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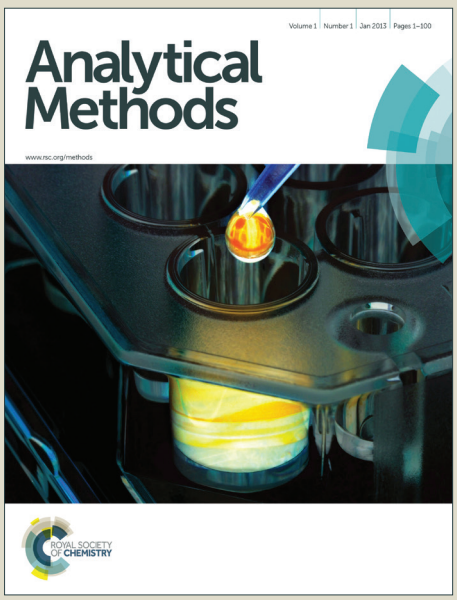

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Ultra–fast Microwave–Assisted Digestion in Choline Chloride–Oxalic Acid Deep Eutectic Solvent for Determining Cu, Fe, Ni and Zn in Marine Biological Samples

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Abstract

A green and very fast method for digestion of biological marine samples in choline chloride–oxalic acid deep eutectic solvent under microwave radiation was developed. These samples were then used for the determination of Cu, Fe, Ni, and Zn by inductively coupled plasma–optical emission spectrometry (ICP–OES). Key parameters that influence analyte recovery were investigated and optimized using the fish protein certified reference material (DORM–3). Features of the sample preparation method include: a) microwaves assisted dissolution of the samples in deep eutectic solvent at atmospheric pressure in just 20 sec, b) addition of 7.0 mL HNO_3 (2.0 M) to the cooled solution, and c) centrifugation, filtration and dilution of the solution to a predetermined volume before being subjected to analysis by ICP–OES. The Student's *t*–test (P = 0.05) showed an excellent agreement between the obtained results and the certified values, the recovery of all the elements being greater than 96.1 %. The proposed method was successfully applied in the determination of analytes in marine samples (fish muscle and liver tissues, and macroalgae). For comparison, a conventional acid digestion method was also used. The simplicity of the procedure, high extraction efficiency, short analysis time, absence of concentrated acids and oxidizing agents, and the use of safe and inexpensive components describe the high potential of the proposed method for routine trace metal analysis in biological samples.

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Keywords: Deep Eutectic Solvent; Choline Chloride–Oxalic acid; Microwave–assisted digestion; Metal; Marine biological sample; Inductively Coupled Plasma–Optical Emission Spectrometry.

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Introduction

Within the framework of green sample preparation methods, microwave–assisted digestion methods occupy a significant place [1]. Microwave heating is a very efficient, energy and cost saving process as the microwaves couple directly with the molecules present in the reaction vessel and lead to a rapid rise in temperature [2, 3]. Two different systems are available for microwave–assisted digestion, pressurized closed–vessel systems and focused open systems (working under atmospheric pressure) [4–6]. In general, microwave–assisted digestions in closed–vessel systems are preferred, since it minimizes possible contamination of the digest and avoids loss of volatile elements [7, 8]. For example, EPA 3052 and AOAC 999.10 are methods that involve digestion in closed–vessel microwave systems and are employed for the determination of heavy metals in a variety of matrices, including soil, sediments, oils, biological and botanical materials [8, 9]. However, a microwave–assisted digestion typically takes about 30–35 min to complete, with an additional 15–30 min cooling time to ensure safe handling [7, 10, 11]. Moreover, along with the high pressure, there is a risk of explosion associated with heating concentrated acids and oxidizing reagents in closed–vessel systems [4–6, 11]. To avoid these problems, focused microwave–induced combustion (FMIC) methods have been developed [12, 13]. These procedures involve the initial digestion of the sample with microwaves while maintaining oxygen flow, which is then followed by refluxing the sample in dilute nitric acid (4M) [14, 15]. However, the consumption of large amounts of oxygen (15 L min^{-1} for 10 min) to complete the combustion of a sample may lead

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to an increase in the cost of the analysis [14]. In contrast, there are some methods such as microwave–assisted alkaline digestion that use mild microwave radiations for relatively fast (about 4 min) digestion of biological samples [16–18], but these methods are usually more suitable for extraction of organometallic compounds such as methylmercury from their matrices.

An alternate approach for the accelerated extraction of analytes from biological samples is the use of ionic liquids (ILs) in combination with microwave irradiation [19]. The widespread use of ILs in both academia and industry is attributed to their unique properties that include negligible vapor pressure, high thermal stability, low/no volatility and ease of handling [20, 21]. The ionic nature of these liquids allow for their effective coupling with microwave energy via mechanisms such as ionic conduction and dipole rotation [19–22]. For example, Ma *et al*. [23] demonstrated that alkaloids from biological samples can be quantitatively extracted from their matrices by microwave–assisted digestion in ILs in a considerably shorter time (2 min) when compared to the time consumed in other conventional methods. Similarly, Jin *et al*. [24] reported a microwaves induced increase in the dissolution rates of medicinal plants in ILs.

However, many reports have highlighted the hazardous nature and poor biodegradability of most ILs [25]. In addition, processes for the synthesis of ILs are not always environment friendly [26].

To overcome the limitations of high price and toxicity of ILs, a new generation of green solvents—deep eutectic solvents (DESs)—have emerged [27]. A DES is generally composed of two or three non–toxic components that are capable of associating with each other through hydrogen bonds [28]. DESs typically have a very high depression in freezing point and are liquids at temperatures ranging from 21 °C to 70 °C [29]. Choline chloride (ChCl), an inexpensive, biodegradable, and non–toxic quaternary ammonium salt is widely used as one

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of the components in the formation of DESs [29]. When combined with non–toxic hydrogen bond donors (HBDs) such as carboxylic acids (e.g., oxalic acid), urea, or polyols (e.g., glycerol), ChCl is capable of rapidly forming a DES [28]. These mixtures can be prepared with high levels of purity and do not react with water, therefore, allowing for easy storage. They are also biodegradable, biocompatible, non–toxic, non–flammable, and inexpensive [29]. Additionally, it is suspected that some DESs are formed in living cells and are responsible for solubilizing, storing, and transporting non–water soluble metabolites [30].

