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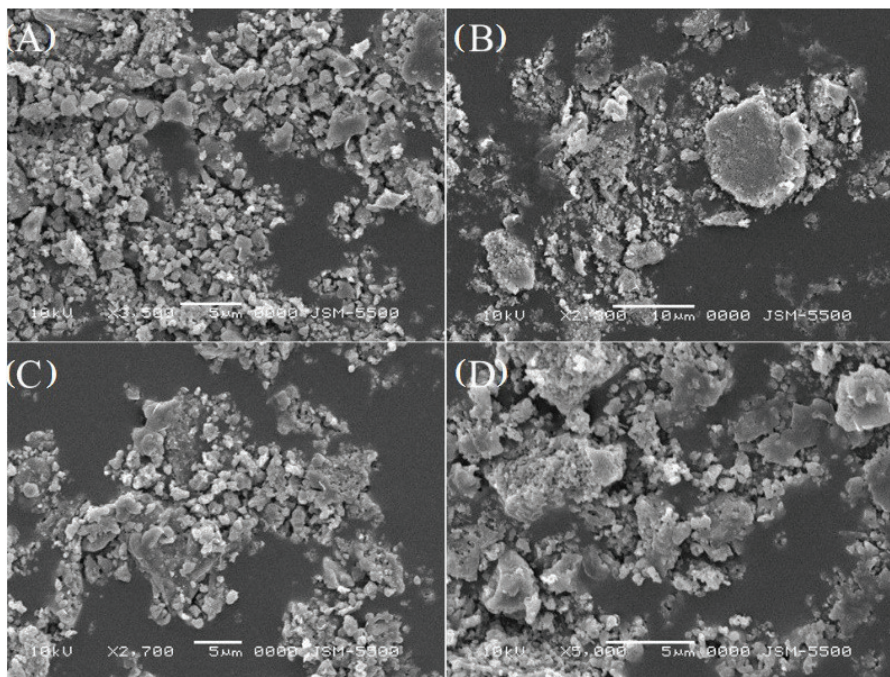
**Morphological characteristics and mitochondrial COI gene
of the green water dragon in Phu Quoc island, Vietnam**

Vietnam Journal of Science, Technology and Engineering (VJSTE) is a quarterly double-blind peer-reviewed academic journal. VJSTE publishes research articles and review articles in all areas of science, technology and engineering with the focus on the physical sciences, life sciences and environmental sciences including the following topics:

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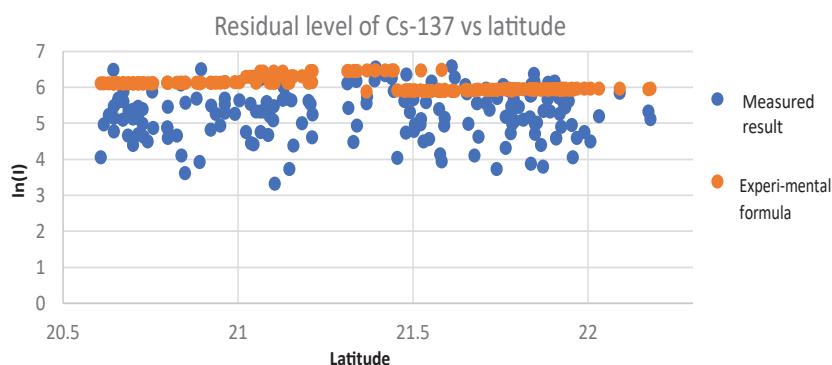
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Luminescence properties of Mn⁴⁺-doped CaAl₂O₄ as a red emitting phosphor for white LEDs

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Abstract:

Akin to the enormous number of discoveries made through luminescence materials, rare earth-doped aluminates are attractive materials for a new class of next generation displays and lighting devices that show merits like high chemical stability, brightness, and flexible industrial processing ability. A series of Mn⁴⁺-activated CaAl₂O₄ (CAO) compounds were synthesized by co-precipitation to seek a candidate for a red-emitting phosphor to be employed in a white LED. The crystal structure, morphology, and fluorescence properties of the as-obtained phosphors were investigated by X-ray diffraction (XRD), scanning electron microscopy (SEM), and photoluminescence (PL). The deep red luminescence of Mn⁴⁺ in CaAl₂O₄ is reported and discussed. The excitation spectrum exhibited broadband emission between 260 and 550 nm with three peaks dominating at 320 nm due to the transition of ⁴A₂→⁴T₁. The emission spectra between 600 to 720 nm displays an overwhelming emission peak at 654 nm owing to the 2E→4A₂ transition of Mn⁴⁺ ion. This research demonstrates the great promise of CaAl₂O₄:Mn⁴⁺ as a commercial red phosphor in warm white LEDs and opens up new avenues for the exploration of novel non-rare-earth red-emitting phosphors.

Keywords: CaAl₂O₄:Mn⁴⁺, co-precipitation, phosphors.

Classification number: 2.1

1. Introduction

Akin to the enormous number of discoveries made through luminescence materials, rare earth-doped aluminates are attractive materials for a new class of next generation displays and lighting devices that show merits like high chemical stability, brightness, and flexible industrial processing ability [1, 2]. Among the alkaline rare earth metal ion-doped aluminate MAI₂O₄ phosphors (M = Ca, Ba, Sr,...), CaAl₂O₄ is regarded as a potential host material for luminescence because of its chemical stability, high resistance to chemical attacks, low thermal expansion coefficient, and environmental friendliness [3]. Recently, most CaAl₂O₄ phosphors have been chiefly studied for their long-lasting luminescence [4] or luminescent properties when doped with expensive rare-earth ions such as Eu³⁺, Dy³⁺, or Ce³⁺ [5-8].

Mn⁴⁺ ions (d³) have exhibited a rather complicated optical spectra that is sensitive to crystalline fields [9] with a strong absorption in the UV and blue spectral ranges and emission of a deep red luminescence when the ions occupy the lattice sites of the host lattice. Thus,

Mn⁴⁺-doped red phosphors excited by blue LED chips can lessen the reabsorption effect when mixed with green or yellow phosphors. Besides, doping with Mn⁴⁺ ions is not likely to cause any significant structural changes due to the similar ionic radius between Al³⁺ and Mn⁴⁺ (Al³⁺:~0.535 Å and Mn⁴⁺:~0.54 Å). Over the years, other phosphors doped with Mn⁴⁺ have also been reported [10-14]. However, it is necessary to carefully study the luminescence properties of these phosphors to meet the requirements for their practical application in white LEDs.

Currently, there are several methods commonly used to prepare photoluminescence materials with small particle sizes such as by co-precipitation at room temperature, the sol-gel method, or through solid-state reactions. The advantage of co-precipitation over these other methods is that little effort is required to control the numerous factors (temperature, calcination time, pH, gelling substance/metal molar ratio, gelling temperature, etc.) that affect the formation of the single-crystalline phase. To our knowledge, CaAl₂O₄:Mn⁴⁺ red phosphors

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applied to white LEDs have been mostly prepared by solid-state reactions [6, 14-16], which is a much more complicated method compared to the previously mentioned methods. After careful consideration, we have chosen to prepare CaAl_2O_4 fluorescent powder-doped Mn^{4+} at low temperature where the combustion process is fast, simple, and safe. Consequently, the $\text{CaAl}_2\text{O}_4:\text{Mn}^{4+}$ phosphors in this work were prepared by co-precipitation and treated at various temperatures as well as under varied doping concentrations of Mn^{4+} to determine the optimum preparation conditions for this material.

2. Experimental

The Mn^{4+} -doped calcium aluminates were synthesized by co-precipitation. The stoichiometric raw materials $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, $\text{Al}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$, $\text{Mn}(\text{NO}_3)_2$, and NH_4OH were all analytical reagent grade. The chemical composition was prepared in the stoichiometric molar ratio of $\text{CaAl}_{2-y}\text{O}_4:\text{Mn}^{4+}$ where y is the doping concentration of the Mn^{4+} ion that ranged from 0.2-5.0 mol.%. The stoichiometric amount of raw materials was weighed, dissolved by DI water, and then stirred to achieve an even mixture. Then, NH_4OH was slowly added to the mixture to precipitate in hydroxide form. The mixture was continuously stirred at room temperature for 2 h until reaction and then a homogeneous solution in the form of a suspension was obtained. This precipitated mixture was dried at 150°C for 5 h to evaporate excess water. The powder was sintered from 900 to 1300°C for 6 h in air. Ultimately, the $\text{CaAl}_2\text{O}_4:\text{Mn}^{4+}$ phosphor samples were obtained.

After annealing, the crystallinity of the prepared powders was analysed by XRD D8-ADVANCE equipment using a Cu tube with K_α radiation of 0.154046 nm wavelength working at 40 kV/30 mA with a scanning speed of 0.005°/s. A scintillation detector was used for the powder samples. The optical properties were measured on a Nanolog, Horiba Jobin Yvon that employed a 450-watt Xenon lamp as the excitation source. The surface morphology of the specimens was examined using a Jeol JSM-5500 SEM. The SEM in this work offers a relatively high spatial resolution ($<5\ \mu\text{m}$ at 10 kV) and can operate over a wide range of acceleration voltages (10 kV). It is possible to operate the SEM in a high vacuum mode for the characterization of conductive materials in addition to low vacuum and environmental SEM (ESEM) modes. Measurements were performed at room temperature unless otherwise specified.

3. Results and discussion

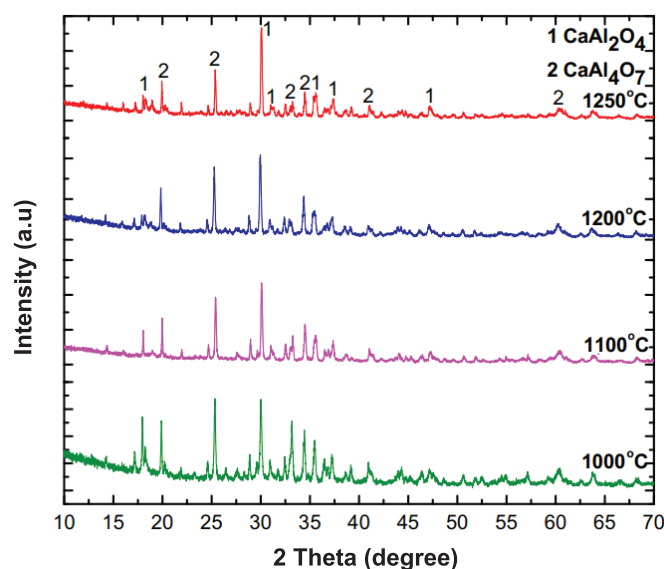


Fig. 1. X-ray patterns of Mn^{4+} -doped CaAl_2O_4 at various temperatures.

The XRD patterns of the $\text{CaAl}_2\text{O}_4:\text{Mn}^{4+}$ phosphors sintered at various temperatures from 1000 to 1250°C are shown in Fig. 1. Most of the diffraction peaks from these samples matched well with the standard Joint Committee on Powder Diffraction Standards (JCPDS) Card No. 70-134 (CaAl_2O_4) and No. 23-1037 (CaAl_4O_7) with the following lattice parameters: $a=8.7\ \text{\AA}$, $b=8.092\ \text{\AA}$, $c=15.191\ \text{\AA}$, and $V=1069.45\ \text{\AA}^3$. The XRD patterns show two crystallization phases: the target product CaAl_2O_4 and a phase of CaAl_4O_7 . The appearance of the CaAl_4O_7 phase remained as the annealing temperature was raised from 1000°C to 1100°C , but the peak intensities decreased. Increasing the calcination temperature to 1250°C led to the most dominant diffraction peaks in terms of sharpness and intensity, which are attributed to the CaAl_2O_4 network. XRD peaks of the CaAl_2O_4 phase increased as a function of the firing temperature because the formation temperature of the CaAl_2O_4 phase is higher than CaAl_4O_7 as shown in Fig. 1.

Besides, doping Mn^{4+} ions into the host does not cause any significant structural changes due to the similar ion radius of Al^{3+} and Mn^{4+} ($\text{Al}^{3+}\sim 0.0535\ \text{nm}$ and $\text{Mn}^{4+}\sim 0.054\ \text{nm}$). Thus, the crystallization temperature of the co-precipitated Mn^{4+} -doped CAO powders is much lower than that of the solid-state reaction method for the same component (1350°C) [14].

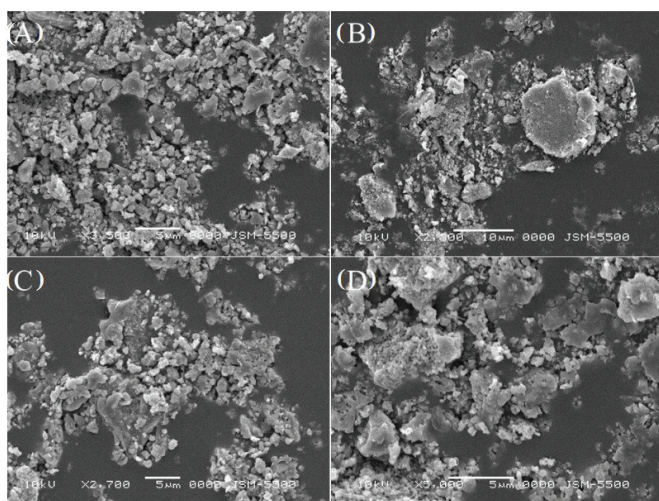


Fig. 2. SEM images of Mn^{4+} doped CaAl_2O_4 annealed at (A) 1000°C, (B) 1100°C, (C) 1200°C, and (D) 1250°C for 4 h.

To determine any morphological changes, SEM was utilized to investigate the effect of sintering temperature. The SEM image in Fig. 2 shows the co-precipitated $\text{CaAl}_2\text{O}_4:\text{Mn}^{4+}$ annealed at 1000, 1100, 1200, and 1250°C for 4 h and after grinding with a pestle. The annealed samples' morphology was similar: they presented an irregular morphology with angularity over various grain sizes. The surface of the phosphor powder annealed at 1000°C consisted of uniform grains with a sphere-like shape and were 300–400 nm in diameter. It is important to note that when the annealing temperature was increased from 1100 to 1250°C, the powder began to exhibit irregularly-shaped particles due to melting and bonding of the sphere-like grains at high annealing temperatures.

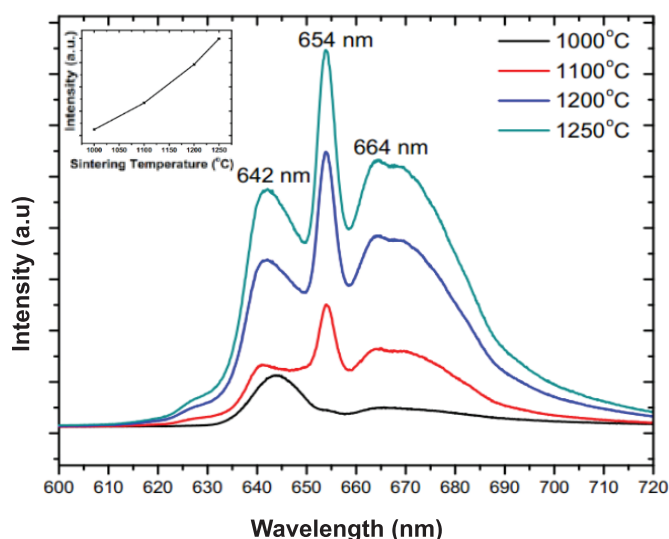


Fig. 3. PL spectra of $\text{CAO}:1\%\text{Mn}^{4+}$ phosphor at different annealing temperature.

To investigate the optical properties of the Mn^{4+} -doped CaAl_2O_4 phosphors, PL spectra were measured and are shown in Figs. 3–5. The PL spectra of the Mn^{4+} -doped CAO phosphors and the relationship between PL peaks' intensity and annealing temperature (1000 to 1250°C) are presented in Fig. 3. At 1000°C, the PL spectrum showed a very weak band around 644 nm while peaks located at 654 and 664 nm were not recognizable. This result can be explained by poor formation of the desired phase. With an increase of the calcining temperature to 1100°C, the emission intensities at 654 and 664 nm became more recognizable indicating the formation of the desired phase (CaAl_2O_4), however, it is still not very intense. The emission band around 654 nm exhibited the highest PL intensity when the calcining temperature grew to 1250°C. According to Fig. 3, the accepted annealing temperature is 1250°C, which is supposed to be the most achievable in this situation.

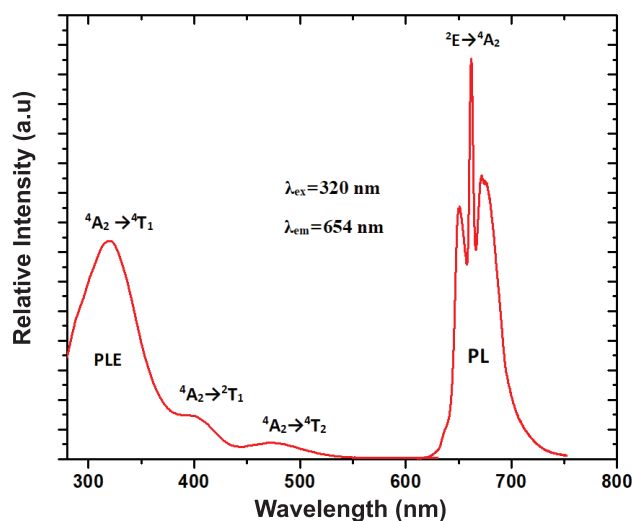


Fig. 4. PLE and PL spectra of $\text{CAO}:\text{Mn}^{4+}$ phosphor annealed at 1250°C for 4 h in air at room temperature ($\lambda_{\text{ex}}=320$ nm, $\lambda_{\text{em}}=654$ nm).

To determine the absorption regions of the phosphor, the normalized room temperature photoluminescence excitation (PLE) spectra of the $\text{CaAl}_{1.99}\text{O}_4:1$ mol.% Mn^{4+} phosphor annealed at 1250°C was monitored at the main emission peak (654 nm), which is plotted in Fig. 4. The excitation spectrum exhibited broadband emission near the visible ultraviolet and blue spectral regions and displays the presence of three peaks that correspond to 320, 390, and 470 nm from the spin-allowed transitions of $^4\text{A}_2 \rightarrow ^4\text{T}_1$, $^4\text{A}_2 \rightarrow ^2\text{T}_1$, and $^4\text{A}_2 \rightarrow ^4\text{T}_2$ of the Mn^{4+} ions in octahedral coordination, respectively [14]. These PLE

spectra indicate that it is possible to produce white light-emitting diodes when combining these phosphors with UV (260-380 nm), near UV (380-420 nm), and blue (420-480 nm) chips.

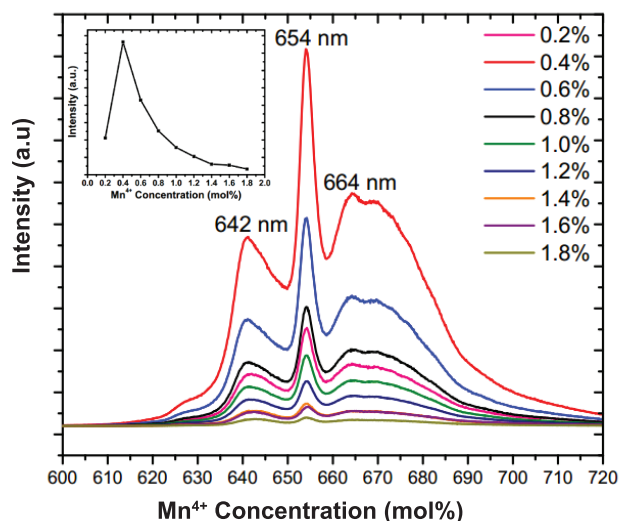


Fig. 5. PL spectra of $\text{CaO}:\text{yMn}^{4+}$ ($0.2 \leq y \leq 1.8$ mol%) phosphors annealed at 1250°C at room temperature ($\lambda_{\text{ex}} = 320$ nm).

To optimize the doping concentration of the phosphor, several $\text{CaAl}_{2-y}\text{O}_4:\text{yMn}^{4+}$ samples with different Mn^{4+} concentration were measured by PL (the concentration step size is 0.2 mol.%) calcinated at 1250°C . Fig. 5 reveals the high-resolution room temperature PL spectrum of phosphor calcined at 1250°C ($\lambda_{\text{ex}} = 320$ nm). The PL band covering 600 to 720 nm shows broadband emission with three primary peaks located at 642, 654, and 664 nm. The PL intensities were enhanced with increasing Mn^{4+} doping concentration in the range of 0.2 mol.% to 0.4 mol.% and decrease when the concentration exceeded 0.4 mol.% due to concentration quenching. This effect is explained by ion-ion interactions via transition metal ions that result in energy transfer and non-radiative relaxation from the emitting state. The cross energy transfer and non-radiative relaxation become dominant when the concentration of Mn^{4+} reaches a certain concentration [11].

Concentration quenching may be related to the mechanisms of the exchange interaction or the multipole-multipole interaction. In order to elucidate the mechanism responsible for the concentration quenching, an estimate of the critical distance (R_c) was performed according to the following relationship proposed by Blasse [9]:

$$R_c \approx 2 \left(\frac{3V}{4\pi X_c N} \right)^{1/3}$$

where V is the volume of the unit cell, X_c represents the critical concentration of the activator ion, and N stands for the number of sites in which the Mn^{4+} ion can be substituted in a unit cell. For $\text{CaAl}_2\text{O}_4:\text{Mn}^{4+}$, $N=4$, $X_c=0.4$, and $V=271.685\text{\AA}^3$, thereupon the R_c value is calculated to be $6.871\text{\AA}$. When the critical distance R_c is smaller than $5\text{\AA}$, the exchange interaction plays a primary role in the energy transfer among activator ions. Otherwise, the energy transfer mechanism belongs to the electric multipole interaction. Therefore, in our case, we infer that concentration quenching principally transpires via the electric multipolar interactions between the Mn^{4+} ions in the CaAl_2O_4 host [3, 10].

According to Ref. [14], the luminescence centre is from the Mn^{4+} ion. The strongest PL band peak located at 654 nm is due to the ${}^2\text{E} \rightarrow {}^4\text{A}_2$ transition of Mn^{4+} ion and the two weak PL band peaks located at 642 and 664 nm are assigned to the anti-Stokes vibronic sidebands associated with the ${}^2\text{E}$ excited state of the Mn^{4+} ion and the vibronic transition of the Mn^{4+} ion with zero-phonon line, respectively [14].

4. Conclusions

In summary, a series of $\text{CaAl}_{2-y}\text{O}_4:\text{yMn}^{4+}$ (0.2 ≤ y ≤ 1.8 mol.%) phosphors were successfully synthesized by co-precipitation with the achievement of the desired phase of CaAl_2O_4 , which was investigated by XRD. The obtained red emission was demonstrated at the 642, 654 and 664 nm peaks of the PL spectrum due to the ${}^2\text{E} \rightarrow {}^4\text{A}_2$ transition of the Mn^{4+} ion. The most efficient doping concentration and sintering temperature was determined to be 0.4 mol.% and 1250°C , respectively. The Mn^{4+} -doped CaAl_2O_4 phosphors with potential for integration as blend phosphors excited at either near UV and/or blue LED chips point out a desirable potential application as components in warm white light-emitting diodes.

CRedit author statement

Thi Kim Chi Nguyen, Duy Hung, Nguyen, Tan Vinh Le,: Data analysis and interpretation, Writing and manuscript revising; Thanh Vu Tran, Chi Nguyen Vo, Thi Kim Trung Pham: Method, Data analysis and Writing.

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COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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Development of new homogeneous photo-Fenton catalytic systems using oxalic acid and ferric ions in very low concentrations

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Abstract:

Despite its contribution to the economic development of many countries over the last few decades, the rapidly growing global textile industry has raised a wide range of environmental problems via the discharge of high amounts of toxic and non-biodegradable dye molecules into the wastewater. In this work, we proposed to combine oxalic acid and ferric ions in very low concentrations to create new, economic, and effective homogeneous photo-Fenton catalytic systems for the degradation of methylene blue. The effects of ferric concentration, $\text{H}_2\text{C}_2\text{O}_4$ concentration, pH, and tert-butanol on the catalytic activity were also investigated. According to the experimental results, Fe^{3+} ions exhibited impressive catalytic performances in the presence of $\text{H}_2\text{C}_2\text{O}_4$ at concentrations below type B (5.0 mg.l⁻¹) from the Vietnam National Technical Regulation on Industrial Wastewater. Specifically, a ferric concentration of 3.0 mg.l⁻¹, $\text{H}_2\text{C}_2\text{O}_4$ concentration of 10–3 mol.l⁻¹, and pH value of 3 were found to be the best conditions for MB degradation under UVA light. Furthermore, owing to a very low concentration of ferric ions, iron sludge formation can be avoided after wastewater treatment, which makes the photo-Fenton process suitable for practical applications.

Keywords: ferric ions, homogeneous photo-Fenton activity, methylene blue, oxalic acid, very low concentration.

Classification number: 2.2

1. Introduction

Despite its contribution to the economic development of many countries over the last few decades, the rapidly growing global textile industry has raised a wide range of environmental problems via the discharge of high amounts of toxic and non-biodegradable dye molecules into the wastewater. This kind of wastewater represents a severe threat to surrounding ecosystems and thus has stimulated the need for effective wastewater treatment technologies. Since the 1990s, homogeneous Fenton reagents that rely on $\text{Fe}^{2+}/\text{Fe}^{3+}$ ions and hydrogen peroxide have been proposed as a powerful method to completely mineralize dye molecules owing to the generation of highly reactive oxygen radicals such as hydroxyl and superoxide anion radicals [1, 2]. Moreover, it has been universally acknowledged that the combination of Fenton's reagents and UV-visible light illumination greatly improves the efficiency of organic compound decomposition [2, 3]. In fact, UV-visible irradiation accelerates the regeneration of Fe^{2+} ions via the photoreduction of Fe^{3+} ions, which ameliorates the production of reactive oxygen species.

Nevertheless, the practical application of the classical homogeneous Fenton technique is still restricted mainly by the enormous quantity of ferric sludge produced during the post-neutralisation process [4]. It is almost impossible to retain these homogeneous catalysts in a continuous system of wastewater treatment since $\text{Fe}^{3+}/\text{Fe}^{2+}$ ions are completely dissolved in the solution. Therefore, large amounts of iron salts must be continuously added to the solution to maintain Fenton reactions that not only increases the content of catalysts, but causes the rise of treatment cost and the formation of ferric sludge as a secondary pollution source [5]. Due to its hazardous features, this solid waste sludge also requires further treatment and thus is considered as a major obstacle blocking the application of Fenton processes on an industrial scale.

To overcome this drawback, numerous efforts have been made to impregnate iron ions into clay [6, 7], porous zeolite [8], and activated carbon [9, 10] or to develop other Fenton-like heterogeneous catalysts such as iron oxides [11, 12] and zero-valent iron [13]. Unfortunately, these

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heterogeneous catalytic systems show lower activity than classical homogeneous $\text{Fe}^{3+}/\text{Fe}^{2+}$ ions. Moreover, most of heterogeneous Fenton-like or photo-Fenton-like catalysts were found to be well dispersed in water due to their nanoscale particle size, which makes the catalyst recovery strenuous in real applications. On the other hand, H_2O_2 , which is traditionally used as the oxidant in photo-Fenton reactions, is found to be unstable and thus able to be decomposed during storage before use.

Therefore, in this work, we proposed to develop a new homogeneous photo-Fenton catalytic system based on the combination of ferric ions in very low concentration and $\text{H}_2\text{C}_2\text{O}_4$ as a radical producing source. Specifically, ferric ions were provided into a solution with concentration below type B (5.0 mg.l^{-1}) from the Vietnam National Technical Regulation on Industrial Wastewater (QCVN 40:2011/BTNMT). Thanks to the very low concentration of Fe^{3+} ions, treatment costs can be reduced and ferric sludge is nearly impossible to form. In addition, according to some previous reports [14, 15], the presence of ferrioxalate complexes produced from the reaction of iron ions and oxalate species can enhance the light absorption and consequently ameliorate catalyst performance. Thus, the replacement of H_2O_2 by $\text{H}_2\text{C}_2\text{O}_4$ in our work may allow us to not only improve the photo-Fenton catalytic activity of ferric ions but also easily store chemicals in the long term due to the high stability of oxalic acid. The influences of ferric concentration, $\text{H}_2\text{C}_2\text{O}_4$ concentration, and pH of solution on the performance of the catalytic system were also investigated.

2. Experimental

2.1. Preliminary tests

The starting materials $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, H_2O_2 , $\text{H}_2\text{C}_2\text{O}_4 \cdot 2\text{H}_2\text{O}$ (>98%, ACS reagent), and methylene blue (MB) as the model pollutant were purchased from Sigma Aldrich (USA). In addition, H_2SO_4 , NaOH and tert-butanol ($\geq 99.5\%$) were obtained from Merck.

To ensure that our hypothesis of the combination of ferric ions in very low concentration and $\text{H}_2\text{C}_2\text{O}_4$ can work as reagents, photo-Fenton tests for MB degradation under ultraviolet A (UVA) light were carried out with two ferric concentrations in the presence of H_2O_2 or $\text{H}_2\text{C}_2\text{O}_4$: a high concentration, 0.1 mol.l^{-1} , and a very low concentration, $9.0 \times 10^{-5} \text{ mol.l}^{-1}$ corresponding to the type B (5.0 mg.l^{-1}) of QCVN 40:2011/BTNMT. In a typical run for the high concentration of ferric ions, an amount

of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ was dissolved in water acidified with a diluted sulphuric acid solution. This ferric solution was poured into a stock solution containing both MB and H_2O_2 or $\text{H}_2\text{C}_2\text{O}_4$. Deionized water was quickly added to obtain a final solution with the volume of 250 ml. The concentrations of Fe^{3+} , MB, and $\text{H}_2\text{O}_2/\text{H}_2\text{C}_2\text{O}_4$ are, respectively, 0.1 , 3×10^{-5} , and $10^{-3} \text{ mol.l}^{-1}$. Then, the final solution was constantly stirred by a mechanic agitator and irradiated by a 9-W Radium 78 light lamp (UVA light), which was hung above the solution. The distance between the UVA light lamp and the solution surface was about 10 cm. During the reaction, the solution temperature was kept at $29\text{--}31^\circ\text{C}$ using a water circulation system. At regular time intervals, 5-ml aliquots of dye solution were taken out and their MB concentration was analysed by an Optima UV/VIS SP-300 spectrophotometer (Optima, Japan) at 664 nm. For the low concentration of ferric ions, the test was carried out in the same procedure but using ferric solution with the concentration of 5 mg.l^{-1} instead of 0.1 mol.l^{-1} .

To preliminarily investigate the mechanism of our catalytic system, three blank tests were effectuated: (i) a test for the photo-degradation of MB in the presence of $\text{H}_2\text{C}_2\text{O}_4$ without ferric ions, (ii) a test for the photo-degradation of MB in the presence of ferric ions without $\text{H}_2\text{C}_2\text{O}_4$, and (iii) a test for the catalytic degradation of MB in the presence of both $\text{H}_2\text{C}_2\text{O}_4$ and ferric ions without light illumination. Moreover, as a scavenger of hydroxyl radicals, tert-butanol was added into the solution containing MB, $\text{H}_2\text{C}_2\text{O}_4$, and ferric ions (5 mg.l^{-1}) to study the role of hydroxyl radicals.

2.2. Investigation of factors affecting the photo-Fenton process

The influences of ferric concentration, $\text{H}_2\text{C}_2\text{O}_4$ concentration, and pH on the photo-Fenton performance of our homogeneous catalytic system were studied via the same MB degradation procedure with different ferric concentrations (0.3 , 0.5 , 1.0 , 3.0 , 5.0 , 10.0 mg.l^{-1}), $\text{H}_2\text{C}_2\text{O}_4$ concentrations (10^{-4} , 5×10^{-4} , $10^{-3} \text{ mol.l}^{-1}$), and pH values (1 , 3 , 5 , 7 , 9 , 11), respectively. H_2SO_4 (1.0 mol.l^{-1}) and NaOH (1.0 mol.l^{-1}) solutions were used to adjust pH.

3. Results and discussion

3.1. Preliminary tests

As mentioned above, preliminary tests were carried out to verify whether the combination of ferric ions in very

low concentration and $\text{H}_2\text{C}_2\text{O}_4$ can effectively degrade MB under UVA light. Fig. 1 compares the $\text{Ln}(C_0/C)$ versus time plots of MB degradation (C_0 and C are the MB concentrations (mol.l^{-1}) at the beginning and at time t , respectively) under 3 different experimental conditions: (i) H_2O_2 ($10^{-3} \text{ mol.l}^{-1}$), Fe^{3+} ions (0.1 mol.l^{-1}); (ii) $\text{H}_2\text{C}_2\text{O}_4$ ($10^{-3} \text{ mol.l}^{-1}$), Fe^{3+} ions (0.1 mol.l^{-1}), and (iii) $\text{H}_2\text{C}_2\text{O}_4$ ($10^{-3} \text{ mol.l}^{-1}$) and Fe^{3+} ions (5.0 mg.l^{-1}). All the plots are quasi-perfectly linear, which proves that MB degradation over all our catalytic systems fits to a pseudo-first-order kinetic model. Hence, the photo-Fenton activity of the three different catalytic systems can be evaluated through an apparent rate constant (k , Table 1). At high ferric concentration (0.1 mol.l^{-1}), the combination of H_2O_2 and

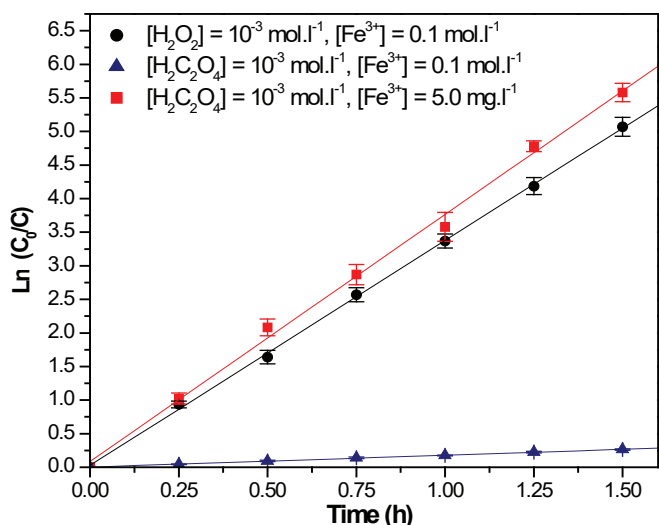


Fig. 1. Kinetic plot of UVA-induced MB degradation in different catalytic solutions.

Table 1. Rate constants (k) of UVA-induced MB degradation in different catalytic solutions.

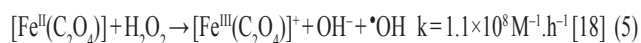
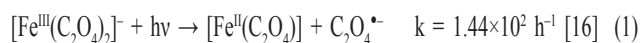
Ferric concentration	0.1 mol.l^{-1}	0.1 mol.l^{-1}	$9.0 \times 10^{-5} \text{ mol.l}^{-1}$ (5.0 mg.l^{-1})
Radical producing source	$[\text{H}_2\text{O}_2] = 10^{-3} \text{ mol.l}^{-1}$	$[\text{H}_2\text{C}_2\text{O}_4] = 10^{-3} \text{ mol.l}^{-1}$	$[\text{H}_2\text{C}_2\text{O}_4] = 10^{-3} \text{ mol.l}^{-1}$
$k \text{ (h}^{-1}\text{)}$	3.35	0.18	3.68

Table 2. Evolution of MB concentration versus time in three blank tests.

Experimental conditions	Time (h)	0	0.25	0.5	0.75	1.0
Blank test 1 $[\text{H}_2\text{C}_2\text{O}_4] = 10^{-3} \text{ mol.l}^{-1}$; UVA without Fe^{3+} ions	[MB] (mol.l^{-1})	2.62×10^{-5}	2.68×10^{-5}	2.65×10^{-5}	2.67×10^{-5}	2.67×10^{-5}
Blank test 2 $[\text{Fe}^{3+}] = 5.0 \text{ mg.l}^{-1}$; UVA without $\text{H}_2\text{C}_2\text{O}_4$	[MB] (mol.l^{-1})	2.41×10^{-5}	2.54×10^{-5}	2.24×10^{-5}	2.25×10^{-5}	2.13×10^{-5}
Blank test 3 $[\text{H}_2\text{C}_2\text{O}_4] = 10^{-3} \text{ mol.l}^{-1}$; $[\text{Fe}^{3+}] = 5.0 \text{ mg.l}^{-1}$ without UVA light	[MB] (mol.l^{-1})	2.70×10^{-5}	2.74×10^{-5}	2.83×10^{-5}	2.67×10^{-5}	2.69×10^{-5}

Fe^{3+} ions ($k=3.35 \text{ h}^{-1}$) had a much higher performance than that of $\text{H}_2\text{C}_2\text{O}_4$ and Fe^{3+} ions ($k=0.18 \text{ h}^{-1}$) proving that H_2O_2 is the better radical producing source than $\text{H}_2\text{C}_2\text{O}_4$ in the photo-Fenton process. However, in practical applications, the recovery of ferric ions at high concentration is very difficult. Furthermore, using a large amount of Fe^{3+} ions may cause ferric sludge as a secondary pollution source. Interestingly, in the presence of $\text{H}_2\text{C}_2\text{O}_4$, when the ferric concentration was decreased to type B of QCVN 40:2011/BTNMT (5 mg.l^{-1} , which is about 1120 times lower than a ferric concentration of 0.1 mol.l^{-1}), the MB rate constant was enhanced by a factor of 20.6 ($k=3.68 \text{ h}^{-1}$) in comparison with the rate constant for a ferric concentration of 0.1 mol.l^{-1} ($k=0.18 \text{ h}^{-1}$). This result demonstrates that a very low amount of ferric ions can effectively play as a photo-Fenton catalyst. Especially, since the ferric concentration is equal to type B of QCVN 40: 2011/BTNMT, the solution after reactions does not require any post-treatment and does not cause sludge.

Table 2 presents the results of the 3 blank tests used to preliminarily investigate the catalytic nature of Fe^{3+} ions in low concentration in the presence of $\text{H}_2\text{C}_2\text{O}_4$. Without Fe^{3+} ions or $\text{H}_2\text{C}_2\text{O}_4$ or light illumination, the MB concentration remained unchanged after 1 h. This confirms that the ferric ions, $\text{H}_2\text{C}_2\text{O}_4$, and UVA light must be involved in a catalytic mechanism for a methylene blue decomposition reaction. According to some previous reports [16-20], when ferrioxalate complexes are dispersed in a solution, UVA light can transform these complexes into many radicals like hydroxyl radicals through the following reactions (Eqs. 1-5):



Then, the generated hydroxyl radicals as highly oxidative reagents cause the complete mineralization of MB molecules. From the suggested mechanism, it was observed that the MB decomposition only takes place in the presence of all three agents, namely, the Fe(III) component, the oxalate species, and UV light, which is consistent with the results of our three blank tests. In our work, although we did not use ferrioxalate complexes, the addition of ferric ions (in very low concentration) into the solution containing $\text{H}_2\text{C}_2\text{O}_4$ is likely to form these complexes and then promote the above reactions to occur.

Moreover, when we added tert-butanol with the increasing concentration into the reaction mixture, we noticed that the performance of our photo-Fenton catalytic system gradually reduced (Fig. 2 and Table 3). Generally, tert-butanol has been widely acknowledged as a $\text{OH}^{\bullet}$ scavenger [21, 22], thus the decline in catalytic activity of MB degradation after adding tert-butanol proves that the hydroxyl radicals are the main agent for the degradation of organic molecules.

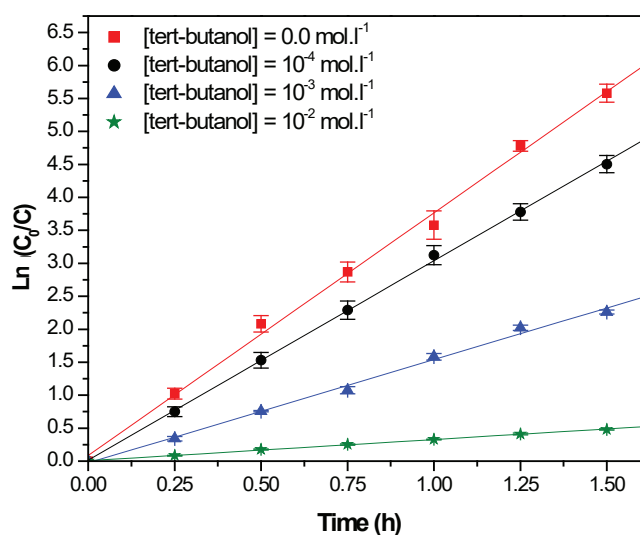


Fig. 2. Kinetic plot of UVA-induced MB degradation in the presence of Fe^{3+} ions (5.0 mg.l^{-1}), $\text{H}_2\text{C}_2\text{O}_4$ ($10^{-3} \text{ mol.l}^{-1}$) and tert-butanol with different concentrations.

Table 3. Rate constants (k) of UVA-induced MB degradation in the presence of Fe^{3+} ions (5.0 mg.l^{-1}), $\text{H}_2\text{C}_2\text{O}_4$ ($10^{-3} \text{ mol.l}^{-1}$) and tert-butanol with different concentrations.

[tert-butanol] (mol.l ⁻¹)	0.0	10 ⁻⁴	10 ⁻³	10 ⁻²
k (h ⁻¹)	3.68	3.02	1.57	0.32

3.2. Investigation of factors affecting the photo-Fenton process

To better understand the possibility of using ferric ions in low concentrations as a homogeneous photo-Fenton catalyst, we sequentially investigated the influence of ferric concentration, $\text{H}_2\text{C}_2\text{O}_4$ concentration, and pH on the catalytic performance of MB degradation.

3.3. Influence of ferric concentration

Figure 3 and Table 4 show the MB degradation profiles over our catalytic systems with different ferric concentrations (0.3, 0.5, 1.0, 3.0, 5.0, 10.0 mg.l^{-1}) in the presence of $\text{H}_2\text{C}_2\text{O}_4$ ($10^{-3} \text{ mol.l}^{-1}$). At the lowest concentration, the ferric ions exhibited the lowest photo-Fenton catalytic activity for MB degradation ($k=0.87 \text{ h}^{-1}$). This can be explained by the fact that when the concentration of Fe^{3+} ions is only 0.3 mg.l^{-1} , these ions hardly react with $\text{H}_2\text{C}_2\text{O}_4$. As a result, very few ferrioxalate complexes were formed, which leads to a small number of hydroxyl radicals generated by illumination with UVA light. In contrast, when the ferric concentration was up to 3.0 mg.l^{-1} , the rate constant quickly increased to 3.87 h^{-1} due to the facile formation of ferrioxalate complexes. However, for ferric concentrations ranging from 5.0 to 10.0 mg.l^{-1} , the catalytic performance slightly declined. It should be reminded that the catalytic performance was dramatically reduced to 0.18 h^{-1} at a ferric concentration of 0.1 mol.l^{-1} . The decrease in photo-Fenton catalytic activity may be related to the self-scavenging of $\text{OH}^{\bullet}$ radicals by Fe^{2+} . In fact, it was reported that radicals such as $\text{COO}^{\bullet}$, $\text{O}_2^{\bullet-}$, and $\text{HO}_2^{\bullet-}$ can act as both oxidants and reductants [23]. At the high ferric concentration (0.1 mol.l^{-1}), after the formation of ferrioxalate complexes, Fe^{3+} ions were still abundant and able to react with many radicals such as $\text{COO}^{\bullet}$, $\text{O}_2^{\bullet-}$, $\text{HO}_2^{\bullet-}$, and even H_2O_2 to form Fe^{2+} ions (Eqs. 6-7) [23, 24]. Then, according to several works [25, 26], these ferrous ions can consume hydroxyl radicals (Eq. 8), which leads to the slow decrease in MB concentration.

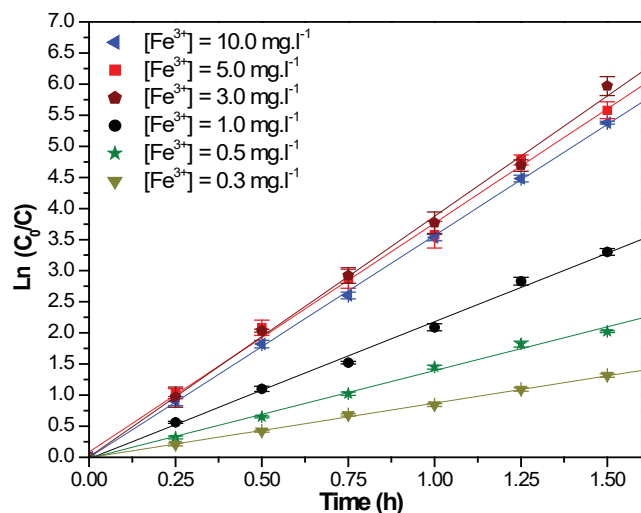


Fig. 3. Kinetic plot of UVA-induced MB degradation with different ferric concentrations in the presence of $\text{H}_2\text{C}_2\text{O}_4$ ($10^{-3} \text{ mol.l}^{-1}$).

Table 4. Rate constants (k) of UVA-induced MB degradation with different ferric concentrations in the presence of $\text{H}_2\text{C}_2\text{O}_4$ ($10^{-3} \text{ mol.l}^{-1}$).

