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Quality control methods for some Zingiberaceous plants from Indonesia using liquid chromatography combined with chemometrics

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September 2013
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with chemometrics

A dissertation submitted to the Gifu University in
partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Material Engineering

By
MOHAMAD RAFI

September 2013
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Preface

Over thousands of years herbal medicines are used for relieving symptoms or treating diseases. Nowadays herbal medicines are still used by a large population of the world due to the general fact that herbal medicines have lower side effects than synthetic drugs. However, the negative effects could also be observed if the herbal medicines used are contaminated with heavy metals or have been added synthetic compounds with the same pharmacological activity at high doses, substitution one or more herbal ingredients with some closely related medicinal plants (same appearance but differ in pharmacological activity) and process in the preparation of herbal medicines from its raw material to the final products is not standardized. So quality control of herbal medicines from upstream to downstream is necessary to ensure the efficacy, safety and quality of herbal medicines.

Quality control of herbal medicines is very important to ensure the quality, efficacy and safety because, among other reasons, the variability of the active compounds in herbs is very large. In 2000, the World Health Organization (WHO) indicated the lack of adequate or accepted research methodology for evaluating herbal medicines. Since then, much effort is put in the quality control and research of herbal products in order to give consistency in their pharmacological effects.

Quality control of herbal medicines is more difficult than that of synthetic drugs due to the chemical complexity of the ingredients. In general, there are two approaches in the development of quality control method by chemicals for plant identification and quality, namely component-based or compound-oriented approach and pattern-based or
pattern-oriented approach. The two approaches are widely used in the world and are accepted by the WHO.

In this study, quality control methods using liquid chromatography (LC) as capillary LC or high performance liquid chromatography alone or in combination with some chemometrics have been proposed for quantitative analysis of some bioactive compounds and discrimination of a closely related species for some Zingiberaceous plants origin from Indonesia.
Chapter 1 Introduction

1.1 Herbal medicines

Herbal medicines have been practiced over a thousand years for prevention or treatment of human diseases [1]. The use of herbal medicines in the last decade has remarkably increased for improving health and treating various diseases in the world. World Health Organization (WHO) estimated about 75-80% of the world population have been using herbal medicines, especially in the developing countries [2,3]. An estimation of the global market value for herbal medicines was about US $83 billion in 2008 with the growth rate between 3-12% per year [4,5].

According to the WHO, herbal medicine is a plant-derived material or preparation with therapeutic or other human health benefits which contains either raw or processed ingredients from one or more plants. About 25% of modern medicines (from the data of WHO) are derived from plants for example aspirin, atropine, digoxin, ephedrine, morphine, quinine, reserpine, taxol, warfarin and vincristine [6]. There are other terms to define medicines derived from plants such as phytomedicines, herbs, herbal materials, botanicals and botanical drugs [7]. Three groups exist in the classification of herbal medicines, i.e. (1) herbal materials (raw or processed herbal materials e.g. powder, slice), (2) traditional herbal products (decoctions, tablets, capsules containing crude herbal materials or extracts) and (3) standardized herbal products (formulations containing standardized extracts or purified substances) [5].

Among all the traditional medicine systems, medicinal herbs are the most widely used. Traditional Chinese Medicine (TCM) is the most leading traditional medicine system in the world with 30-50% of the medical consumption in China, followed by
Indian traditional medicine such as *Ayurveda, Siddha* and *Unani* [7]. Other known traditional medicine systems are traditional Korean medicine from Korea, *kampo* from Japan and *jamu* from Indonesia. Indonesia is known as the second largest country in natural resource diversity as well as inherited indigenous knowledge of traditional medicine (*jamu*) for over hundred years. Many medicinal plants are used in the *jamu* prescription with the plants from the Zingiberaceae family are widely used.

1.2 Plant from Zingiberaceae family

Zingiberaceae plants are perennial and rhizomatous herbs and are one of the largest plant families comprising of 4 subfamilies and 6 tribes with 53 genera and over 1200 species [8,9]. The important genera from this family are *Alpinia, Amomum, Costus, Curcuma, Elettaria, Hedychium, Kaempferia*, and *Zingiber*. Volatile oils and oleoresins were the major products from Zingiberaceae plants. Many of them are important natural resources for food, spices, medicines, dyes, perfumes etc.

Zingiberaceae plants are mostly distributed in tropical and subtropical region with the center of distribution is in Southeast Asia, South Asia and China [10]. Indonesia is one of the major countries in the production of some Zingiberaceae plants such as ginger, turmeric and java turmeric. Zingiberaceae family is mostly found or cultivated in Java and Sumatra islands.

Plants from Zingiberaceae are commonly used as spices, flavor and medicines in Indonesia. Most of *jamu* prescription used one or more plants of this family, for examples ginger (*Zingiber officinale*), turmeric (*Curcuma longa*), java turmeric (*C. xanthorrhiza*), galangal (*Alpinia galanga*), aromatic ginger (*Kaempferia galanga*), cassumunar ginger
(Z. montanum), shampoo ginger (Z. zerumbet) and java cardamom (Amomum compactum).

1.3 Quality control of herbal medicinal products

Quality control of herbal medicines has a direct link to the safety and efficacy of their products because, among other reasons, the variability of the active compounds in herbs is very large. Depending on the species and the environmental factors of growth, such as the cultivating area, climate (temperature, humidity, light and wind) and the harvest time, the active compounds and their concentration vary. Differences are also caused in the postharvest process e.g. the way of drying, washing, crushing and pulverizing, the storage and the conservation and in the process of extraction for making the final products [11,12]. As the harvest quality depends on the climate, it is likely that ‘first quality’ herbs are scarce and that herb cultivated in different places or ‘lookalikes’, containing non-therapeutic concentrations of compounds, or even contaminations (like pesticides, heavy metals, etc.), are sold. Manufacturers also may include illegal adulterations to herbal medicinal products [13,14]. Furthermore, confusion between different herbs may occur because of translations [15]. This becomes a crucial task in the production of herbal medicines from cultivation of the medicinal plant up to manufacturing of the products.

Traditionally, the identification and authentication as a part of quality evaluation of herbal medicines mainly depend on the difference in the appearance of the plants, which could only be figured out by the experienced herbalist. The identification becomes more difficult if they are present in powdered forms. Therefore, the development of analytical strategies to obtain information about authenticity of the plant material is
crucial in order to ensure the quality, safety and efficacy of the raw material and extract before it is converted into the final product. It is well known that the therapeutic effects of herbal medicines come not only from one compound but at least two or more compounds involved [16]. Knowing the chemical composition in herbal medicines still needs to be developed because we know herbal medicines are a multi-compound system and most of the compounds are unknown. This fact leads us to face a problem and meet a challenging task in the quality control of herbal medicines in order to have good reproducibility in the use of herbal medicines.

As we know, quality control of herbal medicines is more difficult than that of synthetic drugs due to the chemical complexity of the ingredients. Hundreds of compounds or even more could be present in herbal medicines and these compounds mostly unique or species-specific compounds. In general, there are two approaches in the development of quality control methods by chemicals for plant identification and quality namely component-based or compound-oriented approach and pattern-based or pattern-oriented approach [17,18]. One or more chemical compounds present in the plant as a raw material or extract were usually used in the compound-oriented approach. The pattern-oriented approach known as fingerprint analysis, will evaluate all detectable compounds of a given plant from an analytical instrument without necessarily characterizing all the compounds. Limitations and advantages of the two approaches should be well understood while embarking on identification and authentication of medicinal plant as a part of quality control of the plant material. In a number of examples, both approaches complement each other in arriving at species identification [18]

In the compound-oriented approach, one or more marker compounds as a major compound or having pharmacological activity are employed for evaluating the quality
and authenticity of the complex herbal medicine or preparations in traditional standards. Even though this is an acceptable method for quality control, sometimes the presence of the chemical markers does not guarantee that the products contain the actual herbs stated on the label. In other word because of the complexity of chemical composition and concentration of herbs makes this approach sometimes insufficient for the quality control of the global herb [19].

The chemical compounds in herbal medicines are the basis of medical efficiency so the efficiency will vary because of some variation in the chemical content and composition. Since the efficiency of herbal medicines depends on the synergistic action of many components, the contents of part compounds sometimes cannot represent its integral quality [20]. Recently, development of quality control methods relies on the utilization of information on all components (fingerprint analysis) to evaluate the quality of herbal medicines [21].

Among all the quality control systems, fingerprint analysis has gained the most attention due to their ability to give more detailed characteristics profile or pattern which chemically represents the sample [11]. An herbal fingerprint can be developed for three main reasons, i.e. identification, classification and calibration. Identification confirms that a sample is originated from the herb expected and not from another source, thus it is able to attain better quality control of the herbs. Classification or clustering can be performed to classify samples according to, for example, their origin. A multivariate calibration can be performed when the herb or its extract can also be characterized by an activity, such as an antioxidant or cytotoxic activity. The activity can then be modeled as a function of a complete chromatogram. The goal of the modeling can be either to build models that can predict the activity for future samples based on the chromatogram, for
example, the antioxidant activity of green tea, or to identify the main compounds/peaks responsible for a given activity [22].

In general, chromatography and spectroscopy are frequently analytical instrumentation used in the quality control methods of herbal medicines and related products. Types of techniques used in chromatography are thin layer chromatography (TLC), liquid chromatography (LC) and gas chromatography (GC), while for spectroscopy, ultraviolet-visible (UV-Vis) spectroscopy, infrared (IR) spectroscopy, mass spectrometer (MS), nucleic magnetic resonance (NMR) spectrometers are often used. Another analytical instrumentation that is also used is capillary electrophoresis (CE). Recently, hyphenated techniques such as LC-MS, GC-MS, CE-MS, LC-NMR, LC-IR etc. are also found in major laboratories [22,24].

1.4. Chromatography

1.4.1 History and principle of chromatography

Chromatography is one of the main branches in the analytical chemistry as physical techniques to separate organic and inorganic compounds so that they can be further analyzed and studied. The word chromatography derives from Greek word chroma and graphy which means “color writing”. Chromatography was invented by Mikhail Semenovich Tswett, a Russian botanist in 1903 when he worked with the separation of plant pigments. The separation of the colored plant pigments was performed in chalk, alumina, inulin (polysaccharides), sucrose, etc. by adsorptive mechanism [25,26].

The chromatographic method was not appreciated among the scientists at the time of the discovery until two decades later, Kuhn and Lederer in 1931 used the techniques
for isolation of another plant pigments such as carotenoids and xanthophylls [27]. In 1941 Martin and Synge established the importance of liquid–liquid partition chromatography and led to the development of a theory for chromatographic separations and they were awarded the 1952 Nobel Prize in chemistry for their work [28]. Since then the development of chromatography has become more sophisticated in the theory, instrumentation and application.

Tswett defined in his earlier paper that chromatography is a method in which the components of a mixture are separated on an adsorbent column in a flowing system [29]. Another definition with a broader scope was come out from the International Union of Pure and Applied Chemistry (IUPAC) defines chromatography as a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other (the mobile phase) moves in a definite direction [30]. Basically, in chromatography, a sample containing a mixture of components is placed onto a stationary phase (which may be either solid or liquid). A mobile phase (which may be either liquid or gas) is then passed through or over the stationary phase, which causes components in the sample to move along the stationary phase. The diverse physical and chemical properties of individual components within the sample allow components to separate out because they migrate along the stationary phase at different rates and this migration or separation process is referred to as elution.

Chromatography also permits a great flexibility in the technique itself. The flow of the mobile phase might be controlled by gravity, pressure, capillary action and electroosmosis. The separation may be carried out over a wide temperature range and sample size can vary from a few atoms to many kilograms. Also, the shape of the system in which the separation takes place can be varied, using columns of various lengths and
diameter, or flat plates. Through all this evolution chromatography has been transformed from an essentially batch technique into an automated, instrumental method [31].

