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MICROWAVE-ASSISTED EXTRACTION OF MAJOR BIOACTIVE PHENOLIC COMPOUNDS FROM OLIVE INDUSTRIAL BYPRODUCTS USING NATURAL DEEP EUTECTIC SOLVENTS

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Abstract

Developing environmentally friendly extraction techniques for natural products is an interesting research issue in the interdisciplinary fields of technology, biology, and applied chemistry. This study applied a novel and sustainable approach to extract and derivatize valuable bioactive phenolic compounds from olive oil industry by-products. Microwave-assisted extraction (MAE) was used to investigate the extraction of phenolic components from olive oil industry by-products by several naturally nontoxic, ecologically friendly Deep eutectic solvents (DESs) and their mixtures with water. The quantification of phenolic compounds was established by high-performance liquid chromatography (HPLC) analysis. The effects of the solvent type were studied and evaluated for the oleuropein, demethyloleuropein, oleacine(3,4-DHPEA-EDA), and hytrosol content in olive oil industry by-products. Among all the solvents studied, the findings of this study suggest that glycerol-based NADES-2 is an excellent solvent in terms of its effectiveness in extracting phenolic compounds, especially oleuropein after 10 minutes. Generally, among all polyphenolic compounds, the best extraction result with NADESs (with and without water) was recorded for dimethyloleuropine. Furthermore, the process of extraction is simple, accurate, and safe. Since the solvents used are obtained from natural sources, it satisfies most of the requirements needed to be considered a sustainable

Keywords

Natural deep eutectic solvents, microwave-assisted extraction, phenolic compounds, olive oil industry byproducts

method with several advantages of microwave-assisted extraction (MAE), including shorter extraction times, decreased solvent consumption, and more efficient extraction capacity

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Introduction. Natural raw materials, including plant material, frequently include "extra nutritional" substances called bioactive compounds. In plants, these compounds are formed as secondary metabolites that have valuable effects on human health. Four broad categories may be used to classify the bioactive compounds found in plants: phenolic compounds, sulfur-containing compounds, terpenes and terpenoids, and alkaloids [1]. To take advantage of these bioactive chemicals, a range of extraction procedures, including unconventional techniques, have been developed and investigated. These processes have produced high-value products. Furthermore, the increasing demand for eco-friendly products throughout the world emphasizes how important "green" extraction methods are to the food, pharmaceutical, and cosmetic industries [2].

In the Mediterranean area, olive trees, also called *Olea europaea*, are a widespread crop. Owing to the many health advantages of olive oil, recent cultivation of this tree has received more attention worldwide [3]. However, several wastes are created during the harvesting or refining process, such as olive leaves, pomace, wastewater from olive mills, and other industrial by-products [4]. These inexpensive olive wastes may be applied to animal feed, nutraceuticals, medicines, and cosmetics. Many phenolic compounds, which are generated by plants as secondary metabolites and present in all plant tissues, may be detected in these wastes. Because these substances have been proven to improve human health, researchers are very interested in finding a way to extract them [5].

The most well-known phenolic chemical found in olive leaves is oleuropein, and the secoiridoid glucosides that give olive leaves and fruits their organoleptic qualities are called ligstrosides. Due to its important pharmacological activities, oleuropein is one of the olive polyphenols that attracts the most attention in the available scientific literature [6, 7]. An abundant supply of bioactive phenolic compounds with several health advantages may be found in olive oil byproducts. These consist of hepatoprotective, antiviral, anti-inflammatory, anti-atherogenic, and anti-carcinogenic properties [8, 9].

Methanol, dichloromethane, ethanol, hexane, ethyl acetate, and acetone are common organic solvents employed in a procedure known as maceration

to extract phenolic compounds from olive fruits and leaves. The extraction process requires extended treatment times and high temperatures. Even though these solvents give high-quality extracts with high yields, the environment, and human health may be harmed by the solvents utilized. Furthermore, the extracted compounds must also be purified before use [10, 11].

Nowadays, one of the main principles of green chemistry is to replace or reduce the use of hazardous solvents. In the last few years, a novel class of environmentally friendly solvents has been developed for use in extraction procedures and synthetic transformations [12–14]. These natural deep eutectic solvents (NDESs) possess some great qualities, including being very viscous, water-miscible, non-flammable, and having very little volatility at room temperature. These solvents have drawn significant attention in chemistry because, in comparison to conventional organic solvents, they offer a more sustainable and environmentally friendly method of extracting and separating specific bioactive compounds from a variety of natural substances [15, 16].

