

DETERMINATION OF ALPHA- AND BETA-ASARONE IN
TRADITIONAL PRODUCTS CONTAINING
Acorus calamus RHIZOME BY
GAS CHROMATOGRAPHY MASS SPECTROMETRY

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KUALA LUMPUR

2017

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GAS CHROMATOGRAPHY MASS SPECTROMETRY**

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**DISSERTATION SUBMITTED IN PARTIAL
FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF BIOTECHNOLOGY**

**INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR**

2017

UNIVERSITY OF MALAYA
ORIGINAL LITERARY WORK DECLARATION

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Name of Degree: Master of Biotechnology

Title of Project Paper/Research Report/Dissertation/Thesis (“this Work”):

Determination of Alpha- and Beta-Asarone in Traditional Products Containing
Acorus calamus Rhizome by Gas Chromatography Mass Spectrometry

Field of Study: Herbal product

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ABSTRACT

The study was conducted to develop an analytical method to identify and quantify alpha- and beta-asarone from traditional products that contain *Acorus calamus* rhizome. Alpha- and beta-asarone are the major active compounds in rhizome of *Acorus calamus* which are responsible for the plants' therapeutic effects such as relieving stomach cramps, dysentery, asthma, antimicrobial, anthelmintic, insecticides, tonic and stimulants. However, there were reports of mammalian toxicity and carcinogenicity of asarones that discourage the medicinal use of this plant. International regulatory authorities have set different restrictions for *Acorus calamus* as some countries have complete prohibition of its use while other countries enforce content limitation. Alpha- and beta-asarone were extracted from traditional products by sonication method using methanol as solvent. Gas chromatography mass spectrometry was chosen for the determination of alpha- and beta-asarone because the analysis is reliable, cost effective and rapid. Standards of alpha- and beta-asarone have shown distinct separation and elute at about six minutes with this method. Mass spectra of alpha- and beta-asarone were obtained after running scan mode and these mass spectra were selected in Selected Ionizing Monitor (SIM) mode for quantitation purposes. Nine samples in different dosage form (three capsulated powder, one tablet and five oils) have been collected from current market for screening of alpha- and beta-asarone. Only one sample of capsulated powder form was found to contain beta-asarone. Alpha- and beta-asarone were not detected in the oil form products. Negative results for alpha- and beta-asarone might be due to the heterogeneous nature of herbal substances in traditional product and lack of efficiency of the extraction method. Overall, the developed method is appropriate to quantify the level of alpha- and beta-asarone in traditional products containing *Acorus calamus* rhizome.

ABSTRAK

Penyelidikan ini dijalankan untuk membangunkan satu kaedah analisa kimia untuk mengenal pasti dan menyatakan kuantiti alfa- dan beta-asarone dalam produk tradisional yang mengandungi rizom *Acorus calamus*. Alfa- dan beta-asarone adalah bahan aktif utama dalam rizom *Acorus calamus* yang boleh memberi kesan terapeutik seperti melegakan kekejangan perut, disentri, asma, antimikrob, antelmintik, racun serangga, tonik dan perangsang. Walau bagaimanapun, terdapat laporan ketoksikan mamalia dan kekarsinogenan daripada penggunaan alfa- dan beta-asarone yang menyebabkan penggunaan tumbuhan ini tidak digalakkan. Terdapat perbezaan pendapat tentang penggunaan *Acorus calamus* di antara pihak berkuasa antarabangsa dimana ada negara yang mengenakan sekatan penuh terhadap penggunaannya dan ada negara yang menguatkuasakan had kandungan bahan aktif tersebut. Alfa- dan beta-asarone diekstrak daripada produk tradisional secara sonifikasi dengan menggunakan metanol sebagai pelarut. Gas kromatografi-spektrometri jisim (GC-MS) telah digunakan untuk penentuan alfa- dan beta-asarone kerana keupayaan mesin tersebut, kos efektif dan cepat. Piawai rujukan alfa- dan beta-asarone telah menunjukkan pemisahan yang jelas dan *elute* pada minit ke-enam. Melalui *scan mode*, spektrum jisim alfa- dan beta-asarone diperoleh dan digunakan dalam *Selected Ionizing Monitor (SIM) mode* untuk mengira kandungan alfa- dan beta-asarone. Sembilan sampel dalam bentuk dos yang berbeza (tiga kapsul, satu tablet dan lima minyak) telah dikumpulkan dari pasaran untuk mengenal pasti kandungan alfa- dan beta-asarone. Hanya satu sampel dalam kapsul didapati mengandungi beta-asarone. Alfa- dan beta-asarone tidak dikesan dalam produk minyak. Keputusan negatif mungkin disebabkan oleh sifat heterogenik kandungan bahan-bahan herba dalam sampel dan kaedah pengekstrakan yang kurang berkesan. Secara keseluruhan, kaedah yang dibangunkan sesuai untuk mengira kandungan alfa- dan beta-asarone dalam produk tradisional yang mengandungi rizom *Acorus calamus*.

ACKNOWLEDGEMENTS

Thanks to Almighty Allah for giving me the courage and patience to complete this dissertation.

I would like to acknowledge the guidance of my Supervisors Dr. Zul Ilham Zulkiflee Lubes and Pn Zakiah Abd. Ghafar through the process of dissertation.

I would like to acknowledge Ministry of Health Malaysia for financial support throughout my degree. I owe my profound gratitude to laboratory staff of Research Section, Centre of Quality Control, National Pharmaceutical Regulatory Agency (NPRA) especially Dr. Tajuddin Akasah, Cik Nik Juzaimah, Mohamed Shahrizan Shahrir, Dinash Aravind for their keen interest on my project work and support till the completion of my project work by providing all necessary information and providing the facilities throughout the project. The author would also like to thank University of Malaya for continuous support and the research funding (Project No. PO008-2015A).

I would also like to thank my family and friends for this unconditional support throughout the process of this research.

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LIST OF SYMBOLS AND ABBREVIATIONS

| | |
|----------------|---|
| AA | : <i>Aristolochic acid</i> |
| AFC | : Antifungal compounds |
| BBB | : Blood Brain Barrier |
| CEFS | : The Council of Europe Committee of Experts on Flavouring Substances |
| CMEC | : Complementary Medicines Evaluation Committee |
| EMA | : European Medicine Agency |
| FDA | : The Food and Drug Administration |
| GC-MS | : Gas Chromatography Mass Spectrometry |
| HPLC | : High Performance Liquid Chromatography |
| HPLC-MS/MS | : Liquid chromatography–tandem mass spectrometric |
| HPTLC | : High Performance Thin Layer Chromatography |
| ICH | : International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use |
| MDMA | : Methylenedioxyamphetamine |
| MEKC | : Micellar electrokinetic capillary chromatography |
| NHPD | : Natural Health Products Directorate |
| NIST | : National Institute of Standards and Technology |
| NPRA | : National Pharmaceutical Regulatory Agency |
| R ² | : Coefficient Of Determination |
| SIM | : Selected Ion Monitoring |
| SPE | : Solid phase extraction |
| TGA | : Therapeutic Goods Administration |
| TLC | : Thin Layer Chromatography |

CHAPTER 1: INTRODUCTION

1.1 General Introduction

Acorus calamus which is commonly known as sweet flag or Jerangau, can be found naturally in Asia, Europe and Northern America. The leaves, rhizome and stem are parts of the plant that have been used traditionally as medicine. The rhizome has been used to treat diseases such as fever, asthma, bronchitis, digestive problems, epilepsy, coma and hysteria. The major compositions of the rhizome have been identified as beta-asarone and alpha-asarone (Venskutonis, 2003). These two asarones are cis-trans isomers which possess important pharmacological properties. It is widely known that both alpha- and beta-asarone exhibit strong antimicrobial activities and possess neuroprotective effects (Sharma, 2014). Reports suggested that beta-asarone possess anti-diabetic properties, anti-cancer activity and showed potent antiviral activities while alpha-asarone possesses hypolipidemic properties and anti-spasmodic properties (Rajput et al., 2014).

On the other hand, mammalian toxicity and carcinogenicity of alpha- and beta-asarones have also been reported. Genotoxicity and hepatocarcinogenicity have been demonstrated in rodents that were exposed to alpha- and beta-asarone (European Medicine Agency, 2005). In a chronic feeding study, beta-asarone produced a dose-dependent increase in leiomyosarcomas in the intestine of male rats which result to severe toxicity and increased mortality. In *vitro* studies also demonstrate that asarones are likely to induce cytotoxicity to hepatocytes (Patel et al., 2015; Unger & Melzig, 2012). Reports of toxicities have raised the concern whether the benefits of *Acorus calamus* outweigh the risk.

Based on the reported toxic effects of alpha- and beta-asarone compiled by The Joint FAO/WHO Expert Committee on Food Additives (JECFA) (1981), national regulatory authorities have taken safety measurements by either imposing exposure limits of beta-

asarone in herbal product or banned the herb completely from being an ingredient of food or medicinal product (European Commission, 2002; US Food & Drug Administration, 2014). Thus there is a need to quantify the content of alpha- and beta-asarone in products containing *Acorus calamus*.

Traditional products containing *Acorus calamus* has been produced in many dosage forms such as tablet, powder, capsule, oil, cream and ointment. Given the nature of traditional products, usually it contains more than one herb in one preparation. These other herb will affect the extraction process of alpha- and beta-asarone from the product (Sasidharan et al., 2011).

Alpha- and beta-asarone has been successfully extracted from *Acorus calamus* by sonication, steam distillation, vortex and decoction (Patel et al., 2015; Malik et al., 2014; Chen et al., 2009; Zuba & Bryska, 2012). Alpha- and beta-asarones has been identified, separated and quantified using various chromatographic methods such as thin layer chromatography (TLC), high performance liquid chromatography (HPLC), high performance thin layer chromatography (HPTLC) and gas chromatography-mass spectrometry (GC-MS) (Shailajan et al., 2015). GC-MS is preferred due to its many advantages such as precision, the ease to conduct the test, less time consuming and most importantly because of its sensitivity. The intrinsic nature of alpha- and beta-asarone themselves are volatile compound which made them readily identified and quantified with GC-MS (Dong et al., 2014).

This study will develop an improved GC-MS method in order to determine and quantify alpha- and beta-asarone in traditional products containing *Acorus calamus*.

1.2 Research Background

In Malaysia, prior to being released into the market, traditional products are tested for heavy metals, microbial contamination, disintegration, uniformity of weight and screening for adulteration by the Centre For Quality Control of the National Pharmaceutical Regulatory Agency (Ministry Of Health Malaysia, 2015). These tests are important to ensure the safety of traditional products. With regards to the complexity and nature of herbal preparation, some plants are toxic and require additional testing. Thus, the development of this method may assist the regulatory bodies to ensure that asarones content is within the validated safety limit when tested in traditional products.

1.3 Problem Statement

In the past decade, the use of herbal medicinal products which include traditional products have seen significant increase with more than 80% of world population preferring them as primary treatment of choice (Ekor, 2013). The situation is similar in Malaysia. People tend to have the perception that traditional products are totally safe due to its natural properties. This lack of awareness and understanding of the potential toxicity and side effects led to uncontrolled intake, misuse and inappropriate doses which eventually lead to poisoning and health problems (Wachtel-Galor & Benzie, 2011). In case of traditional product containing *Acorus calamus*, its major active compounds of alpha- and beta-asarone have the potential to cause genotoxicity and mutagenicity. In Europe, the regulatory authorities have set a limit of exposure to beta-asarone of 115 µg per kg per day. Thus it is important that the amount of alpha- and beta-asarone is quantified in traditional products that contain *Acorus calamus*. Determination of asarones in plant materials has been performed by chromatographic methods. GC-MS method was found to be very specific and precise while having short run time in the separation of each asarones.

1.4 Objectives of Proposed Study

- i. To analyse the extraction method of alpha- and beta-asarone from traditional products containing *Acorus calamus* rhizome.
- ii. To analyse a suitable analytical method to identify and quantify alpha- and beta-asarone in traditional products containing *Acorus calamus* rhizome by Gas Chromatography Mass Chromatogram that can be used as in-house method for National Pharmaceutical Regulatory Agency (NPRA).
- iii. To screen traditional products containing *Acorus calamus* rhizome for alpha- and beta-asarone.

1.5 Scope of the Work

This study will focus on the development of a method to extract alpha- and beta-asarone from products containing *Acorus calamus* rhizome and then to identify, separate, and quantify their contents using gas chromatography mass spectrometry (GC-MS). Therefore, consideration should be given to the factors that can affect the extraction process particularly the matrix of the product. This study will consider methods that can extract alpha- and beta-asarone rapidly and directly without significant loss of the compound during the extraction process. The literature has demonstrated that GC-MS is one of the most frequently used techniques to separate and identify isomer compounds like alpha- and beta-asarone. Based on the identification results, specific ions that were identified correspond to each of the asarones are used for quantification purposes. This study will screen and quantify products that contain *Acorus calamus* from current market for alpha- and beta-asarone. The results of the screening will be able to give an overview on the content of alpha- and beta-asarone in traditional products and furthermore gauge the potential safety concerns to current consumers or users.

1.6 Dissertation Outline

There are five chapters in the dissertation which begin with:

Chapter 1, provides comprehensive introduction, the background, problems, purposes and aims of the study.

Chapter 2, highlights some of the study related to *Acorus calamus* rhizome, alpha- and beta-asarone, and safety of traditional products.

Chapter 3, describes the methods used to extract alpha- and beta-asarone and the GC-MS method developed to analyses samples.

Chapter 4. The results of this study is discussed in this chapter and the relevance of the new method developed for future use and comparison with previous studies.

The summary of this study is concluded in Chapter 5.

CHAPTER 2: LITERATURE REVIEW

2.1 Safety of Traditional Products

Herbal medicines could be found in various forms including herbs, herbal materials, herbal preparations and finished herbal products. These products may contain active ingredients such as parts of plants, mixture of other plant materials, or combinations of both. Traditional use of herbal medicines refers to the long historical use of these medicines. Their use is well established and widely acknowledged to be safe and effective, and may be accepted by national authorities (World Health Organization, 2000). In Malaysia, traditional products is defined as any product used in the practice of indigenous medicine, in which the drug consist solely of one or more naturally occurring substances of a plant, animal or mineral, of parts thereof, in the unextracted or crude extract form, and a homeopathic medicine (Sales of Drug Act, 1952).

There has been tremendous increase in the usage of herbal medicine with the estimation of 80% of worldwide population preferring herbal medicines as their primary treatment (Ekor, 2014). In Malaysia, being an adult female, Malay, and having higher income are more likely in favor of using herbal medicines (Aziz & Tey, 2009). From a survey of 460 women, 73% of them used herbal medicine during labour while 34.3% of them used herbal medicine during pregnancy (Kim Sooi & Lean Keng, 2013). Patients have opted for complimentary medicines as an additional treatment to their current treatment plan and they have perceptions that herbal medicines are reliable and safe as compared to conventional drugs (Hasan et al., 2011).

The quality, efficacy and safety of herbal medicines are still questionable even though the statistics show growing popularity and usage among consumers. Quality issues in herbal medicines are usually associated with contamination, adulteration, misidentification, complexity and non-uniformity nature of herbs (Dog, 2010).

Misidentification of herbs can occur due to use of similar names, similar appearances, and confusion of terminologies and different languages of different countries (Efferth & Kaina, 2011). Herbal medicines are also subjected to contamination by heavy metals, soils, pesticides, microbes and other foreign matter (Murali, 2017). Adulteration in herbal medicines is usually reported due to the addition of active pharmaceutical ingredients such as steroids and sildenafil or by substitution with other plant materials (Zhang et al., 2012). On the other hand, the complexity and non-uniformity of plant extracts have contributed to varying bioavailability and bioactivity which consequently affect the quality of herbal medicines (Mukherjee, 2015). These quality issues will subsequently affect the safety and efficacy of the herbal medicines (Wachtel-Galor & Benzie, 2011).

