ENRICHMENT OF α-GLUCOSIDASE INHIBITORY ACTIVITY CONTAINING COMPOUNDS IN *PHALERIA MACROCARPA* PLANT EXTRACT BY SUBCRITICAL CARBON DIOXIDE SOXHLET FRACTIONATION

BY

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A thesis submitted in fulfillment of the requirement for the degree of Doctor of Philosophy in Pharmaceutical Sciences

Kulliyyah of Pharmacy
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AUGUST 2016

ABSTRACT

Phaleria macrocarpa, also known as "Mahkota Dewa," is a common medicinal plant with enhance vitality. It is widely used as anti-diabetic remedies in Malaysia. The Subcritical Carbon dioxide Soxhlet ($SubCO_2$), an advanced extraction technology which functions at a low temperature, was investigated for the fractionation of *α*-glucosidase inhibitory activity of *P. macrocarpa* in this study. The yields; enzyme activity and contents of potent inhibitory compounds of the extracts were examined. The yields of sonication and $SubCO_2$ extraction method were approximately 10.2±1.0% to 25.4±0.6% and 0.9±0.1% to 2.0±1.0%, respectively. However, inhibitory activity of the extract (IC_{50}) using $SubCO_2$ (4.0±0.3 µg/mL) was higher than the sonication method (7.4±1.7 µg/mL). In addition, inhibitory compounds extracted using $SubCO_2$ contained approximately 5-times more inhibitory compounds compared with sonication-extraction. Overall, $SubCO_2$ extraction represents a promising method to produce highly potent inhibitory compounds contained extracts. In this study, inhibitory ability against *α*-glucosidase of different parts of *P. macrocarpa* was screened. Methanol and *n*-hexane extracts, obtained by solvent extraction, were evaluated for *invitro* inhibition properties. The active compounds were identified using gas chromatography-mass spectrometry (GC-MS). The methanol extract of fruit flesh had the highest yield (25.6±0.5%), whereas *n*-hexane extract of stem is more effective against *α*-glucosidase activity (0.8±0.1 µg/mL). The fruit flesh (1.3±0.2 µg/mL) and leaves (1.6±0.6 µg/mL) had also well effectively. The identified metabolites are D-fructose, squalene, *α*-linolenic acid, and *α*-D-glucopyranoside. In this research, a rapid and simple analytical method was developed for herbal quality control to investigate inhibitory activity of *P. macrocarpa* extract by multi component analyses using a Fourier transform infrared (FTIR) spectroscopy based fingerprinting. A total of thirty six extracts of different ethanol concentrations were prepared and tested on inhibitory potential, and fingerprinted by FTIR spectroscopy, coupled with chemometrics of orthogonal partial least square (OPLS) at 4000-400 cm^{-1} frequency region and resolution 4 cm^{-1}. The OPLS model generated the highest regression co-efficient with $R^2Y=0.98$, $Q^2Y=0.70$, lowest root mean square error estimation (RMSEE) = 17.17 and root mean square error of cross validation ($RMSE_{CV}$) = 57.29, respectively. A five components (1+4+0) predictive model was developed to correlate FTIR spectra with activity and the responsible functional groups such as -CH, -C=O, -NH, -COOH and -OH were identified for the bioactivity. A successful multivariate model was constructed using FTIR-ATR as a simple and rapid technique to predict the inhibition activity. In this study, inhibitory potential against *α*-glucosidase of various ethanolic extracts (water, 20%, 40%, 60%, 80%, and 100% ethanol) of *P. macrocarpa* were assessed using GC-MS and multivariate data analysis (MVDA). OPLS combined with GC-MS analysis was applied to correlate the inhibition of enzyme activity of various extracts to various compounds profiles of *P. macrocarpa*. The obtained score scatter plot of OPLS showed a distinct and remarkable separation of 6 different ratios of ethanolic extract into 6 clusters. GC-MS along with MVDA was used to identify the metabolites that inhibit the enzyme activity. In addition, myo-inositol, squalene, palmitic acid, and *α*-D-glucopyranoside metabolites were identified and exhibit the potential inhibition activity of *P. macrocarpa*.