The majority of NADESs consist of naturally occurring, unbound hydrogen bond donors (HBDs), such as amides, sugars, alcohols, and polyols, combined with non-toxic quaternary ammonium salts like choline chloride. These HBDs are classified based on their functional groups, which determine their ability to form hydrogen bonds and influence extraction efficiency. For instance, alcohols and polyols provide hydroxyl groups for multiple hydrogen bonds, while amides can act as both hydrogen bond donors and acceptors. These solvents are inexpensive, biodegradable, and widely available [17, 18].

NADESs are simply prepared and provide a variety of combinations that can be employed for different purposes. They are called “design solvents” because of their structural flexibility and capacity to customize their physical-chemical characteristics [19]. The extraction of phenolic compounds and natural products for use in pharmaceuticals is the well-studied application of DES [20]

NDES-assisted extraction methods for phenolic compounds have been the subject of several investigations; it has been demonstrated that these methods dissolve phenolic compounds more successfully than conventional and alternative solvents [21, 22]. Because NADES can donate and accept protons and electrons, it may create hydrogen bonds, increasing its dissolution ability. Recently, microwave-assisted extraction (MAE) has become more popular because of its benefits, which include faster extraction times, reduced solvent consumption, and higher extraction efficiency [23]. Thus, this study aims to create a sustainable process for extracting phenolic compounds from olive oil industry by-products by using NADESs in conjunction with microwave activation.

Materials and methods for solving problems, accepted assumptions. Ripe olive fruits used as raw materials during the extraction process were collected from a *Coratina cultivar* (*Olea europaea L.*) of olive trees grown in 87036 Rende (CS), Italy. The CREA-Research Centre (Rende, CS, Italy) supplied the chemicals, reagents, standards, and matured olive drupes (*Leccino cultivar*) utilized in this investigation. Numerous trees were used to collect random samples, which were transported directly to the lab. After washing with distilled water, they were dried at 50 °C for 48 hours, or until a consistent weight was reached. Ripe olives were then crushed and kept at – 20 °C until they were extracted.

NADEs preparation. For the preparation of NADESs, first, choline chloride was placed with various hydrogen donors in a round-bottle flask. Then, the components were heated to 80 °C in a water bath with vigorous stirring until a homogeneous, transparent solution of the colorless liquid was formed. Finally, further investigation was carried out by adding 20 % v/v water to the NADES solutions.

Extraction of phenolic compounds from ripened olive drupes with NADESs. The extraction process was conducted using a MAE system. Ripe olive fruits were first crushed in a mortar with a pestle. In a Pyrex round-bottom flask equipped with a jacketed coiled condenser, 1 g of the prepared olive sample was combined with 8 mL of NADES. The mixture was subjected to microwave heating at a constant power of 800 W. The extraction temperature was maintained at 80 °C, and the extraction time was either 10 or 30 min, depending on the experimental condition. Following the MAE process, the extracts were centrifuged at 1000 rpm for 10 min to separate the solid residue. The supernatant was carefully recovered and subsequently filtered under vacuum to remove any residual NADES. The filtered extracts were then diluted with ethanol and prepared for analysis using the HPLC technique.

These extraction parameters were chosen based on studies demonstrating that a power level of 800 W effectively yields high concentrations of polyphenolic compounds while minimizing thermal degradation. The use of NADESs further enhances both the efficiency and sustainability of the process. Together, these conditions ensure effective and environmentally friendly phenolic compound extraction while preserving their bioactive properties [24, 25].

Microwave heating mechanism. Microwave heating differs fundamentally from conventional heating methods. While conventional heating relies on thermal conduction, where heat is transferred from the outer layers to the inner core of the material, microwave heating utilizes electromagnetic waves to induce dipole

rotation and ionic conduction. This process leads to rapid, localized heating and efficient energy transfer directly to the molecules in the medium, resulting in faster and more uniform heating. The use of microwave activation in this study enhances the extraction efficiency of phenolic compounds from olive oil industry by-products, contributing to a more sustainable and energy-efficient process [26].