$[\text{Fe}^{3+}]$ (mg.l^{-1})	0.3	0.5	1.0	3.0	5.0	10.0
k (h^{-1})	0.87	1.40	2.19	3.87	3.68	3.58

3.4. Influence of $\text{H}_2\text{C}_2\text{O}_4$ concentration

Besides the ferric concentration, the concentration of $\text{H}_2\text{C}_2\text{O}_4$ is also a factor affecting the photo-Fenton activity of our homogeneous catalytic systems. Therefore, we also investigated MB degradation in a solution containing Fe^{3+} ions (3.0 mg.l^{-1}) and $\text{H}_2\text{C}_2\text{O}_4$ with different concentrations (10^{-4} , 5×10^{-4} , 10^{-3} , $5 \times 10^{-3} \text{ mol.l}^{-1}$) (Fig. 4 and Table 5). The pH values of these solutions are also shown in Table 5. The rate constant of MB degradation strongly rose when the $\text{H}_2\text{C}_2\text{O}_4$ concentration varied from $10^{-4} \text{ mol.l}^{-1}$ to $5 \times 10^{-4} \text{ mol.l}^{-1}$ but slightly increased at a $\text{H}_2\text{C}_2\text{O}_4$ concentration of $10^{-3} \text{ mol.l}^{-1}$ and then decreased when the $\text{H}_2\text{C}_2\text{O}_4$ concentration was upped to $5 \times 10^{-3} \text{ mol.l}^{-1}$. This evolution can be partly explained by the dependence of the formation of ferrioxalate complexes on $\text{H}_2\text{C}_2\text{O}_4$ concentration. At a concentration of $10^{-4} \text{ mol.l}^{-1}$, the quantity of $\text{H}_2\text{C}_2\text{O}_4$ molecules is not sufficient to produce a significant amount of ferrioxalate complexes, resulting in a very low photo-Fenton activity. When the $\text{H}_2\text{C}_2\text{O}_4$ concentration increased to $5 \times 10^{-3} \text{ mol.l}^{-1}$ and $10^{-3} \text{ mol.l}^{-1}$, more Fe^{3+} ions were transformed into ferrioxalate

complexes, which effectively enhanced the catalytic performance. However, at a higher $\text{H}_2\text{C}_2\text{O}_4$ concentration ($5 \times 10^{-3} \text{ mol.l}^{-1}$), $\text{H}_2\text{C}_2\text{O}_4$ molecules may compete with MB molecules for the consumption of $\text{OH}^{\bullet-}$ radicals leading to the decrease in MB degradation rate. It should be noted that oxalate species may be related to kidney stone formation [27]. Therefore, in all our experiments, $\text{H}_2\text{C}_2\text{O}_4$ was always used in low concentration (from 5×10^{-4} to $5 \times 10^{-3} \text{ mol.l}^{-1}$). Besides, according to Eqs. 1-5, the oxalate species will be consumed during the photo-Fenton reactions, which lowers the risk of oxalate pollution. However, any remaining oxalate species must still be controlled and the post-treatment of the solution after the photo-Fenton reactions also needs to be investigated in the near future.

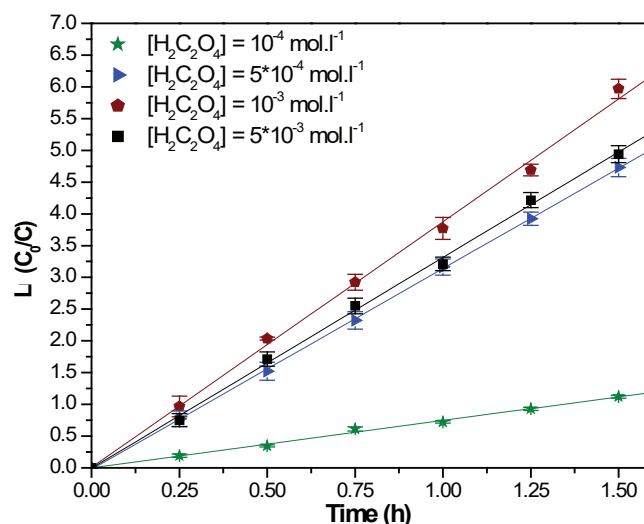


Fig. 4. Kinetic plot of UVA-induced MB degradation with Fe^{3+} ions (3.0 mg.l^{-1}) and $\text{H}_2\text{C}_2\text{O}_4$ at different concentrations.

Table 5. Rate constants (k) of UVA-induced MB degradation with Fe^{3+} ions (3.0 mg.l^{-1}) and $\text{H}_2\text{C}_2\text{O}_4$ at different concentrations.

$[\text{H}_2\text{C}_2\text{O}_4]$ (mol.l^{-1})	10^{-4}	5×10^{-4}	10^{-3}	5×10^{-3}
pH	3.9	3.6	3.2	2.6
k (h^{-1})	0.74	3.13	3.87	3.32

3.5. Influence of pH

Figure 5 and Table 6 display the MB degradation profiles at various pH (1, 3, 5, 7, 9, 11) in the presence of Fe^{3+} ions (3.0 mg.l^{-1}) and $\text{H}_2\text{C}_2\text{O}_4$ ($10^{-3} \text{ mol.l}^{-1}$). In the literature, it was reported that the optimal pH for classical Fenton reactions with H_2O_2 as an oxidant is about 2.8-3.0 [12, 28]. Our study exhibited the same results with

the highest efficiency ($k=3.70 \text{ h}^{-1}$) at pH 3. According to the proposed photo-Fenton mechanism of ferrioxalate complexes (Eqs. 1-5), H^+ ions are necessary to promote the formation of hydroxyl radicals, which is the main reagent for the oxidation of MB molecules. Thus, at pH values greater than 3, catalytic activity was quickly suppressed. However, at pH below 3, the catalytic performance of our system also reduced. This may be attributed to the stability of oxonium ions produced from the reaction between H_2O_2 and H^+ ions (Eq. 9) [29]. In fact, H_2O_2 can be generated by the interactions of ferrioxalate complexes, oxygen molecules, and H^+ ions under UVA light. If the pH is too low, a large amount of H^+ ions can easily react with H_2O_2 to form stable oxonium ions (H_3O_2^+), which makes the conversion of H_2O_2 to hydroxyl radicals more difficult.

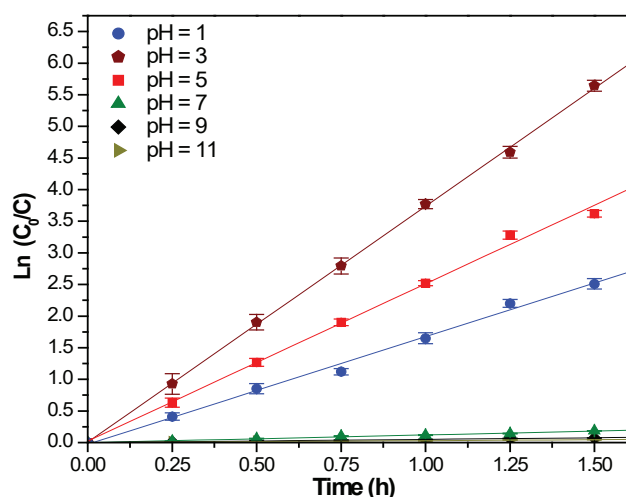


Fig. 5. Kinetic plot of UVA-induced MB degradation in the presence of Fe^{3+} ions (3.0 mg.l^{-1}) and $\text{H}_2\text{C}_2\text{O}_4$ ($10^{-3} \text{ mol.l}^{-1}$) at different pH values.

Table 6. Rate constants (k) of UVA-induced MB degradation in the presence of Fe^{3+} ions (3.0 mg.l^{-1}) and $\text{H}_2\text{C}_2\text{O}_4$ ($10^{-3} \text{ mol.l}^{-1}$) at different pH values.

pH	1	3	5	7	9	11
$k \text{ (h}^{-1}\text{)}$	1.68	3.71	2.49	0.12	0.05	0.03

4. Conclusions

In summary, we successfully developed a new and effective homogeneous photo-Fenton catalytic system for the degradation of dye molecules based on a mixture of $\text{H}_2\text{C}_2\text{O}_4$ and ferric ions in very low concentration (below the type B (5.0 mg.l^{-1}) of QCVN 40:2011/BTNMT). The very low concentration of ferric ions allows us to reduce

the cost of treatment and nearly avoid the formation of sludge as a secondary pollution source. Moreover, using $\text{H}_2\text{C}_2\text{O}_4$ instead of classical H_2O_2 permits long-time storage of chemicals owing to the stability of $\text{H}_2\text{C}_2\text{O}_4$. The influences of ferric concentration, $\text{H}_2\text{C}_2\text{O}_4$ concentration, and pH on the photo-Fenton performance of our catalytic system were also investigated. Among different experimental conditions, the ferric concentration of 3.0 mg.l^{-1} , the $\text{H}_2\text{C}_2\text{O}_4$ concentration of $10^{-3} \text{ mol.l}^{-1}$, and pH 3 were found to be the best conditions for efficient catalytic MB degradation.

CRedit author statement

Quoc Viet Bui: Data analysis and interpretation, Writing and manuscript revising; Thuy Vy Phan, Tien Khoa Le: Method, Data analysis and Writing.

COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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Metal- and solvent-free preparation of benzo[4,5]imidazo[1,2-a]pyrimidine derivatives catalysed by deep eutectic solvents

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Abstract:

Deep eutectic solvents (DESs) were first reported by Abbott and co-workers in 2001 and has since been widely applied to electrolytes, electrodeposition, electropolishing, ionothermal synthesis, gas adsorption, and biotransformation. DESs are classified as a new class of ionic liquids that present many outstanding characteristics such as low vapor pressure, nonflammability, easy preparation, low cost, and large recovery. DESs are classified as a new class of ionic liquids that present many outstanding characteristics such as low vapor pressure, nonflammability, easy preparation, low cost, and large recovery. These characteristics make DESs well-known as green solvents for organic transformations. These characteristics make DESs well-known as green solvents for organic transformations. The Biginelli reaction, first reported in 1891, played an important role in the synthesis of pyrimidine derivatives. A straightforward method for the preparation of benzo[4,5]imidazo[1,2-a]pyrimidine derivatives catalysed by an acidic deep eutectic solvent from choline chloride (ChCl) and phenol via the Biginelli reaction of 2-aminobenzimidazole, ethyl acetoacetate, and aldehydes has been developed under metal- and solvent-free conditions. The desired products were obtained in moderate to good yield (49-79%) and the DESs were known as an economical and environmentally friendly catalyst.

Keywords: Biginelli reaction, deep eutectic solvents, green catalyst, green chemistry.

Classification number: 2.2

1. Introduction

Deep eutectic solvents (DESs) were first reported by Abbott and co-workers in 2001 and has since been widely applied to electrolytes [1], electrodeposition [2], electropolishing [3], ionothermal synthesis [4], gas adsorption [5], and biotransformation [6]. DESs are classified as a new class of ionic liquids that present many outstanding characteristics such as low vapor pressure, nonflammability, easy preparation, low cost, and large recovery [7]. These characteristics make DESs well-known as green solvents for organic transformations.

The P. Biginelli reaction, first reported in 1891, played an important role in the synthesis of pyrimidine derivatives [8]. Benzo[4,5]imidazo[1,2-a]pyrimidine and other compounds containing a pyrimidine framework have been since recognized for their significant biological activities. Many different catalysts including silica sulfuric acid [9], ionic liquids [10, 11], $H_3PO_4-Al_2O_3$ [12], and $\alpha-Zr(CH_3)_{1.2}(O_3PC_6H_4SO_3H)_{0.8}$ [13] have been investigated for the efficient synthesis of these compounds. However, the drawbacks of these protocols are toxicity, expensive agents, and environmental harm. Herein, we describe an effective method for the preparation of benzo[4,5]imidazo[1,2-a]pyrimidine using a deep eutectic solvent as an efficient and reusable catalyst. This is the first time DES

has been used as a catalyst for the synthesis of benzo[4,5]imidazo[1,2-a]pyrimidine.

2. Experimental

All chemicals were purchased from Sigma-Aldrich. Solvents were supplied by ChemSol (Vietnam). ¹H and ¹³C nuclear magnetic resonance (NMR) spectra were performed on a Bruker Advance II 500 MHz NMR spectrometer (Fig. 1).

A typical procedure for the synthesis DES: A mixture of ChCl and phenol with a 1:2 molar ratio was magnetically stirred at 100°C until a colourless liquid was obtained. After cooling the reaction mixture to room temperature, the product was washed with diethyl ether and then dried in vacuum at 60°C for 6 h before use in experiments. The purity of the DES was confirmed by ¹H-NMR and ¹³C-NMR spectroscopy. Further characterization of DES by our group is reported in the previous paper [14].

DES ChCl:phenol (1:2): Colorless liquid. ¹H-NMR (500 MHz, DMSO-*d*₆) δ 9.49-9.35 (m, 2H), 7.14 (t, *J*=7.8 Hz, 4H), 6.85-6.63 (m, 6H), 5.52-5.45 (m, 1H), 3.82 (td, *J*=4.9, 2.4 Hz, 2H), 3.43 (m, 2H), 3.13 (s, 9H). ¹³C-NMR (125 MHz, DMSO-*d*₆) δ 157.85 (s), 129.78 (s), 119.20 (s), 115.73 (s), 67.57-67.27 (m), 55.57 (s), 53.77-53.46 (m).

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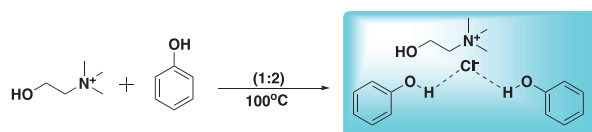


Fig. 1. Preparation of DES from choline chloride and phenol.

A typical procedure for the synthesis of benzo[4,5]imidazo[1,2-a]pyrimidine: 2-aminobenzimidazole (1.0 mmol), ethyl acetoacetate (1.0 mmol), aldehydes (1.0 mmol), and DES [ChCl][Phenol]₂ (10% mmol) were reacted at 100°C for 60-180 min under solvent-free magnetic stirring, then checked for completion by thin - layer chromatography (TLC). The crude products were recrystallized from hot ethanol to afford the desired products. The purity and identity of the products were confirmed by ¹H-NMR and ¹³C-NMR. The catalyst was washed with diethyl ether and activated by heating under reduced vacuum at 80°C for 30 min and then ready for the next recycle.

3. Results and discussion

Deep eutectic solvent-catalysed Biginelli reactions under green conditions is our ambition for the preparation of benzo[4,5]imidazo[1,2-a]pyrimidine derivatives, which are widely applied in pharmaceuticals. To evaluate the catalytic activity, we investigated DESs as homogeneous catalysts. A good yield was obtained from DES [ChCl][phenol]₂ (Table 1, entry 1). The reaction did not proceed in the absence of DES. The [ChCl][Glucose] provided the desired product in low yield due to high viscosity, which affected the diffusion of starting materials in the reaction mixture. The [Emim]Cl ionic liquid gave an acceptable yield (57%) and the best yield was obtained with [ChCl][Phenol]₂.

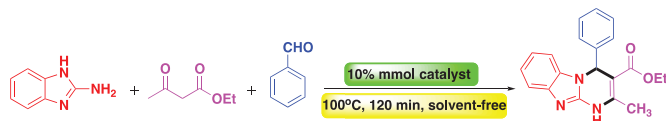


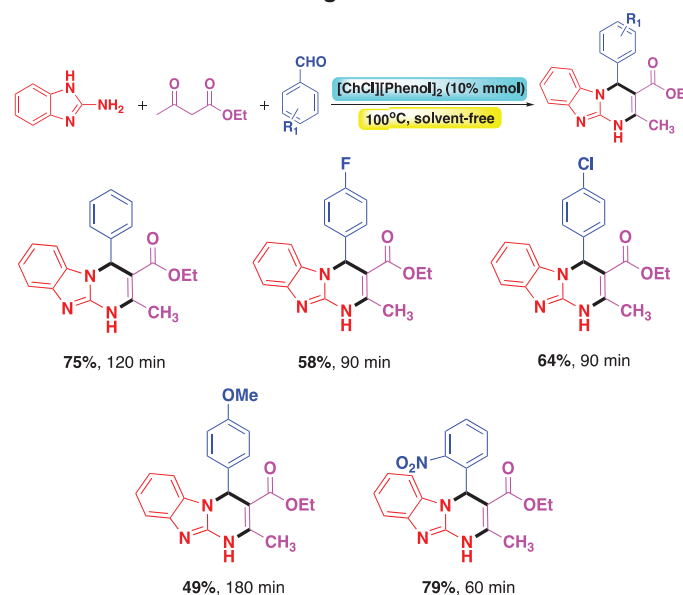
Table 1. Effect of homogeneous catalysts on the condensation reaction.

Entry	Homogeneous catalyst	Isolated yield (%)
1	[ChCl][Phenol] ₂	75
2	-	0
3	[ChCl][Glucose]	39
4	[Emim]Cl	57

Reaction condition: 2-aminobenzimidazole (1 mmol), ethyl acetoacetate (1 mmol), benzaldehyde (1 mmol).

To study the scope of aldehydes on this condensation reaction, aldehydes containing either electron-donating or electron-withdrawing substituents were applied (Table 2). The reaction achieved a higher yield with electron-withdrawing substituents such as -NO₂, -F and -Cl and a lower yield and long reaction time with an electron-donating group like -OMe. At room temperature, this reaction occurred despite a very low yield (~12%).

Table 2. Different substrates of aldehydes on the condensation reaction using DES.



Reaction condition: 2-aminobenzimidazole (1 mmol), ethyl acetoacetate (1 mmol), aldehydes (1 mmol).

To characterize the structure of benzo[4,5]imidazo[1,2-a]pyrimidine derivatives, ¹H-NMR and ¹³C-NMR were recorded on a Bruker Advance II 500 MHz NMR spectrometer using DMSO-*d*₆ as solvent (Table 3).

In the ¹H-NMR spectrum, similar patterns for proton resonance were also observed in five compounds. A triplet at δ 1.05-1.15 ppm (3H) and a multiplet at δ 4.21-3.76 ppm (2H) displayed the protons from the -OC₂H₅ group. Three protons from -CH₃ on the pyrimidine ring had a singlet at δ 2.46-2.42 ppm while the other proton was found as a singlet at δ 6.41-6.37 ppm. The protons from the two phenyl rings gave signals in the aromatic area. Besides, the three protons from the substituent -OMe of compound 4 had a singlet at δ 3.66 ppm. The ¹³C-NMR spectrum given in Table 3 had all signals match to all the carbon atoms.

Table 3. The spectral data of benzo[4,5]imidazo[1,2-a]pyrimidine derivatives.

Compound	R ₁	¹ H-NMR	¹³ C-NMR
1 [15]	H	(500 MHz, DMSO- <i>d</i> ₆) δ 7.35 (m, 3H), 7.25 (m, 3H), 7.17 (t, J=7.3 Hz, 1H), 7.04 (t, J=7.5 Hz, 1H), 6.94 (t, J=7.5 Hz, 1H), 6.41 (s, 1H), 4.07-3.96 (m, 2H), 2.45 (s, 3H), 1.13 (t, J=7.3 Hz, 3H).	(125 MHz, DMSO- <i>d</i> ₆) δ 165.7, 146.9, 146.1, 142.7, 142.5, 132.0, 128.8, 128.2, 128.2, 127.5, 127.5, 122.2, 120.6, 117.2, 110.3, 98.4, 59.8, 56.4, 19.6, 14.5.
2 [15]	F	(500 MHz, DMSO- <i>d</i> ₆) δ 7.40 (m, 2H), 7.34 (d, J=8 Hz, 1H), 7.26 (d, J=8 Hz, 1H), 7.11-7.01 (m, 3H), 6.95 (m, 1H), 6.45 (s, 1H), 4.12-3.90 (m, 2H), 2.45 (s, 3H), 1.14 (t, J=7.0 Hz, 3H).	(125 MHz, DMSO- <i>d</i> ₆) δ 165.6, 162.8 (d, J=244.2 Hz), 160.9, 147.0, 145.9, 142.7, 138.8, 131.9, 129.7, 122.3, 120.7, 117.4, 115.7, 115.5, 110.4, 98.3, 59.8, 55.7, 19.1, 14.5.
3 [15]	Cl	(500 MHz, DMSO- <i>d</i> ₆) δ 7.46 (m, 1H), 7.36 (d, J=8.0 Hz, 1H), 7.32-7.19 (m, 4H), 7.10-7.03 (m, 1H), 7.00-6.93 (m, 1H), 6.46 (s, 1H), 4.21-3.76 (m, 2H), 2.46 (s, 3H), 1.15 (t, J=7.0 Hz, 3H).	(125 MHz, DMSO- <i>d</i> ₆) δ 165.5, 147.5, 145.9, 144.9, 142.7, 133.3, 131.9, 130.9, 128.3, 127.6, 126.1, 122.4, 120.8, 117.4, 110.3, 97.8, 59.9, 55.8, 19.1, 14.5.
4 [15]	OMe	(500 MHz, DMSO- <i>d</i> ₆) δ 7.33 (d, J=7.5 Hz, 1H), 7.25 (m, 3H), 7.06-7.01 (m, 1H), 6.98-6.90 (m, 1H), 6.80 (d, J=8.5 Hz, 2H), 6.37 (s, 1H), 4.14-3.91 (m, 2H), 3.66 (s, 3H), 2.44 (s, 3H), 1.15 (t, J=7.3 Hz, 3H).	(125 MHz, DMSO- <i>d</i> ₆) δ 165.7, 159.1, 146.6, 146.1, 142.8, 134.6, 132.0, 128.7, 128.7, 122.2, 120.6, 117.2, 114.1, 114.1, 110.3, 98.7, 59.8, 55.8, 55.4, 19.0, 14.6.
5 [16]	NO ₂	(500 MHz, DMSO- <i>d</i> ₆) δ 7.91 (dd, J=8.0, 1.0 Hz, 1H), 7.59-7.54 (m, 1H), 7.43 (m, 3H), 7.26 (dd, J=8.0, 1.0 Hz, 1H), 7.11 (s, 1H), 7.10-7.07 (m, 1H), 7.00-6.95 (m, 1H), 4.03-3.97 (m, 1H), 3.94-3.88 (m, 1H), 2.42 (s, 3H), 1.05 (t, J=7.0 Hz, 3H).	(125 MHz, DMSO- <i>d</i> ₆) δ 165.4, 148.3, 147.8, 145.9, 137.3, 134.7, 134.7, 132.2, 129.7, 129.5, 124.5, 122.7, 121.3, 117.6, 110.0, 97.9, 60.1, 51.1, 19.4, 14.5.

4. Conclusions

In conclusion, we reported an effective and green procedure for the preparation of benzo[4,5]imidazo[1,2-a]pyrimidine via the condensation of 2-aminobenzimidazole, ethyl acetoacetate, and aldehydes under mild reaction conditions, short reaction time, and in environmentally-friendly media.

CRedit author statement

Thuy-Duy Thi Nguyen: Data analysis and interpretation, Writing and manuscript revising; Anh Vy Truong, Hoang Phuong Tran: Method, Data analysis and Writing.

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COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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Study on optimization of ternary complex of piroxicam- β -cyclodextrin-lysine inclusion in supercritical CO₂

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Abstract:

A variety of pharmaceutical agents including piroxicam (PC), a non-steroidal anti-inflammatory drug, have inaccessible bioavailability and dissolution. Piroxicam is a bioactive compound classified as a non-steroidal anti-inflammatory drug (NSAID). However, its low solubility in water imposes a serious limitation for its application in the pharmaceutical industry. Using cyclodextrins to form complexes can enhance the dissolution rate, solubility, and bioavailability of piroxicam. In this study, piroxicam/ β -cyclodextrin complexes are prepared in supercritical carbon dioxide (SC-CO₂) in the solid state and the process was optimized using response surface methodology (RSM). UV-Vis spectroscopy, differential scanning calorimetry (DSC), Fourier-transform infrared spectroscopy (FTIR), and dissolution profile in water were used to characterized the complex under optimized temperature, residence time, moisture, and ternary agent. Finally, the maximum reaction yield of the inclusion complex was predicted to be 95% at the optimal conditions of 266 bar, 136°C, 1.84:1 ratio of cyclodextrin:piroxicam, and 1.5:1 ratio of lysine:piroxicam. Large scale production of inclusion complexes can be developed from the results of this work on optimizing conditions.

Keywords: β -cyclodextrin, CO₂ supercritical fluid, piroxicam, piroxicam solubility.

Classification number: 2.2

1. Introduction

A variety of pharmaceutical agents including piroxicam (PC), a non-steroidal anti-inflammatory drug, have inaccessible bioavailability and dissolution. Molecular encapsulation is one approach available to produce a higher solubility compound, such as with the use of cyclodextrins (CDs), through the formation of inclusion complexes [1, 2]. CDs, also known as cycloamyloses and cycloglucans, are cyclic oligosaccharides composed of α (1, 4)-linked glucose units that have the shape of a truncated cone or torus as illustrated in Fig. 1, left [3, 4]. The cavity of the CD molecule is relatively non-polar compared to water, while the outer edge is polar due to the presence of hydroxyl groups on the faces of the structure. The chemical structure of the three native CDs α -, β -, and γ -cyclodextrin, are composed of 6, 7, and 8 glucose units, respectively. Due to their unique chemical structure, CDs are interesting host molecules for hydrophobic guest molecules in aqueous solutions [5]. In an inclusion compound or complex, a hydrophobic guest molecule can move into the cavity of the CD molecule (see Fig. 1, left). The hydrophilic character of the CD

molecule's surface, when replaced by the complex, can lead to improved aqueous solubility. When the inclusion complex is placed in an aqueous solution, the guest molecules are released from the CD's internal cavity through equilibrium association/dissociation, which drives the continuous release of the guest molecule [1, 6].

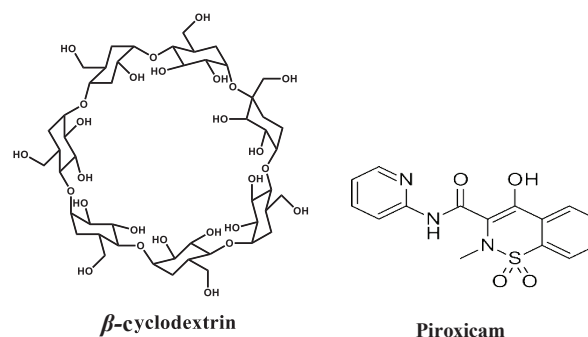


Fig. 1. Chemical structure of β -cyclodextrin (left) and piroxicam (right).

The formation procedures of CD-drug inclusion complexes use a diversity of techniques including methods that require considerable quantities of water

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rather than toxic organic solvents [3]. To be specific, kneading, coprecipitation, and methods that freeze dry or spray dry can provide high inclusion efficiencies but come along with a substantial amount of toxic solvents [4]. However, applying SC-CO₂ to form an inclusion complex can significantly reduce the amount of organic solvents used in the process. Another advantage of using CO₂ is its nontoxicity at ambient conditions and mild toxicity at critical conditions, which simplifies the problem of solvent residues. Supercritical CO₂ is known to be non-flammable, relatively non-toxic, and inert [7-9], which is ideal for a “green solvent” media that is suitable for the preparation and modification of a drug-carrier. Processes that generate particles using supercritical fluids (SCF), rapid expansion of a supercritical solution (RESS), supercritical anti-solvent (SAS), and particles from a gas-saturated solution (PGSS), may show higher rates of powder dissolution by increasing the specific surface area. However, techniques using SC-CO₂ to form inclusion complexes are reported to be limited by solubility of the drug in CO₂. Ternary agents, such as L-lysine, have been investigated as additions in the solid state to promote complex formation [10, 11].

In the present study, SC-CO₂ was used as a synthesis solvent to prepare a piroxicam/lysine/ β -cyclodextrin complex. The reaction was conducted in batch conditions using a high pressure induced autoclave. The effects of process and reaction parameters such as reaction ratio, reaction temperature, and pressure were investigated and analysed by RSM. The addition of L-lysine and water showed a significant influence on reaction yield. A UV-Vis spectrometer, FTIR, and DSC instruments were used to measure the reaction yield, product solubility, and related properties.

2. Materials and methods

2.1. Materials

Piroxicam (PC, 99,5%), β -cyclodextrin (β -CD, 98%), L-lysine (98,5%) from Sigma-Aldrich, methanol (HPLC grade, Merck), acetonitrile (HPLC grade, Merck), double distilled de-ionised water (DI), and CO₂ (99.99%, air liquid) were purchased and used as received. All other reagents and solvents were of analytical grade.

2.2. Synthesis of ternary complex in CO₂ media

In each experiment, the amounts of all related solvents were calculated by the supercritical density as well as the volume of reaction. Supercritical density was determined by the NIST REFPROP database from Aspen Plus. The PC: β -CD: lysine ternary complex was synthesized

from a mixture composed of piroxicam, lysine, and β -cyclodextrin in a molar ratio of reaction materials that varied depending on the experimental design conditions. The equipment included a volumetric pump, a 100-ml stainless steel autoclave, and a depressurisation system at the designed pressure and temperature. In a typical experiment, piroxicam (0.33 g), β -cyclodextrin (2.27 g), and lysine (0.146 g) were physically mixed and ground by a mortar and pestle and the molar ratio of reaction was 1:2:1, respectively. The white powder mixture was transferred to the autoclave. Then, 5 ml of deionized water (DDW) was added and stirred by glass rod until it became a homogeneous suspension. The autoclave was carefully closed and connected to the experimental system. The reactor was filled with SC-CO₂. The heat was slowly increased to 110°C, the pressure was fixed, and a magnetic stirrer ensured continuous stirring. After 3 h of reaction time, the reaction was slowly cooled down and the pressure was vented before collecting the product. Then, it was freeze dried.

2.3. Determination of the reaction yields and solubility

The reaction yield was calculated based on the relative amount of insoluble piroxicam in product and in the solid material mixture. Specifically, 15 mg of powder product was dissolved in 10 ml of DDW with sonication for 30 s. The solution was then centrifuged at 9,000 rpm for 5 min and then the supernatant was decanted. This was repeated two more times until there was no more product to be dissolved. The remaining solid in the centrifuge tube was dissolved by acetonitrile acidified with 0.05 M HCl (2.08 ml of 12 M HCl in 500 ml of acetonitrile). Solution is transferred to the volumetric flask and solvent was added to 25 ml. UV absorption at 334 nm is measured in the solution and the amount of PC was calculated based on a premade calibration of PC.

The reaction yield is then calculated based on the equation:

$$H(\%) = \frac{PC_{tot} - PC_{fr}}{PC_{tot}} \times 100$$

where PC_{tot} and PC_{fr} are the amount of piroxicam in the solid material before the reaction and amount of piroxicam insoluble in the product, respectively.

The water solubility of piroxicam in a complex product was measured by dissolving 25 mg of powder product in 10 ml of DDW with sonication for 30 s. The solution was then centrifuged at 9,000 rpm for 5 min and the saturation solution was decanted. All the clear supernatant solution was collected and DDW was added to 25 ml in a volumetric flask. The measurement was conducted on an HPLC equipped with a Zorbax Eclipse

Plus C18 (4.6x250, 5 μ m) column using a DAD detector and the amount of piroxicam was calculated based on a premade calibration of PC.

2.4. Characterization of complex product

Thermal analysis: Approximately 3 mg of sample was measured by Differential Scanning Calorimetry (DSC, Model 204 F1 Phoenix, NETZSCH, Germany) from 40 to 220°C at a heating rate of 5°C/min under an N₂ gas stream.

FTIR spectroscopy: Study of the piroxicam and product were conducted with an FTIR Tensor 27, Bruker, Germany spectrophotometer. The powder (10 mg) was kneaded with KBr (150 mg) and analysed using 16 scans, resolution of 4 cm⁻¹, and scan from 400 to 4,000 cm⁻¹.

2.5. Optimization of process parameters

RSM was chosen as the system to start the development, improvement, and optimization of complex production stages. According to the results from single-factor experiments, the effects of three factors, namely, temperature, pressure, and ratio of β -CD/PC, were chosen for further optimization. The inclusion product was evaluated by employing RSM based on the Box-Behnken design. The factor levels were coded as -1 to +1 as presented in Table 1. The input ratio of 1.5:1 LL:PC was selected based on preliminary experiments carried out in the laboratory [10, 11]. The optimum combination and the effect of process parameters on reaction yields were the target of the experiment and was performed through CCD. The relations between the response and process variables were observed after fitting the model, which was expressed as a second-order polynomial. The following equation was fit to the model:

$$y = b_0 + \sum_{i=1}^3 b_i X_i + \sum_{i=1}^3 b_{ii} X_i^2 + \sum_{i=1}^2 \sum_{j=i+1}^3 b_{ij} X_i X_j \quad (1)$$

where y is the expected response; X_i, X_j denoted the independent variables; b_0 is the model constant; b_i is the coefficient of the linear parameter; b_{ii} is the coefficient of the quadratic parameter; b_{ij} is the coefficient of the crossed parameter, and $\sum$ is the residual associated with the experiments.

Table 1. Experimental ranges and levels of the independent variables for RSM study.

Factors	Units	-1	0	+1
Temperature	°C	110	120	130
Pressure	Bar	200	250	300
Ratio β -CD/PC	-	1	1.5	2

Finally, the validity of the model was evaluated by the coefficient of determination (R^2) and its adjusted value. In addition, the significance of the obtained model and its terms were verified by using the analysis of variance (ANOVA).

3. Results and discussion

3.1. Characterization of ternary complex

Solubility: The solubility of the piroxicam complexation product in water is presented in Table 2. Under different reaction conditions, the solubility of piroxicam is 0.7 mg/ml in DI water corresponding to 2.1×10^{-3} mol/l, while trace amounts of free piroxicam was obtained in DI water. Based on the results, the water solubility of piroxicam complex is approximately 30.4 times more than free piroxicam.

Table 2. Box-Behnken design for inclusion PC complex and the observed yield for each experiment.

Experiment no.	Temperature (°C) X_1	Pressure (MPa) X_2	β -CD/PC X_3	% Yield actual	% Yield predict
1	140	250	1	52.9	55.03
2	130	250	1.5	82.3	84.00
3	140	300	1.5	79.8	81.24
4	130	250	1.5	80.1	84.00
5	120	200	1.5	35.5	34.06
6	120	300	1.5	40.8	42.12
7	130	200	1	22.3	21.49
8	130	250	1.5	88.1	84.00
9	130	200	2	38.7	42.28
10	140	200	1.5	45.5	44.18
11	130	250	1.5	85.5	84.00
12	120	250	1	36.8	39.05
13	130	300	1	41.9	38.29
14	130	300	2	69.8	70.62
15	140	250	2	92.5	90.23
16	130	250	1.5	84	84.00
17	120	250	2	59.1	56.97

UV-Vis absorption spectrum: The complexation obviously changed the solubility as well as the spectrum of the product with respect to that of free piroxicam. In contrast, the complexation product in DDW was quickly dissolved and formed a clear-to-slightly green coloured solution. The UV-Vis absorption spectrum of the product in Fig. 2 clearly showed the characteristic absorption peak of piroxicam at 358 nm and the shoulder at 291 nm assigned as the interaction of the piroxicam molecule with a protic solvent. In the case of free piroxicam and cyclodextrin, the spectra did not show absorption in the visible region [12, 13].

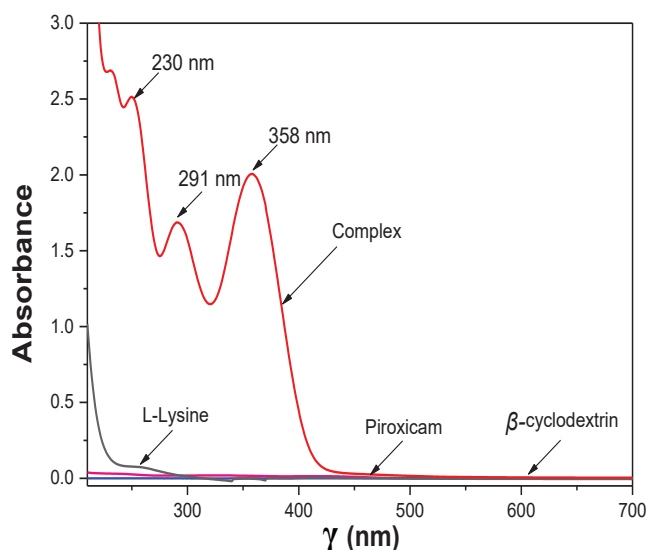


Fig. 2. The UV-Vis absorption spectra of piroxicam/ β -cyclodextrin complex, free piroxicam, β -cyclodextrin, L-lysine in H_2O solvent as indicated.

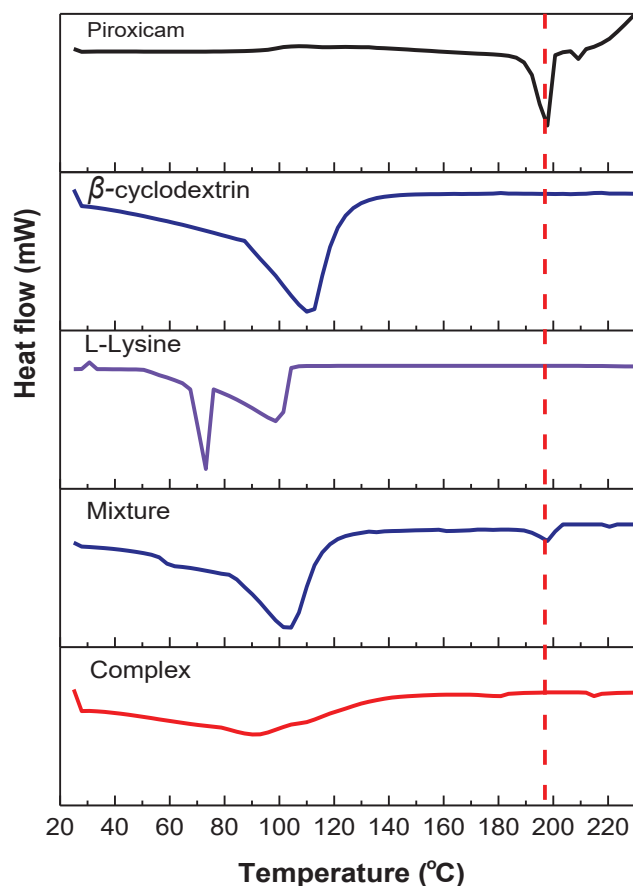


Fig. 3. The DSC curve of L-lysine, piroxicam, β -cyclodextrin, physical mixing and complex.

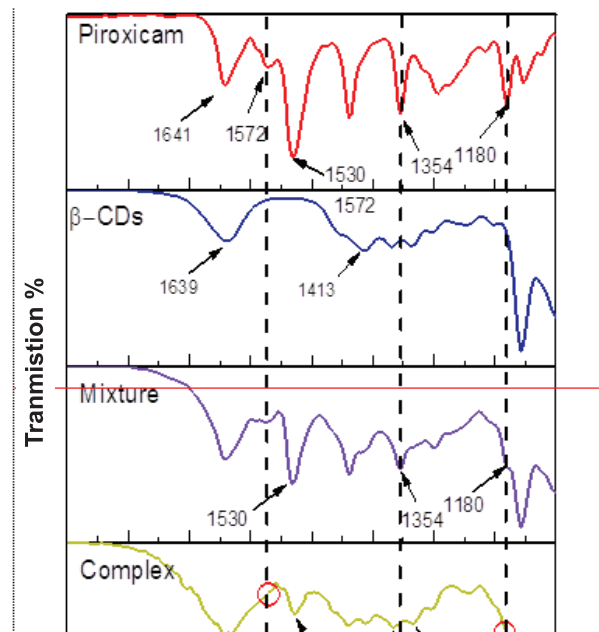


Fig. 4. The FTIR absorption spectra of piroxicam, β -cyclodextrin, their physical mix, and the complex.

Differential Scanning Calorimetry: DSC curves were analysed for pure piroxicam, a physically mixed sample, and a complexation product with a 1:2 ratio of piroxicam/ β -CD, which are shown in Fig. 3. The thermogram of L-lysine showed an endothermic peak starting from 70 to 100°C corresponding to water loss. Fig. 3 clearly showed an endothermic peak in free piroxicam that appeared as a sharp peak at 200-205°C, which is the melting point of the high crystallinity solid. The DSC profile of piroxicam may indicate a cubic crystal polymorph that can be confirmed by FT-IR analysis [14]. Similar to piroxicam, β -CD is a slightly hydrophobic compound. The results did not show the peak as water loss in both cases. However, the thermogram of β -CD showed two thermal phase transfer peaks at 132 and 166°C. In case of the physically mixed sample, a broad peak appeared in the region of 70-100°C and another peak at same position as the free piroxicam case.

With the complexation product, this thermogram is almost flat and the endothermic peak due to molten piroxicam has disappeared. This result reveals that the initial crystalline structure of piroxicam had collapsed. On the molecular level, the piroxicam molecules in the product are in a disordered arrangement. This is consistent with the above discussion in regard to the inclusion of a piroxicam molecule into the cavity of β -CD [7, 14, 15].

Fourier transform infrared spectrometry: Fig. 4 represents a graph of the FTIR analysis of piroxicam, β -cyclodextrin, their physical mix, and the complex.

Characteristic peaks of the amide group is suspected in the crystal form of piroxicam exhibited by the FT-IR spectrum at 3,338.94 cm^{-1} and 1,641 cm^{-1} due to N-H and C=O stretching, respectively. This result confirms the cubic crystal polymorph that was supposed above according to the DSC curve [10]. However, those peaks are broader and lower in intensity in both the case of PC/ β -CD complex. The vibration peak of C=C of the aromatic ring and methyl group in free piroxicam and the physical mix occur at 1,572 cm^{-1} and 1,354 cm^{-1} , which are unobserved in the case of the complex. Moreover, the peak at 1,180 cm^{-1} due to the vibration mode of S(=O)₂ stretching is clearly seen in the spectra of piroxicam and the physical mix, however, in the complex, the vibration signal of S(=O)₂ clearly decreases suggesting an interaction of PC with β -CD during complex formation. Based on the characterization of the spectra, it is highly possible that piroxicam molecules are encompassed in the cyclodextrin cavity [14, 16]. This inclusion then reduces the flexibility and hinders the vibration of the aromatic ring.

3.2. Effect process parameter

The influence of temperature on the reaction was investigated and is shown in Figs. 5A, 5B. The reaction yield increased with increasing the temperature. However, when the reaction temperature was further increased, a dark colour residue formed beside the bright suspension. The dark-coloured product was insoluble in water. For other later investigations, the reaction temperature was chosen from 110-130°C. Pressure was built up inside the reactor due to the occupancy of SC-CO₂ over all the reactor's volume. The pressure rapidly increased with increase in temperature. The influence of pressure on the reaction yield is shown in Figs. 5A, 5C. When the reaction without using SC-CO₂ was carried out and the reaction temperature was 120°C, the observations did not indicate increased pressure on the pressure gauge. At the same time, the yield of the reaction was rather low. However, in the presence of SC-CO₂, the reaction yields dramatically increased. Further, when the pressure was controlled in the range of 200 to 300 bar, reaction yields slightly increased. This result was quite consistent with the reported work in [7]. The influence of the β -cyclodextrin-to-piroxicam molar ratio in the starting materials on the reaction yield is shown in Figs. 5B, 5C. When increasing the amount of cyclodextrin from a ratio of 1.0 to 2.0, the yield almost increases further. This result reveals that there is a high possibility that PC complexation with piroxicam was successful, where piroxicam is contained within a cyclodextrin cavity.

Table 3. Box-Behnken design for inclusion PC complex and the observed yield for each experiment.

Source	Sum of squares	df	Mean square	F value	p-Value
Model	8,327.88	9	925.32	71.24	<0.0001
A-Temperature	1,212.29	1	1,212.29	93.34	<0.0001
B-Pressure	1,018.58	1	1,018.58	78.42	<0.0001
BCD:PC	1,410.60	1	1,410.60	108.60	<0.0001
AB	210.25	1	210.25	16.19	0.0050
AC	74.65	1	74.65	5.75	0.0477
BC	33.35	1	33.35	2.57	0.1531
A ²	284.76	1	284.76	21.92	0.0023
B ²	2,711.39	1	2,711.39	208.75	<0.0001
C ²	1,005.88	1	1,005.88	77.44	<0.0001
Residual	90.92	7	12.99		
Lack of fit	53.76	3	17.92	1.93	0.2666
Pure error	37.16	4	9.29		
Cor total	8,418.80	16			

3.3. Optimization of inclusion complex

RSM and Box-Behnken experimental design were used in this work to analyse the data when applying process parameters to an inclusion complex using SC-CO₂. The yield of the complex formation obtained in each experiment is presented in Table 2. Temperature (X₁), pressure (X₂) and piroxicam/ β -cyclodextrin ratio (X₃) are presented as independent variables in the second-order equation of Eq. (2):

$$\% \text{ Yield} = 84 + 12.31X_1 + 11.2838 X_2 + 13.2788 X_3 + 7.25X_1X_2 + 4.32X_1X_3 + 2.8875X_2X_3 - 8.22375X_1^2 - 25.3762X_2^2 - 15.4562X_3^2 \quad (2)$$

The analysis of variance (ANOVA) was used to confirm the model's reliability. Table 3 illustrates the results of supercritical inclusion on the quadratic model. Because $p < 0.001$, the model is statistically significant. The lack-of-fit value of the model was 0.2666 and the coefficient of determination (R²) and its adjusted value (R² adjusted) were 0.9753 and 0.8909, respectively, which point out the similarity between the experimental data and the model. Obviously, this model was proven to be a proper means of estimating the yield of the product. Moreover, the significance of the different terms belonging to the model can be identified using an ANOVA table. As shown in Table 3, the linear terms of temperature, pressure, and ratio of β -CD/PC are highly significant ($p < 0.001$). The effects of process parameters are factors that have been optimized for further study.