Liquid ChCl mixtures have been used in applications related to various fields, including drug solubilization [31], biodiesel purification [32], electrodeposition of metals [29], and extraction of bioactive compounds [33].

In 2012, Singh et al. [34] reported, for the first time, a combination of DES and ultrasonic radiation for clean and efficient synthesis of oxazole derivatives. However, to the best of our knowledge, there is no report pointing out the effect of microwave radiation on the dissolution of samples in DESs. For a better determination of some biologically significant elements (e.g., Cu, Fe, Ni, and Zn) in specific environmental matrices, it is important to develop more convenient and accurate methodologies. Herein, we present the first report of a safe, efficient, simple, and low–cost method for the quick dissolution of marine biological samples in choline chloride–oxalic acid (ChCl–Ox) DES under microwave radiation at atmospheric pressure. This dissolution procedure was followed by the addition of dilute nitric acid to the sample for completing the extraction of Cu, Fe, Ni, and Zn from their matrices prior to their estimation by ICP–OES. Key parameters (temperature, time, volume and composition of the DES) that influence the dissolution of a marine sample were optimized using the certified reference material (CRM), DORM–3.

Experimental

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Reagents and solutions. All reagents were analytical grade and used without further purification. Choline chloride ($C_5H_{14}NClO$, 98.0%) was purchased from Sigma (St. Louis, MO, USA). High–purity oxalic acid (Ox) and HNO_3 (65%) were supplied by Merck (Darmstadt, Germany). Deionized water (DW) was used throughout the experimental work. A multi–element standard solution (PerkinElmer) containing all of the examined elements, each at a concentration of 10 μg mL^{-1}, in 5% (v/v) HNO_3 solution was used for ICP–OES calibration. Fresh solutions used for calibration were prepared each day from stock solutions. Fish protein CRM for trace metals (DORM–3) was obtained from the National Research Council Canada (NRCC, Ontario, Canada). To minimize the risk of metal contamination, all glassware was soaked in HNO_3 (5 M) for a minimum of 24 h, rinsed with DW, and dried in a laminar flow hood before use.

Instrumentation. The measurements were performed using a PerkinElmer Optima 7300DV ICP–OES instrument (Shelton, CT, USA) equipped with WinLab32 (Version 4.0) software for simultaneous measurement of all analyte wavelengths of interest. The operating conditions for ICP–OES and metal ion emission lines are presented in Table 1. An ETHOS One laboratory microwave system (Milestone, Sorisole, Italy) equipped with temperature and pressure feedback controls, magnetic stirring capability and operating at a maximum exit power of 1800 W was employed for the digestion and extraction processes. Ten high–pressure Teflon reaction vessels (100 mL inner volume) were cleaned with concentrated nitric acid (5 mL) before each digestion. The fish and algae samples were dried in a Zirbus freeze–drier (Zirbus VaCo 2–E, Bad Grund, Germany) with a condenser temperature of −50 °C and a chamber pressure $P < 0.08$ mbar.

<Table 1>

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Sampling and sample pre–treatment. A sample of the fish Kafshak (*Platichthys flesus*) was bought fresh from a local fish market in Khorramshahr, Iran. These fish are usually caught from the Musa estuary (northwestern region of the Persian Gulf, Iran); they are widely consumed by the people in this region. The sample was transported to the laboratory in an icebox. In the laboratory, the fish was washed thoroughly with DW. Different tissues, including the muscle and liver, were separated and cut into small pieces. Marine green macroalgae samples (*Enteromorpha intentinalis*) were freshly collected from Bushehr, Persian Gulf, Iran. They were rinsed thoroughly with DW. Then, all samples (fish tissues and algae) were freeze–dried for about 14 h, ground to a fine powder and sieved through a 125 mesh. The processed samples were preserved in clean polyethylene bottles at 4 °C.

Preparation of ChCl–Ox eutectic mixtures. There is no limit to the number or type of DESs that can be prepared from available chemicals, because there are a large number of salts and hydrogen bond donors that can be used to prepare these solvent mixtures [27, 28]. Our preliminary experiments indicated that ChCl–Ox DES systems have more ability to dissolve marine biological samples and extraction the elements from their matrices than other common eutectic solvents such as ChCl–urea and ChCl–glycerol. Therefore, in this work, the eutectic mixtures of ChCl–Ox were prepared at molar ratios of 2:1, 1:1, 1:1.5, 1:2, and 1:2.5, and utilized as solvents for the extraction of the metal ions form the biological samples. To prepare these eutectic mixtures, ChCl was mixed with Ox in a 100 mL microwave vessel and heated by microwaves at 300 W for 1 min. The temperature was continuously monitored with a thermometer inside the vessel, and the two components were stirred together till the formation of a homogeneous, colorless liquid. During this process, the temperature of the DES raised to 70 ± 3 °C. Since the obtained DES has very low vapor pressure, no pressure change was observed.

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Microwave–assisted digestion by ChCl–Ox (general procedure). About 0.10 g of the fish protein sample CRM was added to a pre–determined volume (1.5–3.5 mL) of the prepared ChCl–Ox DES. The sample was mixed thoroughly by stirring at 120 rpm for 1 min, following which; it was placed in the microwave oven at a pre–determined power for 20–65 sec, to reach the maximum temperature (150 °C) for fast dissolution. During this stage, the entire sample dissolved and resulted in a relatively uniform solution. After 5 min, the temperature of the sample was decreased to 80 °C and the door of the microwave instrument was opened. Then, 7 mL HNO_3 (0.5–2.5 M) was added to the vessel. The sample was centrifuged for 4 min at 8000 rpm. The supernatant was filtered through a 0.45 µm membrane filter and diluted in a volumetric flask with DW to 10 mL. These solutions were stored at 4 °C until analysis (usually within 48 h). With each series of extractions, a similar blank procedure was also conducted.

