Supercritical CO□ Extraction and HPLC Quantification of Carnosic Acid from the leaves of Rosemaryofficinalis: A Green Approach to Bioactive Compound Purification

Abstract

This study explores the extraction and purification of carnosic acid, a potent antioxidant found in rosemary (Rosmarinus officinalis), using a green and sustainable method involving supercritical CO□. The extraction was conducted at 45°C and 350 bar, with three cycles lasting 45 minutes each. The process resulted in the collection of 75 grams of oleoresin from 1 kg of dried rosemary leaves. The oleoresin underwent further purification via hexane precipitation, yielding a carnosic acid powder with a purity of 40% as determined by Gradient elution of Mobile phase A: Methanol: Water + Phosphoric acid solution, Mobile phase B: Methanol: Acetonitrile + Phosphoric acid solution by high-performance liquid chromatography (HPLC). Comparisons with a 60% purity standard highlight the potential of supercritical CO□ extraction in obtaining bioactive compounds, though additional optimization is necessary to achieve higher purity.

Introduction

Carnosic acid, a naturally occurring phenolic diterpene predominantly found in rosemary (Rosmarinus officinalis) and sage (Salvia officinalis), has garnered significant attention due to its potent antioxidant activity [1]. This compound's applications span multiple industries, including food preservation, nutraceuticals, and cosmetics [1].

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Carnosic acid's industrial applications are diverse. In the food industry, it acts as a natural preservative, helping to extend the shelf life of products by preventing oxidative rancidity in fats and oils [2]. Its antioxidant properties are particularly beneficial in processed foods, where it helps maintain flavour and nutritional quality [2]. In nutraceuticals, carnosic acid is incorporated into dietary supplements aimed at promoting health and wellness, leveraging its potential benefits for various health conditions [2]. The cosmetics industry also utilizes carnosic acid for its skin-protective properties, incorporating it into formulations designed to combat oxidative stress and improve skin health [2].

Research highlights the health benefits of carnosic acid, particularly its role as an antioxidant, which helps neutralize free radicals and reduce oxidative stress [3]. This property is beneficial in the prevention and management of several diseases. For instance, carnosic acid has shown potential in neuroprotection, with studies suggesting it may help in preventing neurodegenerative diseases such as Alzheimer's by protecting neuronal cells from oxidative damage[3,4]. Additionally, its anti-inflammatory properties may assist in managing conditions like arthritis and other chronic inflammatory diseases[5]. Emerging research also indicates that carnosic acid could play a role in metabolic health, with potential benefits in weight management and glucose metabolism, making it a valuable supplement for individuals at risk of metabolic syndrome[3, 4, 5, 6].

The extraction of carnosic acid is crucial for maximizing its benefits; however, conventional extraction methods often rely on organic solvents, which present various challenges. Among the traditional techniques, solvent extraction remains one of the most commonly used methods, employing solvents such as ethanol, methanol, and hexane [7,8.9]. While this

method is cost-effective and straightforward, concerns arise regarding solvent toxicity and the potential for residual contaminants in the final product. Moreover, the low selectivity of solvent extraction can lead to the co-extraction of unwanted compounds, which may compromise the quality and stability of the extract.

Carnosic acid extraction from rosemary leaves can be effectively achieved through various methods, including water-solvent mixtures, organic solvents, and enzymatic aqueous extraction [10, 11. 12].Maceration involves soaking plant material in a solvent for extended periods, allowing the solvent to dissolve desired compounds. While it is simple and cost-effective, maceration has long extraction times and similar risks of solvent toxicity and degradation. Using a water-solvent mixture combines the benefits of polar and non-polar solvents, enhancing the extraction efficiency by dissolving a wider range of compounds, including carnosic acid [10]. This method can improve yields while reducing the harshness of using solely organic solvents. In contrast, organic solvent extraction, which employs solvents like ethanol [13] or methanol, can produce higher concentrations of carnosic acid, but it often involves concerns regarding residual solvents and environmental impact.Protease and Hemicellulase offers a more sustainable alternative by utilizing specific enzymes to break down cell walls, thereby facilitating the release of carnosic acid without the need for harsh chemicals [12]. Each method presents distinct advantages, making the choice dependent on factors such as desired yield, purity, and environmental considerations.

