

APPLICATION OF ULTRASONIC-ASSISTED ENZYMATIC DIGESTION SYSTEM
FOR DIGESTION OF COCKLES

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Specially dedicated to my beloved family and friends

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ABSTRACT

Sample preparation is the most time consuming part of elemental analysis where homogeneous samples that are almost free from organic matter is necessary. Therefore, preliminary operations such as separation of interferences and preconcentration of the analytes are required prior to analysis. Ultrasonic digestion recently proposed as an efficient method for extraction of trace metals from solid samples. The ultrasonic digestion was optimized by using two parameters whilst another two other parameters were used in enzymatic digestion. Conventional one-variable-at-a-time (OVAT) optimization method was applied to both digestion techniques. Fe and Zn were extracted from the samples and were determined by using flame atomic absorption spectrometry (FAAS). The combination of ultrasonic and enzymatic digestion techniques is known as Ultrasonic-Assisted Enzymatic Digestion (USAED) method. The ultrasonication equipment used was ultrasonic bath. Four parameters studied for optimization were sample mass, sonication time, protease enzyme volume and incubation time. The optimum condition of parameters for sample mass was 0.2 g, sonication time of 30 min whilst for enzyme volume was 1000 μ L and for incubation time of 24 hours. For the kinetic study, these reactions had undergone zero order reaction. This USAED method was successfully applied for the digestion and kinetic study of the cockle samples.

ABSTRAK

Penyediaan sampel bagi analisis unsur memerlukan masa yang panjang bagi memastikan semua sampel adalah bebas daripada kebanyakan bahan organik. Oleh sebab itu, proses permulaan sebelum analisis seperti proses pemisahan gangguan dan kepekatan analit adalah sangat diperlukan. Kaedah penghadaman ultrasonik dalam masa sekarang dicadangkan sebagai cara paling berkesan bagi mengekstrak logam daripada sampel pepejal. Proses pengoptimuman turut dijalankan untuk mendapatkan hasil penghadaman yang terbaik. Dua parameter di kaji dengan menggunakan teknik penghadaman ultrasonik manakala dua parameter lagi digunakan bagi teknik penghadaman enzim. Kaedah biasa iaitu kaedah satu pemboleh ubah bagi satu masa diaplikasikan bagi kedua-dua teknik penghadaman. Logam Fe dan Zn diekstrak daripada sampel dan ditentukan dengan menggunakan spektroskopi serapan atom nyalaan (FAAS). Hasil gabungan teknik penghadaman ultrasonik dan penghadaman enzim dikenali sebagai kaedah penghadaman berbantuan ultrasonik dan enzim (USAED). Peralatan yang digunakan untuk ultrasonic ialah mandian ultrasonik. Empat parameter yang dikaji untuk pengoptimuman ialah berat sampel, masa sonikasi, isipadu enzim dan masa inkubasi. Keadaan optimum bagi parameter berat sampel ialah 0.2 g, masa sonikasi ialah 30 minit manakala bagi isipadu enzim ialah 1000 μ L dan masa inkubasi ialah 24 jam. Bagi kajian kinetik pula, tindak balas menepati tertib sifar. Kaedah USAED ini telah berjaya diaplikasikan untuk proses penghadaman dan kajian kinetik bagi sampel kerang.

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LIST OF SYMBOLS

cm	-	Centimeter
°C	-	Degree Celcius
°	-	Degree
g	-	Gram
kHz	-	Kilohertz
L	-	Liter
μL	-	Microliter
mL	-	Mililiter
m	-	Meter
mg	-	Miligram
min	-	Minute
μm	-	Micrometer
M	-	Molar
rpm	-	Rotation per minute
ppm	-	Parts per million
h	-	Hour
v/v	-	Volume per volume
w/w	-	Weight per weight
W	-	Watt
GHz	-	Gigahertz

LIST OF ABBREVIATIONS

AAS	-	Atomic Absorption Spectrometry
FAAS	-	Flame Atomic Absorption Spectrometry
HPLC	-	High Performance Liquid Chromatography
ICP-MS	-	Inductively Couple Plasma-Mass Spectrometry
OVAT	-	One-variable-at-a-time
USAED	-	Ultrasonic-Assisted Enzymatic Digestion
US	-	Ultrasound
UP	-	Ultrasonic probe
USASTD	-	Ultrasonic-Assisted Strong Digestion
NIES	-	National Institute of Environmental Studies
NIST	-	National Institute of Standard and Technology
S. D	-	Standard Deviation

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CHAPTER 1

INTRODUCTION

1.1 Introduction

This research is carried out to investigate the digestion of solid biological sample by using Ultrasonic-Assisted Enzymatic Digestion System (USAED) method. This project covered the optimization of parameters by using the one-variable-at-a-time (OVAT) technique. In this study, ultrasonic bath is used for the digestion of solid samples in presence of enzyme.

1.2 Background of Study

These days, most of studies that dealing with metals are focus on use of chemical. This is because, most of the metals have different toxicity depending on the oxidation state (J. Moreda-Piñeiro, *et al.*, 2010). Toxicity and nutritional effects of heavy metals in environmental, biological and food samples has drawn significant attention to determine all these heavy metals. Copper (Cu) is widely distributed in nature and is an essential trace element for humans. Cu functions as a cofactor and is required for structural and catalytic properties of a variety of important enzymes, including cytochrome c oxidase, tyrosinase, phydroxyphenylpyruvate hydrolase and dopamine b hydroxylase (Jurado, Bermejo-Barrera, *et.al.*,2007).