The percentage recovery of each metal ion was calculated and compared with the certified amounts (DORM–3) using the following equation:

$$\text{Recovery (\%)} = \frac{\text{Obtained value (µg g}^{-1})}{\text{Certified value (µg g}^{-1})} \times 100$$

After optimization, this method was applied to determine the prevalence of the 4 heavy metals in the algae sample and fish tissues.

Conventional acid digestion. A conventional acid digestion (CAD) method was also used for the determination of the elements in the fish tissues and the algae sample. To do this, 1.0 g of each sample was placed in a polytetrafluoroethylene (PTFE) digestion tube and 10 mL of concentrated HNO_3 was added. The sample was initially heated at 90 °C for 30 min, following which, the temperature was raised to 140 °C and the heating continued for 4 h or

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until a clear solution was obtained. The interior walls of the tube were washed down with a minimum quantity of DW. After cooling, the solution was filtered through a 0.45 μm filter and then transferred quantitatively to a 25 mL volumetric flask and diluted with DW to make up the remaining volume [35]. The concentrations of the metals in the marine samples were determined by ICP–OES.

Results and discussion

Optimization of microwave conditions. Time, temperature, pressure, and irradiation power in a microwave–assisted digestion method are the basic parameters that should be carefully controlled and optimized for the best extraction efficiencies as well as to ensure risk–free microwave operation [7–11]. Our previous work showed that the complete dissolution of the marine biological samples in ChCl–Ox DES occurred when samples were heated for 45 min in a conventional oil bath at temperatures above 100 °C; at lower temperatures, the dissolution of the CRM was incomplete, leading to relatively low extraction efficiency and precision [36].

In the current method, the temperature and time required for optimal dissolution were studied in detail at 4 power levels: 500, 800, 1000, and 1500 W. To optimize these parameters, 0.10 g of the CRM DORM–3 sample and 2.5 mL ChCl–Ox DES were used in procedures. The extractions were repeated in triplicate (n = 3). Preliminary experiments at each power level indicated that the maximum temperature allowed for dissolution of the samples in ChCl–Ox could not be greater than 150 °C, since at higher temperatures (>160 °C), some of the dissolved sample was carbonized and stuck to the walls of the vessel. The carbonized sample could not be re–dissolved in dilute acid. Therefore, we selected 150 °C as the optimum temperature for fast and complete dissolution of the samples. This temperature is about 30 to 70 °C lower than those typically used in the microwave–based digestion methods [11–15,

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37–40]. In Table 2, the time required to reach this temperature at each power level is presented (n = 5). As can be seen, by increasing the irradiation power of the microwave system from 500 to 1500 W, the time needed to reach the desired temperature decreases.

<Table 2>

Next, to determine the best level of microwave power to be used in the extraction procedure, the recovery of elements at each level of microwave power was investigated. For these studies, the time needed to reach the maximum temperature was kept constant in accordance with the values in Table 2. As shown in Fig. 1, reproducible (n = 3) extraction recoveries of the elements were greater than 94.1% at 500 and 800 W but, with a slight increase, reached to maximum levels (96.0–98.1%) at 1000 and 1500 W. Therefore, for highest extraction recoveries as well as for the shortest dissolution time, a power of 1500 W and its corresponding time (20 sec) were used in the remainder of this work. Typically, complete dissolution and digestion of a sample in microwave–assisted digestion methods takes about 35 min [7–11, 37–41], therefore it can be concluded that the current method of digestion in ChCl–Ox DES is at least 100 times faster. The observed fast rates for the dissolution of the biological samples in ChCl–Ox DES are likely the result of the good absorption of microwaves by the eutectic solvent mixture.

The initial pressure inside the vessel was constant at 1 bar and did not change during the microwave heating, consistent with the low vapor pressure characteristic of DESs. The lack of high pressure during operation and the lack of use of concentrated acids together with the non–toxicity of ChCl–Ox offer a safe and green method of sample dissolution. Additionally, reaction vessels of several types of materials, such as borosilicate glass, quartz, and PTFE, can be used as the dissolution takes place at atmospheric pressure.

<Fig. 1>

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Effect of ChCl–Ox composition. DESs are a unique class of multi–component solvent systems with varying physico–chemical properties [29]. Therefore, changing the type and/or the composition of the DES can significantly impact the dissolution of the metals and their efficient extraction from the matrices. For example, Abbott *et al.* [27] demonstrated that carboxylic acid–based DESs exhibit a much higher ability than other DESs, such as ChCl–urea and ChCl–ethylene glycol, for dissolving metal oxides. This is likely due to the protons from the carboxylic acids acting as good oxygen acceptors from the metal complexes, leading to the formation of chlorometalate species. Therefore, the effect of varying the composition of ChCl–Ox mixture on the recovery of the metals from 0.10 g of the CRM was evaluated under the optimized microwave heating conditions. To do this, ChCl was mixed with Ox at different molar ratios to obtain 5 different ChCl–Ox compositions. As shown in Fig. 2, by increasing the content of Ox (ChCl: Ox, 1:2), >96 % of the metals were recovered after the extraction in all cases. This property was relatively unchanged when the relative composition of Ox was increased up to 1:2.5 (ChCl:Ox). It should be noted here that with an increase in the molar ratio of Ox, the formation of the liquid eutectic ChCl–Ox solvent from its solid constituents takes place at higher temperatures. Therefore, the molar ratio of 1:2 (ChCl:Ox) was selected as the optimal composition of DES for the microwave–assisted fast dissolution of metals in marine biological samples. This method was then used for further optimization of other parameters.

