). Differences among treatment means were assessed using Tukey's test at a significance level of 5% ($p \leq 0.05$).

RESULTS AND DISCUSSION

Deep ionic liquids, commonly known as NADES, typically consist of a hydrogen bond acceptor (HBA) and a hydrogen bond donor (HBD). Organic acids, polyols, amides, and sugars are frequently used as HBDs, while quaternary ammonium bases, amino acids, and metal ions are commonly employed as HBAs [17]. Among NADES, those based on amides, acids, and alcohols demonstrate greater efficiency in extracting phenolic compounds compared to sugar-based NADES [18].

In this specific study, experiments were conducted using NADES incorporating alcohol-, acid-, and sugar-based components, containing seven HBDs (citric acid, lactic acid, malic acid, glucose, and glycerol), together with the HBA (choline chloride). The composition of NADES determines their physicochemical properties, which in turn influence their extraction efficiency. For this reason, the performance of various NADES, commonly reported in the literature and prepared using ultrasound-assisted methods, was compared in this study for the removal of bioactive compounds from *T. speciosum* flowers. In total, seven systems based on one group of HBA components (the quaternary amine choline chloride) and four types of HBDs (carboxylic acids, alcohols, and sugars) were tested. Among all these trials, only three systems resulted in satisfactory eutectic mixtures.

The variety of combinations between hydrogen bond acceptors (HBA) and hydrogen bond donors (HBD) in the context of NADES are crucial factors impacting the physicochemical properties of these ionic liquids. This explains why the compounds presented in Figure 1 exhibit different concentration values in each extraction.

Extraction is the process by which a solvent removes a desired compound from a solute. The selectivity of different solvents for target compounds arises from their distinct physical and chemical properties. ST2 has a pH in the range of 5–6 and exhibits a slightly acidic characteristic. An appropriate pH can accelerate the rate at which a solvent disrupts

the cell wall, facilitating the dissolution of compounds [19]. The four target compounds were not extracted as efficiently when glucose was used as the HBD.

NADES are increasingly being used for the extraction of anthocyanins with remarkable efficiency. Acid-based DES containing 20–30% water have proven to be the most effective in extracting anthocyanins from samples, outperforming sugar-based systems. Recently, interest in DES for the extraction of anthocyanins from cereals has significantly increased, alongside their use in vegetables, flowers, and herbs. This is due to their outstanding potential to produce plant extracts intended for direct application in the food industry [20]. The extraction of bioactive compounds most commonly utilizes low-frequency ultrasound, resulting in an overall increase in yield of 54% [21].

Conventional liquid-phase extraction using a water/glycerol mixture demonstrated high yields in extracting polyphenols such as chlorogenic acids and quercetin glycosides from *Hypericum perforatum* [22]. Conventional liquid-phase extraction using a water/glycerol mixture demonstrated high yields in extracting polyphenols such as chlorogenic acids and quercetin glycosides from *Hypericum perforatum* [23]. Flavonoids including quercetin, isoquercetin, rutin, trifolin, astragalin, hyperoside, and kaempferol were extracted from *Ribes mandshuricum* leaves using choline chloride/lactic acid [24]. Rutin extraction from flower buds of *Sophora japonica* was carried out using 11 NADES combinations as potential solvents, with choline chloride/glycerol showing the highest extraction efficiency [25]. Polyphenols are well known as antioxidants, and their health-related activities are linked to this property [26].

Carotenoids Content

Choosing a low temperature (50°C) and a short extraction time is an effective strategy to prevent oxidative degradation and isomerization of carotenoids, as well as the formation of free radicals, which can be triggered by high temperatures and cavitation. This selected condition should be easily applicable, with low-cost requirements, to other food sources or food by-products rich in carotenoids, especially β-carotene [27].