خلاصة البحث

نبات *Phaleriamacrocarpa*المعروف أيضاً بـMahkota Dewa، هو نبات طبي شائع. يستخدم كخافض للسكر في ماليزيا. في هذه الدراسة تم استخدام نظام ثاني أوكسيد الكربون تحت الحرج $SubCO_2$، وهو طريقة استخلاص متقدمة تعمل بدرجات حرارة منخفضة، لتجزئة ألفا-غلوكوزيداز من هذا النبات. تم تحليل المردود وفعالية الانزيم ومكونات المركبات المثبطة القوية من الخلاصة. المردود باستخدام الأمواج فوق الصوتية و$SubCO_2$ كان تقريبا -10.2±1.0% 25.4±0.6% و 0%1.0±2.0-0.1±9. على الترتيب. لكن الفعالية المثبطة للخلاصة باستخدام ($SubCO_2$(4.0±0.3 µg/mL كانت اعلى من طريقة الامواج فوق الصوتية (7.4±1.7 µg/mL). إضافة الى لك فإن المكربات المستخلصة باستخدام نظام $SubCO_2$ تضمنت تقريبا 5 مرات مركبات مثبطة اكثر من طريقة الامواج فوق الصوتية. عموما فإن الاستخلاص بطريقة $SubCO_2$ يمثل طريقة واعدة لإنتاج خلاصات محتوية على مركبات مثبطة قوية. في هذه الدراسة تمت دراسة التأثير المثبط للالفا غلووزيداز من الاجزاء المتعددة من النبات. تم تقييم خلاصات المتانول والهكسان المستخلصة بطريقة المحل من اجل تأثيرها المثبط. تم التعرف على المركبات الفعالة باستخدام gas chromatography-mass Spectrometry (GC-MS). أعطت خلاصة المتانول للفواكه المردود الاعلى 25.6±0.5%)، بينما خلاصة الهكسان للساق كانت اكثر فعالية ضد الفا غلوكوزيداز (0.8±0.1 µg/mL). للفواكه 1.3±0.2) µg/mL) والاوراق (1.6±0.6 µg/mL) فعالية جيدة ايضا. المستقلبات المتعرف عليها هي -D fructose, Squalene, α-linolenic acid, and α-D-glucopyranoside. في هذا البحث تم تطوير طريقة تحليل سريعة وبسيطة للتحليل الكمي للفعالية المثبطة للخلاصة باستخدام التحليل الكمي المتعد بالبصمة المعتمدة على الاشعة تحت الحمراء. تم تحضير 36 خلاصة بتراكيز متعددة في الايتانول واختبارها بـorthogonal partial least square (OPLS) بطول موجة 4000-400 cm^{-1}. اعطى النموذج المعاملات التالية highest regression co-efficient with $R^2Y=0.98$, $Q^2Y=0.70$, lowest root mean square error estimation (RMSEE) = 17.17 and root mean square error of cross validation ($RMSE_{CV}$) = 57.29). نموذج توقعي لخمس مكونات تم بناؤه لموافقة الأطياف بالفعالية والمجموعات الوظيفية مثل CH,-C=O, -NH, -COOH and -OH. تم بناء نموذج متعدد العوامل باستخدام FTIR-ATR كطريقة بسيطة وسريعة لتوقع الفعالية المثبطة. في هذه الدراسة القدرة المثبطة ضد الفا-غلوكوزيداز للخلاصات الايتانولية المتعددة (water, 20%, 40%, 60%, 80%, and 100% ethanol) تم تقييمها باستخدام GC–MS و multivariate data analysis (MVDA). تم تطبيق تحليل OPLS مع GC-MS لربط تثبيط الانزيم من قبل الخلاصات المتعددة ببروفايلات المركبات المتعددة. اظهرت النتائج انفصال واضح لستة نسب مختلفة للخلاصات الايتانولية في ستة مجموعات. تم استخدام GC-MS مع MVDA لتحديد المستقلبات التي تثبط فعالية الانزيم. اضافة الى ذلك، تم التعرف على مستقلبات myo-inositol, squalene, palmitic acid, and α-D-glucopyranoside واظهرت فعلاية مثبطة محتملة من نبات *P. macrocarpa*.