<Fig. 2>

Effect of the DES volume. The volume of ChCl–Ox (1:2) has a significant effect on the microwave–assisted dissolution of the biological samples and its constituent metals. Different volumes (1.5–3.5 mL) of ChCl–Ox (1:2) were added to 0.10 g of the CRM and the samples were processed according to the general procedure. As can be seen from Fig. 3, the best

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metal–extraction efficiencies and precisions were achieved when 2.5–3.5 mL of the DES was added to the CRM. However, an increase in the volume of ChCl–Ox (1:2) above 2.5 mL led to an increase in the time required to reach the maximum temperature (150 °C) during the microwave heating. The time required at 1500 W was close to 30 sec for 3.5 mL of ChCl–Ox (1:2). Therefore, we used 2.5 mL of the liquid ChCl–Ox (1:2) in all assays for fast dissolution of the metals contained in biological samples. Lower volumes of ChCl–Ox (1:2) were insufficient for complete dissolution of the biological samples and led to lower extraction efficiency and precision.

<Fig. 3>

Effect of acid addition on recovery. After microwave–assisted dissolution of the fish protein CRMs in 2.5 mL ChCl–Ox (1:2) under the optimized conditions, varying concentrations (0.5–2.5 M) of 7.0 mL HNO_3 were added to the solutions in the microwave vessel. As a control, we also tested the effect of diluting the solution with DW. Addition of the aqueous solution, either DW or acid, led to the appearance of some suspended solid particles in the solution. A similar observation was reported when an aqueous solution was added to dissolve cellulose in ILs [40]. These solids were removed from the solution by centrifugation at 8000 rpm for 4 min and the resulting supernatant was used for analysis. As shown in Fig. 4 (at zero concentration of HNO_3), the extraction recoveries of all elements were 87.0–90.7% when the sample was diluted with DW. These relatively high extraction recoveries may be related to the formation of very soluble chlorometalate complexes in the ChCl–Ox DES system which then can be easily extracted from their matrices to DW. The extraction recoveries of Cu could not exceed 91.6% when samples were diluted with 0.5 and 1.0 M HNO_3; however, the recoveries of Fe, Ni, and Zn were in the 92.2 to 95.9 % range. This variation may be related to the different binding affinities of Zn, Cu, Ni, and Fe for

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nitrogen– and sulfur–containing proteins and other cellular macromolecules [41]. By increasing the concentration of HNO_3 to 1.5 M, >95 % recovery of all metals could be obtained. This level of recovery was maintained through a concentration increase up to 2.5 M HNO_3. While the levels of extraction recoveries in 1.5 to 2.5 M HNO_3 range were relatively constant, more reproducible results were obtained in the 2.0 to 2.5 M range (Fig. 4). Therefore, an optimum concentration of 2.0 M HNO_3 was used in all subsequent studies. To ensure proper mixing of 2.0 M HNO_3 with the dissolved sample in the DES and the release of the remaining metal ions bound to the solid matrix or stuck to the walls of the microwave vessel, it was necessary to add 7.0 mL of HNO_3. Lower volumes of the acid led to a decrease in the recovery and precision. Compared to other microwave–assisted acid digestion methods, the concentration of the acid consumed in this method is considerably lower and is required primarily for completing the extraction of the target analytes from their matrices after complete microwave–assisted dissolution [8–15, 38–40].

<Fig. 4>

Effect of the amount of the sample. The amount of the CRM sample (0.05, 0.10, 0.15, and 0.20 g) that can be dissolved in 2.5 mL ChCl–Ox (1:2) was also investigated under the pre–determined optimal conditions (Fig. 5). We found that, although the concentration of the metals in the final solution increased with increasing amounts of the sample, the highest recovery for all elements was obtained from either 0.05 g or 0.10 g CRM samples. Therefore, 0.10 g of the CRM or marine biological sample was used throughout.

<Fig. 5>

Analytical performance

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Precision and accuracy. The precision and accuracy of the procedure were assessed by determining the concentration of Cu, Fe, Ni, and Zn ions in the fish protein CRM, DORM–3. Five–replicates of the CRM sample were subjected to the ChCl–Ox (1:2)–based microwave digestion procedure followed by the ICP–OES analysis for the determination of trace metals. The data are presented in Table 3. As can be seen, the recoveries of elements are 96.1–98.0%. Comparison of the mean values by applying the Student's *t*–test (95% confidence range) verified that there was no significant difference between the results obtained from the proposed method and the certified values (Table 3). These results indicate that the established ChCl–Ox (1:2)–based microwave digestion procedure combined with ICP–OES is potentially applicable to the analysis of biological samples.

<Table 3>

Method detection limits. The method detection limit (MDL) was defined as $3S_b$, where S_b is the standard deviation corresponding to 10 blank measurements by the proposed method [42]. The MDLs of Cu, Fe, Ni, and Zn were calculated to be 0.08, 0.56, 0.04, and 0.23 μg g^{-1} for Cu, Fe, Ni, and Zn, respectively. The calculated MDLs for the proposed method were sufficiently low to allow for the determination of these elements in certified and test or other biological samples.

Application. The proposed method was used to determine the levels of Cu, Fe, Ni, and Zn in different parts (i.e., muscle, liver) of a marine fish, *Platichthys flesus*, and the marine macroalgae *Enteromorpha intentinalis*. All samples were collected and processed following the procedure described in the section of sample pretreatment, to give fine powders. Five replicates of each sample were then subjected to the microwave–assisted digestion by ChCl–Ox (1:2) followed by analysis on ICP–OES. The results are presented in Table 4. As can be

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seen, the concentration of Ni is considerably lower than the concentration of other metals in all the samples, but the reproducibility of the results (expressed as RSD %) was in the range of 0.2 to 6.1 %. Interestingly, the same level of reproducibility was observed in determining the concentrations of the most abundant metals in the samples, i.e. Fe and Zn in the fish–liver tissue and the macroalgae sample. Therefore, it can be concluded that the proposed microwave–assisted digestion by ChCl–Ox can successfully prepare samples for estimation of metals in various biological tissues.