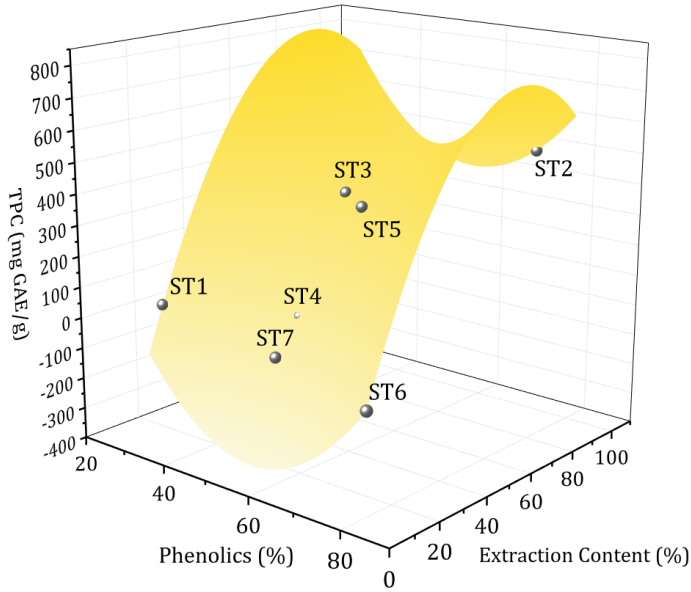

Figure 1: Phenolic compounds content in NADES systems.

The optimization of the β-carotene extraction process from pumpkin was conducted using the ANN model developed to maximize its production, ensuring the highest yield and optimal processing conditions, resulting in a high-quality and high-yield product. The optimal extraction conditions for β-carotene were determined to be a temperature of 60°C and ultrasound power of 60% (52.5 W/cm³) [28].

Based on the ANN model results, the maximum β-carotene content reached 135.39 μg/mL. The validation of the developed ANN model was performed through two independent experiments under the process conditions established to obtain the minimum and maximum β-carotene contents [29].

Based on the results, NADES ST2 stood out as the most efficient in carotenoid extraction. Extraction efficacy is influenced by various experimental parameters such as temperature, ultrasound power, and solvent-to-solid ratio. The carotenoid content in *T. speciosum* flowers may vary depending on the extraction technique used and the experimental conditions. In this research, a significant variation in carotenoid yield was observed, ranging from 15.2 to 247.5 μg/mL, depending on different experimental extraction conditions.

One of the main parameters to be optimized in an extraction process, aiming at energy efficiency and waste minimization, is the matrix-to-solvent ratio. This is because this ratio influences energy consumption and directly impacts the amount of waste produced [30]. Current trends in sustainable extraction aim to reduce solvent use, which contrasts

with our results, as larger volumes of NADES did not increase efficiency due to dilution effects.

Color and Thermal Evaluation

Quantitative color measurements, as shown in Table 2, reveal differences in parameters among the natural deep eutectic solvents. The lightness (L*) values of the samples ranged from 9 to 34, with the lowest L* values recorded for ST1 dissolved in citric acid. Positive values for the green/red parameter (a*) were obtained for all samples. All samples exhibited a* values greater than or equal to 15, and their red color intensity followed the order ST7 > ST6 > ST3 > ST5 > ST2 > ST1 > ST4. All these samples showed higher a* values than a distilled water solution tested for comparison.

Samples	L	a*	b*	ΔE
ST1	9.0 ± 0.1 ^{g}	19.4 ± 0.1 ^{e}	13.0 ± 0.1 ^{g}	44.0 ± 0.1 ^{f}
ST2	10.4 ± 0.1 ^{e}	21.7 ± 0.1 ^{d}	14.1 ± 0.1 ^{f}	47.3 ± 0.1 ^{e}
ST3	9.9 ± 0.1 ^{f}	22.4 ± 0.1 ^{c}	15.8 ± 0.1 ^{e}	48.8 ± 0.1 ^{d}
ST4	34.2 ± 0.1 ^{a}	16.6 ± 0.1 ^{f}	22.6 ± 0.1 ^{d}	35.5 ± 0.1 ^{g}
ST5	24.7 ± 0.2 ^{b}	21.6 ± 0.3 ^{d}	30.0 ± 0.1^{b}	49.2 ± 0.2 ^{c}
ST6	23.0 ± 0.1 ^{c}	31.4 ± 0.1 ^{b}	31.7 ± 0.1 ^{a}	58.3 ± 0.1 ^{b}
ST7	17.1 ± 0.1 ^{d}	38.2 ± 0.1 ^{a}	27.8 ± 0.1 ^{c}	63.6 ± 0.1 ^{a}