Figure 5 shows 3D and contour plots that illustrate the interactive influence of different variables on the yield of complex formation. Fig. 5A illustrates that both negative and

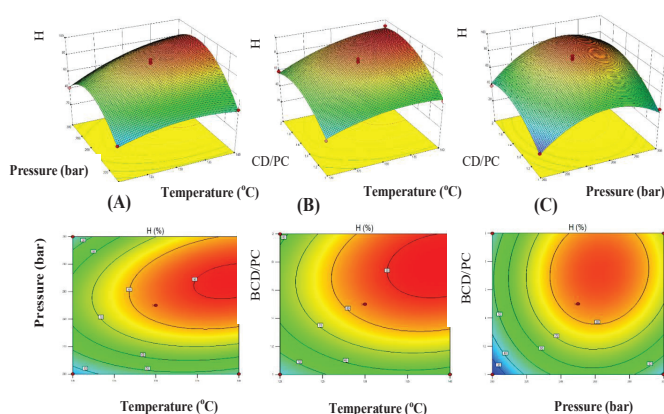


Fig. 5. 3D (top) and contour (bottom) representation of response surface method for supercritical inclusion yield demonstrates the interactive effects of (A) temperature and pressure; (B) temperature and β -CD/PC ratio; (C) pressure and β -CD/PC.

positive effects of pressure and temperature are retained on the yield throughout the studied range. Figs. 5B, 5C demonstrated the interactive effects of temperature and pressure with β -CD/PC ratio. In these figures, despite the similarity of the impacts of temperature, pressure, and β -CD/PC ratio, the β -CD/PC ratio is not a significant factor and its effect on yield is relatively lower than the two other variables. The crossed effect of pressure and β -CD/PC ratio was the only interaction term that was significant. The rising trend of inclusion yield related to supercritical temperature is shown in Fig. 5B to be outstanding at higher levels of β -CD.

Surveying the results of RSM and Design Expert optimization, the ideal condition was a temperature of 136.5°C, pressure of 266 bar, and β -CD/PC ratio at or below 1.84 where the reaction yield was proved to reach to 95.5%. By repeating the process at these optimum conditions, an inclusion yield of about 95% was achieved, which suggests the model's validity and precision.

4. Conclusions

The liquid dissolution of hydrophobic drugs has been shown to improve by making complexes with β -cyclodextrin. Specifically, SC- CO_2 was used in this experiment to prepare an inclusion complex of PC with β -CD. SC- CO_2 was pressurized to submit the powder through a fixed procedure. L-lysine was used as a ternary agent in cooperation with a considerable amount of water and a temperature above 130°C, which were combined to achieve a high inclusion yield. Finally, a complete inclusion was achieved after 3h at 266 bar and 136°C with a 1.84 and 1.5 molar ratio of β -CD and L-lysine, respectively. The characterization of the complexation product was studied by UV-Vis, FTIR, and DSC. The aqueous dissolution rate of the ternary-complex made in SC- CO_2 was determined to be significantly higher than that of the physical mixture of the components by more than 30.4 times, which has a high potential to bring this complexation product to practical application.

CRedit author statement

Phan Minh Vuong,: Data analysis and interpretation, Writing and manuscript revising; Do Huu Duy Khoa, Phan Thanh Thao: Method, Data analysis and Writing.

COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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Synthesis and characterization of alkyl-methacrylate-maleic anhydride copolymer for use as a biodiesel flow improver

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Abstract:

Due to its many advantages such as renewable raw materials and small negative impact on the environment, biodiesel has the most potential as an alternative to petro-diesel. However, one weakness of biodiesel is due to its origin of vegetable oil or animal fat. This is because during the cold season, when the ambient temperature is lower than 15°C, the viscosity of biodiesel increases and produces precipitation that causes difficulties for engine operation. Methacrylic acid was esterified with four alcohols: 1-decanol, 1-dodecanol, 1-tetradecanol, and 1-cetanol. Afterwards, the obtained monomers were copolymerized with maleic anhydride. The synthesized monomers and copolymers were characterized by ¹H-NMR (Nuclear magnetic resonance) and Fourier transform infrared (FTIR) spectroscopy. The ability of the obtained copolymers to reduce the pour point of waste cooking oil-based biodiesel was investigated. The results showed that the polymer additive with the alkyl chain C₁₄H₂₉ demonstrated the best flow improvement performance. At a concentration of 1000 ppm, this polymer additive reduced the pour point of waste cooking oil-based biodiesel from 12 to 5°C. In this study, the effect of alkyl chain length, molecular weight, as well as the concentration of the additives on the pour point of biodiesel was also discussed.

Keywords: cold flow improver, cooking oil-based biodiesel, copolymer, maleic anhydride, pour point, waste.

Classification number: 2.2

1. Introduction

Due to its many advantages such as renewable raw materials and small negative impact on the environment, biodiesel has the most potential as an alternative to petro-diesel. However, one weakness of biodiesel is due to its origin of vegetable oil or animal fat. This is because during the cold season, when the ambient temperature is lower than 15°C, the viscosity of biodiesel increases and produces precipitation that causes difficulties for engine operation [1]. To overcome this weakness, one of the best solutions is the application of a flow - improving biodiesel additive called a cold flow improver. A small amount of cold flow improver added to biodiesel has been shown to greatly improve its fluidity. Among the polymers used as flow improvers for biodiesel, vinyl compound-co-maleic anhydride copolymers are commonly used [2].

It is well known that the properties of a copolymer depend strongly on its composition [3, 4]. The creation of a copolymer is successful when its composition and molecular mass are well controlled. The composition of a copolymer depends on the reactivity of its monomers. In fact, the monomer molar ratio of an obtained copolymer

is often different from that of its initial monomers due to a difference in monomer reactivity [5]. Two of the most commonly used methods to determine the composition content ratio of a polymer are FTIR [6] and NMR spectroscopy [5].

While there have been numerous studies on the manufacture and use of a cold flow improver for petro-diesel [7], studies related to cold flow improvers for biodiesel are hardly available. Therefore, the purpose of this study was to synthesize a cold flow improver suitable for biodiesel derived from waste cooking oil. More specifically, four monomers were synthesized. They were esters of methacrylic acid and the alcohols 1-decanol, 1-dodecanol, 1-tetradecanol, and 1-cetanol. These four monomers were then copolymerized in turn with maleic anhydride. The synthesized monomers and copolymers were characterized by ¹H-NMR and FTIR spectroscopy. The ability of the four obtained copolymers to reduce the pour points of biodiesel was investigated. The effect of alkyl chain length, molecular weight, as well as the concentration of copolymer additives on their ability to improve the flow of biodiesel was discussed.

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2. Experiment

2.1. Chemicals

The chemicals used in this study are listed in the Table 1. Before use, all alcohols were properly vacuum dried at 50°C for 6h. The others were used without purification.

Table 1. Chemicals.

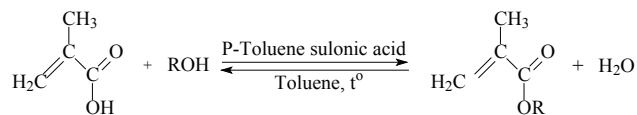
Chemicals	Company	Country	Appearance
1-decanol (C ₁₀ H ₂₁ OH)	Wako	Japan	Liquid
1-dodecanol (C ₁₂ H ₂₅ OH)	Wako	Japan	Liquid
1-tetradecanol (C ₁₄ H ₂₉ OH)	Wako	Japan	Solid
1-cetanol (C ₁₆ H ₃₃ OH)	Wako	Japan	Solid
Benzoyl peroxide	Wako	Japan	Solid
Maleic anhydride	Wako	Japan	Solid
Methyl-methacrylic acid	Merck	Germany	Liquid
p-toluene sulfonic acid	Wako	Japan	Solid
Hydroquinone	Wako	Japan	Solid

The biodiesel was received from the Key Laboratory of Bioenergy development, VNU, University of Science, Hanoi. It was made from waste cooking oil with a conversion of 98%, moisture content of 400 ppm, and solidifying temperature (pour point) of 12°C [8].

2.2. Preparation methods

Synthesis of alkyl methacrylate monomer:

Alkyl methacrylate is synthesized by the reaction as described in Scheme 1:



Scheme 1. Preparation of alkyl methacrylate monomer.

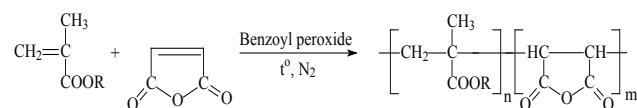
Where R is alkyl chains in alcohol (R=C₁₀H₂₁-, C₁₂H₂₃-, C₁₄H₂₉- and C₁₆H₃₃-).

To obtain a methacrylate monomer, alcohol reacts with methacrylic acid. The reaction was carried out in a three-necked and round-bottomed flask. A Dean - Stark trap was used to remove water. Toluene was used as a solvent and hydroquinone as an inhibitor. P-toluene sulfonic acid served as a catalyst. The molar ratio of alcohol to methacrylic acid was 1.0/1.1. The reaction proceeded for 6 h. After the reaction was completed, toluene was distilled off. Then, the reaction mixture was neutralized with a saturated sodium bicarbonate solution to a pH=6.5-7.0. The excess acids and catalysts were removed by washing the mixture with distilled water three times. The upper

organic layer was separated and collected. The obtained ester was dried by sodium sulfate and next by vacuum drying for 6 h. The prepared monomers had a colour from light-yellow to brown-yellow. The yield of the reaction ranged from 75 to 80%.

Copolymerization of alkyl-methacrylate with maleic anhydride:

The copolymerization reaction is described in Scheme 2:



Scheme 2. Synthesis of AMA copolymers.

Maleic anhydride and alkyl methacrylate were completely dissolved in toluene. After that, benzoyl peroxide (1 wt. %) was added to the reaction mixture. The reaction mixture was bubbled with nitrogen for 10 min, stirred vigorously, and heated to 90°C. The reaction proceeded for 4 h. The resulting copolymer was purified by the precipitation of its toluene solution with excess methanol. After filtration, the product was dried in a vacuum oven at 40°C for 6 h. The yield of the reaction ranged from 75-90%.

2.3. Research methods

Infrared spectroscopy (FTIR): Infrared spectroscopy was used to detect the presence of functional groups in the research compound, thereby contributing to its structural confirmation. An FT/IR-6300 spectrometer was used to record the FTIR spectra in the wave range of 600-4000 cm⁻¹ and with a resolution of 4 cm⁻¹.

Proton Nuclear Magnetic Spectroscopy (¹H-NMR): Proton NMR spectroscopy is an effective tool to confirm the structure of research compounds. A Bruker Avance 400 MHz FT-NMR spectrometer was used to record the NMR spectra. CDCl₃ and DMSO-d₆ were used as solvents. TMS was used as an internal standard.

3. Results and discussion

3.1. FTIR spectrum of alkyl methacrylate and maleic anhydride copolymer

The structure of all alkyl methacrylate - maleic anhydride copolymers were proven by the FTIR spectra. The FTIR spectra of the four studied copolymers (denoted AMA-10, AMA-12, AMA-14, and AMA-16) look similar to one another. Fig. 1 shows the IR spectrum of AMA-10. It can be seen that the bands at 1845.8 and 1780.3 cm⁻¹ are due to >C=O of the anhydride groups. The strong

peak at 1722.43 cm^{-1} is due to $>\text{C}=\text{O}$ of the ester groups. The vibration of the C-O bond in the ester group absorbs at 1165 cm^{-1} . The stretching vibration of C-H (in CH_3 and CH_2) corresponds to the bands at 2852.72 , 2920.23 , and 2954.95 cm^{-1} . A summary of important signals of the groups in the expected copolymers is given in Table 2. We see that all typical groups in the alkyl-methacrylate-maleic anhydride copolymers are fully characterized by FTIR spectra.

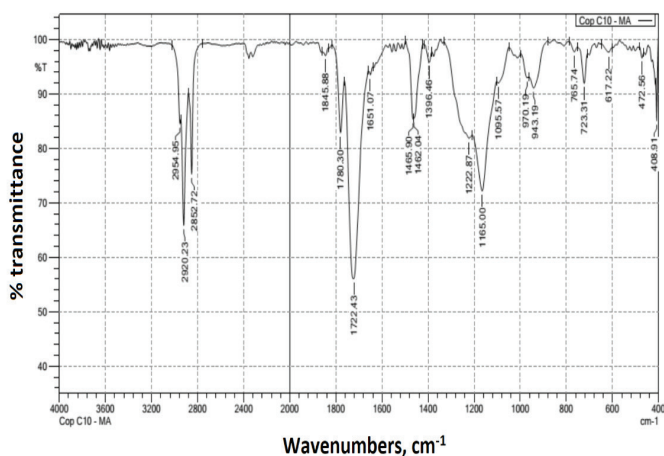


Fig. 1. IR spectrum of copolymer AMA-10.

Table 2. Typical peaks of copolymers of in FTIR spectra.

Group	AMA-10 (cm^{-1})	AMA-12 (cm^{-1})	AMA-14 (cm^{-1})	AMA-16 (cm^{-1})
C=O of ester	1722.43	1722.43	1732.08	1731.09
Stretching vibration of C-O bond in ester	1165	1149.57	1165	1150.55
C=O of MA	1780.30 1845.88	1780.30 1847.81	1772.58	1775.07 1846.36
Stretching vibration of C-H (in CH_3 and CH_2)	2852.72 2920.23 2954.95	2852.72 2921.16 2954.02	2852.72 2920.23 2953.02	2852.72 2921.18 2953.05

3.2. $^1\text{H-NMR}$ spectra of alkyl methacrylate - maleic anhydride copolymers

The structure of alkyl methacrylate - maleic anhydride copolymers is also confirmed by $^1\text{H-NMR}$ spectroscopy. For example, the $^1\text{H-NMR}$ patterns of the AMA-10 copolymer is given in Fig. 2 and all resonance signals are listed in Table 3. From Fig. 2, the methine protons ($-\text{CH}-$) of the maleic anhydride moiety appears at 2.689 ppm while the methylene protons ($-\text{CH}_2-$) of the alkyl chains appear at 1.291 ppm . The $-\text{CH}_3$ protons of the alkyl chains are seen at 0.906 ppm . The chemical shift

of 4.106 ppm belongs to the proton H of the methylene groups adjacent to the oxygen atom of alkyl alcohol. The proton adjacent to the methylene and connected with the oxygen atom of alkyl alcohol ($-\text{CH}_2-\text{CH}_2-\text{O}-$) shows a chemical shift of 1.639 ppm . Thus, the structure of the AMA copolymers is also confirmed by $^1\text{H-NMR}$ spectra.

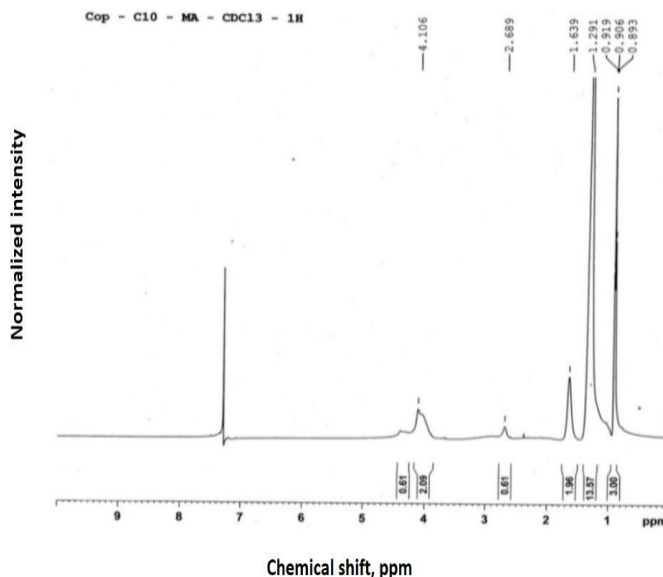


Fig. 2. $^1\text{H-NMR}$ spectrum of copolymer AMA-10.

Table 3. Typical peaks of AMA copolymers in $^1\text{H-NMR}$ spectra.

Groups	Denote	Peak (ppm)			
		AMA-10	AMA-12	AMA-14	AMA-16
$-\text{CH}_3$ (of alkyl chain)		0.906	0.813	0.655	0.803
$-\text{CH}_2-$ (in alkyl chain)		1.291	1.195	1.033-1.073	1.223
$-\text{CH}_2-\text{CH}_2-\text{O}-$ (acrylate)		1.639	1.552	1.384	1.549
$-\text{CH}_2-\text{O}-$ (acrylate)	δ_1	4.106	3.943	3.886	3.925
$-\text{CH}-$ (maleic anhydride)	δ_2	2.689	2.439	2.742	2.788

3.3. Determination of mol fraction of monomer units in copolymers

By controlling the molar ratio of the starting materials under the same conditions mentioned above, the AMA copolymers can be prepared. However, the real molar ratio of maleic anhydride (MA) in the copolymer product may not match that of the initial raw materials. Thus, determining the actual molar ratio of MA in the AMA copolymers is very important because that molar ratio can directly affect the copolymer structure and consequently affect their performance to improve flowability of liquid fuels. In this research, the FTIR technique was used to

determine the real compositions of the copolymers. To determine the MA and alkyl-methacrylate (AA) units in the AMA copolymer, FTIR spectroscopy method was used. The absorption bands for the characteristic groups are 1780 cm^{-1} (asymmetric C=O group of MA) and 1722 cm^{-1} (C=O group of methacrylate). By using the FTIR spectra, the mole fractions (mol.%) of the comonomer units (m_1 and m_2) in the AMA copolymers were calculated. For the calculation, the following equation was used [9]. The obtained results are shown in Table 3.

$$m_1 = \frac{A_{1780}/M_{MA}}{A_{1780}/M_{MA} + A_{1722}/M_{AA}}$$

$$m_2 = \frac{A_{1722}/M_{AA}}{A_{1780}/M_{MA} + A_{1722}/M_{AA}}$$

And $A = \log (I/T)$

where A =absorbance, T =% transmittance, and M_{MA} and M_{AA} are the molecular weights of MA and AA monomer units, respectively.

The real molar ratio of MA and AA in the copolymers was also calculated based on the $^1\text{H-NMR}$ signals using the following equation [10]:

$$\%MA = [S_2/(S_2+S_1)] \times 100$$

where S_1 and S_2 are the peak areas of the resonance at δ_1 ($-\text{CH}_2\text{-O-}$, alkyl methacrylate) and δ_2 (2H in $-\text{CH-CH-}$ of maleic anhydride), respectively. The results are shown in Table 4.

Table 4. Monomer mol fraction obtained by FTIR and $^1\text{H-NMR}$ analysis.

Copolymer	Monomer feed (mol.%)		FTIR results		NMR results	
	[MA]	[AA]	[MA]	[AA]	[MA]	[AA]
AMA-10	50.00	50.00	39.23	60.77	44.10	55.90
AMA-12	50.00	50.00	34.74	65.26	40.34	59.66
AMA-14	50.00	50.00	25.46	74.54	33.82	66.18
AMA-16	50.00	50.00	23.45	76.55	30.82	69.18

It can be seen from the Table 4 that, although the feed molar amount of the both MA and AA is equal, the molar fraction of MA in the copolymer is always less than that of AA. This is due to a difference in the reactivity ratios of MA and AA. As is well known, when the reactivity ratios of two monomers are different ($r_1 > 1$, $r_2 < 1$), the more active monomer will be more present in the resulting copolymer [11]. For the copolymer AMA, a reactivity ratio of AA is 3.10 meanwhile that of MA is 0.01 [12]. Thus, in the copolymer AMA, MA is present with less than its amount in the reaction mixture. The values in Table 4 also show that it is more difficult for maleic anhydride to react with the methacrylate molecules that have longer alkyl chains. This can be understood as follows: the methacrylate molecules have a high tendency to react with each other ($r=3.10$) and the longer alkyl chains create the environment of higher viscosity making it more difficult for maleic anhydride radicals to combine with methacrylate radicals.

Looking at the Table 4, it shows that the figures received from the FTIR and $^1\text{H-NMR}$ methods are different. This is understandable, because the nature of these two analysis methods is different. The question may be which method would be more accurate? In our case with copolymer AMA, it could be said that the $^1\text{H-NMR}$ method would be more accurate. The reason for this accuracy may come from the separation in peak of $^1\text{H-NMR}$ pattern in comparison with FTIR pattern. Two peaks of FTIR (1780 cm^{-1} and 1722 cm^{-1}) that were used for calculation are overlap each other (see Fig. 1). In contrast, two $^1\text{H-NMR}$ -signals for MA (2.689 ppm) and AA (4.106 ppm) distinguish to each other. Because of that, the results of $^1\text{H-NMR}$ method can be considered more accurate. However, the FTIR method is still useful if the chosen peaks for calculation are more separated. On a further note, the four copolymer additives obtained are copolymers with weak polarity. They are considered to be suitable for use as flow improvers for liquid fuel in general [13].

3.4. Molecular weight determination

The molecular weight of copolymers was determined by viscosity measurement. The solvent used was toluene. After the intrinsic viscosity $[\eta]$ was determined, the molecular weight of copolymers was calculated by Mark-Howink formula [14]:

$$[\eta] = K \cdot M^\alpha$$

where K and α are constants depending on the nature of the solvent and temperature.

In our case, $K=0.000078$ and $\alpha=0.697$ [14]. The results are presented in Table 5.

Table 5. Molecular weight of copolymers.

Copolymer	Molecular weight (g/mol)
AMA-C10	13190
AMA-C12	18549
AMA-C14	18896
AMA-C16	12084

In general, under the condition of the copolymer synthesis reaction as mentioned in the experimental section, the molecular weights of the copolymers are in the range of 12000 to 18000. Experiments show that the molecular weight of polymer additive is suitable for each specific oil in terms of flow improvement [15]. It is possible that the molecular weight range obtained above is suitable to improve the flow performance of biodiesel derived from waste cooking oil. It has been also shown that, both molecular weight and polydispersity index of polymer additives that significantly affect their ability to reduce the solidifying temperature of biodiesel [16] This

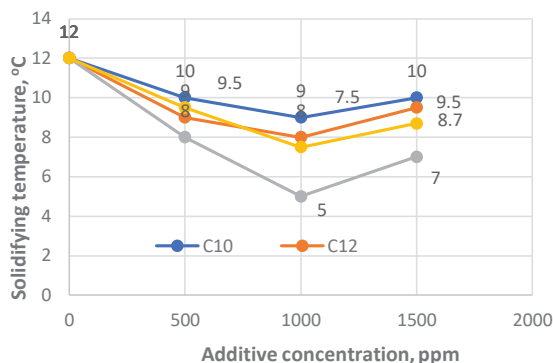
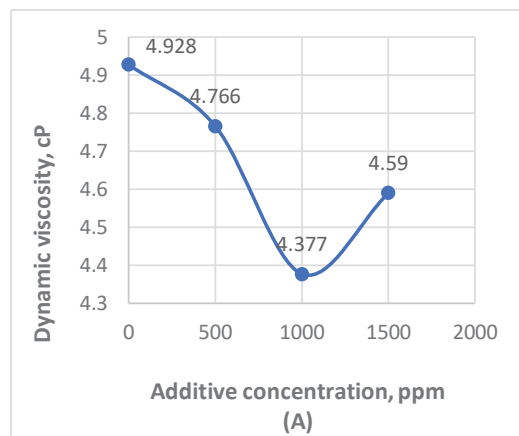


Fig. 3. Solidifying temperature of copolymers.



issue is very interesting and will be further investigated.

3.5. Determination of solidifying temperature of biodiesel

The determination of the solidifying temperatures (pour point) of oils complied with the procedure in ASTM D97-09 [17]. The pour point of the biodiesel was 12°C. As shown in Fig. 3, when adding the additives AMA-C10, AMA-C12, AMA-C14, and AMA-C16 at different concentrations, AMA-C14 had the best solid point depression (SPD=7°C, reduction from 12 to 5°C). According to [14], it is possible that the $n\text{-C}_{14}\text{H}_{29}$ - alkyl chain is an optimal fit to the composition of the wax in the biodiesel selected for this study. In the studies made by some authors on improvers for petro-diesel, similar results have been noted [18]. Fig. 3 also shows that the ability of additives to reduce the pour point of biodiesel strongly depends on their concentration. Thus, the four types of research additives tend to reduce the pour points as their content increases. Specifically, at an additive concentration of 1000 ppm, their pour points are the lowest, however, if their content continues to increase, the pour points tend to increase as well. This trend is understandable because increasing the contents of additives means reducing their solubility in biodiesel.

3.6. Determination of dynamic viscosity of biodiesel

Dynamic viscosity was determined according to the ASTM D445-11 standard using a Giltmol viscometer [19]. The results are shown in Fig. 4A. It can be seen that the viscosity decreases with increasing additive concentration until it reaches a minimal value of 4.377 cP at an additive concentration of 1000 ppm. As the additive concentration continues to increase, the viscosity decreases.

The increase of the additive concentration after reaching the threshold of 1000 ppm reduces their

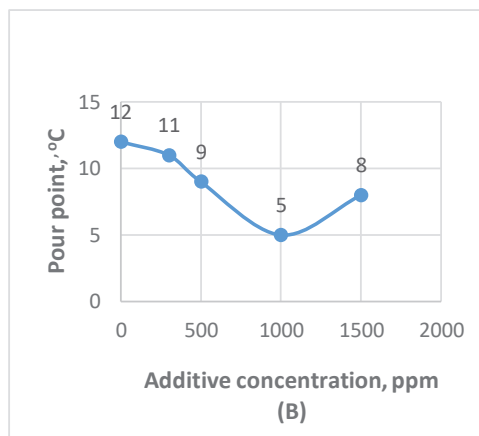


Fig. 4. The change in dynamic viscosity (A) and pour point (B) along with the change in additive concentration.

solubility in biodiesel, which results in the reduction of flow performance of biodiesel. This finding is supported by the variation trend of the biodiesel's pour point along with the change of additive concentration, as shown in Fig. 4B. Indeed, the variation of viscosity and pour point of biodiesel upon the change of additive concentration is completely similar.

4. Conclusions

Four copolymers based on maleic anhydride and alcohols containing the alkyl chains $C_{10}H_{21}$ -, $C_{12}H_{25}$ -, $C_{14}H_{29}$ -, and $C_{16}H_{31}$ - were developed. Their structure was confirmed by FTIR and 1H -NMR spectroscopy methods.

For the first time, these copolymers were studied for use as cold flow improvers for waste cooking oil-based biodiesel.

It has been found that the polymer additive with the alkyl chain $C_{14}H_{29}$ - showed the best flow improvement performance. This additive could reduce the pour point of a waste cooking oil-based biodiesel from 12°C to 5°C at a concentration of 1000 ppm.

The effect of the alkyl chain length, molecular weights, as well as the concentration of additives on the pour point of biodiesel was investigated.

CRedit author statement

Ngoc Lan Pham, Van Boi Luu: Data analysis and interpretation, Writing and manuscript revising; Thi Tuyet Mai Phan, Thi Son Nguyen: Method, Data analysis and Writing.

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COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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Evaluation of removal efficiency of Fe(III) and Al(III) ions in acid sulfate water using agarose-based magnesium oxide composite

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Abstract:

Agarose/MgO composite adsorbents were developed through interspersing MgO nanoparticles with agarose to create an adsorbent. The elimination capacity of the composite towards iron (Fe), aluminium (Al), and arsenate (As) in acid sulfate water was evaluated by means of batch method at room temperature. The constituents of the composite were characterized by thermal gravimetric analysis (TGA). The removal efficiency was determined through inductively coupled plasma (ICP) mass spectrometry. The composite adsorbent exhibited an excellent adsorption capacity towards three types of ions and heavy metals that are found in acid sulfate water. After treating with agarose/MgO, the concentrations of Fe and Al decreased from 60.28 and 604.84 µg/l, respectively, to under 3.42 and 1.78 µg/l, respectively. These exceptional results reveal the potential uses of agarose/MgO composites as adsorbents in the treatment of acid sulfate water. Although the ion concentrations were extremely high in the practical solution, the material still showed the great removal efficiency especially towards the Al ion. The results indicate that the agarose/MgO composite shows potential as a great adsorbent for the elimination of harmful ions and heavy metals from acid sulfate water, especially in water from the Mekong delta region.

Keywords: acid sulfate water, agarose composite, MgO adsorption, treatment.

Classification number: 2.3

1. Introduction

Over 40 percent of the soils in the Mekong river delta are acid sulfate soils affected by acidity [1]. Groundwater and surface water from these areas are primary contaminated by iron, aluminium, and other heavy metals. These contaminants affect agriculture and daily life. Besides, industrial development in the area has resulted in significant environmental pollution that has large impacts on human health as the accumulation of heavy metals in the human body causes serious diseases [2]. Thus, it is necessary to remove these toxic elements from water. There exists various conventional techniques for the elimination of ions and heavy metals in water such as ion-exchange [3, 4], chemical precipitation [5, 6], membrane filtration [7, 8], coagulation [9], reverse osmosis [10, 11], electrochemical processes [12, 13], and adsorption [14-17], which is an effective method owing to its simple technique, excellent removal performance, and cost effectiveness [18-20]. Due to their high

specific surface area, metal oxide nanoparticles have a considerable adsorption capacity and thus have attracted the attention of researchers for heavy metal removal applications. S. Mahdavi, et al. (2013) [21] investigated the removal of some heavy metals from aqueous solutions using metal oxide nanoparticles such as titanium dioxide (TiO₂), magnesium oxide (MgO), and alumina (Al₂O₃). Among these sorbents, MgO nanoparticles hold great removal efficiency compared to others, especially in pH-independent adsorption. Furthermore, MgO is non-toxic, noncorrosive, thermally stable, and environmentally friendly [22-24]. Being useful in practical application, small MgO nanoparticles should be immobilized into rigid supports for easy recovery after treatment. Also an environmentally-friendly material, agarose is a biopolymer comprised of D-galactose with 3,6-anhydro-L-galactopyranose that can be formed into a hydrogel of any desired shape [25-27]. Hence, agarose is an excellent matrix to trap nanoparticles for practical applications and the porous feature of agarose has positive influences on

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contaminated fluid diffusion.

In this study, we advance the ideas from our previous work [28] to fabricate agarose-based adsorbents that eliminate ions from contaminated solutions. MgO nanoparticles performed as the main sorbents, which are entrapped in the porous agarose structure. We further investigate the composition of the agarose/MgO composite by thermal gravimetric analysis (TGA). In addition, we evaluate the adsorption efficiency towards various ions and heavy metals like iron (Fe), aluminium (Al), and arsenate (As), which are found in acid sulfate water collected from the Mekong delta.

2. Materials and methods

The details of the agarose/MgO synthesis procedure can be found in the previous work [28]. Briefly, MgO nanoparticles (0.1, 0.2, and 0.25 g) were first slowly added into distilled water (28.5 g) following by sonication for 15 min at room temperature. Then, agarose powder (1.5 g) was slowly added. After 15 min of sonication, the homogeneous solution was heated to 100°C and was kept at that temperature for 1 h until transparent. The obtained gel mixture, at room temperature, was placed in a glass petri dish and then cut to cylindrical units (5×3 mm) (Fig. 1).

For further characterization from the previous work [28], thermal gravimetric analysis (TGA) using a TA Instruments Q-500 instrument, was carried out under airflow at temperatures ranging from room temperature to 800°C.

The adsorption experiments were followed similar to our previous work [28]. Agarose/MgO (5 g) was added into a conical flask of ion solution (100 ml, 100 ppm), which was diluted from a stock solution (1000 ppm). The flask was continuously shaken (250 rpm) using a shaker (Lab Companion, IST-3075R). At certain intervals, 1 ml of aqueous solution was sampled for concentration measurements. Inductively coupled plasma-optical emission spectrometry (ICP-OES) was used to measure the ion concentration. The following equation was used to calculate removal efficiency:

$$\text{Removal efficiency (\%)} = (C_0 - C_t) / C_0 \times 100\%$$

where C_0 and C_t are the original concentration and sampling time concentration, respectively.

The same experiment was also conducted using acid sulfate water (100 ml) with the above procedure.

3. Results and discussion

Cylindrical agarose/MgO composite units and bare agarose units were created with 3×5 mm shape. The transparent bare agarose unit changed to an opalescent colour due to the presence of white MgO nanoparticles (Fig. 1).



Fig. 1. Photograph of agarose and cylindrical agarose/MgO units.

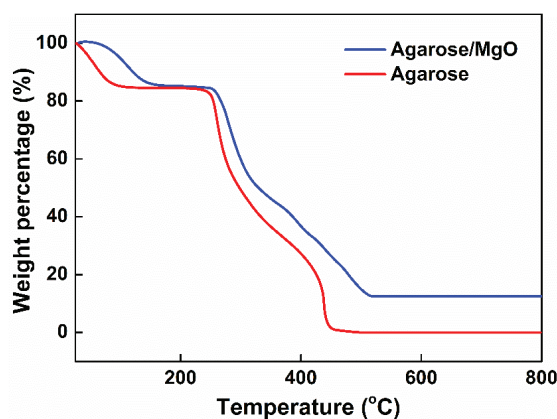


Fig. 2. Thermal gravimetric analysis of agarose/MgO compared with the agarose unit.

The composition of the composite adsorbent was investigated. As can be seen from the TGA result shown in Fig. 2, both samples present two weight loss stages. The first stage occurred around 100°C, which may result from the loss of water in the porous structures. The weight decrement in the agarose sample is approximately 15%, which is higher than that of the composite sample, which was approximately 5%. The second stage of weight loss happened at the range of 200–400°C that corresponds to the evaporation of glycerol, which is the result of charring agarose [29, 30]. The difference between the two samples is the mass of analysis residues. There is no residue for the bare agarose sample while that of the composite sample is approximately 12.6%, which corresponds to MgO nanoparticles entrapped in the agarose matrix.

From our previous work [28], the agarose/MgO composite showed high adsorption capacity towards Fe(III), Al(III) and As(V) in both single-component solutions as well as multi-component solutions (Table 1). In the single-component solution, the capacity varied depending on the ion types: the highest capacity was with Fe(III) and the lowest capacity was with As(V). In contrast, agarose/MgO showed the highest elimination efficiency towards As(V) in the multi-component aqueous solution.

Table 1. Removal efficiency of agarose/MgO composite towards some ions and heavy metal in single- and multi-component solution.

	Removal efficiency (%)		
	<i>Fe</i>	<i>Al</i>	<i>As</i>
Single-component solution	90.69	54.90	14.66
Multi-component solution	50.70	50.05	61.14

We further evaluated the efficiency toward these ions and heavy metals in an acid sulfate water sample with the agarose/MgO composite. The acid sulfate water sample was collected in the An Cu hamlet, Tan Hoa commune, Tieu Can town, Tra Vinh province (Fig. 3) of Vietnam. The adsorption capacities of the bare agarose and agarose/MgO samples with different MgO concentrations were evaluated by the batch method.

Table 2. Concentration of ions in the acid sulfate water and after treatment with various agarose/MgO composites.

	Fe (µg/l)	Al (µg/l)	As (µg/l)
Initial water	60.28	604.84	N/A
Treated with agarose	11.54	387.84	N/A
Treated with agarose/MgO-0.1	0.342	0.033	N/A
Treated with agarose/MgO-0.2	3.42	1.78	N/A
Treated with agarose/MgO-0.25	1.046	0.113	N/A

The results are presented in Table 2 and Fig. 4. The initial concentration of iron in the acid sulfate water was lower than that of the solution prepared in the laboratory in the previous work, however, the concentration of aluminium was significantly higher. All the agarose/MgO samples showed a significantly high adsorption capacity. After 24 h of contact time, the concentrations of Fe and Al decreased from 60.28 and 604.84 µg/l to under 3.42 and 1.78 µg/l, respectively, when the agarose/MgO composites were used. It is clear that the removal efficiency towards the Al ion was much higher than that of the Fe ion. This could be due to the competitive adsorption between various components in the aqueous solution, although the activity towards the Fe and Al ions was similar to the laboratory's multi-component solution. In this case, without the presence of the As ion, the composite material removed Al more completely. The near-complete removal activity indicates that the addition of MgO nanoparticles is beneficial. The concentration of MgO influenced the adsorption behaviour insignificantly due to low ion concentration of the aqueous solution. These results demonstrate that the agarose/MgO composite is effective for acid sulfate water.

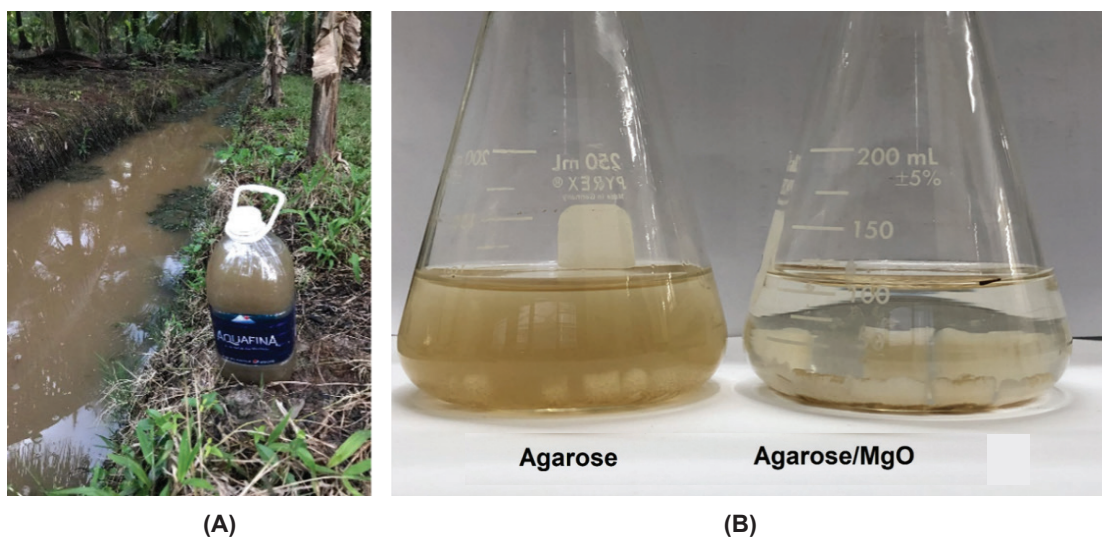


Fig. 3. Photographs of (A) acid sulfate water sample and (B) experimental batches after treatment with agarose and agarose/MgO units.

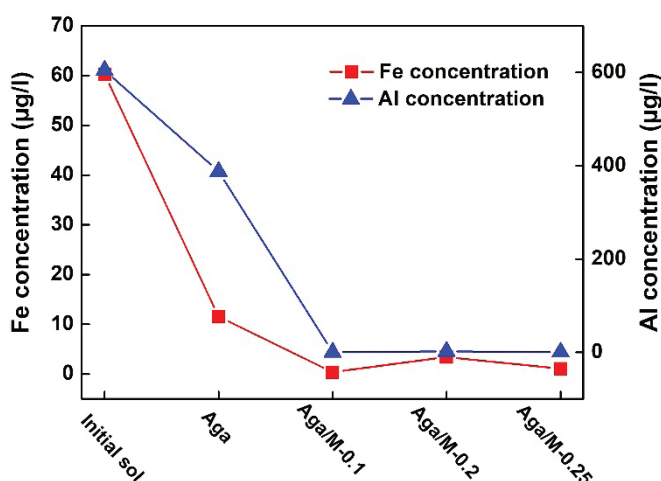


Fig. 4. Adsorption capacity of bare agarose and agarose/MgO units toward Fe and Al ions in the acid sulfate water sample.

4. Conclusions

In summary, we evaluated the removal efficiency of a composite created by trapping MgO nanoparticles in a porous agarose matrix. The presence of MgO nanoparticles in the composite was demonstrated by TGA analysis. The adsorption capacity towards Fe(III), Al(III), and As(V) was found to have different behaviours depending on the aqueous solution, either single- or multi-component. Especially noteworthy is the activity of the agarose/MgO composite showed excellent ion removal efficiency in acid sulfate water, which was collected from the Mekong delta region. Although the ion concentrations were extremely high in the practical solution, the material still showed the great removal efficiency especially towards the Al ion. The results indicate that the agarose/MgO composite shows potential as a great adsorbent for the elimination of harmful ions and heavy metals from acid sulfate water, especially in water from the Mekong delta region.

CRedit author statement

Ngoc Xuan Dat Mai, Tan Le-Hoang Doan: Data analysis and interpretation, Writing and manuscript revising; Le Nguyen Bao Thu, Bach Thang Phan: Method, Data analysis and Writing.

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COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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Degradation of the insecticides fipronil and cypermethrin in green onions (*Allium fistulosum*) and mustard greens (*Brassica juncea*)

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Abstract:

Pesticide dissipation on foods like vegetables, fruits, and cereals is one of the most concerning aspects in pesticide toxicology as it involves risks to human health and food safety. In light of this, a laboratory-scale study was conducted for two separate systems, green onions (*Allium fistulosum*) and mustard greens (*Brassica juncea*), in order to identify the dissipation of fipronil and cypermethrin, two commonly used insecticides, under a tropical climate. After the pesticide application, vegetable samples from these microcosms were collected each day for 7 days to measure fipronil and cypermethrin (n=3) residues. The analytical method was validated and showed repeatability and trueness. Besides, the decay equations fit well to first-order kinetics with a correlation coefficient of $R^2 > 0.93$ and $p < 0.0005$. The calculated half-life values of fipronil were 2.9 d for green onions and 3.2 d for mustard greens while those of cypermethrin were, respectively, 4.5 d and 3.2 d. To meet the maximum residue levels (MRL) of fipronil (0.02 mg/kg) and cypermethrin (0.7 mg/kg) on vegetables, the estimated pre-harvest intervals should be updated to 23 d and 7.5 d, respectively.

Keywords: cypermethrin, dissipation, fipronil, half-life.

Classification number: 3.1

1. Introduction

After Paul Hermann Müller's success in synthesizing the insecticide DDT in 1948 [1], synthetic pesticides have been widely applied to prevent pests and diseases, protect crops, and to improve overall agricultural productivity [2]. Nowadays, a new generation of synthetic pesticides such as pyrethroids, phenyl pyrazoles, neonicotinoids, etc., have been developed and introduced to the market as replacements for prohibited organochlorine pesticides or restricted organophosphate and carbamate pesticides. These new synthetic pesticides are chemically designed to have high selectivity and to decompose faster than the previous generation of pesticides.

Vietnam is an agricultural-based country under a typical tropical climate and is known as the third largest vegetable producer in the world in 2016 [3]. Among the vegetables produced, mustard greens (*Brassica juncea*)

and green onions (*Allium fistulosum*) are cultivated year-round in every province of the country. However, the abuse and misuse (e.g. overdose, high frequency application, etc.), as well as poor toxicity awareness (e.g. shortening the pre-harvest interval), of pesticides used in Vietnam [4-9] leads to high pesticide residue levels in vegetables. This implies a high risk to human health in terms of pesticide intake. Because both green onions and mustard greens can be consumed as a processed or raw food, the latter may bring a higher risk of pesticide intake to human body assuming pesticide residues can be decomposed or volatilized by heat from cooking as hypothesized in [10].

Two of the most frequently applied insecticides for vegetable cultivation in Vietnam are fipronil and cypermethrin, as determined from household interviews (data not shown). Fipronil is a member of a relatively new class of pesticides known as phenyl-pyrazole insecticides,

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while cypermethrin (including its 8 isomers) belongs to a group of synthetic pyrethroid insecticides derived from naturally-occurring pyrethrins that are taken from the pyrethrum of dried *Chrysanthemum* flowers [11]. The toxicology of both these pesticides are classified as class II-moderately hazardous [12].

After their application on plants, pesticides will be partly deposited on the surrounding environment while the remainder will persist on the plants and subsequently degrade. The residue of a pesticide on a plant is defined by its applied dose and its degradation rate, which are strongly affected by environmental conditions such as temperature, rainfall, wind, sunlight amount, and the crop itself [13]. Under a tropical climate, pesticides are likely to dissipate faster than in temperate regions [14]. Several publications exist on the dissipation of pesticides under different climate conditions worldwide, for instance, in Canada [15], Spain [16], or on different cultivars such as Chinese cabbage [17], mango [18], cowpea [19], tomato [20], and mustard greens [21]. A study from L. Chai, et al. (2009) [22] provided evidence of differing pesticide dissipation rates under various weather conditions with the same application technique onto the plant. Accordingly, an exponential decay equation helped to significantly predict the half-life of the individual pesticide as well as to determine the theoretical residue of the target pesticide on a plant at a specific time with the goal of recommending a suitable pre-harvest interval. Noticeably, no systematic study has been conducted on the dissipation of pesticides on plants in Vietnam - an agricultural country located in a tropical climate region. Therefore, in this study, a mesocosm experiment was conducted to define the the dissipation rate of fipronil and cypermethrin insecticides on green onions and mustard greens as well as to compare the effect of the plant itself on each target pesticide.

2. Materials and methods

2.1. Chemicals and reagents

Fipronil and cypermethrin standards, surrogate standard p,p'-DDT, and internal standard fluorene-D10 (>97% purification) were obtained from Sigma Aldrich (USA). Stock solutions (1000 µg/ml) were prepared in acetone and stored at -20°C. Working solutions were prepared in toluene. HPLC-grade solvents were ordered from J.T. Baker (Deventer, Netherlands) including n-hexane, acetone, toluene. Glass fibre filters (Whatman, 47 mm, pore size 1.6 µm) and florisil (1 g/6 ml) silica-based reversed phase cartridges from Sigma Aldrich (USA), activated carbon from Merck (Darmstadt, Germany) were used. General physical-chemical properties of fipronil and cypermethrin are demonstrated in Table 1.