<Table 4>

Comparison with conventional acid digestion. For the sake of comparison, the concentrations of the metals in the marine samples were also determined in a process that constituted an initial dissolution by CAD followed by ICP–OES analysis. The results are presented in Table 4. The results show a good agreement in the determined concentrations when the sample dissolution is conducted either by CAD or microwave–assisted ChCl–Ox. However, in most cases the results obtained from the ChCl–Ox (1:2)–based microwave digestion procedure are more reproducible (expressed as RSD %, n = 5) than the CAD method. The proposed method is also safer as it involves neither high pressure nor concentrated acids. Moreover, quantities of reagents consumed and acid–waste generated are considerably lesser than those in the CAD method. In addition, the time taken for the digestion of the sample in the ChCl–Ox (1:2)–based microwave digestion method (about 20 sec) was significantly lower than the time typically used in the CAD method (about 4.5 h). These advantages highlight the high efficiency of the ChCl–Ox (1:2)–based microwave digestion method in dissolution and extraction of metal ions from biological matrices.

Conclusion

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In this study, we developed a green and efficient digestion method, involving a quick microwave–assisted dissolution of biological samples in ChCl–Ox (1:2) DES, for extraction of Cu, Fe, Ni, and Zn ions prior to their estimation by ICP–OES. This is the first application that incorporates microwave heating with ChCl–Ox DES for dissolution of biological samples. This new method provides operational advantages, such as simplicity of the experimental procedure, use of low–cost materials, and relatively high speed of sample preparation. Moreover, because of the non–toxic nature and low vapor pressure of the DESs along with the absence of concentrated acids or oxidizing agents in the digestion mixture, the microwave–assisted digestion of the marine samples can be conducted under safe operating conditions. The sufficiently low detection limits (0.04–0.56 μg g^{-1}) are well–suited for quantitation of these metals in environmental samples. Agreement of the results obtained from the digestion of the fish protein CRM at optimized conditions with the certified values validated the precision and accuracy of this method. This method was successfully applied in the digestion of different marine samples (muscle and liver tissues from a marine fish, and macroalgae) possessing a broad range of metal concentrations. The results of digestion of the samples with CAD corresponded well with those obtained by the proposed method. The time taken for the ChCl–Ox (1:2)–based microwave digestion method of a marine sample is at least 100 times lower when compared to that consumed in other digestion methods, thereby, leading to reduction in the consumption of both time and energy. It is expected that a broad range of metals in varied biological matrices can be determined by employing this method. Moreover, considering the unique properties of DESs, the proposed method will have a broad applicability as an environmental friendly technique of sample preparation.

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Figure captions

Fig. 1. Effect of the microwave power on the recovery of Cu, Fe, Ni, and Zn (n = 3). *Conditions:* volume of ChCl:Ox, 2.5 mL; temperature, 150 °C; dissolution time, 20 sec; amount of sample, 0.10 g; concentration of HNO_3, 2.0 M.

Fig. 2. Effect of the composition of ChCl:Ox on the recovery of Cu, Fe, Ni, and Zn (n = 3). *Conditions:* volume of ChCl:Ox, 2.5 mL; microwave power, 1500 W; temperature, 150 °C; dissolution time, 20 sec; amount of sample, 0.10 g; concentration of HNO_3, 2.0 M.

Fig. 3. Effect of the volume of ChCl:Ox (1:2) on the recovery of Cu, Fe, Ni, and Zn (n = 3). *Conditions:* microwave power, 1500 W; temperature, 150 °C; dissolution time, 20 sec; amount of sample, 0.10 g; concentration of HNO_3, 2.0 M.

Fig. 4. Effect of the concentration of HNO_3 added to dissolved sample on the recovery of Cu, Fe, Ni, and Zn (n = 3). *Conditions:* volume of ChCl:Ox (1:2), 2.5 mL; microwave power, 1500 W; temperature, 150 °C; dissolution time, 20 sec; amount of sample, 0.10 g.

Fig. 5. Effect of the amount of the sample on the recovery of Cu, Fe, Ni, and Zn (n = 3). *Conditions:* volume of ChCl:Ox (1:2), 2.5 mL; microwave power, 1500 W; temperature, 150 °C; dissolution time, 20 sec; concentration of HNO_3, 2.0 M.