Table 2: Results are expressed as mean ± standard deviations (n = 3).

Regarding the blue/yellow parameter (b*), all samples exhibited positive and high b* values, indicating solutions with yellowish hues. The addition of a third HBD component to the natural deep eutectic solvent strongly influenced this color parameter, as the highest and lowest b* values were observed for ST6 and ST7, respectively. The shift toward a less intense yellow value (which could indicate an increase in blue hues) may be attributed to the presence of acid in ST1, ST2, and ST3.

The calculated maximum color intensity values (ΔE) for the samples indicate that ST6 and ST7 exhibit greater (and similar) color intensity compared to the ST4 solution. This suggests that ST1, ST2, and ST3 result in darker samples (lower L* values) with more vivid color (higher ΔE). In other words, the lower chroma (ΔE) values observed for ST6 and ST7 indicate less color vibrancy, which could suggest a faster degradation of anthocyanins in the presence of citric acid.

According to the literature, anthocyanins exhibit a variety of colors in aqueous solutions depending on pH. At very low pH (approximately pH 1), only the red flavylium cation is present. However, as pH increases, different chemical species can coexist in a

complex chemical equilibrium. In this equilibrium, the colorless hemiketal form becomes thermodynamically stable and is mainly responsible for the fading of anthocyanin color [31].

Anthocyanins are highly polar compounds with better solubility in water than in nonpolar solvents. Their chemical form and stability vary according to pH. At pH below 2, anthocyanins predominantly exist as the stable flavylium cation. However, as the pH increases, their structure changes, and degradation occurs above pH 7 [32]. Organic acid-based natural deep eutectic solvents (NADES) have the potential to fully meet the requirements for anthocyanin isolation [33]. Accordingly, seven different NADES were prepared and characterized, with the highest total anthocyanin concentrations (the sum of anthocyanins identified by HPLC) extracted using ST2 > ST3 > ST5 > ST4 > ST6 > ST7 > ST1 (Figure 2). Although polarity values [34, 35 and pH have been previously identified as crucial solvent characteristics for anthocyanin extraction, no obvious correlation was observed, since pH values ranged from 1.70 ± 0.02 to 6.80 ± 0.01.