1.4.2 Classification of chromatography

Chromatography may be classified in three ways, by the physical state of the mobile phase and stationary phase, by the method of contact between the mobile phase and stationary phase or by the chemical or physical mechanism responsible for separating the sample constituents. The mobile phase is usually a liquid or a gas, and the stationary phase, when present, is a solid or a liquid film coated on a solid surface. Chromatographic techniques are often named by listing the type of mobile phase, followed by the type of stationary phase. Thus, in gas liquid chromatography the mobile phase is a gas and the stationary phase is a liquid. If only one phase is indicated, as in gas chromatography, it is assumed to be the mobile phase. Table 1-1 showed the classification of chromatography techniques.

1.4.3 High performance liquid chromatography

Nowadays HPLC has become a standard separation technique to separate non-volatile components. HPLC arose in the early 1960s by a combination of the experiences gained with both gas chromatography and ordinary column chromatography. Major experimental advances that led to practical HPLC systems were made by Horvath et al. in 1967, Huber and Hulsman also in 1967 and Kirkland in 1969, although many of the theoretical advantages were first described by Giddings in 1965.
Table 1-1 Classification of chromatography

<table>
<thead>
<tr>
<th>Type of mobile phase</th>
<th>Type of stationary phase</th>
<th>Mechanism of separation</th>
<th>Apparatus for stationary phase</th>
<th>Name of chromatography</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gas</td>
<td>Liquid</td>
<td>Partition</td>
<td>Column</td>
<td>Gas-Liquid Chromatography (GLC)</td>
</tr>
<tr>
<td>Solid</td>
<td>Adsorption</td>
<td>Column</td>
<td>Gas-Solid Chromatography (GSC)</td>
<td></td>
</tr>
<tr>
<td>Liquid</td>
<td>Liquid</td>
<td>Partition</td>
<td>Planar</td>
<td>Thin Layer Chromatography (TLC)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Column</td>
<td>Classical Liquid-Liquid</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chromatography (CLLC)</td>
<td></td>
</tr>
<tr>
<td>Bonded-liquid</td>
<td>Modified partition</td>
<td>Planar</td>
<td>High Performance Thin Layer</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chromatography (HPTLC)</td>
<td></td>
</tr>
<tr>
<td>Solid</td>
<td>Adsorption</td>
<td>Planar</td>
<td>Thin Layer Chromatography (TLC)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>High Performance Thin Layer</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chromatography (HPTLC)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Paper Chromatography (PC)</td>
<td></td>
</tr>
<tr>
<td>Ion exchange</td>
<td>Column</td>
<td>Ion Exchange</td>
<td>Size Exclusion Chromatography</td>
<td></td>
</tr>
<tr>
<td>Exclusion</td>
<td>Column</td>
<td></td>
<td>Chromatography (SEC)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Gel Permeation Chromatography</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(GPC)</td>
<td></td>
</tr>
</tbody>
</table>
In HPLC the mobile phase is liquid and in contrast to GC, HPLC is a suitable technique for the analysis of compounds with a wide range of polarities, high molecular weights, and thermally unstable or could ionize in solution. The popularity of HPLC came from its flexibility, both in terms of the diverse nature of the components that can be separated and the different modes that can be used (reverse phase, ion exchange, analytical, preparative, etc.), and also a reliable and reproducible qualitative and quantitative analytical method. In the development of analytical methods with HPLC we must determine many variables to have a good separation (solvents, stationary phases, flow rates, gradients, temperature, etc.) [32].

An HPLC system (Fig 1-1) consists of a pump to deliver the solvent (mobile phase) with the desired velocity, an injector valve for delivering a sample, a separation column that the separation of the components in the sample will occur, a detector to detect the separated individual components and a data processor for recording the separation result (chromatogram).

In 1967 Horvarth et al. firstly introduced the use of capillary tubes in their LC system [33] and since then the era of miniaturization has begun. Nevertheless, capillary
columns were firstly developed for GC, which have several advantages such as fast, efficient, sensitive and versatile. Over the next decade, numerous research groups made significant improvements in the reduction of the column size, column construction materials and packing materials [34]. In 1974, Ishii and co-workers in Nagoya University, Japan also started the study of miniaturization of LC system and used the term “micro-LC” (μLC). Table 1-2 showed the development of capillary based separation methods and their related techniques. μLC has some advantages over the conventional HPLC such as lower consumption of stationary and mobile phase, small amount of sample needed especially for biological samples which are often available in limited amounts and improved mass sensitivity [35,36].

<table>
<thead>
<tr>
<th>Year</th>
<th>Separation methods and their related techniques</th>
</tr>
</thead>
<tbody>
<tr>
<td>1974</td>
<td>Micro-HPLC</td>
</tr>
<tr>
<td>1978</td>
<td>Open-tubular capillary LC</td>
</tr>
<tr>
<td>1978</td>
<td>Packed microcapillary LC</td>
</tr>
<tr>
<td>1979</td>
<td>Fused-silica capillary</td>
</tr>
<tr>
<td>1981</td>
<td>Capillary zone electrophoresis</td>
</tr>
<tr>
<td>1985</td>
<td>Electrokinetic chromatography</td>
</tr>
<tr>
<td>1987</td>
<td>Capillary electrochromatography</td>
</tr>
<tr>
<td>1998</td>
<td>Monolithic silica capillary column</td>
</tr>
</tbody>
</table>
HPLC is one of analytical techniques that extensively used for qualitative and quantitative analysis of components presents in herbal medicines to evaluate the quality of its products. Recently there some new separation-based techniques were developed to reduce analysis time or environmentally friendly, for example separation in monolithic column, use of the capillary LC or ultra-performance liquid chromatography (UPLC) [37-40].

LC was the most frequent analytical instruments used in the quality control method of herbal medicines with compound-oriented and pattern-oriented approaches. For pattern-oriented better known as chromatographic fingerprint are usually given complex chromatograms that represent the overall chemical characteristics of a sample. The development and optimization of a methodology seem to be very important in order to create good chromatographic fingerprints. Sample preparation is the most important step in the development of chromatographic fingerprints, because the herbal medicines contain very complex metabolites that differ widely in their chemical nature and amount, so the optimum extraction conditions will be different for different types of components. The next step is to determine the amount detectable components present in the sample and it is important to ensure that the separation is complete to resolve the components present in the sample [41].

Chromatographic fingerprint will contain a huge data, so we need the help from chemometrics to deal with it. Chemometrics will be used for preprocessing chromatogram (data pretreatment) and pattern recognition (data handling) to build an authentication, discrimination and identification of the samples. Development of quality control methods using LC alone or in combination with chemometrics for evaluating herbal medicines has been done by many researchers recently [42-54].
1.5. Chemometrics

In the late 1960s, T.L. Isenhour from the University of Washington, Seattle, USA was the person to use multivariate analysis methods in chemistry for the first time in a series of papers mainly the classification method “learning machine,” described in a booklet by N. J. Nilsson [55], has been applied to chemical problems. Since then some scientists followed in the forthcoming years. Svante Wold, a chemist from Sweden considered for the first time to use the term of chemometrics in 1972 [56]. In 1974 together with Bruce R. Kowalski, an American chemist and mathematician founded the International Chemometrics Society [57]. One year later in 1975, Bruce R. Kowalski presented a first overview of the contents and aims for a new chemical discipline chemometrics [58]. The early history of chemometrics is documented by published interviews with Bruce R. Kowalski, D. Luc Massart, and Svante Wold who can be considered as the originators of modern chemometrics [57].

So, chemometrics has been used extensively since the 1970s to describe the application of statistical analysis especially multivariate analysis in the chemical data [59]. The International Chemometrics Society (ICS) defines chemometrics as the science of relating measurements made on a chemical system or process to the state of the system via application of mathematical or statistical method [60]. Chemometrics methods became routinely applied tools in chemistry. Typical problems that can be successfully handled by chemometrics methods are: (1) Determination of the concentration of a compound in a complex mixture (often from infrared data), (2) Classification of the origins of samples (from chemical analytical or spectroscopic data), (3) Prediction of a property or activity of a chemical compound (from chemical structure data), (4) Recognition of presence and absence of substructures in the chemical structure of an
unknown organic compound (from spectroscopic data) and (5) Evaluation of the process status in chemical technology (from spectroscopic and chemical analytical data) [57].

Basically chemometrics could be classified into two main categories, pattern recognition methods (exploratory data analysis, supervised and unsupervised) used in qualitative purpose and multivariate calibration for quantitative purpose [61]. Some examples in the pattern recognition that are widely used in analyzing chemical data are principal component analysis (PCA), similarity analysis (SA), hierarchical cluster analysis (HCA), discriminant analysis (DA), soft independent modeling of class analogy (SIMCA), k-nearest neighbor (k-NN) and for multivariate calibration such as multiple linear regression (MLR), principal component regression (PCR) and partial least square regression (PLSR).

1.6. Objective of the present research

Since over hundred or even thousand years, herbal medicines are used to relieve symptoms or to treat diseases in human civilization. Nowadays herbal medicines gain a broader interest in prevention or treatment of diseases or in the search for new drugs. Unfortunately, WHO still indicated the lack of adequate research methodology for evaluating herbal medicines [11]. Recently, much effort is put in the development of quality control and research of herbal medicines to ensure the quality, safety and efficacy of its product. Consistency in the quality, safety and efficacy of herbal medicines become a major task to guarantee the safety in the use of herbal medicines.

The main purpose of this research is to develop a quality control method of some Zingiberaceous plants that are widely used in jamu, a traditional Indonesian medicine using liquid chromatography (LC) alone or in combination with some chemometrics
methods. Two common approaches will use in the development this quality control method namely compound-oriented (simultaneous determination of major components) and pattern-oriented (fingerprint analysis). The methods will give valid and accurate information about authenticity of the raw materials and also could detect if there are fake herbs used in herbal medicinal products. With the presence of this method, the consistency in the quality, safety and efficacy of herbal medicines will be guaranteed.

In chapter 2, the use of capillary LC to determine simultaneously four major components, namely 6-, 8-, 10-gingerol and 6-shogaol found in ginger (*Z. officinale*) was described. With the use of C30 as the stationary phase and 60% acetonitrile as the mobile phase with the flow rate 5 µL/min gave a good baseline separation of all analytes with a resolution higher than 1.5. As the evaluation of method validation, a linear regression of the four components was obtained within the tested range with correlation coefficients \( \geq 0.9995 \). The limits of detection and quantitation were between 0.034-0.039 µg/mL and 0.112-0.129 µg/mL, respectively. Intra- and inter-day precision expressed as relative standard deviations (RSD) were less than 3.1%, and the accuracy based on recovery test was ranging from 97-105%. Stability of the analytes within 1 day was found in the range between 1.34–2.93% (RSD). In addition, based on the amount of these four compounds combining with the DA, a reliable and accurate method was developed for discrimination of three ginger varieties used. The results indicated that the developed method could be used as quality control for ginger raw material and its related products.

In chapter 3, a fingerprint analysis using capillary LC for discrimination of *Z. montanum* from its related species such as *Z. americans* and *Z. zerumbet* was developed. By comparing the fingerprint chromatograms of the three species, we could identify and discriminate *Z. montanum* samples from *Z. americans* and *Z. zerumbet* using their
marker peaks. We also combined the capillary LC fingerprint and multivariate analysis such as PCA and DA as an additional proved method and showed the three species were discriminate successfully. The result indicated that capillary LC fingerprint analysis in combination with PCA and DA could be used for discrimination of Z. montanum samples from its related species.