High-performance liquid chromatography analysis. High-performance liquid chromatography (HPLC) (Rodano, MI, Italy) *Dionex Ultimate 3000* was used to identify and quantify oleuropein, demethyloleuropein, oleacine(3,4-DHPEA-EDA), and hydroxytyrosol. High-performance liquid chromatography was equipped with a 25 cm × 4.6 mm *Thermo Scientific Hypersil GOLD C18* column and a particle size of 5 μm. A gradient elution combining solvents A (H_2O/trifluoroacetic acid, pH = 2.46) and B (acetonitrile) was employed to separate the phenolic compounds in DESs. The column was initially equilibrated with 95 % solvent A and 5 % solvent B. Then a linear gradient of solvent B was increased to 60 % over 17 min, remained isocratic for 2 min, then increased to 95 % over 6 min, before returning to 5 % over 3 min and equilibrating for 5 min (total time 33 min). The chromatograms were obtained at 30 °C with an elution flow rate of 1 mL/min at 280 nm, and changes in absorbance were monitored at 280 nm. *Chromeleon* software was used for the instrumentation performance, chromatograms, and preliminary data processing.

To determine the amount of phenolic compounds present in the olive oil industry byproduct extracts, five standard solutions were prepared: 2000 ppm for pure oleeuropein, 2000 ppm for its aglycone form (3,4-DHPEA-EA), 2000 ppm for hydroxytyrosol, 2000 ppm for oleacein (3,4-DHPEA-EDA), and 2000 ppm for demethyloleuropein. The solutions were prepared in ethanol at 10, 25, 50, 75, 100, and 125 ppm. Each NADES extract was diluted in 500 μL of ethanol, which is a miscible solvent in all the deep eutectic solvents used in this study. Ethanol is the preferred solvent to prevent the formation of acetals from oleacein [2]. The HPLC analysis and quantification were performed on 20 μL of diluted extracts based on standard samples and calibration curves under the same circumstances.

Research results and discussion. The chemical and physical characteristics of NADESs are determined by their structure, which in turn affects how well bioactive compounds are extracted. Natural deep eutectic solvents are often made of affordable, biodegradable, and natural substances like chlorine chloride. The most often used hydrogen bond suppliers are polyols derived from renewable sources, carboxylic acids, and urea [23].

For this study, we selected five distinct NADESs, namely choline chloride in conjunction with urea (NADES-1), glycerol (NADES-2), lactic acid (NADES-3), ethylene glycol (NADES-4), and citric acid (NADES-5), to represent the different "classes" of hydrogen bond donors (HBDs) commonly used in NADES (Table 1). The solutions were prepared by heating to 80 °C in a water bath with continuous and vigorous stirring until a homogeneous, transparent colorless liquid was formed between 30 and 120 min. Because of the high NADESs viscosity, five kinds of aqueous NADES solutions were investigated from the starting solvents by adding 20 % v/v of water to each solution.

To prepare NADES, the two components were mixed continuously at 80 °C for 2 hours to form the desired liquid. The produced NADES were utilized as extraction solvents for phenolic compounds in the industry by-products of olive oil without the need for purification.

Table 1

Compositions and abbreviations of different choline chloride-based NADESs used in this study, molar ratio 1 : 1

Solvents composition	Addition of water, %	Physical color expectation	Abbreviation
ChCl: Urea	0	Colorless transparent oil	NADES-1
ChCl: Urea–water	20		NADES-1-w
ChCl: Glycerol	0		NADES-2
ChCl: Glycerol–water	20		NADES-2-w
ChCl: Lactic acid	0		NADES-3
ChCl: Lactic acid–water	20		NADES-3-w
ChCl: Ethylene glycol	0		NADES-4
ChCl: Ethylene glycol	20		NADES-4-w
ChCl: Citric acid	20	Pale yellow semisolid	NADES-5
ChCl: Citric acid–water	20	Pale yellow oil	NADES-5-w

The process of extracting phenols from plant matrices can be accelerated and enhanced with the use of MAE. This innovative technology has been reported in previous studies to significantly reduce extraction time compared to traditional methods, as well as to promote higher yield and quality of the extracted product. While this study did not directly compare MAE with traditional extraction methods, the observed efficiency of phenolic compound recovery using MAE aligns with findings from prior research. By incorporating green solvents and energy-efficient techniques like MAE, a more sustainable and envi-

ronmentally friendly extraction process can be developed. Additionally, the effectiveness of the microwave extraction process is strongly influenced by the optimization of key parameters, including extraction temperature [27–29]. Generally, increasing the temperature increases the solvent power, which is significant for viscous solvents like NADES. This is due to the reduction in viscosity and diffusivity. Increasing the temperature also helps to reduce surface tension, which improves desorption and dissolution of the target compound in the solvent by reducing the interaction between sample matrix and the target compound. However, higher temperatures can lead to thermal degradation of phenols. Consequently, 80 °C mild temperatures and a maximum microwave power of 800 W were employed in this work. This was done to prevent the extraction mixture from overheating, which might cause the degradation of phenolic chemicals. Compared to the conventional water extraction approach, a 10-minute extraction period is sufficient to achieve a greater extraction of oleuropein and maximal yields of phenolic compounds [30, 31].

