European Journal of Clinical Pharmacy
Print ISSN: 2385-409X **Online ISSN**: 3105-0409
Website: https://farmclin.com/

Original Research Article

Green Extraction of Bioactive Compounds from Spinach Using Eco-Friendly Solvents

Article History:

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Received: 03-11-2025
Revised: 11-11-2025
Accepted: 26-11-2025
Published: 16-12-2025

Abstract: This study focuses on the green recovery of valuable bioactive compounds from spinach (Spinacia oleracea) through the use of Natural Deep Eutectic Solvents (NADES) combined with Ultrasound-Assisted Extraction (UAE). Traditional extraction methods use dangerous chemicals like hexane, which are harmful to the environment and human health. A new NADES formulation comprising Choline Chloride: Citric Acid achieved a maximum Total Phenolic Content of 42.5 mg GAE/g which is much higher than that of the standard acidified ethanol. The UAE conditions were set at 45°C for 30 minutes, which provided a good balance between maximum mass transfer and the preservation of labile pigments like lutein and chlorophyll. The presence of these solvents led to a significantly higher stability of the compounds in the NADES matrix when compared to volatile organic solvents, as determined by HPLC analysis. Furthermore, the green solvent was found to be very recyclable over three cycles with a maximum efficiency reduction of only (less than 12%). Thus, the results affirm the sustainable and large-scale production of " clean-label " nutraceuticals, applying the most rigorous standards of green chemistry and environmental safety.

Keywords: Spinacia oleracea, Ultrasound-Assisted Extraction, Natural Deep Eutectic Solvents, Choline Chloride, HPLC Analysis

INTRODUCTION

Background: Nutritional Significance and Extraction Bottlenecks

Spinach (Spinacia oleracea) is a globally known superfood due to the content of bioactive phytonutrients which ranks it among the healthiest foods. The nutritional composition is largely depicted by pigments that dissolve in fat, mainly chlorophylls and carotenoids like lutein and zeaxanthin . Lutein is considered a major contributor to eye health since it is one of the factors preventing age-related macular degeneration. Besides, spinach comes with very rich amounts of mucilage-soluble polyphenols and flavonoids including, but not limited to, spinacetin and patuletin that show strong antioxidant and anti-inflammatory activity.

In the past, the isolation of these chemicals relied almost entirely on conventional solvent extraction (CSE) techniques using petroleum-derived solvents like hexane, chloroform, and dichloromethane. While the solvent extraction method is very effective in extracting always chemical compounds from plants, it still poses significant hazards. Hexane is recognized as a neurotoxic and a hazardous air pollutant; chloroform is classified as a probable carcinogen with a high degree of environmental persistence. Not only that, but these solvents also require a great deal of energy for their evaporation, and they often leave behind toxic residues in the final extract, rendering it unsuitable for "clean-label" food or pharmaceutical applications that demand high purity . One more drawback is the thermal degradation of heat-sensitive antioxidants taking place during the lengthy conventional heating process, which however is minor yet significant.

The "Green" Rationale: A Shift Toward Sustainable Extraction

Green Chemistry adoption in phytochemistry has been marked by a transition to eco-friendly solvents and the broad application of the "12 Principles" in the process. The term "Green Extraction" applies to a method that is not only energy-efficient, but also makes it possible to use renewable natural materials, solvents, and even the extraction of substances that are both safe and of high quality.

The adoption of eco-friendly solvents—such as Natural Deep Eutectic Solvents (NADES), ethanol, or supercritical fluids—has been justified by their biodegradability, low toxicity, and solubility that is exactly what is needed. NADES, which are based on natural primary metabolites that are sugars and organic acids, can copy the original cellular milieu of the plant, and often result in better stabilization of the extracted pigments as compared to volatile organic compounds (VOCs).

There is a significantly less explored area of research on the optimization of ternary NADES system for the joint extraction of both polar (polyphenols) and non-polar (lutein) fractions in spinach. This research aims to investigate the effectiveness of specially designed eco-friendly solvents through Ultrasound-Assisted Extraction (UAE) to achieve high output and low environmental impact, thereby providing a large-scale model for sustainable nutraceuticals production.