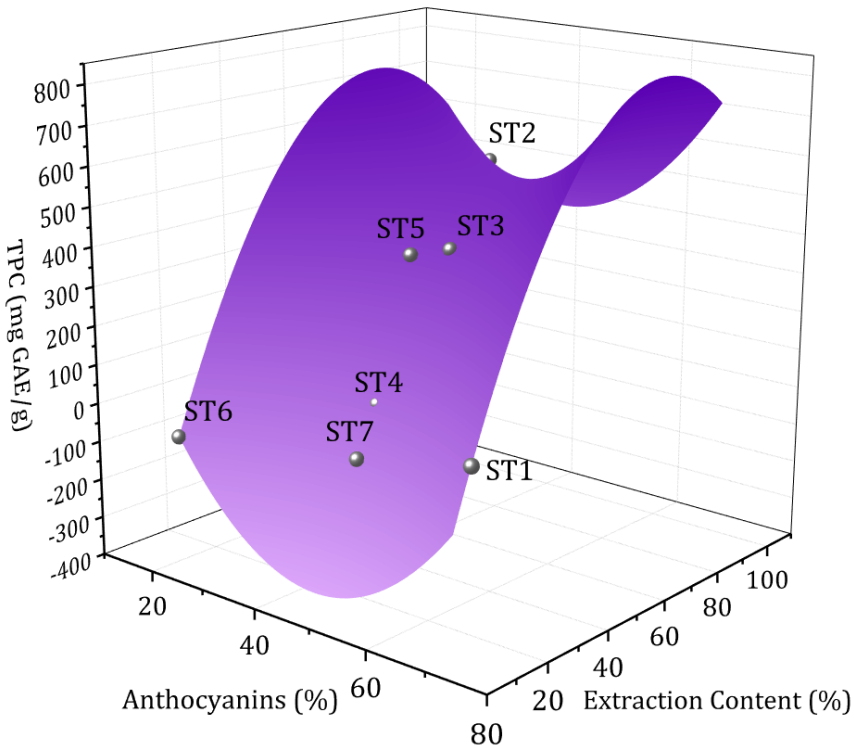

Figure 2: Anthocyanin content in NADES systems.

Similar findings have been reported, indicating that acid-based NADES exhibited lower pH values compared to alcohol- and sugar-based NADES, resulting in higher efficiency in anthocyanin extraction from blackberries [36].

This observation has been corroborated by several studies in which NADES were formed with different components [37]. NADES composed of choline chloride-sugar, choline chloride-organic acid, and choline chloride-alcohol exhibited pH ranges of 2.29 ± 0.02 to 5.05 ± 0.01, 0.76 ± 0.01 to 1.32 ± 0.01, and 5.36 ± 0.02 to 7.04 ± 0.01, respectively [38]. Acid-based solvents showed the lowest pH values, followed by alcohol- and sugar-based NADES [39].

Antioxidant Capacity

The extraction efficiencies of different types of NADES on the total phenolic content (TPC) and antioxidant capacity are illustrated in Table 3. The antioxidant activity of *T. speciosum* flower extracts prepared with NADES was assessed using the DPPH, ABTS, and FRAP methods. Our results showed that all NADES extracts exhibited DPPH and ABTS radical scavenging effects at higher concentrations than the aqueous extract (1031 μmol TE g^{-1} **and 1193 μmol TE g^{-1}, respectively) reported by** Moreira et al. (2021). The highest antioxidant power was observed with Choline chloride: Lactic acid (1401.8 μM Trolox/mL and 1811.8 μM Trolox/mL) > Choline chloride: Malic acid (776.8 μM Trolox/mL and 964.1 μM Trolox/mL) > Choline chloride: Glycerol (747.6 μM Trolox/mL and 830.8 μM Trolox/mL) for the DPPH and ABTS radicals, respectively. The greater antioxidant activity of NADES extracts compared to the aqueous extract is likely attributable to intermolecular interactions, especially hydrogen bonding that stabilizes phenolic compounds [40]. It is important to note that the investigated NADES systems, when evaluated individually, did not cause solution discoloration, suggesting that these NADES solvents cannot donate hydrogen atoms to these radicals.

Overall, a good positive correlation was observed between ABTS and DPPH, with the exception of Choline chloride: Citric acid: Glucose (1:1:1). Differences in antioxidant assay results, as determined by two distinct tests, can likely be attributed to differing mechanisms of action. ABTS assays are based on hydrogen atom transfer, which quantifies the capacity for donating hydrogen atoms [41].