2.2. Experimental design

The mesocosm experiment was conducted in the garden of the University of Sciences, Hue University (16°27'28.32" N, 107°35'29.98" E) from June to July 2018, with the aim of exposing the systems to the natural weather conditions of the tropical climate such as copious sunlight, high temperature, high air humidity, and mild wind speed. The experiment included 4 microcosms: one control (no pesticide application) and three replicated microcosms with pesticide application on both green onions and mustard greens. Each microcosm was a foam box (90x60x30 cm) with small holes at the bottom for water drainage. Porous alluvial topsoil (free of fipronil and cypermethrin) dug from the Huong An commune, a traditional cultivation area in Thua Thien Hue province, was filled in the box. The surface soil of 3 cm height was mixed with NPK fertilizer (free of fipronil and cypermethrin, ca. 10 g/box). Seedlings of green onions (15 days old) and mustard greens (10 days old) were transplanted in rows with a distance of 10 cm in between. The plants were watered and weeds were removed on a daily basis. The experiment started on 1

Table 1. General physical-chemical properties [23] of fipronil and cypermethrin.

Compounds	Chemical class	Formula	Vapour pressure (mmHg, 20°C)	Solubility in water (mg/l, 20°C)	Log (K _{ow})	DT ₅₀ ^(*) in soil (days)	Toxicity ^(**)	MRL ^(***) (mg/kg)
Fipronil	Phenyl pyrazole	C ₁₂ H ₄ Cl ₂ F ₆ N ₄ OS (M=437.2)	3.7 10 ⁻⁴	3.78	3.7	142	II	0.02 (brassicas)
Cypermethrin	Pyrethroid	C ₂₂ H ₁₉ Cl ₂ NO ₃ (M=416.3)	3.1 10 ⁻⁹ (25°C)	0.009	5.3	60	II	0.7 (leafy vegetables)

Note: *: half-life; **: toxicity data announced by WHO [12]; ***: maximum residue level [24].

June 2018 and the systems were maintained for 30 days before pesticide application. Tungent 5 SC (fipronil 50 g/l) and Appencyper 35 EC (cypermethrin 35% w/w) each in amounts of 0.5 ml were mixed and dissolved in 1 l water resulting in 0.025 mg fipronil/l and 0.175 mg cypermethrin/l to spray onto the 3 replicated microcosms of green onions or mustard greens. The total sprayed area was 3.24 m² and the control microcosms were not sprayed. The pre-harvest time suggested for fipronil is 7 d while for cypermethrin is 14 d. The vegetable samples were collected from each system on the first day, denoted as day 0, approximately 1 hour before pesticide application and each day after pesticide application (days 1-6). The weather conditions during the experiment were provided by the Hydrometeorological forecasting centre of Thua Thien Hue province (Table 2).

Table 2. Meteorological information during the experimental period.

Month	Avg. air temp. (°C) (day/night)	Avg. sunshine (h)	Avg. rainfall (mm)	Humidity (%)	Evaporation (mm)
June 2018	34.7/25.3	191	161.9	80	81.7
July 2018	33.4/25.1	163	158.2	84	68.8

2.3. Pesticide analysis

Fipronil and cypermethrin analytical protocol was adopted from the study of N.D.G. Chau, et al. (2017) [25] with modification. Specifically, the vegetable samples (5 g) were chopped, mixed with 60 ml acetone and 20 g Na₂SO₄ and then homogenized by a metal blender (Philips, Netherlands) before ultrasonic extraction in 15 min with 3 replicates. Five-hundred nanograms of the surrogated p,p'-DDT was applied to the sample at the very beginning. The extract, after filtering (Watmann glass fibre filter,

England), was rotary evaporated (Buchi, Switzerland) to 10 ml before the solid phase was extracted, including a charcoal-activated carbon packed column (1 g, Merck, Germany) with a 40 ml acetone:toluene (v:v, 1:1) elution, and then a florisil cartridge (1 g/6 ml, Supelco, Sigma Aldrich, USA) with a 25 ml acetone:n-hexane (v:v, 1:5) elution. The extract was then evaporated to nearly dry, then taken by toluene to a 1 ml amber vial containing 100 ng fluorene-D10 as internal standard before they were filled by toluene to 1 ml and stored at -20°C until analysis. Fipronil and cypermethrin were measured using GC/MS QP2010 plus system (Shimadzu, Japan) employing Rtx-CL pesticide capillary column (fused silica, 30x0.25 mm, film thickness 0.25 µm, Restek, USA). The temperature program was as the following: initial temperature was 70°C for 1 min, then increased to 180°C at a rate of 10°C/min, then to 240°C at a rate of 5°C/min and held at 240°C for 5 min, then increased to 280°C at a rate of 10°C/min and held at 280°C for 15 min. The target ions of fipronil, cypermethrin, p,p'-DDT, and fluorene-D10 were 367-213-143, 183-165-181, 235-237-165, 121-150-122, respectively. A typical chromatogram of 500 ppb analytes mixture is shown in Fig. 1.

2.4. Quality control

Home-grown green onions and mustard greens were used as control samples to measure the laboratory contamination. The instrument limit of detection (LOD) for each target compound was calculated from seven replicated injections of the standard solution at the lowest concentration in the calibration curve (5 ppb in this study), which was delivered by multiplying the *t-distribution* by the determined standard deviation (SD), for example, LOD=3.14xSD, where 3.14 is the *t* value for a 99% confidence interval with six degrees of freedom) [26]. Accordingly, the respective limit of

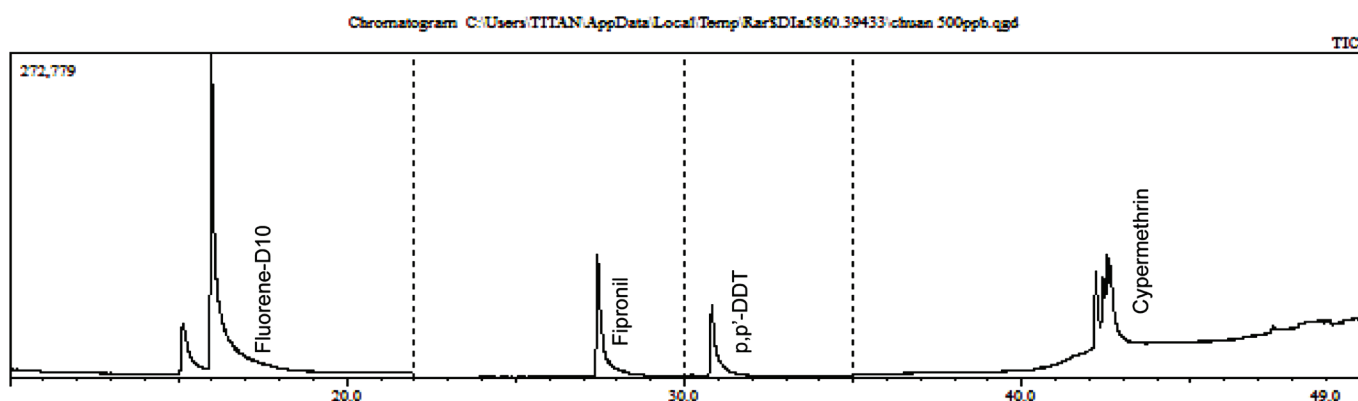


Fig. 1. Chromatogram of standard mixture at 500 ppb.

quantification (LOQ) of the method for an individual compound is $LOQ=10 \times SD$. Only detected concentrations that are greater than the specific LOQ values were used for further assessment. The calibration curves for the studied compounds were developed from seven levels (5, 10, 50, 100, 200, 500, 1000 ppb). The trueness (Rev%) and the repeatability (RSD%) of the analytical method were checked by three replicates with a fortification level of 100 ng of each target pesticide onto the green onions samples (home-grown). In addition, this study accepted a recovery of the surrogate p, p'-DDT from 80 to 120%.

2.5. Data analysis

The pesticide dissipation data were fit to the following exponential decay equation:

$$C_t = C_0 \cdot e^{-kt}$$

where C_t : the concentration of pesticide at time t (ng/g); C_0 : the initial concentration (ng/g); e: the base e; k: the dissipation rate constant (d^{-1}); t: the elapsed time (d).

The equation was derived by applying a non-linear least-squares regression analysis of concentration against time. The dissipation half-lives (DT50), which is the time required for 50% of the initial concentration to dissipate, were calculated from the exponential equation to be $DT50 = (\ln(2))/k$. For persistence comparisons, 99% dissipation times were also calculated.

Sigma Plot 11.0 (Systat Software Inc., San Jose, California, USA) was employed for data analysis.

3. Results and discussion

3.1. Quality control of the analytical method

The quantification limits of fipronil, cypermethrin, and surrogate p,p'-DDT were defined by 3.8, 6.1 and 2.5 ng (Table 3). The trueness (n=3, fortification level of 100 ng) of analytical method varied from 106 to 115% for fipronil and from 79 to 104% for cypermethrin with repeatability (RSD%, n=3) fluctuating from 5 to 6%, which absolutely met the AOAC (Association of Official Analytical Chemists) guideline ($RSD < 21\%$ for the measured concentration from 10 to 100 ng) [27].

3.2. Dissipation of fipronil

One hour after application, the fipronil residue level in green onions and mustard green reached 4.2 and 3.9 mg/kg, respectively (Table 4). Fipronil dissipated exponentially with time (Fig. 2) following first-order kinetics, in which the dissipation equations achieved a relative coefficient of $R^2=0.96$ and $p=0.0001$.

One day after pesticide application onto the mesocosm, 23% (in green onions) and 16% (in mustard greens) of the initial fipronil amount decomposed. The half-lives or time required for 50% of a chemical compound to

Table 3. Limit of LOD, limit of LOQ, Rev, and RSD of the analytical method.

Compounds	LOD (ng) (n=7)	LOQ (ng) (n=7)	Rev (%) (n=3)	RSD (%) (n=3)	Calibration curves; (R^2)
Fipronil	1.1	3.8	106-115	4.7	$y=410^{-5}x+710^{-4}$; (0.997)
Cypermethrin	1.8	6.1	79-104	5.9	$y=310^{-5}x+910^{-4}$; (0.996)
p,p'-DDT	0.7	2.5	87-98	6.4	$y=610^{-4}x-7610^{-4}$; (0.999)

Injection concentration for LOD, LOQ determination was 5 ng. Spiked concentration in real vegetable samples for recovery and repeatability tests was 100 ng.

Table 4. Fipronil dissipation in green onions and mustard greens.

Days after application	Green onions		Mustard greens	
	Avg. quantified concentration (mg/kg) (n=3)	Dissipation rate (%) (n=3)	Avg. quantified concentration (mg/kg) (n=3)	Dissipation rate (%) (n=3)
0	4.2±0.17	-	3.9±0.20	-
1	3.2±0.11	22.9	3.3±0.14	15.8
2	2.2±0.08	48.1	2.3±0.18	40.8
3	1.8±0.07	56.6	2.1±0.11	45.9
4	1.4±0.08	65.1	1.3±0.09	66.8
5	1.4±0.07	66.3	1.5±0.05	60.7
6	1.3±0.09	68.8	1.2±0.06	69.4

Concentrations of fipronil in vegetables were calculated based on fresh weight.

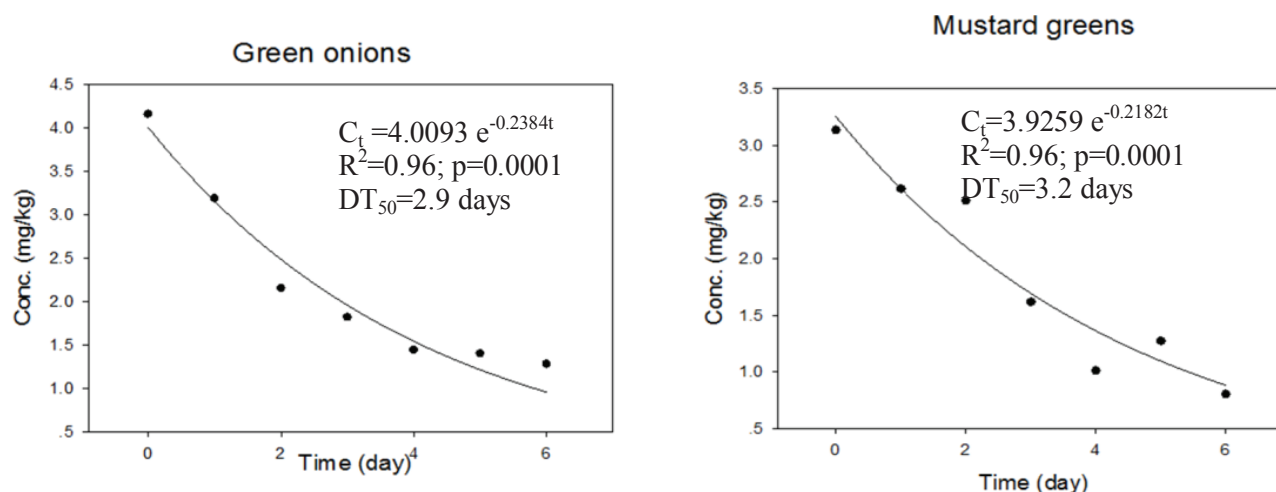


Fig. 2. Dissipation curves of fipronil in green onions and mustard greens.

degrade from its parent compound for fipronil were from 2.9 and 3.2 days for green onion and mustard greens, respectively. Although there were rainfall events on days 2 and 5 during the experiment, at the end of the experiment (7 d after application), nearly 1.3 mg/kg of fipronil were still quantified in green onions and mustard greens, respectively, corresponding to ca. 69% of the original amount dissipated. Based on the defined exponential equation, theoretically 99% of fipronil should be decomposed after 19 d. To meet the MRL of fipronil (in brassicas: 0.02 mg/kg, [24]), a period of 22 d (green onions) or 24 d (mustard greens) is required, which should be considered to replace the currently recommended pre-harvest interval of 7 d. In comparison with a previous study from Z. Pei, et al. (2004) [17], fipronil was reported to have a half-life of 2.6 days in mustard greens and it was mentioned the main processes affecting fipronil dissipation were oxidation and hydrolysis. The work of D.R. Kadam, et al. (2014) [21] on the degradation of fipronil in pomegranate fruits concluded that fipronil persisted up to 3 and 5 d in arils, and 7 and 10 d in whole fruits at the recommended and higher doses, respectively.

3.3. Dissipation of cypermethrin

Cypermethrin is a chiral compound that includes 8 isomers. In this study, the cypermethrin concentration was calculated from all cypermethrin isomers. After application, cypermethrin residue at a level of 2.2 mg/kg was found in green onions, while this figure was recorded higher in mustard greens at 3.1 mg/kg. An interpretation of this difference could be due to the larger and rough leaf surface of mustard greens, which captures more

pesticides. These initial residue levels of cypermethrin, in both green onions and mustard greens after spraying, were significantly lower than that of fipronil ($p < 0.05$) even though the spraying concentration of cypermethrin was seven times higher than that of fipronil (0.175 mg/l versus 0.025 mg/l). This low deposition of cypermethrin can be explained by the following facts: cypermethrin has very low vapour pressure (3.1×10^{-9} mmHg, 25°C) and is almost insoluble in water (solubility 0.009 mg/l at 20°C) with high $\log K_{ow}$ (5.3), which facilitates a high loss of cypermethrin after application. Meanwhile, fipronil's vapour pressure is much higher (3.7×10^{-4}) and its solubility is higher (3.78 mg/l) with a lower $\log K_{ow}$ (3.7) (Table 1). In addition, fipronil is a systemic insecticide [28, 29], which causes the fast absorption of fipronil into the tissues of the leaves after application.

However, the mentioned chemical-physical properties of fipronil and cypermethrin are unlikely to affect their dissipation profiles. The dissipation data of cypermethrin fit well to a first-order degradation kinetic ($R^2 = 0.99$, $p < 0.0001$ for green onions and $R^2 = 0.93$, $p = 0.0005$ for mustard greens, see Fig. 3). Cypermethrin half-lives in mustard greens and green onions were 4.5 d and 3.2 d, respectively. At the end of the experiment, 59% of cypermethrin in green onions and 74% in mustard greens decomposed. It takes 30 d for 99% of cypermethrin to be degraded in green onions, while this period in mustard greens is 23.3 days. The required period for cypermethrin to dissipate in both green onions and mustard greens to reach the MRL 0.7 mg/kg [24] was 7.5 d, much shorter than that of fipronil (see Table 5).

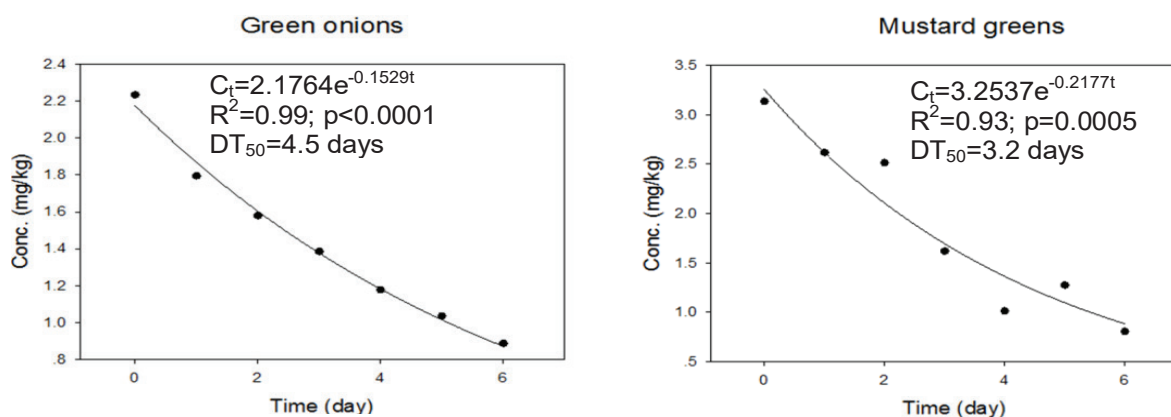


Fig. 3. Dissipation curves of cypermethrin in green onions and mustard greens.

Table 5. Cypermethrin dissipation in green onions and mustard greens.

Days after application	Green onions		Mustard greens	
	Avg. quantified concentration (mg/kg) (n=3)	Dissipation rate (%) (n=3)	Avg. quantified concentration (mg/kg) (n=3)	Dissipation rate (%) (n=3)
0	2.2±0.17	-	3.1±0.23	-
1	1.8±0.12	18.2	2.6±0.18	16.1
2	1.6±0.16	27.3	2.5±0.11	19.4
3	1.4±0.10	36.4	1.6±0.13	48.4
4	1.2±0.06	45.5	1.0±0.12	67.7
5	1.0±0.08	54.5	1.3±0.04	58.1
6	0.9±0.06	59.1	0.8±0.03	74.2

Concentration of cypermethrin in vegetables were calculated based on fresh weight.

A study from L. Chai, et al. (2009) [22] showed the half-life of cypermethrin in mustard greens fluctuated from 1.6 to 2.5 d at different experimental locations in Malaysia. The half-life for cypermethrin in this study (3.2-4.5 d) was similar to those reported for cabbage (2.6-4.9 d) [30-32], okra (4.1 d) [19], and head lettuce (2.8-3.3 d) [15] grown in a temperate climate, which suggests that climate conditions were not the main impact factors for the dissipation of cypermethrin in vegetables.

4. Conclusions

Study of the dissipation of pesticides in vegetables provides key information for pesticide risk assessment and toxicology. The dissipation rates of fipronil and cypermethrin in both green onions and mustard greens could all be fit to first-order kinetics. Half-lives of the studied pesticides varied from 2.9 d (fipronil on green onions) to 4.5 d (cypermethrin on green onions). Chemical-physical characteristics of a pesticide strongly affect its

deposition onto plants after application, which proposes that a high application dose should be considered for a non-systemic pesticide with low vapour pressure and low solubility in water (such as cypermethrin). The pre-harvest interval of 7 d is appropriate for cypermethrin; however, 7 d is inadequate to ensure a safe residue level of fipronil (MRL 0.02 mg/kg) in vegetables to be consumed. This study proposes either a lower spraying dose of fipronil or a longer pre-harvest interval. Otherwise, fipronil should not be applied on vegetables, especially for vegetables that could be directly consumed without cooking such as green onions or mustard greens.

CRedit author statement

Dang Giang Chau Nguyen, Thi Lan Anh Tran: Data analysis and interpretation, Writing and manuscript revising; Thi Thanh Lam Tran, Thi My Duyen Ngo, Thai Long Hoang, Van Hop Nguyen, Kurt Kalcher: Method, Data analysis and Writing.

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COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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Effects of nano-bubbles on seed germination of muskmelon

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Abstract:

Nano-bubbles (NBs) have emerged as promising tools in agriculture, aquaculture, food processing, and wastewater treatment. This study explored the impact of air NBs on muskmelon seed germination. Distilled water was infused with NBs for durations of 10, 15, and 20 minutes, while untreated distilled water served as the control. Significant differences in seed germination rates were observed among the treatments. Growth parameters including stem height, root length, and leaf area were assessed 14 days post-treatment with NB-infused water. Although differences in dry weight among treatments were minimal, statistical analysis revealed their significance. The findings underscore the potential of air NBs in influencing seed germination processes and subsequent plant growth. This research contributes to understanding the application of NB technology in enhancing agricultural practices and may pave the way for further exploration in optimizing seed germination protocols and plant growth strategies.

Keywords: muskmelon, nano-bubbles, seed germination.

Classification number: 3.1

1. Introduction

Applications of nano-bubbles (NBs) have been investigated across fields like agriculture, industry, and medicine. The unique properties recently discovered of these micro- and nano-scale bubbles in solutions present many intriguing characteristics such as long residence time, high efficiency of gas mass transfer, or the ability to increase the internal pressure and contact area between a liquid and a gas [1-3]. These properties lead to efficient chemical reactions and the dissolution and transfer of oxygen, nitrogen, ozone, or carbon dioxide in liquid environments, all of which are necessary for agriculture, aquaculture, food production, and environmental protection [4-6].

In agriculture, characteristics of NBs have been shown to outperform previous methods. One application that benefits most from studying NBs is seed germination [7-9]. Both in soil and soilless cultures, the germination stage is the first and most fundamental stage, which plays an important role in the growth, development, and vitality of plants. Many techniques applied to seed treatment research aims to improve the efficiency of a seed's emergence ability and also the quality of crops initiated from a seedling.

Methods such as using bio-agents, chemical treatments, and heat treatments have been applied. Each technique includes advantages and disadvantages in particular aspects. While the NB technique was developed as a physical treatment, it can also affect the chemical characteristics of objects. Therefore, NBs have a rich potential to promote the sustainability of agriculture and the environment.

This experiment was conducted to evaluate the efficiency of NBs technique in seed treatments, and, in particular, the germination rate of hybrid muskmelon compared with traditional methods to identify the most effective formula. The disadvantages of the technique are also discussed.

2. Materials and methods

The seeds of the "Madam orange" hybrid muskmelon (Known-You Seed Co., LTD) were selected to test the germination rate under different nano-bubble treatments. There are four treatments with four replications of 20 seeds each. The seeds were placed on absorbent paper in Petri dishes and filled with different formulae of water. The four treatments are shown in Table 1.

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Table 1. List of treatments used in this study.

Treatment 1	Control, distilled water.
Treatment 2	Distilled water was generated by nano-bubbles in 10 minutes.
Treatment 3	Distilled water was generated by nano-bubbles in 15 minutes.
Treatment 4	Distilled water was generated by nano-bubbles in 20 minutes.

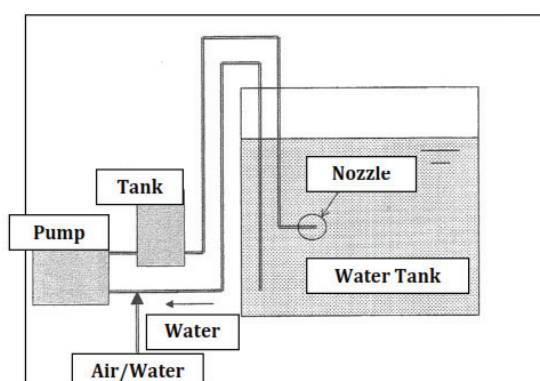


Fig. 1. Schematic diagram and image of the nano-bubble generator. The blue pipe is an inlet pipe in which water from the water tank and air from outside are absorbed. The black pipe is an outlet pipe, in which water carrying nano-bubble particles are released.

The water in Treatments 2, 3, and 4 was treated by a nano-bubble generator (modified and manufactured by Faculty of Engineering, Rajamangala University of Technology Lanna, Chiang Mai, Thailand) following the time as indicated in the formula of each treatment (Fig. 1).

Every 24 h, the water and absorbent papers were changed. After 7 d, the seeds were collected to observe the germination rate while the growth indicators were measured after 14 d (Fig. 2). The treatments were conducted following CRD (completely randomized design). Statistical analysis of variance was computed followed by an LSD multiple range test at $p \leq 0.05$.

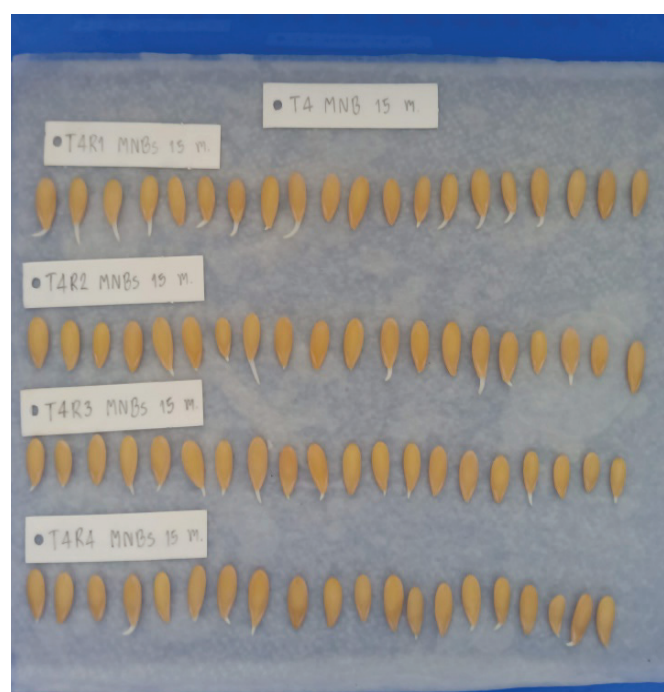


Fig. 2. Seeds treated with NBs.

3. Results and discussion

Before and after treatment with NBs-generated water, factors such as electrical conductivity (EC), water temperature, dissolved oxygen (DO), and pH did not significantly change. In general, EC, water temperature, and DO tended to increase, however, the differences were not significant (Fig. 3).

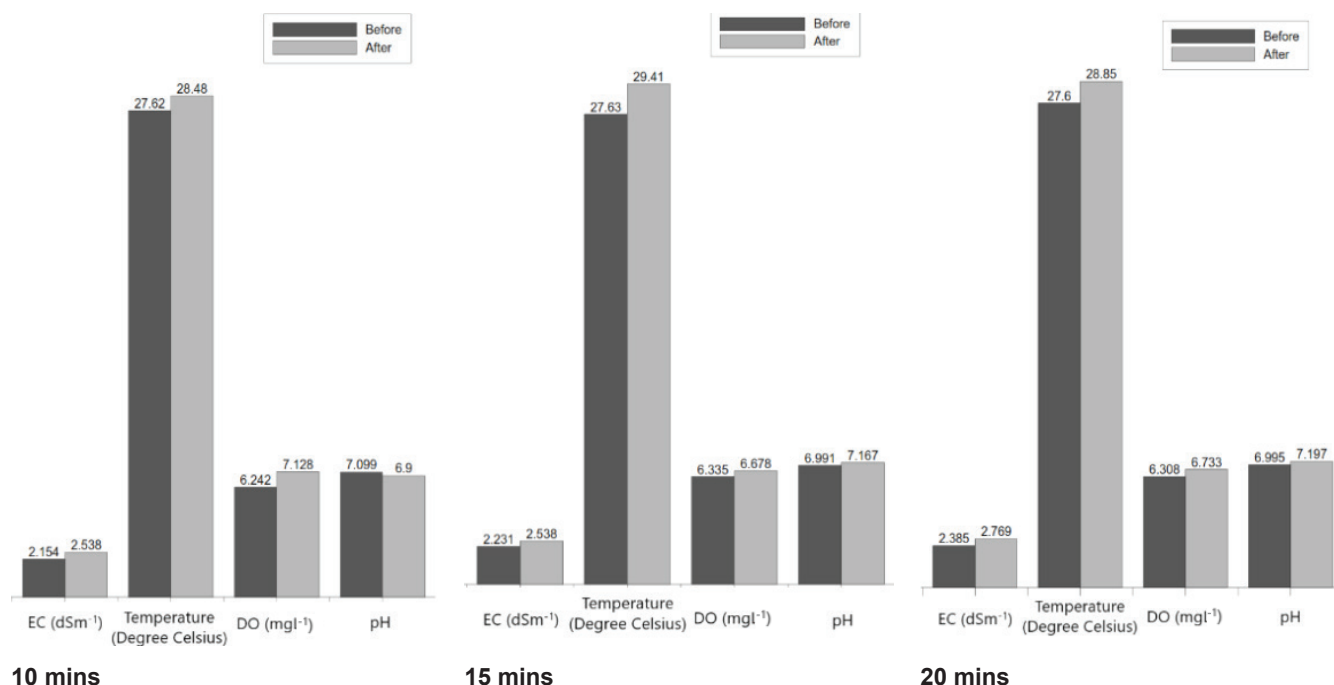


Fig. 3. Water factors before and after treatment with water generated with NBs for different generation times.

Dissolved oxygen plays an important role in agricultural applications such as in hydroponics and aquaculture. In crop science, seed germination is strongly influenced by oxygen concentration in soil and substrate. For seedling emergence, almost all seeds require a high and stable concentration of oxygen for respiration, except for some special cases in which seeds germinate anaerobically. Y. Muhamad, et al. (2016) [10] indicated that various types of vegetable seed were sensitive to decreasing oxygen concentrations, which leads to reduced speed and percentage of germination. Moreover, the existence of nano-bubbles has proven that the residual time in solutions could last up to 2 weeks [11] and it can be considered as an advanced benefit in seedling treatment.

Figure 4 shows that the four treatments with water generated by NBs promoted the germination rate of muskmelon. After 7 d of submersion, the muskmelon germination rate reached more than 90% ($p \leq 0.05$) for all treatments. As a whole, the dormancy rates among the treatments were not significantly different, which was approximately 10% for all of treatments. The progress of the germination of the muskmelon seed is listed in the following stages: dormancy > emerge > root appearance > cotyledon appearance > stem appearance.

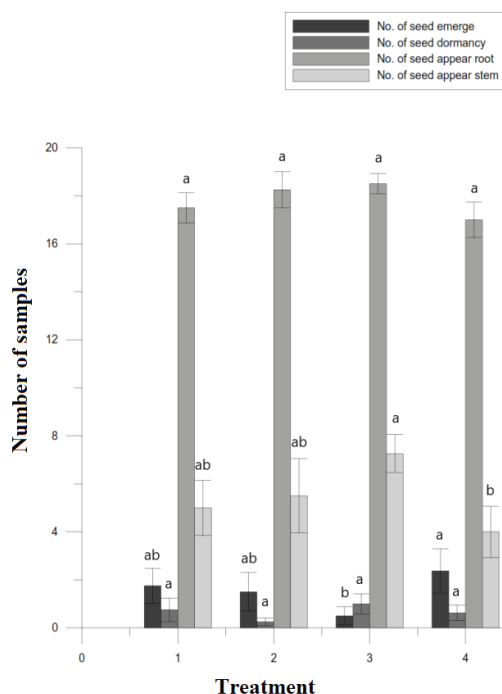


Fig. 4. Characteristics of samples observed after 7 d. A cluster bar chart shows the total number of seeds on the 7th day that existed in each of the four stages of germination. For example, all seeds that appeared to already possess a stem-initiated root were recorded in the column for “No. of seed appear root” and so on. The mean $\pm$ standard deviation is shown along with letters that label a significant difference according to the LSD multiple range test at $p \leq 0.05$.

The final state, stem appearance, obtained the highest observation rate with Treatment 3, i.e., 15 min water generation with NBs. The same result was observed in the emerge rate for all treatments. Conversely, with the number of observed stems, the emerge rate for Treatment 3 was the lowest and the highest belonged to Treatment 4. There was no significant difference in the observed emerge rate between Treatments 1 and 2. The emerge rate of the seeds depend on the quality of the seeds and the germination treatment. Also, if the state of emergence takes too long, the seeds will not have good development.



Fig. 5. Observed samples after 14 d.

Figures 5, 6 presents the growth indicators of muskmelon after 14 d under different treats of NBs. In comparison to the distilled water treatment, most growth indicators from NBs treatments show that there were significant differences ($p \leq 0.05$). The most significant difference belongs to Treatment 3 in terms of stem height, root length, and cotyledon area. In these cases, all the growth indicators showed significantly higher values. However, the stem diameter measurements did not show any significant differences between treatments. In general, treatment with NBs gave indicators that were equal or higher than the treatment with distilled water.

In term of cotyledon area, previous studies indicated that cotyledons could be used as a bioassay to determine the response of plant growth regulators (PGRs) such as kinetin, GA_3 , and IAA under both light and dark germination conditions [12]. When homogenous seed material was used in experiment without the supplement of any type of PGRs, the specificity of the responses of plant hormones only provide the efficiency of internal PGRs among different treatments. Fig. 6 shows the highest cotyledon area belongs to Treatment 3. By linking this with other results from this experiment, the highest development indices appear in samples with Treatment 3 as well. This confirms the hypothesis that NBs not only impact physical factors, but also affect the biochemical activities of plants.

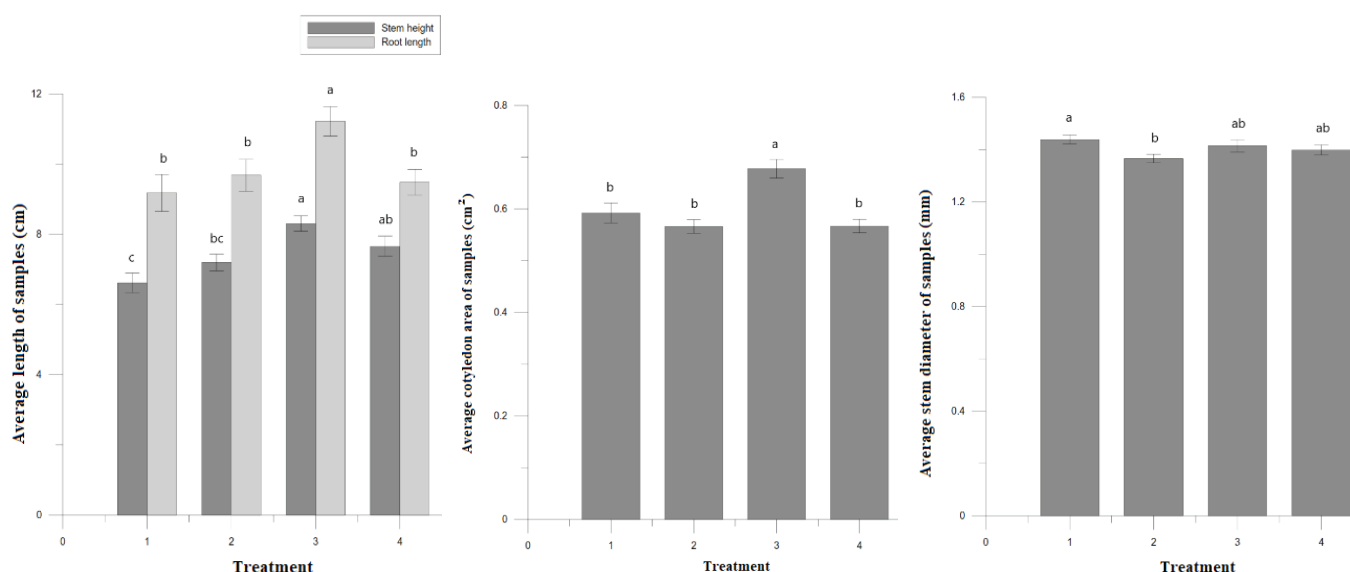


Fig. 6. Growth indicators of samples after 14 d. The mean $\pm$ standard deviation within treatments are followed by different letters that label a significant difference according to the LSD multiple range test at $p \leq 0.05$.

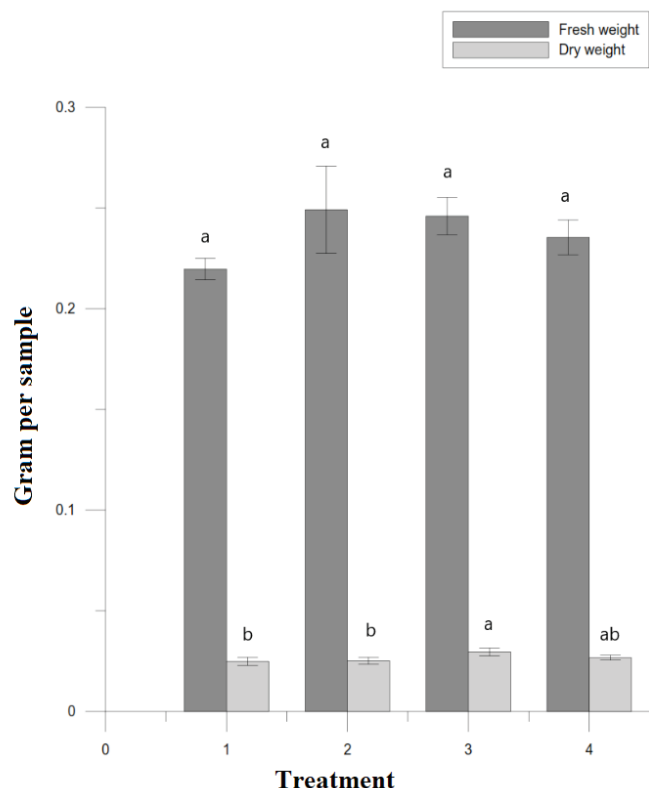


Fig. 7. Average weight of samples after 14 d. The mean $\pm$ standard deviation within treatments are followed by different letters that label a significant difference according to the LSD multiple range test at $p \leq 0.05$.

After 14 d, the weights of the samples were measured and the results are shown in Fig. 7. Even though there were significant differences in the growth indicators among treatments, the weights of the samples between treatments were quite similar. Statistical analysis results showed that there were differences in the dry weight of after each treatment where the largest difference was seen in Treatment 3, which was much higher than Treatments 1 and 2. However, the average amounts from real measurements were quite similar as significant differences are difficult to recognize without statistical analysis.

4. Conclusions

The influences of NBs on the seed germination of lettuce, carrot, fava bean, and tomato were investigated in other studies. Seeds in water containing NBs were found to exhibit 6-25% higher germination rates. Those treated with nitrogen NBs exhibited considerable effects in seed

germination, whereas air and carbon dioxide NBs did not significantly promote germination. The growth of stem length and diameter, leaf number, and leaf width were promoted by NBs (except air) [13]. However, NBs were shown to enhance the growth of plants by improving the oxygen supply as nutrient elements [14]. Moreover, according to a previous study [15], rice growth did not differ between those irrigated with NBs water (water saturated by oxygen NBs) and those irrigated with control water (without NBs). Experiments have shown that there were differences in DO concentration of water before and after treatment of distilled water with NBs. However, DO is not the sole factor deciding the success of germination. Results from the research of germination rate of barley seeds showed that seeds submerged in water treated with nitrogen NBs and air NBs had higher germination rates (15-25%) in comparison to seeds treated in distilled water under the same concentration of DO [9]. In this case, an explanation of NBs carrying oxygen in the solution can provide the most convincing answer [11]. Even though the NBs in this experiment were ANBs (air nano-bubbles), treatments using ANBs showed significant differences in some growth indicators and development stages in the germination of muskmelon seeds. This suggests that the effects of NBs on the physiological progress of plants needs more investigation.

CRedit author statement

Quy Tung Le: Data analysis and interpretation, Writing and manuscript revising; Chiti Sritontip: Method, Data analysis and Writing.

COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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A procedure to detect 6 basic STSs and 11 extended STSs in the AZF region using multiplex PCR

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Abstract:

Microdeletions of Y chromosomes frequently occur in 3 subregions of the AZF, namely, AZFa, AZFb, and AZFc, with 6 basic STS marker sequences, which are sY84, sY86 (AZFa), sY127, sY134 (AZFb), and sY254, sY255 (AZFc). According to EAA/EMQN guidelines, 11 additional AZFabc marker sequences should be used to determine the extent of the microdeletion in the AZF region of infertile men, which is known as 11 extended STSs. By applying mPCR, the authors develop an optimal detection procedure for the 6 basic STS and 11 extended STS using 3 multiplex PCR reactions. The first multiplex PCR reaction includes 6 basic STS plus the 2 control sequences sex-determining region Y (SRY) and zinc finger protein X/Y-linked (ZFX/Y). The second multiplex PCR reaction includes the 6 extended STS sY88, sY1182, sY105, sY121, sY1191, and sY1291 and the 2 control sequences SRY and ZFX/Y. The third multiplex PCR reaction includes the 5 extended STS sY153, sY160, sY82, sY143, and sY83 and the 2 control sequences SRY and ZFX/Y. Six basic primer sequences and eleven extended primer sequences are redesigned to simultaneously pair and amplify STS in the same multiplex reaction: set of 8 primers for 6 basic STS: 6 basic STS + 2 (SRY, ZFX/Y), 8 extension primers set E1: 6 extended STS + 2 (SRY, ZFX/Y), and 7 extension primers set E2: 5 extended STS + 2 (SRY, ZFX/Y). We successfully designed primer pairs with high specificity and stability and successfully amplified 6 basic STS and 11 extended STS, which ensures that the STSs have the correct sequence as recommended by EAA/EMQN and are consistent with the NCBI gene bank. This study has successfully developed a procedure to simultaneously detect 17 STSs, including 6 basic STSs and 11 extended STSs in the AZF region using 3 multiplex PCR reactions.

Keywords: AZF, azoospermia, male infertility, microdeletion, multiplex PCR, oligospermia.

Classification number: 3.2

1. Introduction

Microdeletions of Y chromosomes account for about 10-15% of male infertility [1-3]. On the long arm of the male sex Y chromosome (Yq11) exists a region called AZF (azoospermia factor) that can be classified into three subregions, AZFa, AZFb, and AZFc, which contain genes that regulate sperm production and differentiation. Microdeletion mutations frequently occur in these three subregions. To diagnose microdeletions on the AZF region of the Y chromosome, the European Academy

of Andrology (EAA) and European Molecular Genetics Quality Network (EMQN) recommend using at least 2 sequences in each subregion: AZFa (sY84, sY86), AZFb (sY127, sY134), and AZFc (sY254, sY255). These are the basic sequences to examine and determine any microdeletions are present. In addition to these basic STS sequences, examination of extended STS sequences are also recommended, namely, 4 additional sequences in the AZFa subregion, 4 additional sequences in the AZFb subregion, and 3 additional sequences in AZFc subregion [4, 5]. The eleven recommended STSs are sY88, sY1182,

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sY105, sY121, sY1191, sY1291, sY153, sY160, sY82, sY143, and sY83. The use of multiple sequences in the AZF subregions helps to avoid missing microdeletion mutations when determining the cause of male infertility. The present techniques applied to simultaneously find multiple STSs are capillary electrophoresis and multiplex PCR. While capillary electrophoresis requires a sequencing system, multiplex PCR is simpler and does not require a lot of expensive equipment. However, several problems arise when pairing multiple primers to simultaneously detect many STSs because the difference between STS sizes is insignificant. Therefore, this study deployed a new design to primer sequences and simultaneously amplify multiple STSs in a multiplex PCR reaction. This study intends to integrate 17 STSs into three multiplex PCR reactions. The first multiplex PCR reaction includes 6 basic STSs plus the 2 control sequences SRY and ZFX/Y. The second multiplex PCR reaction includes the 6 extended STSs: sY88, sY1182, sY105, sY121, sY1191, and sY1291 and the 2 control sequences SRY and ZFX/Y. The third multiplex PCR reaction includes 5 extended STSs, namely, sY153, sY160, sY82, sY143, and sY83 and the 2 control sequences SRY and ZFX/Y.

2. Materials and methods

2.1. Materials

The primers help the amplification of the STSs on the AZF region of the Y chromosome. DNA samples of normal men and normal woman were extracted from EDTA anticoagulant blood.

DNA samples were extracted from EDTA anticoagulant blood of male patients with severe oligospermia (sperm density ≤ 5 million/ml semen) and azoospermia (no sperm in semen). The samples were analysed to detect microdeletions with Sweden's Devyser v2 and the Extension test kit, and then compared to the result after applying the multiplex PCR technique.