In this study, 1g of crushed ripe olive fruits was heated in 8 mL of DES at 80 °C and 800 W for MAE. Following centrifugation, the extracts were diluted in ethanol and HPLC analysis was performed.

It is difficult to identify extracted phenolic compounds since many structures are involved. One highly helpful technique for characterizing natural compounds is HPLC. According to published data, olive oil industry by-products mainly contain three bioactive compounds, namely oleeuropein, demethyloleiropein, and oleacine. It is important to note that oleuropein is the primary phenolic compound detected in all extracts, which was initially discovered in olive leaves alongside its isomer [32, 33]. Considering all the extracted phenolic compounds in this study, the result of the HPLC analysis showed that the detected amount of hydroxytyrosol is below the detection limit. However, this study indicates that the most common type of phenolic compound extracted from olive oil industry by-products is demethyloleiropein, compared to fresh or dry olive leaves under the same conditions [34]. Followed by oleacine; however, the yield was low, with the maximum extracted amount using NADES-5 as solvent reaching only 10.55 ppm after 10 min. While the highest amount of oleuropein (2270.25 ppm) was extracted using NADES-2 as a solvent, an extract high in oleuropein was produced after only 10 min, then its amount decreased gradually, and in some green solvents, it was not even detected. Furthermore, Table 2 and Fig. 1 demonstrated that after 30 min, there was a decline in the oleuropein content and a rise in the demethyloleuropein levels, with outstanding results achieved in the extraction process of demethyloleuropein using NADES-3 after 30 min.

Table 2

The HPLC analysis results of the phenolic compound content of ripe oliveextracted in NADESs*

Green solvents	Time, min	Oleuropein, ppm (Mean ± SD)	Demethyloleuropein, ppm (Mean ± SD)	Oleacine(3,4-DHPEA-EDA, ppm (Mean ± SD)
NADES-1	10	ND	ND	ND
	30			
NADES-2	10	2270.25 ± 5.3	7.32 ± 0.5	4.02 ± 0.1
	30	752 ± 0.5	14.57 ± 0.3	6.86 ± 0.2
NADES-3	10	754.61 ± 0.2	21.93 ± 0.7	ND
	30	ND	100.1 ± 0.9	4.3
NADES-4	10	ND	18.92± 0.1	4.81 ± 0.05
	30		14.98 ± 0.1	ND
NADES-5	10	ND	ND	10.55 ± 0.1
	30		10.99 ± 0.1	ND

* Hytrosol is in an amount not detectable by HPLC; ND = Not Detected.

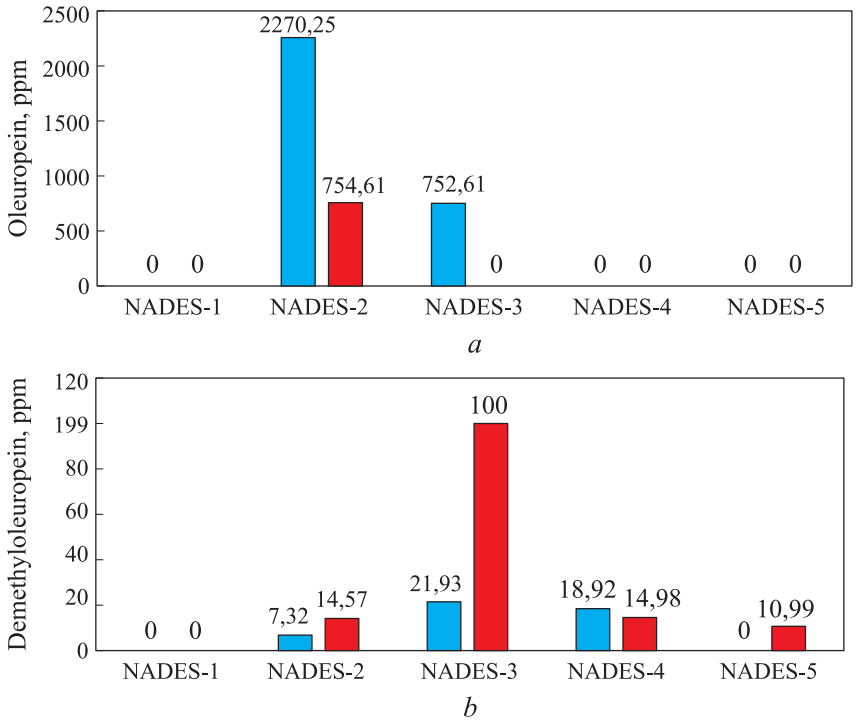
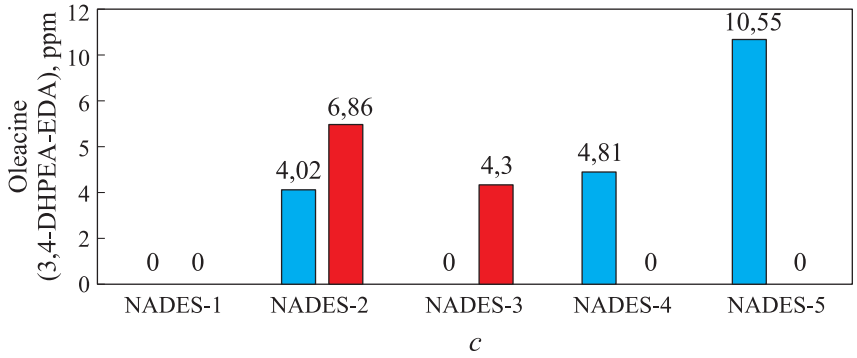