One possible antioxidant mechanism is the reduction of metal ions. In the presence of antioxidant compounds, ferric ion reduction to ferrous ion occurs following a single electron transfer (SET) mechanism [42]. The FRAP method was applied in our study to evaluate the potential of NADES extracts to act as reducing agents. The highest potential to reduce Fe^{3+} to Fe^{2+} was observed with Choline chloride: Lactic acid (1186.8 μM Fe(II)/mL), followed by Choline chloride: Malic acid (726.1 μM Fe(II)/mL), while the aqueous extract showed a value of 1121 μmol Fe(II) g^{-1} **for Fe^{3+}** to Fe^{2+} reduction [14]. The Choline chloride: Citric acid extract showed the lowest electron transfer capacity (244.6 μM Fe(II)/mL). Pure NADES systems tested as controls showed no ability to reduce ferric ions. A study by Jeong et al., (2018) showed that the FRAP assay is highly correlated with total flavonoid content,

while a lower correlation was observed with the DPPH assay, indicating that flavonoids and phenolic compounds are attributed to the antioxidant activity of *T. speciosum* flowers.

Samples	DPPH (μM Trolox/mL)	ABTS (μM Trolox/mL)	TPC (mg GAE/g)	FRAP (μMFe(II)/mL)
ST1	474.3 ± 8.0 [d]	507.4 ± 12.6 [e]	39.8 ± 0.7 [e]	244.6 ± 4.0 [g]
ST2	1401.8 ± 10.4 [a]	1811.8 ± 5.1 [a]	480.1 ± 1.9 [a]	1186.8 ± 3.8 [a]
ST3	776.8 ± 8.0 [b]	964.1 ± 10.7 [b]	392.3 ± 12.1 [b]	726.1 ± 5.7 [b]
ST4	581.8 ± 11.8 [c]	601.9 ± 10.7 [d]	52.9 ± 2.3 [d]	335.4 ± 4.6 [d]
ST5	747.6 ± 12.8 [bc]	830.8 ± 6.9 [c]	369.9 ± 2.5 [c]	616.5 ± 4.2 [c]
ST6	476.8 ± 11.2 [d]	129.7 ± 8.8 [g]	32.1 ± 1.1 [e]	292.8 ± 4.2 [e]
ST7	278.5 ± 11.4 [e]	167.4 ± 13.5 [f]	33.5 ± 2.5 [e]	279.8 ± 5.4 [f]

Table 3: Results are expressed as mean ± standard deviations (n = 3).

The ABTS radical scavenging showed a correlation with the TPC values, similar to that observed in the DPPH activity assay. The results demonstrating the extraction performance of the NADES provided TPC values ranging from 32.1 ± 1.1 to 480.1 ± 1.9 mg GAE/g, while the aqueous extract showed a TPC value of 272.6 ± 0.7 mg GAE/g [14]. NADES exhibited higher TPC than water, methanol, and ethanol, except for ST1, ST4, ST6, and ST7, which showed slightly lower yields. The low extraction efficiency observed in these systems can be mainly attributed to their high viscosity. Dai et al. (2016) observed that the high viscosity of NADES hinders diffusion rates and mass transfer due to the formation of an extensive hydrogen bond network between the acidic hydrogen bond donor (HBD) and the basic hydrogen bond acceptor (HBA).

The effectiveness of the interaction between NADES and phenolic compounds is related to the availability of hydrogen bonds, such as OH and Cl groups [45]. Thus, NADES with a higher capacity to form hydrogen bonds may be more efficient in extraction, even if they exhibit higher viscosity. NADES formed with choline chloride and glycerol, lactic acid, malic acid, ethylene glycol, or acetic acid demonstrated a significant ability to establish multiple hydrogen bond networks with polar compounds, such as phenolic compounds, as reported in previous studies on phenolic compound extraction [46].

Thermogravimetric Analysis (TGA) of NADES

For the future application of natural deep eutectic solvents (NADES), it is essential to determine their thermal stability and other thermal properties. The thermal stability of NADES and their pure components was evaluated through thermogravimetric analysis (TGA), and the results are presented as weight loss versus temperature curves. The TGA curves of NADES containing different HBDs as hydrogen bond donors are summarized in the Figure 3.

