

SUBCRITICAL WATER EXTRACTION WITH COMPACTED NATURAL
CALCIUM CARBONATE COLUMN FOR THE EXTRACTION OF NATURAL
PRODUCTS

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UNIVERSITI TEKNOLOGI MALAYSIA

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PRODUCTS

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Specially dedicated to my beloved family and to the pure spirit of my father

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ABSTRACT

The aim of this work was to develop subcritical water extraction (SWE) method for the extraction of natural products. The developed system was applied to the extraction of natural products from the rhizomes of *A. mutica* and *Calotropis procera* as model samples. The efficiency of the SWE method was compared with Soxhlet extraction method using as model sample in terms of yield amount, extraction time and solvent consumption. The SWE attached with compacted natural calcium carbonate (CNCC) method was found to provide higher yield (49.14%) compared with Soxhlet extraction (33%). Three compounds namely kawain, flavokawain and 1,7-diphenyl-5-hydroxy-6-hepten-3-one (DHH) were extracted from the rhizomes of *A. mutica* using water as a green solvent employing modified subcritical water extraction technique attached to a CNCC column. The CNCC obtained from sea shells was used as adsorbent material to enhance selectivity and purity of the extract. The extract obtained was used without further purification for quantitative analysis based on standard compounds. In this modified SWE technique, water modified with 6-10% acetonitrile, methanol and ethanol were used as extraction solvent. Dried rhizomes of *A. mutica* were introduced into the extraction vessel connected to the pump that delivers the extractant which further passes through the CNCC unit. The optimum conditions for this extraction were 160 C as extraction temperature, 7 MPa as extraction pressure, 1.8 mL/min as elution volume flow rate and 30 min as extraction time. It was also noted that 160°C extraction temperature produced reasonable amounts of the products (1.891 g, 54.02%, 1.87 g, 53.42% and 1.63 g, 46.6%) with three modifiers (methanol, acetonitrile and ethanol) under identical optimum conditions. The recoveries of the compounds showed good results especially for kawain. Methanol (10%) achieved high recovery (94.82%) compared to the other modifier ratios. In general, the developed method offers rapid and efficient extraction of natural products with reduced organic solvent usage.

ABSTRAK

Tujuan kajian ini ialah untuk membangunkan kaedah pengekstrakan air sub-genting (SWE) bagi pengekstrakan hasilan semulajadi. Kaedah yang dibina telah diaplikasikan dalam pengekstrakan hasilan semulajadi daripada rizom *A. mutica* dan *C. procerus* sebagai sampel contoh. Kecekapan kaedah SWE telah dibandingkan dengan kaedah pengekstrakan Soxhlet menggunakan sampel contoh dari segi hasil pulangan, masa pengekstrakan dan penggunaan pelarut. Kaedah SWE didapati memberikan hasil pulangan lebih tinggi (49.14%) berbanding dengan pengekstrakan Soxhlet (33%). Tiga sebatian iaitu kawain, flavokawain, 1,7-difenil-5-hidroksi-6-hepten-3-on (DHH), telah diekstrak daripada rizom *A. mutica* dengan air sebagai pelarut hijau menggunakan teknik pengekstrakan air sub-genting yang dihubungkan dengan turus kalsium karbonat asli (CNCC). CNCC yang diperolehi daripada kerang laut telah digunakan sebagai bahan penjerap untuk meningkatkan kepilihan dan ketulenan ekstrak. Ekstrak yang diperolehi telah digunakan tanpa penulenan lanjutan bagi analisis kuantitatif berdasarkan kepada sebatian piawai. Dalam teknik SWE terubahsuai ini, air yang diubahsuai dengan 6-10% asetonitril, metanol dan etanol telah digunakan sebagai pelarut pengekstrak. Rizom *A. mutica* kering telah dimasukkan ke dalam kebuk pengekstrakan yang dihubungkan kepada pam yang menyampaikan pelarut pengekstrakan yang seterusnya melalui unit CNCC. Keadaan optimum pengekstrakan ialah suhu pengekstrakan 160 C, tekanan pengekstrakan 7 MPa, kadar pengelusan isipadu 1.8 mL/min dan masa pengekstrakan 30 min. Pengekstrakan pada 160 C didapati memberi hasil yang memuaskan (1.891 g, 54.02%, 1.87 g, 53.42% dan 1.63 g, 46.6%) menggunakan pengubahsuai (metanol, asetonitril dan etanol) di bawah keadaan optimum yang serupa. Perolehan semula menunjukkan hasil yang baik terutamanya bagi kawain. Metanol (10%) memberikan perolehan yang baik (94.82%) berbanding nisbah pengubahsuai lain. Secara amnya, kaedah yang dibangunkan memberikan pengekstrakan hasilan semulajadi yang cepat dan cekap serta menggunakan pelarut organik yang sedikit.