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Figure 1

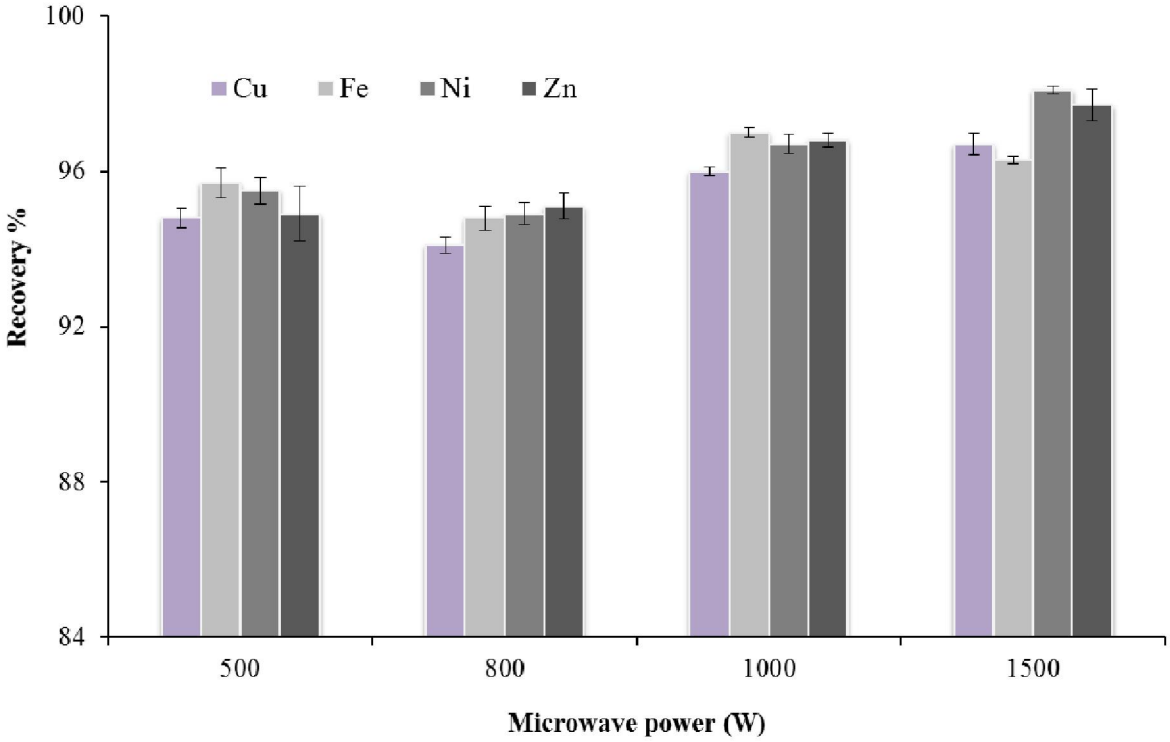

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Figure 2

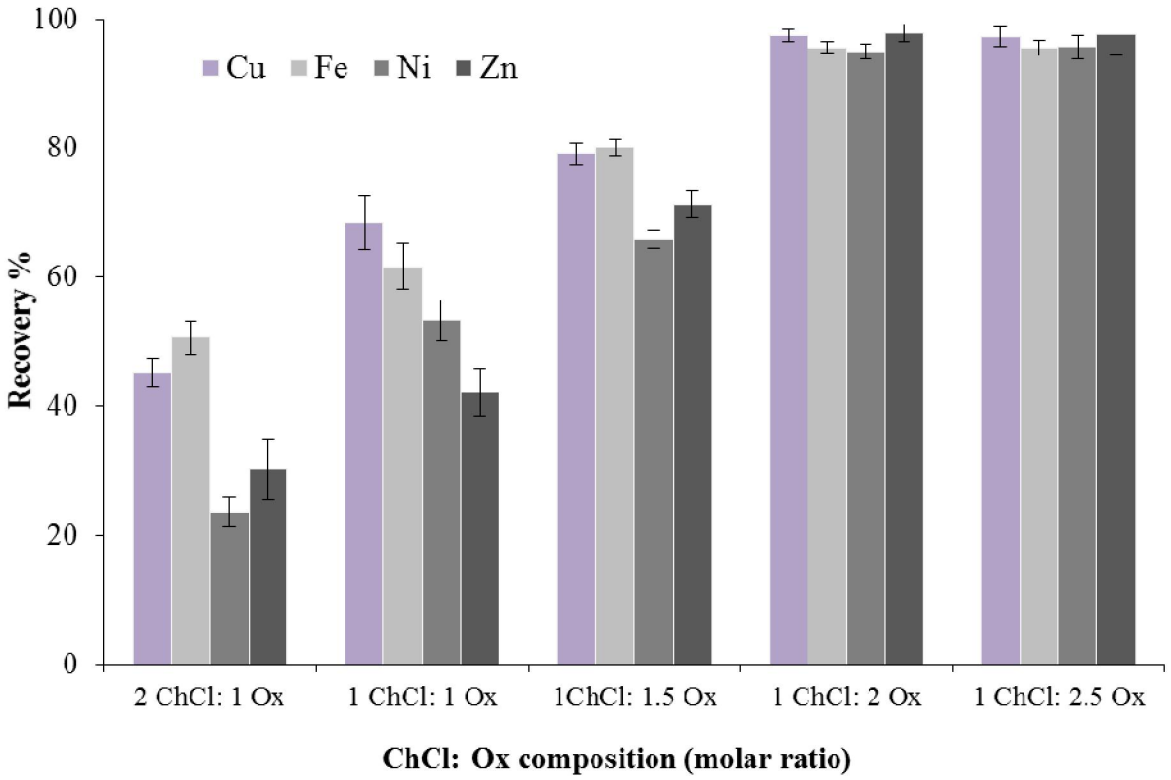

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Figure 3

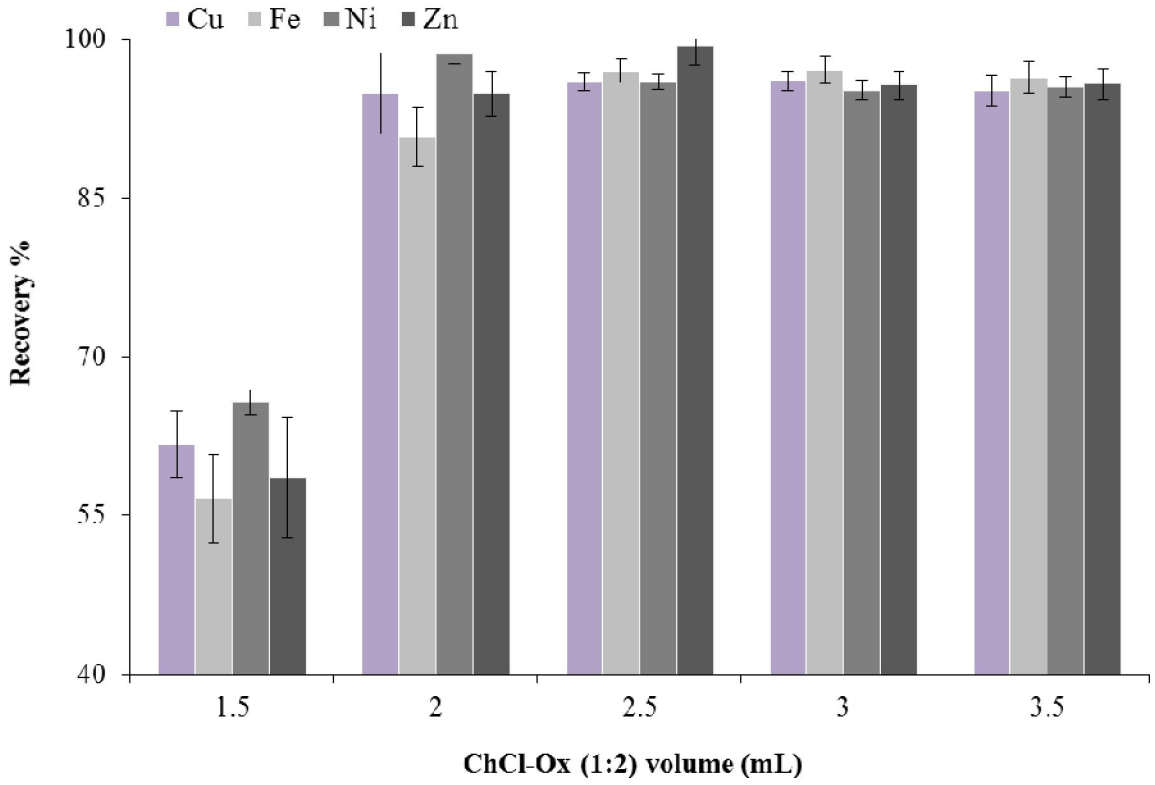

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Figure 4

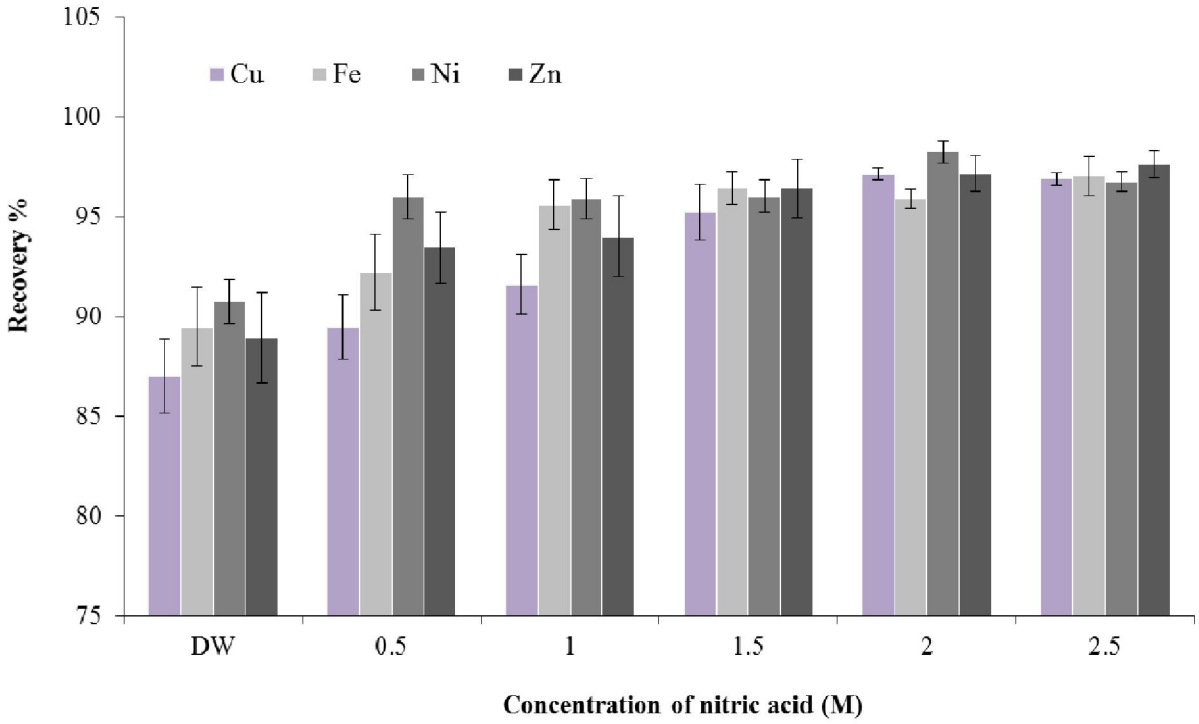

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Figure 5

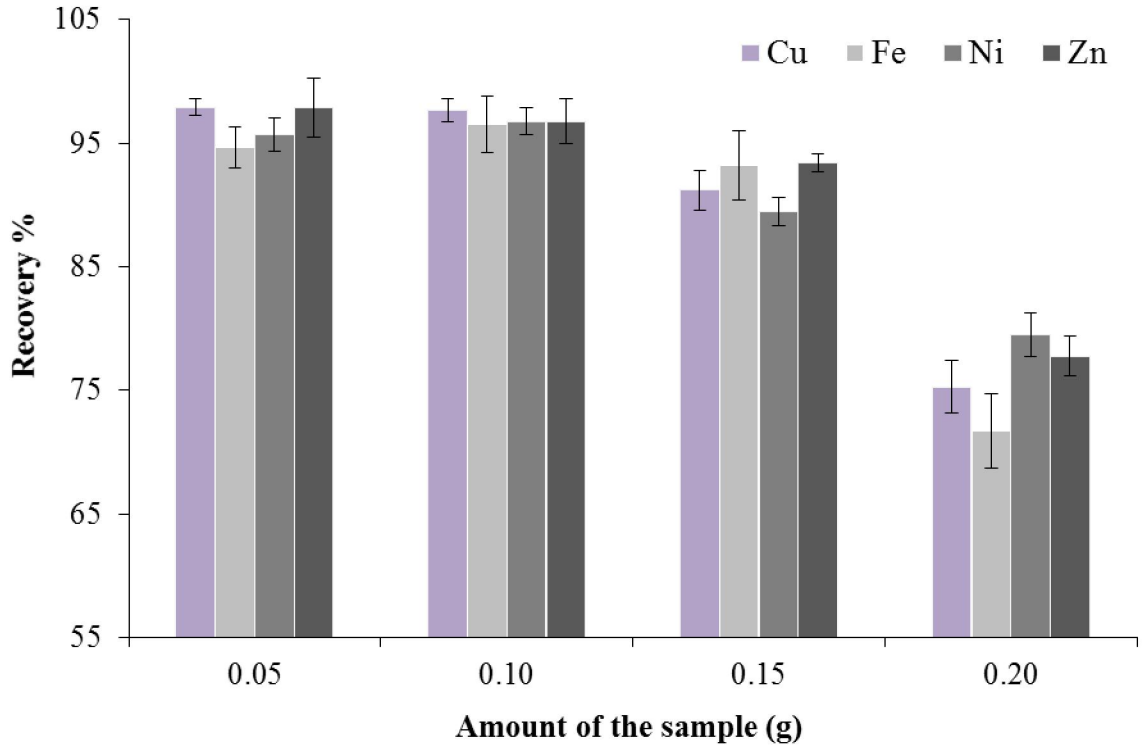

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Table 1

The operating parameters for determination of elements by ICP–OES.

Sample flow rate (mL min^{-1})	1.50
Plasma gas flow rate (L min^{-1})	15.0
Auxiliary gas flow rate (L min^{-1})	0.5
Nebulizer gas flow rate (L min^{-1})	0.65
RF generator power (W)	1450
Analytical lines (nm)	Cu (324.756), Fe (259.934) Ni (231.602), Zn (213.855)

Table 2