2.2. Methods

One set of seventeen primers has been designed for multiplex PCR. Six primers were used to amplify

the six basic STSs including sY84 and sY86 (AZFa), sY127 and sY134 (AZFb), sY254 and sY255 (AZFc). Then, 11 primers were used to amplify 11 extended STSs including sY82, sY83, sY88, and sY1182 (in AZFa), sY105, sY121, sY143, and sY153 (in AZFb), sY160, sY1191, and sY1291 (in AZFc), and 2 primers to amplify 2 sequences used as internal controls, namely, SRY and ZFX/Y. The primer sequences refer to the design of EAA/EMQN. These primers are divided into 3 groups to create 3 multiplex PCR reactions. The first multiplex PCR reaction includes the 6 basic STSs plus the 2 control sequences SRY and ZFX/Y. The second multiplex PCR reaction includes the 6 extended STSs: sY88, sY1182, sY105, sY121, sY1191, and sY1291 and the 2 control sequences SRY and ZFX/Y. The third multiplex PCR reaction includes the 5 extended STSs: sY153, sY160, sY82, sY143, and sY83 and the 2 control sequences SRY and ZFX/Y. To check the accuracy of the primers, a single primer PCR reaction was used. With a total volume of 20 μ l, the Master Mix included a forward primer, reverse primer (2 μ l), template DNA (2 μ l), Master Mix 2X (10 μ l), and dH₂O (6 μ l). A heat cycle of 95°C for 15 min, then 35 cycles of [95°C-30 s; 57°C-30 s; 72°C-45 s], then 72°C for 10 min.

Three multiplex PCR reactions of 11 STSs in the AZF region and the 2 internal control sequences (SRY and ZFX/Y) were optimised.

Nineteen sequences (17 STSs in the AZF region and the 2 internal control sequences SRY and ZFX/Y) were transferred into the cloning vector. The vector pBT, of *E. coli* DH5 α [end A1 rec A1 hsd R17 sup E44 gyp A96 thi-1 relA1 Δ lac U169 (ϕ 80 lacZM15)] from the Invitrogen company was used to clone. After the purified PCR products are transferred into the cloning vector, the plasmid DNA is transformed into *E. coli* cells by heat shock. Then, PCR is directly performed from the *E. coli* colony and the plasmids are extracted from the *E. coli* cells.

The 17 STSs of the AZF region and 2 internal control sequences SRY and ZFX/Y were sequenced from the plasmids.

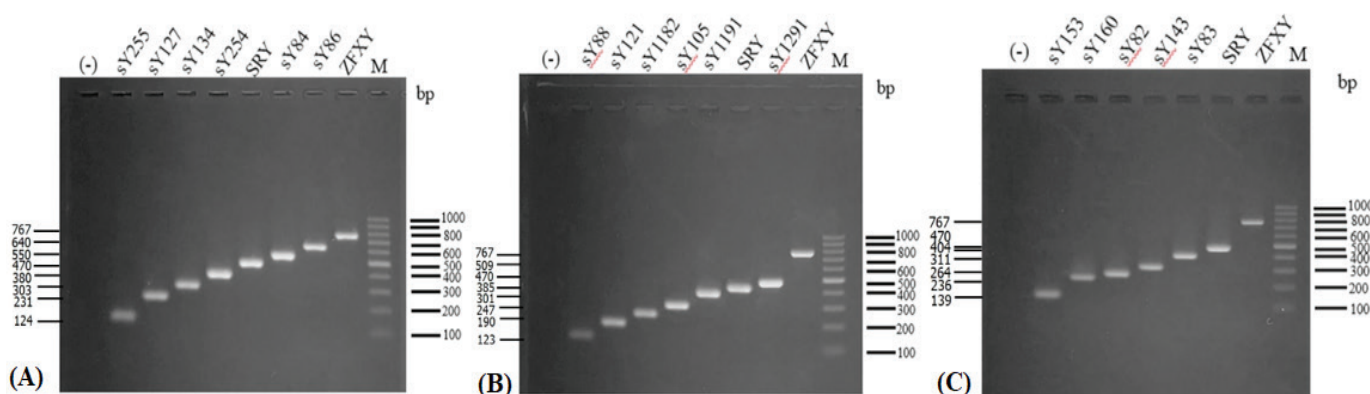


Fig. 1. Agarose gel electrophoresis image of 8 STS sequences after single primer PCR.

Notes: M: marker, DNA 100 bp ladder, (-): negative control, distilled water. **(A):** set of 6 single basic STSs primer and 2 control sequences: sY255: 124 bp, sY127: 231 bp, sY134: 303 bp, sY254: 470 bp, SRY: 470 bp, sY84: 550 bp, sY86: 640 bp, ZFX/Y: 767 bp; **(B):** extended set E1 primers with 6 extended STSs and 2 control sequences: sY88: 123 bp, sY121: 190 bp, sY1182: 247 bp, sY105: 301 bp, sY1191: 385 bp, sY1291: 509 bp, SRY: 470 bp, ZFX/Y: 767 bp; **(C):** extensive set E2 primers with 5 extended STSs and 2 control sequences: sY153: 139 bp, sY160: 236 bp, sY82: 264 bp, sY143: 311 bp, sY83: 404 bp, SRY: 470 bp, ZFX/Y: 767 bp.

A set of 3 multiplex PCR reactions were completed to simultaneously detect microdeletions in the 17 STSs of the AZF region.

DNA samples from normal men, normal women, and severe oligospermia or azoospermia men were used to complete the AZF microdeletion(s) detection procedure.

Main chemicals and equipment required: QIAgen HotStarTaq DNA polymerase (5000U); dNTP Mix, PCR Grade (200 µl); QIAquick PCR purification kit (250); QIAquick gel extraction kit (250); GelPilot Plus Ladder 100 bp, Devyser v2 and Extension test kit (Sweden). A PCR system (BioRad CX96), basic agarose electrophoresis system, agarose gel camera, and an ABI3500 system were the equipment used.

3. Results

3.1. The accuracy of the primers sequences

Nineteen primers designed for amplification were optimised by single primer reactions for each sequence of the 17 AZF STSs and the 2 internal controls SRY and ZFX/Y. The AZF sequences were amplified by single primer PCR, which are the 6 basic STS of the AZFabc region: sY84 and sY86 (AZFa), sY127 and sY134 (AZFb), sY254 and sY255 (AZFc) and 11 extended STS

of the AZFabc region: sY82, sY83, sY88, and sY1182 (in AZFa), sY105, sY121, sY143, and sY153 (in AZFb), and sY160, sY1191, and sY1291 (in AZFc).

The results from the 3 designed primer sets showed that 19 PCR products (including the 17 STSs in AZF region and the 2 control sequences SRY and ZFX/Y) were obtained when electrophoresis on agarose gel were of the correct sizes (Fig. 1). Initially, the newly designed primer sequences are suitable to amplify the 17 STS sequences of AZF region and the 2 internal controls SRY and ZFX/Y.

3.2. Transfer of sequences into cloning vector

The entire PCR product of the 8 STSs, after being purified, was transferred into the pBT cloning vector by a splicing reaction. Then, the product was transformed into *E. coli* DH5α cell. The results of the PCR products directly from the colonies were obtained through electrophoresis images as seen in Fig. 2.

The electrophoresis results showed that the obtained PCR products were of correct size. Thus, 17 STSs were successfully cloned in three PCR reaction sets. The recombinant pBT plasmids were then extracted, purified, and used to sequence the STSs.

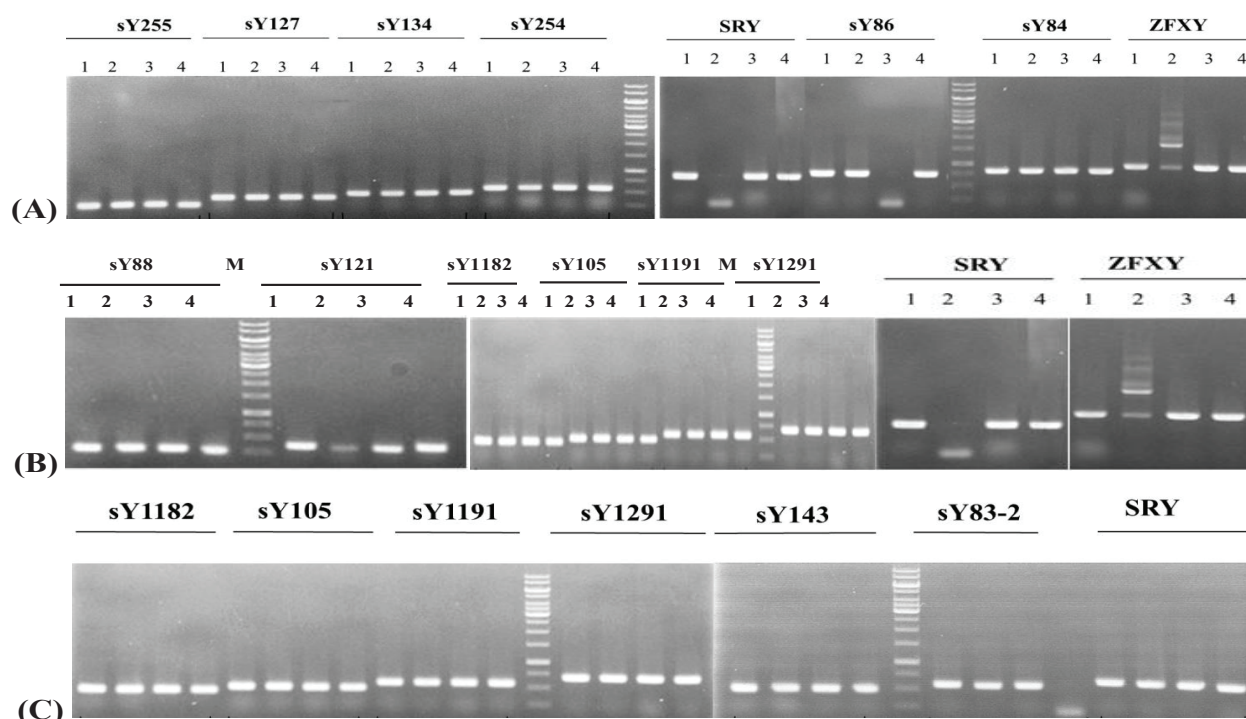


Fig. 2. Electrophoresis image of PCR products from colonies of 17 STSs.

Notes: **(A)**: cloning of 6 basic STSs: sY255, sY127, sY134, sY254, sY84, sY86; **(B)**: cloning of 6 extended STSs: sY88, sY121, sY1182, sY105, sY1191, sY1291; **(C)**: cloning of 5 extended STSs: sY153, sY160, sY82, sY143, sY83.

3.3. Sequencing the sequences transferred into the cloning vector

PCR products from the above cloning sequences were purified and then sequencing was performed on ABI 3500 system. Comparing the above sequences with gene bank references, the obtained sequences are found to be consistent with the reference sequences of the AZF region from the NCBI GenBank (National Center for Biotechnology Information) [6].

3.4. Optimising the detection of STSs in a single multiplex PCR reaction

The first multiplex PCR reaction consisted of the basic primer set of the 8 primers sY84, sY86 (AZFa); sY127, sY134 (AZFb); sY254, sY255 (AZFc); SRY and ZFX/Y in 1 multiplex mixture and then the detection of 8 sequences in 1 multiplex PCR reaction was optimised.

The second multiplex reaction consisted of the E1 extension primer set that allows the combination of the 8 primers sY88; sY121; sY1182; sY105; sY1191; sY1291; SRY and ZFX/Y in a multiplex mixture and then the

detection of 8 sequences in 1 multiplex PCR reaction was optimised.

The third multiplex PCR reaction consisted of the E2 extension primer set that combines the 7 primers sY153; sY160; sY82; sY143; sY83; SRY and ZFX/Y in 1 multiplex mixture and then the detection of 7 sequences in 1 multiplex PCR reaction was optimised.

The primers to amplify the 17 STSs and 2 internal controls were combined with equal concentration. The optimal annealing temperature was 57°C for 70-90 s. The primers were diluted to 40 μM using Quiagen's Master Mix, which has all the basic ingredients for PCR (dNTPs, Taq polymerase, enzyme buffer). Mixtures for multiplex PCR reactions include: 200 ng of template DNA (4.0-6.5 μl), Master Mix (18.5 μl), and just enough distilled water to ensure the volume of multiplex PCR reaction mixture is 25 μl. The optimal thermal cycle is 95°C for 15 min, then a cycle of (94°C/30 s; 57°C/90 s; 72°C/60 s) for 35 cycles, and finally 72°C for 15 min. The PCR products were electrophoresis on 3% agarose gel at a voltage of 100 V for 85 min. The results are shown in Fig. 3.

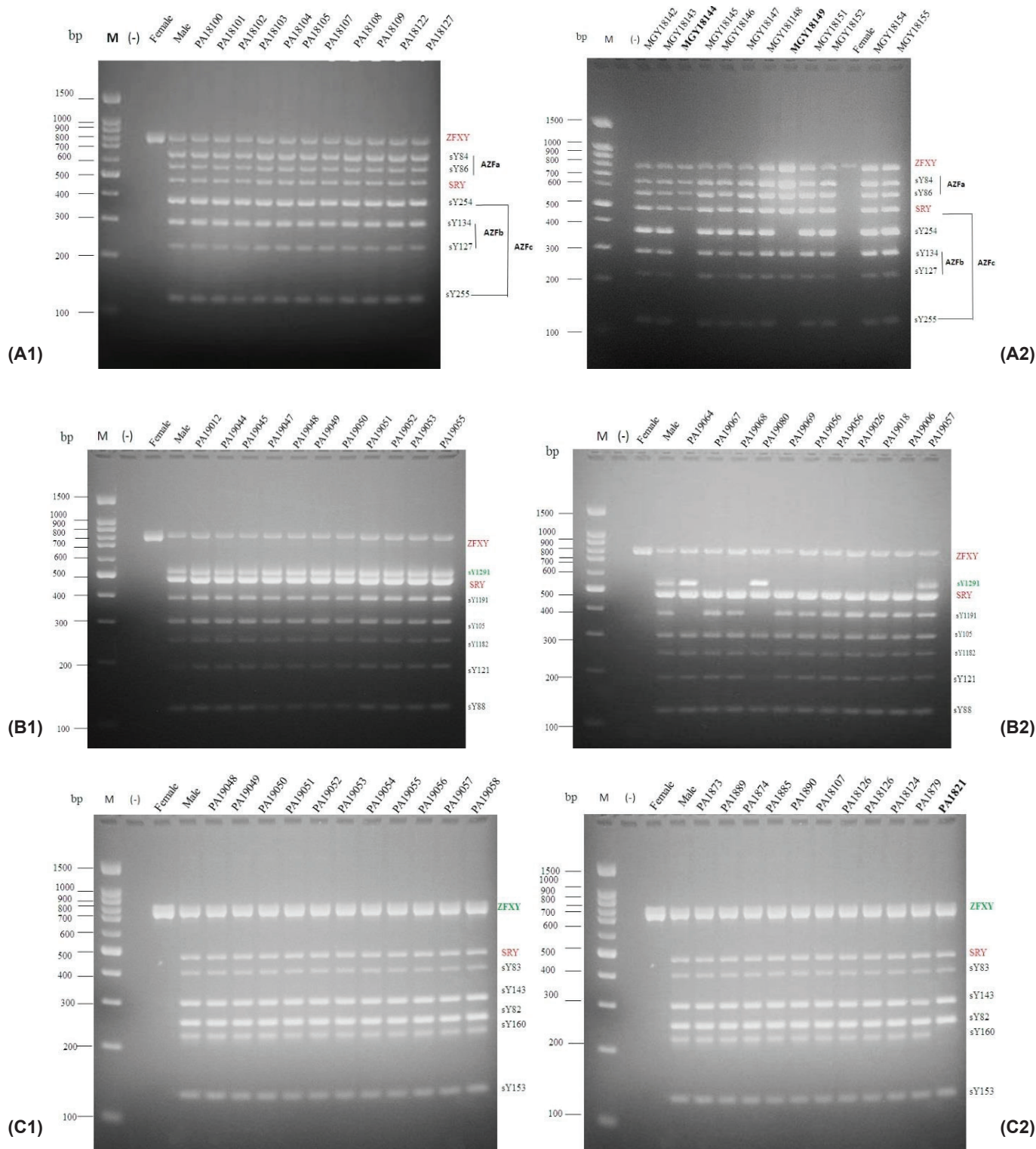


Fig. 3. Electrophoresis image of 3 multiplex PCR products: 17 STSs and 2 control sequences, SRRY, ZFX/Y.

Notes: M: marker, 100 bp DNA ladder; bp: base pair; (-): negative control (distilled water), female: normal female DNA; male: normal male DNA; **(A1, A2)**: 1st multiplex PCR reaction, samples PA 18100-18105, 18107-18109, 18122, 18127, MGY18142-18144, 18145-18149, 18151-18152, 18154-18155. **(B1, B2)**: 2nd multiplex reaction, samples: PA19012, PA19044, PA19045, PA19047, PA19048, PA19049, PA19050, PA19051, PA19052, PA19053, PA19055, PA19064, PA19067, PA19068, PA19080, PA19069, PA19060, PA19056, PA19026, PA19018, PA19006, PA19057. **(C1, C2)**: 3rd multiplex reaction: samples PA19048-19058, PA1873-74, PA1889, PA1885, PA1890, PA18107, PA18126, PA18124, PA1879, PA1821.

For each multiplex reaction, electrophoresis images of the multiplex PCR products showed electrophoresis bands that are clearly separated, the image is sharp, and is consistent with the theoretical calculated size. Each multiplex reaction is performed with the following controls: positive control (male), female control (female), negative control (distilled water). The results obtained indicate the positive control is male with all STSs, female control only has ZFX, and no product existing in the negative control.

For the first multiplex PCR reaction, 23 infertile male samples were analysed with 21 samples having 8 bands corresponding to the 8 sequences sY84, sY86, sY127, sY134, sY254, sY255, SRY, and ZFX/Y (without AZF microdeletion). The sample MGY18144 lacked electrophoresis bands at the sY127, sY137, sY254, and sY255 positions, indicating an AZFbc microdeletion, while sample MGY18149 lacked electrophoresis bands at sY254 and sY255 positions, which indicated an AZFc microdeletion.

For the second multiplex PCR reaction, 22 infertile male samples were analysed with 12 samples PA19012, PA19044, PA19045, PA19047, PA19048, PA19049, PA19050, PA19051, PA19052, PA19053, PA19055, and PA19057, which were similar to the male control and had 8 bands corresponding to the 8 sequences sY88, sY121, sY1182, sY105, sY1191, sY1291, SRY, and ZFX/Y. Samples PA19067, PA19068, PA19069, PA19060, PA19056, PA19026, PA19018, and PA19006 lacked electrophoresis bands at the sY1291 position, which demonstrates a sY1291 microdeletion. Sample PA19064 lacked an electrophoresis band at the sY1191 position, which indicated a sY1191 microdeletion. Sample PA19080 lacked an electrophoresis band at the sY1191 and sY121 positions, which indicated sY1191 and sY121 microdeletions.

For the third multiplex PCR reaction, 22 infertile male samples were analysed. There were 19 samples similar to the male control with 7 electrophoresis bands corresponding to the 7 sequences sY153, sY160, sY82, sY143, sY83, SRY, and ZFX/Y. Samples PA1889 and PA1879 lacked an electrophoresis band at the sY143 position and sample PA1821 lacked an electrophoresis band at the sY160 position.

These samples (67 samples with male and female

controls) were analysed for AZF microdeletions using the Swedish Devyser v2 and Extension test kits. The results were similar between the two methods in their ability to detect and classify the microdeletions.

4. Discussion

PCR is the leading technique employed in molecular genetics and can be easily applied in many laboratories. Each multiplex PCR reaction currently being used in diagnostics can only detect up to 4 or 5 sequences [4]. The idea of this study is to design a multiplex PCR procedure to detect as many AZF-region STSs as possible in the diagnosis of Y chromosome microdeletions in order to solve the problem. These sequences are just the basic STSs of AZF regions a, b, and c [7]. Combining multiple primers in a multiplex PCR reaction requires the primers to have a suitable melting temperature and the primers should not have a complementary sequence to each other, especially at the 3' end. The DNA bands must also be significantly different in size to be easily separated on electrophoresis gel, but the differences should not be too large to be easily amplified under the same PCR cycle. The capillary electrophoresis technique can overcome the shortcomings of multiplex PCR as it can pair multiple primers to simultaneously detect multiple sequences, but this method requires a genetic sequencing system and thus has an expensive test cost.

Since 1999, according to the guidelines of the EAA and EMQN, the diagnosis of AZFa, b, and c microdeletions must use the 6 sequences sY84, sY86, sY127, sY134, sY254, and sY255 along with the two control sequences SRY and ZFX/Y, the latter being a gene found on both male and female sex chromosomes (X and Y). These 8 sequences are then combined into 2 multiplex PCR reactions (multiplex A and B) and the electrophoresis products took about 24 h not including sample preparation and PCR. Because the sequence sizes are close to each other, when under electrophoresis, a low voltage and a long separation time are required to form PCR products [4]. Since 2010, the Hanoi Medical University, based on the method of EAA/EMQN, improved the multiplex PCR method to detect 6 STSs of AZF by dividing them into 3 multiplex PCR reactions (multiplex 1, 2 and 3), which separates similarly size sequences into different multiplexes for the purpose of shortening the electrophoresis time to only 30-45 min

each. Thus, these multiplex reactions can amplify the maximum simultaneous number of 3 STSs [8]. In 2015, the University of Science, Vietnam National University, Ho Chi Minh city, redesigned the primers of the above 6 sequences into 2 multiplex reactions (multiplex A and multiplex B). However, when redesigned, the sequences were significantly different in size and could be clearly separated in the same electrophoresis with the maximum of 3 STSs in a multiplex reaction [7].

Our study is the first study in Vietnam succeeding in optimising 17 STSs simultaneously in 3 multiplex PCR reactions with 5 to 6 STSs in each multiplex PCR reaction. This doubles the number of STSs in multiplex reactions compared to previous studies. For the 6 basic STSs of the AZF region, previous studies had to divide them into 2-3 multiplex PCR reactions [4, 7, 8], in this study, all 6 STSs were detected with only 1 multiplex PCR reaction. For the extended STSs, no studies exist using multiplex PCR to detect these, however, we have also successfully built 2 reactions to simultaneously detect these 11 extended STSs in the AZFabc region as recommended by EAA/EMQN. The use of extended STSs in the diagnosis of AZF microdeletions is recommended for infertile patients especially those with moderate and severe oligospermia. The STSs are selected so that they can be combined together. The most difficult part of this study was the redesign of the 17 STSs amplification primer sequences and the two control sequences, SRY and ZFX/Y, to ensure that each STS had a clear, well separated band on the electrophoresis gel.

In this study, the authors referenced several primer pairs designed in the basic and extended AZF test kits as recommended by EAA/EMQN. We also redesigned several primer pairs to easily identify STSs after electrophoresis. The principle of the primer redesign is to fit in all the respective sequences recommended by EAA/EMQN. According to EAA/EMQN guidelines, the basic and extended AZF test kits should have 17 STSs in the AZFa, AZFb, AZFc subregions and the 2 control sequences SRY and ZFX/Y. To develop a basic and extensive AZF test kit, the following sequences are chosen: sY84 (472 bp); sY86 (318 bp); sY127 (274 bp); sY134 (301 bp); sY254 (380 bp); sY255 (123 bp); sY88 (123 bp); sY121 (190 bp); sY1182 (247 bp); sY105 (301 bp); sY1191 (385 bp); sY1291 (527 bp), sY153 (139 bp); sY160 (236 bp); sY82 (264 bp); sY143 (311 bp); sY83

(275 bp); SRY (472 bp); ZFX/Y (495 bp) [4]. However, the sizes (shown in parentheses) of some STSs are not significantly different, making separation on agarose gel electrophoresis difficult.

Several STS primers sequences were redesigned to amplify as clearly identified products on electrophoresis gel. The sequences are detailed as follows: sY84 (640 bp); sY86 (550 bp); sY127 (231 bp); sY134 (303 bp); sY254 (380 bp); sY255 (124 bp); sY88 (123 bp); sY121 (190 bp); sY1182 (247 bp); sY105 (301 bp); sY1191 (385 bp); sY1291 (509 bp); sY153 (139 bp); sY160 (236 bp); sY82 (264 bp); sY143 (311 bp); sY83 (404 bp).

The separation of the two control sequences SRY (472 bp) and ZFX/Y (495 bp) was difficult, so ZFX/Y was redesigned with a 767 bp size. Some sequences had insignificant differences in size from reference, for example, SRY is now 470 bp (2 nucleotides different) and sY1291 is now 509 bp (18 nucleotide different). The single STS PCR products tested on 1.5% agarose gel in Fig. 1 show successful amplifications and clear electrophoresis bands for each sequence. However, to ensure that these amplified sequences are the STSs in the AZF region and the two control sequences SRY and ZFX/Y, they were purified and transferred into a pBT cloned vector and then transformed into *E. coli* DH5α cells to clone the sequences. The results in Fig. 2 show that we successfully cloned 17 STSs in the AZF region and the 2 control sequences. The electrophoresis results of the PCR products obtained are of the correct theoretical size (STS size plus 164 bp, 164 bp is the general size of the plasmid sequence). Thus, the STSs present in the basic and extended AZF test kits were successfully cloned. The recombinant pBT plasmids were extracted, purified, and used to sequence STSs in the basic and extended AZF test kit. These sequences were then compared with the NCBI GenBank references and found to be completely similar to those references sequences.

The authors successfully combined 19 primers, including 17 primers to amplify the 17 STSs and 2 primers to amplify the 2 internal control sequences SRY and ZFX/Y, into 3 multiplex PCR reactions. When optimised, the redesigned sequence sizes are relatively different from each other, so after amplification and electrophoresis in 3% agarose gel, the STSs are clearly separated in each reaction (Fig. 3). The negative control

(distilled water) was bandless after electrophoresis and the normal female had 1 band of ZFX (767 bp) while the normal male had 8 basic AZF electrophoresis bands, 8 electrophoresis bands of AZF Extension set E1, and 7 electrophoresis bands of AZF Extension set E2. The 67 infertile male samples were analysed for microdeletions in the AZF region with the Devyser v2 and Extension test kit (Sweden). The obtained results were similar to our study's results. Thus, the optimised multiplex PCR technique to simultaneously detect 17 STSs in 3 multiplex PCR reactions is similar to the detection of AZF microdeletions by gene segment size analysis on an ABI 3500 sequencing system. The AZF region sequences were correctly detected and were stable when performed with patient and control DNA samples.

5. Conclusions

The authors have successfully designed primer pairs with high specificity and stability according to amplified 17 STSs that were clearly separated on agarose gel. Eight plasmids were also cloned successfully to carry DNA sequences from the AZF region, as well as SRY and ZFX/Y, which were used as reference DNA samples for the detection of microdeletions on Y chromosome.

A procedure to simultaneously detect 17 STSs in 3 multiplex PCR reactions was successfully developed with each multiplex PCR reaction designed to simultaneously detect up to 6 STSs and the 2 control sequences SRY and ZFX/Y. The first multiplex PCR reaction detects 6 basic STSs: sY84, sY86 (AZFa); sY127, sY134 (AZFb); sY254, sY255 (AZFc); SRY and ZFX/Y in a multiplex mixture and optimises detection of 8 sequences in a multiplex PCR reaction. The second multiplex reaction detected 6 extended STSs, namely, sY88, sY121, sY1182, sY105, sY1191, and sY1291 along with SRY and ZFX/Y in a multiplex PCR reaction and 8 sequences were optimised and detected in each. The third multiplex PCR reaction detected 5 extended STSs, namely, sY153, sY160, sY82, sY143, and sY83, as well as SRY and ZFX/Y in a multiplex PCR reaction and 7 sequences were optimised detected in each.

After testing 67 DNA samples of infertile males, a normal male, and a normal female, this procedure

detected the above described AZF regions microdeletions with high sensitivity, specificity, and stability. The results were similar to that of using a sequencing system.

CRedit author statement

Thi Lan Anh Luong, Thu Lan Hoang, Minh Ngoc Nguyen: Data analysis and interpretation, Writing and manuscript revising; Ngoc Dung Nguyen: Method, Data analysis and Writing.

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COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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Formulation of enteric coated microspheres containing pantoprazole

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Abstract:

Pantoprazole, a primary proton pump inhibitor used to manage gastric acid secretion disorders, is recognized for its minimal adverse effects and limited drug interactions. Enhancing its stability within gastric acid, delayed-release microspheres containing pantoprazole were developed through emulsification-solvent evaporation. This process utilized a polymer blend comprising hydroxypropyl cellulose (HPC) and ethyl cellulose (EC), subsequently coated with alginate and Eudragit® L100. The microspheres' morphology was assessed using SEM, while laser diffraction facilitated the determination of particle size distribution. The physical state of the drug substance was analyzed through Fourier-transform infrared spectroscopy (FTIR) and differential scanning calorimetry (DSC), alongside in vitro drug release studies. Selected microsphere formulations exhibited an average size of 100 μm . Dissolution profiling revealed that less than 10% of the drug was released after 120 minutes in 0.1-M HCl, whereas over 75% of the drug was released within 45 minutes in a phosphate buffer with a pH of 6.8. This research demonstrates the potential of tailored microsphere formulations to optimize drug release kinetics and improve therapeutic outcomes for pantoprazole administration.

Keywords: delayed release, enteric coated microsphere, pantoprazole.

Classification number: 3.3

1. Introduction

Gastric acid is vital for food digestion. However, excess gastric acid can cause irritation, inflammation of the oesophagus, heartburn, and stomach ulcers [1]. Many drugs have been used to treat excess gastric acid like proton pump inhibitors (PPIs), which are the first line of treatment for stomach ulcers. Pantoprazole, a third-generation PPI, has the highest efficacy and lowest side effects and drug interactions among other PPIs [2, 3]. However, pantoprazole is unstable in a gastric acid environment. There are few products containing pantoprazole for treatment of stomach ulcers in the Vietnamese market and most of them are formulated as pellets or enteric coated tablets.

In recent years, microspheres have become an effective solution to a variety of pharmaceutical challenges that include particle size, drug solubility, drug-drug interactions, drug stability, and controlled drug release [4]. Therefore, in this study, the formulation of enteric-coated microspheres containing pantoprazole by emulsification and solvent evaporation was carried out.

2. Materials and methods

2.1. Materials

Pantoprazole sodium sesquihydrate (USP 41) was procured from Mac-Chem Products, India. Hydroxypropyl cellulose (HPC-LMM) (EP7) and ethyl cellulose (EC 45-55 cps) were supplied by Nisso, Japan. Sodium alginate was purchased from Sigma Aldrich, Germany and Eudragit® L100 was distributed by Evonik, Germany. Ethanol, acetone, liquid paraffin, tween 20, span 80, n-hexane, and magnesium oxide (MgO) were procured from Xilong, China and were of industrial grade.

2.2. Methods

Formulation and preparation of pantoprazole microspheres: The emulsification and solvent evaporation method was used to prepare the microspheres. Briefly, polymers were weighted and dissolved in 12.5 ml of a 1:1 acetone-ethanol mixture to form a 2.5% (w/v) solution. Pantoprazole, having a 1:3 ratio with the polymers, was then dissolved in this solution. MgO was dispersed into the solution and 100 ml of paraffin containing 1% (w/v)

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Table 1. Formulations of microspheres preparation.

Formulation	Polymer concentration (%)	HPC:EC ratio	Pantoprazole (%)	MgO (%)	Tween: Span HLB=5.5 (%)
F1	2.5	0:1	0.83	0.5	1
F2	2.5	1:2	0.83	0.5	1
F3	2.5	1:1	0.83	0.5	1

emulsifier (span 80 and tween 20) was then added to this mixture and keep stirring at 500 rpm for 3 hours. The microspheres were collected by allowing the suspension to settle. Paraffin was then removed from the microspheres by centrifugation and washed three times with 30 ml of n-hexane. The microspheres were dried at 50°C for 30 min and stored in a dark bottle for further experiments. The formulation used in this study are described in Table 1.

Evaluation of microsphere: The microspheres were characterised by common methods described in Ref. [5]. Encapsulation yield of pantoprazole in the microspheres: briefly, the microspheres were dissolved in 0.1-M NaOH. The filtrate was then diluted with a phosphate buffer of pH 6.8. Pantoprazole was quantified by UV-Vis at a wavelength of 288 nm. The quantification process was validated according to ICH guidelines. The encapsulation yield of pantoprazole in the microspheres was calculated per the following formula (this experiment was repeated three times):

$$\text{Encapsulation yeild (\%)} = \frac{\text{Amount of PAN in MS}}{\text{Amount of MS}} \times 100$$

- Particle size distribution: The size distribution of the microspheres was determined by laser diffraction (Mastersizer 3000, Malvern).

- Microsphere morphological characteristics: The morphology of the prepared microspheres was analysed by scanning electron microscopy (SEM) (SEM JEOL JSM-6400, Japan). For SEM, the samples were prepared by lightly sprinkling microsphere powder on double-sided adhesive tape, which was placed on an aluminium stub. The stubs were then coated with carbon using a fine coat ion sputter.

- DSC: to determine whether there are interactions between pantoprazole and the polymer, a DSC scan was carried out for pantoprazole, a microsphere sample, and a physically mixed sample with the same proportion of microspheres. Each sample was measured over a temperature range of 30-350°C and heating speed of 10°C/min.

- FTIR: the microspheres and the physically mixed samples with the same proportion of microsphere compositions were prepared and characterised by FTIR scans over a wavenumber range of 3000 to 500 cm⁻¹.

- *In vitro* release of microspheres: A microsphere sample containing 1.6 mg pantoprazole was added to 40 ml of a 6.8-pH phosphate buffer in a 50 ml falcon tube. The falcon tube was incubated at 37°C and shaken horizontally at 125 rpm for 45 min. At an appropriate time, this mixture was then centrifuged at 5000 rpm for 5 min. Then, the supernatant was collected using a 0.45-µm filter and the released pantoprazole was quantified by UV spectrophotometry at a wavelength of 288 nm.

Formulation and preparation of delayed-release microspheres containing pantoprazole: 100 mg of microspheres (<180 µm) was dispersed in a 2% (w/v) alginate solution containing various quantities of L100 (Table 2) at 50°C. This mixture was quickly added dropwise to 50 ml of 5% CaCl₂ from a height of 5 cm above the solution surface. This mixture was then stirred at 150 rpm for 5 min. The microspheres were collected and washed with water and then dried at 50°C. The microspheres were stored in a dark bottle for more experiments. The *in vitro* dissolution studies were carried out in a shaking bath with uncoated microspheres in pH 1.2 for 120 min and then in pH 6.8 for 45 min.

Table 2. Formulations of delayed release pantoprazole microspheres.

Formulation	Uncoated microspheres type	Alginate (%)	L100 (%)	Microsphere (%)
F4	F3	2	0	2
F5	F3	2	5	2
F6	F2	2	5	2

Table 3. Drug encapsulation yield and *in vitro* pantoprazole released in the pH 6.8 of uncoated microspheres of pantoprazole sodium.

Formulation	Drug encapsulation yield (%)	Drug release after 45 mins (%)
F1	9.63±0.12	31.66±0.36
F2	10.61±0.20	81.74±1.46
F3	11.47±0.09	99.53±1.18

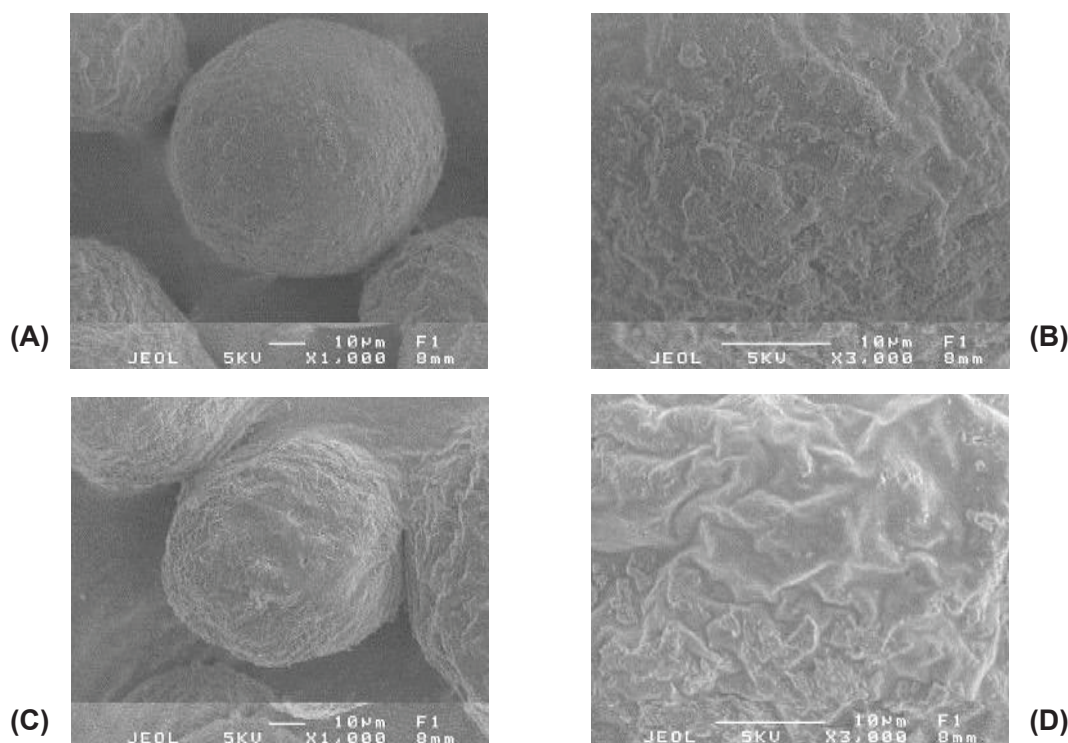


Fig. 1. SEM micrograph of microspheres F1: (A) 1000x, (B) 3000x and F3: (C) 1000x, (D) 3000x.

3. Results and discussion

3.1. Evaluation of microsphere formulation

The encapsulation yield and *in vitro* pantoprazole release of the prepared microspheres are described in Table 3.

The encapsulation yield increased with increasing HPC proportion. In other word, the more HPC in the formula, the greater achieved encapsulation yield. This is because adding a hydrophilic polymer maintains hydrophilic API in its dispersed phase and out of its continuous phase [6].

The drug release from the F1 formulation was relatively low, whereas the F2 and F3 formulations had a higher *in vitro* drug release profile in the pH 6.8 buffer. Therefore, increasing HPC concentration can increase the ability of pantoprazole release.

The microsphere's morphological characteristics are shown in Fig. 1. Microspheres from both formulae have a spherical shape and are an average of 100 μm in size. Although the surface of the F1 particle is smoother than F3, both formulations have a wrinkled surface.

The particle size distribution of the F3 microsphere is illustrated in Fig. 2. The average size is about 100±23 μm.

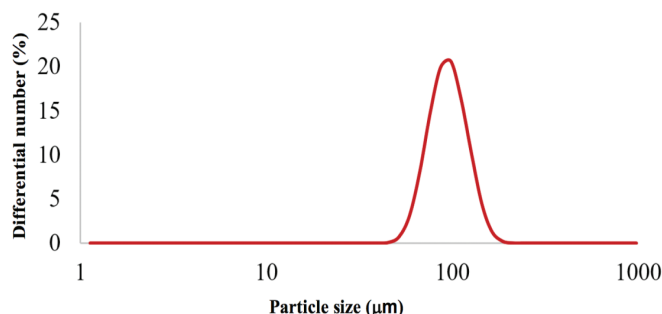


Fig. 2. Particle size distribution of the F1 microspheres.

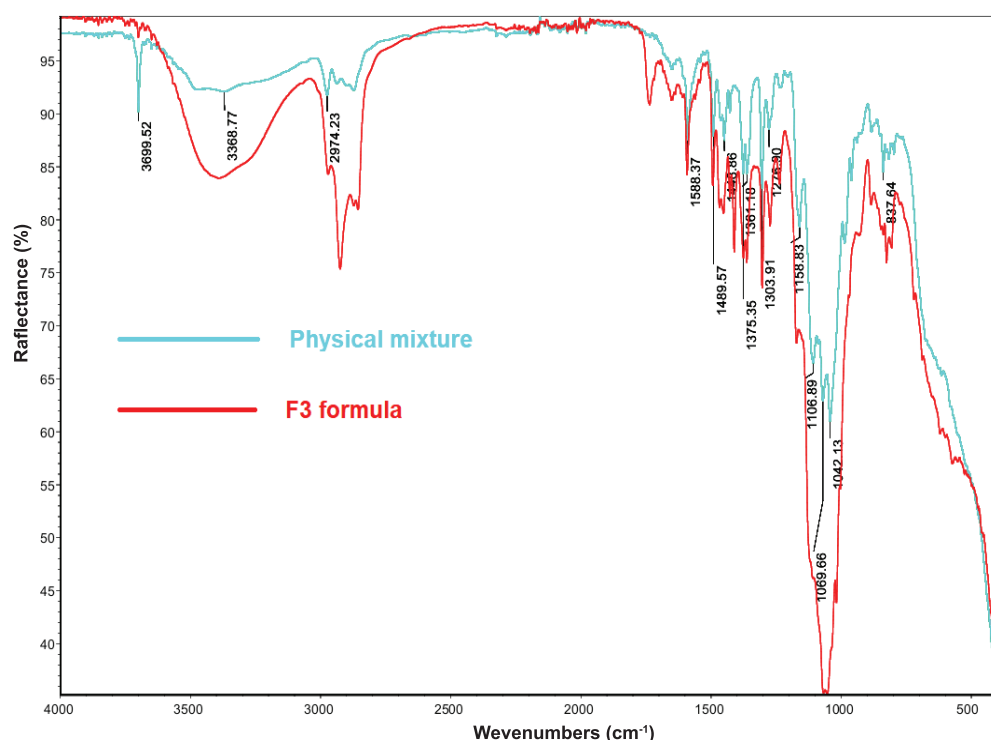


Fig. 3. The FTIR spectra of microsphere F3 (red) and physical mixture (blue).

The IR spectrum of the F3 microspheres and the physically mixed sample are illustrated in Fig. 3.

There is no significant difference between the IR spectra of the F3 formula and the physically mixed sample. Therefore, it is inferred that no chemical interaction exists between pantoprazole and the polymer-made microsphere. The absence of F3's main peak is the effect of being covered by the polymer due its large proportion in this formulation.

The DSC diagrams of the microspheres and different formulation compositions are illustrated in Fig. 4.

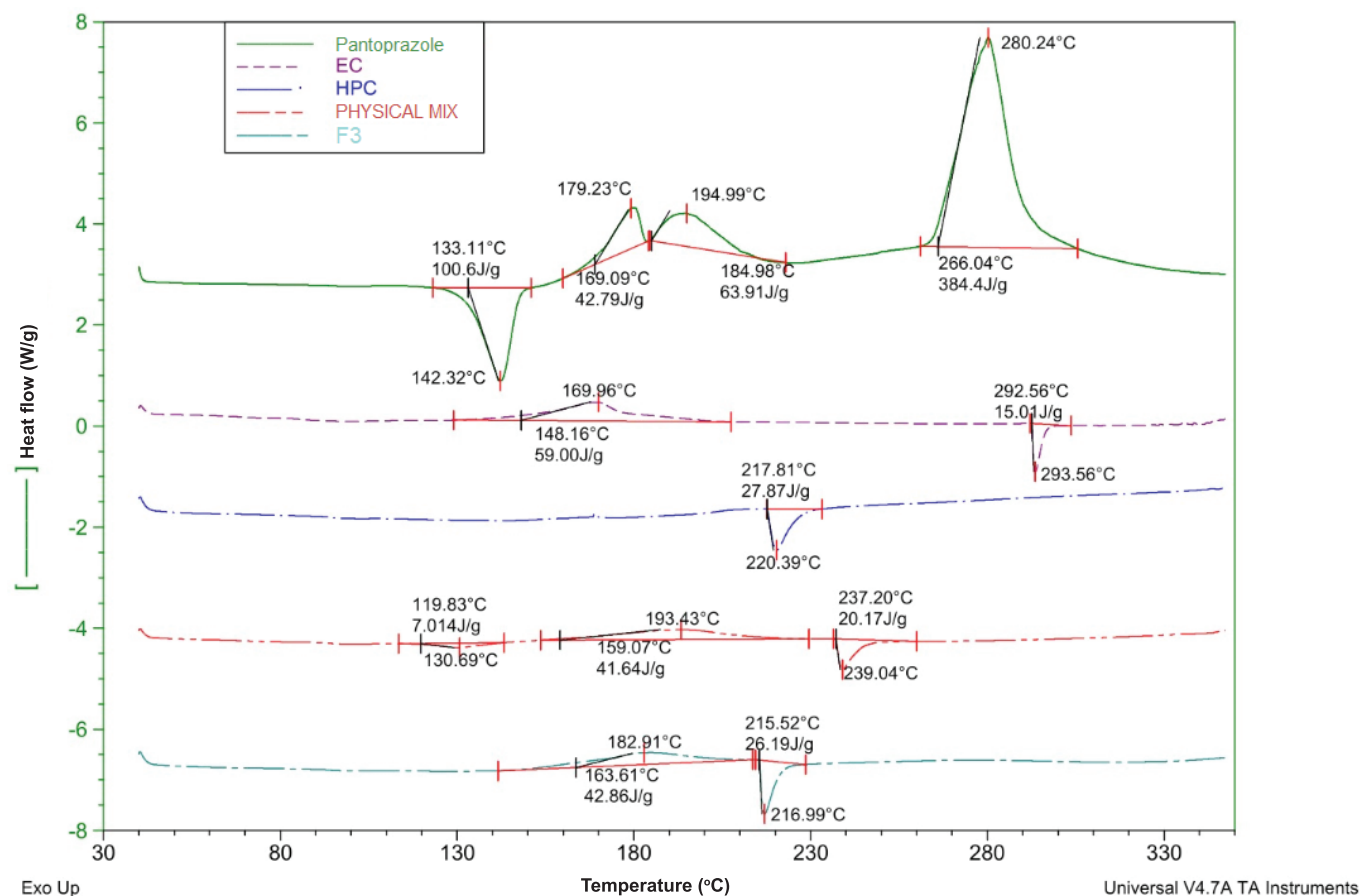


Fig. 4. The DSC thermograms of components in the formula F3.