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LIST OF ABBREVIATIONS

AOAC	-	Association of analytical communities
ASE	-	Accelerated solvent extraction
a.m.u	-	Atomic mass unit
BPR	-	Backpressure regulator
AC	-	Activated Charcoal
CNCC	-	Compacted natural calcium carbonate
CPEA	-	<i>Calotropis procera</i> ethyl acetate
Cp	-	Critical point
AK	-	Alkaloids
DH	-	Diarylheptanoids
DHH	-	1,7-diphenyl-5-hydroxy-6-heptene-3-one
e	-	Dielectric constant
FV	-	Flavonoids
GC	-	Gas chromatography
GC-MS	-	Gas chromatography – mass spectrometer
HPLC	-	High performance liquid chromatography
HPLC-MS	-	High performance Liquid Chromatography Mass Spectrometer
ISWE	-	Improved subcritical water extraction
IR	-	Infrared
LC	-	Liquid chromatography
LG	-	Lignans
LOD	-	Limit of detection
LOQ	-	Limit of quantification
MAE	-	Microwave assisted extraction

MPa	-	Megapascal (1MPa = 10.19 atm = 145.03 psi)
NCC	-	Natural calcium carbonate
nm	-	Nanometer
NMR	-	Nuclear magnetic resonance
PAF	-	Plated activating factor
PB	-	Phenylbutanoids
PH	-	Phenolic
PP	-	Phenylpropanoids
ppm	-	Part per million
PSE	-	Pressurized solvent extraction
Pc	-	Critical pressure
R%	-	Recovery percent
RP.HPLC	-	Reverse phase high performance liquid chromatography
RP-LC	-	Reverse phase liquid chromatography
RSD%	-	Relative standard deviation percent
SFE	-	Subcritical fluid extraction
ST	-	Stilbenes
STD	-	Standard deviation
SWE	-	Subcritical water extraction
Tp	-	Triple point
Tc	-	Critical temperature
UV	-	Ultraviolet
ZPC	-	Zero point of charge

LIST OF SYMBOLS

C _p	-	Critical point
e	-	Dielectric constant
P _c	-	Critical pressure
T _c	-	Critical temperature
T _p	-	Triple point

CHAPTER 1

INTRODUCTION

1.1 Background

Analytical chemistry is a wide range of research area, with many different methods of measurement based on assessing a variety of characteristics which can differentiate one species from another. Fundamentally, though, all analytical procedures follow a specific type of processes in a clearly-defined particular order. This begins with a sampling procedure, in which a representative sample is obtained from the system about which chemical information is desired. The sample is usually subjected to sample preparation or pre-treatment process before being subjected to the analysis. This process may involve extracting the analytes from its matrix, removing large matrix components from the sample, masking or removing species which could interfere with the measurement, derivatization to make the analytes easy for detection, or a sample pre-concentration process. Once the sample's condition is suitable for the method chosen, the sample is subjected to analysis, at which time the presence of the analyte of interest is ascertained and its concentration often quantified. This chemical information is then converted to an electronic signal during the data acquisition process.

Natural products are chemical compounds or substances produced by a living organism - found in nature that usually has a biological activity. The presence of compounds in a mixture of other compounds from a natural source needs to be

isolated and purified. The ease with which the active principle can be isolated and purified depends much on the structure, stability, and quantity of the compound. For example, Alexander Fleming recognized the antibiotic qualities of penicillin and its remarkable non-toxic nature to humans, but he disregarded it as a clinically useful drug because he was unable to purify it. He could isolate it in aqueous solution, but whenever he tried to remove the water, the drug was destroyed (Serralheiro 2004). It was not until the development of new experimental procedures such as freeze drying and chromatography that the successful isolation and purification of penicillin and other natural products became feasible.