Materials and Methods

Raw Materials and Characterization

Untreated spinach (Spinacia oleracea), free from synthetic pesticides and thus not affecting the extraction profile, was sourced from a certified organic farm in Delhi, India. To provide a consistent phytochemical matrix, the leaves were initially sifted to remove any unhealthy portions, followed by washing with deionized water to eliminate all surface contaminants.

The influence of thermal processing on bioactive integrity was assessed between two different drying techniques: freeze-drying (lyophilization) and conventional oven-drying. The samples were pre-frozen at -80°C and subjected to sublimation at -50°C under a vacuum of 0.05 mbar for 48 hours during freeze-drying . The oven-drying procedure was openly controlled at a temperature of 40°C and lasted until a stable weight was reached, thus reducing the thermal loss of chlorophyll to the least possible extent.

The following move was to convert the dehydrated samples into ultra-fine powder (particle size less than 0.5 mm) & keep it in amber containers sealed at -20°C so that lutein could be protected from light-induced oxidation.

Solvent Preparation: Eco-Friendly Systems

The study utilized Acidified Aqueous Ethanol and Natural Deep Eutectic Solvents (NADES) as the two primary green solvent systems . The preparation of the aqueous ethanol system included food-grade ethanol blending with ultrapure water (70:30 v/v) and the addition of 0.1% citric acid, which helped prevent the degradation of anthocyanins and polyphenols during extraction, for the stabilization of the compounds at the same time.

In the case of NADES, the preparation of three different eutectic mixtures, which possessed high affinity to polar and semi-polar compounds, was done in the following manner:

- Choline Chloride : Citric Acid (ChCl:CA) in a molar ratio of 1:1.
- Choline Chloride : Urea (ChCl:U) in a molar ratio of 1:2.
- Glucose : Citric Acid (Glu:CA) in a molar ratio of 1:1.

The synthesis was done using the "heating and stirring" method. The individual components were weighed to an accuracy of ±0.01 grams and heated to a temperature of 80°C while stirring continuously until a clear homogeneous liquid was obtained. To make the high viscosity of NADES less of a problem and to improve mass transfer, 20% (w/w) of deionized water was added to each mixture. These solvents were chosen for their "designer" properties that permitted the tailoring of the extraction of the complex profile of secondary metabolites of spinach.

Extraction Procedure: Ultrasound-Assisted Extraction (UAE)

Ultrasound-Assisted Extraction (UAE) was the method that was used for the extraction of bioactives, and it was a very effective technique because it produced acoustic cavitation which in turn made the rupturing of the plant cell walls happen faster than by the traditional stirring process. The ultrasonic bath that was working at a constant frequency of 40 kHz and providing 250 W ultrasonic power was the tool to implement the entire extraction process.

The method specified a Solid-to-Solvent (S/L) ratio of 1:20 (w/v), thus signifying that the spinach powder used was 1 g and the solvent was 20 mL. The extraction conditions were refined in the following way:

1. Temperature: Set to 45°C, which resulted in increased kinetical energy and at the same time avoiding the degradation of pigments which are sensitive to heat.
2. Time: 30 minutes of continuous sonication.
3. Atmosphere: The extraction vessels were covered with aluminum foil to create a dark environment, thus protecting the light-sensitive lutein and chlorophyll.

After sonication, the mixtures were evaporated at $8,000 rpm for 15 minutes at 4°C. The supernatant was then decanted and filtered through a 0.45 mum PTFE syringe filter . The crude extracts were subsequently kept under nitrogen gas at 4°C for later analytical quantification.

Analytical Techniques and Quantification

The Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) were determined by means of spectrophotometric techniques, namely, the Folin-Ciocalteu and Aluminum Chloride methods, respectively. The absorbance was measured at 765 nm for Total Phenolic Content (TPC), reported as mg of Gallic Acid Equivalents per gram, and at 510 nm for Total Flavonoid Content (TFC), expressed as mg of Quercetin Equivalents per gram.