Fig. 1 (beginning). Quantitative analysis of ripe olives extracted with NADESs using HPLC to measure the levels of oleuropein (*a*), demethyloleuropein (*b*) with extraction durations of 10 ( ) and 30 min ( )



c

Fig. 1 (ending). Quantitative analysis of ripe olives extracted with NADESs using HPLC to measure the levels of oleuropein 3,4-DHPEA-EDA (*c*) with extraction durations of 10 ( ) and 30 min ( )

However, the ability of the desired compound to exit the natural matrix will be limited by the high viscosity of NADES. The high NADESs' viscosity compared with traditional solvents could make extraction solvent handling and efficiency more difficult. Since the interactions (such as hydrogen bonding) between the DES's components are weakened in the presence of water, adding water is essential to lowering the viscosity of the mixture [35]. To address this problem, extraction was carried out using NADES aqueous solutions. Previous studies indicate that a water concentration of 20 % can effectively lower NADES's viscosity while preserving the hydrogen-bonding network. Increased water content may progressively lessen the interactions between the NADES's constituent parts [23].

To perform this test, five aqueous NADES solutions were prepared by adding 20 % water to the respective weight ratios of the green solvents. Among all NADES, only NADES-2 could extract oleeuropein for a duration of 30 min. While demethyloleuropein could be extracted with all of the tested NADES except NADES-1, oleacine was extracted when NADES-2 and NADES-3 were used as extracted solvents (Table 3, Fig. 2).

Table 3

The HPLC analysis results of the phenolic compound content of ripe olive extracted in NADESs diluted with 20 % water*

Solvents	Time, min	Oleuropein, ppm (Mean ± SD)	Demethyloleuropein, ppm (Mean ± SD)	Oleacine(3,4-DHPEA-EDA), ppm (Mean ± SD)
NDES-1-w	10	ND	ND	ND
	30			

End of the Table 3

Solvents	Time, min	Oleuropein, ppm (Mean ± SD)	Demethyloleuropein, ppm (Mean ± SD)	Oleacine(3,4-DHPEA-EDA), ppm (Mean ± SD)
NDES-2-w	10	ND	35.92 ± 0.1	5.44 ± 0.5
	30	5.52 ± 0.4	18.88 ± 0.1	5.52 ± 0.3
NDES-3-w	10	ND	16.88 ± 0.2	13.96 ± 0.1
	30		8.69 ± 0.1	11.27 ± 0.2
NDES-4-w	10	ND	17.23 ± 0.2	ND
	30		13.45 ± 0.1	
NDES-5-w	10	ND	31.28	ND
	30		30.37	

* Hytrosol is in an amount not detectable by HPLC. ND = Not Detected.