APPROVAL PAGE

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DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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ACKNOWLEDGEMENTS

Praise be to Allah, The Most Gracious, The Most Merciful, it is only by His Blessing that I could finish this doctoral thesis.

I would like to express my gratitude to my supervisor Professor Dr. Md. Zaidul Islam Sarker who provided me with the opportunity to pursue my goals and the direction to achieve them.

Furthermore, I would like to express my sincere appreciation to the rest of my supervisory committee: Associate Professor Dr. Alfi Khatib, Assistant Professor Dr. Juliana Bnt Md Jaffri, and Assistant Professor Dr. Sahena Ferdosh for their encouragements, advices and knowledge related to this research.

I would like to acknowledge the financial support provided by International Islamic University Malaysia. Grateful acknowledges are extended to all the staff members and all my friends from Faculty of Pharmacy, IIUM for their assistance and co-operation.

Finally, it is my utmost pleasure to dedicate this work to my dear parents and my family, my husband Tareq, my sons Ethane and Methane who granted me the gift of their unwavering belief in my ability to accomplish this goal: thank you for your support and patience.

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ABBREVIATIONS

$SubCO_2$	Subcritical Carbon dioxide Soxhlet
GC-MS	Gas chromatography-mass spectrometry
FTIR	Fourier transform infra red spectroscopy
OPLS	Othogonal partial least square
RMSEE	Root mean square error of estimation
$RMSE_{CV}$	Root mean square error of cross validation
LC-MS	Liquid chromatography-mass spectrometry
MVDA	Multi variate data analysis
WHO	World health organization
NMR	Nuclear magnetic resonance
NHMS	Malaysian national health and morbidity servey
SFE	Supercritical fluid extraction
MOH	Ministry of health
PCR	Principle component analysis
PLS	Partial least square
MLR	Multiple linear regression
ATR	Attenuated total reflectance
DPPH	2, 2-diphenyl-1-picryl- hydroxyl
LDL	Low density lipoprotein
HDL	High density lipoprotein
PCSK	Pro-protien converters kexin
iNOS	Inducible nitric oxide
AC	Adriamycin cyclophosphamide
FDA	Food and drug administration
RSM	Response surface methodology
CCD	Central composite design
HPLC	High performance liquid chromatography
UAE	Ultra-sound assisted extraction
MAE	Microwave-assisted extraction
SCF	Supercritical fluid
SWE	Subcritical water extraction
pNPG	4-nitrophenyl-α-D-glucopyranoside
CE	Capillary electrophoresis
UPLC	Ultra-high performance liquid chromatography
FTICR	Fourier transform ion cyclotron resonance
TOF	Time of flight
NIR	Near infra red spectroscopy
MIR	Mid infra red spectroscopy
FIR	Far infra red spectroscopy
ICS	International chemometrics society
K	Number of variables
N	Number of observation
DA	Discriminant analysis

HCA	Hierarchical cluster analysis
ICA	Independent component analysis
PC	Principle component
SOM	Self-organizing map
RF	Radom forest
GA	Genetic algorithm
VIP	Variable importance on projection
AOCS	American oil chemist's society
MSTFA	N-methyl-N-(trimethylsillyl)-trifluoroacetamide
MSD	Mass selective detector
ACD	Advanced chemistry development
IS	Internal standard
Std	Standard
Rt	Retention time
RT	Room temperature
hpf	Hours post fertilizations
MSC	Multiplicative signal correction
SNV	Standard normal variant
UV	Ultra violate spectroscopy
MW	Molecular weight
KDa	Kilo Dalton
hr	hour
min	minute
g	gram
mg	miligram
l	litre
mL	mili litre
mg/L	milligram/litre
µL	microlitre
mM	milimol
µg	microgram
cm^{-1}	Per centimetre
MPa	megapascale
LD	Lethal density

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

For many decades, plants have been used as a source of food only. At present, plants are widely used as a natural source of medicinal agents, food additives, cosmetics and neutraceuticals (Hendra et al., 2011a). Recently, researches on medicinal plants are receiving more attention. A large variety of structures and functionalities of natural bioactive compounds gives an excellent pool of molecules that produce essential functional foods, neutraceuticals, food additives and pharmaceuticals for human health benefits. More than 80% of the world's population depends on traditional medicine in treating ailments, as reported by the World Health Organization (WHO) (Shasidharan et al., 2011).