Specialized quantification of Lutein and Chlorophyll was conducted by High-Performance Liquid Chromatography (HPLC). The setup consisted of a C18 reversed-phase column (250 \times 4.6 mm) and a Diode Array Detector (DAD). The gradient of acetonitrile, methanol, and ethyl acetate was used in the mobile phase and the flow rate was set at 1.0 mL/min.

The research on spinach extracts provided a quantitative study of the pigments and the total antioxidant activity of the extracts. The total phenolic content (TPC) and total flavonoid content (TFC) were estimated through the spectrophotometric assays that were conducted based on the Folin-Ciocalteu and Aluminum Chloride techniques, respectively . Measurement of absorbance was done at 765 nm for Total PIn order to assess the performance of the green solvents in a functional way, antioxidant tests of two types were conducted:

1. DPPH Free Radical Scavenging Assay: The extinction here of the DPPH radical was traced at 517 nm structure.
2. ABTS Radical Cation Decolorization Assay: The absorbance was measured at 734 nm. henolic Content (TPC), which was expressed as mg of Gallic Acid Equivalents per gram, and at 510 nm for Total Flavonoid Content (TFC), which was expressed as mg of Quercetin Equivalents per gram.

The results obtained were based on a Trolox standard curve and expressed as Trolox Equivalents (TE) per gram of dry weight.

RESULTS AND DATA ANALYSIS

Comparative Solvent Efficiency and Yield Analysis

The primary goal was to determine how well three Natural Deep Eutectic Solvents (NADES) and acidified aqueous ethanol worked in comparison with Hexane, the established benchmark. The study found that the Choline Chloride: Citric Acid (ChCl:CA) system not only equaled but indeed surpassed all other solvents in terms of the recovery of Total Phenolic Content (TPC). The amount for ChCl:CA was 42.5 mg of Gallic Acid Equivalents (GAE) per gram of dry weight. On the other hand, acidified aqueous ethanol gave only 31.2 mg GAE per gram while the Hexane control just 4.8 mg GAE per gram[1]. The extraordinary difference demonstrates the polar-interaction ability of organic-acid-based eutectic mixtures to a far greater extent.

Solvent	**TPC Yield (mg GAE/g)**	**Pigment**	**Hexane**	**ChCl:U**	**Temperature (C)**	**Yield**	**Time (min)**	**Yield**	**Cycle**	**Efficiency (%)**	
Hexane	4.8	Chlorophyll a	8.2	12.4	25	30.5	10	22	Cycle 1	100	
Acidified Ethanol	31.2	Chlorophyll b	3.4	5.1	35	38.2	20	35.5	Cycle 2	94.5	
ChCl:CA	42.5				45	42.5	30	42.5	Cycle 3	88.2	
					55	36.1	40	42.2			
							50	41.5			

[1] Aade, S. B., Pandey, J. P., Sharma, A. K., & Singh, A. (2022). Ultrasound-assisted extraction of bioactive compounds from spinach (*Spinacia oleracea* L.). *Journal of Food Science and Technology, 59*(2), 642-651.

Table 1:Extraction Data, Source: Author Generated

The Choline Chloride: Urea (ChCl:U) combination was very selective as it particularly took up all the pigment part. The amount of Chlorophyll a and Chlorophyll b present in ChCl:U were established to be 12.4 mg/g and 5.1 mg/g respectively. This was significantly higher than the Hexane production yields of 8.2 mg per gram and 3.4 mg per gram. It is assumed that the better effectiveness of ChCl:U is due to its ability to form very strong hydrogen bonds with the magnesium-porphyrin ring of the chlorophyll molecule, thus "pulling" the pigment out of the chloroplast membranes[2].

Detailed Optimization of Ultrasonic Parameters

Through the use of a systematic approach, the current research improved the three basic factors of Ultrasound-Assisted Extraction (UAE): temperature, duration, and the solid-to-solvent ratio.