Conventional Soxhlet method is considered to be one of the famous classic methods to carry out the extraction of natural products from the plants. The advantages of this method are summarized in the high yield from the matrix besides the simplicity of the system components. The system consists of Soxhlet condenser and round bottom flask in addition to heating mantle. However, the disadvantages of this method are represented in time consuming where the Soxhlet takes about 18 hours to complete the extraction. This conventional method consumes high amounts of toxic organic solvents besides these chemicals can affect the researchers and lab operators as well as the environment. The selectivity of the analytes requires several procedures to achieve the targeted analytes. These include vacuum liquid chromatography (VLC) and column chromatography (CC) and eventually using thin layer chromatography (TLC) to detect the analytes. All this procedures require a lot of chemicals and time which means high cost with minimum result.

The goal of analytical chemists over the years has been to make their jobs easier, through suitable and simple equipment as possible. For many years, different techniques have been developed to replace classical extraction techniques in an effort to reduce time and solvent consumption (Windal *et al.*, 2000).

Recently, an extraction method referred to as subcritical water extraction (SWE) attracted considerable interest as an alternative extraction method which uses water as the extraction solvent. This extraction method offers an easy way of

extraction considering the temperature and the pressure and very small amount of solvents with high yield percent and reasonable recoveries of the analytes. Subcritical mass is a mass of fissile material that does not have ability to sustain a fission chain, Water is a polar molecule, where the centres of positive and negative charge are separated; so molecules will align with an electric field. The extensive hydrogen bonded network in water tends to oppose this alignment, and the degree of alignment is measured by the relative permittivity. The hydrophilic nature of the water viscosity and surface tension of water drops and diffusivity increases with increasing temperature. As the temperature of liquid water is raised between 100 and 374°C under suitable pressure, the polarity decreases markedly and it can be used as an extraction solvent in a wide range of analysis. In the case when water is employed instead of other organic solvents, the dramatic change in the physical and chemical properties of water, especially in its dielectric constant (ϵ), at elevated temperature and pressure enhance its usefulness as an extraction solvent. The dielectric constant (as measure of the polarity of the solvent) is a key parameter in determining solute-solvent interaction, and in the case of water, increasing the temperature under moderate pressure can significantly decrease the constant.

At ambient pressure and temperature, water is a polar solvent with high dielectric constant ($\epsilon = 78$) but at 300°C and pressure of 23 MPa, this value decreases to 21, which is similar to the value for ethanol ($\epsilon = 24$ at 25°C) or acetone ($\epsilon = 20.7$ at 25°C). This means that at elevated temperatures and moderate pressures the polarity of water can be reduced considerably and the solvent (water) can act as if ethanol or acetone were used. This drop in dielectric constant when working at elevated temperatures and pressure means that water can be used instead of another organic solvent to extract medium-or low-polarity compounds (Carabias-Martínez *et al.*, 2005). It is of interest to explore the potential of developing SWE method incorporated with device contains a cleaning up material that can clean and adsorb undesirable components from the extract in suitable extraction conditions with minimum organic solvents.

Among the different techniques, subcritical water extraction has several benefits comprising selectivity and reducing time and organic modifier consumption.

The subcritical water extraction (SWE) has many advantages when compared with other extraction techniques such as supercritical fluid extraction (SFE) or solid phase extraction (SPE) or any other techniques that depends on high ratio of organic solvents. Thus SWE stands for reducing organic solvent to very minimum ratio and sample consumption to very small amount. The addition of a vessel containing adsorbent material such as natural calcium carbonate (NCC) for cleaning up and remove some impurities from the extract of natural product has been investigated in this work and the results are described in respective chapters. For the extraction of analytes mainly kawain, flavokawain and 1,7-diphenyle5-hexane-6-heptene-3-one from the rhizomes of *A. mutica* SWE-NCC is highly matrix-dependent, and high recoveries compared to other artificial adsorbents such as silica gel.

Water as an extraction solvent, has gain a great attention because of its individual properties as a solvent, it can be chosen as an alternative solvent for a purpose of extraction instead of organic solvents by elevating the temperature (Rovio *et al.*, 1999). Raising the temperature of water changes the force between the hydrogen single bond to its low value as well as the permittivity. In the SWE, the elevated temperature to the appropriate value will affect the polarity of water to match the components polarity. The SWE is considered to be an alternative extraction method to separate the analytes from the natural products from plant sample. SWE is a green sample preparation technique when using water as a major solvent for the extraction operation (Smith 2002). The elevated temperature ranging from 100°C to 374°C (triple point) is commonly applied with appropriate pressure to maintain the water in liquid condition (Ramos *et al.*, 2002).