Some herbal plants are reported to possess intrinsic toxicity either at a normal dose or over dose. Adverse reactions associated with *Ephedra*, *Aristolochia*, and *Aconitum* have shown that herbs can produce toxicity in humans (Junhua Zhang et al., 2015). *Aconitum* species are useful for treating a diverse range of conditions from rheumatoid arthritis, collapse, gastroenteritis to bronchial asthma but contain highly toxic cardiotoxins. Adverse reactions include numbness of the mouth and tingling of the hands and feet, nausea, vomiting, dizziness, hypotension, ventricular tachycardia, torsades de pointes and heart block which can lead to death (Shaw, 2010). *Aristolochic acid* (AA) found in *Aristolochia* species is nephrotoxic and carcinogenic in humans and animals (Asif, 2012). *Ephedra sinica* contains *ephedrine* alkaloids which is a useful herb for treating asthma, cough and wheezing. Toxic effects include excitability, insomnia, nausea, poor appetite, increased blood pressure, cardiac arrhythmia and convulsions (Lee et al., 2000).

2.2 *Acorus Calamus*

Acorus calamus is a tall, perennial, semi-aquatic plant from the *Acoraceae* family and the genus of *Acorus*. The species is differentiated based on the genome differences (Motley, 1994). There are about 40 species of *Acorus* and one of the most studied species is *Acorus calamus* (Ganjewala, 2011). Other species that have been investigated for its medicinal value includes *Acorus christophii*, *A. tatarinowii* and *A. gramineus* (Rajput et al., 2014; Yang et al., 2013; Kim et al., 2012). It has a long history of use and has numerous traditional and ethnomedicinal applications. It is known by a variety of names, including deringau, jerangau, jerangoh, shicangpu, rat root, cinnamon sedge, flag root, gladdon, myrtle flag, myrtle grass, myrtle sedge, sweet cane, sweet myrtle, sweet root, sweet rush and sweet sedge (Balakumbahan, 2010). Sweet flag can be found throughout the regions of Eurasia, the Americas and Southeast Asia (Jeon et al., 2013).

Since ancient times, it has been used in various systems of medicines such as Ayurveda, Unani, Siddha and Chinese medicine (Rajput et al., 2014). Traditionally it has been used topically as soothing agent or cream to treat fever, joint pain, lumbago, sore eye, malaria and postnatal (Forest Research Institute Malaysia, 2010). It has also been used for the treatment of various ailments like nervous disorders, appetite loss, bronchitis, chest pain, colic, cramps, diarrhoea, digestive disorders, flatulence, gas, indigestion, rheumatism, sedative, cough, fever, bronchitis, inflammation, depression, tumours, haemorrhoids, skin diseases, numbness, general debility and vascular disorders. Various therapeutic potentials of this plant have been attributed to its rhizome (Rajput et al., 2014).

Later on, more studies have been conducted on *Acorus calamus* to discover the specific pharmacological activity that can be obtain from the leaves, roots and stem of the plant (Balakumbahan, 2010). These studies have demonstrated that *Acorus calamus*

exhibit potential pharmacological activities such as sedative, central nervous system depressant, anticonvulsant, antispasmodic, cardiovascular, hypolipidemic, immunosuppressive, anti-inflammatory, cryoprotective, antioxidant, antidiarrheal, antimicrobial, anticancer and antidiabetic (Rajput et al., 2014).

2.3 Beta- and Alpha-asarone

The chemical compound derived from the rhizomes, leaves and essential oil of *Acorus calamus* varies depending on geographical pattern and grade of polyploidy (Wang et al., 1998). Higher level of polyploidy has higher content of beta-asarone (Du et al., 2008, Radušienė et al., 2007). Active compound that has been identified includes essential oil phenylpropanoids (e.g. beta-asarone, alpha-asarone, eusarone, methyleugenol, and asaryldehyde), terpenoids (e.g. caryophyllene, humulene and safrole), fatty acids and phenolic compounds (Rajput et al., 2014; Yu et al., 2011). Most of the plants found in Europe and temperate Asia primarily contain triploid. The essential oils of the tetraploids have higher (70–96%) beta-asarone content than the triploids (5–19%) (Rana et al., 2013). High concentration of beta-asarone can be obtained from the essential oils (9-19%) compared to dried rhizome (0.3%) (European Medicine Agency, 2005). Micellar electrokinetic capillary chromatography (MEKC) extraction of dried rhizome produce 0.2 to 4.4% w/w of beta-asarone (Hanson et al., 2005). Beta-asarone contents from rhizome are 0.23246 µg/g by HPTLC fingerprinting (Malik et al., 2014).

Beta- and alpha-asarones are cis- and trans-isomers as illustrated in Figure 2.1. The trans-asarone or alpha-asarone chemically known as (E)-1,2,4-trimethoxy-5-(prop-1-en-1-yl) benzene while cis-asarone or beta-asarone known as (Z)- 1,2,4-trimethoxy-5-(prop-1-en-1-yl) benzene (Hamil et al., 2016). Beta-asarone is labile and can be easily converted into alpha-asarone by oxidation. In *vitro*, the pure form of beta-asarone is

stable but in aqueous solution it is easily converted into alpha-asarone after leaving the solution at room temperature and in light conditions for 3 months (Sinha et al., 2003). It is not an enzymatic transformation as there are no enzymes present in the beta-asarone solution (Hayat, 2015).

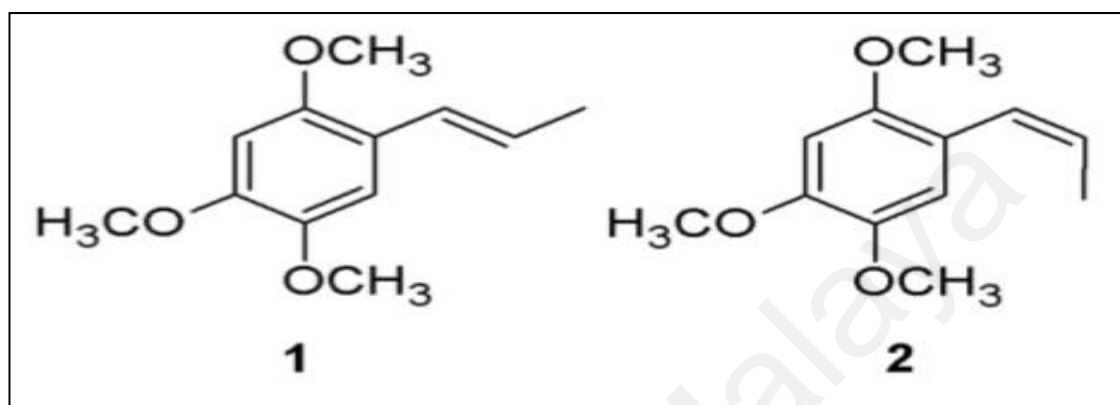


Figure 2.1: Chemical structure of alpha-asarone (1) and beta-asarone (2)

Beta- and alpha-asarone are practically insoluble in water as they are soluble in alcohol, ether, glacial acetic acid, carbon tetrachloride, chloroform and petroleum ether (O'Neil et al., 2013). The beta-asarone is a strong fat-soluble substance with a small molecular weight (208), which can go through the Blood Brain Barrier (BBB) rapidly. These results suggest that beta-asarone might be a potential candidate for the development of a therapeutic agent to manage cognitive impairment associated with conditions such as Alzheimer's disease (Jicheng et al., 2010; Li et al., 2010). The many pharmacological activity observed from the use of *Acorus calamus* rhizomes is mainly because of the action of beta-asarone and alpha-asarone (Rajput et al., 2014).

2.4 Toxicity of alpha- and beta-asarone

Despite of various benefits from the *Acorus calamus* rhizome, there are reports that the main compound of the plant, beta- and alpha-asarone can induce toxicity, carcinogenicity, mutagenicity and cytotoxicity. *In vitro* tests using HepG2 cells (liver hepatocellular cells) demonstrate that beta- and alpha-asarone induce cytotoxicity in the

BrdU (Bromodeoxyuridine) assay (Liu et al., 2013). However only beta-asarone induces genotoxicity in the same report. Alpha-asarone was found to be more cytotoxic compared to beta-asarone (Unger & Melzig, 2012). Beta asarone administered at a dose of 50 mg/kg of body weight was responsible for inducing certain noticeable degenerative changes in histopathological analysis of the mice tissue (Benny et al., 2016). This was supported by altered sperm morphology and hormonal variations when compared to the control groups (Toshimori, 2003). Antioxidant enzyme levels were also found to be decreased (Manikandan & Devi, 2005). Cell-based investigations in THLE-2 cells (human liver cell) confirmed the cytotoxicity of beta-asarone ($IC_{50} = 40.0 \pm 2.0 \mu\text{g/mL}$) which was associated with significant lipid peroxidation and glutathione depletion (Patel et al., 2015). Alpha- and beta-asarone are genotoxic based on the ability of these compounds to induce unscheduled DNA synthesis (UDS) in hepatocytes derived from male Fischer 344 rats (Hasheminejad & Caldwell, 1994). It was demonstrated that beta-asarone has a weak but statistically significant hepatocarcinogenic activity on mice (Wiseman et al., 1987) and mutagenic activity has been detected with *in vitro* studies (Göggelmann & Schimmer, 1983).

In Sweden, intake of herbal preparations containing high concentration of *Acorus calamus* causes prolonged vomiting up to 15 hours in 7 consumers (Björnstad et al., 2009). In one study reported by Zuba and Byrska (2012), a woman experienced symptoms as 'intoxication by excessive dose of amphetamine-like substance' such as tachycardia, dizziness, tremor, irregular breathing, pallor, anxiety, nausea and vomiting after consuming natural herbal products from a traditional Chinese medicine clinic. Toxicological test of tablets and pellets seized revealed alpha- and beta-asarone as major active ingredient instead of suspected drugs such as amphetamine, methamphetamine, cocaine, ecstasy or 3,4-methylenedioxymethamphetamine (MDMA), ephedrine, pseudoephedrine and atropine (Appendino et al., 2014).

2.5 Extraction of alpha- and beta-asarone

Alpha- and beta-asarones are volatile compounds which can be extracted from plant rhizomes and herbal preparation by steam distillation, solvent immersion extraction, solid phase extraction (SPE), and sonication extraction (Deng et al., 2004). Yu et al. (2011) compared three extraction methods which are solvent immersion extraction, sonication extraction, and shaking methods. The study demonstrates that sonication method is the most efficient method which requires shorter extraction time and less processing steps compared to the other methods. Other advantages of sonication method is that it uses less organic solvent for its rapid extraction and no heating is required (Yang et al., 2011).

Besides extraction method, extraction solvent is a critical factor in obtaining good chromatographic fingerprint. Methanol has been used in several studies due to its good dissolving capabilities and high extraction efficiency (Hanson et al., 2005; Patel et al., 2015; Wang et al., 2014; Zuba & Byrska, 2012). Other extraction solvents that have been used to extract beta-asarone from *Acorus calamus* rhizome include hexane, ethanol, petroleum ether, n-hexane, n-butanol, chloroform, ethyl acetate and acetone (Nath & Yadav, 2016; Yu et al., 2011).

2.6 Analytical Method

Analysis of asarone has been performed by thin layer chromatography (TLC), high performance liquid chromatography (HPLC), high performance thin layer chromatography (HPTLC), liquid chromatography–tandem mass spectrometric (HPLC-MS/MS), gas chromatography-mass spectrometry (GC-MS) and micellar electrokinetic capillary chromatography (MEKC) (Hanson et al., 2005; Nandakumar, Menon, & Shailajan, 2013; Patel et al., 2015). Each system is different in terms of sensitivity, cost, complexity, use of derivative agents and total running time (Shailajan et al., 2015).

Recent studies suggest that HPLC and GC-MS are two most frequently used techniques for the analysis of alpha- and beta-asarone.

2.7 Thin Layer Chromatography (TLC)

TLC is a simple, inexpensive and robust means for the chemical and biological screening of plant extracts (Hostettmann et al., 2001). One of the main advantage is the ability for multiple sample analysis to be studied simultaneously in a single run (Kamboj, 2012). However, TLC was found to be unable to differentiate isomers and closely related substances (Zuo et al., 2012). Deficiencies of one method can be improved by hyphenated techniques (Bagetta et al., 2011). For example, isolation and screen of antifungal compounds (AFC) in plant extract was first carried out by TLC. Then the AFC were elucidated and identified by using GC-MS (Lanka, 2015). TLC is more likely to be less sensitive compared to GC-MS as there is need for spots optimization and elimination of interferences (Oprean et al., 1998).

2.8 High Performance Liquid Chromatography (HPLC)

HPLC is a versatile, robust, and widely used technique to identify, quantify and purify multiple components of herbal medicines (Boligon & Athayde, 2014). In the analysis of beta-asarone and alpha-asarone, HPLC has been a popular choice due to its simplicity, cheap and rapidity. Beta- and alpha asarone can be separated and identified within four to five minutes of analysis (Perrett & Whitfield, 1995; Shailajan et al., 2015). However, the run time could also be longer up to 20 minutes as well (Lu et al., 2009; Zuba & Byrska, 2012). In HPLC, the use of single wavelength UV detector will not be able to analyse non-chromophoric compounds in herbal medicines (Kamboj, 2012). Thus, LC-MS techniques can be a viable modern alternative and powerful technique for the characterization of these compounds (Barbarin et al., 2002). The mass

analysis capabilities of LC-MS is used for many applications which has very high sensitivity and selectivity (Kamboj, 2012).

2.9 Gas Chromatography Mass Spectrometry

Selection of method of choice for analysis of desired active compounds depends on its physical and chemical properties. It is well-known that many pharmacologically active components in herbal medicines are volatile chemical compounds including alpha- and beta-asarones (Sharma et al., 2014). GC-MS is an important and frequently used technique for separating and identifying the components of complex volatile mixtures (Folashade et al., 2012; Xie et al., 2013). GC-MS has been widely used due to its high sensitivity and selectivity (Wang et al., 2014; Yu et al., 2011; Zuba & Byrska, 2012). Its high selectivity is due to the use of capillary columns which enables simultaneous separation of many volatile compounds within short period (Srivastava, 2010). High sensitivity of GC-MS is largely due to the use of hyphenation technique mass spectrometry (MS). It improves detection limit and reduces analysis times by using rapid temperature programming and fast data acquisition by flame ionization detector (FID) and high split ratio (Bansal et al., 2014).

Additionally, alpha- and beta-asarone are isomers with similar mass spectra and these characteristic make the identification of these compounds difficult without reference compounds (Oprean et al., 1998). Similar mass spectra will lead to wrong identification. Hence, it is important that these isomers are separated and purified before analysis is performed. This can be achieved with GC-MS which contribute better separation for many organic compounds. The use of typical TLC will not be able to adequately resolve closely related substances and isomers. (Zuo et al., 2012).

Based on literature search, many studies have used GC-MS for the analysis of alpha- and beta-asarone from plant extracts and herbal medicines. The changes made to the

instrument parameters were crucial in getting a rapid analysis. With different system applied in the studies reported, most of the studies preferred using helium as the gas carrier with the flow rate of 1 mL per minute. Most of the system uses capillary column of 30m length, 0.25 μ m column thickness and 0.25mm diameter. Rapid analysis of beta- and alpha-asarone can be achieved by using high rate of temperature increment. At the rate of 25°C per minute, the retention time achieved for beta-asarone is 6.3 minute compared to 40 minute retention time achieved with 5°C increment per minute (Oprean et al., 1998; Patel et al., 2015). Maximum temperature can be set up to 300°C without exceeding the threshold of the column properties and boiling point of asarones. Other parameters that have been seen common among studies include the use of 70 eV of ionisation energy and 40 to 500 amu of mass spectrometry range (Raal et al., 2016).