In chapter 4, a new method using HPLC-DAD was developed for the simultaneous determination of curcumin, demethoxycurcumin and bisdemethoxycurcumin in C. longa and C. xanthorrhiza. The separation of these three compounds was performed using C18 as the stationary phase and a mixture of acetonitrile and 0.5% acetic acid as the mobile phase in gradient program elution mode with a flow rate of 1 mL/min. Separation of the three compounds were achieved within 15 min with a complete baseline resolution. As the evaluation of method validation, a linear regression of the three compounds was obtained within the tested range with correlation coefficients \( \geq 0.9990 \). The limits of detection and quantitation for the three analytes were found between 1.26-1.64 and 4.21-5.47 ng, respectively. Intra- and inter-day precision expressed as RSD were less than 4.7\%, and the average percentages of recovery for curcumin, demethoxycurcumin and bisdemethoxycurcumin were found to be 99, 99 and 98\%, respectively and their RSD values were below 3.7\%. Stability of the analytes within 2 day was found with RSD values ranging between 0.66-2.00\%. About 34 samples consist of 16 samples of C. longa and 18 samples of C. xanthorrhiza were analyzed with the proposed method and it was found that C. longa has higher amount for all curcuminoids compare with C. xanthorrhiza. Based on this result we could discriminate these two closely related species by the amount of curcuminoids present in the samples. To be more convincing, an aid from chemometrics such as PCA and HCA was used.
Combination of HPLC with PCA and HCA gives excellent result with the two species were separated clearly in the PCA plot and HCA dendogram.

Finally in chapter 5, conclusions and future perspective of the present research were elaborated with the late trend and development in quality control of herbal medicine.

1.7 References


26. I. Chinou, Herbal Drugs and the Role of Chromatographic Methods in Their Analysis,


Chapter 2

Simultaneous determination of gingerols and shogaol using capillary liquid chromatography and its application in discrimination of three ginger varieties from Indonesia

2.1 Introduction

Ginger, botanically known as *Zingiber officinale* belongs to the family of Zingiberaceae and is native to Asia. It has been used worldwide as a spice and flavoring agent in foods and beverages as well as dietary supplement and herbal medicines [1,2]. Ginger is extensively used in traditional Chinese medicines, Ayurveda (traditional Indian medicines) and western herbal medicines [2,3] for treating dyspepsia, colic, nausea, vomiting, diarrhea, motion sickness, arthritis, rheumatic disorders and muscular discomfort [3-7]. Recently, ginger has been increasingly used because this rhizome has a broad spectrum of biological activities and low toxicity [5]. Many countries have been included ginger in their national pharmacopeias such as China, India, Japan, Indonesia, the United Kingdom, Germany, the United States, etc.

According to the Food and Agriculture Organization (FAO) of the United Nations, Indonesia ranked 6th in the world for the production of ginger in 2010 [8] and its rhizomes have been cultivated extensively in Java Island. *Jahe* is the Indonesian name for ginger and traditionally used as an ingredient in a Javanese traditional spice drink called *wedang jahe* and it is also widely used in *jamu* (traditional Indonesian medicines). There are three varieties of ginger growth in Indonesia, namely *jahe emprit* (*Z. officinale* var *amarum*, ZOA), *jahe gajah* (*Z. Officinale* var *officinarum*, ZOO) and *jahe merah* (*Z. officinale* var
rubrum, ZOR). ZOA and ZOR are commonly used for dietary supplement or herbal medicines, while the ZOO is usually used for cooking spices or flavor for food and beverages. These three varieties of ginger showed different level in its pungency taste due to the different amount of some homologous phenolic ketones, namely 6-, 8-, 10-gingerol and 6-shogaol that have been identified as the principal pungent compounds in ginger [9]. Differences in the amount of these pungent compounds could be due to species variation, environmental factors of growth (e.g. geographical origin, climate, soil type, and nutrient availability), harvesting, and postharvest processes (e.g. washing, drying, crushing, pulverizing, storing, and extraction for making the final products).

These four compounds especially 6-gingerol are considered to be important ingredients associated for various biological activities of ginger such as antiemetic [10], anticancer [11], antipyretic [12], antioxidant and antiinflammatory [13]. Also they have been utilized as a marker compound for quality evaluation of ginger based dietary supplement and herbal medicines. Differences in the amount of these bioactive compounds also may affect the result of biological activities from each ginger. So, in order to ensure successful use of gingers as a dietary supplement or herbal medicines, accurate quantitation of the bioactive compounds mentioned above and discrimination of the three varieties of ginger to prevent an adulteration have become a crucial task.

Several analytical methods have been developed for qualitative or quantitative analysis of gingerols and shogaol, and in most cases, LC techniques such as HPLC coupled with an ultraviolet detector [1,9,14,15], a mass spectrometer [16] and an electrochemical detector [17] with octyl (C8) or octadecyl (C18) as the stationary phase packed into conventional size column (4.6 mm i.d.) are employed. Nowadays, miniaturization of a chromatographic system such as micro-LC (μLC) is one of the
present trends in the field of separation sciences. μLC or also known as capillary LC was firstly introduced by Horvarth et al. [18], who used a stainless steel packed column with an 0.5-1.0 mm i.d.. Thereafter, Ishii et al. also started using μLC with a slurry packed microcolumn in their works [19]. The main features of capillary LC compared with the conventional-sized LC are the use of columns with smaller i.d. and lower eluent flow rate. With these features, capillary LC offers several analytical advantages such as improved mass sensitivity, reduced consumption of packing materials, mobile phase, and the sample amounts [20]. In recent years, capillary LC has shown significant progress in the instrumentation and applications. There are a number of reported works using capillary LC in the quantitative analysis of phenolic compounds in apple juices [21], cellular flavins [22] iridoid glycosides [23], catechins [24], fat-soluble vitamins and β-carotene [25], quinolones [26], and ochratoxin A [27].

Hydrophobic stationary phases such as C8, C18 and triancontyl (C30) are widely used in reversed phase LC due to their good separation efficiency for a wide range of analytes especially organic molecules. In an early study, C8 and C18 have been used for the separation of gingerols and shogaol. It was found that the elution time for a single chromatographic run with complete separation of these compounds were 40 min using C8 under isocratic elution (65% methanol) [1] and 35 min using C18 with gradient elution as follows: 40% acetonitrile at 0-10 minutes; 40-90% acetonitrile at 10-40 minutes; 100% acetonitrile at 40.5-45 minutes; and 40% acetonitrile at 45.5-50 minutes [9]. C30 phases are more hydrophobic than C8 and C18 and are commonly used for the separation of long chain isomeric analytes such as carotenoids [28], polycyclic aromatic hydrocarbons (PAHs) [29], tocopherols [30] etc. As we can see from the structure of gingerols and shogaol (Fig. 2-1), these compounds have a phenyl group and a long chain structure, C30
is therefore expected to be more suitable for the separation of gingerols and shogaol, and perhaps could reduce the total analysis time by optimizing the experimental conditions.

![Chemical structures of gingerols and shogaol](image)

- n = 4; 6-gingerol
- n = 6; 8-gingerol
- n = 8; 10-gingerol
- 6-shogaol

Fig. 2-1 Chemical structures of 6-, 8-, 10-gingerol and 6-shogaol

To date, to the best of our knowledge, there was no reported work regarding quantitative analysis of gingerols and shogaol using capillary LC with C30 as the stationary phase. So, based on what we described above we developed for the first time a quantitative analysis method of gingerols and shogaol by capillary LC. In addition, we also developed a method for discrimination of three ginger varieties growth in Indonesia based on the amount of gingerols and shogaol present combined with discriminant analysis. The proposed method could be successfully applied for quality control of gingers raw material and its related products.
2.2 Experimental

2.2.1. Chemicals and reagents

6-gingerol and 6-shogaol were obtained from Wako Pure Chemical Industries (Osaka, Japan), while 8-gingerol and 10-gingerol were obtained from Chromadex Inc. (Santa Ana, CA, USA). All solvents used were analytical or HPLC grade and obtained from Kanto Chemicals (Tokyo, Japan), membrane filters of Ekicrodisc 25R (0.45-µm pore size; PTFE; P/N E252) obtained from Gelman Science Japan Co. (Tokyo, Japan) were used for the filtration of the mobile phase and the real samples solution. Develosil C30-UG-5 (C30; mean particle diameter, 5 µm; Nomura Chemical, Seto, Japan) and C18 (L-column2 ODS, Chemical Evaluation and Research Institute, Tokyo, Japan) were used as the stationary phases. These stationary phases were packed into fused-silica tubes (150 mm in length x 0.32 mm i.d. x 0.46 mm o.d.; GL Sciences, Tokyo, Japan). The packing technique employed in this work was almost the same as previously reported [19].

2.2.2 Plant materials

Thirty seven samples from three varieties of gingers were collected from various locations on Java Island, Indonesia (Table 2-1). All of the samples were identified in Herbarium Bogoriense, Research Center for Biology, Indonesian Institute of Sciences, Indonesia and voucher specimens were deposited in Biopharmaca Research Center, Bogor Agricultural University, Indonesia (BMK 2012050001-BMK 2012050037). All samples were sieved, dried and pulverized prior to use.
Table 2-1 Sources of sample

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Sources (sub district, regency, province)</th>
<th>Sample code</th>
<th>Sources (sub district, regency, province)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZOA-1</td>
<td>Arjosari, Pacitan, East Java</td>
<td>ZOO-7</td>
<td>Wonogiri-1, Wonogiri, Central Java</td>
</tr>
<tr>
<td>ZOA-2</td>
<td>Pacitan, Pacitan, East Java</td>
<td>ZOO-8</td>
<td>Wonogiri-2, Wonogiri, Central Java</td>
</tr>
<tr>
<td>ZOA-3</td>
<td>Tegal Ombo, Pacitan, East Java</td>
<td>ZOO-9</td>
<td>Ciampea, Sukabumi, West Java</td>
</tr>
<tr>
<td>ZOA-4</td>
<td>Ponorogo, Ponorogo, East Java</td>
<td>ZOO-10</td>
<td>Nagrak, Sukabumi, West Java</td>
</tr>
<tr>
<td>ZOA-5</td>
<td>Pulung, Ponorogo, East Java</td>
<td>ZOO-11</td>
<td>Ciampea, Bogor, West Java</td>
</tr>
<tr>
<td>ZOA-6</td>
<td>Tawangmangu-1, Karanganyar, Central Java</td>
<td>ZOO-12</td>
<td>Leuwiliang, Bogor, West Java</td>
</tr>
<tr>
<td>ZOA-7</td>
<td>Tawangmangu-2, Karanganyar, Central Java</td>
<td>ZOR-1</td>
<td>Pacitan, Pacitan, East Java</td>
</tr>
<tr>
<td>ZOA-8</td>
<td>Ngadirojo, Wonogiri, Central Java</td>
<td>ZOR-2</td>
<td>Tegal Ombo, Pacitan, East Java</td>
</tr>
<tr>
<td>ZOA-9</td>
<td>Tirtomoyo, Wonogiri, Central Java</td>
<td>ZOR-3</td>
<td>Ponorogo, Ponorogo, East Java</td>
</tr>
<tr>
<td>ZOA-10</td>
<td>Wonogiri-1, Wonogiri, Central Java</td>
<td>ZOR-4</td>
<td>Slahung, Ponorogo, East Java</td>
</tr>
<tr>
<td>ZOA-11</td>
<td>Wonogiri-2, Wonogiri, Central Java</td>
<td>ZOR-5</td>
<td>Wonogiri-1, Wonogiri, Central Java</td>
</tr>
<tr>
<td>ZOA-12</td>
<td>Kutoarjo, Purworejo, Central Java</td>
<td>ZOR-6</td>
<td>Wonogiri-2, Wonogiri, Central Java</td>
</tr>
<tr>
<td>ZOA-13</td>
<td>Ciampea, Bogor, West Java</td>
<td>ZOR-7</td>
<td>Kutoarjo, Purworejo, Central Java</td>
</tr>
<tr>
<td>ZOO-2</td>
<td>Arjosari, Pacitan, East Java</td>
<td>ZOR-8</td>
<td>Purworejo, Purworejo, Central Java</td>
</tr>
<tr>
<td>ZOO-3</td>
<td>Tegal Ombo, Pacitan, East Java</td>
<td>ZOR-9</td>
<td>Ciampea-1, Bogor, West Java</td>
</tr>
<tr>
<td>ZOO-4</td>
<td>Ponorogo, Ponorogo, East Java</td>
<td>ZOR-10</td>
<td>Ciampea-2, Bogor, West Java</td>
</tr>
<tr>
<td>ZOO-5</td>
<td>Slahung, Ponorogo, East Java</td>
<td>ZOR-11</td>
<td>Leuwiliang, Bogor, West Java</td>
</tr>
<tr>
<td>ZOO-6</td>
<td>Tirtomoyo, Wonogiri, Central Java</td>
<td>ZOR-12</td>
<td>Nagrak, Sukabumi, West Java</td>
</tr>
</tbody>
</table>