Iron plays important roles in biological systems. With only a few possible exceptions in the bacterial world, there will be no life without iron. If the iron concentration exceeds the normal level in the body, it may become a potential health hazard. Excess amounts of iron ions in the body cause liver and kidney damages. Some iron compounds are realized to be carcinogens. For this reason, the need to iron ion determination in clinical, medicinal, environmental and different industrial samples has created several methods to measure this analytes. Zinc is an essential trace element of great importance for humans, plants, and animals. Zinc nutrition is based on its quantity and bioavailability in food. For example, red meat is the richest common source of this element, whereas fowl and fish usually provide significantly less zinc (Alaa S.Amin, 2011).

In ultrasonic digestion, the ultrasonic energy can be used to accelerate the digestion of a great variety of samples such as environmental and hygiene samples, for subsequence single element or multi element determination of metals.

It must be pointed out that these days there are many types of ultrasonic devices. For instance, it can be used for sample treatment in analytical chemistry such as ultrasonic probes, ultrasonic baths, sonoreactors, and cup horns are ultrasonic systems. However, a minimum knowledge of their properties is required otherwise unexpected effects can be obtained. For example, the intensity of sonication, which ultimately dictates the properties of cavitation, is considerably different among the above-mentioned systems (Rial-Otero, *et al.*, 2007).

Eventhough ultrasonic bath cannot boost enzymatic kinetics in the same way as the ultrasonic probe or sonoreactor does. The capabilities of the apparatus to speed the enzymatic reactions follow the same order that their respective intensities of sonication. Ultrasonic probe is the highest. Followed by sonoreactor and ultrasonic bath (Rial-Otero, *et al.*, 2007).

Ultrasound-assisted digestion is a promising alternative in the analysis of solid samples when either simple dissolution or direct analysis is not applicable. However, the field of application of ultrasonic sample digestion is still small in comparison with classical digestion alternatives and, particularly, with microwave-assisted digestion.

This fact can be justified by the scant knowledge analytical chemists have about the advantages ultrasonic energy provides to digestion. Among these, the strict control at low temperatures of ultrasound applications allows the implementation of ultrasonic-assisted steps in biochemical analyses.

The use of ultrasound energy (ultrasonic water-baths) has been reported as an interesting approach because increases the efficiency of the extraction and enhances enzymatic kinetics by disrupting cell membranes allowing a direct contact of cytosolic structures and the enzymes. The enzymatic hydrolysis time is then reduced from hours to few minutes. Accelerated ultrasound-enzymatic hydrolysis procedures using ultrasound baths have been proposed for total metal extraction from edible seaweed and mussel tissue, and selenium organic species in krill (J. Moreda-Piñeiro, *et al.*, 2010). When ultrasonic waves cross a liquid solution, different chemical and physical phenomena take place (Vale, Rial-Otero,*et.al.*, 2008).

The use of ultrasounds to speed up enzymatic reactions for chemical speciation was first reported by G. Vale *et al.* This method is named Ultrasonic-assisted enzymatic digestion (USAED) and it meets the requirements of simplicity, robustness and rapidity (Vale, *et al.*, 2008).

Enzymatic hydrolysis or digestion procedures are a group of samples pretreatments that consist of hydrolyzing biomolecules mainly proteins by the action of enzymes. The enzymatic or enzymic hydrolysis breaks down certain bonds of these biomolecules under certain environmental conditions such as pH, temperature and ionic strength. Enzymes are extremely versatile compounds that can catalyze a wide range of chemical reactions. Because of their characteristics, they are ideal catalysts for use in analytical applications. The enzymes most commonly used for digestion are hydrolytic enzymes, where the catalytic effect is based on the insertion of water molecule at a specific bond of the substrate.

1.3 Problem Statements

It is lot of cases that samples obtained from biological and environmental sources are too complex, too dilute and/or incompatible with a suitable detection system for the direct determination of the target compounds of interest. Consequently, preliminary operations such as separation of interferents and preconcentration of the analytes are required prior to analysis.

Although sample pretreatment stages are often the weakest link of the whole analytical method and may be considered as bottlenecks as they are usually very time consuming, often unreliable and ancient in terms of sample/reagent consumption, the importance of these steps is often unseen. Traditional separation processes are usually tedious and time-consuming, involve multiple steps of careful operation and are the main areas of contamination risks, particularly in trace analysis (Luque de Castro *et al.*, 1999). One of the techniques that have shown promise for speeding up and simplifying sample treatment is ultrasonic extraction or digestion.

Ultrasonic digestion enables analytical application to biological materials, where the input factor such as those based on ultrasonic enzymatic digestion in order to increase the selectivity of the method and to avoid loss enzyme activity (Bermejo *et al.*, 2004).

1.4 Significant of Study

This method is very useful and important to define the most effective method that can be used in solid biological samples while keep the integrity of chemicals. So that it will still be conserved. It is also important to find the kinetics study of the reaction. Some requirements of using concentrated mineral acids, high temperature and high pressure were evaded. Ultrasonic-Assisted Enzymatic Digestion System (USAED) method has reduced the length of time that should be needed to do the sample preparation.

1.5 Objectives of Research

The objectives in this study are:

- i. To optimize parameters that effect digestion process of cockle samples using Ultrasonic-Assisted Enzymatic Digestion System (USAED)
- ii. To investigate the kinetics of digestion process in Ultrasonic-Assisted Enzymatic Digestion System (USAED).

1.6 Scope of the Research

Ultrasound-Assisted Enzymatic Digestion (USAED) is a sample preparation method based on the combination of ultrasonication technique and enzymatic digestion technique for the biological samples. Parameters influencing digestion process which are sonication time and sample mass are studied by using the one-variable-at-a-time (OVAT). The determinations of the metal concentrations (Zn and Fe) are carried out by flame atomic absorption spectrometry (FAAS). The kinetic of digestion process was studied using optimized condition by varying the time of digestion process.

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