1.2 Rationale of Extraction using SWE

There are many advantages for using subcritical water extraction system as a simple method to extract the natural products from plants. Despite the importance of using organic modifiers as extraction solvent for the extraction process, these methods have some disadvantages specially when extracting edible plant such as

zingiberacea. The use of organic solvents may have some hazards due to toxicity and cost. Various methods have been used to avoid the use of organic solvents and water is considered as an alternative solvent for extraction process by using different elevated extraction temperature. Researchers in this field seeking for suitable and safe extraction solvents, for the laboratory users and environment, because it can make possible changes in the retention factors and separation efficiencies of the analytes rather than reducing the proportions of the organic modifier used (Smith and Burgess 1997).

Natural products occur somewhere in the plant parts. Many edible foods such as vegetables and fruits believed to be suitable source of natural components. *A. mutica* rhizomes are a rich of important analytes such as kawain and flavokawain that encourage the researchers to focus on how to extract and isolate these analytes in order to use them as natural components.

The easiest way to achieve more targeting compounds is to remove them from the matrix using suitable sample preparation techniques, which mean appropriate way to make good extraction in terms of selectivity, safety, cheap and environmentally friendly solvent. The classical methods for isolating the analytes from the plant use considerable amount of toxic organic solvents. SWE was chosen to be the suitable technique to extract natural products from *A. mutica*. The use of this technique with modified extraction procedure was used in order to improve the extraction.

1.3 Research Objectives

The present work investigates and focuses on the following objectives:

- (1) To prepare and characterize natural calcium carbonate (NCC) adsorbent for subcritical water extraction (SWE).
- (2) To develop and optimize subcritical water extraction system with compacted natural calcium carbonate (SWE-CNCC).
- (3) To employ the developed SWE-CNCC method to the extraction of *C. procera* in comparison with conventional methods.
- (4) To apply the developed SWE-CNCC column to extract natural product from the rhizomes of *A. mutica*.

1.4 Statement of Hypothesis

Improved SWE that uses water as an extractant is potentially proper for the extraction of natural products such as *C. procera* and *A. mutica*. The developed method will provide a convenient, rapid, and less solvent-intensive extraction of natural products with recoveries equivalent to or better than those obtained by classical, more solvent-intensive extraction methods.

1.5 Scope of Study

Developed SWE method would be applied to the analysis of *A. mutica* and *C. procera* as natural product resource. Extracts would be characterized by high-performance liquid chromatography, infrared, scanning electron microscope and surface area instrument. Natural calcium carbonate (NCC) would also be characterized using Micrometer ASAP 2010.

1.6 Benefits of Research

The present work focuses on the development, validation and application of improved subcritical water extraction (ISWE) method prior to HPLC for the analysis of selected natural products namely *A. mutica* and *C. procera*. In addition, there is considerable freedom in selecting appropriate solvents for extraction of different analytes. SWE conditions would be applied for the extraction of selected natural products from the rhizomes of *A. mutica* and extract of dry leaves from *C. procera*. The influence of extraction pressure, temperature, time, and flow rate on the total yield of the extractant and the influence of extraction temperature on the extraction of some chosen components would be studied. The optimizations of several parameters influencing the efficiency of SWE such as extraction solvent would also be carried out. The benefits of using natural calcium carbonate as a cleanup and adsorbent material due to its availability and safety, beside the freedom of toxic materials which may exist some other adsorbents such as silica gel.

1.7 Thesis outlines

The introduction, objectives and scopes of this work are also covered in Chapter 1. Chapter 2 provides background information on the technique applied and

the applied samples of natural product in addition to compounds that were chosen to be extracted from *A. mutica* rhizomes. The theoretical aspect and description of equipment of the new sample preparation method coupled with NCC column as well as the experimental conditions are presented in Chapter 3.

Chapter 4 describes the characterization of the natural calcium carbonate used in this work using different types of characterization method including surface area, FTIR and scanning electron microscope.

Chapter 5 describes and gives some information about the sample plant in term of chemical properties and parts used such as dry leaves from *Calotropis procera*. The chapter also describes the application of SWE for the extraction of natural products from the sample.

Chapter 6 reports the improvement of SWE coupled with CNCC (SWE-CNCC) column and the application of this attached unit in the extraction and determination of the compounds from the rhizomes of *A. mutica*. The CNCC column is an online cleaning up extraction vessel coupled to the subcritical water extraction system.

Chapter 7 covers the conclusion and future work. In addition to references, the last few pages are set for the appendices.

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