2.2.3 Apparatus and chromatographic conditions

An ultrasonication device (USD-3R; As One, Osaka, Japan) was used for sample extraction. Capillary LC system used in this work consists of an L.TEX 8301 Micro Feeder (L.TEX Corp., Tokyo, Japan) equipped with an MS-GAN 050 gas-tight syringe (0.5 mL Ito, Fuji, Japan) as a pump, a model M-435 micro-injection valve (Upchurch...
Scientific, Oak Harbor, WA, USA) with the injection volume of 0.2 µL as an injector, a
0.32 mm i.d. x 150 mm microcolumn and a UV-2705 UV detector (JASCO, Tokyo,
Japan). The data were acquired by a Chromatopac C-R4A data processor (Shimadzu,
Kyoto, Japan). Acetonitrile aqueous solution was used as the mobile phase in the isocratic
elution mode with a flow rate of 5 µL/min and monitored at 280 nm. Peaks of 6-, 8-, 10-gingerol and 6-shogaol were identified by comparing the retention times with the
standards.

2.2.4 Standard solutions and sample preparation

Accurately weighed powdered samples (50 mg) were sonicated with methanol (5
mL) for 1 h at room temperature. The sample extracts were filtered through a 0.45 µm
membrane filter before injected into capillary LC. Standard stock solutions of the 6-, 8-, 10-gingerol and 6-shogaol were prepared in methanol at concentrations of 1600 µg/mL
for 6-gingerol and 1000 µg/mL for 8-gingerol, 10-gingerol and 6-shogaol, respectively.
An appropriate amount of each standard stock solution was mixed and diluted with
methanol to obtain seven concentrations of the working standard solutions of the four
analytes for constructing the calibration curves.

2.2.5 Method validation

Validation of the method was evaluated following the guidelines of the
International Conference on Harmonization (ICH) by determining the linearity of the
calibration curves, limit of detection (LOD), limit of quantification (LOQ), precision,
accuracy and stability [31]. The sample used for the validation tests was ZOA-6.
2.2.6 Data analysis

In this work, discriminant analysis was used to build a model for discrimination of the three ginger varieties and it was performed in XLSTAT software version 2012.2.02 (Addinsoft, New York, USA). The amounts of 6-gingerol, 8-gingerol, 10-gingerol and 6-shogaol were used as the variables.

2.3 Results and Discussions

2.3.1 Optimization of capillary LC conditions

In order to obtain good separation of the analytes in the chromatogram, the type of stationary phase and the mobile phase composition were optimized in this study. For the detection wavelength we used 280 nm according to the previous reference [9]. We examined and compared reversed phase C18 and C30 as the stationary phase with acetonitrile-water as the mobile phase in different composition (60%, 70% and 80%). The flow rate was maintained at 5 µL/min. The resolution of each analytes and the total analysis time were used as the parameters for choosing the optimized chromatographic conditions. Representative chromatograms from the standard solution of analytes are shown in Fig. 2-2.
Fig. 2-2 Capillary LC chromatogram of standard solution of gingerols and shogaol on the C30 and C18 stationary phases. Column: C30 (a) or C18 (b), 150 × 0.32 mm i.d. Mobile phase 60% acetonitrile. Flow-rate: 5.0 µL/min. Sample: 6-gingerol (1), 8-gingerol (2), 6-shogaol (3) and 10-gingerol (4); 10 µg/mL each except for 8-gingerol, 5 µg/mL. Sample injection volume: 0.2 µL. Wavelength of UV detection: 280 nm.

From the results obtained, by using C30 as stationary phase and 60% acetonitrile as mobile phase, a good separation was achieved for all analytes with a separation factor of each peak greater than 1.5 and total analysis time within 25 min. Separation of these four compounds was also achieved using C18 as the stationary phase and 60% acetonitrile in a shorter time compared to C30 with the same mobile phase composition, but the gingerol peak is not pure because there is an impurity peak overlapped with the 6-gingerol peak as we can see in Fig. 2-2.
2.3.2 Validation of the method

Validation of the method was evaluated in terms of linearity of calibration curves, LOD, LOQ, precision, accuracy and stability. Calibration was performed at seven concentration levels with three replicates injection over the concentration range 5-320 µg/mL by plotting the peak areas versus the concentration of each analytes. Linearity of the calibration curves was determined by the correlation coefficient ($r^2$). Good linearity was obtained with a mean correlation coefficient value greater than 0.9995 for all analytes within the test range. LODs (S/N=3) and LOQs (S/N=10) were found to be between 0.034-0.039 µg/mL and 0.112-0.129 µg/mL, respectively. Detailed information for the calibration curves, LOD and LOQ are summarized in Table 2-2.

Table 2-2 Calibration curves, LOD and LOQ for determination of gingerols and shogaol

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Regression equation$^a$ (y = a +bx)</th>
<th>Correlation coefficient ($r^2$)</th>
<th>LOD (µg/mL)</th>
<th>LOQ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-gingerol</td>
<td>-1394.2 + 1640.9x</td>
<td>0.9995</td>
<td>0.034</td>
<td>0.112</td>
</tr>
<tr>
<td>8-gingerol</td>
<td>-46.1 + 1429x</td>
<td>0.9996</td>
<td>0.039</td>
<td>0.129</td>
</tr>
<tr>
<td>6-shogaol</td>
<td>-740.46 + 1523.3x</td>
<td>0.9997</td>
<td>0.036</td>
<td>0.121</td>
</tr>
<tr>
<td>10-gingerol</td>
<td>-2391.5 + 1555.9x</td>
<td>0.9997</td>
<td>0.036</td>
<td>0.118</td>
</tr>
</tbody>
</table>

$^a$ concentration range for all analytes: 5-320 µg/mL (triplicate measurement)

Precision of the method was examined by intra- and inter-day repeatability of six individual samples in each day within three consecutive days. The precision was expressed as relative standard deviation (RSD) and the values obtained for intra- and inter-day were found less than 3.1%, which showed good repeatability of the method. The accuracy of the method was evaluated by carrying out recovery test with three different concentration levels and triplicate measurements at each level. The average percentages
of recovery for all analytes were found to be in the range of 97-105% with RSD values below 4%. These results demonstrated that the established method was reliable and accurate. Stability of analytes in sample solution was evaluated by analyzing the sample solution within 1 day at 0, 4, 8, 12, and 24 h at room temperature. The analytes were found to be stable in the sample solution with RSD values range between 1.34–2.93% for all compounds. Analytical data for precision, accuracy and stability were tabulated in Table 2-3.

Table 2-3 Analytical data for precision, recovery and stability of the proposed method.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Precision (RSD, %)</th>
<th>Recovery&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Stability&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intraday (n =6)</td>
<td>Interday (n=3)</td>
<td>Average recovery (%)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>6-gingerol</td>
<td>Day 1: 2.42</td>
<td>1.69</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>Day 2: 1.98</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Day 3: 2.55</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8-gingerol</td>
<td>Day 1: 2.21</td>
<td>2.49</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>Day 2: 1.34</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Day 3: 2.79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-shogaol</td>
<td>Day 1: 2.48</td>
<td>2.62</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>Day 2: 2.91</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Day 3: 3.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10-gingerol</td>
<td>Day 1: 2.29</td>
<td>2.30</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Day 2: 1.40</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Day 3: 2.24</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> three levels of added standard compounds (6-gingerol: 50, 125 and 200 µg; 8-gingerol: 45, 110, and 175 µg; 10-gingerol: 50, 120 and 190 µg; 6-shogaol: 45, 120, 190 µg) in the sample solution with each level measured in triplicate.

<sup>b</sup> for five measurements at 0, 4, 8, 12 and 24 h after the extraction of the sample.
2.3.3 Quantitative analysis of gingerols and shogaol in three varieties of ginger

The established capillary LC method was applied to the simultaneous determination of gingerols and shogaol in the 37 samples of gingers consisting of 13 ZOA samples, 12 ZOO samples and 12 ZOR samples. Each sample was analyzed with five replicate measurements to determine the mean amount of each analyte. Fig. 2-3 shows representative chromatograms of the three varieties of ginger and the target analytes. It can be seen that the objective components are well separated from coexisting species.

Fig. 2-3 Representative capillary LC chromatogram of real samples on the C30 stationary phase. 6-gingerol (1), 8-gingerol (2), 6-shogaol (3) and 10-gingerol (4). ZOA (a), ZOO (b) and ZOR (c). Operating conditions as in Fig. 2-2.
As shown in Table 2-4, the amounts of 6-, 8-, 10-gingerol and 6-shogaol in all samples were ranging from 2.98-18.83, 0.52-3.07, 0.79-3.54 and 1.24-3.53 μg/g, respectively. 6-gingerol was found to be the most dominant compound, while 8-gingerol was the lowest in amount in majority of the raw material samples investigated. This variation in the amount of gingerols and shogaol may be due to the variations of geographical origin, harvest time, environmental growth conditions and post-harvest processes. Due to these great differences in the amount of gingerols and shogaol in the three varieties of gingers, we could not discriminate them only based on the amount of these four bioactive compounds. Therefore, chemometrics method, which is widely employed for discriminating closely related medicinal plants, was also used.

Table 2-4 The amount of the four bioactive compounds in gingers.