According to diagram, F3's curve has two enthalpy transition points at 182.91°C and 217°C, which is similar to the physically mixed sample. The 130°C peak does not appear in F3's curve. This could be explained due to the cover of nearly peak of excipients (7.0 J/g difference) or Van der Waals effects of hydro connection.

Due to the high *in vitro* drug release profile in the pH 6.8 buffer, the F2 and F3 samples are used in delayed drug release formulation in the next step.

3.2. Formulation and preparation of coated microspheres

The percentage of pantoprazole content, the microparticle encapsulation efficiency, and the *in vitro* release results of the coated microspheres in a pH 1.2 buffer after 120 min are illustrated in Table 4.

The addition of 2% alginate (in formulation F5 and F6) had better API protection from leaking into the acidic medium (9.58 and 2.37%, respectively) than using Eudragit® L100 alone (F4 had 37.11% drug released in the acidic medium). The proportion of HPC and EC also affects the ability of pantoprazole release. The more EC in the formula, the more acidic protection effects (i.e., F5 vs. F6).

Table 4. Percentage content and *in vitro* drug release in the pH 1.2 of coated microsphere.

Formulation	Percentage content (%)	Encapsulation efficiency (%)	Drug release after 2 h in acidic medium (%)
F4	4.83±0.04	97.85	37.11±0.80
F5	2.31±0.03	97.79	9.58±0.08
F6	2.19±0.04	98.45	2.37±0.04

A dissolution test was carried out with the F5 and F6 microspheres and the results are shown in Table 5 and Fig. 5.

Table 5. Result of drug release in pH 6.8.

Formulation	Time of sampling		
	15 min	30 min	45 min
F5	15.09±0.74	53.62±1.96	77.23±1.02
F6	7.28±0.96	24.76±0.54	50.24±1.12

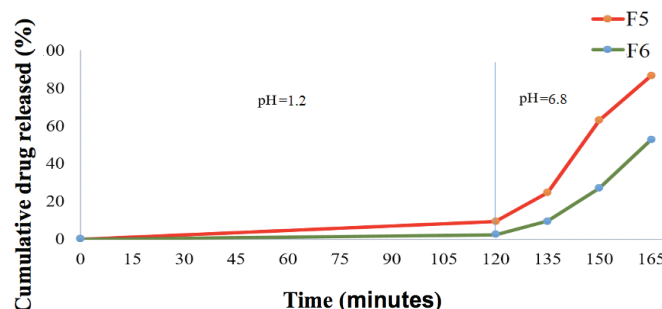


Fig. 5. Chart of *in vitro* drug release of formulations F5 and F6.

F5 showed acid tolerance after 120 min (9.58%). Furthermore, in a pH 6.8 buffer, the F5 formulation showed better API release (77.23%) than F6 (50.24%).

The surface and cross-sectional surface of the F5 formulation are shown in Figs. 6 and 7, respectively. The encapsulated microspheres were protected and intact in the alginate medium. The F3 microspheres are covered by the alginate-L100 coating layer. The cutting edge image shows that alginate gel matrix is integrated with alginate particles and filled by the Eudragit® L100 polymer.

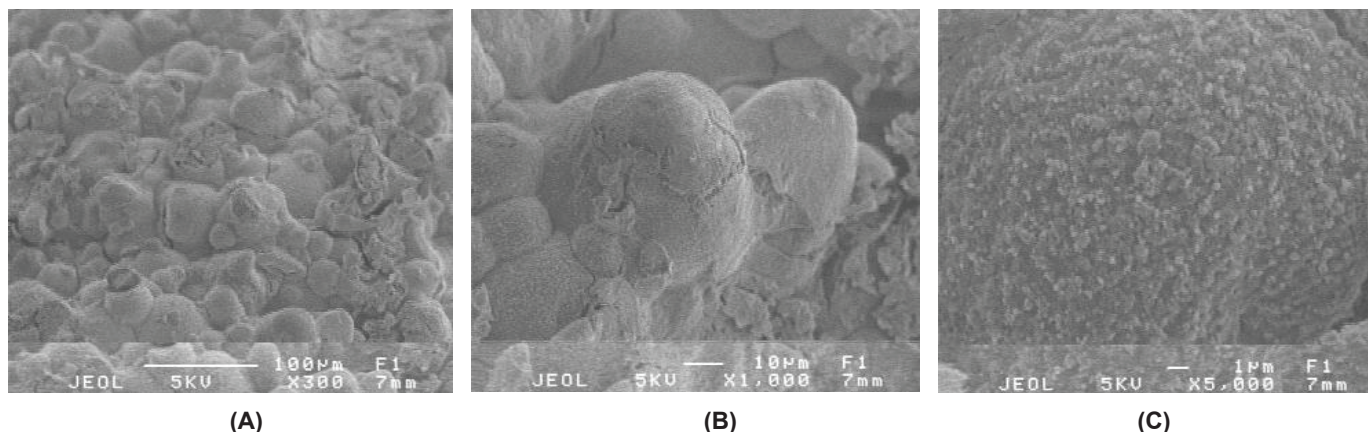


Fig. 6. SEM surface photographs of formulation F5 at magnifications of (A) 300x, (B) 1000x, and (C) 5000x.

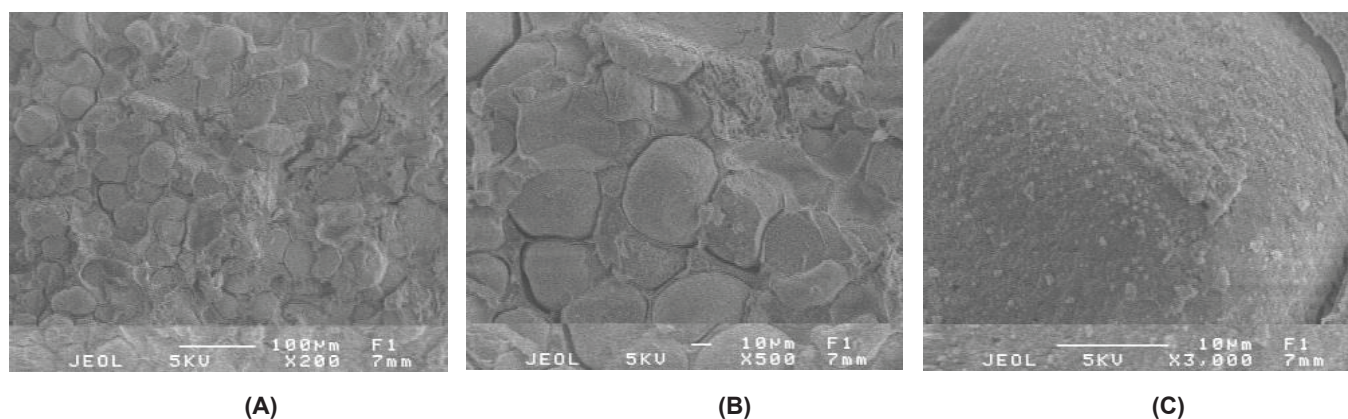


Fig. 7. SEM cross-section surface photographs after cutting formulation F5 at magnifications of (A) 200x, (B) 500x, and (C) 3000x.

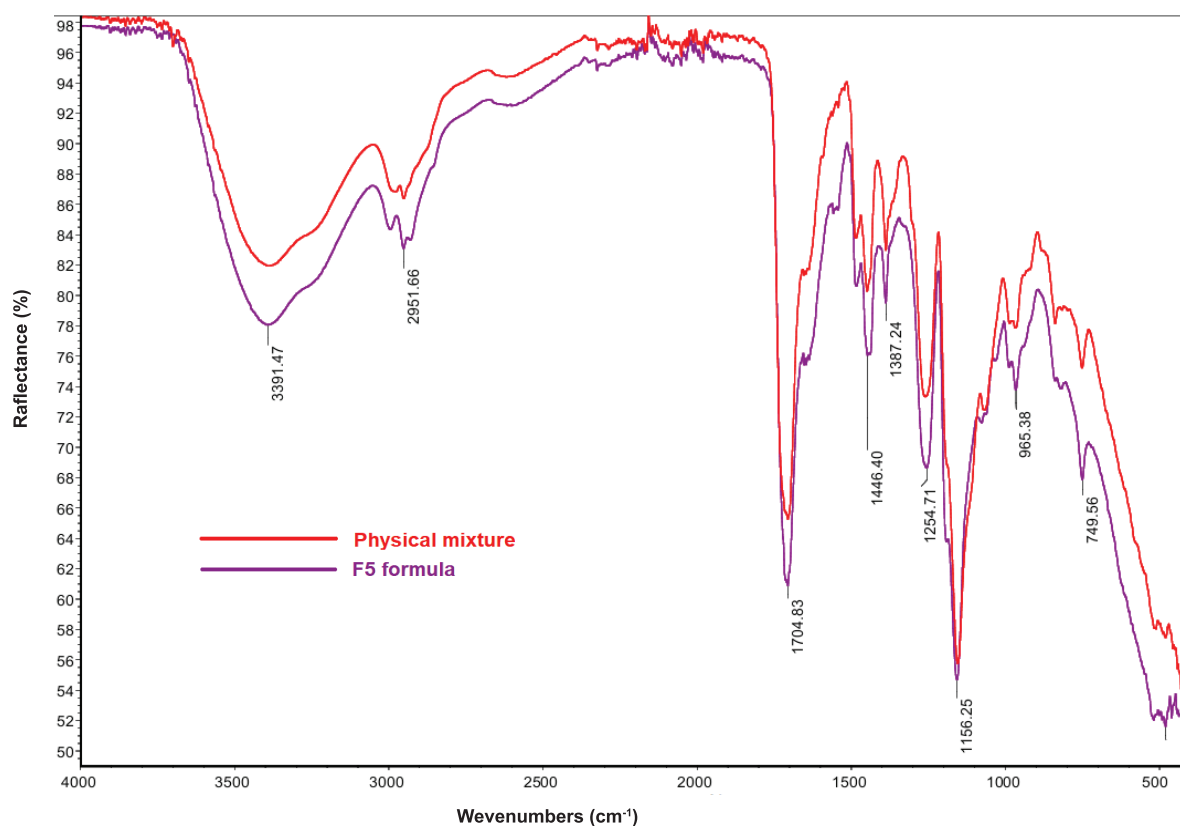


Fig. 8. The FTIR spectra of formulation F5 (purple) and the physically mixed sample (red).

The IR spectra of the F5 microspheres and the physically mixed sample of the same composition are illustrated in Fig. 8.

There are no significant differences between the spectra of the two samples, thus, there are no chemical interactions between the materials in the formulation.

4. Conclusions

Microspheres with 100- μ m average size were successfully prepared with an encapsulation yield of 11.4%. More than 75% of the pantoprazole was released in a pH 6.8 buffer after 45 min. Moreover, the addition of alginate in Eudragite can protect the pantoprazole after

exposure to an acidic environment for 2 h (less 10%) while the release profile in the pH 6.8 buffer was still greater than 75%.

CRediT author statement

Xuan Truong Le, Hue Minh Nguyen, Ngoc Quynh Le: Data analysis and interpretation, Writing and manuscript revising; Thi Thu Loan Trinh, Van Thanh Tran: Method, Data analysis and Writing.

COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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Morphological characteristics and mitochondrial COI gene of the green water dragon in Phu Quoc island, Vietnam

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Abstract:

The green water dragons of Phu Quoc island, Vietnam have been given a VU rating (vulnerable) by the Vietnam red data book and the International Union for conservation of nature and natural resources. A description of the morphological characteristics and sequenced mitochondrial cytochrome oxidase subunit 1 COI gene was completed following a survey of thirty individuals from September 2018 to May 2019. Their body weights ranged from 115 to 850 g and their snout-vent length ranged from 120 to 280 mm. They were identified to have the highest similarity to the species *Physignathus cocincinus* by the barcode of life data system. Their COI gene sequence was compared to GenBank (KM272197.1) and the following alternations were found: C5483T, A5486C, C5537A, A5564G, A5591G, T5633C, G5684A, A5771G, A5849C, C5927T, T5942C, C5993T, G6002A, and A6020G. The neighbourjoining tree of their COI sequence divergence indicated a distance (compared to the same published species) from 2.3 to 2.8%. Thus, it might be possible that the green water dragons of Phu Quoc island are evolving into a specific subspecies. However, more green water dragon specimens from Phu Quoc should be sequenced to ensure a conclusion and further research into farming should be initiated to protect this vulnerable species.

Keywords: COI, gene sequence, morphology, Phu Quoc, *Physignathus cocincinus*.

Classification number: 3.4

1. Introduction

Phu Quoc island, Vietnam, boasts idyllic beaches and evergreen forests. The northern part of the island is relatively untouched due to a National park, which was assessed to have high biodiversity and conservation value. In this park, green water dragons need conservation due to their entry as a vulnerable species in the Vietnam red data book [1] and International Union for Conservation of nature and natural resources [2]. Green water dragons were firstly described by Cuvier (1829) [3]. They belong to the species *Physignathus cocincinus*, genus *Physignathus*, subfamily Amphibolurinae, family Agamidae, suborder Iguania, order Squamata, class Reptilia, phylum Chordata. Their distribution areas are in China, Thailand, Laos, Cambodia, and Vietnam [2]. In Vietnam, they have a wide geographic range that reaches

the mountainous areas of the North, Central, Central Highlands, Eastern South, and Phu Quoc island [4]. They are large in size, have good meat, and an attractive appearance; so much so that they are often hunted for food, medicine, pets, and export [5]. Currently, the green water dragons of central Vietnam have been studied to clarify their morphological characteristics, ecology, behaviour, and diets [6] for breeding and expanding successfully [4]. However, these studies are limited in both their genetic information and sampling of wild populations in different geographies.

The genetic information contained in deoxyribonucleic acid (DNA) sequences of the genome play a decisive role in forming the biological characteristics of each species. The mitochondrial genome evolves about 17 times faster than the nucleus genome, so studies on genetic information

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often originate from the mitochondrial genome [7]. In the mitochondrial genome rests, the mitochondrial cytochrome c oxidase gene subunit 1 (COX-1, COXI or COI) with a size of 1547 bp for *Physignathus cocincinus* (GenBank #KM272197.1). The COI gene is responsible for translating the protein of cytochrome oxidase, which is a component of the respiratory chain that catalyses the reduction of oxygen to water in cells [8, 9]. Alteration of the mitochondrial COI gene would impact cellular respiration, a cell's energy release, and body function. Therefore, the mitochondrial COI gene is often used for taxonomic and phylogenetic analyses [10]. This study aims to provide scientific data on the morphological characteristics and DNA sequence of the mitochondrial COI gene of the green water dragons distributed in Phu Quoc island, Vietnam, for further research on their conservation and breeding.

2. Materials and methods

2.1. Sample collection

The sampling was carried out from September 2018 to May 2019. The green water dragons were collected in National park of Phu Quoc (10°19'30"N, 103°57'0"E), Phu Quoc city, Kien Giang province, Vietnam. There were thirty individuals collected over three collection times (~10 lizards/time) and all samples were licensed by the Forest protection department of Kien Giang province. After coordinating with the staff of the Forest protection the streams (Fig. 1) were surveyed and, when discovering a lizard, a noose was used to catch it and then a code was assigned to manage each individual.

After collecting the samples, photographs of the individuals were recorded, their morphologies measured, and 10% of the lizards were selected randomly to sample tail tissue for DNA isolation [11]. Then, the lizards were handed over to the National park of Phu Quoc. Morphological measurements were conducted according to Schulte II, et al. (2004) [12] including body weight, body length, snout-vent length, tail length, armpit-groin length, head length, head width, head height, front limb length, hindlimb length, snout-eye length, and eye diameter.

2.2. Mitochondrial COI gene sequencing

DNA extraction: The method of DNA isolation was conducted according to Werman, et al. (1996) [13]. Briefly, 50-100 mg of sample was crushed in 300 µl TE, 50 µl SDS 10%, 5 µl proteinase K, and warmed to 65°C for 20 min. Then, 400 µl CTAB 10% in NaCl 0.7 M was added and warmed to 65°C for 20 min. Continuously, 200 µl of chloroform:isoamyl (24:1) was added and centrifuged at 13,000 rpm for 5 min to collect 300 µl supernatant. Next, the supernatant was dissolved in 300 µl Ethanol 95% and centrifuged at 13,000 rpm for 5 min. Then, the supernatant was removed again and washed twice with 500 µl ethanol 70%. Finally, the isolated DNA was dissolved in 100 µl TE for PCR tests or stored at -20°C until analysis. The DNA quality was estimated using the absorbance values at 260 nm by a UV-Vis1800 spectrometer of Shimadzu, Japan.

DNA sequencing: Primers were used to amplify mitochondrial DNA designed with the Primer-BLAST program [14], which is based on the

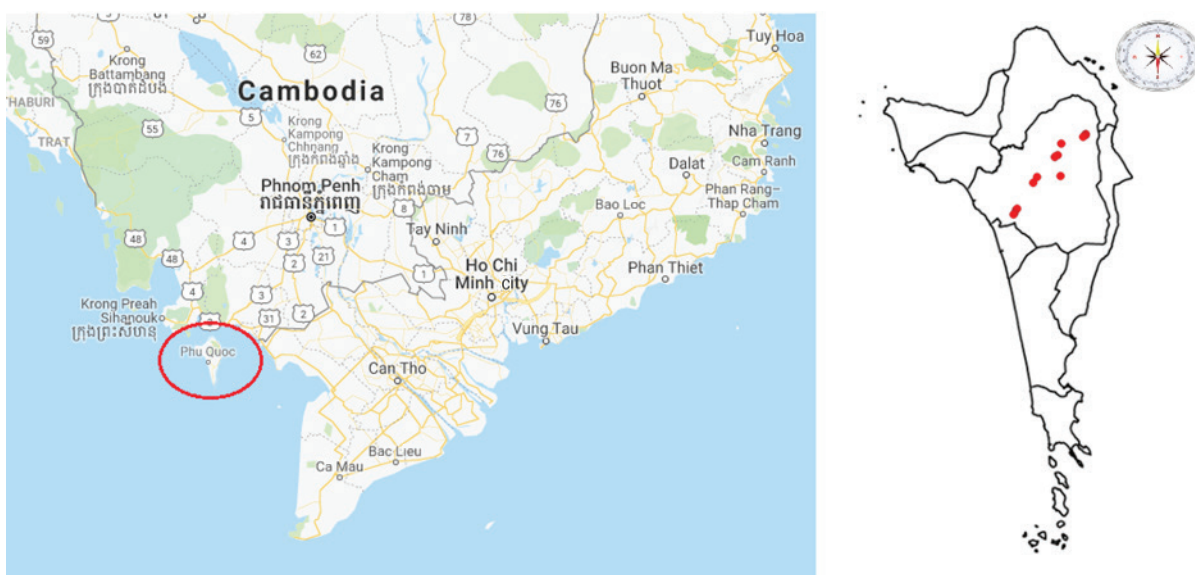


Fig. 1. The collection locations (red dots) for the 30 green water dragons in Phu Quoc island.

DNA sequence of the mitochondrial COI gene of *Physignathus cocincinus* in GenBank (KM272197.1) from base 5169 to 6715. The forward primer was 5'CTAGGTGCCCCAGACATAGC3', and the reverse was 5'GCTCGGGTGTCAATGTCAAG3' with the amplified product of 650 base pairs (bp). The polymerase chain reaction (PCR) was performed in a volume of 50 μ l with components including 25 μ l Master mix 2X, 2 μ l $MgCl_2$ 25 mM, 1 μ l Taq polymerase 5 u/ μ l, 2 μ l genomic DNA, 2.5 μ l forward primer 10 pM, 2.5 μ l reverse primer 10 pM, and nuclease-free water up to 50 μ l. The amplification was performed in a thermocycler programmed at 95°C for 5 min, 35 cycles of 95°C for 30 secs, 58°C for 30 secs, 72°C for 45 secs, and a final extension at 72°C for 5 min. The PCR was done in a GeneAmp® PCR System 9700 of Life Technologies, USA, with 96 wells. The PCR products were confirmed by 1.2% agarose gel electrophoresis. The DNA fragment sequence was determined using Sanger, et al.'s method (1975) [15] and manually edited based on their traces by BioEdit 7.2.5.3 [16].

2.3. Data and DNA analysis

The descriptive statistical parameters of the morphological measurements were calculated in Microsoft Excel 2010. Linear regression analyses of the morphological measurements were run on Minitab 18.1. Translations of the mitochondrial COI gene into amino acids was done by BioEdit 7.0.5.3 [16]. The barcode of life data identification system for COI, which accepts sequences at <http://www.barcodinglife.org>, was used for taxonomy assignment and returns a species-level identification when one was possible [17]. The COI sequence of the samples was checked with GenBank (KM272197.1) for identifying alternations by the basic local alignment search tool for highly similar sequences at <https://blast.ncbi.nlm.nih.gov> [18].

3. Results and discussion

3.1. Morphological characteristics

An external photograph of the green water dragons in Phu Quoc island is given in Fig. 2. Their morphological measurements are shown in Table 1.



Fig. 2. Green water dragons from (A) Phu Quoc island and (B) Thua Thien - Hue province [6].

Table 1. The morphological measurements of the green water dragons in Phu Quoc island and Thua Thien - Hue province.

Traits	Phu Quoc island (n=30)				Thua Thien - Hue [6]
	Min.	Max.	Mean	Standard deviation	
Body weight, g	115	850	326	162	10.4-333
Snout-vent length, mm	120	280	188	38.9	42.4-260
Tail length, mm	300	540	422	76.7	109-550
Head length, mm	31.0	68.0	43.6	9.68	13.1-73.5
Head width, mm	21.0	48.0	30.7	6.32	9.50-38.1
Head height, mm	21.0	46.5	30.2	5.94	7.90-36.8
Front limb length, mm	55.0	120	81.7	17.0	21.6-114
Hindlimb length, mm	95.0	200	145	26.1	16.4-191
Snout-eye length, mm	15.0	31.0	21.2	4.51	5.60-25.3
Armpit-groin length, mm	75.0	160	107	22.0	16.1-111
Eye diameter, mm	7.80	18.0	11.4	2.08	1.70-9.20

Table 2. Linear relationships (n=30) between morphological measurements and snout-vent length of the green water dragons in Phu Quoc Island.

Relationships	Intercept	Slope	R ²	RSD	p
Snout-vent length vs. body weight	116±6.74	0.220±0.019	0.83	16.2	0.001
Snout-vent vs. head length	23.3±11.9	37.7±2.67	0.88	13.9	0.001
Snout-vent length vs. head width	9.36±12.2	58.0±3.88	0.89	13.2	0.001
Snout-vent length vs. head height	0.434±12.3	62.0±4.01	0.90	12.8	0.001
Snout-vent length vs. front limb length	12.8±12.8	21.4±1.54	0.87	14.1	0.001
Snout-vent length vs. hindlimb length	-9.67±16.8	13.6±1.14	0.84	16.0	0.001

Figure 2A shows external observations of the green water dragons from Phu Quoc island. We found that their snout was longer than their eye and that the area of the eye is concave. The eyes were clear, covered with many small scales, and the edges were slightly rough. The body was covered with small scales, evenly spaced, which were smaller than abdominal scales and limbs. Abdominal scales and under limbs were hexagonal. Tail scale size was similar to the abdominal scales and had burrs. The neck area was covered with oval-shaped scales that were small and all facing forward. On each cheek, there were three or four pointed protrusions. The upper body had a row of hard spikes from the neck to the tail. The tail length was nearly 2/3 of the body length. These characteristics were similar to the green water dragons distributed in Thua Thien - Hue province (Fig. 2B) [6].

Table 1 shows that the green water dragons from Phu Quoc have a larger sized body than those observed by N.V. Hoang (2018) [6] in Thua Thien - Hue province. For example, the snout-vent length ranged from 120 to 280 mm (vs. 42.4-260 mm) and the body weight ranged from 115 to 850 g (vs. 10.4-333). However, these figures were the same as the measurements carried out by N.D. Chung, et al. (2007) [19] with an average weight of 355 g (max 650 g). The snout-vent length is a stable morphological index often used to assess species [20]. The relationship analysis of the morphological measurements to the snout-vent length is presented in Table 2.

Table 2 shows that the snout-vent length has a close linear relationship to the body weight, head length, head width, head height, front limb length, and hindlimb length with a coefficient of determination R² from 0.83 to 0.90 (p<0.05). The base-10 logarithm (log) of the snout-vent length had a closer relationship to log (body weight) with R²=0.85 (Fig. 3). Similarly, N.V. Hoang (2018) [6] has identified a relationship of the snout-vent length to other morphological measurements of green water dragons in Thua Thien - Hue, Vietnam with R² ranging from 0.64 to 0.94. In other reptiles, these relationships were also close, e.g., *Oligosoma microlepis* with R²=0.75 [21] and *Euphlyctis cyanophlyctis* with R²=0.84 [22].

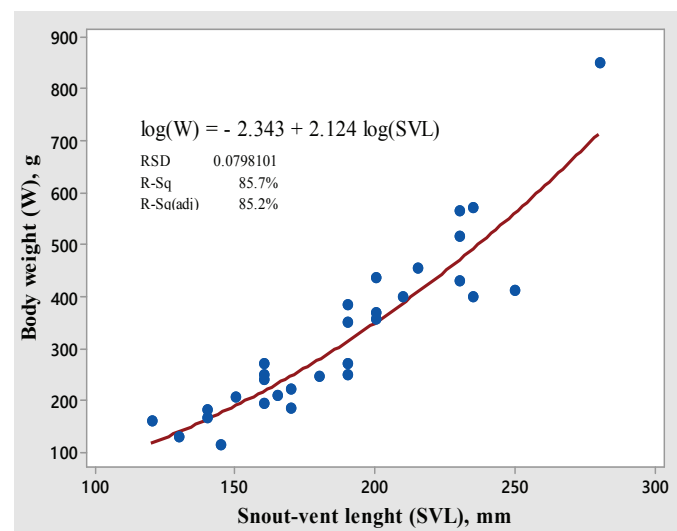


Fig. 3. Relationship between body weight and snout-vent length of green water dragons in Phu Quoc island, Vietnam.

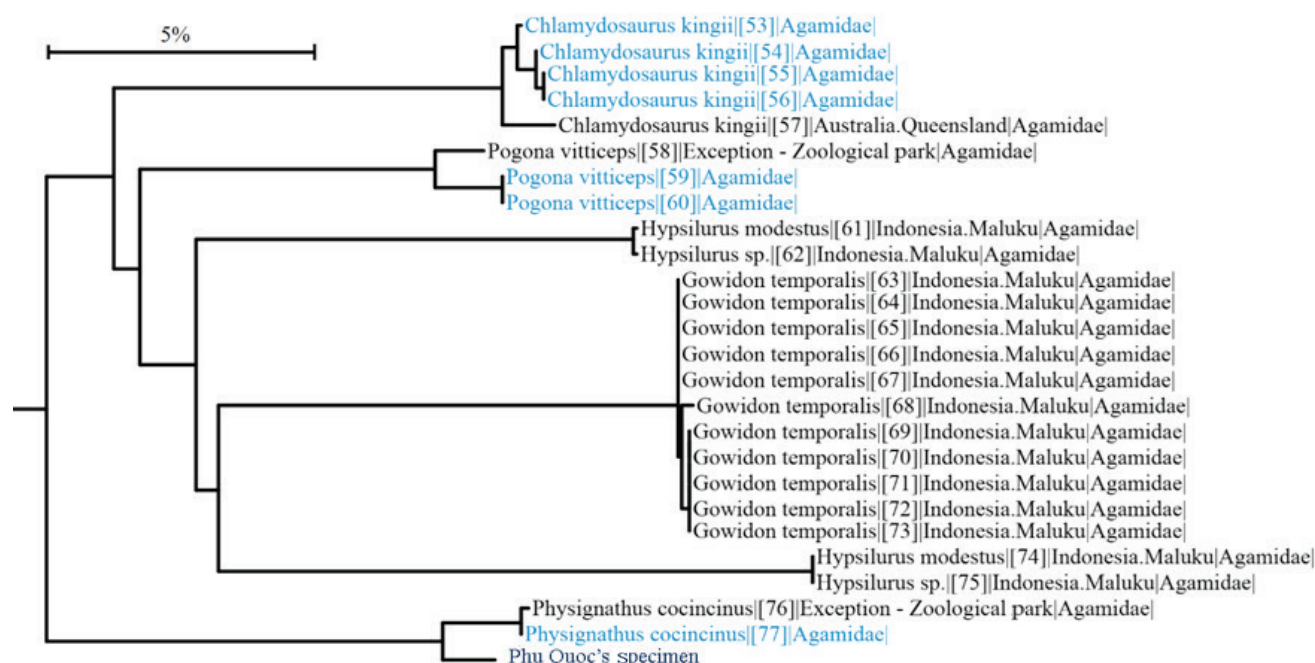


Fig. 4. The neighbor-joining tree for *Physignathus cocincinus* in Phu Quoc island, Vietnam.

3.2. Mitochondrial COI gene

The total isolated DNA was confirmed to be high purity. All samples had an optical density within 1.73-2.01 with high cleanliness that was guaranteed for sequencing. The PCR product analysis on 1.2% agarose gel found the obtained specific DNA fragments had a size of about 650 bp. Sequence reading was done in two dimensions. The PCR products were successfully sequenced and the fluorescence peaks were very clear, which corresponded to each specific alignment of DNA. After eliminating the primer, the DNA sequence of the COI gene was 604 bp for all specimens. This shows that the results of sequencing were highly reliable. The DNA sequence of the COI gene of three individuals of green water dragon in Phu Quoc island were 100% similar. There was no detectable variation of the COI gene in the population of green water dragons in Phu Quoc island, Vietnam. The COI sequences were not identified as a specific species level match in the barcode of life data system, but the nearest match was with *Physignathus cocincinus* at a 97.7% probability of placement.

The neighbor-joining tree is given in Fig. 4 and one can see the distance compared to the same published species is around 2.3 to 2.8%. Consequently, this might

confirm that the green water dragons in Phu Quoc island are evolving into a specific subspecies. Some observations of sequence divergence for intraspecific comparisons of Reptilia (Testudines and Squamata) have been less than 3% [23] or even lower in intraspecific sequence divergences for birds [24], mammals [25], and fishes [26].

The comparison with GenBank (KM272197.1) by the Basic Local Alignment Search Tool for highly similar sequences found 14/604 new alternations in COI sequences of the green water dragons from Phu Quoc Island (Fig. 5). The changes were at localities of C5483T, A5486C, C5537A, A5564G, A5591G, T5633C, G5684A, A5771G, A5849C, C5927T, T5942C, C5993T, G6002A, and A6020G. The protein sequences translated from the COI gene of the green water dragons in Phu Quoc island would alter three amino acids in the cytochrome oxidase I enzyme structure compared to the GenBank. The changes were from three alanine molecules transformed into two glycines and one threonine. Alanine and glycine are nonpolar molecules while threonine is polarized, so it is possible that there is an impact on the function of cytochrome c oxidase I enzyme, which is involved in the electron transport of cellular respiration by reducing oxygen into water [9].

UNVERIFIED: *Physignathus cocincinus* mitochondrion sequence
Sequence ID: [KM272197.1](#) Length: 16451 Number of Matches: 1

Range 1: 5476 to 6079 [GenBank](#) [Graphics](#) [Next Match](#) [Prev Match](#)

Score	Expect	Identities	Gaps	Strand
1038 bits(562)	0.0	590/604(98%)	0/604(0%)	Plus/Plus
Query 1	TCTGACTTCTCCACCATCATACCTCTACTAATATCAACCACCTGATTCAACTCTGGAG	60		
Sbjct 5476	TCTGACTCCTACCACCATCATACCTCTACTAATATCAACCACCTGATTCAACTCTGGAG	5535		
Query 61	TAGGAACAGGATGAACCATTTATCTCCGATATCAGGAAACCTTGACACGCAGGGCCAT	120		
Sbjct 5536	TCGGAACAGGATGAACCATTTATCTCCGATATCAGGAAACCTTGACACGCAGGACCAT	5595		
Query 121	CTATAGACCTAGCCATCTTCTCCCTACATCTAGCAGGCGCATCTCCATTCTAGGCGCAA	180		
Sbjct 5596	CTATAGACCTAGCCATCTTCTCCCTACATCTAGCAGGCGCATCTCCATTCTAGGCGCAA	5655		
Query 181	TTAACTTCATCACAACTGCATCAACATATCCCCACCACACATAACCCCATTCAACTGAC	240		
Sbjct 5656	TTAACTTCATCACAACTGCATCAACATATCCCCACCACACATAACCCCATTCAACTGAC	5715		
Query 241	CCTTATTCGTTTGATCAGTATTCTTAACAGCCACCCTACTCTACTATCATTACCGGTAC	300		
Sbjct 5716	CCTTATTCGTTTGATCAGTATTCTTAACAGCCACCCTACTCTACTATCATTACCGGTAC	5775		
Query 301	TAGCCGCAGCAATCACAATATTACTAACAGACCGAAATCTCAACACAACATTCTTCGACC	360		
Sbjct 5776	TAGCCGCAGCAATCACAATATTACTAACAGACCGAAATCTCAACACAACATTCTTCGACC	5835		
Query 361	CTAATGGTGGAGGCGATCCAGTCTTATCCAACTCTATTCTGATTTTTCGGACACCCAG	420		
Sbjct 5836	CTAATGGTGGAGGAGATCCAGTCTTATCCAACTCTATTCTGATTTTTCGGACACCCAG	5895		
Query 421	AAGTTTACATCCTCATTCTACCAGGATTCGGTATTATCTCCACATCGTCACACACCACG	480		
Sbjct 5896	AAGTTTACATCCTCATTCTACCAGGATTCGGTATTATCTCCACATCGTCACACACCACG	5955		
Query 481	CCAGTAAAAAAGAACCAATTTGGCTACGTCAGCATAGTTTGGGCCATACTAGCAATCACTA	540		
Sbjct 5956	CCAGTAAAAAAGAACCAATTTGGCTACGTCAGCATAGTTTGGGCCATACTAGCAATCACTA	6015		
Query 541	TTCTGGGATTTCATTGTATGAGCACATCACATATTTACAGTAGGACTTGACATTGACACCC	600		
Sbjct 6016	TTCTAGGATTTCATTGTATGAGCACATCACATATTTACAGTAGGACTTGACATTGACACCC	6075		
Query 601	GAGC 604			
Sbjct 6076	GAGC 6079			

Fig. 5. The COI sequences of *Physignathus cocincinus* in Phu Quoc island and GenBank.

4. Conclusions

The present study shows that the green water dragons from Phu Quoc island, Vietnam are lizards with body weights up to 850 g and snout-vent lengths up to 280 mm. The relationships of the body weight to the head length, head width, head height, front limb length, and hindlimb length had coefficients of determination (R^2) from 0.83 to 0.90 ($p < 0.05$). The logarithm of the snout-vent length to base 10 had a closer relationship to log (body weight) with $R^2 = 0.85$. Their COI gene sequence had the highest similarity to the species *Physignathus cocincinus* with a

divergence ranging from 2.3 to 2.8%. Thus, it might be confirmed that the green water dragons from Phu Quoc island are evolving into a specific subspecies, however, there needs to be more sequencing of different specimens to ensure a significant conclusion. More research on farming should be done to contribute to protecting this vulnerable species.

CRedit author statement

Van Thong Ngo, Huu Hanh Tran: Data analysis and interpretation, Writing and manuscript revising; Chi Tao Phan: Method, Data analysis and Writing.

COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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Application of particle image velocimetry (PIV) to measure the displacement of sandy soil in laboratory

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Abstract:

In geotechnical engineering, it is important to measure soil displacement in soil specimens for all laboratory tests, in physical models or in the field. This data is usually recorded using a strain gauge attached to the soil sample during the tests. Particle image velocimetry (PIV) is extensively utilized for measuring displacement and flow velocity in fluid mechanics, yet its application in determining soil displacement during geotechnical laboratory tests is limited. This study seeks to validate PIV's suitability for assessing sandy soil displacement under various saturation levels and grain sizes. Findings indicate that PIV effectively determines soil displacement with a precision of 0.13 mm. Interestingly, the degree of soil saturation did not impact PIV outcomes; however, soil homogeneity, as characterized by grain size distribution, compromised PIV method precision. This research sheds light on PIV's potential in geotechnical applications, emphasizing its efficacy in quantifying soil displacement accurately. Moreover, it underscores the need for considering soil homogeneity when employing PIV for displacement measurements. Expanding PIV's scope to include geotechnical studies could enhance understanding of soil behavior and aid in optimizing soil engineering practices.

Keywords: particle image velocimetry, sandy soil, soil displacement.

Classification number: 4.2

1. Introduction

In geotechnical engineering, it is important to measure soil displacement in soil specimens for all laboratory tests, in physical models or in the field. This data is usually recorded using a strain gauge attached to the soil sample during the tests. With the availability of image analysis software, soil displacement analysis can be carried out easily and inexpensively using various image-based techniques such as X-ray, stereo-photogrammetric techniques, image processing, and PIV and each technique has its own advantages and limitations [1-4]. Among these techniques, PIV is an important non-invasive method to quantify local displacements on a solids' surface [5]. In this method, a series of successive images of an object are captured during the test without using any strain gauge sensors. By comparing the spatial variations on the same patches in these images using image analysis softwares, the displacement data can be obtained [6, 7]. PIV has been evidenced to be an effective technique for observation of stresses and strains of the glass ballotini and soil deformation in creep movement on the slope [4, 6, 8, 9]. In the present study, the PIV technique was adopted to predict the deformation of sandy soil with different degrees of saturation and soil grain size. The precision of the technique was verified using displacement data recorded by a strain gauge during the tests.

2. Materials and methods

2.1. Theory of the PIV method

PIV is an important technique used in fluid dynamics (Fig. 1). It allows to obtain instantaneous velocity measurements and related properties at a specific area, called 'interrogation' areas, in the fluid [10]. PIV technique has its roots from the laser speckle velocimetry technique developed in the late 1970s [11, 12]. In PIV, the displacement of an interrogation area of a pair of digital images is calculated with help of cross-correlation or autocorrelation techniques. The cross-correlation functions are presented in Equations (1) to (3) below.

$$R(\underline{s}) = \sum_U I_{test}(\underline{U}) \cdot I_{search}(\underline{U} + \underline{s}) \quad (1)$$

$$N(\underline{s}) = \sum_U [I_{search}(\underline{U} + \underline{s}) \cdot I_{search}(\underline{U} + \underline{s})] \cdot M(\underline{U}) \quad (2)$$

$$R_n(\underline{s}) = \frac{R(\underline{s})}{N(\underline{s})} \quad (3)$$

where $R(\underline{s})$ = cross-correlation matrix, $N(\underline{s})$ = normalisation matrix, $R_n(\underline{s})$ = normalized cross-correlation matrix, $M(\underline{U})$ = dummy mask matrix, $I_{test}(\underline{U})$ = intensity matrix of test patch, $I_{search}(\underline{U} + \underline{s})$ = intensity matrix of search patch, $\underline{U}$ and $\underline{s}$ = pixel coordinate vector.

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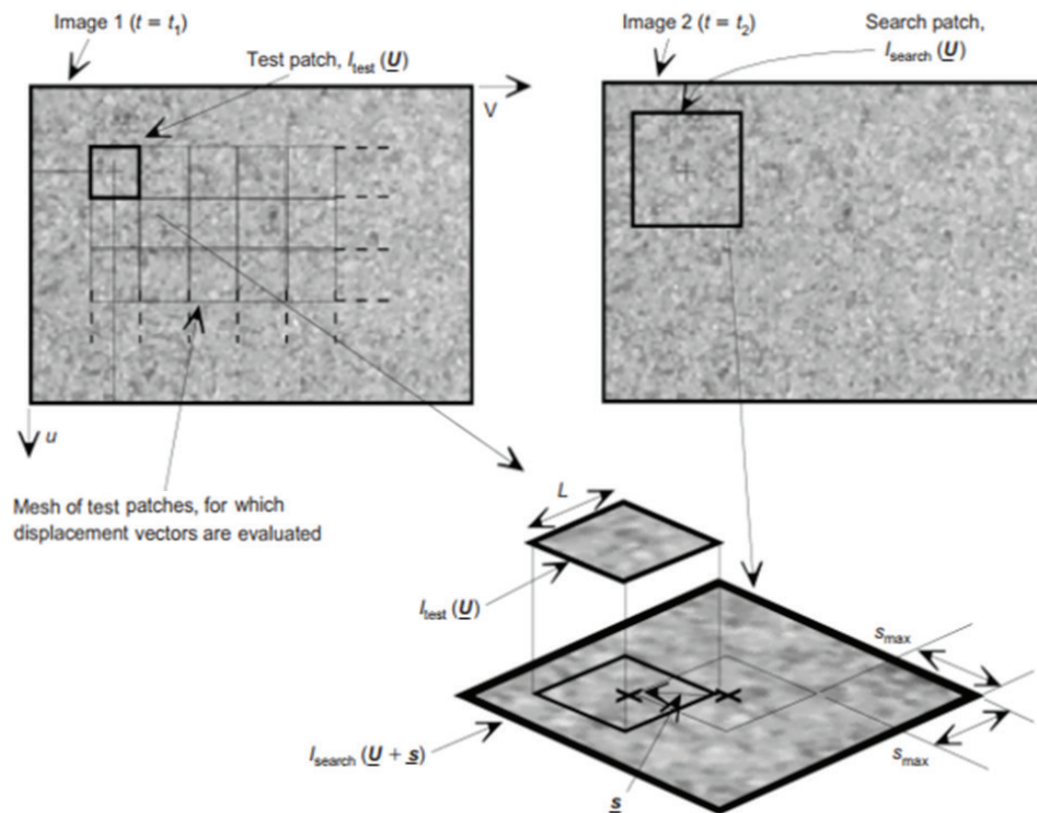


Fig. 1. Principles of image manipulation in PIV analysis [4].

2.2. Testing materials

The soil used in the test was yellow fine sand (Fig. 2A), which was air-dried until its water content of about 0.1%. According to the Unified soil classification system (USCS), the sand was classified as “SP” (i.e., poorly graded sand). The distribution of particle sizes was determined by the sieving method according to ASTM D422, and the grain size distribution curve is shown in Fig. 2B.

Several physical and mechanical properties of the sand are presented in Table 1. The specific gravity was 2.65. The maximum dry density and optimum water content (ASTM D698) were 16.7 kN/m^3 and 14.0%, respectively. The internal friction angle and cohesion (ASTM D3080) were 38° and 0 kPa, respectively. The average hydraulic conductivity of saturated sand at 29°C , i.e., ambient laboratory temperature, for dry unit weight varying from

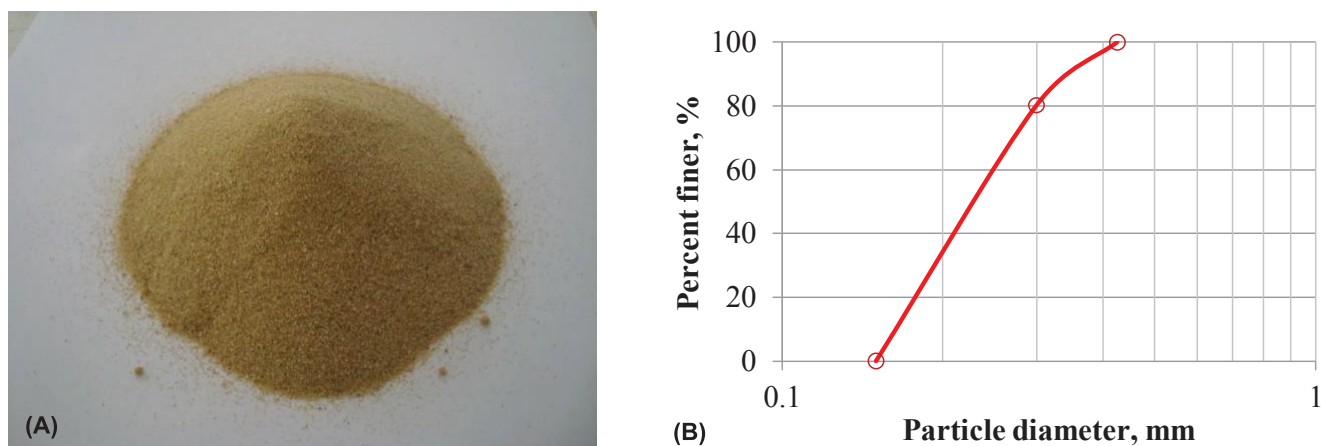


Fig. 2. Image of the poorly graded sand used in testing and its grain size distribution curve.

14.2 to 16.7 kN/m³, which determined by the constant water head method ASTM D2434, was 2.1×10^{-4} m/s and inversely proportional to the dry unit weight (Fig. 3).

Table 1. Physical and mechanical properties of the sand.