2.10 Caffeine (Internal standard)

Internal standard is a compound added into a sample with a known concentration in order to facilitate quantitation of targeted compound in the sample (McNaught & Wilkinson, 1997). It is used to improve the precision and accuracy of results where volume errors are difficult to predict and control. It can significantly improve the precision of a chromatographic method by correcting different sources of volume errors, including injection-to-injection variation, volume errors in sample preparation, and accounts for routine variations in the response of the chromatographic system (Hansen et al., 2015).

The internal standard component must not be contained in the sample and need a good separation between the internal standard and sample components (Zheng et al., 2001). Selected compound used as internal standard should have similar characteristic as to the analyte (Skoog et al., 2017). The signal between the internal standard and the

analyte must elute high similarities of signal yet are readily distinguishable by the instrument (Oliveira et al., 2010).

Several studies have used internal standard in their method for the analysis of beta-asarone. Caffeine as internal standard has been successfully used in the quantitation of beta-asarone from plasma of female rat using HPLC-MS/MS method (Nandakumar et al., 2013). 4-methoxybenzophenone has been used as an internal standard to establish a LC-MS/MS method for the quantitation of alpha-asarone in rabbit plasma (Chen & Zai, 2006). Eugenol which have similar structure properties as asarone has been used in GC-MS analysis of beta-asarone in *herbal medicines* (Oprean et al., 1998; Patel et al., 2015). In determination of alpha-asarone in *Acorus tatarinowii Schott.* rhizome by GC-MS, n-octadecane, has been used as internal standard to improve precision and accuracy of the method (Xi et al., 2012).

It is best to choose internal standard that contains similar functional groups, boiling points, and activity as the target compounds (Karmen et al., 1963; IOFI Working Group on Methods of Analysis, 2011). It is more important that the chosen compound can be applied on to the method developed in such a way that separation can occur within the analysis period and able to provide constant and accurate value during analysis.

2.11 Limit of Alpha- and Beta-asarone.

Due to reported toxicity and carcinogenicity of asarones, national regulatory authorities have taken different approach in regulating the use of asarone particularly in medicines. One of the factors that can affect the ruling on asarone is the availability of the concerned substance in the nation's market. The Complementary Medicines Evaluation Committee (CMEC) of Therapeutic Goods Administration (TGA) of Australia concluded that no regulatory action is required since most of the herbs supplied to Australia do not contain asarone (Therapeutic Goods Administration, 2004).

Some countries have put restrictions or limits on the content of asarone that is allowed in herbal medicines. In 2002, Committee of Experts on Flavouring Substances (CEFS) of the Council of Europe have proposed that the limits of beta-asarone in food and beverages is 0.05 mg/kg and for alcoholic beverages is 0.5 mg/kg (European Commission, 2002). This is due to the findings that beta-asarone was clearly carcinogenic in rodents and potentially genotoxic. Based on the rules that are imposed for food and beverages, the CEFS has applied a limit of exposure for beta-asarone of approximately 115 µg/day (2 µg/kg bw/day) in herbal medicine products (European Medicine Agency, 2005). Similarly, Natural Health Products Directorate (NHPD) of Canada has established that any finished product should not contain more than 10 mg/kg beta-asarone in dried rhizome of *Acorus* species or the daily dose of beta-asarone should not exceed 115 µg (Natural Health Products Ingredients Directorate, 2017).

In the United States of America, it is totally prohibited to use any part of *Acorus calamus* rhizome as an ingredient of food or herbal medicine (US Food & Drug Administration, 2014). The Food and Drug Administration (FDA) is convinced that the toxicity and carcinogenicity of asarone has been established through the reported studies.

Herbal medicine including traditional products in Malaysia is regulated by National Pharmaceutical Regulatory Agency (NPRA). Prior registration, traditional product is tested for quality, heavy metal, disintegration, uniformity of weight and microbial contamination (Ministry Of Health Malaysia, 2015). Currently no ruling is imposed for products containing asarone or *Acorus* species. Traditional product registered in Malaysia may contain *Acorus calamus* and the asarone dose level may exceed 115 µg per day.

CHAPTER 3: METHODOLOGY

3.1 Introduction

Every traditional product that is to be marketed in Malaysia require approval license from the Ministry of Health Malaysia (Ministry Of Health Malaysia, 2015). Information on traditional products that contain *Acorus calamus* has been obtained from the Ministry of Health Malaysia database. Products were purchased from alternative medicine's shops in the city of Selangor and Kuala Lumpur. These products were in the form of capsules, tablets, topical oil and topical ointment. Two raw materials of *Acorus calamus* rhizome in powder form has been purchased from two different stores in Kuala Lumpur. The products and raw materials are subject for extraction by ultrasonication method. Liquid-liquid extraction was also performed on products when direct extraction failed.

GC-MS method has been developed by modification of methods gathered from the literature studies. The products and raw material are screened for alpha- and beta-asarone by running scan mode and then compared with those of reference standards. Caffeine is used in this method as an internal standard. From the mass spectra obtained, three ions specific to alpha- and beta-asarone were selected to be used in Selected Ion Monitoring (SIM) mode for quantification purposes. Summary of methodology is shown in Flowchart in Figure 3.1.

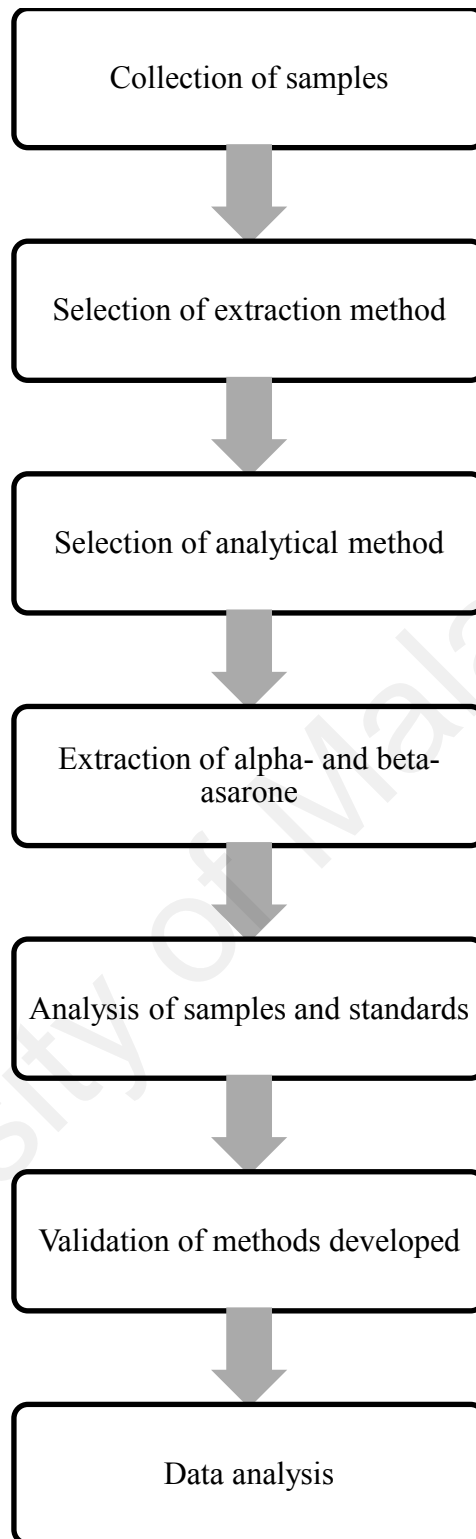


Figure 3.1: Flowchart of methodology

3.2 Chemical and Reagents

Beta-asarone reference standard was purchased from Sigma-Aldrich, Germany. Alpha-asarone reference standard was obtained from Toronto Research Chemical, Canada. Caffeine anhydrous standard is the NPRA secondary reference standard. Methanol used is HPLC Grade from J.T. Baker, USA.

3.3 Samples

Acorus calamus rhizome was purchased from two different traditional Chinese herbal shops located in Kuala Lumpur. The herbs were dried and ground into powder form. The dark green brownish powder is stored in amber bottles and kept refrigerated at 4°C. Nine traditional products that contain alpha- and beta-asarone were purchased in traditional herbal shops around Selangor and Kuala Lumpur within the period of January to June 2015. The selection of samples is simply based on current availability in the market at the time. Database of registered traditional products was acquired from the official website of the National Pharmaceutical Regulatory Agency, Ministry of Health Malaysia. Three products in capsulated powder form, one product in tablet form, three product in oil form and two products in the form of topical ointment were obtained. The content of *Acorus calamus* rhizome in each product varies. The list of samples used for this research is listed in Table 3.1. These products were stored in its original container kept in cabinet at room temperature and protected from light.

Table 3.1: List of samples

| No. | Name of sample | Sample Code | Dosage Form | Content of <i>Acorus calamus</i> (%) |
|-----|---------------------------------|-------------|-------------|--------------------------------------|
| 1. | <i>Acorus calamus</i> rhizome 1 | ACR1 | Powder | - |
| 2. | <i>Acorus calamus</i> rhizome 2 | ACR2 | Powder | - |
| 3. | Kapsul Tongkat Ali Hitam Plus | KTAH | Powder | 20 mg per 250 mg (8%) |
| 4. | Dinars Binti Herbal | DINR | Powder | 30 mg per 350 mg (8.5%) |

| | Formula | | | |
|-----|--|------|--------|-------------------------|
| 5. | Amme Complete | AMM | Powder | 30 mg per 360 mg (8.3%) |
| 6. | Abana Tablets | ABNA | Powder | 5 mg per 700 mg (0.7%) |
| 7. | Minyak Gamat Serai Petani Plus | MGSP | Oil | 5 g per 60 mL (8.3%) |
| 8. | Ubat Tradisional Minyak Gamat Asli Langkawi Plus | MGAL | Oil | 3g per 60mL (5%) |
| 9. | Minyak Angin Petani | MAP | Oil | 1 g per 30 mL (12%) |
| 10. | Bam Gamat Serai Petani Plus | BGSP | Oil | 50 mg per 23 g (0.22%) |
| 11. | Salap Petani | SP | Oil | 2 g per 23 g (8.7%) |

3.4 Standard Preparation

Five milligram of alpha- and beta-asarone standards each was accurately weighed and each standard was transferred to a 10 mL standard volumetric flask. The content of the flask was initially dissolved in a minimum quantity of HPLC grade methanol, followed by sonication and then diluted up to the mark with methanol. A stock solution mixture of alpha- and beta-asarone standard with concentration of 100 µg/mL was prepared. Other standards of solution were prepared by serial dilution of the standard stock solution. Caffeine standard was also prepared with same procedure as the alpha- and beta-asarone stock solution. The working standard solution for caffeine is 100 µg/mL as well. The concentration of caffeine that will be used as internal standard is 10 µg/mL. Stock solution of alpha-asarone, beta-asarone and caffeine were stored in an amber volumetric flask and kept in a refrigerator at 4°C.

3.5 Samples Preparation

Different weight and volume are taken from samples for extraction depending on type and dosage form of samples. Dried rhizome powder of *Acorus calamus* was extracted in methanol in the ratio 0.5:10 (w/v). Tablet form products were crushed using

mortar and pestle. Powder of the tablets and capsule was extracted in methanol in the ratio 2-3: 10 (w/v). In case of oil form products, sample was extracted in methanol in the ratio 1: 10 (v/v). For ointment products, 1 g to 2 g of sample placed on to petri dish and dried in oven (60 °C) up to 4 hours before dissolved with 10 ml of methanol. All sample were extracted for a period of 10 minutes in an ultrasonic bath at room temperature. The sample mixture was then filtered through a 0.45 µm membrane filter and used for GCMS analysis.

3.6 Determination of Alpha- and Beta-asarone by Gas Chromatography-Mass Spectrometry (GC-MS)

Shimadzu gas chromatography-mass spectrometry (Shimadzu GCMS 2010) instrument was used in this study. The column used was non-polar column (ZB-5MS) with the thickness of 0.25 µm, length of 30.0 m and diameter of 0.25 mm. Helium gas was used as carrier set at 1 mL/min. Initially the oven of the GC was set at 80°C and increased to 300°C at the increment rate of 25°C per minute. The final temperature at 300°C was held for 3.2 minutes. The total running time was set at 13 minutes. Sample is injected for 0.2 µL using splitless mode. The mass spectrometer was operated in the electron ionization mode. Mass spectrometer data were recorded between 4.5 – 12 minutes. Full mass spectra, from 50 to 550 *amu* at 1.53 scan s^{-1} , were recorded. From the mass spectra of reference standard, three ions will be selected in SIM mode for each beta asarones, alpha-asarones and caffeine for quantitation. NIST (National Institute of Standards and Technology) library was used for identification of compounds during analysis. The GC-MS parameters are listed in Table 3.2.

Table 3.2: GC-MS parameters for determination of alpha- and beta-asarone

| | | | | | |
|---|---|----------|-----------|------------|----------------|
| Equipment | Shimadzu GCMS-QP 2010 Ultra with Shimadzu GC-2010 Plus | | | | |
| Column | Zebron™ ZB-5MS w/GUARDIAN™, 5m, GC Cap. Column 30m x 0.25mm x 0.25µm | | | | |
| Injection Temperature | 280°C | | | | |
| Carrier Gas | Helium 99.999% | | | | |
| Carrier Gas Flow | 1.0mL/min | | | | |
| Flow Control mode | Linear Velocity | | | | |
| Mode | Splitless | | | | |
| Injection Volume | 0.2µL | | | | |
| Oven Temperature | Oven Ramp | °C / min | Next °C | Hold (min) | Run Time (min) |
| | Initial | - | 80 | 0 | - |
| | Ramp 1 | 25.00 | 280 | 0.00 | - |
| | Post Run | 25.00 | 300 | 3.20 | 13 |
| Diluent | Methanol HPLC grade | | | | |
| MS Parameters: | | | | | |
| Detector Temperature | MS Source | 250°C | Interface | 280°C | |
| EI Ionization Energy | 70 eV | | | | |
| Selective Ion Monitoring Parameters: | | | | | |
| Full Scan Range | 50 to 550m/z | | | | |
| SIM mode | Beta-asarone & Alpha-asarone:208, 193 and 165m/z | | | | |
| | Caffeine:194, 109 and 55m/z | | | | |
| Threshold | 500 | | | | |
| Solvent Delay | 4.2 minute | | | | |
| Detector Voltage | 0.86 kV +0.20 kV | | | | |
| Start Time | 5.31 min | | | | |
| End Time | 7.46 min | | | | |

3.7 Calibration Curve and Quantitation of Alpha- and Beta-Asarone

Linearity and range is determined by constructing calibration curve from the prepared standard solution. Seven standard concentrations of beta-asarone and alpha-asarone (1 µg/mL, 5 µg/mL, 10 µg/mL, 15 µg/mL, 20 µg/mL, 30 µg/mL and 35 µg/mL)

were prepared and injected as per methodology. Caffeine is added to each preparation as an internal standard. The mean area ratio between standard and internal standard is calculated. The mean area ratio of the chromatograms was plotted against the concentration of standards to show the linearity of peak response at this range concentration.

The five concentration of each standard solutions were subjected to regression analysis to calculate calibration equation ($y = ax + b$) and coefficient of determination (R^2), where x is the concentration, y is mean area ratio, a is intercept, and b is slope of the regression line.

Samples are injected as per methodology and quantitation of alpha- and beta-asarone in sample will be calculated by the analytical software based on the ratio of the calibration curve established.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Introduction

This chapter contains the result and discussion part which will discuss on the result of screening for alpha and beta-asarone in traditional products containing *Acorus calamus* rhizome using GC-MS. This chapter will also discuss on the challenges of method extraction used for different form of products. The results presented should be able to verify that this method is suitable for use.