<table>
<thead>
<tr>
<th>Sample code</th>
<th>6-gingerol</th>
<th>RSD (%)</th>
<th>8-gingerol</th>
<th>RSD (%)</th>
<th>10-gingerol</th>
<th>RSD (%)</th>
<th>6-shogaol</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZOA-1</td>
<td>11.53 ± 0.11</td>
<td>0.94</td>
<td>2.47 ± 0.06</td>
<td>2.24</td>
<td>3.07 ± 0.02</td>
<td>0.67</td>
<td>2.56 ± 0.07</td>
<td>2.60</td>
</tr>
<tr>
<td>ZOA-2</td>
<td>9.54 ± 0.16</td>
<td>1.70</td>
<td>2.19 ± 0.05</td>
<td>2.20</td>
<td>2.73 ± 0.11</td>
<td>4.36</td>
<td>2.75 ± 0.09</td>
<td>3.29</td>
</tr>
<tr>
<td>ZOA-3</td>
<td>8.85 ± 0.06</td>
<td>0.62</td>
<td>2.56 ± 0.04</td>
<td>1.56</td>
<td>3.54 ± 0.02</td>
<td>0.62</td>
<td>1.89 ± 0.04</td>
<td>1.98</td>
</tr>
<tr>
<td>ZOA-4</td>
<td>6.48 ± 0.11</td>
<td>1.66</td>
<td>1.44 ± 0.04</td>
<td>2.77</td>
<td>2.13 ± 0.05</td>
<td>2.38</td>
<td>2.34 ± 0.07</td>
<td>2.94</td>
</tr>
<tr>
<td>ZOA-5</td>
<td>9.28 ± 0.30</td>
<td>3.21</td>
<td>1.85 ± 0.05</td>
<td>2.50</td>
<td>2.25 ± 0.09</td>
<td>4.10</td>
<td>2.31 ± 0.06</td>
<td>2.79</td>
</tr>
<tr>
<td>ZOA-6</td>
<td>12.91 ± 0.20</td>
<td>1.55</td>
<td>2.88 ± 0.05</td>
<td>1.62</td>
<td>3.36 ± 0.05</td>
<td>1.59</td>
<td>3.23 ± 0.06</td>
<td>2.03</td>
</tr>
<tr>
<td>ZOA-7</td>
<td>6.78 ± 0.24</td>
<td>3.40</td>
<td>1.52 ± 0.06</td>
<td>3.69</td>
<td>2.18 ± 0.06</td>
<td>2.91</td>
<td>1.75 ± 0.03</td>
<td>1.87</td>
</tr>
<tr>
<td>ZOA-8</td>
<td>14.6 ± 0.22</td>
<td>1.53</td>
<td>2.68 ± 0.05</td>
<td>1.83</td>
<td>2.74 ± 0.05</td>
<td>1.87</td>
<td>3.05 ± 0.09</td>
<td>2.94</td>
</tr>
<tr>
<td>ZOA-9</td>
<td>9.00 ± 0.28</td>
<td>3.11</td>
<td>2.14 ± 0.01</td>
<td>0.63</td>
<td>2.66 ± 0.04</td>
<td>1.35</td>
<td>3.27 ± 0.09</td>
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</tr>
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<td>ZOA-10</td>
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<td>1.97 ± 0.03</td>
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</tr>
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<td>7.29 ± 0.12</td>
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<td>1.56 ± 0.03</td>
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<td>2.05 ± 0.03</td>
<td>1.44</td>
<td>2.47 ± 0.05</td>
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<td>ZOO-5</td>
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<tr>
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<td>9.86 ± 0.37</td>
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<td>2.58 ± 0.07</td>
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<td>3.53 ± 0.12</td>
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<td>2.73</td>
<td>3.49</td>
<td>2.60</td>
<td>2.32</td>
</tr>
</tbody>
</table>
2.3.4 Discrimination of three ginger varieties

In order to develop a method for discriminating the three varieties of ginger, we employed a combination of LC analysis with a chemometrics method. This combination has become one of the most frequently applied approaches for classification, authentication and discrimination of medicinal plants for recognition of geographical origin, detection of adulteration and discrimination of closely related species. Discriminant analysis is one of the multivariate statistical analyses that can be used for this purpose. Discriminant analysis will construct discriminant functions for each group by finding the linear combinations of features that give better separation in two or more groups of observations. In this study, the predictive model of the variety group is built based on the amount of gingerols and shogaol as the variables.

The result of discriminant analysis showed that 100% of each observation groups (ZOA, ZOO and ZOR) were correctly classified (as shown in Fig 2-4), or in other words, the discriminant functions obtained discriminate well between each groups. Cross-validation was used to evaluate the predictive ability of the discriminant model and found 94% from all of the samples were correctly classified with 2 samples (ZOA-13 and ZOR-12) were misclassified. So, based on these results, the amount of gingerols and shogaol are good predictors for discrimination of the three varieties of ginger used in this study.
2.4 Conclusion

The capillary LC method was successfully developed for simultaneous quantitative analysis of 6-, 8-, 10-gingerol and 6-shogaol in raw materials of the three varieties of gingers with good accuracy and reliability. Combination between the amount of gingerols and shogaol determined by capillary LC with discriminant analysis could discriminate the three varieties of ginger tested. The simultaneous quantitative analysis in combination with discriminant analysis was proven to be an efficient method and practically could be applied for the quality control of ginger raw materials and its related products.
2.5 References

Chapter 3

Capillary liquid chromatographic fingerprint analysis used for
discrimination of *Zingiber montanum* from related species

3.1 Introduction

The genus of *Zingiber* is widely spread and used in many Asian countries as a spices, flavor and medicines. One of them is *Z. montanum* known as cassumunar ginger and in Indonesia it is locally known as *bangle*. The rhizome of *Z. montanum* is known for its use in folk medicines to treat various diseases such as asthma, carminative, colic, diarrhea, stomachic, muscle and joint pain [1,2]. Several biological activities have been reported for this plant like antiulcer [2], antioxidant [1,3,4], antiallergic [5], anti-inflammatory [6] as well as antimicrobial activity [4].

There are some related species from the genus of *Zingiber* also available in local Indonesia market and could be used as the fake herbs for *Z. montanum* such as *Z. americans* and *Z. zerumbet*. In Indonesia, *Z. americans* and *Z. zerumbet* are known as *lempuyang emprit* and *lempuyang gajah* respectively. *Z. americans* was reported to have antimicrobial activity [7] while *Z. zerumbet* also reported to have some similar biological activity to *Z. montanum* such as antioxidant, antiinflammatory and antimicrobial activity [8-10]. These three rhizomes look similar in having a pale yellow color and the differences are found in their smell and the size of the rhizome. So physically, to discriminate them is not difficult but it will become more challenging if they are present in powdered forms. Moreover, the price of *Z. montanum* normally is higher than *Z. americans* and *Z. zerumbet*, thus *Z. americans* and *Z. zerumbet* are sometimes found as a
fake herbs for *Z. montanum*. Therefore, an accurate analytical method for the identification of *Z. montanum* is crucial in order to prevent using the fake herbs and also to ensure the quality of herbal medicines that use *Z. montanum*.

The discrimination of some closely related species was commonly used a macroscopical and microscopical analysis as well as marker compound analysis. Macroscopic and microscopic methods sometimes do not provide a precise identification or authentication. Also the marker compound analysis is not always sufficient for this purpose because chemical compositions in medicinal plant are very complex [11]. Moreover, the activity of medicinal plants is often based on synergetic effects of several compounds. The lack of unique chemical compounds (markers) for the identification of certain medicinal plant is also a real problem to qualitatively discriminate them [12].

Fingerprint analysis was recently introduced for the quality control method of herbal medicines with the purpose such as identification, authentication and discrimination of some closely related species. Since the mid 1970's fingerprint analysis have been the subject of literature studies and now gained much attention due to their ability to give more detailed characteristics profile which chemically represents in the sample [13]. So far, fingerprint analysis has been widely used as a feasible and useful method for species identification and authentication. World Health Organization (WHO), Chinese Food and Drug Administration (SFDA), European Medicine Evaluation Agency (EMEA) as well as the United State Food and Drug Administration (US-FDA) have also accepted fingerprint analysis for quality control of medicinal plant [14].

Several analytical instruments have been used for developing a fingerprint analysis and in most cases, chromatography and spectroscopy techniques are widely employed. For chromatography techniques, they used TLC, GC and HPLC. HPLC was
the most frequently employed in fingerprint analysis using octadecylsilica (C18) as the stationary phase packed into conventional size column (4.6 mm i.d.). Fingerprint chromatograms are complex and they represent the chemical characteristics of a sample, so it will contain a large amount of data. To deal with it, we need the help from multivariate analysis. Multivariate analysis is used for data handling such as exploratory data analysis or pattern recognition to build an identification, authentication, or classification of the samples. Combination of chromatographic fingerprint and multivariate analysis has been used extensively for authentication and discrimination of many medicinal plants [15-20].

Currently, there is a trend for miniaturizing an analytical instrumentation in the field of separation sciences and one of them is micro-column liquid chromatography (μLC) or also known as capillary LC. Horvarth et al. [21] was firstly introduced this technique, and a stainless steel packed column with an 0.5-1.0 mm i.d. was used. Thereafter, Ishii et al. also started using μLC with slurry packed micro-column in their works [22]. The main features of capillary LC compared with the conventional-sized LC are the use of columns with smaller i.d. and lower eluent flow rate. With these features, capillary LC offers several analytical advantages such as improved mass sensitivity, reduced consumption of packing materials, mobile phase, and the sample amounts [23]. Capillary LC also has been used in fingerprint analysis studies since the mid 1980’s and continues to develop with rapid development in the instrumentation. Therefore, owing to the advantages by using capillary LC, we used this technique to develop a fingerprint analysis combined with multivariate analysis for identification and discrimination of Z. montanum from its related species.
To date, there was no reported method regarding qualitative analysis to discriminate *Z. montanum* from *Z. americans* and *Z. zerumbet* in order to discriminate *Z. montanum*. So, based on what we described above we developed for the first time a capillary LC fingerprint analysis and in combination with some multivariate analysis such as PCA and DA for discrimination of *Z. montanum*. The proposed method was successfully applied for discrimination of *Z. montanum* from *Z. americans* and *Z. zerumbet*.

### 3.2. Experimental

#### 3.2.1. Chemicals and reagents

All solvents used were obtained from Kanto Chemicals (Tokyo, Japan) as analytical or HPLC grade, membrane filters of Ekicrodisc 25R (0.45-µm pore size; PTFE; P/N E252) obtained from Gelman Science Japan Co. (Tokyo, Japan) were used for the filtration of the mobile phase and samples solution. As the stationary phase, we used C18 (L-column2 ODS-5, Chemical Evaluation and Research Institute, Tokyo, Japan). The stationary phase was packed into fused-silica tube (150 mm in length x 0.32 mm i.d. x 0.46 mm o.d.; GL Sciences, Tokyo, Japan) by a slurry method as previously reported [22].

#### 3.2.2 Plant materials

24 samples consists of 11 samples of *Z. montanum*, 7 samples of *Z. americans* and 6 samples of *Z. zerumbet* were collected from three province in Java Island, Indonesia (Table 3-1). All of the samples were identified in Herbarium Bogoriense, Research Center for Biology, Indonesian Institute of Sciences, Indonesia and voucher specimens were deposited in Biopharmaca Research Center, Bogor Agricultural University,
Indonesia (BMK 2012050038-BMK 2012050061). All samples were sieved, dried and pulverized prior to use.

<table>
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<tr>
<th>Species</th>
<th>Sample code</th>
<th>Sources (regency, province)</th>
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<tbody>
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<td>ZM-1</td>
<td>Karanganyar, Central Java</td>
</tr>
<tr>
<td></td>
<td>ZM-2</td>
<td>Karanganyar, Central Java</td>
</tr>
<tr>
<td></td>
<td>ZM-3</td>
<td>Purworejo, Central Java</td>
</tr>
<tr>
<td></td>
<td>ZM-4</td>
<td>Purworejo, Central Java</td>
</tr>
<tr>
<td></td>
<td>ZM-5</td>
<td>Wonogiri, Central Java</td>
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<tr>
<td></td>
<td>ZM-6</td>
<td>Wonogiri, Central Java</td>
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<td></td>
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<td>Wonogiri, Central Java</td>
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<td></td>
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<td>Ponorogo, East Java</td>
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<td></td>
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<td>Karanganyar, Central Java</td>
</tr>
<tr>
<td></td>
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<td>Pacitan, East Java</td>
</tr>
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</tr>
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<td>Pacitan, East Java</td>
</tr>
<tr>
<td></td>
<td>ZZ-6</td>
<td>Ponorogo, East Java</td>
</tr>
</tbody>
</table>
3.2.3 Instrumentation and chromatographic conditions

An ultrasonication device (USD-3R; As One, Osaka, Japan) was used for samples extraction. Capillary LC system used in this work consists of an L.TEX 8301 micro-feeder (L.TEX Corp., Tokyo, Japan) equipped with an MS-GAN 050 gas-tight syringe (0.5 mL Ito, Fuji, Japan) as a pump, a model M-435 microinjection valve (Upchurch Scientific, Oak Harbor, WA, USA) with the injection volume of 0.2 µL as an injector, a 0.32 mm i.d. x 150 mm micro-column and a UV-2705 UV detector (JASCO, Tokyo, Japan). The data were acquired by a Chromatopac C-R7A data processor (Shimadzu, Kyoto, Japan). 60% Acetonitrile was used as the mobile phase in the isocratic elution mode with a flow rate of 5 µL/min and monitored at 254 nm.