Temperature Dynamics (25 to 55 Degrees Celsius)

Temperature acts as a two-edged sword in extraction kinetics. The first impact was the rise in temperature that caused a corresponding increase in Lutein yield by 28% when the temperature was raised from 25 to 45 degrees Celsius. The main reason behind this is the viscosity of the solvent being reduced and the high vapor pressure in the cavitation bubbles, which in turn increases the "micro-jet" effect on the spinach cell walls. However, when the temperature reached above 50 degrees Celsius, there was a steady drop-in antioxidant activity (the DPPH method was used for the assessment)[3]. The Lutein content was reduced by 15% at 55 degrees Celsius, which indicates that the thermal limitation for spinach carotenoids is quite low. Therefore, 45 degrees Celsius was determined to be the "Goldilocks zone" for maximum yield without any loss in quality.

Temporal Extraction Kinetics (10 to 50 Minutes)

The polyphenol extraction curve showed a rapid increase during the first 20 minutes and subsequently plateaued between 30 and 40 minutes. Approximately 92% of the total recoverable bioactives were already extracted at the 30-minute mark. Extending the time to 50 minutes did not provide a statistically significant increase in yield ($p > 0.05$). The "over-sonication" effect caused the breakdown of some complex flavonoids into plain, low-bioactive phenotype acids. This suggests that 30 minutes is the perfect time for energy-efficient green extraction.

Solid-to-Solvent Ratio Optimization

Grams per milliliter ratios 1:10, 1:20, and 1:30 were tested and compared. The 1:10 ratio led to a slurry with very high viscosity that blocked the ultrasonic vibrations' transmission and thus created "dead zones" in the extraction vessel[4]. The 1:20 ratio was the one that established the proper concentration gradient and thus facilitated the highest possible mass transfer from the plant matrix to the liquid phase. Increasing the ratio to 1:30 resulted in lower returns; the yield per gram increased only by 2%, but the solvent volume required grew up to 50%, thus violating the green chemistry principles of atom economy and waste minimization.

Phytochemical Fingerprinting via HPLC

The HPLC profiles of the spinach extracts gave a qualitative view of the extraction by these green solvents. The chromatogram from the optimized ChCl/CA extract displayed five key peaks, which the first one being:

1. Lutein: The dominant carotenoid species constituting 65% of the whole carotenoid fraction.
2. Chlorophyll b: Isolated at 450 nm due to its characteristic absorption.
3. Chlorophyll a: Registered at 660 nm.
4. Spinacetin Derivatives: Unique flavonoids that occur only in spinach.
5. Ferulic Acid: A very strong phenolic antioxidant.

The application of NADES was a reason for the preservation of the ester bonds in these compounds, whereas mineral acids or high temperature would normally be the cleaving agents in the case of conventional methods. The "molecular shielding" offered by the eutectic network is an extraordinary result as it suggests that the green extracts are chemically closer to the fresh plant than the conventional extracts.

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[2] Bubalo, M. C., Vidovic, S., Redovnikovic, I. R., & Jokic, S. (2018). New solutions for old problems: Natural deep eutectic solvents in natural products extraction. *Separation and Purification Technology, 201*, 328-340.

[3] Rana, S., & Gupta, S. (2020). Impact of different drying methods on the nutritional and antioxidant capacity of green leafy vegetables. *International Journal of Vegetable Science, 26*(5), 452-466.

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Antioxidant Capacity and Radical Scavenging

The extracts were effective according to the DPPH and ABTS test data. The IC50 of ChCl:CA was 42.8 μg/mL, which means that it would take 42.8 μg/mL of this extract for 50% scavenging of free radical. As for the acidified ethanol extract, it was 58.4 μg/mL more which was required to achieve the same results.

A very strong connection ($r = 0.94$) was found between the Total Phenolic Content and the ABTS test for acid-removing action, as expected. Therefore, the eco-friendly polyphenols are the main components of the spinach extract which contribute to its health benefits. Additionally, the ChCl:U extract, which is high in chlorophyll, showed a unique ability to deactivate singlet oxygen radicals, a property that is very much desired in the cosmetics industry for anti-aging products.

Stability and Solvent Recyclability

The solvent lifecycle is an important metric for every "green" process. The solvents used were NADES for the study, and their stability was exceptional. The solvent was recovered after the main extraction by simple filtration, and reused.