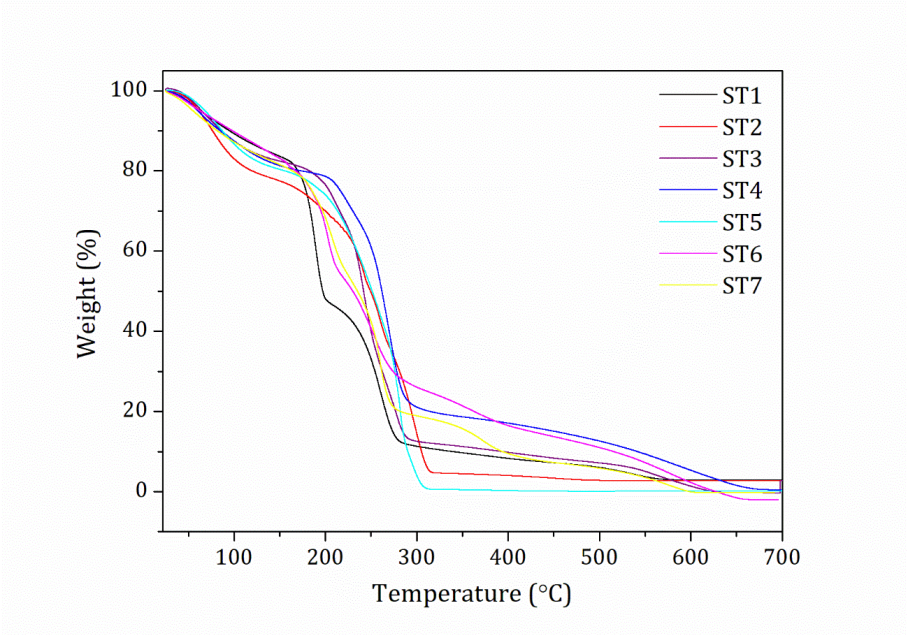

Figure 3: Thermogravimetric Analysis (TGA) Thermograms of NADES

In general, it was observed that when a 5% mass loss occurred at temperatures below 100 ºC, the TGA thermograms mainly showed mass losses associated with the removal of the aqueous component from the NADES. The analysis also indicated that the optimal operating temperatures of the NADES ranged from 190 to 110 ºC, with ST7 and ST1 showing the highest and lowest decomposition temperatures (Tdcp), respectively. The ST7 thermogram revealed a mass loss of 60% between 200 and 280 ºC, while ST1 showed a mass loss of approximately 57% between 290 and 400 ºC, likely due to the decomposition of their corresponding NADES components.

Several factors may be related to the TGA properties, including different HBD and HBA components, the ratio of these components, water content, and the synthesis method. All these elements can influence the interaction and formation of inter- and intramolecular bonds within the complex supramolecular system, thereby contributing to the thermal stability of the solvent [47].

CONCLUSION

In this study, seven methods of NADES preparation, including heating and ultrasound, were applied and compared to extract bioactive compounds from *T. speciosum* flowers. It was observed that NADES provided similar or higher yields of target compounds compared to previously studied aqueous extracts, suggesting that organic solvents can be replaced

by these systems without loss of extraction efficiency. Among the tested NADES, those containing carboxylic acids as hydrogen bond donors showed better extraction capacity for flavonoids and phenolic acids. pH also played an important role and should be considered when interpreting the results. Extracts prepared with NADES exhibited significant antioxidant capacity, as evidenced by DPPH, ABTS, and ferric reducing assays. Based on these results, NADES can be valuable for isolating bioactive compounds from plant sources and have potential for various analytical and chemical applications as sustainable and accessible solvents. Furthermore, given the nature of NADES components, these solvents may be explored in sectors such as food and pharmaceuticals, contributing to the promotion of human health.

AUTHOR CONTRIBUTIONS

J. de A. B. and E.A.S. designed the study and supervised the experiments, J.M.M. and A. de S.C. contributed in methodology, R.Z. de A.N. and S. dos S.C. made contributions in formal analysis, R.F.C. and V.F.K were responsible for data curation, and E.A.S. and P.H.C. assisted in writing – original draft preparation.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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