3.2.4 Sample preparation

Accurately weighed powdered samples (100 mg) were sonicated with methanol (5 mL) for 1 h at room temperature. The samples extracts were filtered through a 0.45 µm membrane filter before injection into capillary LC.

3.2.5 Analytical performance test

Analytical performance tests of the method were evaluated by determining precision, repeatability and stability as expressed as relative standard deviation (RSD) of relative retention time (RRT) and relative peak area (RPA) of the characteristic peaks which are shown in the chromatograms (Fig 3-1). The RRT and RPA were calculated to reference peak number 3. The sample used for these tests was ZM-4.
3.2.6 Data analysis

In this work, principal component analysis (PCA) and discriminant analysis (DA) were used to build a model for discrimination of the *Z. montanum*, *Z. americans* and *Z. zerumbet* and it was performed in XLSTAT software version 2012.2.02 (Addinsoft, New York, USA). We used the peak area of the common peaks obtained from fingerprint chromatograms of *Z. montanum*, *Z. americans* and *Z. zerumbet* as the variables.

3.3 Results and Discussions

3.3.1 Optimization of capillary LC conditions

To develop an optimum capillary LC fingerprint, we examined and compared different composition of acetonitrile-water (50%, 60% and 70%). Other parameters for capillary LC were made fixed such as stationary phase using C18, flow rate at 5 μL min⁻¹, detection wavelength at 254 nm because most organic compound will absorb in this wavelength. Total analysis time, the number of common peaks and resolutions of each common peak were used as the parameters for choosing the optimized capillary LC fingerprint analysis. From the results obtained, by using 60% acetonitrile as mobile phase was found to give a good fingerprint chromatogram with a total analysis time within 30 min (Fig. 3-1).
Fig. 3-1 Representative capillary LC fingerprint chromatograms of *Z. montanum* (a), *Z. americans* (b) and *Z. zerumbet* (c). Column: L-column2 ODS-5 stationary phases (150 × 0.32 mm i.d.), mobile phase 60% acetonitrile, flow-rate: 5 µL/min and wavelength of UV detection: 254 nm.

### 3.3.2 Analytical performance of the method

Analytical performance of the method was evaluated in terms of precision, repeatability and stability. Precision of the method was examined by successive analysis of five injection of the same sample solution. The RSD of RRT and RPA precision of were found less than 1.4% and 2.8%, respectively. Repeatability of the method was performed by injecting five sample solutions prepared independently. The RSD of RRT and RPA of all common peaks in the repeatability test were in the range 1.3-4.8% and
1.4-5.0%, respectively. The stability of the method was evaluated by analyzing the sample solution at 0, 4, 8, and 12 h at room temperature. The RSD of RRT and RPA were found below 1.5% and 4.4%. The result of analytical performance tests indicated that the developed capillary LC fingerprint method was reliable and valid.

### 3.3.3 Establishment of capillary LC fingerprint of *Z. montanum*, *Z. americans* and *Z. zerumbet*

The established capillary LC fingerprint method was applied to the 24 samples consists of 11 *Z. montanum* samples, 7 *Z. americans* samples and 6 *Z. zerumbet* samples. Representative chromatograms from the selected samples of *Z. montanum*, *Z. americans* and *Z. zerumbet* are shown in Fig. 3-1. In each species, the chromatographic profiles were similar. Peaks existing in all samples were assigned as the common peaks. Selection of the common peaks was performed by comparing their retention times and UV spectra of some samples in the HPLC-DAD system. Eight common peaks (Nos. 2, 3, 4, 5, 6, 7, 9 and 10) were chosen as characteristic peaks for the identification of ZM and 4 common peaks (Nos. 1, 2, 3 and 8) for ZA and ZZ. We used a characteristics fingerprints approach with normalization in the retention time and peak area by calculating the relative retention time and peak area to the peak number 3 as the reference peak from each fingerprint chromatogram. Peak number 3 was selected as the reference peak because this peak appears in all samples.

RRT and RPA of each common peak were calculated to the reference peak and summarized in Table 3-2. Since the result indicated that the RRT of each common peak is relatively consistent, the RRT is a suitable parameter for the identification of the samples. However, the RPA is greatly varied in all samples maybe due to different environmental
growth conditions which can result in the difference in chemical composition. By comparing the fingerprint chromatograms of all samples tested, only 2 characteristic peaks (2 and 3) from *Z. montanum* also appears in the *Z. americans* and *Z. zerumbet*, so the six remaining peaks were typical for *Z. montanum*. Therefore, based on the RRT mean value and 6 characteristic peaks (4,5,6,7,9 and 10) we could identify unknown sample as *Z. montanum* if they showed similar chromatographic profile and the RRT value obtained nearly the same with the RRT mean values (Table 3-2).

### 3.3.4 Discrimination of *Z. montanum*, *Z. americans* and *Z. zerumbet* from the chromatographic profile

Fingerprint analysis can be accurately used for the purpose of discrimination of some closely related species. As we mentioned above, visual inspection by comparing the fingerprint chromatograms of *Z. montanum*, *Z. americans* and *Z. zerumbet*, we selected the peak number 4,5,6,7,9 and 10 as the marker peak for *Z. montanum*. By using these marker peaks we could discriminate *Z. montanum* from *Z. americans* and *Z. zerumbet*. Since those peaks are typical in *Z. montanum* chromatogram profile, we could easily use these marker peak to identify *Z. montanum*.

Furthermore, to discriminate *Z. montanum* from *Z. americans* and *Z. zerumbet*, we can employ peak number 8 which only appears in the *Z. americans* and *Z. zerumbet* chromatogram profile. This peak also could be used for the detection if *Z. montanum* sample was replaced with *Z. americans* and *Z. zerumbet*. Discrimination of *Z. americans* from *Z. zerumbet* could not be obtained by using visual inspection on their chromatogram because the chromatogram profiles were very similar, only differ in their peaks intensities. Therefore we need an aid from chemometrics to deal with it.
<table>
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<th>Sample</th>
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<td>1 2 3 4 5 6 7 8 9 10</td>
<td>1 2 3 4 5 6 7 8 9 10</td>
</tr>
<tr>
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<td>- - 1 1.51 1.90 2.57 2.91 -</td>
<td>4.08 4.58 -</td>
</tr>
<tr>
<td>ZM-2</td>
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<td>4.29 4.84 -</td>
</tr>
<tr>
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<td>4.26 4.83 -</td>
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<tr>
<td>ZZ-6</td>
<td>0.79 0.89 1 - - - - 3.12 - -</td>
<td>0.05 0.07 1 - - - -</td>
</tr>
<tr>
<td>Mean</td>
<td>0.78 0.87 - 1.53 1.93 2.62 3.00 3.16 4.20 4.75</td>
<td>0.92 1.12</td>
</tr>
</tbody>
</table>

Table 3-2 Relative retention time and relative peak area of all samples.
3.3.5 Discrimination of *Z. montanum* from *Z. americans* and *Z. zerumbet* by combining capillary LC fingerprint with multivariate analysis

To confirm the result obtained from the visual inspection of the fingerprint chromatogram in discrimination of *Z. montanum, Z. americans* and *Z. zerumbet*, we employed a combination of capillary LC fingerprint analysis with a multivariate analysis. This combination has become one of the most frequently applied approaches for species identification, authentication and classification of medicinal plants for recognition of geographical origin, detection of adulteration and discrimination of closely related species [24, 25]. In this study, we used some techniques in multivariate analysis such as PCA and DA which are commonly used to discriminate medicinal plant species.

PCA is a well-known multivariate analysis tool for reducing data and an extract an information to find a combination of variables or factors for describing major trends in a data set. In our study, PCA was employed to discriminate the samples according to the species based on the peak area of 10 common peaks. Fig 2 showed the score plot derived from PCA analysis using the first two PC. The total variance for this two PC is 84.8%, it means the two PC represent 84.8% of all the data.

As can be seen in Fig 3-2, the samples tested were clustered into three different groups. All *Z. montanum* samples were in the cluster I while *Z. americans* and *Z. zerumbet* samples were in the cluster II and III respectively. So with the PCA analysis we could discriminate *Z. montanum, Z. americans* and *Z. zerumbet*. Although we could discriminate between *Z. americans* and *Z. zerumbet* but the distance between them was so close. This situation occurs due to the chemical profile of *Z. americans* and *Z. zerumbet* was nearly similar as we can see in their fingerprint chromatograms (Fig 3-1b and c).
Another multivariate analysis that can be used for species identification and authentication is discriminant analysis. Discriminant analysis will generate discriminant functions for each group by finding the linear combinations of features that give better separation in two or more groups of observations. The predictive model of the species group is built based on the peak area of 10 common peaks as the variables.

From the result of discriminant analysis, it is clearly showed that 100% of original groups were correctly classified (Fig 3-3), indicating that the discriminant functions obtained discriminate well between Z. montanum, Z. americans and Z. zerumbet. The predictive ability of the discriminant model was evaluated by cross-validation test. About 92% from all of the samples were correctly classified and only 2 samples (ZA-4 and ZZ-4) were misclassified. So, based on these results, peak area of 10 common peaks are
good predictors for discrimination of *Z. montanum*, *Z. americans* and *Z. zerumbet* used in this work.

![Plot of discriminant function of *Z. montanum* (▲), *Z. americans* (■) and *Z. zerumbet* (●)](image)

**3.4 Conclusion**

A valid and reliable capillary LC fingerprint analysis was successfully developed for discrimination of *Z. montanum*, *Z. americans* and *Z. zerumbet*. By visual inspection of the chromatographic profile using its marker peak we could identify and discriminate *Z. montanum* from *Z. americans* and *Z. zerumbet*. Discrimination of *Z. montanum*, *Z. americans* and *Z. zerumbet* could be obtained by using PCA and DA. The capillary LC fingerprint analysis and in combination with PCA and DA was proven to be an efficient method and practically could be applied for the identification and discrimination of *Z. montanum*, *Z. americans* and *Z. zerumbet*. 
3.5 References

Chapter 4

Discrimination of Curcuma longa and Curcuma xanthorrhiza by high performance liquid chromatography combined with chemometrics method

4.1 Introduction

Turmeric (C. longa) and java turmeric (C. xanthorrhiza) which belong to the Zingiberaceae family were extensively used in jamu, i.e. traditional Indonesian medicines. Turmeric is also known as spices (main ingredient in curry), dyes and also used in traditional beverages [1,2]. Turmeric is native to India and distributed throughout tropical and subtropical regions of the world and widely cultivated in Southeast Asia while java turmeric is native to Indonesia and also widely cultivated in Southeast Asia, too [3,4]. The rhizomes of these plants are usually aromatic and carminative, and are used to treat indigestion, hepatitis, jaundice, diabetes, atherosclerosis and bacterial infections [2]. Curcuminoids, diarylheptanoids are the main components in Curcuma species and responsible for their major pharmacological effects [2,5]. Curcuminoids in C. longa and C. xanthorrhiza are represented by curcumin, demethoxycurcumin and bisdemethoxycurcumin (Fig 4-1) with curcumin as the major components [5,6].