Plants contain a wide range of compounds promising as traditional medicine to treat chronic as well as infectious diseases. Thousands of phytochemicals from plants have been considered safe and effective alternatives with fewer side effects. These phytochemicals, which are secondary metabolites of plants including tannins, terpenoids, alkaloids, flavonoids and others, possess many biological remedial benefits such as anticancer, antimicrobial, antioxidant, antidiarrheal, analgesic and wound healing. These bioactive compounds are used to develop either drugs or dietary supplements (Hendra et al., 2011a).

Phaleria macrocarpa (Scheff.) Boerl., belongs to the Thymelaeaceae family, also known as 'Mahkota Dewa', 'God's Crown' and 'Pau'. It is a familiar and widely used medicinal plant in many South Asian countries claimed by informal practitioners

(Harmanto, 2003). *Phaleria macrocarpa* is used to control diabetes mellitus, cancer, hemorrhoids, impotency, allergies, heart and liver diseases, kidney disorders, stroke, blood related diseases, migraine, acne and several skin ailments (Hendra et al., 2011a; Kim et al., 2010).

Every part of this plant from leaves to roots has long been consumed for prevention of diseases with some good results. The stems, leaves and fruits of *P. macrocarpa* have been widely used for medicinal treatments. The constituents of the stem are used to treat bone cancer; seeds are used in treating breast cancer, cervical cancer, lung, liver and heart diseases; while the leaves are used to treat impotence, diabetes mellitus, allergies, tumors and blood diseases (Altaf et al., 2013). This herb has been used in both unprocessed and processed form as a tea, juice and other liquid forms. The unprocessed fruit may have toxicity and sometimes are poisonous (Yosie et al., 2011).

In Malaysia and Indonesia, a large number of people use every part of *P. macrocarpa* plant for many diseases. The existence of scientific evidence on activity of this plant is very important to make it into a standard herb that has long been consumed for treating Type 2 diabetes. Only a few investigators have reported the inhibitory potential against α-glucosidase of *P. macrocarpa* using conventional extraction methods.

Diabetes mellitus is an endocrine disorder that is characterized by hyperglycemia resulting from inadequate insulin secretion (Type 1 diabetes mellitus) or insulin insensitivity (Type 2 diabetes mellitus) (Bastaki et al., 2005; Mitrakou et al., 1992; Porte, 2001). Type 1 diabetes mellitus normally arise in young adults or children and cannot be prevented with diet or a change in lifestyle. Type 1 requires daily insulin injections to balance glucose levels and it is genetically predetermined.

On the other hand, Type 2 diabetes commonly takes place with obesity, and/or a strong family history of diabetes. Type 2 diabetes is easier to handle with diet and lifestyle modifications in its earlier stages. The another type of diabetes, Type 3, a new term proposed by a research team at Brown Medical School and Rhode Island, is an extension of Type 1 and Type 2, and follows a similar path physiology as Type 2, however in the brain. Insulin is needed to help the neurons in the brain absorb glucose for healthy functioning, and if the cells in the brain become insulin-resistant, it can lead to Alzheimer's. Type 2 diabetes makes up approximately 90% of all diabetes. Type 2 diabetes is considered to be a preventable disease; thus it receives more attention than Type 1 diabetes. Post-prandial hyperglycemia has an important function in the development of Type 2 diabetes (Baron, 1998).

According to the International Diabetes Federation, (2012), the prevalence of diabetes is increasing at an alarming rate globally and it is estimated that approximately 592 million people will be affected by Type 2 diabetes within 2035. The Malaysian National Health and Morbidity Survey (NHMS) and projections by the Disease Control Division of the Ministry of Health (MOH) reported that the prevalence of diabetes in Malaysia is projected to rise to 21.6% by the year 2020. Hence, it is essential to control plasma glucose levels to delay or prevent Type 2 diabetes.