In response to the limitations of traditional extraction methods, supercritical CO□ extraction has emerged as a promising alternative. This technique employs carbon dioxide under high pressure and temperature to act as a solvent, offering several advantages. The high selectivity of supercritical CO□ allows for the extraction of specific compounds while minimizing the presence of unwanted substances, resulting in a purer extract [14]. Furthermore, supercritical CO□ is non-toxic and environmentally friendly, leaving no harmful residues, which enhances its appeal for industries prioritizing sustainability [15]. The extraction process is also relatively fast, requiring less time than traditional methods and utilizing higher mass transfer rates to improve yield [14, 15].The UAE (Ultrasound-Assisted Extraction) method for carnosic acid involves using ultrasonic waves to create cavitation bubbles in a solvent, enhancing the extraction efficiency from rosemary leaves [16]. This technique accelerates the release of bioactive compounds while reducing extraction time and solvent usage, making it a more sustainable approach[16].

While traditional extraction methods for carnosic acid have been widely employed, they come with inherent drawbacks that can compromise product quality and safety. Supercritical CO□ extraction presents a superior alternative, offering enhanced efficiency, selectivity, and environmental benefits. With a broad range of industrial applications and numerous health benefits, carnosic acid is poised to play an increasingly important role in food preservation, nutraceuticals, and cosmetics, furthering its significance in promoting health and sustainability.

Carnosic acid, a primary terpene compound in *Rosmarinus officinalis*, has been analysed using various advanced chromatographic techniques. The use of centrifugal partition chromatography for a one-step isolation of carnosic acid and carnosol [17]. The analysis of nanoencapsulation of Rosmarinus officinalis extracts, offering new avenues for enhancing the stability and bioavailability of carnosic acid was studied using chromatographic techniques

[18]. HPLC methods remain the most widely applied for quantification, with validated protocols like the one by for fresh foliage of *Salvia rosmarinus* and *Salvia officinalis* [19, 20]. Various analytical methods, including HPLC and GC-MS, for carnosic acid detection in plants, foods, and biological samples were developed emphasizing HPLC's role in carnosic acid analysis [21, 22, 23, 24].

Materials and Methods

Materials

1. **Plant Material:** Dried rosemary leaves were sourced from Thalavadi farms of Tamil Nadu and prepared for extraction.
2. **Reagents and Solvents:** Analytical-grade n-hexane was used for the precipitation step, while a carnosic acid standard with a purity of ≥90% was obtained from Yangee Biotech, China. All solvents used were of HPLC grade.

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Methods

1. **Supercritical CO Extraction:**

   Procedure: 5LX2 Buffalo extraction equipment was used to run the experiment. 1 kg of dried rosemary leaves was placed in an extraction vessel. The supercritical CO extraction was conducted at 45°C and 350 bar, with three cycles of 45 minutes each. Oleoresin collected from separators yielded a total of 75 grams.

2. **Purification of Carnosic Acid:**

   Precipitation Process: The obtained oleoresin was dissolved in 300 mL of n-hexane and stirred continuously until a yellowish precipitate of carnosic acid formed. The precipitate was filtered, washed with hexane to remove impurities, and then dried to produce 25 grams of carnosic acid powder. Process is explained in **Figure 1**

3. **High-Performance Liquid Chromatography (HPLC) Analysis:**

   Conditions: The method adopted from Stashenko, Elena E., et al [25]. HPLC analysis was performed using a C18 reverse-phase column ((150 mm × 4.6 mm × 5 µm) with a mobile phase consisting of methanol, water, and acetonitrile in a gradient elution. Mobile phase A: Methanol: Water + Phosphoric acid solution, Mobile phase B: Methanol: Acetonitrile + Phosphoric acid solution gradient elution given in **Table 1,** Phosphoric Acid solution: 0.1ml Phosphoric Acid added into 100ml water. The detection wavelength was set at 280 nm, and the flow rate was 1 mL/min. The injection volume was 10 µL, and the column temperature was maintained at 30°C. The system used Prominence I Shimadzu HPLC with UV detector.