Properties	Value	Unit	Standard applied
Grain size distribution: sand - silt - clay	100-0-0	%	ASTM D422
D ₁₀ , D ₃₀ , D ₆₀	0.16-0.19-0.25	mm	-
Coefficient of uniformity, C _u	1.5	-	-
Coefficient of curvature, C _c	0.9	-	-
Classification	SP	-	ASTM D2487
Dry unit weight, γ_d	14.5	kN/m ³	ASTM D7263
Maximum dry unit weight, γ_{dmax}	16.7	kN/m ³	ASTM D698
Optimum water content, W _{opt}	14.0	%	ASTM D698
Specific gravity, G _s	2.65	-	ASTM D854
Cohesion, c'	0	kPa	ASTM D3080
Angle of internal friction, ϕ	38	°	ASTM D3080
Hydraulic conductivity, k _{sat}	2.1×10^{-4}	m/s	ASTM D2434

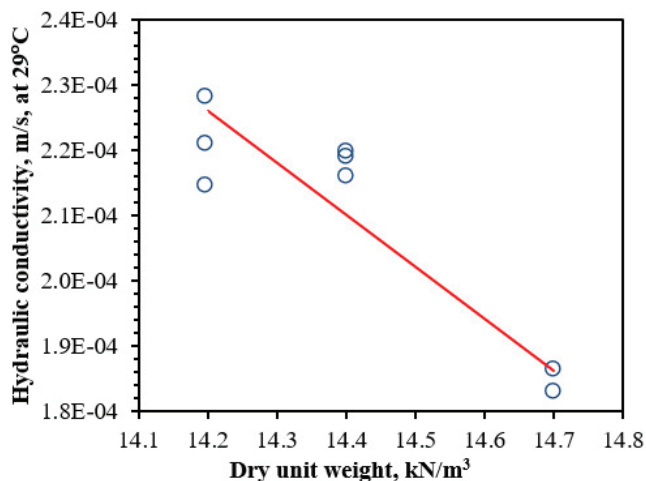


Fig. 3. Hydraulic conductivity variation with dry unit weight of saturated sand.

2.3. Experimental soil box

An experimental soil box made of acrylic was designed and developed. This acrylic box consists of two parts: the upper box and the lower box. The upper box could slide easily while the lower box was attached firmly to the base. A bolt and a down gauge were used to move the upper box in the horizontal direction and the horizontal displacement could be measured (Fig. 4). There was also a guide located on the surface of the lower box to ensure the upper box could be moved easily. In order to calculate the 'scale factor' for PIV analysis, a steel ruler was attached to the lower box surface. The calibration process was also carried out on the same sand at different degrees of saturation from 0 to 90%. The camera Canon EOS REBEL T4i/EOS 650 was used for capturing the photos. The testing process was conducted carefully in a 3-m long, 2-m wide, and 2-m high 'cell' under the light conditions induced by two LED spotlights.

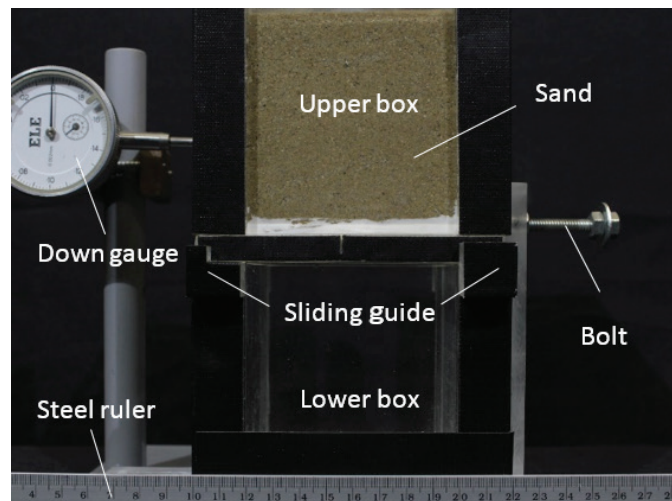


Fig. 4. The acrylic testing soil box.

2.4. Experimental set-up

The initial soil sample mentioned above, soil group A, had a particle size distribution that varied in the range of 0.85-0.15 mm. In order to evaluate the effect of particle distribution on PIV imaging results, group A was divided into 3 groups: group B (size range: 0.85-0.425 mm), group C (size range: 0.425-0.3 mm), and group D (size range: 0.3-0.15 mm). In addition, the effect of moisture on the results of the PIV image analysis were also evaluated for all four soil groups by moistening the soils to achieve the desired degrees of saturation.

A series of tests were carried out for each soil group by using the following experimental set-up:

1. Mix the soil with water to achieve the designed degree of saturation ($S_r=0, 20, 40, 60, 80$, and 90%).

2. Put wet soil samples into the test box, compact the soil until the designed dry weight was attained (14.5 kN/m^3).

3. And then move the upper part of the soil box at different intervals: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 2, 3, 4, 5, 6, 7, and 8 mm and capture photos at every moving step.

Note that the distance from the camera to the test box was fixed at 40 and 80 cm to obtain the images of each condition with 2 different resolutions, 0.05 and 0.12 mm/pixel, respectively. In total, there were 48 experiments conducted for the four groups of soil particles in 6 different degrees of saturation with 2 image resolutions (Table 2).

The images captured after the experiments were analysed using OpenPIV image analysis software, developed by R.N. Taylor, et al. (2010) [2] in MATLAB, to obtain the displacement of the soil. OpenPIV is defaulted to use a given linear scaling factor, which is an input parameter, to convert the output data in pixel to real

sizes (prototype). In other words, the effect of distortion is neglected. Moreover, according to D.J. White (2002) [13], the distortion effect causes a small error compared to the random PIV error so it could be neglected when analysing the displacement measurement. The size of the interrogation area selected was $128 \text{ pixels} \times 128 \text{ pixels}$.

It should be noted that the output data from the OpenPIV are horizontal and vertical velocities. Therefore, the soil's displacement from movement was cumulatively calculated from both velocities multiplied by the displacement time.

3. Results and discussion

3.1. Horizontal displacement

A series of successive images captured during the experiments were analysed using the OpenPIV image analysis software to obtain the soil's horizontal displacement at intervals of 0.1, 1, 2, 3, 4, and 5 mm as presented in Fig. 5. As described previously, only

Table 2. Soil particle size, degree of saturation, and image scaling factor for the 48 experimental set-ups.

Soil type	Scaling factor (mm/pixel)	Degree of saturation, S_r %					
		0	~20	~40	~60	~80	~90
A (0.85-0.15 mm)	0.05	✓	✓	✓	✓	✓	✓
	0.12	✓	✓	✓	✓	✓	✓
B (0.85-0.425 mm)	0.05	✓	✓	✓	✓	✓	✓
	0.12	✓	✓	✓	✓	✓	✓
C (0.425-0.3 mm)	0.05	✓	✓	✓	✓	✓	✓
	0.12	✓	✓	✓	✓	✓	✓
D (0.3-0.15 mm)	0.05	✓	✓	✓	✓	✓	✓
	0.12	✓	✓	✓	✓	✓	✓

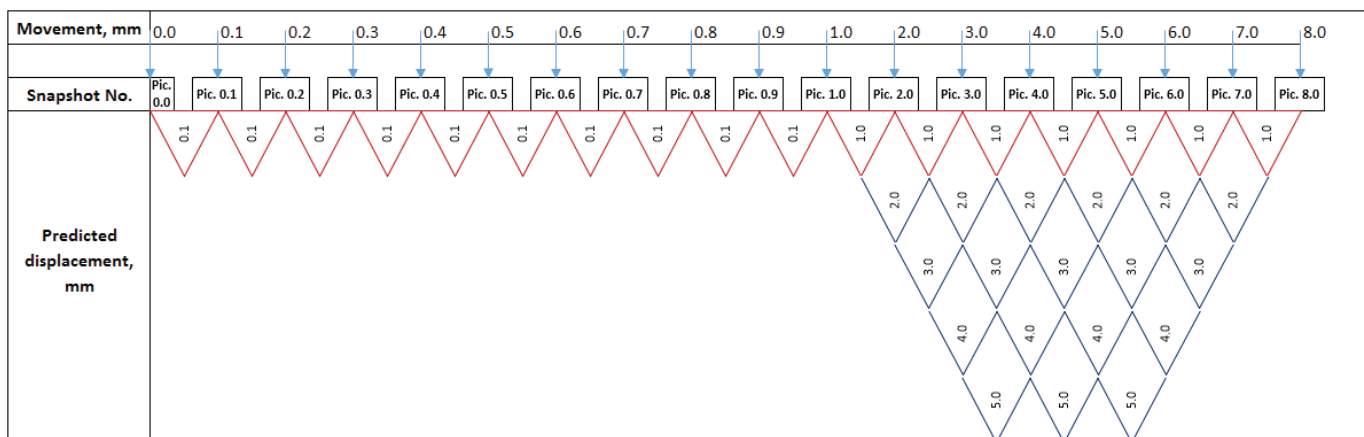


Fig. 5. Scheme of interpreting the horizontal displacement using PIV method.

horizontal deformation in the soil sample was allowed. Therefore, the total displacement is equal to the horizontal displacement. Fig. 6 shows an image of the movement vector of the soil after moving the upper box at a distance of 1 mm in the horizontal direction.

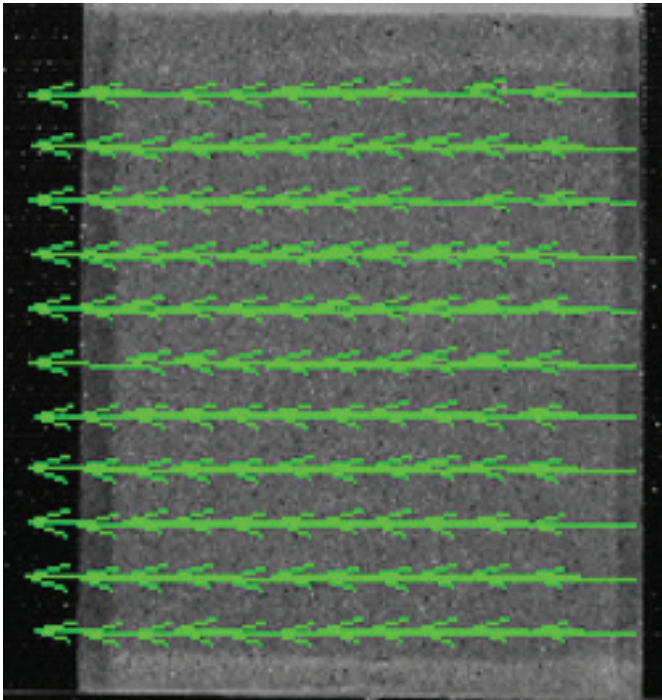


Fig. 6. Horizontal displacement of sand.

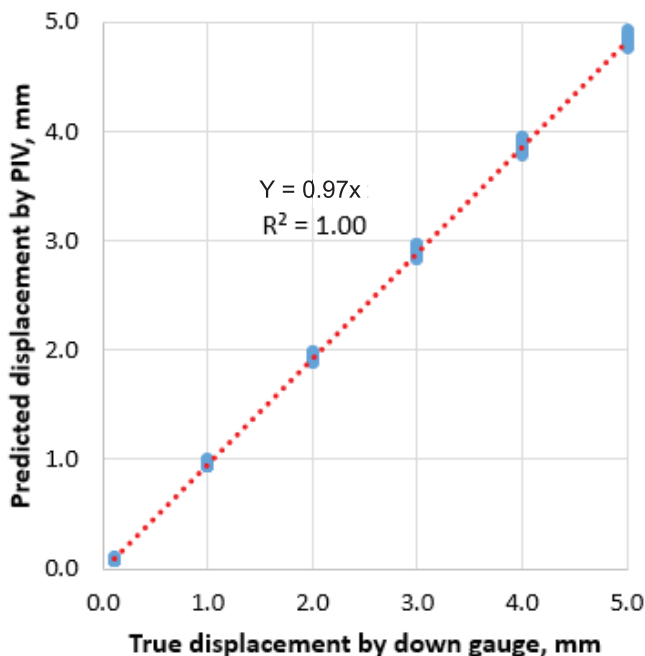


Fig. 7. Correlation between predicted displacement and true displacement.

Figure 7 presents the relationship between the predicted displacement determined from PIV and true displacement measured by the down gauge. Their correlation function was given by Equation (4) with the coefficient of determination $R^2=1.0$, which indicates that the predicted displacement from PIV was equivalent to the true displacement.

$$\text{Predicted displacement} = 0.97 \times \text{True displacement} \quad (4)$$

3.2. Accuracy

The predicted horizontal displacements of the soil sample were also determined by OpenPIV software for interval values ranging from 0.1 to 5 mm corresponding to the four soil groups A, B, C, and D. The accuracy of the predicted displacement was calculated using the following equation:

$$\text{Accuracy} = |\text{True displacement} - \text{Predicted displacement}| \quad (5)$$

For the four soil groups A, B, C, and D, there were no considerable differences in the accuracy of predicted displacement (Fig. 8). The accuracy varied from 0.02 mm (soil A, $S_r=74\%$) to 0.24 mm (soil B in dry condition, i.e. $S_r=0$) with an average accuracy of 0.13 mm.

Considering the accuracy in the same soil group as the degree of saturation was increased, the variation of the accuracy did not show any clear pattern or trend. Therefore, the effect of saturation could be ignored when analysing PIV results.

In all four soil groups A, B, C, and D, with the same test patch size of 128 pixels $\times$ 128 pixels used in the interpretation process, the image with the scaling factor of 0.12 mm/pixel gave a smaller accuracy value than the image with scaling factor of 0.05 mm/pixel. This can be explained by the fact that the area of the test patch of the 0.12 mm/pixel image is larger than the 0.05 mm/pixel image, so the results of PIV interpretation are more accurate.

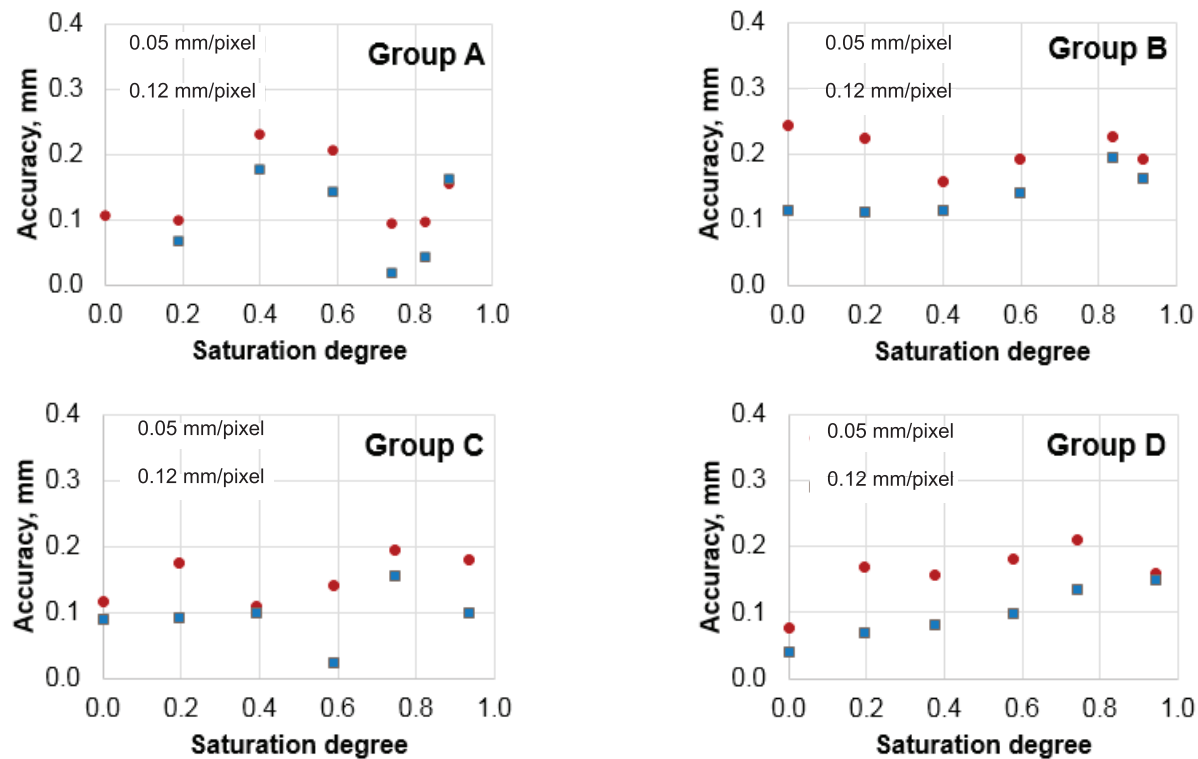


Fig. 8. Change of accuracy with saturation degree for the four sample groups.

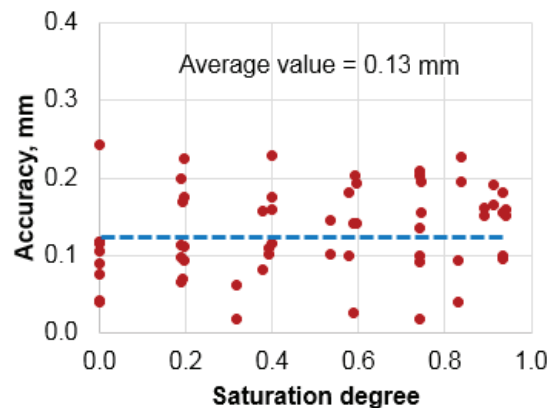


Fig. 9. Average accuracy in the measurement range of 0.1 to 5 mm.

The average accuracies of PIV for the measurement intervals from 0.1 to 5 mm were also determined as the average value of all separate accuracy values. As shown in Fig. 9, the average accuracy was 0.13 mm.

Precision

The precision of the predicted displacement obtained

from PIV was defined as the standard error of the predicted displacement. The precision values for all experimental setups for the four soil groups are shown in Fig. 10. Soil group A, which contained the larger particle grain sizes, presented quite low precision when compared to the other soil groups indicating that the more homogeneous the soil, the more scattered the predicted displacement.

Considering the effect of the degree of saturation of soil for all soil groups, there did not appear to be much difference in the precision as the degree of saturation of soil changed. Therefore, the variation of soil's degree of saturation did not affect the scattering of the results predicted from PIV.

In all four soil groups A, B, C, and D, with the same test patch size of 128 pixels $\times$ 128 pixels used in the interpretation process, the image with scaling factors of 0.05 and 0.12 mm/pixel gave precision values of 0.002 and 0.01 mm, respectively.

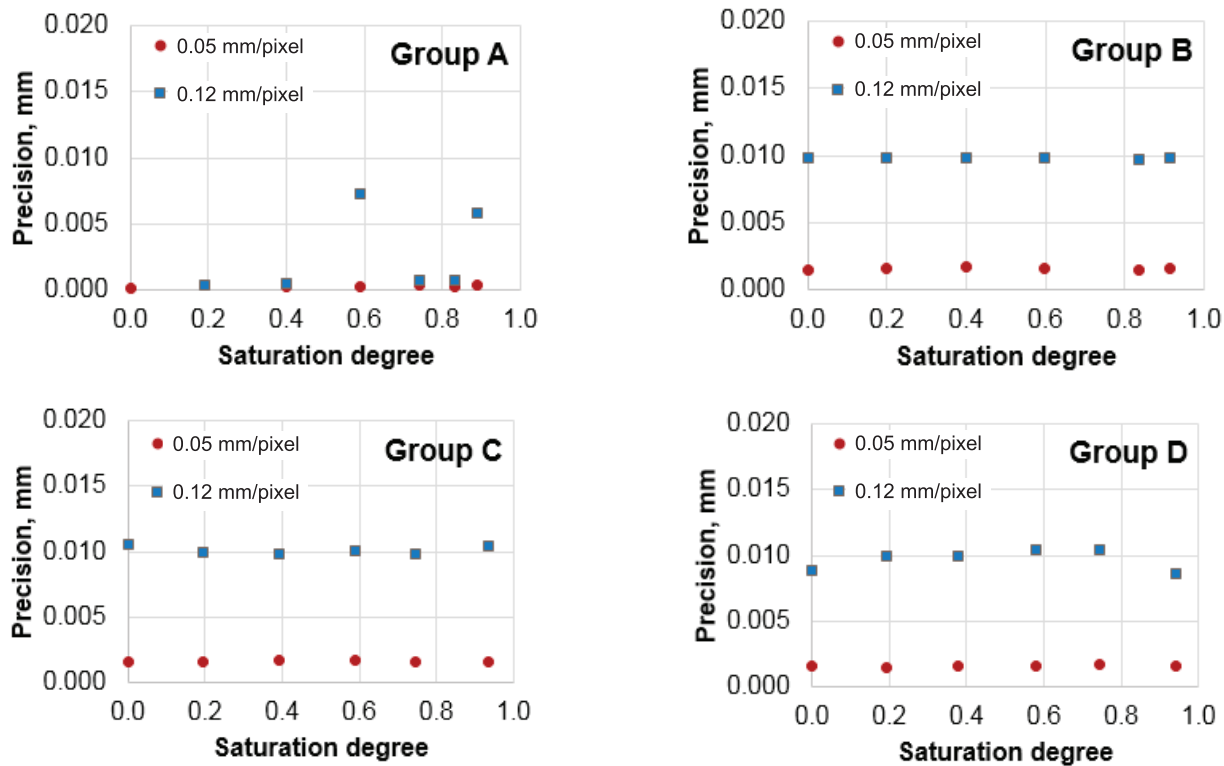


Fig. 10. Change of precision with saturation degree for the four sample groups.

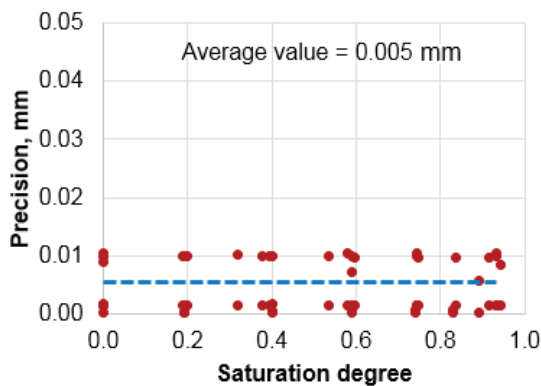


Fig. 11. Average precision in the measurement range of 0.1 to 5 mm.

The average precision values of PIV for measurement intervals from 0.1 to 5 mm were also determined as the average value of all separate precision values. As shown in Fig. 11, the average precision was 0.005 mm.

4. Conclusions

The results showed that in the measurement ranges of 0.1 to 5 mm with image scaling factors of 0.05 and

0.12 mm/pixel, the average values of accuracy and precision were 0.13 and 0.005 mm, respectively. This study also evidenced that PIV can be used to predict the displacement of sandy soil. The results also showed that while the degree of saturation of the soil did not influence the PIV results, and can therefore be ignored when analysing PIV results, the homogeneity of soil could reduce the precision of the PIV method. In other words, PIV works effectively for more heterogeneous soil when grain size distribution is concerned.

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COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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Application of livelihood vulnerability index in evaluating household's livelihood vulnerability to climate change in Can Gio district, Ho Chi Minh city, Vietnam

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Abstract:

Climate change is a global problem; however, its effects vary from region to region, country to country, district to district, and community to community. Climate change has profoundly impacted the livelihoods of households reliant on climatic conditions in Vietnam's coastal regions, both directly and indirectly. This research employs the livelihood vulnerability index (LVI) to gauge household vulnerability to climate change effects in one of Vietnam's most vulnerable regions - the Can Gio coastal district of Ho Chi Minh City. Through a survey encompassing 107 households across six communes and one town within Can Gio, LVI assessments were conducted at both district and commune levels. Findings indicate a moderate vulnerability level for Can Gio district (LVI=0.303), with Ly Nhon commune (LVI=0.334) emerging as the most vulnerable among the surveyed areas. Furthermore, livelihood strategies (0.516), socio-demographic profiles (0.391), and food security (0.385) significantly contribute to determining livelihood vulnerability across the seven surveyed communes. This study underscores the urgency of addressing climate change impacts on coastal livelihoods and highlights the nuanced vulnerabilities within specific communities, providing insights crucial for targeted adaptation and resilience-building efforts in vulnerable regions.

Keywords: Can Gio, climate change, livelihood vulnerability, LVI.

Classification number: 5.2

1. Introduction

Climate change is a global problem; however, its effects vary from region to region, country to country, district to district, and community to community [1]. The negative effects of climate change and climate variability has become an environmental and socio-economic problem that increasingly causes climate-related hazards to people around the world [2]. The poor and farming communities of developing countries are among the most influenced by climate change because they have a low adaptive capacity [3]. Therefore, the improvement of vulnerability assessment methodologies and the resulting adaption policies have become important.

In recent years, many studies using the LVI developed by M.B. Hahn, et al. (2009) [4] has been a popular approach to assess a household's vulnerability to climate change and variability. For example, G.M. Alam (2016) [5] used LVI to assess the vulnerability of riverbank erosion and its impact on the food security of rural households in Bangladesh. D.T. Adu, et al. (2018)

[2] applied LVI to assess the vulnerability of farming households to climate change in the Brong-Ahafo region of Ghana. Additionally, J. Panthi, et al. (2015) [3] assessed the livelihood vulnerability of 543 households living from mixed agro-livestock in Nepal using LVI. Therefore, LVI has been proven as powerful approach to assess household vulnerability to climate change in many regions around the world.

Ho Chi Minh city is regarded as one of the top 10 cities in the world to be severely affected by climate change due to its location in the intra-tropical delta zone and its low elevation [6]. The Can Gio district is a coastal area with mangrove forests, which is a biosphere reserve listed by UNESCO and is an essential part of Ho Chi Minh city. The main livelihoods of the local people are shrimp farming, aquaculture, near-shore fishing, and salt production for the coastal poverty, which attracts about 70% of the commune's workforce [7]. However, this area will become one of the most severely affected areas by climate change and sea level rise [6]. Additionally, the livelihood vulnerability of households to climate

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change is not well documented in this area. Thus, the identification of the livelihood vulnerability of the local people and adaptation strategies must be investigated to respond to these natural hazards.

The objective of this study is to assess the livelihood vulnerability of the local people to climate change in the Can Gio district of Ho Chi Minh city. Seven communes, namely, Ly Nhon, Long Hoa, Tam Thon Hiep, An Thoi Dong, Thanh An, Binh Khanh, and Can Thanh town, were selected for the present study.

2. Study area

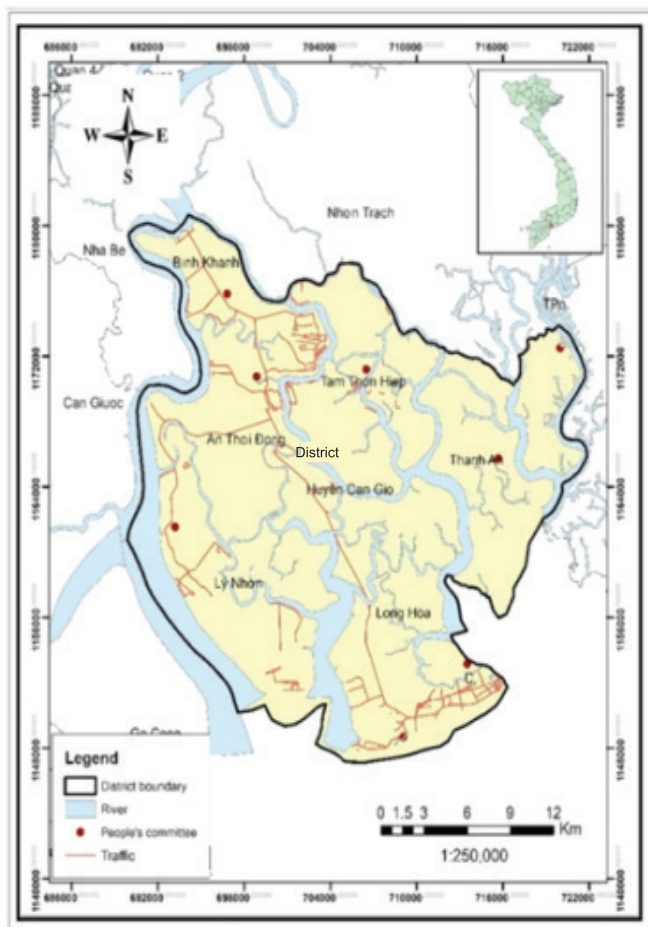


Fig. 1. Location of the study area.

Can Gio, located in the southeast of Ho Chi Minh city, is a suburban coastal district. The district is approximately 50 km away from the city centre. It has an area of 704 km² and it is located between latitudes 10°22'14" to 10°40'00" N and longitudes 106°16'12" to 107°00'50" E (Fig. 1). The Can Gio district has 20 km of coastline and a mangrove forest with a dense river and

canal system that creates a rich, biodiverse ecosystem with many special species. As a result, the local people mainly depend on aquaculture, near-shore fishing, salt production, forest management, and tourist services for their income. Besides, other livelihood activities include orchard and rice farming that are also important to the local people [7]. The weather conditions of the Can Gio district is typical for tropical monsoonal zones and has two distinct seasons: the rainy season and dry season. The annual average temperature is 25.8°C and monthly averages range between 25.5 and 29.0°C.

3. Methodology

3.1. LVI

The LVI developed by M.B. Hahn, et al. (2009) [4] includes seven major components, namely, the socio-demographic profile (SDP), livelihood strategies (LS), social networks (SN), health (H), food (F), water (W), and natural disaster and climate variability (ND). Each major component is made up of several sub-components or indicators. Table 1 presents the major components and sub-components used in this study. As the sub-components were assessed on a different scale, it is therefore necessary to standardize the sub-components as an index using the following equation:

$$\text{Index}S_c = \frac{S_c - S_{\min}}{S_{\max} - S_{\min}} \quad (1)$$

where S_c is the observed sub-component of an indicator for the household and $S_{\min}$ ($S_{\max}$) are the minimum (maximum) values.

After each sub-component is standardized, the major component is calculated by an average of the sub-components following the equation:

$$M_{jc} = \frac{\sum_{i=1}^n \text{index}S_c}{n} \quad (2)$$

where n is the number of sub-components in each major component and M_{jc} is the value of major component, j , for community, c .

The LVI for each commune can be calculated using the following equation:

$$LVI = \frac{\sum_{i=1}^n w_{Mi} M_{ic}}{\sum_{i=1}^n w_{Mi}} \quad (3)$$

where w_{Mi} is the weight of each sub-component that makes up each major component, and are included to ensure that all sub-components contribute equally to the overall LVI. The LVI was scaled to a range from 0 (least vulnerable) to 1 (most vulnerable) [8].

Table 1. Major components and sub-components comprising the LVI developed for the Can Gio district.

Major components	Sub-components	Unit	Explanation of sub-components in relationship with LVI
SDP	Dependency ratio (SDP1)	-	Higher value indicates less capacity to adapt
	Percentage of female-headed households (SDP2)	%	Women typically have less adaptive capacity. Higher value indicates less capacity to adapt
	Percentage of household heads who have not attended school (SDP3)	%	Education makes people more aware and able to adjust to changes in environmental condition. Higher value indicates less capacity to adapt
SN	Percentage of households having access to local governments for assistance in the past 12 months (SN1)	%	The assistance from local government strengthens adaptive capacity. Higher value indicates less vulnerable
	Percentage of households that can borrow money from friends, neighbors, or relatives (SN2)	%	Higher value indicates less vulnerable
	Percentage of households which can't access social networks (SN3)	%	Higher value indicates more vulnerable
LS	Percentage of households with family member working jobs outside their communes (LS1)	%	Income diversification increases adaptive capacity. Higher value reflects more capacity to adapt
	Percentage of households depending solely on one source of income (LS2)	%	Higher value reflects less capacity to adapt
	Livelihood diversification (LS3)	-	Higher value reflects more capacity to adapt
F	Percentage of households depending only on family farms for food (F1)	%	High sensitivity because limited source for food. Higher value indicates more vulnerable
	Crop Diversification Index (F2)	-	Higher value indicates less vulnerable
	Percentage of households struggling for food (F3)	months	Higher value indicates more vulnerable
W	Percentage of households using natural water sources (W1)	%	Higher value indicates more vulnerable
	Water source diversity index (W2)	-	Higher value indicates less vulnerable
	Percentage of households without a consistent water supply (W3)	%	Higher value indicates more vulnerable
	Average water storage volume (W4)	m ³	Higher value indicates less vulnerable
H	Average time to health facility (H1)	min	Higher value indicates more vulnerable
	Percentage of households with family member with chronic illness (H2)	%	Higher value indicates more vulnerable
	Medical expenses in the recent year (H3)	1000 VND/yr	Higher value indicates more vulnerable
ND	Percentage of households not having access to warning system of natural disasters in the past 6 years (ND1)	%	Higher value implies higher exposure
	Percentage of households having family member injured or death as a result of a severe natural disaster in the past 6 years (ND2)	%	Higher value implies higher exposure
	Percentage of households whose house is destroyed or heavily damaged by a natural disaster in the past 6 years (ND3)	%	Higher value implies higher exposure
	Average number of rainy days with extreme rainfall (rainfall amount >50 mm) (ND4)	days	Higher value implies higher exposure
	Mean standard deviation of monthly precipitation. (ND5)	-	Higher value implies higher exposure

Table 2. LVI's major components for six communes and one town in the Can Gio district.

	Binh Khanh	Can Thanh town	Ly Nhon	Thanh An	An Thoi Dong	Tam Thon Hiep	Long Hoa	Can Gio district
SDP	0.396	0.334	0.316	0.441	0.382	0.463	0.402	0.391
SN	0.286	0.256	0.156	0.330	0.222	0.284	0.237	0.254
LS	0.540	0.500	0.556	0.489	0.489	0.568	0.470	0.516
F	0.296	0.363	0.393	0.467	0.374	0.416	0.385	0.385
W	0.006	0.061	0.492	0.259	0.234	0.120	0.080	0.187
H	0.387	0.250	0.272	0.140	0.314	0.316	0.305	0.283
ND	0.168	0.208	0.188	0.198	0.208	0.202	0.228	0.208
LVI	0.286	0.269	0.334	0.318	0.307	0.319	0.288	0.303

3.2. Data collection and analysis

In this study, survey data and rainfall data were collected. The survey data was gathered from a household questionnaire survey developed by the authors. The structured questionnaire was designed based on the LVI's major components and sub-components mentioned in Table 1. The initial questionnaire was pre-tested by interviewing 10 random households in the study area and then the final version of questionnaire was developed to mirror the local realities of the household's livelihood vulnerability to climate change. Lastly, a household survey was conducted with 140 households who were randomly selected in six communes and one town of the Can Gio district in April 2019, where 20 households in each commune were surveyed. Household heads or other experienced members of the selected households were considered for the survey. However, there were 33 visited households who were not eligible or refused to be interviewed. The refusal rate was 24%. Finally, the survey results were analysed based on a total of 107 respondents from the surveyed areas. Rainfall data in the period of 1980-2017 at the Tam Thon Hiep and Can Gio stations were collected from Hydro-Meteorological Data Centre of Vietnam.

Data were inputted, checked, and analysed using Microsoft Excel (Version 2016). Households with missing data were rejected based on a simple deletion approach [9].

4. Results and discussion

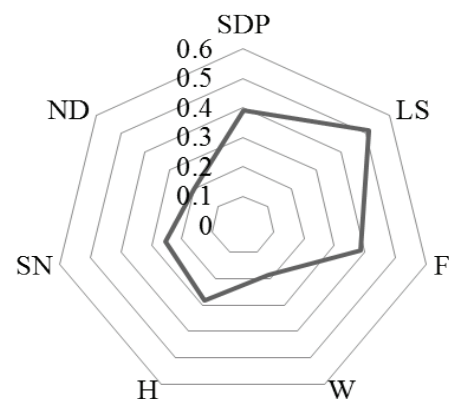
Table 2 shows the results of seven of the LVI's major components and the LVI of the six communes and one town in the Can Gio district. The results indicate that the

vulnerability of the Can Gio district is moderate based on the LVI vulnerability scale of 0 to 0.5. In terms of the commune scale, the LVI values indicated that the most vulnerable commune is Ly Nhon (LVI=0.334), followed by Tam Thon Hiep (LVI=0.319), Thanh An (LVI=0.318), An Thoi Dong (LVI=0.307), Long Hoa (LVI=0.288), Binh Khanh (LVI=0.286), and Can Thanh town (LVI=0.269).

4.1. Livelihood's vulnerability in Can Gio district

Figure 2 illustrates a spider diagram for the seven major components of the LVI for the Can Gio district. There are three major components that increase the livelihood vulnerability of the district, namely, livelihood strategies (0.516), socio-demographic profile (0.391), and food (0.385). In addition, the major component of water (0.187) has the lowest vulnerability.

There were approximately 73.2% households of farmers in the Can Gio district that depend solely on their

**Fig. 2.** Vulnerability spider diagram of LVI major components for Can Gio district.

farm and have a low diversification of income sources. Most of these families are supported by one main job, which is their farm of either fish, shrimp, mollusks, fruit trees, or salt. Therefore, they do not have any other source of income to replace losses due to climate change. The socio-demographic profile is also at an average vulnerability level when the head of household has not completed high school or attended any professional or technical institutions. According to R. Muttarak et al. (2014) [10], education plays an important role in the reduction of adverse effects of extreme climate events in direct and indirect ways. Formal education is considered as an essential method where a person can apprehend knowledge, skills, and abilities that can positively affect their adaptive capacity. Thus, when the head of household has a good education, their adaptive capacity improves, which mitigates their vulnerability in the Can Gio district. Lastly, food is an issue in this district because some households cannot guarantee a food source through a time period where a loss of income occurs due to extreme weather events, for example, due to lack of crop diversification and limitations in government policies for supporting food sources through the extreme weather conditions.

Livelihood vulnerability of 6 communes and 1 town

Figure 3 illustrates in detail the vulnerability of livelihoods in the 6 communes and 1 town. Through this figure, it is easy to recognize three contributing factors to LVI, namely, LS, SDP and F, which need special attention for all the surveyed areas.

Firstly, on livelihood strategies, this contributing factor shows the most vulnerability for all the communes and town surveyed, in which Tam Thon Hiep, Ly Nhon, and Binh Khanh communes are three most vulnerable with a levels of 0.568, 0.556, and 0.540, respectively (Table 2). Particularly, the aspect of livelihood strategies seems to be important in the Can Gio district. Most farmers in the area depend solely on one source of livelihood which makes all farmers vulnerable when climate directly affects their source of income. For example, ocean salinity, temperature changes, and the death of shrimp or fish heavily influence their source of income. Therefore, it is better to have a diverse source of income to ensure the stability of their earnings.

Secondly, on the socio-demographic profile, the figure also reveals that the two most vulnerable communes to this contributing factor are Tam Thon Hiep and Thanh An (0.463 and 0.441, respectively), however, other four communes and town still need attention because their

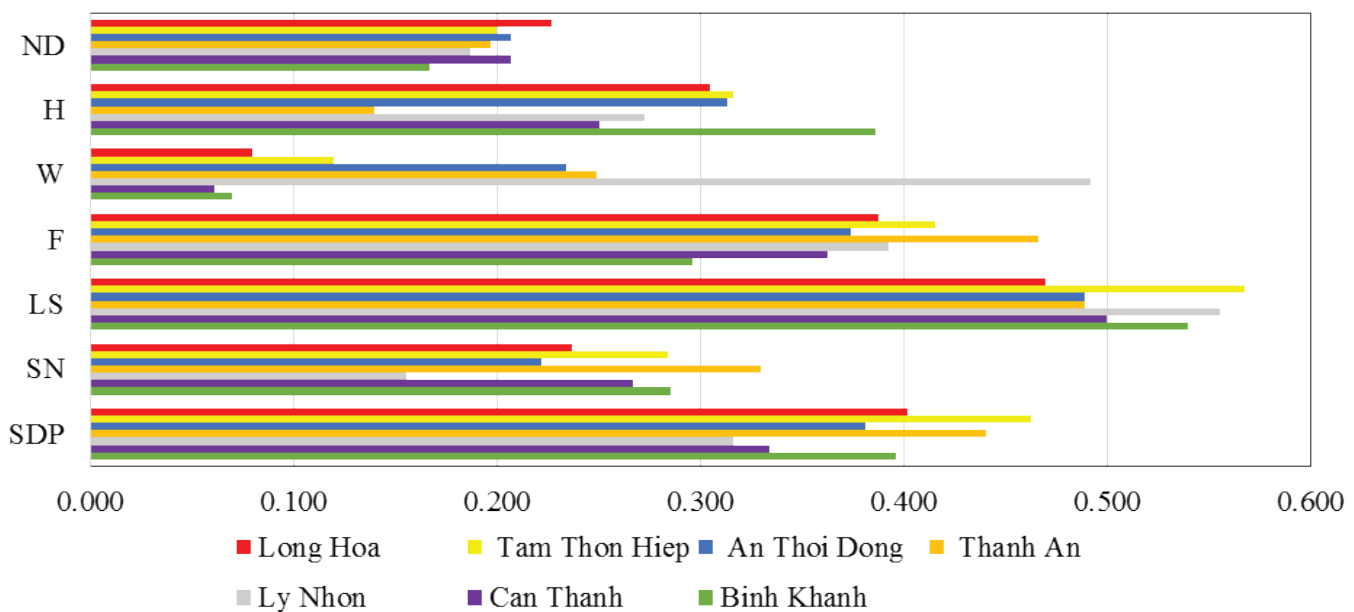


Fig. 3. Contributing factors of LVI for 6 communes and 1 town in Can Gio district.

vulnerability is relatively high. After the interviews, this study showed the key factor influencing socio-demographic profile is that most heads of household didn't finish high school or did not attend any professional institutions. Instead, the participants in the study believed their jobs require experience from the elders of the community that is passed down through generations, and that they need to spend time on their farm all day.

Thirdly, on the contributing factor of food, Fig. 3 shows that all surveyed regions need more attention in this area because their vulnerability reaches the average level, in which Thanh An is considered as the most vulnerable commune. Many households in Thanh An claimed that although they have diverse sources of income to fulfil their living requirements in recent years, their sources of income could not guarantee stability of their food sources. Thanh An is popular for oyster farming. However, oyster death due to high salinization or being a size too small for sale, will put farmers at risk of income loss.

Furthermore, the contributing factor of water seems to be extremely critical in Ly Nhon. In this commune, most households live far from the main street, where the water exists, which leads to a lack of water that severely affects the lives of the local people. Therefore, Ly Nhon is a commune that needs more water support than the others. Additionally, Thanh An and parts of An Thoi Dong commune share the same problems.

Finally, on health and natural disasters, this contributing component shows a low vulnerable level because the local people of all communes and town are well experienced in responding to natural disasters or extreme weather events. Besides, there are many hospitals nearby and good health insurance services. Although the precipitation extremes in terms of intensity and frequency are increasing trends [11], according to interviewed households, there were not many people suffering an injury, death, or severe damage as a result because they had adequate preparation plans by themselves or with help from the local government. Good preparation can help the local people react better to natural disasters.

In general, it is necessary to have appropriate policies

for these study areas. For Ly Nhon, the order of priority for support policies is as follows: livelihood strategies > water > food > socio-demographic profile > health > natural disaster > social networks. For Tam Thon Hiep, the priority is proposed as follows: livelihood strategies > socio-demographic profile > food > health > social networks > natural disasters > water. Likewise, for Thanh An: livelihood strategies > food > socio-demographic profile > social networks > water > natural disasters > health.

5. Conclusions

In conclusion, this study investigated the livelihood vulnerability of households to climate change in Can Gio district, Ho Chi Minh city by using the LVI. The main results can be summarized as follows: (1) the LVI estimates the vulnerability of the Can Gio district to be at a moderate level (0.303); (2) of the 6 communes and 1 town surveyed, Ly Nhon is the most vulnerable commune because of a lack of stable water sources, poor livelihood strategies, and problems with maintaining their food source through extreme weather events; (3) the factor of livelihood strategies is the most vulnerable component over all of the district. This study recommends replicating this research to investigate the change of climate effects in the area over time.

The limitation of the LVI approach is the challenge in the selection of appropriate sub-components used in vulnerability indices [12], and the local environment plays a significant role in forming and designing the indicators [3]. Thus, designing the sub-components of the vulnerability indices requires an extensive literature review, expert consultation, and stakeholder consultation [12]. The LVI approach indicates different sectors and aspects of vulnerability, so this approach can be used with other communities and also to assess the performance of programmatic interventions with goals to reduce vulnerability and highlight potential sectors requiring intervention.

CRedit author statement

Nguyen Phuong Tan Le: Data analysis and interpretation, Writing and manuscript revising; Nguyen Khoi Dao: Method, Data analysis and Writing.

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COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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Removal of heavy metals derived from COD analysis wastewater by electrolysis using graphite electrode

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Abstract:

In this study, electrolysis using graphite electrodes was applied to treat wastewater generated from a COD analysis procedure (referred to as COD wastewater). COD wastewater containing high concentrations of H_2SO_4 , Hg^{2+} , and $\text{Cr}_2\text{O}_7^{2-}$ salts were collected and then treated by electrolysis with graphite electrodes in a lab-scale experiment. The results showed that the electrolysis process was not affected by the electrode's distance or area. The most efficient treatment for all three metals was achieved at a current value of 31.58 mA, which corresponds to a current density of 1.974 mA/cm^2 under a voltage of 3 V, 8-h electrolysis time, wastewater $\text{pH} < 1$, electrode distance of 4 cm, and electrode area of 16 cm^2 . Under these conditions, the concentrations of heavy metals after treatment were 1170.17 mg/l for Hg, 871.20 mg/l for Ag, and 56.3 mg/l for Cr. The treatment efficiencies were 48.15, 66.94, and 50.76%, for Hg, Ag, and Cr, respectively. While this technology is simple, low cost, and achieves a relatively high efficiency, after treatment the COD wastewater still carried a high concentration of heavy metals that exceeded the permissible standards. Therefore, it is necessary to have a further treatment method in place to completely eliminate the heavy metals remaining in wastewater, as well as to recycle and reuse acidic components from wastewater and to treat them up to environmental standards before discharge. In conclusion, electrolysis with graphite electrodes can be applied in practice to treat other sources of wastewater contaminated by heavy metals with low emissions.