4.2 GC-MS Analysis of Alpha- and Beta-asarone Standard

The standard solution of alpha- and beta-asarone was injected into the GC-MS system according to the described methodology. Chromatogram of standard solution showed peaks corresponding to beta-asarone, alpha-asarone and caffeine eluted at about 5.78 minutes, 6.07 minutes and 6.93 minutes respectively which is illustrated in Figure 4.1 and Table 4.1. All three peaks are adequately resolved from each other.

Table 4.1: Retention time of beta-asarone, alpha-asarone and caffeine standard

| | Retention Time (minutes) | | |
|-------------------|--------------------------|---------------|----------|
| | Beta-asarone | Alpha-asarone | Caffeine |
| Standard solution | 5.78 | 6.07 | 6.93 |

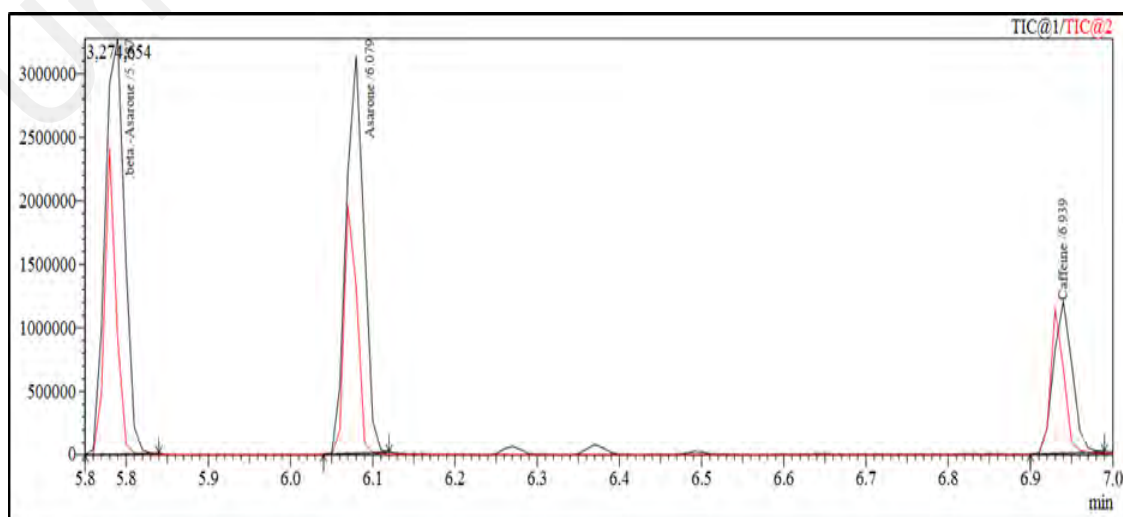


Figure 4.1: Chromatogram of beta-asarone, alpha-asarone and caffeine standard

After running the scan mode, the mass spectrum for each compound was obtained as illustrated in Figure 4.2, Figure 4.3 and Figure 4.4. From the mass spectrum, three ions from each compound were selected to be used in SIM mode for quantitation of each compound. For beta-asarone and alpha-asarone, as they are isomers, the selected ions are the same which are 208, 193 and 165 m/z. For caffeine, the selected ions are 194, 109 and 55 m/z.

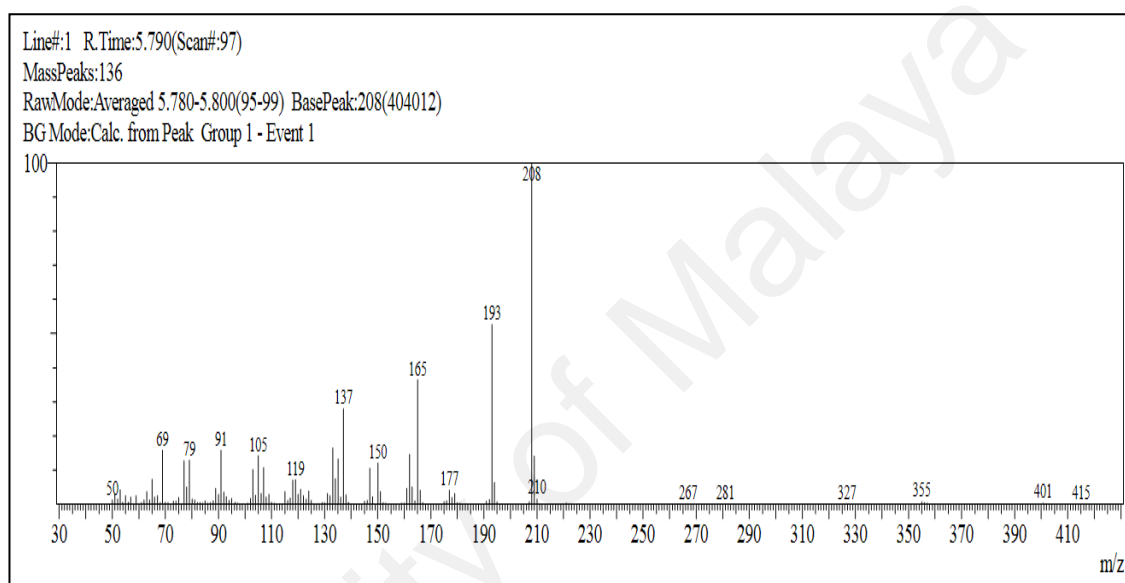


Figure 4.2: Mass spectrum of beta-asarone

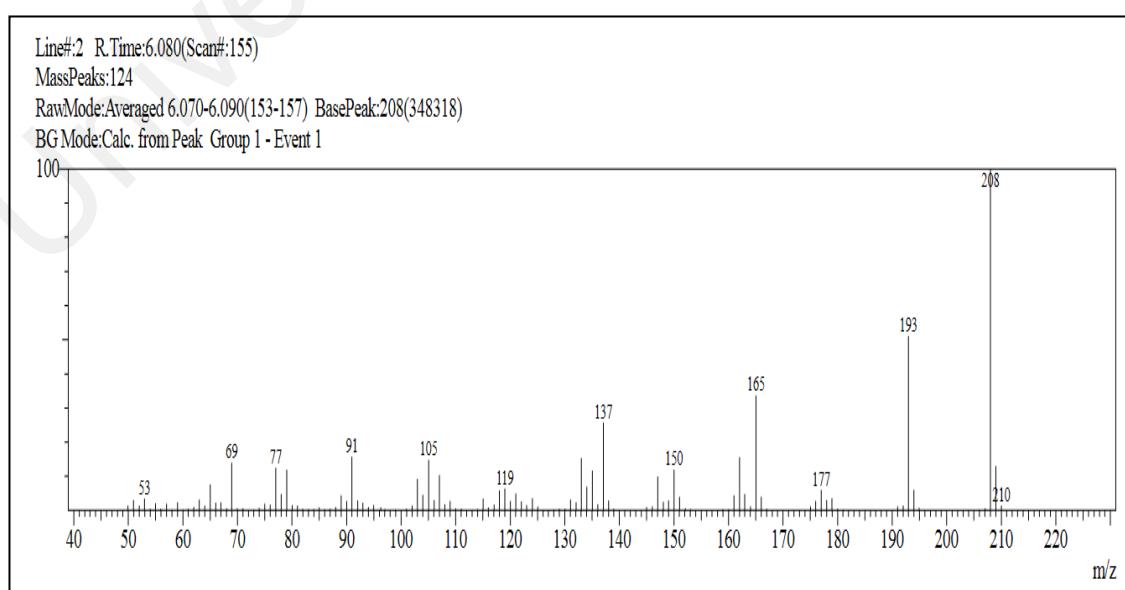


Figure 4.3: Mass spectrum of alpha-asarone

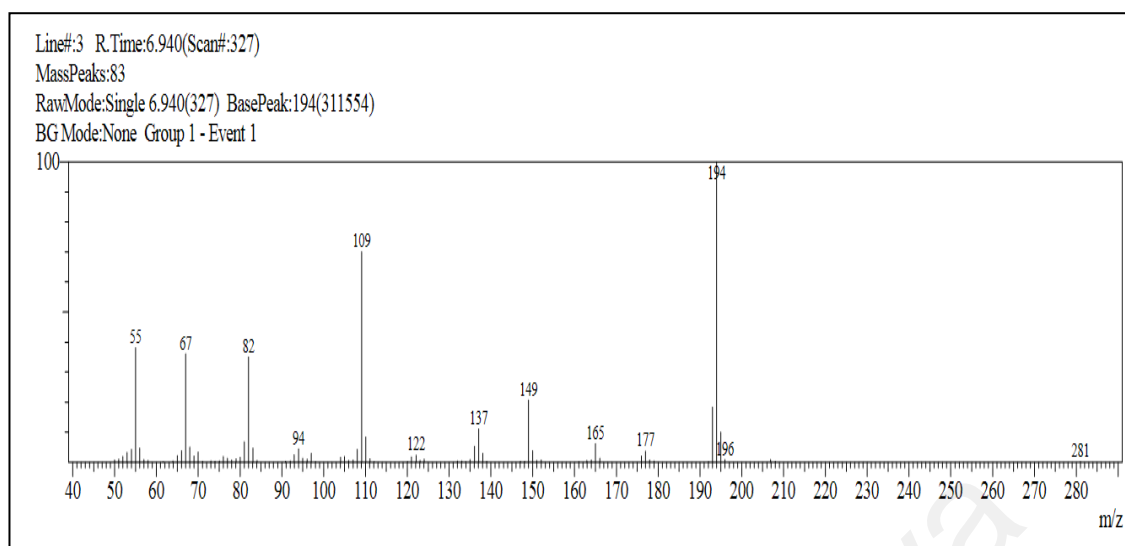


Figure 4.4: Mass spectrum of caffeine

4.3 Calibration Curve of Alpha- and Beta-asarone

The calibration curve constructed for alpha- and beta-asarone show good linearity with the resulting equation of $y = 2.035427x + 0.018018$ and $y = 2.246669x + 0.000896$, respectively. Both equations have the coefficient of determination, R² value of 0.999 that indicates goodness-of-fit of the equation for the concentration of 1 to 35 µg/mL. The results are summarized in Table 4.2 and Table 4.3. The linearity plot is illustrated in Figure 4.5 and Figure 4.6.

Table 4.2: Linearity data for beta-asarone

| No. | Concentration of Beta- asarone (µg/mL) | Concentration ratio | Mean area ratio |
|---|---|---|-----------------|
| 1. | 1 | 0.1 | 0.25 |
| 2. | 5 | 0.5 | 1.15 |
| 3. | 10 | 1.0 | 2.27 |
| 4. | 15 | 1.5 | 3.25 |
| 5. | 20 | 2.0 | 4.56 |
| 6. | 30 | 3.0 | 6.68 |
| 7. | 35 | 3.5 | 7.93 |
| Slope | | 2.035427 | |
| Y-intercept | | 0.000896 | |
| Correlation of determination (R²) | | 0.999 | |
| Acceptance criteria | | Y-intercept must passed zero. The correlation of determination (R ²) is not less than 0.99 | |

Table 4.3: Linearity data for alpha-asarone

| No. | Concentration of Alpha-asarone (µg/mL) | Concentration ratio | Mean area ratio |
|---|--|---|-----------------|
| 1. | 1 | 0.1 | 0.21 |
| 2. | 5 | 0.5 | 1.01 |
| 3. | 10 | 1.0 | 2.09 |
| 4. | 15 | 1.5 | 3.03 |
| 5. | 20 | 2.0 | 4.18 |
| 6. | 30 | 3.0 | 6.05 |
| 7. | 35 | 3.5 | 7.17 |
| Slope | | 2.035427 | |
| Y-intercept | | 0.018018 | |
| correlation of determination (R²) | | 0.999 | |
| Acceptance criteria | | Y-intercept must passed zero. The correlation of determination (R ²) is not less than 0.99 | |

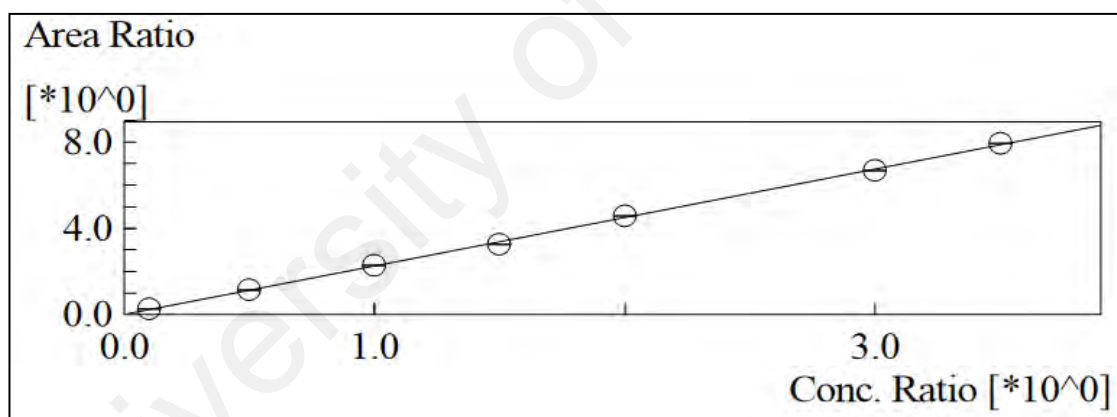


Figure 4.5: Linearity plot for beta-asarone

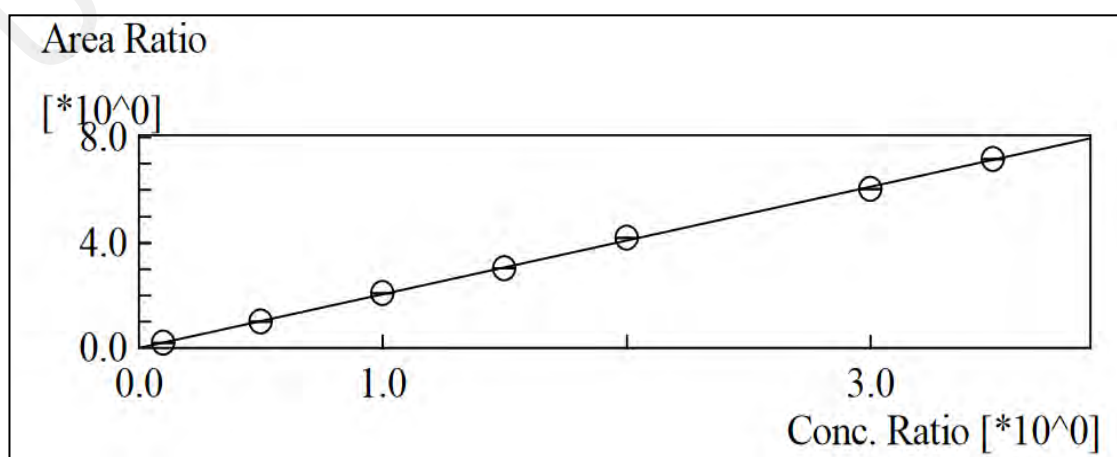


Figure 4.6: Linearity plot for alpha-asarone

4.4 Screening of Alpha- and Beta-asarone in Samples

The result of samples that has been screened and quantified is presented in Table 4.4. From eleven samples screened, alpha- and beta-asarone were only detected in four samples which are two samples of dried *Acorus calamus* rhizome powder and two samples of capsulated powder. Alpha- and beta-asarone was not detected in the oil base samples.