   Analysis Procedure

   1. Preparation of sample solution

   Accurately weigh 140 mg~180 mg of sample (accurate to 0.0001g), dissolve in 20 mL Acetone in a 25 mL volumetric flask, mix well and filtrate through a 0.22 m

microporous membrane, obtain the sample solution.Dissolve in 20 mL Acetone in a 25 mL volumetric flask, mix well and filtrate through a 0.22 m microporous membrane.

2. Drawing of standard curve

The standard solution of mixed gradient was prepared by dissolving the standard substance of carnosic acid with acetone, and the concentration gradient of carnosic acid was 0.010 mg/mL~1.000 mg/mL.Under reference chromatographic conditions, the standard solution was determined and the sample was repeated once.According to the concentration and peak area of the standard solution, draw the standard curve.The linear relationship should be $R2 \geq 0.99$.Record the linear formula for the standard curve $Y = A \times C + B$.Where, C is the concentration of carnosic acid,Y is the peak area corresponding to this concentration, and A and B are the slope and intercept of the standard curve respectively.

3. Determination

From the equation carnosic acid is quantified

$$C1 = \frac{Y1 - b}{a} \times 100\%$$

C1-The concentration % of Carnosic acid, unit: mg/mL
Y1-The peak area of Carnosic acid in sample solution
b-The intercept of standard curve formula of Carnosic acid;
a-The intercept of standard curve formula of Carnosic acid

Results

The extraction and purification of carnosic acid from dried rosemary leaves were performed using supercritical CO□ extraction followed by a precipitation method with n-hexane. The supercritical CO□ extraction process yielded 75 grams of oleoresin from 1 kg of rosemary leaves. This result demonstrates a relatively efficient extraction, indicating that the conditions of 45°C and 350 bar effectively facilitated the release of bioactive compounds from the plant matrix.

The subsequent purification of the oleoresin involved dissolving it in 300 mL of n-hexane, which prompted the formation of a yellowish precipitate of carnosic acid. Continuous stirring allowed for optimal dissolution and the subsequent crystallization of carnosic acid. After filtering and washing the precipitate with hexane to eliminate impurities, the final product was dried, resulting in 25 grams of carnosic acid powder. This purification step is crucial as it isolates the target compound from the matrix and removes undesirable components, thereby enhancing the quality of the final extract.

HPLC analysis was employed to evaluate the purity of the extracted carnosic acid. Using a C18 reverse-phase column and a gradient mobile phase consisting of methanol, water, and acetonitrile, with gradient elution of Mobile phase A: Methanol: Water + Phosphoric acid solution, Mobile phase B: Methanol: Acetonitrile + Phosphoric acid solution the analysis was carried out at a detection wavelength of 280 nm, with a flow rate of 1 mL/min and an injection volume of 10 µL. The results revealed that the carnosic acid content was

approximately 40% when compared to the standard reference (≥60% purity) obtained from Yangee Biotech. This indicates that while the extraction and purification process is functional, there is significant room for optimization to increase the purity level of the extracted carnosic acid.

The lower-than-anticipated purity of carnosic acid suggests that various factors may have influenced the extraction efficiency. These could include the extraction parameters such as temperature, pressure, and duration, which may need further fine-tuning to enhance the selectivity for carnosic acid. Additionally, the hexane precipitation method might require adjustments in solvent volume or washing procedures to maximize the removal of non-target substances and improve the purity of the final product.

Overall, the results indicate that while the supercritical CO□ extraction coupled with n-hexane precipitation is a viable method for isolating carnosic acid, the process requires further refinement. Future studies could explore varying extraction conditions, such as increasing the extraction time or optimizing pressure settings, to improve yield and purity. Additionally, incorporating advanced purification techniques, such as column chromatography or fractional crystallization, may enhance the final product's quality, making it more suitable for industrial applications in food, nutraceuticals, and cosmetics. This research lays the groundwork for further investigations aimed at optimizing the extraction and purification processes to obtain high-purity carnosic acid, which has significant potential for health and wellness applications.