1. Cycle 1: 100% efficiency (baseline).
2. Cycle 2: 94.5% efficiency in TPC recovery.
3. Cycle 3: 88.2% efficiency in TPC recovery.

The slight drop in efficiency in the third cycle was due to the build-up of leftover plant sugars and minerals in the solvent, which changed the molar ratio of the eutectic mixture slightly. However, the fact that the solvent has been reused three times without chemical regeneration is a huge environmental footprint reduction compared to Hexane, which requires energy-consuming distillation for recovery.

Statistical Validation

All the experiments were done three times, and the obtained data were analyzed by One-Way Analysis of Variance (ANOVA). The analysis indicated that the solvent selection had the greatest impact with ($p < 0.01$), whereas the extraction temperature had an impact as well but with a lower significance ($p < 0.05$). Statistical methods found that the time-temperature interaction was also significant, thus at higher temperatures, one could use shorter extraction times to prevent degradation. This statistical validity ensures that the results are not only observable but also supported by reproducibility and sound science.

DISCUSSION

Mechanisms of Green Solvent Interaction with Spinach Matrix

Natural Deep Eutectic Solvents (NADES), particularly the Choline Chloride: Citric Acid (ChCl:CA) combination, are more effective in the extraction of spinach. This occurs due to the incorporation of the solvents into the cell wall components of the spinach. NADES operate through a complicated system of hydrogen bonds, whereas most organic liquids rely on the interactions between hydrophobic and hydrophilic molecules. The hydroxyl and carboxyl groups of citric acid, together with the quaternary ammonium cation of choline chloride, form a highly structured yet flexible liquid structure. The structure mentioned above has a strong interaction with the hydroxyl groups present on the aromatic rings of flavonoids and phenolic acids in spinach .

When these green liquids are incorporated into the spinach matrix, they absorb water and play a role in maintaining the stability of the matrix. The NADES, because of the structure above the molecules, are able to shield the bioactive chemicals from oxidative degradation. The nitrogenous part of the fluid, which is either urea or acid, engages in the formation of hydrogen bonds with the porphyrin ring of chlorophyll. Such interaction facilitates the movement of the pigments into the liquid phase. This is in stark contrast to hexane extraction, where the only type of interaction is through weak London dispersion forces which are not powerful enough to disrupt the strong protein-pigment bond in the chloroplasts.

Synergy Between Ultrasound-Assisted Extraction (UAE) and Green Solvents

In the UAE, the application of green solvents brings about a synergistic effect which not only leads to a significant decrease in the time needed for extraction but also in the number of resources consumed . Acoustic cavitation is the process where micro-bubbles are produced, grow and eventually collapse in a violent manner causing the generation of high-shear forces and micro-jets. The process of making the cell walls of spinach more permeable due to the physical forces is called macroporation, thus it allows the green solvent to quickly penetrate the inner tissues of the leaf.

Our findings showed that the NADES, which are the green solvents, have higher viscosities than ethanol or hexane, but the use of ultrasound completely removes this restriction. Ultrasonic waves generate heat and turbulence in a specific region, resulting in a transient reduction in the flow rate of the NADES at the contact with the plant material. Thus, the mass transfer of lutein and polyphenols into the solvent becomes faster. This synergy explains why we reached the maximum yield in about 30 minutes, where as traditional maceration with the same solvents usually takes 12 to 24 hours. The physical-chemical purity of this combination embodies the principles of green engineering, since it avoids harmful catalysts and the use of high pressure.

Comparative Analysis with Conventional Literature
Our findings revealed 42.5 mg GAE/g for polyphenols, which is among the highest yields reported in the literature for the green extraction of spinach. In contrast, previous studies utilizing acidified methanol reported values ranging from 25 to 35 mg GAE per gram. The 20% to 30% elevation of this study can be credited to the "designer" nature of the ChCl:CA solvent. The molar ratio was changed to 1:1, and the acidity of the medium was increased, which is very important for the preservation of the pigments in spinach like chlorophyll a and b .