Table 4.4: GC-MS Analysis of Samples

| No. | Sample code | Sample concentration | Dilution Factor | Average Concentration of beta-asarone ($\mu\text{g/mL}$) | Average Concentration of alpha-asarone ($\mu\text{g/mL}$) |
|-----|-------------|----------------------|-----------------|--|---|
| 1. | ACR1 | 1.457mg/mL | 1.154 | 12.689 | 2.095 |
| 2. | ACR2 | 1.186mg/mL | 1.154 | 11.480 | 4.089 |
| 3. | KTAH | 25mg/mL | 1.154 | 6.841 | 0.581 |
| 4. | DINR | 35mg/mL | 1.154 | Not detected | Not detected |
| 5. | AMM | 36mg/mL | 1.154 | 0.615 | 0.508 |
| 6. | ABNA | 70mg/mL | 1.154 | Not detected | Not detected |
| 7. | MGSP | 20% v/v | 1 | Not detected | Not detected |
| 8. | MGAL | 20% v/v | 1 | Not detected | Not detected |
| 9. | MAP | 20% v/v | 1 | Not detected | Not detected |
| 10. | BGSP | 246.7mg/mL | 1 | Not detected | Not detected |
| 11. | SP | 155.7mg/mL | 1 | Not detected | Not detected |

The chromatogram indicates that the retention time of the targeted compound in samples are equivalent with the retention time of the standards. GC-MS analysis of each sample is presented in Table 4.5, Table 4.6 and Table 4.7.

Table 4.5: GC-MS Analysis of Sample ACR1

| Compound | Retention Time (min.) | Area | Concentration (µg/mL) |
|-----------------|------------------------------|-------------|------------------------------|
| Beta-asarone | 5.78 | 2119880 | 12.073 |
| Alpha-asarone | 6.07 | 162713 | 5.045 |
| Caffeine | 6.931 | 210908 | 10.000 |

Table 4.6: GC-MS Analysis of Sample ACR2

| Compound | Retention Time (min.) | Area | Concentration (µg/mL) |
|-----------------|------------------------------|-------------|------------------------------|
| Beta-asarone | 5.781 | 1081109 | 9.900 |
| Alpha-asarone | 6.073 | 281670 | 3.510 |
| Caffeine | 6.931 | 210908 | 10.000 |

Table 4.7: GC-MS Analysis of Sample KTAH

| Compound | Retention Time (min.) | Area | Concentration (µg/mL) |
|-----------------|------------------------------|-------------|------------------------------|
| Beta-asarone | 5.779 | 1727328 | 5.603 |
| Alpha-asarone | 6.070 | 24159 | 0.508 |
| Caffeine | 6.931 | 1570533 | 10.000 |

The chromatogram of each sample is illustrated in Figure 4.7, Figure 4.8 and Figure 4.9. The chromatogram shows that resolutions between peaks are good with no intervention of unknown peaks.

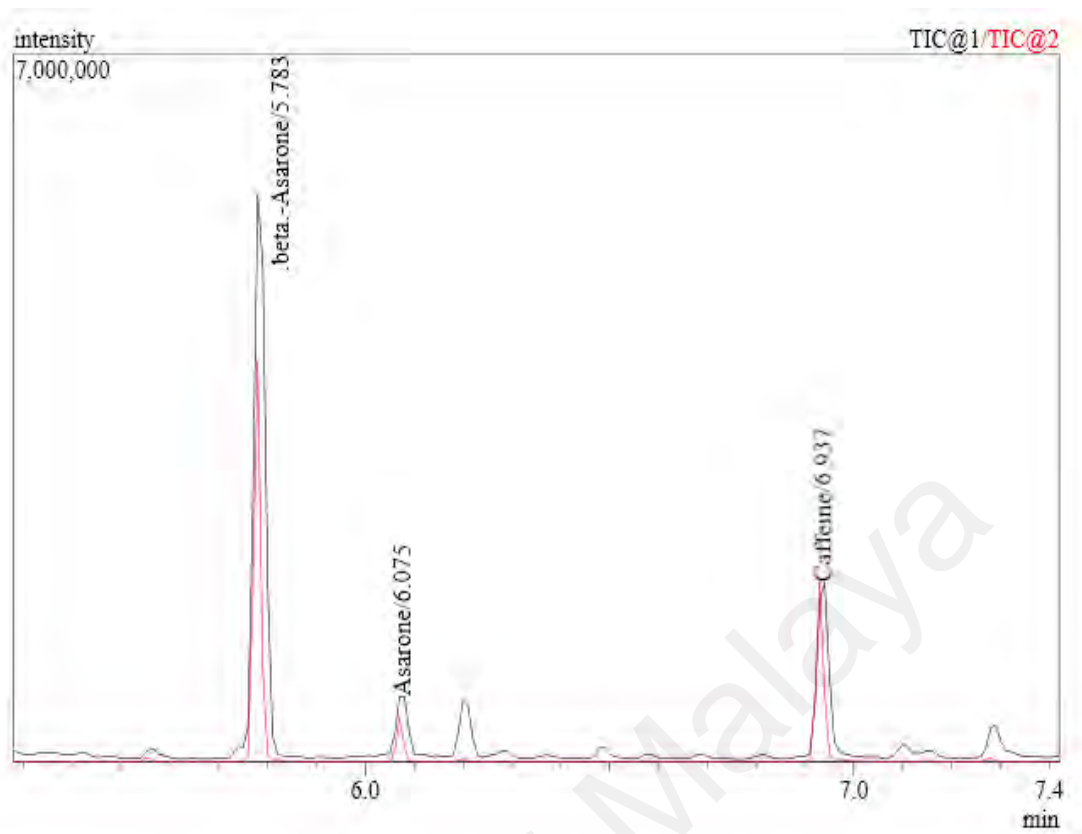


Figure 4.7: Chromatogram of Sample ACR1

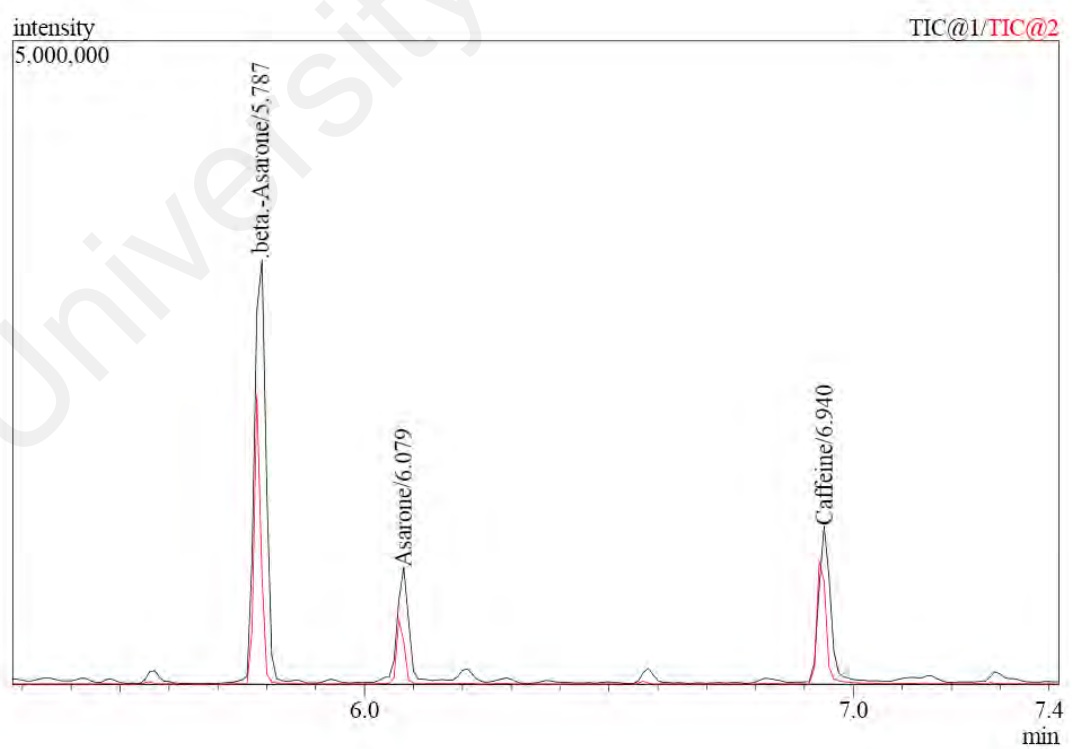


Figure 4.8: Chromatogram of Sample ACR2

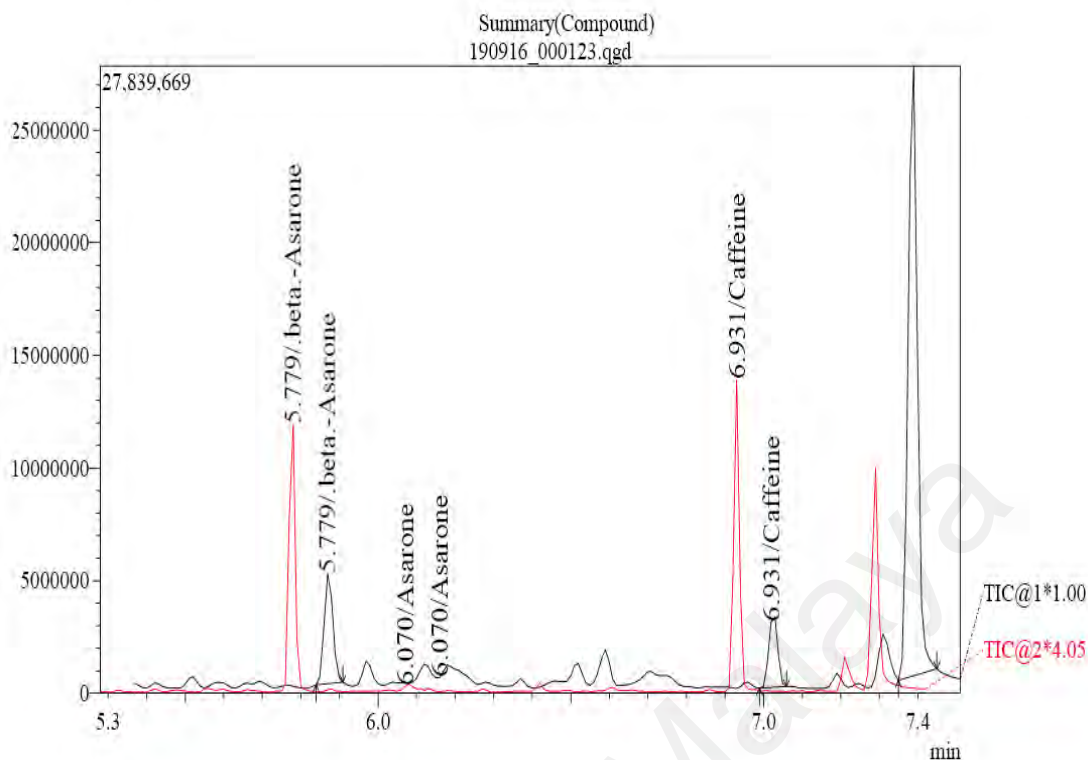


Figure 4.9: Chromatogram of Sample KTAH

The mass spectrum for each active compound was also compared with that of standard as presented in Figure 4.10, Figure 4.11 and Figure 4.12. The three selected ions for each targeted compound has been identified in the mass spectrum of samples and used for quantification.