Keywords: COD analysis, COD wastewater, electrolytic, graphite electrode, heavy metal.

Classification number: 5.3

1. Introduction

BOD (biological oxygen demand) and COD (chemical oxygen demand) are two basic parameters that determine the concentration of organic matters that cause pollution of water sources. BOD is the amount of oxygen required by microorganisms to oxidize biodegradable organic substances, while COD is the amount of oxygen required to oxidize all organic compounds, those that are both difficult and easily biodegradable in water. The main advantage of COD analysis is to provide fast results and a simpler, more accurate process when compared to that of BOD. Therefore, COD is often used to assess the organic pollution of water sources and sometimes it can be used as a substitute for BOD. In addition, the COD indicator is also widely used in water quality testing of all wastewater treatment facilities due to its simplicity and quick analysis time.

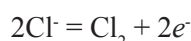
The COD was determined based on the Standard method 5220B-4b [1]. This procedure involves the use of chemical agents such as sulfuric acid (H_2SO_4), dichromate ($\text{Cr}_2\text{O}_7^{2-}$), silver (Ag^+), and mercury (Hg^{2+}). H_2SO_4 is used as a catalyst for the oxidation of organic compounds in wastewater along with the powerful oxidant dichromate ($\text{Cr}_2\text{O}_7^{2-}$). Silver and mercury are used to eliminate agents that interfere with the oxidation reaction. The treated samples continue to heat in a closed reflux at high temperature (150°C) for 2 h. Finally, the obtained samples are used to determine the remaining $\text{Cr}_2\text{O}_7^{2-}$ by titration with Fe^{2+} (FAS solution) or by the colorimetric method. The $\text{Cr}_2\text{O}_7^{2-}$ agent in the COD analysis method oxidizes most organic compounds at high temperature in concentrated acid conditions. Some organic matter, especially straight-chain fatty acids, do not oxidize without the Ag^+ ion catalyst. If the chloride concentration is greater than 2000 mg/l, a major obstacle

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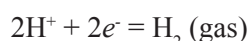
in wastewater treatment, the addition of excess Hg^{2+} ions can overcome the high chloride concentration by forming a chloride complex. Although the amount of wastewater generated from COD analysis is not large (about 50-1000 ml/d), due to the use of toxic chemicals COD wastewater often contains high concentrations of H_2SO_4 , Hg^{2+} , and $\text{Cr}_2\text{O}_7^{2-}$ salts. Thus, if COD wastewater is not treated before discharge, it can cause difficulties in storage as well as serious environmental pollution.

COD wastewater from laboratories has been of interest to domestic and foreign researchers for wastewater treatment via various technologies such as physical-chemical treatments, chemical precipitation, ion exchange, and absorption by chitosan; all of which have been shown to be highly effective in removing heavy metals for some time [2-15]. The advantages of these methods include low cost and ease of operation, however, one significant disadvantage is the creation of secondary waste (i.e. sludge). Thus, it is necessary to employ further treatment before discharging the treated water back into the environment [3, 8, 10, 16]. In recent years, several studies on COD wastewater treatment by more modern and less polluting methods have been conducted using electrochemical processes and membrane filters. Electrolysis employed for the treatment of wastewater containing high concentrations of heavy metal is not a new technology. In fact, it has been utilized since its first application in England in 1889 [3]. The combined use of flocculation and oxidizing electrolysis are used to treat wastewater containing heavy metals and to decompose organic compounds due to its low cost. Further, this process does not create secondary pollution because of the complete oxidation of pollutants in the final product. Therefore, it is especially suitable for the treatment of wastewater with high concentrations of heavy metals up to 1000 mg/l [17]. At the electrodes, cations will be reduced and anions will be oxidized causing separate oxidation and reduction reactions to occur.

For example, at the anode there are the anions (OH^- , Cl^-) and the following electrode-metal (Me_1) oxidation reactions occur:



At the cathode, the following reduction reactions occur:



Due to the existence of high concentrations of Hg, Ag, and Cr ions in COD wastewater, sulfuric acid was selected as the suitable electrolyte agent for electrolysis. Table 1 shows the studies where electrolysis was used to treat COD wastewater and their effectiveness in removing heavy metals.

Table 1. Results on COD wastewater treatment of researchers.

Technology	Results	References
Electrochemical; using iron electrodes	Ag, Cr, Fe met standards discharge, but Hg levels were still high	Pinisakul and Kritayakornpong (2008) [14]
Electrochemical; using platinum anode and copper cathode	47.19% Ag recovery	Djaenudin and Syafila (2009) [15]
Electrolytic with titanium electrode	88% Hg, 89% Ag, and 81% Cr removal	Diem (2016) [16]

The purpose of this study was to use electrolysis with inert graphite electrodes to remove heavy metal ions and to recover valuable components in COD wastewater with high efficiency that is suitable for the application of additional treatment technologies to reduce treatment cost and achieve discharge at environmental standards.

2. Materials and methods

2.1. Materials

COD wastewater was taken at the environmental laboratory of Ho Chi Minh city, University of Food Industry, with the pollution components shown in Table 2.

Table 2. The components of heavy metals in COD wastewater.

Parameter	Concentration (mg/l)	QCVN 07:2009/BTNMT*
Ag	2635.2	5
Hg	2256.7	0.2
Cr^{6+}	114.4	5
pH	<1	≤ 2 or ≥ 12.5

The data in Table 2 shows that COD wastewater has a high acidity with $\text{pH} < 1$ and a very high concentration of heavy metals, which exceeds the permitted threshold according to QCVN 07:2009/BTNMT by several times.

2.2. The experimental model

The lab-scale experiment model is described as in Fig. 1. The electrolysis for COD wastewater treatment used a graphite electrode consisting of three components: (1) a DC power supply; (2) a graphite electrode with 16

*National Technical Regulation on Hazardous Waste Thresholds.

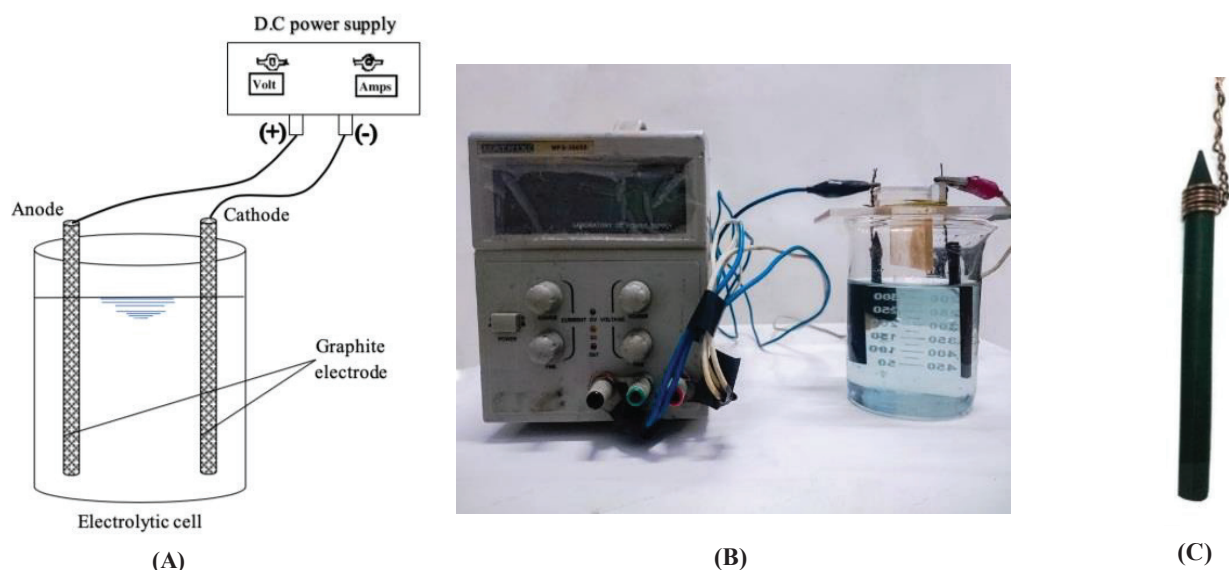


Fig. 1. Electrolytic model of COD wastewater treatment using graphite electrode. (A) drawing, (B) actual model, (C) graphite electrode.

cm² area, diameter (D) and height (h) of 0.7 and 7.5 cm, respectively; and (3) an electrolytic cell (500-ml beaker).

2.3. The experiments of COD wastewater treatment using graphite electrodes

Electrolysis for COD wastewater treatment using graphite electrodes was carried out as follows. First, experiments were conducted to evaluate the efficiency of heavy metal removal under various voltages, then the most suitable voltage for electrolysis with the highest removal and the lowest power consumption was chosen. Then, the effect of electrode distance, electrolysis time, and electrode area on the removal of heavy metals of COD wastewater was investigated.

All experiments were conducted in batch-mode in 500-ml beakers. The samples, after treatment, were

evaluated to be effective by determining pH and the concentration of heavy metal ion residue in wastewater. The pH was measured with a PHS 550 pH meter and the concentrations of Ag, Hg, and Cr were analysed by titration methods [18-20].

3. Results and discussion

3.1. Effect of voltage on the efficiency of COD wastewater treatment

The experiment was carried out under a fixed distance between the two electrodes ($d=4$ cm), an electrode area of 16 cm², and applied voltage in the range between 1-5 V. A ammeter was used to determine the current density running through the system. The results are shown in Figs. 2 and 3.

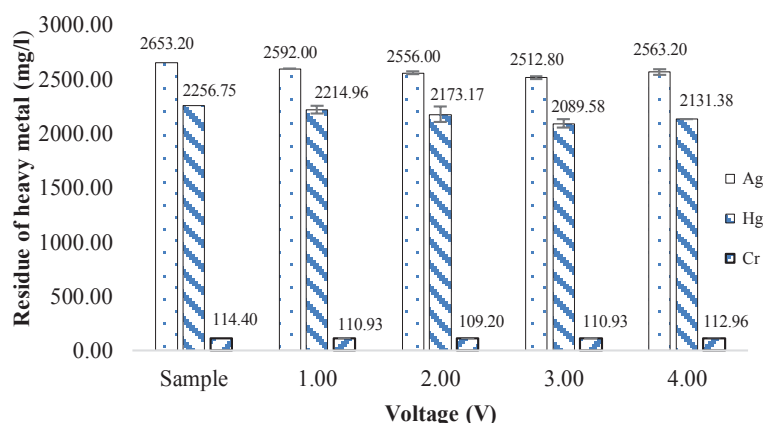
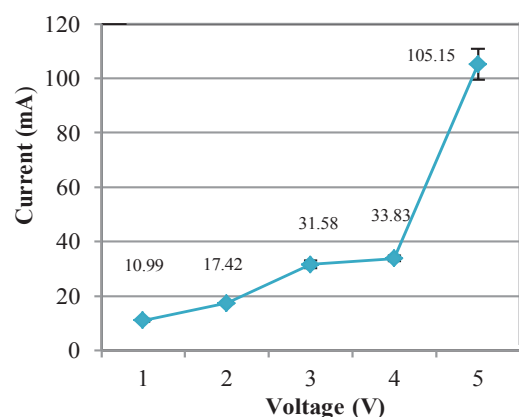


Fig. 2. The correlation between voltage and current. Fig. 3. Effect of voltage on removal of heavy metals.

Figure 2 shows a higher applied voltage induces a larger current. When the voltage was increased from 1 to 3 V, the electrolytic process increased steadily and reached 10.99-31.58 mA. The residual heavy metal concentration is presented in Fig. 3 together with the data from an ANOVA variance analysis, which indicated that the efficiency of heavy metal removal with 1-3 V applied voltage had a significant mean difference at the 0.05 level ($\alpha_{Ag}=0.002$, $\alpha_{Hg}=0.041$, $\alpha_{Cr}=0.039$). These results can be explained with the understanding that the higher the voltage, the greater the current density through the system which caused reduction to occur at the cathode. The amount of metals that attached to the cathode was higher and the colour of solution was lighter than that of the original. However, when the voltage was increased further from 4 to 5 V (the current was 33.83 and 105.15 mA, respectively), electrolysis was strongly affected. However, the results in Fig. 3 show that the heavy metal removal efficiency did not differ significantly from that at 3 V. On the actual image of the setup (Figs. 4 and 5), one

can see the unstable and loose electrolyte product attached to the electrodes that easily fell back into the COD wastewater. This excess electrolyte product forms from the intense electrolysis caused by the high voltage, which then released a large amount of H_2 gas at the cathode. The metals, in porous form, begin to attach unevenly to the electrode causing them to fall off and melt back into the solution thereby reducing the efficiency of the treatment. The evaluation of the efficiency of heavy metal removal as a function of applied voltage (Fig. 3) together with the results from the voltage and current correlation (Fig. 2) show that at a voltage of 3 V, electrolysis for all three metals was the most efficient and thus was used for further electrolysis experiments in this study.

3.2. Effect of the electrode distance on efficiency of COD wastewater treatment

The electrolysis of COD wastewater was carried out for 1 h, at 3 V, in wastewater with $pH < 1$, and an electrode area of 16 cm^2 while the electrode distance was varied to 2, 4, and 6 cm. An ammeter was used to determine the current density running through the system. The changes in current and the corresponding efficiency of heavy metal removal at different electrode distances are described in Fig. 6.

It can be seen from Figs. 6 A, B that when the electrode distance was changed from 2 to 6 cm, the current decreased from 32.85 to 29.16 mA and there were no statistically significant differences seen in the removal efficiency of heavy metals. When comparing the mean values of heavy metal removal at these different electrode distances, there was no the significant mean difference at the 0.05 level in treatment efficiency of Ag, Hg and Cr ($\alpha > 0.05$). These results prove that the electrode distance



Fig. 4. Electrolyte product falling off the electrode.



Fig. 5. Heavy metals attached to electrode.

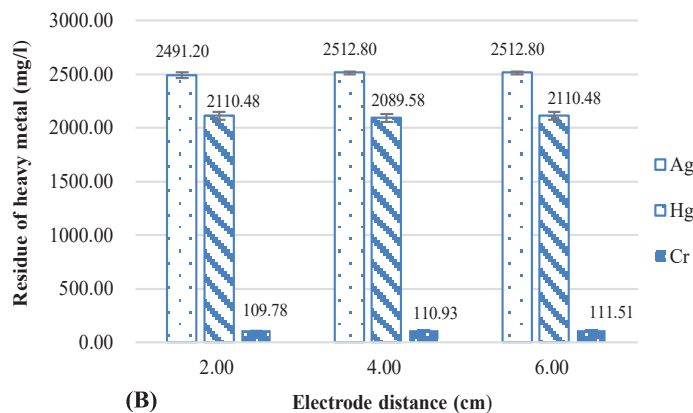
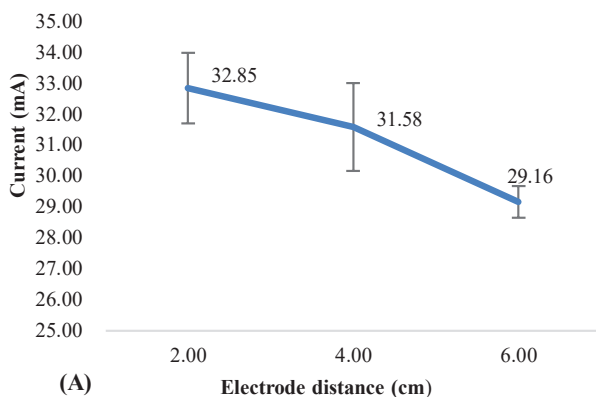


Fig. 6. Effect of the distance between electrodes on (A) the current, (B) the effect of heavy metal removal in COD wastewater treatment.

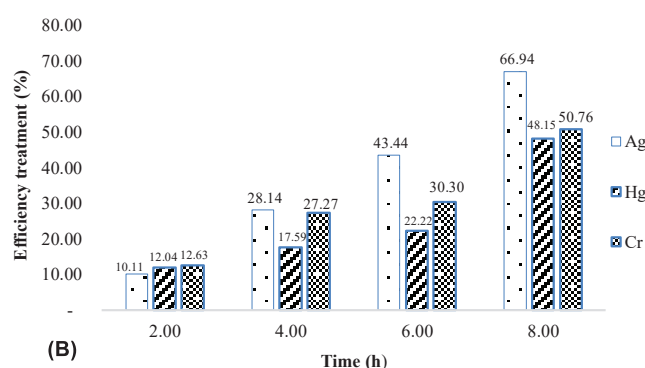
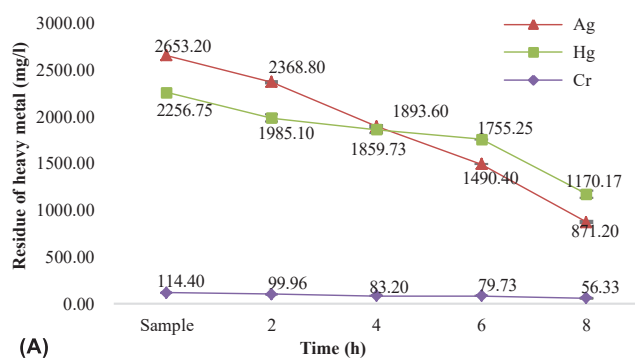


Fig. 7. Effect of electrolysis time on (A) heavy metal concentration and (B) efficiency of removal of heavy metals from COD wastewater.

does not affect the removal of heavy metals in this study. This is because the metals attaching to the electrode cause the ion layer around the cathode to decrease, leading to electrochemical polarization - that is, the discharge rate between the anode and the cathode decreases, which reduces the current [21]. Clearly, the efficiency of the removal of heavy metals was not affected by the change in distance between the two electrodes. Therefore, in this study, it was possible to ignore the effect of the electrode distance and choose an electrode distance fixed at 4 cm for all subsequent experiments.

3.3. Effect of electrolysis time on the efficiency of COD wastewater treatment

Electrolysis time is the basis for determining the efficiency of metal ion reduction and the effectiveness of metal recovery [3, 21]. The following experiment was carried out by fixing the electrode distance at 4 cm, the voltage at 3 V, the wastewater at pH<1, and the electrode area at 16 cm² while varying the electrolysis time from 2-8 h. Fig. 7 illustrates the effect of electrolysis time on the removal of heavy metals.

As shown in Fig. 7, when the electrolysis time was varied from 2 to 8 h, there was a significant mean difference at the 0.05 level ($\alpha=0.027$) in the efficiency of heavy metal removal. The general trend was as electrolysis time was increased, the removal efficiency was higher. The highest heavy metal removal efficiency was achieved at an electrolysis time of 8 h, current density of 1.974 mA/cm² where the removal efficiencies were 66.94, 48.15, and 50.76% for Ag, Hg, and Cr, respectively.

3.4. Effect of electrode area on the efficiency of COD wastewater treatment

The influence of electrode area was investigated by increasing the electrode area to 32 cm² (twice the original electrode area of 16 cm²) and fixing the parameters of distance, voltage, electrolysis time, and pH, to 4 cm, 3 V, 8 h, and 1, respectively. From the results shown in Figs. 8 and 9, it can be seen that the performance trends of the 32-cm² electrode area and that of the 16-cm² area are quite similar. Specifically, as electrolysis time increased, the removal efficiency also rose higher. The most efficient removal was achieved at an 8-h electrolysis time and a current density of 1.66 mA/cm², which yielded removal

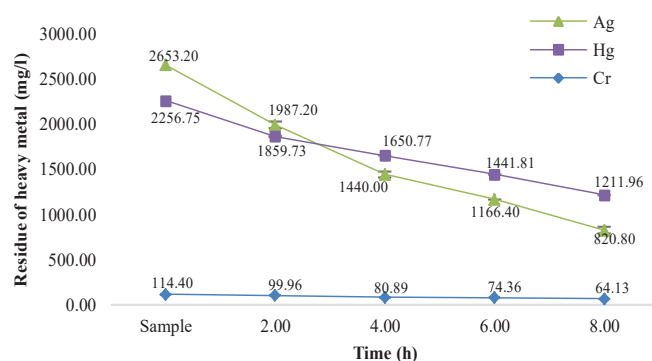


Fig. 8. The change of heavy metal concentration over electrolysis time.

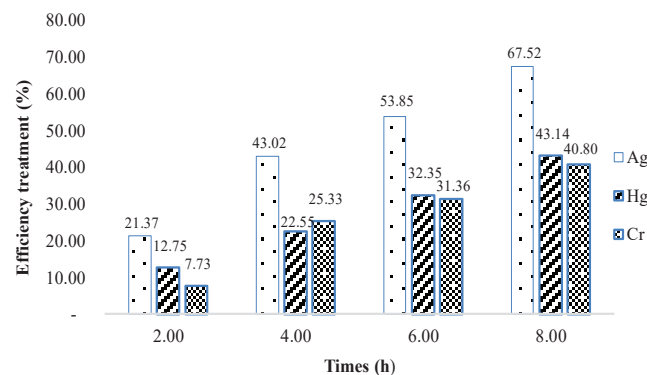


Fig. 9. The removal efficiency of heavy metals over electrolysis time.

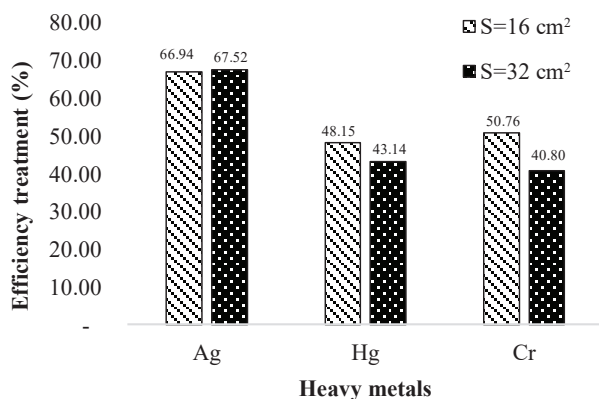


Fig. 10. Comparison of the treatment efficiency of heavy metals between the two electrode areas, 16 and 32 cm², for t=8 h.

efficiencies of 67.52, 43.14, and 40.80% for Ag, Hg, and Cr, respectively. The solution colour was observed to fade over time. However, when comparing the heavy metal removal efficiency between the two electrode areas (Fig. 10), these differences were not significant. In other words, increasing the electrode area by a factor of 2 did not increase the efficiency of heavy metal removal and implies that this is not a main factor affecting COD wastewater treatment efficiency in this study (Fig. 11). This result is similar to the research done on a graphite anode, which also achieved a removal efficiency of about 50-70% and current density of 0.03-0.32 A/m² [22].

These results were also compared with other studies using electrolytic methods as presented in Table 3. Table 3 shows electrolysis treatment methods of COD wastewater using graphite electrodes with similar heavy metals removal efficiency to this study [17]. In addition, graphite is an inexpensive and abundant material and, with efficiency treatment more than 50%, they can be considered as a feasible method in wastewater treatment of heavy metals. Although the concentration of heavy metals after treatment was still higher than the Vietnamese standard (QCVN 07:2009/BTNMT), the heavy metals were significantly reduced from the original wastewater and generate favourable conditions for further treatment, which can still reduce cost as well as increase safety during operation. Moreover, one major disadvantage of chemical precipitation and other physical methods of wastewater treatment is oftentimes secondary waste (sludge) is produced, thus creating the need for continued treatment before discharge back into environment. Because secondary waste increases the cost of treatment, this method poses many advantages in addition to feasibility when applied in practice.



Fig. 11. Wastewater and electrode after treatment process.

Table 3. Comparison of COD wastewater treatment by electrolysis process.

Operating conditions	Electrode type	Removal of heavy metals	References
pH 3.0-3.5, 10 V, electrolysis time 8 h	Fe	Ag, Cr, Fe meet discharge standards but Hg did not	Pinisakul and Kritayakornupong (2008) [14]
6 V, distance 5 cm	Pt (anode, 1x1 cm ²), Cu (cathode, 1x3 cm ²)	47.19% (for Ag)	Djaenudin and Syafila (2009) [15]
30 V, distance: 5 cm, electrolysis time 8 h, electrode area 50 cm ²	Ti	81-89%	Diem (2016) [16]
3 V, distance 4 cm, electrolysis time 8 h, electrode area 50 cm ²	Graphite	66.9% (for Ag), 48.15% (for Hg), 50.7% (for Cr).	This study

4. Conclusions

The electrolysis process using graphite electrodes was successfully employed for COD wastewater treatment with the benefits of low cost, easy operation, and lack of secondary waste. The results showed that the most efficient removal of heavy metals was achieved at a voltage of 3 V, equivalent to a current of 31.58 mA (current density of 1.974 mA/cm²), pH 1 of wastewater, electrolysis time of 8 h, and electrode area of 16 cm². At these optimum conditions, the residual concentration of heavy metals after treatment reached 1170.17 mg/l for Hg, 871.20 mg/l for Ag, and 56.33 mg/l for Cr, which corresponds to treatment efficiencies of 48.15, 66.94, and 50.76% for Hg, Ag, and Cr, respectively. Although the effluent of the treatment process still had a high

concentration of heavy metals and did not reach the Vietnamese standard (QCVN 07:2009/BTNMT), these results make important contributions to the pre-treatment of wastewater contaminated with heavy metals by using a simple technology with low cost and ability to reduce the concentration of pollutants by about 50%, which facilitates further treatment processes and significantly contributes in reducing the cost of an entire treatment process. In addition, the original COD wastewater had high acidity, which is suitable for treatment by electrolysis, and the pH after wastewater treatment remained low so it can be recovered and reused for different destinations.

CRedit author statement

Thi Thanh Diem Ngo: Data analysis and interpretation, Writing and manuscript revising; Minh Tien Tran: Method, Data analysis and Writing.

COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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Developing a database for Cs-137 radioactivity concentration and assessing residual levels in surface soils of Quang Ninh, Hai Phong, and Lang Son provinces

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Abstract:

Cs-137 is an artificial fission product with a relatively long half-life (30 years). As a result, Cs-137 can be used as an indicator of the environment impact of release from a nuclear power plant during normal operation or emergencies. Cs-137, an artificial fission byproduct boasting a relatively extended half-life of 30 years, serves as a valuable indicator of environmental impact stemming from nuclear power plant emissions during normal operations or emergencies. This study analyzed the radioactivity concentration of Cs-137 across 826 soil samples sourced from Hai Phong, Quang Ninh, and Lang Son. Residual Cs-137 levels were meticulously evaluated and juxtaposed against prior publications to discern trends in surface soil Cs-137 concentrations. Findings revealed that the actual average residual Cs-137 level in the soil samples collected for this study stood at 236 ± 146 Bq/m², notably lower than the calculated value of 407 ± 77 Bq/m² derived from the published experimental formula. Consequently, the study confirms the negligible impact of Cs-137 deposition from the Fukushima nuclear accident (2011) within the surveyed area. This research not only contributes to the ongoing monitoring of environmental radiation levels but also underscores the need for precise methodologies in assessing radioactive contaminants for accurate risk evaluation and mitigation strategies.

Keywords: Hai Phong, Lang Son, Quang Ninh, radioactivity of Cs-137, residual level of Cs-137.

Classification number: 5.3

1. Introduction

Cs-137 is an artificial fission product with a relatively long half-life (30 years). As a result, Cs-137 can be used as an indicator of the environment impact of release from a nuclear power plant during normal operation or emergencies. Although the residual level of Cs-137 in soil in Vietnam was studied many years ago, recent developments of nuclear power plants near the North border of Vietnam, especially the operation of Fangchengang Nuclear Power Plant (NPP) at about 54 km from the border, has brought out the need to establish a database of radioactivity concentration (Bq/kg) and a reassessment of residual levels (Bq/m²) of Cs-137 near border areas. In this study, the radioactivity concentration and the residual level of Cs-137 in the soils of the Hai Phong, Quang Ninh, and Lang Son provinces are determined to fulfil this purpose.

2. Materials and methods

A total of 826 soil samples were collected over a period of 3 years from 2017-2019 following standard procedures by VARANS TSC.

To mitigate time constraints, sample processing and analyses were performed simultaneously by three laboratories: VARANS TSC (526 samples; with 100 samples from Quang Ninh and 426 samples from Lang Son), the Institute for Nuclear Science and Techniques (220 samples; including 80 samples from Hai Phong and 140 samples from Quang Ninh), and the Center for Environmental Technology & Treatment - Ministry of Defense (80 soil samples from Quang Ninh).

2.1. Soil sampling and processing method

Soil samples were taken from a flat-surfaced area based on a 4.5 km x 4.5 km grid in all accessible areas to ensure relatively uniform sample density over all three provinces. All the soils, dug from a 30 cm x 30 cm x 30 cm cubic hole, were mixed thoroughly. A depth of 30 cm was chosen to take into account the disturbance processes of soils. Then, the soils were reduced to about 1-1.5 kg by quartering and compartmentalization. Relevant information from the soil samples were also recorded.

The sample processing procedures of all involved organizations were standardized and included the

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following steps: crushing the sample to reduce soil particle size to 1 cm or less; removing foreign materials such as plant roots, pebble, and garbage; spreading the soil sample on tray then heating to about 100-105°C until the samples were completely dried out (weight variation less than 1%); grinding and sieving the dry soil to obtain particle sizes less than 1 mm; and finally weighing and compressing the samples into a confined box for measurements.

2.2. Cs-137 analysis methods and quality assurance measures

Technical specifications of each low-background gamma spectrometer used by each laboratory for sample measurement and analysis are shown in Table 1.

The energy and efficiency calibration of the HPGe detectors were developed using a multipurpose RGU-1 reference source and RGU_IAEA.Lib library provided by IAEA.

The geometry calibration file and correction curve for self-absorption due to sample density and composition were also generated using RGU-1 standard sources. The chemical composition of RGU-1 is similar to that of surface soil samples [1], which indicates that the contribution ratio of chemical elements in the two samples are similar. In addition, the self-absorption of the samples with different chemical compositions mainly influences the energy region of less than 186 keV. For

the higher energy region, self-absorption correction is not necessary and thus was neglected [2]. Therefore, generation of the self-absorption correction curve using RGU-1 is a suitable method.

For VARANS TSC and INST, the quality assurance (QA) for sample analyses were performed by analysing the IAEA reference sample (Sample No.4, provided in the 2018 inter-laboratory comparison conducted by IAEA) with known activity of nuclides including K-40, Cs-137, Tl-208, Pb-210, Pb-212, Pb-214, Bi-214, Ra-226, and Ac-228, which followed IAEA's assessment method in which the trueness, precision of the analysis results is assessed separately [3]. For CTET, the QA was performed by inter-comparison of the analysis results from three laboratories for a set of three samples. The QA results show that all analysis results were evaluated as "acceptable" (the highest grade among three levels of evaluation: A "Acceptable", N "Not acceptable", and W "Warning").

3. Results

3.1. Development of database on Cs-137 radioactivity concentration in surface soil

Figure 1 shows the spatial distribution of Cs-137 radioactivity concentration in soil layers up to 30 cm in depth on the GIS maps of the Hai Phong, Quang Ninh, and Lang Son provinces.

Table 1. Technical specifications of low-background gamma spectrometers used by involved laboratories.

N ^o	Parameter	VARANS TSC	Institute for Nuclear Science and Techniques (INST)	Center for Technology Environmental Treatment (CTET)
1	Lead chamber	ORTEC, Cylindrical, inner diameter: 28.0 cm; outer diameter: 50.4 cm	CANBERRA, Cylindrical, inner diameter: 27.9 cm; outer diameter: 50.8 cm	Cubical lead chamber with outer dimensions: 100 cm x 80 cm and inner dimensions: 50 cm x 50 cm
2	Detector manufacturer	01 HPGe detector made by CANBERRA	02 HPGe detectors made by CANBERRA	01 HPGe detector made by CANBERRA
3	Type of detector and relative efficiency	GX4020, ≥40%	- GC5019, ≥50% - BE6530, ≥65%	GC1520, 15%
4	Resolution	≤2 keV at 1.33 MeV and ≤1.2 keV at 122 keV	- GC5019: ≤1.9 keV at 1.33 MeV and ≤1.0 keV at 122 keV - BE6530: 1.9 keV at 1325 MeV and 0.684 keV at 122 keV	1.66 keV at 1.33 MeV and 0.77 keV at 122 keV
5	Minimum detectable activity (MDA) to Cs-137	0.18 Bq/kg	0.3 Bq/kg	0.6 Bq/kg

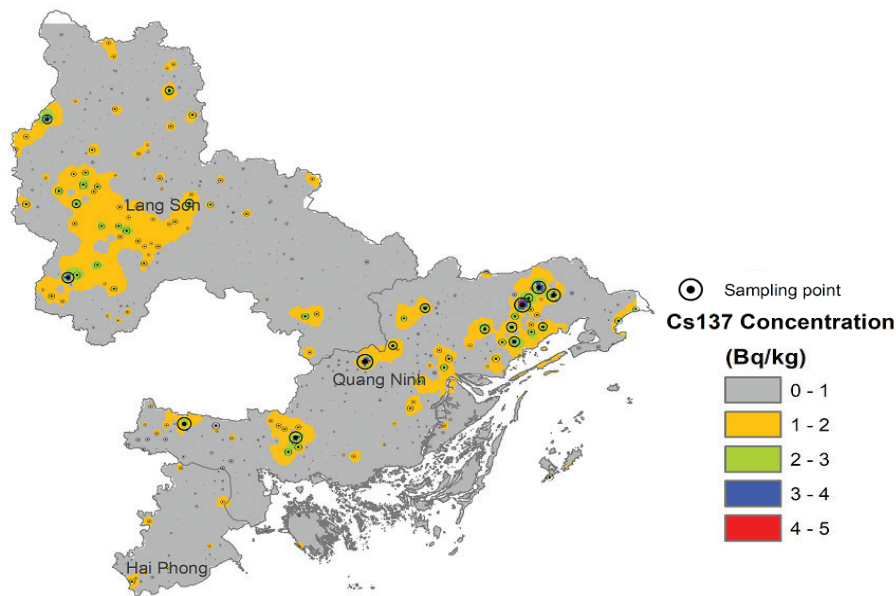


Fig. 1. Spatial distribution of Cs-137 radioactivity concentration in soil samples collected in 03 surveyed provinces.

The measured radioactivity concentrations of Cs-137 in Quang Ninh are higher than that of Hai Phong and Lang Son, albeit not much. The observation of high concentrations of Cs-137 is consistent with the high average annual rainfall in Mong Cai - Quang Ninh in comparison with that of the other surveyed areas [4].

Statistical distributions of Cs-137 radioactivity concentration in the 826 soil samples collected in Hai Phong, Quang Ninh, and Lang Son provinces are shown in Fig. 2.

The statistical parameters of the radioactivity concentration of Cs-137 in the 826 soil samples collected from all 3 provinces are shown in

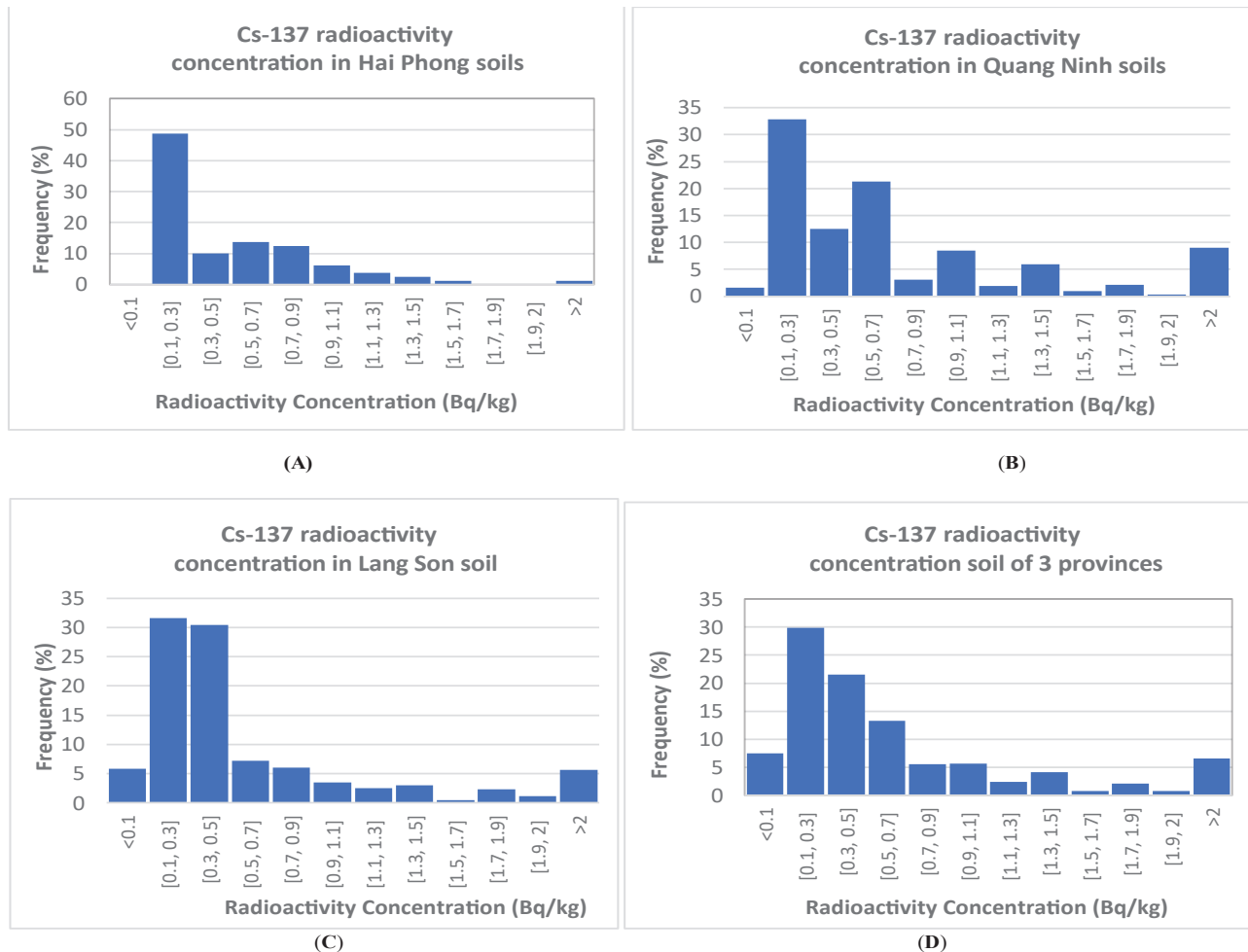


Fig. 2. Statistical distributions of Cs-137 radioactivity concentration in 826 soil samples collected from (A) Hai Phong, (B) Quang Ninh, (C) Lang Son, and (D) the 3 provinces as a whole.

Table 2. Statistical parameters on radioactivity concentration of Cs-137 in 826 soil samples collected in Hai Phong, Quang Ninh, and Lang Son.

Statistical parameters	All 3 provinces	Quang Ninh	Lang Son	Hai Phong
Number of samples	826	320	426	80
Mean (Bq/kg)	0.68	0.84	0.60	0.46
Standard error (Bq/kg)	0.03	0.05	0.03	0.05
Median (Bq/kg)	0.41	0.60	0.37	0.34
Mode (Bq/kg)	0.30	0.30	0.15	0.05
Standard deviation (Bq/kg)	0.75	0.90	0.65	0.46
Kurtosis	9.86	8.64	5.03	2.26
Skewness	2.73	2.71	2.18	1.33
Minimum (Bq/kg)	0.03	0.06	0.03	0.05
Maximum (Bq/kg)	5.70	5.70	4.14	2.32
Confidence level (95%)	0.05	0.10	0.06	0.10

Table 2. For all samples in which Cs-137 radioactivity concentration was below LOD, the LOD values were chosen instead.

3.2. Comparison with results from previous publications

In 2001, Prof. Pham Duy Hien and colleagues published on the residual density of Cs-137 in 292 undisturbed soil samples collected from 292 positions across Vietnam within 9.2-23.0°N latitude [5]. The principle purpose of this study is to assess the current status of Cs-137 in the surveyed area in order to establish a baseline for radioactivity concentration of this nuclide as an initial reference value in case of future emergency. Therefore, the priority for selecting samples is that the sampling points are evenly distributed over all accessible areas of the 3 provinces of interest (latitude from 20.61 to 22.18°N), which will take into account the land use in all localities. Thus, soil disturbance was not considered in our study.

To make an appropriate comparison, only 264 soil samples in our study were taken into account as samples with Cs-137 radioactivity concentration below LOD were neglected.

Radioactivity concentration of Cs-137: According to Ref. [5], the radioactivity concentration of Cs-137 in 292 soil samples varied from 0.5 to 18.0 Bq/kg with an average of 2.03 Bq/kg. In our study, these values ranged between 0.14 to 5.70 Bq/kg with an average of 1.14 Bq/kg, which is 56% of the average value from the previous research. According to the exponential decay

principle, the expected residual levels of Cs-137 should be approximately 60-63% of the published values in 2001 (the addition of Cs-137 due to events such as the Fukushima accident could be ignored*).

The observed difference could be explained by processes that cause Cs-137 to redistribute to deeper layer of soil, i.e., natural erosion or man-made disturbance of the soil from cultivation or road renovation activities.

Residual level of Cs-137: The research by P.D. Hien, et al. (2022) [5] also proposed an experimental formula for determining the residual level of Cs-137 according to the latitude of the sampling location and rainfall:

$$\ln(I) - \varepsilon = (3.53 \pm 0.09) + (0.092 \pm 0.004)L + (0.62 \pm 0.03)AR$$

where I is the residual level of Cs-137 in soil sample (Bq/m²); L is the latitude of the sampling location (degree); AR is the annual rainfall at the sampling location (m).

The variation of $\ln(I)$ in the surveyed area is shown in Fig. 3. The residual level of Cs-137 in soil samples, calculated from the experimental formula with rainfall data taken from the nearest metrological stations, shows a smaller variation than the data collected under our study, although the upper limit of both data sets are quite similar.

*According to the annual measurement data under the Annual Environmental Monitoring Task done by the Atomic Energy Institute of Vietnam, Cs-137 in fallout samples were detected at their Lang Son city and Ha Long city stations over the period from 4/2011 to 9/2011. The residual level at this period was 0.14 Bq/m². After that, the residual levels of Cs-137 at the stations were below LOD.

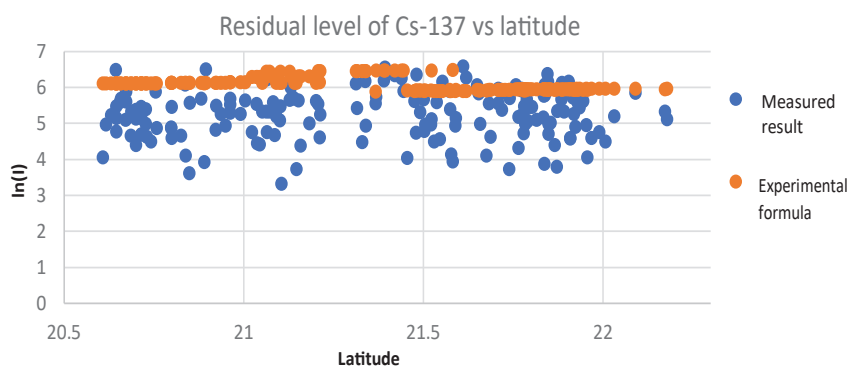


Fig. 3. Comparison of the measured residual level of Cs-137 with calculation results from the experimental formula [5].

The actual average measured residual level of Cs-137 in soil samples collected in this study was 236 ± 146 Bq/m², while the one calculated from the experimental formula was 407 ± 77 Bq/m². Although the measured values were generally more dispersed and lower than the calculated values due to the erosion and disturbance processes as mentioned above, they are still in agreement when one takes into account the standard deviation of both datasets.

4. Discussion and conclusions

This study has provided comprehensive and detailed data on the current background level of Cs-137 radioactivity concentration in soil from Hai Phong, Quang Ninh, and Lang Son provinces. The data could be used as a reference value in case of future significant emergency from nuclear power plants near the north border of Vietnam.

The residual level of Cs-137 in soil samples collected from the surveyed provinces demonstrate a decreasing trend when compared to previous monitoring results, which indicates that the contribution of nuclear accidents that occurred in the past 20 years (including the Fukushima accident) are negligible.

The processes of natural erosion and anthropogenic disturbance of the topsoil are quite strong and widespread in the survey area. The data should be further updated regularly to quantitatively estimate the relationship between these processes and Cs-137 levels to provide a more accurate assessment of Cs-137 supplementary after a significant emergency.

The experimental formula, provided by Prof. Pham Duy Hien and colleagues in his publication in 2001,

is still considered valid for the recent data taking into account the disturbed characteristics of the collected soils.

CRediT author statement

An Trung Nguyen, Thi Cam Ha Nguyen, Tien Thinh Lai: Data analysis and interpretation, Writing and manuscript revising; Hoang Giang Nguyen, Ha Vu, Thanh Thanh Long Nguyen: Method, Data analysis and Writing.

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COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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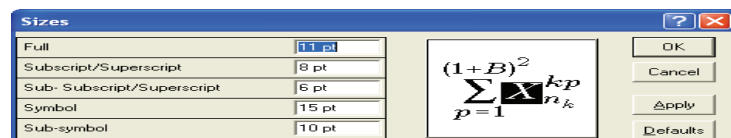


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