These two rhizomes are generally having a yellow or orange color due to the presence of curcuminoids. Physically, to discriminate these two rhizomes is not difficult because the size of C. xanthorrhiza is much bigger than C. longa, but it will become more difficult if they are pulverized as a powder or cut into slices. In Indonesia herbs market, the price of C. xanthorrhiza is normally 3-5 times higher than C. longa [7]. This is one of
the reasons why *C. longa* is sometimes found to be the fake/adulterant of *C. xanthorrhiza*. So it is very important and necessary to discriminate both of them in term of authentication of plant material in order to guarantee the quality of its finished product. To do this task we will need an accurate analytical method for this purpose.

Fig 4-1 Chemical structures of (A) curcumin, (B) demethoxycurcumin and (C) bisdemethoxycurcumin
Several analytical methods have been developed for determination of curcuminoids in some Curcuma species, the most used species is *C. longa* with chromatography techniques were widely used. A number of qualitative and quantitative analysis of curcuminoids using TLC [7-9], HPLC with UV or fluorescence detector [5, 10-16], LC-MS [17,18], micellar electrokinetic chromatography (MEKC) [19] and CE [20] have been reported in various Curcuma samples. HPLC with UV-Vis detection was frequently employed for simultaneous determination of curcuminoids. Previously reported analytical methods for determination of curcuminoids mostly developed for *C. longa* and only a few reported regarding *C. xanthorrhiza*. Furthermore, only one reported analytical method for discrimination of *C. longa* and *C. xanthorrhiza* using CE using the concentration level of bisdemethoxycurcumin to discriminate these two species. Nonetheless, in their study only used one sample of *C. xanthorrhiza* and stability of the standard and sample solutions only 5 hours [20].

In this study, high performance liquid chromatography-diode array detector (HPLC-DAD) was used in the development of quantitative analysis of curcuminoids and also for the purpose to discriminate the *C. longa* and *C. xanthorrhiza*. Discrimination of these two closely related species was achieved by combining the curcuminoids content obtained by HPLC-DAD with some chemometric methods such as PCA and HCA. Satisfactory results were obtained to identify and discriminate this two Curcuma species.

### 4.2 Experimental

#### 4.2.1. Chemicals and reagents

Curcumin, demethoxycurcumin, and bisdemethoxycurcumin were purchased from Chromadex Inc. (Santa Ana, CA, USA). All solvents used were analytical or HPLC
grade and obtained from Merck (Darmstadt, Germany) and for membrane filters of Ekicrodisc 25R (0.45-µm pore size; PTFE; P/N E252) obtained from Gelman Science Japan Co. (Tokyo, Japan) were used for the filtration of samples solutions.

4.2.2 Plant materials

Thirty four samples consist of 16 samples of *C. Longa* and 18 samples of *C. xanthorrhiza* from various locations in three provinces located in Java Island, Indonesia (Table 4-1) were used. All of the samples were identified in Herbarium Bogoriense, Research Center for Biology, Indonesian Institute of Sciences, Indonesia and voucher specimens were deposited at Biopharmaca Research Center, Bogor Agricultural University, Indonesia (BMK 2012050062-BMK 2012050085). All samples were sieved, dried and pulverized prior to use.

4.2.3 Apparatus and chromatographic conditions

The HPLC system used was LC-20A series (Shimadzu, Tokyo, Japan) equipped with a diode array UV detector. A Shim-pack VP-ODS C18 column (150 mm × 4.6 mm i.d.) (Shimadzu, Tokyo, Japan) was used. The mobile phase consisted of acetonitrile (A) and 0.5% acetic acid in water (B) using a gradient elution program of 40–75% (A) in 0–30 min and 100% (A) in 30-40 min with a flow rate of 1 mL/min and monitored at 425 nm. Peaks of curcumin, demethoxycurcumin and bisdemethoxycurcumin were identified by comparing the retention times with the standards.
Table 4-1 Sources of sample

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Sources (subdistrict, regency, province)</th>
<th>Sample code</th>
<th>Sources (sub district, regency, province)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL-1</td>
<td>Ciemias, Sukabumi, West Java</td>
<td>CX-2</td>
<td>Nagrak, Sukabumi, West Java</td>
</tr>
<tr>
<td>CL-2</td>
<td>Ciampea, Bogor, West Java</td>
<td>CX-3</td>
<td>Ciampea, Bogor, West Java</td>
</tr>
<tr>
<td>CL-3</td>
<td>Tanjung Kerta, Sumedang, West Java</td>
<td>CX-4</td>
<td>Leuwiliang, Bogor, West Java</td>
</tr>
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<td>CL-4</td>
<td>Cimalaka, Sumedang, West Java</td>
<td>CX-5</td>
<td>Tanjung Kerta, Sumedang, West Java</td>
</tr>
<tr>
<td>CL-5</td>
<td>Wonogiri, Wonogiri, Central Java</td>
<td>CX-6</td>
<td>Wonogiri (2), Wonogiri, Central Java</td>
</tr>
<tr>
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<td>CX-7</td>
<td>Tirtomoyo, Wonogiri, Central Java</td>
</tr>
<tr>
<td>CL-7</td>
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<td>CX-8</td>
<td>Kutoarjo, Purworejo, Central Java</td>
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<td>CX-9</td>
<td>Purworejo, Purworejo, Central Java</td>
</tr>
<tr>
<td>CL-9</td>
<td>Tawangmangu, Karanganyar, Central Java</td>
<td>CX-10</td>
<td>Tawangmangu, Karanganyar, Central Java</td>
</tr>
<tr>
<td>CL-10</td>
<td>Karangpandan, Karanganyar, Central Java</td>
<td>CX-11</td>
<td>Gondang, Sragen, Central Java</td>
</tr>
<tr>
<td>CL-11</td>
<td>Gondang, Sragen, Central Java</td>
<td>CX-12</td>
<td>Karangpandan, Karanganyar, Central Java</td>
</tr>
<tr>
<td>CL-12</td>
<td>Arjosari, Pacitan, East Java</td>
<td>CX-13</td>
<td>Tegalombo (1), Pacitan, East Java</td>
</tr>
<tr>
<td>CL-13</td>
<td>Tegalombo, Pacitan, East Java</td>
<td>CX-14</td>
<td>Tegalombo (2), Pacitan, East Java</td>
</tr>
<tr>
<td>CL-14</td>
<td>Pulung, Ponorogo, East Java</td>
<td>CX-15</td>
<td>Pacitan, Ponorogo, East Java</td>
</tr>
<tr>
<td>CL-15</td>
<td>Slahung, Ponorogo, East Java</td>
<td>CX-16</td>
<td>Slahung, Ponorogo, East Java</td>
</tr>
<tr>
<td>CL-16</td>
<td>Sine, Ngawi, East Java</td>
<td>CX-17</td>
<td>Ponorogo, Ponorogo, East Java</td>
</tr>
<tr>
<td>CX-1</td>
<td>Ciemias, Sukabumi, West Java</td>
<td>CX-18</td>
<td>Sine, Ngawi, East Java</td>
</tr>
</tbody>
</table>

4.2.4 Preparation of standard and sample solutions

Accurately weighed powdered samples (25 mg) were sonicated with methanol (5 mL) for 1 h at room temperature. After filtration through a 0.45 μm membrane filter, the sample solutions were diluted to 10 mL with methanol before injected into HPLC-DAD. Standard stock solutions of the curcumin, demethoxycurcumin, and
bisdemethoxycurcumin were prepared in methanol at concentrations of 1000 µg/mL. An appropriate amount of each standard stock solution was mixed and diluted with methanol to obtain 12 concentrations ranging from 0.5-100µg/mL for curcumin and 11 concentrations ranging from 0.5-50 µg/mL for demethoxycurcumin and bisdemethoxycurcumin of the working standard solutions of the three analytes for constructing the calibration curves.

4.2.5 Validation of the method

Validation of the method for determination of curcuminoids was evaluated following the guidelines of the International Conference on Harmonization (ICH) by determining the system suitability, linearity of the calibration curves, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy and stability [21]. The sample used for the validation tests was CX-1.

4.2.6 Data analysis

PCA and HCA were used for discrimination of C. longa and C. xanthorrhiza. The analysis was performed in XLSTAT software version 2012.2.02 (Addinsoft, New York, USA). The amounts of each curcuminoids were used as the variables.

4.3 Results and Discussions

4.3.1 Optimization of HPLC conditions

In order to meet the requirement for quantitative analysis and chromatographic fingerprint analysis to have a good baseline separation of the desired analytes in the chromatogram, we examined the mobile phase composition and wavelength for detection
were optimized in this study. The resolution of each analyte and the total analysis time were used as the parameters for choosing the optimized chromatographic conditions. The flow rate was maintained at 1 mL/min.

Fig 4-2  HPLC chromatogram of standard solution of bisdemethoxycurcumin (1), demethoxycurcumin (2) and curcumin (3) with the concentration of each 25 μg/mL. Column: Shim-pack VP-ODS C\textsubscript{18} column (150 mm × 4.6 mm i.d.) (Shimadzu, Tokyo, Japan) was used. The mobile phase consisted of acetonitrile (A) and 0.5% acetic acid in water (B) using a gradient elution program of 40–75% (A) in 0–30 min and 100% (A) in 30-40 min with a flow rate of 1 mL/min and wavelength of UV detection: 425 nm.

The optimum chromatographic separation was achieved using linier gradient elution of 45-75% acetonitrile in 0.5% acetic acid with a resolution of each curcuminoids greater than 1.5 and total analysis time for quantitative analysis within 15 min (Fig 4-2).
For the detection wavelength we selected 425 nm for quantitative analysis of curcuminoids as the maximum wavelength of curcuminoids so it will give higher sensitivity compared to the other wavelength.

4.3.2 Method validation for quantitative analysis of curcuminoids

Validation of the method for quantitative analysis of curcuminoids was evaluated in terms of linearity of calibration curves, LOD, LOQ, precision, accuracy, and stability. Calibration was performed over the concentration range 0.5-100 µg/mL for curcumin and 0.5-50 µg/mL for demethoxycurcumin and bisdemethoxycurcumin by plotting the peak areas versus the concentration of each analyte. Linearity of the calibration curves was determined by the correlation coefficient ($r^2$). Satisfactory linearity was found with a correlation coefficient value greater than 0.9990 for all analytes within the test range. The LOD and LOQ for the three analyte were estimated at signal to noise ratio (S/N) equal to 3 and 10, respectively. The result for LODs and LOQs were found to be between 1.26-1.64 and 4.21-5.47 ng, respectively. This result indicated that the proposed method provide good sensitivity. Table 4-2 summarized the detailed information for the calibration curves, LOD and LOQ.

Table 4-2 Calibration curves, LOD and LOQ for the quantitative analysis of curcuminoids

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Regression equation $(y = a + bx)$</th>
<th>Correlation coefficient ($r^2$)</th>
<th>LOD (ng)</th>
<th>LOQ (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcumin</td>
<td>215,712 x - 152,880</td>
<td>0.990</td>
<td>1.53</td>
<td>5.11</td>
</tr>
<tr>
<td>Demethoxycurcumin</td>
<td>201,458 x - 78,681</td>
<td>0.992</td>
<td>1.64</td>
<td>5.47</td>
</tr>
<tr>
<td>Bisdemethoxycurcumin</td>
<td>261,936 x - 112,290</td>
<td>0.991</td>
<td>1.23</td>
<td>4.21</td>
</tr>
</tbody>
</table>
Table 4-3 showed an analytical data for precision, accuracy and stability of the proposed method. Intra- and inter-day repeatability of six individual samples in each day within three consecutive days were used to determine the precision of the method. The precision was expressed as RSD and the values obtained for intra- and inter-day were found less than 4.7%, which showed good repeatability of the method. The accuracy of the method was evaluated by carrying out recovery test by spiking known amount of curcuminoids standard solutions to a sample test (CX-1) with three different levels of added and triplicate measurements at each level. The average percentages of recovery for curcumin, demethoxycurcumin and bisdemethoxycurcumin were found 99, 99 and 98% respectively and their RSD values below 3.7%. These results demonstrated that the established method was accurate and reliable. Stability of analytes in sample solution was evaluated by analyzing the sample solution within 2 days at 0, 4, 8, 12, 24 and 48 h at room temperature. The analytes were found to be stable in the sample solution with RSD values range between 0.66-2.00% for all compounds.