Discussion

This study investigates the extraction and purification of carnosic acid from rosemary (*Rosmarinus officinalis*) using supercritical CO□ extraction, a method recognized for its efficiency and sustainability. The successful extraction of 75 grams of oleoresin from 1 kg of dried rosemary leaves demonstrates the effectiveness of the selected parameters—specifically, the combination of temperature (45°C) and pressure (350 bar). These conditions facilitate the solvation of bioactive compounds, maximizing the yield of carnosic acid and other valuable constituents.

The subsequent purification process, which employed hexane precipitation, yielded 25 grams of carnosic acid. This method allowed for the effective separation of carnosic acid from the oleoresin. The formation of a yellowish precipitate indicates the successful crystallization of the target compound. The washing and filtration steps were critical in ensuring that impurities were removed, thereby enhancing the purity of the final product. However, the HPLC analysis revealing a purity of approximately 40% compared to the 60% standard highlights the need for further refinement in the extraction and purification processes.

The relatively low purity of carnosic acid suggests several areas for potential optimization. Firstly, the supercritical CO□ extraction parameters could be adjusted; variations in temperature, pressure, and extraction time may significantly influence both yield and purity. For example, increasing the extraction time or adjusting the pressure settings might enhance the extraction of carnosic acid while reducing the co-extraction of less desirable compounds. Furthermore, exploring different solvents or solvent combinations during the precipitation step may also improve the purification process, potentially leading to a higher yield of carnosic acid with greater purity.

Comparatively, the benefits of using supercritical CO□ extraction are significant. This method not only provides a more environmentally friendly alternative to traditional solvent-based extraction techniques but also enhances the overall quality of the extracted compounds. The non-toxic nature of CO□ minimizes the risk of harmful residues in the final product, making it particularly advantageous for applications in the food and cosmetics industries, where product safety and quality are paramount.

The findings also reinforce the versatile industrial applications of carnosic acid, particularly in food preservation, where it functions as a natural antioxidant, extending shelf life and maintaining flavour stability. In nutraceuticals, carnosic acid is recognized for its health-promoting properties, including its potential role in neuroprotection. The ability of carnosic acid to combat oxidative stress suggests its usefulness in the prevention of neurodegenerative diseases like Alzheimer's, underscoring the compound's relevance in health supplements aimed at promoting cognitive function. Furthermore, its anti-inflammatory properties may contribute to its effectiveness in managing chronic conditions, providing a multifaceted approach to health and wellness.

In light of these applications, the ongoing optimization of extraction methods will be crucial. Future research should focus on fine-tuning extraction parameters and exploring additional purification techniques, such as column chromatography or advanced fractional crystallization, to achieve higher purity levels. Such advancements will not only enhance the commercial viability of carnosic acid but also broaden its applications in health-oriented products.

Overall, this study lays a foundational understanding of the extraction and purification of carnosic acid from rosemary, illustrating the potential of supercritical CO□ extraction as a leading method in the field. Continued research in this area will contribute to the development of high-purity carnosic acid, reinforcing its significance in various industries and its potential for promoting health and sustainability.

Conclusion

This research validates supercritical CO□ extraction as a viable approach to extracting carnosic acid from rosemary. Despite achieving a moderate purity level, the process demonstrates significant promise due to its environmentally friendly nature and efficiency. Future studies should focus on optimizing the extraction parameters and exploring complementary purification methods to achieve higher purity levels that meet industry standards.