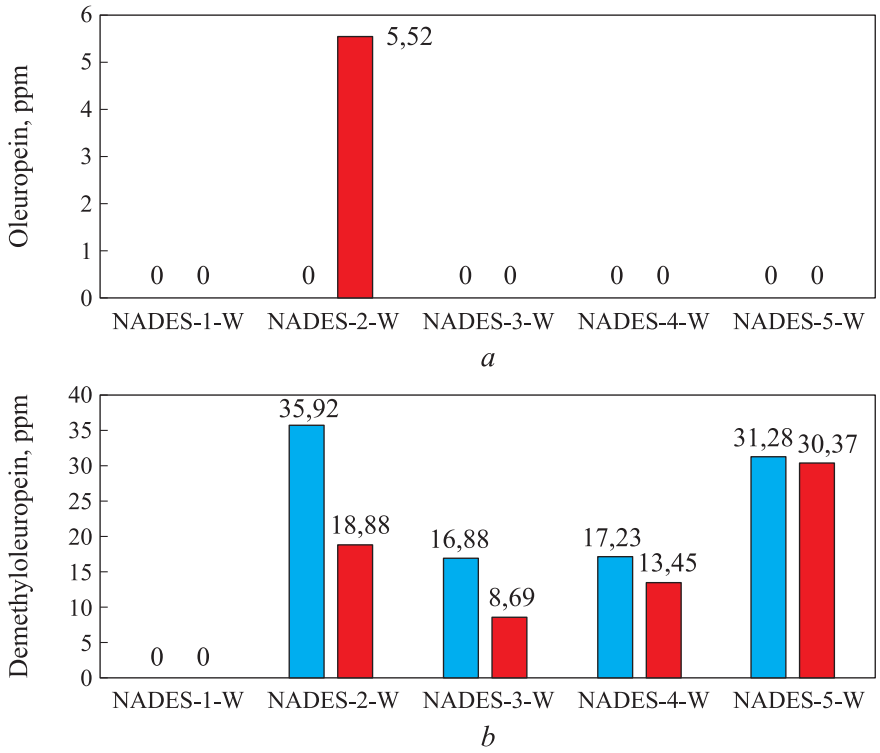


Fig. 2 (beginning). Quantitative analysis of ripe olives extracted with NADESs and water using HPLC to measure the levels of oleuropein (*a*), demethyloleuropein (*b*) with extraction durations of 10 ( ) and 30 min ( )

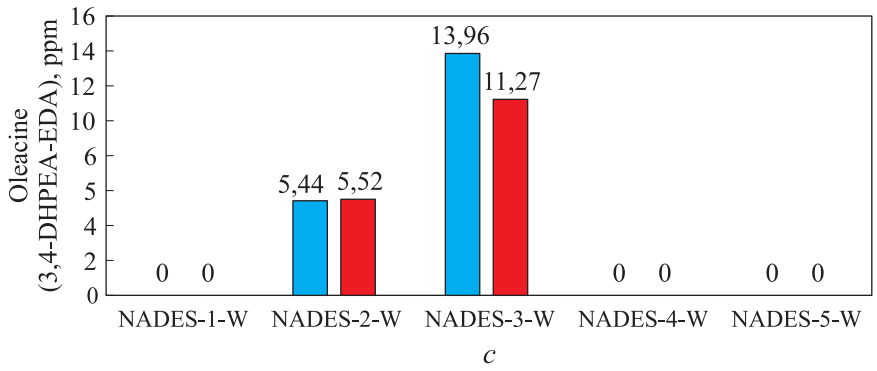


Fig. 2 (ending). Quantitative analysis of ripe olives extracted with NADESs and water using HPLC to measure the levels 3,4-DHPEA-EDA (*c*) with extraction durations of 10 ( ) and 30 min ( )

Conclusion. The study provided data that demonstrate the effectiveness of using sustainable green solvents (NADESs) in conjunction with MAE methods to recover phenolic compounds from olive oil processing by-products. However, the urea-based NADES-1 was discarded due to its low extraction power. Phenolic compounds were effectively extracted from olive drupes using the polyol-based NADESs, NADES-2, and NADES-4, as well as the organic acid-based NADES-3. It has been established that glycerol-based NADES-2 is an excellent solvent in terms of its effectiveness in extracting phenolic compounds, especially oleuropein, after 10 minutes. Generally, among all polyphenolic compounds, the best extraction result with NADESs (with and without water) was recorded for dimethyloleuropine. Finally, the findings showed that the industrial by-products of olive oil still contain significant levels of phenolic compounds that are useful in industrial pharmacies, such as dimethyloleuropine and oleacine(3,4-DHPEA-EDA). In contrast, a minor quantity of oleuropein was detected by NADESs in the olive oil industrial byproduct.

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