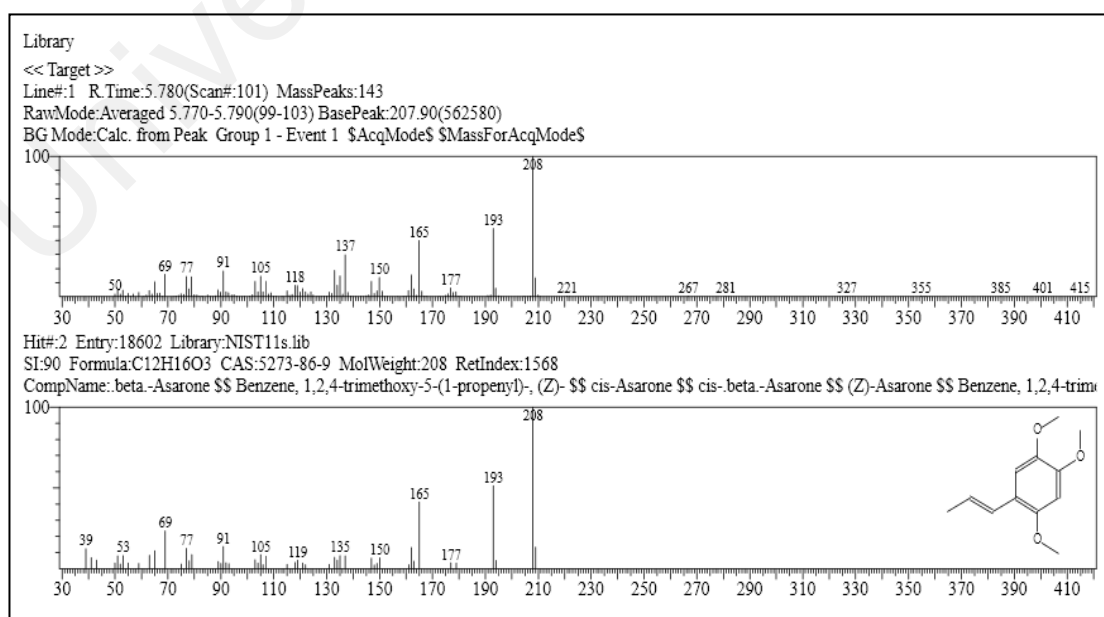


Figure 4.10: Mass Spectrum of Beta-Asarone of Sample KTAH

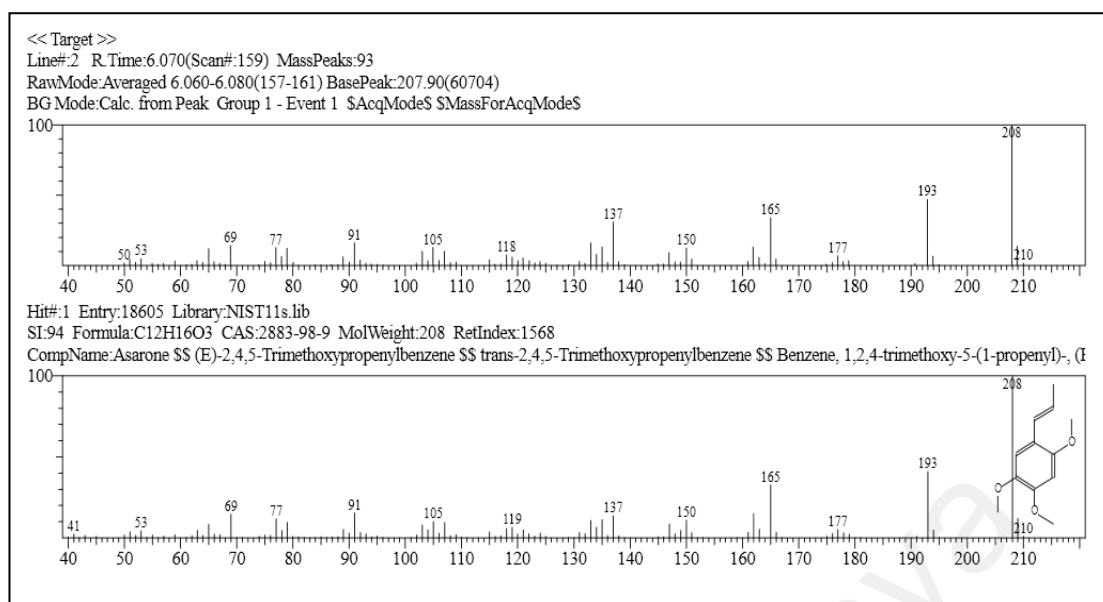


Figure 4.11: Mass Spectrum of Alpha-Asarone of Sample KTAH

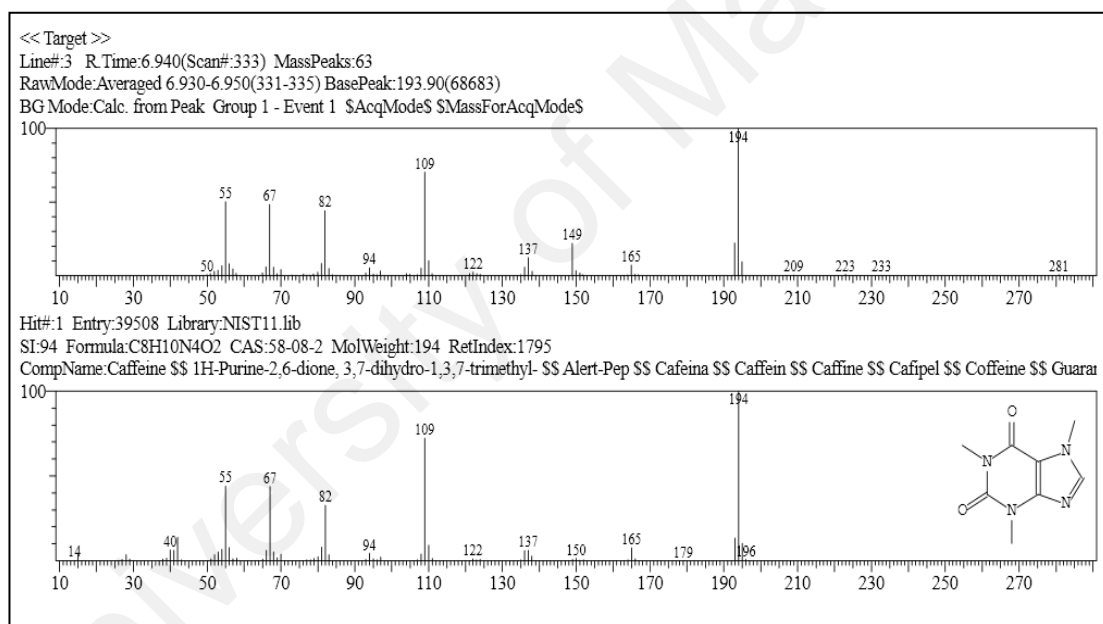


Figure 4.12: Mass Spectrum of Caffeine of Sample KTAH

Based on the GC-MS analysis, beta- and alpha-asarone was detected and quantified in sample ACR1, ACR2, KTAH and AMM. The average concentration of beta-asarone were 12.689 $\mu\text{g/mL}$, 11.480 $\mu\text{g/mL}$, 6.841 $\mu\text{g/mL}$ and 0.615 $\mu\text{g/mL}$, respectively. These concentrations are well within the range of the calibration curve constructed and quantification performed is valid except for the sample AMM. The concentration of beta-asarone in AMM is too low and does not fit into the range of the calibration curve.

Taking into consideration the sample volume and dilution factor, beta-asarone content is calculated as per mg of sample. The average content of beta-asarone per mg of sample was found to be 7.548 μg (0.75%) and 9.68 μg (0.97%) for two samples of dried *Acorus calamus* rhizome powder respectively. This result concurs with results from other studies that *Acorus calamus* rhizome contains between 0.2 to 4.4% w/w of beta-asarone (Hanson et al., 2005). In sample KTAH, the average content of beta-asarone were calculated to be 0.274 μg per mg of sample (0.027%).

For alpha-asarone, the concentration in ACR1, ACR2, KTAH and AMM sample were 2.095 $\mu\text{g/mL}$, 4.089 $\mu\text{g/mL}$, 0.581 $\mu\text{g/mL}$ and 0.508 $\mu\text{g/mL}$. The value obtained from sample KTAH and AMM cannot be used for quantitation as it is lower than the lower limit range of the calibration curve. Thus, the average concentration of alpha-asarone per milligram of sample for two dried *Acorus calamus* rhizome powders calculated are 1.385 μg (0.14%) and 3.320 μg (0.33%).

The total exposure of beta-asarone and alpha-asarone to consumers has also been calculated by taking into account the dose of the sample as stipulated on the label in Table 4.8. As sample ACR1 and ACR2 are raw samples of the *Acorus calamus* rhizome, no specific dosage instruction is included with the sample. However, if applying the limit set by EMA, which is not more than 115 μg of beta-asarone per day, the expected maximum dose of sample 1 and sample 2 are 15.24 mg and 11.88 mg. If sample 1 or sample 2 is used as one of the ingredients of a traditional product, the amount of *Acorus calamus* rhizome included should not exceed the calculated weight. For sample KTAH, the dosage is one capsule per day which equals to 250 mg of sample. It was calculated that the amount of beta-asarone in sample KTAH does not exceed the limit set by EMA (68.4 μg). In Singapore, Patel et al. (2015) reported that ten out of 19 herbal products for children screened contain beta-asarone that exceeds the

115 µg per day limit. The finding suggest that content of beta-asarone is varied in herbal products and consumers in Singapore are high likely to be exposed to beta-asarone compared to Malaysia.

Table 4.8: Estimation of daily exposure levels of beta-asarone in traditional product

| Product | Dose | Daily exposure of beta-asarone based on quantification (µg/day) | Recommended daily allowance as per EMEA guidelines (µg/day) |
|----------------|-----------------------------|--|--|
| ACR1 | Unspecified (15.24mg) | 115 µg/day | 115 µg/day |
| ACR2 | Unspecified (11.88mg) | 115 µg/day | 115 µg/day |
| KTAH | One capsule per day (250mg) | 68.4 µg/day | 115 µg/day |

4.5 Solubility of Alpha- and Beta-asarone

Alpha- and beta-asarone were not detected in the other powder samples and five of the oil base samples. The negative results have led to a series of other small experiments to clarify and discover what may hinder the method from detecting asarones in these products. In order to determine the solubility of asarones in oil, about 100 mg of raw *Acorus calamus* dried rhizome was mixed with Palm oil and placed in water bath (60°C) for 2 hours. This mixture is then filtered and 1mL of the mixture was dissolved with 10mL methanol. The filtered mixture was found to be positive of both asarones as illustrated in Figure 4.13. This result demonstrates that asarone are soluble in oil.

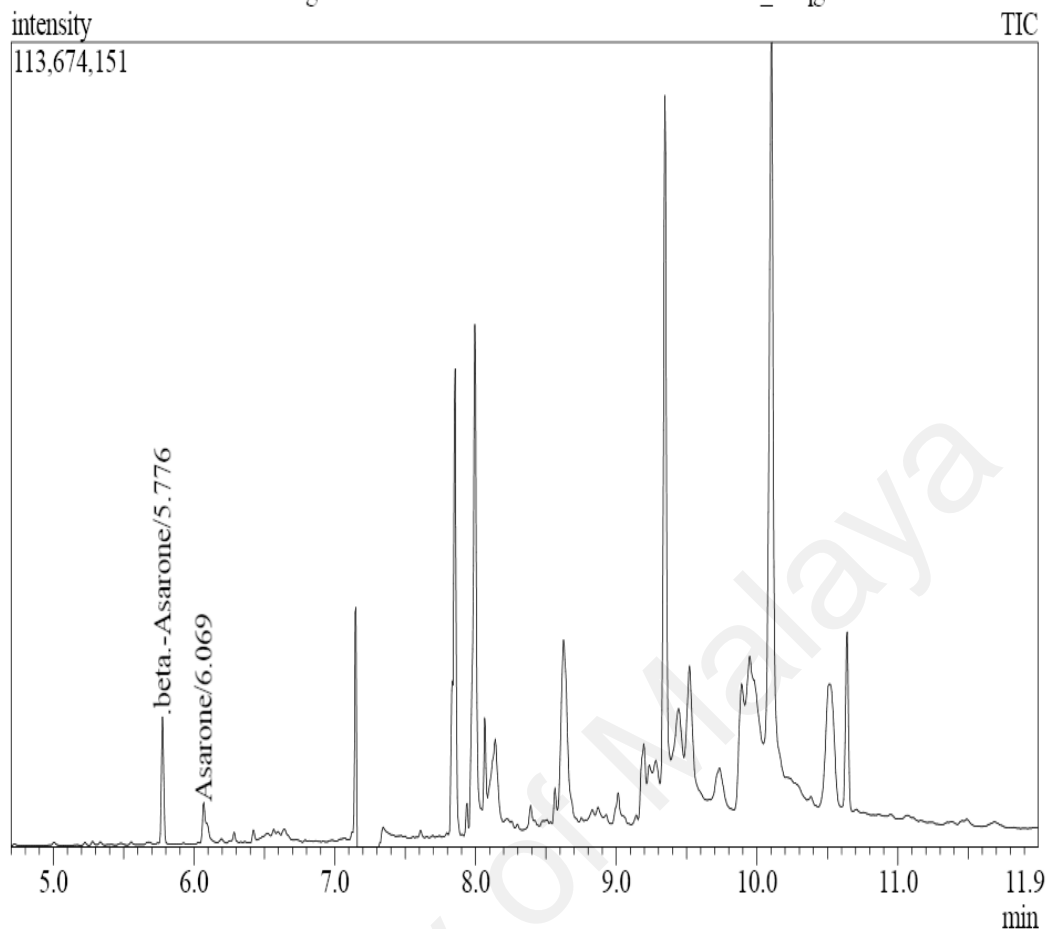


Figure 4.13: Raw *Acorus calamus* rhizome in Palm Oil

This method was also performed using different solvent for extraction which is hexane. Two samples of oil form selected (MGAL and MGSP) were first hydrolysed with 1% v/v of Sulphuric Acid. The hydrolysed sample was then extracted using Liquid-Liquid extraction method with hexane. The collected hexane is then injected as per method. Figure 4.14 and Figure 4.15 shows that the column is saturated which may due to the use of hexane. Beta- and alpha-asarone peak was not detected at the targeted retention times. Thus hexane was excluded as alternative solvent for extraction.

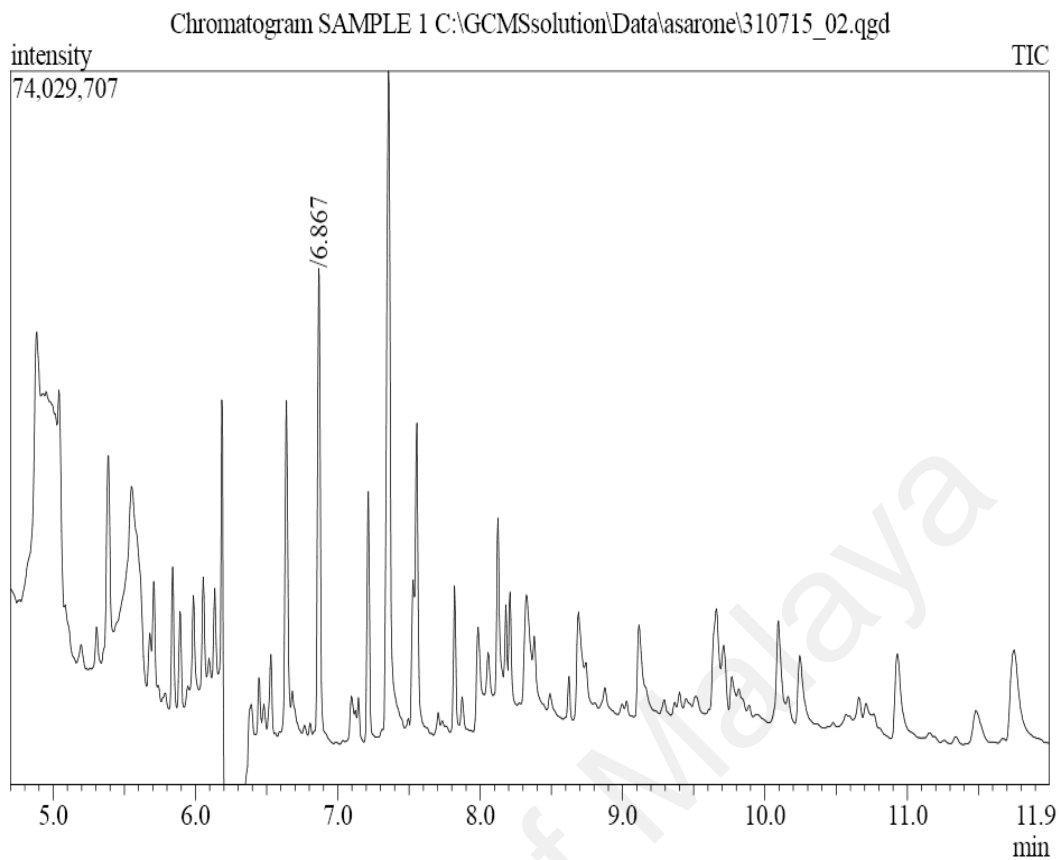


Figure 4.14: Sample 1 in Hexane

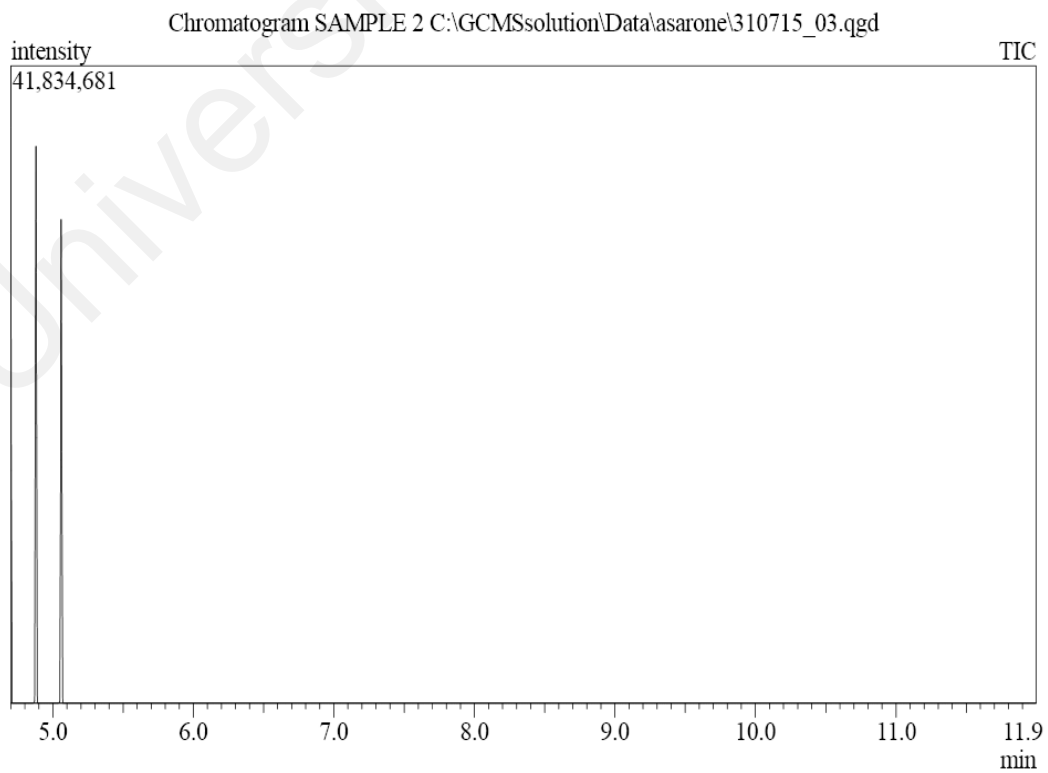


Figure 4.15: Sample 2 in Hexane

4.6 Efficiency of extraction method

Two samples of oil form selected (MGAL and MGSP) were spiked with 5 $\mu\text{g/mL}$ of alpha- and beta-asarones reference standard before extraction. Alpha- and beta-asarone was detected in spiked sample MGAL but not detected in spiked sample MGSP as shown in Figure 4.16 and Figure 4.17. The result suggest that methanol can extract asarone from oil form sample containing *Acorus calamus* rhizome. As reported by Shailajan (2015), oil sample was fractionated in methanol and vortexed for 1 minute before shook at 65 rpm for 6 hours. The mixture was kept at 4°C overnight and filtered through Whattman filter paper no.1 at the following day. Beta-asarone was able to be quantified with this method but longer time for extraction is required. The presence of other ingredient in the sample MGSP may have cause suppression or masking the detection of asarones (Kamboj, 2012).