The α-glucosidase enzyme is responsible for catalyzing the last step in the carbohydrate digestion process by releasing α-D-glucose from the non-reducing side of the sugar before being released into the blood stream (Adisakwattana et al., 2011; Ademiluyi & Oboh, 2013). It has been reported that one of the therapeutic approaches for suppressing postprandial blood glucose is to retard the digestion and absorption of carbohydrates after consumption through inhibition of carbohydrate hydrolyzing

enzymes including *α*-glucosidase (Kim, 2012; Chiasson & Rabasa-Lhoret, 2004; Rabasa-Lhoret & Chiasson, 2003). This approach is considered more efficient than controlling insulin secretion for reasons of economics, convenience and the avoidance of side effects (Porte, 2001).

Currently, a number of *α*-glucosidase inhibitors such as acarbose, miglitol, voglibose and quercetin are used as oral anti-hyperglycaemic drugs and metformin was the most common oral anti-diabetic drug used in Malaysia at 82.5% of Type 2 diabetic patients, followed by 56.9% of patients who were treated with sulphonylurea to manage postprandial hyperglycaemia. However, these inhibitors have many unwanted side effects such as liver disorders, abdominal pain, hepatic injury, abdominal fullness, flatulence, acute hepatitis, renal tumours and diarrhoea (Amarowicz et al., 2005; Fujisawa et al., 2005; Hiroyuki et al., 2001; Madar, 1989; Murai et al., 2002; Shobana et al., 2009). For this reason, finding methods for early control and management of hyperglycemia is crucial and has gained more attention to alternative *α*-glucosidase inhibitor from natural sources, particularly, a food or medicinal plant origin in recent years (Floris et al., 2005; Yuk et al., 2011; Kumar et al., 2011). In addition, these inhibitors can also extenuate diabetes-related complications such as cataracts, neuropathy, retinopathy, angiopathy, nephropathy and keratopathy (Aryangat & Gerich, 2010; Robinson et al., 1983).

For these reasons, *P. macrocarpa* could be the better alternative *α*-glucosidase inhibitor and there is a need to investigate the *α*-glucosidase inhibitory activity of various parts such as fruit flesh, leaves and stem of *P. macrocarpa.* Assuring the identity, safety and quality of herbal products is a grownup concern for the extravagantly complication and unknown mechanism of disease treatment. Besides, the herb and its extracts contain thousands of unknown compounds that can be

different on the basis of some factors such as drying processes, harvesting season, plant origins and so on.

In an herbal material, there are many active components are in miserable amounts and generally their variability exists that cannot be isolated easily. As a result, the classical isolation and measurement methods are laborious, expensive, time consuming, use toxic chemicals and unreliable for the quality control of these herbs (Beek et al., 2009). So, there is a need to use the more crude extracts (polytherapy). Moreover, only a few isolated pharmacologically active principles cannot represent the bioactivity of complex herbal extracts and the activity of herbal preparation arises from combined interactions of thousands of constituents and their synergist/antagonist consequences (Bankova, 2005; Liang et al., 2004).

However, only a few compounds such as phalerin (Altaf et al., 2013), carbohydrates (structure is suspected analogous with myglitol or acarbose) (Sugiwati & Setiasih, 2010), and magniferin (Rabyah et al., 2013) have been reported for the α-glucosidase inhibitory activity of *P. macrocarpa* based on bioassay guided traditional method. Therefore, it is an important issue to develop a rapid and accurate analytical method for ensuring of bioactivity and safety of herbal materials. To overcome this limitation, metabolomics methodologies combined with several common analytical techniques have become an important tool for multi component analysis of herbal extracts where multiple components can be assessed in a single analysis (Cozzolino, 2009).

Metabolomics is a novel, holistic and rapidly-emerging field that provides a comprehensive chemical analysis of all existing metabolites, their variation and metabolic pathway in a biological system ensuring the production of robust and high-quality data in an unbiased way (Madsen et al., 2009; Vander Kooy et al., 2009).

Multivariate data analysis (MVDA) is a suitable statistical tool for handling large data sets found by using spectroscopic tools and is applied in various aspects of chemical and biomedical study areas such as, quality control, chemotaxonomy, biomarker screening, clinical chemistry, environmental metabolism, activity and toxicity investigation by classifying samples based on their phytochemical composition (Dettmer et al., 2007; Liu et al., 2010). Currently, metabolomics along with projection-based multivariate data analysis were successfully employed to manage large data set.