4.3.3 Determination of curcuminoids in C. longa and C. xanthorrhiza

Simultaneous determination of curcuminoids was performed by using the established HPLC-DAD method. About 34 samples consist of 16 samples of C. longa and 18 samples of C. xanthorrhiza have been analyzed. Each sample was analyzed with three replicate measurements to determine the mean amount of each curcuminoids. Fig. 4-3 shows representative chromatograms of the C. longa and C. xanthorrhiza and the target analytes.
Table 4-3 Analytical data for precision, recovery and stability of the proposed method.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Precision (RSD, %)</th>
<th>Recovery&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Stability&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intraday (n =6)</td>
<td>Interday (n=3)</td>
<td>Average recovery (%)</td>
</tr>
<tr>
<td>Curcumin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1: 2.97</td>
<td>0.86</td>
<td>99</td>
<td>3.64</td>
</tr>
<tr>
<td>Day 2: 2.68</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 3: 4.51</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Demethoxycurcumin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1: 3.12</td>
<td>0.90</td>
<td>99</td>
<td>2.93</td>
</tr>
<tr>
<td>Day 2: 2.92</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 3: 4.64</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bisdemethoxycurcumin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1: 0.55</td>
<td>0.51</td>
<td>98</td>
<td>1.07</td>
</tr>
<tr>
<td>Day 2: 0.67</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 3: 1.58</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> three levels of added standard compounds (0.5; 5;50 µg) in the sample solution with each level measured in triplicate.

<sup>b</sup> for six measurements at 0, 4, 8, 12, 24 and 48 h after the extraction of the sample.

As shown in Table 4-4, the amounts of each curcuminoids were varied significantly. The amounts of curcumin, demethoxycurcumin and bisdemethoxycurcumin in all samples were ranging from 0.32-24.32, 0.17-9.91 and 0-7.86 mg/g, respectively. Curcumin was found to be the most dominant compound, while bisdemethoxycurcumin was the lowest in majority of the raw material samples investigated.
Fig 4-3 HPLC chromatogram of bisdemethoxycurcumin (1), demethoxycurcumin (2) and curcumin (3) in *C. longa* (a) and *C. xanthorrhiza* (b). Operating conditions as in Fig. 4-2.

By comparing the amount of all curcuminoids in all samples were found that the amount of curcuminoids in *C. xanthorrhiza* is lower than *C. longa*. Bisdemethoxycurcumin was found very low abundant in *C. xanthorrhiza*. So with this situation we can use the amount of curcuminoids to discriminate the two species because curcuminoids in *C. longa* are much higher than in *C. xanthorrhiza*. To confirm it we can combine with some chemometrics methods.
Table 4-4 The amount of the three curcuminoids in *C. longa* and *C. xanthorrhiza*.

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Amount (mg/g), n = 3</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>curcumin</td>
<td>demethoxycurcumin</td>
<td>Bisdemethoxycurcumin</td>
</tr>
<tr>
<td>CL-1</td>
<td>23.3</td>
<td>8.53</td>
<td>5.83</td>
</tr>
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<td>CL-2</td>
<td>22.12</td>
<td>7.67</td>
<td>6.81</td>
</tr>
<tr>
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<td>19.66</td>
<td>8.14</td>
<td>6.11</td>
</tr>
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<td>CL-4</td>
<td>19.33</td>
<td>8.38</td>
<td>5.19</td>
</tr>
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<td>21.71</td>
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<td>5.65</td>
<td>4.21</td>
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<tr>
<td>CL-13</td>
<td>14.84</td>
<td>6.82</td>
<td>4.59</td>
</tr>
<tr>
<td>CL-14</td>
<td>24.32</td>
<td>9.91</td>
<td>7.86</td>
</tr>
<tr>
<td>CL-15</td>
<td>21.31</td>
<td>6.65</td>
<td>4.66</td>
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<td>CL-16</td>
<td>17.05</td>
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<td>5.12</td>
</tr>
<tr>
<td>CX-1</td>
<td>3.78</td>
<td>1.16</td>
<td>0.21</td>
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<tr>
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<td>1.29</td>
<td>0.42</td>
<td>0.18</td>
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<td>0.27</td>
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<td>0</td>
</tr>
<tr>
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<td>2.35</td>
<td>0.31</td>
</tr>
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<td>1.4</td>
<td>0.23</td>
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<td>0.26</td>
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<tr>
<td>CX-18</td>
<td>2.67</td>
<td>1.09</td>
<td>0.21</td>
</tr>
</tbody>
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4.3.4 Discrimination of *C. longa* and *C. xanthorrhiza*

Discrimination of the *C. longa* and *C. xanthorrhiza* is important to prevent the substitution use of each other in the herbal medicine products contain one of the two
plants. We employed a combination of HPLC analysis with a chemometrics method. This combination has become one of the most frequently applied approaches for classification, authentication and discrimination of medicinal plants for recognition of geographical origin, detection of adulteration and discrimination of closely related species [22,23]. In this study, we used PCA and HCA which are commonly used to discriminate medicinal plant species.

PCA is an unsupervised multivariate statistical method and a well-known multivariate analysis tool for reducing data and extract an information to find a combination of variables or factors for describing major trends in a data set [24]. PCA will transform the original variables into new uncorrelated new variables called principal components (PC). Each PC is a linear combination of the original variables and PCs are chosen to be orthogonal each other [25,26]. PCA plot for the first two PCs is usually used and the most useful in the analysis because both PCs contain the most variation in the data. The closer the PCs value, the greater the similarity between the samples.

In this work, PCA was employed to discriminate the samples according to the species based on the amount of each curcuminoids. Fig 4-3 showed the score plot derived from PCA analysis using the first two PC. The total variance for this two PC is 99.75%, it means the two PC represent 99.75% of all the data. From the PCA plot in Fig 4-3, the two species were clustered into their groups. So with the PCA analysis we could discriminate *C. longa* and *C. xanthorrhiza*. 
Fig. 4-4 The scatter plot from the PC1 and PC2 obtained by PCA for *C. longa* (▲) and *C. xanthorrhiza* (■).

HCA is another unsupervised multivariate analysis which provides a way to classify samples into several groups based on measured characteristics. HCA starts with each sample in a separate cluster and then combines the cluster sequently, reducing the number of clusters at each step until one cluster left. The hierarchical clustering process can be delineated as a tree or dendogram, where each step in the clustering process is illustrated by a joint of the tree [26].

In the HCA analysis, Euclidean distance with single linkage method was chosen as a measurement. A dendogram was generated (Fig. 4-4) which revealed the relationship among the sample tested. Two separate clusters were obtained from the dendogram which represent the cluster of the two species. The result of HCA is similar to that of PCA based on the curcuminoids content.
4.4 Conclusion

In this study simultaneous determination of curcuminoids in *C. longa* and *C. xanthorrhiza* by HPLC-DAD was developed. It was found that curcuminoids content in *C. longa* is higher than *C. xanthorrhiza*. Discrimination these two species also achieved by combining the curcuminoids content determined by HPLC-DAD with PCA and DA. The developed method could be successfully used for quality control of the two plants.

4.5 References


Chapter 5 Conclusions and future perspective

5.1 Conclusion

In the present study, quality control methods for some Zingiberaceous plants origin from Indonesia have been developed. The developed methods use a capillary LC and HPLC alone or in combination with chemometrics methods with compound-oriented and pattern-oriented approaches. Application of the developed method to real samples has been successfully done.

For the first time, a valid method using capillary LC with C30 as the stationary phase has been proposed for simultaneous determination of four bioactive components present in ginger namely 6-, 8-, 10-gingerol and 6-shogaol. The developed method was applied for determination of these components in three ginger varieties, Z. officinale var amarum, Z. officinale var officinarum and Z. officinale var rubrum. From the result, the content of all components varied remarkably in all samples with mostly of Z. officinale var amarum has the highest concentration for all components. In addition, combining the amount of gingerols and shogaol with DA gave satisfactory result in discrimination of these three varieties of ginger.

Capillary liquid chromatography fingerprint analysis was developed in order to discriminate three closely related plants from the Zingiberaceae family i.e. Z. montanum, Z. americans and Z. zerumbet. Fingerprint chromatogram for each sample was obtained by using C18 as stationary phase and 60% acetonitrile as mobile phase. In the fingerprint chromatograms with the elution time within 30 min, there are 10 common peaks were obtained for Z. montanum and 4 for Z. americans and Z. zerumbet. There are 6 typical peaks for Z. montanum that could be used as an identification marker to discriminate from Z. americans and Z. zerumbet. To confirm the identification with marker peaks,
combination of the fingerprint analysis with PCA and also DA, the three species clustered on its own groups. DA gave clearly grouped of the three species.

A new HPLC method was developed for simultaneous determination of three curcuminoids compound present in *C. longa* and *C. xanthorrhiza*. The proposed method gave a good baseline separation of the three compounds within 15 min under gradient elution. *C. longa* has higher amount for all curcuminoids compound compare with *C. xanthorrhiza*. Based on this result we could discriminate this two closely related species. To be more convincing, an aid from chemometrics such as PCA and HCA was used. Combination of HPLC with PCA and HCA gives excellent result with the two species were separated clearly in the PCA plot and HCA dendogram.

### 5.2 Future Perspective

Quality control plays important role in the application and development of herbal medicinal products for evaluation of their quality, safety and efficacy [1]. Quality control could be performed either by chemical entities or biological activity. Evaluation on chemical entities is more often used recently. In terms of chemical entities usually most scientists applied compound-oriented (marker analysis) and pattern-oriented (fingerprint analysis) approach by using an analytical instrumentation such as LC.

LC offers a powerful tool to evaluate the entire compound pattern of complex herbal medicinal products. Recent developments in the quality control of herbal medicines involve miniaturization techniques and multidimensional LC, the use of monolithic and fused core column as well as the use of high temperature in the analysis [2]. Capillary LC as a miniaturized instrument from conventional LC offers some advantages such as improved mass sensitivity, reduced consumption of packing materials,
mobile phase, and the sample amounts because the use of smaller i.d. columns and lower mobile phase flow rate [3]. There is a limitation for capillary LC especially in the pumping system to have an accurate and reproducible at µL/min and also in the case of gradient elution. Herbal medicines are complex mixtures that contain dozens even hundred chemical compounds, so usually gradient elution program is mostly used in the separation with LC. Another limitation is the lifetime of the microcolumn is shorter compared to the conventional column. However, the development of this technique still continues and someday there is a capillary LC system that can overcome this problem.

The second perspective concerns in the complex relationship between the chemical entities with biological activity for determination of the efficacy of herbal medicines. It is well known that the efficacy of herbal medicines has characteristics of a complex mixture of chemical compounds present in the herbs. So, evaluation in the relationship of chemical and biological activity is obviously not a trivial task. Chemical profiles might be linked to the biological activity to provide more assurance of efficacy and consistency of herbal medicines [4]. Recently a new concept named biofingerprint was introduced to know the relationship between the chemical profiles and biological activity. Biofingerprint in LC could give a comprehensive tool in the quality control of herbal medicinal products and also can be used in the area of biological and drug discovery research [5-7].

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