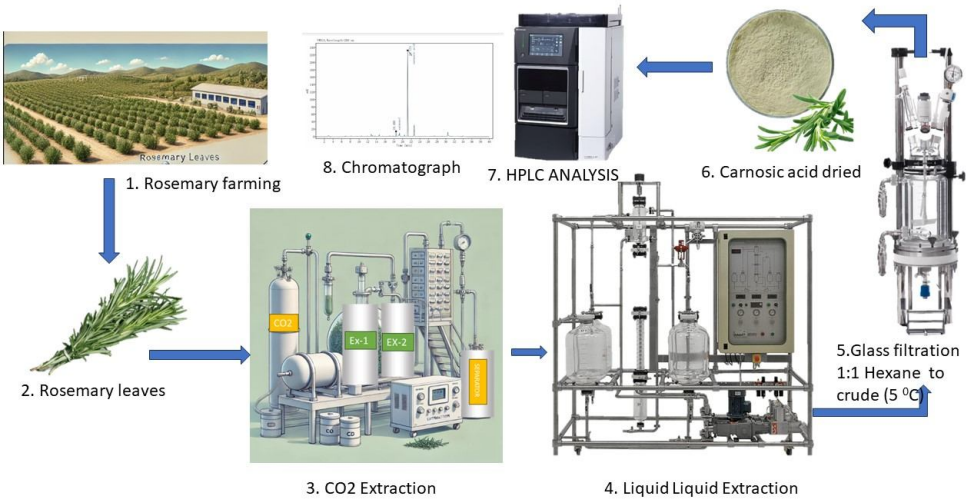
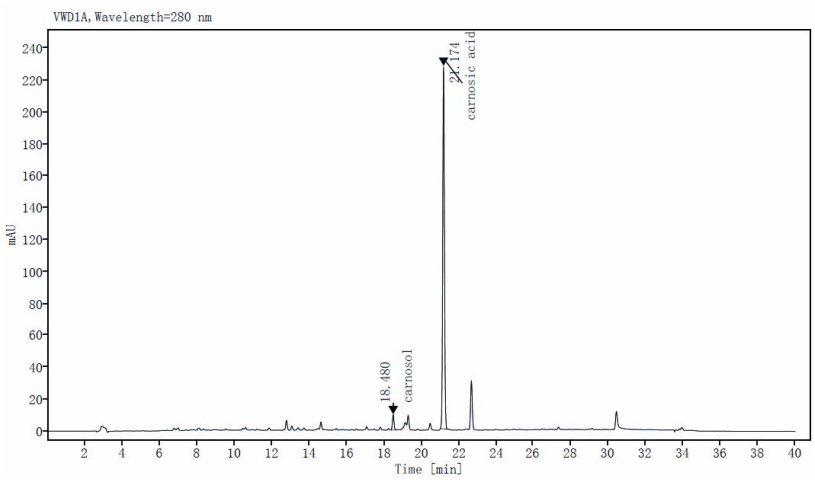


Figure 1: Process flow chart of Super Critical CO2 extraction of Carnosic acid from Rosemary Leaves

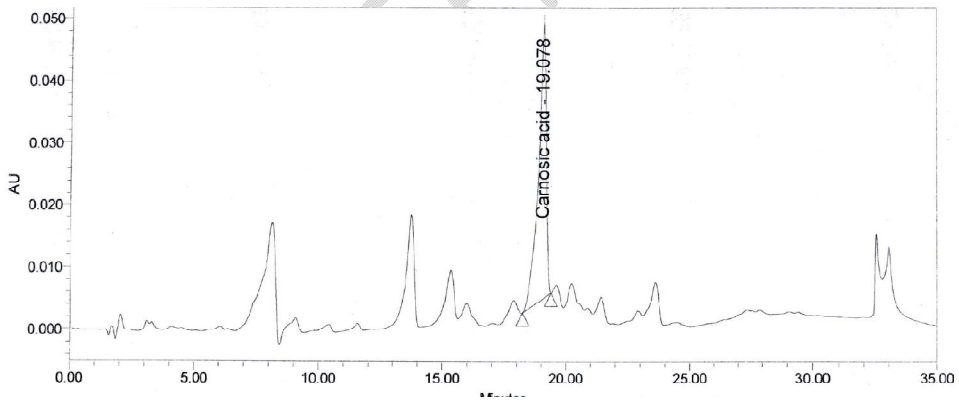
Time/min	Mobile phase A/	Mobile phase B/	Flow Rate /(mL/min)
0.0	77	23	1.0
1.0	77	23	1.0
25.0	0	100	1.0
30.0	0	100	1.0
30.5	77	23	1.0
35.0	77	23	1.0

Table 1: Gradient Elution Condition for chromatographic conditions



Name	keep time [min]	Peak area	Response factor	content [mg/ml]	concentration [%]
Carnosol	18.480	57.7988	2612.17060	0.022	1.68
Carnosic acid	21.174	1443.4228	2482.60746	0.581	60.10

Graph 1:60% standard peak of Carnosic acid



Graph 2: 40% sample peak of Carnosic acid

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