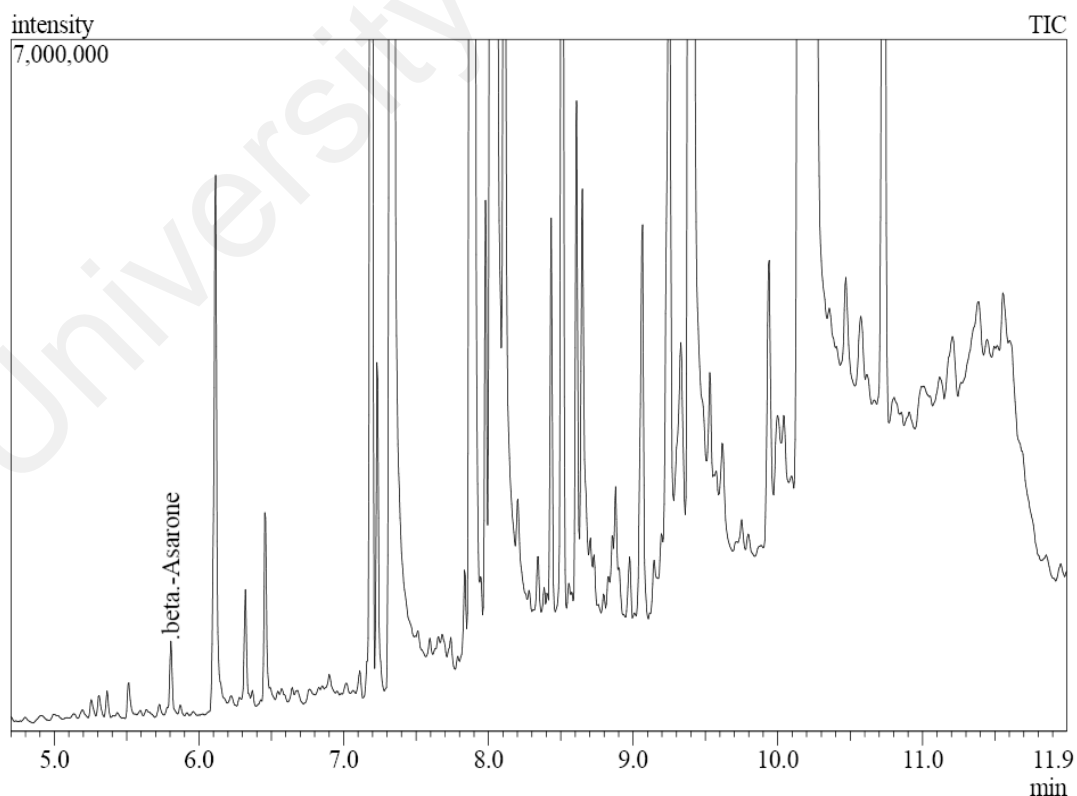


Figure 4.16: Spiked Sample MGAL

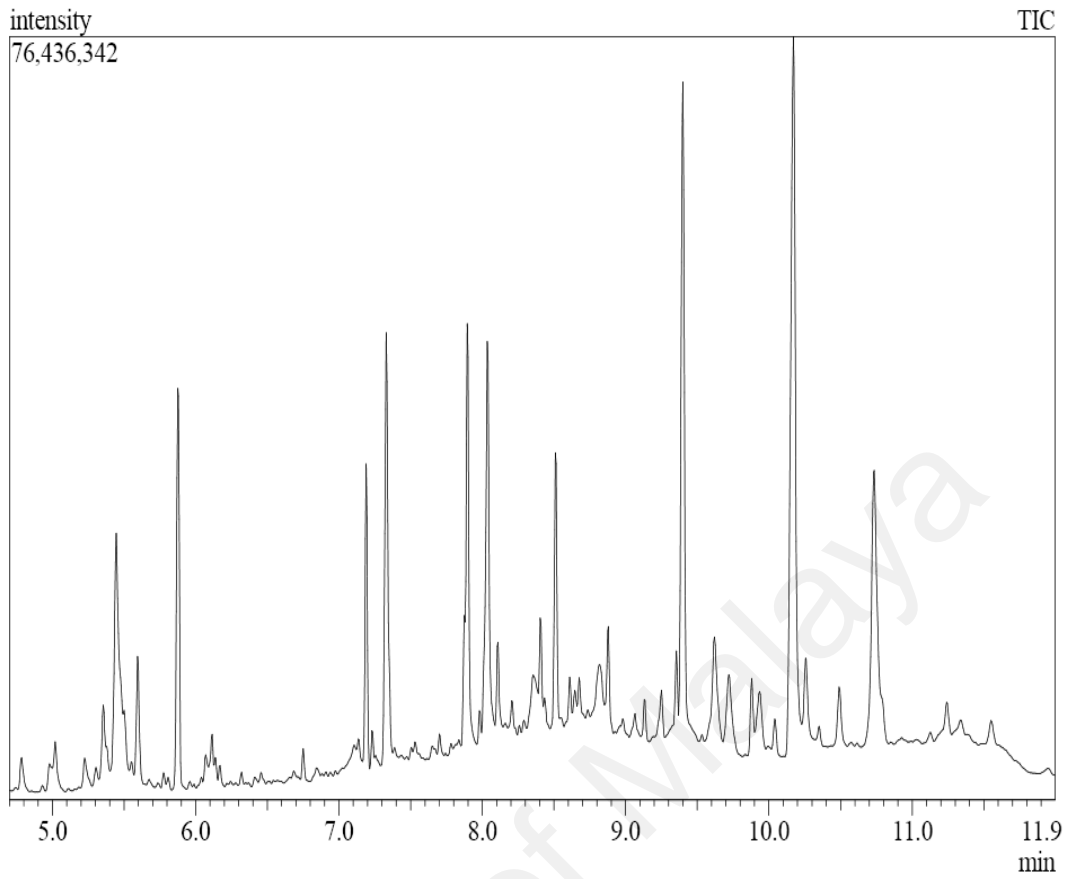


Figure 4.17: Spiked Sample MGSP

CHAPTER 5: CONCLUSION AND RECOMENDATIONS

This study was conducted to analyse a GC-MS method for NPRA using GC-MS to identify and quantify beta- and alpha-asarone in traditional products containing *Acorus calamus* rhizome. The objectives of this research have been fulfilled. This study has demonstrated that alpha- and beta-asarone can be extracted from using sonication method with methanol used as extracting solvent. However, the efficiency of the extraction method varies depending on the dosage form of the samples. The analytical method developed also was able to identify and quantify alpha- and beta-asarone in traditional products containing *Acorus calamus* rhizome that are currently available in the market. It should be noted that alpha- and beta-asarone was only detected in the samples of powder form. Negative results can be due to several factors which include but limited to extraction method and quality of the samples (Huie, 2002; Capasso et al., 2000).

It is preferable that one method can be used to extract alpha- and beta-asarone from all the different form of the samples. However it might not been the case for this research. There are many different extraction methods that can be used on samples of the oil form such as the Soxhlet technique, liquid-liquid extraction method or even the vortex method (Ong, 2004). Currently, most previous studies were performed using powder form samples (Shailajan et al., 2015; Patel et al., 2015). Hence, lack of information regarding the physical and chemical interaction of asarone in oil phase requires a different approach for extraction (Dhanani et al., 2013). It is also possible that due to the volatile nature of the asarone and the content which is too low in the sample, evaporated or loss during extraction process (Charles & Simon, 1990).

Nonetheless, negative result could be partly due to lack of quality control of the sample from the manufacturer. Regulatory requirements for traditional products are

more likely to be less stringent compared to that of pharmaceutical products (Calixto, 2000). Thus this may create loop hole that can be exploited by the manufacturers. One of the scenario could be dishonesty with regard the content of active ingredient where too little or none of the said compound is added in the production perpetuating what has been claimed in the label. Having many different herbs in one product which provide many health benefits is a good selling point when in fact, consumers only receive the placebo effect. The lack of quality control of the raw material may also contribute to this negative result (Patel et al., 2006). Most of the producer or manufacturer of traditional products is small companies, with limited funding and facilities for quality control. Thus, raw material is added in the production without prior testing.

Samples that were identified and quantified did not exceed the beta-asarone limit imposed by EMA. Based on theoretical calculation, current market samples should contain beta-asarone that is within the accepted limit. However, estimation of the content of beta-asarone should not solely based on calculation, as the raw material of *Acorus calamus* rhizome plays a big role in the final concentration of these asarone isomers. Different origin and ploidy of *Acorus calamus* have different value of asarones. Thus, the development of this method is certainly helpful from over exposing the consumers with alpha- and beta-asarones. It is recommended to gather further value of beta-asarone quantified in samples available in Malaysia before setting a limit for traditional product registration requirement.

For future work it is recommended that this method is fully validated based on the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines of Validation Of Analytical Procedures: Text And Methodology Q2 (R1). Linearity for this method has been

demonstrated. This analytical method should be validated to ensure its specificity, accuracy, precision and reliability.

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REFERENCES

- Appendino, G., Minassi, A., & Tagliatela-Scafati, O. (2014). Recreational drug discovery: natural products as lead structures for the synthesis of smart drugs. *Natural Product Reports*, 31(7), 880-904.
- Asif, M. (2012). A brief study of toxic effects of some medicinal herbs on kidney. *Advanced Biomedical Research*, 1(1), 44.
- Aziz, Z., & Tey, N. P. (2009). Herbal medicines: prevalence and predictors of use among Malaysian adults. *Complementary Therapy in Medicine*, 17(1), 44-50.
- Balakumbahan, R., Rajamani, K., & Kumanan, K. (2010). *Acorus calamus*: An overview. *Journal of Medicinal Plants Research*, 4(25), 2740-2745.
- Bagetta, G., Cosentino, M., Corasaniti, M. T., & Sakurada, S. (2011). *Herbal medicines: development and validation of plant-derived medicines for human health* (pp. 108-122). Florida: CRC Press.
- Bansal, A., Chhabra, V., Rawal, R. K., & Sharma, S. (2014). Chemometrics: a new scenario in herbal drug standardization. *Journal of Pharmaceutical Analysis*, 4(4), 223-233.
- Barbarin, N., Henion, J. D., & Wu, Y. (2002). Comparison between liquid chromatography–UV detection and liquid chromatography–mass spectrometry for the characterization of impurities and/or degradants present in trimethoprim tablets. *Journal of Chromatography A*, 970(1), 141-154.
- Benny, B., Doss, C. G. P., Kumar, D. T., & Devi, S. A. (2016). Assessing reproductive toxicity and antioxidant enzymes on beta asarone induced male Wistar albino rats: *In vivo* and computational analysis. *Life Sciences*, 173, 150-160.
- Björnstad, K., Helander, A., Hultén, P., & Beck, O. (2009). Bioanalytical investigation of asarone in connection with *Acorus calamus* oil intoxications. *Journal of Analytical Toxicology*, 33(9), 604-609.
- Boligon, A., & Athayde, M. (2014). Importance of HPLC in analysis of plants extracts. *Austin Chromatography*, 1(3), 1.
- Calixto, J. B. (2000). Efficacy, safety, quality control, marketing and regulatory guidelines for herbal medicines (phytotherapeutic agents). *Brazilian Journal of Medical and Biological Research*, 33(2), 179-189.
- Capasso, R., Izzo, A. A., Pinto, L., Bifulco, T., Vitobello, C., & Mascolo, N. (2000). Phytotherapy and quality of herbal medicines. *Fitoterapia*, 71, S58-S65.
- Charles, D. J., & Simon, J. E. (1990). Comparison of extraction methods for the rapid determination of essential oil content and composition of basil. *Journal of the American Society for Horticultural Science*, 115(3), 458-462.

- Chen, W., & Zai, P. (2006). Study on quantitative determination of α -asarone in rabbit plasma by LC-MS/MS. *Chinese Journal of Pharmaceutical Analysis*, 26(10), 1364-1368.
- Chen, C., Spriano, D., & Meier, B. (2009). Reduction of β -asarone in *Acori rhizoma* by decoction. *Planta Medica*, 75(13), 1448-1452.
- Deng, C., Li, N., & Zhang, X. (2004). Rapid determination of essential oil in *Acorus tatarinowii* Schott. by pressurized hot water extraction followed by solid-phase microextraction and gas chromatography–mass spectrometry. *Journal of Chromatography A*, 1059(1-2), 149-155.
- Dhanani, T., Shah, S., Gajbhiye, N. A., & Kumar, S. (2017). Effect of extraction methods on yield, phytochemical constituents and antioxidant activity of *Withania somnifera*. *Arabian Journal of Chemistry*, 10, S1193-S1199.
- Dog, T. L., Marles, R., Mahady, G., Gardiner, P., Ko, R., Barnes, J., ... & Sarma, N. D. (2010). Assessing safety of herbal products for menopausal complaints: An international perspective. *Maturitas*, 66(4), 355-362.
- Dong, H., Gao, Z., Rong, H., Jin, M., & Zhang, X. (2014). β -asarone reverses chronic unpredictable mild stress-induced depression-like behavior and promotes hippocampal neurogenesis in rats. *Molecules*, 19(5), 5634-5649.
- Du, Z., Clery, R. A., & Hammond, C. J. (2008). Volatiles from leaves and rhizomes of fragrant *Acorus* spp. (*Acoraceae*). *Chemistry & Biodiversity*, 5(6), 887-895.
- Efferth, T., & Kaina, B. (2011). Toxicities by herbal medicines with emphasis to traditional Chinese medicine. *Current Drug Metabolism*, 12(10), 989-996.
- Ekor, M. (2013). The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety. *Frontiers in Pharmacology*, 4(2013), 177.
- European Commission. (2002). *Opinion of the scientific committee on food on the presence of β -asarone in flavourings and other food ingredients with flavouring properties* (SCF/CS/FLAV/FLAVOUR/9 ADD1 Final). Brussel: European Commission.
- European Medicine Agency. (2005). *Public statement on the use of herbal medicinal products containing asarone* (EMA/HMPC/139215/2005). London: European Medicine Agency.
- Folashade, O., Omoregie, H., & Ochogu, P. (2012). Standardization of herbal medicines-A review. *International Journal of Biodiversity and Conservation*, 4(3), 101-112.
- Forest Research Institute Malaysia. (2010). *Malaysian herbal monograph: Jerangau* (pp. 5-10). Forest Research Institute Malaysia.