The time required to reach the maximum temperature at each power

Power (W)	500	800	1000	1500
Temperature (°C)	150	150	150	150
Time (Sec) [a]	65	45	30	20

[a] Based on 5 replications.

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Table 3

Determination of Cu, Fe, Ni and Zn in the fish protein CRM (DORM–3) by the proposed method.

Analytes	Certified values ($\mu g\ g^{-1}$)	Obtained values ($\mu g\ g^{-1}$)	Recovery (%)	$t_{Calc.}$ [a]
Cu	15.50 ± 0.63	15.16 ± 0.40	97.8	1.90
Fe	347 ± 20	333.64 ± 11.14	96.1	2.68
Ni	1.28 ± 0.24	1.25 ± 0.33	97.7	0.21
Zn	51.3 ± 3.1	50.30 ± 1.96 [b]	98.0	1.14

[a] Student's *t*–test. t_{calc} = calculated absolute value, t_{critic} = 2.78 (P = 0.05).
[b] Mean ± standard deviations (%95) based on 5 replications.

Table 4

Analytical results of Cu, Fe, Ni, and Zn in marine biological samples for this method and a conventional acid digestion (CAD) method.

Sample	Analyte	This method		CAD	
		Obtained value ($\mu g\ g^{-1}$)	RSD (%)	Obtained value ($\mu g\ g^{-1}$)	RSD (%)
Fish					
Muscle	Cu	15.40 ± 1.20[a]	6.3	14.08 ± 0.96	5.5
	Fe	63.50 ± 1.76	2.2	58.49 ± 5.49	7.5
	Ni	3.71 ± 0.01	0.2	3.77 ± 0.27	5.8
	Zn	48.82 ± 3.82	6.3	49.90 ± 2.7	4.4
Liver	Cu	94.45 ± 6.90	7.3	85.42 ± 7.23	6.8
	Fe	330.22 ± 6.65	1.6	319.43 ± 21.55	5.4
	Ni	2.10 ± 0.16	6.1	1.73 ± 0.10	4.6
	Zn	1103.86 ± 21.24	1.5	1073.31 ± 51.52	3.9
Macroalgae					
	Cu	8.50 ± 0.51	4.8	8.66 ± 0.58	5.4
	Fe	945.00 ± 18.14	1.5	936.19 ± 21.77	1.8
	Ni	7.75 ± 0.44	4.5	7.94 ± 0.68	6.9
	Zn	840.98 ± 40.58	3.9	821.91 ± 19.84	1.9

[a] Mean ± confidence interval (95%) based on 5 replications.

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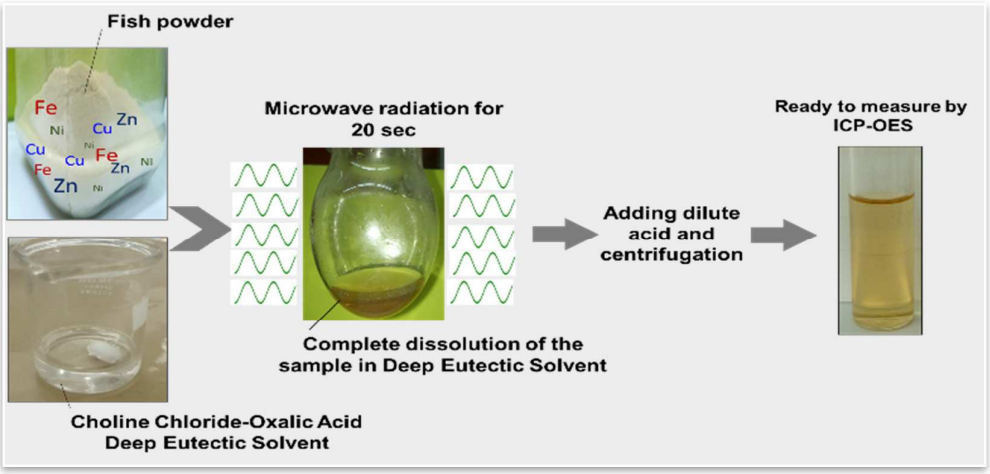
Graphical Abstract

This is the first application that incorporates microwave heating with deep eutectic solvent (DES) for dissolution of biological samples.



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