There are a variety of spectroscopic techniques for herbal fingerprint analysis such as nuclear magnetic resonance spectroscopy (NMR), gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) (Oskman-Caldentey & Saito, 2005). Though NMR, GC-MS and LC-MS are the principal analytical methods applied in plant metabolomics as well as they have some advantages such as comprehensiveness, sample throughput and precision, these systems are not constantly available in developing countries in where the natural products are generally well known.

The application of most available, simple and rapid methods like Fourier Transform Infrared spectroscopy (FTIR) could be an efficient alternate for herb quality control (Sharif et al., 2014). The FTIR spectroscopy, used to determine functional groups, is reagent less, low cost, fast, non-destructive, and suitable for investigations which ensure high reproducibility, specificity, easy and minimal sample preparation (Roggo et al., 2007). In addition, the functional groups absorb the incoming FTIR radiation at specific wavelength and chemical bonds vibrate in characteristic ways such as bending or stretching leading in a spectrum or `fingerprint' (Allwood et al., 2008; Beeks et al., 2007).

On the other hand, the FTIR spectrum is fundamentally compiled of a big set of combination bands and overtones arises from the complicated chemical structure and composition of medicinal products resulting in an extremely convoluted spectrum. Besides, the wavelength dependent ambient effects, scattering effects, instrumental noise, tissue heterogeneities and other origins of variability may further complicated the spectrum. Therefore, it is very hard to specify particular peaks to particular functional groups let alone chemical constituents. The conjunction of FTIR spectroscopy with multivariate techniques including principal component regressions (PCR) and partial least squares (PLS) offers a potent tool for analysis and interpretation of the spectra.

Chemometrics is the application of multivariate statistical techniques used to chemical data. The chemical discipline that applies mathematical and statistical methods, (a) to provide maximum chemical information by analyzing chemical data, and (b) to design or select optimal measurement procedure and experiments. For model calibration, it is needed to take out the information from the complicated compositional attributes of the FTIR spectrum (Cozzolino, 2009). Recently, researches have inserted this idea to manage big data sets to characterize samples. For instance, metabolomics together with projection-based multivariate data analysis including principal component analysis (PCA), partial least square (PLS), or its variant orthogonal partial least square (OPLS) was successfully applied for obtaining reliable result (Yuliana et al., 2001; Mediani et al., 2012).

Chemometrics has a wide scope for data evaluation including descriptive and inference statistics, exploratory data analysis, signal processing, pattern recognition, optimization, modeling, classification, and statistical experimental design. Chemometrics, unlike classic statistics, have multiple variables simultaneously and

allow colinearity (the variation in one variable, or a group of variables, in terms of co-variation with other variables). For developing a calibration, a typical FTIR process of a desirable number of samples with known chemical values (e.g., analyzed by analytical methods) begin from the collection of FTIR spectra (transmittance or reflectance), over the 4000-400 cm^{-1} frequency region.

In this study, a rapid multivariate predictive model was developed based on OPLS method combined with FTIR technique by analyzing a metabolic fingerprint and chemical changes in the different ratios of ethanol-water extracts of *P. macrocarpa*. The model has been established from a correlation between α-glucosidase inhibitory activity and FTIR spectra of *P. macrocarpa*. To the best of our knowledge, this is the first attempt to develop a rapid predictive model by fingerprinting the compositions of this plant under different solvent treatments using FTIR and metabolomics approach.

To investigate the biological activity of all metabolites present in *P. macrocarpa*, comprehensive as well as systematic phytochemical researches should be carried on. Nevertheless, in this herb, no systematic chemical studies have been performed. The biological activity of natural products arises from the synergistic effects of the phytochemical composition of complex plant matrix. Therefore, a wide analytical method is required to analyze all existing metabolites in a natural product. At present, gas chromatography mass spectrometry (GC-MS), high-performance liquid chromatography mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) spectroscopy are the distinctive methods dominating in metabolomics research area with wide-ranging characteristics (Koek et al., 2006; Dunn & Ellis, 2005; Vander Greef et al., 2004; Fiehn & Weckwerth, 2003).