In addition, our lutein recovery rates were on the same level as those obtained with chloroform-methanol mixtures. The absence of halogenated waste in our method contributes to a significant advantage in terms of commercialization. A number of other studies that have dealt with spinach extraction have resorted to high temperatures (above 60 degrees Celsius) to maximize the yield; however, the current research shows that the use of NADES and UAE can still produce significant yields at 45 degrees Celsius. The preservation of the antioxidants' "native state" is an important distinction because heat degradation often results in the formation of artifacts in the extract which can change its biological activity.

Greenness Assessment: The Environmental Factor
We utilized the E-factor (Environmental Factor) measure to support the research's claim of being "eco-friendly," which is characterized by the ratio of waste produced to the mass of the final product. The E-factor for the traditional hexane extraction method of spinach bioactives tends to be high mainly because of the evaporation of the solvent and the toxicity of the waste. However, our UAE-NADES technique showed a significant decline in E-factor.

The main reasons for this eco-friendly position are threefold. The first one is the process of Renewability: the three components choline chloride, citric acid, and glucose are extracted from natural and renewable materials (corn, citrus fruits, and sugar beet, respectively). The second point is low volatility: unlike hexane or ethanol, NADES have a very low vapor pressure, thus, preventing the emission of Volatile Organic Compounds (VOCs) into the air during the extraction process. Lastly, the wading and extraction of waste spinach solids done with NADES are eco-friendly and can either be used as animal feed, or they can be composted since they are free of harmful chemical residues.

The Green Analytical Chemistry (GAC) "Eco-Scale" was the criterion for the assessment of the method. Our method scored 88 out of 100 points, with scores higher than 75 being categorized as "excellent green analysis," primarily due to the energy consumption of the ultrasonic bath.

Stability and Functional Integrity of the Extracts
One of the main concerns regarding green solvents is the possible contamination they may cause during the functionality tests of the isolated compounds. The results obtained from the DPPH and ABTS assays showed that the NADES components (ChCl and Citric Acid) did not alter the radical scavenging process. The citric acid present in the extract showed a combined antioxidant effect by acting as a metal chelator which consequently stopped the pro-oxidative action of the trace minerals present in spinach .

Lutein's extraction in NADES has better stability than that of ethanol. This suggests that the robust hydrogen-bonding network of the eutectic solvent restricts the mobility of the lutein molecule, thus preventing its interaction with remaining oxygen and reducing the rate of photo-oxidation. The result of "in-solvent stability" is very important since it allows the extract to be directly incorporated into liquid food formulations, thus avoiding expensive drying or encapsulation processes.

Potential for Industrial Scale-Up and Limitations
Notably, the ultrasound-assisted extraction process in the laboratory has been an encouragement but the performance at the industrial level still faces some issues that are to be solved specifically. NADES' high viscosity even after ultrasound treatment and the addition of 20% water continues to demand the installation of specialized pumping equipment for the processing of large-scale. The extraction of active compounds from NADES might sometimes lead to a situation where an extract in powdered form is needed as NADES are non-volatile and thus, cannot be evaporated. Extraction of bioactives from the green solvent is via resin adsorption (using macroporous resins) or antisolvent precipitation.

Although in the industries producing liquid nutraceuticals or fortified beverages it is considered to be a "ready-to-add" component. The safety standing of the ingredients (GRAS - Generally

Recognized as Safe) helps the regulatory approval to be faster. The economic modeling of this process should be the main focus of future research comparing the energy costs in the UAE with the solvent recovery costs of the traditional methods to prove the commercial viability of the extraction of green spinach.

Conclusion

The present research clearly shows that the eco-friendly technique of extracting the bioactive components from spinach is not only environmentally friendly but also more productive compared to the conventional ones. The Choline Chloride: Citric Acid system combined with the Ultrasound-Assisted Extraction at 45 degrees Celsius for 30 minutes provided the best combination of effectiveness and stability. We have worked out a process that replaces the use of toxic hydrocarbons with biodegradable, renewable eutectic mixtures, thus fulfilling the world's need for sustainable food technology. The increased recovery of lutein and chlorophyll, together with the inherent stability provided by the NADES matrix, opens up new possibilities for the production of clean-label functional foods.

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