- Göggelmann, W., & Schimmer, O. (1983). Mutagenicity testing of β -asarone and commercial *calamus* drugs with *Salmonella typhimurium*. *Mutation Research Letters*, 121(3-4), 191-194.
- Hamil, M. S. R., Memon, A. H., Majid, A. M. S. A., & Ismail, Z. (2016). Simultaneous determination of two isomers of asarone in *Piper sarmentosum Roxburgh* (*Piperaceae*) extracts using different chromatographic columns. *Tropical Journal of Pharmaceutical Research*, 15(1), 157-165.
- Hansen, S. W., Usher, K. M., Amoo, J. S., Bernstein, A. P., & McNally, M. E. P. (2015). Precision of internal standard and external standard methods in high performance liquid chromatography. *Special Issues*, 33(4), 40–46. Retrieved from <http://www.chromatographyonline.com/precision-internal-standard-and-external-standard-methods-high-performance-liquid-chromatography>
- Hanson, K. M., Gayton-Ely, M., Holland, L. A., Zehr, P. S., & Soderberg, B. C. (2005). Rapid assessment of beta-asarone content of *Acorus calamus* by micellar electrokinetic capillary chromatography. *Electrophoresis*, 26(4-5), 943-946.
- Hasan, S. S., Loon, W. C., Ahmadi, K., Ahmed, S. I., & Bukhari, N. I. (2011). Reasons, perceived efficacy and factors associated with complementary and alternative medicine use among Malaysian patients with diabetes mellitus. *The British Journal of Diabetes & Vascular Disease*, 11(2), 92-98.
- Hasheminejad, G., & Caldwell, J. (1994). Genotoxicity of the alkenylbenzenes α - and β -asarone, myristicin and elemicin as determined by the UDS assay in cultured rat hepatocytes. *Food and Chemical Toxicology*, 32(3), 223-231.
- Hayat, M. A. (2015). *Autophagy: Cancer, other pathologies, inflammation, immunity, infection, and aging: Volume 7- role of autophagy in therapeutic applications* (pp. 150-154). Massachusetts: Academic Press.
- Hostettmann, K., Wolfender, J.-L., & Terreaux, C. (2001). Modern Screening Techniques for Plant Extracts. *Pharmaceutical Biology*, 39(1), 18-32.
- Huie, C. W. (2002). A review of modern sample-preparation techniques for the extraction and analysis of medicinal plants. *Analytical and Bioanalytical Chemistry*, 373(1), 23-30.
- IOFI Working Group on Methods of Analysis. (2011). Guidelines for the quantitative gas chromatography of volatile flavouring substances, from the Working Group on Methods of Analysis of the International Organization of the Flavor Industry (IOFI). *Flavour and Fragrance Journal*, 5(26), 297-299.
- Jeon, S.-H., Kim, H., Nam, J. M., & Kim, J. G. (2013). Habitat characteristics of sweet flag (*Acorus calamus*) and their relationships with sweet flag biomass. *Landscape and Ecological Engineering*, 9(1), 67-75.
- Jicheng, L., Chengchong, L., Guihua, X., Li, Z., Miaoxian, D., Yutao, G., Dejie, Z. (2010). Beta-asarone attenuates neuronal apoptosis induced by Beta amyloid in rat hippocampus. *Yakugaku Zasshi*, 130(5), 737-746.

- Kamboj, A. (2012). Analytical evaluation of herbal drugs. *Drug Discovery Research in Pharmacognosy*, (pp. 23-56). Retrieved from <http://www.intechopen.com/books/drug-discovery-research-in-pharmacognosy/analytical-evaluation-of-herbal-drugs>
- Karmen, A., McCaffrey, I., & Kliman, B. (1963). Derivative ratio analysis: A new method for measurement of steroids and other compounds with specific functional groups using radioassay by gas-liquid chromatography. *Analytical Biochemistry*, 6(1), 31-38.
- Kim, K. H., Moon, E., Kim, H. K., Oh, J. Y., Kim, S. Y., Choi, S. U., & Lee, K. R. (2012). Phenolic constituents from the rhizomes of *Acorus gramineus* and their biological evaluation on antitumor and anti-inflammatory activities. *Bioorganic & Medicinal Chemistry Letters*, 22(19), 6155-6159.
- Kim Sooi, L., & Lean Keng, S. (2013). Herbal medicines: Malaysian women's knowledge and practice. *Evidence-Based Complementary and Alternative Medicine*, 2013(1), 1-10.
- Lanka, S. (2015). TLC Bioautography Guided Detection and Biological Activity of Antifungal Compounds from Medicinal Plant *Acorus calamus* Linn. *Asian Journal of Plant Pathology*, 9(1), 16-26.
- Lee, M. K., Cheng, B. W. H., Che, C. T., & Hsieh, D. P. H. (2000). Cytotoxicity Assessment of Ma-huang (*Ephedra*) under Different Conditions of Preparation. *Toxicological Sciences*, 56(2), 424-430.
- Li, C., Xing, G., Dong, M., Zhou, L., Li, J., Wang, G., Niu, Y. (2010). Beta-asarone protection against beta-amyloid-induced neurotoxicity in PC12 cells via JNK signaling and modulation of Bcl-2 family proteins. *European Journal of Pharmacology*, 635(1), 96-102.
- Liu, L., Wang, J., Shi, L., Zhang, W., Du, X., Wang, Z., & Zhang, Y. (2013). β -Asarone induces senescence in colorectal cancer cells by inducing lamin B1 expression. *Phytomedicine*, 20(6), 512-520.
- Lu, Y.-H., Wang, H.-J., Wang, Z.-T., & Wei, D.-Z. (2009). Rapid analysis of asarone and asarylaldehyde in *fructus carotae* by LC. *Chromatographia*, 69(5-6), 549-553.
- Malik, A., Kurniawan, A., & Najib, A. (2014). Comparative Study of HPTLC fingerprint of β -Asarone content between leaves and rhizome of *Acorus calamus* L. *International Journal of PharmTech Research*, 6(2), 829-833.
- Manikandan, S., & Devi, R. S. (2005). Antioxidant property of α -asarone against noise-stress-induced changes in different regions of rat brain. *Pharmacological Research*, 52(6), 467-474.
- McNaught, A. D., & Wilkinson, A. (1997). *Compendium of chemical terminology* (pp. 748). New Jersey: Blackwell Science Oxford.

- Ministry Of Health Malaysia. (2015). *Drug registration guidance document* (pp. 662). Malaysia: National Pharmaceutical Regulatory Agency, Ministry of Health Malaysia.
- Motley, T. J. (1994). The ethnobotany of sweet flag, *Acorus calamus* (*Araceae*). *Economic Botany*, 48(4), 397-412.
- Mukherjee, P. K. (2015). Evidence based validation of traditional medicine–Farm to Pharma. *Planta Medica*, 81(05), OA40.
- Murali, K., Rajendran, V., & Ramalingam, R. (2017). Indispensability of herbal drug standardization. *Journal of Pharmacognosy and Phytochemistry*, 6(1), 47-49.
- Nandakumar, S., Menon, S., & Shailajan, S. (2013). A rapid HPLC-ESI-MS/MS method for determination of β -asarone, a potential anti-epileptic agent, in plasma after oral administration of *Acorus calamus* extract to rats. *Biomedical Chromatography*, 27(3), 318-326.
- Nath, P., & Yadav, A. K. (2016). Anthelmintic activity of a standardized extract from the rhizomes of *Acorus calamus* Linn. (*Acoraceae*) against experimentally induced cestodiasis in rats. *Journal of Intercultural Ethnopharmacology*, 5(4), 390.
- Natural Health Products Ingredients Directorate. (2017). *Organism - Acorus gramineus*. Health Canada. Retrieved from <http://webprod.hc-sc.gc.ca/nhpid-bdipsn/ingredReq.do?id=5844&lang=eng>
- O'Neil, M., Smith, A., Heckelman, P., & Budavari, S. (2013). *The Merck index: an encyclopedia of chemicals, drugs, and biologicals*. United Kingdom: Royal Society of Chemistry.
- Oliveira, E. C., Muller, E. I., Abad, F., Dallarosa, J., & Adriano, C. (2010). Internal standard versus external standard calibration: an uncertainty case study of a liquid chromatography analysis. *Química Nova*, 33(4), 984-987.
- Ong, E. S. (2004). Extraction methods and chemical standardization of botanicals and herbal preparations. *Journal of Chromatography B*, 812(1), 23-33.
- Oprean, R., Tamas, M., & Roman, L. (1998). Comparison of GC-MS and TLC techniques for asarone isomers determination. *Journal of Pharmaceutical and Biomedical Analysis*, 18(1-2), 227-234.
- Oprean, R., Tamas, M., Sandulescu, R., & Roman, L. (1998). Essential oils analysis. I. Evaluation of essential oils composition using both GC and MS fingerprints. *Journal of Pharmaceutical and Biomedical Analysis*, 18(4-5), 651-657.
- Patel, D. N., Ho, H. K., Tan, L. L., Tan, M. M. B., Zhang, Q., Low, M. Y., ... & Koh, H. L. (2015). Hepatotoxic potential of asarones: *in vitro* evaluation of hepatotoxicity and quantitative determination in herbal products. *Frontiers in Pharmacology*, 6(1), 25.

- Patel, P. M., Patel, N. M., & Goyal, R. K. (2006). Quality control of herbal products. *The Indian Pharmacist*, 5(45), 26-30.
- Perrett, S., & Whitfield, P. J. (1995). Anthelmintic and pesticidal activity of *Acorus gramineus* (Araceae) is associated with phenylpropanoid asarones. *Phytotherapy Research*, 9(6), 405-409.
- Raal, A., Orav, A., & Gretchushnikova, T. (2016). β -Asarone content and essential oil composition of *Acorus calamus* L. rhizomes from Estonia. *Journal of Essential Oil Research*, 28(4), 299-304.
- Radušienė, J., Judžentienė, A., Pečiulytė, D., & Janulis, V. (2007). Essential oil composition and antimicrobial assay of *Acorus calamus* leaves from different wild populations. *Plant Genetic Resources*, 5(1), 37-44.
- Rajput, S. B., Tonge, M. B., & Karuppayil, S. M. (2014). An overview on traditional uses and pharmacological profile of *Acorus calamus* Linn. (sweet flag) and other *Acorus* species. *Phytomedicine*, 21(3), 268-276.
- Rana, T. S., Mahar, K. S., Pandey, M. M., Srivastava, S. K., & Rawat, A. K. S. (2013). Molecular and chemical profiling of 'sweet flag' (*Acorus calamus* L.) germplasm from India. *Physiology and Molecular Biology of Plants*, 19(2), 231-237.
- Sales of Drug Act, (1952). *Cosmetic and drug control regulations 1984, regulation 2:interpretation*. Malaysia: International Law Book Services.
- Sasidharan, S., Chen, Y., Saravanan, D., Sundram, K. M., & Yoga Latha, L. (2011). Extraction, isolation and characterization of bioactive compounds from plants' extracts. *African Journal of Traditional, Complementary, and Alternative Medicines*, 8(1), 1-10.
- Shailajan, S., Menon, S., Swar, G., Singh, D., & Nair, S. (2015). Estimation and quantitation of β -asarone from *Acorus calamus* rhizome and its formulations using validated RP-HPLC method. *Pharmaceutical Methods*, 6(2), 94-99.
- Sharma, V., Singh, I., & Chaudhary, P. (2014). *Acorus calamus* (The Healing Plant): a review on its medicinal potential, micropropagation and conservation. *Natural Product Research*, 28(18), 1454-1466.
- Shaw, D. (2010). Toxicological Risks of Chinese Herbs. *Planta Medica*, 76(17), 2012-2018.
- Sinha, A. K., Joshi, B. P., & Acharya, R. (2003). *U.S. Patent No. 6,590,127*. Virginia: U.S. Patent and Trademark Office.
- Skoog, D. A., Holler, F. J., & Crouch, S. R. (2017). *Principles of instrumental analysis* (pp. 15). Massachusetts: Cengage Learning.
- Srivastava, M. M. (2010). *High-performance thin-layer chromatography (HPTLC)* (pp. 108-109). Berlin, Germany: Springer Berlin Heidelberg.

- Therapeutic Goods Administration. (2004). *Complementary medicines evaluation committee extracted ratified minutes forty-ninth meeting*. Therapeutic Goods Administration, Australian Government.
- Toshimori, K. (2003). Biology of spermatozoa maturation: an overview with an introduction to this issue. *Microscopy Research and Technique*, 61(1), 1-6.
- Unger, P., & Melzig, M. F. (2012). Comparative study of the cytotoxicity and genotoxicity of alpha-and beta-asarone. *Scientia Pharmaceutica*, 80(3), 663-668.
- US Food & Drug Administration. (2014). *Code of Federal Regulations title 21 (21CFR189.110)*. Retrieved from <http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=189.110>
- Venskutonis, P. R., & Dagilyte, A. (2003). Composition of essential oil of sweet flag (*Acorus calamus L.*) leaves at different growing phases. *Journal of Essential Oil Research*, 15(5), 313-318.
- Wachtel-Galor, S., & Benzie, I. F. F. (2011). *Herbal medicine: an introduction to its history, usage, regulation, current trends, and research needs* (pp. 455-459). Florida: CRC Press.
- Wang, H.-Z., Chen, Y.-G., & Fan, C.-S. (1998). Review of studies on chemical constituents and pharmacology of genus *Acorus* in China. *Acta Botanica Yunnanica*, 20, 96-100.
- Wang, Z., Wang, Q., Yang, B., Li, J., Yang, C., Meng, Y., & Kuang, H. (2014). GC-MS method for determination and pharmacokinetic study of four phenylpropanoids in rat plasma after oral administration of the essential oil of *Acorus tatarinowii Schott.* rhizomes. *Journal of Ethnopharmacology*, 155(2), 1134-1140.
- Wiseman, R. W., Miller, E. C., Miller, J. A., & Liem, A. (1987). Structure-activity studies of the hepatocarcinogenicities of alkenylbenzene derivatives related to estragole and safrole on administration to preweanling male C57BL/6J×C3H/HeJ F1 mice. *Cancer Research*, 47(9), 2275-2283.
- World Health Organization. (1981). *Toxicological evaluation of certain food additives. Food Additives Series (FAO/WHO)* (No. 16). Geneva: World Health Organization.
- World Health Organization. (2000). *General guidelines for methodologies on research and evaluation of traditional medicine* (WHO/EDM/TRM/2000.1). Geneva: World Health Organization.
- Xi, Z., Xiaodong, Z., Jun, T., & Shuohua, W. (2012). Assay of α -asarone of volatile oil in *Acorus Tatarinowii Schott.* of four origins. *Guangdong Chemical Industry*, 7, 022.
- Xie, Z., Liu, Q., Liang, Z., Zhao, M., Yu, X., Yang, D., & Xu, X. (2013). The GC/MS analysis of volatile components extracted by different methods from

Exocarpium Citri Grandis. *Journal of Analytical Methods in Chemistry*, 2013(1), 1-8.

- Yang, F. Q., Wang, H. K., Chen, H., Chen, J. D., & Xia, Z. N. (2011). Fractionation of volatile constituents from *Curcuma* rhizome by preparative gas chromatography. *Journal of Analytical Methods in Chemistry*, 2011(1), 1-6.
- Yang, Y. X., Chen, Y. T., Zhou, X. J., Hong, C. L., Li, C. Y., & Guo, J. Y. (2013). Beta-asarone, a major component of *Acorus tatarinowii* Schott., attenuates focal cerebral ischemia induced by middle cerebral artery occlusion in rats. *BMC Complementary and Alternative Medicine*, 13(1), 236.
- Yu, S. M., Kim, E. K., Lee, J. H., Lee, K. R., & Hong, J. K. (2011). Development of fingerprints for quality control of *Acorus* species by gas chromatography/mass spectrometry. *Bulletin of the Korean Chemical Society*, 32(5), 1547-1553.
- Zhang, J., Onakpoya, I. J., Posadzki, P., & Eddouks, M. (2015). The safety of herbal medicine: from prejudice to evidence. *Evidence-Based Complementary and Alternative Medicine*, 2015(1), 1-3.
- Zhang, J., Wider, B., Shang, H., Li, X., & Ernst, E. (2012). Quality of herbal medicines: challenges and solutions. *Complementary Therapies in Medicine*, 20(1), 100-106.
- Zheng, Y. J., Kang, Y., Feng, Y. Z., Zhang, R., & Zhang, W. B. (2001). Addition internal standard method in chromatographic quantitative analysis. *Se pu= Chinese Journal of Chromatography*, 19(5), 464-466.
- Zuba, D., & Byrska, B. (2012). Alpha-and beta-asarone in herbal medicinal products. A case study. *Forensic Science International*, 223(1), e5-e9.
- Zuo, H. L., Yang, F. Q., Zhang, X. M., & Xia, Z. N. (2012). Separation of cis-and trans-asarone from *Acorus tatarinowii* by preparative gas chromatography. *Journal of Analytical Methods in Chemistry*, 2012(1), 1-5.